7. EXPERIMENTAL WORK

7. Experimental Work

Aim of the work was to develop analytical methods for single and binary combination components. The experiment section, therefore, is divided in two parts.

In the first part, the spectroscopic (UV and IR) and chromatographic methods were developed to analyze single component drugs (EZE, PRAVA, ROSU, SIMVA and LOVA) in their formulations. The stability study of these drugs was done as per ICH guidelines using chromatographic methods (HPLC and HPTLC). All developed methods were validated as per ICH guidelines. Bioanalytical method was also developed for the estimation of single component in spiked human plasma to support pharmacokinetic study.

In second part simultaneous estimation was done for EZE in combination with statins (SIMVA, LOVA, ROSU and PRAVA). Some combinations are not available in market but it is well proven that simultaneous prescription is more effective in compare to monothrapy of statins. Both spectroscopic and chromatographic methods were developed. In Spectrophotometric methods (first derivative zero crossing, difference derivative zero crossing, absorbance ratio and absorbance ratio derivative zero crossing) methods, quantitative IR spectroscopy and chemometric approach were tried. In chromatographic methods HPLC and HPTLC methods were tried. In which HPLC method could estimate drug in presence of its degraded products.

7. 1. 1

Ezetimibe

7.1.1 Ezetimibe

Apparatus, instruments and reagents:

Apparatus, instruments and reagents used for the development of spectroscopic and chromatographic methods are mentioned in Chapter 6.

Spectroscopic method

Five spectroscopic methods were developed for the estimation of EZE in bulk drug and pharmaceutical Tablet formulations. First method was simple zero order spectroscopy with the use of methanol as solvent with 231.20 nm as wavelength of absorption. First and second derivative spectroscopy methods employed as advance spectroscopic methods. Forth method was a difference spectrophotometry in that the difference in absorbance (A) between two equimolar solutions of the EZE in 0.1 N NaOH as sample and methanol as reference was developed, which exhibits different spectral characteristics. The Fourier transform infrared spectroscopy applied for quantification of EZE in Tablet formulation as fifth method.

Chromatographic methods:

Three chromatographic methods were developed for the simultaneous estimation of EZE. One was HPLC method, which could estimate EZE in pharmaceutical dosage form and in presence of degradation products. HPLC method could also estimation EZE in human plasma. Two HPTLC methods were developed one for the estimation of EZE in pharmaceutical dosage form and second could estimate EZE in presence of its degraded products.

7.1.1.1 Estimation of EZE by zero order, First order and second order spectrophotometric methods.

Experimental work:

7.1.1.1.1. Preparation of stock solution for EZE

Standard EZE (10 mg) was weighed accurately and transferred to 100 ml volumetric flask. It was dissolved in 50 ml methanol and diluted up to mark with methanol to obtain final concentration of 100 µg/ml. This solution was used as working standard solution for spectrophotometric methods.

7.1.1.1.2. Analytical wavelength

Zero order Spectrophotometric method was developed to analyze the EZE in methanolic solution. 10 µg/ml of methanolic solution of EZE was scanned in the

range 200-300 nm. The curve shows highest absorbance at 231.20 nm (fig 7.1.1.1.1). So 231.20 nm was selected as analytical wavelength.

First and second order derivative spectroscopy method is mathematical conversion of zero order spectra in to a plot of $dA/d\lambda$ vs. λ and $d^2A/d\lambda^2$ vs. λ. Zero order spectra for all development parameters were recoded in UV PC software which was used for the First and second order derivative spectroscopic method. Derivative curves were studied and simultaneous peak and valley was selected so that amplitude difference can give precise correlation coefficient (fig. 7.1.1.1.4 and 7.1.1.1.5).

7.1.1.1.3. Calibration curve for standard EZE:

From the stock solution (100 µg/ml) aliquots of 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 ml were transferred to a series of 10ml volumetric flasks. The volume was adjusted to a mark with methanol to get 1, 5, 10, 15, 20, 25, 30, 35 and 40 µg/ml of solution. The absorbance was measured at λ_{max} or selected wavelength using methanol as a blank.

7.1.1.1.4. Validation Parameters

(I) Linearity:

The absorbance of standard EZE solutions were measured at λ_{max} or selected wavelength using methanol as a blank over the concentration range of 1-40 µg/ml and calibration curve was plotted against concentration and regression equation was calculated. Result should be expressed in terms of Correlation co-efficient.

(II) Precision

a. Repeatability (Intraday and Interday Precision):

The intraday and interday precisions of the proposed methods were determined by estimating the corresponding responses 5 times on the same day and on 5 different day for 10, 20 and 30 µg/ml, which was prepared by transferring 1,2 and 3 ml of stock solution in 10 ml volumetric flask and made up to the volume by methanol. The results are reported in terms of percentage coefficient of variation (%C.V).

b. Reproducibility:

The reproducibility of the method was checked by measuring absorbance of 10, 15 and 20 µg/ml of solution in UV 1700 and UV 1601 for three times. The % CV of the responses for determination of drug was found which reveals the reproducibility of the method.

(III) Accuracy

The accuracy of the methods was determined by recoveries by the standard addition method. For this, previously analyzed 10 µg/ml of drug solution from pharmaceutical formulations were spiked at three levels 50, 100 and 150 % from standard stock solution and made up to the volume with analytical grade methanol and analyzed. The final concentration was found form regression equation.

(IV) Limit of Detection and Quantification

Equation based on standard deviation of the response and the slope is given in Chapter 5, section 5.3.1.6 and 5.3.1.7.

7.1.1.1.3.1 Validation Parameters

(I) Linearity

Linearity range of EZE in methanol was found to be 1-40 µg/ml for all three methods. Correlation co-efficient and % C.V. values are reported in table 7.1.1.1.1.

Table 7.1.1.1.1: Calibration data of EZE by Spectrophotometric methods

Sr. No.	Conc. (µg/ml)	Simple UV Spectro-photometric method	1st derivative Spectrophotometric method			2nd derivative Spectrophotometric method		
		Absorb.* ± %C.V. (N=5)	Absorb. at 223.80 nm	Absorb. at 236.80 nm	Amplitude Difference* ± %C.V. (N=5)	Absorb. at 267.60 nm	Absorb. at 273.2 nm	Amplitude Difference* ± %C.V. (N=5)
1	1	0.030 ± 2.23	0.003	-0.001	0.004 ± 0.58	0.001	0	0.001± 0.30
2	5	0.229 ± 0.34	0.012	-0.006	0.018 ± 0.54	0.003	-0.002	0.005 ± 0.92
3	10	0.517 ± 0.12	0.024	-0.01	0.034 ± 0.63	0.006	-0.004	0.01 ± 0.11
4	15	0.770 ± 0.16	0.032	-0.019	0.051 ± 0.38	0.009	-0.006	0.015 ± 0.91
5	20	1.078 ± 0.19	0.043	-0.026	0.069 ± 0.78	0.012	-0.008	0.02 ± 0.88
6	25	1.351 ± 0.07	0.054	-0.032	0.086 ± 0.41	0.016	-0.01	0.026 ± 0.89
7	30	1.651 ± 0.04	0.063	-0.039	0.102 ± 0.89	0.019	-0.012	0.031 ± 1.15
8	35	1.942 ± 0.23	0.071	-0.046	0.117 ± 0.93	0.021	-0.014	0.035 ± 0.82
9	40	2.202 ± 0.17	0.081	-0.055	0.136 ± 0.46	0.024	-0.016	0.04 ± 1.16

*Mean of five determinations.

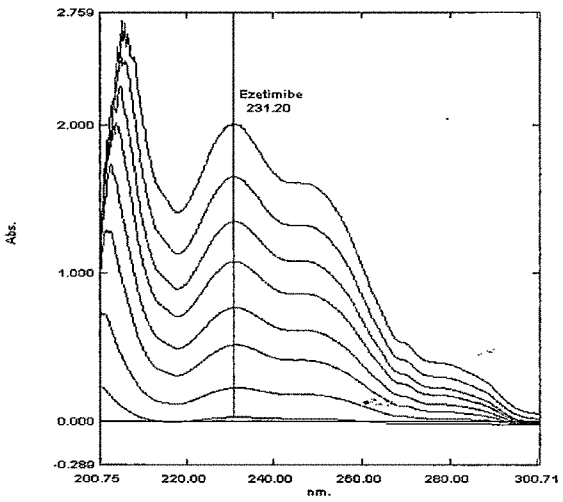


Figure: 7.1.1.1.1 UV spectra of EZE by Spectrophotometric method.

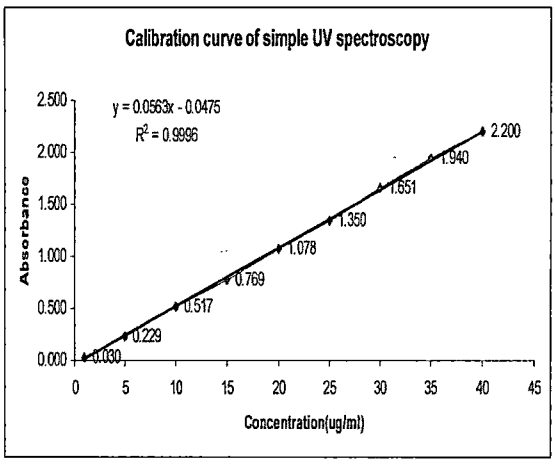


Figure 7.1.1.1.2: Calibration curve of EZE by Spectrophotometric method
Correlation co-efficient = **0.9996**
Slope = 0.0563
Intercept = -0.0475
Regression equation: **Abs. = 0.0563 × Conc. - 0.0475**

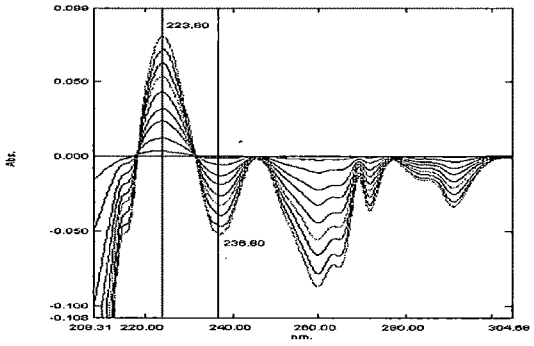


Figure: 7.1.1.1.3.: First derivative UV spectra of EZE

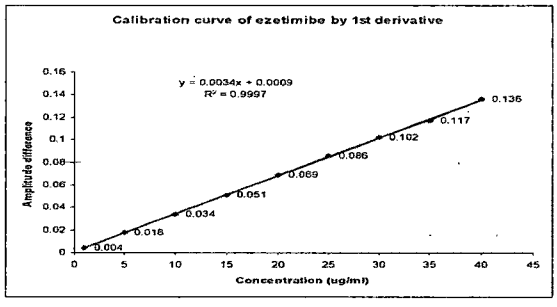


Figure 7.1.1.1.4: Calibration curve of EZE by 1st derivative Spectroscopy
Correlation co-efficient = 0.9997
Slope = 0.0034
Intercept = 0.0009
Regression equation: Abs. = 0.0034 × Conc. + 0.0009

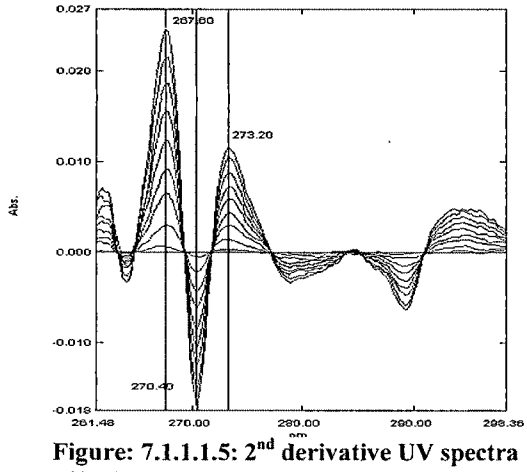


Figure: 7.1.1.1.5: 2nd derivative UV spectra of EZE

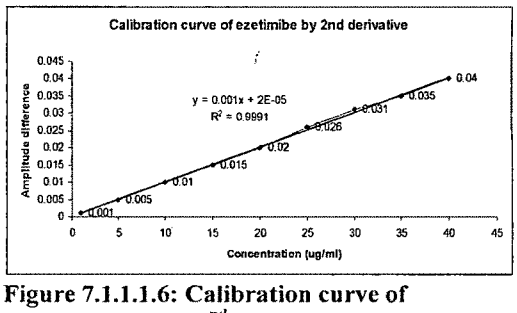


Figure 7.1.1.1.6: Calibration curve of standard EZE by 2nd derivative
Correlation co-efficient = 0.9991
Slope = 0.001
Intercept = 0.00002
Regression equation: Abs. = 0.001 × Conc. + 0.00002

(II) Precision

Intraday and Interday Precision(Repeatability)

Intraday and interday variations of the proposed methods are reported in table 7.1.1.1.2 and 7.1.1.1.1.3.

Table 7.1.1.1.2: Intraday precision data of EZE by Spectrophotometric methods

Conc. (µg/ml)	Simple UV Spectrophotometric method	1st derivative Spectrophotometric method		2nd derivative Spectrophotometric method	
	Absorb.* ± % C.V.) (n=5)	Amplitude difference* ± S.D. (n=5)	% C.V.	Amplitude difference* ± S.D. (n=5)	% C.V.
10	0.518 ± 0.10	0.034 ± 0.000095	0.27	0.011 ± 0.00015	1.15
20	1.080 ± 0.49	0.069 ± 0.00041	0.59	0.020 ± 0.00019	0.95
30	1.653 ± 0.29	0.102 ± 0.00038	0.36	0.029 ± 0.00033	1.13
Table 7.1.1.1.3: Interday precision data of EZE by Spectrophotometric method					
10	0.517 ± 0.88	0.035 ± 0.00015	0.42	0.010 ± 0.00012	0.92
20	1.078 ± 0.45	0.068 ± 0.0063	0.92	0.020± 0.00023	1.15
30	1.651 ± 0.32	0.104 ± 0.0009	0.86	0.030 ± 0.00029	0.96

* Mean of five determinations

Reproducibility:

The reproducibility of the method was determined by using Shimadzu UV 1700 and Shimadzu UV 1601. The % CV of the responses confFTIRms the reproducibility of the method for determination of EZE the data is reported in (Table 7.1.1.1.4).

Table 7.1.1.1.4: Reproducibility data of EZE by Spectrophotometric methods

Conc. (µg/ml)	Absorbance* ± %C.V.					
	Simple UV Spectrophotometric method		1st derivative Spectrophotometric method		2nd derivative Spectrophotometric method	
	UV 1700	UV 1601	UV 1700	UV 1601	UV 1700	UV 1601
10	0.517 ± 0.38	0.520 ± 0.48	0.035 ± 0.28	0.034 ± 0.70	0.011 ± 0.29	0.01 ± 0.92
15	0.770 ± 0.21	0.769 ± 0.21	0.052 ± 0.92	0.050 ± 0.51	0.013 ± 0.83	0.014 ± 0.39
20	1.078 ± 0.77	1.077 ± 0.48	0.065 ± 0.49	0.070 ± 0.88	0.021 ± 0.69	0.02 ± 0.16

*** Mean of three determinations**

(III) Accuracy

Accuracy of the measurement of EZE was determined by standard addition and was found to be in the range of 100.006 – 100.027, 99.18 -100.43 and 98.94 –101.06 %, respectively for zero, First and second order spectroscopic methods.

Table 7.1.1.1.5: Accuracy data of EZE by Spectrophotometric methods

Initial conc. (µg/ml) (A)	Quantity of std. Added (µg/ml) (B)	Total Amount (A + B)	Simple UV Spectrophotometric method		1st derivative Spectrophotometric method		2nd derivative Spectrophotometric method	
			Total quantity Found* ± S.D.	% Recovery ± S.D	Total quantity Found* ± S.D.	% Recovery ± S.D	Total quantity Found* ± S.D.	% Recovery ± S.D
10	5	15	15.03± 0.00516	100.02± 0.55535	14.80± 0.018	99.5 ± 1.39	14.80± 0.018	99.5 ± 1.39
10	10	20	20.02± 0.00497	100.00± 0.36387	20.19± 0.061	100.38 ± 1.96	20.19± 0.061	100.38 ± 1.96
10	15	25	25.026± 0.00533	100.02± 0.27275	25.26 ± 0.11	100.43 ± 1.94	25.26 ± 0.11	100.43 ± 1.94

* Mean of five determinations

(IV) Limit of detection

The minimum detectable concentration of EZE was found to be 0.3454, 0.1822 and 0.101 µg/ml, respectively for zero, First and second order spectroscopic methods.

(V) Limit of quantification

The lowest quantifiable concentration of EZE was found to be 0.915, 0.6076 and 0.3672 µg/ml, respectively for zero, First and second order spectroscopic methods.

7.1.1.1.4. Estimation of EZE in marketed Tablet:

Preparation of test solution:

Two brands of EZE, EZETIB of Unisearch and ZETICA Delta of Torrent pharm, were analyzed by the proposed method. 20 tablets were triturated after taking their average weight. The tablet powder equivalent to one tablet was transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution was sonicated for 10 min and supernatant was filtered through Whatman filter paper no.41. 1 ml from this solution were transferred to 10 ml volumetric flasks and diluted with methanol to get 10 µg/ml concentration. The absorbance of this solution was measured at 231.20 nm and concentration of sample solution was found from regression equation computed from calibration curve of EZE. Experiment was repeated five times and mean was found out. The results of amount found as per labeled claim are reported in Table

7.1.1.1.6.The zero order spectra recorded for these solutions were converted into 1st and 2nd derivative form and the amplitude difference was measured at selected λ_{max} and λ_{min}. The percentage of EZE was found from the regression equation computed form calibration curve of EZE. The result of analysis was reported in Table 7.1.1.1.6.

Table 7.1.1.1.6: Estimation of EZE in Tablet by Spectrophotometric methods

Tablet Formu-lation	Labeled Claim (mg/ Tablet)	Simple UV Spectrophotometric method		1st derivative Spectrophotometric method	2nd derivative Spectrophotometric method		
		Amount Found* (mg/Tablet)	% Assay ± S.D	Amount Found* (mg/Tablet)	% Assay ± S.D	Amount Found* (mg/Tablet)	% Assay± S.D
EZETIB, Unisearch	10	10.05	100.53 ± 0.46	10.38	103.84 ± 0.95	9.976	99.76 ± 0.55
ZETICA, Delta (torrent)	10	10.03	100.25 ± 0.21	10.21	102.11 ± 0.13	10.35	103.58 ± 0.33

* Mean of five determinations

7.1.1.1.5. Summary of Validation parameters:

The summary of the validation parameters is shown in Table 7.1.1.1.7

Table 7.1.1.1.7: Summary of Validation parameters of Spectrophotometric methods

Sr. No	Parameters	Simple UV Spectrophotometric method	1st derivative Spectrophotometric method	2nd derivative Spectrophotometric method
1	λ max (nm)	231.20	223.80	267.60
	λ min (nm)	-	236.80	273.20
2	A (1%, 1cm) (dl gm^{-1} cm^{-1})	502.60	-	-
3	Molar Absoptivity (ε) (L mol^{-1} cm^{-1})	20576.55	-	-
4	Linearity range (µg/ml)	1 -40		
5	Regression equation	Abs. = 0.0563 × Conc. - 0.0475	Abs. = 0.0034 × Conc. - 0.0009	Abs. = 0.001 × Conc. + 0.00002
6	Correlation coefficient (r^2)	0.9996	0.9997	0.9991
7	Intercept	-0.0475	0.0009	0.00002
8	Slope	0.0563	0.0034	0.001
9	Sandell's sensitivity (µg/cm^2/0.001 abs. unit))	0.09948	-	-

10	Assay (%)	100.25 – 100.53	102.11 – 103.84	99.76 – 103.58
11	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements % CV % Recovery	0.10 -0.49 %. 0.32 -0.84 %. 0.16 % 100.00 – 100.02%	0.27-0.59 %. 0.42-0.92 %. 0.79 % 99.5 – 10.43 %	0.95-1.15 %. 0.92-1.15 % 0.88 % 98.97 –101.06 %
12	Limit of detection	0.3454 µg/ml	0.1822 µg/ml	0.101 µg/ml
13	Limit of quantification	0.915 µg/ml	0.6076 µg/ml	0.3672 µg/ml

7.1.1.1.6. Conclusion:

Three simple, accurate and economic methods were developed for the estimation of EZE in pharmaceutical oral dosage form. As summary Table shows method can work in the range of 1 – 40 µg/ml with accuracy of 100.006 – 100.027 % with reproducibility of 0.16 %. The First and second derivative spectroscopic methods were developed for the estimation of EZE in oral dosage form with the recovery of 99.5 – 10.43 % and 98.97 –101.06 %, respectively. Derivative method was developed because it is a useful tool for the estimation of drug in presence of excipients.

7.1.1.2. Estimation of EZE by Difference spectroscopy method.

Experimental work:

7.1.1.2.1. Preparation of standard stock solution:

Procedure for preparation of stock solution was same as per section 7.1.1.1.1.

7.1.1.2.2. Analytical wavelength:

The essential feature of a difference spectrophotometry is that the measured value, the difference in absorbance (A) between two equimolar solutions of the analyte in different chemical forms, which exhibits different spectral characteristics. Here chemical forms selected were 0.1 N NaOH and methanol. Alkaline solution was kept in sample compartment and methanolic solution of EZE was placed in reference compartment. The difference spectrum was obtained by scanning between 220 – 275 nm and the maximum absorbance was measured at 243.80 nm.

7.1.1.2.3. Calibration curve:

From the stock solution (100 µg/ml) aliquots of 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 ml were transferred in duplicate to a series of 10ml volumetric flasks. The volume was adjusted to a mark with methanol and 0.1 M NaOH to get 1, 5, 10, 15, 20, 25, 30, 35

and 40 µg/ml of solution in methanol and NaOH. The absorbance was measured at (λ_{max}) 243.80 nm using methanolic solutions in the reference compartment and 0.1 M NaOH solutions in the sample compartment.

7.1.1.2.4. Validation of analytical method

(I) Linearity:

The absorbance of standard EZE solutions were measured at 243.80 nm using methanol as a blank over the concentration range of 1-40 µg/ml and calibration curve was plotted against concentration and regression equation was calculated. Result should be expressed in terms of Correlation co-efficient.

(II) Precision

a. Repeatability (Intraday and Interday Precision):

The intraday and interday precisions of the proposed methods were determined by estimating the corresponding responses 5 times on the same day and on 5 different day for 10, 20 and 30 µg/ml, which was prepared by transferring 1,2 and 3 ml of stock solution in series of two 10 ml volumetric flask and made up to the volume by methanol and NaOH. The absorbance was taken as proposed method. The results are reported in terms of percentage coefficient of variation (%C.V).

b. Reproducibility:

The reproducibility of the method was checked by measuring absorbance of 10, 15 and 20 µg/ml of solution in UV 1700 and UV 1601 for three times. The % CV of the responses for determination of drug was found which reveals the reproducibility of the method.

(III) Accuracy

The accuracy of the methods was determined by recoveries by the standard addition method. For this, previously analyzed 10 µg/ml of drug solution from pharmaceutical formulations were spiked at three levels 50, 100 and 150 % from standard stock solution in series of two 10 ml volumetric flask and made up to the volume with analytical grade methanol and NaOH and analyzed. The final concentration was found form regression equation.

(IV) Limit of Detection and Quantification

Equation based on standard deviation of the response and the slope is given in Chapter 5, section 5.3.1.6 and 5.3.1.7.

7.1.1.2.5. Results and Discussion:

7.1.1.2.5.1 Validation Parameters

(I) Linearity (Calibration curve for standard EZE):

Linearity range of EZE was found to be 1-40 µg/ml with correlation co-efficient 0.9995 and % C.V. ranging from 0.028 – 0.76 for different concentration.

Table 7.1.1.2.1: Calibration data of EZE by difference UV Spectroscopy

Sr.No.	Concentration (µg/ml)	Absorbance Mean ± S.D. (N=5)	% C.V.
1	1	0.030 ± 0.00446	0.14852
2	5	0.045 ± 0.0347	0.76733
3	10	0.060 ± 0.00348	0.05803
4	15	0.078 ± 0.0261	0.33316
5	20	0.094 ± 0.00882	0.09412
6	25	0.109 ± 0.0751	0.68721
7	30	0.124 ± 0.00348	0.02808
8	35	0.142 ± 0.00525	0.037
9	40	0.158 ± 0.000471	0.0298

* Mean of five determinations

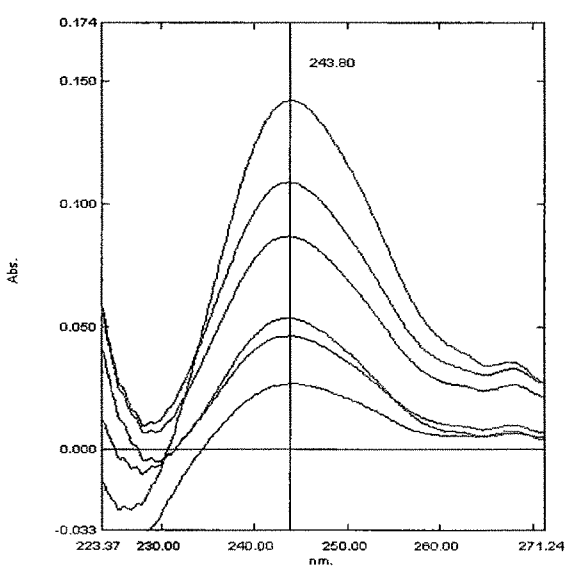


Figure: 7.1.1.2.1 UV spectra of EZE by Difference Spectroscopy

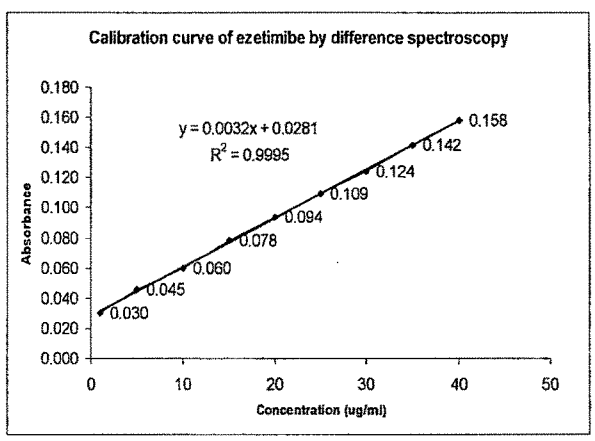


Figure 7.1.1.2.2: Calibration curve of EZE by Difference Spectroscopy
Correlation co-efficient = **0.9995**
Slope = 0.0032
Intercept = 0.028
Regression equation: **Abs. = 0.032 × Conc. + 0.0281**

(II) Precision

a. Repeatability

Intraday and interday variation of the proposed method were 0.12 -0.90 % and 0.32 - 0.98 %.

Table 7.1.1.2.2: Intraday and Interday precision data of EZE by difference spectroscopy

Concentration (µg/ml)	Intraday		Interday	
	Absorbance (Mean* ± S.D.) (n=5)	% C.V.	Absorbance (Mean* ± S.D.) (n=5)	% C.V.
10	0.060 ± 0.0045	0.78	0.059 ± 0.0033	0.45
20	0.094 ± 0.0087	0.90	0.093 ± 0.0043	0.98
30	0.123 ± 0.023	0.12	0.125 ± 0.00899	0.32

* Mean of five determinations

b. Reproducibility:

The reproducibility of the method was determined by using Shimadzu UV 1700 and Shimadzu UV 1601. As per the instrument specification the % C.V. of repeated measurement of the same solution should not more than 1%. Here the % C.V. was found to 0.37(<1%).

Table 7.1.1.2.4: Reproducibility data of EZE method

Concentration (µg/ml)	Absorption*		% CV	
	UV 1700	UV 1601	UV 1700	UV 1601
10	0.061	0.059	0.93	0.73
15	0.079	0.077	0.69	0.28
20	0.094	0.092	0.22	0.88

*** Mean of three determinations**

(III) Accuracy

Accuracy of the measurement of EZE was determined by standard addition and was found to be in the range of 98.8 - 104 %.

Table 7.1.1.2.5: Accuracy data of EZE by Spectrophotometry

Initial conc. (µg/ml) (A)	Quantity of std. Added (µg/ml) (B)	Total Amount (A + B)	Total quantity Found Mean ± S.D.	% Recovery± S.D
10	5	15	14.82 ± 0.0516	98.8 ± 0.535
10	10	20	19.99 ± 0.0497	99.95 ± 0.6387
10	15	25	26 ± 0.0533	104 ± 0.7275

* Mean of five determinations

(IV) Limit of detection

The minimum detectable concentration of EZE was found to be 0.0883 µg/ml.

(V) Limit of quantification

The lowest quantifiable concentration of EZE was found to be 0.194 µg/ml by practical observation.

7.1.1.2.5.2. Estimation of EZE in marketed Tablet:

Preparation of test solution:

The table test solution was prepared as described under section 7.1.1.1.4. 1 ml of test solution was transfer in two 10 ml volumetric flask and made up to 10 ml with methanol and NaOH. The absorbance of this solution was determined using the proposed method at 243.80 nm and concentration of sample solution was found from regression equation obtained form calibration curve of EZE.

Table 7.1.1.2.6: Estimation of EZE in Tablet by Spectrophotometry

Tablet Formulation	Labeled Claim (mg/Tablet)	Amount found (mg/Tablet)	% Recovery ± S.D
EZETIB, Unisearch	10	10.16	101.64 ± 0.32
ZETICA, Delta (torrent)	10	9.982	99.82 ± 0.87

* Mean of five determinations

7.1.1.2.6. Summary of Validation parameters:

The summary of validation parameters is reported in Table 7.1.1.2.7.

Table 7.1.1.2.7: Summary of Validation parameters of Difference Spectroscopy

Sr. No	Parameters	Results
1	λ max (nm)	243.80
2	A (1%, 1cm) (dl gm^{-1} cm^{-1})	79.33
3	Molar Absoptivity (ε) (L mol^{-1} cm^{-1})	3247.92
4	Linearity range (µg/ml)	1 – 40
5	Regression equation	Abs. = 0.0032 × Conc. + 0.0281
6	Correlation coefficient (r^2)	0.9995
7	Intercept	0.0281
8	Slope	0.0032
9	Sandell's sensitivity (µg/cm^2/0.001 abs. unit)	0.63025

10	Assay	99.82 – 101.64 %
11	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements % CV Accuracy (% Recovery)	 0.12 -0.90 %. 0.32 -0.98 %. 0.37 % (<1%) 98.8 – 104 %
12	Limit of detection	0.0883 µg/ml
13	Limit of quantification	0.194 µg/ml

7.1.1.2.7. Conclusion:

Here with use of difference spectroscopic method EZE can be estimated form pharmaceutical dosage form without interference form any excipient with the accuracy of 100.24 %

7.1.1.3. Quantitative estimation of EZE by FT-Infra Red spectroscopic method.

The aim of the present study was to use FT-IR spectroscopy, to investigate the possibility to quantify EZE in pharmaceutical preparation, such as EZEDOC® 10 mg Tablets. The main objective of this work was to develop a chemometric procedure for the fast and accurate determination of EZE using MLR approaches, reducing the sample pre-treatment and providing direct FT-IR measurement. Diffuse reflectance infrared Fourier transform spectroscopy (DRIFT) spectra was scannned between 4000 and 400 cm^{-1}, by averaging 16 scans for each spectrum with a resolution of 4 cm^{-1} (data point resolution per interval 1 cm^{-1}). 1780 to 1640 cm^{-1} and 3275 cm^{-1} was selected for measurement of peak area and peak height at single wave length, respectively. Calibration curves were drawn using the multiple linear regression (MLR) method and the drug amount in sample was computed by QUANT function of FTIR solution software.

7.1.1.3.1. Preparation of standards for EZE

For quantitative FTIR spectroscopic study standard EZE (10 mg) was weighed accurately and made up to 100 mg by adding pure, anhydrous potassium bromide onto the same butter paper to obtain final concentration of 100 µg/mg. This sample was triturated in mortar and pestle and sample was used as working standard sample for FTIR spectroscopic method.

7.1.1.3.2. Calibration curve for EZE

Appropriate fractions were transferred from standard (100 µg/mg) to butter paper and weight adjusted 10 mg with KBr to get minimum transmission. Peak area and peak height was measured at selected wavenumber. Calibration curves were drawn using the multiple linear regression (MLR) method and the drug amount in sample was computed from regression equation.

7.1.1.3.3. Validation Parameters

(I) Linearity:

Peak area and peak height of standard EZE solutions were measured at selected wavenumber using KBR as a blank over the concentration range of 10-150 µg/mg and peak area and height was plotted against concentration and regression equation was calculated. Results are expressed in terms of Correlation co-efficient.

(II) Precision

a. Repeatability (Intraday and Interday Precision):

2, 6 and 1.2 mg of stock sample (100 µg/mg) was transferred to butter paper and mixed up to 10 mg with KBr to get concentration in 20, 60 and 120 µg/mg and DRIFTS spectra were recorded. Intraday precision was determined by analyzing sample five times in the same day. Interday precision was determined by analyzing sample daily for five days and % C.V. was calculated.

b. Reproducibility:

To study the reproducibility of FTIR method two sample compartments having different diameters were used. Compartment of higher diameter was termed as compartment number I and the smaller diameter was termed as compartment number II. Same sample was put in both the compartments and obtained peak area was compared. % CV was reported for average peak area and compared reproducibility.

(III) Accuracy

Accuracy of the method was determined by performing recovery study of pharmaceutical formulation by standard addition method. For this, 30 µg/mg of drug sample form pharmaceutical formulations were spiked at three levels 50, 100 and 150 % from standard stock. The final concentration was found form regression equation.

(IV) Limit of Detection and Quantification

Equation based on standard deviation of the response and the slope is given in Chapter 5, section 5.3.1.6 & 5.3.1.7.

7.1.1.3.3. Results and Discussion:

7.1.1.3.2.1 Validation Parameters

(I) Linearity

Linearity range of EZE in FTIR methanol was found to be 11.00 - 145 µg/mg.

Table 7.1.1.3.1: Calibration data of EZE by Infrared Spectroscopy

Sr. No.	Concentration (µg/mg)	Peak Area	%CV	Peak height	%CV
1	11	0.25531	0.34	0.000223	0.26
2	34	16.5774	0.45	0.196419	0.37
3	41	18.2735	0.24	0.216549	0.11
4	147	68.0076	0.94	1.087013	0.28

* Mean of five determinations

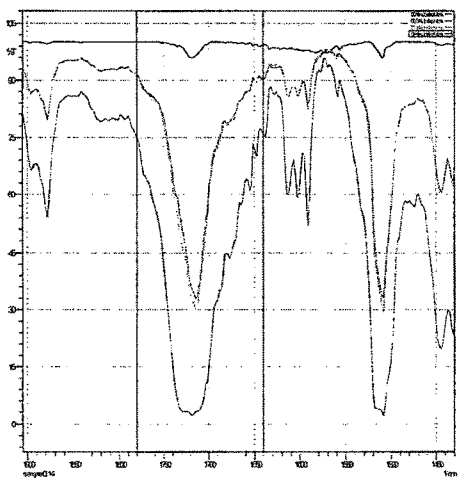


Figure: 7.1.1.3.1 FTIR spectra of EZE by Peak Area

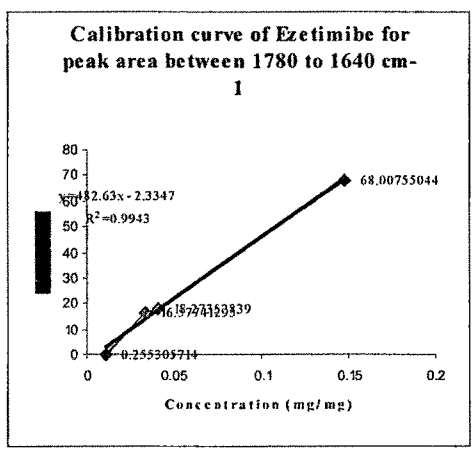


Figure 7.1.1.3.3: Calibration curve of EZE by peak area

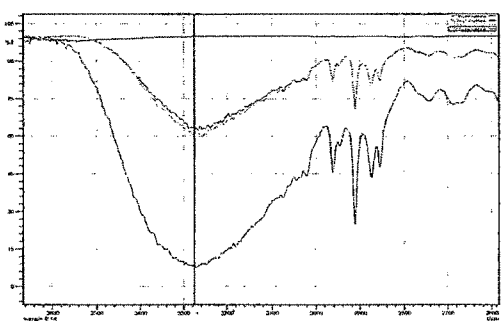


Figure: 7.1.1.3.2: FTIR spectra of EZE by Single Wavelength Number

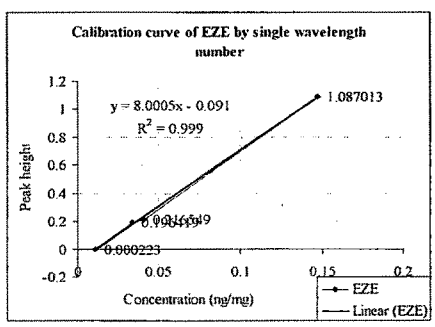


Figure 7.1.1.3.4: Calibration curve of EZE by single wavelength number

(II) Precision

a. Repeatability

Intraday and interday precision are reported in table 7.1.1.3.2.

Table 7.1.1.3.2: Intraday and Interday precision data of EZE by FTIR Spectroscopy

Concentration (µg/mg)	Intraday		Interday	
	Peak Area* ±%C.V.	Peak Height* ±%C.V.	Peak Area* ±%C.V.	Peak Height* ±%C.V.
20	0.46419±0.36	0.00041±0.93	1.46114±0.29	0.00128±0.99
60	29.9258±0.97	0.0829±0.46	29.228±0.65	0.03824±0.18
120	48.8515±0.29	0.524343±0.84	48.586±0.29	0.5243±0.37

* Mean of five determinations

b. Reproducibility:

Observing peak area and height in both the sample compartment checked reproducibility. Table 7.1.1.3.4 shows that %CV value was not more then 1%. So method was reproducible.

Table 7.1.1.3.3: Reproducibility data of EZE by FTIR Spectroscopy (15 µg/mg)

Sample compartment	Peak Area	Peak height
Compartment no I	0.34815	0.000304
	0.34760	0.000306
Compartment no II	0.34920	0.000309
	0.34620	0.000300
S.D.	0.00108	3.57E-06
%C.V. (<1%)	0.31108	1.16857

* Mean of three determinations

(III) Accuracy

Accuracy of the measurement of EZE was determined by standard addition method.

Table 7.1.1.3.5: Accuracy data of EZE by FTIR Spectroscopy

Quantitative FTIR		EZE		
Initial conc.(µg/mg) (A)		30	30	30
Quantity of std. Added (µg/mg) (B)		15	30	45
Total Amount (A + B)		45	60	75
Peak area (Mean (n=5) ± %C.V.)	Total quantity Found Mean ± S.D.	44.45 ± 0.34	60.03 ± 0.21	74.86 ± 0.32
	% Recovery± S.D	98.77 ± 0.78	100.05 ± 0.87	99.81 ± 0.33
Peak height (Mean (n=5) ± %C.V.)	Total quantity Found Mean ± S.D.	44.69 ± 0.24	60.09 ± 0.34	75.01 ± 0.72
	% Recovery± S.D	99.31 ± 0.11	100.15 ± 0.89	100.01 ± 0.33

* Mean of five determinations

(IV) Limit of detection

The minimum detectable concentration of EZE were found to be 0.0285and 1.4191 µg/mg for peak area and height, respectively.

(V) Limit of quantification

The lowest quantifiable concentration of EZE was found to be 0.0949 and 4.7303 µg/mg by practical observation for peak area and peak height, respectively.

7.1.1.3.2.2. Estimation of EZE in marketed Tablet:

Preparation of test Sample:

EZEDOC from Lupin were analyzed by the proposed method. Twenty tablets were triturated after taking their average weight. The tablet powder equivalent to one tablet was transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution (100 µg/ml) was sonicated for 10 min and supernatant was filtered through whatman filter paper no.41. Filtrate was evaporated and from the residue 1 mg was weighed and made up to 100 mg with KBr on butter paper and triturated in mortar pestle and DRIFT spectra was scanned between 4000 and 400 cm^{-1}, by averaging 16 scans for each spectrum with a resolution of 4 cm^{-1} (data point resolution per interval 1 cm^{-1}). 1780 to 1640 cm^{-1} and 3275 cm^{-1} were selected for measurement of peak area and peak height at single wave length, respectively. Concentration of sample was found from regression equation of calibration curve of EZE.

Table 7.1.1.3.6: Estimation of EZE in Tablet by FTIR Spectroscopy

Formulation	Labeled Claim (mg/tab)	Peak Area		Wavelength Number	
		Amount found*	% Assay	Amount found*	% Assay
EZEDOC (Lupin)	10	10.43	104.32	10.03	100.3

***Average of three determinations.**

7.1.1.3.2 Summary of Validation parameters:

The summary of validation parameters is reported in Table 7.1.1.3.9.

Table 7.1.1.3.7: Summary of Validation parameters of Spectrophotometry

Sr. No	Parameters	Peak area	Peak Height
1	Wavenumber (cm^{-1})	1780 to 1640	3275

2	Linearity range (µg/mg)	11.00 to 145	
3	Regression equation	Abs. = 482.63× Conc. -2.3347	Abs. = 8.0005× Conc. - 0.091
4	Correlation coefficient (r^2)	0.9943	0.999
5	Intercept	-2.3347	-0.091
6	Slope	482.63	8.0005
7	Assay (%)	104.32 %	100.30 %
8	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements %CV % Recovery	 0.29 -0.97% 0.29 – 0.97% 0.31% 98.77 – 100.05	 0.46 – 0.93% 0.18 – 0.99% 1.16% 99.3 – 100.15
9	Limit of detection (µg/mg)	0.0285	1.4191
10	Limit of quantification (µg/mg)	0.0949	4.7303

7.1.1.3.3. Conclusion:

From the above summary it can be concluded that proposed quantitative FTIR spectroscopic method was specific method for estimation of EZE from solid samples without any pretreatment and solvent consumption.

7.1.1.4. Stability Indicating Reverse Phase High Performance Liquid Chromatographic (HPLC) method for estimation of EZE.

Accordingly, the aims of the present study was to establish inherent stability of EZE through stress studies under a variety of ICH recommended test conditions and to develop a stability-indicating assay for an oral Tablet containing 10 mg drug.

Chromatographic conditions were established during the initial studies to get the chromatogram of EZE having the optimum retention time.

7.1.1.4.1. Optimization of method:

The chromatographic method was developed optimizing the following parameters

Determination of solvent for sample preparation:

The solubility of EZE was tested in many solvents (Table 7.1.6.3). Initial HPLC studies were done using EZZ solutions in methanol and ACN, but chromatographic peaks of methanolic solution of EZE showed tailing effects, so ACN was selected as the solvent for EZE.

Determination of analytical wavelength: EZE shows reasonably good response at 248 nm (fig 6.6) in ACN. So this wavelength was selected as detection wavelength in dual channel UV detector.

Determination of mobile phase:

Different combinations of Mobile phase were tried at flow rate 1 ml/min and column C_{18} Phenomenex. The observations are summarized in Table 7.1.1.4.1.

Table 7.1.1.4.1. Determination of mobile phase

Mobile phase combination	Ratio (V/V)	Retention time (min)	Peak shape
Methanol: Water	70:30	6.990	Broad peak and tailing at the base
Methanol: Water	80:20	5.463	Broad peak and tailing at the base
Acetonitrile: Water	70:30	4.512	Sharp peak but tailing at the base
Acetonitrile: 0.1 % Formic acid	60:40	5.317	Sharp peak but tailing at the base
Acetonitrile: 0.1 % Formic acid	70:30	6.754	Sharp peak but tailing at the base
Acetonitrile: 0.1 % Formic acid	40:60	18.654	Sharp peak but tailing at the base
Acetonitrile: 0.2 % Formic acid	60:40	6.865	Sharp peak but tailing at the base
Acetonitrile: 0.5 % Formic acid	**60:40**	**6.142**	**Sharp peak**
Acetonitrile: 0.5 % Formic acid	65:35	4.47	Sharp peak
Acetonitrile: 0.5 % Formic acid	80:20	2.25	Sharp peak

It is evident that mobile phase combination of acetonitrile: 0.5 % formic acid in proportion of 60:40 was most suitable for the development of RP-HPLC method. Further work was done using this mobile phase.

Determination of Flow rate

The effect of flow rate on the retention time of EZE was studied. The data is presented in Table 7.1.1.4.2.

Table 7.1.1.4.2. Determination of Flow rate

Flow rate (ml/min)	Retention time (min)
0.5	12.354
1.0	6.142
1.2	4.253

Most suitable Retention time is between 4 to 8 min so the flow rate was selected was 1 ml/min,

Column

Two type of C18 column were tried, to study the effect on retention time and peak shape. The observations are reported in Table 7.1.1.4.3.

Table 7.1.1.4.3. Selection of column

Column (C18, 250 X 4.60 mm)	Retention time (min)	Peak Shape
	EZE	EZE
Hypersil	6.245	Sharp peak
Phenomenex	6.142	Sharp peak

So both the column were found to be suitable for development of HPLC analytical method. Here C18 Phenomenex 240 X 4.6 mm was used further. The main aim of the method was to resolve the compounds in presence of degradation products and impurities, Phenomenex C_{18} column (250 mm x 4.6 mm i.d., 5 µm particle size was preferred as it has high carbon loading with very closely packed material to give high resolution.

Method:

Chromatographic condition:

- **Column:** C_{18} (size-250 x 4.60 mm, I.D-5 µm) (Phenomenex)
- **Mobile Phase:** Acetonitrile : 0.5% Formic acid (60:40 v/v)
- **Detection:** UV detection at 248 nm
- **Flow rate:** 1.0 ml/minute
- **Injection volume:** 20 µl

Preparation of Mobile Phase:

Mobile phase was prepared by mixing 600 ml of Acetonitrile with 400 ml of 0.5 % formic acid (60: 40, V/V). The mobile phase was filtered through nylon 0.45 µm, 47 mm membrane filter and degassed in ultrasonic bath prior to use for 30 min.

7.1.1.4.2. Preparation of stock solution for EZE

For chromatographic study standard EZE (100 mg) was weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml ACN properly and diluted up to mark with ACN to obtain final concentration of 1000 µg/ml.

7.1.1.4.3. Validation Parameters

(I) Linearity (Calibration curve for EZE):

From the stock solution (1000 µg/ml), aliquots of 0.1, 0.5, 1, 1.5, 2, 2.5, and 3 ml were transferred to a series of 10 ml volumetric flasks. The volume was adjusted up to the mark with ACN to get final concentrations of 10, 50, 100, 150, 200, 250, and 300 µg/ml of solution. 20 µl of all these solutions were injected separately into HPLC column and the peak area of each solution was measured at selected wavelength. The calibration curve was constructed by plotting peak area vs. concentration (µg/ml) over the concentration range and regression equation was calculated.

(II) Precision

The precision is measure of either the degree of reproducibility or repeatability of analytical method.

Repeatability

For that 0.6, 1.2 and 2.4 ml aliquot of stock solution (1000 µg/ml) was transferred to 10 ml volumetric flask to get 60, 120 and 240 µg/ml concentration. 20 µl of this solution was injected in HPLC column and the peak area was measured at selected wavelength. Intraday precision was determined by analyzing 60, 120 and 240 µg/ml for five times in the same day and % C.V. was calculated. Interday precision was determined by analyzing 60, 120 and 240 µg/ml daily for five days and % C.V. was calculated.

Reproducibility:

The reproducibility of the method was checked by repeatedly injecting (n=3) standrd solution of 150 µg/ml in C18 phenomenex and hypersile column. The result are reported in term of % C.V.

(III) Accuracy

Accuracy of the method was determined by performing recovery study on Tablet test solution by standard addition method. To a 100 µg/ml preanalyzed sample of Table test solution, spiking was done at three levels 50, 100 and 150 % of stock solution, respectively and final concentration was found form regression equation.

(IV) Limit of Detection and Quantification

LOD and LOQ were calculated using the equation given in Chapter 5.

(VI) Specificity : The specificity of the method was ascertained by analyzing standard drugs and sample of EZE in presence of the its degradation products. The peak purity of EZE was assessed by comparing the retention time (T_R) of sample with standard EZE.

(V) System suitability parameters (SSP):

System suitability parameters were obtained from standard solution of drugs that should be uniform through out the study. SSP was checked by software by selecting SSP parameters in analyzed sequence.

7.1.1.4.2. Results and Discussion:

7.1.1.4.2.1. Validation Parameters

(I) Linearity

Linearity range of EZE was found to be 5–300 µg/ml with correlation co-efficient and % CV is 0.9973 and 0.18–0.87 for peak area.

Table 7.1.1.4.4: Calibration data of EZE by HPLC with UV detection

Sr. No.	Concentration (µg/ml)	Peak Area *	%CV	RT*
1	5	1185992	0.25	6.14
2	10	2471980	0.62	6.12
3	50	11758923	0.87	6.03
4	100	23719843	0.29	6.08
5	150	35579765	0.36	6.09
6	200	47439686	0.74	6.09
7	250	59299608	0.65	6.18
8	300	76179711	0.59	6.14

***Average of five readings**

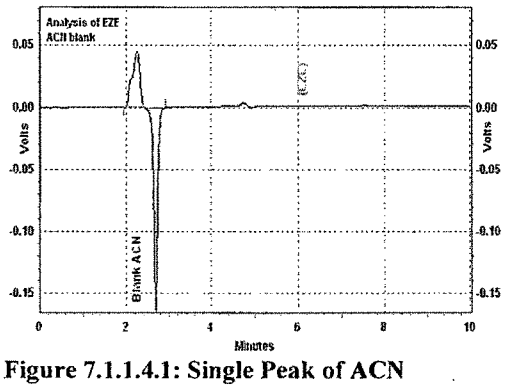


Figure 7.1.1.4.1: Single Peak of ACN Blank by HPLC with UV detection

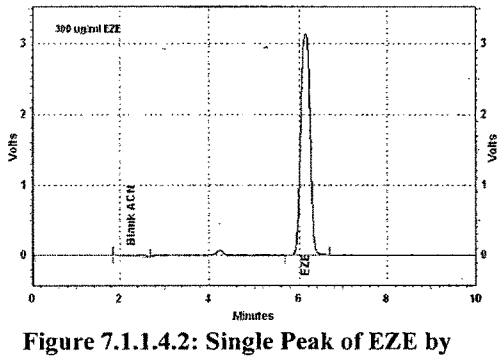


Figure 7.1.1.4.2: Single Peak of EZE by HPLC with UV detection

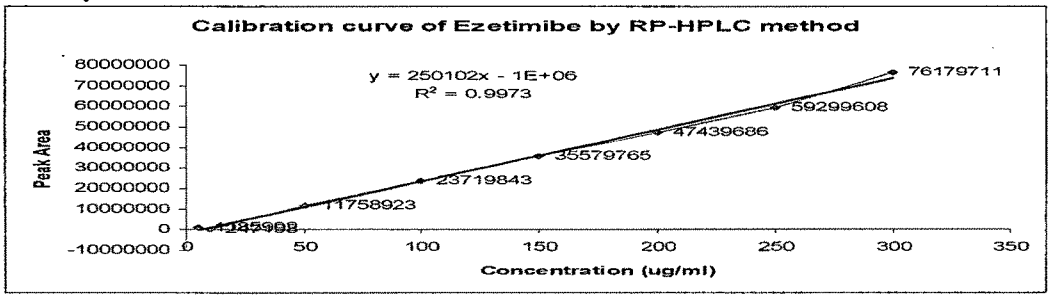


Figure 7.1.1.4.3: Calibration curve of EZE by HPLC (Peak Area)

(II) Precision

Intraday and Interday Precision (Repeatability)

Intraday and interday variation of the proposed method was 0.39-0.73 % and 0.45-0.95 % for peak area.

Table 7.1.1.4.5: Intraday and interday precision data of EZE by HPLC

Sr. No.	Concentration (µg/ml)	Intraday		RT*	Interday		RT*
		Peak area*	%CV		Peak area*	%CV	
1	60	18758923	0.39	6.19	18438725	0.63	6.10
2	120	28463812	0.69	6.19	27995674	0.45	6.18
3	240	56927623	0.73	6.19	56685966	0.95	6.19

***Average of five readings**

b. Reproducibility:

The % C.V. of repeated measurement of the same solution was not more than 1%.

Table 7.1.1.4.6: Repeatability data of EZE by HPLC

C18 Column	Hypersil		Phenomenex	
	Peak Area*	RT	Peak Area*	RT
150 µg/ml	35579765	6.19	35896797	6.15
	35678499	6.12	34784695	6.17
	36748578	6.12	33392235	6.19
%CV	0.36	0.95	0.52	0.89

* Mean of three determinations

(III) Accuracy

Accuracy of the measurement of EZE was determined by standard addition and was found to be in the range of 99.87-100.12 % for peak area.

Table 7.1.1.4.7: Accuracy data of EZE by HPLC with UV detection

Initial conc. (µg/ml) (A)	Quantity of std. Added (µg/ml) (B)	Total Amount (A + B)	Peak Area	
			Total quantity Found* ± S.D.	%Recovery ± S.D
100	50	150	150.51±0.65	100.12±0.49
100	100	200	199.87±0.92	99.97±1.25
100	150	250	249.23±0.58	99.87±0.32

* Mean of five determinations

(IV) Limit of detection

The minimum detectable concentration of EZE was found to be 0.03157 µg/ml for peak area.

(V) Limit of quantification

The lowest quantifiable concentration of EZE was found to be 0.1052 µg/ml for peak area.

7.1.1.4.2.2. Applicability of the method:

(A) Analysis of Tablet formulation

Two brands of EZE, EZETIB of Unisearch and EZEDOC of Lupin were analyzed by the proposed method. Twenty tablets were accurately weighed and triturated to fine powder then the tablet powder equivalent to one Tablet was transferred into a 100 ml volumetric flask containing 50 ml ACN, sonicated for 15 min and further diluted to 100 ml with ACN. The resulting solution was sonicated for 10 min and supernatant was filtered through Whatman filter paper no.41. 20 µl of filtrate was injected in HPLC column and peak area was measured at selected wavelength and concentration of sample solution was found from regression equation computed from calibration curve of EZE. The result of amount found as per labeled claim was reported in Table 7.1.1.4.8.

Table 7.1.1.4.8: Estimation of EZE in Tablet by HPLC with UV detection

Tablet Formulation	Labeled Claim (mg/Tablet)	Amount Found* (mg/Tablet)	% Assay ± S.D
EZEDOC (Lupin)	10	10.22	102.24 ± 0.95
EZETIB (Unisearch)	10	09.96	99.61 ± 0.13

* Mean of five determinations

(B) Analysis of degradation products

Procedure for forced degradation study

Forced degradation study was done in different conditions. Solvents tried were 30% H_2O_2 at room temperature, water at neutral pH 7 at reflux condition, for acidic condition 0.1 N HCl for reflux and room temperature and 1 N HCl for reflux condition and in alkaline condition 0.1 N NaOH room temperature and reflux and 1 N NaOH for reflux condition. Approximately 25 mg drug was weighed accurately and transferred to 25 ml volumetric flask. The drug was dissolved in 5 ml of ACN then diluted with the solvent selected for degradation. Similarly solid-state stability study

was done by exposing 25 mg of drug to 80°C in a stability oven and 25 mg drug to a photo stability chamber. Samples were collected for analysis at two stages, at 0 min (as soon as sample was prepared), after 24 hrs and after 48 hrs of exposure to degradation condition for room temperature and for sample under reflux condition aliquots were withdrawn after 0, 10, 30 min to 1 hr interval up to 5 hrs. Samples were prepared by taking 2 ml of degraded solution in 10 ml volumetric flask and made up to 10 ml with ACN (200ug/ml) and 20µl of that was injected in HPLC column. Figs 7.1.1.4.4 to 7.1.1.4.9 show the chromatograms of forced degraded samples.

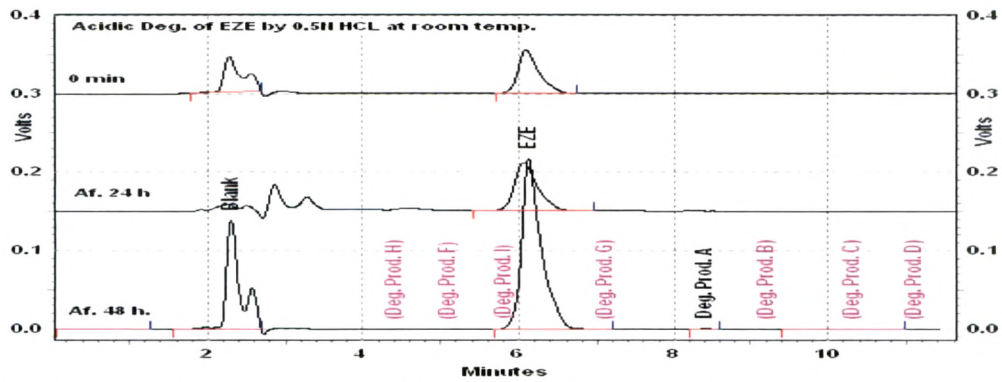


Figure 7.1.1.4.4(A): Chromatograms of EZE in 0.5 N HCL at 0 min, after 24 hrs and after 48 hrs at room temperature

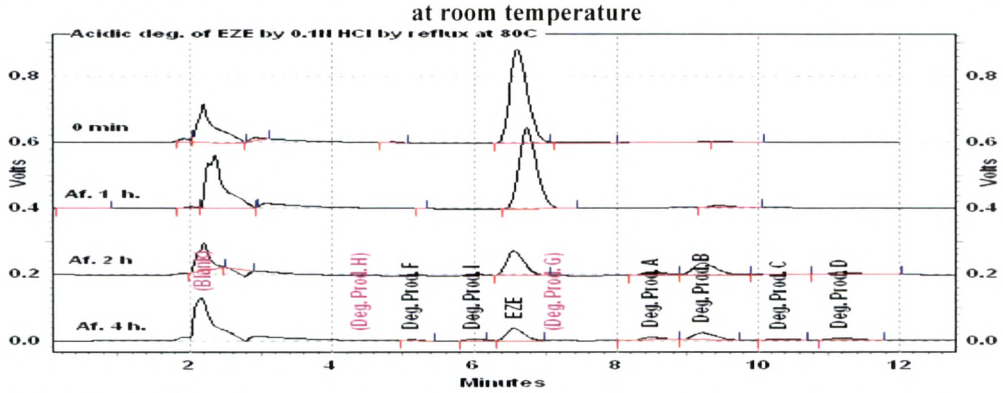


Figure 7.1.1.4.4(B): Chromatograms of EZE in 0.1 N HCL at 0 min, after 1 hrs , after 2 hrs and after 4 hrs at reflux at 80 C

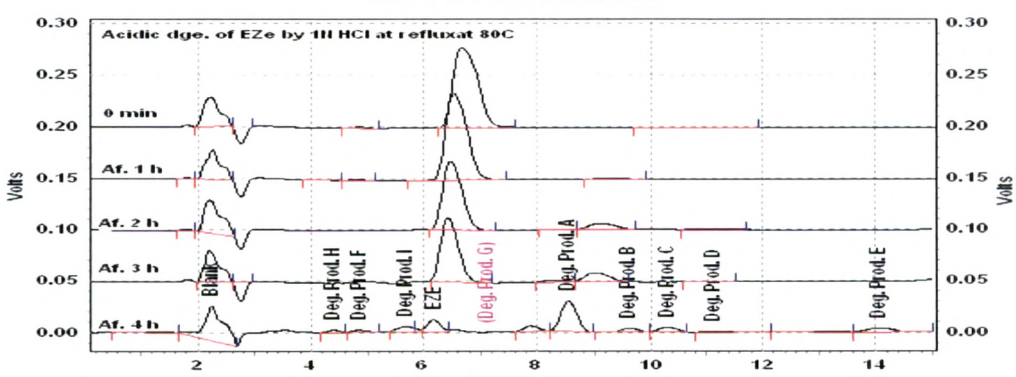


Figure 7.1.1.4.4(C): Chromatograms of EZE in 1 N HCL at 0 min, after 1 hrs , 2 hrs and 3 hrs. after 4 hrs at reflux at 80 C

Figure 7.1.1.4.4: Chromatograms of acid hydrolysis- degraded EZE

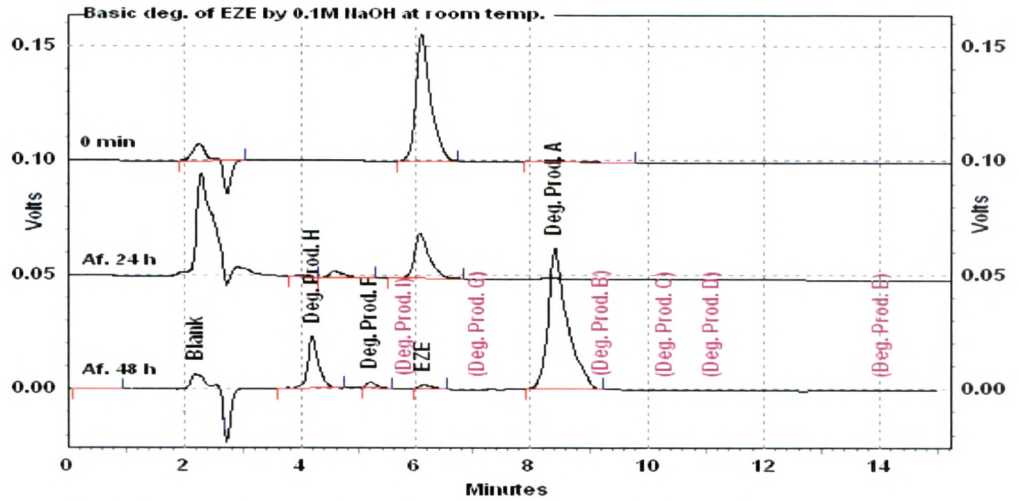


Figure 7.1.1.4.5(A): Chromatograms of EZE in 0.1N NaOH at 0 min, after 24 hr and after 48 hrs at room temperature.

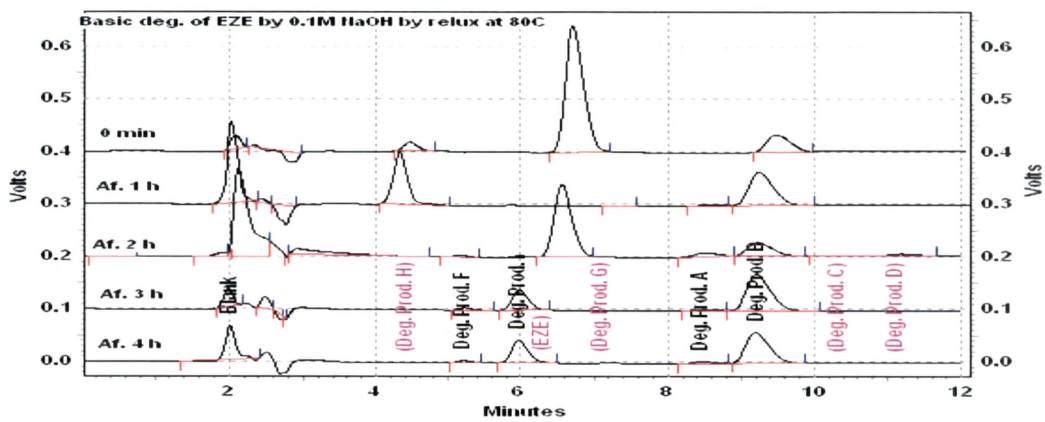


Figure 7.1.1.4.5(B): Chromatograms of EZE in 0.1N NaOH at 0 min, after 1 hr, 2 hr, 3 hr and after 4 hrs at reflux at 80 C.

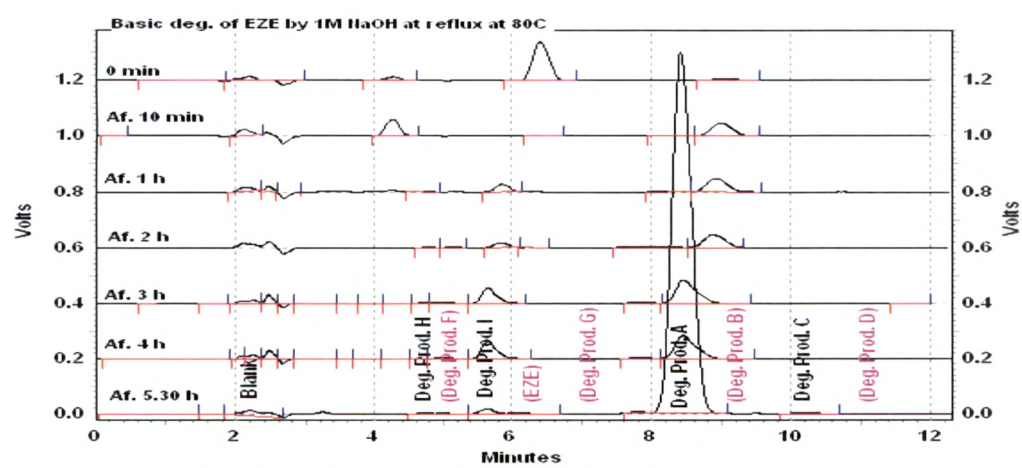


Figure 7.1.1.4.5(C): Chromatograms of EZE in 1N NaOH at 0 min, 10 min after 1 hr, 2 hr, 3 hr,4 hr and after 5.30 hrs reflux at 80 C

Figure 7.1.1.4.5: Chromatograms of base hydrolysis-degraded EZE

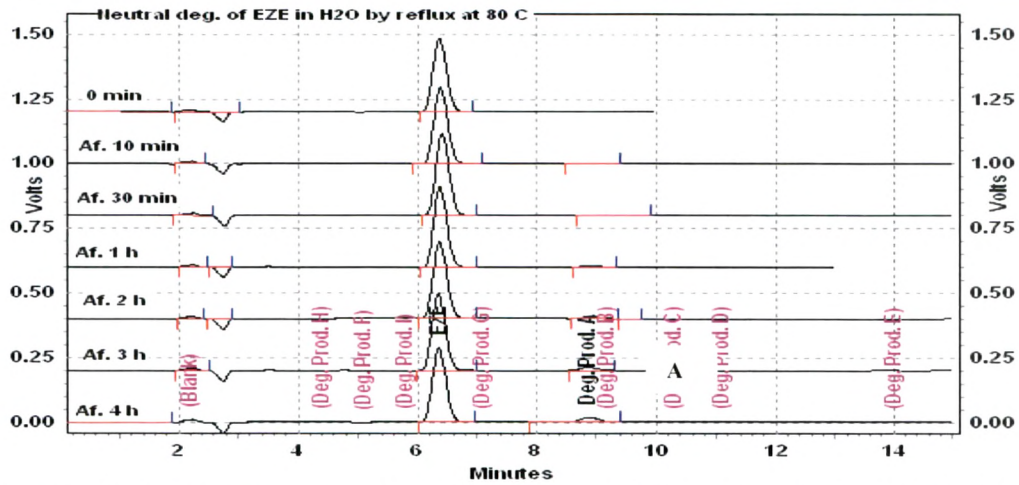


Figure 7.1.1.4.6: Chromatograms of 100 ug/ml EZE in Neutral condition standard , 0 min, 10 min, 30 min, 60 min, 120 min, 180 min and 240 min (4hr) reflux at 80 C

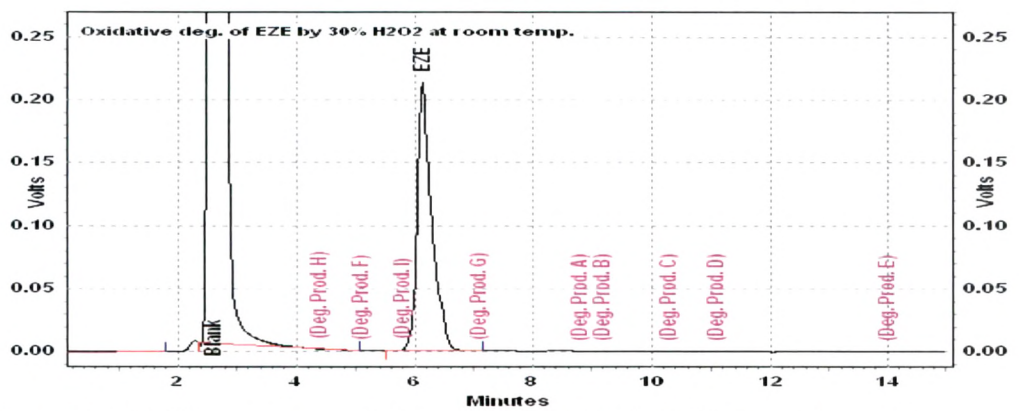


Figure 7.1.1.4.7: Chromatograms of 100 ug/ml EZE in 30 % H2O2 after 48 hrs at room temperature

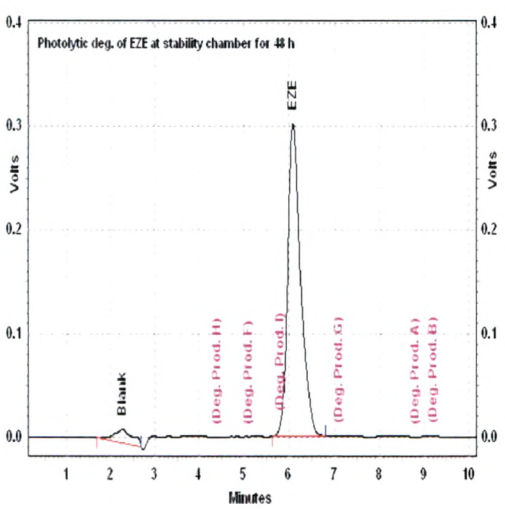


Figure 7.1.1.4.8: Chromatograms of EZE in UV/vis after 48 hrs in photostability chamber

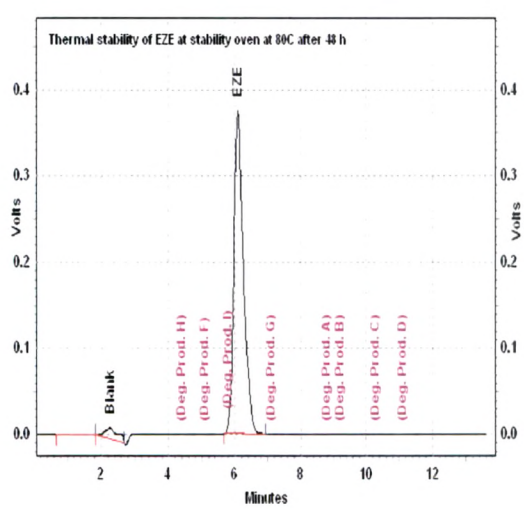


Figure 7.1.1.4.9: Chromatograms of EZE in thermal 80C after 48 hrs in Stability oven

Table 7.1.1.4.9: Percentage degradation of EZE by force degradation.

Sr. No	Parameters (Stress condition /duration/state)	% of undergrad EZE	% of individual Degradation products									Total % Deg.
			A	B	C	D	E	F	G	H	I	
1	Neutral/H2Oat pH 7/4 h/ sol./Ref.	93	7	-	-	-	-	-	-	-	-	7
2	Acidic/0.1 N HCl/4 h/ sol./Ref.	42	11	33	4	11	10	-	-	-	-	**58**
	Acidic/1 N HCl/4 h/ sol./Ref.	7	7	58	8	9	10	14	-	-	-	**93**
	Acidic/0.5 N HCl/48 h/ sol./RT	99	1	-	-	-	-	-	-	-	-	**1**
3	Alkali/0.1N NaOH/4 h/ sol./Ref.	33	3	62	-	-	-	2	-	-	-	**67**
	Alkali/1N NaOH/4 h/ sol./Ref.	-	70	-	-	-	-	2	10	1	18	**100**
	Alkali/0.1N NaOH/48 h/ sol./RT	2	80	-	-	-	-	2	16	-	-	**98**
4	Oxidative/30% H2O2/48 h/ sol., Thermal/80 C/48 h/solid, Photo/uv254 and Vis/366 nm/48 h/solid	100	No degradation found									

Table 7.1.1.4.10: System suitability parameters of EZE.

Sr. No	System Suitability Parameters	EZE	Degradation products								
			A	B	C	D	E	F	G	H	I
1	Retention time (minutes)	**6.145**	**8.883**	**9.208**	**10.308**	**11.158**	**14.025**	**5.125**	**7.158**	**4.467**	**5.83**
2	Theoretical plates	11055.41	7568.362	24823.43	52452.84	33316.52	31304.84	10347.49	10105.12	13609.81	28957.86
3	Resolution	**1.094**	**4.98**	**1.02**	**1.36**	**0.95**	**1.01**	**1.09**	**0.73**	-	**1.77**
4	Asymmetry	0.64	1.09	0.98	1.92	1.03	1.04	0.65	0.08	1.16	1.11
5	Width at ½ peak height	0.87	1.52	0.87	0.67	0.91	1.18	0.75	1.06	0.57	0.51
6	Tailing factor	1.05	1.09	1.05	1.92	1.30	1.08	0.67	0.09	1.14	1.12
7	Capacity Factor	1.90	3.04	3.19	3.69	4.07	5.38	1.14	2.76	1.03	1.77

7.1.1.4.3. Summary of Validation parameters:

The summary of validation parameters is reported in Table 7.1.1.4.11.

Table 7.1.1.4.11: Summary of Validation parameters by HPLC with UV detection

Sr. No	Parameters	HPLC
1	Detection wavelength (nm)	248
2	Retention time (minutes)	6.145
3	Linearity range (µg/ml)	1–300
4	Regression equation	Y = 25775 x conc.+ 954063
5	Correlation coefficient (r^2)	0.9973
6	Intercept	984063
7	Slope	25775
8	Assay	99.61 – 102.24 %
9	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements %CV % Recovery	 0.39-0.73 % 0.45-0.95 % 0.36 (<1%) 99.87 – 100.12 %
10	Limit of detection (µg/ml)	0.03157
11	Limit of quantification (µg/ml)	0.1052

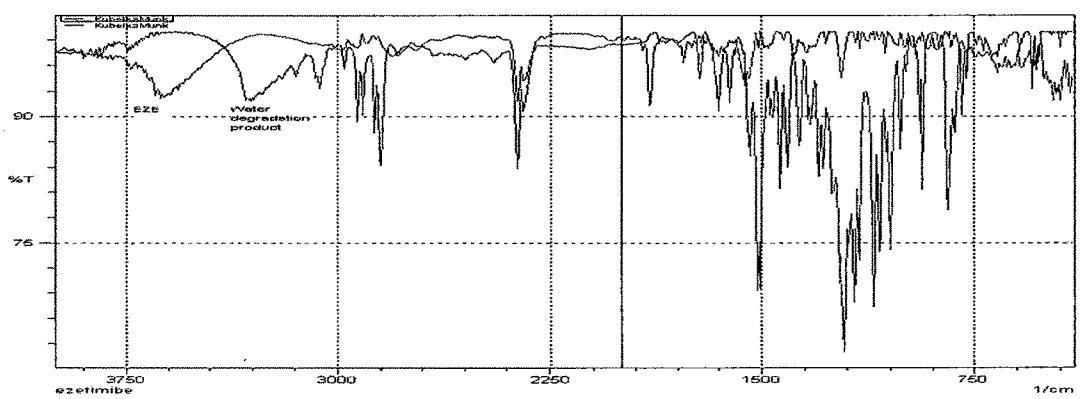


Figure 7.1.1.4.10: Overlain FTIR spectra of water degraded product and EZE standard

Water was evaporated form water degraded product and dissolve in methanol and recrystalized by evaporating methanol at room temperature. The FTIR spectra of degraded product show that peak at 3500 cm-1 is disappear and shift to 3200 cm-1 which indicate degradation at hydroxyl group. Peak at 1600 to 1430 also shift to 3300 to 3400 which indicate tertiary amine converted into secondary amine (fig. 7.1.1.4.10).

7.1.1.4.4. Conclusion:

Singh et al[2] have reported a stability indicating RP-HPLC method for the determination of EZE in presence of its degradation product. Using this methods it was possible to separate EZE and the degradation products generated during forced degradation studies. But the run time is around 90 min so solvent consumption is more and also, method is tedious, so it was attempted to develop a method, which is simple and easy, but with less run time. Developed HPLC method can estimate EZE in pharmaceutical dosage form and also in presence of it degraded products. In acidic condition it was degraded in degraded product A, B, C, D, E and F at RRT 8.883, 9.208,10.308, 11.158, 14.025 and 5.125(fig. 7.1.1.4.4(A) to (C)). The drug was found to be highly labile to alkaline hydrolysis. The reaction in 1 N NaOH at 80°C reflux whole of the drug was degraded (fig. 7.1.1.4.5(C)). Drug degradation was associated with rise in a major degradation product at RRT 8.883 (degradation product A). Upon heating the drug solution in water for 7 hr almost complete degradation of drug was observed with the corresponding rise in the major degradation peak at RRT 8.883 (degradation product A, fig. 7.1.1.4.7). From the degradation study, we can conclude that EZE degraded to one major product that was degradation product A which have RRT 8.883. EZE was stable against thermal and photolytic conditions. The rate of hydrolysis in acid was lower as compare to that of alkali or water. % degradation and system suitability parameters were reported in table 7.1.1.4.9 and 7.1.1.4.10.

It may be pertinent to add here that the main degradation product at RRT 8.883 (Fig. 7.1.1.4.6.(C)) formed almost as a single compound in alkaline and water was isolated and characterized as (*2 *,3 *,6 **)-,6- bis(4-fluorophenyl)-2-(4-hydroxyphenyl)-3,4,5,6-tetrahydro-*2*-pyran-3-carboxamid through crystallographic studies[1].

The method proved to be simple, accurate, precise, specific and selective. Hence it is recommended for analysis of the drug and degradation products in stability samples by the industry.

7.1.1.5. Reverse Phase High Performance Liquid Chromatography (HPLC) method for estimation of EZE in human plasma

HPLC method developed for the estimation of EZE in pharmaceutical formulation was tried and found to be successful for the analysis of EZE in human plasma. The method was repeated with plasma spiked EZE samples and analyzed by protein precipitation method. Human plasma was obtained from blood bank on request.

7.1.1.5.1. Preparation of stock solution for EZE

For chromatographic study standard EZE (10 mg) was weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml ACN and diluted up to mark with ACN to obtain final concentration of 100 µg/ml.

7.1.1.5.2. Calibration curve for standard Drug

From the stock solution (100 µg/ml) aliquots of 0.01, 0.1, 1, 3, 6 and 9 µl were transferred to a series of sample tubes. Then 90 µl of human plasma was added to each tube. The tubes were vortexed for 1 min on Vortex shaker. The volume was adjusted to a 1000 µl with acetonitrile to get final concentrations of 1, 10, 100, 300, 600 and 900 ng/ml of solution. The solutions were centrifuged for 5 min at 4000 rpm. 20 µl of supernatant were injected into HPLC column and the peak area of each solution was measured at selected wavelength.

7.1.1.5.3. Validation Parameters

(I) Linearity:

The calibration curve was constructed by plotting peak area vs. concentration (µg/ml) over the concentration range and regression equation was computed.

(II) Precision

The precision is measure of either the degree of reproducibility or repeatability of analytical method.

a. Repeatability of measurements by HPLC with UV detection:

For that 0.5, 2.5 and 5 ml aliquot of stock solution (100 µg/ml) was transferred to series of sample tubes. Then 90 µl of human plasma was added to each tube. The tubes were vortexed for 1 min on Vortex shaker. The volume was adjusted to a 1000 µl with ACN to get final concentrations of 50, 250 and 500 ng/ml concentration. The solutions were centrifuged for 5 min at 4000 rpm. 20 µl of this solution was injected in HPLC column and the peak area was measured at selected wavelength.

Intraday precision was determined by analyzing five times in the same day and Interday precision was determined for five days and % C.V. was calculated.

b. Reproducibility :

The reproducibility of the method was determined by using two different column (Phenomenex and Hypersil, C18) for taking spectra of same solution with repetition of five times. For that 1.5 µl aliquot of stock solution (100 µg/ml) was transferred to sample tube. Add 90 µl of plasma sample. Vortex for 1 min on vortex shaker. The volume was adjusted to a 1000 µl with ACN to get final concentrations of 150 ng/ml of solution. Centrifuge for 5 min at 4000 rpm. 20 µl of supernatant were injected into HPLC column the peak area was measured at 248 nm for five times and % C.V. was calculated.

(III) Accuracy

Accuracy of the method was determined by performing recovery study on Tablet test solution by standard addition method. To a previously analyzed 120 ng/ml sample of Tablet solution was spiked at three levels (50, 100 and 150 %), respectively and final concentration was found form calibration curve.

(IV) Limit of Detection and Quantification

LOD and LOQ were calculating using the equation given in Chapter 5.

(VI) Stability and matrix effect:

Procedure was followed as per described in Chapter 5, Section 5.4.2 (4). **Sample**: Low concentration 15 ng/ml and high concentration 680 ng/ml.

Acceptance criteria

- The back-calculated concentrations of all LQC and HQC samples were within 85.00 - 115.00% of their nominal concentration.
- 67.00% of QC samples were within above-mentioned criteria at each LQC and HQC levels.
- % Mean change were within ± 15.00%.

7.1.1.5.4. Results and Discussion:

7.1.1.5.4.1. Validation Parameters

(I) Linearity

Linearity range of EZE was found to be 0.1–945 ng/ml with correlation of 0.9973co-efficient and % CV is 0.11 to 0.97 % for peak area.

Table 7.1.1.5.1: Calibration data of EZE by HPLC with UV detection

Sr. No.	Concentration (ng/ml)	Peak Area		RT *
		Mean*	%CV	
1	0.105	179	0.12	6.18
2	10.5	1410	0.23	6.18
3	100.5	13594	0.45	6.13
4	315	42327	0.97	6.18
5	420	56436	0.37	6.09
6	630	84654	0.11	6.09
7	945	116731	046	6.17

***Average of five readings**

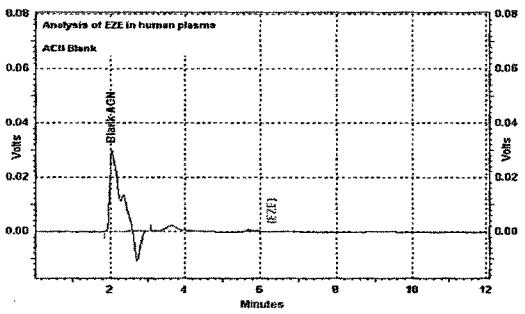


Figure 7.1.1.5.1: Single Peak of ACN blank by HPLC with UV detection

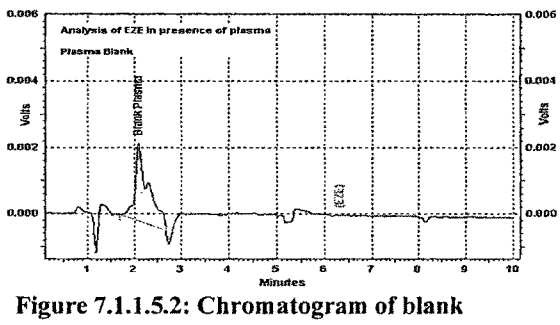


Figure 7.1.1.5.2: Chromatogram of blank plasma by HPLC

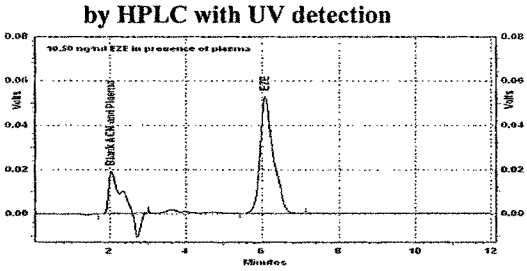


Figure 7.1.1.5.3: Chromatogram of EZE in plasma by HPLC

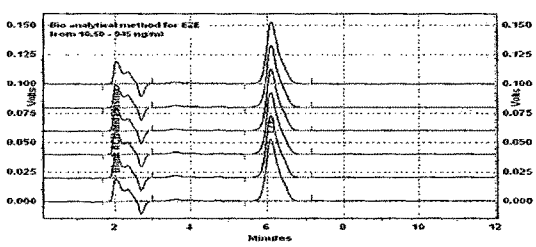


Figure 7.1.1.5.3: Overlain chromatogram of EZE in plasma by HPLC

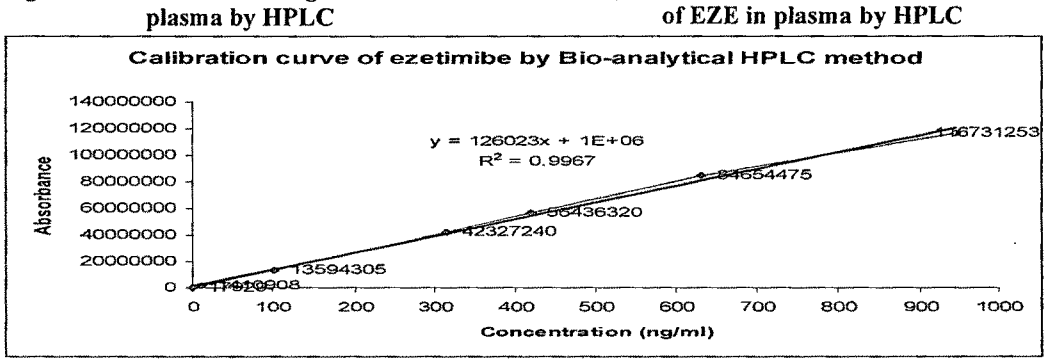


Figure 7.1.1.5.4: Calibration curve of EZE by HPLC (Peak Area)

(II) Precision

Intraday and Interday Precision (Repeatability)

Intraday and interday variation of the proposed method was 0.34-0.93 % and 0.59-0.92 % for peak area.

Table 7.1.1.5.2: Intraday precision data of EZE by HPLC with UV detection

Sr. No.	Concentration (ng/ml)	Intraday		RT*	Interday		RT*
		Peak Area *	%CV		Peak Area *	%CV	
1	50	6275693	0.34	6.17	6183	0.63	6.19
2	250	31753498	0.93	6.18	30881	0.92	6.17
3	500	61957934	0.12	6.18	62384	0.59	6.19

***Average of five readings**

b. Reproducibility:

As per the instrument specification the % C.V. of repeated measurement of the same solution should not more than 1%.

Table 7.1.1.5.3: Reproducibility data of EZE (150 ng/ml) by HPLC

Time	Peak Area*	RT
Phenomenex C18	20155	6.18
	20145	6.17
	19983	6.19
Hypesil C18	19938	6.18
	20179	6.19
	20080	6.082
S.D.	111036.2	0.0083
%CV	0.55 (<1%)	0.13 (<1%)

***Average of five readings**

(III) Accuracy

Accuracy of the measurement of EZE was determined by standard addition and was found to be in the range of 99.34-100.12 % for peak area.

Table 7.1.1.5.4: Accuracy data of EZE by HPLC with UV detection

Initial conc. (ng/ml) (A)	Quantity of std. Added (ng/ml) (B)	Total Amount (A + B)	Peak Area	
			Total quantity Found* ± S.D.	%Recovery ± S.D
120	60	180	180.51±0.34	100.12±0.67
120	120	240	239.48 ± 0.43	99.34 ± 0.19
120	200	320	320.18 ± 0.82	100.02 ± 0.23

***Average of five reading**

(IV) Limit of detection

The minimum detectable concentration of EZE was found to be 0.1218 ng/ml for peak area.

(V) Limit of quantification

The lowest quantifiable concentration of EZE was found to be 0.06040 ng/ml for peak area.

(VI) Stability

1. Freeze and Thaw Stability

Samples were prepared at LQC and HQC levels, aliquoted and frozen. Six samples from each concentration were subjected to three freeze and thaw cycles (stability samples). These samples were processed after 3rd cycle and analyzed along with freshly prepared calibration standards, LQC and HQC samples (comparison samples). Concentrations were calculated to determine % mean change after 3rd cycle. Frozen EZE was found to be stable in LQC and HQC samples after 3rd cycle with % mean change of -6.35 and 2.87 respectively (Table No. 7.1.1.5.5)

Table 7.1.1.5.5: Freeze and thaw stability.

Sr. No.	LQC (15.000 ng/ml)				HQC (680.000 ng/ml)			
	Comparison samples		Stability samples		Comparison samples		Stability samples	
	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.
1	18.545*	123.63*	14.857	99.05	690.920	101.61	723.747	106.43
2	16.130	107.53	13.706	91.37	764.297	112.40	692.907	101.90
3	18.081*	120.54*	13.795	91.97	709.853	104.39	686.339	100.93
4	14.230	94.86	13.579	90.53	630.800	92.76	671.961	98.82
5	14.694	97.96	14.228	94.85	815.155*	119.88*	764.793	112.47
6	15.402	102.68	14.762	98.41	659.203	96.94	713.032	104.86
N	**4**		**6**		5		6	
Mean	15.114		14.154		691.015		708.797	
% Mean Change	-6.35				2.87			
* Not included in the calculation								

2. Short-Term Temperature Stability

LQC and HQC samples were spiked in human plasma and were kept at room temperature for 11.0 hours and were processed and analyzed along with freshly prepared calibration standards, LQC and HQC samples. Concentrations were calculated to determine % mean change during stability period.

EZE was found to be stable in LQC and HQC samples for 11.0 hours at room temperature with % mean change of 3.13 and 3.63 respectively (Table No. 7.1.1.5.6).

Table 7.1.1.5.6: Short term temperature stability

Sr. No.	LQC (15.000 ng/ml)				HQC (680.000 ng/ml)			
	Comparison samples		Stability samples		Comparison samples		Stability samples	
	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.
1	16.030	106.87	15.668	104.45	711.864	104.69	746.871	109.83
2	15.109	100.73	15.054	100.36	740.536	108.90	726.994	106.91
3	15.262	101.75	15.619	104.13	707.448	104.04	696.617	102.44
4	15.580	103.87	16.578	110.52	683.246	100.48	685.800	100.85
5	15.999	106.66	16.487	109.92	672.597	98.91	694.157	102.52
6	14.630	97.53	16.107	107.38	662.434	97.42	776.406	114.18
N	4		6	14.154				
Mean	15.435		15.919		696.354		721.641	
% Mean Change	3.13				3.63			

3. Long-Term Stability

EZE was found to be stable in human plasma at below -20°C after 45 Days in LQC, and HQC samples with % Mean Change of -0.33 and -1.76 respectively, (Table No. 7.1.1.5.7).

Table 7.1.1.5.7: Long term stability

Sr. No.	LQC (15.000 ng/ml)				HQC (680.000 ng/ml)			
	Comparison samples		Stability samples		Comparison samples		Stability samples	
	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.
1	18.852*	125.68*	14.152	114.35	707.754	104.08	704.918	103.66

2	17.064	113.76	16.022	106.82	732.187	107.67	715.365	105.20
3	16.174	107.83	15.376	102.51	727.455	106.98	741.381	109.03
4	16.851	112.34	16.209	108.06	755.293	111.07	716.767	105.41
5	15.635	104.23	16.672	111.15	756.428	111.24	752.746	110.70
6	15.765	105.10	16.030	106.86	750.699	110.40	720.629	105.97
N								
Mean	16.298		16.244		738.303		725.301	
% Mean Change	-0.33				**-1.76**			
* Not included in the calculation								

4. Stock Solution Stability

Stock solution stability was determined by comparing the peak areas of freshly prepared solutions (comparison samples) with stability samples.

EZE stock solution was found to be stable at room temperature for 13 hours with % mean change of 2.41 (Table No. 7.1.1.5.8).

Table 7.1.1.5.8: Stock solution stability

Sr.No.	EZE	
	Comparison samples	**Stability samples**
1	2461424	2634757
2	2617147	2658581
3	2650749	2670768
4	2661869	2723646
5	2648859	2672790
6	2665102	2723604
Mean	2617525	2680691
SD	78336.950	35906.176
%CV	2.99	1.34
Mean % Change	2.41	

(VII) Matrix effect

In order to ensure the effect of matrix through out the application of the method, plasma blanks obtained from two different lots were spiked with EZE at LQC and HQC level. Three quality control samples at each level along with the set of

calibration standards were analyzed and the % nominal concentration of the samples analyzed was represented in (Table No. 7.1.1.5.9) for EZE.

Table 7.1.1.5.9: Matrix effect

Sample ID	Calculated Conc. (ng/µl)	% Nominal Conc.	Sample ID	Calculated Conc. (ng/µl)	% Nominal Conc.
I	LQC	14.989	I	HQC	708.337
	LQC	16.110		HQC	680.493
	LQC	14.231		HQC	685.456
II	LQC	15.232	II	HQC	697.032
	LQC	15.479		HQC	684.288
	LQC	15.314		HQC	694.180

7.1.1.5.1.2. Estimation of EZE in marketed Tablet:

The Tablets were analyzed in presence of plasma by proposed method and the percentage of EZE was found from the calibration curve of EZE. It was found that excipients as well as plasma do not interfere with the method.

The contents of 20 Tablets were weighed and their mean weight determined and finely powdered. An equivalent weight of the are Tablet content was transferred into a 10 ml volumetric flask containing 5 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution was sonicated for 10 min and supernatant was filtered through whatman filter paper no.41. 1 µl from this solution was transferred to sample tube. 90 µl of plasma sample was added add and this was vortexed for 1 min on vortex shaker. The volume was adjusted to 1000 µl with ACN to get final concentrations of 100 ng/ml of solution. This was centrifuged for 5 min at 4000 rpm and 20 µl of supernatant were injected into HPLC column and the Peak area of each solution was measured at selected wavelength. The concentration of sample solution was found from regression equation and result of amount found as per labeled claim was reported in Table 7.1.1.5.11.

Table 7.1.1.5.11: Estimation of EZE in Tablet formulation by HPLC

Tablet Formulation	Labeled Claim (mg/Tablet)	Amount Found* (mg/Tablet)	% Assay ± S.D
EZEDOC (Lupin)	10	9.97	99.73 ± 0.11
EZETIB (Unisearch)	10	09.96	99.61 ± 0.16

*Mean of five determinaitons

7.1.1.5.2. Summary of Validation parameters:

The summary of validation parameters is reported in Table 7.1.1.5.12.

Table 7.1.1.5.12: Summary of Validation parameters by HPLC with UV detection

Sr. No	Parameters	Peak Area
1	Analytical wavelength (nm)	248
2	Retention time (minutes)	6.183
3	Theoretical plates	43,758.54
4	Asymmetry	1.04
5	USP width (at ½ peak height)	0.44
6	Linearity range (ng/ml)	0.1- 945
7	Regression equation	Y = 126023 x conc.+ 1E+6
8	Correlation coefficient (r^2)	0.9967
9	Intercept	126023
10	Slope	1E+6
11	Assay	99.61 – 99.73 %
12	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements %CV % Recovery	 0.34-0.93 % 0.59-0.92 % 0.55 (<1%) 99.34 – 100.12 %
13	Limit of detection (ng/ml)	0.1218
14	Limit of quantification (ng/ml)	0.06040

7.1.1.8.3. Conclusion:

Developed HPLC method could successfully estimate EZE in presence of human plasma. It have LOD 0.0121 ng/ml and LOQ 0.06040 ng/ml. So method is applicable to evaluation of pharmacokinetic profiles of EZE in human volunteers. The method is suitable for routine analysis of EZE in human serum in bioavailability and bioequivalence studies.

7.1.1.6. High Performance Thin Layer Chromatography (HPTLC).

Two HPTLC methods were developed for the estimation of EZE. Method A was developed for the estimation of EZE in oral dosage form showing no interference of excipients in estimation. But this method was not able to separate degradation products of EZE from the EZE standard. So the method B was developed which can estimate EZE in presence of degraded products.

7.1.1.6.1. Optimization of method:

For good chromatographic method it is necessary that mobile phase have good polarity to separate compounds, proper selection of solvent and wavelength therefore is required.

Determination of solvent for sample preparation and λmax:

Different solvents were tried to study the solubility of EZE (Table 6.3). EZE is soluble in methanol so methanol was selected for the preparation of drug solutions. Wavelength was selected by scanning standard solutions of both drugs over 200 nm to 400 nm wavelengths. Drug has λ max at **231 nm** and significant absorbance at 250 nm as illustrated in Fig. 7.1.1.1.1. So these wavelengths were selected in densitometer UV detector.

Determination of mobile phase:

Various combinations of mobile phases were tried for sharp peak of EZE on.silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 μm thickness) (E. Merck). The results are reported in Table 7.1.1.6.1.

Table 7.1.1.6.1: Determination of mobile phase

Mobile phase combination	Ration (V/V/V)	Peak separation
Chloroform: Methanol	8: 2	Broad peak
Chloroform: Methanol	5: 5	Broad peak
Benzene: Methanol	8: 2	Sharp peak
Benzene: Methanol	5: 5	Broad peak
Ethyl acetate : Toluene: Methanol	3: 4: 3	Broad peak
Ethyl acetate: Toluene: Methanol	4: 4: 2	Broad peak
Ethyl acetate: Benzene: Methanol	3: 4: 3	Broad peak
Ethyl acetate: Toluene: Methanol: Formic acid	**5: 5: 0.5: 0.5**	**Sharp peak and separate degraded products.**
Chloroform: Toluene: Methanol: Glacial acetic acid	3: 4 : 4: 0.05	Broad peak
Chloroform: Toluene: Methanol: Glacial acetic acid	4: 4: 2: 0.2	Broad peak

It is evident from the data that mobile phase combination of **Benzene: Methanol** in proportion of 8:2 v/v was selected as method A for estimation of EZE in dosage form and **Ethyl acetate: Toluene: Methanol: Formic acid** in proportion of **5: 5: 0.5: 0.5** v/v/v/v was selected as method B for separation of EZE form it degraded products. The chromatographic condition for spotting on plate are given in Chapter 6.

7.1.1.6.2. Chromatographic condition:

Method A

- **Mobile phase:** Benzene: Methanol (8:2 v/v)
- **Scanning Wave length:** 250 nm

Method B

- **Mobile phase:** Ethylacetate: Toluene: Methanol: formic acid (5:5:0.5:0.5 v/v/v/v)
- **Scanning Wave length:** 231 nm (good separation of degraded product)

- Other chromatographic conditions are mentioned in chapter 6
- Pre washing of plate was done as mentioned in chapter 6.

7.1.1.6.3. Preparation of stock solution for EZE

For chromatographic study, standard EZE (100 mg) was weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml methanol and diluted up to mark with methanol to obtain final concentration of 1000 µg/ml.

7.1.1.6.4. Validation Parameters

(I) Linearity (Calibration curve for EZE):

Analysis was performed on Pre-coated silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 µm thickness) (E. Merck) pre-washed with methanol and then dried for 30 minute at 50°C. From the stock solution (1000 µg/ml) aliquots of 0.3, 0.6, 0.9, 1.8, 2.7 and 3.6 µl were spotted on the TLC plate under nitrogen stream using Desaga Applicator, AS30win to obtain final concentration range 300 - 3600 ng/spot.

The plate was dried in air, and then the plate was developed in Twin trough developing chamber (100 × 100) with stainless steel lid, previously saturated with the mobile phase for 30 min. The plate was removed from the chamber, dried in air and was scanned and quantified at 250 and 231 nm in Re-emission/Excitation mode with Desaga TLC scanner, Proquant. The calibration curve was constructed by plotting

peak area vs. concentration (ng/spot) and peak height vs. concentration (ng/spot) corresponding to each spot and regression equation was calculated.

(II) Precision

The precision is measure of either the degree of reproducibility or repeatability of analytical method.

a. Repeatability of measurements:

0.5, 1.5 and 2 µl aliquot of stock solution (1000 µg/ml) was spotted on the TLC plate under nitrogen stream using Desaga Applicator, AS30win, was developed and scanned five times without changing plate position at selected wavelenght in Reemission/Excitation mode with Desaga TLC scanner, Proquant and % C.V. was calculated. Intraday and interday precision were determined by analyzing 500, 1500 and 2000 ng/spot for five times in the same day and daily for five days and % C.V. was calculated. Interday precision was determined by analyzing 500, 1500 and 2000 ng/spot.

b. Reproducibility of measurements:

0.4 µl aliquot of stock solution (1000 µg/ml) was spotted on the aluminum and glass TLC plate under nitrogen stream using Desaga Applicator, AS30win, was developed and scanned five times without changing plate position at selected wavelength in Reemission/Excitation mode with Desaga TLC scanner, Proquant and % C.V. was calculated.

(III) Accuracy

It was determined by calculating the recovery of drug by Standard addition method. 1 ml of previously analyzed sample (1000 µg/ml) was transferred in a series of three volumetric flasks. In that 50, 100 and 200 % of spiking was done with stock solution. Form final concentration of 150, 200 and 300 µg/ml 5 µl was spotted on TLC plate under nitrogen stream using Desaga Applicator, AS30win which was developed and scanned five times without changing plate position at selected wavelength in Reemission/Excitation mode with Desaga TLC scanner, Proquant and % C.V. was calculated.

(IV) Limit of Detection and Quantification

LOD and LOQ were calculated using the equation given in Chapter 5.

7.1.1.6.3. Results and Discussion:

7.1.1.6.3.1. Validation Parameters

(I) Linearity

Linearity range of EZE was found to be 306 – 3610 ng/spot with correlation co-efficient and % C.V. were reported in table 7.1.1.6.1.

Table 7.1.1.6.1: Calibration data of EZE by HPTLC with UV detection

Sr. No.	Concentration (ng/spot)	Method A					Method B		
		Peak Area		Peak Height		R_f*	Peak Area		R_f*
		Mean ± SD	%CV	Mean ± SD	%CV		Mean	%CV	
1	306	44.00 ± 0.66	0.30	33.00 ±0.42	0.72	0.43	130	0.34	0.60
2	810	141.00 ±1.37	0.37	141.00 ±0.29	0.31	0.44	288	0.58	0.59
3	918	244.00 ±0.85	0.17	191.00 ±0.86	0.65	0.43	329	0.79	0.61
4	1836	479.47 ±1.42	0.20	390.61 ±0.56	0.34	0.43	569	0.41	0.59
5	2754	737.00 ±1.78	0.22	583.00 ±0.61	0.31	0.43	825	0.29	0.60
6	3610	964.52 ±0.99	0.10	760.00 ±0.93	0.43	0.43	991	0.73	0.60

***Average of five readings**

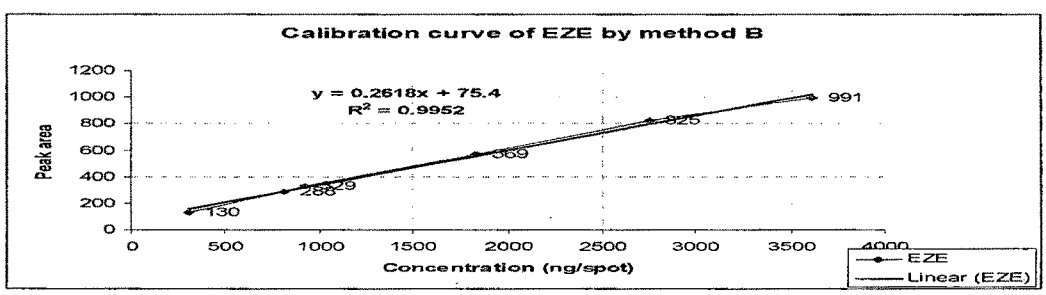


Figure 7.1.1.6.1: Calibration curve of EZE by HPTLC by method B (Peak Area)

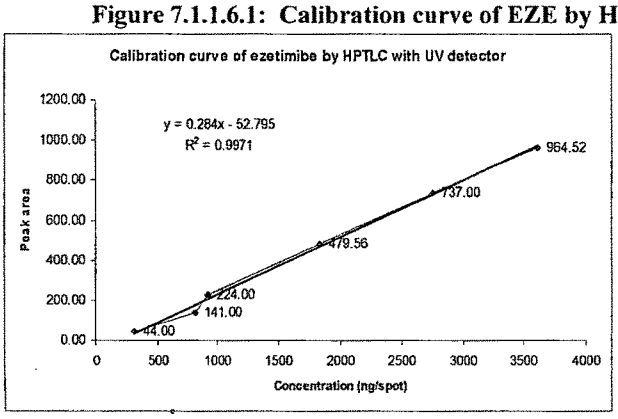


Figure 7.1.1.6.2: Calibration curve of EZE by HPTLC by method A. (Peak Area)

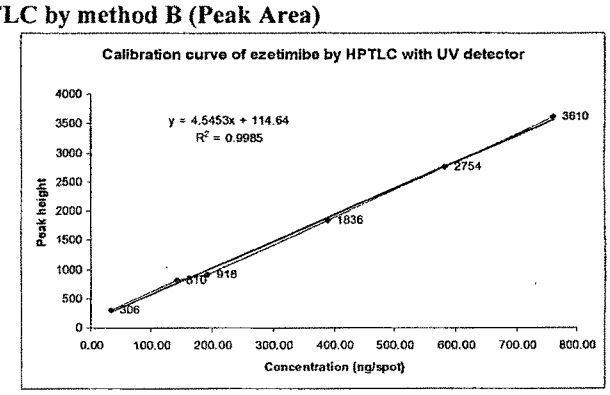


Figure 7.1.1.6.3: Calibration curve of EZE by HPTLC by method B. (Peak Height)

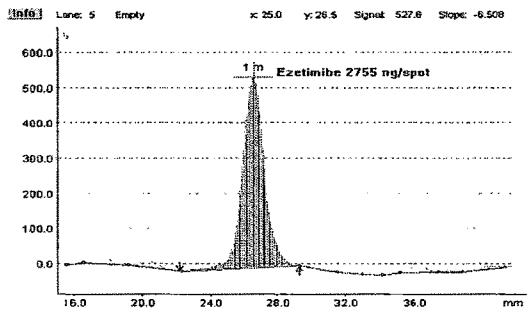


Figure 7.1.1.6.4: Single Spectrum of EZE by HPTLC by method A

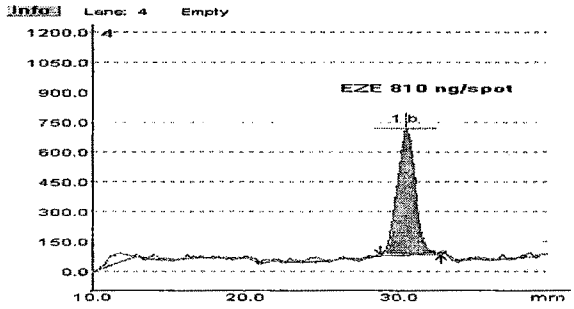


Figure 7.1.1.6.5: Single Spectrum of EZE by HPTLC by method B

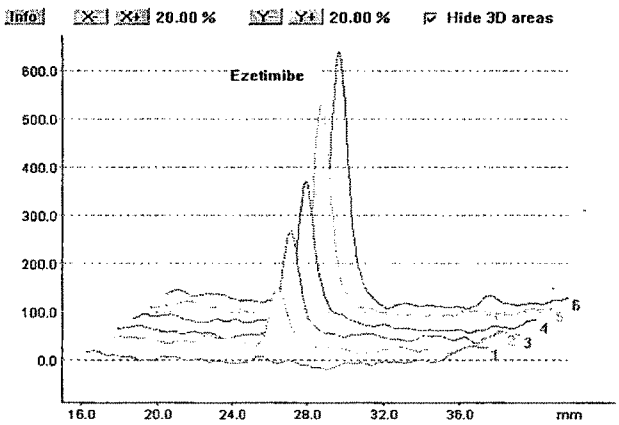


Figure 7.1.1.6.6: 3D Spectra of EZE by HPTLC by method A

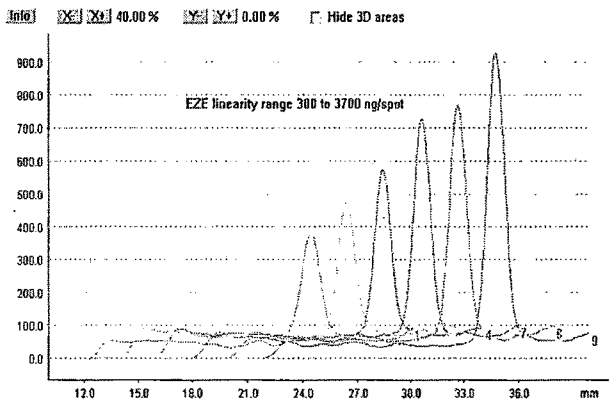


Figure 7.1.1.6.7: 3D Spectra of EZE by HPTLC by method B

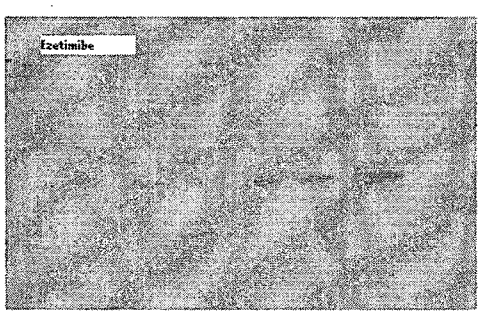


Figure: 7.1.1.6.8: Chromatogram of EZE with UV detection (After detection)

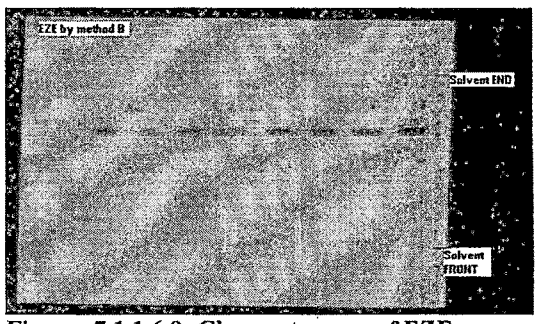


Figure: 7.1.1.6.9: Chromatogram of EZE with UV detection (After detection)

(II) Precision

Reproducibility

The % C.V. of repeated measurement of the same solution was not more than 1%.

Table 7.1.1.6.2: Reproducibility data of EZE by HPTLC with UV detection (400ng/ml)

Time	Method A			Method B	
	Peak Area	Peak Height	Rf	Peak Area	R_f
Aluminum plate	63.14	72.35	0.43	157.451	0.59
	64.20	73.00	0.42	158.69	0.60
	64.00	72.00	0.43	157.39	0.60

Glass plate	63.65	73.98	0.43	158.03	0.60
	63.98	73.00	0.43	157.45	0.61
	63.79	72.87	0.428	157.93	0.59
S.D.	0.85	0.86	0.0044	0.46025	0.00687
%CV	0.17 (<1%)	0.65 (<1%)	1.02 (>1%)	0.36007	1.1485

***Average of five readings**

Repeatability:

Intraday and Interday Precision

Intraday and interday variation of the proposed method are reported in table 7.1.1.6.3.

Table 7.1.1.6.3: Intraday precision data of EZE by HPTLC

Sr. No.	Concentration (ng/spot)	Method A					Method B		
		Peak Area		Peak Height		R_f*	Peak Area		R_f*
		Mean ± SD	%CV	Mean ± SD	%CV		Mean	%CV	
1	500	87.04±0.49	0.22	88.27±0.41	0.72	0.42	177.77	0.48	0.60
2	1500	366.01±1.13	0.23	312.09±0.64	0.65	0.43	537.58	0.29	0.59
3	2000	522.40±0.97	0.12	425.07±0.93	0.47	0.43	619.82	0.72	0.60
Table 7.1.1.6.4: Interday precision data of EZE by HPTLC									
1	500	81.90±0.89	0.40	83.92±0.31	0.52	0.42	172.42	0.64	0.60
2	1500	360.77±0.77	0.16	315.79±0.39	0.29	0.43	533.33	0.39	0.60
3	2000	532.56±1.39	0.17	421.05±0.71	0.36	0.43	696.78	0.73	0.61

***Average of five readings**

(III) Accuracy

Accuracy of the measurement of EZE is reported in table 7.1.1.6.5.

Table 7.1.1.6.5: Accuracy data of EZE by HPTLC with UV detection

Spoted Amount (A + B) (ng/spot)	Method A				Method B	
	Peak Area		Peak Height		Peak Area	
	Total quantity Found*± S.D.	%Recovery ± S.D	Total quantity Found* ± S.D.	%Recovery ± S.D	Total quantity Found*	%Recovery
750	698.73±1.32	98.92±1.88	698.80±1.22	98.80±1.67	749.87	100.02
1000	997.91±0.91	99.70±1.61	994.34±0.86	99.43±1.65	1049.32	95.30
1500	1499.52±1.19	99.51±1.79	1500.56±0.98	100.31±1.54	1503.63	99.76

***Average of five reading**

(IV) Limit of detection

The minimum detectable concentration of EZE found from method A was 0.6580 ng/spot for peak area and 0.5712 ng/spot for peak height and from Method B 0.582 ng/spot by peak area.

(V) Limit of quantification

The lowest quantifiable concentration of EZE found from method A was 2.1936 ng/spot for peak area and 1.9042 ng/spot for peak height of method A and from Method B 2.42 ng/spot by peak area.

7.1.1.6.3.2.. Estimation of EZE in marketed Tablet:

(A) Preparation of test solution:

20 Tablets were triturated after taking their average weight. The Tablet powder equivalent to one Tablet was transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution was sonicated for 10 min and supernatant was filtered through whatman filter paper no.41. From the filtrate 0.5 µl was spotted on TLC plate under nitrogen stream using Desaga Applicator, AS30win this was developed and scanned five times without changing plate position at selected wavelength in Reemission/Excitation mode with Desaga TLC scanner, Proquant and concentration of sample solution was found from regression equation computed from calibration curve of EZE.

Table 7.1.1.6.6: Estimation of EZE in Tablet by HPTLC with UV detection

Tablet Formulation	Labeled Claim (mg/Tablet)	Method A				Method B	
		Peak Area		Peak Height		Peak Area	
		Amount found (mg/Tablet)	% Recovery ± S.D	Amount found (mg/Tablet)	% Recovery ± S.D	Amount found (mg/Tablet)	% Recovery ± S.D
EZEDOC (Lupin)	10	9.99	99.98	10.24	102.43	10.29	102.83
EZETIB (Unisearch)	10	10.12	101.21	10.09	100.98	10.08	100.81

***Average of five readings**

(B) Applicability of method B:

Method B can easily estimate EZE in pharmaceutical dosage form but it can also estimate of EZE in presence of its degradation products. It was evident from the stability studies of EZE that EZE was stable against thermal, UV and oxidative condition and degraded in Water, alkaline and acidic conditions.

Procedure for forced degradation study

Forced degraded samples which was obtained at the end of degradation study under neutral (reflux) acidic (1 N reflux) and alkaline (1 N reflux) were used over here for the dFTIRect spotting. Sample was applied by dFTIRect spotting of 1 µl on prewashed TLC plate. Fig 7.1.1.6.10 to 7.1.1.6.14 show the chromatograms of forced degraded samples.

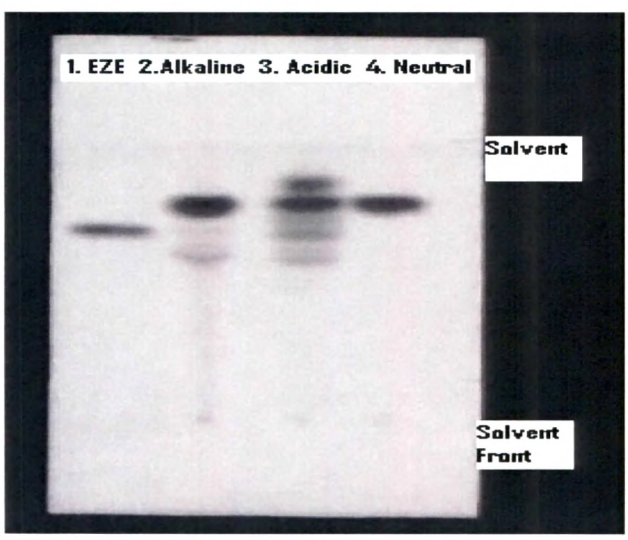


Figure: 7.1.1.6.10: TLC plate of EZE and its alkaline, acidic and neutral degradation product in UV (after develope)

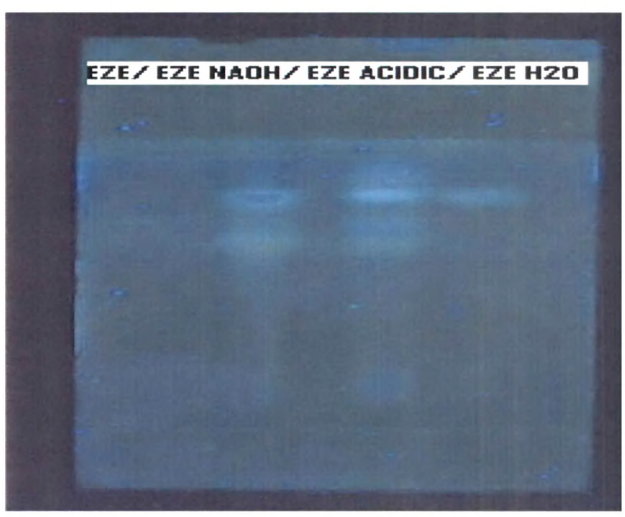


Figure 7.1.1.6.11: TLC plate of EZE in it alkaline, acidic and neutral degradation product in florescence light (after develope)

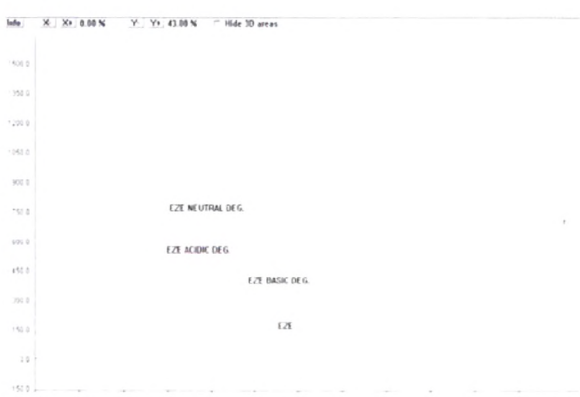


Figure 7.1.1.6.12: overlain Chromatograph of EZE in it alkaline, acidic and neutral degradation product in UV

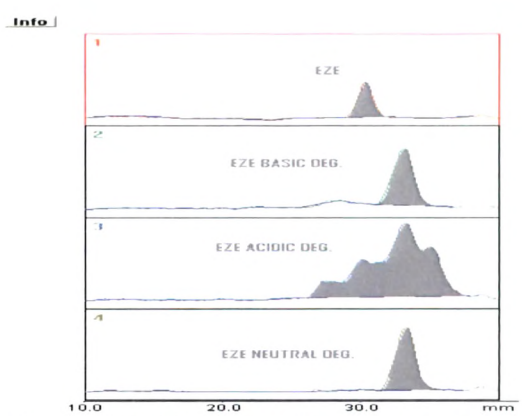


Figure 7.1.1.6.13: vertical Chromatograph of EZE in it alkaline, acidic and neutral degradation product in UV.

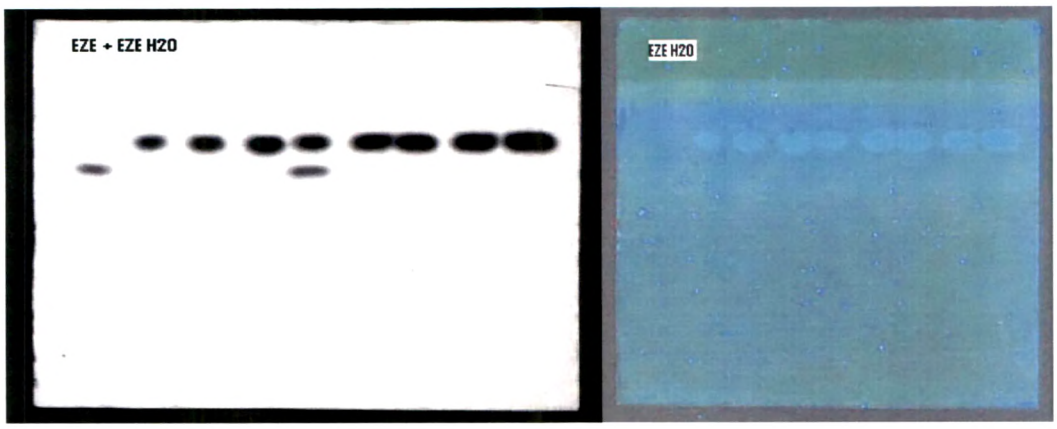


Figure 7.1.1.6.14: Photograph of TLC plate of EZE and its water degraded product in UV (left) and florescent light (right).

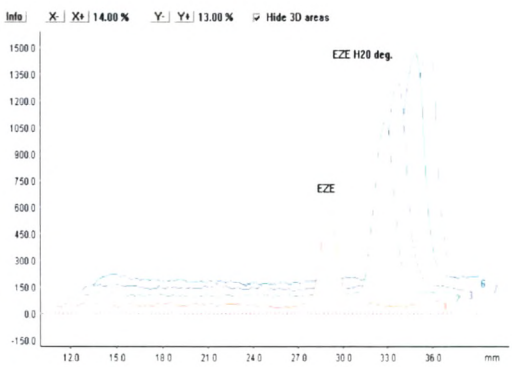


Figure 7.1.1.8.15: overlain Chromatograph of EZE and it neutral degradation product in UV

Figure 7.1.1.8.16: Chromatograph of neutral degradation of EZEproduct in UV

7.1.1.8.4. Summary of Validation parameters:

The summary of validation parameters is reported in Table 7.1.1.8.7.

Table 7.1.1.8.7: Summary of Validation parameters by HPTLC.

Sr. No	Parameters	Method A		Method B
		Peak Area	Peak Height	Peak Area
1	Analytical wavelength (nm)	250		231
2	Linearity range (ng/spot)	306 – 3672		306 – 3610
3	Regression equation	Y = 0.284 x conc.– 2.97	Y= 4.553 x conc. + 44.64	Y = 0.2618 x conc.+75.4
4	Correlation coefficient (r^2)	0.9971	0.9985	0.9952
5	Intercept	-52.97	144.64	75.4
6	Slope	0.284	4.553	0.2658

7	Assay	99.98 – 101.21 %	100.98 – 102.43 %	100.81 – 102.83 %
8	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements %CV % Recovery	 0.12-0.23 % 0.16-0.40% 0.17 (<1%) 98.92 – 99.70	 0.47-0.72% 0.29-0.52% 0.65 (<1%) 98.31 – 100.31	 0.59 – 0.60 % 0.60 – 0.61 % 0.46 99.30 – 100.42
9	Limit of detection (ng/spot)	0.6580	0.5712	0.582
10	Limit of quantification (ng/spot)	2.1936	1.9042	2.42

7.1.1.8.6. Conclusion:

Two HPTLC methods were developed for the estimation of EZE in pharmaceutical dosage form. Method B could estimate EZE in presence of it degraded products. As per degradation study and HPTLC method in acidic condition it was degraded in four degraded product (fig. 7.1.1.8.9 to 8.10). In alkaline condition initially degraded in two products and then after converted in to main impurity (fig. 7.1.1.8.9). In neutral condition when reflux it was fully converted in main impurity which was fluorescent in nature with R_f of 0.70(fig 7.1.1.8.11 to 7.1.1.8.13).

Reference:

1. Rosenblum S B, Huynh T, Afonso A, Davis H R, Yumibe N, Clader J W, Burnett D A. Discovery of 1-(4-fluorophenyl)-(3R)-[3-(4-fluorophenyl)-(3S)-hydroxypropyl]-(4S)-(4 -hydroxyphenyl)-2-azetidinone (SCH 58235): a designed, potent, orally active inhibitor of cholesterol absorption. J Med Chem. 1998; 12: 41: 6: 973-980.

7. 1. 2

Pravastatin

7.1.2 Pravastatin (PRAVA)

Apparatus, instruments and reagents:

Apparatus, instruments and reagents used for the development of spectroscopic and chromatographic methods are mentioned in chapter 6.

Spectrophotometery

Six spectroscopic methods were developed for the estimation of PRAVA in oral dosage form. Simple zero orders spectroscopic method was developed for routine analysis of PRAVA. To study specific spectra characterization, first and second derivative spectroscopic methods were tried. PRAVA shows different spectral characteristics in methanol and alkaline medium so a difference spectroscopic method was developed. The 3 wavelengths method was tried which gives very reliable results for estimation of oral dosage form. The quantitative FTIR spectroscopic method could also be used for accurate estimation of PRAVA in tablet dosage form.

Chromatography

Chromatographic methods were developed as SIAM methods. HPLC method was developed which can study PRAVA in presence of degraded products. HPTLC methods was developed which can estimate PRAVA form pharmaceutical formulation and second HPTLC method was developed which can estimate PRAVA in presence of its acidic degraded products and also from pharmaceutical formulation. To study the pharmacokinetic data HPLC method was developed for the estimation of PRAVA in spiked human plasma.

7.1.2.1. Estimation of PRAVA by zero order spectrophotometry, first order and second order derivative Spectrophotometric methods.

Zero order spectra for a series of standard solutions were recoded in UV PC software, which was used for the first order derivative spectroscopic method.

Experimental work:

7.1.2.1.1. Preparation of Stock solution

Standard PRAVA (10 mg) was weighed accurately and transferred to 100 ml volumetric flask. It was dissolved in 50 ml methanol and diluted up to mark with methanol to obtain .final concentration of 100 µg/ml. This solution was used as working standard solution for Spectrophotometric methods.

7.1.2.1.2. Analytical wavelengths:

Simple UV spectroscopic method was developed in methanol as solvent. PRAVA shows maximum absorbance at 237.20 nm, which was used for the further estimation of PRAVA. Zero order spectra were converted in to a plot of dA/dλ vs. λ(first derivative). First order derivative curves were studied and maximum 235.20 nm and minimum at 240.30 nm were selected as analytical wavelengths. Similarly for second order derivative curves were studied maximum 242.60 nm and minimum at 246.70 nm were selected for further study. The amplitude difference was measured for each concentration of standard solution.

7.1.2.1.3. Calibration curve of PRAVA

From the stock solution (100 µg/ml) aliquots of 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 ml were transferred to a series of 10 ml volumetric flasks. The volume was adjusted to a mark with methanol to get 1, 5, 10, 15, 20, 25, 30, 35 and 40 µg/ml of solution. The absorbance was measured at λ_{max} using methanol as a blank over the concentration range of 1-40 µg/ml and was plotted against concentration and regression equation was calculated. Result were expressed in terms of Correlation co-efficient.

7.1.2.1.3. Validation of method:

Similar procedure was followed for validation of analytical method as described in Chapter 7.1.1.1 and Section 7.1.1.1.4.

7.1.2.1.4. Results and Discussion:

7.1.2.1.4.1. Validation Parameters

(I) Linearity

Linearity range of PRAVA in methanol was found to be 1-40 µg/ml for all three methods. Correlation co-efficient were found to be 0.9997, 0.9992 and 0.9957, respectively for zero, first and second order spectroscopic method. The % C.V. range are reported in table 7.1.2.1.1.

Table 7.1.2.1.1: Calibration data of PRAVA by Spectrophotometry methods

Sr. No.	Conc. (µg/ml)	Simple UV Spectrophotometric method	1st derivative Spectrophotometric method			2nd derivative Spectrophotometric method		
		Absorb.*± %CV (N=5)	Absorb. at 235.20 nm	Absorb. at 240.30 nm	Amplitude Difference* ± % C.V. (N=5)	Absorb. at 242.60 nm	Absorb. at 246.70 nm	Amplitude Difference (N=5)*

1	1	0.130 ± 0.267	0.002	-0.018	0.020 ± 0.926	0.002	-0.002	0.004 ± 0.235
2	5	0.32 ± 0.105	0.021	-0.024	0.045 ± 0.203	0.017	-0.009	0.025 ± 0.107
3	10	0.579 ± 0.0068	0.022	-0.046	0.068 ± 0.101	0.017	-0.023	0.04 ± 0.064
4	15	0.82 ± 0.138	0.036	-0.061	0.097 ± 0.067	0.027	-0.03	0.057 ± 0.043
5	20	1.07 ± 0.304	0.038	-0.082	0.120 ± 0.050	0.03	-0.04	0.07 ± 0.032
6	25	1.29 ± 0.08718	0.046	-0.097	0.143 ± 0.038	0.035	-0.048	0.083 ± 0.026
7	30	1.517 ± 0.0026	0.054	-0.116	0.170 ± 0.033	0.042	-0.057	0.099 ± 0.021
8	35	1.78 ± 0.199	0.069	-0.13	0.199 ± 0.029	0.054	- 0.0671	0.1211 ± 0.018
9	40	2 ± 5119	0.07	-0.152	0.222 ± 0.025	0.054	-0.081	0.135 ± 0.016

* Mean value of five determinations

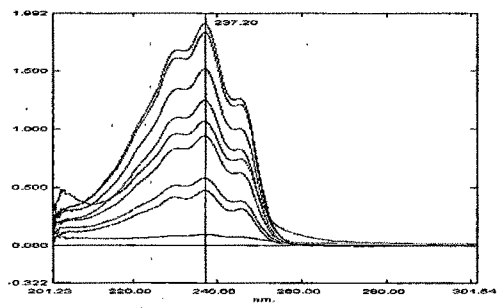


Figure: 7.1.2.1.1. UV spectra of PRAVA by Simple UV Spectrophotometry

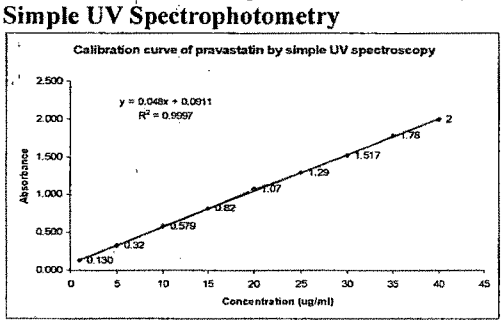


Figure 7.1.2.1.2: Calibration curve of PRAVA by Simple UV Spectrophotometry

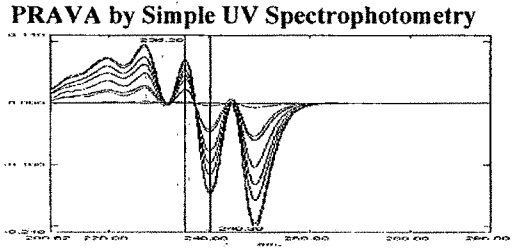


Figure: 7.1.2.1.3: First derivative UV spectra of PRAVA

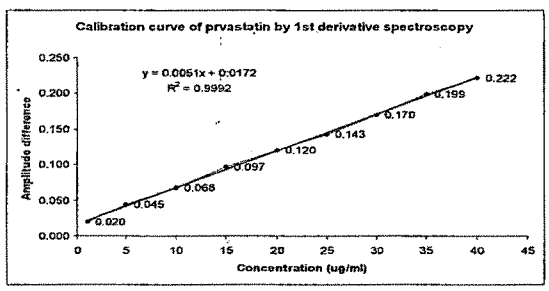


Figure 7.1.2.1.4: Calibration curve of PRAVA by 1st derivative Spectrophotometry

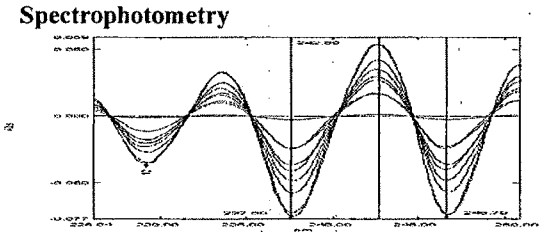


Figure: 7.1.2.1.5: 2nd derivative UV spectra of PRAVA

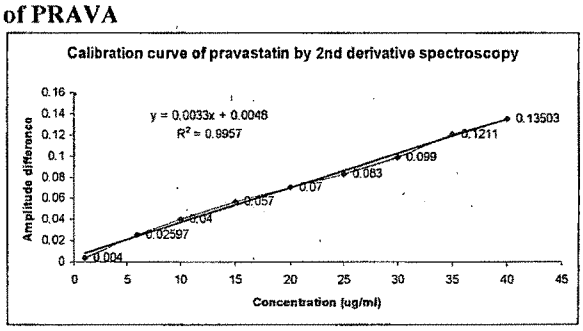


Figure 7.1.2.1.6: Calibration curve of standard PRAVA by 2nd derivative

(II) Precision

a. Intraday and Interday Precision (Repeatability)

Intraday and interday variation of the proposed method was reported in table 7.1.2.1.2.

Table 7.1.2.1.2: Intraday precision data of PRAVA by Spectrophotometric method

Conc. (µg/ml)	Simple UV Spectrophotometric method	1st derivative Spectrophotometric method		2nd derivative Spectrophotometric method	
	Absorb.*± % C.V. (n=5)	Amplitude difference* ± S.D. (n=5)	% C.V.	Amplitude difference* ± S.D. (n=5)	% C.V.
10	0.57817 ± 0.00978	0.067 ± 0.0095	0.67	0.043 ± 0.38	0.15
20	1.08308 ± 0.00459	0.119 ± 0.00341	0.29	0.070 ± 0.29	0.95
30	1.54583 ± 0.00365	0.170 ± 0.00098	0.46	0.093 ± 0.25	0.13
Table 7.1.2.1.3: Interday precision data of PRAVA by Spectrophotometric method					
10	0.57683 ± 0.00917	0.065 ± 0.00055	0.21	0.048 ± 0.40	0.82
20	1.07325 ± 0.00481	0.118 ± 0.00073	0.62	0.075 ± 0.99	0.11
30	1.59045 ± 0.00321	0.168 ± 0.000059	0.32	0.098 ± 0.35	0.29

* Mean value of five determinations

b. Reproducibility:

The reproducibility of the method was determined by using Shimadzu UV 1700 and Shimadzu UV 1601. The % CV of the responses confirms the reproducibility of the method for determination of PRAVA, the data is reported in Table 7.1.2.1.4.

Table 7.1.2.1.4: Reproducibility data of PRAVA

Conc. (µg/ml)	Absorbance* ± %C.V.					
	Simple UV Spectrophotometric method		1st derivative Spectrophotometric method		2nd derivative Spectrophotometric method	
	UV 1700	UV 1601	UV 1700	UV 1601	UV 1700	UV 1601
10	0.579±0.85	0.568±0.93	0.068±0.29	0.066 ± 0.87	0.04 ± 0.87	0.042 ± 0.37
15	0.82 ± 0.13	0.819±0.63	0.097 ± 0.17	0.102 ± 0.56	0.057 ± 0.36	0.055 ± 0.19
20	1.07± 0.79	1.07 ±0.71	0.120±0.46	0.11±0.39	0.071± .46	0.073 ± 0.71

* Mean of three determinations

(III) Accuracy

Accuracy of the measurement of PRAVA was determined by standard addition and was found to be in the range of 100.08 – 100.03, 100.12 -100.17 and 100.20 – 100.30 %, respectively for zero, first and second order spectroscopic method.

Table 7.1.2.1.5: Accuracy data of PRAVA by Spectrophotometry methods

Initial conc. (µg/ml) (A)	Quantity of std. Added (µg/ml) (B)	Total Amount (A + B)	Simple UV Spectrophotometric method		1st derivative Spectrophotometric method		2nd derivative Spectrophotometric method	
			Total quantity Found * ± S.D.	% Recovery ± S.D	Total quantity Found* ± S.D.	% Recovery ± S.D	Total quantity Found* ± S.D.	% Recovery ± S.D
10	5	15	15.30 ± 0.0016	100.03 ± 0.62985	15.027 ± 0.34	100.178 ± 0.51	15.046 ± 0.31	100.304 ± 0.81
10	10	20	20.002 ± 0.0097	100.08 ± 0.46487	20.029 ± 0.51	100.144 ± 0.31	20.049 ± 0.11	100.247 ± 0.76
10	15	25	25.36 ± 0.0033	100.03 ± 0.41356	25.030 ±0.15	100.121 ± 0.34	25.052 ± 0.92	100.208 ± 0.60

* Mean of five determinations

(IV) Limit of detection

The minimum detectable concentration of PRAVA was found to be 0.18, 0.098 and 0.11 µg/ml, respectively for zero, first and second order spectroscopic method.

(V) Limit of quantification

The lowest quantifiable concentration of PRAVA was found to be 0.60, 0.3179 and 0.39 µg/ml, respectively for zero, first and second order spectroscopic method.

7.1.2.1.4.2. Estimation of PRAVA in marketed Tablet:

Preparation of test solution:

PRAVATOR of SOLUS contained 10 mg of PRAVA per Tablet were analyzed by the proposed method. 20 tablets were triturated after taking their average weight. The Tablet powder equivalent to one Tablet was transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution was sonicated for 10 min and supernatant was filtered through Whatman filter paper no.41. 1 ml from this solution were transferred to 10 ml volumetric flasks and diluted with methanol to get 10 µg/ml concentration. The absorbance of this solution was measured at 237.20 nm and concentration of sample solution was found from regression equation. Zero order spectrum of the

solution was converted in first and second order and the absorbance was measured at λ_{maxima} and λ_{minima}. The concentration of sample solution was found from regression equation computed from calibration curve of PRAVA. Result of the analysis are mentioned in table 7.1.2.1.6 in terms of label claim.

Table 7.1.2.1.6: Estimation of PRAVA in tablet by Spectrophotometry

Tablet Formu-Lation	Labeled Claim (mg/Tablet)	Simple UV Spectrophotometric method		1st derivative Spectrophotometric method		2nd derivative Spectrophotometric method	
		Amount Found* (mg/Tablet)	% Assay ± S.D	Amount Found* mg/Tablet)	% Assay ± S.D	Amount Found* (mg/Tablet)	% Assay ± S.D
PRAVATOR (SOLUS)	10	10.08	100.89 ±0.399	10.12	101.21 ± 0.84	9.998	99.98 ±0.05

* Mean of five determinations

7.1.2.1.5. Summary of Validation parameters:

The summary of validation parameters is given in Table 7.1.2.1.7

Table 7.1.2.1.7: Summary of Validation parameters of Spectrophotometry

Sr. No	Parameters	Simple UV Spectrophotometric method	1st derivative Spectrophotometric method	2nd derivative Spectrophotometric method
1	λ max (nm)	237.20	235.20	242.60
1	λ min (nm)	-	240.30	246.70
2	A (1%, 1cm) (dl gm^{-1} cm^{-1})	402.65	-	-
3	Molar Absoptivity (ε) (L mol^{-1} cm^{-1})	2897.65(>100)	-	-
4	Linearity range (µg/ml)	1 -40	1 – 40	1 -40
5	Regression equation	Abs. = 0.048 × Conc. + 0.0911	Abs. = 0.051 × Conc. + 0.0172	Abs. = 0.0033 × Conc. + 0.0048
6	Correlation coefficient (r^2)	0.9997	0.9992	0.9957
7	Intercept	0.0911	0.051	0.0033
8	Slope	0.048	0.0172	0.0048
9	Sandell's sensitivity (µg/cm^2/0.001 abs. unit))	0.9456	-	-
10	Assay	100.89 %.	101.21 %.	99.98 %.
11	Precision Intra day % CV (n = 5)			0.13 – 0.95 %.

	Inter day % CV (n = 5) Reproducibility of measurements % CV % Recovery	0.003 -0.009 %. 0.0032 -0.0091 %. 0.66 % (<1%) 100.00 – 100.03 %	0.29-0.67 %. 0.21-0.62 %. 0.057 % 100.12 -100.17 %.	0.11-0.82 % 0.39 % 100.20 – 100.30 %.
12	Limit of detection	0.1801 µg/ml	0.098 µg/ml	0.11 µg/ml
13	Limit of quantification	0.6003 µg/ml	0.3179 µg/ml	0.39 µg/ml

7.1.2.1.5. Conclusion:

Simple, accurate and economic methods were developed for the estimation of PRAVA in pharmaceutical oral dosage form. As summary table shows, method can work in the range of 1 – 40 µg/ml with accuracy of 100.00 % with %CV of reproducibility was 0.66 %.

The first and second derivative spectroscopic methods were developed for the estimation of PRAVA in oral dosage form with the recovery of 100.20 – 100.30 %. Derivative method was developed because it is a useful tool for the estimation of drug in presence of excipients.

7.1.2.2 Estimation of PRAVA by 3 wavelength & Difference Spectrophotometric methods

Preparation of stock solution, and test solution were same as described 7.1.2.1.

7.1.2.2.1. Experimental work:

Three-wavelength method:

In order, to determine the concentration of PRAVA, the 3 wavelengths selected are as follows: WL1 = 234.50 nm, WL2 = 237.30 nm and WL3 = 241.80 nm (Fig. 7.1.2.2.3) The absorbance of all the solutions was measured at the three selected wavelengths (fig. 7.1.2.2.3.) and net absorbance of PRAVA was determined using the equation mentioned under Chapter 5, Section 5.1.2. A graph of Net absorbance vs. concentration of standard PRAVA was plotted and the regression equation was calculated.

Difference Spectrophotometric method:

The essential feature of a difference spectrophotometry is that the measured value, the difference in absorbance (A) between two equimolar solutions of the analyte in different chemical forms, exhibits different spectral characteristics. Here chemical forms selected were 0.1 N NaOH and methanol. Alkaline solutions were placed in

sample compartment and methanolic solutions of PRAVA were kept in reference compartment to obtain the difference spectra. The maximum absorbance was measured at 250.50 nm (Fig. 7.1.2.2.1).

Preparation of stock solution, calibration curve and validation parameters:

Procedure for validation of analytical method was similar as described in Section 7.1.1.3.

7.1.2.2.2 Results and Discussion:

7.1.2.2.2.1 Validation Parameters

(I) Linearity:

Linearity range of PRAVA for both the methods was found to be 1 - 40µg/ml. Correlation co-efficient and % C.V. range are reported in to table 7.1.2.2.1.

Table 7.1.2.2.1: Calibration data of PRAVA by spectrophotometric methods

Sr. No.	Conc. (µg/ml)	Difference spectrophotometry		Three wavelength spectrophotometry			
		Absorbance* ± S.D. (N=5)	% C.V.	Absorbance at 234.50 nm	Absorbance at 237.30 nm	Absorbance at 241.80 nm	Net Absorbance (N=5)*± % C.V.
1	1	0.024 ± 0.00068	0.068	0.067	0.082	-0.0512	0.038 ±0.38
2	5	0.045 ± 0.00078	0.016	0.295	0.41	0.242	0.282±0.32
3	10	0.077 ± 0.00065	0.006	0.366	0.512	0.032	0.284±0.45
4	15	0.105 ± 0.00121	0.008	0.585	0.832	0.263	0.506±0.46
5	20	0.131 ± 0.01599	0.080	0.677	0.96	0.149	0.547±0.81
6	25	0.166 ± 0.0209	0.084	0.76	1.1	0.048	0.585±0.52
7	30	0.194 ± 0.02657	0.089	0.933	1.343	0.078	0.723±0.46
8	35	0.223 ± 0.02619	0.075	1.119	1.61	0.169	0.886±0.72
9	40	0.245 ± 0.02922	0.073	1.16	1.675	-0.067	0.859±0.58

* Mean value of five determinations

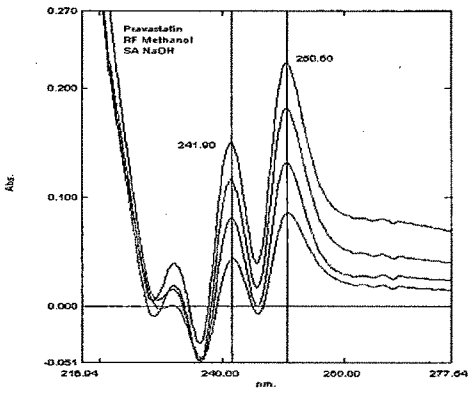


Figure: 7.1.2.2.1 Difference spectra of PRAVA

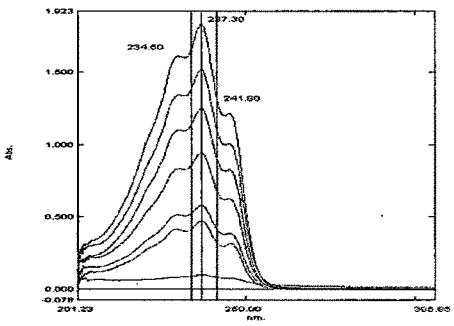


Figure: 7.1.2.2.3 UV spectra of PRAVA by 3-wavelength method

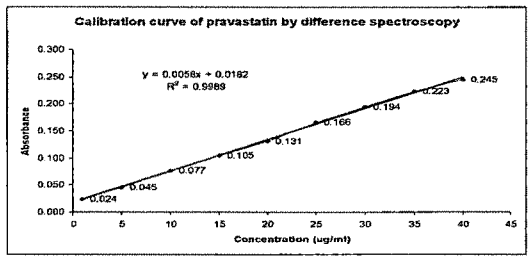


Figure 7.1.2.2.2: Calibration curve of PRAVA by Difference Spectrophotometry
Correlation co-efficient = **0.9989**
Slope = 0.0058
Intercept = 0.0182
Regression equation: **Abs. = 0.0058 × Conc. + 0.0182**

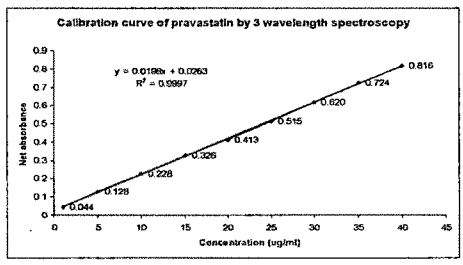


Figure 7.1.2.2.4: Calibration curve of PRAVA by 3-wavelength method
Correlation co-efficient = **0.9997**
Slope = 0.0198
Intercept = 0.0263
Regression equation: **Abs. = 0.0198 × Conc. + 0.0263**

(II) Precision

a. Intraday and Interday Precision (Repeatability)

Intraday and interday variation of the proposed method are reported in table 7.1.2.2.2.

Table 7.1.2.2.2: Intraday precision data of PRAVA by spectrophotometric method

Conc. (µg/ml)	Absorbance Mean ±% C.V. (n=5)			
	Difference spectrophotometry		Three wavelength spectrophotometry	
	Intraday	Interday	Intraday	Interday
10	0.078 ± 0.92	0.076 ± 0.35	0.228 ± 0.95	0.227 ± 0.90
20	0.106 ± 0.21	0.104 ± 0.23	0.413 ± 0.66	0.411 ± 0.53
30	0.132 ± 0.42	0.130 ± 0.82	0.620 ± 0.69	0.619 ± 0.56

* Mean of five determinations

b. Reproducibility: The experiment was repeated using Shimadzu UV 1700 and Shimadzu UV 1601. The % CV of the responses confirms the reproducibility of the method for determination of PRAVA. The data is reported in Table 7.1.2.2.3.

Table 7.1.2.2.3: Reproducibility data of PRAVA

Conc. (µg/ml)	Difference spectrophotometry				Three wavelength spectrophotometry			
	Absorption		% CV		Absorption		% CV	
	UV 1700	UV 1601	UV 1700	UV 1601	UV 1700	UV 1601	UV 1700	UV 1601
10	0.077	0.075	0.25	0.79	0.284	0.283	0.29	0.35
15	0.105	0.0102	0.63	0.6	0.506	0.501	0.37	0.62
20	0.132	0.030	0.17	0.13	0.547	0.540	0.15	0.87

*** Mean of three determinations**

(III) Accuracy

Accuracy of the measurement of PRAVA was determined by standard addition and the recovery was found to be in the range of 100.06 – 100.23 % for

Table 7.1.2.2.4: Accuracy data of PRAVA by spectrophotometry

Initial conc. (µg/ml) (A)	Quantity of std. Added (µg/ml) (B)	Total Amount (A + B)	Difference spectrophotometry		Three wavelength spectrophotometry	
			Total quantity Found Mean ± S.D.	% Recovery ± S.D	Total quantity Found Mean ± S.D.	% Recovery ± S.D
10	5	15	15.036 ± 0.0044	100.238 ± 4.91884	15.02 ± 0.61	100.05 ± 1.22
10	10	20	20.013 ± 0.0042	100.064 ± 3.79702	20.1 ± 0.20	100.20 ± 1.95
10	15	25	25.058 ± 0.0045	100.231 ± 3.21382	24.95 ± 0.91	99.91 ± 0.99

* Mean value of five determinations

(IV) Limit of detection

The minimum detectable concentration of PRAVA was found to be 0.138 and 0.062 µg/ml, respectively.

(V) Limit of quantification

The lowest quantifiable concentration of PRAVA was found to be 0.46 and 0.207 µg/ml by practical observation, respectively.

7.1.2.2.2.2. Estimation of PRAVA in marketed Tablet:

Preparation of test solution:

Test solution was prepared as per procedure given in chapter 7.1.2.1.4.2. The absorbance of this solution was measured at 234.50, 237.30 and 241.80 nm and net absorbance calculated. Concentration of sample solution was found from the regression equation. Test solution prepared in methanol and in 0.1N NaOH as per procedure given in chapter 7.1.1.2.5.2. The absorbance of this solution was measured at 250.50 nm and concentration of sample solution was found from regression equation. The result of PRAVA estimation in dosage form is shown in table 7.1.2.2.5.

Table 7.1.2.2.5: Estimation of PRAVA in tablet by spectrophotometry methods

Tablet Formulation	Labeled Claim (mg/tablet)	Difference spectrophotometry		Three wavelength	
		Amount Found (mg/tablet)	% Assay ± S.D	Amount found (mg/tablet)	% Assay± S.D
PRAVATOR (SOLUS), Unisearch	10	10.017	100.17 ± 3.97	9.987	99.67 ± 0.81

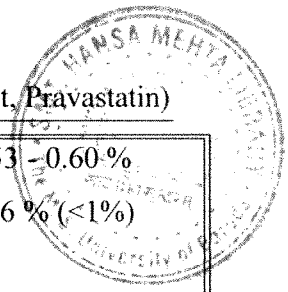
* Mean value of five determinations

7.1.2.2.3. Summary of Validation parameters:

The summary of validation parameters is given in table 7.1.2.2.6.

Table 7.1.2.2.6: Summary of Validation parameters of spectrophotometry

Sr. No	Parameters	Difference	Three wavelengths
1	Analytical wavelength (nm)	250.50	234.50, 237.30, 241.80
2	Linearity range (µg/ml)	1 – 40	
3	Regression equation	Abs. = 0.0058 × Conc. + 0.0182	Abs. = 0.0198 × Conc. + 0.0263
4	Correlation coefficient (r^2)	0.9989	0.9997
5	Intercept	0.0182	0.0263
6	Slope	0.0058	0.0198
7	Assay	100.17 ± 3.97 %	99.67 ± 0.81
8	Precision Intra day % CV (n = 5)	0.01 – 0.92 %.	0.66 - 0.95 %



	Inter day % CV (n = 5) Reproducibility of measurements % CV % Recovery	0.23 – 0.82 %. 0.11 % (<1%) 100.06 – 100.23 %	0.53 -0.60 % 0.26 % (<1%) 99.91 -100.2 %.
9	Limit of detection	0.138 µg/ml	0.0623 µg/ml
10	Limit of quantification	0.46 µg/ml	0.2079 µg/ml

7.1.2.2.5. Conclusion:

With use of difference spectroscopic method, PRAVA can be estimated from pharmaceutical dosage form without interference from any excipient with the accuracy of 100.24 %. Three wavelengths is specific method for estimation of PRAVA.

7.1.2.3 Infra Red spectroscopy method.

Experimental work:

7.1.2.3.1. Preparation of standards for PRAVA

For quantitative IR spectroscopic study standard PRAVA (10 mg) was weighed accurately and make up to 100 mg in same butter paper to obtain final concentration of 100 µg/mg. This sample was triturated with mortar and pestle and sample was used as working standard sample for FTIR spectroscopic method.

7.1.2.3.2. Selection of wave number:

Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) spectra were applied for the analysis of PRAVA at 875 to 854.41 cm^{-1} and 1726.17 cm^{-1} as a Peak area and peak height at single wavelength, respectively. Calibration curves were drawn using the multiple linear regression (MLR) method and the drug amount in sample was computed by QUANT function of IR solution software.

7.1.2.3.3. Calibration curve for PRAVA

Approximate fractions were transferred from standard (100 µg/mg) to butter paper and weight adjusted 10 mg with KBr to get minimum transmission. Peak area and peak height was measured at selected wavenumber. Calibration curves were drawn using the multiple linear regression (MLR) method and the drug amount in sample was computed from regression equation.

7.1.2.3.4. Validation Parameters

Validation of method was done as per procedure given in Chapter 7.1.1.3.

7.1.2.3.5 Results and Discussion:

7.1.2.3.5.1 Validation Parameters

(I) Linearity

Linearity range of PRAVA was found to be 13 -71 µg/mg.

Table 7.1.2.3.1: Calibration data of PRAVA by Infrared Spectroscopy

Sr. No.	Concentration (mg/mg)	Peak Area		Peak height	
		% Transmission	% CV	% Transmission	% CV
1	0.0130	1.926	0.38	0.340	0.48
2	0.0309	4.630	0.27	0.940	0.39
3	0.0419	6.540	0.87	1.319	0.73
4	0.0548	8.340	0.17	1.760	0.68
5	0.0718	11.160	0.45	2.403	0.29

* Mean value of five determinations

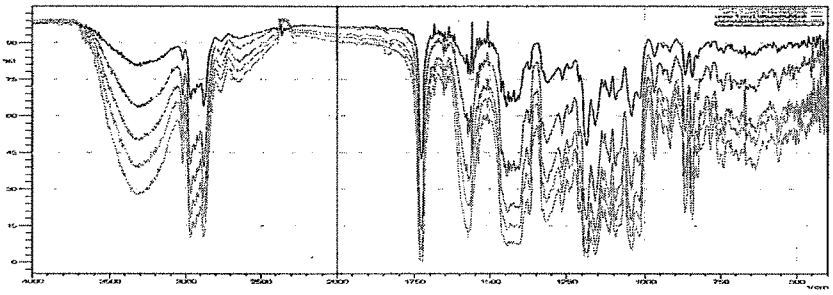


Figure: 7.1.2.3.1: Overlain IR spectra of PRAVA

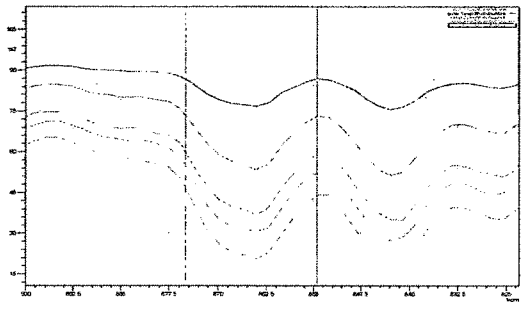


Figure: 7.1.2.3.2: IR spectra of PRAVA of Peak Area

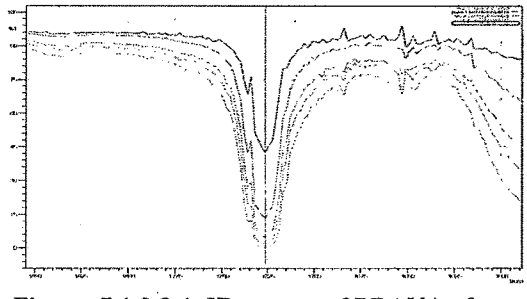


Figure: 7.1.2.3.4: IR spectra of PRAVA of Single Wavelength Number

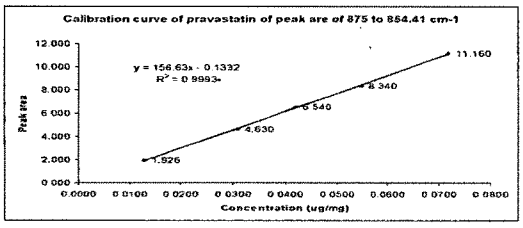


Figure 7.1.2.3.3: Calibration curve of PRAVA by peak area

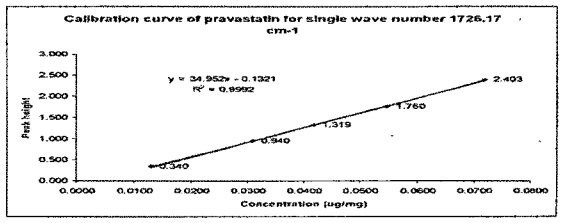


Figure 7.1.2.3.5: Calibration curve of PRAVA by single wavelength number

(II) Precision

a. Intraday and Interday Precision (Repeatability)

The % C.V. of intraday and interday measurements of the same sample was not more than 1%.

Table 7.1.2.3.2: Intraday precision data of PRAVA by FTIR Spectroscopy

Concentration (µg/mg)	Intraday		Interday	
	Peak Area* ±%C.V.	Peak Height* ±%C.V.	Peak Area* ±%C.V.	Peak Height* ±%C.V.
20	2.96419±0.29	0.531±0.59	2.97197±0.45	0.52465±0.29
60	4.65795±0.83	0.98697±0.12	4.60655±0.15	0.9132±0.54
120	5.94393±0.19	1.04929±0.11	6.01823±0.93	1.06241±0.32

* Mean value of five determinations

b. Reproducibility:

As per the instrument specification the % C.V. of repeated measurement of the same solution should not more than 1%.

Table 7.1.2.3.4: Reproducibility data of PRAVA (50 µg/mg) by FTIR **Spectroscopy**

Time	Peak Area	Peak height
	% Transmission	% Transmission
Bigger sample comp.	7.42992	1.61162
	7.46261	1.61739
	7.45072	1.61529
Smallest sample comp.	7.75683	1.66933
	7.45369	1.61581
	7.43437	1.6124
S.D.	0.00238	0.00887
%C.V. (<1%)	0.60774	0.88565

* Mean value of five determinations

(III) Accuracy

Accuracy of the measurement of PRAVA was determined by standard addition method.

Table 7.1.2.3.5: Accuracy data of PRAVA by peak area

Quantitative IR		PRAVA		
Initial conc.(µg/ml) (A)		30	30	30
Quantity of std. Added (µg/ml) (B)		15	30	40
Total Amount (A + B)		45	60	70
Peak area (Mean (n=5) ± %C.V.)	Total quantity Found Mean ± S.D.	39 ± 0.34	61 ± 0.89	70.9 ± 0.38
	% Recovery± S.D	97.5 ± 0.23	101.667 ± 0.32	99.86 ± 0.67
Single wavelength number (Mean (n=5) ± %C.V.)	Total quantity Found Mean ± S.D.	41.2 ± 0.67	60.1 ± 0.46	70.3 ± 0.36
	% Recovery± S.D	103 ± 0.39	100.167± 0.29	100.4 ± 0.12

* Mean value of five determinations

(IV) Limit of detection

The minimum detectable concentration of PRAVA was found to be 0.0745and 0.9631 µg/mg for peak area and peak height, respectively.

(V) Limit of quantification

The lowest quantifiable concentration of PRAVA was found to be 0.248 and 1.550 µg/mg by practical observation for peak area and peak height, respectively.

7.1.2.3.3.2. Estimation of PRAVA in marketed Tablet:

Preparation of test Sample:

The contents of 20 Tablets were weighed and their mean weight determined and finely powdered. An equivalent weight of one Tablet was transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution (100 µg/ml) was sonicated for 10 min and supernatant was filtered through Whatman filter paper no.41. Filterate was evaporated and from the residue 1 mg was taken and made up to 100 mg with KBr on butter paper and triturated in mortar pestle. The DRIFT spectra was scannned between 4000 and 400 cm^{-1}, by averaging 16 scans for each spectrum with a resolution of 4 cm^{-1} (data point resolution per interval 1 cm^{-1}). 875 to 854.41 cm^{-1} and 1726.17 cm^{-1} was selected for measurement of Peak area and peak height at single wave length, respectively. Concentration of sample was found from regression equation. The result of analysis of pharmaceutical formulation is reported in table 7.1.2.3.6.

Table 7.1.2.3.6: Estimation of PRAVA in tablet by FTIR Spectroscopy

Formulation	Labeled Claim (mg/tab)	Peak Area		Single Wavelength Number	
		*Amount found	% Assay	*Amount found	% Assay
PRAVATOR (solus)	10	10.32	103.21	10	100

***Average of three determinations.**

7.1.2.3.4. Summary of Validation parameters:

The summary of validation parameters is given in table 7.1.2.3.7.

Table 7.1.2.3.7: Summary of Validation parameters of FTIR Spectroscopy

Sr. No	Parameters	Peak area	Peak Height
1	Wave number (1/cm)	875 to 854.41 cm^{-1}	1726.17
2	Linearity range (µg/mg)	13-70 µg/mg	
3	Regression equation	Y = 156.6x + 0.1332	Y = 34.59x - 0.232
4	Correlation coefficient (r^2)	0.9993	0.9992
5	Intercept	0.1332	-0.232
6	Slope	156.6	34.59
7	Assay	103.21	100.00
8	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements % CV % Recovery	 0.19-0.83 0.15-0.93 0.60 97.5-101.66	 0.11-0.59 0.29-0.54 0.88 100.16-103
9	Limit of detection (µg/mg)	0.0745	0.9631
10	Limit of quantification (µg/mg)	0.248	1.550

7.1.2.3.5. Conclusion:

FTIR method can estimate PRAVA with the accuracy of 95 to 103 %. The lowest quantifiable concentration of PRAVA was found to be 0.248 and 1.550 µg/mg by practical observation for peak area and Peak height single wavelength number.

7.1.2.4 Stability Indicating Reverse Phase High Performance Liquid Chromatography (HPLC) with UV detection.

7.1.2.4.1. Optimization of method:

Determination of solvent for sample preparation:

Different solvents were used depending upon its solubility. Drug solution was prepared in methanol and further dilutions were done with acetonitrile.

Determination of detection wavelength:

Detector wavelength was selected by scanning standard solutions of PRAVA over 200 nm to 400 nm wavelengths.

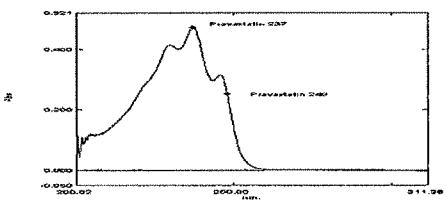


Figure 7.1.2.4.1. Simple UV spectrum of PRAVA in acetonitrile

Drug shows reasonable high λmax 237 nm and good response at 248 nm. So both the wavelengths were selected in dual channel UV detector. But validation was done at 248nm for better resolution of degradation products.

Determination of mobile phase:

Different combinations of Mobile phase were used at flow rate 1 ml/min and column C18 Phenomenex.

Table 7.1.2.4.1. Determination of mobile phase

Mobile phase combination	Ration (V/V)	Retention time (min)	Peak shape
Methanol: Water	70:30	2.990	Merge with blank peak
Methanol: Water	80:20	3.463	Merge with blank peak
Acetonitrile: Water	70:30	2.512	Merge with blank peak
Acetonitrile: 0.1 % Formic acid	80:30	2.512	Merge with blank peak
Acetonitrile: 0.1 % Formic acid	40:60	2.839	Merge with blank peak
Acetonitrile: 0.1 % Formic acid	45:55	4.400	Not merge with blank peak but tailing observed
Acetonitrile: 0.2 % Formic acid	40:60	5.601	Sharp peak but tailing at the base
Acetonitrile: 0.5 % Formic	**40:60**	**5.678**	**Sharp peak**

acid			
Acetonitrile: 0.5 % Formic acid	65:35	4.47	Sharp peak
Acetonitrile: 0.5 % Formic acid	80:20	2.25	Sharp peak

From the data, it was evident that mobile phase combination of acetonitrile: 0.5 % Formic acid in proportion of 40:60 was most suitable for the development of RP-HPLC method.

Table 7.1.2.4.2. Determination of Flow rate

Flow rate (ml/min)	**Retention time (min)**
	PRAVA
0.5	11.354
1.0	**5.642**
1.2	3.253

Most suitable Retention time is between 4 to 8 min so flow rate was selected as 1 ml/min,

Column

Two type of C18 column were tried.

Table 7.1.2.4.3 column

Column (C18, 250 X 4.60 mm)	**Retention time (min)**	**Peak hape**
	PRAVA	**PRAVA**
Hypersil	5.545	Sharp peak
Phenomenex	5.642	Sharp peak

So both the columns were suitable for development of HPLC analytical method. Here C18 Phenomenex 240X4.6 mm was selected

Method:

Chromatographic condition:

The optimized chromatographic conditions were:

- **Column:** C_{18} (size-238 x 4.60 mm, I.D-5 µm) (Phenomenex)
- **Mobile Phase:** Acetonitrile : 0.5% Formic acid (40:60 v/v)
- **Detection:** UV detection at 248 nm
- **Flow rate:** 1.0 ml/minute
- **Application volume:** 20 µl

Preparation of Mobile Phase:

Mobile phase was prepared by mixing 600 ml of Acetonitrile with 400 ml of 0.5 % formic acid (40:60, V/V). The mobile phase was filtered through nylon 0.45 µm, 47 mm membrane filter and degassed in ultrasonic bath prior to use for 30 min.

7.1.2.4.2. Preparation of stock solution:

For chromatographic study standard PRAVA (100 mg) was weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml ACN properly and diluted up to mark with ACN to obtain final concentration of 1000 µg/ml.

7.1.2.4.3. Calibration curve:

From the stock solution (1000 µg/ml), aliquots of 0.1, 0.5, 1, 1.5, 2, 2.5, and 3 ml were transferred to a series of 10 ml volumetric flasks. The volume was adjusted up to the mark with ACN to get final concentrations of 10, 50, 100, 150, 200, 250, and 300 µg/ml of solution. 20 µl of all these solutions were injected separately into HPLC column and the peak area of each solution was measured at selected wavelength. The calibration curve was constructed by plotting peak area vs. concentration (µg/ml) over the concentration range and regression equation was calculated.

7.1.2.4.4.Validaiton parameters:

Validation parameter procedures followed are described in 7.1.1.6.3.

7.1.2.4.5. Results and Discussion:

7.1.2.4.5.1. Validation Parameters

(I) Linearity

Linearity range of PRAVA was found to be 1.43–715 µg/ml with correlation co-efficient and % CV is 0.9990 and 0.19–0.88 for peak area.

Table7.1.2.4.4: Calibration data of PRAVA by HPLC with UV detection

Sr. No.	Concentration (µg/ml)	Peak Area		Rt*
		Mean*	%CV	
1	1.43	103377	0.88	5.625
2	14.3	1000317	0.42	5.617
3	21.45	2334466	0.19	5.642
4	71.5	5257915	0.43	5.667
5	214.5	12900000	0.84	5.675
6	326	19000000	0.84	5.633
7	715	41664552	0.47	5.692

***Average of five reading**

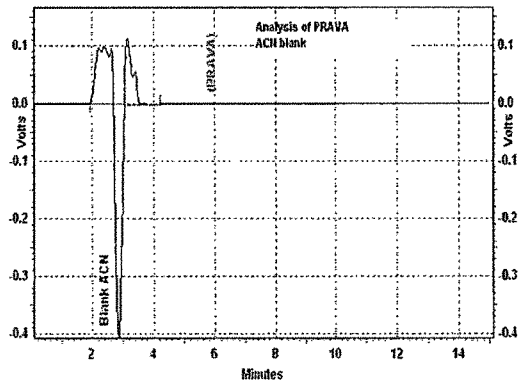


Figure 7.1.2.4.2: Single Peak of ACN Blank by HPLC with UV detection

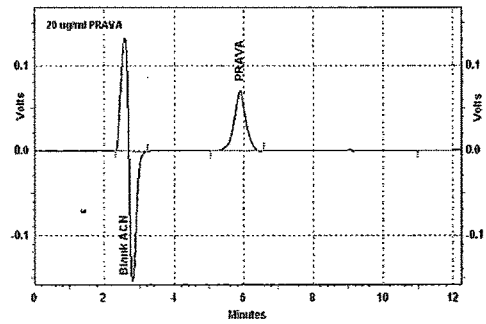


Figure 7.1.2.4.3: Single Peak of PRAVA by HPLC with UV detection

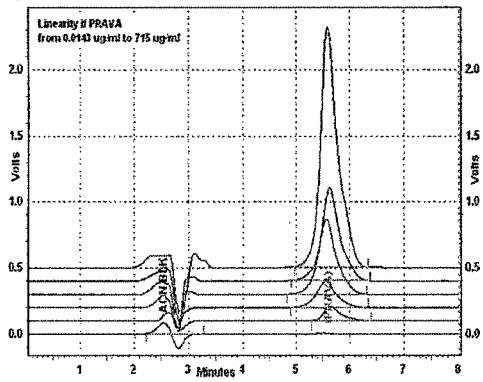


Figure 7.1.2.4.4: Overlain Peak of PRAVA by HPLC with UV detection

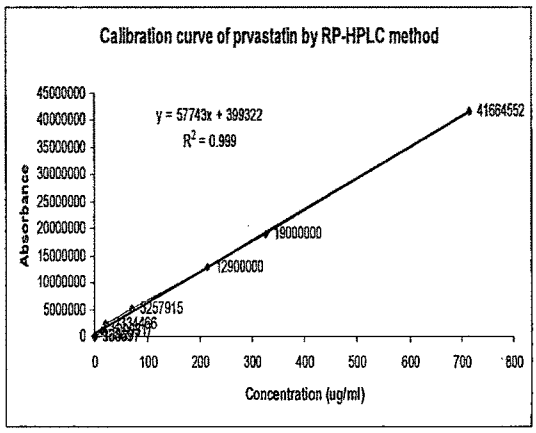


Figure 7.1.2.4.5: Calibration curve of PRAVA by HPLC

(II) Precision

A. Intraday and Interday Precision (Repeatability)

Intraday and interday variation of the proposed method was reported in table 7.1.2.4.5.

Table 7.1.2.4.5: Intraday precision data of PRAVA by HPLC with UV detection

Sr. No.	Concentration (µg/ml)	Intraday		RT*	Interday		RT*
		Peak area*	%CV		Peak area*	%CV	
1	60	3504512	0.47	5.625	3515483	0.38	5.675
2	120	6969092	0.92	5.625	6967937	0.28	5.633
3	240	13898252	0.40	5.617	13898829	0.92	5.692

***Average of five readings**

b. Reproducibility:

The % C.V. of repeated measurement of the same solution was not more than 1%.

Table 7.1.2.4.7: Reproducibility data of PRAVA (150 µg/ml) by HPLC

Time	Peak Area	RT
Hypersil C18	8701382	5.642
	8700805	5.667
	8753928	5.675
Phenomenex C18	8731986	5.633
	8710043	5.692
	8719629	5.6018
%CV	0.26 (<1%)	0.60 (<1%)

***Average of five readings**

(III) Accuracy

Accuracy of the measurement of PRAVA was determined by standard addition and was found to be in the range of 99.87-100.12 % for peak area.

Table 7.1.2.4.8: Accuracy data of PRAVA by HPLC with UV detection

Initial conc. (µg/ml) (A)	Quantity of std. Added (µg/ml) (B)	Total Amount (A + B)	Peak Area	
			Total quantity Found Mean ± S.D.	%Recovery ± S.D
100	50	150	150.51±0.65	100.12±0.49
100	100	200	199.87±0.92	99.97±1.25
100	150	250	249.23±0.58	99.87±0.32

***Average of five readings**

(IV) Limit of detection

The minimum detectable concentration of PRAVA was found to be 0.0286 µg/ml for peak area.

(V) Limit of quantification

The lowest quantifiable concentration of PRAVA was found to be 0.245 µg/ml for peak area.

7.1.2.4.5.2. Estimation of PRAVA in marketed Tablet:

(A) Preparation of test solution:

PRAVATOR of SOLUS contained 10 mg of PRAVA per Tablet were analyzed by the proposed method. 20 tablets were triturated after taking their average weight. The Tablet powder equivalent to one Tablet content was transferred into a 100 ml

volumetric flask containing 50 ml ACN, sonicated for 15 min and further diluted to 100 ml with ACN. The resulting solution was sonicated for 10 min and supernatant was filtered through Whatman filter paper no.41. 20 µl of filtrate was injected in HPLC column and peak area was measured at 248 nm. The concentration of sample solution was found from regression equation. The results are shown in table 7.1.2.4.11.

Table 7.1.2.4.9: Estimation of PRAVA in tablet by HPLC with UV detection

Tablet Formulation	Labeled Claim (mg/tablet)	Amount Found* (mg/tablet)	% Assay ± S.D
PRAVATOR (SOLUS)	10	09.96	99.61 ± 0.13

***Average of five reading**

(B) Procedure for forced degradation study

Forced degradation study was done in different condition. Solvent selected was 30% H_2O_2, water at neutral pH 7, 0.5 N HCl and 0.1 N NaOH. Approximate 25 mg drug was weighed, transferred to 25 ml volumetric flask and dissolved with 5 ml of methanol. The volume was then made up with the solvent selected for degradation. Similarly solid state stability was done after exposing 25 mg of the drug to 80^0 in a stability oven and in a photostability chamber. Samples were collected for analysis at three stages at 0 min (as soon as sample prepared), after 24 hrs and after 48 hrs of exposure to degradation condition. Fig 7.1.2.4.7 to 7.1.2.4.9 show the chromatograms of forced degraded samples.

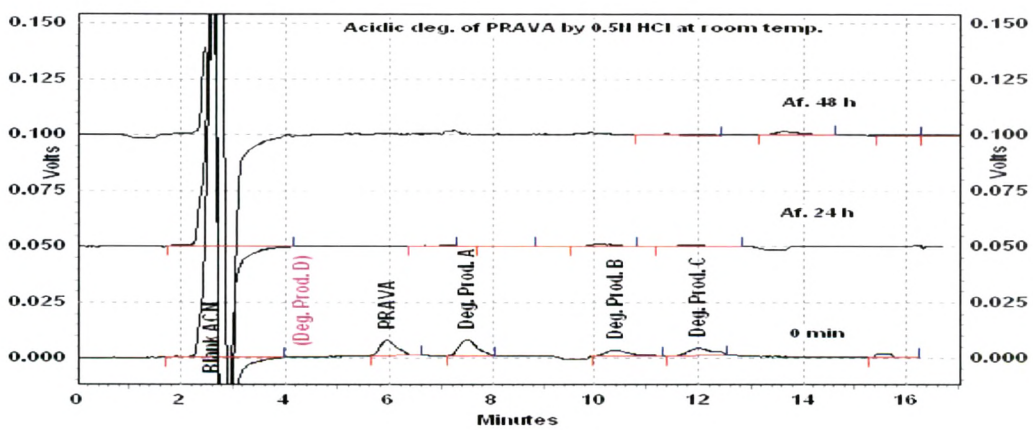


Figure 7.1.2.4.6: Chromatograms of acid hydrolysis of PRAVA in 0.5 M HCl at 0 min, 24 hrs and 48 hrs

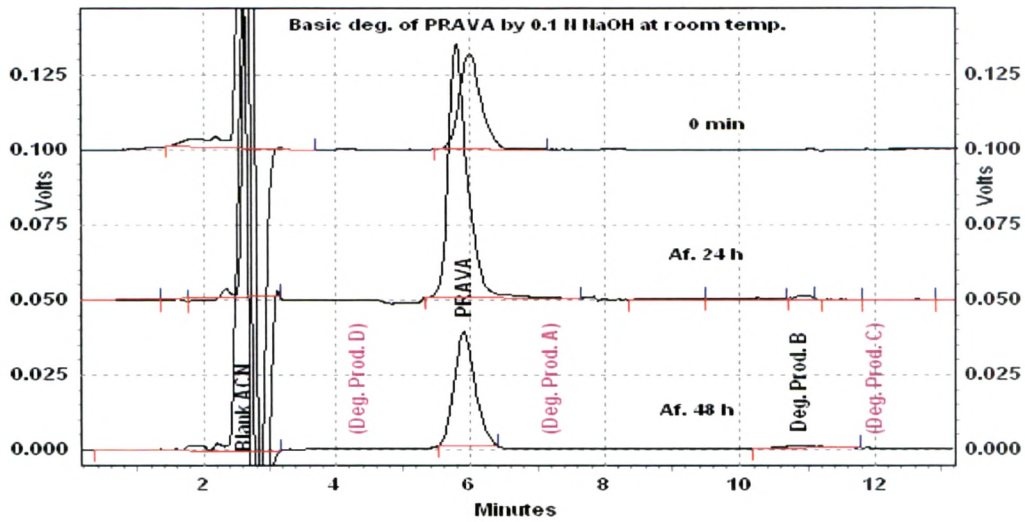


Figure 7.1.2.4.7: Chromatograms of base hydrolysis of PRAVA in 0.1 M NaOH at 0 min, at 24 hrs and 48 hrs

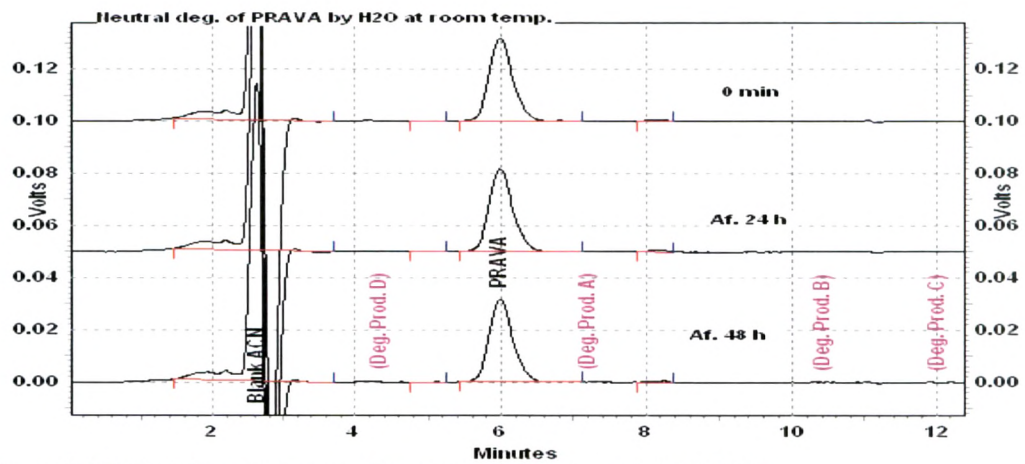


Figure 7.1.2.4.8: Chromatograms of neutral (H2O) – degraded PRAVA

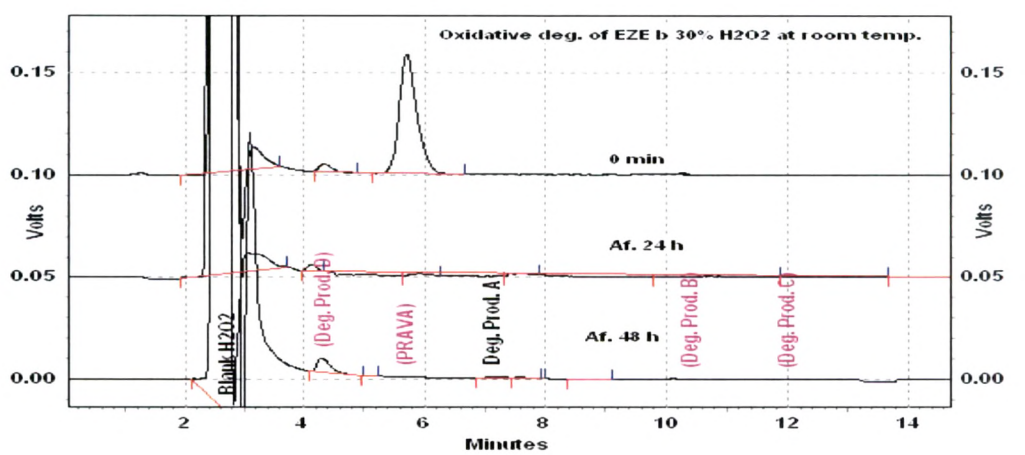


Figure 7.1.2.4.9: Chromatograms of oxidative of PRAVA in 30 % H2O2 at 0 min, 24 hrs and at 48 hrs

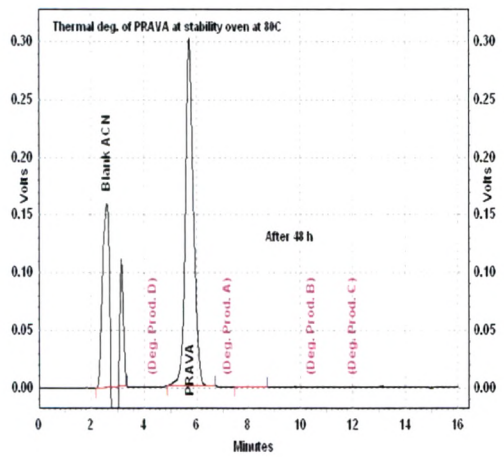


Figure 7.1.2.4.10: Chromatograms of thermal 80C at stability oven -degraded PRAVA

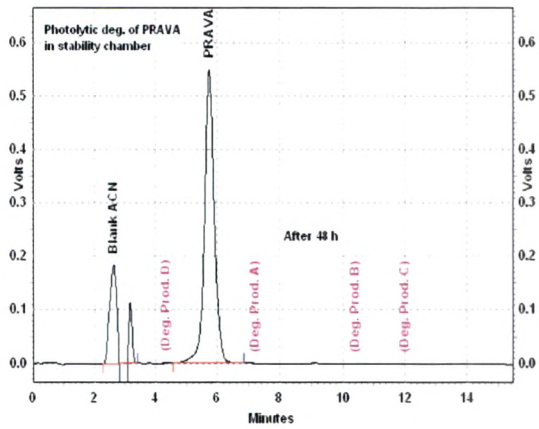


Figure 7.1.2.4.11: Chromatograms of UV/VIS photo stability chamber degraded PRAVA

Table 7.1.2.4.10: System suitability parameters of PRAVA

Sr. No	System suitability parameters	PRAVA	Degradation products			
			A	B	C	D
1	Retention time (minutes)	**5.983**	**7.267**	**10.40**	**12.008**	**4.325**
2	Theoretical plates	13821.51	27037.54	44986.02	42257.79	2031.132
3	Resolution	**6.20**	**9.22**	**3.68**	**1.73**	**3.91**
4	Asymmetry	0.97	0.78	1.29	1.32	1.21
5	Width at ½ peak height	1.32	1.52	1.37	1.15	0.70
6	Tailing factor	1.02	1.00	1.18	1.21	0.98
7	Capacity Factor	5.42	2.46	3.95	-4.72	0.97

Table 7.1.2.4.11: Percentage degradation of PRAVA by force degradation

Sr. No	Parameters (Stress condition /duration/state)	% of undergrad PRAVA	% of individual Degradation products				Total % Deg.
			A	B	C	D	
1	Neutral/H2Oat pH 7/48 hrs/ solution	100	No degradation found				
2	Acidic/0.5 N HCl/48 hrs/ solution	-	2	6	92	-	**100**
3	Alkali/0.1N NaOH/48 hrs/ solution	96	-	4	-	-	**4**
4	Oxidative/30% H2O2/48 hrs/ solution	100	-	-	-	100	**100**
5	Thermal/80 C/48 hrs/solid	100	No degradation found				
6	Photo/uv254 and Vis/366 nm/48 hrs/solid	100	No degradation found				

7.1.2.4.6. Summary of Validation parameters:

The summary of validation parameters is given in table 7.1.2.4.12.

Table 7.1.2.4.12: Summary of Validation parameters by HPLC

Sr. No	Parameters	Peak Area
1	Detection wavelength (nm)	248
2	Retention time (minutes)	5.642
3	Linearity range (µg/ml)	1.43 - 715
4	Regression equation	y = 57743x +399322
5	Correlation coefficient (r^2)	0.999
6	Intercept	399322
7	Slope	57743
8	Assay	99.61
9	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements %CV % Recovery	 0.40-0.92 0.38-0.92 0.26 99.87-100
10	Limit of detection (µg/ml)	0.0286
11	Limit of quantification (µg/ml)	0.245

7.1.2.4.7. Conclusion:

Developed HPLC method can estimate PRAVA in pharmaceutical dosage form and in presence of its degraded product. HPLC study on PRAVA under different degradation conditions suggests that PRAVA is highly degraded in acidic (fig. 7.1.2.4.6) and oxidative (fig. 7.1.2.4.9) condition. PRAVA was stable in neutral (fig. 7.1.2.4.7) and alkaline (fig. 7.1.2.4.8) condition at room temperature. There is no effect of thermal and photolytic condition on PRAVA. Main degradation was under acidic conditions where it was degraded into three minor products (fig. 7.1.2.4.6). % degradation and system suitability parameters were reported in table 7.1.2.4.10 and 7.1.2.4.11. The method can be applied even to the analysis of stability samples obtained during accelerated stability experiments.

7.1.2.5. Bio Analytical Reverse Phase High Performance Liquid Chromatography (HPLC) method for estimation of PRAVA in human plasma with UV detection.

HPLC method developed for the estimation of PRAVA in pharmaceutical formulation was tried and found to be successful for the analysis of PRAVA in human plasma. The method was repeated with plasma spiked PRAVA samples and analyzed by protein precipitation method. Human plasma was obtained from blood bank on request.

7.1.2.5.1. Preparation of stock solution for PRAVA

For chromatographic study standard PRAVA (10 mg) was weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml ACN and diluted up to mark with ACN to obtain final concentration of 100 µg/ml.

7.1.2.5.2. Calibration curve for standard Drug

From the stock solution (100 µg/ml) aliquots of 0.01, 0.4, 2, 4, 6, 8 and 10 µl were transferred to a series of sample tubes. Then 90 µl of human plasma was added to each tube. The tubes were vortexed for 1 min on Vortex shaker. The volume was adjusted to a 1000 µl with acetonitrile to get final concentrations of 1, 40, 200, 400, 600, 800 and 1000 ng/ml of solution. The solutions were centrifuged for 5 min at 4000 rpm. 20 µl of supernatant were injected into HPLC column and the peak area of each solution was measured at selected wavelength.

7.1.2.5.3. Validation Parameters

Validation procedure is given in Section 7.1.1.7.3.

7.1.2.5.4 Results and Discussion:

7.1.2.5.4.1 Validation Parameters

(I) Linearity

Linearity range of PRAVA was found to be 1.68–1000 ng/ml with correlation co-efficient and % CV is 0.9999 and 0.02 – 0.73 for peak area.

Table 7.1.2.5.1: Calibration data of PRAVA by HPLC with UV detection

Sr. No.	Concentration (ng/ml)	Peak Area		RT*
		Mean*	%CV	
1	1.68	115	0.02	5.792
2	40.1	1682	0.19	5.758
3	200	8393	0.46	5.775
4	400	16787	0.28	5.732

5	600	25181	0.58	5.775
6	800	35758	0.73	5.758
7	1000	19698	0.37	5.75

***Average of five readings**

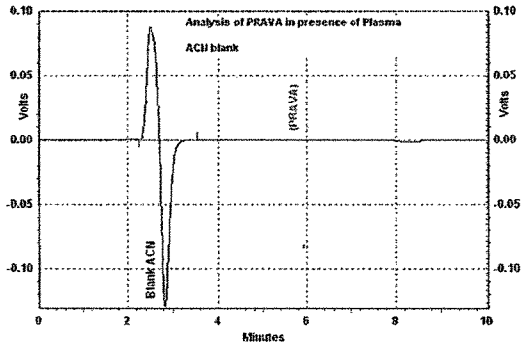


Figure 7.1.2.5.1: Single Peak of ACN blank by HPLC with UV detection

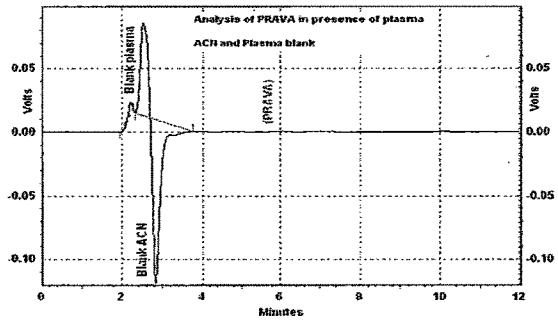


Figure 7.1.2.5.2: Single Peak of plasma blank by HPLC with UV detection

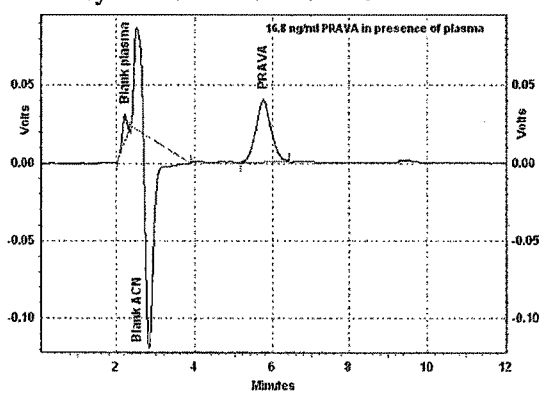


Figure 7.1.2.5.3: Single Peak of PRAVA by HPLC with UV detection

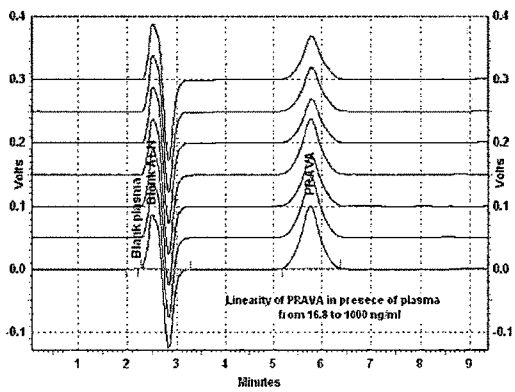


Figure 7.1.2.5.4: Overlay Peak of PRAVA by HPLC with UV detection

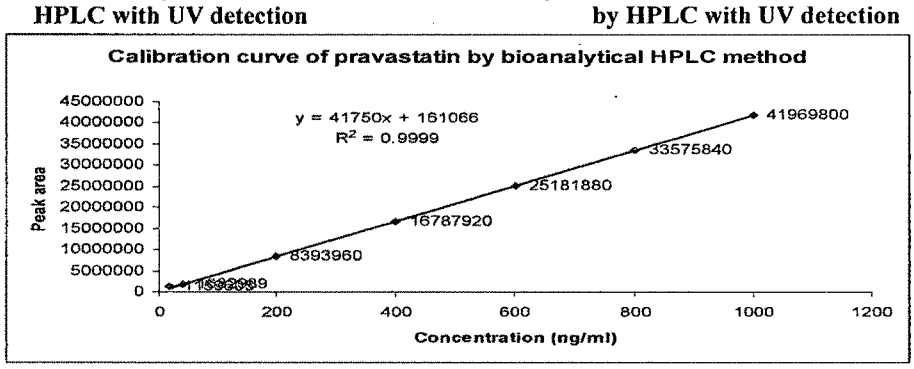


Figure 7.1.2.5.5: Calibration curve of PRAVA by HPLC with UV detection (Peak Area)

(II) Precision

Repeatability

The % C.V. of repeated measurement of the same solution was not more than 1%.

Table 7.1.2.5.2: Reproducibility data of PRAVA (150 ng/ml) by HPLC with UV detection

Column	Peak Area	RT	Column	Peak Area	RT
Hypesile C18	6295	5.775	Phenomenex C18	6296	5.758
	6307	5.732		6311	5.75
	6295	5.775		6301	6
S.D.	3.224128	0.12	S.D.	2.55	0.3115

***Average of three readings**

Intraday and Interday Precision

Intraday and interday variation of the proposed method were 0.34 – 0.48 % and 0.38 – 0.46 % for peak area.

Table 7.1.2.5.3: Intraday precision data of PRAVA by HPLC with UV detection

Sr. No.	Concentration (ng/ml)	Intraday		RT*	Interday		RT*
		Peak Area *	%CV		Peak Area *	%CV	
1	50	2098	0.34	5.775	2099	0.38	5.775
2	250	9988	0.28	5.732	9990	0.37	5.758
3	500	20984	0.48	5.775	20999	0.46	5.75

***Average of five readings**

(III) Accuracy

Accuracy of the measurement of PRAVA was determined by standard addition and was found to be in the range of 99.7-100.32 % for peak area.

Table 7.1.2.5.5: Accuracy data of PRAVA by HPLC with UV detection

Initial conc. (ng/ml) (A)	Quantity of std. Added (ng/ml) (B)	Total Amount (A + B)	Peak Area	
			Total quantity Found Mean ± S.D.	%Recovery ± S.D
120	60	180	180.23±0.34	100.32±0.20
120	120	240	240.07±0.12	100.29±0.25
120	200	320	319.92±0.18	99.97±0.12

***Average of five readings**

(IV) Limit of detection

The minimum detectable concentration of PRAVA was found to be 0.0889 ng/ml for peak area.

(V) Limit of quantification

The lowest quantifiable concentration of PRAVA was found to be 0.29661 ng/ml for peak area.

(VI) Stability

1. Freez and Thaw Stability

Samples were prepared at LQC and HQC levels, aliquoted and frozen. Six samples from each concentration were subjected to three frePRAVA and thaw cycles (stability samples). These samples were processed after 3rd cycle and analyzed along with freshly prepared calibration standards, LQC and HQC samples (comparison samples). Concentrations were calculated to determine % Mean change after 3rd cycle.

PRAVA was found to be stable in LQC and HQC samples after 3rd cycle after frozen with % Mean change of 1.67 and -0.05 respectively (Table No. 7.1.2.5.6)

Table 7.1.2.5.6: FrePRAVA and thaw stability of PRAVA

Sr. No.	LQC (15.000 ng/ml)				HQC (680.000 ng/ml)			
	Comparison samples		Stability samples		Comparison samples		Stability samples	
	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.
1	14.982	99.88	14.146	94.31	680.12	100.02	681.03	100.15
2	15.028	100.19	15.723	104.82	679.34	99.90	678.98	99.85
3	15.239	101.59	15.023	100.15	680.23	100.03	680.23	100.03
4	14.973	99.82	14.279	95.19	680.03	100.00	680.63	100.09
5	15.087	100.58	14.792	98.61	679.34	99.90	680.17	100.03
6	15.047	100.31	14.887	99.25	679.47	99.92	679.67	99.95
N	6		6		6		6	
Mean	15.06		14.81		679.76		680.12	
% Mean Change	1.67				-0.05			

2. Short-Term Temperature Stability

LQC and HQC samples were spiked in human plasma and were kept at room temperature for 11.0 hours and were processed and analyzed along with freshly prepared calibration standards, LQC and HQC samples. Concentrations were calculated to determine % Mean change during stability period.

PRAVA was found to be stable in LQC and HQC samples for 11.0 hours at room temperature with % Mean change of -0.83 and -0.21 respectively (Table No. 7.1.2.5.7).

Table 7.1.2.5.7: Short term temperature stability

Sr. No.	LQC (15.000 ng/ml)				HQC (680.000 ng/ml)			
	Comparison samples		Stability samples		Comparison samples		Stability samples	
	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.
1	14.82	98.80	16.146	107.64	680.14	100.02	678.93	99.84
2	15.08	100.53	15.23	101.53	679.784	99.97	689.34	101.37
3	15.39	102.60	15.02	100.13	681.23	100.18	680.39	100.06
4	14.73	98.20	14.29	95.27	679.34	99.90	680.03	100.00
5	14.087	93.91	15.792	105.28	679.01	99.85	680.97	100.14
6	15.747	104.98	14.12	94.13	681.04	100.15	679.47	99.92
N	6		6		6		6	
Mean	14.98		15.10		680.09		681.52	
% Mean Change	-0.83				-0.21			

3. Long-Term Stability

PRAVA was found to be stable in human plasma at below -20°C after 45 Days in LQC, MQC and HQC samples with % Mean Change of 2.12 and -0.01 respectively, (Table No. 7.1.2.5.8).

Table 7.1.2.5.8: long term stability

Sr. No.	LQC (15.000 ng/ml)				HQC (680.000 ng/ml)			
	Comparison samples		Stability samples		Comparison samples		Stability samples	
	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.
1	14.32	95.47	16.16	107.73	680.44	100.06	679.34	99.90
2	15.03	100.20	14.23	94.87	679.74	99.96	679.34	99.90
3	15.79	105.27	14.02	93.47	680.23	100.03	680.78	100.11
4	14.89	99.27	14.29	95.27	679.94	99.99	680.33	100.05
5	14.07	93.80	15.02	100.13	678.01	99.71	680.04	100.01
6	16.047	106.98	14.52	96.80	681.06	100.16	679.97	100.00

N	6		6		6		6	
Mean	15.02		14.71		679.90		679.97	
% Mean Change	2.12				-0.01			

4. Stock Solution Stability

Stock solution stability was determined by comparing the peak areas of freshly prepared solutions (comparison samples) with stability samples.

PRAVA stock solution was found to be stable at 2-8°C for 7 days with % Mean change of 2.41 (Table No. 7.1.2.5.9).

Table 7.1.2.5.9: Stock solution stability

Sr No.	PRAVA(800.000 ng/ml)	
	Compe. sampl area	Stability sample area
1	35758	35854
2	36144	36262
3	35770	35762
4	34931	35854
5	35481	35292
6	35758	35854
N	6	6
%CV	0.134	0.1030
Mean % Change	2.41	

(VII) Matrix effect

In order to study the effect of matrix through out the application of the method, plasma blanks obtained from two different lots were spiked with PRAVA at LQC and HQC level. Three quality control samples at each level along with the set of calibration standards were analyzed and the % nominal concentration of PRAVA in the samples analyzed was represented in (Table No. 7.1.2.5.10).

Table 7.1.2.5.10: Matrix effect for PRAVA

Sample ID		Calculated Conc. (ng/ml)	% Nominal Conc.	Sample ID		Calculated Conc. (ng/ml)	% Nominal Conc.
I	LQC	14.98	99.87	I	HQC	680.34	100.05

	LQC	15.05	100.33		HQC	680.34	100.05
	LQC	15.09	100.60		HQC	679.78	99.97
II	LQC	14.99	99.93	II	HQC	680.72	100.11
	LQC	14.67	97.80		HQC	680.14	100.02
	LQC	15.77	105.13		HQC	680.04	100.01

7.1.2.5.1.2. Estimation of PRAVA in marketed Tablet:

The Tablets were analyzed in presence of plasma by proposed method and the percentage of PRAVA was found from the calibration curve of PRAVA. It was found that excipients as well as plasma do not interfere with the method.

The contents of 20 Tablets were weighed and their mean weight determined and finely powdered. An equivalent weight of the are Tablet content was transferred into a 10 ml volumetric flask containing 5 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution was sonicated for 10 min and supernatant was filtered through whatman filter paper no.41. 1 µl from this solution was transferred to sample tube. 90 µl of plasma sample was added add and this was vortexed for 1 min on vortex shaker. The volume was adjusted to 1000 µl with ACN to get final concentrations of 100 ng/ml of solution. This was centrifuged for 5 min at 4000 rpm and 20 µl of supernatant were injected into HPLC column and the Peak area of each solution was measured at selected wavelength. The concentration of sample solution was found from regression equation and result of amount found as per labeled claim was reported in Table 7.1.1.5.11.

Table 7.1.2.5.11: Estimation of PRAVA in tablet by HPLC with UV detection

Tablet Formulation	Labeled Claim (mg/tablet)	Amount found (mg/tablet)	% Assay ± S.D
PRAVATOR(SOLUS)	10	10.02	100.24 ± 0.56

***Average of five readings**

7.1.2.5.2. Summary of Validation parameters:

The summary of validation parameters is reported in table 7.1.2.5.12.

Table 7.1.2.5.12: Summary of Validation parameters by HPLC with UV detection

Sr. No	Parameters	Peak Area
1	Detection wavelength (nm)	248

2	Retention time (minutes)	5.983
3	Theoretical plates	13821.51
4	Asymmetry	0.93
5	USP width (at ½ peak height)	1.32
6	Linearity range (ng/ml)	16.8 - 1000
7	Regression equation	Y = 41750x + 161066
8	Correlation coefficient (r^2)	0.9999
9	Intercept	161066
10	Slope	41750
11	Assay	100.24
12	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Repeatability of measurements %CV % Recovery	 0.34-0.48 0.38-0.46 0.12 99.97 – 100.32
13	Limit of detection (ng/ml)	0.0889
14	Limit of quantification (ng/ml)	0.2966

7.1.2.5.3. Conclusion:

The developed HPLC method could be successfully used to determine PRAVA in presence of human plasma. It help in study of PRAVA in human volunteers.

7.1.2.6. High Performance Thin Layer Chromatography (HPTLC) with UV detection.

Two HPTLC methods were developed for the estimation of PRAVA. Method A was developed for the estimation of PRAVA in oral dosage form without any interference of excipients. This method was not able to separate degradation products of PRAVA from the PRAVA standard. So the method B was developed as the stability indicating assay method (SIAM).

7.1.2.6.1. Optimization of method:

The chromatographic parameters were optimized to achieve the best resolution.

Determination of solvent and analytical wavelength:

Different solvents were tried to study the solubility of PRAVA as stated in table 7.1.1.8.1. PRAVA was soluble in methanol so methanol was selected for the preparation of drug solutions. Wavelength was selected by scanning standard solutions of the drug over 200 nm to 400 nm wavelengths. Drug shows significant absorbance at **237 nm** as illustrated in fig. 7.1.2.1.1. So this wavelength was selected in densitometer UV detector.

Determination of mobile phase:

Various combinations of mobile phases were tried for sharp peak of PRAVA on silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 μm thickness) (E. Merck). The results are reported in Table 7.1.2.6.1.

Table 7.1.2.6.1: Determination of mobile phase

Mobile phase combination	Ration (V/V/V)	Peak separation
Chloroform: Methanol	8: 2	Broad peak
Chloroform: Methanol	5: 5	Broad peak
Benzene: Methanol	8: 2	Broad peak
Benzene: Methanol	5: 5	Broad peak
Toluene: Methanol	**7: 3**	**Sharp peak**
Hexane: Methanol	5: 5	Broad peak
Ethyl acetate : Toluene: Methanol	3: 4: 3	Broad peak
Ethyl acetate: Toluene: Methanol	4: 4: 2	Broad peak
Ethyl acetate: Benzene: Methanol	3: 4: 3	Broad peak
Ethyl acetate: Toluene: ACN : Formic acid	**6: 3.5: 0.5: 0.2**	**Sharp peak**
Chloroform: Toluene: Methanol: Glacial acetic acid	3: 4 : 4: 0.05	Broad peak
Chloroform: Toluene: Methanol: Glacial acetic acid	4: 4: 2: 0.2	Broad peak

- It is evident from the data that mobile phase combination of **Toluene: Methanol** is suitable and was selected as solvent method A for estimation of PRAVA in dosage form and **Ethyl acetate: Toluene: ACN: Formic acid** in proportion of **6: 3.5: 0.5: 0.2** v/v/v/v was selected in method B for separation of PRAVA form its acidic degradates. The chromatographic condition for spotting on plate are given in Chapter 6.

7.1.2.6.2. Chromatographic conditions:

Method A

- **Mobile phase:** Toluene : Methanol (7:3 v/v)
- **Scanning Wave length:** 238 nm

Method B

- **Mobile phase:** Ethylacetate: Toluene: Acetonitrile: formic acid (6:3.5:0.5:0.2 v/v/v/v)
- **Scanning Wave length:** 237 nm (showed better resolution in presence of degraded products)
- Other chromatographic conditions are mentioned in chapter 6.

7.1.2.6.3. Preparation of stock solution for PRAVA

For chromatographic study, standard PRAVA (100 mg) was weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml methanol and diluted up to mark with methanol to obtain final concentration of 1000 µg/ml.

7.1.2.6.4. Calibration curve for PRAVA:

Analysis was performed on Pre-coated silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 µm thickness) (E. Merck) pre-washed with methanol and then dried for 30 minute at 50°C. From the stock solution (1000 µg/ml) aliquots of 0.3, 0.6, 0.9, 1.8, 2.7 and 3.8 µl were spotted on the TLC plate under nitrogen stream using Desaga Applicator, AS30win to obtain final concentration range 300 - 3800 ng/spot.

The plate was dried in a FTIR, and then the plate was developed in Twin trough developing chamber (100 × 100) with stainless steel lid, previously saturated with the mobile phase for 30 min. The plate was removed from the chamber, dried in a FTIR and was scanned and quantified at 238 and 237 nm in Re-emission/Excitation mode with Desaga TLC scanner, Proquant. The calibration curve was constructed by plotting peak area vs. concentration (ng/spot) and peak height vs. concentration (ng/spot) corresponding to each spot and regression equation was calculated.

7.1.2.6.4. Validation of method:

Validation of method was done as per prescribed earliar.

7.1.2.6.5. Results and Discussion:

7.1.2.6.5.1 Validation Parameters

(I) Linearity: Linearity range of PRAVA was found to be 318-3816 ng/spot for both methods. Correlation co-efficient and % C.V. for method A was 0.9995 and 0.12 –

0.89 for peak area, 0.9999 and 0.14-0.78 for peak height and for method B was 0.9988 and 0.38-0.72%, respectively.

Table 7.1.2.6.2: Calibration data of PRAVA by HPTLC with UV detection

Sr. No.	Conc. (ng/spot)	Method A					Method B		
		Peak Area		Peak Height		R_f*	Peak Area		R_f*
		Mean	%CV	Mean	%CV		Mean	%CV	
1	318	10.16	0.89	46.00	0.14	0.27	263.92	0.48	0.49
2	636	96.19	0.12	80.00	0.45	0.23	627.49	0.82	0.49
3	1060	196.69	0.34	126.34	0.78	0.23	1284.92	0.72	0.48
4	1480	276.05	0.46	173.62	0.45	0.23	2192.34	0.38	0.50
5	2862	605.56	0.36	315.80	0.67	0.24	3940.98	0.89	0.49
6	3816	823.21	0.25	416.42	0.47	0.25	5329.43	0.41	0.49

***Average of five readings**

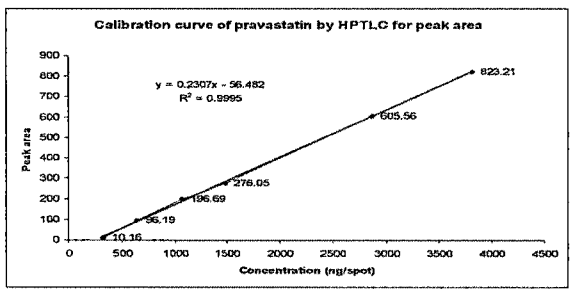


Figure 7.1.2.6.1: Calibration curve of PRAVA by HPTLC by method A (Peak Area)

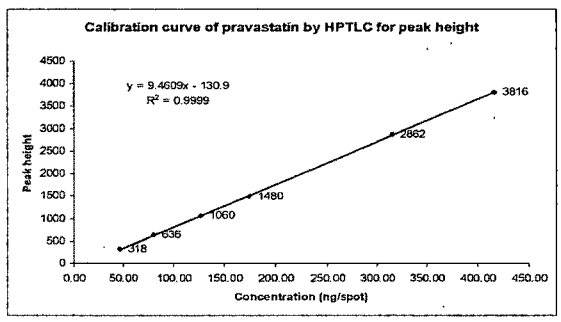


Figure 7.1.2.6.2: Calibration curve of PRAVA by HPTLC by method A (Peak Height)

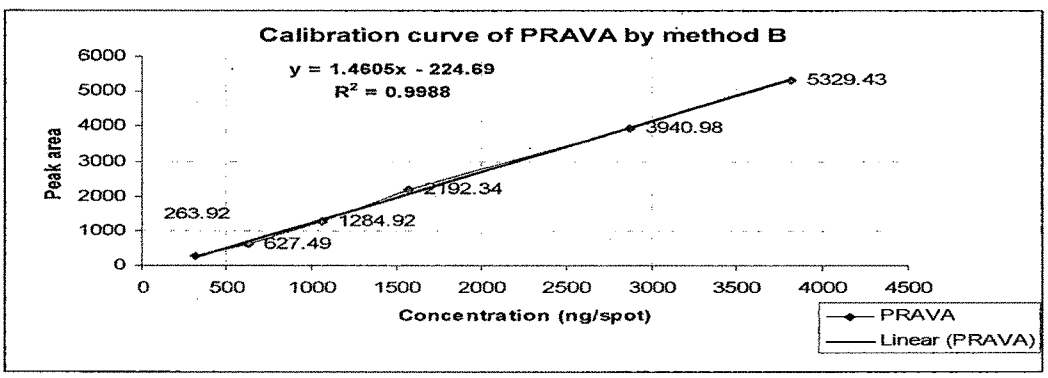


Figure 7.1.2.6.3: Calibration curve of PRAVA by HPTLC by method B (Peak Area)

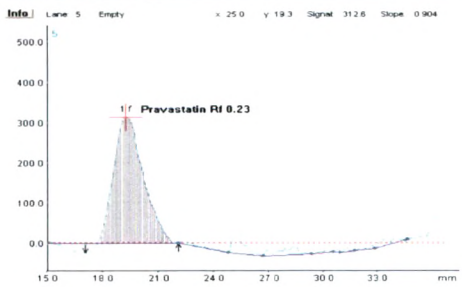


Figure 7.1.2.6.4: Single Spectrum of PRAVA by HPTLC by method A

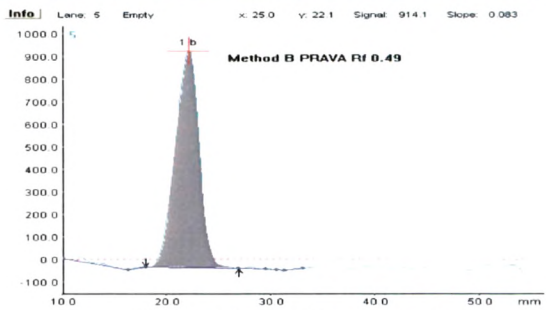


Figure 7.1.2.6.5: Single Spectrum of PRAVA by HPTLC by method B

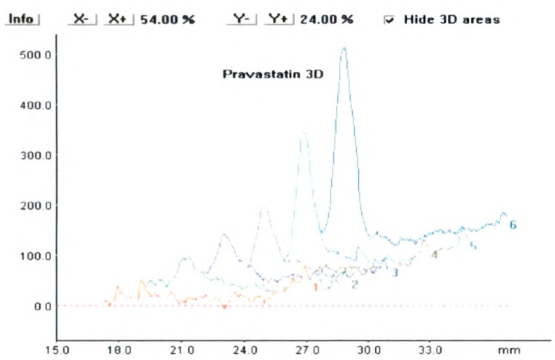


Figure 7.1.2.6.6: 3D Spectra of PRAVA by HPTLC by method A

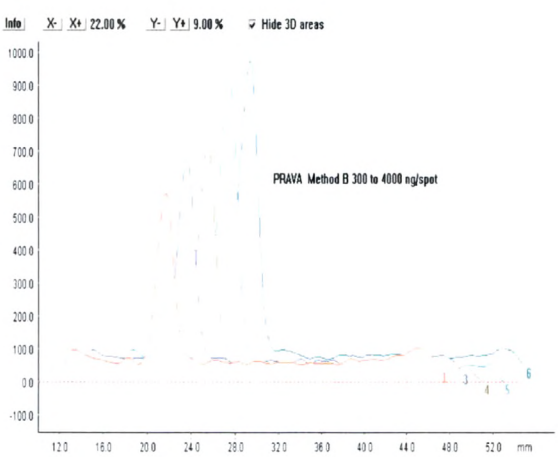


Figure 7.1.2.6.7: 3D Spectra of PRAVA by HPTLC by method B

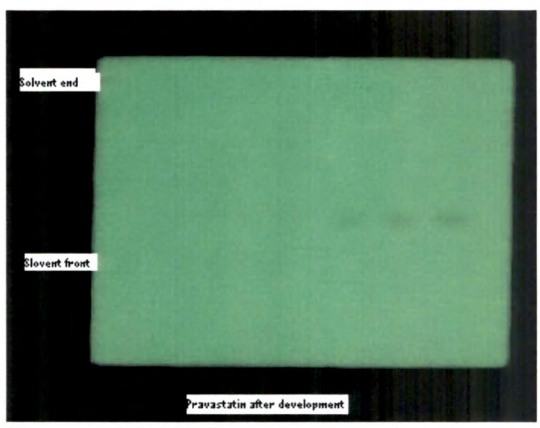


Figure: 7.1.2.6.8: Chromatogram of PRAVA by method A (After detection)

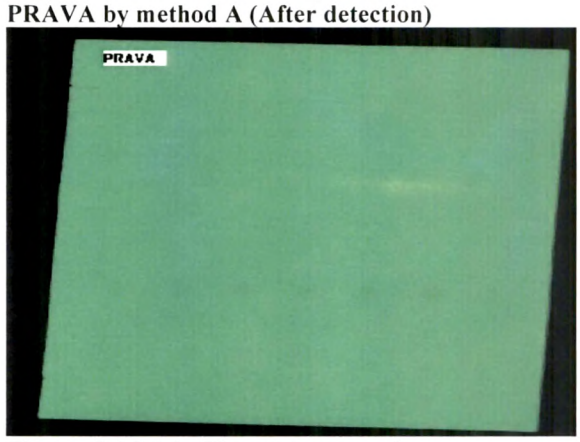


Figure: 7.1.2.6.9: Chromatogram of PRAVA by method B (After detection)

(II) Precision

a. Repeatability

Intraday and Interday Precision

Intraday and interday variation of the method A and B were reported in table 7.1.2.6.3.

Table 7.1.2.6.3: Intraday precision data of PRAVA by HPTLC

Sr. No.	Conc. (ng/spot)	Method A					Method B		
		Peak Area		Peak Height		R_f*	Peak Area		R_f*
		Mean ± SD	%CV	Mean ± SD	%CV		Mean ± SD	%CV	
1	500	87.04±0.49	0.22	58.27±0.41	0.72	0.22	414.969	0.89	0.49
2	1500	366.01±1.13	0.23	192.09±0.64	0.65	0.23	2479.93	0.32	0.48
3	2000	522.40±0.97	0.12	225.07±0.93	0.47	0.23	2924.38	0.41	0.49
Table 7.1.2.6.4: Interday precision data of PRAVA by HPTLC									
1	500	87.90 ± 0.34	0.23	54.58 ± 0.23	0.67	0.27	416.094	0.48	0.49
2	1500	363.64 ± 0.23	0.15	193.71 ± 0.25	0.29	0.23	2494.59	0.21	0.48
3	2000	531.23 ± 0.34	0.24	228.14 ± 0.23	0.98	0.23	2954	0.56	0.48

b. Reproducibility:

The % C.V. of repeated measurement of the same solution was not more than 1%.

Table 7.1.2.6.5: Reproducibility data of PRAVA by HPTLC with UV detection(400 ng/ml)

Time	Method A			Method B	
	Peak Area	Peak Height	Rf	Peak Area	R_f
Aluminum plate	42.69	58.87	0.23	348.98	0.49
	42.69	58.87	0.23	355.39	0.49
	42.65	58.84	0.23	349.58	0.48
Glass plate	42.72	58.90	0.24	348.94	0.49
	42.68	58.87	0.25	359.30	0.49
	42.69	**58.87**	**0.24**	348.69	0.49
S.D.	0.0242	0.0201	0.0089	4.47	0.004
%CV	0.11 (<1%)	0.11 (<1%)	3.79 (>1%)	1.77	0.84

(III) Accuracy

Accuracy of the measurement of PRAVA was determined by standard addition method. Accuracy was found to be in the range of 99.98-101.23 % for peak area and 99.98-100.04 % for peak height by method A and 99.99-103.97 % by method B.

Table 7.1.2.6.6: Accuracy data of PRAVA by HPTLC with UV detection

Spotted Amount (A + B)	Method A				Method B	
	Peak Area		Peak Height		Peak Area	
	Total quantity Found Mean ± S.D.	%Recovery ± S.D	Total quantity Found Mean ± S.D.	%Recovery ± S.D	Total quantity Found Mean	%Recovery
750	750.07 ± 0.34	100.07 ± 0.89	749.98 ± 0.98	99.98 ± 0.26	749.93	99.99
1000	999.98 ± 0.54	99.98 ± 0.45	1000.34 ± 0.35	100.04 ± 0.37	1039.74	103.97
1500	1501.11 ± 0.35	101.23 ± 0.46	1499.98 ± 0.75	99.99 ± 0.48	1524.32	101.62

(IV) Limit of detection

The minimum detectable concentration of PRAVA was found to be 3.825 ng/spot for peak area and 0.284 ng/spot for peak height for method A and for method B was 5.298 ng/spot of peak area.

(V) Limit of quantification

The lowest quantifiable concentration of PRAVA was found to be 12.752 ng/spot for peak area and 0.948 ng/spot for peak height for method A and for method B was 8.392 ng/spot of peak area.

7.1.2.6.5.2. Estimation of PRAVA in marketed Tablet:

(A) Preparation of test solution:

Taking their average weight triturated 20 Tablets. The Tablet powder equivalent to one Tablet was transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution was sonicated for 10 min and supernatant was filtered through Whatman filter paper no.41. From the filterate 0.5 µl was spotted on TLC plate under nitrogen stream using Desaga Applicator, AS30win, was developed and scanned three times without changing plate position at selected wavelength in Reemission/Excitation mode with Desaga TLC scanner, Proquant and concentration of sample solution was found from regression equation computed from calibration curve of drugs. The plate was removed from the chamber, dried in air and was scanned and quantified at 238 and 267 nm in Reemission/Excitation mode with Desaga TLC scanner, Proquant. The concentration of sample solution was found from regression equation. Results are reported in the table 7.1.2.6.6.

Table 7.1.2.6.7: Estimation of PRAVA in tablet by HPTLC with UV detection

Tablet Formulation	Labeled Claim (mg/tablet)	Method A				Method B	
		Peak Area		Peak Height		Peak Area	
		Amount found (mg/tablet)	% Recovery ± S.D	Amount found (mg/tablet)	% Recovery ± S.D	Amount found (mg/tablet)	% Recovery ± S.D
PRAVATOR (SOLUS)	10	10.32	103.21	10.09	100.98	9.93	99.30

***Average of five readings**

(B) Applicability of method B:

Method B can easily estimate PRAVA in pharmaceutical dosage form but it could estimate of PRAVA in presence of degradation products. From the developed SIAM HPLC method and study of inherent character of PRAVA it was evident that PRAVA was stable against thermal, photostable, neutral and alkaline conditions. So degradation was done only under oxidative and acidic stress conditions. No specific degradation product available in oxidative condition. So HPTLC method was tried for the acidic condition only.

Procedure for forced degradation study

Forced degradation study was done in acidic condition only. For acidic condition concentrated HCl was used at room. Approximate 25 mg drug was accurate weighed and transferred to 25 ml volumetric flask. The drug was dissolved in 5 ml of methanol then diluted with concentrated HCl for degradation. Samples were colleted for analysis at two stages, at 0 min (as soon as sample was prepared), after 1 hrs and after 4 hrs of exposure to degradation condition for room temperature. Sample was applied by direct spotting of 1 µl on prewashed TLC plate. Fig 7.1.2.6.9 to 7.1.2.6.11 shows the chromatograms of forced degraded samples.

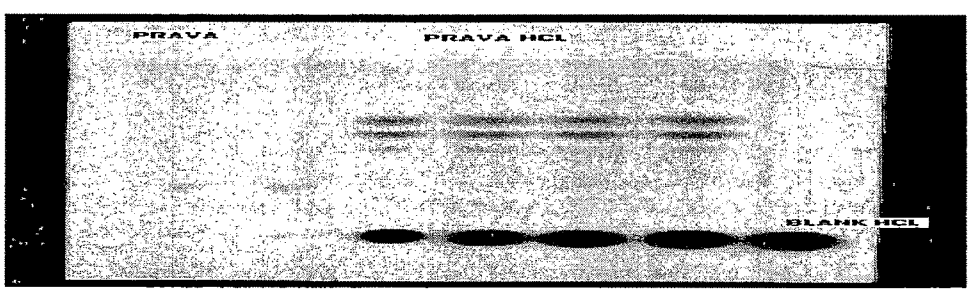


Figure: 7.1.2.6.10: Chromatogram of PRAVA in acidic degradation with UV detection (After detection)

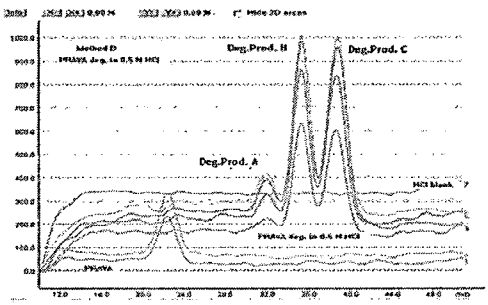


Figure: 7.1.2.6.11: Chromatogram of PRAVA in acidic degradation (acidic degradation show three degradation product)

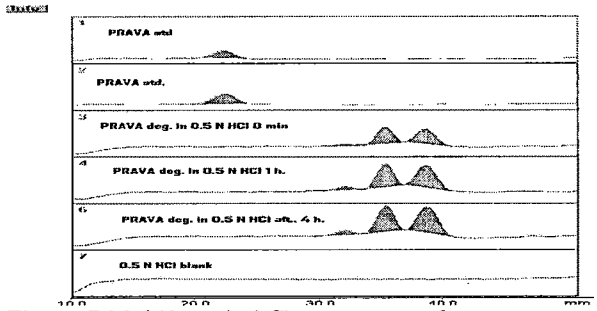


Figure: 7.1.2.6.12:vertical Chromatogram of PRAVA in acidic degradation (acidic degradation show three degradation products)

7.1.2.6.6. Summary of Validation parameters:

The summary of validation parameters is given in table 7.1.2.6.8

Table 7.1.2.6.8: Summary of Validation parameters by HPTLC

Sr. No	Parameters	Method A		Method B
		Peak Area	Peak Height	Peak Area
1	Detection wavelengths (nm)	238	238	237
2	Linearity range (ng/spot)	100 – 3672		
3	Regression equation	Y = 0.2307 x conc.– 56.482	Y= 9.4609 x conc.+ 130.9	Y= 1.4605 x conc.+ 224.69
4	Correlation coefficient (r^2)	0.9995	0.9999	0.9988
5	Intercept	-56.482	130.9	224.69
6	Slope	0.2307	9.4609	1.4605
7	Assay	103.21 %	100.98 %	99.30 %
8	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measureme %CV (<1%) % Recovery	 0.12-0.23 % 0.15-0.24% 0.11 99.98-101.2	 0.47-0.72% 0.29-0.98% 0.11 99.98-100.04	 0.32-0.89 0.21-0.56 1.77 99.99-103.97
9	Limit of detection (ng/spot)	3.825	0.284	5.298
10	Limit of quantification (ng/spot)	12.752	0.948	8.392

7.1.2.6.7. Conclusion:

HPTLC method was used to estimate PRAVA in dosage form and to estimate PRAVA in presence of its acid degradation products. The fig. 7.1.2.6.8 to 7.1.2.6.12 show that three main degradation products, study help in understanding degradation and synthetic pathway of PRAVA.

7. 1. 3

Rosuvastatin

7.1.3 Rosuvastatin (ROSU)

Here in present investigation spectrophotometric and chromatographic methods were developed. Spectrophotometric methods were used to study the ROSU in pharmaceutical tablet dosage form. Chromatographic methods were developed as stability indication methods after separating degradation products from ROSU standard.

Spectrophotometric methods:

Four spectrophotometric methods were developed. First was simple spectrophotometric method in methanol. From the zero order spectrophotometry, a derivative spectrophotometric method was tried. In derivative spectrophotometry only first derivative method was workable as the second derivative spectra were complicated and with low absorbance. Three wavelengths absorbance method was also tried. FTIR spectroscopic method was also developed for estimation of ROSU from pharmaceutical formulation as forth method.

Chromatography

To study the stability of ROSU, stress degradation was done. HPLC method was developed which can study ROSU in presence of degraded products. HPTLC methods were developed which can estimate ROSU form pharmaceutical formulation and also in preparation of degradation product. To study the pharmacokinetic data HPLC method was developed for the estimation of ROSU in spiked human plasma.

Apparatus, instruments and reagents:

Apparatus, instruments and reagents used for the development of spectroscopic and chromatographic methods are mentioned in chapter 6.

7.1.3.1 Estimation of ROSU by zero order, first order derivative and three wavelength Spectrophotometric methods.

Experimental work:

7.1.3.1.1. Preparation of stock solution for ROSU

Standard ROSU (10 mg) was weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml methanol and diluted up to mark with methanol to obtain final concentration of 100 µg/ml. This solution was used as working standard solution for simple zero order, three wavelengths and derivative methods.

7.1.3.1.2. Selection of analytical wavelengths:

Zero order Spectrophotometric method was developed to analyze the ROSU in methanolic solution. 10 ug/ml of methanolic solution of ROSU was scanned in UV PC software which shows highest absorbance at 243 nm (fig 7.1.3.1.1). So 243 nm was selected for further study. All spectra were stored in UV PC software for further study of derivative spectrophotometry. Zero order spectral curves were transformed in derivative command by transformation function of UV PC software. First order derivative curves were studied and maximum at 234.00 nm and minimum at 251.20 nm were selected for further studies. The amplitude difference was measured for the each concentration of standard (Fig. 7.1.3.1.2). The three analytical wavelengths selected were WL1 = 230.20 nm, WL2 = 243.00 nm and WL3 = 273.20 nm. The absorbance of all the solutions was measured at these wavelengths and net absorbance (Fig. 7.1.3.1.3) of ROSU was measured using the equation.

7.1.3.1.3. Calibration curve:

From the stock solution (100 µg/ml) aliquots of 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 ml were transferred to a series of 10ml volumetric flasks. The volume was adjusted to a mark with methanol to get 1, 5, 10, 15, 20, 25, 30, 35 and 40 µg/ml of solution. The absorbance was measured at λ_{max} or selected wavelength using methanol as a blank.

7.1.3.1.4. Validation Parameters

Validation of analytical method was done as per procedure given in section 7.1.1.1.2. Aliquots was taken form the stock solution of ROSU

7.1.3.1.5. Results and Discussion:

7.1.3.1.3.1 Validation Parameters

(I) Linearity (Calibration curve for standard ROSU):

Linearity range of ROSU in methanol was found to be 1-40 µg/ml with correlation co-efficient 0.9997, 0.9997 and0.9997, respectively for zero order, first order derivative and three wavelength Spectrophotometric methods and % C.V. ranging from 0.15 – 0.93, 0.10 - 0.86 and 0.28 - 0.98, respectively for zero order, first order derivative and three wavelength Spectrophotometric methods.

Table 7.1.3.1.1: Calibration data of ROSU by Spectrophotometry

Sr. No.	Conc. (µg/ml)	Simple UV Spectro-photometric method	1st derivative Spectrophotometric Method			Three wavelength Spectrophotometric Method			
		Absorb.* ± %CV (N=5)	Absorb. at 234.00 nm	Absorb. at 251.20 nm	Amplitude Difference* ± CV	Absorb. at 230.20 nm	Absorb. at 243.00 nm	Absorb. at 273.20 nm	Net Absorb.* ± CV (N=5)*
1	1	0.030 ± 0.23	0.003	-0.001	0.004 ± 0.34	-	-	-	-
2	5	0.198 ± 0.15	0.012	-0.006	0.018 ± 0.56	0.150	0.198	0.071	0.072 ± 0.34
3	10	0.396 ± 0.26	0.024	-0.01	0.034 ± 0.86	0.301	0.396	0.145	0.141 ± 0.28
4	15	0.577 ± 0.83	0.032	-0.019	0.051 ± 0.39	0.427	0.577	0.210	0.215 ± 0.87
5	20	0.754 ± 0.38	0.043	-0.026	0.069 ± 0.29	0.538	0.735	0.239	0.286 ± 0.34
6	25	0.942 ± 0.49	0.054	-0.032	0.086 ± 0.10	0.691	0.942	0.343	0.355 ± 0.28
7	30	1.179 ± 0.93	0.063	-0.039	0.102 ± 0.11	0.856	1.179	0.480	0.435 ± 0.98
8	35	1.346 ± 0.49	0.071	-0.046	0.117 ± 0.19	0.995	1.368	0.528	0.512 ± 0.76
9	40	1.532 ± 0.39	0.081	-0.055	0.136 ± 0.84	1.113	1.532	0.565	0.582 ± 0.78

***Average of five readings**

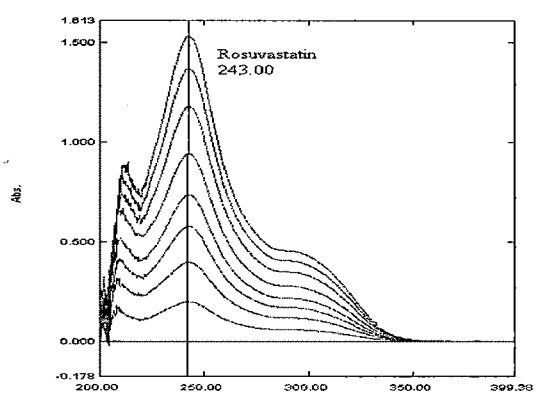


Figure: 7.1.3.1.1 UV spectra of ROSU by Simple UV Spectrophotometry

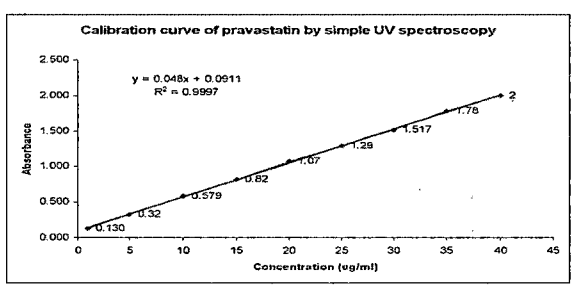


Figure 7.1.3.1.2: Calibration curve of ROSU by Simple UV Spectrophotometry
Correlation co-efficient = **0.9997**
Slop = 0.048
Intercept = 0.0911
Regression equation: **Abs. = 0.048 × Conc. + 0.0911**

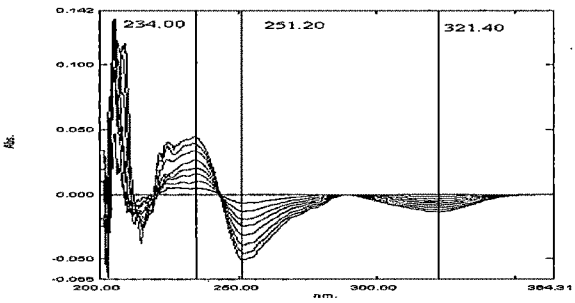


Figure: 7.1.3.1.3: First derivative UV spectra of ROSU

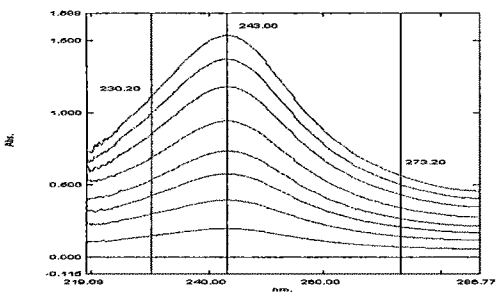


Figure 7.1.3.1.5: UV spectra of ROSU by 3-wavelength method

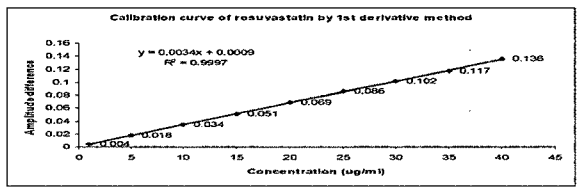


Figure 7.1.3.1.4: Calibration curve of ROSU by 1st derivative Spectrophotometry

Correlation co-efficient = 0.9997

Slope = 0.00034

Intercept = 0.0009

Regression equation: Abs. = 0.00034 × Conc. + 0.0009

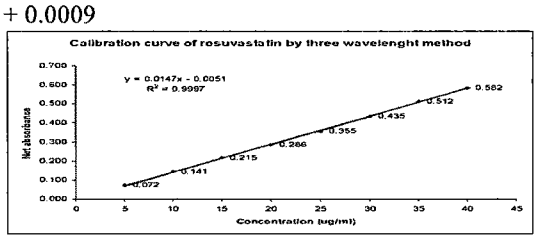


Figure 7.1.3.1.6: Calibration curve of ROSU by 3-wavelength method

Correlation co-efficient = **0.9997**

Slop = 0.0147

Intercept =- 0.0051

Regression equation: **Abs. = 0.00147 × Conc. - 0.0051**

(II) Precision

a. Intraday and Interday Precision (Repeatability)

Intraday and interday variation of the proposed method were reported in table 7.1.3.1.2 and 7.1.3.1.3.

Table 7.1.3.1.2: Intraday precision data of ROSU by Spectrophotometry

Conc. (µg/ml)	Simple UV Spectrophotometric method	1st derivative Spectrophotometric method		Three wavelength Spectrophotometric method	
	Absorb. * ± % C.V. (n=5)	Amplitude difference* ± S.D. (n=5)	% C.V.	Amplitude difference* ± S.D. (n=5)	% C.V.
10	0.397 ± 0.034	0.0340 ± 0.038	0.93	0.143 ± 0.023	0.12
20	0.793 ± 0.12	0.0680 ± 0.29	0.29	0.285 ± 0.34	0.38
30	1.178 ± 0.59	0.1020 ± 0.96	0.48	0.489 ± 0.87	0.28
Table 7.1.3.1.3: Interday precision data of ROSU by Spectrophotometry					
10	0.409 ± 0.85	0.0341 ± 0.057	0.15	0.143 ± 0.12	0.98
20	0.791 ± 0.29	0.0686 ± 0.73	0.48	0.287 ± 0.28	0.39
30	1.179 ± 0.57	0.1020 ± 0.29	0.29	0.481 ± 0.87	0.76

***Average of five readings**

b. Reproducibility:

The reproducibility of the method was determined by using Shimadzu UV 1700 and Shimadzu UV 1601. The % CV of the responses confirms the reproducibility of the method for determination of ROSU. Data is reported in Table 3.2.3.1.4.

Table 7.1.3.1.4: Reproducibility data of ROSU method

Conc. (µg/ml)	Simple UV Spectrophotometric method		1st derivative Spectrophotometric method		Three wavelength Spectrophotometric method	
	Absorbance* ± %C.V.					
	UV 1700	UV 1601	UV 1700	UV 1601	UV 1700	UV 1601
10	0.396 ± 0.39	0.396 ± 0.27	0.034 ± 0.86	0.031 ± 0.21	0.141 ± 0.28	0.140 ± 0.98
15	0.577 ± 0.53	0.572 ± 0.44	0.051 ± 0.39	0.049 ± 0.91	0.215 ± 0.87	0.212 ± 0.21
20	0.754 ± 0.75	0.751 ± 0.97	0.069 ± 0.29	0.067 ± 0.28	0.286 ± 0.34	0.284 ± 0.25

*** Mean value of three determinations**

(III) Accuracy

Accuracy of the measurement of ROSU was determined by standard addition and was found to be in the range of 99.90 -100.27, 100.20 -100.33 and 100.00-101.33 %.

Table 7.1.3.1.5: Accuracy data of ROSU by Spectrophotometry

Initial conc. (µg/ml) (A)	Quantity of std. Added (µg/ml) (B)	Total Amount (A + B)	Simple UV Spectrophotometric method		1st derivative Spectrophotometric method		Three wavelength Spectrophotometric method	
			Total quantity Found * ± S.D.	% Recovery ± S.D	Total quantity Found* ± S.D.	% Recovery ± S.D	Total quantity Found* ± S.D.	% Recovery ± S.D
10	5	15	15.04 ± 0.39	100.27 ± 0.84	15.0509 ± 0.03	100.33 ± 0.29	15.2 ± 0.39	101.33 ± 0.11
10	10	20	19.98 ± 0.57	99.90 ± 0.29	20.0501 ± 0.23	100.25 ± 0.31	20 ± 0.65	100 ± 0.87
10	15	25	24.98 ± 0.72	99.92 ± 0.38	25.0503 ± 0.89	100.20 ± 0.51	25.11 ± 0.28	100.44 ± 0.23

***Average of five readings**

(IV) Limit of detection

The minimum detectable concentration of ROSU was found to be 1.6406, 1.82229 and 0.0321 µg/ml, respectively for zero order, first order derivative and three wavelength Spectrophotometric methods.

(V) Limit of quantification

The lowest quantifiable concentration of ROSU was found to be 5.468, 6.076 and 0.1072 µg/ml, respectively for zero order, first order derivative and three wavelength Spectrophotometric methods

7.1.3.1.3.2. Estimation of ROSU in marketed Tablet:

Preparation of test solution:

Two brands of ROSU, NOVASTAT of Lupin and FORTIUS of Nic. piramal, were analyzed by the proposed method. Twenty tablets were taken and average weight was determined. The tablets were triturated and the tablet powder equivalent to one tablet content was transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution was sonicated for 10 min and supernatant was filtered through whatman filter paper no.41. 1 ml from this solution were transferred to 10 ml volumetric flasks and diluted with methanol. The absorbance of this solution was measured at 243 nm and concentration of sample solution was found from regression equation. . Zero order spectrum was converted in first derivative and the absorbance was measured at λ_{maxima} = 234.00 nm and λ_{minima} = 251.20 nm. The amplitude difference was measured and concentration of sample solution was found from regression equation. The result of tablet estimated is given in Table 7.1.3.1.6. in terms of label claim.

Table 7.1.3.1.6: Estimation of ROSU in tablet by Spectrophotometric methods

Tablet Formu-lation	Labeled Claim (mg/Tablet)	Simple UV Spectrophotometric method		1st derivative Spectrophotometric method		Three wavelength Spectrophotometric method	
		Amount Found* (mg/Tablet)	% Assay ± S.D	Amount Found* mg/Tablet)	% Assay ± S.D	Amount Found* (mg/Tablet)	% Assay ± S.D
NOVASTAT (LUPIN)	5	5.09	101.81 ± 0.37	5.09	101.81 ± 0.37	4.98	99.96 ± 0.34
	10	10.09	100.92 ± 0.24	10.09	100.92 ± 0.24	10.13	101.39 ± 0.19
	20	20.69	103.45 ± 0.98	20.69	103.45 ± 0.98	20.16	100.53 ± 0.89
FORTIUS (NIC. PIRAMAL)	10	10.15	101.52 ± 0.69	10.15	101.52 ± 0.69	10.49	104.90 ± 0.30
	20	20.58	102.40 ± 0.39	20.58	102.40 ± 0.39	20.37	101.82 ± 0.90

***Average of five readings**

7.1.3.1.4 Summary of Validation parameters:

Summary of validation parameters is given in table 7.1.3.1.7.

Table 7.1.3.1.7: Summary of Validation parameters of Spectrophotometric methods

Sr. No	Parameters	Simple UV Spectrophotometric method	1st derivative Spectrophotometric method	Three wavelength Spectrophotometric method
1	Analytical wavelengths (nm)	243	234.00, 251.20	230.20, 243.00, 273.20
2	A (1%, 1cm) (dl gm^{-1} cm^{-1})	356.42	-	-
3	Molar Absoptivity (ε) (L mol^{-1} cm^{-1})	5438.76(>100)	-	-
4	Linearity range (µg/ml)	5 – 40	1 – 40	5 - 40
5	Regression equation	Abs. = 0.0147 × Conc. -0.0051	Abs. = 0.0004 × Conc. + 0.0009	Abs. = 0.0147 × Conc. -0.0051
6	Correlation coefficient (r^2)	0.9997	0.9997	0.9997
7	Intercept	-0.0051	0.0009	-0.0051
8	Slope	0.0147	0.0034	0.0147
9	Sandell's sensitivity (µg/cm^2/0.001 abs. unit))	1.0856	-	-
10	Assay	99.96 –104.90 %.	100.92 -102.40 %.	99.96 –104.90 %.
11	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements % CV % Recovery	 0.12 - 0.38 % 0.39 – 0.98 % <1% 100.00 – 101.33 %	 0.29-0.93 %. 0.15-0.48 %. <1 % 100.20 -100.33%	 0.12 - 0.38 % 0.39 – 0.98 % <1% 100.00 – 101.33 %
12	Limit of detection	1.6406 µg/ml	1.822 µg/ml	0.03217 µg/ml
13	Limit of quantification	5.468 µg/ml	6.076 µg/ml	0.1072 µg/ml

7.1.3.1.5. Conclusion:

Simple spectrophotometric methods were developed for the estimation of ROSU in pharmaceutical oral dosage forms. Three methods can estimate ROSU in dose of 5 – 20 mg with the accuracy of 99.90 -100.27% to assay in the range of 100.92 -103.45%. The coefficient of variation was 0.9997. All three methods can estimate ROSU in the range of 99.96 to 104.90 %. % recovery of method was around 100 %.

7.1.3.2 Estimation of ROSU by FT-Infra Red spectroscopy method.

The aim of the present study was to use FT-IR spectrometry, to investigate the possibility to quantify ROSU in pharmaceutical preparation, such as ROSUTOR® 10 mg tablets. The main objective of this work was to develop a chemometric procedure for the fast and accurate determination of ROSU using MLR approaches, reducing the sample pre-treatment and providing direct FT-IR measurement.

Experimental work:

7.1.3.2.1. Preparation of stock solution for ROSU

For quantitative FT-IR spectroscopic study standard ROSU (10 mg), weighed accurately and make up to 100 mg in same butter paper to obtain final concentration of 100 µg/mg. This sample was triturated in mortar and pestle and sample was used as working standard sample for FTIR spectroscopic method.

Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) spectra was applied for the analysis of ROSU at 990 to 930 cm^{-1} and 1510.00 cm^{-1} was selected for measurement of Peak area and peak height at single wave length, respectively. Calibration curves were drawn using the multiple linear regression (MLR) method and the drug amount in sample was computed by QUANT function of FT-IR solution software.

7.1.3.2.2. Calibration curve

Appropriate fractions were transferred from standard (100 µg/mg) to butter paper and weight adjusted 10 mg with KBr to get minimum transmission. Peak area and peak height was measured at selected wavenumber. Calibration curves were drawn using the multiple linear regression (MLR) method and the drug amount in sample was computed from regression equation.

7.1.3.2.3. Validation Parameters

Procedure for validation of analytical method was given in section 7.1.1.5.2.

7.1.3.2.4. Results and Discussion:

7.1.3.2.4.1 Validation Parameters

(I) Linearity

Linearity range of ROSU was found to be 28 – 295 µg/mg .

Table 7.1.3.2.1: Calibration data of ROSU by Infrared Spectroscopy

Sr.No.	Concentration (µg/mg)	Peak Area ±SD	%CV	Peak height ±SD	%CV
1	28	2.688 ± 0.97	0.29	0.0544 ± 0.68	0.029
2	68	4.721 ± 0.241	0.63	0.1166 ± 0.49	0.17
3	295	13.89 ± 0.092	0.86	0.6187 ± 0.39	0.72

***Average of five reading**

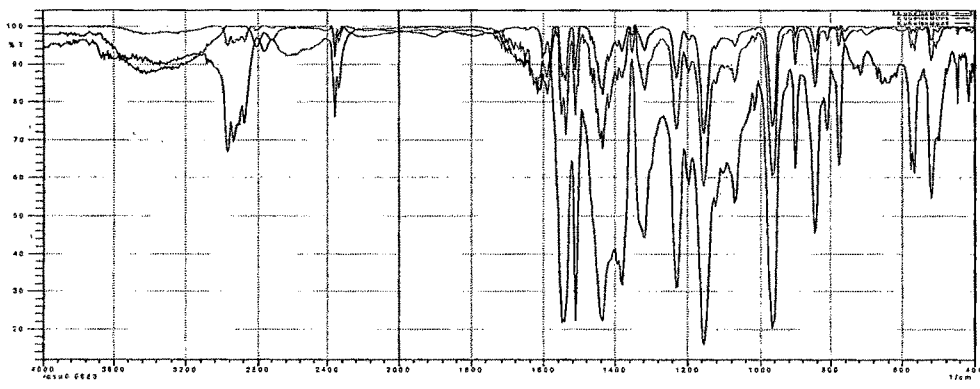


Figure: 7.1.3.2.1 Overlain FT-IR spectra of ROSU

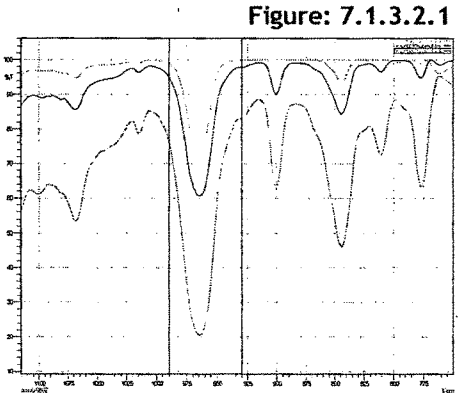


Figure: 7.1.3.2.3 FT-IR spectra of ROSU by peak area

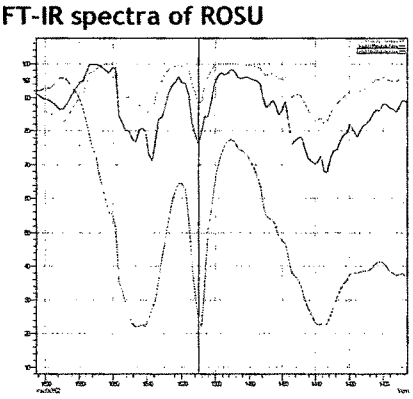


Figure: 7.1.3.2.4 FT-IR spectra of ROSU by peak height

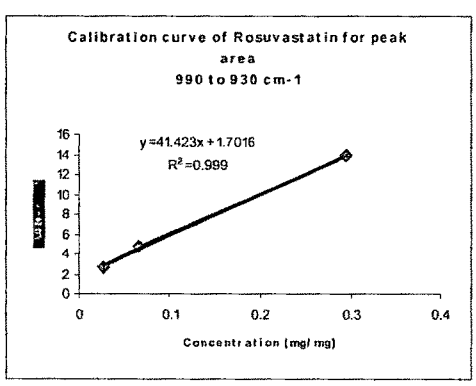


Figure 7.1.3.2.5: Calibration curve of ROSU by peak area

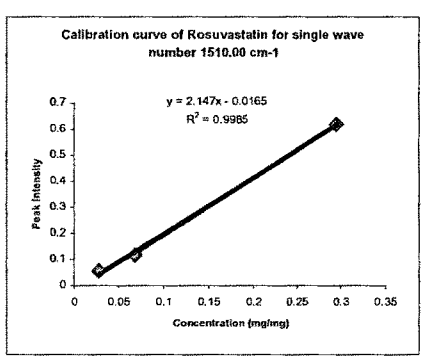


Figure 7.1.3.2.6: Calibration curve of ROSU by peak height

(II) Precision

a. Intraday and Interday Precision (Repeatability)

Intraday and interday variation of the proposed method were reported in table 7.1.3.2.2.

Table 7.1.3.2.2: Intraday precision data of ROSU by FT-IR Spectroscopy

Concentration (µg/mg)	Peak Area* ± SD	%CV	Peak height* ± SD	%CV
50	3.810 ± 0.98	0.34	0.0975 ± 0.22	0.84
100	8.272 ± 0.29	0.82	0.2081 ± 0.30	0.28
150	10.496 ± 0.39	0.11	0.2937 ± 0.78	0.84
Table 7.1.3.2.3: Interday precision data of ROSU by FT-IR Spectroscopy				
50	3.762 ± 0.39	0.67	0.0965 ± 0.87	0.48
100	8.192 ± 0.89	0.87	0.2045 ± 0.65	0.29
150	10.381 ± 0.73	0.18	0.2914 ± 0.72	0.19

***Average of five readings**

b. Reproducibility:

As per the instrument specification the % C.V. of repeated measurement of the same solution should not more than 1%. Here the % C.V. was found to 0.94 and 0.95(<1%) for peak area and peak height at single wavelength number, respectively.

Table 7.1.3.2.4: Reproducibility data of ROSU (50µg/mg) by FT-IR Spectroscopy

Time	Peak Area*	Peak Height*
Bigger sample com.	3.800	0.0973
	3.896	0.0992
	3.848	0.0982
Small sample com.	3.781	0.0969
	3.810	0.0975
	3.827	0.098
S.D.	0.04574	0.00093
%C.V.	0.9476	0.95

***Average of five readings**

(III) Accuracy

Accuracy of the measurement of ROSU was determined by standard addition and was found to be in the range of 99.92- 100.50 % for peak area and for perk height as 99.99 – 100.42 %.

Table 7.1.3.2.5: Accuracy data of ROSU by peak area

HPLC		ROSU		
Initial conc.(µg/ml) (A)		100	100	100
Quantity of std. Added (µg/ml) (B)		**50**	**100**	**150**
Total Amount (A + B)		**150**	**200**	**250**
Peak area (Mean (n=5) ± %C.V.)	**Total quantity Found Mean ± S.D.**	149.9 ± 0.32	201.02 ± 0.72	249.89 ± 0.67
	% Recovery± S.D	99.93 ± 0.28	100.50 ± 0.87	99.92 ± 0.29
Peak height (Mean (n=5) ± %C.V.)	**Total quantity Found Mean ± S.D.**	150.03 ± 0.94	200.83 ± 0.29	249.98 ± 0.19
	% Recovery± S.D	100.02 ± 0.19	100.42 ± 0.29	99.99 ± 0.18

***Average of five readings**

(IV) Limit of detection

The minimum detectable concentration of ROSU was found to be 0.2958 and 0.9861 µg/mg for peak area and height, respectively.

(V) Limit of quantification

The lowest quantifiable concentration of ROSU was found to be 5.449 and 18.644 µg/mg by practical observation for peak area and height, respectively.

7.1.3.2.3.2. Estimation of ROSU in marketed Tablet:

Preparation of test Sample:

Test sample was prepared as per procedure given in section 7.1.1.5.2.2. DRIFT spectra was scanned between 4000 and 400 cm^{-1}, by averaging 16 scans for each spectrum with a resolution of 4 cm^{-1} (data point resolution per interval 1 cm^{-1}). 990 to 930 cm^{-1} and 1510.00 cm^{-1} was selected for measurement of Peak area and peak height at single wave length, respectively. Concentration of samples were found from regression equation of ROSU.

Table 7.1.3.2.6: Estimation of ROSU in tablet by FTIR Spectroscopy

Formulation	Labeled Claim (mg/tab)	Peak Area		Peak Height	
		*Amount found	% Assay	*Amount found	% Assay
NOVASTAT (Lupin)	5	5.09	102.8	5.05	100.04
	10	10.08	100.81	10.09	100.92
	20	20.59	104.18	19.98	99.90

***Average of three determinations.**

7.1.3.2.4. Summary of Validation parameters:

The summary of validation parameters is given in table 7.1.3.2.7.

Table 7.1.3.2.7: Summary of Validation parameters of FTIR Spectroscopy

Sr. No	Parameters	Peak area	Peak area
1	wavenumber (cm^{-1})	990-930	1510
2	Linearity range (µg/mg)	28 – 295	
3	Regression equation	41.42× **Conc.** +1.701	**2.147 × Conc. – 0.0165**
4	Correlation coefficient (r^2)	0.999	0.9985
5	Intercept	1.701	0.0165
6	Slope	41.42	2.147
7	Assay	100.81-102.8	99.90-100.92
8	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements % CV % Recovery	 0.11-0.82 0.18-0.87 0.94 99.92-100.50	 0.28-0.84 0.19-0.48 0.95 99.99-100.42
9	Limit of detection (µg/mg)	0.2958	0.981
10	Limit of quantification (µg/mg)	5.449	18.644

7.1.3.2.5. Conclusion:

FTIR spectroscopic method was developed for the estimation of ROSU in pharmaceutical oral dosage forms. This method can estimate ROSU in dose of 5 –20 mg. The coefficient of variation was 0.999 that show method can estimate ROSU in bulk drug. An FT-IR spectrum of compound is characteristic of that compound. So estimation by FTIR is one of the specific analytical method for respective compounds.

7.1.3.3 Stability Indicating Reverse Phase High Performance Liquid Chromatography (HPLC) with UV detection.

As literature review reveals that around 9 methods were reported to analyzed ROSU in tablet dosage form and in human plasma. But no SIA method was reported so Accordingly, the aims of the present study were to establish inherent stability of ROSU through stress studies under a variety of ICH recommended test conditions and to develop a stability-indicating assay for an oral table

7.1.3.3.1. Optimization of method:

Determination of solvent for sample preparation:

Different solvents were tried depending upon its solubility (chapter 6)

ROSU is soluble in methanol and acetonitrile but the absorbance was higher in ACN so ACN was selected as the main solvent.

Determination of detection wavelength

Wavelength was selected by scanning standard solutions of both drugs over 200 nm to 400 nm wavelengths. The drug shows reasonably good response at 248 nm (fig. 6.8). So the wavelength was selected in dual channel UV detector.

Determination of mobile phase:

Different combinations of Mobile phase were tried at flow rate 1 ml/min and column C18 Phenomenex.

Table 7.1.3.3.1: Determination of mobile phase

Mobile phase combination	**Ration (V/V)**	**Retention time (min)**	**Peak shape**
		ROSU	**ROSU**
Methanol: Water	70:30	2.990	Merge with blank peak
Acetonitrile: Water	70:30	2.512	Sharp but merge with blank peak
Acetonitrile: 0.1 % Formic acid	60:40	4.219	Sharp peak but tailing at the base
Acetonitrile: 0.1 % Formic acid	40:60	14.654	Sharp peak but tailing at the base
Acetonitrile: 0.2 % Formic acid	40:60	13.091	Sharp peak but tailing at the base
Acetonitrile: 0.5 % Formic acid	**50:50**	**6.728**	**Sharp peak**
Acetonitrile: 0.5 % Formic acid	65:35	4.47	Sharp peak
Acetonitrile: 0.5 % Formic acid	40:60	12.982	Sharp peak

So from above data, it was evident that mobile phase combination of acetonitrile: 0.5 % Formic acid in proportion of 50:50 was most suitable for the development of RP-HPLC method.

Table 7.1.3.3.2: Determination of Flow rate

Flow rate (ml/min)	Retention time (min)
	ROSU
0.5	12.354
1.0	**6.728**
1.2	4.253

Most suitable Retention time is between 4 to 8 min so that flow rate selected was 1 ml/min,

Column

Two type of C18 columns were tried.

Table 7.1.3.3.3: Column

Column (C18, 231 X 4.60 mm)	Retention time (min)	Peak shape
	ROSU	ROSU
Hypersil	6.429	Sharp peak
Phenomenex	6.728	Sharp peak

So both the columns were suitable for development of HPLC analytical method. Here C18 Phenomenex 240X4.6 mm was selected. The main aim of the method was to resolve the compounds in presence of degradation products and impurities, Phenomenex C 18 column (231 mm x 4.6 mm i.d., 5 µm particle size was preferred as it has high carbon loading with very closely packed material to give high resolution. To develop a precise, accurate, specific and suitable stability indication RP-HPLC method for the estimation of ROSU. After thorough optimization procedures, following parameters were found appropriate for the quantitative determination of ROSU in presence of degradation products.

Method:

Chromatographic condition:

- **Column:** C_{18} (size-231 x 4.60 mm, I.D-5 µm) (Phenomenex)
- **Mobile Phase:** Acetonitrile : 0.5% Formic acid (50 : 50, v/v)
- **Detection:** UV detection at 248 nm
- **Flow rate:** 1.0 ml/minute
- **Application volume:** 20 µl

Under these conditions, T_R for ROSU was obtained at 6.742 min.

Preparation of Mobile Phase:

Mobile phase was prepared by mixing 500 ml of Acetonitrile with 500 ml of 0.5 % formic acid (50: 50, V/V). The mobile phase was filtered through nylon 0.45 µm, 47 mm membrane filter and degassed in ultrasonic bath prior to use for 30 min.

7.1.3.3.2. Preparation of stock solution for ROSU

For chromatographic study standard ROSU (100 mg), weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml ACN properly and diluted up to mark with ACN to obtain final concentration of 1000 µg/ml.

7.1.3.3.3. Calibration curve

From the stock solution (1000 µg/ml), aliquots of 0.1, 0.5, 1, 1.5, 2, 2.5, and 3 ml were transferred to a series of 10 ml volumetric flasks. The volume was adjusted up to the mark with ACN to get final concentrations of 10, 50, 100, 150, 200, 250, and 300 µg/ml of solution. 20 µl of all these solutions were injected separately into HPLC column and the peak area of each solution was measured at selected wavelength. The calibration curve was constructed by plotting peak area vs. concentration (µg/ml) over the concentration range and regression equation was calculated.

7.1.3.3.4. Validation Parameters

Procedure for validation of analytical method was given in section 7.1.1.6.3. Aliquots was taken form the stock solution of ROSU.

7.1.3.3.5. Results and Discussion:

7.1.3.3.5.1 Validation Parameters

(I) Linearity

Linearity range of ROSU was found to be 5–300 µg/ml with correlation co-efficient and % CV is 0.9999 and 0.18–0.73 for peak area.

Table 7.1.3.3.4: Calibration data of ROSU by HPLC with UV detection

Sr. No.	Concentration (µg/ml)	Peak Area		RT*
		Mean*	%CV	
1	5	400000	0.38	6.817
2	10	817803	0.19	6.733
3	50	3948689	0.73	6.733
4	100	7756336	0.37	6.733
5	200	14982397	0.67	6.629
6	300	22473596	0.18	6.817

***Average of five readings**

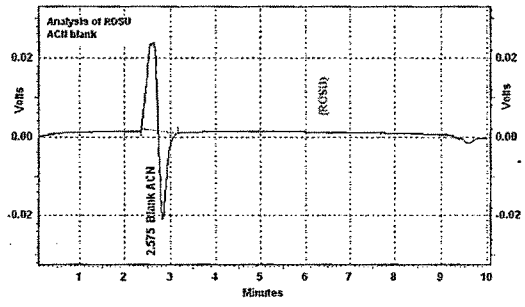


Figure 7.1.3.3.2: Single Peak of ACN Blank by HPLC with UV detection

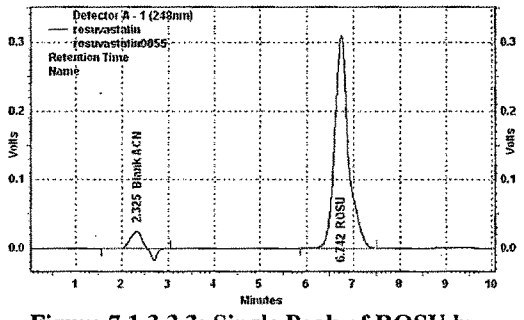


Figure 7.1.3.3.3: Single Peak of ROSU by HPLC with UV detection

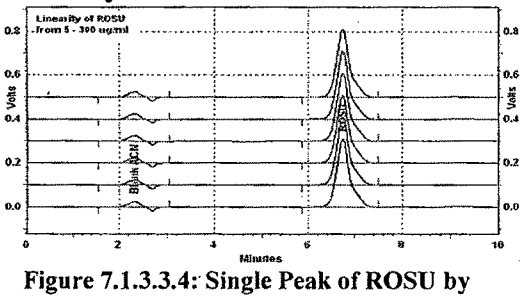


Figure 7.1.3.3.4: Single Peak of ROSU by HPLC with UV detection

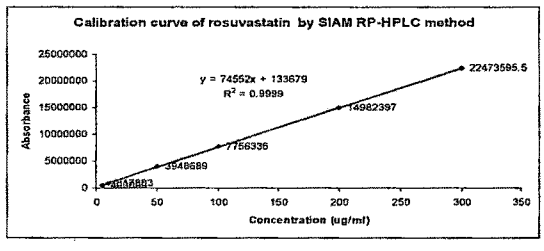


Figure 7.1.3.3.5: Calibration curve of ROSU by HPLC with UV detection (Peak Area)

(II) Precision

a. Intraday and Interday Precision (Repeatability)

Intraday and interday variation of the proposed method were reported in table 7.1.3.3.5

Table 7.1.3.3.5: Intraday and interday precision data of ROSU by HPLC

Sr. No.	Concentration (µg/ml)	Intraday		RT*	Interday		RT*
		Peak area*	%CV		Peak area*	%CV	
1	60	4660782	0.38	6.733	4653026	0.48	6.733
2	120	9314584	0.87	6.715	9316911	0.29	6.733
3	240	18544624	0.23	6.7495	18618309	0.84	6.715

***Average of five readings**

b. Reproducibility:

The % C.V. of repeated measurement of the same solution was not more than 1%.

Table 7.1.3.3.6: Reproducibility data of ROSU(150 µg/ml) by HPLC

C18 Column	Hypersile		Phenomenex	
	Peak Area*	RT	Peak Area*	RT
150 µg/ml	11634504	6.817	11643812	6.715
	11624421	6.733	11636055	6.7495
	11636831	6.733	11635125	6.7495
%CV	0.059	0.58	0.67	0.15

(III) Accuracy

Accuracy of the measurement of ROSU was determined by standard addition and was found to be in the range of 99.51-100.66 % for peak area.

Table 7.1.3.3.7: Accuracy data of ROSU by HPLC with UV detection

Initial conc. (µg/ml)(A)	Quantity of std. Added (µg/ml)(B)	Total Amount (A + B)	Peak Area	
			Total quantity Found*± S.D.	%Recovery ± S.D
100	50	150	149.67 ± 0.48	99.78 ± 0.38
100	100	200	201.32 ± 0.39	100.66 ± 0.54
100	150	250	248.78 ± 0.29	99.512 ± 0.57

***Average of five readings**

(IV) Limit of detection

The minimum detectable concentration of ROSU was found to be 0.0905 µg/ml for peak area.

(V) Limit of quantification

The lowest quantifiable concentration of ROSU was found to be 0.318 µg/ml for peak area.

7.1.3.3.4.2. Applicability of method

(I) Estimation of ROSU in marketed Tablet:

Twenty tablets were taken and average weight found. Then the tablets were and triturated the powder equivalent to one Tablet was transferred into a 100 ml volumetric flask containing 50 ml ACN, sonicated for 15 min and further diluted to 100 ml with ACN. The resulting solution was sonicated for 10 min and supernatant was filtered through whatman filter paper no.41. 20 µl of this solution was injected into HPLC column and the peak area was measured at 248 nm. The concentration of sample solution was found from regression equation of ROSU. Result of tablet formulation in term of % assay is given in table 7.1.3.3.8.

Table 7.1.3.3.8: Estimation of ROSU in tablet by HPLC with UV detection

Tablet Formulation	Labeled Claim (mg/tablet)	Amount found (mg/tablet)	% Assay ± S.D
NOVASTAT (Lupin)	5	5.09	100.18 ± 0.77
	10	10.02	100.21 ± 0.67
	20	20.09	100.45 ± 0.23

***Average of five readings**

(II) Procedure for forced degradation study

Forced degradation study was done under different conditions. Solvents selected were 30% H_2O_2 at room temperature and reflux at 80 °, water at neutral pH 7 room temperature and reflux at 80 °, 0.5 N HCl room temperature and 1 N HCl reflux at 80 ° and 1 N NaOH at room temperature and reflux at 80 °. Approximate 25 mg drug was weighed and transferred to 25 ml volumetric flask and dissolved with 5 ml of ACN and then the volume was made upto the mark with the solvent used for degradation. Same ways solid state stability was performed by putting 25 mg of drug to direct exposure to 80°C temperature and UV/254nm and Vis/366 nm. Samples were colleted t two stages at 0 min (as soon as sample was prepared) and after 48 hrs of exposure to degradation condition.

FIGS 7.1.3.3.5 – 7.1.3.3.10 show the chromatograms of forced degraded samples.

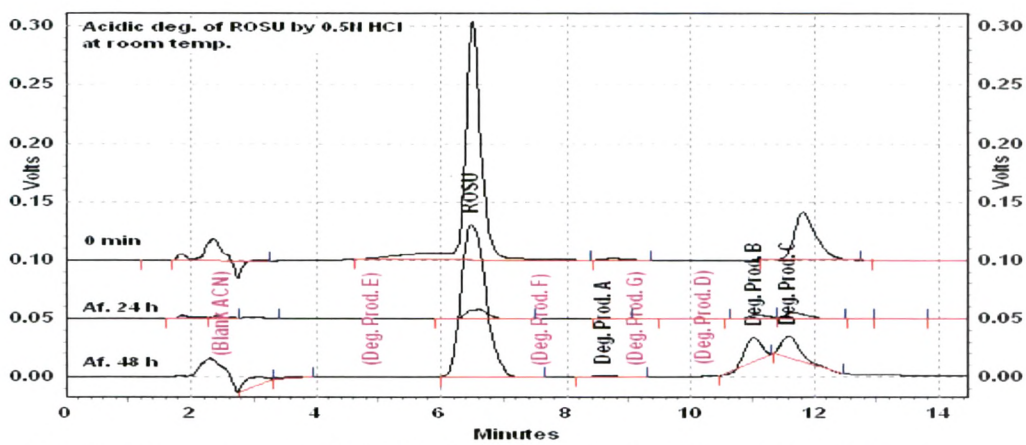


Figure 7.1.3.3.5(A): Chromatograms of ROSU in 0.5 N HCl at 0 min, 24 hr and after 48 hrs at room temperature

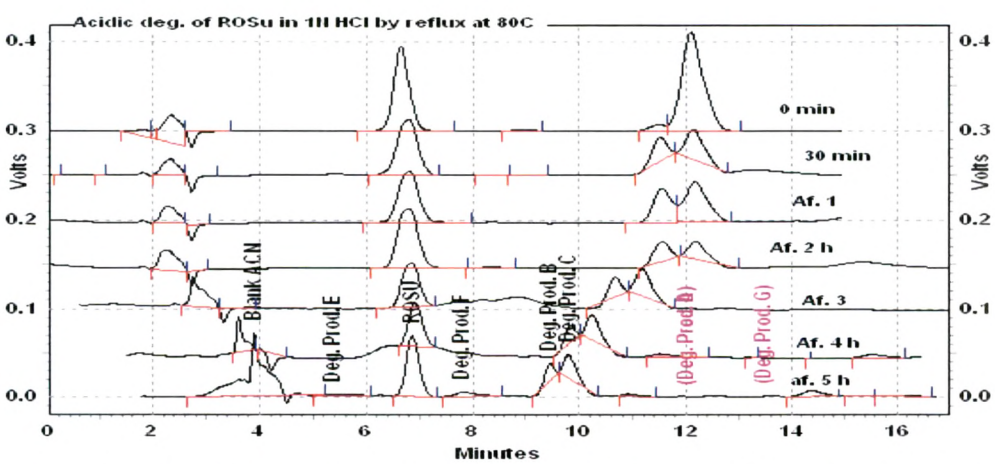


Figure 7.1.3.3.5(A): Chromatograms of ROSU in 1 N HCl at 0 min, 30 min,1 hr, 2 hr, 3 hr, 4 hr and after 5 hrs reflux at 80

Figure 7.1.3.3.5: Chromatograms of acid hydrolysis- degraded ROSU

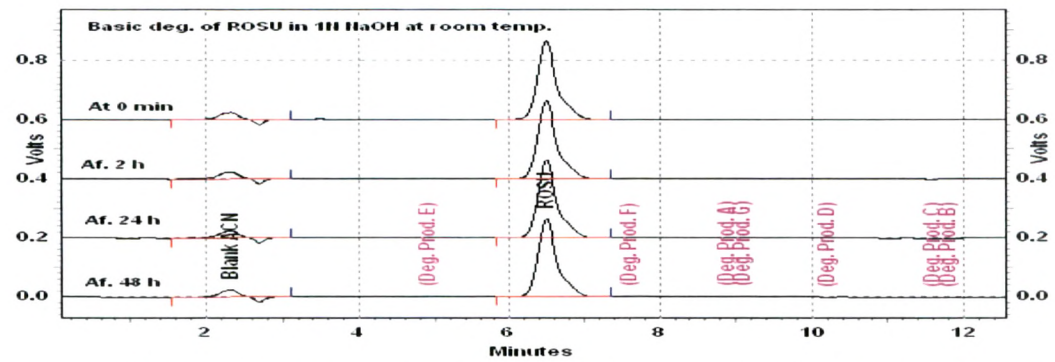


Figure 7.1.3.3.6(A): Chromatograms of ROSU in 1 N NaOH at 0 min, 2 hr, 24 hr and after 48 hrs at room temperature

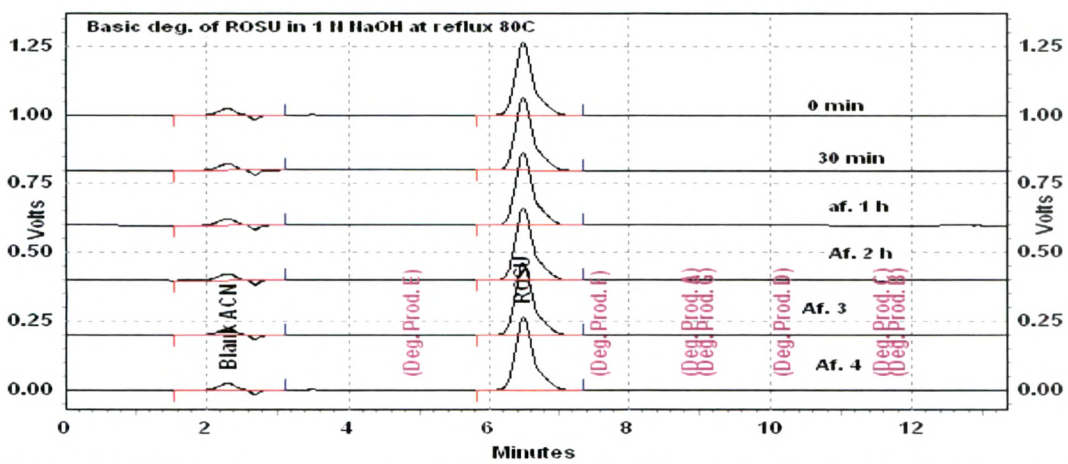


Figure 7.1.3.3.6(A): Chromatograms of ROSU in 1 N NaOH at 0min, 30 min, 1 hr, 2 hr, 3 hr and after 4 hrs reflux at 80 C

Figure 7.1.3.3.6: Chromatograms of base hydrolysis-degraded ROSU

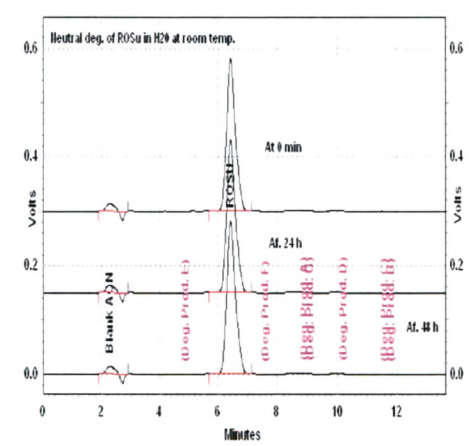


Figure 7.1.3.3.7(A): Chromatograms of ROSU in neutral at 0 min, 24 hr and after 48 hrs at room temperature

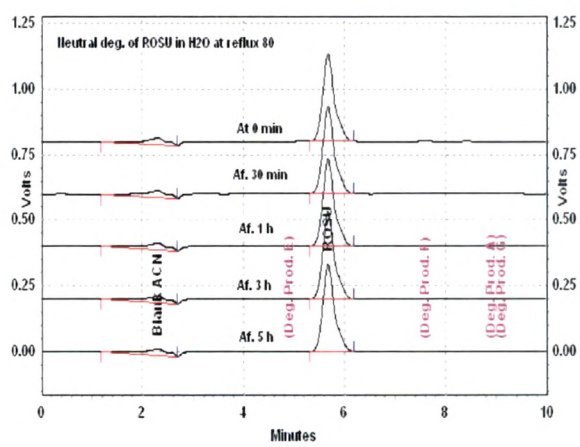


Figure 7.1.3.3.7(A): Chromatograms of ROSU in neutral at 0 min, 30 min, 1 h, 3 hr and after 5 hrs reflux at 80 C

Figure 7.1.3.3.7: Chromatograms of neutral (H2O) – degraded ROSU

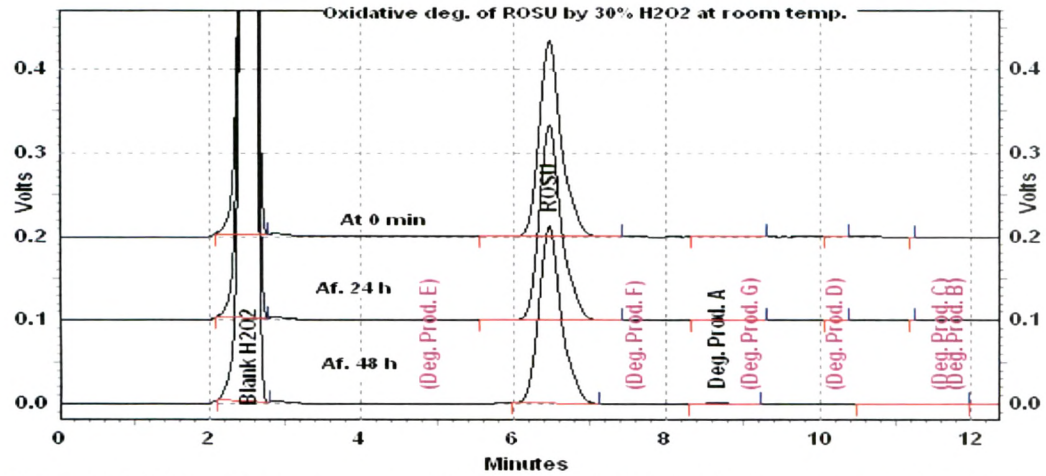


Figure 7.1.3.3.8(A): Chromatograms of ROSU in 30 % H2O2 at 0 min, 24 hr and after 48 hrs at room temperature

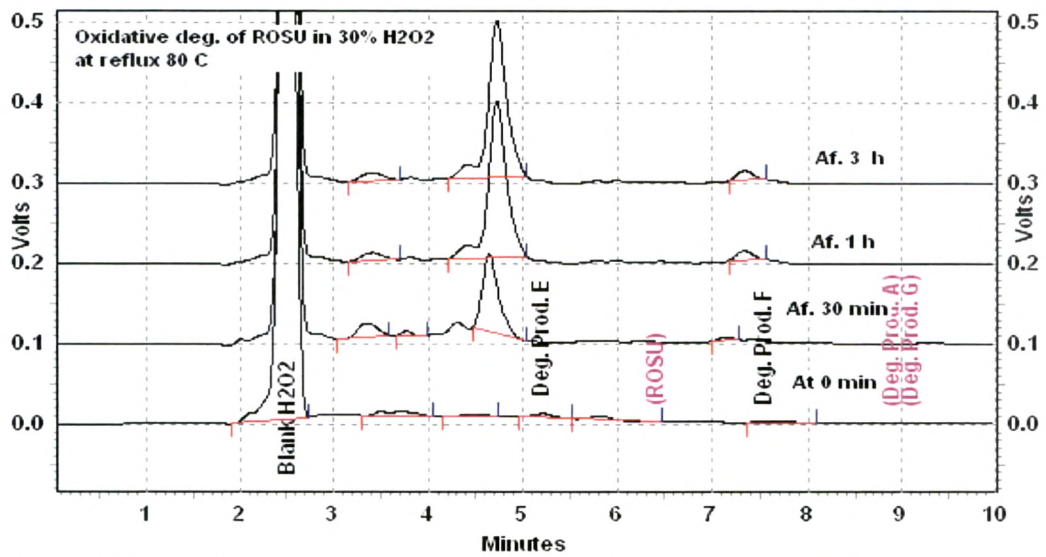


Figure 7.1.3.3.8(A): Chromatograms of ROSU in 30 % H2O2 at0 min, 30 min, 1 h and after 3 hrs reflux at 80 C

Figure 7.1.3.3.8: Chromatograms of oxidative-degraded ROSU

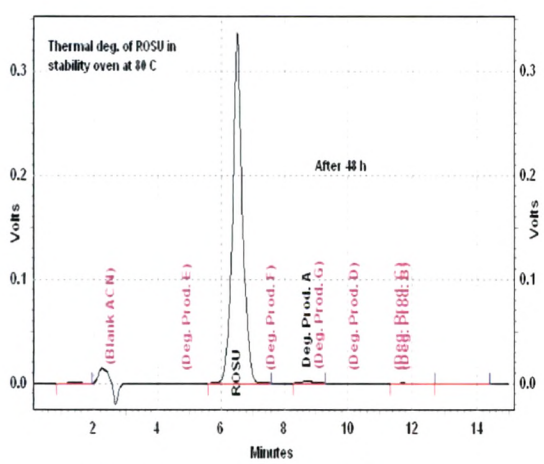


Figure 7.1.3.3.9: Chromatograms of Thermal-degraded ROSU

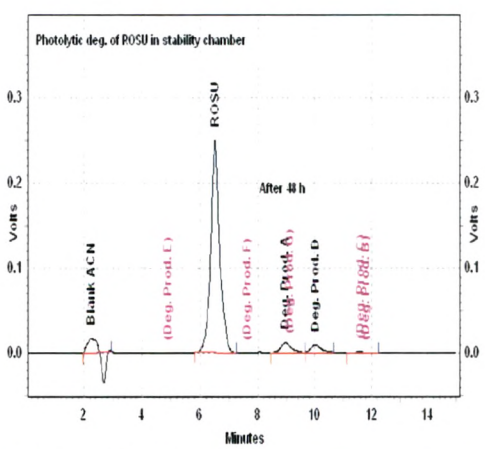


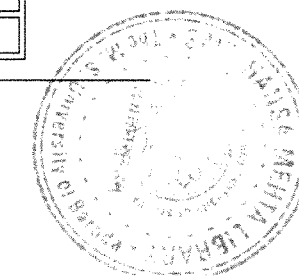
Figure 7.1.3.3.10: Chromatograms of UV/254 and Vis/366-degraded ROSU

Table 7.1.3.3.9: Percentage degradation of ROSU by force degradation.

Sr. No	Parameters (Stress condition /duration/state)	% Unchanged ROSU	% degradation of individual Degradation products							Total % Deg.
			A	B	C	D	E	F	G	
1	Neutral/H_2Oat pH 7/**48 h**/ sol./**RT**	100	No degradation found							
	Neutral/H_2Oat pH 7/**3 h**/ sol./**Ref.**	100	No degradation found							
2	Acidic/0.5 N HCl/**72 h**/ sol./**RT**	68	2	12	17	-	-	-	-	**32**
	Acidic/ 1 N HCl/**5 h**/ sol./**Ref.**	48	-	15	17	-	8	12		**52**
3	Alkali/ 1N NaOH/**48 h**/ sol./**RT**	100	No degradation found							
	Alkali/ 1N NaOH/**4 h**/ sol./**Ref.**	100	No degradation found							
4	Oxidative/30% H_2O_2/**48 h**/ sol./**RT**	70	15	-	-	15	-	-	-	**30**
	Oxidative/30% H_2O_2/**5 h**/ sol./**Ref.**	-	-	-	-	53	47	-	-	**100**
5	Thermal/80 C/**48 h**/solid/**RT**	86	14	-	-	-	-	-	-	**14**
6	Photo/uv254 and Vis/366 nm/**48 h**/solid/**RT**	69	12	-	4	13	-	-	2	**31**

Table 7.1.3.3.11: System suitability parameters of ROSU.

Sr. No	System suitability parameters	ROSU	Degradation products						
			A	B	C	D	E	F	G
1	Retention time (minutes)	**6.517**	**8.925**	**11.825**	**11.667**	**10.200**	**4.933**	**7.675**	**8.025**
2	Theoretical plates	16398.84	165283.3	594456.8	37165	305473	8291.543	3134.142	7194.101
3	Resolution	**7.28**	**3.90**	**4.92**	**2.31**	**8.77**	**7.58**	**2.34**	**1.70**
4	Asymmetry	0.88	1.09	1.43	1.21	1.13	1.31	0.82	1.12
5	USP width	1.32	3.06	4.38	1.11	3.64	1.24	0.49	0.71
6	Tailing factor	0.81	1.10	1.38	1.20	1.15	1.30	0.83	1.07
7	Capacity Factor	1.96	0.77	1.63	4.30	1.16	0.81	3.15	3.15



7.1.3.3.3 Summary of Validation parameters:

The summary of validation parameters is reported in table 7.1.3.3.11.

Table 7.1.3.3.11: Summary of Validation parameters by HPLC with UV detection

Sr. No	Parameters	Peak Area
1	Analytical wavelengths (nm)	248
2	Linearity range (µg/ml)	5 - 300
3	Regression equation	Y = 74552xcon.+133679
4	Correlation coefficient (r^2)	0.9999
5	Intercept	133679
6	Slope	74552
7	Assay	100.18 – 100.45%
8	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements %CV % Recovery	 0.23 – 0.87% 0.29 – 0.84% 0.059 99.51 – 100.66 %
9	Limit of detection (µg/ml)	0.0905
10	Limit of quantification (µg/ml)	0.318

7.1.3.3.4. Conclusion:

Developed HPLC method can estimate ROSU in pharmaceutical dosage form and also in presence of its degradation products. Degradation study suggests that ROSU is stable in alkaline and neutral condition (fig. 7.1.3.3.6 and 7.1.3.3.7). In acidic medium it forms two main degradation products as shown in fig. 7.1.3.3.5. ROSU undergoes oxidative degradation as indicated by reduction in peak height and area but no new peak could be obtained in the chromatogram (fig. 7.1.3.3.8). Upon thermal and photolytic exposure it converted into two products (fig. 7.1.3.3.9 to 10). So this method can be used as specific SIAM to study ROSU in presence of its degradation products.

7.1.3.4 Bio Analytical Reverse Phase High Performance Liquid Chromatography (HPLC) method for estimation in human plasma with UV detection.

HPLC method developed for estimation of ROSU in pharmaceutical formulation was used for the analysis of ROSU in human plasma. This is invitro method of analysis of ROSU in human plasma. ROSU was not administrated orally it was spiked in human plasma and analyzed by protein precipitation method. Human plasma was obtained form blood bank as request for research. The method was validated for some additional parameters.

7.1.3.4.1. Preparation of stock solution for ROSU

For chromatographic study standard ROSU (100 mg), weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml ACN properly and diluted up to mark with ACN to obtain final concentration of 1000 µg/ml.

7.1.3.4.2. Calibration curve for ROSU:

From the stock solution (1000 µg/ml) aliquots of 0.005, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 µl were transferred to a series of sample tubes. Then 90 µl of human plasma was added to each tube. The tubes were vortexed for 1 min on Vortex shaker. The volume was adjusted to a 1000 µl with acetonitrile to get final concentrations of 0.55, 50, 100, 200, 300, 400, 500 and 600 ng/ml of solution. The solutions were centrifuged for 5 min at 4000 rpm. 20 µl of supernatant were injected into HPLC column and the peak area of each solution was measured at selected wavelength. The calibration curve was constructed by plotting peak area vs. concentration (µg/ml) over the concentration range and regression equation was computed.

7.1.3.4.3. Validation of method:

Procedure for rest of validation parameters were given in section 7.1.1.6.2.

7.1.3.4.4. Results and Discussion:

7.1.3.4.4.1 Validation Parameters

(I) Linearity

Linearity range of ROSU was found to be 0.55 – 600 ng/ml with correlation co-efficient and % CV is 0.9997 and 0.18–0.87 for peak area.

Table 7.1.3.4.1: Calibration data of ROSU by HPLC with UV detection

Sr. No.	Concentration (ng/ml)	Peak Area		RT*	Theoretical plates*	Asymmetry*
		Mean*	%CV			
1	0.55	662	0.67	6.975	2898.67	1.98
2	50	2872	0.34	6.933	2943.76	1.67
3	100	5127	0.56	6.9	2896.45	1.56
4	200	10254	0.67	6.833	2967.09	1.65
5	300	15381	0.23	6.75	2845.98	1.45
6	400	20508	0.18	6.758	2960.55	1.67
7	500	25635	0.87	6.755	2945.09	1.55
8	600	30762	0.96	6.792	2865.09	1.45

***Average of five readings**

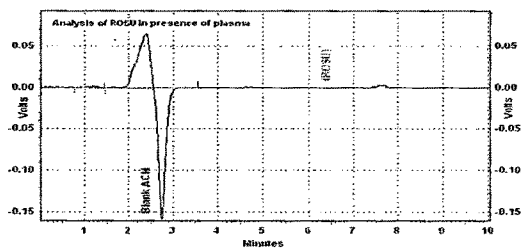


Figure 7.1.3.4.1: Single Peak of ACN blank by HPLC with UV detection

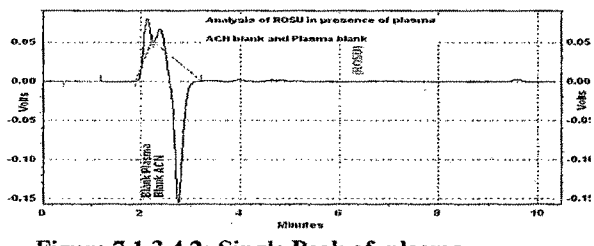


Figure 7.1.3.4.2: Single Peak of plasma blank by HPLC with UV detection

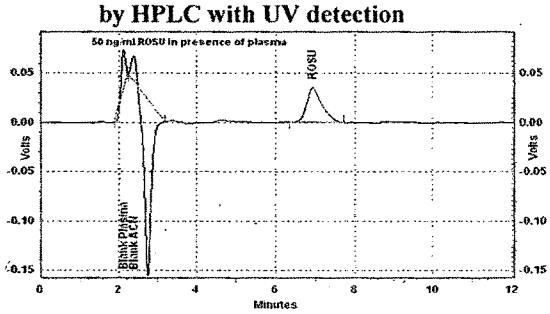


Figure 7.1.3.4.3: Single Peak of ROSU by HPLC with UV detection

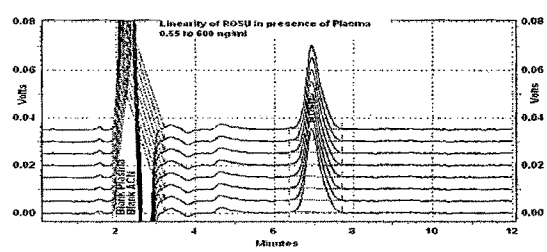


Figure 7.1.3.4.4: Single Peak of ROSU by HPLC with UV detection

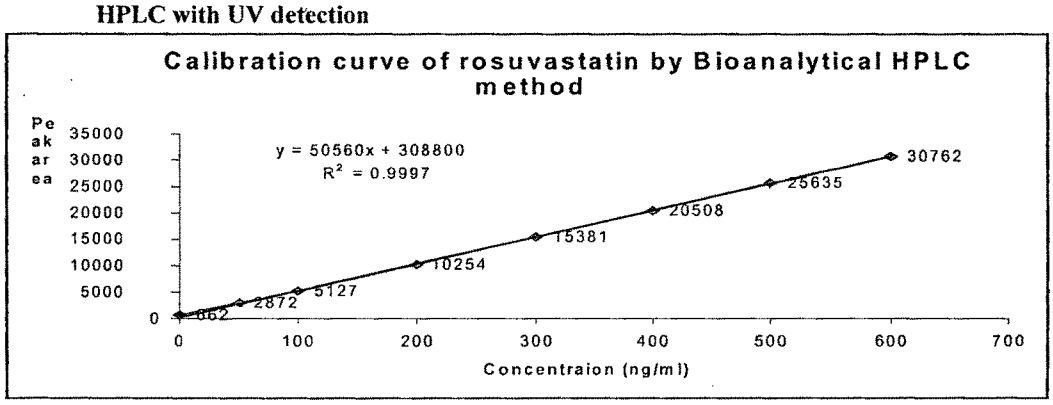


Figure 7.1.3.4.5: Calibration curve of ROSU by bioanalytical method

(II) Precision

a. Intraday and Interday Precision (Repeatability)

Intraday and interday variation of the proposed method were reported in table 7.1.3.4.2.

Table 7.1.3.4.2: Intraday precision data of ROSU by HPLC with UV detection

Sr. No.	Concentration (µg/ml)	Intraday		RT*	Interday		RT*
		Peak area*	%CV		Peak area*	%CV	
1	60	2863	0.34	6.75	2862	0.98	6.758
2	120	12817	0.28	6.758	12805	0.33	6.725
3	240	25635	0.98	6.725	25579	0.29	6.792

***Average of five readings**

b. Reproducibility:

As per the instrument specification the % C.V. of repeated measurement of the same solution should not more than 1%.

Table 7.1.3.4.4: Reproducibility data of ROSU (150 ng/ml) by HPLC

C18 Column	Hypersil		Phenomenex	
	Peak Area*	RT	Peak Area*	RT
150 ng/ml	7690	6.833	7695	6.625
	7692	6.75	7627	6.592
	7689	6.658	7679	6.692
%CV	0.37	0.56	0.42	0.97

(III) Accuracy

Accuracy of the measurement of ROSU was determined by standard addition and was found to be in the range of 99.87-100.12 % for peak area.

Table 7.1.3.4.5: Accuracy data of ROSU by HPLC with UV detection

Initial conc. (ng/ml) (A)	Quantity of std. Added (ng/ml) (B)	Total Amount (A + B)	Peak Area	
			Total quantity Found * ± S.D.	%Recovery
120	60	180	181.39	100.772
120	120	240	239.59	99.8292
120	200	320	319.56	99.8625

***Average of five readings**

(IV) Limit of detection

The minimum detectable concentration of ROSU was found to be 0.09837 ng/ml for peak area.

(V) Limit of quantification

The lowest quantifiable concentration of ROSU was found to be 0.1498 ng/ml for peak area.

(VI) Stability

Freeze and Thaw Stability

Samples were prepared at LQC and HQC levels, aliquoted and frozen. Six samples from each concentration were subjected to three freez and thaw cycles (stability samples). These samples were processed after 3rd cycle and analyzed along with freshly prepared calibration standards, LQC and HQC samples (comparison samples). Concentrations were calculated to determine % mean change after 3rd cycle. ROSU was found to be stable in LQC and HQC samples after 3rd cycle after frozen with % mean change of 0.10 and –0.33 respectively (Table No. 7.1.3.4.6)

Table 7.1.3.4.6: Freeze and thaw stability

Sr. No.	LQC (15.000 ng/ml)				HQC (680.000 ng/ml)			
	Comparison samples		Stability samples		Comparison samples		Stability samples	
	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.
1	14.98	99.87	15.02	100.13	679.02	99.86	672.03	98.83
2	15.02	100.13	14.29	95.27	682.98	100.44	684.92	100.72
3	14.37	95.80	15.28	101.87	680.89	100.13	688.22	101.21
4	15.39	102.60	14.98	99.87	679.22	99.89	689.39	101.38
5	15.29	101.93	14.98	99.87	678.23	99.74	679.22	99.89
6	14.92	99.47	15.33	102.20	680.22	100.03	680.09	100.01
N	6		6		6		6	
Mean	15.00		14.98		680.09		682.31	
% Mean Change	0.01				-0.33			

2. Short-Term Temperature Stability

LQC and HQC samples were spiked in human plasma and were kept at room temperature for 11.0 hours and were processed and analyzed along with freshly prepared calibration standards, LQC and HQC samples. Concentrations were calculated to determine % mean change during stability period.

ROSU was found to be stable in LQC and HQC samples for 11.0 hours at room temperature with % mean change of 0.98 and -0.35, respectively (Table No. 7.1.3.4.7).

Table 7.1.3.4.7: Short term temperature stability

Sr. No.	LQC (15.000 ng/ml)				HQC (680.000 ng/ml)			
	Comparison samples		Stability samples		Comparison samples		Stability samples	
	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.
1	14.34	95.60	15.22	101.47	702.00	103.24	688.09	101.19
2	15.03	100.20	14.29	95.27	689.22	101.36	672.29	98.87
3	14.93	99.53	14.22	94.80	689.22	101.36	694.00	102.06
4	15.22	101.47	15.02	100.13	679.34	99.90	684.99	100.73
5	15.00	100.00	15.00	100.00	678.29	99.75	712.84	104.83
6	14.88	99.20	14.77	98.47	680.23	100.03	680.33	100.05
N	**6**		**6**		**6**		**6**	
Mean	14.90		14.75		686.38		688.76	
% Mean Change	0.98				-0.35			

3. Long-Term Stability

ROSU was found to be stable in human plasma at below -20°C after 45 Days in LQC and HQC samples with % Mean Change of 0.28 and 0.63 respectively, (Table No. 7.1.3.4.8.).

Table 7.1.3.4.8: Long term stability

Sr. No.	LQC (15.000 ng/ml)				HQC (680.000 ng/ml)			
	Comparison samples		Stability samples		Comparison samples		Stability samples	
	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.	Sample conc. (ng/ml)	% Nominal Conc.
1	14.22	94.80	15.10	100.67	680.33	100.05	693.22	101.94

2	15.29	101.93	14.89	99.27	705.98	103.82	708.33	104.17
3	14.99	99.93	14.98	99.87	698.34	102.70	680.33	100.05
4	15.09	100.60	15.03	100.20	683.29	100.48	677.33	99.61
5	15.22	101.47	15.22	101.47	679.22	99.89	669.38	98.44
6	14.99	99.93	14.33	95.53	680.22	100.03	673.29	99.01
N	**6**		**6**		**6**		**6**	
Mean	14.97		14.93		687.90		683.65	
% Mean Change	0.28				**0.63**			

4. Stock Solution Stability

Stock solution stability was determined by comparing the peak areas of freshly prepared solutions (comparison samples) with stability samples.

ROSU stock solution was found to be stable at room temperature for 13 hours with % mean change of 2.51 (Table No. 7.1.3.4.9).

Table 7.1.3.4.9: Stock solution stability

Sr.No.	ROSU (400 ng/µl)	
	Com. Sample area	Stability sample area
1	20508	21892
2	21123	20405
3	20455	19393
4	19955	19955
5	21147	20559
6	20779	21635
Mean	20661	20640
SD	4533	9635
%CV	2.19	4.67
% Mean Change	2.51	

(VII) Matrix effect

In order to ensure the effect of matrix through out the application of the method, plasma blanks obtained from two different lots were spiked with ROSU and LQC and HQC

level. Three quality control samples at each level along with the set of calibration standards were analyzed and the % nominal concentration of the samples analyzed was represented in (Table No. 7.1.3.4.10) for ROSU.

Table 7.1.3.4.10: Matrix effect

Sample ID	Calculated Conc. (ng/µl)	% Nominal Conc.	Calculated Conc. (ng/µl)	% Nominal Conc.
I	LQC	14.92	HQC	693.22
	LQC	15.02	HQC	708.33
	LQC	14.29	HQC	680.33
II	LQC	16.99	HQC	677.33
	LQC	15.98	HQC	669.38
	LQC	14.78	HQC	673.29

7.1.3.4.4.2 Estimation of ROSU in marketed Tablet:

Preparation of test solution:

Test solution prepared as per procedure given in chapter 7.1.1.6.10.2. From the stock solution (1000 µg/ml) aliquots of 1 was transferred to a series of sample tubes. Then 90 µl of human plasma was added to each tube. The tubes were vortexed for 1 min on Vortex shaker. The volume was adjusted to a 1000 µl with acetonitrile to get final concentrations of 100 ng/ml of solution. The solutions were centrifuged for 5 min at 4000 rpm. 20 µl of supernatant were injected into HPLC column and the Peak area of each solution was measured at 248 nm. The concentration of sample solution was found from regression equation. The result of tablet was given in table 7.1.3.4.11.

Table 7.1.3.4.11: Estimation of ROSU in tablet by HPLC with UV detection

Tablet Formulation	Labeled Claim (mg/tablet)	Amount Found* (mg/tablet)	% Recovery ± S.D
NOVASTAT (Lupin)	5	5.35	104.18 ± 0.44
	10	10.05	100.42 ± 0.38
	20	20.02	100.43 ± 0.92

7.1.3.4.4 Summary of Validation parameters:

The summary of validation parameters is given in table 7.1.3.4.12.

Table 7.1.3.4.12: Summary of Validation parameters by HPLC with UV detection

Sr. No	Parameters	Peak Area
1	Detection wavelength (nm)	248
2	Retention time (minutes)	6.758
3	Theoretical plate	2758.39
4	Linearity range (ng/ml)	0.55-600
5	Regression equation	Y = 50560x con. +30880
6	Correlation coefficient (r^2)	0.9997
7	Intercept	50560
8	Slope	30880
9	Assay	100.42-104.18
10	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements %C % Recovery	 0.28-0.98 0.29-0.98 0.37 99.82-100.77
11	Limit of detection	0.09837 ng/ml
12	Limit of quantification	0.1498 ng/ml

7.1.3.4.5. Conclusion:

This HPLC method can estimate ROSU in human plasma with lower limit of detection as 0.0983 ng/ml.

7.1.3.5 High Performance Thin Layer Chromatography (HPTLC) with UV detection.

Two HPTLC methods were developed for the estimation of ROSU. Method A was developed for the estimation of ROSU in oral dosage form. There is no interference of excipient in analytical method. But this method was not able to separate degradation products of ROSU from the ROSU standard. So the method B was developed which can estimate ROSU and its degraded product.

7.1.3.5.1. Optimization of method:

For good chromatographic method it should be necessary that mobile phase have good polarity to separate compound for that proper selection of solvent and wavelength is required.

Determination of solvent and wavelength:

Different solvents were tried to study the solubility of ROSU (Chapter 6) and methanol was selected for the preparation of drug solutions .Wavelength was selected by scanning standard solutions of ROSU over 200 nm to 400 nm wavelengths. Drug shows significant absorbance at **231 and 243 nm** as illustrated in fig. 7.1.3.1.1. So these wavelengths were selected in densitometer UV detector.

Determination of mobile phase:

Various combinations of mobile phases were tried for sharp peak of ROSU on silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 μm thickness) (E. Merck). The results are reported in Table 7.1.3.5.1.

Table 7.1.3.5.1: Determination of mobile phase

Mobile phase combination	Ration (V/V/V)	Peak separation
Chloroform: Methanol	8: 2	Broad peak
Chloroform: Methanol	5: 5	Broad peak
Toluene: Methanol	**7: 3**	**Sharp peak**
Benzene: Methanol	5: 5	Broad peak
Ethyl acetate : Toluene: Methanol	3: 4: 3	Broad peak
Ethyl acetate: Toluene: Methanol	4: 4: 2	Broad peak
Ethyl acetate: Benzene: Methanol	3: 4: 3	Broad peak
Ethyl acetate: Toluene: Methanol: Formic acid	**6: 3: 0.5: 0.2**	**Sharp peak and separate degraded products.**
Chloroform: Toluene: Methanol: Glacial acetic acid	3: 4 : 4: 0.05	Broad peak
Chloroform: Toluene: Methanol: Glacial acetic acid	4: 4: 2: 0.2	Broad peak

It is evident from the data that mobile phase combination of **toluene: methanol** in proportion of **7: 3** v/v suitable and was selected as solvent in methd A which was used to estimate ROSU from dosage form. The e**thyl acetate: toluene: ACN: FA** in ratio of **6: 3: 0.5: 0.2** v/v/v/v was selected as mobile phase in method B which was used as SIAM. The general chromatographic parameters used for analysis are given in Chapter 6.

7.1.3.5.2. Chromatographic conditions:

Method A

- **Mobile phase:** Toluene : Methanol (7:3 v/v)
- **Scanning Wave length:** 231 nm

Method B

- **Mobile phase:** Ethylacetate: Toluene: ACN: formic acid (6:3.5:0.5:0.2 v/v/v/v)
- **Scanning Wave length:** 243 nm (good separation of degraded product)

7.1.3.5.3. Preparation of stock solution for ROSU

For chromatographic study standard ROSU (100 mg), weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml methanol properly and diluted up to mark with methanol to obtain final concentration of 1000 µg/ml.

7.1.3.5.4. Calibration curve

Analysis was performed on Pre-coated silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 µm thickness) (E. Merck) pre-washed with methanol and then dried for 30 minute at 50°C. From the stock solution (1000 µg/ml) aliquots of 0.3, 0.6, 0.9, 1.8, 2.7 and 3.9 µl were spotted on the TLC plate under nitrogen stream using Desaga Applicator, AS30win to obtain final concentration range 300 - 3900 ng/spot.

The plate was dried in a FTIR, and then the plate was developed in Twin trough developing chamber (100 × 100) with stainless steel lid, previously saturated with the mobile phase for 30 min. The plate was removed from the chamber, dried in a FTIR and was scanned and quantified at 231 and 243 nm in Re-emission/Excitation mode with Desaga TLC scanner, Proquant. The calibration curve was constructed by plotting peak area vs. concentration (ng/spot) and peak height vs. concentration (ng/spot) corresponding to each spot and regression equation was calculated

7.1.3.5.5. Validation Parameters

Procedure for the validation of analytical method was given in section 7.1.1.8.4.

7.1.3.5.6 Results and Discussion:

7.1.3.5.6.1 Validation Parameters

(I) Linearity

Linearity range of ROSU was found to be 318 - 3816 ng/spot for both the method. Correlation co-efficient and % C.V. for method A was 0.9985 and 0.32 – 0.93 for peak

area, 0.9993 and 0.33-0.93 for peak height and for method B was 0.9993 and 0.31 – 0.87.

Table 7.1.3.5.2: Calibration data of ROSU by HPTLC with UV detection

Sr. No.	Concentration (ng/spot)	Method A					Method B		
		Peak Area		Peak Height		R_f*	Peak Area		R_f*
		Mean ± SD	%CV	Mean ± SD	%CV		Mean	%CV	
1	318	130 ± 0.87	0.92	195 ± 0.20	0.83	0.37	213.00	0.59	0.84
2	636	370 ± 0.23	0.73	302 ± 0.38	0.53	0.38	280.00	0.31	0.83
3	954	510 ± 0.89	0.83	371 ± 0.98	0.92	0.37	373.88	0.66	0.84
4	1908	970 ± 0.47	0.53	618 ± 0.37	0.45	0.37	590.00	0.38	0.84
5	2862	1510 ± 0.19	0.93	849 ± 0.87	0.93	0.36	833.42	0.87	0.84
6	3816	1980 ± 0.37	0.32	1089 ± 0.57	0.33	0.38	1042.22	0.42	0.85

***Average of five readings**

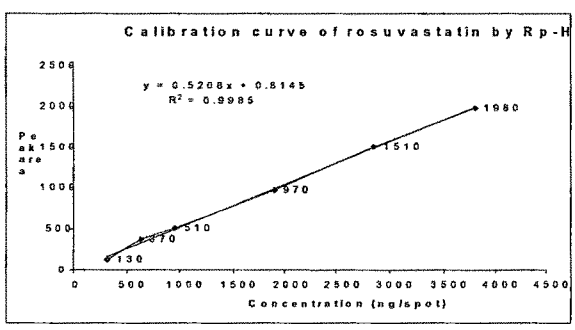


Figure 7.1.3.5.1: Calibration curve of ROSU by HPTLC by method A (Peak Area)

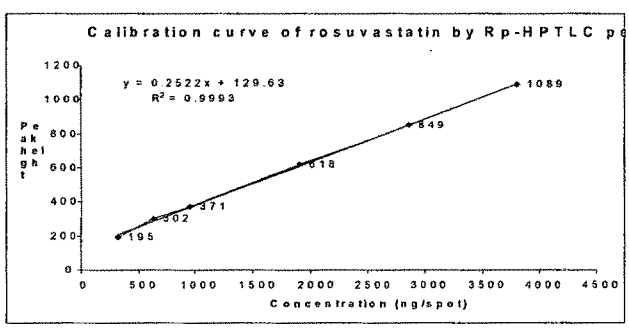


Figure 7.1.3.5.2: Calibration curve of ROSU by HPTLC by method A (Peak Height)

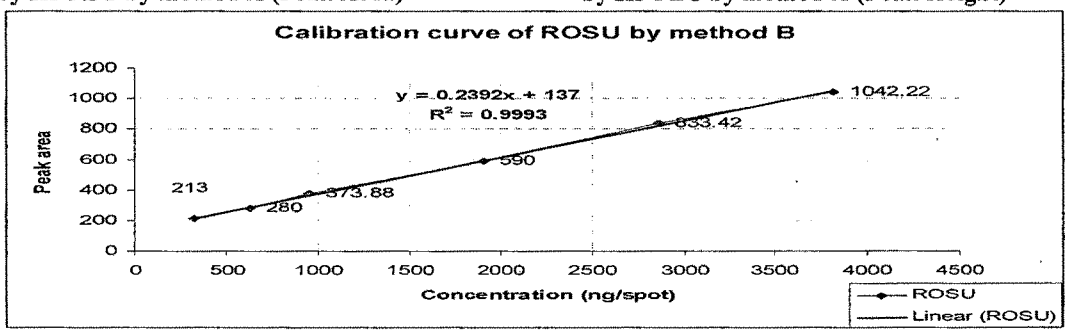


Figure 7.1.3.5.3: Calibration curve of ROSU by HPTLC by method B(Peak Area)

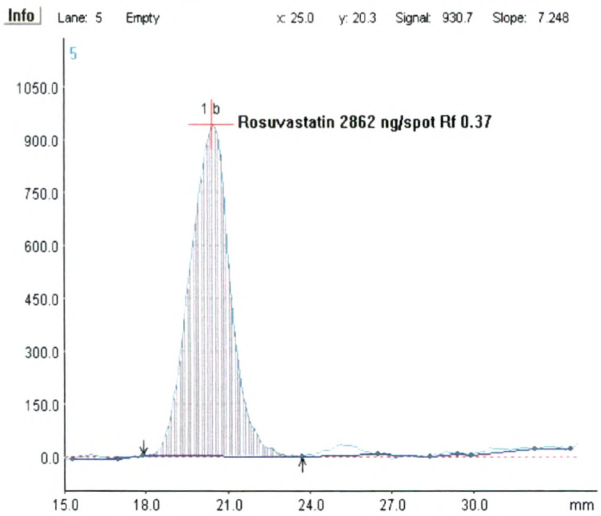


Figure 7.1.3.5.4: Single Spectrum of ROSU by HPTLC by method A

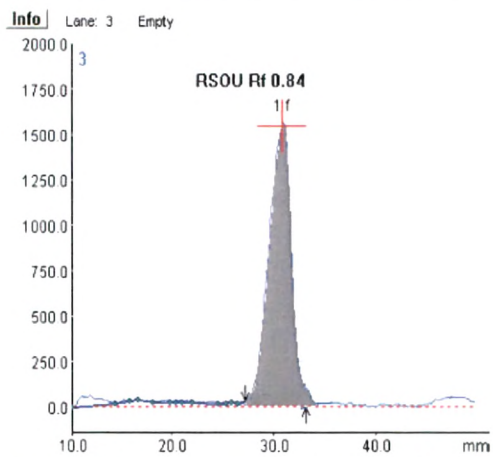


Figure 7.1.3.5.5: Single Spectrum of ROSU by HPTLC by method B

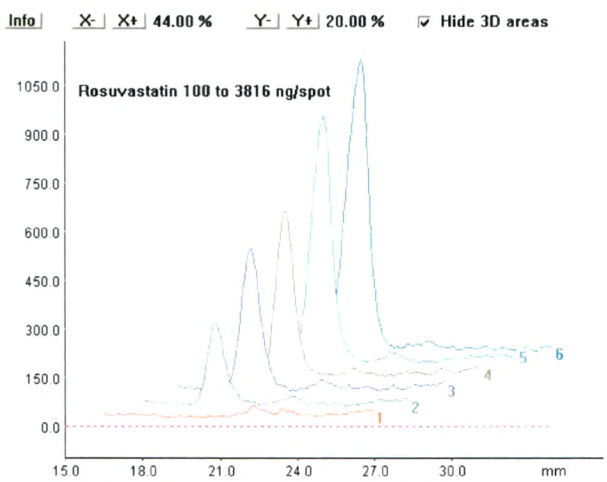


Figure 7.1.3.5.6: 3D Spectra of ROSU by HPTLC by method A

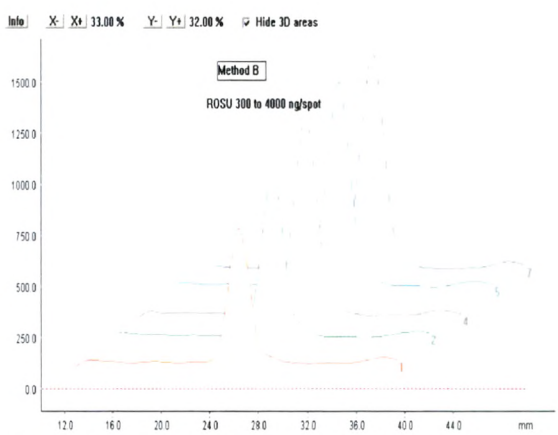


Figure 7.1.3.5.7: 3D Spectra of ROSU by HPTLC by method B

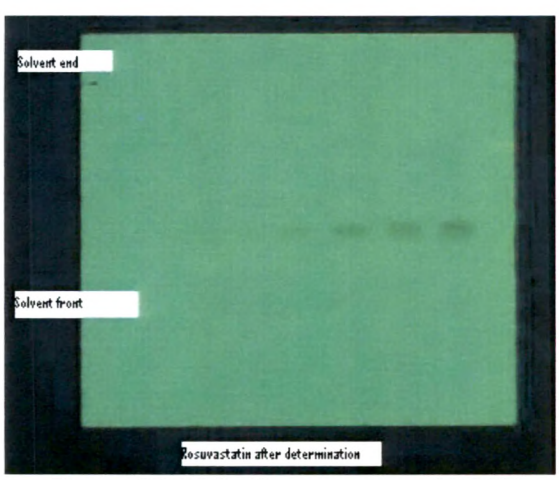


Figure: 7.1.3.5.8: Chromatogram of ROSU by method A (After detection)

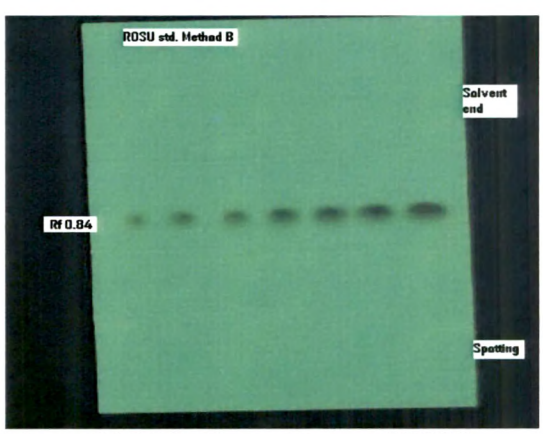


Figure: 7.1.3.5.9: Chromatogram of ROSU by method B (After detection)

(II) Precision

a. Repeatability

Intraday and Interday Precision

Intraday and interday variation of the method A and B were reported in table 7.1.3.5.3.

Table 7.1.3.5.3: Intraday precision data of ROSU by HPTLC with UV detection

Sr. No.	Conc. (ng/spot)	Method A					Method B		
		Peak Area		Peak Height		R_f*	Peak Area		R_f*
		Mean ± SD	%CV	Mean ± SD	%CV		Mean	%CV	
1	500	267 ± 0.39	0.76	274 ± 0.67	0.23	0.38	245.95	0.93	0.84
2	1500	763 ± 0.28	0.56	486 ± 0.28	0.83	0.37	463.83	0.48	0.84
3	2000	1057 ± 0.47	0.38	694 ± 0.48	0.29	0.38	582.40	0.66	0.83
Table 7.1.3.5.4: Interday precision data of ROSU by HPTLC with UV detection									
1	500	267 ± 0.56	0.28	274 ± 0.46	0.91	0.37	240.13	0.48	0.84
2	1500	763 ± 0.67	0.67	486 ± 0.34	0.10	0.38	487.86	0.24	0.83
3	2000	1055 ± 0.36	0.17	693 ± 0.99	0.31	0.38	568.45	0.77	0.84

***Average of five readings**

b. Reproducibility:

As per the instrument specification the % C.V. of repeated measurement of the same solution should not more than 1%.

Table 7.1.3.5.5: Reproducibility data of ROSU by HPTLC (400 ng/spot)

Time	Method A			Method B	
	Peak Area	Peak Height	Rf	Peak Area	R_f
Aluminum TLC plate	192.64	215.14	0.37	210.943	0.84
	192.72	215.17	0.38	212.075	0.84
	193.05	215.30	0.38	217.572	0.83
Glass TLC plate	192.14	215.93	0.37	212.7673	0.84
	194.00	215.69	0.37	217.3606	0.84
	192.91	215.25	0.38	211.9355	0.85
S.D.	0.69	0.28	0.12	0.78	0.38
%CV	0.23 (<1%)	0.98 (<1%)	0.89 (>1%)	0.44	0.00632

***Average of five readings**

(III) Accuracy

Accuracy of the measurement of ROSU was determined by standard addition and was found to be in the range of 99.99 – 102.38 % for peak area and100.49 – 101.40% for peak height of method A and 99.77 – 103.94 % of method B.

Table 7.1.3.5.6: Accuracy data of ROSU by HPTLC with UV detection

Spott Amount (A + B)	Method A				Method B	
	Peak Area		Peak Height		Peak Area	
	Total quantity Found Mean ± S.D.	%Assay ± S.D	Total quantity Found Mean ± S.D.	% Assay ± S.D	Total quantity Found	% Assay
750	749.98 ± 0.84	99.99 ± 0.33	759.29 ± 0.53	101.23 ± 0.39	748.29	99.77
1000	1023.84 ± 0.47	102.38 ± 0.34	1004.98 ± 0.83	100.49 ± 0.38	1039.43	103.94
1500	1503.08 ± 0.38	100.20 ± 0.35	1521.09 ± 0.54	101.40 ±0.86	1523.94	101.60

***Average of five readings**

(IV) Limit of detection

The minimum detectable concentration of ROSU was found to be 0.6580 ng/spot for peak area and 0.5712 ng/spot for peak height by method A and 0.986 ng/spot for method B.

(V) Limit of quantification

The lowest quantifiable concentration of ROSU was found to be 2.1936 ng/spot for peak area and 1.9042 ng/spot for peak height for method A and 2.682 ng/spot for method B.

7.1.3.5.7.2. Estimation of ROSU in marketed Tablet:

Preparation of test solution:

Test solution was prepared as per procedure given in section 7.1.3.1.10.2. 0.5 µl from this solution was spotted on the TLC plate under nitrogen stream using Desaga Applicator, AS30win. The plate was dried in air, and then the plate was developed in Twin trough developing chamber (100 × 100) with stainless steel Lid, previously saturated with the mobile phase for 30 min of both method. The plate was removed from the chamber, dried in air and was scanned and quantified at 231 nm and 248 nm in Reemission/Excitation mode with Desaga TLC scanner, Proquant. The concentration of sample solution was found from regression equation. Result of the analysis are reported in table 7.1.3.5.6

Table 7.1.3.5.7: Estimation of ROSU in tablet by HPTLC with UV detection

Tablet Formulation	Labeled Claim (mg/tablet)	Method A				Method B	
		Peak Area		Peak Height		Peak Area	
		Amount found (mg/tablet)	% Recovery ± S.D	Amount found (mg/tablet)	% Recovery ± S.D	Amount found (mg/tablet)	% Recovery
NOVASTAT (Lupin)	5	5.29	104.18	5.12	102.42	-	-
	10	10.04	100.42	10.12	101.23	10.29	102.93
	20	20.08	100.43	20.12	100.51	-	-

***Average of five readings**

Applicability of method B:

Method B can easily estimate ROSU in pharmaceutical dosage form but it could estimate ROSU in presence of its degradation products. The results of forced degradation study of ROSU (table) suggested that ROSU was stable against thermal, photolytic, neutral and alkaline conditions. So it was degraded under oxidative and acidic condition only.

Procedure for forced degradation study

Forced degradation study was done in acidic condition only. 25 mg drug was accurately weighed and transferred to 25 ml volumetric flask. The drug was dissolved in 5 ml of methanol then diluted with con. HCl. The degradation was done at room temperature. Samples were colleted for analysis at various stages, at 0 min (as soon as sample was prepared), after 1 hrs and after 4 hrs of exposure to degradation condition for room temperature. Sample was applied by direct spotting of 1 µl on prewashed TLC plate. Fig 7.1.3.5.10 to 7.1.3.5.11 shows the chromatograms of forced degraded samples.

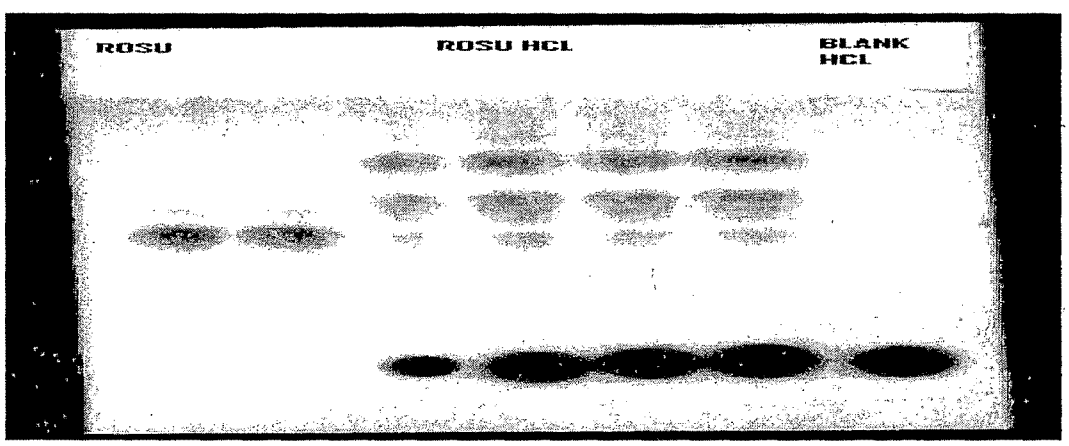


Figure: 7.1.3.5.10: HPTLC Chromatogram of ROSU and its acidic degradation in UV detection.

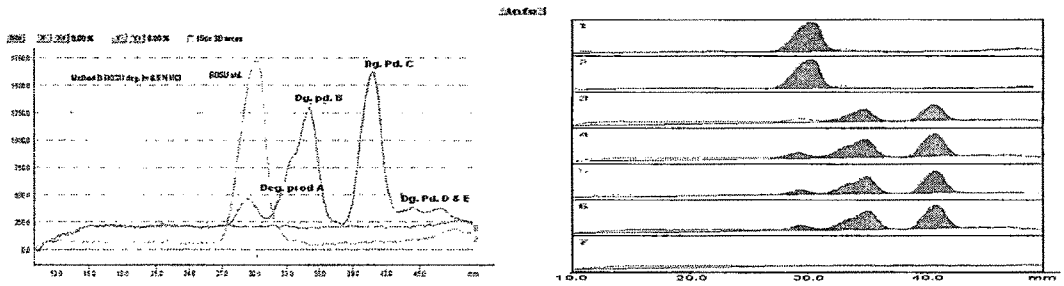


Figure: 7.1.3.5.11: Overlain and vertical HPTLC Chromatogram of ROSU and its acidic degradation products.

7.1.3.5.8 Summary of Validation parameters:

The summary of validation parameters is reported in table 7.1.3.5.8.

Table 7.1.3.5.8: Summary of Validation parameters by HPTLC

Sr. No	Parameters	Method A		Method B
		Peak Area	Peak Height	Peak Area
1	Detection wavelengths (nm)	231	231	243
2	Linearity range (ng/spot)	100 – 3672		
3	Regression equation	Y = 0.5208 x conc.+0.8145	Y= 0.2522 x conc.+ 129.63	Y = 0.2392 x conc.+ 137
4	Correlation coefficient (r^2)	0.9985	0.9993	0.9993
5	Intercept	0.8145	129.63	137
6	Slope	0.5208	0.2522	0.2392
7	Assay	100.18-100.42	100.51-102.42	102.93
8	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements %CV % Recovery	 0.38-0.76 0.17-0.67 0.23 99.99-102.38	 0.23-0.83 0.10-0.91 0.98 100.49-101.40	 0.48-0.93 0.24-0.77 0.44 99.77-103.94
9	Limit of detection	0.6580	0.5712	0.986
10	Limit of quantification	2.1936	1.9042	2.682

7.1.3.5.9. Conclusion:

HPTLC method was developed for the estimation of ROSU in pharmaceutical dosage form and in presence of its acid degradates. Degradation study in acidic condition showed that ROSU was degraded to five degraded products (fig. 7.1.3.5.9 to 11). Upon prolong degradation five degradation products may converted in to two main degradation product as seen in HPLC study and seen in TLC plate after development.

7. 1. 4

Simvastatin

7.1.4. Simvastatin

The parent drug is a pharmacologically inactive lactone (prodrug form), which is absorbed from the stomach, extracted by the liver, and largely converted to several active metabolites. The most notable is SIMVA hydroxy acid (Fig. 1)[1-5]. The active metabolites tend to concentrate in the liver, a major site of cholesterol endogenous synthesis. The inhibition of HMG-CoA reductase results in a moderate reduction in cholesterol synthesis.

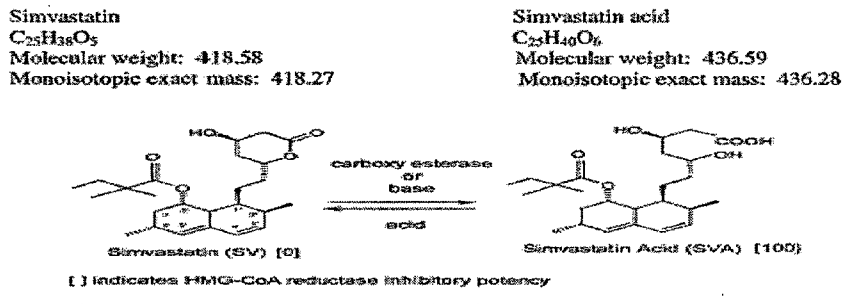


Fig. 1. Structure of simvastatin (SV) and simvastatin acid (SVA).

Spectrophotometry and chromatographic methods were developed for the estimation of SIMVA in pharmaceutical dosage form. Degradation study was done to study the inherent character of drug. Bio analytical method was developed for pharmacokinetic and bioavailability study.

Spectrophotometry

Five Spectrophotometric methods were developed for the estimation of SIMVA in oral dosage form. Simple zero order spectrophotometric method was developed for routine analysis of SIMVA. To study specific spectra characterization first and second derivative spectrophotometric method was tried. One of new approach of 3 wavelengths was tried which gave very reliable results for estimation of oral dosage form. Advance quantitative FTIR spectroscopic method also could accurately estimate SIMVA in tablet dosage form.

Chromatography

To study the inherent stability characters of SIMVA, stress degradation study was done. HPLC method was developed which can estimate SIMVA in presence of its degraded products. To study the pharmacokinetic data developed HPLC method was used for the estimation of SIMVA in spiked human plasma. HPTLC methods were developed to estimate SIMVA form pharmaceutical ,formulation and to estimate

SIMVA in presence of its degraded products and also from pharmaceutical formulation.

Apparatus, instruments and reagents:

Apparatus, instruments and reagents used for the development of spectroscopic and chromatographic methods are mentioned in chapter 6.

7.1.4.1 Estimation of SIMVA by zero order spectrophotometry, first order and second order derivative Spectrophotometric methods.

Experimental Work:

7.1.4.1.1. Preparation of stock solution for SIMVA

Standard SIMVA (10 mg) was weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml methanol and diluted up to mark with methanol to obtain final concentration of 100 µg/ml. This solution was used as working standard solution for simple zero order, three wavelengths, derivative and difference spectroscopic methods.

7.1.4.1.2. Selection of analytical wavelength

Simple UV spectroscopic method was developed in methanol as solvent. SIMVA shows maximum absorbance at 237.40 nm (Fig. 7.1.4.1.1), which was used for the further estimation. All spectra were transformed in derivative command by transformation function of UV PC software. First order derivative curves were studied and simultaneous peak and valley were selected so that amplitude difference can give precise correlation coefficient. The maximum 235.60 nm and minimum 240 nm (Fig. 7.1.4.1.2), at which r^2 was 0.9997, were selected as analytical wavelengths for further study. The amplitude difference was measured for each concentration of standard.

Second order derivative curves were studied and maximum 242.60 nm and minimum 246.60 nm (Fig. 7.1.4.1.3), at which r^2 was 0.9995, were selected for further study. The amplitude difference was measured for each concentration of standard.

7.1.4.1.3. Calibration curve:

From the stock solution (100 µg/ml) aliquots of 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 ml were transferred to a series of 10ml volumetric flasks. The volume was adjusted to a mark with methanol to get 1, 5, 10, 15, 20, 25, 30, 35 and 40 µg/ml of solution. The absorbance was measured at λ_{max} or selected wavelength using methanol as a blank

7.1.4.1.4. Validation Parameters

Procedure for validation of method was given in chapter 7.1.1.1.2. Aliquots were taken form stock solution of SIMVA.

7.1.4.1.5 Results and Discussion:

7.1.4.1.5.1 Validation Parameters

(I) Linearity

Linearity range of SIMVA in methanol was found to be 5-40 µg/ml for all 3 methods correlation co-efficient 0.9998, 0.9997 and 0.9995, respectively for zero order spectrophotometry, first and second derivative spectrophotometry and % C.V. ranging from 0.11 – 0.96, 0.14 – 0.92 and 0.19-0.63 %, respectively for zero order spectrophotometry, first and second derivative spectrophotometry.

Table 7.1.4.1.1: Calibration data of SIMVA by Spectrophotometry

Sr. No.	Conc. (µg/ml)	Simple UV Spectrophotometric method	1st derivative Spectrophotometric method			2nd derivative Spectrophotometric method		
		Absorb.* ± %CV	Absorb. at 235.60 nm	Absorb. at 240.00 nm	Amplitude Difference* ± S.D	Absorb. at 242.60 nm	Absorb. at 246.60 nm	Amplitude Difference* ± S.D
1	5	0.357 ± 0.11	0.007	-0.043	0.05 ± 0.83	0.014	-0.021	0.035 ± 0.48
2	10	0.644± 0.84	0.02	-0.078	0.098 ± 0.18	0.028	-0.042	0.07 ± 0.29
3	15	0.946 ± 0.19	0.032	-0.113	0.145 ± 0.37	0.042	-0.064	0.106 ± 0.63
4	20	1.21 ± 0.57	0.042	-0.154	0.196 ± 0.89	0.055	-0.084	0.139 ± 0.19
5	25	1.529 ± 0.39	0.052	-0.194	0.246 ± 0.22	0.068	-0.104	0.172 ± 0.59
6	30	1.83 ± 0.76	0.061	-0.228	0.289 ± 0.92	0.081	-0.122	0.203 ± 0.39
7	35	2.13 ± 0.82	0.066	-0.279	0.345 ± 0.14	0.09	-0.147	0.237 ± 0.37
8	40	2.422 ± 0.96	0.081	-0.311	0.392 ± 0.44	0.108	-0.167	0.275 ± 0.45

*** Mean of five determinations**

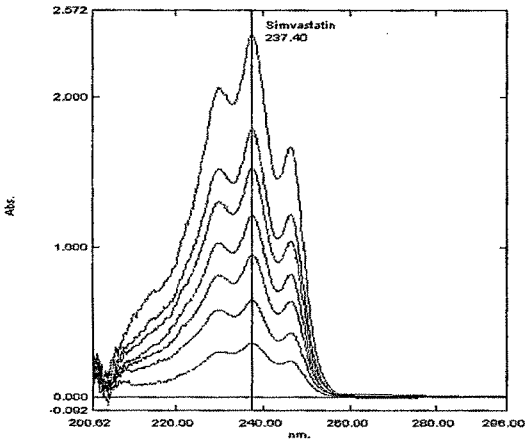


Figure: 7.1.4.1.1 UV spectra of SIMVA by Simple UV Spectrophotometry

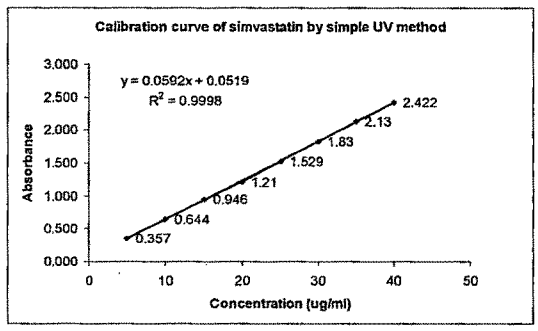


Figure 7.1.4.1.2: Calibration curve of SIMVA by Simple UV Spectrophotometry
Correlation co-efficient = **0.9998**
Slop = 0.0592
Intercept = 0.0519
Regression equation: **Abs. = 0.0592 × Conc. + 0.0519**

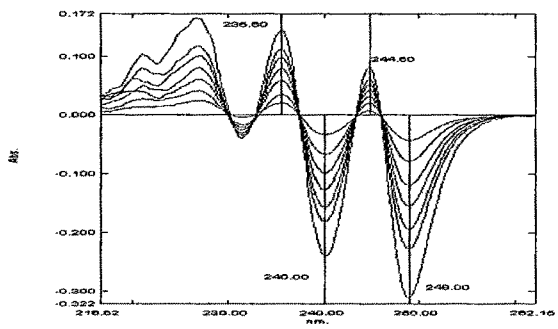


Figure: 7.1.4.1.3: First derivative UV spectra of SIMVA

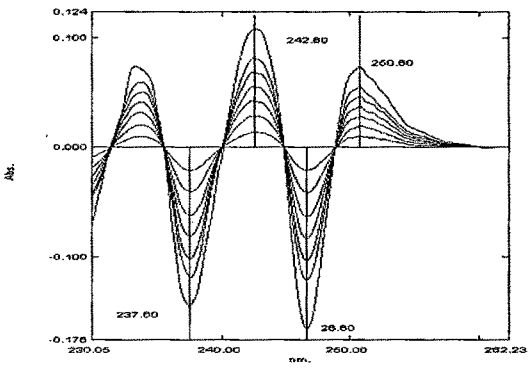


Figure: 7.1.4.1.4: 2nd derivative UV spectra of SIMVA

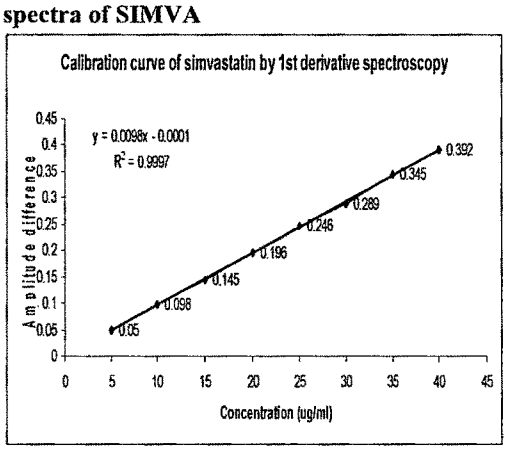


Figure 7.1.4.1.5: Calibration curve of SIMVA by 1st derivative Spectrophotometry
Correlation co-efficient = 0.9997
Slope = 0.0098
Intercept = -0.0001
Regression equation: Abs. = 0.0098 × Conc. - 0.0001

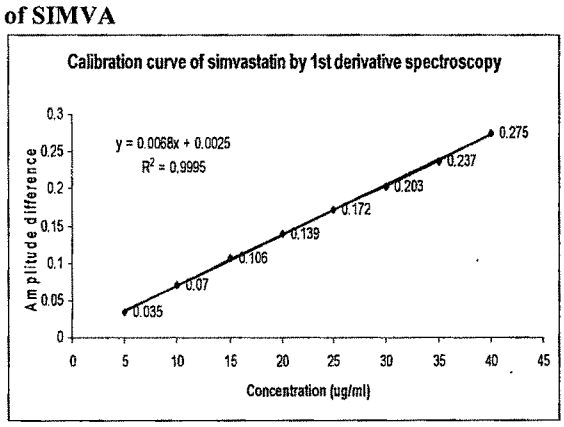


Figure 7.1.4.1.6: Calibration curve of standard SIMVA by 2nd derivative
Correlation co-efficient = 0.9995
Slope = 0.0068
Intercept = 0.0025
Regression equation: Abs. = 0.0068 × Conc. + 0.0025

(II) Precision a. Intraday and Interday Precision (Repeatability)

Intraday variation of the proposed method was 0.28 – 0.67, 0.28 – 0.58 and 0.19 – 0.75 %, respectively for zero order spectrophotometry, first and second derivative spectrophotometry.

Interday variation of the proposed method was 0.12 – 0.98, 0.29 – 0.50 0.29 – 0.84 %, respectively for zero order spectrophotometry, first and second derivative spectrophotometry.

Table 7.1.4.1.2: Intraday precision data of SIMVA by Spectrophotometry

Conc. (µg/ml)	Simple UV Spectrophotometric method	1st derivative Spectrophotometric method		2nd derivative Spectrophotometric method	
	Absorb. * ± % C.V.) (n=5)	Amplitude difference* ± S.D. (n=5)	% C.V.	Amplitude difference* ± S.D. (n=5)	% C.V.
10	0.629 ± 0.48	0.097 ± 0.28	0.33	0.071 ± 0.39	0.28
20	1.273 ± 0.28	0.193 ± 0.038	0.58	0.142 ± 0.46	0.75
30	1.890 ± 0.67	0.290 ± 0.82	0.28	0.209 ± 0.29	0.19
Table 7.1.4.1.3: Interday precision data of SIMVA by Spectrophotometry					
10	0.651 ± 0.98	0.097 ± 0.67	0.48	0.072 ± 0.29	0.29
20	1.260 ± 0.12	0.193 ± 0.52	0.29	0.143 ± 0.72	0.84
30	1.872 ± 0.47	0.292 ± 0.43	0.50	0.208 ± 0.46	0.32

*** Mean value of five determinations**

b. Reproducibility:

The % C.V. of repeated measurement of the same solution was not more than 1%.

Table 7.1.4.1.4: Reproducibility data of SIMVA by Spectrophotometry

Conc. (µg/ml)	Simple UV Spectrophotometric method		1st derivative Spectrophotometric method		2nd derivative Spectrophotometric method	
	Absorbance* ± %C.V.					
	UV 1700	UV 1601	UV 1700	UV 1601	UV 1700	UV 1601
10	0.644 ± 0.47	0.643 ± 0.25	0.098 ± 0.42	0.094 ± 0.74	0.07 ± 0.43	0.03 ± 0.97
15	0.946 ± 0.21	0.943 ± 0.82	0.145 ± 0.69	0.142 ± 0.59	0.106 ± 0.29	0.103 ± 0.48
20	1.21 ± 0.78	1.233 ± 0.99	0.196 ± 0.33	0.193 ± 0.29	0.143 ± 0.39	0.142 ± 0.29

*** Mean value of three determinations**

(III) Accuracy

Accuracy of the measurement of SIMVA was determined by standard addition and was found to be in the range of 99.90 – 102.33, 99.16 -101.65 and 99.27 – 100.65%, respectively for zero order spectrophotometry, first and second derivative spectrophotometry.

Table 7.1.4.1.5: Accuracy data of SIMVA by Spectrophotometry

Initial conc. (µg/ml) (A)	Quantity of std. Added (µg/ml) (B)	Total Amount (A + B)	Simple UV Spectrophotometric method		1st derivative Spectrophotometric method		2nd derivative Spectrophotometric method	
			Total quantity Found * ± S.D.	% Recovery ± S.D	Total quantity Found* ± S.D.	% Recovery ± S.D	Total quantity Found* ± S.D.	% Recovery ± S.D
10	5	15	15.35 ± 0.023	102.33 ± 0.087	14.98 ± 0.48	99.87 ±0.14	14.89 ± 0.49	99.27 ± 0.11
10	10	20	19.98 ± 0.068	99.90 ± 0.038	20.33 ± 0.39	101.65 ± 0.87	20.13 ± 0.28	100.65 ± 0.54
10	15	25	25.08 ± 0.085	100.32 ± 0.067	24.79 ± 0.98	99.16 ± 0.57	24.98 ± 0.58	99.92 ± 0.49

*** Mean value of five determinations**

(IV) Limit of detection

The minimum detectable concentration of SIMVA was found to be 0.31, 0.088 and 0.047 µg/ml, respectively for zero order spectrophotometry, first and second derivative spectrophotometry.

(V) Limit of quantification

The lowest quantifiable concentration of SIMVA was found to be 0.903, 0.298 and 0.151 µg/ml, respectively for zero order spectrophotometry, first and second derivative spectrophotometry.

7.1.4.1.3.2. Estimation of SIMVA in marketed Tablet:

Preparation of test solution:

Two brands of SIMVA, IFISTATIN of JB chemicals and STATIN of Unisearch, were analyzed by the proposed method. The Tablet powder equivalent one Tablet content was transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution was sonicated for 10 min and supernatant was filtered through whatman filter paper no.41. 1 and 2 ml from this solution were transferred to 10 ml volumetric flasks and diluted with methanol to get 10 and 20 µg/ml concentration. The absorbance of this solution

was measured at 237.40 nm and concentration of sample solution was found from regression equation.

Zero order save data were converted in to derivative mode and the absorbance was measured at λ_{maxima} = 235.20 nm and λ_{minima} = 240.00 nm. The amplitude difference was measured and concentration of sample solution was found from regression equation.

Zero order spectra of test samples were converted in to 2nd derivative and the absorbance was measured at λ_{maxima} = 242.60 nm and λ_{minima} = 246.60 nm. The amplitude difference was measured and concentration of sample solution was found from regression equation.Result of tablets are given in table 7.1.4.1.1.6 in term of % assay.

Table 7.1.4.1.1.6: Estimation of SIMVA in tablet by Spectrophotometry

Tablet Formu-lation	Labeled Claim (mg/Tablet)	Simple UV Spectrophotometric method		1st derivative Spectrophotometric method		2nd derivative Spectrophotometric method	
		Amount Found* (mg/Tablet)	% Assay ± S.D	Amount Found* mg/Tablet)	% Assay ± S.D	Amount found (mg/Tablet)	% Assay ±S.D
IFISTATIN (JB chemicals)	5	5.01	100.2	5.12	102.4	5.09	101.8
	10	10.19	101.9	9.98	99.8	9.99	99.9
	20	20.62	103.1	20.49	102.45	20.98	104.9
STATIN (Unisearch)	10	10.09	100.9	10.12	101.2	10.29	102.9
	20	20.45	102.25	20.68	103.4	20.33	101.65

*** Mean value of five determinations**

7.1.4.1.4 Summary of Validation parameters:

The summary of validation parameters is given in table 7.1.4.1.7.

Table 7.1.4.1.7: Summary of Validation parameters of Spectrophotometry

Sr. No	Parameters	Simple UV Spectrophotometric method	1st derivative Spectrophotometric method	2nd derivative Spectrophotometric method
1	λ max (nm)	237.20	235.20	242.60
	λ max (nm)	-	240.00	246.60
2	A (1%, 1cm) (dl gm^{-1} cm^{-1})	579.31	-	-
3	Molar Absoptivity (ε) (L mol^{-1} cm^{-1})	6715.98(>100)	-	-
4	Linearity range (µg/ml)	5 -40	5 – 40	5 -40

5	Regression equation	Abs. = 0.0592 × Conc. + 0.0519	Y = 0.0098x Con. –0.0001	Y = 0.0068X con. + 0.0025
6	Correlation coefficient (r^2)	0.9998	0.9992	0.9995
7	Intercept	0.0519	0.0001	0.0025
8	Slope	0.0592	0.0098	0.0068
9	Sandell's sensitivity (µg/cm^2/0.001 abs. unit))	0.9453	-	-
10	Assay	100.2 – 102.25 %.	99.8 – 103.4 %.	99.9 – 104.9
11	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements % CV % Recovery	0.28-0.67 %. 0.12-0.98 %. <1% 99.90 – 102.33	0.28-0.58 %. 0.29-0.850%. < 1 % 99.16 –101.65 %.	0.19 – 104.9 % 0.29 – 0.84 % < 1 % 99.27 – 100.65 %
12	Limit of detection	0.31 µg/ml	0.088	0.047
13	Limit of quantification	0.903 µg/ml	0.298	0.151

7.1.4.1.5. Conclusion:

Simple, accurate and economic method was developed for the estimation of SIMVA in pharmaceutical oral dosage form. As summary table shows method can work in the range of 5 – 40 µg/ml with accuracy of 99.90 – 102.33 % with %CV of reproducibility was < 1 %.

Both the first and second derivative spectroscopic methods were developed for the estimation of SIMVA in oral dosage form with the recovery of 99.16 -101.65% and 99.27 – 100.65 %, respectively. Derivative method was developed because it is a useful tool for the estimation of drug in presence of excipients.

7.1.4.2 3-wavelength Photometric method

The three analytical wavelengths selected after scanning a standard solution in the range f 220 – 250 nm were **WL1 = 234.20 nm, WL2 = 237.40 nm, WL3 = 241.20 nm.** The net absorbance of SIMVA was determined by using the equation described in chapter 5.

Preparation of stock solution, calibration curve and test solution were same as described earlier.

7.1.4.2.1 Results and Discussion:

7.1.4.2.1.1 Validation Parameters

(I) Linearity

Linearity range of SIMVA in methanol was found to be 1 - 40µg/ml with correlation co-efficient 0.9986 and % C.V. ranging from 0.18 – 0.83% for different concentrations.

Table 7.1.4.2.1: Calibration data of SIMVA by 3-wavelength

Sr. No.	Concentration (µg/ml)	Absorbance at 243.20 nm	Absorbance at 237.40 nm	Absorbance at 241.20 nm	Net Absorbance (N=5)*	% C.V.
1	5	0.298	0.357	0.254	0.079	0.28
2	10	0.544	0.644	0.437	0.149	0.53
3	15	0.775	0.946	0.642	0.232	0.29
4	20	0.984	1.21	0.831	0.296	0.67
5	25	1.248	1.529	1.051	0.371	0.83
6	30	1.464	1.786	1.219	0.434	0.58
7	35	2	2.376	1.740	0.495	0.18
8	40	2.008	2.422	1.678	0.565	0.49

*** Average of five readings**

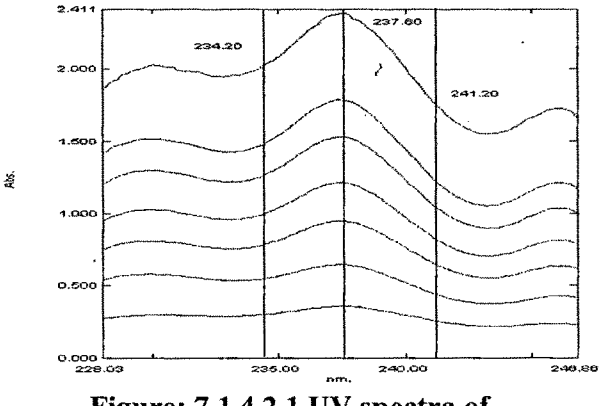


Figure: 7.1.4.2.1 UV spectra of SIMVA for 3-wavelength method

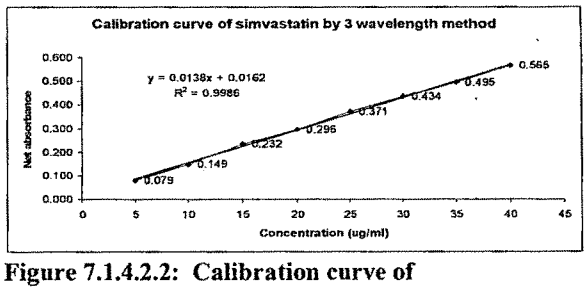


Figure 7.1.4.2.2: Calibration curve of SIMVA by 3-wavelength method
Correlation co-efficient = **0.9986**
Slope = 0.0138
Intercept = 0.0162
Regression equation: **Abs. = 0.0138 × Conc. + 0.0162**

(II) Precision

a. Repeatability

Intraday and Interday Precision

Intraday and interday variation of the proposed method were reported in table 7.1.4.2.2.

Table 7.1.4.2.2: Intraday precision data of SIMVA by 3-wavelength method

Concentration (µg/ml)	Intraday		Interday	
	Absorbance (Mean* ± S.D.) (n=5)	% C.V.	Absorbance (Mean* ± S.D.) (n=5)	% C.V.
10	0.155 ± 0.0037	0.39	0.156 ± 0.0037	0.39
20	0.299 ± 0.0083	0.94	0.297 ± 0.0058	0.64
30	0.464 ± 0.0029	0.48	0.467 ± 0.0028	0.73

*** Mean value of five determinations**

b. Reproducibility:

The reproducibility of the method was determined by using Shimadzu UV 1700 and Shimadzu UV 1601. The % CV of the responses confirms the reproducibility of the method for determination of SIMVA the data is reported in (Table 7.1.4.3.3.2).

Table 7.1.4.2.3: Reproducibility data of SIMVA

Concentration (µg/ml)	Absorbance*		% CV	
	UV 1700	UV 1601	UV 1700	UV 1601
10	0.149	0.142	0.38	0.85
15	0.232	0.235	0.45	0.22
20	0.296	0.299	0.69	0.78

*** Mean value of three determinations**

(III) Accuracy

Accuracy of the measurement of SIMVA was determined by standard addition and was found to be in the range of 99.16 – 100.40 %.

Table 7.1.4.2.4: Accuracy data of SIMVA by 3-wavelength method

Initial conc. (µg/ml) (A)	Quantity of std. Added (µg/ml) (B)	Total Amount (A + B)	Total quantity Found* ± S.D.,	% Recovery± S.D
10	05	15	15.00 ± 0.23	100.00 ± 0.28
10	10	20	20.08 ± 0.38	100.40 ± 0.43
10	15	25	24.79 ± 0.75	99.16 ± 0.83

*** Mean value of five determinations**

(IV) Limit of detection

The minimum detectable concentration of SIMVA was found to be 0.014 µg/ml.

(V) Limit of quantification

The lowest quantifiable concentration of SIMVA was found to be 0.482 µg/ml by practically observation.

7.1.4.2.1.2. Estimation of SIMVA in marketed Tablet:

Test solution prepared as per procedure given in section 7.1.4.1.3.2. The percentage of SIMVA was found from the regression equation.

Table 7.1.4.2.5: Estimation of SIMVA in tablet by 3-wavelength method

Tablet Formulation	Labeled Claim (mg/tablet)	Amount Found* (mg/tablet)	% Assay ± S.D
IFISTATIN (JB chemicals)	5	4.92	98.46 ± 0.043
	10	10.30	102.95 ± 0.043
	20	20.19	100.93 ± 0.32
STATIN (unisearch)	10	9.85	98.54 ± 0.83
	20	19.94	99.71 ± 0.31

*** Mean value of five determinations**

7.1.4.2.2. Summary of Validation parameters

The summary of validation parameters is given in table 7.1.4.2.6.

Table 7.1.4.2.6: Summary of Validation parameters by 3-wavelength method

Sr. No	Parameters	Results
1	WL1 (nm)	234.20
2	WL2 (nm)	237.40
3	WL3 (nm)	241.20
4	Linearity range (µg/ml)	5 – 40
5	Regression equation	Y = 0.0138Xcon. + 0.0162
6	Correlation coefficient (r^2)	0.9986
7	Intercept	0.0162
8	Slope	0.0138
9	Assay (%)	98.46 – 102.95
10	Precision	
	Intra day % CV (n = 5)	0.39 – 0.94
	Inter day % CV (n = 5)	0.39 – 0.73
	Reproducibility of measurements % CV	< 1%
	% Recovery	99.16 – 100.40

11	Limit of detection (µg/ml)	0.014
12	Limit of quantification (µg/ml)	0.48

7.1.4.2.3. Conclusion:

Three wavelengths spectroscopic method was developed for the estimation of SIMVA in pharmaceutical oral dosage forms. This method can estimate SIMVA in dose of 5 – 20 mg with the accuracy of 99.16 – 100.40%.

7.1.4.3. Estimation of SIMVA by FT-Infra Red spectroscopy method.

Experimental work:

7.1.4.3.1. Preparation of stock solution for SIMVA

For quantitative FT-IR spectroscopic study standard SIMVA (10 mg), weighed accurately and make up to 100 mg in same butter paper to obtain final concentration of 100 µg/mg. This sample was triturated in mortar and pestle and sample was used as working standard sample for FTIR spectroscopic method.

7.1.4.3.2. Selection of analytical wave number:

Diffuse reflectance infrared fourier transform spectroscopy (DRIFTS) spectra were applied for the analysis of SIMVA at 1800 to 1650 cm^{-1} and 1267 cm^{-1} was selected for measurement of Peak area and peak height at single wave length, respectively. Calibration curves were drawn using the multiple linear regression (MLR) method and the drug amount in sample was computed by QUANT function of FT-IR solution software.

7.1.4.3.3. Calibration curve:

Appropriate fractions were transferred from standard (100 µg/mg) to butter paper and weight adjusted 10 mg with KBr to get minimum transmission. Peak area and peak height was measured at selected wavenumber. Calibration curves were drawn using the multiple linear regression (MLR) method and the drug amount in sample was computed from regression equation.

7.1.4.3.4. Validation Parameters

The solid standards in the range of 14 – 130 µg/mg were prepared by mixing with KBr, details of the procedure of validation of analytical methods was given in section 7.1.1.5.2.

7.1.4.3.3.Results and Discussion:

7.1.4.3.4.1 Validation Parameters

(I) Linearity

Linearity range of SIMVA in methanol was found to be 14 – 130 µg/mg .

Table 7.1.4.3.1: Calibration data of SIMVA by FTIR Spectroscopy

Sr.No.	Concentration (mg/mg)	Peak Area ± SD	%CV	Peak height ± SD	%CV
1	**14.71**	8.268418 ± 0.028	0.46	0.214303 ± 0.068	0.58
2	41.026	25.10534 ± 0.027	0.28	0.478404 ± 0.082	0.71
3	87.774	49.33128 ± 0.38	0.16	0.912752 ± 0.19	0.48
4	130.594	73.39718 ± 0.026	0.84	1.341738 ± 0.31	0.92

*** Mean value of five determinations**

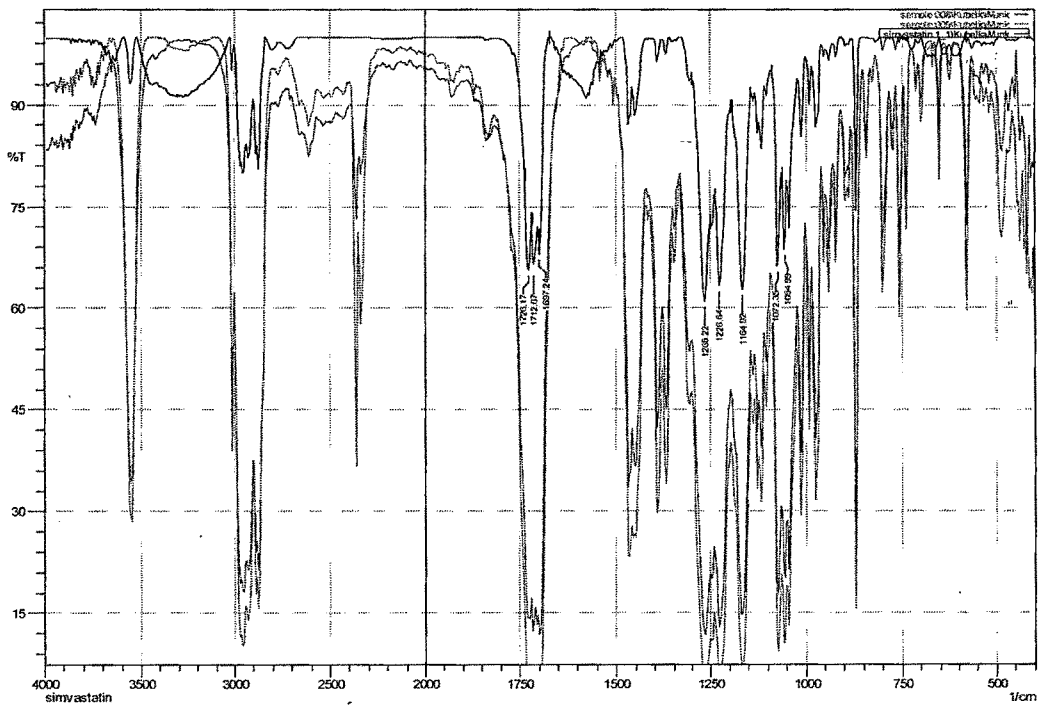


Figure: 7.1.4.3.1 Overlay FT-IR spectra of SIMVA

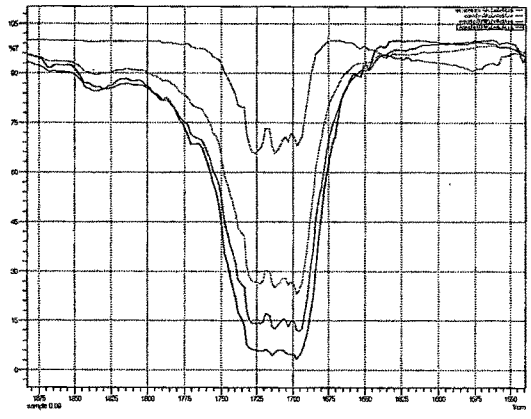


Figure: 7.1.4.3.2. FT-IR spectra of SIMVA by Peak Area and peak height

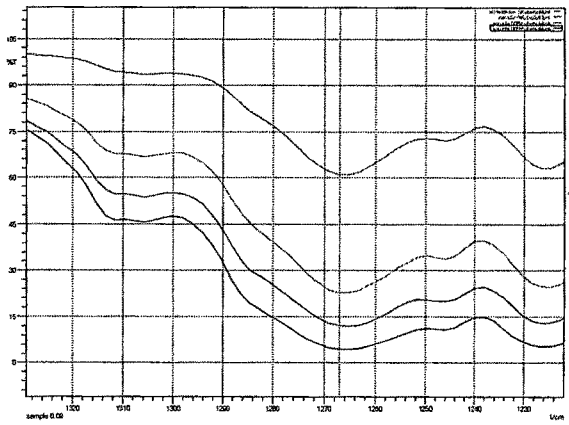


Figure: 7.1.4.3.3. FT-IR spectra of SIMVA by Peak height

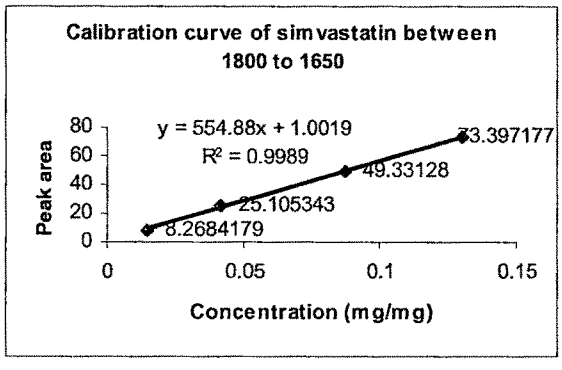


Figure 7.1.4.3.4: Calibration curve of SIMVA by peak area
Correlation co-efficient = **0.9989**
Slope = 554.88
Intercept = 1.0019
Regression equation: **Abs. = 554.88 × Conc. +1.0019**

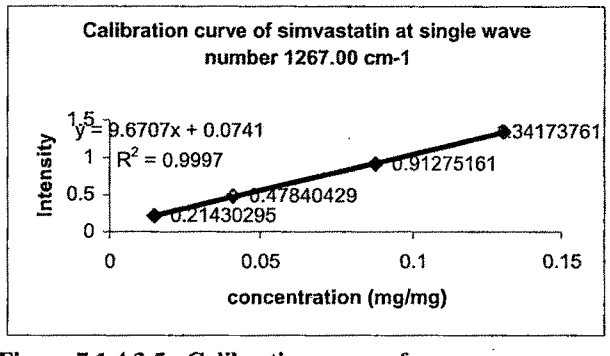


Figure 7.1.4.3.5: Calibration curve of SIMVA by peak height
Correlation co-efficient = **0.97**
Slope = 9.6707
Intercept = 0.0741
Regression equation: **Abs.** = 9.6707× **Conc.** +0.0741

(II) Precision

a. Intraday and Interday Precision (Repeatability)

Table 7.1.4.3.2: Intraday and interday precision data of SIMVA by FTIR Spectroscopy

Concentration (µg/mg)	Intraday		Interday	
	Peak Area* ±%C.V.	Peak Height* ±%C.V.	Peak Area* ±%C.V.	Peak Height* ±%C.V.
20	11.4933±0.38	0.2979±0.27	12.0441±0.46	0.2923 ± 0.38
60	34.53±0.3860	0.6951±0.027	33.2265±0.058	0.6824 ± 0.088
120	38.8473 ±0.042	0.8844±0.28	30.6980±0.027	0.8732 ± 0.052

*** Mean value of five determinations**

b. Reproducibility:

As per the instrument specification the % C.V. of repeated measurement of the same solution should not more than 1%.

Table 7.1.4.3.4: Reproducibility data of SIMVA by FT-IR Spectroscopy (15 µg/mg)

Sample compar-tment	Peak Area	Peak height
I	8.4977	0.2202
	8.4269	0.2184
	8.5595	0.2218
II	8.6438	0.2240
	8.6157	0.2233
	8.5488	0.2216
S.D.	0.0882	0.0023
%C.V.	1.031	0.34

*** Mean value of three determinations**

(III) Accuracy

Accuracy of the measurement of SIMVA was determined by standard addition method.

Table 7.1.4.3.5: Accuracy data of SIMVA by peak area

Quantitative FT-IR		**SIMVA**		
Initial conc.(µg/mg) (A)		**30**	**30**	**30**
Quantity of std. Added (µg/mg) (B)		**15**	**30**	**45**
Total Amount (A + B)		**45**	**60**	**75**
Peak area (Mean (n=5) ± %C.V.)	Total quantity Found Mean ± S.D.	44.87 ± 0.36	60.12 ± 0.18	75.49 ± 0.78
	% Recovery± S.D	99.71 ±0.27	100.20 ± 0.22	100.65 ± 0.38
Peak height (Mean (n=5) ± %C.V.)	Total quantity Found Mean ± S.D.	45.12 ± 0.87	60.27 ± 0.58	74.61 ± 0.62
	% Recovery± S.D	100.27 ± 0.61	100.45 ± 0.29	99.48 ± 0.87

*** Mean value of five determinations**

(IV) Limit of detection

The minimum detectable concentration of SIMVA was found to be 0.0232 and 1.3021 µg/mg for peak area and peak height, respectively.

(V) Limit of quantification

The lowest quantifiable concentration of SIMVA was found to be 0.0777 and 4.304 µg/mg by practical observation for peak area and peak height, respectively.

7.1.4.3.3.2. Estimation of SIMVA in marketed Tablet:

Preparation of test Sample:

The contents of 20 Tablets were weighed and their mean weight determined and finely powdered. An equivalent weight of the Tablet content was transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution (100 µg/ml) was sonicated for 10 min and supernatant was filtered through whatman filter paper no.41. Filterate was evaporated and from the residue 1 mg weigh and make up to 100 mg with KBr in butter paper and triturated in mortar pestle and DRIFT spectra was scannned between 4000 and 400 cm^{-1}, by averaging 16 scans for each spectrum with a resolution of 4 cm^{-1} (data point resolution per interval 1 cm^{-1}). 1800 to 1650 cm^{-1} and 1267 cm^{-1} was selected for measurement of Peak area and peak height at single wave length, respectively. Concentration of sample was found from regression equation and result of % assay are given in table 7.1.4.3.5.

Table 7.1.4.3.5: Estimation of SIMVA in tablet by FTIR Spectroscopy

Formu-lation	Labeled Claim (mg/tab)	Peak Area		PH at SWN	
		*Amount found	% Assay	*Amount found	% Assay
STATIN (unisearch)	10	10.09	100.98	9.99	99.90
	20	20.21	101.05	19.98	99.90

***Average of three determinations.**

7.1.4.3.4. Summary of Validation parameters:

The summary of validation parameters was given in table 7.1.4.3.6.

Table 7.1.4.3.6: Summary of Validation parameters of FTIR Spectroscopy

Sr. No	Parameters	Peak area	Peak height
1	Wavelength number (1/cm)	1800 to 1650	1267

2	Linearity range (µg/mg)	14 – 17	
3	Regression equation	Y = 554.88 × Conc. +1.0019	Y = 9.6707× Conc. +0.0741
4	Correlation coefficient (r^2)	0.9989	0.9997
5	Intercept	1.0019	0.0741
6	Slope	554.88	9.6707
7	Assay	100.98-101.05	99.90
8	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements % CV % Recovery	 0.29-0.74 0.28-0.83 1.03 99.71-100.65	 0.18-0.84 0.33-0.76 0.34 99.48-100.45
9	Limit of detection (µg/mg)	0.0232	1.3021
10	Limit of quantification (µg/mg)	0.0777	4.304

7.1.4.3.5. Conclusion:

FTIR spectroscopic method was developed for the estimation of SIMVA in pharmaceutical oral dosage forms. This method can estimate SIMVA in dose of 5 –20 mg. An FT-IR spectrum of compound is characteristic of that compound. So estimation by FTIR is one of the specific analytical methods for respective compounds.

7.1.4.4 High Performance Thin Layer Chromatography (HPTLC) with UV detection.

Two HPTLC methods were developed for the estimation of SIMVA. Method A was developed for the estimation of SIMVA in oral dosage form. There was no interference of excipients in analytical method but the method was not able to separate degradation products of SIMVA from the SIMVA standard. So the method B was developed which could estimate SIMVA in presence of its degraded products.

7.1.4.4.1. Optimization of method:

The chromatographic procedure was optimized for efficient separation of degradate peaks in the chromatogram.

Determination of solvent and wavelength:

Different solvents were tried to study the solubility of SIMVA as stated in table 6.3. SIMVA was soluble in methanol and as such was selected for the preparation of drug solutions .Wavelength was selected by scanning standard solutions of SIMVA over 200 nm to 400 nm wavelengths. Drug show significant absorbance at **238 nm** as illustrated in fig. 7.1.4.1.1. So this wavelength was selected in densitometer UV detector.

Determination of mobile phase:

Various combinations of mobile phases were tried for sharp peak of SIMVA on silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 µm thickness) (E. Merck). The results are reported in Table 7.1.4.4.1

Table 7.1.4.4.1: Determination of mobile phase

Mobile phase combination	Ration (V/V/V)	Peak separation
Chloroform: Methanol	8: 2	Broad peak
Chloroform: Methanol	5: 5	Broad peak
Benzene: Methanol	8: 2	Sharp peak
Toluene: Methanol	**7: 3**	**Sharp peak**
Ethyl acetate : Toluene: Methanol	3: 4: 3	Broad peak
Ethyl acetate: Toluene: Methanol	4: 4: 2	Broad peak
Ethyl acetate: Benzene: Methanol	3: 4: 3	Broad peak
Ethyl acetate: ACN: Formic acid	**8: 1: 0.1**	**Sharp peak and separate degraded products.**
Chloroform: Toluene: Methanol: Glacial acetic acid	3: 4 : 4: 0.05	Broad peak
Chloroform: Toluene: Methanol: Glacial acetic acid	4: 4: 2: 0.2	Broad peak

- It is evident from the data that mobile phase combination of **Toluene: Methanol** in proportion of 7: 3 v/v was efficient and was selected as mobile phase in method A for estimation of SIMVA in dosage form and **Ethyl acetate: ACN: Formic acid** in proportion of **8: 1: 0.1** v/v/v was selected as mobile phase in method B for separation of SIMVA form it degraded products. The chromatographic condition for spotting on plate are given in Chapter 6.

7.1.4.4.2. Chromatographic condition:

Method A

- **Mobile phase:** Toluene : Methanol (7:3 v/v)
- **Scanning Wave length:** 238 nm

Method B

- **Mobile phase:** Ethylacetate: Acetonitrile: formic acid (8:1: 0.1 v/v/v)
- **Scanning Wave length:** 237 nm (good separation of degraded product)

- Other chromatographic conditions are mentioned in chapter of preliminary work (Chapter 6)
- Pre washing of plate also mentioned in chapter 6.

7.1.4.4.3. Preparation of stock solution for SIMVA

For chromatographic study standard SIMVA (100 mg), weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml methanol properly and diluted up to mark with methanol to obtain final concentration of 1000 µg/ml.

7.1.4.4.4. Calibration curve:

Analysis was performed on Pre-coated silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 µm thickness) (E. Merck) pre-washed with methanol and then dried for 30 minute at 50°C. From the stock solution (1000 µg/ml) aliquots of 0.3, 0.6, 0.9, 1.8, 2.7 and 3.9 µl were spotted on the TLC plate under nitrogen stream using Desaga Applicator, AS30win to obtain final concentration range 300 - 3900 ng/spot.

The plate was dried in a FTIR, and then the plate was developed in Twin trough developing chamber (100 × 100) with stainless steel lid, previously saturated with the mobile phase for 30 min. The plate was removed from the chamber, dried in a FTIR and was scanned and quantified at 250 and 231 nm in Re-emission/Excitation mode with Desaga TLC scanner, Proquant. The calibration curve was constructed by plotting peak area vs. concentration (ng/spot) and peak height vs. concentration (ng/spot) corresponding to each spot and regression equation was calculated.

7.1.4.4.5. Validation Parameters

Procedure for validation of analytical method was given in chapter 7.1.8.4.

7.1.4.4.6 Results and Discussion:

7.1.4.4.6.1 Validation Parameters

(I) Linearity

Linearity range of SIMVA was found to be 318 – 3816 ng/spot with correlation co-efficient and % C.V. 0.9996 and 0.19 – 0.97 for peak area, 0.9986 and 0.39-0.97 for peak height by method A and 0.9916 and 0.28 –0.83 % CV by method B.

Table 7.1.4.4.2: Calibration data of SIMVA by HPTLC with UV detection

Sr. No.	Concentration (ng/spot)	Method A					Method B		
		Peak Area		Peak Height		R_f*	Peak Area		R_f*
		Mean ± SD	%CV	Mean ± SD	%CV		Mean	%CV	
1	318	315 ± 0.038	0.48	286 ± 0.042	0.39	0.38	214.32	0.32	0.82
2	636	370 ± 0.048	0.22	312 ± 0.029	0.49	0.35	428.93	0.83	0.82
3	954	417 ± 0.083	0.49	371 ± 0.018	0.73	0.37	560.73	0.52	0.83
4	1908	565 ± 0.039	0.93	485 ± .083	0.92	0.37	859.44	0.60	0.82
5	2862	710 ± 0.78	0.19	604 ± 0.48	0.97	0.38	1134.98	0.28	0.82
6	3816	845 ± 0.073	0.38	735 ± 0.19	0.82	0.38	1492.84	0.83	0.82

***Average of five readings**

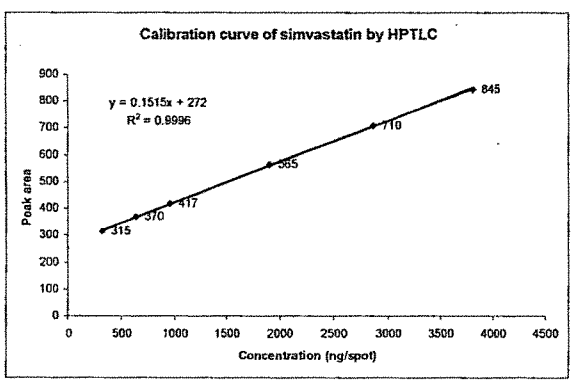


Figure 7.1.4.4.1: Calibration curve of SIMVA by HPTLC method A (Peak Area)

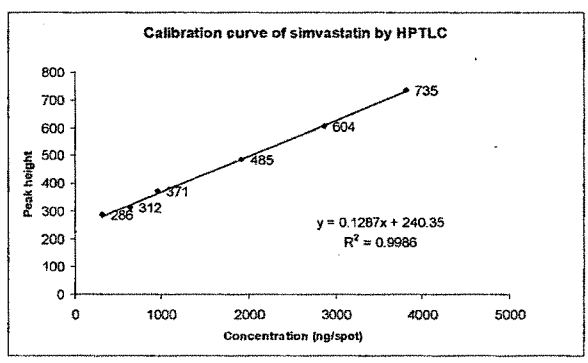


Figure 7.1.4.4.2: Calibration curve of SIMVA by HPTLC method A (Peak Height)

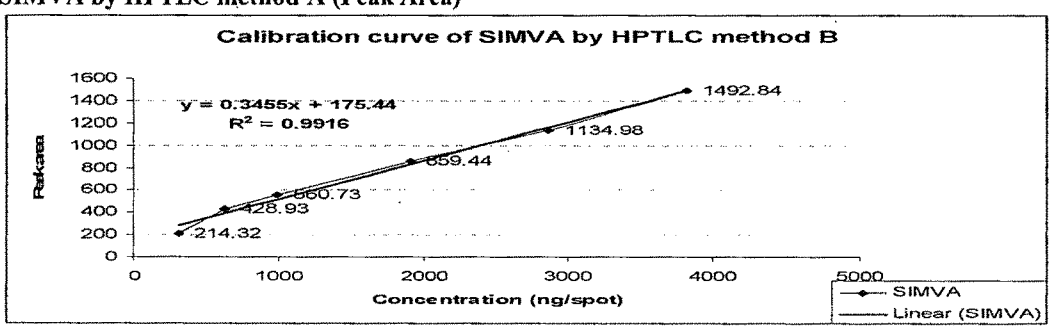


Figure 7.1.4.4.3: Calibration curve of SIMVA by HPTLC method B(Peak Area)

(II) Precision

Intraday and Interday Precision (Repeatability)

Intraday variation of the method A was 0.23 – 0.49 % for peak area and 0.19-0.82% for peak height and by method B was 0.29 – 0.93.

Interday variation of the method A was 0.19 – 0.75% for peak area and 0.13 – 0.39% for peak height and by method B was 0.34 – 0.52.

Table 7.1.4.4.3: Intraday precision data of SIMVA by HPTLC with UV detection

Sr. No.	Conc. (ng/spot)	Method A					Method B		
		Peak Area		Peak Height		R_f*	Peak Area		R_f*
		Mean ± SD	%CV	Mean ± SD	%CV		Mean	%CV	
1	500	365 ± 0.38	0.28	305 ± 0.29	0.33	0.372	336.98	0.93	0.81
2	1500	468 ± 0.29	0.49	349 ± 0.84	0.82	0.378	672.63	0.27	0.81
3	2000	5081 ± 0.29	0.23	517 ± 0.12	0.19	0.381	894.79	0.38	0.81
Table 7.1.4.4.4: Interday precision data of SIMVA by HPTLC with UV detection									
1	500	365 ± 0.034	0.29	309 ± 0.11	0.13	0.372	334.20	0.37	0.80
2	1500	461 ± 0.078	0.75	398± 0.29	0.39	0.371	675.66	0.52	0.81
3	2000	508 ± 0.29	0.19	512 ± 0.84	0.23	0.379	893.14	0.34	0.81

*** Mean value of five determinations**

b. Reproducibility:

As per the instrument specification the % C.V. of repeated measurement of the same sample should not more than 1%.

Table 7.1.4.4.5: Reproducibility data of SIMVA (300 ng/spot) by HPTLC

Time	Method A			Method B	
	Peak Area	Peak Height	Rf	Peak Area	R_f
Aluminum plate	329	290	0.379	252.19	0.81
	328	298	0.378	252.33	0.80
	326	291	0.372	259.92	0.81
Glass plate	329	290	0.37	255.13	0.81
	324	299	0.379	258.97	0.80
	329	290	0.37	257.36	0.81
S.D.	1.174	1.066	0.004	0.37	0.81

%CV	-	-	0.379	-	0.01

*** Mean value of five determinations**

(III) Accuracy

Accuracy of the measurement of SIMVA was determined by standard addition and was found to be in the range of 99.32 – 100.17 % for peak area and 98.80-100.31% for peak height and for method B was 100.24 – 100.56%.

Table 7.1.4.4.6: Accuracy data of SIMVA by HPTLC with UV detection

Spotted Amount (A + B)	Method A				Method B	
	Peak Area		Peak Height		Peak Area	
	Total quantity Found Mean ± S.D.	%Recovery ± S.D	Total quantity Found Mean ± S.D.	%Recovery ± S.D	Total quantity Found	%Recovery
750	745 ± 0.48	99.33 ± 0.83	749 ± 0.97	99.87 ± 0.29	751.83	100.24
1000	998.92 ± 0.29	99.89 ± 0.48	1004.82 ± 0.19	100.48 ± 0.39	1043.92	104.39
1500	1502.49 ± 0.84	100.17 ± 0.29	1499.78 ± 0.48	99.99 ± 0.77	1508.39	100.56

*** Mean value of five determinations**

(IV) Limit of detection

The minimum detectable concentration of SIMVA was found to be 0.1835 ng/spot for peak area and 1.3955 ng/spot for peak height by method A and 2.658 ng/spot by method B.

(V) Limit of quantification

The lowest quantifiable concentration of SIMVA was found to be 0.6119 ng/spot for peak area and 4.6510 ng/spot for peak height by method A and 5.317 ng/spot by method B.

7.1.4.4.6.2. Estimation of SIMVA in marketed Tablet:

Preparation of test solution:

Test solution was prepared as per procedure given in section 7.4.1.2.2. The plate was removed from the chamber, dried in air and was scanned and quantified at 238 and 237 nm in Reemission/Excitation mode with Desaga TLC scanner, Proquant. The concentration of sample solution was found from regression equation and result of % assay is reported in table 7.1.4.4.7.

Table 7.1.4.4.7: Estimation of SIMVA in tablet by HPTLC with UV detection

Tablet Formulation	Labeled Claim (mg/tablet)	Method A				Method B	
		Peak Area		Peak Height		Peak Area	
		Amount found (mg/tablet)	% Recovery ± S.D	Amount found (mg/tablet)	% Recovery ± S.D	Amount found (mg/tablet)	% Recovery ± S.D
STATIN (unisearch)	10	10.05	100.52	10.08	100.82	10.39	103.92
	20	20.09	100.45	20.18	100.54	19.93	99.38

*** Mean value of five determinations**

Applicability of method B:

Method B can easily estimate SIMVA in pharmaceutical dosage form but it could estimate of SIMVA in presence of degradation products. From the developed SIAM HPLC method (Chapter 7.2.7) and study of inherent stability character of SIMVA it was evident that SIMVA was stable against thermal, photostable, and oxidative condition and degraded in neutral, basic and acidic conditions. It was converted in to simvastatin hydroxy acid[1-5]. In acidic and neutral condition it was slowly converted to simvastatin hydroxy acid and again back to in slight amount in simvastain. So alkaline condition was used for the degradation study.

Procedure for forced degradation study

Forced degradation study was done in alkaline condition only. For alkaline degradation concentrated NaOH was used at room temperature. Approximate 25 mg drug was accurately weighed and transferred to 25 ml volumetric flask. The drug was dissolved in 5 ml of methanol then diluted with the conc. NaOH. Samples were colleted for analysis at two stages, at 0 min (as soon as sample was prepared), after 1 hrs and after 4 hrs of exposure to degradation condition at room temperature. Sample was applied by direct spotting of 1 µl on prewashed TLC plate. Fig 7.1.4.4.11 to 7.1.4.4.13 shows the chromatograms of forced degraded samples.

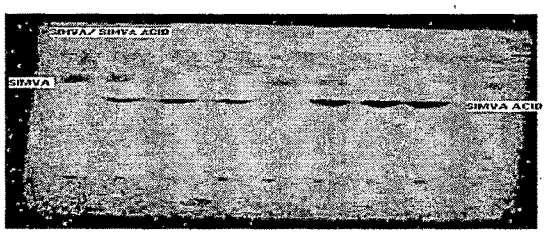


Figure: 7.1.4.4.B: TLC plate of SIMVA & SIMVA ACID by method A (After detection)

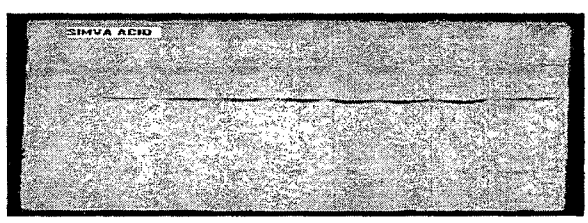


Figure: 7.1.4.4.11: TLC plate of SIMVA & SIMVA ACID by method B (After detection)

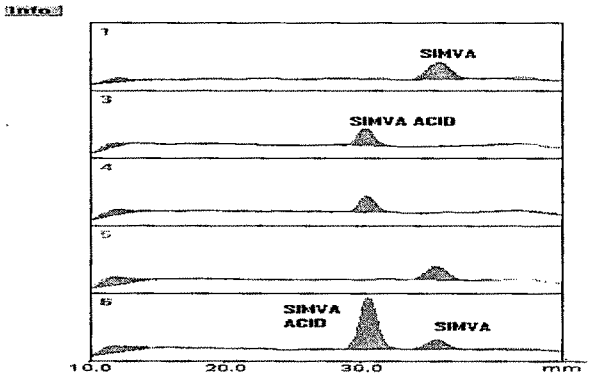


Figure: 7.1.4.4.12: Vertical chromatogram of SIMVA and SIMVA acid

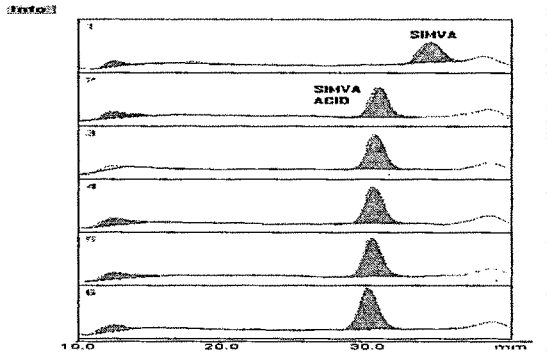
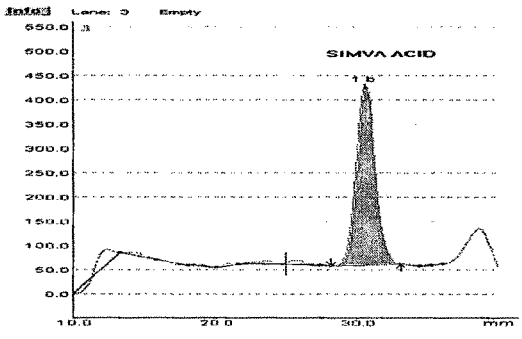


Figure: 7.1.4.4.13: Vertical chromatogram of SIMVA and SIMVA acid



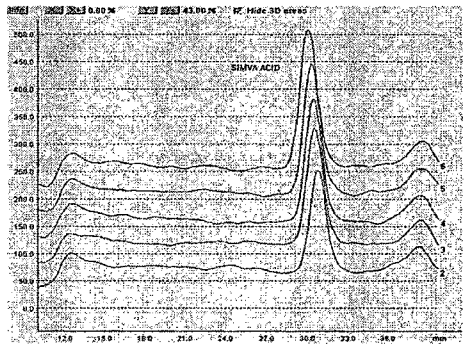


Figure: 7.1.4.4.14: Chromatogram of SIMVA acid by method B

7.1.4.4.7 Summary of Validation parameters:

The summary of validation parameters is reported in table 7.1.4.4.8.

Table 7.1.4.4.8: Summary of Validation parameters by HPTLC

Sr. No	Parameters	Method A		Method B
		Peak Area	Peak Height	Peak Area
1	Detection Wavelengths (nm)	238	238	237
2	Linearity range (ng/spot)	318 – 3816		
3	Regression equation	Y = 0.1515 X conc. + 272	Y = 0.1287 X conc. + 240	Y = 0.3455 X conc. + 175.44
4	Correlation coefficient (r^2)	0.9996	0.9986	0.9916
5	Intercept	272	240	175.44
6	Slop	0.1515	0.1287	0.3455
7	Assay	100.45-100.52	100.54-100.82	99.38-103.92
8	Precision Intra day % CV (n = 5) Inter day % CV (n = 5)	0.23-0.49	0.19-0.82	0.29-0.93

	Reproducibility of measurements %CV % Recovery	0.19-0.75 0.004 99.33-100.17	0.23-0.39 0.004 99.87-100.48	0.34-0.52 0.81 100.24-100.56
9	Limit of detection (ng/spot)	0.1835	1.3955	2.658
10	Limit of quantification (ng/spot)	0.6119	4.6510	5.317

7.1.4.4.8. Conclusion:

Developed HPTLC methods A and B could estimate SIMVA in pharmaceutical dosage form. Method B can estimate SIMVA in presence of it hydrolyzed product SIMVA acid as illustrated in fig. 7.1.4.4.9 to 7.1.4.4.11. So methods B can be used as selective and specific SIAM HPTLC method. Developed HPTLC method can estimate SIMVA acid. So method can be used for the pharmacokinetic and equivalence study.

Reference:

1. Martindale. The Extra-Pharmacopoeia, 30th ed., The Royal Pharmaceutical Society, 1993, 993–994.
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7. 1. 5

Lovastatin

7.1.5. Lovastatin

Spectrophotometry and chromatographic methods were developed for the estimation of LOVA in pharmaceutical dosage form. Degradation study was done for estimate inherent stability character of drug. Bio analytical method was developed for pharmacokinetic and bioavailability study.

Spectrophotometry

Five spectrophotometric methods were developed for the estimation of LOVA in oral dosage form. Zero order, 1st derivative, 2nd derivative and 3 wavelength methods were developed as UV spectrophotometric method. Advance quantitative FTIR spectroscopic method was developed for accurate estimation of LOVA in tablet dosage form.

Chromatography

HPLC method was developed which can estimate LOVA in presence of its degraded products. Two HPTLC methods were developed, one could estimate LOVA form pharmaceutical formulation and second HPTLC method could estimate LOVA in presence of its degraded products and also from pharmaceutical formulation. To study the pharmacokinetic data HPLC method was developed for the estimation of LOVA in spiked human plasma.

Apparatus, instruments and reagents:

Apparatus, instruments and reagents used for the development of spectroscopic and chromatographic methods are mentioned in chapter 6.

7.1.5.1 Estimation of SIMVA by zero order spectrophotometry, first order and second order derivative Spectrophotometric methods.

Experimental work:

7.1.5.1.1. Preparation of stock solution for LOVA

Standard LOVA (10 mg) was weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml methanol and diluted up to mark with methanol to obtain final concentration of 100 µg/ml. This solution was used as working standard solution for simple zero order, three wavelengths, derivative and difference spectroscopic methods.

7.1.5.1.2. Selection of analytical wavelength:

Simple UV spectroscopic method was developed in methanol as solvent. LOVA shows maximum absorbance at 237.60 nm (Fig.7.1.5.1.1) which was used for the further estimation. All saved data of calibration curve solution, validation parameters and test solutions were used. All data were transformed in derivative command by transformation function of UV PC software. First order derivative curves were studied and maximum at 244.60 nm and minimum at 240 nm (Fig. 7.1.5.1.3) s was selected for further study. Zero order spectra for all development parameters were recoded in UV PC software which was used for the second order derivative spectroscopic method. Second order derivative curves were studied maximum 242.60 nm and minimum at 246.60 nm (Fig.7.1.5.1.3) were selected as analytical wavelengths.

7.1.5.1.3. Calibration curve:

From the stock solution (100 µg/ml) aliquots of 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 ml were transferred to a series of 10ml volumetric flasks. The volume was adjusted to a mark with methanol to get 1, 5, 10, 15, 20, 25, 30, 35 and 40 µg/ml of solution. The absorbance was measured at λ_{max} or selected wavelength using methanol as a blank.

7.1.5.1.4. Validation Parameters

Procedure for validation of method was given in Chapter 7.1.1.1.2. Aliquots were taken form stock solution of LOVA.

7.1.5.1.5. Results and Discussion:

7.1.5.1.5.1 Validation Parameters

(I) Linearity

Linearity range of LOVA in methanol was found to be 5-40 µg/ml with correlation co-efficient 0.9998, 0.9989 and 0.9997, respectively for zero order spectrophotometry, first and second derivative spectrophotometry.

Table 7.1.5.1.1: Calibration data of LOVA by Spectrophotometry

Sr. No.	Conc. (µg/ml)	Simple UV Spectrophotometric method	1st derivative Spectrophotometric method			2nd derivative Spectrophotometric method		
		Absorb. Mean ± %CV (N=5)	Absorb. at 244.60 nm	Absorb. at 240.00 nm	Amplitude Difference Mean ± % C.V. (N=5)	Absorb. at 242.60 nm	Absorb. at 246.60 nm	Amplitude Difference Mean ± % C.V. (N=5)*
1	5	0.330 ± 0.92	0.012	-0.035	0.047 ± 0.84	0.015	-0.023	0.038 ± 0.028

2	10	0.62 ± 0.48	0.022	-0.067	0.089 ± 0.73	0.028	-0.043	0.071 ± 0.19
3	15	0.946 ± 0.19	0.033	-0.103	0.136 ± 0.18	0.043	-0.066	0.109 ± 0.39
4	20	1.255 ± 0.67	0.044	-0.133	0.177 ± 0.33	0.055	-0.088	0.143 ± 0.81
5	25	1.571 ± 0.82	0.055	-0.166	0.221 ± 0.72	0.07	-0.11	0.18 ± 0.11
6	30	1.882 ± 0.43	0.065	-0.198	0.263 ± 0.55	0.084	-0.131	0.215 ± 0.72
7	35	2.173 ± 0.63	0.075	-0.224	0.299 ± 0.84	0.097	-0.154	0.251 ± 0.22
8	40	2.474 ± 0.77	0.085	-0.253	0.338 ± 0.22	0.108	-0.174	0.282 ± 0.62

*Average of five readings

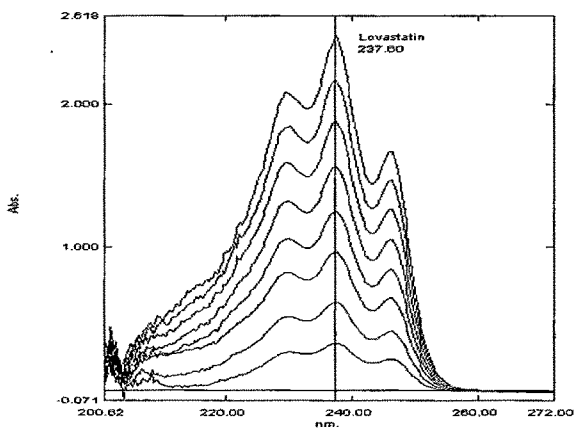

Figure: 7.1.5.1.2 UV spectra of LOVA by Simple UV Spectrophotometry

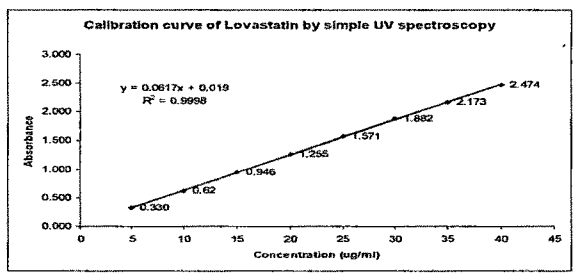

Figure 7.1.5.1.3: Calibration curve of LOVA by Simple UV Spectrophotometry
Correlation co-efficient = **0.9998**
Slope = 0.0617
Intercept = 0.019
Regression equation: **Abs. = 0.0617 × Conc. + 0.019**

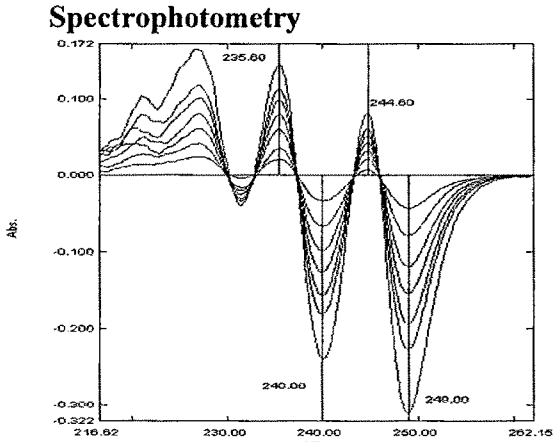

Figure: 7.1.5.1.4: First derivative UV spectra of LOVA

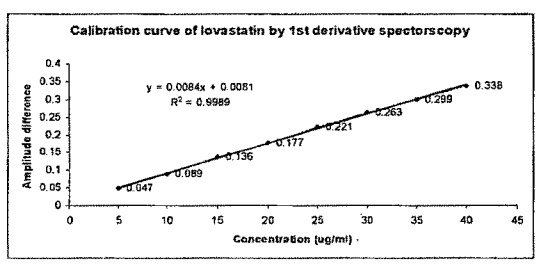

Figure 7.1.5.1.5: Calibration curve of LOVA by 1st derivative Spectrophotometry
Correlation co-efficient = 0.9989
Slope = 0.0084
Intercept = 0.0081
Regression equation: Abs. = 0.0084 × Conc. + 0.0081

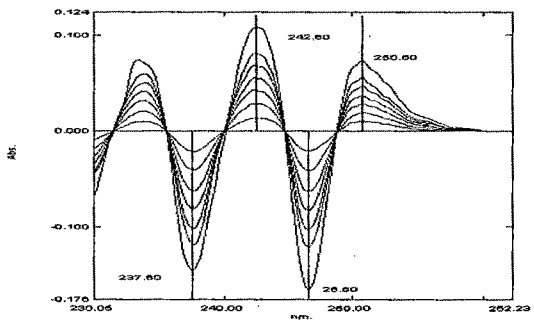

Figure: 7.1.5.1..6: 2nd derivative UV spectra of LOVA

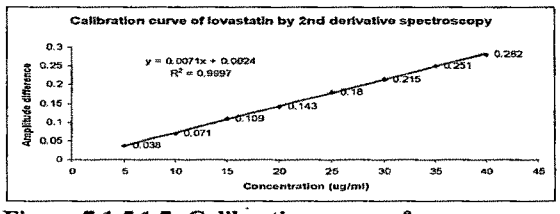

Figure 7.1.5.1.7: Calibration curve of standard LOVA by 2nd derivative
Correlation co-efficient = 0.9997
Slope = 0.0071
Intercept = 0.0024
Regression equation: Abs. = 0.0071 × Conc. + 0.0024

(II) Precision

a. Intraday and Interday Precision (Repeatability)

Intraday and interday variation of the proposed method were reported in table 7.1.5.1.2.

Table 7.1.5.1.2: Intraday precision data of LOVA by Spectrophotometry

Conc. (µg/ml)	Simple UV Spectrophotometric method	1st derivative Spectrophotometric method		2nd derivative Spectrophotometric method	
	Absorb. * ± % C.V.) (n=5)	Amplitude difference* ± S.D. (n=5)	% C.V.	Amplitude difference* ± S.D. (n=5)	% C.V.
10	0.636 ± 0.33	0.089 ± 0.032	0.39	0.073 ± 0.082	0.82
20	1.259 ± 0.54	0.171± 0.049	0.14	0.145 ± 0.022	0.73
30	1.880 ± 0.78	0.243 ± 0.033	0.72	0.218 ± 0.65	0.62
Table 7.1.5.1.3: Interday precision data of LOVA by Spectrophotometry					
10	0.639 ± 0.49	0.089 ± 0.033	0.48	0.073 ± 0.028	0.72
20	1.258 ± 0.36	0.174 ± 0.083	0.29	0.146 ± 0.066	0.39
30	1.886 ± 0.75	0.261 ± 0.021	0.33	0.216 ± 0.038	0.63

***Average of five readings**

b. Reproducibility:

As per the instrument specification the % C.V. of repeated measurement of the same solution should not more than 1%.

Table 7.1.5.1.4: Reproducibility data of LOVA by Spectrophotometry

Conc. (µg/ml)	Simple UV Spectrophotometric method		1st derivative Spectrophotometric method		2nd derivative Spectrophotometric method	
	Absorb.* ± %C.V.					
	UV 1700	UV 1601	UV 1700	UV 1601	UV 1700	UV 1601
10	0.62 ± 0.94	0.619 ±	0.089 ± 0.43	0.083 ±	0.071 ± 0.94	0.73 ±

		0.53		0.88		0.93
15	0.946 ± 0.32	0.943 ± 0.69	0.136 ± 0.75	0.132 ± 0.28	0.109 ± 0.72	0.102 ± 0.75
20	1.255 ± 0.69	1.254 ± 0.29	0.177 ± 0.29	0.177 ± 0.84	0.143 ± 0.65	0.142 ± 0.28

*** Mean value of three determinations**

(III) Accuracy

Accuracy of the measurement of LOVA was determined by standard addition and was found to be in the range of 99.87 – 102.45, 99.86 – 103.96 and 99.13 – 103.65 %, respectively for zero order spectrophotometry, first and second derivative spectrophotometry.

Table 7.1.5.1.5: Accuracy data of LOVA by Spectrophotometry

Initial conc. (µg/ml) (A)	Quantity of std. Added (µg/ml) (B)	Total Amount (A + B)	Simple UV Spectrophotometric method		1st derivative Spectrophotometric method		2nd derivative Spectrophotometric method	
			Total quantity Found * ± S.D.)	% Recovery ± S.D	Total quantity Found* ± S.D.	% Recovery ± S.D	Total quantity Found* ± S.D.	% Recovery ± S.D
10	5	15	14.98 ± 0.33	99.87 ±0.39	14.98 ± 0.29	99.86 ± 0.67	14.87 ± 0.02	99.13 ± 0.088
10	10	20	20.49 ± 0.24	102.45 ± 0.19	20.33 ± 0.39	101.65 ± 0.29	20.73± 0.067	103.65 ± 0.036
10	15	25	25.12 ± 0.19	100.48 ± 0.67	25.99 ± 0.29	103.96 ± 0.39	25.83 ± 0.038	103.32 ± 0.087

***Average of five readings**

(IV) Limit of detection

The minimum detectable concentration of LOVA was found to be 0.0863, 0.17 and 0.49 µg/ml, respectively for zero order spectrophotometry, first and second derivative spectrophotometry.

(V) Limit of quantification

The lowest quantifiable concentration of LOVA was found to be 0.2878, 0.57 and 0.6164 µg/ml, respectively for zero order spectrophotometry, first and second derivative spectrophotometry.

7.1.5.1.3.2. Estimation of LOVA in marketed Tablet:

Preparation of test solution:

Two brands of LOVA, AZTATIN of Sun and LOVACARD of Cipla, were analyzed by the proposed method. The Tablet powder equivalent one Tablet content was

transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution was sonicated for 10 min and supernatant was filtered through whatman filter paper no.41. 1 and 2 ml from this solution were transferred to 10 ml volumetric flasks and diluted with methanol to get 10 µg/ml concentration. The absorbance of this solution was measured at 237.60 nm and concentration of sample solution was found from regression equation. Zero order save data were converted in to derivative and the absorbance was measured at λ_{maxima} = 244.60 nm and λ_{minima} = 240.00 nm. The amplitude difference was measured and concentration of sample solution was found from regression equation.Zero order data of the methanolic solution was converted in to derivative and the absorbance was measured at λ_{maxima} = 242.60 nm and λ_{minima} = 246.60 nm. The amplitude difference was measured and concentration of sample solution was found from regression equation.

The result of estimation of tablet formulation is reported in table 7.1.5.1.6 in term of % assay.

Table 7.1.5.1.6: Estimation of LOVA in tablet by Spectrophotometry

Tablet Formu-lation	Labeled Claim (mg/Tablet)	Simple UV Spectrophotometric method		1st derivative Spectrophotometric method		2nd derivative Spectrophotometric method	
		Amount Found* (mg/Tablet)	% Assay ± S.D	Amount Found* (mg/Tablet)	% Assay ± S.D	Amount Found* (mg/Tablet)	% Assay ± S.D
AZTATIN (Sun)	10	10.09	100.9 ± 0.39	10.34	103.49	10.25	102.51
	20	20.13	100.51 ± 0.72	20.09	100.18	19.99	99.98
LOVACARD (Cipla)	20	20.09	100.45 ± 0.55	19.99	99.98	20.04	100.21

***Average of three determinations.**

7.1.5.1.4 Summary of Validation parameters:

The summary of validation parameters is given in table 7.1.5.1.7.

Table 7.1.5.1.7: Summary of Validation parameters of Spectrophotometry

Sr. No	Parameters	Simple UV Spectrophotometric method	1st derivative Spectrophotometric method	2nd derivative Spectrophotometric method
1	λ max (nm)	237.60	244.60	242.60

	λ max (nm)	-	240.00	246.60
2	A (1%, 1cm) (dl gm^{-1} cm^{-1})	298.46	-	-
3	Molar Absoptivity (ε) (L mol^{-1} cm^{-1})	2896.17(>100)	-	
4	Linearity range (µg/ml)	5 -40	5 – 40	5 -40
5	Regression equation	Y= 0.0617 X con. +0.019	Y=0.0084xcon. + 0.0081	Y = 0.0071x Con. + 0.0024
6	Correlation coefficient (r^2)	0.9998	0.9989	0.9997
7	Intercept	0.019	0.0081	0.0024
8	Slope	0.0617	0.0084	0.0071
9	Sandell's sensitivity (µg/cm^2/0.001 abs. unit))	1.275	-	-
10	Assay	100.45-100.90	99.48 – 103.49	99.98 – 102.51
11	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurem % Recovery	 0.33-0.78 0.36-0.75 0.29-0.69 99.87 – 102.45	 0.14 – 0.72 0.29 – 0.48 < 1 % 99.86 – 103.96 %	 0.62 – 0.82 0.39 –0.72 < 1 % 99.13 – 103.65
12	Limit of detection	0.0863 µg/ml	0.17 µg/ml	0.49 µg/ml
13	Limit of quantification	0.2878 µg/ml	0.579 µg/ml	0.6164 µg/ml

7.1.5.1.5. Conclusion:

Simple, accurate and economic method was developed for the estimation of LOVA in pharmaceutical oral dosage form. As summary table shows method can work in the range of 5 – 40 µg/ml with accuracy of 99.48 – 103.49 %.

7.1.5.2 Determination of LOVA in Bulk Drug and Pharmaceutical Dosage Form by 3-wavelength Photometric method

The absorbance of methanolic solution of LOVA was measured at three selected wavelengths, 234.60 nm, 237.60 nm and 241.20 nm. The net absorbance was calculated using the equation given in chapter 5.

Procedure for preparation of stock solution, calibration curve and test solution were same as described earlier.

7.1.5.2.1 Results and Discussion:

7.1.5.2.1.1 Validation Parameters

(I) Linearity:

The absorbance was measured at (λ_{max}) 234.60, 237.60 and 241.20 nm using methanol as a blank. Linearity range of LOVA in methanol was found to be 5 - 40µg/ml with correlation co-efficient 0.9991 and % C.V. ranging from 0.012 – 0.98% for different concentration.

Table 7.1.5.2.1: Calibration data of LOVA by 3-wavelength photometric method

Sr. No.	Concentration (µg/ml)	Absorbance at 234.60 nm	Absorbance at 237.60 nm	Absorbance at 241.20 nm	Net Absorbance (N=5)*	% C.V.
1	5	0.269	0.329	0.218	0.083	0.98
2	10	0.511	0.619	0.410	0.154	0.012
3	15	0.809	0.946	0.598	0.233	0.18
4	20	1.039	1.254	0.836	0.307	0.72
5	25	1.3	1.567	1.036	0.387	0.53
6	30	1.52	1.876	1.296	0.458	0.45
7	35	1.806	2.165	1.458	0.517	0.82
8	40	2.051	2.474	1.656	0.603	0.72

*** Average of five readings**

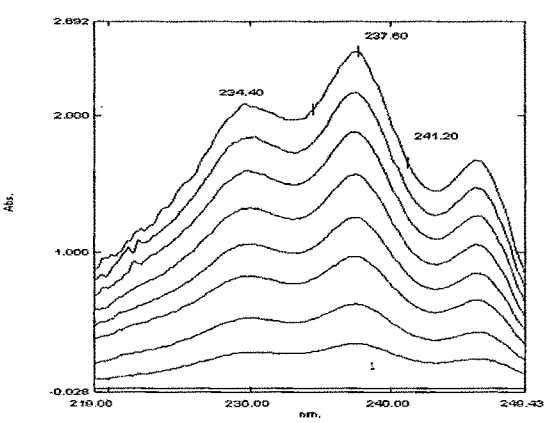

Figure: 7.1.5.2.1 UV spectra of LOVA by 3-wavelength method

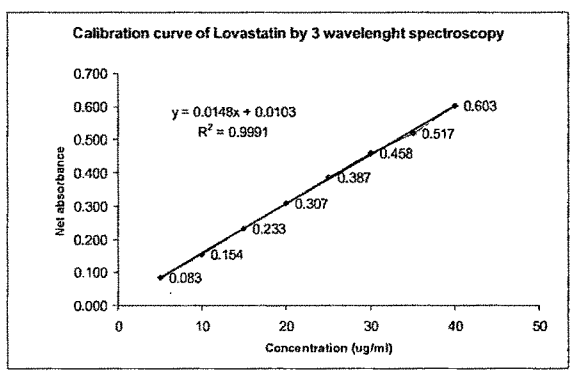

Figure 7.1.5.2.2: Calibration curve of LOVA by 3-wavelength method

Correlation co-efficient = **0.9991**
Slope = 0.0148
Intercept = 0.0103
Regression equation: **Abs. = 0.0148 × Conc. + 0.0103**

(II) Precision

a. Intraday and Interday Precision (Repeatability)

Intraday and interday variation of the proposed method were reported in table 7.1.5.2.2.

Table 7.1.5.2.2: Intraday precision data of LOVA by 3-wavelength method

Concentration (µg/ml)	Intraday		Interday	
	Absorbance (Mean* ± S.D.) (n=5)	% C.V.	Absorbance (Mean* ± S.D.) (n=5)	% C.V.
10	0.154 ± 0.033	0.29	0.158 ± 0.022	0.74
20	0.301 ± 0.028	0.58	0.307 ± 0.064	0.38
30	0.452 ± 0.048	0.84	0.458 ± 0.087	0.18

***Average of five readings**

b. Reproducibility:

The reproducibility of the method was determined by using Shimadzu UV 1700 and Shimadzu UV 1601. The % CV of the responses confirms the reproducibility of the method for determination of LOVA the data is reported (Table 7.1.5.2.4).

Table 7.1.5.2.3: Reproducibility data of LOVA

Concentration (µg/ml)	Absorbance*		% CV	
	UV 1700	UV 1601	UV 1700	UV 1601
10	0.154	0.144	0.75	0.32
15	0.233	0.239	0.38	0.53
20	0.307	0.304	0.94	0.78

*** Mean value of three determinations**

(III) Accuracy

Accuracy of the measurement of LOVA was determined by standard addition and was found to be in the range of 99.45 – 102.60 %.

Table 7.1.5.2.4: Accuracy data of LOVA by 3-wavelength method

Initial conc. (µg/ml) (A)	Quantity of std. Added (µg/ml) (B)	Total Amount (A + B)	Total quantity Found*± S.D.	% Recovery± S.D
10	05	15	15.39 ± 0.037	102.60 ± 0.28
10	10	20	19.89 ± 0.028	99.45 ± 0.52
10	15	25	25.3 ± 0.071	101.20 ± 0.62

***Average of five readings**

(IV) Limit of detection

The minimum detectable concentration of LOVA was found to be 0.1592 µg/ml.

(V) Limit of quantification

The lowest quantifiable concentration of LOVA was found to be 0.5309 µg/ml by practically observation.

7.1.5.2.1.2. Estimation of LOVA in marketed Tablet:

Preparation of test solution:

Test solution prepared as per procedure given in section 7.1.5.1.1.2. The absorbance of this solution was measured at 234.60, 237.60 and 241.20 nm and concentration of sample solution was found from Regression equation and result in term of % assay are reported in table 7.1.5.2.5.

Table 7.1.5.2.5: Estimation of LOVA in tablet by 3-wavelength method

Tablet Formulation	Labeled Claim (mg/tablet)	Amount found (mg/tablet)	% Assay ± S.D
AZTATIN (Sun)	10	10.00	100.00
	20	20.01	100.05
LOVACARD (Cipla)	20	19.93	99.65

***Average of five readings**

7.1.5.2.2 Summary of Validation parameters

The validation parameters are reported in it table 7.1.5.2.6.

Table 7.1.5.2.6: Summary of Validation parameters by 3-wavelength method

Sr. No	Parameters	Results
1	WL1 (nm)	234.60
2	WL2 (nm)	237.60
3	WL3 (nm)	241.20
4	Linearity range (µg/ml)	5 – 40
5	Regression equation	Y = 0.0148x Con. + 0.0103
6	Correlation coefficient (r^2)	0.9901
7	Intercept	0.0103
8	Slope	0.0148

9	Assay	99.65 – 100.05
10	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements % C % Recovery	 0.29-0.84 0.18-0.74 < 1% 99.45-102.60
11	Limit of detection	0.159 µg/ml
12	Limit of quantification	0.5309 µg/ml

7.1.5.2.3. Conclusion:

Three wavelengths spectroscopic method was developed for the estimation of LOVA in pharmaceutical oral dosage forms. This method can estimate LOVA in dose of 5 – 20 mg with the accuracy of 99.45 – 102.60%. The coefficient of variation was 0.9991.

7.1.5.3 Estimation of LOVA by FT-Infra Red spectroscopy method.

Experimental work:

7.1.5.3.1. Preparation of standards for EZE

For quantitative FTIR spectroscopic study standard EZE (10 mg) was weighed accurately and made up to 100 mg by adding pure, anhydrous potassium bromide onto the same butter paper to obtain final concentration of 100 µg/mg. This sample was triturated in mortar and pestle and sample was used as working standard sample for FTIR spectroscopic method.

7.1.5.3.2. Selection of analytical wavenumber:

Diffuse reflectance infrared fourier transform spectroscopy (DRIFTS) spectra were applied for the analysis of LOVA at 3585 to 3510 cm^{-1} and 3542.00 cm^{-1} was selected for measurement of Peak area and peak height at single wave length, respectively. Calibration curves were drawn using the multiple linear regression (MLR) method and the drug amount in sample was computed by QUANT function of FT-IR solution software.

7.1.5.3.3. Calibration curve:

Appropriate fractions were transferred from standard (100 µg/mg) to butter paper and weight adjusted 10 mg with KBr to get minimum transmission. Peak area and peak height was measured at selected wavenumber. Calibration curves were drawn using

the multiple linear regression (MLR) method and the drug amount in sample was computed from regression equation.

7.1.5.3.4. Validation of method:

Procedure for validation of method was given in Chapter 7.1.1.3.4.

7.1.5.3.5. Results and Discussion:

7.1.5.3.5.1 Validation Parameters

(I) Linearity

Linearity range of LOVA was found to be 6 – 80 µg/mg .

Table 7.1.5.3.1: Calibration data of LOVA by Infrared Spectroscopy

Sr.No.	Concentration (mg/mg)	Peak Area ± SD	%CV	Peak height ± SD	%CV
1	6.3	0.46033 ± 0.034	0.37	0.03804 ± 0.048	0.22
2	22.3	2.29752 ± 0.083	0.82	0.08554 ± 0.059	0.62
3	42.1	4.97991 ± 0.072	0.28	0.18854 ± 0.37	0.92
4	80.1	9.5236 ± 0.084	0.77	0.35311 ± 0.18	0.68

***Average of five readings**

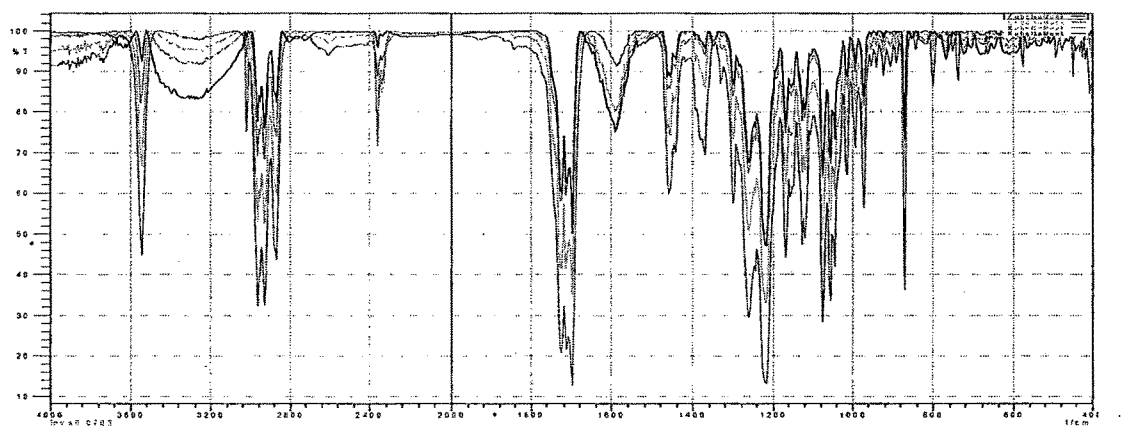

Figure: 7.1.5.3.1 Overlay FT-IR spectra of LOVA

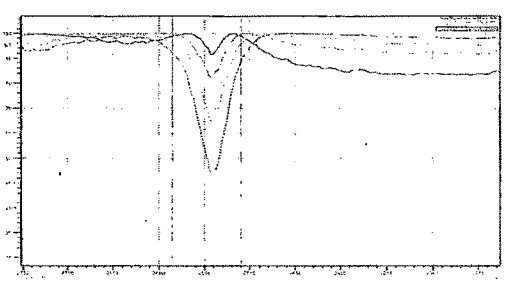

Figure: 7.1.5.3.2 FT-IR spectra of LOVA by Peak Area

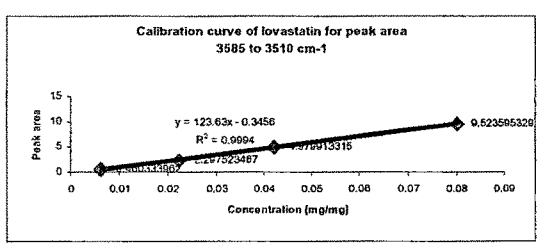

Figure 7.1.5.3.3: Calibration curve of LOVA by peak area

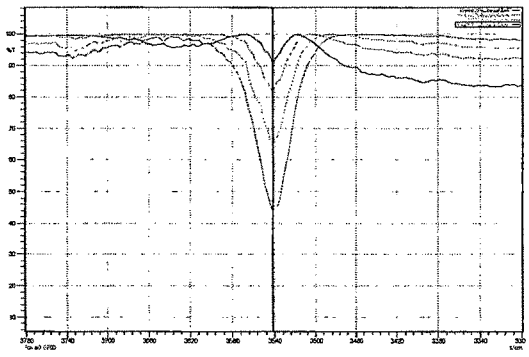

Figure: 7.1.5.3.4: FT-IR spectra of LOVA by Single Wavelength Number

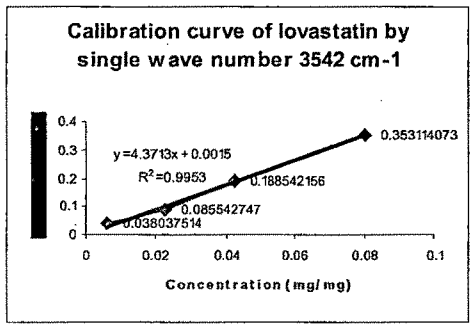

Figure 7.1.5.3.5: Calibration curve of LOVA by single wavelength number

(II) Precision

a. Intraday and Interday Precision (Repeatability)

Table 7.1.5.3.2: Intraday and interday precision data of LOVA by FTIR Spectroscopy

Concentration (µg/mg)	Intraday		Interday	
	Peak Area* ±%C.V.	Peak Height* ±%C.V.	Peak Area* ±%C.V.	Peak Height* ±%C.V.
20	4.1314 ± 0.74	0.1538 ± 0.48	4.1108 ± 0.87	0.1531 ± 0.59
60	6.3671 ± 0.48	0.2371 ± 0.18	6.1920 ± 0.47	0.2305 ± 0.83
120	9.4725 ± 0.87	0.3452 ± 0.69	9.4961 ± 0.65	0.3498 ± 0.77

***Average of five readings**

b. Reproducibility:

As per the instrument specification the % C.V. of repeated measurement of the same solution should not more than 1%.

Table 7.1.5.3.4: Reproducibility data of LOVA (50 µg/mg) by FT-IR Spectroscopy

Sample comp.	Peak Area	Peak height
I	5.1514	0.1918
	5.4605	0.2033
	5.8623	0.2183
II	5.1359	0.1912
	5.1823	0.1930
	5.3585	0.1995
S.D.	0.31132	0.01159
%C.V.	0.98	0.57

***Average of three readings**

(III) Accuracy

Table 7.1.5.3.5: Accuracy data of LOVA by peak area

Quantitative IR		**SIMVA**		
Initial conc.(µg/ml) (A)		**30**	**30**	**30**
Quantity of std. Added (µg/ml) (B)		**15**	**30**	**45**
Total Amount (A + B)		**45**	**60**	**75**
Peak area **(Mean (n=5) ± %C.V.)**	**Total quantity Found Mean ± S.D.**	45.87 ± 0.55	59.98 ± 0.33	74.67 ± 0.84
	% Recovery± S.D	101.93 ± 0.57	99.97 ± 0.64	99.56 ± 0.41
Peak height **(Mean (n=5) ± %C.V.)**	**Total quantity Found Mean ± S.D.**	45.08 ± 0.59	61.12 ± 0.32	74.98 ± 0.39
	% Recovery± S.D	100.18 ± 0.63	101.87 ± 0.44	99.97 ± 0.29

***Average of five readings**

(IV) Limit of detection

The minimum detectable concentration of LOVA was found to be 0.947 and 1.2840 µg/mg for peak area and peak height, respectively.

(V) Limit of quantification

The lowest quantifiable concentration of LOVA was found to 0.6865 and 4.2801 µg/mg by practical observation for peak area and peak height, respectively.

7.1.5.3.1.2. Estimation of LOVA in marketed Tablet:

Preparation of test Sample:

Test sample was prepared as per procedure given in section 7.1.4.5.1.2. DRIFT spectra was scannned between 4000 and 400 cm^{-1}, by averaging 16 scans for each spectrum with a resolution of 4 cm^{-1} (data point resolution per interval 1 cm^{-1}). 3585 to 3510 cm^{-1} and 3542.00 cm^{-1} was selected for measurement of Peak area and peak height at single wave length, respectively. Concentration of sample was found from regression equation and result reported in table 7.1.5.3.6 in term of % labeled claim.

Table 7.1.5.3.6: Estimation of LOVA in tablet by FTIR Spectroscopy

Formulation	**Labeled Claim (mg/tab)**	**Peak Area**		**Peak height**	
		***Amount found**	**% Assay**	***Amount found**	**% Assay**
LOVACARD (Cipla)	20	20.08	100.41	20.01	100.05

***Average of three determinations.**

7.1.5.3.2 Summary of Validation parameters:

The validation parameters are reported in table 7.1.5.3.7.

Table 7.1.5.3.7: Summary of Validation parameters of FTIR Spectroscopy

Sr. No	Parameters	Peak area	Peak height
1	Wavelength number (1/cm)	3585-3510	3545
2	Linearity range (µg/mg)	6-80	
3	Regression equation	Y=123.63xCon. -0.345	Y=4.3713xCon. +0.0015
4	Correlation coefficient (r^2)	0.9994	0.9953
5	Intercept	-0.345	0.0015
6	Slope	123.63	4.3713
7	Assay	100.41	100.05
8	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements % C % Recovery	 0.48-0.87 0.47-0.87 0.98 99.56-101.93	 0.18-0.69 0.59-0.83 0.57 99.97-101.87
9	Limit of detection	0.947	1.284
10	Limit of quantification	0.6865	4.280

7.1.5.3.3. Conclusion:

FTIR spectroscopic method was developed for the estimation of LOVA in pharmaceutical oral dosage forms. This method can estimate LOVA in dose of 5 –20 mg. An FT-IR spectrum of compound is characteristic of that compound. So estimation by FTIR is one of the specific analytical method for respective compounds.

7.1.5.4 High Performance Thin Layer Chromatography (HPTLC) with UV detection.

Two HPTLC methods were developed for the estimation of LOVA. Method A was developed for the estimation of LOVA in oral dosage form, there was no interference of excipients in analytical method. But this method was not able to separate degradation product of LOVA from the LOVA standard. So the method B was developed which could estimate LOVA in presence of its degraded product.

7.1.5.4.1. Optimization of method:

For good chromatographic method it is be necessary that mobile phase have good polarity to separate compound and for that proper selection of solvent and wavelength is required.

Determination of solvent for sample preparation and λmax:

Different solvents were tried to study the solubility of LOVA as stated in table 6.3. LOVA is soluble in methanol so methanol was selected for the preparation of drug solutions .Wavelength was selected by scanning standard solutions of LOVA over 200 nm to 400 nm wavelengths. Drug shows significant absorbance at **239 +237 nm** as illustrated in fig. 7.1.7.1.5.3. So this wavelength was selected in densitometer UV detector.

Determination of mobile phase:

Various combinations of mobile phases were tried for sharp peak of LOVA on silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 µm thickness) (E. Merck). The results are reported in Table 7.1.5.4.1

Table 7.1.5.4.1: Determination of mobile phase

Mobile phase combination	Ration (V/V/V)	Peak separation
Chloroform: Methanol	8: 2	Broad peak
Chloroform: Methanol	5: 5	Broad peak
Benzene: Methanol	8: 2	Sharp peak
Toluene: Methanol	**7: 3**	**Sharp peak**
Ethyl acetate : Toluene: Methanol	3: 4: 3	Broad peak
Ethyl acetate: Toluene: Methanol	4: 4: 2	Broad peak
Ethyl acetate: Benzene: Methanol	3: 4: 3	Broad peak
Ethyl acetate: ACN: Formic acid	**8: 1: 0.1**	**Sharp peak and separate degraded products.**
Chloroform: Toluene: Methanol: Glacial acetic acid	3: 4 : 4: 0.05	Broad peak
Chloroform: Toluene: Methanol: Glacial acetic acid	4: 4: 2: 0.2	Broad peak

- It is evident from the data that mobile phase combination of **Toluene: Methanol** in proportion of 7: 3 v/v was suitable so was selected for method A for estimation of LOVA in dosage form and **Ethyl acetate: ACN: Formic acid** in proportion of **8: 1: 0.1** v/v/v was selected as mobile phase in method B for separationof LOVA from its degraded products.

7.1.5.4.2. Chromatographic condition:

Method A

- **Mobile phase:** Toluene : Methanol (7:3 v/v)
- **Scanning Wave length:** 239 nm

Method B

- **Mobile phase:** Ethylacetate: Acetonitrile: formic acid (8:1: 0.1 v/v/v)
- **Scanning Wave length:** 237 nm (good separation of degraded product)
- Pre washing of plate is also mentioned in chapter 6.

7.1.5.4.3. Preparation of stock solution for LOVA

For chromatographic study standard LOVA (100 mg) was weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml methanol and diluted up to mark with methanol to obtain final concentration of 1000 µg/ml.

7.1.5.4.4. Validation Parameters

Procedure for validation of analytical method was given in chapter 7.1.8.4.

7.1.5.4.5. Results and Discussion:

7.1.5.4.5.1 Validation Parameters

(I) Linearity

Linearity range of LOVA was found to be 306 – 3672 ng/spot for both method. Correlation co-efficient and % C.V. 0.9997 and 0.22 – 0.93 for peak area, 0.9976 and 0.39 – 0.97 for peak height for method A and for method B was 0.9975 r^2 and 0.37 – 0.93 %CV.

Table 7.1.5.4.2: Calibration data of LOVA by HPTLC with UV detection

Sr. No.	Concentration (ng/spot)	Method A					Method B		
		Peak Area		Peak Height		R_f*	Peak Area		R_f*
		Mean ± SD	%CV	Mean ± SD	%CV		Mean	%CV	
1	306	315 ± 0.038	0.48	286 ± 0.042	0.39	0.38	301.65	0.93	0.80
2	612	370 ± 0.048	0.22	312 ± 0.029	0.49	0.35	371.92	0.52	0.81
3	918	417 ± 0.083	0.49	371 ± 0.018	0.73	0.37	420.57	0.78	0.80
4	1836	565 ± 0.039	0.93	485 ± .083	0.92	0.37	582.76	0.41	0.80
5	2836	710 ± 0.78	0.19	604 ± 0.48	0.97	0.38	739.93	0.37	0.80
6	3672	845 ± 0.073	0.38	735 ± 0.19	0.82	0.38	944.33	0.55	0.80

***Average of five readings**

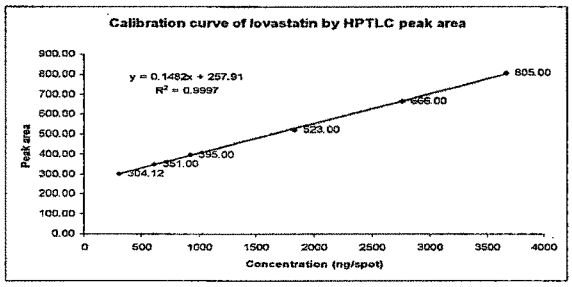

Figure 7.1.5.4.1: Calibration curve of LOVA by HPTLC by method (Peak Area)

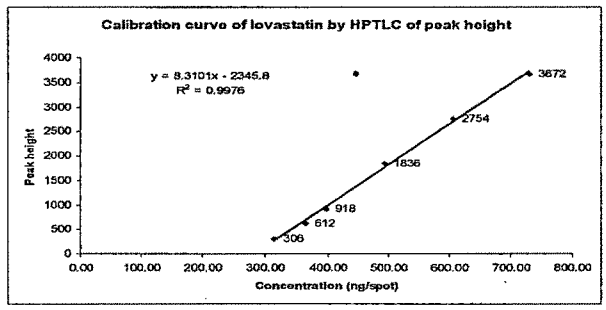

Figure 7.1.5.4.2: Calibration curve of LOVA by HPTLC by method A (Peak Height)

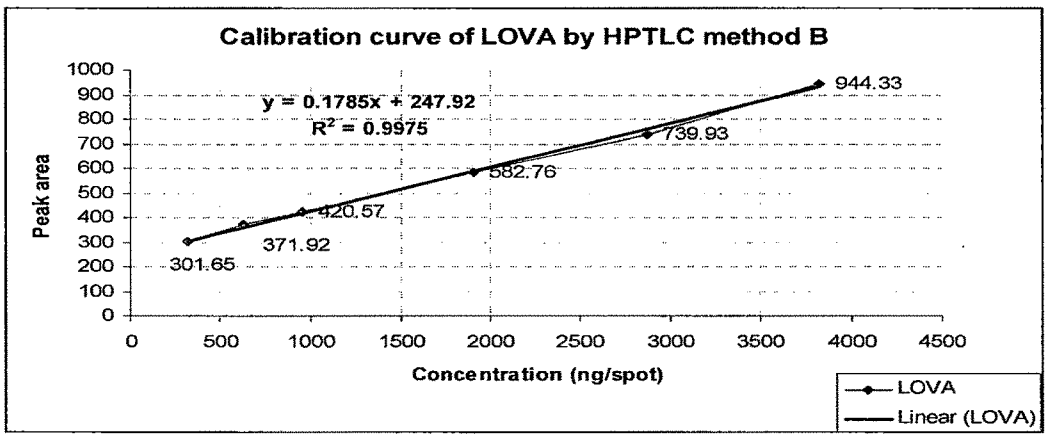

Figure 7.1.5.4.3: Calibration curve of LOVA by HPTLC by method B (Peak Area)

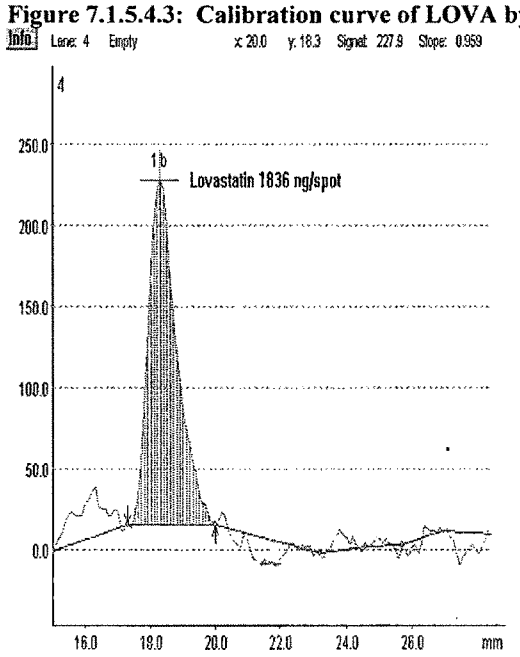

Figure 7.1.5.4.4: Single Spectrum of LOVA by HPTLC by method A

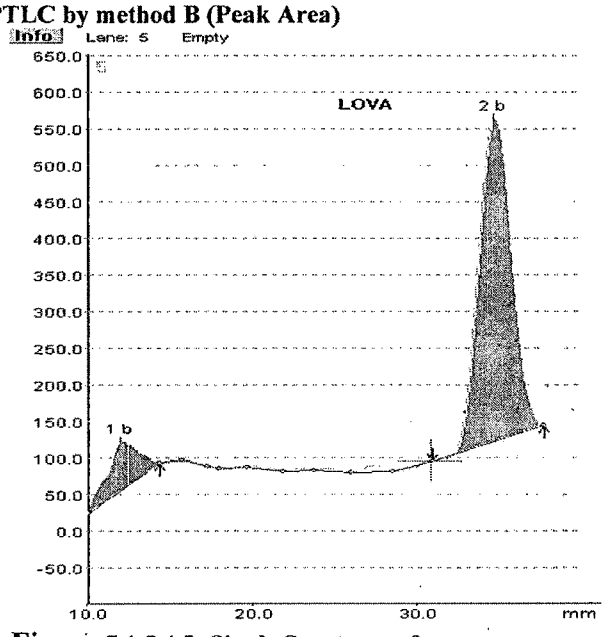

Figure 7.1.5.4.5: Single Spectrum of LOVA by HPTLC by method B

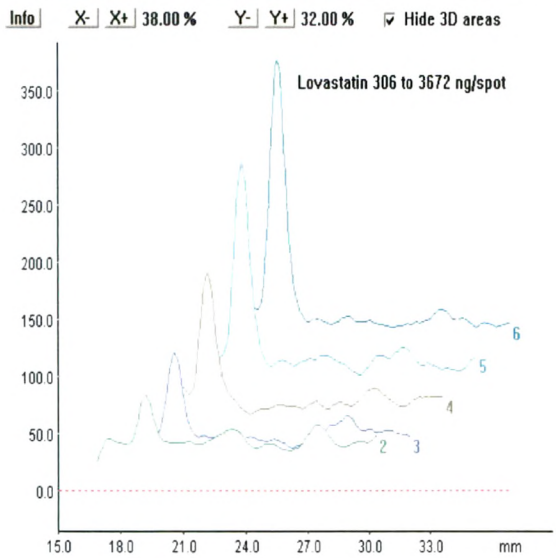

Figure 7.1.5.4.6: 3D Spectra of LOVA by HPTLC by method A

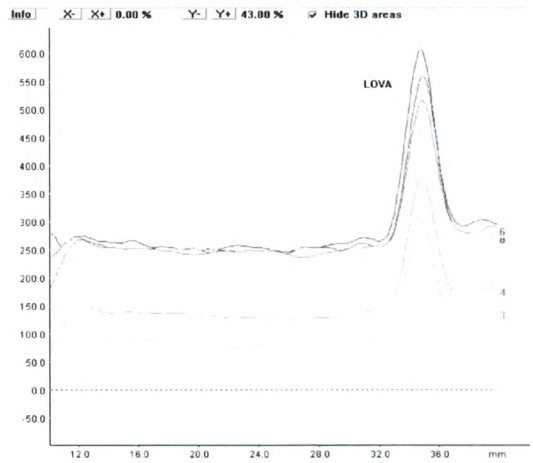

Figure 7.1.5.4.7: 3D Spectra of LOVA by HPTLC by method B

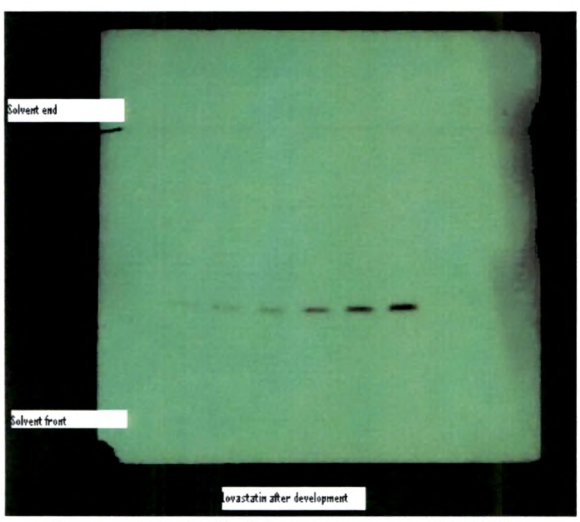

Figure: 7.1.5.4.8: Chromatogram of LOVA by method A (After detection)

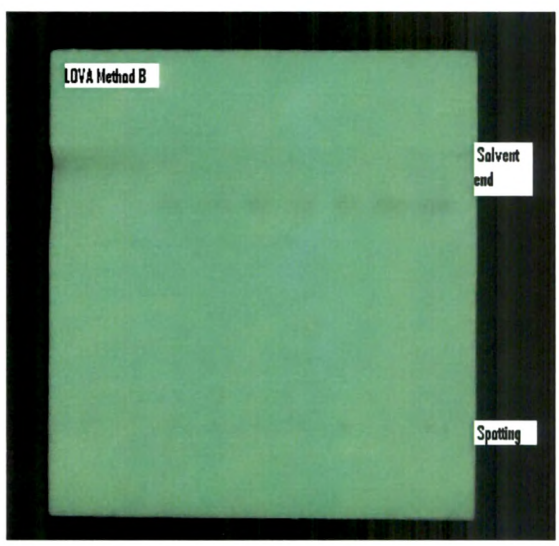

Figure: 7.1.5.4.9: Chromatogram of LOVA (After detection) by method B

(II) Precision

a. Intraday and Interday Precision (Repeatability)

Intraday variation of the method A was 0.19-0.92 % for peak area and 0.44-0.82% for peak height and for method B was 0.22 – 0.83.

Interday variation of the method A was 0.29 – 0.56 % for peak area and 0.50 – 0.82% for peak height and for method B was 0.22 – 0.79.

Table 7.1.5.4.3: Intraday precision data of LOVA by HPTLC with UV detection

Sr. No.	Conc. (ng/spot)	Method A					Method B		
		Peak Area		Peak Height		R_f*	Peak Area		R_f*
		Mean ± SD	%CV	Mean ± SD	%CV		Mean	%CV	
1	500	496.91 ± 0.23	0.92	513.38 ± 0.82	0.44	0.60	474.29	0.83	0.80
2	1500	1480.52 ± 0.82	0.63	1529.59 ± 0.83	0.82	0.60	877.17	0.58	0.81
3	2000	2036.66 ± 0.72	0.19	2104.16 ± 0.82	0.77	0.60	881.70	0.22	0.80
Table 7.1.5.4.4: Interday precision data of LOVA by HPTLC with UV detection									
1	500	496.93 ± 0.38	0.38	513.40 ± 0.29	0.52	0.61	220.42	0.53	0.80
2	1500	1490.78 ± 0.29	0.29	1540.20 ± 0.92	0.82	0.60	458.14	0.79	0.80
3	2000	1987.71 ± 0.82	0.56	2053.59 ± 0.39	0.50	0.60	517.07	0.22	0.80

***Average of five readings**

b. Reproducibility:

As per the instrument specification the % C.V. of repeated measurement of the same solution should not more than 1%.

Table 7.1.5.4.5: Reproducibility data of LOVA (400 ng/spot) by HPTLC

Time	Method A			Method B	
	Peak Area	Peak Height	Rf	Peak Area	R_f
Aluminum plate	298.16	308.04	0.62	284.58	0.81
	300.97	310.95	0.61	289.42	0.80
	298.00	307.87	0.60	280.48	0.81
Glass plate	300.87	310.84	0.60	284.93	0.79
	316.87	327.37	0.60	283.95	0.80
	302.97	313.02	0.61	281.63	0.80
S.D.	0.85	0.86	0.0044	3.11	0.01
%CV	0.28 (<1%)	0.39 (<1%)	1.47 (>1%)	1.10	0.94

***Average of three readings**

(III) Accuracy

Accuracy of the measurement of LOVA was determined by standard addition and was found to be in the range of 99.50 – 104.82 % for peak area and 99.88 – 100.33% for peak height by method A and 99.86 – 108.40% by method B.

Table 7.1.5.4.6: Accuracy data of LOVA by HPTLC with UV detection

Total Amount (A + B)	Method A				Method B	
	Peak Area		Peak Height		Peak Area	
	Total quantity Found Mean ± S.D.	%Recovery ± S.D	Total quantity Found Mean ± S.D.	%Recovery ± S.D	Total quantity Found	%Recovery
750	746.22 ± 0.39	99.50 ± 0.28	751.3 ± 0.29	100.17 ± 0.72	748.92	99.86
1000	1048.2 ± 0.29	104.82 ± 0.93	1003.29 ± 0.72	100.33 ± 0.81	1084.02	108.40
1500	1538.22 ± 0.29	102.55 ± 0.29	1498.22 ± 0.38	99.88 ± 0.72	1510.93	100.73

***Average of five readings**

(IV) Limit of detection

The minimum detectable concentration of LOVA was found to be 19.91 ng/spot for peak area and 0.3532 ng/spot for peak height by method A and 5.902 ng/spot by method B.

(V) Limit of quantification

The lowest quantifiable concentration of LOVA was found to be 66.39 ng/spot for peak area and 1.1776 ng/spot for peak height by method A and 9.419 ng/spot by method B.

7.1.5.4.5.2. Estimation of LOVA in marketed Tablet:

Preparation of test solution:

Test solution was prepared as per procedure given in chapter 7.1.4.8.2.2. The concentration of sample solution was found from regression equation. Result in term of % assay was reported in to table 7.1.5.4.7.

Table 7.1.5.4.7: Estimation of LOVA in tablet by HPTLC with UV detection

Tablet Formulation	Labeled Claim (mg/tablet)	Method A				Method B	
		Peak Area		Peak Height		Peak Area	
		Amount found (mg/tablet)	% Assay ± S.D	Amount found (mg/tablet)	% Assay ± S.D	Amount found (mg/tablet)	% Assay ± S.D
LOVACARD (Cipla)	20	20.01	100.05	20.20	101.00	20.29	102.94

***Average of five readings**

Applicability of method B:

Method B can easily estimate LOVA in pharmaceutical dosage form and it could estimate of LOVA in presence of its degradation products. From the developed SIAM HPLC method and study of inherent stability characters of LOVA, it was evident that LOVA was stable against thermal, photostable, and oxidative conditions. LOVA degraded in neutral, basic and acidic condition. It was converted in to LOVA hydroxy acid[1-5]. In acidic and neutral condition it was slowly converted in LOVA hydroxy acid and again back to in slight amount in LOVA. So alkaline conditions was used for the study.

Procedure for forced degradation study

Forced degradation study was done in alkaline condition only. For alkaline condition concentrated NaOH was used at room temperature. Approximately 25 mg drug was accurately weighed and transferred to 25 ml volumetric flask. The drug was dissolved in 5 ml of methanol then diluted with the conc. NaOH. Samples were collected for analysis at two stages, at 0 min (as soon as sample was prepared), after 1 hrs and after 4 hrs of exposure to NaOH at room temperature. Sample was applied by direct spotting of 1 µl on prewashed TLC plate. Fig 7.1.5.4.9 to 7.1.5.4.13 show the chromatograms of forced degraded samples.

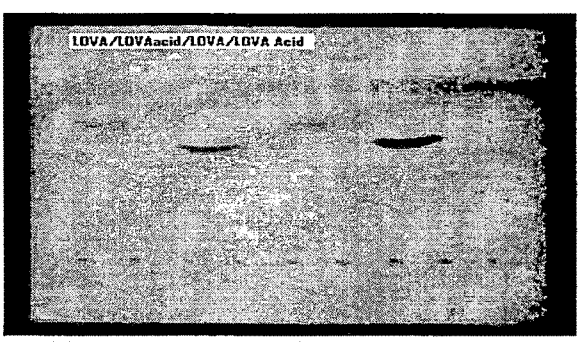

Figure: 7.1.5.4.10: TLC plate of LOVA by method a (After detection)

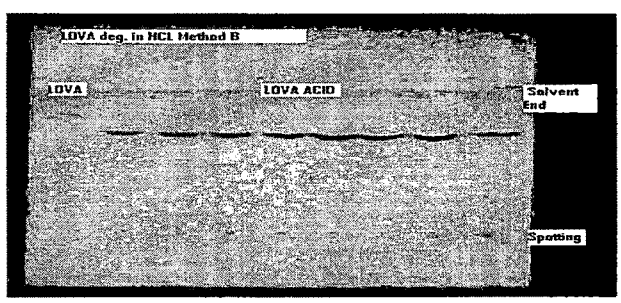

Figure: 7.1.5.4.11: TLC plate of LOVA by method B (After detection)

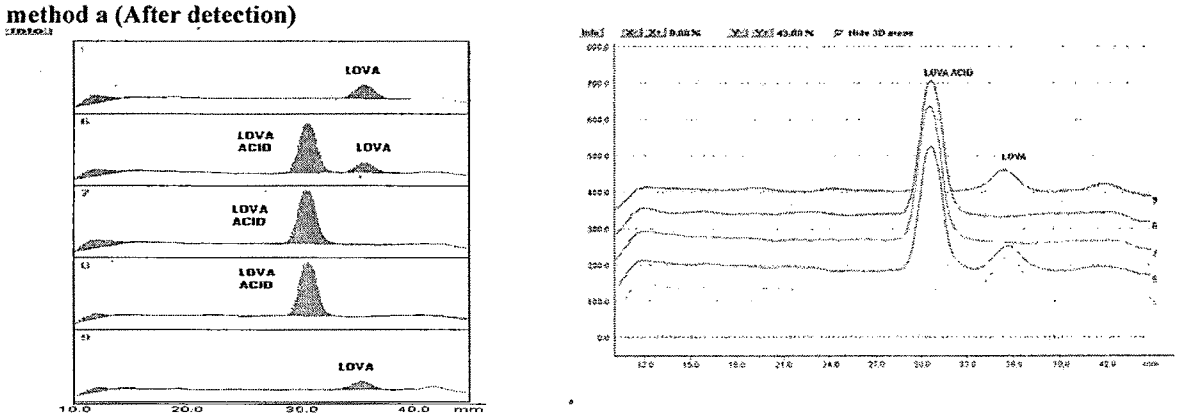

Figure: 7.1.5.4.12: Vertical chromatogram of LOVA and LOVA acid

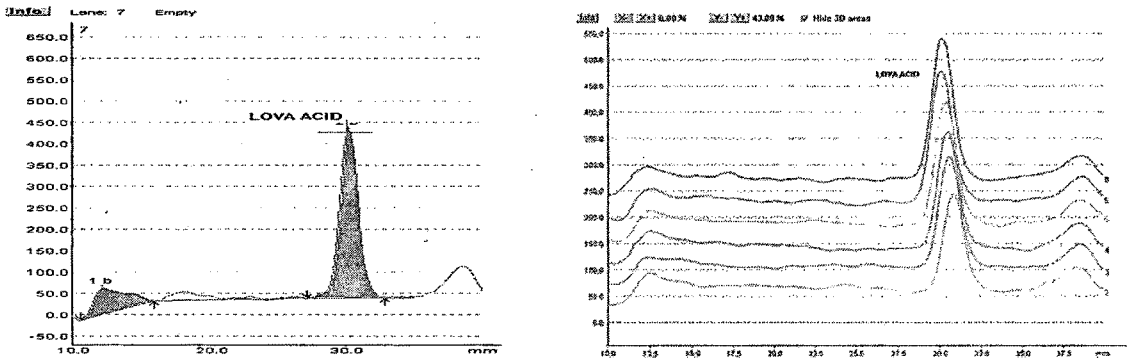

Figure: 7.1.5.4.13: Chromatogram of LOVA acid

7.1.5.4.6 Summary of Validation parameters:

The summary of validation parameters are reported in table 7.1.5.4.8.

Table 7.1.5.4.8: Summary of Validation parameters by HPTLC with UV detection

Sr. No	Parameters	Method A		Method B
		Peak Area	Peak Height	Peak Area
1	Detection wavelengths (nm)	238	238	237
2	Linearity range (ng/spot)	306 - 3672		
3	Regression equation	Y = 0.148X conc. + 257.91	Y = 8.3101 X conc. –234.58	Y = 0.1785 X conc. + 247.9
4	Correlation coefficient (r^2)	0.9977	0.9976	0.9975
5	Intercept	257.91	–234.58	247.9
6	Slop	0.148	8.3101	0.1785
7	Assay	100.05	101.00	102.94
8	Precision			
	Intra day % CV (n = 5)	0.19-0.92	0.44-0.82	0.22-0.83
	Inter day % CV (n = 5)	0.29-0.56	0.50-0.82	0.22-0.74
	Reproducibility of measurements			
	%CV	0.85	0.86	0.11
	% Recovery	99.50-104.82	99.88-100.33	99.88-108.40
9	Limit of detection (ng/spot)	19.91	0.3532	5.902
10	Limit of quantification (ng/spot)	66.39	1.1776	9.419

7.1.5.4.7. Conclusion:

Developed HPTLC methods A and B could estimate LOVA in pharmaceutical dosage form. Method B can estimate LOVA in presence of it hydrolyzed product LOVA acid as illustrated in fig. 7.1.5.4.9 to 7.1.5.4.13. So methods B can be selective and specific SIAM HPTLC method.

Reference:

1. Martindale. The Extra-Pharmacopoeia, 30th ed., The Royal Pharmaceutical Society, 1993, 993–994.
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7. 2. 1

Ezetimibe and Simvastatin

7.2.1. Development of analytical methods for simultaneous estimation of Ezetimibe and Simvastatin:

In Indian market combined commercial formulations of EZE and SIMVA contain EZE and SIMVA in a ratio of 10mg: 10mg (SIMVAS-EZ). Here for the EZE and SIMVA spectrophotometric, chromatographic and chemometric methods were developed.

Spectrophotometric Methods:

Spectrophotometric methods were developed for simultaneous estimation of EZE and SIMVA. Individual spectra of EZE and SIMVA were superimposed to obtain suitable working wavelengths that could be used for simultaneous estimation (fig. 7.2.1.1.1). Simultaneous equation method was not found to be applicable in this case due to extensive overlap of the two spectra. But the absorbance ratio (Q- analysis) method was tried for the quantitative analysis using λmax of one drug and isoabsorptive point of both the drugs (fig. 7.2.1.1.1) was not give proper ratio. The 1^{st} and 2^{nd} order derivative spectrophotometric was tried in which 1^{st} order derivative zero crossing method was possible as per fig. 7.2.1.1.2. Ratio derivative zero crossing method was also developed to analyze EZE and SIMVA as per fig. 7.2.1.1.6 to 7.2.1.1.13. Fourth one was quantitative IR method (Chapter 7.2.1.2.).

Chromatographic methods:

Two chromatographic methods were developed for the simultaneous estimation of EZE and SIMVA. HPLC method was developed which could estimate EZE and SIMVA in pharmaceutical dosage form and in presence of their degradation products. HPTLC method was developed for the estimation of EZE and SIMVA in pharmaceutical dosage form.

Chemometric methods

Chemometric approach was tried with the spectrophotometric methods. Two chemometric methods, ILS and CLS were developed for the simultaneous estimation of EZE and SIMVA.

Apparatus, instruments and reagents:

Apparatus, instruments and reagents used for the development of spectrophotometric and chromatographic methods are mentioned in Chapter 6.

7.2.1.1. Simultaneous spectrophotometric estimation of EZE and SIMVA by first derivative zero crossing (FDZC) and absorbance ratio derivative zero crossing (RDZC)methods:

Experimental Work:

7.2.1.1.1. Preparation of binary stock solution:

Standard EZE and SIMVA (10 mg) were weighed accurately and transferred to 100 ml volumetric flask. It was dissolved in 50 ml methanol and diluted up to mark with methanol to obtain final concentration of 100 µg/ml each of EZE and SIMVA. This solution was used as working standard solution spectrophotometric methods.

7.2.1.1.2. Selection of analytical wavelength:

(I) First derivative zero crossing spectrophotometric method:

The selection of zero crossing point, where absorbance of one component is zero while other is having significant absorbance is crucial. The zero crossing point should be valid for the entire concentration range in which analysis is carried out. From the overlain 1st derivative spectra of EZE and SIMVA (fig. 7.2.1.1.2), 265.20 nm was selected as zero crossing point for SIMVA as at this wavelength EZE has absorbance. Whereas, 245.40 nm was selected as zero crossing point for EZE as at this wavelength, SIMVA shows absorbance.

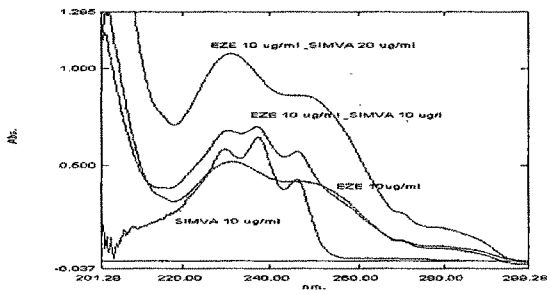


Figure: 7.2.1.1.1: Overlain zero order spectra of EZE and SIMVA and binary mixture 1:1.

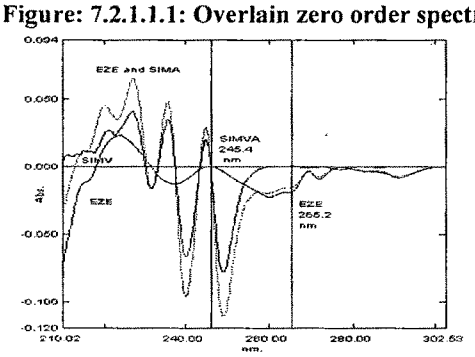


Figure: 7.2.1.1.2: Overlain 1st order UV spectra of EZE and SIMVA

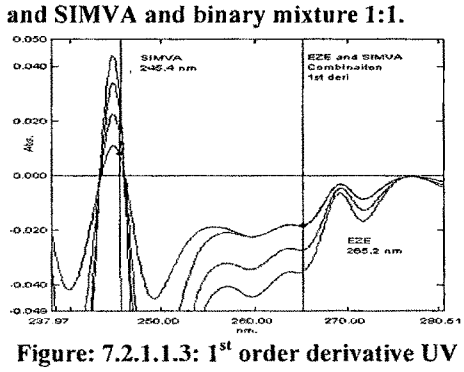


Figure: 7.2.1.1.3: 1st order derivative UV spectra of EZE and SIMVA

(II) For ratio derivative spectrophotometric method:

The zero order UV spectra of EZE and SIMVA for their respective standard solutions (5 -40 µg/ml) were obtained and stored. Stored spectra of standard EZE solutions were divided wavelength by wavelength by standard spectra of SIMVA (5 – 40 µg/ml) to select the best divisor concentration of SIMVA.

Then the first derivative of above ratio spectra was recorded and the values of the derivatives were measured at suitably selected wavelengths. The calibration curve was prepared by plotting the amplitude against the concentration.

The similar procedure was followed to determine the appropriate divisor concentration for SIM when EZE was used as divisor in the same way as described above. The calibration curve was obtained by plotting absorbance versus drug concentration.

Concentration of 40 µg/ml of SIMVA and 10 µg/ml of EZE as divisor gave best results in terms of signal to noise ratio and highest correlation coefficient values, being an indication of the quality of fitting of the data to the straight line (Table 7.2.1.1.1 and 7.2.1.1.2. and fig. 7.2.1.1.4 and 7.2.1.1.5).

Table 7.2.1.1.1: Optimization of divisor concentration of EZE for SIMVA

Compound	Divisor con. Of SIMVA*	λ (nm)	R^2
EZE (5-40 µg/ml)	20	242.8	0.9945
	40	**240.80**	**0.9974**

Table 7.2.1.1.2: Optimization of divisor concentration of SIMVA for EZE

Compound	Divisor con. Of EZE*	λ (nm)	R^2
SIMVA (5 – 40 µg/ml)	5	249.80	0.9934
	10	**249.00**	**0.9990**
	15	248.00	0.9983
	20	248.30	0.9932
	30	249.00	0.9943
	35	248.80	0.9962
	40	249.60	0.9925

Effect of derivative intervals on derivative ration spectra:

Δλ, the width of the boundaries over which the derivative is calculated was tested for Δλ = 2 nm, 4 nm and 8 nm.

The value of $\Delta\lambda$ = 2 nm was found optimal in connection with both slit width and wavelength interval.

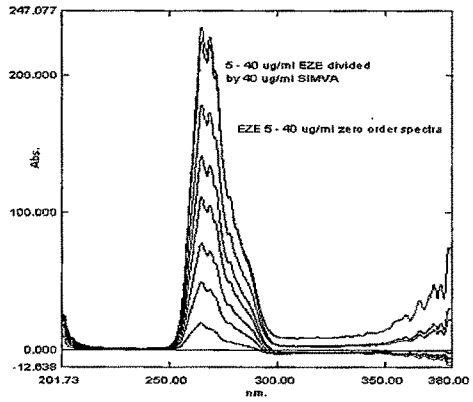


Figure: 7.2.1.1.4: Ratio spectra of EZE (40 µg/ml SIMVA as divisor)

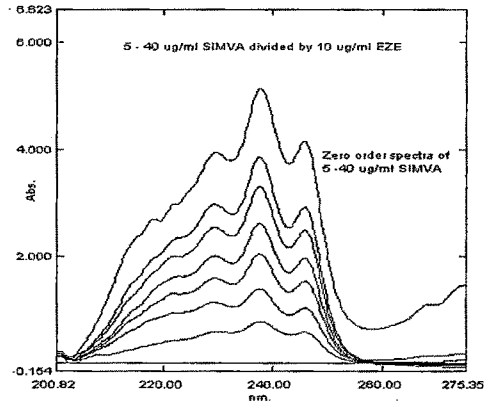


Figure: 7.2.1.1.5: Ratio spectra of SIMVA (10 µg/ml EZE as divisor)

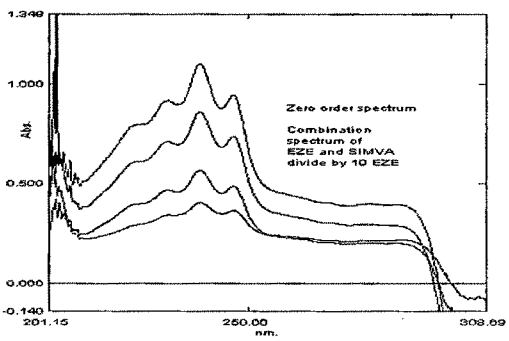


Figure: 7.2.1.1.6: Ratio spectra of EZE and SIMVA (10 µg/ml EZE as divisor)

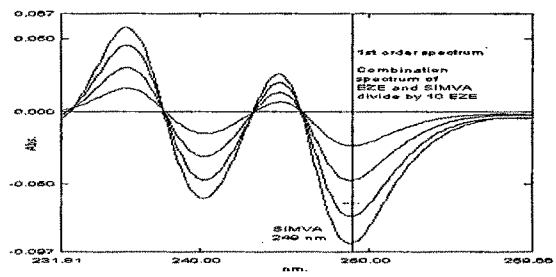


Figure: 7.2.1.1.7: Ratio derivative spectra of EZE and SIMVA (10 µg/ml EZE as divisor)

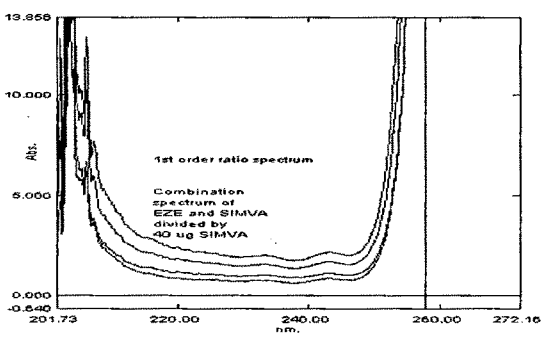


Figure: 7.2.1.1.8: ratio spectra of EZE and SIMVA (40 µg/ml SIMVA as divisor)

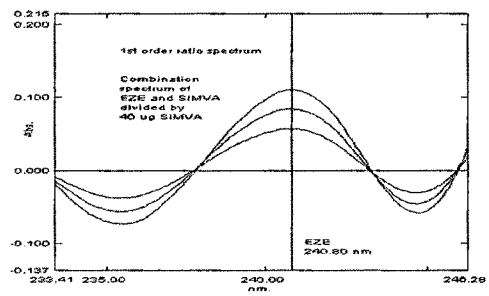


Figure: 7.2.1.1.9: Ratio derivative spectra of EZE and SIMVA(40 µg/ml SIMVA as divisor)

The absorption spectra of 10 µg/ml of EZE and 40 µg/ml of SIMVA were recorded in the range of 220 nm to 400 nm and stored in the memory of the instrument as divisor spectra. The absorption spectra of the binary mixture solutions of EZE and SIMVA were recorded in the range of 200 to 400 nm and were stored in the memory of the

software. The stored standard spectra of binary mixture were divided by a previously stored divisor spectrum of 40 µg/ml SIMVA to get the ratio spectra (fig. 7.2.1.1.7 and 7.2.1.1.9). The first derivative of the ratio spectra was traced with λ = 2 interval and amplitude at 240.8 nm were plotted against respective concentration of EZE. Similarly stored binary mixture was divided by a 10 µg/ml of EZE (fig 7.2.1.1.6) and the first derivative (fig. 7.2.1.1.8) of the ratio spectra were traced λ = 2 and the amplitude 249.0 nm were then plotted against the respective concentrations of SIMVA.

7.2.1.1.3. Calibration curve:

From the binary stock solution (100 µg/ml) of EZE and SIMVA aliquots of 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 ml were transferred to a series of 10ml volumetric flasks. The volume was adjusted to a mark with methanol to get 1, 5, 10, 15, 20, 25, 30, 35 and 40 µg/ml each of EZE and SIMVA.

7.2.1.1.4. Validation parameter

(I) Linearity:

EZE and SIMVA found to be linear in the concentration rang of 5 -40 µg/ml for FDZC and Ratio derivative spectrophotometric methods and calibration curve was plotted against concentration and regression equation was calculated. Result should be expressed in terms of Correlation co-efficient.

(II) Precision:

a. Repeatability (Intraday and Interday Precision):

The intraday and interday precisions of the proposed methods were determined by estimating the corresponding responses 5 times on the same day and on 5 different day for 10, 20 and 30 µg/ml, which was prepared by transferring 1,2 and 3 ml of stock solution in 10 ml volumetric flask and made up to the volume by methanol. The results are reported in terms of percentage coefficient of variation (%C.V).

b. Reproducibility:

The reproducibility of the method was checked by measuring absorbance of 10, 15 and 20 µg/ml of solution in UV 1700 and UV 1601 for three times. The % CV of the responses for determination of drug was found which reveals the reproducibility of the method.

(III) Accuracy

The accuracy of the methods was determined by recoveries by the standard addition method. For this, previously analyzed 10 µg/ml of drug solution from pharmaceutical formulations were spiked at three levels 50, 100 and 150 % from standard stock solution and made up to the volume with analytical grade methanol and analyzed. The final concentration was found form regression equation.

(IV) Limit of Detection and Quantification

Equation based on standard deviation of the response and the slope is given in Chapter 5, section 5.3.1.6 and 5.3.1.7.

7.2.1.1.5. Result and discussion

7.2.1.1.5.1. Validation parameters

(I) Linearity

Zero order spectra were converted in to 1st order derivative spectrum and absorbance was taken at selected analytical wavelengths. The derivative spectrophotometry method showed good linearity for EZE in the range of 5- 40 µg/ml at 265.2 nm (zero crossing of SIMVA) with co-relation co-efficient, of 0.9993 as seen in fig. 7.2.1.1.10. For SIMVA the line of best fit was obtained at 245.40 nm (zero crossing of EZE) with correlation coefficients of 0.9923 as seen in fig. 7.2.1.1.11. Calibration data was given in Table 7.2.1.1.3.

Table 7.2.1.1.3: Calibration data of EZE and SIMVA by FDZC method

Sr.No.	Concentration (µg/ml)	Absorbance Mean ± S.D. (N=5)		% C.V.	
		EZE at 265.2 nm	SIMVA at 245.4 nm	EZE	SIMVA
1	5	-0.009±0.00035	0.004 ± 0.00028	0.92	0.42
2	10	-0.019 ± 0.00042	0.014 ± 0.00072	0.43	0.33
3	15	-0.027 ± 0.00025	0.022 ± 0.00049	0.72	0.58
4	20	-0.037 ± 0.00014	0.029 ± 0.00028	0.59	0.26
5	25	-0.046 ± 0.00063	0.0362 ± 0.002	0.82	0.41
6	30	-0.055 ± 0.00026	0.041 ± 0.00072	0.48	0.14
7	35	-0.0655 ± 0.00028	0.048 ± 0.00023	0.72	0.24
8	40	-0.073 ± 0.00071	0.0543 ± 0.0083	0.33	0.83

* Mean value of five determinations.

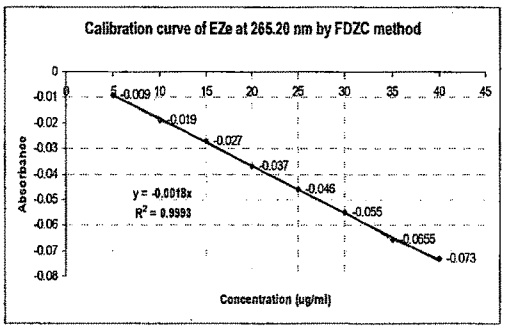


Figure 7.2.1.1.10: Calibration curve of EZE at 265.2 nm by FDZC method

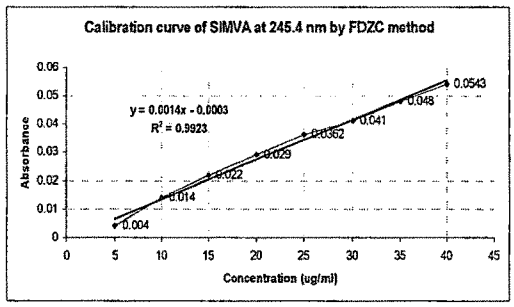


Figure 7.2.1.1.11: Calibration curve of SIMVA at 245.4 nm by FDZC method

The ratio derivative spectrophotometry method showed good linearity for EZE in the range of 5 - 40 µg/ml at 240.80 nm with co-relation co-efficient, of 0.9997 as seen in fig. 7.2.1.1.12. For SIMVA the line of best fit was obtained at 249.00 nm with correlation coefficients of 0.9994 as seen in fig. 7.2.1.1.12. Calibration data is given in Table 7.2.1.1.4.

Table 7.2.1.1.4: Calibration data of EZE and SIMVA by Ratio derivative method

Sr. No.	Concentration (µg/ml) (EZE and SIMVA)	Absorbance Mean (N=5)		% C.V.	
		EZE at 240.8 nm	SIMVA at 249.0 nm	EZE	SIMVA
1	5	0.011	-0.045	0.42	0.27
2	10	0.021	-0.085	0.62	0.81
3	15	0.0315	-0.1275	0.84	0.38
4	20	0.0402	-0.182	0.61	0.62
5	25	0.05025	-0.2275	0.88	0.44
6	30	0.0603	-0.273	0.52	0.12
7	35	0.07035	-0.3185	0.63	0.89
8	40	0.0804	-0.364	0.78	0.31

* Mean of five determinations.

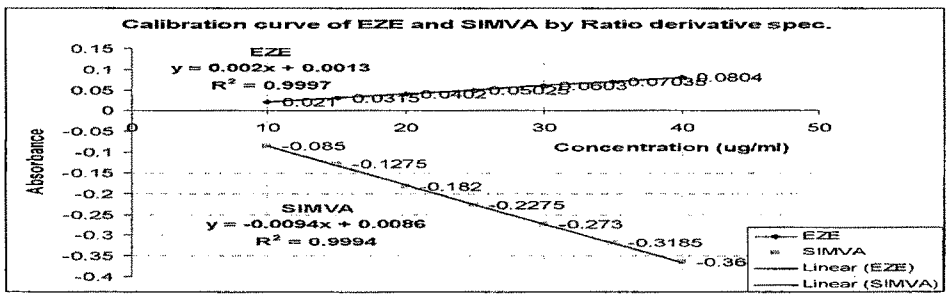


Figure 7.2.1.1.12: Calibration curve of EZE and SIMVA ratio derivative method

(II) Precision

- **Repeatability**

Intraday and interday variation for proposed methods were reported in table 7.2.1.1.5 and 7.2.1.1.6.

Table 7.2.1.1.5: Intraday precision data of EZE and SIMVA by Spectrophotometric methods.

Concentration (µg/ml)		First derivative zero crossing method (Mean (n=5) ± %C.V.)		Ratio derivative spectrophotometric method (Mean (n=5) ± %C.V.)	
EZE	**SIMVA**	**EZE at 265.2 nm**	**SIMVA at 245.4 nm**	**EZE at 240.80 nm**	**SIMVA at 249.00 nm**
10	10	-0.019±0.38	0.014±0.83	0.024±0.93	-0.088±0.62
20	20	-0.038±0.92	0.028±0.46	0.0408±0.072	-0.186±0.48
30	30	-0.055±0.43	0.041±0.62	0.0504±0.66	-0.226±0.22
Table 7.2.1.1.6: Interday precision data of EZE and SIMVA by Spectrophotometric methods.					
10	10	-0.018±0.37	0.013±0.82	0.023 ± 0.67	-0.084±0.66
20	20	-0.037±0.62	0.023±0.77	0.0401 ± 0.37	-0.181±0.38
30	30	-0.054±0.18	0.041±0.84	0.0505 ± 0.76	-0.223±0.29

***Mean of five determinations**

Reproducibility:

The reproducibility of the method was determined by using different instruments Shimadzu UV 1700 and Shimadzu UV 1601. The % CV of the responses confirms the reproducibility of the method for determination of EZE and SIMVA. The data is reported in (Table 7.2.1.1.7).

Table 7.2.1.1.7: Reproducibility data of EZE and SIMVA by Spectrophotometric methods.

Spectrophotometric Methods		Concentrations (µg/ml)					
		10		15		20	
		UV 1700	UV 1601	UV 1700	UV 1601	UV 1700	UV 1601
First derivative zero crossing method (Mean (n=5) ± %C.V.)	EZE at 265.2 nm	-0.019± 0.93	-0.020± 0.28	-0.029± 0.29	-0.029± 0.48	-0.038± 0.47	-0.038± 0.22
	SIMVA at 245.4 nm	0.014± 0.41	0.013± 0.38	0.022± 0.38	0.020± 0.28	0.028± 0.51	0.026± 0.44
Ratio derivative spectrophotometric method (Mean (n=5) ± %C.V.)	EZE at 240.8 nm	0.023± 0.93	0.024± 0.72	0.015± 0.29	0.016± 0.84	0.0406± 0.75	0.0408± 0.93
	SIMVA at 249.0 nm	-0.085± 0.83	-0.088± 0.39	-0.128± 0.93	-0.122± 0.59	-0.184± 0.48	-0.185± 0.35

***Mean of three determinations**

(III) Accuracy

Accuracy of the method was determined by performing recovery study on laboratory prepared synthetic mixture by standard addition method. The result showed % mean recovery values reported in table 7.2.1.1.8.

Table 7.2.1.1.8: Accuracy data of EZE and SIMVA by Spectrophotometric methods.

Spectrophotometric methods		EZE	SIMVA	EZE	SIMVA	EZE	SIMVA
Initial conc.(µg/ml) (A)		10	10	10	10	10	10
Quantity of std. Added (µg/ml) (B)		5	5	10	10	15	15
Total Amount (A + B)		15	15	20	20	25	25
First derivative zero crossing method (Mean (n=5) ± %C.V.)	Total quantity Found Mean	14.98	15.02	19.92	20.04	25.03	25.06
	% Recovery± S.D	99.87 ±0.38	100.13 ± 0.29	99.60 ±0.39	100.20 ± 0.79	100.20 ± 0.29	100.20 ± 0.52
Ratio derivative spectrophotometric method (Mean (n=5) ± %C.V.)	Total quantity Found Mean	14.98	15.03	19.98	20.03	25.03	24.99
	% Recovery± S.D	99.87	100.20	99.90	100.15	100.12	99.96

*** Mean value of five determinations.**

(IV) Limit of detection and quantification

The minimum detectable and quantifiable concentration of EZE and SIMVA were reported in table 7.2.1.1.10.

7.2.1.1.5.2. Applicability of the method for the analysis of commercial tablet formulation:

SIMVAS-EZ from Ranbaxy was analyzed by the proposed method. Twenty tables were triturated after taking their average weigh. The Tablet powder equivalent to one tablet was transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution was sonicated for 10 min and supernatant was filtered through Whatman filter paper no. 41. 1 ml from this solution were transferred to 10 ml volumetric flasks and diluted with methanol to get 10 µg/ml concentration. The absorbance of the

prepared solution was measured at 240.80 nm and 265.2 nm in FDZC method and the absorbance of the prepared solution was measured at 240.80 nm and 249.0 nm in ratio derivative method. The concentrations of EZE and SIMVA in tablets were calculated using the corresponding regression equation in ratio derivative method. Results obtained are reported in Table 7.2.1.1.9.

Table 7.2.1.1.9: EZE and SIMVA in tablet by Spectrophotometric methods.

Formu-lation	Drugs	Labeled Claim (mg/tab)	FDZC		Ratio derivative	
			*Amount found	% Assay	*Amount found	% Assay
SIMVAS-EZ	EZE	10	10.08	100.92	10.08	100.81
	SIMVA	10	10.12	101.31	10.12	101.21
Laboratory pre. mix	EZE	10	10.20	102.18	10.20	101.01
	SIMVA	10	10.12	103.21	10.12	101.23

*** Mean of five determinations.**

7.2.1.1.6. Summary of Validation parameters:

A summary of validation parameters is reported in Table 7.2.1.1.10.

Table 7.2.1.1.10: Summary of Validation parameters by Spectrophotometric methods.

Sr. No	Parameters	FDZC		Ratio derivative	
		EZE	SIMVA	EZE	SIMVA
1	Analytical wavelengths (nm)	265.20	245.40	240.8	249.0
2	Linearity range (µg/ml)	5 to 40 µg/ml			
3	Regression equation	Y=-0.00182x +0.0012	Y = -0.0014x -0.0003	Y=0.002x +0.0013	Y=-0.0094x +0.0086
4	Correlation coefficient (r^2)	0.9993	0.9923	0.9997	0.9994
5	Intercept	0.0012	0.0003	0.002	0.0094
6	Slope	0.0018	0.0014	0.0013	0.0086
7	% Assay	100.92	101.31	100.81	101.21
8	Accuracy and precision				
	Intra day % CV (n = 5)	0.38 –0.92 %	0.46 – 0.83 %	0.66 – 0.93 %	0.22 – 0.62%
	Inter day % CV (n = 5)	0.18 – 0.62 %	0.77 – 0.84 %	0.37 – 0.76 %	0.29 – 0.66%
	Reproducibility of measurements % CV	< 1 %			
	% Recovery	99.60 -100.20	100.13 -100.20	99.87 -100.12	99.96 -100.20
9	Limit of detection (µg/ml)	0.39	0.12	0.0128	0.012
10	Limit of quantification(µg/ml)	1.10	0.40	0.427	0.398

7.2.1.1.7. CONCLUSION

1st order zero crossing spectrophotometric method is a simple and specific technique for the reliable analysis of commercial formulations containing combinations of EZE and SIMVA (Table 7.2.1.1.10).

Ratio derivative spectrophotometric method is a comparably new technique for the reliable analysis of commercial formulations containing combinations of EZE and SIMVA (Table 7.2.1.1.10).

7.2.1.2. Infra Red spectroscopic method.

Experimental work:

7.2.1.2.1. Preparation of stock sample

For **quantitative IR** spectroscopic study 10 mg of both standard drugs were weighed accurately and made up to 100 mg on same butter paper to obtain final concentration of 100 µg/mg of each drug. This sample was triturated in mortar and pestle and sample was used as working standard sample for FTIR spectroscopic method.

7.2.1.2.2. Selection of analytical wavenumber

The individual IR spectrum of both the drugs and their mixture were overlain (fig. 7.2.1.2.1) to select the zero absorbance point. It was observed that 1614 cm^{-1} is the zero absorbance point for SIMVA so EZE was estimated at this frequency where as SIMVA was estimated at 3550 cm^{-1} at which EZE showed zero absorbance.

7.2.1.2.3. Calibration curve

Appropriate fractions were transferred from standard (100 µg/mg) to butter paper and weight adjusted 10 mg with KBr to get minimum transmission. At 1614 cm^{-1} and 3550 cm^{-1} peak height was measured for EZE and SIMVA, respectively. Calibration curves were drawn using the multiple linear regression (MLR) method and the drug amount in sample was computed from regression equation.

7.2.1.2.4. Validation parameters:

(I) Linearity:

Peak height of standard EZE and SIMVA sample were measured at selected wavenumber using KBR as a blank over the concentration range of 10-150 µg/mg and height was plotted against concentration and regression equation was calculated. Results are expressed in terms of Correlation co-efficient.

(II) Precision

a. Repeatability (Intraday and Interday Precision):

1.5, 3 and 6 mg of stock sample (100 µg/mg) was transferred to butter paper and mixed up to 10 mg with KBr to get concentration in 15, 30 and 60 µg/mg and DRIFTS spectra were recorded. Intraday precision was determined by analyzing sample five times in the same day. Interday precision was determined by analyzing sample daily for five days and % C.V. was calculated.

b. Reproducibility:

To study the reproducibility of FTIR method two sample compartments having different diameters were used. Compartment of higher diameter was termed as compartment number I and the smaller diameter was termed as compartment number II. Same sample was put in both the compartments and obtained peak height was compared. % CV was reported for average peak area and compared reproducibility.

(III) Accuracy

Accuracy of the method was determined by performing recovery study of pharmaceutical formulation by standard addition method. For this, 10 µg/mg of drug sample form pharmaceutical formulations were spiked at three levels 50, 100 and 150 % from standard stock. The final concentration was found form regression equation.

(IV) Limit of Detection and Quantification

Equation based on standard deviation of the response and the slope is given in Chapter 5, section 5.3.1.6 & 5.3.1.7.

7.2.1.2.5. Results and Discussion:

7.2.1.2.5.1 Validation Parameters

(I) Linearity

The IR spectrophotometry method showed good linearity for EZE in the range of 11.2 to 147 µg/mg at 1614 cm^{-1} (zero absorbance point of SIMVA) with co-relation co-efficient of 0.9993 as seen in fig. 7.2.1.2.4. For SIMVA the line of best fit was obtained at 3550 cm^{-1} (zero absorbance point of EZE) with correlation coefficients of 0.9999 in linearity range of 10.3 to 94 µg/mg as seen in fig. 7.2.1.2.5. Calibration data is given in Table 7.2.1.2.1.

Table 7.2.1.2.1: Calibration data of EZE and SIMVA by IR Spectroscopy

Sr. No.	Concentration (µg/mg)		Peak heihgt* ± S.D. (N=5)		% C.V.	
	EZE	SIMVA	EZE at 1614 1/cm	SIMVA 3550 1/cm	EZE	SIMVA
1	11.2	10.3	0.0023 ± 0.00043	0.0641 ± 0.00073	0.38	0.83
2	34.3	42.4	0.0247 ± 0.00053	0.245 ± 0.00068	0.72	0.29
3	41.2	86.4	0.0314 ± 0.00022	0.793 ± 0.00035	0.48	0.62
4	147	94	0.15 ± 0.00086	0.895 ± 0.00082	0.58	0.48

*Mean value of five determinations.

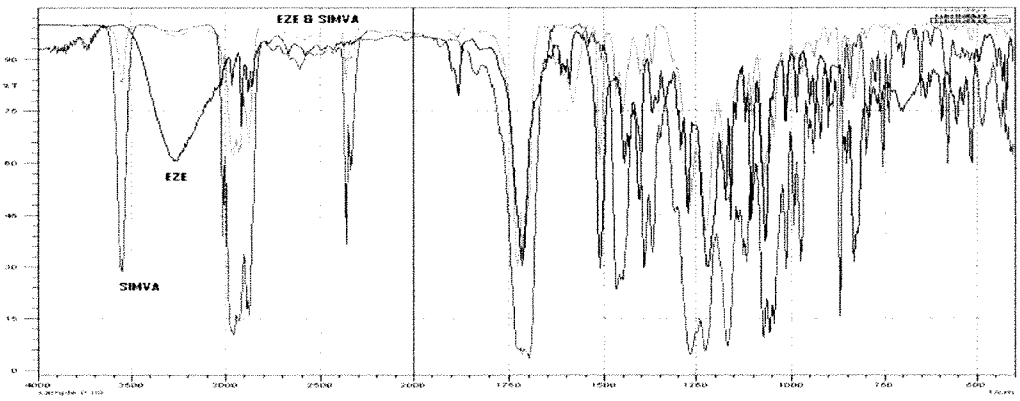


Figure: 7.2.1.2.1: Overlain IR spectra of EZE and SIMVA

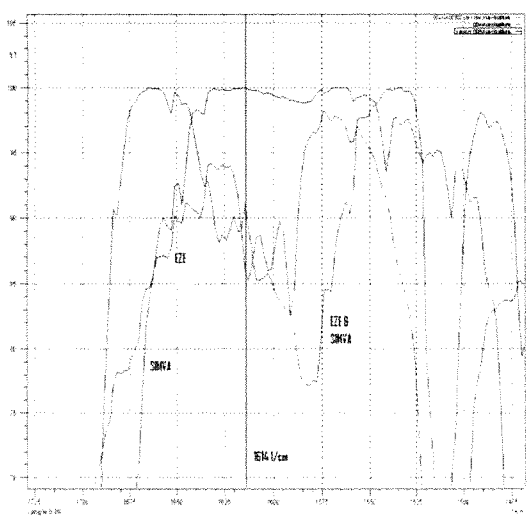


Figure: 7.2.1.2.2: Overlain IR spectra of SIMVA and EZE at 1614 cm^{-1}

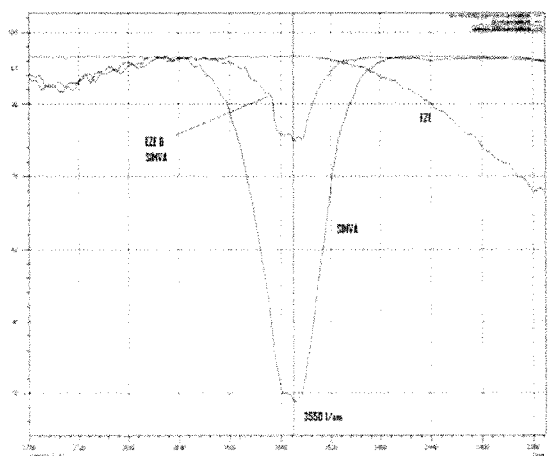


Figure: 7.2.1.2.3: Overlain IR spectra of SIMVA and EZE at 3550 cm^{-1}

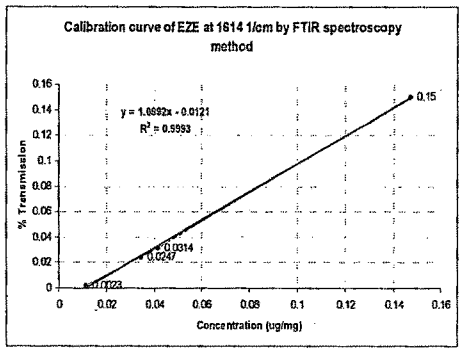


Figure 7.2.1.2.4: Calibration curve of EZE at 1614cm^{-1} by IR method

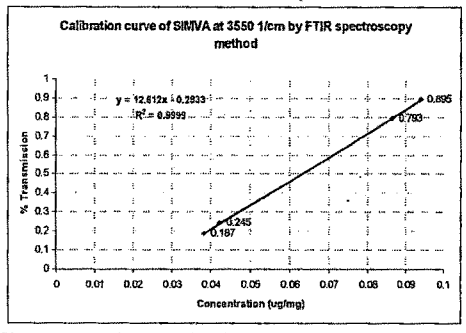


Figure 7.2.1.2.5: Calibration curve of SIMVA at 3550 cm^{-1} by IR method

(II) Precision

Repeatability

The average % CV values were in the range of 0.25 – 0.71 % and 0.28 – 0.93 % for EZE and 0.35 – 0.81 % and 0.25 – 0.71 % for SIMVA (Table 7.2.1.2.2 and 7.2.1.2.3), respectively.

Table 7.2.1.2.2: Intraday precision data of EZE and SIMVA by IR Spectroscopy

Concentration (µg/mg)		Peak height* ± S.D. (n=5)		% C.V*.	
EZE	SIMVA	EZE at 1614 cm^{-1}	SIMVA at 3550 cm^{-1}	EZE	SIMVA
15	15	0.0031±0.00048	0.0732±0.00058	0.49	0.48
30	30	0.0216±0.00024	0.1733±0.00028	0.25	0.35
60	60	0.0529±0.00062	0.6507±0.00077	0.71	0.81
Table 7.2.1.2.3: Interday precision data of EZE and SIMVA by IR Spectroscopy					
15	15	0.0032±0.00048	0.0767±0.00086	0.93	0.25
30	30	0.0218±0.00024	0.1768±0.00035	0.58	0.71
60	60	0.0536±0.00082	0.6755±0.00074	0.28	0.44

* Mean value of five determinations.

Reproducibility:

The % CV of the responses for determination of EZE and SIMVA revealing the reproducibility of the method are reported in Table 7.2.1.2.4.

Table 7.2.1.2.4: Reproducibility data of EZE and SIMVA by IR Spectroscopy

Concentration (µg/mg)		Peak heihgt ±%CV*			
EZE	SIMVA	EZE at 1614 cm^{-1} ± %CV		SIMVA at 3550 cm^{-1} ± %CV	
		Sample compartment I	Sample compartment II	Sample compartment I	Sample compartment II
15	15	0.0038 ± 0.73	0.0037 ± 0.25	0.0749 ± 0.85	0.0767 ± 0.41

30	30	0.0219 ± 0.82	0.0211 ± 0.85	0.1799 ± 0.99	0.1707 ± 0.17
45	45	0.0459 ± 0.59	0.0465 ± 0.36	0.3357 ± 0.25	0.3285 ± 0.83

***Mean value of five determinations**

(III) Accuracy

The method showed % mean recovery for EZE in the range of 99.97 – 100.09 % and for SIMVA it was 99.97 – 100.36 % in synthetic mixture (Table 7.2.1.2.5).

Table 7.2.1.2.5: Accuracy data of EZE and SIMVA by IR Spectroscopy

Initial conc. (µg/mg) (A)		Quantity of std. Added (µg/mg) (B)		Total Amount (A + B)		Total quantity Found Mean*		% Recovery± C.V*	
EZE	SIMVA	EZE	SIMVA	EZE	SIMVA	EZE at 1614 cm^{-1}	SIMVA at 3550 cm^{-1}	EZE	SIMVA
10	10	5	5	15	15	14.98	15.03	99.87 ± 0.43	100.20 ± 0.38
10	10	15	15	25	25	24.98	25.09	99.92 ± 0.28	100.36 ± 0.78
10	10	20	20	30	30	30.03	30.98	100.09 ± 0.21	99.97 ± 0.18

*** Mean value of three determinations**

(IV) Limit of detection

The minimum detectable concentration of EZE and SIMVA were found to be 10.7044 µg/mg and 0.9872 µg/mg, respectively.

(V) Limit of quantification

The lowest quantifiable concentration of EZE and SIMVA were found to be 35.2241 µg/mg and 3.2241 µg/mg, respectively by practically observation.

7.2.1.2.3.2. Analysis of commercial tablet formulation:

The contents of 20 Tablets were weighed and their mean weight determined and finely powdered. An equivalent weight of one Tablet was transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution (100 µg/ml) was sonicated for 10 min and supernatant was filtered through Whatman filter paper no.41. Filterate was evaporated and from the residue 1 mg weigh and make up to 100 mg with KBr in butter paper and triturated in motor pestle and DRIFT spectra was scannned between 4000 and 400 cm^{-1}, by averaging 16 scans for each spectrum with a resolution of 4 cm^{-1} (data point resolution per interval 1 cm^{-1}). % Transmission was recorded at 1614 cm^{-1} and 3550

cm^{-1}. Concentration of sample was found from respective regression equation of EZE and SIMVA.

Table 7.2.1.2.6: Estimation of EZE and SIMVA in tablet by IR spectrosopy.

Formulation	DRUGS	Labeled Claim (mg/tab)	QIR	
			*Amount found	% Assay ± %CV
SIMVAS-EZ	EZE	10	10.09	100.92 ± 0.48
	SIM	10	10.32	103.21 ± 0.28
Laboratory pre. mixture	EZE	10	10.15	100.52 ± 0.51
	SIM	10	10.01	100.12 ± 0.92

*** Mean value of five determinations.**

7.2.1.2.4. Summary of Validation parameters:

The validation parameter of analytical method is reported in Table 7.2.1.2.7

Table 7.2.1.2.7: Summary of Validation parameters of IR method

Sr. No	Parameters	Results	
		EZE	SIMVA
1	Wavenumber (cm^{-1})	1614	3550
2	Linearity range (µg/mg)	11.2 – 14.7	10.3 -94
3	Regression equation	Y=1.0992x-0.0121	Y=12.612x - 0.2933
4	Correlation coefficient (r^2)	0.9993	0.9999
5	Intercept	-0.0121	0.2933
6	Slope	1.0992	12.612
7	Assay	100.92	103.21
8	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Repeatability of measurements % CV % Recovery	 0.25-0.71 0.28-0.93 < 1 % 99.97-100.07	 0.35-0.81 0.25-0.71 < 1% 99.97-100.36
9	Limit of detection	10.7044 µg/mg	0.9872 µg/mg
10	Limit of quantification	35.2241 µg/mg	3.2241 µg/mg

7.2.1.2.5. CONCLUSION

The proposed method is simple, precise and accurate and can be used as a method for quality control of pharmaceuticals. This technique extends the use of a standard IR spectrophotometer typically used for identification purposes, to the reliable quantification of EZE and SIMVA.

7.2.1.3. Simultaneous estimation of EZE and SIMVA by Chemometric Methods:

In this section the Chemometric methods have been described for EZE and SIMVA. The concept of mathematical modeling is used to provide maximum relevant chemical information in order to analyze the drugs simultaneously.

The two-chemometric techniques ILS and CLS were developed for the simultaneous determination of the titled drugs in their binary mixture.

7.2.1.3. Inverse Least Square (ILS) and Classical Least Square (CLS) technique

It is the application of multiple linear regressions (MLR) to the inverse expression of the Beer-Lambert Law of spectrophotometry.

7.2.1.3.1. Preparation of calibration (training) set of the EZE and SIMVA:

A training set was consisting of 24 binary mixture (Table 7.2.1.3.1) solutions in the possible combinations of linearity range. The zero order absorbance spectra for all the solutions were measured and stored in the computer. To develop the ILS and CLS models for the training set, the computer was fed with absorbance and concentration matrices, and then calculations were carried out using MATLAB software.

7.2.1.3.2. Preparation of the validation set of the EZE and SIMVA:

15 binary mixtures of the drugs were prepared by diluting different volumes of EZE and SIMVA from the standard stock solutions in 10 ml measuring flasks. The composition of the solutions is given in the table 7.2.1.3.4.

7.2.1.3.3. Selection of the spectral region:

In order to develop ILS and CLS method for simultaneous estimation of EZE and SIMVA, light absorption study was done. Individual spectrum of methanolic solutions of both the drugs and their binary mixture were overlain.

Although the ILS and LCS are the full spectrum method, 21 wavelengths were selected between 230 nm to 250 nm with the interval of $\Delta\lambda$ = 1 nm in the zero order spectra as shown in fig. 7.2.1.3.1.

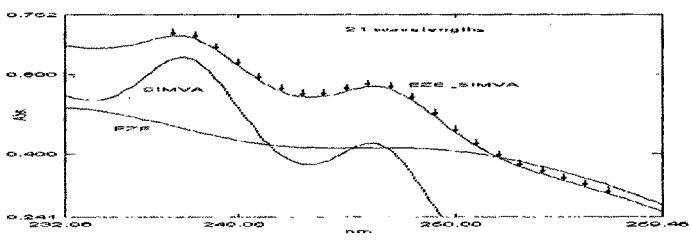


Figure 7.2.1.3.1: Overlain spectra of EZE, SIMVA and their binary mixture showing spectral region 237 nm to 268 nm (21 wavelengths)

7.2.1.3.4. Measurement of the absorbance:

The absorbance matrices were produced by measuring absorbance of binary mixture at 21 wavelengths. In this calibration was obtained by measuring absorbance data matrix and concentration data matrix to predict the concentration of EZE and SIMVA in their binary mixtures and tablets. The numerical calculations were performed using MATLAB 6.1 software and excel.

7.2.1.3.5. Equation for ILS and CLS method:

The mathematical expression of ILS is C = P x A.

The calibration coefficient (P) was obtained from the linear equation system using the absorbance data and the concentration taken in training set (Table 7.2.1.3.1 and 7.2.1.3.2)

The absorbance values (Table 7.2.1.3.3) of the sample solutions at 21 wavelengths were placed in the above equation and the amounts of EZE and SIMVA in the synthetic mixture and formulation were found as shown in Table 7.2.1.3.4.

Table 7.2.1.3.1: Composition of Calibration (training) set for EZE and SIMVA:

Mixture No.	EZE (µg/ml)	SIMVA (µg/ml)	Mixture No.	EZE (µg/ml)	SIMVA (µg/ml)
1	0	5.35	13	16.5	16.05
2	2.2	10.7	14	16.5	17.5
3	5.5	5.35	15	16.5	32.1
4	5.5	19.26	16	16.5	32.1
5	6.05	16.58	17	16.5	19.26
6	8.8	16.05	18	19.8	24.2
7	11	10.7	19	22	19.26
8	11	10.7	20	22	21.4
9	11	19.26	21	24.2	32.1
10	13.2	21.4	22	27.5	19.26
11	15.95	8.01	23	30.8	16.05
12	16.5	10.7	24	35.2	10.7

Table 7.2.1.3.2: Absorbance data for the calibration set at 21 wavelengths:

Absorbance	Mi No.	Wavelength																			
		237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257
	1	0.235	0.235	0.213	0.18	0.149	0.126	0.118	0.125	0.142	0.155	0.147	0.117	0.076	0.037	0.009	-0.011	-0.023	-0.031	-0.035	-0.036	-0.037
	2	0.619	0.614	0.565	0.492	0.426	0.379	0.362	0.375	0.407	0.431	0.41	0.344	0.259	0.178	0.116	0.074	0.045	0.028	0.016	0.009	0.004
	3	0.783	0.773	0.739	0.695	0.653	0.623	0.608	0.611	0.624	0.637	0.627	0.595	0.549	0.503	0.463	0.432	0.407	0.386	0.367	0.348	0.33
	4	1.263	1.247	1.155	1.023	0.904	0.819	0.786	0.805	0.861	0.9	0.863	0.743	0.588	0.443	0.333	0.256	0.202	0.167	0.143	0.125	0.111
	5	1.195	1.187	1.108	0.994	0.888	0.808	0.772	0.783	0.826	0.861	0.83	0.729	0.594	0.466	0.366	0.295	0.245	0.212	0.188	0.17	0.156
	6	1.183	1.169	1.09	0.981	0.883	0.812	0.786	0.802	0.847	0.88	0.848	0.752	0.625	0.507	0.414	0.348	0.302	0.27	0.245	0.226	0.209
	7	1.058	1.051	0.999	0.923	0.85	0.793	0.765	0.768	0.795	0.82	0.805	0.744	0.658	0.573	0.504	0.452	0.412	0.382	0.358	0.337	0.318
	8	1.122	1.106	1.056	0.99	0.93	0.886	0.866	0.869	0.89	0.908	0.894	0.844	0.775	0.706	0.648	0.604	0.568	0.538	0.511	0.484	0.457
	9	1.59	1.582	1.489	1.351	1.218	1.119	1.071	1.081	1.133	1.178	1.147	1.029	0.867	0.713	0.592	0.504	0.44	0.395	0.362	0.335	0.313
	10	1.668	1.645	1.538	1.393	1.26	1.164	1.128	1.149	1.206	1.249	1.204	1.075	0.905	0.746	0.623	0.535	0.469	0.424	0.389	0.36	0.335
	11	0.549	0.542	0.513	0.471	0.434	0.405	0.392	0.396	0.41	0.422	0.412	0.378	0.333	0.288	0.253	0.226	0.206	0.191	0.179	0.168	0.157
	12	1.234	1.215	1.153	1.069	0.995	0.942	0.922	0.934	0.966	0.991	0.97	0.906	0.817	0.73	0.66	0.607	0.564	0.531	0.501	0.472	0.444
	13	1.494	1.473	1.388	1.272	1.169	1.096	1.066	1.083	1.129	1.162	1.131	1.035	0.907	0.784	0.687	0.616	0.562	0.521	0.487	0.456	0.427
	14	1.785	1.759	1.65	1.499	1.366	1.267	1.23	1.251	1.311	1.354	1.311	1.182	1.011	0.85	0.724	0.632	0.565	0.516	0.477	0.444	0.415
	15	2.193	2.153	2.025	1.828	1.654	1.528	1.477	1.502	1.577	1.633	1.578	1.41	1.186	0.977	0.816	0.698	0.613	0.555	0.509	0.471	0.439
	16	2.502	2.455	2.307	2.074	1.868	1.721	1.662	1.697	1.783	1.85	1.782	1.576	1.311	1.063	0.874	0.737	0.638	0.572	0.521	0.48	0.445
	17	1.825	1.803	1.702	1.559	1.428	1.328	1.285	1.299	1.352	1.394	1.355	1.234	1.073	0.919	0.799	0.708	0.641	0.592	0.553	0.518	0.487
	18	2.296	2.258	2.118	1.926	1.756	1.634	1.586	1.613	1.687	1.741	1.682	1.512	1.294	1.09	0.929	0.812	0.725	0.664	0.615	0.574	0.536
	19	1.959	1.939	1.838	1.701	1.57	1.473	1.43	1.442	1.492	1.53	1.495	1.38	1.225	1.076	0.954	0.865	0.795	0.742	0.697	0.657	0.619
	20	2.132	2.109	1.991	1.831	1.686	1.58	1.532	1.549	1.605	1.649	1.608	1.477	1.301	1.133	0.997	0.898	0.822	0.764	0.716	0.673	0.634
	21	2.847	2.808	2.631	2.405	2.187	2.038	1.974	2.008	2.097	2.167	2.089	1.876	1.603	1.348	1.149	1.001	0.894	0.819	0.757	0.705	0.659
	22	2.529	2.486	2.371	2.177	2.01	1.884	1.826	1.843	1.912	1.965	1.916	1.76	1.553	1.353	1.195	1.078	0.987	0.919	0.863	0.813	0.765
	23	2.057	2.018	1.925	1.804	1.695	1.618	1.587	1.602	1.648	1.681	1.648	1.552	1.424	1.298	1.196	1.117	1.05	0.995	0.944	0.895	0.845
	24	2.241	2.197	2.114	2.019	1.925	1.865	1.842	1.852	1.882	1.906	1.885	1.815	1.724	1.63	1.547	1.476	1.41	1.352	1.292	1.23	1.165

Table 7.2.1.3.3: Absorbance data for the calibration set at 21 wavelengths:

Absorbance	Mi No.	Wavelength																			
		237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257
	1	1.615	1.604	1.48	1.298	1.132	1.007	0.953	0.974	1.045	1.101	1.047	0.88	0.662	0.457	0.301	0.193	0.12	0.075	0.046	0.028	0.019
	2	0.42	0.414	0.395	0.369	0.345	0.327	0.319	0.321	0.329	0.336	0.33	0.311	0.283	0.256	0.234	0.217	0.203	0.191	0.18	0.17	0.16
	3	1.144	1.136	1.063	0.954	0.856	0.78	0.746	0.756	0.796	0.829	0.801	0.707	0.581	0.459	0.365	0.298	0.25	0.218	0.195	0.177	0.163
	4	1.782	1.771	1.648	1.471	1.305	1.179	1.125	1.142	1.209	1.264	1.215	1.056	0.842	0.638	0.483	0.372	0.295	0.246	0.211	0.187	0.17
	5	1.609	1.598	1.474	1.294	1.128	1.003	0.949	0.969	1.039	1.096	1.041	0.875	0.658	0.454	0.299	0.191	0.118	0.074	0.045	0.028	0.018
	6	1.388	1.372	1.29	1.176	1.07	0.991	0.957	0.967	1.009	1.043	1.012	0.914	0.782	0.655	0.557	0.484	0.431	0.393	0.364	0.339	0.318
	7	1.398	1.383	1.297	1.175	1.062	0.978	0.941	0.953	0.998	1.034	1.003	0.9	0.758	0.622	0.517	0.44	0.384	0.345	0.316	0.292	0.272
	8	1.061	1.048	0.991	0.91	0.836	0.78	0.755	0.763	0.792	0.815	0.795	0.728	0.638	0.551	0.481	0.428	0.388	0.359	0.335	0.314	0.295
	9	1.016	0.999	0.961	0.911	0.867	0.834	0.818	0.821	0.836	0.848	0.838	0.803	0.753	0.703	0.661	0.625	0.595	0.568	0.542	0.515	0.488
	10	1.646	1.623	1.536	1.417	1.307	1.223	1.187	1.198	1.239	1.274	1.244	1.145	1.01	0.882	0.779	0.7	0.64	0.596	0.559	0.525	0.494
	11	1.512	1.49	1.423	1.335	1.255	1.197	1.171	1.177	1.204	1.228	1.208	1.141	1.05	0.959	0.884	0.825	0.775	0.735	0.697	0.661	0.626
	12	1.997	1.975	1.876	1.749	1.632	1.545	1.506	1.517	1.558	1.596	1.561	1.46	1.321	1.185	1.075	0.989	0.921	0.868	0.821	0.776	0.732
	13	1.745	1.721	1.644	1.539	1.448	1.38	1.349	1.356	1.389	1.416	1.393	1.316	1.211	1.106	1.018	0.949	0.893	0.846	0.803	0.761	0.719
	14	2.073	2.049	1.951	1.829	1.711	1.627	1.591	1.599	1.639	1.673	1.644	1.542	1.41	1.276	1.167	1.082	1.012	0.956	0.906	0.859	0.813
	15	1.342	1.314	1.285	1.26	1.24	1.226	1.219	1.217	1.217	1.219	1.219	1.216	1.209	1.194	1.175	1.149	1.118	1.084	1.043	0.998	0.949

Table 7.2.1.3.4: Composition of Validation set for EZE and SIMVA:

Mixture No.	EZE (µg/ml)	SIMVA (µg/ml)	Mixture No.	EZE (µg/ml)	SIMVA (µg/ml)
1	0	26.75	9	16.5	16.05
2	5.5	3.21	10	22	10.7
3	5.5	16.05	11	24.2	16.05
4	5.5	26.75	12	24.75	12.3
5	9.9	16.05	13	27.2	16.05
6	9.9	16.05	14	33	0
7	11	10.7	15	35.2	16.05
8	16.5	5.35	-	-	-

Introducing (P) into the linear equation system, the calibration for ILS was obtained as fig. 7.2.1.3.2.

The absorbance values of the samples, the 21 wavelengths in the spectral region from 230 nm to 250 nm, were placed in the linear equation and the amounts of EZE and SIMVA in the synthetic mixture and tablets were found as shown in Table 7.2.1.3.6.

$$\begin{bmatrix} C_{EZE} \\ \cdot \\ C_{ROSU} \end{bmatrix} \approx 1.0e+003 * \begin{pmatrix} -0.8330 & -0.0811 \\ -0.8330 & -0.0811 \\ 0.6683 & -0.6940 \\ -1.2664 & 0.0066 \\ 0.1163 & -0.8955 \\ 0.7257 & 0.9417 \\ -0.5894 & -0.0703 \\ 0.5647 & 0.3972 \\ 1.3319 & 1.0458 \\ -1.4246 & -2.4106 \\ -1.0143 & -0.1653 \\ 1.2513 & 1.9554 \\ 0.4785 & 2.1786 \\ -3.8533 & -4.2378 \\ 4.4847 & 1.9633 \\ -0.0176 & -0.2911 \\ -2.1481 & -4.1148 \\ 3.6028 & 3.6003 \\ -6.3383 & -2.8380 \\ 6.3336 & 3.0817 \\ -3.1423 & 0.1393 \end{pmatrix} \times \begin{pmatrix} A_1 \\ A_2 \\ A_3 \\ A_4 \\ A_5 \\ A_6 \\ A_7 \\ A_8 \\ A_9 \\ A_{10} \\ A_{11} \\ A_{12} \\ A_{13} \\ A_{14} \\ A_{15} \\ A_{16} \\ A_{17} \\ A_{18} \\ A_{19} \\ A_{20} \\ A_{21} \end{pmatrix}$$

Fig 7.2.1.3.2: Equation of ILS

7.2.1.3.6. Equation for CLS method:

The mathematical expression of ILS is A = K x C.

In this method, the calibration coefficient (K) was obtained from the linear equation system using the absorbance data and the training set (Table 7.2.1.3.1 and 7.2.1.3.2)

The absorbance values (Table 7.2.1.3.3) of the samples at 21 wavelengths were placed in the above equation and the amounts of EZE and SIMVA in the synthetic mixture and formulation were found as shown in Table 7.2.1.3.4.

Introducing (K) into the linear equation system, the calibration for ILS was obtained as fig. 7.2.1.3.2.3.

The absorbance values of the samples, the 21 wavelengths in the spectral region from 230 nm to 250 nm were placed in the linear equation and the amounts of EZE and SIMVA in the synthetic mixture and tablets were found as shown in Table 7.2.1.3.7.

$$\begin{pmatrix} A1 \\ A2 \\ A3 \\ A4 \\ A5 \\ A6 \\ A7 \\ A8 \\ A9 \\ A10 \\ A11 \\ A12 \\ A13 \\ A14 \\ A15 \\ A16 \\ A17 \\ A18 \\ A19 \\ A20 \\ A21 \end{pmatrix} \approx \begin{pmatrix} 0.0044 & 0.0534 \\ 0.0433 & 0.0529 \\ 0.0423 & 0.0486 \\ 0.0414 & 0.0421 \\ 0.0404 & 0.0362 \\ 0.0398 & 0.0319 \\ 0.0396 & 0.0302 \\ 0.0395 & 0.0311 \\ 0.0397 & 0.0338 \\ 0.0398 & 0.0358 \\ 0.0397 & 0.0338 \\ 0.0394 & 0.0278 \\ 0.0390 & 0.0199 \\ 0.0383 & 0.0127 \\ 0.0374 & 0.0072 \\ 0.0365 & 0.0034 \\ 0.0355 & 0.0010 \\ 0.0343 & -0.0005 \\ 0.0330 & -0.0014 \\ 0.0316 & -0.0019 \\ 0.0300 & -0.0021 \end{pmatrix} \times \begin{pmatrix} CEZE \\ CROSU \end{pmatrix}$$

Fig 7.2.1.3.3: Equation of CLS

7.2.1.3.7. Validation Parameters

(I) Accuracy

Accuracy of the method was determined by performing recovery study on laboratory prepared synthetic mixture. The ILS method showed % mean recovery for EZE and SIMVA in the range of 92.56 – 103.14 % and 97.60 – 105.39 % from synthetic mixture (Table 7.2.1.3.6).

The CLS method showed % mean recovery for EZE and SIMVA in the range of 98.05 – 105.89% and 94.23 – 101.76 % from synthetic mixture (Table 7.2.1.3.2.7).

(II) Precision

The precision was determined by means of one way ANOVA. The experiments were repeated five times in a day for intra day and on five different days for inter day precision for 10 synthetic mixtures. F values below the tabulated levels were obtained and there was no significant difference between the results obtained in the determination of each drug in the presence of other on different days.

Table 7.2.1.3.5: Data for precision study using one-way ANOVA

Parameters	CLS		ILS	
	EZE	SIMVA	EZE	SIMVA
Between days variance	4.70	3.98	4.03	2.67
Within days variance	2.01	1.83	1.73	1.33
F ratio	2.34	2.18	2.33	2.01

Note the between day and within day degrees of freedom are 4 and 45 respectively. The critical F ratio value for 4 and 45 df and the confidence level of 95% is 2.53.

(III) Limit of detection

The minimum detectable concentration for ILS of EZE and SIMVA was found to be 0.069 µg/ml and 0.173 µg/ml, respectively. The minimum detectable concentration for CLS of EZE and SIMVA was found to be 0.0329 µg/ml and 0.281 µg/ml, respectively.

(IV) Limit of quantification

The lowest quantifiable concentration for ILS of EZE and SIMVA was found to be 0.492 µg/ml and 0.946 µg/ml, respectively by practical observation. The lowest quantifiable concentration for CLS of EZE and SIMVA was found to be 0.238 µg/ml and 0.772 µg/ml, respectively by practical observation.

(V) Predicted versus known concentration plot:

The predicted concentrations of the validation samples were plotted against the known concentration values.

This tool is used to determine whether the model accounts for the concentration variation in the validation set or not. Plots were expected to fall on a straight line with a slope of 1 and o intercept. The predicted versus known concentration plots of the prepared concentration plots of the prepared validation samples.

Table 7.2.1.3.6: Actual, predicted and residual values for ILS.

SR. No.	Actual Conc.		Predicted conc.		Residual		% Recovery	
	EZE	SIMVA	EZE	SIMVA	EZE	SIMVA	EZE	SIMVA
1	0	26.75	0	26.5449	0	0.2051	100.00	99.23
2	5.5	3.21	5.6726	3.2363	-0.1726	-0.0263	103.14	100.82
3	5.5	16.05	5.091	16.8064	0.409	-0.7564	92.56	104.71
4	5.5	26.75	5.3567	26.6255	0.1433	0.1245	97.39	99.53
5	9.9	16.05	9.9705	16.9152	-0.0705	-0.8652	100.71	105.39
6	9.9	16.05	9.9995	16.0306	-0.0995	0.0194	101.01	99.88
7	11	10.7	11.0384	10.5008	-0.0384	0.1992	100.35	98.14
8	16.5	5.35	16.1567	5.2215	0.3433	0.1285	97.92	97.60
9	16.5	16.05	16.9567	16.2602	-0.4567	-0.2102	102.77	101.31
10	22	10.7	22.465	10.475	-0.465	0.225	102.11	97.90
11	24.2	16.05	24.6418	16.6657	-0.4418	-0.6157	101.83	103.84
12	24.75	12.3	24.0439	12.7781	0.7061	-0.4781	97.15	103.89
13	27.2	16.05	27.9361	16.3088	-0.7361	-0.2588	102.71	101.61
14	33	0	33.3017	0	-0.3017	0	100.91	100.00
15	35.2	16.05	35.7791	16.794	-0.5791	-0.744	101.65	104.64
16	0	26.75	0	26.5449	0	0.2051	100.00	99.23
17	5.5	3.21	5.6726	3.2363	-0.1726	-0.0263	103.14	100.82
18	5.5	16.05	5.091	16.8064	0.409	-0.7564	92.56	104.71
19	5.5	26.75	5.3567	26.6255	0.1433	0.1245	97.39	99.53
20	9.9	16.05	9.9705	16.9152	-0.0705	-0.8652	100.71	105.39
21	9.9	16.05	9.9995	16.0306	-0.0995	0.0194	101.01	99.88

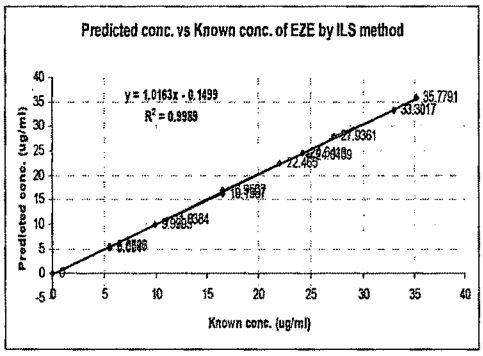


Fig 7.2.1.3.4: Linearity plots of EZE and SIMVA for validation set.

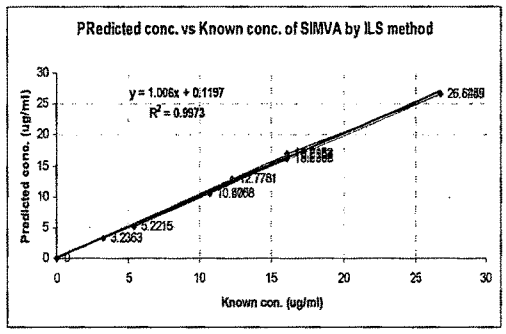


Fig 7.2.1.3.5: Linearity plots of EZE and SIMVA for validation set.

It was noticed that both EZE and SIMVA in all samples lay on a straight line and the equations of these lines are shown on the graphs fig. 7.2.1.3.4 and 7.2.1.3.5 by ILS method and fig. 7.2.1.3.6 and 7.2.1.3.7 by CLS method.

This indicates that the prediction ability of the validation set is good.

Table 7.2.1.3.7: Actual, predicted and residual values for CLS.

SR. No.	Actual Conc.		Predicted conc.		Residual		Percentage	
	EZE	SIMVA	EZE	SIMVA	EZE	SIMVA	EZE	SIMVA
1	0	26.75	0	26.1323	0	0.6177	100.00	97.69
2	5.5	3.21	5.6043	3.2279	-0.1043	-0.0179	101.90	100.56
3	5.5	16.05	5.6153	16.036	-0.1153	0.014	102.10	99.91
4	5.5	26.75	5.5975	27.222	-0.0975	-0.472	101.77	101.76
5	9.9	16.05	9.9526	16.0454	-0.0526	0.0046	100.53	99.97
6	9.9	16.05	9.7066	16.3187	0.1934	-0.2687	98.05	101.67
7	11	10.7	11.6477	10.0827	-0.6477	0.6173	105.89	94.23
8	16.5	5.35	16.6281	5.2857	-0.1281	0.0643	100.78	98.80
9	16.5	16.05	16.6116	16.239	-0.1116	-0.189	100.68	101.18
10	22	10.7	22.5708	10.4763	-0.5708	0.2237	102.59	97.91
11	24.2	16.05	24.5374	16.3547	-0.3374	-0.3047	101.39	101.90
12	24.75	12.3	24.8265	12.1487	-0.0765	0.1513	100.31	98.77
13	27.2	16.05	28.1392	15.6593	-0.9392	0.3907	103.45	97.57
14	33	0	33.5476	0	-0.5476	0	101.66	100.00
15	35.2	16.05	35.278	15.771	-0.078	0.279	100.22	98.26
16	0	26.75	0	26.1323	0	0.6177	100.00	97.69

17	5.5	3.21	5.6043	3.2279	-0.1043	-0.0179	101.90	100.56
18	5.5	16.05	5.6153	16.036	-0.1153	0.014	102.10	99.91
19	5.5	26.75	5.5975	27.222	-0.0975	-0.472	101.77	101.76
20	9.9	16.05	9.9526	16.0454	-0.0526	0.0046	100.53	99.97
21	9.9	16.05	9.7066	16.3187	0.1934	-0.2687	98.05	101.67

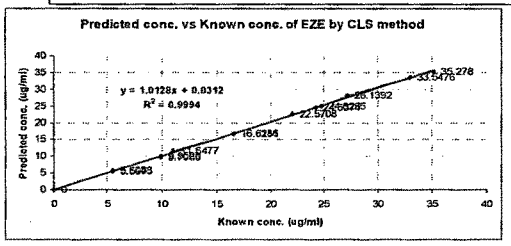


Fig 7.2.1.3.6: Linearity plots of EZE for validation set.

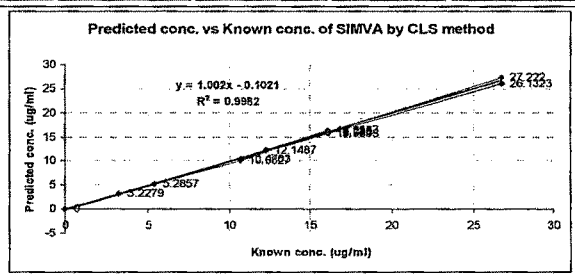


Fig 7.2.1.3.7: Linearity plots of SIMVA for validation set.

It was noticed that both EZE and SIMVA in all samples lay on a straight line and the equations of these lines are shown on the graphs fig. 7.2.1.3.2.2 and 7.2.1.3.2.3.

(VI) Residuals concentration versus actual concentration plot:

The difference between the known and predicted concentration (residuals) were plotted against the actual concentrations for the validation samples.

This tool is used to determine whether the model accounts for the concentration variation in the validation set and it also provides information about how well the method will predict the future samples.

For the validation set of ILS it can be found that the residual values are more close to zero and are more randomly distributed as illustrated in fig. 7.2.1.3.8 and 7.2.1.3.9.

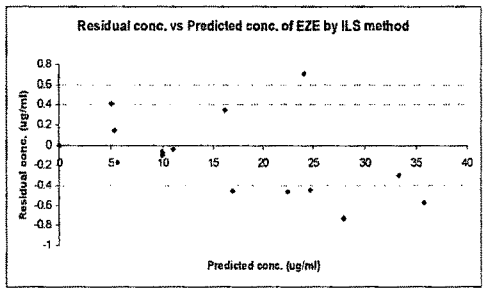


Fig 7.2.1.3.8: Residual vs. predicted concentration plot for EZE and SIMVA.

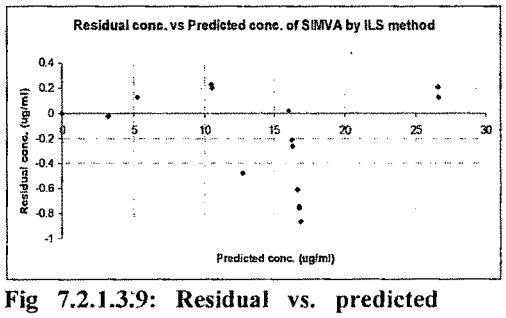


Fig 7.2.1.3.9: Residual vs. predicted concentration plot for EZE and SIMVA.

For the validation of CLS set it can be found that the residual values are more close to zero and are more randomly distributed as illustrated in fig. 7.2.1.3.2.10 and 7.2.1.3.2.11.

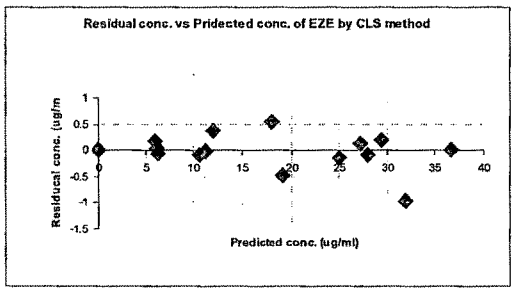


Fig 7.2.1.3.10: Residual vs. predicted concentration plot for EZE.

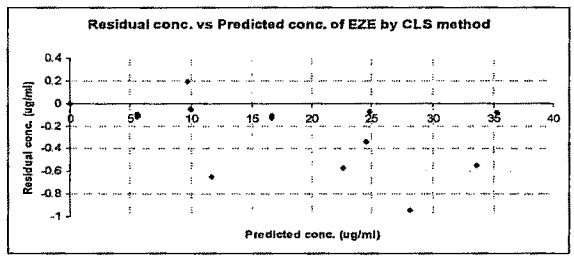


Fig 7.2.1.3.11: Residual vs. predicted concentration plot for SIMVA.

(VII) Root Mean Square Error of Prediction:

RMSEP was calculated using the equation mentioned in Chapter 5.

Table 7.2.1.3.10: RMSEP values for EZE and SIMVA.

Compound	RMSEP	
	ILS	CLS
EZE	0.41736	0.39241
SIMVA	0.44389	0.3267

7.2.1.3.8. Preparation of sample solutions:

Procedure for preparation for the sample is given in Chapter 7.2.1.1. The absorbance of the prepared solution was measured between 231.0 nm and 265.2 nm. The concentrations of EZE and SIMVA in tablets were calculated using the developed model. Results are shown in Table 7.2.1.3.9.

Table 7.2.1.3.9: Estimation of EZE and SIMVA in tablet by CLS method

Formulation	DRUGS	Labeled Claim (mg/tab)	ILS		CLS	
			*Amount found	% Labeled Claim	*Amount found	% Labeled Claim
SIMVAS-EZ	EZE	10	9.9	99.00	9.98	99.80
	SIM	10	10.04	100.40	10.03	100.30
STARSTAT-EZ	EZE	20	10.08	100.40	20.38	101.90
	SIM	10	10.11	101.10	10.33	103.30

*** Mean value of five determinations.**

7.2.1.3.9. Summary of Validation parameters:

The summary of validation parameters were reported in Table 7.2.1.3.10

Table 7.2.1.3.10: Summary of Validation parameters by ILS and CLS methods.

Sr. No	Parameters	ILS		CLS	
		EZE	SIMVA	EZE	SIMVA
1	Calibration design	24			
2	Validation design	15			
3	Spectral region	237 -257			
4	Linearity range (µg/ml)	1 – 40 µg/ml			
5	Regression equation	Y=1.0136x -0.1499	Y=1.006x +0.1197	Y=1.0128x +0.0312	Y=1.002x -0.1021
6	Correlation coefficient (r^2)	0.9989	0.9973	0.9994	0.9982
7	Intercept	-0.1499	0.1197	0.0312	0.1021
8	Slope	1.0163	1.006	1.0128	1.002
9	RMSEP	0.417	0.443	0.392	0.326
10	Assay	99.00	100.40	99.80	100.30
11	Precision				
12	% Recovery	99.61-103.54	99.31-102.00	96.58-103.69	99.64-103.58
12	Limit of detection (µg/ml)	0.069	0.173	0.0329	0.281
13	Limit of quantification(µg/ml)	0.429	0.946	0.238	0.772

7.2.1.10. CONCLUSION

The proposed ILS and CLS methods were found to be precise and accurate. The mathematical calculations for the proposed ILS and CLS methods could be effectively performed using MATLAB and Excel. The simplicity of the method can be explained on the basis of direct out put of the data in terms of unknown concentration on providing the concentration matrix and absorbance matrix of the calibration set to the software. The method does not require any time consuming separation or sample preparation step as used in HPLC.

The ILS method can be strongly applied to a routine analysis, quality control of binary mixtures and commercial products containing these two drugs.

7. 2. 2

Ezetimibe and Pravastatin

7.2.2. Development of Analytical methods for simultaneous estimation of Ezetimibe and Pravastatin:

The combined regimen (**EZE** 10mg plus **PRAVA** 10, 20, or 40mg) also produced significant LDL-C reductions compared with each corresponding and higher dose of **PRAVA** alone[1]. In spite of this, no binary combination containing both EZE and PRAVA is not available in Indian market. The study has been done after mixing the commercially available tablet formulation of EZE and PRAVA.

UV Spectrophotometric Methods:

In order to develop UV Spectrophotometric method for simultaneous estimation of EZE and PRAVA, light absorption study was done. Individual spectra of methanolic solutions of both the drugs were superimposed to obtain suitable working wavelengths (fig. 7.2.2.1.1) that could be used for simultaneous estimation methods. Simultaneous equation method was not found to be applicable in this case as due to extensive overlap of the two spectra. Application of the Q analysis method was tried for the quantitative analysis using λmax of one drug and isosbestic point of both the drugs taken in 1:1 ratio. But a consistent isosbestic point with the change in concentration was not obtained. As a result the determination of these two compounds was not possible by Q-analysis for reliable direct absorbance measurement. The 1st and 2nd order derivative spectroscopies were tried in which 1st order derivative zero crossing method was possible as per fig. 7.2.2.1.2. The individual zero order difference spectra of both the drugs were also superimposed but best result were obtained in difference derivative zero crossing method as seen in fig. 7.2.2.1.5 and 7.2.2.1.6.

Chromatographic methods:

Two chromatographic methods were developed for the simultaneous estimation of EZE and PRAVA. HPLC method was developed which could estimate EZE and PRAVA in pharmaceutical dosage form and in presence of degradation products. HPTLC method was developed for the estimation of EZE and PRAVA in pharmaceutical dosage form.

Chemometrix methods

Chemometrix approach was tried with the spectrophotometric methods. Here two chemometrix methods ILS and CLS were developed.

Apparatus, instruments and reagents:

Apparatus, instruments and reagents used for the development of spectrophotometric and chromatographic methods are mentioned in Chapter 6.

7.2.2.1. Simultaneous spectrophotometric estimation of EZE and PRAVA by first derivative zero crossing (FDZC) and difference derivative zero crossing (DDZC) methods:

Experimental Work:

7.2.2.1.1. Preparation of binary stock solution:

Standard EZE and PRAVA (10 mg) were weighed accurately and transferred to 100 ml volumetric flask. It was dissolved in 50 ml methanol and diluted up to mark with methanol to obtain final concentration of 100 µg/ml each of EZE and PRAVA. This solution was used as working standard solution spectrophotometric methods.

7.2.2.1.2. Selection of analytical wavelength:

7.2.2.1.2.1. First derivative zero crossing method:

The selection of zero crossing point, where absorbance of one component is zero while other having significant absorbance. The zero crossing point should be valid for the entire concentration range in which analysis in carried out. From the overlain 1st derivative spectra of EZE and PRAVA (fig. 7.2.2.1.2), 237.2 nm was selected as zero crossing point for PRAVA as at this wavelength EZE has negligible absorbance. Whereas, 218.2 nm was selected as zero crossing point for EZE as at this wavelength, PRAVA shows negligible absorbance. The data of this absorbance is shown in Table 7.2.2.1.1.

7.2.2.1.2.2. Difference derivative zero crossing method:

The difference derivative spectra was taken using methanolic solutions in the reference compartment and 0.1 M NaOH solutions in the sample compartment of standard PRAVA and EZE (1 – 40 µg/ml) were overlain. (difference spectra were taken as per procedure given in Chapter 7.1.1.2 and 7.1.2.2.) The 250.40 nm and 243.60 nm wavelengths were selected as ZCP for PRAVA and EZE as the drugs have absorbance at these wavelengths (fig. 7.2.2.1.6). So 250.40 nm and 243.60nm were selected for estimation for EZE and PRAVA, respectively.

Effect of derivative intervals on derivative:

$\Delta\lambda$, the width of the boundaries over which the derivative is calculated was tested for $\Delta\lambda$ = 2 nm, 4 nm and 8 nm. The values of $\Delta\lambda$ = 2 nm was found optimal in connection with both slit width and wavelength interval.

7.2.2.1.3. Calibration curve for standard EZE and PRAVA

From the stock solution (100 µg/ml) aliquots of 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 ml were transferred in duplicate to a series of 10ml volumetric flasks. The volume was adjusted to a mark with methanol and 0.1 M NaOH to get 1, 5, 10, 15, 20, 25, 30, 35 and 40 µg/ml solutions of EZE and PRAVA in methanol and NaOH. The absorbance was measured at 237.2 nm and 218.20 nm of first derivative spectrum of methanolic solutions. The absorbance was measured at 250.40 and 243.60 nm of first derivative of difference spectra. Linearity range of EZE and PRAVA was found with correlation co-efficient.

7.2.2.1.4. Validation parameters:

Method was validated by procedure given in chapter 7.2.1 for FDZC and 7.1.1 for DDZC.

7.2.2.1.5. Results and Discussion:

7.2.2.1.5.1 Validation Parameters

(I) Linearity

The derivative spectrophotometry method showed good linearity for EZE and PRAVA in the range of 5- 40 µg/ml. Calibration data was given in Table 7.2.2.1.5.

Table 7.2.2.1.1: Calibration data of EZE and PRAVA by FDZC and DDZC methods

Sr. No.	Conc. (µg/ml)	FDZC		DDZC	
		Absorbance (Mean ± % C.V. (N=5))			
		EZE at 237.2 nm	PRAVA at 218.2 nm	EZE at 250.4 nm	PRAVA at 243.6 nm
1	5	-0.0007 ± 0.29	0.0067 ± 0.92	-0.001 ± 0.83	-0.01 ± 0.48
2	10	-0.006 ± 0.84	0.0164 ± 0.33	-0.0018 ± 0.29	-0.0162 ± 0.33
3	15	-0.013 ± 0.19	0.027 ± 0.64	-0.0027 ± 0.63	-0.0222 ± 0.84
4	20	-0.0204 ± 0.29	0.036 ± 0.83	-0.0035 ± 0.72	-0.028 ± 0.28
5	25	-0.0273 ± 0.28	0.0467 ± 0.49	-0.0043 ± 0.28	-0.034 ± 0.62
6	30	-0.0347 ± 0.84	0.057 ± 0.28	-0.0051 ± 0.54	-0.039 ± 0.65
7	35	-0.0416 ± 0.20	0.0655 ± 0.82	-0.006 ± 0.33	-0.045 ± 0.81
8	40	-0.0489 ± 0.83	0.074 ± 0.28	-0.0069 ± 0.11	-0.051 ± 0.15

* Mean of five determinations.

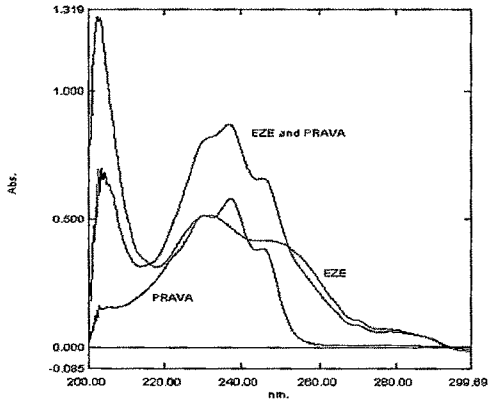


Figure: 7.2.2.1.1: Overlain zero order UV spectra of EZE and PRAVA

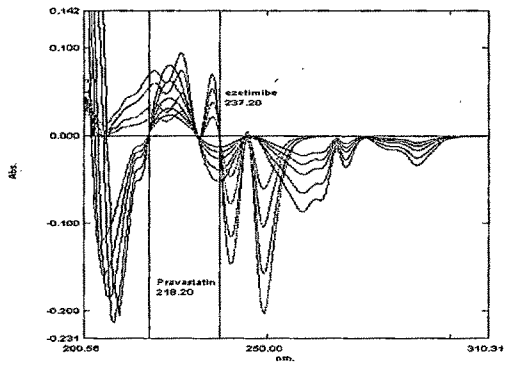


Figure: 7.2.2.1.2: Overlain 1st order derivative UV spectra of EZE and PRAVA

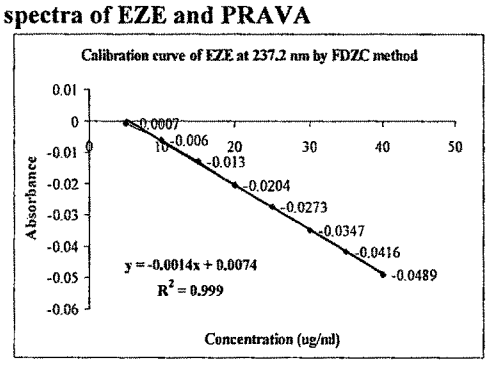


Figure 7.2.2.1.3: Calibration curve of EZE at 237.2 nm by FDZC method

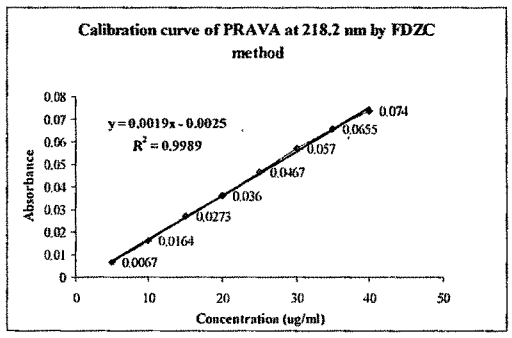


Figure 7.2.2.1.4: Calibration curve of PRAVA at 218.2 nm by FDZC method

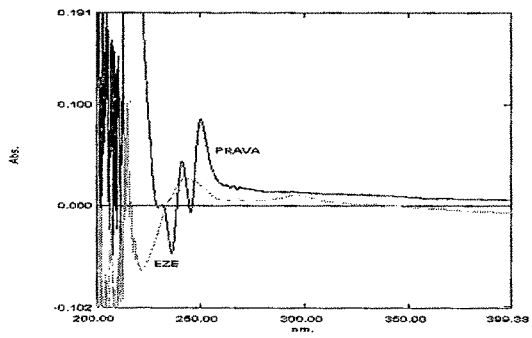


Figure: 7.2.2.1.5: Overlain zero order difference spectra of EZE and PRAVA

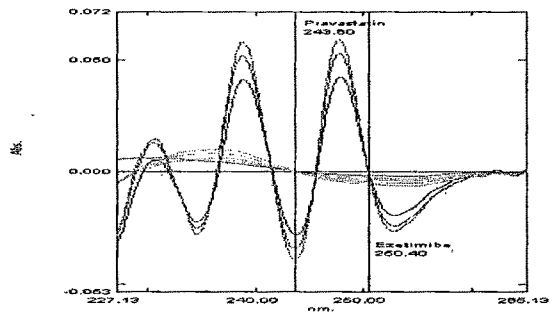


Figure: 7.2.2.1.6: Overlain difference 1st derivative spectra of EZE and PRAVA

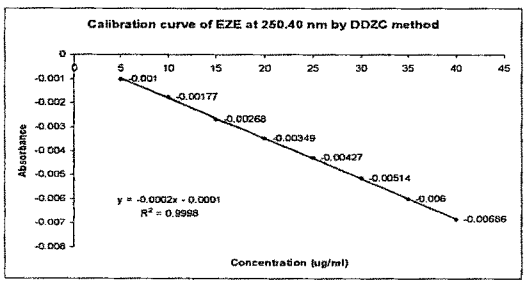


Figure 7.2.2.1.7: Calibration curve of EZE at 250.4 nm by DDZC method

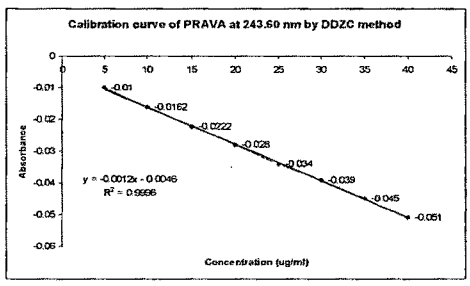


Figure 7.2.2.1.8: Calibration curve of PRAVA at 243.6 nm by DDZC method

(II) Precision

Repeatability

Intraday and inter day reported in Table 7.2.2.1.3 and 7.2.2.1.4.

Table 7.2.2.1.3: Intraday precision data of EZE and PRAVA by spectrophotometeric method

Concentration (µg/ml)		FDZC method (Mean (n=5) ± %C.V.)		DDZC method (Mean (n=5) ± %C.V.)	
EZE	PRAVA	EZE at 237.2 nm	PRAVA at 218.2 nm	EZE at 250.4 nm	PRAVA at 243.6 nm
10	10	-0.006 ± 0.38	0.0163 ± 0.77	-0.0018 ± 0.73	-0.0161 ± 0.83
20	20	-0.0206 ± 0.29	0.0364 ± 0.48	-0.0035 ± 0.62	-0.0283 ± 0.57
30	30	-0.0347 ± 0.86	0.0570 ± 0.72	-0.0053 ± 0.44	-0.0397 ± 0.22
Table 7.2.2.1.4: Interday precision data of EZE and PRAVA by spectrophotometeric method					
10	10	-0.006 ± 0.93	0.0165 ± 0.84	-0.0018 ± 0.66	-0.0163 ± 0.20
20	20	-0.0209 ± 0.29	0.0366 ± 0.66	-0.0036 ± 0.39	-0.0289 ± 0.63
30	30	-0.0348 ± 0.73	0.0573 ± 0.31	-0.0053 ± 0.44	-0.0392 ± 0.19

***Mean of five determinations**

Reproducibility:

The reproducibility of the method was determined by using different instruments Shimadzu UV 1700 and Shimadzu UV 1601. The % CV of the responses confirms the reproducibility of the method for determination of EZE and PRAVA. The data is reported in Table 7.2.2.1.5.

Table 7.2.2.1.5: Reproducibility data of EZE and PRAVA by spectrophotometeric method

Spectrophotometric methods		Concentrations (µg/ml)					
		10		15		20	
		UV 1700	UV 1601	UV 1700	UV 1601	UV 1700	UV 1601
First derivative zero crossing method(Mean (n=5) ± %C.V.)	EZE at 265.2 nm	-0.006 ± 0.38	-0.006 ± 0.28	-0.012 ± 0.72	-0.012 ±0.47	-0.202 ± 0.48	-0.202 ±0.82
	PRAVA at 245.4 nm	0.0164 ±0.47	0.0164 ±0.82	0.0277 ±0.19	0.0276 ±0.93	0.0369 ±0.28	0.0368 ±0.18
Difference derivative zero crossing method (Mean (n=5) ± %C.V.)	EZE (250.40 nm)	-0.0018 ± 0.57	-0.0018 ± 0.74	-0.0026 ± 0.28	-0.0027 ± 0.29	-0.0036 ± 0.48	-0.0037 ± 0.53
	PRAVA (243.60 nm)	-0.0161 ± 0.82	-0.0162 ± 0.64	-0.0223 ± 0.58	-0.0221 ± 0.22	-0.0288 ± 0.33	-0.0289 ± 0.93

***Mean of three determinations**

(III) Accuracy

Accuracy of the method was determined by performing recovery study on laboratory prepared synthetic mixture by standard addition method. The results of % mean recovery values reported Table 7.2.2.1.6.

Table 7.2.2.1.6: Accuracy data of EZE and PRAVA spectrophotometeric method

Spectrophotometic methods		EZE	PRAVA	EZE	PRAVA	EZE	PRAVA
Initial conc.(µg/ml) (A)		10	10	10	10	10	10
Quantity of std. Added (µg/ml) (B)		5	5	10	10	15	15
Total Amount (A + B)		15	15	20	20	25	25
First derivative zero crossing method (Mean (n=5) ± %C.V.)	Total quantity Found Mean ± S.D.	14.82 ± 0.028	15.02 ± 0.083	20.03 ± 0.084	19.62 ± 0.043	24.73 ± 0.033	25.22 ± 0.063
	% Recovery± S.D	98.8 ± 0.38	100.13± 0.83	100.15 ± 0.83	98.11 ± 0.48	98.92 ± 0.83	100.88 ± 0.72
Difference derivative zero crossing method (Mean (n=5) ± %C.V.)	Total quantity Found Mean ± S.D.	14.88	15.03	19.83	19.87	25.22	25.03
	% Recovery± S.D	99.2 ± 0.0245	100.2 ± 0.0197	99.15 ± 0.058	99.35 ± 0.124	100.88 ± 0.0987	100.12 ± 0.037

***Mean of five determinations**

(IV) Limit of detection

The minimum detectable concentration of EZE and PRAVA were found to be 0.213 µg/ml and 0.658 µg/ml, 0.164µg/ml and 0.214 µg/ml respectively for FDZC and DDZC.

(V) Limit of quantification

The lowest quantifiable concentration of EZE and PRAVA were determined by practical observation and found to be 0.710 µg/ml and 0.893 µg/ml, 0.546 µg/ml and 0.431 µg/ml respectively for FDZC and DDZC.

7.2.2.1.4.2. Applicability of the method for the analysis of commercial tablet formulation:

Ten tablets of **EZEDOC** and ten tablets of **PRAVATOR** were taken and average weigh was found out. Then powder was made in a mortar and pestle. The Tablet powder equivalent to one tablet was transferred from powder of both tablets into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 15 min and

further diluted to 100 ml with methanol. The resulting solution was sonicated for 10 min and supernatant was filtered through Whatman filter paper no. 41. A synthetic mixture was prepared having PRAVA and EZE in ratio of 1:1. Prepared solution (100 µg/ml) was used as standard test solution. From that, 1 ml was diluted to 10 ml with methanol. Zero order spectrum of the solution was obtained and converted to derivative cure. The absorbance was measured at 237.2 nm and 218.2 nm in order to determine the concentrations of EZE and PRAVA from their respective calibrations curves and regression equations. Similarly 1 ml of the solution was transferred into a 10 ml volumetric flask and diluted with 0.1 N NaOH. The Methanol and NaOH solutions were used for difference derivative method. The concentrations of EZE and PRAVA were calculated using the corresponding regression equation. Results obtained are reported in Table 7.2.2.1.7.

Table 7.2.2.1.7: Estimation of EZE and PRAVA in tablet by spectrophotometeric method

Formu-lation	Drugs	Labeled Claim (mg/tab)	FDZC		DDZC	
			*Amount found	% Assay	*Amount found	% Assay
EZEDOC + PRAVATOR	EZE	10	10.92	109.21	10.22	102.21
	PRAVA	10	9.98	99.98	9.96	99.68
LAB. MIX.	EZE	10	10.09	100.91	10.01	100.11
	PRAVA	10	10.24	102.41	10.04	100.41

*** Mean value of five determinations.**

7.2.2.1.5. Summary of Validation parameters:

A summary of the results of validation parameters is reported in Table 7.2.2.1.8.

Table 7.2.2.1.8: Summary of Validation parameters of spectrophotometeric method

Sr. No	Parameters	FDZC		DDZC	
		EZE	PRAVA	EZE	PRAVA
1	Analytical wavelengths (nm)	237.20	218.20	250.40	243.60
2	Linearity range (µg/ml)	5 to 40 µg/ml			
3	Regression equation	Abs. = -0.0014 × Conc. + 0.0074	Abs. = 0.0019 × Conc. - 0.0025	**Abs. = -0.0002 × Conc. - 0.0001**	**Abs. = -0.0012 × Conc. - 0.0046**
4	Correlation coefficient (r^2)	0.999	0.9989	**0.9996**	**0.9996**
5	Intercept	0.0074	-0.0025	-0.0001	-0.0046

6	Slope	-0.0014	0.0019	-0.0002	-0.0012
7	% Assay	100.91-109.21%	99.89 – 102.41 %	100.11-102.21%	99.68–100.41%
8	Accuracy & Precision				
	Intra day % CV (n = 5)	0.29 –0.86	0.48 – 0.77	0.44 – 0.73 %	0.22 – 0.83 %
	Inter day % CV (n = 5)	0.29 – 0.93	0.31 – 0.84	0.39 – 0.66 %	0.19 – 0.63 %
	Repeatability of measurements % CV	0.28 – 0.82	0.28 –0.82	0.28 – 0.74	0.22 – 0.93
	% Recovery	98.80 – 100.15	98.11 – 100.88	98.80 – 100.15	98.11 – 100.88
9	Limit of detection (µg/ml)	0.213	0.656	0.164	0.214 1
10	Limit of quantification (µg/ml)	0.710	0.893	0.5468	0.4314

7.2.2.1.6. CONCLUSION

1st order zero crossing spectrophotometric method is a suitable technique for the reliable analysis of simultaneous estimation of EZE and PRAVA. The most striking features of DDZC method are its simplicity, sensitivity and specificity, which render it suitable for the routine analysis of EZE and PRAVA in quality control laboratories from their combination dosage forms. High % recoveries greater than 99 % shows that both FDZC and DDZC methods are free from the interference of excipients used in the formulation (Table 7.2.2.1.8).

7.2.2.2. Infra Red spectroscopic method.

7.2.2.2.1. Preparation of stock sample

For **quantitative IR** spectroscopic study both standard drugs were weighed accurately (about 10 mg) and made up to 100 mg on same butter paper to obtain final concentration of 100 µg/mg of each drug. This sample was triturated in mortar and pestle and sample was used as working standard sample for FTIR spectroscopic method.

7.2.2.2.2. Selection of analytical wavenumber

Individual IR spectrum of both the drugs and their mixture were overlain (fig. 7.2.2.2. 1) to select the zero absorbance point. It was observed that 2354.92 cm^{-1} is the zero absorbance point for PRAVA so EZE was estimated at this frequency where as PRAVA was estimated at 1566.38 cm^{-1} at which EZE showed zero absorbance.

7.2.2.2.3. Calibration curve

Appropriate fractions were transferred from standard (100 µg/mg) to butter paper and weight adjusted 10 mg with KBr to get minimum transmission. At 2354.92 cm^{-1} and 1566.38 cm^{-1} peak height was measured for EZE and PRAVA, respectively. Calibration curves were drawn using the multiple linear regression (MLR) method and the drug amount in sample was computed from regression equation.

7.2.2.2.4. Validation parameters:

Method was validated as per procedure given in Chapter 7.1.2.2.4.

7.2.2.2.5. Results and Discussion:

7.2.2.2.5.1 Validation Parameters

(I) Linearity

The IR spectrophotometry method showed good linearity for EZE in the range of 12.9 to 136 µg/mg at 2354.92 cm^{-1} (zero crossing of PRAVA) with co-relation co-efficient of 0.9995 as seen in fig. 7.2.2.2. 4. For PRAVA the line of best fit was obtained at 1566.38 cm^{-1} (zero crossing of EZE) with correlation coefficients of 0.9998 as seen in fig. 7.2.2.2. 5. Calibration data is given in Table 7.2.2.2.1.

Table 7.2.2.2.1: Calibration data of EZE and PRAVA by IR Spectroscopy

Sr.No.	Concentration (µg/mg)		Peak height * ± S.D. (N=5)		% C.V.	
	EZE	PRAVA	EZE at 2354.92 1/cm	PRAVA 1566.38 1/cm	EZE	PRAVA
1	12.9	13	0.0029±0.00046	0.029±0.00049	0.73	0.38
2	34.3	30.9	0.021±0.00028	0.376±0.00028	0.29	0.28
3	43.2	41.9	0.0273±0.00028	0.609±0.00082	0.57	0.73
4	136	54.8	0.096±0.00022	0.865±0.00038	0.47	0.54
5	-	71.8	-	1.22±0.00062	-	0.19

*Mean value of five determinations.

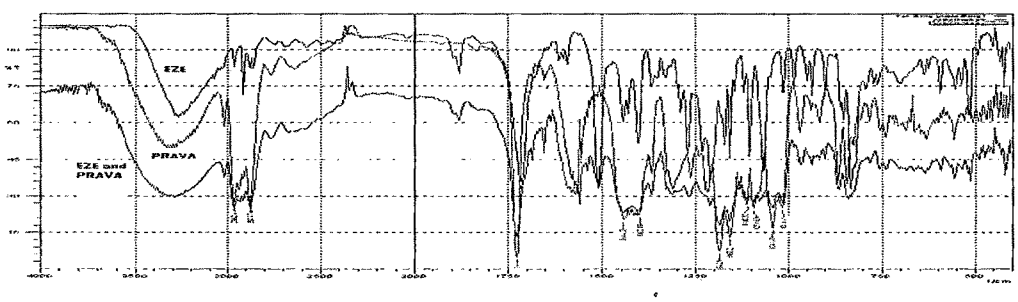


Figure: 7.2.2.2.1: Overlain IR spectra of EZE and PRAVA

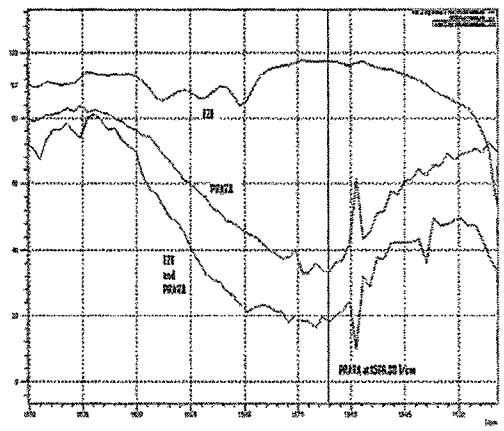


Figure: 7.2.2.2.2: Overlain IR spectra of PRAVA and EZE at 1566.38 cm^{-1}

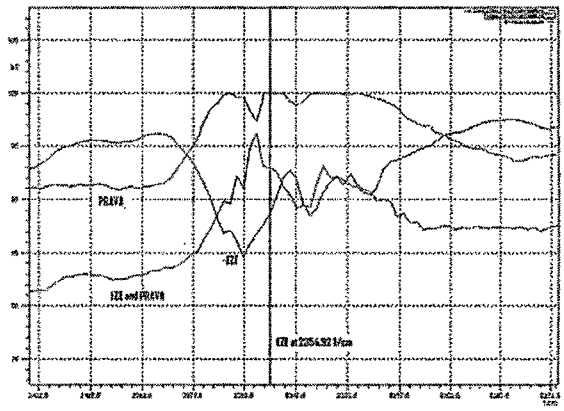


Figure: 7.2.2.2.3: Overlain IR spectra of PRAVA and EZE at 2354.92 cm^{-1}

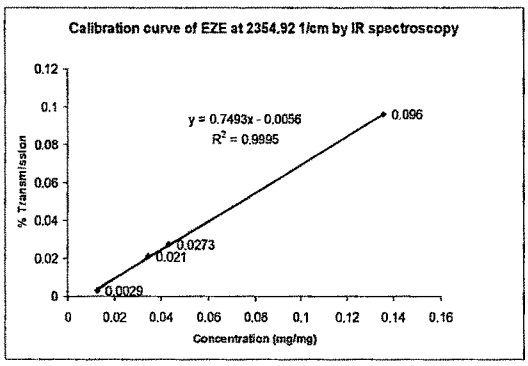


Figure 7.2.2.2.4: Calibration curve of EZE at 2354.92cm^{-1} by IR method

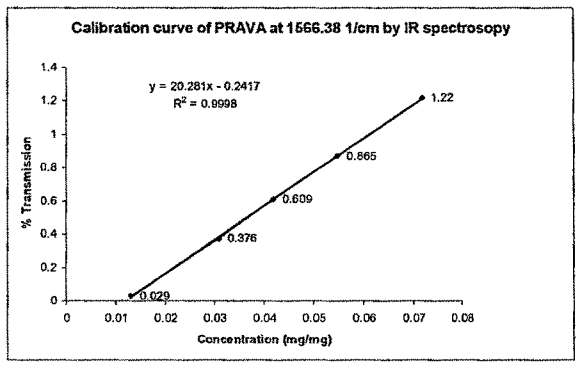


Figure 7.2.2.2.5: Calibration curve of PRAVA at 1566.38 cm^{-1} by IR method

(II) Precision

Repeatability

The method was found to be precise on intra day and inter day basis as the average % CV values were reported in Table 7.2.2.2.2

Table 7.2.2.2.2: Intraday and Interday precision data of EZE and PRAVA by IR Spectroscopy

Concentration (µg/mg)		Peak height* ± % C.V*. (n=5)			
		Intraday		Interday	
EZE	PRAVA	EZE at 2354.92 cm^{-1}	PRAVA at 1566.38 cm^{-1}	EZE at 2354.92 cm^{-1}	PRAVA at 1566.38 cm^{-1}
15	15	0.00894 ± 0.72	0.17072 ± 0.52	0.00883 ± 0.38	0.17392 ± 0.72
30	30	0.01953 ± 0.51	0.37834 ± 0.88	0.0199 ± 0.59	0.38219 ± 0.19
45	45	0.02875 ± 0.47	0.74636 ± 0.45	0.02864 ± 0.29	0.7526 ± 0.38

* Mean value of five determinations.

Reproducibility:

The % CV of the responses for determination of EZE and PRAVA revealing the reproducibility of the method is reported in Table 7.2.2.2.3.

Table 7.2.2.2.3: Reproducibility data of EZE and PRAVA by IR Spectroscopy

Concentration (µg/mg)		Peak height* ± %CV*			
EZE	PRAVA	EZE at 2354.92 cm^{-1} ± %CV		PRAVA at 1566.38 cm^{-1} ± %CV	
		Sample compartment I	Sample compartment II	Sample compartment I	Sample compartment II
15	15	0.00824 ± 0.41	0.00877 ± 0.29	0.1746 ± 0.82	0.7113 ± 0.61
30	30	0.01971 ± 0.24	0.01908 ± 0.02	0.38211 ± 0.62	0.3863 ± 0.92
45	45	0.02852 ± 0.22	0.02889 ± 0.82	0.7558 ± 0.91	0.75281 ± 0.19

***Mean value of five determinations**

(III) Accuracy

The method showed % mean recovery for EZE in the range of 99.53 – 102.63 % and for PRAVA it was 99.20 – 100.60 % in synthetic mixture (Table 7.2.2.2.4).

Table 7.2.2.2.4: Accuracy data of EZE and PRAVA by IR Spectroscopy

Initial conc. (µg/mg) (A)		Quantity of std. Added (µg/mg) (B)		Total Amount (A + B)		Total quantity Found Mean ± S.D.		% Recovery± S.D*	
EZE	PRAVA	EZE	PRAVA	EZE	PRAVA	EZE at 2354.92 cm^{-1}	PRAVA at 1566.38 cm^{-1}	EZE	PRAVA
10	10	5	5	15	15	14.93 ± 0.038	14.88 ± 0.061	99.53 ± 0.021	99.20 ± 0.051
15	15	15	15	30	30	29.89 ± 0.072	30.18 ± 0.038	99.63 ± 0.052	100.60 ± 0.72
10	10	25	25	35	35	35.92 ± 0.071	34.98 ± 0.072	102.63 ± 0.081	99.94 ± 0.051

***Mean value of three determinations**

(IV) Limit of detection

The minimum detectable concentration of EZE and PRAVA were found to be 1.5449 µg/mg and 0.4405 µg/mg, respectively.

(V) Limit of quantification

The lowest quantifiable concentration of EZE and PRAVA were found to be 5.14992 µg/mg and 1.4683 µg/mg, respectively by practical observation.

7.2.2.2.3.2. Analysis of commercial tablet formulation:

The procedure for preparation of test sample is given in Chapter 7.2.2.1.4.2. A synthetic mixture was prepared having PRAVA and EZE in ratio of 1:1. Filtrate was evaporated and from the residue 1 mg was weighed and made up to 100 mg with KBr on butter paper and triturated in mortar pestle and The DRIFT spectrum was scanned between 4000 and 400 cm^{-1}, by averaging 16 scans for each spectrum with a resolution of 4 cm^{-1} (data point resolution per interval 1 cm^{-1}). 2354.92 cm^{-1} and 1566.38 cm^{-1} were selected for estimation for EZE and PRAVA, at single wavelength, respectively. Concentration of sample was found from calibration curve of EZE and PRAVA.

Table 7.2.2.2.5: Estimation of EZE and PRAVA in Tablet by IR spectrosopy.

Formulation	DRUGS	Labeled Claim (mg/tab)	QIR	
			*Amount found ± SD	% Assay ± SD
EZEDOC & PRAVATOR	EZE	10	10.03 ± 0.032	100.32 ± 0.062
	PRAVA	10	10.09 ± 0.072	100.91 ± 0.019
LAB. MIX.	EZE	10	10.12 ± 0.061	101.23 ± 0.051
	PRAVA	10	10.39 ± 0.071	103.92 ± 0.041

*** Mean value of five determinations.**

7.2.2.2.4. Summary of Validation parameters:

The summary of validation parameters is given in Table 7.2.2.2.6.

Table 7.2.2.2.6: Summary of Validation parameters of IR spectroscopic method

Sr. No	Parameters	Results	
		EZE	PRAVA
1	Wave number (cm^{-1})	2354.92	1566.38
2	Linearity range (µg/mg)	12.9-136	13-71.8
3	Regression equation	Y=0.7493x -0.0056	Y=20.281x -0.2417
4	Correlation coefficient (r^2)	0.9995	0.9998
5	Intercept	-0.0056	-0.2417
6	Slope	0.7493	20.281
7	Assay	100.32	100.91

8	Precision		
	Intra day % CV (n = 5)	0.47-0.72	0.45-0.88
	Inter day % CV (n = 5)	0.29-0.59	0.19-0.72
	Repeatability of measurements % CV	< 1%	< 1 %
	% Recovery	99.53 – 102.63	99.20-100.60
9	Limit of detection (µg/mg)	1.5449	0.4405
10	Limit of quantification(µg/mg)	5.149	1.468

7.2.2.2.5. CONCLUSION

The proposed method is simple, precise and accurate and can be used as a method for quality control of pharmaceuticals. This technique extends the use of a standard IR spectrophotometer typically used for identification purposes, to the reliable quantification of EZE and PRAVA. The present method opens the possibility of applying IR spectroscopy to quantify other active ingredients.

7.2.2.3. Stability Indicating Reverse Phase High Performance Liquid Chromatography (HPLC) with UV detection.

Stability indicating HPLC method was developed for the estimation of EZE and PRAVA in pharmaceutical dosage form and also in presence of its degraded products.

7.2.2.3.1. Optimization method

Determination of solvent for sample preparation:

Different solvents were tried depending upon the solubility of the drugs as per Chapter 6. Both the drugs were soluble in methanol and ACN, but with methanol as a solvent, large tailing effects were observed so ACN was selected as a solvent to prepare drug solutions.

Determination of λmax

Detection wavelength was selected by scanning standard solutions of the drugs over 200 nm to 400 nm wavelengths. The 248 nm was selected as detection wavelength .

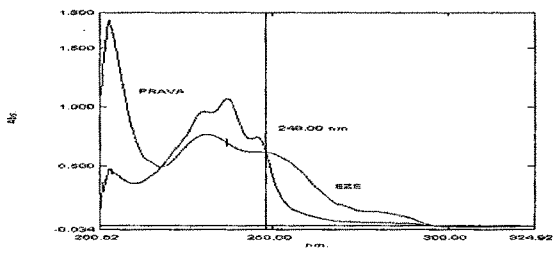


Figure 7.2.2.3.1: overlain UV spectrum of EZE and PRAVA in acetonitrile

Optimization of mobile phase:

Various combinations of Mobile phase were tried at flow rate 1 ml/min and column C18 Phenomenex. The observations are shown in Table 7.2.2.3.1.

Table 7.2.2.3.1: Determination of mobile phase

Mobile phase combination	Ratio (V/V)	Retention time (min)		Peak shape	
		EZE	PRAVA	EZE	PRAVA
Acetonitrile: 0.1 % Formic acid	45:55	21.5	4.6	Slight broad	Still Merge with blank
Acetonitrile: 0.1 % Formic acid: Methanol (1.2 ml/min)	**40:50:10**	**15.5**	**4.3**	**Sharp**	**Sharp**
Acetonitrile: 0.1 % Formic acid: Methanol (1 ml/min)	40:50:10	18.825	5.292	Sharp	Sharp
Acetonitrile: 0.2 % Formic acid	55:45	11.182	3.02	Peak become slight Sharp	Merge with blank
Acetonitrile: 0.5 % Formic acid	50:50	12.017	3.89	Broad peak	Merge with blank
Acetonitrile: 0.5 % Formic acid	60:40	6.1	3.1	Broad peak	Merge with blank
Acetonitrile: 0.5 % Formic acid: Methanol	50:40:10	6.2	3.1	Broad peak	Merge with blank
Acetonitrile: 0.5 % Formic acid: Methanol	30:50:20	19.592	3.375	Broad peak	Merge with blank

It is evident from the data that mobile phase combination of acetonitrile: 0.1 % Formic acid: Methanol in proportion of 40:50:10 v/v/v with 1.2 ml/min flow rate was most suitable for the development of RP-HPLC method.

Column

Two type of C18 column were tried.

Table 7.2.2.3.2: Selection of column

Column (C18, 250 X 4.60 mm)	Retention time (min)		Peak Shape	
	EZE	PRAVA	EZE	PRAVA
Hypes	15.11	4.23	Sharp	Sharp
Phenomenex	**15.5**	**4.3**	**Sharp**	**Sharp**

Both the columns were suitable for development of HPLC analytical method, but C18 Phenomenex 240 X 4.6 mm was selected

After optimizing all the parameters, following chromatographic condition were used for the quantitative determination of EZE and PRAVA in presence of its degradation products, formulation excipients and impurities.

Chromatographic condition:

- **Column:** C_{18} (size-250 x 4.60 mm, I.D-5 µm) (Phenomenex)
- **Mobile Phase:** Acetonitrile: 0.1% Formic acid: Methanol (40:50:10 v/v/v)
- **Detection:** UV detection at 248 nm
- **Flow rate:** 1.2 ml/minute
- **Application volume:** 20 µl

Preparation of Mobile Phase:

Mobile phase was prepared by mixing 400 ml of Acetonitrile with 500 ml of 0.1 % formic acid and 100 ml of methanol (40:50:10, V/V/V). The mobile phase was filtered through nylon (0.45 µm, 41 mm) membrane filter and degassed in ultrasonic bath prior to use for 30 min.

7.2.2.3.2. Preparation of binary stock solution for EZE and PRAVA

For chromatographic study standard EZE and PRAVA (100 mg) were weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml ACN and diluted up to mark with ACN to obtain final concentration of 1000 µg/ml.

7.2.2.3.3. Calibration curve of EZE and PRAVA

From the stock solution (1000 µg/ml), aliquots of 0.1, 0.5, 1, 1.5, 2, 2.5, and 3 ml were transferred to a series of 10 ml volumetric flasks. The volume was adjusted up to the mark with ACN to get final concentrations of 10, 50, 100, 150, 200, 250, and 300 µg/ml of solution. 20 µl of all these solutions were injected separately into HPLC column and the peak area of each solution was measured at selected wavelength. The calibration curve was constructed by plotting peak area vs. concentration (µg/ml) over the concentration range and regression equation was calculated.

7.2.2.3.4. Validation parameters:

Procedure for validation of analytical method was given in Chapter 7.1.1.6.3.

7.2.2.3.5. Results and Discussion:

7.2.2.3.5.1. Validation Parameters

(I) Linearity

The RP-HPLC method showed good linearity for EZE and PRAVA in the range of 5 - 300 µg/ml. Calibration data is given in Table 7.2.2.3.3.

Table 7.2.2.3.3: Calibration data of EZE by HPLC with UV detection

Sr. No.	Concentration (µg/ml)		EZE			PRAVA		
			Peak Area		RT*	Peak Area		RT*
	EZE	PRAVA	Mean*	%CV		Mean*	%CV	
1	5	5	236318	0.235	15.325	169977	0.65	4.395
2	22	20	1039801	0.24	15.332	679906	0.17	4.398
3	55	50	2306804	0.89	15.245	1481273	0.98	4.356
4	110	100	4608872	0.65	15.398	2123704	0.56	4.298
5	150	150	6284825	0.789	15.426	3185556	0.14	4.344
6	200	200	8379767	0.45	15.265	4247408	0.87	4.397
7	250	250	10474709	0.87	15.326	5309260	0.65	4.356
8	300	300	12569651	0.69	15.258	6371112	0.16	4.389

*Average of five readings

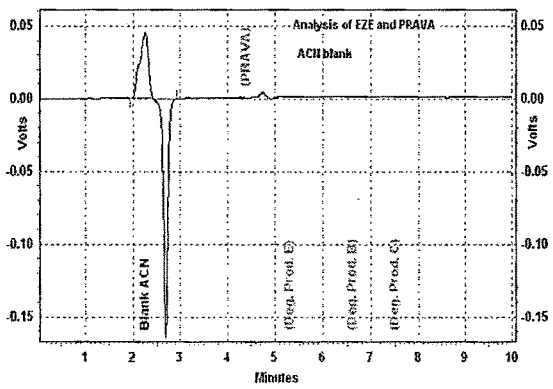


Figure 7.2.2.3.2: Peak of ACN Blank by HPLC with UV detection

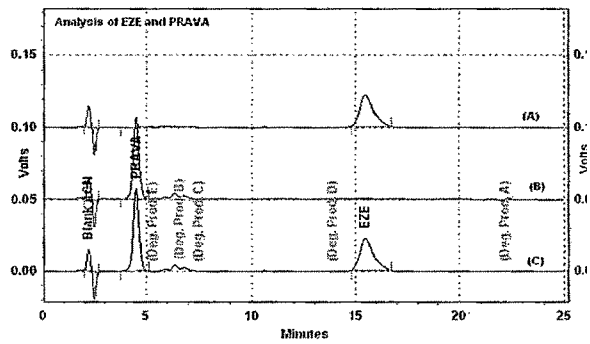


Figure 7.2.2.3.3:(A) Peak of EZE(22 µg/ml) and (B) PRAVA(22 µg/ml) and (C) combination of EZE and PRAVA by HPLC with UV detection

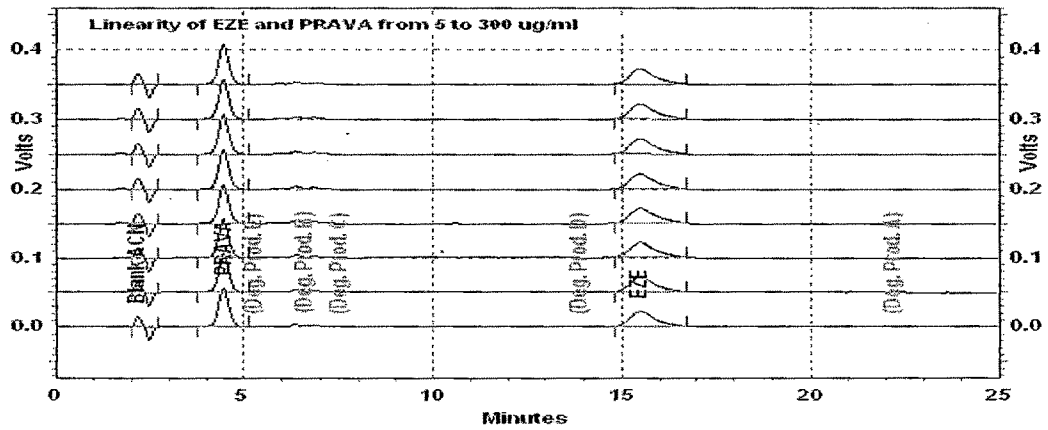


Figure 7.2.2.3.4: Overlain Peaks of EZE and PRAVA by HPLC with UV detection

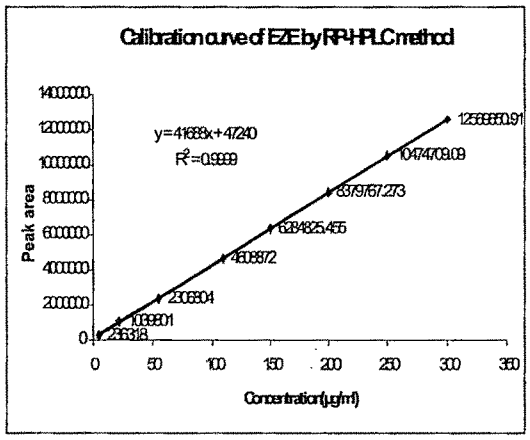


Figure 7.2.2.3.5: Calibration curve of EZE by HPLC with UV detection (Peak Area)
Correlation co-efficient = **0.9999**
Slope = 41688
Intercept = 47240
Regression equation: **Abs. = 41688 × Conc. +47240**

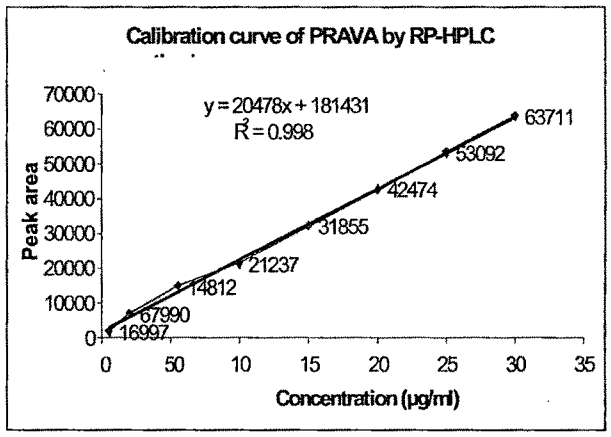


Figure 7.2.2.3.6: Calibration curve of PRAVA by HPLC with UV detection (Peak Area)
Correlation co-efficient = **0.998**
Slope = 20478
Intercept = 181431
Regression equation: **Abs. = 20478 × Conc. 181431**

(II) Precision

Repeatability

The method was found to be precise on intra day and inter day basis as the average % CV value for the determination of EZE and PRAVA were reported in table 7.2.2.3.4.

Table 7.2.2.3.4: Intraday and interday precision data of EZE and PRAVA by RP-HPLC method

Concentration (µg/ml)		Peak area (Mean ± % C.V.) (n=5)			
		Intraday		Interday	
EZE	PRAVA	EZE	PRAVA	EZE	PRAVA
60	60	2513930 ± 0.28	1574222 ± 0.17	2539769 ± 0.45	1536633 ± 0.14
120	120	5027860 ± 0.45	2548445 ± 0.45	5246991 ± 0.61	2559515 ± 0.98
240	240	10055721 ± 0.65	5096890 ± 0.62	10124854 ± 0.87	5131931 ± 0.46

*Mean of five determinations.

Reproducibility:

The % CV of the responses for determination of EZE and PRAVA are mentioned in Table 7.2.2.3.5.

Table 7.2.2.3.5: Reproducibility data of EZE and PRAVA by RP-HPLC method

Concentration (µg/ml)		Peak area ± % CV			
EZE	PRAVA	EZE		PRAVA	
		Hypersil	Phenomenex	Hypersil	Phenomenex
60	60	2524405 ± 0.23	2445216 ± 0.65	1579532 ± 0.85	1539394 ± 0.56
120	120	5041268 ± 0.87	5109563 ± 0.78	2555241 ± 0.14	2589857 ± 0.45
240	240	10109351 ± 0.54	10012984 ± 0.18	5124073 ± 0.47	5075228 ± 0.35

(III) Accuracy

The method showed % mean recovery for EZE in the range of 99.57 – 105.29% from synthetic mixture. For PRAVA % recovery lies between 100.67 – 104.16% for synthetic mixture (Table 7.2.2.3.6).

Table 7.2.2.3.6: Accuracy data of EZE and PRAVA by RP-HPLC method

Initial conc. (µg/ml) (A)		Quantity of std. Added (µg/ml) (B)		Total Amount (A + B)		Total quantity Found Mean ± S.D.		% Recovery± S.D	
EZE	PRAVA	EZE	PRAVA	EZE	PRAVA	EZE at 237.2 nm	PRAVA at 218.2 nm	EZE	PRAVA
100	100	50	50	150	150	149.36 ± 0.024	151.23 ± 0.045	99.57 ± 0.017	100.82 ± 0.032
100	100	100	100	200	200	210.58 ± 0.054	208.32 ± 0.014	105.29 ± 0.057	104.16 ± 0.047
100	100	150	150	250	250	257.65 ± 0.041	251.68 ± 0.065	103.06 ± 0.098	100.67 ± 0.078

(IV) Limit of detection

The minimum detectable concentration of EZE and PRAVA were found to be 0.0689 µg/ml and 0.187 µg/ml, respectively.

(V) Limit of quantification

The lowest quantifiable concentration of EZE and PRAVA were found to be 0.482 µg/ml and 0.954 µg/ml, respectively by practical observation.

7.2.2.3.4.2. Applicability of the method:

(A) Analysis of tablet formulation

The procedure for preparation of test solutions was given in Chapter 7.2.2.2.4.2 with use of ACN. A synthetic mixture was prepared having PRAVA and EZE in ratio of

1:1. 20 µl of this solution was injected into HPLC column and the peak area was measured at 248 nm. The concentration of EZE and PRAVA were found from regression equation of EZE and PRAVA. The results are shown in Table 7.2.2.3.7.

Table 7.2.2.3.7: Estimation of EZE and PRAVA in tablet by RP-HPLC method.

Formulation	DRUGS	Labeled Claim (mg/tab)	HPLC	
			*Amount found ± S.D.	% Assay ± S.D.
EZEDOC & PRAVATOR	EZE	10	9.98 ± 0.025	99.80 ± 0.014
	PRAVA	10	10.21 ± 0.015	102.10 ± 0.078
LAB. MIX.	EZE	10	10.32 ± 0.089	103.20 ± 0.014
	PRAVA	10	9.97 ± 0.047	99.70 ± 0.052

*** Mean value of five determinations.**

(B) Analysis of drug in degradation products

Efforts were made to analyze EZE and PRAVA in presence of their degradation products using the proposed method. Though conditions used for forced degradation were attenuated to achieve degradation in the range of 20 – 80 % this could not be achieved in some cases even after stress degradation for prolonged duration.

Procedure for forced degradation study

Forced degradation study was done in different conditions at room temperature. Solvents tried were 30% H_2O_2, water at neutral pH 7, 0.5 N HCl and 0.1 N NaOH. Approximate 25 mg drug was accurately weighed and transferred to 25 ml volumetric flask. The drug was dissolved in 5 ml of methanol then diluted with the solvent selected for degradation. Similarly solid-state stability was studied by exposing 25 mg of drug to 80°C in a stability oven and 25 mg drug to photostability oven. Samples were collected for analysis at two stages, at 0 min (as soon as sample was prepared), after 24 hrs and after 48 hrs of exposure to degradation condition. Sample was prepared by taking 2 ml of degraded solution in 10 ml volumetric flask and made up to 10 ml with ACN and 20µl of that was injected in HPLC column. Fig 7.2.2.3.6 to 7.2.2.3.11 show the chromatograms of forced degraded samples.

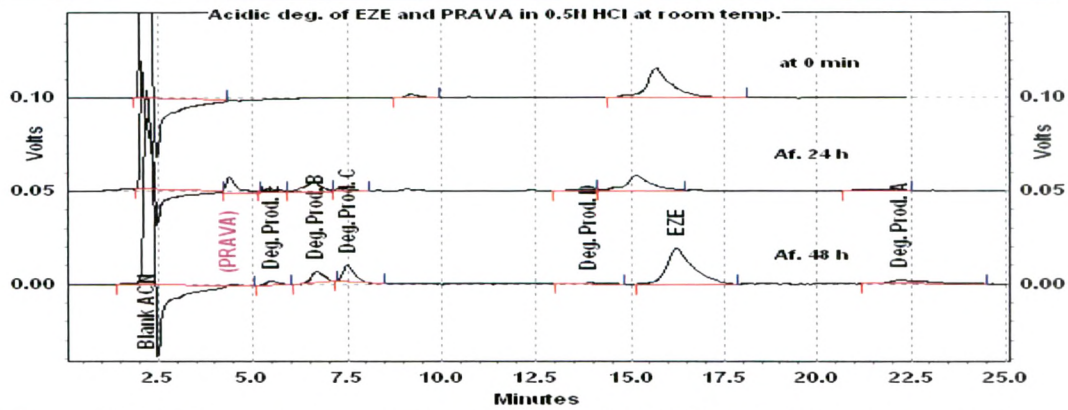


Figure 7.2.2.3.6: Chromatograms of acid hydrolysis in 0.5 N HCL after 0 min , 24 hrs and after 48 hrs degraded EZE and PRAVA

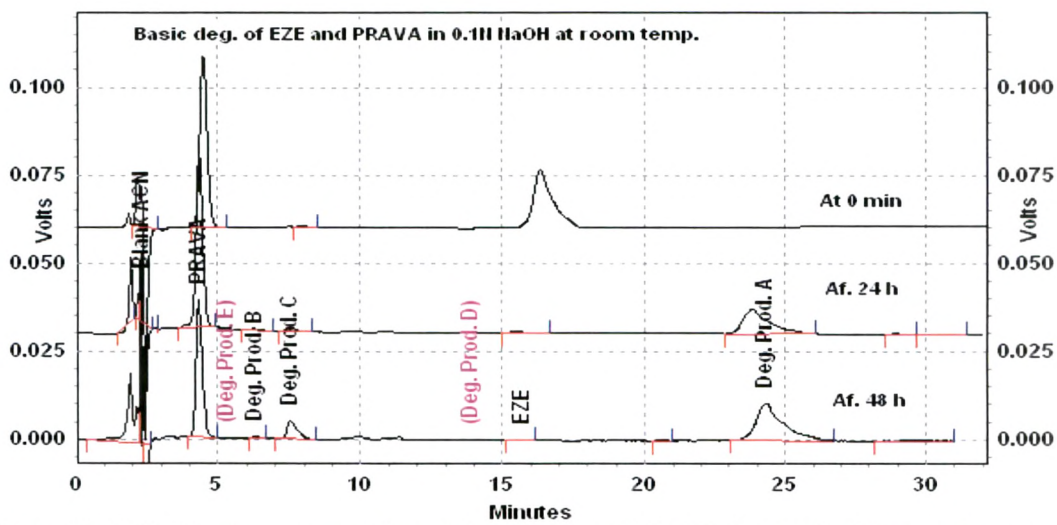


Figure7.2.2.3.7: Chromatograms of base hydrolysis in 0.1N NaOH after 0 min, 24 hrs and after 48 hrs degraded -degraded EZE and PRAVA

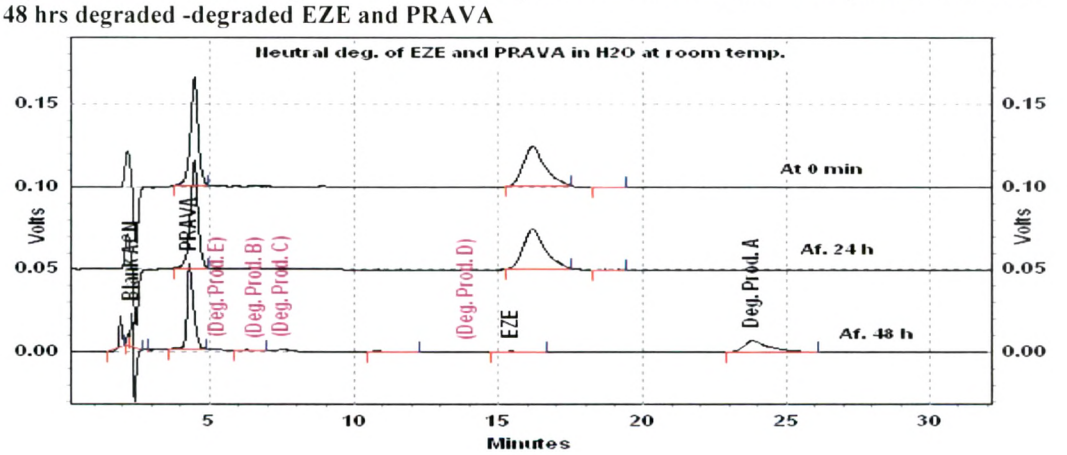


Figure7.2.2.3.8: Chromatograms of Neutral hydrolysis in 0.1N NaOH after 0 min, 24 hrs and after 48 hrs degraded -degraded EZE and PRAVA

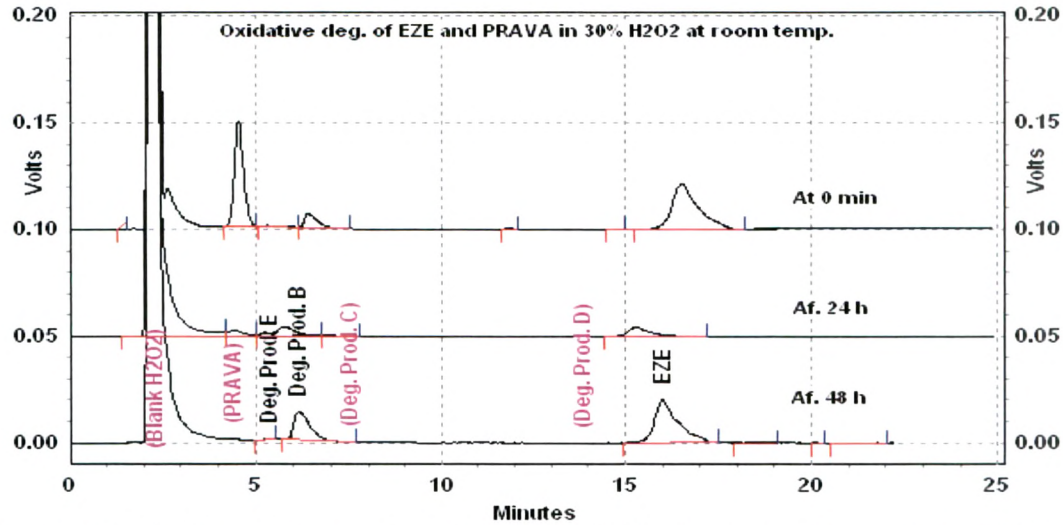


Figure 7.2.2.3.9: Chromatograms of oxidative in 30 % H2O2 after 0 min, 24 hrs and after 48 hrs -degraded EZE and PRAVA

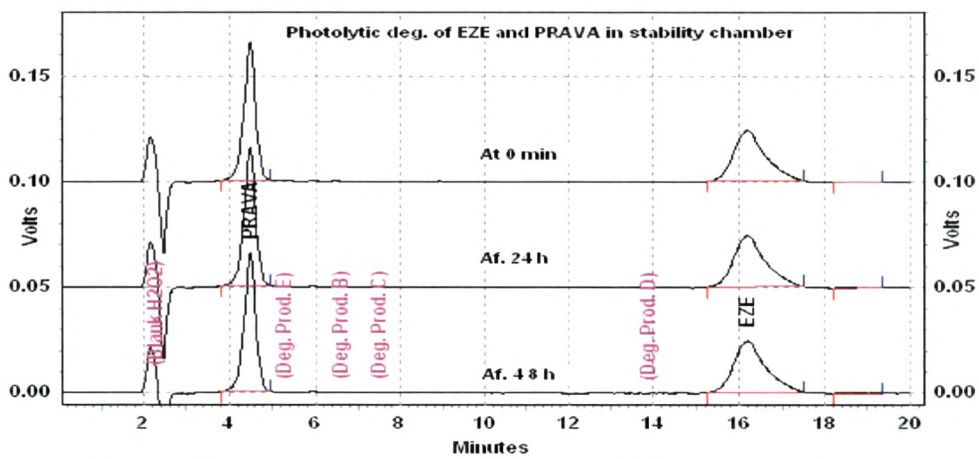


Figure 7.2.2.3.10: Chromatogram of EZE (30 µg/ml) and PRAVA (40 µg/ml) in UV/vis after 48 hrs in photostability chamber

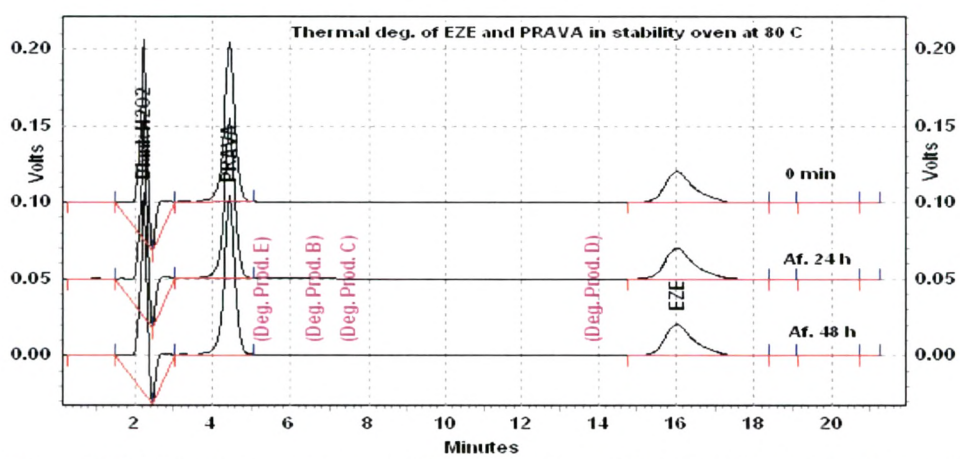


Figure 7.2.2.3.11: Chromatogram of EZE (26 µgml) and PRAVA (20 µg/ml) in thermal 80C after 48 hrs in Stability oven

Table 7.2.2.3.8: Percentage degradation of EZE and PRAVA

Sr. No	Parameters (Stress condition /duration/state)	% of undergrad		% of individual Degradation products					Total % Deg.	
		EZE	PRAVA	A	B	C	D	E	EZE	PRAVA
1	Neutral/H2Oat pH 7/4 h/ sol./RT	63	100	37	-	-	-	-	37	No Deg.
2	Acidic/0.5 N HCl/48 h/ sol./RT	84	36	16	19	27	18	-	16	64
3	Alkali/0.1N NaOH/48 h/ sol./RT	53	48	47	19	33	-	-	53	52
4	Oxidative/30% H2O2/48 h/ sol./RT	100	-	-	83	-	-	17	No Deg.	100
5	Thermal/80 C/48 h/solid	100	100	No degradation found						
6	Photo/uv254 and Vis/366 nm/48 h/solid	100	100	No degradation found						

A- EZE degraded product. B, C, D and E – PRAVA degraded products.

Table 7.2.2.3.9: System suitability parameters of EZE and PRAVA

Sr. No	System Suitability Parameters	EZE	PRAVA	Degradation products				
				A	B	C	D	E
1	Retention time (minutes)	15.475	4.483	22.225	6.683	7.517	13.908	5.333
2	Theoretical plates	141862.2	2029.153	298003.4	3387.072	8522.495	34720.35	1737.127
3	Resolution	12.35	2.39	1.31	3.33	1.84	6.91	1.46
4	Asymmetry	1.38	1.01	1.87	2.19	1.62	1.07	1.72
5	USP width	3.27	1.35	3.30	1.17	1.65	1.80	1.05
6	Tailing factor	1.41	1.05	2.03	1.99	1.64	1.15	1.73
7	Capacity Factor	6.37	1.11	9.57	2.18	2.58	5.62	2.36

7.2.2.3.5. Summary of Validation parameters:

The summary of validation parameters is reported in Table 7.2.2.3.10.

Table 7.2.2.3.10: Summary of Validation parameters by HPLC

Sr. No	Parameters	EZE	PRAVA
1	Analytical wavelength (nm)	248	
2	Retention time (minutes)	15.3	4.3
3	Linearity range (µg/ml)	5-300	
4	Regression equation	Y=41688x +47240	Y=20478x +181431

5	Correlation coefficient (r^2)	0.9999	0.9980
6	Intercept	47240	181431
7	Slope	41688	20478
8	Assay (%)	99.80	102.10
9	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements %CV % Recovery	 0.28-0.65 0.45-0.87 <1% 99.57-105.29	 0.17-0.62 0.14-0.98 < 1 % 100.67 – 104.16
10	Limit of detection	0.0689	0.187
11	Limit of quantification	0.482	0.954

7.2.2.3.6. Conclusion:

For specificity, placebo solution, sample solutions and forced degraded sample were analyzed. No peak was observed at the retention time of EZE and PRAVA in the placebo chromatogram. There were no co-eluting peaks of interference form excipients, impurities and degradation products due to variable stress components, thus establishing the specificity of assay method. Developed method can be use for the analysis of EZE and PRAVA in presence of each other.

7.2.2.4. High Performance Thin Layer Chromatography (HPTLC) with UV detection.

7.2.2.4.1. Optimization of method:

For the simultaneous analysis of EZE and PRAVA by HPTLC, efforts were made to select a common solvent and a common absorption wavelength.

Determination of solvent for sample preparation:

Different solvents were tried to study the solubility of both the drugs as stated in Table 7.2.2.3.1 and methanol was selected for the preparation of drug solutions.

Determination of detection wavelength:

Wavelength was selected by scanning standard solutions of both drugs over 200 nm to 400 nm wavelengths. Drugs show high absorbance at **238 nm** as illustrated in fig. 7.2.2.3.1. So this wavelength was selected in densitometer UV detector.

Determination of mobile phase:

Various combinations of mobile phases were tried for effective separation of EZE and PRAVA on silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 µm thickness) (E. Merck). The results are reported in Table 7.2.2.1.1.

Table 7.2.2.4.1: Determination of mobile phase

Mobile phase combination	Ration (V/V/V)	Peak separation
Chloroform: Methanol	8: 2	No separation band
Chloroform: Methanol	5: 5	No separation band
Benzene: Methanol	8: 2	No separation
Benzene: Methanol	5: 5	No separation
Toluene: Methanol	8: 2	Spot not separated
Hexane: Methanol	5: 5	Spot not separated
Chloroform: Toluene: Methanol	3: 4: 3	Slight separation
Chloroform: Toluene: Methanol	4: 4: 2	Good separation but broad band
Chloroform: Benzene: Methanol	3: 4: 3	Adjocent spots
Chloroform: Toluene: Methanol: Glacial acetic acid	**4: 4: 2: 0.1**	**Good separation with sharp band**
Chloroform: Toluene: Methanol: Glacial acetic acid	4: 4: 2: 0.05	Good separation but band was broad
Chloroform: Toluene: Methanol: Glacial acetic acid	4: 4: 2: 0.2	Merged no separation

It is evident from the data that mobile phase combination of **Chloroform: Toluene: Methanol: Glacial acetic acid** in proportion of **4: 4: 2: 0.1** v/v/v was most suitable for the development of HPTLC method.

7.2.2.4.2. Preparation of binary stock solution:

For chromatographic study, standard EZE and PRAVA (100 mg) were weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml methanol and diluted up to mark with methanol to obtain final concentration of 1000 µg/ml of EZE and PRAVA.

7.2.2.4.3. Calibration curve:

Analysis was performed on Pre-coated silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 µm thickness) (E. Merck) pre-washed with methanol and then dried for 30 minute at 50°C. From the stock solution (1000 µg/ml) aliquots of 0.1, 0.2, 0.4, 0.8, 1.2 and 1.6 µl were spotted on the TLC plate under nitrogen stream using Desaga Applicator, AS30win to obtain final concentration range 100 - 1800 ng/spot.

The plate was dried in air, and then the plate was developed in Twin trough developing chamber (100 × 100) with stainless steel lid, previously saturated with the mobile phase for 30 min. The plate was removed from the chamber, dried in air and was scanned and quantified at 2381 nm in Re-emission/Excitation mode with Desaga TLC scanner, Proquant. The calibration curve was constructed by plotting peak area vs. concentration (ng/spot) and peak height vs. concentration (ng/spot) corresponding to each spot and regression equation was calculated.

7.2.2.4.4. Validation parameter:

Validation of method was done as per procedure give in Chapter 7.1.1.8.4.

7.2.2.4.5. Results and Discussion:

7.2.2.4.5.1. Validation Parameters

(I) Linearity

The HPTLC method showed good linearity for EZE in the range of 135 to 1620 ng/spot. Peak height and area were taken for each concentration was reported in table 7.2.2.4.2 and 7.2.2.4.3.for EZE and PRAVA.

Table 7.2.2.4.2: Calibration data of EZE by HPTLC with UV detection

Sr. No.	Concentration (ng/spot)	Peak Area		Peak Height		Rf*
		Mean* ± SD	%CV	Mean* ± SD	%CV	
1	135	135.91 ± 0.089	0.54	87.86 ± 0.098	0.84	0.77
2	270	139.00 ± 0.098	0.32	93.73 ± 0.045	0.71	0.78
3	405	144.50 ± 0.054	0.18	96.12 ± 0.021	0.52	0.75
4	810	163.00 ± 0.041	0.65	112.00 ± 0.041	0.34	0.77
5	1215	181.17 ± 0.024	0.44	130.01 ± 0.085	0.41	0.78
6	1620	201.00 ± 0.078	0.98	142.92 ± 0.064	0.55	0.75

***Average of five readings**

Table 7.2.2.4.3: Calibration data of PRAVA by HPTLC with UV detection

Sr. No.	Concentration (ng/spot)	Peak Area		Peak Height		Rf*
		Mean* ± SD	%CV	Mean* ± SD	%CV	
1	159	128.09 ± 0.015	0.12	113.91 ± 0.045	0.65	0.54

2	318	135.00 ± 0.095	0.34	125.00 ± 0.065	0.71	0.52
3	477	145.00 ± 0.021	0.56	131.50 ± 0.074	0.52	0.54
4	954	170.00 ± 0.064	0.14	150.00 ± 0.065	0.42	0.55
5	1431	193.62 ± 0.012	0.27	175.00 ± 0.044	0.84	0.53
6	1908	220.20 ± 0.032	0.98	193.58 ± 0.052	0.55	0.54

***Average of five readings**

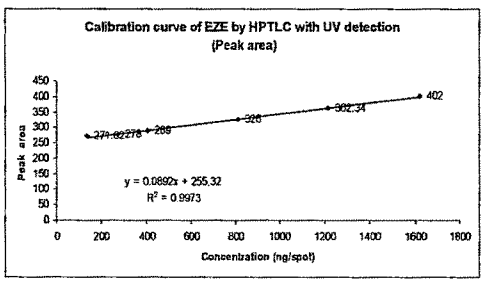


Figure 7.2.2.4.1: Calibration curve of EZE by HPTLC with UV detection (Peak Area)

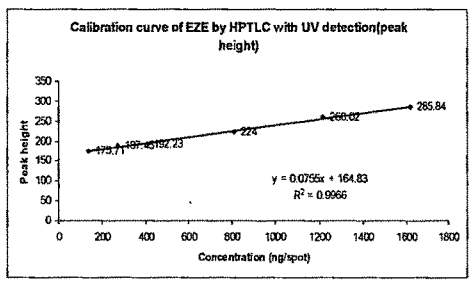


Figure 7.2.2.4.2: Calibration curve of EZE by HPTLC with UV detection (Peak Height)

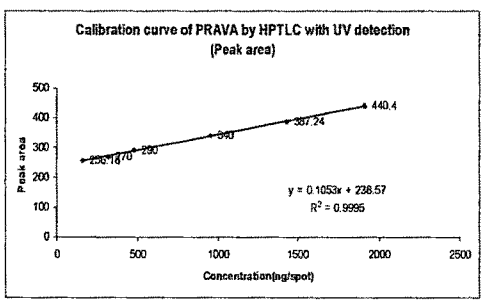


Figure 7.2.2.4.3: Calibration curve of PRAVA by HPTLC with UV detection (Peak Area)

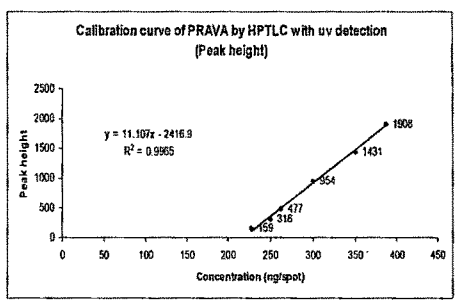


Figure 7.2.2.4.4: Calibration curve of PRAVA by HPTLC with UV detection (Peak Height)

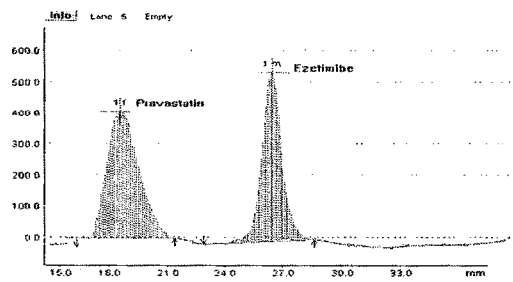


Figure 7.2.2.4.5: Spectrum of EZE and PRAVA by HPTLC with UV detection

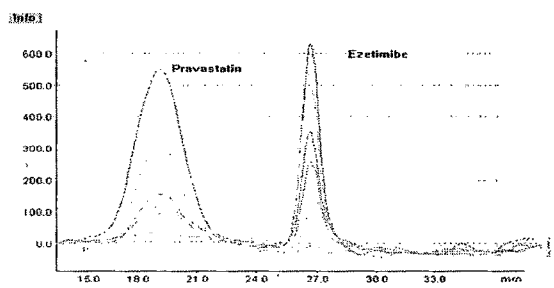


Figure 7.2.2.4.6: Overlain Spectra of EZE and PRAVA by HPTLC with UV detection

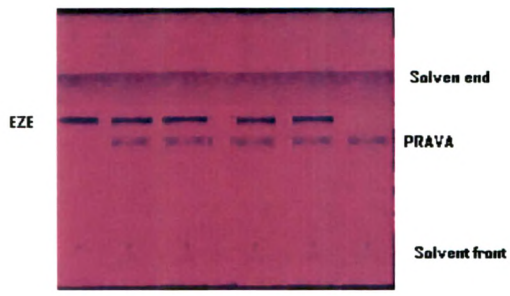


Figure 7.2.2.4.7: TLC plate of EZE and PRAVA with UV detection (After detection)

(II) Precision

Repeatability

The method was found to be precise on intra day and inter day basis as the average % CV value for the determination of EZE and PRAVA was found to be < 1 %. (Table 7.2.2.4.4 and 7. 2.2.4.5).

Table 7.2.2.1.4: Intraday and interday precision data of EZE and PRAVA by HPTLC (peak area)

Concentration (ng/spot)		Intraday		Interday	
		Peak area Mean* ± % C.V. (n=5)			
EZE	PRAVA	EZE	PRAVA	EZE	PRAVA
250	250	138.20 ± 0.23	129.00 ± 0.19	138.33 ± 0.85	129.92 ± 0.56
750	750	158.93 ± 0.58	158.65 ± 0.82	158.70 ± 0.47	159.92 ± 0.19
1000	1000	175.11 ± 0.74	184.30 ± 0.54	177.00 ± 0.65	186.29 ± 0.74

*** Mean of five determinations.**

Table 7.2.2.4.5. Intraday and interday precision data of EZE and PRAVA by HPTLC (peak height)

Concentration (ng/spot)		Intraday		Interday	
		Peak height Mean* ± % C.V. (n=5)			
EZE	PRAVA	EZE	PRAVA	EZE	PRAVA
250	250	88.26±0.23	119.62±0.16	89.65±0.65	119.65±0.73
750	750	102.61±0.12	142.85±0.28	101.45±0.41	144.22±0.91
1000	1000	128.62±0.85	161.85±0.64	127.95±0.98	159.96±0.54

*** Mean of five determinations.**

Reproducibility:

The % CV of the responses for determination of EZE and PRAVA are mentioned in Tables 7.2.2.4.6, which reveal the reproducibility of the method .

Table 7.2.2.4.6: Reproducibility data of EZE and PRAVA by HPTLC

Concentration (ng/spot)		Peak area ± % CV*				Peak height ± % CV*			
EZE	PRAVA	EZE		PRAVA		EZE		PRAVA	
		Glass plate	Aluminum plate	Glass plate	Aluminum plate	Glass plate	Aluminum plate	Glass plate	Aluminum plate
250	250	138.16 ± 0.87	137.46 ± 0.74	129.51 ± 0.65	130.96 ± 0.47	88.99 ± 0.98	86.52 ± 0.57	117.47 ± 0.91	118.52 ± 0.28
750	750	158.58 ± 0.56	158.65 ± 0.23	158.17 ± 0.41	158.11 ± 0.23	103.57 ± 0.42	102.44 ± 0.63	146.49 ± 0.22	147.77 ± 0.47
1000	1000	177.23 ± 0.74	177.64 ± 0.71	182.12 ± 0.51	184.23 ± 0.55	128.80 ± 0.12	129.72 ± 0.18	162.40 ± 0.84	168.92 ± 0.35

*** Mean of five determinations.**

(III) Accuracy

The method showed % mean recovery for EZE in the range of 99.81 - 101.17 % and for PRAVA it was 99.07 – 103.83 % in synthetic mixture (Table 7.2.2.4.8) for peak area. Same way for peak height % mean recovery for EZE in the range of 100.39 - 100.95 % and for PRAVA 99.81 – 102.86 % in synthetic mixture (Table 7.2.2.4.7)

Table 7.2.2.4.7: Accuracy data of EZE and PRAVA by HPTLC

Spotted Amount (A + B)		Peak area				Peak height			
		Total quantity Found (Mean*)		% Recovery		Total quantity Found (Mean*)		% Recovery	
EZE	PRAVA	EZE	PRAVA	EZE	PRAVA	EZE	PRAVA	EZE	PRAVA
750	750	748.56	749.66	99.81	99.95	752.89	748.56	100.39	99.81
1000	1000	1011.74	1038.27	101.17	103.83	1009.47	1022.98	100.95	102.30
1500	1500	1497.32	1485.98	99.82	99.07	1508.47	1542.87	100.56	102.86

* Mean of three determinations.

(IV) Limit of detection and quantification

Table 7.2.2.4.8: LOD and LOQ data of EZE and PRAVA by HPTLC

Parameter	RP-HPTLC			
	Peak area		Peak Height	
	EZE	PRAVA	EZE	PRAVA
LOD (ng/spot)	7.7757	17.4615	21.1748	0.2125
LOQ (ng/spot)	8.1776	58.2051	25.9192	0.7085

7.2.2.4.2.2. Analysis of tablet formulation:

The procedure for preparation of test solutions was given in Chapter 7.2.2.4.2. A synthetic mixture was prepared having PRAVA and EZE in ratio of 1: 1. From the filtrate 0.5 µl was spotted on TLC plate under nitrogen stream using Desaga Applicator, AS30win this was developed and scanned five times without changing plate position. The concentrations of EZE and PRAVA were computed from regression equation of EZE and PRAVA and found to be in the range of 99.86 – 101.93 % and 99.43 – 103.02 %, respectively (Table 7.2.2.1.10).

Table 7.2.2.4.10: Estimation of EZE and PRAVA in tablet by RP-HPTLC method

Formulation	DRUGS	Labeled Claim (mg/tab)	HPTLC			
			Peak Area		Peak Height	
			*Amount found	% Assay	*Amount found	% Assay
EZEDOC + PRAVATOR	EZE	10	10.09	100.92	10.15	101.52
	PRAVA	10	9.98	99.86	9.94	99.43
LAB. MIX.	EZE	10	10.16	101.69	10.10	101.07
	PRAVA	10	10.29	102.93	10.30	103.02

*** Mean value of five determinations.**

7.2.2.4.3. Summary of Validation parameters:

The summary of validation parameters is reported in Table 7.2.2.4.11.

Table 7.2.2.4.11: Summary of Validation parameters by HPTLC with UV detection

Sr. No	Parameters	EZE		PRAVA	
		Peak Area	Peak Height	Peak Area	Peak Height
1	Detection wavelength (nm)	238		238	
2	Linearity range (ng/spot)	135-1620		159 - 1908	
3	Regression equation	Y=0.0892x +255.32	Y=0.0755x +164.83	Y=0.1053x +238.57	Y=11.107x -2416.4
4	Correlation coefficient (r^2)	0.9973	0.9966	0.9995	0.9965
5	Intercept	255.32	164.83	238.57	-2416.4
6	Slope	0.0892	0.0755	0.1053	11.107
7	Assay (%)	100.92	101.52	99.86	99.43
8	Precision				
	Intra day % CV (n = 5)	0.23-0.74	0.12-0.85	0.19-0.82	0.16-0.64
	Inter day % CV (n = 5)	0.47-0.85	0.41-0.98	0.19-0.74	0.54-0.91
	Repeatability of measurements %CV	< 1 %			

	% Recovery	99.81-101.17	100.39-100.95	99.07-103.83	99.81-102.86
9	Limit of detection (ng/spot)	7.7757	21.17	17.46	0.2125
10	Limit of quantification (ng/spot)	8.1776	25.9192	58.20	0.7085

7.2.2.4.4. Conclusion:

Developed HPTLC method can well separate and analyze EZE and PRAVA with the accuracy of 99.07 to 103.83 % by peak area and peak height.

7.2.2.5. Employing Chemometric Methods:

In this section the Chemometric methods have been described for EZE and PRAVA. The concept of mathematical modeling is used to provide maximum relevant chemical information in order to analyze the drugs simultaneously.

The two chemometric techniques ILS and CLS are developed for the simultaneous determination of the titled drugs in their binary mixture.

7.2.2.5. Inverse Least Square (ILS) and Classical Least Square (CLS) techniques:

It is the application of multiple linear regressions (MLR) to the inverse expression of the Beer-Lambert Law of spectrophotometry.

7.2.2.5.1. Preparation of calibration and validation set:

Calibration and validation set were prepared in the same manner as mentioned in Chapter 7.2.1.

7.2.2.5.2. Selection of the spectral region:

In order to develop ILS and CLS methods for simultaneous estimation of EZE and PRAVA, light absorption study was done. Individual spectrum of both the drugs and their binary mixture were overlain.

Although the ILS is the full spectrum method, 21 wavelengths were selected between 230 nm to 250 nm with the interval of $\Delta\lambda$ = 1 nm in the zero order spectra as shown in fig. 7.2.2.5.1.

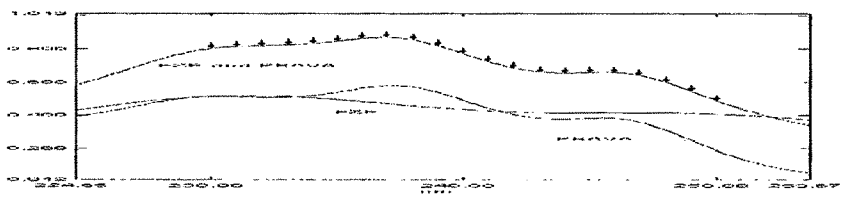


Fig 7.2.2.5.1: Overlain spectra of EZE , PRAVA and their binary mixture showing spectral region 230 nm to 250 nm (21 wavelengths)

7.2.2.5.3. Equation for ILS method and CLS method:

The mathematical expression of ILS is C = P x A.

The calibration coefficient (P) was obtained from the linear equation system using the absorbance data and the concentration taken in training set (Table 7.2.2.5.1 and 7.2.2.5.2)

The absorbance values (Table 7.2.2.5.3) of the samples at 21 wavelengths were placed in the above equation and the amounts of EZE and PRAVA in the synthetic mixture and formulation were found as shown in Table 7.2.2.5.4.

Introducing (P) into the linear equation system, the calibration for ILS was obtained as fig. 7.2.2.5.2.

$$\begin{bmatrix} C_{EZE} \\ C_{PRAVA} \end{bmatrix} \approx 1.0e+003 * \begin{pmatrix} -0.9748 & 0.2986 \\ 1.0726 & -0.7975 \\ 0.1458 & 0.1442 \\ 0.4027 & 0.5871 \\ -0.0251 & 0.1181 \\ -0.8772 & -0.4664 \\ 0.0040 & 0.4087 \\ 0.6723 & -0.3598 \\ -0.2728 & 0.1501 \\ 0.2157 & 0.4264 \\ -0.3488 & -1.2410 \\ 0.1383 & 0.8654 \\ -0.6352 & -0.4218 \\ -0.0677 & 0.4188 \\ -0.3637 & 0.1467 \\ 1.8208 & -1.3334 \\ -0.3824 & 1.4790 \\ -1.0413 & 0.5950 \\ 0.2963 & -1.2039 \\ 0.4874 & 0.0962 \\ -0.2810 & 0.0789 \end{pmatrix} \times \begin{pmatrix} A_1 \\ A_2 \\ A_3 \\ A_4 \\ A_5 \\ A_6 \\ A_7 \\ A_8 \\ A_9 \\ A_{10} \\ A_{11} \\ A_{12} \\ A_{13} \\ A_{14} \\ A_{15} \\ A_{16} \\ A_{17} \\ A_{18} \\ A_{19} \\ A_{20} \\ A_{21} \end{pmatrix}$$

Fig 7.2.2.5.2: Equation of ILS

Equation of CLS

The mathematical expression A = K x C in the matrix is given in section .

In this method, the calibration coefficient (K) was obtained from the linear equation system using the absorbance data and the training set (Table 7.2.2.5.1 and 7.2.2.5.2)

The absorbance values (Table 7.2.2.5.3) of the samples at 21 wavelengths were placed in the above equation and the amounts of EZE and PRAVA in the synthetic mixture and formulation were found as shown in Table 7.2.2.5.4.

Introducing (K) into the linear equation system, the calibration for ILS was obtained as fig. 7.2.2.1.2.1.

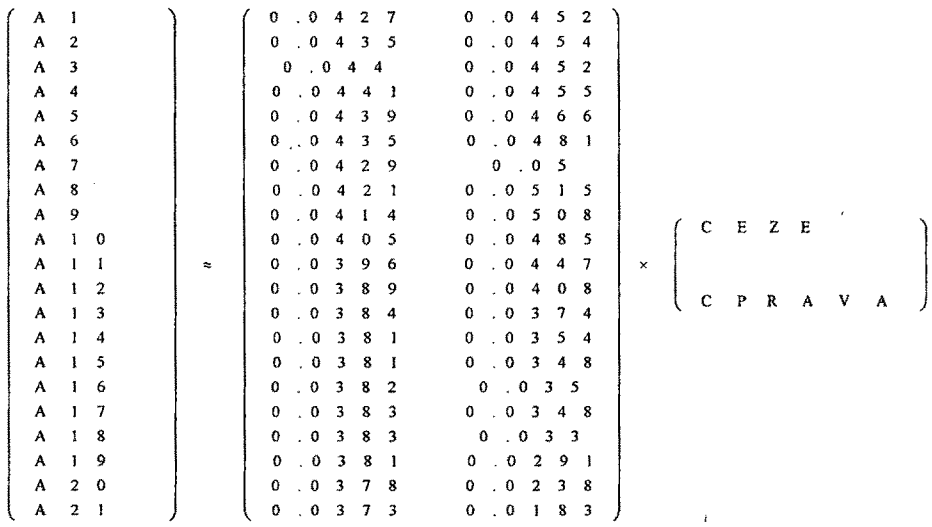


Fig 7.2.2.5.3: Equation of CLS

Table 7.2.2.5.1: Composition of Calibration (training) set for EZE and PRAVA:

Mixture No.	EZE (µg/ml)	PRAVA (µg/ml)	Mixture No.	EZE (µg/ml)	PRAVA (µg/ml)
1	0	5	**13**	15	15
2	2	10	**14**	15	18
3	5	10	**15**	15	20
4	5	18	**16**	15	25
5	5.5	15.5	**17**	15	30
6	8	15	**18**	18	25
7	10	5	**19**	20	18
8	10	10	**20**	20	20
9	10	18	**21**	22	30
10	12	20	**22**	25	18
11	14.5	7.5	**23**	28	35
12	15	10	**24**	38	0

Table 7.2.2.5.2: Absorbance data for the calibration set at 21 wavelengths:

Absorbance	Mi No.	Wavelength																				
		230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250
	1	0.214	0.217	0.216	0.217	0.224	0.235	0.248	0.256	0.254	0.24	0.217	0.194	0.174	0.163	0.161	0.163	0.164	0.155	0.134	0.104	0.074
	2	0.502	0.507	0.508	0.51	0.521	0.538	0.556	0.566	0.559	0.532	0.493	0.453	0.42	0.401	0.396	0.398	0.396	0.377	0.339	0.289	0.237
	3	0.694	0.694	0.69	0.687	0.69	0.701	0.714	0.72	0.707	0.674	0.627	0.582	0.542	0.516	0.507	0.506	0.503	0.483	0.445	0.392	0.337
	4	0.937	0.947	0.95	0.955	0.972	1.004	1.04	1.061	1.048	0.997	0.923	0.848	0.783	0.743	0.731	0.735	0.733	0.699	0.63	0.534	0.433
	5	0.87	0.88	0.884	0.889	0.905	0.931	0.96	0.976	0.963	0.917	0.853	0.787	0.73	0.696	0.687	0.691	0.689	0.66	0.6	0.518	0.431
	6	0.897	0.907	0.909	0.913	0.927	0.952	0.977	0.99	0.975	0.931	0.868	0.806	0.754	0.723	0.715	0.719	0.716	0.688	0.63	0.553	0.471
	7	0.594	0.606	0.613	0.617	0.621	0.627	0.631	0.631	0.62	0.598	0.571	0.545	0.524	0.511	0.508	0.51	0.511	0.503	0.484	0.457	0.427
	8	0.802	0.815	0.821	0.825	0.834	0.849	0.864	0.87	0.857	0.822	0.773	0.726	0.686	0.662	0.656	0.659	0.659	0.641	0.603	0.548	0.489
	9	1.186	1.198	1.201	1.203	1.217	1.243	1.271	1.285	1.265	1.209	1.131	1.053	0.984	0.943	0.931	0.935	0.933	0.9	0.831	0.735	0.636
	10	1.167	1.179	1.181	1.185	1.203	1.235	1.272	1.289	1.271	1.212	1.128	1.045	0.975	0.933	0.922	0.927	0.923	0.885	0.808	0.701	0.59
	11	0.961	0.977	0.985	0.989	0.994	1.002	1.007	1.004	0.987	0.953	0.911	0.872	0.839	0.819	0.814	0.817	0.817	0.805	0.775	0.733	0.686
	12	1.032	1.048	1.054	1.058	1.066	1.078	1.09	1.092	1.074	1.036	0.983	0.934	0.893	0.869	0.863	0.866	0.867	0.849	0.811	0.754	0.693
	13	1.3	1.317	1.324	1.329	1.34	1.36	1.382	1.389	1.37	1.316	1.244	1.175	1.116	1.08	1.07	1.075	1.073	1.047	0.988	0.905	0.816
	14	1.419	1.432	1.433	1.432	1.442	1.463	1.486	1.495	1.471	1.411	1.33	1.248	1.179	1.138	1.127	1.13	1.129	1.096	1.026	0.931	0.829
	15	1.479	1.495	1.499	1.503	1.518	1.547	1.578	1.593	1.569	1.506	1.416	1.328	1.251	1.207	1.193	1.198	1.196	1.159	1.082	0.974	0.86
	16	1.692	1.711	1.719	1.724	1.745	1.783	1.825	1.844	1.82	1.745	1.633	1.522	1.432	1.377	1.359	1.366	1.362	1.315	1.218	1.083	0.94
	17	1.876	1.895	1.897	1.899	1.928	1.967	2.02	2.045	2.018	1.936	1.811	1.689	1.583	1.517	1.497	1.503	1.499	1.443	1.33	1.174	1.007
	18	2.779	2.801	2.801	2.817	2.872	2.883	2.925	2.985	2.948	2.883	2.723	2.566	2.447	2.379	2.37	2.386	2.389	2.34	2.198	1.993	1.78
	19	1.618	1.64	1.644	1.654	1.665	1.688	1.71	1.713	1.687	1.627	1.54	1.456	1.386	1.344	1.333	1.335	1.333	1.299	1.231	1.137	1.031
	20	1.615	1.637	1.645	1.652	1.666	1.692	1.718	1.729	1.701	1.638	1.547	1.46	1.385	1.341	1.329	1.333	1.332	1.298	1.224	1.121	1.008
	21	2.665	2.691	2.684	2.703	2.73	2.768	2.825	2.853	2.784	2.684	2.562	2.452	2.346	2.278	2.263	2.265	2.263	2.214	2.096	1.934	1.757
	22	1.837	1.864	1.878	1.88	1.892	1.912	1.934	1.938	1.91	1.843	1.754	1.668	1.597	1.554	1.541	1.544	1.541	1.508	1.436	1.336	1.225
	23	2.525	2.557	2.566	2.581	2.613	2.635	2.677	2.696	2.665	2.586	2.448	2.312	2.197	2.117	2.104	2.111	2.104	2.037	1.91	1.728	1.537
	24	1.623	1.655	1.67	1.673	1.665	1.653	1.635	1.604	1.575	1.539	1.503	1.474	1.454	1.443	1.442	1.445	1.448	1.447	1.44	1.425	1.401

Table 7.2.2.5.3: Absorbance data for the calibration set at 21 wavelengths:

Absorbance	Mi No.	Wavelength																				
		230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250
	1	1.581	1.585	1.58	1.58	1.607	1.657	1.722	1.759	1.737	1.649	1.523	1.399	1.293	1.234	1.22	1.233	1.232	1.174	1.052	0.889	0.722
	2	0.327	0.331	0.333	0.333	0.335	0.337	0.339	0.338	0.333	0.322	0.309	0.295	0.284	0.278	0.275	0.275	0.275	0.27	0.26	0.245	0.229
	3	0.892	0.898	0.898	0.9	0.914	0.938	0.965	0.979	0.966	0.922	0.859	0.795	0.741	0.708	0.698	0.701	0.698	0.667	0.606	0.526	0.442
	4	1.339	1.348	1.346	1.348	1.371	1.415	1.461	1.486	1.466	1.397	1.291	1.19	1.103	1.05	1.036	1.04	1.034	0.981	0.882	0.75	0.613
	5	1.133	1.141	1.139	1.14	1.153	1.177	1.203	1.217	1.198	1.147	1.073	0.999	0.939	0.903	0.893	0.896	0.892	0.858	0.79	0.699	0.604
	6	1.042	1.05	1.051	1.053	1.065	1.088	1.113	1.124	1.107	1.059	0.993	0.928	0.874	0.842	0.832	0.836	0.833	0.803	0.743	0.661	0.576
	7	0.87	0.879	0.881	0.884	0.891	0.903	0.917	0.92	0.906	0.871	0.824	0.779	0.74	0.718	0.711	0.713	0.712	0.692	0.653	0.599	0.542
	8	0.855	0.866	0.871	0.872	0.873	0.875	0.875	0.869	0.854	0.827	0.796	0.767	0.743	0.729	0.725	0.727	0.726	0.716	0.695	0.665	0.632
	9	1.39	1.404	1.405	1.407	1.417	1.437	1.46	1.466	1.442	1.386	1.312	1.24	1.181	1.144	1.134	1.138	1.134	1.103	1.038	0.95	0.857
	10	1.326	1.338	1.343	1.345	1.349	1.357	1.364	1.36	1.337	1.292	1.235	1.182	1.139	1.113	1.106	1.108	1.106	1.085	1.042	0.982	0.917
	11	1.619	1.637	1.64	1.642	1.65	1.665	1.683	1.679	1.653	1.595	1.519	1.446	1.387	1.35	1.339	1.343	1.339	1.31	1.249	1.166	1.075
	12	1.474	1.492	1.498	1.501	1.503	1.512	1.521	1.518	1.491	1.442	1.377	1.319	1.271	1.242	1.234	1.237	1.235	1.213	1.167	1.102	1.03
	13	1.692	1.701	1.714	1.712	1.724	1.741	1.755	1.755	1.724	1.663	1.583	1.511	1.448	1.411	1.4	1.404	1.401	1.371	1.309	1.222	1.129
	14	1.217	1.227	1.229	1.231	1.242	1.263	1.286	1.293	1.273	1.222	1.154	1.086	1.028	0.993	0.983	0.986	0.982	0.952	0.891	0.807	0.719
	15	1.57	1.588	1.592	1.588	1.573	1.552	1.527	1.496	1.464	1.432	1.402	1.38	1.364	1.354	1.352	1.353	1.353	1.354	1.349	1.341	1.323

Table 7.2.2.5.4: Composition of Validation set for EZE and PRAVA:

Mixture No.	EZE (µg/ml)	PRAVA (µg/ml)	Mixture No.	EZE (µg/ml)	PRAVA (µg/ml)
1	0	25	9	15	15
2	5	3	10	20	10
3	5	15	11	22	15
4	5	25	12	22.5	11.5
5	8.5	16.5	13	25	15
6	9	15	14	32	15
7	10	10	15	35	0
8	15	5	-	-	-

7.2.2.5.4. Validation Parameters

(I) Accuracy

Accuracy of the ILS and CLS methods was determined by performing recovery study on laboratory prepared synthetic mixture. The % mean recovery values are reported in Table 7.2.2.5.6 and 7.2.2.5.7.

(II) Precision

The precision was determined by means of a one way ANOVA including experiments were repeated five times in a day for intra day and on five different days for inter day precision for 10 synthetic mixtures. F values below the tabulated levels were obtained and there was no significant difference between the results obtained in the determination of each drug in the presence of other on different days.

Table 7.2.2.5.5: Data for precision study using one way ANOVA

Parameters	ILS		CLS	
	EZE	PRAVA	EZE	PRAVA
Between days variance	3.89	3.64	3.17	4.22
Within days variance	1.93	1.99	1.43	1.83
F ratio	**2.01**	**1.83**	**2.22**	**2.31**

Note the between day and within day degrees of freedom are 4 and 45 respectively. The critical F ratio value for 4 and 45 df and the confidence level of 95% is 2.53.

(III) Limit of detection

The minimum detectable concentration by ILS method of EZE and PRAVA was found to be 0.429µg/ml and 0.248µg/ml and CLS method was found to be 0.324µg/ml and 0.429 µg/ml, respectively.

(IV) Limit of quantification

The lowest quantifiable concentration by ILS of EZE and PRAVA was found to be 0.813µg/ml and 0.634µg/ml and CLS method was found to be 0.889µg/ml and 0.932µg/ml, respectively by practical observation.

(V) Predicted versus known concentration plot:

The predicted concentrations of the validation samples were plotted against the known concentration values.

This tool is used to determine whether the model accounts for the concentration variation in the validation set or not.

Plots were expected to fall on a straight line with a slope of 1 and o intercept. The predicted versus known concentration plots of the prepared concentration plots of the prepared validation samples are shown in fig. 7.2.2.5.4 and 7.2.2.5.5 by ILS method and fig. 7.2.2.5.6. and 7.2.2.5.7 by CLS method

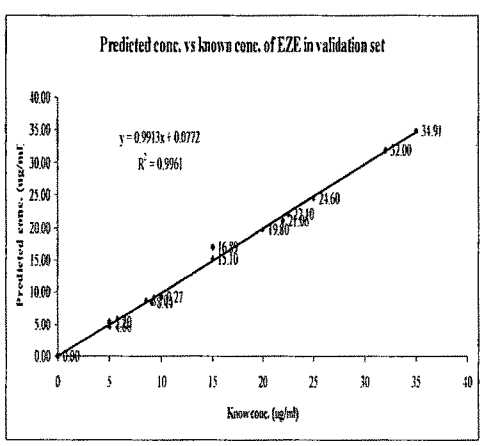


Fig 7.2.2.5.4 : Linearity plots of EZE and PRAVA for validation set.

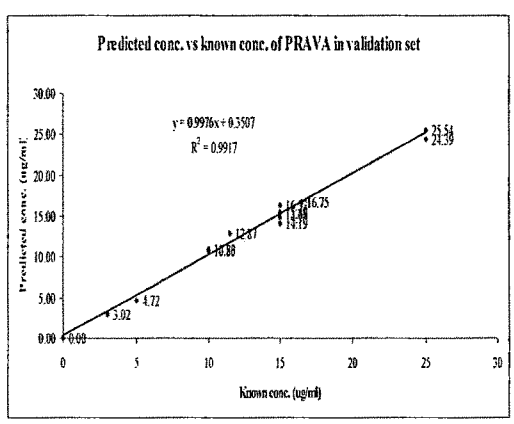


Fig 7.2.2.5.5 : Linearity plots of EZE and PRAVA for validation set.

Table 7.2.2.5.6: Actual, predicted and residual values.

SR. No.	Actual Conc.		Predicted conc.		Residual		Percentage	
	EZE	PRAVA	EZE	PRAVA	EZE	PRAVA	EZE	PRAVA
1	0	25	0.00	24.39	0.00	0.61	100.00	97.56
2	5	3	4.60	3.02	0.40	-0.02	92.00	100.67
3	5	15	5.10	15.33	-0.10	-0.33	102.00	102.20
4	5	25	5.20	25.54	-0.20	-0.54	104.00	102.17
5	8.5	16.5	8.70	16.75	-0.20	-0.25	102.35	101.52
6	9	15	8.44	15.49	0.56	-0.49	93.81	103.27
7	10	10	9.27	10.80	0.73	-0.80	92.66	107.96
8	15	5	15.10	4.72	-0.10	0.28	100.67	94.33
9	15	15	15.89	15.49	-0.89	-0.49	105.92	103.26
10	20	10	19.80	10.88	0.20	-0.88	99.00	108.84
11	22	15	21.06	14.19	0.94	0.81	95.71	94.63
12	22.5	11.5	22.10	10.87	0.40	0.63	98.22	94.53
13	25	15	24.60	15.41	0.40	-0.41	98.40	102.73
14	32	15	32.00	14.90	0.00	0.10	100.00	99.33
15	35	0	34.91	0.00	0.09	0.00	99.74	100.00
16	0	25	0.00	24.39	0.00	0.61	100.00	97.56
17	5	3	4.60	3.02	0.40	-0.02	92.00	100.67
18	5	15	5.10	15.33	-0.10	-0.33	102.00	102.20
19	5	25	5.20	25.54	-0.20	-0.54	104.00	102.17
20	8.5	16.5	8.70	16.75	-0.20	-0.25	102.35	101.52
21	9	15	8.44	15.49	0.56	-0.49	93.81	103.27

Table 7.2.2.5.7: Actual, predicted and residual values.

SR. No.	Actual Conc.		Predicted conc.		Residual		Percentage	
	EZE	PRAVA	EZE	PRAVA	EZE	PRAVA	EZE	PRAVA
1	0	25	0.00	24.68	0	0.3226	100.00	98.71
2	5	3	4.82	2.64	0.1794	0.3598	96.41	88.01

3	5	15	4.92	15.87	0.0844	-0.8681	98.31	105.79
4	5	25	5.34	25.21	-0.336	-0.2125	106.72	100.85
5	8.5	16.5	8.38	15.70	0.1246	0.8015	98.53	95.14
6	9	15	8.69	15.57	0.3072	-0.5731	96.59	103.82
7	10	10	9.42	10.20	0.5821	-0.1993	94.18	101.99
8	15	5	14.34	5.19	0.6597	-0.1871	95.60	103.74
9	15	15	14.85	15.39	0.1515	-0.3947	98.99	102.63
10	20	10	19.24	10.76	0.7552	-0.7608	96.22	107.61
11	22	15	21.13	15.46	0.8732	-0.4559	96.03	103.04
12	22.5	11.5	21.78	11.71	0.7219	-0.2098	96.79	101.82
13	25	15	25.38	15.84	-0.3768	-0.8373	101.51	105.58
14	32	15	31.35	15.91	0.6501	-0.9104	97.97	106.07
15	35	0	35.29	0.00	-0.2857	0	100.82	100.00
16	0	25	0.00	24.68	0	0.3226	100.00	98.71
17	5	3	4.82	2.64	0.1794	0.3598	96.41	88.01
18	5	15	4.92	15.87	0.0844	-0.8681	98.31	105.79
19	5	25	5.34	25.21	-0.336	-0.2125	106.72	100.85
20	8.5	16.5	8.38	15.70	0.1246	0.8015	98.53	95.14
21	9	15	8.69	15.57	0.3072	-0.5731	96.59	103.82

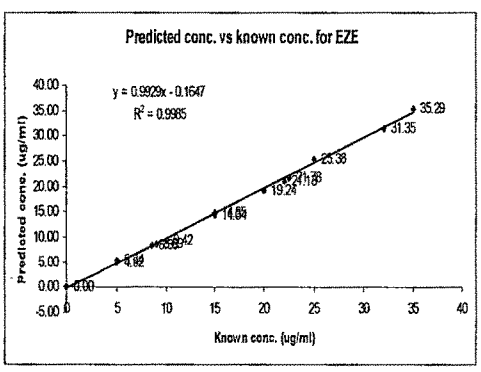


Fig 7.2.2.5.6: Linearity plots of EZE for validation set.

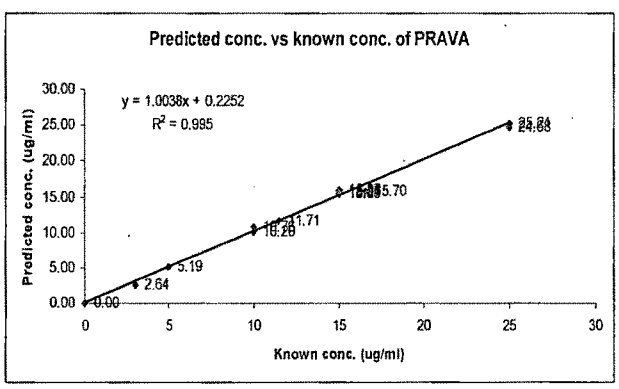


Fig 7.2.2.5.7: Linearity plots of PRAVA for validation set.

This indicates that the prediction ability of the validation set is good.

(VI) Residuals concentration versus actual concentration plot:

The difference between the known and predicted concentration (residuals) were plotted against the actual concentrations for the validation samples. This tool is used to determine whether the model accounts for the concentration variation in the validation set and it also provides information about how well the method will predict the future samples. For the validation set it can be found that the residual values are more close to zero and are more randomly distributed as illustrated in fig. 7.2.2.5.8 and 7.2.2.5.9 by ILS method and fig. 7.2.2.5.10 and fig 7.2.2.5.10 by CLS method.

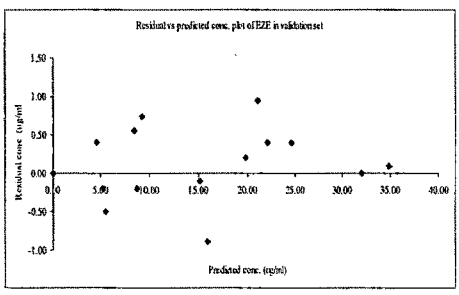


Fig 7.2.2.5.8: Residual vs. predicted conc. plot for EZE and PRAVA.

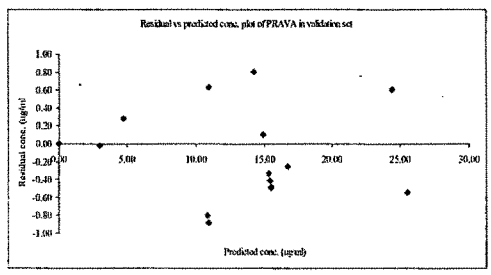


Fig 7.2.2.5.9: Residual vs. predicted conc. plot for EZE and PRAVA.

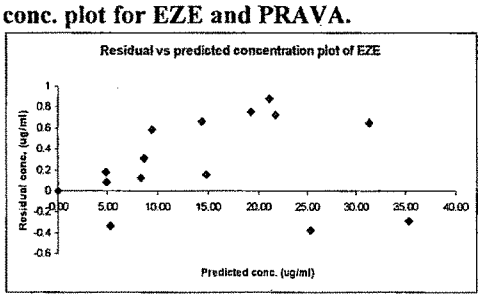


Fig 7.2.2.5.10: Residual vs. predicted conc. plot for EZE.

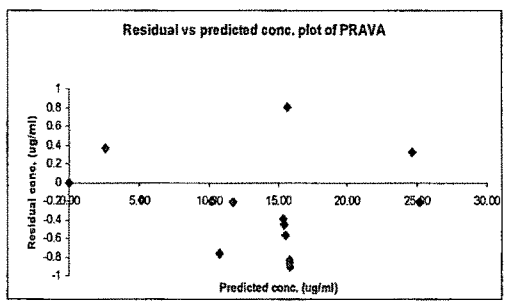


Fig 7.2.2.5.11: Residual vs. predicted conc. plot for PRAVA.

(VII) Root Mean Square Error of Prediction:

The RMSEP values calculated as per equation given in Chapter 5 are reported in Table 7.2.2.5.8.

Table 7.2.2.5.8: RMSEP values for EZE and PRAVA.

Compound	ILS	CLS
	RMSEP	RMSEP
EZE	0.49261	0.50422
PRAVA	0.53706	0.57285

7.2.2.5.5. Preparation of sample solutions:

The concentration of EZE and PRAVA in tablets was calculated using the equation computed from the data. Procedure of preparation of sample mixture is given in previous section. Results are shown in Table 7.2.2.5.9.

Table 7.2.2.5.9: Estimation of EZE and ROSU in tablet by ILS & CLS methods

Formulation	DRUGS	Labeled Claim (mg/tab)	CLS		ILS	
			*Amount found	% Labeled Claim	*Amount found	% Labeled Claim
EZEDOC + PRAVATOR	EZE	10	10.11	101.1	9.98	99.8
	PRAV	10	9.87	98.7	10.21	102.1
LAB. MIX.	EZE	10	10.29	102.9	11.28	112.8
	PRAVA	10	10.07	100.7	9.76	97.6

*** Mean value of five determinations.**

7.2.2.5.6. Summary of Validation parameters:

The summary of validation parameters were reported in table 7.2.2.5.10.

Table 7.2.2.5.10: Summary of Validation parameters of CLS method.

Sr. No	Parameters	ILS		CLS	
		EZE	PRAVA	EZE	PRAVA
1	Calibration design	25			
2	Validation design	15			
3	Spectral region	230 – 250			
4	Linearity range (µg/ml)	1-40			
5	Regression equation	Y=0.9913x +0.0772	Y=0.9976x +0.3507	Y=0.9928x -0.1647	Y=1.0038x +0.2252
6	Correlation coefficient (r^2)	0.9961	0.9917	0.9985	0.9951
7	Intercept	0.0772	0.3507	-0.1647	0.2252
8	Slope	0.9913	0.9976	0.9928	1.0038
9	RMSEP	0.49	0.50	0.504	0.572
10	Assay	99.8	102.10	101.1	98.7
11	Precision				
12	% Recovery	97.55-106.17	96.08-109.20 .	98.54-102.57	98.91-106.00

13	Limit of detection (µg/ml)	0.429	0.248	0.324	0.429
14	Limit of quantification(µg/ml)	0.893	0.634	0.889	0.932

7.2.2.5.7. CONCLUSION

The proposed ILS and CLS methods were found to be precise and accurate. The mathematical calculations for the proposed ILS and CLS methods can be effectively performed using MATLAB and Excel. The simplicity of the method can be explained on the basis of direct out put of the data in terms of unknown concentration on providing the concentration matrix and absorbance matrix of the calibration set to the software. The method does not require any time consuming separation or sample preparation step as used in HPLC.

The ILS method can be strongly applied to a routine analysis, quality control of binary mixtures and commercial products containing these two drugs.

Reference:

1) Lorenzo M., Richard M., David H., Robert L., Leslie L., Alexandre Le B., Ramachandran S., Pabak M., Enrico V. Efficacy and safety of EZE coadministered with PRAVA in patients with primary hypercholesterolemia: a prospective, randomized, double-blind trial. European Heart Journal. 2003; 24(8):717-728.

7. 2. 3

Ezetimibe and Rosuvastatin

7.2.3. Development of Analytical methods for Ezetimibe and Rosuvastatin:

The rosuvastatin-ezetimibe combination reduced LDL cholesterol levels significantly more than did rosuvastatin alone (69.8% vs 57.1%)[1]. The study has been done after mixing the commercially available tablet formulation of EZE and ROSU.

UV Spectrophotometric Methods:

In order to develop UV Spectrophotometric method for simultaneous estimation of EZE and ROSU, light absorption study was done. Individual spectra of both the drugs were superimposed to obtain suitable working wavelengths (fig. 7.2.3.1.1) that could be used for simultaneous estimation methods. Simultaneous equation method was not found to be applicable in this case due to extensive overlap of the two spectra. Absorbance ratio method was tried for the quantitative analysis using λmax of one drug and isoabsorptive point of both the drugs taken in 1:1 ratio but method was not giver precise results. The 1st and 2nd order derivative spectroscopies were tried in which 1st order derivative zero crossing method was possible as per fig. 7.2.3.1.2. Ratio spectrophotomertry could not be developed as no suitable divisor value could be obtained.

Chromatographic methods:

Three chromatographic methods were developed for the simultaneous estimation of EZE and ROSU. Two HPLC methods were developed. One could estimate EZE and ROSU in pharmaceutical dosage form and second could estimate EZE and ROSU in pharmaceutical dosage form and in presence of degradation products. HPTLC method was developed for the estimation of EZE and ROSU in pharmaceutical dosage form.

Chemometric methods

Chemometric approach was tried with the spectrophotometric methods. Here two Chemometric methods were developed, first is ILS and other is CLS.

Apparatus, instruments and reagents:

Apparatus, instruments and reagents used for the development of spectrophotometric and chromatographic methods are mentioned in Chapter of preliminary work (Chapter 6).

7.2.3.1. Simultaneous Spectrophotometric estimation of EZE and ROSU by First derivative zero crossing (FDZC) method.

Experimental work:

7.2.3.1.1. Preparation of stock solution:

Standard EZE and ROSU (10 mg) were weighed accurately and transferred to 100 ml volumetric flask. It was dissolved in 50 ml methanol and diluted up to mark with methanol to obtain final concentration of 100 µg/ml each of EZE and ROSU. This solution was used as working standard solution for spectrophotometric methods.

7.2.3.1.2. Selection of analytical wavelength:

The selection of zero crossing point, where absorbance of one component is zero while other is having significant absorbance in crucial. The zero crossing point should be valid for the entire concentration range in which analysis is carried out. From the overlain 1st derivative spectra of EZE and ROSU (fig. 7.2.3.1.2), 290 nm was selected as zero crossing point for ROSU as at this wavelength EZE has negligible absorbance. Whereas, 245.6 nm was selected as zero crossing point for EZE as at this wavelength, ROSU shows negligible absorbance. The absorbance data is given in Table 7.2.3.1.3.

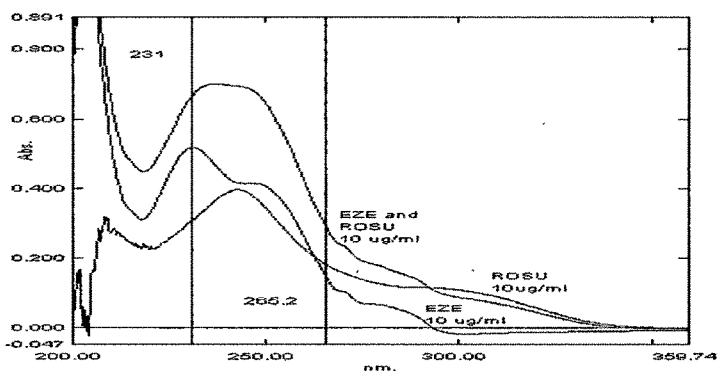


Figure: 7.2.3.1.1: Overlain zero order spectra of EZE and ROSU and binary mixture (1:1).

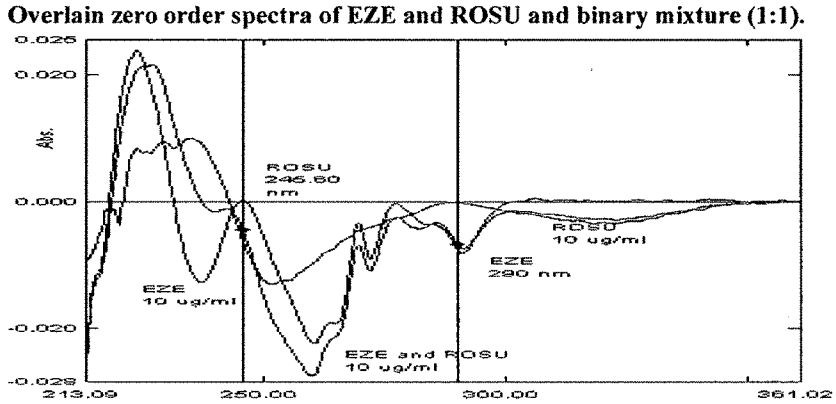


Figure: 7.2.3.1.2: Overlain 1st order derivative spectra of EZE and ROSU

Effect of derivative intervals on derivative:

$\Delta\lambda$, the width of the boundaries over which the derivative is calculated was tested for $\Delta\lambda$ = 2 nm, 4 nm and 8 nm.

The values of $\Delta\lambda$ = 2 nm was found optimal in connection with both slit width and wavelength interval.

7.2.3.1.3. Calibration curve:

From the binary stock solution (100 µg/ml) of EZE and ROSU aliquots of 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 ml were transferred to a series of 10ml volumetric flasks. The volume was adjusted to a mark with methanol to get 1, 5, 10, 15, 20, 25, 30, 35 and 40 µg/ml each of EZE and ROSU.

7.2.3.1.4. Validation of method:

Procedure for validation of method is given in Chapter 7.2.1.1.4.

7.2.3.1.5. Result and discussion

7.2.3.1.5.1. Validation parameters

(I) Linearity

The derivative spectrophotometry method showed good linearity for EZE in the range of 5- 40 µg/ml at 290.0 nm (zero crossing of ROSU) with co-relation co-efficient, of 0.9993 as seen in fig. 7.2.3.2.3. For ROSU the line of best fit was obtained at 245.60 nm (zero crossing of EZE) with correlation coefficients of 0.9935 as seen in fig. 7.2.3.2.4. Calibration data is given in Table 7.2.3.2.1.

Table 7.2.3.1.1: Calibration data of EZE and ROSU by FDZC method

Sr.No.	Concentration (µg/ml)	Absorbance * ± S.D. (N=5)		% C.V.	
		EZE at 290.0 nm	ROSU at 245.6 nm	EZE	ROSU
1	5	-0.003 ± 0.038	-0.003 ± 0.028	0.29	0.63
2	10	-0.007 ± 0.082	-0.006 ± 0.081	0.57	0.83
3	15	-0.011 ± 0.072	-0.008 ± 0.094	0.39	0.58
4	20	-0.016 ± 0.021	-0.011 ± 0.064	0.54	0.29
5	25	-0.02 ± 0.18	-0.014 ± 0.072	0.02	0.81
6	30	-0.024 ± 0.021	-0.017 ± 0.066	0.73	0.58
7	35	-0.028 ± 0.072	-0.02 ± 0.077	0.56	0.83
8	40	-0.032 ± 0.82	-0.021 ± 0.062	0.91	0.66

* Mean value of five determinations.

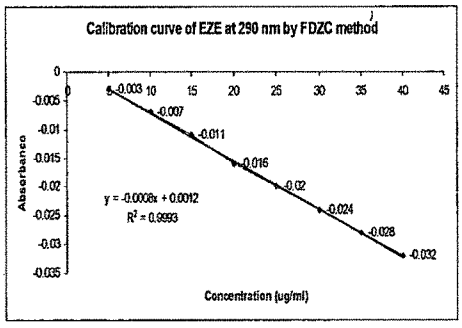


Figure 7.2.3.1.3: Calibration curve of EZE at 290.0 nm by FDZC method

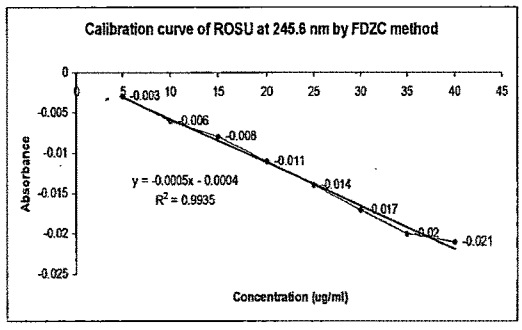


Figure 7.2.3.1.4: Calibration curve of ROSU at 245.6 nm by FDZC method

(II) Precision

Repeatability

Three different binary mixtures were prepared of EZE and ROSU, respectively and the experiments were repeated five times a day for intra day and on five different days for inter day precision. The %C.V. was reported in table 7.2.3.1.2.

Table 7.2.3.1.2: Intraday precision data of EZE and ROSU by FDZC Method

Concentration (µg/ml)		Intraday		Interday	
EZE	ROSU	EZE at 290.0 nm	ROSU at 245.6 nm	EZE at 290.0 nm	ROSU at 245.6 nm
10	10	-0.007 ± 0.83	-0.005 ± 0.74	-0.007 ± 0.48	-0.005 ± 0.74
20	20	-0.016 ± 0.57	-0.011 ± 0.27	-0.016 ± 0.66	-0.011 ± 0.64
30	30	-0.024 ± 0.38	-0.017 ± 0.43	-0.024 ± 0.28	-0.017 ± 0.21

***Mean value of five determinations**

Reproducibility:

The % CV of the responses confirms the reproducibility of the method for determination of EZE and ROSU. The data is reported in Table 7.2.3.1.3.

Table 7.2.3.1.3: Reproducibility data of EZE and ROSU by FDZCMethod

Spectrophototmetric methods		Concentrations (µg/ml)					
		10		15		20	
		UV 1700	UV 1601	UV 1700	UV 1601	UV 1700	UV 1601
First derivative zero crossing method(Mean	EZE (290.00 nm)	-0.006± 0.29	-0.006 ±0.48	-0.014 ±0.47	-0.014 ±0.14	-0.016 ±0.32	-0.016 ±0.68

(n=5) ± %C.V.)	**ROSU (245.60 nm)**	-0.006 ±0.82	-0.006 ±0.73	-0.008 ±0.56	-0.008 ±0.83	-0.011 ±0.38	-0.011 ±0.62

*** Mean value of three determinations**

(III) Accuracy

Accuracy of the method was determined by performing recovery study on laboratory prepared synthetic mixture by standard addition method. The result of % mean recovery values obtained is reported in table 7.2.3.1.4.

Table 7.2.3.1.4: Accuracy data of EZE and ROSU by spectrophotmetery **method**

Spectrophotometic methods		**EZE**	**ROSU**	**EZE**	**ROSU**	**EZE**	**ROSU**
Initial conc.(µg/ml) (A)		**10**	**10**	**10**	**10**	**10**	**10**
Quantity of std. Added (µg/ml) (B)		**5**	**5**	**10**	**10**	**15**	**15**
Total Amount (A + B)		**15**	**15**	**20**	**20**	**25**	**25**
First derivative zero crossing method (Mean (n=5) ± %C.V.)	**Total quantity Found Mean ± S.D.**	15.00 ± 0.032	14.88 ± 0.073	20.43 ± 0.052	20.21 ± 0.082	24.88 ± 0.29	25.33 ± 0.083
	% Recovery± S.D	100.00 ± 0.073	99.20 ± 0.32	102.15 ± 0.20	101.05 ± 0.83	99.52 ± 0.022	101.32 ± 0.54

*** Mean value of five determinations.**

(IV) Limit of detection

The minimum detectable concentration of EZE and ROSU were 0.43 µg/ml and 0.69 µg/ml, respectively by FDZC method.

(V) Limit of quantification

The lowest quantifiable concentrations of EZE and ROSU by practical observation were 1.44 µg/ml and 2.89 µg/ml by FDZC method.

7.2.3.1.4.2. Applicability of the method for the analysis of commercial tablet formulation:

Ten tablets of **EZEDOC** and ten tablets of **NOVASTAT** were taken and average weight was found out. Then powder was made in a mortar and pestle. The Tablet powder equivalent to one tablet was transferred from powder of both tablets into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution was sonicated for 10

min and supernatant was filtered through Whatman filter paper no. 41. The absorbance for FDZC method was observed at 290and 245.6 nm. The concentrations of EZE and ROSU in tablets were calculated using the equation mentioned earlier (7.2.3.1.2). Results obtained are reported in Table 7.2.3.1.5.

Table 7.2.3.1.5: Estimation of EZE and ROSU in tablet by FDZC method.

Formu-lation	Drugs	Labeled Claim (mg/tab)	FDZC	
			*Amount found	% Labeled Claim
EZEDOC + NOVASTAT	EZE	10	10.03	100.32
	ROSU	10	10.01	104.12
LAB. MIX.	EZE	10	9.99	99.98
	ROSU	10	10.02	100.21

* Mean value of five determinations.

7.2.3.1.5. Summary of Validation parameters:

A summary of the results of validation parameters is reported in Table 7.2.3.1.9.

Table 7.2.3.1.9: Summary of Validation parameters of FDZC method

Sr. No	Parameters	FDZC	
		EZE	ROSU
1	Analytical wavelengths (nm)	290.00	245.60
2	Linearity range (µg/ml)	5 to 40	
3	Regression equation	Y=-0.0008x +0.0012	Y=-0.0005x -0.0004
4	Correlation coefficient (r^2)	0.9993	0.9935
5	Intercept	0.0012	-0.0004
6	Slope	-0.0008	-0.0005
7	% Assay	100.32	104.12
8	Accuracy and precision		
	Intra day % CV (n = 5)	0.38-0.83	0.27-0.74
	Inter day % CV (n = 5)	0.48-0.66	0.21-0.74
	Repeatability of measurements % CV	<1%	<1%
	% Recovery	99.52-102.15	99.20-101.32

10	Limit of detection (µg/ml)	0.43	0.69
11	Limit of quantification (µg/ml)	1.44	2.89

7.2.3.1.6. CONCLUSION

1st order zero crossing spectrophotometric method is a suitable technique for the reliable analysis of commercial formulations containing combinations of EZE and ROSU. High % recovery greater than 98 % shows that the method is free from the interference of excipients used in the formulation (Table 7.2.3.2.9).

7.2.3.2 Infra Red spectroscopic method.

Experimental work:

7.2.3.2.1. Preparation of standard stock for EZE and ROSU

For **quantitative IR** spectroscopic study 10 mg of both standard drugs were weighed accurately and made up to 100 mg on same butter paper to obtain final concentration of 100 µg/mg of each drug. This sample was triturated in mortar and pestle and sample was used as working standard sample for FTIR spectroscopic method.

7.2.3.2.2. Selection of analytical wavenumber:

Determination of zero absorbance point for analysis: Individual IR spectrum of both the drugs and their mixture were overlain (fig. 3.2.1.3.1) to select the zero absorption point. It was observed that 1879cm^{-1} is the zero absorbance point for ROSU so EZE was estimated at this frequency where as ROSU was estimated at 965cm^{-1} at which EZE showed zero absorbance.

7.2.3.2.3. Calibration curve:

Appropriate fractions were transferred from standard (100 µg/mg) to butter paper and weight adjusted 10 mg with KBr to get minimum transmission. At 1879cm^{-1} and 965cm^{-1} peak height was measured for EZE and ROSU, respectively. Calibration curves were drawn using the multiple linear regression (MLR) method and the drug amount in sample was computed from regression equation.

7.2.3.2.4. Validation of method:

Procedure for validation of method was given in Chapter 7.2.1.2.4.

7.2.3.2.5. Results and Discussion:

7.2.3.2.5.1 Validation Parameters

(I) Linearity

The IR spectrophotometry method showed good linearity for EZE in the range of 12.9 to 147 µg/mg at 1879 cm^{-1} (zero absorbance point of ROSU) with co-relation co-efficient of 0.9995 (fig. 7.2.3.2.4). For ROSU the line of best fit was obtained at 965 cm^{-1} (zero absorbance point of EZE) with correlation coefficients of 0.9998 in linearity range of 2.75 to 86.2 µg/ml (fig. 7.2.3.2.5). Calibration data is given in Table 7.2.3.2.1.

Table 7.2.3.2.1: Calibration data of EZE and ROSU by IR Spectroscopy

Sr.No.	Concentration (µg/mg)		Peak height* ± S.D. (N=5)		% C.V.	
	EZE	ROSU	EZE at 1879 1/cm	ROSU 965 1/cm	EZE	ROSU
1	0.0129	0.00275	0.0011±0.00032	0.045±0.00038	0.59	0.39
2	0.0343	0.0413	0.0229±0.0059	0.366±0.00028	0.25	0.65
3	0.0412	0.0683	0.0304±0.0029	1.068±0.00053	0.19	0.21
4	0.147	0.0862	0.157±0.00048	1.571±0.00072	0.82	0.50

*Mean value of five determinations.

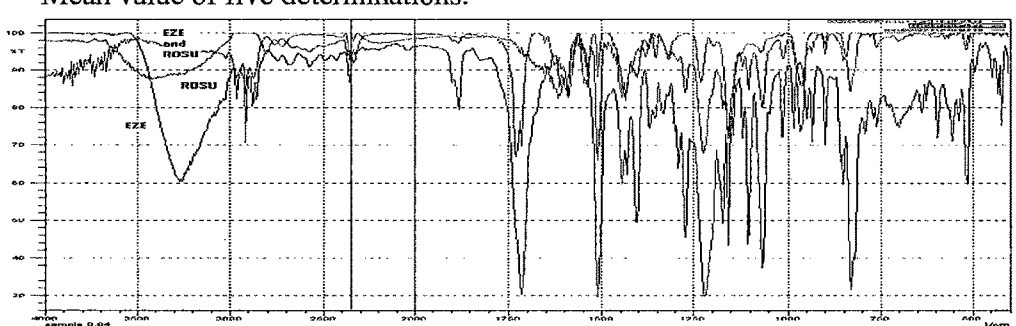


Figure: 7.2.3.2.1: Overlain IR spectra of EZE and ROSU

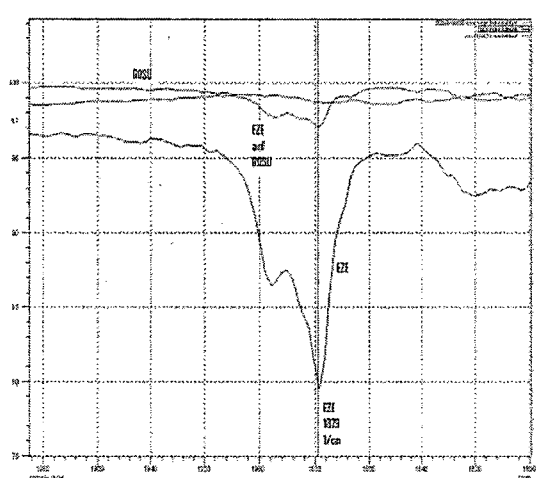


Figure: 7.2.3.2.2: Overlain IR spectra of ROSU and EZE at 1879 cm^{-1}

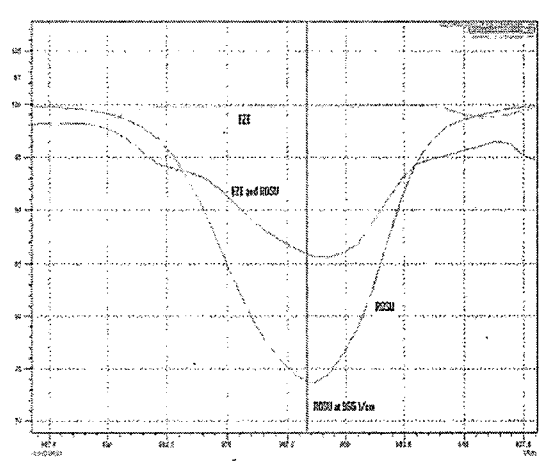


Figure: 7.2.3.2.3: Overlain IR spectra of ROSU and EZE at 965 cm^{-1}

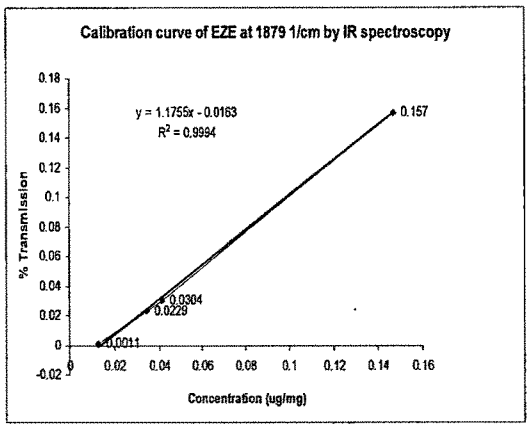


Figure 7.2.3.2.4: Calibration curve of EZE at 1879cm^{-1} by IR method
Correlation co-efficient = **0.9994**
Slope = 1.1755
Intercept = -0.0163
Regression equation: **Abs. = 1.1755 × Conc. -0.0163**

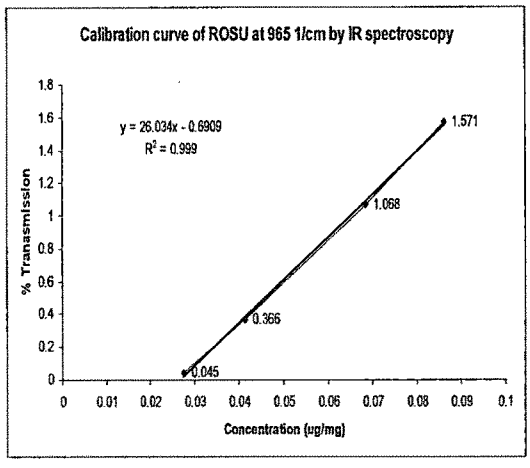


Figure 7.2.3.2.5: Calibration curve of ROSU at 965 cm^{-1} by IR method
Correlation co-efficient = **0.999**
Slope = 26.034
Intercept = -0.6909
Regression equation: **Abs. = 26.034 × Conc. -0.6909**

(II) Precision

Repeatability

The method was found to be precise on intra day and inter day basis as the average % CV values were in the range of 0.48 – 0.72 % and 0.36 – 0.81% for EZE and 0.53 – 0.82 % and 0.33 – 0.88 % for ROSU (Table 7.2.3.2.2), respectively.

Table 7.2.3.2.2: Intraday precision data of EZE and ROSU by IR Spectroscopy

Concentration (µg/mg)		Peak height±% C.V*.			
		Indraday		Interday	
EZE	ROSU	EZE at 1879 cm^{-1}	ROSU at 965 cm^{-1}	EZE at 1879 cm^{-1}	ROSU at 965 cm^{-1}
15	15	0.00131±0.52	0.1312±0.53	0.00138±0.52	0.1374±0.82
30	30	0.0220±0.48	0.2816±0.82	0.0221±0.81	0.2660±0.33
45	45	0.0702±0.72	0.7238±0.75	0.0788±0.36	0.722±0.74

* Mean value of five determinations.

Reproducibility:

The % CV of the responses for determination of EZE and ROSU revealing the reproducibility of the method is reported in Table 7.2.3.2.3.

Table 7.2.3.2.3: Reproducibility data of EZE and ROSU by IR Spectroscopy

Concentration (µg/mg)		Peak height ± SD*			
EZE	ROSU	EZE at 1879 cm^{-1} ± %CV		ROSU at 965 cm^{-1} ± %CV	
		Sample compartment I	Sample compartment II	Sample compartment I	Sample compartment II
15	15	0.00138 ± 0.62	0.00139 ± 0.92	0.1374 ± 0.045	0.1338 ± 0.44
30	30	0.0225 ± 0.42	0.0221 ± 0.51	0.2660 ± 0.52	0.2832 ± 0.91
45	45	0.0708 ± 0.72	0.0702 ± 0.14	0.722 ± 0 .77	0.7165 ± 0.18

***Mean value of five determinations**

(III) Accuracy

The method showed % mean recovery for EZE in the range of 98.80 – 100.06 % and for ROSU it was 99.40 – 100.13 % in synthetic mixture (Table 7.2.3.2.4).

Table 7.2.3.2.4: Accuracy data of EZE and ROSU by IR Spectroscopy

Initial conc. (µg/mg) (A)		Quantity of std. Added (µg/mg) (B)		Total Amount (A + B)		Total quantity Found Mean ± S.D.		% Recovery± S.D*	
EZE	ROSU	EZE	ROSU	EZE	ROSU	EZE at 1879 cm^{-1}	ROSU at 965 cm^{-1}	EZE	ROSU
10	10	5	5	15	15	14.82 ± 0.051	15.02 ± 0.033	98.80 ± 0.043	100.13 ± 0.055
10	10	15	15	25	25	24.92 ± 0.074	24.98 ± 0.021	99.68 ± 0.062	99.92 ± 0.14
10	10	25	25	35	35	35.02 ± 0.071	34.79 ± 0.066	100.06 ± 0.82	99.40 ± 0.013

*** Mean value of three determinations**

(IV) Limit of detection

The minimum detectable concentration of EZE and ROSU were found to be 7.8034 µg/mg and 0.4521 µg/mg, respectively.

(V) Limit of quantification

The lowest quantifiable concentration of EZE and ROSU were found to be 10.6780 µg/mg and 1.5070 µg/mg, respectively by practically observation.

7.2.3.2.1.2. Applicability of the method for the analysis of commercial tablet formulation:

The procedure for preparation of binary mixture was given in Chapter 7.2.3.1.5.2. A synthetic mixture was prepared having EZE and ROSU in ratio of 1:1. DRIFT spectra

were scanned between 4000 and 400 cm^{-1}, by averaging 16 scans for each spectrum with a resolution of 4 cm^{-1} (data point resolution per interval 1 cm^{-1}). 1879 cm^{-1} and 965 cm^{-1} were selected for estimation for EZE and ROSU, at single wavelength, respectively. Concentration of sample was found from calibration curve of EZE and ROSU.

Table 7.2.3.2.5: Estimation of EZE and ROSU in tablet by IR spectroscopy.

Formulation	DRUGS	Labeled Claim (mg/tab)	QIR	
			*Amount found ± SD	% Assay ± SD
EZEDOC & NOVASTAT	EZE	10.00	100.41 ± 0.057	10.04 ± 0.033
	ROSU	10.00	101.12 ± 0.022	10.11 ± 0.027
LAB. MIX.	EZE	10.00	100.92 ± 0.048	10.09 ± 0.046
	ROSU	10.00	102.30 ± 0.073	10.23 ± 0.021

*** Mean value of five determinations.**

7.2.3.2.2. Summary of Validation parameters:

The summary of validation parameters were reported in table 7.2.3.2.6.

Table 7.2.3.2.6: Summary of Validation parameters of IR method

Sr. No	Parameters	Results	
		EZE	ROSU
1	Wave number (cm^{-1})	1879	965
2	Linearity range (µg/mg)	12.9-147	2.75-86.2
3	Regression equation	Y=1.175x -0.0163	Y=26.03x -0.6909
4	Correlation coefficient (r^2)	0.9994	0.9990
5	Intercept	-0.0163	-0.6909
6	Slope	1.175	26.03
7	Assay %	100.41-100.92	101.12-102.30
8	Precision		
9	Intra day % CV (n = 5)	0.48-0.72	0.53-0.82
	Inter day % CV (n = 5)	0.36-0.81	0.33-0.88

	Reproducibility of measurements % CV	< 1 %	
	% Recovery	98.80-100.06	99.40-100.13
10	Limit of detection (µg/mg)	7.8034	0.4521
11	Limit of quantification (µg/mg)	10.67	1.5070

7.2.3.2.3. CONCLUSION

The proposed method is simple, precise and accurate and can be used as a method for quality control of pharmaceuticals. This technique extends the use of a standard IR spectrophotometer typically used for identification purposes, to the reliable quantification of EZE and ROSU. The present method opens the possibility of applying IR spectroscopy to quantify other active ingredients.

7.2.3.3. Stability Indicating Reverse Phase High Performance Liquid Chromatography (HPLC) with UV detection.

7.2.3.3.1. Optimization of method:

Determination of solvent for sample preparation:

Different solvent were tried to assess the solubility of the drugs as per Chapter 6. Both the drugs were soluble in methanol and ACN, but with methanol as a solvent, large tailing effect were observed. So ACN was selected as a solvent to prepare drug solution.

Determination of detection wavelength

Detection wavelength was selected by scanning standard solutions of the drugs over 200 nm to 400 nm wavelengths. The overlain spectra of ROSU and EZE (fig.) showed thatboth the drugs have significant absorption in the wavelength region between 225-255 nm, and 248 nm was selected as detection wavelength.

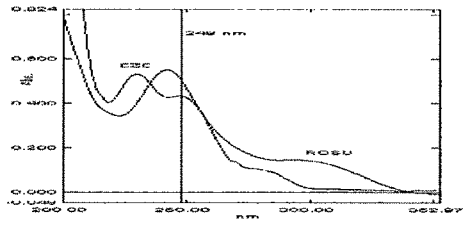


Figure 7.2.3.3.1: overlain UV spectrum of EZE and ROSU in acetonitrile

Optimization of mobile phase:

Various combinations of mobile phase were tried at a flow rate of 1 ml/min on a C18 Phenomenex column. The observations are shown in Table 7.2.3.3.2.

Table 7.2.3.3.1: Determination of mobile phase

Mobile phase combination	Ration (V/V)	Retention time (min)		Peak shape	
		EZE	ROSU	EZE	ROSU
Acetonitrile: 0.1 % Formic acid	60: 40	10.978	4.5	Slight broad	Near to blank and broad
Acetonitrile: 0.5 % Formic acid	60: 40	9.672	3.311	Sharp	Sharp but merge with blank
Acetonitrile: 0.5 % Formic acid	**40:60**	**23.325**	**9.458**	**Sharp**	**Sharp**
Acetonitrile: 0.5 % Formic acid	**50:50**	**12.075**	**6.525**	**Sharp**	**Sharp**
Acetonitrile: 0.2 % Formic acid	50:50	11.182	6.423	Peak become slight broad	Peak become slight broad

Both these mobile phased were tried for the separation of degradate and the resolution was found to be better with the mobile phase of ACN:FA (40 : 60) so it was selected as the mobile phase for SIA method.

Column

Two type of C18 column were tried.

Table 7.2.3.3.2: Selection of column

Column (C18, 250 X 4.60 mm)	Retention time (min)		Peak Shape	
	EZE	ROSU	EZE	ROSU
Hypersil	23.183	9.521	Sharp	Sharp
Phenomenex	**23.325**	**9.458**	**Sharp**	**Sharp**

Both the columns were suitable for development of HPLC analytical method, but C18 Phenomenex 240 X 4.6 mm was selected. The main aim of the method was to resolve the drugs in presence of degradation products. So Phenomenex C_{18} column (250 mm x 4.6 mm i.d., 5 µm particle size) was preferred as it has high carbon loading with very closely packed material to give high resolution.

Method:

After optimizing all the parameters, following chromatographic conditions were used for the development of a precise, accurate, specific and suitable stability indicating RP-HPLC methods for the estimation of EZE and ROSU.

Chromatographic conditions:

For method A:

- **Column:** C_{18} (size-250 x 4.60 mm, I.D-5 µm) (Phenomenex)
- **Mobile Phase**: **Method A**: Acetonitrile: 0.5% Formic acid (50:50 v/v)
  Method B: Acetonitrile: 0.5% Formic acid (40:60 v/v)
- **Detection:** UV detection at 248 nm
- **Flow rate: Method A** :1.0 ml/minute
  Method B: 1.5 ml/min
- **Application volume:** 20 µl

7.2.3.3.2. Preparation of stock solution:

For chromatographic study standard EZE and ROSU (100 mg) were weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml ACN and diluted up to mark with ACN to obtain final concentration of 1000 µg/ml.

7.2.2.3.3. Calibration curve of EZE and ROSU

From the stock solution (1000 µg/ml), aliquots of 0.1, 0.5, 1, 1.5, 2, 2.5, and 3 ml were transferred to a series of 10 ml volumetric flasks. The volume was adjusted up to the mark with ACN to get final concentrations of 10, 50, 100, 150, 200, 250, and 300 µg/ml of solution. 20 µl of all these solutions were injected separately into HPLC column and the peak area of each solution was measured at selected wavelength. The calibration curve was constructed by plotting peak area vs. concentration (µg/ml) over the concentration range and regression equation was calculated.

7.2.2.3.4. Validation parameters:

Procedure for validation of analytical method was given in Chapter 7.1.1.6.3.

7.2.2.3.5. Results and Discussion:

7.2.3.3.5.1. Validation Parameters for method A and B:

(I) Linearity

The RP-HPLC method showed good linearity for EZE in the range of 10-312 µg/ml with co-relation co-efficient, of 0.9999 as seen in fig. 7.2.3.3.4. For ROSU the linearity range was 11.3 – 339 µg/ml and the line of best fit was obtained with

correlation coefficient of 0.998 as seen in fig. 7.2.3.3.5. Calibration data is given in Table 7.2.3.3.4 by method A

Table 7.2.3.3.3: Calibration data of EZE and ROSU by HPLC method A

Sr. No.	Concentration (µg/ml)		EZE			ROSU		
			Peak Area		RT*	Peak Area		RT*
	EZE	ROSU	Mean*	%CV		Mean*	%CV	
1	10.4	11.3	478093	0.75	12.072	45141	0.32	6.741
2	50.2	56.5	2307719	0.32	12.084	234274	0.54	6.723
3	104	113	4780932	0.53	12.083	448549	0.55	6.745
4	208	226	9561864	0.22	12.083	907098	0.73	6.752
5	312	339	14342796	0.93	12.095	1245647	0.82	6.758

***Average of five readings**

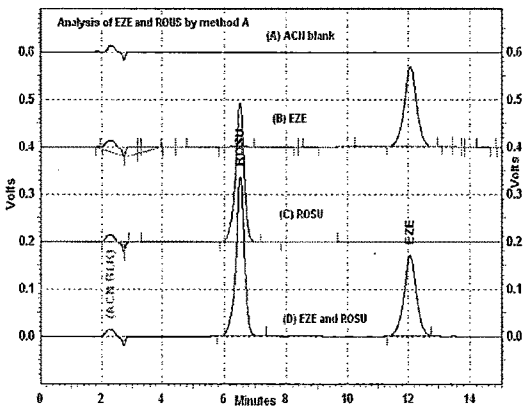


Figure 7.2.3.3.2: Peak of EZE(104 µg/ml) and ROSU(113 µg/ml) by HPLC Method A

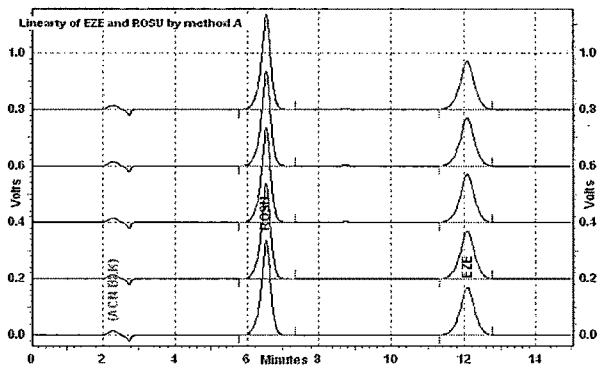


Figure 7.2.3.3.3: Peak of EZE(104 µg/ml) and ROSU(113 µg/ml) by HPLC Method A

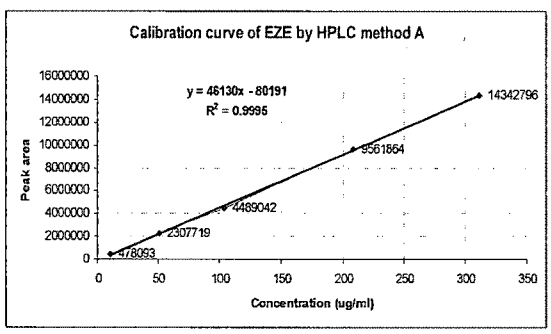


Figure 7.2.3.3.4: Calibration curve of EZE by HPLC (Peak Area)

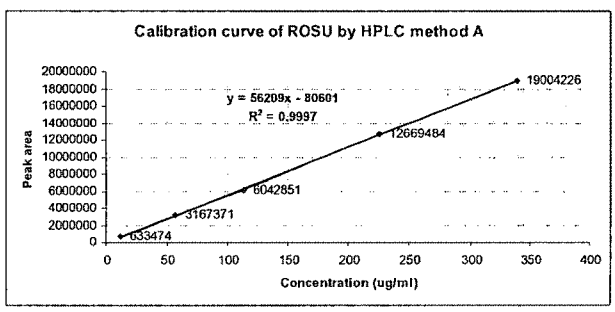


Figure 7.2.3.3.5: Calibration curve of ROSU by HPLC (Peak Area)

7.2.3.3.1.1. Validation Parameters for method B:

(I) Linearity

The RP-HPLC method showed good linearity for EZE in the range of 10.4-312 µg/ml with co-relation co-efficient, of 0.9998 as seen in fig. 7.2.3.3.6. For ROSU the linearity range was 113 – 339 µg/ml and the line of best fit was obtained with correlation coefficient of 0.9962 as seen in fig. 7.2.3.3.7. Calibration data is given in Table 7.2.3.3.4.

Table 7.2.3.3.4: Calibration data of EZE and ROSU by HPLC method B

Sr. No.	Concentration (µg/ml)		EZE			ROSU		
			Peak Area		RT*	Peak Area		RT*
	EZE	ROSU	Mean*	%CV		Mean*	%CV	
1	10.4	11.3	332242	0.84	23.425	45141	0.43	9.459
2	50.2	56.5	1643224	0.73	23.427	234274	0.65	9.458
3	104	113	3321421	0.63	23.425	448549	0.24	9.458
4	208	226	6698762	0.28	23.425	907098	0.55	9.459
5	312	339	9864263	0.47	23.428	1245647	0.83	9.458

***Average of five readings**

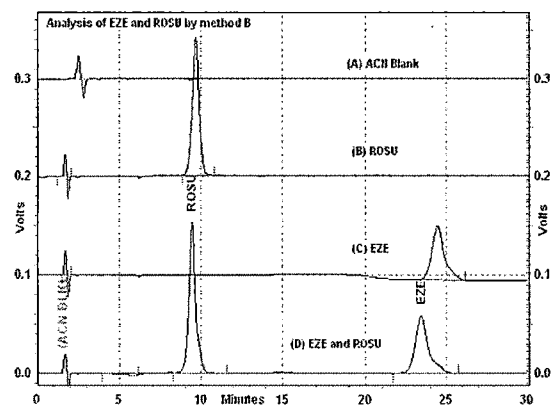


Figure 7.2.3.3.6: (A) Peak of EZE(104 µg/ml) and (B) ROSU(113 µg/ml) and (C) combination of EZE and ROSU by HPLC with UV detection

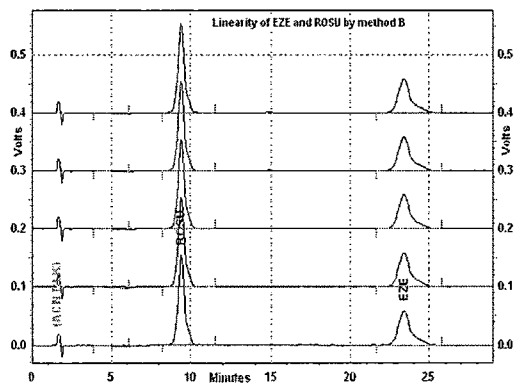


Figure 7.2.3.3.7: Peaks of binary mixture of EZE and ROSU by HPLC By method B

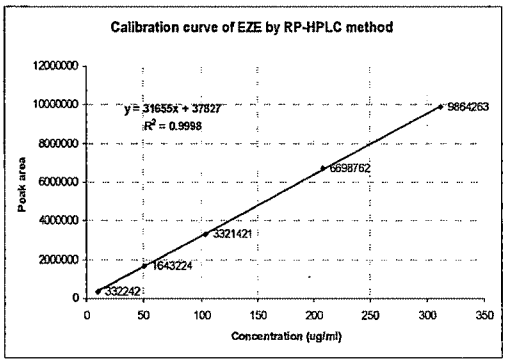


Figure 7.2.3.3.8: Calibration curve of EZE by HPLC (Peak Area) method B

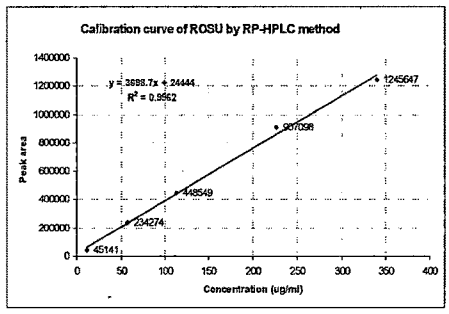


Figure 7.2.3.3.9: Calibration curve of ROSU by HPLC (Peak Area) Method B

(II) Precision

Repeatability

The intraday and interday precision data were reported in table 7.2.3.3.5 and 7.2.3.3.6 for method A and B.

Table 7.2.3.3.5: Intraday precision data of EZE and ROSU by RP-HPLC method

Concentration (µg/ml)		Peak area (Mean ± % C.V.) (n=5)			
		Method A		Method B	
EZE	ROSU	EZE	ROSU	EZE	ROSU
60	60	2758230 ± 0.56	336358 ± 0.75	1916781 ± 0.64	269687 ± 0.63
120	120	5516460 ± 0.83	672716 ± 0.32	3928025 ± 0.74	497573 ± 0.38
240	240	11032920 ± 0.22	984543 ± 0.43	7664818 ± 0.38	952670 ± 0.41
Table 7.2.3.3.6: Interday precision data of EZE and ROSU by RP-HPLC method					
60	60	2726970 ± 0.76	336470 ± 0.88	1914947 ± 0.57	268343 ± 0.82
120	120	5647016 ± 0.82	677929 ± 0.29	3928025 ± 0.82	498932 ± 0.67
240	240	11075673 ± 0.21	985115 ± 0.17	7664818 ± 0.59	953482 ± 0.99

***Average of five readings**

Reproducibility:

The % CV of the responses for determination of EZE and ROSU are mentioned in Table 7.2.3.3.7, respectively.

Table 7.2.3.3.7: Reproducibility data of EZE and ROSU by RP-HPLC method

Spectrophototmetric methods		Peak area ± % CV					
		60		120		240	
		Hypersil	Pheno menex	Hypersil	Pheno menex	Hypersil	Pheno menex
Method A	EZE	2736049 ± 0.46	2753445 ± 0.83	5572897 ± 0.83	5501842 ± 0.27	11518699 ± 0.35	11183203 ± 0.27
	ROSU	339137 ± 0.82	345646 ± 0.51	673747 ± 0.16	676418 ± 0.62	988232 ± 0.81	988799 ± 0.22
Method B	EZE	1913878 ± 0.37	1993452 ± 0.72	3928025 ± 0.16	3942115 ± 0.17	7664818 ± 0.27	7634212 ± 0.38
	ROSU	268948 ± 0.27	260846 ± 0.22	497573 ± 0.87	499124 ± 0.71	954123 ± 0.16	952789 ± 0.41

***Average of three readings**

(III) Accuracy

The % mean recovery for EZE and ROSU were reported in table 7.2.3.3.8.

Table 7.2.3.3.8: Accuracy data of EZE and ROSU by RP-HPLC method

		EZE	ROSU	EZE	ROSU	EZE	ROSU
Initial conc.(µg/ml) (A)		100	100	100	100	100	100
Quantity of std. Added (µg/ml) (B)		50	50	100	100	150	150
Total Amount (A + B)		150	150	200	200	250	250
Method A	Total quantity Found Mean ± S.D.	149 ± 0.032	148 ± 0.035	200.93 ± 0.053	201 ± 0.025	248.95 ± 0.053	251.03 ± 0.022
	% Recovery± %CV	99.33 ± 0.22	98.67± 0.15	100.47 ± 0.59	100.50± 0.74	99.58 ± 0.39	100.41± 0.82
Method B	Total quantity Found Mean ± S.D.	149.5 ± 0.023	150.34 ± 0.062	204.03 ± 0.084	200.42 ± 0.082	251.42 ± 0.072	250.33 ± 0.025
	% Recovery± %CV	99.67 ± 0.82	100.23 ± 0.24	102.02 ± 0.52	100.21± 0.32	100.57 ± 0.17	100.13 ± 0.82

***Average of five readings**

(IV) Limit of detection

The minimum detectable concentration of EZE and ROSU were found to be 0.064 µg/ml and 0.028 µg/ml and 0.281 µg/ml and 0.592 µg/ml, respectively by method A and B.

(V) Limit of quantification

The lowest quantifiable concentration of EZE and ROSU were found to be 0.487 µg/ml and 0.117 µg/ml and 0.425 µg/ml and 0.993 µg/ml, respectively by method A and B.

7.2.3.3.1.2. Applicability of the method:

The procedure for preparation of binary mixture is given in Chapter 6. A synthetic mixture was prepared having EZE and ROSU in ratio of 1:1. That was used for both methods.

(A) Analysis of tablet formulation

20 µl of the prepared solution was injected into HPLC column and the peak area was measured at 248 nm. The concentration of EZE and ROSU were found from regression equation of EZE and ROSU for both the method A and B. The results are shown in Table 7.2.3.3.9.

Table 7.2.3.3.9: Estimation of EZE and ROSU in tablet by RP-HPLC method A.

Formu-lation	DRUGS	Labeled Claim (mg/tab)	Method A		Method B	
			*Amount found ± S.D.	% Assay ± S.D.	*Amount found ± S.D.	% Assay ± S.D.
EZEDOC & NOVASTAT	EZE	10	9.98±0.034	99.81±0.054	10.02±0.072	100.24±0.052
	ROSU	10	10.04±0.062	100.45±0.062	10.30±0.083	103.03±0.029
LAB. MIX.	EZE	10	10.05±0.024	100.52±0.082	9.98±0.037	99.83±0.073
	ROSU	10	10.13±0.062	101.35±0.083	10.04±0.073	100.42±0.066

*** Mean value of five determinations.**

(B) Analysis of drug in degradation products by method A and B

Efforts were made to analyze EZE and ROSU in presence of their degradation products using the proposed method. The conditions used for forced degradation were attenuated to achieve degradation in the range of 20 – 80 % to establish the stability indicating.

Procedure for forced degradation study

Forced degradation study was done in different conditions. Solvents tried were 30% H_2O_2, water at neutral pH 7, 0.5 N HCl and 0.1 N NaOH. Approximate 25 mg drug was accurately weighed and transferred to 25 ml volumetric flask. The drug was dissolved in 5 ml of methanol then diluted with the solvent selected for degradation. Similarly solid-state stability was studied by exposing 25 mg of drug to 80°C stability oven and 25 mg drug to photostablity chamber. Samples were collected for analysis at two stages, at 0 min (as soon as sample was prepared), after 24 hrs and after 48 hrs of

exposure to degradation condition. Sample was prepared by taking 2 ml of degraded solution in 10 ml volumetric flask and made up to 10 ml with ACN and 20µl of that was injected in HPLC column. Fig 7.2.3.3.10 to 7.2.3.3.17 shows the chromatograms of forced degraded samples.

Degradation Peak by method A:

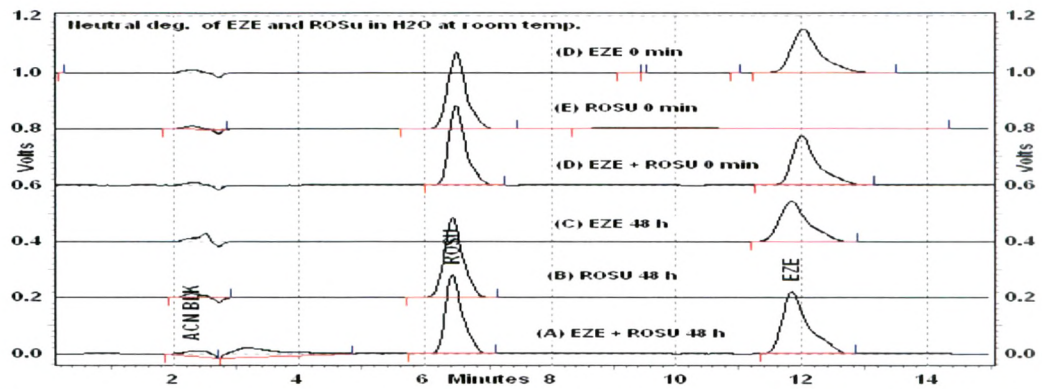


Figure 7.2.3.3.10: Chromatograms of neutral degradation at Rt of EZE and ROSU

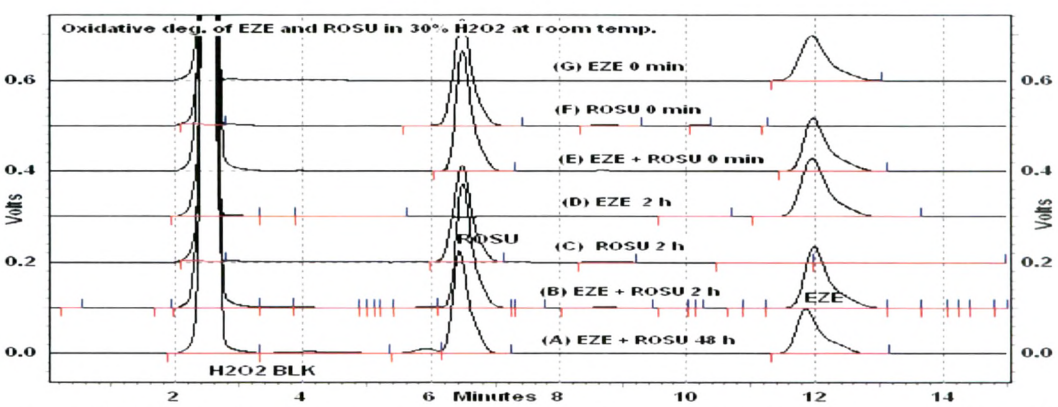


Figure 7.2.3.3.11: Chromatograms of oxidative-degraded of EZE and ROSU at RT

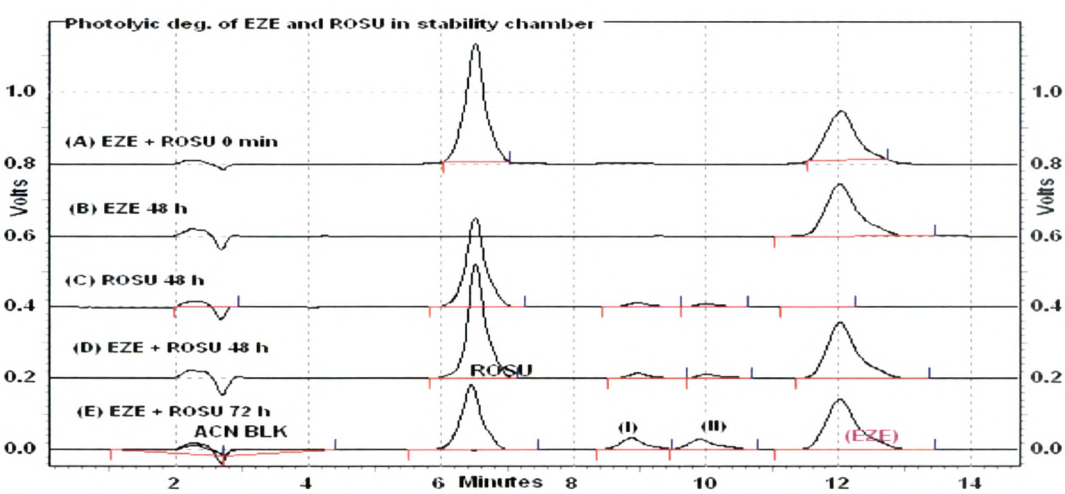


Figure 7.2.3.3.12: Chromatogram of EZE and ROSU in photostability chamber

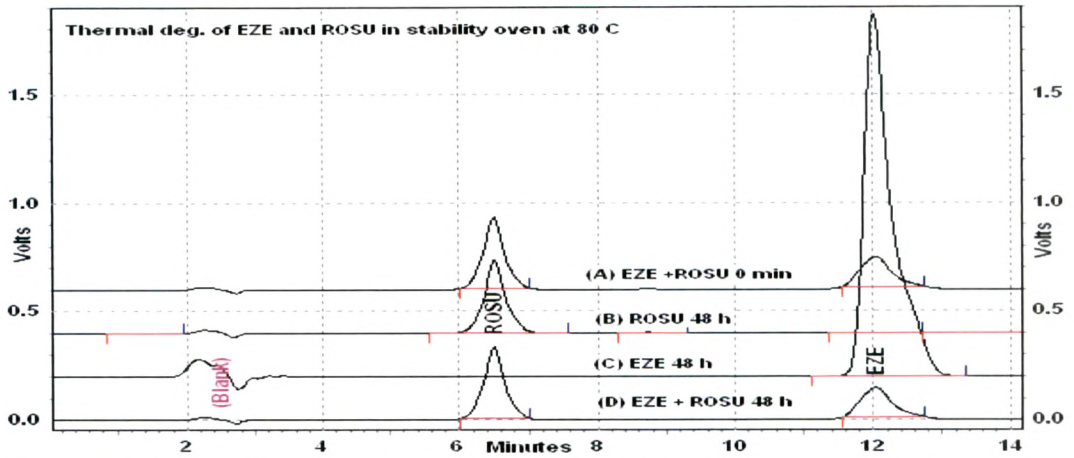


Figure 7.2.3.3.13: Chromatogram of EZE and ROSU in thermal 80C after 48 hrs in Stability oven

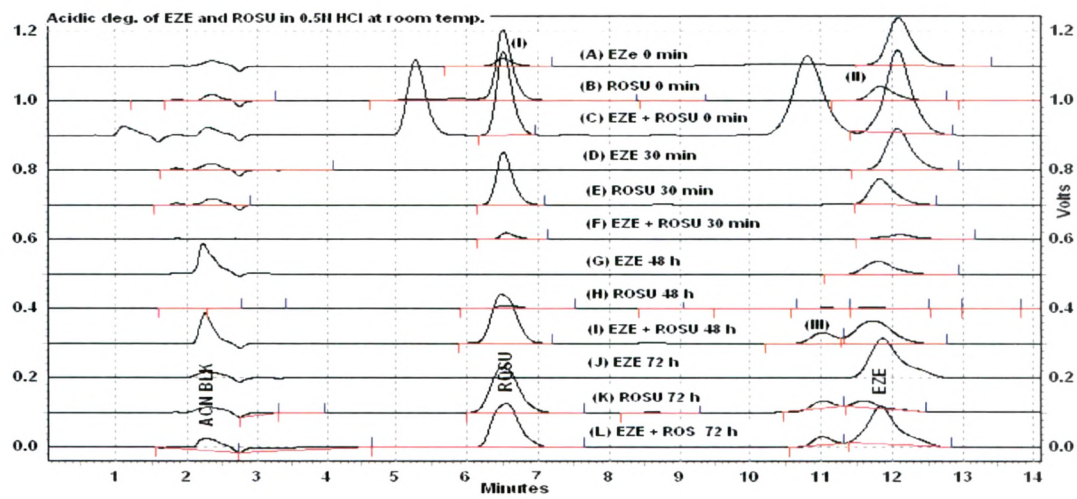


Figure 7.2.3.3.14: Chromatograms of acid hydrolysis of EZE and ROSU at RT

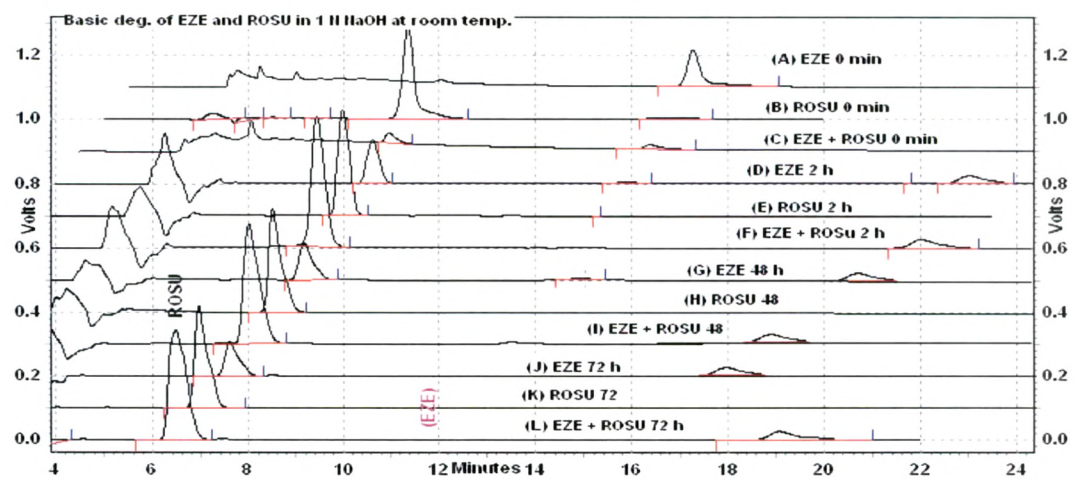


Figure7.2.3.3.15: Chromatograms of base hydrolysis of EZE and ROSU at RT

Degradation Peak by method B :

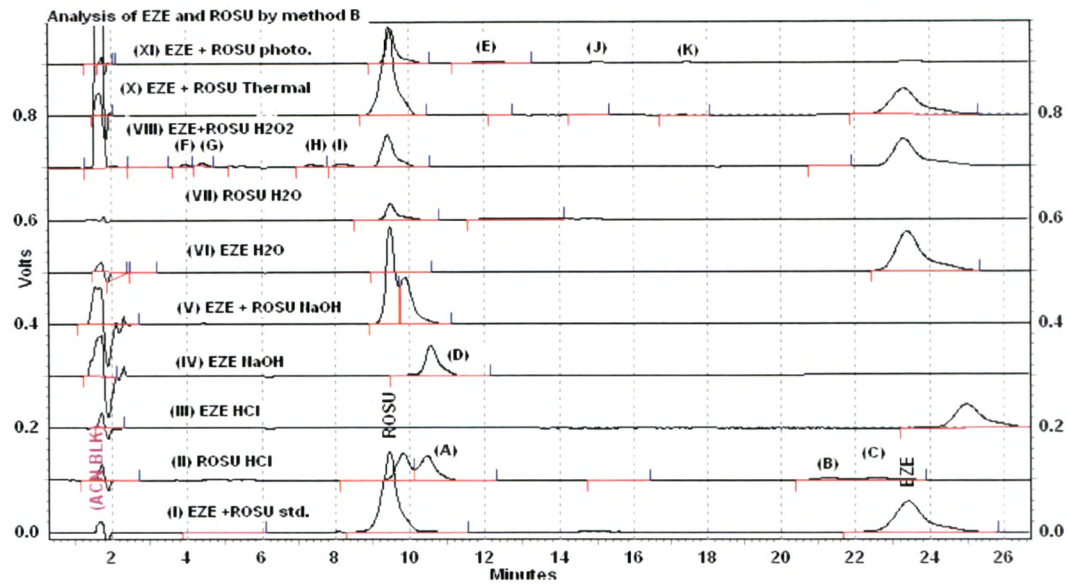


Figure 7.2.3.3.16: Chromatogram of degradation of EZE and ROSU in all condition by method B

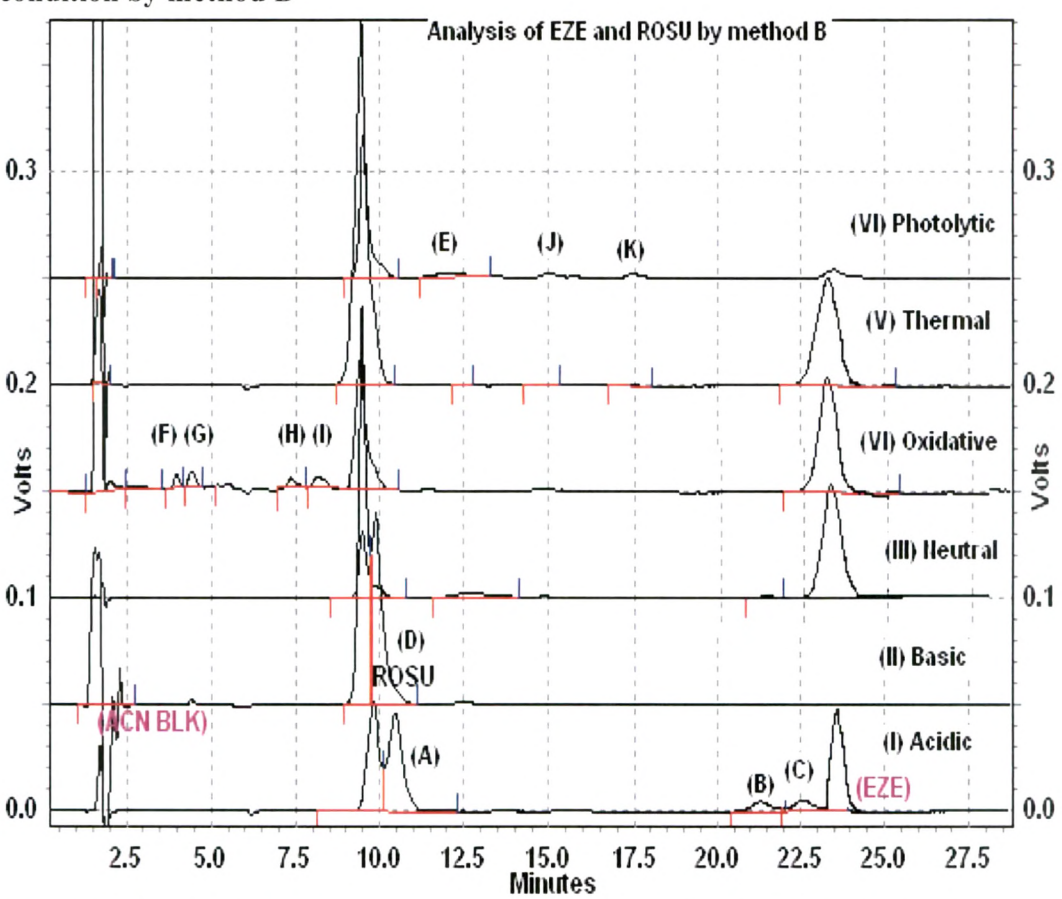


Figure 7.2.3.3.17 : Chromatogram of degradation of EZE and ROSU in all condition by method B after 48 hrs

Table 7.2.3.3.10: Percentage degradation of EZE and ROSU

Sr. No	Parameters (Stress condition /duration/state)	% of undergrad		% of individual Degradation products										Total % Deg.	
		EZE	ROSU	A	B	C	D	E	F	G	H	I	J	K	EZE	ROSU
1	Neutral/H2Oat pH 7/4 h/ sol./RT	-	100	-	-	-	100	-	-	-	-	-	-	-	100	No Deg.
2	Acidic/0.5 N HCl/48 h/ sol./RT	98	42	43	7	8	2	-	-	-	-	-	-	-	2	38
3	Alkali/0.1N NaOH/48 h/ sol./RT	-	100	-	-	-	100	-	-	-	-	-	-	-	100	No Deg.
4	Oxidative/30% H2O2/48 h/ sol./RT	100	80	-	-	-	-	-	4	5	5	6	-	-	No Deg.	20
5	Thermal/80 C/48 h/solid	100	97	-	-	-	-	1	-	-	-	-	1	1	No Deg.	3
6	Photo/uv254 and Vis/366 nm/48 h/solid	100	85	-	-	-	-	8	-	-	-	-	3	4	No Deg.	15

D- EZE degraded product. A to C and E to K- ROSU degraded products.

Table 7.2.3.3.11: System suitability parameters of EZE and ROSU

Sr. No	System Suitability Parameters	EZE	ROSU	Degradation products					
				A	B	C	D	E	F
1	Retention tim	24.450	9.683	10.475	21.275	22.575	10.567	12.692	3.975
2	Theoretical p	254113.1	20777.32	29154.53	67442.96	106259.7	44430.66	58485.72	218.8387
3	Resolution	14.60	6.31	1.79	10.87	1.12	15.68	7.46	2.48
4	Asymmetry	1.35	0.96	0.81	1.28	1.31	1.08	1.31	0.99
5	USP width	2.77	2.00	2.19	1.64	1.94	2.68	2.56	0.50
6	Tailing factor	1.33	0.96	0.83	1.27	1.30	1.43	1.30	0.99
7	Capacity Fact	8.78	2.87	3.19	7.51	8.03	6.34	16.77	1.62
	System Suitability Parameters			G	H	I	J	K	
1	Retention time (minutes)			4.423	7.342	8.133	15.008	17.433	
2	Theoretical plates			270.9466	2157.627	2345.262	43635.37	45837.6	
3	Resolution			3.14	4.85	0.96	2.02	3.19	-
4	Asymmetry			0.87	0.92	0.81	0.35	1.85	
5	USP width			0.50	0.85	0.80	1.87	1.65	
6	Tailing factor			0.87	0.91	0.81	0.34	0.58	
7	Capacity Factor			1.96	3.89	4.42	9.01	10.62	

7.2.3.3.2. Summary of Validation parameters:

The summary of validation parameters were reported in table 7.2.3.3.12.

Table 7.2.3.3.12: Summary of Validation parameters by HPLC with UV detection

Sr. No	Parameters	Method A		Method B	
		EZE	ROSU	EZE	ROSU
1	Analytical wavelenght (nm)	248			
2	Retention time (minutes)	12.687	6.785	24.450	9.683
3	Linearity range (µg/ml)	10.4-312	11.3-339	10.4-312	11.3-339
4	Regression equation	Y=46130x -80191	Y=56209x -80601	Y=31655x +37827	Y=36987x +24444
5	Correlation coefficient (r^2)	0.9995	0.9997	0.9998	0.996
6	Intercept	-80191	-80601	37827	24444
7	Slope	46130	56209	31655	36987
8	Assay (%)	99.81	100.45	100.24	103.03
9	Precision				
	Intra day % CV (n = 5)	0.22-0.83	0.32-0.75	0.38-0.74	0.38-0.63
	Inter day % CV (n = 5)	0.21-0.82	0.17-0.88	0.57-0.82	0.67-0.99
	Repeatability of measurements %CV	< 1 %			
	% Recovery	99.33-100.47	98.67-100.50	99.67-102.02	100.13-100.23
10	Limit of detection (µg/ml)	0.064	0.028	0.281	0.592
11	Limit of quantification (µg/ml)	0.487	0.117	0.425	0.99

7.2.3.3.3. Conclusion:

The degradation study of ROSU and EZE concluded that, ROSU undergo acidic hydrolysis, oxidation and it also affected by light and heat. Which EZE undergo hydrolysis through acid, base and neutral conditions. Fig. 7.2.3.3.14 show that acid degradation products of ROSU merge with EZE standard. Fig 7.2.3.3.15 shows that basic degradation product of EZE merge with ROUS standard. SO method B was used for degradation study in that all degradation products of EZE and ROSU well separated with each other. SO method A and B can used for the simultaneous estimation of EZE and ROSU while method B can be used as SIA method for EZE and ROSU.

7.2.3.4. High Performance Thin Layer Chromatography (HPTLC) with UV detection.

For the simultaneous analysis of EZE and ROSU by HPTLC, efforts were made to select a common solvent and a common absorption wavelength.

7.2.3.4.1. Optimization of method:

Determination of solvent for sample preparation:

Different solvents were tried to study the solubility of both the drugs as stated in table 7.2.3.3.1 and methanol was selected for the preparation of drug solutions.

Determination of analytical wavelength:

Wavelength was selected by scanning standard solutions of both drugs over 200 nm to 400 nm wavelengths. Drugs show high absorbance at **248 nm** as illustrated in fig. 7.2.3.3.1. So this wavelength was selected in densitometer UV detector.

Determination of mobile phase:

Various combinations of mobile phases were tried for effective separation of EZE and ROSU on silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 µm thickness) (E. Merck). The results are reported in Table 7.2.3.4.1.

Table 7.2.3.4.1: Determination of mobile phase

Mobile phase combination	Ratio (V/V/V)	Peak separation
Chloroform: Methanol	8: 2	No separation band
Chloroform: Methanol	5: 5	No separation band
Benzene: Methanol	8: 2	No separation
Benzene: Methanol	5: 5	No separation
Toluene: Methanol	8: 2	Spot not separated
Hexane: Methanol	5: 5	Spot not separated
Chloroform: Toluene: Methanol	3: 4: 3	Slight separation
Chloroform: Toluene: Methanol	4: 4: 2	Good separation but broad band
Chloroform: Benzene: Methanol	3: 4: 3	Adjacent spots
Chloroform: Toluene: Methanol	**3: 4: 4**	**Good separation with sharp band**
Chloroform: Toluene: Methanol: Glacial acetic acid	3: 4 : 4: 0.05	Good separation but band was broad
Chloroform: Toluene: Methanol: Glacial acetic acid	4: 4: 2: 0.2	Merged no separation

It is evident from the data that mobile phase combination of **Chloroform: Toluene: Methanol** in proportion of **3: 4: 4** v/v/v was most suitable for the development of HPTLC method. The chromatographic separation was achieved under following conditions;

- **Stationary phase:** Pre-coated silica gel aluminum Plate 60F–254 (20 × 10 cm
  Mobile phase: Chloroform: Toluene: Methanol: Glacial acetic acid (3: 4: 4 v/v/v)
- **Scanning Wave length:** 248nm

7.2.3.4.2. Preparation of binary stock solution:

For chromatographic study, standard EZE and ROSU (100 mg) were weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml methanol and diluted up to mark with methanol to obtain final concentration of 1000 µg/ml of EZE and ROSU.

7.2.3.4.3. Calibration curve:

Analysis was performed on Pre-coated silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 µm thickness) (E. Merck) pre-washed with methanol and then dried for 30 minute at 50°C. From the stock solution (1000 µg/ml) aliquots of 0.1, 0.2, 0.4, 0.8, 1.2 and 1.6 µl were spotted on the TLC plate under nitrogen stream using Desaga Applicator, AS30win to obtain final concentration range 100 - 1800 ng/spot.

The plate was dried in air, and then the plate was developed in Twin trough developing chamber (100 × 100) with stainless steel lid, previously saturated with the mobile phase for 30 min. The plate was removed from the chamber, dried in air and was scanned and quantified at 2381 nm in Re-emission/Excitation mode with Desaga TLC scanner, Proquant. The calibration curve was constructed by plotting peak area vs. concentration (ng/spot) and peak height vs. concentration (ng/spot) corresponding to each spot and regression equation was calculated.

7.2.3.4.4. Validation parameter:

Validation of method was done as per procedure give in Chapter 7.1.1.8.4.

7.2.3.4.5. Results and Discussion:

7.2.3.4.5.1. Validation Parameters

(I) Linearity

The HPTLC method showed good linearity for EZE in the range of 153 - 1836 ng/spot with co-relation co-efficient, of 0.9973 as seen in fig. 7.2.3.4.1 for peak area and 0.9966

as seen in fig. 7.2.3.4.2 for peak height. For ROSU the line of best fit was obtained with correlation coefficients of 0.9995 as seen in fig. 7.2.3.4.3 for peak area and 0.9965 as seen in fig. 7.2.3.4.4. Calibration data was given in Table 7.2.3.4.2.

Table 7.2.3.4.2: Calibration data of EZE by HPTLC with UV detection

Sr. No.	Concentration (ng/spot)	Peak Area		Peak Height		Rf*
		Mean* ± SD	%CV	Mean* ± SD	%CV	
1	153	271.82 ± 0.058	0.85	175.71 ± 0.021	0.97	0.53
2	306	278 ± 0.097	0.54	187.45 ± 0.041	0.25	0.52
3	459	289 ± 0.075	0.45	192.23 ± 0.034	0.48	0.52
4	918	326 ± 0.157	0.79	224 ± 0.11	0.79	0.51
5	1377	362.34 ± 0.0642	0.86	260.02 ± 0.047	0.23	0.52
6	1836	402 ± 0.0125	0.12	285.84 ± 0.0712	0.86	0.52

*Average of five reading

Table 7.2.3.4.3: Calibration data of ROSU by HPTLC with UV detection

Sr. No.	Concentration (ng/spot)	Peak Area		Peak Height		Rf*
		Mean* ± SD	%CV	Mean* ± SD	%CV	
1	168	256.18 ± 0.078	0.77	227.82 ± 0.054	0.24	0.73
2	336	270 ± 0.056	0.65	250 ± 0.098	0.77	0.7
3	504	290 ± 0.098	0.18	263 ± 0.033	0.58	0.71
4	1008	340 ± 0.074	0.42	300 ± 0.074	0.44	0.71
5	1512	387.24 ± 0.98	0.62	350 ± 0.044	0.63	0.71
6	2016	440.4 ± 0.074	0.44	387.15 ± 0.011	0.88	0.71

*Average of five reading

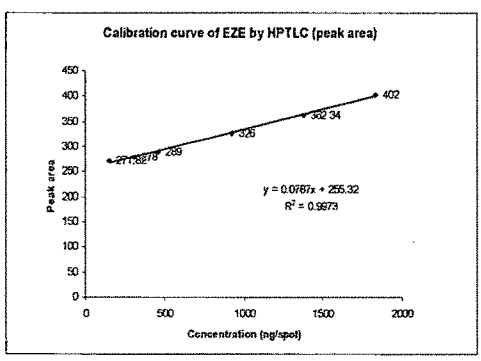


Figure 7.2.3.4.1: Calibration curve of EZE by HPTLC with UV detection(Peak Area)

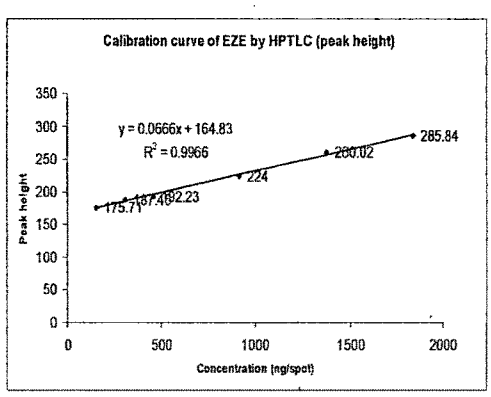


Figure 7.2.3.4.2: Calibration curve of EZE by HPTLC with UV detection(Peak Height)

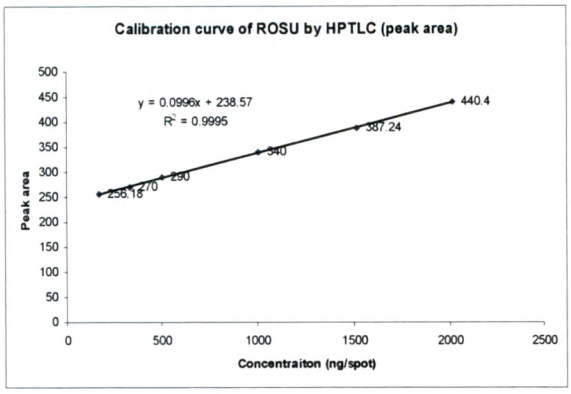


Figure 7.2.3.4.3: Calibration curve of ROSU by HPTLC with UV detection (Peak Area)

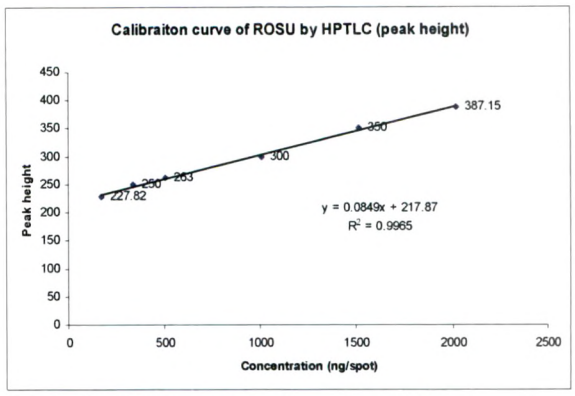


Figure 7.2.3.4.4: Calibration curve of ROSU by HPTLC with UV detection (Peak Height)

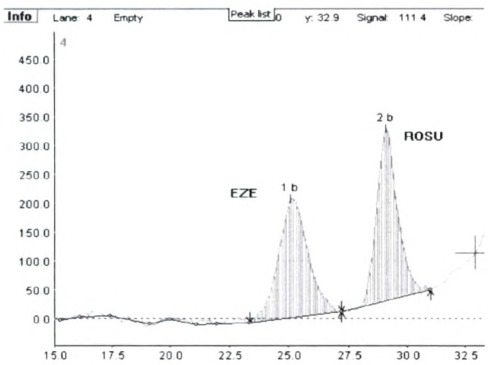


Figure 7.2.3.4.5: Single Spectrum of EZE and ROSU by HPTLC with UV detection

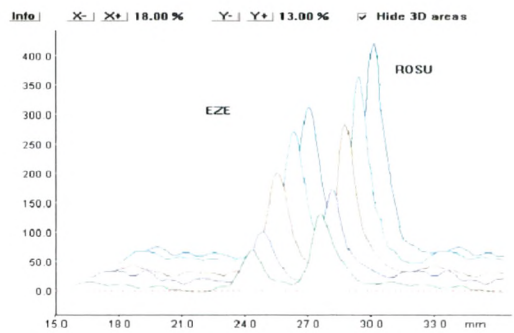


Figure 7.2.3.4.6: Overlain Spectra of EZE and ROSU by HPTLC with UV detection

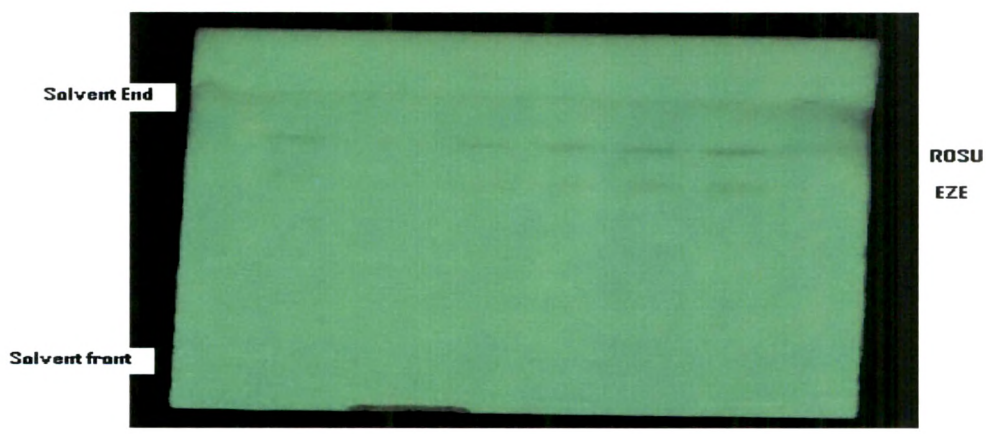


Figure 7.2.3.4.7: Chromatogram of EZE and ROSU with UV detection (After detection)

(II) Precision

Repeatability

The method was found to be precise on intra day and inter day basis as the average % CV value for the determination of EZE and ROSU were reported in table 7.2.3.4.4 and 7.2.1.4.5.

Table 7.2.3.4.4: Intraday precision data of EZE and ROSU by HPTLC

Concentration (ng/spot)		Interaday			
		Peak area* ± % C.V. (n=5)		Peak height* ± % C.V. (n=5)	
EZE	ROSU	EZE	ROSU	EZE	ROSU
250	250	275.12 ± 0.28	266.89 ± 0.41	181.70 ± 0.52	238.46 ± 0.62
750	750	302.22 ± 0.65	331.55 ± 0.28	204.01 ± 0.41	284.21 ± 0.77
1000	1000	355.12 ± 0.41	337.30 ± 0.88	236.83 ± 0.22	294.48 ± 0.11

*** Mean of five determinations.**

Table 7.2.3.4.5: Interday precision data of EZE and ROSU by HPTLC

Concentration (ng/spot)		Interday			
		Peak area* ± % C.V. (n=5)		Peak height* ± % C.V. (n=5)	
EZE	ROSU	EZE	ROSU	EZE	ROSU
250	250	275±0.25	266±0.65	181±0.29	236±0.13
750	750	302±0.98	328±0.18	204±0.81	284±0.78
1000	1000	355±0.78	337±0.56	236±0.64	294±0.65

*** Mean of five determinations.**

Reproducibility:

The % CV of the responses for determination of EZE and ROSU are mentioned in Tables 7.2.3.4.6 and 7.2.3.4.7, which reveal the reproducibility of the method (Table 7.2.3.3.6 and 3.2.4.4.7).

Table 7.2.3.4.6: Reproducibility data of EZE and ROSU by HPTLC (peak area)

Concentration (ng/spot)		Peak area ± % CV*			
EZE	ROSU	EZE		ROSU	
		Glass plate	Aluminum plate	Glass plate	Aluminum plate
250	250	275.11 ± 0.25	275.22 ± 0.44	269.81 ± 0.11	261.42 ± 0.28
750	750	305.52 ± 0.14	301.37 ± 0.87	333.97 ± 0.41	334.51 ± 0.41
1000	1000	350.00 ± 0.52	355.07 ±0.74	341.16 ± 0.84	338.35 ± 0.44

Table 7.2.3.4.7: Reproducibility data of EZE and ROSU by HPTLC (peak height)

250	250	184.69 ± 0.12	185.21± 0..11	237.75 ± 0.16	230.80 ± 0.15
750	750	201.28 ± 0.14	202.68 ± 0.61	284.47 ± 0.56	282.74 ± 0.68
1000	1000	237.38 ± 0.21	235.80 ±.0.69	294.13 ± 0.21	297.40 ± 0.64

*** Mean of five determinations.**

(III) Accuracy

The method showed % mean recovery for EZE and ROSU were reported in table 7.2.3.4.8.

Table 7.2.3.4.8: Accuracy data of EZE and ROSU by HPTLC

Spotted Amount (A + B)		Peak area				Peak height			
		Total quantity Found (Mean*)		% Recovery		Total quantity Found (Mean*)		% Recovery	
EZE	ROSU	EZE	ROSU	EZE	ROSU	EZE	ROSU	EZE	ROSU
500	500	751.23	754	100.16	100.53	749.55	750.98	99.94	100.13
500	500	1065	1006	106.50	100.60	1005.36	1052.39	100.54	105.24
500	500	1523	1509	101.53	100.60	1502.28	1510.23	100.15	100.68

*** Mean of three determinations.**

(IV) Limit of detection and quantification

LOD and LOQ reported in table 7.2.3.4.10.

7.2.3.4.5.2. Analysis of tablet formulation:

The procedure for preparation of binary mixture is given in Chapter 7.2.3.1.5.2. A synthetic mixture was prepared having EZE and ROSU in ratio of 1:1. From the filtrate 0.5 µl was spotted on TLC plate under nitrogen stream using Desaga Applicator, AS30win this was developed and scanned five times without changing plate position. The concentrations of EZE and ROSU were computed from their respective calibration curves and found to be in the range of 99.86 – 101.93 % and 99.43 – 103.02 %, respectively (Table 7.2.3.4.9).

Table 7.2.3.4.9: Estimation of EZE and ROSU in tablet by RP-HPTLC method

Formulation	DRUGS	Labeled Claim (mg/tab)	HPTLC			
			Peak Area		Peak Height	
			*Amount found	% Labeled Claim	*Amount found	% Labeled Claim
EZEDOC + NOVASTAT	EZE	10	10.03	100.31	10.09	100.91
	ROSU	10	10.24	102.41	10.18	101.8
LAB. MIX.	EZE	10	10.05	100.52	10.21	102.19
	ROSU	10	10.02	100.21	10.00	100.00

*** Mean value of five determinations.**

7.2.3.4.2. Summary of Validation parameters:

Table 7.2.3.4.10: Summary of Validation parameters by HPTLC methods

Sr. No	Parameters	EZE		ROSU	
		Peak Area	Peak Height	Peak Area	Peak Height
1	Analytical wavelengths (nm)	248			
2	Linearity range (ng/spot)	153 – 1836		168 - 2016	
3	Regression equation	Y=0.0787x +255.32	Y=0.0666x +164.83	Y=0.09996x +238.57	Y=0.849x +215.87
4	Correlation coefficient (r^2)	0.9973	0.9966	0.9995	0.9965
5	Intercept	255.32	164.83	238.57	215.87
6	Slope	0.0787	0.0666	0.09996	0.849
7	Assay (%)	100.31	100.91	102.41	101.80
8	Precision				
	Intra day % CV (n = 5)	0.28-0.65	0.22-0.52	0.28-0.88	0.11-0.62
	Inter day % CV (n = 5)	0.25-0.98	0.29-0.81	0.18-0.65	0.13-0.78
	Repeatability of measurements %CV	< 1 %			
	% Recovery	100.16-106.50	99.94-100.54	100.53-100.60	100.23-105.24
9	Limit of detection (ng/spot)	55.6641	5.3674	9.0900	14.9082
10	Limit of quantification (ng/spot)	85.54	17.8915	30.3003	49.6941

7.2.3.4.3. Conclusion:

Developed HPTLC method could estimate both EZE and ROUS form their binary mixture with the accuracy of 99.94 to 106.50 %.

7.2.3.5. Employing Chemometric Methods:

In this section the Chemometric methods have been described for EZE and ROSU. The concept of mathematical modeling is used to provide maximum relevant chemical information in order to analyze the drugs simultaneously.

The two-chemometric techniques ILS and CLS are developed for the simultaneous determination of the titled drugs in their binary mixture.

7.2.3.5. Inverse Least Square (ILS) and Classical Least Square (CLS) technique:

It is the application of multiple linear regressions (MLR) to the inverse expression of the Beer-Lambert Law of spectrophotometry.

7.2.3.5.1. Selection of the spectral region:

In order to develop ILS method for simultaneous estimation of EZE and ROSU, light absorption study was done. Individual spectrum of both the drugs and their binary mixture were overlain.

Although the ILS is the full spectrum method, 21 wavelengths were selected between 230 nm to 250 nm with the interval of $\Delta\lambda$ = 1 nm in the zero order spectra as shown in fig. 7.2.3.5.1.

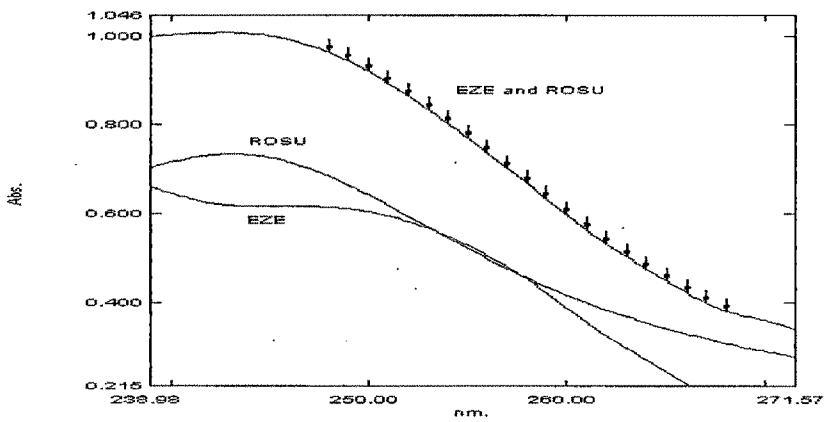


Fig 7.2.3.5.1: Overlain spectra of EZE, ROSU and their binary mixture showing spectral region 248 nm to 268 nm (21 wavelengths)

7.2.3.5.2. Measurement of the absorbance:

The absorbance matrices were produced by measuring absorbencies of binary mixture at 21 wavelengths. In this calibration was obtained by measuring absorbance data matrix and concentration data matrix to predict the concentration of EZE and ROSU in their binary mixtures and tables. The numerical calculations were performed using MATLAB 6.1 software and excel.

7.2.3.5.3. Equation for ILS method:

The mathematical expression of ILS is C = P x A.

The calibration coefficient (P) was obtained from the linear equation system using the absorbance data and the concentration taken in training set (Table 7.2.3.5.1 and 7.2.3.5.2)

The absorbance values (Table 7.2.3.5.3) of the samples at 21 wavelengths were placed in the above equation and the amounts of EZE and ROSU in the synthetic mixture and formulation were found as shown in table 7.2.3.5.4.

Table 7.2.3.5.1: Composition of Calibration (training) set for EZE and ROSU:

Mixture No.	EZE (µg/ml)	ROSU (µg/ml)	Mixture No.	EZE (µg/ml)	ROSU (µg/ml)
1	0	5.2	13	18.6	18.72
2	6.2	5.2	14	18.6	20.8
3	6.2	18.72	15	18.6	26
4	6.82	16.12	16	18.6	31.2
5	9.92	15.6	17	22.32	26
6	12.4	10.2	18	24.8	10.4
7	12.4	5.4	19	24.8	18.72
8	12.4	18.72	20	24.8	20.8
9	14.48	20.8	21	31	18.72
10	17.98	7.8	22	34.72	15.6
11	18.6	10.4	23	39.68	10.4
12	18.6	15.6	24	18.6	18.72

Table 7.2.3.5.2: Absorbance data for the calibration set at 21 wavelengths:

Absorbance	Mi No.	Wavelength																				
		248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268
	1	1.721	1.704	1.688	1.658	1.625	1.582	1.533	1.478	1.414	1.344	1.268	1.183	1.089	0.999	0.917	0.84	0.768	0.693	0.616	0.554	0.514
	2	0.171	0.166	0.16	0.154	0.147	0.141	0.135	0.129	0.122	0.117	0.111	0.106	0.101	0.097	0.093	0.089	0.085	0.082	0.079	0.077	0.074
	3	0.417	0.41	0.401	0.39	0.379	0.366	0.354	0.339	0.324	0.309	0.293	0.277	0.26	0.244	0.228	0.214	0.201	0.188	0.174	0.163	0.155
	4	0.962	0.939	0.912	0.884	0.854	0.822	0.79	0.757	0.722	0.69	0.658	0.626	0.594	0.565	0.538	0.512	0.488	0.464	0.441	0.421	0.405
	5	0.906	0.886	0.863	0.836	0.809	0.779	0.749	0.718	0.685	0.655	0.623	0.592	0.561	0.531	0.503	0.477	0.453	0.429	0.405	0.385	0.369
	6	0.966	0.947	0.922	0.896	0.867	0.836	0.805	0.772	0.737	0.703	0.669	0.633	0.597	0.563	0.532	0.503	0.475	0.448	0.421	0.398	0.381
	7	0.861	0.847	0.828	0.806	0.783	0.756	0.729	0.7	0.67	0.638	0.606	0.572	0.536	0.502	0.471	0.442	0.415	0.387	0.36	0.337	0.321
	8	0.675	0.666	0.654	0.639	0.623	0.604	0.584	0.562	0.537	0.511	0.484	0.454	0.422	0.393	0.365	0.339	0.316	0.292	0.267	0.247	0.232
	9	1.188	1.163	1.134	1.102	1.066	1.028	0.99	0.949	0.907	0.867	0.824	0.78	0.736	0.694	0.655	0.62	0.586	0.553	0.52	0.491	0.469
	10	1.364	1.335	1.302	1.264	1.224	1.182	1.139	1.092	1.044	0.996	0.947	0.896	0.845	0.795	0.75	0.708	0.668	0.629	0.591	0.558	0.533
	11	0.99	0.977	0.959	0.938	0.914	0.886	0.858	0.825	0.789	0.752	0.712	0.669	0.625	0.583	0.543	0.507	0.473	0.438	0.402	0.372	0.351
	12	1.13	1.113	1.09	1.064	1.035	1.002	0.966	0.929	0.889	0.848	0.803	0.755	0.705	0.657	0.614	0.574	0.536	0.497	0.457	0.425	0.402
	13	1.264	1.244	1.216	1.186	1.152	1.115	1.074	1.032	0.987	0.942	0.893	0.843	0.79	0.74	0.694	0.651	0.611	0.571	0.531	0.497	0.472
	14	1.412	1.386	1.354	1.318	1.278	1.235	1.191	1.143	1.093	1.043	0.99	0.936	0.88	0.826	0.776	0.73	0.686	0.643	0.6	0.564	0.537
	15	1.56	1.53	1.495	1.455	1.411	1.364	1.314	1.261	1.206	1.151	1.093	1.032	0.97	0.911	0.859	0.808	0.761	0.714	0.667	0.627	0.598
	16	1.691	1.655	1.613	1.569	1.519	1.465	1.412	1.354	1.293	1.235	1.176	1.113	1.048	0.987	0.932	0.881	0.833	0.785	0.737	0.696	0.665
	17	1.901	1.862	1.816	1.763	1.706	1.645	1.582	1.518	1.452	1.385	1.318	1.248	1.178	1.113	1.052	0.995	0.943	0.89	0.838	0.794	0.759
	18	1.821	1.785	1.743	1.697	1.645	1.589	1.532	1.471	1.406	1.341	1.275	1.203	1.133	1.066	1.003	0.945	0.892	0.838	0.784	0.738	0.704
	19	0.493	0.48	0.466	0.45	0.434	0.417	0.4	0.383	0.366	0.349	0.333	0.317	0.301	0.286	0.273	0.26	0.248	0.236	0.225	0.215	0.207
	20	1.617	1.591	1.557	1.519	1.474	1.427	1.377	1.322	1.265	1.207	1.145	1.08	1.011	0.947	0.887	0.831	0.779	0.726	0.672	0.629	0.598
	21	1.739	1.71	1.672	1.628	1.581	1.529	1.478	1.418	1.355	1.293	1.227	1.157	1.085	1.016	0.952	0.893	0.839	0.783	0.728	0.681	0.647
	22	1.897	1.861	1.825	1.783	1.734	1.68	1.619	1.557	1.49	1.421	1.346	1.267	1.184	1.105	1.033	0.964	0.902	0.838	0.774	0.72	0.682
	23	1.864	1.84	1.808	1.767	1.719	1.666	1.608	1.547	1.48	1.41	1.334	1.254	1.171	1.09	1.015	0.946	0.882	0.815	0.747	0.692	0.654
	24	1.916	1.895	1.862	1.826	1.781	1.729	1.674	1.609	1.539	1.466	1.386	1.301	1.208	1.119	1.038	0.96	0.891	0.817	0.742	0.681	0.64

Table 7.2.3.5.3: Absorbance data for the Validaiton set at 21 wavelengths:

Absorbance	Mi No.	Wavelength																			
		248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268
	1	0.888	0.861	0.832	0.801	0.769	0.736	0.704	0.672	0.641	0.612	0.585	0.558	0.533	0.509	0.487	0.467	0.449	0.431	0.414	0.399	0.385
	2	0.354	0.349	0.343	0.335	0.325	0.315	0.304	0.291	0.278	0.265	0.25	0.234	0.218	0.202	0.186	0.172	0.16	0.147	0.134	0.122	0.115
	3	0.872	0.852	0.828	0.802	0.774	0.744	0.715	0.684	0.653	0.623	0.593	0.563	0.533	0.505	0.478	0.453	0.43	0.407	0.386	0.367	0.352
	4	1.307	1.273	1.235	1.195	1.152	1.107	1.061	1.015	0.969	0.926	0.884	0.841	0.799	0.759	0.723	0.688	0.656	0.626	0.597	0.571	0.55
	5	1.097	1.073	1.045	1.013	0.98	0.943	0.907	0.869	0.83	0.792	0.752	0.711	0.67	0.63	0.595	0.561	0.53	0.499	0.469	0.442	0.421
	6	0.75	0.736	0.719	0.699	0.678	0.655	0.63	0.604	0.577	0.55	0.52	0.489	0.457	0.427	0.398	0.373	0.349	0.325	0.3	0.28	0.264
	7	0.88	0.864	0.845	0.823	0.797	0.769	0.741	0.71	0.678	0.645	0.612	0.577	0.54	0.504	0.47	0.439	0.411	0.382	0.354	0.329	0.312
	8	0.877	0.866	0.852	0.834	0.812	0.787	0.76	0.73	0.697	0.663	0.626	0.587	0.544	0.503	0.465	0.429	0.396	0.362	0.328	0.3	0.281
	9	1.347	1.323	1.293	1.26	1.222	1.181	1.137	1.091	1.042	0.994	0.942	0.89	0.834	0.78	0.731	0.684	0.641	0.599	0.556	0.519	0.491
	10	1.291	1.273	1.248	1.221	1.188	1.151	1.111	1.068	1.02	0.97	0.919	0.862	0.803	0.745	0.692	0.643	0.597	0.552	0.504	0.464	0.436
	11	1.508	1.486	1.459	1.428	1.389	1.344	1.298	1.248	1.194	1.138	1.075	1.01	0.94	0.875	0.813	0.754	0.701	0.648	0.593	0.546	0.513
	12	1.983	1.948	1.907	1.861	1.808	1.748	1.683	1.617	1.546	1.476	1.402	1.322	1.242	1.165	1.092	1.024	0.962	0.9	0.837	0.781	0.741
	13	1.795	1.772	1.736	1.697	1.651	1.599	1.541	1.486	1.419	1.354	1.281	1.205	1.125	1.047	0.976	0.909	0.849	0.786	0.722	0.668	0.63
	14	1.352	1.344	1.33	1.308	1.28	1.246	1.209	1.165	1.115	1.059	0.996	0.927	0.853	0.781	0.714	0.651	0.595	0.536	0.473	0.421	0.387
	15	1.963	1.932	1.907	1.867	1.818	1.765	1.706	1.64	1.57	1.496	1.416	1.329	1.237	1.149	1.065	0.987	0.916	0.844	0.768	0.704	0.66

Table 7.2.3.5.4: Composition of Validation set for EZE and ROSU:

Mixture No.	EZE (µg/ml)	ROSU (µg/ml)	Mixture No.	EZE (µg/ml)	ROSU (µg/ml)
1	0	26	9	18.6	15.6
2	6.2	3.12	10	24.8	10.4
3	6.2	15.6	11	27.9	11.96
4	6.2	26	12	27.28	22.88
5	10.54	17.16	13	31	15.6
6	11.16	9.36	14	37.2	0
7	12.4	10.4	15	29.68	12.48
8	18.6	5.2	-	-	-

Introducing (P) into the linear equation system, the calibration for ILS was obtained as fig. 7.2.3.5.2.

$$\begin{bmatrix} CEZE \\ \\ CROSU \end{bmatrix} \approx 1.0e+004 * \begin{bmatrix} 0.1624 & 0.0004 \\ 0.4686 & 0.0116 \\ 0.3526 & 0.0040 \\ 0.1452 & 0.0134 \\ -1.5908 & -0.0435 \\ -0.0910 & 0.0348 \\ -1.2263 & -0.0140 \\ 1.5930 & 0.0231 \\ 0.3379 & -0.0162 \\ -0.4205 & -0.0152 \\ -0.6585 & -0.0041 \\ 1.0786 & 0.0137 \\ -0.5968 & -0.0247 \\ 0.2333 & 0.0401 \\ 0.7779 & -0.0291 \\ -0.7567 & -0.0103 \\ 0.0108 & -0.0130 \\ 1.1229 & 0.0119 \\ 1.2334 & 0.0100 \\ -2.2097 & -0.0055 \\ 0.0168 & 0.0128 \end{bmatrix} \times \begin{bmatrix} A1 \\ A2 \\ A3 \\ A4 \\ A5 \\ A6 \\ A7 \\ A8 \\ A9 \\ A10 \\ A11 \\ A12 \\ A13 \\ A14 \\ A15 \\ A16 \\ A17 \\ A18 \\ A19 \\ A20 \\ A21 \end{bmatrix}$$

Fig 7.2.3.5.2: Equation of ILS

7.2.3.5.4. Equation for CLS method:

The mathematical expression of ILS is A = K x C.

In this method, the calibration coefficient (K) was obtained from the linear equation system using the absorbance data and the training set (table 7.2.3.5.1 and 7.2.3.5.2)

The absorbance values (table 7.2.3.5.3) of the samples at 21 wavelengths were placed in the above equation and the amounts of EZE and ROSU in the synthetic mixture and formulation were found as shown in table 7.2.3.5.4.

Introducing (K) into the linear equation system, the calibration for ILS was obtained as fig. 7.2.3.5.3.

The absorbance values of the samples, the 21 wavelengths in the spectral region from 230 nm to 250 nm were placed in the linear equation and the amounts of EZE and ROSU in the synthetic mixture and tables were found as shown in table

$$\begin{pmatrix} A_1 \\ A_2 \\ A_3 \\ A_4 \\ A_5 \\ A_6 \\ A_7 \\ A_8 \\ A_9 \\ A_{10} \\ A_{11} \\ A_{12} \\ A_{13} \\ A_{14} \\ A_{15} \\ A_{16} \\ A_{17} \\ A_{18} \\ A_{19} \\ A_{20} \\ A_{21} \end{pmatrix} \approx \begin{pmatrix} 0.0345 & 0.0404 \\ 0.0342 & 0.0393 \\ 0.0338 & 0.0380 \\ 0.0333 & 0.0366 \\ 0.0326 & 0.0352 \\ 0.0317 & 0.0338 \\ 0.0307 & 0.0324 \\ 0.0296 & 0.0310 \\ 0.0284 & 0.0296 \\ 0.0270 & 0.0283 \\ 0.0254 & 0.0271 \\ 0.0237 & 0.0258 \\ 0.0219 & 0.0247 \\ 0.0201 & 0.0237 \\ 0.0184 & 0.0227 \\ 0.0169 & 0.0218 \\ 0.0154 & 0.0210 \\ 0.0139 & 0.0195 \\ 0.0124 & 0.0188 \\ 0.0113 & 0.0182 \end{pmatrix} \times \begin{pmatrix} C_{EZE} \\ C_{ROSU} \end{pmatrix}$$

Fig 7.2.3.5.3: Equation of CLS

7.2.3.5.5. Validation Parameters

(I) Accuracy

Accuracy of the method was determined by performing recovery study on laboratory prepared synthetic mixture by standard addition method. The ILS method showed % mean recovery for EZE and ROSU in the range of 96.12 – 104.71 % and 96.43-104.89% from synthetic mixture (Table 7.2.3.5.6).

The CLS method showed % mean recovery for EZE and ROSU in the range of 97.00 – 103.13 % and 97.28 – 103.51 % from synthetic mixture (Table 7.2.3.5.5).

(II) Precision

The precision was determined by means of a one way ANOVA including experiments were repeated five times in a day for intra day and on five different days for inter day precision for 10 synthetic mixtures. F values below the tabulated levels were obtained and there was no significant difference between the results obtained in the determination of each drug in the presence of other on different days.

Table 7.2.3.5.5: Data for precision study using one way ANOVA

Parameters	ILS		CLS	
	EZE	ROSU	EZE	ROSU
Between days variance	2.95	1.69	4.22	4.09
Within days variance	1.36	1.02	1.73	1.93
F ratio	**2.17**	**1.66**	**2.44**	**2.12**

Note the between day and within day degrees of freedom are 4 and 45 respectively. The critical F ratio value for 4 and 45 df and the confidence level of 95% is 2.53.

(III) Limit of detection

The minimum detectable concentration of EZE and ROSU was found to be 0.487 µg/ml and 0.284 µg/ml and 0.56 µg/ml and 0.38 µg/ml, respectively by ILS and CLS methods.

(IV) Limit of quantification

The lowest quantifiable concentration of EZE and ROSU was found to be 1.294 µg/ml and 1.463 µg/ml and 1.44 µg/ml and 1.06 µg/ml, respectively by ILS and CLS methods.

(V) Predicted versus known concentration plot:

The predicted concentrations of the validation samples were plotted against the known concentration values. This tool is used to determine whether the model accounts for the concentration variation in the validation set or not. Plots were expected to fall on a straight line with a slope of 1 and o intercept. The predicted versus known concentration plots of the prepared concentration

plots of the prepared validation samples are shown in fig. 7.2.3.5.4 and 7.2.3.5.5 by ILS and fig. 7.2.3.5.6. and 7.2.3.5.7 by CLS method.

Table 7.2.3.5.6: Actual, predicted and residual values.

SR. No.	Actual Conc.		Predicted conc.		Residual		Percentage	
	EZE	ROSU	EZE	ROSU	EZE	ROSU	EZE	ROSU
1	0.000	26.000	0.000	26.496	0.000	-0.496	**100.00**	**101.91**
2	6.200	3.120	6.308	3.062	-0.108	0.058	**101.74**	**98.15**
3	6.200	15.600	6.284	15.766	-0.084	-0.166	**101.35**	**101.06**
4	6.200	26.000	6.075	27.096	0.125	-1.096	**97.99**	**104.22**
5	10.540	17.160	10.407	17.307	0.133	-0.147	**98.73**	**100.85**
6	11.160	9.360	11.099	9.818	0.061	-0.458	**99.45**	**104.89**
7	12.400	10.400	12.984	10.426	-0.584	-0.025	**104.71**	**100.25**
8	18.600	5.200	18.173	5.793	0.428	-0.593	**97.70**	**111.40**
9	18.600	15.600	18.315	15.044	0.285	0.557	**98.47**	**96.43**
10	24.800	10.400	23.837	10.098	0.963	0.302	**96.12**	**97.10**
11	27.900	11.960	27.373	12.015	0.527	-0.054	**98.11**	**100.46**
12	27.280	22.880	28.145	23.607	-0.865	-0.727	**103.17**	**103.18**
13	31.000	15.600	31.833	15.507	-0.833	0.093	**102.69**	**99.40**
14	37.200	0.000	37.089	0.000	0.111	0.000	99.70	100.00
15	29.680	12.480	29.905	12.404	-0.225	0.076	**100.76**	**99.39**
16	0.000	26.000	0.000	26.496	0.000	-0.496	**100.00**	**101.91**
17	6.200	3.120	6.308	3.062	-0.108	0.058	**101.74**	**98.15**
18	6.200	15.600	6.284	15.766	-0.084	-0.166	**101.35**	**101.06**
19	6.200	26.000	6.075	27.096	0.125	-1.096	**97.99**	**104.22**
20	10.540	17.160	10.407	17.307	0.133	-0.147	**98.73**	**100.85**
21	11.160	9.360	11.099	9.818	0.061	-0.458	**99.45**	**104.89**

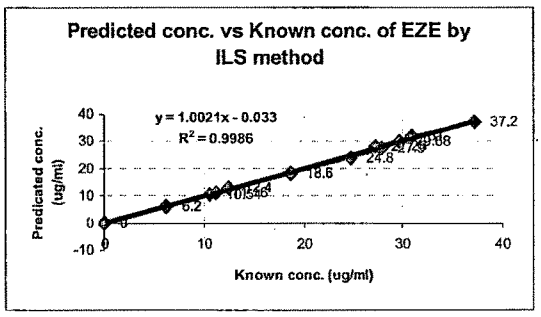


Fig 7.2.3.5.4: Linearity plots of EZE and ROSU for validation set.

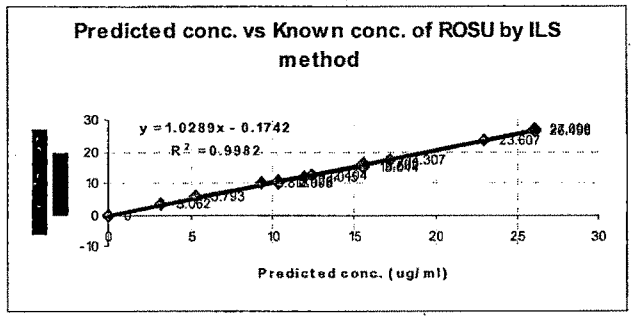


Fig 7.2.3.5.5: Linearity plots of EZE and ROSU for validation set.

Table 7.2.3.5.7: Actual, predicted and residual values.

SR. No.	Actual Conc.		Predicted conc.		Residual		Percentage	
	EZE	ROSU	EZE	ROSU	EZE	ROSU	EZE	ROSU
1	0.000	26.000	0.000	25.4166	0.000	0.583	100.00	97.76
2	6.200	3.120	6.0308	3.0848	0.169	0.035	97.27	98.87
3	6.200	15.600	6.1611	15.3194	0.039	0.281	99.37	98.20
4	6.200	26.000	6.2571	26.4171	-0.057	-0.417	100.92	101.60
5	10.540	17.160	10.6387	17.0907	-0.099	0.069	100.94	99.60
6	11.160	9.360	11.18994	9.2783	-0.030	0.082	100.27	99.13
7	12.400	10.400	12.0275	10.685	0.373	-0.285	97.00	102.74
8	18.600	5.200	18.058	5.2253	0.542	-0.025	97.09	100.49
9	18.600	15.600	19.0698	15.1764	-0.470	0.424	102.53	97.28
10	24.800	10.400	24.9489	10.7652	-0.149	-0.365	100.60	103.51
11	27.900	11.960	27.9989	11.7252	-0.099	0.235	100.35	98.04
12	27.280	22.880	27.1556	22.3336	0.124	0.546	99.54	97.61
13	31.000	15.600	31.9691	15.4533	-0.969	0.147	103.13	99.06
14	37.200	0.000	36.7552	0.000	0.000	0.000	98.80	100.00
15	29.680	12.480	29.4831	12.3614	0.197	0.119	99.34	99.05
16	0.000	26.000	0.000	25.4166	0.000	0.583	100.00	97.76
17	6.200	3.120	6.0308	3.0848	0.169	0.035	97.27	98.87
18	6.200	15.600	6.1611	15.3194	0.039	0.281	99.37	98.20
19	6.200	26.000	6.2571	26.4171	-0.057	-0.417	100.92	101.60

20	10.540	17.160	10.6387	17.0907	-0.099	0.069	100.94	99.60
21	11.160	9.360	11.18994	9.2783	-0.030	0.082	100.27	99.13

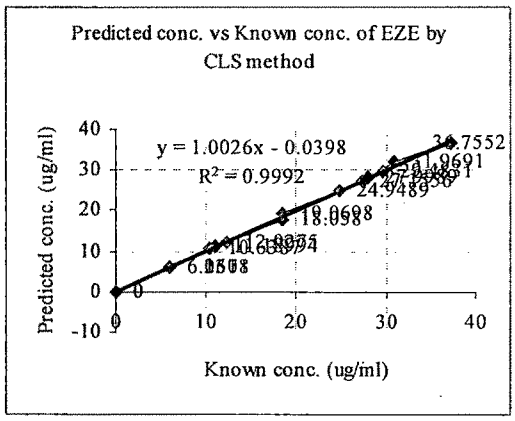


Fig 7.2.3.5.6: Linearity plots of EZE for validation set.

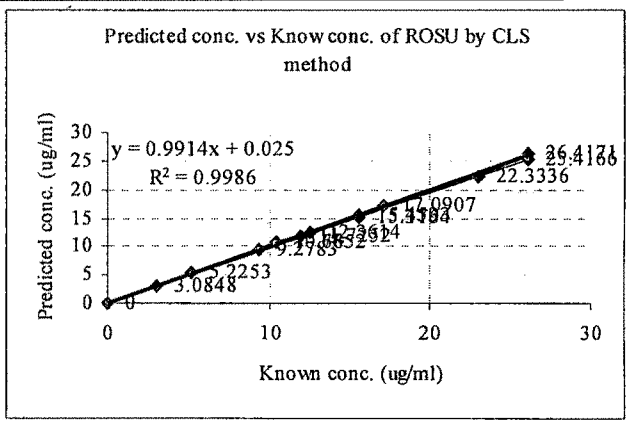


Fig 7.2.3.5.7: Linearity plots of ROSU for validation set.

This indicates that the prediction ability of the validation set is good.

(VI) Residuals concentration versus actual concentration plot:

The difference between the known and predicted concentration (residuals) were plotted against the actual concentrations for the validation samples.

This tool is used to determine whether the model accounts for the concentration variation in the validation set and it also provides information about how well the method will predict the future samples.

For the validation set it can be found that the residual values are more close to zero and are more randomly distributed as illustrated in fig. 7.2.3.5.8 and 7.2.3.5.9.

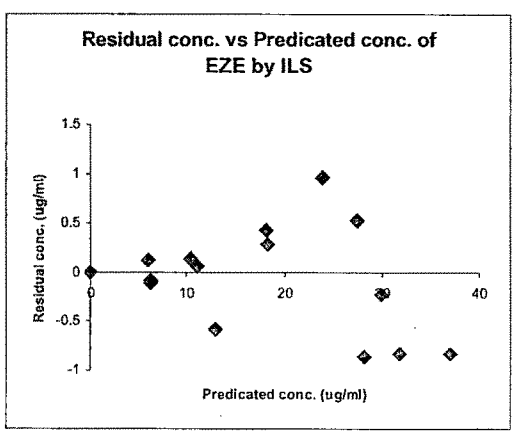


Fig 7.2.3.5.8: Residual vs. predicted concentration plot for EZE and ROSU.

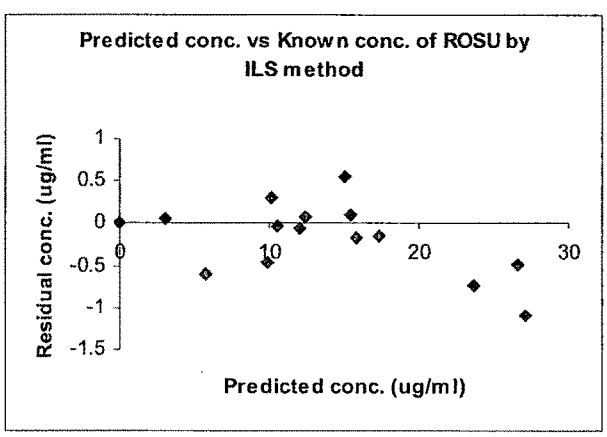


Fig 7.2.3.5.9: Residual vs. predicted concentration plot for EZE and ROSU.

For the validation set it can be found that the residual values are more close to zero and are more randomly distributed as illustrated in fig. 7.2.3.5.10 and 7.2.3.5.11.

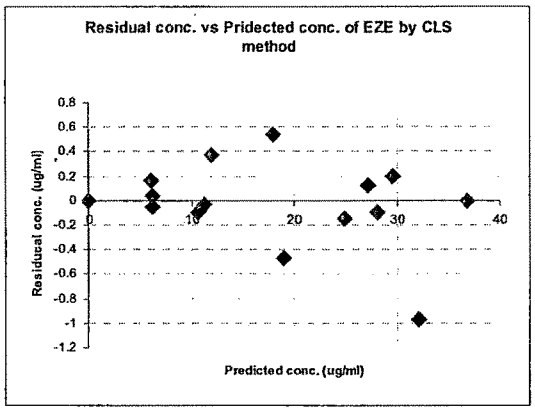


Fig 7.2.3.5.10: Residual vs. predicted concentration plot for EZE.

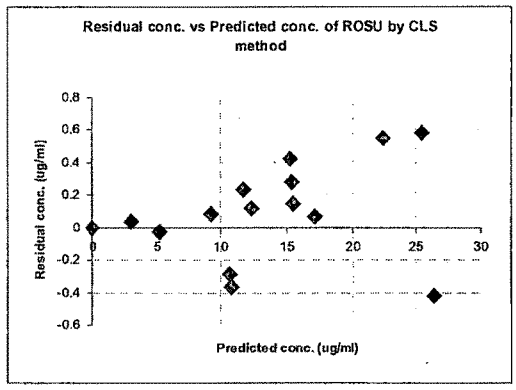


Fig 7.2.3.5.11: Residual vs. predicted concentration plot for ROSU.

(VII) Root Mean Square Error of Prediction:

RMSEP was calculated using the equation mentioned earlier.

Table 7.2.3.5.8: RMSEP values for EZE and ROSU.

Compound	RMSEP	RMSEP
EZE	0.4947	0.3746
ROSU	0.5805	0.3775

7.2.3.5.4. Preparation of sample solutions:

The concentration of ezetimibe and rosuvastatin in tablets were calculated using the equation computed form data. Procedure of preparation of sample mixture is given in previous section. Results are shown in Table 7.2.3.5.9.

Table 7.2.3.5.9: Estimation of EZE and ROSU in tablet by CLS method

Formulation	DRUGS	Labeled Claim (mg/tab)	CLS		ILS	
			*Amount found	% Labeled Claim	*Amount found	% Labeled Claim
EZEDOC + NOVASTAT	EZE	10	10.24	102.40	9.98	99.8
	ROSU	10	9.98	99.80	10.12	101.2
LAB. MIX.	EZE	10	10.15	101.50	10.02	100.2
	ROSU	10	10.65	106.50	10.24	102.4

*** Mean value of five determinations.**

7.2.3.5.5. Summary of Validation parameters:

The summary of validation parameters were reported in table 7.2.3.5.10.

Table 7.2.3.5.10: Summary of Validation parameters of CLS method.

Sr. No	Parameters	ILS		CLS	
		EZE	ROSU	EZE	ROSU
1	Calibration design	24			
2	Validation design	15			
3	Spectral region	248-268			
4	Linearity range (µg/ml)	1-40			
5	Regression equation	Y=1.0021x -0.033	Y=1.0289x -0.1748	Y=1.002x -0.0398	Y=0.99914x +0.025
6	Correlation coefficient (r^2)	0.9986	0.9982	0.9992	0.9986
7	Intercept	-0.033	-0.1748	-0.0398	0.025
8	Slope	1.0021	1.0289	1.002	0.99914
9	RMSEP	0.49	0.58	0.37	0.37
10	Assay %	99.8	101.2	102.40	99.80
11	Precision				
12	% Recovery	99.91-102.29	100.50-103.42	99.77-103.40	97.73-103.05
13	Limit of detection (µg/ml)	0.487	0.284	0.56	0.38
14	Limit of quantification (µg/ml)	1.294	1.463	1.44	1.06

7.2.3.5.6. CONCLUSION

The proposed ILS and CLS methods were found to be precise and accurate. The simplicity of the method can be explained on the basis of direct out put of the data in terms of unknown concentration on providing the concentration matrix and absorbance matrix of the calibration set to the software. The ILS method can be strongly applied to a routine analysis, quality control of binary mixtures and commercial products containing these two drugs.

Reference:

1) Rosuvastatin-ezetimibe combination dramatically cuts LDL cholesterol. *Am J Cardiol.* 2007; 99: 673-680.

7. 2. 4

Ezetimibe and Lovastatin

7.2.4. Development of Analytical methods for Ezetimibe and Lovastatin:

Lovastatin (LOVA), an HMG-CoA reductase inhibitor, and ezetimibe (EZE), a selective cholesterol absorption inhibitor are prescribed in combination because Ezetimibe/Lovastatin 10/10 to 10/80 milligrams (mg) administered daily in the evening is effective for reducing LDL-C from 27 to 41% in place of given Lovastatin alone(Chapter 2). A combined commercial formulation for EZE and LOVA is not yet available in Indian market in spite of many favourable clinical reports. So individual tablet of EZE and LOVA were taken, powdered together and analyzed. For that LOVA tablet selected was AZTATIN (SUN) in dose of 10 mg/tablet and for EZE tablet selected was EZEDOC (lupin) in dose of 10 mg/tablet.

UV Spectrophotometric Methods:

In order to develop UV Spectrophotometric method for simultaneous estimation of EZE and LOVA, light absorption study was done. Individual spectra of both the drugs were superimposed to obtain suitable working wavelengths that could be used for simultaneous estimation methods (fig. 7.2.4.1.1). First simultaneous equation method was tried but it was not found to be applicable in this case as due to extensive overlap of the two spectra. Q analysis method was also tried for the quantitative analysis using λmax of one drug and isoabsorptive point of both the drugs. As a result the determination of these two compounds is not possible for reliable direct absorbance measurement by Q- analysis method as seen in fig. 7.2.4.1.1. The 1^{st} and 2^{nd} order derivative spectroscopie were tried in which 1^{st} order derivative zero crossing method was possible as per fig. 7.2.4.1.3. The difference spectra of the LOVA was not obtained so simultaneous estimation was not possible by difference spectrophotometry of EZE and LOVA. Ratio derivative zero crossing method was also developed to analyze EZE and LOVA as per fig. 7.2.4.1.4.

Chromatographic methods:

Two chromatographic methods were developed for the simultaneous estimation of EZE and LOVA. HPLC method was developed which could estimate EZE and LOVA in pharmaceutical dosage form and in presence of its degradation products. HPTLC method was developed for the estimation of EZE and LOVA in pharmaceutical dosage form.

Chemometric methods

Chemometric approach was tried with the spectrophotometric methods. Here two chemometric methods ILS and CLS were developed.

Apparatus, instruments and reagents:

Apparatus, instruments and reagents used for the development of spectrophotometric and chromatographic methods are mentioned in Chapter 6.

7.2.4.1. Simultaneous spectrophotometric estimation of EZE and LOVA by First derivative zero crossing and Absorbance ratio derivative zero crossing methods:

Three simple, rapid, accurate and economical methods have been developed for the simultaneous estimation of EZE and LOVA in pharmaceutical formulation. First method is based on the Q-absorbance ratio; absorbances of both the drugs were determined at 231 nm (λ max of EZE) and at isoabsortive point (240.80 nm). Second method is base on first derivative spectrophotometry, using zero crossing technique. Third method was base on ratio derivative spectrophotometry.

7.2.4.1.1. Preparation of stock solution:

Standard EZE and LOVA (10 mg) were weighed accurately and transferred to 100 ml volumetric flask. It was dissolved in 50 ml methanol and diluted up to mark with methanol to obtain final concentration of 100 µg/ml each of EZE and LOVA. This solution was used as standard stock solution for all the spectrophotometric methods.

7.2.4.1.2. Selection of analytical wavelength:

(I) For first derivative zero crossing spectrophotometric method:

The selection of zero crossing point, where absorbance of one component is zero while other is having significant absorbance. The zero crossing point should be valid for the entire concentration range in which analysis is carried out. From the overlain 1st derivative spectra of EZE and LOVA (fig. 7.2.4.1.2), 265.2 nm was selected as zero crossing point for LOVA as at this wavelength EZE has negligible absorbance. Whereas, 245.40 nm was selected as zero crossing point for EZE as at this wavelength, LOVA shows negligible absorbance. The data of this absorbance is given in Table 7.2.4.1.1.

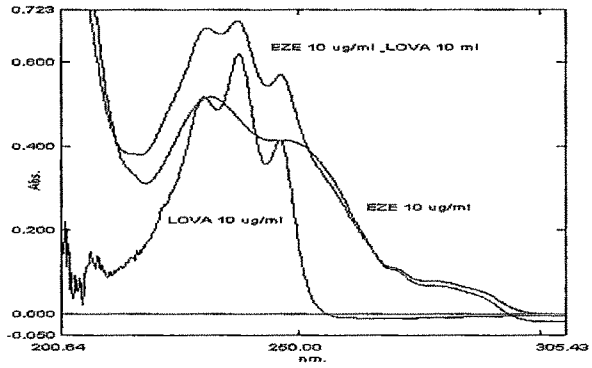


Figure: 7.2.4.1.1: Overlain zero order spectra of EZE and LOVA and binary mixture 1:1

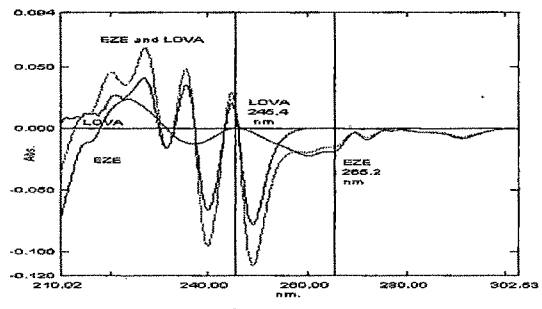


Figure: 7.2.4.1.2: Overlain 1st order UV spectra of EZE and LOVA

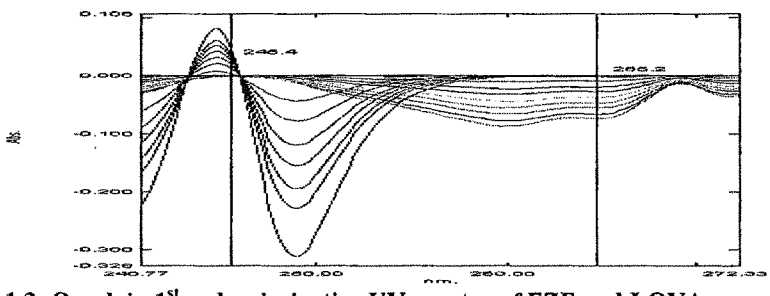


Figure: 7.2.4.1.3: Overlain 1st order derivative UV spectra of EZE and LOVA

(II) Ratio derivative spectrophotometric method:

In a preliminary investigation, for selecting the standard solutions as divisor at an appropriate concentration of EZE and LOVA in the range of 5 – 40 µg/ml were tested respectively. Stored spectra of standard EZE solutions were divided wavelength by wavelength by standard spectra of LOVA (5 – 40 µg/ml)

Then the first derivative of above ratio spectra was recorded and the values of the derivatives were measured at suitably selected wavelengths and plotting against the corresponding concentration to obtain the calibration graph.

The similar procedure was followed for the solutions of different concentration of LOVA when EZE was used as divisor in the same way as described above. The calibration curve was obtained by plotting absorbance versus drug concentration.

The standard solutions of 5 µg/ml of LOVA and 10 µg/ml of EZE could be used as divisor. Using these divisors, highest correlation coefficient values were obtained indicating the quality of fitting of the data to the straight line.

Effect of derivative intervals on derivative ratio spectra:

$\Delta\lambda$, the width of the boundaries over which the derivative is calculated was tested for $\Delta\lambda$ = 2 nm, 4 nm and 8 nm.

The value of $\Delta\lambda$ = 2 nm was found optimal in connection with both slit width and wavelength interval.

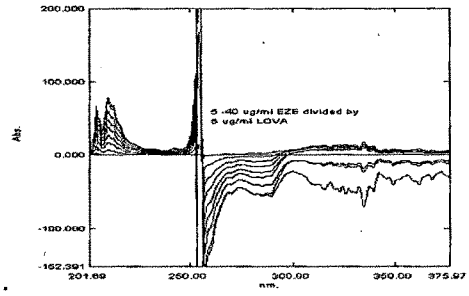


Figure: 7.2.4.1.5: Overlain ratio spectra of EZE (5 µg/ml LOVA as divisor)

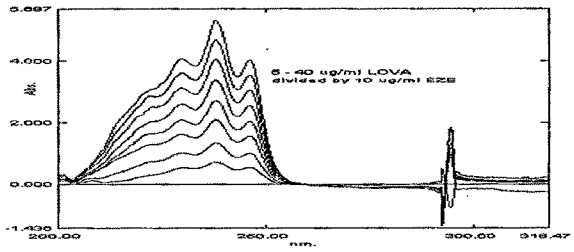


Figure: 7.2.4.1.6: Overlain ratio spectra of LOVA (10 µg/ml EZE as divisor)

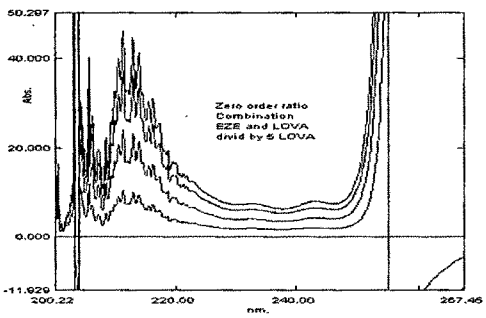


Figure: 7.2.4.1.7: Overlain ratio spectra of EZE (5 µg/ml LOVA as divisor)

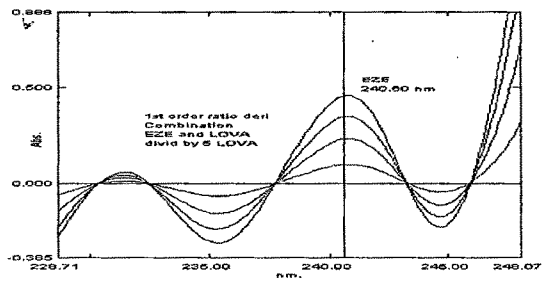


Figure: 7.2.4.1.8: Overlain ratio derivative spectra of EZE (5 µg/ml LOVA as divisor)

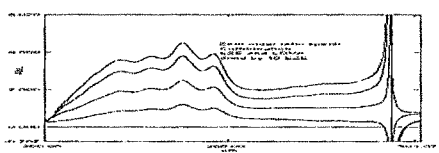


Figure: 7.2.4.1.9: Overlain ratio spectra of LOVA (10 µg/ml EZE as divisor)

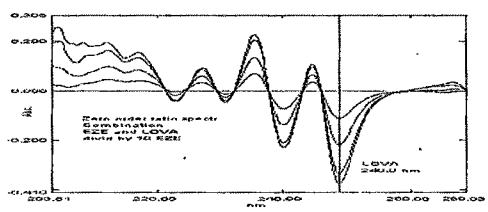


Figure: 7.2.4.1.10: Overlain ratio derivative spectra of LOVA (10 µg/ml EZE as divisor)

The absorption spectra of 10 µg/ml of EZE and 5 µg/ml of LOVA were recorded in the range of 220 nm to 400 nm and stored in the memory of the instrument as divisor spectra. The absorption spectra of the binary mixture solutions of EZE and LOVA were recorded in the range of 200 to 400 nm and were stored in the memory of the software. The stored standard spectra of binary mixture were divided by a previously stored divisor spectrum of 5 µg/ml LOVA to get the ratio spectra (Fig. 7.2.4.1.7). The first derivative of the ratio spectra were traced with λ = 2 interval (fig. 7.2.4.1.8) and the spectra of binary mixture were similarly treated and amplitude at 240.8 nm (fig. 7.2.4.1.8) were plotted against respective concentration of EZE. Similarly stored

binary mixture were divided by a 10 µg/ml of EZE (Fig. 7.2.4.1.9) and the first derivative of the ratio spectra (fig. 7.2.4.1.10) were traced λ = 2. The spectra of binary mixture were similarly treated and the amplitude 249.0 nm (Fig: 7.2.4.1.10) were then plotted against the respective concentrations of LOVA.

7.2.4.1.3. Calibration curve of EZE and LOVA

From the binary stock solution (100 µg/ml) aliquots of 0.1, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 ml were transferred to a series of 10ml volumetric flasks. The volume was adjusted to a mark with methanol to get 1, 5, 10, 15, 20, 25, 30, 35 and 40 µg/ml each of EZE and LOVA.

7.2.4.1.4. Validation of method:

Procedure for validation of method is given in Chapter 7.2.1.1.4.

7.2.4.1.5. Result and Discussion

7.2.4.1.5.1 Validation Parameters

(I) Linearity

The derivative spectrophotometry method showed good linearity for EZE in the range of 5- 40 µg/ml at 265.2 nm (zero crossing of LOVA) with co-relation co-efficient, of 0.9993 as seen in fig. 7.2.4.3.3. For LOVA the line of best fit was obtained at 245.40 nm (zero crossing of EZE) with correlation coefficients of 0.9985 as seen in fig. 7.2.4.3.4. Calibration data is given in Table 7.2.4.3.1.

Table 7.2.4.1.1: Calibration data of EZE by FDZC method

Sr.No.	Concentration (µg/ml)	Absorbance Mean ± S.D. (N=5)		% C.V.	
		EZE at 265.2 nm	LOVA at 245.4 nm	EZE	LOVA
1	5	-0.009 ± 0.035	0.007 ± 0.048	0.92	0.38
2	10	-0.019± 0.00042	0.014± 0.00029	0.43	0.72
3	15	-0.027± 0.00025	0.02 ± 0.00082	0.72	0.48
4	20	-0.037± 0.00014	0.027± 0.00021	0.59	0.29
5	25	-0.046± 0.00063	0.033± 0.00072	0.82	0.41
6	30	-0.055± 0.00026	0.038± 0.00048	0.48	0.89
7	35	-0.0655± 0.00028	0.046± 0.00092	0.72	0.21
8	40	-0.073 ± 0.00071	0.051 ± 0.00048	0.33	0.69

* Mean value of five determinations.

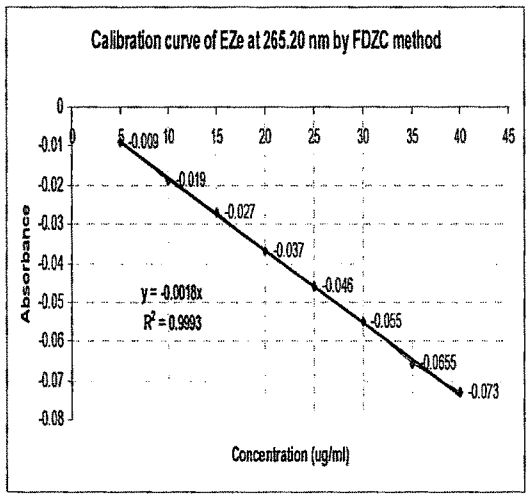


Figure 7.2.4.1.11: Calibration curve of EZE at 265.2 nm by FDZC method
Correlation co-efficient = **0.9993**
Slope = - 0.0018
Intercept =0.0012
Regression equation: **Abs. = -0.0018 × Conc. + 0.0012**

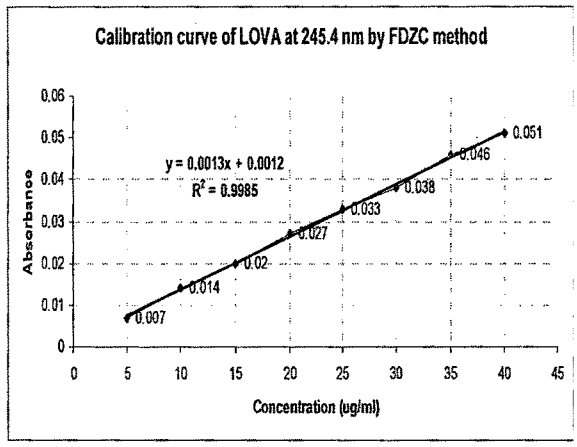


Figure 7.2.4.1.12: Calibration curve of LOVA at 245.4 nm by FDZC method
Correlation co-efficient = **0.9985**
Slope = 0.0013
Intercept = 0.0012
Regression equation: **Abs. = 0.0013 × Conc. + 0.0012**

The spectrophotometry method showed good linearity for EZE in the range of 5 - 40 µg/ml at 240.80 nm (λ max of EZE) with co-relation co-efficient, of 0.9974 as seen in fig. 7.2.1.2.5.

For LOVA the line of best fit was obtained at 249.00 nm (isoabsorptive point of EZE and LOVA) with correlation coefficients of 0.9990 as seen in fig. 7.2.1.2.6. Calibration data is given in Table 7.2.1.2.2.

Table 7.2.4.1.3: Calibration data of EZE and LOVA by RDZC method

Sr. No.	Concentration (µg/ml) (EZE and LOVA)	Absorbance Mean (N=5)		% C.V.	
		EZE at 240.6 nm	LOVA at 248.8 nm	EZE	LOVA
1	10	0.011	-0.055	0.72	0.37
2	15	0.024	-0.095	0.51	0.51
3	20	0.032	-0.127	0.88	0.15
4	25	0.040	-0.158	0.38	0.22
5	30	0.048	-0.190	0.59	0.74
6	35	0.056	-0.222	0.26	0.58
7	40	0.064	-0.253	0.44	0.63

* Mean value of five determinations.

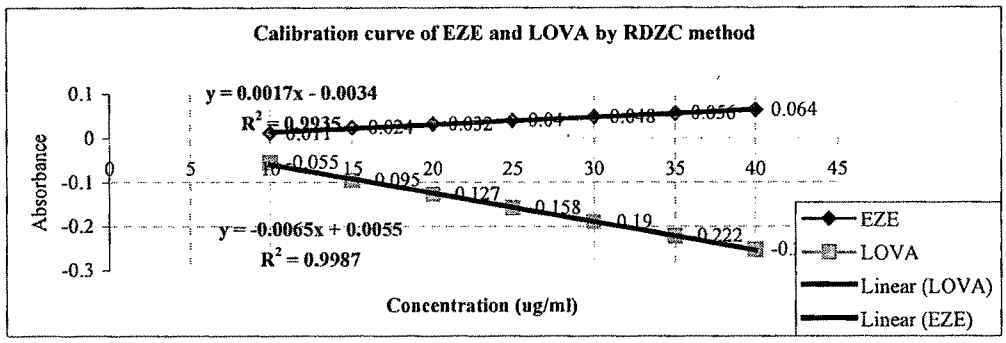


Figure 7.2.4.1.13: Calibration curve of EZE and LOVA by RDZC method

(II) Precision

Repeatability

The intraday and interday precision of EZE and LOVA were reported in table 7.2.4.1.3 and 7.2.4.1.4.

Table 7.2.4.1.5.3: Intraday precision data of EZE and LOVA by spectrophotmetery Method

Concentration (µg/ml)		Ratio derivative spectrophotometric method (Mean (n=5) ± %C.V.)		First derivative zero crossing method (Mean (n=5) ± %C.V.)	
EZE	LOVA	EZE at 240.60 nm	LOVA at 248.80 nm	EZE at 265.2 nm	LOVA at 245.4 nm
10	10	0.014 ± 0.83	-0.055 ± 0.29	-0.019 ± 0.38	0.013 ± 0.28
20	20	0.038 ± 0.50	-0.121 ± 0.42	-0.038 ± 0.92	0.026 ± 0.85
30	30	0.044 ± 0.32	-0.157 ± 0.79	-0.055 ± 0.43	0.053 ± 0.31
Table 7.2.4.1.6: Interday precision data of EZE and LOVA by spectrophotmetery Method					
10	10	0.012 ± 0.53	-0.057 ± 0.63	-0.019 ± 0.37	0.015 ± 0.52
20	20	0.035 ± 0.79	-0.125 ± 0.58	-0.038 ± 0.62	0.029 ± 0.69
30	30	0.042 ± 0.84	-0.155 ± 0.22	-0.056 ± 0.18	0.059 ± 0.48

***Mean value of five determinations**

Reproducibility:

The % CV of the responses confirms the reproducibility of the method for determination of EZE and LOVA the data is reported in (Table 7.2.4.1.5).

Table 7.2.4.1.5: Reproducibility data of EZE and LOVA by spectrophotometry Method

Spectrophototmetric methods		Concentrations (µg/ml)					
		10		15		20	
		UV 1700	UV 1601	UV 1700	UV 1601	UV 1700	UV 1601
Ratio derivative spectrophotometric method (Mean (n=5) ± %C.V.)	EZE (240.6 nm)	0.013 ± 0.213	0.014 ±0.97	0.020 ±0.320	0.022 ±0.48	0.037 ±0.427	0.038 ±0.79
	LOVA (248.8 nm)	-0.053 ±0.92	-0.056 ±0.44	-0.094 ±0.48	-0.098 ±0.68	-0.125 ±0.52	-0.122 ±0.47
First derivative zero crossing method (Mean (n=5) ± %C.V.)	EZE (265.20 nm)	-0.019 ±0.93	-0.020 ±0.28	-0.029 ±0.29	-0.029 ±0.48	-0.038 ±0.47	-0.038 ±0.22
	LOVA (245.40 nm)	0.013 ±0.38	0.016 ±0.25	0.020 ±0.78	0.021 ±0.48	0.025 ±0.25	0.026 ±0.82

*** Mean value of three determinations**

(III) Accuracy

The result showed % mean recovery values for EZE and LOVA were reported in Table 7.2.4.3.6.

Table 7.2.4.1.6: Accuracy data of EZE and LOVA by spectrophotometry method

Spectrophotometic methods		EZE	LOVA	EZE	LOVA	EZE	LOVA
Initial conc.(µg/ml) (A)		10	10	10	10	10	10
Quantity of std. Added (µg/ml) (B)		5	5	10	10	15	15
Total Amount (A + B)		15	15	20	20	25	25
Ratio derivative spectrophotometric method (Mean (n=5) ± %C.V.)	Total quantity Found Mean ± S.D.	15.03	15.04	20.42	20.18	24.98	25.04
	% Recovery± S.D	100.20	100.25	102.10	100.92	99.92	100.15
First derivative zero crossing method (Mean (n=5) ± %C.V.)	Total quantity Found Mean ± S.D.	15.26	14.98	20.04	19.98	24.98	25.29
	% Recovery± S.D	101.73 ± 0.49	99.87 ± 0.94	100.20 ± 0.24	99.90 ± 0.89	99.87 ± 0.94	100.97 ± 0.21

*** Mean value of five determinations.**

(IV) Limit of detection and quantification

The minimum detectable and quantifiable concentration of EZE and LOVA were reported in table 7.2.4.1.8.

7.2.4.1.5.2. Analysis of commercial tablet formulation:

Ten tablets of **EZEDOC** and ten tablets of **AZTATIN** were taken and average weigh was found out. Then powder was made in motor pistol. The Tablet powder equivalent one tablet content was transferred from both tablets into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution was sonicated for 10 min and supernatant was filtered through Whatman filter paper no. 41. This solution was used as the sample solutions for the developed analytical method.

The absorbance of the prepared sample solution was measured at 231 nm and 240.8 nm. The concentrations of EZE and LOVA in tablets were calculated using the equation for Q analysis. The absorbance of the prepared solution was measured at 240.60 nm and 248.8 nm. The concentrations of EZE and LOVA in tablets were calculated using the regression equation for FDZC. The absorbance of the prepared solution was measured at 265.20 nm and 245.40 nm. The concentrations of EZE and LOVA in tablets were calculated using the regression equation for RDZC. Results obtained are reported in Table 7.2.4.1.7.

Table 7.2.4.1.9: Estimation of EZE and LOVA in tablet by spectrophotometric methods.

Formu-lation	Drugs	Labeled Claim (mg/tab)	FDZC		Ratio derivative	
			*Amount found	% Assay	*Amount found	% Assay
EZEDOC + AZTATIN	EZE	10	10.31	103.12	10.03	100.31
	LOV	10	9.97	99.78	10.19	101.94
LAB. MIX.	EZE	10	10.42	104.21	10.21	102.12
	LOV	10	9.98	99.82	10.42	104.24

*** Mean value of five determinations.**

7.2.4.1.6. Summary of Validation parameters:

A summary of the results of validation parameters is reported in Table 7.2.4.1.8.

Table 7.2.4.1.8: Summary of Validation parameters of spectrophotmetery method

Sr. No	Parameters	FDZC		Ratio derivative	
		EZE	LOVA	EZE	LOVA
1	λ max (nm)	265.20	245.40	240.6	248.8
2	Linearity range (µg/ml)	5 to 40 µg/ml			
3	Regression equation	Y=-0.0018 +0.0012	Y=-0.013x +0.0012	Y=0.0017x -0.0034	Y=-0.0065x +0.0057
4	Correlation coefficient (r^2)	0.9993	0.9985	0.9935	0.9987
5	Intercept	0.0012	0.0012	-0.0034	0.0057
6	Slope	-0.0018	-0.0013	0.0017	-0.0065
7	% Assay	100.31	101.14	103.12	99.78
8	Accuracy and precision				
	Intra day % CV (n = 5)	0.30-0.92	0.46-0.82	0.32-0.83	0.29-0.79
	Inter day % CV (n = 5)	0.28-0.85	0.48-0.82	0.53-0.85	0.22-0.63
	Repeatability of measurements % CV	< 1 %			
	% Recovery	99.87 to 101.37	99.87 to 100.97	99.92 to 102.10	100.15 to 100.92
9	Limit of detection	0.39	1.30	0.099	0.0271
10	Limit of quantification	0.12	0.41	0.332	0.902

7.2.4.1.7. CONCLUSION

Derivative spectrophotometric method is a suitable technique for the reliable analysis of commercial formulations containing combinations of EZE and LOVA. (Table 7.2.4.3.7).

7.2.4.2. Infra Red spectroscopic method.

Experimental work:

7.2.4.2.1. Preparation of stock sample

For **quantitative IR** spectroscopic study both standard drugs were weighed accurately about 10 mg and make up to 100 mg on the same butter paper to obtain final concentration of 100 µg/mg of each drug. This sample was triturated in mortar and pestle and sample was used as working standard sample for FTIR spectroscopic method.

7.2.4.2.2. Selection of analytical wavenumber:

Individual IR spectrum of both the drugs and their mixture were overlain (fig. 7.2.4.2.1) to select the zero absorbance point or transmission point. It was observed that 1510.16 cm^{-1} is the zero absorbance point for LOVA so EZE was estimated at this frequency where as LOVA was estimated at 3542.99cm^{-1} at which EZE showed zero absorbance.

7.2.4.2.3. Calibration curve

From the stock standard (100 µg/mg) fractions were transferred to butter paper and the weight was adjusted to 10 mg with KBR to get concentration in the range of 11-150 µg/mg and DRIFTS spectra were recorded. The spectra were scanned between 4000 and 400 cm^{-1}, by averaging 16 scans for each spectrum with a resolution of 4 cm^{-1} (data point resolution per interval 1 cm^{-1}). At selected wave number measurement of Peak area, peak height and peak height at single wave length was done for the EZE and LOVA. Calibration curves were drawn using the multiple linear regression (MLR) method and the drug amount in sample was computed from regression equation.

7.2.4.2.4. Validation of method:

Procedure for validation of analytical method is given in Chapter 7.2.1.2.4.

7.2.4.2.5. Results and Discussion:

7.2.4.2.5.1 Validation Parameters

(I) Linearity

The IR spectrophotometry method showed good linearity for EZE in the range of 11.2 to 147 µg/mg at 1510.16 cm^{-1} (zero absorbance point of LOVA) with co-relation co-efficient of 0.9998 as seen in fig. 7.2.4.2.4. For LOVA the line of best fit was obtained at 3542.99cm^{-1} (zero absorbance point of EZE) with correlation coefficients of 0.9990 in linearity range of 28 to 77.5 µg/mg as seen in fig. 7.2.4.2.5. Calibration data was given is Table 7.2.4.2.1.

Table 7.2.4.2.1: Calibration data of EZE and LOVA by IR Spectroscopy

Sr. No.	Concentration (µg/mg)		Peak height* ± S.D. (N=5)		% C.V.	
	EZE	LOVA	EZE at 1510.16 1/cm	LOVA 3542.991/cm	EZE	LOVA
1	11.2	8.0	0.001 ± 0.00043	0.003 ± 0.00092	0.38	0.85
2.	35.2	37.6	0.592 ± 0.00028	0.017 ± 0.00074	0.29	0.39

3	41.2	61.3	0.727 ± 0.00078	0.0585 ± 0.00081	0.78	0.92
4	147	77.5	200 ± 0.00038	0.088 ± 0.00058	0.43	0.18

*Mean value of five determinations.

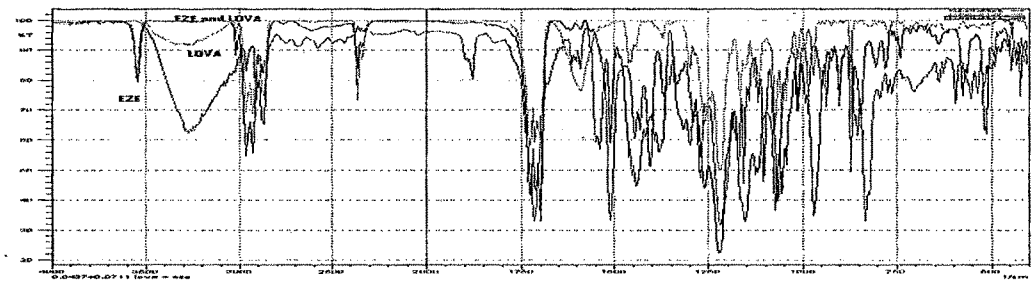


Figure: 7.2.4.2.1: Overlain IR spectra of EZE and LOVA

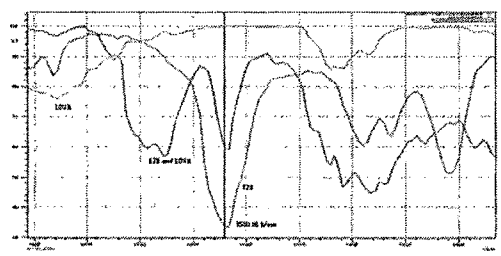


Figure: 7.2.4.2.2: Overlain IR spectra of LOVA and EZE at 1510.16 cm^{-1}

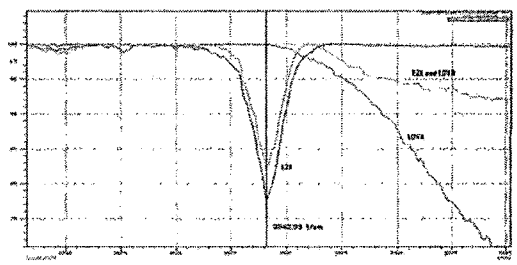


Figure: 7.2.4.2.3: Overlain IR spectra of LOVA and EZE at 3542.99cm^{-1}

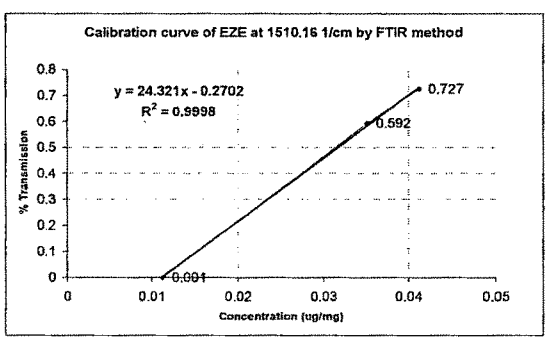


Figure 7.2.4.2.4: Calibration curve of EZE at 1510.16cm^{-1} by IR method
Correlation co-efficient = **0.9998**
Slope = 24.321
Intercept = -0.2702
Regression equation: **Abs. = 24.321 × Conc. - 0.2702**

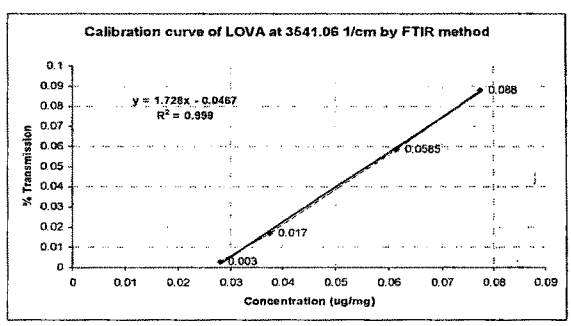


Figure 7.2.4.2.5: Calibration curve of LOVA at 3542.99cm^{-1} by IR method
Correlation co-efficient = **0.9990**
Slope = 1.728
Intercept = -0.0467
Regression equation: **Abs. = 1.728× Conc. - 0.0467**

(II) Precision

Repeatability

Three different aliquot were prepared and the experiments were repeated five times a day for intra day and on five different days for inter day precision. The method was found to be precise on intra day and inter day basis as the average % CV values were

in the range of 0.28 – 0.83 % and 0.18 - 0.75% for EZE and 0.21 – 0.79 % and 0.18 – 0.94 % for LOVA (Table 7.2.4.2.2 and 7.2.4.2.3), respectively.

Table 7.2.4.2.2: Intraday precision data of EZE and LOVA by IR Spectroscopy

Concentration (µg/mg)		Peak height (Mean ± S.D.) (n=5)		% C.V*.	
EZE	LOVA	EZE at 1510.16 cm^{-1}	LOVA at 3542.99cm^{-1}	EZE	LOVA
15	15	0.00134 ± 0.00053	0.00661 ± 0.00093	0.53	0.48
30	30	0.50455 ± 0.028	0.01356 ± 0.0027	0.75	0.21
60	60	1.12937 ± 0.073	0.05726 ± 0.0079	0.28	0.79
Table 7.2.4.2.3: Interday precision data of EZE and LOVA by IR Spectroscopy					
15	15	0.00132 ± 0.00053	0.0067 ± 0.00066	0.28	0.57
30	30	0.50541 ± 0.0028	0.01376 ± 0.0039	0.75	0.94
60	60	1.13731 ± 0.0052	0.05777 ± 0.0025	0.18	0.18

* Mean value of five determinations.

Reproducibility:

To study the reproducibility of IR method two sample compartments having different diameter were used. Compartment of higher diameter was termed as compartment number I and of smaller diameter was termed as compartment number II. Same sample was put in both compartments and peak area was compared. The % CV of the responses for determination of EZE and LOVA revealing the reproducibility of the method is reported in Table 7.2.4.2.4.

Table 7.2.4.2.4: Reproducibility data of EZE and LOVA by IR Spectroscopy

Concentration (µg/mg)		Peak height*			
EZE	LOVA	EZE at 1510.16 cm^{-1}		LOVA at 3542.99cm^{-1}	
		Sample compartment I	Sample compartment II	Sample compartment I	Sample compartment II
15	15	0.00134	0.00134	0.00661	0.00661
30	30	0.50455	0.50455	0.01313	0.01313
45	45	0.79405	0.79405	0.04294	0.04294

***Mean value of five determinations**

(III) Accuracy

Accuracy of the method was determined by performing recovery study on laboratory prepared synthetic mixture by standard addition of 50, 100 and 150%. The method

showed % mean recovery for EZE in the range of 98.66 – 101.09 % and for LOVA it was 100.61 – 102.19 % in synthetic mixture (Table 7.2.4.2.5).

Table 7.2.4.2.5: Accuracy data of EZE and LOVA by IR Spectroscopy

Initial conc. (µg/mg) (A)		Quantity of std. Added (µg/mg) (B)		Total Amount (A + B)		Total quantity Found*		% Recovery± C.V*	
EZE	LOVA	EZE	LOVA	EZE	LOVA	EZE at 1510.16 cm^{-1}	LOVA at 3542.99cm^{-1}	EZE	LOVA
10	10	5	5	15	15	14.80	15.33	98.66 ± 0.52	102.19 ± 0.74
10	10	15	15	25	25	25.03	25.37	100.14 ± 0.89	101.48 ± 0.29
10	20	25	50	35	70	35.38	70.43	101.09 ± 0.24	100.61 ± 0.22

*** Mean value of three determinations**

(IV) Limit of detection

The minimum detectable concentration of EZE and LOVA were found to be 0.4835 µg/mg and 7.1248 µg/mg, respectively.

(V) Limit of quantification

The lowest quantifiable concentration of EZE and LOVA were found to be 1.616 µg/mg and 23.7494 µg/mg, respectively by practical observation.

7.2.4.2.3.2. Applicability of the method for the analysis of commercial tablet formulation:

Procedure for prepateation of test sample is given in Chapter 7.2.4.1. Filtrate was evaporated and from the residue 1 mg was made up to 100 mg with KBr on butter paper. This was triturated in mortar and pestle and DRIFT spectra was scannned between 4000 and 400 cm^{-1}, by averaging 16 scans for each spectrum with a resolution of 4 cm^{-1} (data point resolution per interval 1 cm^{-1}). 1510.16 cm^{-1} and 3542.99cm^{-1} were used for estimation for EZE and LOVA, at single wavelength, respectively. A synthetic mixture was prepared having PRAVA and EZE in ratio of 1:1. Concentration of sample was found from regression equation of EZE and LOVA.

Table 7.2.4.2.6: Estimation of EZE and LOVA in tablet by IR spectrosopy.

Formulation	DRUGS	Labeled Claim (mg/tab)	QIR	
			*Amount found	% Assay ± %CV
EZEDOC + AZTATIN	EZE	10	10.01	100.12
	LOV	10	10.21	102.19
LAB. MIX.	EZE	10	10.32	103.20
	LOV	20	19.99	99.91

*** Mean value of five determinations.**

7.2.4.2.4. Summary of Validation parameters:

The summary of validation parameter is reported in Table 7.2.4.2.7.

Table 7.2.4.2.7: Summary of Validation parameters of FDZC method

Sr. No	Parameters	Results	
		EZE	LOVA
1	Wavenumber (cm^{-1})	1510.16	3542.90
2	Linearity range (µg/mg)	11.2-147	2.8-77.5
3	Regression equation	Y=24.321x -0.2702	Y=1.728x -0.0467
4	Correlation coefficient (r^2)	0.9998	0.999
5	Intercept	-0.2702	-0.0467
6	Slope	24.321	1.728
7	Assay	101.12	102.4
8	Precision		
	Intra day % CV (n = 5)	0.28-0.83	0.21-0.79
	Inter day % CV (n = 5)	0.18-0.75	0.18-0.94
	Reproducibility of measurements % CV	< 1 %	
	% Recovery	98.66-101.09	100.61-102.19
10	Limit of detection (µg/mg)	0.4835	0.71248
11	Limit of quantification(µg/mg)	1.616	2.37494

7.2.4.2.5. CONCLUSION

The proposed method is simple, precise and accurate and can be used as a method for quality control of pharmaceuticals. This technique extends the use of a standard IR spectrophotometer typically used for identification purposes, to the reliable quantification of EZE and LOVA. The present method opens the possibility of applying IR spectroscopy to quantify other active ingredients.

7.2.4.3. Simultaneous estimation of EZE and LOVA by Chemometric Methods:

In this section the Chemometric methods have been described for EZE and LOVA. The concept of mathematical modeling is used to provide maximum relevant chemical information in order to analyze the drugs simultaneously.

The two-chemometric techniques ILS and CLS are developed for the simultaneous determination of the titled drugs in their binary mixture.

7.2.4.3. Inverse Least Square (ILS) and Classical Least Square (CLS) technique:

It is the application of multiple linear regressions (MLR) to the inverse expression of the Beer-Lambert Law of spectrophotometry.

Calibration (training) and validation set of the EZE and LOVA were prepared as per procedure given in Chapter 7.2.1.3.1.

7.2.4.3.1. Result and discussion:

7.2.4.3.1.1. Selection of the spectral region:

In order to develop ILS method for simultaneous estimation of EZE and LOVA, light absorption study was done. Individual spectrum of both the drugs and their binary mixture were overlain.

Although the ILS is the full spectrum method, 21 wavelengths were selected between 230 nm to 250 nm with the interval of $\Delta\lambda = 1$ nm in the zero order spectra as shown in fig. 7.2.4.3.1.

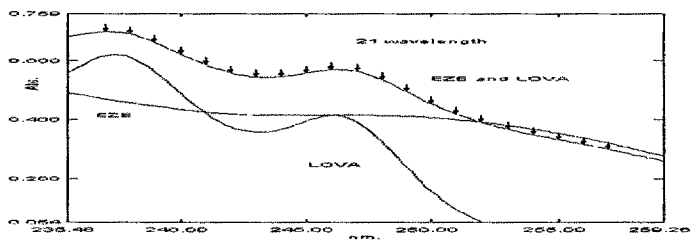


Fig 7.2.4.3.1: Overlain spectra of EZE, LOVA and their binary mixture showing spectral region 237 nm to 258 nm (21 wavelengths)

7.2.4.3.1.2. Measurement of the absorbance:

The absorbance matrices were produced by measuring absorbencies of binary mixture at 21 wavelengths. In this calibration was obtained by measuring absorbance data matrix and concentration data matrix to predict the concentration of EZE and LOVA in their binary mixtures and tables. The numerical calculations were performed using MATLAB 6.1 software and excel.

7.2.4.3.1.3. Equation for ILS method:

The mathematical expression of ILS is C = P x A.

The calibration coefficient (P) was obtained from the linear equation system using the absorbance data and the concentration taken in training set (Table 7.2.4.3.1 and 7.2.4.3.2)

The absorbance values (Table 7.2.4.3.3) of the samples at 21 wavelengths were placed in the above equation and the amounts of EZE and LOVA in the synthetic mixture and formulation were found as shown in Table 7.2.4.3.4.

Table 7.2.4.3.1: Composition of Calibration (training) set for EZE and LOVA:

Mixture No.	EZE (µg/ml)	LOVA (µg/ml)	Mixture No.	EZE (µg/ml)	LOVA (µg/ml)
1	0	5.8	14	16.95	23.2
2	2.26	11.6	15	16.95	29
3	5.65	5.8	16	16.95	34.8
4	5.65	20.86	17	20.34	29
5	6.22	20.3	18	22.6	20.88
6	11.3	5.8	19	22.6	23.2
7	11.3	11.6	20	22.6	23.2
8	11.3	20.38	21	24.86	34.8
9	13.56	23.2	22	28.25	20.88
10	16.39	8.7	23	36.16	11.6
11	16.95	11.6	24	36.64	17.4
12	16.95	17.4	25	42.95	0
13	16.95	20.8	-	-	-

Table 7.2.4.3.2: Absorbance data for the calibration set at 21 wavelengths:

Absorbance	Mi No.	Wavelength																				
		237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257
	1	0.299	0.297	0.275	0.24	0.208	0.185	0.175	0.18	0.194	0.206	0.196	0.164	0.122	0.082	0.052	0.033	0.02	0.013	0.008	0.005	0.004
	2	0.786	0.776	0.718	0.636	0.562	0.509	0.487	0.498	0.531	0.555	0.53	0.454	0.355	0.266	0.198	0.151	0.119	0.099	0.085	0.076	0.069
	3	0.559	0.549	0.515	0.469	0.429	0.4	0.39	0.397	0.415	0.429	0.415	0.375	0.323	0.276	0.239	0.213	0.193	0.179	0.167	0.156	0.146
	4	1.476	1.454	1.343	1.195	1.061	0.965	0.927	0.948	1.008	1.051	1.001	0.861	0.682	0.519	0.397	0.313	0.255	0.219	0.193	0.174	0.159
	5	0.643	0.632	0.592	0.538	0.489	0.455	0.442	0.45	0.472	0.489	0.472	0.424	0.36	0.303	0.259	0.228	0.204	0.188	0.175	0.163	0.152
	6	0.761	0.748	0.71	0.663	0.622	0.593	0.582	0.588	0.605	0.618	0.606	0.568	0.518	0.469	0.431	0.401	0.377	0.358	0.34	0.322	0.304
	7	1.162	1.142	1.073	0.984	0.905	0.849	0.827	0.839	0.873	0.897	0.871	0.793	0.69	0.597	0.524	0.471	0.43	0.401	0.376	0.354	0.333
	8	1.714	1.687	1.569	1.419	1.281	1.185	1.147	1.168	1.227	1.27	1.219	1.078	0.897	0.731	0.607	0.518	0.455	0.412	0.378	0.352	0.329
	9	1.405	1.384	1.291	1.163	1.048	0.966	0.934	0.95	0.999	1.035	0.994	0.879	0.729	0.592	0.487	0.413	0.36	0.325	0.299	0.278	0.26
	10	1.145	1.123	1.065	0.993	0.93	0.886	0.869	0.878	0.905	0.923	0.903	0.844	0.765	0.692	0.633	0.589	0.553	0.524	0.497	0.47	0.444
	11	1.378	1.355	1.281	1.188	1.105	1.045	1.021	1.033	1.067	1.093	1.065	0.985	0.882	0.784	0.707	0.649	0.604	0.57	0.539	0.51	0.482
	12	1.765	1.735	1.63	1.495	1.373	1.287	1.253	1.271	1.322	1.358	1.315	1.195	1.04	0.896	0.784	0.702	0.641	0.597	0.561	0.527	0.496
	13	1.971	1.939	1.818	1.659	1.517	1.416	1.375	1.399	1.46	1.502	1.449	1.303	1.116	0.943	0.812	0.716	0.646	0.597	0.556	0.522	0.49
	14	2.048	2.014	1.889	1.722	1.571	1.468	1.425	1.448	1.51	1.555	1.501	1.352	1.157	0.974	0.837	0.736	0.663	0.612	0.57	0.535	0.502
	15	2.422	2.385	2.232	2.035	1.849	1.719	1.665	1.691	1.774	1.828	1.756	1.561	1.309	1.077	0.9	0.774	0.683	0.622	0.574	0.535	0.501
	16	2.71	2.69	2.53	2.307	2.091	1.936	1.871	1.905	1.997	2.067	1.982	1.75	1.451	1.175	0.965	0.817	0.711	0.641	0.588	0.545	0.509
	17	2.533	2.494	2.332	2.136	1.948	1.814	1.763	1.787	1.866	1.923	1.854	1.665	1.42	1.193	1.017	0.891	0.8	0.735	0.682	0.639	0.6
	18	2.107	2.073	1.956	1.802	1.663	1.564	1.524	1.542	1.598	1.64	1.594	1.459	1.279	1.114	0.984	0.888	0.817	0.764	0.717	0.676	0.637
	19	1.957	1.927	1.8	1.629	1.475	1.365	1.322	1.343	1.409	1.458	1.401	1.241	1.04	0.855	0.714	0.613	0.543	0.494	0.455	0.424	0.397
	20	2.091	2.052	1.942	1.787	1.649	1.552	1.515	1.535	1.591	1.632	1.58	1.448	1.272	1.109	0.983	0.889	0.819	0.766	0.72	0.679	0.64
	21	2.961	2.882	2.777	2.562	2.346	2.189	2.139	2.167	2.253	2.328	2.237	2.011	1.716	1.443	1.232	1.081	0.97	0.893	0.832	0.779	0.73
	22	2.361	2.343	2.21	2.054	1.913	1.814	1.771	1.792	1.85	1.891	1.84	1.699	1.514	1.339	1.203	1.101	1.022	0.962	0.909	0.859	0.811
	23	2.033	1.993	1.914	1.81	1.718	1.654	1.626	1.637	1.671	1.696	1.668	1.589	1.485	1.381	1.297	1.226	1.166	1.116	1.065	1.013	0.96
	24	2.304	2.263	2.152	2.017	1.894	1.804	1.769	1.787	1.836	1.869	1.829	1.713	1.557	1.41	1.292	1.202	1.129	1.071	1.016	0.964	0.912
	25	0.676	0.663	0.648	0.636	0.626	0.62	0.617	0.617	0.618	0.621	0.621	0.62	0.616	0.608	0.599	0.586	0.571	0.554	0.534	0.51	0.485

Table 7.2.4.3.3: Absorbance data for the validation set at 21 wavelengths:

Absorbance	Mi No.	Wavelength																			
		237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257
	1	1.708	1.682	1.535	1.33	1.15	1.02	0.973	1.006	1.09	1.147	1.074	0.877	0.628	0.403	0.239	0.128	0.056	0.013	-0.012	-0.026	-0.033
	2	0.378	0.372	0.351	0.326	0.303	0.287	0.282	0.286	0.297	0.305	0.299	0.277	0.249	0.223	0.201	0.184	0.171	0.162	0.153	0.144	0.135
	3	1.206	1.186	1.096	0.969	0.858	0.779	0.749	0.769	0.821	0.856	0.813	0.694	0.546	0.411	0.31	0.241	0.193	0.162	0.141	0.125	0.113
	4	1.936	1.903	1.755	1.55	1.363	1.231	1.183	1.214	1.296	1.354	1.281	1.082	0.834	0.608	0.439	0.324	0.246	0.198	0.165	0.143	0.127
	5	1.431	1.408	1.305	1.168	1.046	0.959	0.928	0.95	1.005	1.043	0.998	0.871	0.708	0.561	0.449	0.371	0.318	0.281	0.255	0.235	0.217
	6	1.407	1.384	1.286	1.157	1.041	0.958	0.929	0.95	1.001	1.037	0.994	0.876	0.723	0.583	0.477	0.403	0.351	0.315	0.288	0.267	0.248
	7	1.029	1.013	0.946	0.858	0.778	0.722	0.702	0.716	0.752	0.777	0.751	0.671	0.569	0.474	0.401	0.35	0.314	0.287	0.266	0.249	0.232
	8	1.766	1.736	1.63	1.49	1.368	1.28	1.247	1.269	1.322	1.359	1.316	1.192	1.032	0.885	0.773	0.691	0.63	0.585	0.548	0.516	0.484
	9	1.007	0.989	0.943	0.888	0.839	0.804	0.791	0.799	0.819	0.834	0.82	0.775	0.718	0.662	0.616	0.58	0.55	0.523	0.498	0.472	0.446
	10	1.55	1.523	1.446	1.345	1.257	1.194	1.171	1.185	1.221	1.247	1.218	1.135	1.027	0.925	0.844	0.782	0.733	0.692	0.656	0.621	0.587
	11	2.02	1.982	1.875	1.728	1.606	1.52	1.486	1.505	1.557	1.593	1.551	1.431	1.273	1.126	1.01	0.924	0.858	0.807	0.761	0.719	0.678
	12	1.71	1.677	1.591	1.483	1.39	1.321	1.295	1.311	1.35	1.379	1.349	1.258	1.142	1.03	0.941	0.875	0.822	0.777	0.737	0.698	0.659
	13	2.169	2.133	2.019	1.881	1.756	1.667	1.633	1.651	1.705	1.738	1.696	1.575	1.42	1.271	1.154	1.066	0.996	0.94	0.891	0.843	0.796
	14	1.367	1.338	1.309	1.283	1.262	1.248	1.242	1.242	1.246	1.25	1.25	1.245	1.238	1.222	1.201	1.175	1.144	1.108	1.066	1.019	0.968
	15	2.418	2.367	2.256	2.116	1.99	1.905	1.871	1.889	1.944	1.971	1.931	1.813	1.659	1.51	1.393	1.3	1.226	1.165	1.107	1.051	0.994

Table 7.2.4.3.4: Composition of Validation set for EZE and LOVA:

Mixture No.	EZE (µg/ml)	LOVA (µg/ml)	Mixture No.	EZE (µg/ml)	LOVA (µg/ml)
1	0	29	9	16.95	5.8
2	5.65	3.48	10	22.6	11.6
3	5.65	17.4	11	24.86	17.4
4	5.65	29	12	25.43	13.34
5	9.61	19.14	13	28.25	17.4
6	10.17	17.4	14	33	0
7	11.3	11.6	15	36.16	17.4
8	16.95	17.4	-	-	-

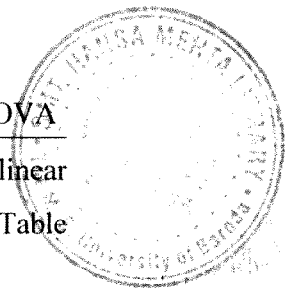
Introducing (P) into the linear equation system, the calibration for ILS was obtained as fig. 7.2.4.3.

$$\begin{bmatrix} C_{EZE} \\ C_{ROSU} \end{bmatrix} \approx 1.0e+004 * \begin{pmatrix} 0.1470 & -0.2913 \\ -0.0681 & 0.1439 \\ -0.0869 & -0.1538 \\ -0.5632 & 1.3282 \\ 0.8184 & -1.3599 \\ 0.1081 & -0.2344 \\ -0.1445 & 0.1237 \\ -0.2483 & -0.4280 \\ -0.3257 & 0.9662 \\ 0.3111 & -0.1115 \\ -0.0660 & -0.1082 \\ 0.3773 & 0.3670 \\ 0.0011 & -0.7387 \\ -0.9359 & 1.2044 \\ 0.8144 & -0.2949 \\ -0.1844 & -1.7372 \\ 0.9435 & -0.2111 \\ -1.3914 & 1.1338 \\ 0.9642 & -1.1156 \\ -1.2263 & 2.4934 \\ 0.7696 & -0.8527 \end{pmatrix} \times \begin{pmatrix} A_1 \\ A_2 \\ A_3 \\ A_4 \\ A_5 \\ A_6 \\ A_7 \\ A_8 \\ A_9 \\ A_{10} \\ A_{11} \\ A_{12} \\ A_{13} \\ A_{14} \\ A_{15} \\ A_{16} \\ A_{17} \\ A_{18} \\ A_{19} \\ A_{20} \\ A_{21} \end{pmatrix}$$

Fig 7.2.4.3.2: Equation of ILS

7.2.4.3.3. Equation for CLS method:

The mathematical expression of ILS is A = K x C.



In this method, the calibration coefficient (K) was obtained from the linear equation system using the absorbance data and the training set (Table 7.2.4.3.1 and 7.2.4.3.2)

The absorbance values (Table 7.2.4.3.3) of the samples at 21 wavelengths were placed in the above equation and the amounts of EZE and LOVA in the synthetic mixture and formulation were found as shown in Table 7.2.4.3.4.

Introducing (K) into the linear equation system, the calibration for ILS was obtained as fig. 7.2.4.3.1.

$$\begin{pmatrix} A1 \\ A2 \\ A3 \\ A4 \\ A5 \\ A6 \\ A7 \\ A8 \\ A9 \\ A10 \\ A11 \\ A12 \\ A13 \\ A14 \\ A15 \\ A16 \\ A17 \\ A18 \\ A19 \\ A20 \\ A21 \end{pmatrix} \approx \begin{pmatrix} 0.0292 & 0.0634 \\ 0.0286 & 0.0625 \\ 0.0277 & 0.0582 \\ 0.0269 & 0.0521 \\ 0.0263 & 0.0464 \\ 0.0259 & 0.0423 \\ 0.0257 & 0.0407 \\ 0.0257 & 0.0416 \\ 0.0259 & 0.0441 \\ 0.026 & 0.0459 \\ 0.0259 & 0.0437 \\ 0.0256 & 0.0375 \\ 0.0251 & 0.0297 \\ 0.0245 & 0.0225 \\ 0.024 & 0.0171 \\ 0.0233 & 0.0134 \\ 0.0226 & 0.0108 \\ 0.0219 & 0.0092 \\ 0.0211 & 0.008 \\ 0.0201 & 0.0073 \\ 0.0191 & 0.0066 \end{pmatrix} \times \begin{pmatrix} CEZE \\ \\ CROSU \end{pmatrix}$$

Fig 7.2.4.3.3: Equation of CLS

7.2.4.3.4. Validation Parameters

(I) Accuracy

Accuracy of the method was determined by performing recovery study on laboratory prepared synthetic mixture by standard addition method. % mean recovery for EZE and LOVA in the range of 90.46 – 102.13 % and 98.12 – 104.00 % from synthetic mixture (Table 7.2.4.3.5)by ILS method and 96.95-107.73 % and 90.57-106.59% by CLS method

(II) Precision

The precision was determined by means of a one way ANOVA including experiments were repeated five times in a day for intra day and on five different days for inter day precision for 10 synthetic mixtures. F values below

the tabulated levels were obtained and there was no significant difference between the results obtained in the determination of each drug in the presence of other on different days.

Table 7.2.4.3.5: Data for precision study using one way ANOVA

Parameters	ILS		CLS	
	EZE	ROSU	EZE	ROSU
Between days variance	2.59	3.12	4.65	2.84
Within days variance	1.29	1.48	1.93	1.42
F ratio	**2.01**	**2.11**	**2.41**	**2.01**

Note the between day and within day degrees of freedom are 4 and 45 respectively. The critical F ratio value for 4 and 45 df and the confidence level of 95% is 2.53.

(III) Limit of detection

The minimum detectable concentration of EZE and LOVA was found to be 0.069 µg/ml and 0.173 µg/ml and 0.0329 µg/ml and 0.281 µg/ml, respectively by ILS and CLS methods.

(IV) Limit of quantification

The lowest quantifiable concentration of EZE and LOVA was found to be 0.492 µg/ml and 0.946 µg/ml and 0.238 µg/ml and 0.772 µg/ml, respectively by ILS and CLS method.

(V) Predicted versus known concentration plot:

The predicted concentrations of the validation samples were plotted against the known concentration values.

This tool is used to determine whether the model accounts for the concentration variation in the validation set or not.

Plots were expected to fall on a straight line with a slope of 1 and o intercept. The predicted versus known concentration plots of the prepared concentration plots of the prepared validation samples are shown in fig. 7.2.4.3.4 and 7.2.4.3.5 by ILS method and fig. 7.2.4.3.6 and 7.2.4.3.7 by CLS method.

Table 7.2.4.3.6: Actual, predicted and residual values.

SR. No.	Actual Conc.		Predicted conc.		Residual		Percentage	
	EZE	LOVA	EZE	LOVA	EZE	LOVA	EZE	LOVA
1	0	29	0	29.7591	0	-0.7591	0.00	102.62
2	5.65	3.48	5.1109	3.5335	0.5391	-0.0535	90.46	101.54
3	5.65	17.4	5.6614	17.2415	-0.0114	0.1585	100.20	99.09
4	5.65	29	5.5742	28.555	0.0758	0.445	98.66	98.47
5	9.61	19.14	9.8146	19.5792	-0.2046	-0.4392	102.13	102.29
6	10.17	17.4	9.9484	17.2654	0.2216	0.1346	97.82	99.23
7	11.3	11.6	11.7336	11.3815	-0.4336	0.2185	103.84	98.12
8	16.95	17.4	17.1131	17.6874	-0.1631	-0.2874	100.96	101.65
9	16.95	5.8	17.2315	5.757	-0.2815	0.043	101.66	99.26
10	22.6	11.6	22.7635	12.0637	-0.1635	-0.4637	100.72	104.00
11	24.86	17.4	25.0023	17.3221	-0.1423	0.0779	100.57	99.55
12	25.43	13.34	25.6297	13.3028	-0.1997	0.0372	100.79	99.72
13	28.25	17.4	28.4971	17.0385	-0.2471	0.3615	100.87	97.92
14	33	0	33.667	0	-0.667	0	102.02	0.00
15	36.16	17.4	36.3861	17.4646	-0.2261	-0.0646	100.63	100.37
16	0	29	0	29.7591	0	-0.7591	0.00	102.62
17	5.65	3.48	5.1109	3.5335	0.5391	-0.0535	90.46	101.54
18	5.65	17.4	5.6614	17.2415	-0.0114	0.1585	100.20	99.09
19	5.65	29	5.5742	28.555	0.0758	0.445	98.66	98.47
20	9.61	19.14	9.8146	19.5792	-0.2046	-0.4392	102.13	102.29
21	10.17	17.4	9.9484	17.2654	0.2216	0.1346	97.82	99.23

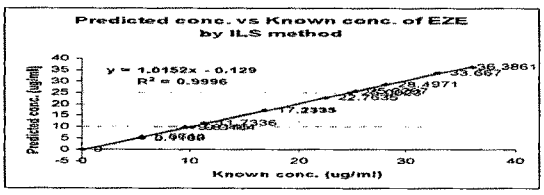


Fig 7.2.4.3.4: Linearity plots of EZE and LOVA for validation set.

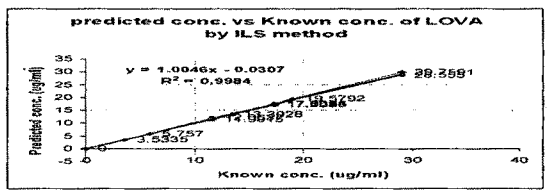


Fig 7.2.4.3.5: Linearity plots of EZE and LOVA for validation set.

Table 7.2.4.3.7: Actual, predicted and residual values.

SR. No.	Actual Conc.		Predicted conc.		Residual		Percentage	
	EZE	LOVA	EZE	LOVA	EZE	LOVA	EZE	LOVA
1	0	29	0	29.6478	0	-0.6478	100.00	102.23
2	5.65	3.48	6.0865	3.1517	-0.4365	0.3283	107.73	90.57
3	5.65	17.4	5.7689	17.1218	-0.1189	0.2782	102.10	98.40
4	5.65	29	5.7888	29.3366	-0.1388	-0.3366	102.46	101.16
5	9.61	19.14	10.1635	20.4012	-0.5535	-1.2612	105.76	106.59
6	10.17	17.4	10.2763	17.0745	-0.1063	0.3255	101.05	98.13
7	11.3	11.6	11.807	11.5077	-0.507	0.0923	104.49	99.20
8	16.95	17.4	16.6211	17.0927	0.3289	0.3073	98.06	98.23
9	16.95	5.8	17.3791	5.6281	-0.4291	0.1719	102.53	97.04
10	22.6	11.6	22.4974	11.174	0.1026	0.426	99.55	96.33
11	24.86	17.4	24.1007	18.2849	0.7593	-0.8849	96.95	105.09
12	25.43	13.34	25.995	13.0477	-0.565	0.2923	102.22	97.81
13	28.25	17.4	28.467	17.82	-0.217	-0.42	100.77	102.41
14	33	0	33.5762	0	-0.5762	0	101.75	100.00
15	36.16	17.4	36.1787	16.8177	-0.0187	0.5823	100.05	96.65
16	0	29	0	29.6478	0	-0.6478	100.00	102.23
17	5.65	3.48	6.0865	3.1517	-0.4365	0.3283	107.73	90.57
18	5.65	17.4	5.7689	17.1218	-0.1189	0.2782	102.10	98.40
19	5.65	29	5.7888	29.3366	-0.1388	-0.3366	102.46	101.16
20	9.61	19.14	10.1635	20.4012	-0.5535	-1.2612	105.76	106.59
21	10.17	17.4	10.2763	17.0745	-0.1063	0.3255	101.05	98.13

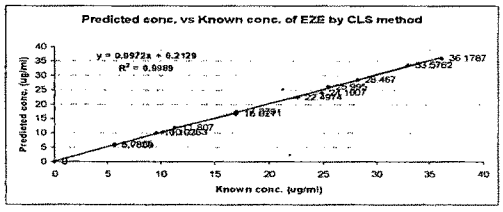


Fig 7.2.4.3.6: Linearity plots of EZE for validation set.

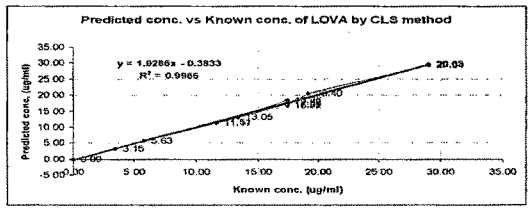


Fig 7.2.4.3.7: Linearity plots of LOVA for validation set.

This indicates that the prediction ability of the validation set is good.

(VI) Residuals concentration versus actual concentration plot:

The difference between the known and predicted concentration (residuals) were plotted against the actual concentrations for the validation samples.

This tool is used to determine whether the model accounts for the concentration variation in the validation set and it also provides information about how well the method will predict the future samples.

For the validation set it can be found that the residual values are more close to zero and are more randomly distributed as illustrated in fig. 7.2.4.3.8 and 7.2.4.3.9 by ILS method and fig. 7.2.4.3.10 and 7.2.4.3.11 by CLS method.

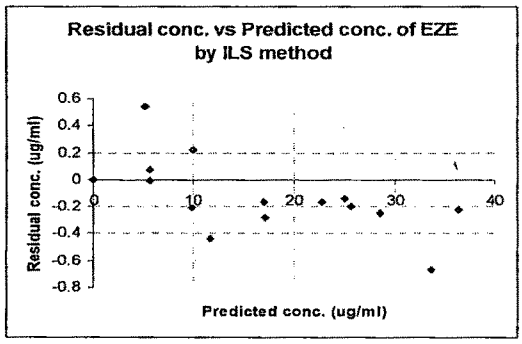


Fig 7.2.4.3.8: Residual vs. predicted concentration plot for EZE and LOVA.

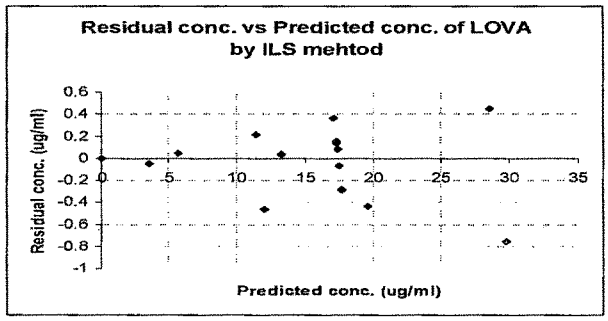


Fig 7.2.4.3.9: Residual vs. predicted concentration plot for EZE and LOVA.

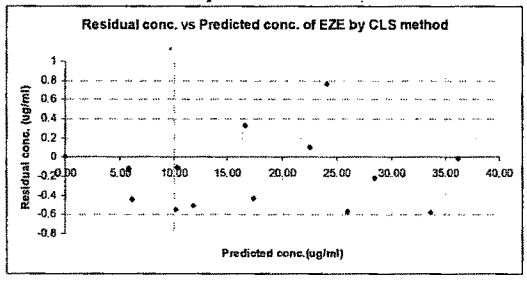


Fig 7.2.4.3.10: Residual vs. predicted concentration plot for EZE.

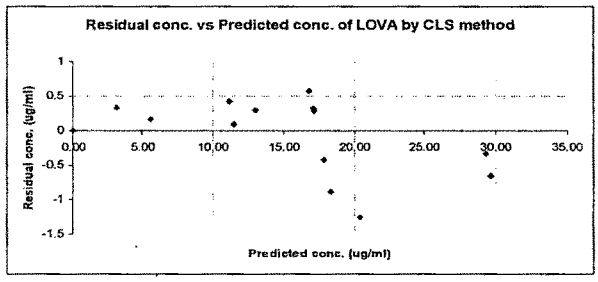


Fig 7.2.4.3.11: Residual vs. predicted concentration plot for LOVA.

(VII) Root Mean Square Error of Prediction:

RMSEP was calculated using the equation mentioned in Chapter 5.

Table 7.2.4.3.8: RMSEP values for EZE and LOVA.

Compound	RMSEP	RMSEP
EZE	0.1879	0.2471
LOVA	0.3097	0.1830

7.2.4.3.5. Preparation of sample solutions:

Procedure for preparation for the sample is given in Chapter 7.2.1.1. The absorbance of the prepared solution was measured between 231.0 nm and 265.2 nm. The concentrations of EZE and LOVA in tablets were calculated using the developed model. Results are shown in Table 7.2.1.3.2.9.

Table 7.2.1.3.9: Estimation of EZE and LOVA in tablet by CLS method

Formulation	DRUGS	Labeled Claim (mg/tab)	ILS		CLS	
			*Amount found	% Labeled Claim	*Amount found	% Labeled Claim
LOVAS-EZ	EZE	10	9.94	99.43	9.98	99.82
	SIM	10	10.26	102.56	10.32	103.24
STARSTAT-EZ	EZE	20	10.44	104.40	10.44	104.38
	SIM	10	10.43	104.30	9.90	99.00

*** Mean value of five determinations.**

7.2.4.3.6. Summary of Validation parameters:

The summary of validation parameters were reported in Table 7.2.1.3.1.10

Table 7.2.4.3.10: Summary of Validation parameters by ILS and CLS methods.

Sr. No	Parameters	ILS		CLS	
		EZE	LOVA	EZE	LOVA
1	Calibration design	24			
2	Validation design	15			
3	Spectral region	237 -257			
4	Linearity range (µg/ml)	1 – 40 µg/ml			
5	Regression equation	Y=1.0152x -0.129	Y=1.0046x -0.0307	Y=0.9972x +0.2129	Y=1.0286x -0.3833
6	Correlation coefficient (r^2)	0.9996	0.9984	0.9989	0.9966
7	Intercept	-0.129	-0.0307	0.2129	-0.3833
8	Slope	1.0152	1.0046	0.9972	1.0286
9	RMSEP	0.18	0.30	0.24	0.18
10	Assay	99.43	102.56	99.82	103.24
11	Precision				
12	% Recovery	99.91-102.29	100.50-103.40	99.77-103.40	97.73-103.05
12	Limit of detection (µg/ml)	0.069	0.173	0.0329	0.281
13	Limit of quantification(µg/ml)	0.492	0.946	0.283	0.772

7.2.4.3. CONCLUSION

The proposed ILS and CLS methods were found to be precise and accurate. The mathematical calculations for the proposed ILS and CLS methods could be effectively performed using MATLAB and Excel. The simplicity of the method can be explained on the basis of direct out put of the data in terms of unknown concentration on providing the concentration matrix and absorbance matrix of the calibration set to the software. The method does not require any time consuming separation or sample preparation step as used in HPLC.

The ILS method can be strongly applied to a routine analysis, quality control of binary mixtures and commercial products containing these two drugs.

7. 2. 5

Simvastatin and Nicotinic acid

7.2.5. Development of HPTLC method for Simvastatin and Nicotinic acid:

Simvastatin (SIMVA) and nicotinic acid (NICO) were given as antihyperlipidemic agent in combination[1, 2]. In Indian market combined commercial formulations available was SIMVOTIN TAB Ranbaxy Pharmaceuticals containing SIMVA and NICO in the ratio of 0.5:1.25, respectively. HPTLC method was developed which could separate SIMVA and NICO with the R_f of 0.66 and 0.44, respectively.

7.2.5.1 Apparatus, Instruments and reagent:

Apparatus, instruments and reagents used for estimation of HPTLC method is mentioned in chapter 6.

7.2.5.2 Procedure of preparation of stock of SIMVA and NICO

Standard SIMVA (5 mg) and NICO (125mg) were weighed accurately and transferred in to two 10 ml volumetric flask. It was dissolved in 5 ml methanol properly and diluted up to mark with methanol to obtain final concentration of 500 µg/ml of SIMVA and 12500 µg/ml NICO. Varied aliquots from these solutions were transferred to other 10 ml volumetric flasks and diluted up to the mark with methanol to obtain a series of standard binary mixtures.

7.2.5.3. Optimization of method:

For the simultaneous analysis of SIMVA and NICO by HPTLC, efforts were made to select a common solvent and a common absorption wavelength.

Determination of solvent for sample preparation and λmax:

Different solvents were tried to study the solubility of both the drugs as stated in table 6.4. So methanol was selected for the preparation of drug solutions which also good for HPTLC method development. Wavelength was selected by scanning standard solutions of both drugs over 200 nm to 400 nm wavelengths. Drugs show significant absorbance at **237 nm.** So this wavelength was selected in densitometer UV detector.

Determination of mobile phase:

Various combinations of mobile phases were tried for effective separation of SIMVA and NICO on silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 µm thickness) (E. Merck). The results are reported in Table 7.2.5.1.

Table7.2.5.1: Determination of mobile phase

Mobile phase combination	Ration (V/V/V)	Peak separation
Chloroform: Methanol	5: 5	No separation band
Benzene: Methanol	8: 2	No separation
Benzene: Methanol	5: 5	No separation
Toluene: Methanol	8: 2	Spot not separated
Hexane: Methanol	5: 5	Spot not separated
Ethyl acetate : Toluene: Methanol	3: 4: 3	Slight separation
Ethyl acetate: Toluene: Methanol	4: 4: 2	Good separation but broad band
Ethyl acetate: Benzene: Methanol	3: 4: 3	Adjacent spots
Ethyl acetate: Toluene: Methanol: Formic acid	**7: 2: 1: 0.2**	**Good separation with sharp band**
Chloroform: Toluene: Methanol: Glacial acetic acid	3: 4 : 4: 0.05	Good separation but band was broad
Chloroform: Toluene: Methanol: Glacial acetic acid	4: 4: 2: 0.2	Merged no separation

- It is evident from the data that mobile phase combination of **Ethyl acetate: Toluene: Methanol: Formic acid** in proportion of **7: 2: 1: 0.2** v/v/v/v was most suitable for the development of HPTLC method. The chromatographic condition for spotting on plate is given in Chapter 6.

7.2.5.4.Calibration curve for SIMVA and NICO:

Analysis was performed on Pre-coated silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 µm thickness) (E. Merck) pre-washed with methanol and then dried for 30 minute at 50°C. From the binary stock solution aliquots of 0.1, 0.15, 0.2, 0.25, and 0.3 µl were spotted on the TLC plate under nitrogen stream using Desaga Applicator, AS30win.

The plate was dried in air, and then the plate was developed in Twin trough developing chamber (100 × 100) with stainless steel lid, previously saturated with the mobile phase for 30 min. The plate was removed from the chamber, dried in air and was scanned and quantified at selected wavelength in Re-emission/Excitation mode with Desaga TLC scanner, Proquant. The calibration curve was constructed by plotting peak area vs. concentration (ng/spot) corresponding to each spot and regression equation was calculated.

7.2.5.5. Results and Discussion:

7.2.5.5.1. Validation Parameters

(I) Linearity

The HPTLC method showed linearity for SIMVA in the range of 50 to 150 ng/spot with co-relation co-efficient, of 0.9996 as seen in fig. 7.2.5.1 for peak area. For NICO linearity was in the range of 1250 to 3750 ng/spot with the correlation coefficients of 0.9997 as seen in fig. 7.2.5.3 for peak area. Calibration data is given in Table 7.2.5.2.

Table7.2.5.2: Calibration data of SIMVA and NICO by HPTLC method

Sr. No.	Concentration (ng/spot)		Peak Area				R_f*	
	SIMVA	NICO	SIMVA (Mean*)	%CV	NICO (Mean*)	%CV	SIMVA	NICO
1	50	1250	103	0.25	76	0.25	0.66	0.44
2	75	1875	154	0.65	114	0.96	0.65	0.42
3	100	2500	205	0.98	157	0.13	0.63	0.44
4	125	3125	263	0.79	196	0.98	0.66	0.42
5	150	3750	315	0.15	235	0.11	0.64	0.43

***Average of five reading**

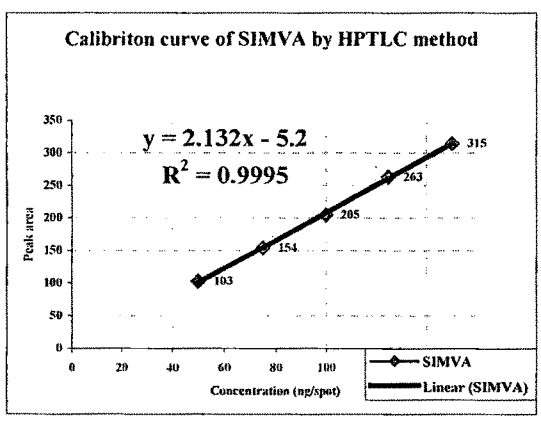


Figure7.2.5.1: Calibration curve of SIMVA by HPTLC (Peak Area).

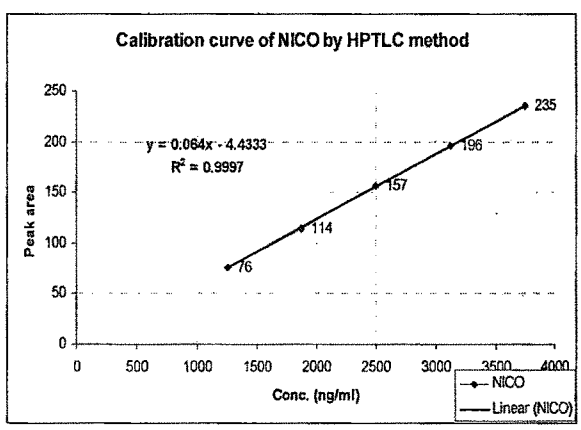


Figure7.2.5.2: Calibration curve of NICO by HPTLC (Peak Area).

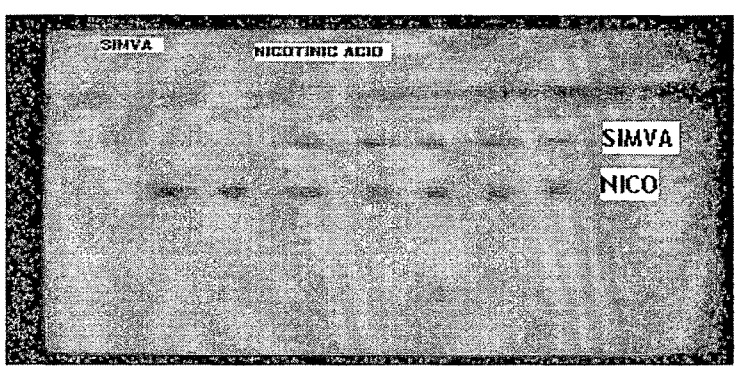


Figure7.2.5.3: Chromatogram of SIMVA and NICO (After detection)

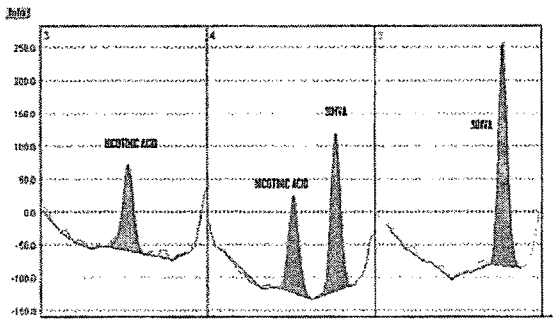


Figure7.2.5.4: Single Spectrum of SIMVA and NICO by HPTLC

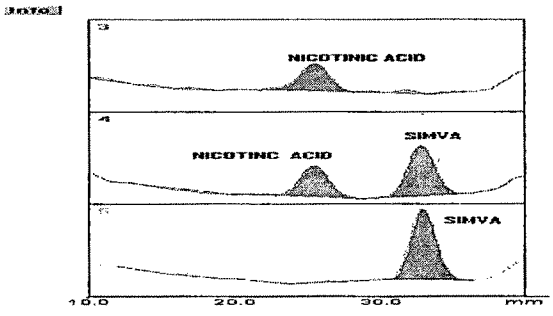


Figure7.2.5.5: Vertical Spectra of SIMVA and NICO by HPTLC

(II) Precision

Repeatability

The method was found to be precise on intra day and inter day basis. The average % CV value for the determination of SIMVA and NICO were reported in table 7.2.5.3.

Table7.2.5.3: Intraday and interday precision data of SIMVA and NICO by HPTLC (peak area)

Concentration (ng/spot)		Intraday		Interday	
		Peak area (Mean* ± % C.V. (n=5))			
SIMVA	NICO	SIMVA	NICO	SIMVA	NICO
50	1250	104 ± 0.23	78 ± 0.26	101 ± 0.98	75 ± 0.85
75	1875	152 ± 0.58	115 ± 0.48	155 ± 0.12	113 ± 0.21
100	2500	208 ± 0.12	158 ± 0.96	209 ± 0.85	154 ± 0.74

* Mean of three determinations.

Reproducibility:

The % CV of the responses for determination of SIMVA and NICO are mentioned in Tables7.2.5.4, which reveal the reproducibility of the method.

Table7.2.5.4: Reproducibility data of SIMVA and NICO by HPTLC (peak area)

Concentration (ng/spot)		Peak area ± % CV*			
SIMVA	NICO	SIMVA		NICO	
		Glass plate	Aluminum plate	Glass plate	Aluminum plate
50	1250	102 ± 0.26	75 ± 0.85	108 ± 0.85	75 ± 0.87
75	1875	154 ± 0.98	113 ± 0.23	152 ± 0.14	112 ± 0.84
100	2500	202 ± 0.32	152 ± 0.15	204 ± 0.96	152 ± 0.85

* Mean of three determinations.

(III) Accuracy

The method showed % mean recovery for SIMVA in the range of 100.17 – 102.73 % and for NICO it was 100.02 – 100.57% (Table 7.2.5.5).

Table7.2.5.5: Accuracy data of SIMVA and NICO by HPTLC (peak area)

Spotted Amount (A + B)		Total quantity Found (Mean*)		% Recovery	
SIMVA	NICO	SIMVA	NICO	SIMVA	NICO
100	2500	205.45	2514.36	102.73	100.57
75	1875	150.26	1879.36	100.17	100.23
150	3750	302.56	3750.96	100.85	100.03

*** Mean of five determinations.**

(IV) Limit of detection and quantification

LOD and LOQ were found by empirical method. For SIMVA it was 2.35 and 5.210 ng/spot respectively. Same way for the NICO it was found 52.65 and 124.63 ng/spot, respectively.

7.2.5.5.3. Analysis of tablet formulation:

SIMVOTIN from Ranbaxy was selected for the study. 20 Tablets were triturated after taking their average weight. The Tablet powder equivalent to one Tablet content was transferred into a 100 ml volumetric flask containing 50 ml methanol, sonicated for 15 min and further diluted to 100 ml with methanol. The resulting solution was sonicated for 10 min and supernatant was filtered through whatman filter paper no.41. From the filterate 0.5 µl was spotted on TLC plate under nitrogen stream using Desaga Applicator, AS30win, was developed and scanned three times without changing plate position at selected wavelength in Reemission/Excitation mode with Desaga TLC scanner, Proquant and concentration of sample solution was found from regression equation computed from calibration curve of drugs. The concentrations of SIMVA and NICO were computed from calibration curve of SIMVA and NICO and reported in to Table7.2.5.6.

Table7.2.5.6: Estimation of SIMVA and NICO in tablet by RP-HPTLC method

Formulation	DRUGS	Labeled Claim (mg/tab)	HPTLC	
			*Amount found	% Labeled Claim ± CV
SIMVOTIN TAB (Ranbaxy)	SIMVA	5	5.24	102.45
	NICO	125	124.68	99.62

*** Mean of three determinations.**

7.2.5.4. Summary of Validation parameters:

The summary of validation parameters is reported in to table7.2.5.7.

Table7.2.5.7: Summary of Validation parameters by HPTLC

Sr. No	Parameters	SIMVA	NICO
1	Detection wavelength (nm)	237 nm	
2	Linearity range (ng/spot)	50-150	1250-3750
3	Regression equation	Y=1.0675xcon.-5.75	Y=0.064xCon.-4.433
4	Correlation coefficient (r^2)	0.9996	0.9997
5	Intercept	-5.75	-4.433
6	Slope	1.0675	0.064
7	Assay	102.45	99.62
8	Precision Intra day % CV (n = 5) Inter day % CV (n = 5) Reproducibility of measurements %CV % Recovery	 0.12-0.58 0.12-0.98 <1% 100.17-102.73	 0.26 – 0.96 0.21 – 0.85 <1% 100.02 – 100.57
9	Limit of detection (ng/spot)	2.35	52.65
10	Limit of quantification (ng/spot)	5.210	124.63

7.2.5.5. Conclusion:

Developed analytical method was very good for the separation of SIMVA and NICO with accuracy of 100.17 -102.73 % for SIMVA and 100.02 – 100.57% for NICO.

Reference:

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2. Kolovou G. D, Salpea K. D, Mihas C, Malakos I, Kafaltis N, Bilianou H. G, Adamopoulou E. N, Mykoniatis M, Cokkinos D. V. Comparison of simvastatin and nicotinic acid administration in alcohol-treated Wistar rats. Hellenic J Cardiol. 2008; 49: 2: 79-85.

7. 2. 6

Ezetimibe & Simvastatin and Ezetimibe & Lovastatin

7.2.6. Development of High Performance Thin Layer Chromatography (HPTLC) method for EZE and SIMVA and EZE and LOVA

HPTLC methods were developed for the simultaneous estimation of EZE-SIMVA and EZE-LOVA combinations. Both the methods were developed separately as independent methods, but are described together in the following section.

7.2.6.1. Optimization of method:

For the simultaneous analysis of EZE and SIMVA and EZE and LOVA by HPTLC, efforts were made to select a common solvent and a common absorption wavelength.

Determination of solvent for sample preparation and detection wavelength:

Different solvents were tried to study the solubility of both the drugs as stated in Chapter 6. Methanol was selected for the preparation of drug solutions, which is a good solvent for HPTLC method development. Wavelength was selected by scanning standard solutions of both drugs over 200 nm to 400 nm wavelengths. Drugs show significant absorbance at **231 nm** as illustrated in fig. 7.2.1.4.1. So this wavelength was selected in densitometer UV detector.

Determination of mobile phase:

Various combinations of mobile phases were tried for effective separation of EZE and SIMVA on silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 µm thickness) (E. Merck). The results are reported in Table 7.2.6.1.

Table 7.2.6.1: Determination of mobile phase

Mobile phase combination	Ration (V/V/V)	Peak separation
Chloroform: Methanol	8: 2	No separation band
Benzene: Methanol	8: 2	No separation
Benzene: Methanol	5: 5	No separation
Toluene: Methanol	8: 2	Spot not separated
Hexane: Methanol	5: 5	Spot not separated
Ethyl acetate : Toluene: Methanol	3: 4: 3	Slight separation
Ethyl acetate: Toluene: Methanol	4: 4: 2	Good separation but broad band
Ethyl acetate: Benzene: Methanol	3: 4: 3	Adjacent spots
Ethyl acetate: Toluene: Methanol: Formic acid	**6: 3.5: 0.5: 0.2**	**Good separation with sharp band**
Chloroform: Toluene: Methanol: Glacial acetic acid	3: 4 : 4: 0.05	Good separation but band was broad
Chloroform: Toluene: Methanol: Glacial acetic acid	4: 4: 2: 0.2	Merged no separation

- It is evident from the data that mobile phase combination of **Ethyl acetate: Toluene: Methanol: Formic acid** in proportion of **6: 3.5: 0.5: 0.2**v/v/v was most suitable for the development of HPTLC method. The chromatographic condition for spotting on plate are given in Chapter 6.

7.2.6.2. Preparation of binary stock solution:

For chromatographic study, standard EZE and SIMVA (100 mg) were weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml methanol and diluted up to mark with methanol to obtain final concentration of 1000 µg/ml of EZE and SIMVA.

Similarly stock solution of EZE and LOVA was prepared.

7.2.6.3. Calibration curve:

Analysis was performed on Pre-coated silica gel aluminum Plate 60F–254 (20 × 10 cm with 250 µm thickness) (E. Merck) pre-washed with methanol and then dried for 30 minute at 50°C. From the stock solution (1000 µg/ml) aliquots of 0.1, 0.2, 0.4, 0.8, 1.2 and 1.6 µl were spotted on the TLC plate under nitrogen stream using Desaga Applicator, AS30win to obtain final concentration range 100 - 1800 ng/spot.

The plate was dried in air, and then the plate was developed in Twin trough developing chamber (100 × 100) with stainless steel lid, previously saturated with the mobile phase for 30 min. The plate was removed from the chamber, dried in air and was scanned and quantified at 2381 nm in Re-emission/Excitation mode with Desaga TLC scanner, Proquant. The calibration curve was constructed by plotting peak area vs. concentration (ng/spot) and peak height vs. concentration (ng/spot) corresponding to each spot and regression equation was calculated. Same procedure was use for calibration of EZE and LOVA.

7.2.6.4. Validation parameter:

Validation of method was done as per procedure give in Chapter 7.1.1.8.4.

7.2.6.5. Results and Discussion:

7.2.6.5.1. Validation Parameters

(I) Linearity

The HPTLC method showed good linearity for EZE, SIMVA and LOVA in the range of 150 to 1800 ng/spot. Calibration data is reported in table 7.2.6.2.

Table 7.2.6.2: Calibration data of EZE and SIMVA by HPTLC method

Sr. No.	Concentration (ng/spot)			Peak Area			R_f*		
	EZE	SIMVA	LOVA	EZE (Mean* ± %CV)	SIMVA (Mean* ± %CV)	LOVA (Mean*) %CV	EZE	SIMVA	LOVA
1	150	150	150	108.33 ±0.43	69.26 ±0.84	73.48 ±0.57	0.80	0.64	0.64
2	300	300	300	191.67 ±0.68	93.33 ±0.29	99.53 ±0.79	0.80	0.64	0.64
3	450	450	450	325.00 ±0.83	140.00 ±0.58	149.29 ±0.41	0.81	0.65	0.65
4	900	900	900	575.00 ±0.18	216.00 ±0.98	243.93 ±0.93	0.80	0.66	0.66
5	1350	1350	1350	815.12 ±0.62	290.17 ±0.31	317.24 ±0.27	0.81	0.65	0.65
6	1800	1800	1800	1086.83 ±0.52	386.89 ±0.47	422.99 ±0.48	0.81	0.64	0.64

***Average of five readings**

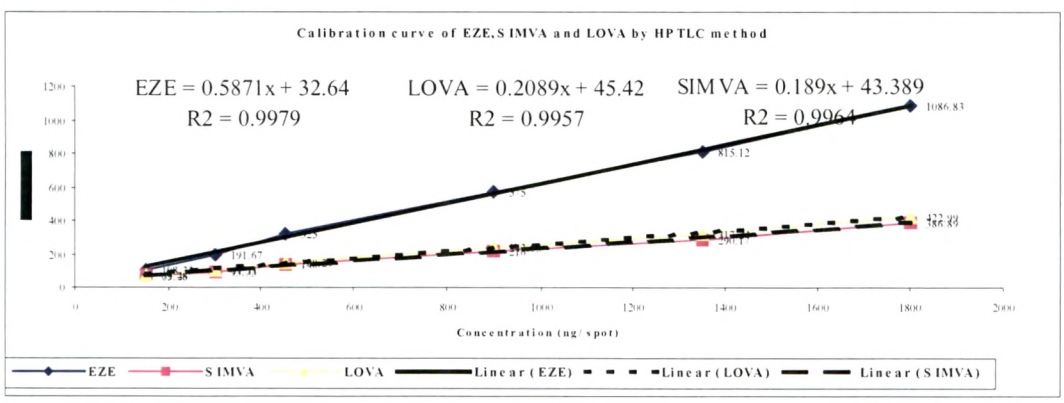

Figure 7.2.6.1: Calibration curve of EZE, SIMVA and LOVA by HPTLC (Peak Area).

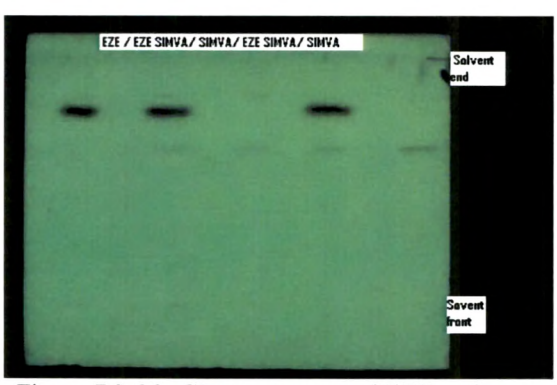

Figure 7.2.6.2: Chromatogram of EZE and SIMVA with UV detection (After detection)

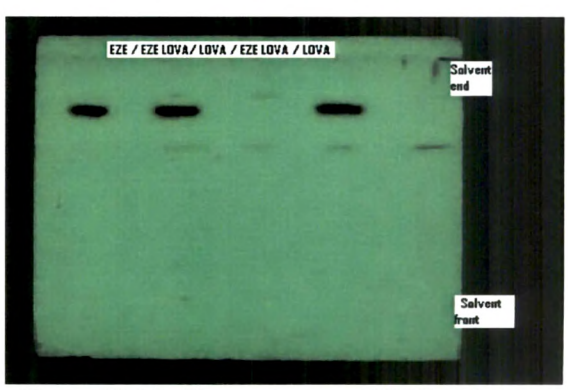

Figure 7.2.6.3: Chromatogram of EZE and LOVA with UV detection (After detection)

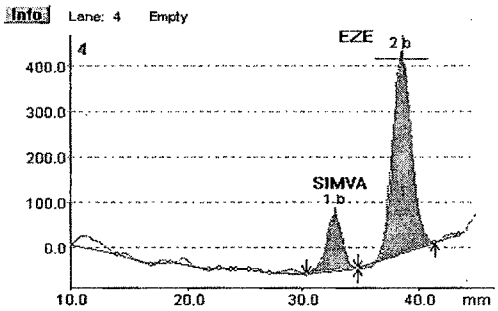

Figure 7.2.6.4: Single Spectrum of EZE and SIMVA by HPTLC

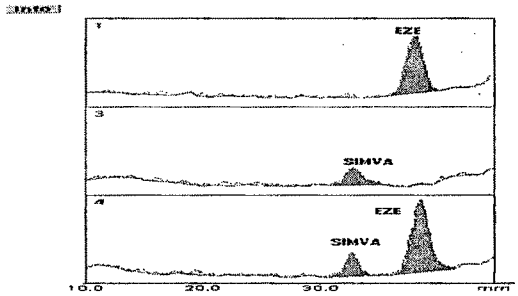

Figure 7.2.6.5: Vertical Spectra of EZE and SIMVA by HPTLC

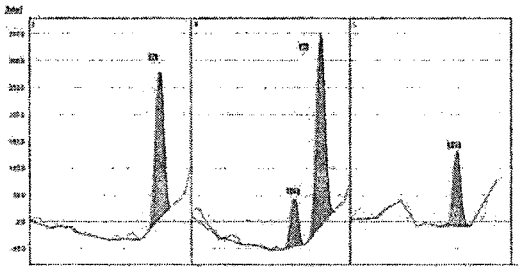

Figure 7.2.6.6: Single Spectrum of EZE and LOVA by HPTLC

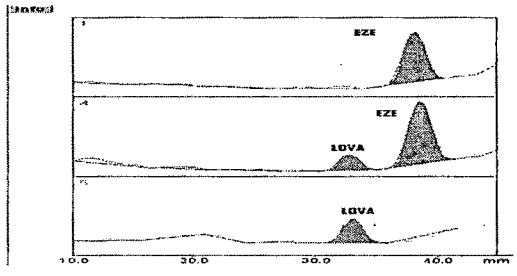

Figure 7.2.6.7: Vertical Spectra of EZE and LOVA by HPTLC

(II) Precision

Repeatability

The method was found to be precise on intra day and inter day basis as the average % CV value for the determination of EZE, SIMVA and LOVA were reported in table 7.2.6.3.

Table 7.2.6.3: Intraday precision data of EZE and SIMVA by HPTLC

Concentration (ng/spot)			Intraday			Interday		
			Peak area (Mean* ± % C.V. (n=5))					
EZE	SIMVA	LOVA	EZE	SIMVA	LOVA	EZE	SIMVA	LOVA
250	250	250	159.72 ± 0.42	77.78 ± 0.83	82.94 ± 0.62	158.87 ± 0.23	76.78 ± 0.18	81.97 ± 0.64
750	750	750	541.67 ± 0.39	233.33 ± 0.77	248.82 ± 0.44	544.82 ± 0.78	231.65 ± 0.15	247.36 ± 0.45
1000	1000	1000	638.89 ± 0.82	240.00 ± 0.57	271.02 ± 0.72	637.85 ± 0.85	241.82 ± 0.91	272.85 ± 0.76

*** Mean of five determinations.**

Reproducibility:

The % CV of the responses for determination of EZE and SIMVA are mentioned in Table 7.2.6.4, which reveal the reproducibility of the method.

Table 7.2.6.4: Reproducibility data of EZE and SIMVA by HPTLC (peak area)

Concentration (ng/spot)			Peak area ± % CV*					
			EZE		SIMVA		LOVA	
EZE	SIMVA	LOVA	Glass plate	Aluminum plate	Glass plate	Aluminum plate	Glass plate	Aluminum plate
250	250	250	159.56 ± 0.28	159.72 ±0.69	75.43 ± 0.24	78.00 ±0.48	81.47 ±0.82	82.76 ±0.12
750	750	750	549.17 ± 0.89	545.84 ±0.52	233.33 ± 0.47	233.21 ±0.88	248.82 ± 0.69	246.24 ±0.34
1000	1000	1000	632.22 ± 0.21	633.79 ±0.44	241.11 ± 0.19	244.94 ±0.23	2711.76 ± 0.25	274.99 ±0.22

*** Mean of five determinations.**

(III) Accuracy

The method showed % mean recoveries for EZE and SIMVA and EZE and LOVA are reported in table 7.2.6.5.

Table 7.2.6.5: Accuracy data of EZE and SIMVA by HPTLC (peak area)

Spotted Amount (A + B)			EZE and SIMVA				EZE and LOVA			
			Total quantity Found (Mean*)		% Recovery		Total quantity Found (Mean*)		% Recovery	
EZE	SIMVA	LOVA	EZE	SIMVA	EZE	SIMVA	EZE	LOVA	EZE	LOVA
750	750	750	749.02	748.29	99.87	99.77	745.02	756.29	99.34	100.84
1000	1000	1000	1038.93	1002.44	103.89	100.24	1035.93	1032.44	103.59	103.24
1500	1500	1500	1528.38	1520.82	101.89	101.39	1523.38	1529.82	101.56	101.99

*** Mean of three determinations.**

(IV) Limit of detection and quantification

LOD and LOQ are reported in table 7.2.6.9.

7.2.6.5.2. Analysis of tablet formulation:

Procedure for preparation of the sample is given in Chapter 7.2.2.1 and 7.2.2.4. Prepared sample solution of EZE and SIMVA was analyzed by the developed method. The concentrations of EZE and SIMVA and EZE and LOVA were computed from calibration curve and reported in to Table 7.2.6.6.

Table 7.2.6.6: Estimation of EZE and SIMVA in tablet

Formulation	DRUGS	Labeled Claim (mg/tab)	HPTLC	
			*Amount found	% Labeled Claim ± CV
SIMVAS-EZ	EZE	10	9.96	99.64 ± 0.52
	SIMVA	10	10.08	100.83 ± 0.82
LOVAS-EZ	EZE	10	9.97	99.71
	LOVA	10	10.09	100.88

7.2.6.6. Summary of Validation parameters:

The summary of validation parameters is reported in table 7.2.6.7.

Table 7.2.6.7: Summary of Validation parameters by HPTLC

Sr. No	Parameters	EZE	SIMVA	LOVA
1	Detection wavelength (nm)	231		
2	Linearity range (ng/spot)	150 - 1800		
3	Regression equation	Y=0.5871x +32.641	Y=0.189x +43.39	Y=0.2089x +45.418
4	Correlation coefficient (r^2)	0.9979	0.9964	0.9957
5	Intercept	32.641	43.39	45.418
6	Slope	0.5871	0.189	0.2089
7	Assay	99.64-99.71	100.83	100.88
8	Precision			
	Intra day % CV (n = 5)	0.39-0.82	0.57-0.83	0.44-0.72
	Inter day % CV (n = 5)	0.23-0.85	0.15-0.91	0.45-0.76
	% Recovery	99.34-103.89	99.77-101.39	100.84-103.24
9	Limit of detection (ng/spot)	1.540	2.912	4.637
10	Limit of quantification (ng/spot)	14.497	27.407	43.651

7.2.6.7. Conclusion:

Developed analytical methods showed good separation of EZE and SIMVA and EZE and LOVA but cannot be used for the separation of SIMVA and LOVA. Method has good recovery to analyzed EZE and SIMVA and EZE and LOVA. In TLC plate 7.2.6.2 and 7.2.6.3 middle spot have two band upper one is SIMVA acid and LOVA acid. Which show that this HPTLC method could estimate EZE in presence of SIMVA and LOVA. SIMVA and LOVA can estimated in presence of its metabolites. So this method could use for bioequivalence study also.

7. 2. 7

Ezetimibe, Simvastatin and Lovastatin

7.2.7. Stability Indicating Reverse Phase High Performance Liquid Chromatography (HPLC) for the estimation of EZE, SIMVA and LOVA.

The parent drug stability test guideline Q1A (R2) issued by International Conference on harmonization (ICH) suggests that stress studies should be carried out on a drug to establish its inherent stability characteristics, leading to identification of degradation products and hence supporting the suitability of the proposed analytical procedures. It also requires that analytical test procedures for stability samples should be stability indicating and they should be fully validated. Accordingly, the aims of the present study were to establish inherent stability of EZE, SIMVA and LOVA through stress studies under a variety of ICH recommended test conditions and **estimate EZE, SIMVA and LOVA as single, binary combination of EZE and SIMVA or EZE and LOVA and all three together.**

Apparatus, instruments and reagents:

Apparatus, instruments and reagents used for the development of chromatographic methods are mentioned in chapter 6.

7.2.7.1. Light absorption study (Optimization of method):

For development of HPLC method in combination it is necessary that drugs are soluble in a common solvent and detection wavelength selected should be such at which drugs show significant absorbance. Mobile phase was selected in a manner that drugs are showing well resolved peaks without any merging with the other peaks.

Determination of solvent for sample preparation:

Different solvents were tried depending upon the solubility of the drugs as mentioned in chapter 6 (table 6.4). All three drugs were soluble in methanol and ACN, but with methanol as a solvent, large tailing effects were observed. So ACN was selected as a solvent to prepare drug solution.

Determination of Detection wavelength

Detection wavelength was selected by scanning standard solutions of the drugs over 200 nm to 400 nm wavelengths. The overlain spectra (fig. 7.2.7.1) of EZE, SIMVA and LOVA showed that the three drugs show significant absorbance in the spectral region between 215 nm to 255 nm. After some preliminary studies, 237 nm was selected as detection wavelength.

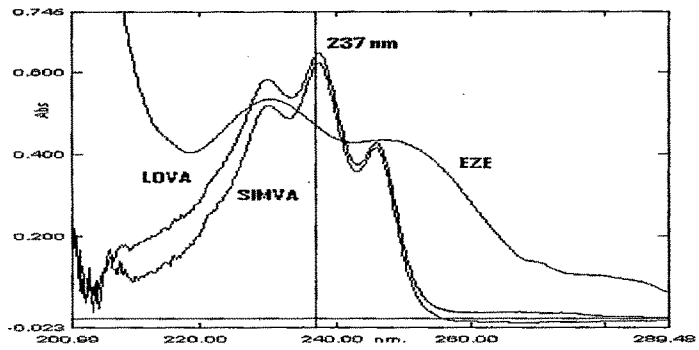

Figure 7.2.7.1: overlain UV spectrum of EZE, SIMVA and LOVA in ACN

Optimization of mobile phase:

Various combinations of mobile phases were tried at a flow rate 1 ml/min and column C_{18} Phenomenex. The observations are shown in Table 7.2.7.1.

Table 7.2.7.1: Determination of mobile phase

Mobile phase combination	Ratio (V/V)	Retention time (min)			Peak shape		
		EZE	SIMVA	LOVA	EZE	SIMVA	LOVA
Methanol: Water	70:30	4.52	15.368	12.368	Broad peak but tailing at the base	Broad peak but tailing at the base	Broad peak but tailing at the base
Methanol: Water	80:20	3.422	9.123	6.123	Broad peak but tailing at the base	Broad peak but tailing at the base	Broad peak but tailing at the base
Acetonitrile: Water	80:20	3.417	9.233	6.433	Sharp peak but tailing at the base	Sharp peak but tailing at the base	Sharp peak but tailing at the base
Acetonitrile: 0.1 % Formic acid	80:20	3.425	9.258	6.058	Sharp peak	Sharp peak	Sharp peak
Acetonitrile: 0.1 % Formic acid	**80:30**	**3.954**	**12.675**	**9.725**	**Sharp peak**	**Sharp peak**	**Sharp peak**
Acetonitrile: 0.2 % Formic acid	80:20	3.425	9.258	6.258	Sharp peak	Sharp peak but tailing at the base	Sharp peak but tailing at the base
Acetonitrile: 0.5 % Formic acid	50:50	12.365	34.350	19.350	Sharp peak	Broad peak and tailing at the base	Broad peak and tailing at the base

Acetonitrile: 0.5 % Formic acid	60:40	9.865	25.230	15.230	Sharp peak	Broad peak and tailing at the base	Broad peak and tailing at the base
Acetonitrile: 0.5 % Formic acid	70:30	4.47	15.550	12.550	Sharp peak	Broad peak and tailing at the base	Broad peak and tailing at the base
Acetonitrile: 0.5 % Formic acid	80:20	2.25	9.008	5.008	Sharp peak	Broad peak and merge with blank	Broad peak and tailing at the base

It is evident from the data that mobile phase combination of acetonitrile: 0.1 % Formic acid in proportion of 80: 30 v/v with 1 ml/min flow rate was most suitable for the development of RP-HPLC method.

Column

Two type of C_{18} column were tried.

Table 7.2.7.2: Selection of column

Column (C_{18}, 250 X 4.60 mm)	Retention time (min)			Peak Shape		
	EZE	SIMVA	LOVA	EZE	SIMVA	LOVA
Hypersil	3.968	12.965	9.372	Sharp peak	Sharp peak	Sharp peak
Phenomenex	**3.954**	**12.658**	**9.728**	**Sharp peak**	**Sharp peak**	**Sharp peak**

Both the columns were suitable for development of HPLC analytical method, but C_{18} Phenomenex 240 X 4.6 mm was selected. The main aim of the method was to resolve the drugs in presence of degradation products. So Phenomenex C_{18} column (250 mm x 4.6 mm i.d., 5 µm particle size) was preferred as it has high carbon loading with very closely packed material to give high resolution.

Method:

After optimizing all the parameters, following chromatographic condition were used for the development of a precise, accurate, specific and suitable stability indicating RP-HPLC methods for the estimation of EZE, SIMVA and LOVA.

Chromatographic condition:

For method:

- **Column:** C_{18} (size-250 x 4.60 mm, I.D-5 µm) (Phenomenex)
- **Mobile Phase:** Acetonitrile: 0.1% Formic acid (80:30 v/v)
- **Detection:** UV detection at 237 nm

- **Flow rate:** 1.0 ml/minute
- **Application volume:** 20 µl

Preparation of Mobile Phase:

Mobile phase was prepared by mixing 800 ml of acetonitrile with 300 ml of 0.1 % formic acid. The mobile phase was filtered through nylon (0.45 µm, 41 mm) membrane filter and degassed in ultrasonic bath prior to use for 30 min.

7.2.7.2. Preparation of stock solution

Standard EZE (100 mg) was weighed accurately and transferred to 100ml volumetric flask. It was dissolved in 50 ml ACN and diluted up to mark with ACN to obtain final concentration of 1000 µg/ml. This solution was used as working standard solution. Similarly stock solutions of SIMVA and LOVA were prepared in ACN.

Procedure for

7.2.7.3. Preparation of sample solution of SIMVA acid and LOVA acid:

A methanolic solution of SIMVA acid (100 ppm) was obtained from Torrent Research Center, Chataral as gift sample. 1 µl were transferred to a sample tubes. Then 90 µl of human plasma was added to tube. The tubes were vortexed for 1 min on Vortex shaker. The volume was adjusted to a 1000 µl with ACN to get final concentrations of 100 ng/ml of solution. The solutions were centrifuged for 5 min at 4000 rpm. 20 µl of supernatant were injected into HPLC column. The Rt was observed at 7.932 min.

7.2.7.4. Calibration curve:

From the stock solution (1000 µg/ml), aliquots of 0.1, 0.5, 1, 1.5, 2, 2.5, and 3 ml were transferred to a series of 10 ml volumetric flasks. The volume was adjusted up to the mark with ACN to get final concentrations of 10, 50, 100, 150, 200, 250, and 300 µg/ml of solution. 20 µl of all these solutions were injected separately into HPLC column and the peak area of each solution was measured at selected wavelength. The calibration curve was constructed by plotting peak area vs. concentration (µg/ml) over the concentration range and regression equation was calculated.

7.2.7.5. Validation parameters

Procedure for validation of method is given in Chapter 7.1.1.7.

7.2.7.5. Results and Discussion:

7.2.7.5.1. Validation of method:

(I) Linearity

The RP-HPLC method showed good linearity in the range of 1-250 µg/ml. Calibration data for EZE, SIMVA and LOVA is given in Tables 7.2.7.3, 7.2.7.4 and 7.2.7.5, respectively.

Table 7.2.7.3: Calibration data of EZE by HPLC with UV detection

Sr. No.	Concentration (µg/ml)	Peak Area		RT*
		Mean*	%CV	
1	1	66780	0.46	3.995
2	20	1340678	0.19	3.95
3	54	3219445	0.67	3.949
4	96	6434565	0.28	3.947
5	104	6970779	0.58	3.978
6	120	8143267	0.29	3.958
7	145	9839781	0.58	3.984
8	200	13572112	0.18	3.994
9	250	16965140	0.92	3.942

***Average of five readings**

Table 7.2.7.4: Calibration data of SIMVA by HPLC with UV detection

Sr. No.	Concentration (µg/ml)	Peak Area		RT*
		Mean*	%CV	
1	1	84536	0.28	12.67
2	20	1568753	0.48	12.639
3	54	4235634	0.75	12.673
4	96	6879175	0.42	12.693
5	120	8598969	0.79	12.678
6	134	9662198	0.28	12.673
7	145	10455364	0.57	12.683
8	200	14421191	0.28	12.672
9	250	18026489	0.48	12.677

***Average of five readings**

Table 7.2.7.5: Calibration data of LOVA by HPLC with UV detection

Sr. No.	Concentration (µg/ml)	Peak Area		RT*
		Mean*	%CV	
1	1	68886	0.64	9.725
2	20	1377712	0.78	9.733
3	54	3285373	0.28	9.638
4	96	6613016	0.95	9.625
5	120	7283311	0.64	9.004
6	134	8266270	0.38	9.263
7	145	9836532	0.58	9.041

8	200	13777116	0.83	9.639
9	250	16883452	0.22	9.743

***Average of five readings**

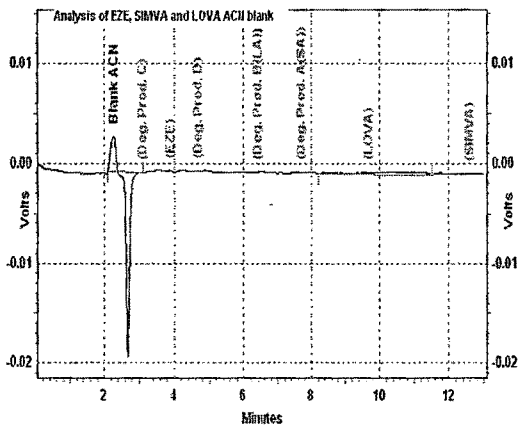

Figure 7.2.7.2: Peak of ACN Blank by HPLC with UV detection

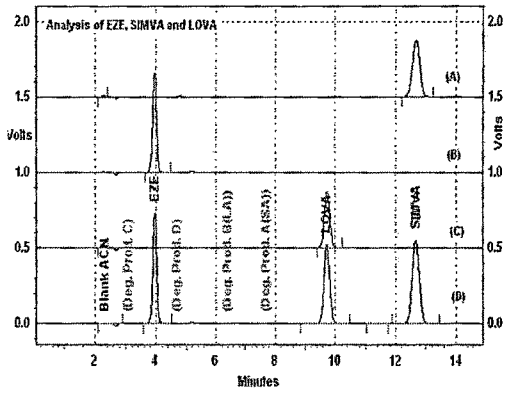

Figure 7.2.7.3: Peak of (A) SIMVA, (B) EZE, (C) LOVA and (D) EZE, SIMVA and LOVA by HPLC with UV detection.

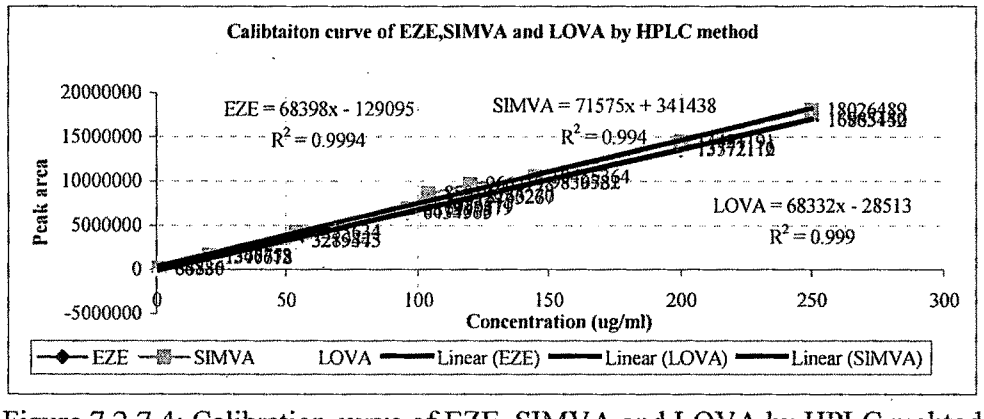

Figure 7.2.7.4: Calibration curve of EZE, SIMVA and LOVA by HPLC mehtod

(II) Precision

Repeatability

The method was found to be precise on intra day and inter day basis as the average % CV value for the determination of EZE, SIMVA and LOVA were found to be 0.39 – 0.92 %, 0.28 – 0.49 % and 0.29 – 0.79% and 0.29 – 0.85%, 0.33 – 0.85 % and 0.21 – 0.75 % , respectively (Table 7.2.7.6 and 7.2.7.7).

Table 7.2.7.6: Intraday precision data of EZE, SIMVA and LOVA by RP-HPLC method

Concentration (µg/ml)			Peak area (Mean ± S.D.) (n=5)			% C.V.		
EZE	SIMVA	LOVA	EZE	SIMVA	LOVA	EZE	SIMVA	LOVA
60	60	60	4021603 ±0.058	4687339 ± 0.037	4133135 ± 0.038	0.39	0.28	0.79
120	120	120	8143207 ±0.038	2500391 ± 0.025	7500829 ± 0.074	0.58	0.49	0.51
200	200	200	13582894 ± 0.028	14421192 ± 0.082	13777116 ± 0.062	0.92	0.33	0.64
Table 7.2.7.7: Interday precision data of EZE, SIMVA and LOVA by RP-HPLC method								
60	60	60	4022034 ±0.038	4606259 ± 0.075	4098003 ± 0.022	0.53	0.33	0.69
120	120	120	8154322 ±0.028	9412520 ± 0.022	7266270 ± 0.049	0.85	0.85	0.53
200	200	200	13592491 ±0.058	14331615 ± 0.085	13168048 ± 0.019	0.29	0.42	0.75

***Average of five readings**

Reproducibility:

The % CV of the responses for determination of EZE, SIMVA and LOVA are mentioned in Table 7.2.7.8.

Table 7.2.7.8: Reproducibility data of EZE and SIMVA by RP-HPLC method

Concentration (µg/ml)			Peak area ± % CV					
			EZE		SIMVA		LOVA	
EZE	SIMVA	LOVA	Hypersil	Phen.	Hypersil	Phen.	Hypersil	Phen.
60	60	60	4077161 ± 0.93	4077161 ± 0.73	4679812 ± 0.18	4689413 ± 0.18	4179009 ± 0.18	4150414 ± 0.38
120	120	120	8143206 ± 0.39	8144286 ± 0.78	8591314 ± 0.79	8592431 ± 0.79	7201336 ± 0.79	7263253 ± 0.62
240	240	240	16086413 ± 0.69	16189413 ± 0.39	16831893 ± 0.48	16732431 ± 0.48	16869269 ± 0.48	16807641 ± 0.99

***Average of three readings (Phen. is phenomenex)**

(III) Accuracy

The method showed % mean recovery for EZE, SIMVA and LOVA in the range of 99.93 – 100.33 %, 99.56 – 100.02 and 99.82 – 101.85%, respectively (Table 7.2.7.9).

Table 7.2.7.9: Accuracy data of EZE and SIMVA by RP-HPLC method

DRUG	EZE	SIMVA	LOVA
Initial conc. (µg/ml) (A)	100	100	100

Quantity of std. Added (µg/ml) (B)		50	100	150
Total Amount (A + B)		150	200	250
Quantity Found (Mean*)	EZE	150.03	149.89	250.82
	SIMVA	149.98	200.04	248.9
	LOVA	149.73	203.69	250.93
% Recovery± %CV	EZE	100.02 ± 0.32	99.93 ± 0.79	100.33 ± 0.52
	SIMVA	99.99 ± 0.60	100.02 ± 0.52	99.56 ± 0.66
	LOVA	99.82	101.85	100.37

***Average of five readings**

(IV) Limit of detection

The minimum detectable concentration of EZE, SIMVA and LOVA were found to be 0.0154 µg/ml, 0.0223 µg/ml and 0.0546, respectively.

(V) Limit of quantification

The lowest quantifiable concentration of EZE, SIMVA and LOVA were found to be 0.1519 µg/ml, 0.744 µg/ml and 0.3822, respectively.

7.2.7.6.2. Applicability of the method:

(A) Analysis of tablet formulation

STATIN of Unisearch was analyzed by the proposed method. Twenty tablets were weighed and average weight was determined. All twenty tablets were triturated and the tablet powder equivalent one tablet content was transferred into a 100 ml volumetric flask containing 50 ml ACN, sonicated for 15 min and further diluted to 100 ml with ACN. The resulting solution was sonicated for 10 min and supernatant was filtered through Whatman filter paper no.41. 20 µl of this solution was injected into HPLC column and the peak area was measured at 237 nm. The concentration of sample solution was found from regression equation and results are reported in table 7.2.7.10.

Table 7.2.7.10: Estimation of SIMVA in tablet by HPLC with UV detection

Tablet Formulation	Labeled Claim (mg/tablet)	Amount found (mg/tablet)	% Recovery ± S.D
STATIN (Unisearch)	10	10.09	100.92
	20	20.18	100.54

*** Mean value of five determinations**

LOVACARD of Cipla was analyzed by the proposed method. Twenty tablets were weighed and average weight was determined. Similar procedure was followed as mentioned above. The concentration of sample solution was found from regression equation and results are reported in table 7.2.7.11.

Table 7.2.7.11: Estimation of LOVA in tablet by HPLC with UV detection

Tablet Formulation	Labeled Claim (mg/tablet)	Amount found (mg/tablet)	% Recovery ± S.D
LOVACARD (Cipla)	20	19.98	99.98

***Average of five readings**

SIMVAS-EZ and **STARSTAT-EZ** were analyzed by the proposed method. Twenty tablets were weighed and average weight was determined. Similar procedure was followed as mentioned above. The concentration of sample solution was found from regression equation and results are reported in table 7.2.7.12.

Table 7.2.7.12: Estimation of EZE and SIMVA in tablet by RP-HPLC method A.

Formulation	DRUGS	Labeled Claim (mg/tab)	FDZC	
			*Amount found	% Labeled Claim ± CV
SIMVAS-EZ	EZE	10	10.04	100.41 ± 0.78
	SIM	10	10.21	102.19 ± 0.52
STARSTAT-EZ	EZE	10	10.19	100.51 ± 0.63
	SIM	20	20.32	103.20 ± 0.38

*** Mean value of five determinations.**

Ten tablets of EZEDOC and ten tablets of AZTATIN were weighed and average weight was determined. All ten tablets were triturated and the tablet powder equivalent to one tablet content of EZEDOC and AZTATIN was transferred into a 100 ml volumetric flask containing 50 ml ACN. Optimized chromatographic procedure was followed as mentioned above. The concentration of sample solution was found from regression equation and results are reported in table 7.2.7.13.

Table 7.2.7.13: Estimation of EZE and LOVA in tablet by RP-HPLC method A.

Formulation	DRUGS	Labeled Claim (mg/tab)	HPLC	
			*Amount found	% Labeled Claim ± CV
EZEDOC + AZTATIN	EZE	10	10.09	100.90
	LOV	10	10.12	101.23
LAB. MIX.	EZE	10	9.98	99.84
	LOV	20	20.01	100.12

* Mean value of five determinations.

(B) Analysis of drug in degradation products

Efforts were made to analyze EZE, SIMVA and LOVA in presence of their degradation products using the proposed method.

Procedure for forced degradation study

Forced degradation study was done in different conditions. Solvents tried were 30% H_2O_2, water at neutral pH 7, 0.5 N HCl and 0.1 N NaOH. Approximate 25 mg drug was accurate weighed and transferred to 25 ml volumetric flask. Each drug was dissolved in 5 ml of methanol then diluted with the solvent selected for degradation. Similarly solid-state stability was studied by exposing 25 mg of drug to 80°C stability oven and 25 mg drug to stability chamber. Samples were collected for analysis at three stages, at 0 min (as soon as sample was prepared), after 24 hrs and after 48 hrs of exposure to degradation condition. Samples were prepared by taking 2 ml of degraded solution in 10 ml volumetric flask and made up to 10 ml with ACN and 20µl of that was injected in HPLC column. Fig 7.2.7.8 to 7.2.7.39 show the chromatograms of forced degraded samples.

Fig. 7.2.7.5 to 7.2.7.10: Chromatograms of degradation of SIMVA under different conditions at 0 min, 24 hr and 48 hrs at room temperature

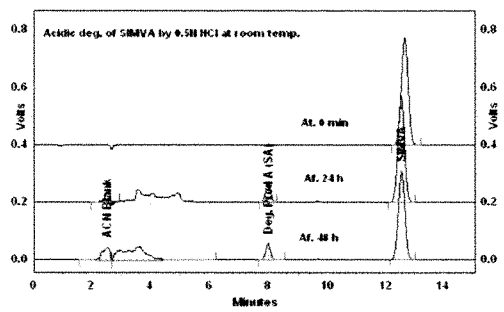

Figure 7.2.7.5: Chromatogram of SIMVA in acidic condition

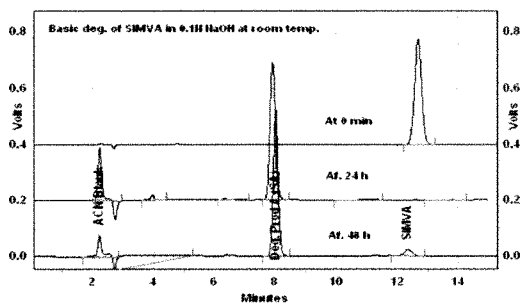

Figure 7.2.7.6: Chromatogram of SIMVA in basic condition

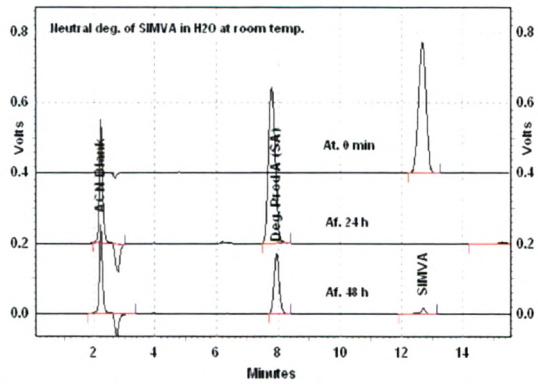

Figure 7.2.7.7: Chromatogram of SIMVA in Neutral condition

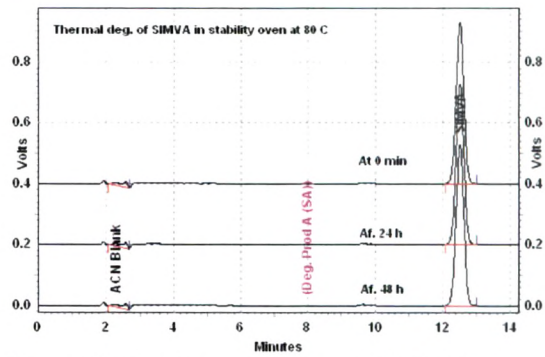

Figure 7.2.7.9: Chromatogram of SIMVA in thermal condition

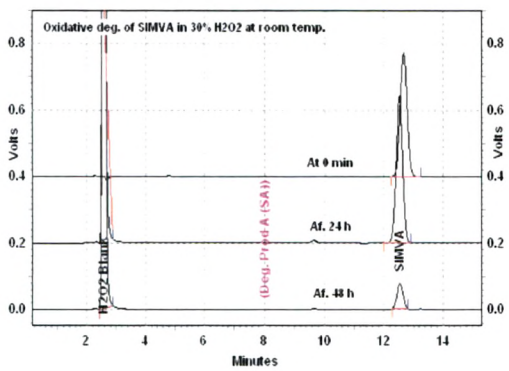

Figure 7.2.7.8: Chromatogram of SIMVA in oxidative

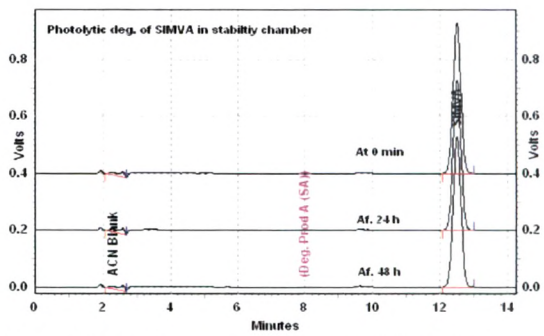

Figure 7.2.7.10: Chromatogram of SIMVA in photolytic condition

Fig. 7.2.7.11 to 7.2.7.16: Chromatograms of degradation of EZE and SIMVA under different conditions (A) 0 min EZE and SIMVA (B) 0 min SIMVA (C), 0 min EZE (D) 48 h SIMVA (E) 48 h EZE and (F) 48 h EZE and SIMVA.

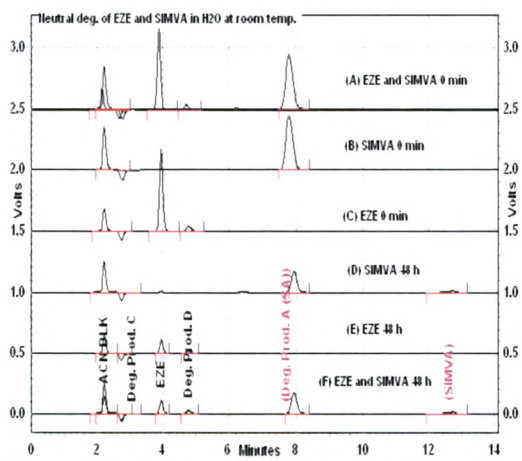

Figure 7.2.7.11: Chromatogram of EZE and SIMVA in neutral condition (water) at 7 pH.

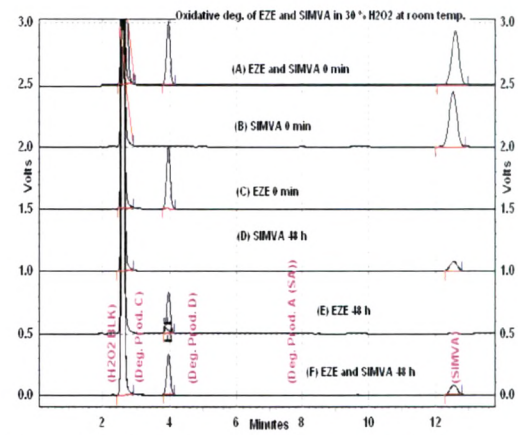

Figure 7.2.7.12: Chromatogram of EZE and SIMVA in 30 % H2O2 at room temperature

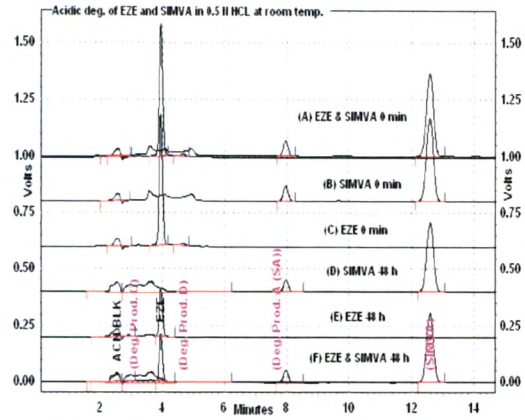

Figure 7.2.7.13: Chromatogram of EZE and SIMVA in 0.5 N HCL

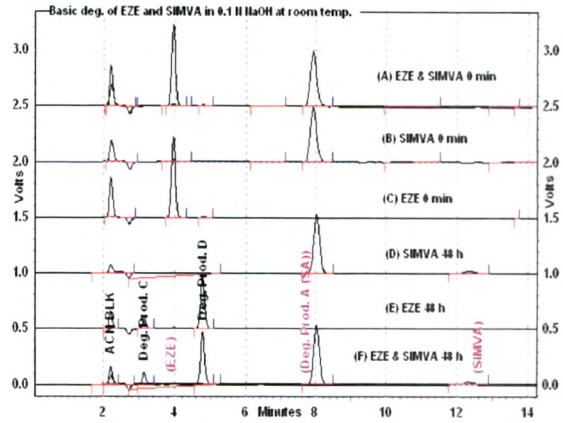

Figure 7.2.7.14: Chromatogram of EZE and SIMVA in 0.1N NaOH

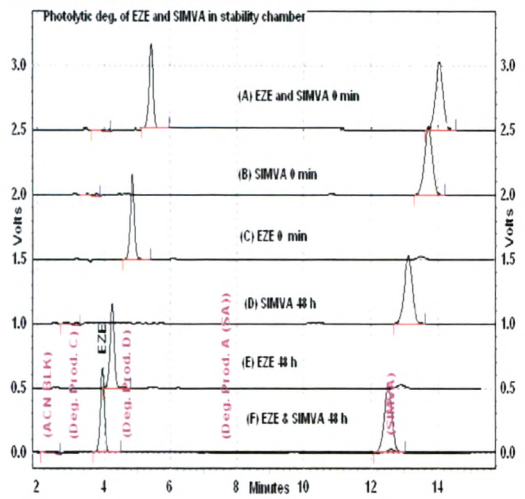

Figure 7.2.7.15: Chromatogram of EZE and SIMVA in photostability chamber

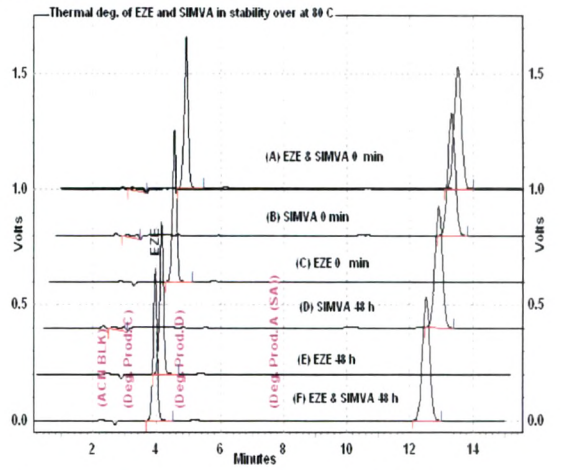

Figure 7.2.7.16: Chromatogram of EZEand SIMVA in Stability oven

Fig. 7.2.7.17 to 7.2.7.22: Chromatograms of degradation of LOVA under different conditions at 0 min, 24 hr and 48 hrs at room temperature.

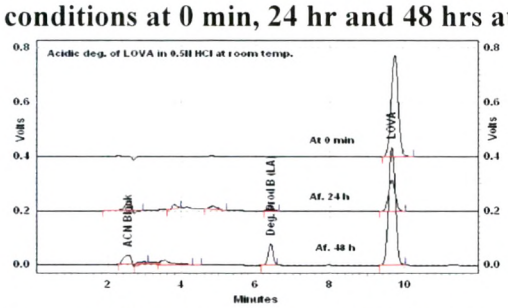

Figure 7.1.5.17: Chromatogram of LOVA in 0.5 N HCl .

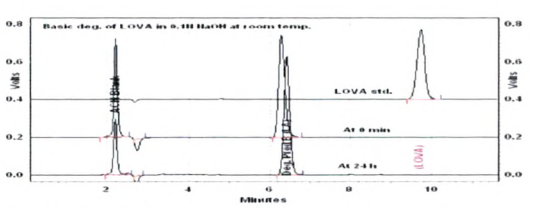

Figure 7.1.5.18: Chromatogram of LOVA in 0.1 N NaOH.

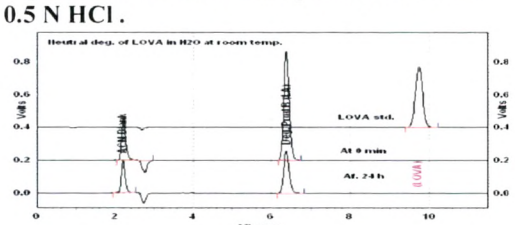

Figure 7.1.5.19: Chromatogram of LOVA in Neutral .

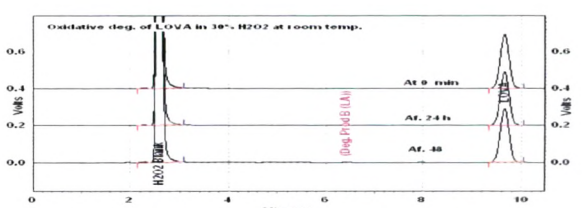

Figure 7.1.5.20: Chromatogram of LOVA in 30% H2O2 .

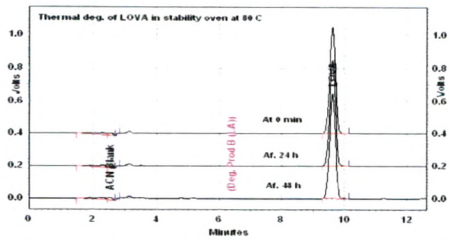

Figure 7.1.5.21: Chromatogram of LOVA in stability oven

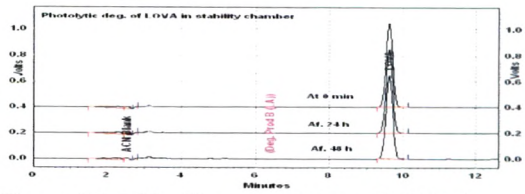

Figure 7.1.5.22: Chromatogram of LOVA in photo stability condition

Fig. 7.2.7.23 to 7.2.7.28: Chromatograms of degradation of EZE and LOVA under different conditions (A) 0 min EZE and LOVA, (B) 0 min LOVA, (C), 0 min EZE, (D) 48 h LOVA, (E) 48 h EZE and (F) 48 h EZE and LOVA

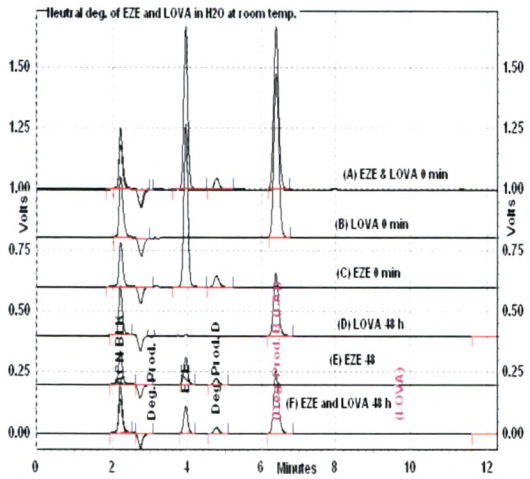

Figure 7.2.7.23: Chromatogram of EZE and LOVA in neutral condition (water) at 7 pH.

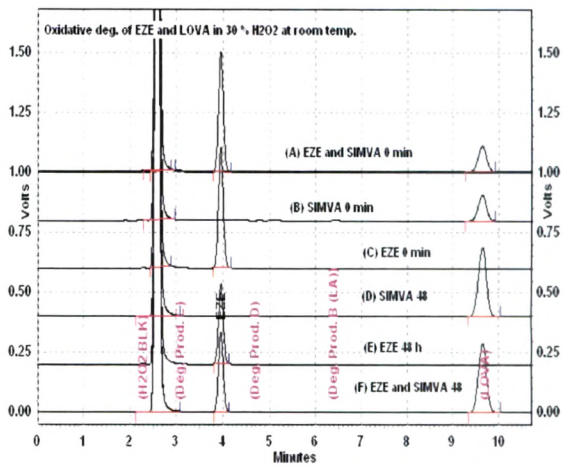

Figure 7.2.7.24 : Chromatogram of EZE and LOVA in 30 % H2O2 at room temperature

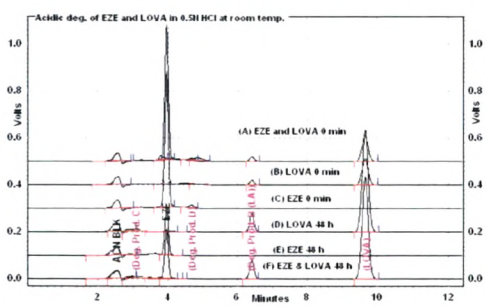

Figure 7.2.7.25 : Chromatogram of EZE and LOVA in 0.5 N HCL

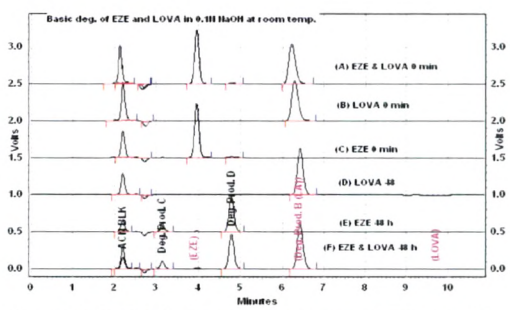

Figure 7.2. 5.26: Chromatogram of EZE and LOVA in 0.1N NaOH

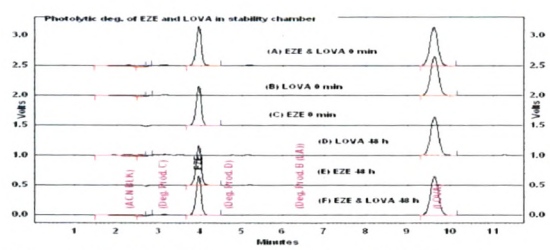

Figure 7.2.7.27: Chromatogram of EZE and LOVA in photostability chamber

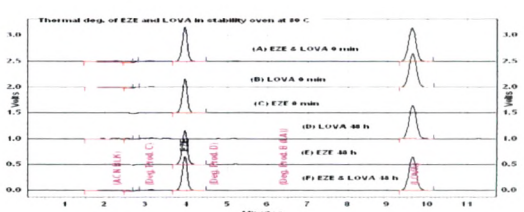

Figure 7.2.7.28: Chromatogram of EZE and LOVA in Stability oven

Fig. 7.2.7.29 to 7.2.7.36: Chromatograms of degradation of EZE, SIMVA and LOVA under different conditions.

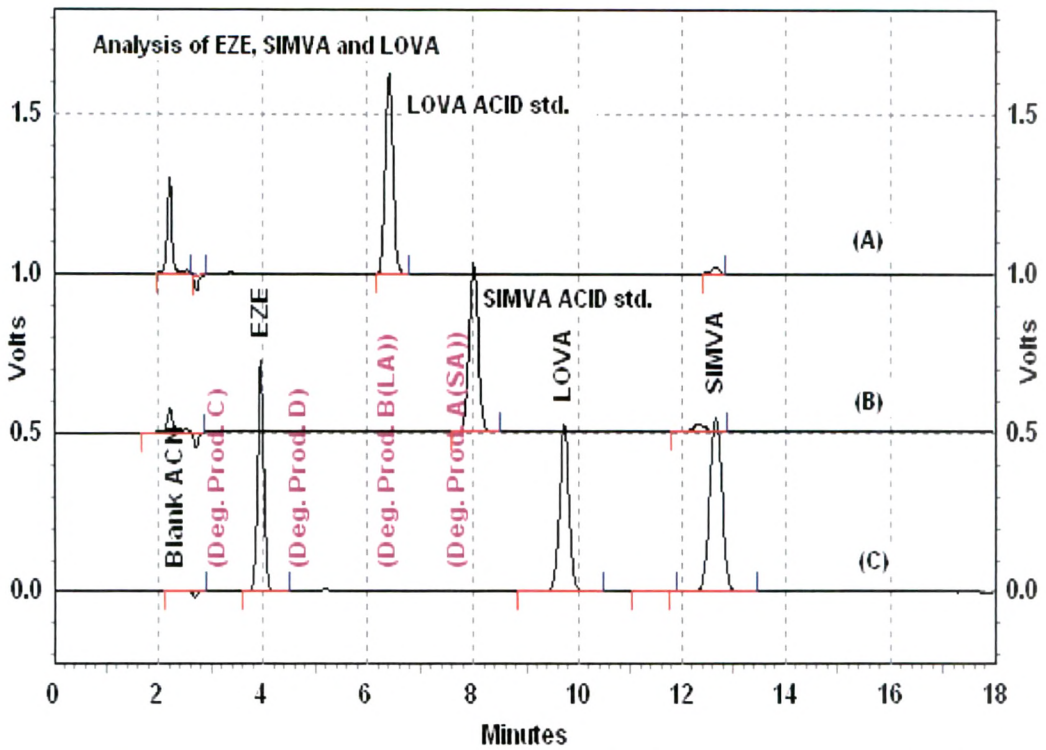

Figure 7.2.7.29: Chromatogram of (A) LOVA acid std. (B) SIMVA acid std, and (C) EZE, SIMVA and LOVA std.

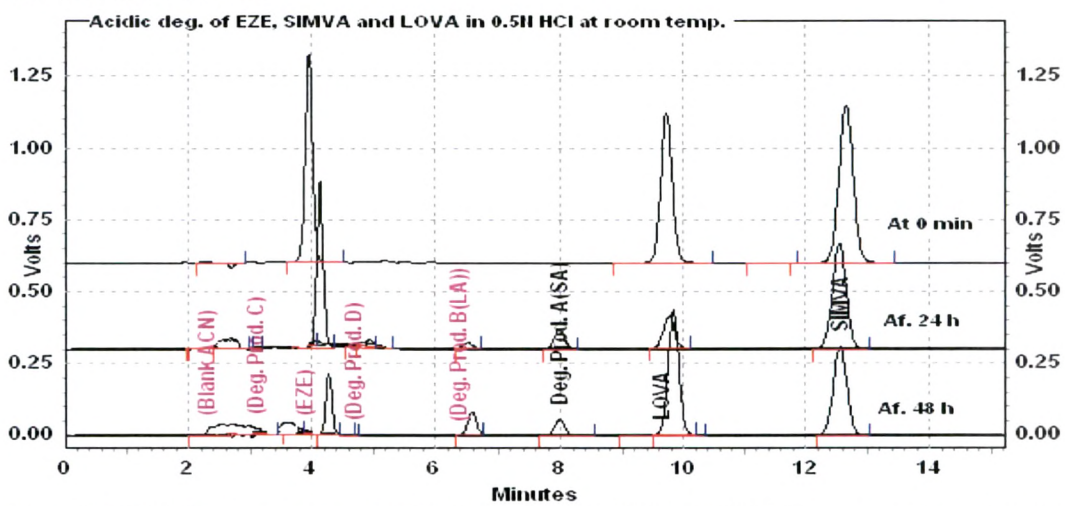

Figure 7.2.7.30: Chromatogram of EZE, SIMVA and LOVA in 0.5N HCL at room temp. at 0min, 24 h and 48 h.

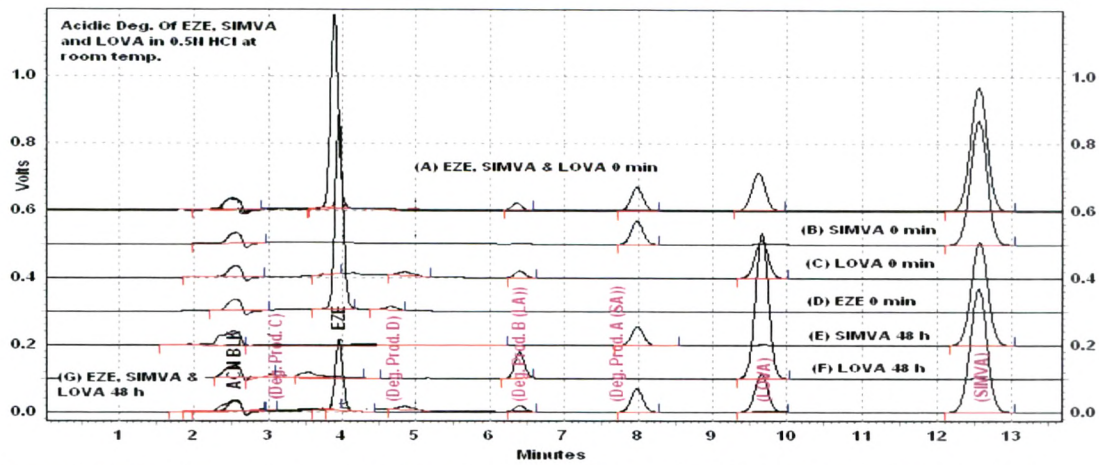

Figure 7.2.7.31: Chromatogram of (A) 0 min EZE, SIMVA and LOVA, (B) 0 min SIMVA, (C) 0 min LOVA, (D) 0 min EZE, (E) 48 h SIMVA, (F) LOVA 48 h and (G) 48 h EZE, SIMVA and LOVA in 0.5 N HCl at room temp.

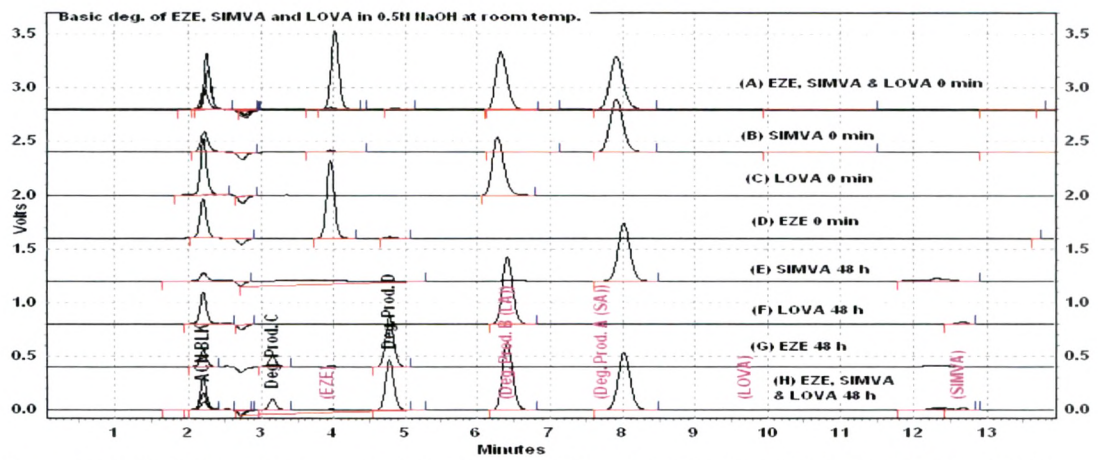

Figure 7.2.7.32: Chromatogram of (A) 0 min EZE, SIMVA and LOVA, (B) 0 min SIMVA, (C) 0 min LOVA, (D) 0 min EZE, (E) 48 h SIMVA, (F) LOVA 48 h and (G) 48 h EZE, SIMVA and LOVA in 0.1 N NaOH at room temp.

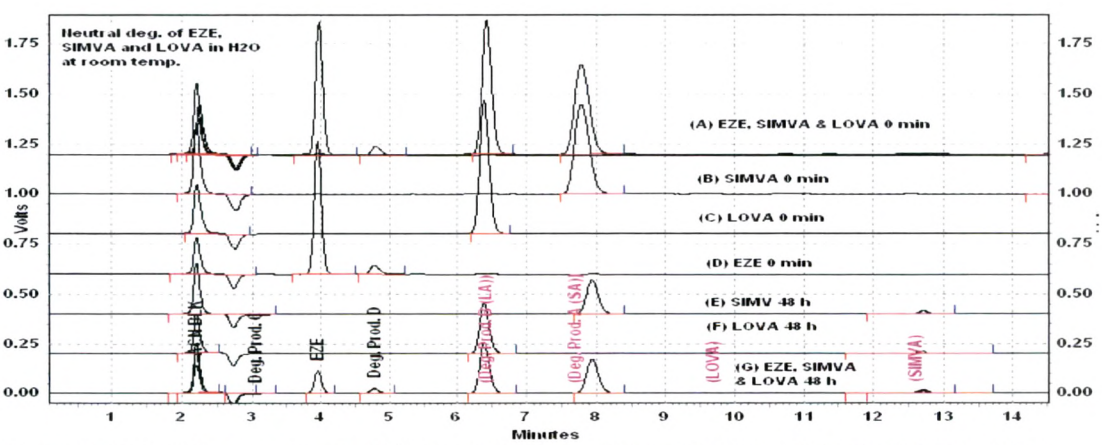

Figure 7.2.7.33: Chromatogram of (A) 0 min EZE, SIMVA and LOVA, (B) 0 min SIMVA, (C) 0 min LOVA, (D) 0 min EZE, (E) 48 h SIMVA, (F) LOVA 48 h and (G) 48 h EZE, SIMVA and LOVA in H2O at room temp.

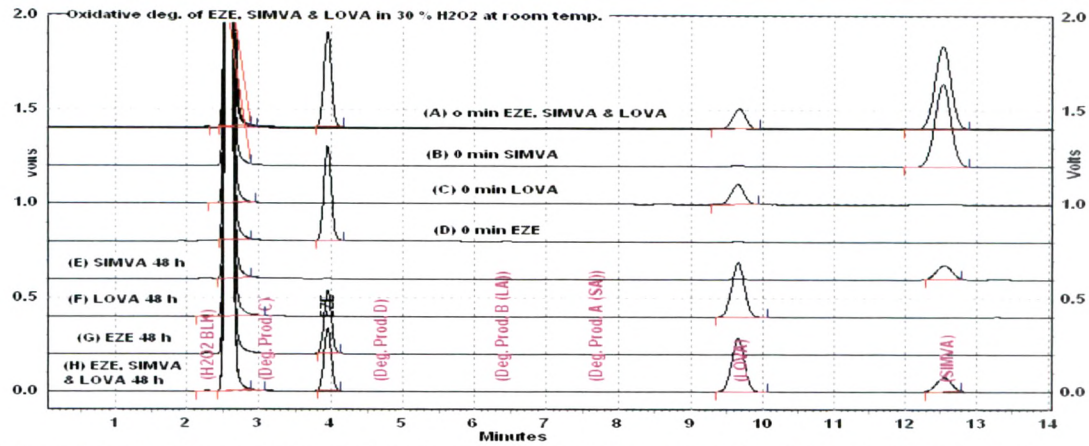

Figure 7.2.7.34: Chromatogram of (A) 0 min EZE, SIMVA and LOVA, (B) 0 min SIMVA, (C) 0 min LOVA, (D) 0 min EZE, (E) 48 h SIMVA, (F) LOVA 48 h and (G) 48 h EZE, SIMVA and LOVA in 30% H2O2 at room temp

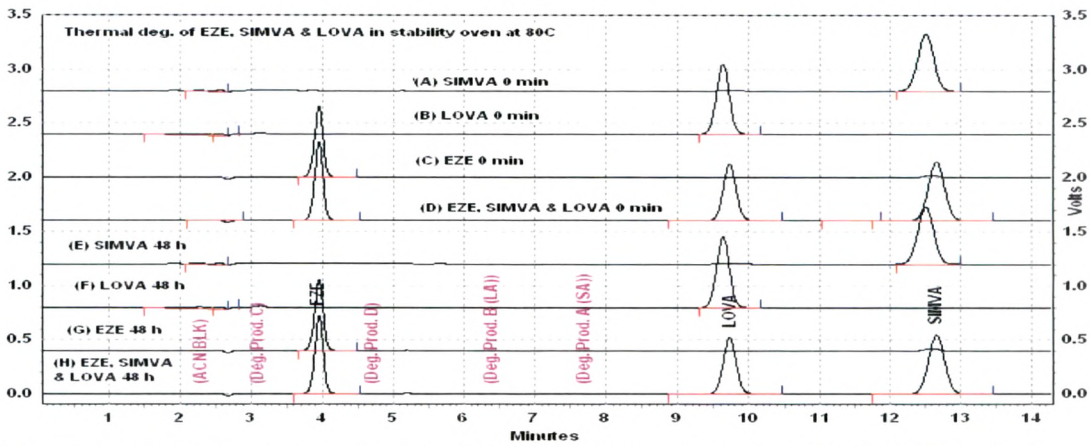

Figure 7.2.7.35: Chromatogram of (A) 0 min EZE, SIMVA and LOVA, (B) 0 min SIMVA, (C) 0 min LOVA, (D) 0 min EZE, (E) 48 h SIMVA, (F) LOVA 48 h and (G) 48 h EZE, SIMVA and LOVA in stability oven at 80C

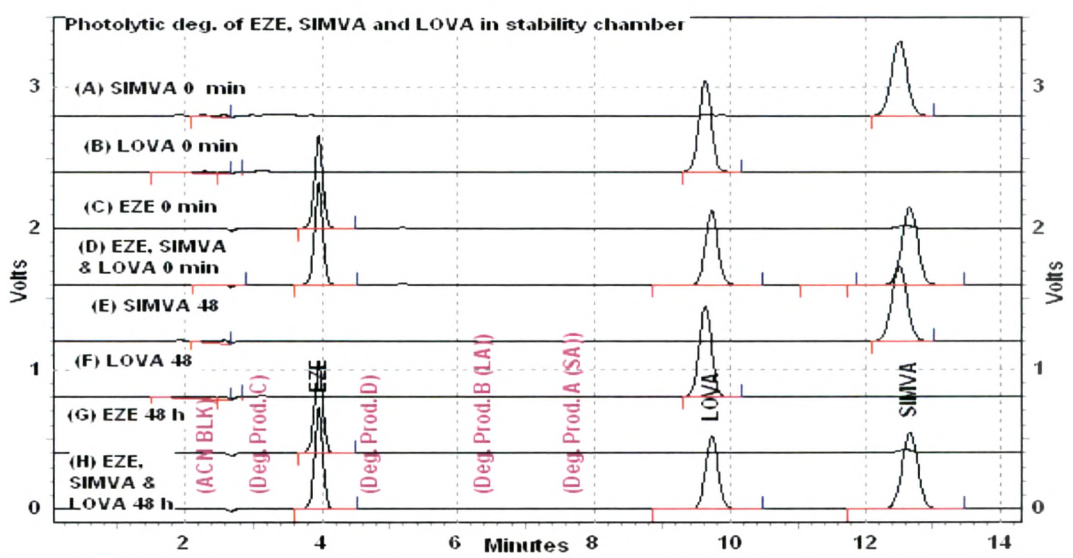

Figure 7.2.7.36: Chromatogram of (A) 0 min EZE, SIMVA and LOVA, (B) 0 min SIMVA, (C) 0 min LOVA, (D) 0 min EZE, (E) 48 h SIMVA, (F) LOVA 48 h and (G) 48 h EZE, SIMVA and LOVA in stability chamber

Table 7.2.7.14: Percentage degradation of EZE, SIMVA and LOVA by force degradation.

Sr. No	Parameters (Stress condition /duration/state)	% of undergrad			% of individual Degradation products				Total % Deg.		
		EZE	SIMVA	LOVA	A	B	C	D	EZE	SIMVA	LOVA
1	Neutral/H_2Oat pH 7/48 h/ sol./RT	63	-	8	100	92	-	37	**37**	**100**	**92**
2	Acidic/0.5 N HCl/48 h/ sol./RT	71	76	76	24	24	-	29	**29**	**24**	**24**
3	Alkali/0.1N NaOH/48 h/ sol./RT	53	-	-	100	100	12	35	**47**	**100**	**100**
4	Oxidative/30% H_2O_2/48 h/ sol./RT	100	100	100	No degradation found						
5	Thermal/80 C/48 h/solid	100	100	100	No degradation found						
6	Photo/uv254 and Vis/366 nm/48 h/solid	100	100	100	No degradation found						

A- SIMVA ACID. B- LOVA ACID. C, D- degraded product of EZE.

Table 7.2.7.15: System suitability parameters of EZE, SIMVA and LOVA

Sr. No	System Suitability Parameters	EZE	SIMVA	LOVA	Degradation products			
					A	B	C	D
1	Retention time (minutes)	**3.950**	**12.675**	**9.725**	**7.792**	**6.383**	**3.142**	**4.775**
2	Theoretical plates	13830.06	370460.8	7985.81	16247.44	17910.3	11299.96	18685.72
3	Resolution	**3.39**	**9.84**	**6.21**	**3.18**	**2.31**	**2.84**	**3.71**
4	Asymmetry	0.98	1.12	1.03	1.21	0.99	1.03	0.99
5	USP width	0.50	0.31	1.62	0.91	0.71	0.44	0.52
6	Tailing factor	0.99	1.13	1.02	1.20	0.98	1.03	0.99
7	Capacity Factor	5.21	7.12	6.32	3.21	5.21	3.98	4.19

7.2.7.6. Summary of Validation parameters:

The summary of validation parameters is given in table 7.2.7.15

Table 7.2.7.16: Summary of Validation parameters by HPLC with UV detection

Sr. No	Parameters	HPLC		
		EZE	SIMVA	LOVA
1	Analytical wavelength(nm)	237		
2	Retention time (minutes)	3.950	12.675	9.725
3	Linearity range (µg/ml)	1-250		
4	Regression equation	Y=6839x -129095	Y=71582x -167816	Y=68332x -28514
5	Correlation coefficient (r^2)	0.9994	0.9992	0.9990
6	Intercept	-129095	-167816	-28514
7	Slope	6839	71582	68332
9	Precision			
	Intra day % CV (n = 5)	0.39-0.92	0.28-0.49	0.29-0.79
	Inter day % CV (n = 5)	0.29-0.85	0.33-0.85	0.21-0.75
	Reproducibility of measureme %CV	< 1 %		
	% Recovery	99.93-100.33	99.56-100.02	99.82-101.85
10	Limit of detection (µg/ml)	0.0154	0.0223	0.0546
11	Limit of quantification (µg/ml)	0.1519	0.744	0.3822

7.2.7.7. Conclusion:

Developed HPLC method has an advantage that it can analyze EZE, SIMVA and LOVA individually or together in presence of their degradation products. SIMVA and LOVA are structurally similar. The only difference is the additional methyl group present in the molecular structure of SIMVA. The developed HPLC method offer an advantage of separating these two drugs of similar properties. Though the ternary combination is a hypothetical combination but method can be used to analyze each drug separately or together in presence of their different degradation products.

Degradation study of SIMVA shows that it is converted in to SIMVA acid in alkaline and neutral conditions. Conversion of SIMVA into SIMVA acid is slower in acidic medium. There is no effect of thermal and photolytic condition on SIMVA. So this method can be Selective and Specific SIAM for SIMVA (fig. 5.2.5.5 to 5.2.5.10)

Degradation study of LOVA shows that it is converted in to LOVA acid in alkaline and neutral conditions. Conversion of LOVA into LOVA acid is slower in acidic medium.

There is no effect of thermal and photolytic condition on LOVA. So this method can be Selective and Specific SIAM for LOVA (fig. 5.2.5.17 to 5.2.5.22).

EZE can also degraded in D and C form in acidic, alkaline and neutral condition. There is no effect of oxidative, thermal and photolytic condition on EZE, LOVA and SIMVA. So this method can be Selective and Specific SIAM for EZE, SIMVA and LOVA. (Fig. 5.2.5.29 to 5.2.5.36).

There were no co-eluting peaks of interference form excipients, impurities and degradation products due to variable stress components, thus establishing the specificity of assay method.

7. 2. 8

Simvastatin

Lovastatin

7.2.8. Bio Analytical Reverse Phase High Performance Liquid Chromatography (HPLC) method for estimation of SIMVA and LOVA in human plasma.

The developed HPLC method for the estimation of SIMVA (Chapter 7.2.5) was tried to analyze SIMVA in presence of human plasma. This is invitro method of analysis of SIMVA in human plasma. SIMVA was spiked with human plasma and analyzed by protein precipitation method. Human plasma was obtained form blood bank as request for research.

7.2.8.1. Preparation of stock solution for SIMVA and its metabolite

For chromatographic study, standard SIMVA (10 mg) was weighed accurately and transferred to 100 ml volumetric flask. It was dissolved in 50 ml ACN and diluted up to the mark with ACN to obtain final concentration of 100µg/ml.

A methanolic solution of SIMVA acid (100 ppm) was obtained from Torrent Research Center, Chataral as gift sample. 1 µl were transferred to a sample tubes. Then 90 µl of human plasma was added to tube. The tubes was vortexed for 1 min on Vortex shaker. The volume was adjusted to a 1000 µl with ACN to get final concentrations of 100 ng/ml of solution. The solutions were centrifuged for 5 min at 4000 rpm. 20 µl of supernatant were injected into HPLC column. The Rt was observed at 7.932 min.

7.2.8.2. Preparation of calibration curve for SIMVA

From the stock solution (100 µg/ml) aliquots of 0.1, 0.4, 2, 4, 6, 8 and 10 µl were transferred to a series of sample tubes. Then 90 µl of human plasma was added to each tube. The tubes were vortexed for 1 min on Vortex shaker. The volume was adjusted to a 1000 µl with acetonitrile to get final concentrations of 1, 40, 200, 400, 600, 800 and 1000 ng/ml of solution. The solutions were centrifuged for 5 min at 4000 rpm. 20 µl of supernatant were injected into HPLC column and the peak area of each solution was measured at selected wavelength. The calibration curve was constructed by plotting peak area vs. concentration (µg/ml) over the concentration range and regression equation was computed.

A parallel method was also developed for the analysis of LOVA in presence human plasma. The analytical procedure is analogous to above mentioned procedure.

7.2.8.3. Validation of analytical method:

Procedure for other validation parameters was given in chapter 7.1.1.7.2.

7.2.8.4. Results and Discussion:

7.2.8.4.1 Validation Parameters

(I) Linearity

Linearity range of SIMVA and LOVA was found to be 10.8 – 1000 ng/ml. Calibration data reported in table 7.2.8.1 and 7.2.8.2.

Table 7.2.8.1: Calibration data of SIMVA by HPLC with UV detection

Sr. No.	Concentration (ng/ml)	Peak Area		RT*
		Mean*	%CV	
1	10.8	215	0.75	12.673
2	40.1	513	0.42	12.693
3	200	2485	0.79	12.678
4	400	4970	0.28	12.673
5	600	8452	0.57	12.683
6	800	11269	0.28	12.672
7	1000	14087	0.48	12.677

***Average of five readings**

Table 7.2.8.2: Calibration data of LOVA by HPLC with UV detection

Sr. No.	Concentration (ng/ml)	Peak Area		RT*
		Mean*	%CV	
1	10.8	528	0.28	9.638
2	40.1	1260	0.95	9.625
3	200	6286	0.64	9.004
4	400	12571	0.38	9.263
5	600	18857	0.58	9.041
6	800	25143	0.83	9.639
7	1000	32512	0.22	9.743

***Average of five readings**

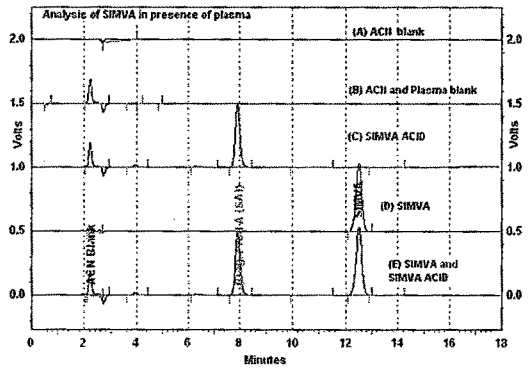

Figure 7.2.8.1: ACN, plasma blank, SIMVA and SIMVA acid by HPLC with UV detection

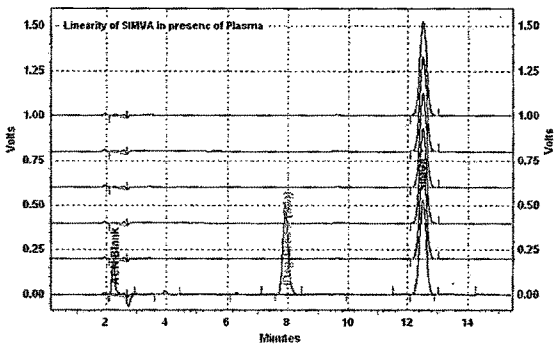

Figure 7.2.8.2: Linearity of SIMVA by HPLC with UV detection

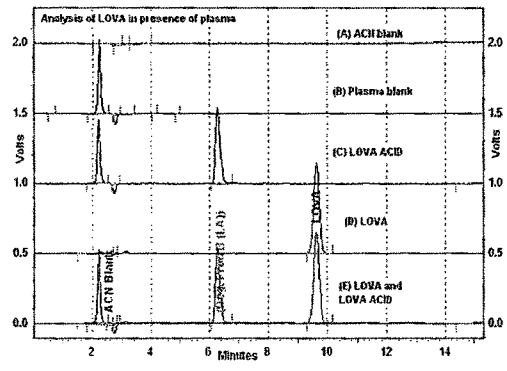

Figure 7.2.8.3: ACN, plasma, LOVA and LOVA acid by HPLC with UV detection

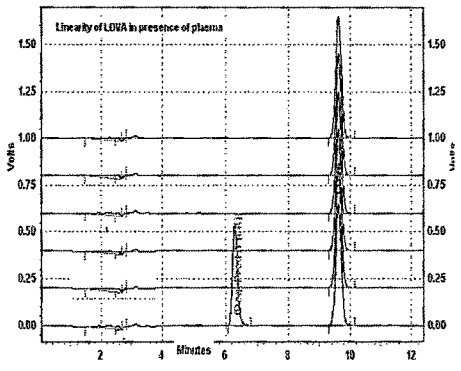

Figure 7.2.8.4: Linearity of LOVA by HPLC with UV detection

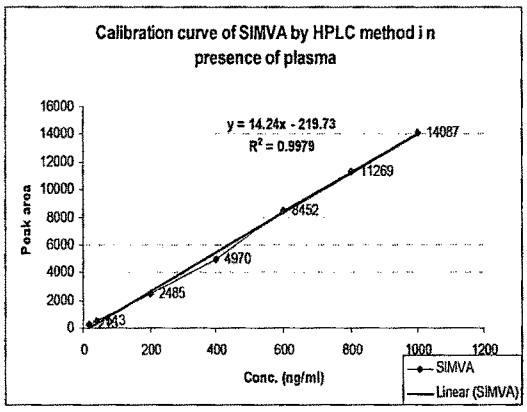

Figure 7.2.8.5: Calibration curve of SIMVA by HPLC with UV detection (Peak Area)

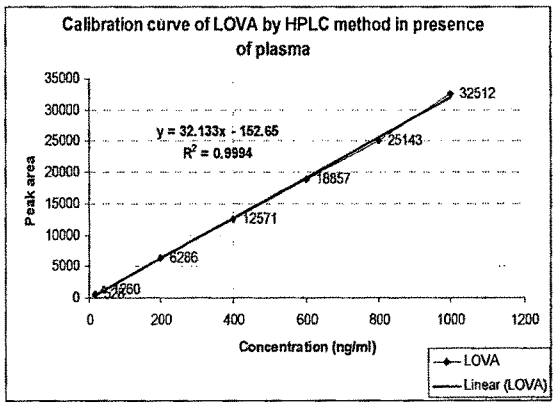

Figure 7.2.8.6: Calibration curve of LOVA by HPLC with UV detection (Peak Area)

(II) Precision

a. Repeatability

Intraday and Interday Precision

Intraday and interday variation of the proposed method was reported in table 7.2.8.4.

Table 7.2.8.3: Intraday precision data of SIMVA by HPLC with UV detection

Sr. No.	Concentration (ng/ml)	Intraday			Interday		
		Peak Area		RT*	Peak Area		RT*
		Mean*	%CV		Mean*	%CV	
1	50	640	0.25	12.675	704	0.25	12.568
2	250	3199	0.65	12.657	3522	0.89	12.698
3	500	6796	0.87	12.698	7705	0.54	12.677

***Average of five readings**

Intraday and interday variation of the proposed method was reported in table 7.2.8.5.

Table 7.2.8.4: Intraday precision data of LOVA by HPLC with UV detection

Sr. No.	Concentration (ng/ml)	Intraday			Interday		
		Peak Area		RT*	Peak Area		RT*
		Mean*	%CV		Mean*	%CV	
1	50	1550	0.25	9.658	1575	0.96	9.642
2	250	7843	0.69	9.614	7900	0.36	9.601
3	500	16092	0.12	9.645	15754	0.15	9.614

***Average of five readings**

b. Reproducibility:

As per the instrument specification the % C.V. of repeated measurement of the same solution should not more than 1%.

Table 7.2.8.5: Reproducibility data of SIMVA and LOVA(150 ng/ml) by HPLC

SIMVA			LOVA		
Time	**Peak Area**	**RT**	**Time**	**Peak area**	**RT**
Hypersil C18	1864	12.657	Hypesil C18	4714	9.602
	1839	12.754		4711	9.645
	1764	12.785		4788	9.600
Phenomenex C18	1864	12.698	Phenomenex C18	4721	9.631
	1889	12.645		4660	9.635
	1889	12.784		4753	9.610
S.D.	7.26	4.25	S.D.	5.6	4.39
%CV	0.56	0.69	%CV	0.28	0.56

*** Mean value of three determinations**

(III) Accuracy

Accuracy of the measurement of SIMVA was determined by standard addition and was found to be in the range of 99.77-100.51 % for peak area.

Table 7.2.8.6: Accuracy data of SIMVA and LOVA by HPLC with UV detection

Initial conc. (ng/ml) (A)	Quantity of std. Added (ng/l) (B)	Total Amount (A + B)	SIMVA		LOVA	
			Peak Area		Peak Area	
			Total quantity Found Mean ± S.D.	%Recovery ± S.D	Total quantity Found Mean ± S.D.	%Recovery ± S.D
120	60	180	180.56 ± 0.23	100.31 ± 0.44	179.85 ± 0.23	99.92 ± 0.89
120	120	240	241.23 ± 0.58	100.51 ±0.54	245.32 ± 0.96	102.22 ± 0.51
120	200	320	319.25 ± 0.65	99.77 ± 0.12	320.56 ± 0.62	100.18 ± 0.41

*** Mean value of five determinations**

(IV) Limit of detection

The minimum detectable concentration of SIMVA and LOVA was found to be 0.149 ng/ml and 0.964 ng/ml, respectively.

(V) Limit of quantification

The lowest quantifiable concentration of SIMVA was found to be 5.231ng/ml and 8.425 ng/ml, respectively.

(VI) Stability

1. Freez and Thaw Stability

SIMVA was found to be stable in LQC and HQC samples after 3rd cycle after frozen with % mean change of 9.0 and -0.05 (Table No. 7.2.8.7). LOVA was found to be stable in LQC and HQC samples after 3rd cycle after frozen with % mean change of 0.58 and 0.10 (Table No. 7.2.8.7)

Table 7.2.8.7: Freeze and thaw stability of SIMVA and LOVA

Sr.No.	LQC (15.000 ng/ml)							
	Comparison samples				Stability samples			
	Sample conc.(ng/ml)		% Nominal Conc.		Sample conc.(ng/ml)		% Nominal Conc.	
	SIMVA	LOVA	SIMVA	LOVA	SIMVA	LOVA	SIMVA	LOVA
1	14.98	14.98	99.8667	99.87	15.23	14.98	101.533	99.87
2	15.02	15.02	100.133	100.13	14.28	15.23	95.2	101.53
3	14.89	15.22	99.2667	101.47	15.22	15.04	101.467	100.27

4	15.23	15.04	101.533	100.27	15.02	14.99	100.133	99.93
5	14.98	15.23	99.8667	101.53	14.89	15.26	99.2667	101.73
6	15	15.09	100	100.60	15.02	14.56	100.133	97.07
N	6	6	100.111	100.64	6	6	91.1048	100.07
Mean	15.0167	15.10			14.94333	15.01		
% Mean Change	9.00635 (SIMVA)							
	0.58 (LOVA)							
Sr.No.	**HQC (680.000 ng/ml)**							
1	680.12	681.02	100.02	100.15	681.03	680.23	100.15	100.03
2	679.34	680.23	99.90	100.03	678.98	681.22	99.85	100.18
3	680.23	682.12	100.03	100.31	680.23	679.23	100.03	99.89
4	680.03	680.45	100.00	100.07	680.63	680.56	100.09	100.08
5	679.34	679.55	99.90	99.93	680.17	678.23	100.03	99.74
6	679.47	681.22	99.92	100.18	679.67	681.2	99.95	100.18
N	6	6	99.96	100.11	6	6	100.02	100.02
Mean	679.76	680.77			680.12	680.11		
% Mean Change	-0.05 (SIMVA)							
	0.10 (LOVA)							

2. Short-Term Temperature Stability

SIMVA was found to be stable in LQC and HQC samples for 11.0 hours at room temperature with % mean change of -2.14 and -0.21 respectively (Table No. 7.2.8.8). LOVA was found to be stable in LQC and HQC samples for 11.0 hours at room temperature with % mean change of -0.77 and -0.45 respectively (Table No. 7.2.8.8).

Table 7.2.8.8: Short term temperature stability of SIMVA and LOVA

Sr.No.	LQC (15.000 ng/ml)							
	Comparison samples				Stability samples			
	Sample conc.(ng/ml)		% Nominal Conc.		Sample conc.(ng/ml)		% Nominal Conc.	
	SIMVA	LOVA	SIMVA	LOVA	SIMVA	LOVA	SIMVA	LOVA
1	15.23	14.96	101.53	99.73	15.23	15.11	101.53	100.73
2	15.02	15.02	100.13	100.13	15.45	14.98	103.00	99.87
3	14.95	15.22	99.67	101.47	14.36	15.23	95.73	101.53
4	15.03	15.09	100.21	100.60	15.28	15.66	101.87	104.40
5	15.23	15.12	101.53	100.80	15.98	15.12	106.53	100.80
6	14.98	15.22	99.87	101.47	15.88	15.22	105.87	101.47
N	6.00	6	100.28	100.70	6.00	6	102.42	101.47
Mean	15.07	15.11			15.36	15.22		
% Mean Change	-2.14 (SIMVA)							
	-0.77 (LOVA)							
Sr.No.	HQC (680.000 ng/ml)							
1	680.14	681.23	99.73	100.02	678.93	681.56	100.73	99.84
2	679.784	679.39	100.13	99.97	689.34	689.36	99.87	101.37
3	681.23	680.88	101.47	100.18	680.39	675.36	101.53	100.06
4	679.34	681.65	100.60	99.90	680.03	688.36	104.40	100.00
5	679.01	684.23	100.80	99.85	680.97	688.69	100.80	100.14
6	681.04	678.36	101.47	100.15	679.47	680.59	101.47	99.92
N	6	6	100.70	100.01	6	6	101.47	100.22
Mean	680.09	680.96			681.52	683.99		
% Mean Change	-0.21 (SIMVA)							
	-0.45 (LOVA)							

3. Long-Term Stability

SIMVA was found to be stable in human plasma at below -20°C upto 45 Days in LQC and HQC samples with % Mean Change of 2.12 and -0.04 respectively, (Table No. 7.2.8.9). LOVA was found to be stable in human plasma at below -20°C upto 45 Days in LQC and HQC samples with % Mean Change of -0.37 and 0.10 respectively, (Table No. 7.2.8.9).

Table 7.2.8.9: long term stability of SIMVA and LOVA

Sr.No.	**LQC (15.000 ng/ml)**							
	Comparison samples				**Stability samples**			
	Sample conc.(ng/ml)		**% Nominal Conc.**		**Sample conc.(ng/ml)**		**% Nominal Conc.**	
	SIMVA	LOVA	SIMVA	LOVA	SIMVA	LOVA	SIMVA	LOVA
1	14.32	15.23	95.47	101.53	16.16	15.09	107.73	100.60
2	15.03	15.02	100.20	100.13	14.23	15.47	94.87	103.13
3	15.79	15.09	105.27	100.60	14.02	15.02	93.47	100.13
4	14.89	15.23	99.27	101.53	14.29	15.98	95.27	106.53
5	14.07	15.44	93.80	102.93	15.02	15.09	100.13	100.60
6	16.047	15.97	106.98	106.47	14.52	15.66	96.80	104.40
N	6	6	100.16	102.20	6	6	98.04	102.57
Mean	15.02	15.33			14.71	15.39		
% Mean Change	2.12 (SIMVA)							
	-0.37 (LOVA)							
Sr.No.	**HQC (680.000 ng/ml)**							
1	680.23	681.56	100.03	100.23	681.69	687.96	100.25	101.13
2	681.55	680.55	100.23	100.08	681.66	680.26	100.24	100.00
3	680.22	684.69	100.03	100.69	682.36	678.56	100.35	99.75
4	681.36	679.3	100.20	99.90	680.12	682.36	100.02	100.31
5	680.65	688.31	100.10	101.22	680.33	682.64	100.05	100.35
6	680.24	679.96	100.04	99.99	680.17	680.26	100.03	100.00
N	6.00	6	100.12	100.35	6.00	6	100.16	100.26
Mean	680.71	682.40			681.06	682.01		
%	-0.04 (SIMVA)							

Mean Change	0.10 (LOVA)

4. Stock Solution Stability

Stock solution stability was determined by comparing the peak areas of freshly prepared solutions (comparison samples) with stability samples.

SIMVA stock solution was found to be stable at 2-8°C for 7 days with % mean change of 1 (Table No. 7.2.8.10). SIMVA stock solution was found to be stable at 2-8°C for 7 days with % mean change of 1.35 (Table No. 7.2.8.10).

Table 7.2.8.10: Stock solution stability of SIMVA and LOVA

Sr No.	SIMVA(800.000 ng/ml)		LOVA(800.000 ng/ml)	
	Comp. Sample area	Stability sample area	Comp. sample area	Stability sample area
1	10238	10174	25143	25214
2	10264	10238	25223	25634
3	9990	9915	26515	25311
4	10089	10126	25217	25165
5	11241	11255	25362	25182
6	11100	11467	25636	25322
Mean	10487	10529	25516	25305
SD	540.8	656.8	519.61	174.09
%CV	5.2	6.2	2.04	0.69
Mean % Change	1		1.35	

(VII) Matrix effect

In order to ensure the effect of matrix through out the application of the method, plasma blanks obtained from two different lots were spiked with SIMVA at LQC and HQC level. Three quality control samples at each level along with the set of calibration standards were analyzed and the % nominal concentration of the samples analyzed was represented in Table No. 7.2.8.11 for SIMVA and Table No. 7.2.811 for LOVA.

Table 7.2.8.11: Matrix effect for SIMVA and LOVA

Sample ID		SIMVA		LOVA	
		Calculated Conc. (ng/ml)	% Nominal Conc.	Calculated Conc. (ng/ml)	% Nominal Conc.
I	LQC	15.23	101.53	15.23	101.53
	LQC	15.02	100.13	15.02	100.13
	LQC	15.00	100.00	15.29	101.93
II	LQC	15.47	103.13	15.69	104.60
	LQC	15.92	106.13	15.02	100.13
	LQC	15.33	102.20	15.69	104.60
I	HQC	680.23	100.03	681.65	100.24
	HQC	681.22	100.18	679.69	99.95
	HQC	680.25	100.04	681.69	100.25
II	HQC	681.55	100.23	679.33	99.90
	HQC	680.22	100.03	680.54	100.08
	HQC	684.22	100.62	679.66	99.95

7.2.8.4.2. Estimation of SIMVA in marketed Tablet:

The contents of 20 Tablets were weighed and their mean weight determined and finely powdered. An equivalent weight of the Tablet content was transferred into a 10 ml volumetric flask containing 5 ml methanol, sonicated for 15 min and further diluted to 10 ml with methanol. The resulting solution was sonicated for 10 min and supernatant was filtered through whatman filter paper no.41. 1 and 2 µl from this solution were transferred to sample tube. Add 90 µl of plasma sample. Vortex for 1 min on vortex shaker. The volume was adjusted to a 1000 µl with ACN to get final concentrations of 100 and 200 ng/µl of solution. Centrifuge for 5 min at 4000 rpm. 20 µl of supernatant were injected into HPLC column and the Peak area of each solution was measured at 248 nm. The concentration of SIMVA and LOVA were found from regression equation and result of the % assay was given in table 7.2.8.12 and 7.2.8.12, respectively.

Table 7.2.8.12: Estimation of SIMVA in tablet by HPLC with UV detection

Tablet Formulation	Labeled Claim (mg/tablet)	Amount found (mg/tablet)	% Recovery ± S.D
SIMVATOR	10	10.23	102.14 ± 0.26

(SOLUS)	20	19.99	99.99 ± 0.44
LOVACARD (Cipla)	10	9.98	99.85 ± 0.35
	20	19.98	99.84 ± 0.34

*** Mean value of five determinations**

7.2.8.5. Summary of Validation parameters:

The summary of validation parameters was given in table 7.2.8.13.

Table 7.2.8.13: Summary of Validation parameters by HPLC

Sr. No	Parameters	Peak Area	
		SIMVA	LOVA
1	Detection wavelength (nm)	237	
2	Retention time (minutes)	12.67	9.638
3	Linearity range (ng/ml)	10.8 - 1000	
4	Theoretical plate	8505.637	5470.101
5	Regression equation	Y=14.25x -219.73	Y=32.133x -152.65
6	Correlation coefficient (r^2)	0.9979	0.9994
7	Intercept	-219.73	-152.65
8	Slope	14.24	32.133
9	Assay	99.99-102.14	99.84-99.85
10	Precision		
	Intra day % CV (n = 5)	0.25-0.87	0.12-0.69
	Inter day % CV (n = 5)	0.25-0.89	0.15-0.96
	Repeatability of measurements %CV	< 1 %	
	% Recovery	99.77-100.51	99.92-102.22
11	Limit of detection (ng/ml)	0.149	0.964
12	Limit of quantification (ng/ml)	5.231	8.425

7.2.8.6. Conclusion:

Method development to estimate drug solute in vitro extrapolated to determine drug concentration in biological fluids.