4

DEVELOPMENT AND VALIDATIONS OF ANALYTICAL METHODS FOR RITONAVIR

4. Development and Validation of Analytical Methods

4.1 Development and Validation of Analytical Methods for Ritonavir

Literature reported different analytical methods was used for the estimation of the ritonavir (RTV) in bulk API and finished formulation and also from biological samples like HPTLC[1-6], UV spectroscopy[7-9], LC/MS-MS[10-12] and HPLC[13-28] etc. Simple RP-HPLC analytical method was developed for the various studies in present investigation. The methods are described in the following sections.

4.1.1 UV-Spectrophotometric Method for Ritonavir

4.1.1.1 Materials and methods

Ritonavir API was received as a gift sample from Hetero Pharmaceuticals, Hyderabad. HPLC grade Methanol was procured from Rankem Pvt Ltd, India. Double distilled water was prepared by double distillation assembly and filtered through 0.22µ Millipore filter paper (Millipore, India) before use. UV-Spectrophotometer (UV-1700) Shimadzu, Digital pH meter (Picco^{+} Lab India Pvt. Ltd, India) and digital weighing balance (AX 120) Shimadzu, Japan etc instruments were used in experiments.

Stock Solution of RTV

The standard stock solution of RTV (1000µg/ml) was prepared by dissolving 10 mg accurately weighed RTV in 10ml of methanol.

Calibration curve of RTV in methanol

The λmax of RTV was determined by scanning 10µg/ml solution of RTV in methanol in UV Spectrophotometric range 200-400nm. From the above prepared standard stock solution suitable aliquots were pipette out in 10ml volumetric flask and volume was made up to 10ml with methanol to get final concentration range from 5-100 µg/ml. The solutions were mixed gently and measure the absorbance at λmax using methanol as blank in UV-Visible Spectrophotometer (Shimadzu 1700) and calibration curve was plotted. The same procedure was repeated three times. The overlay spectra for different solutions and Calibration curve of RTV in methanol are showing in figures 4.1 and 4.2 respectively.

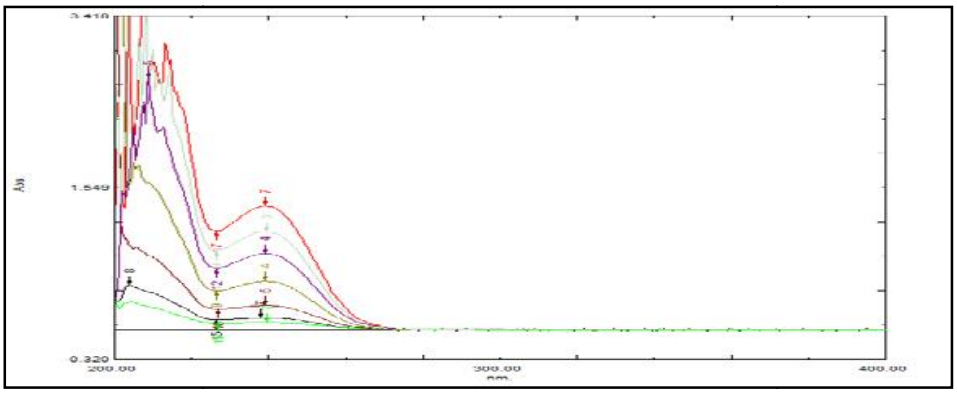

Figure 4.1 Overlay UV-Spectra of RTV in methanol (5-100 µg/ml)

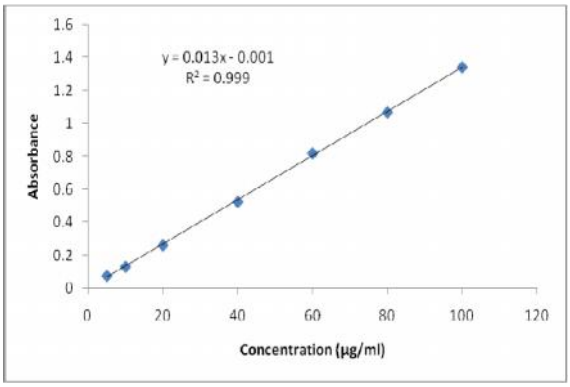

Figure 4.2 Calibration Curve of RTV in methanol (5-100 µg/ml)

The results show that the calibration curve for RTV in methanol was linear in 5-100µg/ml concentration range and the slope, intercept and correlation coefficients were obtained by linear least square treatment of the results**.**

4.1.1.2 Analytical Method Validation

Linearity

The linearity of proposed analytical method is its ability to obtain test results which are directly proportional to the concentration or amount of analyte in the sample.[29-30] For evaluation of the linearity parameter of the developed UV method for RTV, series of six different concentrations were prepared at 5-100 µg/ml and absorbances of samples were taken at 240 nm. The results shows proposed UV spectroscopic method obeys the Beer's law in 5 to 100µg/ml concentration range. The regression equation was Y= 0.013x+0.001 with correlation coefficient (R2) = 0.999 that indicate linearity of the plot. The overlay spectra of UV and Calibration curve of RTV in methanol are showing in Figure 4.1 and 4.2 respectively

Accuracy

The various excipients like a binder, diluents, disintegrating agents, flavoring agents and fillers etc are present in the finished formulation may affect or produce interface

during the analysis. Recovery studies were executed for confirming the accuracy of the developed analytical method.[31] Recovery studies were carried out on the finished formulation. A fixed amount of pre-analyzed sample was taken and standard drug solution was added at 80%, 100% and 120% level.

Accurately weighted ten tablets of RTV were taken in mortar and pestle and triturated to make a fine powder. Equivalent quantity of powder of 50 mg of RTV was taken and transfers in to a beaker. 20ml of methanol was added and stirred slowly for RTV to solubilise in methanol. The solution was filtered through Whatman filter paper no.42 and collected in 50ml volumetric flask. Rinsing the filter paper 2-3 times with 5 ml of methanol and combining all the methanol in 50 ml volumetric flask. the volume was made up to 50ml with methanol (1000µg/ml). 5ml of aliquot was taken and diluted with 0.1N HCl solution up to 50ml, to get concentration of 100µg/ml of RTV and this was formulation working solution for RTV. The same procedure was used for preparation of 1000µg/ml standard drug solution.

Then aliquots of 1ml were taken in 10 ml volumetric flask from the prepared formulation working solution and each of them was spiked with standard drug solution at three levels (80%, 100% and 120%) by addition of aliquots of 0.8, 1, 1.2 ml. The analysis was carried out in triplicate. The amount of RTV recovered was calculated by the equation obtained by calibration curve. The results of accuracy are showing in table 4.1

Table 4.1 Recovery study of RTV

% spiked	Actual Conc. of RTV (µg/ml)	Amount of RTV added (µg/ml)	Amount of RTV Recovered (µg/ml)	% recovered ± SD
80%	10	8	18.38	101.92 ± 1.56
100%	10	10	20.28	100.51 ± 1.75
120%	10	12	22.12	99.14 ± 0.97

*Mean value of three determinations.

Precision

The precision study of developed analytical method was confirmed by Interday and Intra-day study.

Intraday

Series of seven different concentrations were prepared at 5-100 µg/ml and absorbances of samples were taken at λmax. The experiment was repeated three times in a day and the % RSD was calculated for each concentration level.

Inter-day

In the interday precision experiment series of seven different concentrations were prepared at 5-100 µg/ml and absorbances of samples were taken at 240 nm and it was repeated on three different days. The percentage RSD was calculated at each concentration level. The obtained results are given in Table 4.2

Table4.2 Precision studies for estimation of RTV in methanol by UV spectroscopy

Conc. of Sample (µg/ml)	Intraday Precision (% RSD)	Interday Precision (%RSD)
5	0.962	0.202
10	0.740	0.425
20	0.377	0.579
40	0.582	0.503
60	0.610	0.394
80	0.341	0.295
100	0.490	0.322

*Mean value of three determinations

Limit of detection (LOD) and limit of quantification (LOQ)

Calibration curve of RTV in methanol was repeated for three times and the standard deviation (SD) of the intercepts was calculated. Then Limit of detection (LOD) and limit of quantification (LOQ) were calculated as follows and results are given in table 4.3.

LOD= 3.3 * S.D./slope of calibration curve

LOQ= 10 * S.D./slope of calibration curve

Where, S.D. = Standard deviation of intercepts.

Table 4.3 LOD and LOQ of RTV

Parameter	RTV
Limit of detection (LOD)	0.253 (µg/ml)
Limit of quantification (LOQ)	0.769 (µg/ml)

Table4.4 Summarized data for the developed method

Parameter	Results
λ max (nm)	240
Beer's law limit (µg/ml)	5 to 100
Regression equation (Y=mX+c)	0.013x - 0.001
Slope	0.013
Intercept	0.001
Limit of detection (µg/ml)	0.253
Limit of quantification (µg/ml)	0.769
Coefficient of determination	0.9996
% RSD	< 2%
Accuracy	> 99%

4.1.2 HPLC Method for Ritonavir

4.1.2.1 Materials and methods

RTV was gifted from Hetero Pharmaceuticals, Hyderabad. Marketed tablet formulation, containing 100 mg of RTV was procured from commercial marketed source. Methanol and acetonitrile (both HPLC grade) were purchased from Rankem India. Double Double distilled water was prepared by double distillation assembly and prepared water filtered through 0.22µ Millipore filter paper (Millipore, India) and then used for the further. All the other reagents and solvents were of HPLC grade and were used as such.

The instruments used for the estimation of RTV include Digital weighing balance (Ax 120, Shimadzu, Japan) pH meter (Picco+, Lab India, India), High performance liquid chromatography with UV detector (HPLC Isocratic system with LC-20AT pump, Software-LC solution, Shimadzu).

4.1.2.2 Method for RTV by RP-HPLC

The RP-HPLC method for Estimation of RTV was developed and validated as per ICH guideline. In this method mobile phase consisted of mixture of methanol, Acetonitrile and Ammonium Formate Buffer pH 4.5 (40:35:25). The Ammonium Formate buffer was prepared by dissolving 1.26gm Ammonium Formate in 1000 ml of deionised water and adjust the pH between 4.4-4.5 by using formic acid. Then organic solvent and buffer was mix thoroughly and sonicated for 15 min and filtered through 0.22µ nylon filter. The mobile phase was ready for the further use.

Shimadzu isocratic HPLC with a UV-visible detector was used for HPLC analysis. The Kromasil C18 (250 X 4.6 mm, 5 µm), column was used for the separation at ambient temperature. The injection volume was 20µl, the flow rate was 1 ml/min, run time was 10 min and detection wavelength was 210 nm. The RP-HPLC Chromatographic conditions are given in Table 4.5

Table 4.5 RP-HPLC Chromatographic conditions for RTV

Drug	**Ritonavir**
Column	Kromasil
Mobile Phase	Methanol: Acetonitrile :pH 4.5 Ammonium Formate Buffer(40:35:25)
Flow rate	1 ml/ min
Detector	UV-Visible detector (SPD-20A)
Temperature	Ambient temperature
Detection Wavelength	210 nm

Preparation of standard stock solution of RTV

A stock solution of RTV was prepared by dissolving 10 mg of RTV in 10 ml of mobile phase. (1000µg/ml)

Calibration curve of RTV in mobile phase

Accurate aliquot 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5ml were transferred in 10ml calibrated volumetric flasks and diluted up to the volume with mobile phase to get the final concentrations range 5-50 µg/ml.

4.1.2.3 RP-HPLC Analytical Method Validation

Linearity

The calibration curve was found linear in the concentration range of 5 to 50µg/ml. The linearity regression equation was Y= 112.01x+3.14 with correlation coefficient (R2) = 0.999 that indicate linearity of the plot. (Fig. 4.3 and 4.4)

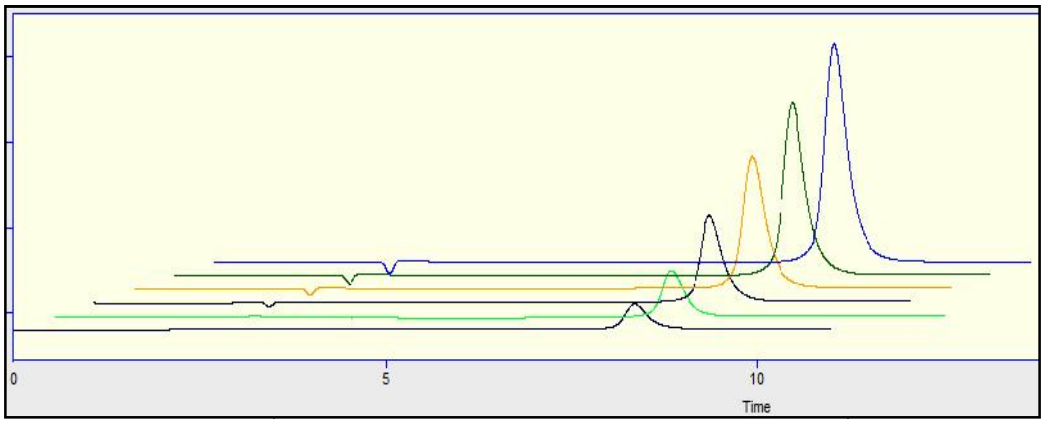

Figure4.3 Overlay Chromatogram of Ritonavir

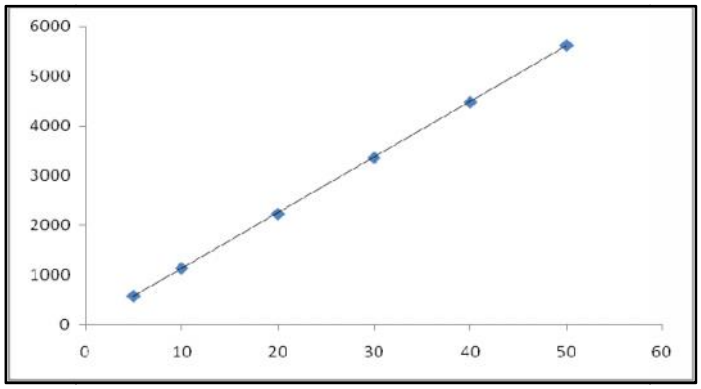

Figure 4.4 Calibration Curve of RTV

Precision

Three different concentration solution of RTV were prepared with mobile phase. The experiment was repeated 3 times in a day and 3 different day for intraday and interday respectively. The result was calculated as % RSD and it was found within the limit. The results are shown in tables 4.6 and 4.7 respectively.

Table 4.6 Intraday-precision

RTV concentration (µg/ml)	Peak Area			mean	SD	% RSD
7.5	878.56	876.23	879.45	878.23	1.015	0.115
25	2956.59	2957.58	2955.25	2956.47	1.677	0.056
45	5356.89	5354.22	5353.28	5354.91	2.605	0.048
Average						0.073

*Mean value of three determinations.

Table 4.7 Interday-precision

RTV concentration (µg/ml)	Peak area			mean	SD	% RSD
7.5	876.58	875.23	874.45	875.58	1.726	0.197
25	2955.12	2956.28	2957.69	2956.51	1.133	0.038
45	5352.96	5354.58	5354.12	5353.88	0.075	0.014
Average						0.083

*Mean value of three determinations.

Recovery study

Accurately weighed five tablets of RTV were taken in mortar and triturated to make a fine powder. Then equivalent quantity of powder of 50 mg of RTV was taken and transfer in a beaker. 20ml of mobile phase was added and stirred slowly for RTV to solubilise in mobile phase. Solution was filtered through Whatman filter paper no.42 and collected in 50ml volumetric flask. Rinsing the filter paper 2-3 times with 5ml of mobile phase and all fraction were collected in 50 ml volumetric flask and the volume up to 50ml with mobile phase (1000µg/ml). From this 5ml of aliquot was taken and diluted with mobile phase up to 50ml, to get 100µg/ml of RTV and this was formulation working solution for RTV. The same procedure was used for preparation of 100µg/ml standard drug solution. Then aliquots of 1ml were taken in 10 ml volumetric flask from the prepared formulation working solution and each of them was spiked with standard drug solution at three levels (80%, 100% and 120%) by addition of aliquots of 0.8, 1, 1.2 ml. Then volume was made up to 10ml with mobile phase. The analysis was carried out in triplicate. The amount of RTV recovered was calculated by the equation obtained by calibration curve and the results are shown in tables 4.8

Table 4.8 Recovery study of RTV

% spiking	Actual Conc. of RTV (µg/ml)	Amount of RTV added (µg/ml)	Amount of RTV Recovered (µg/ml)	% recovered ± SD
80	10	8	18.13	101.63 ± 0,076
100	10	10	19.84	98.84 ± 0.049
120	10	12	22.21	101.30 ± 0.079

*Mean value of three determinations.

Estimation of RTV in Formulation

Developed RP-HPLC method was successfully applied for the estimation of RTV in marketed finished formulation. Aliquots of 1ml were taken in 10 ml volumetric flask from the above prepared formulation working solution (100µg/ml) and made-up the volume up to 10 ml with mobile phase. Six such solutions were prepared and inject in HPLC. Their peak area was measured at 210nm wavelength and % assay was measured. The results are shown in tables 4.9

Table4.9 Analysis of marketed formulation

Concentration of Sample (µg/ml)	Concentration obtained (µg/ml)	% of label claimed
10	10.14	100.90%

*Mean value of three determinations

LOD and LOQ

Calibration curve was repeated for 3 times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were calculated as follows and results are given in table 4.10.

LOD = 3.3 * S.D./slope of calibration curve
LOQ = 10 * S.D./slope of calibration curve
Where, S.D. = Standard deviation of intercepts,

Table 4.10 LOD and LOQ of RTV by RP-HPLC method

Parameter	RTV
Limit of detection (LOD)	0.0153 (µg/ml)
Limit of quantification (LOQ)	0.0459 (µg/ml)

Table 4.11 Summarized data for the developed method of RTV

Parameters	RTV
Detection Wavelength	210nm
Linearity Range	5-50µg/ml
Accuracy	100.85
Intraday precision (% RSD)	0.496
Interday Precision (% RSD)	0.306
Regression Equation	Y=112.1+3.141
LOD	0.0153 (µg/ml)
LOQ	0.0459 (µg/ml)

4.1.3 HPLC Method for Estimation of RTV in Plasma

Simple bio analytical RP-HPLC method used for the determination and estimation of RTV using Efavirenz as an internal standard was developed and validated as per regulatory requirements. The protein precipitation technique was used for the sample preparation and chromatographic separation on a reverse phase Kromasil C18 column. The above developed RP-HPLC method was used for the estimation of RTV in plasma samples. The mobile phase consisted of mixture of Methanol Acetonitrile and ammonium formate buffer pH 4.5 (40:35:25) at a flow rate of 1 ml/min. The wavelength used for the detection of RTV was 210 nm with a total run time of 12 minutes. The retention times of RTV and efavirenz were found to be 8.3 and 10.2 minutes respectively. The method was developed and tested for the linearity range of 100ng/ml to 10000ng/ml. The method was validated for accuracy, precision, linearity, recovery and stability in compliance to international regulatory guidelines.

4.1.3.1 Experimental

Primary and secondary stock solution

To prepare primary stock solution 100 mg of RTV was transferred into 100 mL of volumetric flask. Add 50 ml acetonitrile and sonicated for about 5 min and volume made upto 10ml with methanol (1000µg/ml). The secondary stock solution was prepared from primary stock solution, 0.05, 0.15, 0.3, 0.4, 0.5, 1, 2, 3 and 5 ml of primary stock solution was transferred into 10mL volumetric flask and volume made upto 10ml with Acetonitrile: water (50:50) that prepare 5, 15, 30, 40, 50, 100, 200, 300 and 500µg/ml solution.

Plasma sample preparation

0.1 ml of from secondary stock solution of each concentration and 0.05 ml of Efavirenz as IS was added in 2 mL polypropylene centrifuge tube. Add 0.85 ml of drug-free human plasma. Mixed the plasma and drug solution thoroughly for 5 minutes on vortex shaker.

Sample extraction Procedure

Protein precipitation technique was used for drug extraction from plasma sample. 0.1 ml of drug spiked with 0.85ml plasma was taken in 1ml centrifuge tube and 0.4 ml of acetonitrile was added to precipitate plasma proteins. Mixed thoroughly for 5 minutes on vortex shaker. Then tubes were centrifuged at 10000 RPM for 10 min in high speed homogenizer (Sigma Hi Speed Homogenizer, Japan). Supernatant from

each tube was separated using micropipettes and 20 µl solutions were injected into HPLC system.

4.1.3.2 Bio-analytical Method Validation

System Suitability

System suitability parameter was performed by injecting six replicates of MQC of RTV. The mean standard deviation and % RSD for the peak area ratio and for the retention time of analyte and IS were calculated. Acceptance limit for retention time (Rt) deviation and area deviation 2% and 5 %RSD respectively were passed

Calibration Curve

The linearity of the developed method was determined over calibration curve range of 100-10000ng/ml. The calibration standards were prepared by spiking known concentration of RTV working standard solution. A linearity curve containing nine non-zero concentrations was analyzed. Back-calculated the concentrations of each level and plot the graph of back-calculated concentration against drug area ratio. Calculate the slope, y-intercept and correlation coefficient curve by suitable linear regression analysis.

Precision and Accuracy

Accuracy and precision were measured on the samples spiked with known amounts of the analyte. Accuracy and precision were determined by replicate analysis of six determinations of four concentration levels which covers the calibration range: LLOQ, LQC, MQC, and HQC. These QC samples were analyzed against the calibration curve and obtained concentrations compared with the nominal value. Precision is expressed as the % coefficient of variation (% CV). The accuracy and precision were evaluated as within batch and between-batch.

1) **Between-batch (Inter-day) accuracy and precision**: The inter-day accuracy and precision were assessed by measuring the concentration of RTV in three aliquots of four different concentrations LLOQ QC, LQC, MQC and HQC on three consecutive days.

2) **Within-batch (Intra-day) accuracy and precision**: The intra-day accuracy and precision were assessed by measuring the concentration of RTV in three aliquots of four different concentrations LLOQ QC, LQC, MQC and HQC on a single day

Recovery of Analyte:

Recovery of RTV in plasma was evaluated by comparing the mean peak responses of at least six injection of each LQC, MQC and HQC quality control sample,

prepared in plasma, to mean peak responses of non-spiked samples prepared in elution solvent and external spiked matrix extracted sample.

Recovery of IS (internal standard):

Recovery of Internal standard in plasma was evaluated by comparing the mean peak responses of at least six medium quality control samples, prepared in plasma, to mean peak responses of non-spiked samples prepared in elution solvent and external spiked matrix extracted sample

4.1.3.3 Stability study of RTV samples

Bench Top Stability

Bench top stability of spiked samples was performed at LQC and HQC level over three replicates were kept at room temperature for 12 hrs. These samples were preferred as stability samples. Stability was carried out by comparing these samples against the freshly spiked LOC and HQC samples in three replicate.

Freeze Thaw Stability

The freeze and thaw stability of analyte was determined after three Freeze and Thaw cycles. The three sets of LQC and HQC samples were stored at -70±5°C and subjected to three freeze thaw cycles at interval of 24 hrs. After the completion of three cycles of 24 hrs the samples were analyzed. Stability was carried out by comparing these samples against the freshly spiked LOC and HQC samples in three replicate

Long Term Stability

Long term stability was performed at LQC and HQC level. Three replicates of LQC and HQC were kept at -70°C for 14 days. After 14 days these samples were used as stability samples. Stability was carried out by comparing these samples against the freshly spiked LOC and HQC samples in three replicate.

4.1.4 Results and Discussion

System Suitability:

The % RSD of system suitability was observed in the range of **0.00 to 0.99%** for retention time of drug, **0.00 to 0.89%** for retention time of IS and area ratio in the range of **0.9 to 2.0%** which is not more than 5.00% as per the acceptance criteria. The results are shown in table 4.12 and 4.13.

Table 4.12 System Suitability Parameters

Sample	Area ratio	Analyte RT	IS RT
MQC-1	0.5414	8.21	10.3
MQC-2	0.5416	8.35	10.2
MQC-3	0.5513	8.20	10.3
MQC-4	0.5373	8.37	10.2
MQC-5	0.5384	8.45	10.2
MQC-6	0.5406	8.38	10.4
MEAN	0.5418	8.28	10.266
SD	0.0049	0.075	0.081
% RSD	0.917	0.908	0.795

Table 4.13 System Suitability Parameters

Day	% RSD		
	Analyte RT	IS RT	Area ratio
Day 1	0.908	0.795	0.917
Day 2	1.281	0.733	1.405
Day 3	0.678	1.141	1.874

Calibration curve of RTV

Calibration curves were found to be consistently accurate and precise for RTV over 100-10000ng/ml. The correlation coefficient was greater than or equal to 0.990. RTV concentration at each calibration level was back calculated from the calibration curves. The results obtained are shown in Table 4.14 and were meeting the acceptance criteria of $r^2 \geq 0.98$. Figure 4.5 describes the chromatogram of blank human plasma, also the figure 4.6 and figure 4.7 describes the overlay chromatograms of RTV and IS in plasma and calibration curve of RTV in plasma respectively.

Table 4.14 Linearity of RTV

Conc. (µg/ml)	Back Calculated Conc.(µg/ml)			Mean	% RSD
100	96.24528	97.37736	102.3774	98.66667	3.307119
300	266.9057	278.0377	286.2453	277.0629	3.503391
600	559.1698	573.6038	578.7925	570.522	1.782199
800	794.1698	815.3962	759.8302	789.7987	3.550236
1000	997.4717	957.0943	968.4151	974.327	2.137657
2000	1903.038	1921.906	1940.774	1921.906	0.98173
4000	3966.528	3888.132	3915.208	3923.289	1.014915
6000	6086.057	6043.887	6068.226	6066.057	0.348966
10000	9918.132	9853.132	9888.981	9886.748	0.329304
Slope	105.9	104.8	105.4		
Intercept	2.218	1.647	2.010		
r^2	0.999	0.998	0.999		

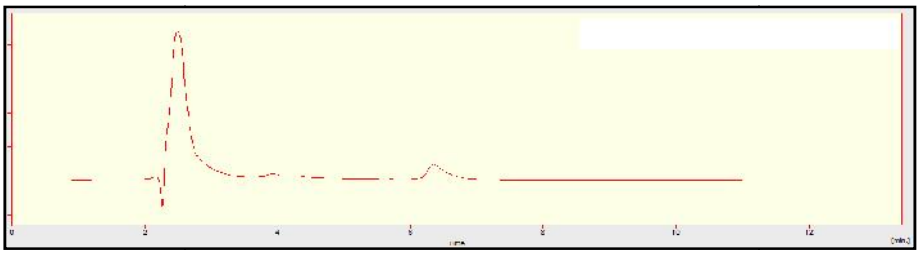

Figure 4.5 Chromatogram of Blank (unspiked) Human Plasma (PB)

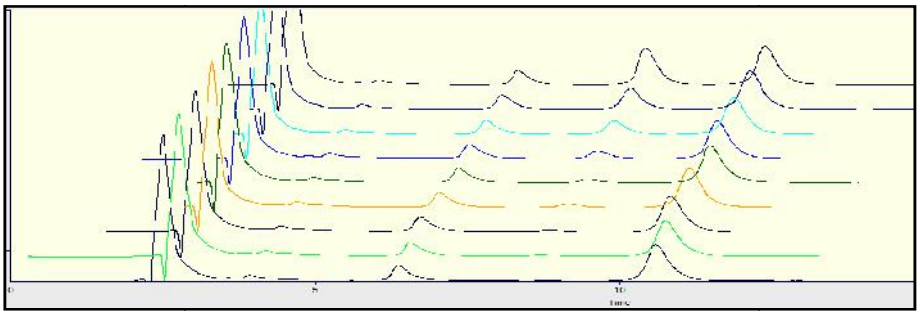

Figure 4.6 Chromatogram of RTV in Plasma sample with IS

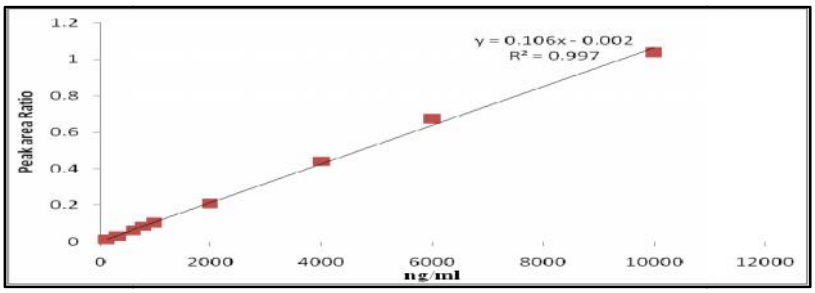

Figure4.7 Calibration curve of RTV in human plasma

Precision and Accuracy

Inter batch (between-batch) precision and accuracy:

The between batch % coefficients of variation for RTV was ranged between 0.7 % to 4 % and % accuracy was ranged between 94 % to 100 %.The results are within ± 15 % and the results are presented in table 4.15.

Table4.15 Inter Day Precision and Accuracy of RTV

	LLOQ	LQC	MQC	HQC
Conc. (ng/ml)	**100**	**500**	**5000**	**8000**
Estimated conc.	98.03	484.16	4926.15	7718.32
	98.22	472.47	4919.35	7636.15
	97.84	490.20	4942.37	7738.88
	95.39	470.30	4925.11	7816.05
	95.86	456.05	4919.45	7739.07
	97.84	495.49	5036.52	7748.79
Mean	97.20	478.11	4944.83	7732.88
SD	1.234	14.589	45.705	57.990
RSD	1.270	3.051	0.924	0.749
% accuracy	97.20	95.62	98.89	96.66

Intra batch (within batch) precision and accuracy:

The within batch % coefficient of variation for RTV was ranged between 0.5% to 2% and % accuracy was ranged between 95 % to 99 %. The results are within ± 15 % the results are presented in table 4.16

Table 4.16 Intra Day Precision and Accuracy of RTV

	LLOQ	LQC	MQC	HQC
Conc. (ng/ml)	**100**	**500**	**5000**	**8000**
Estimated conc.	100.30	481.90	5041.81	7739.92
	98.32	492.47	4949.54	7768.41
	99.16	475.01	4927.75	7728.60
	98.32	473.88	5033.03	7743.22
	99.07	482.37	4955.96	7750.39
	97.66	493.41	4926.43	7855.20
MEAN	98.80	483.17	4972.42	7764.29
SD	0.919	8.32	51.75	46.44
% RSD	0.930	1.72	1.040	0.598
% accuracy	98.80	96.63	99.44	97.05

Absolute Recovery
Recovery of analyte

Recovery of analyte were evaluated by injecting three replicates of the un-extracted QC samples at each LQC, MQC and HQC concentrations and three replicates of the extracted QC samples at each LQC, MQC and HQC concentrations. Comparing both samples area and calculate the absolute recovery of the RTV. Results are presented in table 4.17.

Table 4.17 Recovery study of RTV

	LQC		MQC		HQC	
Sr. No	**Extracted peak Area**	**Unextracted peak Area**	**Extracted peak area**	**Unextracted peak area**	**Extracted peak Area**	**Unextracted peak area**
1	55.03	57.6	553.87	598.34	836.88	874
2	47.21	56.95	544.54	595.12	828.23	880
3	56.32	57.45	559.34	585.23	818.12	888
Mean	52.85	57.33	552.58	592.89	827.74	880.66
SD	4.92	0.34	7.48	6.83	9.38	3.70
% RSD	9.32	0.59	1.35	1.15	1.13	0.42
% Recovery	91.18%		93.20		93.99	

Recovery of Internal Standard

Recovery of IS were evaluated by injecting three replicates of the un-extracted QC samples of HQC concentration and three replicates of the extracted QC samples of

HQC concentrations. Comparing both samples area and calculate the absolute recovery of the analyte Results are presented in table 4.18

Table 4.18 Recovery study of IS

HQC	
Extracted peak Area	**Unextracted peak area**
983.13	1045.25
993.34	1050.14
998.65	1060.13
991.70	1051.84
7.88	7.58
0.79	0.72
94.28	

Bench Top Stability

The bench top stability of RTV was checked by kept three sets of LQC and HQC at room temperature for 12 hours. The % mean stability for LQC and HQC of RTV concentration was found to be 97.82 and 98.40% respectively. The results are within the acceptable limit, Acceptance criteria is at least 67% QC samples should pass acceptance limit of 85.00–115.00 % and not more than 50% at each QC level should fail and it is presented in table 4.19

Freeze-Thaw Stability

Freeze thaw stability in plasma was evaluated by analyzed three replicates of LQC and HQC samples previously frozen at -70 ± 5 º C and thawed at room temperature over three cycles. The % mean stability for LQC and HQC of RTV concentration was found to be 98.97 and 98.59% respectively. The results are within the acceptable limit, Acceptance criteria is at least 67% QC samples should pass acceptance limit of 85.00–115.00 % and not more than 50% at each QC level should fail and it is presented in table 4.19.

Long Term Stability

Long term stability of the spiked quality control samples was determined after stored at -70°C for 14 days. Stability was assessed by comparing them against the freshly spiked calibration standards. The percentage mean stability of LQC & HQC was found to be 96.73 & 98.83%, for RTV, which is within the acceptance limit. Acceptance criteria is at least 67% QC samples should pass acceptance limit of 85–

115 % and not more than 50% at each QC level should fail. Results are summarized in table 4.19 for RTV

Table 4.19 Stability study

QC samples RTV	Mean conc. Observed (ng/ml)	Fresh prepared (ng/ml)	Mean conc. Observed (%)
Bench top stability (n=6) (after 12 h)			
LQC (500ng/mL)	480.85	491.52	97.82
HQC (8000ng/mL)	7785.44	7911.33	98.40
Freeze-thaw cycle (3 cycle) (n=6)			
LQC (500ng/mL)	488.61	493.69	98.97
HQC (8000ng/mL)	7753.38	7864.16	98.59
Long term stability (after 14 days) (n=6)			
LQC (500ng/mL)	477.28	493.41	96.73
HQC (8000ng/mL)	7741.13	7824.83	98.83

4.1.5 Conclusion

A simple, precise and sensitive RP-HPLC method has been developed for the quantification of RTV from human plasma using protein precipitation method. The method shows good linearity over 100-10000 ng/ml concentration range. Sensitivity of developed analytical method was enough for detecting and quantifying RTV in human plasma. Along with these features it gives high recovery and fast and simple sample preparation technique make this method suitable for application in clinical and preclinical pharmacokinetic, pharmacodynamics and metabolite study.

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DEVELOPMENT AND VALIDATIONS OF ANALYTICAL METHODS FOR LOPINAVIR

4.2 Development and Validation of Analytical Methods for Lopinavir

Literature reported different analytical methods for the estimation of the lopinavir (LPV) in bulk API and finished formulation and also from biological samples like UV spectroscopy,[1-3] HPTLC,[4-6] LC/MS-MS[7-9] and HPLC[10-27] etc. A simple UV spectrophotometric and RP-HPLC analytical methods were developed for various studies in present investigation. The methods are described in the following section.

4.2.1 UV-Spectrophotometric Method for Lopinavir

4.2.1.1 Materials and methods

Lopinavir API was received as gift sample from Emcure Pharmaceuticals, Pune. Acetonitrile (HPLC grade) was procured from Rankem Pvt Ltd, India. Double distilled water was prepared by double distillation assembly and filtered through 0.22µ Millipore filter paper (Millipore, India) before use. UV-Spectrophotometer (UV-1700) Shimadzu, Digital pH meter (Picco$^+$ Lab India Pvt. Ltd, India) and digital weighing balance (AX 120) Shimadzu, Japan etc instruments were used in experiments.

Stock Solution of LPV:

The standard stock solution of LPV (1000µg/ml) was prepared by dissolving 10mg accurately weighed LPV in 10ml of acetonitrile.

Calibration curve of LPV in Acetonitrile

The λmax of LPV was determined by scanning 10µg/ml solution of LPV in acetonitrile in UV Spectrophotometer in range 200-400nm. As the Lopinavir spectra shows maximum absorption at 220 nm, but on that wavelength various solvents and excipients shows absorption therefore the zero order spectrum was converted into second derivative spectrum using scaling factor-10 and delta-λ was set 2 in UV probe software. The wavelength, at which maximum absorption (220 nm) for lopinavir shows, that wavelength was selected for further analysis. From the prepared standard stock solution suitable aliquots were pipetted out in 10ml volumetric flasks and volume was made up to 10ml with acetonitrile to get final concentration range from 5-30 µg/ml. Then absorbance was measured at λmax using acetonitrile as blank in UV-Visible Spectrophotometer (Shimadzu 1700) and calibration curve was plotted. The same procedure was repeated three times. The overlay spectra for different solution and Calibration curve of LPV in acetonitrile are shown in Figure 4.8 and 4.9 respectively.

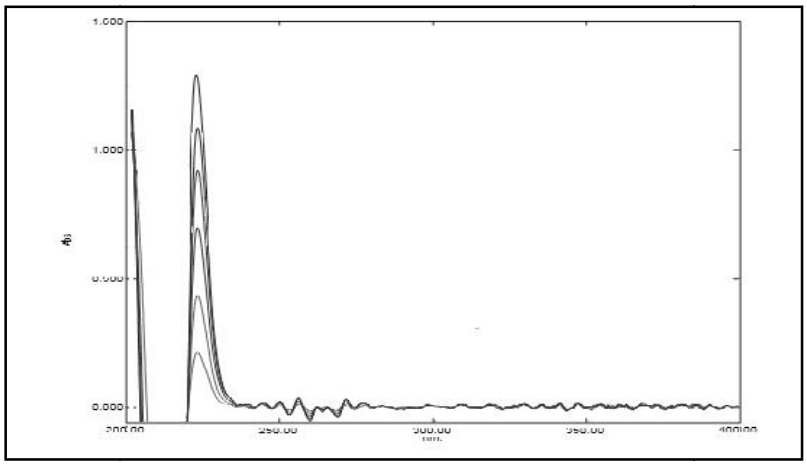

Figure 4.8 Overlay of UV-Spectra of LPV (5-30 µg/ml)

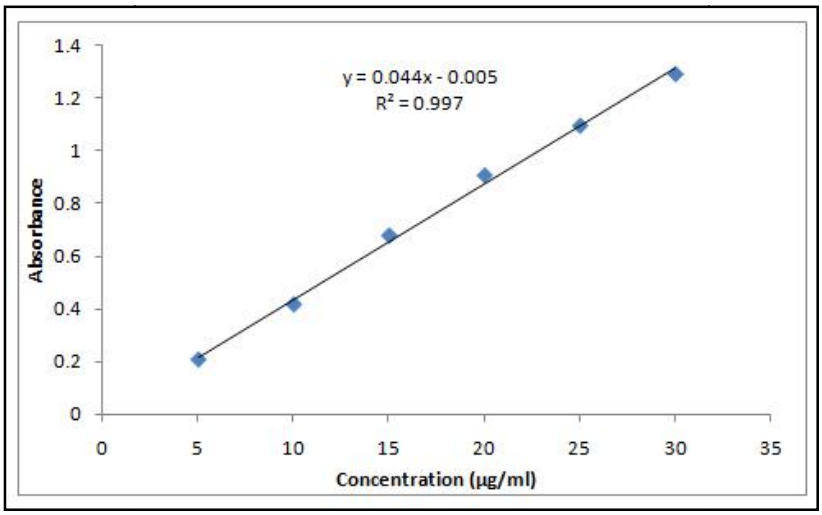

Figure 4.9 Calibration Curve of LPV (5-30 µg/ml)

The results show that the calibration curve for LPV in acetonitrile was linear in the concentration range of 5-30µg/ml. The correlation coefficients, slopes and intercepts were obtained by linear least square treatment of the results.

4.2.1.2 Analytical Method Validation

Linearity

A series of six different concentrations of LPV were prepared at 5-30 µg/ml and absorbances of samples were taken at 220 nm. The results shows proposed UV method obeys Beer's law in the concentration range of 5 to 30µg/ml. The linearity regression equation was Y=0.044x-0.005 with correlation coefficient (r^2) = 0.999 that indicate linearity of the plot. The overlay spectra of UV and Calibration curve of LPV in ACN are shown in Figure 4.8 and 4.9

Accuracy

Recovery studies on finished formulation were executed for confirming the accuracy of the developed analytical method. A fixed amount of pre-analyzed sample was taken and standard drug solution was added at 80%, 100% and 120% level.

Accurately weighted ten tablets of LPV were taken in mortar and triturated to make fine powder. Then equivalent quantity to 50 mg of LPV was taken and transferred in to beaker. 20ml of methanol was added and stirred slowly to dissolve LPV in ACN. The solution was filtered through Whatman filter paper no.42 and collected in 50ml volumetric flask. The filter paper was washed 2-3 times with 5 ml of ACN collecting the ACN fraction in 50 ml volumetric flask. The volume was made up to 50ml with ACN (1000µg/ml) and from this 5ml of aliquot was diluted with ACN to 50ml, to get concentration of 100µg/ml of LPV to be used as working solution for LPV. The same procedure was used for preparation of 1000µg/ml standard drug solution.

Then aliquots of 1ml were taken in 10 ml volumetric flask from the prepared formulation working solution and each of them was spiked with standard drug solution at three levels (80%, 100% and 120%) by addition of aliquots of 0.8, 1, 1.2 ml. The analysis was carried out in triplicate. The amount of LPV recovered was calculated by the equation obtained by calibration curve. The results of accuracy are showing in table 4.20

Table 4.20 Recovery study of LPV

% spiked	Actual Conc. of LPV (µg/ml)	Amount of LPV added (µg/ml)	Amount of LPV Recovered (µg/ml)	% recovered ± SD
80%	10	8	17.53	99.42 ± 0.99
100%	10	10	20.61	101.13 ± 1.20
120%	10	12	22.64	100.19 ± 0.39

*Mean value of three determinations.

Precision

The precision study of developed analytical method was confirmed by Interday and Intra-day study.

Intraday

Series of seven different concentrations were prepared at 5-30 µg/ml and absorbances of samples were taken at λmax. The experiment was repeated three times in a day and the % RSD was calculated for each concentration level.

Inter-day

In the interday precision experiment series of seven different concentrations were prepared at 5-30 µg/ml and absorbances of samples were taken at 220 nm and it was repeated on three different days. And the percentage RSD was calculated at each concentration level. The obtained results are given in Table 4.21

Table 4.21 Precision studies for estimation of LPV in ACN by UV spectroscopy

Conc. of Sample (µg/ml)	Intraday Precision (% RSD)	Interday Precision (%RSD)
5	1.308974	1.569204
10	1.148478	1.445288
15	0.83299	0.915099
20	0.43406	0.460277
25	0.546696	0.783662
30	0.451923	0.615929

. *Mean value of three determinations

Limit of detection (LOD) and limit of quantification (LOQ)

Calibration curve of LPV in ACN was repeated for three times and the standard deviation (SD) of the intercepts was calculated. Then Limit of detection (LOD) and limit of quantification (LOQ) were calculated as follows and results are given in table 4.22

LOD= 3.3 * S.D./slope of calibration curve

LOQ= 10 * S.D./slope of calibration curve

Where, S.D. = Standard deviation of intercepts.

Table 4.22 LOD and LOQ of LPV

Parameter	**LPV**
Limit of detection (LOD)	0.298 (µg/ml)
Limit of quantification (LOQ)	0.894 (µg/ml)

Table 4.23 Summarized data for the developed method of LPV

Parameter	**Results**
λ max (nm)	220
Beer's law limit (µg/ml)	5 -30
Regression equation (Y=mX+c)	0.044x - 0.005
Slope	0.044
Intercept	0.005
Limit of detection (µg/ml)	0.298
Limit of quantification (µg/ml)	0.894
Coefficient of determination	0.997
% RSD	< 2%
Accuracy	> 99%

4.2.2 HPLC method for Lopinavir

4.2.2.1 Materials and methods

LPV was gifted from Emcure Pharmaceuticals, Pune. Marketed tablet formulation, containing 200 mg of LPV was procured from commercial marketed source. Methanol and acetonitrile (both HPLC grade) were purchased from Rankem India. Double distilled water was prepared by double distillation assembly and prepared water filtered through 0.22µ Millipore filter paper (Millipore, India) and then used for the further. All the other reagents and solvents were of HPLC grade and were used without further purification.

The instruments used for the estimation of LPV include Digital weighing balance (Ax-120, Shimadzu, Japan) pH meter (Picco+, Lab India, India), High performance liquid chromatography with UV detector (HPLC Isocratic system with LC-20AT pump, Software-LC solution, Shimadzu).

4.2.2.2 Method of LPV by RP-HPLC

The RP-HPLC method for estimation of LPV was developed and validated as per ICH guideline. In this method mobile phase consisted of mixture of methanol, Acetonitrile and Ammonium Formate Buffer pH 4.5(40:40:20). The organic solvent and buffer were mixed thoroughly and sonicated for 15 min and filtered through 0.22 µ nylon filter.

Shimadzu isocratic HPLC with a UV-visible detector was used for HPLC analysis. The C18 (250 X 4.6 mm, 5 µm), Thermo column was used for the separation at ambient temperature. The injection volume was 20µl, the flow rate was 1 ml/min, run time was 10 min and detection wavelength was 210 nm. The RP-HPLC Chromatographic conditions are given in Table 4.24

Table 4.24 RP-HPLC Chromatographic conditions for LPV

Drug	Lopinavir
Column	Thermo
Mobile Phase	Methanol: Acetonitrile: Ammonium Formate Buffer pH 4.5(40:40:20)
Flow rate:	1 ml/ min
Detector	UV-Visible detector (SPD-20A)
Temperature	Ambient temperature
Detection Wavelength	210 nm

Preparation of standard stock solution of LPV

A stock solution of LPV was prepared by dissolving 10 mg of LPV in 10 ml of mobile phase. (1000µg/ml)

Calibration curve of LPV in mobile phase

From the above prepared stock solution suitable and accurate aliquots were transferred in 10ml calibrated volumetric flasks and diluted up to the volume with mobile phase to get the final concentrations range 10-50 µg/ml.

4.2.2.3 RP-HPLC Analytical Method Validation

Linearity

The calibration curve was found linear in the concentration range of 10 to 50µg/ml. The linearity regression equation was Y= 119.9x-22.45 with correlation coefficient (R2) = 0.999 that indicate linearity of the plot.(Fig 4.10 and 4.11)

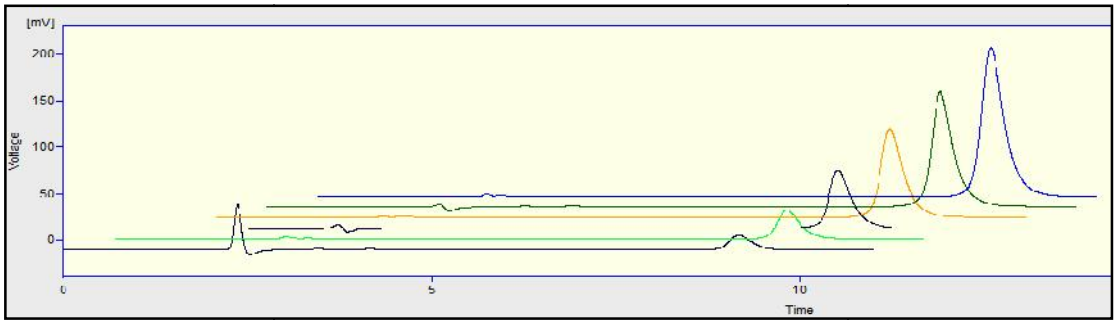

Figure 4.10 Chromatogram of Lopinavir

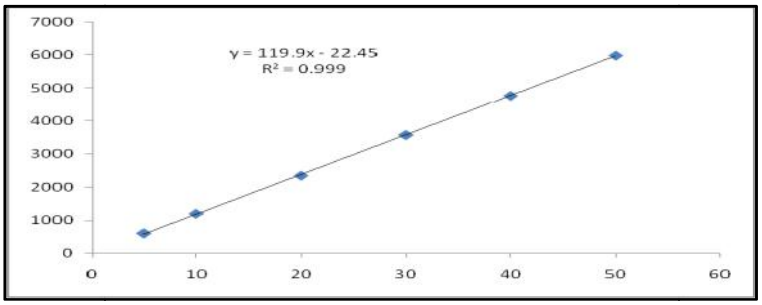

Figure 4.11 Calibration Curve of LPV in ACN: methanol: Buffer (40:40:20) by HPLC

Precision

Three different concentration solution of LPV were prepared with mobile phase. The experiment was repeated three times in a day and three different days for intraday and interday respectively. The result was calculated as % RSD and it was found within the limit. The results shows in tables 4.25 and 4.26

Table 4.25 Intraday-precision study

LPV concentration (µg/ml)	Peak Area			mean	SD	% RSD
7.5	878.56	876.23	879.45	878.23	1.015	0.115
25	2956.59	2957.58	2955.25	2956.47	1.677	0.056
45	5356.89	5354.22	5353.28	5354.91	2.605	0.048
Average						**0.073**

*Mean value of three determinations.

Table 4.26 Interday-precision study

LPV concentration (µg/ml)	Absorbance			mean	SD	% RSD
7.5	876.58	875.23	874.45	875.58	1.726	0.197
25	2955.12	2956.28	2957.69	2956.51	1.133	0.038
45	5352.96	5354.58	5354.12	5353.88	0.075	0.014
Average						**0.083**

*Mean value of three determinations.

LOD and LOQ

Calibration curve was repeated for 3 times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were calculated as follows and results are given in table 4.27

LOD = 3.3 * S.D./slope of calibration curve

LOQ = 10 * S.D./slope of calibration curve

Where, S.D. = Standard deviation of intercepts.

Table 4.27 LOD and LOQ of LPV by RP-HPLC method

Parameter	LPV
Limit of detection (LOD)	0.0171 (µg/ml)
Limit of quantification (LOQ)	0.0518 (µg/ml)

Recovery study

Accurately weighted five tablets of LPV were taken in mortar and triturated to make a fine powder. Then equivalent quantity of powder of 50 mg of LPV was taken and transfer in beaker. 20ml of mobile phase was added and stirred slowly for LPV to solubilise in mobile phase. The solution was filtered through Whatman filter paper no.42 and collected in 50ml volumetric flask, rinsing the filter paper 2-3 times with 5ml of mobile phase. All fraction were collected in the 50 ml volumetric flask and the volume was made up to 50ml with mobile phase (1000µg/ml). From this solution take 5ml of aliquot was taken and diluted with mobile phase up to 50ml, to get 100µg/ml of LPV and this was formulation working solution for LPV. The same procedure was used for preparation of 100µg/ml standard drug solution. Then aliquots of 1ml were taken in 10 ml volumetric flask from the prepared formulation working solution and each of them was spiked with standard drug solution at three levels (80%, 100% and 120%) by addition of aliquots of 0.8, 1, 1.2 ml. Then volume was made up to 10ml with mobile phase. The analysis was carried out in triplicate. The amount of LPV recovered was calculated by the equation obtained by calibration curve. The obtained results are given in table 4.28

Table 4.28 Recovery study of LPV

% spiking	Actual Conc. of LPV (µg/ml)	Amt. of LPV added (µg/ml)	Amt. of LPV Recovered (µg/ml)	% recovered ± SD
80	10	8	17.77	98.72 ± 1.71
100	10	10	19.99	99.97 ± 1.37
120	10	12	22.17	101.47 ± 1.55

*Mean value of three determinations.

Estimation of LPV in Formulation

Developed RP-HPLC method was successfully applied for the estimation of LPV in marketed finished formulation. Aliquots of 1ml were taken in 10 ml volumetric flask from the above prepared formulation working solution (100µg/ml) and made-up the volume up to 10 ml with mobile phase. Six such solutions were prepared and inject in HPLC. Their peak area was measured at 210nm wavelength and % assay was measured. The results are shown in tables 4.29

Table 4.29 Analysis of marketed formulation

Concentration of Sample (µg/ml)	Concentration obtained (µg/ml)	% of label claimed
10	9.96	99.62%

*Mean value of six determinations

Table 4.30 RP-HPLC Chromatographic conditions for LPV

Parameters	LPV
Detection Wavelength	210nm
Linearity Range	5-50µg/ml
Accuracy	100.85
Intraday precision (% RSD)	0.073
Interday Precision (% RSD)	0.083
Regression Equation	Y=119.9x-22.45
LOD	0.0171 (µg/ml)
LOQ	0.0518 (µg/ml)

4.2.3 HPLC Method for Estimation of LPV in Plasma

Simple bio analytical RP-HPLC method used for the determination and estimation of LPV using nelfinavir as an internal standard (IS) was developed and validated as per regulatory requirements. The protein precipitation technique was used for the sample preparation and chromatographic separation on a reverse phase Kromasil C18 column. The above developed RP- HPLC method was used for analysis of LPV in plasma samples. The mobile phase consisted of mixture of Methanol Acetonitrile and ammonium formate buffer pH 4.5 (40:40:20) at a flow rate of 1 ml/min. The wavelength used for the detection of LPV was 210 nm with a total run time of 13

minutes. The retention times of LPV and IS were found to be 9.2 and 11.2 minutes respectively. The method was developed and tested for the linearity range of 100-10000ng/ml. The method was validated for accuracy, precision, linearity, recovery and stability in compliance to international regulatory guidelines.

4.2.3.1 Application of developed RP-HPLC method for Bio analytical study

Primary and secondary stock solution

To prepare primary stock solution 100 mg of LPV was transferred into 100 ml of volumetric flask. Add 50 ml acetonitrile and sonicated for about 5 min and volume made upto 10ml with methanol (1000µg/ml). The secondary stock solution was prepared from primary stock solution, 0.05, 0.15, 0.3, 0.4, 0.5, 1, 2, 3 and 5 ml of primary stock solution was transferred into 10mL volumetric flask and volume made upto 10ml with Acetonitrile: water (50:50) that prepare 5, 15, 30, 40, 50, 100, 200, 300 and 500µg/ml solution.

Plasma sample preparation

0.1 ml of from secondary stock solution of each concentration and 0.05 ml of nelfinavir as IS was added in 2 ml polypropylene centrifuge tube. Add 0.85 ml of drug-free human plasma. Mixed the plasma and drug solution thoroughly for 5 minutes on vortex shaker.

Sample extraction Procedure

Protein precipitation technique was used for drug extraction from plasma sample. From each centrifuge tube 0.1 ml of drug contained plasma was taken in 1ml centrifuge tube and added 0.4 ml of acetonitrile was added to precipitate plasma proteins. Mix thoroughly for 5 minutes on vortex shaker. Then Centrifuge tubes were centrifuged at 10000 RPM for 10 min in high speed homogenizer (Sigma Hi Speed Homogenizer, Japan). Supernatant from each tube was separated using micropipettes and 20 µl solutions were injected into HPLC system.

4.2.3.2 Bio analytical Method Validation

Bio analytical method was validated as per ICH guideline and its procedure was follow as per mention in bio analytical method validation for ritonavir.

4.2.3.3 Results and Discussion:

System Suitability:

The % RSD of system suitability was observed in the range of **0.00 to 0.980%** for retention time of drug, **0.00 to 1.20%** for retention time of ISTD and area ratio in the

range of **1.0 to 1.5%** which is not more than 5.00% as per the acceptance criteria. The results are shown in table 4.31 and 4.32.

Table 4.31 System Suitability Parameters

Sample	Area ratio	Analyte RT	IS RT
MQC-1	0.620	9.2	11.4
MQC-2	0.616	9.3	11.2
MQC-3	0.622	9.2	11.3
MQC-4	0.611	9.3	11.2
MQC-5	0.659	9.4	11.3
MQC-6	0.599	9.3	11.2
MEAN	0.621	9.28	11.26
SD	0.020	0.075	0.081
% RSD	1.283	0.810	0.723

Table 4.32 System Suitability Parameters

Day	% RSD		
	Analyte RT	IS RT	Area ratio
Day 1	0.810	0.723	1.283
Day 2	0.884	0.673	1.430
Day 3	0.973	1.126	1.231

Calibration Model

Calibration curves were found to be consistently accurate and precise for LPV over 100-10000 ng/ml. The correlation coefficient was greater than or equal to 0.990. LPV concentration at each calibration level was back calculated from the calibration curves. The results obtained are shown in Table 4.33 and were meeting the acceptance criteria of $r_2 \geq 0.98$. Fig. 4.12 describes the chromatogram of blank human plasma, also the Fig. 4.13 and Fig. 4.14 describes the overlay chromatograms of LPV and IS in plasma and calibration curve of LPV in plasma respectively.

Table 4.33 Linearity of LPV

Conc.(ng/ml)	Back Calculated Conc.(ng/ml)			Mean	% RSD
100	98.42742	91.33065	102.0565	97.27151	5.608562
300	300.7661	294.1532	317.2984	304.0726	3.920637
600	582.8629	595.2016	575.4435	584.5027	1.707533
800	751.4919	777.7016	762.4597	763.8844	1.72314
1000	997.8629	1011.734	1021.411	1010.336	1.171517
2000	1949.395	1965.524	1981.653	1965.524	0.820597
4000	3753.669	3726.976	3766.25	3748.965	0.534955
6000	5767.137	5811.734	5832.54	5803.804	0.575743
10000	9688.105	9713.185	9743.831	9715.04	0.287278
Slope	0.119	0.120	0.120		
Intercept	0.573	0.039	0.422		
R^2	0.998	0.997	0.998		

Figure 4.12 Chromatogram of Blank Human Plasma

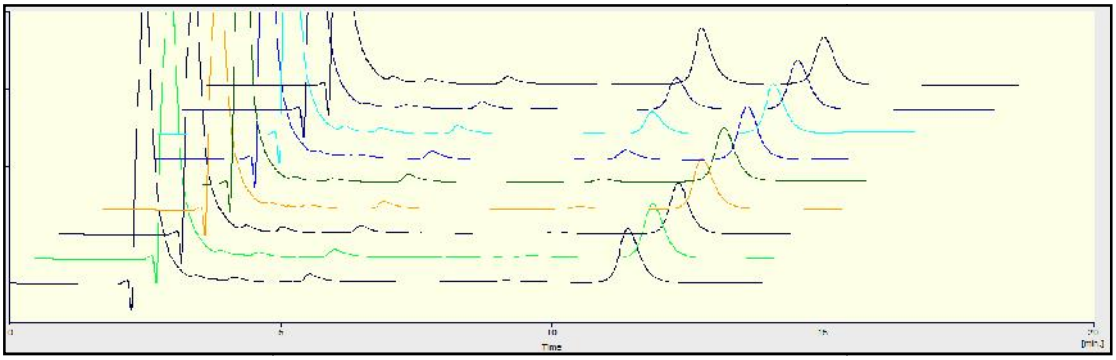

Figure 4.13 Chromatogram of LPV in Plasma sample with IS

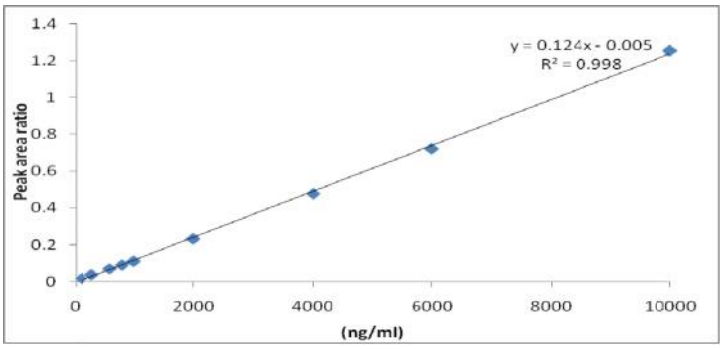

Figure 4.14 Calibration curve of LPV in human plasma

Precision and Accuracy

Inter batch (between-batch) precision and accuracy

The between batch % coefficients of variation for LPV was ranged between 1% to 7% and % accuracy was ranged between 94% to 101%.The results are within ± 15%, the results are presented in table 4.34

Table 4.34 Inter Day Precision and Accuracy of LPV

	LLOQ	**LQC**	**MQC**	**HQC**
Conc.	**100**	**500**	**5000**	**8000**
Estimated conc.	103.3468	470.3629	4783.669	7705.524
	98.91129	476.4919	4785.927	7693.669
	102.0565	499.7177	4700.766	7787.218
	85.44355	490.7661	4766.653	7769.718
	92.8629	502.7823	4705.363	7685.363
	98.18548	480.0403	4781.25	7804.153
Mean	96.80108	486.6935	4753.938	7740.941

SD	6.653381	13.11557	40.00422	52.0448
RSD	6.873251	2.694831	0.841496	0.672332
% accuracy	96.80108	97.33871	95.07876	96.76176

Intra batch (within batch) precision and accuracy:

The within batch % coefficient of variation for LPV was ranged between 1% to 11% and % accuracy was ranged between 98% to 106%.The results are within ± 15% the results are presented in table 4.35

Table 4.35 Intra Day Precision and Accuracy of LPV

	LLOQ	**LQC**	**MQC**	**HQC**
Conc.	**100**	**500**	**5000**	**8000**
Estimated Conc.	100.84	472.13	4939.15	7883.589
	92.54	510.84	4906.49	7791.492
	96.73	484.87	4772.13	7870.121
	100.68	491.65	4706.00	7741.089
	104.63	492.37	4862.46	7770.282
	100.60	504.23	4958.75	7855.847
MEAN	99.34	492.68	4857.5	7818.737
SD	4.16	13.766	99.57	58.89415
RSD	4.19	2.79	2.05	0.753244
% accuracy	99.34	98.53	97.15	97.73

Absolute Recovery
Recovery of analyte

Recovery of analyte were evaluated by injecting three replicates of the aqueous QC samples at each LQC, MQC and HQC concentrations and three replicates of the extracted QC samples at each LQC, MQC and HQC concentrations. Results are presented in table 4.36

Table 4.36 Absolute Recovery of LPV

	LQC		**MQC**		**HQC**	
Sr. No	**Extracted peak Area**	**Unextracted peak area**	**Extracted peak area**	**Unextracted peak Area**	**Extracted peak Area**	**Unextracted peak area**
1	62.13	68.34	612.47	658.34	976.28	1044.23
2	65.41	66.95	634.23	655.45	948.34	1020.32
3	59.82	65.45	649.41	665.32	958.21	1028.43
Mean	62.18	67.01	631.98	659.58	961.01	1030.66
SD	2.94	1.57	18.44	5.16	14.11	1.51
% RSD	4.74	2.35	2.91	0.78	1.46	0.14
% Recovery	92.81%		95.81		93.24	

Recovery of Internal Standard

Recovery of IS were evaluated by injecting three replicates of the aqueous QC samples of HQC concentrations and three replicates of the extracted QC samples of HQC concentrations. Results are presented in table 4.37

Table 4.37 Absolute Recovery of IS

HQC	
Extracted peak area	**Unextracted peak area**
1009.83	1075.52
1003.34	1060.42
998.65	1080.33
1003.94	1051.84
5.61	10.42
0.55	0.97
93.66	

Bench Top Stability

The bench top stability of LPV at room temperature was examined by keeping three sets of LQC and HQC at room temperature for 12 hours. The % mean stability for LQC and HQC of LPV concentration was found to be 97.81 and 98.99% respectively. The results are within the acceptable limit, Acceptance criteria is at least 67% QC samples should pass acceptance limit of 85.00–115.00 % and not more than 50% at each QC level should fail and it is presented in table 4.38

Freeze-Thaw Stability

Freeze thaw stability in plasma was assessed by analyzing three replicates of LQC and HQC samples previously frozen at -70 ± 5 º C and thawed at room temperature over three cycles. The % mean stability for LQC and HQC of LPV concentration was found to be 97.04 and 99.66% respectively. The results are within the acceptable limit, Acceptance criteria is at least 67% QC samples should pass acceptance limit of 85.00–115.00 % and not more than 50% at each QC level should fail and it is presented in table 4.38

Long Term Stability

Long term stability of the spiked quality control samples was determined after stored at -70°C for 14 days. Stability was assessed by comparing them against the freshly spiked calibration standards. The % mean stability for LQC & HQC was found to be 96.72 & 99.36%, for LPV, which is within the acceptance limit. Acceptance criteria

is at least 67% QC samples should pass acceptance limit of 85–115% and not more than 50% at each QC level should fail. Results are summarized in table 4.38 for LPV

Table 4.38 Stability Data of LPV

QC samples LPV	Mean conc. Observed (ng/ml)	at 0 h (ng/ml)	Mean conc. Observed (%)
Bench top stability (n=6) (after 6 h)			
LQC (500ng/mL)	476.21	486.65	97.81
HQC (8000ng/mL)	7916.74	7995.34	98.99
Freeze-thaw cycle (3 cycle) (n=6)			
LQC (500ng/mL)	479.00	493.58	97.04
HQC (8000ng/mL)	7970.25	7996.81	99.66
Long term stability (after 20 days) (n=6)			
LQC (500ng/mL)	482.08	498.42	96.72
HQC (8000ng/mL)	7938.27	7988.75	99.36

4.2.4 Conclusion

A simple, sensitive and precise RP-HPLC method has been developed for the quantification of LPV from human plasma using protein precipitation method. The method shows good linearity over the concentration range 100-10000 ng/ml. Selectivity and sensitivity of method were sufficient for detecting and quantifying LPV in human plasma. Along with these features it gives high recovery and fast and simple sample preparation technique make this method suitable for application in clinical and preclinical pharmacokinetic, pharmacodynamics and metabolite study.

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