3.1 Introduction

A UV-Visible spectrophotometric method in solvent mixture methanol and dichloromethane (DCM) was developed for determination of Darunavir and Atazanavir sulfate loaded in nanoparticles. 7:3 ratio of methanol: dichloromethane was selected among different ratio since in this proportion, DCM was sufficient to allow complete solubility of the lipid content and not to allow re-precipitation of the lipid during dilution. Dichloromethane was selected since the solid lipids used during development of nanoparticles are soluble in this solvent and it forms a homogenous phase with the methanol, the other solvent. The interference of the various solid lipids and sodium oleate (as surfactant) was checked using UV interference study for both the drugs individually. For determination of drug during *in-vitro* drug release study, UV methods were developed for Darunavir and Atazanavir sulfate in simulated gastric fluid (SGF): methanol (1:0.5) and simulated intestinal fluid (SIF): methanol (1:0.5). HPLC and LCMS method (in plasma and spleen) were developed for Darunavir determination during cell line studies (internalization pathways and intestinal permeability) and *in-vivo* studies respectively. HPLC methods were developed for determination of Atazanavir sulfate during cell line studies, pharmacokinetics and organ distribution studies.

3.2 Materials

Darunavir and Atazanavir sulfate were kindly gifted by Matrix laboratories, Hyderabad. Glyceryl behenate (Compritol® 888 ATO) and Glyceryl palmitostearate (Precirol® ATO 5) were gifted by Gattefosse (Germany). Trimyristin (Dynasan® 114) and Tripalmitin (Dynasan® 116) were gifted by Sasol Germany GmbH (Hamburg, Germany). Hydrogenated castor oil (HCO) was obtained as gift sample from Meril Life Sciences Pvt. Ltd. (Gujarat, India). Dichloromethane and methanol were purchased from spectrochem (Mumbai, India). Sodium hydroxide, hydrochloric acid, oleic acid, sodium chloride, sodium lauryl sulphate, monobasic potassium phosphate and dimethyl sulphoxide (DMSO) were purchased from S.D.Fine chem Ltd. (Mumbai, India). Acetonitrile (HPLC grade) was purchased from Spectrochem, India. Pepsin (800-2500

units per mg protein) and Pancreatin were purchased from HiMedia, India. Double distilled water was prepared in laboratory.

3.3 Methods for Darunavir estimation

3.3.1 UV spectroscopic method of Darunavir in methanol: DCM (7:3)

3.3.1.1 Preparation of calibration plot

Methanol: DCM mixture was prepared in ratio of 7:3. 50 mg of drug (Darunavir) was weighed accurately, dissolved in 20-30 ml of solvent mixture and stirred gently through sphinx. The final volume was made up to 50 ml with the same solvent mixture to give a stock solution-A of 1000 µg/ml. From this solution, 1 ml was pipetted out accurately in a 10 ml volumetric flask and final volume was made up to mark to give stock solution-B of 100 µg/ml. Appropriate aliquots of the stock solution-B were diluted to get the solutions of concentration ranging from 3-21 µg/ml. The absorbance maxima (λ_{max}) was determined by scanning the solution between 200-400 nm using UV-spectrophotometer. The prepared solutions were analyzed by the UV spectrophotometer (Schimadzu 1700) at the $\lambda_{max.}$ All the measurements were made in triplicate.

3.3.1.2 Analytical method validation

The method was validated for linearity, precision, accuracy, limit of detection and limit of quantitation.

Linearity: The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations (quantities) of the analyte in the sample (1). Linearity of a light absorption determination should be examined to ensure that Beer's law operates over the range of interest. For evaluation of the linearity, the mean absorbance was plotted against concentration to get a calibration plot. Least square regression method was used to determine the regression coefficient, r^2 and the equation for the best fitting line. The method can said to be linear if r^2 is near to 1.

Precision: It refers to the extent of variability of a group of measurements observed under similar conditions. Precision provides an indication of random errors and is

generally subdivided into two cases: repeatability and reproducibility, which were determined by calculating RSD (Relative standard deviation) of inter-day and intra-day determinations. The inter-day and intra-day precisions were measured in terms of % Relative Standard Deviation (% RSD). The 3 concentration were selected and experiment was repeated 3 times in a day for intra-day and on 3 different days for inter-day precision. Criteria: % RSD ≤ 2 % as per the requirements of ICH guidelines (2, 3).

Accuracy: Accuracy refers to the closeness of an individual observation or mean of the observations to true value (4). Accuracy was performed by standard addition method at three levels: 80%, 100% and 120% of working concentration (three replicates for each concentration) into a standard solution and by calculating the percent recovery of active ingredient from the placebo solution. The percent recovery was calculated for nine determinations.

Limit of detection and limit of quantitation: Calibration plot was repeated for 3 times and the standard deviation (SD) of the intercepts was calculated. The average of the slope of calibration plot was calculated. Then LOD and LOQ were measured as follows:

$$LOD = 3.3 * \left(\frac{\sigma}{S}\right)$$

$$LOQ = 10 * \left(\frac{\sigma}{S}\right)$$

Where, σ = Standard deviation of intercepts

S = Slope of calibration plot

3.3.2 Analytical interference study for Darunavir

To study interference of the formulation excipients in the estimation of the Darunavir, 10 µg/ml solution of Darunavir was prepared in solvent mixture (7:3 ratio of methanol: DCM) and scanned in UV spectrophotometer. Other solutions containing 10 µg/ml Darunavir and each solid lipid (100 µg/ml) or sodium oleate (100 µl) were scanned in UV spectrophotometer to detect any change in the spectra due to presence of excipient.

3.3.3 UV spectroscopic method of Darunavir in simulated gastric fluid: methanol (1:0.5)

3.3.3.1 Preparation of simulated gastric fluid (SGF), pH 1.2 with 0.5 %w/v SLS (5)

2 g sodium chloride and 3.2 g pepsin were accurately weighed and dissolved in 500 ml distilled water. 7 ml hydrochloric acid and 5 g sodium lauryl sulphate was added to it. Final volume was made up to 1000 ml with distilled water. 0.5 % w/v sodium lauryl sulphate (SLS) was added to SGF to maintain sink conditions.

3.3.3.2 Preparation of calibration plot

Calibration plot of Darunavir in SGF: methanol (1:0.5) was prepared in the concentration range of 3-21 µg/ml. The stock solution of 50µg/ml was prepared by dissolving drug in solvent mixture. Appropriate aliquots of the stock solution were diluted to get the solutions of concentration ranging from 3-21µg/ml. The absorbance maxima (λ_{max}) was determined. The prepared solutions were analyzed by the UV spectrophotometer (Schimadzu 1700) at the $\lambda_{max.}$ All the measurements were made in triplicate. Analytical method was validated for different parameters as described in section 3.3.1.2.

3.3.4 UV spectroscopic method of Darunavir in simulated intestinal fluid: methanol (1:0.5)

3.3.4.1 Preparation of simulated intestinal fluid (SIF), pH 7.4 (5)

6.8 g monobasic potassium phosphate was dissolved in 250 ml distilled water. 77 ml 0.2 N sodium hydroxide was added to 500 ml distilled water. Both these solutions were mixed and 10 g pancreatin as well as 5 g sodium lauryl sulphate was added in to it. pH was adjusted to 6.8 with 0.2 N NaOH or 0.2 N HCl. The final volume was made up to 1000 ml. 0.5 % w/v sodium lauryl sulphate was added to SIF to maintain sink conditions.

3.3.4.2 Preparation of calibration plot

Calibration plot of Darunavir in SIF: methanol (1:0.5) was prepared in the concentration range of 5-30 µg/ml**.** The stock solution of 100 µg/ml was prepared by dissolving drug in solvent mixture. Appropriate aliquots of the stock solution were diluted to get the solutions of concentration ranging from 5-30 µg/ml. The absorbance maxima (λ_{max}) was determined. The prepared solutions were analyzed by the UV spectrophotometer (Schimadzu 1700) at the $\lambda_{max.}$ All the measurements were made in triplicate. Analytical method was validated for different parameters as described in section 3.3.1.2.

3.3.5 HPLC method development of Darunavir

3.3.5.1 Sample preparation

25 mg of Darunavir was accurately weighed and dissolved in 1 ml DMSO and final volume was made to 25 ml with the solvent mixture acetonitrile: water (50:50) to get concentration of 1000 µg/ml (stock solution). From this solution, 1ml was pippeted out and diluted to 100 ml using solvent mixture to get stock solution-B having concentration 10 µg/ml. Appropriate aliquots of the stock solution-B were diluted with the solvent mixture to get concentrations 2, 4, 6 and 8 µg/ml.

3.3.5.2 HPLC analysis

Shimadzu isocratic HPLC with a UV-visible detector was used for HPLC analysis. The separation was done on SUPELCO C_{18} column (5µm particle size, 4.6 x 250 mm internal diameter; sigma aldrich) with a C_{18} guard column: under the following conditions: mobile phase- acetonitrile: water (50:50) with pH adjusted to 3 using formic acid, flow rate 1ml/min. The mobile phase was degassed using bath sonicator before use. The detection wavelength was 267 nm. The column temperature of 40ºC was maintained using column heater. The required parameters were programmed using software (Spinchrom). 20 µl of the prepared solutions (2, 4, 6, 8 and 10 µg/ml) were injected and the area was obtained. All the measurements were made in triplicate. Analytical method was validated for different parameters as described in section 3.3.1.2.

3.3.6 LCMS method for determination of Darunavir in plasma

During *in-vivo* study, the concentration of drug in the biological samples is very low. Hence, LCMS method of Darunavir estimation was developed, which is more sensitive method than the developed HPLC method.

3.3.6.1 Preparation of solutions

The blank plasma samples (obtained from Suraktam blood bank, vadodara) were spiked with the stock solution of Darunavir (2 µg/ml) prepared in acetonitrile to get concentrations in range of 25-1000 ng/ml. Chilled acetonitrile was added into these sample to precipitate protein (ratio of plasma: acetonitrile was taken as 1:1). The samples were centrifuged at 3000 rpm, 5 min to separate the precipitates. Supernatant was taken and used for analysis.

3.3.6.2 LCMS Analysis

The chromatographic system consisted of an Ekspert ultraLC 100 HPLC system (Eksigent- AB sciex, USA) with SUPLECO C_{18} column (5 µm particle size, 4.6 x 250 mm internal diameter; sigma aldrich) and C_{18} guard column. The temperature of column was set at 40ºC. The mobile phase consisted of acetonitrile: water (60:40) at the flow rate of 1ml/min. LC/MS measurements were achieved using 3200 QTRAP mass spectrometer (AB sciex, USA) equipped with an electrospray ionization (ESI) source. Samples for analysis were fed into 1.5 ml PTFE vials (9 mm septa) and put in autosampler. Injection volume was 20 µl and sample run time was 15 min. Data were acquired using Analyst 1.6.2 software (Applied biosystems).

3.3.7 LCMS method for determination of Darunavir in spleen

The spleen homogenate samples (organ concentration 10% w/v in double distilled water, centrifuged at 5000rpm, 15min) were spiked with the stock solution of Darunavir (2 µg/ml) prepared in acetonitrile to get concentrations in range of 25-1000 ng/ml. Protein precipitation was carried out using acetonitrile and the precipitates were removed

by centrifugation at 3000 rpm, 5 min. Supernatant was taken and used for LCMS analysis as per procedure and parameters mentioned in section 3.3.6.2.

3.4 Methods for Atazanavir sulfate estimation

3.4.1 UV spectroscopic method of Atazanavir sulfate (ATZ) in methanol: DCM (7:3)

Calibration plot of Atazanavir sulfate (ATZ) in methanol: DCM (7:3) was prepared in the concentration range of 10-60 µg/ml. The stock solution-A of 1mg/ml was prepared by dissolving ATZ in solvent mixture. From this solution, 1ml was pippeted out and diluted to 10 ml using solvent mixture to get stock solution-B having concentration 100 µg/ml. Appropriate aliquots of the stock solution-B were diluted to get the solutions of concentration ranging from 10-60 µg/ml. The absorbance maxima (λ_{max}) was determined. The prepared solutions were analyzed by the UV spectrophotometer (Schimadzu 1700) at the $\lambda_{max.}$ All the measurements were made in triplicate. Analytical method was validated for different parameters as described in section 3.3.1.2.

3.4.2 Analytical interference study for Atazanavir sulfate

To study interference of the formulation excipients in the estimation of the ATZ, 50 µg/ml solution of ATZ was prepared in solvent mixture (7:3 ratio of methanol: DCM) and scanned in UV spectrophotometer. Other solutions containing 50 µg/ml ATZ and each solid lipid (500 µg/ml) or sodium oleate (200 µl) were scanned in UV spectrophotometer to detect any change in the spectra due to presence of excipient.

3.4.3 UV spectroscopic method of ATZ in simulated gastric fluid: methanol (1:0.5)

Simulated gastric fluid containing SLS was prepared as per procedure mentioned in section 3.3.3.1. Calibration plot of Atazanavir sulfate (ATZ) in SGF: methanol (1:0.5) was prepared in the concentration range of 10-50 µg/ml. 50 mg ATZ was dissolved in 10 ml solvent mixture in volumetric flask and final volume made up to 50ml to get concentration of 1 mg/ml as stock solution. From this solution, 2.5ml was pippeted out and diluted to 25 ml using solvent mixture to get stock solution-B having concentration 100 µg/ml. Appropriate aliquots of the stock solution-B were diluted to get the solutions

of concentration ranging from 10-50 µg/ml. The absorbance maxima (λ_{max}) was determined. The prepared solutions were analyzed by the UV spectrophotometer (Schimadzu 1700) at the $\lambda_{max.}$ All the measurements were made in triplicate. Analytical method was validated for linearity, precision, and accuracy as described in section 3.3.1.2.

3.4.4 UV spectroscopic method of ATZ in simulated intestinal fluid: methanol (1:0.5)

Simulated intestinal fluid containing SLS was prepared as per procedure mentioned in section 3.3.4.1. Calibration plot of Atazanavir sulfate (ATZ) in SIF: methanol (1:0.5) was prepared in the concentration range of 10-50 µg/ml. 50 mg ATZ was dissolved in 10 ml solvent mixture in volumetric flask and final volume made up to 50ml to get concentration of 1 mg/ml as stock solution. From this solution, 2.5ml was pippeted out and diluted to 25 ml using solvent mixture to get stock solution-B having concentration 100 µg/ml. Appropriate aliquots of the stock solution-B were diluted to get the solutions of concentration ranging from 10-50 µg/ml. The absorbance maxima (λ_{max}) was determined. The prepared solutions were analyzed by the UV spectrophotometer (Schimadzu 1700) at the $\lambda_{max.}$ All the measurements were made in triplicate. Analytical method was validated for linearity, precision, and accuracy as described in section 3.3.1.2.

3.4.5 HPLC method development of Atazanavir sulfate

3.4.5.1 Sample preparation

1 mg of ATZ was accurately weighed and dissolved in sufficient methanol in a 100 ml volumetric flask. The final volume was made up to mark with methanol to obtain stock solution of 10 µg/ml (stock solution). From this solution, 10 ml was pippeted out and diluted to 100 ml using mobile phase to get stock solution-B having concentration 1000 ng/ml. Appropriate aliquots of the stock solution-B were diluted with the mobile phase to get concentrations of 50, 100, 150 and 250 ng/ml.

3.4.5.2 HPLC analysis

The calibration plot of Atazanavir sulfate by HPLC was developed after modification of a reported method (6). HPLC system, column and guard column were used as mentioned in section 3.3.5.2. Mobile phase consisted of 45 % water, 20 % methanol and 35 % acetonitrile with pH adjusted to 3.55 using acetic acid. Flow rate was kept as 0.5 ml/min. The mobile phase was degassed using bath sonicator before use. Run time was kept as 10 min. The detection wavelength was 249 nm. 20 µl of the prepared solutions were injected and the area was obtained. All the measurements were done in triplicate. Analytical method was validated for different parameters as described in section 3.3.1.2.

3.4.6 HPLC method for determination of Atazanavir sulfate in plasma

The blank plasma samples (obtained from Suraktam blood bank, vadodara) were spiked with the stock solution of Atazanavir sulfate (10 µg/ml) prepared in acetonitrile to get concentrations in range of 50-1000 ng/ml. Chilled acetonitrile was added into these sample to precipitate protein (ratio of plasma: acetonitrile was taken as 1:1). The samples were centrifuged at 3000 rpm, 5 min to separate the precipitates. Supernatant was taken and used for analysis. All procedures for analysis were followed as mentioned in section 3.4.5.2. Analytical method was validated for different parameters as described in section 3.3.1.2.

Mobile phase: 45 % water, 20 % methanol and 35 % acetonitrile with pH adjusted to 3.55 using acetic acid; Flow rate: 0.5 ml/min, run time: 10 min, detection wavelength: 249 nm.

3.4.7 HPLC method for determination of Atazanavir sulfate in spleen

The spleen homogenate samples (organ concentration 10% w/v in double distilled water, centrifuged at 5000rpm, 15min) were spiked with the stock solution of Atazanavir sulfate (10 µg/ml) prepared in acetonitrile to get concentrations in range of 50-250 ng/ml. Protein precipitation was carried out using acetonitrile and the precipitates were removed by centrifugation at 3000 rpm, 5 min. Supernatant was taken and used for analysis. All

procedures for analysis were followed as mentioned in section 3.4.5.2. Analytical method was validated for different parameters as described in section 3.3.1.2.

Mobile phase: 45 % water, 20 % methanol and 35 % acetonitrile with pH adjusted to 3.55 using acetic acid; Flow rate: 0.5 ml/min, run time: 10 min, detection wavelength: 249 nm.

3.5 Results and discussion for methods of estimation of Darunavir

3.5.1 UV spectroscopic method of Darunavir in methanol: DCM (7:3)

Darunavir in solvent mixture of methanol and DCM showed a characteristic spectrum when scanned in the ultraviolet range between 200 and 400 nm. The scan showed absorption maximum at 267 nm (Figure 3.1) and this wavelength was chosen as the analytical wavelength. Beer's law was obeyed between 3 and 21 µg/ml (Table 3.1). The UV spectrum for Darunavir is shown in Figure 3.2. Regression analysis was performed on the experimental data. Regression equation for standard plot was y =0.038x- 0.0009 (Figure 3.3). Correlation coefficient was found to be 0.9999 signifying that a linear relationship existed between absorbance and concentration of the drug.

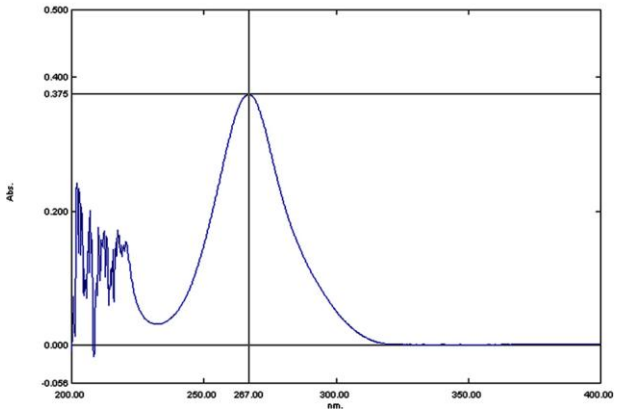

Figure 3.1 UV spectrum of Darunavir (10 ppm)

Table 3.1 Data for calibration plot of Darunavir in Methanol: DCM (7:3)

Sr. No.	Concentration (µg/ml)	Absorbance ± SD
1	3	0.111 ± 0.002
2	6	0.230 ± 0.001
3	9	0.342 ± 0.004

4	12	0.455 ± 0.002
5	15	0.568 ± 0.003
6	18	0.678 ± 0.001
7	21	0.803 ± 0.004

*n=3

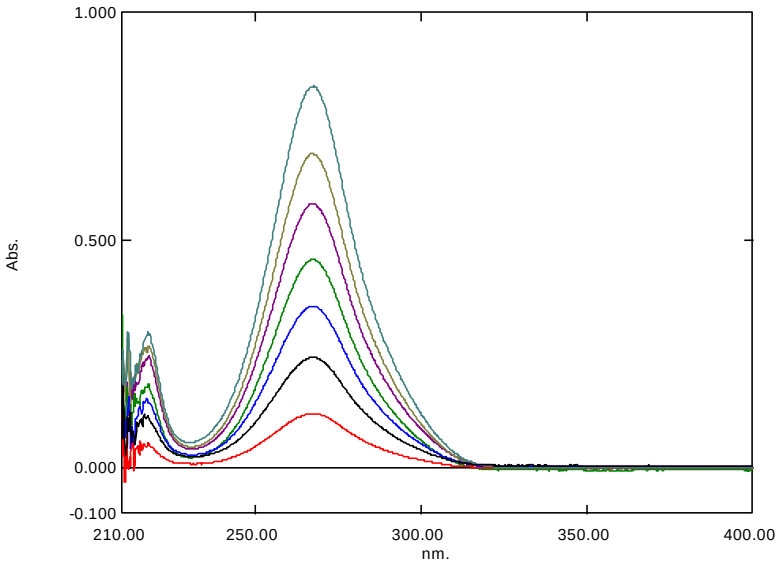

Figure 3.2 Overlay Spectra of Darunavir

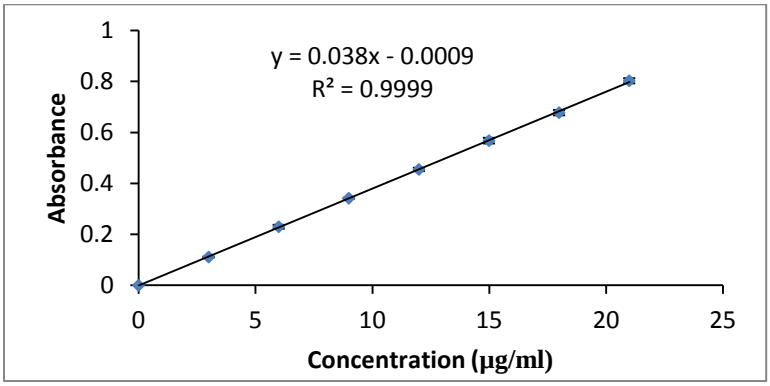

Figure 3.3 Calibration plot of Darunavir in Methanol: DCM (7:3) at 267 nm

Precision: The mean absorbances (n=3) observed intra-day and inter-day are given in following Table 3.2. The average % RSD of intra-day and inter-day measurements for determination of Darunavir were 1.472 % and 1.165 % respectively at 267 nm. The values confirm the precision of the method.

Table 3.2 Intra-day and inter-day precision for determination of Darunavir in methanol: DCM (7:3)

Sr. No.	Darunavir (µg/ml)	Mean absorbance values (n=3)				% RSD
		1	2	3	Average	
Intra-day precision						
1	3	0.111	0.115	0.112	0.112	1.847
2	12	0.448	0.455	0.461	0.454	1.431
3	21	0.815	0.797	0.803	0.805	1.138
Average % RSD						1.472
Sr. No.	**Darunavir (µg/ml)**	**Mean absorbance values (n=3)**				**% RSD**
		Day 1	**Day 2**	**Day 3**	**Average**	
Inter-day precision						
4	3	0.112	0.116	0.114	0.114	1.754
5	12	0.461	0.451	0.457	0.456	1.102
6	21	0.803	0.800	0.810	0.804	0.637
Average % RSD						1.165

Accuracy: Accuracy of the method was confirmed by recovery study from prepared laboratory sample at three level of standard addition (80%, 100% and 120%) of 10µg/ml. The results are given in Table 3.3. Recovery greater than 98% with low relative standard deviation (RSD) justified the accuracy of the method. The **LOD** and **LOQ** obtained were 0.133 and 0.404 µg/ml. The final parameters are listed in Table 3.4.

Table 3.3 Determination of accuracy of the method

Sr. No.	Concentration level	Theoretical content (µg/ml)	Mean amount recovered (µg/ml)	% Recovery ± RSD
1	80 %	18	17.821	99.00±1.27
2	100 %	20	19.632	98.15±2.26
3	120 %	22	21.729	98.76±1.38

*n=3

Table 3.4 Optical characteristics of Darunavir in methanol: DCM (7:3)

Sr. No.	Parameter	Optimized parameter
1	Solvent	Methanol: DCM (7:3)
2	Scanning range and speed	200 - 400 nm, Fast speed
3	λ_{max}	267 nm
3	Beer's range	3-21 µg/ml
4	LOD	0.133 µg/ml
5	LOQ	0.404 µg/ml
6	Molar absorptivity	18.3 x 10^3 lit/mole/cm

3.5.2 Analytical interference study for Darunavir

The —— (brown) colored spectra line in following spectra (Figure 3.4-3.6) represents plain drug solution while other represents drug and lipid/surfactant mixture. There was no difference found in the spectra of Darunavir in presence of lipid/surfactant indicating no interference and suggesting that the developed analytical method could be used for analysis of Darunavir in the formulation.

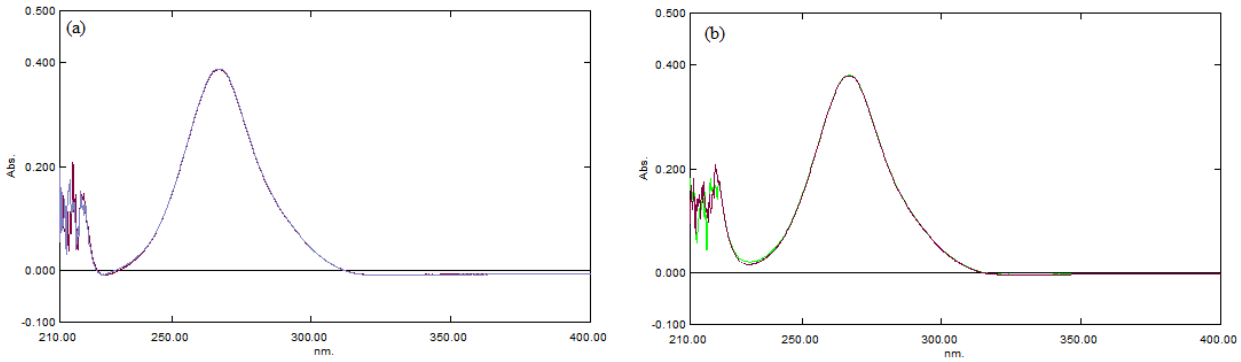

Figure 3.4 UV interference result of (a) Darunavir solution and mixture of Darunavir and glyceryl behenate and (b) Darunavir solution and mixture of Darunavir and glyceryl palmitostearate

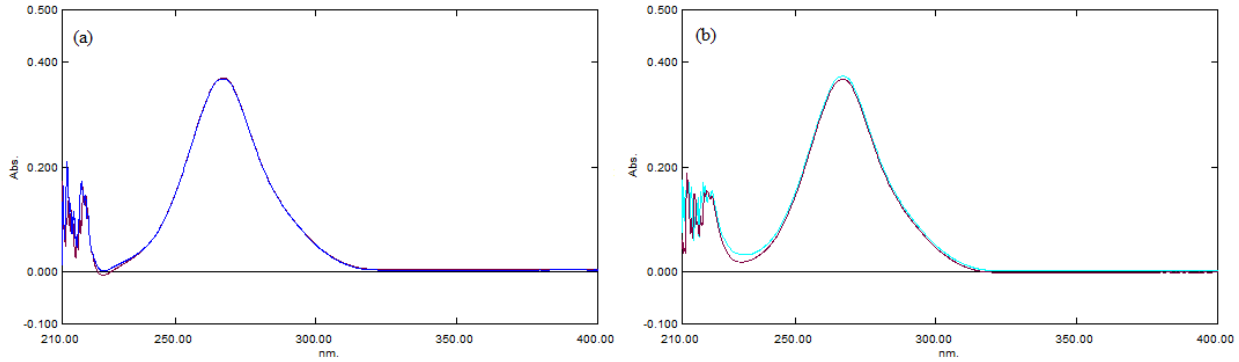

Figure 3.5 UV interference result of (a) Darunavir solution and mixture of Darunavir and hydrogenated castor oil and (b) Darunavir solution and mixture of Darunavir and trimyristin

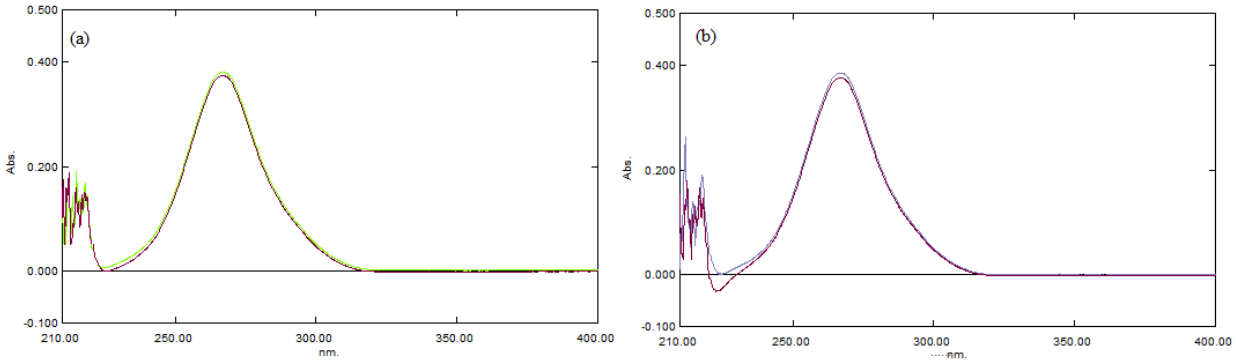

Figure 3.6 UV interference result of (a) Darunavir solution and mixture of Darunavir and tripalmitin and (b) Darunavir solution and mixture of Darunavir and sodium oleate

3.5.3 UV method for estimation of Darunavir in SGF: methanol

Darunavir showed absorption maximum at 267 nm in solvent mixture SGF: methanol (1:0.5). Beer's law was obeyed in range of 3-21 µg/ml (Table 3.5). The UV spectrum for Darunavir is shown in Figure 3.7. Regression analysis was performed on the experimental data. Regression equation for standard plot was y =0.0317x + 0.0073 (Figure 3.8). Correlation coefficient was found to be 0.9997 signifying `that a linear relationship existed between absorbance and concentration of the drug.

Table 3.5 Data for calibration plot of Darunavir in SGF: methanol (1:0.5)

Sr. No.	Concentration (µg/ml)	Absorbance ± SD
1	3	0.111 ± 0.003
2	6	0.198 ± 0.004
3	9	0.293 ± 0.001
4	12	0.386 ± 0.004
5	15	0.484 ± 0.003
6	18	0.576 ± 0.002
7	21	0.671 ± 0.003

*n=3

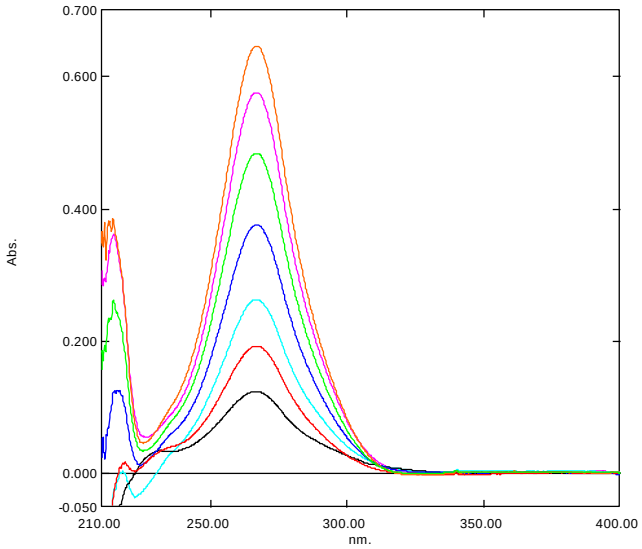

Figure 3.7 Overlay Spectra of Darunavir

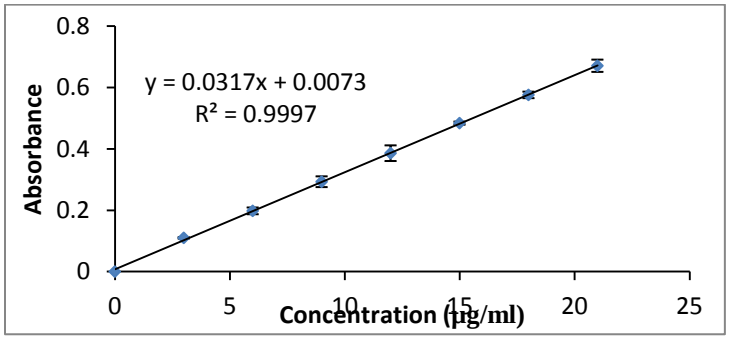

Figure 3.8 Calibration plot of Darunavir in SGF: methanol (1:0.5) at 267 nm

Precision: The average % RSD of intra-day and inter-day measurements for determination of Darunavir were 1.462 % and 1.131 % respectively at 267 nm (Table 3.6). The values confirm the precision of the method.

Table 3.6 Intra-day and inter-day precision for determination of Darunavir in SGF: methanol (1:0.5)

Sr. No.	Darunavir (µg/ml)	Mean absorbance values (n=3)				% RSD
		1	2	3	Average	
Intra-day precision						
1	3	0.112	0.113	0.109	0.111	1.86
2	12	0.387	0.386	0.376	0.383	1.58
3	21	0.664	0.676	0.673	0.671	0.93
Average % RSD						1.462
Sr. No.	**Darunavir (µg/ml)**	**Mean absorbance values (n=3)**				**% RSD**
		Day 1	**Day 2**	**Day 3**	**Average**	
Inter-day precision						
4	3	0.112	0.110	0.110	0.111	1.043
5	12	0.387	0.391	0.380	0.386	1.44
6	21	0.664	0.674	0.675	0.671	0.907
Average % RSD						1.131

Accuracy: The results of the determination of accuracy by standard addition method are given in Table 3.7. Recovery greater than 98% with low RSD justified the accuracy of the method. The **LOD** and **LOQ** obtained were 0.7002 and 2.12 µg/ml and all the optimized method parameters are listed in Table 3.8.

Table 3.7 Determination of accuracy of the method

Sr. No.	Concentration level	Theoretical content (µg/ml)	Mean amount recovered (µg/ml)	% Recovery ± RSD
1	80 %	18	18.022	100.12 ± 1.36
2	100 %	20	19.824	99.12 ± 1.36

3	120 %	22	22.45	102.04 ± 1.42

*n=3

Table 3.8 Optical characteristics of Darunavir in SGF: methanol (1:0.5)

Sr. No.	Parameter	Optimized parameter
1	Solvent	SGF: Methanol (1:0.5)
2	Scanning range and speed	200 - 400 nm, Fast speed
3	λ_{max}	267 nm
4	Beer's range	3-21 µg/ml
5	LOD	0.7002 µg/ml
6	LOQ	2.12 µg/ml
7	Molar absorptivity	15.3 x 10^3 lit/mole/cm

3.5.4 UV method for estimation of Darunavir in SIF: methanol

Darunavir showed absorption maximum at 267 nm in solvent mixture SIF: methanol (1:0.5). Beer's law was obeyed in range of 5-30 µg/ml (Table 3.9). The UV spectrum for Darunavir is shown in Figure 3.9. Regression analysis was performed on the experimental data. Regression equation for standard plot was y =0.0324x - 0.0024 (Figure 3.10). Correlation coefficient was found to be 0.9997 signifying that a linear relationship existed between absorbance and concentration of the drug.

Table 3.9 Data for calibration plot of Darunavir in SIF:methanol (1:0.5)

Sr. No.	Concentration (µg/ml)	Absorbance ± SD
1	5	0.164 ± 0.005
2	10	0.319 ± 0.006
3	15	0.481 ± 0.004
4	20	0.641 ± 0.008
5	25	0.802 ± 0.005
6	30	0.980 ± 0.006

*n=3

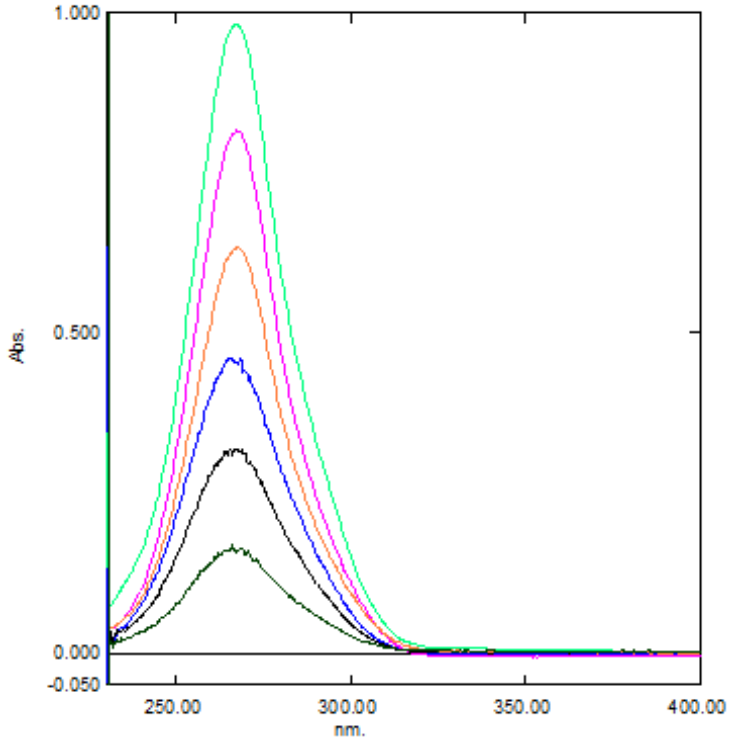

Figure 3.9 Overlay Spectra of Darunavir

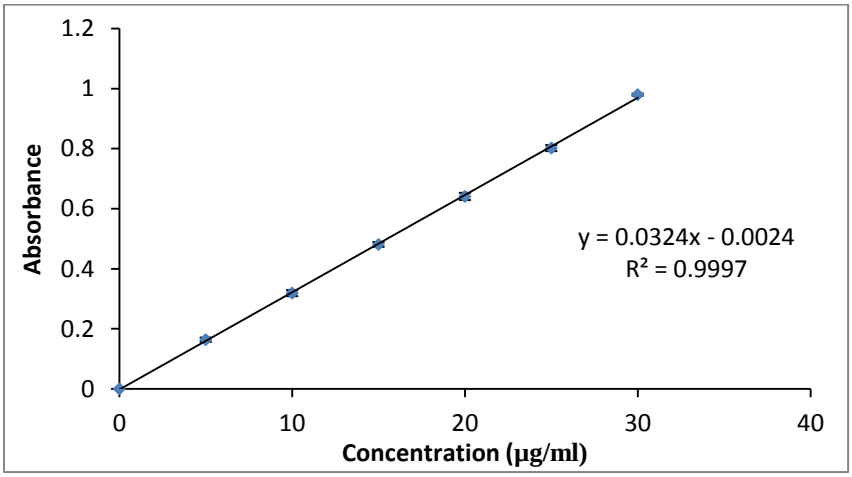

Figure 3.10 Calibration plot of Darunavir in SIF: methanol (1:0.5) at 267 nm

Precision: The average % RSD of intra-day and inter-day measurements for determination of Darunavir were 1.065 % and 0.649 % respectively at 267 nm (Table 3.10). The values confirm the precision of the method.

Table 3.10 Intra-day and inter-day precision for determination of Darunavir in SIF:methanol (1:0.5)

Sr. No.	Darunavir (µg/ml)	Mean absorbance values (n=3)				% RSD
		1	2	3	Average	
Intra-day precision						
1	10	0.319	0.315	0.326	0.320	1.73
2	20	0.640	0.645	0.654	0.646	1.097
3	30	0.984	0.980	0.977	0.980	0.350
Average % RSD						1.065
Sr. No.	**Darunavir (µg/ml)**	**Mean absorbance values (n=3)**				**% RSD**
		Day 1	**Day 2**	**Day 3**	**Average**	
Inter-day precision						
4	10	0.315	0.322	0.320	0.319	1.130
5	20	0.645	0.641	0.641	0.645	0.359
6	30	0.980	0.985	0.989	0.984	0.457
Average % RSD						0.649

Accuracy: The results of the determination of accuracy by standard addition method are given in Table 3.11. Recovery greater than 98% with low RSD justified the accuracy of the method. The **LOD** and **LOQ** obtained were 0.625 and 0.206 µg/ml.

Table 3.11 Determination of accuracy of the method

Sr. No.	Concentration level	Theoretical content (µg/ml)	Mean amount recovered (µg/ml)	% Recovery ± RSD
1	80 %	18	18.133	100.63±1.62
2	100 %	20	19.196	100.98±1.78
3	120 %	22	21.57	98.07±1.94

*n=3

Table 3.12 Optical characteristics of Darunavir in SGF: methanol (1:0.5)

Sr. No.	Parameter	Optimized parameter
1	Solvent	SIF: Methanol (1:0.5)
2	Scanning range and speed	200 - 400 nm, Fast speed
3	λ_{max}	267 nm
3	Beer's range	5-30 µg/ml
4	LOD	0.625 µg/ml
5	LOQ	0.206 µg/ml
6	Molar absorptivity	17.8 x 10^3 lit/mole/cm

3.5.5 HPLC method development of Darunavir

Figure 3.11 shows the HPLC chromatogram of Darunavir. It is evident that the retention time obtained was 7.1 min. Beer's law was obeyed in range of 2-10 µg/ml (Table 3.13). Regression equation for standard plot was y = 21.438x + 0.233. Correlation coefficient of 0.9999 was obtained indicating a linear relationship between the concentration and peak area.

Table 3.13 Data for calibration plot of Darunavir using HPLC

Sr. No.	Concentration (µg/ml)	Area ± SD
1	2	44.32 ± 1.92
2	4	85.09 ± 4.02
3	6	128.91 ± 5.04
4	8	173.60 ± 8.53
5	10	217.14 ± 9.64

*n=3

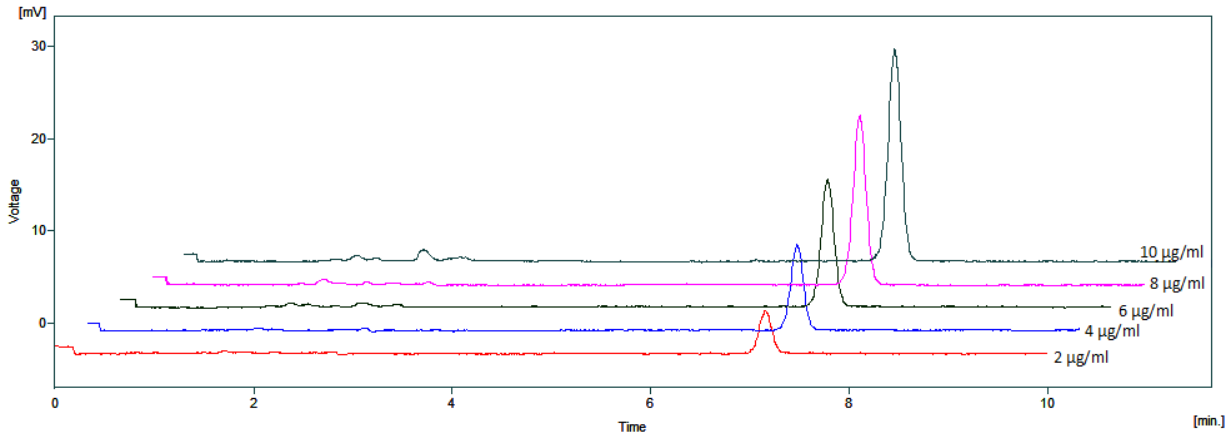

Figure 3.11 Chromatogram of Darunavir using HPLC

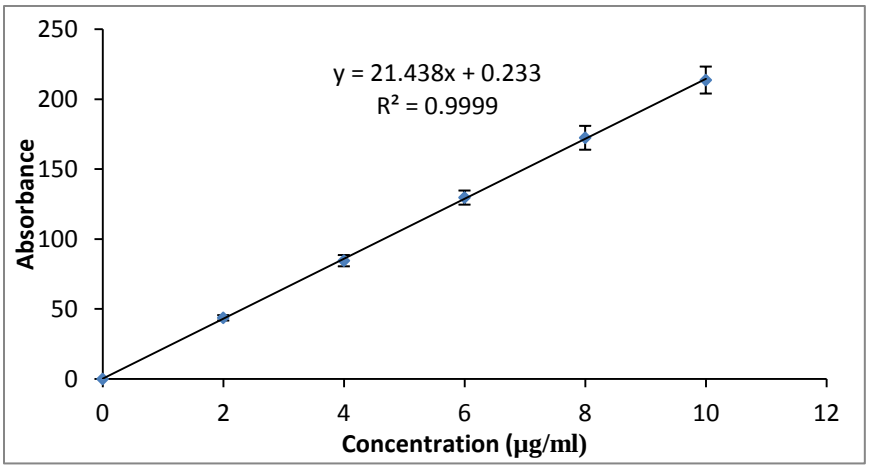

Figure 3.12 Calibration plot of Darunavir using HPLC

Precision: The average % RSD of intra-day and inter-day measurements for determination of Darunavir were 1.827 % and 1.589 % respectively at 267 nm (Table 3.14). The values confirm the precision of the method.

Table 3.14 Intra-day and inter-day precision for determination of Darunavir using HPLC

Sr. No.	Darunavir (µg/ml)	Mean Area values (n=3)				% RSD
		1	2	3	Average	
Intra-day precision						
1	2	43.75	44.74	45.47	44.65	1.933
2	6	125.54	129.36	126.35	127.08	1.583

3	10	213.93	219.58	222.36	218.62	1.964
Average % RSD						1.827
Sr. No.	**Darunavir (µg/ml)**	**Mean Area values (n=3)**				**% RSD**
		Day 1	**Day 2**	**Day 3**	**Average**	
Inter-day precision						
4	2	43.75	45.44	44.78	44.65	1.907
5	6	125.54	125.36	129.45	126.78	1.822
6	10	213.93	216.36	218.42	216.23	1.039
Average % RSD						1.589

Accuracy: Accuracy was determined by addition of 80 %, 100 % and 120% of 5 µg/ml. The results are shown in Table 3.15. The **LOD** and **LOQ** obtained were 0.45 and 0.67 µg/ml.

Table 3.15 Accuracy determination of method

Sr. No.	Concentration level	Theoretical content (µg/ml)	Mean amount recovered (µg/ml)	% Recovery ± RSD
1	80 %	9	9.25	102.77±1.45
2	100 %	10	9.83	98.30 ±1.85
3	120 %	11	11.23	102.02±1.86

*n=3

Table 3.16 Optical characteristics of Darunavir using HPLC method

Sr. No.	Parameter	Optimized parameter
1	Mobile phase	Acetonitrile: water (50:50) with pH adjusted to 3 using formic acid
2	Retention time	7.1 min
3	λ_{max}	267 nm
3	Beer's range	2-10 µg/ml
4	LOD	0.45 µg/ml
5	LOQ	0.67 µg/ml

3.5.6 LCMS method for determination of Darunavir in plasma

The mass spectrum of Darunavir is shown in Figure 3.13. The energy was optimized at m/z 548.1 → m/z 156.20 Da and the samples for calibration were analyzed. The chromatograms obtained are shown in Figure 3.14 and Figure 3.15. The Beer's plot was obtained in range of 25-1000 ng/ml (Table 3.17). Regression equation for standard plot was $y = 567x + 6.2 \times 10^3$ with correlation coefficient of 0.9982.

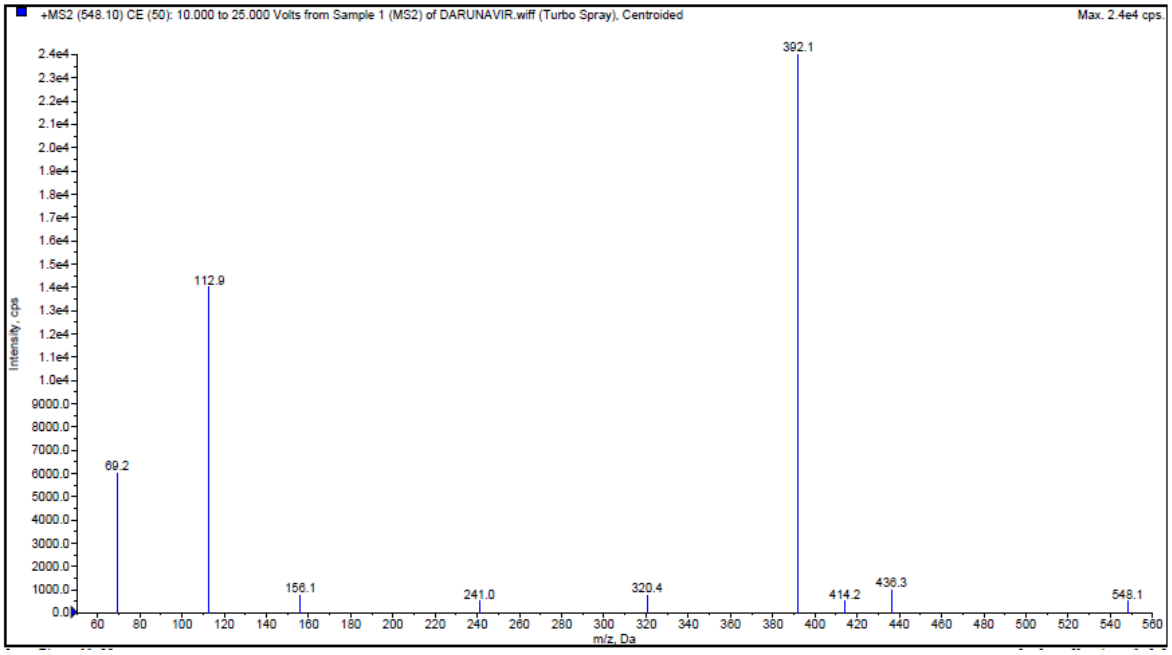

Figure 3.13 Mass spectrum of Darunavir

Table 3.17 Calibration plot of Darunavir in plasma using LC/MS

Sr. No.	Concentration (ng/ml)	Area
1	25	1.99×10^4
2	50	3.51×10^4
3	100	6.74×10^4
4	250	1.53×10^4
5	500	2.83×10^4
6	750	4.02×10^4
7	1000	5.64×10^4

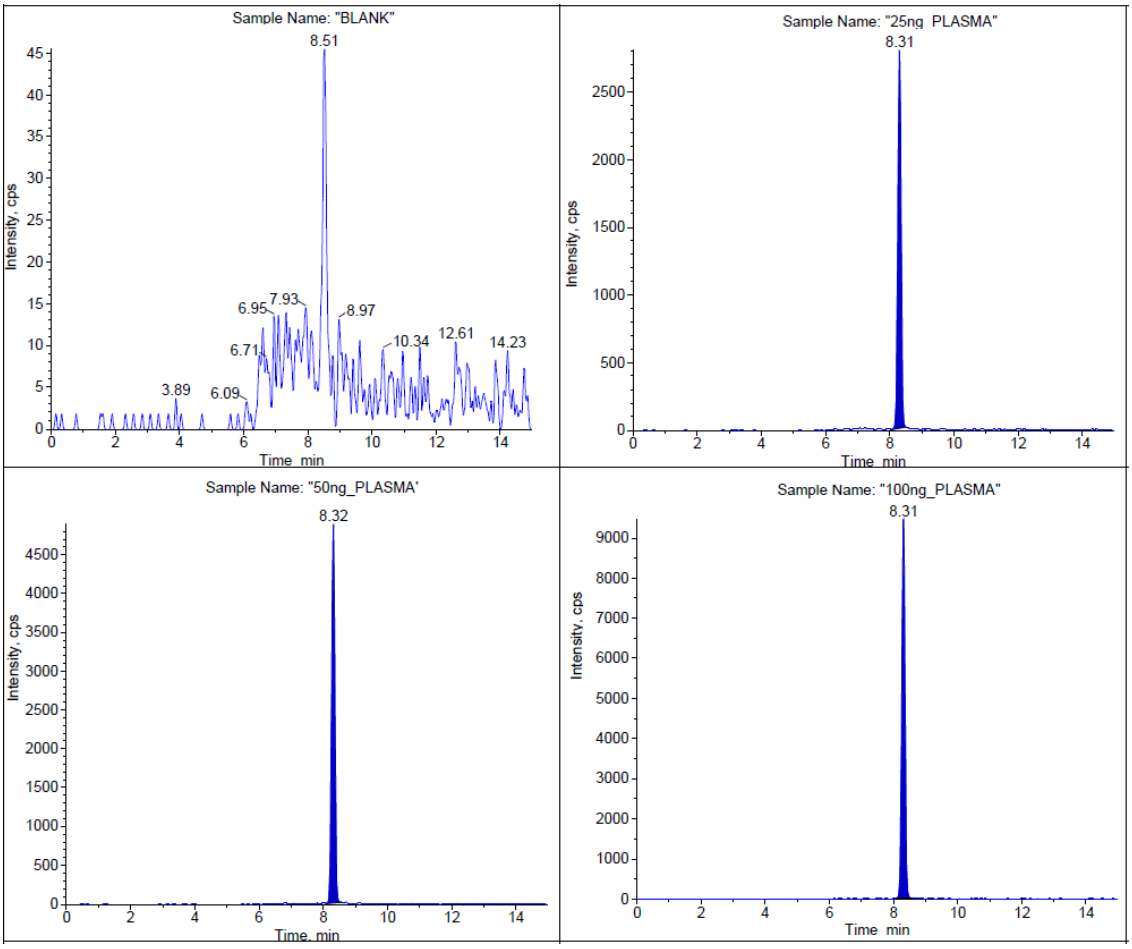

Figure 3.14 Standard peaks obtained from LCMS analysis (blank sample, 25, 50 and 100 ng/ml Darunavir samples)

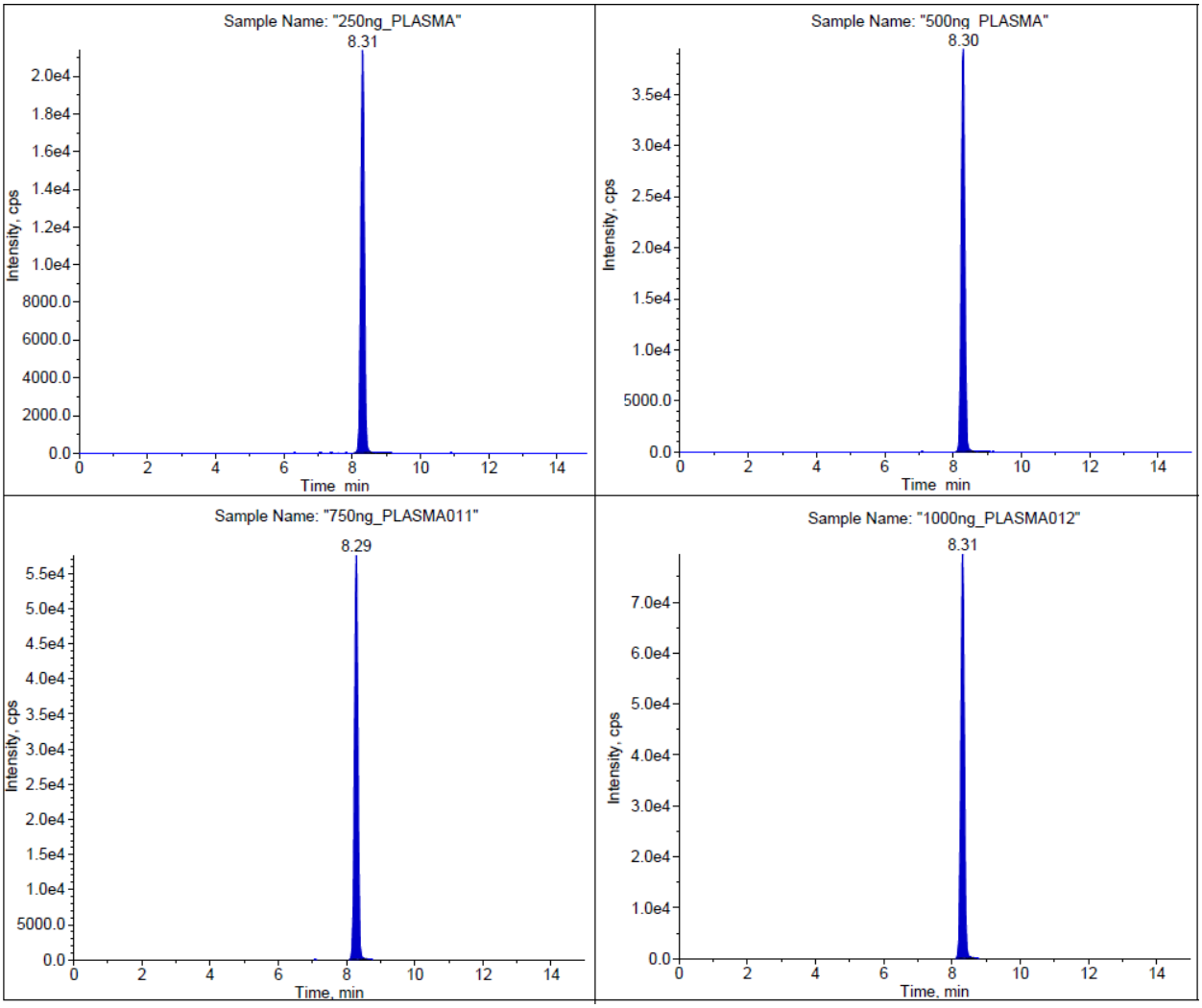

Figure 3.15 Standard peaks obtained from LCMS analysis (250, 500, 750 and 1000 ng/ml Darunavir samples)

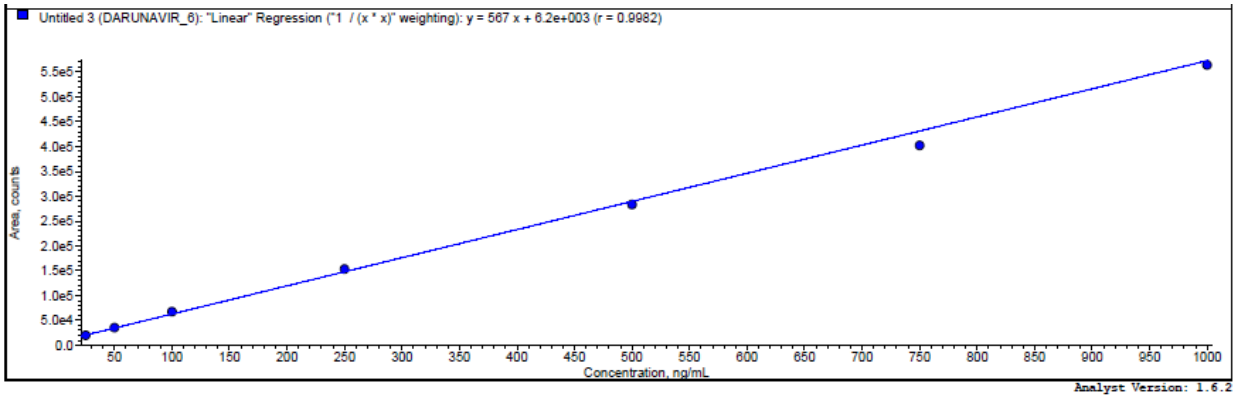

Figure 3.16 Calibration plot of Darunavir using LCMS in plasma

Table 3.18 Optical characteristics of Darunavir in plasma using LCMS

Sr. No.	Parameter	Optimized parameter
1	Mobile phase	Acetonitrile: water (60:40)
2	Retention time	7.1 min
3	Run time	15 min
4	Energy	m/z 548.1 → m/z 156.20 Da
5	Beer's range	25-1000 μg/ml

3.5.7 LCMS method for determination of Darunavir in spleen

The chromatograms obtained are shown in Figure 3.17. The energy was optimized at m/z 548.1 → m/z 241.40 Da and the samples for calibration were analyzed. The retention time obtained was 8.2 min. Regression equation for standard plot was $y = 109x + 1.21 \times 10^4$. Correlation coefficient of 0.9992 was obtained indicating a linear relationship between concentration and area.

Table 3.19 Data for calibration plot of Darunavir in spleen using LC/MS

Sr. No.	Concentration (ng/ml)	Area
1	25	1.45×10^4
2	100	2.35×10^4
3	250	4.10×10^4
4	500	6.78×10^4
5	1000	1.18×10^5

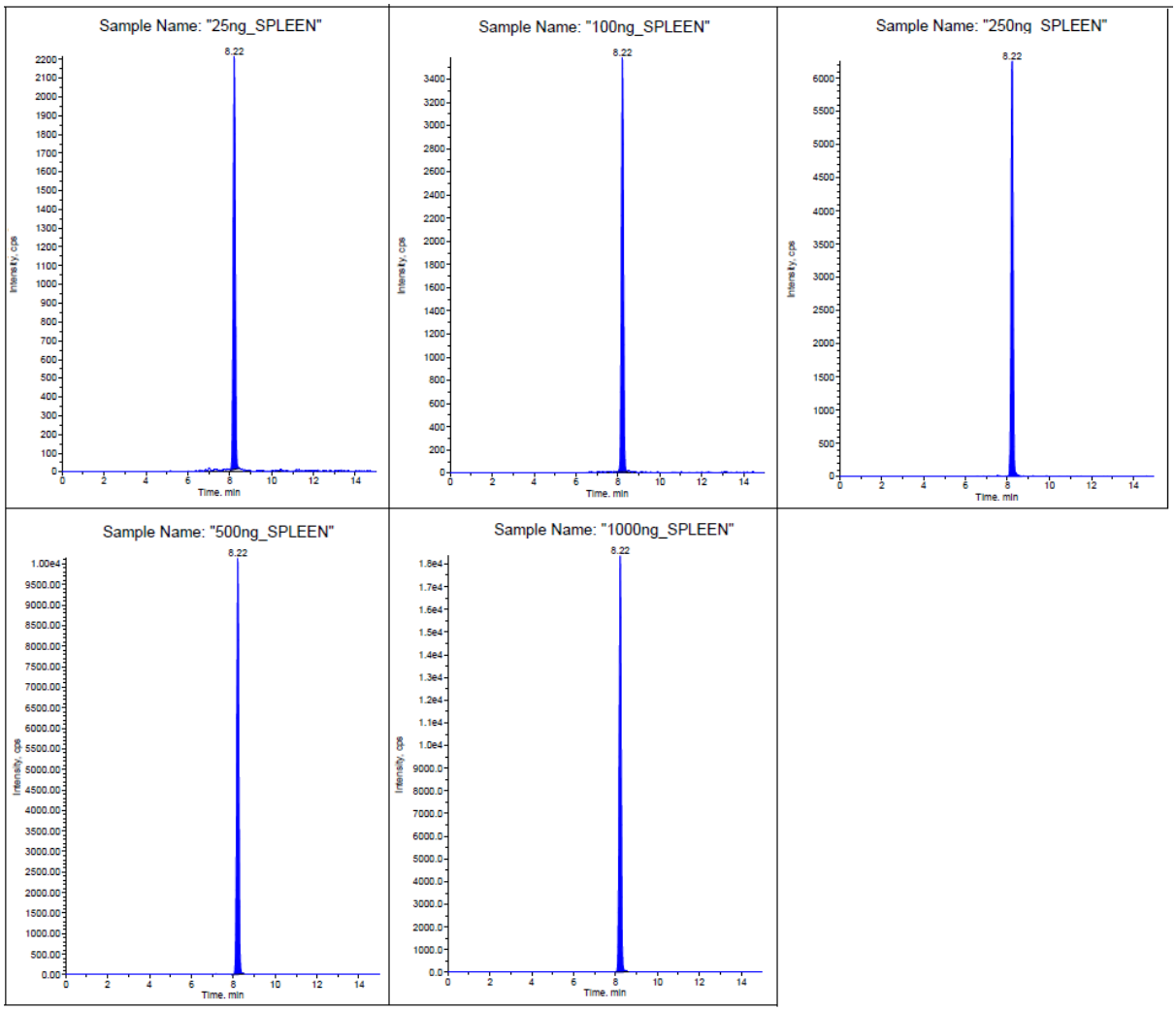

Figure 3.17 Standard peaks obtained from LCMS analysis of Darunavir in spleen

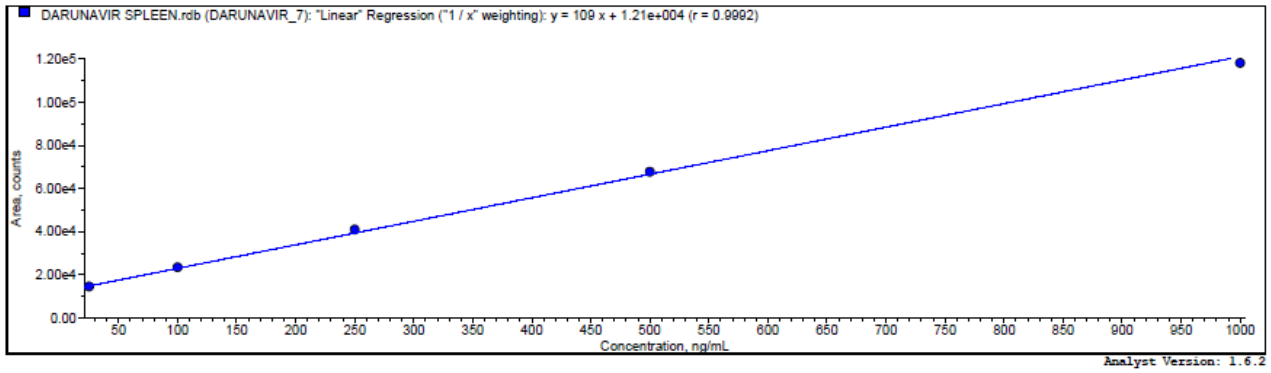

Figure 3.18 Calibration plot of Darunavir using LCMS in spleen

Table 3.20 Optical characteristics of Darunavir in spleen using LCMS

Sr. No.	Parameter	Optimized parameter
1	Mobile phase	Acetonitrile: water (60:40)
2	Retention time	8.2 min
3	Run time	15 min
4	Energy	m/z 548.1 → m/z 241.40 Da
5	Beer's range	25-1000 µg/ml

3.6 Results and discussion for methods of estimation of Atazanavir sulfate

3.6.1 UV spectroscopic method of Atazanavir sulfate (ATZ) in methanol: DCM (7:3)

ATZ showed absorption maxima at 249 nm (other maxima peaks were also seen but 249 nm was selected based on literature review (7)) in solvent mixture methanol: DCM (7:3) as shown in Figure 3.19. Beer's law was obeyed in range 10-60 µg/ml (Table 3.21). The UV spectrum for ATZ is shown in Figure 3.20. Regression analysis was performed on the experimental data. Regression equation for standard plot was y =0.0167x+0.01 (Figure 3.21). Correlation coefficient was found to be 0.9993 signifying that a linear relationship existed between absorbance and concentration of the drug.

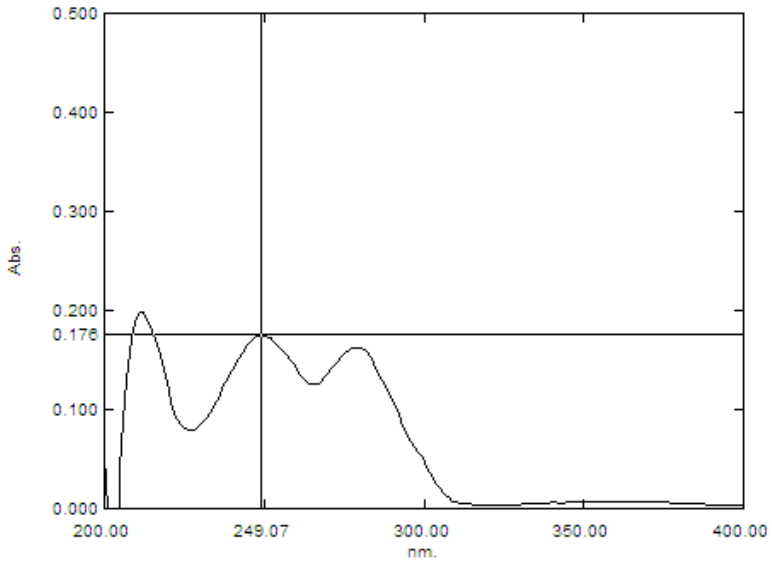

Figure 3.19 UV spectrum of ATZ (10 µg/ml)

Table 3.21 Data for calibration plot of ATZ in methanol: DCM (7:3)

Sr. No.	Concentration (µg/ml)	Absorbance ± SD
1	10	0.174 ± 0.008
2	20	0.351 ± 0.006
3	30	0.519 ± 0.009
4	40	0.691 ± 0.008
5	50	0.836 ± 0.005
6	60	1.002 ± 0.007

*n=3

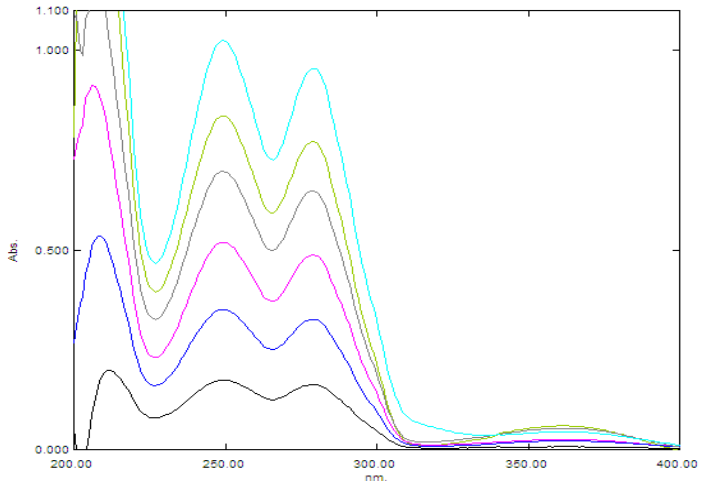

Figure 3.20 Overlay Spectra of ATZ

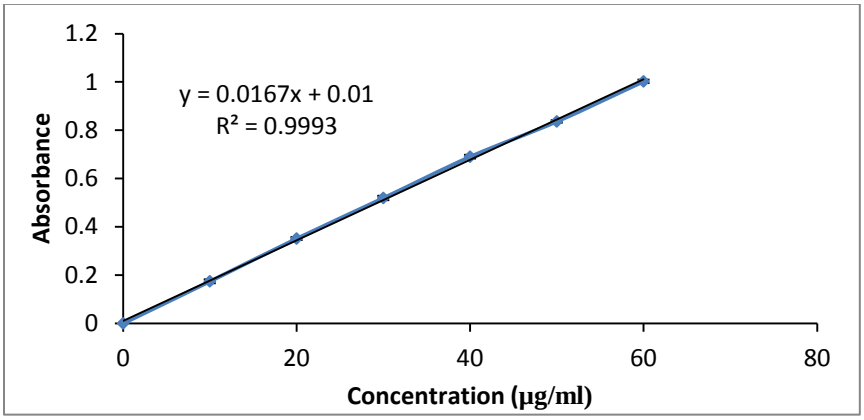

Figure 3.21 Calibration plot of ATZ in Methanol: DCM (7:3) at 267 nm

Precision: The average % RSD of intra-day and inter-day measurements for determination of ATZ were 1.315 % and 1.259 % respectively at 249 nm (Table 3.22). The values confirm the precision of the method.

Table 3.22 Intra-day and inter-day precision for determination of ATZ in methanol: DCM (7:3)

Sr. No.	ATZ (µg/ml)	Mean absorbance values (n=3)				% RSD
		1	2	3	Average	
Intra-day precision						
1	10	0.173	0.175	0.179	0.175	1.739
2	30	0.513	0.523	0.515	0.517	1.023
3	50	0.834	0.844	0.854	0.844	1.184
Average % RSD						1.315
Sr. No.	**ATZ (µg/ml)**	**Mean absorbance values (n=3)**				**% RSD**
		Day 1	**Day 2**	**Day 3**	**Average**	
Inter-day precision						
4	10	0.173	0.171	0.177	0.173	1.759
5	30	0.513	0.525	0.513	0.517	1.340
6	50	0.834	0.842	0.831	0.835	0.680
Average % RSD						1.259

Accuracy: The results of the determination of accuracy by standard addition method are given in Table 3.23. Recovery greater than 98% with low RSD justified the accuracy of the method. The **LOD** and **LOQ** obtained were 0.721 and 2.187 µg/ml.

Table 3.23 Determination of accuracy of the method

Sr. No.	Concentration level	Theoretical content (µg/ml)	Mean amount recovered (µg/ml)	% Recovery
1	80 %	18	17.893	99.40±1.42
2	100 %	20	20.124	100.62±1.73
3	120 %	22	22.313	101.42±1.46

*n=3

Table 3.24 Optical characteristics of ATZ in methanol: DCM (7:3)

Sr. No.	Parameter	Optimized parameter
1	Solvent	Methanol: DCM (7:3)
2	Scanning range and speed	200 - 400 nm, Fast speed
3	λ_{max}	249 nm
4	Beer's range	10-60 µg/ml
5	LOD	0.721 µg/ml
6	LOQ	2.187 µg/ml
7	Molar absorptivity	13.97 x 10^3 lit/mole/cm

3.6.2 Analytical interference study for Atazanavir sulfate

The blue colored spectra line in following spectra (Figure 3.22-3.24) represents plain drug solution while other represents drug and lipid/surfactant mixture. There was no difference found in the spectra of ATZ in presence of lipid/surfactant no interference suggests that the developed analytical method could be used for analysis of ATZ in the formulation.

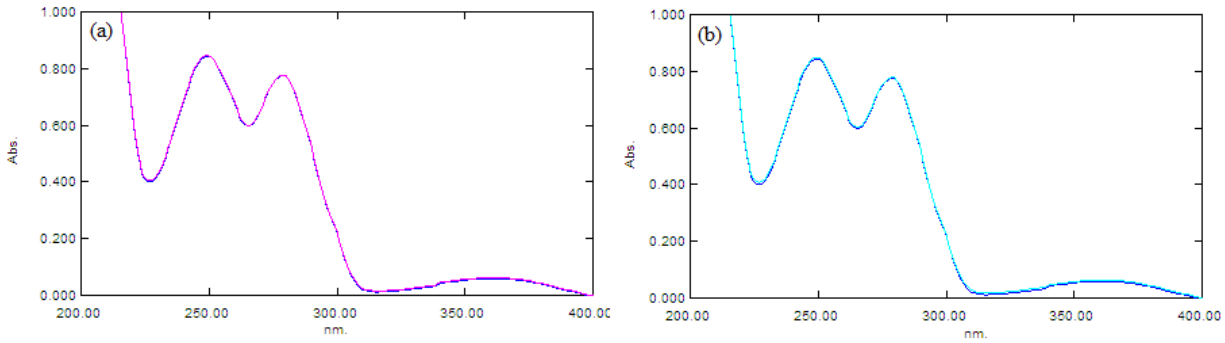

Figure 3.22 UV interference result of (a) ATZ solution and mixture of ATZ and glyceryl behenate and (b) ATZ solution and mixture of ATZ and glyceryl palmitostearate

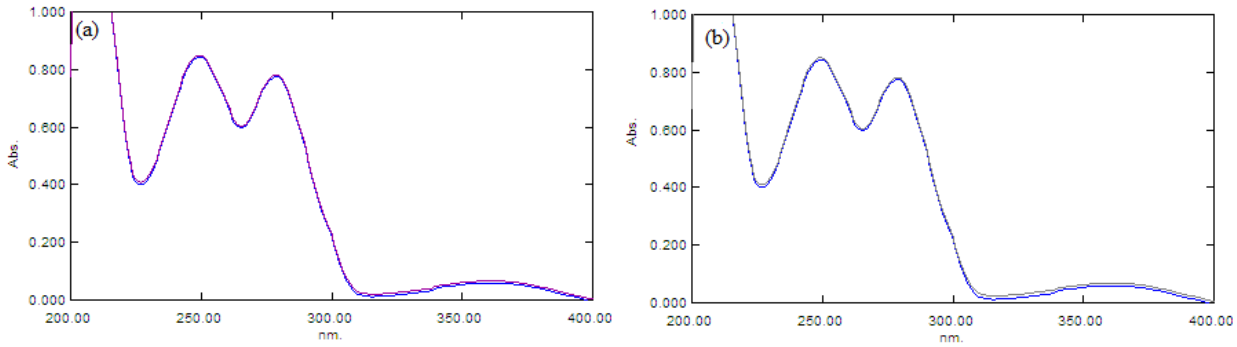

Figure 3.23 UV interference result of (a) ATZ solution and mixture of ATZ and hydrogenated castor oil and (b) ATZ solution and mixture of ATZ and trimyristin

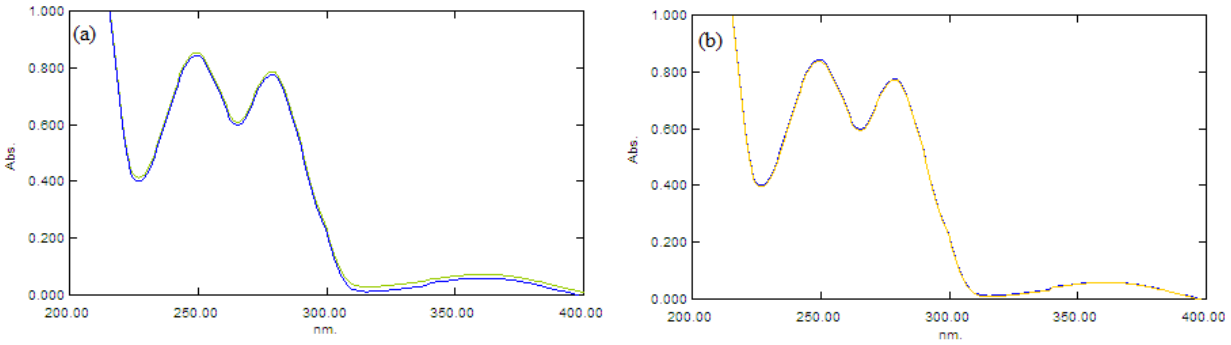

Figure 3.24 UV interference result of (a) ATZ solution and mixture of ATZ and tripalmitin and (b) ATZ solution and mixture of ATZ and sodium oleate

3.6.3 UV spectroscopic method of ATZ in simulated gastric fluid: methanol (1:0.5)

ATZ showed absorption maximum at 249 nm in solvent mixture SGF: methanol (1:0.5). Beer's law was obeyed in range of 10-50 µg/ml (Table 3.25). The UV spectrum for ATZ is shown in Figure 3.25. Regression equation for standard plot was y =0.0208x+0.0122 (Figure 3.26) with correlation coefficient of 0.9992.

Table 3.25 Data for calibration plot of ATZ in SGF:methanol (1:0.5)

Sr. No.	Concentration (µg/ml)	Absorbance ± SD
1	10	0.239 ± 0.008
2	20	0.432 ± 0.008
3	30	0.626 ± 0.008

4	40	0.845 ± 0.007
5	50	1.048 ± 0.007

*n=3

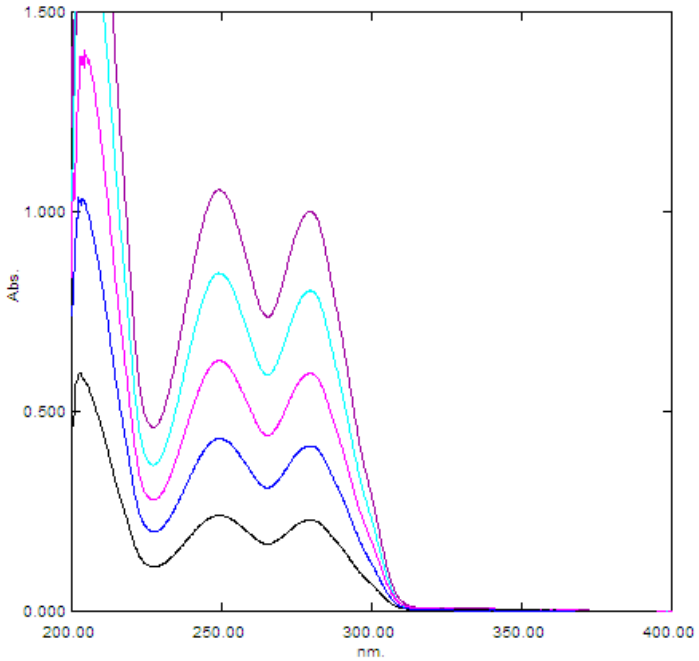

Figure 3.25 Overlay spectra of ATZ in Simulated gastric fluid

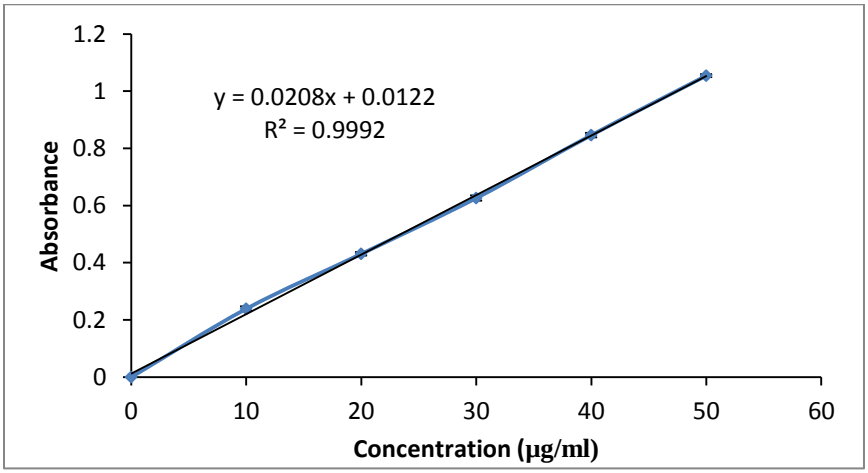

Figure 3.26 Calibration plot of ATZ in SGF: methanol (1:0.5) at 249 nm

Precision: The average % RSD of intra-day and inter-day measurements for determination of Atazanavir sulfate were 1.187 % and 0.659 % respectively at 249 nm (Table 3.26). The values confirm the precision of the method.

Table 3.26 Intra-day and inter-day precision for determination of ATZ in SGF:methanol (1:0.5)

Sr. No.	Atazanavir sulfate (µg/ml)	Mean absorbance values (n=3)				% RSD
		1	2	3	Average	
Intraday precision						
1	10	0.234	0.243	0.241	0.239	1.974
2	30	0.633	0.620	0.624	0.625	1.064
3	50	1.047	1.058	1.053	1.052	0.523
Average % RSD						1.187
Sr. No.	**Atazanavir sulfate (µg/ml)**	**Mean absorbance values (n=3)**				**% RSD**
		Day 1	**Day 2**	**Day 3**	**Average**	
Interday precision						
4	10	0.234	0.236	0.240	0.236	1.29
5	30	0.633	0.632	0.632	0.632	0.091
6	50	1.047	1.044	1.056	1.049	0.595
Average % RSD						0.659

Accuracy: The accuracy was determined by addition of 80, 100 and 120 % to 20 µg/ml concentration and the results obtained are given in Table 3.27. Recovery between 97-103 % with low RSD justified the accuracy of the method. The **LOD** and **LOQ** obtained were 0.231 and 0.701 µg/ml.

Table 3.27 Determination of accuracy of the method

Sr. No.	Concentration level	Theoretical content (µg/ml)	Mean amount recovered (µg/ml)	% Recovery ± RSD
1	80 %	36	35.432	98.42±1.63
2	100 %	40	41.054	102.63±0.83
3	120 %	44	44.642	101.45±1.73

*n=3

Table 3.28 Optical characteristics of ATZ in SGF: methanol (1:0.5)

Sr. No.	Parameter	Optimized parameter
1	Solvent	SGF: methanol (1:0.5)
2	Scanning range and speed	200 - 400 nm, Fast speed
3	λ_{max}	249 nm
4	Beer's range	10-50 µg/ml
5	LOD	0.231 µg/ml
6	LOQ	0.701 µg/ml
7	Molar absorptivity	19.2 x 10^3 lit/mole/cm

3.6.4 UV spectroscopic method of ATZ in simulated intestinal fluid: methanol (1:0.5)

ATZ showed absorption maximum at 249 nm in solvent mixture SGF: methanol (1:0.5). Beer's law was obeyed in range of 10-50 µg/ml (Table 3.29). The UV spectrum for ATZ is shown in Figure 3.27. Regression equation for standard plot was y =0.022x - 0.008 (Figure 3.28) with correlation coefficient of 0.9992.

Table 3.29 Data for calibration plot of ATZ in SIF:methanol (1:0.5)

Sr. No.	Concentration (µg/ml)	Absorbance ± SD
1	10	0.217 ± 0.007
2	20	0.423 ± 0.005
3	30	0.641 ± 0.005
4	40	0.862 ± 0.007
5	50	1.110 ± 0.02

*n=3

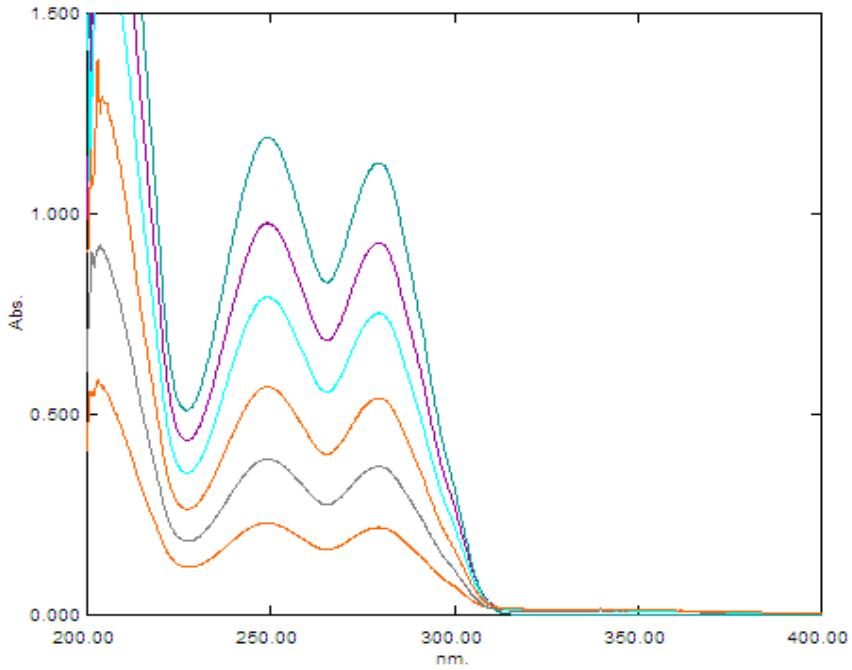

Figure 3.27 Overlay spectra of ATZ in simulated intestinal fluid: methanol (1:0.5)

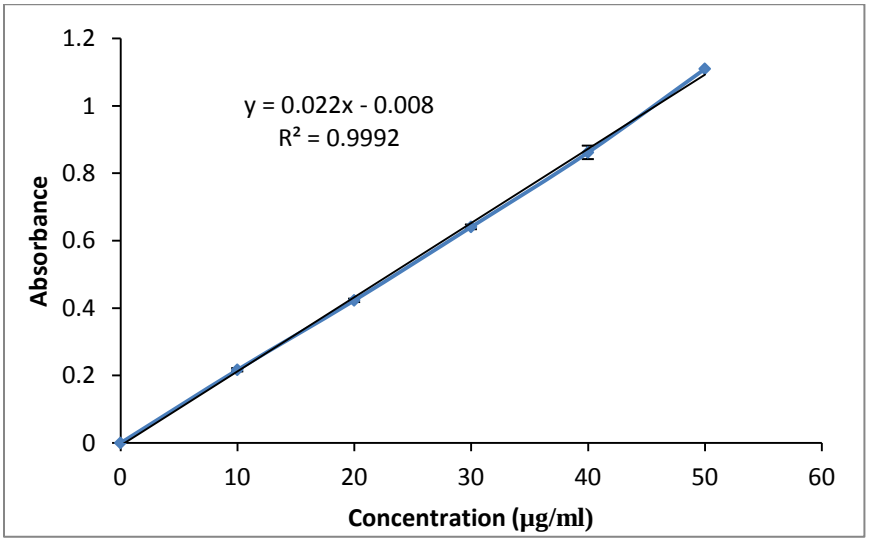

Figure 3.28 Calibration plot of ATZ in SIF: methanol (1:0.5) at 249 nm

Precision: The average % RSD of intra-day and inter-day measurements for determination of Atazanavir sulfate were 0.646 % and 0.909 % respectively at 249 nm (Table 3.30). The values confirm the precision of the method.

Table 3.30 Intra-day and inter-day precision for determination of ATZ in SGF:methanol (1:0.5)

Sr. No.	Atazanavir sulfate (µg/ml)	Mean absorbance values (n=3)				% RSD
		1	2	3	Average	
Intra-day precision						
1	10	0.216	0.214	0.216	0.215	0.536
2	30	0.638	0.643	0.649	0.643	0.856
3	50	1.118	1.117	1.107	1.114	0.546
Average % RSD						0.646
Sr. No.	**Atazanavir sulfate (µg/ml)**	**Mean absorbance values (n=3)**				**% RSD**
		Day 1	**Day 2**	**Day 3**	**Average**	
Inter-day precision						
4	10	0.216	0.217	0.223	0.218	1.731
5	30	0.638	0.646	0.642	0.642	0.623
6	50	1.118	1.112	1.110	1.113	0.373
Average % RSD						0.909

Accuracy: The accuracy was determined by addition of 80, 100 and 120 % to 20 µg/ml concentration and the results obtained are given in Table 3.31. Recovery between 97-103 % with low relative standard deviation (RSD) justified the accuracy of the method. The **LOD** and **LOQ** obtained were 0.393 and 1.19 µg/ml.

Table 3.31 Determination of accuracy of the method

Sr. No.	Concentration level	Theoretical content (µg/ml)	Mean amount recovered (µg/ml)	% Recovery ± RSD
1	80 %	36	36.752	102.02±1.75
2	100 %	40	41.036	102.59±1.17
3	120 %	44	42.738	97.13±0.83

*n=3

Table 3.32 Optical characteristics of ATZ in SIF: methanol (1:0.5)

Sr. No.	Parameter	Optimized parameter
1	Solvent	SIF: methanol (1:0.5)
2	Scanning range and speed	200 - 400 nm, Fast speed
3	λ_{max}	249 nm
3	Beer's range	10-50 µg/ml
4	LOD	0.393 µg/ml
5	LOQ	1.19 µg/ml
6	Molar absorptivity	17.4 x 10^3 lit/mole/cm

3.6.5 HPLC method development of Atazanavir sulfate

The HPLC chromatogram for Darunavir (Figure 3.29) shows a sharp peak at the retention time of 8.1 min. Beer's law was obeyed in range of 50-250 ng/ml (Table 3.33). Regression equation for standard plot was y = 2.8827x – 15.613 with correlation coefficient of 0.9976.

Table 3.33 Data for calibration plot of Atazanavir sulfate using HPLC

Sr. No.	Concentration (ng/ml)	Area ± SD
1	50	111.67 ± 6.64
2	100	276.72 ± 6.98
3	150	401.49 ± 11.17
4	200	568.66 ± 12.51
5	250	709.81 ± 12.47

*n=3

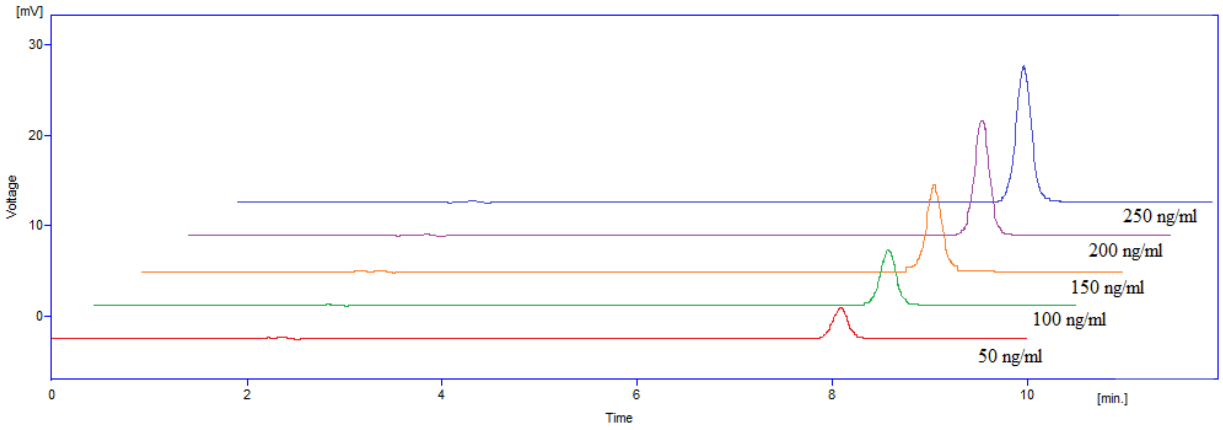

Figure 3.29 HPLC Chromatogram of Atazanavir sulfate

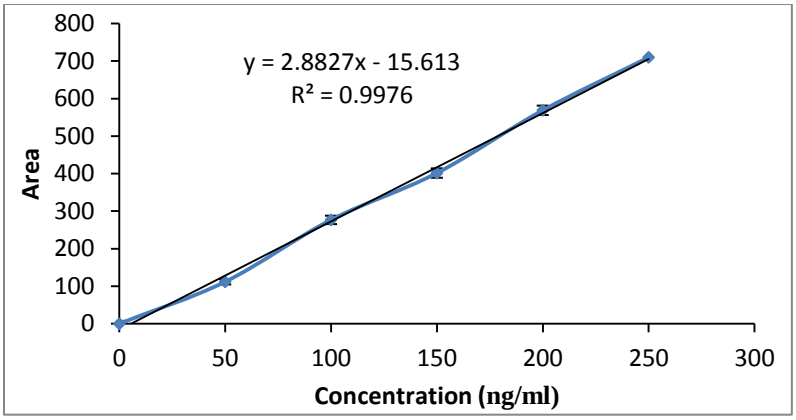

Figure 3.30 Calibration plot of Atazanavir sulfate using HPLC

Precision: The average % RSD of intra-day and inter-day measurements for determination of Atazanavir sulfate were 1.48 % and 1.99 % respectively at 249 nm (Table 3.34). The values confirm the precision of the method.

Table 3.34 Intra-day and inter-day precision for determination of Atazanavir sulfate using HPLC

Sr. No.	Atazanavir sulfate (ng/ml)	Mean Area values (n=3)				% RSD
		1	2	3	Average	
Intra-day precision						
1	50	113.78	111.58	113.27	112.87	1.02
2	150	402.85	411.93	396.7	403.82	1.89

3	250	722.56	707.8	701.26	710.54	1.53
Average % RSD						1.48
Sr. No.	**Atazanavir sulfate (ng/ml)**	**Mean Area values (n=3)**				**% RSD**
		Day 1	**Day 2**	**Day 3**	**Average**	
Inter-day precision						
4	5	113.78	114.67	110.23	112.89	2.08
5	150	402.85	412.23	396.34	403.80	1.97
6	250	722.56	719.25	697.24	713.01	1.93
Average % RSD						1.99

Accuracy: Accuracy was determined by addition of 80 %, 100 % and 120 % of 100 ng/ml. The results are shown in Table 3.35. The **LOD** and **LOQ** obtained were 1.90 and 5.77 ng/ml.

Table 3.35 Accuracy determination of method

Sr. No.	Concentration level	Theoretical content (ng/ml)	Mean amount recovered (ng/ml)	% Recovery ± RSD
1	80 %	180	184.8	102.66 ± 0.45
2	100 %	200	195.6	97.8 ± 0.31
3	120 %	220	213.5	97.04 ± 0.47

*n=3

Table 3.36 Optical characteristics of ATZ using HPLC

Sr. No.	Parameter	Optimized parameter
1	Mobile phase	45 % water, 20 % methanol and 35 % acetonitrile with pH adjusted to 3.55 using acetic acid
2	Retention time	8.1 min
3	λ_{max}	249 nm
4	Beer's range	50-250 ng/ml
5	LOD	1.90 ng/ml

6	LOQ	5.77 ng/ml

3.6.6 HPLC method for determination of Atazanavir sulfate in plasma

The HPLC chromatogram for Darunavir is shown in Figure 3.31. Retention time was obtained as 8.1 min. Beer's law was obeyed in range of 50-1000 ng/ml (Table 3.37). Regression equation for standard plot was y = 2.3116x + 22.305 with correlation coefficient of 0.9985 indicating linearity in the range of 50-1000 ng/ml.

Table 3.37 Data for calibration plot of Atazanavir sulfate in plasma

Sr. No.	Concentration (ng/ml)	Area ± SD
1	50	97.13 ± 4.77
2	100	248.36 ± 7.71
3	200	526.39 ± 12.43
4	300	749.02 ± 16.68
5	500	1193.88 ± 20.34
6	1000	2309.88 ± 37.10

*n=3

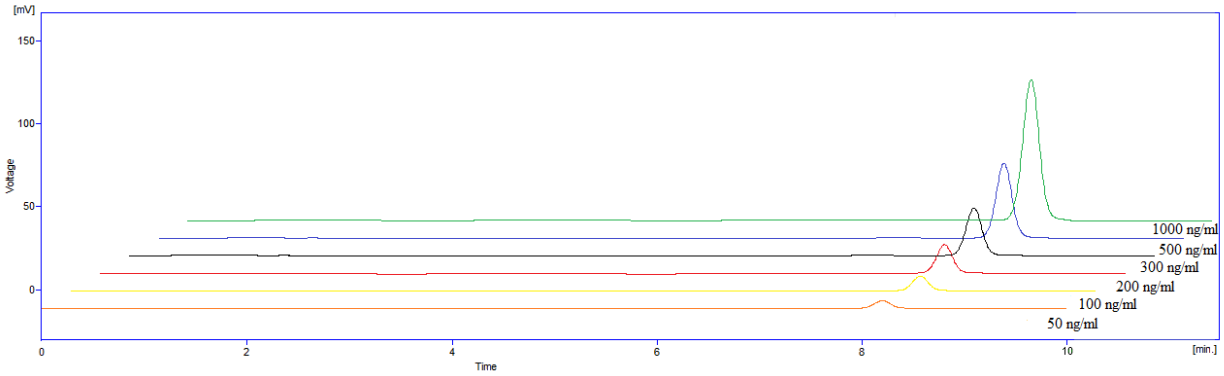

Figure 3.31 Chromatogram of Atazanavir sulfate in plasma

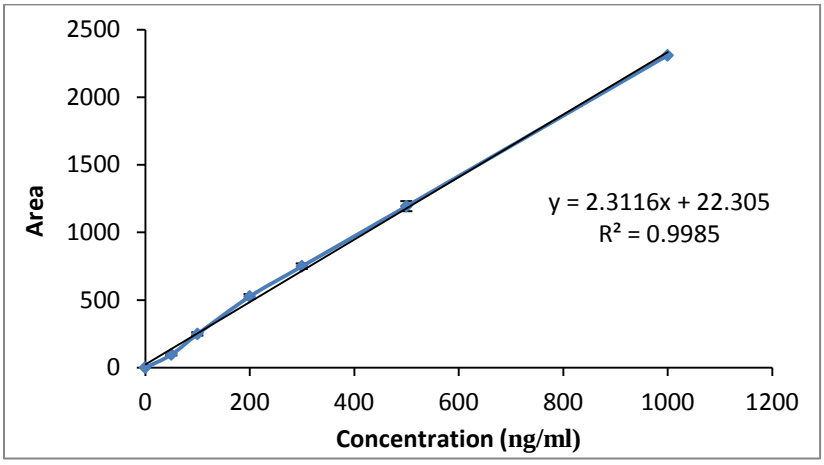

Figure 3.32 Calibration plot of Atazanavir sulfate in plasma

Precision: The average % RSD of intra-day and inter-day measurements for determination of Atazanavir sulfate were 1.89 % and 1.78 % respectively at 249 nm (Table 3.38). The values confirm the precision of the method.

Table 3.38 Intra-day and inter-day precision for determination of Atazanavir sulfate in plasma using HPLC

Sr. No.	Atazanavir sulfate (ng/ml)	Mean Area values (n=3)				% RSD
		1	2	3	Average	
Intra-day precision						
1	50	98.28	99.34	102.26	99.89	1.95
2	300	755.25	735.23	762.34	750.94	1.87
3	1000	2304.56	2277.46	2362.38	2314.8	1.87
Average % RSD						1.89
Sr. No.	**Atazanavir sulfate (ng/ml)**	**Mean Area values (n=3)**				**% RSD**
		Day 1	**Day 2**	**Day 3**	**Average**	
Inter-day precision						
4	50	98.28	95.37	98.23	97.29	1.71
5	300	755.25	760.23	734.28	749.92	1.83
6	1000	2304.56	2353.92	2270.34	2309.60	1.81
Average % RSD						1.78

Accuracy: Accuracy was determined by addition of 80 %, 100 % and 120 % of 200 ng/ml. The results are shown in Table 3.39. The **LOD** and **LOQ** obtained were 1.50 and 4.55 ng/ml.

Table 3.39 Accuracy determination of method

Sr. No.	Concentration level	Theoretical content (ng/ml)	Mean amount recovered (ng/ml)	% Recovery ± RSD
1	80 %	360	370.8	103 ± 0.15
2	100 %	400	409.6	102.4 ± 0.28
3	120 %	440	452.9	102.93 ± 0.38

*n=3

Table 3.40 Optical characteristics of ATZ in plasma using HPLC

Sr. No.	Parameter	Optimized parameter
1	Mobile phase	45 % water, 20 % methanol and 35 % acetonitrile with pH adjusted to 3.55 using acetic acid
2	Retention time	8.1 min
3	λ_{max}	249 nm
4	Beer's range	50-1000 ng/ml
5	LOD	1.50
6	LOQ	4.55

3.6.7 HPLC method for determination of Atazanavir sulfate in spleen

The HPLC chromatogram for Darunavir is shown in Figure 3.33. Retention time was obtained as 8.1 min. Beer's law was obeyed in range of 50-250 ng/ml (Table 3.41). Regression equation for standard plot was y = 2.9612x – 7.1351 with correlation coefficient of 0.9991.

Table 3.41 Data for calibration plot of Atazanavir sulfate for estimation in spleen

Sr. No.	Concentration (ng/ml)	Area ± SD

1	50	127.57 ± 4.51
2	100	294.94 ± 9.54
3	150	433.69 ± 12.33
4	200	591.64 ± 11.46
5	250	730.21 ± 8.05

*n=3

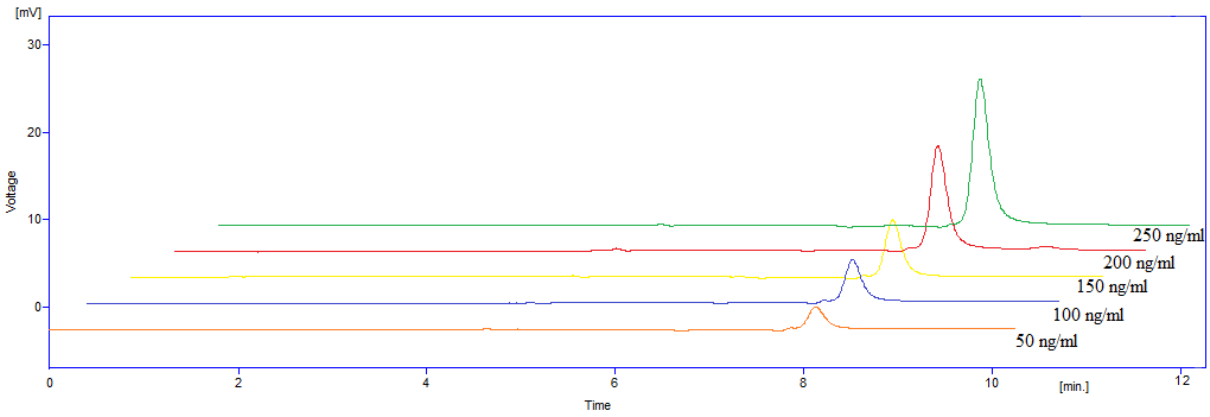

Figure 3.33 Chromatogram of Atazanavir sulfate in spleen

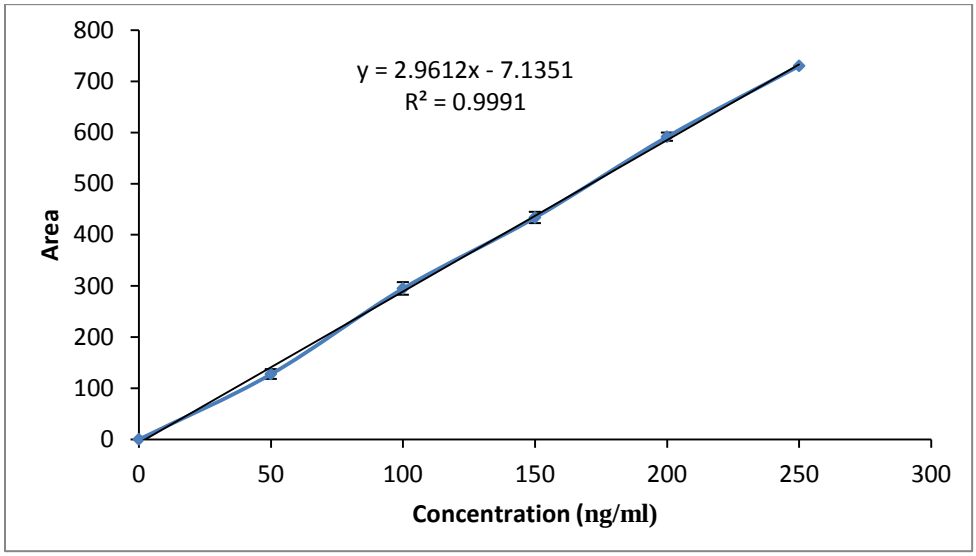

Figure 3.34 Calibration plot of Atazanavir sulfate in spleen

Precision: The average % RSD of intra-day and inter-day measurements for determination of Atazanavir sulfate were 0.96 % and 0.98 % respectively at 249 nm (Table 3.42). The values confirm the precision of the method.

Table 3.42 Intra-day and inter-day precision for determination of Atazanavir sulfate in spleen using HPLC

Sr. No.	Atazanavir sulfate (ng/ml)	Mean Area values (n=3)				% RSD
		1	2	3	Average	
Intra-day precision						
1	50	125.67	128.53	129.26	127.82	1.48
2	150	430.26	437.39	433.62	433.75	0.82
3	250	731.38	735.27	726.48	731.04	0.60
Average % RSD						0.969
Sr. No.	**Atazanavir sulfate (ng/ml)**	**Mean Area values (n=3)**				**% RSD**
		Day 1	**Day 2**	**Day 3**	**Average**	
Inter-day precision						
4	50	125.67	128.32	130.12	128.03	1.74
5	150	430.26	433.13	437.12	433.50	0.79
6	250	731.38	733.13	729.19	730.56	0.41
Average % RSD						0.98

Accuracy: Accuracy was determined by addition of 80 %, 100 % and 120 % of 100 ng/ml. The results are shown in Table 3.43. The **LOD** and **LOQ** obtained were 1.62 and 4.91 ng/ml.

Table 3.43 Accuracy determination of method

Sr. No.	Concentration level	Theoretical content (ng/ml)	Mean amount recovered (ng/ml)	% Recovery ± RSD
1	80 %	180	185.2	102.88 ± 0.64
2	100 %	200	201.34	100.67 ± 1.38
3	120 %	220	217.36	98.8 ± 1.47

*n=3

Table 3.44 Optical characteristics of ATZ in spleen using HPLC

Sr. No.	Parameter	Optimized parameter
1	Mobile phase	45 % water, 20 % methanol and 35 % acetonitrile with pH adjusted to 3.55 using acetic acid
2	Retention time	8.1 min
3	λ_{max}	249 nm
3	Beer's range	50-250 ng/ml

3.7 References

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