Chapter 3

ANALYTICAL METHODS

3.1 Introduction

Analytical methods are intended to establish the identity, purity, physical characteristics and potency of the drugs. Methods are developed to support drug testing against specifications during manufacturing and quality operations, as well as during long-term stability studies. Methods may also support safety and characterization studies or evaluations of drug performance.

The most widely used methods for quantitative determination of drugs and metabolites in different solvents, media and biological matrices such as blood, serum, plasma, or urine includes UV- Spectrophotometry, Gas chromatography (GC), High-performance liquid chromatography (HPLC) [1,2], Thin layer chromatography (TLC), combined GC and LC mass spectrometric (MS) procedures such as LC-MS [3,4], LC-MS-MS [5,6], GC-MS [7,8], and GC-MS- MS, techniques like NMR is used for structure identification.

Validation is an important requirement in the practice of an analytical process. The goal of validation of an analytical method is to ensure that every future measurement/analysis will be close enough to the unknown true value for the content of the analyte in the sample. [9-15] The various parameters of method validation include Linearity, Accuracy, Precision, Range, Robustness, Ruggedness, Limit of Quantification and Limit of Detection [16-19]

The primary focus of this chapter was to develop and validate UV spectrophotometric and LCMS-MS methods for the estimation of drugs (Leuprolide acetate and Raloxifene Hydrochloride) for the various studies like % drug entrapment, loading efficiency (%w/w), in- vitro drug release and in- vivo pharmacokinetic studies. UV Spectrophotometry is the simplest instrumentation method capable of drug estimation in micrograms. Whereas, LCMS-MS technique is more sophisticated method and is used for the estimation of samples with very low quantity of the drug, especially in the biological samples. An LCMS-MS is a highly sensitive hyphenated system consisting of HPLC system with a mass spectroscopy detector [20]

3.2 Materials and Equipment

Reagents used:

Raloxifene Hydrochloride (RLX) was kindly gifted by Aarti Drugs Ltd. (Mumbai, India). Leuprolide Acetate (LA) was a gift sample from Sun Pharma Advanced Research Centre, Vadodara, India. HPLC grade Choloroform, Methanol, Formic acid, Acetonitrile, Ammonium formate and Propionic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). Millipore Milli-Q gradient purified water (Molsheim, France) was used throughout the study. Supelco solid extraction tubes were purchased from Sigma Aldrich, USA. Sodium Hydroxide, Potassium Hydroxide, Calcium Hydroxide, Hydrochloric acid and Acetic acid were obtained from Loba Chemie, Mumbai, India. Bovine Serum Albumin, Lactic acid and Glycerol were procured from Merck Specialities Pvt. Ltd. Mumbai, India. Sodium Chloride, Urea and Glucose were purchased from Qualigens Fine Chemicals, Mumbai, India. Distilled water was obtained from in-house distillation assembly. All other chemicals used were obtained from authentic source and were of Analytical Reagent grade.

Equipment used:

- Electronic Balance (Shimadzu, Japan)
- UV-Visible Spectrophotometer (UV1800, Shimadzu, Japan)
- Magnetic Stirrer (Remi Instruments, Mumbai, India)
- Vortex Mixer (Spinix-Vortex Shaker, Tarsons, India)
- Cooling centrifuge (Remi Equipments, Mumbai, India)
- Ultrasonic Bath 120W (Vibronics Co. Pvt. Ltd., Mumbai, India)
- LCMS-MS (ekspert^{TM} ultraLC with ekspert^{TM} ultraLC 100 pump system (eksigent-AB Sciex, Framingham, MA) coupled with 3200 QTRAP mass spectrometer (AB Sciex, Framingham, MA)

3.3 Analytical Methods for estimation of Leuprolide acetate

3.3.1 UV-Visible Spectrophotometric Method in Distilled Water

Preparation of stock solution: Accurately weighed 10 mg of drug was taken in 10 ml volumetric flask. It was initially dissolved in 4-5 ml of distilled water. Then the volume was made up to 10 ml with distilled water to get a stock solution of 1mg/ml (1000 µg/ml).

Determination of Absorbance maxima (λ_{max}): From the stock solution, an aliquot of 0.1 ml was withdrawn and transferred to a 10 ml volumetric flask. It was diluted with distilled water up to the mark to obtain 10 µg/ml solution of Leuprolide acetate .UV spectrum of the solution was recorded using UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan) between the range of 200-400 nm and the wavelength of maximum absorbance was determined.

Preparation of calibration plot: From the stock solution of 1000 µg/ml, aliquots of 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 were withdrawn and further diluted up to 10 ml with distilled water to obtain a concentration range of 40-100 µg/ml. The absorbance of these solutions was measured at the λ_{max} of 279 nm. A graph of concentration vs. absorbance was plotted. Experiment was done in triplicate.

3.3.2 UV-Visible Spectrophotometric Method in Simulated Vaginal Fluid (pH 4.2)

Preparation of Simulated Vaginal Fluid (SVF): SVF was prepared as reported: 3.51 g/l NaCl, 1.40 g/l KOH, 0.222 g/l Ca(OH)2, 0.018 g/l bovine serum albumin (BSA), 2 g/l lactic acid, 1 g/l CH3COOH, 0.16 g/l glycerol, 0.4 g/l urea and 5 g/l glucose. The pH was adjusted to 4.2 with HCl 0.1N. [21]

Preparation of stock solution in SVF: Accurately weighed 10 mg of drug was taken in 10 ml volumetric flask. It was initially dissolved in 4-5 ml of SVF. Then the volume was made up to 10 ml with SVF to get a stock solution of 1 mg/ml (1000 µg/ml).

Determination of Absorbance maxima (λ_{max}): From the stock solution, an aliquot of 0.1 ml was withdrawn and transferred to a 10 ml volumetric flask. It was diluted with SVF up to the mark to obtain 10 µg/ml solution of Leuprolide acetate .UV spectrum of the solution

was recorded using UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan) between the range of 200-400 nm and the wavelength of maximum absorbance was determined.

Preparation of calibration plot: From the stock solution of 1000 µg/ml, aliquots of 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1.0 were withdrawn and further diluted up to 10 ml with SVF to obtain a concentration range of 40-100 µg/ml. The absorbance of these solutions was measured at the λ_{max} of 280 nm. A graph of concentration vs. absorbance was plotted.

3.3.3 Method Validation

The method was validated according to ICH Q2B guideline for validation of analytical procedures in order to determine the linearity, accuracy, precision, limit of detection, and limit of quantification of the analyte. Both, the UV Spectrophotometry methods developed for estimation of Leuprolide acetate in distilled water and SVF, were validated.

3.3.3.1 Linearity

Calibration plot of Leuprolide acetate both in distilled water and SVF was done in the range of 40-100 µg/ml. The graphs obtained by plotting the absorbance versus the concentration data were treated by linear regression analysis. Measurements were done in triplicate.

3.3.3.2 Accuracy

The accuracy of the method was determined by calculating the recoveries of the analyte by the method of standard additions. Known amounts of standard drug (80%, 100% and 120%) were added to the pre-analyzed samples and the absorbance were measured and % recovery was calculated.

3.3.3.3 Precision

The precision was determined by repeatability: intraday and inter-day precision and reported as % RSD for a statistically significant number of replicate measurements. The inter-day precision was studied by comparing the assays on three different days and the results are documented as the standard deviation (SD) and % RSD.

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

LOD and LOQ were based on the standard deviation of the response and the slope of the corresponding plot using the following equations-

LOD = 3 SD/m; LOQ = 10 SD/m

Where SD, is the standard deviation of the blank and m is the slope of the related calibrations graphs.

3.3.4 Analytical Interference study

In order to ascertain the non-interference of the excipients in estimation of Leuprolide acetate, solutions containing known concentration of each excipient were prepared in distilled water. The prepared solutions were scanned in the UV range between 200-400 nm using distilled water as blank. To study the interference in the presence of drug, Leuprolide acetate solution (100 µg/ml) in distilled water was spiked with known concentrations of each excipient (DSPC and Cholesterol) and scanned in the UV range between 200 nm- 400 nm.

3.3.5 Estimation of Leuprolide acetate in Rabbit plasma using LCMS-MS method

Estimation of Leuprolide acetate in rabbit plasma was done using LC-MS/MS method with slight modifications in the method reported by Yan Zhan et al. [22] A liquid chromatography/tandem mass spectrometry (LC- MS/MS) method was developed and validated for the determination of LA in plasma samples. The method was validated in accordance with current acceptance criteria (ICH guidelines).

A) Chromatographic conditions:

- Instrument: LCMS-MS (ekspert^{TM} ultraLC with ekspert^{TM} ultraLC 100 pump system (eksigent-AB Sciex, Framingham, MA) coupled with 3200 QTRAP mass spectrometer (AB Sciex, Framingham, MA)
- Software: Analyst version 1.6.2
- Column: Sigma Supelco 516-C-18-DB (25 cm x 4.6 mm, 5 mm) (Sigma Aldrich, USA)

- SecurityGuard C_{18} guard column (4mm x 3.0mm i.d.; Phenomenex, Torrance, CA, USA)
- Mobile Phase: A mixture of acetonitrile–water–propionic acid (20:80:0.05 v/v/v)
- Elution Pattern: Isocratic
- Flow rate: 0.50 ml/min
- Selected Reaction Monitoring (SRM) Transitions for Leuprolide acetate: m/z 605.5 → m/z (221.0 + 249.0)
- Ionization mode: Positive electrospray
- Injection volume: 20 µL

B) Preparation of Mobile Phase:

All the three solvents i.e. Acetonitrile, Water and Propionic acid were mixed in the ratio of 20:80:0.05 v/v/v. The prepared mobile phase was then bath sonicated for 10 min to remove the air bubbles.

C) Preparation of Stock Solution:

Stock Solution: A stock solution of Leuprolide acetate with a concentration of 100 µg/ml was prepared by dissolving 1 mg of the drug in 10 ml of Methanol: Water (50:50 v/v) solution. From this stock solution, 1 ml aliquot was withdrawn and transferred to 10 ml volumetric flask and diluted to the mark with Methanol: Water mixture (50:50 v/v) to get a second stock of 10 µg/ml. From the 10 µg/ml stock solution, an aliquot of 1 ml was withdrawn and transferred to 10 ml volumetric flask and diluted to the mark with Methanol: Water mixture (50:50 v/v) to get a final stock of 1 µg/ml.

D) Standard working solutions

A series of standard working solutions with concentrations in the range of 2–10 ng/ml for leuprolide were obtained by further dilution of the 1 µg/ml stock solution with methanol–water (50:50, v/v).

E) Preparation of Calibration Plot and samples for method validation:

Calibration plot was prepared by spiking 40 µL of the appropriate standard solution into

200 µL of rabbit serum. Samples for method validation were similarly prepared at concentrations of 4.8, 6, 7.2 ng/ml in rabbit serum, by a separate weighing of the reference compound. All of the solutions were stored at -20°C and were brought to room temperature before use.

F) Sample extraction procedure:

Frozen rabbit serum samples from the rabbits were thawed to room temperature prior to preparation. 40 µL of methanol–water (50:50, v/v), and 200 µL methanol were added to 200 µL of serum sample into the pre-labeled polypropylene vials. The mixture was vigorously vortexed for 1 min and centrifuged (Remi Equipments, Mumbai, India) at 11,300 × g for 5 min at 4°C. The supernatant was mixed with 300 µL water and then transferred to solid-phase extraction tubes (Supelco, Sigma Aldrich, St. Louis, USA) that had been pre-treated sequentially with 2× 1ml of methanol and 2× 1ml of water. After loading the serum sample, the cartridge was washed with 1 ml methanol–water solution (10:90, v/v), then leuprolide was eluted with 2× 1 ml of methanol containing 1% formic acid. The eluate was evaporated to dryness at 40 °C under a gentle stream of nitrogen (Turbovap apparatus (Speedovap, India), and the residue was reconstituted by addition of 100 µL of acetonitrile–water–propionic acid (20:80:0.05, v/v/v). A 20 µL aliquot was injected onto the LC–MS/MS system for analysis. [22]

G) Method Validation:

The method was validated by calculating the validation parameters like Linearity, Accuracy, and LOQ.

Linearity: The standard curve was fitted to linear regression and the equation $y = mx + c$ of the calibration curve obtained was calculated by the quantitative module of Analyst software, where y represents the Leuprolide acetate peak area and x represents the plasma concentration of the Leuprolide acetate. In addition, a blank (no leuprolide) serum sample was also run to eliminate the presence of interferences.

Accuracy: Accuracy of the method was assessed by the determination of samples at three concentration levels (4.8, 6, 7.2 ng/ml) in three replicates. Accuracy was expressed as

relative error (RE), i.e. (observed concentration − nominal concentration)/(nominal concentration)×100%.

LOQ: The lower limit of quantification (LLOQ), taken, as the lowest concentration on the calibration plot that could be measured with acceptable accuracy was determined in three replicates.

3.4 Analytical Methods for estimation of Raloxifene Hydrochloride

3.4.1 UV-Visible Spectrophotometric Method in Methanol: Choloroform (1:9)

Preparation of stock solution: Accurately weighed 10mg of drug was taken in 10 ml volumetric flask. It was initially dissolved in 3-4 ml of Methanol: Chloroform (1:9). Then the volume was made up to 10 ml with the same solvent mixture to get a stock solution of 1 mg/ml (1000 µg/ml).

Determination of Absorbance maxima (λ_{max}): From the stock solution, an aliquot of 0.1 ml was withdrawn and was transferred to a 10 ml volumetric flask. It was diluted with the solvent mixture up to the mark to obtain 10 µg/ml solution of Raloxifene HCl .UV spectrum of the solution was recorded using UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan) between the range of 200-400 nm. Wavelength of maximum absorbance was determined.

Preparation of calibration plot: From the stock solution of 1000 µg/ml, an aliquot of 1.0 ml was withdrawn and further diluted up to 10 ml with the solvent mixture to obtain a concentration of 100 µg/ml. From this stock solution, aliquots of 0.2, 0.4, 0.6, 0.8 and 1 ml were withdrawn and transferred in to separate 10 ml volumetric flasks and further diluted up to the mark with solvent mixture to get the concentration range of 2-10 µg/ml. The absorbance of these solutions was measured at the λ_{max} of 287 nm. A graph of concentration vs. absorbance was plotted.

3.4.2 UV-Visible Spectrophotometric Method in Simulated Vaginal Fluid (pH 4.2)

Preparation of stock solution: Accurately weighed 10mg of drug was taken in 10ml volumetric flask. It was initially dissolved in 2-3ml of Methanol: Chloroform (1:9). Then

the volume was made up to 10 ml with SVF to get a stock solution of 1 mg/ml (1000 µg/ml).

Determination of Absorbance maxima (λ_{max}): From the stock solution, an aliquot of 0.1 ml was withdrawn and was transferred to a 10 ml volumetric flask. It was diluted with the SVF up to the mark to obtain 10 µg/ml solution of Raloxifene HCl .UV spectrum of the solution was recorded using UV-Vis spectrophotometer (UV-1800, Shimadzu, Japan) between the range of 200-400 nm. Wavelength of maximum absorbance was determined.

Preparation of calibration plot: From the stock solution of 1000 µg/ml, an of aliquot of 1.0 ml was withdrawn and further diluted up to 10 ml with SVF to obtain a concentration of 100 µg/ml. From this stock solution, aliquots of 0.2, 0.4, 0.6, 0.8 and 1 ml were withdrawn and transferred in to separate 10ml volumetric flasks and further diluted up to the mark with SVF get the concentration range of 2-10 µg/ml. The absorbance of these solutions was measured at the λ_{max} of 287 nm. A graph of concentration vs. absorbance was plotted.

3.4.3 Method Validation

The methods, both in Methanol: Chloroform (1:9) and in SVF were validated according to ICH Q2B guideline for validation of analytical procedures in order to ensure the linearity, sensitivity (LOD and LOQ), precision and accuracy in analysis of the drug.

3.4.4 Analytical Interference study

In order to ascertain the non-interference of the excipients in estimation of Raloxifene Hydrochloride, solutions containing known concentration of each excipient were prepared in Methanol: Chloroform (1:9). The prepared solutions were scanned in the UV range between 200-400 nm using same solvent mixture as blank. To study the interference in the presence of drug, Raloxifene Hydrochloride solution (10 µg/ml) in Methanol: Chloroform (1:9) was spiked with known concentrations of each excipient (DSPC and Cholesterol) and scanned in the UV range between 200 nm- 400 nm.

3.4.5 Estimation of Raloxifene Hydrochloride in Rabbit plasma using LCMS-MS method

Estimation of Raloxifene Hydrochloride in rabbit plasma was done using LC-MS/MS method with modifications made in the method reported by Jadhav and Ramaa 2012. [23] A liquid chromatography/tandem mass spectrometry (LC- MS/MS) method was developed and validated for the determination of RLX in plasma samples. The method was validated in accordance with current acceptance criteria (ICH guidelines).

A) Chromatographic conditions:

- Instrument: LCMS-MS (ekspert^{TM} ultraLC with ekspert^{TM} ultraLC 100 pump system (eksigent-AB Sciex, Framingham, MA) coupled with 3200 QTRAP mass spectrometer (AB Sciex, Framingham, MA)
- Software: Analyst version 1.6.2
- Column: Sigma Supelco 516-C-18-DB (25 cm x 4.6 mm, 5 mm) (Sigma Aldrich, USA)
- SecurityGuard C_{18} guard column (4mm x 3.0mm i.d.; Phenomenex, Torrance, CA, USA)
- Mobile Phase: Solvent A) 10 mM ammonium formate with 0.1 % formic acid in water; Solvent B) Acetonitrile
- Elution Pattern: Gradient (Gradient flow (T (min)/ % solvent B): 0.01/30, 0.3/30, 0.8/85, 2.25/85, 2.5/30, 4.0/30
- Flow rate: 0.8 ml/min
- Selected Reaction Monitoring (SRM) Transitions for Raloxifene Hydrochloride: m/z 474.30 (precursor ion) and 112.06 (product ion)
- Ionization mode: Positive electrospray
- Injection volume: 10 µL

B) Preparation of Mobile Phase:

Solvent A: Accurately weighed 630.0 mg of ammonium formate was dissolved in distilled water in a 1000 ml volumetric flask. 1.0 ml of formic acid was added and volume was made up to the mark with distilled water. The prepared buffer was then sonicated for 10 min in a bath sonicator to remove the air bubbles.

Solvent B: Acetonitrile (ACN)

C) Preparation of Stock Solution

Stock solution: A stock standard containing a 100 µg/ml Raloxifene Hydrochloride solution was prepared in a 10 ml volumetric flask using ACN: water: (80:20).

D) Standard working solutions

Working solutions of Raloxifene Hydrochloride were prepared in diluent by appropriate dilutions of the standard stock solution. From the standard stock solution of 100 µg/ml, an aliquot of 1 ml was withdrawn and transferred to 10 ml volumetric flask. The solution was then diluted with ACN: water: (80:20) up to the mark to get a stock solution of 10 µg/ml. From the 10 µg/ml stock solution, appropriate aliquots were withdrawn and diluted with ACN: water: (80:20) to give working solutions of 0.05, 0.25, 1.0, 2.5, 5.0, 10.0, and 20.0 µg/ml. All working standard solutions were stored at 4 ^{o}C until further use.

E) Preparation of Calibration Plot and samples for method validation

The calibration plot in rabbit plasma was prepared by spiking 10 µL of working solution of Raloxifene in to rabbit plasma (90 µL). The standard calibration plot, in rabbit plasma, was prepared by using 5, 25, 100, 250, 500, 1000 and 2000 ng/ml plasma concentrations of Raloxifene Hydrochloride. The samples were prepared at three concentrations of 80, 100 and 120 ng/ml Raloxifene Hydrochloride. All the samples were stored at -20 °C until required.

F) Sample extraction procedure

Before extraction, Calibration and samples for validation of method were removed from freezer and thawed at room temperature. 100 µL of plasma samples were added to 50 µL of 0.1% Formic acid solution into the pre-labeled polypropylene vials that were centrifuged for 5 min at 14000 rpm at 4ºC. The supernatant were then subjected to a solid phase extraction (SPE) procedure using Supelco Solid Phase Extraction tubes (Sigma Aldrich, USA). Before the samples were loaded, the SPE cartridges were sequentially conditioned with 1 ml of methanol and 1 ml of water. The Cartridges were washed sequentially with 1 ml water and 1 ml of 10% methanol in water, followed by drying with nitrogen gas for 2 min (25 psi). The elution was performed with 1ml of acetonitrile: methanol (50:50, v/v). The eluants were dried in a stream of nitrogen at 40°C in a Turbovap apparatus (Speedovap, India). The dried samples were reconstituted with 300 µL Acetonitrile: Water (10:90, v/v). 10 µL of the sample was injected into the chromatographic system. [23]

G) Method Validation:

The method was validated by calculating the validation parameters like Linearity, Accuracy, and LOQ.

Linearity: The standard curve was fitted to linear regression and the equation $y = mx + c$ of the calibration curve obtained was calculated by the quantitative module of Analyst software, where y represents the Raloxifene Hydrochloride peak area and x represents the plasma concentration of the Raloxifene Hydrochloride. In addition, a blank (no Raloxifene) serum sample was also run to eliminate the presence of interferences.

Accuracy: Accuracy of the method was assessed by the determination of samples at three concentration levels (80, 100, 120 ng/ml) in three replicates. Accuracy was expressed as relative error (RE), i.e. (observed concentration − nominal concentration)/(nominal concentration)×100%.

LOQ: The lower limit of quantification (LLOQ), taken, as the lowest concentration on the calibration curve that could be measured with acceptable accuracy was determined in three replicates.

3.5 Analytical Method for simultaneous estimation of Leuprolide acetate and Raloxifene Hydrochloride in Simulated Vaginal Fluid

UV-Spectrophotometry method was developed and validated for simultaneous estimation of LA and RLX for determination of both the drug concentrations of the dual drug loaded liposomal formulation during various characterization studies like % Entrapment Efficiency, % Loading and In Vitro drug release.

Concentration of drugs was found by using the simultaneous estimation equation as given below:

Cx (LA) = (A2*ay1- A1*ay2) / (ax2*ay1 – ax1*ay2).....(1)

Cy (RLX) = (A1*ax2- A2*ax1) / (ax2*ay1 – ax1*ay2).....(2)

A1 &A2 = Absorbance of LA & RLX At 280 nm & 287 nm respectively

Cx & Cy = Conc. of LA & RLX respectively, in samples.

ax1 & ax2 = Absorptivity of LA at 280 nm & 287 nm respectively

ay1 & ay2 = Absorptivity of RLX at 280 nm & 287 nm respectively

Standard solutions of LA and RLX in the concentration range of 40-100 µg/ml and 2-10 µg/ml respectively were prepared according to the method described previously in sections 3.3.2 and 3.4.2 Calibration curves were plotted to verify the Beer's law and the absorptivity values were calculated at the respective wavelengths for both the drugs.

Absorptivity= Absorbance/Concentration of drug

To check the accuracy of the proposed method, recovery studies were carried out by standard addition method at three different levels according to ICH guidelines. A series of solutions of LA and RLX at 80%, 100%, and 120% of the standard preparation were prepared and checked for accuracy by determining the absorbance values at λmax of 280 nm and 287 nm respectively. Precision studies and determination of LOD and LOQ was also carried out as per the methods described in section 3.3.3 and 3.4.3.

3.6 Results and Discussion

3.6.1 Estimation of Leuprolide acetate

3.6.1.1 UV- Spectrophotometric Method for estimation of LA in Distilled Water

Leuprolide acetate solution in distilled water shows the absorption maxima at 279 nm. Linearity was observed in the concentration range of 40 to 100 µg/ml. Figure 3.1 shows the calibration plot Figure 3.2 shows the overlay plot of spectra at 279 nm. The results of the same have been mentioned in Table 3.1.

Table 3.1: Calibration data for LA in distilled water

Concentration in µg/ml	**Absorbance ±SD***
0	0
40	0.190±0.005
50	0.231±0.005
60	0.282±0.001
70	0.324±0.002
80	0.367±0.005
90	0.417±0.003
100	0.469±0.006

* experiment was done in triplicate (n=3)

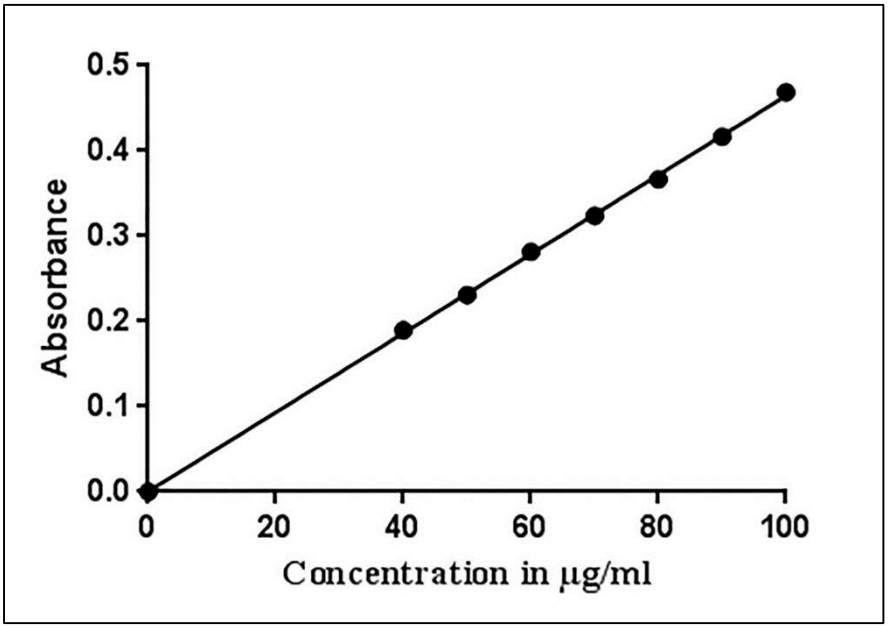

Figure 3.1 Calibration plot of LA in distilled water

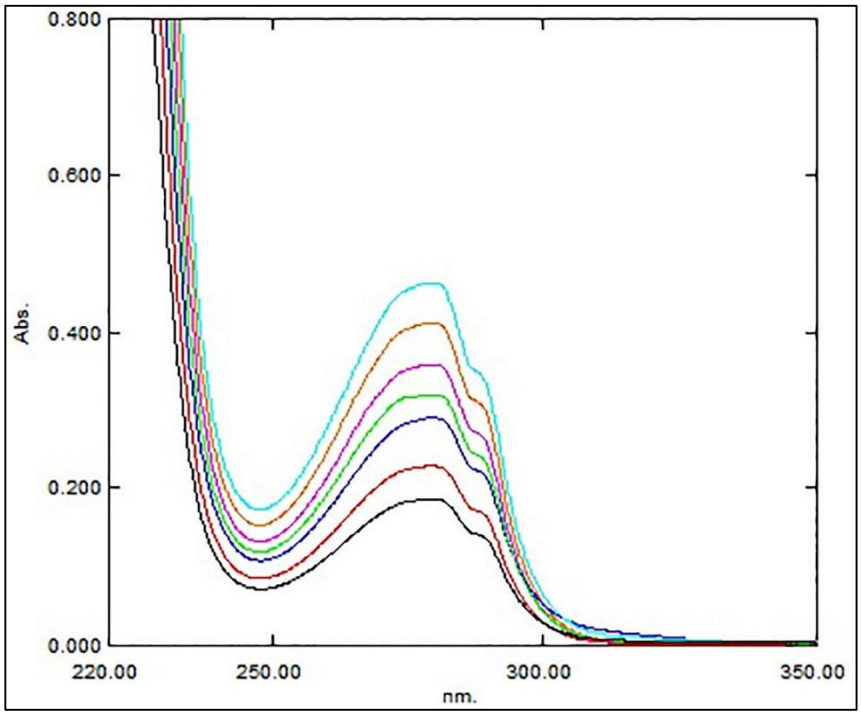

Figure 3.2 Overlay plot of LA in distilled water in 279 nm

The regression analysis of the plot using the method of least squares was made to evaluate the intercept, slope and correlation coefficient (R^2). The high value of correlation coefficient of the regression equation and the negligible value of intercept confirm the linearity of calibration plot. The parameters of the regression analysis have been mentioned in Table 3.2.

Table 3.2 Parameters for estimation of LA in distilled water by UV spectrophotometry

Parameters	**Results**
λmax	279 nm
Linearity range	40-100 µg/ml
Regression Equation	y = 0.0046x + 0.0008
Correlation Co-efficient	0.9998
Molar absorptivity	5865.92 l $mol^{-1}cm^{-1}$

3.6.1.2 UV- Spectrophotometric Method for estimation of LA in SVF

Leuprolide acetate solution in SVF shows the absorption maxima at 280 nm. Linearity was observed in the concentration range of 40 to 100 µg/ml. Figure 3.3 shows the calibration plot and Figure 3.4 shows the overlay spectra at 280 nm. The results of the same have been depicted in Table 3.3.

Table 3.3: Calibration data for LA in SVF

Concentration in µg/ml	**Absorbance ± SD ***
0	0
40	0.186±0.001
50	0.241±0.003
60	0.287±0.005
70	0.335±0.006
80	0.377±0.005
90	0.425±0.002
100	0.472±0.001

* experiment was done in triplicate (n=3)

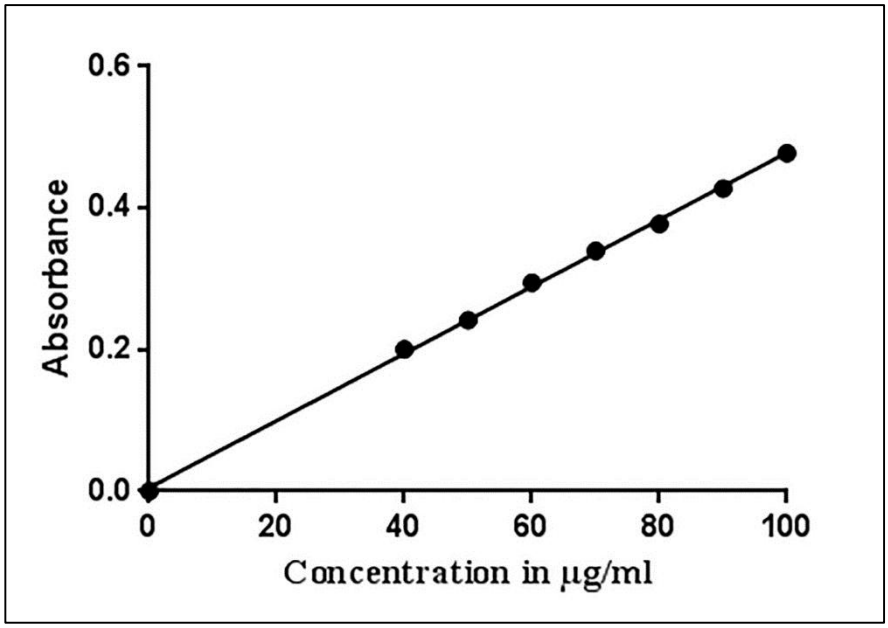

Figure 3.3 Calibration plot of LA in SVF pH 4.2

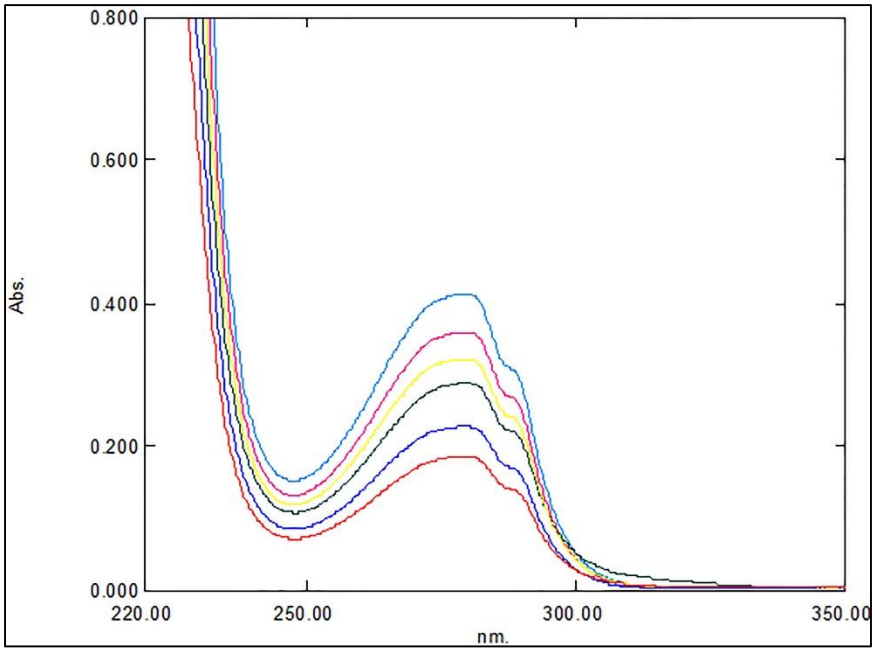

Figure 3.4 Overlay plot of LA in SVF pH 4.2 at 280 nm

The regression analysis of the plot using the method of least squares was made to evaluate the intercept, slope and correlation coefficient (R^2). The high value of correlation coefficient of the regression equation and the negligible value of intercept confirm the linearity of calibration plot. The parameters of the regression analysis have been mentioned in Table 3.4.

Table 3.4 Parameters for estimation of LA in SVF by UV spectrophotometry

Parameters	Results
λmax	280 nm
Linearity range	40-100 µg/ml
Regression Equation	y = 0.0047x + 0.005
Correlation Co-efficient	0.9990
Molar absorptivity	6119.85 l $mol^{-1}cm^{-1}$

3.6.1.3 Results of Method Validation

The methods were validated for Linearity, Accuracy, Precision, LOD and LOQ.

Accuracy: The results of accuracy are shown in Table 3.5 and 3.6 for the methods developed in distilled water and SVF respectively. % Relative Standard Deviation (RSD) was found be less than 2 %, which suggest that, the methods were accurate. There was no significant difference between true values and observed values at all the concentrations levels.

Table 3.5: Accuracy results for estimation of LA in distilled water

Quantity of Leuprolide acetate added	Actual conc. (µg/ml)	Obtained conc. (µg/ml)	% Recovery	%RSD
80%	56	57.8±0.001	103.21	1.24
100%	70	72.8±0.001	104	0.32
120%	84	86.8±0.002	100.9	0.26

*experiment was done in triplicate (n=3)

Table 3.6: Accuracy results for estimation of LA in SVF

Quantity of Leuprolide acetate added	Actual conc. (µg/ml)	Obtained conc. (µg/ml)	% Recovery	%RSD
80%	64	65.5±0.001	102.34	1.06
100%	80	79.1±0.002	98.87	0.63
120%	96	98.3±0.002	103.39	1.62

* experiment was done in triplicate (n=3)

Precision: The results of precision are given in Table 3.7 and 3.8 for the methods developed in distilled water and SVF respectively. As seen in the tables less than 2 % RSD values indicates the precision of method.

Table 3.7: Precision results for estimation of LA in distilled water

Actual Concentration (µg/ml)	Intraday precision			Interday precision		
	Observed Conc. µg/ml	% Recovery	%RSD	Observed Conc. µg/ml	% Recovery	% RSD
80	80.03±0.001	100.03	0.02	79.95±0.002	99.93	0.13
90	90.12±0.003	100.13	0.09	89.87±0.004	99.85	0.10
100	101.5±0.002	101.5	1.05	99.97±0.001	99.97	0.02

* experiment was done in triplicate (n=3)

Table 3.8: Precision results for estimation of LA in SVF

Actual Concentration (µg/ml)	Intraday precision			Interday precision		
	Observed Conc. µg/ml	% Recovery	%RSD	Observed Conc. µg/ml	% Recovery	%RSD
80	80.93±0.002	101.16	0.82	80.15±0.002	100.18	0.13
90	90.25±0.001	100.27	0.20	91.03±0.003	101.14	0.80
100	101.87±0.002	101.87	1.31	101.95±0.002	101.95	1.37

* experiment was done in triplicate (n=3)

LOD and LOQ: Limit of Detection and Limit of Quantification was calculated as per the formula given below

LOD = 3*(S.D/m) ; LOQ= 10*(S.D/m), where SD, is the standard deviation of the blank and m is the slope of the calibrations plot.

LA could be precisely detected and quantified at 0.652 µg/ml and 2.17 µg/ml in distilled water. Where LOD and LOQ values of LA estimation in SVF were 1.27 µg/ml and 4.25 µg/ml.

3.6.1.4 Analytical Interference Studies of LA

Concentration of excipients were taken at approximate level at which they are present in final formulation. The absorbance values of DSPC, Cholesterol, Mannitol and Gelatin excipients at 279 nm were very negligible indicating that these excipients would not interfere in the estimation of Leuprolide acetate. Absorbance values of 100 µg/ml solution of LA with and without excipients is shown in Table 3.9

Table 3.9: Results of Analytical Interference Studies for LA

Leuprolide acetate solution (100 µg/ml) without excipients	**Leuprolide acetate solution (100 µg/ml) with excipients (DSPC and Cholesterol)**
0.470±0.002	0.470±0.104

* experiment was done in triplicate (n=3)

3.6.1.5 Estimation of Leuprolide acetate in rabbit plasma by LCMS-MS method

In this study, sharp peak of Leuprolide acetate was obtained with mobile phase of the mixture acetonitrile–water–propionic acid (20:80:0.05 v/v/v). The retention time of LA was found to be 3.72 min. The results of the standard calibration plot have been shown in Table 3.10. Figure 3.5 shows the calibration plot of estimation of LA in rabbit plasma by LCMS-MS method. Blank serum was also run to see the interference in the analysis of drug. The chromatograms for the standard calibration plot are shown in Figure 3.6. Figure

explains that the blank serum did not show any peak during the run time of 8 min which suggest the non-interference during the estimation of drug.

Table 3.10 Calibration data for LA in rabbit plasma by LCMS-MS

Concentration (ng/ml)	**Peak Area ± SD**
0	0
2	230636±1939.11
4	481272±1182.17
6	771908±1859.30
8	992544±1740.69
10	1153180±3881.21

* experiment was done in triplicate (n=3)

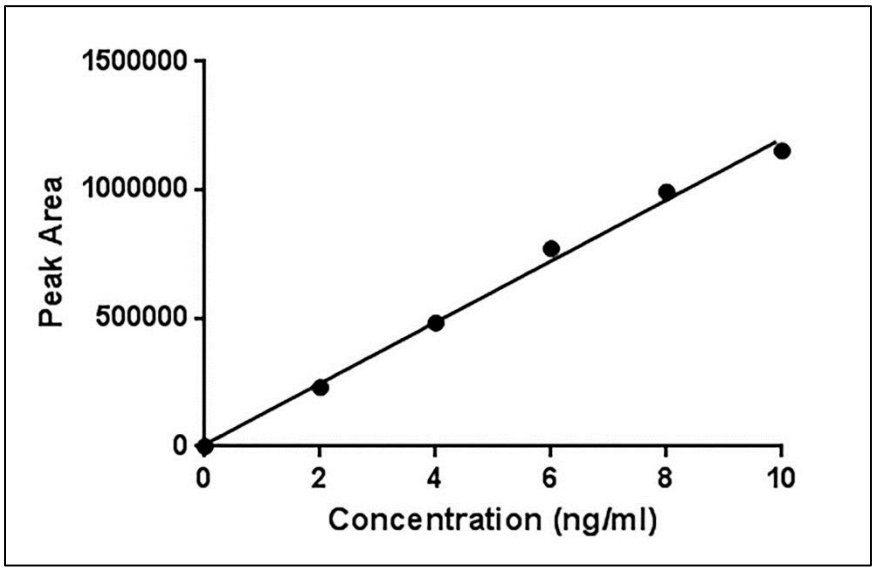

Figure 3.5 Standard Calibration plot of LA in rabbit plasma by LCMS-MS method

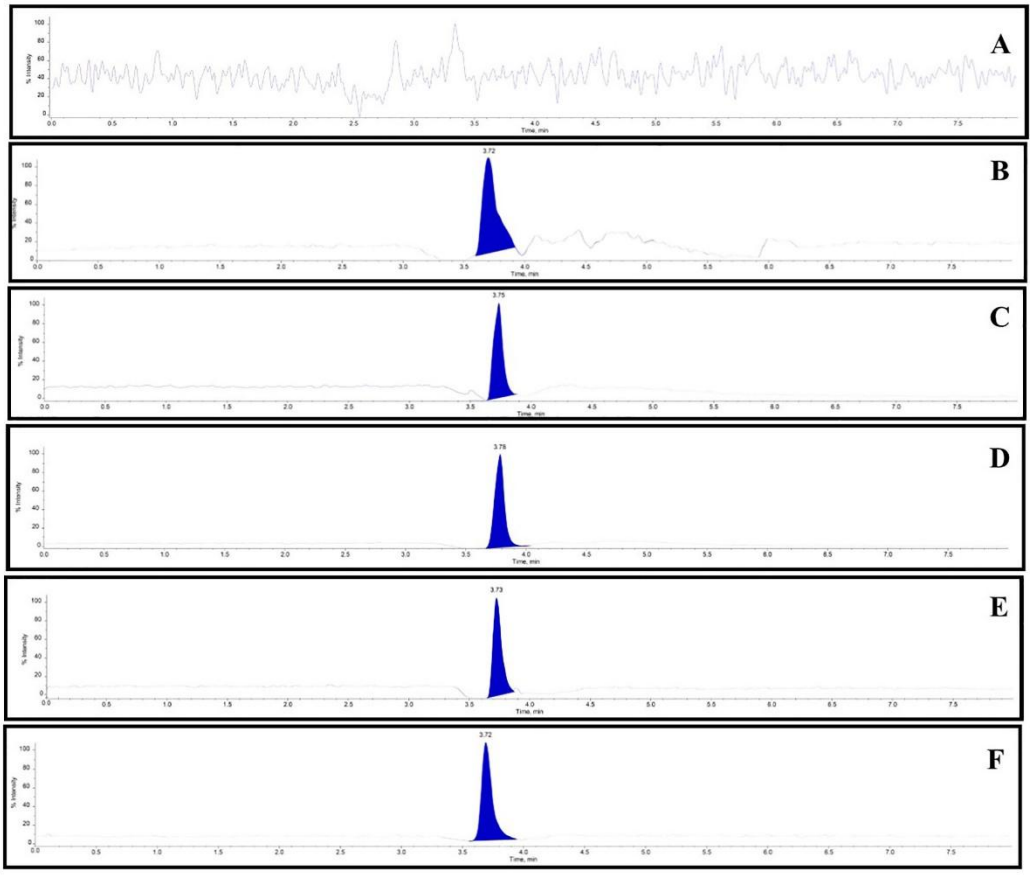

Figure 3.6 Chromatograms of calibration plot of LA showing retention time of 3.72-3.78 min (A- Blank, B- 2 ng/ml, C- 4ng/ml, D- 6 ng/ml, E- 8ng/ml, F- 10ng/ml)

The method developed was validated for Linearity, Accuracy and LOQ. The results of Linearity are shown in Table 3.11. High value of R^2 0.994 indicates that the method was linear for the range 2-10 ng/ml.

Table 3.11 Parameters for estimation of LA in rabbit plasma by LCMS-MS

Parameters	Results
Linearity range	2-10 ng/ml
Regression Equation	y = 119175x + 9047.6
Correlation Co-efficient	0.99416

Results of accuracy as seen in Table 3.12 suggest that the method was accurate with % RSD less than 2%.

Table 3.12 Accuracy results for estimation of LA by LCMS-MS

Actual conc. (ng/ml)	Retention Time	Peak Area± SD	Obtained Conc.	% Recovery	% RSD
4.8	3.77	617526±1024.45	4.87	101.45	1.02
6	3.78	771908±1859.30	6.03	100.5	0.35
7.2	3.71	926289±1456.27	7.29	101.25	0.88

* experiment was done in triplicate (n=3)

LA could be precisely detected and quantified at 2 ng/ml in rabbit plasma, which was the lower limit of quantification by the method developed.

3.6.2 Estimation of Raloxifene Hydrochloride

3.6.2.1 UV- Spectrophotometric Method for estimation of RLX in Methanol: Chloroform (1:9)

Raloxifene Hydrochloride solution in methanol: chloroform shows the absorption maxima at 287 nm. Linearity was observed in the concentration range of 2 to 10 µg/ml. Figure 3.7 shows the calibration plot and Figure 3.8 shows the overlay plot at 287 nm. The results of the same have been mentioned in Table 3.13.

Table 3.13: Calibration data for RLX in Methanol: Chloroform (1:9)

Concentration (µg/ml)	Absorbance ± SD *
0	0
2	0.113±0.001
4	0.245±0.002
6	0.380±0.001
8	0.531±0.003
10	0.672±0.002

* experiment was done in triplicate (n=3)

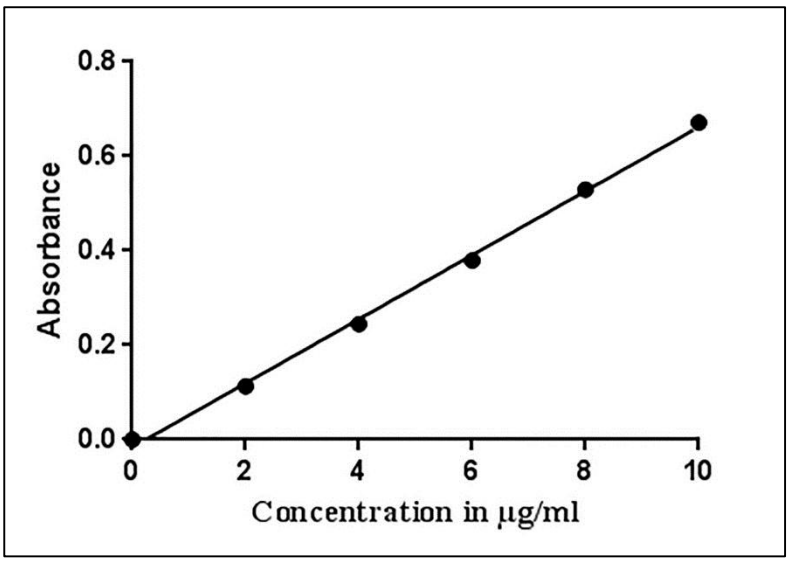

Figure 3.7 Calibration plot of RLX in Methanol: Chloroform (1:9)

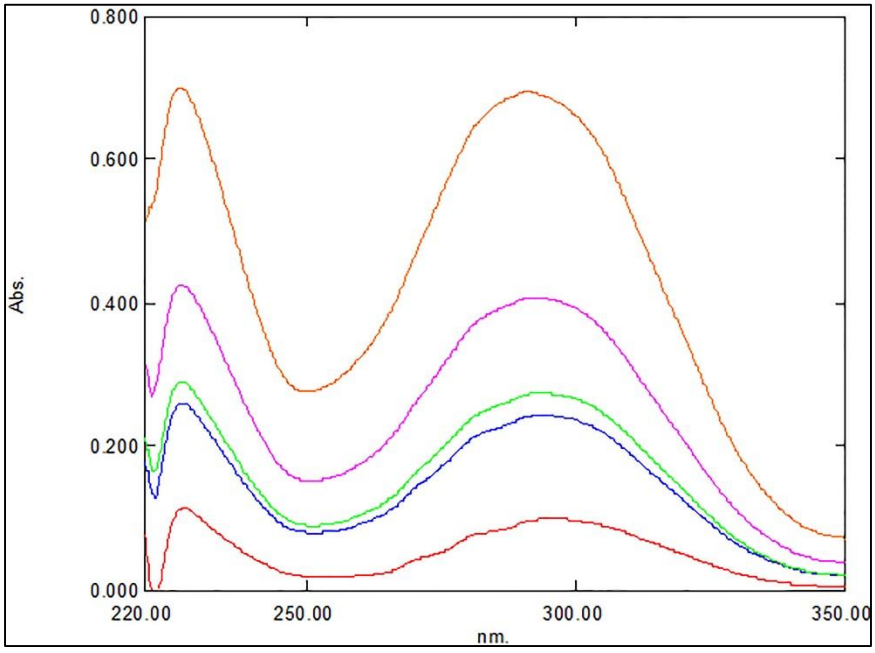

Figure 3.8 Overlay plot of RLX in Methanol: Chloroform (1:9) at 287 nm

The regression analysis of the plot using the method of least squares was made to evaluate the intercept, slope and correlation coefficient (R^2). The high value of correlation coefficient of the regression equation and the negligible value of intercept confirm the linearity of calibration plot. The parameters of the regression analysis have been mentioned

in Table 3.14.

Table 3.14 Parameters for estimation of RLX in Methanol: Chloroform (1:9)

Parameters	Results
λmax	287 nm
Linearity range	2-10 µg/ml
Regression Equation	Y= 0.0678x - 0.0157
Correlation Co-efficient	0.9980
Molar absorptivity	31250 l $mol^{-1}cm^{-1}$

3.6.2.2 UV- Spectrophotometric Method for estimation of RLX in SVF

RLX solution in SVF shows the absorption maxima of 287 nm. There was no change in the λmax of the drug. Linearity was observed in the concentration range of 2 to 10 µg/ml. Figure 3.9 shows the calibration plot and Figure 3.10 shows the overlay plot at 287 nm. The results of the same have been shown in Table 3.15.

Table 3.15: Calibration data for RLX in SVF

Concentration (µg/ml)	Absorbance ± SD *
0	0
2	0.119±0.002
4	0.254±0.003
6	0.387±0.005
8	0.523±0.001
10	0.662±0.001

* experiment was done in triplicate (n=3)

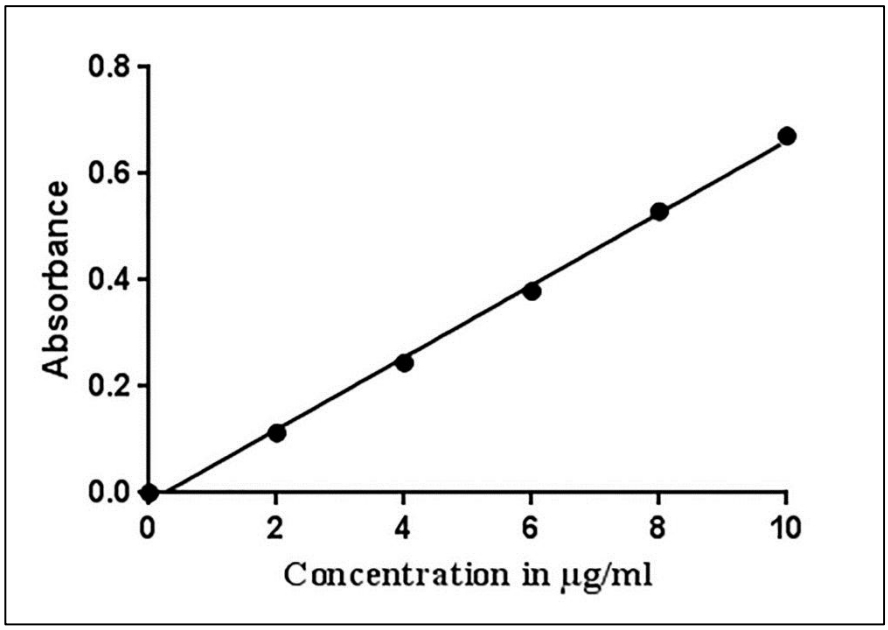

Figure 3.9 Calibration plot of RLX in SVF pH 4.2

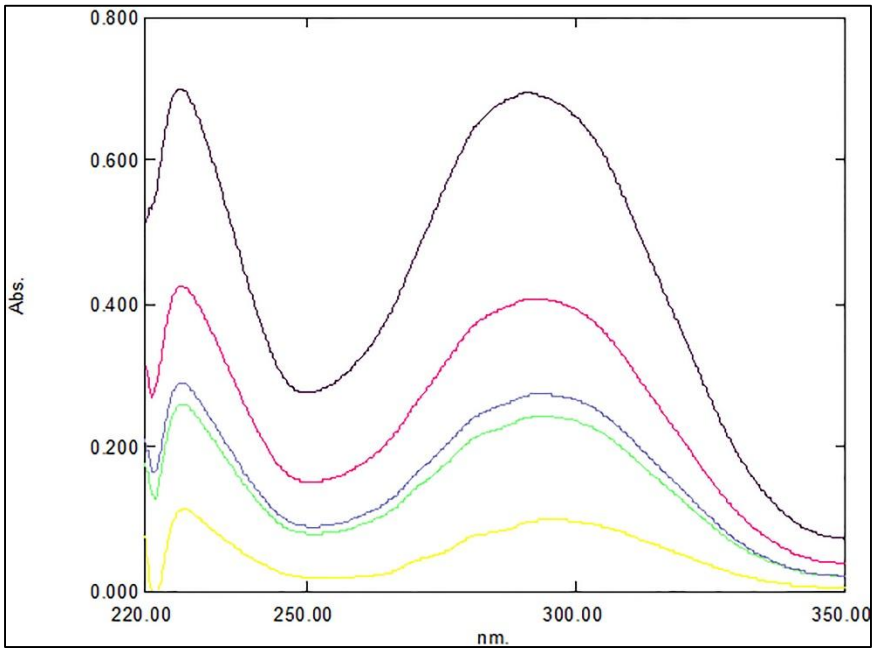

Figure 3.10 Overlay plot of RLX in SVF pH 4.2 at 287 nm

The regression analysis of the plot using the method of least squares was made to evaluate the intercept, slope and correlation coefficient (R^2). The high value of correlation coefficient of the regression equation and the negligible value of intercept confirm the linearity of calibration plot. The parameters of the regression analysis have been shown in Table 3.16.

Table 3.16 Parameters for estimation of RLX in SVF by UV spectrophotometry

Parameters	Results
λmax	287 nm
Linearity range	2-10 µg/ml
Regression Equation	y = 0.0665x-0.0083
Correlation Co-efficient	0.99949
Molar absorptivity	31250 l $mol^{-1}cm^{-1}$

3.6.2.3 Results of Method Validation

The methods were validated for Linearity, Accuracy, Precision, LOD and LOQ.

Accuracy: The results of accuracy are given in Table 3.17 and 3.18 for the methods developed in Methanol: Chloroform (1:9) and SVF respectively. % Relative Standard Deviation (RSD) was found be less than 2 %, which suggest that, the methods were accurate. There was no significant difference between true values and observed values at all the concentrations levels.

Table 3.17: Accuracy results for estimation of RLX in Methanol: Chloroform (1:9)

Quantity of Raloxifene Hydrochloride added	Actual conc. (µg/ml)	Obtained conc. (µg/ml)	% Recovery	%RSD
80%	8	7.97±0.001	99.62	0.19
100%	10	10.13±0.001	101.3	0.41
120%	12	12±0.002	100	0.01

* experiment was done in triplicate (n=3)

Table 3.18: Accuracy results for estimation of RLX in SVF

Quantity of Leuprolide acetate added	Actual conc. (µg/ml)	Obtained conc. (µg/ml)	% Recovery	%RSD
80%	8	8.12±0.001	101.5	1.05
100%	10	10.10±0.002	101	0.70
120%	12	12.05±0.002	100.41	0.29

*experiment was done in triplicate (n=3)

Precision: The results of precision study are given in Table 3.19 and 3.20 for the methods developed in Methanol: Chloroform (1:9) and SVF respectively. As seen in the tables less than 2 % RSD values indicates the precision of method.

Table 3.19: Precision results for estimation of RLX in Methanol: Chloroform (1:9)

Actual Concentration (µg/ml)	Intraday precision			Interday precision		
	Observed Conc. µg/ml	% Recovery	%RSD	Observed Conc. µg/ml	% Recovery	%RSD
2	2.02±0.001	101	0.70	2.03±0.002	101.5	1.05
6	6.05±0.003	100.83	0.59	6.14±0.004	102.33	1.63
10	10.06±0.002	100.6	0.42	10.05±0.001	100.5	0.35

* experiment was done in triplicate (n=3)

Table 3.20: Precision results for estimation of RLX in SVF

Actual Concentration (µg/ml)	Intraday precision			Interday precision		
	Observed Conc. µg/ml	% Recovery	%RSD	Observed Conc. µg/ml	% Recovery	%RSD
2	2.01±0.001	100.5	0.35	2.02±0.002	101	0.70
6	6.07±0.003	101.16	0.82	6.08±0.003	101.33	0.94
10	10.04±0.002	100.4	0.28	10.03±0.003	100.3	0.21

* experiment was done in triplicate (n=3)

LOD and LOQ: Limit of Detection and Limit of Quantification was calculated as per the formula given below

LOD = 3*(S.D/m) ; LOQ= 10*(S.D/m), where SD, is the standard deviation of the blank and m is the slope of the calibrations plot.

RLX could be precisely detected and quantified at 0.044 µg/ml and 0.147 µg/ml in Methanol: Chloroform (1:9). Where LOD and LOQ values of RLX estimation in SVF were 0.045 µg/ml and 0.150 µg/ml.

3.6.2.4 Analytical Interference Studies of RLX

Concentration of excipients were taken at approximate level at which they are present in final formulation. The absorbance values of DSPC, Cholesterol, Mannitol and Gelatin excipients at 287 nm were very negligible indicating that these excipients would not interfere in the estimation of Raloxifene Hydrochloride. Absorbance values of 10 µg/ml solution of RLX with and without excipients is given in Table 3.21

Table 3.21: Results of Analytical Interference Studies of RLX

Raloxifene Hydrochloride solution (10 µg/ml) without excipients	**Raloxifene Hydrochloride solution (10 µg/ml) with excipients (DSPC and Cholesterol)**
0.672±0.002	0.671±0.102

* experiment was done in triplicate (n=3)

3.6.2.5 Estimation of Raloxifene Hydrochloride in rabbit plasma by LCMS-MS method

A sharp peak of Raloxifene Hydrochloride was obtained with a mobile phase consisting of Solvent A) 10 mM ammonium formate with 0.1 % formic acid in water; Solvent B) Acetonitrile run in gradient elution pattern. Under the present chromatographic conditions, the retention time of RLX was found to be 1.68-1.79 min. The results of the standard calibration plot have been given in Table 3.22. Figure 3.11 shows the calibration plot of estimation of RLX in rabbit plasma by LCMS-MS method. Blank serum was also run to see the interference in the analysis of drug. The chromatograms for the standard calibration plot are shown in Figure 3.12. Figure explains that the blank serum did not show any peak during the run time of 8 min, which suggest the non-interference during the estimation of drug.

Table 3.22 Calibration data for RLX in rabbit plasma by LCMS-MS

Concentration (ng/ml)	**Peak Area ± SD**
0	0
5	83108±347.285
25	420540±9830.544
100	1522160±1657.792
250	4005400±2489.318
500	8610800±2533.699
1000	17221600±715.549
2000	32443200±2545.883

* experiment was done in triplicate (n=3)

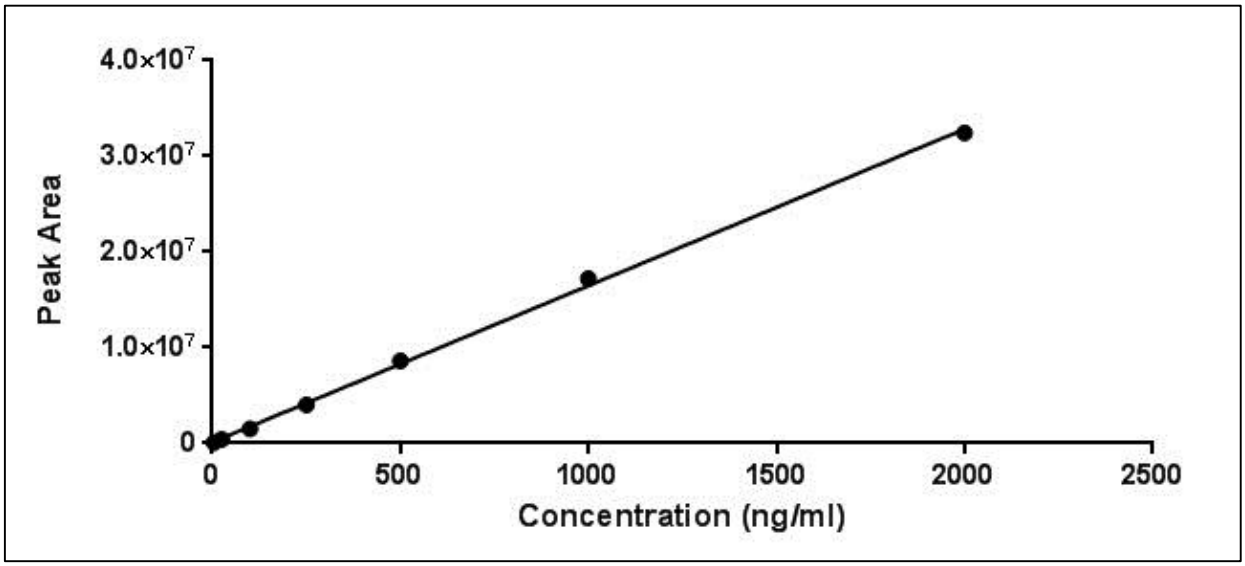

Figure 3.11 Standard Calibration plot of RLX in rabbit plasma by LCMS-MS method

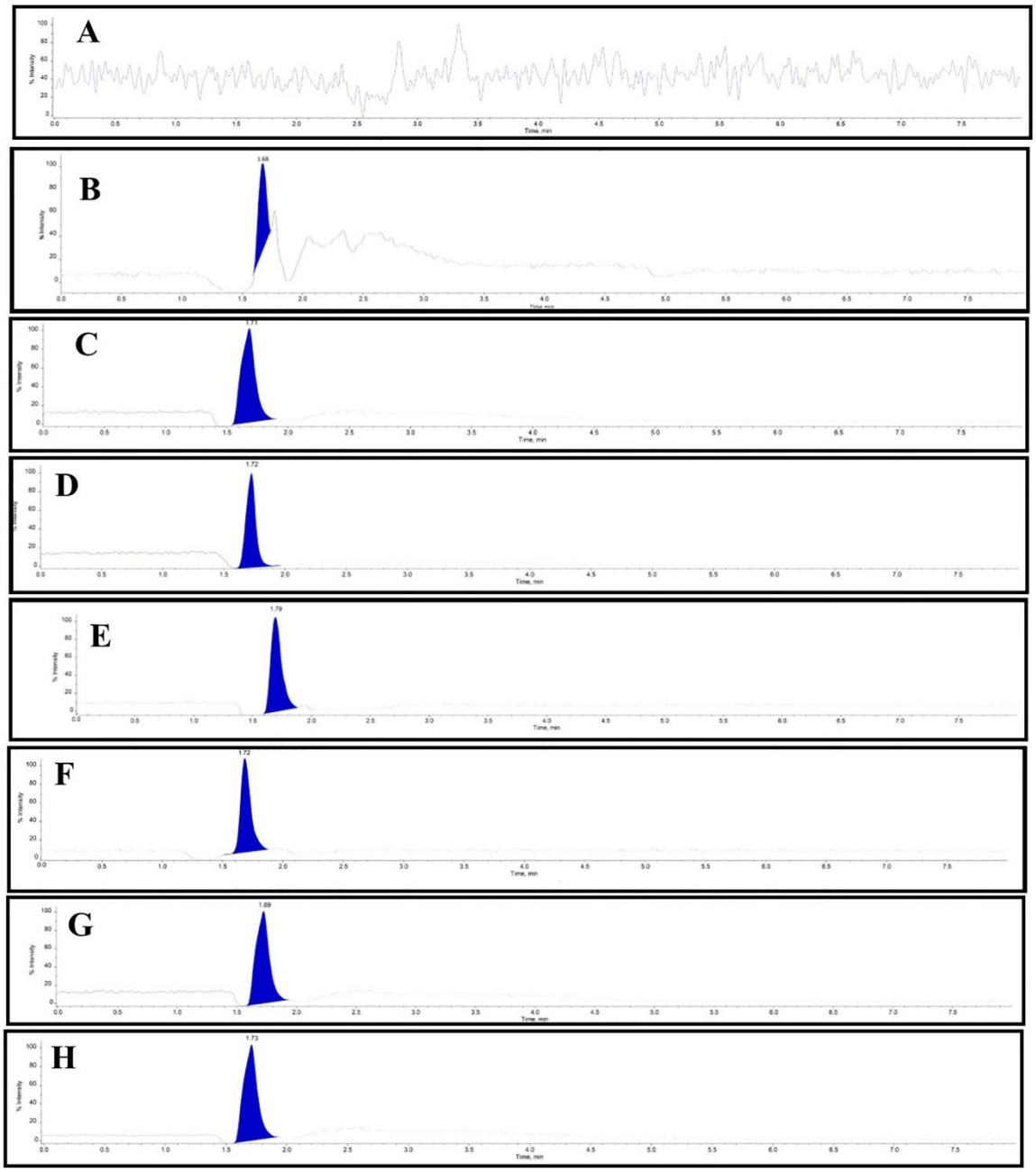

Figure 3.12 Chromatograms of calibration plot of RLX showing retention time of 1.68-1.79 min (A-Blank, B- 5 ng/ml, C- 25 ng/ml, D- 100 ng/ml, E- 250 ng/ml, F- 500 ng/ml, G- 1000 ng/ml, H- 2000 ng/ml)

The method developed was validated for Linearity, Accuracy and LOQ. The results of Linearity have been given in Table 3.23. High value of R^2 0.9989 indicates that the method was linear for the range 5-2000 ng/ml.

Table 3.23 Parameters for estimation of RLX in rabbit plasma by LCMS-MS

Parameters	Results
Linearity range	5-2000 ng/ml
Regression Equation	y = 16370x + 113139
Correlation Co-efficient	0.9989

Results of accuracy as seen in Table 3.24 suggest that the method was accurate with % RSD less than 2%.

Table 3.24 Accuracy results for estimation of RLX by LCMS-MS method

Actual conc. (ng/ml)	Retention Time	Peak Area	Obtained Conc.	% Recovery	% RSD
80	1.68	1217728±1145.890	80.57	100.71	0.50
100	1.72	1522160±1657.792	100.13	100.13	0.09
120	1.74	1826592±2345.765	120.09	100.07	0.05

* experiment was done in triplicate (n=3)

RLX could be precisely detected and quantified at 5 ng/ml in rabbit plasma, which was the lower limit of quantification by the method developed.

3.6.3 Simultaneous estimation of LA and RLX in SVF by UV-Spectrophotometry

The results of simultaneous estimation of LA and RLX revealed that the drugs didn't interfere in the absorbance of each other. Table 3.25 and 3.26 shows the data for linearity of LA and RLX in SVF for simultaneous estimation, respectively. Figure 3.13 and 3.14 shows the calibration plots of LA and RLX in SVF pH 4.2 for simultaneous estimation.

Table 3.25 Calibration plot of LA in SVF for Simultaneous estimation

Concentration (µg/ml)	**Absorbance at 280 nm**	**Absorbance at 287 nm**	**Absorptivity at 280 nm**	**Absorptivity at 287 nm**
0	0	0	0	0
40	0.185±0.005	0.031±0.002	0.0046	0.0007
50	0.246±0.004	0.012±0.001	0.0049	0.0002
60	0.292±0.002	0.015±0.003	0.0048	0.0002
70	0.344±0.001	0.021±0.002	0.0049	0.0003
80	0.385±0.002	0.010±0.003	0.0048	0.0001
90	0.430±0.003	0.023±0.001	0.0047	2.5e-4
100	0.480±0.001	0.041±0.003	0.0048	0.00041

* experiment was done in triplicate (n=3)

Table 3.26 Calibration plot of RLX in SVF for Simultaneous estimation

Concentration (µg/ml)	**Absorbance at 287 nm**	**Absorbance at 280 nm**	**Absorptivity at 287 nm**	**Absorptivity at 280 nm**
0	0	0	0	0
2	0.115±0.005	0.01±0.002	0.057	0.0050
4	0.250±0.004	0.03±0.001	0.062	0.0075
6	0.383±0.002	0.05±0.003	0.063	0.0083
8	0.525±0.001	0.02±0.001	0.065	0.0025
10	0.659±0.002	0.04±0.003	0.066	0.0040

* experiment was done in triplicate (n=3)

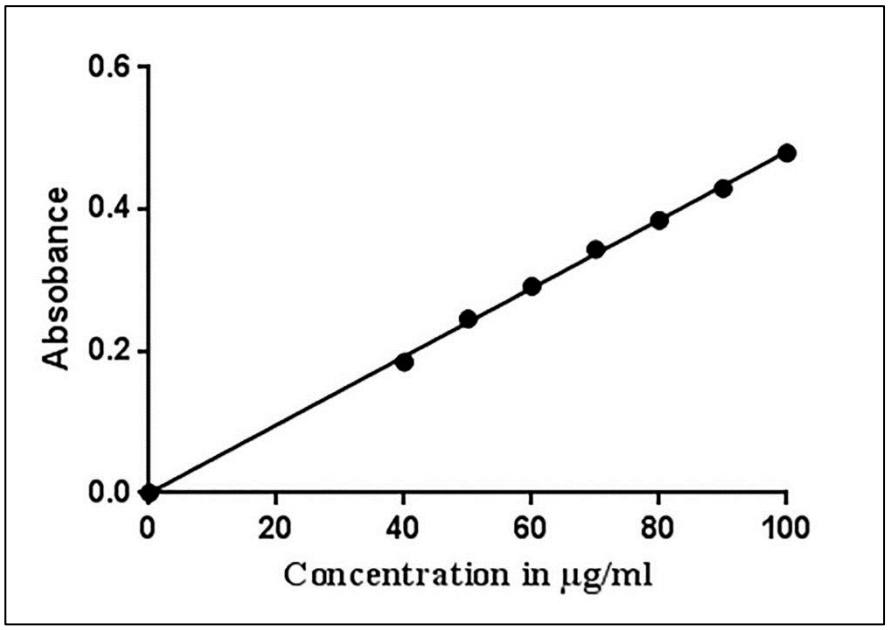

Figure 3.13 Calibration plot of LA in SVF pH 4.2 (for simultaneous estimation)

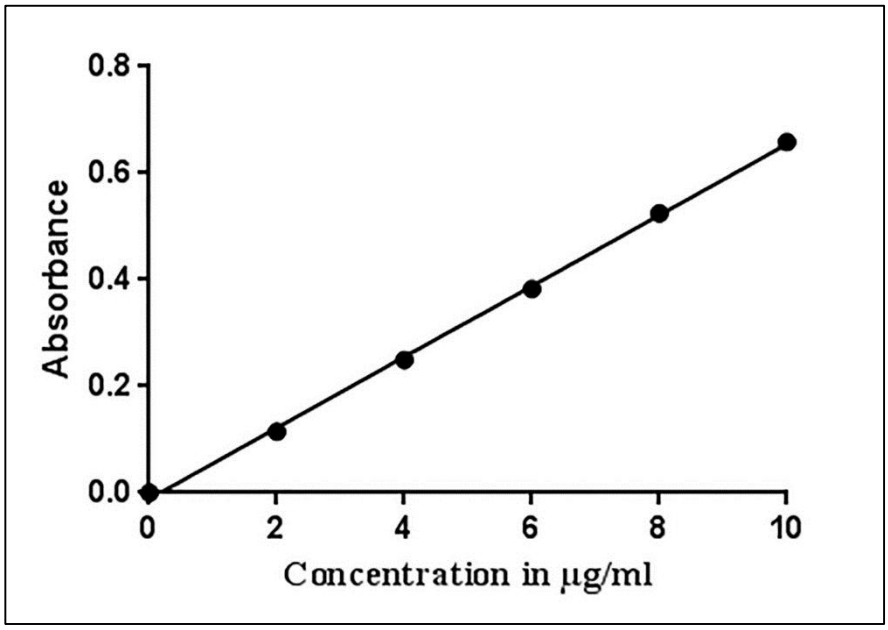

Figure 3.14 Calibration plot of RLX in SVF pH 4.2 (for simultaneous estimation)

Results of Accuracy are given in Table 3.27. % RSD was less than 2 %, which suggest that the method was accurate.

Table 3.27 Accuracy results for simultaneous estimation of LA and RLX in SVF

Levels	Actual concentration (µg/ml)		Observed concentration (µg/ml)		% Recovery		% RSD	
	LA	RLX	LA	RLX	LA	RLX	LA	RLX
80%	64	8	64.22±0.001	8.05±0.002	100.34	100.62	0.24	0.44
100%	80	10	80.13±0.003	10.10±0.001	100.16	101	0.11	0.70
120%	96	12	97.45±0.003	12.14±0.002	101.51	101.16	1.06	0.82

* experiment was done in triplicate (n=3)

Table 3.28 and 3.29 shows the Intraday and Interday precision results for LA and RLX respectively for simultaneous estimation in SVF. % RSD values were less than 2 % indicating the precision of the method.

Table 3.28 Precision results for simultaneous estimation of LA in SVF

Actual Concentration (µg/ml)	Intraday precision			Interday precision		
	Observed Conc. µg/ml	% Recovery	%RSD	Observed Conc. µg/ml	% Recovery	%RSD
80	80.11±0.002	100.13	0.1	80.15±0.002	100.18	0.13
90	90.07±0.001	100.07	0.05	90.14±0.001	100.15	0.11
100	100.04±0.003	100.04	0.28	100.24±0.003	100.24	0.17

* experiment was done in triplicate (n=3)

Table 3.29 Precision results for simultaneous estimation of RLX in SVF

Actual Concentration (µg/ml)	Intraday precision			Interday precision		
	Observed Conc. µg/ml	% Recovery	%RSD	Observed Conc. µg/ml	% Recovery	%RSD
2	2.02±0.005	101	0.70	2.05±0.003	102.5	1.75
6	6.07±0.004	101.16	0.82	6.09±0.002	101.5	1.05
10	10.04±0.003	100.4	0.28	10.14±0.004	101.4	0.98

* experiment was done in triplicate (n=3)

The developed method for simultaneous estimation of LA and RLX in SVF pH 4.2 could precisely detect and quantify 1.30 µg/ml and 4.34 µg/ml of LA while 0.140 µg/ml and 0.476 µg/ml of RLX.

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