4. ANALYTICAL METHOD DEVELOPMENT

4.1 INTRODUCTION

Analytical method development is the process of finding a suitable approach for a certain application. It is often used to figure out a formulation's composition. It is the process of showing that an analytical method is suitable for measuring the concentration of future samples (1). The important parameters that may be considered during method development are linearity, range, accuracy, precision, Limits of Detection (LOD) and Limit of Quantitation (LOQ) (2).

4.2 ANALYSIS OF FULVESTRANT BY UV VISIBLE SPECTROSCOPY

4.2.1 Calibration plot of Fulvestrant in Acetonitrile

Standard stock solution was prepared by dissolving 100 mg of Fulvestrant in 100 ml of Acetonitrile (1000 µg/ml). Consequent aliquots were prepared by dilutions accordingly to make different concentrations ranging from 25 – 300 µg/ml. The absorbance of these solutions was measured using UV – Visible Spectrophotometer (UV – 1900, Shimadzu).

4.2.2 Calibration plot of Fulvestrant in Methanol

Standard stock solution was prepared by dissolving 100 mg of Fulvestrant in 100 ml of Methanol (1000 µg/ml). Consequent aliquots were prepared by dilutions accordingly to make different concentrations ranging from 25 – 300 µg/ml. The absorbance of these solutions was measured using UV – Visible Spectrophotometer (UV – 1900, Shimadzu).

4.2.3 Calibration plot of Fulvestrant in Tetrahydrofuran

Standard stock solution was prepared by dissolving 100 mg of Fulvestrant in 100 ml of Tetrahydrofuran (1000 µg/ml) (3). Consequent aliquots were prepared by dilutions accordingly to make different concentrations ranging from 10 – 60 µg/ml. The absorbance of these solutions was measured using UV – Visible Spectrophotometer (UV – 1900, Shimadzu).

4.2.4 Calibration plot of Fulvestrant in Acetate Buffer pH 5.5

Standard stock solution was prepared by dissolving 100 mg of Fulvestrant in 100 ml of 10% Methanolic acetate buffer pH 5.5 (1000 µg/ml). Consequent aliquots were prepared by dilutions accordingly to make different concentrations ranging from 30 – 210 µg/ml. The

absorbance of these solutions was measured using UV – Visible Spectrophotometer (UV – 1900, Shimadzu).

4.2.5 Calibration plot of Fulvestrant in Phosphate Buffer pH 7.4

Standard stock solution was prepared by dissolving 100 mg of Fulvestrant in 100 ml of 10% Methanolic phosphate buffer pH 7.4 (1000 µg/ml). Consequent aliquots were prepared by dilutions accordingly to make different concentrations ranging from 30 – 210 µg/ml. The absorbance of these solutions was measured using UV – Visible Spectrophotometer (UV – 1900, Shimadzu).

4.3 ANALYSIS OF FULVESTRANT by High Performance Liquid Chromatography

4.3.1 HPLC Conditions

Quantitative identification of Fulvestrant was done by HPLC as described in literature with slight modifications. The quaternary HPLC system (Thermo Scientific, Germany) composed of a Diode Array Detector (DAD). The separation was performed using a C18 HPLC column (Hypersil Gold, Fischer Scientific, Netherlands). A filtered and degassed mobile phase having Methanol: Acetonitrile: DDW (65:15:20) was used as mobile phase. The run time was 12 min, and the retention time was 5.7 min. The mobile phase was delivered at a flow rate of 1ml/min, the injection volume was 10 µl and the effluent was monitored at Diode array Detector, wavelength 280 nm (4). Data processing was done using the Chromoleon 7.3 software (Thermo Scientific, Germany).

4.3.2 Preparation of standard stock solution

Standard stock solution was prepared by dissolving 10 mg of Fulvestrant in 10 ml of Methanol (1000 µg/ml). From this stock solution, working standard of 100 µg/ml was prepared by dissolving 1 ml of stock solution and marking up the volume up to 10ml. This solution was further used for preparation of dilutions (5).

4.3.3 Calibration plot of Fulvestrant in Mobile phase

Suitable aliquots were prepared by dilution of working standard to prepare the dilutions ranging from 0.2 – 10 µg/ml to get concentration in the range. These standards were analyzed by HPLC at the detection wavelength of 280 nm and the mobile phase flow rate of 1.0 ml/min. The calibration curve was plotted for the measured area against the drug concentration.

4.4 HPLC ESTIMATION OF FULVESTRANT IN RAT PLASMA

Rat Plasma was collected from the allotted animal under the protocol approved by IAEC committee, The Maharaja Sayajirao University of Baroda, Vadodara, India. Calibration plot of Fulvestrant in the range of 50 -500 ng/ml. The blank plasma 200 µl was spiked with the stock solution prepared in acetonitrile (100 µg/ml). The protein precipitation was conducted by addition of sufficient amount of acetonitrile. The samples were centrifuged at 5000 rpm, 10 min at 4°C, the supernant was filtered using 0.22µ Millipore syringe filter. The sample was further injected into HPLC system by HPLC auto injector at 100µl volume (6).

The mobile phase consisted of methanol: acetonitrile: DDW (65:15:20). The run time was 12 min, and the retention time was 5.7 min. the mobile phase was delivered at a flow rate of 1ml/min, the injection volume was 100 µl and the effluent was monitored at detection wavelength of 280 nm. Data processing was done using Chromoleon 7.3 software. The calibration curve was plotted between area and drug concentration.

4.5 ANALYSIS OF EXEMESTANE BY UV VISIBLE SPECTROSCOPY

4.5.1 Calibration plot of Exemestane in Acetonitrile

Standard stock solution was prepared by dissolving 100 mg of Exemestane in 100 ml of Acetonitrile (1000 µg/ml). From this stock solution, working standard of 100 µg/ml was prepared by dissolving 1 ml of stock solution and marking up the volume up to 10ml (7). This solution was further used for preparation of dilutions Consequent aliquots were prepared by dilutions accordingly to make different concentrations ranging from 3 – 30 µg/ml. The absorbance of these solutions was measured using UV – Visible Spectrophotometer (UV – 1900, Shimadzu).

4.5.2 Calibration plot of Exemestane in Methanol

Standard stock solution was prepared by dissolving 100 mg of Exemestane in 100 ml of Methanol (1000 µg/ml). From this stock solution, working standard of 100 µg/ml was prepared by dissolving 1 ml of stock solution and marking up the volume up to 10ml. This solution was further used for preparation of dilutions Consequent aliquots were prepared by dilutions accordingly to make different concentrations ranging from 3 – 30 µg/ml. The absorbance of these solutions was measured using UV – Visible Spectrophotometer (UV – 1900, Shimadzu).

4.5.3 Calibration plot of Exemestane in Tetrahydrofuran

Standard stock solution was prepared by dissolving 100 mg of Exemestane in 100 ml of Tetrahydrofuran (1000 µg/ml). From this stock solution, working standard of 100 µg/ml was prepared by dissolving 1 ml of stock solution and marking up the volume up to 10ml. This solution was further used for preparation of dilutions Consequent aliquots were prepared by dilutions accordingly to make different concentrations ranging from 3 – 30 µg/ml. The absorbance of these solutions was measured using UV – Visible Spectrophotometer (UV – 1900, Shimadzu).

4.5.4 Calibration plot of Exemestane in Acetate Buffer pH 5.5

Standard stock solution was prepared by dissolving 100 mg of Exemestane in 100 ml of 10% Methanolic acetate buffer pH 5.5 (1000 µg/ml). From this stock solution, working standard of 100 µg/ml was prepared by dissolving 1 ml of stock solution and marking up the volume up to 10ml. This solution was further used for preparation of dilutions Consequent aliquots were prepared by dilutions accordingly to make different concentrations ranging from 5 – 40 µg/ml. The absorbance of these solutions was measured using UV – Visible Spectrophotometer (UV – 1900, Shimadzu).

4.5.5 Calibration plot of Exemestane in Phosphate Buffer pH 7.4

Standard stock solution was prepared by dissolving 100 mg of Exemestane in 100 ml of 10% Methanolic phosphate buffer pH 7.4 (1000 µg/ml). From this stock solution, working standard of 100 µg/ml was prepared by dissolving 1 ml of stock solution and marking up the volume up to 10ml. This solution was further used for preparation of dilutions Consequent aliquots were prepared by dilutions accordingly to make different concentrations ranging from 5 – 40 µg/ml. The absorbance of these solutions was measured using UV – Visible Spectrophotometer (UV – 1900, Shimadzu).

4.6 ANALYSIS OF EXEMESTANE by High Performance Liquid Chromatography

4.6.1 HPLC Conditions

Quantitative identification of Exemestane was done by HPLC as described in literature with slight modifications. The quaternary HPLC system (Thermo Scientific, Germany) composed of a Diode Array Detector (DAD). The separation was performed using a C18 HPLC column (Hypersil Gold, Fischer Scientific, Netherlands). A filtered and degassed mobile phase

having Methanol: Acetonitrile: DDW (65:15:20) was used as mobile phase (8). The run time was 10 min, and the retention time was 7.6 min. The mobile phase was delivered at a flow rate of 1ml/min, the injection volume was 10 µl and the effluent was monitored at Diode array Detector, wavelength 243 nm. Data processing was done using the Chromoleon 7.3 software (Thermo Scientific, Germany).

4.6.2 Preparation of standard stock solution

Standard stock solution was prepared by dissolving 10 mg of Exemestane in 10 ml of Methanol (1000 µg/ml). From this stock solution, working standard of 100 µg/ml was prepared by dissolving 1 ml of stock solution and marking up the volume up to 10ml. This solution was further used for preparation of dilutions.

4.6.3 Calibration plot of Exemestane in Mobile phase

Suitable aliquots were prepared by dilution of working standard to prepare the dilutions ranging from 50 – 600 ng/ml to get concentration in the range. These standards were analyzed by HPLC at the detection wavelength of 243 nm and the mobile phase flow rate of 1.0 ml/min. The calibration curve was plotted for the measured area against the drug concentration.

4.7 HPLC ESTIMATION OF EXEMESTANE IN RAT PLASMA

Rat Plasma was collected from the allotted animal under the protocol approved by IAEC committee, The Maharaja Sayajirao University of Baroda, Vadodara, India. Calibration plot of Exemestane in the range of 50 -600 ng/ml. The blank plasma 200 µl was spiked with the stock solution prepared in acetonitrile (100 µg/ml). The protein precipitation was conducted by addition of sufficient amount of acetonitrile (9). The samples were centrifuged at 5000 rpm, 10 min at 4°C, the supernant was filtered using 0.22µ Millipore syringe filter. The sample was further injected into HPLC system by HPLC auto injector at 100µl volume.

The mobile phase consisted of methanol: acetonitrile: DDW (65:15:20). The run time was 10 min, and the retention time was 7.6 min. the mobile phase was delivered at a flow rate of 1ml/min, the injection volume was 100 µl and the effluent was monitored at detection wavelength of 243 nm. Data processing was done using Chromoleon 7.3 software. The calibration curve was plotted between area and drug concentration. Analytical method was validated for linearity, accuracy and precision as described in section 4.9.

4.8 ESTIMATION OF TOTAL PHOSPHOLIPID CONTENT BY STEWART METHOD

4.8.1 Principle

Through Stewart's Colorimetric Technique, phospholipid content can be measured. This approach for the identification of phospholipids is based on the ability of phospholipids to create a stable compound with ammonium ferro-thiocyanate. Ammonium ferro-thiocyanate is an inorganic, red-colored compound that appears to stay insoluble in chloroform. However, with phospholipids it can form stable complexes and those complexes are soluble in chloroform. Therefore, when at room temperature, a chloroform solution consisting of phospholipids were mixed with ammonium ferro-thiocyanate, a red-colored complex is formed which partitions and gets solubilized in chloroform phase. Via colorimetry, the absorption of the colored soluble complex in chloroform was calculated at 462 nm (10).

4.8.2 Preparation of ammonium ferro-thiocyanate solution:

In de-ionized distilled water, 27.03 g ferric chloride hexahydrate (FeCl3.6H2O) and 30.4 g ammonium thiocyanate (NH4SCN) were dissolved and made up to 1 litre volume to form ammonium ferro-thiocyanate solution.

4.8.3 Calibration plot of phospholipids:

The glass equipment used for the analysis were washed with chromic acid solution prior to use to evade the possible corrosion from surface active washing agents. The lipids used to prepare the stock solution were included with their respective molar ratios in the final optimized formulation, i.e., 105 µl SPC – 3, 15 µl of Phospholipon 90G, 12 µL DSPE-PEG2000. (The above lipid amount was taken from the separate stock lipid solution in chloroform with condensed lipids). Then 3 ml of chloroform was added. After 5 min, the biphasic mixture was completely mixed, and the lower chloroform layer of high density was removed using syringe. The optical density of the segregated chloroform layer was determined against pure chloroform as a blank at a wavelength of 462 nm. The average of optical densities obtained was studied and results were plotted in a graph against overall lipid concentration.

4.9 ANALYTICAL METHOD VALIDATION

4.9.1 Linearity

It is defined as its ability to elicit tests that directly, or by a well-defined mathematical transformation proportional to the concentration of analyte in samples within a given range. The linearity was determined by analyzing independent levels of calibration curve in the selected concentration range. Calibration curve of absorbance vs concentration was plotted and correlation coefficient and regression line equation for was determined.

4.9.2 Precision assay

It is defined as the degree of agreement among individual test results when the method is applied repeatedly to multiple sampling of a homogenous sample. The precision of assay was determined by repeatability (intraday), intermediate precision (interday) and reported as the % relative standard deviation (%RSD). %RSD of the data obtained were calculated with the formula:

$$\%\boldsymbol{RSD} = \frac{\boldsymbol{Standard\ Deviation}}{\boldsymbol{Average}} \times \mathbf{100}$$

4.9.3 Accuracy

It is defined as the closeness of the test results obtained by that method to the true value. Accuracy was determined using following equation:

$$\%\boldsymbol{Accuracy} = \frac{\boldsymbol{Mean\ observed\ concentration}}{\boldsymbol{Actual\ concentration}} \times \mathbf{100}$$

4.9.4 Limit of Detection (LOD)

The limit of detection (LOD) is the lowest amount of analyte in a sample that can be detected, but not necessarily quantified, under standard experimental condition.

LOD was calculated using the following formula:

$$\boldsymbol{LOD} = \mathbf{3.3}\ (\boldsymbol{\sigma} / \boldsymbol{S})$$

Where, σ is the standard deviation of the y- intercept and S is the slope of the calibration curve.

4.9.5 Limit of Quantitation (LOQ)

The limit of quantification (LOQ) is the lowest amount of analyte in a sample that can be determined with acceptable precision and accuracy under standard experimental condition.

LOQ was calculated using the following formula:

$$\boldsymbol{LOD = 10\ (\sigma\ /\ S)}$$

Where, σ is the standard deviation of the y- intercept and S is the slope of the calibration curve.

4.10 RESULTS AND DISCUSSION

4.10.1 ESTIMATION OF FULVESTRANT BY UV SPECTROSCOPY

4.10.1.1 Calibration Curve in Acetonitrile

Fulvestrant in acetonitrile showed a characteristic spectrum when scanned in ultraviolet range between 200 – 400 nm. The absorption maxima (λ_{max}) were found at 280 nm in acetonitrile and Beer's law was obeyed between 25 – 200 µg/ml (Table 4.1). The overlay plot of Fulvestrant in acetonitrile is shown in figure 4.1. Regression analysis was performed on the experimental data. Regression equation for standard curve was $y = 0.005\,x - 0.004$ and correlation coefficient (R^2) was found to be 0.9983 signifying that a linear relationship existed between absorbance and concentration of the drug (figure 4.2).

Table 4.1 Standard Calibration data of Fulvestrant in Acetonitrile

Sr. No.	Concentration (µg/ml)	Absorbance	%RSD
1.	25	0.117±0.008	1.170
2.	50	0.256±0.013	0.773
3.	75	0.345±0.011	0.471
4.	100	0.501±0.013	0.694
5.	125	0.637±0.006	0.743
6.	150	0.750±0.014	0.942
7.	175	0.864±0.010	1.184
8.	200	0.995±0.0142	1.263

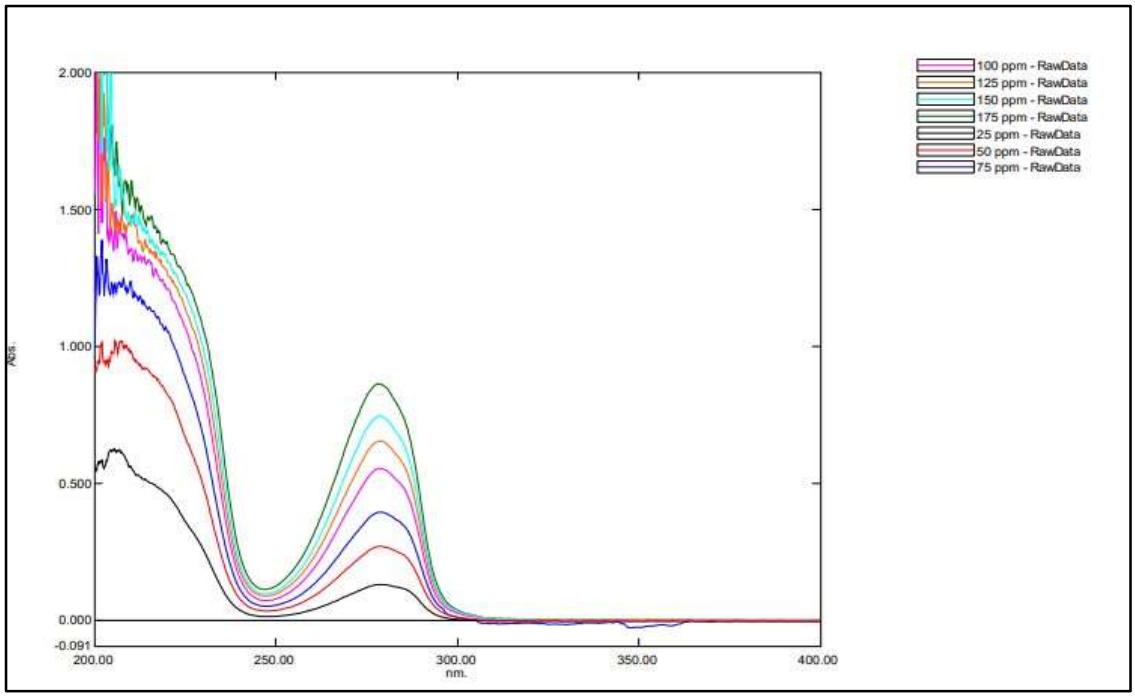

Figure 4.1 Overlay plot of Fulvestrant in Acetonitrile

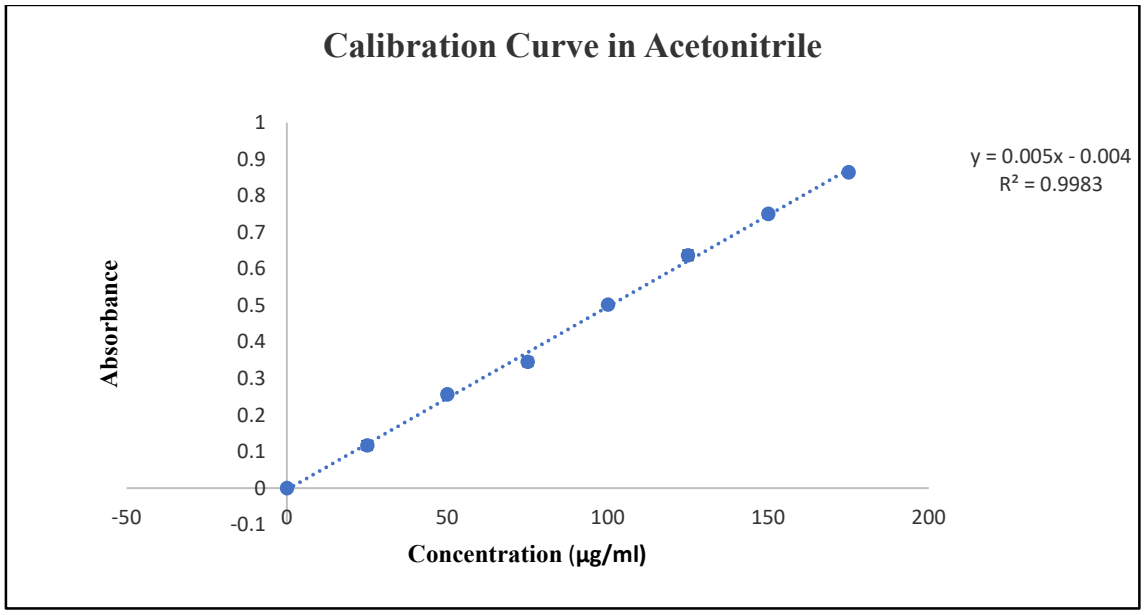

Figure 4.2 Standard calibration curve of Fulvestrant in Acetonitrile

4.10.1.1.1 VALIDATION

The analytical method for estimation of Fulvestrant in acetonitrile was validated for different parameters of analytical method validation.

4.10.1.1.1.1 Linearity

Linear correlation was obtained as per Beer-Lambert law between absorbance and concentrations of Fulvestrant in concentration range of 25 to 200 µg/ml for acetonitrile. The summarized parameters for regression equation and correlation are given in table 4.2.

Table 4.2 Regression analysis of Fulvestrant in acetonitrile

Parameters	Results
λ_{max}	280 nm
Linearity range	25 to 200 µg/ml
Regression equation (y= a + bc)	$y = 0.005x - 0.003$
Correlation coefficient (R^2)	0.9983

4.10.1.1.1.2 Accuracy and Precision assay

The results of accuracy and precision are shown in Table 4.3. The results reveal that the proposed method is accurate and precise.

Table 4.3 Accuracy and Precision for Fulvestrant in acetonitrile

Standard concentration (µg/ml)		Precision (%RSD)		Accuracy
Actual	Observed	Interday	Intraday	(%)
25	24.87 ± 0.124	0.498	0.867	99.48
75	75.13 ± 0.186	0.247	0.368	100.17
150	149.94 ± 0.218	0.145	0.286	99.96

4.10.1.1.1.3 LOD and LOQ

The LOD and LOQ for Fulvestrant in acetonitrile was found to be 1.192 µg/ml and 3.611 µg/ml respectively.

4.10.1.2 Calibration curve of Fulvestrant in methanol

Fulvestrant in methanol showed a characteristic spectrum when scanned in ultraviolet range between 200 – 400 nm. The absorption maxima (λ_{max}) were found at 280 nm in methanol and Beer's law was obeyed between 25 – 200 µg/ml (Table 4.4). The overlay plot of Fulvestrant in methanol is shown figure 4.3. Regression analysis performed on the

experimental data. Regression equation for standard curve was $y = 0.0049\,x + 0.0073$ and correlation coefficient (R^2) was found to be 0.9996 signifying that a linear relationship existed between absorbance and concentration of the drug (figure 4.4).

Table 4.4 Standard Calibration data of Fulvestrant in methanol

Sr. No.	Concentration (µg/ml)	Absorbance	%RSD
1.	25	0.132±0.014	1.136
2.	50	0.261±0.019	1.076
3.	75	0.376±0.011	1.090
4.	100	0.499±0.013	1.257
5.	125	0.634±0.010	0.836
6.	150	0.748±0.015	1.253
7.	175	0.864±0.010	1.380
8.	200	0.987±0.018	1.809

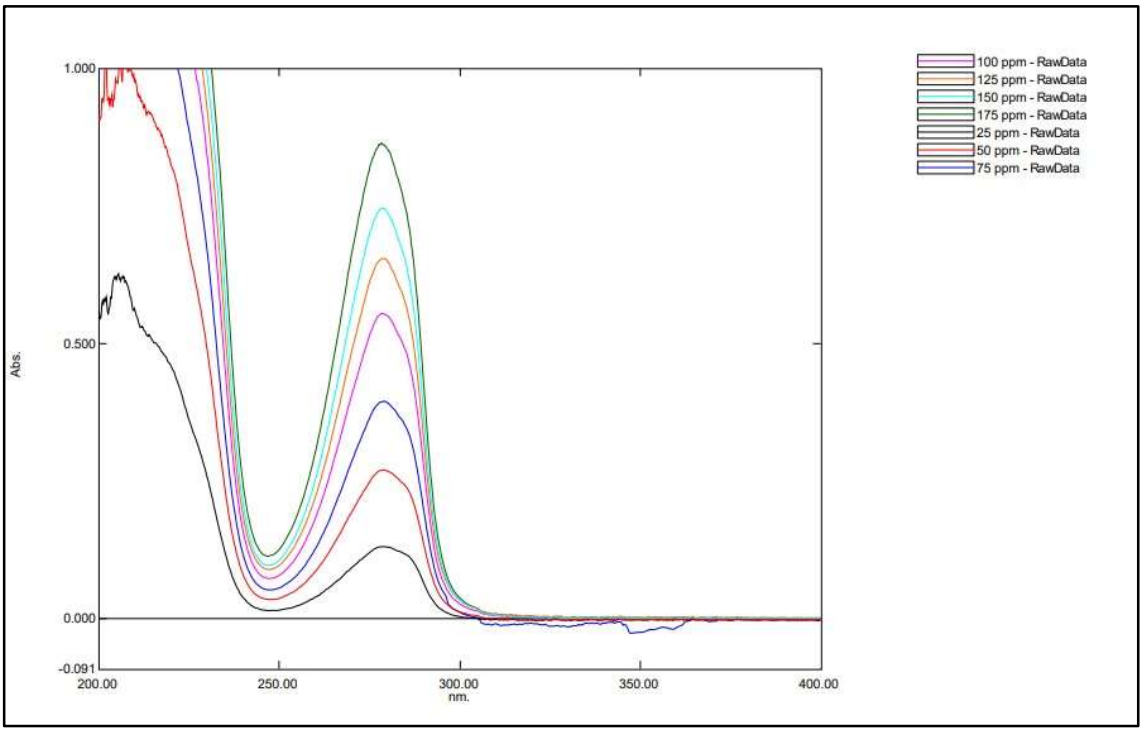

Figure 4.3 Overlay plot of Fulvestrant in methanol

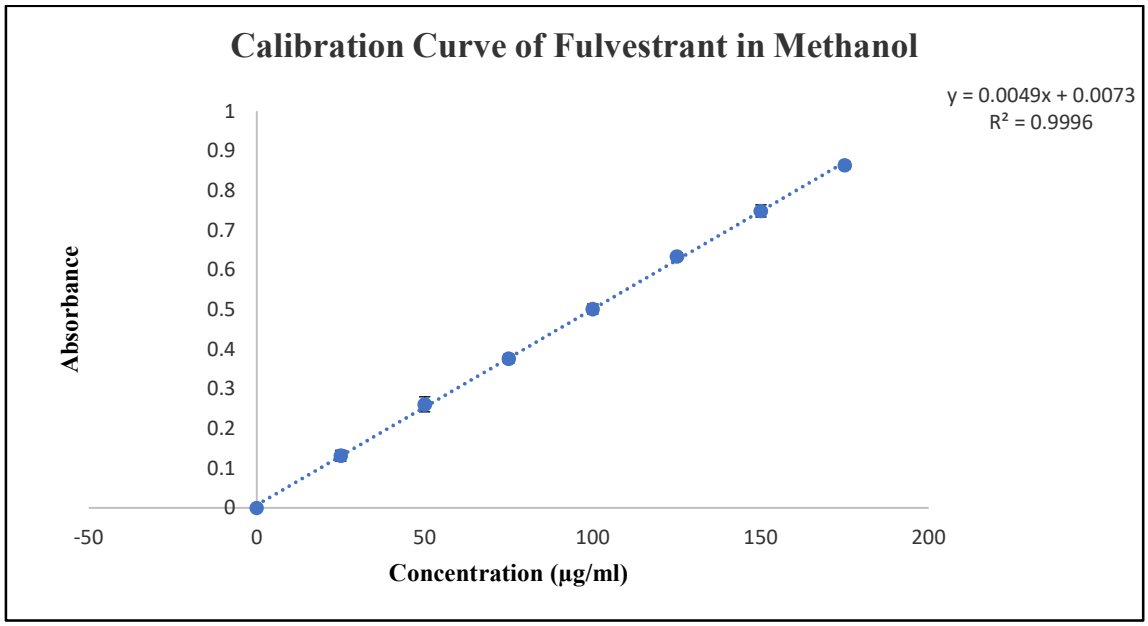

Figure 4.4 Standard calibration curve of Fulvestrant in methanol

4.10.1.2.1 VALIDATION

The analytical method for estimation of Fulvestrant in methanol was validated for different parameters of analytical method validation.

4.10.1.2.1.1 Linearity

Linear correlation was obtained as per Beer-Lambert law between absorbance and concentrations of Fulvestrant in concentration range of 25 to 200 µg/ml for methanol. The summarized parameters for regression equation and correlation are given in Table 4.5.

Table 4.5 Regression analysis of Fulvestrant in methanol

Parameters	Results
λ_{max}	280 nm
Linearity range	25 to 200 µg/ml
Regression equation (y= a + bc)	$y = 0.0049\,x + 0.0073$
Correlation coefficient (R^2)	0.9996

4.10.1.2.1.2 Accuracy and Precision assay

The results of accuracy and precision are shown in Table 4.6. The results reveal that the proposed method is accurate and precise.

Table 4.6 Accuracy and Precision for Fulvestrant in methanol

Standard concentration (µg/ml)		Precision (%RSD)		Accuracy
Actual	**Observed**	**Interday**	**Intraday**	**(%)**
25	24.97 ± 0.114	1.214	0.986	99.88
75	74.88 ± 0.206	0.628	0.719	99.84
150	150.34 ± 0.164	0.846	0.538	100.22

4.10.1.2.1.3 LOD and LOQ

The LOD and LOQ for Fulvestrant in methanol was found to be 4.10 µg/ml and 12.4 µg/ml respectively.

4.10.1.3 Calibration curve of Fulvestrant in Tetrahydrofuran

Fulvestrant in tetrahydrofuran showed a characteristic spectrum when scanned in ultraviolet range between 200 – 400 nm. The absorption maxima (λ_{max}) was found at 280 nm in tetrahydrofuran and Beer's law was obeyed between 10 – 80 µg/ml (Table 4.7). The overlay plot of Fulvestrant in tetrahydrofuran is shown figure 4.5. Regression analysis performed on the experimental data. Regression equation for standard curve was $y = 0.0116\,x - 0.0156$ and correlation coefficient (R^2) was found to be 0.9953 signifying that a linear relationship existed between absorbance and concentration of the drug (figure 4.6).

Table 4.7 Standard Calibration data of Fulvestrant in Tetrahydrofuran

Sr. No.	Concentration (µg/ml)	Absorbance	%RSD
1.	10	0.122±0.004	0.849
2.	20	0.204±0.006	0.596
3.	30	0.321±0.006	0.626
4.	40	0.437±0.007	0.779
5.	50	0.531±0.003	1.221
6.	60	0.684±0.010	1.216
7.	80	0.942±0.014	1.644

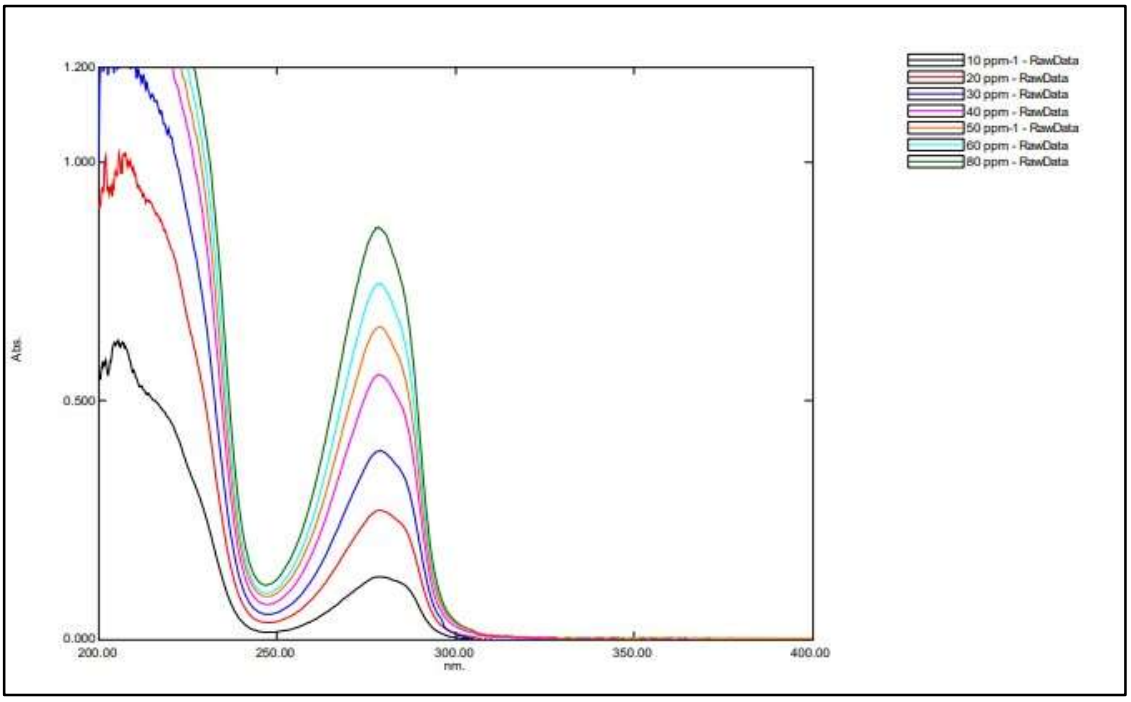

Figure 4.5: Overlay plot of Fulvestrant in Tetrahydrofuran

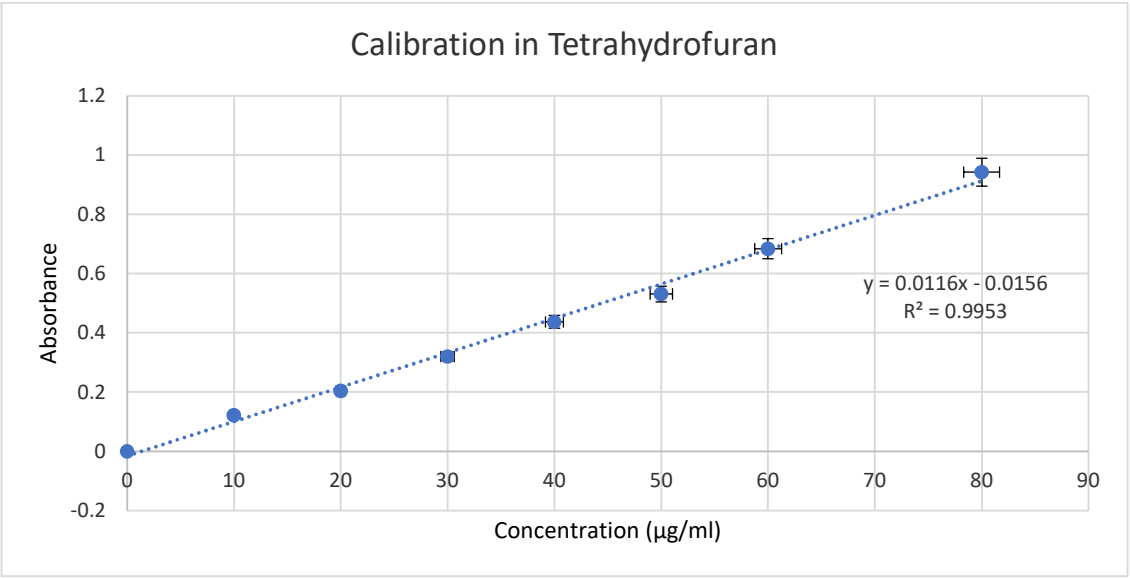

Figure 4.6 Standard calibration curve of Fulvestrant in Tetrahydrofuran

4.10.1.3.1 VALIDATION

The analytical method for estimation of Fulvestrant in tetrahydrofuran was validated for different parameters of analytical method validation.

4.10.1.3.1.1 Linearity

Linear correlation was obtained as per Beer-Lambert law between absorbance and concentrations of Fulvestrant in concentration range of 10 to 80 µg/ml for tetrahydrofuran. The summarized parameters for regression equation and correlation are given in Table 4.8.

Table 4.8 Regression analysis of Fulvestrant in tetrahydrofuran

Parameters	Results
λ_{max}	280 nm
Linearity range	10 to 80 µg/ml
Regression equation (y= a + bc)	$y = 0.0116\,x - 0.0156$
Correlation coefficient (R^2)	0.9953

4.10.1.3.1.2 Accuracy and Precision assay

The results of accuracy and precision are shown in Table 4.9. The results reveal that the proposed method is accurate and precise.

Table 4.9 Accuracy and Precision for Fulvestrant in tetrahydrofuran

Standard concentration (µg/ml)		Precision (%RSD)		Accuracy
Actual	Observed	Interday	Intraday	(%)
10	9.92 ± 0.141	1.068	1.607	99.20
30	30.16 ± 0.104	0.344	0.408	100.53
60	59.88 ± 0.114	0.190	0.223	99.80

4.10.1.3.1.3 LOD and LOQ

The LOD and LOQ for Fulvestrant in tetrahydrofuran was found to be 1.98 µg/ml and 6.0 µg/ml respectively.

4.10.1.4 Calibration curve of Fulvestrant in Acetate buffer pH 5.5

Fulvestrant in tetrahydrofuran showed a characteristic spectrum when scanned in ultraviolet range between 200 – 400 nm. The absorption maxima (λ_{max}) was found at 280 nm in 10% methanolic acetate buffer pH 5.5 and Beer's law was obeyed between 30 – 210 µg/ml (Table 4.10). The overlay plot of Fulvestrant in 10% methanolic acetate buffer 5.5 is shown figure

4.7. Regression analysis performed on the experimental data. Regression equation for standard curve was $y = 0.0049\,x + 0.0095$ and correlation coefficient (R^2) was found to be 0.9998 signifying that a linear relationship existed between absorbance and concentration of the drug (figure 4.7).

Table 4.10 Standard Calibration data of Fulvestrant in 10% methanolic acetate buffer pH 5.5

Sr. No.	Concentration (µg/ml)	Absorbance	%RSD
1.	30	0.161±0.0011	0.683
2.	60	0.310±0.0027	0.870
3.	90	0.447±0.0052	1.163
4.	120	0.598±0.0076	1.270
5.	150	0.737±0.0093	1.261
6.	180	0.885±0.0120	1.355
7.	210	1.033±0.0170	1.645

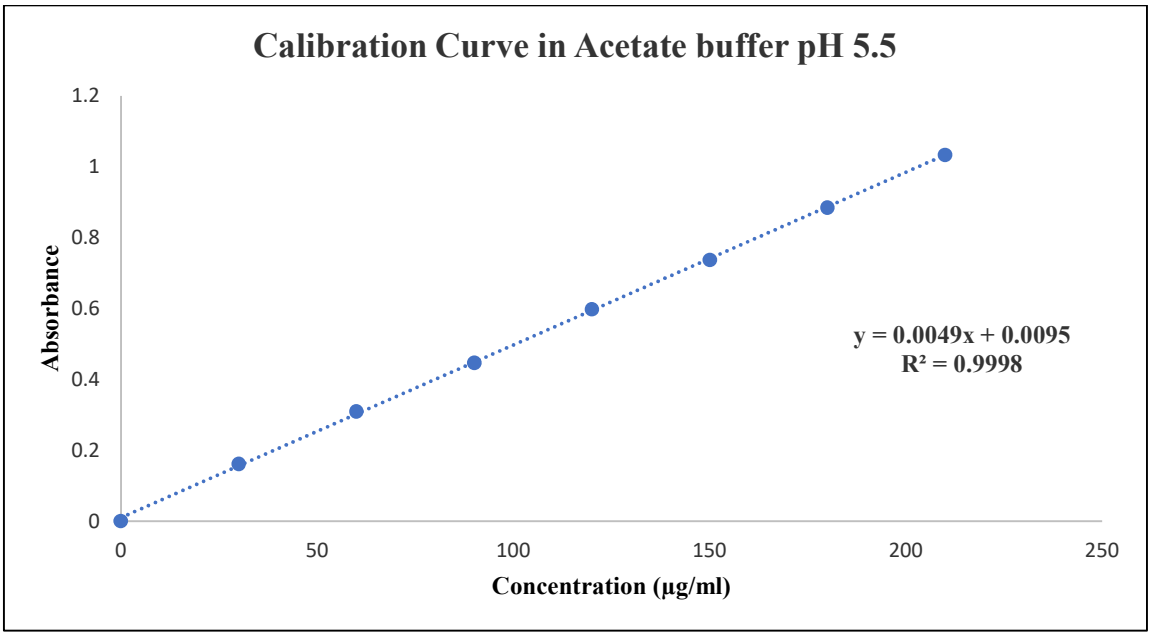

Figure 4.7 Standard calibration curve of Fulvestrant in 10% methanolic acetate buffer pH 5.5

4.10.1.4.1 VALIDATION

The analytical method for estimation of Fulvestrant in 10% methanolic acetate buffer pH 5.5 was validated for different parameters of analytical method validation.

4.10.1.4.1.1 Linearity

Linear correlation was obtained as per Beer-Lambert law between absorbance and concentrations of Fulvestrant in concentration range of 30 to 210 µg/ml for 10% methanolic acetate buffer pH 5.5. The summarized parameters for regression equation and correlation are given in Table 4.11.

Table 4.11 Regression analysis of Fulvestrant in 10% methanolic acetate buffer pH 5.5

Parameters	Results
λ_{max}	280 nm
Linearity range	30 to 210 µg/ml
Regression equation (y= a + bc)	$y = 0.0049\,x + 0.0095$
Correlation coefficient (R^2)	0.9998

4.10.1.4.1.2 Accuracy and Precision assay

The results of accuracy and precision are shown in Table 4.12. The results reveal that the proposed method is accurate and precise.

Table 4.12 Accuracy and Precision for Fulvestrant in 10% methanolic acetate buffer pH 5.5

Standard concentration (µg/ml)		Precision (%RSD)		Accuracy
Actual	**Observed**	**Interday**	**Intraday**	**(%)**
30	29.81 ± 0.131	0.439	0.802	99.36
90	89.86 ± 0.194	0.215	0.340	99.84
150	151.28 ± 0.536	0.354	0.688	100.85

4.10.1.4.1.3 LOD and LOQ

The LOD and LOQ for Fulvestrant in 10% methanolic acetate buffer pH 5.5 was found to be 6.40 µg/ml and 19.38 µg/ml respectively.

4.10.1.5 Calibration of Fulvestrant in Phosphate buffer pH 7.4

Fulvestrant in tetrahydrofuran showed a characteristic spectrum when scanned in ultraviolet range between 200 – 400 nm. The absorption maxima (λ_{max}) was found at 278 nm in 10% methanolic phosphate buffer pH 7.4 and Beer's law was obeyed between 30 – 210 µg/ml (Table 4.13). The overlay plot of Fulvestrant in 10% methanolic phosphate buffer 7.4 is shown figure 4.9. Regression analysis performed on the experimental data. Regression equation for standard curve was $y = 0.0042\,x + 0.0034$ and correlation coefficient (R^2) was found to be 0.9997 signifying that a linear relationship existed between absorbance and concentration of the drug (figure 4.8).

Table 4.13 Standard Calibration data of Fulvestrant in 10% methanolic phosphate buffer pH 7.4

Sr. No.	Concentration (µg/ml)	Absorbance	%RSD
1.	30	0.126±0.0011	0.876
2.	60	0.258±0.0027	1.046
3.	90	0.384±0.0043	1.119
4.	120	0.513±0.0071	1.384
5.	150	0.637±0.0098	1.538
6.	180	0.767±0.0110	1.434
7.	210	0.876±0.0160	1.826

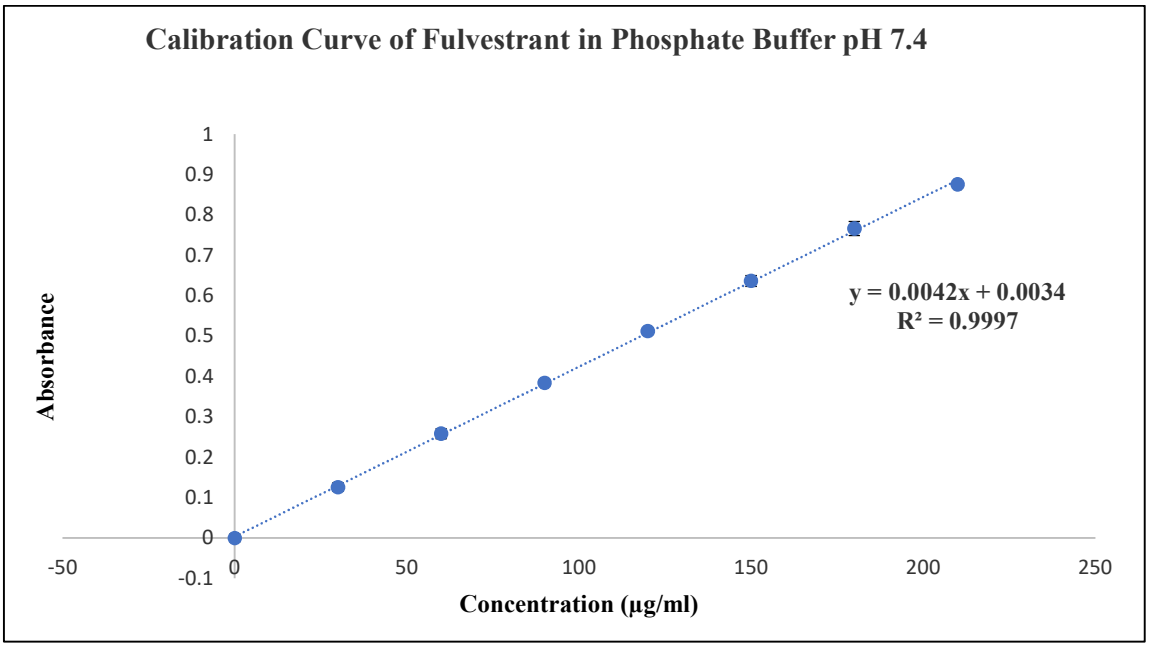

Figure 4.8 Standard calibration curve of Fulvestrant in 10% methanolic phosphate buffer pH 7.4

4.10.1.5.1 VALIDATION

The analytical method for estimation of Fulvestrant in 10% methanolic phosphate buffer pH 7.4 was validated for different parameters of analytical method validation.

4.10.1.5.1.1 Linearity

Linear correlation was obtained as per Beer-Lambert law between absorbance and concentrations of Fulvestrant in concentration range of 30 to 210 µg/ml for 10% methanolic phosphate buffer pH 7.4. The summarized parameters for regression equation and correlation are given in Table 4.14.

Table 4.14 Regression analysis of Fulvestrant in 10% methanolic phosphate buffer pH 7.4

Parameters	Results
λ_{max}	278 nm
Linearity range	30 to 210 µg/ml
Regression equation (y= a + bc)	$y = 0.0042\,x + 0.0034$
Correlation coefficient (R^2)	0.9997

4.10.1.5.1.2 Accuracy and Precision assay

The results of accuracy and precision are shown in Table 4.15. The results reveal that the proposed method is accurate and precise.

Table 4.15 Accuracy and Precision for Fulvestrant in 10% methanolic phosphate buffer pH 7.4

Standard concentration (µg/ml)		Precision (%RSD)		Accuracy (%)
Actual	Observed	Interday	Intraday	
30	30.31 ± 0.368	1.214	1.372	101.03
90	89.76 ± 0.514	0.572	0.699	99.73
150	148.78 ± 1.631	1.096	1.163	99.18

4.10.1.5.1.3 LOD and LOQ

The LOD and LOQ for Fulvestrant in phosphate buffer pH 7.4 was found to be 2.67 µg/ml and 8.09 µg/ml respectively.

4.10.2 HPLC METHOD DEVELOPMENT OF FULVESTRANT

4.10.2.1 Calibration curve of Fulvestrant in HPLC

The retention time of Fulvestrant was found to be 5.7 min. The standard plot of Fulvestrant in methanol is shown in Table 4.16. The overlay plot of HPLC chromatogram is shown in figure 4.11. Regression equation for standard curve was $y = 82.066\,x - 0.2363$ and correlation coefficient (R^2) was found to be 0.9995 signifying that a linear relationship existed between peak area and concentration of the drug (figure 4.10).

Table 4.16 Standard Calibration data for estimation of Fulvestrant in HPLC

Sr. No.	Concentration (µg/ml)	Peak Area (µAU*s)	Retention time (min)	%RSD
1.	0.2	16.23±0.158	5.72	0.973
2.	0.4	32.88±0.275	5.68	0.836
3.	0.6	49.17±0.471	5.74	0.957
4.	0.8	65.62±0.785	5.72	1.196
5.	1.0	81.59±1.096	5.71	1.343

6.	1.2	98.97±1.113	5.70	1.124
7.	10	846.32±9.356	5.72	1.105

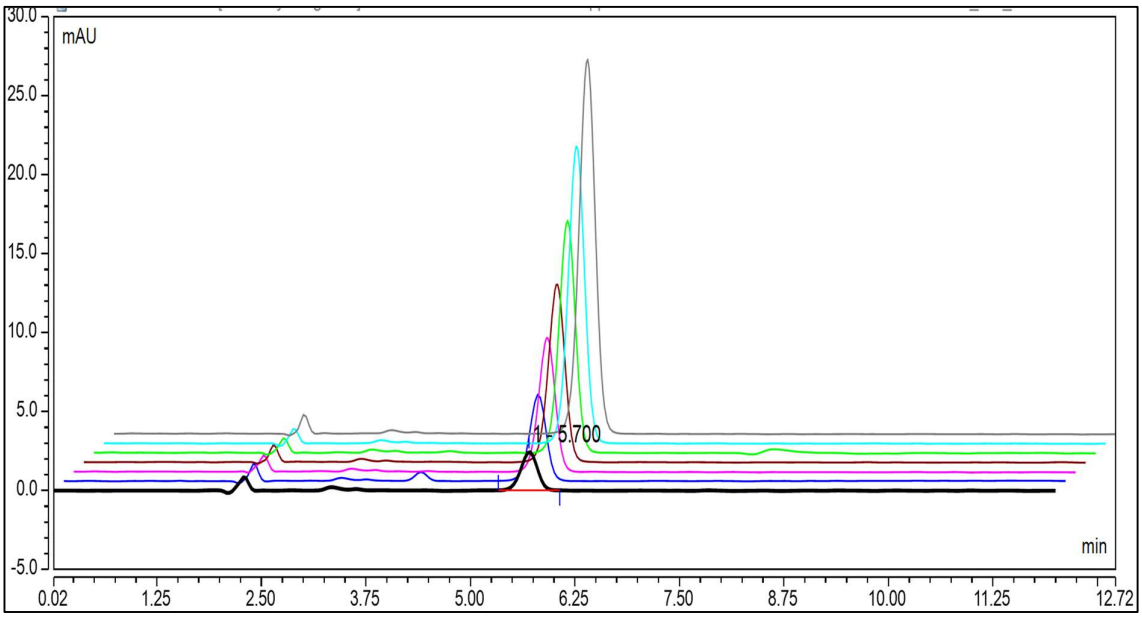

Figure 4.9 Overlay RP – HPLC spectrum of Fulvestrant in methanol

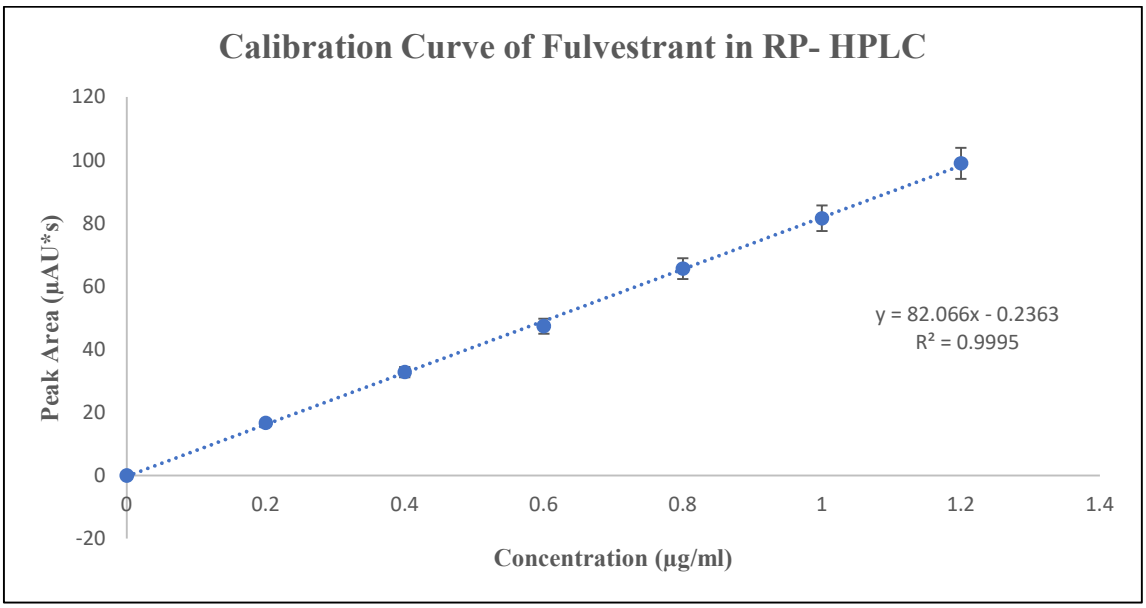

Figure 4.10 Calibration plot of Fulvestrant by RP – HPLC

4.10.2.2 VALIDATION

The analytical method for estimation of Fulvestrant in RP – HPLC in methanol was validated for different parameters of analytical method validation.

4.10.2.2.1 Linearity

Linear correlation was obtained for Fulvestrant in concentration range of 0.2 to 10 (µg/ml). The summarized parameters for regression equation and correlation are given in Table 4.17.

Table 4.17 Parameters from calibration plot of Fulvestrant in mobile phase

Parameters	Results
λ_{max}	280 nm
Linearity range	200 ng/ml to 10 µg/ml
Regression equation (y= a + bc)	$y = 82.066\,x - 0.2363$
Correlation coefficient (R^2)	0.9995
Mobile Phase	Methanol: Acetonitrile: Water (65:15:20)

4.10.2.2.2 Accuracy

The percentage recoveries for lower, intermediate, and higher concentration are given in Table 4.18 in mobile phase using RP – HPLC. Their outcome indicates that the suggested analytical approach will reliably assess and predict any slight shift in the drug concentration in the solution.

Table 4.18 Accuracy of the method in mobile phase

Level	Expected Concentration (µg/ml)	Observed Concentration (µg/ml)	%Drug Recovered
80%	0.32	0.315±0.24	98.43%
100%	0.4	0.407±0.37	101.75%
120%	0.48	0.476±0.51	99.16%

4.10.2.2.3 Precision

The precision analysis for intraday and interday was conducted in the same operating condition. The number of RSD values obtained in the precision analysis was less than 2.0

percent, suggesting that these approaches have good accuracy and reproducibility. Percent RSD from Table 4.19 reveals that the method is reliable and there is no intraday and interday variability in the system.

Table 4.19 Intraday and Interday precision of Fulvestrant in mobile phase using RP – HPLC

Concentration (µg/ml)	Observed Concentration (µg/ml)		%RSD	
	Intraday	Interday	Intraday	Interday
0.3	0.304±0.002	0.297±0.004	0.157	0.207
0.5	0.507±0.008	0.495±0.006	0.213	0.256
1.0	0.992±0.010	1.02±0.016	0.251	0.310

4.10.2.2.4 Limit of detection and Limit of quantitation

Table 4.20 LOD and LOQ of Fulvestrant in mobile phase

Limit of Detection (ng/ml)	Limit of Quantitation (ng/ml)
10.04	31.80

4.10.3 Calibration plot of Fulvestrant by RP – HPLC in rat plasma

The Fulvestrant calibration plot was obtained in the 50 – 600 ng/ml range. The linear curve regression equation was found to agree with $y = 106.50\,x + 0.2217$. It was noticed that the correlation coefficient for the system was 0.9999, meaning the presence of a linear relationship between the peak region and the drug concentration. There was a retention time of 5.71 minutes.

Table 4.21 RP – HPLC calibration curve values of Fulvestrant in Rat Plasma

Sr. No.	Concentration (ng/ml)	Peak Area (µAU*s)	Retention time (min)	%RSD
1.	50	5.53±0.097	5.72	1.754
2.	100	10.91±0.153	5.68	1.402
3.	200	21.86±0.322	5.74	1.473
4.	300	32.36±0.514	5.72	1.588

5.	400	42.39±0.646	5.71	1.523
6.	500	53.76±0.884	5.70	1.644
7.	600	63.99±0.495	5.72	1.208

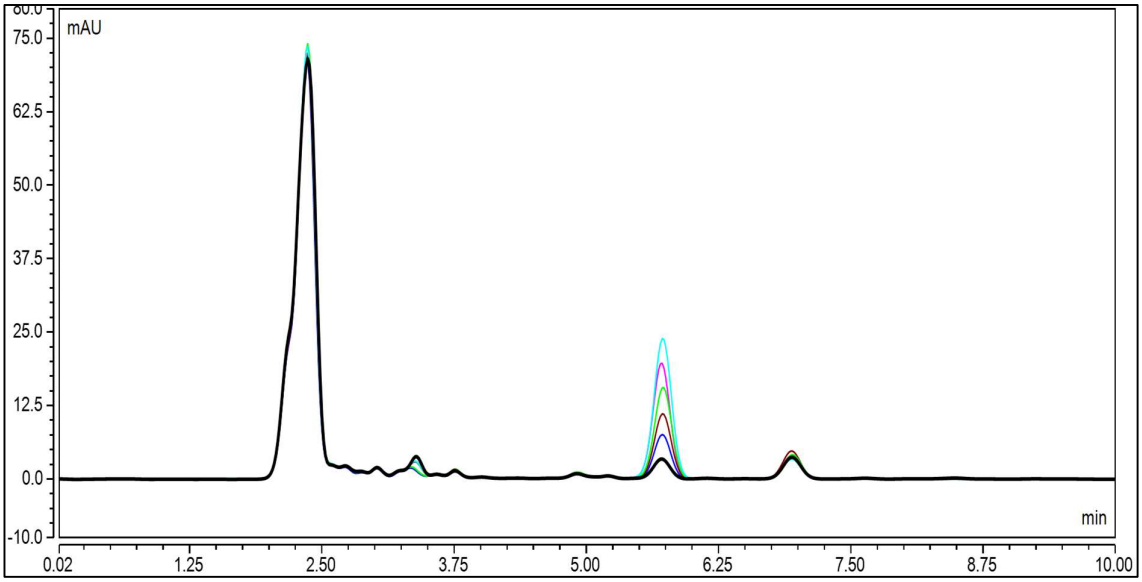

Figure 4.11 Overlay chromatogram of Fulvestrant in rat plasma by RP – HPLC

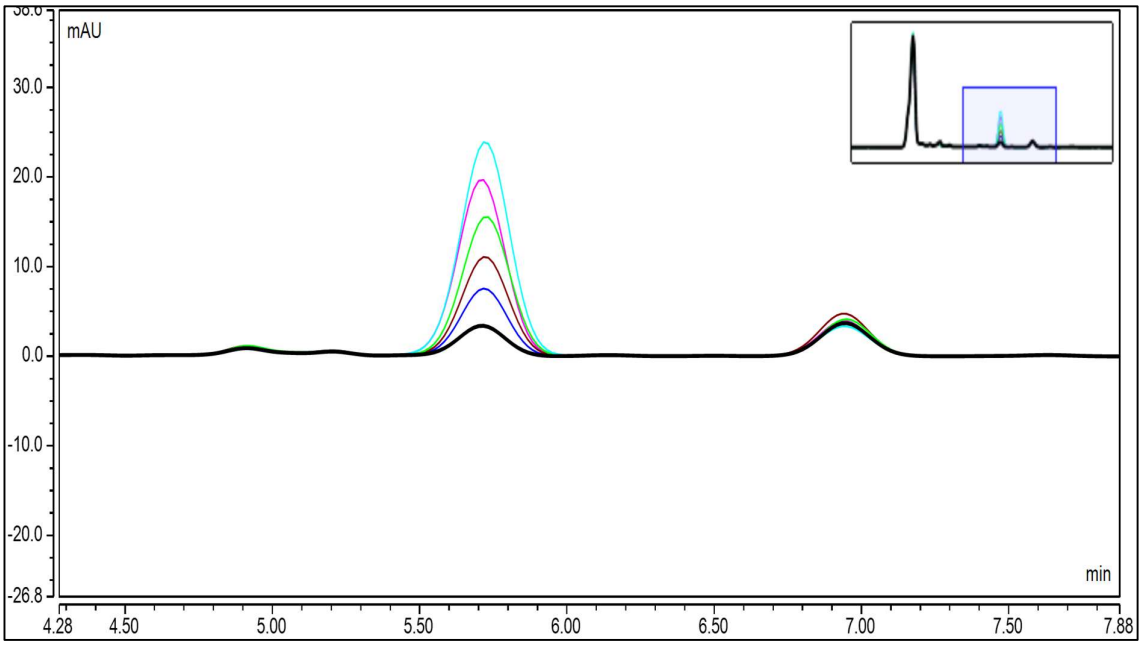

Figure 4.12 Overlay chromatogram of Fulvestrant in rat plasma by RP – HPLC

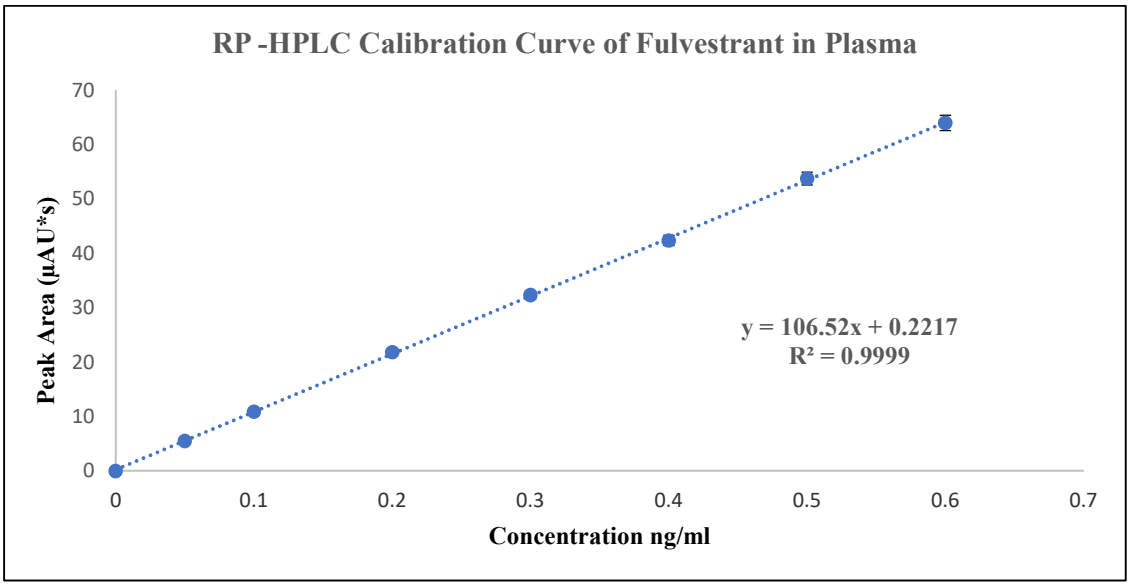

Figure 4.13 Calibration plot of Fulvestrant by RP – HPLC in rat plasma

4.10.3.1 VALIDATION

The analytical method for estimation of Fulvestrant in RP – HPLC in methanol was validated for different parameters of analytical method validation.

4.10.3.1.1 Linearity

Linear correlation was obtained for Fulvestrant in concentration range of 0.05 to 0.6 (µg/ml). The summarized parameters for regression equation and correlation are given in Table 4.21.

Table 4.21 Parameters from calibration plot of Fulvestrant in mobile phase

Parameters	Results
λ_{max}	280 nm
Linearity range	50 to 600 ng/ml
Regression equation (y= a + bc)	$y = 106.52\,x + 0.2217$
Correlation coefficient (R^2)	0.9999
Mobile Phase	Methanol: Acetonitrile: Water (65:15:20)

4.10.3.1.2 Accuracy

The percentage recoveries for lower, intermediate and higher concentration are given in Table 4.22 in mobile phase using RP – HPLC. Their outcome indicates that the suggested

analytical approach will reliably assess and predict any slight shift in the drug concentration in the solution.

Table 4.22 Accuracy of the method in mobile phase

Level	Expected Concentration (µg/ml)	Observed Concentration (µg/ml)	%Drug Recovered
80%	0.24	0.237±0.24	98.75%
100%	0.30	0.296±0.37	98.66%
120%	0.36	0.365±0.51	101.36%

4.10.3.1.3 Precision

The precision analysis for intraday and interday was conducted in the same operating condition. The number of RSD values obtained in the precision analysis was less than 2.0 percent, suggesting that these approaches have good accuracy and reproducibility. Percent RSD from Table 4.23 reveals that the method is reliable and there is no intraday and interday variability in the system.

Table 4.23 Intraday and Interday precision of Fulvestrant in rat plasma using RP – HPLC

Concentration (µg/ml)	Observed Concentration (µg/ml)		%RSD	
	Intraday	Interday	Intraday	Interday
0.2	0.203±0.002	0.197±0.003	0.985	1.522
0.3	0.304±0.005	0.295±0.003	1.644	1.016
0.5	0.506±0.008	0.495±0.009	1.581	1.818

4.10.3.1.4 Limit of detection and Limit of quantitation

Table 4.24 LOD and LOQ of Fulvestrant in rat plasma

Limit of Detection (ng/ml)	Limit of Quantitation (ng/ml)
6.86	20.81

4.10.4 Estimation of total phospholipid content by Stewart method

As observed in Figure 4.16, the calibration plot of the total phospholipid blend in chloroform was calculated and plotted with regression coefficient value (R^2) of 0.9997 in the concentration range of 20 – 100 µg/ml, which shows that the phospholipids follow Beer's rule.

Table 4.25 Calibration data for estimation of total phospholipid content

Sr. No.	Concentration (µg/ml)	Absorbance	%RSD
1.	0	0	0
2.	20	0.185±0.0012	0.648
3.	40	0.375±0.0028	0.746
4.	60	0.564±0.0064	1.134
5.	80	0.765±0.0083	1.084
6.	100	0.966±0.0122	1.262

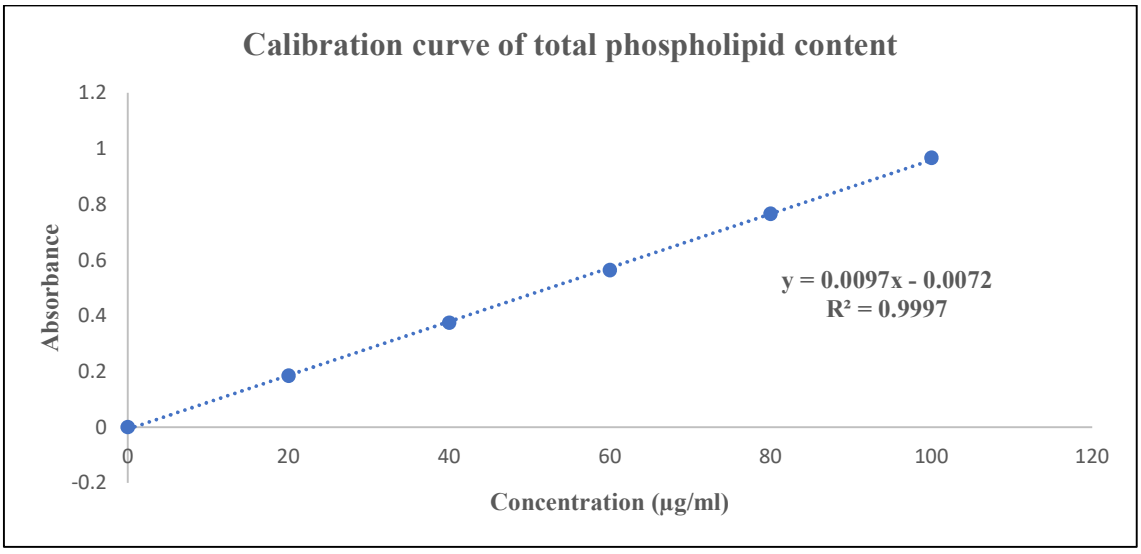

Figure 4.14 Calibration plot of total phospholipids mixture in chloroform

4.10.4.1 VALIDATION

4.10.4.1.1 Linearity

Linear correlation was obtained for Fulvestrant in concentration range of 20 to 100 µg/ml. The summarized parameters for regression equation and correlation are given in Table 4.26.

Table 4.26 Parameters from calibration plot of total phospholipid mixture

Parameters	Results
λ_{max}	462 nm
Linearity range	20 to 100 µg/ml
Regression equation (y= a + bc)	$y = 0.0097x - 0.0072$
Correlation coefficient (R^2)	0.9997
Solvent	Chloroform

4.10.4.1.2 Accuracy

The percentage recoveries for lower, intermediate, and higher concentration are given in Table 4.27 with chloroform using UV–Vis spectrophotometry. Their outcome indicates that the suggested analytical approach will reliably assess and predict any slight shift in the drug concentration in the solution.

Table 4.27 Accuracy of the phospholipid mixture in chloroform

Level	Expected concentration (µg/ml)	Observed concentration (µg/ml)	%RSD
80%	48	47.31±0.21	98.56
100%	60	61.08±0.43	101.80
120%	72	71.37±0.84	99.13

4.10.4.1.3 Precision

The precision analysis for intraday and interday was conducted in the same operating condition. The number of RSD values obtained in the precision analysis was less than 2.0 percent, suggesting that these approaches have good accuracy and reproducibility. Percent RSD from Table 4.28 reveals that the method is reliable and there is no intraday and interday variability in the system.

Table 4.28 Intraday and Interday precision for total phospholipid content in chloroform

Concentration (µg/ml)	Observed Concentration (µg/ml)		%RSD	
	Intraday	Interday	Intraday	Interday
30	29.24±0.241	30.19±0.217	0.824	0.718
50	50.61±0.183	49.61±0.378	0.361	0.761
70	69.24±0.631	70.48±0.664	0.911	0.942

4.10.4.1.4 Limit of Detection and Limit of Quantitation

Table 4.29 LOD and LOQ of total phospholipid content in chloroform

Limit of Detection (µg/ml)	Limit of Quantitation (µg/ml)
2.24	7.42

4.10.5 Analytical Interference study

Analytical interference studies are carried out to understand the potential interactions that can occur between drug and excipients which can lead to chemical as well as physical changes in drug's native state. The interaction cane be studied by different methods, mainly for chemical interaction UV visible spectroscopy is employed to check whether due to interaction is there any change in wavelength or the absorbance of drug. This provides the initial idea about chemical interaction which can be further confirmed by employing other analytical studies.

There was no peak observed for PLGA in the graph of UV analysis. Even mixture of Fulvestrant and formulation excipients shows almost overlaying peaks which suggest negligible interference of the excipients in the analysis of Fulvestrant using UV-Vis spectrophotometer. Reading of absorbance maxima did remain constant during analytical interference study which suggest no analytical interference of excipients with Fulvestrant.

Table 4.30 Interference study Fulvestrant and Excipients

Sr. No.	Name of Ingredients	Absorbance
1.	Fulvestrant	0.274±0.012
2.	PLGA	---
3.	Fulvestrant + PLGA	0.281±0.019

4.	Fulvestrant + PLGA + SPC-3 + DSPE PEG_{2000}	0.270±0.024
5.	Fulvestrant + PLGA + SPC-3 + DSPE PEG_{2000} + Trehalose	0.284±0.027

4.10.6 ESTIMATION OF EXEMESTANE BY UV SPECTROSCOPY

4.10.6.1 Calibration Curve in Acetonitrile

Exemestane in acetonitrile showed a characteristic spectrum when scanned in ultraviolet range between 200 – 400 nm. The absorption maxima (λ_{max}) was found at 243 nm in acetonitrile and Beer's law was obeyed between 25 – 200 µg/ml (Table 4.31). The overlay plot of Exemestane in acetonitrile is shown in figure 4.18. Regression analysis was performed on the experimental data. Regression equation for standard curve was $y = 0.0403\,x + 0.0085$ and correlation coefficient (R^2) was found to be 0.9999 signifying that a linear relationship existed between absorbance and concentration of the drug (figure 4.16).

Table 4.31 Standard Calibration data of Exemestane in Acetonitrile

Sr. No.	Concentration (µg/ml)	Absorbance	%RSD
1.	0	0	0
2.	3	0.133±0.0014	1.052
3.	6	0.256±0.0021	0.821
4.	9	0.375±0.0042	1.121
5.	12	0.494±0.0063	1.275
6.	15	0.613±0.0074	1.207
7.	18	0.732±0.0096	1.311
8.	21	0.854±0.016	1.873

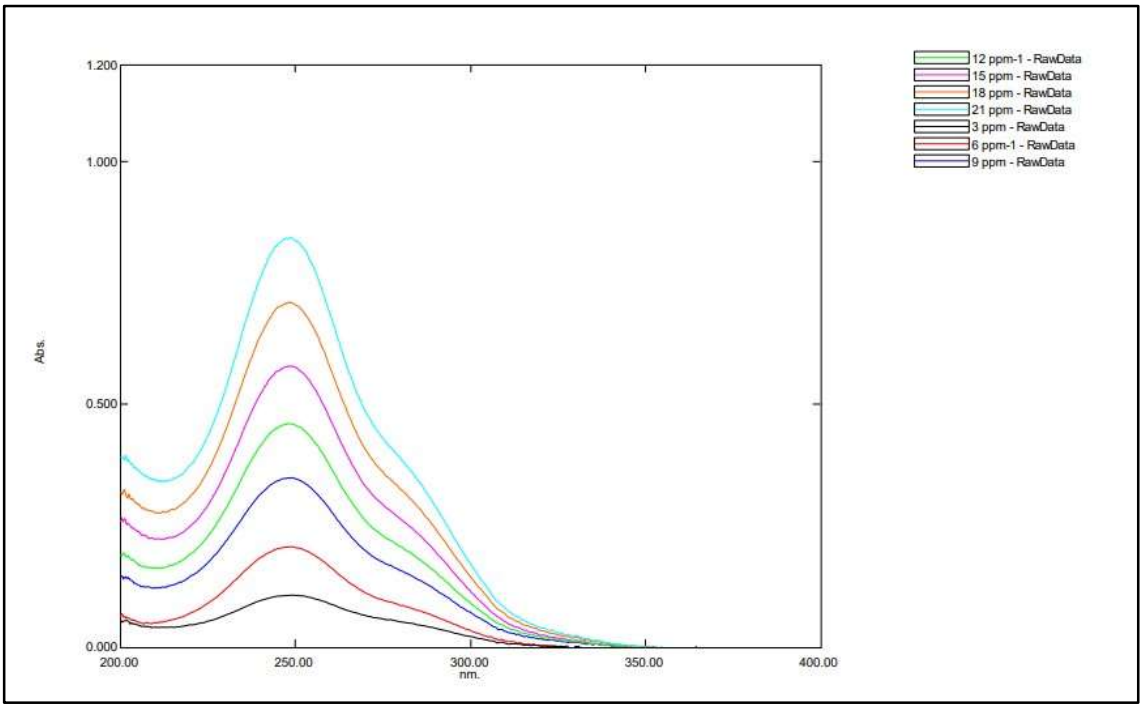

Figure 4.15 Overlay plot of Exemestane in Acetonitrile

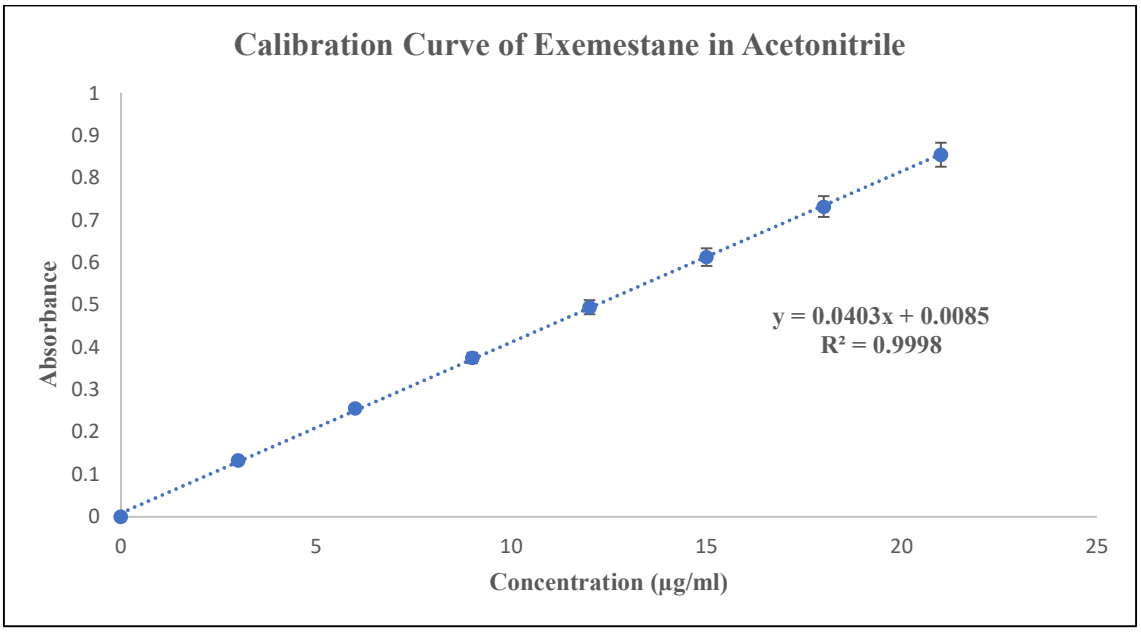

Figure 4.16 Standard calibration curve of Exemestane in Acetonitrile

4.10.6.2 VALIDATION

The analytical method for estimation of Exemestane in acetonitrile was validated for different parameters of analytical method validation.

4.10.6.2.1 Linearity

Linear correlation was obtained as per Beer-Lambert law between absorbance and concentrations of Exemestane in concentration range of 3 to 30 µg/ml for acetonitrile. The summarized parameters for regression equation and correlation are given in table 4.32.

Table 4.32 Regression analysis of Exemestane in acetonitrile

Parameters	Results
λ_{max}	243 nm
Linearity range	3 to 30 µg/ml
Regression equation (y= a + bc)	$y = 0.0403\,x + 0.0085$
Correlation coefficient (R^2)	0.9998

4.10.6.2.2 Accuracy and Precision assay

The results of accuracy and precision are shown in Table 4.33. The results reveal that the proposed method is accurate and precise.

Table 4.33 Accuracy and Precision for Exemestane in acetonitrile

Standard concentration (µg/ml)		Precision (%RSD)		Accuracy
Actual	**Observed**	**Interday**	**Intraday**	**(%)**
9	8.97 ± 0.124	0.658	1.082	99.66
12	12.06 ± 0.186	0.812	1.228	100.50
15	14.93 ± 0.218	1.216	1.546	99.33

4.10.6.2.3 LOD and LOQ

The LOD and LOQ for Exemestane in acetonitrile was found to be 0.69 µg/ml and 2.11 µg/ml respectively.

4.10.7 Calibration curve of Exemestane in methanol

Exemestane in methanol showed a characteristic spectrum when scanned in ultraviolet range between 200 – 400 nm. The absorption maxima (λ_{max}) was found at 243 nm in methanol and Beer's law was obeyed between 3 – 30 µg/ml (Table 4.34). The overlay plot of Exemestane in methanol is shown figure 4.20. Regression analysis performed on the experimental data.

Regression equation for standard curve was $y = 0.0405\,x + 0.0057$ and correlation coefficient (R^2) was found to be 0.9999 signifying that a linear relationship existed between absorbance and concentration of the drug (Figure 4.18).

Table 4.34 Standard Calibration data of Exemestane in methanol

Sr. No.	Concentration (µg/ml)	Absorbance	%RSD
1.	0	0	0
2.	3	0.128±0.0012	0.937
3.	6	0.254±0.0031	1.220
4.	9	0.371±0.0049	1.321
5.	12	0..498±0.0062	1.224
6.	15	0.608±0.0074	1.217
7.	18	0.732±0.0110	1.502
8.	21	0..856±0.0151	1.764

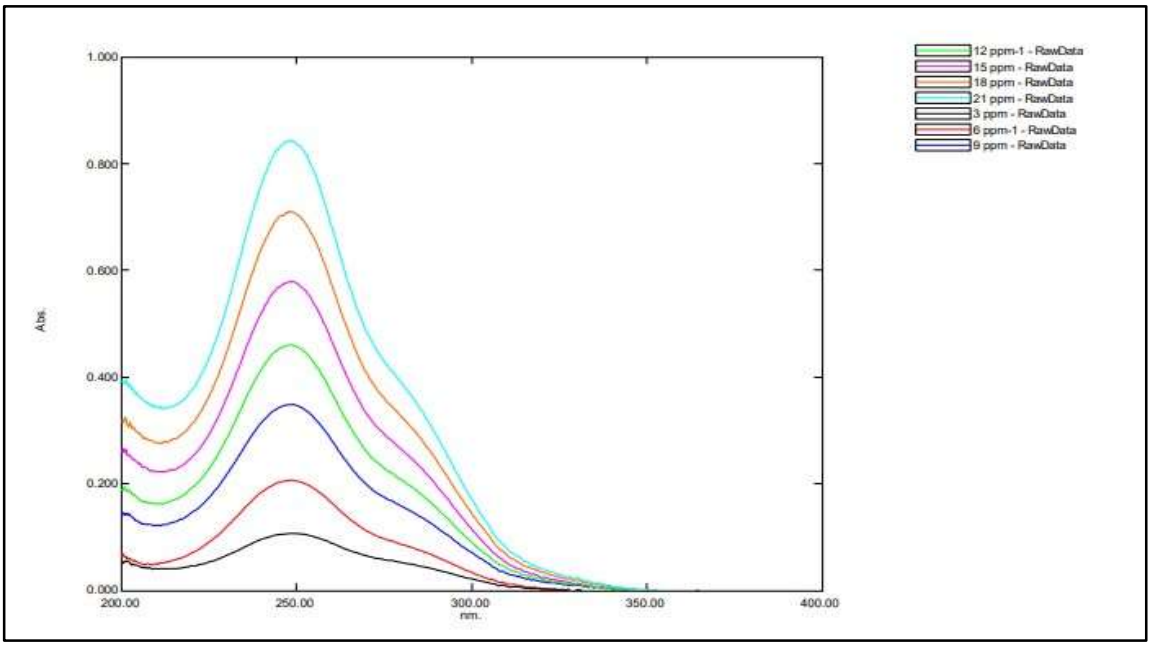

Figure 4.17 Overlay plot of Exemestane in methanol

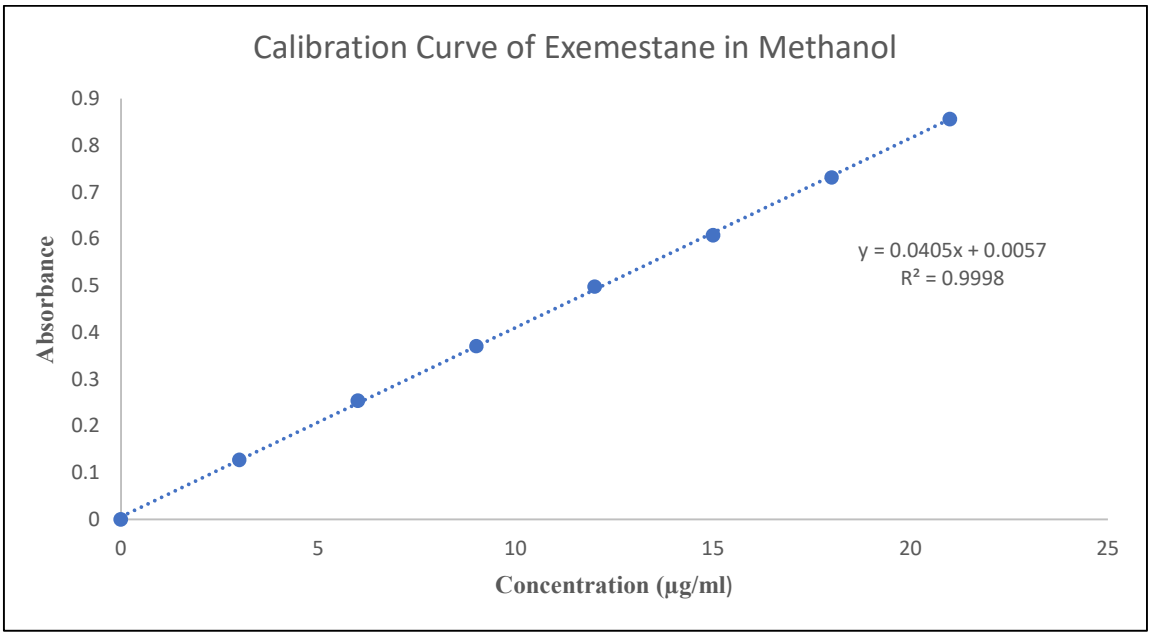

Figure 4.18 Standard calibration curve of Exemestane in methanol

4.10.7.1 VALIDATION

The analytical method for estimation of Exemestane in methanol was validated for different parameters of analytical method validation.

4.10.7.1.1 Linearity

Linear correlation was obtained as per Beer-Lambert law between absorbance and concentrations of Exemestane in concentration range of 3 to 30 µg/ml for methanol. The summarized parameters for regression equation and correlation are given in Table 4.35.

Table 4.35 Regression analysis of Exemestane in methanol

Parameters	Results
λ_{max}	243 nm
Linearity range	3 to 30 µg/ml
Regression equation (y= a + bc)	$y = 0.0405\,x + 0.0057$
Correlation coefficient (R^2)	0.9998

4.10.7.1.2 Accuracy and Precision assay

The results of accuracy and precision are shown in Table 4.36. The results reveal that the proposed method is accurate and precise.

Table 4.36 Accuracy and Precision for Exemestane in methanol

Standard concentration (µg/ml)		Precision (%RSD)		Accuracy
Actual	Observed	Interday	Intraday	(%)
9	9.05 ± 0.114	0.986	1.259	100.55
12	11.93 ± 0.206	0.719	1.726	99.41
15	15.11 ± 0.164	0.538	1.087	100.73

4.10.7.1.3 LOD and LOQ

The LOD and LOQ for Exemestane in methanol was found to be 0.46 µg/ml and 1.41 µg/ml respectively.

4.10.8 Calibration curve of Exemestane in Tetrahydrofuran

Exemestane in tetrahydrofuran showed a characteristic spectrum when scanned in ultraviolet range between 200 – 400 nm. The absorption maxima (λ_{max}) was found at 243 nm in tetrahydrofuran and Beer's law was obeyed between 3 – 30 µg/ml (Table 4.37). The overlay plot of Exemestane in tetrahydrofuran is shown figure 4.22. Regression analysis performed on the experimental data. Regression equation for standard curve was $y = 0.0484\,x - 0.0157$ and correlation coefficient (R^2) was found to be 0.9993 signifying that a linear relationship existed between absorbance and concentration of the drug (figure 4.20).

Table 4.37 Standard Calibration data of Exemestane in Tetrahydrofuran

Sr. No.	Concentration (µg/ml)	Absorbance	%RSD
1.	3	0.167±0.0012	0.718
2.	6	0.310±0.0027	0.871
3.	9	0.459±0.0043	0.937
4.	12	0.609±0.0075	1.231
5.	15	0.738±0.0097	1.314
6.	18	0.881±0.0136	1.534

7.	21	1.029±0.0187	1.817

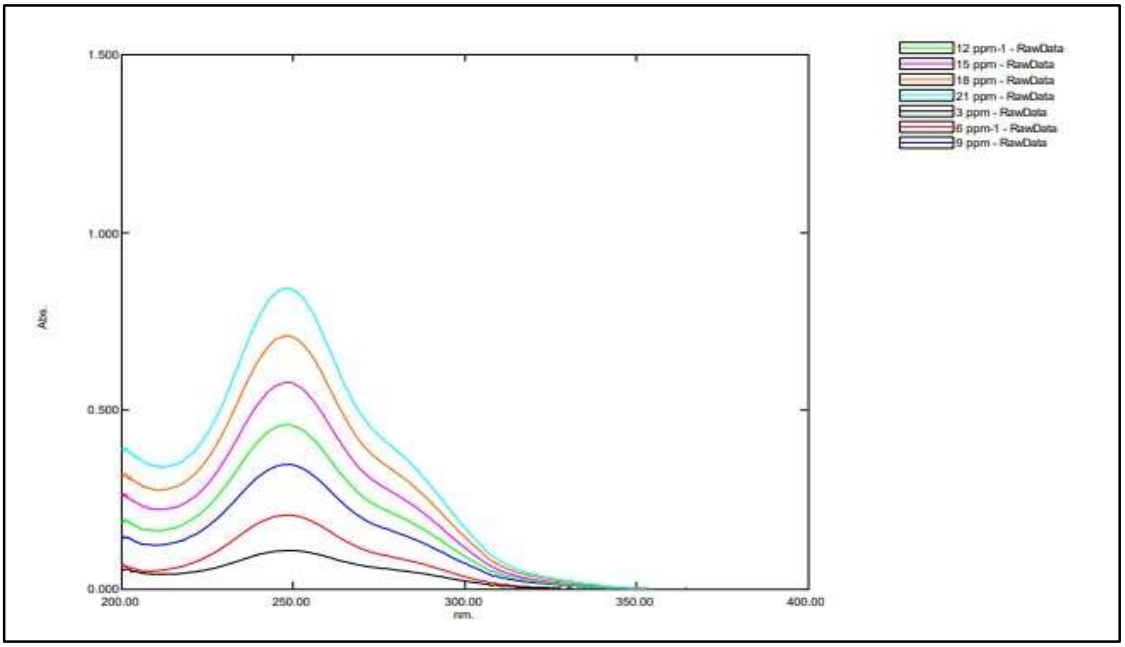

Figure 4.19: Overlay plot of Exemestane in Tetrahydrofuran

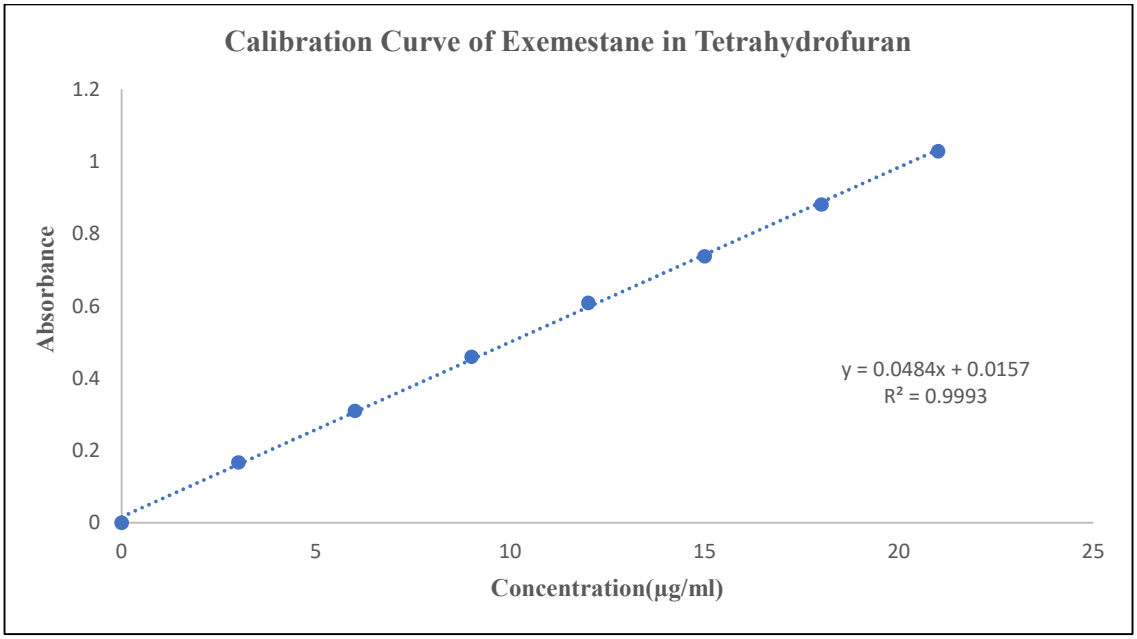

Figure 4.20 Standard calibration curve of Exemestane in Tetrahydrofuran

4.10.8.1 VALIDATION

The analytical method for estimation of Exemestane in tetrahydrofuran was validated for different parameters of analytical method validation.

4.10.8.1.1 Linearity

Linear correlation was obtained as per Beer-Lambert law between absorbance and concentrations of Exemestane in concentration range of 3 to 30 µg/ml for tetrahydrofuran. The summarized parameters for regression equation and correlation are given in Table 4.38.

Table 4.38 Regression analysis of Exemestane in tetrahydrofuran

Parameters	Results
λ_{max}	243 nm
Linearity range	3 to 30 µg/ml
Regression equation (y= a + bc)	$y = 0.0484\,x - 0.0157$
Correlation coefficient (R^2)	0.9993

4.10.8.1.2 Accuracy and Precision assay

The results of accuracy and precision are shown in Table 4.39. The results reveal that the proposed method is accurate and precise.

Table 4.39 Accuracy and Precision for Exemestane in tetrahydrofuran

Standard concentration (µg/ml)		Precision (%RSD)		Accuracy
Actual	**Observed**	**Interday**	**Intraday**	**(%)**
9	9.06 ± 0.101	1.114	1.556	100.66
12	11.89 ± 0.124	1.042	1.314	99.08
15	15.17 ± 0.143	0.942	1.229	101.33

4.10.8.1.3 LOD and LOQ

The LOD and LOQ for Exemestane in tetrahydrofuran was found to be 1.07 µg/ml and 3.24 µg/ml respectively.

4.10.9 Calibration curve of Exemestane in Acetate buffer pH 5.5

Exemestane in acetate buffer pH 5.5 showed a characteristic spectrum when scanned in ultraviolet range between 200 – 400 nm. The absorption maxima (λ_{max}) was found at 243 nm in 10% methanolic acetate buffer pH 5.5 and Beer's law was obeyed between 5 – 40 µg/ml (Table 4.40). The overlay plot of Exemestane in 10% methanolic acetate buffer 5.5 is shown figure 4.24. Regression analysis performed on the experimental data. Regression equation for standard curve was $y = 0.0254\,x - 0.0011$ and correlation coefficient (R^2) was found to be 0.9998 signifying that a linear relationship existed between absorbance and concentration of the drug (figure 4.21).

Table 4.40 Standard Calibration data of Exemestane in 10% methanolic acetate buffer pH 5.5

Sr. No.	Concentration (µg/ml)	Absorbance	%RSD
1.	5	0.120±0.0011	0.916
2.	10	0.252±0.0027	1.071
3.	15	0.386±0.0052	1.347
4.	20	0.508±0.0076	1.496
5.	25	0.636±0.0093	1.462
6.	30	0.767±0.0120	1.564
7.	35	0.882±0.0156	1.768

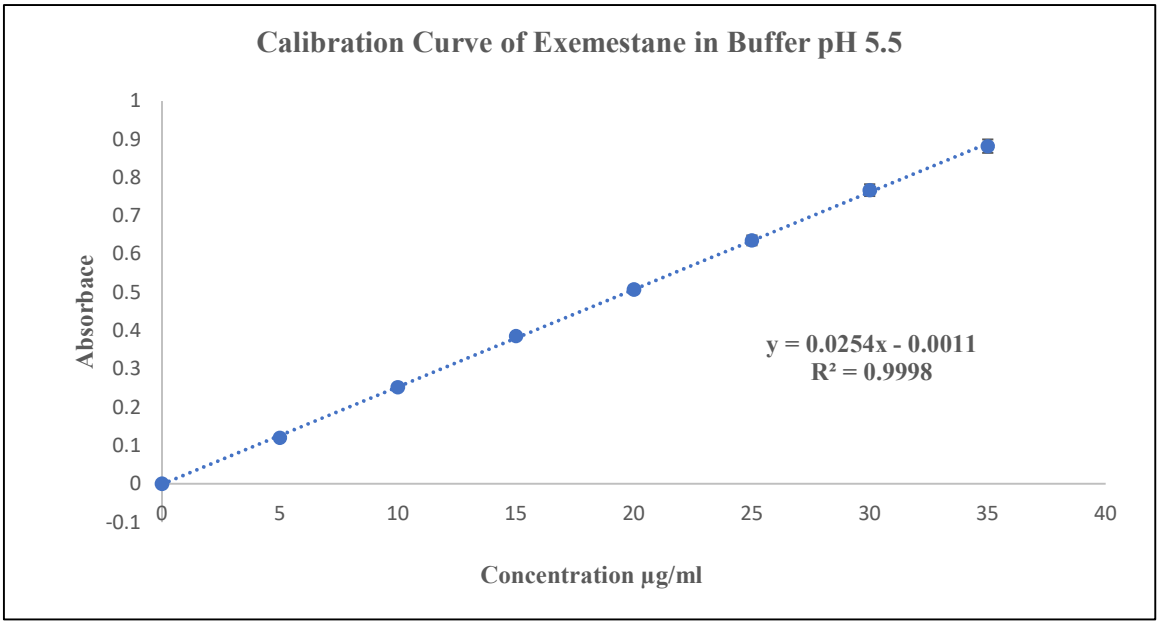

Figure 4.21 Standard calibration curve of Exemestane in 10% methanolic acetate buffer pH 5.5

4.10.9.1 VALIDATION

The analytical method for estimation of Exemestane in 10% methanolic acetate buffer pH 5.5 was validated for different parameters of analytical method validation.

4.10.9.1.1 Linearity

Linear correlation was obtained as per Beer-Lambert law between absorbance and concentrations of Exemestane in concentration range of 5 to 40 µg/ml for 10% methanolic acetate buffer pH 5.5. The summarized parameters for regression equation and correlation are given in Table 4.41.

Table 4.41 Regression analysis of Exemestane in 10% methanolic acetate buffer pH 5.5

Parameters	Results
λ_{max}	243 nm
Linearity range	5 to 40 µg/ml
Regression equation (y= a + bc)	$y = 0.0254\,x - 0.0011$
Correlation coefficient (R^2)	0.9998

4.10.9.1.2 Accuracy and Precision assay

The results of accuracy and precision are shown in Table 4.12. The results reveal that the proposed method is accurate and precise.

Table 4.42 Accuracy and Precision for Exemestane in 10% methanolic acetate buffer pH 5.5

Standard concentration (µg/ml)		Precision (%RSD)		Accuracy
Actual	**Observed**	**Interday**	**Intraday**	**(%)**
15	14.91 ± 0.181	0.757	1.213	99.40
20	20.26 ± 0.234	0.868	1.154	101.30
25	24.83 ± 0.336	0.761	1.353	99.32

4.10.9.1.3 LOD and LOQ

The LOD and LOQ for Exemestane in 10% methanolic acetate buffer pH 5.5 was found to be 6.40 µg/ml and 19.38 µg/ml respectively.

4.10.10 Calibration of Exemestane in Phosphate buffer pH 7.4

Exemestane in tetrahydrofuran showed a characteristic spectrum when scanned in ultraviolet range between 200 – 400 nm. The absorption maxima (λ_{max}) was found at 245 nm in 10% methanolic phosphate buffer pH 7.4 and Beer's law was obeyed between 5 – 40 µg/ml (Table 4.26). The overlay plot of Exemestane in 10% methanolic phosphate buffer 7.4 is shown figure 4.9. Regression analysis performed on the experimental data. Regression equation for standard curve was $y = 0.0231\,x + 0.0062$ and correlation coefficient (R^2) was found to be 0.9994 signifying that a linear relationship existed between absorbance and concentration of the drug (figure 4.21).

Table 4.43 Standard Calibration data of Exemestane in 10% methanolic phosphate buffer pH 7.4

Sr. No.	Concentration (µg/ml)	Absorbance	%RSD
1.	5	0.130±0.0014	1.076
2.	10	0.246±0.0025	1.016
3.	15	0.345±0.0041	1.118
4.	20	0.463±0.0057	1.231
5.	25	0.581±0.0072	1.239
6.	30	0.706±0.0094	1.331
7.	35	0.827±0.0118	1.426

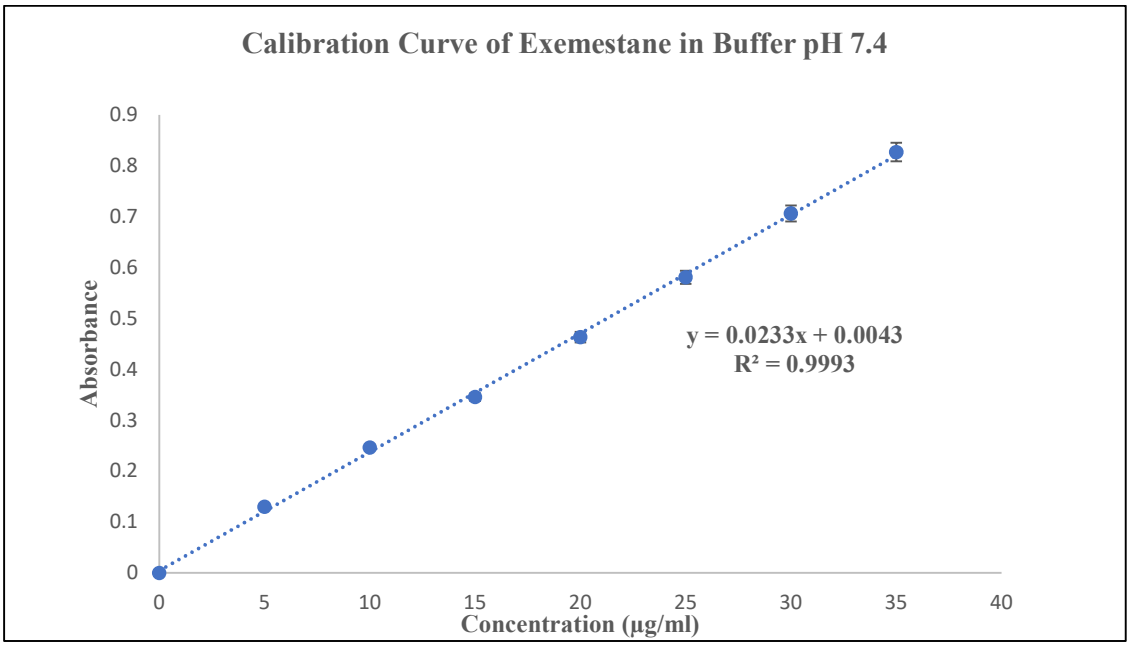

Figure 4.21 Standard calibration curve of Exemestane in 10% methanolic phosphate buffer pH 7.4

4.10.10.1 VALIDATION

The analytical method for estimation of Exemestane in 10% methanolic phosphate buffer pH 7.4 was validated for different parameters of analytical method validation.

4.10.10.1.1 Linearity

Linear correlation was obtained as per Beer-Lambert law between absorbance and concentrations of Exemestane in concentration range of 5 to 40 µg/ml for 10% methanolic phosphate buffer pH 7.4. The summarized parameters for regression equation and correlation are given in Table 4.44.

Table 4.44 Regression analysis of Exemestane in 10% methanolic phosphate buffer pH 7.4

Parameters	Results
λ_{max}	245 nm
Linearity range	5 to 40 µg/ml
Regression equation (y= a + bc)	$y = 0.0233\,x + 0.0043$
Correlation coefficient (R^2)	0.9993

4.10.10.1.2 Accuracy and Precision assay

The results of accuracy and precision are shown in Table 4.45. The results reveal that the proposed method is accurate and precise.

Table 4.45 Accuracy and Precision for Exemestane in 10% methanolic phosphate buffer pH 7.4

Standard concentration (µg/ml)		Precision (%RSD)		Accuracy
Actual	Observed	Interday	Intraday	(%)
15	15.11 ± 0.168	0.832	1.112	100.73
20	19.84 ± 0.214	0.704	1.078	99.20
25	25.28 ± 0.314	0.881	1.242	101.12

4.10.10.1.3 LOD and LOQ

The LOD and LOQ for Exemestane in phosphate buffer pH 7.4 was found to be 0.62 µg/ml and 1.84 µg/ml respectively.

4.10.11 HPLC METHOD DEVELOPMENT OF EXEMESTANE

4.10.11.1 Calibration curve of Exemestane in HPLC

The retention time of Exemestane was found to be 7.61 min. The standard plot of Exemestane in methanol is shown in Table 4.46. The overlay plot of HPLC chromatogram is shown in figure 4.28. Regression equation for standard curve was $y = 580.74\,x - 1.6983$ and correlation coefficient (R^2) was found to be 0.9999 signifying that a linear relationship existed between peak area and concentration of the drug (figure 4.23).

Table 4.46 Standard Calibration data for estimation of Exemestane in HPLC

Sr. No.	Concentration (ng/ml)	Peak Area (µAU*s)	Retention time (min)	%RSD
1.	50	28.77±0.251	7.61	0.872
2.	100	54.41±0.387	7.57	0.710
3.	200	113.05±1.411	7.62	1.248
4.	300	170.90±1.829	7.55	1.070
5.	400	231.30±2.324	7.62	1.004

6.	500	289.55±2.718	7.61	0.938
7.	600	347.04±4.356	7.62	1.255

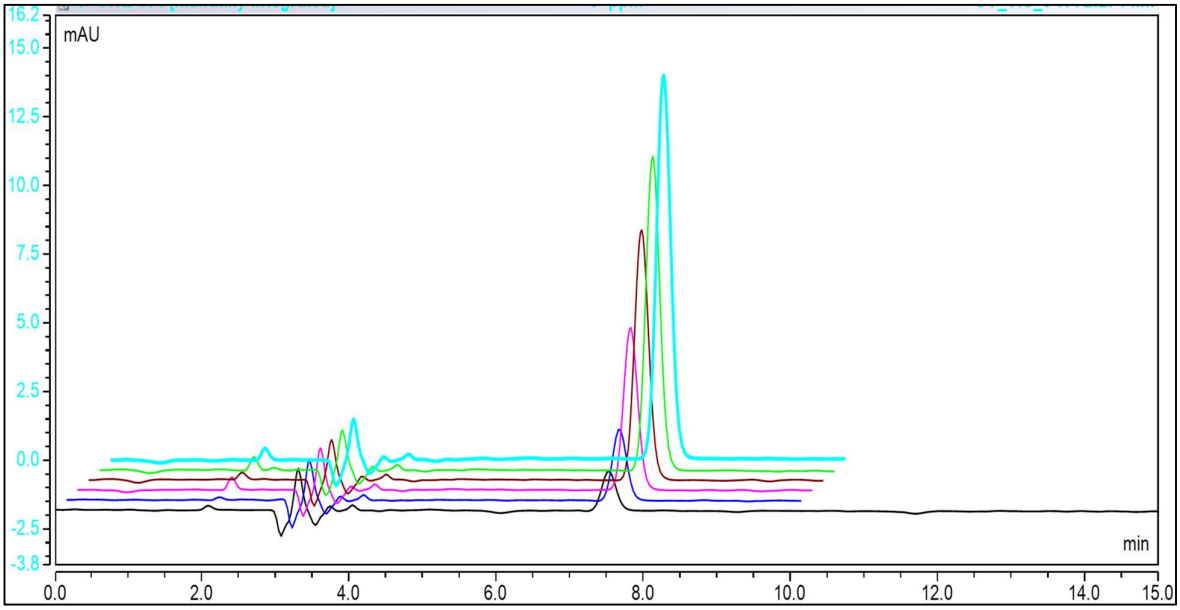

Figure 4.22 Overlay RP – HPLC spectrum of Exemestane in methanol

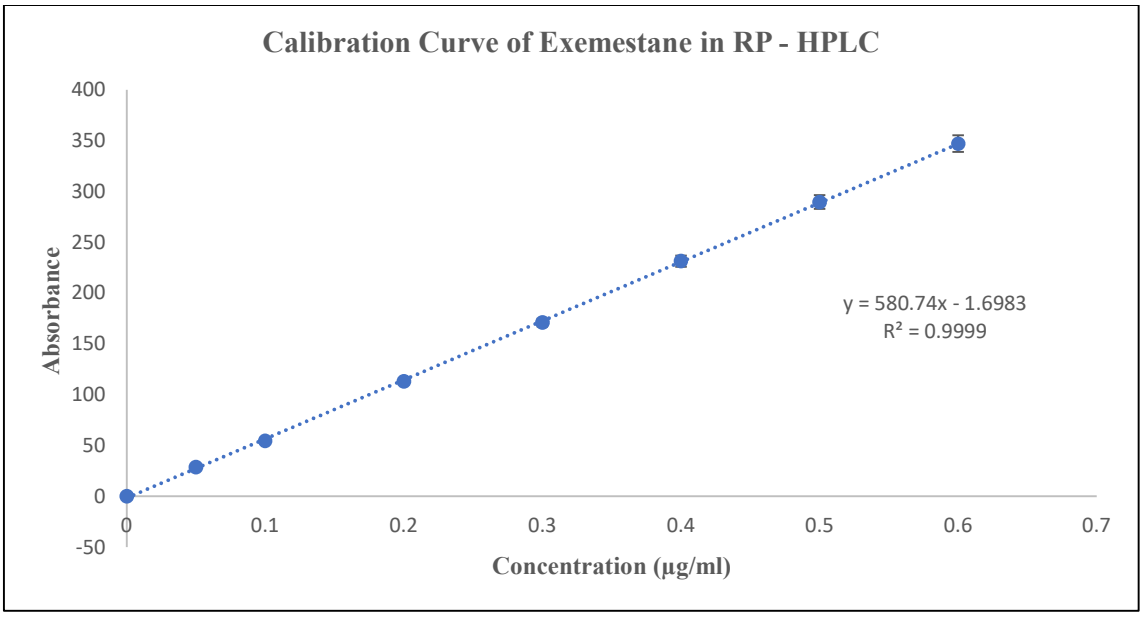

Figure 4.23 Calibration plot of Exemestane by RP – HPLC

4.10.11.1.1 VALIDATION

The analytical method for estimation of Exemestane in RP – HPLC in methanol was validated for different parameters of analytical method validation.

4.10.11.1.1 Linearity

Linear correlation was obtained for Exemestane in concentration range of 50 to 600 (ng/ml). The summarized parameters for regression equation and correlation are given in Table 4.47.

Table 4.47 Parameters from calibration plot of Exemestane in mobile phase

Parameters	Results
λ_{max}	243 nm
Linearity range	50 to 600 ng/ml
Regression equation (y= a + bc)	$y = 580.74\,x - 1.6983$
Correlation coefficient (R^2)	0.9999
Mobile Phase	Methanol: Acetonitrile: Water (65:15:20)

4.10.11.1.2 Accuracy

The percentage recoveries for lower, intermediate, and higher concentration are given in Table 4.48 in mobile phase using RP – HPLC. Their outcome indicates that the suggested analytical approach will reliably assess and predict any slight shift in the drug concentration in the solution.

Table 4.48 Accuracy of the method in mobile phase

Level	Expected Concentration (µg/ml)	Observed Concentration (µg/ml)	%Drug Recovered
80%	0.24	0.237±0.0024	98.75%
100%	0.30	0.306±0.0037	102%
120%	0.36	0.353±0.0051	98.05%

4.10.11.1.3 Precision

The precision analysis for intraday and interday was conducted in the same operating condition. The number of RSD values obtained in the precision analysis was less than 2.0

percent, suggesting that these approaches have good accuracy and reproducibility. Percent RSD from Table 4.49 reveals that the method is reliable and there is no intraday and interday variability in the system.

Table 4.49 Intraday and Interday precision of Exemestane in mobile phase using RP – HPLC

Concentration (µg/ml)	Observed Concentration (µg/ml)		%RSD	
	Intraday	Interday	Intraday	Interday
0.2	0.204±0.0014	0.197±0.0023	0.686	1.167
0.3	0.294±0.0027	0.307±0.0046	0.918	1.498
0.4	0.408±0.0043	0.393±0.0054	1.053	1.374

4.10.11.1.4 Limit of detection and Limit of quantitation

Table 4.50 LOD and LOQ of Exemestane in mobile phase

Limit of Detection (ng/ml)	Limit of Quantitation (ng/ml)
0.91	2.92

4.10.12 Calibration plot of Exemestane by RP – HPLC in rat plasma

The Exemestane calibration plot was obtained in the 50 – 600 ng/ml range. The linear curve regression equation was found to agree with $y = 0.1998\,x + 0.2168$. It was noticed that the correlation coefficient for the system was 0.9999, meaning the presence of a linear relationship between the peak region and the drug concentration. There was a retention time of 7.61 minutes.

Table 4.41 RP – HPLC calibration curve values of Exemestane in Rat Plasma

Sr. No.	Concentration (ng/ml)	Peak Area (µAU*s)	Retention time (min)	%RSD
1.	50	10.24±0.097	7.61	0.947
2.	100	20.19±0.153	7.64	0.757
3.	200	40.18±0.322	7.63	0.801
4.	300	59.87±0.514	7.58	0.858

5.	400	81.03±0.646	7.62	0.797
6.	500	100.41±0.884	7.61	0.880
7.	600	119.43±1.495	7.58	1.251

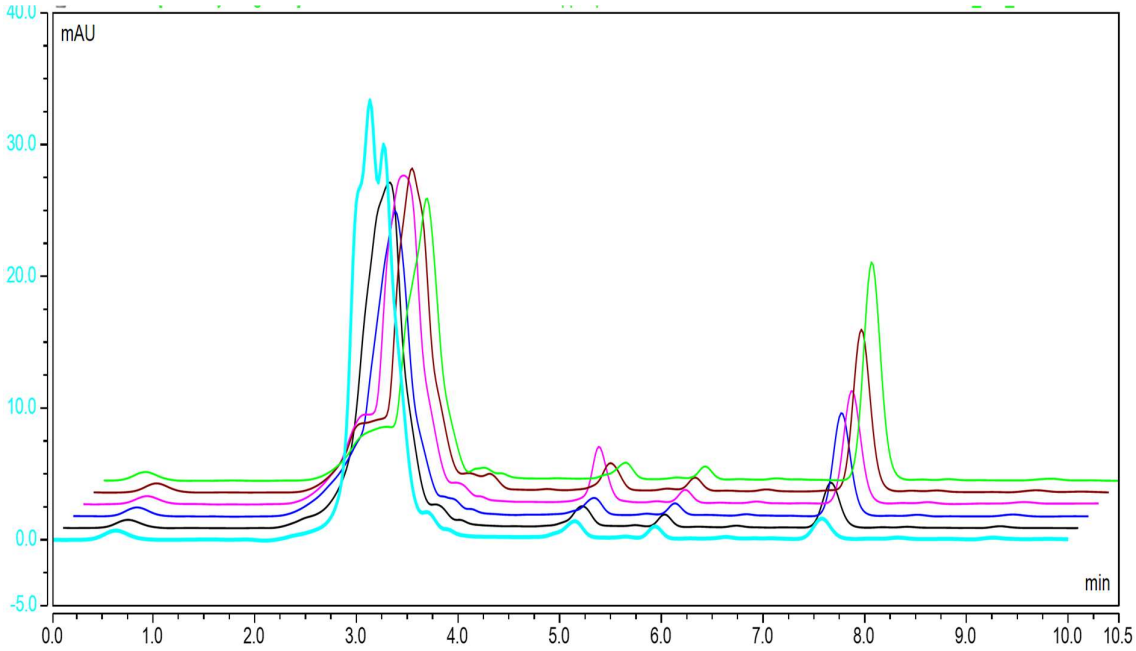

Figure 4.24 Overlay chromatogram of Exemestane in rat plasma by RP – HPLC

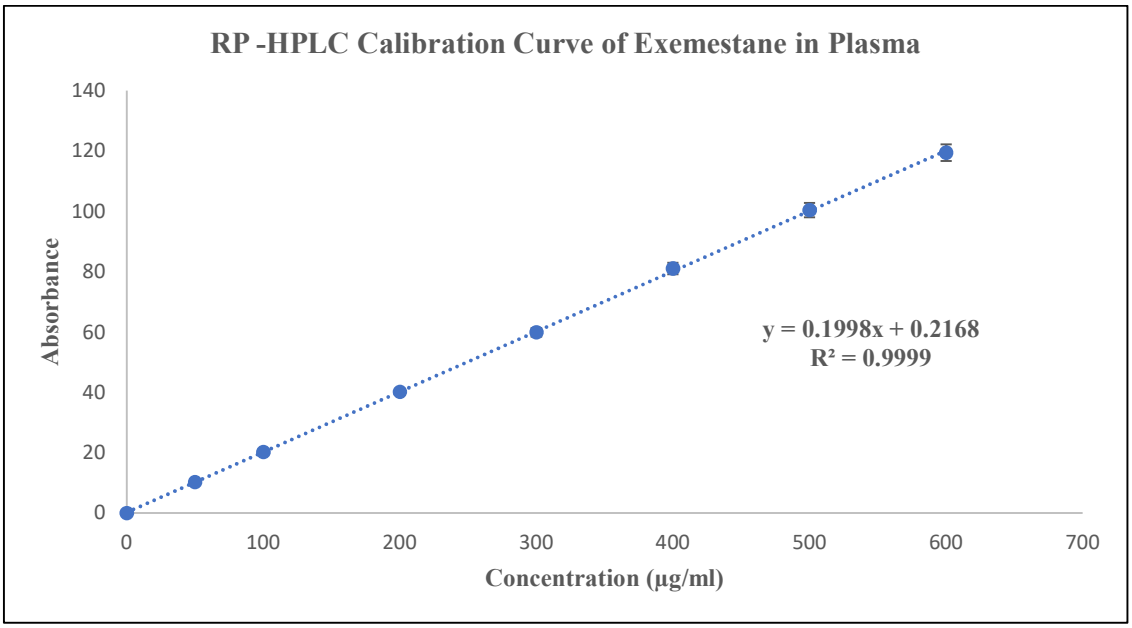

Figure 4.25 Calibration plot of Exemestane by RP – HPLC in rat plasma

4.10.12.1 VALIDATION

The analytical method for estimation of Exemestane in RP – HPLC in methanol was validated for different parameters of analytical method validation.

4.10.12.1.1 Linearity

Linear correlation was obtained for Exemestane in concentration range of 0.05 to 0.6 (µg/ml). The summarized parameters for regression equation and correlation are given in Table 4.51.

Table 4.51 Parameters from calibration plot of Exemestane in mobile phase

Parameters	Results
λ_{max}	243 nm
Linearity range	0.05 to 0.6 µg/ml
Regression equation (y= a + bc)	$y = 0.1998\,x + 0.2168$
Correlation coefficient (R^2)	0.9999
Mobile Phase	Methanol: Acetonitrile: Water (65:15:20)

4.10.12.1.2 Accuracy

The percentage recoveries for lower, intermediate, and higher concentration are given in Table 4.52 in mobile phase using RP – HPLC. Their outcome indicates that the suggested analytical approach will reliably assess and predict any slight shift in the drug concentration in the solution.

Table 4.52 Accuracy of the method in mobile phase

Level	Expected Concentration (µg/ml)	Observed Concentration (µg/ml)	%Drug Recovered
80%	0.24	0.238±0.027	99.16
100%	0.30	0.307±0.037	102.33
120%	0.36	0.365±0.051	101.38

4.10.12.1.3 Precision

The precision analysis for intraday and interday was conducted in the same operating condition. The number of RSD values obtained in the precision analysis was less than 2.0

percent, suggesting that these approaches have good accuracy and reproducibility. Percent RSD from Table 4.53 reveals that the method is reliable and there is no intraday and interday variability in the system.

Table 4.53 Intraday and Interday precision of Exemestane in rat plasma using RP – HPLC

Concentration (µg/ml)	Observed Concentration (µg/ml)		%RSD	
	Intraday	Interday	Intraday	Interday
0.2	0.203±0.0017	0.197±0.0023	0.837	1.167
0.3	0.305±0.0034	0.296±0.0043	1.114	1.452
0.4	0.407±0.0056	0.393±0.0069	1.375	1.775

4.10.12.1.4 Limit of detection and Limit of quantitation

Table 4.54 LOD and LOQ of Exemestane in rat plasma

Limit of Detection (ng/ml)	Limit of Quantitation (ng/ml)
3.58	10.85

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