Chapter 3

ANALYTICAL METHODS

3.1 Introduction

Analytical methods are an important tool in identification and authentication of drugs also, used to assess their purity, potency and physical characteristics. Analytical methods are established for testing the drugs for their specification in order to confirm the quality standards in manufacturing, as well as, during the period of long-term stability assessment. Moreover, methods can be useful to support characterization or evaluation study and safety of the pure drugs or drugs incorporated in pharmaceutical formulation.

Analytical methods can be used for both qualitative as well as for quantitative purpose. Qualitative determination of drugs is performed in various solvents or media and in different biological fluids like plasma, blood, serum or urine. Several techniques such as UV-Visible Spectrophotometry, High-performance liquid chromatography (HPLC), Gas chromatography (GC) [1, 2], Thin layer chromatography [3], Sophisticated tandem chromatographic-spectrophotometric methods like GC-Mass spectrophotometry (GC-MS) [4], LC-MS [5, 6], LC-MS-MS [7, 8] and GC-MS-MS and Nuclear magnetic resonance (NMR) spectroscopy are used in structure identification.

Analytical process is required to be validated in order to ensure the statistical significance of the developed method. The aim of validating an analytical method is to confirm that every single analysis performed will reproduce similar or close enough values or results of the analyte present in the sample [9-12]. Analytical method validation includes various parameters such as Linearity, Accuracy, Precision, Range, Robustness, Ruggedness, Limit of Quantification (LOQ) and Limit of Detection (LOD) [13-15].

This chapter focuses on development and validation of UV-Visible spectrophotometric and LCMS-MS methods for drug estimation (Gemcitabine hydrochloride and Vinorelbine tartrate). The developed analytical method will be used to determine the concentration of drug in sample in studies like entrapment efficiency, % loading (%w/w), *in vitro* drug release study, *in vivo* pharmacokinetic study and stability studies. UV-Visible Spectrophotometry is the convenient method to estimate the drug concentration in micrograms. While, highly sensitive method like LCMS-MS is used to determine very low drug concentration in nano grams, specifically in biological samples. Highly sensitive LCMS-MS technique, consists hyphenated HPLC system with mass spectroscopy, which acts as detector system.

3.2 Materials and Equipments

3.2.1 Materials

Gemcitabine HCl was obtained as a gift sample from Sun Pharmaceutical Industries Ltd., Vadodara, India. Vinorelbine tartrate was obtained as a gift sample from Cipla Ltd. Mumbai, India. Cholesterol, Mannitol and Potassium oleate along with HPLC grade Methanol, Formic acid, Acetonitrile and Ammonium acetate were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium chloride, Potassium chloride, Disodium phosphate and monobasic potassium phosphate were purchased from Loba Chemie, Mumbai, India. For LCMS analysis, Direct-Q® Water Purification System, Millipore (Bedford, MA, USA) was used throughout the study. In house double distilled water was used for UV-Visible spectrophotometric analysis. All other chemicals used were obtained from authentic source and were of Analytical Reagent grade.

3.2.2 Equipments

- Analytical Weighing Balance (ATX 224, 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 Methods

3.3.1 Analytical Methods for estimation of Gemcitabine hydrochloride

3.3.1.1 UV-Visible Spectrophotometric Method in Distilled Water

A) Standard stock solution: Accurately weighed 10 mg of drug was transferred to a 10 mL volumetric flask. It was dissolved in small quantity of distilled water, followed by making up the volume up to 10 mL with distilled water to get the final concentration of 1 mg/mL (1000µg/mL).

B) Determination of analytical wavelength (λmax): From the stock solution, 0.1 mL was transferred to a 10 mL volumetric flask using a micropipette. Dilution with distilled water was done up to the mark to obtain 10 µg/mL solution of Gemcitabine Hydrochloride. The UV spectrum of the resulting solution was recorded using UV-Visible spectrophotometer (UV-

1800, Shimadzu, Japan) in the range of 200-400 nm. The wavelength at which maximum absorbance was obtained was selected as analytical wavelength.

C) Preparation of calibration plot: From the stock solution of 1000 µg/mL, 1 mL aliquot was diluted in 10 mL distilled water to get the concentration of 100 µg/mL. From this solution 0.5, 1, 1.5, 2, 2.5 and 3 mL aliquots were withdrawn and diluted in 10 mL distilled water to get the working concentrations of 5, 10, 15, 20, 25, 30 µg/mL, respectively. The absorbance of these solutions was measured at the λmax of 266 nm. A concentration vs. absorbance calibration plot was plotted. Each reading was taken in triplicate (n=3).

3.3.1.2 UV-Visible Spectrophotometric Method in Phosphate-buffered saline (PBS) pH 7.4

A) Preparation of Phosphate-buffered saline (PBS) pH 7.4

8 g of Sodium chloride, 0.2 g of Potassium chloride, 1.44g of Disodium phosphate and 0.24 g of monobasic potassium phosphate were dissolved in 800 mL distilled water, followed by pH adjustment to 7.4 using 0.1 N Hydrochloric acid. The volume was made up to 1000 mL (1 *l*) with distilled water [16].

B) Standard stock solution: Accurately weighed 10 mg of drug was transferred to a 10 mL volumetric flask. It was dissolved in small quantity of distilled water, followed by making up the volume up to 10 mL with distilled water to get the final concentration of 1 mg/mL (1000µg/mL).

C) Determination of Absorbance maxima (λmax): From the stock solution, 0.1 mL was transferred to a 10 mL volumetric flask using a micropipette. Dilution with distilled water was done up to the mark to obtain 10 µg/mL solution of Gemcitabine Hydrochloride. The UV spectrum of the resulting solution was recorded using UV-Visible spectrophotometer (UV-1800, Shimadzu, Japan) in the range of 200-400 nm. The wavelength at which maximum absorbance was obtained was selected as analytical wavelength.

D) Preparation of calibration plot: From the stock solution of 1000 µg/mL, 1 mL aliquot was diluted in 10 mL distilled water to get the concentration of 100 µg/mL. From this solution 0.5, 1, 1.5, 2, 2.5 and 3 mL aliquots were withdrawn and diluted in 10 mL distilled water to get the working concentrations of 5, 10, 15, 20, 25, 30 µg/mL, respectively. The absorbance of these solutions was measured at the λmax of 269 nm. A concentration vs. absorbance calibration plot was plotted. Each reading was taken in triplicate (n=3).

3.3.1.3 Method Validation [17]

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 Gemcitabine Hydrochloride in distilled water and PBS pH 7.4, were validated.

3.3.1.3.1 Linearity

Calibration plot of Gemcitabine Hydrochloride both in distilled water and PBS pH 7.4 was performed in the range of 5-30 µg/mL. The graphs obtained by plotting the absorbance versus the concentration data were treated by linear regression analysis. Measurements were done in triplicate.

3.3.1.3.2 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 (15 mg as 80%, 20 mg as 100% and 25 mg as 120%) were spiked in the pre-analyzed samples. The absorbance of the resulting solution were measured and calculation of % recovery was done. Statistically analyzed by applying t test.

3.3.1.3.3 Precision

The precision was determined by measuring the repeatability of. Both intraday and inter-day precision were determined and reported as % RSD for replicate (n=3) measurements. For determining inter-day precision, the results of the assays done on three different days were compared and standard deviation (SD) and % RSD were reported.

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

LOD and LOQ were calculated using the 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 calibration plot.

3.3.1.4 Analytical Interference study

Drug-Excipient analytical interference study was performed to confirm their non-interference during estimation of the drug. Study was carried out by preparing drug solution along with known concentration of excipients separately in distilled water, resulting solution was scanned in the UV range i.e. 200-400 nm, using distilled water as blank. Briefly, to Gemcitabine Hydrochloride solution (20 µg/mL) in distilled water, known concentrations of Soyabean

Phophatidylcholine (SPC), Cholesterol (Chol), Mannitol (MN) and Potassium oleate (PO) were added and spectra was run in the UV range between 200-400 nm.

3.3.1.5 Estimation of Gemcitabine Hydrochloride using LCMS-MS method

Gemcitabine hydrochloride was estimated in rat plasma by using LC-MS/MS method [18]. A method was developed for drug estimation from rat plasma by liquid chromatography/tandem mass spectrometry (LC- MS/MS). Method validation was performed as per acceptance criteria reported in ICH guideline.

A) Chromatographic conditions:

- Instrumentation: 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: 10% Methanol in 10 mM ammonium acetate buffer (pH 6.8); (10:90 v/v)
- Elution Pattern: Isocratic
- Flow rate: 0.50 mL/min
- Selected Reaction Monitoring (SRM) Transitions for Gemcitabine hydrochloride: m/z 226 → m/z 112
- Ionization mode: Positive electrospray
- Injection volume: 10 µL

B) Preparation of Mobile phase:

10 mM ammonium acetate buffer (pH 6.8) was prepared by adding 0.77 g of ammonium acetate (CH_3COONH_4) in 400 mL of distilled water and mixed thoroughly until all the salt get dissolved. Resulting solution was passed through 0.2 µm membrane filter (Himedia lab Pvt. Ltd., Mumbai, India), followed by addition of water till 950 mL and pH of the solution was checked. Acetic acid was used to adjust the pH to 6.8 and volume was made up to 1000 mL (1 *l*). This solution was degassed using bath sonicator.

10 parts of methanol was mixed with 90 parts of 10 mM ammonium acetate buffer (pH 6.8), followed by bath sonication and this mixure was used as mobile phase for the elution of Gemcitabine hydrochloride.

C) Preparation of Stock Solution

Stock solution of Gemcitabine hydrochloride was prepared in methanol. Briefly, 1 mg of drug was dissolved in 10 mL of methanol in a volumetric flask to get the drug concentration of 100 µg/mL. Stock solution was stored at -20 ºC until further dilution.

D) Preparation of Calibration plot

Standard working solutions were prepared by withdrawing appropriate aliquots of stock solution followed by dilution with water (Direct-Q® Water Purification System, Millipore, Bedford, MA, USA) to obtain the concentration range viz. 20, 100, 250, 500, 1000, 2000 ng/mL. Briefly, 20 µl aliquot was withdrawn from stock solution of 100 µg/mL and diluted in 100 mL water to obtain 20 ng/mL concentration. Subsequently, 100, 250, 500, 1000 and 2000 ng/mL concentrations were obtained by withdrawing 100, 250, 500, 1000 and 2000 µl of aliquots respectively from stock solution and diluted to 100 mL with water.

E) Sample extraction procedure:

Blood from Sprague-Dawley rat was withdrawn and collected in 1.5 mL of heparinized eppendorf tubes. Plasma was separated from blood sample by centrifuging (Remi Equipments, Mumbai, India) the eppendorf tube for 5 minutes at 2200 ×g at 4 ºC. Separated plasma was obtained as supernatant. It was collected and stored at -20 ºC till further analysis.

Frozen rat plasma samples were thawed at room temperature. Micro protein precipitation (mPPT) with acetonitrile was performed to precipitate out the plasma proteins. Briefly, 5 µL plasma, 10 µL water and 45 µL acetonitrile were taken in a 1.5 mL eppendorf tube. Immediately the tube was vortexed for 1 minute, followed by centrifugation for 5 minutes at 9200 ×g at 4 ºC. 50 µL of supernatant was taken in fresh eppendorf tube and dried under nitrogen, followed by reconstitution with 50 µL of mobile phase.

F) Preparation of Calibration plot in rat organ homogenate

Calibration plot in various organ homogenate was performed in order to estimate the drug concentration in biodistribution study. Highly perfused organs like Heart, Lungs, Liver, Spleen and Kidneys were isolated from Sprague Dawley rat and used for the analysis. Sample from rat organs was extracted by homogenization of the organ, followed by centrifugation for 5 minutes at 2200 ×g at 4 ºC. Supernatant was collected and Micro protein precipitation (mPPT) with acetonitrile was performed to precipitate out the proteins and sample was prepared with the similar procedure as described above.

3.3.1.6 Method Validation:

Validation of analytical method was performed by calculating validation parameters such as Linearity, Accuracy, and LOQ.

3.3.1.6.1 Linearity: Standard curve was obtained over a range of drug concentration and fitted with linear regression. Equation of a straight line i.e. y = mx + c was calculated, where, y represents the Gemcitabine hydrochloride peak area and x represents the plasma concentration of the Gemcitabine hydrochloride. In order to eliminate the presence of any interference a blank plasma sample was analyzed.

3.3.1.6.2 Accuracy: Samples were prepared at three different concentration levels (150, 300, 450 ng/mL). Accuracy was calculated by using formula: (observed concentration − nominal concentration)/(nominal concentration)×100%. Calculated accuracy was expressed as relative error (RE). the obtained results were analyzed Statistically by applying t test. t value was calculated using Microsoft Excel. The calculated t value was compared to the tabulated t value.

3.3.1.6.3 LOQ: The limit of quantification (LOQ), taken, the lowest concentration on the calibration plot which could be measured accurately was taken as the LOQ.

3.3.2 Analytical Methods for estimation of Vinorelbine Tartrate

3.3.2.1 UV-Visible Spectrophotometric Method in Distilled Water

A) Standard stock solution: Accurately weighed 10 mg of drug was transferred to a 10 mL volumetric flask. It was dissolved in small quantity of distilled water, followed by making up the volume up to 10 mL with distilled water to get the final concentration of 1 mg/mL (1000µg/mL).

B) Determination of Absorbance maxima (λmax): From the stock solution, 0.1 mL was transferred to a 10 mL volumetric flask using a micropipette. Dilution with distilled water was done up to the mark to obtain 10 µg/mL solution of Gemcitabine Hydrochloride. The UV spectrum of the resulting solution was recorded using UV-Visible spectrophotometer (UV-1800, Shimadzu, Japan) in the range of 200-400 nm. The wavelength at which maximum absorbance was obtained was selected as analytical wavelength.

C) Preparation of calibration plot: From the stock solution of 1000 µg/mL, 1 mL aliquot was diluted in 10 mL distilled water to get the concentration of 100 µg/mL. From this solution 0.5, 1, 1.5, 2, 2.5 and 3 mL aliquots were withdrawn and diluted in 10 mL distilled water to get the working concentrations of 5, 10, 15, 20, 25, 30 µg/mL, respectively. The absorbance of these

solutions was measured at the λmax of 271 nm. A concentration vs. absorbance calibration plot was plotted. Each reading was taken in triplicate (n=3).

3.3.2.2 UV-Visible Spectrophotometric Method in Phosphate-buffered saline (PBS) pH 7.4

A) Preparation of Phosphate-buffered saline (PBS) pH 7.4

8 g of Sodium chloride, 0.2 g of Potassium chloride, 1.44g of Disodium phosphate and 0.24 g of monobasic potassium phosphate were dissolved in 800 mL distilled water, followed by pH adjustment to 7.4 using 0.1 N Hydrochloric acid. The volume was made up to 1000 mL (1 *l*) with distilled water [16].

B) Standard stock solution: Accurately weighed 10 mg of drug was transferred to a 10 mL volumetric flask. It was initially dissolved in 4-5 mL of PBS pH 7.4. Then the volume was made up to 10 mL with PBS pH 7.4 to get the final concentration of 1 mg/mL (1000 µg/mL).

C) Determination of Absorbance maxima (λmax): From the stock solution, 0.1 mL was transferred to a 10 mL volumetric flask using a micropipette. Dilution with distilled water was done up to the mark to obtain 10 µg/mL solution of Gemcitabine Hydrochloride. The UV spectrum of the resulting solution was recorded using UV-Visible spectrophotometer (UV-1800, Shimadzu, Japan) in the range of 200-400 nm. The wavelength at which maximum absorbance was obtained was selected as analytical wavelength.

D) Preparation of calibration plot: From the stock solution of 1000 µg/mL, 1 mL aliquot was diluted in 10 mL distilled water to get the concentration of 100 µg/mL. From this solution 0.5, 1, 1.5, 2, 2.5 and 3 mL aliquots were withdrawn and diluted in 10 mL distilled water to get the working concentrations of 5, 10, 15, 20, 25, 30 µg/mL, respectively. The absorbance of these solutions was measured at the λmax of 273 nm. A concentration vs. absorbance calibration plot was plotted. Each reading was taken in triplicate (n=3).

3.3.2.3 Method Validation [17]

The method was validated as discussed in the earlier section 3.3.1.3, 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 Vinorelbine Tartrate in distilled water and PBS pH 7.4 were validated.

3.3.2.4 Analytical Interference study

Drug-Excipient analytical interference study was performed to confirm their non-interference during estimation of the drug. Study was carried out by preparing drug solution along with known concentration of excipients separately in distilled water, resulting solution was scanned in the UV range i.e. 200-400 nm, using distilled water as blank. Briefly, to Vinorelbine Tartrate solution (20 µg/mL) in distilled water, known concentrations of Soyabean Phophatidylcholine (SPC), Cholesterol (Chol), Mannitol (MN) and Potassium oleate (PO) were added and spectra was run in the UV range between 200-400 nm.

3.3.2.5 Estimation of Vinorelbine Tartrate using LCMS-MS method

Vinorelbine Tartrate was estimated in rat plasma by using LC-MS/MS method [19]. A method was developed for drug estimation from rat plasma by liquid chromatography/tandem mass spectrometry (LC- MS/MS). Method validation was performed as per acceptance criteria reported in ICH guideline.

A) Chromatographic conditions:

- Instrumentation: 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: 70% Methanol and 30% 10mM ammonium acetate with 0.8% Formic acid
- Elution Pattern: Isocratic
- Flow rate: 0.20 mL/min
- Selected Reaction Monitoring (SRM) Transitions for Vinorelbine Tartrate: m/z 779.2 → m/z 122.0.
- Ionization mode: Positive electrospray
- Injection volume: 5 µL

B) Preparation of Mobile phase:

10 mM ammonium acetate buffer (pH 6.8) was prepared as per the method discussed in section *3.3.1.5*. 0.8 % Formic acid solution was mixed with the above solution and was degassed using bath sonicator.

Mobile phase was prepared by mixing 70 parts of methanol with 30 parts of above prepared

solution i.e. 10 mM ammonium acetate buffer with 0.8 % Formic acid.

C) Preparation of Stock Solution

Vinorelbine tartrate stock solution was prepared in methanol. Briefly, 1 mg of drug was dissolved in 10 mL of methanol in a volumetric flask to get the drug concentration of 100 µg/mL. Stock solution was stored at -20 ºC until further dilution.

D) Preparation of Calibration plot

Standard working solutions were prepared by withdrawing appropriate aliquots of stock solution followed by dilution with water (Direct-Q® Water Purification System, Millipore, Bedford, MA, USA) to obtain the concentration range viz. 5, 10. 50, 100, 200, 500 ng/mL. Briefly, 5 µl aliquot was withdrawn from stock solution of 100 µg/mL and diluted in 100 mL water to obtain 5 ng/mL concentration. Subsequently, for 10, 50, 100, 200 and 500 ng/mL concentrations were obtained by withdrawing 10, 50, 100, 200 and 500 µl of aliquots respectively from stock solution and diluted to 100 mL with water.

E) Sample extraction procedure:

Sprague-Dawley rat were used as experimental animals, blood was withdrawn and collected in 1.5 mL of heparinized eppendorf tubes. Plasma was separated from blood sample by centrifuging (Remi Equipments, Mumbai, India) the eppendorf tube for 5 minutes at 2200 ×g at 4 ºC. Separated plasma was obtained as supernatant. It was collected and stored at -20 ºC till further analysis.

Frozen rat plasma samples were thawed at room temperature. Micro protein precipitation (mPPT) with methanol was performed to precipitate out the plasma proteins. Briefly, 240 µL methanol was added in 80 µL plasma in a 1.5 mL eppendorf tube. Immediately the tube was vortexed for 10 minute, followed by centrifugation for 5 minutes at 9200 ×g at 4 ºC. The supernatant was collected in fresh eppendorf tube and dried under nitrogen, followed by reconstitution with 80 µL of methanol. This solution was analyzed by injecting in the LCMS system.

F) Preparation of Calibration plot in rat organ homogenate

Calibration plot in various organ homogenate was performed in order to estimate the drug concentration in biodistribution study. Highly perfused organs like Heart, Lungs, Liver, Spleen and Kidneys were isolated from Sprague Dawley rat and used for the analysis. Sample from rat organs was extracted by homogenization of the organ, followed by centrifugation for 5 minutes

at 2200 ×*g* at 4 ºC. Supernatant was collected and Micro protein precipitation (mPPT) with methanol was performed to precipitate out the proteins and sample was prepared with the similar procedure as described above.

3.3.2.6 Method Validation:

Validation of analytical method was performed by calculating validation parameters such as Linearity, Accuracy, and LOQ as described in earlier section 3.3.1.6.

3.4 Results and Discussion

3.4.1 Analytical method for estimation of Gemcitabine Hydrochloride

3.4.1.1 UV-Visible Spectrophotometric Method for estimation of Gemcitabine Hydrochloride in Distilled Water

Gemcitabine Hydrochloride showed absorption maxima at 266 nm in distilled water. Linearity was performed in the concentration range of 5 to 30 µg/mL. An overlay spectra of Gemcitabine Hydrochloride is seen in Figure 3.1 and respective concentration and their absorbance have been tabulated in Table 3.1. Figure 3.2 shows Absorbance vs. Concentration graph i.e. calibration plot.

Table 3.1: Calibration data for Gemcitabine Hydrochloride in distilled water.

Concentration in µg/mL	Absorbance ±SD*
0	0
5	0.159±0.011
10	0.308±0.012
15	0.469±0.007
20	0.620±0.010
25	0.784±0.010
30	0.941±0.002

* Experiment was done in triplicate (n=3)

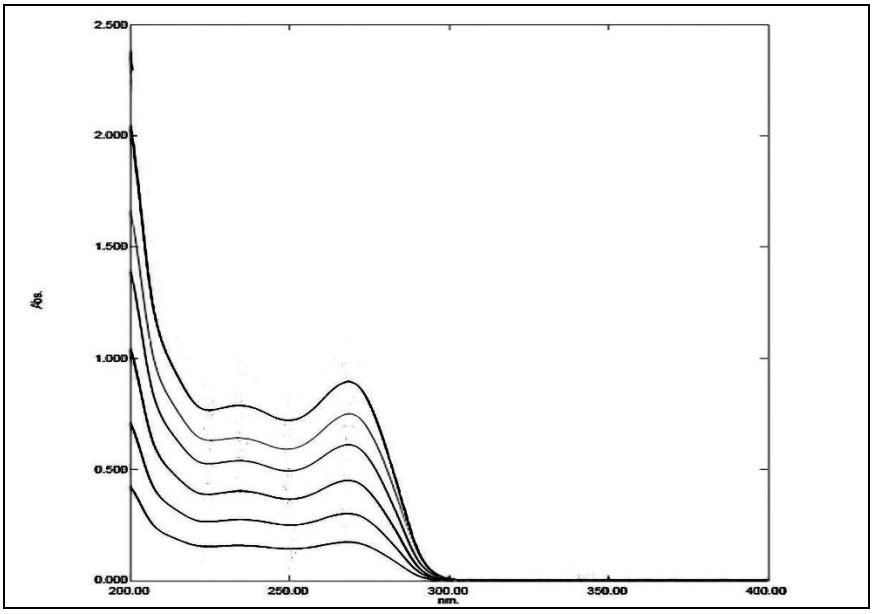

Figure 3.1: Overlay plot of Gemcitabine Hydrochloride in distilled water at 266 nm.

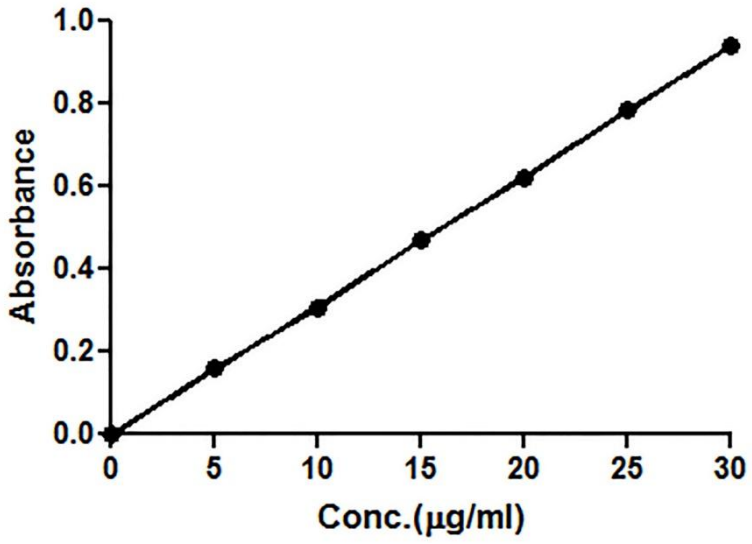

Figure 3.2: Calibration plot of Gemcitabine Hydrochloride in distilled water.

Regression analysis of the calibration plot was done using the method of least squares to assess the intercept, slope and correlation coefficient (R^2). The value of correlation coefficient of the regression equation was found to be near to 1 (0.9999), moreover, the negligible intercept value confirmed the calibration plot was linear, which is in accordance with the Beer-Lambert law. The regression analysis values have been shown in Table 3.2.

Table 3.2: Parameters for estimation of Gemcitabine Hydrochloride in distilled water by UV Spectrophotometry.

Parameters	Results
λmax	266 nm
Linearity range	5-30 µg/mL
Regression Equation	y = 0.0313x-0.001
Correlation Co-efficient	0.9999

3.4.1.2 UV-Visible Spectrophotometric Method for estimation of Gemcitabine Hydrochloride in PBS pH 7.4

Gemcitabine Hydrochloride showed absorption maxima at 269 nm in PBS pH 7.4. Linearity was performed in the concentration range of 5 to 30 µg/mL. An overlay spectra of Gemcitabine Hydrochloride is seen in Figure 3.3 and respective concentration and their absorbance have been tabulated in Table 3.3. Figure 3.4 shows Absorbance vs. Concentration graph i.e. calibration plot.

Table 3.3: Calibration data for Gemcitabine Hydrochloride in PBS pH 7.4.

Concentration in µg/mL	Absorbance ±SD*
0	0
5	0.149±0.010
10	0.288±0.026
15	0.444±0.038
20	0.589±0.052
25	0.738±0.069
30	0.886±0.096

* Experiment was done in triplicate (n=3)

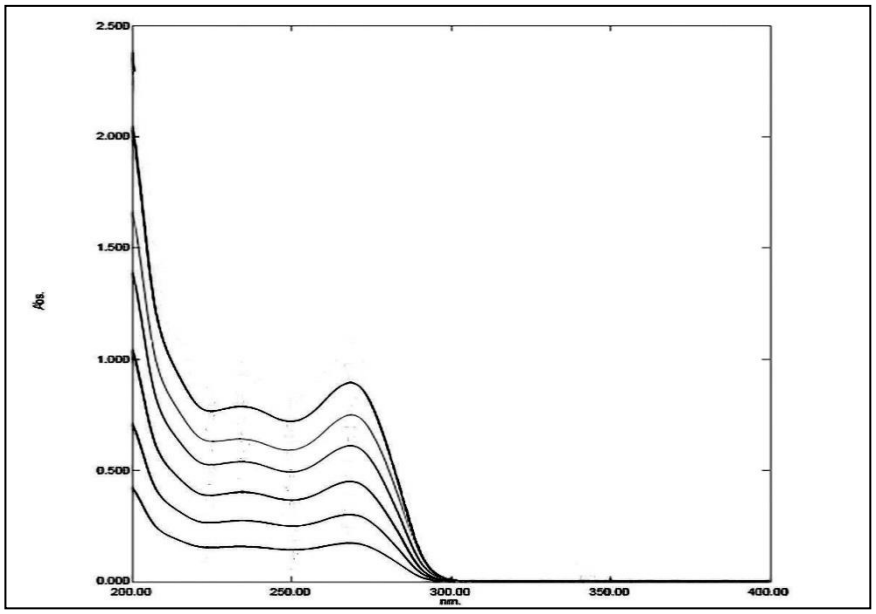

Figure 3.3: Overlay plot of Gemcitabine Hydrochloride in PBS pH 7.4 at 269 nm.

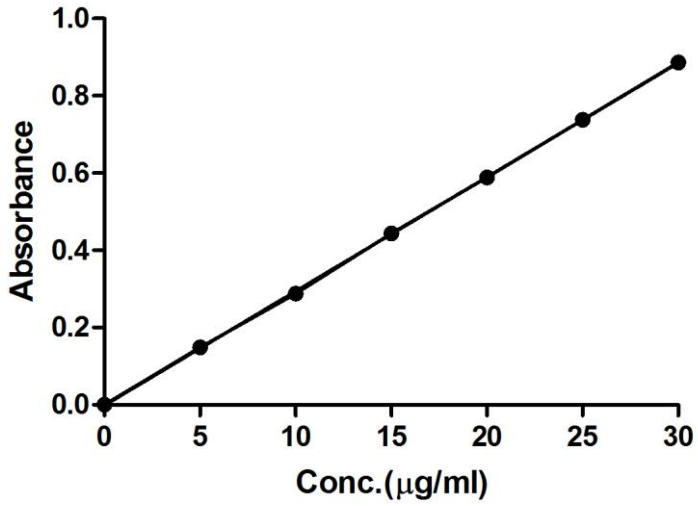

Figure 3.4: Calibration plot of Gemcitabine Hydrochloride in PBS pH 7.4.

Regression analysis of the calibration plot was done using the method of least squares to assess the intercept, slope and correlation coefficient (R^2). The value of correlation coefficient of the regression equation was found to be near 1 (0.9999), moreover, the negligible intercept value confirmed the calibration plot was linear, which is in accordance with the Beer-Lambert law. The regression analysis values have been shown in Table 3.4.

Table 3.4: Parameters for estimation of Gemcitabine Hydrochloride in PBS pH 7.4 by UV-Visible Spectrophotometry.

Parameters	Results
λmax	269 nm
Linearity range	5-30 µg/mL
Regression Equation	y =0.0296x-0.0012
Correlation Co-efficient	0.9999

3.4.1.3 Method Validation

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

3.4.1.3.1 Accuracy

The results of accuracy for developed analytical method in both distilled water and PBS pH 7.4 are shown in Table 3.5 and Table 3.6 respectively. Method was found to be accurate as the % Relative Standard Deviation (RSD) was less than 2 %. No significant difference was found between true values and observed values at all the concentration levels. Unpaired t test was applied to assess the statistical significance for accuracy results of Gemcitabine Hydrochloride in distilled water and it was found to be non-significant as t tabulated value (t_{tab}=2.77) was much higher than t calculated value (t_{cal}=0.13) where, p=0.9024 with 95% confidence interval (CI). Accuracy results of Gemcitabine Hydrochloride in PBS pH 7.4 were also found statistically insignificant as t_{tab}=2.77 was much higher than t_{cal}=0.14 where, p=0.8889 with 95% CI.

Table 3.5: Accuracy results for estimation of Gemcitabine Hydrochloride in distilled water.

Quantity of Gemcitabine Hydrochloride added	Actual conc. (µg/mL)	Obtained conc. (µg/mL)	% Recovery	%RSD
80%	15	15.4±0.04	102.67	0.26
100%	20	20.8±0.07	104.00	0.34
120%	25	25.4±0.03	101.60	0.12

*Experiment was done in triplicate (n=3)

Table 3.6: Accuracy results for estimation of Gemcitabine Hydrochloride in PBS pH 7.4.

Quantity of Gemcitabine Hydrochloride added	Actual conc. (µg/mL)	Obtained conc. (µg/mL)	% Recovery	%RSD
80%	15	14.87±0.02	99.13	0.13
100%	20	21.30±0.04	106.50	0.19
120%	25	25.74±0.09	102.96	0.35

* Experiment was done in triplicate (n=3)

3.4.1.3.2 Precision

The results of precision for developed analytical method in both distilled water and PBS pH 7.4 are shown in Table 3.7 and 3.8 respectively. Method was found to be accurate as the % Relative Standard Deviation (RSD) was less than 2 %. No significant difference was found between true values and observed values at all the concentration levels.

Table 3.7: Precision results for estimation of Gemcitabine Hydrochloride in distilled water.

Actual Concentration (µg/mL)	Intraday precision			Interday precision		
	Observed Conc. (µg/mL)	% Recovery	%RSD	Observed Conc. (µg/mL)	% Recovery	% RSD
15	14.87±0.02	99.13	0.13	14.89±0.09	99.27	0.60
20	21.3±0.04	106.50	0.19	19.95±0.18	99.75	0.90
25	25.74±0.09	102.96	0.35	24.97±0.07	99.88	0.28

* Experiment was done in triplicate (n=3)

Table 3.8: Precision results for estimation of Gemcitabine Hydrochloride in PBS pH 7.4.

Actual Concentration (µg/mL)	Intraday precision			Interday precision		
	Observed Conc. µg/mL	% Recovery	%RSD	Observed Conc. µg/mL	% Recovery	%RSD
15	15.19±0.04	101.27	0.26	15.20±0.03	101.33	0.20
20	20.54±0.09	102.70	0.44	19.85±0.15	99.25	0.76
25	25.37±0.12	101.48	0.47	24.91±0.07	99.64	0.28

* Experiment was done in triplicate (n=3)

3.4.1.3.3 LOD and LOQ: Limit of Detection and Limit of Quantification was calculated for developed analytical method. Gemcitabine Hydrochloride was found to be precisely detected and quantified at 0.67 µg/mL and 2.24 µg/mL in distilled water, while in PBS pH 7.4 LOD and LOQ values were 0.81 µg/mL and 2.70 µg/mL respectively.

3.4.1.4 Analytical Interference Study

All the excipients viz. Soyabean Phosphatidylcholine, Cholesterol, Potassium oleate and Mannitol were taken at concentration which are used in the final formulation. The absorbance values of excipients at 266 nm were found to be negligible showing that there was no interference in the estimation of Gemcitabine Hydrochloride. Table 3.9 shows the absorbance values of 20 µg/mL solution of Gemcitabine Hydrochloride with and without excipients.

Table 3.9: Results of Analytical Interference Studies for Gemcitabine Hydrochloride.

Absorbance of Gemcitabine Hydrochloride solution (20 µg/mL) without excipients	**Absorbance of Gemcitabine Hydrochloride solution (20 µg/mL) with excipients (Soyabean Phosphatidylcholine, Cholesterol, Potassium oleate and Mannitol)**
0.620±0.004	0.620±0.011

*Experiment was done in triplicate (n=3)

3.4.1.5 Estimation of Gemcitabine Hydrochloride using LCMS-MS method

3.4.1.5.1 Calibration plot of Gemcitabine Hydrochloride in rat plasma

Gemcitabine Hydrochloride was estimated precisely by LCMS-MS method. Mobile phase used for elution of Gemcitabine Hydrochloride was Methanol in 10 mM ammonium acetate buffer (pH 6.8); (10:90 v/v). Retention time was found to be 2.89 min. Table 3.10 shows the peak area with its respective concentration and chromatograms acquired from analyzing the standard calibration plot samples are shown in Figure 3.5. As it is observed the blank plasma sample did not showed any peak over the total run time of 8 minutes, suggesting there was no interference during the drug estimation. Figure 3.6 depicts the standard calibration plot of Gemcitabine Hydrochloride estimated from rat plasma by LCMS-MS analytical method. Interference study was also performed to ensure the accurate analysis of drug. Briefly, blank plasma sample was injected into LCMS-MS system and the obtained chromatogram was studied for any interference.

Table 3.10: Calibration data for Gemcitabine Hydrochloride in rat plasma by LCMS-MS.

Concentration (ng/mL)	**Peak Area ± SD**	**Average RSD**
0	0	0.11
20	2.02E+05 ± 615.73	
100	1.10E+06 ± 707.55	
250	2.56E+06 ± 958.08	
500	5.21E+06 ± 2824.43	
1000	9.97E+06 ± 3478.01	
2000	2.03E+07 ± 36097.03	

* Experiment was done in triplicate (n=3)

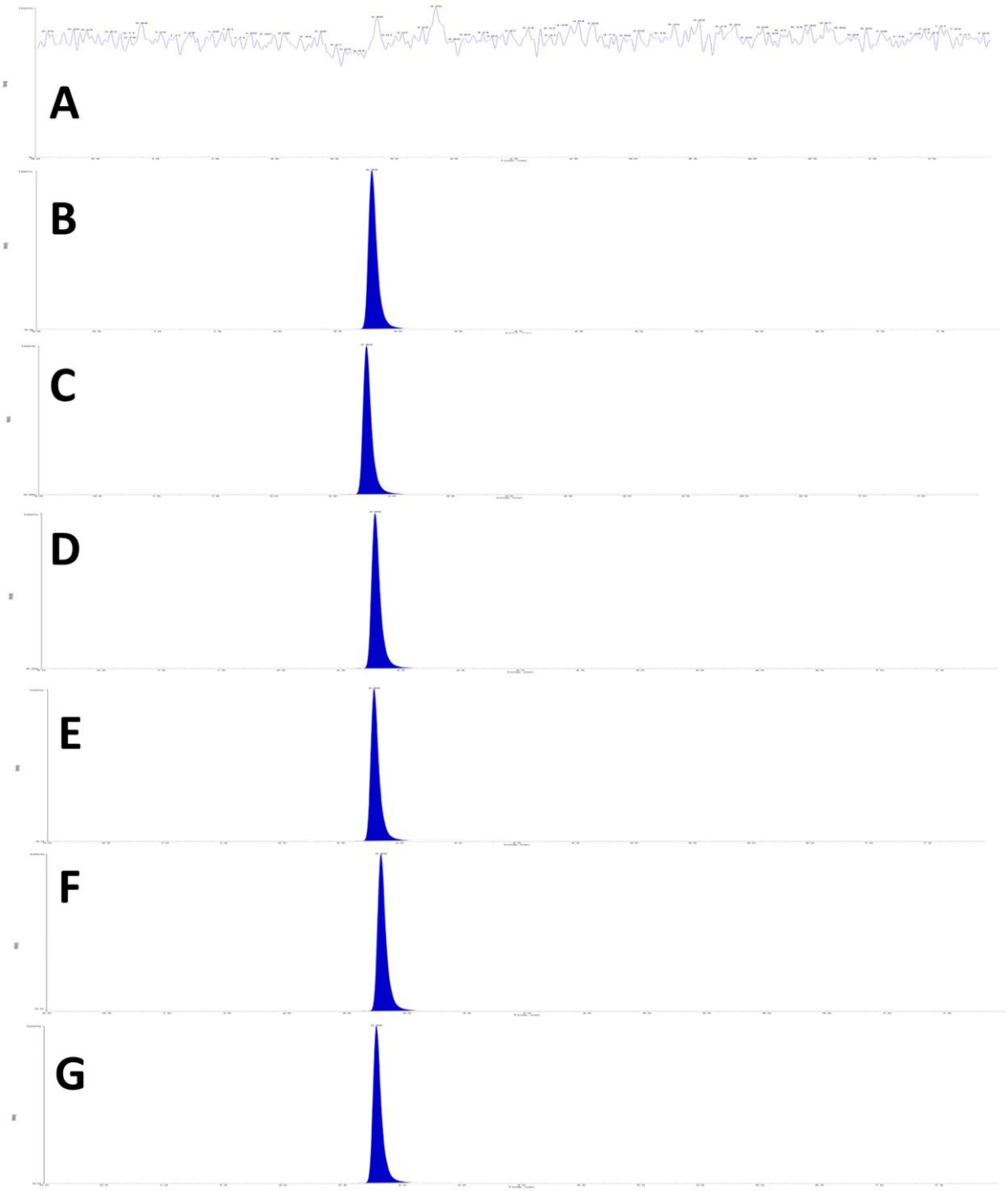

Figure 3.5: Chromatograms of Gemcitabine Hydrochloride calibration plot showing retention time of 2.89 min (A- Blank, B- 20 ng/mL, C- 100 ng/mL, D- 250 ng/mL, E- 500 ng/mL, F- 1000 ng/mL, G- 2000 ng/mL).

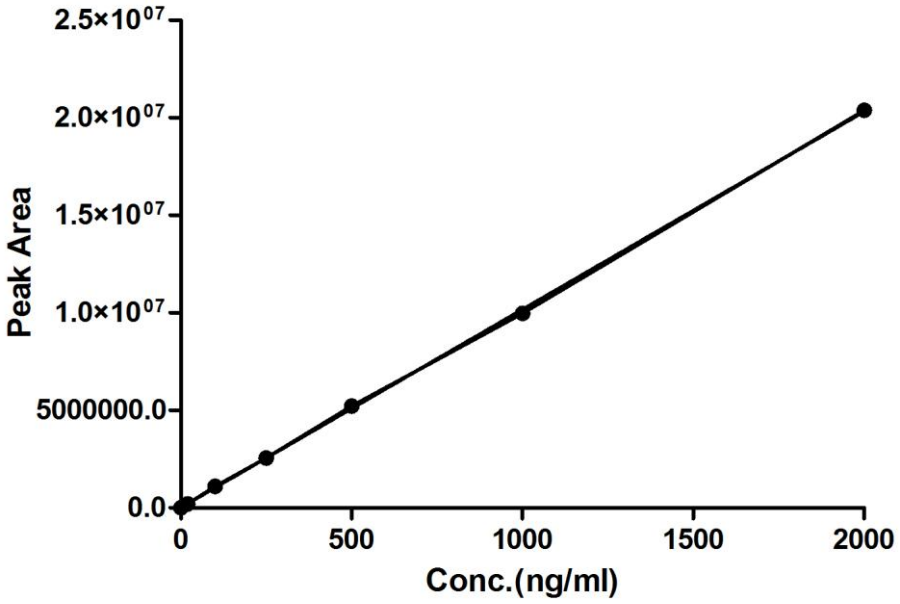

Figure 3.6: Standard Calibration plot of Gemcitabine Hydrochloride in rat plasma by LCMS MS method.

The developed analytical method was validated by calculating linearity, Accuracy and LOQ. Regression equation for standard plot was y = 10140x+33208. R^2 value was found to be 0.9998 indicating the method obeyed Beer's law in the concentration range of 20-2000 ng/mL showing the linear relationship between concentration and peak area.

Table 3.11 shows the results of accuracy, indicating the developed method was accurate with % RSD was found to be less than 2%. Unpaired t test was applied to assess the statistical significance for accuracy results of Gemcitabine Hydrochloride in rat plasma and it was found to be non-significant as t_{tab}=2.77 was much higher than t_{cal}=0.004 where, p=0.9963 with 95% CI.

Table 3.11: Accuracy results for estimation of Gemcitabine Hydrochloride in rat plasma by LCMS-MS.

Actual conc. (ng/mL)	Retention Time (min)	Peak Area± SD	Obtained Conc.	% Recovery	% RSD
150	2.89	1.53E+06 ± 1766.17	148.2	98.80	0.11
300	2.89	3.04E+06 ± 1649.00	297.3	99.10	0.05
450	2.89	4.66E+06 ± 8457.10	456.3	101.40	0.18

* Experiment was done in triplicate (n=3)

Gemcitabine Hydrochloride was precisely detected and quantified at 20 ng/mL in rat plasma which was found to be the lower limit of quantification by the developed method.

3.4.1.5.2 Calibration plot of Gemcitabine Hydrochloride in Heart homogenate

Gemcitabine Hydrochloride calibration plot was carried out in heart homogenate. Blank heart homogenate sample was run and a chromatogram was obtained, indicating no prominent peak in the region of drugs principal peak. Table 3.12 shows the peak area with its respective concentration and Figure 3.7 depicts the standard calibration plot of Gemcitabine Hydrochloride estimated from rat heart homogenate by LCMS-MS analytical method.

Table 3.12 Calibration data for Gemcitabine Hydrochloride extracted from rat heart homogenate by LCMS-MS.

Concentration (ng/mL)	Peak Area ± SD	Average RSD
0	0	0.13
100	1.25E+06 ± 2474.96	
250	3.24E+06 ± 5463.21	
500	6.48E+06 ± 6390.24	
1000	1.21E+07 ± 11059.92	
2000	2.51E+07 ± 26281.21	

*Experiment was done in triplicate (n=3)

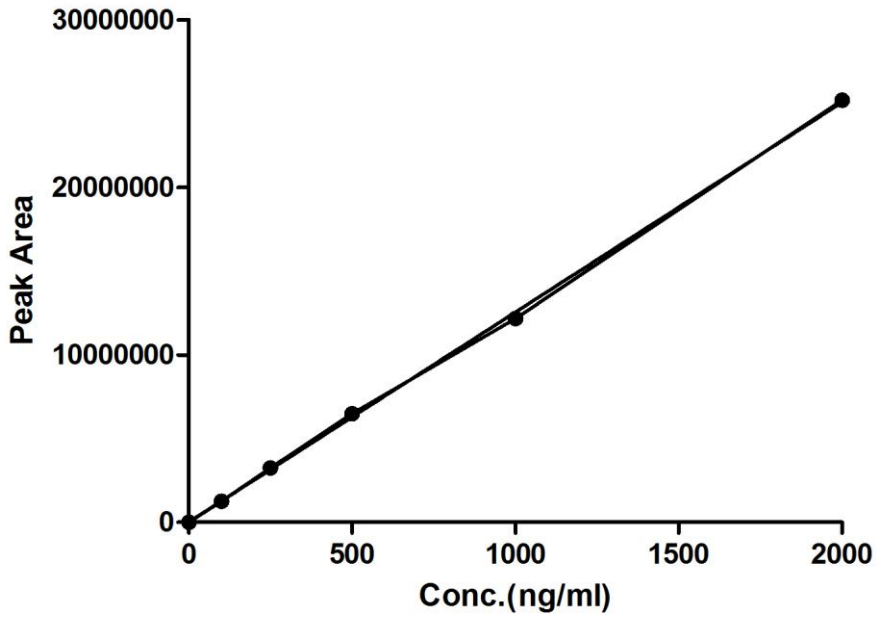

Figure 3.7: Standard Calibration plot of Gemcitabine Hydrochloride extracted from rat heart homogenate by LCMS-MS method.

The developed analytical method was validated by calculating linearity and Accuracy. Regression equation for standard plot was y = 12523x+24416. R^2 value was found to be 0.9995 indicating the method obeyed Beer's law in the concentration range of 100-2000 ng/mL

showing the linear relationship between concentration and peak area.

Table 3.13 shows the results of accuracy, indicating the developed method was accurate with % RSD was found to be less than 2%. Unpaired t test was applied to assess the statistical significance for accuracy results of Gemcitabine Hydrochloride in rat heart homogenate and it was found to be non-significant as t_{tab}=2.77 was much higher than t_{cal}=0.001 where, p=0.9989 with 95% CI..

Table 3.13: Accuracy results for estimation of Gemcitabine Hydrochloride from rat heart homogenate by LCMS-MS method.

Actual conc. (ng/mL)	Retention Time (min)	Peak Area± SD	Obtained Conc.	% Recovery	% RSD
150	2.87	1.89E+06 ± 2477.20	149.3	99.53	0.13
300	2.89	3.81E+06 ± 3372.99	302.7	100.90	0.09
450	2.90	5.64E+06 ± 3261.52	448.5	99.67	0.06

* Experiment was done in triplicate (n=3)

3.4.1.5.3 Calibration plot of Gemcitabine Hydrochloride in Lungs homogenate

Gemcitabine Hydrochloride Calibration plot was carried out in lungs homogenate. Blank lungs homogenate sample was run and a chromatogram was obtained, indicating no prominent peak in the region of drugs principal peak. Table 3.14 shows the peak area with its respective concentration and Figure 3.8 depicts the standard calibration plot of Gemcitabine Hydrochloride estimated from rat lungs homogenate by LCMS-MS analytical method.

Table 3.14: Calibration data for Gemcitabine Hydrochloride extracted from rat lungs homogenate by LCMS-MS.

Concentration (ng/mL)	Peak Area ± SD	Average RSD
0	0	0.08
100	1.12E+06 ± 2627.77	
250	2.81E+06 ± 1520.77	
500	5.63E+06 ± 1935.30	
1000	1.22E+07 ± 5780.71	
2000	2.25E+07 ± 2241.61	

*Experiment was done in triplicate (n=3)

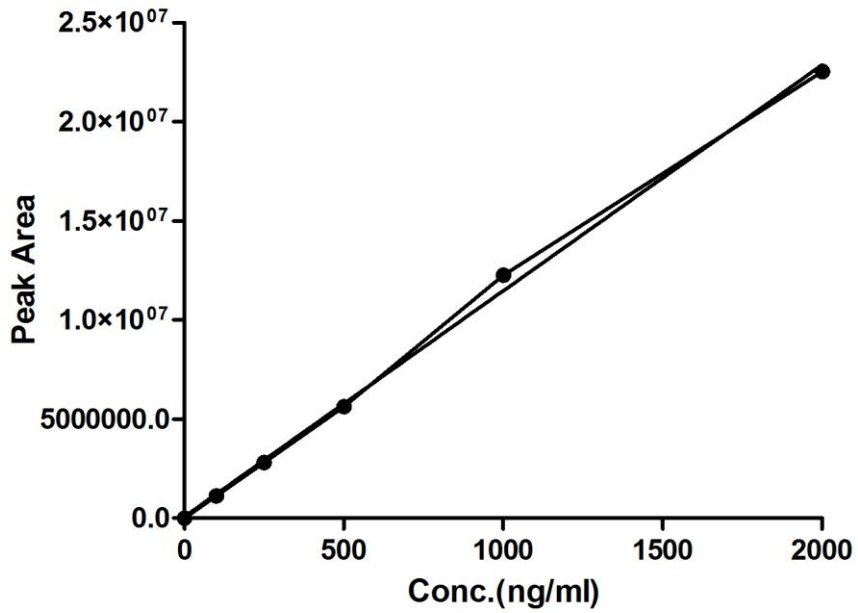

Figure 3.8: Standard Calibration plot of Gemcitabine Hydrochloride extracted from rat lungs homogenate by LCMS-MS method.

The developed analytical method was validated by calculating linearity and Accuracy. Regression equation for standard plot was y = 11395x+85734. R^2 value was found to be 0.9979 indicating the method obeyed Beer's law in the concentration range of 100-2000 ng/mL showing the linear relationship between concentration and peak area.

Table 15 shows the results of accuracy, indicating the developed method was accurate with % RSD was found to be less than 2%. Unpaired t test was applied to assess the statistical significance for accuracy results of Gemcitabine Hydrochloride in rat lungs homogenate and it was found to be non-significant as t_{tab}=2.77 was much higher than t_{cal}=0.01 where, p=0.9924 with 95% CI..

Table 3.15: Accuracy results for estimation of Gemcitabine Hydrochloride from rat lungs homogenate by LCMS-MS method.

Actual conc. (ng/mL)	Retention Time (min)	Peak Area± SD	Obtained Conc.	% Recovery	% RSD
150	2.89	1.80E+06 ± 1633.71	150.7	100.47	0.09
300	2.90	3.55E+06 ± 2148.32	304.3	101.43	0.06
450	2.89	5.19E+06 ± 5654.67	448.7	99.71	0.11

*Experiment was done in triplicate (n=3)

3.4.1.5.4 Calibration plot of Gemcitabine Hydrochloride in Liver homogenate

Gemcitabine Hydrochloride Calibration plot was carried out in liver homogenate. Blank liver homogenate sample was run and a chromatogram was obtained, indicating no prominent peak

in the region of drugs principal peak. Table 3.16 shows the peak area with its respective concentration and Figure 3.9 depicts the standard calibration plot of Gemcitabine Hydrochloride estimated from rat heart homogenate by LCMS-MS analytical method.

Table 3.16: Calibration data for Gemcitabine Hydrochloride extracted from rat liver homogenate by LCMS-MS.

Concentration (ng/mL)	Peak Area ± SD	Average RSD
0	0	0.22
100	9.36E+05 ± 5050.12	
250	2.14E+06 ± 6979.74	
500	4.03E+06 ± 7028.85	
1000	7.29E+06 ± 3991.15	
2000	1.35E+07 ± 3621.10	

*Experiment was done in triplicate (n=3)

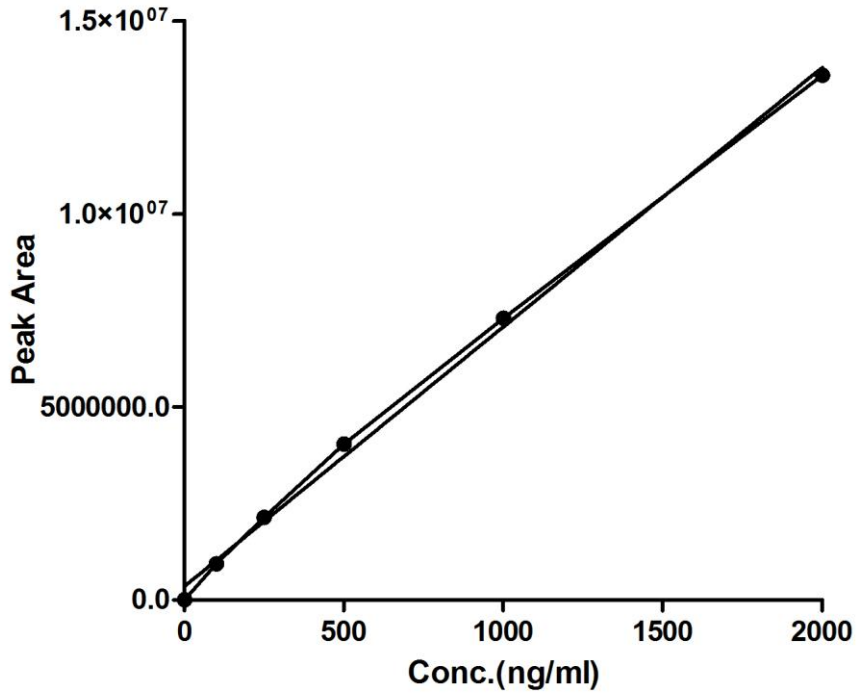

Figure 3.9: Standard Calibration plot of Gemcitabine Hydrochloride extracted from rat liver homogenate by LCMS-MS method.

The developed analytical method was validated by calculating linearity and Accuracy. Regression equation for standard plot was y = 6719.7x+355878. R^2 value was found to be 0.9974 indicating the method obeyed Beer's law in the concentration range of 100-2000 ng/mL showing the linear relationship between concentration and peak area.

Table 3.17 shows the results of accuracy, indicating the developed method was accurate with

% RSD was found to be less than 2%. Unpaired t test was applied to assess the statistical significance for accuracy results of Gemcitabine Hydrochloride in rat liver homogenate and it was found to be non-significant as t_{tab}=2.77 was much higher than t_{cal}=0.018 where, p=0.9859 with 95% CI..

Table 3.17: Accuracy results for estimation of Gemcitabine Hydrochloride from rat liver homogenate by LCMS-MS method.

Actual conc. (ng/mL)	Retention Time (min)	Peak Area± SD	Obtained Conc.	% Recovery	% RSD
150	2.88	1.35E+06 ± 14055.09	148.1	98.73	1.04
300	2.89	2.35E+06 ± 1258.81	297.4	99.13	0.05
450	2.89	3.36E+06 ± 2380.23	447.6	99.47	0.07

* Experiment was done in triplicate (n=3)

3.4.1.5.5 Calibration plot of Gemcitabine Hydrochloride in Kidney homogenate

Gemcitabine Hydrochloride Calibration plot was carried out in kidney homogenate. Blank kidney homogenate sample was run and a chromatogram was obtained, indicating no prominent peak in the region of drugs principal peak. Table 3.18 shows the peak area with its respective concentration and Figure 3.10 depicts the standard calibration plot of Gemcitabine Hydrochloride estimated from rat heart homogenate by LCMS-MS analytical method.

Table 3.18: Calibration data for Gemcitabine Hydrochloride extracted from rat kidney homogenate by LCMS-MS.

Concentration (ng/mL)	Peak Area ± SD	Average RSD
0	0	0.08
100	1.25E+06 ± 2262.11	
250	3.29E+06 ± 4130.16	
500	6.28E+06 ± 2686.09	
1000	1.36E+07 ± 4251.02	
2000	2.71E+07 ± 3421.35	

*Experiment was done in triplicate (n=3)

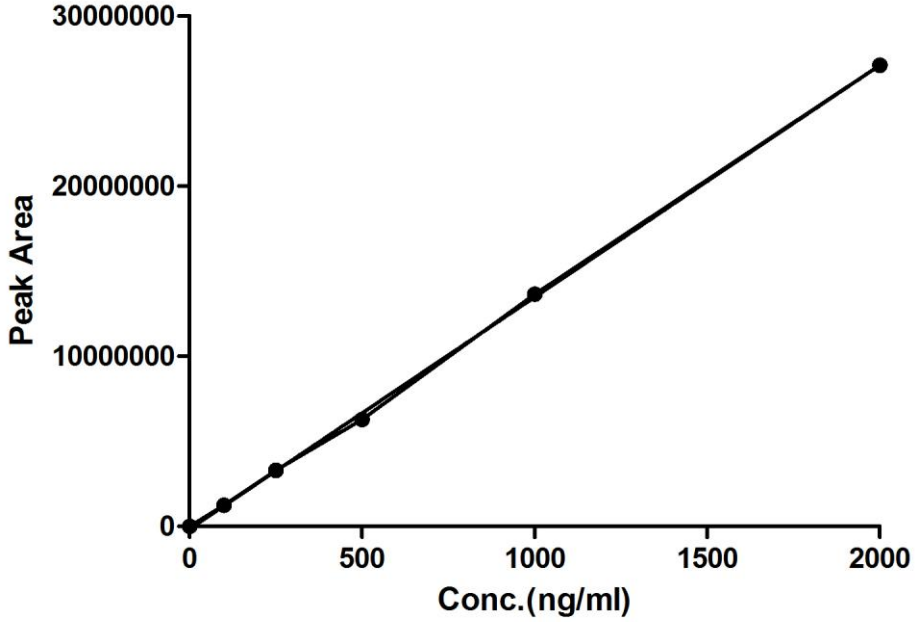

Figure 3.10: Standard Calibration plot of Gemcitabine Hydrochloride extracted from rat kidney homogenate by LCMS-MS method.

The developed analytical method was validated by calculating linearity and Accuracy. Regression equation for standard plot was y = 13626x-143160. R^2 value was found to be 0.9996 indicating the method obeyed Beer's law in the concentration range of 100-2000 ng/mL showing the linear relationship between concentration and peak area.

Table 3.19 shows the results of accuracy, indicating the developed method was accurate with % RSD was found to be less than 2%. Unpaired t test was applied to assess the statistical significance for accuracy results of Gemcitabine Hydrochloride in rat kidney homogenate and it was found to be non-significant as t_{tab}=2.77 was much higher than t_{cal}=0.011 where, p=0.9914 with 95% CI..

Table 3.19: Accuracy results for estimation of Gemcitabine Hydrochloride from rat kidney homogenate by LCMS-MS method.

Actual conc. (ng/mL)	Retention Time (min)	Peak Area± SD	Obtained Conc.	% Recovery	% RSD
150	2.89	1.91E+06 ± 2541.39	151.4	100.93	0.13
300	2.89	3.92E+06 ± 3167.77	298.7	99.57	0.08
450	2.90	6.04E+06 ± 3051.17	454.1	100.91	0.05

* Experiment was done in triplicate (n=3)

3.4.1.5.6 Calibration plot of Gemcitabine Hydrochloride in Spleen homogenate

Gemcitabine Hydrochloride Calibration plot was carried out in spleen homogenate. Blank spleen homogenate sample was run and a chromatogram was obtained, indicating no prominent

peak in the region of drugs principal peak. Table 3.20 shows the peak area with its respective concentration and Figure 3.11 depicts the standard calibration plot of Gemcitabine Hydrochloride estimated from rat heart homogenate by LCMS-MS analytical method.

Table 3.20: Calibration data for Gemcitabine Hydrochloride extracted from rat spleen homogenate by LCMS-MS.

Concentration (ng/mL)	Peak Area ± SD	Average RSD
0	0	0.13
100	1.08E+06 ± 4054.59	
250	2.98E+06 ± 3590.93	
500	5.73E+06 ± 2717.75	
1000	1.17E+07 ± 10894.30	
2000	2.51E+07 ± 7874.61	

*Experiment was done in triplicate (n=3)

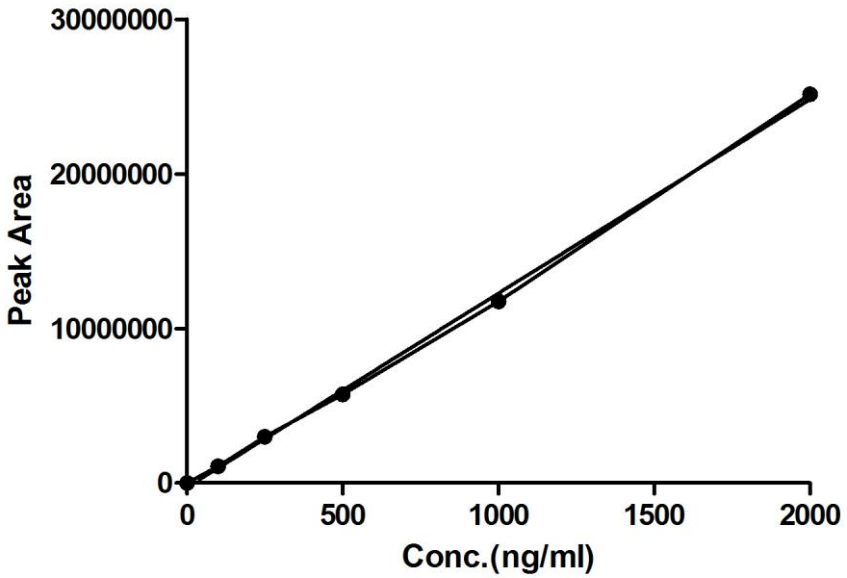

Figure 3.11: Standard Calibration plot of Gemcitabine Hydrochloride extracted from rat spleen homogenate by LCMS-MS method.

The developed analytical method was validated by calculating linearity and Accuracy. Regression equation for standard plot was y = 12551x-269539. R^2 value was found to be 0.9988 indicating the method obeyed Beer's law in the concentration range of 100-2000 ng/mL showing the linear relationship between concentration and peak area.

Table 3.21 shows the results of accuracy, indicating the developed method was accurate with % RSD was found to be less than 2%. Unpaired t test was applied to assess the statistical significance for accuracy results of Gemcitabine Hydrochloride in rat spleen homogenate and it

was found to be non-significant as t_{tab}=2.77 was much higher than t_{cal}=0.022 where, p=0.9831 with 95% CI.

Table 3.21: Accuracy results for estimation of Gemcitabine Hydrochloride from rat spleen homogenate by LCMS-MS method.

Actual conc. (ng/mL)	Retention Time (min)	Peak Area± SD	Obtained Conc.	% Recovery	% RSD
150	2.88	1.62E+06 ± 2246.35	151.2	100.80	0.14
300	2.90	3.53E+06 ± 2092.88	302.9	100.90	0.06
450	2.89	5.43E+06 ± 2258.34	454.2	100.93	0.04

* Experiment was done in triplicate (n=3)

3.4.2 Analytical method for estimation of Vinorelbine Tartrate

3.4.2.1 UV-Visible Spectrophotometric Method for estimation of Vinorelbine Tartrate in Distilled Water

As shown in figure 3.12, Vinorelbine Tartrate showed absorption maxima at 271 nm in distilled water. Table 3.22 shows the absorbance at different concentration and it was found that Vinorelbine tartrate obeys Beer Lamberts law between 5 to 30 µg/mL. Figure 3.13 shows Absorbance vs. Concentration graph i.e. calibration plot. An overlay spectra of Vinorelbine Tartrate is seen in Figure 3.12.

Table 3.22: Calibration data for Vinorelbine Tartrate in distilled water.

Concentration in µg/mL	Absorbance ±SD*
0	0
5	0.202±0.001
10	0.306±0.001
15	0.436±0.005
20	0.565±0.003
25	0.720±0.005
30	0.834±0.001

* Experiment was done in triplicate (n=3)

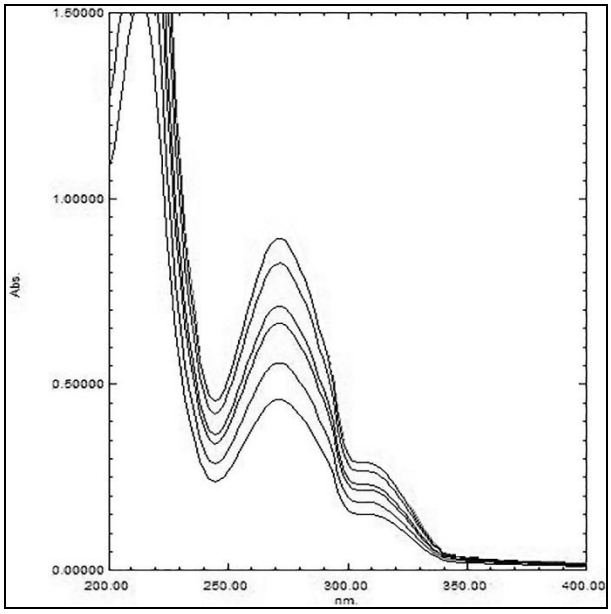

Figure 3.12: Overlay plot of Vinorelbine Tartrate in distilled water at 271 nm.

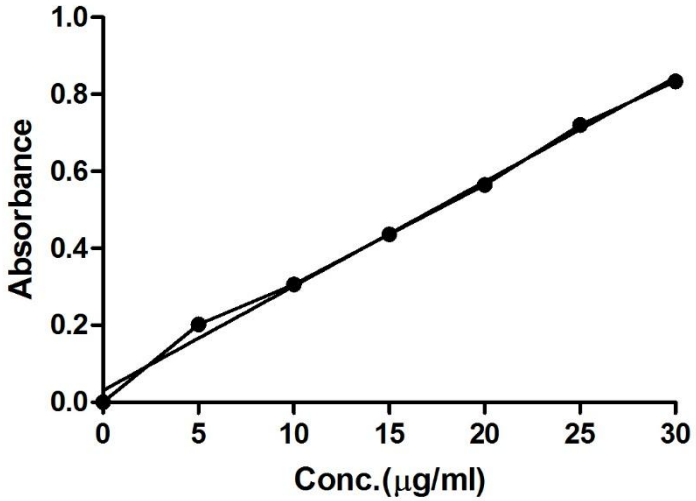

Figure 3.13: Calibration plot of Vinorelbine Tartrate in distilled water.

Regression analysis of the calibration plot was done using the method of least squares to assess the intercept, slope and correlation coefficient (R^2). The value of correlation coefficient of the regression equation was found to be near 1 (0.9951), moreover, the negligible intercept value confirmed the calibration plot was linear, which is in accordance with the Beer-Lambert law. The regression analysis values have been shown in Table 3.23.

Table 3.23: Parameters for estimation of Vinorelbine Tartrate in distilled water by UV-Visible spectrophotometry.

Parameters	Results
λmax	271 nm
Linearity range	5-30 µg/mL
Regression Equation	y = 0.0271x - 0.0308
Correlation Co-efficient	0.9951

3.4.2.2 UV-Visible Spectrophotometric Method for estimation of Vinorelbine Tartrate in PBS pH 7.4

Vinorelbine Tartrate showed absorption maxima at 273 nm in PBS pH 7.4. An overlay spectra of Vinorelbine Tartrate is seen in Figure 3.14 and respective concentration and their absorbance have been tabulated in Table 3.24. Figure 3.15 shows Absorbance vs. Concentration graph i.e. calibration plot.

Table 3.24: Calibration data for Vinorelbine Tartrate in PBS pH 7.4.

Concentration in µg/mL	Absorbance ±SD*
0	0
5	0.203±0.003
10	0.324±0.001
15	0.459±0.001
20	0.579±0.006
25	0.706±0.004
30	0.829±0.001

* Experiment was done in triplicate (n=3)

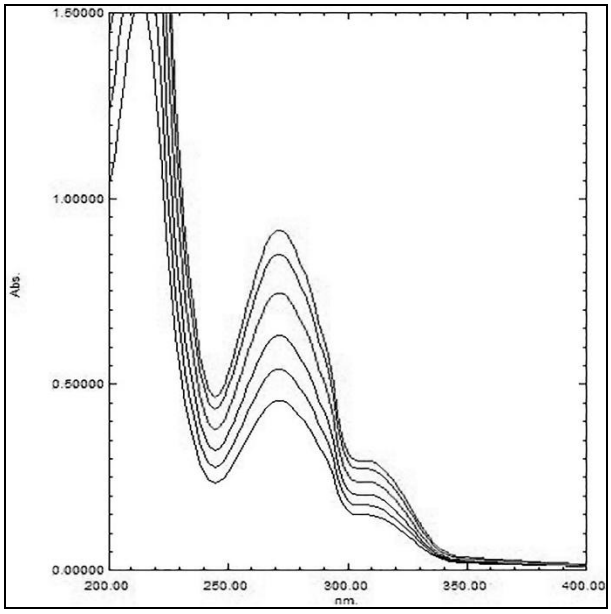

Figure 3.14: Overlay plot of Vinorelbine Tartrate in PBS pH 7.4 at 273 nm.

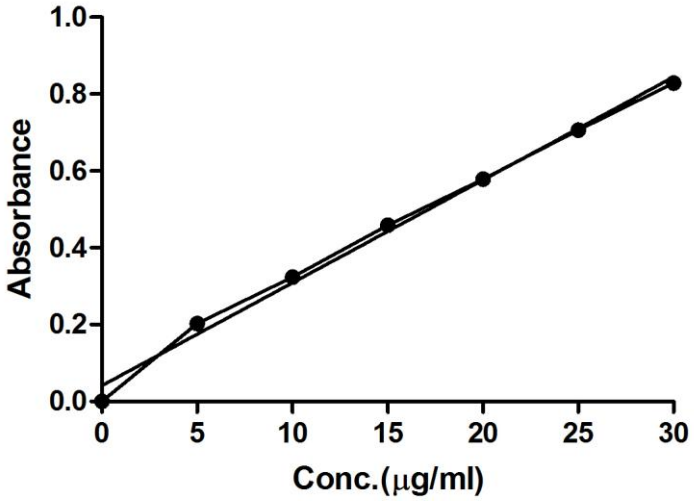

Figure 3.15: Calibration plot of Vinorelbine Tartrate in PBS pH 7.4.

Regression analysis of the calibration plot was done using the method of least squares to assess the intercept, slope and correlation coefficient (R^2). The value of correlation coefficient of the regression equation was found to be near 1 (0.9936), moreover, the negligible intercept value confirmed the calibration plot was linear, which is in accordance with the Beer-Lambert law. The regression analysis values have been shown in Table 3.25.

Table 3.25: Parameters for estimation of Vinorelbine Tartrate in PBS pH 7.4 by UV-Visible spectrophotometry.

Parameters	Results
λmax	273 nm
Linearity range	5-30 µg/mL
Regression Equation	y = 0.0268x + 0.0413
Correlation Co-efficient	0.9936

3.4.2.3 Method Validation

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

3.4.2.3.1 Accuracy

The results of accuracy for developed analytical method in both distilled water and PBS pH 7.4 are shown in Table 3.26 and Table 3.27 respectively. Method was found to be accurate as the % Relative Standard Deviation (RSD) was less than 2 %. No significant difference was found between true values and observed values at all the concentration levels. Unpaired t test was applied to assess the statistical significance for accuracy results of Vinorelbine Tartrate in distilled water and it was found to be non-significant as t_{tab}=2.77 was much higher than t_{cal}=0.06 where, p=0.9526 with 95% CI. Accuracy results of Vinorelbine Tartrate in PBS pH 7.4 were also found statistically insignificant as t_{tab}=2.77 was much higher than t_{cal}=0.01 where, p=0.992 with 95% CI.

Table 3.26: Accuracy results for estimation of Vinorelbine Tartrate in distilled water.

Quantity of Vinorelbine Tartrate added	Actual conc. (µg/mL)	Obtained conc. (µg/mL)	% Recovery	%RSD
80%	15	14.93±0.04	99.53	0.27
100%	20	20.51±0.09	102.55	0.44
120%	25	25.35±0.07	101.40	0.28

*Experiment was done in triplicate (n=3)

Table 3.27: Accuracy results for estimation of Vinorelbine Tartrate in PBS pH 7.4.

Quantity of Vinorelbine Tartrate added	Actual conc. (µg/mL)	Obtained conc. (µg/mL)	% Recovery	%RSD
80%	15	15.07±0.11	100.47	0.73
100%	20	19.87±0.19	99.35	0.96
120%	25	25.19±0.07	100.76	0.28

* Experiment was done in triplicate (n=3)

3.4.2.3.2 Precision

The results of precision for developed analytical method in both distilled water and PBS pH 7.4 are shown in Table 3.28 and Table 3.29 respectively. Method was found to be accurate as the % Relative Standard Deviation (RSD) was less than 2 %. No significant difference was found between true values and observed values at all the concentration levels.

Table 3.28: Precision results for estimation of Vinorelbine Tartrate in distilled water.

Actual Concentration (µg/mL)	Intraday precision			Interday precision		
	Observed Conc. (µg/mL)	% Recovery	%RSD	Observed Conc. (µg/mL)	% Recovery	% RSD
15	15.18±0.04	101.20	0.26	15.07±0.07	100.47	0.46
20	20.14±0.15	100.70	0.74	19.87±0.15	99.35	0.75
25	24.19±0.09	99.64	0.36	25.32±0.11	101.28	0.43

*Experiment was done in triplicate (n=3)

Table 3.29: Precision results for estimation of Vinorelbine Tartrate in PBS pH 7.4.

Actual Concentration (µg/mL)	Intraday precision			Interday precision		
	Observed Conc. µg/mL	% Recovery	%RSD	Observed Conc. µg/mL	% Recovery	%RSD
15	14.93±0.07	99.53	0.47	15.37±0.05	102.47	0.33
20	20.24±0.11	101.20	0.54	19.97±0.13	99.85	0.65
25	24.85±0.14	99.40	0.56	25.41±0.19	101.64	0.75

*Experiment was done in triplicate (n=3)

3.4.2.3.3 LOD and LOQ: Limit of Detection and Limit of Quantification was calculated for developed analytical method. Vinorelbine Tartrate was found to be precisely detected and quantified at 0.22 µg/mL and 0.74 µg/mL in distilled water, while in PBS pH 7.4 LOD and LOQ values were 0.22 µg/mL and 0.75 µg/mL respectively.

3.4.2.4 Analytical Interference Study

All the excipients viz. Soyabean Phosphatidylcholine, Cholesterol, Potassium oleate and Mannitol were taken at concentration which are used in the final formulation. The absorbance values of excipients at 271 nm were found to be negligible showing that there was no interference in the estimation of Vinorelbine Tartrate. Table 3.30 shows the absorbance values of 20 µg/mL solution of Vinorelbine Tartrate with and without excipients.

Table 3.30: Results of Analytical Interference Studies for Vinorelbine Tartrate.

Absorbance of Vinorelbine Tartrate solution (20 µg/mL) without excipients	**Absorbance of Vinorelbine Tartrate solution (20 µg/mL) with excipients (Soyabean Phosphatidylcholine, Cholesterol, Potassium oleate and Mannitol)**
0.566±0.004	0.565±0.011

*Experiment was done in triplicate (n=3)

3.4.2.5 Estimation of Vinorelbine tartrate using LCMS-MS method

3.4.2.5.1 Calibration plot of Vinorelbine tartrate in rat plasma

Vinorelbine tartrate was estimated precisely by LCMS-MS method. Mobile phase used for elution of Vinorelbine tartrate was 70% Methanol and 30% 10mM ammonium acetate with 0.8% Formic acid. Retention time was found to be 2.72 min. Table 3.31 shows the peak area with its respective concentration and chromatograms acquired from analyzing the standard calibration plot samples are shown in Figure 3.16. As it is observed the blank plasma sample did not showed any peak over the total run time of 8 minutes, suggesting there was no interference during the drug estimation. Figure 3.17 depicts the standard calibration plot of Vinorelbine tartrate estimated from rat plasma by LCMS-MS analytical method. Interference study was also performed to ensure the accurate analysis of drug. Briefly, blank plasma sample was injected into LCMS-MS system and the obtained chromatogram was studied for any interference.

Table 3.31: Calibration data for Vinorelbine tartrate in rat plasma by LCMS-MS.

Concentration (ng/mL)	**Peak Area ± SD**	**Average RSD**
0	0	0.21
5	2.17E+06 ± 16927.76	
10	5.01E+06 ± 11156.91	
50	2.34E+07 ± 20946.04	
100	4.39E+07 ± 29335.75	
200	9.26E+07 ± 58066.67	
500	2.35E+08 ± 42878.98	

*Experiment was done in triplicate (n=3)

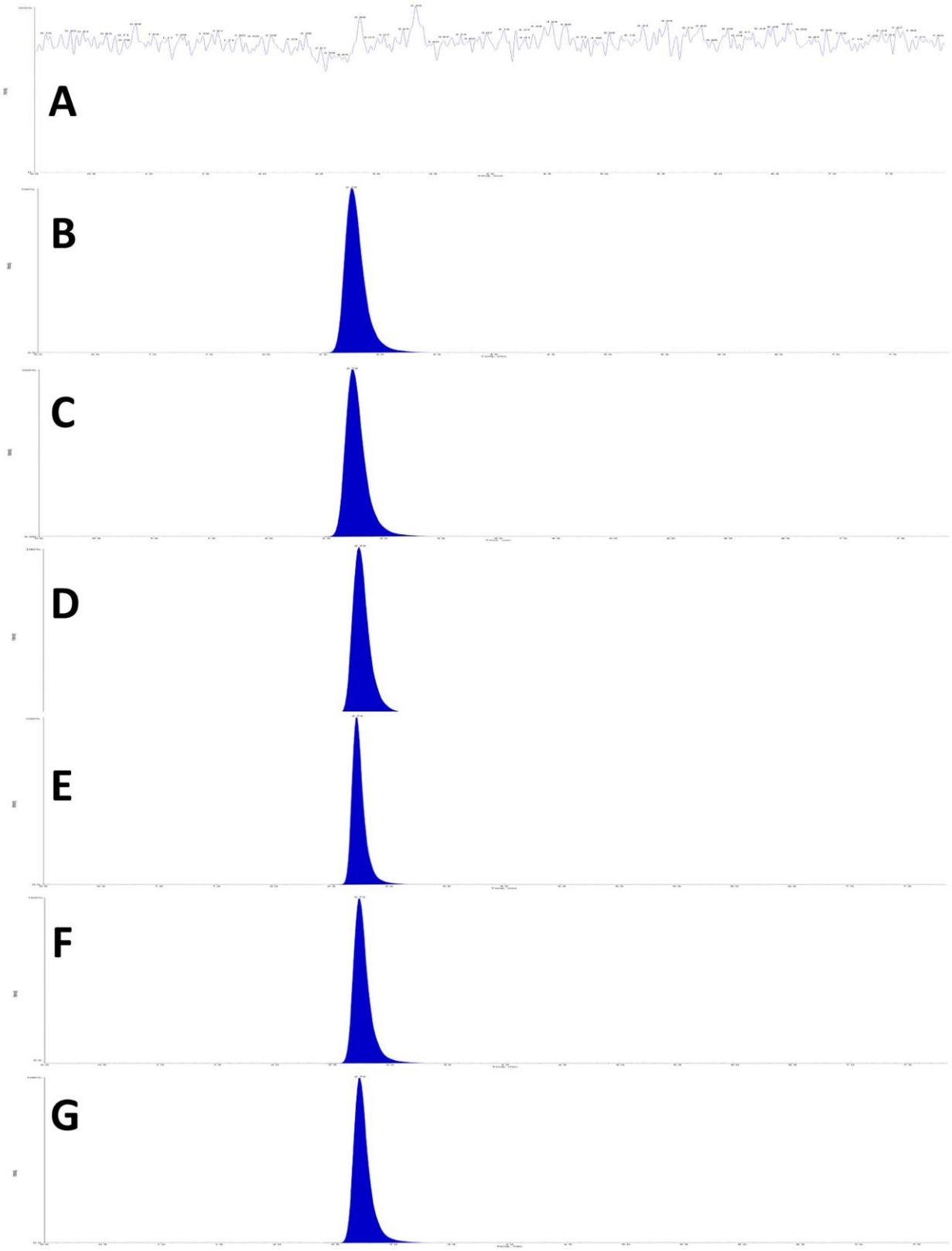

Figure 3.16: Chromatograms of Vinorelbine tartrate calibration plot showing retention time of 2.72 min (A- Blank, B- 5 ng/mL, C- 10 ng/mL, D- 50 ng/mL, E- 100 ng/mL, F- 200 ng/mL, G- 500 ng/mL).

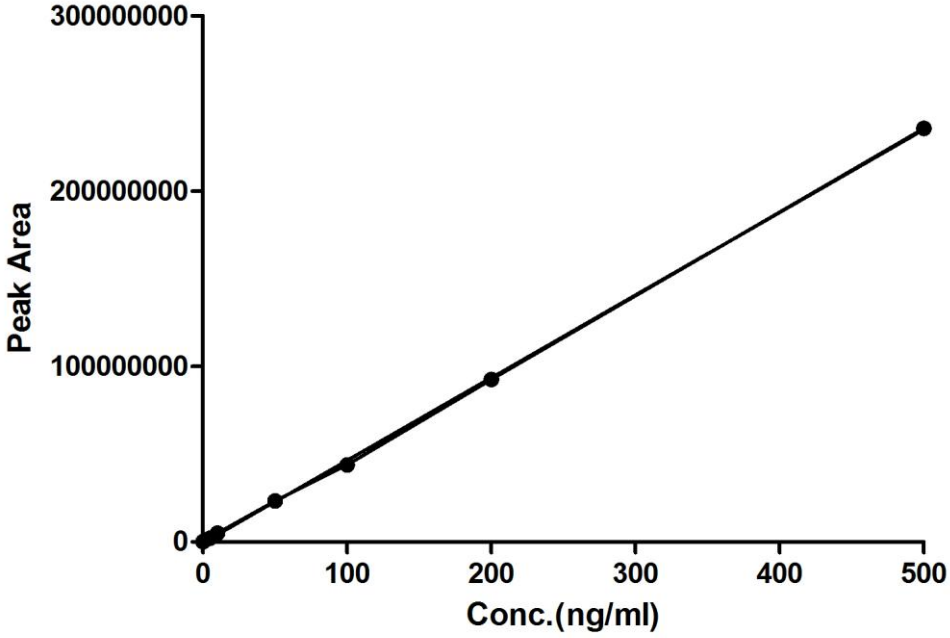

Figure 3.17: Standard Calibration plot of Vinorelbine tartrate in rat plasma by LCMS-MS method.

The developed analytical method was validated by calculating linearity, Accuracy and LOQ. Regression equation for standard plot was y = 471520x-667397. R^2 value was found to be 0.9998 indicating the method obeyed Beer's law in the concentration range of 5-500 ng/mL showing the linear relationship between concentration and peak area.

Table 3.32 shows the results of accuracy, indicating the developed method was accurate with % RSD was found to be less than 2%. Unpaired t test was applied to assess the statistical significance for accuracy results of Vinorelbine tartrate in rat plasma and it was found to be non-significant as t_{tab}=2.77 was much higher than t_{cal}=0.014 where, p=0.9887 with 95% CI.

Table 3.32: Accuracy results for estimation of Vinorelbine tartrate in rat plasma by LCMS-MS.

Actual conc. (ng/mL)	Retention Time (min)	Peak Area± SD	Obtained Conc.	% Recovery	% RSD
150	2.72	7.17E+07 ± 47060.65	153.6	102.40	0.07
300	2.72	1.40E+08 ± 110081.51	299.2	99.76	0.08
450	2.72	2.12E+08 ± 126408.44	452.7	100.60	0.06

*Experiment was done in triplicate (n=3)

Vinorelbine tartrate was precisely detected and quantified at 5 ng/mL in rat plasma which was found to be the lower limit of quantification by the developed method.

3.4.2.5.2 Calibration plot of Vinorelbine tartrate in Heart homogenate

Vinorelbine tartrate calibration plot was carried out in heart homogenate. Blank heart homogenate sample was run and a chromatogram was obtained, indicating no prominent peak in the region of drugs principal peak. Table 3.33 shows the peak area with its respective concentration and Figure 3.18 depicts the standard calibration plot of Vinorelbine tartrate estimated from rat heart homogenate by LCMS-MS analytical method.

Table 3.33: Calibration data for Vinorelbine tartrate extracted from rat heart homogenate by LCMS-MS.

Concentration (ng/mL)	Peak Area ± SD	Average RSD
0	0	0.12
5	2.07E+06 ± 8742.06	
10	5.16E+06 ± 9784.05	
50	2.22E+07 ± 11079.90	
100	4.36E+07 ± 10072.68	
200	9.42E+07 ± 19597.46	
500	2.27E+08 ± 50968.68	

*Experiment was done in triplicate (n=3)

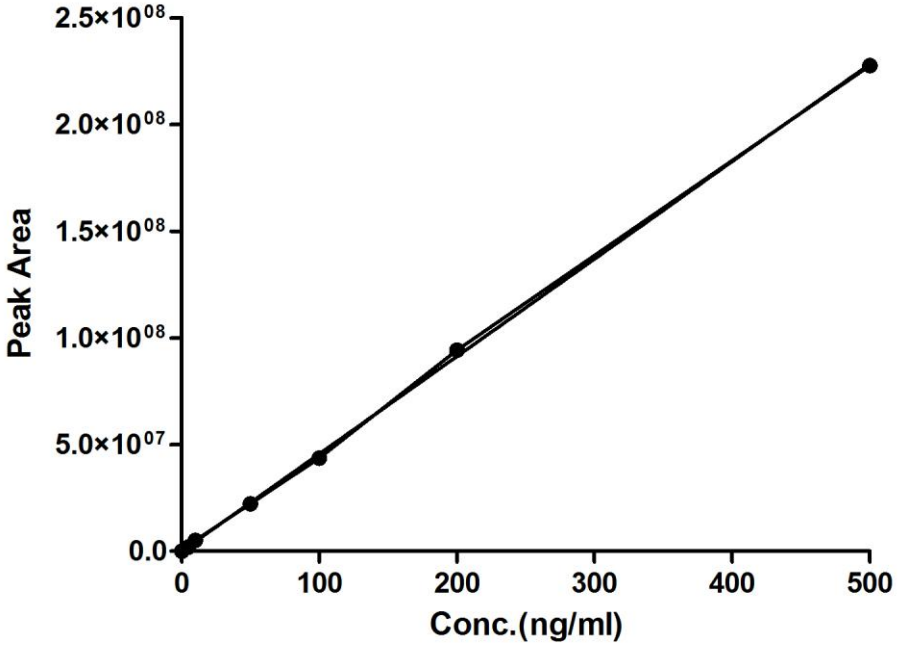

Figure 3.18: Standard Calibration plot of Vinorelbine tartrate extracted from rat heart homogenate by LCMS-MS method.

The developed analytical method was validated by calculating linearity and Accuracy. Regression equation for standard plot was y = 466966x-511197. R^2 value was found to be 0.9985 indicating the method obeyed Beer's law in the concentration range of 5-500 ng/mL showing the linear relationship between concentration and peak area.

Table 3.34 shows the results of accuracy, indicating the developed method was accurate with % RSD was found to be less than 2%. Unpaired t test was applied to assess the statistical significance for accuracy results of Vinorelbine tartrate in rat heart homogenate and it was found to be non-significant as t_{tab}=2.77 was much higher than t_{cal}=0.003 where, p=0.9971 with 95% CI.

Table 3.34: Accuracy results for estimation of Vinorelbine tartrate from rat heart homogenate by LCMS-MS method.

Actual conc. (ng/mL)	Retention Time (min)	Peak Area± SD	Obtained Conc.	% Recovery	% RSD
150	2.72	6.89E+07 ± 49304.97	148.7	99.13	0.07
300	2.72	1.39E+08 ± 48363.33	300.3	100.10	0.03
450	2.71	2.10E+08 ± 152644.20	452.4	100.53	0.07

*Experiment was done in triplicate (n=3)

3.4.2.5.3 Calibration plot of Vinorelbine tartrate in Lungs homogenate

Vinorelbine tartrate calibration plot was carried out in lungs homogenate. Blank lungs homogenate sample was run and a chromatogram was obtained, indicating no prominent peak in the region of drugs principal peak. Table 3.35 shows the peak area with its respective concentration and Figure 3.19 depicts the standard calibration plot of Vinorelbine tartrate estimated from rat lungs homogenate by LCMS-MS analytical method.

Table 3.35: Calibration data for Vinorelbine tartrate extracted from rat lungs homogenate by LCMS-MS.

Concentration (ng/mL)	Peak Area ± SD	Average RSD
0	0	0.52
5	1.92E+06 ± 15646.46	
10	4.99E+06 ± 86446.56	
50	2.07E+07 ± 63794.92	
100	5.16E+07 ± 72996.90	
200	8.94E+07 ± 89332.54	
500	2.28E+08 ± 71121.16	

*Experiment was done in triplicate (n=3)

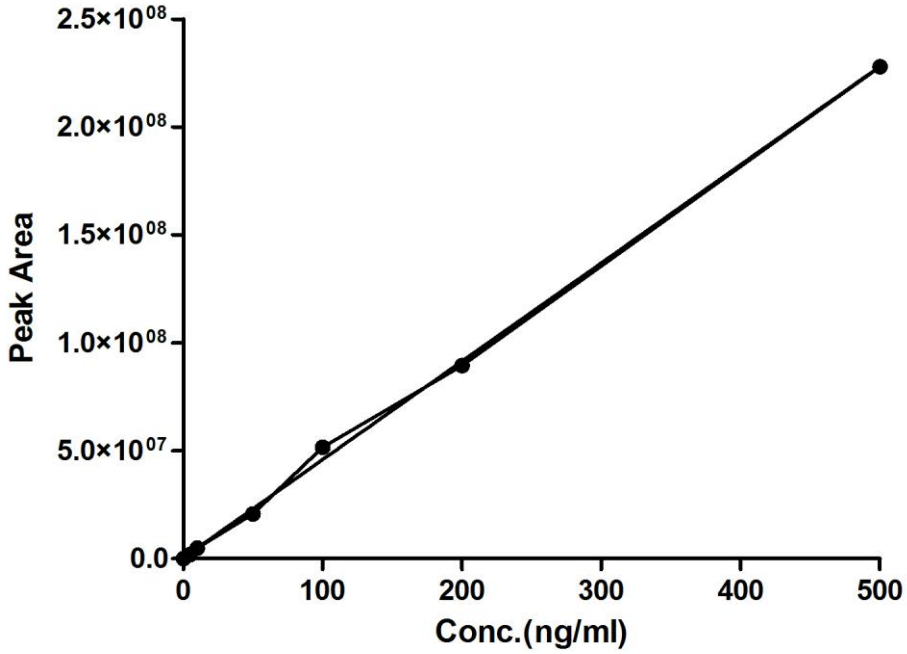

Figure 3.19: Standard Calibration plot of Vinorelbine tartrate extracted from rat lungs homogenate by LCMS-MS method.

The developed analytical method was validated by calculating linearity and Accuracy. Regression equation for standard plot was y = 455217x+430116. R^2 value was found to be 0.9989 indicating the method obeyed Beer's law in the concentration range of 5-500 ng/mL showing the linear relationship between concentration and peak area.

Table 3.36 shows the results of accuracy, indicating the developed method was accurate with % RSD was found to be less than 2%. Unpaired t test was applied to assess the statistical significance for accuracy results of Vinorelbine tartrate in rat lungs homogenate and it was found to be non-significant as t_{tab}=2.77 was much higher than t_{cal}=0.012 where, p=0.9903 with 95% CI.

Table 3.36: Accuracy results for estimation of Vinorelbine tartrate from rat lungs homogenate by LCMS-MS method.

Actual conc. (ng/mL)	Retention Time (min)	Peak Area± SD	Obtained Conc.	% Recovery	% RSD
150	2.72	6.98E+07 ± 95823.00	152.4	101.60	0.14
300	2.73	1.38E+08 ± 51303.15	302.4	100.80	0.04
450	2.72	2.05E+08 ± 78460.02	449.9	99.98	0.04

*Experiment was done in triplicate (n=3)

3.4.2.5.4 Calibration plot of Vinorelbine tartrate in Liver homogenate

Vinorelbine tartrate calibration plot was carried out in liver homogenate. Blank liver homogenate sample was run and a chromatogram was obtained, indicating no prominent peak in the region of drugs principal peak. Table 3.37 shows the peak area with its respective concentration and Figure 3.20 depicts the standard calibration plot of Vinorelbine tartrate estimated from rat liver homogenate by LCMS-MS analytical method.

Table 3.37: Calibration data for Vinorelbine tartrate extracted from rat liver homogenate by LCMS-MS.

Concentration (ng/mL)	Peak Area ± SD	Average RSD
0	0	0.59
5	1.89E+06 ± 41582.91	
10	4.73E+06 ± 40588.51	
50	2.08E+07 ± 29288.14	
100	4.92E+07 ± 114620.29	
200	8.94E+07 ± 96199.52	
500	2.38E+08 ± 66972.53	

*Experiment was done in triplicate (n=3)

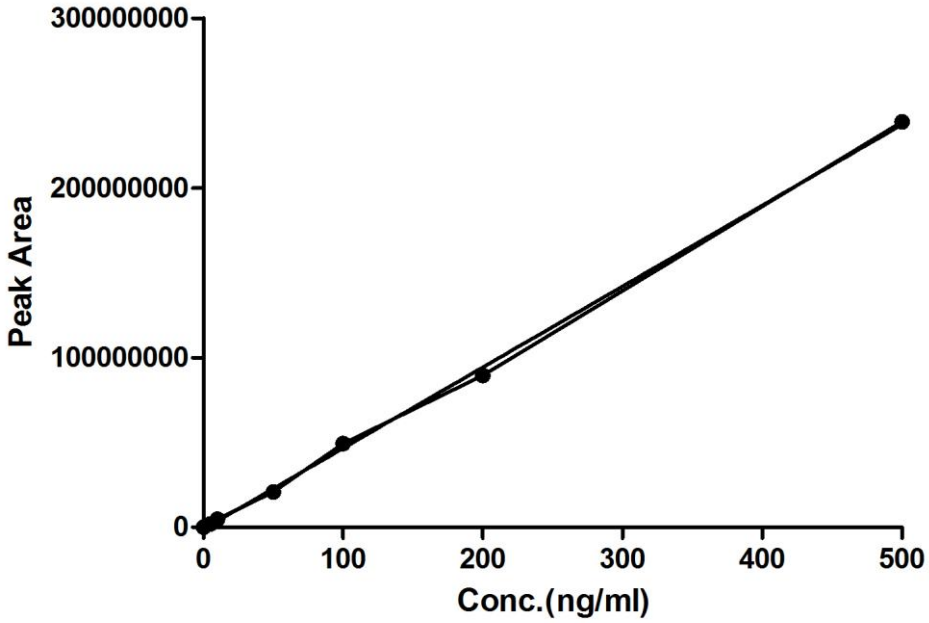

Figure 3.20: Standard Calibration plot of Vinorelbine tartrate extracted from rat liver homogenate by LCMS-MS method.

The developed analytical method was validated by calculating linearity and Accuracy.

Regression equation for standard plot was y = 476724x-1E+06. R^2 value was found to be 0.9991 indicating the method obeyed Beer's law in the concentration range of 5-500 ng/mL showing the linear relationship between concentration and peak area.

Table 3.38 shows the results of accuracy, indicating the developed method was accurate with % RSD was found to be less than 2%. Unpaired t test was applied to assess the statistical significance for accuracy results of Vinorelbine tartrate in rat liver homogenate and it was found to be non-significant as t_{tab}=2.77 was much higher than t_{cal}=0.006 where, p=0.9951 with 95% CI.

Table 3.38: Accuracy results for estimation of Vinorelbine tartrate from rat liver homogenate by LCMS-MS method.

Actual conc. (ng/mL)	Retention Time (min)	Peak Area± SD	Obtained Conc.	% Recovery	% RSD
150	2.72	6.93E+07 ± 53669.68	147.6	98.40	0.08
300	2.72	1.43E+08 ± 92755.44	302.1	100.70	0.06
450	2.72	2.14E+08 ± 128393.05	452.7	100.60	0.06

*Experiment was done in triplicate (n=3)

3.4.2.5.5 Calibration plot of Vinorelbine tartrate in Kidney homogenate

Vinorelbine tartrate calibration plot was carried out in kidney homogenate. Blank kidney homogenate sample was run and a chromatogram was obtained, indicating no prominent peak in the region of drugs principal peak. Table 3.39 shows the peak area with its respective concentration and Figure 3.21 depicts the standard calibration plot of Vinorelbine tartrate estimated from rat kidney homogenate by LCMS-MS analytical method.

Table 3.39: Calibration data for Vinorelbine tartrate extracted from rat kidney homogenate by LCMS-MS.

Concentration (ng/mL)	Peak Area ± SD	Average RSD
0	0	0.54
5	2.79E+06 ± 51234.39	
10	4.76E+06 ± 39602.55	
50	2.17E+07 ± 70598.72	
100	4.02E+07 ± 71634.22	
200	9.07E+07 ± 62151.55	
500	2.06E+08 ± 60617.63	

*Experiment was done in triplicate (n=3)

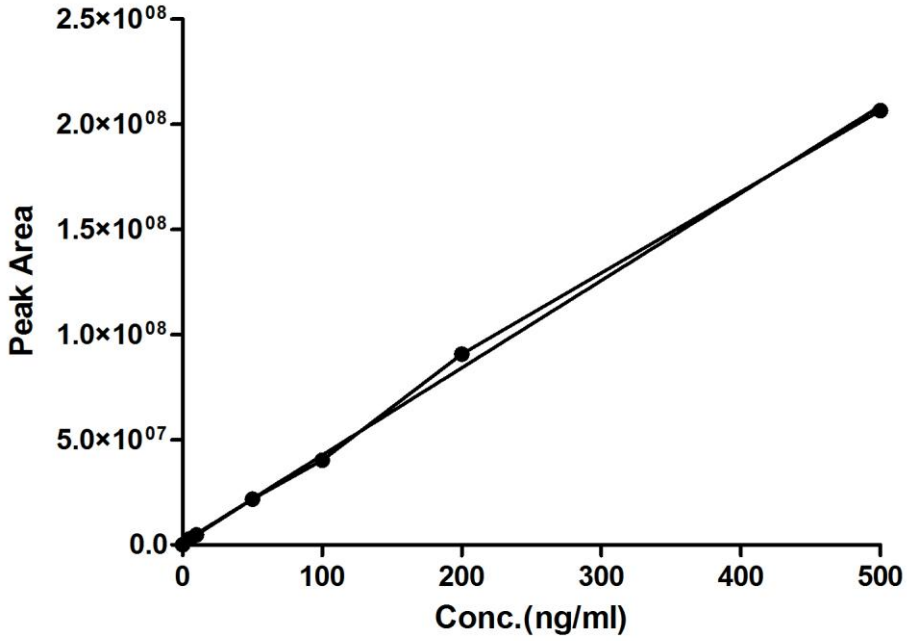

Figure 3.21: Standard Calibration plot of Vinorelbine tartrate extracted from rat kidney homogenate by LCMS-MS method.

The developed analytical method was validated by calculating linearity and Accuracy. Regression equation for standard plot was y = 415069x+1E+06. R^2 value was found to be 0.9984 indicating the method obeyed Beer's law in the concentration range of 5-500 ng/mL showing the linear relationship between concentration and peak area.

Table 3.40 shows the results of accuracy, indicating the developed method was accurate with % RSD was found to be less than 2%. Unpaired t test was applied to assess the statistical significance for accuracy results of Vinorelbine tartrate in rat kidney homogenate and it was found to be non-significant as t_{tab}=2.77 was much higher than t_{cal}=0 where, p=1 with 95% CI.

Table 3.40: Accuracy results for estimation of Vinorelbine tartrate from rat kidney homogenate by LCMS-MS method.

Actual conc. (ng/mL)	Retention Time (min)	Peak Area± SD	Obtained Conc.	% Recovery	% RSD
150	2.73	6.34E+07 ± 188427.82	150.5	100.33	0.30
300	2.72	1.26E+08 ± 79076.04	301.2	100.40	0.06
450	2.72	1.87E+08 ± 196975.97	448.3	99.62	0.11

*Experiment was done in triplicate (n=3)

3.4.2.5.6 Calibration plot of Vinorelbine tartrate in Spleen homogenate

Vinorelbine tartrate calibration plot was carried out in spleen homogenate. Blank spleen homogenate sample was run and a chromatogram was obtained, indicating no prominent peak in the region of drugs principal peak. Table 3.41 shows the peak area with its respective

concentration and Figure 3.22 depicts the standard calibration plot of Vinorelbine tartrate estimated from rat spleen homogenate by LCMS-MS analytical method.

Table 3.41: Calibration data for Vinorelbine tartrate extracted from rat spleen homogenate by LCMS-MS.

Concentration (ng/mL)	Peak Area ± SD	Average RSD
0	0	0.61
5	2.23E+06 ± 40039.81	
10	5.29E+06 ± 50785.31	
50	1.93E+07 ± 101357.74	
100	4.09E+07 ± 70763.97	
200	8.71E+07 ± 125252.28	
500	2.07E+08 ± 168783.13	

*Experiment was done in triplicate (n=3)

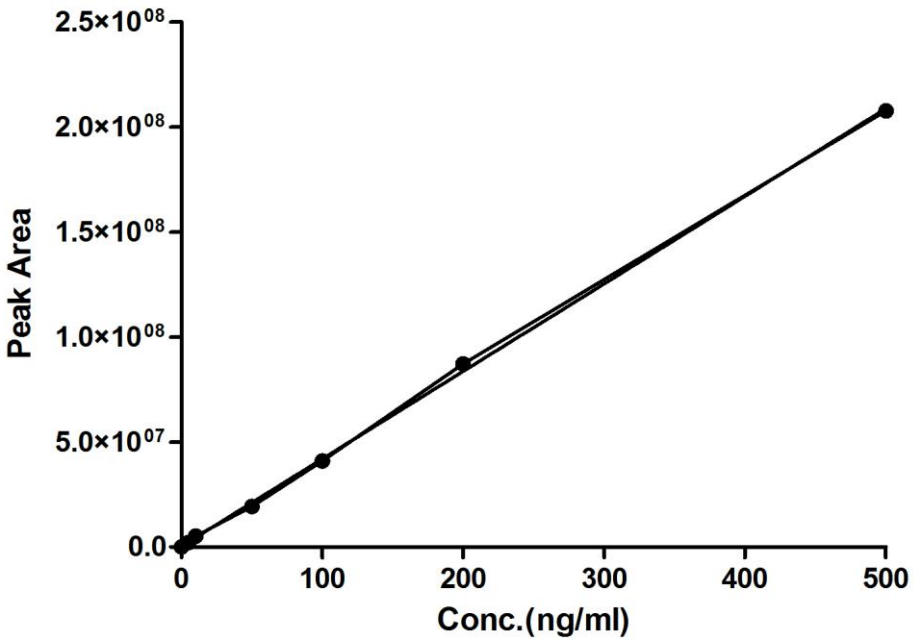

Figure 3.22: Standard Calibration plot of Vinorelbine tartrate extracted from rat spleen homogenate by LCMS-MS method.

The developed analytical method was validated by calculating linearity and Accuracy. Regression equation for standard plot was y = 416737x+308467. R^2 value was found to be 0.9995 indicating the method obeyed Beer's law in the concentration range of 5-500 ng/mL showing the linear relationship between concentration and peak area.

Table 3.42 shows the results of accuracy, indicating the developed method was accurate with % RSD was found to be less than 2%. Unpaired t test was applied to assess the statistical

significance for accuracy results of Vinorelbine tartrate in rat spleen homogenate and it was found to be non-significant as t_{tab}=2.77 was much higher than t_{cal}=0.016 where, p=0.9877 with 95% CI.

Table 3.42: Accuracy results for estimation of Vinorelbine tartrate from rat spleen homogenate by LCMS-MS method.

Actual conc. (ng/mL)	Retention Time (min)	Peak Area± SD	Obtained Conc.	% Recovery	% RSD
150	2.72	6.34E+07 ± 48371.89	151.5	101.00	0.08
300	2.71	1.25E+08 ± 68308.31	301.1	100.37	0.05
450	2.73	1.89E+08 ± 123975.44	453.4	100.76	0.07

*Experiment was done in triplicate (n=3)

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