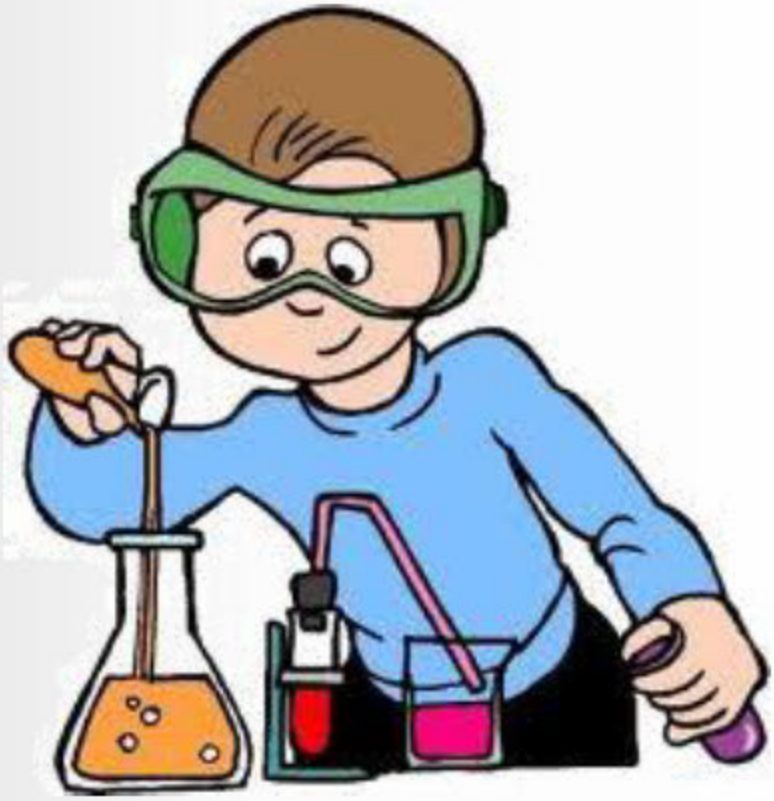
Chapter- 3
Analytical Methods



3.1 Introduction

A validated analytical method is a crucial and fundamental component in formulation development. Analytical method required to estimate paclitaxel during preparation, optimization and characterization of its inclusion complex, conventional PEGylated liposomes, double loaded PEGylated liposomes and immunoliposomes was developed and partially validated for aptness of the analysis. The methods for analysis and estimation of paclitaxel, phospholipids, plasma proteins, cell lysate proteins, sulfhydryl groups and 6-coumrin have been discussed in this chapter.

Analytical Method Validation

It is essential to authenticate the performance of an analytical method for acceptable estimation of an analyte. In following conditions, it is mandatory to validate analytical method; (1) when for a definite problem a new method is established; (2) when results of already established method are varying with time; (3) when a developed method is improved or extended for additional indication; (4) when the developed method is performed in a diverse laboratories by different instruments and analyst; (5) when the equality of a new and a conventional standard method has to be proved (1). As per the recommendation of various regulatory agencies accuracy, precision, linearity, selectivity, specificity, limit of detection, limit of quantification, robustness and ruggedness are suggested validation parameters (2, 3).

Accuracy

Accuracy expresses the closeness of estimated results to the standard or already accepted value. It is also referred to as trueness.

Precision

Under defined set of conditions, the degree of agreement between values obtained from multiple measurements measured for a single homogenous sample is termed as precision. Repeatability, reproducibility and intermediate precision are the three levels of precision. The precision of an analytical procedure is usually expressed as the variance, standard deviation (SD) or coefficient of variation (CV) of a series of measurements.

Linearity and Range

The ability of an analytical procedure to obtain results for the test substance that are in direct proportion to the analyte concentration is termed as linearity. When an analytical procedure has been demonstrated to be precise, accurate and linear, in that case the interval between the highest and lowest amount of analyte in test sample is termed as range.

Selectivity

The ability of an analytical method to measure only particular analyte when multiple analytes are to be detected in a given sample is termed as its selectivity.

Specificity

It is the capability of the method to estimate the analyte precisely in the presence of all possible sample or formulation components such as excipients, impurities, degradation products etc. (1, 2). The specificity of an analytical method can be tested by estimating the analyte in a test sample followed by its comparison with standard solution that only contains an analyte. Any variation in the response of analyte in the test sample as compare to the standard solution suggests non-specificity of the analytical method.

Limit of Detection (LOD)

It is the lowest amount of sample that can be detected using the analytical procedure employed but not necessarily be quantitated.

Limit of Quantification (LOQ)

Under a suitable level of precision and accuracy, the lowest concentration of analyte in a sample that can be accurately quantified using the analytical procedure is termed as LOQ. It is of high importance for determination of impurity / degradation product in a sample.

Robustness/Ruggedness

When an analytical method employed is subjected to small but deliberate variations in the parameters, the capacity of the method not to get affected by such changes is termed as robustness.

Materials

Sr No	Chemicals/Materials	Source/Manufacturer
1.	Paclitaxel	Sun Pharmaceutical Industries. Ltd. (Vadodara, India) – Gift sample
2.	HSPC	Lipoid GMBH (Ludwigshafen, Germany
3.	Egg PC	Lipoid GMBH (Ludwigshafen, Germany
4.	DSPE-mPEG2000	Lipoid GMBH (Ludwigshafen, Germany
5.	Cholesterol extra pure	Sigma-Aldrich, Mumbai, India.
6.	Coumarin 6	Sigma-Aldrich, Mumbai, India.
7.	Bradford reagent	Sigma-Aldrich, Mumbai, India.
8.	Ellman's reagent	Sigma-Aldrich, Mumbai, India.
9.	Bicinchoninic Acid (BCA) assay kit	Bangalore Genei, Bangalore, India
10.	L-Cysteine hydrochloride monohydrate	Himedia, Mumbai, India.

All other chemicals used were of analytical reagent grade and were used without any further purification.

Instruments

Sr No	Instruments	Company
1.	Model 680 Microplate Reader	Bio-Rad
2.	Spectrofluorometer	Shimadzu, Japan
3.	UV-visible spectrophotometer	Shimadzu 1800, Japan
4.	RP-HPLC	Shimadzu LC-20AT, Japan
5.	pH Meter	Labindia Instrument
6.	Weighing Balance	ATX-224, Shimadzu, Japan
7.	Bath Sonicator	Sartorius, India

3.2 Methods

3.2.1 Estimation of Paclitaxel by RP-HPLC method

Estimation of PTX content in inclusion complexes was performed by reverse-phase HPLC method/RP-HPLC (Shimadzu LC-20AT, Japan) using C18 ODS (octa decyl silane) column (250 mm *4.6 mm* 5 µm, Thermo scientific) at ambient temperature. The mobile phase Acetonitrile: Methanol: Water (45:30:25), pH adjusted to ~3 with 1% v/v of glacial acetic acid was run at a flow rate of 1 ml/min. Samples were prepared by appropriate dilutions of PTX in methanol and final dilution was

done with mobile phase. Concentrations from 1 to 50 µg/ml were estimated to prepare calibration curve. 20 µl of each solution was injected into HPLC and chromatogram was run for 10 minutes. Estimation of PTX was done using UV-visible detector at wavelength of 227 nm. Optimization and validation of the HPLC method were performed according to ICH guidelines.

3.2.2 Estimation of total phospholipid content by Stewart method

Principle:

Phospholipid content may be estimated by Stewart's Colorimetric Method (4). This method for the detection of phospholipids relies on the ability of phospholipids to form a stable complex with ammonium ferrothiocyanate. Ammonium ferrothiocyanate is a red coloured inorganic compound which has tendency to remain insoluble in chloroform. However, it can form stable complexes with phospholipids and those complexes are soluble in chloroform. Thus, when a chloroform solution consisting of phospholipids was mixed with ammonium ferrothiocyanate at room temperature, a red coloured complexes are formed which partitions in the chloroform phase and gets solubilized. The absorbance of the coloured soluble complex in chloroform can be determined at 472 nm wave length through colorimetry.

Preparation of ammonium ferrothiocyanate solution:

The standard solution of ammonium ferrothiocyanate was prepared by following method;

27.03 g of ferric chloride hexahydrate ($FeCl_3.6H_2O$) and 30.4 g ammonium thiocyanate (NH_4SCN) was dissolved in deionised distilled water and volume was made up to 1 litre.

Calibration Curve of Phospholipids:

All the glass apparatus used for the study were washed with chromic acid solution prior to use to evade the probable contamination from surface active cleansing agents. A stock solution of 51.3 mg lipid mixture was prepared by solubilizing all the lipids in 100 ml of chloroform leading to final lipid concentration of 513 µg/ml. The lipids used to prepare stock solution were those lipids used in the final optimized formulation with their respective molar ratios i.e. 13.3 mg of HSPC,

20.9 mg of Egg PC and 9.4 mg of DSPE mPEG2000. As the final formulation also contains cholesterol, it was added to in the stock solution (7.9 mg). The stock solution was diluted 5.7 times with chloroform to achieve final lipid concentration of 90 µg/ml. From this stock solution, 0.2, 0.4, 0.8, 1.2, 1.6 & 2 ml volumes were taken which has 15, 36, 72, 108, 144 and 180 µg/ml amount of lipids respectively and were added to 3 ml of ammonium ferrothiocyanate solution. Then chloroform was made up to the volume of 3 ml. The biphasic mixture was mixed thoroughly for 5 min and the high density lower chloroform layer was separated. The optical density of separated chloroform layer was measured at wave length of 472 nm by taking native chloroform as a blank. The average of optical densities obtained was plotted in a graph against total lipid concentration.

3.2.3 Estimation of plasma proteins by BCA protein estimation method

Principle:

Bicinchoninic acid (BCA) protein estimation method or Smith's assay is the most selective, sensitive and detergent compatible method for quantitative estimation of total protein in solution by colorimetry (5). This method is an amalgamation of widely explored biuret reaction and colorimetric technique. The first step is based on the biuret reaction in which reduction of Cu+2 to Cu+1 takes place by protein in an alkaline condition. The number of Cu+2 reduced is proportional to the quantity of protein present in the solution. The second step relies on reaction of cuprous ions (Cu+1) with BCA sodium salt that can form an intense purple coloured complex (Figure 3.1). The resulted water soluble stable complex shows a strong absorbance at 562 nm.

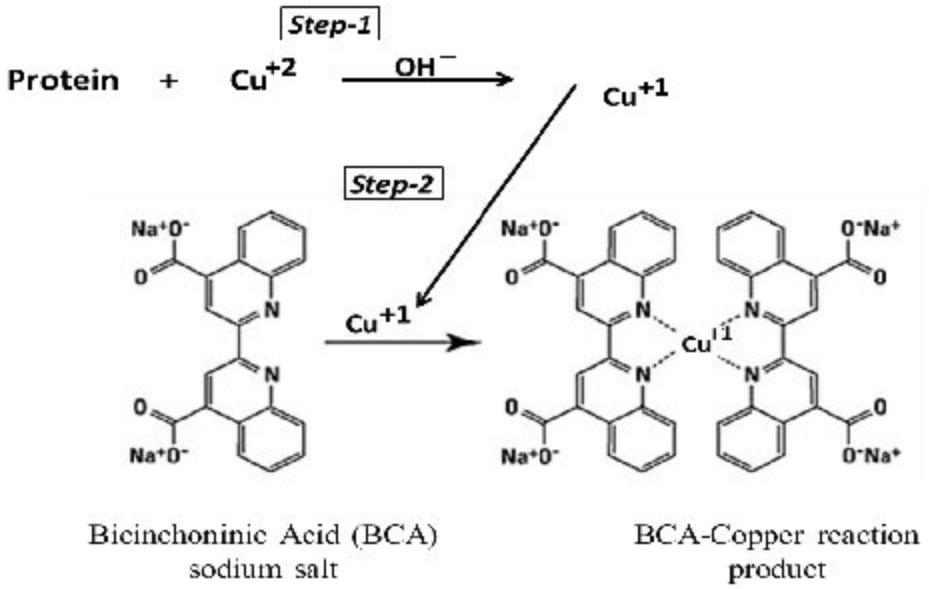


Figure 3.1: Principe and Reaction of BCA protein estimation method

Preparation of standard protein solution:

Bovine Serum Albumin (BSA) was taken as a standard protein for the purpose of constructing calibration curve. The standard protocol provided with BCA protein estimation kit (Bangalore Genei, Bangalore) was followed.

Preparation of working reagent:

To prepare this reagent, reagent A (50 parts) and reagent B (1 part) supplied with the BCA protein estimation kit were mixed properly to yield an intense green coloured reagent solution. Reagent A contains bicinchoninic acid, sodium carbonate, sodium bicarbonate, and sodium tartrate in 0.1 M sodium hydroxide. While reagent B is copper sulphate solution.

Procedure:

1. From each standard or test protein samples, 0.2 ml was pipetted in previously labelled test tubes. The solvent alone without any protein sample was taken as a blank. The standard calibration curve range was selected as 12.5-150 µg/ml.
2. To each test tube, 0.2 ml of working reagent was added and mixed well.
3. All the test tubes were then incubated for 30 min at 60 ^{0}C for completion of the reaction between protein sample and working reagent.
4. After incubation for specified time, all the test tubes were allowed to cool and attain room temperature.

5. Absorbance of all the test tube samples was measured at 562 nm against water as a reference.
6. Absorbance of blank was also measured at a same wave length which was then subtracted from the average absorbance of each standard or test samples.
7. A calibration curve was plotted by taking concentration of BSA standard protein concentrations on x-axis and their average absorbance (corrected by subtracting blank) on y-axis.

3.2.4 Estimation of cell lysate protein by Bradford method

Principle:

Binding of proteins to a pH sensitive dye allows for quantitative estimation of the protein content. Here, the dye employed for the assay is Coomasine Brilliant Blue G-250 which exists in three forms: red when cationic, green when neutral and blue when anionic. Conversion of dye from a double protonated state (in acidic state) to unprotonated state occurs when dye binds to protein and red color of the dye changes to give blue colour having a λ_{max} of 595 nm (Figure 3.2), that can be detected using a microplate reader or spectrophotometer. This dye is used for specific estimation of aromatic amino acid residues and basic amino acids like arginine. The assay has high sensitivity and specificity as the complex formed has a high molar extinction coefficient that does not change even on ten-fold dilution. The only important point is the selection of an appropriate ratio of dye solution to sample amount and by selection of such ratio Beer's law will be followed for accurate estimation. Interference may arise during estimation due to presence of non-protein components, due to change in equilibrium levels of the three forms of the dye as for e.g. the neutral dye state gets stabilized by presence of flavonoids, detergents and basic protein buffers due to direct binding or by shifting pH.

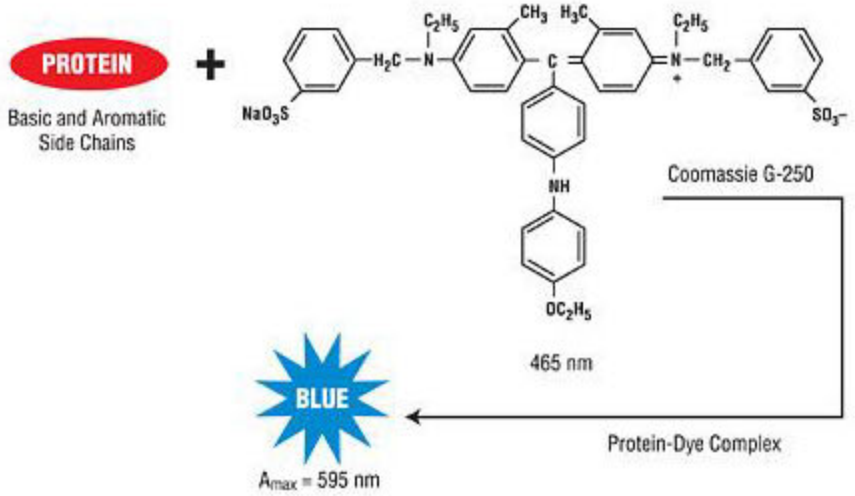


Figure 3.2 Principle of Bradford Assay

Protocol:

Standard solution of Bovine serum albumin solution in milliQ was prepared at a concentration of 0.5mg/ml. From this standard solution a dilution were made to obtain concentration range of 0.125-1 µg/ml of protein and was accurately placed in triplicate in 96 well plate. The volume in each well was kept constant. To each well 100µl of Bradford's reagent was added to each well and absorbance was taken at 595 nm using ELISA plate reader. The estimation was carried out in triplicate and the concentration of protein was determined using software automatically using BSA as standard.

3.2.5 Estimation of Cysteine by Ellman's Assay

Principle:

For quantification of free sulfhydryl group in solution, a water-soluble compound DTNB was introduced in 1959 by Ellman. DTNB stands for 5,5'-dithio-*bis*-(2-nitrobenzoic acid), is highly specific for -SH group and it reacts with free sulfhydryl group to produce yellow colour for solution at neutral pH. The reaction time is short as well as the reaction proceeds with high molar extinction coefficient.

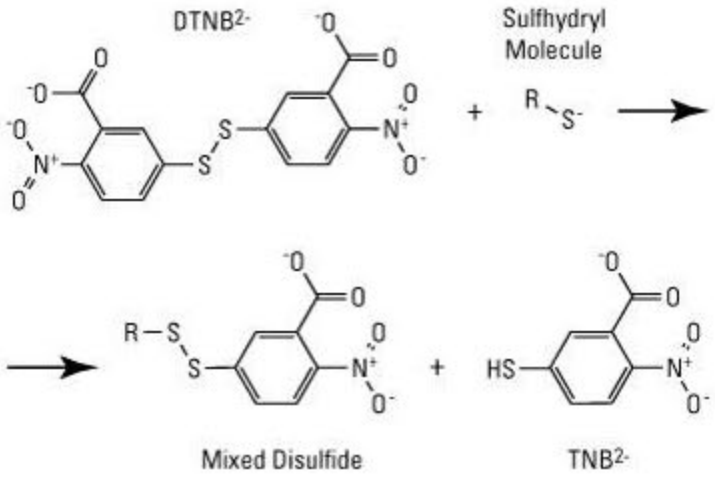


Figure 3.3 Reduction of Ellman's Reagent

As shown in the above figure 3.3, reaction of DTNB with free sulfhydryl group yields a mixture of mixed disulphide and TNB (2-nitro-5-thiobenzoic acid). Several factors such as reaction pH, pKa of sulfhydryl group as well as steric and electrostatic effects affect the rate of reaction. The TNB products possess a high molar extinction coefficient of 14,150 $M^{-1}cm^{-1}$ at 412 nm in the visible range. The standard used for the reaction is a solution of cysteine prepared at a known concentration and the comparison is done after plotting calibration curve. Another method that may be used is by comparison of the extinction coefficient of TNB.

Preparation of reaction buffer:

0.1M sodium phosphate buffer (pH 8.0) with 1mM EDTA.

Standard solution: Cysteine hydrochloride (M.W. = 175.6)

Ellman's reagent solution: 1 ml of reaction buffer was taken to which 4 ml of Ellman's reagent was added and dissolved.

Protocol:

1. Cysteine hydrochloride monohydrate was dissolved in reaction buffer to prepare a set of cysteine standards in concentration range of 0.1 mM to 1.0 mM.
2. A set of test tubes was prepared containing 50µl of Ellman's reagent solution and 2.5 ml reaction buffer.

3. To each of the above tube 250 µl of standard/test sample solution were added (Note: the dilutions for the test sample solution are so prepared so as to fall in the working range of standard curve prepared)
4. The above solutions were incubated at room temperature for 15 minutes after mixing.
5. Absorbance of above solutions was measured using UV-visible spectrophotometer at 412 nm.
6. Concentrations of the test samples were determined from the standard curve. This method was similarly used for estimation of -SH group of Fab′ fragment prior to conjugating with liposomes.

3.2.6 Estimation of coumarin-6 by spectrofluorometer

To estimate the uptake / distribution of coumarin-6 loaded liposomes in cell line, a spectrofluorometric method was used for quantification of coumarin-6 during preparation and optimization of liposomes. The solvent system used was Chloroform: Methanol (1:1);

Fluorescence measurements were made on a Shimadzu RF-1501 spectrofluorometer (Japan), equipped with a 150 W xenon lamp and using 1.0- cm quartz cells.

Preparation of stock solutions

A Stock solution of Coumarin-6 was prepared by dissolving 1 mg of Coumarin-6 in 1 mL of Chloroform: Methanol (1:1) to get final concentration of 1000 µg/mL.

Calibration curve

Calibration curve of Coumarin 6 was developed in solvent system- Chloroform: Methanol (1:1). From the stock solution, suitable dilutions were made to obtain solutions of concentrations 1000-5000 pg/mL in Chloroform: Methanol (1:1). Blank solution was prepared by using with respective media. Analysis was done by using Spectrofluorometer (RF-5301PC, Shimadzu) at excitation and emission wavelengths of 458 and 505 nm, respectively for coumarin-6.

3.2.7 Estimation of Paclitaxel from plasma samples by RP-HPLC method

Estimation conditions:

All the chromatographic conditions for estimation of drug in plasma were kept same as done for estimation of drug by RP-HPLC. However, for extraction of drug protein precipitation method was employed and was carried out as follows:

Method:

Preparation of drug stock solution

40 mg of Paclitaxel was dissolved in 10 ml of methanol to obtain stock solution of 4 mg/ml. Dilutions were made in methanol of the above stock solution to obtain concentration of 8, 80, 160, 320, 480 and 640 µg/ml and made to a total volume of 10 ml with methanol.

Incorporation of drug solution in Plasma (Rat Plasma):

From the above prepared dilutions of drug in methanol, 20µl was pipetted from each and 380µl of plasma was added to achieve concentration of 0.4, 4, 8, 16, 24 and 32 µg/ml of drug in plasma.

Extraction of drug from Plasma by precipitating protein:

From the above plasma samples containing drug in range of 0.4 - 32 µg/ml; 100 µl volume of plasma was spiked in 300µl volume of methanol. The above mixture was centrifuged at 5000 rpm at 4°C for 10 min to separate the precipitated plasma from the clear solution of drug in methanol in supernatant. The final concentration of drug in supernatant that were injected in RP-HPLC for analysis thus was 0.1, 1, 2, 4, 6 and 8 µg/ml. A volume of 20µL of clear supernatant was syringed out and injected in RP-HPLC for analysing the drug content. The analysis was carried out in triplicate. The chromatographic conditions were kept same as described in Estimation of Paclitaxel by RP-HPLC method.

3.3 Result and Discussion

3.3.1 Estimation of Paclitaxel by RP-HPLC method

For estimation of Paclitaxel, a partially validated RP-HPLC method was used. The calibration curve was prepared in the concentration range of 1 – 50 µg/mL and the method was verified for interday and intraday precision and accuracy. The data

indicates that the method employed was precise and accurate and can be reliably employed for estimation of drug (Figure 3.4, Figure 3.5 and Table 3.1, Table 3.2).

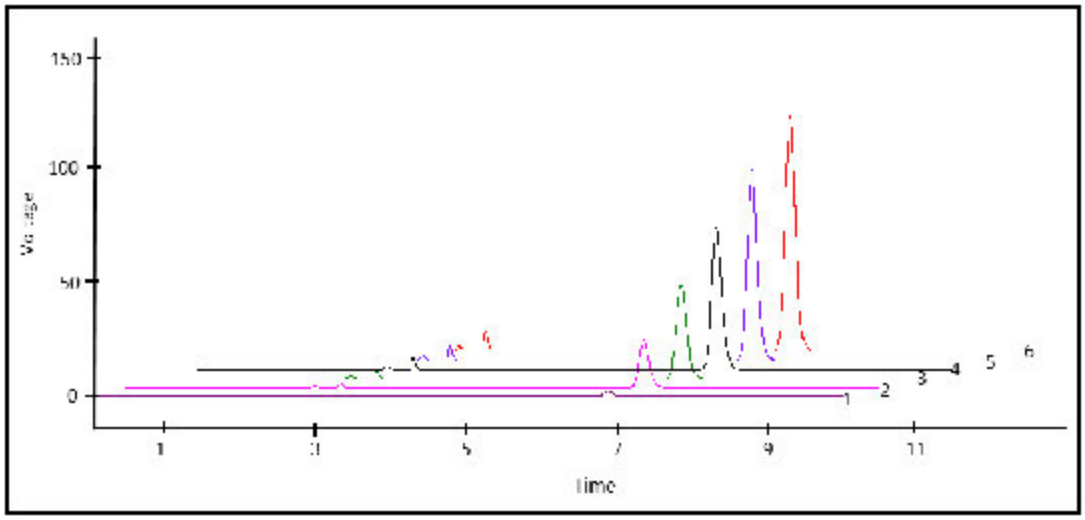


Figure 3.4 RP-HPLC chromatogram overlay of Paclitaxel in methanol at λmax 227 nm.

Table 3.1 Calibration curve values of Paclitaxel in methanol at λmax 227 nm

Sr No.	*Concentration (µg/mL)*	*Mean Area[a] ± SD (mV.s)*	*%RSD[b]*
1	1	23.27 ± 0.46	1.96
2	10	211.26 ± 1.37	0.64
3	20	417.70 ± 7.06	1.69
4	30	637.87 ±5.93	0.92
5	40	850.68 ±5.20	0.61
6	50	1056.38 ±6.27	0.59

a The values represented are mean±SD, n=3

b (% CV) = (Standard deviation/Mean concentration) *100

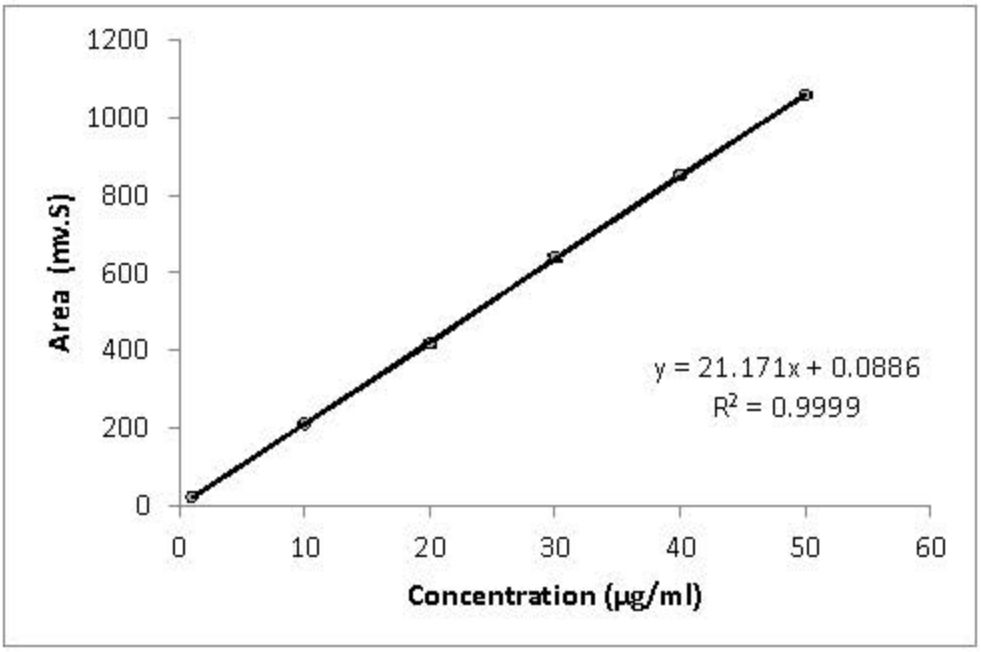


Figure 3.5 RP-HPLC calibration curve of paclitaxel in methanol at λmax 227 nm.

Table 3.2 Parameters for Estimation of PTX by RP-HPLC method.

Parameters	Results
λmax	227 nm
Linearity range	1-50 µg/mL
Regression equation	Y=21.171X + 0.0886
Correlation coefficient (r^2)	0.9999
Retention time	6.8 min

Accuracy and precision of Intraday and Interday experimentation of Paclixatel was performed and the data are provided in Table 3.3 and Table 3.4. Precision of the method was confirmed by the value of coefficient of variance that was observed less than 2% along with insignificant difference in true and observed concentration values indicating accuracy (6, 7).

Table 3.3 Intraday precision and accuracy data for estimation of Paclitaxel by RP-HPLC

Concentration (µg/ml)		*Precision*	*Accuracy*
Actual	*Observed*	*(% CV)* [a]	*(%Relative error)* [b]
1	1.02±0.012	1.18	-2.00
10	10.06±0.098	0.97	-0.6
50	50.09±0.169	0.34	-0.18

Table 3.4 Interday precision and accuracy data for estimation of Paxlitaxel by RP-HPLC

Concentration (µg/ml)		*Precision*	*Accuracy*
Actual	*Observed*	*(% CV)* [a]	*(%Relative error)* [b]
1	1.04±0.021	2.01	-4.00
10	10.09±0.151	1.50	-0.90
50	50.07±0.212	0.42	-0.14

[a] Precision (% CV) = (Standard deviation/Mean concentration) *100

[b] Accuracy (% Relative error) = (Actual value – Observed value/Actual value) *100

3.3.2 Estimation of total phospholipid content by Stewart method

This method was used to quantify the total amount of lipids in liposomes (4). All the lipids that were used in final composition for preparation of liposomes were dissolved in chloroform to prepared stock solution. Dilutions of the stocks were prepared and colorimetric estimation of ammonium-ferrothiocyanate: lipid complex was performed to obtain calibration curve (Figure 3.6, Table 3.6 and Table 3.7).

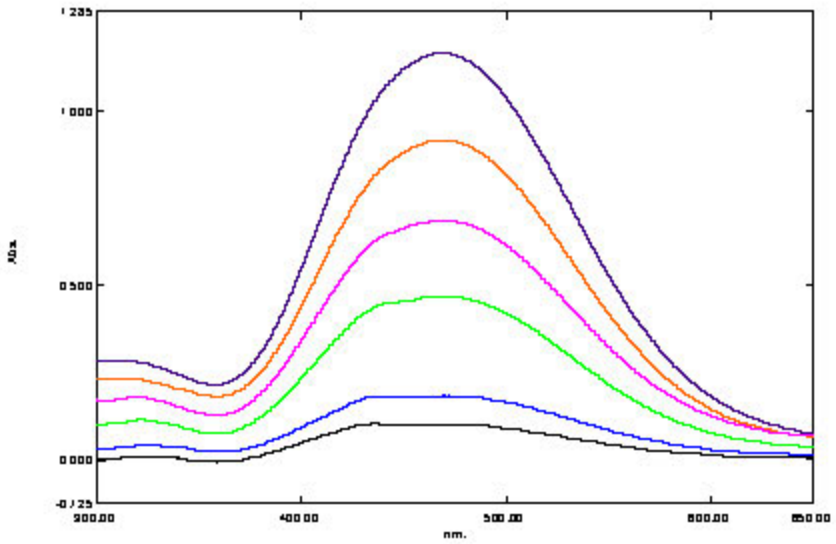


Figure 3.6 UV absorbance scans of total phospholipid mixture in chloroform at λmax 472 nm.

Table 3.5 Calibration curve values of total phospholipid mixture in chloroform at λmax 472 nm

Sr. No.	*Concentration (µg/mL)*	**Mean Absorbance Absorbance ± SD*
1	18	0.097 ± 0.006
2	36	0.195 ± 0.010
3	72	0.440 ± 0.017
4	108	0.689 ± 0.027
5	144	0.925 ± 0.024
6	180	1.175 ± 0.042

***The values represented are Mean±SD, n=3**

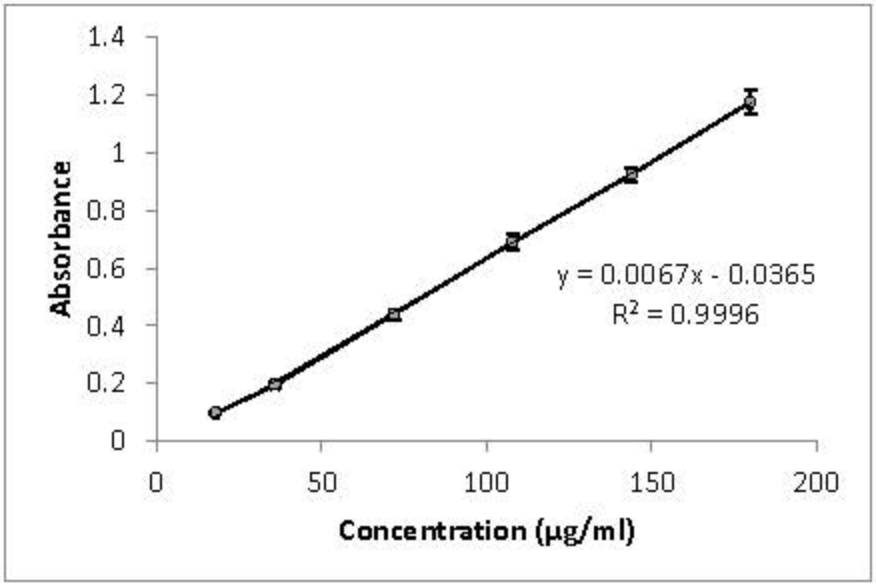


Figure 3.7 Calibration curve of total phospholipid mixture in chloroform at λmax 472 nm.

Table 3.6 Parameters for Estimation of total phospholipid mixture by Stewart method

Parameters	Results
λmax	472 nm
Linearity range	18-180 µg/mL
Regression equation	Y=0.0067X - 0.0365
Correlation coefficient (r^2)	0.9996

3.3.3 Estimation of plasma proteins by BCA protein estimation method

The significance of this method lies for estimation of plasma proteins that may get associated with liposomal formulation. It is a reliable in-vitro estimation method wherein Bovine Serum Albumin (BSA) was taken as a standard. The complex of BSA with Cu^{+1} ion showed absorbance at reported λmax of 562 nm (Figure 3.8, Table 3.7 and Table 3.8)

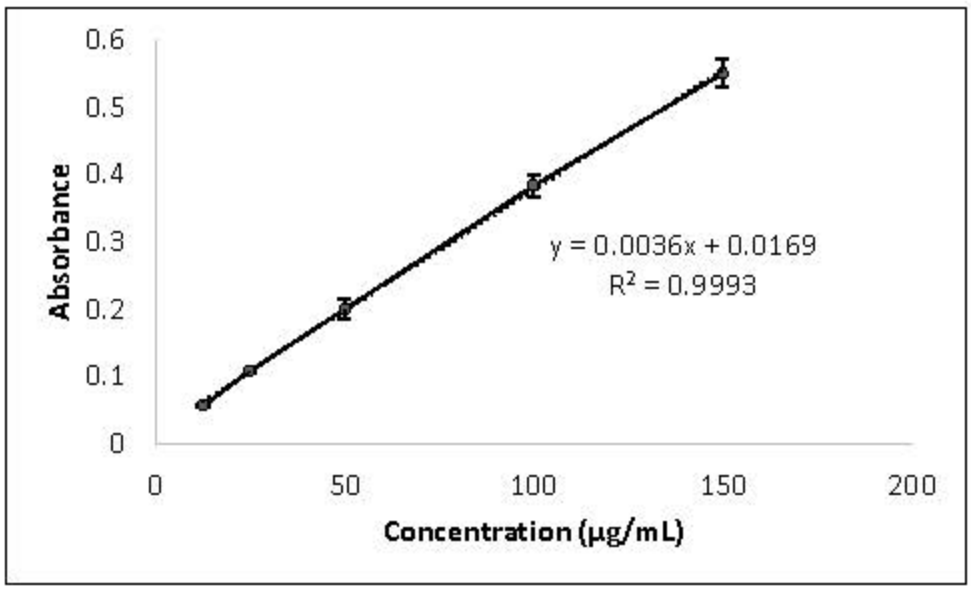


Figure 3.8 Calibration curve of BSA at λmax 562 nm

Table 3.7 Calibration curves values of BSA at λmax 562 nm

Sr. No.	*Concentration (µg/mL)*	**Mean Absorbance Absorbance ± SD*
1	12.5	0.056 ± 0.002
2	25	0.108 ± 0.002
3	50	0.199 ± 0.015
4	100	0.383 ± 0.017
5	150	0.550 ± 0.021

***The values represented are Mean±SD, n=3**

Table 3.8 Parameters for Estimation of protein by BCA protein estimation method

Parameters	Results
λmax	562 nm
Linearity range	12.5-150 µg/mL
Regression equation	Y= 0.0036 ± 0.0169
Correlation coefficient (r^2)	0.9993

3.3.4 Estimation of cell lysate protein by Bradford method

To estimate the protein content in cell lysate, Bradford method was used. As used in BCA method, here too Bovine Serum Albumin was used as standard for preparation of calibration curve. The dye-protein complex exhibited λmax of 595 nm and the method was found to be linear and accurate (Table 3.9, Table 3.10 and Figure 3.9).

Table 3.9 Calibration curve values of BSA at λmax 595 nm

Sr. No.	*Concentration (µg/mL)*	**Mean Absorbance Absorbance ± SD*
1	0.125	0.079 ± 0.002
2	0.25	0.152 ± 0.004
3	0.5	0.292 ± 0.005
4	0.75	0.410 ± 0.008
5	1	0.546 ± 0.010

***The values represented are Mean±SD, n=3**

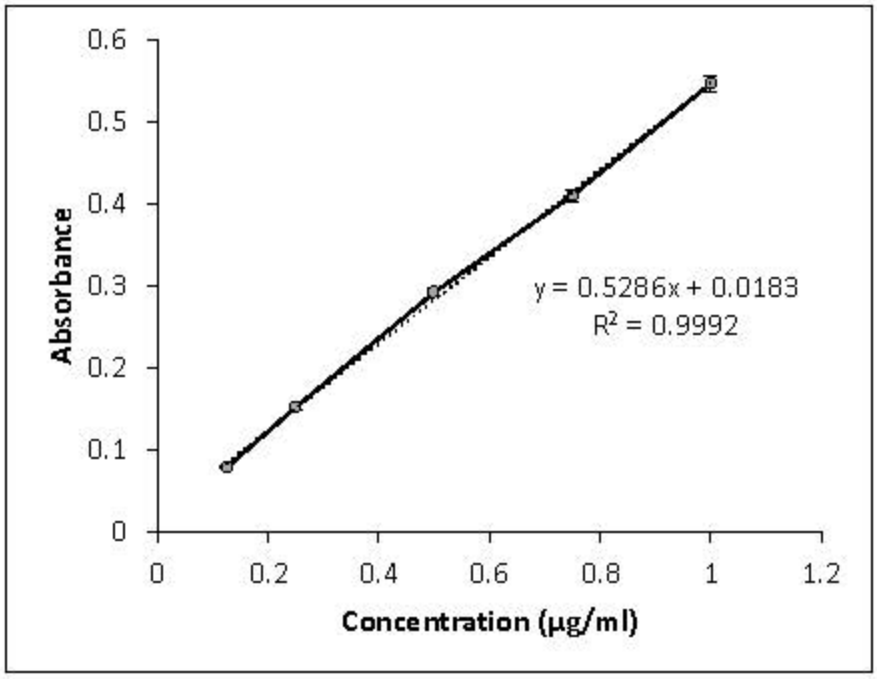


Figure 3.9 Calibration curve of BSA at λmax 595 nm

Table 3.10 Parameters for Estimation of protein by Bradford's method

Parameters	Results
λmax	595 nm
Linearity range	0.125-1 µg/mL
Regression equation	Y=0.5286X + 0.0183
Correlation coefficient (r^2)	0.9992

3.3.5 Estimation of cysteine by Ellman's Assay

Cysteine was used as a standard for Ellman's assay and the assay method was partially validated and data are presented (Figure 3.10, Figure 3.11, Table 3.11 and Table 3.12).

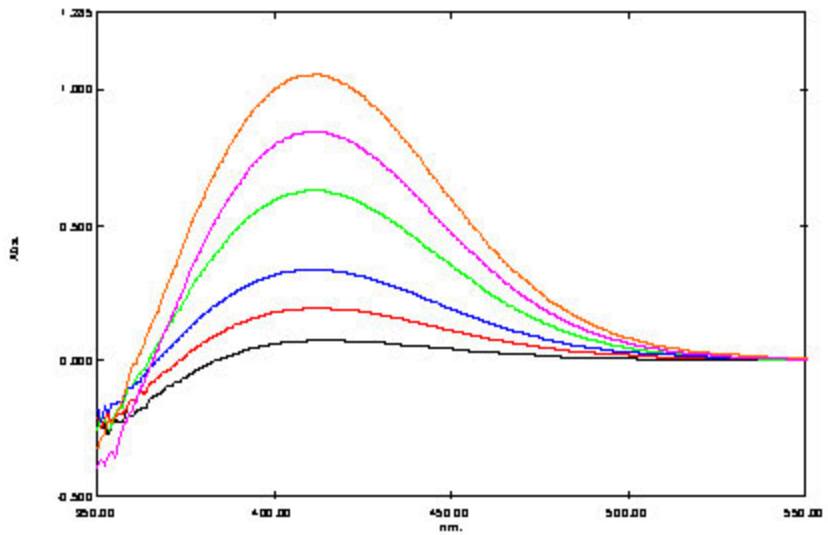


Figure 3.10 UV absorbance scans of cysteine at λmax 412 nm

Table 3.11 Calibration curve values of cysteine at λmax 412 nm

Sr. No.	*Concentration (mM)*	**Mean Absorbance Absorbance ± SD*
1	0.1	0.076 ± 0.001
2	0.2	0.196 ± 0.003
3	0.4	0.413 ± 0.006
4	0.6	0.627 ± 0.015
5	0.8	0.847 ± 0.021
6	1	1.057 ± 0.029

***The values represented are Mean±SD, n=3**

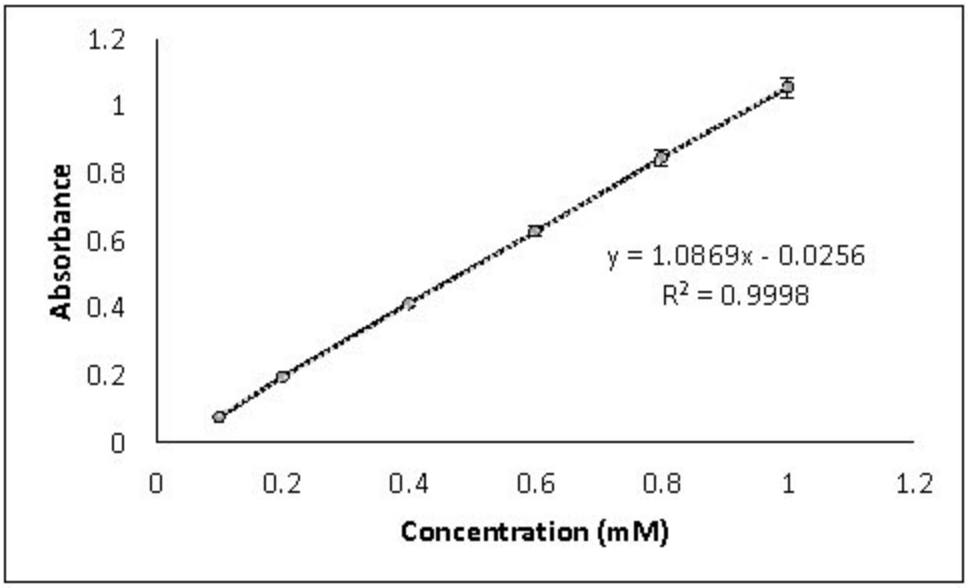


Figure 3.11 Calibration curve of cysteine at λmax 412 nm

Table 3.12 Parameters for Estimation of cysteine by Ellman's assay

Parameters	Results
λmax	412 nm
Linearity range	0.1-1 mM
Regression equation	Y=1.0869x + 0.0256
Correlation coefficient (r^2)	0.9998

3.3.6 Estimation of 6-coumarin by spectrofluorometer

The Spectrofluorimetric method was employed to estimate coumarin 6 in liposomes (8-10). Calibration curve was developed in Chloroform:Methanol (1:1). For determining the encapsulation efficiency of coumarin 6 in liposomes calibration curve was prepared in Chloroform:Methanol (1:1). Fluorescence intensity values for different concentrations are shown Table 3.13. At all concentration levels the SD was low and the % RSD did not exceed 7.94. (Figure 3.12, Figure 3.13 and Table 3.13)

Table 3.13 Calibration data for Coumarin 6 in Chloroform: Methanol (1:1)

Conc. (pg/mL)	Coumarin 6	
	Mean Fluorescence intensity[a] (± SD)	**% RSD [b]**
1000	108.15 ± 8.59	7.94
2000	172.83 ± 8.87	5.13
3000	240.35 ± 17.46	7.26
4000	303.39 ± 22.66	7.47
5000	385.52 ± 15.78	4.09
6000	468.42 ± 29.68	6.33
7000	545.94 ± 38.68	7.08
8000	620.64 ± 31.11	5.01
Linearity (pg/ml)	1000-8000 pg/mL	
Regression coefficient (R^2)	0.9978	
Regression Equation	Fluorescence Intensity = [0.074× conc.(pg/mL)] + 22.459	

[a] Each value is mean of three independent determinations
[b] Percentage relative standard deviation

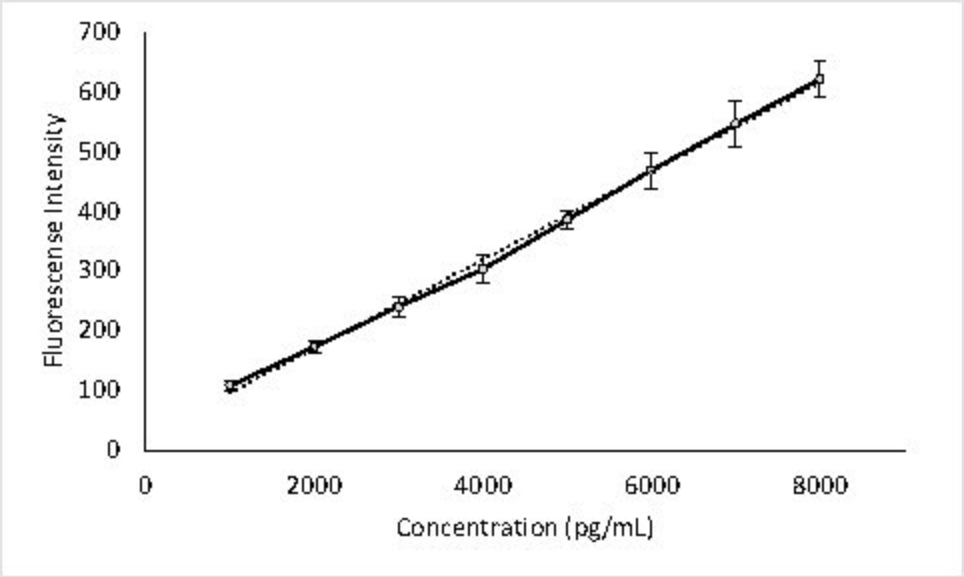


Figure 3.12 Calibration curve of Coumarin 6 in Chloroform:Methanol (1:1)

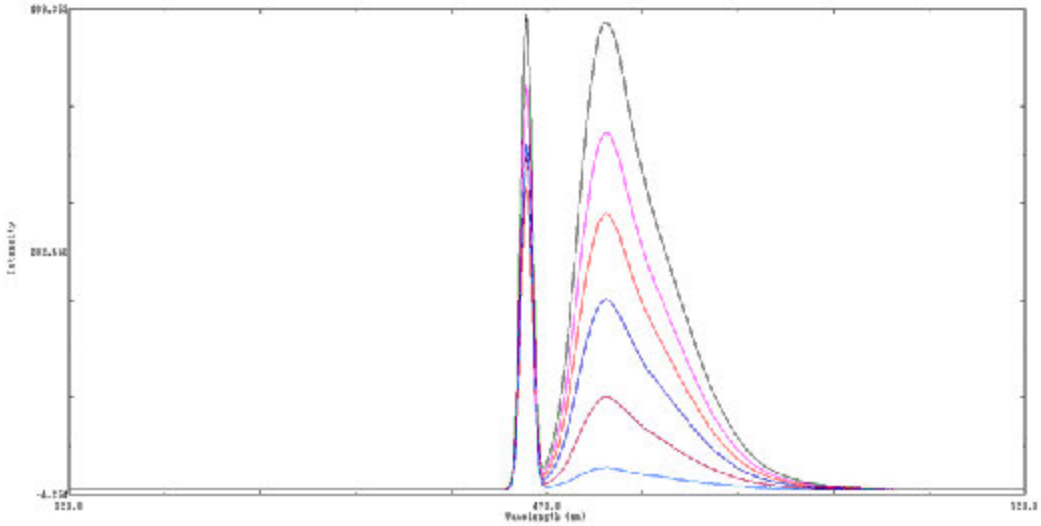


Figure 3.13 Spectra of different concentration of Coumarin 6 in Chloroform:Methanol (1:1)

3.3.7 Estimation of Paclitaxel from plasma samples by RP-HPLC method

Estimation of drug concentration in plasma was carried out by protein precipitation method. As paclitaxel as such is insoluble in plasma, it was first solubilized in minimum quantity of methanol. So, stock solution of drug in methanol was prepared followed by diluting it suitably to a level wherein precipitation of plasma does not occur. The volume of first dilution of stock that was successfully spiked in plasma (380µl) was 20µl i.e. each plasma sample contained the required concentration of drug in only 5% methanol, which was inadequate to precipitate proteins. For protein precipitation from plasma samples excess amount of methanol (3

times the volume of plasma samples) was added to the plasma sample i.e. 100µl of plasma volume was added in 300µl methanol, wherein complete precipitation of plasma protein was observed. It was observed that the percentage recovery of drug from the supernatant after centrifugation was greater than 95% in all cases (Figure 3.14 and Figure 3.15).

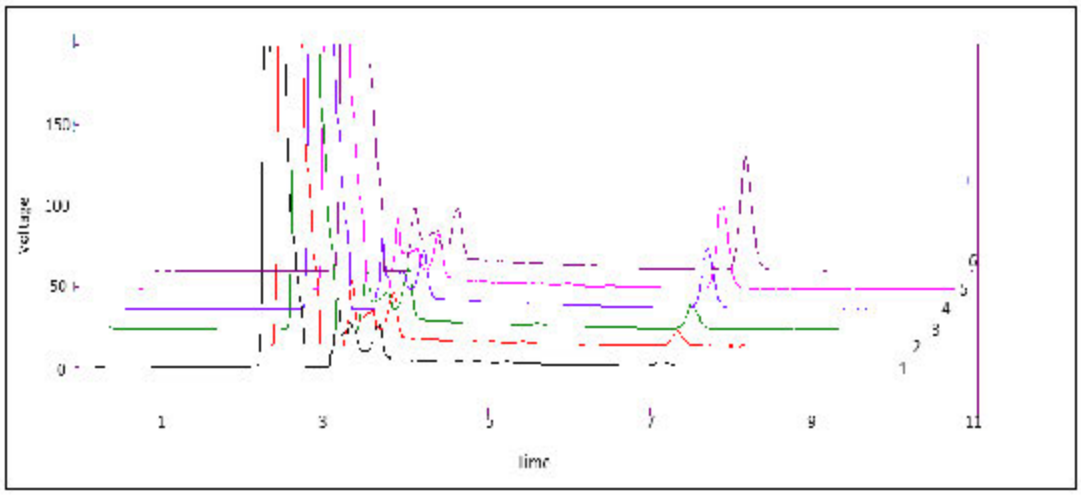


Figure 3.14 RP-HPLC chromatogram overlay of paclitaxel in plasma at λmax at 227 nm.

Table 3.14 RP-HPLC calibration curve values of paclitaxel in plasma at λmax at 227 nm

Sr. No.	*Concentration (µg/mL)*	**Mean Area (mV.s) ± SD*
1	0.1	4.74 ± 0.46
2	1	19.56 ± 1.37
3	2	34.67 ± 7.06
4	4	73.26 ±5.93
5	6	108.87 ±5.20
6	8	142.37 ±6.27

***The values represented are Mean±SD, n=3**

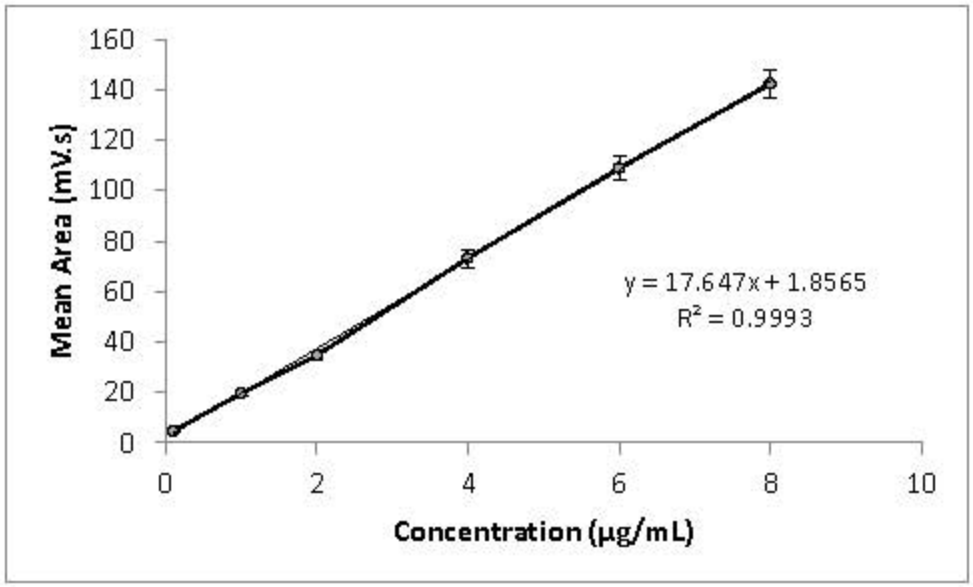


Figure 3.15 RP-HPLC calibration curve of paclitaxel in plasma at λmax at 227 nm

Table 3.15 Parameters for Estimation of PTX in plasma by RP-HPLC method.

Parameters	Results
λmax	227 nm
Linearity range	0.1-8 µg/mL
Regression equation	Y=17.647X + 1.8565
Correlation coefficient (r^2)	0.9993
Retention time	7.1 min

Accuracy and precision of Intraday and Interday experimentation of Paclixatel in plasma was performed and the data are provided in Table 3.16 and Table 3.17. Precision of the method was confirmed by the value of coefficient of variance that was observed less than 2% along with insignificant difference in true and observed concentration values indicating accuracy.

Table 3.16 Intraday precision and accuracy data for estimation of Paclitaxel in plasma by RP-HPLC

Concentration (µg/ml)		*Precision*	*Accuracy*
Actual	*Observed*	*(% CV)* [a]	*(%Relative error)* [b]
0.1	0.108 ± 0.005	4.62	-8.00
1	1.055 ± 0.058	5.49	-5.50
8	8.081 ± 0.352	4.35	-1.00

Table 3.17 Interday precision and accuracy data for estimation of Paxlitaxel in plasma by RP-HPLC

Concentration (µg/ml)		*Precision*	*Accuracy*
Actual	*Observed*	*(% CV)* [a]	*(%Relative error)* [b]
0.1	0.106 ± 0.004	3.77	-6.00
1	1.061 ± 0.049	4.62	-6.1
8	8.093 ± 0.371	4.58	-3.1

[a] Precision (% CV) = (Standard deviation/Mean concentration) *100

[b] Accuracy (% Relative error) = (Actual value – Observed value/Actual value) *100

3.4 References

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