3 ANALYTICAL METHODS

3.1 INTRODUCTION

Analytical processes have gained particular position in the production of formulation. It provides the basis for the measurement of performance, assay and stability studies for encapsulation. In this chapter, the method for estimating Docetaxel in PLHNCs is optimized. Docetaxel calculation was conducted with various physiological parameters e.g., blood pH 7.4 etc. for serum consistency and Docetaxel calibration with homogenate in particular organs was performed for pharmacokinetic tests.

For quantification of pDNA in PLHNCs gel electrophoresis and UV spectrophotometric analysis were chosen as method of choice. Gel electrophoresis would provide the immediate idea about the complexation of anionic charged shRNA pDNA with cationic charged lipid present in PLHNCs. UV spectrometric analysis gives precise idea about the purity of shRNA pDNA.

3.2 ANALYTICAL METHOD VALIDATION

3.2.1 Linearity

The linearity of an analytical approach is its ability to retrive test results that are directly proportional to the concentration of the analyte in samples within a given range (1). Lambert-Beer's rule states that absorbance of analyte is relative to the concentration of absorbing species. As a function of an dependent variable (absorbance, Y-axis), a calibration curve is prepared by plotting a independent variable (concentration, X-axis). This relationship is found with set of measurements, which are also a linear in nature.

3.2.2 Accuracy

An analytical method's precision is its closeness to the true value for the test results obtained (2). An analytical method's accuracy is commonly expressed as the standard deviation or confidence limit. Three quality control tests (10µg/mL, 20µg/mL and 30µg/mL) of acetonitrile were used to determine the precision of the process. The samples selected were such that the entire spectrum of the normal curve, i.e. lower, intermediate, and higher range concentrations, were reflected. Accuracy was determined by analysis of 3 sample replicates.

3.2.3 Precision

The precision of an analytical technique is the degree of conformity between the actual test findings as the procedure is consistently extended to several homogenous sample scans (2).

Precision may be an indicator of either the degree of repeatability or reproducibility under standard operating conditions of the analytical process.

3.2.4 Limit of Detection and Limit of Quantification:

The Limit of Detection (LoD) is a quantitative parameter. It is the lowest analyte concentration in a sample that can be detected under specified experimental conditions with reasonable accuracy and precision. The Limit of Quantification (LoQ) is the lowest analyte concentration that can be quantitatively detected with a stated accuracy and precision (3). It is expressed as the analyte's concentration in the sample. In terms of μg/mL, ng/mL, pg/mL, the cap is expressed etc. For a particular set of experimental conditions, LoD values are specific. The identification limits can be altered by parameters that affects a method's susceptibility, including the instrument, sample preparation, etc.

3.3 EXPERIMENTAL WORK

3.3.1 Materials

Table 3-1 List of materials used with their sources

Sr. No.	Materials	Source
1.	Docetaxel	Gift by Sun Pharmaceutical Industries. Ltd. (Vadodara, India)
2.	Egg Phosphatidylcholine (EPC)	Gift from Lipoid GMBH (Ludwigshafen, Germany)
3.	Cholesterol extra pure	Purchased from Sigma-Aldrich
4.	DOTAP	Gift from Lipoid GMBH (Ludwigshafen, Germany)
5.	1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine [methoxy (polyethyleneglycol)-2000] (DSPE-mPEG2000)	Gift from Lipoid GMBH (Ludwigshafen, Germany)
6.	DPPC	Gift from Lipoid GMBH (Ludwigshafen, Germany)
7.	PLA-PCL	Gift from evonik
8.	Methanol AR Grade	Thermo fisher, India
9.	Methanol HPLC Grade	Astron, India
10.	Acetonitrile HPLC Grade	Astron, India
11.	Sodium Hydroxide	Fisher Scientific, India
12.	Sodium dihydrogen phosphate	Fisher Scientific, India
13.	Disodium hydrogen phosphate	Fisher Scientific, India
14.	Tween 80	S.D.Fine Chemical Laboratory
15.	Double distilled Water	Prepared in laboratory (G.H.Patel pharmacy building)

16.	DNAse free water	Prepared in laboratory(G.H.Patel pharmacy building)
17.	Gel loading buffer (6X)	Himedia Laboratories,India.
18.	ABCB1 shRNA plasmid	SCBT, USA

3.3.2 Equipment

Table 3-2 List of Equipments Used

Sr. No.	Equipment	Company
1.	Single pan electronic balance	Type AX 120 & ELB 300, Shimadzu
2.	UV Visible Spectrophotometer	UV-1800, Pharm Spec, Shimadzu, Japan
3.	HPLC	Agilent Technologies 1260 infinity II
4.	Bath sonicator	Sartorius, India
5.	NanoDrop 2000	NanoDrop,Germany
6.	GeNei mini sub system	Merk-Millipore-GeNei Techware, India.
7.	GelDocTM XR+ Imaging System	Bio-Rad, USA

3.4 ESTIMATION OF DOCETAXEL BY UV SPECTROPHOTOMETRY

3.4.1 λmax determination

The maximal absorption (λ max) of Docetaxel was calculated by scanning 10 μg/mL of Docetaxel solution against acetonitrile as a reagent blank in spectrum mode between 200 and 400 nm.

3.4.2 Preparation of stock solution for calibration

Primary stock solution of Docetaxel in acetonitrile (0.1mg/1mL) was prepared by dissolving 10 mg Docetaxel in 100mL acetonitrile. The primary stock solution was stored at 2-8ºC.

3.4.3 Preparation of the Docetaxel calibration curve in acetonitrile

Appropriate aliquots of the Docetaxel stock solution (1mg/10mL) were transferred to 10mL volumetric flasks and diluted with acetonitrile up to the level. Using the UV-VIS spectrophotometer, the absorption of all prepared solutions (5, 10, 15, 20, 25, 30, 35, 40 and 45 μg/mL) was then measured at an absorbance limit of 229 nm against the reagent blank (acetonitrile). The readings were taken in triplicates. The mean value (n=3) along with the standard deviation (SD) are reported. Regressed absorption values were graphically plotted

against the concentrations. The stability of Docetaxel solutions in acetonitrile was defined by observing changes in solution absorption at the analytical wavelength over a 24-hour span at room temperature.

3.4.4 Docetaxel calibration plot in phosphate buffer pH 7.4

For in-vitro drug release tests, the calibration plot of Docetaxel in phosphate buffer with pH 7.4 (pH of the blood and natural tissue) was used. The release of the drug at pH 7.4 would provide insight into the release of Docetaxel from PLHNCs in the blood and inside normal tissue. In water, Docetaxel is insoluble. Thus, 0.1% Tween 80 was formulated as a solubilizing element in PBS 7.4 pH to improve the solubility of Docetaxel in aqueous media.

3.4.4.1 Preparation of Phosphate buffer pH 7.4 (IP-2007)

In a 200 mL volumetric flask, 50.0 mL of 0.2 M potassium dihydrogen phosphate was taken to which 39.1 mL of 0.2 M sodium hydroxide and 0.2 mL tween 80 was added and then purified water was mixed to make up the final volume. To fully dissolve the solid contents, the solution was sonicated for 3 minutes. The pH of the buffer was tested using a pH meter and if necessary, modified using a sodium hydroxide or hydrochloric acid solution (4).

3.4.4.2 Docetaxel stock solution preparation

Transferred 10 mg of precisely weighed Docetaxel to 100 mL volumetric flask. To ensure complete solubilization of the compound, limited amounts of phosphate buffer pH 7.4 were added to the volumetric flask. Finally, volume was made up with the phosphate buffer of pH 7.4, resulting in a stock solution with concentration of 100µg/mL.

3.4.4.3 Calibration plot of Docetaxel in phosphate buffer pH 7.4.

Docetaxel standard solution (100µg/mL) in phosphate buffer pH 7.4, was taken in aliquots of 1, 2, 3, 4 and 5 mL with help of a pipette and transferred to 10 mL volumetric flasks and volume was made up by phosphate buffer pH 7.4 up to mark to bring homogeneous solution of 10, 20, 30, 40 and 50µg/mL. The absorption of all the formulated solutions was measured using the phosphate buffer pH 7.4 as a blank at an absorption limit of 229 nm.

3.4.5 Docetaxel calibration plot in Phosphate buffer pH 6.6

For in-vitro drug release tests, the calibration plot of Docetaxel in phosphate buffer pH 6.6 (pH of the cancer tissue) was used. An idea about release of docetaxel in cancer tissue interstitium is provided by the drug release at pH 6.6. Docetaxel is insoluble in water. Thus, 0.1% Tween 80 was applied as a solubilizing agent in PBS with 6.6 pH to improve the solubility of Docetaxel in phosphate buffer pH 6.6.

3.4.5.1 Preparation of Phosphate buffer pH 6.6 (IP, 2007)

In a 200 mL volumetric flask, 50.0 mL of 0.2 M potassium dihydrogen phosphate was added, in which 16.4 mL of 0.2 M sodium hydroxide and 0.2 mL tween 80 were introduced to the above solution and then purified water was added to achieve the final quantity. The solution was sonicated until the solute dissolved completely. The pH of a buffer was tested using a pH meter and if necessary, modified using a sodium hydroxide or hydrochloric acid solution (4).

3.4.5.2 Preparation of Docetaxel stock solution

10 mg of precisely measured Docetaxel were taken to 100 mL volumetric flask and complete dissolution of Docetaxel was ensured with pre-measured amounts of phosphate buffer pH 6.6. Finally, volume was made up to level using the Phosphate buffer pH 6.6, resulting in a standard stock solution of 100µg/mL concentration.

3.4.5.3 Calibration plot of Docetaxel in phosphate buffer pH 6.6

Aliquots of 1, 2, 3, 4 and 5 mL were made from the Docetaxel stock solution (100µg/mL) and diluted with the phosphate buffer pH 6.6 through pipette and transferred to 10 mL volumetric flasks and the volume was made up by phosphate buffer pH 6.6 to give final concentrations of 10, 20, 30, 40 and 50µg/mL. At the absorption limit of 229 nm, the absorbance of all formulated solutions was measured using the phosphate buffer pH 6.6 as a blank.

3.4.5.4 Calibration plot of Docetaxel in acetate buffer pH 5.5

For in-vitro drug release tests, the calibration plot of Docetaxel in phosphate buffer pH 5.5 (pH within cancer cells vary from 5-6) was used. The release of drugs at pH 5.5 can provide an understanding of the release of Docetaxel within cancer cells until cancer cells internalize PLHNCs.

3.4.5.5 Preparation of acetate buffer pH 5.5 (IP, 2007)

In a 250 mL volumetric flask, 200 mL of distilled water was taken.1.93 g of Sodium Acetate was added to the solution. After that 0.088 g of Acetic Acid was added to the solution. Solution was adjusted to final desired pH using HCl or NaOH. Distilled water up to 250 mL was added in 250 mL volumetric flask. (4).

3.4.5.6 Preparation of Docetaxel stock solution

10 mg of precisely measured Docetaxel was offloaded to a 100 mL volumetric flask. Measured amount of pH 5.5 acetate buffer were added to the volumetric flask to ensure complete dissolution of Docetaxel. Finally, volume was made up by acetate buffer pH 5.5, resulting in a standard solution of 100 µg/mL concentration.

3.4.5.7 Calibration plot of Docetaxel in acetate buffer pH 5.5

Aliquots of 1, 2, 3, 4 and 5 mL were accurately collected from the Docetaxel standard solutions (100 µg/mL) in the acetate buffer pH 5.5 through pipette and shifted to specific 10 mL volumetric flasks and the volume was made up to level with acetate buffer pH 5.5 to give final concentrations of 10, 20, 30, 40 and 50µg/mL. At the absorption limit of 231 nm, the absorbance of all primed solution was determined using the acetate buffer pH 5.5 as a blank.

3.5 ESTIMATION OF DOCETAXEL BY HIGH PERFORMANCE LIQUID CHOMATOGRAPHY.

The High-Performance Liquid Chromatography (HPLC) approach is being used for determining Docetaxel in biological materials due to its sensitivity in analysing small quantities of drugs.

3.5.1 Estimation of Docetaxel by RP-HPLC method

In order to calculate the Docetaxel content in PLHNCs using the C18 ODS (octa decyl silane) column (250 mm * 4.6 mm* 5 µm, Thermo scientific) at ambient temperature, the reverse-phase HPLC method/RP-HPLC (Agilent Technologies 1260 infinity II) was used. The mobile phase acetonitrile: water (60:40) was managed with a flow rate of 1 mL/min. For the samples, appropriate dilutions of Docetaxel in methanol were prepared and a final mobile phase dilution was performed. With 20 µl of each solution, the chromatogram ran for 10 minutes in HPLC. The Docetaxel estimation was performed at a wavelength of 231 nm using a UV-visible detector (5).

3.5.2 Calibration curve of Docetaxel by HPLC method

3.5.2.1 Preparation of Stock Solution

Docetaxel 10 mg was accurately measured and transferred to a 10 ml volumetric flask containing. Premeasured quantity of methanol was added step by step to measured Docetaxel till the complete solubility was achieved. Then volume was made up to 10 ml to obtained concentration of 1000 µg/ml.

3.5.2.2 Preparation of Working Standard Solutions

In a 10 mL volumetric flask, 0.1 ml of the above solution was placed and made up to 10 mL with the mobile phase to obtain 10 µg/ml of working standard solution.

3.5.2.3 Calibration curve of docetaxel in Acetonitrile: Water (60:40)

Samples spanning from 0.2, 0.4, 0.6, 0.8, 1.0 ml were obtained from the working solution in 1 mL Eppendorf and mixed with a mobile phase up to 1 ml at a final concentration

of 200, 400, 600, 800, 1000 ng/ml. 20 µl of each sample was infused for each concentration, and a chromatogram was taken under condition mentioned in Table 3-3. The calibration curve was constructed by plotting peak area versus Docetaxel intensity and the regression analysis was computed.

Table 3-3 Process Parameters for estimation of Docetaxel in Acetonitrile: Water (60:40) by HPLC

System Parameter	Value
HPLC	Agilent Technologies 1260 infinity II
Column	250 mm *4.6 mm* 5 µm, Thermo scientific
Wavelength	231 nm
Flow-rate	1 mL/min.
Run-time	10 min
Injection volume	Rhenodyne 7725 injector valve with fixed loop at 20 µl
Mobile Phase	ACN: Water (60:40)
Retention time	7.6 mins.

3.5.3 Calibration curve of Docetaxel in rat plasma by HPLC method

3.5.3.1 Blood collection and separation of plasma

Blood from the retro-orbital plexus of Sprague dawley rats weighing 200-250 gm was collected using glass capillaries in vial containing 10 IU heparin as anticoagulant. Using the REMI cooling centrifuge, transparent supernatant plasma was isolated from blood after centrifugation at 5000 rpm at 4℃ for 10 minutes. Before further study, samples were held at -40℃.

3.5.3.2 Preparation of Stock Solutions:

The stock solution of Docetaxel (1000µg/mL) was prepared in 10 mL volumetric flask by dissolving 10 mg of the Docetaxel into 10 mL of methanol. The final standard concentration of the solution was 1000 µg/ml.

3.5.3.3 Preparation of working standard solutions:

20, 40, 60, 80, 100µl was separated from the above regular stock solution and water was added up to 1 mL to achieve homogeneous solution of 20, 40, 60, 80, 100 µg/ml.

3.5.3.4 Preparation of drug-plasma solution

In order to achieve concentrations of 1-5 µg/mL, the plasma calibration standard solutions were prepared by spiking 20 µl above working standard of Docetaxel in 380µl drug-

free plasma. 20 µl of the mobile phase into 380 µl of plasma was spiked, and used as blank sample. All solutions were kept at 2-8°C until further use.

3.5.3.5 Calibration curve of Docetaxel in rat plasma by liquid-liquid extraction technique

For the extraction of Docetaxel from plasma, a basic single step protein precipitation procedure has been adopted (6) . 1200 µl ice cold acetonitrile was introduced to the above plasma drug solution to achieve a concentration of 250-1250 ng/ml. Samples were vortexed for 2 min and centrifuged for 10 min at 5000 rpm. Supernatant was extracted and screened via a 0.22 µm nylon syringe filter. 20 µl of sample was injected into HPLC and evaluated using the parameters referred in section 3.5.

3.5.4 Calibration curve of Docetaxel in lung homogenate by HPLC method

3.5.4.1 Preparation of Lung Homogenate:

Using a high-speed homogenizer, 2.5 gm of lung tissue was homogenized in 25 mL purified water in order to generate 10 percent of lung homogenate in ice bath to sustain temperature at 4℃. Further it was centrifuged at 5000 rpm at 4℃ for 10 min. The transparent supernatant of the homogenate was isolated and stored at -70℃.

3.5.4.2 Preparation of Stock Solutions:

Docetaxel's stock solution (1000µg/mL) was prepared by dissolving 10 mg of Docetaxel in 10 mL volumetric flasks using methanol. The final standard inventory solution concentration was 1000 µg/ml.

3.5.4.3 Preparation of working standard solutions:

30, 50, 70, 90 and 110 µl of the above final regular stock solution was isolated and diluted to 1 mL to achieve final concentrations of 30, 50, 70, 90 and 110 µg/mL.

3.5.4.4 Preparation of lung homogenate drug solution

In order to achieve concentrations of 30-110µg/mL, plasma calibration standards were prepared by spiking 20 µl of above working standard of Docetaxel in 380 µl of drug-free lung homogenate obtained from controlled/Non-treated animals. A blank sample was prepared by spiking 20 µl of the mobile phase into 380 µl of lung homogenate. Before further use all the solutions were stored at 2-8℃.

3.5.4.5 Calibration curve of Docetaxel in lung homogenate by liquid-liquid extraction technique

For the extraction of Docetaxel from lung tissue homogenate, a basic single stage protein precipitation procedure was adopted (6). 1200 µl of ice-cold Acetonitrile was added to

the above lung homogenate drug solution to achieve a concentration of 300-1100 ng/mL Samples were then vortexed for 2 min and centrifuged for 10 min at 5000 rpm at 4℃. Supernatant was obtained and screened via a 0.22 µm nylon syringe filter. 20 µl of this was injected into HPLC and evaluated using the parameters referred in section 3.5 .

3.5.5 Method validation

The proposed approach was validated by HPLC as per the ICH guideline for the content of Docetaxel in sample. The method was validated for its precision, linearity, range, accuracy and clarity in order to demonstrate that the method is suitable for its intended use as per the ICH Q2 (R1) guideline (7). Validation of the protocol was obligatory according to the ICH Q2(R1) instructions if i) new observations were seen over time ii) the method developed was used for extended application iii) the method developed was performed at different locations with different instruments. Accuracy, precision, linearity, selectivity, specificity, limit of detection, limit of quantification, robustness and robustness, as defined in ICH Q2, are suggested as parameter values for the validation parameter (R1) (8).

3.5.6 Estimation of total phospholipid content by Stewart method

3.5.6.1 Principle

Through Stewart's Colorimetric Technique, phospholipid content can be measured. This approach for the identification of phospholipids is based on the ability of phospholipids to create a stable compound with ammonium ferro-thiocyanate. Ammonium ferro-thiocyanate is an inorganic, red-colored compound that appears to stay insoluble in chloroform. However, with phospholipids it can form stable complexes and those complexes are soluble in chloroform. Therefore, when at room temperature, a chloroform solution consisting of phospholipids was mixed with ammonium ferro-thiocyanate, a red colored complex is formed which partitions and gets solubilized in chloroform phase. Via colorimetry, the absorption of the colored soluble complex in chloroform was calculated at 462 nm (9).

3.5.6.2 Preparation of ammonium ferro-thiocyanate solution:

In de-ionized distilled water, 27.03 g ferric chloride hexahydrate ($FeCl_3.6H_2O$) and 30.4 g ammonium thiocyanate (NH_4SCN) were dissolved and made up to 1 liter volume to form ammonium ferro-thiocyanate solution.

3.5.6.3 Calibration plot of Phospholipids:

All the glass equipment used for the analysis were washed with chromic acid solution prior to use to evade the possible corrosion from surface active washing agents. A stock

solution of 21.90 mg lipid mixture was prepared by solubilizing all the lipids in 21.90 ml of chloroform leading to final lipid concentration of 1000 µg/ml. The lipids used to prepare the stock solution were included with their respective molar ratios in the final optimized formulation, i.e. 105 µl DPPC, 15 µl of DOTAP, 12 µl DSPE mPEG2000. (The above lipid amount was taken from the separate stock lipid solution in chloroform with condensed lipids). Then 3 ml of chloroform was added. After 5 min, the biphasic mixture was completely mixed and the lower chloroform layer of high density was removed using syringe. The optical density of a segregated chloroform layer was determined by taking pure chloroform as a blank at a wave length of 462 nm. The average of optical densities obtained was studied and results were plotted in a graph against overall lipid concentration.

3.5.7 Analytical Interference study

Interference testing is undertaken to assess excipient interference in drug detection. In brief, 10 mg of Docetaxel, appropriately measured, was added to a 10 ml volumetric flask containing 5 ml of acetonitrile and was fully dissolved. A volume was made up to 10 ml to produce a 1000 ppm acetonitrile solution. To offer a solution of 100µg/ml, this stock was sufficiently diluted. The fixed amount was removed from this solution and transferred to another volumetric flask containing correctly weighed polymer and lipid amounts, i.e. In the 1:1 ratio [drug: polymer], PEG-PCL (50:50) and DPPC, DOTAP, DSPE-PEG2000. The volume was made up with acetonitrile up to the mark. The absorption of this solution was reported as blank at 229 nm using methanol. The absorption of the stock solution of Docetaxel was also reported as blank at 229 nm using acetonitrile.

3.6 ANALYTICAL METHODS USED FOR QUANTIFICATION OF pDNA

Two techniques, namely the UV spectrophotometric method and gel electrophoresis, were used for quantitative analysis of pDNA. The process of gel electrophoresis would provide an immediate understanding of the efficiency of complexation as well as assist in identifying the pDNA. In other tests, such as serum stabilization studies as well as complexation efficiency, UV spectrophotometric technique can provide precise assessments.

3.6.1 UV Spectrophotometric Analysis of pDNA

It is possible to measure the pDNA by calculating the ultraviolet absorbance. For DNA chronology, 260 nm, 280 nm and 230 nm are the three primary wavelengths of interest. The 260 nm absorbance provides an estimate of the amount of DNA found in the specimen. Concentration can be measured using a wavelength reading of 260 nm and a conversion factor

dependent on the nucleic acid extinction coefficient. Measurements of absorption at the wavelength at 280 nm will concurrently be used to measure the volume of protein contaminant in the sample depending on the presence of aromatic amino acids that absorb light at 280 nm. It is possible to use a test at 230 nm to assess the quantity of shRNA pDNA that could be found in the samples. In addition, some light-scattering materials in the sample take into consideration an absorbance reading at 320 nm. The 320 nm wavelength reading is subtracted as a background from the 260 nm, 280 nm and 230 nm readings (10). Good linearity and reproducibility were provided by the results of the known DNA solution dilution. In further tests, this spectrum of linearity was used to figure out the concentration of shRNA pDNA.

3.6.2 Method for UV analysis of shRNA pDNA

A UV spectrophotometric approach was used to calculate pDNA (11). The pDNA solution absorption was tested by taking the absorbance values at four wavelengths, i.e., 230 nm, 260 nm, 280 nm and 320 nm using NanoDrop 2000 (NanoDrop, Germany). Absorbance values at all wavelengths were corrected by subtracting the absorbance value at 320 nm for scattering from the sample (correction performed automatically by software). By testing the ratios of A260/A280 and A260/A230, the purity of the pDNA was calculated. Once its purity was validated by, the calibration curve was built. The 1 μg/μL pDNA stock solution in DNAse free water (DFW) and pDNA solutions with differing concentrations between 2 ng/μL and 200 ng/μL were prepared with sufficient dilutions. At 260 nm using NanoDrop UV spectrophotometer, the absorbance values of these solutions were reported. The pDNA content was determined by a corrected absorbance at 260 nm, i.e. A260-A320 and multiplying the dilution factor reading and using the 1.0 = 50 μg dsDNA reference for A260. The whole experiment was carried out in triplicates. To assess the linearity of the particular concentration spectrum and the reproducibility of the data, a graph of measured concentration versus real concentration of DNA was plotted.

3.7 GEL ELECTROPHORESIS OF pDNA

Agarose gel electrophoresis was used for relative quantification of pDNA in which the movement of free pDNA under the influence of electrical potential occurs. This approach makes use of an ethidium bromide (EtBr) Ligand - binding dye that intercalates at the minor groove of the nucleic acid double strand that helps the depiction of the nucleic acid on the gel and the DNA's complexation performance with the cationic polymer (12). The underlying theory is that the nucleic acid is isolated according to its size. As the potential differential is added, smaller molecules move quicker relative to larger molecules from the cathode to the

anode in the electrophoretic chamber. Boc-protected amino acids (tert-butoxycarbonyl protecting group) modified lipids and cationic liposomes were efficiently prepared to complex with DNA to make them useful for effective DNA delivery (13). The interaction between the phospholipid and the DNA cause delay in migration of gel plate while naked DNA travels faster on gel plate, providing a direct idea of the complexity of the sum relative to the amount of free DNA template.

3.7.1 Preparation of TAE buffer (Tris/Acetate/EDTA buffer, pH 8.4)

For 50X TAE buffer: In 70 mL of double distilled water, 24.2 g Tris free base and 1.861 g Disodium EDTA were dissolved. 5.71 mL of acetic acid was added and the solution was balanced using double distilled water to a 100 mL level. The buffer was kept in the refrigerator at 2-8 °C until further dilution.

For 1X TAE buffer: The 1X TAE buffer was prepared with filtered double distilled water by diluting the 50X TAE buffer up to 50 times. The final buffer would have a pH of ~8.6. The final composition of the 1X TAE buffer is 40 mM Tris, 20 mM Acetate and 1 mM EDTA after dilution.

3.7.2 1.2% Agarose gel

By dispersing agarose powder (1.2 g) into 100 mL of 1X TAE buffer, agarose gel was prepared. To completely dissolve agarose powder in buffer, the scattered agarose was heated at 90 °C with occasional shaking for few minutes. Agarose solution was left at room temperature to bring a consistency that can be quickly poured. After pouring up to 4-6 mm thick slab, poured gel enclosed in the casting tray. To build reservoirs for loading pDNA samples, a comb was inserted in the gel. The gel was allowed to solidify completely at room temperature for 30-40 minutes. Without distorting the wells, combs were removed properly.

3.7.3 EtBr stock solution (10 mg/mL)

The stock solution of Ethidium Bromide (EtBr) was prepared by dissolving EtBr concentrate powder in double-distilled water. As an intercalating/staining dye (EtBr) that intercalates into the main DNA groove and fluoresces in UV absorbance maxima at 300 and 360 nm, and an emission maximum at 590 nm. EtBr fluorescence increases 25 times when DNA is present.

3.7.4 Tank buffer:

The tank buffer was primed by applying the EtBr stock solution with final concentration of 0.5 μg/mL of 1X TAE buffer in tank.

3.7.5 Loading buffer:

For loading pDNA samples, a 6X loading buffer was used at 2 μL per well. The gel filling buffer used bromphenol blue as a monitoring dye to watch the electrophoresis and sucrose flow to maximize the sample density such that the sample sinks to the bottom of each well.

3.7.6 Method of analysis.

The tank buffer was loaded with gel electrophoresis tanks (GeNei Mini Sub System, Merck-Millipore-GeNei Techware, Banglore, India) and the electrodes were mounted in the tank. Electrodes were connected to the voltage supplier (GeNei Electrophoresis Power Supplies, Merck-Millipore-GeNei Techware, Banglore, India). In the tank buffer, the gel was immersed 2-3 mm below the buffer depth and 1.2 % agarose gel in the gel tray was mounted with gel-end having wells towards to the negative electrode and other ends of gel towards the positive electrode. 3μL of 6X loading dye was combined with each DNA sample and then placed in to the wells of gel. Depending on the distance between the electrodes, electrophoresis was carried out at 5 V/cm for 45 min to 60 min. The gel was extracted and the pDNA in the agarose gel was visualized using the GelDocTM XR+ Imaging System under UV light at 254 nm (Bio-Rad, USA). Gel images were taken on ImageLab Software (Version 4.0 Build 16, Bio-Rad Laboratories, USA). For quantification, Gel images were processed by ImageJ software ((ImageJ Ver. 1.49, NIH, USA) and relative density compared to 200ng of pDNA standard was estimated.

3.8 RESULTS AND DISCUSSION

3.8.1 Estimation of Docetaxel by UV-VIS spectrophotometry

3.8.1.1 Determination of λ_{max}

The maximum absorbance corresponding wavelength (λ_{max}) was determined to be 229 nm (Figure 3-1). Obtained λ_{max} was further utilized, to receive a calibration plot of Docetaxel.

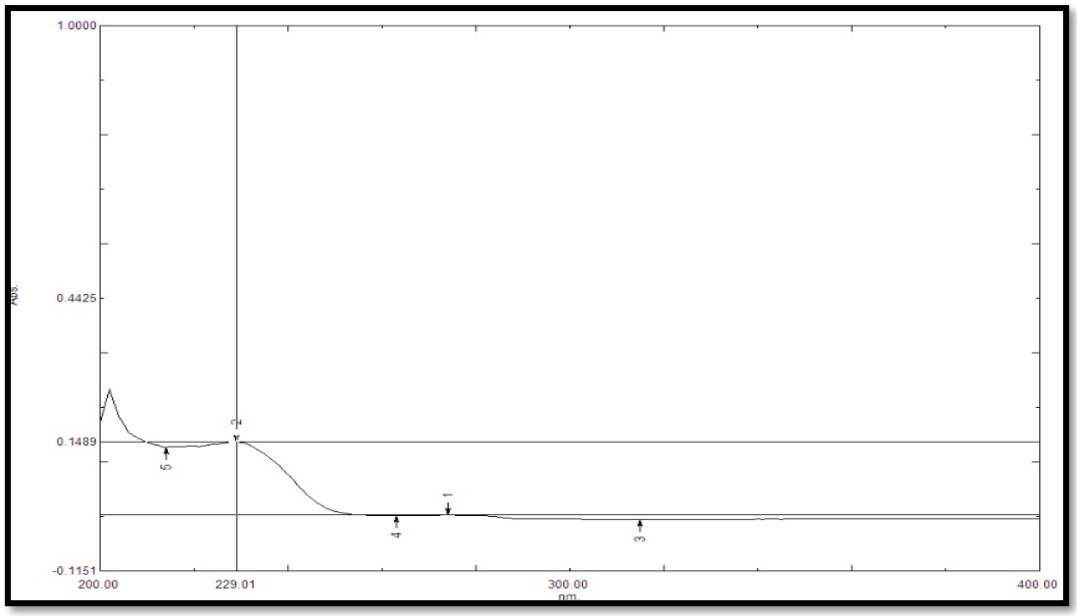

Figure 3-1 Docetaxel λ_{max} determination

3.8.1.2 Calibration plot of Docetaxel in Acetonitrile

As seen in Figure 3-3, the calibration plot of Docetaxel in Acetonitrile was found to be linear in the 10-50 ppm concentration range with a high regression coefficient value (R^2 = 0.9991), showing that Docetaxel solution complies with Beer's law between the 10-50 ppm concentration range.

Table 3-4 Calibration data for estimation of Docetaxel in Acetonitrile

Concentration (ppm)	Absorbance ± SD (n=3)	%RSD
0	0.000±0.0000	0.0000 %
10	0.214± 0.0023	1.6990 %
20	0.402± 0.0035	0.7788 %
30	0.638±0.0027	0.8906 %
40	0.845± 0.0041	0.4964 %
50	1.03± 0.0056	0.7788 %

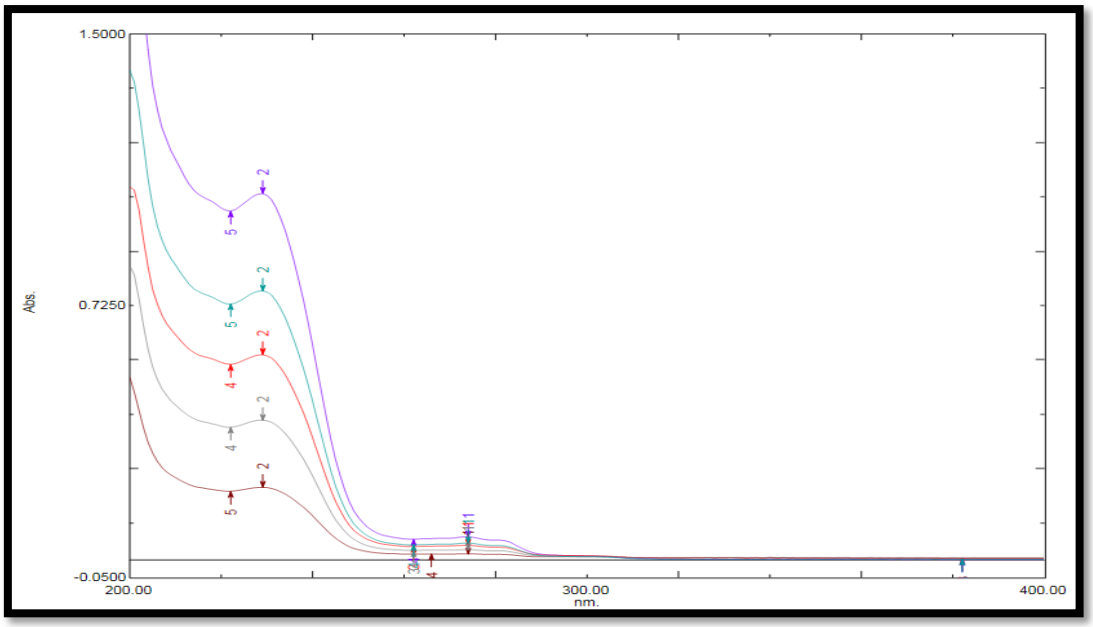

Figure 3-2 Calibration plot of Docetaxel in Acetonitrile by UV-VIS spectrophotometry

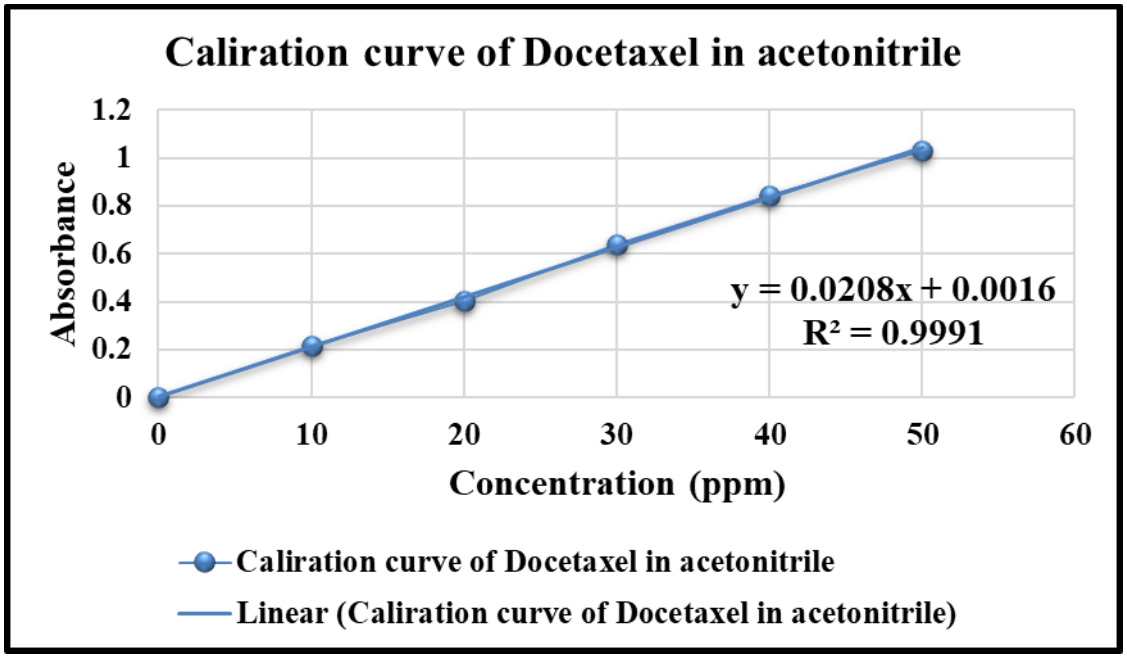

Figure 3-3 Calibration plot of Docetaxel in Acetonitrile

Table 3-5 Parameters from calibration plot of Docetaxel in Acetonitrile

λ max	Solvent	Concentration Range	Regression Equation	Regression Coefficient
229 nm	Acetonitrile	10-50 ppm	y = 0.0208x + 0.0016	0.9991

3.8.1.2.1 Accuracy

The percentage recoveries for lower, intermediate, and higher concentration are given in Table 3-6 for Acetonitrile. Their outcome indicates that the suggested analytical approach will reliably assess and predict any slight shift in the drug concentration in the solution.

Table 3-6 Accuracy of the method in Acetonitrile

Level	Expected concentration (ppm)	Observed Concentration (ppm) ±SD (n=3)	% Drug recovered
80%	24	23.67±0.0031	98.71%
100%	30	29.84±0.0042	99.29%
120%	36	36.11±0.0023	100.23%

3.8.1.2.2 Precision

The precision analysis for intraday and interday was conducted in the same operating condition. The number of RSD values obtained in the precision analysis was less than 2.0 percent, suggesting that these approaches have good accuracy and reproducibility. Percent RSD from Table 3-7 reveals that the method is reliable as such and there is no intraday and interday variability in the system.

Table 3-7 Intraday and Interday precision of Docetaxel in Acetonitrile.

Concentration (ppm)	Observed Concentration(ppm) ±SD (n=3)		%RSD	
	Intraday	Interday	Intraday	Interday
10	10.13±0.0031	9.98±0.0026	0.0325%	0.0202%
30	28.98±0.0026	31.21±0.0034	0.0126%	0.0803%
50	49.73±0.0029	49.99±0.0044	0.0161%	0.0792%

3.8.1.2.3 Limit of detection & Limit of quantification

Table 3-8 LOD and LOQ of Docetaxel in Acetonitrile.

LOD [ppm]	LOQ [ppm]
0.762	2.100

3.8.1.3 Calibration plot of Docetaxel in Phosphate buffer 7.4

The calibration plot of Docetaxel in Phosphate buffer 7.4, as seen in Figure 3-5 and Table 3-9, was found to be linear in the concentration range from 10-50 ppm with a high regression coefficient value (R^2 = 0.9991), demonstrating that docetaxel complies with Beer's rule between 10-50 ppm concentration range.

Table 3-9 Calibration data for estimation of Docetaxel in Phosphate buffer 7.4

Concentration (ppm)	Absorbance ±SD (n=3)	%RSD
0	0.000± 0.0000	0.000 %
10	0.231± 0.0025	1.890 %
20	0.422± 0.0035	0.762 %
30	0.633±0.0041	0.621 %
40	0.835± 0.0026	0.395 %
50	1.125± 0.0013	0.224 %

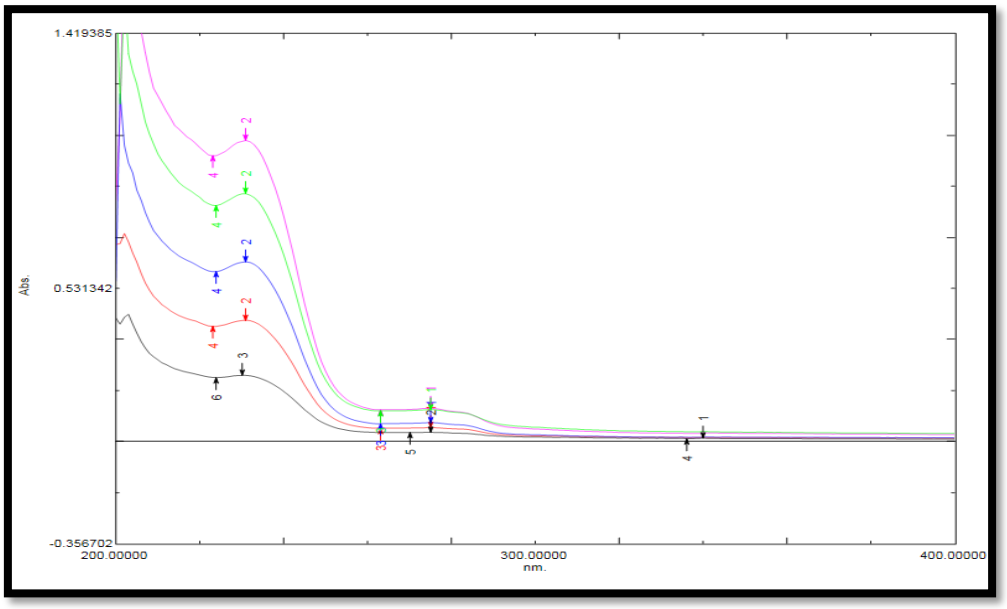

Figure 3-4 Overlay Calibration plot of Docetaxel in Phosphate buffer 7.4by UV spectrophotometry

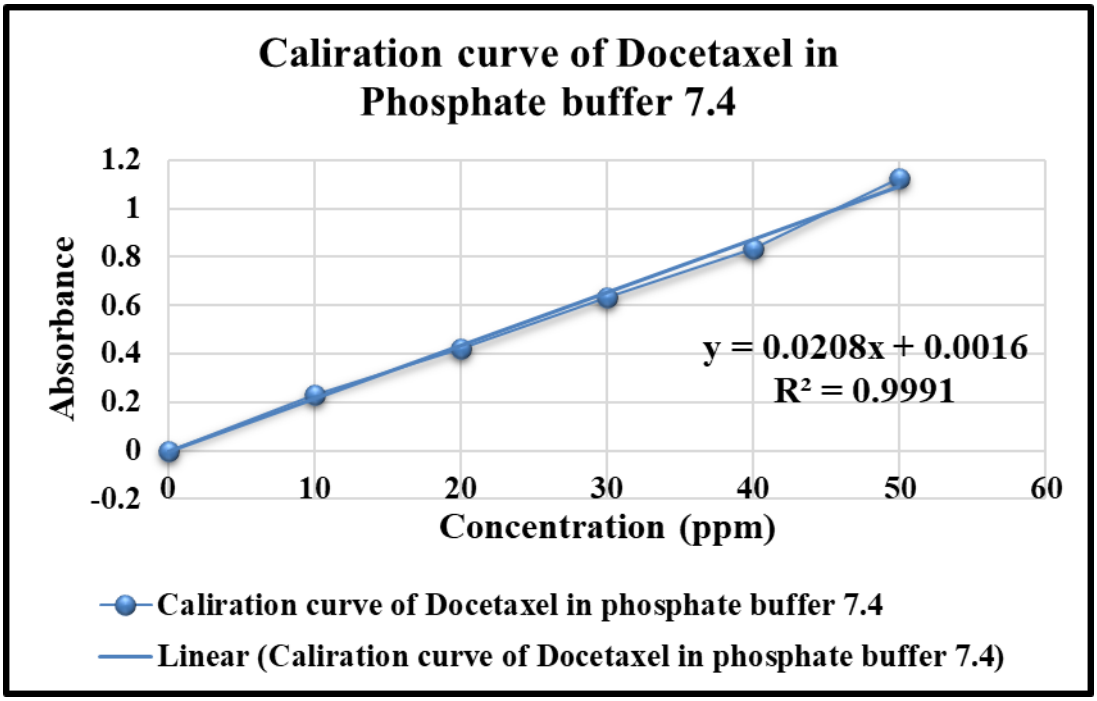

Figure 3-5 Calibration plot of Docetaxel in Phosphate buffer 7.4

Table 3-10 Parameters from calibration plot of Docetaxel in Phosphate buffer 7.4

λ max	Solvent	Concentration Range	Regression Equation	Regression Coefficient
229 nm	Phosphate buffer 7.4	10-50 ppm	y =0.0208x + 0.0016	0.9991

3.8.1.3.1 Accuracy

In Table 3-11 for Phosphate buffer 7.4, the percentage recoveries for lower intermediate, and higher concentrations are given. Their result indicates that any small shift in the drug concentration in the solution may be correctly measured and calculated by the analytical approach suggested.

Table 3-11 Accuracy of the method in Phosphate buffer 7.4

Level	Expected concentration (ppm)	Observed Concentration (ppm) ±SD (n=3)	% Drug recovered
80%	24	23.78±0.0022	98.64%
100%	30	30.12±0.0034	100.40%
120%	36	35.92±0.0038	99.78%

3.8.1.3.2 Precision

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

percent, suggesting that these approaches have good accuracy and reproducibility. Percent RSD from Table 3-12 reveals that the system is reliable as such and the method does not have intraday and interday variability.

Table 3-12 Intraday and Interday precision of Docetaxel in Phosphate buffer 7.4

Concentration (ppm)	Observed Concentration(ppm) ±SD (n=3)		%RSD	
	Intraday	Interday	Intraday	Interday
10	10.24±0.0023	10.52±0.0022	0.0522%	0.0112%
30	29.64±0.0050	30.36±0.0042	0.012%	0.0226%
50	49.54±0.0045	51.12±0.0034	0.0015%	0.0048%

3.8.1.3.3 Limit of detection & Limit of quantification

Table 3-13 LOD and LOQ of Docetaxel in Phosphate buffer 7.4

LOD [ppm]	LOQ [ppm]
0.482	1.589

3.8.1.4 Calibration plot of Docetaxel in Phosphate buffer 6.6

As seen in Figure 3-7, the calibration plot of Docetaxel in Phosphate Buffer 6.6 was observed to be linear in the 10-50 ppm concentration range with a high regression coefficient value (R^2 = 0.9993), demonstrating that docetaxel complies with the law of Beer between the 10-50 ppm concentration range.

Table 3-14 Calibration data for estimation of Docetaxel in Phosphate buffer 6.6

Concentration (ppm)	Absorbance ±SD (n=3)	%RSD
0	0.000± 0.0000	0.000 %
10	0.196± 0.0032	1.235 %
20	0.372± 0.0034	0.542 %
30	0.535±0.0026	0.421 %
40	0.733± 0.0054	0.652 %
50	0.923± 0.0028	0.954 %

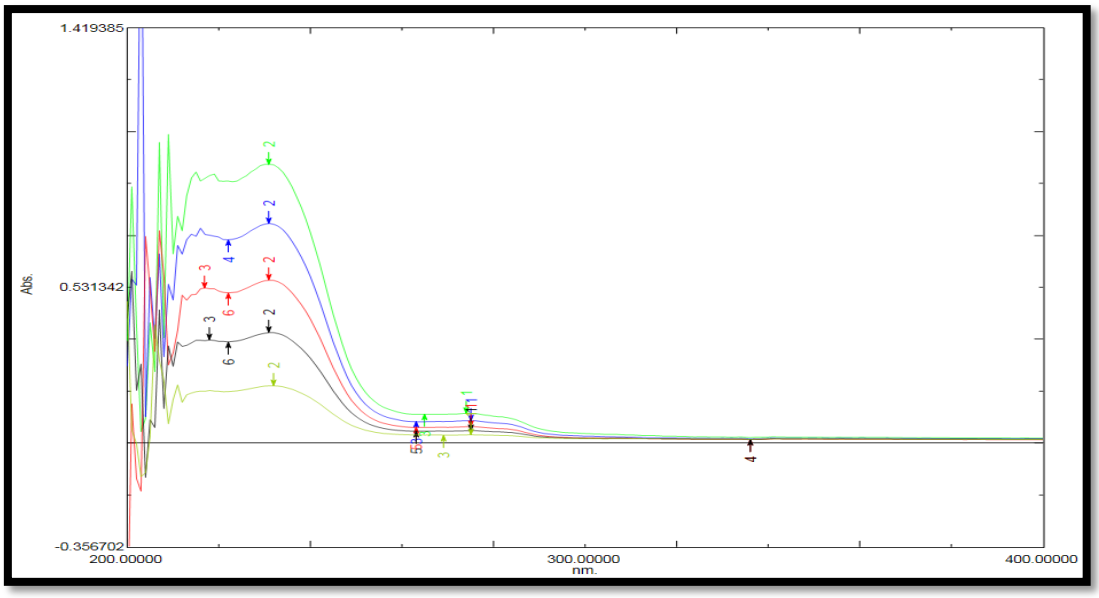

Figure 3-6 Overlay Calibration plot of Docetaxel in Phosphate buffer 6.6 by UV spectrophotometry

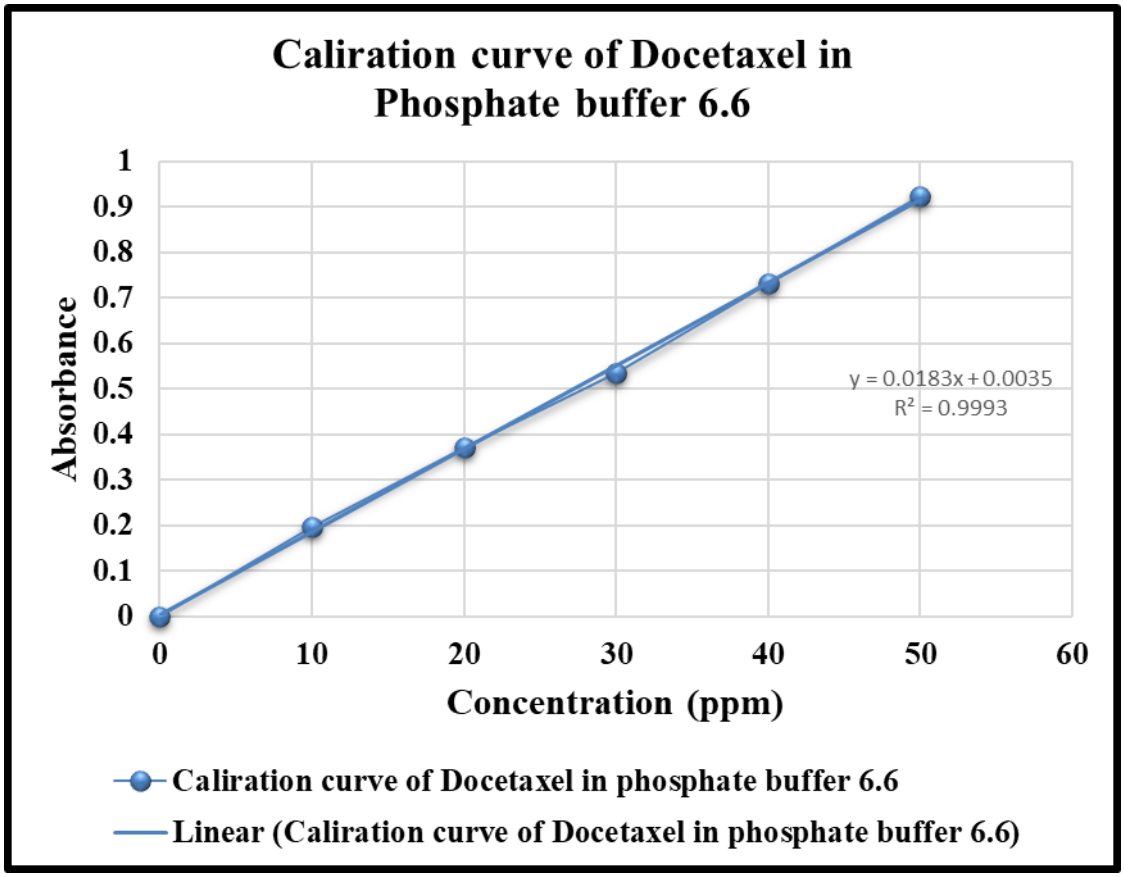

Figure 3-7 Calibration plot of Docetaxel in Phosphate buffer 6.6

Table 3-15 Parameters from calibration plot of Docetaxel in Phosphate buffer 6.6

λ max	Solvent	Concentration Range	Regression Equation	Regression Coefficient
229 nm	Phosphate buffer 6.6	10-50 ppm	y = 0.0183x - 0.0035	0.9993

3.8.1.4.1 Accuracy

In Table 3-16, the percent recoveries for lower intermediate, and higher concentrations are given for Phosphate buffer 6.6. Their result indicates that any small shift in the drug concentration in the solution may be correctly measured and calculated by the analytical approach proposed.

Table 3-16 Accuracy of the method in Phosphate buffer 6.6

Level	Expected concentration (ppm)	Observed Concentration (ppm) ±SD (n=3)	% Drug recovered
80%	24	23.87±0.0068	99.64%
100%	30	29.68±0.0069	99.40%
120%	36	35.82±0.0026	99.56%

3.8.1.4.2 Precision

The precision analysis for intraday and interday was conducted in the same operating condition. The number of RSD values obtained in the precision analysis was less than 2.0 percent, suggesting that these approaches have good accuracy and reproducibility. Percent RSD from Table 3-17 reveals that the system is reliable as such and that the method does not have intraday and interday variability.

Table 3-17 Intraday and Interday precision of Docetaxel in Phosphate buffer 6.6

Concentration (µg/ml)	Observed Concentration (µg/ml) ±SD (n=3)		%RSD	
	Intraday	Interday	Intraday	Interday
10	9.96±0.0053	10.02±0.0012	0.0542%	0.0128%
30	30.02±0.0030	30.68±0.0017	0.0106%	0.0112%
50	49.24±0.0056	49.14±0.0062	0.0124%	0.0123%

3.8.1.4.3 Limit of detection & Limit of quantification

Table 3-18 LOD and LOQ of Docetaxel in Phosphate buffer 6.6

LOD [ppm]	LOQ [ppm]
0.660	2.201

3.8.1.5 Calibration plot of Docetaxel in Acetate buffer 5.5

As seen in Figure 3-9, the Docetaxel calibration plot in Acetate buffer 5.5 was observed to be linear in the 10-50 ppm concentration range with a high regression coefficient value (R2 = 0.9993), which shows that Docetaxel complies with Beer's law between the 10-50 ppm concentration range.

Table 3-19 Calibration data for estimation of Docetaxel in Acetate buffer 5.5

Concentration (ppm)	Absorbance ±SD (n=3)	%RSD
0	0	0
10	0.201± 0.0018	0.598 %
20	0.389± 0.0022	0.314 %
30	0.586±0.0062	1.365 %
40	0.765± 0.0035	0.425 %
50	0.989± 0.0064	0.290 %

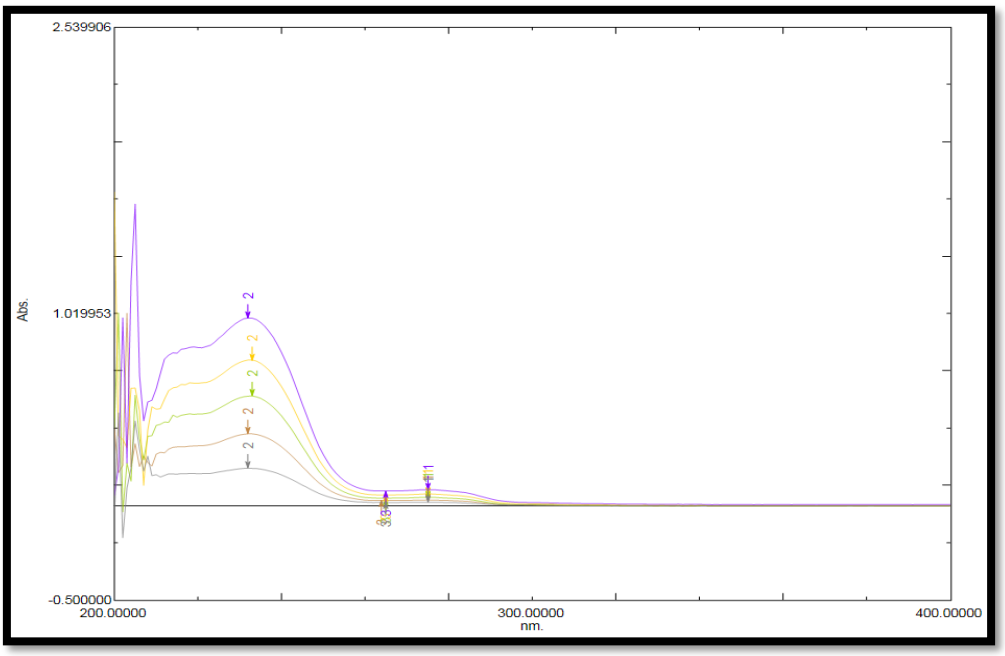

Figure 3-8 Overlay Calibration plot of Docetaxel in Acetate buffer 5.5 by UV spectrophotometry

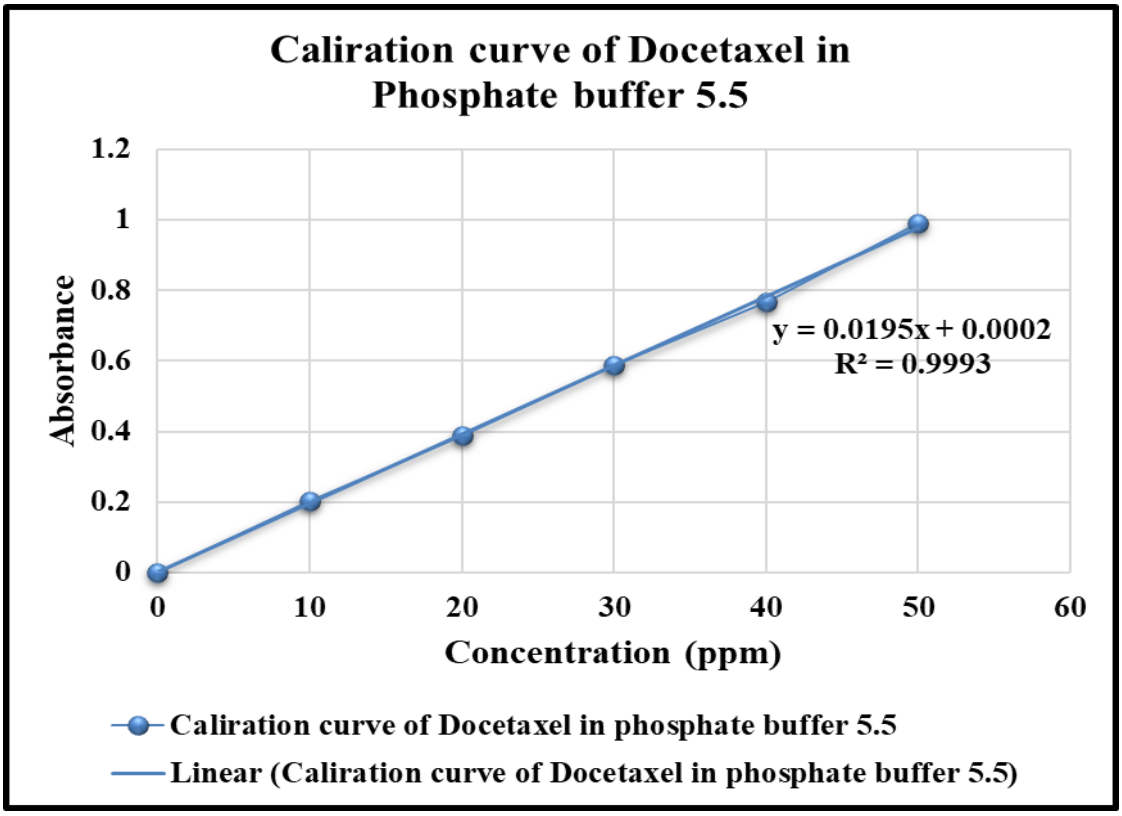

Figure 3-9 Calibration plot of Docetaxel in Acetate buffer 5.5

Table 3-20 Parameters from calibration plot of Docetaxel in Acetate buffer 5.5

λ max	Solvent	Concentration Range	Regression Equation	Regression Coefficient
229 nm	Acetate buffer 5.5	10-50 ppm	y = 0.0195x + 0.0002	0.9993

3.8.1.5.1 Accuracy

In Table 3-21, for Acetate buffer 5.5, the percentage recoveries for lower intermediate, and higher concentrations are given. Their result indicates that any small shift in the drug concentration in the solution may be correctly measured and calculated by the analytical method suggested.

Table 3-21 Accuracy of the method in Acetate buffer 5.5

Level	Expected concentration (ppm)	Observed Concentration (ppm) ±SD (n=3)	% Drug recovered
80%	24	23.87±0.0025	99.62%
100%	30	29.92±0.0036	99.79%
120%	36	36.22±0.0042	100.22%

3.8.1.5.2 Precision

The precision analysis for intraday and interday was conducted in the same operating condition. The number of RSD values obtained in the precision analysis was less than 2.0 percent, suggesting that these approaches have good accuracy and reproducibility. Percent RSD from Table 3-22 reveals that the system is reliable and that the method does not have intraday and interday variations.

Table 3-22 Intraday and Interday precision of Docetaxel in Acetate buffer 5.5

Concentration (ppm)	Observed Concentration (ppm) ±SD (n=3)		%RSD	
	Intraday	Interday	Intraday	Interday
10	9.86±0.0023	10.18±0.0013	0.0559%	0.0120%
30	29.76±0.0035	30.25±0.0016	0.0110%	0.0064%
50	49.65±0.0064	49.34±0.0026	0.0100%	0.0124%

3.8.1.5.3 Limit of detection & Limit of quantification

Table 3-23 LOD and LOQ of Docetaxel in Acetate buffer 5.5

LOD [ppm]	LOQ [ppm]
0.432	1.44

3.8.2 Estimation of Docetaxel using High Performance Liquid Chromatography (HPLC)

3.8.2.1 Calibration plot of Docetaxel by RP-HPLC

A Docetaxel calibration plot in the range of 200-1000 ng/ml was obtained. The regular curve regression equation was found to be y = 545.06x - 9156.9. It was observed that the correlation coefficient for the system was 0.999, suggesting the presence of a linear relationship between peak area and Docetaxel concentration. The retention period was 7.55 minutes.

Table 3-24 RP-HPLC calibration curve values of Docetaxel in Acetonitrile: Water (60: 40)

Concentration (ng/ml)	Area (µV*s)±SD (n=3)
0	0
200	48475 ± 1856.24
400	177456 ± 1162.45
600	295468 ± 1123.54
800	414687 ± 1489.65
1000	537156 ± 1212.28

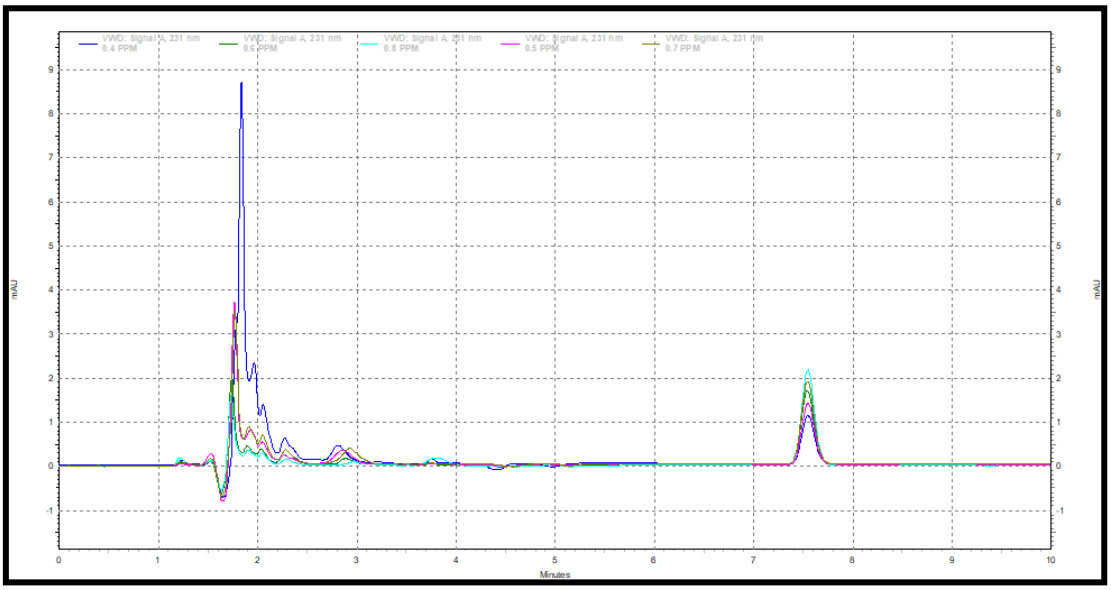

Figure 3-10 Overlay Chromatogram of Docetaxel by RP-HPLC

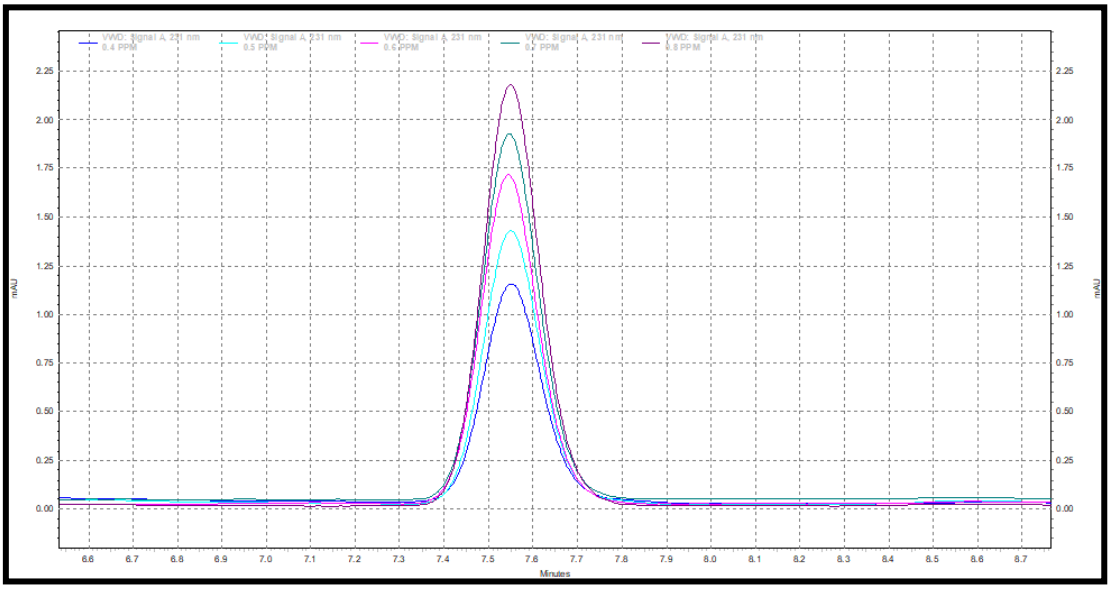

Figure 3-11 Overlay Chromatogram of Docetaxel by RP-HPLC

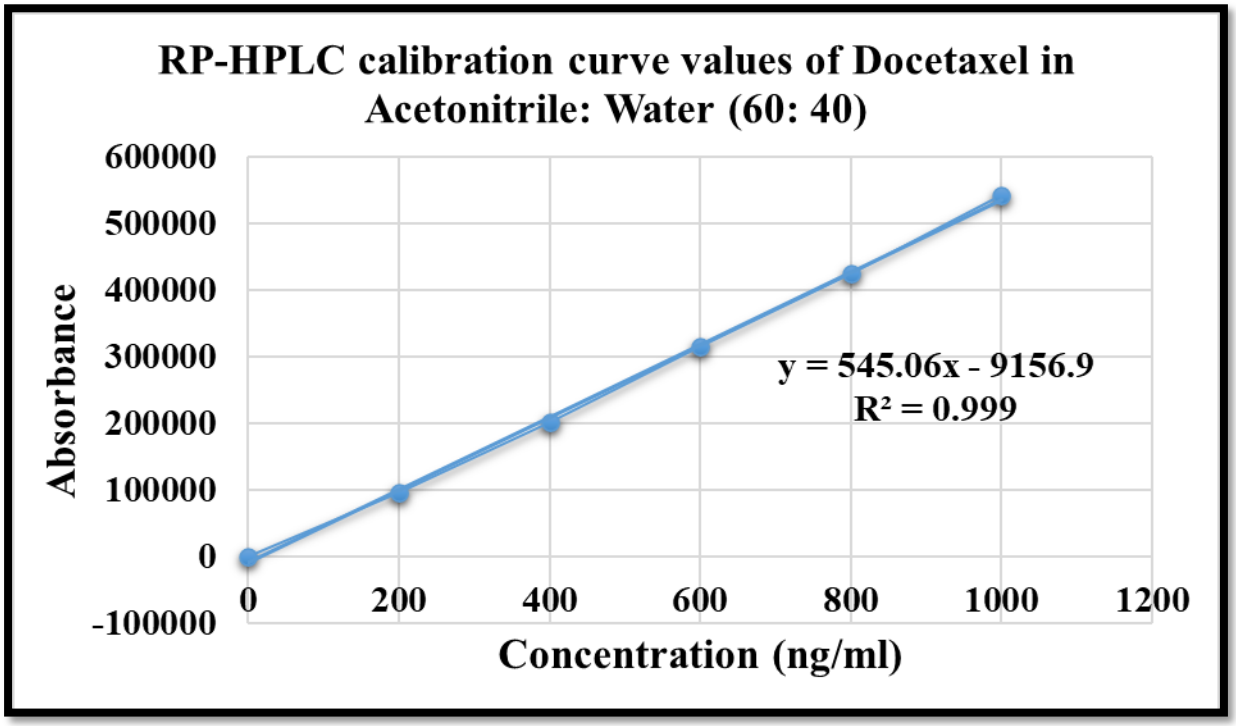

Figure 3-12 Calibration plot of Docetaxel by RP-HPLC

Table 3-25 Parameters from calibration plot of Docetaxel in Acetonitrile: Water (60: 40)

λ max	Solvent	Concentration Range	Regression Equation	Regression Coefficient
231 nm	Acetonitrile: Water (60: 40)	200-1000 ng/ml	y = 545.06x - 9156.9	R^2 = 0.999

3.8.2.1.1 Accuracy

The percentage recoveries for lower, intermediate, and higher concentration are given in Table 3-26 in Acetonitrile: Water as mobile phase using RP-HPLC. Their outcome indicates that the suggested analytical approach will reliably assess and predict any slight shift in the drug concentration in the solution.

Table 3-26 Accuracy of the method in Acetonitrile: Water as mobile phase

Level	Expected concentration (ng/ml)	Observed Concentration (ng/ml)±SD (n=3)	% Drug recovered
80%	160	159.67±0.92	98.79%
100%	200	201.84±0.42	100.92%
120%	240	239.11±0.23	99.62%

3.8.2.1.2 Precision

The precision analysis for intraday and interday was conducted in the same operating condition. The number of RSD values obtained in the precision analysis was less than 2.0 percent, suggesting that these approaches have good accuracy and reproducibility. Percent RSD from Table 3-27 reveals that the method is reliable as such and there is no intraday and interday variability in the system.

Table 3-27 Intraday and Interday precision of Docetaxel in Acetonitrile: water (60:40) using RP-HPLC.

Concentration (ng/ml)	Observed Concentration(ng/ml) ±SD (n=3)		%RSD	
	Intraday	Interday	Intraday	Interday
100	100.3±0.12	99.8±0.26	0.119%	0.260%
300	298.8±0.26	301.1±0.34	0.087%	0.112%
500	497.3±0.29	499.9±0.44	0.058%	0.220%

3.8.2.1.3 Limit of detection & Limit of quantification

Table 3-28 LOD and LOQ of Docetaxel in Acetonitrile: Water (60: 40)

LOD [ng/ml]	LOQ [ng/ml]
6.92	21.08

3.8.2.2 Calibration plot of Docetaxel by RP-HPLC in rat plasma

The Docetaxel calibration plot was obtained in the 250-1250 ng/ml range. The regular curve regression equation was found to agree with y =219.6x + 239.24. It was noticed that the correlation coefficient for the system was 0.9994, meaning the presence of a linear relationship between the peak region and the drug concentration. There was a retention time of 7.96 minutes.

Table 3-29 RP-HPLC calibration curve values of Docetaxel in rat plasma

Concentration (ng/ml)	Area (µV*s)±SD (n=3)
0	0
250	53824 ± 1456.27
500	112546 ± 1267.54
750	162542 ± 1869.23
1000	223546 ± 1964.23
1250	272459 ± 2354.68

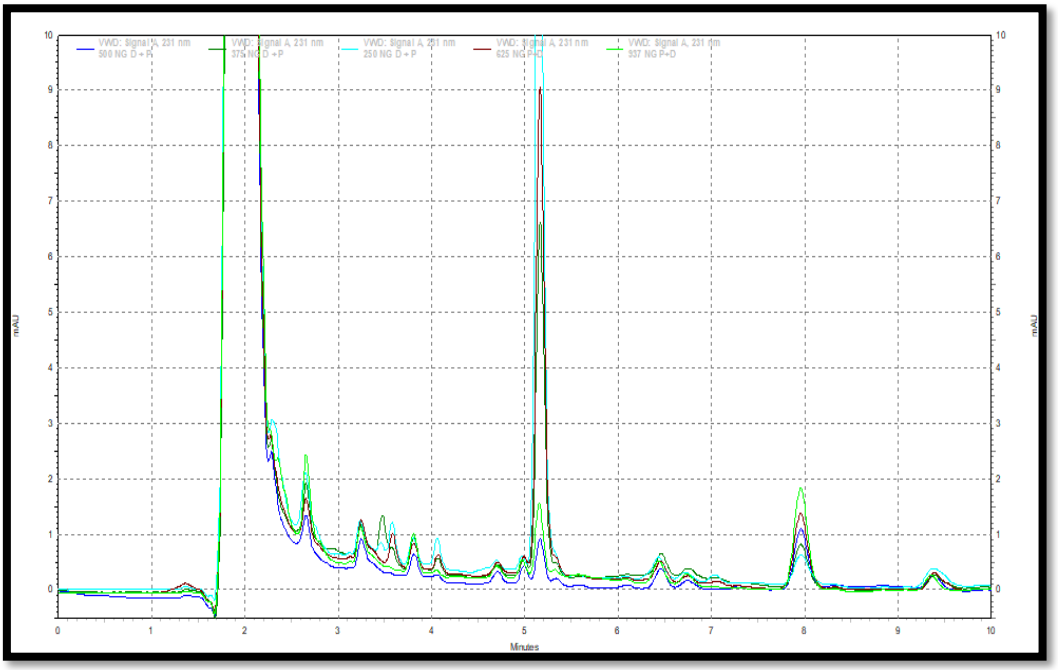

Figure 3-13 Overlay Chromatogram of Docetaxel in rat plasma by RP-HPLC

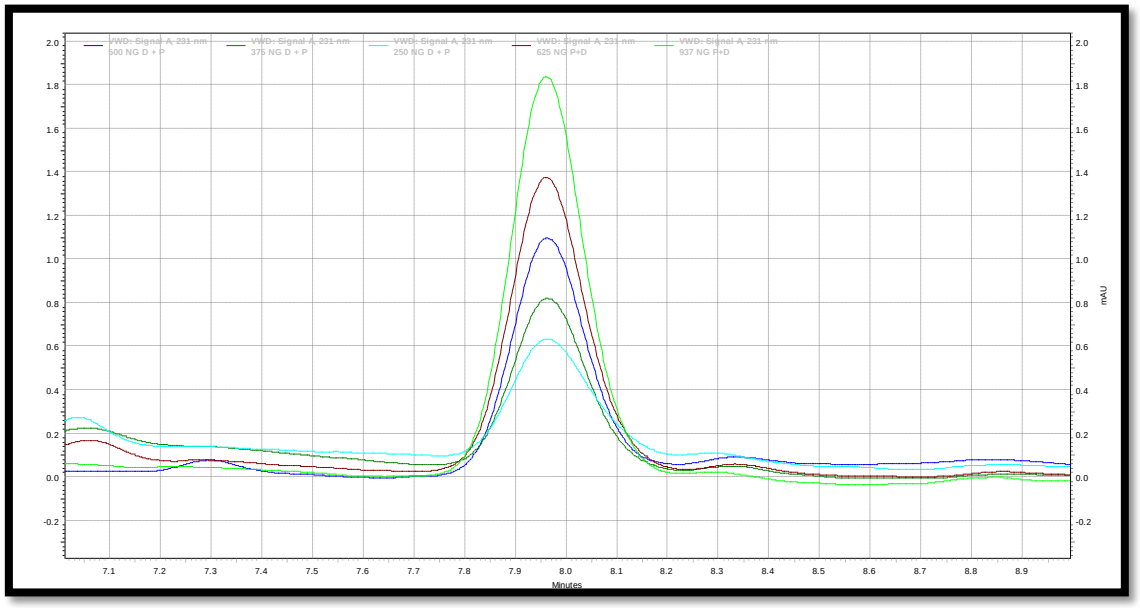

Figure 3-14 Overlay Chromatogram of Docetaxel in rat plasma by RP-HPLC

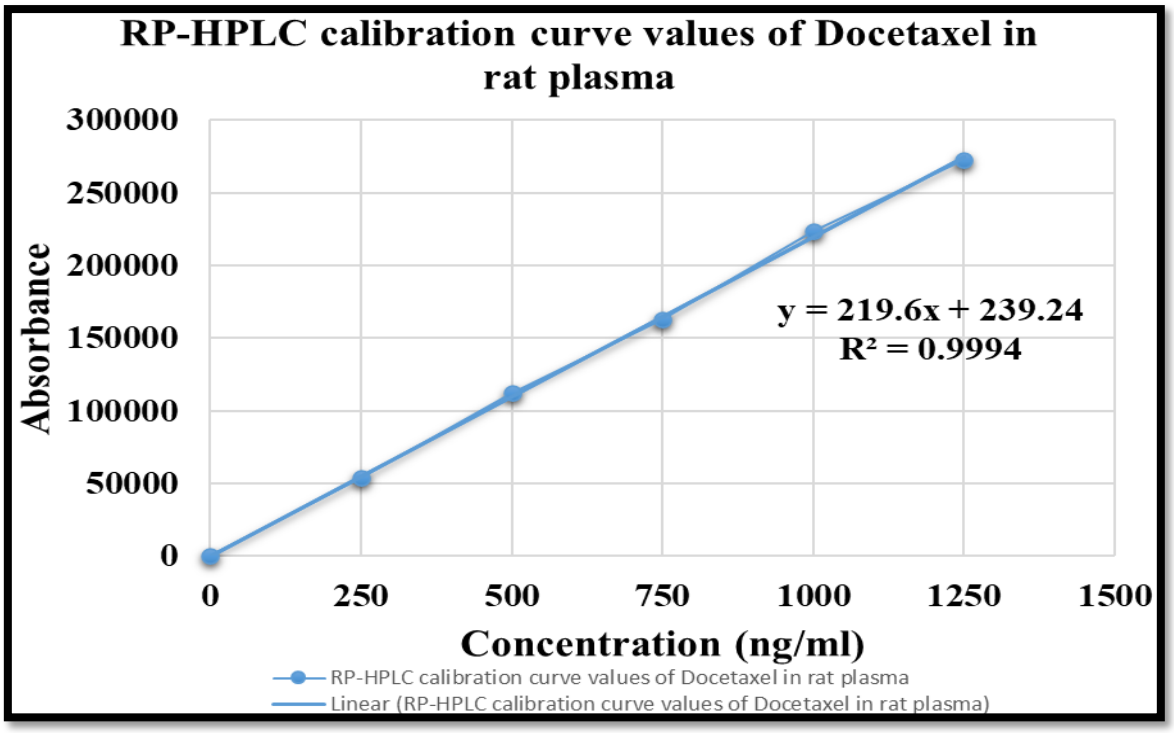

Figure 3-15 Calibration plot of Docetaxel by RP-HPLC in rat plasma

Table 3-30 Parameters from calibration plot of Docetaxel in Rat plasma

λmax	Solvent	Concentration Range	Regression Equation	Regression Coefficient
231 nm	Acetonitrile: Water (60: 40)	250-1250 ng/ml	y =219.6x + 239.24	R^2 = 0.9994

3.8.2.2.1 Accuracy

The percentage recoveries for lower, intermediate, and higher concentration are given in Table 3-31 with rat plasma using RP-HPLC. Their outcome indicates that the suggested analytical approach will reliably assess and predict any slight shift in the drug concentration in the solution.

Table 3-31 Accuracy of the method in rat plasma

Level	Expected concentration (ng/ml)	Observed Concentration (ng/ml)±SD (n=3)	% Drug recovered
80%	200	199.59±0.12	99.79%
100%	250	251.26±0.22	100.50%
120%	300	299.34±0.53	99.78%

3.8.2.2.2 Precision

The precision analysis for intraday and interday was conducted in the same operating condition. The number of RSD values obtained in the precision analysis was less than 2.0 percent, suggesting that these approaches have good accuracy and reproducibility. Percent RSD from Table 3-32 reveals that the method is reliable as such and there is no intraday and interday variability in the system.

Table 3-32 Intraday and Interday precision of Docetaxel in rat plasma using RP-HPLC.

Concentration (ng/ml)	Observed Concentration(ppm) ±SD (n=3)		%RSD	
	Intraday	Interday	Intraday	Interday
100	100.8±0.12	99.6±0.26	0.119%	0.261%
300	299.8±0.26	300.7±0.34	0.086%	0.113%
500	498.9±0.29	500.9±0.44	0.058%	0.087%

3.8.2.2.3 Limit of detection & Limit of quantification

Table 3-33 LOD and LOQ of Docetaxel in Rat plasma

LOD [ng/ml]	LOQ [ng/ml]
20.45	64.28

3.8.2.3 Calibration plot of Docetaxel by RP-HPLC in rat lung homogenate

A Docetaxel calibration plot in the range of 300-1100ng/ml was obtained. The standard curve regression equation was found to be y = 213.5x + 2558.4. It was noticed that the correlation coefficient for the system was 0.9988, suggesting the presence of a linear relationship between absorption and drug concentration. The storage time was 7.86 minutes.

Table 3-34 RP-HPLC calibration curve values of Docetaxel in rat lung homogenate

Concentration (ng/ml)	Area (µV*s)±SD (n=3)
0	0000
300	62543 ± 1542.65
500	112547 ± 1423.54
700	162354 ± 1967.24
900	220541 ± 2239.13
1100	264784 ± 1839.32

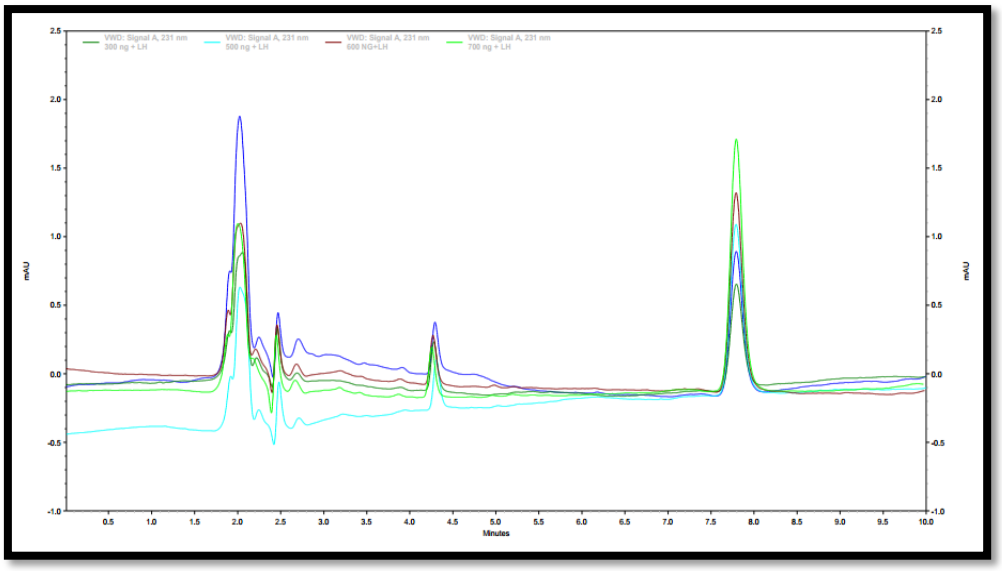

Figure 3-16 Overlay Chromatogram of Docetaxel in rat Lung homogenate by RP-HPLC

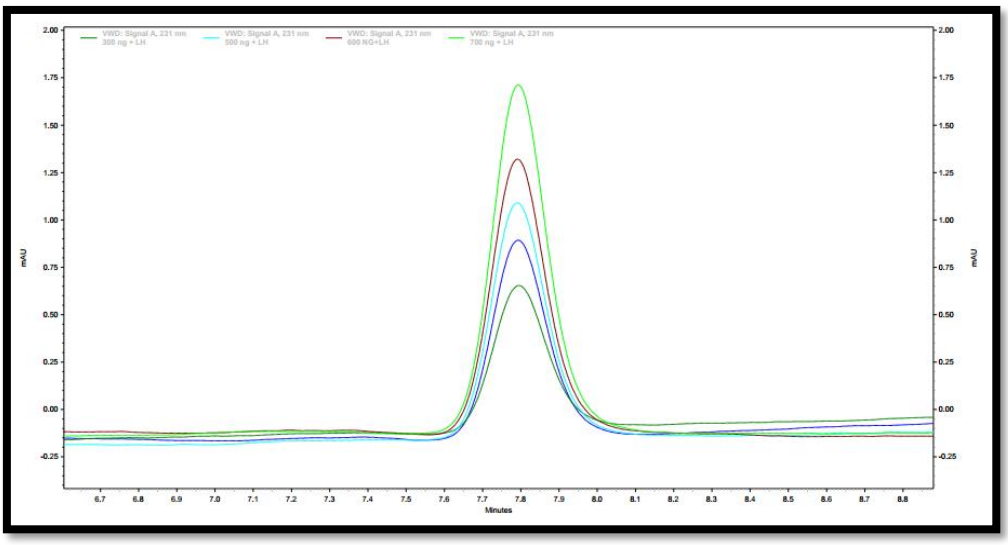

Figure 3-17 Overlay Chromatogram of Docetaxel in rat Lung homogenate by RP-HPLC

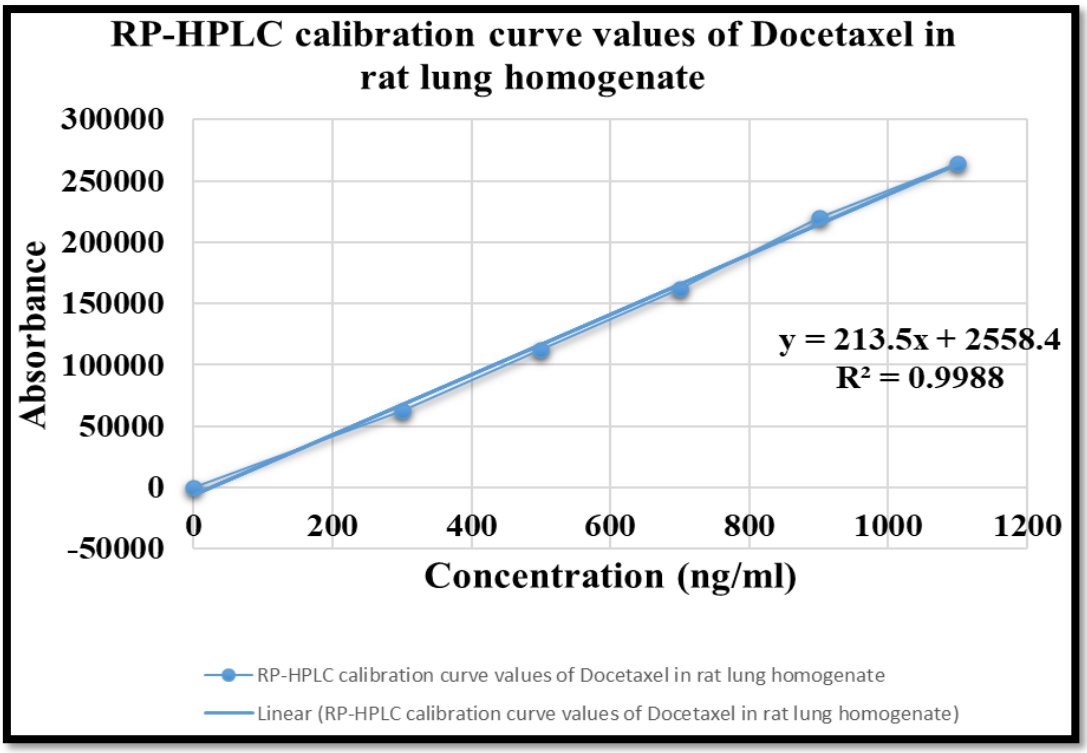

Figure 3-18 Calibration plot of Docetaxel by RP-HPLC in rat Lung homogenate

Table 3-35 Parameters from calibration plot of Docetaxel in rat lung homogenate

λ max	Solvent	Concentration Range	Regression Equation	Regression Coefficient
231 nm	Acetonitrile: Water (60: 40)	300-1100 ng/ml	y = 213.5x + 2558.4	R^2 = 0.9988

3.8.2.3.1 Accuracy

The percentage recoveries for lower, intermediate, and higher concentration are given in Table 3-36 with lung homogenate using RP-HPLC. Their outcome indicates that the suggested analytical approach will reliably assess and predict any slight shift in the drug concentration in the solution.

Table 3-36 Accuracy of the method in rat lung homogenate

Level	Expected concentration (ng/ml)	Observed Concentration (ng/ml)±SD (n=3)	% Drug recovered
80%	240	238.98±0.28	99.57%
100%	300	301.58±0.21	100.52%
120%	360	359.84±0.33	99.95%

3.8.2.3.2 Precision

The precision analysis for intraday and interday was conducted in the same operating condition. The number of RSD values obtained in the precision analysis was less than 2.0 percent, suggesting that these approaches have good accuracy and reproducibility. Percent RSD from Table 3-37 reveals that the method is reliable as such and there is no intraday and interday variability in the system.

Table 3-37 Intraday and Interday precision of Docetaxel in lung homogenate using RP-HPLC.

Concentration (ng/ml)	Observed Concentration(ppm) ±SD (n=3)		%RSD	
	Intraday	Interday	Intraday	Interday
100	99.8±0.11	100.6±0.26	0.110%	0.258%
300	298.9±0.34	299.6±0.37	0.113%	0.123%
500	501.9±0.18	500.1±0.25	0.035%	0.049%

3.8.2.3.3 Limit of detection & Limit of quantification

Table 3-38 LOD and LOQ of Docetaxel in rat lung homogenate

LOD [ng/ml]	LOQ [ng/ml]
30.36	95.28

3.8.3 Estimation of total phospholipid content by Stewart method

3.8.3.1 Calibration plot of Phospholipids

As seen in Figure 3-20, the calibration plot of the total phospholipid blend in chloroform was calculated and plotted with a high regression coefficient value (R^2 = 0.9989) in the concentration range of 20-100 ppm, which shows that the phospholipids follow Beer's rule between 20-100 ppm.

Table 3-39 Calibration data for estimation of total phospholipid content

Concentration (ppm)	Absorbance ±SD (n=3)	%RSD
0	0.000 ± 0.0000	0.000 %
20	0.228 ± 0.0021	0.609 %
40	0.421 ± 0.0012	0.342 %
60	0.638 ±0.0024	1.290 %
80	0.824 ± 0.0023	0.310 %
100	1.012 ± 0.0029	0.180 %

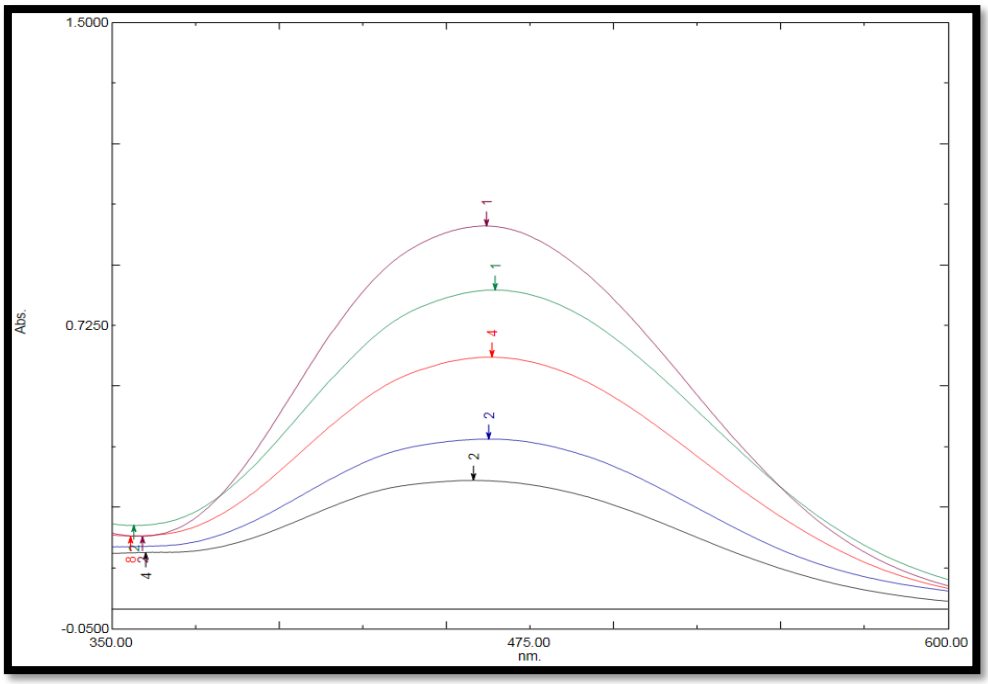

Figure 3-19 Overlay Calibration plot of total phospholipids mixture in chloroform

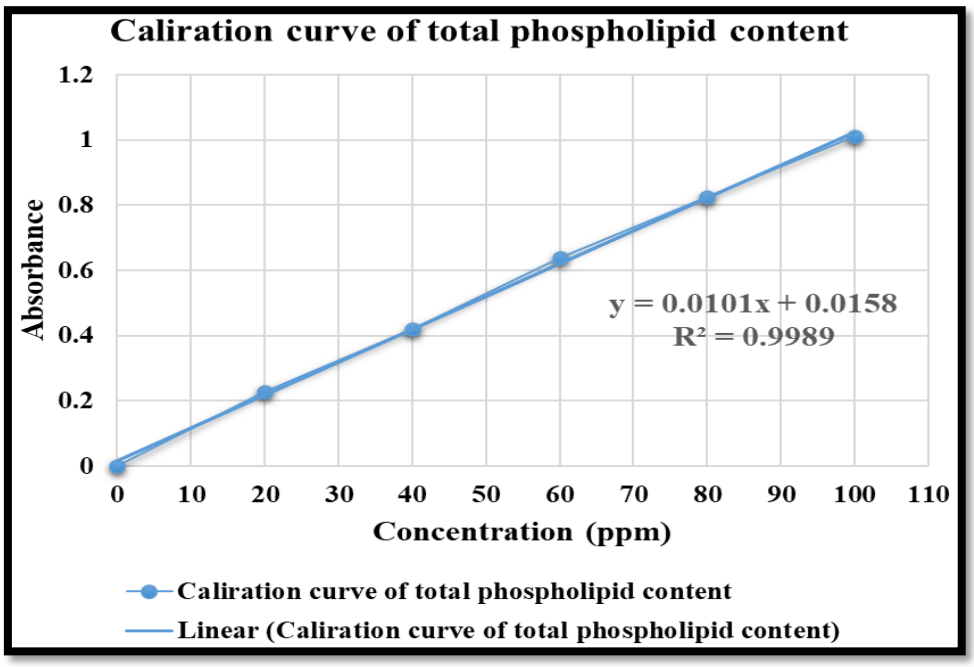

Figure 3-20 Calibration plot of total phospholipids mixture in chloroform

Table 3-40 Parameters from calibration plot of total phospholipids mixture

λ max	Solvent	Concentration Range	Regression Equation	Regression Coefficient
462 nm	Chloroform	20-100 ppm	y = 0.0101x+ 0.0158	0.9989

3.8.3.1.1 Accuracy

The percentage recoveries for lower, intermediate, and higher concentration are given in Table 3-41 with chloroform using UV-VIS spectrophotometry. Their outcome indicates that the suggested analytical approach will reliably assess and predict any slight shift in the drug concentration in the solution.

Table 3-41 Accuracy of the method in chloroform

Level	Expected concentration (ppm)	Observed Concentration (ng/ml) ±SD (n=3)	% Drug recovered
80%	40	39.61±0.14	99.02%
100%	50	51.34±0.26	102.68%
120%	60	59.36±0.49	98.93%

3.8.3.1.2 Precision

The precision analysis for intraday and interday was conducted in the same operating condition. The number of RSD values obtained in the precision analysis was less than 2.0 percent, suggesting that these approaches have good accuracy and reproducibility. Percent RSD from Table 3-42 reveals that the method is reliable as such and there is no intraday and interday variability in the system.

Table 3-42 Intraday and Interday precision of total lipid mixture in chloroform.

Concentration (ng/ml)	Observed Concentration(ppm) ±SD (n=3)		%RSD	
	Intraday	Interday	Intraday	Interday
25	24.8±0.19	25.6±0.21	0.766%	0.820%
50	49.6±0.22	50.7±0.37	0.443%	0.729%
75	74.9±0.31	74.9±0.24	0.413%	0.320%

3.8.3.1.3 Limit of detection & Limit of quantification

Table 3-43 LOD and LOQ of total phospholipids mixture in Chloroform

LOD [ppm]	LOQ [ppm]
0.993	3.313

3.8.4 Analytical Interference study

As seen from the Figure 3-21, there was no peak observed for PEG-PCL in the graph of UV analysis. Even mixture of Docetaxel and formulation excipients shows almost overlaying peaks which suggest negligible interference of the excipients in the analysis of Docetaxel using UV detector. Reading of absorbance maxima was remain constant during

analytical interference study which suggest no analytical inference of excipients in Docetaxel estimation.

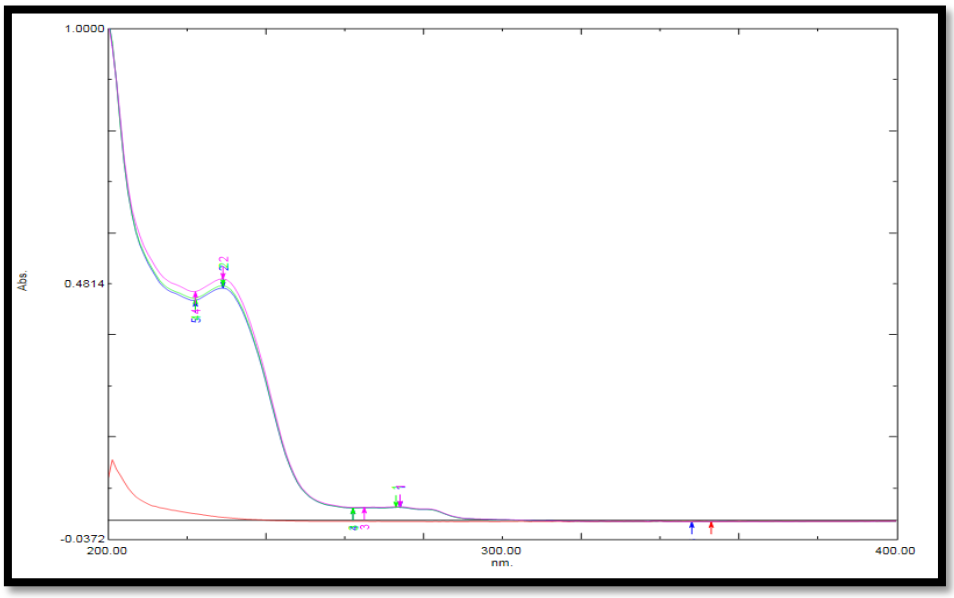

Figure 3-21 Graph of analytical interference study of Docetaxel and Formulation excipients

Table 3-44 Change in absorbance of interference Study

Sr No.	Name of Ingredients	Absorbance± SD (n=3)
1.	Docetaxel	0.489 ±0.0045
2.	PEG-PCL	-
3.	Docetaxel + PEG-PCL	0.482 ±0.0014
4.	Docetaxel+DPPC+DOTAP+DSPE-PEG_{2000}	0.486 ±0.0065
5.	Docetaxel+ DPPC+DOTAP+DSPE-PEG_{2000}+Cryoprotectant	0.479±0.0013

3.8.5 Estimation of shRNA pDNA using UV spectrophotometry

3.8.5.1 Purity of the pDNA

Figure 3-22 and Table 3-45 demonstrate the absorption profile of the isolated pDNA. The ratios of A260 to A280 and A230 were perceived as core parameters to estimate the purity of pDNA. The average ratio of A260/A280 or pure pDNA is between 1.8-2.0, while the ratio of A260/A230 is usually 2.0-2.2. The values of A260/A280 and A260/A230 were 1.92 and 2.10 respectively, indicating high pDNA purity.

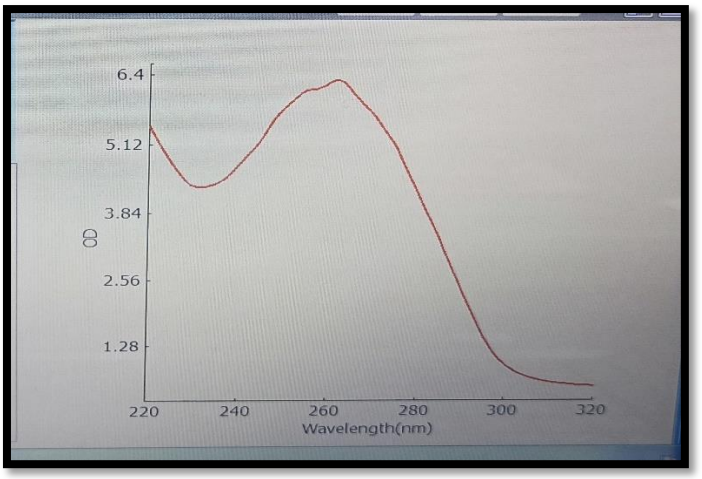

Figure 3-22 UV absorption profile of isolated pDNA

Table 3-45 Absorbance profile of pDNA

Wavelength (nm)	Mean optical density (Absorbance)
230	0.094
260	0.198
280	0.103
320	-0.062
A_{260}/A_{280}	1.92
A_{260}/A_{230}	2.10

3.8.5.2 Calibration curve of pDNA

In order to check the association between specific concentrations and measured concentrations, calibration of pure pDNA was performed at 260 nm wavelength using NanoDrop UV spectrophotometer. The observed responses displayed a linear relationship of an R^2 value being 1 between 2 ng/µL and 200 ng/µL. (Figure 3-23). This was further used to document the linearity spectrum that will be helpful in further studies to assess pDNA concentration.

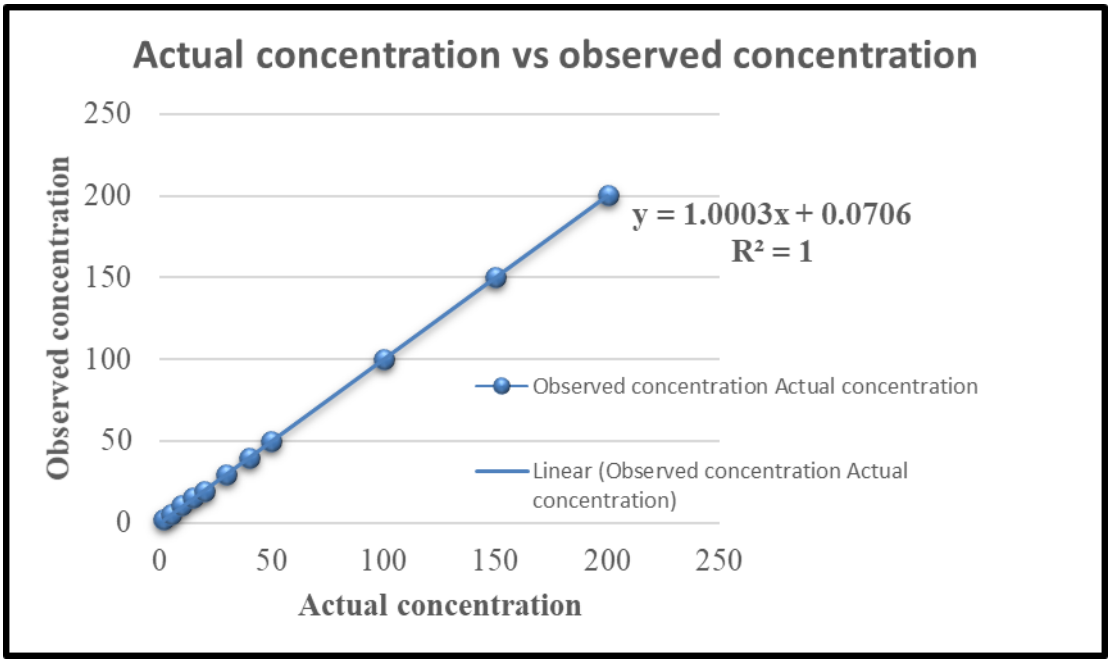

Figure 3-23 Correlation of actual concentration of pDNA vs observed concentration

Absorbance was registered for each solution and the percent recovery was determined according to the following formula.

$$\%\ DNA\ recovered = \frac{Content\ of\ shRNA\ pDNA\ after\ being\ recovered}{Theoritical\ content\ of\ the\ shRNA\ pDNA\ in\ sample\ solution}\ X\ 100$$

3.8.5.3 Accuracy and Precision of the method

The estimation of the reproducibility of the system was calculated from the absorption and measurement of the RSD of each sample at various points in time. The Table 3-46 and Table 3-47 reflect the method's precision, intraday and interday accuracy respectively. It can be observed, the percent recovery was found to be between 98.0% to 100.0% and less than 2% of the percent RSD values complied with the criteria of the ICH guidance (14, 15).

Table 3-46 Accuracy of the UV spectrophotometric method

Actual Concentration (ng/µL)	Observed Concentration (ng/µL)	Standard Deviation (SD)	%Recovery
10	09.92	0.026	99.2
100	99.56	0.148	99.56
200	198.2	0.286	99.1

*Values are represented as mean ± SD, n=3.

Table 3-47 Interday and intraday precision of the UV spectrophotometric method

Actual Concentration (ng/µL)	Observed Concentration ± SD		%Relative Standard Deviation	
	Intraday precision(ng/µL)	Interday Precision(ng/µL)	Intraday precision	Interday Precision
10	9.94±0.024	9.89±0.02	0.23	0.21
100	99.12±0.32	98.97±0.11	0.26	0.14
200	198.86±0.26	199.01±0.26	0.11	0.16

*Values are represented as mean ± SD, n=3.

3.8.6 Determination of detectable range of pDNA for Gel Retardation Assay

As shown in Figure 3-24, pDNA concentrations of ≥50 ng were observable and quantifiable after UV visualization of Gel plate. A faint band of 25 ng shows that concentration of 25 ng and below were not quantifiable with gel electrophoresis method.

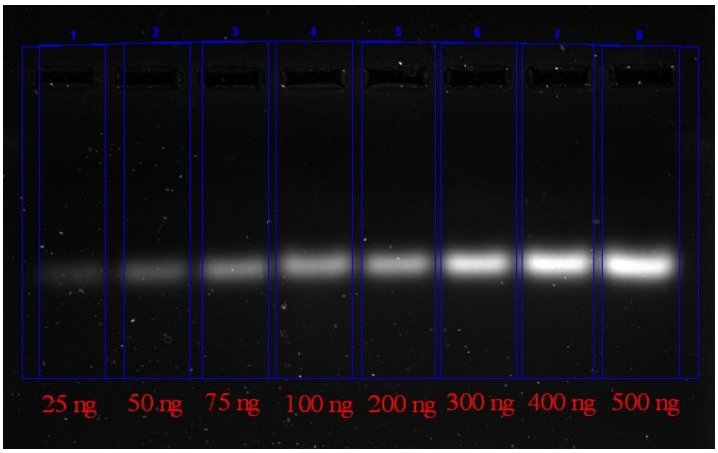

Figure 3-24 Determination of quantifiable range of pDNA
(pDNA/well, lane 1-25 ng, lane 2-50 ng, lane 3-75 ng, lane 4-100 ng, lane 5-200 ng, lane 6-300 ng, lane 7-400 ng, lane 8-500 ng)

3.8.7 Relative Quantification

Band densitometry has been evaluated to see its suitability to serve as a reference band for relative quantification, the 200 ng band was tested by repetitive examination. The gel was filled with concentrations of 200 ng pDNA in 6 wells. Using one band as a reference band, relative band densities were calculated (Figure 3-25 and Table 3-48). Relative band densities were found to be in harmony with 3% RSD, suggesting the method's precision and accuracy. The pDNA recovery percentage was found to be 99.4 ± 1.344 %.

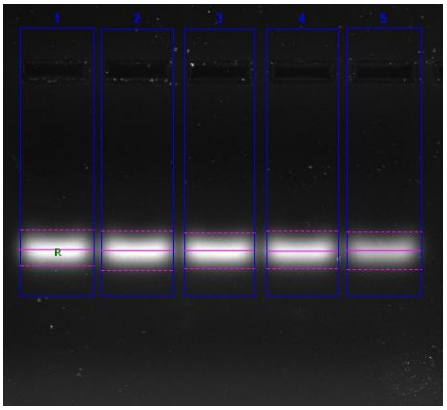

Figure 3-25 Band densities at 200 ng Lane 1 to Lane 6: 200 ng pDNA.

Table 3-48 Relative band density of 200 ng pDNA

Band No	Relative and Density	pDNA recovery (ng)	% recovery
1	**1**	200	100
2	**0.989**	197.8	98.9
3	**0.987**	198.4	99.2
4	**1.008**	201.6	100.8
5	**0.968**	193.6	96.8
Mean	**0.9904**	198.28	99.14
SD	**0.013**	2.688	1.344
%RSD	**1.454**	1.354	1.265

By taking the band density at 200 ng concentration as 1.00 and measuring other band densities relative to this concentration, the calibration curve for the relative quantification of pDNA was constructed. Briefly, pDNA solutions of varying concentrations (10-200 ng) were prepared and as mentioned above, combined with a gel loading buffer and gel electrophoresis. The study was repeated three times, and the measurement error was measured (as a standard deviation). In relation to the 200 ng concentration, Table 3-49 displays band densities obtained at the above pDNA concentrations as well as their relative band densities.

The calibration curve (Figure 3-26) of the observed pDNA concentrations against the concentrations taken has been developed using the values shown in Table 3-49. With a maximum deviation of ~5 % of the present pDNA standard, the technique would be exact. This will allow complex pDNA to be easily calculated with adequate precision and accuracy to make the necessary conclusions. UV spectrophotometric for chosen formulations that are considered to be more reliable and precise approaches would help to validate the decision.

Table 3-49 Relative band densities at different pDNA concentrations

pDNA taken (ng)	Relative band densities		pDNA observed (ng)		%RSD
	Mean	SD	Mean	SD	
10	0.048	0.004	9.6	0.342	5.205
20	0.091	0.012	18.2	2.442	2.446
40	0.204	0.011	40.8	1.752	3.532
50	0.246	0.008	49.2	1.421	3.614
100	0.492	0.007	98.4	0.798	4.577
200	1.000	-	200.0	-	-

*Values are represented as mean±SD, n=3.

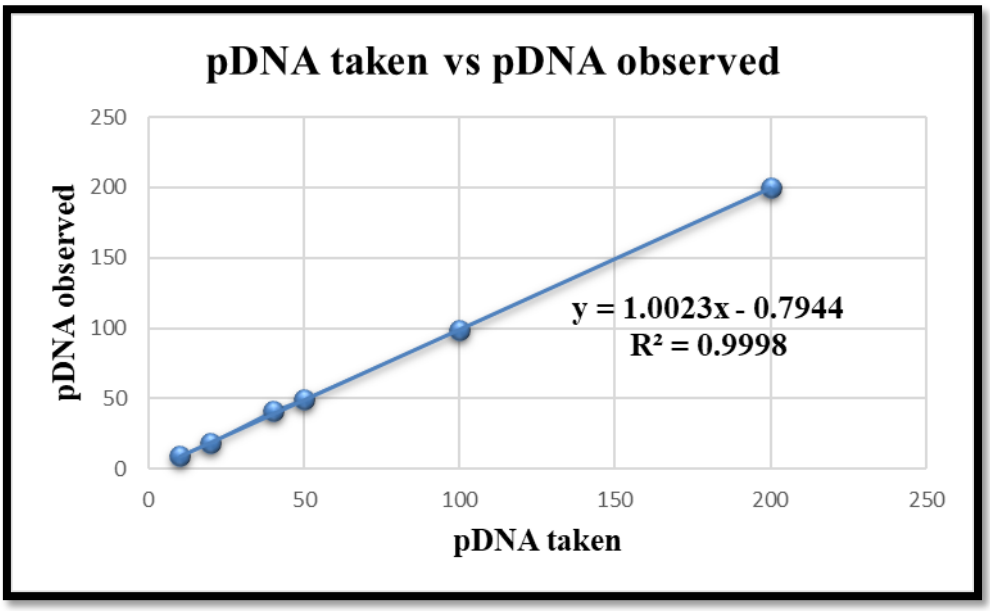

Figure 3-26 Calibration plot of DNA gel retardation.

The percentage of recovery and the percentage of relative standard deviation of the method were found to be 99.14 ± 1.344 % and 1.265 % respectively. The data reflets the method's precision and reproducibility. Therefore, for routine estimations, the proposed analytical approach for quantification of pDNA was found to be accurate.

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