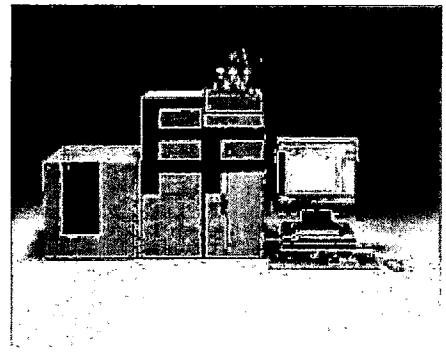
Chapter - 3

Analytical Methods



3.1. Introduction

The analytical methods employed in the preparation and optimization of docetaxel loaded conventional liposomes, PEGylated liposomes and immunoliposomes are discussed below. The method for analysis of process impurities, for estimation of plasma proteins and antibody, for estimation of phospholipid content, for estimation of sulphydryl groups of modified Fab' fragments has been discussed.

3.2. Experimental

Materials

Docetaxel Trihydrate, 7-epidocetaxel, and 10-Oxo-7-epidocetaxel were obtained as gift sample from Fresenius Kabi Oncology Limited, GURGAON, India. HSPC, DDPC, DPPG, DSPE-mPEG$_{2000}$, were kindly gifted by Lipoid GMBH (Ludwigshafen, Germany). Cholesterol Extra Pure, Ellman's Reagent and Bradford reagent were purchased from Sigma Aldrich, Mumbai, India. BCA kit was purchased from Bangalore Genei, Bangalore, India. All other reagents used were of analytical reagent grade and were used without further purification.

Instruments:

UV-visible Spectrophotometer (Shimadzu 1601, Japan), RP-HPLC (Shimadzu LC-20AT, Japan) and Automated ELISA Reader (Molecular Devises, SPECTRUM AX190).

3.3. Methods

3.3.1. Estimation of docetaxel by UV-visible spectrophotometer

Standard stock solution (1000µg/ml) was prepared by dissolving 10 mg of docetaxel in 10ml methanol. The UV-visible spectrophotometric method of analysis was developed by scanning solution of docetaxel (10µg/ml) to determine its λ_{max}. Further dilutions were made and calibration curve was plotted between 2 to18 µg/ml.

3.3.2. Estimation of docetaxel and their impurities by RP-HPLC method

Standard stock solution (1000µg/ml) was prepared by dissolving 10mg of drug in 10ml methanol. Further dilutions were made between 0.1µg/ml to 100µg/ml and calibration curve was developed at the determined λ_{max} of 231nm using RP-HPLC (Kumar et al., 2007; Mallikarjuna Rao et al., 2006).

Standard stock solutions of 7-epidocetaxel (600µg/mL) and 10-oxo-7-epidocetaxel (900µg/mL) were prepared in methanol. Further dilutions were made between 0.15µg/mL to 60µg/mL for 7-epidocetaxel and 0.45µg/mL to 90µg/mL for 10-oxo-7epidocetaxel and calibration curves were developed at the λ_{max} of 231nm using RP-HPLC. The chromatographic condition includes mobile phase: acetonitrile: water (60:40, v/v); Kromasil C18 column (150×4.6mm, 5 micron, Merck); flow rate: 1mL/minute; column temperature: 30 °C; UV-visible detector; λ_{max}: 231nm.

3.3.3. Estimation of total phospholipid content by Stewart method

Principle:

Phospholipids may be measured calorimetrically, without conventional acid digestion and color development procedures, by forming a complex with ammonium ferrothiocyanate (Stewart 1980). The red inorganic compound ammonium ferrothiocyanate is insoluble in chloroform, but forms a complex with phospholipids which is freely soluble in chloroform. When a solution of chloroform containing phospholipid is mixed intimately with ammonium ferrothiocyanate at room temperature, a coloured complex (A_{max}=472nm) is formed which partitions in the chloroform phase.

Preparation of ammonium ferrothiocyanate solution:

Throughout the work a standard solution of ammonium ferrothiocyanate was used. It was prepared by dissolving 27.03g ferric chloride hexahydrate ($FeCl_3 \cdot 6H_20$) and 30.4g ammonium thiocyanate (NH_4SCN) in deionized distilled water and the volume was made up to 1 liter.

Calibration curve of lipid mixture:

All glassware's used in the study were washed with chromic acid solution (5g sodium dichromate was dissolved in 5mL deionised water in a 250mL beaker and to this solution 100mL of concentrated sulphuric acid was slowly added with constant stirring and allowed the mixture to attain room temperature) to avoid the possible contamination from surface active cleansing agents. A stock solution containing 90mg of lipids mixture (HSPC:35mg; DPPC:35mg; DPPG: 8.6mg; DSPE-$mPEG_{2000}$: 11.4mg) was prepared in 100ml chloroform (900µg/mL). The stock solution also contains cholesterol of 13.5mg as one of the component of the final formulation. The 2.5mL of the above stock solution was diluted up to 25mL with chloroform to obtain a concentration of 90µg/mL solution. From the 90µg/mL stock solution 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 and 2mL were pipetted off (9µg to 180µg) and added to 3.0mL ammonium ferrothiocyanate solution in a test tube. Then the enough chloroform was added to the test tubes to make the final chloroform volume 3.0 ml. The biphasic system was then vigorously mixed for 3min. The lower chloroform phase was separated with a syringe and the optical density of the chloroform phase was read at λ_{max} 472nm against chloroform as a blank and the average optical density was plotted against concentration.

3.3.4. Estimation of plasma proteins (BCA protein estimation method)

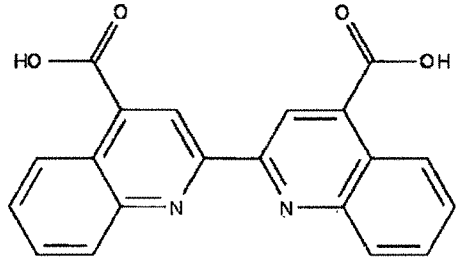
Principle

Protein Assay based on bicinchoninic acid (BCA) is a most sensitive and detergent compatible method for the colorimetric detection and quantitation of total protein. This method is a combination of the well-known biuret reaction, the reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium and the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{1+}) with reagent containing bicinchoninic acid (Smith et al., 1985). The purple coloured reaction product of this assay is formed by the

chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm.

BCA-Protein Reaction:

1. **Protein (peptide bonds) + Cu^{2+} tetradentate → Cu^{1+} complex**
2. **Cu^{1+} + 2 Bicinchoninic Acid → BCA-Cu^{1+} complex (purple coloured, read at 562 nm).**



Bicinchoninic acid [2-(4-carboxyquinolin-2-yl) quinoline-4-carboxylic acid]

Preparation of standard BSA solution

The standard calibration curve of BSA was prepared as per the standard protocol provided along with BCA protein estimation kit (Bangalore Genei, Bangalore).

Preparation of the BCA working reagent (BWR)

To prepare BWR mixed 50 parts of Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartarate in 0.1 M sodium hydroxide) with 1 part of Reagent B (copper sulphate solution). When Reagent A is initially added to Reagent B, turbidity is observed that quickly disappears upon mixing to yield a clear-green BWR. Prepared sufficient volume of BWR based upon the number of tests to be done.

Protocol (Sample to BWR ratio = 1:10):

1. Pipetted 0.2 ml of each standard or unknown sample into appropriately labeled test tubes. The 0.2 ml of the diluent was used as blank.
2. Added 2.0 ml of the BCA working reagent (BWR) to each tube and mixed well.
3. Incubated all the tubes at the selected temperature and time, Enhanced Protocol: 60°C for 30 minutes (Standard Assay range = 6.25-500µg/ml)
3. After incubation, cooled all tubes to room temperature (RT).
4. Measured the absorbance at 562 nm (A-562) of each tube against water as reference.
5. Subtracted the average A-562 reading of the blanks from the A-562 reading for each standard or unknown sample.
6. Prepared a standard curve by plotting the average blank corrected A-562 reading for each BSA standard against its concentration in µg/ml.

3.3.5. Estimation of cell lysate protein by Bradford method

Principle:

The Bradford assay is a protein determination method that involves the binding of Coomassie Brilliant Blue G-250 dye (Figure 3.1) to proteins (Bradford 1976). The dye exists in three forms: cationic (red), neutral (green), and anionic (blue) (Compton and Jones 1985). Under acidic conditions, the dye is predominantly in the doubly protonated red cationic form (λ_{max} = 470nm). However, when the dye binds to protein, it is converted to a stable unprotonated blue form (λ_{max} = 595nm) (Reisner et al., 1975). This blue protein-dye form is detected at 595nm using a spectrophotometer or microplate reader. Work with synthetic polyamino acids indicates that Coomassie Brilliant Blue G-250 dye binds primarily to basic (especially arginine) and aromatic amino acid residues (Compton and Jones 1985). Spector (Spector 1978) found that the extinction coefficient of a dye-albumin complex solution was constant over a 10-fold concentration range. Thus, Beer's law may be applied for accurate quantitation of protein by selecting an appropriate ratio of dye volume to sample concentration. Certain chemical-protein and chemical-dye interactions interfere with the assay. Interference from non-protein compounds is due to their ability to shift the equilibrium levels of the dye among the three coloured species. Known sources of interference, such as some detergents, flavonoids, and basic protein buffers, stabilize the green neutral dye species by direct binding or by shifting the pH (Compton and Jones 1985; Fanger 1987).

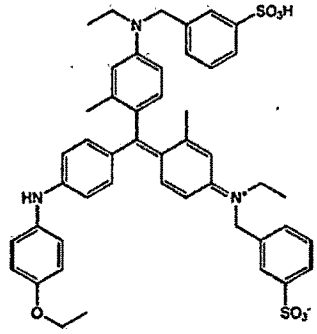


Figure 3.1. Chemical structure of Coomassie Brilliant Blue G-250

Protocol:

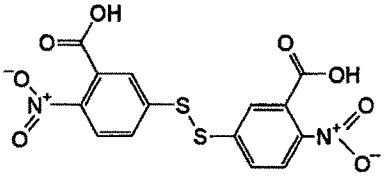
Standard 0.5mg/ml Bovine serum albumin (BSA) solution in milliQ water was prepared. 1.25, 2.5, 3.75 and 5µg of BSA from standard solution (2.5µl, 5µl, 7.5µl and 10µl, respectively) were transferred to 96 well plate in triplicate. Volume was made up to 10µl with milliQ water. 10µl water was kept as blank. 100µl of Bradford's reagent was added to all sample and blank wells and absorbance was taken at 595nm using ELISA reader with SoftMax Pro software. The estimation was done in triplicate. Protein concentration was automatically determined by the software, using the BSA standard.

3.3.6. Estimation of cysteine by Ellman's Assay

Principle:

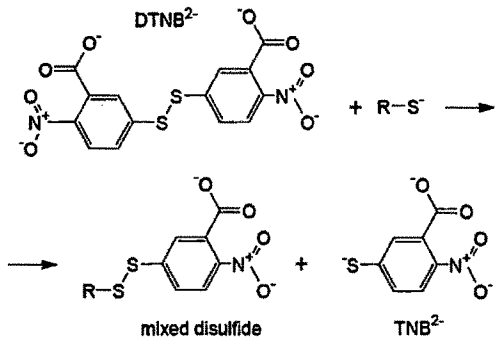
In 1959 Ellman (Ellman 1959) introduced 5,5'-dithio-*bis*-(2-nitrobenzoic acid), also known as DTNB (Figure 3.2a), as a versatile water-soluble compound for quantitating free sulfhydryl groups in solution. A solution of this compound produces a measurable yellow coloured product when it reacts with sulfhydryls. Consequently, Ellman's Reagent is very useful as a sulfhydryl assay reagent because of its specificity for -SH groups at neutral pH, high molar extinction coefficient and short reaction time.

DTNB reacts with a free sulfhydryl group to yield a mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB; Figure 3.2b). The target of DTNB in this reaction is the conjugate base ($R\text{-}S^-$) of a free sulfhydryl group. Therefore, the rate of this reaction is dependent on several factors (Riddles et al., 1983): 1) the reaction pH, 2) the pKa' of the sulfhydryl and 3) steric and electrostatic effects. TNB is the "coloured" species produced in this reaction and has a high molar extinction coefficient in the visible range. The molar extinction coefficient of TNB was originally reported to be 13,600 $M^{-1}cm^{-1}$ at 412nm and pH 8.0. Consequently, this value has been cited frequently in the literature. Later studies have shown, however, that the molar extinction coefficient is more accurately reflected by a value of 14,150 $M^{-1}cm^{-1}$ at 412 nm (Riddles et al., 1983; Riddles et al., 1979). Sulfhydryl groups may be estimated in a sample by comparison to a standard curve composed of known concentrations of a sulfhydryl-containing compound such as cysteine. Alternatively, sulfhydryl groups may be quantified with reference to the extinction coefficient of TNB.



Ellman's Reagent;
5,5'-Dithio-*bis*-(2-nitrobenzoic acid)
$C_{14}H_8N_2O_8S2$
Mol. Wt.: 396.35

Figure 3. 2(a). Structure of Ellman's reagent



3.2(b). Reduction of Ellman's reagent

Material preparation:

Reaction Buffer: 0.1 M sodium phosphate, pH 8.0, containing 1 mM EDTA

Cysteine Hydrochloride Monohydrate (M.W. = 175.6) in reaction buffer

Ellman's Reagent Solution: Dissolved 4mg Ellman's Reagent (5,5'-dithio-bis-(2-nitrobenzoic acid; DTNB) in 1 ml of Reaction Buffer.

Protocol:

1. Prepared a set of cysteine standards by dissolving Cysteine Hydrochloride Monohydrate in reaction buffer (0.0mM to 1.5mM).
2. Prepared a set of test tubes, each containing 50µl of Ellman's Reagent Solution and 2.5 ml of Reaction Buffer.
3. Added 250µl of each standard or unknown to the separate test tubes prepared in step 2.

   Note: For the unknown(s), make dilutions so that the 250µl sample applied to the assay reaction has a sulfhydryl concentration in the working range of the standard curve (0.1-1.0 mM is ideal).
4. Mixed and incubated at room temperature for 15 minutes.
5. Measured the absorbance at 412 nm using UV-visible spectrophotometer.
6. Plotted the values obtained for the standards to generate a standard curve and the experimental sample concentrations were determined from this curve. This curve was used for estimation of sulphydryl groups of Fab' fragments before conjugation to liposomes.

3.4. Results and Discussion

3.4.1. Estimation of docetaxel by UV-visible spectrophotometer

Docetaxel in methanol yields characteristic curve when scanned in UV wavelength range. The scan (Figure 3.3) shows absorption maxima at 231nm. The linearity was observed between absorbance and concentration of docetaxel in the range of 2-18µg/mL (Table 3.1 and Figure 3.4). The regression analysis of the plot using the method of least squares was made to evaluate the intercept (-0.001), slope (0.020) and correlation coefficient (r^2) (0.999). The high value of correlation coefficient (greater than 0.999) of the regression equation and the negligible value of intercept confirm the linearity of calibration plot. This method was used to determine the concentration of docetaxel loaded in the liposome. The estimation of docetaxel loaded in liposomes was carried out against blank (methanol containing other constituents of liposomes at the appropriate level at which they are present in the liposomes). The presence of low concentration lipids showed no significant interference in the estimation of docetaxel present in the liposome. Hence, no interference was observed with our experimental condition.

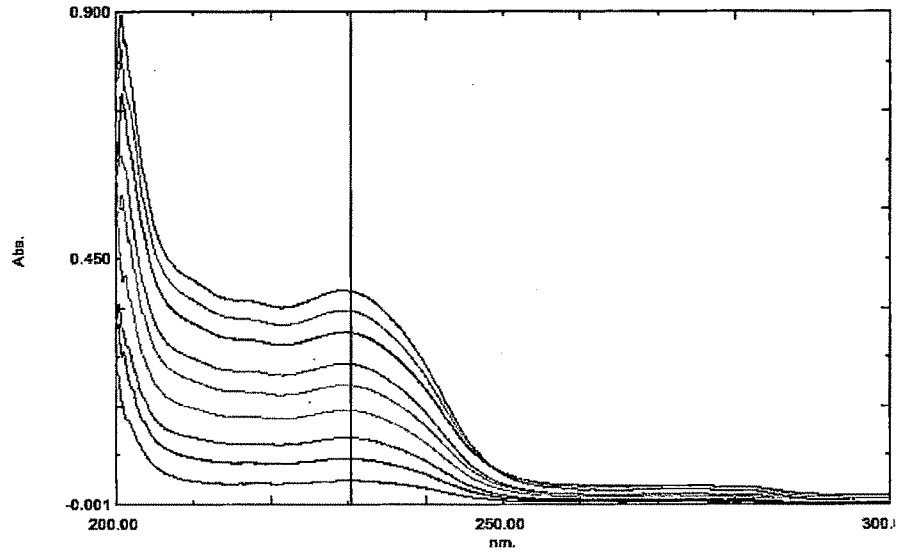


Figure 3.3. UV absorbance scans of docetaxel in methanol

Table 3.1. Calibration curve values of docetaxel in methanol at λ_{max} of 231nm

Concentration (µg/mL)	*Mean Absorbance±SD (231nm)
2	0.044±0.004
4	0.079±0.006
6	0.12±0.007
8	0.159±0.009
10	0.202±0.003
12	0.241±0.005
14	0.287±0.003
16	0.323±0.006
18	0.369±0.005

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

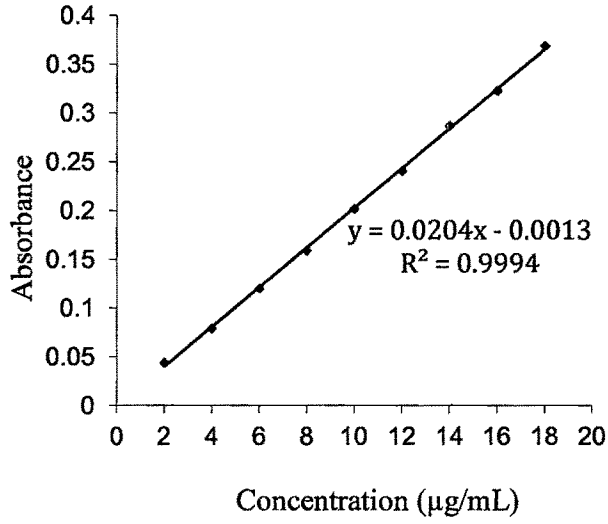


Figure 3.4. Calibration curve of docetaxel in methanol at λ_{max} 231nm

3.4.2. Estimation of docetaxel and its impurities by RP-HPLC method

The degradation of docetaxel drug substance and its injection formulation has been investigated. The majority of impurities were observed in a base degradation study and all five degradation products were characterized. Based on the spectral data, these were characterized as 10-deacetyl baccatin III, 7-epi-10-deacetyl baccatin III, 7-epi-10-oxo-10-deacetyl baccatin III, 7-epidocetaxel and 10-oxo-7-epidocetaxel, respectively. The last two impurities were also detected in the stability study of docetaxel formulation (Kumar et al., 2007; Mallikarjuna Rao et al., 2006). The monitoring of these impurities is important for pharmaceutical drug development and quality control of drug substance and its formulation. As these impurities are unable to distinguish from docetaxel using UV-visible spectrophotometer, we used reported RP-HPLC method to identify and quantify the impurities formed during liposome preparation. Using HPLC method we determined the actual amount docetaxel loaded in liposomes. Also, we determined total amount of impurities generated during liposome preparation and actual amount of impurities loaded in liposomes along with docetaxel using calibration curves generated using standard impurities.

Standard docetaxel, 7-epidocetaxel and 10-oxo-7-epidocetaxel were dissolved in methanol and analysed using RP-HPLC with UV-visible detector (at 231nm) and acetonitrile and water (60:40) as mobile phase. Figures 3.5, 3.7, 3.9 represent the HPLC absorbance peaks of docetaxel, 7-epidocetaxel and 10-oxo-7-epidocetaxel, respectively. Table 3.2, 3.3 and 3.4 represent the calibration curve values of docetaxel, 7-epidocetaxel and 10-oxo-7-epidocetaxel, respectively. Figures 3.6, 3.8 and 3.10 represent the calibration curves of docetaxel, 7-epidocetaxel and 10-oxo-7-epidocetaxel, respectively. Table 3.5 compares the retention time, linearity range, slope, and regression coefficients of docetaxel, 7-epidocetaxel and 10-oxo-7-epidocetaxel.

3.4.2.1. Estimation of docetaxel

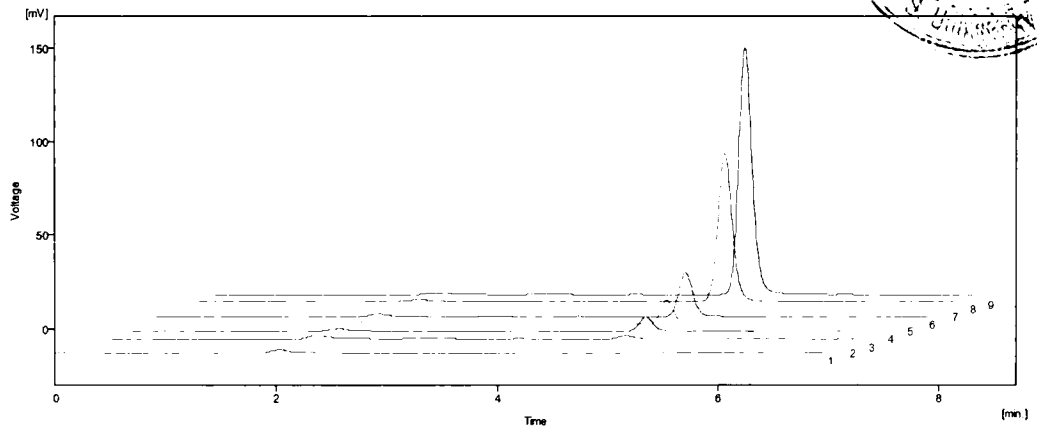


Figure 3.5. RP-HPLC absorbance peaks of docetaxel in methanol at λ_{max} of 231nm

Table 3.2. RP-HPLC calibration curve values of docetaxel in methanol at λ_{max} of 231nm

Concentration (µg/mL)	*Mean Area (mV.s)±SD
0.1	1.886±0.36
0.5	6.558±0.48
1	12.785±1.31
5	65.641±3.54
10	135.809±5.12
15	204.587±8.43
25	309±4.43
50	639.83±6.33
100	1300.008±10.22

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

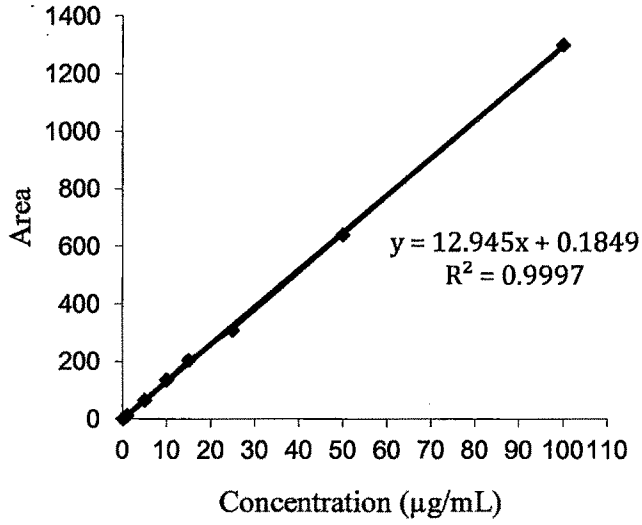


Figure 3.6. RP-HPLC calibration curve of docetaxel in methanol at λ_{max} of 231nm

3.4.2.2. Estimation of 7-epidocetaxel

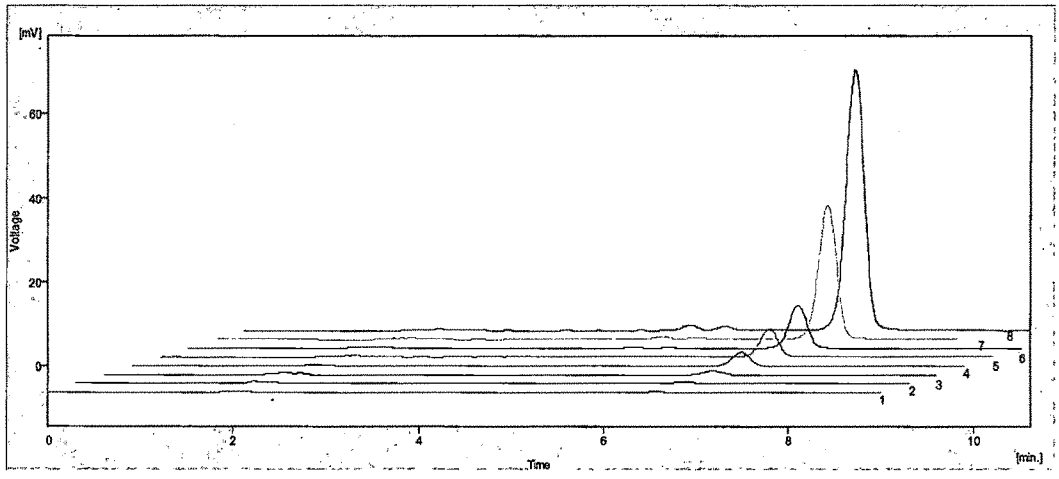


Figure 3.7. RP-HPLC absorbance peaks of 7-epidocetaxel in methanol at λ_{max} of 231nm

Table 3.3. RP-HPLC calibration curve values of 7-epidocetaxel in methanol at λ_{max} of 231nm

Concentration (µg/mL)	*Mean Area(mV.s)±SD
0.15	2.01±0.14
0.3	3.656±0.546
0.9	10.879±1.41
3	38.7±4.25
6	93.115±3.23
9	136.304±6.44
18	263.178±5.54
30	440.433±8.87
60	878.117±11.53

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

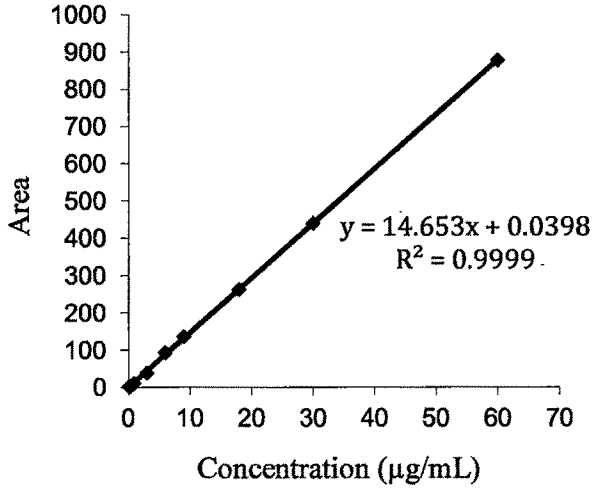


Figure 3.8. RP-HPLC calibration curve of 7-epidocetaxel in methanol at λ_{max} of 231nm

3.4.2.3. Estimation of 10-oxo-7-epidocetaxel

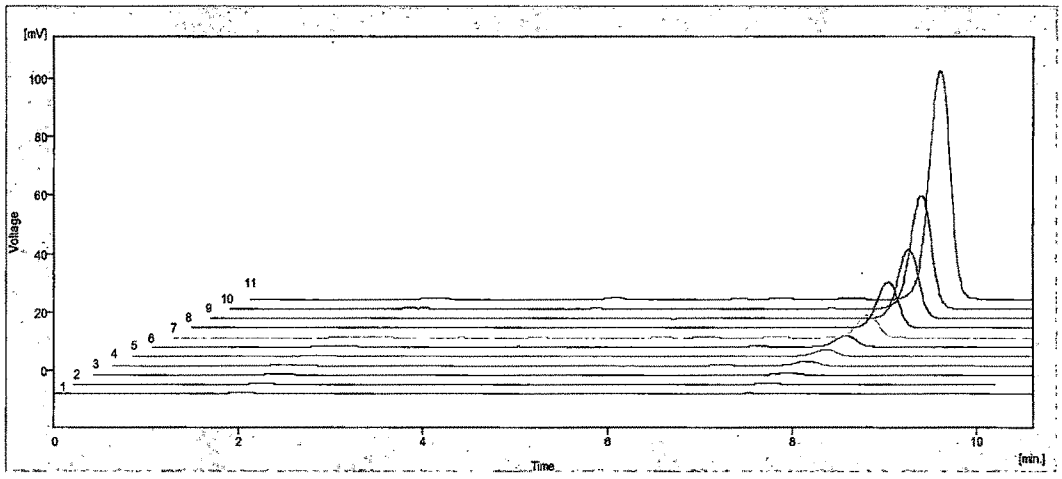


Figure 3.9. RP-HPLC absorbance peaks of 10-oxo-7-epidocetaxel in Methanol at λ_{max} of 231nm

Table 3.4. RP-HPLC calibration curve values of 10-oxo-7-epidocetaxel in methanol at λ_{max} of 231nm

Concentration (µg/mL)	*Mean Area(mV.s)±SD
0.45	6.331±0.82
0.9	11.924±1.21
1.8	26.29±1.29
2.7	38.436±3.32
4.5	63.248±4.23
9	126.265±3.43
18	257.013±6.23
27	385.903±9.19
45	637.21±7.26
90	1270.187±12.91

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

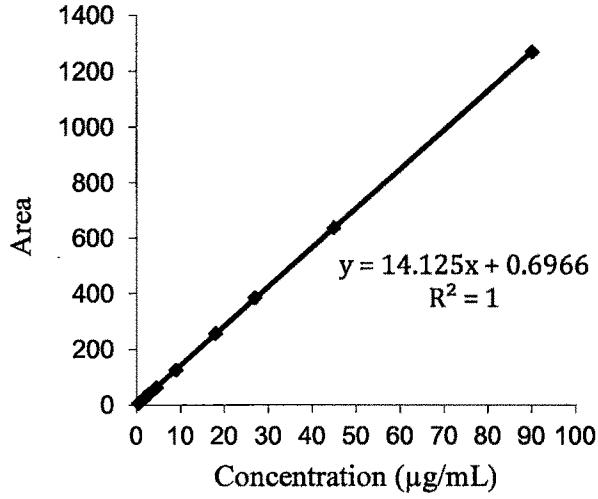


Figure 3.10. RP-HPLC calibration curve of 10-oxo-7-epidocetaxel in methanol at λ_{max} of 231nm

Table 3.5. Comparison of HPLC data of docetaxel and its impurities

Drug	Retention time (min)	Linearity range (µg/mL)	Slope	Regression coefficient (r^2)
Docetaxel	4.820±0.076	0.1-100	12.94	0.999
7-Epidocetaxel	6.587±0.054	0.15-60	14.65	0.999
10-Oxo-7-epidocetaxel	7.573±0.042	0.45-90	14.12	1.0

3.4.3. Estimation of total phospholipid concentration by Stewart colorimetric method

The standard calibration curve, to quantify total phospholipid content of liposome, was developed as per Stewart method (Stewart, 1980) with slight modifications. We prepared the standard stock solution containing phospholipids (HSPC, DPPC, DSPE-$mPEG_{2000}$) and cholesterol at the concentration used for final formulation in chloroform. The serial dilutions were made from the main stock solution and used to develop standard calibration curve. The coloured phospholipid-ammonium ferrothiocyanate complex in chloroform yields characteristic curve when scanned in colorimetric wavelength range. The scan (Figure 3.11) shows absorption maxima at 472nm. The linearity was observed between absorbance and concentration of phospholipid in the range of 9-180µg/mL (Table 3.6 and Figure 3.12). The regression analysis of the plot using the method of least squares was made to evaluate the intercept (+0.01514), slope (0.00406) and correlation coefficient (r^2) (0.99914). The high value of correlation

coefficient (greater than 0.999) of the regression equation and the negligible value of intercept confirm the linearity of calibration plot. This method was used to determine the total phospholipid content of prepared final formulation.

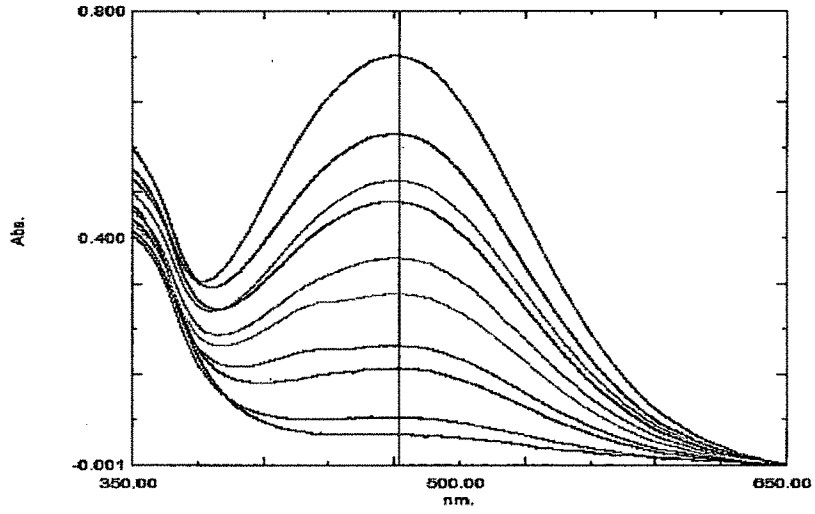


Figure 3.11. UV absorbance scans of phospholipid mixture in chloroform at λ_{max} of 472nm

Table 3.6. Calibration curve values of phospholipid mixture in chloroform at λ_{max} of 472nm

Concentration (µg/mL)	*Mean Absorbance±SD (472nm)
9	0.054±0.006
18	0.092±0.008
36	0.163±0.012
54	0.2235±0.013
72	0.3045±0.015
90	0.378±0.021
108	0.468±0.023
126	0.5205±0.016
144	0.5995±0.028
180	0.747±0.042

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

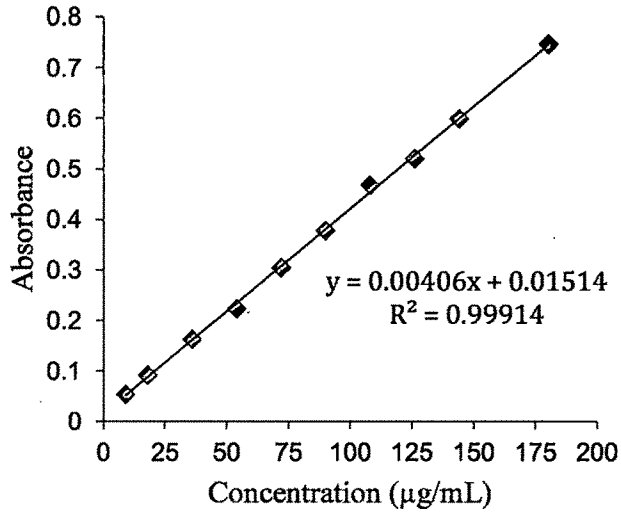


Figure 3.12. Calibration curve of phospholipid mixture in chloroform at λ_{max} of 472nm

3.4.4. Estimation of bovine serum albumin (BSA) by bicinchoninic acid (BCA) method

Estimation of protein was performed using bovine serum albumin as standard protein. Cu^{1+}- BCA complex shows absorbance at 562nm with correlation coefficient of 0.99978 and the slope of the regressed line was 0.0038 (Table 3.7 and Figure 3.13) which indicates that absorbance and concentration of protein are linear. This method of estimation was used for estimation of total serum proteins (opsonins) associated with conventional and PEGylated liposomes after separation using Sepharose CL-4B column.

Table 3.7. Calibration curve values of BSA at 562nm

Concentration (µg/mL)	*Mean Absorbance±SD (562nm)
12.5	0.0555±0.0021
25	0.1095±0.017
50	0.1965±0.011
100	0.393±0.015
150	0.5855±0.022

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

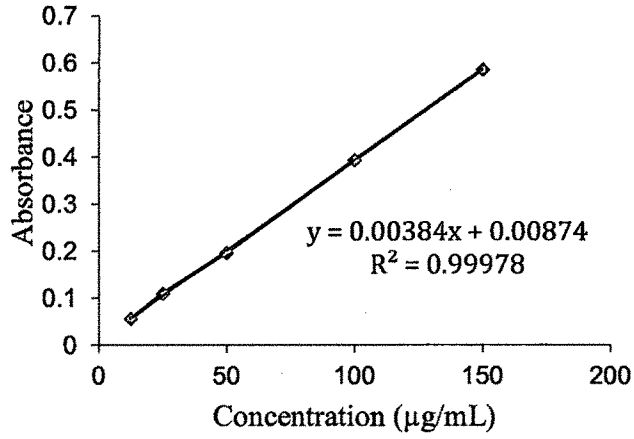


Figure 3.13. Calibration curve of BSA at λ_{max} of 472nm

3.4.5. Estimation of cell lysate protein using Bradford method

Bradford protein estimation method was used to estimate the protein content of cell lysates (B16F10 and A549 lung cell lines) prepared in modified RIPA buffer. The calibration curve was developed using BSA as standard protein. The protein- Coomassie Brilliant Blue G-250 dye complex shows absorbance at 595nm with correlation coefficient of 0.997 and the slope of the regressed line was 0.514 (Table 3.8 and Figure 3.14) which indicates that absorbance and concentration of protein are linear.

Table 3.8. Calibration curve values of BSA at λ_{max} of 595nm

Concentration (µg/mL)	*Mean Absorbance±SD (595nm)
0.125	0.087±0.004
0.25	0.161±0.006
0.375	0.219±0.006
0.5	0.282±0.009

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

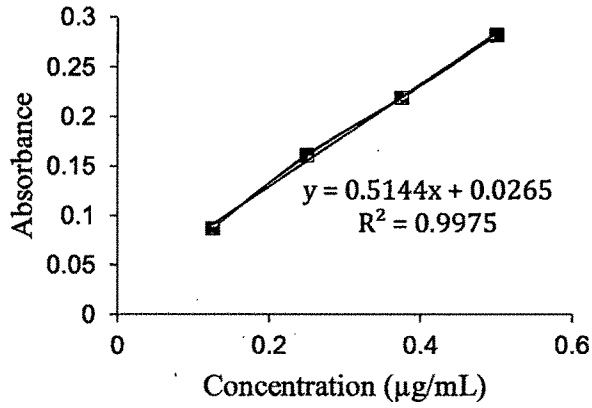


Figure 3.14. Calibration curve of BSA at λ_{max} of 595nm

3.4.6. Estimation of cysteine by Ellman's assay

Estimation of sulphydryl group was done as per the protocol given by thermo scientific with slight modifications. Calibration curve was developed using cysteine hydrochloride monohydrate as standard. The yellow colored product shows absorbance at 412nm with correlation coefficient of 0.999 and the slope of the regressed line was 1.55 (Table 3.9 and Figure 3.15) which indicates that absorbance and concentration of sulphydryl group (cysteine) are linear. This calibration curve (Figure 3.15) was used to identify and quantify the sulphydryl groups generated on Fab' fragments prepared by pepsin digestion of intact antibody followed by reduction of $F(ab')_2$ fragments with dithiothreitol. Also, it was used to confirm the insertion of DSPE-$mPEG_{2000}$-Maleimide on liposomes by back estimating the unreacted cysteine after incubating functional liposomes overnight with excess cysteine.

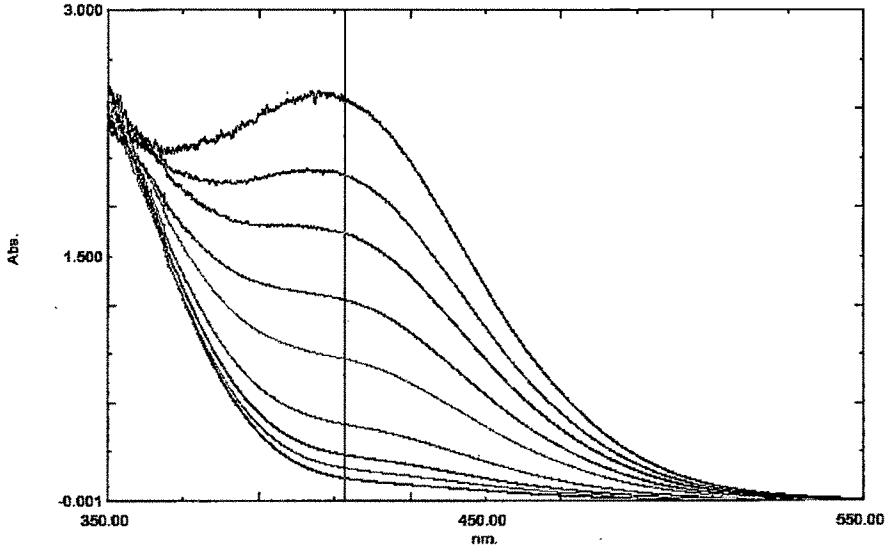


Figure 3.15. UV absorbance scans of cysteine at λ_{max} of 412nm

Table 3.9. Calibration curve values of cysteine at λ_{max} of 412nm

Concentration (mM)	*Mean Absorbance±SD (412nm)
0.03125	0.045±0.001
0.0625	0.107±0.005
0.125	0.188±0.009
0.25	0.376±0.011
0.5	0.777±0.016
0.75	1.139±0.028
1	1.55±0.017
1.25	1.905±0.025
1.5	2.351±0.033

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

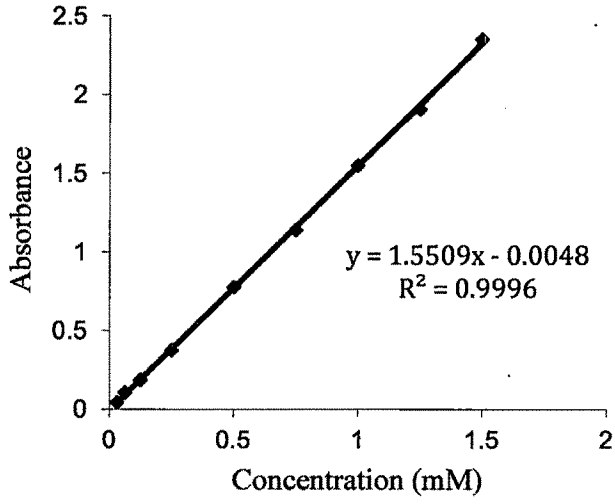


Figure 3.16. Calibration curve of cysteine at λ_{max} of 412nm

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