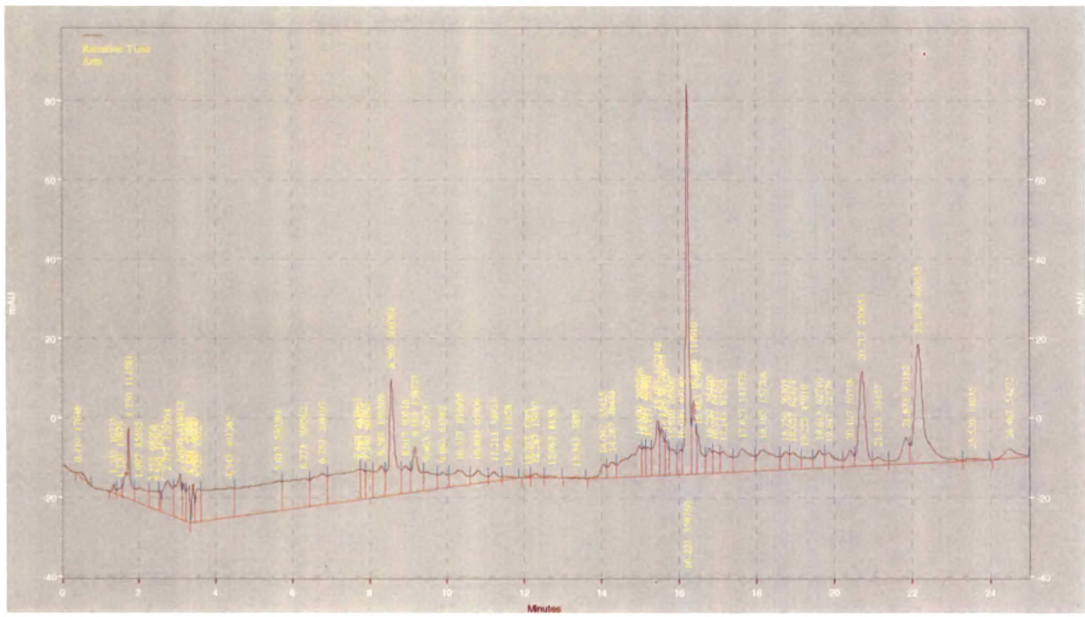
Chapter 3
Analytical Methods



3.1 Introduction

The analytical methods employed in the optimization, preparation and characterization of unconjugated and RGD conjugated nanoparticles of docetaxel are discussed below. The nanoparticles were characterized for particle size, zeta potential, surface morphology, in-vitro drug release, DSC, FTIR, H^1NMR, residual PVA and residual dichloromethane. BT-20 and MDA-MB-231 cells were used to assess the cellular drug levels, in-vitro antiproliferative activity and cellular uptake of the NPs. The stability studies of unconjugated and conjugated NPs were conducted to determine the particle size, zeta potential, % EE, *in- vitro* drug release and the physical changes like caking and discoloration.

3.2 Materials and Equipments

Material	Source
Docetaxel (DC)	Gift samples from Dabur research ltd., Gaziabad, India.
Water (distilled)	Prepared in laboratory by distillation
PLGA (50:50)	Gift samples from gift sample from Boehringer Ingelheim, Germany
Bichinconinic acid (BCA) protein Assay Kit	Banglore Genei, India
6-Coumarin	Gift sample from Neelikon dyes, Mumbai, India
Glacial acetic acid, potassium dihydrogen phosphate, disodium hydrogen phosphate, potassium chloride, potassium hydroxide, sodium chloride, sodium hydroxide.	S.D.Fine chemicals, Mumbai, India
HPLC grade methanol, dichloromethane, acetonitrile	Loba Chemicals, India.
Nuclepore Polycarbonate membrane 2 µm 25mm	Whatman, USA
Polyvinyl alcohol(PVA)	S.D.Fine chemicals, Mumbai, India

Equipments	Source
Calibrated pipettes of 1.0 ml, 5.0 ml and 10.0 ml, volumetric flasks of 10 ml, 25 ml, 50 ml and 100 ml capacity, Funnels (i.d. 5.0 cm), beakers (250 ml) and other requisite glasswares	Schott & Corning (India) Ltd., Mumbai
Analytical balance	Precisa 205A SCS, Switzerland
pH meter	Systronics 335, India
Cyclomixer, three blade stirrer	Remi Scientific Equipments, Mumbai
Cooling Centrifuge	3K 30, Sigma Laboratory centrifuge, Osterode, GmBH.
Lyophilizer, DW1, 0-60E	Heto Drywinner, Denmark
Stability oven	Shree Kailash Industries, Vadodara
UV-Visible Spectrophotometer	Shimadzu UV-1601, Japan
Vacuum Pump F16	Bharat Vacuum pumps, Bangalore
Bath sonicator	INCO, Ambala
Malvern particle size analyser	Malvern Master sizer 2000 SM, U.K.
Scanning electron microscope	JSM-840 SEM, Jeol, Japan
Differential Scanning Calorimeter	Mettler DSC 20, Mettler Toledo, Switzerland
HPLC system	Dionex HPLC with Chromleon 6.5 data processing software

3.3 Chemical analysis

3.3.1 Estimation of Docetaxel

3.3.1.1 Estimation of DC

The drug content was determined using a Dionex HPLC system (Dionex Softron GmbH, Germany). The HPLC system was composed of a pump (P-680, Dionex), a simple 10-µl loop injector (Reodyne 7125) and a UV-visible spectrophotometric detector (UVD 170U, Dionex).The separation was carried out on a 14 cm Kromasil C 18 150-4.6 HPLC column (Merck) having particle size of 5µm. Mobile phase for DC consisted of acetonitrile and water mixture with gradient system. The run time of the assay was 30 min and the retention time of docetaxel was 16.2 min. UV detection wavelength was 227nm and mobile phase flow rate 1 ml/min.

Preparation of standard stock solutions of docetaxel in methanol

50 mg of DC was accurately weighed using single pan electronic balance and transferred to 50 ml volumetric flask. 25 ml of AR grade methanol was accurately measured and transferred to the above volumetric flask, the drug was dissolved properly and then the final volume of the flask was made up to 50 ml with AR grade methanol to produce 1000 µg per ml of DC.

10 ml of the above solution was accurately measured by graduated pipette and transferred to the 100 ml volumetric flask. The final volume was made up to 100 ml with AR grade methanol to prepare stock solution of 100 µg per ml of docetaxel.

Preparation of calibration curve of docetaxel

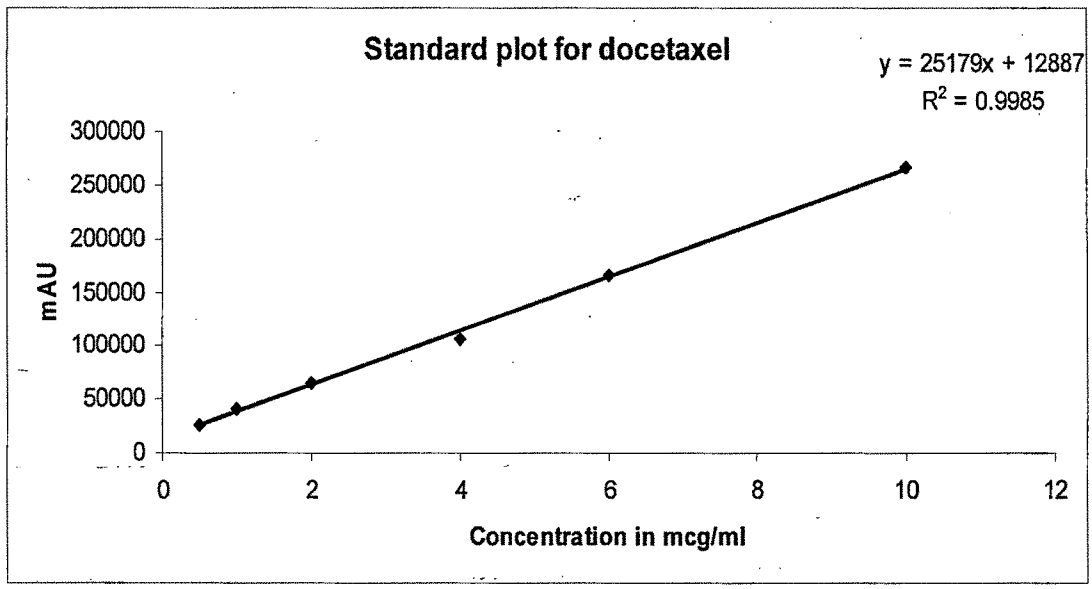
Suitable aliquots of standard stock solution were accurately measured and transferred to the 10 ml of volumetric flasks. The final volume was made up to 10 ml with HPLC grade methanol to give final concentrations of 0.5, 1, 2, 4, 6, 8 and 10 µg/ml and analyzed in above mentioned HPLC system. The above procedure was repeated three times. Raw data was recorded in Table: 3.1 along with standard deviation and plotted graphically in Figure: 3.1.

Table: 3.1. Calibration for Docetaxel

Concentration (µg / ml)	Mean area(mAU)* ± S.D
0	0
0.5	25336 ±21
1	40538 ±28
2	64861 ±35
6	106573 ±42
10	166045 ±31

Regression equation** Y= 25179x + 12887; Correlation coefficient = 0.9985
*Mean of 3 values

Figure: 3.1. Regressed calibration curve for estimation of Docetaxel



3.3.1.2 Estimation of docetaxel in NPs

To determine the amount of DC entrapped in the NPs, 2mg of NPs were added to 2 ml of acetonitrile and subjected to shaking at room temperature for 4hrs complete dissolution of PLGA. The resulting solution was diluted with mobile phase and centrifuged at 5000rpm for 15mins to remove the precipitated components. The supernatant was analyzed in above explained HPLC system.

3.3.1.3 Estimation of docetaxel for in-vitro release

The release studies for docetaxel nanoparticles in phosphate buffer saline pH 7.4 + 0.1%w/v polysorbate-80. Nanoparticles equivalent to 1mg drug were suspended in 10 ml of release medium in a screw capped tubes, which were placed in a horizontal shaker bath maintained at 37°C and shaken at $60min^{-1}$. At specific time intervals following incubation samples were taken out and centrifuged at 25000rpm for 30min. The residue (settled nanoparticles) were collected and dissolved in acetonitrile and analyzed as per the method above. The amount of the drug released was calculated using the following equation:

$$\% \text{ Drug released} = 1 - \frac{\text{Amount of drug in nanoparticles settled}}{\text{Amount of drug initially taken}} \times 100$$

3.4 Estimation of residual PVA

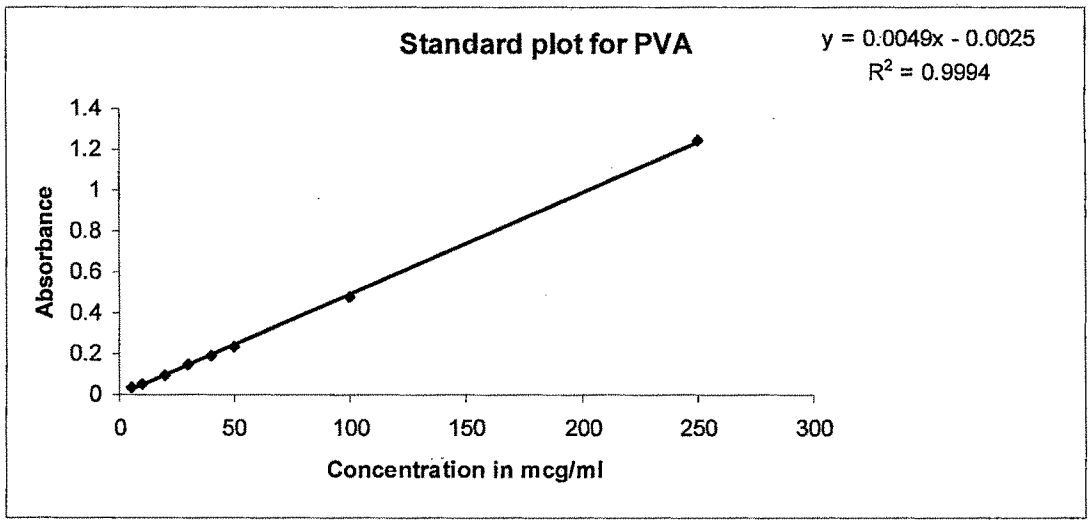
The amount of PVA associated with nanoparticles was determined by a colorimetric method based on the formation of a colored complex between two adjacent hydroxyl groups of PVA and an iodine molecule (Joshi, D.P. et al., 1979). Briefly 10mg of PVA was dissolved in 10ml of distilled water to yield 1000 µg/ml stock solution. From the stock solution, different aliquots were taken and to each sample, 3 ml of a 0.65 M solution of boric acid, 0.5 ml of a solution of I_2/KI (0.05 M/0.15 M), and 1.5 ml of distilled water were added to yield final concentration of 10-250 µg/ml. Finally, the absorbance of the samples was measured vs. water treated in same manner at 690 nm after 15 min incubation. The above procedure was repeated three times and the mean absorbance was determined. The data was recorded in Table: 3.8 along with standard deviation. Figures: 3.7 show calibration curve of PVA in water.

Table: 3.2. Calibration Curve of PVA (λmax= 690nm)

Concentration (µg/ml)	Mean Absorbance ± SD (n=3)
5	0.035 ± 0.006
10	0.051 ± 0.005
20	0.098 ± 0.004
30	0.146 ± 0.02
40	0.195 ± 0.04
50	0.236 ± 0.05
100	0.474 ± 0.04
250	1.242 ± 0.12

Regression equation** Y= 0.0049X-0.0025; Correlation coefficient R^2= 0.9994
*Mean of 3 values

Figure: 3.2. Calibration plot for PVA



3.5 Determination of residual dichloromethane in NPs

As per USP, residual solvents are tested under General Chapter <467> "Organic Volatile Impurities." Dichloro methane (DCM)s belongs to class 2 solvents. Class 2 solvents are non-genotoxic animal carcinogens. Solvents of this class should be limited in pharmaceutical products because of their inherent toxicity. Limit for DCM is 600 ppm and permissible daily exposure is 6 mg/day. DCM was analyzed by Gas chromatography

coupled with static head space sampling, the operating parameters of which are listed in Table: 3.3.

Table: 3.3. Headspace Operating Parameters

	Headspace Operating Parameter Sets		
	1	2	3
Equilibration temperature °C	80	105	80
Equilibration time (min.)	60	45	45
Transfer-line temperature °C	85	110	105
Carrier gas: nitrogen or helium at an appropriate pressure			
Pressurization time (s)	30	30	30
Injection volume (mL)	1	1	1

3.6 Determination of peptide (RGD) by BCA method

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^{2+}) with reagent containing Bichinconinic acid (Smith et al). The macromolecular structure of protein, the number of peptide bonds and the presence of four amino acids (cysteine, cystine, tryptophan and tyrosine) (Wiechelman et al., 1988). The purple-colored 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. A series of dilutions of known concentration are prepared from the protein and assayed alongside the unknown(s) before the concentration of each unknown is determined based on the standard curve. The BCA reagent does not reach a true end point, color development continues even after cooling to RT, but because the color development is slow at room temperature, no significant error is introduced if readings of all the test tubes are done within 10 min.

BCA-Protein Reaction

1. protein (peptide bonds) + Cu^{2+} tetradentate- $Cu1^{+}$ complex
2. $Cu1^{+}$ + 2 Bichinchoninic Acid BCA- Cu^{1+} complex (purple colored, read at 562nm)

Materials:

Genei's BCA Protein Assay kit KT-31

Distilled water containing 0.05-0.1 % sodium azide.

UV-Visible spectrophotometer

Methods:

1. The powder in the standard vial of the Genei's BCA Protein Assay kit KT-31 was dissolved in distilled water containing 0.05 % sodium azide to yield 5 mg/ml of Transferrin stock solution.
2. A fresh set of standard solutions was prepared from this stock solution by diluting it according to Table: 3.4.
3. To prepare BCA working Reagent (BWR), 50 parts of Reagent A was mixed with 1 parts of Reagent B. Upon addition of reagent A to reagent B, initially turbidity is observed that quickly disappears upon mixing to yield a clear green BWR. This BWR is stable for at least 24 hours when stored in a closed container at room temperature.
4. 0.2 ml of each standard or unknown sample was taken into labeled test tubes. 0.2 ml of the diluent (distilled water) was taken for blank reading.
5. 2 ml of the BWR was added to each test tube and mixed well.
6. All the test tubes were incubated at 60°C for 30 min.
7. The test tubes were then cooled down to room temperature and the absorbance measured at 562 nm Vs a water reference used as a blank and recorded in Table: 3.5.
8. A standard curve was prepared by plotting the average absorbance reading for each RGD standard vs. its concentration in µg/ml and plotted as shown in Figure: 3.3.
9. Using this standard curve, the protein concentration for each unknown sample was determined.

Table: 3.4. Preparation of diluted RGD standards

Volume of the RGD solution	Volume of Diluent	Final RGD concentration
300 µl of stock	1200 µl	1000 µg/ml (**A**)
375 µl of (A)	125 µl	750 µg/ml (**B**)
250 µl of (A)	250 µl	500 µg/ml (**C**)
125 µl of (A)	375 µl	250 µg/ml (**D**)
75 µl of (A)	425 µl	150 µg/ml (**E**)
50 µl of (A)	450 µl	100 µg/ml (**F**)
25 µl of (A)	475 µl	50 µg/ml (**G**)
12.5 µl of (A)	487.5 µl	25 µg/ml (**H**)
6.25 µl of (A)	493.75 µl	12.5 µg/ml (**I**)

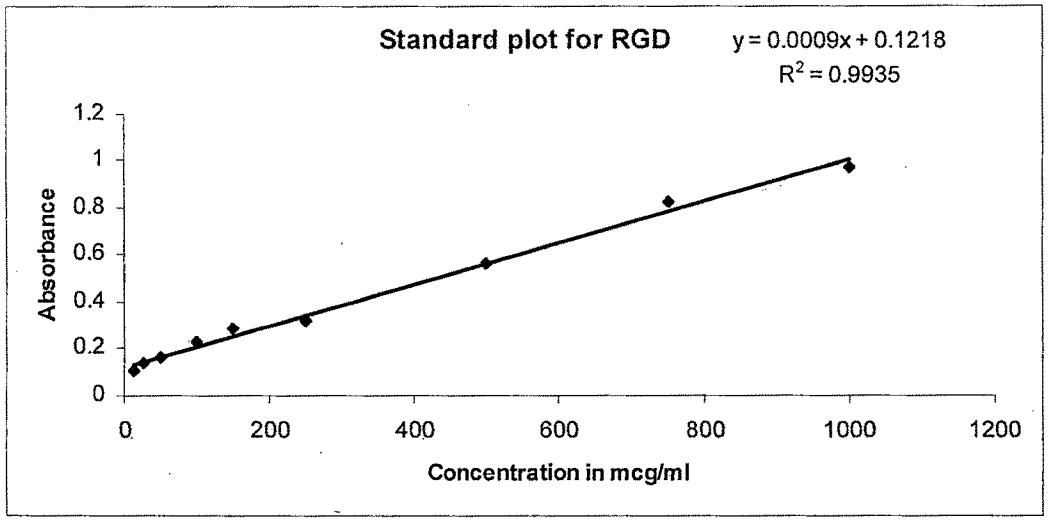
Table: 3.5. Calibration of RGD by BCA method

Sample	Concentration µg/ml	Mean Absorbance* ± S.D at 562nm
A	1000	0.972 ± 0.21
B	750	0.824 ± 0.18
C	500	0.563 ± 0.06
D	250	0.315 ± 0.04
E	150	0.283 ± 0.05
F	100	0.227 ± 0.06
G	50	0.166 ± 0.03
H	25	0.135 ± 0.03
I	12.5	0.110 ± 0.02

*Mean of 3 values;

Regression equation; $Y = 0.0009x + 0.1218$ Correlation coefficient; $R^2 = 0.9935$

Figure: 3.3. Calibration plot for RGD



3.6.1 Estimation of Phosphatidyl Choline

The Stewart assay (Stewart et al., 1980) was used for estimating phosphatidyl choline in liposomes. This method utilizes the ability of phospholipids to form a complex with ammonium ferrothiocyanate in organic solution.

3.6.1.1 Solutions

1. Ammonium ferrothiocyanate solution (0.1M) was prepared by dissolving 27.03g of ferric chloride hexahydrate and 30.4g of ammonium thiocyanate in double distilled water and making up the volume of the resulting solution to 1 litre.
2. Stock solution of phosphatidyl choline in chloroform (0.1mg/ml) was prepared by dissolving 50mg of phosphatidyl choline in 10ml of chloroform. 2ml of this solution was diluted 100 times to yield a solution of the required concentration.
3. Saturated sodium chloride solution: Sodium chloride was dissolved in distilled water with heating to form a supersaturated solution. This solution was then cooled to room temperature and filtered to give the required saturated solution.

3.6.1.2 Procedure for calibration curve

Suitable aliquots (0.1 – 1.5 ml) of the stock solution of phosphatidyl choline were transferred to 10ml centrifuge tubes. Appropriate quantities of chloroform were then added such that the total volume of the contents of the tubes was 3ml. To each tube, 2ml of ammonium ferrothiocyanate solution (0.1M) was then added. The contents of each tube were mixed by vigorous vortexing on a cyclomixer for 15sec. The tubes were then

spun for 5min at 1800 rpm in a tabletop centrifuge. The lower, organic colored layer was then removed using a syringe and long needle (18 gauge) and transferred to a test tube. The absorbance of these solutions was measured at 485 nm on a Shimadzu 1601 UV-Visible spectrophotometer with glass cells of 10mm path length using a blank prepared in the same manner omitting the phospholipids.

The above procedure was repeated six times. Mean absorbance values along with the regressed values and the regression equation obtained are shown in Table: 3.6.

3.6.1.3 Stability and selectivity

Stability of the colored solutions, prepared above for the calibration curve of phosphatidyl choline, was ascertained by observing the changes in absorbance of the solutions over a period of 4 h. The selectivity of the method for phosphatidyl choline was investigated by carrying out the procedure detailed above in the presence of potential interferences such as cholesterol, Sucrose, etc., at the levels at which these materials were included in the liposomes.

Table: 3.6. Calibration for estimation of phosphatidyl choline (Stewart assay)

Concentration (µg/3ml)	Mean Absorbance* ± S.E.M	Regressed value
10	0.026 ± 0.004	0.031
20	0.055 ± 0.002	0.056
40	0.103 ± 0.004	0.106
60	0.160 ± 0.002	0.156
80	0.211 ± 0.007	0.206
100	0.263 ± 0.003	0.256
150	0.373 ± 0.004	0.381

Regression equation** Y= 0.0025 X + 0.0057
Correlation coefficient = 0.9979
*Mean of 6 values

3.6.1.4 Estimation of phosphatidyl choline from liposomes/ supernatant

The Bligh-Dyer two-phase extraction method (New et al., 1990) was modified for estimating phosphatidyl choline from liposomes. Briefly, 0.1ml of liposomal dispersion or 0.5ml of the supernatant was taken in a centrifuge tube and to this 1ml of saturated sodium chloride solution was added. 2ml of chloroform was then added to the contents followed by vigorous vortexing on a cyclomixer for 30 sec and centrifugation at 1800 rpm for 5 min in a tabletop centrifuge. The lower chloroform layer was separated using a syringe and needle (18 gauge) and passed over a bed of anhydrous sodium sulfate into a 10ml volumetric flask. The process was repeated with a further 2ml and 1ml of chloroform. The chloroform layers were then pooled and the volume made up to 10ml with chloroform. To 0.5ml of this chloroform extract in a centrifuge tube, 2.5ml of chloroform and 2ml of 0.1M ammonium ferrothiocyanate solution was added. The contents were then subjected to the same procedure as detailed above for the standards (Section 3.6.1.2). Duplicate estimations were performed and the mean absorbance was used to determine the amount of phosphatidyl choline in the liposomes or supernatant using the regression equation.

3.6.2 Estimation of cholesterol

The Zlatkis, Zak and Boyle's method was used for cholesterol estimation. This method utilizes the ability of cholesterol in acetic acid to form a complex with ferric chloride and sulphuric acid (Zlatkis et. al., 1953).

3.6.2.1 Solutions

1. Ferric chloride solution: A 0.05% w/v solution of ferric chloride hexahydrate in glacial acetic acid was prepared by dissolving 50mg of ferric chloride hexahydrate in 100ml glacial acetic acid.
2. Stock solution of cholesterol: A 0.5mg/ml solution of cholesterol in glacial acetic acid was prepared by dissolving 25mg of cholesterol in 50ml of glacial acetic acid.
3. Saturated sodium chloride solution: It was prepared in the same manner as for the estimation of phosphatidyl choline from liposomes (Section 3.6.1.1)

3.6.2.2. Procedure for calibration curve

Suitable aliquots of the stock solution of cholesterol (0.1 to 1ml) were transferred accurately into 10ml volumetric flasks. To each flask, 4ml of ferric chloride solution and 4ml of concentrated sulphuric acid was added. The contents were mixed, made up to the volume with glacial acetic acid and allowed to stand for 30 min. The absorbance of the resulting coloured solutions of the complex, formed between cholesterol, ferric chloride and sulphuric acid was measured at 550nm using Shimadzu 1601 UV-Visible spectrophotometer with glass cells of 10mm path length, against a blank prepared in the same manner as the standard solutions except cholesterol. The above procedure was repeated six times. The experimental data along with the results of the statistical evaluation of the data are shown in Table: 3.4.

3.6.2.3 Stability and selectivity

Stability of the solutions of the complex, prepared for obtaining the calibration curve of cholesterol, was ascertained by observing the changes in their absorbance over a period of 24 h. Cholesterol was estimated in the presence of phosphatidyl choline, Sucrose, etc., at the same concentrations at which these materials were included in the liposomes to ascertain the selectivity of the method.

3.6.2.4. Estimation of cholesterol from liposomes/ supernatant

1ml of the chloroform extract, obtained as described before in the estimation of phosphatidyl choline form liposomes or supernatant (Section 3.6.1.4) was taken in a 10ml volumetric flask and evaporated to dryness by heating at 90°C in a thermostatically controlled, electrically heated water bath. The dried contents were then subjected to the same procedure as discussed previously (Section 3.6.2.2). The amount of cholesterol in the liposomes or supernatant was then obtained using the regression equation of the calibration curve.

Table: 3.7. Calibration for estimation of cholesterol (Zlatkis, Zak and Boyle's method)

Concentration (µg/ml)	Mean Absorbance* ± S.E.M	Regressed value
5	0.065 ± 0.001	0.069
10	0.154 ± 0.0021	0.155
20	0.344 ± 0.003	0.327
30	0.475 ± 0.004	0.499
40	0.695 ± 0.003	0.671
50	0.836 ± 0.004	0.843

Regression equation** Y= 0.0172X –0.0173
Correlation coefficient = 0.9966
*Mean of 6 values

3.7 Determination of Particle Size, Polydispersity and Zeta potential

The particle size was measured with Malvern zetasizer NanoZS. The instrument is based on the principle of dynamic light scattering (DLS), also sometimes referred to as photon correlation spectroscopy (PCS) or quasi elastic light scattering. DLS is a technique of measuring the size of particles typically in the sub-micron region and is usually applied to the measurement of particle suspended within a liquid. The technique measures particle diffusion due to Brownian motion and relates this to the size of the particles. Brownian motion is the random movement of particles due to the bombardment by the solvent molecules that surround them. The parameter calculated is defined as the translational diffusion coefficient. The particle size is then calculated from the translational diffusion coefficient using the Strokes-Einstein equation.

Malvern zetasizer NanoZS was used to measure the zeta potential of the particles based on the electrophoresis and electrical conductivity of the formed nanoparticle dispersion. The electrophoretic mobility (µm/s) of the particles was converted to the zeta potential by in-built software based on Helmholtz- Smoluchowski equation.

A 2.0 mg sample of nanoparticles was suspended in distilled water, and the particle size, polydispersity index and zeta potential were measured using the principle of laser light scattering with zeta sizer (Nano-ZS, Malvern Instruments, UK).

3.8 Morphological characterization

The morphology of the nanoparticles was analyzed using EDAX (Energy dispersion analysis by X-ray) Scanning Electron Microscopy (SEM) and TEM (Transmission Electron Microscopy). Aqueous nanoparticle suspensions were layered on the SEM stubs, and they were allowed to dry at room temperature. Samples were then observed with Phillips SEM 51S set at 10 kV.

Aqueous liposomal suspension was negatively stained with phosphotungstic acid (0.5%) while nanoparticles were analyzed without any staining. Samples were then observed with Morgagni, Philips, Eindhoven, Netherlands

3.9 Differential scanning calorimetry

The DSC of samples was carried out by scanning the samples using differential scanning colorimeter (Mettler). Thermograms were analyzed using Mettler Toledo star SW 7.01. An empty aluminium pan was used as the reference for all measurements. During each scan, 2 to 3 mg of sample was heated, in a hermetically sealed aluminium pan, at a heating rate of 10° C/min, from 35° C to 300° C, under a nitrogen atmosphere.

3.10. Discussions

DC in nanoparticles, liposomes and in-vitro release medium was estimated by HPLC method. RGD was estimated by BCA protein assay and amino acid analysis. The estimation in the nanoparticles, liposomes and *in vitro* release studies for DC was performed by HPLC method. The calibration curve of DC was established in acetonitrile: water gradient system by HPLC at 227nm. The linearity of DC was found to be 0.5-10μg/ml (R^2=0.9985). To determine the amount of drug entrapped nanoparticles were dissolved in acetonitrile. The resulting system was centrifuged to remove the precipitated components and the supernant was diluted with the solvent system and subjected to analysis.

The invitro release study was performed using the tube shaking method. At different time intervals, the samples were removed and centrifuged at 25000 rpm and the settled

nanoparticles were dissolved in acetonitrile and analyzed for the drug remaining in the nanoparticles as the same method for entrapment efficiency. The drug released was calculated by taking the difference of the drug taken initially and the drug remaining in the nanoparticles.

The estimation of RGD conjugation was carried out using BCA protein estimation. The calibration curve was established at 12.5-1000 µg/ml (R^2=0.9935). The amount of PVA associated with nanoparticles was determined by a colorimetric method based on the formation of a colored complex between two adjacent hydroxyl groups of PVA and an iodine molecule. A standard plot for known concentrations of PVA was established at 5-250 µg/ml. (R^2=0.9994). Residual solvents in pharmaceuticals, commonly known as organic volatile impurities (OVIs), are chemicals that are either used or produced during the manufacture of active pharmaceutical ingredients (APIs), excipients and drug products. OVIs are classified into three classes on the basis of their toxicity level and the degree to which they can be considered an environmental hazard. DCM belongs to class 2 solvents which are non-genotoxic animal carcinogens. Solvents of this class should be limited in pharmaceutical products because of their inherent toxicity. Limit for DCM is 600 ppm and recommended daily exposure is 6 mg/day.

The Stewart assay was used for estimating phosphatidyl choline in liposomes (Stewart, 1980). This method is based on complex formation between ammonium ferrothiocyanate and phospholipids in organic solution. The complex in chloroform exhibits maximum absorbance at 485nm. The advantage of this method is that the presence of inorganic phosphate does not interfere with the assay. A disadvantage, however, is that this method is not applicable to samples where mixtures of unknown phospholipids may be present (New et al., 1990a). A correlation coefficient of 0.9979 indicated a linear relationship between absorbance and concentration of phosphatidyl choline taken for complex formation. Beer's law was found to be obeyed between 10 – 150 µg/3ml.

The Zlatkis, Zak and Boyle method (Zlatkis et. al., 1953) was used for estimating cholesterol in liposomes. Here, cholesterol in acetic acid forms a colored complex with ferric chloride and sulphuric acid, which exhibits maximum absorbance at 550nm. Linearity of the method was observed from a correlation coefficient of 0.9966. Beer's law was found to be obeyed between 5 – 50 µg/ml. The regression equation obtained was $Y = 0.0172 X + 0.0146$. Monitoring of the absorbance of the solutions, used for preparing

the calibration curve revealed that the color of the complex was retained as its original intensity for only 2h.

3.11. Rreferences

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