Chapter – 3

Analytical Methods

Chapter 3 Analytical Methods

In the current investigation, Hyaluronic acid (HA) grafted liposomal Dry Powder Inhalers (DPIs) of Etoposide and Docetaxel were prepared for site specific delivery of both the drugs in treatment of lung cancer. In this chapter, analytical methods used in preparation, characterization and optimization of HA grafted liposomal DPIs of Etoposide and Docetaxel are discussed. The prepared liposomes were characterized for their particle size, zeta potential, percentage drug entrapment, in vitro drug release, drug excipients interaction or incompatibility by DSC. The liposomal DPIs of both the drugs were characterized for physical tests like flowability, residual moisture content, volume mean diameter, mass mean aerodynamic diameter, in vitro drug release and in vitro lung deposition. The optimized liposomal DPI formulations were assessed for their stability for 12 months and characterization was conducted in terms of particle size, zeta potential, percentage drug entrapment and physical changes like discolouration etc.

A549 cell lines were used to determine cellular drug levels, in vitro cytotoxicity, cell uptake and effect on cell cycle analysis.

Chapter 3 Analytical Methods

3.1 MATERIALS AND METHODS

Materials	Source
Etoposide	Cadila Pharma Ltd., Ahmedabad, India.
Docetaxel	Sun Pharma Advanced Research Centre, Vadodara,India
Methanol, Acetonitrile, Chloroform, ammonium hydroxide, methylene chloride (All reagents used were of Analytical grade (AR), spectroscopic grade /HPLC grade and used as purchased without any modification.	S.D.Fine Chemicals Ltd., Mumbai,India
Polysorbate 80	S.D.Fine Chemicals Ltd., Mumbai,India
UV Visible Spectrophotometer	UV 1601, Shimadzu,Japan.
HPLC	Dionex Softron Gmbh,Germany.
Spectrofluorimeter	RF-540, Shimadzu,Japan.
Particle Size Analyzer	Malvern Instruments, U.K.
Malvern Zeta Sizer Analyzer	Malvern Instruments, U.K.
DSC	Mettler, DSC 20, Mettler Toledo, Switzerland.
Analytical Balance	Precisa 205A SCS, Switzerland
Bath Sonicator	INCO, Ambala,India

Stability oven	Shree kailash Industries, Vadodara,India
pH meter	Pico+ Labindia,Mumbai,India.
Vacuum Pump	Bharat Vacuum Pumps, Bengaluroo,India
Materials/Glassware	Source
Nuclepore Polycarbonate membrane, 2 μ, 25 mm	Whatmann, USA.
Glassware (25 ml graduated pipettes, 10 ml pipettes, serological pipettes, 100 ml volumetric flasks, 250 ml volumetric flasks, 10 ml volumetric flask, 1 ml pipettes, 100 ml beakers, 25 ml beakers, 250 ml conical flasks)	Schott and Corning,Mumbai,India
Bichinconinic acid (BCA) protein assay kit	Genei,Bengaluroo,India
Deionized water	Prepared in the lab

3.2 CHEMICAL ANALYSIS

3.2.1 Estimation of Etoposide (ETP) by UV Spectrophotometric method:

UV spectrophotometric method was mainly developed to estimate drug content in solution, developed liposomal formulations in terms of percentage drug entrapment and in vitro drug release. Drug content was estimated by dissolving Etoposide in Acetonitirile (spectroscopic grade). The stock solution was further used to prepare a series of lower concentrations and absorbance for each serial dilutions was measured at 285 nm using UV 1601, Shimadzu Japan.

3.2.1.1 Estimation of Etoposide (ETP) by HPLC method:

For estimation of ETP in cell lysates, biological fluids and in vitro cell uptake, HPLC method was developed. Drug content was determined using an Octadecylated silicon ODS Hypersil Si-10 column (300x4 mm internal diameter, 10μ). The guard column ODS-E (Shimadzu) was 10 mm long with internal diameter of 4 mm and particle size of 5 μ. The column flow rate was set at 2 ml/minute and column as well as pre column temperature was maintained at 40^0C. The mobile phase constituted of Methanol with 1 % ammonium hydroxide: Methylene chloride (2:98v/v) mixture and determinations were performed at UV detection wavelength of 254 nm. The run time for assay was 15 minutes and retention time was of 4.6 minutes

Preparation of standard stock solutions of Etoposide in Acetonitirile

50 mg of Etoposide was accurately weighed using single pan electronic balance and transferred to 50 ml volumetric flask. 25 ml of AR grade acetonitrile was accurately transferred to the above volumetric flask, the drug was dissolved carefully and completely and then the final volume was adjusted to 50 ml with acetonitrile to produce 1 mg/ml of Etoposide.

20 ml of the above solution was diluted to 100 ml with acetonitirile to get 200 μg/ml of Etoposide.

Preparation of Calibration Curve of Etoposide

Suitable aliquots of standard stock solution were accurately measured and transferred to the 10 ml volumetric flasks. The final volume was adjusted to 10 ml with AR grade acetonitirile to give concentrations of 5, 10, 20, 40,60, 80, 100 and 120 μg/ml concentrations and analyzed in UV Visible spectrophotometer at λmax=285 nm. The above procedure was repeated in triplicate and results were recorded in Table 3.1 along with standard deviation and plotted graphically in Fig 3.1.

Table 3.1 Calibration for Etoposide by UV spectrophotometric method

Concentration in µg/ml	Absorbance (unit)* ± S.D.
5	0
10	0.039 ±0.003
20	0.086 ±0.004
40	0.152 ±0.011
60	0.359 ±0.004
80	0.436 ±0.004
100	0.585 ±0.021
120	0.74 ±0.019

Regression equation: 0.0074x+0.0106, R^2=0.9956 *-n=3

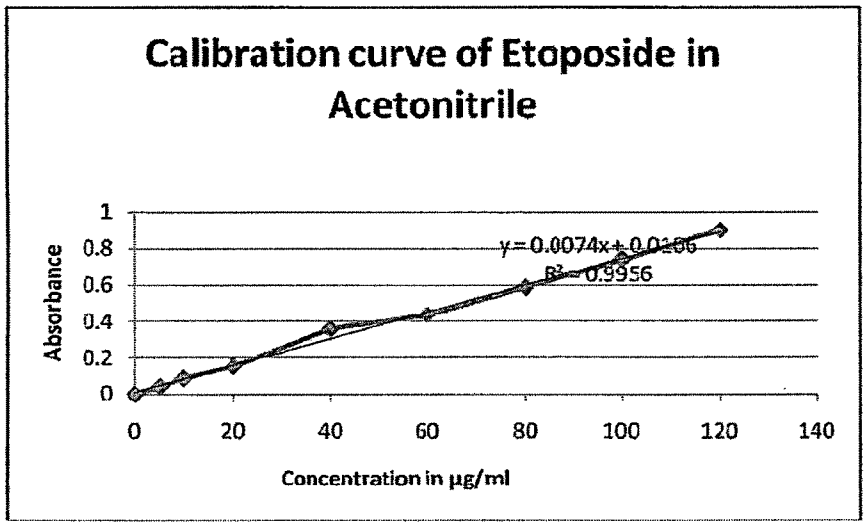


Fig 3.1 Calibration curve for estimation of Etoposide by UV spectrophotometric method.

Table 3.2 Calibration for Etoposide using HPLC

Concentration(ng/ml)	mAU*±S.D.
0	0
10	0.0133 ±0.002
20	0.0229 ±0.003
40	0.0498 ± 0.004
60	0.0435 ±0.001
80	0.0747 ±0.001
100	0.0954 ±0.003
200	0.1468 ±0.004
400	0.3298 ±0.004
800	0.6541 ±0.008

*-n=3,

** Regression equation: y=0.0008X+0.0047, R^2=0.9979

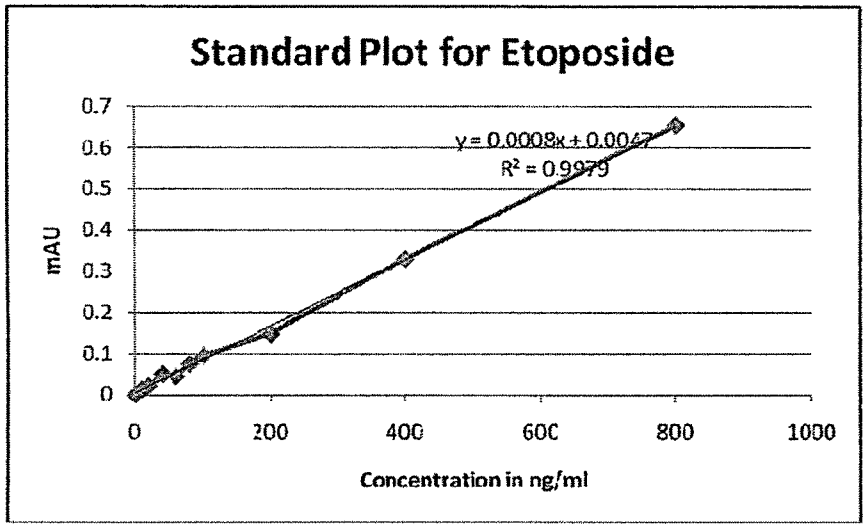


Fig 3.2 Calibration curve for estimation of Etoposide by HPLC.

3.2.1.2 Estimation of Etoposide in Liposomes (non grafted and grafted)

The unentrapped drug from liposomes was removed by subjecting the preparation to ultracentrifugation at 25,000 rpm for 30 minutes after treating the liposomes. The liposomes were lysed using 0.02% Triton X solution followed by their dissolution in Methanol and drug content was spectrophotometrically estimated at 285 nm using UV-Visible spectrophotometer (UV 1601,Shimadzu, Japan). All the determinations were performed in triplicate.

3.2.1.3 Estimation of Etoposide in diffusion medium (PBS,pH=7.4)

Aliquots were withdrawn at different time intervals by removing the diffusion medium containing 0.1 % Polysorbate 80. This was conducted in triplicate. The amount of drug in diffusion medium was quantified using UV Spectrophotometric technique as explained above.

3.2.1.4 Estimation of Etoposide in biological samples (Cell lysates)

Procedure for Calibration Curve

Appropriate aliquots of the stock solution of Etoposide were transferred to 5 ml volumetric flasks and diluted with biological samples to give final concentrations of 10, 20, 40, 60, 80, 100, 200, 400 and 800 ng/ml. Diphenylhydantoin was added as an internal standard (Kato et al, 2003). The resultant solutions were mixed well and then extracted with methanol 5 ml twice. The

methanolic samples were evaporated to dryness and reconstituted with 1 ml of mobile phase; centrifuged at 5000 rpm for 20 minutes and supernatant was analyzed by HPLC method discussed above. The above procedure was repeated thrice and results were recorded in Table 3.3 for cell lysates.

Stability and Selectivity

Stability of ETP in biological samples, used for preparing the calibration curve, was assessed by observing the changes in their mAU at the analytical wavelength, over a period of 24 h stored at 2-8^{0}C. Blank biological samples were analyzed to ensure the selectivity of the method.

Extraction Efficiency

The recovery of ETP from biological samples was evaluated at low, medium and high concentrations of 40, 200 and 800 ng/ml respectively. The recovery of ETP was calculated by comparing the peak area obtained from the extracts of spiked biological samples with the peak area obtained from the direct injection of known amounts of standard drug solutions. The overall extraction yields of ETP in cell lysates are shown in Table 3.4

Table 3.3 Calibration of ETP in lung lysates

Concentration in ng/ml	mAU*±S.D.
0	0
40	0.0357 ± 0.021
60	0.0532 ± 0.01
80	0.0688 ± 0.041
100	0.0831 ± 0.036
200	0.1179 ±0.018
400	0.202 ± 0.034
800	0.337 ± 0.067

*-n=3 Regression equation: y=0.0004X+0.0281; R^2= 0.9811

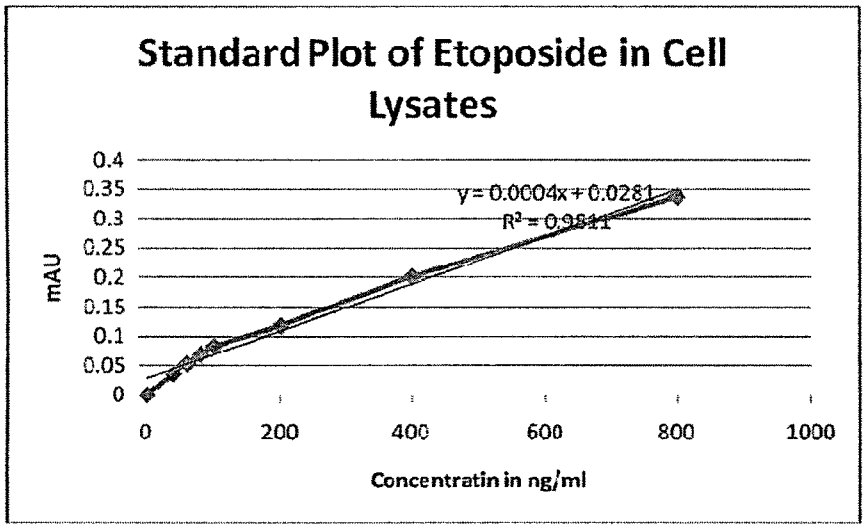


Fig 3.3 Calibration curve of ETP in Cell Lysate

Table 3.4 Extraction efficiency of ETP in cell lysates (n=3)

Sample	Concentration in ng/ml	Percentage Recovery ±S.D.
Cell Lysate	40	95 ±0.06
	200	96.1 ± 0.03
	800	98.4 ± 0.02

3.2.2 Estimation of Docetaxel (DOC)

3.2.2.1 Estimation of Docetaxel in solution by UV spectrophotometry

UV spectrophotometric method was mainly developed to estimate drug content in solution, developed liposomal formulations in terms of percentage drug entrapment and in vitro drug release. Drug content was estimated by dissolving Docetaxel in ethanol. The stock solution was

further used to prepare a series of lower concentrations and absorbance for each serial dilutions was measured at 230 nm using UV 1601, Shimadzu Japan.

3.2.2.1.1 Estimation of DOC by HPLC

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 (Rheodyne 7125) and a UV-visible Spectrophotometric detector (UVD 170U, Dionex). The separation was carried out on 14 cm Kromasil C18 150-4.6 HPLC column (Merck, Germany) with particle size of 5 µ. Mobile phase for DOC comprised of deionized water and HPLC grade acetonitrile with 34 % acetonitrile in deionized water held for 5 minutes, then line gradient to 58 % acetonitrile in 16 minutes, linear gradient to 70% acetonitrile in 2 minutes and linear gradient to 34 % acetonitirile in 4 minutes and 34 % acetonitirile for 5 minutes at a flow rate of 1 ml/minute and wavelength detection at 228 nm.

Preparation of standard stock solutions of DOC

50 mg of Docetaxel was accurately weighed using single pan electronic balance and transferred to 50 ml volumetric flask. 25 ml of ethanol was accurately transferred to the above volumetric flask, the drug was dissolved carefully and completely and then the final volume was adjusted to 50 ml with ethanol to produce 1 mg/ml of Docetaxel.

20 ml of the above solution was diluted to 100 ml with ethanol to get 200 µg/ml of DOC.

Preparation of Calibration Curve of DOC

Suitable aliquots of standard stock solution were accurately measured and transferred to the 10 ml volumetric flasks. The final volume was adjusted to 10 ml with ethanol give concentrations of 5, 10, 20, 40,60, 80, 100 and 120 µg/ml concentrations and analyzed in UV Visible spectrophotometer at λmax=230 nm. The above procedure was repeated in triplicate and results were recorded in Table 3.5 along with standard deviation and plotted graphically in Fig 3.4 and Table 3.5.

Table 3.5 Calibration of DOC by UV spectrophotometric method

Concentration in µg/ml	Absorbance ±S.D.*
0	0
10	0.047 ± 0.001
20	0.084 ± 0.002
40	0.171 ± 0.021
60	0.312 ± 0.015
80	0.412 ± 0.008
100	0.537 ± 0.025
120	0.601 ± 0.057

*-n=3, **Regression equation: y=0.0052X-0.0112, R^2= 0.9847

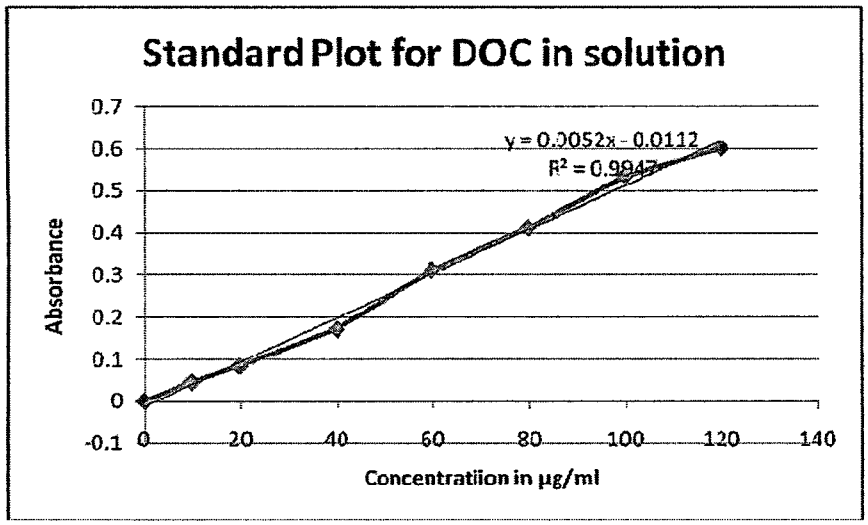


Fig 3.4 Calibration curve for DOC by UV spectrophotometric method

Table 3.6 Calibration for DOC by HPLC

Concentration in ng/ml	mAU* ± S.D.
0	0
50	0.0231 ± 0.02
100	0.0689 ± 0.01
200	0.1768 ± 0.034
400	0.412 ± 0.021
600	0.7018 ± 0.009
800	0.9932 ± 0.003

*-n=3

Regression equation: y=0.0013x-0.0467; R^2=0.9929

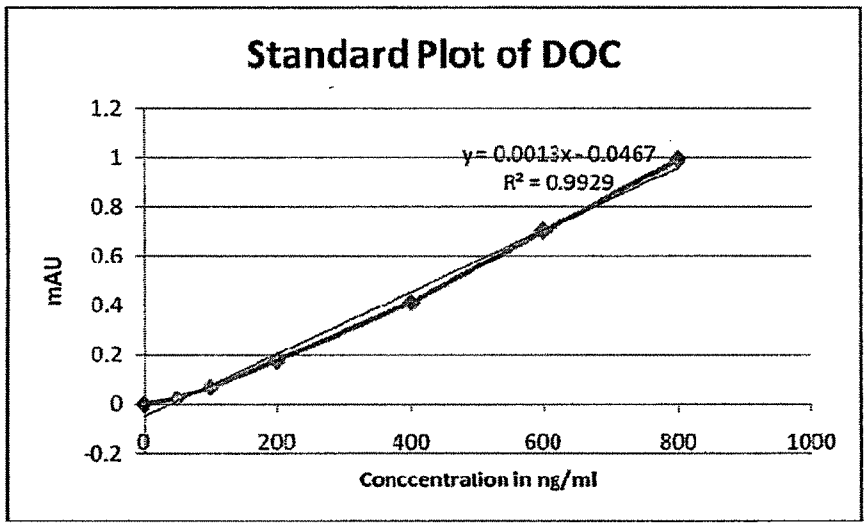
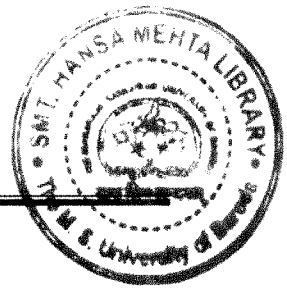


Fig 3.5 Calibration curve for DOC by HPLC



3.2.3 Estimation of DOC in Liposomes (non grafted and grafted)

The unentrapped drug from liposomes was removed by subjecting the preparation to ultracentrifugation at 25,000 rpm for 30 minutes after treating the liposomes. The liposomes were lysed using 0.02% Triton X solution followed by their dissolution in ethanol and drug content was spectrophotometrically estimated at 230 nm using UV-Visible spectrophotometer (UV 1601,Shimadzu, Japan). All the determinations were performed in triplicate.

3.2.3.1 Estimation of DOC in diffusion medium (PBS, pH=7.4)

Aliquots were withdrawn at different time intervals by removing the diffusion medium containing 0.1 % Polysorbate 80. This was conducted in triplicate. The amount of drug in diffusion medium was quantified using UV Spectrophotometric technique as explained above.

3.2.3.2 Estimation of Docetaxel in biological samples (Cell lysates)

Procedure for Calibration Curve

Appropriate aliquots of the stock solution of Docetaxel were transferred to 5 ml volumetric flasks and diluted with biological samples to give final concentrations of 10, 20, 40, 60, 80, 100, 200, 400 and 800 ng/ml. The resultant solutions were mixed well and then extracted with methanol 5 ml twice. The methanolic samples were evaporated to dryness and reconstituted with 1 ml of mobile phase; centrifuged at 5000 rpm for 20 minutes and supernatant was analyzed by HPLC method discussed above. The above procedure was repeated thrice and results were recorded in Table 3.7 for cell lysates.

Table 3.7 Calibration for DOC in cell lysates

Concentration in ng/ml	mAU* ± S.D.
0	0
50	0.0312 ± 0.002
100	0.0612 ± 0.002
200	0.1595 ± 0.011
400	0.2976 ± 0.013
600	0.5578 ± 0.027
800	0.6911 ± 0.004

*-n=3

Regression equation:0.0009x-0.0178, R^2=0.9915

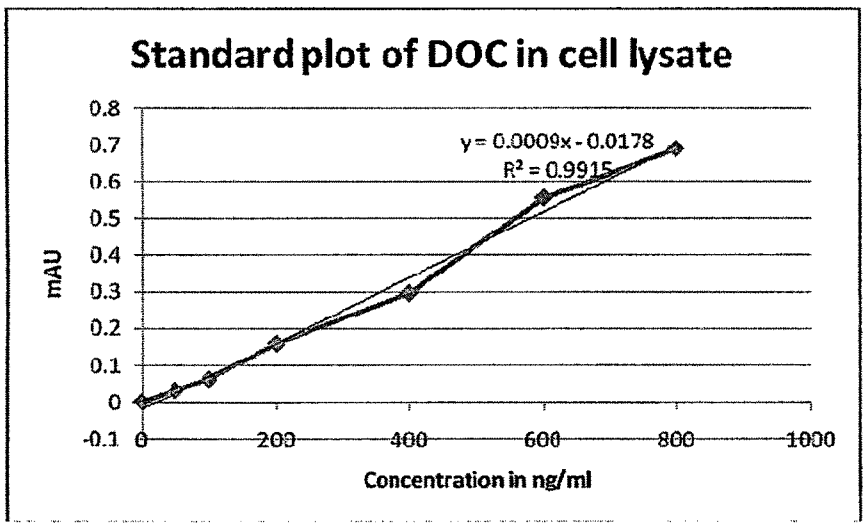


Fig 3.6 Calibration curve for DOC in cell lysates.

Stability and Selectivity

Stability of DOC in biological samples, used for preparing the calibration curve, was assessed by observing the changes in their mAU at the analytical wavelength, over a period of 24 h stored at 2-8^{0}C. Blank biological samples were analyzed to ensure the selectivity of the method.

Extraction Efficiency

The recovery of DOC from biological samples was evaluated at low, medium and high concentrations of 40, 200 and 800 ng/ml respectively. The recovery of DOC was calculated by comparing the peak area obtained from the extracts of spiked biological samples with the peak area obtained from the direct injection of known amounts of standard drug solutions. The overall extraction yields of ETP in cell lysates are shown in Table 3.8.

Table 3.8 Extraction efficiency of DOC in cell lysates (n=3)

Sample	Concentration in ng/ml	Percentage Recovery ±S.D.
Cell Lysate	40	94.1 ±0.06
	200	97.3 ± 0.06
	800	98.7 ± 0.02

3.3 DETERMINATION OF PROTEIN (PROTEIN IN CELL LYSATES) BY BCA METHOD

Protein Assay based on bichinconinic acid (BCA) is one of the most sensitive, accurate methods for colourimetric detection and quantification of total protein. The method is based on the combined principles of biuret reaction involving reduction of Cu^{2+} to Cu^{1+} by protein in an alkaline medium and colourimetric detection of cuprous cation (Cu^{1+}) with reagent containing Bichinconninic acid (Smith, 1985). The purple coloured reaction product during the assay is formed by chelation of two molecules of BCA with one cuprous ion. This water soluble, purple coloured complex exhibits a strong absorbance at 562 nm.

BCA-Protein Reaction

Protein (Peptide bonds) + Cu^{2+} tetradentate—Cu^{1+} complex

Cu^{1+} + 2 BCA- Cu^{1+} complex (Purple coloured, absorbance measured at 562 nm)

Methods:

A 2 mg/ml bovine serum albumin (BSA) solution was prepared freshly in protease inhibitor. A protein standard curve was prepared from the BSA stock with dilutions made in protease inhibitor.

The working reagent was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B.

200 µl of working reagent (BWR) is required for each well of a 96-well plate used.

25 µl of each standard or unknown sample was pipetted in duplicate onto a 96-well plate. 200 µl of working reagent was added to each well and the plate was mixed thoroughly on a plate shaker for 30 seconds.

The plate was covered and incubated at 37^0C for 30 minutes and absorbance was read at 562 nm.

The average 562 nm absorbance of the blank was subtracted from the 562 nm measurements of all other individual standard and unknown replicates.

A standard curve was prepared by plotting the average blank corrected 562 nm absorbance for each BSA standard versus its concentration in µg/ml. The standard curve was then used to determine the protein concentration of each unknown sample.

Table 3.9 Calibration of protein by BCA method

Sample	Concentration in µg/ml	Mean Absorbance* ± S.D. at 562 nm	Absorbance-Blank
Blank	____	0.191 ± 0.002	------
1	1000	1.687 ± 0.005	1.496
2	750	1.489 ± 0.003	1.298
3	500	1.068 ± 0.005	0.877
4	250	0.759 ± 0.004	0.568
5	200	0.625 ± 0.001	0.434
6	150	0.513 ± 0.004	0.322
7	100	0.436 ± 0.005	0.245
8	50	0.371 ± 0.008	0.180
9	20	0.319 ± 0.009	0.128
10	10	0.273 ± 0.005	0.082

***-n=3, Regressiion equation: y=0.0015x+0.0993, R^2=0.9851**

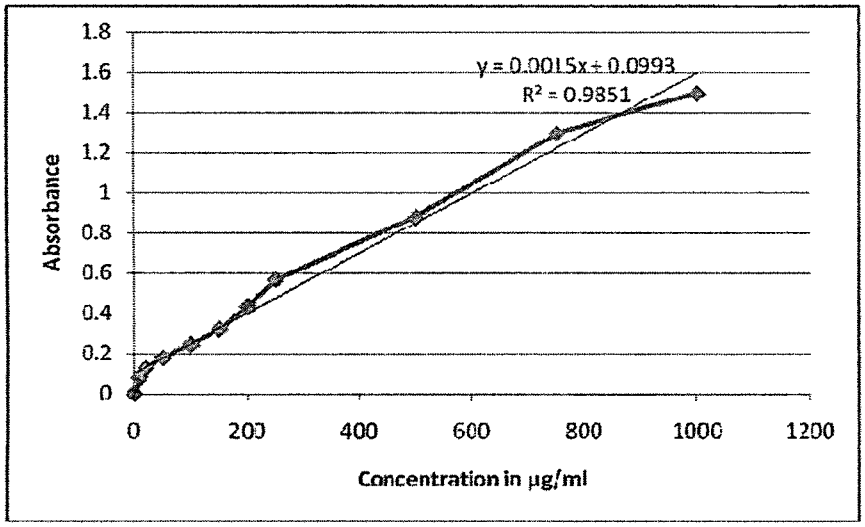


Fig 3.7 Calibration curve of protein by BCA method

3.4 SPECTROPHOTOMETRIC DETERMINATION OF HYALURONIC ACID (HA)

This method was based on modified Uronic acid carbazole reaction of Bitter and Muir (Bitter, 1962) based on DISCHE's carbazole reaction.(Dische, 1947) Serial dilutions of HA ranging from standard concentrations of 0.5, 1,10, 20, 30, 40 and 50 µg/ml were prepared in double distilled water.

First, 3 ml of 0.025 M sodium tetraborate in sulphuric acid was added to tubes and cooled at 4^0 C. 0.25 ml of different standard concentrations were added slowly to tubes followed by initial vigorous agitation of the tubes to ensure complete reaction followed by constant cooling. Later on, the tubes were heated for 10 minutes in water bath and cooled to ambient temperature followed by addition of 0.1 ml of 0.125% carbazole in ethanol, tubes were agitated moderately and heated for 20 minutes in a heated water bath followed by cooling at ambient room temperature and absorbance was measured spectrophotometrically at 530 nm using UV-Visible Spectrophotometer-UV1601 (Shimadzu, Japan) using Double Distilled water as a blank.

3.5 PHYSICAL CHARACTERIZATION

3.5.1 Determination of Particle size and Polydispersity

Particle size was determined by laser diffractometry (Malvern Mastersizer, 2000 series, Malvern Instruments, U.K.) using the Hydro 2000 SM sampling unit. Each sample was dispersed in sufficient quantity of deionized water to achieve obscuration range between 5-10%. Samples were stirred using a cyclomixer at 1000 rpm to maintain the particles in dispersed form and measurements were recorded for volume mean diameter (VMD), which is related to the mass median diameter by the density of the particles.

Polydispersity Index (PDI) of powder was defined from span (Chew, 2002).

$$\text{Span} = \frac{[D(V,_{90}) - D(V,_{10})]}{[D(V,50)]} \qquad \text{--------(1)}$$

D (V,90, V,50 and V,10) are equivalent volume diameters at 90, 50 and 10 % cumulative volume respectively.

3.5.2 Determination of zeta potential

Liposomal dispersion was suspended in deionized water followed by measurement of particle size and zeta potential based on the principle of laser light scattering with zeta sizer (Nano-ZS,Malvern Instruments, U.K.).

3.5.3 Morphological Characterization

Surface morphology of the prepared liposomal Dry Powder Inhalers (DPIs) was analyzed using EDAX (Energy dispersion analysis by X-rays) scanning electron microscopy (SEM). Lyophilized DPIs were observed using scanning electron microscope (JSM-5610 LV, JEOL, Japan). Powder samples were adhered to sample stubs using double sided adhesive tape, and then viewed using an accelerating voltage of 15 kV at the magnification of ×250 to ×5,000.

3.5.4 Differential Scanning Calorimetry

DSC study was performed in order to assess the physical state of drugs (Etoposide-ETP and Docetaxel-DOC) in liposomes as well as to study drug excipient interaction that can precipitate out incompatibility, if any. DSC of samples was carried out by scanning the samples using Differential Scanning Calorimeter. Thermograms were analyzed using Mettler Toledo star SW 20. An empty aluminium pan was used as the reference for all determinations. 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 25 to 300 C under a nitrogen atmosphere.

3.5.5 Fourier Transform Infra Red Analysis (FTIR)

The reason for subjecting the drug delivery system to FTIR was to confirm the grafting of HA to drug loaded Liposomes. Liposomal samples of non grafted and HA grafted drug loaded Liposomes were mixed with potassium bromide and compressed on a hydraulic press to make pellets. Spectra were obtained by scanning the samples between 400 and 4000 cm^{-1} on a FTIR (Perkin Elmer).

3.6 DISCUSSION

ETP and DOC in solution, liposomes, in vitro drug release medium and liposomal DPIs were estimated by UV Spectrophotometric method, Cell lysates, diffusion medium and other biological fluids by HPLC and BCA protein method.

Calibration curve was established for ETP and DOC which was observed to be linear in the concentration range of 5-120 μg/ml for UV spectrophotometry and 50-800 ng/ml for HPLC. The drug concentration in diffusion media was estimated by withdrawing the aliquots of diffusion medium (PBS, pH=7.4 with 0.1% Polysorbate 80) and estimated by UV Spectrophotometric technique.

To establish calibration curves in biological samples like cell lysates, appropriate aliquots of the stock solution of drugs were taken and concentrations in range of 50-800 ng/ml were finally obtained. The resulting solutions were extracted using methanol and evaporated to dryness. The dried residue was reconstituted with mobile phases; centrifuged at 5000 rpm for 20 minutes and supernatant was analyzed by HPLC. Stability of ETP and DOC in biological samples was

ascertained by observing changes in their area under peak at the analytical wavelength, over a period of 24 h stored at 2-8 C. Blank biological samples were analyzed to assess the selectivity of the method.

The recovery of ETP from biological samples was evaluated at low, medium and high concentrations of 40, 200 and 800 ng/ml. The extraction efficiency was more than 95% in all the cases and calibration curves were linear in concentration range of 40-200 ng/ml.

Calibration curve was established for BCA protein assay and was found to be linear in concentration range of 10 to 1,000 µg/ml with regression coefficient of 0.9851.

The surface density of HA grafted on liposomal surface can be determined by modified uronic acid carbazole reaction of Bitter and Muir based on DISCHE's carbazole reaction (Dische, 1947). Briefly, treatment of HA based carriers with 0.025 M sodium tetraborate in sulphuric acid and 0.125 % carbazole in absolute ethanol, resulted in disintegration of HA into glucoronic acid. This glucoronic acid yielded stable violet color with carbazole which can be quantified at 530 nm.

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