CHAPTER 3

ANALYTICAL METHODS

3. ANALYTICAL METHODS

The analytical methods for preparation, optimization and characterization of unconjugated and Tf conjugated PLGA nanoparticles of etoposide and temozolomide are employed in this section. The prepared nanoparticles were characterized for particle size, surface charge in the form of zeta potential, surface morphology by transmission electron microscopy, in-vitro drug release, DSC, ^{1}H-NMR, residual PVA and conjugation efficiency of Tf. The prepared formulations were investigated for cell line studies on C6 rat glioma cells. In vivo studies in animals include the tissue bio-distribution in the different body organs or tissues. The biodistribution studies were carried out after radiolabeling the drug and the nanoparticle formulation with ^{99m}Tc. (Chapter 9) The drug delivery to the brain was confirmed by gamma scintigraphy technique. The stability studies of unconjugated and conjugated NPs were conducted to determine the particle size, zeta potential, % EE, and the physical changes like caking and discoloration.

Table 3.1 Materials and Equipments

Material	Source
Etoposide (ETP)	Gift samples from Cadila Pharmaceuticals, Ahmadabad, India
Temozolomide (TMZ)	Gift samples from V B Shilpa, Raichur, Karnataka, 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, acetonitrile, acetic acid	S.D. Fine chemicals, India.
Nuclepore Polycarbonate membrane 2 μm 25mm	Whatman, USA
Polyvinyl alcohol	Sigma chemicals, USA

Equipments	Source/Make
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	AX 120, EL 8300, Shimadzu Corp., Japan
pH meter	Pico^{+} Labindia, Mumbai, India
Cyclomixer, magnetic 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
HPLC system	LC 20-AT prominence, Shimadzu Corp., Japan
UV-Visible Spectrophotometer	Shimadzu UV-1601, Japan
Spectrofluorimeter	RF 540, Shimadzu Corp., Japan
Vacuum Pump F16	Bharat Vacuum pumps, Bangalore
Bath sonicator	DTC 503, Ultra Sonics
Malvern particle size analyser	Malvern zeta sizer NanoZS, U.K.
Transmission electron microscope	Morgagni, Philips, Netherlands
Differential Scanning Calorimeter	Mettler DSC 20, Mettler Toledo, Switzerland

3.1 Estimation of Etoposide

3.1.1 Estimation of Etoposide in solution

Etoposide shows strong absorbance in UV-Visible region. Hence, the estimation of etoposide was performed by UV-visible spectrophotometry. A common method for estimation of drug content, entrapment efficiency and invitro release was developed. The method was developed in acetonitrile.

Preparation of standard stock solutions of Etoposide in Acetonitrile

50 mg of Etoposide was accurately weighed using single pan electronic balance and transferred to 50 ml volumetric flask. 25 ml of Acetonitrile AR grade 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 Acetonitrile AR grade to produce 1000 µg per ml of Etoposide.

20 ml of the above solution was accurately measured by calibrated graduated pipette and transferred to the 100 ml volumetric flask. The final volume was made up to 100 ml with Acetonitrile AR grade to prepare stock solution of 200 µg per ml of Etoposide.

Calibration curve of Etoposide

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 Acetonitrile AR grade to give final concentrations of 5, 10, 20, 40, 60, 80, 100, 120 µg/ml and analyzed by UV spectrophotometry at 283nm. The above procedure was repeated three times. The data was recorded in Table 3.2 along with standard deviation. Figures 3.1, 3.2 show the UV spectra and calibration curve respectively of Etoposide in acetonitrile.

Figure 3.1: UV spectrophotometric scan of Etoposide in Acetonitrile

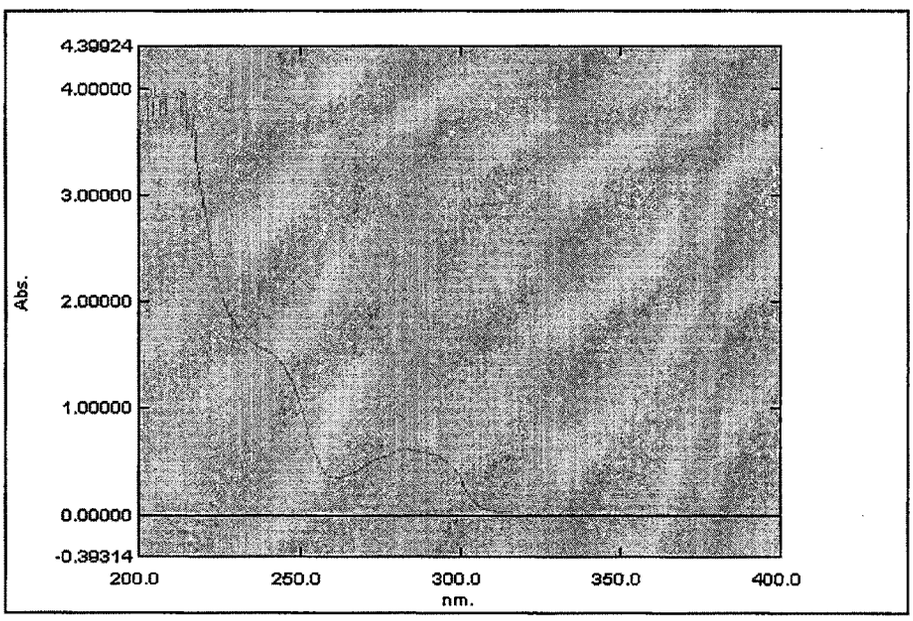


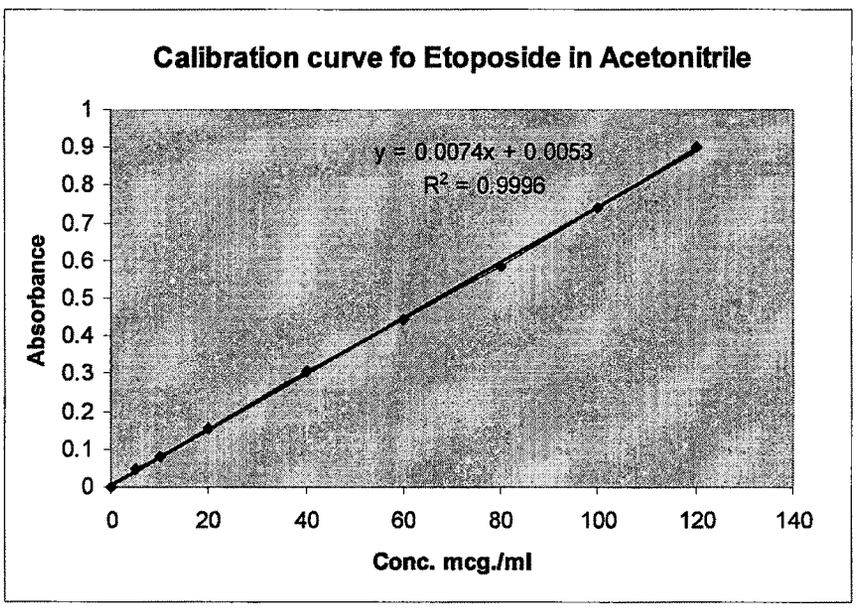
Table 3.2: Calibration for Etoposide in Acetonitrile

Concentration (µg/ ml)	Mean Absorbance* ± S.D
0	0
5	0.045±0.004
10	0.082±0.005
20	0.158±0.013
40	0.305±0.003
60	0.442±0.003
80	0.585±0.016
100	0.740±0.013
120	0.901±0.020

Regression equation** Y= 0.0074X + 0.0053; Correlation coefficient = 0.9996

*Mean of 3 values

Figure 3.2: Regressed calibration curve for estimation of Etoposide



Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of ETP (10µg/mL, 60µg/mL and 100µg/mL) were subjected to recovery studies as per the procedure described above. The results obtained are tabulated in table 3.3.

Table 3.3: Evaluation of accuracy and precision of the estimation method of Etoposide

Theoretical Concentration of ETP (µg/mL)	Determined Value (µg/mL)	Coefficient of variance (CV)	Relative mean error	Confidence limits*
10	10.13	1.6867	0.0853	10.13 ± 0.2715
60	61.30	0.5186	0.1554	61.30 ± 0.4943
100	99.23	0.3203	0.1601	99.23 ± 0.5090

** At 95% Confidence level; t_{tab} = 3.18 for 4 degrees of freedom*

3.1.2 Estimation of Etoposide in NPs

To determine the amount of ETP entrapped in the NPs, 2mg of NPs were added to 2 ml of acetonitrile and subjected to shaking at room temperature for 4hrs for complete dissolution of PLGA for extraction of the drug from the nanoparticles. The resulting system was centrifuged at 5000rpm for 15mins to remove the precipitated components. The supernant was further diluted with acetonitrile and estimated at 283nm.

3.1.3 Estimation of Etoposide for in-vitro release

The release studies for etoposide 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 15000rpm 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:

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

3.2 Estimation of Temozolomide

3.2.1 Estimation of Temozolomide in solution

The estimation of temozolomide was performed by HPLC method suggested by Huang G. et. al, 2007. The drug was estimated using a Shimadzu HPLC system (Shimadzu, Japan). The HPLC system was composed of a pump (LC-20AT prominence, Shimadzu) , a sample 20-µl loop injector (Rheodyne 7725) and a UV-visible spectrophotometric detector (SPD-20A prominence, Shimadzu).The separation was carried out on a Phenomenax C 18 250 x 4.6 mm HPLC column (Phenomenax) having particle size of 5µm. Mobile phase for Temozolomide consisted of methanol: acetic acid (0.5%) (10:90), UV detection wavelength was 330nm and mobile phase flow rate 1 ml/min. The retention time of temozolomide was 9.3mins.

Preparation of standard stock solutions of Temozolomide in mobile phase

50 mg of Temozolomide was accurately weighed using single pan electronic balance and transferred to 50 ml volumetric flask. 25 ml of methanol: acetic acid (0.5%) (10:90) [mobile phase] 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 mobile to produce 1000 µg per ml of Temozolomide.

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 mobile phase to prepare stock solution of 100 µg per ml of Temozolomide.

Preparation of calibration curve of Temozolomide:

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 1, 2, 4, 6, 8, 10, 20 and 25 µg/ml and analyzed in above mentioned HPLC system. The above procedure was repeated three times. The data was recorded in Table 3.4 along with standard deviation. Figures 3.3, 3.4 show the HPLC chromatogram and calibration curve respectively for Temozolomide in mobile phase.

Figure 3.3: HPLC Chromatogram for Temozolomide

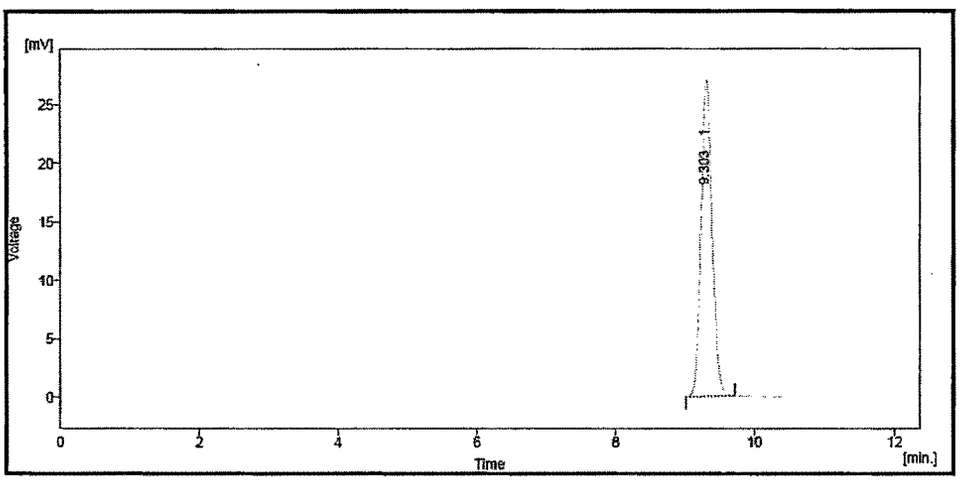


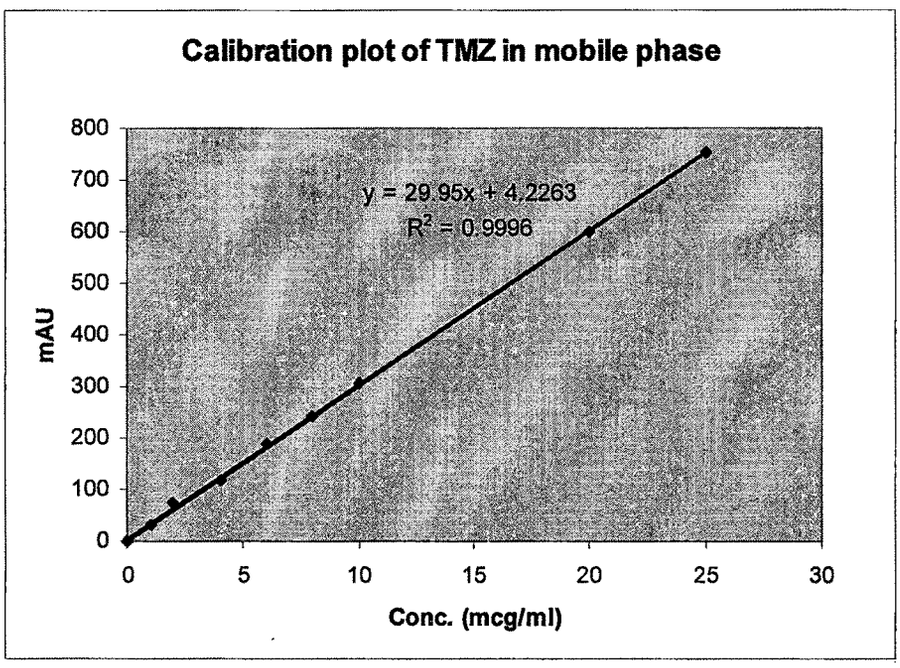
Table 3.4: Calibration for Temozolomide

Concentration (µg/ml)	Mean area(mAU)* ± S.D
0	0
1	31.46 ± 0.026
2	74.22 ± 0.012
4	117.6 ± 0.017
6	188.64 ± 0.010
8	241.62 ± 0.013
10	305.82 ± 0.012
20	601.27 ± 0.012
25	753.61 ± 0.014

*Mean of 3 values

Regression equation** Y= 29.95x + 4.2263; Correlation coefficient R^2= 0.9996

Figure: 3.4 Regressed calibration curve for estimation of Temozolomide



Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of TMZ (2µg/mL, 8µg/mL and 20µg/mL) were subjected to recovery studies as per the procedure described above. The results obtained are tabulated in table 3.5.

Table 3.5: Evaluation of accuracy and precision of the estimation method of Temozolomide

Theoretical Concentration of TMZ (µg/mL)	Determined Value (µg/mL)	Coefficient of variance (CV)	Relative mean error	Confidence limits*
2	2.11	4.4742	0.0471	2.11 ± 0.1499
8	8.07	1.6163	0.0652	8.07 ± 0.2074
20	19.85	0.6503	0.0645	19.85 ± 0.2052

** At 95% Confidence level; t_{tab} = 3.18 for 4 degrees of freedom*

3.2.2 Estimation of Temozolomide in NPs

To determine the amount of TMZ 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.

3.2.3 Estimation of Temozolomide for in-vitro release

The release studies were conducted as per method reported by Zhang H, Gao S 2007. The release studies for temozolomide nanoparticles in phosphate buffer saline pH 7.4. 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 15000rpm for 30min. The residue (settled nanoparticles) were collected and dissolved in acetonitrile and analyzed as per the method above

3.3. Estimation of 6-Coumarin

3.3.1 Estimation of 6-coumarin in solution

All fluorimetric estimations were performed on a Shimadzu RF-540 spectrofluorometer (Shimadzu Coporation, Japan) equipped with a xenon lamp. The ordinate scale was kept at setting 1 and the abscissa scale at 2. The speed of the scanning was kept at fast. The excitation was set at 430nm and the emission was scanned in the range of 450-550nm and determined to be 485 nm.

Preparation of Calibration Plot in acetonitrile

Principal stock solution (100µg/ml) of 6-coumarin in acetonitrile was prepared by accurately weighing 5mg of 6-coumarin in 50mL of solvent mixture. After ensuring that the 6-coumarin has totally dissolved, different aliquots were transferred to 10ml volumetric flasks. The volume was made upto 10ml to yield a final concentration of 0.02, 0.05, 0.08, 0.1, 0.15, 0.2µg/ml. The

contents were shaken well and the relative fluorescence intensity was measured setting the $\lambda_{excitation}$ at 430nm and the corresponding $\lambda_{emission}$ peak intensity was measured at 485 (slit widths as mentioned above) using a Shimadzu RF-540 spectrofluorometer (Shimadzu Corporation, Japan) against suitable blank. The above procedure was repeated three times and the mean relative fluorescence intensity values were determined. The data was recorded in Table 3.6 along with standard deviation. Figures 3.5 show fluorimetry curve and calibration curve respectively for 6-coumarin in acetonitrile.

Figure 3.5: Fluorimetry curve for λemission for 6-coumarin

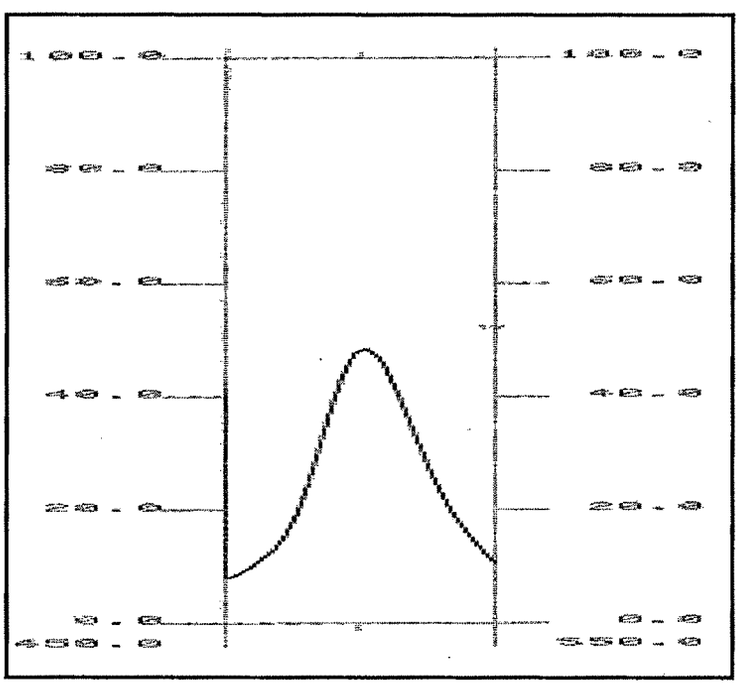


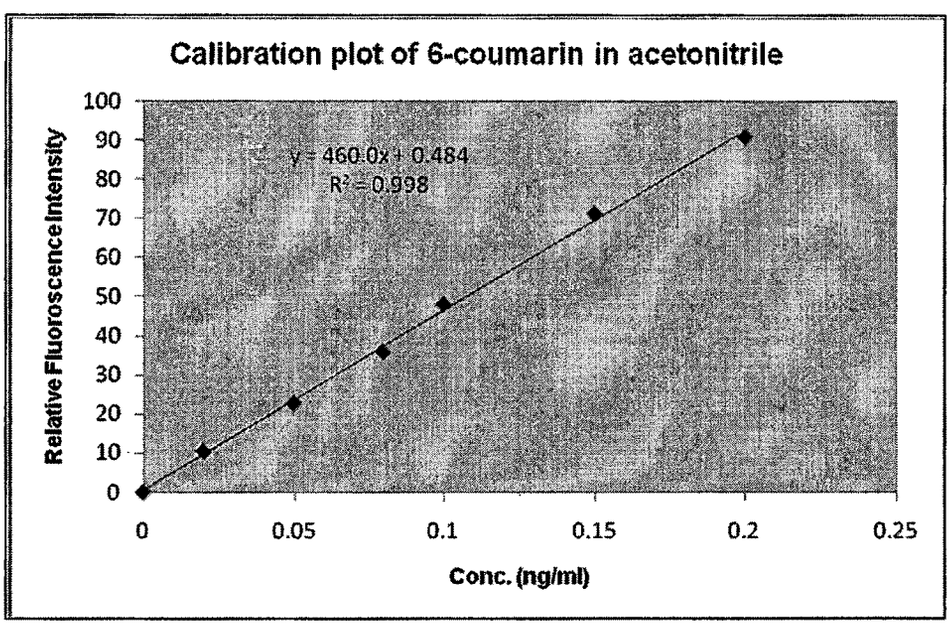
Table 3.6: Calibration for 6-Coumarin in Acetonitrile

Concentration (μg/ml)	Mean relative fluorescence intensity* ±S.D
0	0
0.02	10.4 ± 0.200
0.05	22.80 ± 0.557
0.08	35.10 ± 0.700
0.1	48.13 ± 0.551
0.15	71.17 ± 1.405
0.2	92.73 ± 1.922

*Mean of 3 values

Regression equation** Y= 460.0X + 0.484; Correlation coefficient R^2= 0.998

Figure 3.6: Regressed calibration curve for estimation of 6-coumarin



Accuracy and Precision

In order to determine the accuracy and precision of the developed method, known amounts of 6-coumarin (0.02ng/mL, 0.08ng/mL and 0.15ng/mL) were subjected to recovery studies as per the procedure described above. The results obtained are tabulated in table3.7

Table 3.7: Evaluation of accuracy and precision of the estimation method of 6-coumarin

Theoretical Concentration of 6-coumarin (ng/mL)	Determined Value (ng/mL)	Coefficient of variance (CV)	Relative mean error	Confidence limits*
0.02	0.022	5.655307	0.0007	0.021 ± 0.002
0.08	0.081	1.558273	0.0006	0.081± 0.002
0.15	0.148	1.692047	0.0012	0.148 ± 0.004

** At 95% Confidence level; t_{tab} = 3.18 for 4 degrees of freedom*

3.3.2 Estimation of 6-coumarin in NPs

To determine the amount of 6-coumarin entrapped in the NPs, 2mg of NPs were added to 2 ml of acetonitrile and subjected to shaking at room temperature for 4hrs for complete dissolution of PLGA. The resulting solution was centrifuged at 5000rpm for 15mins to remove the precipitated components. The supernant was further diluted with acetonitrile and estimated as per the fluorimetric method developed above.

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 (D.P. Joshi 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. .

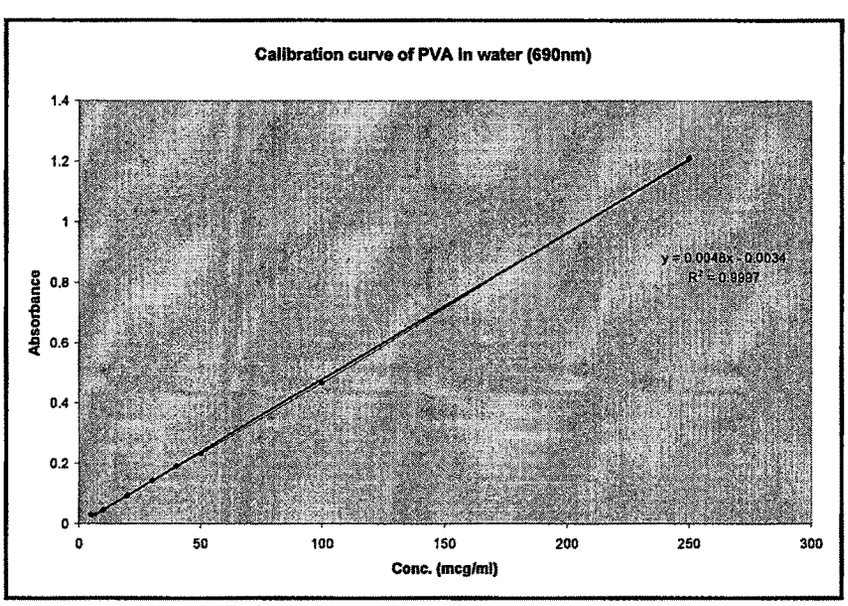
Table 3.8: Calibration Curve of PVA (λmax= 690nm)

Concentration (µg/ml)	Mean Absorbance ± SD (n=3)
5	0.030 ± 0.004
10	0.045 ± 0.00536
20	0.094 ± 0.007
30	0.143 ± 0.01
40	0.191 ± 0.003
50	0.232 ± 0.006
100	0.468 ± 0.006
250	1.210 ± 0.03

*Mean of 3 values

Regression equation** Y= 0.0048X-0.0034; Correlation coefficient R^2= 0.9997

Figure 3.7: Regressed calibration curve for determination of residual PVA



3.5 Determination of protein (transferrin) 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). 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 3.9.

2. 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.

3. 0.2 ml of each standard or unknown sample was taken into labeled test tubes. 0.2 ml of the diluent (dist water) was taken for blank reading.

4. 2 ml of the BWR was added to each test tube and mixed well.

5. All the test tubes were incubated at 60°C for 30 min.

6. 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 3.10

7. A standard curve was prepared by plotting the average absorbance reading for each Transferrin standard vs. its concentration in µg/ml and plotted as shown in figure 3.8

8. Using this standard curve, the protein concentration for each unknown sample was determined.

Table: 3.9 Preparation of diluted Transferrin standards

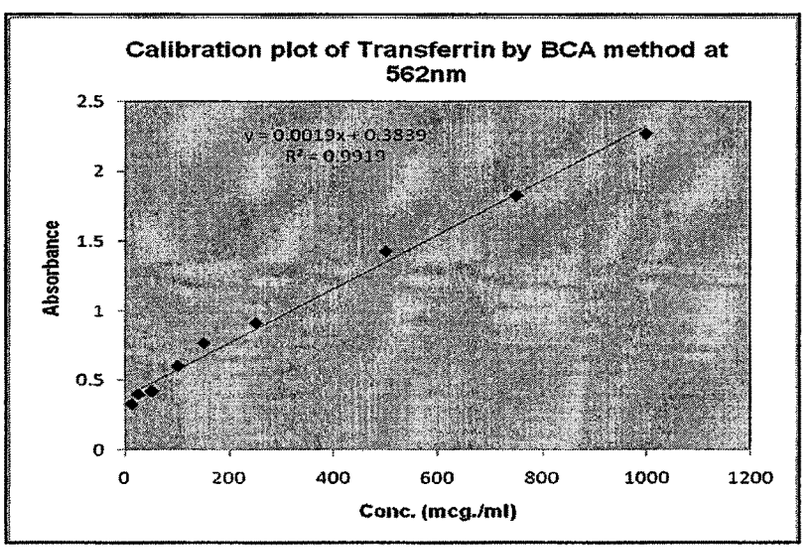
Volume of the Transferrin solution	**Volume of Diluent**	**Final Transferrin concentration**
300 µl of stock	1200 µl	1000 µg/ml (**A**)
375 µl of (A)	125 µl	750 µg/ml (**B**)
250 µl of (A)	250 µl	500 µg/ml (**C**)
125 µl of (A)	375 µl	250 µg/ml (**D**)
75 µl of (A)	425 µl	150 µg/ml (**E**)
50 µl of (A)	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: 3.10 Calibration of transferrin by BCA method

Sample	Concentration µg/ml	Mean Absorbance* ± S.D at 562nm
A	1000	1.584 ± 0.18
B	750	1.302 ± 0.26
C	500	1.025 ± 0.093
D	250	0.551 ± 0.072
E	150	0.347 ± 0.077
F	100	0.247 ± 0.064
G	50	0.170 ± 0.049
H	25	0.120 ± 0.023
I	12.5	0.099 ± 0.010

*Mean of 3 values;
Regression equation; Y = 0.0019x + 0.3839
Correlation coefficient; R^2 = 0.9919

Figure 3.8 Regressed calibration curve of Transferrin by BCA method



3.6 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).

3.7 Morphological characterization

The morphology of the nanoparticles was analyzed using TEM (Transmission Electron Microscopy). Aqueous nanoparticle suspension was negatively stained with phosphotungstic acid (0.5%). Samples were then observed with Morgagni, Philips, Eindhoven, Netherlands

3.8 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.

3.9 Discussion

ETP in nanoparticles and in-vitro release medium was estimated by UV spectrophotometry method. TMZ in nanoparticles, invitro release medium was estimated using HPLC method. Transferrin was estimated by BCA protein assay. The estimation of 6-coumarin in nanoparticles was performed using spectrofluorimetry.

The estimation in the nanoparticles, invitro release studies for ETP and TMZ was performed by UV spectrophotometry and HPLC method respectively. The calibration curve of Etoposide was established in acetonitrile by UV spectrophotometry at 283nm. The linearity of Etoposide was found to be 5-120µg/ml (R^2=0.9996). The recovery studies for accuracy and precision were carried out at 10, 60 and 100 µg/ml and the recovery was found to be more than 90%, indicating the reliability of the method. 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 15000 rpm and the settled nanoparticles were dissolved in acetonitrile and analysed 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.

For Temozolomide, the calibration curve was established using HPLC with methanol: acetic acid (0.5%) (10:90) as mobile phase and detection at 330nm. The linearity of Temozolomide was found to be 1-25 µg/ml (R^2=0.9996). The recovery studies for accuracy and precision were carried out at 2, 8 and 20 µg/ml and the recovery was found to be more than 90%, indicating the reliability of the method. The drug entrapment efficiency and the invitro drug release were determined in the same manner as Etoposide, except that the drug was analysed using HPLC.

For cellular uptake experiments, a fluorescent dye 6-coumarin was incorporated into the PLGA nanoparticles and further conjugated with transferrin for enhanced uptake in the cell. 6-coumarin in the nanoparticles was estimated using spectrofluorimetric technique with excitation at 435nm and emission at 485nm. The calibration curve was established in acetonitrile and linearity was observed between 0.02-0.2 μg/ml (R^2= 0.998). The recovery studies at 0.02, 0.08 and 0.15 μg/ml showed greater than 90% recovery, indicating the reliability of the method.

The estimation of transferrin conjugation was carried out using BCA protein estimation. The calibration curve was established at 12.5-1000 μg/ml (R^2=0.9919). 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=0.9997)

References

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