3.1. Materials

Temozolomide (TMZ) and Lenalidomide (LND) were obtained as gift sample from Cipla Ltd., Mumbai, India and Apicore Pharmaceuticals Pvt. Ltd., Vadodara, India respectively. Methanol and Acetonitrile of HPLC grade, hydrochloric acid, glacial acetic acid, phosphoric acid and Coomassie Brilliant Blue G-250 were purchased from Spectrochem, Mumbai, India. Disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium chloride, sodium acetate, sodium hydroxide, potassium sulphate, Tris-hydrochloride, dipotassium EDTA dihydrate and cetyltrimethylammonium bromide (CTAB) were purchased from SD fine chemicals Pvt. Ltd., Mumbai, India. Fluorescamine and chondroitin sulphate (CS) was purchased from Himedia, Mumbai, India. Hyaluronic acid was obtained as gift sample from Novozymes, Denmark. All chemical and reagents were of analytical grade. Plasma was procured from Suraktam Blood Bank, Vadodara, India.

3.2. Equipments

- UV Visible spectrophotometer 1800 (Shimadzu, Japan)
- Spectrofluorophotometer RF-5301 (Shimadzu, Japan)
- Digital Analytical Balance (Shimadzu, SCS, Switzerland)
- pH meter (Lab India, India)
- High pressure liquid chromatography system (HPLC) (Shimadzu, Shimadzu Corporation, Kyoto, Japan)
- Model 680 XR microplate reader (Bio-Rad Laboratories (India) Pvt.Ltd., India)

3.3 Preparation of reagents (1)

3.3.1 Phosphate buffer saline (PBS) pH 7.4

Dissolved 2.38g of disodium hydrogen phosphate , 0.19g of potassium dihydrogen phosphate and 8.0g of sodium chloride in sufficient water to produce 1000ml. Adjusted the pH to 7.4, if necessary by adding 0.1N HCL or 0.1N NaOH.

3.3.2 Sodium acetate buffer pH 5.5

Dissolved 272 g of sodium acetate in500 ml of water by heating to 35º, cooled and added slowly 50 ml of glacial acetic acid and sufficient water to produce 1000 ml. Adjusted the pH, if necessary.

3.3.3 Phosphate buffer pH 5.5

Solution I — Dissolved 13.61 g of potassium dihydrogen phosphate in sufficient water to produce 1000 ml.
Solution II — Dissolved 35.81 g of disodium hydrogen phosphate in sufficient water to produce 1000 ml.
Then 96.4 ml of solution I and 3.6 ml of solution II were mixed to produce 100 ml of buffer.

3.4 Estimation of TMZ by UV-visible spectrophotometry

3.4.1 Calibration curve of TMZ in water

TMZ (10 mg) was dissolved in 10 ml of double distilled water (DD water) to obtain standard stock solution of 1000 µg/ml. From this stock solution, working standard solution (100µg/mL) was prepared by taking 1 ml of aliquot and volume was made up to 10 ml with water. Aliquots of TMZ in range of 0.2 ml-1.6 ml were transferred to 10 ml volumetric flask and the volume was adjusted upto mark with DD water to get concentration ranging from 2.0 µg/ml- 16.0 µg/ml. The absorbance of the above solutions was taken at 330 nm against DD water as the blank. A graph of absorbance vs. concentration was plotted. The readings were recorded in triplicate (n=3).

3.4.2 Calibration curve of TMZ in acetate buffer pH 5.5

TMZ (10 mg) was dissolved in 10 ml of acetate buffer (pH 5.5) to obtain standard stock solution of 1000 µg/ml. From this stock solution, working standard solution (100µg/ml) was prepared by taking 1 ml of aliquot and volume was made up to 10 ml with buffer. From working standard solution, aliquots of TMZ in range of 0.2 ml-1.2 ml were transferred to 10 ml volumetric flask and the volume was adjusted upto mark with buffer to get concentration ranging from 2.0 µg/ml-12.0 µg/ml. The absorbance of the above solutions was taken at 330 nm against acetate buffer as

the blank. A graph of absorbance vs. concentration was plotted. The readings were recorded in triplicate (n=3).

3.5 Estimation of TMZ in plasma by double salting out assisted liquid- liquid extraction high pressure liquid chromatography (SALLE-HPLC)

Various techniques have been used for estimation of TMZ in plasma or biological samples like high-performance liquid chromatography with UV detection (HPLC-UV), micellar electrokinetic capillary chromatography (MKEC) and liquid chromatography coupled to mass spectroscopy (LC-MS). Irrespective of the technique used for estimation of the drug; drug extraction from plasma is performed by using precipitation, Solid Phase Extraction (SPE) and Liquid-Liquid Extraction (LLE) techniques (2,3). However; with the above methods several drawbacks like low recovery, low sensitivity, low resolution or high cost involvement are also associated. So for overcoming the draw backs of existing method, Jain et al. (2014) (4) developed and validated a cost effective, robust and low plasma component interfering HPLC method using salting out liquid–liquid extraction (SALLE) technique and same method was utilized in current research to estimate TMZ in plasma samples.

3.5.1 Instrument and chromatographic condition

(A) HPLC System

HPLC: Shimadzu (Shimadzu Corporation, Kyoto, Japan) chromatographic system equipped with Shimadzu LC-20AT pump
Detector: Shimadzu SPD-20AV absorbance detector.
Software: LC-Solution

(B) Chromatographic conditions

Mode of detection: Reverse phase high performance Liquid chromatography
Columns: ODS- Hypersil (Particle size 5µm, 4.6 mm x 250mm) and ODS- Hypersil guard column
Mobile Phase: Acetonitrile: 0.1% v/v acetic acid (10:90)
λ max: 255 nm

Injection volume: 20µl (Rheodyne 7725 injector valve with fixed loop at 20µl)

Flow rate: 1ml/min

(C) Mobile phase preparation

Solvent A was Acetonitrile and solvent B was 0.1% v/v acetic acid. Solvent A and Solvent B were mixed in a ratio 10:90 and were degassed via ultrasonic water bath prior to use.

3.5.2 Double salting out assisted liquid-liquid extraction (SALLE)

In this method, two salts (sodium chloride and potassium sulfate) were utilized to extract drug from plasma samples. Briefly, 0.5 ml of plasma was spiked with 1 ml of TMZ solution (10µg/ml) solubilized in 0.1% acetic acid solution in a 10 ml glass tube. Then 0.5 ml of sodium chloride (1 M solution) was added to this solution and subjected to vortex for 2 min. In the next step, 2 ml of acetonitrile was added and centrifuged at 5000 rpm for 10 min and obtained supernatant was separated. Then in the supernatant, 0.5 ml potassium sulfate (1 M solution) was added and subjected to vortex for 2 min and centrifuged at 5000 rpm for 20 min to separate plasma proteins and other residual materials. Then supernatant was collected and injected into HPLC system with above mentioned chromatographic conditions to analyze the samples (4).

3.6 HPLC method II

Preparation of calibration curve of TMZ in (0.5% glacial acetic acid V/V) 90 ml: 10 ml (methanol) mobile phase

3.6.1 Instrument and chromatographic condition

(A) Instrument

HPLC: Shimadzu (Shimadzu Corporation, Kyoto, Japan) chromatographic system equipped with Shimadzu LC-20AT pump

Detector: Shimadzu SPD-20AV absorbance detector.

Software: LC-Solution

(B) Chromatographic conditions:

Column: C8 Oyster column (250mm x 4.6mm)(5 µm)

Flow rate: 1ml/min

Run time: 10min

ƛmax : 330nm, 254nm

Mode: isocratic

Injection volume: 20µl **(**Rheodyne 7725 injector valve with fixed loop at 20 µl)

Diluent: (0.5% Glacial acetic acid V/V) 90ml: 10ml (methanol)

(C) Mobile phase preparation

Solution A: 0.5% V/V Glacial acetic acid

Solution B: Methanol

Mix solution A and B in ratio of 90:10

3.6.2 Preparation of calibration curve of TMZ in plasma

TMZ (10 mg) was dissolved in 10ml of acetate buffer (pH 5.0) to prepare stock solution. Suitable aliquots (0.4 ml, 0.8ml, 1.2ml, 1.6ml and 2.0ml) of the stock solution were pipetted into 10 ml of volumetric flasks and the volume was made up to 10 ml with buffer to give final concentrations. Then from above solution, 20 µl aliquots were pipetted into 1.5 ml of microcentrifuge tubes and 180µl plasma was added in each tube. In the next step, 200 µl acetonitrile was added in all tubes to precipitate plasma, vortex and centrifuged. Then 20 µl of each supernatant was injected in HPLC and analyzed using above mentioned chromatographic conditions (3).

3.6.3 Preparation of calibration curve of TMZ in brain homogenate

TMZ (10 mg) was dissolved in 10ml of acetate buffer (pH 5.0) to prepare stock solution. Suitable aliquots (0.4 ml, 0.8ml, 1.2ml, 1.6ml and 2.0ml) of the stock solution were pipetted into 10 ml of volumetric flasks and the volume was made up to 10 ml with buffer to give final concentrations. Then from above solution, 20 µl aliquots were pipetted into 1.5 ml of microcentrifuge tubes and 180µl brain homogenate was added in each tube. In the next step, 200 µl acetonitrile was added in all tubes to precipitate plasma, vortex and centrifuged. Then 20 µl of each supernatant was injected in HPLC and analyzed using above mentioned chromatographic conditions (3).

3.7 Estimation of LND by UV-visible spectrophotometer

3.7.1 Calibration curve of LND in methanol: water (50:50)

LND (10 mg) was dissolved in 10 ml of methanol: water (50:50) mixture to obtain standard stock solution of 1000 µg/ml. From this stock solution, working standard solution (100µg/ml) was prepared by taking 1 ml of aliquot and volume was made up to 10 ml with methanol: water (50:50) mixture. From working standard solution, aliquots of LND in range of 0.5 ml-5.5 ml were transferred to 10 ml volumetric flask and the volume was adjusted upto mark with methanol: water (50:50) mixture to get concentration ranging from 5.0 µg/ml- 55.0 µg/ml. The absorbance of the above solutions was taken at 305 nm against methanol: water (50:50) mixture as the blank. A graph of absorbance vs. concentration was plotted. The readings were recorded in triplicate (n=3).

3.8 Estimation of LND by Spectrofluorophotometer

For the estimation of LND, various analytical methods have been developed viz. capillary electrophoresis method with photodiode array detector (5), High-performance liquid chromatography (6), LC–MS/MS (7) and HPLC with fluorescence detector (8). Apart from all these methods, spectrofluorometry method has been also reported and found to be useful for the determination of trace amounts of LND in different pharmaceuticals along with several advantages such as low detection limit, high sensitivity and the use of conventional instrument (9). Darwish *et al.* also developed spectroflurometric method for determination of LND in distilled water which is highly sensitive (10). Hence, for determination of LND in different buffers (PBS pH 7.4 and Phosphate buffer pH 5.5) same method was utilized with slight modification.

3.8.1 Preparation of stock solution and working stock solution of LND

About 5 mg of LND was accurately weighed and transferred into 5 ml of volumetric flask, dissolved in buffer (PBS pH 7.4 and phosphate buffer pH 5.5) and made up volume with the same buffer to produce a stock solution of 1000 µg/ml. From the stock solution, working stock solution of LND (100 µg/ml) was prepared.

3.8.2 Fluorescamine (FLC) solution

FLC (2.5 mg) was weighed and transferred into a 10-ml volumetric flask, dissolved in acetone and completed to volume with the same solvent to produce a stock solution of 0.025% (w/v). The solution was freshly prepared, stored at -20°C in amber colored container and used within seven days.

3.8.3 Procedure

Different aliquots of LND working stock solution (100 μg/ml) were transferred into separate 10-mL volumetric flasks to obtain a series of LND standard solutions covering the working range of 0.01–5.0 μg/ml in the final solution. Then 1 ml of FLC solution (0.025% w/v) was added to each flask and kept for 10 min at room temperature (25 ± 2°C) to forwarded the reaction, and then volume was made up to 10 ml with buffer (PBS pH 7.4 and phosphate buffer pH 5.5). The resulting solutions were analyzed by spectrofluorometer by measuring the fluorescence intensity at 494 nm after excitation at 381 nm against a reagent blank (10).

3.9 Estimation of HA and CS using CTAB turbidimetric method

CTAB turbidity method for estimation of HA was firstly described by (Scott, 1960). The method was based on the formation of insoluble complex between HA and organic ammonium cations such as CTAB. This insoluble complex produced turbidity which was related to polymer concentration and can be titrated by spectrophotometry (11). The CTAB turbidimetric method was even demonstrated to be more accurate, sensitive and specific than the carbazole method (12). So for the estimation of free HA and CS, this method is utilized.

3.9.1 Procedure

Estimation of free HA and CS after conjugation with nanoparticles were determined by previously reported CTAB turbidimetric method (13,14) with slight modifications. The CTAB (10 mM) was dissolved in 100 ml of NaOH (2% w/v) to prepare reagent. In the next step, 50 μl of HA/CS standard solutions (0.1- 1.0 mg/ml) were added into 96 well plates filled with 50 μl of 0.2 M sodium acetate buffer pH 5.5. The plates were then incubated at 37°C for 15 min. Subsequently, 100 μl of CTAB reagent at 37°C was added to each well which gave final concentration range 25 μg/ml – 250 μg/ml and absorbance was read at 570 nm within 10 min.

against the blank (HA/CS solution replaced by 0.2 M sodium acetate buffer pH 5.5) using microplate reader (Model 680 XR Microplate reader). The obtained absorbance was plotted against HA concentrations to generate calibration curve. The slope of the standard curve was obtained by the linear regression analysis. The amount of free HA and CS was later determined using regression equation.

3.10 Estimation of Lactoferrin (Lf) using Bradford Assay

3.10.1 Preparation of reagents (15)

(A) Bradford reagent

Coomassie Brilliant Blue G-250 (5.0 mg) was dissolved in 5 ml of methanol and 10 ml of 85% w/v phosphoric acid was added in it. The acid solution was slowly added into 85 ml of double distilled water and allowed the dye to dissolve completely. Then the dye solution was filtered using Whatman filter paper to remove precipitates just before use. The dye solution was stirred in dark bottle at 4°C till use.

(B) 1M Tris hydrochloride (Tris-HCl) solution (pH 7.4)

Tris-HCl (1.576 g) was dissolved in 8 ml of double distilled water and adjusted the pH if required and volume was made up to 10 ml with double distilled water.

(C) 0.5 M EDTA solution

Dipotassium EDTA dihydrate (2.02g) was dissolved in 8 ml of double distilled water. pH of the solution was adjusted to 7.4 using NaOH and finally volume was make up to 10 ml with double distilled water.

(D) Tris-EDTA buffer (pH 7.4)

Measured out 1 ml of 1M Tris-HCl solution (pH 7.4) and added to 100 ml of bottle. Then 0.2 ml of 0.5M EDTA solution (pH 7.4) was added in the bottle and volume was made up to 100 ml with double distilled water to prepare Tris-EDTA buffer (pH 7.4).

3.10.2 Procedure

Lf standard stock solution (2 mg/ml) was prepared in Tris-EDTA buffer (pH 7.4). From the stock solution, working standard solution in range of 0.05 – 1.5 mg/ml was prepared. Then 100 µl of working solutions were taken in different volumetric flasks and 3 ml of Bradford reagent was added in each sample, vortex and incubated at room temperature for5-45 min for the formation of protein- dye complex. Then absorbance was measured at 595 nm within one hr using UV-visible spectrophotometer (15,16) .

3.11 Estimation of albumin (BSA) using Bradford protein assay

BSA standard stock solution (2 mg/ml) was prepared in Tris-EDTA buffer (pH 7.4). From the stock solution, working standard solution in range of 0.05 – 1.5 mg/ml was prepared. Then 100 µl of working solutions were taken in different volumetric flasks and 3 ml of Bradford reagent was added in each sample, vortex and incubated at room temperature for5-45 min for the formation of protein- dye complex. Then absorbance was measured at 595 nm within one hr using UV-visible spectrophotometer (15) .

3.12. Results and discussion

3.12.1 Estimation of TMZ by UV-visible spectrophotometry

3.12.1.1 Calibration curve of TMZ in water

TMZ showed a characteristic spectrum when scanned in ultraviolet range between 200-400 nm. The λmax was found to be 330 nm and linearity range was found to be 2.0 – 16.0 µg/ml (Table 3.1). Regression analysis was performed on the experimental data and results are shown in table 3.2 and figure 3.1. Results of regression analysis indicated linear relationship between absorbance and concentration of TMZ.

Table 3.1: Calibration curve of TMZ in DD water by UV-visible spectrophotometer

Sr. No.	Concentration (µg/ml)	Absorbance mean ± SD
1	2.0	0.17572 ± 0.0015
2	4.0	0.27827 ± 0.0020
3	6.0	0.37439 ± 0.0023
4	8.0	0.47171 ± 0.0029
5	10.0	0.59511 ± 0.0031
6	12.0	0.71262 ± 0.0035
7	14.0	0.84122 ± 0.0029
8	16.0	0.93266 ± 0.0031

Table 3.2: Calibration data of TMZ in DD water

Solvent	Λmax	Range	Regression Equation	Regression Coefficient (R^2)
Water	330 nm	2-16 µg/ml	y = 0.055x + 0.052	R^2=0.997

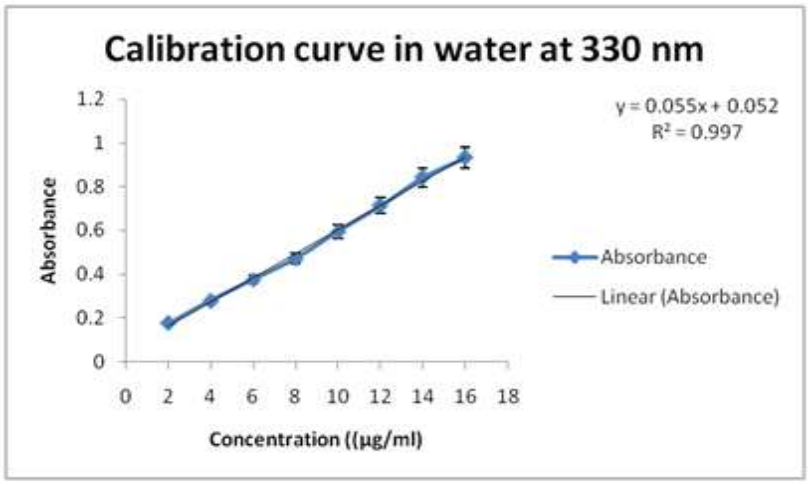

Figure 3.1: Calibration plot of TMZ in DD water

3.12.1.2 Calibration curve of TMZ in acetate buffer pH 5.5

Calibration curve of TMZ in acetate buffer pH 5.5 was prepared using UV –visible spectrophotometer and TMZ showed a characteristic spectrum when scanned in ultraviolet range between 200-400 nm. The λmax was found to be 330 nm and linearity range was found to be 2.0

– 12.0 µg/ml (Table 3.3). Regression analysis was performed on the experimental data and results are shown in table 3.4 and figure 3.2. Results of regression analysis indicated linear relationship between absorbance and concentration of TMZ.

Table 3.3: Calibration curve of TMZ in acetate buffer (pH 5.5) by UV-visible spectrophotometer

Sr. No.	Concentration (µg/ml)	Absorbance mean ± SD
1	2.0	0.24505 ± 0.0012
2	4.0	0.38183 ± 0.0017
3	6.0	0.50853 ± 0.0019
4	8.0	0.64175 ± 0.0021
5	10.0	0.77801 ± 0.0018
6	12.0	0.950108 ± 0.0017

Table 3.4: Calibration data of TMZ in acetate buffer pH 5.5

Solvent	**λmax**	**Range**	**Regression Equation**	**Regression Coefficient (R^2)**
Acetate buffer pH 5.5	330 nm	2-12 µg/ml	y = 0.055x + 0.052	R^2=0.997

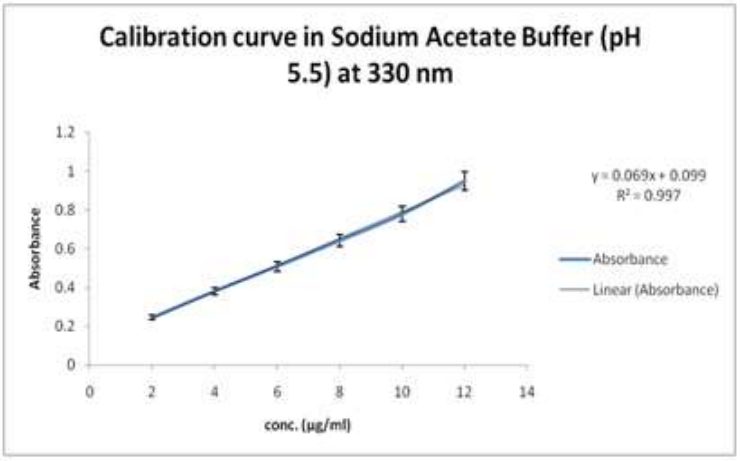

Figure 3.2: Calibration plot of TMZ in acetate buffer pH 5.5

3.12.2 Estimation of TMZ in plasma by SALLE-HPLC

For estimation of TMZ in plasma, SALLE-HPLC method was utilized and linearity range for the same was found to be 0.1 – 20.0 µg/ml (Table 3.5). Regression analysis was performed on the experimental data and results are shown in table 3.6 and figure 3.3. Results of regression analysis indicated linear relationship between area under curve and concentration of TMZ.

Table 3.5: Calibration curve of TMZ in plasma by SALLE-HPLC

Sr. No.	Concentration (µg/ml)	Area mean ± SD
1	0.1	4215.35 ± 225.87
2	0.2	4546.51 ± 300.95
3	0.3	4950.87 ± 380.51
4	0.4	5524.29 ± 285.39
5	0.5	6983.37 ± 280.41
6	1.0	7813.33 ± 236.81
7	2.0	10180.79 ± 295.91
8	10.0	70085.25 ± 640.49
9	20.0	144143.39 ± 773.29

Table 3.6: Calibration data of TMZ in plasma by SALLE-HPLC

Sample	**λmax**	**Range**	**Regression Equation**	**Regression Coefficient (R^2)**
Plasma	255 nm	0.1-20.0 µg/ml	y = 7044x + 1712	R^2=0.997

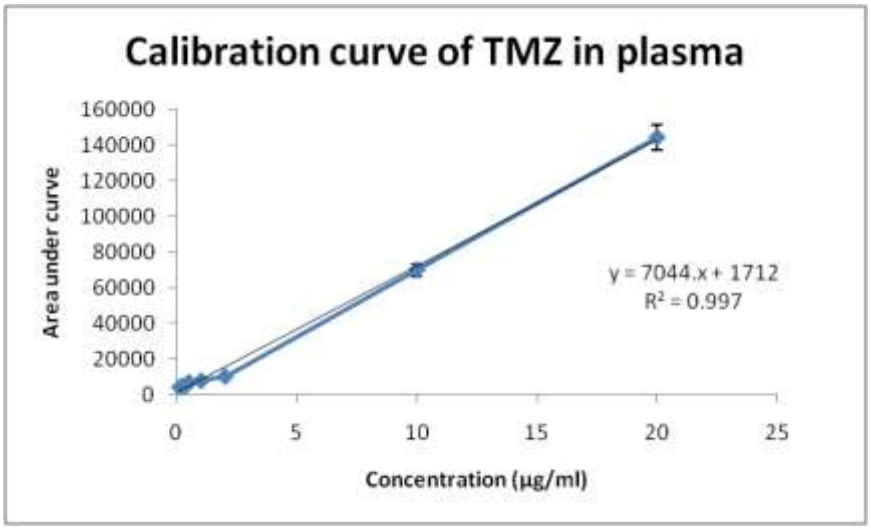

Figure 3.3: Calibration curve of TMZ in plasma by SALLE-HPLC

3.12.3 Estimation of TMZ in plasma and brain homogenate by HPLC method II

3.12.3.1 Estimation of TMZ in plasma by HPLC method II

For estimation of TMZ in plasma, another HPLC method was also utilized in which linearity range was found to be 2.0 – 10.0 µg/ml (Table 3.7). Regression analysis was performed on the experimental data and results are shown in table 3.8 and figure 3.4. Results of regression analysis indicated linear relationship between area under curve and concentration of TMZ.

Table 3.7: Calibration curve of TMZ in plasma by HPLC method II

Sr. No.	Concentration (µg/ml)	Area mean ± SD
1	2.0	138687±264.7288
2	4.0	240735±566.9095
3	6.0.	336801±565.3436
4	8.0	475439±396.1519
5	10.0	595472±268.0902

Table 3.8: Calibration data of TMZ in plasma by HPLC method II

Sample	λmax	Range µg/ml	Regression Equation	Regression Coefficient (R^2)
Plasma	330 nm	2.0-10.0	y = 57414x + 12945	R^2=0.995

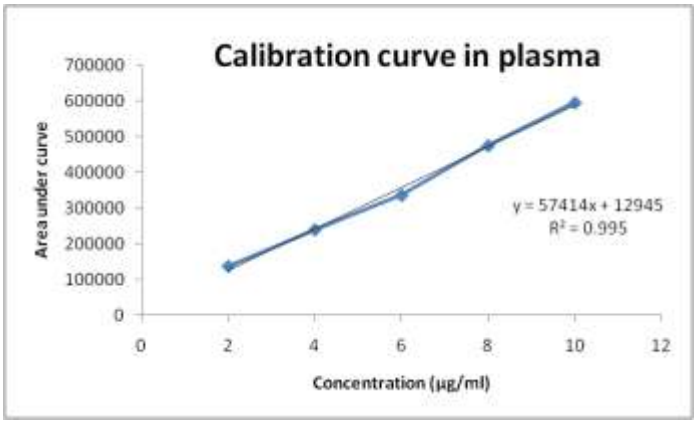

Figure 3.4: Calibration curve of TMZ in plasma by HPLC method II

3.12.3.2 Estimation of TMZ in brain homogenate by HPLC method II

For estimation of TMZ in brain homogenate, HPLC method was utilized in which linearity range was found to be 200.0– 1200.0 ng/ml (Table 3.9). Regression analysis was performed on the experimental data and results are shown in table 3.10 and figure 3.5. Results of regression analysis indicated linear relationship between area under curve and concentration of TMZ.

Table 3.9: Calibration curve of TMZ in brain homogenate by HPLC method II

Sr. No.	Concentration (ng/ml)	Area mean ± SD
1	200	9213±362.576
2	400	23438±267.176
3	600	31798±275.4312
4	800	43803±262.8884
5	1000	57337±422.855
6	1200	74981±254.225

Table 3.10: Calibration data of TMZ in brain homogenate HPLC method II

Sample	λmax	Range ng/ml	Regression Equation	Regression Coefficient (R^2)
Brain homogenate	330 nm	2000-12000	y = 63.22x -4159.2	R^2=0.9893

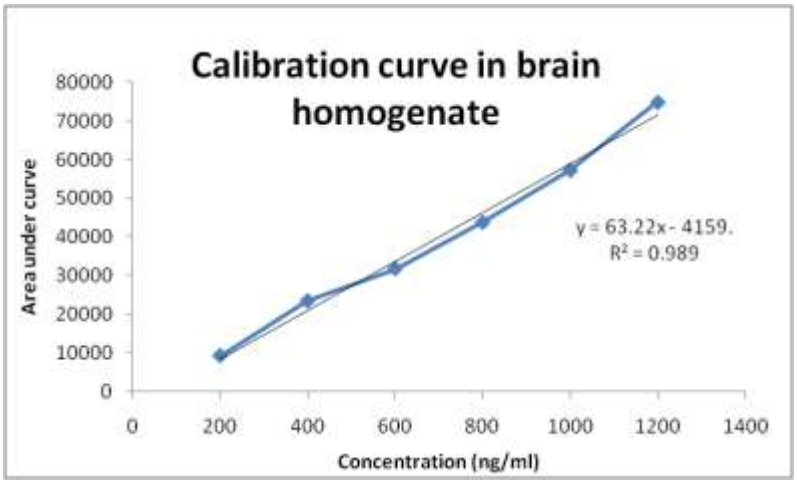

Figure 3.5 Calibration curve of TMZ in brain homogenate HPLC method II

3.12.4 Estimation of LND by UV-visible spectrophotometer

3.12.4.1 Calibration curve of LND in methanol: water (50:50) by UV-visible spectrophotometer

For estimation of LND, calibration curve of LND in methanol: water (50:50) was prepared using UV-visible spectrophotometer. LND showed a characteristic spectrum when scanned in ultraviolet range between 200-400 nm. The λmax was found to be 305 nm and linearity range was found to be 5.0 – 55.0 µg/ml (Table 3.11). Regression analysis was performed on the experimental data and results are shown in table 3.12 and figure 3.6. Results of regression analysis indicated linear relationship between absorbance and concentration of LND.

Table 3.11: Calibration curve of LND in methanol: water (50:50) by UV-visible spectrophotometer

Sr. No.	Concentration (µg/ml)	Absorbance mean ± SD
1	5.0	0.053 ± 0.009
2	10.0	0.109 ± 0.011
3	15.0	0.168 ± 0.013
4	20.0	0.225 ± 0.007
5	25.0	0.280 ± 0.013
6	30.0	0.333 ± 0.007
7	35.0	0.394 ± 0.015
8	40.0	0.456 ± 0.011
9	45.0	0.505 ± 0.013
10	50.0	0.562 ± 0.009
11	55.0	0.614 ± 0.015

Table 3.12: Calibration data of LND in methanol: water (50:50)

Solvent	λmax	Range	Regression Equation	Regression Coefficient (R^2)
Methanol: water	305 nm	5-55 µg/ml	y = 0.011x - 0.002	R^2=0.999

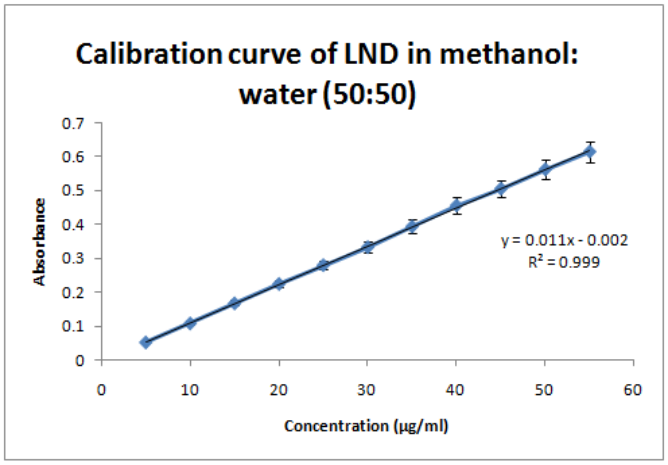

Figure 3.6: Calibration curve of LND in methanol: water (50:50)

3.12.5 Estimation of LND by Spectrofluorophotometer

LND does not contain any native fluorescence group, so for its fluorimetric determination derivatization of LND with fluorogenic reagent was necessary. As FLC forms highly fluorescent derivatives with primary amines under relatively mild reaction conditions it was chosen as a derivatizing reagent. LND reacts with FLC and forms a highly fluorescent derivative that exhibited maximum fluorescence intensity (λem) at 494 nm after excitation at wavelength (λex) of 381 nm (figure 3.7) (10).

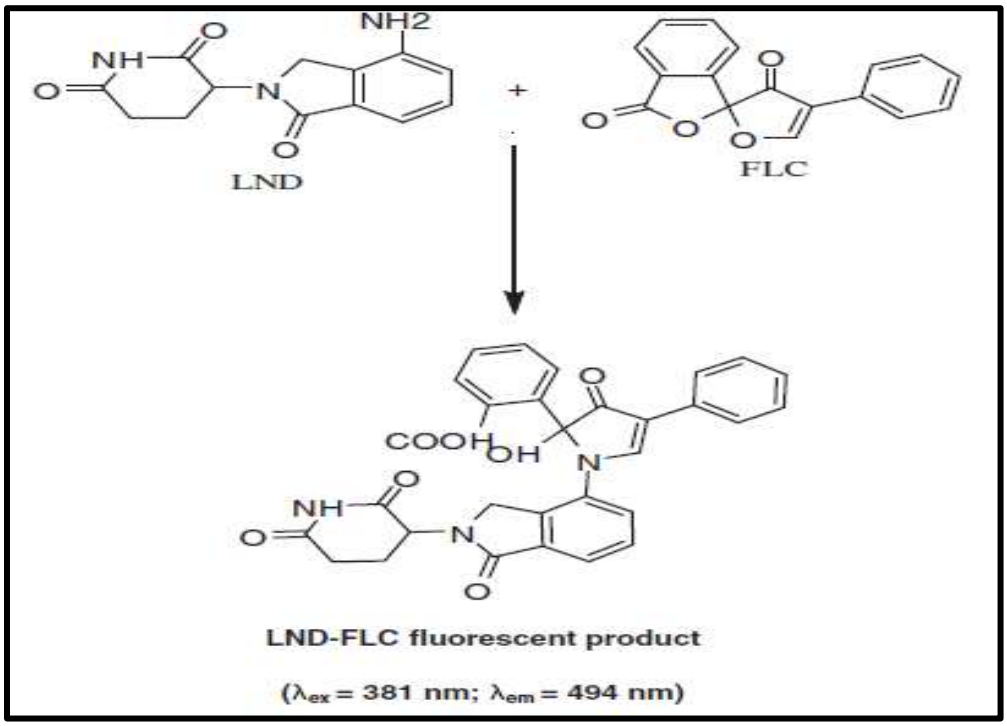

Figure 3.7: Scheme for the reaction pathway between LND and FLC.

3.12.5.1 Calibration curve of LND in PBS 7.4 by Spectrofluorophotometer

For estimation of LND in release media, calibration curve of LND in PBS 7.4 was prepared using Spectrofluorophotometer. The linearity range was found to be 0.05 – 5.0 µg/ml (Table 3.13). Regression analysis was performed on the experimental data and results are shown in table 3.14 and figure 3.8 Results of regression analysis indicated linear relationship between fluorescence intensity and concentration of LND.

Table 3.13: Calibration curve of LND in PBS 7.4 by Spectrofluorophotometer

Sr. No.	Concentration (µg/ml)	Fluorescence intensity (mean ± SD)
1	0.05	23.382 ± 1.019
2	0.1	25.183 ± 1.211
3	0.5	51.746 ± 1.423
4	1.0	85.173 ± 1.571
5	2.0	143.252 ± 1.475
6	3.0	200.312 ± 1.671
7	4.0	260.186 ± 1.549
8	5.0	304.796 ± 1.876

Table 3.14: Calibration data of LND in PBS pH 7.4

Solvent	**Emission**	**Range**	**Regression Equation**	**Regression Coefficient (R^2)**
PBS pH 7.4	490 nm	0.055-5.0 µg/ml	y = 57.56x + 24.66	R^2=0.997

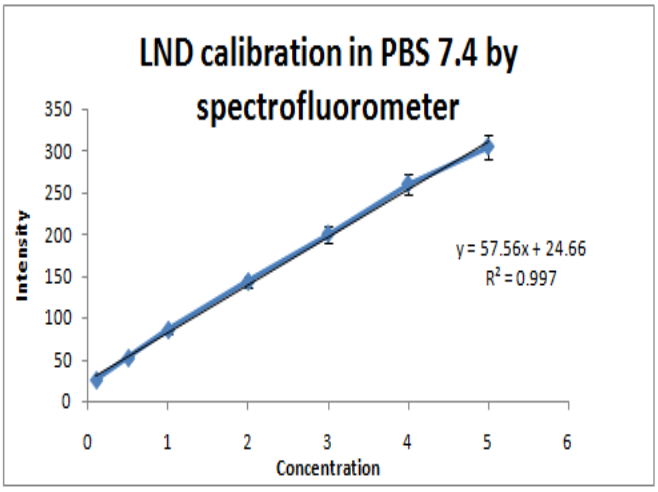

Figure 3.8: Calibration curve of LND in PBS pH 7.4 by Spectrofluorophotometer

3.12.5.2 Calibration curve of LND in phosphate buffer pH 5.5 by Spectrofluorophotometer

For estimation of LND in (tumor environment mimicking) release media, calibration curve was prepared in phosphate buffer pH 5.5 using Spectrofluorophotometer. The linearity range was found

to be 0.1 – 4.0 µg/ml (Table 3.15). Regression analysis was performed on the experimental data and results are shown in table 3.16 and figure 3.9. Results of regression analysis indicated linear relationship between fluorescence intensity and concentration of LND.

Table 3.15: Calibration curve of LND in phosphate buffer pH 5.5 by Spectrofluorophotometer

Sr. No.	Concentration (µg/ml)	Fluorescence intensity (mean ± SD)
1	0.1	26.303 ± 1.019
2	0.5	72.887 ± 1.211
3	1.0	150.465 ± 1.423
4	2.0	308.525 ± 1.571
5	3.0	427.489± 1.475
6	4.0	577.53 ± 1.671
7	5.0	541.89 ± 1.549

Table 3.16: Calibration data of LND in phosphate buffer pH 5.5

Solvent	Emission	Range (µg/ml)	Regression Equation	Regression Coefficient (R^2)
Phosphate buffer pH 5.5	490 nm	0.1-4.0	y = 141.9x + 9.698	R^2=0.998

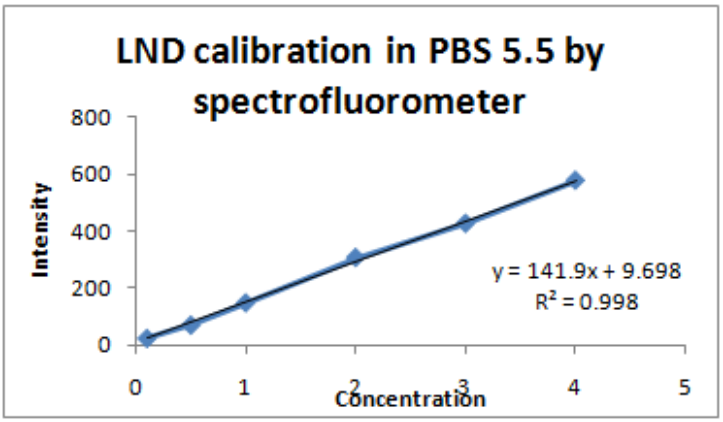

Figure 3.9: Calibration curve of LND in phosphate buffer pH 5.5 by Spectrofluorophotometer

3.12.6 Estimation of HA and CS using CTAB turbidimetric method

For estimation of free HA and CS after conjugation with nanoparticles, CTAB turbidimetric method was utilized and calibration curve was prepared in which linearity range of HA and CS was found to be 25.0– 250.0 µg/ml (Table 3.17 and Table 3.19 respectively). Regression analysis was performed on the experimental data and results are shown in table 3.18 and figure 3.10 (for HA) and table 3.20 and figure 3.11 (for CS). Results of regression analysis indicated linear relationship between absorbance due to turbidity of complex and concentration of polymer (HA and CS)

Table 3.17: Calibration curve of HA by CTAB turbidimetric method

Sr. No.	Concentration (µg/ml)	Absorbance (mean ± SD)
1	25	0.110 ± 0.019
2	50	0.193 ± 0.011
3	75	0.288 ± 0.023
4	100	0.378 ± 0.011
5	125	0.433 ± 0.017
6	150	0.508 ± 0.015
7	175	0.566 ± 0.019
8	200	0.654 ± 0.021
9	225	0.718 ± 0.017
10	250	0.785 ± 0.013

Table 3.18: Calibration data of HA by CTAB turbidimetric method

Solvent	**λmax**	**Range (µg/ml)**	**Regression Equation**	**Regression Coefficient (R^2)**
Acetate buffer	570 nm	25-250	y = 0.003x + 0.056	R^2=0.996

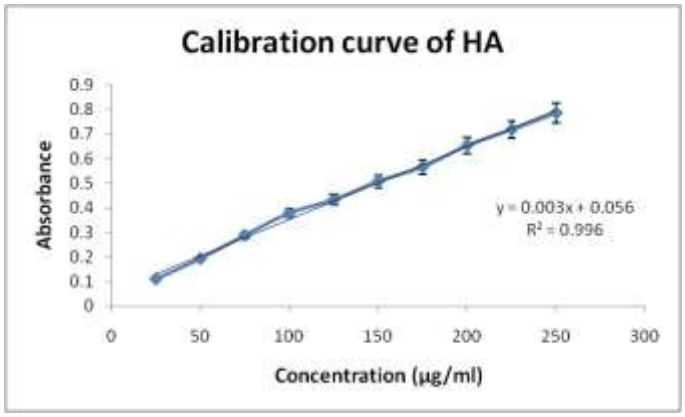

Figure 3.10: Calibration curve of HA by CTAB turbidimetric method

Table 3.19: Calibration curve of CS by CTAB turbidimetric method

Sr. No.	Concentration (µg/ml)	Absorbance (mean ± SD)
1	25	0.073 ± 0.019
2	50	0.121 ± 0.021
3	75	0.165 ± 0.023
4	100	0.226 ± 0.024
5	125	0.282 ± 0.027
6	150	0.355 ± 0.025
7	175	0.411 ± 0.029
8	200	0.459 ± 0.025
9	225	0.505 ± 0.027
10	250	0.554 ± 0.023

Table 3.20: Calibration data of CS by CTAB turbidimetric method

Solvent	**λmax**	**Range (µg/ml)**	**Regression Equation**	**Regression Coefficient (R^2)**
Acetate buffer	570 nm	25-250	y = 0.002x + 0.011	R^2=0.997

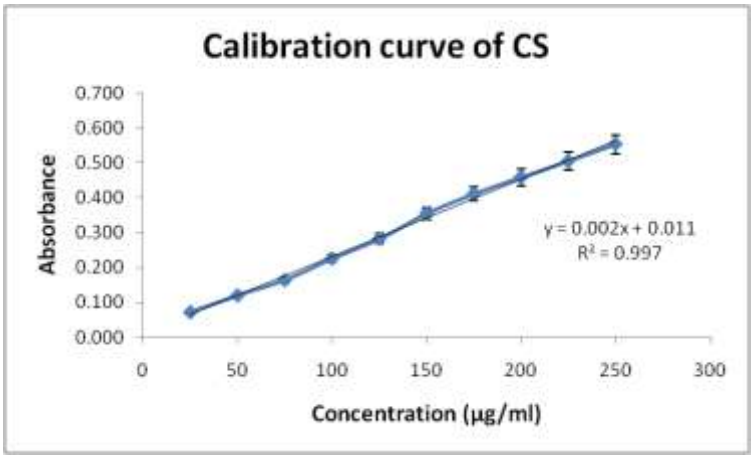

Figure 3.11: Calibration curve of CS by CTAB turbidimetric method

3.12.7 Estimation of Lf and BSA by Bradford protein assay method

The Bradford protein assay method is used to determine the concentration of total protein in a sample and invented by Bradford in 1976. This assay is based on the principal that the binding of protein molecules to Coomassie dye under acidic conditions results in a color change from brown to blue. This method actually measures the presence of the basic amino acid residues, arginine, lysine and histidine, which contributes to formation of the protein-dye complex (15).

3.12.7.1 Estimation of Lf using Bradford protein assay

Lf was estimated using Bradford protein assay and calibration curve was generated in which linearity range was found to be 0.05- 1.0 mg/ml (Table 3.21). Regression analysis was performed on the experimental data and results are shown in table 3.22 and figure 3.12. Results of regression analysis indicated linear relationship between absorbance and concentration of Lf.

Table 3.21: Calibration curve of Lf using Bradford protein assay

Sr. No.	Concentration (µg/ml)	Absorbance (mean ± SD)
1	0.05	0.079 ± 0.015
2	0.1	0.122 ± 0.018
3	0.2	0.199 ± 0.021
4	0.4	0.330 ± 0.023
5	0.6	0.464 ± 0.019
6	0.8	0.583 ± 0.017
7	1	0.719 ± 0.021

Table 3.22: Calibration data of Lf using Bradford protein assay

Solvent	λmax	Range (mg/ml)	Regression Equation	Regression Coefficient (R^2)
Tris-EDTA buffer	595 nm	0.05-1.0	y = 0.664x + 0.057	R^2=0.999

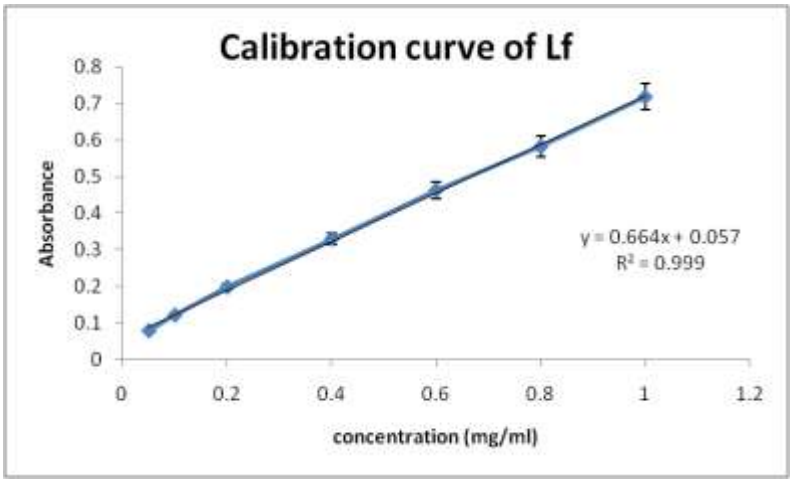

Figure 3.12 Calibration curve of Lf using Bradford protein assay

3.12.7.2 Estimation of BSA using Bradford protein assay method

BSA was estimated using Bradford protein assay and calibration curve was generated in which linearity range was found to be 0.05- 1.5 mg/ml (Table 3.23). Regression analysis was performed on the experimental data and results are shown in table 3.24 and figure 3.13. Results of regression analysis indicated linear relationship between absorbance and concentration of BSA.

Table 3.22: Calibration curve of BSA using Bradford protein assay

Sr. No.	Concentration (µg/ml)	Absorbance (mean ± SD)
1	0.05	0.059 ± 0.015
2	0.1	0.122 ± 0.017
3	0.2	0.181 ± 0.019
4	0.4	0.299 ± 0.021
5	0.6	0.395 ± 0.019
6	0.8	0.499 ± 0.017
7	1	0.616 ± 0.013
8	1.5	0.889 ± 0.018

Table 3.19: Calibration data of BSA using Bradford protein assay

Solvent	**λmax**	**Range (mg/ml)**	**Regression Equation**	**Regression Coefficient (R^2)**
Tris-EDTA buffer	595 nm	0.05-1.5	y = 0.555x + 0.059	R^2=0.997

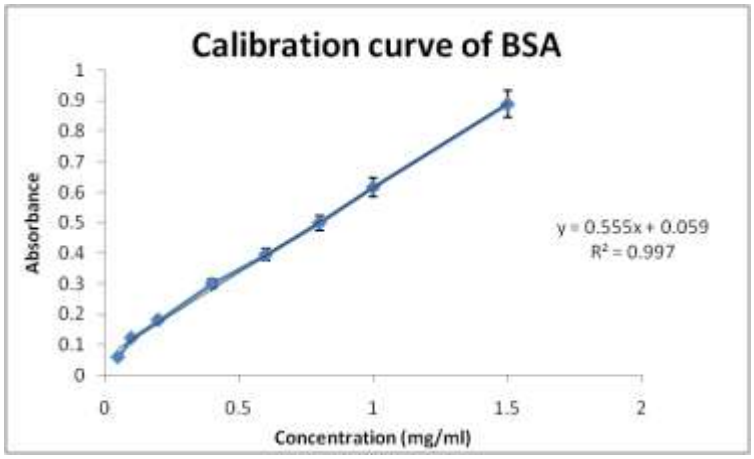

Figure 3.13 Calibration curve of BSA using Bradford protein assay

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