3.1. Materials.

Reagent	Supplier
Methanol AR grade	Merck,India
Acetonitrile AR grade	Merck,India
Sodium dihydrogen phosphate AR grade	LOBA Chemie, India
Potassium dihydrogen phosphate AR grade	LOBA Chemie, India
Sodium chloride AR grade	LOBA Chemie, India
Hydrochloric acid AR grade	LOBA Chemie, India
Florescamine, Fluram (>99%)	Sigma Aldrich, USA.
Double distilled water	Prepared in house
0.22 µ 45 mm filter	Merck Millipore, USA

3.2. Development of UV spectroscopic method for estimation of lenalidomide.

Lenalidomide was obtained as a gift sample from FTF pharmaceuticals Pvt. Ltd., Ahmedabad, India. Methanol and Acetonitrile AR grade were purchased from Merck, India.

3.2.1. Equipments.

UV spectrophotometer	UV-1800,Shimadzu,Japan
Weighing balance	ATX-224,Shimadzu,Japan

3.2.2. Preparation of Calibration curve.

5 mg of lenalidomide was weighed accurately using a weighing paper and transferred immediately to a centrifuge tube and dissolved in 1 ml of the Methanol: Acetonitrile (1:1) solvent system and vortexed gently to get a clear solution. The resultant solution was referred as stock solution with concentration of 5000µg/ml. The stock solution was diluted 10 times with the solvent system to get working stock of 500 µg/ml using a calibrated micropipette. From the working stock, dilutions with resultant concentrations of 10, 20, 30….70 µg/ml were prepared in centrifuge tubes. The blank correction was made using only solvent system and samples were analysed in spectrum mode after determination of lambda max for lenalidomide in Methanol: Acetonitrile (1:1). The figure 3.1 shows the lambda max for lenalidomide at 310±1nm.

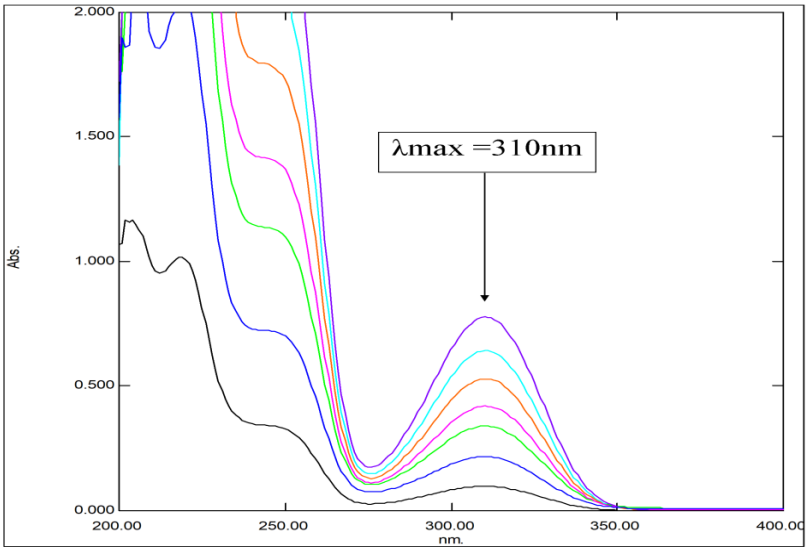


Figure 3.1: UV spectrum of lenalidomide in Methanol: Acetonitrile (1:1).

3.2.3. Validation of analytical method for estimation of lenalidomide.

The validation for the analytical method was carried out as per the International Conference on Harmonization(ICH), Geneva Q2(R1) guidelines (1-3).

3.2.3.1. Linearity and Range.

A seven point calibration curve was prepared and the graph of concentration v/s absorbance was plotted to check the linearity and the regression coefficient and equation was determined as shown in figure 3.2 and table 3.1.The linearity of the calibration curve was found to be 0.9994.

Table 3.1: Data of calibration curve of lenalidomide in Methanol: Acetonitrile.

Concentration (µg/ml)	Absorbance (Average±S.d.) (n=3)
10	0.085±0.001
20	0.202±0.001
30	0.331±0.003
40	0.437±0.006
50	0.554±0.009
60	0.679±0.012
70	0.780±0.005

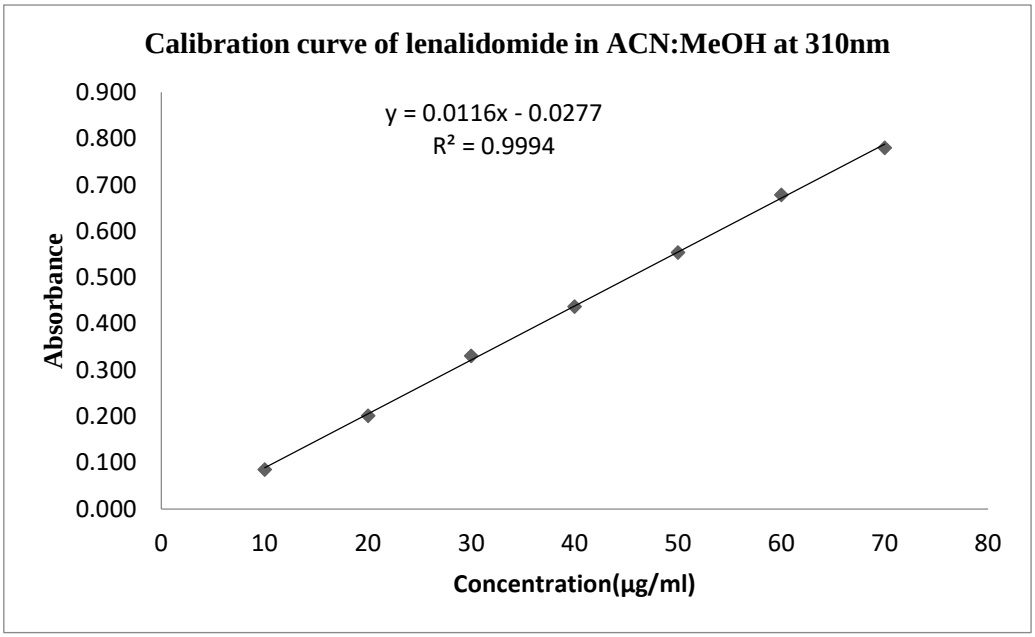


Figure 3.2: Calibration curve of lenalidomide in Methanol:Acetonitrile.

3.2.3.2. Limit of detection (LOD) and Limit of quantification (LOQ).

The limit of detection (LOD) is the minimum amount of the analyte which can be detected from the sample but not necessarily be quantified to the exact value may be expressed as:

LOD= (3.3 σ)/S

Where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte.

The quantitation limit (LOQ) is the minimum amount which can be quantified with suitable accuracy and precision may be expressed as:

LOQ= (10 σ)/S

The LOD and LOQ for the method were found to be 0.88 µg /ml and 2.67 µg/ml which indicate high sensitivity of the method.

3.2.3.3. Precision.

Precision refers to the closeness to the exact value or repeatability. Precision was determined by using 3 replicates of three concentrations. Intraday and Interday precisions were determined by measurement of absorbances of the selected three concetrations within the same day and at each day for three days. Table 3.2 represents the results of the precision study. It can be seen from the results that the relative standard deviation is less than 2% which is within acceptable limits.

Table 3.2: Precision of the analytical method.

Intraday precision						
Concentration (µg/ml)	**I**	**II**	**III**	**Average**	**S.D.**	**%RSD**
30	0.332	0.321	0.324	0.326	0.006	1.691
40	0.447	0.455	0.457	0.453	0.005	1.150
50	0.566	0.554	0.564	0.561	0.006	1.144
Interday precision						
Concentration (µg/ml)	**I**	**II**	**III**	**Average**	**S.D.**	**%RSD**
30	0.3315	0.3272	0.333	0.331	0.003	0.91072
40	0.4384	0.4429	0.4308	0.437	0.006	1.398329
50	0.5612	0.556	0.5442	0.554	0.009	1.572935

3.2.3.4. Accuracy.

Accuracy is defined as the closeness to true value. It is generally measured by the %recovery after addition of known amount of analyte in the sample. Table 3.3 shows the results of the accuracy assessment of the method. The results show that the %recovery for the selected concentrations is within the acceptable range of 98-102% indicating the accuracy of the method.

Table 3.3: Results of accuracy test.

Accuracy level	**C_{actual} (µg/ml)**	**C_{added} (µg/ml)**	**%Recovery**
0	30	0	100.233±1.401
80	30	24	100.400±0.794
100	30	30	100.310±1.372
120	30	36	100.367±1.250

3.2.3.5. Interference study/Specificity.

It is the ability of the method to detect the analyte in presence of components which are expected to remain present or may be the ingredients of the formulation. The study was done by taking spectra of solutions of PLGA, PVA, 50µg/ml lenalidomide, plain uncoated metallic nanoparticles and their mixture as shown in figure 3.3. It can be concluded that PVA and PLGA do not have any interference in the region of 310nm while the metallic nanoparticles showed very high absorbance throughout the spectrum and they must be removed after extraction of drug with solvent system by centrifugation or magnetic separation to avoid interference.

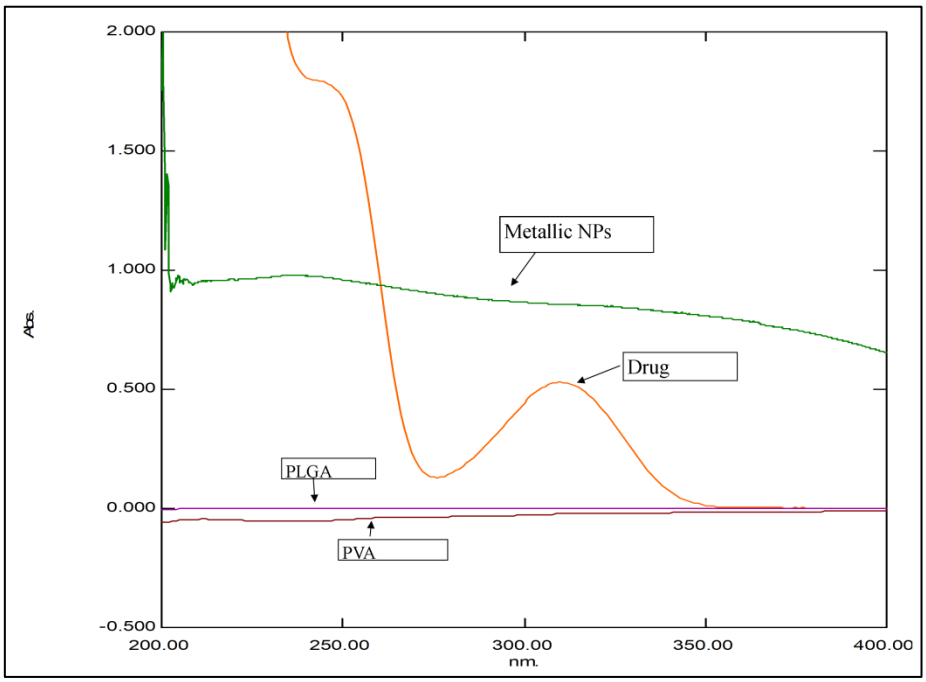


Figure 3.3: Interference study.

3.3. Estimation of lenalidomide in methanolic phosphate buffer saline pH 7.4 for *in vitro* drug release studies.

3.3.1. Equipments

UV spectrophotometer	UV-1800,Shimadzu,Japan
Weighing balance	ATX-224,Shimadzu,Japan
pH meter	Pico+, LABINDIA, India
Bath sonicator	Inseref,India

3.3.2. Preparation of methanolic phosphate buffer saline pH 7.4.

It was prepared as per the method given in Indian Pharmacopoeia (4). 2.83 gm of sodium dihydrogen phosphate, 0.19 gm potassium dihydrogen phosphate and 8 gm of sodium chloride were dissolved in double distilled water to make 1000 ml of the buffer. pH was adjusted to 7.4 using 0.1M HCl. After adjustment of pH, the buffer was filtered through 0.22µ nylon filter using vacuum filtration. In a beaker, 100 ml of methanol was added using a measuring cylinder and volume was made up using PBS 7.4 upto 1000ml.

3.3.3. Preparation of calibration curve.

5 mg of lenalidomide was carefully weighed, transferred to a centrifuge tube and 1 ml of the methanolic PBS was added to it drug and sonicated for 3 minutes to get the stock solution. The stock solution was diluted 10 times to get working stock solution of 500µg/ml from which aliquots of 10, 20, 30... 60µg/ml were prepared.

The prepared aliquots were analysed after blank correction with only methanolic PBS 7.4.The spectrum was obtained to determine lambda max and the calibration curve was prepared after measuring the absorbance at same wavelength. Figure 3.4 shows the spectrum of lenalidomide in methanolic PBS 7.4 which shows lambda max of 302nm.

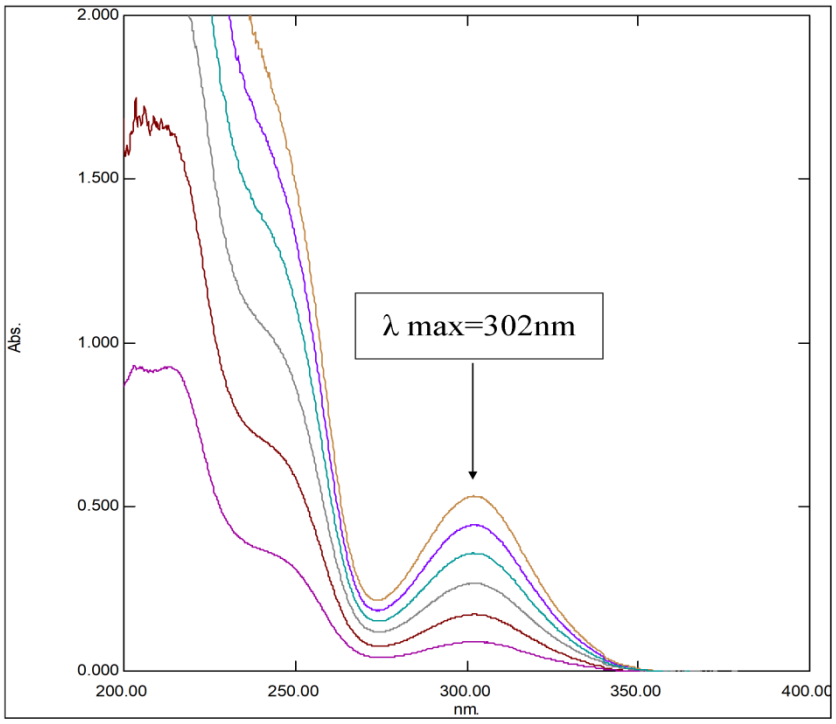


Figure 3.4: Spectrum of lenalidomide in methanolic PBS pH 7.4.

3.3.4. Range and linearity.

A calibration curve was prepared for lenalidomide using the absorbance obtained by the concentrations of aliquots as shown in figure 3.5 and table 3.4. The figure 3.5 shows good linearity of the plot with regression coefficient of 0.9996. and table 3.5 shows that the results have %RSD < 2% which is acceptable for any analytical method as per the ICH guidelines (5).

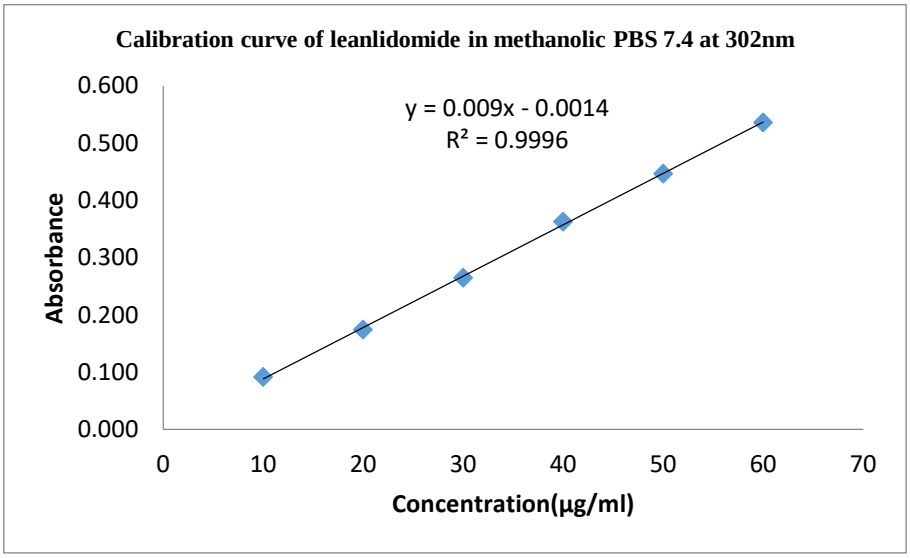


Figure 3.5: Calibration curve of lenalidomide in methanolic PBS pH 7.4.

Table 3.4: Data of calibration curve of lenalidomide in methanolic PBS pH 7.4.

Concentration (µg/ml)	Absorbance (Average±S.d.) (n=3)	%RSD
10	0.091±0.002	1.672
20	0.174±0.001	0.575
30	0.265±0.004	1.327
40	0.362±0.002	0.422
50	0.447±0.002	0.466
60	0.536±0.003	0.494

3.4. Bioanalytical method for estimation of lenalidomide in plasma and brain.

3.4.1. Equipments.

Spectroflorimeter	RF-5301, Shimadzu, Japan.
High speed cooling centrifuge	C-24 plus, REMI, India.

3.4.2. Principle of the analytical method.

The florescamine dye is chemically 4′-phenylspiro [2-benzofuran3,2′-furan]−1,3′-dione which binds to primary amines and yields a florescent product as shown in figure 3.6. Several drugs have been reported to undergo this reaction to yield florescent product which can be analysed at 472-476 nm after excitation at 355-402 nm. The solvent used for the reaction can affect the florescent intensity. Solvents such as buffers, ethanol, methanol, acetonitrile etc.. can be used depending on the solubility of the analyte and the dye (6). Drugs containing primary amine including lenalidomide can also be determined in biological fluids by this method without use of sophisticated methods such as HPLC (7, 8).

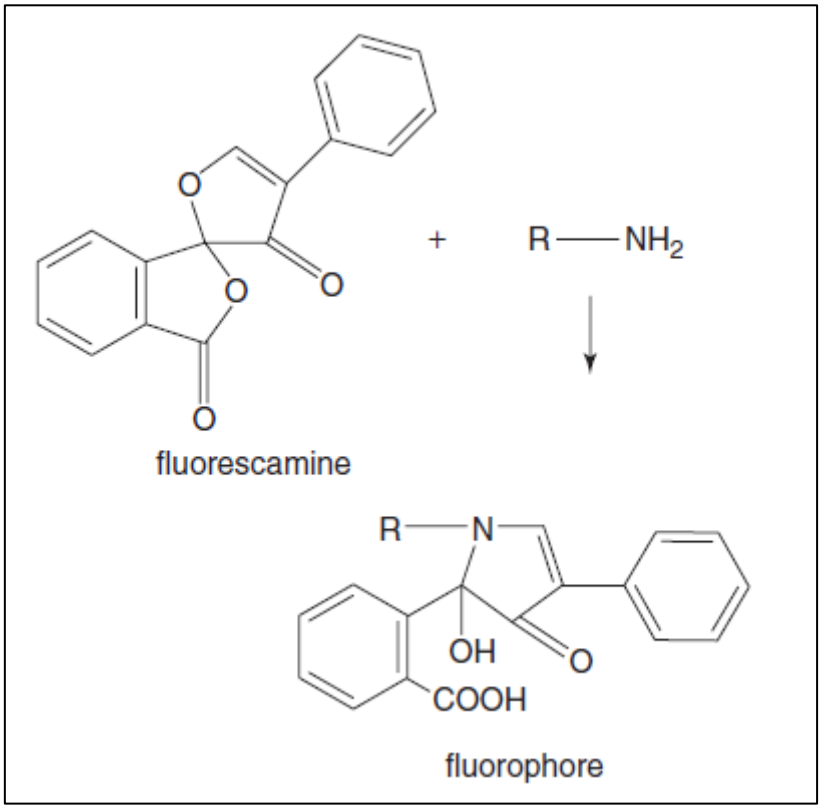
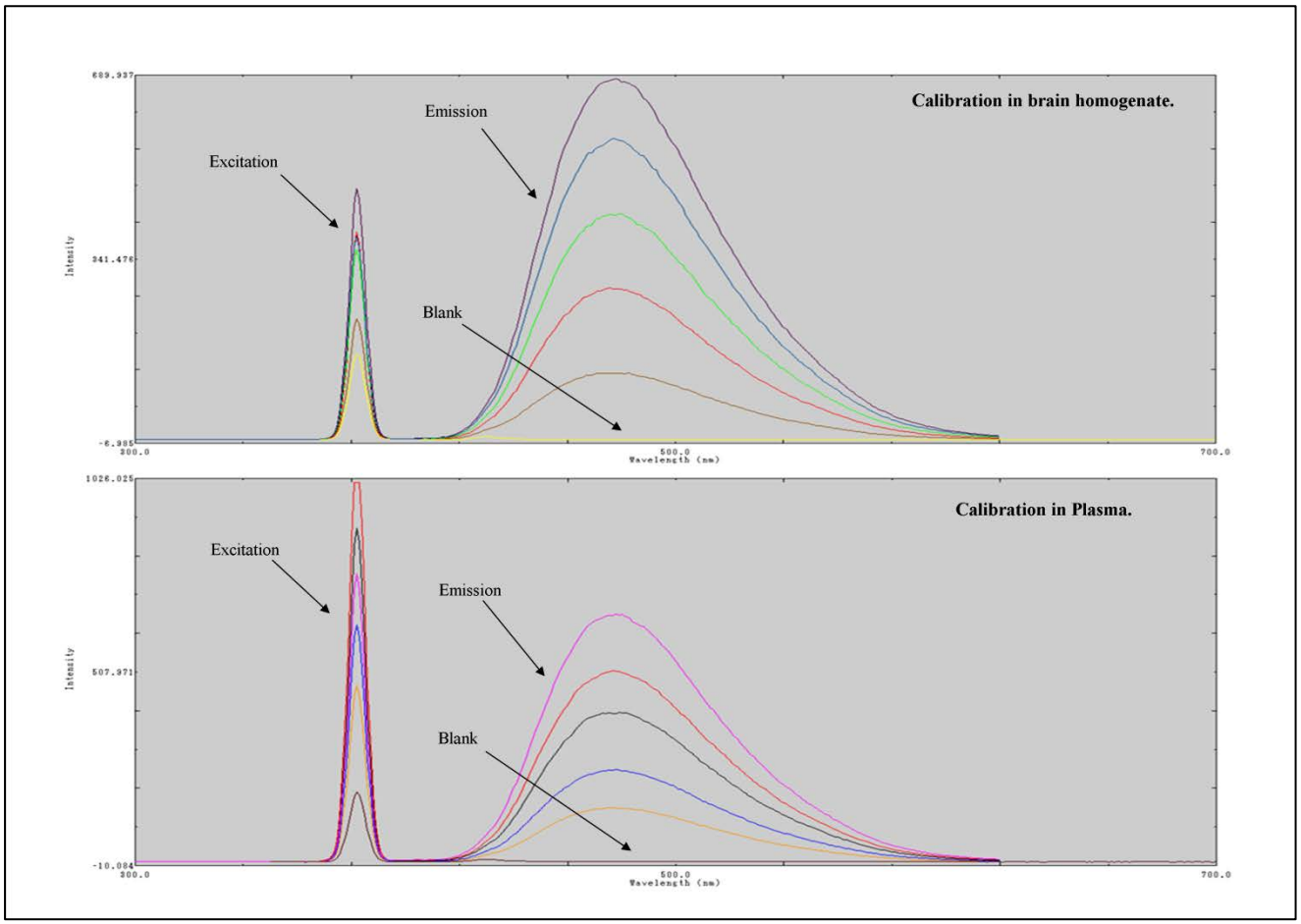


Figure 3.6: Reaction of florescamine with primary amine (9).

3.4.3. Preparation of standard samples in plasma and brain homognenate.

The plasma and brain were isolated after humanely sacrifice of rats and 10% w/v brain homogenate in double distilled water was prepared using organ homogenizer and stored in -20°C till further use. 5µg/ml of working stock solution was spiked in 100µl plasma and brain homogenate separately in centrifuge tubes. After spiking, the precipitation of plasma and brain protein was done with addition of ice cold methanol upto 1ml in a centrifuge tube. The samples were subjected to centrifugation at 15000rpm for 10 minutes at 4°C. To the supernatant, 1 ml of 0.05% of florescamine dye in methanol was added following incubation in the dark for 10minutes to allow the reaction to complete. The samples were further diluted with methanol to get the florescence in detection range. The resultant aliquots were subjected to analysis by spectroflorometer at excitation wavelength of 381nm and emission wavelength of 494nm after blank correction as shown in figure 3.7.

Figure 3.7: Spectra of lenalidomide in brain homogenate and plasma.



The figure 3.8 and table 3.5 show the calibration data for the bio analytical method developed for lenalidomide in brain homogenate and plasma. Good linearity is observed in both the cases with regression coefficient of respectively indicating linear relationship between drug concentration and florescence. The %RSD value is also less than 2% in both the cases which is within the acceptable limits for the developed method.

Figure 3.8: Calibration curves for bio analytical method.

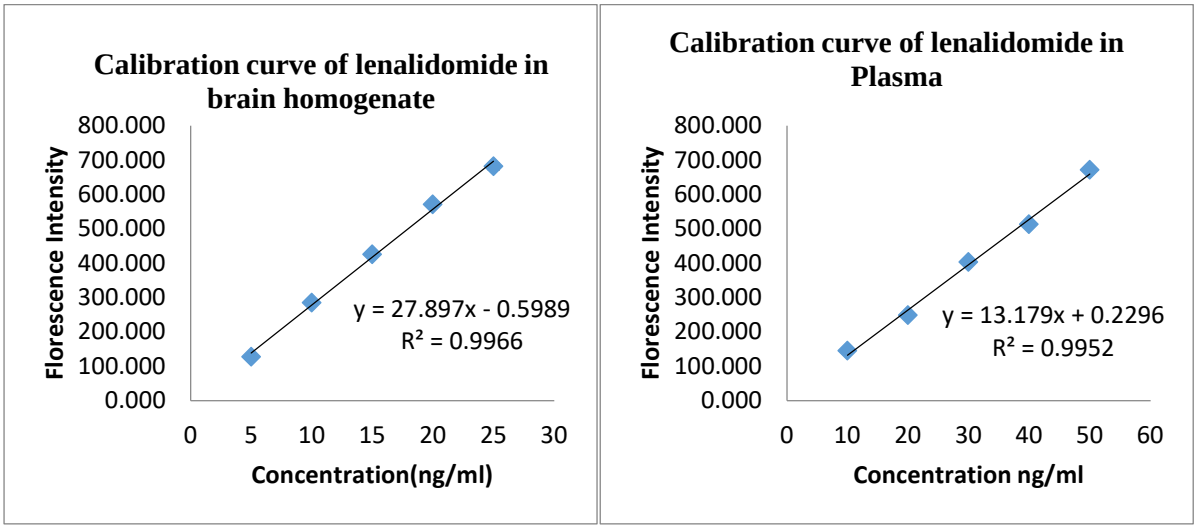


Table 3.5: Data of bio analytical method.

Brain homogenate		
Concentration (ng/ml)	**Florescence Intensity (Average±S.d.) (n=3)**	**%RSD**
5	126.881±1.496	1.179
10	284.747±4.066	1.428
15	425.550±4.360	1.024
20	570.892±7.259	1.272
25	681.245±9.798	1.438
Plasma		
10	144.354±2.525	1.749
20	247.650±2.905	1.173
30	402.730±6.486	1.610
40	512.328±8.019	1.565
50	670.980±10.135	1.511

3.5. References.

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