3.1. Materials

Lenalidomide (LND) was obtained as gift sample from Apicore Pharmaceuticals Pvt. Ltd., Vadodara,India. Methanol, Acetonitrile and Hydrochloric acid were of AR grade and purchased from Spectrochem, Mumbai, India. Sodium nitrite, Ammonium Sulphamate, N-(1-napthyl) ethylenediaminedihydrochloride, Fluorescamine, Ferric chloride hexahydrate and Copper acetate were purchased from Himedia, Mumbai, India.Plasma was procured from Suraktam Blood Bank, Vadodara, India.

3.2. Equipments

UV Visible spectrophotometer 1700 (Shimadzu, Japan)
Spectrofluorophotometer RF-5301 (Shimadzu, Japan)
Digital Analytical Balance (Shimadzu, SCS, Switzerland)

3.3. Estimation of Lenalidomide (LND) by UV Visible Spectrophotometer

- **Reagents preparation:**
  1. *Sodium nitrite solution (0.2% w/v):* 200 mg of sodium nitrite was dissolved in distilled water and made up to 100 ml.
  2. *Hydrochloric acid (5N):* 425 ml of conc. HCl was taken and diluted to 1000 ml with distilled water.
  3. *Ammonium sulphamate solution (0.5 %w/v):* 500 mg of ammonium sulphate was dissolved in distilled water and made up to 100 ml.
  4. *N-(1-napthyl) ethylenediaminedihydrochloride solution (0.1 % w/v):* 100 mg of N-(1-napthyl) ethylenediaminedihydrochloride was dissolved in 100 ml of distilled water.
- **Standard preparation:**
  About 10 mg of LND was accurately weighed and dissolved in 100 ml of methanol to get 100 μg/ml standard solution.

3.4. Procedure for Calibration curve:

Aliquots of LND solution (100 μg/ml) ranging from 0.1 to 0.5 ml were transferred into a series of 10 ml volumetric flasks and total volume in all flasks was adjusted to 1.0 ml with methanol. To each flask, 1 ml of 5N hydrochloric acid and 1 ml of sodium nitrite solution were added and allowed to stand for five minutes.1 ml of ammonium sulphamate solution

was then added, mixed and allowed to stand for two minutes. To this solution, 1 ml of N-(1-napthyl) ethylenediaminedihydrochloride (B.M reagent) solution was added and mixed well. The final volume was made up to 10 ml with different buffers. The absorbance of pink coloured chromogen was measured at 542 nm and 548nm for Phosphate buffer saline (PBS) pH 7.4 and Citro-phosphate buffer (CPB) pH5.5 respectively against reagent blank(Buffers without addition of drug) [1].

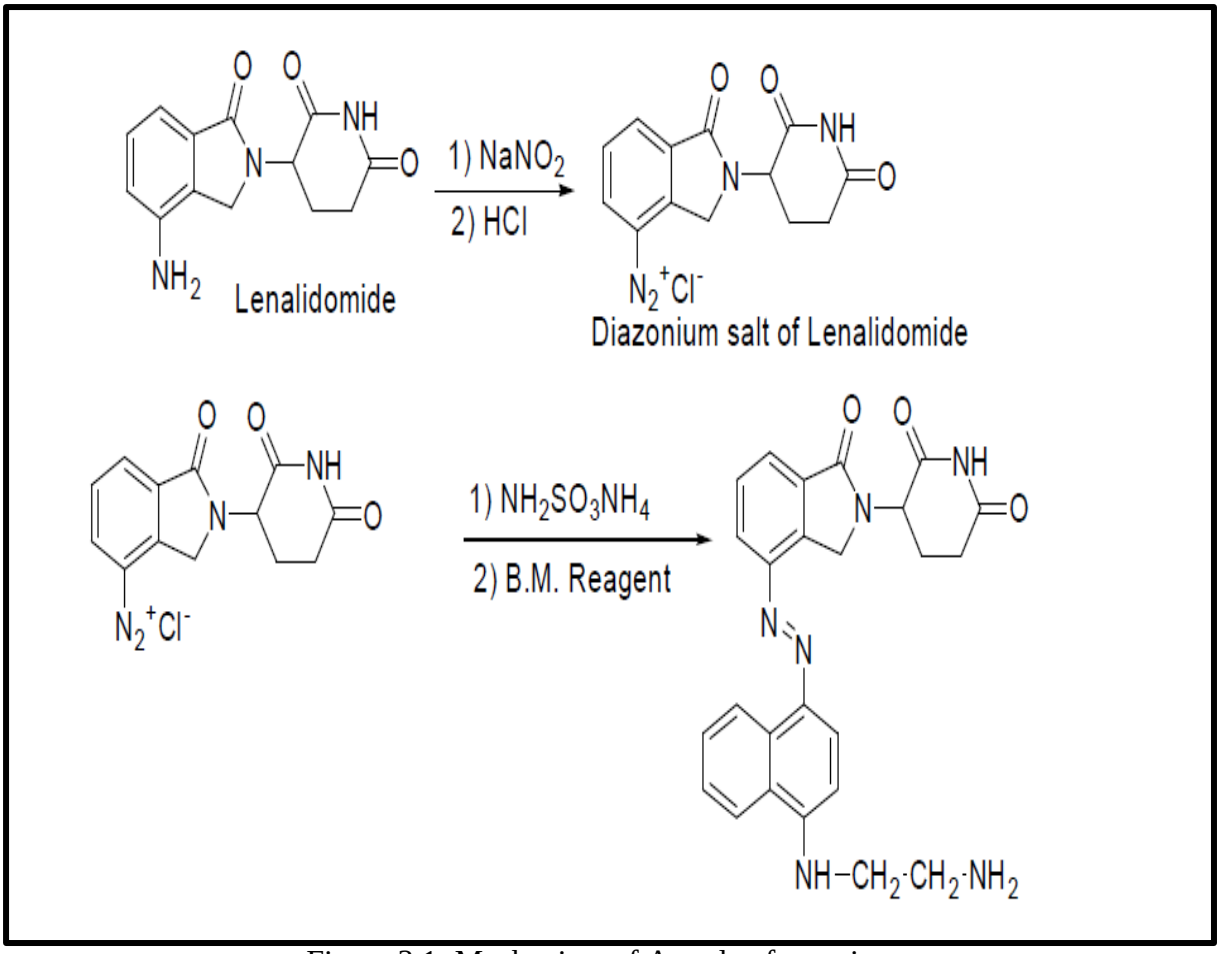

Figure 3.1: Mechanism of Azo-dye formation

a. Calibration curve in PBS (pH 7.4)

The regression of the plot using the method of least squares was made to evaluate the intercept,slope and correlation coefficient(R^2) as shown in figure 3.1. The high value of correlation coefficient of the regression equation and the negligible value of intercept confirmthe linearity of calibration plot. Parameters indicating linearity for the developed UV spectrometric method of analysis for LND are shown in Table 3.1.

Table 3.1: Calibration curve for LND in PBS (pH 7.4) by UV Visible spectrophotometer

Sr. No.	Concentration(µg/ml)	Mean Absorbance ± SD	%RSD
1	0	0.000±0.0000	-
2	1	0.148±0.0015	1.0297
3	2	0.223±0.0020	0.9320
4	3	0.362±0.0026	0.7189
5	4	0.486±0.0050	1.0349
6	5	0.581±0.0100	1.7230
7	6	0.672±0.0126	1.8852

Mean SD	Mean %RSD	LOD	LOQ	R^2
0.0057	1.2207	0.166µg/ml	0.504µg/ml	0.9945

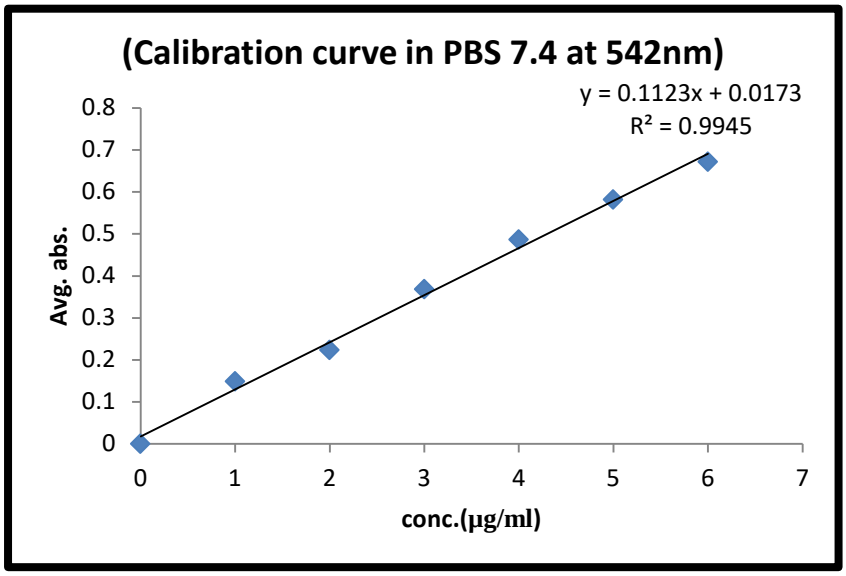

Figure 3.2: Standard plot of LND in PBS 7.4 by UV visible spectroscopy

b. Calibration curve in CPB (pH 5.5)

Table 3.2: Calibration curve for LND in CPB (pH 5.5) by UV visible spectrophotometer

Sr. No.	Concentration(µg/ml)	Mean Absorbance ± SD	%RSD
1.	0	0.000±0.0000	-
2.	1	0.109 ± 0.0015	1.376
3.	2	0.253 ± 0.0045	1.779
4.	3	0.375 ± 0.0061	1.627
5.	4	0.484 ± 0.0099	1.978
6.	5	0.608 ± 0.0085	1.398
7.	6	0.699 ± 0.0041	0.587

Mean SD	Mean %RSD	LOD	LOQ	R^2
0.0058	1.458	0.161µg/ml	0.486µg/ml	0.9968

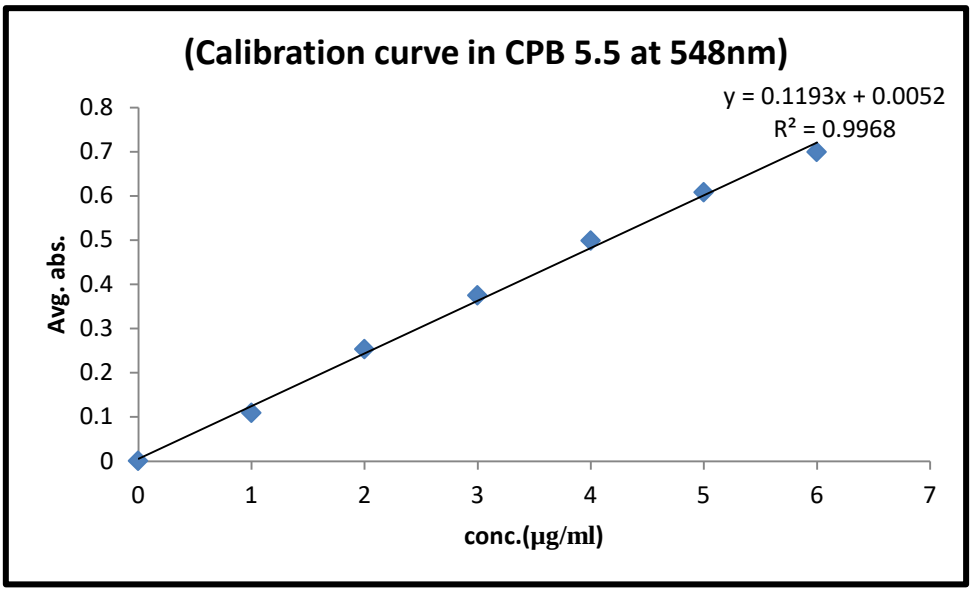

Figure 3.3: Standard plot of LND in CPB 5.5 by UV visible spectroscopy

3.4.1. Method validation

Validation of the given method is necessary because, the reported method gives calibration curve of drug in distilled water and no data is available about the calibration curve of drug in physiological buffers.

3.4.2. Accuracy

It was performed by calculating % recovery of LND from PBS 7.4 and CPB 5.5 at three levels of standard addition (80,100,120%) of 2µg/ml LND solution and it was analyzed for drug content [2].

Table 3.3.Accuracy of analytical method in PBS 7.4 and CPB 5.5

Amount of LND in sample (PBS& CPB) (µg/ml)	**Amount of std. LND added (µg/ml)**	**Total amount of LND added (µg/ml)**	**Total amount of LND found in PBS ± SD (µg/ml)**	**% Mean Recovery in PBS7.4**	**Total amount of LND found in CPB± SD (µg/ml)**	**%Mean Recovery in CPB 5.5**
2.0	1.2	3.2	3.18±0.013	99.37%	3.25±0.015	101.56%
2.0	2.0	4.0	3.92±0.016	98.00%	4.01±0.019	100.25%
2.0	2.8	4.8	4.86±0.021	101.25%	4.82±0.011	100.41%

% Recovery of LND was found to be 98.00-101.25% and 100.25-101.56% from PBS and CPB respectively as shown in the table 3.3.Recovery greater than 98% and less than 102% in both the solvents justified the accuracy of the method [2].

3.4.3. Precision

Intraday deviation and interday deviation was checked for the three concentrations of calibration range. To study intraday deviation, the solutions of all given concentrations were prepared and analyzed for three different times during the same day. To study interday deviation, the solutions of all concentrations were prepared and analyzed for three consecutive days. The experiments were performed in triplicates and the mean concentrations and %RSD were calculated.

Table 3.4: Precision of analytical method in PBS 7.4

Concentration added (µg/ml)	**Observed parameters in PBS 7.4**			
	Concentration±SD (µg/ml)		%RSD	
	Intraday	Interday	Intraday	Interday
2	1.85 ± 0.0020	1.79±0.0012	0.9320	0.9985
4	4.19 ± 0.0050	4.21±0.0074	1.0349	1.0581
6	5.85±0.0126	5.98±0.0130	1.8852	1.4325

Table 3.5: Precision of analytical method in CPB 5.5

Concentration added (µg/ml)	**Observed parameters in PBS 7.4**			
	Concentration ± SD (µg/ml)		%RSD	
	Intraday	Interday	Intraday	Interday
2	2.08±0.0017	2.16±0.0021	0.6846	0.7985
4	4.13±0.0098	4.56±0.0016	1.9784	0.2917
6	5.82±0.0055	5.96±0.0023	0.7783	0.3216

From table 3.4 and 3.5, it can be seen that %RSD for intraday and interday analysis was not above 2%. Hence, the above method was accurate and precise [2].

3.5. Calibration curve of LND in Methanol

About 10 mg of LND was accurately weighed and dissolved in 100 ml of methanol to get 100 µg/ml standard solution. Aliquots of LND solution (100 µg/ml) ranging from 0.1 to 0.5 ml were transferred into a series of 10 ml volumetric flasks and total volume in all flasks was adjusted to 10.0 ml with methanol. The absorbance of all the prepared solutions was then measured at the absorbance maxima, 220 nm against reagent blank (Distilled water). The readings were recorded in triplicate.

Table 3.6: Calibration curve of LND in Methanol by UV visible spectrophotometer

Sr. No.	Concentration(µg/ml)	Mean Absorbance ± SD	%RSD
1.	0	0.000 ± 0.0000	-
2.	1	0.160 ± 0.0012	0.7500
3.	2	0.301 ± 0.0025	0.8306
4.	3	0.452 ± 0.0023	0.5088
5.	4	0.643 ± 0.0047	0.7309
6.	5	0.805 ± 0.0056	0.6956

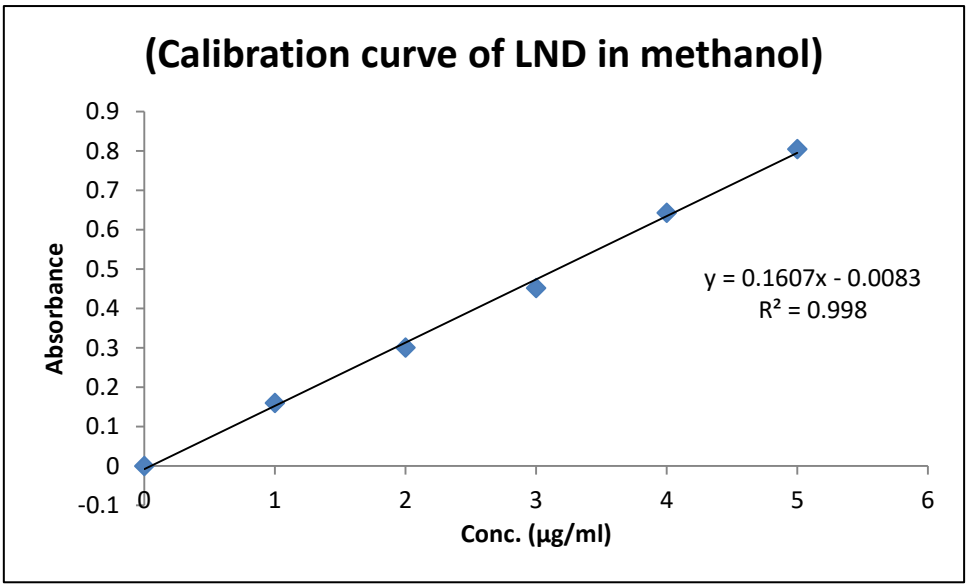

Figure 3.4: Standard plot of LND in Methanol by UV visible spectroscopy

3.6. Estimation of Iron by UV spectroscopy

121mg of Iron Chloride Hexahydrate ($FeCl_3.6H_2O$) was accurately weighed and dissolved in 100 ml of distilled water to get 0.25mg/ml Iron stock solution. This solution was further diluted using distilled water to obtain the final concentration of 5, 10,15, 20, 25µg/ml for the preparation of calibration curve. The absorbance of all the prepared solutions was then measured at the absorbance maxima, 545nm against reagent blank (Distilled water).The readings were recorded in triplicate [3].

Table 3.7: Calibration curve of Iron in distilled water by UV visible spectrophotometer

Sr. No.	Concentration (µg/ml)	Mean Absorbance ± SD	%RSD
1.	0	0.000±0.0000	-
2.	5	0.086±0.0014	1.6279
3.	10	0.162±0.0032	1.9753
4.	15	0.255±0.0042	1.6470
5.	20	0.330±0.0041	1.2424
6.	25	0.424±0.0030	0.7075

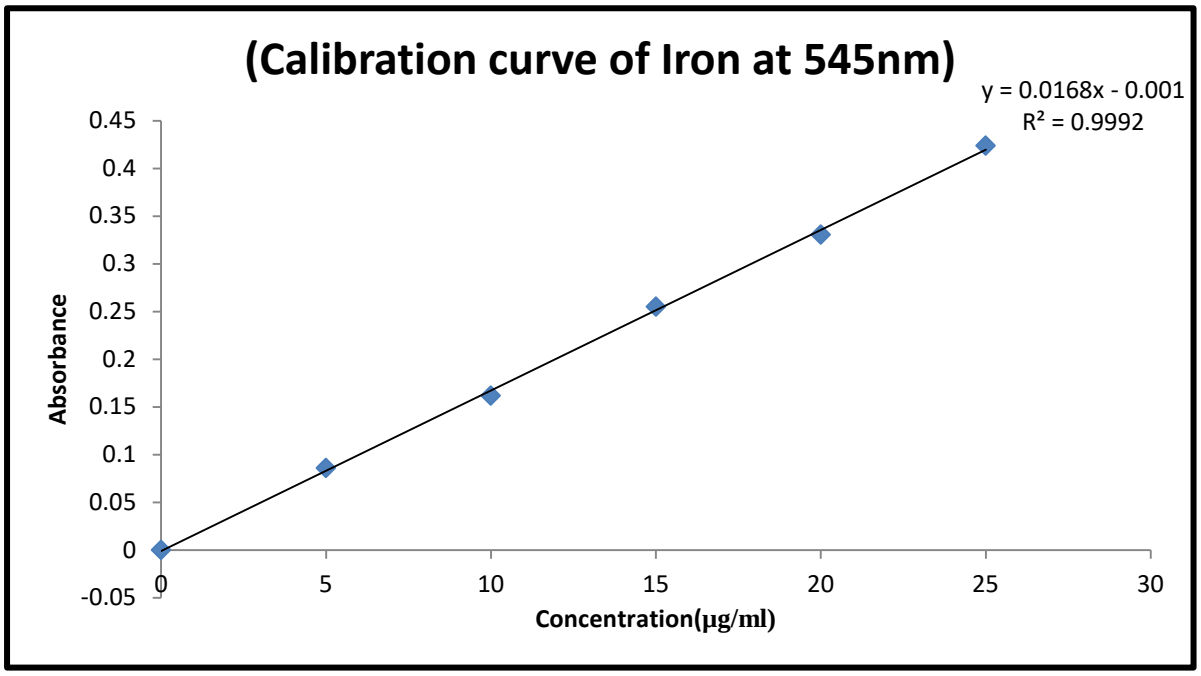

Figure 3.5: Standard plot of Iron in distilled water by UV visible spectroscopy

3.7. Estimation of LND by spectrofluorimetry in plasma

Various analytical methods have been developed for the estimation of LND. For example, Capillary electrophoresis method with photodiode array detector [4], High-performance liquid chromatography [5], LC–MS/MS[6] and HPLC with fluorescence detector [7]. Spectrofluorometry has been found to be useful for the determination of trace amounts of different pharmaceuticals, showing several advantages such as low detection limit, high sensitivity and the use of conventional instrument [8]. A spectrofluorometric method has

been developed by Darwish *et al.* for determination of LND in distilled water which is highly sensitive [9]. Hence, for determination of drug in plasma, fluorescent intensities at excitation (λ_{ex}) and emission (λ_{em}) wavelength was measured after spiking plasma with drug solution.

- **Lenalidomide (LND) standard solution**

An accurately weighed amount of LND was quantitatively transferred into a calibrated volumetric flask, dissolved in methanol and completed to volume with the same solvent to produce a stock solution of 1 mg/ml and kept in the refrigerator. On the day of analysis, the stock solution was further diluted stepwise with water to obtain a working standard solution containing 1.0 µg/ml.

- **Fluorescamine(FLC) solution**

An accurately weighed amount (5 mg) of Fluorescamine was transferred into a 10-mlVolumetric flask, dissolved in acetonitrile and completed to volume with the same solvent to produce a stock solution of 0.05% (w/v). The solution was freshly prepared and kept at -20°C protected from light to be used within seven days.

- **Preparation of the sample for analysis**

Accurately measured aliquots of LND working stock solution (1.0 µg/ml) ranging from 25–350µl were transferred into eight separate eppendorfs each containing 500 µl plasma and 100 µl of a 0.2% solution of copper acetate. The volumes in all eppendorfs were adjusted, as necessary, to 1.0 ml with water. The eppendorfs were heated in a boiling water-bath for 15 min. and cooled. 1.0 ml of acetonitrile was added to each eppendorf and centrifuged for 15 min at 5000 rpm. Portions of 500 µl of the supernatant solutions were transferred into each of a set of eight eppendorfs followed by 300 µl of water. 200 µl of Fluorescamine solution (0.05% w/v) was added to all eppendorfs. This resulted in a series of LND standard solutions covering the working range of 0.25-35 ng/mL. In the final reaction mixture, 1ml of each solution was taken in volumetric flasks and diluted up-to 10ml with distilled water to get the final concentration of 0.25-3.5ng/ml. The reaction in each flask was allowed to proceed at room temperature for 5 min. before analysis. The resulting solutions were estimated by spectrofluorimetry at λ_{ex} =381nm and λ_{em}=494nm.All the readings were taken in triplicate [9, 10].

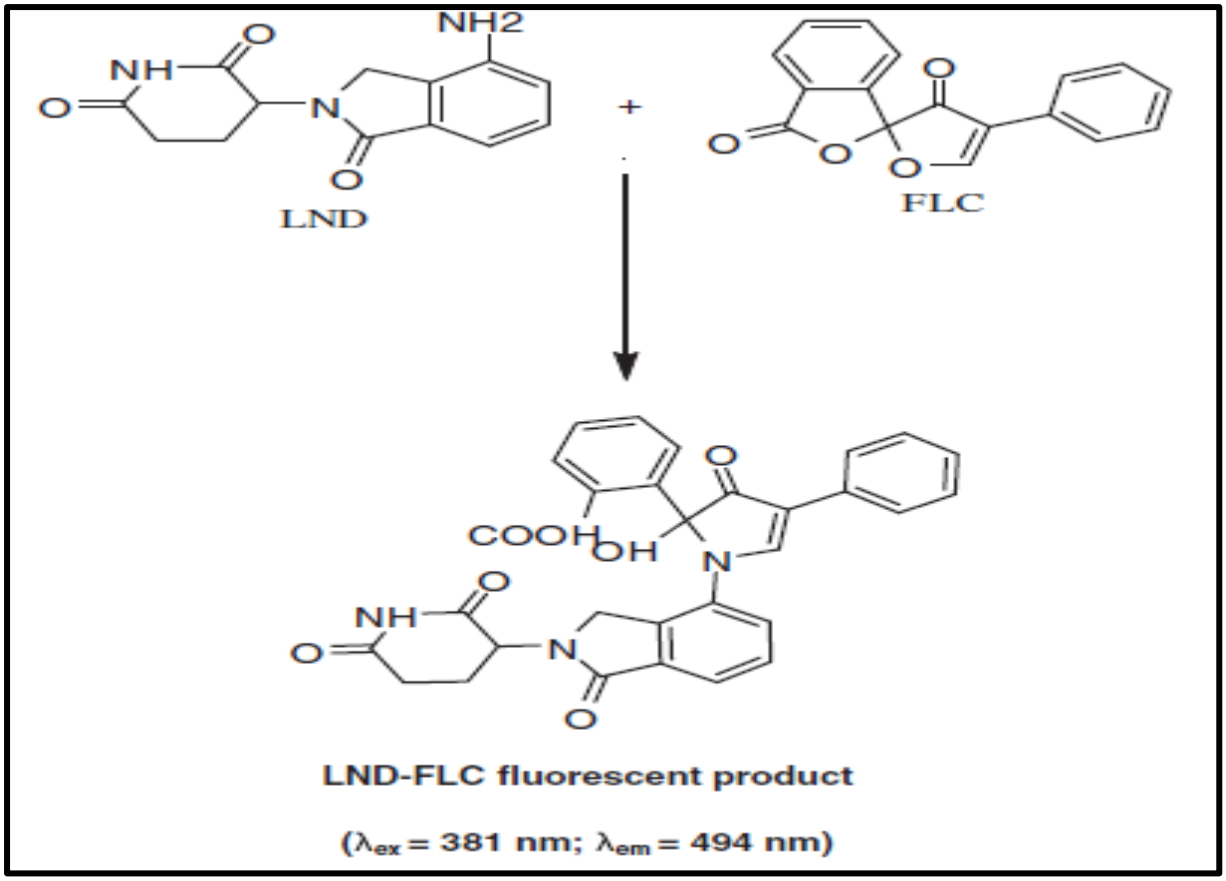

Figure 3.6: Scheme for the reaction pathway between LND and Fluorescamine.

Table 3.8: Calibration curve of LND in plasma by spectrofluorimetry

Sr. No.	**Concentration (ng/ml)**	**Mean Intensity ± SD**	**%RSD**
1.	0.00	00.0±0.000	-
2.	0.25	12.3±0.198	1.60
3.	0.50	19.2±0.297	1.54
4.	0.75	22.3±0.262	1.17
5.	1.00	55.4±1.081	1.95
6.	1.50	81.6±1.469	1.80
7.	2.00	96.2±1.842	1.91
8.	3.00	129.6±2.521	1.94

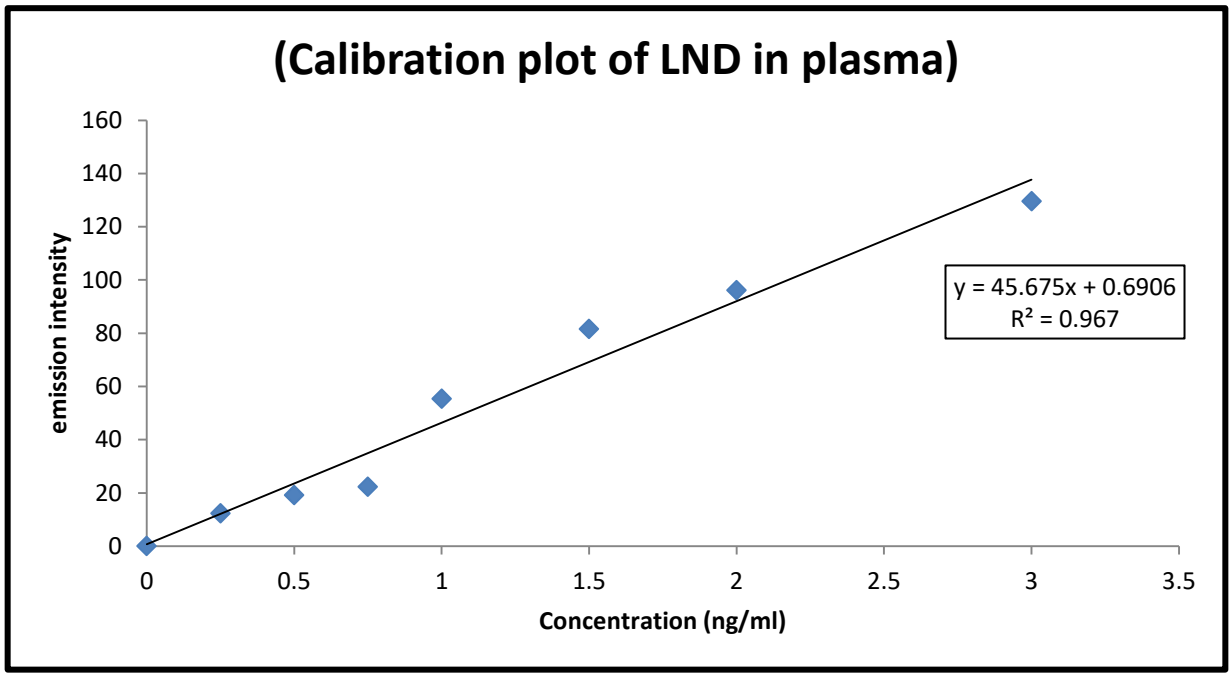

Figure 3.7: Standard plot of LND in plasma by spectrofluorimetry

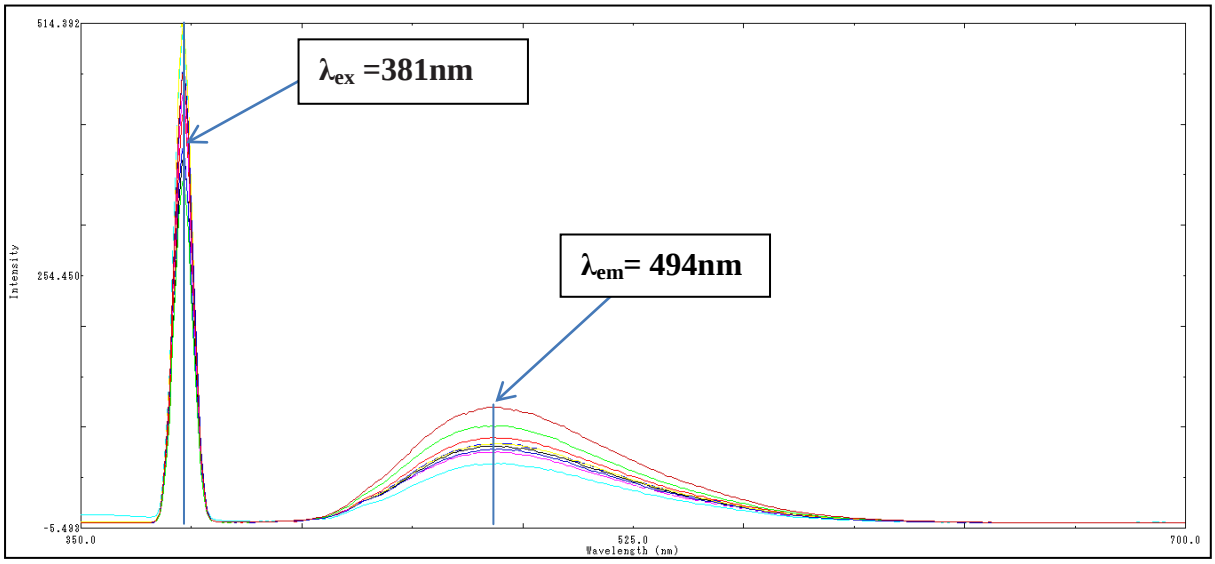

Figure 3.8: Overlay spectra of LND in plasma by spectrofluorimetry

3.8. Interference study by UV Visible spectrophotometer

The interference study of Lenalidomide (LND) and excipients Hyaluronic acid (HA) and Carboxymethylated Hyaluronic Acid(CMHA) was done by UV Visible spectrophotometer in PBS 7.4.

Stock solution of LND, HA and CMHA was prepared by dissolving them in PBS 7.4 to get stock solution of (100 µg/mL). From the stock solutions (100 µg/mL) of drug, HA and CMHA in PBS 7.4, all 3 were diluted with the same solvent to get the concentration of 5µg/ml. The absorbance of 5µg/ml LND solution, 1:1 mixture of LND:HA (5µg/ml : 5µg/ml) and 1:1 mixture of LND:CMHA (5µg/ml : 5µg/ml) was taken for analysis and absorbance was measured at 542 nm in PBS 7.4.

Table 3.9: Interference study data of LND,HA and CMHA

Sr. no.	Name of Ingredient	Absorbance±SDSD305
1.	Lenalidomide (LND)	0.598±0.015
2.	Lenalidomide (LND)+Hyaluronic Acid(HA)	0.618±0.021
3.	Lenalidomide (LND)+Carboxy-methylated HA(CMHA)	0.623±0.017

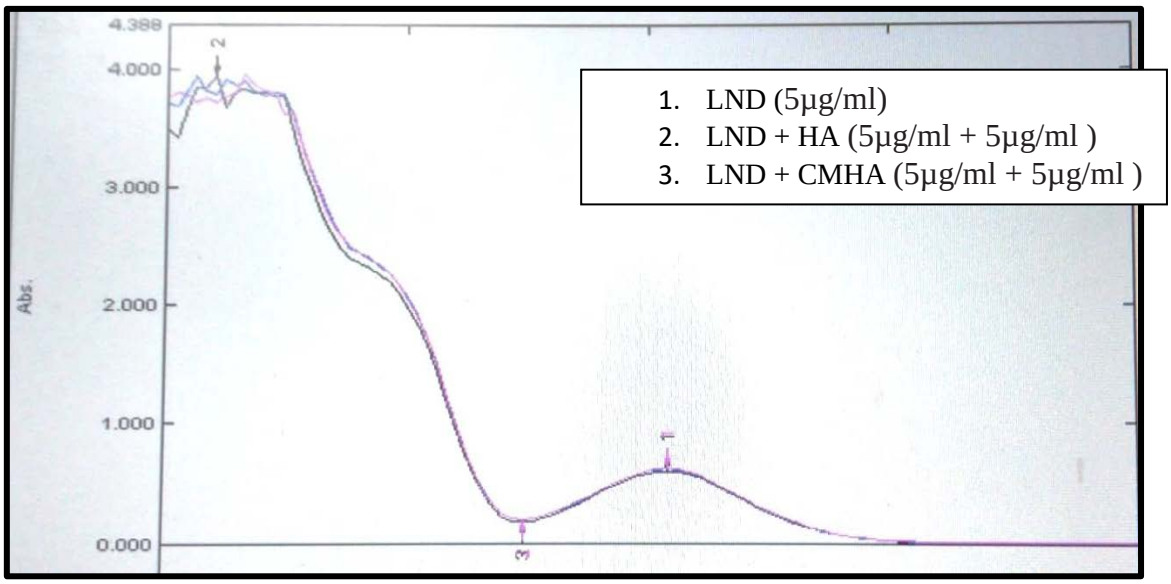

Figure 3.9: UV spectra of (1) LND, (2) Mixture of LND and HA and (3) Mixture of LND and CMHA in PBS 7.4 for interference study.

There was no major difference in the absorbance value of the drug alone and the drug with polymers which shows that HA and CMHA did not interfere with the analysis of LND.

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