CHAPTER 3

ANALYTICAL METHODS

3 ANALYTICAL METHODS

3.1 Introduction

Analytical methods are important tools to estimate the drug content in the formulations and assess the stability of the drugs in the formulations over the period of time. The analytical methods are of volumetric methods and instrumental methods. Instrumental methods have advantages over volumetric methods because of their sensitivity, low sample requirement and accuracy. UV spectrophotometric method is the simplest instrumentation method capable of drug estimation in micrograms.

Analytical methods for Tramadol (TMD) and Lamotrigine (LTG) were developed in acetonitrile, methanol and PBS pH 5 with surfactant using UV spectrophotometry because TMD and LTG show strong absorbance in UV region. Methods were developed for the assay and invitro drug release study. The method of both drugs in acetonitrile was developed for determination %EE of Nanoparticles (NPs). While, for the assay of Microemulsion (ME) and nanoemulsion (NE) of both drugs, the method was developed in methanol. A method was developed in PBS pH 5 with 2% Tween 80 and in phosphate buffer saline (PBS) pH 5 with 1% SLS respectively for *in vitro* drug diffusion study of emulsion containing TMD and LTG. Assay method of NPs is employed for determination of drug release. Surface hydrophilicity of NPs is imparted by polyvinyl alcohol (PVA). The concentration of residual PVA associated with NPs is determined using colorimetric iodine reaction and estimated spectrophotometrycally. So, calibration of PVA in water was incorporated in this section. The NPs were intended to be conjugated with proteins (Transferrin (Tf) and Lavtoferrin (Lf)). Method is used for estimation of protein content of NPs. Hence, standared plot of bicinchoninic acid (BCA) is employed in this section.

3.2 Methods

3.2.1 Estimation of Tramadol

Estimation of TMD was performed by UV spectrophotometry. A common method was developed in acetonitrile for %EE and release study of drug from NPs. The method was developed in methanol for assay of drug and in PBS pH 5 with 2% Tween-80 for release study of drug in ME and NE (emulsions).

3.2.1.1 Estimation of Tramadol in acetonitrile

Preparation of standard stock solutions of TMD in acetonitrile

50 mg of TMD was accurately weighed using single pan electronic balance and transferred to 100 ml volumetric flask. Approximately 30-40 ml of acetonitrile was transferred to the above volumetric flask, the drug was dissolved properly and then the final volume of the flask was made up to 100 ml with acetonitrile to prepare stock solution of 500 µg per ml of TMD.

Calibration curve of TMD in acetonitrile

Suitable aliquots of standard stock solution were accurately pipetted and transferred to the 10 ml of volumetric flasks. The final volume was made up to 10 ml with acetonitrile to give final concentrations of 25, 50, 75, 100, 125, 150 µg/ml and analyzed by UV spectrophotometry at 278nm. The above procedure was repeated three times. The data was recorded in Table 3.2 along with standard deviation. Fig. 3.1 shows calibration curve of TMD in acetonitrile.

3.2.1.2 Estimation of Tramadol in methanol

Preparation of standard stock solutions of TMD in methanol

50 mg of TMD was accurately weighed using single pan electronic balance and transferred to 100 ml volumetric flask. Approximately 30-40 ml of methanol was transferred to the above volumetric flask, the drug was dissolved properly and then the final volume of the flask was made up to 100 ml with methanol to prepare stock solution of 500 µg per ml of TMD.

Calibration curve of TMD in methanol

Suitable aliquots of standard stock solution were accurately pipetted and transferred to the 10 ml of volumetric flasks. The final volume was made up to 10 ml with methanol to give final concentrations of 25, 50, 75, 100, 125, 150 µg/ml and analyzed by UV spectrophotometry at 278nm. The above procedure was repeated three times. The data was recorded in Table 3.7 along with standard deviation. Fig. 3.2 shows calibration curve of TMD in methanol.

3.2.1.3 Estimation of Tramadol in PBS pH 5 with 2% Tween-80

Preparation of standard stock solutions of TMD in PBS pH 5 with 2% Tween-80

50 mg of TMD was accurately weighed using single pan electronic balance and transferred to 100 ml volumetric flask. Approximately 30-40 ml of acetonitrile of 0.2M PBS pH 5 with 2% Tween-80 was transferred to the above volumetric flask, the drug was dissolved properly and

then the final volume of the flask was made up to 100 ml with 0.2M PBS pH 5 with 2% Tween-80 to prepare stock solution of 500 µg/ml of TMD.

Calibration curve of TMD in PBS pH 5 with 2% Tween-80

Suitable aliquots of standard stock solution were accurately pipetted and transferred to the 10 ml of volumetric flasks. The final volume was made up to 10 ml with 0.2M PBS pH 5 with 2% Tween-80 to give final concentrations of 25, 50, 75, 100, 125, 150, 175, 200 µg/ml and analyzed by UV spectrophotometry at 278nm. The above procedure was repeated three times. The data was recorded in Table 3.12 along with standard deviation. Fig. 3.3 shows the calibration curve of TMD in 0.2M PBS pH 5 with 2% Tween-80.

3.2.1.4 Analytical method Validation

Linearity

The Linearity of an analytical method is its ability to elicit, test results that are directly or by a well defined mathematical transformation proportional to the concentration of analyte in samples with a given range (Rifino CB, 2003). Linearity of an analytical method for TMD in acetonitrile, methanol and PBS pH5 with 2% Tween-80 was established by the regression coefficient as shown in Table 3.3, 3.8, 3.13 respectively.

Accuracy

Accuracy of an analytical method is the closeness of test results obtained by that method to true value (USP30-NF25, 2007). Accuracy is calculated from the test results as the percentage of analyte recovered by assay. Accuracy was calculated by analysis of three replicate samples for the above described methods. The observed concentrations of the drug were then back calculated using the equation of standard calibration curve and compared with actual concentrations. Accuracy of method for analysis of TMD in acetonitrile, methanol and PBS pH5 with 2% Tween-80 was show in Table 3.4, 3.9, 3.14 respectively.

Precision

Precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple sampling of homogenous sample (USP30-NF25, 2007). Precision may be measure of either the degree of reproducibility or of repeatability of the analytical method under normal operating conditions. The precision of an analytical method is usually expressed as the standard deviation or confidence limit.

The intra- and inter day precision of the assay were calculated by replicate analysis of the solutions of known concentrations of TMD at three control concentration (low, medium, high). The observed concentrations of the drug were then back calculated (from absorbance) using the equation of standard calibration curve. The variations between the observed concentrations were determined by calculating the % RSD (Rifino CB, 2003).

Intra-day Precision of the Assay

Primary stock solutions were appropriately diluted using suitable solvent to obtain final concentration. Three different sets of primary stock solutions were prepared and diluted in the similar manner. The absorbance was measured at specific λ_{max} using a UV-Visible spectrophotometer (Shimadzu, UV-1700, Pharmaspec, Japan) against suitable solvent blank three times on the same day. The solutions were prepared freshly on each time. The % RSD was calculated and the results are recorded in Table 3.5, 3.10, 3.15 for TMD in acetonitrile, methanol and PBS pH5 with 2% Tween-80 respectively.

Inter-day Precision of the Assay

Primary stock solutions were appropriately diluted using suitable solvent to obtain final concentration. Three different sets of primary stock solutions were prepared and diluted in the similar manner. The absorbance was measured at specific λ_{max} using a UV-Visible spectrophotometer (Shimadzu, UV-1700, Pharmaspec, Japan) against suitable solvent on three consecutive days. The solutions were prepared freshly on each time. The % RSD was calculated and the results are recorded in Table 3.6, 3.11, 3.16 for TMD in acetonitrile, methanol and PBS pH 5 with 2% Tween-80 respectively.

3.2.2 Estimation of Lamotrigine

Estimation of LTG was performed by UV spectrophotometry. A common method was developed in acetonitrile for %EE and release study of drug from NPs. The method was developed in methanol for assay of drug and in PBS pH 5 with 1% SLS for release study of drug in ME and NE.

3.2.2.1 Estimation of Lamotrigine in acetonitrile

Preparation of standard stock solutions of LTG in acetonitrile

50 mg of LTG was accurately weighed using single pan electronic balance and transferred to 50 ml volumetric flask. Approximately 20-25 ml of acetonitrile was 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 to produce 1000 µg/ml of acetonitrile.

10 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 to prepare stock solution of 100 µg/ml of LTG.

Calibration curve of LTG in acetonitrile

Suitable aliquots of standard stock solution were accurately pipetted and transferred to the 10 ml of volumetric flasks. The final volume was made up to 10 ml with acetonitrile to give final concentrations of 1, 2.5, 5, 10, 15, 20, 25, 30 µg/ml and analyzed by UV spectrophotometry at 307nm. The above procedure was repeated three times. The data was recorded in Table 3.17 along with standard deviation. Fig. 3.4 shows the calibration curve of LTG in acetonitrile.

3.2.2.2 Estimation of Lamotrigine in methanol

Preparation of standard stock solutions of LTG in methanol

50 mg of LTG was accurately weighed using single pan electronic balance and transferred to 50 ml volumetric flask. Approximately 20-25 ml of methanol was 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 methanol to produce 1000 µg/ml of methanol.

10 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 methanol to prepare stock solution of 100 µg/ml of LTG.

Calibration curve of LTG in methanol

Suitable aliquots of standard stock solution were accurately pipetted and transferred to the 10 ml of volumetric flasks. The final volume was made up to 10 ml with methanol to give final concentrations of 0.5, 5, 10, 15, 20, 30, 35 µg/ml and analyzed by UV spectrophotometry at 307 nm. The above procedure was repeated three times. The data was recorded in Table 3.22 along with standard deviation. Fig. 3.5 shows the calibration curve of LTG in methanol.

3.2.2.3 Estimation of Lamotrigine in PBS pH 5 with 1% SLS

Preparation of standard stock solutions of LTG in PBS pH 5 with 1% SLS

50 mg of LTG was accurately weighed using single pan electronic balance and transferred to 50 ml volumetric flask. Approximately 20-25 ml of 0.05M PBS pH 5 with 1% SLS was

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 0.05M PBS pH 5 with 1% SLS to prepare stock solution of 1000 µg/ml of LTG.

10 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 0.05M PBS pH 5 with 1% SLS to prepare stock solution of 100 µg/ ml of LTG.

Calibration curve of LTG in PBS pH 5 with 1% SLS

Suitable aliquots of standard stock solution were accurately pipetted and transferred to the 10 ml of volumetric flasks. The final volume was made up to 10 ml with 0.05M PBS pH 5 with 1% SLS to give final concentrations of 2.5, 5, 10, 15, 20, 25, 30, 35 µg/ml and analyzed by UV spectrophotometry at 307nm. The above procedure was repeated three times. The data was recorded in Table 3.27 along with standard deviation. Fig. 3.6 shows the calibration curve of LTG in PBS pH 5 with 1% SLS.

3.2.2.4 Analytical method Validation

Linearity

The Linearity of an analytical method is its ability to elicit, test results that are directly or by a well defined mathematical transformation proportional to the concentration of analyte in samples with a given range (Rifino CB, 2003). Linearity of an analytical method for LTG in acetonitrile, methanol and PBS pH5 with 1%SLS was established by the regression coefficient as shown in Table 3.18, 3.23, 3.28 respectively.

Accuracy

Accuracy of an analytical method is the closeness of test results obtained by that method to true value (USP30-NF25, 2007). Accuracy is calculated from the test results as the percentage of analyte recovered by assay. Accuracy was calculated by analysis of three replicate samples for the above described methods. The observed concentrations of the drug were then back calculated using the equation of standard calibration curve and compared with actual concentrations. Accuracy of method for analysis of LTG in acetonitrile, methanol and PBS pH5 with 1% SLS was show in Table 3.19, 3.24, 3.29 respectively

Precision

Precision of an analytical method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple sampling of homogenous sample (USP30-NF25, 2007). Precision may be measure of either the degree of reproducibility or of repeatability of the analytical method under normal operating conditions. The precision of an analytical method is usually expressed as the standard deviation or confidence limit.

The intra- and inter day precision of the assay were calculated by replicate analysis of the solutions of known concentrations of LTG at three concentration (low, medium, high). The observed concentrations of the drug were then back calculated (from absorbance) using the equation of standard calibration curve. The variations between the observed concentrations were determined by calculating the % RSD (Rifino CB, 2003).

Intra-day Precision of the Assay

Primary stock solutions were appropriately diluted using suitable solvent to obtain final concentration. Three different sets of primary stock solutions were prepared and diluted in the similar manner. The absorbance was measured at specific λ_{max} using a UV-Visible spectrophotometer (Shimadzu, UV-1700, Pharmaspec, Japan) against suitable solvent blank three times on the same day. The solutions were prepared freshly on each time. The % RSD was calculated and the results are recorded in Table 3.20, 3.25, 3.30 for LTG in acetonitrile, methanol and PBS pH5 with 1% SLS respectively.

Inter-day Precision of the Assay

Primary stock solutions were appropriately diluted using suitable solvent to obtain final concentration. Three different sets of primary stock solutions were prepared and diluted in the similar manner. The absorbance was measured at specific λ_{max} using a UV-Visible spectrophotometer (Shimadzu, UV-1700, Pharmaspec, Japan) against suitable solvent on three consecutive days. The solutions were prepared freshly on each time. The % RSD was calculated and the results are recorded in Table 3.21, 3.26, 3.31 for LTG in acetonitrile, methanol and PBS pH5 with 1% SLS.

3.2.3 Estimation of residual PVA

The amount of PVA associated with NPss 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 (Joshi DP 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 10-200 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 3.32 along with standard deviation. Fig. 3.7 shows calibration curve of PVA in water. Linearity of method for estimation of PVA in water was recorded in Table 3.33.

3.2.4 Determination of proteins (Tf and Lf) by BCA method

Protein assay based on bicinchoninic acid (BCA) is a most sensitive 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 PK et al., 1985). The purple-colored product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. (Wiechelman K et al., 1988) 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- Cu^{+} complex
2. Cu^{+} + 2 Bichinchoninic Acid BCA- Cu^{+} complex (purple colored, read at 562nm)

Procedure

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 3.1.

3. To prepare BCA working Reagent (BWR), 50 parts of reagent A was mixed with 1 part 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.
4. 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.
5. 2 ml of the BWR was added to each test tube and mixed well.
6. All the test tubes were incubated at 60°C for 30 min.
7. 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 3.34.
8. A standard curve was prepared by plotting the average absorbance reading for each Tf and Lf standard vs. its concentration in µg/ml and plotted as shown in Fig. 3.8
9. Using this standard curve, the protein concentration for each unknown sample was determined. Linearity of BCA method for estimation of Tf and Lf was recorded in Table 3.35.

Table 3.1: Preparation of diluted Tf and Lf standards

Volume of the Tf and Lf solution	Volume of Diluent	Final Tf and Lf concentration
300 µl of stock	1200 µl	1000 µg/ml (A)
87.5 µl of (A)	412.5 µl	175 µg/ml (B)
75 µl of (A)	425µl	150 µg/ml (C)
62.5 µl of (A)	437.5 µl	125 µg/ml (D)
50 µl of (A)	450 µl	100 µg/ml (E)
37.5 µl of (A)	462.5 µl	75 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)	493.75µl	12.5 µg/ml (I)

3.3 Results and Discussion

3.3.1 Estimation of Tramadol

3.3.1.1 Calibration of Tramadol in acetonitrile

Table 3.2: Calibration of TMD in acetonitrile

Concentration (µg/ ml)	Mean Absorbance*	SD	%RSD
12.5	0.079	0.001	1.266
25	0.178	0.001	0.562
50	0.346	0.003	0.867
75	0.519	0.002	0.385
100	0.678	0.003	0.442
125	0.846	0.009	1.064
150	0.996	0.006	0.602

*n=3

Figure 3.1: Regressed calibration curve for estimation of TMD in acetonitrile

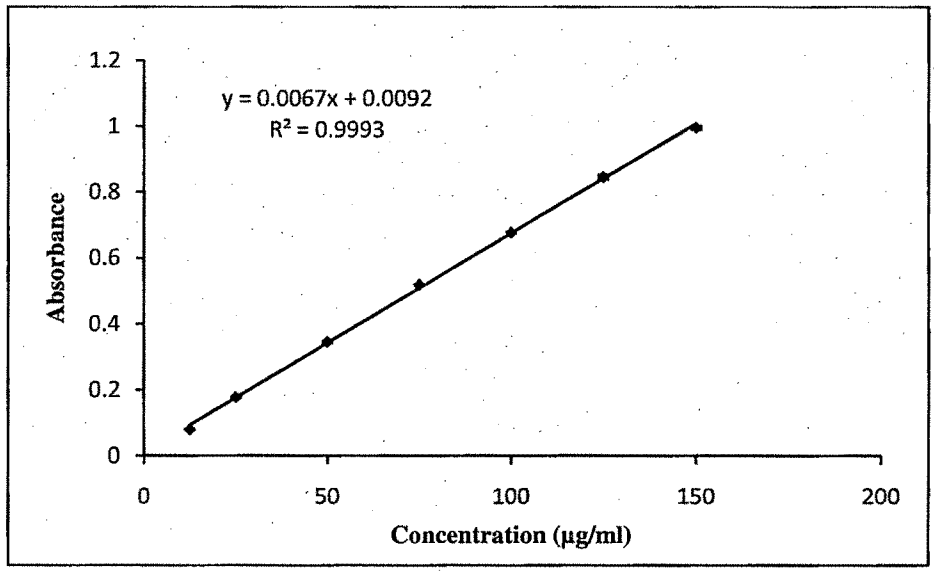

Table 3.3: Linearity of method for estimation of TMD in acetonitrile

Parameter	Value
Wavelength (nm)	278
Linearity range (µg/ ml)	12.5 - 150
Regression equation	y = 0.0067x + 0.0092
Regression coefficient (R^2)	0.9993

Table 3.4: Accuracy of the developed method for TMD in acetonitrile

% of TMD added	Actual Concentration (µg/ml)	Mean of observed Conc.* (µg/ml)	% Accuracy*
50	37.5	37.50 ± 0.11	99.99 ± 0.28
100	75	75.40 ± 0.23	100.54 ± 0.31
150	112.5	112.22 ± 0.21	99.75 ± 0.19

*Values are represented as mean ± SD, n= 6

Table 3.5: Intraday precision for TMD determination in acetonitrile

TMD Concentration (µg/ml)		Mean of observed conc.* (µg/ml)	SD	Precision as %RSD
		Set-I		
Low	12.5	12.58	0.065	0.517
Medium	75	74.99	0.124	0.165
High	150	150.02	0.327	0.218
		Set-II		
Low	12.5	12.61	0.005	0.040
Medium	75	75.03	0.108	0.144
High	150	149.98	0.526	0.351
		Set-III		
Low	12.5	12.48	0.056	0.449
Medium	75	75.01	0.213	0.284
High	150	150.12	0.165	0.110

*n=6

Table 3.6: Inter day precision for TMD determination in acetonitrile

TMD Concentration (µg/ml)		Mean of observed conc.* (µg/ml)	SD	Precision as %RSD
		Day-I		
Low	12.5	12.54	0.009	0.072
Medium	75	74.84	0.232	0.310
High	150	149.92	0.421	0.281
		Day –II		
Low	12.5	12.52	0.064	0.511
Medium	75	74.9	0.342	0.457
High	150	150.08	0.217	0.145

		Day –III		
Low	12.5	12.46	0.054	0.433
Medium	75	74.89	0.235	0.314
High	150	150.16	0.423	0.282

*n=6

3.3.1.2 Calibration of Tramadom in methanol

Table 3.7: Calibration of TMD in methanol

Concentration (µg/ ml)	Mean Absorbance*	SD	%RSD
12.5	0.086	0.0007	0.814
25	0.174	0.001	0.575
50	0.343	0.004	1.166
75	0.504	0.002	0.397
100	0.663	0.006	0.905
125	0.825	0.007	0.848
150	0.98	0.011	1.122

*n=3

Figure 3.2: Regressed calibration curve for estimation of TMD in methanol

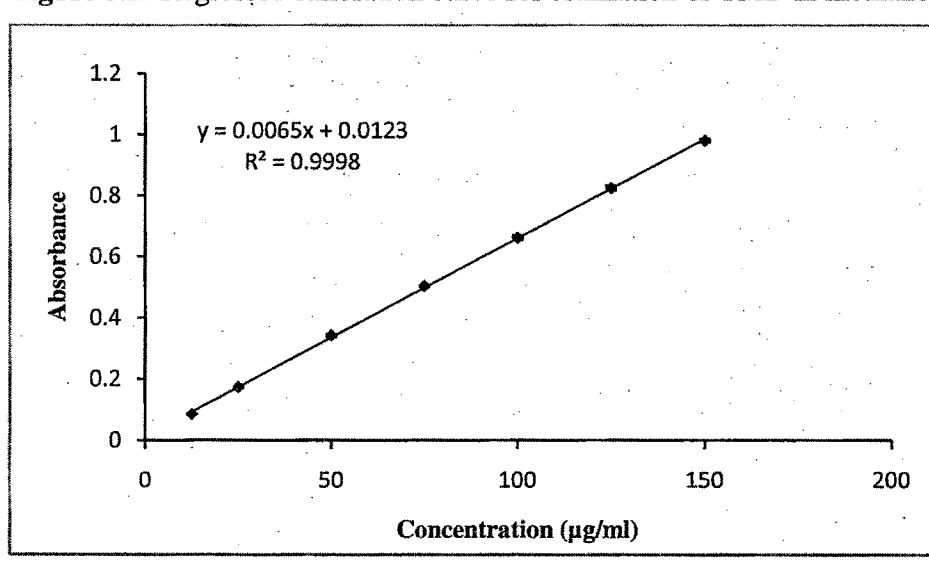

Table 3.8: Linearity of method for estimation of TMD in methanol

Parameter	Value
Wavelength (nm)	278
Linearity range (µg/ ml)	12.5-150
Regression equation	y = 0.0065x + 0.0123
Regression coefficient (R^2)	0.9998

Table 3.9: Accuracy of the developed method for TMD in methanol

% of TMD added	Actual Concentration (µg/ml)	Mean of observed Conc.* (µg/ml)	% Accuracy*
50	37.5	37.50 ± 0.12	100.01 ± 0.32
100	75	74.98 ± 0.33	99.97 ± 0.44
150	112.5	112.40 ± 0.29	99.91 ± 0.26

*Values are represented as mean ± SD, n= 6

Table 3.10: Intraday precision for TMD determination in methanol

TMD Concentration (µg/ml)		Mean of observed conc.* (µg/ml)	SD	Precision as %RSD
		Set-I		
Low	12.5	12.62	0.054	0.428
Medium	75	75.04	0.272	0.362
High	150	149.98	0.137	0.091
		Set-II		
Low	12.5	12.56	0.025	0.199
Medium	75	75.12	0.312	0.415
High	150	150.03	0.511	0.341
		Set-III		
Low	12.5	12.55	0.006	0.048
Medium	75	74.98	0.178	0.237
High	150	150.01	0.247	0.165

*n=6

Table 3.11: Inter day precision for TMD determination in methanol

TMD Concentration (µg/ml)		Mean of observed conc.* (µg/ml)	SD	Precision as %RSD
		Day-I		
Low	12.5	12.47	0.076	0.609
Medium	75	75.13	0.136	0.181
High	150	149.79	0.321	0.214
		Day –II		
Low	12.5	12.49	0.042	0.336
Medium	75	75.06	0.323	0.430
High	150	149.89	0.137	0.091
		Day –III		
Low	12.5	12.58	0.064	0.509
Medium	75	74.99	0.156	0.208
High	150	150.11	0.231	0.154

*n=6

3.3.1.3 Calibration of Tramadol in PBS pH 5 with 2% Tween-80

Table 3.12: Calibration of TMD in PBS pH 5 with 2% Tween-80

Concentration (µg/ ml)	Mean Absorbance*	SD	%RSD
25	0.102	0.001	0.980
50	0.219	0.003	1.370
75	0.335	0.002	0.597
100	0.448	0.005	1.116
125	0.539	0.005	0.928
150	0.663	0.003	0.452
175	0.781	0.005	0.640
200	0.902	0.008	0.887

*n=3

Figure 3.3: Regressed calibration curve for estimation of TMD in PBS pH 5 with 2% Tween-80

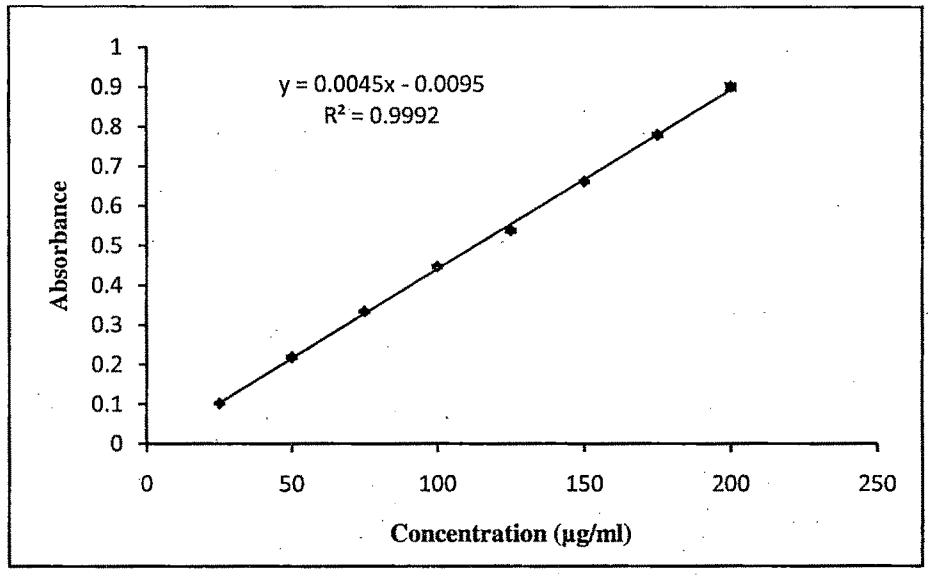

Table 3.13: Linearity of method for estimation of TMD in PBS pH5 with 2% Tween-80

Parameter	Value
Wavelength (nm)	278
Linearity range (µg/ ml)	25-200
Regression equation	y = 0.0045x - 0.0095
Regression coefficient (R^2)	0.9992

Table 3.14: Accuracy of the developed method for TMD in PBS pH 5 with 2% Tween-80

% of TMD added	Actual Concentration (µg/ml)	Mean of observed Conc.* (µg/ml)	% Accuracy*
50	50	49.88 ± 0.24	99.75 ± 0.48
100	100	100.11 ± 0.26	100.11 ± 0.29
150	150	150.46 ± 0.50	100.30 ± 0.33

*Values are represented as mean ± SD, n= 6

Table 3.15: Intra day precision for TMD determination in PBS pH 5 with 2% Tween-80

TMD Concentration (µg/ml)		Mean of observed conc.* (µg/ml)	SD	Precision as %RSD
		Set-I		
Low	25	25.04	0.096	0.383
Medium	125	125.13	0.232	0.185
High	200	199.89	0.386	0.193
		Set-II		
Low	25	25.12	0.045	0.179
Medium	125	124.89	0.322	0.258
High	200	200.03	0.286	0.143
		Set-III		
Low	25	24.93	0.078	0.313
Medium	125	125.08	0.385	0.308
High	200	199.96	0.358	0.179

*n=6

Table 3.16: Inter day precision for TMD determination in PBS pH 5 with 2% Tween-80

TMD Concentration (µg/ml)		Mean of observed conc.* (µg/ml)	SD	Precision as %RSD
		Day-I		
Low	25	25.1	0.005	0.020
Medium	125	125.21	0.312	0.249
High	200	200.14	0.475	0.237
		Day –II		
Low	25	25.02	0.101	0.404
Medium	125	124.95	0.321	0.257
High	200	200.11	0.417	0.208
		Day –III		
Low	25	24.99	0.101	0.404
Medium	125	125.1	0.326	0.261
High	200	200.01	0.456	0.228

*n=6

The UV spectroscopic method was used for the TMD estimation in acetonitrile, methanol and PBS pH 5 with 2% Tween-80. The measurement was done at 278nm for all three solvents. There was no interference observed with any excipient used. The method was validated for

linearity, accuracy and precision. The validation parameters were found to meet the "readily pass criteria" specified in the USP and % RSD were found less than 1%.

The absorbance for TMD in acetonitrile was found to be linear in the range of 12.5-150 µg /ml with r^2 value of 0.9993 (Fig. 3.1). The % recovery of 99.75% to 100.54% (Table 3.4) showed the method was accurate to estimate TMD in that 12.5-150 µg/ml range. The repeatability of the measurement was expressed in terms of %RSD and the %RSD for intra-day and inter-day of TMD at 3 different concentration levels were shown in Table 3.5 and 3.6 respectively.

The absorbance for TMD in methanol was found to be linear in the range of 12.5-150 µg/ml with r^2 value of 0.9998 (Fig. 3.2). The % recovery of 99.91% to 100.01% (Table 3.9) showes the method was accurate to estimate TMD in that 12.5-150 µg/ml range. The repeatability of the measurement was expressed in terms of %RSD and the %RSD for intra-day and inter-day of TMD at 3 different concentration levels were shown in Table 3.10 and 3.11 respectively.

The absorbance for TMD in PBS pH 5 with 2% Tween 80 was found to be linear in the range of 25-200 µg/ml with r^2 value of 0.9992 (Fig. 3.3). The % recovery of 99.75% to 100.30% (Table 3.14) showes the method was accurate to estimate TMD in that 25-200 µg/ml range. The repeatability of the measurement was expressed in terms of %RSD and the %RSD for intra-day and inter-day of TMD at 3 different concentration levels were shown in Table 3.15 and 3.16 respectively. Results of accuracy and precision are indicating the reliability of the developed method.

3.3.2 Estimation of Lamotrigine

3.3.2.1 Calibration of Lamotrigine in acetonitrile

Table 3.17: Calibration of LTG in acetonitrile

Concentration (µg/ ml)	Mean Absorbance*	SD	%RSD
1	0.054	0.0006	1.111
2.5	0.084	0.0008	0.952
5	0.17	0.001	0.588
10	0.338	0.003	0.888
15	0.497	0.002	0.402
20	0.653	0.004	0.613
25	0.832	0.007	0.841
30	0.979	0.008	0.817

*n=3

Figure 3.4: Regressed calibration curve for estimation of LTG in acetonitrile

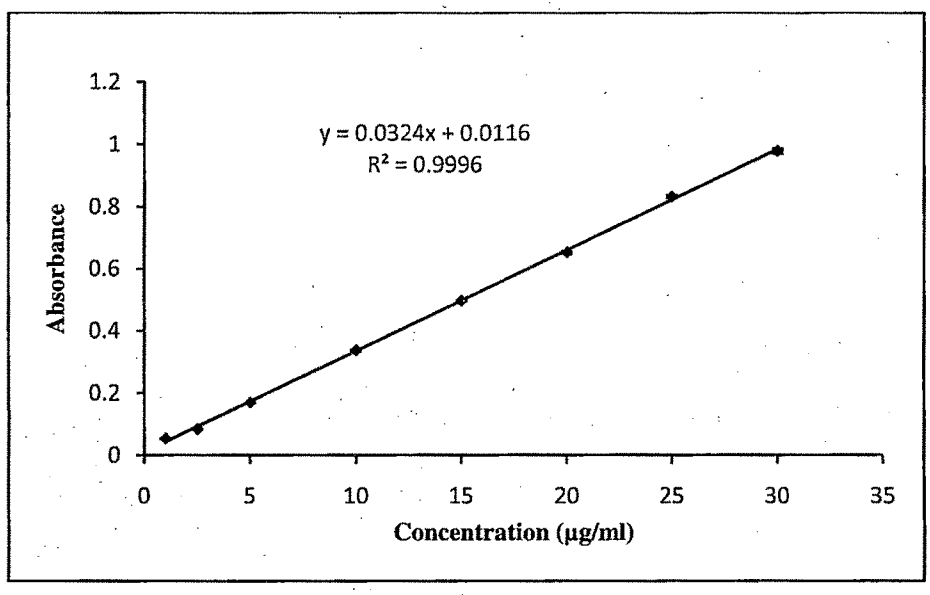

Table 3.18: Linearity of method for estimation of LTG in acetonitrile

Parameter	Value
Wavelength (nm)	307
Linearity range (µg/ ml)	1-30
Regression equation	y = 0.0324x + 0.0116
Regression coefficient (R^2)	0.9996

Table 3.19: Accuracy of the developed method for LTG in acetonitrile

% of LTG added	Actual Concentration (µg/ml)	Mean of observed Conc.* (µg/ml)	% Accuracy*
50	7.5	7.53 ± 0.04	100.35 ± 0.54
100	15	14.89 ± 0.07	99.25 ± 0.45
150	22.5	22.65 ± 0.08	100.68 ± 0.36

*Values are represented as mean ± SD, n= 6

Table 3.20: Intraday precision for LTG determination in acetonitrile

TMD Concentration (µg/ml)		Mean of observed conc.* (µg/ml)	SD	Precision as %RSD
		Set-I		
Low	1	1.02	0.003	0.294
Medium	15	14.95	0.092	0.615
High	30	29.92	0.125	0.418
		Set-II		
Low	1	0.99	0.002	0.202
Medium	15	15.07	0.103	0.683
High	30	30.05	0.178	0.592
		Set-III		
Low	1	1.12	0.004	0.357
Medium	15	15.17	0.087	0.574
High	30	30.08	0.203	0.675

*n=6

Table 3.21: Inter day precision for LTG determination in acetonitrile

TMD Concentration (µg/ml)		Mean of observed conc.* (µg/ml)	SD	Precision as %RSD
		Day-I		
Low	1	1.1	0.006	0.545
Medium	15	15.14	0.073	0.482
High	30	29.89	0.113	0.378
		Day –II		
Low	1	0.94	0.003	0.319
Medium	15	14.87	0.088	0.592
High	30	29.9	0.167	0.559
		Day –III		
Low	1	0.98	0.002	0.204
Medium	15	15.01	0.067	0.446
High	30	30.1	0.205	0.681

*n=6

3.3.2.2 Calibration of Lamotrigine in methanol

Table 3.22: Calibration for LTG in methanol

Concentration (µg/ ml)	Mean Absorbance*	SD	%RSD
2.5	0.064	0.0006	0.938
5	0.146	0.002	1.370
10	0.295	0.001	0.339
15	0.425	0.005	1.176
20	0.562	0.004	0.712
25	0.716	0.005	0.698
30	0.848	0.008	0.943
35	0.976	0.006	0.615

*n=3

Figure 3.5: Regressed calibration curve for estimation of LTG in methanol

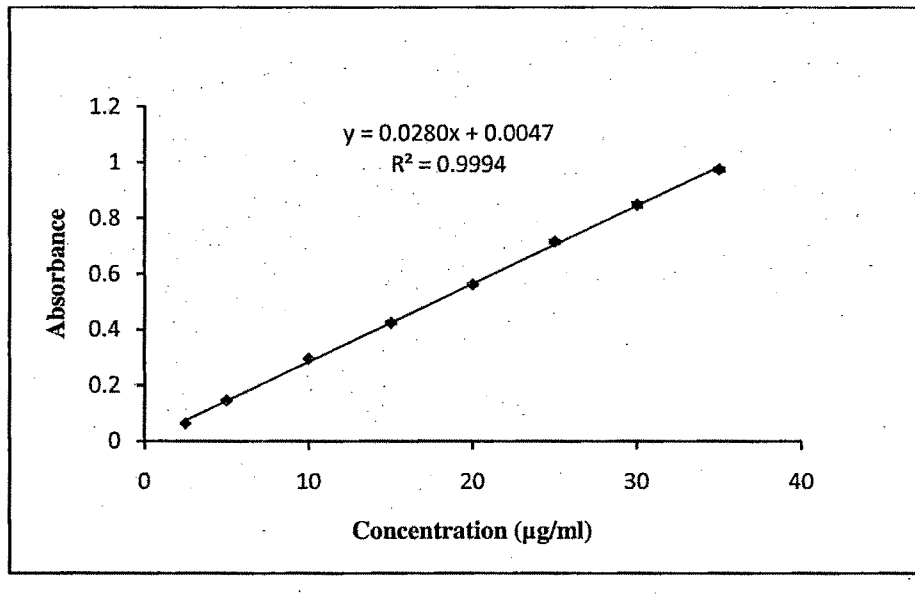

Table 3.23: Linearity of method for estimation of LTG in methanol

Parameter	Value
Wavelength (nm)	307
Linearity range (µg/ ml)	2.5-35
Regression equation	y = 0.0280x + 0.0047
Regression coefficient (R^2)	0.9994

Table 3.24: Accuracy of the developed method for LTG in methanol

% of LTG added	Actual Concentration (µg/ml)	Mean of observed Conc.* (µg/ml)	% Accuracy*
50	10	10.07 ± 0.05	100.74 ± 0.46
100	20	19.90 ± 0.03	99.49 ± 0.15
150	30	30.27 ± 0.11	100.88 ± 0.38

*Values are represented as mean ± SD, n= 6

Table 3.25: Intraday precision for LTG determination in methanol

TMD Concentration (µg/ml)		Mean of observed conc.* (µg/ml)	SD	Precision as %RSD
		Set-I		
Low	2.5	2.48	0.008	0.323
Medium	20	20.19	0.121	0.599
High	35	34.87	0.134	0.384
		Set-II		
Low	2.5	2.55	0.009	0.353
Medium	20	20.05	0.087	0.434
High	35	35.03	0.237	0.677
		Set-III		
Low	2.5	2.57	0.012	0.467
Medium	20	19.97	0.108	0.541
High	35	35.11	0.092	0.262

*n=6

Table 3.26: Inter day precision for LTG determination in methanol

TMD Concentration (µg/ml)		Mean of observed conc.* (µg/ml)	SD	Precision as %RSD
Day-I				
Low	2.5	2.39	0.003	0.126
Medium	20	20.03	0.054	0.270
High	35	35.01	0.216	0.617
Day –II				
Low	2.5	2.42	0.007	0.289
Medium	20	19.89	0.108	0.543
High	35	34.92	0.065	0.186
Day –III				
Low	2.5	2.51	0.004	0.159
Medium	20	19.92	0.103	0.517
High	35	34.99	0.217	0.620

*n=6

3.3.2.3 Calibration of Lamotrigine in PBS pH 5 with 1% SLS

Table 3.27: Calibration of LTG in PBS pH 5 with 1% SLS

Concentration (µg/ ml)	Mean Absorbance*	SD	%RSD
2.5	0.089	0.0008	0.899
5	0.171	0.001	0.585
10	0.283	0.003	1.060
15	0.417	0.002	0.480
20	0.536	0.007	1.306
25	0.658	0.004	0.608
30	0.794	0.009	1.134
35	0.922	0.007	0.759

*n=3

Figure 3.6: Regressed calibration curve for estimation of LTG in PBS pH 5 with 1% SLS

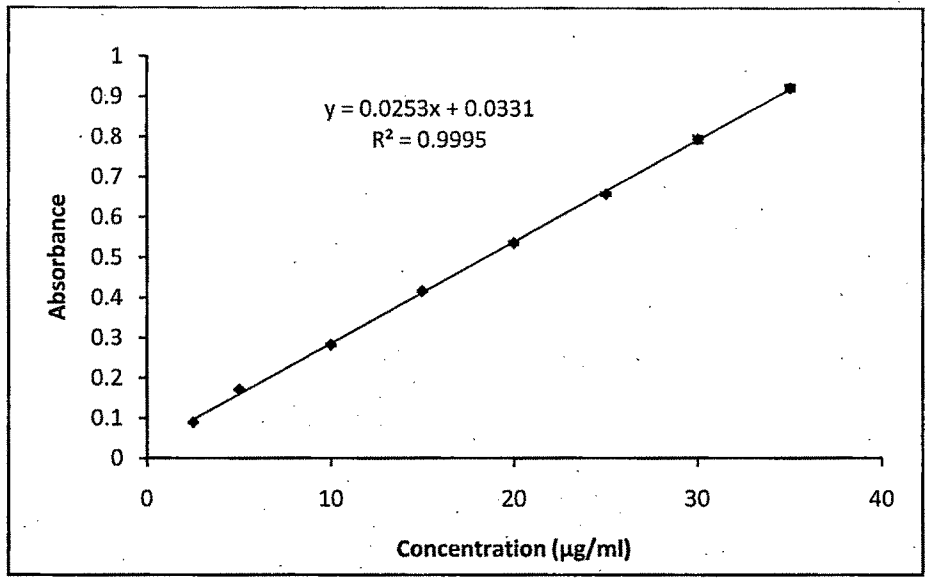

Table 3.28: Linearity of method for estimation of LTG in PBS pH5 with 1% SLS

Parameter	Value
Wavelength (nm)	307
Linearity range (µg/ ml)	2.5-35
Regression equation	y = 0.0253x + 0.0331
Regression coefficient (R^2)	0.9995

Table 3.29: Accuracy of the developed method for LTG in PBS pH 5 with 1% SLS

% of LTG added	Actual Concentration (µg/ml)	Mean of observed Conc.* (µg/ml)	% Accuracy*
50	10	9.99 ± 0.05	99.86 ± 0.51
100	20	20.13 ± 0.07	100.64 ± 0.36
150	30	29.85 ± 0.07	99.49 ± 0.24

*Values are represented as mean ± SD, n= 6

Table 3.30: Intraday precision for LTG determination in PBS pH 5 with 1% SLS

TMD Concentration (µg/ml)		Mean of observed conc.* (µg/ml)	SD	Precision as %RSD
		Set-I		
Low	2.5	2.53	0.009	0.356
Medium	20	19.96	0.109	0.546
High	35	34.98	0.017	0.049
		Set-II		
Low	2.5	2.47	0.012	0.486
Medium	20	20.12	0.123	0.611
High	35	35.07	0.111	0.317
		Set-III		
Low	2.5	2.48	0.007	0.282
Medium	20	20.07	0.112	0.558
High	35	35.1	0.056	0.160

*n=6

Table 3.31: Inter day precision for LTG determination in PBS pH 5 with 1% SLS

TMD Concentration (µg/ml)		Mean of observed conc.* (µg/ml)	SD	Precision as %RSD
		Day-I		
Low	2.5	2.51	0.004	0.159
Medium	20	19.86	0.057	0.287
High	35	34.92	0.102	0.292
		Day –II		
Low	2.5	2.56	0.012	0.469
Medium	20	20.1	0.138	0.687
High	35	35.02	0.226	0.645
		Day –III		
Low	2.5	2.52	0.012	0.476
Medium	20	19.05	0.0756	0.397
High	35	35.08	0.132	0.376

*n=6

The UV spectroscopic method was used for the LTG estimation in acetonitrile, methanol and PBS pH 5 with 1% SLS. The measurement was done at 307 nm for all three solvents. There was no interference observed with any excipient used. The method was validated for linearity,

accuracy and precision. The validation parameters were found to meet the "readily pass criteria" specified in the USP and % RSD were found less than 1%.

The absorbance for LTG in acetonitrile was found to be linear in the range of 1-30 µg /ml with r^2 value of 0.9996 (Fig. 3.4). The % recovery of 99.25% to 100.68% (Table 3.19) showed the method was accurate to estimate LTG in that 1-30 µg/ml range. The repeatability of the measurement was expressed in terms of %RSD and the %RSD for intra-day and inter-day of LTG at 3 different concentration levels were shown in Table 3.20 and 3.21 respectively.

The absorbance for LTG in methanol was found to be linear in the range of 2.5-35 µg/ml with r^2 value of 0.9994 (Fig. 3.5). The % recovery of 99.49% to 100.88% (Table 3.24) showed the method was accurate to estimate LTG in that 2.5-35 µg/ml range. The repeatability of the measurement was expressed in terms of %RSD and the %RSD for intra-day and inter-day of LTG at 3 different concentration levels were shown in Table 3.25 and 3.26 respectively.

The absorbance for LTG in PBS pH 5 with 1% SLS was found to be linear in the range of 2.5-35 µg/ml with r^2 value of 0.9995 (Fig. 3.6). The % recovery of 99.49% to 100.64% (Table 3.29) showed the method was accurate to estimate LTG in that 1-6 µg/ml range. The repeatability of the measurement was expressed in terms of %RSD and the %RSD for intra-day and inter-day of LTG at 3 different concentration levels were shown in Table 3.30 and 3.31 respectively. Results of accuracy and precision are indicating the reliability of the developed method.

3.3.3 Estimation of PVA

Table 3.32: Calibration of PVA in water

Concentration (µg/ ml)	Mean Absorbance*	SD	%RSD
10	0.048	0.0005	1.042
15	0.065	0.0006	0.923
25	0.115	0.001	0.870
50	0.232	0.002	1.293
100	0.437	0.002	0.458
150	0.652	0.005	0.767
200	0.867	0.007	0.807

*n=3

Figure 3.7: Regressed calibration curve for estimation of residual PVA in water

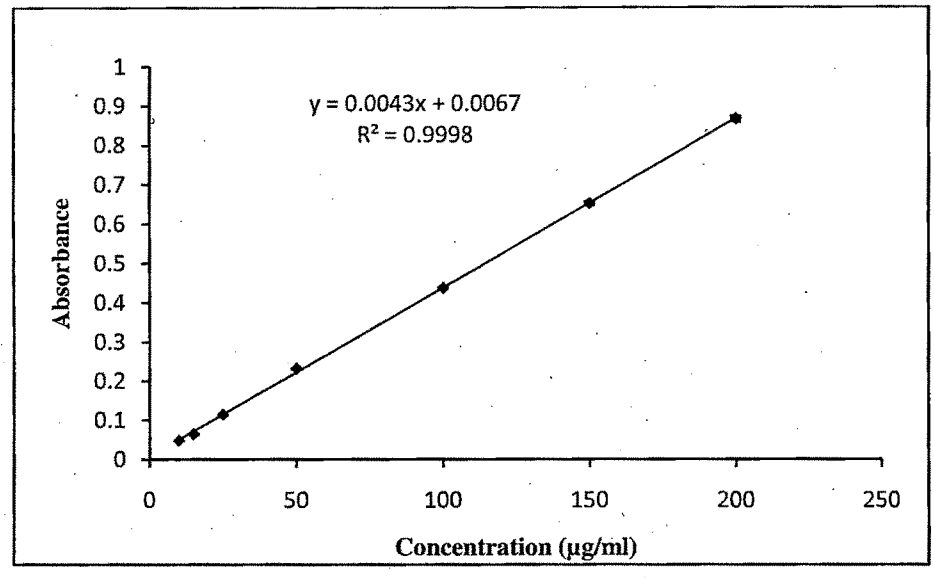

Table 3.33: Linearity of method for estimation of PVA in water

Parameter	Value
Wavelength (nm)	690
Linearity range (µg/ ml)	10-200
Regression equation	y = 0.0043x + 0.0067
Regression coefficient (R^2)	0.9998

3.3.4 Estimation of Tf and Lf

Table 3.34: Calibration of Tf and Lf by BCA method

Sample	Concentration (µg/ ml)	Mean Absorbance*	SD	%RSD
B	175	0.055	0.0002	0.364
C	150	0.107	0.0001	0.093
D	125	0.197	0.001	0.508
E	100	0.284	0.002	0.704
F	75	0.393	0.004	1.018
G	50	0.471	0.003	0.637
H	25	0.586	0.005	0.853
I	12.5	0.679	0.003	0.442

*n=3

Figure 3.8: Regressed calibration curve of Tf and Lf by BCA method

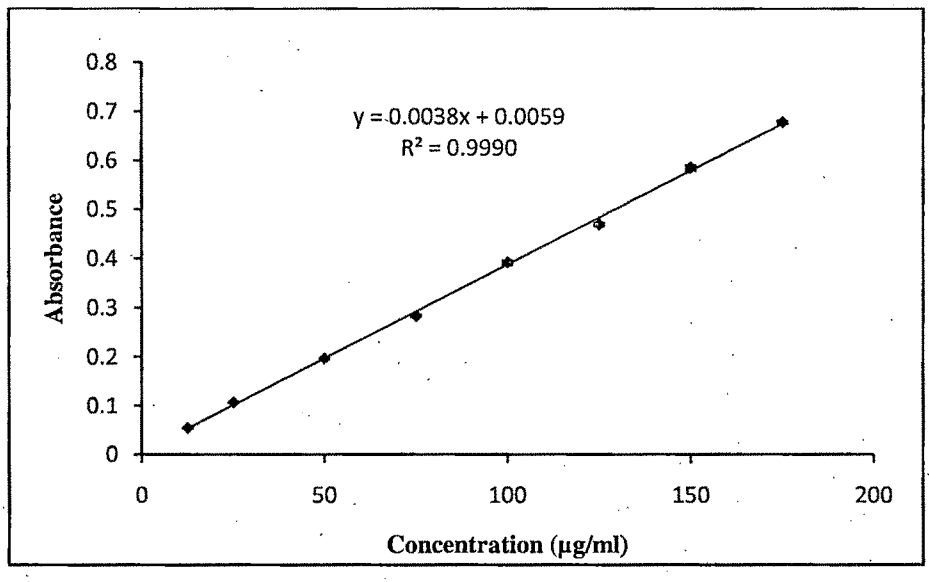

Table 3.35: Linearity of BCA method for estimation of Tf and Lf

Parameter	Value
Wavelength (nm)	562
Linearity range (µg/ ml)	12.5-175
Regression equation	y = 0.0038x + 0.0059
Regression coefficient (R^2)	0.9990

3.4 Conclusion

The UV spectrophotometric method was employed for estimation of drug content and drug released from NPs, ME and NE. The calibration curve of TMD/LTG was established in acetonitrile and methanol for estimation of drug content in NPs and ME/NE respectively. For the estimation of TMD and LTG in diffusion sample of ME/NE the method was developed in PBS pH 5 + 2% Tween-80 and PBS pH 5 + 1% SLS using UV spectrophotometry respectively. The methods for TMD and LTG in all solvents were developed by UV spectrophotometry at 278 and 307 nm respectively.

Results of accuracy and precision are indicating the reliability of the developed methods for both the drugs. There was no interference observed with any excipients used. The methods were found linear, accurate and precise. The validation parameters were complies with USP.

3.5 References

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