

Development and Validation of Spectrophotometric Methods for the Sensitive and Selective Determination of Lamotrigine in Pharmaceuticals Using Bromocresol Purple

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ABSTRACT

Three simple, selective and sensitive methods have been developed and validated for the determination of lamotrigine (LMT) in pure drug and in tablets. The first method (method A) is based on the formation of ion-pair complex between LMT and the dye, bromocresol purple (BCP) at $\text{pH } 2.40 \pm 0.01$ which was extracted into dichloromethane (DCM) and the absorbance of yellow ion-pair complex was measured at 410 nm. In the second and third methods (method B and method C), the drug-dye ion-pair was dissolved either in ethanol and the resulting acid form of the dye was measured at 410 nm or in ethanolic potassium hydroxide and the resulting base form of the dye was measured at 600 nm. Under the optimized conditions, Beer's law was obeyed over 2.0-20.0 $\mu\text{g/mL}$, 150-1500 ng/mL and 50-600 ng/mL for method A, method B and method C, respectively, and the corresponding molar absorptivity values were 1.018×10^4 , 1.43×10^5 and 4.21×10^5 L/mol/cm . The Sandell sensitivity values of 0.0252, 0.0018 and 0.0006 $\mu\text{g/cm}^2$ for method A, method B and method C, respectively, and the corresponding values for limits of detection and quantification were also reported for all three methods. The molar ratio of the formed ion-pair complex was found to be 1: 1 as deduced by Job's method for method A, and the calculated stability constant was also reported. Over the linear ranges applicable, the accuracy and precision of the methods were evaluated on intra-day and inter-day basis; the reported mean accuracy values are $99.50 \pm 1.09\%$, $99.99 \pm 1.11\%$ and $100.72 \pm 0.62\%$ for method A, method B, and method C, respectively; the relative error (RE) was $\leq 2.57\%$ and the relative standard deviation (RSD) was $\leq 2.01\%$. Application of the proposed methods to bulk powder and commercial pharmaceutical tablets are also presented.

Key words: lamotrigine, bromocresol purple, pharmaceuticals, spectrophotometry, determination

INTRODUCTION

Lamotrigine (LMT), [6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine], is an anticonvulsant drug used in the treatment of epilepsy and bipolar disorder as monotherapy or as an adjunct with other antiepileptics for the treatment of partial and generalized toxic-clonic seizures. It is also used to treat neurological lesions and as a tranquilizer^(1,2). LMT is not official in any pharmacopoeia. Most methods for lamotrigine analysis utilized HPLC⁽³⁻¹⁰⁾, HPTLC⁽¹¹⁾ and GC⁽¹²⁾ techniques in biological fluids. Various analytical techniques that have been reported for the determination of this drug in pharmaceuticals include planar chromatography⁽¹³⁾,

TLC and HPLC⁽¹⁴⁾, HPLC and GC⁽¹⁵⁾, capillary electrophoresis^(16,17) and immunoassay⁽¹⁸⁾. Talekar *et al.*⁽¹⁹⁾ have described a UV-spectrophotometric method for the determination of LMT in tablets where the tablet extract in 0.1 M sodium hydroxide was measured at 305 nm. In spite of its simplicity, fair selectivity and sensitivity, and reasonable accuracy and precision, visible spectrophotometry has not been utilized for the assay of LMT except one report⁽¹⁴⁾. Recently Youssef and Taha⁽¹⁴⁾ have reported the application of the technique for the determination of LMT using chloranilic acid as a chromogen. The reported method is less sensitive with a linear range of 10-200 $\mu\text{g/mL}$ and the molar absorptivity of 1.28×10^3 L/mol/cm . Though the method is claimed to be selective, any N-containing basic moiety would definitely interfere with the assay.

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Many of the other reported methods are sensitive and selective but they are time consuming, requires expensive instrumental setup, and some require preliminary sample treatment⁽¹³⁻¹⁸⁾. Adsorptive stripping voltammetric method is highly complicated and is reported to be less precise (RSD ~10%)⁽²⁰⁾. Considering these drawbacks, there was a need to develop more advantageous spectrophotometric method for the determination of LMT in bulk powder and commercial dosage forms.

This paper described three highly sensitive, rapid, simple, selective and economical spectrophotometric methods for the determination of LMT in bulk powder and in tablets by exploiting its basic nature and its ability to form ion-pair complex with bromocresol purple. The first method (method A) is based on the formation of an ion-pair complex between drug and dye bromocresol purple (BCP) at $\text{pH } 2.40 \pm 0.01$ followed by extraction of the complex into dichloromethane (DCM), and the absorbance of yellow drug-dye complex was measured at 410 nm. In the second (method B) and third (method C) methods, the drug-dye ion-pair was broken either in ethanol and the resulting acid form of the dye was measured at 410 nm or in ethanolic alkali and the blue colour of the base form of the dye was measured at 600 nm. The methods (B and C) offered two highly sensitive approaches for determination of LMT in bulk drug and in tablets.

MATERIALS AND METHODS

I. Instruments

A Systronic model 106 digital spectrophotometer (Ahmadabad, India) with matched 1-cm quartz cells was used for absorbance measurements. A digital pH meter Model Elico L1 120 was used for pH measurements.

II. Chemicals and Reagents

Chemicals used were of analytical grade. The solvents used were of the spectroscopic grade. Distilled water was used throughout the investigation.

Zero point one molar concentration Sulphuric acid (S.D. Fine Chem, Mumbai, India, Sp. gr. 1.84), 0.25% bromocresol purple (S.D. Fine Chem Ltd, Mumbai, India), 0.5 M sodium acetate (Merck Specialities Pvt Ltd, Mumbai, India) and sodium acetate-hydrochloric acid buffer of pH 2.4 (hydrochloric acid, Merck Specialities Pvt Ltd, Mumbai, India, Sp. gr. 1.18) were prepared usually in water. A 1% (w/v) ethanolic KOH (S.D. Fine Chem Ltd, Mumbai, India) was prepared in ethanol.

(I) Standard Drug Solution

Pharmaceutical grade LMT (99.88% pure) was procured from Cipla India Ltd, Mumbai, India, as a gift,

and was used as received. Lamotrigine standard solution was prepared as 40 $\mu\text{g/mL}$ solution in 0.1 M H_2SO_4 and used in method A.

III. Recommended Procedures

(I) Method A

Aliquots of LMT standard solution (40 $\mu\text{g/mL}$) containing 1-20 $\mu\text{g/mL}$ LMT were transferred into a series of 125-mL separating funnels and the total volume was brought to 5 mL by adding 0.1 M H_2SO_4 . To each funnel 15 mL of H_2O , 2 mL of 0.5 M sodium acetate (NaOAc) and 5 mL of NaOAc-HCl buffer (pH 2.4) were added. Contents were mixed and 5 mL of 0.25% dye solution was added to each separating funnel, mixed well and kept aside for 5 min. The drug-dye ion-pair was then extracted with 10 mL of dichloromethane by shaking for 30 sec and the layers were allowed to separate. The organic layer was then passed over anhydrous sodium sulphate and absorbance was measured at 410 nm against the reagent blank.

(II) Method B

Into a series of 10-mL volumetric flasks, volumes of LMT-BCP complex (3 $\mu\text{g/mL}$, prepared in method A) equivalent to 150-1500 ng/mL with respect to LMT were transferred. The total volume in each flask was brought to 5 mL by adding dichloromethane. After the addition of 1 mL ethanol, the flasks were kept aside for 5 min, then diluted up to the mark with ethanol and absorbance was measured at 410 nm against the reagent blank.

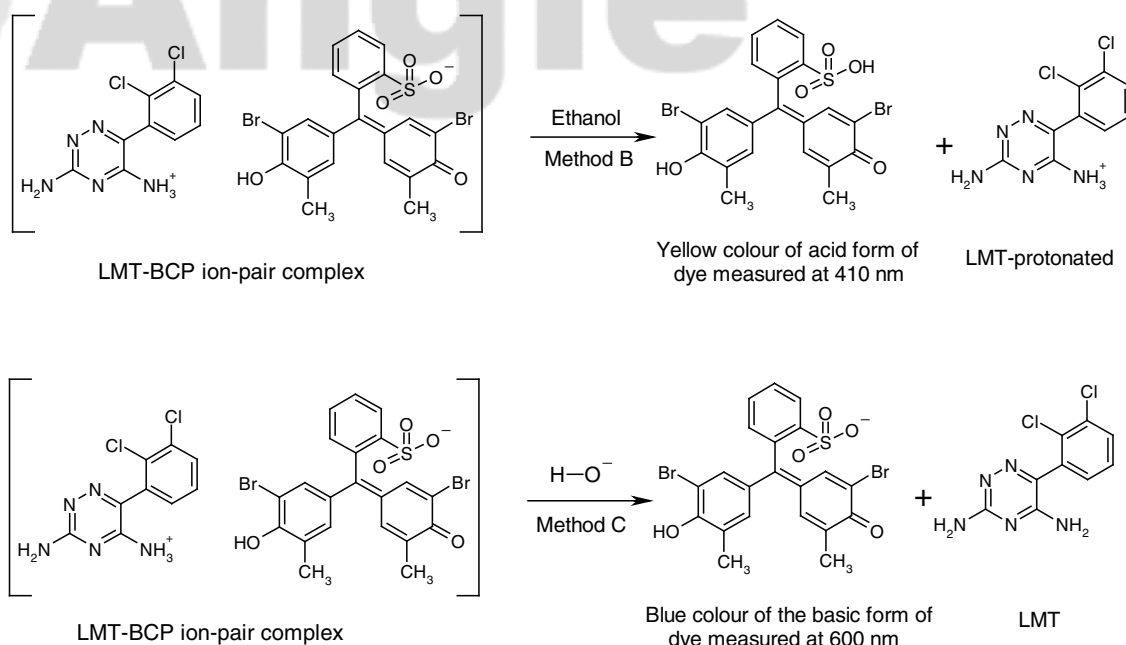
(III) Method C

Varying aliquots of LMT-BCP complex (1 $\mu\text{g/mL}$, prepared by following the procedure described in method A) equivalent to 50-600 ng/mL with respect to LMT were transferred into a series of 10-mL standard flasks and the total volume was brought to 6 mL by adding dichloromethane. To each flask, 1 mL of alcoholic KOH was added, the content was mixed and kept aside for 5 min. Finally, the volume was made up to the mark with ethanol and the absorbance was measured at 600 nm against the reagent blank as reference.

In all the three methods, standard graph was prepared by plotting the absorbance *versus* drug concentration, and the concentration of the unknown was read from the calibration graph or computed from the respective regression equation derived using the absorbance-concentration data.

IV. Analysis of Pharmaceutical Formulations

Lamosyn 100 and Lamosyn 25 (Sun Pharmaceuticals Ltd, Mumbai, India) and Lametec 50 DT (Cipla India Ltd,



Scheme 2. Reaction details about the formation of free dye in ethanolic and alkaline media.

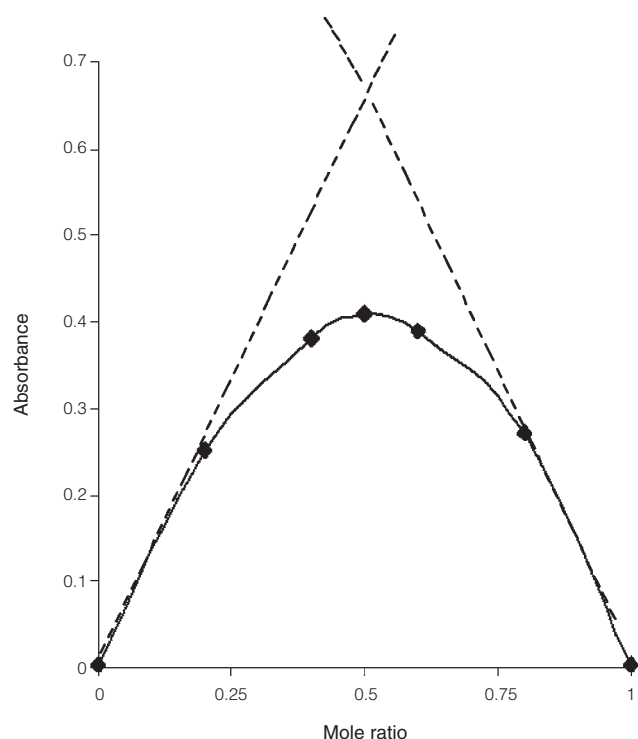


Figure 2. Job's method of equimolar solutions for the LMT-BCP complex in dichloromethane.

experiments were performed by measuring the absorbance at 410 nm in method A and method B, and at 600 nm in method C by varying one and fixing the other parameters in each case.

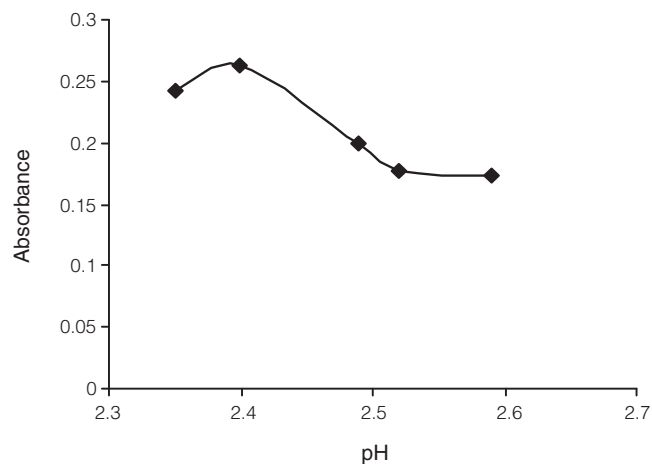


Figure 3. Effect of pH of aqueous phase on the formation and extraction of ion-pair complex.

I. Method Development: Optimization of Experimental Variables

(I) Method A

(1) Effect of pH

In order to establish the optimum pH range, 5 mL of LMT solution in 0.1 M H₂SO₄ was diluted with 5, 10, 15, 20 and 25 mL of water before mixing with 5 mL of the dye solution in the presence of 2 mL of 0.5 M NaOAc and 5 mL buffer, and the effective pH of the resulting

aqueous solution ranged from 2.35 ± 0.01 to 2.59 ± 0.01 . Figure 3 shows that a higher absorbance was observed at pH 2.40, and the absorbance of the reagent blank was negligible at this pH range. Above and below this pH, the sensitivity decreases. There was no complete separation of the organic and aqueous phase at pH values less than 2.40 due to emulsion formed between the two layers. It was also found that a ratio of 3:1 of aqueous to organic phases was required for efficient extraction of the coloured species. Hence, an aqueous phase of 32 mL which includes 15 mL of H₂O was used in all subsequent work.

(2) Effect of Sodium Acetate, BCP and Buffer Concentrations

Various amounts of NaOAc were added to the acidic solution of lamotrigine to bring the pH to the optimum value and measurements were carried out as recommended. Maximum absorbance was observed when the volume of NaOAc was 2 mL (0.5 M) in a total volume of 32 mL. The effect of BCP concentration was investigated by varying the volume of dye solution, and using a fixed amount of drug. The complex formation and its extraction were unaffected in the range of 4.0 to 8.0 mL of 0.25% BCP solution. Hence, 5 mL of 0.25% BCP solution was fixed in a total volume of 32 mL of aqueous phase.

Various amounts of buffer solution were used in the investigation to establish its effect on the absorbance. There was almost no influence on the absorbance up to 10 mL, but an amount less than 4 mL resulted in unsatisfactory separation of the organic phase during the extraction. So 5 mL of buffer was used throughout the investigation.

(3) Reaction Time

After the addition of dye, the effect of standing time was studied in the time range 5-30 min before extraction. After a contact time of 5 min, measured absorbance of the complex after extraction into dichloromethane, showed almost constant absorbance values from 5-30 min. So a contact time of 5 min was adequate to form the complex.

(4) Effect of Shaking Time

Shaking times ranging from 30 to 60 seconds, produced no change in absorbance, by maintaining all other parameters constant. So a 30 sec shaking time was fixed.

(5) Choice of Organic Solvents

In the preliminary experiments, chloroform, benzene, carbon tetrachloride, cyclohexane, hexane were tried as extraction solvents. None of these systems showed better sensitivity compared to dichloromethane. Hence, dichloromethane was chosen as the solvent for extraction.

(6) Effect of Number of Extractions

Under optimum conditions, the drug-dye complex in the aqueous phase was extracted with three 10 mL portions of DCM and absorbance was measured each time. After the second extraction, the absorbance of the organic layer was negligibly small. Hence, a single extraction with 10 mL of DCM was selected for the extraction because of complete recovery of the complex.

(7) Equilibration Time and Stability of the Coloured Complexes

The organic and aqueous phases were clearly separated in less than 1 min. The drug-dye ion-pair complex was stable for more than 12 h at laboratory temperature ($30 \pm 2^\circ\text{C}$).

(8) Effect of Order of Addition of Reactants

The sequence of order of addition of the reactants prior to extraction had small change in the absorbance values. So the order of addition of reactants should be in the described manner. The complexing ratio of LMT and BCP in method A was examined by Job's continuous variations method⁽²²⁾. The concentration of the aqueous dye and the drug was 7.81×10^{-4} M. Six solutions containing LMT and BCP in various molar ratios, with a total volume of 5 mL, in addition to 15 mL H₂O, 2 mL of 0.5 M NaOAc and 5 mL buffer solution were prepared. The extraction was performed using 10 mL of dichloromethane and the absorbance subsequently was measured at 410 nm. The graph of the results obtained (Figure 2) gave a maximum at a molar ratio of $X_{\max} = 0.5$ which indicated the formation of a 1:1 LMT-BCP complex. The conditional stability constant (K_f) of the ion-association complex was calculated from the continuous variation data using the following equation⁽²³⁾:

$$K_f = \frac{A/A_m}{[1 - A/A_m]^{n+2} C_M (n)^n}$$

Where A and A_m are the observed maximum absorbance and the absorbance value when all the drug present is associated, respectively. C_M is the mole concentration of drug at the maximum absorbance and n is the stoichiometry which BCP ion associates with drug. The log K_f value was found to be 4.730.

II. Method Validation

(I) Linearity, Sensitivity, Limits of Detection and Quantification

Calibration graphs were constructed from ten, eight and eight points covering the concentration ranges 2.0-20.0 µg/mL, 150-1500 ng/mL and 50-600 ng/mL for

method A, method B and method C, respectively. Regression analysis of the Beer's law data indicated a linear relationship between absorbance and concentration (Table 1) which is corroborated by high values (close to unity) of the correlations coefficients. A plot of *log* absorbance and *log* concentration, yielded straight lines with slope equal to 1.024, 1.023 and 1.002 for method A, method B and method C, respectively, further establishing the linear relation between the two variables. The calculated molar absorptivity and Sandell sensitivity⁽²⁴⁾ values are summarized in Table 1. The limits of detection (LOD) and quantification (LOQ) were calculated using the formulae:

$$\text{LOD} = 3.3 S/b \text{ and } \text{LOQ} = 10 S/b,$$

(where S is the standard deviation of blank absorbance values, and b is the slope of the calibration plot), calculated according to the ICH guidelines⁽²⁵⁾, are also summarized in Table 1. The high values of ϵ and low values of Sandell sensitivity and LOD indicate the high sensitivity of the proposed methods.

(II) Precision and Accuracy

The assays described under "General Procedures" were repeated seven times within the day to determine the repeatability (intra-day precision) and five times on

different days to determine the intermediate precision (inter-day precision) of the methods. These assays were performed for three levels of analyte. The results of this study are summarized in Table 2. The percentage relative standard deviation (% RSD) values were $\leq 1.39\%$ (intra-day) and $\leq 2.01\%$ (inter-day) indicating high precision of the methods. The accuracy of the methods was determined by the percent mean deviation from known concentration, bias % = [(concentration found - known concentration) \times 100 / known concentration]. Bias was calculated at each concentration and these results are also presented in Table 2. Percent relative error (% RE) values $\leq 1.33\%$ demonstrate the high accuracy of the proposed methods.

(III) Selectivity

A systematic study was performed to determine the effect of matrix by analyzing the placebo blank and synthetic mixture containing LMT. A placebo blank of the composition: starch (10 mg), acacia (15 mg), hydroxyl cellulose (10 mg), sodium citrate (10 mg), talc (20 mg), magnesium stearate (15 mg) and sodium alginate (10 mg) was obtained and its solution was prepared as described under 'tablets', and then subjected to analysis. The absorbance of the placebo solution in each case was almost

Table 1. Sensitivity and regression parameters

Parameter	Method A	Method B	Method C
λ_{max} (nm)	410	410	600
Color stability	> 12 h	> 24 h	> 24 h
Linear range	2.0-20 $\mu\text{g/mL}$	150-1500 ng/mL	100-600 ng/mL
Molar absorptivity(ϵ) (L/mol/cm)	1.018×10^4	1.433×10^5	4.212×10^5
Sandell sensitivity* ($\mu\text{g/cm}$)	0.0252	0.0018	0.0006
Limit of detection (LOD) ($\mu\text{g/mL}$)	0.27	0.02	0.01
Limit of quantification (LOQ) ($\mu\text{g/mL}$)	0.80	0.06	0.02
Regression equation, Y**			
Intercept (a)	-0.0147	-0.0053	0.0030
Slope (b)	0.0415	0.5701	1.6343
Standard deviation of a (S_a)	0.0998	0.0065	0.0052
$\pm tS_a/\sqrt{n}$	0.0713	0.0046	0.0033
Standard deviation of b (S_b)	0.0055	0.0048	0.0087
$\pm tS_b/\sqrt{n}$	0.0039	0.0034	0.0055
Variance (S_a^2)	1.0×10^{-3}	4.3×10^{-5}	2.70×10^{-5}
Regression coefficient (r)	0.9988	0.9997	0.9999

*Limit of determination as the weight in μg per mL of solution, which corresponds to an absorbance of $A = 0.001$ measured in a cuvette of cross-sectional area 1 cm^2 and $l = 1 \text{ cm}$.

** $Y = a + bX$, where Y is the absorbance; X is concentration in $\mu\text{g/mL}$; a is intercept; b is slope; $\pm tS_a/\sqrt{n}$ = confidence limit for intercept; $\pm tS_b/\sqrt{n}$ = confidence limit for slope.

equal to the absorbance of the blank which revealed no interference. To assess the role of the inactive ingredients on the assay of LMT, a synthetic mixture was separately prepared by adding 10 mg of LMT to the placebo mentioned above. The drug was extracted and solution prepared as described under the general procedure for tablets. The solution after appropriate dilution were analyzed following the recommended procedures. The absorbance resulting from 15 µg/mL (method A), 1000 ng/mL (method B) and 400 ng/mL (method C) were nearly the same as those obtained for pure LMT solutions of identical concentrations. This unequivocally demonstrated the non-interference of the inactive ingredients in the assay of LMT. Further, the slopes of the calibration plots prepared from the synthetic mixture solutions were about the same as those prepared from pure drug solutions.

(IV) Robustness and Ruggedness

The robustness of the methods was evaluated by making small incremental changes in volume of H₂O/ethanol/ethanolic KOH and contact time and the effect of the changes was studied on the absorbance of the coloured systems. The changes had negligible influence on the results as revealed by small intermediate precision values expressed as % RSD (< 1.5%).

Method ruggedness was demonstrated by having the analysis done by four analysts, and also by a single analyst performing analysis on four different instruments in the same laboratory. Intermediate precision values (% RSD) in both instances were in the range 0.56-2.65% indicating acceptable ruggedness. The results are presented in Table 3.

Table 2. Evaluation of intra-day and inter-day accuracy and precision

Method	LMT taken*	Intra-day accuracy and precision (n = 7)			Inter-day accuracy and precision (n = 5)		
		LMT found* ± CL	% RE	% RSD	LMT found* ± CL	% RE	% RSD
A	6.0	6.07 ± 0.07	1.10	1.25	6.06 ± 0.15	1.0	2.01
	12.0	11.9 ± 0.15	0.83	1.39	12.01 ± 0.23	0.08	1.56
	18.0	17.8 ± 0.11	1.1	0.64	17.96 ± 0.23	0.22	1.02
B	300	302 ± 3.75	0.67	1.34	302 ± 4.94	0.66	1.32
	900	895 ± 8.88	0.59	1.07	901 ± 11.30	0.11	1.01
	1500	1480 ± 12.61	1.33	0.92	1498 ± 36.2	0.13	1.95
C	200	201 ± 1.36	0.25	0.73	201 ± 1.97	0.50	0.79
	400	404 ± 1.42	0.97	0.38	401.1 ± 4.72	0.28	0.95
	600	604 ± 4.14	0.59	0.74	602.1 ± 7.83	0.35	1.05

% RE: Percent relative error; % RSD: relative standard deviation and CL.

Confidence limits were calculated from: CL = ± tS/√n (The tabulated value of t is 2.45 and 2.77 for six and four degrees of freedom respectively, at the 95% confidence level; S = standard deviation and n = number of measurements).

*The values are in µg/mL in method A and ng/mL in method B and method C.

Table 3. Method robustness and ruggedness expressed as intermediate precision (% RSD)

Method	LMT taken*	Robustness		Ruggedness	
		Parameters altered		Inter-analysts (% RSD), (n = 4)	Inter-instruments (% RSD), (n = 4)
		Volume of H ₂ O/Ethanol / Ethanolic KOH**	Reaction/Breaking time [‡]		
A	15	1.26	0.85	0.72	2.65
B	1000	0.85	1.06	0.69	1.97
C	400	0.76	0.92	0.56	2.36

*The values are in µg/mL in method A and ng/mL in method B and method C.

**In method A, the volume of H₂O was 13, 15 and 17 mL; in method B, the volumes of ethanol added were 0.8, 1.0 and 1.2 mL and in method C, the volumes of ethanolic KOH added were 0.8, 1.0 and 1.20 mL.

[‡]In method A, the reaction times were 3, 5 and 7 min and in method B and method C the breaking times were 3, 5 and 7 min.

Table 4. Results of analysis of tablets by the proposed methods and statistical comparison of the results with the reference method

Tablet brand name ^ψ	Nominal amount (mg/tablet)	Found* (Percent of label claim ± SD)			
		Reference method	Method A	Method B	Method C
Lamosyn-100 ^a	100	98.48 ± 0.65	99.12 ± 0.86	98.76 ± 0.54	98.14 ± 0.45
			t = 1.34	t = 0.74	t = 0.98
Lamosyn-25 ^a	25	100.4 ± 0.56	100.8 ± 0.92	101.2 ± 0.72	100.1 ± 0.36
			F = 1.75	F = 1.45	F = 2.09
Lamotec-50 DT ^b	50	102.3 ± 0.74	102.7 ± 1.04	101.7 ± 0.86	101.1 ± 0.53
			t = 0.85	t = 1.98	t = 1.03
			F = 2.70	F = 1.65	F = 2.42
			t = 0.71	t = 1.18	t = 2.99
			F = 1.98	F = 1.35	F = 1.95

*Mean value of 5 determinations.

(Tabulated t-value at the 95 % confidence level and for four degrees of freedom is 2.77).

(Tabulated F-value at the 95 % confidence level and for four degrees of freedom is 6.39).

^ψMarketed by: ^aSun pharmaceuticals; ^bCipla India Ltd, Mumbai.

(V) Application

The proposed methods were applied for the quantification of LMT in commercial tablets. The results were compared with these obtained by a published method⁽¹⁹⁾. The method consisted of the measurement of the absorbance of the tablet extract in 0.1 M NaOH at 305 nm. Statistical analysis of the results did not detect any significant difference between the performance of the proposed methods and reference method with respect to accuracy and precision as revealed by the Student's t-value and variance ratio F-value⁽²⁶⁾. The results of assay are given in Table 4.

(VI) Recovery Study

To further assess the accuracy of the methods, recovery experiments were performed by applying the standard-addition technique. The recovery was assessed by determining the agreement between the measured standard concentration and added known concentration to the sample. The test was done by spiking the pre-analysed tablet powder with pure LMT at three different levels (50, 100 and 150% of the content present in the tablet powder (taken) and the total was found by the proposed methods. Each test was repeated three times. In all the cases, the recovery percentage values ranged between 96.36 and 103.7 with standard deviation in the range 0.42-1.25%. Closeness of the results to 100% showed the fairly good accuracy of the methods. The results are shown in Table 5.

CONCLUSIONS

The presented work describes validated spectrophotometric methods for the determination of lamotrigine in bulk drug and in tablets. The proposed methods are highly sensitive and are 10-300-fold more sensitive than the only visible spectrophotometric method reported. The sensitivity offered by the proposed methods surpasses even that of the existing HPLC methods in terms of linear range and quantification limits. The methods are quite selective as the drug contains basic moiety which preferentially interacts with bromocresol purple, and the drug-dye ion-pair is extracted into the organic solvent before measurement. The methods are free from interferences from the common excipients and additives. The statistical parameters and the recovery data reveal good accuracy and precision of the methods. Hence, methods can be used in routine analysis of drug in quality control laboratories.

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Table 5. Results of recovery study via standard-addition method

Tablet studied	Method A			Method B			Method C					
	LMT in tablet (µg/mL)	Pure LMT added (µg/mL)	Total found (µg/mL)	Pure LMT recovered (Percent ± SD*)	LMT in tablet (ng/mL)	Pure LMT added (ng/mL)	Total found (ng/mL)	Pure LMT recovered (Percent ± SD*)	LMT in tablet (ng/mL)	Pure LMT added (ng/mL)	Total found (ng/mL)	Pure LMT recovered (Percent ± SD*)
Lamosyn-100	5.95	3.0	9.02	102.3 ± 1.25	493.8	250	737.8	97.56 ± 0.86	196.3	100	295.7	99.37 ± 0.56
	5.95	6.0	12.05	101.6 ± 0.92	493.8	500	975.6	96.36 ± 1.04	196.3	200	396.5	100.1 ± 0.72
	5.95	9.0	15.28	103.7 ± 0.73	493.8	750	1239.5	99.43 ± 0.63	196.3	300	498.4	100.7 ± 0.42

*Mean value of three determinations.

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