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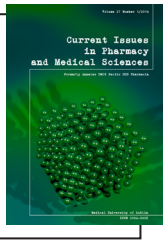

Comparative validation of HPLC, densitometric and videodensitometric determination of lamotrigine in pharmaceutical

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ABSTRACT

Simple, sensitive, precise and accurate HPLC, densitometric and videodensitometric methods for determination of lamotrigine in tablet forms were developed and validated. The HPLC method was carried out using a Symmetry C8 column and a mobile phase acetonitrile-phosphate buffer pH 2.80 (25:75, v/v), with a flow rate of 1 mL/min, and UV detection at 210 nm. Ethosuximide was used as the internal standard. Densitometric and videodensitometric analysis was performed on silica gel 60 F254 plates, in horizontal chambers, with methanol-chloroform-ammonia (25%) 1.5:7.5:1, (v/v) as mobile phase. Densitometric detection was performed at 225 nm and at 315 nm, and videodeoscanning at 254 nm. Calibration plots were constructed in the range 0.5-10 μg/spot, with good correlation coefficients $r > 0.99$ for both methods. The precision and accuracy of all elaborated methods were compared. Finally, the developed methods were applied for the quality control of lamotrigine tablets.

INTRODUCTION

Lamotrigine, 6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine (Fig.1) is an anticonvulsant drug used in the treatment of partial and generalized epileptic seizures. It is also used as a mood stabilizer and for the treatment of bipolar depression. Lamotrigine inhibits the voltage-dependent sodium channels, and, thus, prevents the release of excitatory neurotransmitters. In addition, lamotrigine blocks the high voltage-activated calcium channels [2]. Moreover, it is a lipophilic weak base, and it is well absorbed after oral administration.

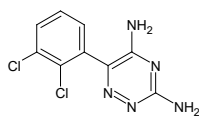

Figure 1. Structure of lamotrigine

In the literature, some concerns are expressed regarding the quantitative analysis of lamotrigine when in pharmaceutical dosage forms. The content of the drug in tablets is normally determined by spectrophotometric [1,7,10,12], spectrofluorimetric [4], HPLC [5,7], HPTLC [7] and capillary electrophoresis [9] methods. The ion-selective electrode method [6] and two different voltammetric techniques: differential pulse adsorptive stripping voltammetry [3,11] and square wave adsorptive stripping voltammetry [3] have also been proposed. In addition, HPLC and TLC methods [13] were normally employed to quantify lamotrigine in the presence of its related impurities, while HPLC [8] alone was used for the evaluation of drug stability under stress conditions.

Lamotrigine is frequently used in the therapy of epilepsy and of bipolar disorder. It is necessary to control the content of lamotrigine in pharmaceuticals, and, therefore, more perfect methods for its determination are still being developed.

The aim of this work was to develop a new, sensitive and precise methods for the routine quantity control of lamotrigine in pharmaceutical products, by way of using HPLC, densitometry and videodensitometry. TLC methods reduce the consumption of reagents and their harmful effects on human health and the environment.

MATERIALS AND METHODS

Chemicals. Lamotrigine and ethosuximide were purchased from Sigma (St. Louis, MO, USA). Lamotrix tablets

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containing 25 mg lamotrigine per tablet were obtained commercially. Methanol, acetonitrile (Merck, Darmstadt, Germany) and chloroform, 25% ammonia solution (POCH, Gliwice, Poland) were of analytical reagent grade. The salts used to prepare the phosphate buffer (KH_2PO_4 and Na_2HPO_4) were of "Ultrapure Bioreagent" (JT Baker, UK) grade. The water used in all experiments was fresh double distilled.

HPLC conditions. The Waters HPLC system (Milford, USA) was employed. This consisted of a Waters 515 isocratic pump, the variable wavelength detector Waters 2487 at 210 nm and a Rheodyne injection valve (20 µL loop). In addition, the column Symmetry C8 (5 µm, 4.6 mm × 250 mm) and a mixture of acetonitrile-phosphate buffer pH 2.80 (25:75, v/v) as a mobile phase (with a flow rate 1 mL/min), were used. Ethosuximide was applied as an internal standard.

TLC conditions. All analyses were performed on 20 cm × 10 cm silica gel 60 F_{254} TLC plates (Merck, Darmstadt, Germany), using methanol-chloroform-ammonia (25%) 1.5:7.5:1 (v/v) as the mobile phase. These plates were then developed at room temperature (20°C) to a distance of 9 cm, in horizontal Teflon DS chambers (Chromdes, Lublin, Poland). Spots were applied 15 mm apart and 10 mm from the lower edge of the plate, by means of a Desaga AS-30 applicator equipped with a Hamilton (USA) syringe.

Densitometry was carried out by means of a Desaga (Heidelberg, Germany) CD 60 densitometer, controlled by Desaga ProQuant software. Spots were scanned at $\lambda = 225$ nm and at $\lambda = 315$ nm, with slit dimensions of 0.04 mm × 6.0 mm.

Videodensitometry was performed at 254 nm, using the Desaga VD40 system comprising of Cab UV-Vis, in conjunction with a high-resolution Mitsubishi color-video CCD camera. The Desaga video-documentation system (Pro-ViDoc, version 3.02), and Desaga software for quantitative analysis (ProResult, version 3.00), were subsequently employed.

Calibration for the HPLC method. The stock solutions of lamotrigine and internal standard (ethosuximide) (1mg/mL) were prepared in methanol. The working standard solutions of lamotrigine at a concentration of 0.1 mg/mL were obtained by dilution of stock solutions, using methanol. A series of calibration solutions of lamotrigine were prepared in 10 mL volumetric flasks, by the appropriate dilution of the working solutions with methanol to reach the concentration range of 0.5-8.0 µg/mL. For each sample, 0.2 mL of the stock solution of ethosuximide was added as an internal standard (20 µg/mL). Each solution was then injected and a chromatogram was recorded. Calibration was performed 5-fold, each time with independent weighing and solvent dilution. The chromatogram obtained from the analysis of lamotrigine calibration solutions is presented in Figure 2.

Calibration for densitometric and videodensitometric methods. Stock solutions (1mg/mL) of lamotrigine were prepared in methanol. The working standard solutions (0.5 mg/mL) of lamotrigine were made up by appropriate dilutions of stock solutions. Methanolic solutions corresponding to 0.5-10 µg of lamotrigine (1-20 µL working solutions) per spot were applied on the plate to perform calibration. Calibration was performed 5-fold, each time with independent weighing and solvent dilution.

Assay in tablets. The average mass of 20 Lamotrix tablets was first determined. The tablets were then ground in a mortar to a fine powder, and the accurately weighted amounts of about 0.1 g were transferred to 25-mL volumetric flasks containing approximately 15 mL methanol. After adding the appropriate volume of internal standard (if needed), the mixtures were shaken mechanically for 15 min, diluted to volume with methanol, and subsequently filtered. The resulting solutions were used for densitometric and videodensitometric analysis. Five micro-liters of each solution was applied to TLC plates. The peak areas were then recorded. For HPLC analysis, the filtrate was appropriately diluted before application to the chromatography column. For each elaborated method, the procedure was repeated five times, individually weighing the tablet powder each time.

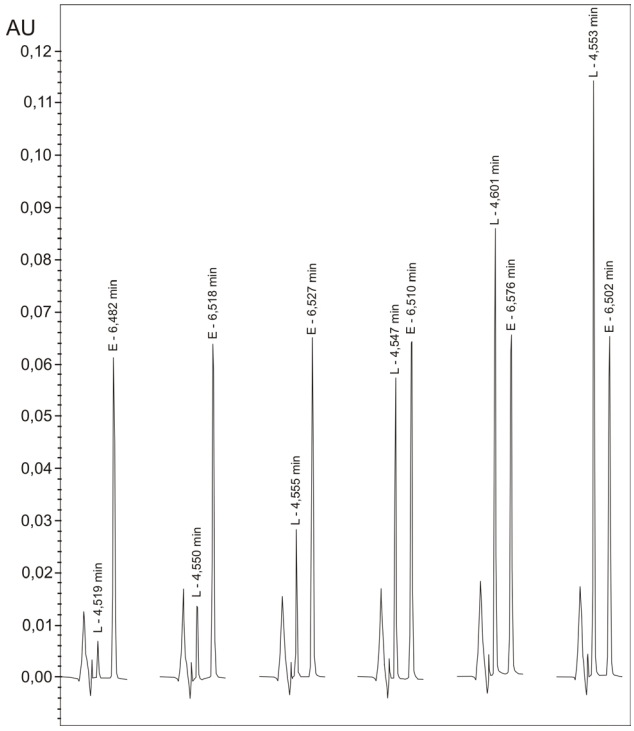

Figure 2. The HPLC chromatogram obtained from analysis of lamotrigine calibration solutions in the range of 0.5 – 8.0 µg/mL; L – lamotrigine, E – ethosuximide (internal standard)

RESULTS AND DISCUSSION

The development of the methods described in this paper required pre-optimization analysis conditions, and different chromatographic conditions were examined (data not shown). The study took into account the best separation of the analytes, the shape of the peaks / spots, selectivity, execution time analysis, linearity, ease of sample preparation, as well as reagents consumption.

A reversed-phase isocratic procedure was proposed as a suitable method for the analysis of lamotrigine in tablet form. Through our research, a mixture of acetonitrile-phosphate buffer pH 2.80 (25:75, v/v) at a flow rate 1 mL/min was found to be an appropriate mobile phase, as this allowed adequate and rapid separation of the lamotrigine and the internal standard (retention times 4.55 and 6.52 min, respectively). Ethosuximide, chosen as an internal standard, was

clearly separated from lamotrigine, and the total analysis time was 7.0 min. As shown in Figure 5, the substances, once eluted, formed well-shaped, symmetrical single peaks, well-separated from the solvent front. In the HPLC methods previously presented for the analysis of lamotrigine in tablet forms [5,7] and for the analysis of the presence of impurity [13] and for stability tests under stress conditions [8], an internal standard was not used. This study uses an internal standard – ethosuximide. The addition of the internal standard to the sample before proceeding with the preparation of the samples for chromatographic analysis allows researchers to correct the loss of the analyte. The use of internal standard method also enables independence to be achieved with regard to the results obtained from fluctuations in the amount of injected sample.

Lamotrigine has two maxima of light absorption: a strong one at 210 nm and a weak one at 308 nm. The wavelength of 210 nm (at which the best detector response for the analyte was obtained) was selected for the determination of lamotrigine by HPLC.

For the calibration graph, five (5) independent determinations were performed at each of six (6) concentrations. The relationship was subsequently constructed between the peak area ratio of lamotrigine to that of the internal standard and the corresponding concentration, by a linear regression equation:

$$y = 0.14283(\pm 0.00054)\, x - 0.00228(\pm 0.00241)$$
$$r = 0.9999.$$

The results indicate a good linear proportionality between the detector response and the concentration of lamotrigine.

Our work also developed TLC methods (densitometry at 225 nm and 315 nm and videodensitometry at 254 nm) for the quantitative determination of lamotrigine in tablet form.

In the densitometric method, the appropriate wavelength for the determination of lamotrigine was determined experimentally using spectrodensitometry. The absorption spectrum of lamotrigine, taken directly from the silica gel plate, showed two absorbance maxima at approximately 225 and 315 nm; these wavelengths were therefore selected for a densitometric evaluation of the drug.

A mobile phase consisting of methanol-chloroform-ammonia (25%) (1.5:7.5:1, v/v) was selected as an optimum, and was utilised to obtain sharp and well-defined spots without tailing at $hR_F = 42.82 \pm 0.57$ (mean ± SD; n = 20).

Calibration was carried out using seven (7) points. For each point, five (5) measurements were made, the data were averaged and calibration curves were calculated. The plot of the peak area versus concentration of lamotrigine was found to be linear in the range of 0.5-10 µg per spot. The calibration curves were represented by the following linear regression equations:

$$y_{\text{Dens 225 nm}} = 320.427(\pm 15.0698)\, x + 301.401(\pm 84.7229)$$
$$(r = 0.9945)$$

and

$$y_{\text{Dens 315 nm}} = 410.132(\pm 20.9601)\, x + 643.001(\pm 117.838)$$
$$(r = 0.9935)$$

and

$$y_{\text{Videodens 254 nm}} = 1138.64(\pm 63.2532)\, x + 1087.71(\pm 355.610)$$
$$(r = 0.9929).$$

The densitogram and video-chromatogram recorded for the calibration solutions are presented in Figures 3A and 4.

An analysis of tablets was performed for five (5) independently weighed samples. Each of these was applied to the plate five (5) times. The results from the analysis of lamotrigine in tablet forms were evaluated statistically; the results are presented in Table 1.

Total recoveries from tablets are 99.78 ± 0.34% for HPLC, 100.32 ± 0.37% for densitometry 225 nm, 99.80 ± 0.56% for densitometry 315 nm and 100.63 ± 0.54% for videodensitometry 254 nm; the results do not differ from the declared contents (verified by way of the Student's t-test). There was also no interference from the excipients present in the formulation. The HPLC chromatogram of the tablets extract is shown in Figure 5. Densitogram and video-chromatogram obtained during analysis of pharmaceutical formulation are shown in Figures 3B and 4A.Limits to detection (LOD) and quantification (LOQ) of lamotrigine were obtained in both the HPLC and TLC-methods experimentally, taking into account the signal to noise ratio. HPLC shows the best sensitivity; the LOD and LOQ were 0.10 µg/mL and 0.25 µg/mL, respectively. The LOD of densitometry, the amount for which the signal-to-noise ratio was 3:1, was 0.01 µg and 0.005 µg per spot for densitometry 225 nm and densitometry 315 nm, respectively; the LOQ, the amount for which the signal-to-noise ratio was 10:1, was 0.02 µg

Table 1. Statistical evaluation of results obtained from determination of lamotrigine in pharmaceutical preparations

	HPLC	Densitometry 225 nm	Densitometry 315 nm	Videodensitometry 254 nm
Amount claimed [mg]	25	25	25	25
Mean amount found [mg]	24.9460	25.0806	24.9495	25.1579
Recovery [%]	99.78	100.32	99.80	100.63
Variance	0.00733	0.00857	0.01921	0.01829
Standard deviation [mg/tablet]	0.0856	0.0926	0.1386	0.1352
Relative standard deviation [%]	0.34	0.37	0.56	0.54
95% Confidence interval	24.8397–25.0523	24.9656–25.1956	24.7289–25.1701	24.9899–25.3259
Difference between the declared and found amounts (t-Student test)	TV = -1.411 < $t_{95\%,4}$ = 2.776	TV = 1.948 < $t_{95\%,4}$ = 2.776	TV = -0.729 < $t_{95\%,4}$ = 2.776	TV = 2.610 < $t_{95\%,4}$ = 2.776

TV = the tested value

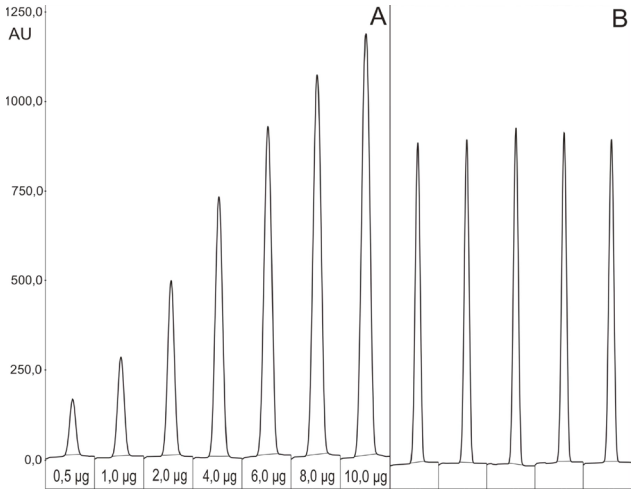

Figure 3. Densitograms obtained from the analysis of lamotrigine standard solutions in the calibration range of 0.5 – 10.0 µg per spot (A), and of tablet samples (B)

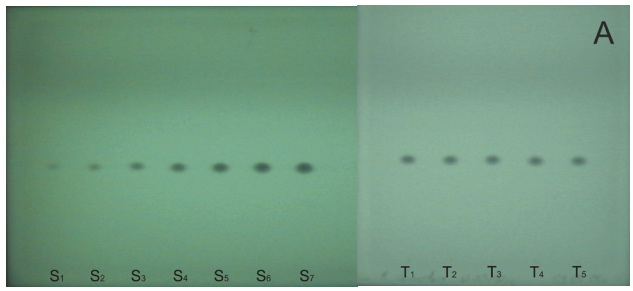

Figure 4. Video-chromatograms obtained from the analysis of lamotrigine calibration solutions (S1 – S7), and of tablet samples T1 – T5 (A)

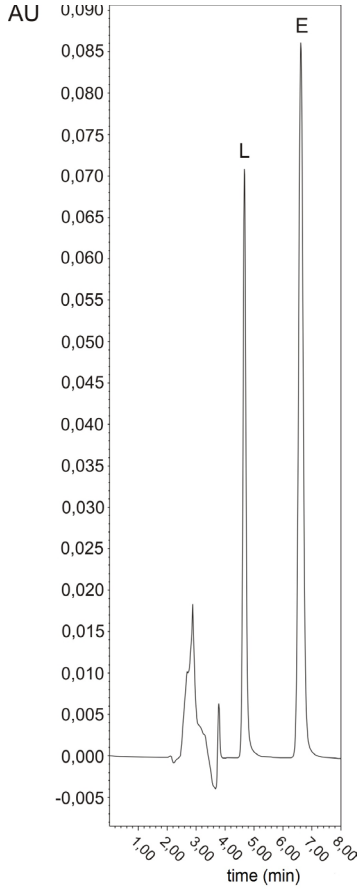

Figure 5. The HPLC chromatogram of lamotrigine (L) and ethosuximide (E) – internal standard, after extraction from Lamotrix tablets

per spot for both densitometric methods. In the videodensitometry, the LOD and LOQ were 0.05 µg and 0.2 µg per spot, respectively.

The precision of the analyzes are expressed using RSD. The best precision was observed in HPLC, and it is higher (RSD < 0.4%) than the precision given in current literature regarding the HPLC method when used for determination of lamotrigine in tablets (RSD < 1.8% [5]; RSD < 1.86% [7]). The precision of the HPLC method reported by Martins et al. [8] had RSD in the range 0.53 to 1.71%, and by Youssef and Taha [13], RSD < 1.15%. Densitometric and videodensitometric methods also resulted in good precision (RSD < 1%), and this result is higher than the precision of employed TLC methods as described previously (RSD = 2.19% [7]; RSD = 1.30% [13]).

The accuracy of the elaborated methods was assessed on the basis of the determination of lamotrigine in fortified samples at three (3) levels of addition (50, 100, and 150 % of the drug concentration in tablets). For HPLC, the recovery results ranged from 97.69 to 99.77 %, with RSD values ranging from 0.97 to 0.51 %. For densitometry, the recovery results ranged from 98.24 % (RSD of 2.18%) to 101.46 % (RSD of 1.93%) at 225 nm, and from 95.12 % (RSD of 2.78 %) to 101.95 % (RSD of 3.04 %) at 315 nm. For videodensitometry, the recovery results ranged from 97.27 to 103.54 %, with RSD values ranging from 2.05 to 3.17 %. Results are the average of five (5) determinations at each level.

The elaborated methods were compared in respect of accuracy and precision, together (Bartlett, ANOVA and

Table 2. Comparison of precision (Bartlett and F-test) and accuracy (ANOVA, Kruskal-Wallis and Wilcoxon tests) of elaborated methods

Compared Methods	F- Snedecor		t-Student		Wilcoxon	
	F	p	t	p	W	p
Densitometry 225 nm – densitometry 315 nm	5,3315	0,1339	0,3281	0,75510	11	0,83403
Densitometry 225 nm – videoscanning	1,1000	0,9286	-1,5559	0,15842	5	0,15079
Densitometry 315 nm – videoscanning	4,8468	0,1555	-2,3943	0,05680	2	0,03614
Densitometry 225 nm - HPLC	1,1744	0,8799	2,3894	0,04408	22	0,05555
Densitometry 315 nm - HPLC	4,5397	0,1720	2,8389	0,03145	23	0,03614
Videoscanning - HPLC	1,0676	0,9509	4,0706	0,00358	25	0,00793
Bartlett test	$K^2 = 2,56931$, $p = 0,46289$					
ANOVA test	$F = 6,68818$, $p = 0,00389$					
Kruskal-Wallis test	$X^2 = 11,37427$, $p = 0,00986$					

Kruskal-Wallis tests) and pairwise using the F-Snedecor, t-Student or Wilcoxon test. The results are presented in Table 2.

There are no differences in precision of all compared methods (Bartlett test). Precision of the methods, compared pairwise show no differences (F-Snedecor test) and the accuracy is significantly different only between: densitometry 315 nm and HPLC, videodensitometry and HPLC.

CONCLUSION

The proposed HPLC, densitometric and videodensitometric methods are simple, sensitive and precise. They were successfully applied for the determination of lamotrigine in tablet forms. High recoveries of analyte and low coefficients of variation confirm the suitability of the elaborated methods for the routine analysis of lamotrigine in pharmaceutical products.

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