Development and Validation of Spectrophotometric, TLC and HPLC Methods for the Determination of Lamotrigine in Presence of Its Impurity

Nadia Fayek YOUSSEF* and Elham Anwer TAHA

National Organization for Drug Control and Research "NODCAR"; 6 Abou Hazem St.Pyramids Ave. P.O. Box-29 Cairo, Egypt. Received October 17, 2006; accepted January 6, 2007

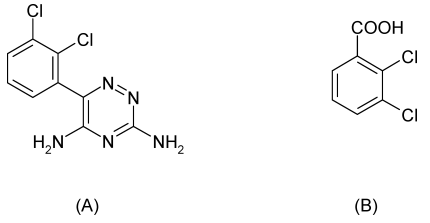
Three reliable, rapid and selective methods have been developed and validated for the determination of lamotrigine in the presence of its impurity, 2,3-dichlorobenzoic acid. The first method is spectrophotometric method using *p*-chloranilic acid forming a colored product with λ_{max} 519±2 nm. All variables affecting the reaction have been investigated and the conditions were optimized. Beer's law was obeyed over a concentration range of 10—200 μg ml^{-1} with mean accuracy 100.13±0.44%. The molar ratio of the formed ion-association complex is found to be 1 : 1 as deduced by Job's method. The conditional stability constant (K_f), standard free energy (ΔG), molar absorptivity(ε), and sensitivity index were evaluated. The second method is based on TLC separation of the cited drug (Rf=0.75±0.01) from its impurity (Rf=0.23±0.01) followed by densitometric measurement of the intact drug spots at 275 nm. The separation was carried on silica gel plates using ethyl acetate : methanol : ammonia 35% (17 : 2 : 1 v/v/v) as a mobile phase. The linearity range was 0.5—10 μg/spot with mean accuracy 99.99±1.33%. The third method is accurate and sensitive stability-indicating HPLC method based on separation of lamotrigine from its impurity on a reversed phase C_{18} column, using a mobile phase of acetonitrile : methanol : 0.01 M potassium orthophosphate (pH 6.7±0.1) (30 : 20 : 50 v/v/v) at ambient temperature 25±5 °C and UV detection at 275 nm in an overall analysis time of about 6 min., based on peak area. The injection repeatability, intraday and interday repeatability were calculated. The procedure provided a linear response over the concentration range 1—12 μg ml^{-1} with mean accuracy of 99.50±1.30%. The proposed methods were successfully applied for the determination of lamotrigine in bulk powder, in dosage form and in presence of its impurity. The results obtained were analyzed by ANOVA to assess that no significant difference between each of the three methods and the reported one. The validation was performed according to USP guidelines.

Key words lamotrigine; 2,3-dichlorobenzoic acid; *p*-chloranilic acid; TLC; HPLC

Lamotrigine [6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine], is a broad-spectrum antiepileptic, used as monotherapy and as an adjunct with other antiepileptics for treatment of partial and generalized tonic-clonic seizures. Its use to treat neurological lesions and as a tranquilizer also has been studied.[1,2]

Lamotrigine is not official in any pharmacopoeia, two stability indicating HPLC methods with UV detection at 210 nm have been reported with either longer analysis time[3] or at definite temperature 40 °C.[4] The present HPLC method differs from those reported previously[3,4] in terms of mobile phase, pH and temperature, so the main advantage is that the analysis was proceeded at ambient temperature (25±5 °C) not at definite temperature 40 °C. Moreover, an UV spectrophotometric method using 0.1 M sodium hydroxide was reported for its determination in tablets.[5] But no work seems to have been done on colorimetric assay.

Most methods for lamotrigine analysis utilized HPLC technique in biological fluids.[6—12] Few methods have been published for its determination including thin layer chromatography which needs 2 h for tank saturation and can not determine lamotrigine in presence of its impurity,[5] gas chromatography,[13] capillary electrophoresis[14,15] and immunoassay.[16,17] In the present study we suggest a simple selective and validated spectrophotometric method using *p*-chloranilic acid which is used as chromogenic agent for different drugs, it gives an immediate purple chromogen at room temperature.[18] Also TLC and HPLC methods that can identify and quantitate lamotrigine in presence of its impurity 2,3-dichlorobenzoic acid, in bulk and dosage form.



(A) (B)

Fig. 1. Structure of Lamotrigine (A) and 2,3-Dichlorobenzoic Acid (B)

Experimental

Instrumentation Shimadzu UV/VIS spectrophotometer 1601. Shimadzu dual wave length flying spot densitomerter, Model CS-9301, photomode=reflection, scan mode=zigzag and swing width=10. TLC plates (10×10 cm) aluminium plates precoated with 0.25 mm silica gel F254, were purchased from E. Merck. UV lamp–short wave length 254 nm. The HPLC system (Waters Co.) consisted of a Model 600 LC series pump and 600 controller unit, 486 tunable absorbance detector, an injector valve with a 20 μl constant loop, and 745 data module. A 250×4.6 mm Alltech Adsorbosphere C_{18}, 10 μm column was used for separation and quantification.

Materials and Reagents Lamotrigine working standard, was supplied by Delta Pharma Co. (India), the purity of the sample was found to be 99.68±0.97% according to the reported specifications.[3] 2,3-Dichlorobenzoic acid (99.90%), was purchased from Riedel-de Haem Pestanal. Lamictal tablets were purchased from Welcome Co. Egypt, labeled to contain 25 mg lamotrigine per tablet. *p*-Chloranilic acid (E. Merck), 42 mg ml^{-1} in acetone. Methanol, acetonitrile and ethylacetate (Lab-scan, Ireland). Potassium orthophosphate (El-Nasr Co., Egypt). All chemicals are of spectroscopic and chromatographic grade.

Standard Solutions Lamotrigine (1 mg ml^{-1}) was prepared in acetone for spectrophotometric method, and in methanol for TLC and HPLC methods. 2,3-Dichlorobenzoic acid (1 mg ml^{-1}) was prepared in acetone for colorimetric method and in methanol for TLC and HPLC methods. The standard solution of HPLC method was subsequently used to prepare working standard solution (0.04 mg ml^{-1}) in the mobile phase.

* To whom correspondence should be addressed. e-mail: gomagdy@yahoo.com

All solutions were kept in a refrigerator at 4 °C and were stable for one week.

Synthetic Mixtures Aliquots of standard solutions of 2,3-dichlorobenzoic acid equivalent to 0.1—10% were added to the standard solutions of lamotrigine containing 2 mg (for spectrophotometric and TLC method) and 0.08 mg (for HPLC method), and the volumes were completed with the appropriate solvents to 10 ml for sspectrophotometric, HPLC methods and to 5 ml for TLC.

Sample Preparation Ten tablets were accurately weighed and finely powdered. An amount equivalent to 100 mg lamotrigine was transferred into 100 ml volumetric flask, and dissolved in 50 ml acetone or methanol. The solution was stirred with magnetic stirrer for 10 min, filtered and the volume was completed to the mark.

Chromatographic Conditions For TLC method, the plates were developed in ethyl acetate : methanol : ammonia 35% (17 : 2 : 1 v/v/v) as the mobile phase. For detection and quantification, 10 μl each of the sample solutions and standard solutions of different concentrations within the linearity range were applied as separate compact spots 10 mm apart and 15 mm from the bottom of the TLC plate using 25 μl Hamilton microsyringe. The chromatographic tank was saturated with the mobile phase for 30 min before development of the plates. The plates were developed up to 8 cm in the usual ascending way, air-dried, and scanned for lamotrigine at 275 nm by using the instrumental parameters mentioned above.

For HPLC method, the mobile phase was prepared by mixing acetonitrile : methanol : 0.01 M potassium orthophosphate adjusted with phosphoric acid to pH 6.7±0.1, in the proportions of (30 : 20 : 50 v/v/v). It was filtered by using a 0.45 μm membrane filter and degassed in an ultrasonic bath before use. The samples were also filtered by using 0.45 μm Teflon filters. The flow rate was set at 1.5 ml/min and UV detector at 275 nm. The column was conditioned for ≥30 min. All determinations were performed at ambient temperature 25±5 °C and the injection volume was 20 μl.

Procedures. Calibration for Spectrophotometric Method Aliquots of standard solution (1 mg ml^{-1}) equivalent to (0.1—2 mg) lamotrigine were transferred into 10 ml volumetric flasks. *p*-Chloranilic acid solution (3 ml) was added and the volume was completed to the mark with acetone. Absorbance of the colored product was measured at λ_{max} 519 nm. The calibration curve was plotted and the regression equation was recorded.

Calibration for TLC Method Aliquots of standard solution (1 mg ml^{-1}) equivalent to (0.25—5 mg) lamotrigine were transferred into a series of 5-ml volumetric flasks and the volume was completed with methanol. Ten microliters of each solution was applied to TLC plate and developed as described under chromatographic conditions previously mentioned under 'Chromatographic Conditions.' The plates were visualized at 254 nm and scanned at 275 nm by densitometer. Calibration curve was plotted representing the relationship between the average peak area and concentration and the regression equation was recorded.

Calibration for HPLC Method Aliquots of standard solution (0.04 mg ml^{-1}) equivalent to (0.01—0.12 mg) lamotrigine were transferred into 10-ml volumetric flasks and the volume was completed to the mark with the mobile phase. Triplicate 20 μl injections were made of each concentration. The average peak areas were calculated and plotted *versus* concentrations, linear relationship was obtained and the regression equation was recorded.

Application to Tablets The above producers were applied to the analysis of lamictal tablets, using sample preparation as mentioned under 'Sample Preparation.' The concentration of lamotrigine was calculated from the recorded regression equations.

Results and Discussion

Method Development. Spectrophotometric Method The UV absorption spectra of lamotrigine and its impurity 2,3-dichlorobenzoic acid display complete overlap from 200—320 nm in methanol and dilute acid with broad maximum absorption at 275 nm (Fig. 2). The reaction of lamotrigine with *p*-chloranilic acid yields an immediate pink color at an ambient temperature 25±5 °C with a characteristic λ_{max} 519±2 nm (Fig. 3). This bathochromic shift is due to the formation of ion-association complex between lamotrigine and *p*-chloranilic acid which confirmed by IR spectra, while 2,3-dichlorobenzoic acid fails to give this reaction. So, this method could be used as stability-indicating assay. All factors affecting the formation of ion-association complex namely, reagent concentration, solvent used, temperature and reaction time were thoroughly studied. The optimum conditions were incorporated into the general procedure. Beer's law was over concentration range 10—200 μg/ml and the parameters of the regression equation are shown in Table 1. Several organic solvents as methanol, ethanol, dichloromethane, acetonitrile and acetone were tried. Acetone was found to be the best solvent for lamotrigine and *p*-chloranilic

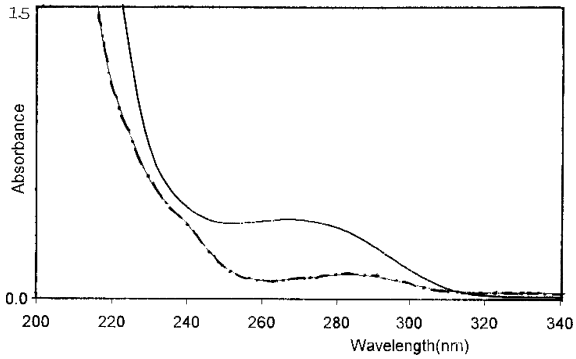


Fig. 2. Absorption Spectra of Lamotrigine (10 μg ml^{-1}) —— and 2,3-Dichlorobenzoic Acid (10 μg ml^{-1}) –·–·–

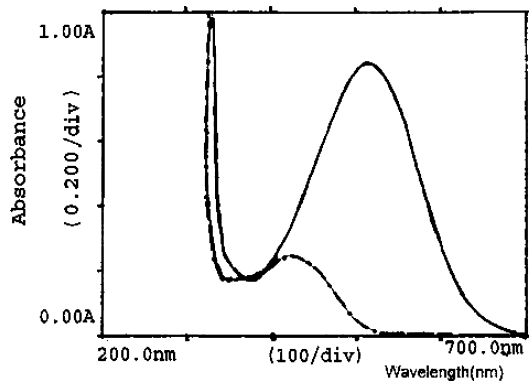


Fig. 3. Absorption Spectra of Ion-association Complex between Lamotrigine (160 μg ml^{-1}) and *p*-Chloranilic Acid (250 μg ml^{-1}) —— and Blank Experiment –·–·–

Table 1. Validation Report of Spectrophotometric, TLC and HPLC Methods for the Determination of Lamotrigine[22)]

Parameters	Colorimetric method	TLC method	HPLC method
Linearity range	10—200 μg ml^{-1}	0.5—10 μg/spot	1—12 μg ml^{-1}
Regression equation			
Slope	0.005	1065.2	27668
S.D. of slope	0.000	10.97	15.28
Intercept	0.0198	388.12	38.97
S.D. of intercept	1.28×10^{-3}	11.24	568
Correlation Coefficient (*r*)	0.9991	0.9984	0.9993
S.D. of (*r*)	1.16×10^{-4}	0.000	2.3×10^{-4}
Precision±RSD%			
Intra-day[a)]	100.09±0.44	99.93±1.30	99.48±1.15
Inter-day[a)]	99.98±0.80	99.59±1.75	99.42±1.34
Accuracy (mean[b)]±S.E.)	100.13±0.18	99.99±0.54	99.50±0.53

a) Average of $n=9$. *b*) Average of $n=6$.

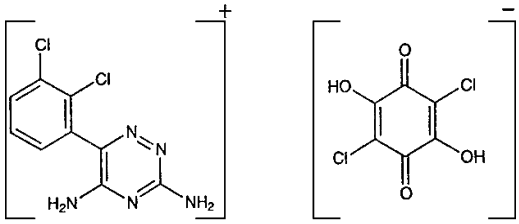


Chart 1. Proposal of the Reaction Pathway between Lamotrigine and *p*-Chloranilic Acid

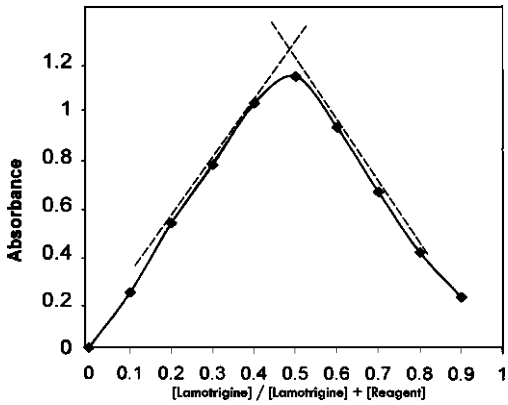


Fig. 4. Determination of the Stoichiometry of the Reaction of Lamotrigine with *p*-Chloranilic Acid by Continuous Variation Method Using 4×10^{-3} M Solutions

acid ion-association complex formation because it has a high relative permittivity which ensure the maximum yield of ion-association complex. The possibility of ion-association complex formation was that, both lamotrigine and *p*-chloranilic acid became partially positive and negative charged ions, respectively in acetone as shown in Chart 1. The composition of ion-pair association complex was established by applying Job's method of continuous variation[19] the plot reached a maximum value at a mole fraction of 0.5 which indicated the formation of 1 : 1 (drug : reagent) complex as shown in (Fig. 4). The conditional stability constant (K_f) of the ion-association complex was calculated from the continues variation data using the following equation[20]:

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

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 *p*-chloranilic acid ion associates with drug. The $\log K_f$ value was found to be 5.83.

The free energy changes (ΔG) of the complexation[21] between the ions of drug and reagent is related to the overall stability constant, K_f by the following relationship:

$$\Delta G = -2.303 RT \log K_f,$$

where R is the universal gas constant, 1.987 cal/mol; T is absolute temperature in Kelvin and ΔG is Gibb's free energy. The calculated association energy of the complex is -7.91 cal mol^{-1}. The indication is that the complex can be

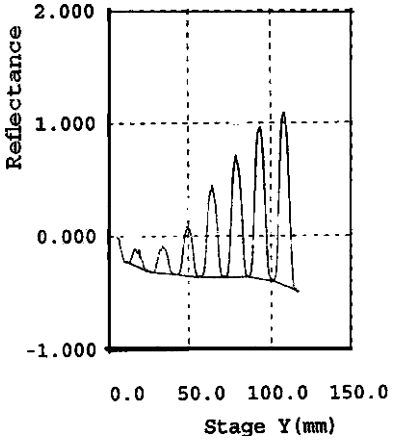


Fig. 5. TLC Scanning Profile of Lamotrigine (0.5—10 μg/Spot)

formed without an external supply of energy. The molar absorptivity (mol^{-1} cm^{-1}) and sensitivity index (μg cm^{-2}) were found to be 1.28×10^3 and 1.91×10^{-3}, respectively.

Thin Layer Chromatographic Method In this work, TLC densitometric method was used for the lamotrigine determination in presence of its main impurity, 2,3-dichlorobenzoic acid depending on the difference in *Rf* values. The experimental conditions for TLC method such as mobile phase composition, scan mode and wavelength of detection were optimized to provide accurate, precise and reproducible results. The chosen scan mode was zigzag mode and the wavelength of scanning was chosen to be 275 nm (Fig. 5). Complete separation was obtained using ethylacetate : methanol : ammonia 35% (17 : 2 : 1 v/v/v) as a mobile phase. The *Rf* values of the drug and its impurity were 0.75 ± 0.01 and 0.23 ± 0.01, respectively. A linear calibration curve was obtained in the concentration range 0.5—10 μg/spot with mean accuracy $99.99\pm1.33\%$. The parameters of regression equation are shown in Table 1.

HPLC Method The developed HPLC method was applied to the determination of lamotrigine in the presence of its impurity 2,3-dichlorobenzoic acid. To optimize HPLC assay parameters, the mobile phase composition and pH were studied. A satisfactory separation was obtained with a mobile phase of acetonitrile : methanol : 0.01 M potassium orthophosphate pH 6.7 ± 0.1 (30 : 20 : 50 v/v/v) using C_{18} column at ambient temperature. The analysis was carried out by isocratic elution with flow rate 1.5 ml/min and detection at 275 nm (Fig. 6). A linear range of 1—12 μg ml^{-1} was obtained with mean accuracy $99.50\pm1.30\%$ as show in Table 1.

The system suitability tests of HPLC method were evaluated Table 2.

Methods Validation. Linearity/Range Seven solutions were prepared for the linearity test. Each solution was measured (or injected) three times and linear regression analysis of lamotrigine was driven (Table 1).

Precision The precision of the methods were assessed by determining RSD values of intra-day and inter-day analysis ($n=9$) of lamotrigine standard solutions over 3 d (Table 1). The injection repeatability for HPLC method was determined (Table 2).

Accuracy The accuracy of the three suggested methods was estimated based on the mean percentage accuracy of measured concentrations ($n=6$) to the actual concentration as

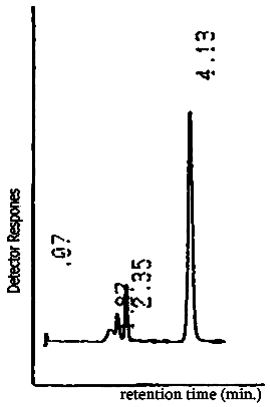


Fig. 6. HPLC Chromatogram of Lamotrigine (4 μg ml^{-1}, t_R=4.13) and 2,3-Dichlorobenzoic Acid (4 μg ml^{-1}, t_R=2.35)

Table 2. Results of System Suitability Tests of HPLC Method[22)]

Parameters	Value	Comments
Retention time	4.1±0.04	±Standard deviation
Injection repeatability	0.58%	RSD for five injections
K	2.82	Capacity factor
Selectivity factor (α)	2.40	Separation factor calculated as K_2/k_1
Tailing factor	0.74±0.01	Calculated at 5% of peak height.
Theoretical plates (N)	6822	Column efficiency plate/column.
HETP	0.04	Height equivalent theoretical plate.
Resolution	11.87	Calculated by $2(t_2-t_1)/w_2+w_1$

Table 3. Determination Lamotrigine in Presence of Its Impurity in the Synthetic Mixtures

Sample No.	2,3-Dichloro benzoic acid (% w/w)	Spectrophotometric method (Found %)	TLC method (Found %)	HPLC method (Found %)
1	0.10	100.02	101.20	101.25
2	0.20	99.52	98.53	99.25
3	0.50	100.71	98.70	100.38
4	1.00	99.02	97.74	98.00
5	5.00	101.02	101.11	98.13
6	7.50	100.02	99.86	100.00
7	10.00	99.88	99.35	99.75
Mean		100.03	99.50	99.54
RSD		0.68	1.32	1.19
S.E.		0.26	0.50	0.45

shown in (Table 1). The calculated *t*-test and *F*-test are not exceeding their theoretical values at p=0.05, indicating that there is no significant difference between each method and the reported one. Moreover, one-way Analysis of Variance (ANOVA) has been calculated for the proposed methods and the reported one. Source of variation as treatments (between columns) and residuals (within column), shows no significant difference among them.

Specificity Synthetic mixtures of the intact drug and its impurity 2,3-dichlorobenzoic acid in different proportions 0.1—10% were analyzed as shown in Table 3. The acceptance criteria set forth by the manufacturers for this impurity is less than reported limit of the impurity is less than 0.2%.[3)]

Standard Addition Technique The proposed methods were applied for the analysis of the drug in pharmaceutical dosage form. The validity of the methods was assessed by applying the standard addition technique. The results in (Table 4) indicate no interference from tablets excipients such as calcium carbonate, hydroxypropyl cellulose, aluminium magnesium silicate, povidone, sodium starch glycolate, saccharin sodium and magnesium stearate. Moreover, 2,3-dichlorobenoic acid was found to be within the specified limit 0.2%.

Table 4. Statistical Comparison of the Proposed Methods and the Reported Method for the Determination of Lamotrigine in Lamictal Tablets

Item	Spectrophotometric method	TLC method	HPLC method	Reported[c)] method
Mean[a)]	98.03	97.98	98.28	97.95
S.D.	0.60	0.51	0.60	0.48
S.E.	0.27	0.23	0.27	0.22
Variance	0.36	0.26	0.36	0.23
$t(2.306)^{b)}$	0.23	0.09	0.95	
$F(6.39)^{b)}$	1.57	1.13	1.57	

a) Average of n=5. *b*) The theoretical value *t* and *F*-test at p=0.05. *c*) Spectrophotometric method (supplied by Welcome Co.) by measuring the absorbance at 307 nm in 0.1 M sodium hydroxide against a lamotrigine standard.

Conclusion

The presented work describes validated spectrophotometric; TLC and HPLC methods for the assay of lamotrigine in presence of its impurity. The three suggested methods are simple, selective, accurate and can be used for the routine quality control analysis of the cited drug either in bulk or in dosage form without any interference from common excipients. The spectrophotometric method is rapid with low cost for both identification and quantification, moreover no color reaction has been reported up till now for its analysis.

References and Notes

1) Gilman A. G., Hardman J. G., Limbird L. E., "Goodman and Gilman's the Pharmacological Basis of Therapeutics," 10th ed., McGraw Hill, New York, U.S.A., 2001, p. 539.
2) Sean C., Sweetman; Martindale, The Complete Drug Reference 34th ed. Pharmaceutical Press, London, 2005, p. 363.
3) HPLC Manufacturer procedure supplied by Dr. Reddy's Co. India, 2002.
4) Emami J., Ghassami N., Ahmadi F., *J. Pharm. Biomed. Anal.*, **40**, 999—1005 (2006).
5) Dreassi E., Corbini G., Corti P. M., Ulivelli M., Rocchi R., *J. AOAC*, **79**, 1277—1280 (1996).
6) Cociglio M., Alric R., Bouvier O. J., Chromatogr., Biomed. Appl., 110 1—2, *J. Chromatogr.*, **572**, 269—276 (1991).
7) Lensmeyer G. L., Gidal B. E., Wiebe D. A., *Ther. Drug Monit.*, **19**, 292—300 (1997).
8) Angelis-Stoforidis P., Morgan D. J., O'Brien T. J., Vajda F. J. E., *J. Chromatogr. B*, *Biomed. Appl.*, **727**, 113—118 (1999).
9) Croci D., Salmaggi A., de Grazia U., Bernardi G., *Ther. Drug Monit.*, **23**, 665—668 (2001).
10) Castel-Branco M. M., Almeida A. M., Falcao A. C., Macedo T. A., Caramona M. M., Lopez F. G., *J. Chromatogr. B*, *Biomed. Appl.*, **755**, 119—127 (2001).
11) Cheng C. L., Chou C. H., Hu O. Y. P., *J. Chromatogr. B*, *Anal. Technol. Biomed. Life Sci.*, **817**, 199—206 (2005).
12) Matar K. M., Nicholls P. J., Bawazir S. A., Al-Hassan M. I., Tekle A., *J. Pharm. Biomed. Anal.*, **17**, 525—531 (1998).
13) Hallbach J., Vogel H., Guder W. G., *Eur. J. Clin. Chem. Clin. Biochem.*, **35**, 755—759 (1997).
14) Shihabi Z. K., Oles K. S., *J. Chromatogr. B*, *Biomed Appl.*, **683**, 119—123 (1996).
15) Theurillat R., Kuhn M., Thormann W., *J. Chromatogr. A.*, **979**, 353—368 (2002).
16) Biddlecombe R. A., Dean K. L., Smith C. D., Jeal S. C., *J. Pharm.*

Biomed. Anal., **8**, 691—694 (1990).
17) Sailstad J. M., Findlay J. W. A., *Ther. Drug Monit.*, **13**, 433—442 (1991).
18) Abdel-hey M. H., Sabry S. M., Barary M. H., Belal T. S., *Anal. Lett.*, **37**, 247—262 (2004).
19) Douglas A. S., Donald M. W., "Principels of Instrumental Analysis," Holt, Rinhart and Winston, New York, 1971, p. 104.
20) Erk N., *Anal. Lett.*, **36**, 1183—1196 (2003).
21) Al-Ghannam S. M., *J. Pharm. Biomed. Anal.*, **40**, 151—156 (2006).
22) The United States Pharmacopeia. The National Formulary USP 29 United States Pharmacopoeial Convection Inc., 2006, p. 3050.