Journal of Pharmaceutical and Biomedical Analysis 56 (2011) 763–770

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Development and validation of an analytical method based on high performance thin layer chromatography for the simultaneous determination of lamotrigine, zonisamide and levetiracetam in human plasma

Letizia Antonilli[a], Valentina Brusadin[a], Francesca Filipponi[a], Renzo Guglielmi[b], Paolo Nencini[a,*]

[a] Dipartimento di Fisiologia e Farmacologia, "V. Erspamer" – "Sapienza" University of Rome and Centro Antidroga and Farmacologia Clinica Policlinico Umberto I, Italy
[b] Dipartimento di Neurologia e Psichiatria, "Sapienza" University of Rome, Italy

ARTICLE INFO

Article history:
Received 28 March 2011
Received in revised form 6 July 2011
Accepted 13 July 2011
Available online 23 July 2011

Keywords:
Antiepileptics
HPTLC
LC–MS/MS
HPLC
Plasma concentrations

ABSTRACT

Methods based on HPLC technology are the most frequently adopted for monitoring blood levels of novel antiepileptics. Here a rapid method based on HPTLC was developed for quantitative determination of lamotrigine (LTG), zonisamide (ZNS) and levetiracetam (LVT) in human plasma and compared with HPLC and LC–MS/MS methods. Chromatographic separation was achieved on silical gel $60F_{254}$ plates using ethylacetate:methanol:ammonia (91:10:15 v/v/v) as mobile phase. Quantitative analysis was carried out by densitometry at a wavelength of 312, 240 and 210 nm for LTG, ZNS and LVT, respectively. Calibration curves were linear over range of 0–200 ng for LTG and ZNS and 0–400 ng for and LVT. The limit of quantification of LTG, ZNS and LTV was found to be 3.69, 3.7 and 6.85 μg/ml, respectively. Intra and inter-assay precision provided relative standard deviations lower than 10% for all three analytes. Correlation and Bland–Altman plot showed general agreement between HPTLC and LC–MS/MS quantification, with a mean bias of −0.25, −0.46 and 0.5 μg/ml for LTG ZNS and LVT, respectively. Likewise, comparison between HPLC–UV and LC–MS/MS showed good agreement for all the three compounds analyzed. In conclusion, the proposed HPTLC method is simple, rapid, precise and accurate. It therefore is appropriate for the routine quantification of therapeutic levels of LTG, ZNS and LVT in human plasma.

1. Introduction

Prevention of recurrent, unprovoked seizures is the goal of the pharmacological therapy of epilepsy. In fact, this apparently simple goal is not easily reached, since 30–40% of patients continue to have seizure in spite the use of antiepileptic drugs, either alone or in combination [1].

Moreover, prevention of seizures depends on the continuous maintenance of effective blood concentrations of the drug, but, at these concentrations, traditional antiepileptics drugs such as carbamazepine, valproate, phenytoin, and phenobarbital, are burden by an array of often intolerable side-effects [2]. Hence, it has been welcome the introduction, in the last two decades, of new antiepileptics with a much better therapeutic profile in terms of both spectrum of activity and safety. Lamotrigine, zonisamide, and levetiracetam are among the most used of these new antiepileptic drugs [3].

Yet, these new antiepileptics also show a narrow therapeutic index and consequently they require a careful monitoring of adherence to therapy [4–6] Moreover, since the 70 s it has been realized that pharmacokinetic variability is a major determinant of differences in the clinical response to antiepileptic drugs [7]. Dissimilarity of age or gender, co-administration of other drugs, genetic polymorphism for biotransforming enzymes are major determinants of this pharmacokintetic variability [8,9]. Accordingly, it is believed that plasma drug concentrations better than drug daily doses correlate with clinical response [10]. Hence the interest to develop simple but reliable analytical methods to monitoring blood concentrations of antiepileptic drugs. In the case of the traditional compounds, such as carbamazepine, phenobarbital, phenytoin, and valproic acid, immunoassays are commercially available for quantitation of their blood concentrations. In contrast, therapeutic monitoring of newer antiepileptic drugs mainly relies on chromatographic assays. The attention has been addressed in particular to high performance liquid chromatography (HPLC) either associated with mass spectrometry or not. However, these methods are technically complex, rather expensive and time consuming. High performance thin layer chromatography (HPTLC) with direct ultraviolet measure provides a potentially alternative method for its capability to handle multiple samples per plate and

* Corresponding author at: Department of Physiology and Pharmacology, Sapienza University of Rome, P.le A. Moro 5, 00185 Rome, Italy. Tel.: +39 06 4450618; fax: +39 06 4450618.
E-mail address: paolo.nencini@uniroma1.it (P. Nencini).

0731-7085/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2011.07.018

then to quantitate blood concentrations of many drugs simultaneously, cutting considerably analysis time and cost. For these reasons HPTLC is gaining substantial interest as an important tool in the assay of drugs in biological fluids. Nevertheless, few studies have been addressed to the use of HPTLC for quantitation of antiepileptic drugs in blood [10–14].

In the present study, we compared the assay performance of HPTLC and HPLC with uv detector, with that obtained with HPLC associated with tandem mass spectrometry (LC–MS/MS), in dosing blood concentrations of lamotrigine (LTG), zonisamide (ZNS), levetiracetam (LVT), in patients daily treated with one of these drugs.

2. Experimental

2.1. Chemicals and materials

Lamotrigine, zonisamide and levetiracetam were kindly provided by GlaxoSmithKline Great West Road, Brentford, Middlesex (UK), Dainippon Sumitomo Pharma Co., Ltd. Suzuka (Japan) and UCB Pharma Sector, S.A. (Belgium), respectively. Acetonitrile, methanol, both gradient grades were purchased from Merck (Darmstadt, Germany) and ammonium formate from Sigma Aldrich (Milan, Italy). Ultrapure water was prepared using a Millu-Q system (Millipore, MA USA). Human blank serum samples used for development and validation of the procedure were obtained from volunteers of laboratory staff.

2.2. Instrumentation and chromatographic conditions

2.2.1. HPTLC

High performance thin layer chromatography (HPTLC) was performed on glass plates silica gel $60F_{254}$ (10×10 cm) 250 μm thicknesses (Merck, Darmstadt, Germany). The plates were first washed with methanol and then activated at100 °C for 15 min before use.

Sample application was done by means of a Linomat IV applicator (Camag, Muttenz, Switzerland) in the narrow band of 4 mm length at a constant flow rate of 7 s μl^{-1} by using a nitrogen aspirator. Plates were left to equilibrate for 10 min in a 10×10 cm horizontal chamber (Camag) and then developed at a distance of 80 mm using ethylacetate:methanol:ammonia (90:10:1.5 v/v/v) as mobile phase. Separation was obtained within 15 min and hence plates were dried in hot air for 1 min.

Densitometric scanning was carried out using a TLC Scanner II (Camag) in the absorbance/reflectance mode at 312, 240 and 210 nm for lamotrigine, zonisamide and levetiracetam respectively. Calculation of retention factor (R_f), integration of chromatogram and acquisition of UV absorption spectra were performed by Camag CATS 3 software (version 3.12). This part of the procedure took no more than 10 min.

2.2.2. HPLC diode array

Chromatographic analyses were carried out using a HPLC system equipped with automatic sampler (model L-7250), pump (model L-7100), diode array detector (model L-7455), all purchased from Merck Hitachi. Data were stored and processed using appropriate software (D-7000 HPLC System Manager Ver.3.1, Hitachi).

Separation of compounds was achieved using a reversed phase LiChroCART-Purospher 100 RP-18, 5 μm, 250×4 mm with LiChroCART–LiCrospher 100 RP-18, 5 μm (Merck) as guard column. The mobile phase was a mixture of aqueous 30 mM potassium phosphate buffer (adjusted to pH 3.7 with 5% phosphoric acid) and acetonitrile (65:35) at flow rate 1 ml/min. Detection was monitored at 270 nm for lamotrigine and zonisamide and at 210 nm for levetiracetam.

2.2.3. Liquid chromatography–mass spectrometry

The HPLC system consisted of a Perkin Elmer 200 series binary pump and autosampler (Perkin Elmer, Norwalk,CT). The chromatographic separation was performed on a reversed phase LiChroCART-Purospher 100 RP-18, 5 μm, 150×4.6 mm with LiChroCART–LiCrospher 100 RP-18, 5 μm (Merck) as guard column.

Isocratic elution was performed using a mixture of 5 mM ammonium formate and acetonitrile. Flow rate was kept constant at 0.8 ml/min an then was split 1:20 before entering the electrospray interface (Turbo Ionspray®) of an API 2000 triplo quadrupole mass spectrometry system (Applied Biosystem – Sciex, Ont., Canada). Ions were created in the positive ion mode under atmospheric pressure at 4.5 kV and at a source temperature of 350 °C. The nitrogen curtain gas was adjusted to a constant value of 35 units, Gas 1 (GS1) and Gas 2 (GS2) was set to 25 and 30 units, respectively. Multiple reaction monitoring (MRM) was used for drug quantification.

Selected ion masses of the protonated precursors and fragmented ions were 256/185 *m*, 213/132 *m* and 171/126 *m* for lamotrigine, zonisamide and levetiracetam, respectively. Chromatographic peaks were integrated using Analysts™ software (version 1.4.1 Sciex).

2.3. Calibration standards and quality control samples

Stock solutions of lamotrigine, zonisamide and levetiracetam were prepared by dissolving 10 mg of each compound in 1 ml methanol. The working solutions were prepared by appropriate dilution of the stock solution with methanol for HPTLC and mobile phase for LC–MS/MS. Each working solution of LTG, LVT and ZNS was used to spike drug-free human serum to provide calibration standards from 0 to 20 μg/ml for LTG and ZNS and from 0 to 40 μg/ml for LVT.

The quality control samples (QC) used in validation study were prepared in the same way as the calibration standard, by spiking drug free human serum with appropriate volume of working solutions to obtain three different concentrations, near the low, middle and high values of concentration curve.

2.4. Serum sample preparation

To 0.25 ml of serum sample (calibration standards, quality control and patients' samples) 0.75 ml of acetonitrile was added. The mixture was vortexed for 1 min, and then centrifuged for 10 min at $2500 \times g$. The supernatant was recovered and evaporated to dryness under a stream of nitrogen. The residue was dissolved in the mobile phase that in our analytical conditions resulted more appropriate for each of the three different chromatographic methods, i.e. methanol (100 μl) for HPTLC analysis, formate buffer (1 ml) for LC–MS/MS, and potassium phosphate buffer (1 ml) for HPLC.

2.5. Method validation

The methods were validated for selectivity, precision, accuracy, linearity, limit of detection, limit of quantification and recovery, according to the International Conference on Harmonization (ICH) [15] and Food and Drug Admistration (FDA) guidelines [16].

2.5.1. Selectivity

Interferences of endogenous compounds with the analytes were investigated by analysis of blank serum sample of three different volunteers (unspiked and spiked with the analytes at LLOQ level). For HPTLC the selectivity was ascertained by analyzing and comparing chromatograms of both blank serum samples and spiked LLOQ

samples to ensure the absence of any possible interference at *Rf* of lamotrigine, levetiracetam and zonisamide. In particular, selectivity was investigated (i) by comparing the *Rf* of the testing samples with that of the reference samples; (ii) by performing the spectral analysis of the testing sample peak; (iii) by performing the spectral analysis at the start, the apex and the end of the peak. The latter analysis also allowed to evaluate peak purity. We also investigated whether or not the presence of other co-eluting antiepileptic drugs interfered with both resolution and detection of the analytes of our interest. Accordingly, standard solutions containing phenobarbital, carbamazepine, and valproic acid were submitted to HPTLC analysis, being these drugs the more frequent in the polytherapeutic regimen prescribed to the patients herein studied.

For HPLC and LC/MS–MS the selectivity was investigated by analyzing the chromatograms to determine whether endogenous constituents co-elueted with the analytes of interest.

2.5.2. Precision and accuracy

Intra and inter-day precision and accuracy of the methods were determinate by performing replicate ($n = 6$) analyses of quality control samples in plasma (low, medium and high level) against a calibration curve. This procedure was replicated on different days ($n = 3$). Precision was calculated from the percentage relative standard deviation (R.S.D. %) for the repeated measurements whereas accuracy was expressed as % of recovery.

According to the FDA criteria [15], precision and accuracy determined at each concentration level should not exceed 15% except for the LOQ, which should not deviate by more than 20%.

2.5.3. Linearity

For HPTLC assay calibration samples of 0–200 ng/spot for LTG and ZNS and 0–400 ng/spot for LVT, were analyzed in triplicate in three different days. Calibration curves were calculated by least-square method. The coefficient of correlation (r) was determined to assess linearity of calibration curves. For the LC/MS–MS the linearity was determined by analysis of calibration samples of 0–20 and 0–40 μg/ml for LTG and ZNS and 0–50 μg/ml for LVT. For the HPLC diode array detection the calibration ranged from 0 to 40 μg/ml for all analytes. Calibration curve were run in triplicate on each analysis day ($n = 3$).

2.5.4. Limit of detection and limit of quantization

For HPTLC and LC/MS–MS, in order to estimate the limit of detection (LOD) and limit of quantization (LOQ), blank sample was analyzed nine times following the same method described above and the standard deviation (σ) of the analytical response was determined. The LOD was expressed as 3.3σ/slope of the calibration curve, whereas LOQ was expressed as 10σ/slope of the calibration curve of LTG, LVT and ZNS.

2.5.5. Recovery

Recovery was determined by spiking LTG, LVT and ZNS in drug free human plasma to obtain three different concentrations covering the low, medium and higher ranges of calibration curve. The sample were then extracted and analyzed as described earlier. The recovery was calculated by comparing the peak areas of quality control samples with those obtained extracting analytes from blank serum to which they have been added at the same nominal concentration.

2.6. Application

2.6.1. Therapeutic drug monitoring

The validated HPTLC method was applied to determine the plasma concentration of LTG, ZNS and LVT in 80 patients (38 males and 42 females, aged 7–71 years, mean 40 ± 15) under daily oral

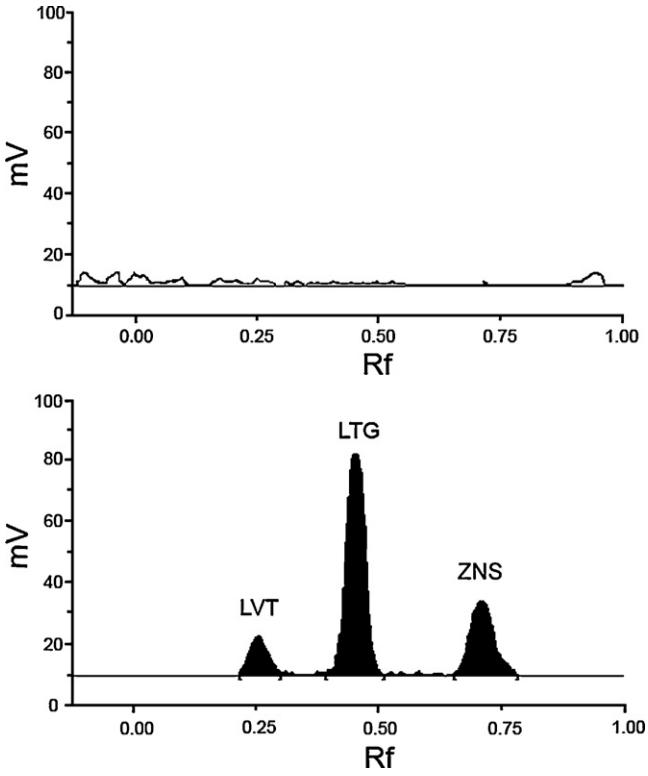

Fig. 1. HPTLC chromatograms of drug-free plasma (upper panel) and plasma spiked with of LEV, ZNS and LTG (100ng/spot) (lower panel).

treatment for epileptic conditions at the Neurology Department of Policlinico Umberto I of Rome.

The collection of the samples was done in the morning, 12 h after the last drug administration of LTG (25–400 mg/day) ZNS (150–400 mg/day) and LVT (1000–3000 mg/day).

Venous blood samples were drawn from patients and transferred into heparinized tubes. Plasma was separated by centrifugation at 3000 rpm for 10 min and stored at −20 °C prior to analysis.

2.7. Method comparison

Results from the HPTLC and HPLC assay were compared with those obtained by LC–MS/MS method. Correlation between methods was assessed using linear regression analysis (GraphPad Prism version 4.0). Differences between techniques were plotted according to the method described by Bland–Altman [17].

3. Results

3.1. Selectivity and specificity

LVT, LGT and ZNS were efficiently separated under HPTLC conditions with Rf values of 0.26, 0.45 and 0.71, respectively. Neither resolution nor detection of the three analytes were affected by phenobarbitone, carbamazepine and valproic acid, these antiepileptics showing *Rf* values of 0.78, 0.50 and 0, respectively. Likewise, the blank plasma samples did not yield peaks at the *Rf*'s of the analytes when their chromatograms were compared with those obtained from spiked samples, indicating the absence of matrix interferences and the high selectivity of the method (Fig. 1).

The identity of LVT, LGT and ZNS in the samples was confirmed by overlaying their UV absorption spectra with those of reference standards, as well as by the coincidence of their respective *Rf* values using a TLC densitometric analysis. Absorption maxima (λ max)

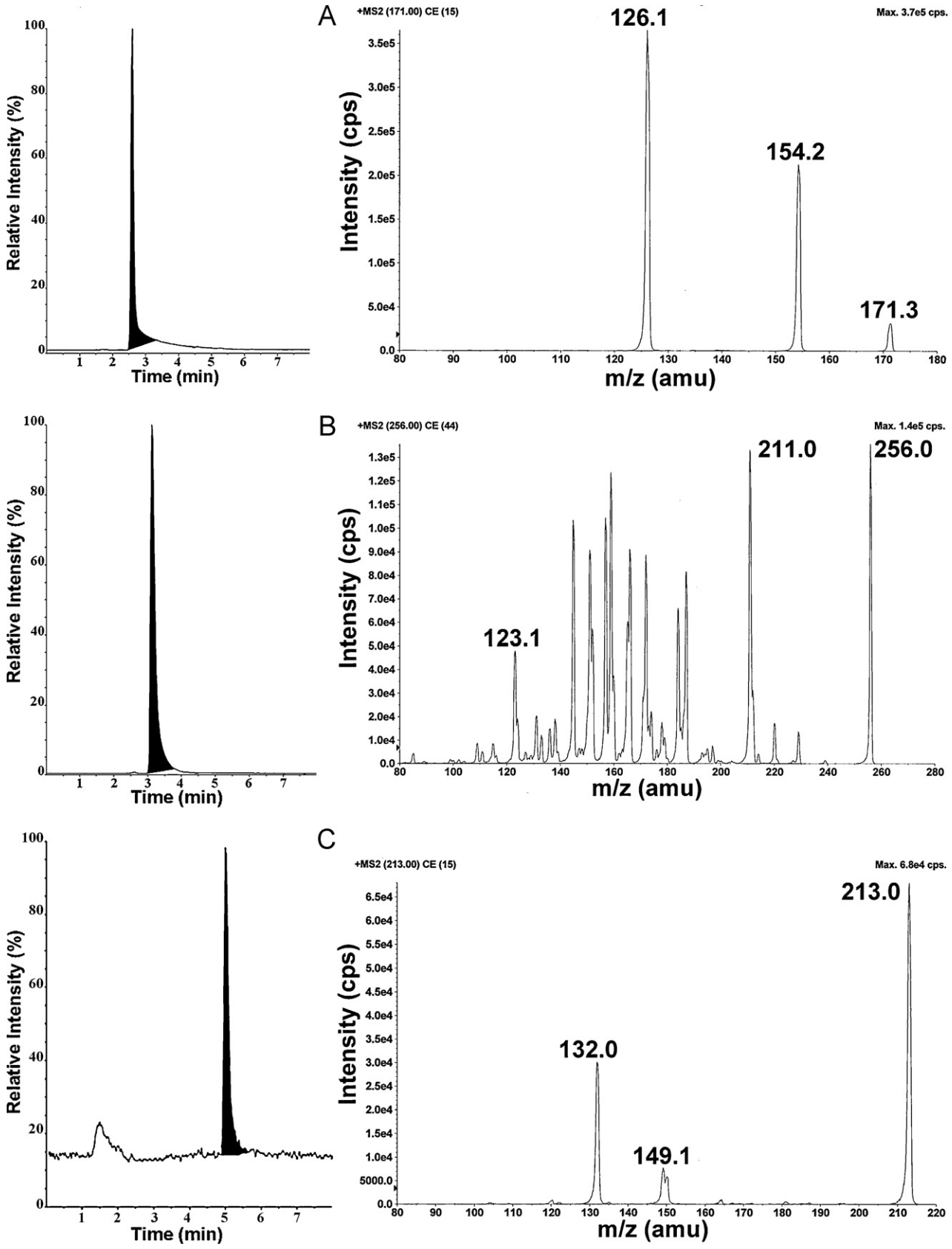

Fig. 2. Representative chromatograms and product ions spectrum with fragmentation pathway, in plasma spiked with LVT (A), LTG (B) and ZNS (C).

Table 1
Precision and accuracy parameters of the assay.

Drug	Nominal concentration	Intraday ($n = 6$)			Inter-day ($n = 18$)		
		Calculated concentration (mean ± S.D.)	Precision (R.S.D. %)	Accuracy %	Calculated concentration (mean ± S.D.)	Precision (R.S.D. %)	Accuracy %
HPTLC							
LTG	10 (ng/spot)	9.96 ± 0.33	3.31	−0.4	9.88 ± 0.31	3.14	−1.2
	40	42.17 ± 1.10	2.61	5.4	41.45 ± 1.90	4.59	3.6
	100	103.90 ± 1.59	1.53	3.8	104.1 ± 1.77	1.70	4.1
ZNS	40	37.64 ± 1.16	3.08	−5.9	39.61 ± 3.64	9.18	−1.0
	80	77.20 ± 4.83	6.26	−3.5	77.91 ± 4.96	6.38	−2.6
	200	191.00 ± 11.61	6.08	−4.5	195 ± 11.95	6.13	−2.5
LVT	10	10.04 ± 0.36	3.59	0.4	10.69 ± 0.98	9.17	6.9
	60	60.78 ± 2.43	4.00	1.3	58.60 ± 3.75	6.40	−2.3
	400	438.10 ± 13.13	3.00	9.5	408.00 ± 28.75	7.04	2.0
LC–MS/MS							
LTG	1.0 (μg/ml)	1.01 ± 0.03	2.97	1.0	1.02 ± 0.05	4.90	2
	5.0	4.96 ± 0.09	1.80	−0.8	5.01 ± 0.09	5.78	0.2
	20.0	20.8 ± 0.51	2.45	4.0	21.3 ± 0.61	2.86	6.5
ZNS	2.0	1.98 ± 0.10	5.00	−1.0	1.95 ± 0.18	9.23	−2.5
	10.0	9.95 ± 0.32	3.21	−0.5	9.91 ± 0.45	4.54	−0.9
	20.0	21.3 ± 0.74	3.47	6.5	20.07 ± 0.85	4.23	0.3
LVT	5	5.17 ± 0.23	4.45	3.3	4.89 ± 0.32	6.54	−2.2
	25	24.92 ± 0.52	2.08	−0.3	24.56 ± 0.48	1.95	−1.7
	50	49.61 ± 0.65	1.31	−0.8	51.06 ± 0.95	1.86	2.2
HPLC							
LTG	0.5 (μg/ml)	0.51 ± 0.04	7.85	2.0	0.46 ± 0.02	4.35	−8.0
	5.0	5.11 ± 0.05	1.05	2.2	5.27 ± 0.25	4.78	5.4
	40	39.34 ± 1,17	2.97	−1.6	39.52 ± 0.86	2.17	−1.2
ZNS	0.5 (μg/ml)	0.57 ± 0.01	1.75	14	0.55 ± 0.04	7.27	10
	5.0	4.65 ± 0.19	4.09	−7.0	5.01 ± 0.03	0.59	0.2
	40	40.41 ± 0.81	2.02	1.0	40.2 ± 0.08	0.19	0.5
LVT	0.5 (μg/ml)	0.49 ± 0.02	4.08	−2.0	0.48 ± 0.04	8.3	−4
	5.0	5.01 ± 0.21	4.19	0.2	5.02 ± 0.12	2.4	0.4
	40	40.1 ± 0.43	1.07	0.25	41.2 ± 0.75	1.8	3.0

of 312, 240 and 210 nm for LTG, ZNS and LVT, respectively, were thus confirmed. The purity of each compound was confirmed by analyzing the UV spectrum at the start, apex and end of the peak. The derived peak purity index (P) ranged from 0.9954 to 0.9999 for the three compounds.

In the cases of HPLC and LC–MS/MS methods, selectivity was evaluated by comparing chromatograms of three extracted blank plasma samples of different sources, with those of plasma samples spiked with LTG, LVT and ZNS. Under the conditions described above, HPLC analysis did not show interfering peaks at the retention time of 3.04 ± 0.04, 5.53 ± 0.06 and 5.17 ± 0.03 for LTG, LVT and ZNS, respectively. Likewise, LC–MS/MS analysis did not show interfering peaks at the retention time of 3.42 ± 0.02, 2.61 ± 0.01, and 5.13 ± 0.06 for LTG, LVT and ZNS, respectively.

Representative chromatograms and tandem mass spectrometry spectra of extracted blank sera and extracted sera spiked with ZNS, LTG and LVT are shown in Fig. 2.

3.2. Precision and accuracy

The HPTLC method was validated for intra and inter-day precision and accuracy at three different concentrations of LTG, LVT and ZNS (low, medium and high level of QC). The relative standard deviation (RSD) ranged from 1.53 to 6.26 and from 1.70 to 9.18 for intra and inter-day assays, respectively. In the case of HPLC diode array detection, the RSD for the intra-day precision ranged from 1.05 to 7.85, whereas it ranged from 0.19 to 7.27 for the inter-day precision. In the case of LC–MS/MS method, the RSD for the intra-day precision ranged from 1.31 to 5.00, whereas it ranged from 1.86 to 9.23 for the inter-day precision (Table 1).

Precision and accuracy for the three methods were consistent with the FDA criteria [15]. Moreover, the restricted range of RSD values suggests that the present HPTLC procedure provides highly reproducible determinations of LTG, LVT and ZNS concentrations in plasma samples.

3.3. Linearity and sensitivity

For HPTLC method calibration curves were constructed testing six increasing drug concentrations within their respective therapeutic range, i.e. 0–20 μg/ml (0–200 ng/spot) for LTG and ZNS and 0–50 μg/ml (0–400 ng/spot) for LVT. These curves were linear in the range of concentrations assayed and accordingly, their respective

Table 2
Detection and quantitation limits of HPTLC and LC–MS/MS for lamotrigine, zonisamide and levetiracetam.

Method	LOD			LOQ		
	LTG	ZNS	LEV	LTG	ZNS	LEV
HPTLC (μg/ml)	1.3	1.2	2.25	3.69	3.7	6.85
LC–MS/MS (ng/ml)	0.34	11.7	5.6	1.98	36.5	24.2
HPLC (μg/ml)	0.21	0.59	0.6	0.37	0.89	1.95

Table 3
Ranges of concentrations detected in human plasma samples (numbers between parentheses represent the median values).

Drug	Concentration (μg/ml)		
	LC–MS/MS	HPTLC	HPLC
LTG ($n = 38$)	0.14–12.21 (3.88)	0–13.19 (3.88)	0–24.84 (4.8)
ZNS ($n = 20$)	0.61–14.93 (4.20)	0–15.79 (4.31)	0–15.76 (4.80)
LVT ($n = 22$)	2.75–46.30 (15.90)	0–40.45 (13.81)	1.65–29.40 (12.65)

Table 4
Correlation and agreement statistics.

Drug	Pearson correlation (r)	Linear regression		Bland–Altman bias analysis		
		Slope	Intercept	Bias	Limit of agreement	
					Lower 95%	Upper 95%
HPTLC vs. LC–MS/MS						
LTG	0.9447	1.125 ± 0.065	−0.834 ± 0.379	−0.25	−3.04	2.55
ZNS	0.9626	1.142 ± 0.075	−1.233 ± 0.522	−0.46	−3.43	2.51
LVT	0.8864	1.242 ± 0.148	−3.256 ± 2.635	0.50	−11.34	12.34
HPLC vs. LC–MS/MS						
LTG	0.8297	1.104 ± 0.125	0.277 ± 0.739	0.77	−4.41	5.95
ZNS	0.9795	1.013 ± 0.050	0.041 ± 0.343	0.11	−1.67	1.89
LVT	0.9334	0.945 ± 0.083	−0.175 ± 1.479	−0.99	−7.28	5.29

mean equations ($n = 9$) and correlation coefficients (r) were the following: $y = 17.47(\pm 0.14) + 38.89$ (± 12.81) and $r^2 = 0.9997$ for LTG; $y = 4.88$ (± 0.29) $- 48.66$ (± 29.40) and $r^2 = 0.9971$ for ZNS; $y = 0.99$ (± 0.012) $+ 5.44$ ($\pm$ 2.140) and $r^2 = 0.9989$ for LVT.

Linearity of the calibration curves was also tested by plotting residuals against the quantities added [18]. Since residuals were randomly distributed around the regression function without any trend, the calibration function can therefore be regarded as linear.

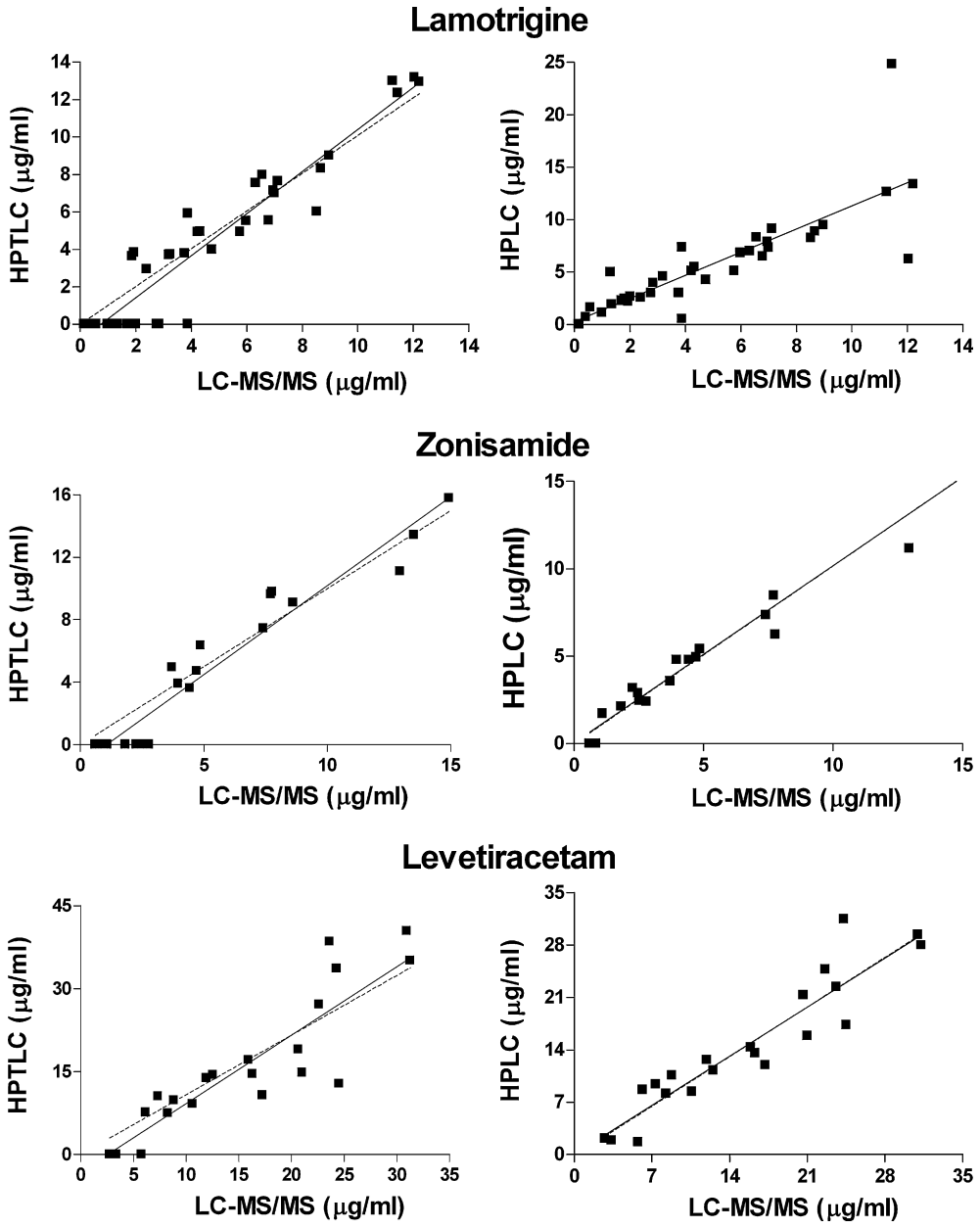

Fig. 3. Correlation between LTG, ZNS and LEV concentrations in patient samples measured by LC/MS-MS and HPTLC (left panels) or LC/MS- MS and HPLC (right panels). Further details on the regression lines and the degree of correlation are given in Table 4.

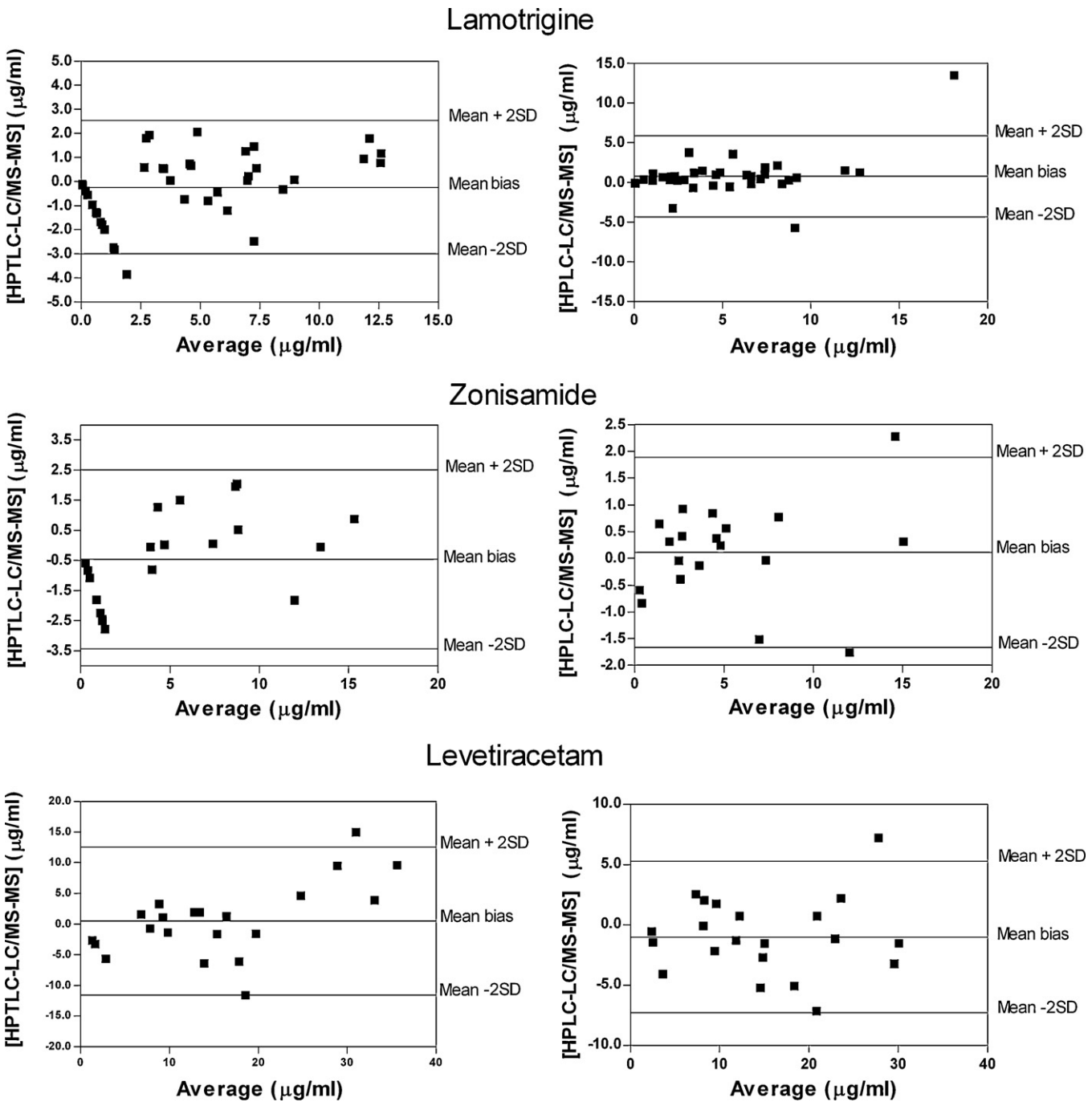

Fig. 4. Bland-Altman plots comparing HPTLC vs LC/MS-MS and HPLC vs LC/MS-MS methods for the measurements of LTG, ZNS and LVT in patient samples. The 95% limits of agreement are shown (mean bias±SD).

Limits of quantification (LOQ) were 3.69 μg/ml, 3.75 μg/ml and 6.85 μg/ml for LTG, ZNS and LVT respectively, indicating the adequate sensitivity of the method with respect to therapeutic needs. For HPLC LOQs were 0.37 μg/ml, 0.89 μg/ml and 1.95 μg/ml for LTG, ZNS and LVT respectively. In the case of LC–MS/MS these limits were 1.98 ng/ml, 36.5 ng/ml and 24.2 ng/ml for LTG, ZNS and LVT, respectively (Table 2).

3.4. Application

The assay was applied to the determination of serum concentrations of LTG (38 samples), LVT (22 samples) and ZNS (20 samples) in patients daily treated with these therapeutics.

3.5. Method comparison

The ranges of plasma concentrations of the three analytes obtained with HPTLC, HPLC or LC–MS/MS are shown in Table 3. Note that analyte concentrations in all the samples were above the LOQ of LC–MS/MS. In the case of HPTLC, concentrations of LTG resulted below the LOQ in 12 out of 38 samples, although LC–MS/MS analysis revealed that all these samples had LTG concentrations below the therapeutic range (4–10 μg/ml). LVT concentrations resulted below the LOQ (6.85 μg/ml) in 2 out of 22 samples and again, LC–MS/MS analysis revealed that LVT concentrations in these two samples were below the therapeutic range (10–37 μg/ml). In the case of ZNS, HPTLC assay revealed that 8 samples had blood concentra-

tions below the LOQ, but LC–MS/MS showed that in only 4 of these 8 samples ZNS concentrations were below the therapeutic range (2.3–12 μg/ml).

When blood samples were submitted to HPLC analysis, only two of them showed LTG and ZNS concentrations below the LOQ, whereas all samples had LVT concentrations within the therapeutic range.

Table 4 and Fig. 3 show high Person correlation indexes for all the three analytes when results obtained with either HPTLC or HPLC were compared with those obtained with LC–MS/MS. As firstly observed by Bland and Altman, in clinical measurement comparisons the use of correlation coefficients may be misleading and a plot of the difference between the methods against their mean are considered more informative. Accordingly, we assessed the agreement between the results obtained with HPTLC or HPLC and those obtained with LC–MS/MS using Bland–Altman test. As shown in Fig. 4 HPTLC and LC–MS/MS provided fairly consistent results for concentration of LTG and ZNS between 2.5 and 10 μg/ml with a randomized distribution around the mean. For concentration below 2.5 μg/ml deviations were systematically negative due to a different sensitivity of the two methods. Above 10 μg/ml deviation values became positive, as expected from the higher specificity of LC–MS/MS. A better level of agreement for all the three analytes was obtained between HPLC and LC–MS/MS as far as the Bland–Altman test yielded random distributions of the differences between the results obtained by the two methods (see Fig. 4).

4. Discussion

Blood concentrations of the drug represent a direct measure of the adherence of the patient to antiepileptic therapy. Searching for optimization of the balance between cost and benefit of monitoring plasma or seric concentrations of antiepileptics, a wide array of analytical procedure has been proposed. Curiously enough, little attention has been paid to the possible use of HPTLC methodology for dosing antiepileptic drugs in the clinical setting. For instance, at the best of our knowledge, we are aware of a single study dedicated to the HPTLC determination of seric LTG [12], whereas there are no reports concerning the development of a HPTLC method for dosing LVT and ZNS in the plasma matrix. Here we filled this gap reporting a HPTLC method for the determination of LTG, ZNS and LVT, which appears simple, accurate and rapid. Simple because it avoids cumbersome extractive procedures, being the sample preparation restricted to a single step liquid–liquid extraction with acetonitrile. It is important to note that simplifying the extractive procedure did not jeopardize the accuracy of the analysis. In fact, besides preserving very good levels of recovery, the single step extraction brought the sample to HPTLC analysis in optimal conditions of resolution as demonstrated by the absence of biological matrix effects in the elution intervals concerning the analytes here studied. Time saving is another merit of our HPTLC method for dosing plasmatic levels of LTG, ZNS and LVT. The single step extraction is not the only reason for time saving, a major contribution being provided by the possibility provided by HPTLC technology to run up to ten samples at the same time.

When the results were compared with those obtained with the benchmark method of LC–MS/MS, HPTLC showed clear limits in term of sensitivity, in particular in the cases of LTG and ZNS. HPTLC LOQ was however far beneath the lower limit of the therapeutic range of these drugs, making clinically unimportant the underevaluation of blood concentrations that in any case were therapeutically insufficient. These limits in sensitivity did not emerge when the same samples were analyzed using the HPLC method. On this basis, HPLC appears a better surrogate of LC–MS/MS than HPTLC for research purposes, for instance when an accurate determination of the terminal half-life of these antiepileptics is required. Yet, the possibility of performing up to ten quantitations in the same plate makes HPTLC time saving and relatively cheap, two important merits in clinical routine.

References

[1] P. Kwan, M.J. Brodie, Drug treatment of epilelpsy: when does it fail and how to optimize its use? CNS Spectr. 9 (2004) 110–119.
[2] S.R. Winkler, M.S. Luer, Antiepileptic drug review: part 1, Surg. Neurol. 49 (1998) 449–452.
[3] S.M. LaRoche, S.L. Helmers, The new antiepileptics drugs: clinical applications, JAMA 291 (2004) 615–620.
[4] B. De Spiegeleer, B.L. Van Hoorebeke, A. De Spiegeleer, P. Castelein, L. Van Bortel, The paradox of scored tablets: a cost-saving risk, Pharmazie 64 (2009) 550–552.
[5] R. Sankar, T.A. Glauser, Understanding therapeutic equivalence in epilepsy, CNS Spectr. 15 (2010) 112–123.
[6] M.D. Krasowski, Therapeutic drug monitoring of the newer anti-epilepsy medications, Pharmaceuticals (Basel) 3 (2010) 1909–1935.
[7] E.F. Hvidberg, M. Dam, Clinical pharmacokinetics of anticonvusants, Clin. Pharmacokinet. 1 (1976) 161–188.
[8] U. Klotz, The role of pharmacogenetics in the metabolism of antiepileptic drug: pharmacokinetic and therapeutic implications, Clin. Pharmacokinet. 46 (2007) 271–279.
[9] E. Perucca, Age-related changes in pharmacokinetics: predictability and assessment methods, Int. Rev. Neurobiol. 81 (2007) 183–199.
[10] G.D. Anderson, Pharmacokinetic, pharmacodynamic and pharmacogenetic targeted therapy of antiepileptic drugs, Ther. Drug Monit. 30 (2008) 173–180.
[11] P. Corti, A. Cenni, G. Corbini, E. Dreassi, C. Murratzu, A.M. Caricchia, Thin-layer chromatography and densitometry in drug assay: comparison of methods for monitoring valproic acid in plasma, J. Pharm. Biomed. Anal. 8 (1990) 431–436.
[12] K.M. Patil, S.L. Bodhankar, High-performance thin-layer chromatographic determination of lamotrigine in serum, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 823 (2005) 152–157.
[13] M. Wójciak-Kosior, A. Skalska, G. Matysik, M. Kryska, Quantitative analysis of phenobarbital in dosage form by thin-layer chromatography combined with densitometry, J. AOAC Int. 89 (2006) 995–998.
[14] S. Mennickent, R. Fierro, M. Vega, M. de Diego, C.G. Godoy, Instrumental planar chromatographic method for determination of carbamazepine in human serum, J. Sep. Sci. 32 (2009) 1454–1458.
[15] US Department of Health and Human Services, Food and Drug Administration, Center for Biologics Evaluation and Research, Guidance for Industry: Bioanalytical Method Validation, Rockville, MD, 2001, http://www.fda.gov/downloads/Drugs/Guidance ComplianceRegulatoryInformation/Guidances/UCM070107.pdf (accessed 21.01.2010).
[16] International Conference on Harmonization Tripartite Guideline, ICH Topic Q2, Validation of Analytical Procedures: Text and Methodology, Geneva, 2005, http://www.ich.org/cache/compo/276-254-1.html (accessed 21.01.2010).
[17] J.M. Bland, D.G. Altman, Statistical methods for assessing agreement between two methods of clinical measurement, Lancet 8476 (1986) 307–310.
[18] B. Renger, Z. Végh, K. Ferenczi-Fodor, Validation of thin layer and high performance thin layer chromatographic methods, J. Chromatogr. A 1218 (2011) 2712–2721.