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Short communication

Validated high-performance liquid chromatographic method for the determination of lamotrigine in human plasma

Nádia Rezende Barbosa, Antônio Flávio Mídio*

Departamento de Análises Clínicas e Toxicológicas, Faculdade de Ciências Farmacêuticas da Universidade de São Paulo, Av. Prof. Lineu Prestes, 580 CEP 05508-900, São Paulo, S.P., Brazil

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Abstract

A high-performance liquid chromatographic (HPLC) procedure for lamotrigine was developed and validated. Lamotrigine (LTG) and an internal standard were extracted from plasma using liquid–liquid extraction under alkaline conditions into an organic solvent. The method was linear in the range 0.78–46.95 μmol/l, with a mean coefficient of correlation (r)≥0.99923. The limit of detection (LOD) and limit of quantification (LOQ) were 0.19 and 0.58 μmol/l, respectively. Within- and between-run precision studies demonstrated C.V.<3% at all tested concentrations. LTG median recovery was 86.14%. Antiepileptic drugs tested did not interfere with the assay. The method showed to be appropriate for monitoring LTG in plasma samples.

Keywords: Lamotrigine

1. Introduction

Lamotrigine, 3,5-diamino-6-(2,3 diclorophenyl)-1,2,4-triazine, is a recently developed broad-spectrum antiepileptic drug, unrelated chemically to other anticonvulsants in current use [1–3]. Its mode of action is believed to be the inhibition of the release of glutamate and aspartate and possibly an involvement in the blockade of voltage-dependent sodium channels [4]. Its efficacy against partial and generalized seizures has been demonstrated in several studies [5,6].

Lamotrigine (LTG) is well absorbed after oral dosing and approximately 55% is bound to proteins in plasma. It is eliminated by glucuronidation and renal excretion, displaying first-order linear kinetics. Wide inter-individual differences exist in serum/plasma lamotrigine levels achieved at any given dose, largely because of its pharmacokinetic interactions with concurrently prescribed anticonvulsants. A large variation has been reported in the elimination half-life of LTG: 14 h in patients treated with carbamazepine; 23–28 h in patients on monotherapy and 70 h during concomitant valproic acid therapy. Thus, when LTG is used as an add-on therapy, dosage depends on the type of concomitantly administered drugs (hepatic enzyme inducers or inhibitors) [6].

A tentative target range of LTG in plasma (3.90–15.60 μmol/l) [1,7,8] has been proposed. Further studies are required to assess the relationship between concentration and clinical effect and to determine the changes of LTG kinetics in relation to the type of comedication [9]. For this purpose,

*Corresponding author.

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sensitive and specific methods are needed for its quantification in biological fluids.

In the present work a high-performance liquid chromatographic (HPLC) assay for LTG for therapeutic drug monitoring (TDM) was developed and validated.

2. Experimental

2.1. Chemicals

Standards: BW725C (internal standard) and lamotrigine (LTG) Cardiff Bioanalytical Services; carbamazepine: Biogalênica; phenobarbital: Rhodia S.A.; phenytoin and primidone: UCI-Farma. Solvents and reagents: methanol and ethyl acetate (Ohminisolv®): EM Science; triethylamine (analytical grade), potassium dihydrogen orthophosphate (analytical grade) and sodium hydroxide (analytical grade): Merck; deionized water Milli-Q®: Millipore. Solutions: 0.1 *M* potassium dihydrogen orthophosphate solution, methanol and triethylamine in the proportions 560:435:0.1 (v/v/v), respectively (mobile phase); working internal standard solution BW725C 20 μg/ml sodium hydroxide 5 *M*; standard solutions in drug-free human plasma: lamotrigine (390 μmol/l); carbamazepine (52.87 μmol/l); carbamazepine-10,11-epoxide (9.90 μmol/l); primidone (114.50 μmol/l); phenytoin (59.40 μmol/l); phenobarbital (53.87 μmol/l). Standard solutions in methanol–internal standard solution BW725C (20 μg/ml); lamotrigine (3.90, 39 and 390 μmol/l). Calibrator samples: dilute aliquots of LTG standard solution with drug-free human plasma. The calibration curve prepared spans the concentration range 1.95–39 μmol/l. Quality control samples: a separate stock solution of LTG (390 μmol/l) was prepared by dissolving the pure compound in methanol and dilute aliquots of this solution with plasma to obtain a quality control samples (QC) at three different concentrations (1.17; 15.60 and 35.10 μmol/l).

2.2. Instrumentation

The HPLC system (LKB 2152-LC) was equipped with a 20 μl loop; 2151 ultraviolet variable-wavelength detector set a 306 nm; an LKB 2220 integrator; an LKB 2150 pump. The analytical column was a Supelcosil LC-18 (150×4.6 mm), 5 μm particle size column protected with a precolumn Supelguard (20×4.6 mm) (Supelco).

2.3. Analytical procedure

To 1 ml of plasma in a 10 ml glass vial, the internal standard (50 μl) and 10 ml of ethyl acetate were added. After mixing for 15 min, the sample was centrifuged for 10 min at 1800×*g*. The aqueous phase was discarded, and the organic phase was evaporated to dryness under a flow of air at 45°C. The residue was dissolved in 100 μl of mobile phase, and a 50 μl aliquot was injected into the HPLC column at a flow-rate of 0.8 ml/min. The analyte was detected at 306 nm.

2.4. Validation of the analytical method

Calibration curves and linearity were performed in plasma samples and methanol in which LTG and internal standard were spiked to obtain concentration ranges 1.95–39 μmol/l and 0.78–46.95 μmol/l, respectively. They were constructed by plotting peak-area ratios of LTG to internal standard as a function of the drug concentration in the calibrators. The equation of the calibration line was calculated by least-squares linear regression and was used to calculate the drug concentration in the unknowns.

Routine quality control was assessed by use of in-house lamotrigine controls analyzed in each run.

QC samples used to assess within- and between-day precisions were freshly prepared and assayed with each calibration curve. Sample stability was evaluated by calibrators (3.90 μmol/l) at room temperature, 4°C and −20°C.

Precision and stability tests were performed in six replicates for three days and in three replicates, respectively.

Recoveries of LTG and internal standard were determined by spiking drug-free human plasma with known amounts of the LTG (calibrator samples at concentrations of 3.90 and 39 μmol/l) and internal standard (1 μg/ml). The samples were extracted and analyzed in 10 replicates, as described elsewhere. The recoveries from plasma were determined by comparing the resulting peak-areas with those ob-

tained from direct injection of the pure standards in methanol solutions.

Assay specificity was established by analyzing a pool of drug-free human plasma. In addition, antiepileptic drugs were spiked in drug-free human plasma and were tested for potential interferences. Carbamazepine (52.87 μmol/l); phenytoin (59.40 μmol/l); carbamazepine-10,11-epoxide (9.90 μmol/l); phenobarbital (53.87 μmol/l); primidone (114.50 μmol/l) were included.

Accuracy was assessed by comparing results of eight lyophilized serum samples from 'Heath Control External Quality Assessment Scheme for Therapeutic Drug Assays' Cardiff, Wales, Bioanalytical Services (HEQAS), UK, comparing the present method and the average LTG concentrations obtained and established as reference method used by a group of eight different laboratories.

3. Results and discussion

LTG has been measured in biological fluids by gas chromatography–NPD [10] and by immunoassays [11]. Several HPLC methods have been reported for the determination of LTG concentration in human plasma/serum. These include normal and reversed-phase procedures after organic-phase extraction under alkaline conditions [11–15], as well as reversed-phase ion-pairing assay after solid-phase extraction [16,17]. Some of them permit even the simultaneous determination of other antiepileptic drugs [15–18]. Reversed-phase chromatographic assays after solvent demixing sample preparation have also been reported [19]. More recently, procedures based on direct HPLC analysis after sample deproteinization with acetonitrile or perchloric acid have been reported [18,20–22]. However some of them fail to resolve metabolites of coadministered drugs or require dual-wavelength monitoring to determine each component [18] and do not use internal standards [20].

A method for the determination of LTG associated with carbamazepine, phenytoin and phenobarbital was described [15] in which LTG coeluted with carbamazepine giving broad tailing peaks. Other methods were presented [23,24] in which LTG was determined efficiently in the presence of other antiepileptic drugs. However, one of them [23] is time consuming and rather expensive as it involves solid-phase extraction of the drugs from plasma contrary to conventional extraction with organic solvents. A very recent micro-method for the simultaneous determination of LTG with ethosuximide, primidone, phenytoin, phenobarbital, carbamazepine and two metabolites of carbamazepine (carbamazepine-diol and carbamazepine-10,11-epoxide) was described for 100 μl plasma samples [25]. The method showed to be suitable for therapeutic drug monitoring of antiepileptic drugs, particularly in pediatric patients where small sample size is mandatory.

In the present method LTG was extracted with ethyl acetate and separated on a reversed-phase HPLC column.

During the phase of optimization of the assay, different mobile phases were evaluated for their performance in separating LTG, its internal standard and endogenous compounds. The mixture already described (0.1 *M* potassium dihydrogen orthophosphate solution, methanol and triethylamine, 560:435:0.1, v/v/v) produced optimal separation with retention times (t_R) of 8.10 and 3.70 min for LTG and the internal standard, respectively. Typical chromatograms obtained with extracted drug-free human plasma, sample of plasma spiked with LTG (21.06 μmol/l) and internal standard (1 μg/ml) and one patient's plasma treated with LTG (400 mg/day)

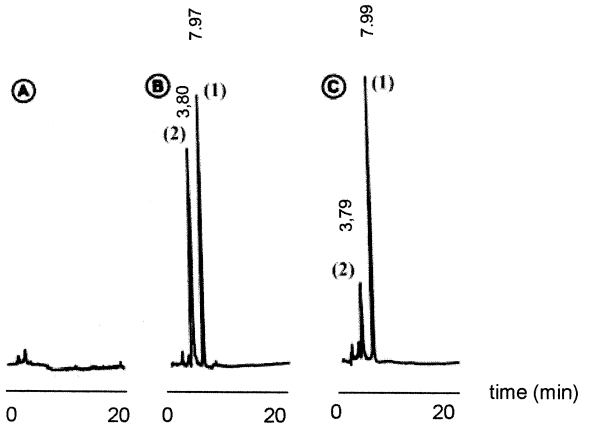

Fig. 1. Chromatographic profile of (A) a drug-free human plasma (without internal standard), (B) a sample of plasma spiked with (1) LTG (21.06 μmol/l) and (2) internal standard (1 μg/ml) and (C) patient's plasma treated with LTG (400 mg/day).

Table 1
Coefficients of variation within-run and between-run of plasma LTG concentrations in spiked samples[a]

Concentration (μmol/l)	*N*	SD	C.V. (%)
Within-run			
1.17	6	0.004	2.22
15.60	6	0.060	2.18
35.10	6	0.132	2.06
Between-run			
1.17	18	0.004	2.11
15.60	18	0.058	2.12
35.10	18	0.143	2.26

[a] *N*, number of analyzed samples; SD, standard deviation; C.V., coefficient of variation.

Table 2
Absolute and relative retention times of the antiepileptic drugs tested by HPLC[a]

Compound	t_R (min)	R_{RT}
Internal standard	3.70	–
Lamotrigine	8.10	2.19
Carbamazepine	21.00	5.68
Carbamazepine-10,11-epoxide	ND	–
Phenobarbital	ND	–
Phenytoin	ND	–
Primidone	ND	–

[a] t_R, retention time; R_{RT}, relative retention time; ND, not detected.

and spiked with internal standard are illustrated in Fig. 1.

Under optimized conditions, calibration curve in plasma showed the following straight line equation: $y=0.6343x-0.04241$ and $r=0.99986$.

The present method showed good linearity in a concentration range 0.78–46.95 μmol/l ($y=0.63145x-0.03973$ and $r=0.99923$) which permits to determine plasma LTG concentrations in patients receiving therapeutic dosages of the drug.

The limit of detection (LOD) was 0.19 μmol/l of LTG determined by progressive dilution method [26]. The limit of quantification (LOQ) was 0.58 μmol/l with coefficient of variation (C.V.) of 2.10% and standard deviation (SD) of 0.002 for five replicates.

The coefficients of variation ranged from 2.06 to 2.22% for within-run and from 2.11 to 2.26% for between-run precision studies, as shown in Table 1. The precision of the assay is fully satisfactory with a C.V.<3% at all tested concentrations.

No significant loss of LTG (≤10%) was observed after storage of plasma: at room temperature for 24 h, which enables postal samples to be accepted for analysis; at 4°C for 15 days and at −20°C for 2 months, which proves LTG stability under routine storage conditions.

A 100% recovery value was attributed to methanol solutions spiked with LTG and internal standard. Extraction efficiency was demonstrated by LTG median recovery of 86.14% whereas the absolute recovery of the internal standard was 83.90%.

Normal plasma constituents do not interfere in the assay. The following drugs have been shown not to interfere using the conditions above specified: pheno-

Table 3
Comparison of LTG concentration obtained by the present method with those Health Control External Quality Assessment Scheme for Therapeutic Drug Assays (HEQAS), Cardiff)

Sample (code 1292)	Concentration spiked (μmol/l)	Mean concentration ($n=8$) (μmol/l)	Our results (μmol/l)	Bias
AA	0.00	0.00	0.00	0
BB	33.04	31.19	31.09	−0.32
CC	3.78	4.02	4.07	+1.24
DD	14.17	14.40	14.40	0
EE	1.89	2.31	2.33	+0.87
FF	11.34	11.36	11.35	−0.09
GG	49.04	46.96	47.05	+0.19
HH	0.95	1.21	0.97	−19.8

barbital; carbamazepine-10,11-epoxide; phenytoin; primidone. Carbamazepine eluted with a retention time 2.6 times longer than lamotrigine in this procedure. Results of absolute and relative retention times of these compounds shown in Table 2 demonstrate the appropriate selectivity of Supelcosil LC-18 column for LTG determination. Because of its specificity, the method may be usefully applied to evaluate plasma LTG levels in patients taking comedication. This aspect is particularly important because LTG is used mostly as an add-on drug.

The results obtained with this method were satisfactory at all tested concentrations. They can also compare with those (HPLC) used in the eight different participating laboratories in the HEQAS (Table 3).

The method is appropriate for TDM of LTG in human plasma and is competitive with some HPLC procedures reported in the literature in terms of precision and sensitivity.

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