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Short communication

Simultaneous liquid chromatographic determination of lamotrigine, oxcarbazepine monohydroxy derivative and felbamate in plasma of patients with epilepsy

Manuela Contin *, Monica Balboni, Erica Callegati, Carmina Candela, Fiorenzo Albani, Roberto Riva, Agostino Baruzzi

Laboratory of Clinical Neuropharmacology, Neurology Clinic, Department of Neurological Sciences, University of Bologna, Via Foscolo 7, 40123 Bologna, Italy

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Abstract

A very simple and fast method has been developed and validated for simultaneous determination of the new generation antiepileptic drugs (AEDs) lamotrigine (LTG), oxcarbazepine's (OXC) main active metabolite monohydroxycarbamazepine and felbamate in plasma of patients with epilepsy using high-performance liquid chromatography (HPLC) with spectrophotometric detection. Plasma sample (500 μL) pre-treatment was based on simple deproteinization by acetonitrile. Liquid chromatographic analysis was carried out on a Synergi 4 μm Hydro-RP, 150 mm × 4 mm I.D. column, using a mixture of potassium dihydrogen phosphate buffer (50 mM, pH 4.5) and acetonitrile/methanol (3/1) (65:35, v/v) as the mobile phase, at a flow rate of 1.0 mL/min. The UV detector was set at 210 nm. Calibration curves were linear (mean correlation coefficient >0.999 for all the three analytes) over a range of 1–20 μg/mL for lamotrigine, 2–40 μg/mL for monohydroxycarbamazepine and 10–120 μg/mL for felbamate. Both intra and interassay precision and accuracy were lower than 7.5% for all three analytes. Absolute recoveries ranged between 100 and 104%. The present procedure describes for the first time the simultaneous determination of these three new antiepileptic drugs. The simple sample pre-treatment, combined with the fast chromatographic run permit rapid processing of a large series of patient samples.

Keywords: Lamotrigine; Oxcarbazepine main active metabolite; Felbamate; HPLC analysis; Therapeutic drug monitoring

1. Introduction

In the last decade, several new antiepileptic drugs (AEDs) have been licensed around the world [1]. Although the experimental evidence does not always allow for a definite conclusion [2], sufficient clinical and pharmacological data support the usefulness of therapeutic drug monitoring (TDM) for some of these compounds [1]. Demands for TDM of different new AEDs in our laboratory have increased in the last few years, partly reflecting the increased clinical use of these agents and the attitude of many clinicians to use AED assay as part of patient therapeutic management [3].

Among the newer AEDs, lamotrigine (LTG), [3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4 triazine], (Lamictal®, GlaxoSmithKline, Brentford, UK) shows a broad spectrum of clinical efficacy [4]. LTG interindividual kinetic variability is pronounced, and is further amplified by age, comedication, pregnancy and disease states [1]. Oxcarbazepine (OXC), (10,11-dihydro-10-oxo-5H-dibenzo[b,f]azepine-5-carboxamide), (Trileptal®, Tolep®, Novartis Pharma, Basel, Switzerland) is a 10-ketoanalogue of carbamazepine registered for partial seizures and generalized tonic-clonic seizures in children and adults [4]. In humans OXC is rapidly metabolized to its therapeutically active metabolite 10,11-dihydro-10-hydroxy-5H-dibenzo[b,f]azepine-5-carboxamide (MHD) which reaches steady-state plasma concentrations about a hundred-fold higher than those of the parent drug [5]. MHD is the main active compound during chronic OXC therapy and the moiety routinely determined for OXC TDM. In spite of the notion of a low susceptibility of OXC to drug interactions, plasma concentrations of MHD are reduced by coadministration of AED inducers (phenytoin, phenobarbital) [6,7]. Felbamate (FBM),

* Corresponding author. Tel.: +39 051 2092752; fax: +39 051 2092751.
E-mail address: manuela.contin@unibo.it (M. Contin).

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(2-phenyl-1,3-propanediol dicarbamate), (Taloxa®, Schering-Plough, Kenilworth, NJ, USA) is a broad spectrum AED [4]. Because of the risk of aplastic anemia and hepatotoxicity its prescription is restricted to patients with partial seizures and Lennox Gastaut syndrome refractory to other AEDs [4]. Although it is not commonly prescribed, measurement of FBM plasma concentrations may be helpful in optimizing dosing schedule in these severely affected patients [1]. Co-prescription of these newer AEDs, namely LTG and OXC, even in association with older antiepileptic agents is used, especially in patients with partial-onset and generalized tonic-clonic refractory seizures [4]. To implement our long-standing AED TDM service, we offset out to devise simple and rapid analytical procedures, which could process a large series of patient samples in a single analytical session by minimizing plasma specimen preparation steps and grouping different newer AEDs in the same assay.

Many high-performance liquid chromatography (HPLC) methods for the determination of the old and new AEDs in human plasma have been developed [8,9]. Different simple HPLC–UV procedures based on direct HPLC injection after sample deproteinization or even without sample pretreatment have been reported for the individual determination of LTG [10–13], MHD [14] and FBM [15,16] in human plasma. Shortcomings of these methods for TDM in patients with epilepsy include the need for dual-wavelength monitoring [10] or a gradient elution [15] to eliminate interferences, low analyte extraction efficiency [14], inadequate limit of quantitation (LOQ) [11], inappropriate choice of the internal standard [16] and short guard column [14] or column life [12]. Only one recently published method allows for the simultaneous determination of LTG and MHD [17], but it requires laborious and expensive sample pre-treatment. No method has been proposed so far for the simultaneous monitoring of LTG, MHD and FBM in patients with epilepsy.

Here we describe a new fast and simple HPLC method with spectrophotometric detection for the simultaneous measurement of LTG, MHD and FBM in plasma of patients with epilepsy which is suitable for application in a routine AED TDM setting.

2. Experimental

2.1. Reagents and standards

LTG was kindly provided by GlaxoSmithKline (Brentford, UK); MHD by Novartis Pharma (Basel, Switzerland); FBM by Schering-Plough (Kenilworth, NJ, USA); 4-methylprimidone (internal standard, I.S.) was purchased from Carlo Erba Reagenti (Milan, Italy). Methanol, acetonitrile, both gradient grade, and potassium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). Ultrapure water was obtained from a MilliQ Gradient A10 apparatus (Millipore, Billerica, MA, USA).

Stock solutions (1 mg/mL) and subsequent dilutions (500, 200, 100 and 50 μg/mL) of the drugs were prepared by dissolving LTG in acetronile and MHD, FBM and 4-methylprimidone in methanol. All solutions were prepared monthly and stored at 4 °C.

Plasma standards for the calibration curves of 1.0, 2.0, 4.0, 10.0 and 20.0 μg/mL for LTG; 2.0, 4.0, 10.0, 20.0 and 40.0 μg/mL for MHD; 10.0, 20.0, 40.0, 80.0 and 120.0 μg/mL for FBM were prepared by pipetting suitable amounts of the three drug standard solutions to 500 μL aliquots of blank pooled plasma and then treated exactly as patients' specimens.

2.2. Chromatographic apparatus and conditions

The HPLC system consisted of a Series 200 liquid chromatograph, a Series 200 UV–vis spectrophotometric detector, set at 210 nm, and a Series 200 autosampler connected by a model 600 link chromatography interface to the TotalChrom chromatography workstation. All the equipment was purchased from Perkin Elmer, Norwalk, CA, USA.

Chromatographic separations were performed with a Synergi 4 μm Hydro-RP, 150 mm × 4 mm I.D. column (Phenomenex, Torrance, CA, USA) protected by a C_{18} Securityguard precolumn (Phenomenex) and a graphite filter (ESA, Chelmsford, MA, USA). The mobile phase was a mixture of potassium dihydrogen phosphate buffer (50 mM, pH 4.5), filtered through a 0.22 μm membrane filter (GS type, Millipore) and acetonitrile/methanol (3/1) (65:35, v/v). The mobile phase was sparged with helium and the flow rate was set at l.0 mL/min.

2.3. Blood sampling and plasma processing

Venous blood samples (5 mL) were drawn from patients at 8 a.m., before their first morning dose of AEDs, transferred into heparinized tubes (8 IU heparin/mL blood) and centrifuged at 1500 × *g* for 10 min at 4 °C. Plasma was separated, transferred into test tubes and stored at −20 °C until analysis. Five hundred microliter plasma aliquots were spiked with 25 μL of I.S. solution (1 mg/mL), deproteinized by addition of 1.5 mL acetonitrile, vortexed for 20 s and then centrifuged at 2500 × *g* at 4 °C for 10 min. Five microliters of the clean upper layer were injected directly into the chromatographic system.

2.4. Method specificity

Standard solutions of several commonly co-prescribed AEDs, their metabolites and benzodiazepines were injected to check for possible interferences (Table 1). Blank plasma from 10 pools was tested for endogenous interferences. Furthermore, a series of plasma samples from patients with epilepsy not taking LTG, MHD, FBM and treated with commonly prescribed AED and non-AED cotherapies (including antidepressants, hypnotics, antipsychotics, different types of antibiotics, antiinflammatory and cardiac drugs) were analyzed to check for drugs which could potentially interfere with the three AEDs determination.

2.5. Method validation

Standard curves were run on each analysis day ($n = 10$) over 4 months. The analyte to I.S. peak area ratios were plotted against each drug-matched concentration added to the blank plasma. The calibration curves were calculated by the least square method.

Table 1
List of drugs checked as potential assay interferences ($n = 6$)

Compound	Concentration (μg/mL)	Retention time (min)
AEDs		
Carbamazepine	10	12.36 ± 0.02
Carbamazepine-diol	2	2.90 ± 0.01
Carbamazepine-epoxide	5	5.71 ± 0.01
Ethosuximide	50	3.02 ± 0.02
Gabapentin	10	n.d.
Levetiracetam	20	1.76 ± 0.01
Oxcarbazepine	5	7.06 ± 0.01
Phenobarbital	20	5.98 ± 0.02
Phenytoin	10	12.96 ± 0.03
Primidone	10	2.98 ± 0.01
Tiagabine	0.1	n.d.
Topiramate	5	n.d.
Valproic acid	50	n.d.
Vigabatrin	2	n.d.
Benzodiazepines		
Clobazam	1	n.d.
Clonazepam	1	n.d.
Diazepam	0.1	n.d.
Lorazepam	0.1	n.d.
Nitrazepam	1	n.d.
Norclobazam	2	n.d.

n.d., not detectable.

Linearity was assessed by determining the coefficient of correlation (r) of the points of the curves.

For assay precision and accuracy assessment, spiked blank plasma pools were prepared at three concentrations (i.e., 1.0, 4.0 and 20.0 μg/mL for LTG; 2.0, 10.0 and 40.0 μg/mL for MHD; 10.0, 40.0 and 120.0 μg/mL for FBM) corresponding to the lower, middle and upper points of each calibration curve, separated into 500 μL aliquots and stored frozen at −20 °C.

The precision of the method was assessed by determining the relative standard deviation (R.S.D. = 100 × S.D./mean) at the three plasma concentrations chosen for the three drugs within the same analysis ($n = 6$, intra-day precision) and in triplicate over a series of six analyses on different days ($n = 18$, inter-day precision).

The accuracy of the method was determined by comparing the means of the calculated concentrations at the three plasma concentrations chosen for each drug with the nominal concentrations (percentage differences) within the same analysis ($n = 6$, intra-day accuracy) and in triplicate over a series of six analyses on different days ($n = 18$, inter-day accuracy).

The absolute recovery of the three AEDs and the I.S. was calculated for each analyte as the ratio of the drug peak area from deproteinized blank plasma spiked with LTG, MHD and FBM, at the three abovementioned concentrations, and with the I.S. (50 μg/mL) to the peak area obtained from the injection of LTG, OXC, FBM and I.S. standard solutions, at the same theoretical concentrations, reconstituted in mobile phase.

The lower limit of quantitation (LOQ) was defined as the lowest quantifiable concentration with an associated R.S.D. and inaccuracy <20%. The precision and accuracy at the LOQ were determined both intraday ($n = 6$) and interday (triplicate samples over six analyses on different days, $n = 18$).

The lower limit of detection (LOD) was calculated as three times the baseline noise.

3. Results and discussion

During the optimization phase of the assay, different mobile phases were evaluated. The mixture already described (potassium dihydrogen phosphate buffer, 50 mM, pH 4.5 and acetonitrile/methanol 3/1, 65:35, v/v) provided optimal separation of the three analytes and I.S. with mean ± S.D. ($n = 6$) retention times of 3.20 ± 0.01 min for LTG, 3.51 ± 0.01 min for FBM, 3.93 ± 0.02 min for MHD, and 4.44 ± 0.02 min for I.S. (Fig. 1). There were no endogenous interferences in the assayed analytes' elution region for any of the blank pools tested. None of the possibly co-prescribed drugs tested interfered in the analysis: elution times of the agents checked over a 20-min run are reported in Table 1. Moreover, from plasma analyses of patients with epilepsy not taking LTG, OXC, FBM and treated with commonly prescribed AED and non-AED cotherapies no interfering peak was detected.

Calibration curves showed a linear and reproducible correlation between the three AEDs plasma concentrations and matched analyte to I.S. peak area ratios; correlation coefficients were >0.998 for all curves (Table 2).

The results of precision and accuracy analyses are reported in Table 3. The R.S.Ds. for both intra and interassay precision were below 7.5% for the whole concentration range for all the compounds. Similarly, deviation of the mean of the measured concentrations from their nominal concentrations (intra and interassay accuracy) was below 7.5% for all drugs. The LOQ was set at 0.5 μg/mL for LTG, 1.0 μg/mL for MHD and 5.0 μg/mL for FBM (Table 3). The LOD was 0.25 μg/mL for LTG, 0.5 μg/mL for MHD and 2.5 μg/mL for FBM. The absolute recovery ranged between 100 and 104% for the three AEDs and I.S. (Table 4).

From the analyses of 902 plasma specimens of 622 patients with epilepsy referred to our laboratory over 6 months, treated with LTG (dosage range, 25–600 mg/day, $n = 560$), OXC (300–2400 mg/day, $n = 290$), FBM (600–3600 mg/day, $n = 52$), combined with different AED cotherapy, we found steady-state AEDs plasma trough concentrations of 0.5–22.9 μg/mL for LTG, 3.1–48.6 μg/mL for MHD and 5.1–86.8 μg/mL for FBM. LTG was associated with OXC in 60 out of 622 patients; FBM was co-prescribed with OXC in 6 patients and with LTG in 9 patients.

Table 2
AEDs calibration lines parameters (mean ± S.D., $n = 10$)

Drug	Intercept (a)	Slope (b)	r
LTG	0.0134 ± 0.0041	0.0684 ± 0.0049	0.9994 ± 0.0007
MHD	−0.0200 ± 0.0055	0.0494 ± 0.0044	0.9995 ± 0.0005
FBM	−0.0265 ± 0.0088	0.0121 ± 0.0010	0.9996 ± 0.0003

Equation of the regression line: $y = a + bx$, where x is the analyte concentration, expressed in μg/mL, and y is the analyte to I.S. peak area ratio, expressed in arbitrary area units; r = coefficient of correlation.

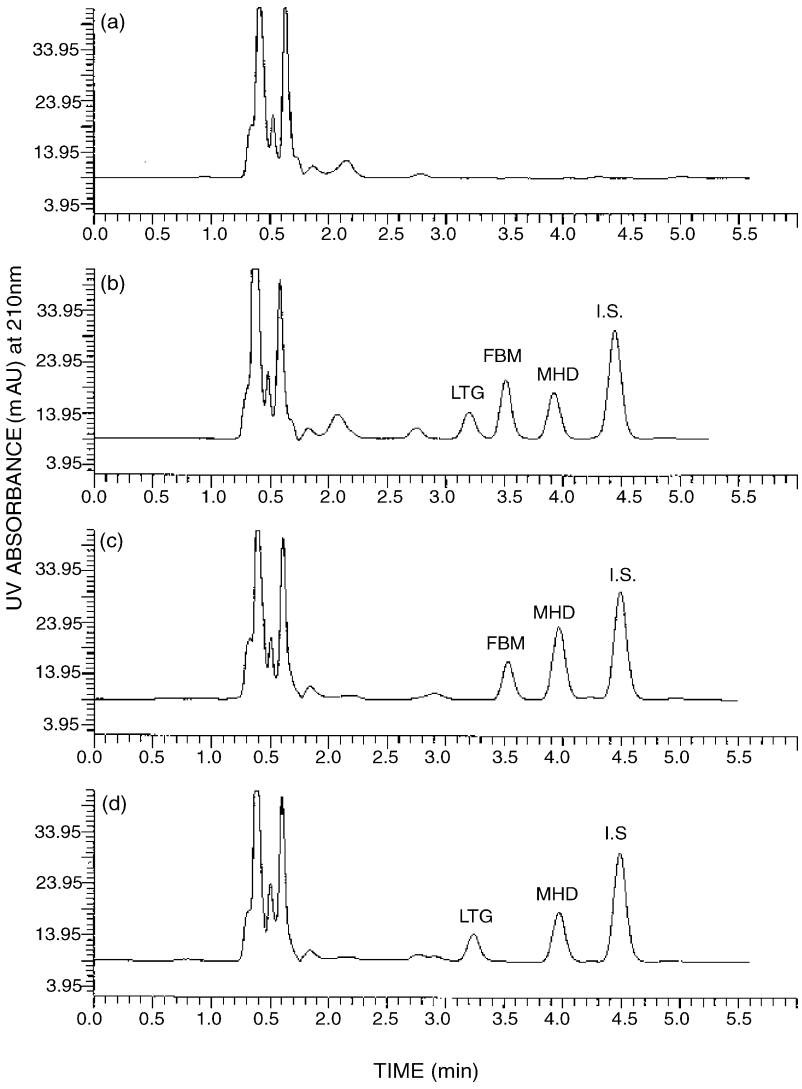

Fig. 1. Chromatograms obtained by injecting 5 μl of: (a) deproteinized blank plasma; (b) blank plasma spiked with LTG, 4.0 μg/mL, FBM, 40.0 μg/mL, MHD, 10.0 μg/mL, I.S., 50.0 μg/mL; (c) plasma specimen of a child treated with felbamate (720 mg/die) and oxcarbazepine (600 mg/die): FBM, 30.0 μg/mL, MHD, 17.3 μg/mL and (d) plasma specimen of an adult patient treated with lamotrigine (300 mg/die) and oxcarbazepine (900 mg/die): LTG, 3.9 μg/mL, MHD, 10.8 μg/mL. LTG, lamotrigine; FBM, felbamate; MHD, monohydroxycarbamazepine; I.S., internal standard.

One of the main advantage of the present analysis is the simple and fast procedure of sample pre-treatment, allowing a large series of plasma specimens to be processed in a short time. LC detection is highly selective and the chromatographic separation very rapid, allowing LTG, MHD and FBM determination in plasma of patients also receiving complex AED co-medication in about 5 min. No interfering peaks were observed in any of the samples tested to date with this method (about 900 patients' samples). The injection of a very low aliquot of deproteinized specimens and the adoption of the graphite filter combined with the guard column provides highly effective protection of the analytical system: a change of graphite filter after about 300 injections and the preguard column after 500 injections avoids increased column back pressure and maintains an excellent chromatographic separation (about 1800 deproteinized samples injected to date).

Compared with the only HPLC method for the simultaneous LTG and MHD plasma analysis published so far [17], this assay significantly simplifies sample purification by omitting time-consuming and expensive solid-phase extraction and drying steps, with reduced risks of analytical errors. The method quantitation range chosen for the three analytes proved to be adequate for TDM purposes even in patients receiving low daily dosages, especially during clinically recommended LTG slow titration regimens [18]. The LOQs of the assay are well above the lower concentration values of currently proposed tentative "optimal" ranges for LTG (3–14 μg/mL) [3], MHD (12–35 μg/mL) and FBM (30–80 μg/mL) [19]. Finally, the statistical validation shows a good intra and inter assay precision and accuracy within the whole concentration range for all the three analytes and an optimal extraction efficiency.

In conclusion, the proposed method proved to possess adequate specificity, sensitivity, accuracy and precision for a reliable simultaneous determination of LTG, MHD and FBM in patients with epilepsy. By minimizing plasma preparation steps and grouping different new AEDs in the same assay the method

Table 3
Precision and accuracy parameters of the assay

Drug	Amount added to blank plasma (μg/mL)	Intraday($n = 6$)			Interday ($n = 18$)		
		Calculated concentration (mean ± S.D.) (μg/mL)	Precision (R.S.D.%)	Accuracy (%)	Calculated concentration (mean ± S.D.) (μg/mL)	Precision (R.S.D.%)	Accuracy (%)
LTG	0.5 (LOQ)	0.48 ± 0.02	4.2	−4.0	0.43 ± 0.06	13.9	−14.0
	1.0	0.96 ± 0.02	2.1	−4.0	0.96 ± 0.06	6.2	−4.0
	4.0	4.08 ± 0.09	2.2	2.0	4.20 ± 0.09	2.1	5.0
	20.0	20.13 ± 0.38	1.9	0.6	20.29 ± 0.63	3.1	1.4
MHD	1.0 (LOQ)	1.16 ± 0.06	5.2	16.0	1.11 ± 0.19	17.1	11.0
	2.0	1.98 ± 0.10	5.0	−1.0	2.14 ± 0.16	7.4	7.0
	10.0	9.82 ± 0.30	3.0	−1.8	10.05 ± 0.32	3.2	0.5
	40.0	40.06 ± 0.65	1.6	0.1	38.81 ± 1.72	4.4	−3.0
FBM	5.0 (LOQ)	4.72 ± 0.23	4.9	−5.6	4.97 ± 0.58	11.7	−0.6
	10.0	9.67 ± 0.47	4.9	−3.3	10.53 ± 0.41	3.9	5.3
	40.0	41.56 ± 0.55	1.3	3.9	39.85 ± 0.65	1.6	−0.4
	120.0	120.1 ± 1.96	1.6	0.1	118.8 ± 3.39	2.8	−1.0

Precision (R.S.D.%) = 100 × S.D./mean; Accuracy (%) = 100× (mean concentration found − known concentration)/ known concentration); Interday ($n = 18$) = triplicate samples, over a series of six analyses on different days; LOQ: limit of quantitation.

Table 4
Recovery assays ($n = 6$)

Drug	Amount added to blank plasma (μg/mL)	Absolute recovery (mean % ± S.D.)
LTG	1.0	103.7 ± 0.02
	4.0	100.2 ± 0.03
	20.0	100.8 ± 0.03
MHD	2.0	104.4 ± 0.03
	10.0	102.9 ± 0.06
	40.0	102.2 ± 0.05
FBM	10.0	103.8 ± 0.03
	40.0	102.2 ± 0.06
	120.0	103.5 ± 0.05
4-Methyl-primidone (I.S.)	50.0	104.0 ± 0.03

allows a large series of patient samples to be processed in a single analytical session, a task which can be very advantageous in a TDM setting.

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