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New micromethod for the determination of lamotrigine in human plasma by high-performance liquid chromatography

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Abstract

An extraction procedure and reversed-phase high-performance liquid chromatographic assay is described and validated for the determination of lamotrigine in human plasma. The method involves extraction with chloroform–isopropanol after alkalinization with a carbonate buffer, back-extraction into 0.05% phosphoric acid and separation by reversed-phase HPLC using a 5-μm Supelco diphenyl column (150×4.6 mm I.D.). Quantitation was performed by measurement of the UV absorbance at a wavelength of 265 nm. This method was carried out on 50–200 μl samples of plasma, depending on whether they were pediatric or adult samples. The lower limit of quantitation was 0.2 μg/ml using 200 μl of plasma. A linear response was tested from 0.5 to 20 μg/ml. Within- and between-day accuracy and precision were always below 10.0% at all analysed concentrations. The method selectivity towards the most used antiepileptic drugs has been proven. Satisfactory performances were obtained in the evaluation of samples from epileptic patients.

Keywords: Lamotrigine

1. Introduction

Lamotrigine, 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine (Fig. 1), is a new antiepileptic drug structurally and pharmacologically unrelated to currently used antiepileptic medications. It acts by stabilising presynaptic neuronal membranes by blockade of voltage-dependent sodium channels, thus preventing the release of excitatory neurotransmitters, in particular glutamate and aspartate.

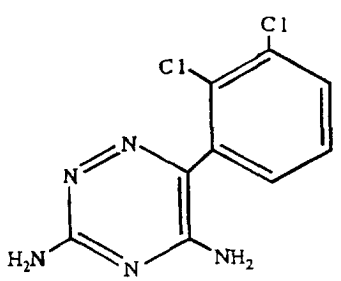

Fig. 1. Chemical structure of lamotrigine.

Efficacy has been demonstrated for lamotrigine (LTG) as add-on therapy in seizure control on patients with intractable partial seizures and in patients with secondarily generalised tonic–clonic seizures [1].

Lamotrigine is metabolized by glucuronidation to a 2-N-glucuronide, which accounts for approximately 75 to 90% of the amount present in urine after a single oral dose.

The steady-state elimination half-life ($t_{1/2}$) of

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lamotrigine in healthy young adults is approximately 25 to 30 h. This value is halved in epileptic patients who are receiving concomitant administration of enzyme-inducing drugs, such as carbamazepin and phenytoin, and it noticeably increases to 59 h in the presence of valproic acid [2]. On the other hand, LTG itself does not influence the plasma concentrations of concomitant antiepileptic drugs, but it does cause an increase in the 10,11-epoxide metabolite of carbamazepine.

Toxic symptoms have been reported [3] when plasma LTG concentrations exceeded 18 μg/ml. Thus, it is important to monitor patients' plasma levels and to relate the analytical data to clinical efficacy. This requires the use of a sensitive and specific method for the quantitation of this antiepileptic drug. Moreover, a valid analytical method is the first step to try to define a therapeutic window range.

Analytical methods for the quantitation of LTG in human plasma by high-performance liquid chromatography (HPLC) have been briefly described in some clinical reports [4–6], but these methods either involve extensive sample preparation with large amounts of extraction solvents, or give minimal validation data. Among the methodological reports published [7,8], the most complete validated assay was the one by Cociglio et al. [7]: otherwise, this method is time-consuming, resulting in longer retention times for LTG and its internal standard.

As regards the glucuronide conjugate of LTG, it is extremely polar. Its determination can be achieved through two methods: differential enzymatic or chemical hydrolysis to the aglycone (indirect method); by extraction at low pH with ethyl acetate or solid-phase extraction [9], as well as by employing an ion-pairing agent followed by either liquid–liquid or solid-phase extraction (direct method) [10]. However, the 2-N-glucuronide metabolite seems to be undetectable in human plasma [11].

The aim of this study is to define and validate a new extraction procedure and reversed-phase HPLC separation method for the determination of LTG in human plasma, which could be simple, rapid, sensitive, reproducible and well suited to routine measurements. This method is not capable of quantifying the glucuronide conjugate of LTG.

2. Experimental

2.1. Chemicals and reagents

Lamotrigine and its internal standard, 3,5-diamino-6-(2-methoxyphenyl)-1,2,4-triazine were a generous gift from the Wellcome Foundation (London, UK). HPLC-grade acetonitrile was obtained from Carlo Erba (Milan, Italy) and phosphoric acid was from Aldrich Chemie (Steinheim, Germany). All other chemicals were of the highest commercial grade available.

2.2. Standard solutions

Standard stock solutions of LTG and of its internal standard were prepared by dissolving pure, accurately weighed, compounds in methanol, thus obtaining concentrations of 10 mg/ml. The stock standard solutions were stored at 4°C. Working standard solutions were prepared by dilution of the stock solutions in methanol in order to give a final concentration of 0.1 mg/ml. Calibration standards were prepared by adding appropriate volumes of LTG working standard solution to drug-free human plasma to give the concentrations 0.5, 1.0, 5.0, 10.0 and 20.0 μg/ml. The spiked plasma samples were prepared just before use.

2.3. Instrumentation and chromatographic conditions

The method was developed by using a Perkin Elmer Series 3B liquid chromatograph connected to a LC-85 spectrophotometric detector. The analytical column was a diphenyl column, 15 cm×4.6 mm I.D., 5 μm particle size (Supelco, Bellefonte, PA, USA). The chromatographic separation was carried out at room temperature and at a solvent flow-rate of 1.2 ml/min. The mobile phase was prepared by mixing acetonitrile and 0.3% phosphoric acid (30:70, v/v) and by adjusting the final pH to 3.5 using 6 *M* NaOH. The mobile phase was filtered before use using a Millipore vacuum filter system equipped with a 0.45-μm filter. The detector was set at a wavelength of 265 nm and at a range of 0.02 AUFS. Analyte concentrations were calculated from peak-

height ratios of LTG to the internal standard, using a HP 3395 integrator (Hewlett-Packard, Avondale, PA, USA).

2.4. Sample preparation

Blood samples were collected in heparinized tubes and centrifuged at 4°C (2200 *g*) for 5 min. The plasma was stored at 4°C until analysis (within one week). A 10-μl volume of internal standard (I.S.) working solution was added to 200 μl of plasma (standard or patients' plasma); then, the samples were alkalinized with 50 μl of carbonate buffer ($NaHCO_3$–Na_2CO_3, pH 9.2) and extracted with 2 ml of chloroform–isopropanol (80:20, v/v) by vortex-mixing for 5 min. After shaking, samples were centrifuged at 2200 *g* for 3 min (4°C). The organic layer was transferred to another tube and back-extracted by shaking with 200 μl of 0.05% phosphoric acid for 5 min. The samples were centrifuged at 4°C (2200 *g*) for 3 min and 25 μl of the upper layer were injected into the HPLC system.

2.5. Linearity and selectivity

The linearity of the method was tested on calibration curves ranging from 0.5 to 20 μg/ml.

The selectivity of the analytical procedure was determined by analysing a lyophilized reference serum of animal origin (Seronorm Pharmaca; Nycomed Pharma) containing carbamazepine (13.3 μg/ml), clonazepam (120.5 μg/ml), ethosuximide (128.8 μg/ml), phenobarbitone (39.1 μg/ml), phenytoin (24.8 μg/ml), primidone (14.8 μg/ml), valproic acid (125.8 μg/ml) and gentamycin (7.8 mg/l).

2.6. Extraction efficiency

The extraction efficiency (recovery) of LTG and IS was calculated as follows: we have compared the peak heights of drug-free plasma spiked with known amounts of drug submitted to the sample preparation procedures with the peak heights of the same concentrations of standards prepared in 0.05% phosphoric acid and directly injected onto the analytical column.

2.7. Accuracy and precision

The accuracy and precision of the present method were evaluated by analyzing plasma samples spiked with different concentrations of LTG. The within-day reproducibility was determined by using replicates of spiked plasma pools at five different concentrations, i.e. 0.2, 2, 5, 10, 20 μg/ml, which were analyzed ten times on the same day. The between-day reproducibility was determined by evaluating the analysis results of five independently spiked plasma pools (0.2, 2, 5, 10, 20 μg/ml) assayed ten times over a period of fifteen days.

3. Results

3.1. Chromatography and selectivity

Typical chromatograms of human drug-free plasma, of human plasma spiked with LTG and I.S. and of a plasma sample from a patient receiving LTG and spiked with I.S. are shown in Fig. 2. The retention times of LTG and I.S. were approximately 4.3 and 2.8 min, respectively. No interfering peaks were found at the retention times of the compounds of interest, when comparing the chromatogram of the reference serum (Seronorm) with chromatograms of spiked plasma and of patients' plasma.

3.2. Sensitivity

Under the described experimental conditions, the detection limit (LOD) was approximately 0.1 μg/ml, at a signal-to-noise ratio of three. The lower limit of quantitation (LOQ), defined as the lowest concentration of the standard curve that can be measured with acceptable accuracy, precision and variability, was 0.2 μg/ml, with the coefficient of variation and bias <15%.

3.3. Linearity

The equation of the regression line was: $y = -0.1872 + 0.21988x$ ($n=5$, $r=0.999$, $r^2=0.998$, standard error=0.132).

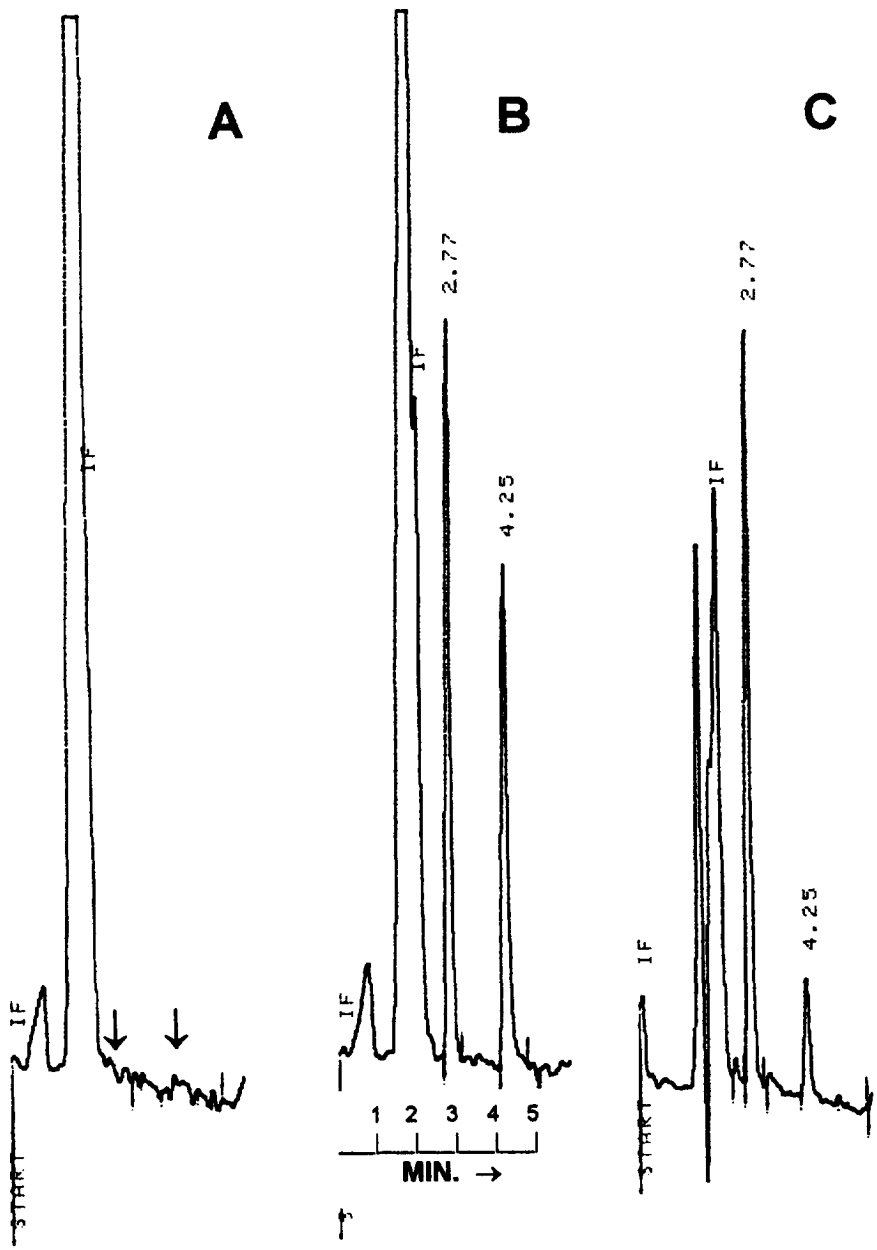

Fig. 2. Chromatograms of an extracted blank plasma (A), an extracted plasma sample spiked with 10 μg/ml lamotrigine (B) and a plasma sample from a patient treated with lamotrigine (C). The peak at 2.77 min is the I.S. and that at 4.25 min is lamotrigine. The arrows in panel A indicate the retention time of LTG and the I.S. Conditions: diphenyl column (150×4.6 mm, 5 μm); mobile phase, acetonitrile–0.3% phosphoric acid, pH 3.5 (30:70, v/v); flow-rate, 1.2 ml/min; detection wavelength, 265 nm; graph scale=2×0.01 AUFS.

3.4. Extraction efficiency and recovery

The extraction recovery was determined in replicate (n=5) at three concentrations (2, 5 and 10 μg/ml) for LTG, and only at the working concentration for the I.S. The extraction recovery remained constant across the concentration range studied, being 59.8% (±0.66) for LTG and 60%

Table 1
Within-day and between-day accuracy and precision of the HPLC method for the determination of lamotrigine in human plasma

Concentration (μg/ml)		n	C.V. (%)	Bias
Actual	Found (mean±S.D.)			
Within-day				
0.2	0.21±0.02	10	7.70	+4.0
2	2.13±0.10	10	4.80	+6.5
5	5.47±0.14	10	2.60	+9.4
10	10.27±0.09	10	0.87	+2.7
20	20.59±0.76	10	3.70	+2.9
Between-day				
0.2	0.23±0.03	10	12.90	+16.0
2	2.30±0.17	10	7.30	+15.0
5	5.76±0.17	10	3.0	+15.0
10	10.41±1.09	10	10.0	+4.0
20	20.86±0.67	10	3.0	+4.0

(±0.83) for the I.S. These values are justified if the double extraction performed in the complete procedure is considered.

3.5. Accuracy and precision

As shown in Table 1, the within-day coefficients of variation (C.V.) were below 5% at all concentrations, except at the LOQ. The accuracy, as expressed by the bias, varied between +2.7 and +9.4%. The between-day coefficients of variation were within 10%, except at the LOQ. The accuracy of the method, expressed by the bias, varied between +4 and +16%.

3.6. Method application

The method is currently in use for the analysis of samples from patients receiving therapeutic doses (50–350 mg/day) of LTG. The assay was usually performed by using 200 μl of plasma samples, but in cases where the patients were children, it was also performed with 50 μl of plasma, with the same analytical performances being obtained (data not shown).

Fig. 3 shows the steady-state concentrations of LTG in two patients. Patient 1 was a nine-year-old child receiving chronic treatment with valproic acid. Patient 2 was a 34-year-old adult receiving chronic treatment with carbamazepine and phenobarbital. These results are examples of how useful the present method can be for monitoring LTG plasma concentrations, if we consider the effects of age or the coadministration of other antiepileptic drugs on LTG pharmacokinetics, as well.

4. Discussion and conclusions

The therapeutic efficacy of an antiepileptic drug depends on the achievement of well-defined plasma concentrations. A validated analytical method is essential to yield results that satisfactorily allow the monitoring of patients during therapy.

On the other hand, a requirement of a routine laboratory where a large number of samples have to be analyzed every day is to have shorter analysis times, simple instrumentation and chromatographic

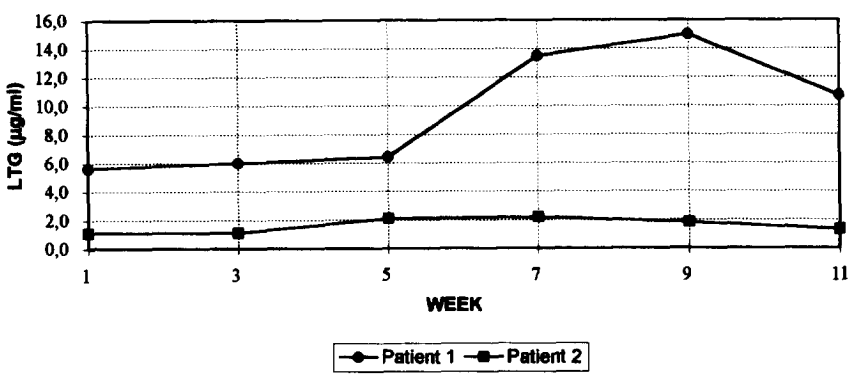

Fig. 3. Steady-state concentrations of lamotrigine in two different patients. Patient 1 was treated with lamotrigine dosages of 50 mg/day and patient 2 received dosages of 200 mg/day. Each dot represents the concentration of the drug just before intake of the morning dose.

conditions, as well as an easily applicable technique, without affecting the accuracy and reproducibility of the analytical methods.

Most published methods to quantify LTG in human plasma involved tedious extraction and separation steps, a long assay time and complex chomatography conditions. In particular, the use of large amounts of extraction solvents, of analytical columns that require frequent maintenance and of chromatographic apparatus equipped with different components such as a column thermostat, is a problem for a routine laboratory management [7,8,10].

In order to simplify all the analytical steps mentioned, we have developed a method that combines rapid and easy assay procedure with a simple technical apparatus. Plasma samples were extracted in two simple steps with minimal reagent volumes, without affecting validation parameters. The use of an acetonitrile–aqueous mobile phase, instead of using buffers, together with the choice of an analytical column that exhibited greater selectivity than the other reversed-phase columns tested (alkyl-type bonded phases), results in a shorter analysis time (5 min) and easier maintenance of the HPLC equipment (optimum column performance and maximum life). Furthermore, the small sample size required (as little as 50 μl) is particularly suitable for pediatric microsamples.

In other words, the present method turns out to be technically simple and quite good with respect to all validation parameters (linearity, accuracy and precision), thus being suitable for large therapeutic monitoring programmes performed in a routine laboratory.

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