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Determination of lamotrigine in human plasma by high-performance liquid chromatography

Pela Angelis-Stoforidis[a,*], Denis J. Morgan[b], Terence J. O'Brien[a], Frank J.E. Vajda[a]

[a]*Australian Centre for Clinical Neuropharmacology, St. Vincent's Hospital, 41 Victoria Parade, Fitzroy, Victoria 3065, Australia*
[b]*Department of Pharmaceutics, Victorian College of Pharmacy, Melbourne, Australia*

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Abstract

The method involves precipitation of plasma proteins with acetonitrile and analysis of the supernatant by high-performance liquid chromatography using a 5 μm Zorbax C8 column. Quantitation was performed by measurement of the UV absorbance at a wavelength of 306 nm. The method was linear in the range of 1–20 μg/ml, with a mean coefficient of determination (r^2=0.998). The limit of detection was 0.6 μg/ml and the lower limit of quantitation was 1 μg/ml using 200 μl of plasma. Within- and between-day accuracy and precision were below 6% at all analysed concentrations except at the limit of quantitation. No interfering peaks were found by commonly monitored antiepileptic drugs. Recovery was found to be ≥99%. Satisfactory performance was obtained in the evaluation of epileptic patient samples, whose results of plasma concentration measurements are briefly discussed. We conclude that this is a reliable method for the routine monitoring of lamotrigine concentration in plasma in the clinical setting.

Keywords: Lamotrigine

1. Introduction

Lamotrigine (LTG) is a new antiepileptic agent, chemically unrelated to other anticonvulsants, which is increasingly used in the management of partial and generalized epilepsies. Although initially developed as a potential anti-folate drug, its mechanism of action is complex. In therapeutic concentrations it is thought to bind slowly to fast inactivated sodium channels [1] and exhibit a use- and voltage dependent block of these channels [2]. Although this may also be associated with preventing glutamate release, most recent studies suggest that this action of lamotrigine may not be significant at physiological concentrations [3].

Although LTG is usually well tolerated, its clinical use is complicated by a large interpatient variability in dosage requirements [4]. This is largely related to wide differences in LTG pharmacokinetics, especially among patients also receiving antiepileptic drugs (AED) that inhibit or induce LTG metabolism. Valproic acid decreases the rate of metabolism [5], resulting in reduced LTG clearance, whilst enzyme-inducing drugs such as phenytoin (PTH), phenobarbital and carbamazepine, increase LTG clearance [6]. Phenytoin, which shares with LTG the cytochrome P_{450} isoenzyme responsible for its metabolism, is particularly potent in inducing the hepatic phase of LTG metabolism and withdrawal of PTH may result

*Corresponding author. Tel.: +61-3-9288-3555; fax: +61-3-9288-3527.

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in dramatic increases in LTG plasma concentrations [7,8].

The putative therapeutic plasma concentration of LTG was estimated to be in the range of 1–4 μg/ml [9], but subsequent observations indicated that some patients may tolerate much higher therapeutic concentrations (>10 μg/ml) without any adverse effects. Although the value of monitoring plasma LTG levels has been questioned [9] further studies are required to assess the relationship between plasma concentration and clinical effect. Therapeutic monitoring of lamotrigine may also be important in detecting non-compliers, confirming toxicity, confirming drug interactions and guiding withdrawal of other AED in the management of patients with epilepsy.

Earlier methods of LTG analysis in human plasma utilised high-performance liquid chromatography (HPLC), which requires tedious extraction and separation steps, a long assay time and complex chromatographic conditions [10–12], making it less suitable for therapeutic monitoring.

The objective of this study was to develop and validate a specific, rapid and simple HPLC assay for measuring LTG levels that is free of interference from other commonly administered anticonvulsant drugs, requires a small specimen volume and minimal handling for sample preparation. We also suggest a novel approach in timing of blood sampling which is more practical. This involves measuring LTG levels mid-way between morning and evening doses, as the drug is usually prescribed in a b.d. regimen. Mid-dose samples are convenient to obtain and the patient does not risk an early morning seizure by waiting for a trough sample to be available before the next dose is taken.

2. Experimental

2.1. Chemicals and reagents

Lamotrigine and its internal standard (I.S), 3,5-diamino-6-(2-methoxyphenyl)-1,2,4-triazine were generous gifts from the Wellcome Foundation (London, UK). Ethosuximide and gabapentine were gifts of Parke Davis (NSW, Australia) while carbamazepine-10,11-epoxide and 5-(p-hydroxyphenyl)-5-phenylhydantoin were kindly donated by Dr. David Rutherford (Alfred Hospital, VIC, Australia). Methanol and acetonitrile (HPLC grade) were obtained from Biolab Scientific (Melbourne, Victoria, Australia). Sodium dihydrogen orthophosphate ($NaH_2PO_4.2H_2O$) and di-sodium hydrogen orthophosphate dodecahydrate ($Na_2HPO_4.12H_2O$) were analytical grade and purchased from Ajax Laboratory Chemicals (Sydney, NSW, Australia).

2.2. Standard solutions

A standard stock solution of LTG (10 mg/ml) was prepared by dissolving 100 mg of pure substance in 10 ml methanol in a 10 ml volumetric flask and stored at 4°C. The six working standard solutions (200, 150, 100, 50, 25, 10 μg/ml) were produced by appropriately diluting the stock solution with either water or plasma. A range of standards for calibration curves (LTG 20, 15, 10, 5, 2.5, and 1 μg/ml) were obtained by further diluting the working standards, 1 in 10, in either water or plasma.

A stock standard of I.S. was prepared by dissolving the pure compound in methanol to obtain a concentration of 1 mg/ml.

2.3. Instrumentation and chromatographic conditions

The HPLC system was equipped with a Waters model 510 pump (Waters, Millford, MA, USA), a Shimadzu C-R5A Chromatopac Integrator (Kyoto, Japan), a Rheodyne Activon manual injector (California, USA) and a Waters 484 Tunable Absorbance Detector (Waters, Millford, MA, USA) set at 306 nm and AUFS of 0.1. The analytical column was a Zorbax C8 column, 250×4.6 mm I.D., 5 μm particle size (Activon, Thornleigh, NSW, Australia) protected with a 15×3.2-mm Brownlee New Guard cartridge (Alltech Associates, Baulkham Hills, NSW, Australia) of the same material. The chromatographic separation was carried out at room temperature and at a solvent flow-rate of 1.6 ml/min.

The mobile phase was prepared by mixing 10 ml of a 0.5 *M* phosphate buffer (pH 6.5), 790 ml of water and 200 ml of acetonitrile. Before use the mobile phase was degassed by using a Millipore vacuum filter system equipped with a 0.45μm filter.

The 0.5 *M* phosphate buffer was prepared by dissolving 23.4 g $NaH_2PO_4.2H_2O$ and 35.8 g $Na_2HPO_4.12H_2O$ in 500ml of water.

2.4. Sample preparation

Blood samples were collected in heparinized tubes (patient samples were collected 6 hrs after the morning dose of lamotrigine) and centrifuged at 2200 g for 10 min. The plasma was then removed and stored at 4°C until analysis (within 1 week). To 200 μl of plasma were added internal standard solution (20 μl) and acetonitrile (400 μl) and the mixture was vortex mixed for 5 min followed by centrifugation in a Beckman microfuge 12 at maximum speed for 5 min. 25 μl of the supernatant was injected into the HPLC system.

2.5. Validation

The linearity was tested by calibration curves ranging from 1 μg/ml to 20 μg/ml. Calibration data were plotted as a graph of peak area versus drug concentration and the plot was then subjected to linear regression analysis.

The selectivity of the analytical procedure was determined by analysing plasma samples received for therapeutic drug monitoring from patients who were receiving carbamazapine, phenytoin, vigabatrin, and valproate. Interference by ethosuximide, gabapentine, phenobarbital, carbamazapine-epoxide and 5-(p-hydroxyphenyl)-5-phenylhydantoin was determined by spiking drug free plasma samples with the relevant drug.

The accuracy and precision of the 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 three different concentrations, i.e. 1, 5, 20 μg/ml which were analysed six times on the same day. The between-day reproducibility was determined by analysing three independently spiked plasma pools (1, 5, 20 μg/ml) in duplicate on at least 6 different days.

Quality controls were created by adding an known amount of analyte to plasma and storing in small aliquots (1–2 mls) under the same conditions and for the same duration as the actual samples.

3. Results

3.1. Chromatography and selectivity

Fig. 1 shows 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. The retention times of LTG and I.S were 9.3 and 3.7 min, respectively. The following drugs at the concentrations listed did not give any chromatographic peak when analyzed as unknowns in this procedure: carbamazepine (45 μmol/l), phenytoin (37 μmol/l), vigabatrin, valproate (259 μmol/l), ethosuximide (708 μmol/l), phenobarbitone (172 μmol/l), gabapentine (584 μmol/l), carbamazepine-epoxide (40 μmol/l) and 5-(p-hydroxyphenyl)-5-phenylhydantoin (70 μmol/l). At higher than pharmacological concentrations, ethosuximide (2124 μmol/l) and phenobarbitone(516 μmol/l) gave small peaks at 4.7 min and 11 min respectively. These peaks did not interfere with those of LTG or I.S.

3.2. Sensitivity

Under the experimental conditions used, the detection limit (LOD) was approximately 0.6 μg/ml, at a signal-to-noise ratio of 3:1. The lower limit of quantitation (LOQ) is the lowest amount of analyte which can be measured with defined precision and accuracy. This was found to be 1 μg/ml, with the coefficient of variation $<8\%$.

3.3. Linearity

For linearity, regression analysis gave the following equation: $y=-0.0486+0.0925x$ ($n=3$, $r=0.999$, $r^2=0.998$, standard error of slope$=0.002$).

3.4. Accuracy and precision

As shown in Table 1, the within-day coefficients of variation were below 5% at all concentrations, except at the LOQ . The accuracy of the method, expressed by the bias, varied between −6% and +19% (LOQ). The between-day coefficients of variation (Table 1) were below 7% at all concen-

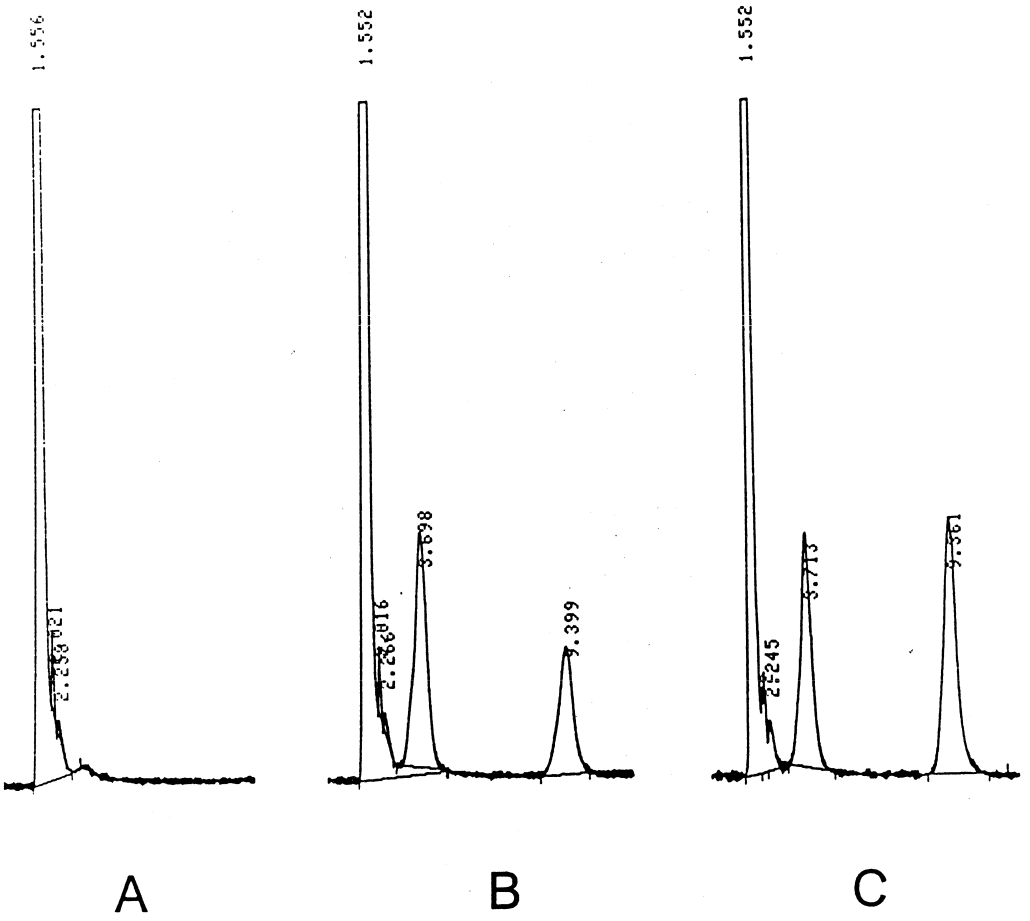

Fig. 1. (A) Chromatograms of supernatant from blank plasma (without I.S.); (B) plasma spiked with 10μg/ml lamotrigine; (C) a plasma sample from a patient receiving lamotrigine. The peak at 3.7 min is the I.S. and that at 9.3 min is lamotrigine.

Table 1
Within and between day precision (C.V.) and accuracy (bias) of the HPLC assay

Theoretical LTG concentration (μg/ml)	Measured LTG concentration (μg/ml) mean±SD	*n*	C.V. (%)	Bias
Within-day				
1	1.19±0.09	6	7.54	+19
5	4.71±0.15	6	3.28	−6
20	20.1±0.34	6	1.68	0
Between-day				
1	1.25±0.08	6	6.1	+25
5	5.07±0.21	6	4.2	+1
20	20.5±0.40	6	1.97	+2.5

trations analysed. The accuracy of the method varied between +1% and +25% (LOQ).

3.5. Recovery

The recovery was determined in triplicate ($n=3$) at four concentrations (1, 5, 10, 20 μg/ml) and was found to be ≥99%.

3.6. Method application

This method is currently in use in our laboratory for monitoring mid-dose lamotrigine levels in epileptic patients receiving therapeutic doses (150–600 mg/day).

Table 2
Patient demographic data

Serial number	Gender	Age (yrs)	Epilepsy type	Duration of disorder (yrs)	Other AED	LTG dose (mg/day)	LTG level (μg/ml)
1	F	45	primary generalised	40	sodium valproate, phenytoin, clonazepam, carbamazepine	400	8.13
2	F	32	Lennox–Gastaut syndrome	16	sodium valproate	600	27.2
3	F	46	primary generalised	40	sodium valproate	150	6.8
4	F	46	Lennox–Gastaut syndrome	30	sodium valproate	250	13.7
5	F	23	primary generalised	11	ethosuximide	300	5.36
6	F	28	complex partial	15	carbamazepine, clobazam, gabapentin	200	3.02
7	F	40	primary generalized	35	–	600	7.95
8	M	27	complex partial	27	carbamazepine, gabapentin	300	4.59
9	F	40	complex partial	28	carbamazepine	300	4.28
10	M	31	primary generalised	29	sodium valproate	400	13.7

Results are presented of the first 10 patients who presented chronologically for utilisation of this method of measurement and in clinical practice. These patients all had well documented diagnosis of epilepsy and their clinical and demographic details are shown in Table 2.

Patients evaluated comprised 8 women and 2 men (mean age 35.8 yrs; range 23–46 yrs). The epilepsy syndromes comprised complex partial epilepsy (3), Lennox–Gastaut syndrome (2), and primary generalised epilepsy (5). The mid-dose plasma concentration of LTG ranged from 4.2 to 27.2 μg/ml and included one patient on monotherapy, four on concomittant valproate, four on concomittant enzyme inducers and one on a combination of sodium valproate and enzyme inducing agents (Table 2).

These results are examples of how useful the present method can be for monitoring mid-dose LTG plasma concentrations, considering the effect of age, dose and coadministration of other antiepileptics on LTG pharmacokinetics.

4. Discussion

Several HPLC methods are available for the analysis of lamotrigine in human plasma. However, most require extensive extraction and separation steps, and complex chromatography conditions. In these assays, evaporation of the organic solvent and reconstitution of the resulting residue [11,13–17] are time consuming, whereas solid-phase extraction is expensive, and the use of ion-pairing reagents [12] often requires elevated column temperatures, longer assay time, and problems with rapid column deterioration.

Procedures based on direct HPLC analysis after sample deproteinization with perchloric acid or acetonitrile [10,18–21] have also been reported. Some of these however also require both addition of excess Na_2CO_3 for the extraction and dilution of the acetonitrile supernatant before injection, which is time-consuming [10], give minimal explanation of the method and validation data [19], while others do not use an internal standard [18]. Ren et al. [20] report on a one-step protein precipitation procedure for sample preparation, however omit to take into consideration the possible interference from other AEDs.

Several other groups have published procedures for the simultaneous measurement of lamotrigine and other anticonvulsants. Ramachandran et al. [21] used dual-wavelength monitoring to measure other AEDs and their metabolites. Lensmeyer et al. [22] reported on an optimized method for the concomitant determination of lamotrigine, phenytoin, carbamazepine and carbamazepine epoxide which used solid-phase extraction followed by HPLC using a stabilized cyanopropyl column.

Our method offers a number of significant advantages. Sample preparation is simple and is based on deproteinization with 2 volumes of acetonitrile fol-

lowed by direct injection of the supernatant. Chromatographic conditions are simple, solvent evaporation and sample reconstitution was avoided, potential interference from other AEDs was considered, the required sensitivity was maintained without concentrating the sample, and recovery was complete (≥99%).

The execution of this procedure at room temperature, the composition of the eluant, and the relatively short analysis time (9 min) allow maximum column life while maintaining good separation. Chromatograms were found to be relatively free of any interference from commonly coadministered anti-epileptic medication and their metabolites. The within- and between-day accuracy and precision of the assay were found to be acceptable, even though the relative recovery (accuracy) of LTG at the LOQ was around 120%. In our experience, recovery of minimally detectable concentrations of pharmaceutical substances appears to be less consistent than recovery of more significant amounts under identical chromatographic conditions.

In conclusion, our method was found to be highly satisfactory for determining mid-dose LTG concentrations in patients receiving therapeutic doses of the drug. This method will be utilised clinically to monitor mid-dose plasma LTG levels which may be useful in guiding dosage, determining compliance, confirming toxicity, confirming drug interactions and guiding withdrawal of other AED in the management of epileptic patients.

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References

[1] C.C. Kuo, L. Lu, Br. J. Pharmacol. 121 (1997) 1231.
[2] D.A. Coulter, Journal of Child Neurology 12 (1997) S2.
[3] P.C. Waldmeier, P.A. Baumann, P. Wicki, J.J. Feldtrauer, C. Stierlin, M. Schmutz, Neurology 45 (1995) 1907.
[4] J.P. Leach, M.J. Brodie, in: R.H. Levy, R.H. Mattson (Eds.), Antiepileptic Drugs, Raven Press, New York, 1995, p. 889.
[5] A.W. Yuen, G. Land, B.C. Weatherley, A.W. Peck, Br. J. Clin. Pharmacol. 33 (1992) 511.
[6] S. George, A.J. Wood, R.A. Braithwaite, Ann. Clin. Biochem. 32 (1995) 584.
[7] P.M. Edelbroek, J.P. Segers, K.G.P.M. Gilissen et al., Epilepsia 37 (suppl. 4) (1996) 64.
[8] P.N. Patsalos, in: Merritt–Putnam Symposium Proceedings, Adis International, Sydney, 1998, p. 14.
[9] M.J. Brodie, Lancet 339 (1992) 1397.
[10] M. Cociglio, R. Alric, O. Bouvier, J. Chromatogr. 572 (1991) 269.
[11] A. Fazio, C. Artesi, M. Russo, R. Trio, G. Oteri, F. Pisani, Ther. Drug Monit. 14 (1992) 509.
[12] M.W. Sinz, R.P. Remmel, J. Chromatogr. 571 (1991) 217.
[13] J. Posner, A.F. Cohen, G. Land, C. Winton, A.W. Peck, Br. J. Clin. Pharmacol. 28 (1989) 117.
[14] A.D. Fraser, W. MacNeil, A.F. Isner, P.R. Camfield, Ther. Drug Monit. 17 (1995) 174.
[15] A.F. Cohen, G.S. Land, D.D. Breimer, W.C. Yuen, C. Winton, A.W. Peck, Clin. Pharmacol. Ther. 42 (1987) 535.
[16] B.C. Sallustio, G.M. Morris, Ther. Drug Monit. 19 (1997) 688.
[17] A.P. Hart, S. Mazarr–Proo, W. Blackwell, A. Dasgupta, Ther. Drug Monit. 19 (1997) 431.
[18] J. Boutagy, M. Dell'Anna, Ther. Drug Monit. 17 (1995) 410.
[19] J.O. Svensson, Ther. Drug Monit. 17 (1995) 408.
[20] S. Ren, M.L. Scheuer, W. Zheng, Ther. Drug Monit. 20 (1998) 209.
[21] S. Ramachandran, S. Underhill, S.R. Jones, Ther. Drug Monit. 16 (1994) 75.
[22] G.L. Lensmeyer, B.E. Gidal, D.A. Wiebe, Ther. Drug Monit. 19 (1997) 292.