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Lamotrigine analysis in plasma by gas chromatography–mass spectrometry after conversion to a *tert.*-butyldimethylsilyl derivative

Amitava Dasgupta*, Amy P. Hart

Department of Pathology, University of New Mexico Health Sciences Center, 2211 Lomas Blvd N.E., Albuquerque, NM 87106, USA

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Abstract

Lamotrigine (lamictal) is a new anticonvulsant drug recently approved by the FDA for clinical use. Therapeutic monitoring of lamotrigine is useful for patient management (therapeutic range 1–4 μg/ml). Here we describe a gas chromatography–mass spectrometric identification and quantitation of lamotrigine after extraction from human serum and derivatization. Lamotrigine was extracted from alkaline serum with chloroform and derivatized with *N*-methyl-*N*-(*tert.*-butyldimethysilyl) trifluoroacetamide containing 2% *tert.*-butyldimethylchlorosilane. Oxazepam-d_5 was used as an internal standard. The *tert.*-butyldimethylsilyl derivative of lamotrigine showed distinct molecular ions at *m/z* 483 and 485 as well as other peaks at *m/z* 426, 370 and 334 for unambiguous identification. The base peak was observed at *m/z* 199. Similarly, the *tert.*-butyldimethysilyl derivative of oxazepam-d_5 showed molecular ions at *m/z* 519 and 521 along with other characteristic peaks at *m/z* 462, 376 and 318. For the analysis of lamotrigine, the mass spectrometer was operated in the selective ion monitoring mode. The within-run and between-run precisions were 4.3% (mean=3.01, S.D.=0.13 μg/ml) and 5.1% (mean=2.93, S.D.=0.15 μg/ml), respectively at a serum lamotrigine concentration of 3.0 μg/ml. The within-run and between-run precisions were 8.2% (mean=0.49, S.D.=0.04 μg/ml) and 10.6% (mean=0.47, S.D.=0.05 μg/ml), respectively at a serum lamotrigine concentration of 0.5 μg/ml. The assay was linear for serum lamotrigine concentrations of 0.5–20 μg/ml. The detection limit was 0.25 μg/ml. The assay was free from interferences from common tricyclic antidepressants, benzodiazepines, other common anticonvulsants, salicylate and acetaminophen.

Keywords: Lamotrigine

1. Introduction

Lamotrigine (lamictal) is a new anticonvulsant drug approved by the FDA. The drug belongs to the phenyltriazine class and is structurally unrelated to any currently used anticonvulsants such as phenytoin, carbamazepine, valproic acid and phenobarbital. The chemical name of lamotrigine is 6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine with a molecular mass of 256.09. Lamotrigine is recommended as adjunctive therapy in the treatment of partial seizure in adults. The pharmacokinetics of lamotrigine has been extensively investigated in adults [1–3]. The average elimination half life was 14.4 h in single dose and 12.6 h with multiple dosing. The clearance of lamotrigine is affected by the co-administration of other antiepileptic drugs. The major

*Corresponding author.

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metabolite is an inactive 2-*N*-glucuronide conjugate. The drug is only 55% bound to serum protein and the protein binding is not affected by other anticonvulsants.

Although a therapeutic serum concentration of lamotrigine has not been established, a tentative therapeutic range of 1–4 μg/ml has been proposed based on comparison with phenytoin in animal studies [4]. However, another investigator demonstrated that individual patients may tolerate a higher dose [5]. In case of a lamotrigine overdose, the plasma concentration was 52 μg/ml. The patient was comatose and remained comatose for 12 h. Therefore, therapeutic monitoring of lamotrigine is beneficial for patient management.

Lamotrigine has been assayed in biological fluids by high-performance liquid chromatography (HPLC) and immunoassays. Cohen et al. [6] were the first to report an HPLC assay for lamotrigine using ethyl acetate as an extraction solvent and normal-phase chromatography using a silica gel column. Other investigators have reported solid-phase extraction or liquid-liquid extraction and reversed-phase HPLC for the analysis of lamotrigine [7–12]. Fraser et al. described an isocratic HPLC protocol for the analysis of lamotrigine after liquid–liquid extraction from serum [13].

Although several HPLC assays for lamotrigine have been described, gas chromatography–mass spectrometric (GC–MS) analysis of lamotrigine after conversion to *tert.*-butyldimethylsilyl derivative has not been described in the literature. One advantage of GC–MS analysis is the unambiguous identification of lamotrigine based on the mass spectral fragmentation pattern.

Most investigators used BW725C78 as an internal standard which is only available from Burroughs Wellcome company as a gift on request. Recently, medazepam was used as an internal standard for HPLC analysis of lamotrigine [14]. We used oxazepam-d_5 as an internal standard because the compound has two functional groups suitable for double derivatization like lamotrigine. Since oxazepam is clinically used and also is a metabolite of diazepam, we used oxazepam-d_5 as an internal standard and used selective ion monitoring for our GC–MS assay.

2. Experimental

Lamotrigine was obtained from Burroughs Wellcome company (Research Triangle Park, NC, USA). The internal standard, oxazepam-d_5 was purchased from Radian Laboratory (Austin, TX, USA). The derivatizing agents *N*-methyl-*N*-(*tert.*-butyldimethylsilyl) trifluoroacetamide and *tert.*-butyldimethyl chlorosilane were procured from Pierce (Rockford, IL, USA).

Stock solutions of lamotrigine and oxazepam-d_5 were prepared in methanol. Pooled human plasma was obtained from a local blood bank. We supplemented plasma (tested negative for the presence of lamotrigine) with various concentrations of lamotrigine. We added 50 μl of internal standard stock solution to 0.5 ml of plasma (placed in a 15-ml screw capped glass tube) to achieve a concentration of 5.0 μg/ml of the internal standard. Then 4 ml borate buffer was added to plasma in order to make it alkaline. We extracted lamotrigine along with the internal standard from plasma using 10 ml of chloroform. The extraction of lamotrigine along with the internal standard was achieved by mixing the aqueous layer with chloroform in a Multipurpose Rotator, Model 151 (Scientific Industries, Bohemia, NY, USA) for 10 min. The organic phase (bottom layer) was separated from the aqueous layer by centrifugation at 1500 *g* for 5 min using a Safety Head Centrifuge (Clay Adam, a Division of Becton-Dickinson, Parsippany, NY, USA). After discarding the aqueous phase, the organic phase was transferred to another 15-ml screw capped conical glass tube. The chloroform extract was evaporated to dryness under nitrogen. We added 50 μl of *N*-methyl-*N*-(*tert.*-butyldimethylsilyl) trifluoroacetamide containing approximately 2% *tert.*-butyldimethylchlorosilane to the dried extract. The reaction mixture was incubated at 85°C for 30 min, and further concentrated under nitrogen to approximately 25 μl while in the same screw capped conical tube. We injected 2 μl into the GC–MS system. For the recovery study only, the reaction mixture after incubation was not further concentrated.

The GC–MS analysis was performed by a Model 5890 series II gas chromatograph coupled to a 5972 mass selective detector (Hewlett-Packard, Palo Alto,

CA, USA). An Ultra 1 column (25 m×0.2 mm I.D.) also obtained from Hewlett-Packard was used in the gas chromatograph. The inside of the column was coated with methylsilicone with a film thickness of 0.33 μm. The initial oven temperature of the gas chromatograph was 200°C. The oven temperature was increased at a rate of 15°C/min to reach an oven temperature of 300°C. Then the oven temperature was increased at a rate of 25°C/min to reach a final oven temperature of 310°C. The final temperature was maintained for an additional 8 min with a total run time of 15.07 min. Both derivatized internal standard and lamotrigine eluted from the column minutes after the column temperature reached 310°C. Therefore, rapid heating of the column from 300°C to 310°C did not change the retention times of either derivatized internal standard or derivatized lamotrigine. The carrier gas was helium. The column head pressure of helium gas was 69 kPa with a linear velocity of 31.5 cm/s. The injector port temperature was 275°C and the solvent delay was 8 min. We used splitless injection for our GC–MS analysis. The mass spectrometer was operated in selective ion monitoring mode using electron ionization. The ions being monitored were *m/z* 519, 483, 468, 462, 426, 199 and 172. The ionization energy was 70 eV.

The high-performance thin layer chromatographic analysis was carried out using HPTLC silica gel plates (10 cm×10 cm) obtained from EM separation (Gibbstown, NJ, USA). The mobile phase composition was ethyl acetate–methanol–ammonium hydroxide (1:1:0.5, v.v). The bands were visualized by spraying first with 2% mercuric sulfate containing 10% sulfuric acid followed by 1% diphenyl carbazone in ethanol. Then in order to visualize the lamotrigine peak further, we sprayed with 4% copper sulfate solution containing 30% concentrated phosphoric acid. The bands were intensified after heating the HPTLC plate.

3. Results and discussion

3.1. Reaction conditions for derivatization

The time course of reaction was followed using high-performance thin layer chromatography. The reaction was complete in 30 min because we did not observe any spot corresponding to the lamotrigine standard. Similarly, we observed no spot for the unreacted internal standard when the reaction mixture was incubated for 30 min at 85°C. A shorter incubation time showed the presence of unreacted lamotrigine along with a minor spot which may be the mono derivative. Although the reaction appeared to be quantitative in 30 min based on HPTLC analysis, two to three minor peaks were observed (less than 5% area compared to derivatized internal standard and lamotrigine) in the total ion chromatogram. Therefore, the presence of trace amounts of mono *tert.*-butyldimethylsilyl derivatives of lamotrigine and internal standard in the chromatogram is possible.

3.2. Mass spectral characteristics of derivatized lamotrigine and oxazepam-d_5

We observed a baseline separation between derivatized lamotrigine and the internal standard. The total ion chromatogram obtained by using selective ion monitoring from a patient receiving lamotrigine is given in Fig. 1. Because both derivatized lamotrigine and the internal standard elute at a high temperature, our analysis is free from interferences from volatile components of the plasma matrix.

The *tert.*-butyldimethylsilyl derivative of lamotrigine (with both amino group derivatized) showed molecular ions at *m/z* 483 (relative abundance: 4.6%) and *m/z* 485 (relative abundance: 3.4%) because organic compounds containing chlorine display a group of molecular ions according to their natural isotopic abundance of the two chlorine isotopes with masses 35 and 37. Another peak at *m/z* 468 (relative abundance: 4.9%) was observed, probably due to loss of a methyl group from the *tert.*-butyldimethylsilyl moiety. A very strong peak at *m/z* 426 (relative abundance: 93.3%) was formed due to loss of the tertiary butyl group from *tert.*-butyldimethyl silyl moiety. The presence of such a peak is characteristic of a *tert.*-butyldimethylsilyl derivative (Fig. 2).

The *tert.*-butyldimethylsilyl derivative of oxazepam-d_5 showed molecular ions at *m/z* 519 (relative abundance: 8.1%) and *m/z* 521 (relative

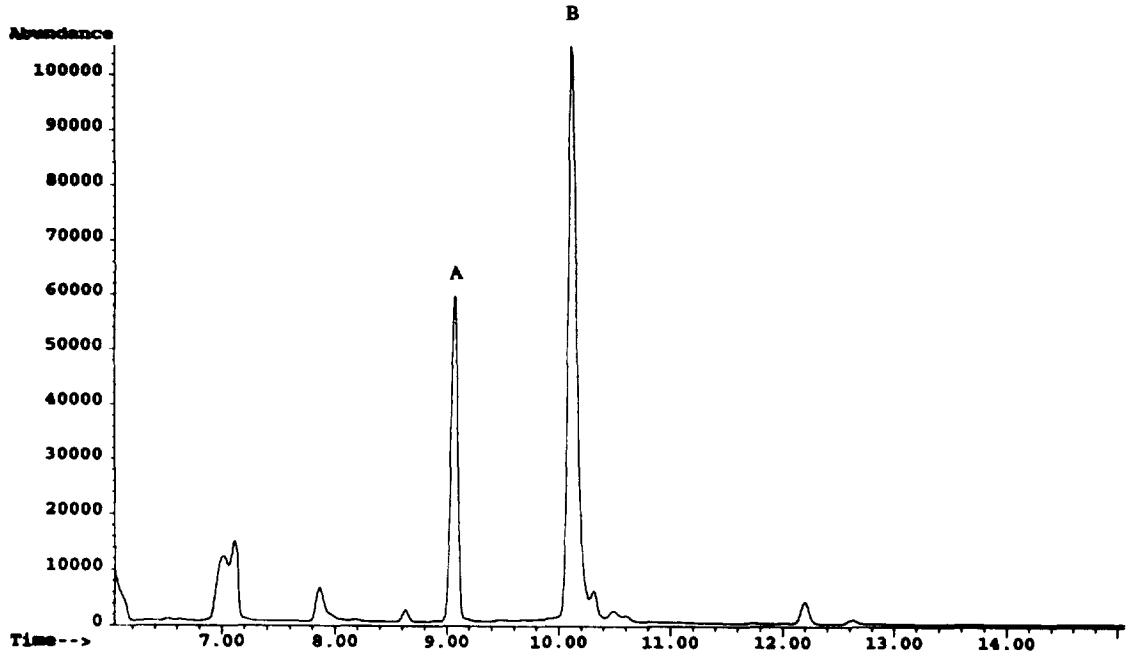

Fig. 1. Total ion chromatogram showing separation between oxazepam-d_5, the internal standard (A) and lamotrigine (B) after derivatization. The patient was receiving lamotrigine. The concentration of lamotrigine was reported as 9.1 μg/ml by the reference laboratory. The concentration of lamotrigine measured by the GC–MS assay was 10.1 μg/ml. The concentration of the internal standard in the GC–MS assay was 5.0 μg/ml.

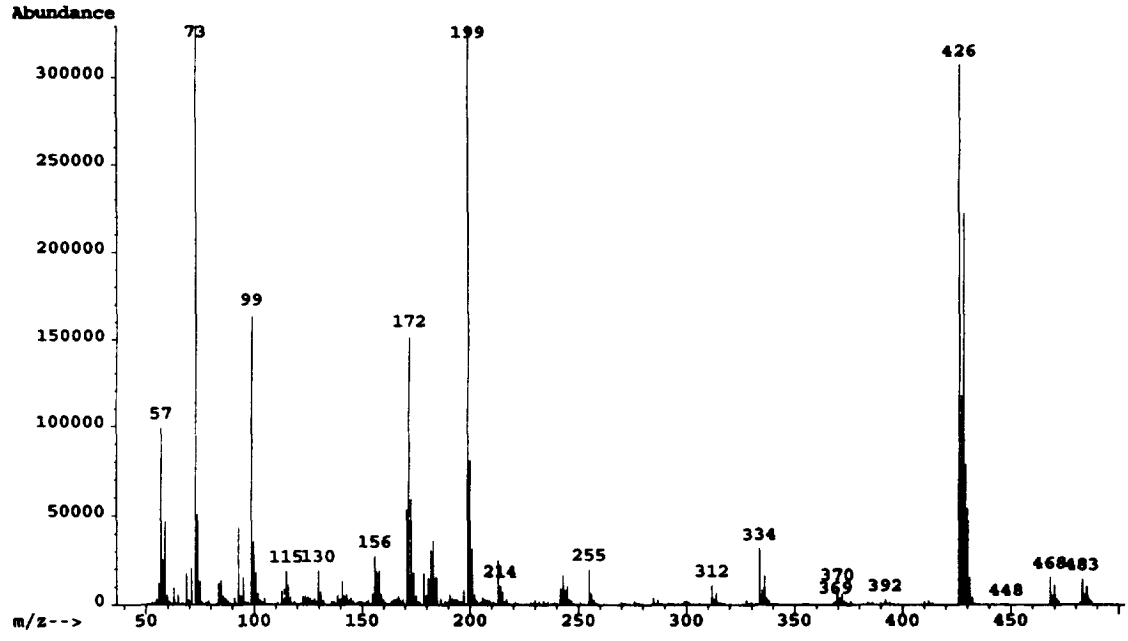

Fig. 2. Electron ionization mass spectral fragmentation pattern of *tert.*-butyldimethylsilyl derivative of lamotrigine.

abundance: 3.0%) because oxazepam-d_5 also contains one chlorine atom. A characteristic strong peak was observed at m/z 462 (relative abundance: 45.4%) due to the loss of the *tert.*-butyl group from the *tert.*-butyldimethylsilyl moiety as expected. The base peak was observed at m/z 73 (Fig. 3). Therefore, we selected ions at m/z 519, 483, 468, 462, 426, 199 and 172 for monitoring elution of peaks in our mass spectrometric analysis.

3.3. Precision, linearity detection limit and recovery

The precisions of the assay were determined using two plasma standards containing 3.0 μg/ml and 0.5 μg/ml of lamotrigine, respectively. The 3.0 μg/ml standard represents the approximate mid point of the therapeutic concentration while the 0.5 μg/ml standard represents the lower end of linearity and also the sub therapeutic concentration of lamotrigine. The C.V. for the within-run precision using 3.0 μg/ml standard was 4.3% (mean=3.01, S.D.=0.13 μg/ml, n=6), while the C.V. for the between-run precision was 5.1% (mean=2.93, S.D.=0.15 μg/ml, n=7). The within-run precision was 8.2% (mean=0.49, S.D.=0.04 μg/ml, n=6) using the 0.5 μg/ml standard. The corresponding between-run precision was 10.6% (mean=0.47, S.D.=0.05 μg/ml, n=5). The linearity of the assay was determined using serum standards containing 0.5, 1.0, 2.5, 5.0, 10.0, 20.0 and 40.0 μg/ml of lamotrigine. The assay was linear for a serum lamotrigine concentration of 0.5–20 μg/ml. Using the x-axis as the target concentration and the y-axis as the observed concentration, we obtained the following regression equation:

$$y = 1.08x + 0.83 \quad (r = 0.99, Sy/x: 0.46)$$

The detection limit was 0.25 μg/ml of serum lamotrigine concentration. The detection limit was determined by observing a distinct peak which can be separated from the baseline by visual inspection as well as by observing the characteristic fragmentation pattern.

The average recoveries of lamotrigine at a concentration of 3.0 μg/ml and 0.5 μg/ml were 85.0% (S.D.=2.3, duplicate measurement) and 81.2% (S.D.=2.0, duplicate measurement), respectively. The recovery of the internal standard was 81.4% (S.D.=1.9, duplicate measurement).

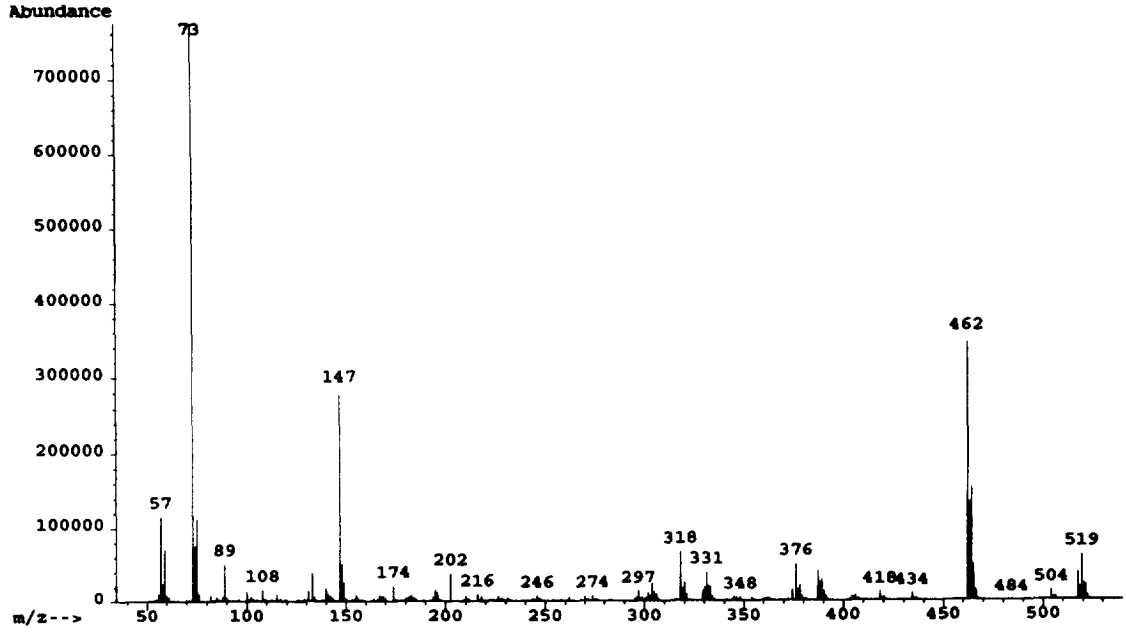

Fig. 3. Electron ionization mass spectral fragmentation pattern of *tert.*-butyldimethylsilyl derivative of oxazepam-d_5.

3.4. Interference study

Because lamotrigine is often used as an adjunctive therapy in the treatment of partial seizure in adults along with other anticonvulsants, we extensively studied the possibility of interferences in our assay from commonly administered drugs. We observed no interference from other anticonvulsants, valproic acid, phenytoin, carbamazepine and phenobarbital in our assay. Commonly used tricyclic antidepressant, amitriptyline, nortriptyline, doxepine, nordoxepin, imipramine and desipramine also did not cause any interference in our assay. Commonly used barbiturates secobarbital, pentobarbital, butabarbital and commonly used benzodiazepines valium and librium did not cause any interference. Our assay is also free from interference from salicylate and acetaminophen in serum.

3.5. Analysis of sera from patients receiving lamotrigine

In order to establish the validity of our GC–MS assay, we analyzed six serum specimens from patients receiving lamotrigine. The specimens were obtained from ARUP (Associated Regional University Pathologists) reference laboratory located in Salt Lake City, Utah. Each specimen was given a code number to protect patient's identity and the concentration of lamotrigine obtained by the reference laboratory using a HPLC assay. We analyzed these specimens using our GC–MS assay and obtained a good correlation with the HPLC assay for lamotrigine used by the reference laboratory (Table 1).

Table 1
Concentration of lamotrigine in six patients determined by the GC–MS assay and HPLC assay used by ARUP[a] reference laboratory

Patient ID	Lamotrigine concentration (μg/ml)	
	HPLC assay	GC–MS assay
1	2.4	2.3
2	17.0	18.4
3	16.0	14.6
4	4.1	3.9
5	3.2	2.6
6	9.1	10.1

[a] ARUP: Associated Regional University Pathologists, a national reference laboratory located in Salt Lake City, Utah, USA.

Again using x-axis as lamotrigine concentration obtained by the reference laboratory and y-axis as the lamotrigine concentration obtained by our GC–MS analysis, we observed the following regression equation;

$$y = 1.03x - 0.25 \quad (r = 0.99, Sy/x: 1.1)$$

3.6. Application of the new GC–MS assay

The therapeutic monitoring of lamotrigine is recommended for proper patient management especially because the clearance of the drug is affected by the presence of other anticonvulsants. Coadministration of phenytoin or carbamazepine resulted in a higher clearance of lamotrigine [15]. In contrast, valproic acid significantly increases half life of lamotrigine [16]. Although death has not been reported from lamotrigine overdose, an adverse medical outcome has resulted from lamotrigine overdose. The presently used HPLC protocols for monitoring lamotrigine concentration in serum identify the drug based on retention time only. In our new GC–MS assay, positive identification of lamotrigine as well as quantitation can be achieved in one run. Moreover, the oxazepam-d_5, the internal standard we used is commercially available unlike BW725C78, the internal standard used by most investigators for HPLC assay of lamotrigine. Cano et al recently used medazepam as an internal standard to determine the concentration of lamotrigine in serum by HPLC [14]. We used oxazepam-d_5 because the molecule has two functional groups (amine and hydroxyl) which can be derivatized like lamotrigine (two amine groups). On the other hand medazepam has no derivatizable functional group. The unambiguous identification of lamotrigine based on mass spectral fragmentation pattern is essential in investigating any medical–legal situation involving lamotrigine overdose. Our assay uses a simple extraction and derivatization technique that can be easily adopted in a clinical laboratory.

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