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Measurement of serum lamotrigine by high performance liquid chromatography using a phenyltriazine as internal standard

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Lamotrigine, an anti-epileptic drug with a phenyltriazine molecular structure, is commonly measured for therapeutic drug monitoring purposes by high performance liquid chromatography (HPLC) or gas liquid chromatography (GLC). A convenient internal standard is the structurally related phenyltriazine compound BWA725C previously obtainable from the Wellcome Foundation, UK. Irsogladine is also structurally similar to lamotrigine and was therefore tested as a possible replacement for BWA725C.

A GLC procedure with thermionic detection (NPD) has been utilized routinely for lamotrigine in our drug monitoring facility. Irsogladine was unsuitable, however, because the retention times of irsogladine and a co-prescribed drug, carbamazepine, were very similar. An HPLC method utilizing a Prodigy Phenomenex ODS3 column performed well using either of the internal standards. The pH of the mobile phase had a distinct impact on the spectra of lamotrigine and BWA725C. A mobile phase at pH 3, with detection at 225 nm was required to effectively resolve lamotrigine from sulthiame and irsogladine from phenobarbitone. Comparison of the HPLC and the existing GLC method with routine patient specimens (n = 43) gave an equation, $y = 0.9382x + 0.8238$, $R^2 = 0.9862$. Irsogladine was found to be a suitable internal standard for an HPLC analysis of lamotrigine.

Keywords: Lamotrigine; HPLC; Internal standard; Irsogladine

Introduction

Lamotrigine, 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine (Figure 1), is a phenyltriazine anti-epileptic drug used for generalized seizures, refractory partial seizures, and bipolar disorders. Its molecular structure is unrelated to other anti-epileptics. It is licensed for use as monotherapy[1] or co-prescribed with carbamazepine, valproic acid or phenytoin for control of partial seizures in adults and children.

The therapeutic range of lamotrigine is not clearly defined. Earlier levels of 4–16 µmol/L have been discarded because they were associated with suboptimal therapeutic response.[2] Johannessen[3] has employed a range of 10–60 µmol/L. A therapeutic range of 6–39 µmol/L was suggested by Hirsch.[4] This range is currently used in our laboratory.

Plasma levels are not influenced significantly by plasma albumin concentration but clearance is significantly affected by co-prescribed inducers and inhibitors.[5] For example, valproic acid is a well-known enzyme inhibitor which increases the half-life of lamotrigine.[6] The wide inter-patient variability in lamotrigine dosage, required to attain a desired serum concentration, and the variability in clearance caused by concomitant dosing with carbamazepine, valproate, and phenytoin, make therapeutic monitoring of lamotrigine desirable.

At our own laboratory we had previously quantified serum lamotrigine using a gas liquid chromatography (GLC) method based on that described by Watelle *et al.*[7] except that a phenyltriazine, 3,5-diamino-6-(2-methoxyphenyl)-1,2,4-triazine (BWA725C) (Figure 2) is used as the internal standard. This compound is a structural analogue of lamotrigine. Other laboratories have also used this compound with HPLC procedures.[8–12] The continued supply of this compound from The Wellcome Foundation has been in doubt and supplies are now scarce. For this reason we investigated an alternative phenyltriazine compound called irsogladine (2,4-diamino-6-(2,5-dichlorophenyl)-1,3,5-triazine) (Figure 3). Other papers describe the use of prazepam,[7] acetanilide,[13] thiopental,[14] felbamate,[15] 4-methylprimidone,[16] cyheptamide,[17] thipentone,[18] and nortriptyline[19] as an internal standard. None of these compounds has a phenyltriazine molecular structure. This paper presents the use of irsogladine, a structural analogue of lamotrigine, as an internal standard in the HPLC-UV (ultraviolet detector) analysis of serum lamotrigine.

Material and methods

Reagents and stock solutions

Authentic lamotrigine and BWA725C were provided by Wellcome Foundation (London, UK). Irsogladine was provided as a gift from Dr Takashi Kyoi, Research Laboratories, Nippon Shinyaku Co., Ltd (Kyoto, Japan) and also purchased from Richland Chemicals Ltd (Shenzhen, China). Butyl acetate, acetonitrile, phosphoric acid, and methanol, HPLC grade, were purchased from Scharlau or BDH, Global Science and Technology (Auckland, New Zealand).

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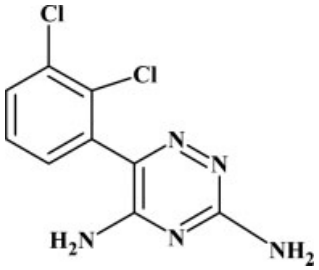

Figure 1. Chemical structure of lamotrigine.

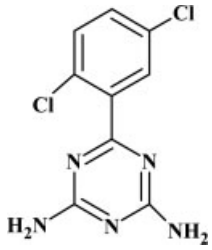

Figure 2. Chemical structure of BWA725C.

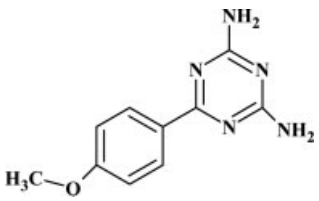

Figure 3. Chemical structure of Irsogladine.

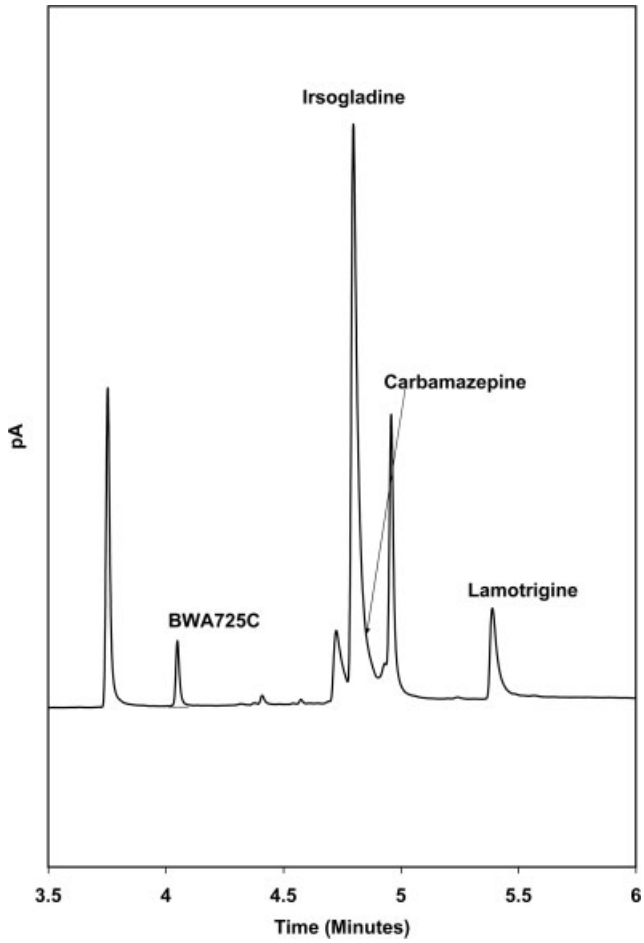

Figure 4. GLC Chromatogram with HP-5 MS (5% phenyl) column.

Stock 5 mmol/L lamotrigine (64 mg/50 ml), 3.9 mmol/L BWA725C (50 mg/50 ml) and 6.7 mmol/L irsogladine (86 mg/50 ml) solutions were prepared in methanol. Five-point calibrators (10–60 µmol/L) were prepared by spiking blank plasma with a 1 mmol/L lamotrigine working solution. The internal standard solution contained 4 mmol/L of irsogladine and BWA725C combined in methanol.

Specimens in the UK-NEQAS (external quality assessment scheme) were obtained from Cardiff Bioanalytical Services Ltd (Cardiff, Wales). Clinchek levels I and II which contained only lamotrigine and sulthiame were obtained from Recipe Instruments and Chemicals (Munich, Germany) and were used as low and high controls. An in-house control containing 6 µmol/L of lamotrigine was also run.

Sample preparation procedure for both HPLC and GLC

Plasma (0.5 ml), the mixed internal standard (20 µL) containing both irsogladine and BWA725C and 0.1 M bicarbonate buffer (0.5 ml, pH 11) were extracted into butyl acetate (0.3 ml) by rotary mixing for 15 min and centrifuged. The supernatant butyl acetate was transferred directly into 0.2 ml inserts placed into 1.5 ml auto sampler vials. These extracts were dried with nitrogen gas to approximately 75 µL prior to GLC analysis. For HPLC analysis, the butyl acetate extract was evaporated to dryness and reconstituted with mobile phase (200 µL).

Instruments

HPLC method was performed using a Dionex Ultimate ISO 3100A pump, Dionex Ultimate WP 3000SL autosampler, and Waters 996 photodiode array detector. The system was controlled by Chromeleon version 6.80 client server. Separations were performed with a Phenomenex Prodigy ODS3 column, 150 mm × 4.6 mm, 5 µm particle size with guard column at 35 °C in a Dionex STH 585 column oven. The mobile phase was phosphate buffer 0.1 M either pH 3.0 or pH 7.0 and acetonitrile (66% : 33% *v*/*v*) at a flow rate 1.0 ml/min.

Results

The routine GLC procedure provided adequate separation of BWA725C from lamotrigine with respective retention times of 4.76 and 6.98 min. Unfortunately, the irsogladine internal standard could not be employed with this routine GLC procedure because of the co-elution with carbamazepine (Figure 4). It was therefore necessary to disestablish this routine procedure if irsogladine was to be used as an internal standard. The use of irsogladine with our GLC system was not assessed further.

Consequently an HPLC method was investigated in detail using both BWA725C and irsogladine as internal standards. Mobile phase conditions were altered to test the separation of lamotrigine from sulthiame, irsogladine, phenobarbitone, and

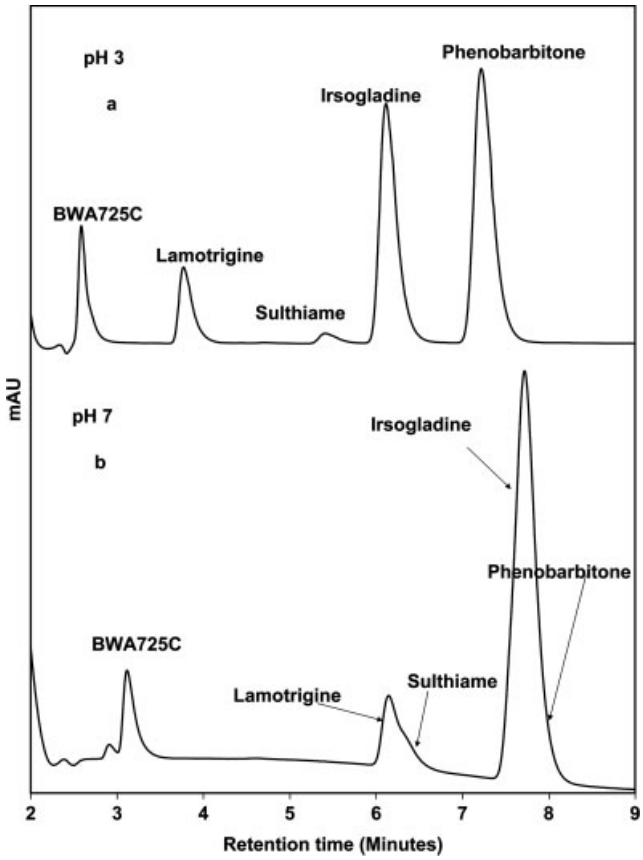

Figure 5. HPLC Chromatograms at pH 7 and 3.

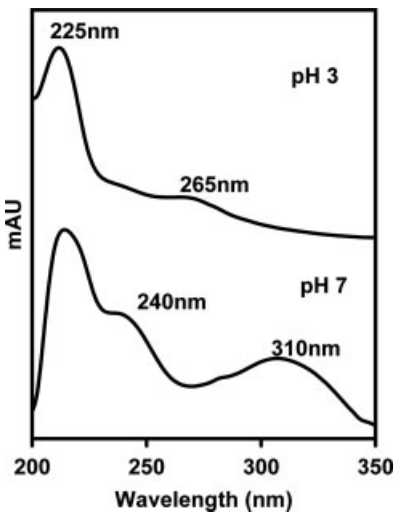

Figure 6. Lamotrigine spectra at pH 3 and pH 7.

BWA725C (Figure 5). Mobile phase pH had a strong influence on the relative retention times and especially the resolution of lamotrigine from sulthiame and phenobarbitone from irsogladine (Figure 5). BWA725C was well resolved and carbamazepine eluted much later and does not appear on the chromatograms. Mobile phase pH also had a profound affect on the spectra of lamotrigine (Figure 6) and BWA725C (Figure 7), but no effect on irsogladine (Figure 8).

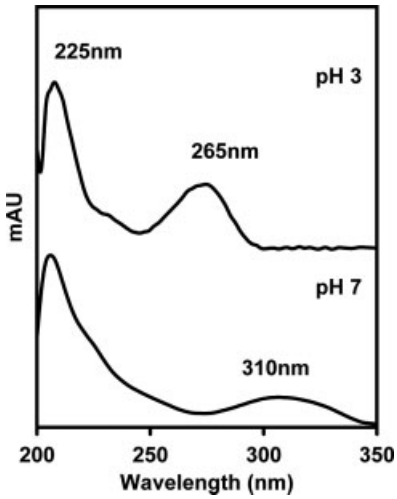

Figure 7. BWA725C spectra at pH 3 and pH 7.

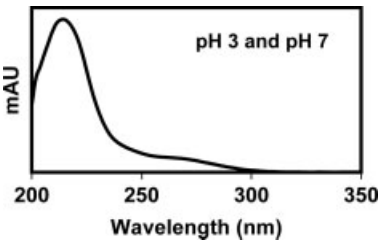

Figure 8. Irsogladine spectra at pH 3 and pH 7.

Table 1. Analytical imprecision for measuring lamotrigine by HPLC (Between batch)

Mean (μmol/L)	n	%CV	Range (μmol/L)
6.6	8	9.6	5.9–7.5
14.9	8	6.1	13.7–16.3
45.8	8	3.8	42.6–49.5

Calibration was performed with each batch for HPLC along with a blank and quality control samples. Calibration curves were linear across the range 5–60 μmol/L with average correlation coefficient of >0.98. Slopes over 8 non-consecutive days varied within ±6.5%.The quality control samples exhibited satisfactory imprecision at the three levels as shown in Table 1 (between day). Within-batch imprecision (n = 6) at three levels (mean values) were 5.4% at 7 μmol/L, 2.3% at 15 μmol/L and 2.1% at 45 μmol/L. The limit of quantitation with S/N ratio ≤3 was 0.2 μmol/L. Injection volumes between 5 μL and 50 μL had no discernible effect on the peak quality.

Absolute recoveries were determined by comparing the peak heights of standards taken through the extraction process with the peak heights of standards dissolved in mobile phase and injected directly. The absolute recoveries for lamotrigine, irsogladine, and BWA725C were found to be 79.1%, 81.0%, and 76.2%, respectively.

Comparative data of HPLC with the mean levels in 12 UK-NEQAS specimens (Figure 9) gave Eqn 1.

$$y = 0.9126x + 0.6885,\ R^2 = 0.966 \quad (1)$$

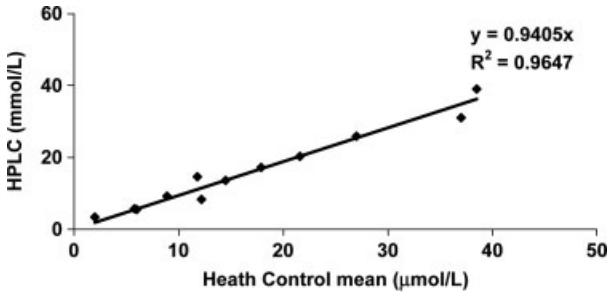

Figure 9. Performance on UK-NEQAS; External Quality Control Programme.

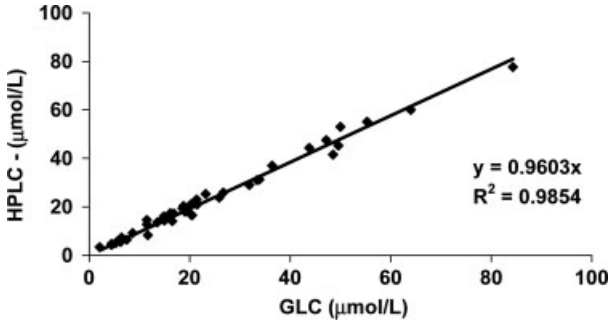

Figure 10. Comparisons of HPLC and GLC methods as regression plot.

Forty-three clinical samples from patients taking lamotrigine were compared by the HPLC-UV method and GLC method for lamotrigine. All the GLC data presented is with BWA725C as the internal standard and HPLC with irsogladine as the internal standard. These results are shown in Figure 10 as regression plot with Eqn 2.

$$y = 0.9382x + 0.8238,\ R^2 = 0.9862 \quad (2)$$

The mean bias for the HPLC samples when compared with GLC samples was -0.5 ± 2.1 µmol/L.

Discussion

With the HPLC method, mobile phase pH is the most important parameter for successful performance. Mobile phase at pH 7 inadequate resolution was observed between sulthiame and lamotrigine and between phenobarbitone and irsogladine (Figure 5b). Conversely, at mobile phase pH 3, all the peaks were well resolved (Figure 5a). Irsogladine is the preferred internal standard because it has a better relative retention time versus lamotrigine and can be used as a replacement for BWA725C.

Mobile phase pH had a substantial effect on the spectra of lamotrigine (Figure 6) and BWA725C (Figure 7). It had minimal effect on irsogladine Spectra (Figure 8). At pH 7, lamotrigine detection is excellent at 310 nm which is a far more selective wavelength. However, mobile phase pH 3 is preferred for better chromatography which requires the chromatograms to be collected at this lower (less selective) wavelength.

The GLC method has been heavily validated over several years of clinical service. The equivalence of the HPLC and GLC lamotrigine data allows for direct transfer of methodology between the two chromatographic systems with no compromise to patient results. The accuracy of both chromatographic systems is rated against the UK-NEQAS medians.

Conclusion

Irsogladine is a good replacement for BWA725C in the therapeutic drug monitoring of lamotrigine by HPLC, in the likely event that BWA725C is not available. The advantage is that it has a similar phenyltriazine structure to lamotrigine. Further it is not commonly used in human therapeutics, and is therefore an ideal internal standard.

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