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Performance analysis of a reversed-phase liquid chromatographic assay of lamotrigine in plasma using solvent-demixing extraction

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ABSTRACT

A reversed-phase column liquid chromatographic assay is described and validated for lamotrigine, a new anticonvulsant drug. The drug and its internal standard were extracted from plasma into acetonitrile according to a previously described solvent-demixing procedure, separated on LiChrospher 100CN, and measured by ultraviolet absorption at 280 nm. The assay performance was evaluated through analysis of variance and of regression with our usual validation design. The method detects *ca.* 2 ng (55 μg/l × 30 μl) and shows a linear response with a constant 5% coefficient of variation from 1 to 10 mg/l. It is easy and robust, and seems well suited to therapeutic drug monitoring.

INTRODUCTION

Lamotrigine is a novel anticonvulsant acting by a phenytoin-like membrane-stabilizing mechanism, namely blockade of voltage-sensitive sodium channels and inhibition of glutamate release [1]. The drug is an aryl-substituted diaminotriazine and as a cationic compound it is unusual among anticonvulsants. It has been successfully used in resistant epilepsies at a dosage of 50–200 mg twice a day, resulting in mean trough plasma levels of 3.0 ± 0.6 mg/l [2]. It showed linear kinetics in long-term oral therapy, the mean apparent clearance ranging from 1 to 1.4 l/kg per day (coefficient of variation 20–40%) after two to four months of treatment in the same group of four patients [2]. The mean plasma half-life was 35.7 ± 9.3 h after a 300-mg dose [3], decreasing to 8–33 h in the presence of carbamazepine or phenytoin and increasing to 30–90 h in the presence of sodium valproate [4].

Thus the metabolic clearance of lamotrigine, which changes during the first two months and is significantly affected by other anticonvulsants, requires monitoring during therapy. The column liquid chromatographic method presented in this paper has been validated for this purpose. The only other available method [5] involves ethyl acetate extraction, drying and normal-phase column liquid chromatography; its validation seems not to have been published.

EXPERIMENTAL

Reagents and solvents

Frozen human plasma (ref. 25258, batch 081) was obtained from the Centre de Transfusion Sanguine (Montpellier, France). Acetic acid, ammonium acetate and sodium carbonate were Rectapur analytical-grade reagents from Prolabo (Paris, France). Acetonitrile (HPLC grade S) was from Rathburn (Walkerburn, UK) and methanol from Carlo Erba (Milan, Italy).

Lamotrigine (batch 23005) and 3,5-diamino-6-(2-methoxyphenyl)-1,2,4-triazine (compound A725C, batch 86713), the latter used as internal standard, were gifts from the Wellcome Foundation (London, UK).

Thirty human plasma samples spiked with ten concentrations ranging from 0 to 14.7 mg/l (55.33 μmol/l) were provided by Cardiff Bioanalytical Services (Cardiff, UK).

Working solutions

The extraction solution consisted of the internal standard (1 mg/l) in acetonitrile, and the concentration of the lamotrigine stock solution was 1.0 g/l in methanol.

Chromatographic apparatus

A Model SP8810 pump and a Model Spectra100 variable-wavelength UV–Vis detector from Spectra-Physics France (Les Ulis, France) were used. The autosampler Model 655A-40 and the D-2000 chromatointegrator were from Merck (Darmstadt, Germany).

The column was a LiChrocart 250 mm × 4 mm I.D. cartridge filled with LiChrospher 100CN, particle size 5 μm, from Merck.

Solvent-demixing extraction [6]

To a 5-ml glass tube, one volume (0.2–1 ml) of sample (water solution or plasma) and one volume of the extraction solution (I.S. in acetonitrile) were added. After brief vortex-mixing, excess Na_2CO_3 was added and the mixture was vortex-mixed vigorously to saturation. After centrifugation (6°C, 1500 g for 10 min), the extraction mixture separated into (from bottom upwards) excess salt, a saturated aqueous layer, a compact disc of plasma proteins and an acetonitrile layer of nearly one volume.

Chromatography

The acetonitrile supernatant was diluted by half with water in the autosampler vial and 30 μl were usually injected. The isocratic mobile phase was acetonitrile–0.01 M ammonium acetate buffer pH 3.5 (55:45, v/v). The flow-rate was 1.5 ml/min, the detection wavelength 280 nm, and the elution was performed at room temperature.

Validation design and calculations (laboratory samples)

The height and area of the lamotrigine peak were measured, then divided by the height or area of the I.S. peak (peak-height ratio and peak-area ratio, respectively). Measurements were taken both from aqueous solutions and from spiked plasma samples at three concentrations overlapping the therapeutic range: 0.25, 1.0 and 4.0 mg/l (0.975, 3.9 and 15.6 μM, respectively).

The factorial design thus involved two factors: concentration (columns, three levels) and extraction matrix (rows, two levels) resulting in six cells. Each sample was extracted twice and each extract was injected twice, according to our usual nested within-cell design [7]. The nested design allows us to estimate variances both of the chromatographic measurements and of the extraction process.

Analysis of variance (Anova) and linear regression were performed with a spreadsheet program (PC-ABACUS, Psion, 1987). All Anova calculations were made from napierian logarithms of peak heights, peak areas and peak ratios, from the guess (to be checked) that measurement error is nearer to constant coefficient of variation than to constant variance, and that consequently logarithmic transformation brings nearer to homoscedasticity. Following transformation, the expected proportionality relationship between measurements m and concentrations C:

$$m = a\,C$$

results in

$$\ln m = \ln a + \ln C$$

an expected straight line of slope 1 which was estimated by unweighted linear regression of $\ln m$ vs. $\ln C$.

Anova was carried out as follows:

(1) Homoscedasticity of the twelve pairs of duplicates was checked first through Bartlett's test [8]. If not significant at the $P = 0.1$ level, analysis was performed further.

(2) Following factorial analysis, F tests were applied to (between-row, one degree of freedom) extraction difference from either matrix and to (between-column, two degrees of freedom) linear regression in both matrices and common non-linearity; the concentration–matrix interaction term (column–row, twice one degree of freedom) was split into non-parallelism and opposite non-linearity of regressions in the two matrices. In the present design limited to fixed factors, the denominator of F tests was taken as the within-cell mean square (eighteen degrees of freedom) [9]. The regression coefficient b ("slope") was compared to the theoretical value with a Student's t-test of $(b - 1)/s_b$, s_b being calculated as usual as the within-cell mean square times the sum of squares of $\ln C$.

(3) the within-cell mean square was split into a within-extract (measurement) variance estimate and a between-extract (extraction + measurement) variance estimate.

Logarithmic standard deviations $s_{\ln}$ directly provide approximations [6] of the corresponding coefficients of variation (C.V.) s/m: $s_{\ln} \approx d \ln m = dm/m \approx s/m$, provided that s/m is small enough, say $\leq 20\%$.

Accuracy check (external samples)

The spiked plasma samples provided by Cardiff Bioanalytical Services were measured blind. Following communication of the target values, unweighted logarithmic regression of obtained *versus* target values was carried out.

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms obtained from (I) a 2 mg/l calibration plasma sample, and (II) a sample from a patient treated with lamotrigine and carbamazepine. The limit of detection [10], defined as five times the baseline noise, was 55 μg/l.

No interference was found with the five major anticonvulsants currently associated with lamotrigine (phenobarbitone, phenytoin, carbamazepine, valproic acid and clonazepam). In fact, only carbamazepine absorbs UV significantly at 280 nm, and its retention time on the cyanopropyl stationary phase is much shorter.

Validation design

The demixing recovery of lamotrigine was directly calculated by comparing
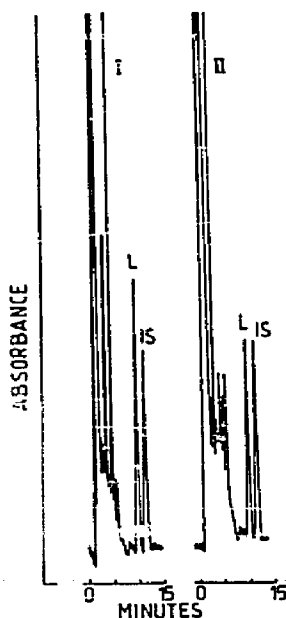

Fig. 1. Chromatograms of (I) a plasma sample spiked with lamotrigine (2.0 mg/l), and (II) a plasma sample from a patient treated with lamotrigine (200 mg per day) and carbamazepine (800 mg per day). Peaks: L = lamotrigine; IS = internal standard. Graph scale: 4 · 10^{-3} a.u.f.s.

mean peak heights with those obtained from the stock solution properly diluted, and it was found to be 97% both from aqueous and from plasma samples. However, lamotrigine peak-height ratios were significantly higher from plasma, due to the 30% lower extraction recovery of I.S. The I.S. provided by the Wellcome Foundation is the 2-methoxyphenyl [5], instead of 2,3-dichlorophenyl, analogue of lamotrigine, and is presumably a much more basic molecule owing to the opposite electronic inductive effects of the respective substituents. It is current bioanalytical knowledge that strongly cationic species have a strong affinity for plasma proteins, even solvent-denaturated. Thus the available I.S. is probably not the best suited to our technique, but this defect is of little consequence owing to the high reproducibility of the demixing recovery.

Fig. 2 is a logarithmic calibration graph from the validation design samples. Table I provides the full analysis of the variance of peak-height ratios. Peak-area ratio measurements showed lower precision (C.V. 4.5% instead of 2.5%), which is theoretically unexpected but currently observed. The peak height is liable to vary more than the peak area if the column performance (retention time and/or efficiency) changes during the assay session, the main factor being temperature. Imperfect design and/or parametrization of integration algorithms expose small errors in the localization of the peak baseline, which are likely to affect areas more
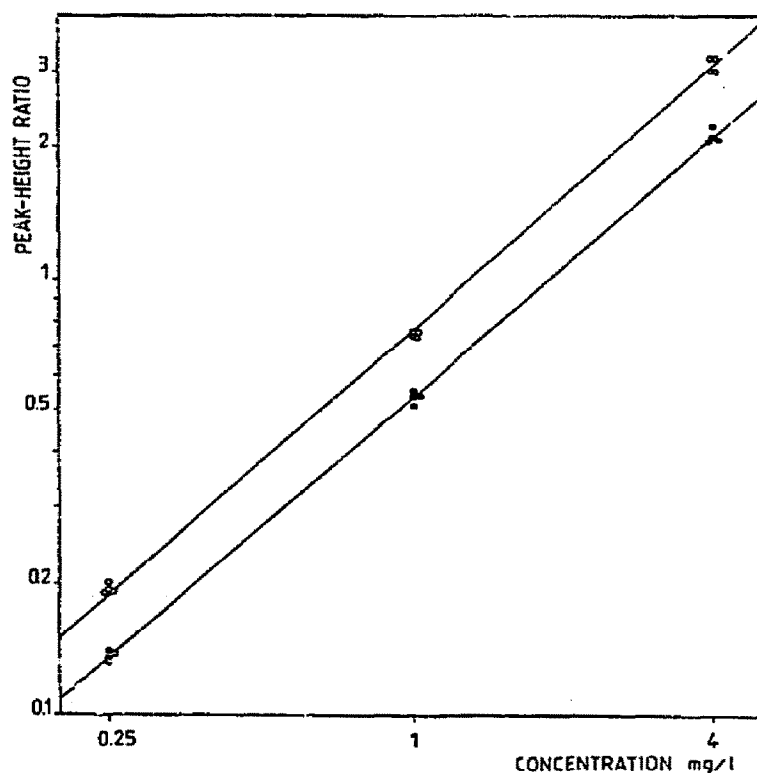

Fig. 2. Logarithmic calibration graph: horizontal axis, lamotrigine nominal concentrations (mg/l); vertical axis, lamotrigine/internal standard peak-height ratios. (●) Aqueous extracts; (○) spiked plasma extracts.

TABLE I

ANALYSIS OF VARIANCE OF THE VALIDATION DESIGN USING NAPIERIAN LOGA-
RITHMS OF PEAK-HEIGHT RATIOS

$\sqrt{}$ MS — square roots of mean squares; P = significance levels of one-sided F comparisons of mean squares
to the "within-cell" MS (bold type figures); HS = highly significant; NS = non-significant at the P = 0.1
level.

Anova component	Degrees of freedom	Sums of squares	Mean squares	$\sqrt{}$ MS (difference)	P
Total	23	31.2879692			
Within cell	(18)	0.01451705	**0.0008065**		
Between-measurement	12	0.00770159	0.00064180	0.0253	
Between-extract	6	0.00681546	0.00113591	0.0337	$0.1 < P < 0.25$
Between-cell	(5)	31.2734521			
Between-matrix	1		0.75998074	(35.6%)[a]	HS
Between-concentration	(2)	30.51176642			
Linear regression	1		30.5076549		HS
Common curvature	1		0.00411152		$0.025 < P < 0.05$
Interaction	(2)	0.00170493			
Opposed curvature	1		0.00097930		NS
Non-parallelism	1		0.00072563		NS

[a] Percentage mean difference between peak-height ratio measurements in plasma relative to measurements
in aqueous solutions.

than heights, in a more random way. Presumably the second cause of error often
exceeds the first.

Bartlett's test of the twelve pairs of logarithmic values was not significant
(χ^2 = 12.05, $0.25 < P < 0.5$), which justifies the logarithmic transformation. Be-
tween-measurement and between-extract variance estimates did not differ
($F[6/12]$ 1.77, $0.1 < P < 0.25$), which underlines once again the high reproducibil-
ity [11] of the solvent-demixing extraction technique. The corresponding C.V.
were 2.5 and 3.4%, respectively. This leads to an estimate of the C.V. of single
measurements on single extracts as 3.4% within the present range of concentra-
tions, and a prediction of the between-session C.V. of assay results as 3.4–5.0%,
depending on the number of calibration points in each session.

The logarithmic regression slope was 0.996, not different from the theoretical
unity value (t = 0.42, $0.6 < P < 0.7$): the response relationship of the assay is thus
not different from $m = aC^1$, which means arithmic linearity with no intercept.

The overall logarithmic non-linearity had to be considered significant accord-
ing to our rule: $F[1/18]$ = 5.1, $0.05 < P < <0.10$. Logarithmic non-linearity, to-
gether with unit slope, would occur in some special cases of both arithmetic
non-linearity and intercept: inspection of Figs. 2 and 3 suggests that the imperfec-
tion is of little practical consequence: moreover, multiple comparisons do not

conserve the probability level of individual tests and favour the occurrence of significant deviations by chance.

External accuracy check

Fig. 3 is the logarithmic correlation graph from Cardiff Bioanalytical's spiked samples, including a plot of logarithmic residuals taken as estimates of residual C.V.

Linearity holds up to 55 μM (ca. 15 mg/l). Residuals were evenly distributed, and the residual C.V. calculated as the square root of the residual mean square of logarithmic regression was 5.3%, a little larger than the predicted 3.5%. The sole anomaly was a consistent − 10% bias in relation to target values. This can hardly be imputed to intrinsic factors of the method. Provided that parameters such as extraction pH (fixed by water saturation by sodium carbonate) or the ratio of plasma to solvent volumes are kept constant, as they were, uncontrolled factors such as variations of I.S. recovery among sera should result in random imprecision, which was not observed among the set of 24 reference samples. The systematic bias is most probably due to discrepancy between our calibrators and those
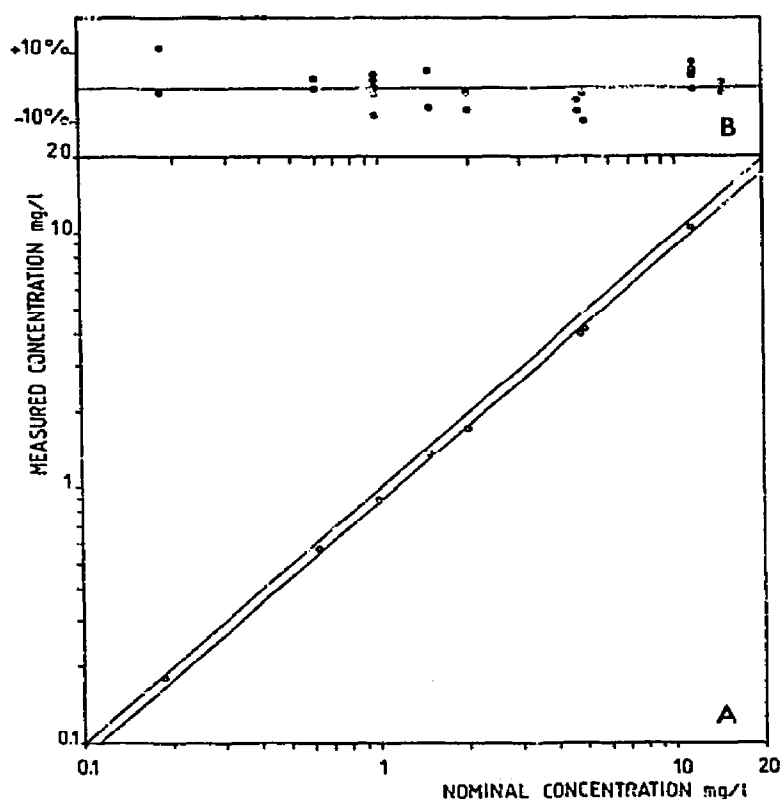

Fig. 3. (A) Bilogarithmic correlation graph of measurements (vertical axis) versus nominal concentrations (horizontal axis), both in mg/l. The lower line is the calculated regression line, the upper one is the identity line. (B) Diagram of regression residuals at the same horizontal scale, expressed as percentage deviations.

of the reference laboratory, presumably because of ours. The accuracy of calibrators can be ascertained only by the agreement of several independent standards and is often beyond the capacity of the commonplace bioanalytical laboratory, which makes external quality control essential.

CONCLUSION

Therapeutic drug monitoring of lamotrigine seems desirable, since its mechanism of action suggests a concentration-related activity, and most associated drugs have been shown to modify its kinetics. The present reversed-phase method is technically simpler than the published normal-phase method [5], and it shows comparable sensitivity and acceptable precision. Solvent-demixing extraction is easy and has been repeatedly shown to be highly reproducible. The method thus seems suitable for routine therapeutic monitoring.

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