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Short communication

Simultaneous determination of lamotrigine and its glucuronide and methylated metabolites in human plasma by automated sequential trace enrichment of dialysates and gradient high-performance liquid chromatography

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Abstract

The use of the system, automated sequential trace enrichment of dialysates (ASTED), to prepare plasma samples for the estimation of lamotrigine, its glucuronide and methylated metabolites in plasma prior to gradient high-performance liquid chromatography (HPLC) is described. Using this technique the procedure was observed to be specific for all three compounds and linear over the range 0.04 to 10 μg/ml for lamotrigine and the glucuronide metabolite and 2 to 500 ng/ml for the methylated metabolite. The within-run precision (C.V.) at four different supplemented plasma lamotrigine concentrations of 0.04, 0.10, 2.5 and 10.0 μg/ml was 6.21, 5.17, 1.29 and 0.73%, respectively, and the between-run precision (C.V.) estimated to be 13.49, 6.08, 1.95 and 1.78%, respectively. The overall accuracy (% bias) of the procedure was estimated to be 12.50, 0.00, 2.80 and 1.80%, respectively. The glucuronide and methylated metabolites in plasma showed similar assay performance. © 1997 Elsevier Science B.V.

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1. Introduction

The antiepileptic drug lamotrigine [3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine] is metabolised and eliminated mainly by glucuronidation [1,2]. Another minor N^2-methylated metabolite has been found to have cardioactive properties. It is desirable, particularly during pharmacokinetic studies, that lamotrigine (430C78), its major glucuronide metabolite (166C89) and its cardioactive methylated metabolite (583C80) are estimated simultaneously.

Previous assays for the estimation of lamotrigine (430C78) and these two metabolites have used three different methods including liquid–liquid extraction [3], solid-phase extraction (SPE) [4] and radio immunoassay (RIA) [5]. In order to obtain the necessary pharmacokinetic and safety data the use of such a plethora of assays is time consuming and expensive with reduced sample throughput. However the simultaneous estimation of all three compounds

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is difficult since the polarities of the compounds differ greatly. Furthermore the analytical ranges of plasma lamotrigine and lamotrigine glucuronide differ greatly from the methylated lamotrigine metabolite.

Automated sequential trace enrichment of dialysates (ASTED) configurations with improved sample preparation capabilities have previously been described in earlier publications [6,7]. This paper reports the development of a further extension to the ASTED system for the determination of 430C78 and its metabolites, 166C89 and 583C80 in human plasma. This procedure utilises automatic tandem injection of enriched sample dialysates, gradient elution and wavelength switching to overcome the difficulties of differing molecular polarities and analytical calibration ranges.

2. Experimental

2.1. Instrumentation

Unless otherwise stated the HPLC and ASTED units were obtained from Anachem (Luton, UK).

2.1.1. HPLC

The gradient HPLC system consisted of Models 306 and 307/5SC pumps, a Gilson 118 UV detector and a Rheodyne 7010 injection valve fitted on the ASTED unit. Control of the HPLC system, integration of chromatographic peaks and communication with the ASTED system (via Gilson Medical Electronics GSIOC) was made using a 715, V1.2, system controller (IBM PS1 with hard disc, EGA graphic card, mouse, MS DOS and Windows software V3.11).

2.1.2. Sample preparation (ASTED) unit

The software and hardware modifications to an ASTED unit to enhance its capabilities (e.g., "heart-cutting") have been described previously [6]. The modified ASTED unit (Fig. 1) comprised a 231 auto-sampling injector; two 401 dilutors fitted with 1 ml syringes (controlling sample pre-treatment on the donor side and dialysate flow on the recipient side of the dialyser); a universal valve switching module to isolate the dialyser unit from the trace enrichment device to enable "heart-cutting" routines; a Kel F dialyser unit with a 370 μl donor volume fitted with a 15 kD Cuprophan membrane, and a stainless-steel trace enrichment cartridge (Prelute, Anachem) packed with 70 mg of 10 μm Hypersil ODS (Shandon Southern Products, Runcorn, UK) to replace the loop on the Rheodyne 7010 injection valve. Control of the ASTED operations was made using Process 4 V1.20 software (Clinical Innovations, Kenilworth, UK).

2.2. Reagents

2.2.1. General reagents

Unless otherwise stated, all chemicals were analytical grade obtained from Sigma (Poole, UK). HPLC grade water, prepared using a Purite (Thame, UK) system, was used for all reagent preparations. HPLC solvents were obtained from Romil (Cambridge, UK). The following reagents were prepared: ammonium hydrogen orthophosphate buffer (500 mmol/l, pH 4.15, 2.5 and 7.0); 10% (v/v) acetonitrile in water; 500 mmol/l monochloroacetic acid (MCA) dissolved in ammonium orthophosphate buffer (500 mmol/l, pH 2.5). 1.0 and 5.0 mmol/l of ammonium phosphate buffer (pH7.0) were dispensed by the Gilson 401 dilutors for liquid movement on the donor and recipient sides of the dialyser, respectively.

2.2.2. Standard preparations

430C78 (isethionate salt), 166C89 (trifluoroacetate dihydrate salt) and 583C80 (mesylate salt) were obtained from Glaxo Wellcome Research Labs. (Beckenham, UK). Separate stock solutions containing 400 μg/ml 430C78 and 166C89 and 50 μg/ml 583C80 were prepared in 10% (v/v) methanol–water. From the stock standard solutions working standards containing 1.0:1.0:0.05, 25:25:1.25 and 100:100:5.0 μg/ml of 430C78–166C89–583C80 in 10% (v/v) methanol–water were prepared. Calibration standards ranging from 0.04 to 10.0 μg/ml for 430C78 and 166C89 and from 2.0 to 500.0 ng/ml for 583C80 were prepared by supplementing blank plasma with the working standards.

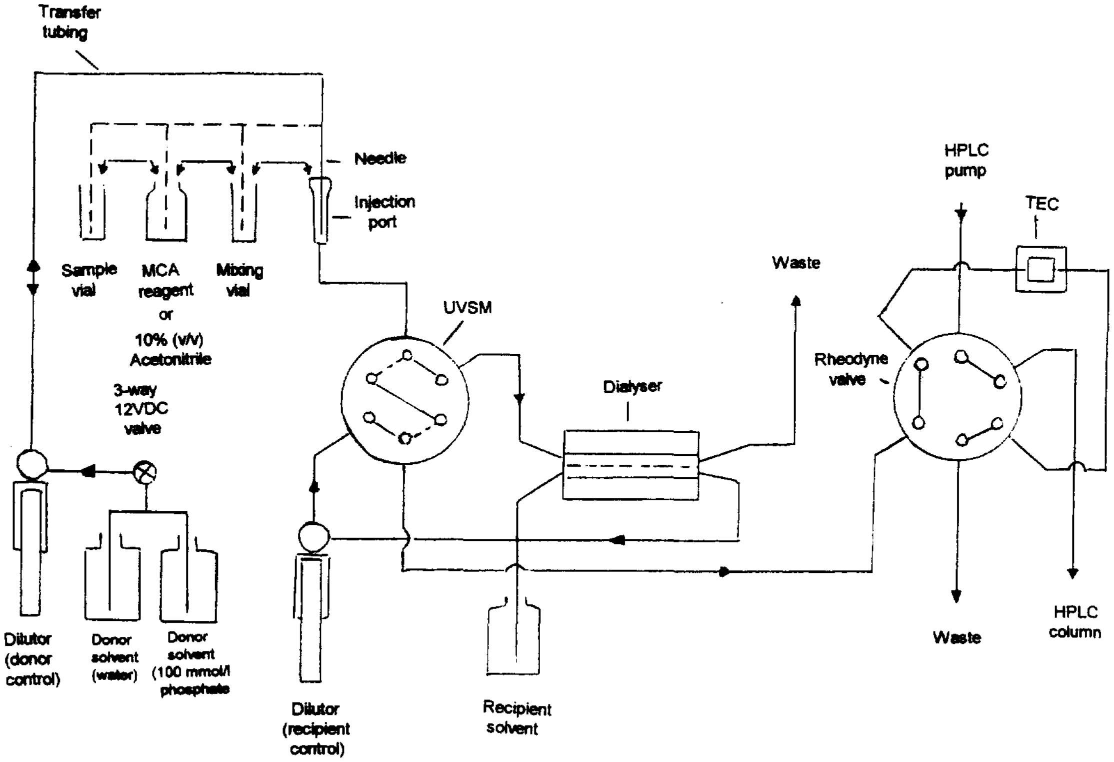

Fig. 1. Schematics of the ASTED system. The arrows indicate direction of reagent flow.

2.3. Chromatographic conditions

A gradient HPLC mobile phase was utilised comprising:

Solvent A: 50 mmol/l ammonium phosphate buffer (pH 4.15) containing 20 mmol/l of diethylamine hydrochloride (DEA)

Solvent B: acetonitrile–ammonium phosphate (500 mmol/l, pH 4.15)–DEA (200 mmol/l) buffer–water (60:10:30, v/v/v). The gradient conditions were at time 0 min 14% B; at 3.0 min 14% B; at 3.2 min 31% B; at 13.4 min 31% B; at 13.5 min 100% B; at 15 min 100% B; at 15.2 min 14% B; at 19 min loop to time 0 min; at a flow-rate of 1.5 ml/min. The HPLC column (150×4.6 mm I.D.). was packed with 5 μm particles of Kromasil C_8 (Technicol, Stockport, UK). No guard column was employed and the analytical column run at ambient temperature. The detector was set at time 0 min, 270 nm wavelength and 0.006 a.u.f.s. and at time 12.5 min, 215 nm and 0.003 a.u.f.s..

2.4. Sample preparation

Sample preparation was carried out in a completely automated manner using the ASTED system previously reported [6] and optimised as follows:

(1) 550 μl of plasma were mixed with 100 μl of MCA–ammonium phosphate buffer and 630 μl of this mixture delivered exactly to the exit of the donor channel of the dialyser. After 4 min a further 300 μl of mixture was pulsed into the donor channel. Two pulses of the sample were made to obtain the required analytical sensitivity.

(2) 2000 μl of recipient solvent (5.0 mmol/l, pH 7.0 potassium phosphate buffer) were moved through to the Prelute cartridge in an 8 min time period.

(3) Following enrichment the donor tubing and dialyser channel was purged with 1500 μl of donor solvent and 500 μl of the donor solvent (1.0 mmol/l, pH 7.0 potassium phosphate buffer) moved (via valve switching) through the Prelute cartridge.

(4) The Rheodyne high-pressure valve was

switched to the inject position and 166C89 eluted onto the HPLC column. After 30 s the high pressure valve was returned to the load position.

(5) 200 μl of 10% acetonitrile was moved though the Prelute cartridge via the ASTED injection port followed by 500 μl of donor solvent.

(6) 4 min into the chromatography cycle the Rheodyne high-pressure valve was again switched to the inject position and the 430C78 and 583C80 compounds eluted onto the HPLC column.

(7) The system was then purged and the Prelute cartridge regenerated with 500 μl of recipient solvent ready for the next sample.

2.5. Quantification

Calibration standards were situated at the beginning and end of each analytical run. A linear regression (weighting $1/X$) was performed on the peak areas and concentrations of both sets of standards. The regression line established was used to calculate test analyte concentrations.

2.6. Quality control

For 430C78 and 166C89 heparinised plasma samples were supplemented with 0.04, 0.10, 2.5 and 10.0 μg/ml of each compound. The same heparinised plasma samples were also supplemented with 2.00, 5.00, 125 and 500 ng/ml of 583C80. These samples were aliquoted and stored at −20°C. The standard solutions used to supplement the plasma were prepared from separate weighings to those solutions used to prepare the calibration standards.

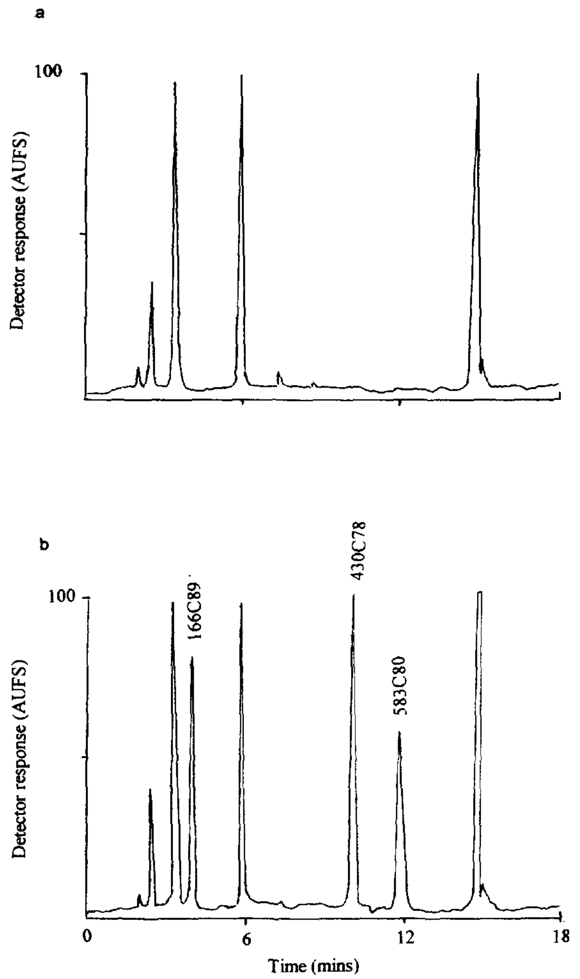

Fig. 2. (a) Chromatogram of blank human plasma; (b) chromatogram of plasma supplemented with 2.5 μg/ml of 430C78 and 166C89 and 125 ng/ml of 583C80.

3. Results

3.1. Optimisation of sample preparation and chromatography conditions

Chromatographic conditions were established using gradient elution with the sample preparation conditions described. Fig. 2a and Fig. 2b show chromatograms of blank plasma, plasma supplemented with 2.5 μg/ml of 430C78 and 166C89 and 125 ng/ml of 583C80. The specificity of the assay was assessed by analysing six plasma samples from volunteers not receiving lamotrigine and for these samples no peaks were detected at the retention time of the analytes under investigation.

3.2. Assay performance

3.2.1. Linearity and limits of quantification

Peak areas varied linearly over the analytical range employed. The limit of quantification was set at the lowest standard concentration on the calibration curve for respective analytes.

3.2.2. Precision and accuracy

The within-run coefficient of variation (C.V.) was estimated by assaying the quality control samples five times in the same analytical run. The between-run precision (C.V.) and accuracy (% bias) was obtained by estimating five replicates each of the same control samples in a further three analytical runs. The results are shown in Table 1.

3.2.3. Matrix effects

To test for matrix differences between samples the relative recovery of the method was obtained by calibrating the assay with aqueous standards and analysing each quality control sample five times within one batch. Compared with aqueous solutions of the analytes the mean relative recovery of 430C78, 166C89 and 583C80 from plasma was 88.4, 78.1 and 93.5% respectively.

3.2.4. Stability of analytes

The stability of the compounds in plasma was assessed over 24 h at room temperature, three freeze–thaw cycles and over 12 months stored at −20°C. No obvious degradation of the compounds was observed.

4. Discussion

4.1. Chromatographic separation and analyte detection

The detection of compounds with widely differing polarities requires the use of gradient elution in order to achieve realistic chromatography times and maintain reasonable sample throughput. Acetonitrile used as the organic solvent modifiers was found to produce the least baseline shifts during the gradient whilst monitoring with UV. The inclusion of DEA improved peak shapes especially that of 583C80 and with the elution pH adopted the choice of Kromasil HPLC column material also minimised peak distortion. The chromatographic resolution was designed to allow adequate time between the emergence of 430C78 and 583C80 for wavelength and absorbance range switching to allow for the differential in the analytical ranges required to be examined for subjects receiving 430C78 therapy.

4.2. Sample preparation

For sample clean-up prior to achieving specificity by chromatography and detection, ASTED operates

Table 1

Within- and between-run precision and accuracy of the ASTED plasma 430C78, 166C89 and 583C80 methods

Analyte	Statistical procedure	*Nominal 430C78* (μg/ml)			
		0.04	0.10	2.50	10.00
430C78	Overall measured mean (μg/ml)	0.045	0.10	2.57	10.18
	Within-run precision (C.V., %)	6.82	5.71	0.60	1.78
	Between-run precision (C.V., %)	13.49	6.08	1.95	1.78
	Between-run accuracy (% bias)	12.50	0.00	2.80	1.80
		Nominal 166C89 (μg/ml)			
166C89	Overall measured mean (μg/ml)	0.042	0.11	2.59	10.36
	Within-run precision (C.V., %)	8.96	4.56	2.90	4.46
	Between-run precision (C.V., %)	8.83	5.78	4.25	4.95
	Between-run accuracy (% bias)	5.00	10.00	3.60	3.60
		Nominal 583C80 (ng/ml)			
		2.00	5.00	125.00	500.00
583C80	Overall measured mean (μg/ml)	2.18	4.65	123.11	507.19
	Within-run precision (C.V., %)	10.27	8.07	0.92	1.90
	Between-run precision (C.V., %)	18.26	11.87	3.29	1.93
	Between-run accuracy (% bias)	9.00	−7.00	−1.51	1.44

two separation processes sequentially i.e., membrane accelerated dialysis and trace enrichment. These variables, combined, provide a powerful tool to achieve extremely clean sample preparations. Discussions concerning the importance of sample preparation and the attributes of on-line systems such as ASTED have been previously published [8]. The efficacy of the membrane to enable regeneration of the trace enrichment material over many hundreds of cycles has been enhanced by the improved specificities obtained using a "heart-cutting" approach on the material used to enrich the dialysates. This has previously been described and utilises specialised software control extending the analytical capabilities of the basic ASTED system [6]. Further enhancements were required to obtain the objectives of the method for 430C78 and metabolites described in this report.

In this procedure the use of Hypersil ODS in this method was necessary to obtain sufficient breakthrough volume of the least retained compound 166C89 for the trace enrichment material. The retention mechanism operates principally by partition but mixed mechanisms including ion-exchange are almost certainly operating for the other more basic compounds 430C78 and 583C80. In view of the low breakthrough volume of 166C89 and to remove extraneous enriched interferences that would interfere with the elution of 166C89 a small volume of donor buffer was moved through the Hypersil ODS material. This however was not sufficient for clean-up of the following more non-polar molecules 430C78 and 583C80. To accomplish this task tandem switching was developed in that after 166C89 was injected onto the column by "heart-cutting", the trace enrichment material was washed with acetonitrile prior to injection of the other two compounds. Fig. 2a and Fig. 2b show the typical chromatograms obtained with such a clean-up procedure and the specificity of the assay was proved to be acceptable within the context of this report and over numerous injections of prepared samples of pre-dose plasma collections from subjects about to receive 430C78 doses.

It has been shown by other workers that the glucuronide metabolite degrades at a pH in excess of 7.0 [2]. This was confirmed during this method development and provided plasma has been stored correctly at −20°C after collection no problems were encountered after thawing and storage at room temperature for 24 h. For the purposes of the ASTED procedure the 24 h room temperature investigations were essential since samples could remain at this temperature for this time period during analysis. In addition no obvious degradation of the analytes in plasma was found when the samples were freeze–thawed over 3 cycles or stored at −20°C over a 12 month period.

4.3. Sample preparation and matrix effects

Although other procedures suffer from the effects of matrix variations many workers consider that the use of membranes could make such procedures more susceptible. Previous reports using the ASTED procedure have recognised this fact [8] and have offered solutions to the potential problems. In this procedure the reduction of plasma pH using the automatic addition of MCA minimises protein binding to avoid between sample matrix variations. Without the addition of MCA only 40% relative recovery was observed for 430C78 and although, after the addition of MCA, the relative recovery of all three compounds did not completely achieve 100% their recoveries were much improved. This, combined with the use of plasma based calibration standards ensures that the accuracy of the assay is not compromised.

4.4. Assay performance

In comparison with the analysis of all three compounds using different methods the procedure described reduces sample analysis times and combined with the concurrent sequential process of preparing a sample during the chromatography of the previous specimen, sample throughput increases markedly although the procedure may appear relatively complex. This is not surprising when considering the difficult objective of analysing three compounds with wide polarity differences and analytical ranges. However the nature of the automated process dictates that once the ASTED system is set-up the procedure becomes a matter of routine with no operator intervention required once the samples have been loaded onto the system. Furthermore the assay has been applied successfully to the analysis of urine samples and has been adapted successfully to the more recent Gilson ASTED XL system. The use of this technique has provided a rapid method for examining the pharmacokinetics of 430C78 and its glucuronide and methylated metabolites and the acceptable assay performance (Table 1) both at the lower limit of quantification and throughout the analytical range has been consistent during the analysis of many hundreds of plasma samples from a variety of clinical situations and disease states.

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