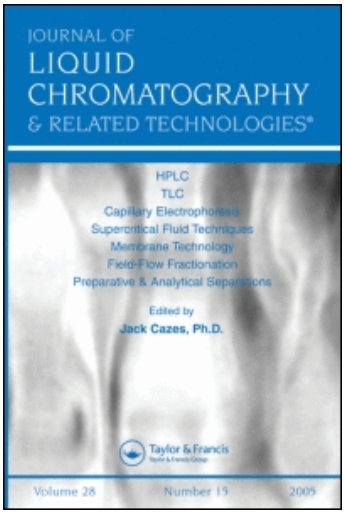

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SOLID-PHASE EXTRACTION OF CARBAMAZEPINE AND TWO MAJOR METABOLITES FROM PLASMA FOR ANALYSIS BY HPLC

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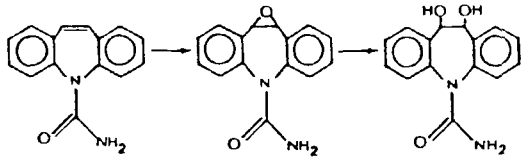
ABSTRACT

A rapid, sensitive and simple to operate HPLC method for the simultaneous determination of carbamazepine, carbamazepine 10,11-epoxide and 10,11-dihydro-10,11-trans-dihydroxycarbamazepine in plasma is described. The drug and its metabolites are extracted from plasma using commercially available reversed-phase octadecylsilane bonded-silica columns (Bond Elut C_{18}, 2.8 ml capacity). Separation was achieved by reversed-phase chromatography, using a mobile phase consisting of acetonitrile - methanol - water (19:37:44) at a flow-rate of 1.8 ml/min in conjunction with a Waters Assoc. Nova-Pak C_{18} column. The analytical column, in Radial-Pak cartridge form, was used in combination with a Waters Assoc. Z-module RCSS and protected by a Waters Assoc. Guard-Pak precolumn module containing a Guard-Pak μBondapak C_{18} insert. Using ultraviolet detection at 214 nm, levels in the region of 50-100 ng/ml for CBZ and its metabolites can be measured with only 250 μl of plasma. The method has been used to determine steady-state concentrations of the drug and its metabolites in paediatric patients.

INTRODUCTION

It is widely recognised that the anticonvulsant drug, carbamazepine (CBZ), provides optimum seizure control when plasma concentrations are maintained in the range 4-12 μg/ml [1]. However, poor correlation between CBZ dose and steady-state plasma levels in long-term epileptic patients [2] necessitates individual drug-level monitoring to maintain target concentrations. This variability may be a consequence of the diverse metabolism of CBZ [3] or, alternatively since CBZ induces its own metabolism [4], it could result from the effects of autoinduction.

The major metabolite of CBZ, carbamazepine 10,11-epoxide (CBZ-EP), exhibits anticonvulsant properties in rats [5] but this activity has not yet been confirmed in man. In simultaneous studies of CBZ and CBZ-EP, steady-state plasma levels of the metabolite correlated more closely with CBZ dose than did plasma levels of the drug itself [6]. The relative levels of CBZ and its epoxide metabolite are altered in patients receiving chronic dosing [7] and in those on combination anticonvulsant therapy [6-8]. It has been suggested, therefore, that induction of the epoxide-diol pathway (see FIG. 1) occurs both in long-term patients [9] and in those receiving combination therapy [10]. This is supported by metabolic studies of CBZ which indicate a higher urinary excretion of 10,11-dihydro-10,11-trans-dihydroxycarbamazepine (CBZ-DIOL) in long-term patients and in those receiving either phenytoin, phenobarbitone, primidone or



CBZ CBZ-EP CBZ-DIOL

FIG. 1. Chemical structures of carbamazepine (CBZ), carbamazepine 10,11-epoxide (CBZ-EP) and 10,11-dihydro-10,11-trans-dihydroxycarbamazepine (CBZ-DIOL).

valproic acid concomitantly [11]. Investigation of the epoxide-diol pathway to-date has chiefly involved measurements of urinary metabolites [9-11]. Consequently, quantitative information regarding plasma levels of CBZ-DIOL is limited [12-14].

Techniques currently available for the simultaneous determination of CBZ, CBZ-EP and CBZ-DIOL in plasma include TLC [15] and HPLC [14,16-18]. CBZ and its metabolites have also been measured in urine using HPLC [11] and GLC-mass spectrometry [9-11,19]. In addition, CBZ-DIOL has been estimated in microsomal suspensions using HPLC [20].

This paper describes a rapid and simple to operate HPLC procedure for quantifying CBZ, CBZ-EP and CBZ-DIOL following solid-phase extraction from plasma.

MATERIALS AND METHODS

Reagents

Ethanol (absolute alcohol AR quality) was purchased from James Burrough, London, UK. Acetonitrile (HPLC S grade) and methanol (HPLC grade) were obtained from Rathburn Chemicals,

Walkerburn, UK. Carbamazepine, carbamazepine 10, 11-epoxide, 10-methoxycarbamazepine and 10,11-dihydro-10,11-trans-dihydroxy-carbamazepine were gifts from Ciba-Geigy, Horsham, UK. Nitrazepam was donated by Roche Products, Welwyn Garden City, UK.

Extraction columns and vacuum apparatus

Rapid sample processing was achieved using Bond Elut C_{18} columns, 2.8 ml capacity, in conjunction with a Vac-Elut vacuum apparatus. These are both manufactured by Analytichem International (Harbor City, CA, USA) and supplied by Jones Chromatography (Llanbradach, UK).

Equipment

The high-performance liquid chromatograph consisted of a Waters Assoc. Model 510 constant-volume pump, U6K injector, Lambda-Max Model 481 variable-wavelength LC spectrophotometer and a Model 730 data module.

Chromatography

A Waters Assoc. Z-Module RCSS was used in conjunction with a Nova-Pak C_{18} Radial-Pak cartridge (10 cm x 8 mm ID, 4-µm spherical, fully capped, C_{18} bonded-silica) and protected by a Waters Assoc. Guard-Pak precolumn module containing a Guard-Pak µBondapak C_{18} insert. Freshly prepared mobile phase consisting of acetonitrile-methanol-water (19:37:44) was filtered through a 0.22 - µm Millipore filter (Durapore type GVWP) and degassed prior to use. Chromatography was performed at ambient temperature using a flow-rate of 1.8 ml/min which produced a back-pressure in the region of 6.82 MPa (1000 psi). A variable-wavelength LC

spectrophotometer (Lambda-Max Model 481) operating at 214 nm with a sensitivity setting of 0.05 a.u.f.s. was used to monitor the eluent. This was linked to a Model 730 data module programmed to provide a chart-speed of 198 mm/hr.

Preparation of Internal Standard Solution

The internal standard, nitrazepam, was dissolved in ethanol (10 mg in 100 ml) to provide the working concentration of 100 µg/ml. This solution was incorporated into the sample by adding a 25 µl aliquot to 250 µl of plasma as described in the extraction procedure.

Extraction Procedure

CBZ, CBZ-EP and CBZ-DIOL were extracted from plasma using reversed-phase octadecylsilane bonded-silica columns (Bond Elut C_{18}, 2.8 ml capacity). These were conditioned immediately prior to use by drawing two column volumes of acetonitrile (2 x 2.8 ml) followed by a similar volume of water through the column under vacuum. On releasing the vacuum, 250 µl of the plasma sample followed by 25 µl of the internal standard solution (nitrazepam, 100 µg/ml in ethanol) were loaded onto the column. The sample containing internal standard was allowed to equilibrate for 1 min before reapplying the vacuum. Following loading, the vacuum was again released and a further equilibration period of 2 min preceded the washing stage. Washing was achieved by drawing one column volume of water followed by a similar volume of water-acetonitrile (95:5) through the column under vacuum. Finally, CBZ, CBZ-EP and CBZ-DIOL together with the internal standard were

eluted from the Bond Elut C_{18} column with 750 µl of acetone. This acetone extract was evaporated to dryness under nitrogen at 55°C and the residue was reconstituted in 250 µl of mobile phase. The reconstituted samples were injected, using 10-25 µl aliquots, directly into the chromatograph.

Preparation of Calibration Standards

Stock solutions of CBZ (1mg/ml and 100µg/ml), CBZ-EP (100 µg/ml) and CBZ-DIOL (100 µg/ml) were made up in acetonitrile. Calibration standards containing all three components were prepared from these by adding appropriate aliquots to plasma to produce concentrations of 0.5, 1, 5, 10 and 20 µg/ml in the case of CBZ and 0.25, 0.5, 1, 2 and 5 for each of the two metabolites.

Extraction Recovery Experiment

Samples were prepared in plasma by adding authentic CBZ, CBZ-EP and CBZ-DIOL to produce concentrations of 0.5 and 5 µg/ml for CBZ and 0.25 and 1 µg/ml for each of the two metabolites CBZ-EP and CBZ-DIOL. Following extraction by the previously described procedure, 10-20 µl samples were injected into the chromatograph. The peak heights obtained were compared with those from injections of standard solutions and the percentage recovery determined.

RESULTS

The chromatogram illustrated in FIG. 2A is that obtained following an injection of authentic components prepared in mobile phase. CBZ, CBZ-EP and CBZ-DIOL are well separated with retention

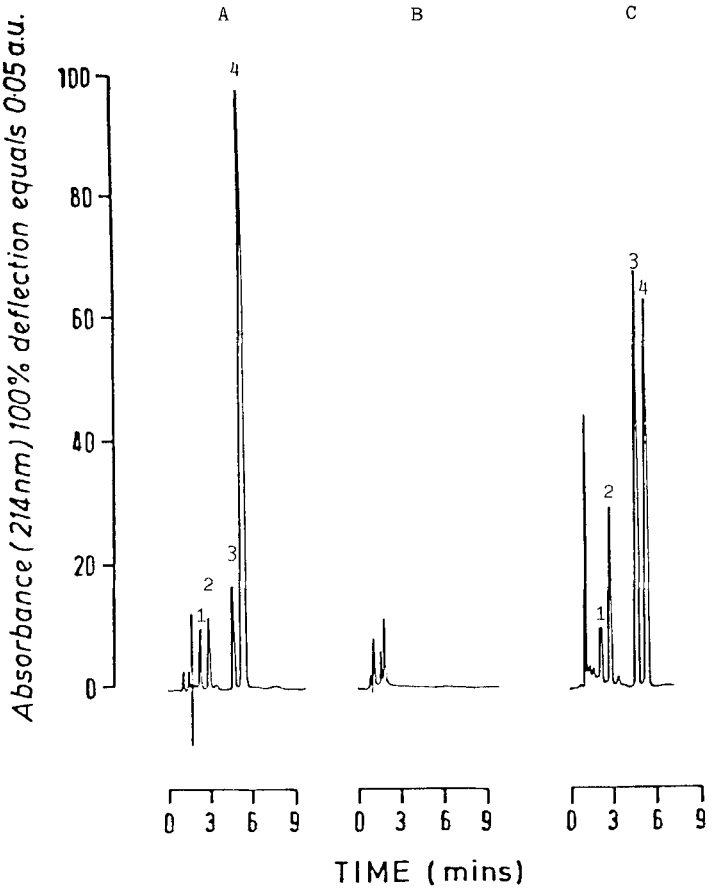


FIG. 2: Chromatogram of (A) authentic components in mobile phase; (B) extracted drug-free plasma; (C) a typical extracted plasma sample from a patient receiving long-term carbamazepine monotherapy. Plasma levels of CBZ, CBZ-EP and CBZ-DIOL were found to be (using a 15 µl injection) 7.13, 2.10 and 0.86 µg/ml respectively. Peaks: 1 = CBZ-DIOL, 2 = CBZ-EP, 3 = CBZ, 4 = nitrazepam (internal standard).

times of 4.93, 3.10 and 2.45 min, respectively. In addition, nitrazepam (the internal standard) has a retention time of 5.56 min. The chromatogram represented in FIG. 2B is typical of extracted plasma samples from paediatric patients prior to receiving CBZ. FIG. 2C is representative of the chromatograms obtained from paediatric patients who have achieved steady-state levels of CBZ. The peak attributable to the parent drug has a retention time of 4.93 min, whilst the two peaks with retention times of 2.45 and 3.10 min correspond to CBZ-DIOL and CBZ-EP, respectively. The internal standard (nitrazepam) is the longest retained component with a retention time of 5.56 min.

Table 1 indicates that the recovery values for CBZ, CBZ-EP and CBZ-DIOL obtained using solid-phase extraction columns are influenced by the composition of the solvent used in the washing procedure. High acetonitrile content (20%) provided good recovery of CBZ and CBZ-EP but had the reverse effect on the more polar CBZ-DIOL. Recovery of CBZ-DIOL was significantly improved by lowering the acetonitrile component in the wash (5%). Aqueous acetonitrile (95:5,v/v) was selected, therefore, for the second stage of the washing procedure since adequate recoveries of all three CBZ-related components (94±2% in all cases), with acceptable reproducibility (coefficients of variation <3% in all cases), are achieved using this approach.

Calibration curves were obtained by plotting graphs of the peak height ratio CBZ or CBZ metabolite/internal standard versus the actual concentration of CBZ or CBZ metabolite in spiked

TABLE 1

Recovery of Carbamazepine and Two Major Metabolites from Plasma using Solid-Phase Extraction

Component	Plasma Conc. µg/ml	2nd stage wash - solvent composition					
		Water		Water - Acetonitrile 95 : 5		Water - Acetonitrile 80 : 20	
		Recovery %	C V %	Recovery %	C V %	Recovery %	C V %
CBZ	10	61.80	4.17	92.98	2.92	88.00	3.31
CBZ-EP	2	64.60	2.68	95.50	2.34	91.58	2.82
CBZ-DIOL	2	85.77	4.43	95.02	2.57	29.90	6.25
Nitrazepam	10	56.30	2.90	87.34	7.08	94.81	3.13

aliquots of plasma. The graphs obtained were linear over the working range 0-20 µg/ml for CBZ and 0-5 µg/ml for each of the two CBZ metabolites. The correlation coefficients (r) and corresponding slope values were 0.994 and 0.155, 0.990 and 0.217, and 0.991 and 0.159 for CBZ, CBZ-EP and CBZ-DIOL, respectively.

Table 2 shows the effects of sample storage on the reproducibility of results obtained using this method. Analyses were carried out on two series of replicate plasma samples containing either 0.5, 0.25 and 0.25 µg/ml or 5, 1 and 1 µg/ml for CBZ, CBZ-EP and CBZ-DIOL, respectively. These were stored at -20°C and analysed at weekly intervals up to 8 weeks followed by a final estimation after 3 months. Recovery values in the range

TABLE 2

Reproducibility of Measurements Obtained from Samples Prepared in Plasma - Effect of Storage at -20°C.

Component	Conc. µg/ml		Recovery	Coefficient of variation	Number of determinations
	Added	Determined	%	%	
CBZ	0.5	0.55	110.0	7.89	14
	5.0	5.00	100.0	6.02	15
CBZ-EP	0.25	0.27	108.0	8.93	14
	1.0	1.00	100.0	6.00	15
CBZ-DIOL	0.25	0.27	108.0	8.93	14
	1.0	1.01	101.0	8.57	15

105±5% and coefficients of variation <8.95% indicate that storage at -20°C for up to 3 months has little effect on the recovery or reproducibility of the method described.

DISCUSSION

The method described permits the simultaneous determination of CBZ, CBZ-EP and CBZ-DIOL in plasma from epileptic patients. Rapid separation of CBZ and its metabolites was achieved by using a ternary mobile phase (acetonitrile-methanol-water) and a short, efficient column (10cm x 8mm. I.D. Waters Assoc. Nova-Pak C_{18}, 4-µm spherical particles). The resolution provided by this combination is apparent from Table 3 which contains the retention data of a variety of drugs commonly used in paediatric medicine.

TABLE 3

Retention Data of Some Drugs Commonly Used in Paediatric Medicine.

Compound	Retention time	Compound	Retention time
Acetazolamide	< 2 min	Ethoxyzolamide	4.07 min
Theophylline	"	Chlormethiazole edisylate	4.32
Caffeine	"	Phenytoin	4.34
Cefuroxime	"	Prednisolone	4.75
Chlorothiazide	"	CBZ	4.93
Acetaminophen	"	Clonazepam	5.37
CBZ-DIOL	2.45	Nitrazepam	5.56
Ampicillin	2.75	10-methoxy CBZ	6.25
Konakion (Vit $K_{1(20)}$)	2.80	Diazepam	13.35
Phenobarbitone	2.92	Netilmicin	N.P.*
CBZ-EP	3.10	Acetylsalicylic Acid	N.P.
Prednisone	3.95	D,L-α-Tocopherol	N.P.

*No peak observed up to 20 min

Phenobarbitone and prednisolone are the only components listed which might interfere with the estimation of CBZ-EP and CBZ, respectively, using this method.

The isocratic approach adopted here is quick and simple to operate compared with the gradient-elution technique employed by Wad [16] or the column switching method with stepped-flow gradient described by Kuhnz and Nau [18]. Two recent papers by Kumps [14] and Kumps et al [17] describe an isocratic approach using a ternary mobile phase to achieve separation of antiepileptic drugs which included CBZ and its metabolites, but in both cases the retention times of CBZ and its metabolites were longer than those reported here. In addition to providing a faster

chromatographic separation, the present method demonstrates better sensitivity towards the metabolites of CBZ than the previously mentioned procedures used by Kumps and co-workers [14,17]. This is because the latter employ UV detection at 254 nm and differences in UV absorbance characteristics at this wavelength, arising from the absence of a double-bond in the 10,11-position of the metabolites, results in poorer sensitivity towards CBZ-EP and CBZ-DIOL. However by selecting a wavelength (214 nm) where the relative responses of CBZ and its metabolites are more equal, it is possible to achieve improved sensitivity and effect a significant reduction in sample requirement. In paediatric patients, where sample volumes are limited, this is particularly important. This approach also obviates the need for a double internal standard to accommodate large differences in peak heights between CBZ and its metabolites [21].

In cases where nitrazepam is co-administered, an alternative internal standard will be required, so 10-methoxycarbamazepine (10-methoxyCBZ) was evaluated for this purpose. This compound exhibits similar extraction characteristics to CBZ, CBZ-EP, CBZ-DIOL and nitrazepam (recovery 92.98%) and has a slightly longer retention time of 6.25 min (see Table 3).

The larger capacity (2.8 ml) Bond Elut C_{18} extraction columns were adopted because there was less risk of blockage from highly viscous plasma samples which are occasionally encountered. Initial trials with the smaller, 1 ml capacity columns had indicated that this could be a problem, particularly with samples

which have been stored frozen for 2-3 months. However, more recently we have demonstrated that highly viscous plasma samples can be managed by simply diluting the plasma with water (1:2). Dilution can be accomplished on the Bond Elut column during sample loading, prior to applying the vacuum. This simple remedy enables 1 ml Bond Elut C_{18} extraction columns to be used routinely. Plasma samples containing 6.33, 1.24, 0.5 and 10 µg/ml of CBZ, CBZ-EP, CBZ-DIOL and nitrazepam, respectively, which were extracted using the smaller capacity extraction columns, produced recoveries of 89.22%, 93.46%, 88.56% and 97.22% for the respective components. In all cases, the coefficients of variation were less than 6.30% (based on 5 determinations). The changes in recovery of the CBZ-related compounds compared with those obtained using 2.8 ml capacity Bond Elut C_{18} columns are not significant and the main advantage of using the smaller capacity extraction columns is the reduced cost factor. However, in order to minimise costs, extraction columns can be regenerated for repetitive use by washing with methanol as suggested by Kabra et al [22].

Validation of the HPLC method by comparison of measured patient plasma levels with results obtained using the EMIT procedure was limited to CBZ determinations since the latter technique does not allow quantitation of the two metabolites. However, analysis of 21 plasma samples with CBZ values which ranged from 2.4 to 10.9 µg/ml as measured by EMIT (mean ± SD = 5.93 ± 1.91 µg/ml) revealed a highly significant correlation between results obtained using both analytical procedures

($r = 0.963$, slope = 1.09, $p < 0.001$) and a paired "t" test showed no significant difference between the two sets of data ($p > 0.8$).

The method is being used to measure steady-state plasma levels of CBZ, CBZ-EP and CBZ-DIOL in children receiving chronic CBZ monotherapy and some preliminary results are included. The mean CBZ dose ± SD received by eight such patients was 7.09 ± 2.48 mg/kg and this was administered at 12 hourly intervals (8.00 a.m. and 8.00 p.m.). Blood specimens were taken prior to each dose and at 4 hours post-dose in all cases. The mean minimum concentrations ± SD determined in pre-dose plasma samples were 5.42 ± 1.34, 1.02 ± 0.33, and 3.03 ± 1.12 µg/ml for CBZ, CBZ-EP and CBZ-DIOL, respectively. The mean maximum concentrations ± SD obtained from post-dose plasma samples were 8.06 ± 1.66, 1.28 ± 0.47 and 2.96 ± 1.15 µg/ml for the same components.

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