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SIMULTANEOUS DETERMINATION OF CARBAMAZEPINE AND ITS EPOXIDE METABOLITE IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple procedure for the simultaneous determination of carbamazepine and its major metabolite, carbamazepine epoxide, in plasma and urine is described. The assay involves two extractions of the drugs and an internal marker, clonazepam, from the alkalinized sample. The extract is evaporated to dryness at 45°C and the residue is redissolved in methanol (30 μl). A 25-μl aliquot is injected into the liquid chromatograph and eluted with acetonitrile—water (40:60, v/v) on a C_{18} pre-column linked to a 5-μm C_8 reversed-phase column. The eluent is detected at 215 nm The method has been used to investigate the steady-state concentrations of carbamazepine and carbamazepine epoxide in the plasma and urine of a manic-depressive patient

INTRODUCTION

Carbamazepine (CBZ), 5H-dibenzo[*b*,*f*]azepine-5-carboxamide, is an effective agent for the control of epileptic seizures and in the treatment of trigeminal neuralgia, and possesses acute and prophylactic antimanic effects in the control of manic-depressive illnesses [1]. Thirty-three metabolites of CBZ have been isolated and characterized in the urine from patients on oral CBZ [2]. Of these metabolites, carbamazepine 10,11-epoxide (CBZ-E) is both qualitatively and quantitatively the most important one from a clinical point of view. CBZ-E is pharmacologically as active (as an anticonvulsant) as the parent compound in experimental animals. In humans the variation of CBZ-E concentrations in the blood, both inter-individually and intra-individually, is greater than that of CBZ [3]. Monitoring the concentrations of CBZ and CBZ-E in plasma in the

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management of epilepsy has been proved useful. Very little is known about the plasma concentrations of these compounds in the treatment of manic depression by CBZ. The purpose of this paper is to report the development of a high-performance liquid chromatographic (HPLC) assay that can determine simultaneously the concentration of CBZ and CBZ-E in plasma and urine from a manic-depressive patient.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a Waters 6000A pump, a U6K injector with a 25-μl loop (Waters Assoc., Milford, MA, U.S.A.) and a variable-wavelength Hitachi 220-S UV detector with a chart recorder (Hitachi, Tokyo, Japan). Analyses were performed on a reversed-phase C_8 column (Hibar, LiChroCart RP-8, 250 mm × 4.6 mm I.D., 5 μm Merck, Darmstadt, F.R.G.) linked to a C_{18} pre-column (30 μm, 75 mm × 4.6 mm I.D., Serva, Heidelberg, F.R.G.). The operating conditions for the HPLC system were: mobile phase, acetonitrile—water (40:60); flow-rate, 1.2 ml/min; temperature, ambient (25 ± 1°C); UV detector wavelength, 215 nm; sensitivity scale, 0—0.01 a.u.f.s.

Other apparatus included 10-ml and 15-ml centrifuge tubes with well fitting screw caps (Sovirel, Levallois-Perret, France), and 15-ml stoppered evaporation tubes with finely tapered bases (50 μl capacity). All glassware was cleaned by soaking overnight in a 5% solution of Extran (Merck) in water, then rinsed thoroughly with methanol and hot tap water followed by distilled water. These tubes were subsequently silanized by rinsing with a 1% solution of Prosil-28 silanizing agent (PRC, Gainesville, FL, U.S.A.) followed by rinsing with distilled water and dried at 150°C overnight. This treatment of glassware was necessary to eliminate possible loss of drug owing to absorption on the glass walls [4]. Hamilton syringes, 10 μl and 25 μl, were used.

Materials

The following materials were used: dichloromethane, diethyl ether and methanol, all of Analar grade (Merck) were freshly distilled before use; sodium hydroxide solutions (5 *M* and 0.1 *M*); water was double-distilled in a glass apparatus; carbamazepine BP and carbamazepine 10,11-epoxide were gifts from Ciba Geigy (Basle, Switzerland) and clonazepam and a midazolam methanol analogue, 8-chloro-6-(2-chlorophenyl)-1-methyl-4H-imidazo-[1,5-*a*][1,4]-benzodiazepine-3-methanol (I, Ro 21-6962) were supplied by Roche (Basle, Switzerland).

Preparation of reagents and standards

Standard solutions calculated as mg base per ml in distilled methanol were made of CBZ and CBZ-E and diluted to the following calibration ranges: 0—5 μg/ml for CBZ and 0—800 ng/ml for CBZ-E. Dilutions were made up in drug-free plasma and urine (final volume, 0.5 ml) and to each were added 8 μl (100 μg/ml) of clonazepam as the internal standard. The midazolam methanol analogue, I, gave two peaks under the running conditions. The eluent of the second HPLC peak corresponding to pure I was collected and used to prepare standard solution.

The mobile phase consisted of a freshly prepared mixture of acetonitrile and distilled water, which was filtered before use through a Millipore filter type AA (pore size, 0.5 μm; Waters Assoc.). Further degassing was found not necessary immediately after filtration.

Extraction of CBZ and CBZ-E from plasma and urine

In a previous study on extraction of five antiepileptic drugs [5], CBZ was extracted under acidic conditions at pH 2. CBZ may be considered as a neutral substance with no basic or acidic functions. But the compound, with its tricyclic structure, is readily soluble in organic solvents. CBZ-E, on the other hand, is relatively polar. A selective extraction procedure was used to extract CBZ, CBZ-E and the internal standard (clonazepam) under alkaline conditions such that other acidic antiepileptic drugs were not co-extracted. The recovery of CBZ, CBZ-E and clonazepam from plasma and urine samples under alkaline conditions was studied. The midazolam analogue (I), with a longer retention time (10 min) could also be used as an internal standard if and when clonazepam was included in patients' therapy. Preliminary results suggested that two extractions with 7 ml of organic solvent mixture gave a better recovery.

General procedure

Into a 15-ml glass centrifuge tube, clonazepam (the internal standard, 800 ng) was added to the drug-containing plasma (0.5 ml) for assay. To precipitate the plasma proteins, methanol (200 μl) was added, followed by distilled water (1.5 ml) and 5 *M* sodium hydroxide (20 μl) to adjust the pH to 12. The basified solution was extracted twice with organic solvent [7 ml of a mixture of dichloromethane and diethyl ether (1:3)] by mixing with the aid of an automatic shaker for 15 min. After centrifugation for 10 min at 2500 *g* to break the emulsion, the organic extract was transferred into a 15-ml evaporation tube; the combined extract was then evaporated to dryness at 45°C in a water-bath. The residue was dissolved in distilled methanol (30 μl) and vortexed for 30 sec. An aliquot (25 μl) was injected into the liquid chromatograph.

To determine CBZ and CBZ-E in urine, 0.5-ml volumes were used and the same procedure was followed.

Quantitation

Calibration graphs were constructed by plotting the peak height ratio of the drugs to the internal standard, against the known concentrations of CBZ and CBZ-E added to drug-free plasma or urine to cover the ranges 0—5 μg/ml and 0—800 ng/ml, respectively. The drug or metabolite was quantitated by relating the respective peak height ratio to obtain the concentration from the calibration graph.

Recovery

To assess the recovery of CBZ and CBZ-E from plasma by the extraction procedure, the drugs were added to drug-free plasma or urine (2000 ng/ml for CBZ and 200 ng/ml for CBZ-E) and assayed with the internal standard as

described. For comparison, the same concentrations of CBZ and CBZ-E and internal standard were prepared in a diethyl ether—dichloromethane solution, evaporated and assayed, but with the extraction step omitted. The corresponding peak height ratios from the plasma and urine extractions and from the diethyl ether—dichloromethane solutions were compared.

Selectivity

Samples of plasma spiked with a variety of antiepileptic drugs [5] were analysed to find out if the latter produced peaks after chromatography that interfered with those of CBZ, CBZ-E and clonazepam.

Precision

Six replicate samples of CBZ and CBZ-E in plasma or urine at 2000 and 200 ng/ml, respectively, were assayed as described under *General procedure*, and the peak height ratios of the drug to the standard were calculated.

Stability on storage

Samples of plasma and urine spiked with drugs or samples from patients were analysed immediately and after storage at 4°C overnight, and at −20°C for seven days and three months.

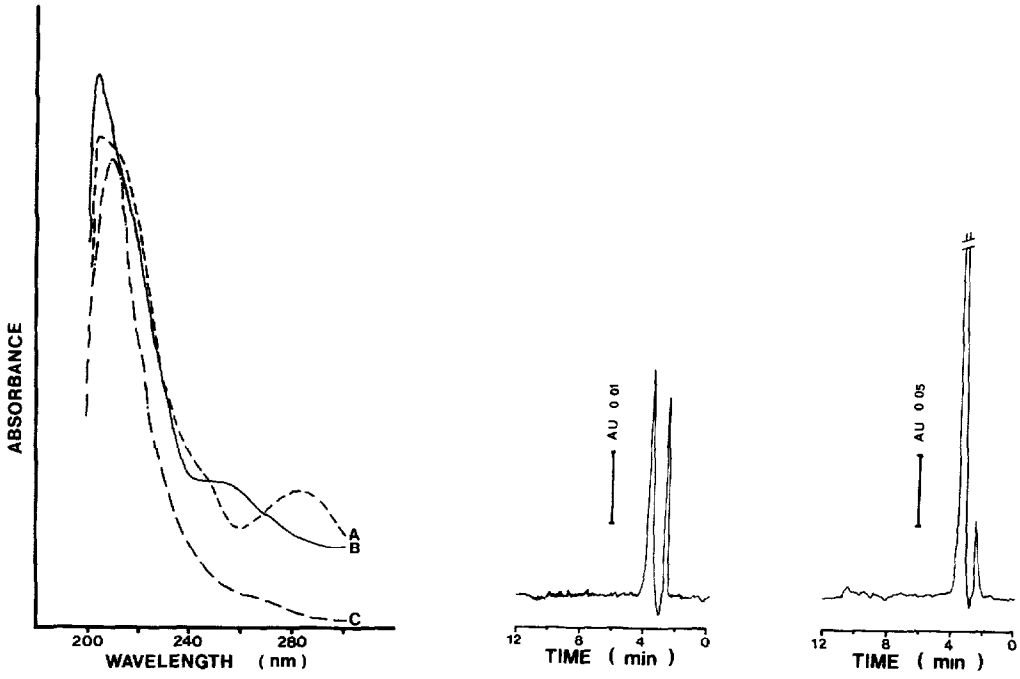

Fig. 1. UV spectra of carbamazepine (A) carbamazepine epoxide (C) and clonazapam (B) in methanol.

Fig. 2. Chromatograms of drug-free plasma (left) and urine (right) extracts.

RESULTS AND DISCUSSION

Performance of the HPLC system

Fig. 1 shows the UV spectra of CBZ, CBZ-E and clonazepam in methanol. The wavelength was set at 215 nm for optimal detection. Fig. 2 illustrates the chromatograms of a drug-free plasma and a drug-free urine extract. Fig. 3 shows the chromatograms of an extract of plasma spiked with CBZ, CBZ-E and internal standard, and plasma and urine samples from a patient treated with CBZ. The performance of the HPLC analytical system is summarized in Table I.

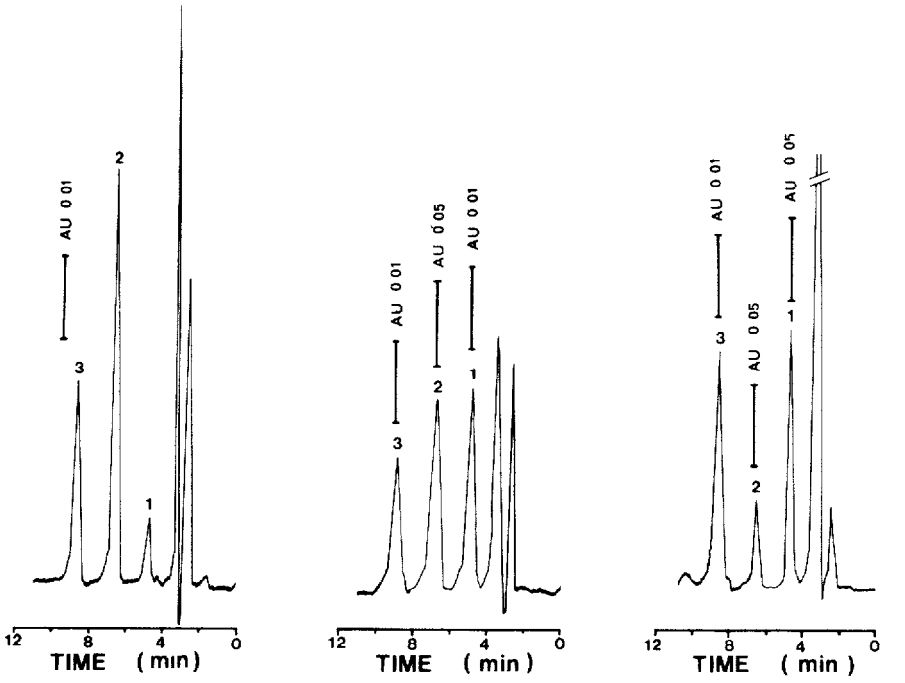

Fig. 3. Chromatograms of (1) carbamazepine epoxide (CBZ-E), (2) carbamazepine (CBZ) and (3) clonazepam (internal standard). Left: a plasma standard extract (CBZ 2.0 μg/ml, CBZ-E 0.2 μg/ml); middle: a plasma extract from a patient (CBZ 7.4 μg/ml, CBZ-E 1.4 μg/ml); right: a urine extract from the same patient (CBZ 1 7 μg/ml, CBZ-E 4.2 μg/ml).

TABLE I

PERFORMANCE OF THE HPLC SYSTEM

Injection volume, 25 μl; mobile phase, acetonitrile- water (40:60); flow-rate, 1 2 ml/min; column, RP C_{18} pre-column and C_8 analytical column, UV detection, 215 nm.

Drug	Retention time (min)	Symmetry factor (0.95—1.05*)	Resolution (> 1.0*)
Carbamazepine epoxide	4.8	1.0	—
Carbamazepine	6.6	0.97	1.38
Clonazepam	8.8	0.95	1.47

*Limits defined by British Pharmacopoeia (1980).

TABLE II

PERCENTAGE RECOVERY OF CARBAMAZEPINE AND CARBAMAZEPINE EPOXIDE FROM PLASMA AND URINE SAMPLES UNDER ALKALINE CONDITIONS

Drug	Plasma samples				Urine samples			
	One extraction	C V * (%)	Two extractions**	C V (%)	One extraction	C V (%)	Two extractions	C V (%)
CBZ (n = 6) (at 2000 ng/ml)	91 75	2 04	96 87	1 66	92 70	3 54	94 58	1 81
CBZ-E (n = 6) (at 200 ng/ml)	67 95	0 45	92 54	0 18	69 11	0 45	92 10	0 91

*C V. = Coefficient of variation (n = 6)

**Plasma proteins were precipitated with 200 µl of methanol, followed by two extractions with dichloromethane–diethyl ether (1 3) at pH 12

All analytical peaks are well resolved and their symmetry factors and resolution between adjacent peaks are within the British Pharmacopoeia limits [6], hence the peak height ratio technique for calibration is justified.

Recovery, extraction and selectivity

The addition of methanol (200 µl) to precipitate plasma proteins improved the recovery of CBZ and CBZ-E from the plasma. Extraction twice with organic solvent under alkaline conditions also improved the recovery (Table II). The extraction procedure has several advantages over published determinations of CBZ. Firstly, interference by acidic antiepileptic drugs, such as phenobarbital and phenytoin, is not encountered. Secondly, CBZ and CBZ-E are simultaneously extracted and assayed. Thirdly, the use of a solvent mixture [diethyl ether—dichloromethane (3:1)] improves recovery after two extractions. Finally, the procedure may possibly reduce the extraction of endogenous contaminants as no interfering peak appears in the chromatograms of a drug-free plasma and a drug-free urine extract (Fig. 2).

Reproducibility, linearity and storage

Repeated assays of plasma samples spiked with CBZ and CBZ-E indicated that the reproducibility of the procedure was satisfactory over the calibration ranges (Tables II and III). The calibration graphs relating the peak height ratios and concentrations of CBZ and CBZ-E added to plasma blanks were linear. The linear regression coefficients, r, were 0.9989 and 0.9999 (Table III).

Samples of plasma, urine and blood, whether fresh or stored at −20°C for one and four weeks, did not give peaks that would interfere with the measurement of peaks corresponding to CBZ, CBZ-E and internal marker (Fig. 2). There was no appreciable loss of the drugs from the samples after storage at 4°C overnight or at −20°C over a period of three months.

Application

The assay procedure was used to determine the plasma steady-state levels of CBZ and its major metabolites CBZ-E in a Chinese manic-depressive patient, who was stabilized on lithium carbonate (2.0 g daily) and carbamazepine (800 mg daily). The plasma concentrations of CBZ over a study period of 5 h were 7.8 µg/ml (overnight-fast value) to 9.5 µg/ml (4 h after oral dose), and the

TABLE III

CORRELATION BETWEEN PEAK HEIGHT RATIO AND CONCENTRATION OF CARBAMAZEPINE EPOXIDE AND CARBAMAZEPINE

Concentration (ng/ml)	CBZ-E		CBZ	
	Peak height ratio (mean ± S.D.)	C.V.* (%)	Peak height ratio (mean ± S.D.)	C.V. (%)
50	0.078 ± 0 005	0.19	—	—
100	0 138 ± 0 009	0.38	—	—
200	0.260 ± 0.011	0.44	—	—
400	0.516 ± 0.020	0.83	—	—
500	—	—	0.518 ± 0.018	0.75
600	0.758 ± 0.015	0.63	—	—
800	1.009 ± 0.023	0.93	—	—
1000	—	—	1.065 ± 0.042	1 70
2000	—	—	2.099 ± 0.035	1.41
3000	—	—	2.982 ± 0 110	4.49
4000	—	—	4.299 ± 0.15	6.12
5000	—	—	5 089 ± 0.14	6.11
Calibration graph	$y = 799.2x - 7\ 8$, $r = 0.9999$		$y = 968.3x - 6.24$, $r = 0.9989$	

*C.V. = Coefficient of variation ($n = 6$).

respective values for CBZ-E were 1.41 and 1.42 μg/ml. The ratio of CBZ-E to CBZ in the plasma was relatively constant: 0.18, 0.17, 0.17, 0.16 and 0.16 over various intervals during the study. Very little CBZ and CBZ-E were recovered in the 24-h urine (2300 ml): 1.7 and 4.2 μg/ml (ca. 0.5 and 1.25%, respectively, of a total daily dose of 800 mg). A programme on monitoring CBZ and CBZ-E in depressive patients of Chinese origin who are on CBZ therapy is being carried out.

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