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Short communication

Simultaneous determination of carbamazepine and its metabolites in plasma from carbon tetrachloride-intoxicated rats using a new reversed-phase chromatographic column of 2-μm porous microspherical silica gel

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Abstract

A high-performance liquid chromatographic method has been developed for the simultaneous analysis of carbamazepine (CBZ) and its two metabolites, carbamazepine-10,11-epoxide (CBZ-E) and carbamazepine-10,11-dihydroxide (CBZ-diOH), using a recently developed reversed-phase column with 2-μm particles and a 2-μl microflow cell equipped with a UV detector. The separation was achieved using two different C_{18} reversed-phase columns (column 1: 100×4.6 mm I.D., particle size 2 μm, TSK gel Super-ODS; column 2: 100×4.6 mm I.D., particle size 5 μm, Hypersil ODS-C_{18}) for comparison. The mobile phase was composed of methanol–water (30:70, v/v), and the flow-rate was 0.4 ml/min for both columns. The absorbance of the eluent was monitored at 210 nm. Retention times with column 1 were shorter than with column 2. When the three compounds were determined, the sensitivity and limit of quantification were about ten times better with column 1 than with column 2. The relative recovery and linearity with column 1 were approximately the same as those with column 2. These results show that the new ODS column packing with a particle size of 2 μm gives a higher sensitivity and shorter analysis time than the conventional ODS column packing

Keywords: Carbamazepine; Carbamazepine-10,11-epoxide; Carbamazepine-10,11-dihydroxide

1. Introduction

Carbamazepine (CBZ) is one of the most widely used antiepileptic drugs and it is often encountered in emergency toxicology screening and forensic medical examinations [1]. CBZ is extensively metabolized and its two metabolites, carbamazepine-10,11-epoxide (CBZ-E) and carbamazepine-10,11-dihydroxide (CBZ-diOH), can represent 10–50% of the administered dose. CBZ-E is almost fully biotransformed to the biologically inactive product CBZ-diOH. Several high-performance liquid chromatographic (HPLC) methods have already been reported for the determination of CBZ and its major metabolite (CBZ-E) [2–14]. However, there have been few reports of the simultaneous determination of CBZ, CBZ-E and CBZ-diOH in human sera by HPLC. Those that have been published use a reversed-phase column with packing 4- and 5-μm in size [9,13,14].

Recently, a new reversed-phase chromatographic column, TSK gel Super-ODS, based on 2-μm silica gel, became commercially available from Toshoh (Tokyo, Japan) [15]. This column showed less ionic

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and metal interactions than previous columns. In addition, the use of the smaller particle size gives a higher column efficiency than other reversed-phase columns for many compounds. Therefore, faster separation and better resolution can be achieved on Super-ODS. A few factors must be considered, however, when using this column: the retention capacity is lower than that of other conventional ODS columns so that the content of organic modifier in the mobile phase should be lowered, and the void volumes in the operating system must be reduced to a minimum.

In this paper, we report on the simultaneous determination of CBZ and its two metabolites in rat plasma using this reversed-phase column in comparison with a conventional column.

2. Experimental

2.1. Regents and materials

CBZ-E and CBZ-diOH were kindly provided by Dr. F.A. Sedlacek of Ciba-Geigy (Basel, Switzerland). CBZ and 5-ethyl-5-*p*-tolylbarbituric acid, used as an internal standard (I.S.), were purchased from Aldrich (Milwaukee, WI, USA) and Sigma (St. Louis, MO, USA), respectively. The mobile phase was prepared by mixing deionized water obtained using a Milli-Q system (Millipore, Bedford, MA, USA) and HPLC-grade organic solvent.

2.2. Extraction procedure

We added 100 μl I.S. (40 μg/ml) and 3 ml dichloromethane to 0.1 ml plasma (or standard aqueous solution) in 15-ml culture tubes. After vortex-mixing for 2 min, the tubes were centrifuged at 1200 *g* for 5 min and the aqueous phase removed by aspiration. The organic phase was transferred to a clean conical tube and evaporated in a water bath at about 40°C under a gentle stream of nitrogen. The residue was dissolved in 100 μl mobile phase and 10-μl aliquots were injected into the HPLC apparatus.

2.3. Standard solutions and calibration

A standard stock solution containing CBZ and its metabolites was prepared in methanol at a concentration of 1 mg/ml of each compound and this remained stable for at least two months at −20°C. Plasma standards were prepared containing 0.05, 0.5, 1 and 5 μg/ml of each compound by diluting appropriate aliquots of stock solution with drug-free serum. A calibration curve was obtained by linear regression of the peak-height ratio versus concentration.

2.4. Apparatus

The HPLC equipment consisted of a pump (Model CCPS, Tosho, Tokyo, Japan) and a variable-wavelength UV detector (Model UV-8000, Tosho, Tokyo, Japan) equipped with a 2-μl (column 1) or 10-μl microflow cell (column 2) without a heat-sink coil. The separation was achieved using a C_{18} reversed-phase column (column 1: 100×4.6 mm I.D., particle size 2 μm, TSK gel Super-ODS; column 2: 100×4.6 mm I.D., particle size 5 μm, Hypersil ODS-C_{18}, Yokogawa, Tokyo, Japan). The mobile phase was composed of acetonitrile–water (30:70, v/v) and the flow-rate was 0.4 ml/min for both columns. The absorbance of the eluent was monitored at 210 nm. All instruments and the two columns were operated at ambient laboratory temperature (ca. 23°C).

2.5. Accuracy and recovery

The accuracy and recovery were calculated by comparing the peak heights of CBZ-spiked samples (0.01, 0.1 and 1 μg/ml) after extraction from plasma with the peak heights of a series of unextracted reference standards.

2.6. Animal study

Male Sprague–Dawley (210–240 g) rats, 7–8 weeks of age, were obtained from CLEA Japan (Tokyo, Japan). The rats were kept in an air-conditioned room (25±1°C, 50–60% humidity) with a

12-h light–dark cycle (8:00 a.m.–20:00 p.m.) and given free access to commercial rat chow (Oriental-MF, Tokyo, Japan) and water. Twenty hours before the experiment, a cannula was inserted into the right jugular vein under ether anaesthesia for drug administration and blood sampling. A polyethylene cannula was inserted and, to avoid removal by the rat during the experiment, it was pulled subcutaneously to the nape of the neck, thus allowing free movement of the animals during the experiment. The rats were fasted overnight in the metabolic cage, given free access to water and injected intravenously with 10 mg/kg CBZ. Normal control rats were given olive oil (2 ml/kg). CCl_4 (0.25 ml/kg) was dissolved in olive oil and administered orally 24 h prior to the intravenous administration of CBZ. Blood samples (about 0.2 ml) were obtained from the jugular vein at 0.08, 0.17, 0.25, 0.5, 1, 1.5, 2 and 3 h after intravenous administration of CBZ.

2.7. Data analysis

The plasma concentration profiles of CBZ after intravenous administration were fitted to the equation below using a non-linear least-squares program NONLIN [16] and the parameters A, B, α, β and k were obtained.

$$C_t = Ae^{-\alpha t} + Be^{-\beta t} \quad (1)$$

where C_t is the plasma concentration of CBZ at time t, A and B are ordinate intercepts, and α and β are the corresponding first-order disposition rate constants. The elimination half-life ($t_{1/2}$) was calculated from $t_{1/2} = 0.693/k$. K is the elimination rate constant and estimated by applying logarithmic regression analysis to the terminal part of the plasma concentration-time profile (AUC). The AUC was calculated using the linear trapezoidal rule up to 3 h and extrapolated to infinity with k. The total body clearance (Cl_{tot}) of the doses was determined from Cl = dose/AUC. The apparent volume of distribution (V_d) was calculated from $V_d = Cl/k$. The results are shown as means ± standard deviation (S.D.). Statistical analysis was performed using the unpaired Student's t-test.

3. Results and discussion

3.1. Retention time

Fig. 1 shows chromatograms of CBZ and its metabolites separated using column 1. The retention times of CBZ-diOH, CBZ-E, I.S. and CBZ were approximately 3.7, 5.6, 8.8 and 9.7 min for column 1 and 4.1, 8.4, 12.9 and 17.0 min for column 2. CBZ and its metabolites and the I.S. were well separated. These results show that the retention times of CBZ and its metabolites depend mainly on the particle size of the reversed-phase column.

No interfering peaks appeared from endogenous material eluted after the drug.

3.2. Limits of quantification

The limit of quantification using column 1 is the lowest concentration on the standard curve which can be measured with acceptable accuracy (a coefficient of variation, C.V., <5%). The lowest practical limit of quantification was 0.01 μg/ml for CBZ and its metabolites (Table 1). The sensitivity for CBZ and its metabolites using the methods of Bnato et al. [9] and Pienimaki et al. [14] was much lower (about 40–50×) than that of our method (column 1). The sensitivity using column 1 was about 10× greater than that with column 2 (data not shown). The quantification limits with both columns are adequate for forensic and clinical analysis (toxic level: >12 μg/ml) [1,2].

3.3. Precision and accuracy

The precision and accuracy obtained using column are shown in Table 2. Within-day reproducibility was assessed by analysing six samples at three different concentrations on the same day. The C.V.s ranged from 1.1 to 3.1%. Between-day reproducibility was determined six times using four different quality control samples over a period of two weeks. The C.V.s ranged from 2.9 to 4.9%.

3.4. Recovery

Several liquid–liquid extraction systems were investigated, including ethyl acetate–chloroform

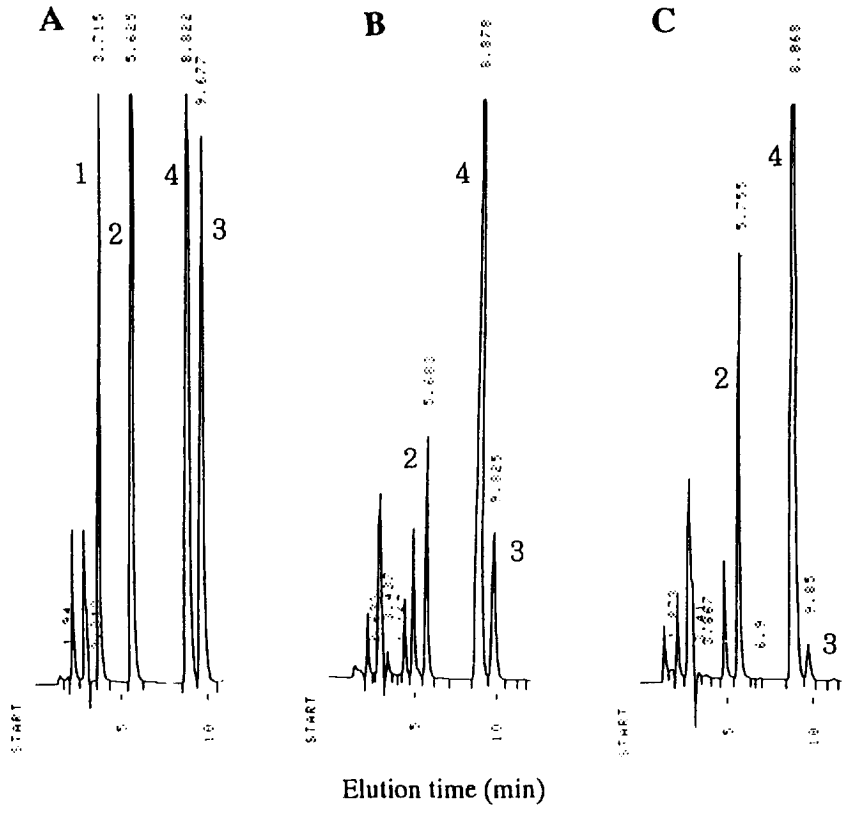

Fig. 1. Chromatograms of carbamazepine and its metabolites in rat plasma. (A) Rat plasma spiked with CBZ, CBZ-E and CB-diOH. The concentrations are 1 μg/ml for CBZ, CBZ-E and CBZ-diOH; (B) rat plasma obtained 2 h after i.p. administration of CZP (10 mg/kg) to carbon tetrachloride (0.25 ml/kg)-intoxicated rats; (C) rat plasma obtained 2 h after i.p. administration of CZP (10 mg/kg) to control rats. Column, 100×4.6mm I.D., particle size 2 μm, TSK gel Super-ODS; mobile phase, acetonitrile-water (30:70, v/v); flow-rate, 0.4 ml/min; detection wavelength, 210 nm. Peaks: 1=carbamazepine-10,11-dihydroxide (CBZ-diOH), 2=carbamazepine-10,11-epoxide (CBZ-E), 3=carbamazepine (CBZ), 4=internal standard (5-ethyl-5-*p*-tolylbarbituric acid).

(1:1) [2,4], dichloromethane–ethyl acetate (2:1) [8] and dichloromethane. Dichloromethane was chosen as the extraction solvent (indicated absolute recoveries 96% for CBZ, 95% for CBZ-E, and 75% for CBZ-diOH) because it afforded a better extraction than other solvents (92–94% for CBZ, 91–93% for CBZ-E and 69–72% for CBZ-diOH).

Relative recovery was calculated from the values obtained using drug-spiked plasma (Table 2). The C.V.s ranged from 2.6 to 4.3%. The recovery with column 1 were approximately the same as that with column 2 (data not shown).

3.5. Linearity

The calibration curves (the ratio between the peak-height of the drugs analyzed and that of the I.S. vs. amount of each drug) were obtained over the concentration range 0.01–1 μg/ml plasma. The equations and r values for the curves were: $y=0.51x+0.27$, $r=0.999$ for CBZ-diOH; $y=0.90x+0.44$, $r=1.000$ for CBZ-E; $y=0.43x+0.20$, $r=0.999$ for CBZ. The linearity with column 1 was approximately the same as that with column 2 (data not shown).

3.6. Animal study

The plasma concentration of CBZ in olive-oil-treated rats decreased according to a two-compartment model and plasma CBZ-E levels reached a

Table 1
Precision of the determination carbamazepine and its metabolite in rat plasma by the present HPLC method using column 1

Drug	Added (μg/ml)	Mean (μg/ml)	Within-day C.V.(%)	Between-day C.V.(%)
CBZ	0.01	0.011	3.6	4.1
	0.1	0.11	2.1	4.6
	1	1.1	3.1	3.2
CBZ-E	0.01	0.010	2.3	3.2
	0.1	0.11	1.1	3.7
	1	1.0	2.4	2.9
CBZ-diOH	0.01	0.010	1.8	4.2
	0.1	0.10	1.7	4.9
	1	1.0	1.4	3.6

$n=6$; column 1: 100×4.6 mm I.D., particle size 2 μm (TSK gel Super-ODS); mobile phase, acetonitrile–water (pH 6) (30:70, v/v); flow-rate, 0.4 ml/min; detection wavelength, 210 nm. All instruments and the column were operated at ambient laboratory temperature (ca. 23°C). CBZ: carbamazepine, CBZ-E: carbamazepine-10,11-epoxide, CBZ-diOH: carbamazepine-10,11-dihydroxide.

peak at around 1.5 h and then decreased gradually. However, no CBZ-diOH was detected after administration of CBZ. The elimination of CBZ in CCl_4 (0.25 ml/kg)-treated rats was delayed and CBZ-E production in the CCl_4-treated group was less than in the olive oil group (Table 3).

An ODS column packing with a particle size of 2 μm (TSKgel Super-ODS) and a pore volume and specific surface area about one third less than that of conventional ODS column packing has recently been developed [15]. This new ODS packing has the following advantages: (1) more rapid determination can be expected even at room temperature and without gradient elution, compared with conventional methods, (2) the amount of organic solvent required is small and (3) more sensitive determination is possible.

Table 3
Pharmacokinetic parameters following the intravenous administration of carbamazepine to carbon tetrachloride-intoxicated rats

Treatment	Control	CCl_4 treatment
A (μg/ml)	15.2±1.12	16.5±0.98
B (μg/ml)	8.14±0.56	10.98±1.12
α (h^{-1})	4.04±0.56	2.27±0.43*
β (h^{-1})	1.27±0.22	0.70±0.15*
$t_{1/2}$ α (h)	0.17±0.03	0.31±0.04*
$t_{1/2}$ β (h)	0.56±0.07	1.01±0.21*
Cl_{tot} (ml/min/kg)	26.32±2.89	10.60±1.43*
V_d (l/kg)	1.27±0.26	0.91±0.06

Pharmacokinetic parameters were calculated based on a 2-compartment model. Control rats received olive oil (2 ml/kg). CCl_4 was administered orally at 0.25 ml/kg, 24 h prior to the intravenous administration of CBZ (10 mg/kg). $t_{1/2}$ = elimination half-life, V_d = apparent volume of distribution, Cl_{tot} = total body clearance, $n=5$, mean±S.D., * $p<0.01$.

The detection and determination of CBZ and its metabolites in rat plasma using a new column packing with a particle size of 2 μm gives a higher sensitivity and shorter analysis time than is possible with conventional ODS column packings.

These results show that the rapid turnaround time and accuracy of this method make it suitable for emergency and forensic toxicology as well as clinical medicine, allowing rapid detection, confirmation and

Table 2
Percentage recovery of CBZ and its metabolites from rat plasma by the present HPLC method using column 1

Drug	Concentration (μg/ml)	Recovery (%)	Coefficient of variation (%)
CBZ	0.01	98.1	3.3
	0.1	99.6	2.6
	1	101.2	2.9
CBZ-E	0.01	98.7	4.3
	0.1	103.1	2.9
	1	99.5	3.7
CBZ-diOH	0.01	71.6	4.2
	0.1	75.2	3.8
	1	70.5	2.9

$n=6$; column 1: 100×4.6 mm I.D., particle size 2 μm (TSK gel Super-ODS); mobile phase, acetonitrile–water (pH 6) (30:70, v/v); flow-rate, 0.4 ml/min; detection wavelength, 210 nm. All instruments and the column were operated at ambient laboratory temperature (ca. 23°C). CBZ: carbamazepine, CBZ-E: carbamazepine-10,11-epoxide, CBZ-diOH: carbamazepine-10,11-dihydroxide.

quantification of CBZ and its metabolites using a single method.

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