Chiral separation of 10,11-dihydro-10,11-*trans*-dihydroxycarbamazepine, a metabolite of carbamazepine with two asymmetric carbons, in human serum

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Abstract

Chiral separation of 10,11-dihydro-10,11-*trans*-dihydroxycarbamazepine (CBZ-diol), a metabolite of carbamazepine (CBZ) with two asymmetric carbons, in serum taken from epileptic patients receiving CBZ alone for a long period, was performed by high-performance liquid chromatography using a polysaccharide stationary phase with n-hexane–ethanol (75:25, v/v) as the mobile phase. The enantiomeric ratio (S,S-/R,R-CBZ-diol) was 10.74 ± 1.13 (mean $\pm$ S.D.), which could demonstrate the presence of the stereospecificity in the hydrolysis of 10,11-dihydro-10,11-epoxycarbamazepine (CBZ-epoxide) to CBZ-diol and/or in the conversion of CBZ-diol to some metabolite such as 9-hydroxymethyl-10-carbamoylacridan. This is the first paper to report the determination of each enantiomer and the enantiomeric ratio of CBZ-diol in serum of epileptic patients who received CBZ.

Keywords: Carbamazepine; 10,11-Dihydro-10,11-*trans*-dihydroxycarbamazepine; Enantiomer separation

1. Introduction

Carbamazepine (CBZ), an iminostilbene derivative, is widely used for the control of epileptic seizures and trigeminal neuralgia. After administration, CBZ is transformed into 10,11-dihydro-10,11-epoxycarbamazepine (CBZ-epoxide), which is further hydrolysed by epoxide hydrolase to 10,11-dihydro-10,11-*trans*-dihydroxycarbamazepine (CBZ-diol), having two

asymmetric centres (Fig. 1) [1]. Recently, we found that CBZ-diol was converted with ring contraction through a pinacol-type rearrangement into 9-hydroxymethyl-10-carbamoylacridan (HMCA), which can be regarded as one of the major metabolites of CBZ in human serum [2].

By HPLC with diastereomeric derivatization, Bellucci et al. [3] isolated the enantiomers of CBZ-diol, which were excreted in the urine of patients receiving CBZ therapy in an enantiomeric excess of ca. 80%. They determined the absolute configurations of the enantiomers by the circular dichroism exciton coupling method to
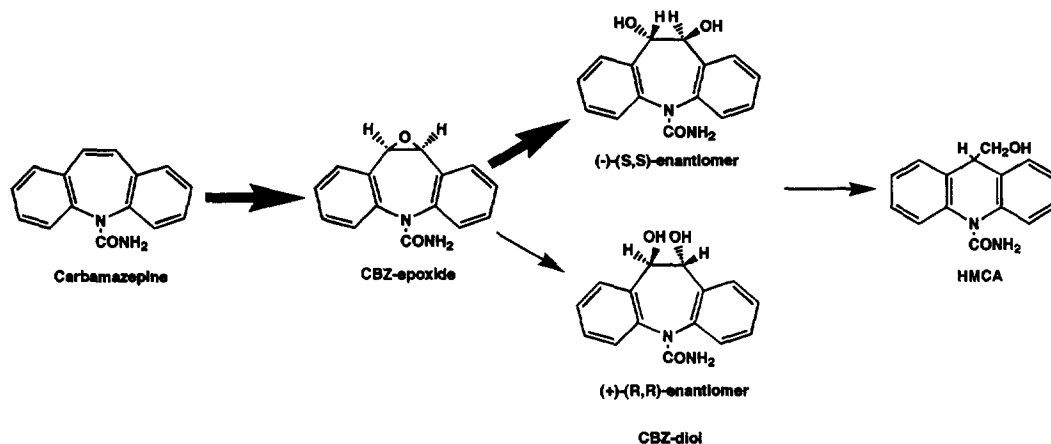
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Fig. 1. Formation of 10,11-dihydroxy-10,11-*trans*-dihydrocarbamazepine enantiomers in carbamazepine metabolism.

prove the prevalent enantiomer having the (−)-*S,S*-form [3].

Although there is a large number of papers dealing with assays of CBZ-diol levels [4–10], no report concerning the chiral determination of the enantiomers in sera from patients has been published. Previously, we have developed an HPLC method for the simultaneous determination of antiepileptic drugs and their metabolites, including chiral compounds, via β-cyclodextrin inclusion complexes, but the analytical conditions were not suitable for the chiral separation of CBZ-diol enantiomers [11]. Consequently, the stereospecific disposition of CBZ-diol in human has not been explored to date.

This paper describes a simple and effective method for the determination of each enantiomer in sera from epileptic patients by HPLC using a trimethylcellulose-type chiral stationary column.

2. Experimental

2.1. Chemicals

Racemic CBZ-diol and CBZ-epoxide were generous gift from Dr. A. Sedlacek of Ciba-Geigy (Basle, Switzerland). The enantiomers of CBZ-diol were obtained by the HPLC separation described in this paper. The configurations were identified on the basis of the values of optical rotation given by Bellucci et al. [3]. CBZ was purchased from Tokyo Kasei (Tokyo, Japan). β-Glucuronidase (type VII-A from *Escherichia coli*) was purchased from Sigma (St. Louis, MO, USA). The solvents for mobile phases were of HPLC grade and all other chemicals were of analytical-reagent grade.

2.2. Apparatus and HPLC conditions

The LC-6A system used (Shimadzu, Kyoto, Japan) consisted of an LC-6AD pump, an SPD-6AD UV detector, an SCL-6B system controller and a C-R4A integrator. The column was Chiralcel OJ (250 × 4.6 mm I.D.) (Daicel, Tokyo, Japan), and was eluted with *n*-hexane–ethanol (75:25, v/v) as the mobile phase. The flow-rate was 0.5 ml/min at room temperature and the eluates were monitored at 210 nm.

2.3. Sample preparation for HPLC

For validation of the assay, standard samples were prepared by the addition of CBZ and its metabolites at different concentrations to drug-free serum obtained from a male who had received no drug. Samples from patients were prepared from sera from epileptic patients receiving CBZ alone for a long period.

To 0.4 ml of a patient's serum was added 0.4 ml of 75 mM phosphate buffer solution (pH 6.8) containing 200 units of β-glucuronidase, and

then the mixture was incubated at 37°C for 30 min. At the end of the incubation, 0.4 ml of 0.5 M NaOH and 10% $ZnSO_4$ were added, and the mixture was shaken well followed by centrifugation at 6700 g for 10 min. Chloroform (2.5 ml) was added to 1.25 ml of the supernatant and the mixture was shaken vigorously and centrifuged at 1000 g for 10 min. The chloroform layer was removed and 1.0 ml of the residual aqueous layer was poured into an Extrelut-1 column (Merck, Darmstadt, Germany). After 10 min, the column was eluted with 3 ml of ethyl acetate and the eluate was dried under a gentle stream of nitrogen. The residue was dissolved in 50 μl of ethanol, then 10-μl aliquots were injected into the chromatograph. The above chloroform layer can be used for the determination of CBZ, CBZ-epoxide, CBZ-diol (as a mixture of enantiomers) and HMCA [2,11].

2.4. Determination

The concentrations of CBZ-diol enantiomers were determined by a direct method using absolute calibration graphs. The precision and recovery for enantiomers were obtained by varying the analytical runs using the standard solutions of CBZ-diol racemate at 0.4, 2 and 4 μg/ml in drug-free human serum. The recovery after the

extraction procedures was estimated by comparing the analytical data for CBZ-diol racemate between an extracted sample from the standard solutions and the non-extracted ethanolic solutions under the same conditions.

3. Results and Discussion

3.1. Chiral separation of CBZ-diol

A Chiralcel OJ column packed with cellulose tris(4-methylbenzoate) coated on silica gel has been utilized for the chiral separation of many compounds [12]. Previously, we resolved 5-(p-hydroxyphenyl)-5-phenylhydantoin (p-HPPH), an optically active metabolite of phenytoin, into enantiomers using this column [13]. CBZ-diol racemate in an ethanolic solution and in a drug-free serum could also be separated effectively into the respective enantiomers on Chiralcel OJ using n-hexane–ethanol (75:25, v/v) (Fig. 2B). Fig. 2A shows a typical chromatogram obtained in the assay of serum from six epileptic patients who were not given CBZ. No peaks disturbed the determination of CBZ-diol enantiomers.

The elution of each enantiomer was accelerated with increasing ethanol concentration in the mobile phase: 50% ethanol solution in n-hexane
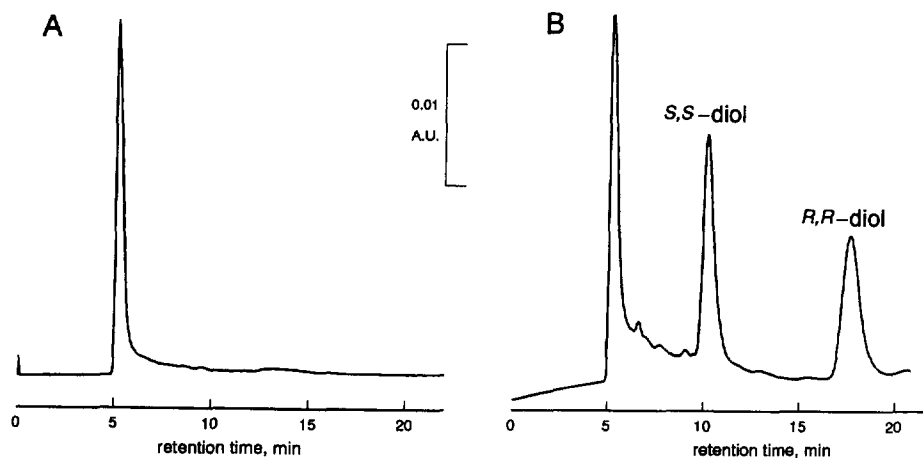

Fig. 2. Optical resolution of CBZ-diol enantiomers using a cellulose tris(4-methylbenzoate) column. (A) Chromatogram of the extract obtained from serum from a male who received no drug; (B) chromatogram of the extract obtained from drug-free serum with racemic CBZ-diol added (14.8 μM).

gave a separation factor of more than 2. However, the pressure in the column increased as the proportion of ethanol increased, and the baseline became unstable. Therefore, we used 75:25 (v/v) n-hexane–ethanol at a flow-rate of 0.5 ml/min, which gave a moderate column pressure (12 bar/cm^2).

The efficiency of the column could be maintained for 500 analyses of CBZ-diol enantiomers in serum. It is essential to check the baseline of the chromatogram before each analysis: occasional drifts can be eliminated by equilibration for 2 h.

3.2. Preparation of serum samples from epileptic patients for HPLC

The assay conditions using a Chiralcel OJ column and n-hexane–ethanol (75:25, v/v) allowed the separation of the peaks due to (S,S)- and (R,R)-CBZ-diol enantiomers from those for CBZ, CBZ-epoxide and HMCA. Under the conditions used, long elution times of >1 h were necessary for CBZ, CBZ-epoxide and HMCA, which was impractical for routine analyses of large numbers of serum samples. Therefore, a selective extraction of CBZ-diol was developed, that is, after the treatment of a serum sample with β-glucuronidase followed by deproteinization with alkaline zinc sulfate solution, CBZ, CBZ-epoxide, HMCA and a small part of CBZ-diol were removed by chloroform extraction.

The aqueous layer separated was applied to an Extrelut-1 column and the extract obtained using ethyl acetate was used for the chiral separation of CBZ-diol. In this case, CBZ and CBZ-epoxide were not detected on the chromatograms. The recoveries of this assay were 27.4 ± 2.2% and 26.8 ± 1.7% (means ± S.D.) for (S,S)-diol and (R,R)-diol, respectively, for the serum samples spiked with racemic CBZ-diol at a concentration of 0.4 μg/ml. The low recovery using serum might depend on deproteinization and chloroform extraction. However, as described below, the assay showed a small variance in the precision for both enantiomers and, of course, both enantiomers were recovered equally with the peak-area ratios (S,S/R,R) of 0.96–1.00 at concentrations of 0.4, 2 and 4 μg/ml of CBZ-diol racemate.

3.3. Concentration–response relationship

The UV absorbance of each CBZ-diol enantiomer varied linearly over the range 0.1–10.0 μg/ml. The calibration graph was as follows: slope = 0.0082 (S.D. 0.00023), intercept = −0.00026 (S.D. 0.00036) and regression coefficient $r = 0.998$ for (S,S)-diol, and slope = 0.0045 (S.D. 0.00018), intercept = −0.00043 (S.D. 0.00028) and $r = 0.995$ for (R,R)-diol.

3.4. Accuracy and precision

The analytical precision was assessed from the intra-day and inter-day variations in the concentrations from serum samples spiked with racemic CBZ-diol at concentrations of 0.4, 2 and 4 μg/ml. The data showed good reproducibility for CBZ-diol enantiomers (Table 1).

3.5. Limits of detection and quantitation

The limits of detection were 24 and 42 pg for the (S,S)- and (R,R)-diol, respectively, with a signal-to-noise ratio for CBZ-diol enantiomers of five. The limits of quantitation were 0.1 μg/ml for both at the lowest concentration of the calibration graph, at which the R.S.D.s were below 10%.

3.6. Determination of CBZ-diol enantiomers in serum

Fig. 3 shows a typical chromatogram for a serum sample taken from an epileptic patient receiving CBZ alone. The complete separation of CBZ-diol enantiomers were achieved within 20 min.

In sera from 54 epileptic patients who were given 100–600 mg of CBZ alone per day over a long period, the S,S/R,R ratio of CBZ-diol was 10.69 ± 1.11 (mean ± S.D.), that is, (S,S)-diol was predominantly observed, representing more than 90%. These results suggest stereoselectivity in

Table 1
Analytical accuracy and precision in the determination of CBZ-diol enantiomers in human serum samples

Enantiomer	Added[a] (μg/ml)	Accuracy (found) (mean ± S.D.) (μg/ml)		Precision (R.S.D.) (%)	
		Intra-day	Inter-day	Intra-day	Inter-day
(−)-(S,S)-Diol	0.2	0.20 ± 0.003	0.19 ± 0.01	1.7	5.9
	1.0	1.03 ± 0.02	1.03 ± 0.04	2.1	4.2
	2.0	2.00 ± 0.03	1.99 ± 0.06	1.5	3.1
(+)-(R,R)-Diol	0.2	0.19 ± 0.005	0.18 ± 0.01	2.5	5.7
	1.0	1.02 ± 0.03	1.01 ± 0.07	2.9	6.7
	2.0	2.01 ± 0.06	2.00 ± 0.10	2.9	5.1

The intra-day precision and accuracy of the assay were estimated by six measurements of each of three different concentrations. The inter-day precision and accuracy of the assay were tested by measurements of three different concentrations on six days over a 2-week period.
[a] Two-fold amounts of the listed amounts of CBZ-diol racemate were added.

the hydrolysis of CBZ-epoxide by epoxide hydrolase and/or further metabolism of CBZ-diol. The relationship between the $S,S/R,R$ ratio and the concentration of CBZ-diol in the case of 54 epileptic patients is shown in Fig. 4. The $S,S/R,R$ ratio remained constant independently of the variation of total CBZ-diol concentration in serum. The intra-day variation of the $S,S/R,R$ ratio was also kept constant at 10.94 ± 0.75 (mean ± S.D.).

4. Conclusion

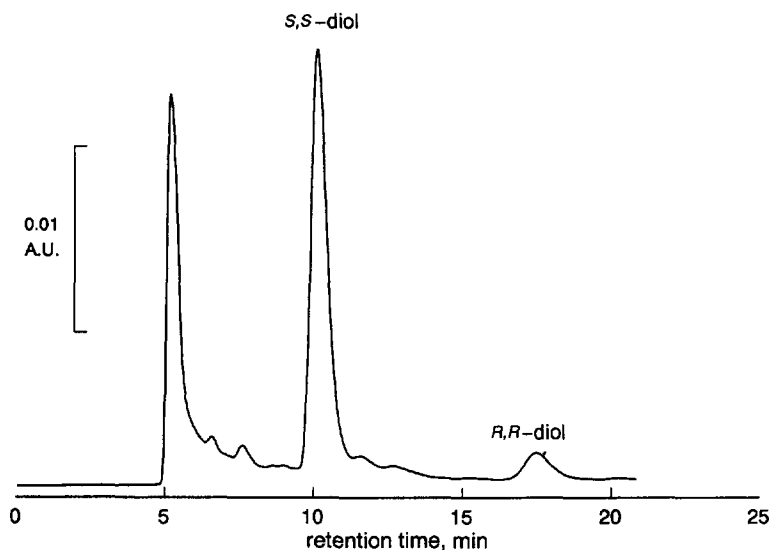A useful HPLC method was developed for the resolution of CBZ-diol enantiomers in serum and

Fig. 3. Typical chromatogram of an extract containing of CBZ-diol enantiomers separated with a cellulose tris(4-methylbenzoate) column. The extract was obtained from serum from a patient administered 600 mg of CBZ over a long period.
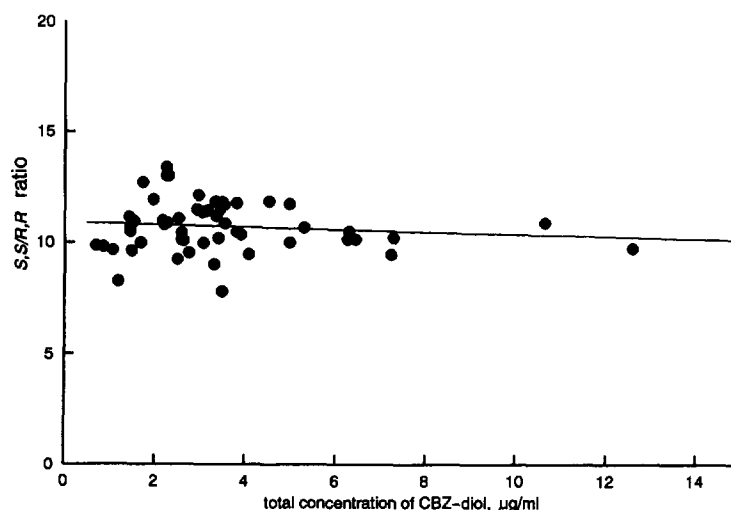
Fig. 4. Relationship between $S,S/R,R$ ratio and the concentration of total CBZ-diol in serum taken from 54 epileptic patients receiving CBZ alone for a long period.

the stereoselective disposition of CBZ-diol in serum from epileptic patients receiving CBZ was elucidated. CBZ-diol is an intermediate metabolite of CBZ, which is biotransformed into HMCA, detected as another major metabolite of CBZ [2].

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