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Journal of Chromatography B, 658 (1994) 385–390

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Simultaneous determination of antiepileptic drugs and their metabolites, including chiral compounds, via β-cyclodextrin inclusion complexes by a column-switching chromatographic technique☆

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First received 17 December 1993; revised manuscript received 6 May 1994

Abstract

Using β-cyclodextrin as a mobile-phase additive, a column-switching chromatographic system equipped with two 25-mm short ODS cartridge columns and two UV detectors was successfully employed for the simultaneous determination of some antiepileptic drugs and their metabolites, including chiral compounds, in human serum. The compounds examined were phenobarbital, zonisamide, phenytoin and its metabolites, *S*- and *R*-5-(*p*-hydroxyphenyl)-5-phenylhydantoin, *R*- and *S*-mephobarbital, carbamazepine and its main metabolites, 10,11-dihydro-10-11-epoxycarbamazepine and *trans*-10,11-dihydroxy-10,11-dihydrocarbamazepine (as a racemate), and allobarbital (as an internal standard).

1. Introduction

Several methods have been developed for the simultaneous determination of mixtures of antiepileptic drugs (AEDs) as well as their metabolites, including the optically active compounds [1]. In these studies, several chiral stationary phases were developed for the optical separation of many pairs of enantiomers [2–6]. Although a β-cyclodextrin (β-CyD) bonded column (Cyclobond I, Astec) is commercially available [2], it has not been applied for the simultaneous analysis of AEDs. The resolution of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH) enantiomers was achieved by the column, however, the column temperature had to be maintained below 22°C by cooling with a water jacket [4]. When we examined separation by this method at room temperature (26°C), an ineffective peak resolution below 0.7 was obtained.

On the other hand, we have previously reported the chiral resolution of *p*-HPPH enantiomers using a mobile phase containing β-CyD and a 150 × 4 mm I.D. ODS column [7]. However, the retention time of phenytoin (PHT) was too long to be practical for routine analysis.

☆ Part of this work has been presented at the 53th International Congress of Pharmaceutical Sciences (FIP), Tokyo, 1993.

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0378-4347/94/$07.00
SSDI 0378-4347(94)00246-2

Using 25 or 50 mm short ODS cartridge columns, we succeeded in the simultaneous separation of the chiral and achiral constituents in a serum sample taken from an epileptic patient, who received tablets containing phenobarbital (PB), PHT and mephobarbital (MPB) [8]. However, the problem of the long retention time of PHT still remained. In this paper, we present a practical column-switching HPLC system equipped with two 25-mm ODS cartridge columns and two UV detectors for the simultaneous separation of typical chiral and achiral AEDs and their metabolites.

2. Experimental

2.1. Chemicals

The structures of the AEDs studied in this work are shown in Fig. 1. Racemic *p*-HPPH was purchased from Aldrich Chem. (Milwaukee, WI, USA), and allobarbital, PHT, PB and β-CyD were from Tokyo Kasei Kogyo (Tokyo, Japan). Zonisamide was obtained from Dainihon Pharmaceutical Co. (Osaka, Japan). Carbamazepine (CBZ), 10,11-dihydro-10,11-epoxycarbamazepine (CBZ-epo) and *trans*-10,11-dihydroxy-10,11-dihydrocarbamazepine (CBZ-diol) racemate were from Ciba-Geigy (Basel, Switzerland). *R*-(+)- and *S*-(−)-*p*-HPPH were obtained by separation of the racemate with a chiral stationary phase as reported previously [6]. Pure enantiomers of MPB were kindly provided by Prof. A. Kuroiwa and Dr. K. Aoki (Showa University, Tokyo, Japan). The β-glucuronidase Type VII-A (from *Escherichia coli*) was obtained from Sigma (St. Louis, MO, USA). Other chemicals were of reagent grade, and the solvents for elution were of HPLC grade. Extrelut-1 was obtained from E. Merck (Darmstadt, Germany).

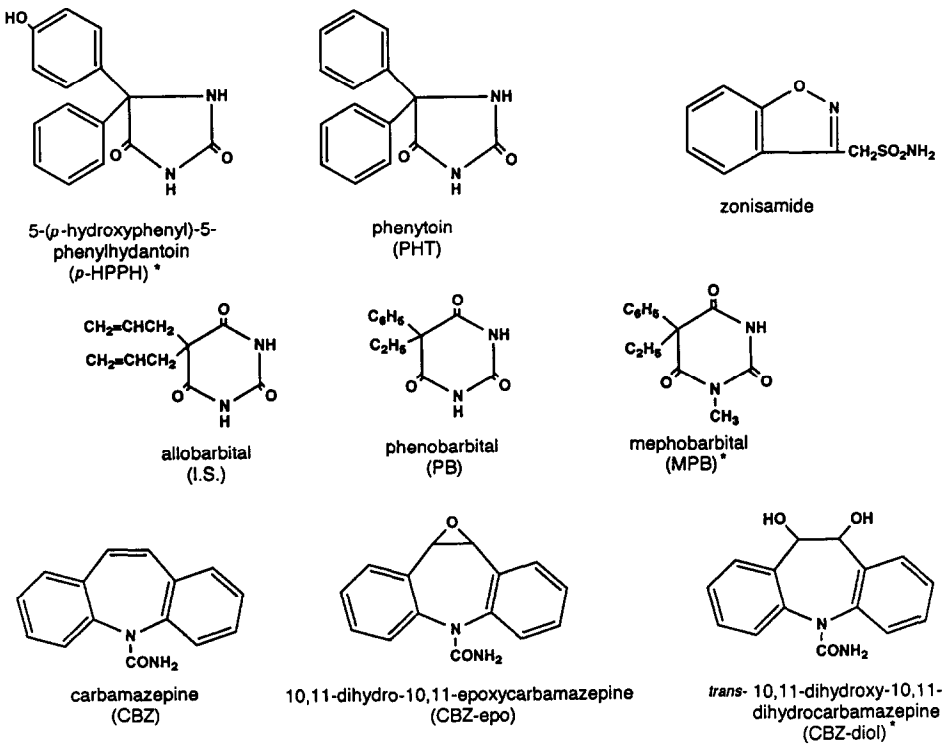

Fig. 1. Structures of the antiepileptic drugs and their main metabolites studied in this work. Compounds with chiral centers are marked with an asterisk.

2.2. Apparatus and HPLC conditions

The column-switching chromatographic system consisted of a Shimadzu LC-6AD HPLC system (Shimadzu, Kyoto, Japan) equipped with an integrator (Shimadzu C-R4A), two UV detectors (Shimadzu SPD-6AV) and a column-switching valve (Shimadzu, FCV-2AH) with a Shimadzu Model SCL-6A system controller. The ODS columns (25 × 4 mm I.D.) were LiChroCART HPLC-Cartridge Superspher RP-18e with 4 μm particle diameter (E. Merck). The column temperature was ambient (ca. 26°C). The mobile phase A was a mixture of 11.2 m*M* β-CyD in 20 m*M* KH_2PO_4 and methanol (95:5, eluent A), and the mobile phase B was 20 m*M* KH_2PO_4 containing 16% acetonitrile (eluent B). The β-CyD solution was filtered through Millicup-LCR membrane filter (0.5-μm, Millipore, Tokyo, Japan) before use. The flow-rate was 0.8 ml/min, and both UV detectors were set at 210 nm.

2.3. Analysis of serum samples from a patient

Before each sample preparation, 0.6 ml of 75 m*M* phosphate buffer solution (pH 6.8) containing allobarbital as an internal standard was added to 0.5 ml of serum taken from an epileptic patient receiving drug therapy. After treatment of the glucuronides of the metabolites in the serum sample with 200 units of β-glucuronidase at 37°C for 30 min, 1.0 ml of the mixed sample was poured onto the Extrelut-1 column. After 10 min, the column was eluted with 2.5 ml of *tert.*-butyl methyl ether. The eluate was dried under a gentle stream of nitrogen, and the residue was dissolved in 50 μl of a 1:1 mixture of methanol and water. Aliquots (10 μl) were injected onto the chromatographic system.

2.4. Recovery and precision

The precision for AEDs and their metabolites, including the enantiomers of *p*-HPPH or MPB, was described by the variation of the standard racemate spiked in healthy drug-free human serum. The average percentage recovery from the Extrelut-1 column was determined by comparing the analytical results of the standard sample in serum with those of the control samples in methanol.

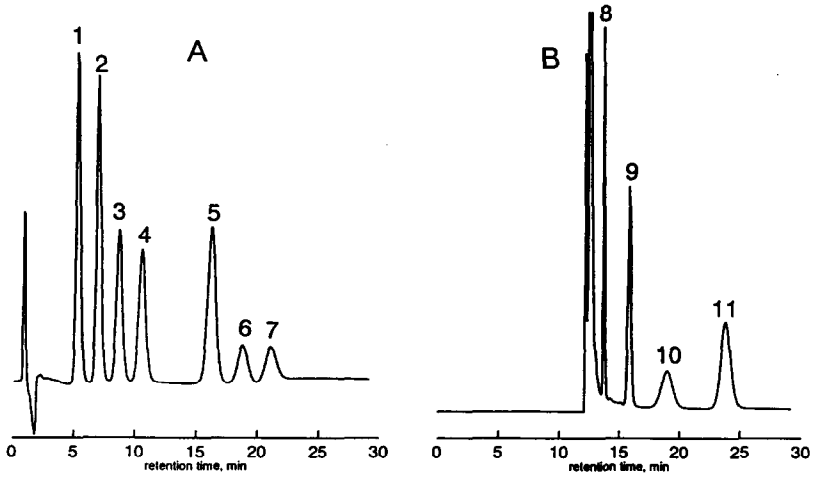

Fig. 2. Chromatograms of antiepileptic drugs and their metabolites obtained by (A) detector A, and (B) detector B with the column-switching system. Peaks: (A) 1 = PB; 2 = zonisamide; 3 = *S*-*p*-HPPH; 4 = *R*-*p*-HPPH; 5 = I.S.; 6 = *R*-MPB; 7 = *S*-MPB. (B) 8 = CBZ-diol; 9 = CBZ-epo; 10 = PHT; 11 = CBZ. Chromatograms were drawn by a personal computer as data of "ASCII" type converted from the chromatographic data obtained by the integrator of the HPLC system.

3. Results and discussion

3.1. Column-switching chromatographic system

When AEDs including PHT, PB, zonisamide, CBZ, CBZ-epo, the enantiomers of *p*-HPPH, MPB and CBZ-diol, and allobarbital (as an internal standard) in human serum were separated by HPLC using a 25-mm ODS cartridge column and β-CyD as a mobile phase additive, PHT, CBZ and their metabolites were eluted after retention times of more than 60 min, and the CBZ and CBZ-epo peaks overlapped. To eliminate these drawbacks we designed a column-switching system equipped with two 25-mm columns and two UV-detectors. Columns A and B were equilibrated with eluent A (20 m*M* KH_2PO_4 solution containing 11.2 m*M* β-CyD and 5% methanol), and then the mixture of AEDs was injected. Since PB, zonisamide, each enantiomer of *p*-HPPH and MPB, and allobarbital (I.S.) were eluted from column A within 12

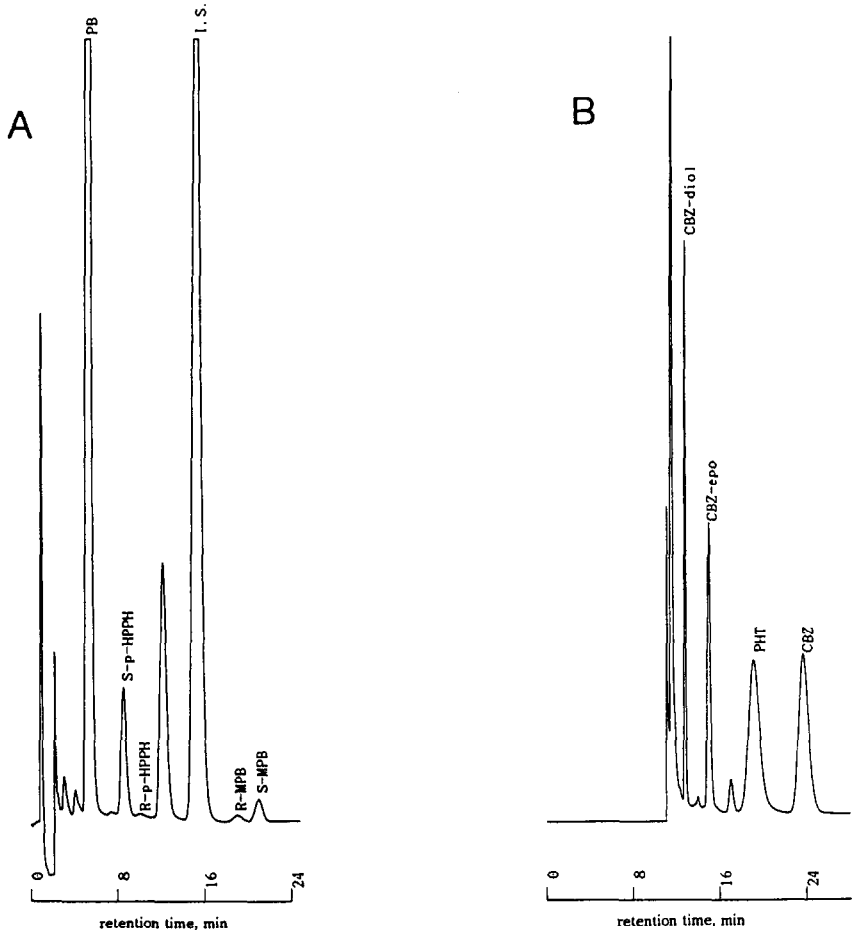

Fig. 3. Chromatograms of the extract of a serum sample taken from an epileptic patient administered 200 mg of CBZ and 100 mg each of PB, MPB and PHT. Chromatograms (A) and (B) were obtained by detector A and detector B with the column-switching system, respectively. The serum sample was drawn 2 h after dosing. The following concentrations were obtained: PB: 34.61 μg/ml; *S*-*p*-HPPH: 2.40 μg/ml; *R*-*p*-HPPH: 0.01 μg/ml; *R*-MPB: 0.48 μg/ml; *S*-MPB: 1.92 μg/ml; CBZ-diol: 1.69 μg/ml; CBZ-epo: 2.13 μg/ml; PHT: 6.85 μg/ml; CBZ: 2.98 μg/ml. Chromatograms were represented as the native data obtained by the integrator of the HPLC-system.

min, the valve was switched to column B at 12 min after a sample injection. The respective enantiomers of *p*-HPPH and MPB could be optically separated in column B by subsequent elution with eluent A, and were monitored with detector A (Fig. 2A). On the other hand, CBZ-diol racemate, CBZ-epo, PHT and CBZ retained on column A were eluted separately with eluent B extruded from pump B, followed by quantitation using detector B (Fig. 2B). After an analytical run, columns A and B were equilibrated again with eluent A for 5 min, and the next run was ready to start.

Chiral resolution of the CBZ-diol enantiomers could not be achieved by this system under the conditions described above.

3.2. Determination of AEDs in the serum of an epileptic patient

This column-switching system could be applied to the analysis of a serum sample taken from an epileptic patient who was given 200 mg of CBZ and 100 mg each of PB, MPB and PHT 2 h before blood sampling. No interfering peaks appeared within 25 min after injection as shown in Fig. 3A and B.

Different concentrations of these compounds spiked in healthy human serum were determined from the peak-height ratios of these compounds to that of the internal standard. The calibration curves exhibited excellent linearity. The correlation coefficient was greater than 0.9999 over the concentration range 0.1–20 μg/ml for each compound. The precision, accuracy, and detection limits for PHT, *S*- and *R*-*p*-HPPH, and other AEDs are summarized in Table 1.

The efficiency of the columns could be maintained for 1000 analyses of AEDs, even in biological fluids. It is essential to elute compounds retained on the columns with a proper mobile phase without any salt to maintain column performance.

Chiral separations of other optically active compounds by this method are now in progress.

Table 1
Analytical precision in the determination of antiepileptic drugs and their metabolites in serum samples

Drugs and metabolites	Within-day variation found (mean ± S.D., $n = 6$) (μg/ml)		R.S.D. (%)		Detection limits (ng/ml)
Concentration added (μg/ml)	0.5	10.0	0.5	10.0	
PB	0.49 ± 0.01	10.22 ± 0.12	2.6	1.3	1.9
Zonisamide	0.50 ± 0.01	9.94 ± 0.04	2.4	0.5	2.1
PHT	0.53 ± 0.03	9.93 ± 0.06	5.4	0.6	3.9
CBZ	0.49 ± 0.004	9.87 ± 0.12	1.0	1.3	2.2
Concentration added (μg/ml)	0.2	4.0	0.2	4.0	
S-*p*-HPPH	0.21 ± 0.003	4.05 ± 0.05	1.7	1.2	4.9
R-*p*-HPPH	0.21 ± 0.007	4.03 ± 0.04	3.9	1.1	5.7
CBZ-epo	0.20 ± 0.003	3.97 ± 0.06	1.7	1.6	3.9
Concentration added (μg/ml)	0.4	4.0	0.4	4.0	
CBZ-diol	0.41 ± 0.02	4.24 ± 0.17	3.9	4.1	2.2
Concentration added (μg/ml)	0.2	5.0	0.2	5.0	
R-MPB	0.22 ± 0.01	4.97 ± 0.03	7.3	0.7	11.6
S-MPB	0.22 ± 0.01	4.88 ± 0.07	4.8	1.6	12.8

Two-fold amounts of racemate of *p*-HPPH and MPB represented in the table were employed in the study.
The detection limits were defined as a signal-to-noise ratio of 3.

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