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Chiral separation of barbiturates and hydantoins by reversed-phase high-performance liquid chromatography using a 25 or 50 mm short ODS cartridge column via β-cyclodextrin inclusion complexes

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ABSTRACT

A high-performance liquid chromatographic method on a 25 or 50 mm short ODS cartridge column has been developed for the resolution of the enantiomers of some optically active barbiturates and hydantoins in human serum. β-Cyclodextrin was used in the mobile phase. This method also seems to be an easy and effective way to test whether β-cyclodextrin would be a useful chiral discriminator for a particular racemate.

INTRODUCTION

In more recent years, several chiral stationary phases have been developed. One of these is a β-cyclodextrin (β-CyD) bonded column (Cyclobond I, Astec) and its ability to separate the enantiomers of anticonvulsants, such as barbiturates and hydantoins, has been demonstrated [1–3]. We have previously determined the enantiomers of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH) formed by the metabolism of phenytoin (PHT) in the isolated rat hepatocyte system on a chiral tris(4-methylbenzoate) column (Daicel, Tokyo, Japan), with a mobile phase of ethanol–water [4].

β-CyD has also been used as a mobile phase component in the reversed-phase high-performance liquid chromatographic (HPLC) resolution of some racemic compounds. Sybilska *et al.* [5] separated the enantiomers of some barbiturates and hydantoins by this simple method. However, the resultant long retention times reduced the applicability of the method to biological samples.

Using a mobile phase containing β-CyD and a 150 mm × 4 mm I.D. ODS column, we also succeeded in a separation of *p*-HPPH enantiomers contained in the incubation mixture of PHT in isolated rat hepatocytes [6]. The precision, reproducibility and simplicity of the procedure were

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excellent, but the retention time was still too long. This disadvantage was now been overcome by using a short ODS cartridge column (50 or 25 mm × 4 mm I.D.) instead of an ordinary one.

EXPERIMENTAL

Materials

The structures of the optically active barbiturates and hydantoins studied in this work are shown in Fig. 1. (±)-*p*-HPPH and (±)-5-(*m*-hydroxyphenyl)-5-phenylhydantoin (*m*-HPPH) were purchased from Aldrich (Milwaukee, WI, USA), and (±)-hexobarbital, allobarbital, phenytoin (PHT), phenobarbital (PB) and β-CyD from Tokyo-Kasei Kogyo (Tokyo, Japan). (±)-Ethotoin and (±)-thiamylal were from Dainihon Pharmaceutical (Tokyo, Japan) and Kyorin Pharm. (Osaka, Japan), respectively. Each enantiomer of *p*-HPPH was obtained by the separation of the racemate with a chiral stationary phase as reported previously [4,6]. The pure enantiomers of hexobarbital and methylphenobarbital (MPB) were kindly provided by Prof. S. Toki and Dr. K. Miyano (Fukuoka University, Fukuoka, Japan) [7] and Prof. A. Kuroiwa and Dr. K. Aoki (Showa University, Tokyo, Japan), respectively. The β-glucuronidase Type VII-A (from *Escherichia coli*) was obtained from Sigma (St. Louis, MO, USA). Other chemicals were of reagent grade, and solvents for elution were of HPLC grade. Extrelut-1 columns were obtained from E. Merck (Darmstadt, Germany).

Apparatus and HPLC conditions

A Shimadzu LC-6A system (Shimadzu, Kyoto, Japan) equipped with an LC-6AD pump and a UV detector (SPD-6AV) was employed. The columns (50 or 25 mm × 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 phases were mixtures of 11.2 m*M* β-CyD in 33.3 m*M* KH_2PO_4 and different amounts of methanol. The flow-rate was 0.8 ml/min, and the eluates were monitored at 228 nm.

Calculation of stability constants

To calculate the inclusion stability constants in the mobile phases containing the appropriate amounts of β-CyD, the retention behaviour of the enantiomers of barbiturates and hydantoins

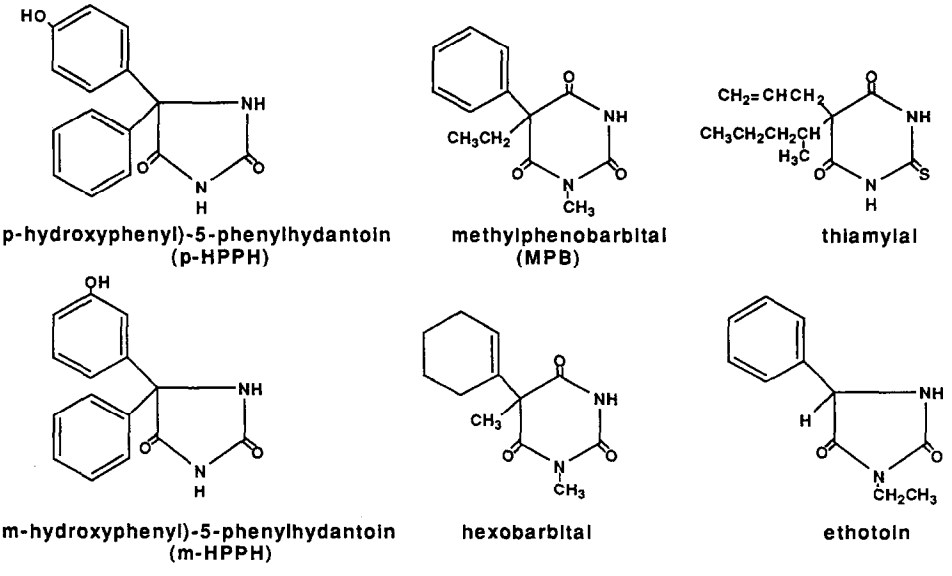

Fig. 1. Structures of the optically active barbiturates and hydantoins studied in this work.

was treated according to the method of Seno *et al.* [8]. The void volume of the column was determined by using reagent-grade potassium nitrate [9].

Procedure for analysis of serum samples

Before each sample purification, 0.6 ml of 100 m*M* phosphate buffer (pH 6.8) containing allobarbital as an internal standard (I.S.) were added to 0.5 ml of serum taken from an epileptic patient. After the treatment of *p*-HPPH glucuronide in the serum sample with 200 U of glucuronidase at 37°C for 30 min, 1.0 ml of the mixture was poured into the Extrelut-1 column. After 10 min, the column was eluted with 2.5 ml of *tert.*-butyl methyl ether. The eluate was dried, then dissolved in 100 μl of methanol; 20-μl aliquots were injected into the chromatograph.

Recovery and precision

The precision for each *p*-HPPH of MPB enantiomer was described by the variation of the standard racemate samples spiked in healthy drug-free human serum. In the case of *p*-HPPH enantiomers, the average percentage recovery from the Extrelut-1 column treatment was determined by comparing the analytical results of the standard sample in serum with those of the control samples in methanol.

RESULTS AND DISCUSSION

Chiral separation

Different organic modifiers (methanol, ethanol and acetonitrile) and different molarities of β-CyD and phosphate buffer were examined for their effects on the chiral separation of the selected barbiturates and hydantoins. The most satisfactory results were obtained when the mobile phase contained 11.2 m*M* β-CyD in a 94:6 (v/v) mixture of 33.3 m*M* KH_2PO_4 and methanol (Figs. 2 and 3A). The best detection sensitivity was obtained at 210 nm for the spiked samples in an organic solvent. However, excessive disturbance occurred at this wavelength in the analysis of serum samples, so 228 nm was used for the monitoring.

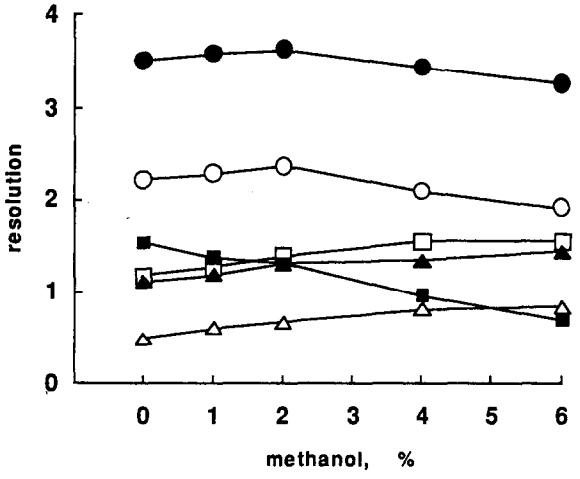

Fig. 2. Resolution of (●) *m*-HPPH, (○) *p*-HPPH, (□) MPB, (▲) hexobarbital, (■) ethotoin and (△) thiamylal enantiomers in 11.2 m*M* β-CyD mobile phase with a given methanol percentage.

A lower resolution of these enantiomers was obtained on a 25 mm ODS cartridge with a mobile phase of 8 m*M* β-CyD, 33.3 m*M* KH_2PO_4 and 1% methanol. The resolution for *p*-HPPH and *m*-HPPH enantiomers was 1.8 and 2.2, respectively, and 1.0 for the enantiomers of both MPB and hexobarbital. However, the enantiomers of thiamylal and ethotoin could not be resolved.

Determination of stability constant of β-CyD inclusion complexes

A more accurate estimate of the stability constants is possible with a shorter column (25 mm), because one can examine the retention of the analytes in the presence and absence of β-CyD as a component of the mobile phase (*cf.* ref. 5). The stability constants of the inclusion complexes between β-CyD and the substrates (*p*-HPPH, *m*-HPPH, MPB enantiomers and achiral PHT) were determined from the relationships between the reciprocal capacity factors and the concentrations of β-CyD (0–8 m*M*) in the mobile phase (Table I). The data indicate clearly that the resolution of the enantiomers is dependent on the magnitude of the difference between the stability constants of each enantiomeric complex with

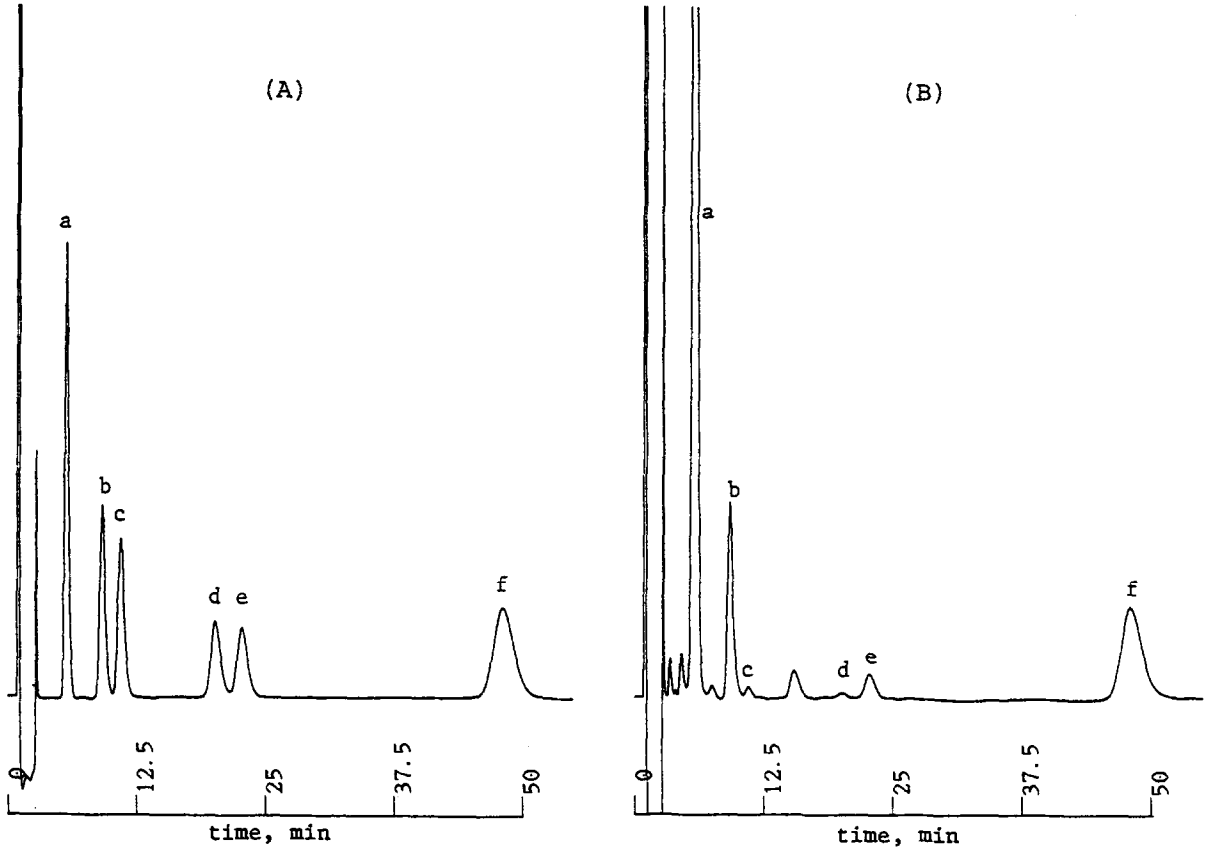

Fig. 3. Typical chromatograms of (a) PB, (b) *S*-*p*-HPPH, (c) *R*-*p*-HPPH, (d) *R*-MPB, (e) *S*-MPB and (f) PHT performed on a 50 mm ODS cartridge column. Mobile phase, 11.2 m*M* β-CyD in 33.3 m*M* KH_2PO_4–methanol (94:6, v/v); UV range, 0.05 a.u.f.s. (A) Chromatogram of methanolic solution of the compounds listed above. (B) Chromatogram of serum sample taken from a patient 2 h after the administration of 50 mg each of PB, MPB and PHT. The serum sample was extracted with *tert.*-butyl methyl ether after deglucuronidation by β-glucuronidase.

β-CyD, and each retention time becomes shorter as the stability of the complex increases.

Determination of p-HPPH and MPB enantiomers in serum samples

As mentioned above, the optimum chiral separation of barbiturates and hydantoins on the 50 mm ODS cartridge column was achieved with a mobile phase containing 11.2 m*M* β-CyD and 33.3 m*M* KH_2PO_4. Under the same conditions, PHT and the enantiomers of *p*-HPPH and MPB spiked in healthy human serum were determined by peak-height ratios using allobarbital as an internal standard (retention time 17 min). All compounds were resolved completely from each other, and the calibration curves exhibited excellent linearity, with a correlation coefficient greater than 0.9999 in the concentration range of 0.07–37 μ*M* for *p*-HPPH enantiomers and 0.08–41 μ*M* for MPB enantiomers, respectively. Fig. 3B shows a typical chromatogram of the serum sample taken from an epileptic patient. In this case, allobarbital (internal standard) was not added. However, no disturbing peaks were observed at *ca.* 17 min.

The lower detection limits of this method, defined as three times the level of baseline noise, were 15 and 18 n*M* for each enantiomer of *p*-HPPH and 47 and 52 n*M* for both enantiomers of MPB, respectively. The analytical precision

TABLE I

STABILITY CONSTANTS OF INCLUSION COMPLEXES

Compound	Stability constant (mmol/l)	Difference[a]	Resolution[b]
p-HPPH, *S*-form	3.2	1.2	1.4–1.8
p-HPPH, *R*-form	2.0		
m-HPPH[c]	6.0	3.4	2.2–2.9
	2.6		
MPB, *R*-form	3.2	0.4	0.6–1.0
MPB, *S*-form	2.8		
PHT	1.4	–	–

[a] These values show the difference between the stability constants of the two enantiomers.

[b] The resolutions were obtained by using 33.3 m*M* KH_2PO_4 mobile phases containing 1–8 m*M* β-CyD.

[c] The peak assignments of *m*-HPPH enantiomers were not investigated.

for each enantiomer of *p*-HPPH and MPB was excellent, as shown with samples prepared in two low concentrations by adding *p*-HPPH and MPB (Table II). At higher concentrations of these compounds (4–40 μ*M*), the precision was also excellent with standard deviations of less than 2%. The average recoveries of *p*-HPPH and MPB enantiomers from serum samples after the treatment by Extrelut-1 column were 75–80% (S.D. less than 3%).

In the serum of epileptic patients who were given 50–300 mg of PHT a day, the recovery of *S*-(−)-*p*-HPPH was dominant as reported by Steiner *et al.* [10]. Fig. 4 shows the representative examples obtained from two outpatients in our university hospital, that is, the *S*/*R* ratios of *p*-HPPH were above 19 and remained constant roughly during PHT administration.

CONCLUSION

We have established a simple and accurate HPLC method for the resolution of the enantiomers of barbiturates and hydantoins on a 25 or 50 mm ODS cartridge column, employing β-CyD as a component of the mobile phase. It also seems of value to make a preliminary examination of the type of drug separation by this method, which is an easy and effective way to test whether β-CyD would be a useful chiral discriminator for a particular racemate.

TABLE II

ANALYTICAL PRECISION IN THE DETERMINATION OF *p*-HPPH AND MPB ENANTIOMERS IN SERUM SAMPLES

Concentration enantiomers[a] (μ*M*)	Within-day variation (mean ± S.D., $n = 6$) (μ*M*)		Relative standard deviation (%)	
p-HPPH	(*S*)-(−)	(*R*)-(+)	(*S*)-(−)	(*R*)-(+)
0.37	0.37 ± 0.01	0.36 ± 0.02	3.3	6.5
1.86	1.89 ± 0.04	1.88 ± 0.04	2.4	2.2
MPB	(*R*)-(−)	(*S*)-(+)	(*R*)-(−)	(*S*)-(+)
0.41	0.45 ± 0.03	0.46 ± 0.02	7.4	4.0
2.03	2.02 ± 0.01	1.98 ± 0.03	0.7	1.6

[a] Two-fold amounts of each racemate represented in the table were employed in the study.

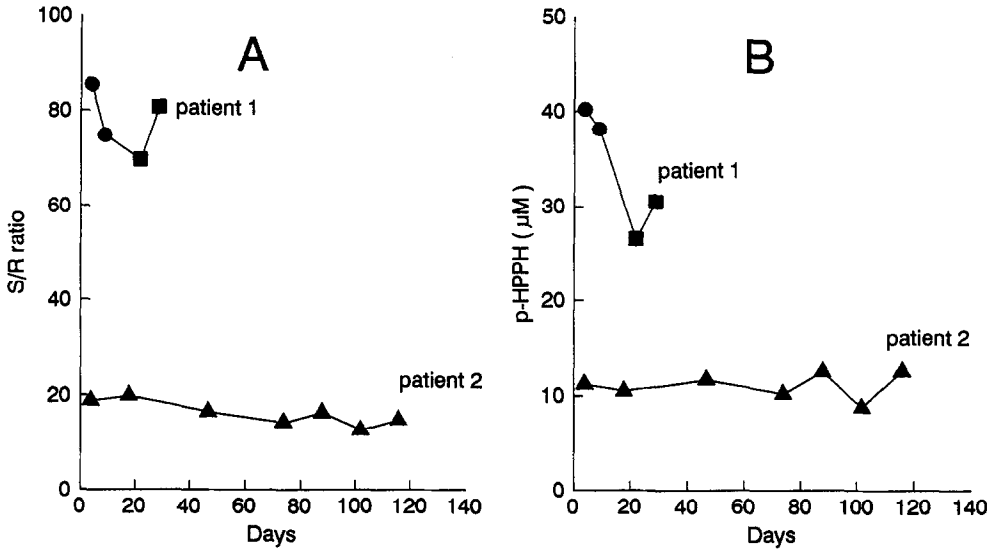

Fig. 4. *S/R* ratio (A) and total concentration (B) of serum *p*-HPPH during the period of PHT administration in two patients. PHT dose: 300 mg per day (●), 200 mg per day (■) in patient 1 and 213 mg per day (▲) in patient 2.

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