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High-performance liquid chromatographic method for direct separation of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin enantiomers using a chiral tris(4-methylbenzoate) column

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ABSTRACT

After simple purification of the incubation mixture of phenytoin in isolated rat hepatocytes, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (p-HPPH), which formed as a major metabolite, was readily resolved to each enantiomer by direct high-performance liquid chromatography on a cellulose tris(4-methylbenzoate) column, with a mobile phase of ethanol–water. It was also observed that the formation of *S*-(−)-p-HPPH was dominant, and the *S*/*R* ratio was 11.5.

INTRODUCTION

5-(*p*-Hydroxyphenyl)-5-phenylhydantoin (p-HPPH) is a main metabolite of the prochiral antiepileptic drug phenytoin (PHT), which was isolated first by Butler in 1957 [1] as almost pure levorotatory p-HPPH after repeated recrystallization from the urine samples of PHT-administered dogs and humans (Fig. 1). Both p-HPPH enantiomers have also been detected in the urine of patients on chronic PHT therapy, and the absolute configurations of the isomers were assigned by Poupaert *et al.* [2] as *S*-(−)- and *R*-(+)-structures for the major and minor urine components, respectively. Steiner *et al.* [3] determined the excretion amounts of *S*-(−)- and *R*-(+)-p-HPPH in the urine of the PHT-administered normal volunteers, and found that *S*-(−)-p-HPPH dominated with an average content of 94%.

The separate determination of *S*-(−)- and *R*-(+)-enantiomers of p-HPPH by high-performance liquid chromatography was reported by McClanahan and Maguire [4], to indicate the method whereby the enantiomeric contents of p-HPPH in human urine samples were determined on a chiral β-cyclodextrin column using

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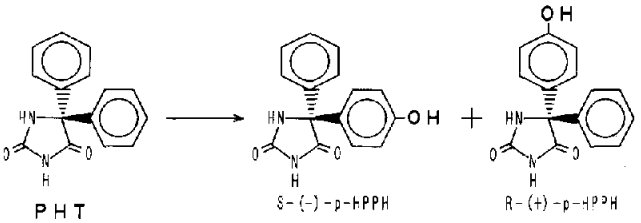

Fig. 1. Stereoselective formation of p-HPPH from PHT.

acetonitrile–water as the eluent. Fritz *et al.* [5] accomplished a chiral ligand-exchange chromatography, a method based on the diastereomeric formation of chiral nickel complexes between both hydantoins and L-prolinamide carrying a C_8 chain as a spacer molecule to the HPLC support. These methods, however, include some tedious prepurification procedures, because they were developed in order to determine p-HPPH enantiomers excreted in the urine of animals or humans.

This paper describes a simple and effective method for the isolation of p-HPPH from the metabolic reaction mixture of PHT in isolated rat hepatocytes. It also illustrates chiral resolution of the enantiomers by HPLC on a cellulose tris(4-methylbenzoate) packed column with a mobile phase of ethanol–water.

EXPERIMENTAL

Materials

PHT and p-HPPH racemate were purchased from Aldrich (Milwaukee, WI, USA) and Tokyo Chemical (Tokyo, Japan), respectively. Ethanol used for eluents was of HPLC grade from E. Merck (Darmstadt, Germany), and the other chemicals were of reagent grade. The Extrelut-1 column was obtained from E. Merck.

Apparatus and HPLC conditions

A Shimadzu (Kyoto, Japan) LC-6AD high-performance liquid chromatograph was equipped with a UV detector (Shimadzu SPD-6AV) and a Chiralcel OJ column packed with cellulose tris(4-methylbenzoate) (250 mm × 4.6 mm I.D., 10 μm particle size, Daicel, Tokyo, Japan). The column temperature was ambient (*ca.* 26°C). The mobile phase was water–ethanol (30:70, v/v), and the flow-rate was 0.5 ml/min. The eluates were monitored at 228 nm. The optical rotations of both enantiomers were measured in methanol at 589 nm and 26°C with a Nihon Bunko (Jasco, Tokyo, Japan) DIP-370 digital polarimeter.

Peak assignment

The fractions of the corresponding enantiomers were collected following re-

peated injections of 20-μl aliquots of an ethanol solution of p-HPPH racemate into the chromatograph. The collected eluates were extracted with *tert.*-butyl methyl ether. After drying over anhydrous sodium sulphate, the solvent was removed and the residues were dissolved in methanol for measuring the optical rotations.

Incubation of PHT in isolated rat hepatocytes

Isolated hepatocytes were prepared from male Wistar rats (200–220 g) according to the method described by Moldéus *et al.* [6]. After addition of 79.3 μM PHT to the hepatocyte suspensions ($3 \cdot 10^6$ cells per ml) the incubation was performed at 37°C under an O_2–CO_2 (95:5) atmosphere for 40 min. When the incubation had ceased, the reaction mixture was frozen immediately at −40°C until analysis. To examine the recovery and separation precision, the hepatocyte suspension was ultrasonicated, and different concentrations of p-HPPH racemate were added, from which the standard hepatocyte suspensions were prepared after treatment as described in *Sample purification.*

Sample purification

The incubated reaction mixtures of isolated rat hepatocytes were ultrasonicated for 1 min and centrifuged (1300 *g*). The supernatants were poured into the Extrelut-1 column (Merck), and after 10 min the column was eluted with 2.5 ml of *tert.*-butyl methyl ether. After drying the eluates, the residues were dissolved in ethanol, and 20-μl aliquots were injected into the chromatograph.

Recovery and precision

The average percentage recovery of p-HPPH enantiomers from standard hepatocyte suspensions spiked with 3.7 or 18.6 μM was determined by comparing the analytical results after work-up with those for the control ethanol solutions without work-up. The precision for each p-HPPH enantiomer was described by the variation of the standard hepatocyte suspensions spiked with 0.93, 1.86 or 9.30 μM p-HPPH after work-up (see the footnote to Table I).

RESULTS AND DISCUSSION

p-HPPH racemate in ethanol solution could be resolved effectively into the enantiomers by HPLC on a column packed with cellulose tris(4-methylbenzoate) (Chiralcel OJ) using ethanol–water eluent (Fig. 2A). The resolution between the enantiomers improved as the proportion of water in the eluent increased (Fig. 3). On the other hand, the pressure in the column increased as the proportion of water in the eluent increased. Columns packed with cellulose derivatives become unstable under higher pressure, so we used a 30:70 mixture of water–ethanol as the mobile phase at a flow-rate of 0.5 ml/min, which gave a moderate column pressure (54 bar/cm^2). Under these conditions, an excellent resolution was attained (resolution factor, $R_s = 2.2$).

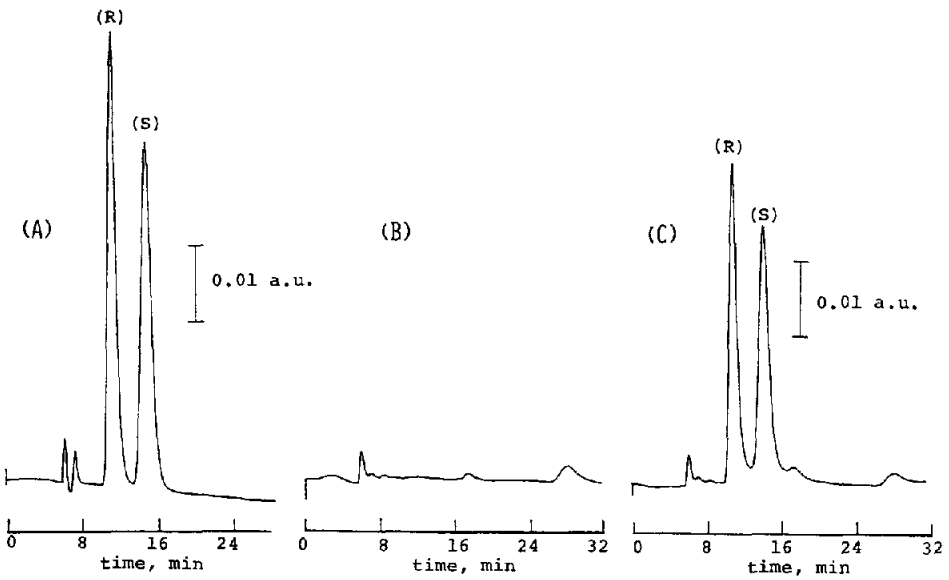

Fig. 2. Optical resolution of p-HPPH enantiomers on a cellulose tris(4-methylbenzoate) column. Mobile phase, ethanol–water (70:30, v/v); flow-rate, 0.5 ml/min. (A) Chromatogram of ethanolic solution of p-HPPH. (B) Chromatogram of sample solution obtained from the standard hepatocyte suspension without addition of p-HPPH. (C) Chromatogram of sample solution obtained from the standard hepatocyte suspension with added racemic p-HPPH (9.3 μM).

For the assignment of the peaks to the *R*-(+)- and *S*-(−)-enantiomers, sufficient of the fractions was collected for the optical rotations to be measured. The analytes corresponding to peaks with retention times of 10.7 and 14.0 min showed angles of rotation $[\alpha]_{589}^{26} = +20.0°$ and $-20.0°$, respectively (2.85 mg/ml in methanol). According to the elucidation of the absolute configuration of p-HPPH enantiomers by Poupaert *et al.* [2], we assigned *R*-(+)-p-HPPH to the isomer corresponding to the former peak and the *S*-(−)-isomer to the latter.

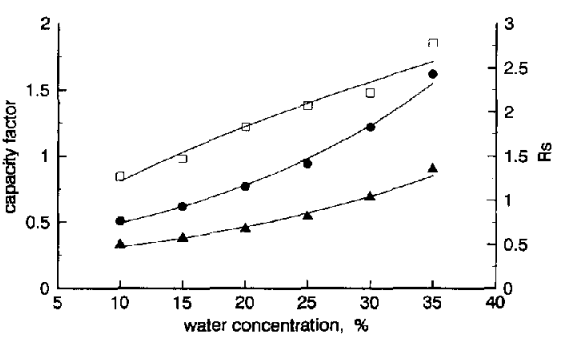

Fig. 3. Effect of water in the ethanolic mobile phase on the resolution of p-HPPH: (□) R_s (resolution); (●) (*S*)-(−)-p-HPPH capacity factor; (▲) (*R*)-(+)-p-HPPH capacity factor.

Both enantiomers were determined separately from p-HPPH-spiked standard hepatocyte suspensions, after sample purification as described in Experimental (Fig. 2C). The analytical precision for each enantiomer was determined for three p-HPPH concentrations (0.93, 1.86 and 9.30 μM) by adding p-HPPH, and the results were excellent (Table I). The standard curves for both enantiomers were linear over the concentration range 0.93–9.3 μM ($r = 0.9999$ for each curve). After each work-up, the average recovery from the standard hepatocyte suspensions spiked with 3.7 and 18.6 μM p-HPPH was 62–68% ($n = 6$, standard deviation 2.3–3.1%).

After addition of 79.3 μM PHT, the isolated rat hepatocyte suspension was incubated for 40 min at 37°C, and the ultrasonicated mixture was purified on the Extrelut-1 column to yield an ethanolic sample solution. The Chiralcel HPLC demonstrated that the main oxidized metabolite of PHT was *S*-(−)-p-HPPH (Fig. 4). The *S*/*R* ratio of p-HPPH formation was *ca.* 11.5, and remained almost constant throughout the incubation period.

Resolution of p-HPPH enantiomers by HPLC was performed previously by McClanahan and Maguire [4] and Fritz *et al.* [5]. Their methods, however, required lengthy pretreatment of samples for purification, such as preseparation by gas chromatography, reversed-phase column HPLC or thin-layer chromatography. Our pretreatment procedure using the Extrelut column is simple, effective and brief.

Okamoto *et al.* [7] and Krstulovic [8] established the optical resolutions of the enantiomers of various chiral compounds on cellulose or amylose derivative columns using aprotic or less-polar eluents. Recently, Rudolph [9] used methanol as a mobile phase on a tris(3,5-dimethylphenylcarbamate) cellulose (Daicel OD) column to resolve completely the enantiomers of saterinone. We first examined some organic solvents as eluents, but the enantiomers of p-HPPH could not be separated sufficiently. However, ethanol–water (70:30) gave an excellent resolution. The stability of the cellulose column to water in the eluent has not yet been examined, and the supplier of the column (Daicel) has recommended that water

TABLE I

ANALYTICAL PRECISION IN THE DETERMINATION OF p-HPPH ENANTIOMERS IN ISOLATED HEPATOCYTE SUSPENSION

p-HPPH enantiomers (μM)[a]	Within-day variation: found (mean ± S.D., $n = 6$) (μM)		R.S.D. (%)	
	(*S*)-(−)-p-HPPH	(*R*)-(+)-p-HPPH	(*S*)-(−)-p-HPPH	(*R*)-(+)-p-HPPH
0.93	0.90 ± 0.06	0.96 ± 0.06	6.5	6.0
1.86	1.86 ± 0.02	1.87 ± 0.02	1.2	1.2
9.30	9.30 ± 0.04	9.31 ± 0.04	0.4	0.4

[a] Two-fold amounts of the listed amounts of p-HPPH racemate were added.

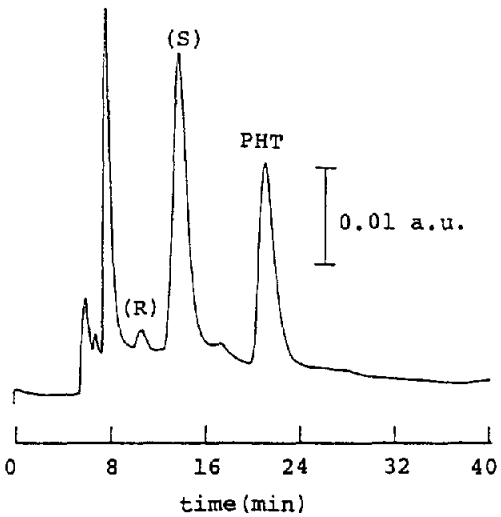

Fig. 4. Chromatogram of a sample prepared after 40 min incubation in isolated rat hepatocytes using 79.3 μM PHT.

be excluded as a component of the eluent [10]. In the first stage of our investigation, the number of theoretical plates for the separation of *R*-(+)-p-HPPH was 3700. However, after the analytical runs were repeated *ca.* 1000 times, the effectiveness of the separation increased and the number reached 15 000. The precise reason is not clear, but it is probable that the phenomenon is related to the swelling of cellulose particles in water contained in the eluent. This can be inferred from the fact that the retention times of p-HPPH enantiomers were increased by up to 3 min from the values observed during the initial examination.

We are also developing a chiral separation method by HPLC on an ODS reversed-phase column using eluents containing β-cyclodextrin, instead of the cellulose column mentioned above [11]. Further studies using these two analytical methods are in progress to elucidate the stereochemical modes of PHT metabolism, not only in isolated rat hepatocyte systems but also in various kinds of biological environment. The details will be reported in near future.

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