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Note

High-performance liquid chromatographic determination of the enantiomeric composition of urinary phenolic metabolites of phenytoin*

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Phenytoin (5,5-diphenylhydantoin, PHT) has been extensively prescribed for treatment of epilepsy since its introduction in 1938, despite toxic reactions associated with its use [1, 2]. Metabolic routes which may be involved in the development of these toxic reactions to PHT include the production of phenolic [5-(4-hydroxyphenyl)-5-phenylhydantoin; p-HPPH] enantiomers and dihydrodiol [5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin; DHD] diastereomers from arene oxide intermediates (Fig. 1). The latter are reactive species which may react with cellular nucleophiles to give covalently bound arenes [3]. As the arene oxides are transient metabolites, their stereoselective production can only be detected by monitoring the stereoselective production of the rearrangement products [(*R*)- and (*S*)-p-HPPH] or the formation of *trans*-dihydrodiols such as (*R*)- and (*S*)-DHD which are derived from (*R*)- and (*S*)-arene oxides of PHT, respectively. The quantitation of the isomeric forms of p-HPPH as described in this report and of DHD by a previously reported procedure [4] will provide an indirect method of identifying the isomeric arene oxides which might be formed by the metabolism of the prochiral phenyl substituents of PHT.

Stereoselective metabolism of the prochiral antiepileptic drug PHT to its major metabolite, p-HPPH, was first described by Butler [5]. The absolute configurations of p-HPPH enantiomers have been assigned as (*R*)-(+)-p-HPPH

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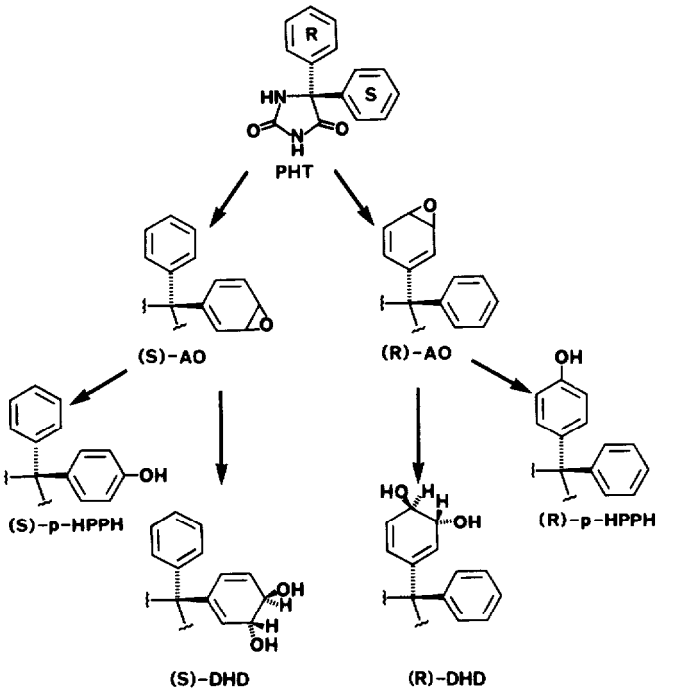

Fig. 1. Metabolism of PHT to p-HPPH enantiomers and DHD diastereomers via (*R*)- and (*S*)-arene oxide (AO) intermediates.

and (*S*)-(−)-p-HPPH [6], and both isomers have been found in the urine of patients on chronic PHT therapy. The high-performance liquid chromatographic (HPLC) resolution of (*R*)-p-HPPH from (*S*)-p-HPPH and from urinary constituents has been reported to occur using chiral ligand exchange chromatography [7]. Resolution of diastereomeric β-D-glucuronides of p-HPPH enantiomers has also been demonstrated [8]. Neither method of separation has proven to be readily applicable in this laboratory. Additionally, efforts to separate the enantiomers of p-HPPH as well as those of its O-acetyl derivative on either a covalent chiral Pirkle column or a N-(3,5-dinitrobenzoyl)-leucine column were unsuccessful [9]. We wish to describe a method whereby p-HPPH samples may be isolated from urine samples and the enantiomeric content determined by HPLC on a chiral β-cyclodextrin column using acetonitrile—water eluents.

EXPERIMENTAL

Chemicals

Samples of (*R*)-(+)-p-HPPH, (*S*)-(−)-p-HPPH and various mixtures of the two were available from previous studies [10], as were (*R*)- and (*S*)-DHD metabolites. Samples of racemic 5-(3-hydroxyphenyl)-5-phenylhydantoin and its enantiomers [(*R*)-(+)- and (*S*)-(−)-m-HPPH] were also available from previous studies [10]. Racemic 5-(2-hydroxyphenyl)-5-phenylhydantoin (o-HPPH) was kindly supplied by Dr. Kenneth H. Dudley (University of North Carolina, Chapel Hill, NC, U.S.A.). The preparation of o-HPPH is described elsewhere [11]. A partially purified solid preparation of β-glucuronidase from Helix

pomatia was purchased from Sigma (St. Louis, MO, U.S.A.). Acetonitrile and methanol, both HPLC grade, as well as all other analytical-reagent-grade chemicals were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). HPLC-grade water was produced from distilled water using a Millipore Norganic filter system from Waters (Milford, MA, U.S.A.).

Apparatus

A gradient system consisting of two Altex Model 110A pumps (Berkeley, CA, U.S.A.) coupled to a Rheodyne Model 7125 injector with a 20-μl loop (Cotati, CA, U.S.A.) and to an ISCO Model V4 variable-wavelength absorbance detector (Lincoln, NE, U.S.A.) was controlled by an Axxiom Model 710 microprocessor from Cole Scientific (Calabasas, CA, U.S.A.). Chromatographic peak areas were determined by a Hewlett-Packard Model 3390A reporting integrator (Avondale, PA, U.S.A.).

Standard urine sample preparation

To blank urine were added 7.7, 10.3, 16.9, 50.0 and 65.0% (*R*)-p-HPPH standard ethanolic solutions to obtain standard urine samples with concentrations of 5, 50 or 150 μg/ml p-HPPH (Table I). A 1.0-ml sample of these preparations was treated as described under *Urinary metabolite purification.*

TABLE I

STANDARD URINE SAMPLE PREPARATION

Concentration of p-HPPH in standard ethanolic solutions (mg/ml)	p-HPPH enantiomeric content [% (*R*)]	Concentration of p-HPPH in standard urine samples (μg/ml)	Ethanol concentration in standard urine samples (volume %)
2.5	10.3	5	0.4
2.5	50.0	5	0.4
2.5	65.0	5	0.4
2.5	7.7	50	4.0
2.5	10.3	50	4.0
2.5	16.9	50	4.0
2.5	50.0	50	4.0
2.5	65.0	50	4.0
7.5	10.3	150	4.0
7.5	50.0	150	4.0
7.5	65.0	150	4.0

Urinary metabolite purification

Human urine samples from pediatric patients on chronic PHT and other antiepileptic therapy were obtained and assayed for total p-HPPH and DHD content by gas chromatography (GC) [12]. After the GC assay, partially purified DHD and p-HPPH fractions were isolated from such urine samples by a previously described method [4]. Briefly, a urine sample was incubated with β-glucuronidase under conditions whereby all metabolite conjugates were released [12]. Following a solid-phase extraction procedure (Waters Sep-Pak C_{18} cartridges), metabolites (DHD and p-HPPH) were eluted and concentrated

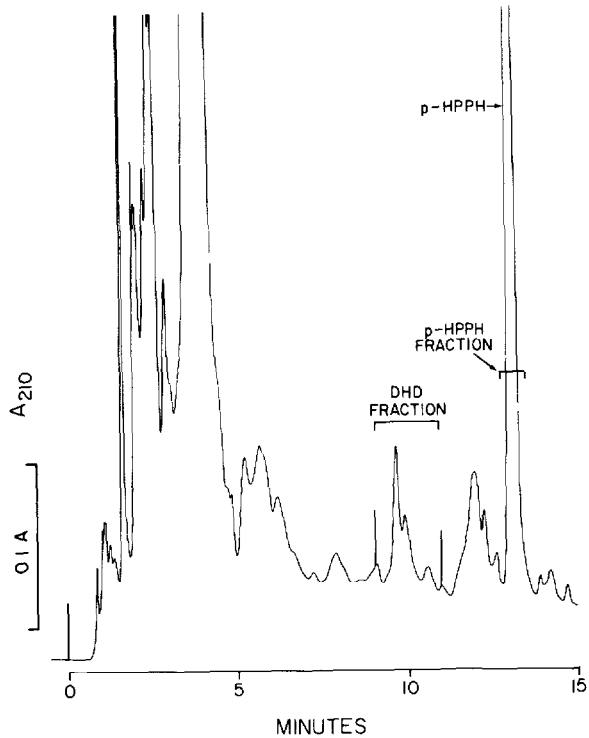

Fig. 2. Reversed-phase gradient separation of p-HPPH and DHD fractions from other urinary constituents after initial purification (Sep-Pak cartridge) of a human urine sample containing PHT metabolites.

prior to reversed-phase (C_{18}) chromatographic separation of DHD and p-HPPH fractions (Fig. 2). Columns, gradient conditions and instrumentation for this initial separation of DHD and p-HPPH samples were identical to those described [4], with the exception that the column was maintained at 22°C with a water jacket and circulating water bath. A fraction containing the DHD diastereomers was collected and the isomeric content was determined as previously described [4]. A 1-ml fraction containing the p-HPPH peak (Fig. 2) was collected, evaporated under reduced pressure, and the residue reconstituted initially in 15 μl of 100% methanol. This mixture was vortexed and diluted with 50 μl of 50% methanol—water.

Chromatographic assay of p-HPPH enantiomeric content

Isocratic chromatography of the purified reconstituted p-HPPH fraction was performed using a 0.5-μm in-line filter and a Cyclobond I column (5 μm, 250 × 4.6 mm I.D.) from Astec (Whippany, NJ, U.S.A.) maintained at 22°C with a water jacket and circulating water bath. The detection wavelength was 254 nm, the eluent was 20% acetonitrile—water at a flow-rate of 0.6 ml/min, and the injection volumes were 1—15 μl. Urinary p-HPPH enantiomeric content was calculated from the integrated areas of both the (*R*)- and (*S*)-p-HPPH peaks from the chromatogram (Fig. 3).

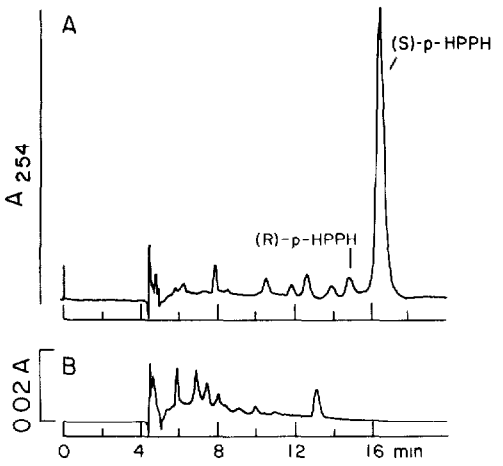

Fig. 3. (A) Chromatogram of the complete assay (β-cyclodextrin column) of a p-HPPH fraction from a human urine sample containing 10.5 μg/ml p-HPPH, which was composed of 95.2% (*S*)-p-HPPH. (B) Chromatogram of the complete assay of blank human urine.

Accuracy, precision and recovery studies

Triplicate samples of each of the eleven standard urine samples described above were treated as outlined under *Urinary metabolite purification*. The p-HPPH fraction was chromatographed on the Cyclobond I column and the enantiomeric content was calculated from the integrated areas of the (*R*)- and (*S*)-p-HPPH peaks. The enantiomeric content of each individual sample within each set of three was determined, allowing the inter-sample assay accuracy to be calculated. Precision was determined by analyzing single samples from each of the eleven sets for enantiomeric content on four consecutive days.

The recovery of urinary p-HPPH from the sample preparation procedure was estimated chromatographically. Upon HPLC analysis of all standard urine samples used to determine the assay accuracy, the peak areas of (*R*)- and (*S*)-p-HPPH in each sample were compared with those resulting from the HPLC analysis of appropriate dilutions of corresponding standard ethanolic solutions of p-HPPH. The recovery was calculated for each enantiomer in each sample as the ratio of the area under the chromatographic curve for that enantiomer in the urinary standard (after the complete assay) to the area under the chromatographic curve for that enantiomer in the standard ethanolic solution.

RESULTS

Baseline separation of (*R*)-(+)-p-HPPH and (*S*)-(−)-p-HPPH was readily achieved on a chiral β-cyclodextrin column (Cyclobond I) with 20% acetonitrile–water [resolution (*R*) = 1.30; capacity factor (k') = 1.70 and 2.0 for (*R*)- and (*S*)-p-HPPH, respectively; separation factor (α) = 1.2]. (*R*)-p-HPPH eluted first (15.0 min) as verified by an authenic sample while (*S*)-p-HPPH had a retention time (t_R) of 16.6 min. As p-HPPH enantiomers have identical UV spectra, integration of chromatographic peaks [(*R*)-(+) and (*S*)-(−) enantiomers] accurately reflected the enantiomeric composition of the urinary

p-HPPH. Aqueous solutions of either acetonitrile or methanol were used initially to obtain resolution of the pure p-HPPH enantiomers. However, with 40% methanol—water as the eluent (under optimum conditions for enantiomeric separation), chromatography of partially purified urinary p-HPPH fractions did not resolve (*R*)-p-HPPH from one endogenous component in human urine. The use of 20% acetonitrile resulted in baseline separation of both enantiomers and of this urinary constituent (t_R = 12.8 min). The majority of compounds in the urine gave no interference with the p-HPPH assay (Fig. 3) as their elution times were much earlier than that of the p-HPPH fraction which was collected in the initial reversed-phase chromatography (Fig. 2). A chromatogram of the complete assay of blank human urine is shown in Fig. 3. Similar results were achieved with blank human urine from three other volunteers.

Other possible interferences with the p-HPPH assay included the enantiomers of m-HPPH [(*R*)-(+) and (*S*)-(−)-m-HPPH] as well as 5-(4-hydroxy-3-methoxyphenyl)-5-phenylhydantoin (MCAT). The retention times for (*R*)- and (*S*)-m-HPPH on the β-cyclodextrin column using 20% acetonitrile—water as the eluent were 14.1 and 18.5 min, respectively (R = 2.60; k' = 1.60 for the (*R*) isomer and 2.40 for the (*S*) isomer; α = 1.50). The retention time for MCAT enantiomers under the same conditions was 10.3 min (R = 0). Thus, the MCAT and the enantiomers of a possible dehydration product of DHD, m-HPPH, did not interfere with the assay, as their retention times did not coincide with those of the p-HPPH enantiomers.

The initial gradient chromatographic procedure for collection of DHD and p-HPPH fractions was altered in the case of those patients treated with PHT and phenobarbital (PB). The latter drug, when present in urinary samples, co-eluted with the p-HPPH fraction during the initial separation of DHD and p-HPPH fractions on a C_{18} column (Fig. 2) and also co-eluted with (*R*)-p-HPPH on the β-cyclodextrin column, complicating quantitation of the enantiomeric content of p-HPPH. To alleviate this complication, the gradient elution program [4] for separation of p-HPPH from DHD on the C_{18} column was modified. The initial hold time at 10% acetonitrile—water was lengthened to 8 min and the subsequent gradient to 50% acetonitrile—water was flattened (10 to 50% in 20 min). These modifications have resolved PB and p-HPPH on the C_{18} column, thereby eliminating the interference of PB with p-HPPH enantiomeric content determination on the β-cyclodextrin column.

Assay accuracy for each p-HPPH enantiomer, as reflected by analysis of sets of three samples at five enantiomeric compositions and p-HPPH concentrations of 5, 50 and 150 μg/ml (values chosen to reflect observed dilute, normal and above normal metabolite concentrations in human urine samples), is shown on Table II. These standard urine samples were prepared from standard ethanolic solutions of p-HPPH with known enantiomeric content [7.7, 10.3, 16.9, 50.0 or 65.0% (*R*)-p-HPPH]. As can be seen from Table II, the enantiomeric content of the standard urine samples as determined by HPLC analysis following their complete assay does not differ significantly from the known enantiomeric content of the samples prior to the assay. This is the case for low p-HPPH concentrations (5 μg/ml), at commonly observed concentrations (50 μg/ml) and at higher concentrations (150 μg/ml).

Recovery of the p-HPPH enantiomers in this assay system was determined

TABLE II

ASSAY ACCURACY AND RECOVERY OF p-HPPH ENANTIOMERS FROM THE COMPLETE ASSAY OF STANDARD URINARY SAMPLES

p-HPPH enantiomeric content [% (*R*)]	Total p-HPPH concentration (μg/ml)	Accuracy (mean ± S D.)		Recovery (mean ± S.D) (%)	
		(*R*)-p-HPPH	(*S*)-p-HPPH	(*R*)-p-HPPH	(*S*)-p-HPPH
7.7	50	8.2 ± 1.0	91.8 ± 1 0	93 4 ± 11 0	101.4 ± 13.6
10 3	5	10.5 ± 1 9	89.5 ± 1 9	105 6 ± 11.5	100 5 ± 1 5
	50	10.6 ± 0 3	89.4 ± 0 3	104 5 ± 12.9	106 6 ± 11 2
	150	10 1 ± 0 7	89 9 ± 0 7	97.4 ± 7.4	103 0 ± 3 6
16.9	50	16 6 ± 0.9	83 4 ± 0 9	103.9 ± 5.4	107 3 ± 8 0
50.0	5	49 5 ± 1.9	50.5 ± 1.9	102 3 ± 13.1	104 8 ± 4.5
	50	49 3 ± 0.6	50.7 ± 0.6	Not determined	Not determined
	150	51.0 ± 0.7	49 0 ± 0 7	100 5 ± 6 8	98 6 ± 6 4
65.0	5	64.6 ± 1 2	35.6 ± 1 2	99 7 ± 9 2	97.7 ± 13.2
	50	63.5 ± 0.4	36.5 ± 0 4	91 3 ± 13 3	93.2 ± 14 2
	150	63.7 ± 0 1	36.3 ± 0 1	100 6 ± 7.2	102 1 ± 10 1

TABLE III

ASSAY PRECISION OF THE ANALYSIS OF p-HPPH ENANTIOMERS FOLLOWING THE COMPLETE ASSAY OF STANDARD URINE SAMPLES

p-HPPH enantiomeric content [% (*R*)]	Total p-HPPH concentration (μg/ml)	Coefficient of variation (%)	
		(*R*)-p-HPPH	(*S*)-p-HPPH
7.7	50	2.6	0.2
10.3	5	10 4	1.1
	50	0.9	0.1
	150	1.7	0.2
16.9	50	1.2	0.2
50.0	5	2.0	2.0
	50	0.4	0.4
	150	0.6	0.6
65.0	5	1.0	3.2
	50	1.4	2.6
	150	0.3	0.5

for all but one set of samples from the accuracy study. The results (Table II) show that the recovery of both enantiomers was essentially quantiative for the three concentrations of p-HPPH examined and for all enantiomeric compositions at those concentrations. Recovery values ranged from 91.3 to 107.3% with an overall average of 99.9 ± 4.7% for (*R*)-p-HPPH and 101.5 ± 4.3% for (*S*)-p-HPPH.

Prescision was determined by day-to-day analysis of enantiomeric content of single samples within each set and the results are summarized in Table III. The precision for the (*R*)-p-HPPH enantiomer at the lowest concentration [5

μg/ml, 10.3% (*R*)] was 10.4%. In all other cases, the coefficient of variation was less than 3.2%. As is shown by this data, the day-to-day variance of the assay results was not significant.

The detection limit for this assay was determined to be 1 μg of total p-HPPH per ml of urine for 10.3% (*R*)-p-HPPH (the commonly observed enantiomeric composition of p-HPPH in patients' urinary samples). Lower p-HPPH concentrations and percentage (*R*) enantiomeric composition could be measured by the use of larger volumes of urine samples. A second alternative to increase sensitivity would be UV monitoring at 210 nm which is approximately seven times more sensitive than UV detection of these enantiomers at 254 nm.

The average stereoselectivities observed for random urine samples from patients (n = 9) on chronic PHT were 10.7 ± 4.0% (*R*) and 26.2 ± 3.0% (*R*) for p-HPPH and DHD metabolites, respectively. Similar results were seen for patients (n = 13) on chronic PHT and PB therapy. In this case, the average stereoselectivities observed were 9.6 ± 2.6% (*R*)-p-HPPH and 28.3 ± 3.7% (*R*)-DHD.

DISCUSSION

The use of a β-cyclodextrin column for the determination of the enantiomeric content of urinary p-HPPH readily resolved the two isomers of this metabolite. The same conditions also resolved the two enantiomers of m-HPPH, a PHT metabolite observed only in the dog. Alteration of the acetonitrile content in the mobile phase to a lower percentage (15%) afforded partial resolution of the o-HPPH enantiomers (R = 0.80; k' = 3.3 and 3.5 for (*R*)- and (*S*)-o-HPPH, respectively; α = 1.1). As has been demonstrated, the use of the β-cyclodextrin column is quite versatile for the enantiomeric separation of the phenolic derivatives of PHT and has the diversity to lend itself well to an entire spectrum of applications.

Originally, methods were desired which would allow the determination of the diastereomeric content of DHD and of the enantiomeric content of p-HPPH as indirect measures of the stereoselective production of arene oxides from PHT metabolism. A method for the separation and determination of the diastereomeric content of the DHD isomers was developed [4]. With the use of the β-cyclodextrin column for the determination of the enantiomeric composition of p-HPPH, differences in the stereoselective metabolism of PHT to arene oxides and the subsequent conversion of the latter to DHD and p-HPPH isomers can be examined.

Previous assays from a study in which volunteers received a single 5 mg/kg intravenous dose of PHT indicated that the stereochemical composition of DHD and of p-HPPH metabolites was not altered with time after dose [13, 14]. Thus, the p-HPPH and DHD isomeric composition observed in random urine samples obtained from patients on chronic PHT therapy should reflect the stereoselective metabolism that would be observed in a 24-h urine collection. Assay of such samples from pediatric patients on chronic PHT or PHT and PB therapy showed decreased stereoselectivity in DHD formation as compared to p-HPPH formation. Possible interpretations of such results are described else-

where [13, 14]. Previous studies of stereoselective PHT metabolism by alternative methods had examined p-HPPH stereochemistry and suggested that the prochiral (*S*)-phenyl substituent of PHT is preferentially metabolized in humans [7, 8, 10, 11]. This is in agreement with the determinations of the stereochemistry of p-HPPH and DHD metabolites in pediatric patients on chronic PHT or PHT and PB (see Results). In both cases the predominant configuration of DHD and p-HPPH was that of the (*S*) isomer.

The methods discussed have provided the opportunity to examine the stereoselective pathways in human PHT metabolism. These studies are continuing and are currently utilized to observe the relationship between human stereoselective metabolism and toxic effects of PHT therapy in order to evaluate the role of PHT metabolic intermediates in PHT toxicities.

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