Journal of Chromatography, 342 (1985) 323—332
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2632

URINARY DIHYDRODIOL METABOLITES OF PHENYTOIN: HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF DIASTEREOMERIC COMPOSITION*

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(First received December 13th, 1984; revised manuscript received March 14th, 1985)

SUMMARY

Diastereomeric dihydrodiol metabolites of phenytoin, (5*S*)-5-[(3*R*,4*R*)-3,4-dihydroxy-1,5-cyclohexadien-1-yl]-5-phenylhydantoin, (*S*)-DHD, and (5*R*)-5-[(3*R*,4*R*)-3,4-dihydroxy-1,5-cyclohexadien-1-yl]-5-phenylhydantoin, (*R*)-DHD, have been resolved from each other and from urinary constituents with reversed-phase HPLC columns and acetonitrile—water gradients. Recoveries of DHD isomers from urine averaged 99.1% and it was demonstrated that known mixtures of DHD diastereomers added to blank urine were not altered by the assay procedures. The relative diastereomeric content of DHD was determined from integration of the chromatographic peaks. Assay of urine samples from patients on chronic phenytoin therapy and from volunteers indicated that both DHD isomers were present in all samples, and stereoselectivity favored the production of (*S*)-DHD.

INTRODUCTION

Metabolism of the prochiral antiepileptic drug phenytoin (5,5-diphenylhydantoin, PHT) to a dihydrodiol metabolite, 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin (DHD), was first demonstrated by Chang et al. [1]. Subsequent studies of DHD isolated from urines of dogs treated with PHT demonstrated the existence of two diastereomeric forms of the metabolite [2]. One of these isomers, designated as (*S*)-DHD, (5*S*)-5-[(3*R*,4*R*)-3,4-di-

*A preliminary report of a portion of this work was presented at the 1984 Annual Meeting of the American Society for Pharmacology and Experimental Therapeutics, Indianapolis, IN, U.S.A., August 20, 1984 [Pharmacologist, 26 (1984) 125].

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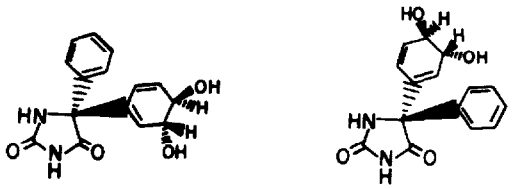

Fig. 1. Structures of (*S*)-DHD and (*R*)-DHD.

hydroxy-1,5-cyclohexadien-1-yl]-5-phenylhydantoin (Fig. 1), was found to be present in the urine of rats treated with PHT, and also in the urine of a patient on chronic PHT therapy. The existence of (*R*)-DHD, (5*R*)-5-[(3*R*,4*R*)-3,4-dihydroxy-1,5-cyclohexadien-1-yl]-5-phenylhydantoin (Fig. 1), in dog urine had been demonstrated, but minor amounts of (*R*)-DHD in rat and human urines could not be confirmed [2]. Previous reports of high-performance liquid chromatographic (HPLC) methodology for the study of DHD and other metabolites of PHT have used reversed-phase columns with methanol–water or acetonitrile–water eluents [3–6]. However, evidence of resolution of possible isomeric DHD by such methods has not been reported. We wish to describe methodology whereby DHD may be extracted from urine and its diastereomeric components successfully resolved and quantitated.

Our interest in the quantitation of the isomeric forms of DHD was to provide an indirect method of identifying the isomeric arene oxides which might be formed by the metabolism of the prochiral phenyl substituents of PHT. As arene oxides are transient metabolites, their presence can usually only be detected by monitoring rearrangement products (phenols) or *trans*-dihydrodiols, such as (*R*)- and (*S*)-DHD, which are derived from enzymatic hydration of putative (*R*)- and (*S*)-arene oxides of phenytoin, respectively.

EXPERIMENTAL SECTION

Chemicals

Samples of various mixtures of (*R*)- and (*S*)-DHD were available from previous studies [2], as were the isomeric racemic metabolites 5-(3-hydroxyphenyl)-5-phenylhydantoin (*m*-HPPH) and 5-(4-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH). The internal standard used for recovery studies, 5-ethyl-5-(4-hydroxyphenyl)hydantoin (EHPH) was available from a previous study [7]. 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.).

Apparatus

Two Altex Model 110A pumps (Berkeley, CA, U.S.A.) were used for solvent delivery and were coupled to a Rheodyne (Cotati, CA, U.S.A.), Model 7125 injector equipped with a 20-μl loop, and to an ISCO (Lincoln, NE, U.S.A.) Model V4 variable wavelength absorbance detector. Gradient programming and

system control were provided by an Axxiom Model 710 microprocessor from Cole Scientific (Calabasas, CA, U.S.A.). A Hewlett-Packard (Avondale, PA, U.S.A.) Model 3390A reporting integrator was used for measuring peak areas. A Millipore Norganic filter system from Waters (Milford, MA, U.S.A.) was used to produce HPLC grade water from distilled water. Sample preparation involved the use of Waters Sep-Pak C_{18} cartridges, Gelman (Ann Arbor, MI, U.S.A.) Acrodisc-CR 0.45-μm filters, and a Buchler (Fort Lee, NJ, U.S.A.) Evapo-Mix vortex evaporator.

Urine samples

Samples from pediatric patients on chronic PHT and other antiepileptic therapy were obtained during regularly scheduled pediatric neurology clinics at Duke University Medical Center (Durham, NC, U.S.A.) and at North Carolina Memorial Hospital (University of North Carolina School of Medicine, Chapel Hill, NC, U.S.A.). Informed consent of the parents was obtained and all studies were approved by the local institutional review boards. Samples from volunteers given a single 5 mg/kg i.v. dose of PHT were supplied by Dr. Gunnar Alván of the Department of Clinical Pharmacology of the Karolinska Institutet, Huddinge, Sweden. Samples of dog urine were obtained by the previously described procedure [2]. Rat urine was obtained following a 50 mg/kg i.p. dose to a female Sprague-Dawley rat, and collection of 0—48 h urine sample [2]. All types of urine sample were assayed for DHD and *p*-HPPH content by a gas—liquid chromatographic (GLC) method [8]. Samples containing 1 to greater than 50 μg DHD/ml of urine were selected for further assay of diastereomeric content of DHD.

Sample preparation

To 1.0 ml of urine in a 16 × 125 mm PTFE-lined screw-cap culture tube were added 1000 units of β-glucuronidase dissolved in 0.5 ml of 1.0 *M* sodium acetate buffer, pH 5.0. After incubation for 18 h at 37°C, the sample was transferred by disposable pipette onto a Sep-Pak cartridge which had previously been washed with 5 ml of methanol and then 5 ml of water. The urine sample was flushed through the cartridge, which was then washed with 5 ml of water. An Acrodisc-CR filter was attached to the bottom of the cartridge and 5 ml of 50% methanol—water was used to elute the sample. The bulk of the solvent was removed on the vortex evaporator, and the sample was completely dried using a stream of nitrogen gas. The residue was reconstituted in 50 μl of 50% methanol—water.

Chromatography

The standard chromatographic assay involved sample purification (Sep-Pak cartridges), gradient chromatography with collection of a DHD fraction (Fig. 2), followed by a second gradient chromatography of the purified DHD sample (Figs. 3 and 4). For both portions of the assay, the detection wavelength was 210 nm, the eluent was acetonitrile—water, and the columns were at ambient temperature (24—25°C).

A LiChrosorb RP-18 column (10 μm, 250 × 4.6 mm I.D.) from E.M. Reagents (Gibbstown, NJ, U.S.A.) and a 100 × 3.2 mm C_{18} guard column

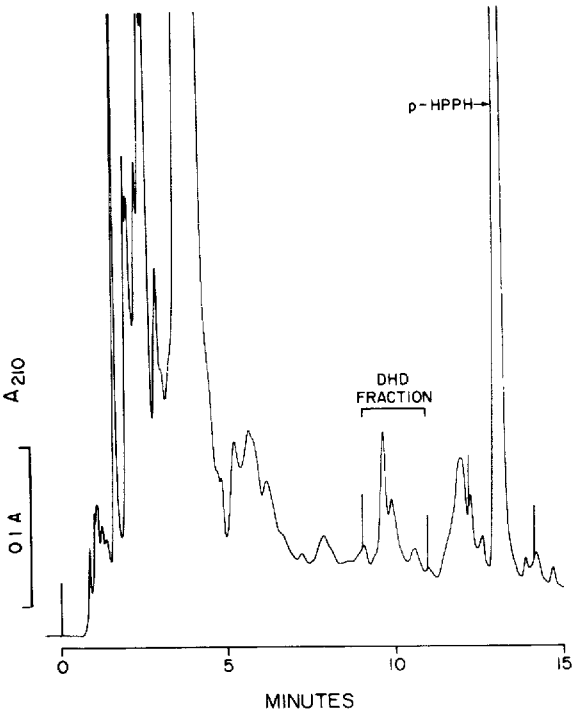

Fig. 2 Reversed-phase gradient separation (Program I) of DHD fraction from other urinary constituents and PHT metabolites after initial purification on a Sep-Pak cartridge. The urine sample contained 19 μg/ml of total DHD and was from a volunteer given a single dose of PHT.

(Whatman, Clifton, NJ, U.S.A.) were used for the initial chromatography (Fig. 2). The injection volume was 20 μl and the flow-rate was 2.0 ml/min. Gradient program I was as follows:

Step	Acetonitrile (vol. %)	Time (min)
1	10	0.00
2	10	4 00
3	50	10.00
4	5	1.00
5	5	2.00
6	10	2.00
7	10	End

A 4-ml fraction containing the DHD peaks (Fig. 2) was collected, evaporated under reduced pressure, and the residue reconstituted in 100 μl 50% methanol—water.

For the analytical determination a 0.5-μm in-line filter and a Microsorb C_{18} column (3 μm, 100 × 4.6 mm I.D.) from Rainin (Woburn, MA, U.S.A.) were used. The injection volume was 5 μl and the flow-rate was 1.0 ml/min.

Gradient program II was as follows:

Step	Acetonitrile (vol. %)	Time (min)
1	10	0.00
2	10	4.00
3	15	7.00
4	15	3.00
5	7	1.00
6	7	1.00
7	10	1.00
8	10	End

Peak verification and recovery studies

In order to verify the chemical content of DHD peaks, 1-ml fractions corresponding to (*S*)- and (*R*)-DHD from patient samples were collected during an analytical run. To each sample was added 0.25 ml of 12 *M* hydrochloric acid, and samples were placed on a steam bath for 30 min to dehydrate the dihydrodiols to their corresponding phenols (*p*-HPPH and *m*-HPPH). Upon cooling, the samples were neutralized with 0.25 ml 10 *M* sodium hydroxide and then extracted with 8 ml diethyl ether saturated with 0.25 *M* sodium phosphate buffer (pH 7.6). Samples (5 ml) of the extracts were evaporated and the residues reconstituted in 50 μl methanol. These samples, along with standard *p*- and *m*-HPPH solutions, were analyzed by HPLC on the 3 μm column using Program I. Retention times of *m*-HPPH and *p*-HPPH were 12.5 and 13.1 min, respectively.

For testing dihydrodiol recovery during sample preparation, two standard solutions were prepared to be 1:1 and 98:2 (*S*)-DHD/(*R*)-DHD, both of concentration 10 μg/ml water. From each of these solutions 1 ml was subjected to the sample preparation procedure while 1 ml was set aside to serve as control. Following the Sep-Pak cartridge elution step, 10 μl of an internal standard solution (100 μg EHPH/ml methanol) was added to both extracted and control samples. The samples were evaporated and the residues reconstituted in 100 μl 50% methanol—water prior to HPLC analysis (Program II). With this method, retention times of EHPH, (*S*)-DHD and (*R*)-DHD were 9.2, 12.5, and 13.4 min, respectively.

RESULTS

Assay development

Resolution of (*S*)-DHD and (*R*)-DHD from a 1:1 mixture of the purified metabolites [2] was achieved on a variety of ODS columns with acetonitrile—water eluents. (*S*)-DHD eluted first as verified with a sample known to contain largely (*S*)-DHD. Baseline resolution was achieved with 12% acetonitrile on a 10 cm, 3 μm spherical ODS column [resolution (R) = 2.2, k' = 14.1 for (*S*)-DHD, α = 1.12]. However, this isocratic system did not allow complete separation of DHD isomers from co-extracted endogenous compounds in human urine, and the following purification procedure was subsequently developed to circumvent this problem.

A urine sample was incubated with β-glucuronidase under conditions whereby all conjugated DHD should be released [8]. Metabolites were then extracted by a C_{18} Sep-Pak cartridge, and subsequent elution of the cartridge with 50% methanol gave a partially purified DHD fraction. This fraction was subjected to gradient chromatography (Program I) on a 25-cm, 10-μm irregular ODS column, and the DHD fraction was collected as indicated in Fig. 2. Subsequent gradient chromatography (Program II) of this concentrated DHD fraction on a 3-μm ODS column allowed separation of (*S*)- and (*R*)-DHD from interfering substances and from each other. Fig. 3A shows the chromatogram of the 1:1 standard mixture [2], and Fig. 3B the result obtained from the complete assay of blank human urine with 10 μg/ml of added 1:1 standard. It has been previously demonstrated that (*R*)- and (*S*)-DHD have identical UV spectra in the 240—270 nm range [2]. Integration of the chromatogram (Fig. 3A) of the 1:1 mixture at detection wavelengths from 210 to 270 nm gave identical 1:1 ratios, indicating the diastereomers have identical UV spectra in this range. The relative composition of DHD mixtures were thus determined from the ratio of peak areas measured by an electronic integrator. The chroma-

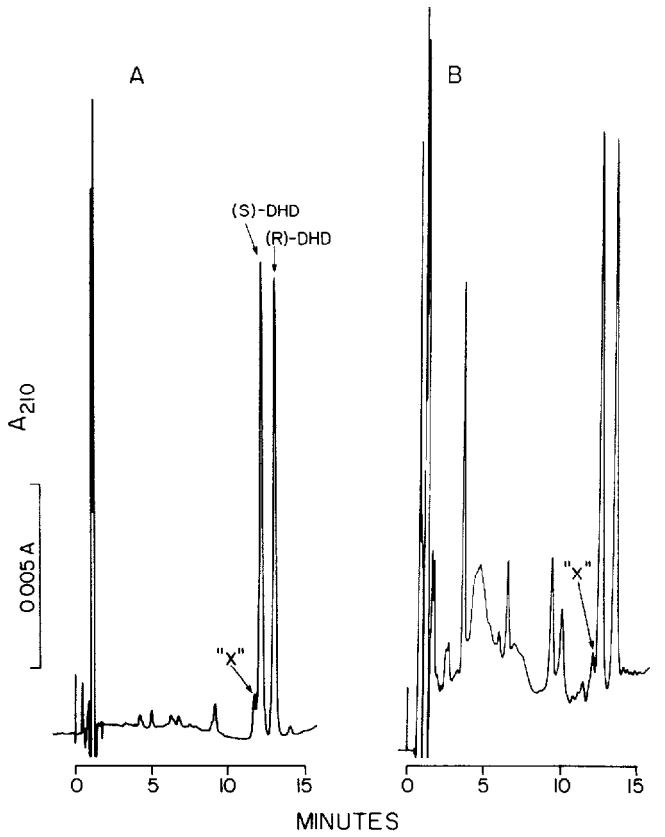

Fig. 3. (A) Chromatogram (Program II) of a 1·1 mixture of (*S*)-DHD/(*R*)-DHD previously isolated from dog urine [2]. "X" is an unidentified contaminant present in the original preparation. (B) Chromatogram of the complete assay of a blank human urine sample with 10 μg/ml of added 1:1 DHD mixture.

togram of an assayed blank urine sample, when monitored at either 210 or 254 nm, did not show any obvious interferences (Fig. 4A). The increased absorbance at 210 nm offered a 5—6 fold increased sensitivity over monitoring at 254 nm and this was the sole reason for chosing the lower wavelength.

Recovery and reproducibility of the assay

As it might be possible to alter inadvertently the ratio of diastereomers by incomplete extraction or elution, conditions were chosen such that maximal extraction of DHD from urine and recovery from the Sep-Pak cartridges were achieved. Recoveries of DHD from aqueous solutions were calculated by comparing peak area ratios of DHDs to an internal standard that was added to the aqueous DHD stock solution and to DHD fractions after Sep-Pak extraction/elution. When 10 μg/ml aqueous solutions of 1:1 and 98:2 mixtures of (*S*)-DHD/(*R*)-DHD were submitted to the Sep-Pak extraction/elution procedure, recoveries of 99.2 ± 0.5% (n = 4) and 99.0 ± 1.2% (n = 4), respectively, were observed. No detectable change in percentage (*S*)-DHD in the two samples could be observed after the extraction/elution procedure. With this procedure, it was not possible to calculate DHD recoveries from urine, as endogenous compounds coeluted with the internal standard. However, when blank urine samples with added 1:1 or 98:2 (*S*)-DHD/(*R*)-DHD mixtures, were assayed by the complete method, values of 50.5 ± 1.6% (*S*)-DHD (coefficient of variation (CV) = 3.2%, n = 4) and 98.4 ± 0.5% (*S*)-DHD (CV = 0.5%, n = 4), respectively, were obtained. These values were within experimental error of those of the corresponding aqueous DHD stock solutions. This evidence suggests that the recovery from urine is equivalent to that from water, and that extraction and collection of DHD through the assay procedure had not compromised the diastereomeric content of the DHD sample.

Evaluation of within-sample variability was performed with urine samples containing 82:18 and 98:2 mixtures of (*S*)-DHD/(*R*)-DHD. Repeated injections of DHD fractions from these two samples gave values of 81.9 ± 1.4% (*S*)-DHD (CV = 1.7%, n = 10) and 97.8 ± 0.8% (*S*)-DHD (CV = 0.8%, n = 10), respectively. Based upon the multiple assay of urines containing known DHD mixtures and the observation of within-sample variability, an error limit of ± 1% would routinely be applied to all samples assayed.

Verification of peak identity

In all human urine samples, both blank and with added DHD, little or no co-eluting endogenous substances have been found which interfere with quantitation of DHD. Fig. 4A is representative of a typical blank urine assay. Selected urine samples of patients or volunteers given PHT were carried through the assay procedure and collections of (*S*)- and (*R*)-DHD peaks were made. These samples were treated with acid to quantitatively dehydrate any DHD to a mixture of the isomeric phenols, *p*-HPPH and *m*-HPPH. The ratio of amounts of phenols produced from (*S*)-DHD to those produced by (*R*)-DHD fractions were within experimental error of the observed ratios for the intact DHD isomers, indicating that no UV-absorbing substances were interfering with DHD quantitation in these samples.

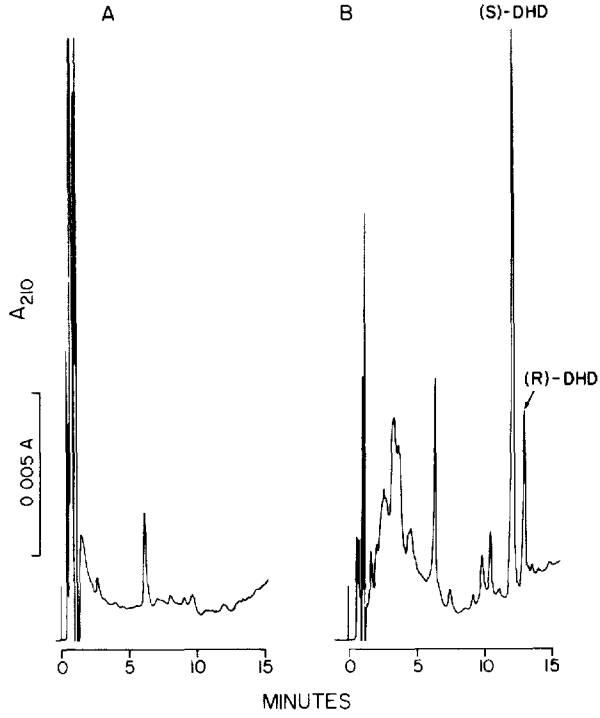

Fig. 4. (A) Chromatogram (Program II) of the complete assay of blank human urine. (B) Complete assay of a human urine sample containing 20 μg/ml of total DHD, which was found to contain 78% (*S*)-DHD.

Applicability of assay

Quantitation of relative amounts of DHD diastereomers was possible in urines of patients treated with PHT alone, and those treated with added valproic acid or phenobarbital. Examples of the stereoselectivity observed are shown in Table I. Patients treated with PHT and carbamazepine consistently had urinary product(s) with retention time(s) of approximately 14 min. Such materials interfered with the elution of (*R*)-DHD and made quantitation virtually impossible. It is assumed that the products represent metabolites of carbamazepine, although this has not been verified. Modification of the gradient elution conditions (longer initial hold time, 8 min) of Program I allowed improved separation of DHD from the urinary products, and the interference with the assay has largely been eliminated. Examples of the DHD composition obtained with the modified purification procedure are also shown in Table I.

It has also proved possible to assay DHD composition in the urine of other mammalian species dosed with PHT, with compositions from rat and dog urine having been found to be 98 and 43% (*S*)-DHD, respectively.

DISCUSSION

The existence of diastereomeric dihydrodiol metabolites of PHT, (*S*)-DHD and (*R*)-DHD (Fig. 1), has been confirmed by resolution of the purified

TABLE I

OBSERVED DIASTEREOMERIC COMPOSITION OF URINARY DHD IN PATIENTS (CHRONIC PHT THERAPY) AND VOLUNTEERS

VPA = Valproic acid; CBZ = carbamazepine; PB = phenobarbital.

Patient group	Average percentage (*S*)-DHD (range)
PHT alone (n = 7)	74 (69—77)
PHT, VPA (n = 3)	72 (68—78)
PHT, CBZ (n = 6)	73* (69—78)
PHT, PB (n = 2)	91 (86—96)
PHT, CBZ, PB (n = 3)	74 (57—88)
Volunteers (n = 7)	77 (75—79)

*Determined with the modified assay procedure (see *Applicability of assay*).

samples with the reversed-phase HPLC methodology reported here. The assayed composition of DHD from rat and dog urines has also provided evidence confirming the compositions that were previously assayed by chemical and instrumental methods [2].

The recovery of DHD isomers from urine and from Sep-Pak cartridges used for purification has been shown to be quantitative, and the assay procedures do not affect the diastereomeric content of known DHD mixtures added to blank human urine. This HPLC method is presently used for quantitation of relative amounts of DHD isomers in urine samples which have been previously assayed for total DHD content by a GLC method [8]. It is possible that the HPLC method could be expanded to allow quantitation of absolute amounts of the DHD isomers providing an appropriate internal standard could be found. The internal standard EHPH used in the recovery studies co-elutes with endogenous urinary constituents, however other elution conditions or other appropriate internal standards might be developed to allow quantitation of total amounts of DHD isomers.

Theoretically there should be four possible *trans*-dihydrodiol metabolites of PHT, (*S*)-DHD and its enantiomer, and (*R*)-DHD and its enantiomer. In the previous study, enantiomers of (*S*)-DHD and (*R*)-DHD were not detected, and this finding was reported to be consistent with the (*R*,*R*) stereochemistry that is prevalent in metabolic dihydrodiols of other aromatic compounds [2]. The chromatographic columns and conditions of this assay cannot differentiate between (*S*)-DHD and its enantiomer, or (*R*)-DHD and its enantiomer. While the results of the urinary DHD diastereomer assay are reported as % (*S*)-DHD (Table I), we cannot presently exclude the possibility that the peaks identified as (*S*)- and (*R*)-DHD may contain enantiomers of the respective compounds.

The results obtained from assay of human urinary DHD (Table I) suggest that both (*S*)- and (*R*)-DHD are being produced, with stereoselectivity favoring (*S*)-DHD. This provides the evidence of formation of two arene oxides of PHT

which are the precursors of the corresponding (*R*)- and (*S*)-DHD. Previous studies of stereoselective PHT metabolism have examined metabolic phenol (*p*-HPPH) stereochemistry and have suggested that the *pro-S*-phenyl substituent of PHT is preferentially metabolized in man [2, 9—11], with approximately 90% of the *p*-HPPH being of the *S*-configuration. The identification of (*S*)-DHD in human urine by this and a previous study [2] is consistent with an (*S*)-arene oxide being an intermediate in the formation of the (*S*)-phenol and (*S*)-DHD. The present study has demonstrated that a second arene oxide, the precursor to (*R*)-DHD, is also being formed in man. The stereoselectivity of DHD formation does not appear to vary for those patients on PHT alone, (74%(*S*)-), PHT and valproic acid (72%(*S*)-), or PHT and carbamazepine (73%(*S*)-) nor does it differ substantially from values obtained from volunteers (77%(*S*)-) given a single dose of PHT (Table I). Differences are observed in two patients on PHT and phenobarbital, and three patients on PHT, phenobarbital, and carbamazepine. This preliminary investigation suggests that some antiepileptic drugs may interfere with the stereoselective metabolism of PHT, and further such studies are in progress.

The HPLC methodology reported here allows the quantitation of diastereomeric content of DHD metabolites and can be used in conjunction with other assay methods for enantiomeric content of the phenolic metabolite (*p*-HPPH) [10, 11]. Application of such methods should allow a more complete study of stereoselective PHT metabolism in man and other species, and allow study of apparent differences in stereoselectivity observed for *p*-HPPH production [2, 9—11] and those presently observed for DHD production (Table I).

ACKNOWLEDGEMENTS

This study was supported in part by a R.J. Reynolds Industries Junior Faculty Development Award (J.H.M.) and by USPHS NIH Grant DE-06541. The authors wish to thank Dr. Gunnar Alván of the Karolinska Institutet for supplying urine samples, and Dr. Thomas C. Butler and Kenneth H. Dudley (Department of Pharmacology, University of North Carolina) for helpful discussions.

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