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Two-dimensional high-performance liquid chromatographic method to assay *p*-hydroxyphenylphenylhydantoin enantiomers in biological fluids and stereoselectivity of enzyme induction in phenytoin metabolism

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ABSTRACT

A two-dimensional high-performance liquid chromatographic method was developed to assay the enantiomers of a major phenytoin metabolite, *p*-hydroxyphenylphenylhydantoin (p-HPPH). Racemic p-HPPH was first separated from phenytoin and other interfering peaks by a reversed-phase column and monitored by an ultraviolet detector. At the retention time of p-HPPH, the racemic p-HPPH peak was automatically transferred to a chiral ligand-exchange column to separate *R*-p-HPPH and *S*-p-HPPH by a time-programmed column-switching valve. The ratio of enantiomers was measured by a second ultraviolet detector. The method can be used to assay *R*- and *S*-p-HPPH enantiomers with reasonable sensitivity and reproducibility. By using this method, the stereoselectivity of enzyme induction and inhibition of phenytoin metabolism was investigated. Male rats were treated with phenobarbital, 3-methylcholanthrene, acetone, Aroclor 1254, pregnenolone-16α-carbonitrile, dexamethasone and isosafrole. Microsomes were prepared from the rat liver and phenytoin hydroxylation was measured. Pretreatment with phenobarbital, pregnenolone-16α-carbonitrile or acetone induced phenytoin metabolism non-stereoselectively. Pretreatment with dexamethasone decreased *R*-p-HPPH formation without affecting the formation of *S*-p-HPPH. Liver microsomes from female rats showed a higher *S*-p-HPPH formation, whereas *R*-p-HPPH formation remained the same. Various inhibitors were added to inhibit phenytoin metabolism by control microsomes. Sulphaphenazole, ketoconazole, 4,4-di(*p*-methoxyphenyl)hydantoin, cimetidine and diazepam inhibited the formation of *R*- and *S*-p-HPPH. Quinidine, tolbutamide and mephenytoin showed no significant inhibitory activity. None of these inhibitors showed stereoselectivity.

INTRODUCTION

The complex metabolism of the antiepileptic drug phenytoin has been extensively studied because of its clinical importance. Several metabolites, including 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (p-HPPH) [1], 5-(*m*-hydroxyphenyl)-5-phenylhydantoin (m-HPPH) [2], 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin (DHD) [3], 5-(3,4-dihydroxyphenyl)-5-phenylhydantoin (diHPPH) [4,5] and 5-(3-methoxy-4-hydroxyphenyl)-5-phenylhydantoin (MeOCat) [5,6] have been reported previously. Phenytoin is a prochiral compound, and hydroxylation occurs on either pro-*S* or pro-*R* phenyl rings, resulting in the formation of enantiomers for each metabolite. In the dog, *R*-m-HPPH is the predominate metabolite [7]. In rats, mice and humans, *S*-p-HPPH [8–10] and *S*-DHD [11] are the major metabolites.

Previous reports [12] have mainly described assays of phenytoin metabolites as their racemates. Two assay methods for p-HPPH and m-HPPH enantiomers have recently been reported [13,14].

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One used thin-layer chromatography to separate p-HPPH from other compounds before ligand-exchange separation [13]; the other used reversed-phase gradient high-performance liquid chromatographic (HPLC) separation and collected the p-HPPH peak to inject onto a β-cyclodextrin column [14]. Both methods required tedious work to collect the racemic p-HPPH peak before enantiomeric separation. In order to study the stereospecificity of enzyme induction and inhibition of p-HPPH formation, we modified the published method into a two-dimensional HPLC method by using a column-switching technique. The modified method allowed us to use an automatic injector to process a large number of samples.

EXPERIMENTAL

Chemicals

Phenytoin, racemic p-HPPH, NADP, glucose-6-phosphate, phenobarbital (PB), 3-methylcholanthrene (3-MC), dexamethasone (DEX), ketoconazole, quinidine, tolbutamide, and *tert.*-butyloxycarbonyl-L-proline (Boc-L-proline) hydroxysuccinimide ester for the synthesis of octyl prolinamide were from Sigma (St. Louis, MO, USA). Aroclor 1254 was obtained from Alltech Assoc. (Deerfield, IL, USA). Sulphaphenazole was obtained from Taiwan Dainippon Pharmaceutical. Mephenytoin was obtained from Sandoz Pharmaceutical (East Hanover, NJ, USA). Diazepam was obtained from Roche Labs. (Nutley, NJ, USA). Cimetidine was obtained from Smith Kline & French Labs. (Philadelphia, PA, USA). Acetonitrile and methanol (LC grade) were from J. T. Baker (Phillipsburg, NJ, USA). Glucose-6-phosphate dehydrogenase was obtained from E. Merck (Darmstadt, Germany). 1-Amino-octane and isosafrole (ISF) were from Fluka (Buchs, Switzerland). Dried tetrahydrofuran (max. 0.01% water) was obtained from E. Merck, and further dried with $LiAlH_4$ (E. Merck) and distilled before the usage. Pregnenolone-16α-carbonitrile (PCN) was a generous gift from Upjohn (Kalamazoo, MI, USA). 5,5-Di(*p*-methoxyphenyl)hydantoin was provided by Dr. H. P. Wang (School of Pharmacy, National Taiwan University). Octyl L-prolinamide was synthesized as described by Fritz *et al.* [13]. In brief, equimolar amounts of 1-amino-octane and of Boc-L-proline hydroxysuccininamide were dissolved in dried tetrahydrofuran and stirred for 3 h. The reaction product, Boc-L-proline-octylamide was extracted and then hydrolysed overnight with 10% trifluoroacetic acid in dichloromethane. The residue after extraction with diethyl ether was used for chiral ligand exchange chromatography. Other chemicals were mostly from E. Merck and were of the highest purity available.

HPLC method

A two-dimensional HPLC method was developed to quantitate *R*-p-HPPH, *S*-p-HPPH, and phenytoin from the microsomal samples. Part of the method was modified from the chiral ligand exchange method reported by Fritz *et al.* [13]. The HPLC system consisted of two pumps (Models 510 and 501, Waters Assoc., Milford, MA, USA), an automated gradient controller (Model 680, Waters Assoc.), an automatic injector (Model 712, Waters Assoc.), a reversed-phase column (LiChrospher RP-18, 150 mm × 4 mm I.D., 5 μm particles; E. Merck) for separating racemic p-HPPH and phenytoin, a ligand-exchange column (LiChrospher RP-18, 150 mm × 4 mm I.D., 5 μm particles, pre-equilibrated with octyl-L-prolinamide in mobile phase; E. Merck) for separating *R*-p-HPPH and *S*-p-HPPH, a temperature-control module (Waters Assoc.) to heat both columns, a column-switching valve (Waters Assoc.), two UV detectors (detector A: Model M490, Waters Assoc.; detector B: Model 757, ABI Analytical, Kratos Division, Ramsey, NJ, USA), and a two-channel integrator (Model 745, Waters Assoc.). The HPLC system is shown diagrammatically in Fig. 1. Between pump B and the switching valve there was a LiChrosorb RP-18 (25–40 μm particles, E. Merck) precolumn to saturate the mobile phase with dissolved silica. The mobile phase for the reversed-phase column was 21% acetonitrile in 35 m*M* monobasic ammonium phosphate at a flow-rate of 1.2 ml/min. The

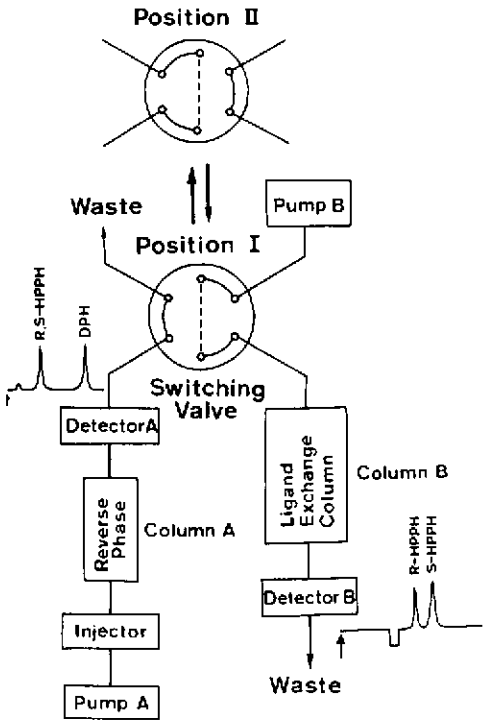

Fig. 1. Schematic diagram of the two-dimensional HPLC system for separating p-HPPH and phenytoin in a reversed-phase column and separating *R*-p-HPPH and *S*-p-HPPH in a ligand-exchange column. At position I, the eluent from column A is fed directly to waste. At position II, the eluent from column A passes through the loop before going to waste. For most of the time, the switching valve is at position I: when the p-HPPH peak passes through detector A, the valve is switched to position II and back to position I.

eluent was monitored at 235 nm in both detectors. The mobile phase for ligand-exchange separation was 0.375 m*M* octyl-L-prolinamide, 0.1 *M* ammonium acetate and 1.5 m*M* nickel(II) acetate in 50% methanol, adjusted to pH 9 with 25% ammonium hydroxide, at a flow-rate of 1.2 ml/min. The column temperature was 35°C for most studies. For reversed-phase separations, the retention times of p-HPPH and phenytoin were 6.3 and 21 min, respectively. The column-switching valve was installed with a 100-μl loop, and the eluent between 6.25 and 6.35 min was trapped in the loop (position II) by the automated time programme of the controller. The contents of the loop were transferred to the ligand-exchange column (position I). The retention times of *R*-p-HPPH and *S*-p-HPPH (time from the injection) were 12.5 and 14.1 min, respectively. The peak areas of racemic p-HPPH and phenytoin were compared with those of standards to calculate the concentrations. The ratio of the peak areas of the p-HPPH enantiomers was compared with that of the standard (racemic p-HPPH) to determine the concentration ratio of the two enantiomers.

Experimental animals and enzyme induction

Male and female Sprague–Dawley rats bred in the Animal Supply Center of National Cheng Kung University were used throughout the study. They were maintained on a standard diet of Purina lab. chow and water *ad libitum*. Rats of 150–200 g were used.

In each enzyme induction study, rats were treated as follows: (1) PB, 75 mg/kg in alkaline water injected intraperitoneally for four days (n = 4); (2) 3-MC, 25 mg/kg in corn oil injected intraperitoneally for four days (n = 10, control group n = 8); (3) isosafrole, 150 mg/kg in corn oil injected intraperitoneally for four days (n = 9, control group n = 10); (4) Aroclor 1254, 300 mg/kg in corn oil injected intraperitoneally for four days (n = 10, control group n = 9); (5) PCN, 250 mg/kg in 16.7% Tween 20 for four days (n = 10, control group n = 9); (6) DEX, 300 mg/kg in 16.7% Tween 20 for four days (n = 9, control group n = 10); (7) acetone, 5% acetone in feeding water *ad libitum* for ten days (n = 11, control group n = 10). For each treatment study, a separate group of animals was treated with vehicles. For a comparison of the sex difference of metabolism, two groups of untreated male (n = 10) and female (n = 11) rats were used.

Preparation and incubation of liver microsomes

Livers of decapitated rats were minced and homogenized at 0°C in four volumes of cold 1.15% KCl solution using a Potter–Elvehjem homogenizer with a PTFE pestle. The homogenate was centrifuged at 9000 *g* for 20 min at 4°C. The supernatant was then centrifuged at 100 000 *g* for 1 h at 4°C to spin down the microsomes. The microsomes was suspended in phosphate buffer (pH

7.4) and 20% glycerol, and stored at −70°C. The protein content of the microsomes was determined by Lowry's method [22], with bovine serum albumin as the standard.

In the enzyme induction studies, 50 μg of phenytoin were added to 1 mg/1.5 ml of microsomal proteins from control or treated animals, together with 1 mg of NADP, 3.4 mg of glucose-6-phosphate, and 1 U of glucose-6-phosphate dehydrogenase. The mixture was topped up with phosphate buffer solution to a final volume of 2.5 ml. The incubation was carried out at 37°C for 30 min. The incubation mixture was extracted with 3 ml of ethyl acetate and then assayed for the formation of *R*- and *S*-p-HPPH. For each determination, the data were averaged from triplicate experiments.

In the enzyme inhibition studies, 1 mg/1.5 ml of microsomes from untreated male animals were used, and 12.5 μg of phenytoin and various concentrations of each inhibitor (ketoconazole, sulphaphenazole, DMPH, cimetidine, diazepam, quinidine, tolbutamide, mephenytoin; $n = 4$ for each concentration) were added with cofactors. The inhibitory constant (K_i) of each inhibitor was determined by Dixon's plot.

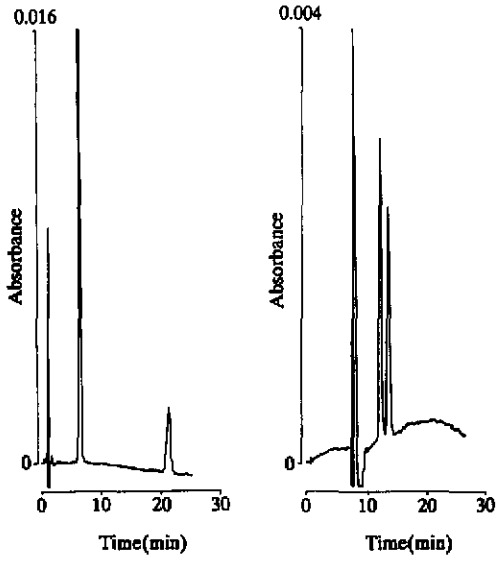

Fig. 2. Chromatograms of the separation of phenytoin and p-HPPH. (Left) Reversed-phase separation: the retention times of p-HPPH and phenytoin are 6.3 and 21 min, respectively. (Right) Ligand-exchange separation: the retention times of *R*-p-HPPH and *S*-p-HPPH are 12.5 and 14.1 min, respectively. The peaks corresponded to 150 ng of racemic p-HPPH, or 75 ng each of *R*- and *S*-p-HPPH.

RESULTS

Two-dimensional HPLC separation of p-HPPH

By using the column-switching valve, the p-HPPH peak in the reversed-phase separation can be automatically transferred to a ligand-exchange column (Fig. 2). The theoretical plate number of the ligand-exchange column can be increased by increasing the column temperature (Fig. 3). However, resolution of the two p-HPPH enantiomers and the selectivity factor decreased at higher temperature (Figs. 4 and 5). Increasing the concentration of octyl-L-prolinamide increased the resolution and the selectivity factor.

The assay was sensitive and reproducible down to 10 ng of each enantiomer injected. The coefficients of variation (C.V.) were 4.2, 2.8 and 12.2% for 15 ng each of racemic, *R*- and *S*-p-HPPH, respectively ($n = 7$), 1.5, 0.8 and 2.2% for 75 ng each of racemic *R*- and *S*-p-HPPH, respectively, and 5.2, 6.8 and 3.6% for 375 ng each of racemic, *R*- and *S*-p-HPPH, respectively.

Effect of enzyme induction and sex difference on the formation of p-HPPH enantiomers

Treatment with PB, PCN or acetone increased the formation of p-HPPH non-stereoselectively

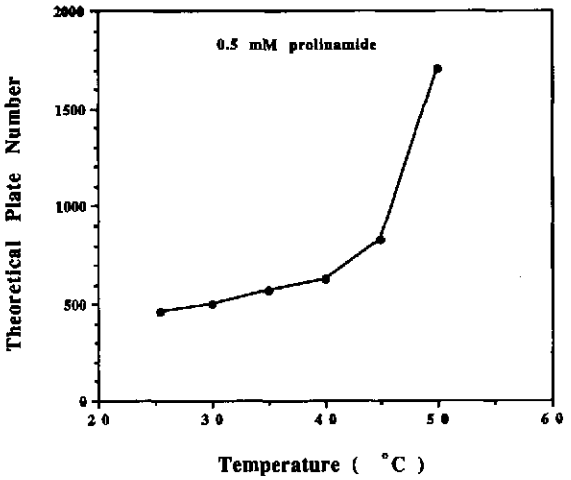

Fig. 3. Temperature dependence of the theoretical plate number of the chiral ligand-exchange column in the analysis of p-HPPH.

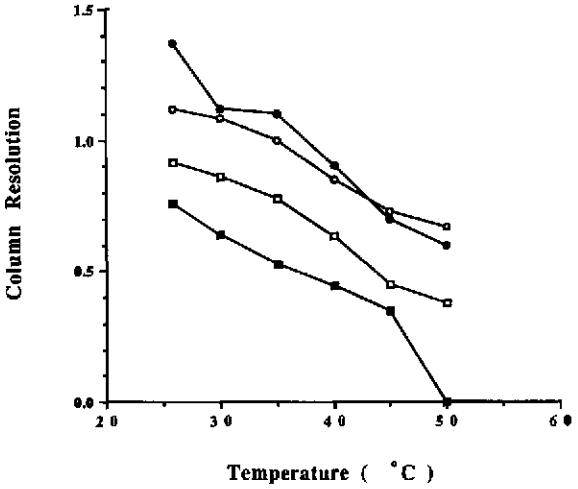

Fig. 4. Effect of column temperature and concentration of octyl-L-prolinamide on the resolution of *R*- and *S*-p-HPPH in the ligand-exchange column: (●) 0.5 m*M* prolinamide; (○) 0.375 m*M* prolinamide; (□) 0.3 m*M* prolinamide; (■) 0.25 m*M* prolinamide.

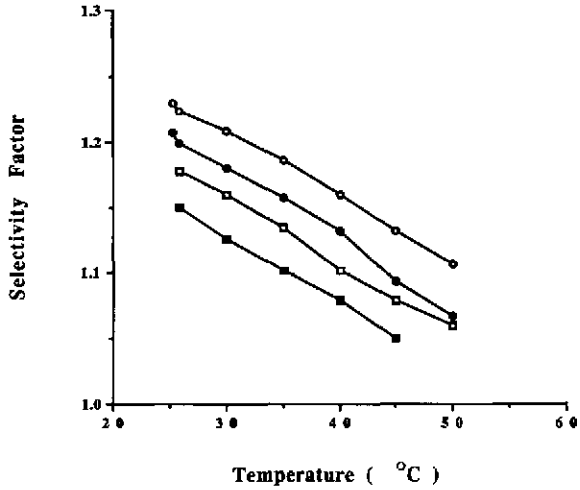

Fig. 5. Effect of column temperature and concentration of octyl-L-prolinamide on the selectivity factor of *R*-p-HPPH: (●) 0.5 m*M* prolinamide; (○) 0.375 m*M* prolinamide; (□) 0.3 m*M* prolinamide; (■) 0.25 m*M* prolinamide.

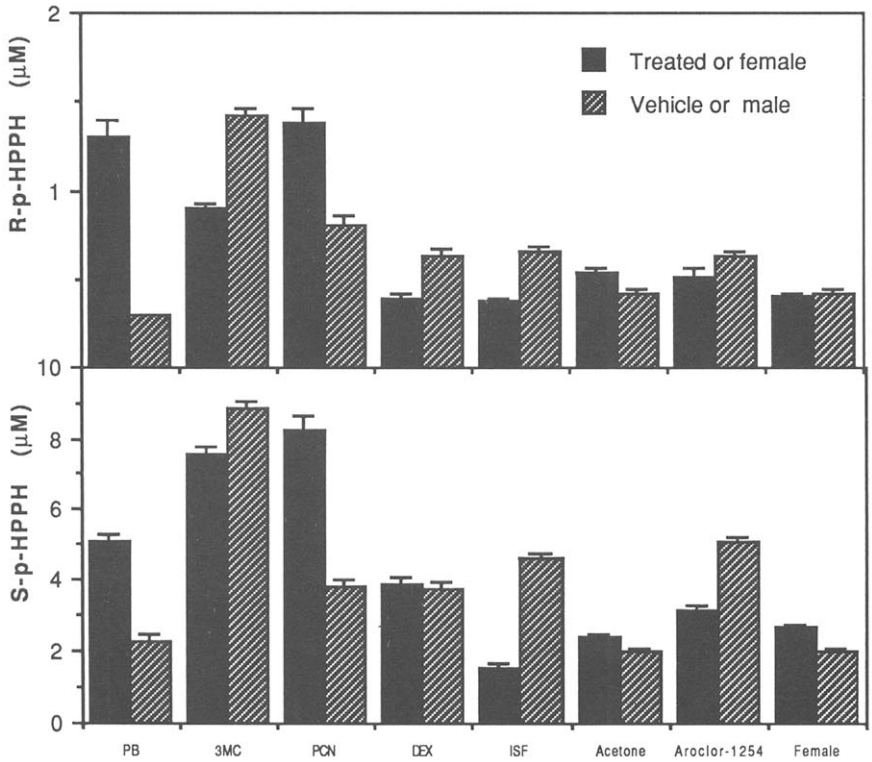

Fig. 6. Effect of enzyme induction and sex difference on formation of *R*- and *S*-p-HPPH: 50 μg of phenytoin were incubated in liver microsomes of male rats treated with various inducers, and in those of untreated female rats.

(Fig. 6). Treatment with 3-MC, Aroclor 1254, or ISF decreased the formation of both p-HPPH enantiomers. Treatment with DEX decreased the formation of *R*-p-HPPH, but not of *S*-p-HPPH. The formation of *S*-p-HPPH is significantly higher in female rats. The formation of *R*-p-HPPH showed no sex difference.

Inhibition of phenytoin metabolism by other drugs

Sulphaphenazole, ketoconazole, DMPH, cimetidine and diazepam inhibited the formation of both p-HPPH enantiomers. The K_i was 450, 6.1, 200, 175 and 22 μM for sulphaphenazole, ketoconazole, DMPH, cimetidine and diazepam, respectively. None of these inhibitors showed stereoselectivity. Quinidine, mephenytoin and tolbutamide showed no inhibitory activity in p-HPPH formation.

DISCUSSION

The use of a ligand-exchange column to separate p-HPPH enantiomers required a complexation agent, octyl-L-prolinamide, which is not commercially available. We tried an alternative method, which uses a β-cyclodextrin column (Astec, Whippany, NJ, USA). This column gave satisfactory results for the separation of mephenytoin enantiomers, but not for p-HPPH enantiomers. We therefore chose the ligand-exchange method to couple with the reversed-phase column separation.

Only a part of the eluent (100 μl) was applied to the ligand-exchange column. The absolute peak area of *R*- and *S*-p-HPPH varied because of a small variation in the retention time of racemic p-HPPH in reversed-phase separation. The peak was not necessarily transferred exactly at the peak time. However, the ratio of the two enantiomers is the same in the whole racemic p-HPPH peak. When the concentration of the two enantiomers was calculated based on the peak area of racemic p-HPPH and the peak-area ratio of p-HPPH enantiomers, a reasonable C.V. was obtained. The C.V. was higher at low concentrations of *S*-p-HPPH, but this was not a significant problem because *S*-p-HPPH is the major enantiomer in rats and in humans.

Although an increased temperature of the ligand-exchange column increased the efficiency of the column, it decreased the resolution of p-HPPH enantiomers because of a decrease in the selectivity factor. Increasing the level of octyl-L-prolinamide increased the resolution, although no further improvement was observed above 0.375 mM. To obtain a reasonable separation in a reasonable time, a prolinamide concentration of 0.375 mM and a temperature of 35°C were selected.

The metabolism of phenytoin can be induced or inhibited by pretreatment with other chemicals. Kutt and Fouts [15] reported that treatment with PB, chlordane or dichlorodiphenyltrichloroethane (DDT) induced p-HPPH formation, whereas 3-MC inhibited it. Pantarotto *et al.* [16] reported that treatment with β-naphthoflavone (BNF) or 3-MC significantly increased p-HPPH formation, whereas treatment with PB was without significant effect. Treatment with isoniazid inhibited the *p*-hydroxylation of phenytoin [17]. Claesen *et al.* [18] found that neither PB nor 3-MC showed an effect on the mechanism of p-HPPH formation. p-HPPH enantiomers both inhibit phenytoin metabolism [19]. It was also shown that oxyphenbutazone inhibits the elimination of phenytoin [20]. Nafimidone and other 1-imidazoles have been shown to be the inhibitors of p-HPPH formation [21] in microsomal preparations. Previous studies on the induction and inhibition of p-HPPH were all based on the measurement of racemic p-HPPH. Because the metabolism is often stereospecific, the previous results might be considered as the sum of two different metabolic pathways, which form *R*-p-HPPH and *S*-p-HPPH.

The hydroxylation of p-HPPH to form diHPPH and MeOCat was reported to be male-specific and appeared to be different from the isozyme of forming p-HPPH [23]. In microsomes from female rats, *S*-p-HPPH formation is higher than in those from male control rats. The formation of *R*-p-HPPH showed no sex difference. These data suggest that the sex difference in the hydroxylation is specific for *S*-p-HPPH. It is not known whether *R*-p-HPPH can be hydroxylated

to *R*-diHPPH. The reaction may be absent or without sex difference.

PCN and DEX are inducers of cytochrome P450p. It is unusual to see a difference in the induction pattern of these two enzyme inducers. The fact that DEX inhibited the formation of *R*- but not *S*-p-HPPH suggests that the two enantiomers may be formed by different isozymes. The pattern of enzyme induction can be used to suggest the specific isozyme responsible for the formation of an enantiomer. The analysis is, however, complicated by the fact that p-HPPH is further metabolized to diHPPH and MeOCat [4–6]. The enzyme metabolizing p-HPPH may be induced or inhibited instead.

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