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Assay of Mephenytoin Metabolism in Human Liver Microsomes by High-Performance Liquid Chromatography

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The metabolism of mephenytoin to its two major metabolites, 4-OH-mephenytoin (4-OH-M) and 5-phenyl-5-ethylhydantoin (nirvanol) was studied in human liver microsomes by a reversed phase HPLC assay. Because of preferential hydroxylation of *S*-mephenytoin *in vivo*, microsomes (5–300 μg protein) were incubated separately with *S*- and *R*-mephenytoin. After addition of phenobarbital as internal standard, the incubation mixture was extracted with dichloromethane. The residue remaining after evaporation was dissolved in water and injected on a 60 × 4.6-mm reversed-phase column (5 μ-C-18). Elution with acetonitrile/methanol/sodium perchlorate (20 mM, pH 2.5) led to almost baseline separation of mephenytoin, metabolites, and phenobarbital. Quantitation was performed by uv-absorption at 204 nm by the internal standard method. Propylene glycol was found to be the best solvent for mephenytoin, but inhibited the reaction noncompetitively. 4-OH-M and nirvanol could be detected at concentrations in the incubation mixture as low as 40 and 80 nM, respectively. The rates of metabolite formation were linear with time and protein concentration. The reaction was found to be substrate stereoselective. At substrate concentrations below 0.5 mM *S*-mephenytoin was preferentially hydroxylated to 4-OH-M, while *R*-mephenytoin was preferentially demethylated to nirvanol at all substrate concentrations tested (25–1600 μM). These data provide a mechanistic explanation for the stereospecific pharmacokinetics in vivo. The dependence of both metabolic reations on NADPH and the inhibition by CO suggest that they are mediated by cytochrome *P*-450-type monooxygenases. These data, together with the recent discovery of a genetic polymorphism causing an exclusive deficiency of the 4-hydroxylation of *S*-mephenytoin, suggest that hydroxylation and demethylation of *S*-mephenytoin are catalyzed by different cytochrome *P*-450 isozymes.

KEY WORDS: HPLC; mephenytoin; drug metabolism; human liver; microsomes; cytochrome *P*-450.

The anticonvulsant drug mephenytoin (3-methyl-5-phenyl-5-ethylhydantoin) is administered as a racemic mixture. However, mephenytoin metabolism in man displays marked stereoselectivity, the *S*- enantiomer being preferentially hydroxylated and excreted as 3-methyl-5-(4-hydroxyphenyl)-5-ethylhydantoin (4-hydroxymephenytoin, 4-OH-M)[1] and the *R*- enantiomer *N*-demethylated and excreted as 5-phenyl-5-ethylhydantoin (PEH, nirvanol; (1,2)). Further hydroxylation of nirvanol to 4-OH-nirvanol occurs to a small extent (3,4). A genetic deficiency affecting the hydroxylation of *S*-mephenytoin and *S*-nirvanol, but not the demethylation (5,6), has been established in several population studies (7–9). It occurs in 2–5% of Caucasian (7–9) and 23% of Japanese subjects (10). In order to study this common genetic polymorphism of drug oxidation we have developed a reversed-phase HPLC assay for the determination of formation of 4-OH-M and nirvanol from mephenytoin in human liver microsomes. Since the metabolism of only one enantiomer appears to be controlled by the polymorphism, the *in vitro* metabolism of each enantiomer,

[1] Abbreviations used: 4-OH-M, 3-methyl-5-(4-hydroxyphenyl)-5-ethylhydantoin; PEH, nirvanol, 5-phenyl-5-ethylhydantoin; 4-OH-nirvanol, 5-(4-hydroxyphenyl)-5-ethylhydantoin; PMSF, phenylmethylsulfonyl fluoride; BHT, butylated hydroxytoluene.

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R- and S-mephenytoin, was investigated separately.

METHOD

Chemicals. Acetonitrile, methanol (LiChrosolv), and dichloromethane (Uvasol) were obtained from Merck (Darmstadt, FRG). Propylene glycol (puriss p.a.) was purchased from Fluka (Buchs, Switzerland). NADP (for biochemical purposes), (*dl*)-isocitrate and isocitrate dehydrogenase (type IV) were obtained through Sigma Chemical Company (St. Louis, Mo.). Aqueous buffers for HPLC were filtered through a 0.45-μm membrane (Millipore, Molsheim, France). All other chemicals were of analytical grade.

Racemic 4-hydroxymephenytoin and 4-hydroxynirvanol as well as the *R*- and *S*- enantiomers of nirvanol were kindly provided by Dr. A. Küpfer and Dr. G. Karlaganis (Department of Clinical Pharmacology, University of Berne, Switzerland). The enantiomers were 96% pure as judged by the optical rotation values (11,12). *R*- and *S*-mephenytoin were synthesized from the corresponding nirvanol enantiomers by methylation of the nitrogen in position 3 of the hydantoin ring (13). To remove remaining amounts of nirvanol, the mixture was dissolved in benzene and extracted with 1 N sodium hydroxide. After evaporation of the benzene layer the residue was dissolved in hot propylene glycol at 50 mg/ml and diluted 10-fold with boiling water. This crystallization yielded chromatographically pure mephenytoin as judged by the HPLC conditions described below. Stock solutions of 160 mM were prepared in propylene glycol and diluted with water immediately before starting incubations.

Preparation of microsomes. Human liver microsomes were prepared from kidney donor livers according to Meier *et al.* (14) with slight modifications. Briefly, liver pieces were thawed and homogenized in 0.15 M potassium chloride containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 20 μM butylated hydroxytoluene (BHT). The microsomal pellet of the 105,000*g* centrifugation for 60 min was washed twice with 100 mM sodium pyrophosphate buffer (pH 7.25, adjusted with HCl at 4°C) containing 1 mM EDTA and PMSF and BHT at the same concentrations as in the homogenization buffer. The final pellet was resuspended in 100 mM potassium phosphate buffer, pH 7.4, and the microsomes were stored in liquid nitrogen. Protein and cytochrome *P*-450 contents were determined according to Lowry *et al.* (15) and Omura and Sato (16), respectively.

Incubation and sample preparation. Microsomes were incubated in a final volume of 250 μl using 37 or 300 μg of microsomal protein for *S*- and *R*-mephenytoin, respectively. The microsomal suspension was preincubated for 5 min at 37°C with 25 μl of a NADPH generating system resulting in a final concentration of 1 unit isocitrate dehydrogenase/ml, 1 mM NADP, 5 mM isocitrate, and 5 mM $MgCl_2$. The reaction was started by the addition of 25 μl of mephenytoin solution of variable concentration as indicated. The incubation was stopped after 60 min with 100 μl of a 2% sodium azide solution containing 2.5 μM sodium phenobarbital (internal standard). The mixture was extracted with 5 ml of dichloromethane and the extract evaporated under a gentle stream of nitrogen. The residue was dissolved in 200 μl of water and a 100-μl aliquot was injected for HPLC analysis.

High-performance liquid chromatography. HPLC was performed on a system equipped with a constant-flow pump (Model 414, Kontron, Zürich, Switzerland) and either a Rheodyne injector (Model 7125, Rheodyne, Berkeley, Calif.) or an ISS-100 autosampler (Perkin–Elmer, Ueberlingen, FRG). A 60 × 4.6-mm steel column was filled at 600 bars with Nucleosil 5-C-18 reversed-phase support (Macherey-Nagel, Düren, FRG) and a Uvikon 725 spectrophotometer (Kontron) was set at 204 nm. The mobile phase consisted of acetonitrile/methanol/20 mM $NaClO_4$ (pH 2.5, adjusted with 60% perchloric acid) in proportions of 6/25/69 by volume and was delivered at 1 ml/min. Chromatograms were recorded on a Shimadzu C-R3A integrator at 4 mAU full

scale and the peak heights quantitated by the internal standard method.

Recovery. Recovery of the extraction was determined by spiking the complete incubation mixture (150 μg microsomal protein, NADPH generating system, sodium azide) with five concentrations (4.4–111.2 μM) of 4-OH-nirvanol, 4-OH-M, nirvanol, and phenobarbital. These solutions were extracted with 5 ml of either dichloromethane or 10% 2-propanol in dichloromethane. The organic layers were evaporated under nitrogen and redissolved in 200 μl of water, of which 40 μl was injected.

Kinetics. Kinetics were analyzed by the Eadie-Hofstee plot (17). The type of inhibition and the K_i values for propylene glycol on mephenytoin oxidation were determined by Dixon and Cornish–Bowden plots (18). All plots were evaluated by linear regression analysis. Substrate concentrations in inhibition experiments ranged from 12.5 to 200 μM and from 50 to 800 μM *S*- and *R*-mephenytoin, respectively.

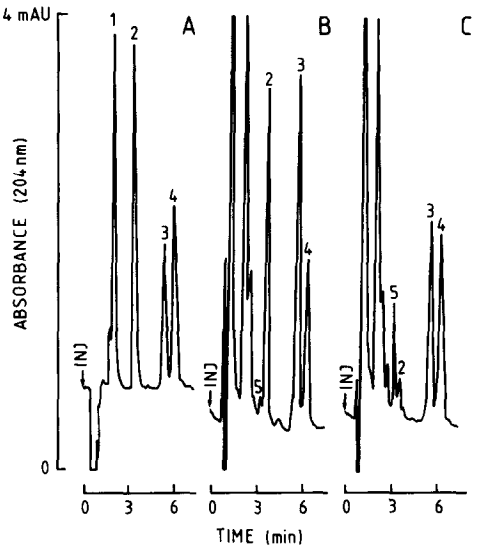

FIG. 1. Typical chromatograms of (A) 100-μl calibration standard containing 873 nM 4-OH-nirvanol (peak 1), 975.5 nM 4-OH-M (peak 2), 1111.8 nM nirvanol (peak 3), and 697.3 nM phenobarbital (peak 4, internal standard); (B,C) 50 μl extracted incubation mixture which contained 50 μg human microsomal protein and 800 μM either *S*-mephenytoin (B) or *R*-mephenytoin (C) in 0.5% propylene glycol (v/v). Unidentified metabolite (peak 5).

RESULTS

The chromatographic conditions applied led to almost baseline separation of all compounds of interest extracted from a biological matrix (Fig. 1). The retention time of mephenytoin was 10.3 min, allowing subsequent analysis of different samples after 12 min. A further unidentified peak (peak 5, Fig. 1) was detected in addition to 4-OH-M and nirvanol, most likely another metabolite of mephenytoin because its occurence was dependent on protein concentration and time of incubation (data not shown). A small amount of the nirvanol formed from *S*-mephenytoin is hydroxylated to 4-OH-nirvanol *in vivo* (3,4,6). 4-OH-nirvanol (peak 1, Fig. 1), could not be detected in microsomal incubations even after incubation of 30 μg of microsomal protein at 500 μM *S*-nirvanol in 0.5% propylene glycol (v/v).

Recovery of the extraction with dichloromethane was linear with concentration (4.4 to 111.2 μM) and greater then 80% for 4-OH-M, nirvanol, and phenobarbital. The recovery of 4-OH-nirvanol could be increased from 18.6 to 81.9% by the use of 10% 2-propanol in dichloromethane as extraction solvent. This however was accompanied by more interferences in the chromatograms. Thus dichloromethane was chosen as the extraction medium. Phenobarbital showed the same properties as 4-OH-M and nirvanol in the extraction procedure as well as a comparable retention time (Fig. 1), qualifying it as appropriate internal standard.

Reinjection of the same 18 samples on two different days with new calibration yielded mean variations of 1.6% for 4-OH-M and 1.0% for nirvanol. The overall reproducibility (incubation, extraction, HPLC) from day to day was 4.5% (SD in percentage of mean, $n = 5$) for the hydroxylation and 19.5% for the demethylation of mephenytoin. The reasons for the large variability of the demethylation reaction are not known. We observed a higher sensitivity of the demethylation activity to

thawing and freezing and not to storage time. The overall intraassay reproducibility in a typical experiment was 3.7 and 5.2% (SD in percentage of mean, $n = 5$) for the formation of 4-OH-M and nirvanol, respectively. The detection limits of the hydroxylated and demethylated metabolite in the incubation mixture were 40 and 80 nM, respectively, with an approximate signal-to-noise ratio of 3.

In Fig. 2 the dependence of microsomal mephenytoin oxidation on protein (A) and incubation time (B) is shown for each enantiomer separately. All activities but the demethylation of *R*-mephenytoin are linear in the range measured.

The low water solubility of mephenytoin represents a major problem in the *in vitro* study of its metabolism. Since methanol, ethanol, dimethyl sulfoxide, glycerol, nonionic detergents (Emulgen 911), and bovine serum albumin were less efficient in dissolving mephenytoin, propylene glycol was chosen as solvent. Jurima *et al.* (19) already demonstrated that 0.25% of propylene glycol (v/v) is sufficient to dissolve racemic mephenytoin at a 500 μM concentration. Because it inhibits the oxidation of mephenytoin *in vitro* as do the first four mentioned solvents (data not shown) we studied the inhibition in more detail. The results are summarized in Table 1. Although propylene glycol inhibited the velocity of the reaction, the K_m values scattered around a mean value which is most compatible with a noncompetitive type of inhibition. The increase of propylene glycol from 0.5 to 8% led to a 10-fold decrease in *S*-mephenytoin oxidation rates. We found that a concentration of 0.125% for 200 μM *S*-mephenytoin is sufficient to work under V_{max} conditions. However, in order to reach saturating concentrations of *R*-mephenytoin higher concentrations of propylene glycol had to be used. Substrate dependence of 4-OH-M and nirvanol formation was determined with both enantiomers (Fig. 3). The rates of metabolite formation were consistent with Michaelis–Menten kinetics except for the demethylation of *S*-mephenytoin. The nirvanol production rate of this reaction was almost linear within the substrate concentration range measured.

The enzymatic reaction exhibited an absolute requirement for NADPH and could not be mediated by NADH. It was inhibited by carbon monoxide and NAD (data not shown).

DISCUSSION

The described assay allows the determination of the formation of 4-OH-M and nirvanol from mephenytoin in only 5 μg of human liver microsomal protein. At least two metabolic reactions, 4-hydroxylation and *N*-demethylation, can be detected in one experiment. Our data suggest that two active sites on one iso-

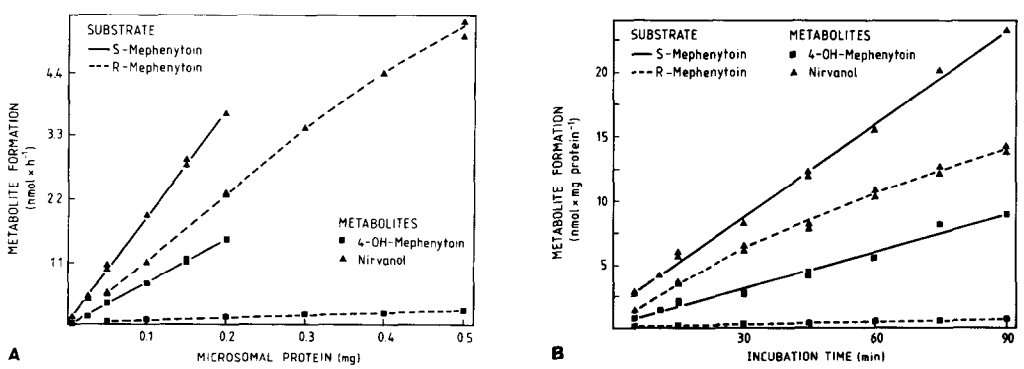

FIG. 2. Protein (A) and time (B) dependence of 4-hydroxylation and *N*-demethylation of mephenytoin enantiomers in human liver microsomes. Incubations were performed with 800 μM mephenytoin in 0.5% propylene glycol (v/v) and microsomes containing 0.59 nmol cytochrome *P*-450/mg protein.

TABLE 1

KINETIC CONSTANTS DERIVED FROM INHIBITION BY PROPYLENE GLYCOL OF METABOLITE FORMATION FROM *R*- AND *S*-MEPHENYTOIN

Propylene glycol		Substrate:	*S*-Mephenytoin		*R*-Mephenytoin			
		Metabolite:	4-OH-Mephenytoin		4-OH-Mephenytoin		Nirvanol	
(% (v/v))	(mM)		K_m[a]	V_{max}[b]	K_m[a]	V_{max}[b]	K_m[a]	V_{max}[b]
0.125	17.1		21.6	11.34				
0.25	34.2		16.5	8.36				
0.5	68.3		12.5	6.83	372	0.84	478	12.09
1.0	136.7		19.6	6.85	492	0.81	609	11.46
1.5	205.0		21.0	5.64	455	0.67	534	9.23
2.0	273.3		22.5	4.59	479	0.62	639	9.32
2.5	341.7				353	0.45	491	6.78
K_i[c]			1–2%		1–2%		1.5–2.5%	
Type of inhibition[c]			Mixed		Noncompetitive		Noncompetitive	

Note. Values given in [a]μM, [b]nmol × mg protein^{-1} × h^{-1}.
[c] Determined by Dixon and Cornish-Bowden plots.

zyme or two different cytochrome *P*-450 isozymes mediate hydroxylation and demethylation of mephenytoin. This is supported by the fact that only hydroxylation is deficient in poor metabolizers of mephenytoin *in vivo* (5,6) and *in vitro* (Meier *et al*, in press). Moreover, the different kinetic parameters of the two pathways (Fig. 3) and the selective induction of only the demethylation reaction in rats (Meier, unpublished observation; (20)) also are in line with this contention. The inhibition of both microsomal reactions by carbon monoxide and NAD as well as the requirement of NADPH provide strong evidence for cytochrome *P*-450 isozymes and its flavoprotein reductase catalyzing mephenytoin oxidation, confirming recent studies in another laboratory (21).

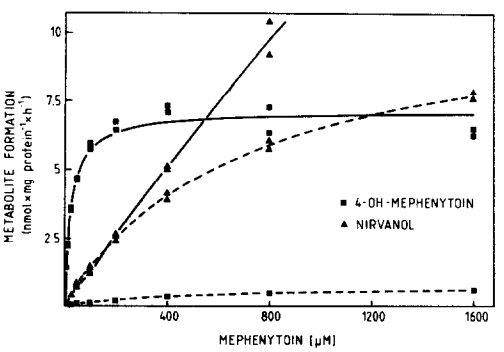

FIG. 3. Kinetics of *R*- (---) and *S*- (—)mephenytoin metabolism in human liver microsomes. Hydroxylation to 4-OH-mephenytoin and *N*-demethylation to nirvanol were measured with 1% propylene glycol (v/v) as solvent in the incubation mixture and with microsomes containing 0.68 nmol cytochrome *P*-450/mg protein. K_m and V_{max} values are given in Table 1.

A third metabolite of mephenytoin apparently was produced in microsomal incubations (peak 5, Fig. 1), the identity of which could not be determined. We compared its chromatographic behavior to the pure standards (Fig. 1A) and to published data on other mephenytoin metabolites (22). We suspect this metabolite to be the aliphatic hydroxylated mephenytoin, 3-methyl-5-(2-hydroxyethyl)-5-phenylhydantoin.

The large differences in the kinetic constants of the hydroxylation of *R*- and *S*-mephenytoin clearly indicate that the use of racemic mephenytoin in microsomal incubations will

predominantly represent the metabolism of the *S*-enantiomer. These differences also demonstrate that the observed stereoselective metabolism of mephenytoin *in vivo* (1,2) is due to substrate stereoselectivity (Fig. 3).

The HPLC assay described here will serve as a powerful tool for further characterizing the mephenytoin hydroxylation polymorphism *in vitro* and to characterize the cytochrome *P*-450 isozyme catalyzing this reaction.

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