

Mephenytoin hydroxylation polymorphism: Characterization of the enzymatic deficiency in liver microsomes of poor metabolizers phenotyped in vivo

The rate of 4-hydroxylation and of *N*-demethylation of *S*- and *R*-mephenytoin was determined in liver microsomes of 13 extensive (EM) and two poor (PM) metabolizers of mephenytoin. Detailed kinetic studies were performed in microsomes of eight EMs and the two PMs. Microsomal mephenytoin metabolism in PMs was characterized by an increased K_m (150.6 and 180.6 vs. a mean [\pm SD] 37.8 ± 9.6 $\mu\text{mol/L}$ *S*-mephenytoin in 8 EMs), a decreased maximum rate of metabolism for *S*-mephenytoin hydroxylation (0.76 and 0.69 vs 4.85 ± 1.65 nmol 4-hydroxymephenytoin per milligram protein per hour), and loss of stereoselectivity for the hydroxylation of the *R*- and *S*-enantiomers of mephenytoin (*R/S* ratio: 1.10 and 0.76 vs. 0.11 ± 0.04 in 13 EMs). The formation of 4-OH-mephenytoin from *R*-mephenytoin and the demethylation reaction remained unaffected. These results support our hypothesis that the mephenytoin polymorphism is caused by a partial or complete absence or inactivity of a cytochrome P-450 isozyme with high affinity for *S*-mephenytoin. (CLIN PHARMACOL THER 1985;38:488-94.)

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A genetic polymorphism of deficient metabolism of mephenytoin (3-methyl-5-phenyl-5-ethylhydantoin) is observed in 2% to 5% of white subjects¹⁻³ and in 23% of Japanese subjects.⁴ This deficiency, first described by Küpfer et al.⁵ in 1979, appears to be inherited as an autosomal recessive trait and concerns one of the two major metabolic pathways of mephenytoin, namely aromatic hydroxylation. The other major pathway, *N*-demethylation, remains unaffected.

Mephenytoin has a center of asymmetry at the 5 position of the hydantoin ring, and its metabolism in man is highly stereoselective. Thus in normal subjects the *S*-enantiomer is almost completely hydroxylated in the *p* position of the phenyl ring and rapidly excreted

as *S*-4-hydroxymephenytoin glucuronide, whereas the *R*-enantiomer is preferentially *N*-demethylated and slowly excreted as *R*-5-phenyl-5-ethylhydantoin (PEH, or nirvanol).^{6,7} In subjects with deficient hydroxylation, the stereoselectivity of metabolite excretion after racemic mephenytoin dosing is virtually absent, suggesting that the deficiency affects only the 4-hydroxylation of *S*-mephenytoin. *S*- and *R*-metabolites are slowly excreted in equal amounts, predominantly as nirvanol.^{8,9} The molecular mechanism responsible for the mephenytoin polymorphism has not been studied at the level of the hepatic enzymes catalyzing mephenytoin metabolism.

Our laboratory and others have provided recent evidence that another common polymorphism of drug metabolism that affects the oxidative metabolism of debrisoquin, sparteine, bufuralol, and several other drugs (debrisoquin/sparteine-type polymorphism) is caused by the deficiency of a specific hepatic cytochrome P-450 isozyme.¹⁰⁻¹⁵ In population and family studies, intrasubject comparisons of the debrisoquin and mephenytoin hydroxylation phenotypes indicated that the two

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Table I. In vivo metabolism of racemic mephenytoin and debrisoquin in 15 unrelated subjects

Subject	Sex	Age (yr)	Mephenytoin			Debrisoquin	
			Urinary 4-OH-mephenytoin* (μmol)	Hydroxylation index	Phenotype	Urinary metabolic ratio*	Phenotype
V. A.†	F	56	1.8	127.3	PM	0.59	EM
R. E.†	F	41	4.3	53.4	PM	0.21	EM
G. M.†	M	64	114.2	2.0	EM	0.55	EM
B. R.	M	48	136.0	1.7	EM	22.80	PM
P. G.	F	67	50.1	4.6	EM	1.46	EM
P. J.†	F	53	121.6	1.9	EM	2.09	EM
W. N.	F	63	114.8	2.0	EM	0.28	EM
V. L.	F	26	115.4	2.0	EM	0.70	EM
M. C.†	F	85	35.7	6.4	EM	2.47	EM
L. P.†	M	55	28.7	8.0	EM	1.73	EM
P. C.	F	52	164.8	1.4	EM	12.60	PM
T. S.†	F	74	128.2	1.8	EM	1.47	EM
S. J.†	F	55	125.4	1.8	EM	1.20	EM
M. M.†	M	64	134.0	1.7	EM	2.79	EM
G. L.†	M	52	144.1	1.6	EM	0.26	EM

F = Female; M = male.

*Urine samples were collected for 8 to 12 hours after oral dosing with 100 mg (458 μmol) mephenytoin and 10 mg debrisoquin.

†Liver microsomes of these subjects were used for kinetic studies (Fig. 2).

polymorphisms occur independently.¹⁻³ This has been supported by the lack of inhibition of sparteine oxidation by mephenytoin in human liver preparations in vitro.¹⁶ As both major reactions of mephenytoin (hydroxylation and demethylation) are catalyzed by cytochrome P-450-dependent monooxygenases,¹⁷ we tested the hypothesis that in subjects with polymorphically deficient mephenytoin metabolism, a cytochrome P-450 isozyme catalyzing the 4-hydroxylation of *S*-mephenytoin is wholly or partly missing or inactive, while the cytochrome P-450 isozyme(s) for demethylation remain(s) intact. For this purpose we developed a sensitive assay for measurement of the metabolites of mephenytoin in urine and in human liver microsomes and investigated the metabolism of *S*- and *R*-mephenytoin in liver biopsy specimens of extensive (EM) and poor (PM) metabolizers of mephenytoin phenotyped in vivo.

METHODS

Our subjects were surgical patients, 26 to 85 years old, at the Geneva University Hospital who were undergoing laparotomy and liver biopsy for therapeutic or diagnostic reasons. Tissue from small wedge biopsy specimens (100 to 500 mg wet weight) not used for histologic examination was immediately frozen in liquid nitrogen and stored at -80°C . The project had the approval of the hospital clinical investigation review board.

Between 1 day and 1 week after surgery, the patients

received a tablet containing 100 mg (458 μmol) racemic mephenytoin and 10 mg debrisoquin.* Urine was collected for the following 8 to 12 hours to determine simultaneously the hydroxylation phenotype of mephenytoin and debrisoquin. The subjects were classified according to their mephenytoin hydroxylation index (micromoles of *S*-mephenytoin administered/micromoles of 4-OH-mephenytoin in urine collected for 8 to 12 hours) and their urinary metabolic ratio of debrisoquin (debrisoquin/4-OH-debrisoquin) as EMs or PMs of the respective polymorphism by the criteria of Wedlund et al.³ and Price Evans et al.¹⁸ Urine samples were stored at -20°C until assayed.

Human liver microsomes were prepared by a micro-method as previously described,¹⁵ with the modification that 0.15 mol/L potassium chloride containing 5 mmol/L EDTA was used instead of 25 mmol/L sucrose. Protein content was determined with the use of bovine serum albumin as standard.¹⁹

Microsomes (0.1 mg protein) were incubated in the presence of an NADPH-generating system¹⁵ at 37°C for 1 hour in a total volume of 250 μl . The final substrate concentration was 1 mmol/L *R*- or *S*-mephenytoin in 1% propylene glycol (v/v) when used for determination of enantiomer ratios. A concentration range from 6.25 to 400 $\mu\text{mol/L}$ *S*-mephenytoin in 0.25% propylene

*Formulation by Drs. Soliva and Büchi, School of Pharmacy, University of Berne, Switzerland.

Table II. Substrate stereoselectivity of in vitro metabolism in liver microsomes from EMs and PMs

	<i>R</i> -mephenytoin (nmol/mg protein/hr)		<i>S</i> -mephenytoin (nmol/mg protein/hr)		<i>R/S</i> ratio	
	4-OH-mephenytoin	Nirvanol	4-OH-mephenytoin	Nirvanol	4-OH-mephenytoin	Nirvanol
EMs						
P. G.	0.42	3.06	5.80	7.01	0.07	0.44
B. R.	0.44	2.37	3.57	4.55	0.12	0.52
P. J.*	0.43	4.30	3.85	7.13	0.11	0.60
W. N.	0.63	5.10	7.70	11.52	0.08	0.44
V. L.	0.16	0.99	1.30	2.42	0.12	0.41
M. C.*	0.42	4.73	5.44	8.88	0.08	0.53
L. P.*	0.26	1.85	3.71	4.65	0.07	0.40
P. C.	0.49	3.19	4.32	6.18	0.11	0.52
T. S.*	0.38	2.59	3.12	5.73	0.12	0.45
S. J.*	0.20	1.65	1.46	3.95	0.14	0.42
M. M.*	0.26	3.13	3.62	10.82	0.07	0.29
G. L.*	0.35	2.95	2.95	7.49	0.12	0.39
G. M.*	0.38	4.47	1.77	10.71	0.21	0.49
\bar{X}	0.37	3.11	3.74	7.00	0.11	0.45
\pm SD	\pm 0.13	\pm 1.26	\pm 1.81	\pm 2.84	\pm 0.04	\pm 0.08
PMs						
V. A.*	0.37	3.87	0.49	7.52	0.76	0.51
R. E.*	0.57	10.14	0.52	18.99	1.10	0.53

*Liver microsomes of these subjects were used in kinetic studies (Fig. 2).

glycol (v/v) was used for kinetic experiments. The reaction was stopped with 100 μ l 2% sodium azide (w/v) containing 2.5 μ mol/L phenobarbital as internal standard, and the mixture was extracted with 5 ml dichloromethane. After evaporation of the solvent, the residue was dissolved in 0.2 ml water and 100 μ l was analyzed by reversed-phase HPLC. The enzymatic reaction showed the characteristics of a cytochrome P-450-catalyzed monooxygenase reaction, namely dependence on NADPH and inhibition by carbon monoxide. Product formation was linear with time and protein concentration. A detailed description and evaluation of the assay will be published elsewhere.²⁰

After the addition of phenobarbital as internal standard, urine samples were treated by acid hydrolysis⁶ and a 100 μ l aliquot of the hydrolysate was extracted with 5 ml dichloromethane. The solvent was evaporated and the residue was dissolved in 1 ml water, of which 100 μ l was injected into the HPLC. The chromatographic system consisted of a constant flow pump, a spectrophotometer set at 204 nm, and a model 7125 injector (Rheodyne Inc.). Columns (60 \times 4.6 mm) were filled with Nucleosil-5-C-18 reversed-phase support (Macherey-Nagel). Chromatography was performed isocratically in 30% methanol/70% potassium phosphate buffer, 10 mmol/L at pH 7.0 (v/v) at a flow rate of 1 ml/min. The chromatograms were analyzed

by the internal standard method. A linear calibration curve was obtained for 4-OH-mephenytoin over the range 1 to 250 μ g/ml urine. The coefficient of variation at 5 μ g/ml urine was 1.2% (n = 6). Retention times for 4-OH-mephenytoin and internal standard were 4.25 and 5.5 minutes, respectively.

R- and *S*-enantiomers of nirvanol and 4-OH-mephenytoin* were at least 96% pure²¹ according to reported optical rotation values.²² *R*- and *S*-mephenytoin were synthesized from the corresponding nirvanol enantiomers by methylation of the nitrogen in the 3 position of the hydantoin ring.^{20,23}

RESULTS

Of 15 unrelated subjects, two (V. A. and R. E.) showed a mephenytoin hydroxylation index consistent with the PM phenotype (Table I). Both PMs of mephenytoin metabolized debrisoquin efficiently, with metabolic ratios reflecting the EM phenotype for this substrate. These results confirm the independence of the two polymorphisms.¹⁻³ An additional advantage of combined dosing with both drugs is that it allows judgment on subject compliance with mephenytoin dosing. As <2% of mephenytoin is excreted unchanged by both

*Provided by Drs. A. K pfer and G. Karlaganis, Department of Clinical Pharmacology, University of Berne, Berne, Switzerland.

EMs and PMs,³ the definition of a metabolic "ratio" is not possible. The age or sex of the subjects had no apparent influence on their phenotype (Table I).

Results of microsomal incubations with *R*- and *S*-mephenytoin are summarized in Table II. In liver microsomes of mephenytoin EMs phenotyped *in vivo*, *S*-mephenytoin was metabolized more efficiently than *R*-mephenytoin by both hydroxylation and demethylation, resulting in a mean (\pm SD) *R/S* ratio of 0.11 ± 0.04 ($n = 13$) and 0.45 ± 0.08 for 4-OH-mephenytoin and nirvanol, respectively. In microsomes of the first mephenytoin PM (V. A.), the expected marked decrease in the hydroxylation rate of *S*-mephenytoin resulted in an increased *R/S* ratio of 0.76, whereas the hydroxylation of *R*-mephenytoin and the nirvanol formation from both enantiomers were comparable with those of the EMs. The microsomal incubations of the second PM (R. E.) also formed metabolites with an increased *R/S* ratio (1.10), but had higher absolute demethylation rates for both enantiomers than those observed in all other subjects. Thus the two subjects with high mephenytoin hydroxylation indices as determined *in vivo* had lost the stereoselectivity for *S*-mephenytoin as manifested by *R/S* ratios of microsomal 4-hydroxylation activity near unity (Fig. 1). It must be realized that at substrate concentrations of 1 mmol/L, *R*-mephenytoin hydroxylation does not reflect conditions of maximal velocity of metabolism (V_{max}) because of the higher Michaelis-Menten constant (K_m) of this reaction.²⁰

The rate of 4-hydroxylation of *S*-mephenytoin in microsomes at different substrate concentrations resulted in apparent simple Michaelis-Menten kinetics for eight EMs and the two PMs (Fig. 2, A). The mean (\pm SD) apparent K_m and V_{max} as determined by Eadie-Hofstee plots²⁴ in the eight EMs were 37.8 ± 9.6 (range 25.6 to 53.8) $\mu\text{mol/L}$ *S*-mephenytoin and 4.85 ± 1.65 (range 2.49 to 7.55) nmol 4-OH-mephenytoin per milligram protein per hour, respectively. Compared with the EMs, the K_m of the PMs was more than fourfold greater and the V_{max} was more than sixfold less (Fig. 2, B).

The substrate dependence of the demethylation of *S*-mephenytoin was almost linear in the concentration range measured (Fig. 2, B). Whereas the demethylation of one PM (V. A.) was within the range of the eight EMs, the other PM (R. E.) showed a two- to threefold increase in nirvanol formation over the entire concentration range (Fig. 2, B).

DISCUSSION

Our study demonstrates that mephenytoin polymorphism results from a deficiency of the hepatic micro-

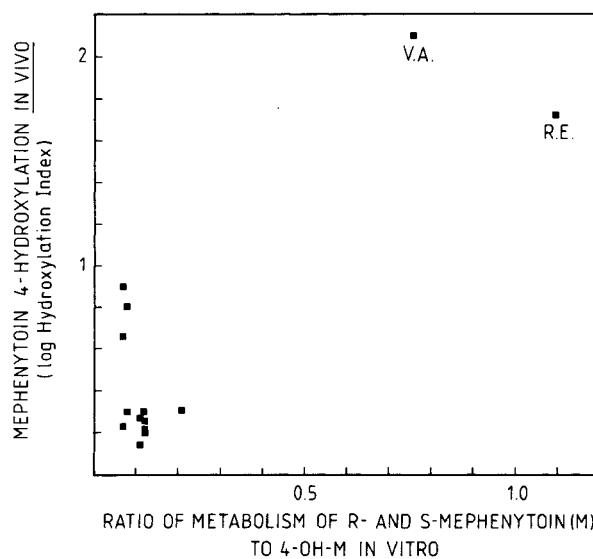


Fig. 1. *In vivo* hydroxylation index plotted against ratio of *in vitro* metabolism of *R*- and *S*-mephenytoin to 4-OH-mephenytoin of 15 unrelated subjects.

somal monooxygenase reaction that catalyzes the 4-hydroxylation of *S*-mephenytoin. In monooxygenase reactions, cytochrome P-450 isozymes are responsible for substrate binding and the stereo- and regioselectivity of the oxidation reactions. Our present findings are therefore consistent with the hypothesis that the absence or functional deficiency of a cytochrome P-450 isozyme with high affinity for this substrate causes the mephenytoin polymorphism. In hepatic microsomes of the two PMs of mephenytoin, there was an increased apparent K_m and a decreased V_{max} for the 4-hydroxylation of *S*-mephenytoin and a striking loss of stereoselectivity for this reaction. These *in vitro* findings correlate well with the *in vivo* data of Wedlund et al.³ Thus in EMs the mean (\pm SD) *S/R* ratio of mephenytoin measured in the 8-hour urine sample was 0.16 ± 0.12 ($n = 152$), indicating preferential metabolism of the *S*-enantiomer, whereas in PMs the ratio was near unity (0.99 ± 0.01 ; $n = 4$). Moreover, demethylation, the other major pathway of mephenytoin metabolism, was not affected in the PMs *in vitro*, consistent with the *in vivo* observation that demethylation is not affected by the polymorphism.⁹ However, our observation of a preference of microsomal *S*-nirvanol production as compared with formation of the *R*-enantiomer (Table II) is not reflected in the urinary excretion pattern^{8,9} or in the plasma concentration-time profiles²⁵ of either enantiomer. This discrepancy may be due to the fact that demethylation is determined at concentrations markedly below K_m *in vivo* (serum concentrations of 70 to 140 $\mu\text{mol/L}$ total

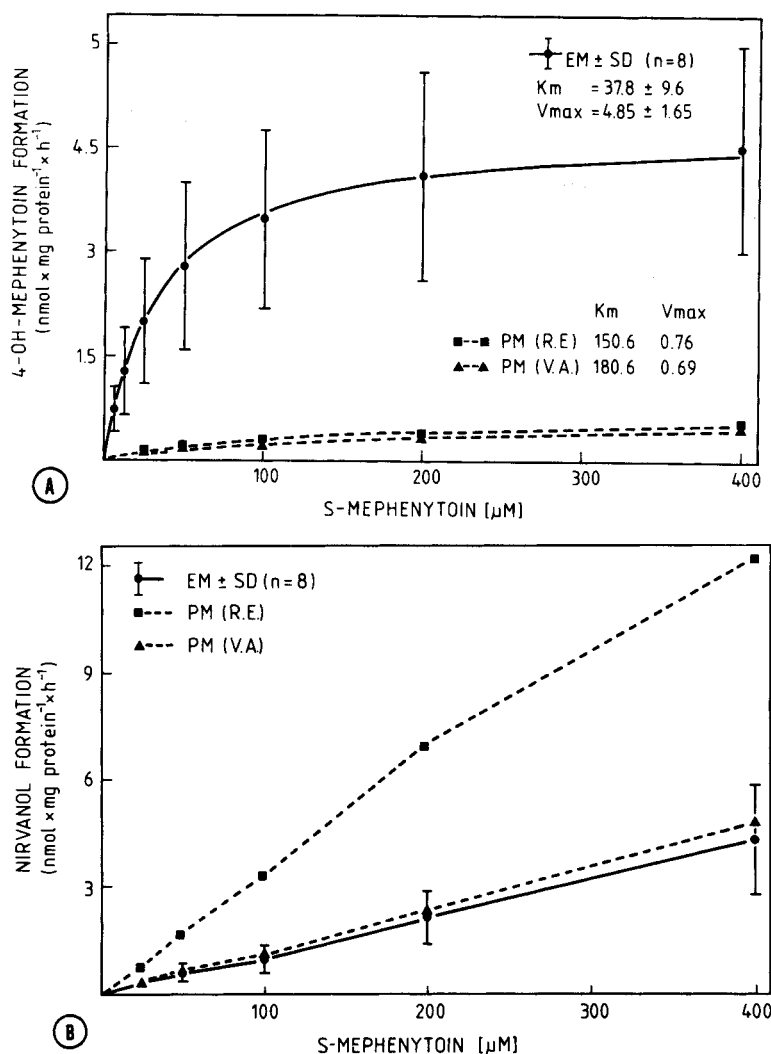


Fig. 2. Kinetics of *S*-mephenytoin 4-hydroxylation (A) and demethylation (B) in human liver microsomes from eight EMs and two PMs. K_m values are given as $\mu\text{mol/L}$ of *S*-mephenytoin, and V_{max} values are given as nanomoles of 4-OH-mephenytoin per milligram protein per hour.

hydantoin have been reported²⁶) as well as in vitro (solubility limitations). Under these conditions comparisons of the stereospecificity of the reaction are of limited value.

Probably for similar reasons, the Eadie-Hofstee plots of the microsomal demethylation reaction indicate a complex kinetic pattern. While the mephenytoin demethylation rate of one PM (V. A.) was within the range of the eight EMs, as expected from in vivo findings,⁹ the other PM (R. E.) showed increased nirvanol formation (Table II; Fig. 2, B). We presume that this subject had an increased activity of the enzyme(s) catalyzing demethylation because of induction by environmental factors. The probability of this induction

is supported by the known inducibility of mephenytoin demethylation in rats²⁷ and by the suggested substrate induction of the reaction in man.^{6,7} This is also reflected by the marked variation in nirvanol formation in the 13 EMs between 2.42 and 11.52 nmol nirvanol per milligram protein per hour (Table II). If the hydroxylation rate in this PM was only low because another metabolic pathway, i.e., demethylation, was increased and was not a result of a genetically determined deficiency, one would expect no change in the apparent K_m and an even higher increase in demethylation compensating such low 4-OH-mephenytoin production in

*Meier UT. Unpublished observations, 1984.

vitro as well as in vivo. It is therefore reasonable to assume that this subject is a genetically determined PM of mephenytoin. Obviously, only family studies allow the unequivocal identification of a subject as a PM.

Because of the small amount of metabolites formed, the K_m and V_{max} values for 4-OH-mephenytoin of the two PMs are based on determinations near the limit of detectability. This is because of the small size of the wedge biopsy specimen and the small concentration range of the substrate, which for reasons of limited solubility did not sufficiently exceed the K_m . Nevertheless, data indicate that the PMs can clearly be distinguished from the EMs in vitro by their markedly increased K_m and decreased V_{max} of hydroxylation (Fig. 2, A).

Interestingly, our findings are very similar to those characterizing the enzymatic deficiency in livers of subjects with the debrisoquin/sparteine-type polymorphism when (+) and (-)-bufuralol are used as substrate.^{13,15} The two independently occurring polymorphisms may thus be due to the same basic molecular mechanism(s) affecting two different genes.

In conclusion, the enzymatic data presented here support the hypothesis of the whole or partial loss or inactivity of a cytochrome P-450 isozyme with high affinity for the *S*-enantiomer of mephenytoin. Of several cytochrome P-450 isozymes recently purified from human liver,^{8,13} one had a high activity for mephenytoin 4-hydroxylation. Preliminary studies with a rabbit antibody raised against this cytochrome P-450 isozyme indeed suggest that the presence of a functionally altered enzyme is the most probable cause of this polymorphism.

*Gut J, Meier UT, Catin T, Meyer UA. Manuscript in preparation.

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