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High-performance liquid chromatographic assay for human liver microsomal omeprazole metabolism

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ABSTRACT

Assays for the measurement of omeprazole metabolites in plasma and urine have been reported, but when applied to the determination of omeprazole metabolites formed by human liver microsomal incubations there were obvious limitations in sensitivity. The present high-performance liquid chromatographic (HPLC) assay, which comprises extraction, evaporation and reconstitution, is several-fold more sensitive with a limit of detection of approximately 2 pmol (2 n*M* in incubate) for omeprazole sulphone and 25 pmol (25 n*M* in incubate) for hydroxyomeprazole. Extraction efficiency is essentially quantitative and is highly reproducible (coefficient of variation = 2.1% for both metabolites). The assay is linear over a wide range of concentrations and the formation of the metabolites is linear with respect to both time (to 15 min) and protein concentration (to 1.5 mg/ml). Two minor metabolites, one of which was identified tentatively as 5-O-desmethylomeprazole, were also formed by human liver microsomes and could be determined by this method. Preliminary studies of the formation of omeprazole sulphone and hydroxyomeprazole showed that the formation kinetics in human liver microsomes were biphasic for both metabolites, suggesting that at least two different cytochrome P450 isoforms are involved in their formation.

INTRODUCTION

Omeprazole, a substituted benzimidazole, is a potent long-acting inhibitor of gastric acid secretion. It interacts directly with the gastric proton pump H^+,K^+-ATPase in the secretory membrane [1,2]. The effective control of acid secretion by omeprazole results in a more rapid healing of acid-related diseases (*i.e.* peptic ulcers and reflux esophagitis) than is observed for H_2-blockers [3–5]. Omeprazole is apparently metabolised completely in the liver by cytochrome P450 (CYP), and the major metabolites found in plasma are

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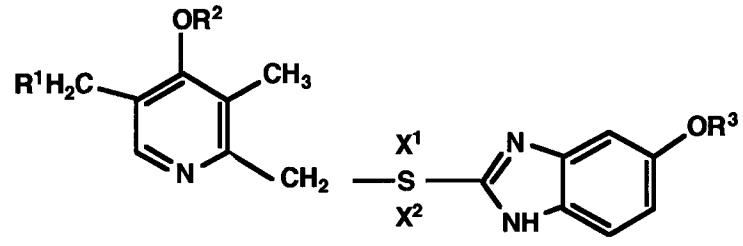

COMPOUND	R^1	R^2	R^3	X^1	X^2
OMEPRAZOLE	H	CH3	CH3	=O	-
OMEPRAZOLE SULPHONE	H	CH3	CH3	=O	=O
HYDROXYOMEPRAZOLE	OH	CH3	CH3	=O	-
OMEPRAZOLE SULPHIDE	H	CH3	CH3	-	-
H 215/02	H	CH3	H	=O	-
H 153/95	H	H(-)	CH3	-	-
H 195/77	OH	CH3	CH3	=O	=O
H 195/69	OH	CH3	CH3	-	-
H 182/68	H	CH3	H	-	-

Fig. 1. Structures of omeprazole, its two major metabolites found in plasma and obtained in incubated human liver microsomes (omeprazole sulphone and hydroxyomeprazole) and a minor demethylated metabolite, 5-O-desmethylomeprazole (H 215/02). The structures of omeprazole sulphide, omeprazole pyridone (H 153/95) and three other synthetic omeprazole derivatives [hydroxysulphone (H 195/77), hydroxyomeprazole sulphide (H 195/69) and 5-O-desmethylomeprazole sulphide (H 182/68)] are also shown.

omeprazole sulphone and hydroxyomeprazole [6] (see Fig. 1). The formation of hydroxyomeprazole has been suggested to be carried out by an isoform of the CYP2C subfamily, mephenytoin hydroxylase [7–11]. This enzyme exhibits polymorphic expression, with about 3% of the Caucasian population being slow metabolisers.

Omeprazole metabolism studies reported to date have been performed in healthy volunteers, and omeprazole biotransformation in man so far has not been studied *in vitro* to confirm the involvement of specific CYP isoforms in the various metabolic pathways. Since previously published assays are appropriate for determination of omeprazole and its metabolites in plasma and urine only [12–16], an assay for omeprazole metabolites formed by human liver microsomes has been developed here. The method is sensitive and precise and has been applied to a study of the kinetics of the formation of omeprazole metabolites in human liver microsomes.

EXPERIMENTAL

Chemicals

Omeprazole (5-methoxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulphinyl]-1*H*-benzimidazole), omeprazole sulphone (5-methoxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]-sulphonyl]-1*H*-benzimidazole), hydroxyomeprazole (5-methoxy-2-[[(4-methoxy-3-methyl-5-hydroxymethyl-2-pyridinyl)methyl]sulphinyl]-1*H*-benzimidazole), H 215/02 or 5-O-desmethylomeprazole (5-hydroxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulphinyl]-1*H*-benzimidazole), H 168/22 or omeprazole sulphide (5-methoxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]-thio]-1*H*-benzimidazole), H 153/95 or omeprazole pyridone (5-methoxy-2-[[(4-hydroxy-3,5-dimethyl-2-pyridinyl)methyl]thio]-1*H*-benzimidazole), H 195/77 or hydroxysulphone (5-methoxy-2-[[(4-methoxy-3-methyl-5-hydroxymethyl-2-pyridinyl)-methyl]sulphonyl]-1*H*-benzimidazole), H 195/69 or hydroxyomeprazole sulphide (5-methoxy-2-[[(4-methoxy-3-methyl-5-hydroxymethyl-2-pyri-

dinyl)methyl]thio]-1*H*-benzimidazole), H 182/68 or 5-O-desmethylomeprazole sulphide (5-hydroxy-2-[[(4-methoxy-3,5-dimethyl-2-pyridinyl)-methyl]thio]-1*H*-benzimidazole) (see Fig. 1 for all structures), and the internal standard (4,6-dimethyl-2-[[(4-methoxy-2-pyridinyl)methyl]sulphinyl]-1*H*-benzimidazole) were obtained from Astra Hässle (Mölndal, Sweden). $NADP^+$, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were purchased from Sigma (St. Louis, MO, USA). All other reagents and solvents were of analytical grade.

HPLC conditions

The assay was developed using a Model LC1100 solvent-delivery system, a Model LC1200 variable-wavelength UV–Vis detector (ICI Instruments, Melbourne, Australia), a Model 7125 Rheodyne injector and a Model SE120 BBC Goetz Metrawatt recorder (Brown-Boveri, Vienna, Austria). Absorbance was monitored at 302 nm. The instrument was fitted with a Superspher SI-60, 4-μm separation column (125 mm × 4.0 mm I.D.; E. Merck, Darmstadt, Germany) and a Brownlee Aquapore silica, 7-μm guard column (15 mm × 3 mm; Brownlee Labs., CA, USA), and operated at ambient temperature. The mobile phase, delivered at a flow-rate of 1.5 ml/min, comprised dichloromethane–5% NH_4OH in methanol–2-propanol (191:8:1).

Microsomal incubations and sample preparation

Microsomes were prepared from human livers of renal transplant donors as previously described [17] and protein concentration was determined according to the method of Lowry *et al.* [18] with bovine serum albumin as standard. Reaction mixtures contained human liver microsomal protein (1 mg), omeprazole (2.5–500 μM) and NADPH-generating system (consisting of 1 mM $NADP^+$, 10 mM glucose 6-phosphate, 2 U of glucose 6-phosphate dehydrogenase and 5 mM $MgCl_2$) in a final volume of 1.0 ml of 0.1 M KH_2PO_4 buffer, pH 7.4. Omeprazole was dissolved in 0.1 M KH_2PO_4 buffer–methanol (10:1) and used fresh. The final concentration of methanol in the incubation mixture was generally less than 1%, and this concentration did not seem to inhibit the metabolism of omeprazole. Omeprazole sulphone, hydroxyomeprazole and internal standard were dissolved in carbonate buffer (25 ml of 0.5 M Na_2CO_3 and 65 ml of 1.0 M $NaHCO_3$ added to 910 ml of deionised water)–methanol (4:1); aliquots of these solutions were kept frozen (−20°C) until used. Reactions were started by placing the reaction tubes in a metabolic shaker (37°C, water bath) immediately after addition of the NADPH-generating system. After 15 min, reactions were terminated by the addition of 2 ml of dichloromethane–butanol (99:1) and by cooling the samples on ice. Thereafter NaH_2PO_4 (100 μl of a 1 M solution) was added to the mixture for the optimal extraction of hydroxyomeprazole, followed by the addition of the assay internal standard. Extraction was performed on a vortex mixer for 1 min immediately followed by centrifugation for 5 min (1000 *g*). A 1.5-ml aliquot of the organic phase was evaporated to dryness under nitrogen. Residues were reconstituted in 150 μl of the mobile phase, and a 50-μl aliquot was injected onto the HPLC column.

Extraction efficiency was determined by comparing peak heights of omeprazole sulphone, hydroxyomeprazole and internal standard for extracted samples ($n = 5$) with those for directly injected samples ($n = 5$ for each compound). The concentrations chosen for this experiment were 2.8, 2.6 and 3.2 μM for omeprazole sulphone, hydroxyomeprazole and the internal standard, respectively. The directly injected samples were prepared as follows: the aqueous solution of each compound was dried in a Speedvac vacuum concentrator for 1 h, redissolved in 1 ml of the mobile phase, vortex-mixed for 30 s, centrifuged for 1 min and duplicate aliquots (100 μl) were injected onto the HPLC column. Within-day assay precision was assessed by investigating the formation of omeprazole metabolites at added omeprazole concentrations of 5 and 500 μM using the same batch of microsomes ($n = 6$).

Standard curves were constructed in the concentration range 0.28–7.0 μM for omeprazole sulphone and 0.26–6.5 μM for hydroxyomeprazole. Unknown concentrations were determined

by comparison of metabolite-to-internal standard peak-height ratio with those of the calibration curve.

RESULTS

Following the microsomal incubations, chromatographic peaks were detected with retention times corresponding to those of omeprazole sulphide (1.3 min), omeprazole sulphone (2 min), omeprazole (2.5 min), internal standard (3.3 min), 5-O-desmethylomeprazole (11 min), hydroxyomeprazole (13.5 min) (Fig. 2) and omeprazole pyridone (16 min). A minor peak, representing an unknown metabolite (metabolite X), was observed 0.5 min after the internal standard. The size of the peak corresponding to omeprazole pyridone indicated that this metabolite was formed only in small amounts during the incubations. The omeprazole sulphide seemed, however, to be formed during the preparation procedures since the blank samples (*i.e.* absence of NADPH-generating system) also contained this metabolite. There was no interference from extracted components of the microsomal incubation system with any of the detected omeprazole metabolites. The retention times of authentic samples of three other potential metabolites, hydroxysulphone (H 195/

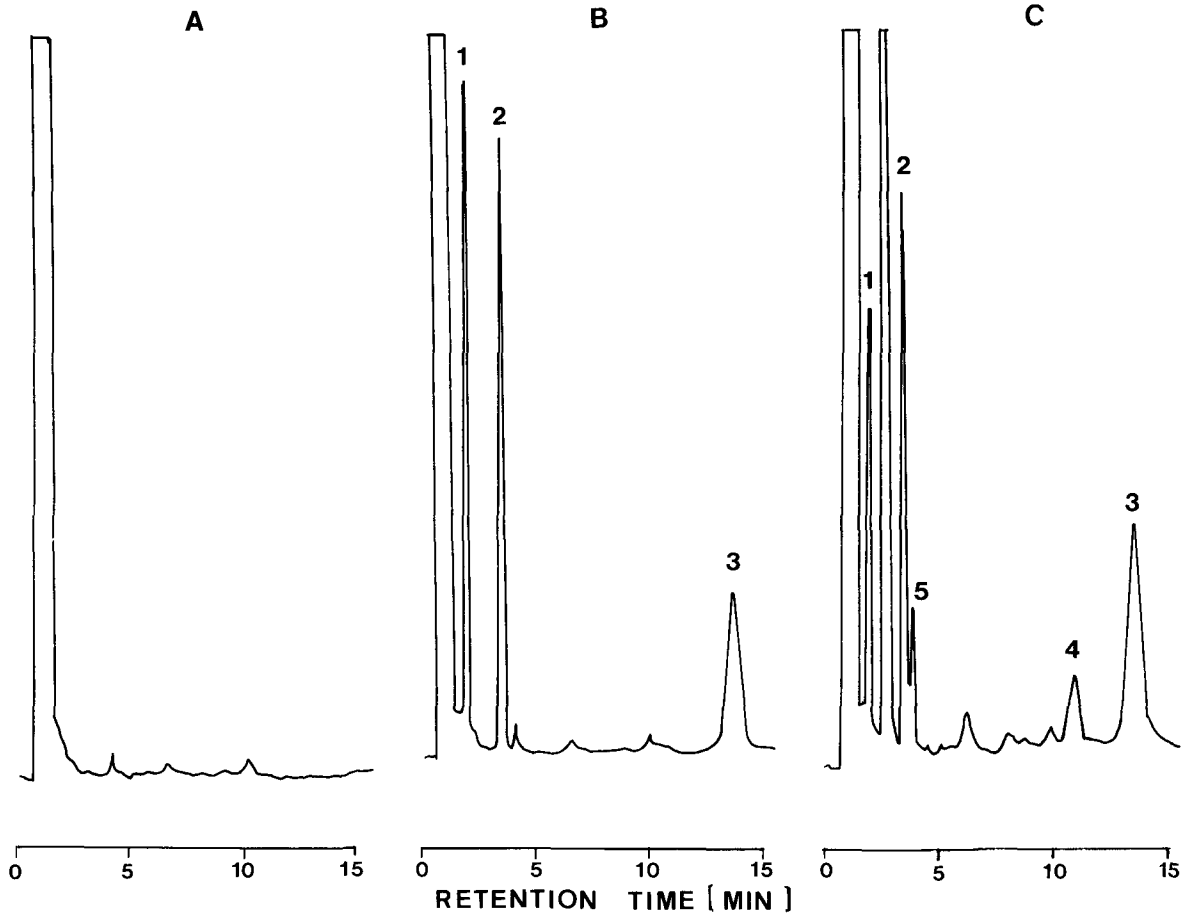

Fig. 2. Chromatogram of extracts. (A) Microsomal incubation without substrate; (B) standard containing omeprazole sulphone (0.18 μM), hydroxyomeprazole (0.65 μM) and internal standard; (C) incubation of human liver microsomes in the presence of omeprazole (5 μM). Peaks: 1 = omeprazole sulphone; 2 = internal standard; 3 = hydroxyomeprazole; 4 = 5-O-desmethylomeprazole or H 215/02; 5 = metabolite X.

77), hydroxyomeprazole sulphide (H 195/69) and 5-O-desmethylomeprazole sulphide (H 182/68), were 9, 5 and 4.3 min, respectively, under the assay conditions. These three compounds were, however, not detected after incubation with omeprazole.

The detection limit of the assay, defined as a signal-to-noise ratio of 5:1, was 2 pmol for omeprazole sulphone (corresponding to an incubation concentration of 2 nM) and 25 pmol for hydroxyomeprazole (corresponding to an incubation concentration of 25 nM). Standard curves for the major metabolites, omeprazole sulphone and hydroxyomeprazole, were linear over the concentration ranges studied (0.28–7.0 μM for omeprazole sulphone and 0.26–6.5 μM for hydroxyomeprazole). Representative regression equations for the standard curves for omeprazole sulphone and hydroxyomeprazole were $y = 0.054 + 0.28x$ ($r = 1.00$) and $y = 0.037 + 0.13x$ ($r = 1.00$), respectively. Linearity was demonstrated further for hydroxyomeprazole concentrations down to 0.065 μM and for omeprazole sulphone concentrations down to 0.018 μM.

Mean extraction efficiencies for the three compounds tested, omeprazole sulphone, hydroxyomeprazole and internal standard, were essentially quantitative: 112 ± 4, 106 ± 3 and $103 \pm 2\%$ (mean $\pm$ S.D.), respectively. In all cases coefficients of variation for extraction efficiencies were $< 3.5\%$. The coefficient of variation for the overall assay within-day precision study was 2.7% for omeprazole sulphone and 6.9% for hydroxyomeprazole at 5 μM omeprazole and 2.1% for both metabolites at 500 μM omeprazole. Corresponding figures for 5-O-desmethylomeprazole and metabolite X were 2.4 and 5.4%, respectively, at 5 μM and 2.5 and 3.3%, respectively, at 500 μM.

The effect of protein concentration and incubation time on the formation of the major omeprazole metabolites is shown in Figs. 3 and 4, respectively. The formation of both omeprazole sulphone and hydroxyomeprazole was linear for microsomal protein concentrations up to 1.5 mg/ml and for incubation times up to at least 15 min. The formation of the two minor metabolites, 5-O-desmethylomeprazole and metabolite X, followed the same pattern as the major metabolites as regards effects of protein concentration and incubation time. A protein concentration of 1 mg/ml and an incubation time of 15 min was therefore utilized in subsequent investigations.

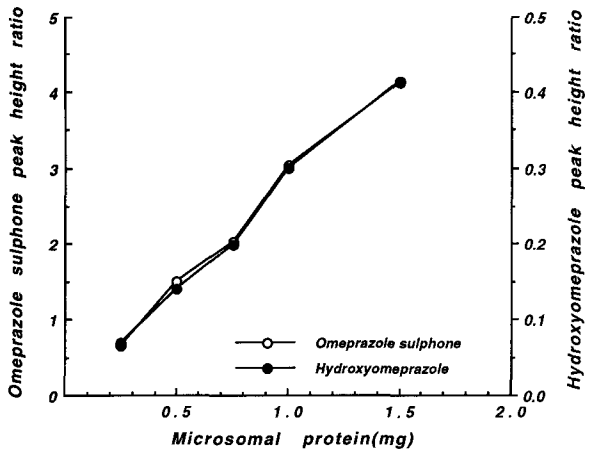

Fig. 3. Effect of protein concentration after 30 min incubation with 500 μM omeprazole on the formation of omeprazole sulphone and hydroxyomeprazole by human liver microsomes.

The formation of omeprazole sulphone and the formation of hydroxyomeprazole by hepatic microsomes from a single donor over the omeprazole concentration range 2.5–500 μM both exhib-

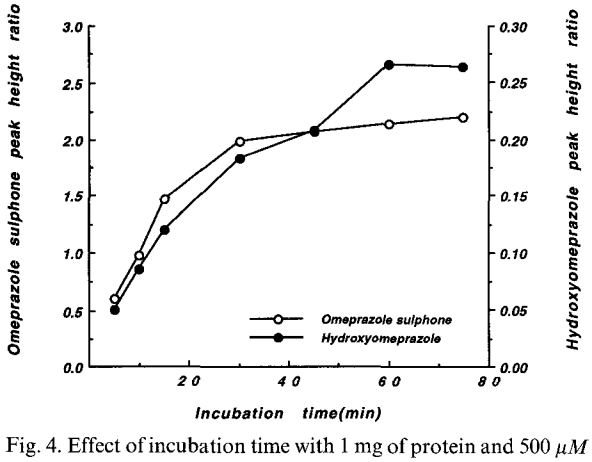

Fig. 4. Effect of incubation time with 1 mg of protein and 500 μM omeprazole on the formation of omeprazole sulphone and hydroxyomeprazole by human liver microsomes.

ited biphasic kinetics (Fig. 5). The apparent Michaelis constant (K_m) and maximum velocity (v_{max}) values for the formation of omeprazole sulphone, calculated by MK Model, an extended least-squares modelling programme [19], were 264 and 30 μM and 0.36 and 0.10 nmol/mg per min, respectively. Corresponding K_m and v_{max} values for the formation of hydroxyomeprazole were 81 and 5.2 μM and 0.24 and 0.04 nmol/mg per min, respectively.

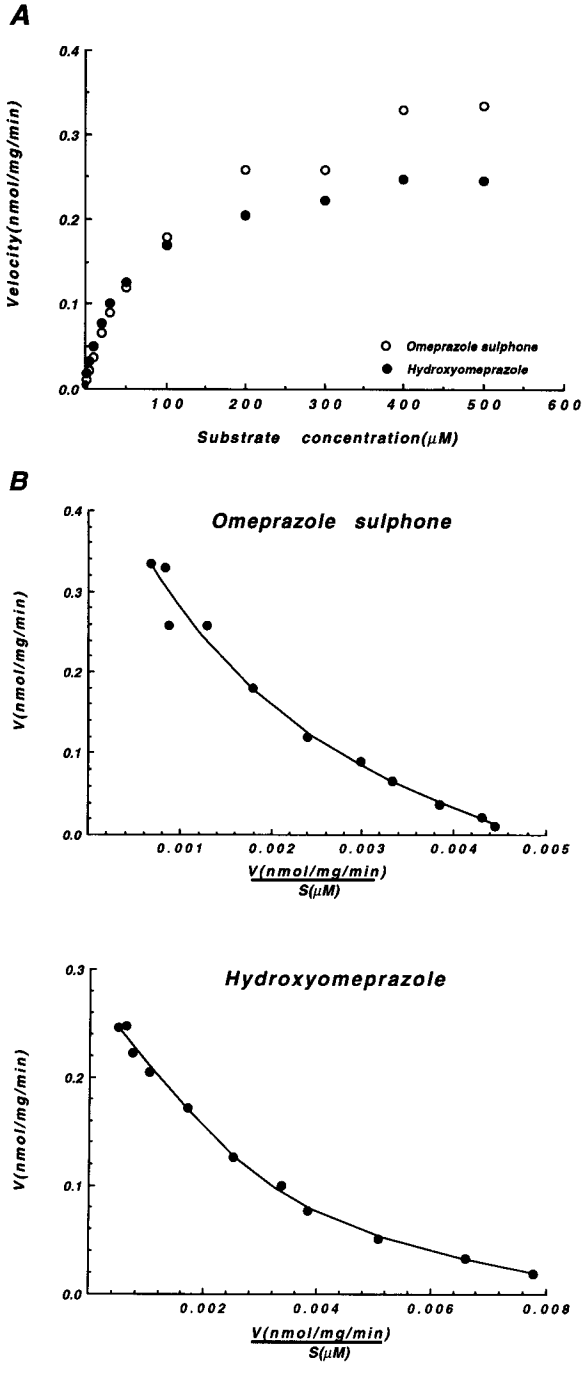

Fig. 5. Velocity *versus* substrate concentration plot (A) and Eadie–Hofstee plots (B) for formation of omeprazole sulphone and hydroxyomeprazole by human liver microsomes.

DISCUSSION

Omeprazole has found widespread application as an anti-ulcer agent since its introduction into clinical practice in the late 1980s. The drug is cleared almost entirely by hepatic metabolism, but as yet there has been no human *in vitro* investigation of the enzymes involved in omeprazole metabolism. Confirmation of the individual enzymes responsible for omeprazole biotransformation is important for characterisation of the regulation of the metabolism of this drug in humans. In the present work, a straightforward HPLC method has been developed for the measurement of omeprazole sulphone and hydroxyomeprazole formation by human liver microsomes. The method is precise and has a chromatography time of about 15 min.

Omeprazole sulphone and hydroxyomeprazole were identified as the main metabolites *in vitro*. Another metabolite, tentatively identified as 5-O-desmethylomeprazole (H 215/02), was also formed to a minor extent by human liver microsomes, although scarcity of authentic compound precluded full assay validation of this compound. An additional metabolite, which eluted just after the internal standard (3.8 min), was also detected but its structure remains unknown at this time. The omeprazole pyridone was also formed during incubation with omeprazole, but in amounts several-fold less than those of 5-O-desmethylomeprazole as indicated by the sizes of the peaks. None of these metabolites were detected in incubations performed in the absence of NADPH-generating system. For the omeprazole sulphide, however, it seemed that this metabolite was formed during the sample preparation procedure. Three other synthetic omeprazole derivatives, the hydroxysulphone (H 195/77),

hydroxyomeprazole sulphide (H 195/69) and 5-O-desmethylomeprazole sulphide (H 182/68), tested for retention times in this assay, were not primary omeprazole metabolites since they could not be detected in any of the samples incubated with omeprazole.

A preliminary study of the formation of omeprazole sulphone and hydroxyomeprazole from omeprazole by human liver microsomes has shown that the formation of both metabolites exhibit biphasic kinetics, suggesting that at least two CYP isoforms are involved in the formation of each of these metabolites. The apparent K_m for the low-affinity component of hydroxyomeprazole formation was close to that for the high-affinity component of omeprazole sulphone formation, suggesting that these reactions may be performed by the same CYP isoform. The other two K_m values were different suggesting that two additional isoforms are probably involved in the formation of these two metabolites. Further studies are underway to define the CYP isoforms in human liver responsible for the formation of different omeprazole metabolites.

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