Biochemical Pharmacology, Vol. 51, pp. 1003–1008, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)2251-1

Cytochromes P450, 1A2, and 2C9 are Responsible for the Human Hepatic O-Demethylation of R- and S-Naproxen

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ABSTRACT. A preliminary report implicated cytochrome P450 (CYP) 2C9 in the human liver microsomal O-demethylation of S-naproxen, suggesting that this pathway may be suitable for investigation of human hepatic CYP2C9 *in vitro*. Kinetic and inhibitor studies with human liver microsomes and confirmatory investigations with cDNA-expressed enzymes were undertaken here to define the role of CYP2C9 and other isoforms in the O-demethylation of R- and S-naproxen. All studies utilised a newly developed sensitive and specific HPLC assay that measured the respective O-desmethyl metabolites of R- and S-naproxen in incubations of human liver microsomes and in COS cell lysates. Microsomal R- and S-naproxen O-demethylation kinetics followed Michaelis-Menten kinetics, with respective mean apparent K_m values of 123 μM and 143 μM. Sulfaphenazole, a specific inhibitor of CYP2C9, reduced the microsomal O-demethylation of R- and S-naproxen by 43% and 47%, respectively, and the CYP1A2 inhibitor furafylline decreased R- and S-naproxen O-demethylation by 38% and 28%, respectively. R,S-Mephenytoin was a weak inhibitor of R- and S-naproxen O-demethylation, but other CYP isoform specific inhibitors (e.g., coumarin, diethyldithiocarbamate, quinidine, troleandomycin) had little or no effect on these reactions. cDNA-expressed CYP2C9 and CYP1A2 were both shown to O-demethylate R- and S-naproxen. Apparent K_m values (92–156 μM) for the reactions catalysed by the recombinant enzymes were similar to those observed for human liver microsomal R- and S-naproxen O-demethylation. The data demonstrate that CYP2C9 and CYP1A2 together account for the majority of human liver R- and S-naproxen O-demethylation, precluding the use of either R- or S-naproxen as a CYP isoform-specific substrate *in vitro* and *in vivo*. BIOCHEM PHARMACOL 51;8:1003–1008, 1996.

KEY WORDS. naproxen; cytochrome P450; human liver; CYP2C9; CYP1A2; drug metabolism

Cytochrome P450 (CYP) is responsible for the oxidative metabolism of a structurally diverse range of drugs, environmental chemicals, and endogenous compounds. This versatility is due to the fact that CYP exists as a superfamily of isoforms. Indeed, more than 220 human CYP genes have been identified and these have been classified into families and subfamilies according to the similarity of their deduced amino acid sequences [1]. Xenobiotic metabolising CYP isoforms characteristically exhibit distinct, but frequently overlapping, patterns of substrate and inhibitor specificities and tend to differ in terms of regulation (induction, repression, tissue expression, polymorphism) [2, 3].

Given the differing regulation of CYP isoforms, prediction of drug interactions and other environmental and genetic factors that influence the metabolism of any drug (or nondrug chemical) necessarily requires identification of the CYP isoform(s) responsible for its metabolism. In recent years, a number of approaches have been developed for identification of the human CYP isoform(s) involved in the metabolism of any xenobiotic *in vitro* [4, 5]. Central to these strategies is the availability of compounds that may be used as isoform-specific substrate and/or inhibitor probes.

There is increasing awareness of the importance of CYP2C subfamily isoforms in human xenobiotic metabolism [6]. In particular, CYP2C9 is responsible for the oxidative metabolism of phenytoin, tolbutamide, torasemide, S-warfarin, and numerous nonsteroidal anti-inflammatory agents (NSAIDs) [6–12]. Recognition of the contribution of CYP2C9 to human drug metabolism has prompted investigation, in this and other laboratories, of xenobiotics that may be used to characterise CYP2C9 activity *in vitro* and *in vivo*. A preliminary report [13] indicated that the human liver microsomal O-demethylation of S-naproxen (Fig. 1), a propionic acid NSAID, was decreased by the CYP2C9-specific inhibitor sulfaphenazole. The plasma unbound clearance of S-naproxen is high, with the O-demethylation pathway accounting for approximately 30% of total clearance [14, 15]. Along with the known ability of CYP2C9 to catalyse the oxidation of a number of NSAIDs, these observations suggested that naproxen may serve as CYP2C9-specific substrate suitable for investigation of the

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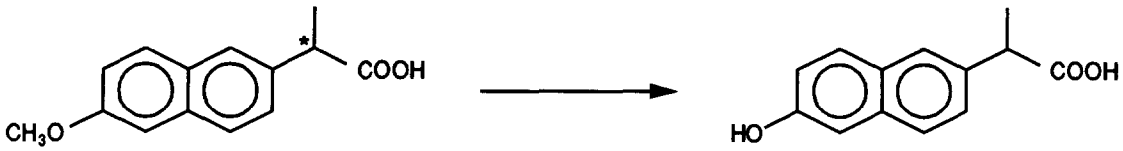
Received 28 June 1995; accepted 9 November 1995.



Naproxen O-Desmethylnaproxen

FIG. 1. The conversion of naproxen to O-desmethylnaproxen; the asterisk shows the position of the chiral carbon atom.

activity of this isoform *in vitro*. Here, we report the results of work that aimed to characterise the involvement of CYP2C9 and other isoforms in human liver microsomal naproxen O-demethylation. Because naproxen contains an asymmetric carbon atom (Fig. 1), studies were undertaken with both the R- and S-enantiomers to investigate the possible CYP isoform stereoselectivity of naproxen O-demethylation.

MATERIALS AND METHODS

Chemicals and Reagents

R- and S-naproxen and R,S,O-desmethylnaproxen were a generous gift from Syntex Discovery Research (Palo Alto, CA). Coumarin, diethyldithiocarbamate, 4-hydroxycoumarin, troleandomycin, NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from the Sigma Chemical Co (St. Louis, MO). Sources of other compounds used here were: furafylline from Dr. R. Gasser (Roche, Basel, Switzerland), R,S-mephenytoin from Sandoz Ltd. (Basel, Switzerland), quinidine sulfate from Burroughs Wellcome (Sydney, Australia), and sulfaphenazole from Ciba-Geigy Australia (Sydney, Australia). All other chemicals and solvents were of analytical reagent grade.

Human Liver Samples and cDNAs

Human liver samples were obtained from renal transplant donors with the approval of next-of-kin and the Flinders Medical Centre Committee on Clinical Investigation. Details of donors of livers (e.g., H10, H24, H30) used in this work have been published previously [16]. Hepatic microsomes were prepared by differential centrifugation, suspended in 0.1 M phosphate buffer (pH 7.4) that contained glycerol (20% v/v) and stored at −70°C until used. Microsomal and COS cell lysate protein concentrations were measured according to Lowry *et al.* [17] with bovine serum albumin as the standard.

The CYP1A2 and CYP2C9 cDNAs were isolated, subcloned into the expression vector pCMV4, and transfected separately into COS-7 cells as described previously [7, 18]. Cells were harvested 48 hr posttransfection and resuspended in 0.1 M phosphate buffer (pH 7.4) containing glycerol (20% v/v). Cells transfected with the respective cDNA in the reverse orientation served as negative controls in the studies investigating naproxen O-demethylase activity by cDNA-expressed CYP1A2 and CYP2C9.

Measurement of the Naproxen O-Demethylation Activity of Human Liver Microsomes and cDNA-Expressed CYP1A2 and CYP2C9

Standard 1 mL incubations contained human liver microsomal (0.2 mg) or COS cell lysate (0.5 mg) protein, NADPH-generating system (1 mM NADP, 100 mM glucose-6-phosphate, 2 IU glucose-6-phosphate dehydrogenase, and 5 mM $MgCl_2$) and R- or S-naproxen (5–500 μM) in phosphate buffer (0.1 M, pH 7.4). Reactions were initiated by the addition of NADPH generating system and were carried out in air at 37°C for 30 min. Incubations were terminated by the addition of 11.3 M HCl (0.2 mL) and cooling on ice. After addition of 4-hydroxycoumarin (2.5 nmol), the assay internal standard, the incubation mixture was centrifuged (3000 × g for 10 min). The supernatant fraction from each incubation was poured into a 15-mL glass culture tube containing ammonium sulfate (1.5 g) and extracted with diethylether (6 mL) using a vortex mixer. The organic and aqueous fractions were separated by centrifugation (3000 × g for 10 min) and a 5-mL aliquot of the organic phase was transferred to a clean, conical tip glass tube. The ether extract was evaporated to dryness under a stream of N_2. Residues were reconstituted in 0.2 mL of the HPLC mobile phase and an aliquot (0.1 mL) was subsequently injected onto the column. The chromatograph employed was fitted with an Ultrasphere ODS column (25 cm × 4.6 mm id, 5-micron particle size; Beckman Instruments, San Ramon, CA). The column was eluted with phosphate buffer (10 mM, pH 7.0)-acetonitrile (90:10) at a flow rate of 1.8 mL/min and peaks were monitored by ultraviolet detection at 235 mm. Standard curves were constructed using authentic R,S-O-desmethylnaproxen over the concentration range 0.1–2.5 μM; calibrators were treated in the same manner as incubation samples. Unknown concentrations were calculated by comparison of the O-desmethylnaproxen/internal standard peak height ratio with those of the standard curve.

Kinetic and Inhibitor Studies

R- and S-naproxen O-demethylation kinetics by human liver microsomes were determined over the concentration range 5–500 μM. The substrate concentration range employed for the kinetic studies with cDNA-expressed CYP1A2 and CYP2C9 was 50–500 μM. The CYP isoform selective inhibitors/substrates [4, 5] coumarin (2A6), diethyldithiocarbamate (2E1), furafylline (1A2), mephenytoin (2C19), quinidine (2D6), sulphaphenazole (2C9), and

troleandomycin (3A4) were screened for effects on human liver microsomal naproxen O-demethylation at a substrate concentration of 150 μM (the approximate apparent K_m for both R- and S-naproxen; see Results). Concentrations of inhibitors are given in Fig. 4. Except for quinidine and diethyldithiocarbamate, which were added to incubations as aqueous solutions, xenobiotic inhibitors were dissolved in DMSO so that the final concentration of solvent in incubations was 0.5% v/v. Control incubations contained an equal volume of DMSO, which was shown to have a negligible effect on microsomal naproxen O-demethylation. A 10-min preincubation was utilised for diethyldithiocarbamate, furafylline, and troleandomycin.

Analysis of Results

Results are presented as mean ± SD. The Michaelis-Menten parameters apparent K_m and V_{max} were calculated by fitting data to MK Model, an extended least-squares modelling program.

RESULTS

Assay for R- and S-Naproxen O-Demethylase Activity

Under the chromatographic conditions employed, retention times for 4-hydroxycoumarin (the internal standard), R- and S-O-desmethylnaproxen and R- and S-naproxen were 4.6, 10.5, and 75 min, respectively (Fig. 2). The large difference in retention times for O-desmethylnaproxen and naproxen allowed 5 successive injections prior to elution of naproxen. Thus, average chromatography time for each sample was in the order of 15 min. Standard curves were linear over the calibration range (0.1–2.5 μM) and passed through the origin. O-Desmethylnaproxen recovery from incubation mixtures over the standard curve concentration range was 92.5 ± 1.4%; internal standard recovery (n = 5) was 85.5 ± 2.0%. Overall assay within-day reproducibility was assessed by investigating the conversion of R- and S-naproxen to R- and S-O-desmethylnaproxen, respectively, at a substrate concentration of 150 μM using the same batch of human liver microsomes. Within-day reproducibilities for R- and S-O-desmethylnaproxen formation (n = 6 estimations) were 4.6% and 6.9%, respectively.

Using the microsomal assay procedure, O-desmethylnaproxen formation (from either R- and S-naproxen) was linear with respect to protein concentration and incubation time to at least 1.0 mg/mL and 50 min, respectively. Incubations performed with the CYP isoform specific inhibitors/substrates coumarin, diethyldithiocarbamate, furafylline, mephenytoin, quinidine, sulfaphenazole, and troleandomycin, in the absence of R- and S-naproxen, indicated that none of these compounds or their metabolites interfered with the chromatography of R- and S-O-desmethylnaproxen.

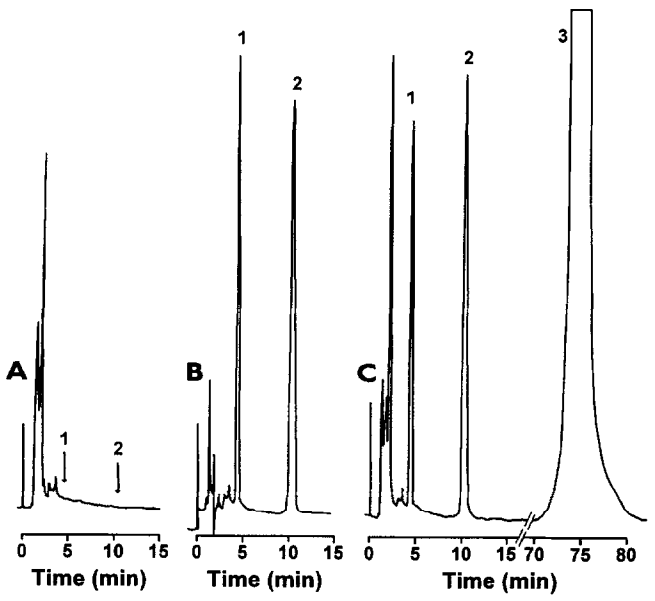


FIG. 2. Representative chromatograms of human liver microsomal extracts. (A) Blank chromatogram: microsomal incubation performed without R- or S-naproxen; (B) Standard containing R,S-O-desmethylnaproxen, 1 μM; and (C) Microsomal incubation performed in the presence of S-naproxen, 50 mM. Peaks 1, 2, and 3 show retention times for 4-hydroxycoumarin, R,S-O-desmethylnaproxen, and R- and S-naproxen. Chromatography conditions as described in Methods.

Kinetics of Human Liver Microsomal R- and S-Naproxen O-Demethylation

R- and S-naproxen O-demethylation kinetics were investigated using microsomes from three human livers. Michaelis-Menten kinetics were observed for the O-demethylation of both R- and S-naproxen in all three livers (Fig. 3). Mean apparent K_m values for R- and S-O-desmethylnaproxen formation were 123 ± 24 μM and 143 ± 41 μM, respectively. The mean V_{max} values for R- and S-O-desmethylnaproxen formation were 0.97 ± 0.30 nmol/min/mg protein and 0.84 ± 0.16 nmol/min/mg protein, respectively. Mean values of the V_{max} to K_m ratios for R-naproxen ($7.9 \times 10^{-3} \pm 2.9 \times 10^{-3}$) and S-naproxen ($6.0 \times 10^{-3} \pm 2.4 \times 10^{-3}$) differed by less than 30%.

Inhibition by CYP Isoform-Specific Probes

Sulfaphenazole, a specific inhibitor of CYP2C9, reduced the conversion of R-naproxen to R-O-desmethylnaproxen by 43% and the conversion of S-naproxen to S-O-desmethylnaproxen by 47% (Fig. 4). The CYP1A2-specific inhibitor furafylline decreased R- and S-naproxen O-demethylation by 38% and 28%, respectively. Although R,S-mephenytoin impaired the formation of both R- and S-O-desmethylnaproxen by 16%, coumarin (CYP2A6), diethyldithiocarbarmate (CYP2E1), quinidine (CYP2D6), and troleandomycin (CYP3A4) had little or no effect on the 2 reactions.

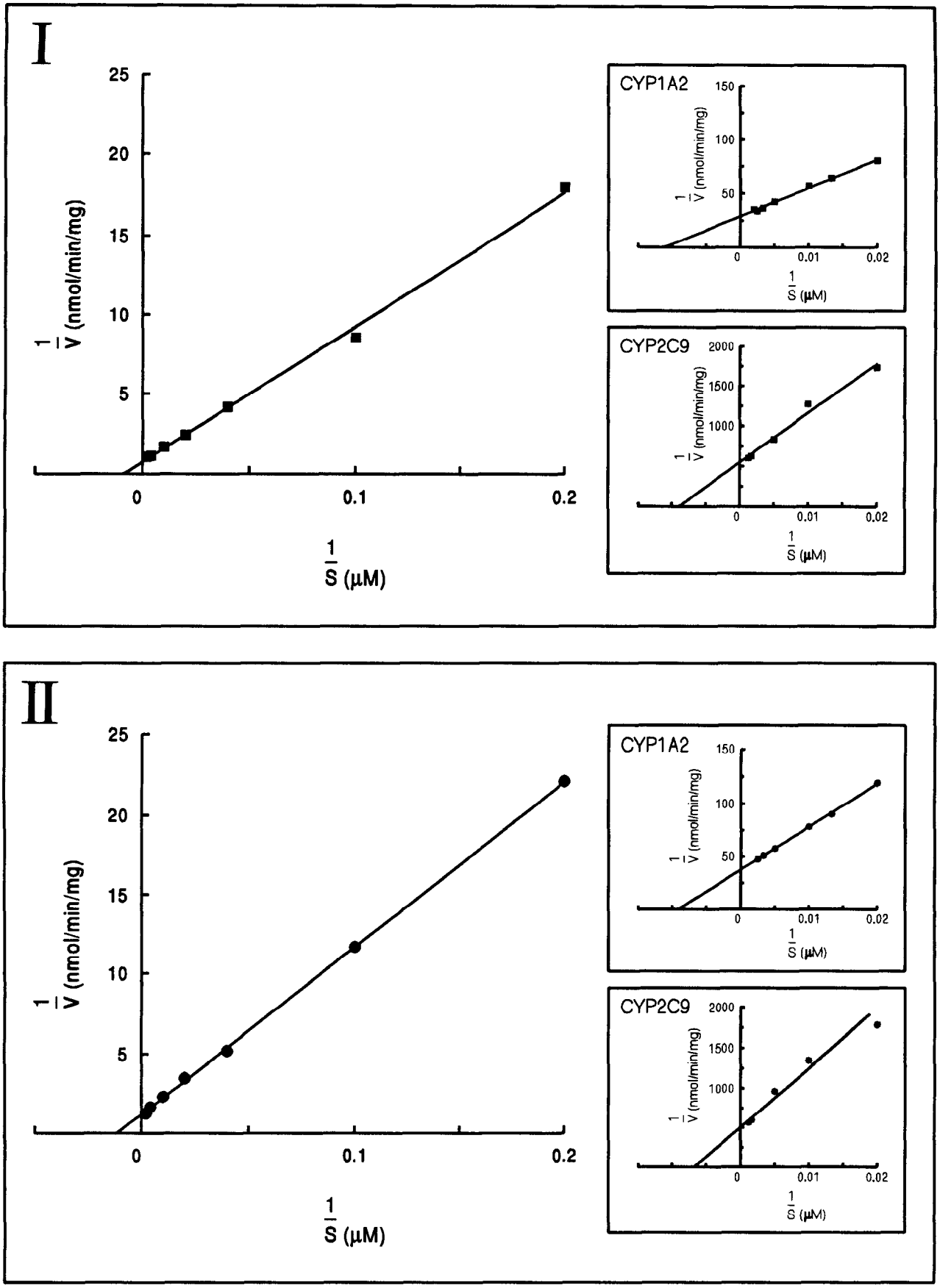


FIG. 3. Representative Lineweaver-Burk plots for the conversion of R-naproxen (panel I) and S-naproxen (panel II) to R- and S-O-desmethylnaproxen, respectively, in human liver microsomes (liver H10). Kinetic plots for the O-demethylation of R- and S-naproxen by cDNA-expressed CYP1A2 and CYP2C9 are shown in the insets in panels I and II. Points are experimentally derived values and the solid lines are the computer-generated curves of best-fit.

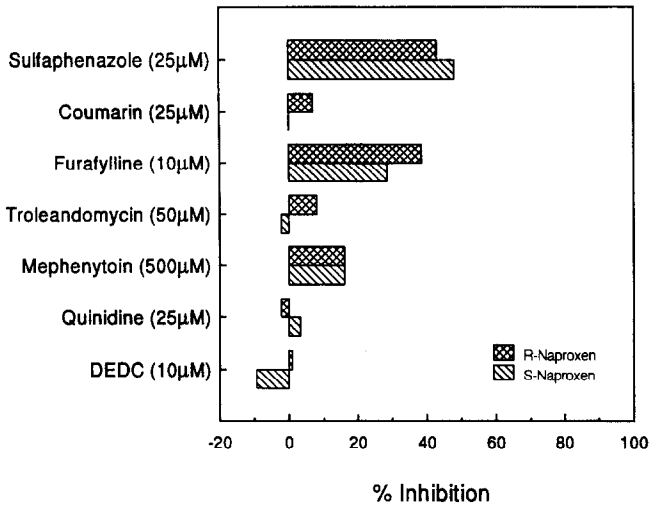


FIG. 4. Effects of CYP isoform-specific xenobiotic probes on human liver microsomal R- and S-naproxen O-demethylation (mean of results from two livers, H10 and H30). The substrate concentration (i.e., R- or S-naproxen) was 150 μM. Concentrations of xenobiotics were as shown. DEDC refers to diethyldithiocarbamate.

R- and S-Naproxen O-Demethylation by cDNA-Expressed CYP1A2 and CYP2C9

Because the inhibition studies indicated a major role for CYP1A2 and CYP2C9 in R- and S-naproxen O-demethylation, the ability of cDNA-expressed CYP1A2 and CYP2C9 to catalyse both reactions was investigated. cDNA-expressed CYP1A2 catalysed the conversion of R- and S-naproxen to their O-demethylated metabolites with respective apparent K_m values of 92 μM and 103 μM (Fig. 3, inset). Similarly, cDNA-expressed CYP2C9 O-demethylated both R- and S-naproxen with apparent K_m values of 126 μM and 156 μM, respectively (Fig. 3, inset).

DISCUSSION

Results presented here demonstrate that CYP2C9 and CYP1A2 are the major CYP isoforms contributing to the O-demethylation of both R- and S-naproxen in human liver. Together, sulfaphenazole and furafylline, which are specific inhibitors of human liver microsomal CYP2C9 and CYP1A2, respectively [19, 20], reduced hepatic R- and S-naproxen O-demethylation by 75–80%. Comparative naproxen O-demethylation kinetic studies using human liver microsomes and cDNA-expressed enzymes were consistent with a major involvement of CYP 2C9 and 1A2 in this biotransformation pathway. Apart from sulfaphenazole and furafylline, R,S-mephenytoin also inhibited R- and S-naproxen O-demethylation to a significant extent (ca. 15%). Isoforms involved in R,S-mephenytoin metabolism, particularly CYP2C19 [21] may, therefore, be responsible for the remainder of R- and S-naproxen O-demethylation.

The present study was undertaken on the basis of a preliminary report [13] linking CYP2C9 to S-naproxen O-demethylation along with the known involvement of this isoform in the microsomal metabolism of a range of NSAIDs. In particular, CYP2C9 is the principal enzyme involved in the 2-hydroxylation of R- and S-ibuprofen and in the 4′-hydroxylation of R- and S-flurbiprofen [11, 12], two propionic acid NSAIDs structurally related to naproxen. The apparent exclusive involvement of CYP2C9 in the hydroxylation of flurbiprofen and ibuprofen is in contrast to the partial contribution of this isoform to naproxen O-demethylation. Interestingly, cDNA-expressed CYP1A2 also catalysed flurbiprofen hydroxylation, although the turnover was less than 1% of that observed for CYP2C9 [11].

Stereoselective elimination is a not an uncommon feature of drugs possessing a chiral carbon atom, and differences in the rates of oxidative metabolism of the enantiomers of a number of NSAIDs have been reported [22]. However, there was no evidence of marked stereoselectivity in the O-demethylation of naproxen in the present work. Mean apparent K_m and V_{max} values (and ratios of V_{max} to K_m) for the human liver microsomal O-demethylation of both R- and S-naproxen were relatively close in value. Similarly, apparent K_m values observed for the CYP2C9- and CYP1A2-catalysed O-demethylation of R- and S-naproxen were all within the range 92–156 μM and effects of the various prototypic CYP isoform inhibitors on the oxidation of each enantiomer were not dissimilar. A lack of stereoselectivity has also been reported for human liver microsomal ibuprofen 2-hydroxylation [12]. These observations are in contrast to the approximately 60% difference in apparent K_m and almost 2-fold difference in the intrinsic clearances (V_{max}/K_m) calculated for the 4′-hydroxylation of the flurbiprofen enantiomers *in vitro* [11].

Despite the involvement of at least two enzymes in microsomal naproxen O-demethylation, this reaction exhibited Michaelis-Menten kinetics *in vitro*. The occurrence of Michaelis-Menten kinetics is frequently used to support the involvement of a single enzyme in a particular metabolic pathway. In this instance, however, affinities of R- and S-naproxen for both CYP2C9 and CYP1A2 were close in value and the contribution of these separate isoforms to the microsomal reaction could not be differentiated kinetically. Such data serve to emphasize the necessity for the application of multiple approaches *in vitro* (e.g., kinetic and inhibition studies, use of cDNA-expressed enzymes, activity correlations) to differentiate and characterise the contribution of individual enzymes to a particular biotransformation pathway [4, 5].

In summary, it has been demonstrated that CYP2C9 and CYP1A2 together account for the majority of R- and S-naproxen O-demethylation in human liver *in vitro*. Thus, the *in vivo* metabolic clearance of S-naproxen, the enantiomer used clinically, will be determined largely by the activities of these two isoforms. The contribution of CYP1A2 to naproxen O-demethylation is in contrast to the apparently exclusive involvement of CYP2C9 in the oxidative metabolism of other NSAIDs (e.g. diclofenac, flurbiprofen, ibuprofen, mefenamic acid, and some oxicams) [6,

11, 12, 23, 24]. The involvement of multiple enzymes in R- and S-naproxen O-demethylation precludes the use of naproxen (R or S) as a CYP isoform-specific substrate *in vitro* or *in vivo*.

Support from the National Health and Medical Research Council of Australia and the United States Public Health Service (grant no. GM 36590) is gratefully acknowledged.

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