Biochemical Pharmacology, Vol. 58, pp. 787–796, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00167-7

Hepatic Metabolism of Diclofenac: Role of Human *CYP* in the Minor Oxidative Pathways

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ABSTRACT. The aim of this study was to re-examine the human hepatic metabolism of diclofenac, with special focus on the generation of minor hydroxylated metabolites implicated in the idiosyncratic hepatotoxicity of the drug. Different experimental approaches were used: human hepatocytes, human microsomes, and engineered cells expressing single human *CYP* (cytochromes P450). Human hepatocytes formed 3′-hydroxy-, 4′-hydroxy-, 5-hydroxy- 4′,5-dihydroxy-, and *N*,5-dihydroxydiclofenac, as well as several lactams. Formation of 4′- and 5-hydroxydiclofenac by human liver microsomes followed a Michaelis–Menten kinetics (K_m 9 ± 1 μM; V_{max} 432 ± 15 pmol/min/mg and K_m 43 ± 5 μM; and V_{max} 15.4 ± 0.6 pmol/min/mg, respectively). Secondary metabolites were detected after incubation of 5-hydroxydiclofenac with human liver microsomes, yielding 4′,5-dihydroxydiclofenac (K_m 15 ± 1 μM; V_{max} 96 ± 3 pmo1/min/mg) and small amounts of *N*,5-dihydroxydiclofenac (non-Michaelis–Menten kinetics). Based on microsome studies and the incubations with human hepatocytes and engineered cells, we estimated that *in vivo* CYP2C9 would be exclusively responsible for the 4′ hydroxylation of diclofenac (>99.5%) as well as 5-hydroxydiclofenac (>97%). CYP2C9 was exclusively responsible for the formation of 3′-hydroxydiclofenac. Multiple regression analysis evidenced that the rate of production of 5-hydroxydiclofenac in human microsomes followed the algorithm: 0.040 × *S*-mephenytoin 4′-hydroxylation + 0.083 × tolbutamide methylhydroxylation, (multiple correlation coefficient = 0.969). However, the incubation of diclofenac with cell lines expressing different human *CYP* suggested that 7 isoforms could be involved. Comparison of data obtained with *CYP*-expressing cells and human hepatocytes suggests that CYP2C8 > CYP2C19 ≅ CYP2C18 ≫ CYP2B6 are the isoforms implicated in the 5-hydroxylation of diclofenac *in vivo*. BIOCHEM PHARMACOL **58**;5:787–796, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. cytochrome P450; diclofenac; *in vitro* metabolism; CYP2C

Diclofenac, an arylacetic non-steroidal anti-inflammatory drug, was developed in the late 1970s and approved for clinical use in treating several rheumatic diseases (rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis) and as an analgesic [1]. Early studies in rat, baboon, dog, monkey, and man [2, 3] showed that diclofenac undergoes an extensive hepatic metabolism involving aromatic hydroxylations and conjugations (Fig. 1). Most diclofenac metabolites are found in animals and man, but their relative abundance differs. In man, 4′-OHDic,¶ 5-OHDic, 4′, 5-diOHDic, 3′-OHDic, and 3′-OH-4′-OCH_3-Dic have been identified in urine, plasma, and/or bile [2, 4].

The use of diclofenac has been associated with occasional hepatic toxicity. Borderline elevation of enzyme markers is found in a significant number of patients taking the drug (15%), and a mild to severe hepatotoxicity has been reported in a small but significant number of patients [5–8]. Several hypotheses have been put forth to explain this idiosyncratic adverse reaction, among them the formation of toxic metabolites and/or reactive intermediates able to bind hepatocyte proteins [5, 6, 9–11] triggering, presumably, an allergic response. Interest in diclofenac metabolism has been increased by indirect experimental evidence suggesting that a minor CYP-generated oxidation product of diclofenac could be involved in the formation of drug–protein adducts in human microsomes [12].

4′-hydroxylation of diclofenac was first attributed to CYP2C enzymes [13] and, more precisely, to CYP2C9 [14].

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¶ *Abbreviations:* 4′-OHDic, 4′-hydroxydiclofenac [2-(2′,6′-dichloro-4′-hydroxy-phenylamine)-phenyl]-acetic acid; 5-OHDic, 5-hydroxydiclofenac [2-(2′,6′-dichlorophenylamine)-5-hydroxyphenyl]-acetic acid; 3′-OHDic, 3′-hydroxydiclofenac [2-(2′,6′-dichloro-3′-hydroxyphenylamine)- phenyl]-acetic acid; 4′,5-diOHDic, 4′,5-dihydroxydiclofenac [2-(2′,6′-dichloro-4′-hydroxy-phenylamine)-5-hydroxyphenyl]-acetic acid; N,5-diOHDic, N,5-dihydroxydiclofenac [2-(2′,6′-dichlorophenylhydroxylamine)-5-hydroxyphenyl]-acetic acid; 3′-OH-4′-OCH_3-Dic, 3′-hydroxy-4′-methoxydiclofenac [2-(2′,6′-dichloro-3′-hydroxy-4′-methoxyphenylamine)-phenyl]-acetic acid; CYP, cytochrome P450; R, regression coefficient; and R_T, retention time.

Received 23 October 1998; accepted 5 March 1999.

Compound	R_1	R_2	R_3	R_4
Diclofenac	H	H	H	H
4'-hydroxydiclofenac	OH	H	H	H
5-hydroxydiclofenac	H	OH	H	H
3'-hydroxydiclofenac	H	H	OH	H
4', 5-dihydroxydiclofenac	OH	OH	H	H
N,5-dihydroxydiclofenac	H	OH	H	OH
3'-hydroxy-4'-methoxydiclofenac	OCH_3	H	OH	H

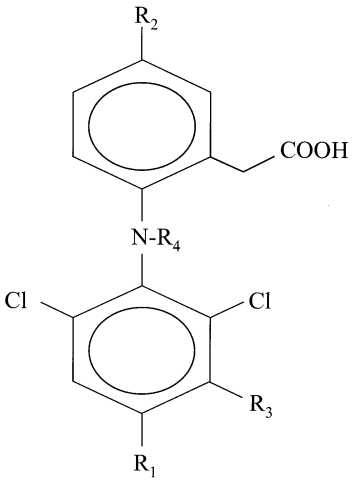

FIG. 1. Structure of diclofenac and its metabolites.

However, no detailed study has yet been performed to identify the precise human isoform(s) involved in the formation of the other oxidised metabolites. Findings from our laboratory suggest that at least one minor hydroxylation metabolic pathway could be at the root of the human hepatic toxicity of diclofenac [15]. This led us to examine in detail the metabolism of diclofenac and to precisely identify the enzymes involved. By using cultured human hepatocytes, human liver microsomes, and genetically engineered cell lines expressing human *CYP*, it was possible to identify the minor pathways of diclofenac biotransformation and to estimate the contribution of the different CYP isoforms to its metabolism *in vivo*.

MATERIALS AND METHODS

Chemicals

Diclofenac, taxol, chlorzoxazone, coumarin, quinidine, troleandomycin, and NADPH were from Sigma. Furafylline and sulfaphenazole were a generous gift from Dr. Alan Boobis. 6α-Hydroxytaxol was obtained from Gentest Corp. S(+)-Mephenytoin and (±)-4′-hydroxymephenytoin was from Ultrafine Chemicals. The enzyme β-glucuronidase/arylsulphatase was from Boehringer Mannheim. Culture media (Ham's F-12, Lebovitz L-15) were from Life Technologies. All other chemicals were of analytical grade. Synthesis of 4′-OHDic, 5-OHDic as well as their lactams and *N*,5-diOHDic has been described in detail elsewhere [15, 16].

Synthesis of 3′-OHDic

2,6-Dichloro-3-nitroaniline was treated with $NaNO_2$ followed by KI to give 2,6-dichloro-3-nitro-iodobenzene. This compound was allowed to react with aniline under xylene reflux to give *N*-phenyl-2,6-dichloro-3-nitroaniline, which was transformed to 1,3-dihydro-*N*-[(2,6-dichloro-3-nitro)-phenyl]-2*H*-indol-2,3-dione by reaction with oxalyl chloride. Reduction by P/HI gave 1,3-dihydro-*N*-[(2,6-dichloro-3-amino)-phenyl]-2*H*-indol-2-one, which by reaction with $NaNO_2$ and $Cu(NO_3)_2$/Cu_2O, gave 1,3-dihydro-*N*-[(2,6-dichloro-3-hydroxy)-phenyl]-2*H*-indol-2-one. The phenolic hydroxyl group of this compound was protected by reaction with benzyl chloride to give 1,3-dihydro-*N*-[(2,6-dichloro-3-benzyloxy)-phenyl]-2*H*-indol-2-one. Hydrolysis of this compound with NaOH to open the ring, and hydrogenolysis to remove the benzyl group yielded 3′-OHDic. The most relevant spectral properties were: ^{1}H-NMR (d_6-acetone), d 3.9 (s, 2H, Ar-CH_2-COO-), 6.9 (s, 1H, -NH-), 7.4-6.8 (m, 6H, aromatic); (KBr tablet), 3400 (NH stretching), 1720 (COOH carbonyl stretching), 1585 (NH deformation), 1580 (C═C stretching); MS m/z (%), 311 (13), 293 (24), 258 (46), 230 (100).

Synthesis of 4′,5-diOHDic

2,6-dichloro-4-methoxy aniline with its amino group protected by reaction with Ac_2O was allowed to react with *p*-bromoanisole to give *N*-acetyl-*N*-(4-methoxy)phenyl-2,6-dichloro-4-methoxyaniline. This compound was transformed to *N*-1, 3-dihydro-*N*-[(2,6-dichloro-4-methoxy)-phenyl]-5-methoxy-2*H*-indol-2,3-dione first by deprotection of the amine group with KOH, followed by reaction with oxalyl chloride. Reduction with P/HI gave the 4′,5-dihydroxydiclofenac lactam. Hydrolysis of this compound with NaOH to open the ring yielded 4′,5-diOHDic. The most relevant spectral features of this compound were: ^{1}H-NMR (d_6-acetone), d 3.78 (s, 2H, Ar-CH_2-COO-), 7.2-6.4 (m, 5H, aromatic); i.r. (KBr tablet), 3410 (NH stretching), 3200 (OH stretching), 1695 (COOH carbonyl stretching), 1585 (NH deformation), 1580 (C═C stretching); MS: m/z (%): 327 (46), 309 (14), 274 (22), 246 (100), 211 (24).

Isolation and Culture of Human Hepatocytes

Human hepatocytes were obtained from surgical liver biopsies (2–4 g) of patients undergoing cholecystectomy, after informed consent. Cells were isolated by microperfusion of the tissue sample with collagenase, as described in detail elsewhere [17]. Cellular viability was assessed by the trypan blue dye exclusion test and was usually 85–90%.

Hepatocytes were seeded on fibronectin-coated plastic dishes (3.5 μg/cm^2) at a density of 8×10^4 viable cells/cm^2 and cultured in Ham's F-12/Lebovitz L-15 (1:1) medium supplemented with 2% new-born bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin, 0.2% BSA, and 10^{-8} M insulin. One hour later the medium was changed, and after 24 hr the cells were shifted to serum-free hormone-supplemented medium (10^{-8} M dexamethasone and insulin). The incubation with the drug was routinely done between 24 and 48 hr of culture.

CYP-Engineered Cell Lines

Immortalised human liver epithelial cells (THLE) were genetically manipulated to express specific *CYP* genes. Cell lines expressing *CYP*1A2 (T5-1A2), *CYP*2A6 (T5-2A6), *CYP*2B6 (T5-2B6), *CYP*2D6 (T5-2D6), *CYP*2E1 (T5-2E1), and *CYP*3A4 (T5-3A4) were cultured on fibronectin/collagen-coated flasks in serum-free medium, as previously described [18, 19]. In addition, *CYP*2C-expressing cells were generated by inserting the following cDNAs by blunt-ended cloning (kindly provided by J. A. Goldstein): 1.9 kb *CYP*2C8 (clone 7b), 1.85 kb *CYP*2C9 (clone 65; $Ile_{359}Gly_{475}$), 2.0 kb *CYP*2C18 (clone 29c-1a;Asp_2Thr_{385}), 1.75 kb *CYP*2C19 (clone 11a) into the BamHI site of the pCMVneo vector (kindly provided by B. Vogelstein), as described elsewhere [20].

Metabolism by Cultured Cells

Cells were incubated with diclofenac (Na salt) at a concentration showing no cytotoxic effect on cells. Samples of the culture media were withdrawn and immediately acidified with acetate buffer 0.1 M, pH 4.5. The metabolites present in the culture medium were enzymatically deconjugated and subjected to HPLC analysis as described below.

Microsome Incubations and Inhibition Studies

Samples of human liver were from the human liver bank maintained by the Section on Clinical Pharmacology, Imperial College School of Medicine, London. Local Ethical Committee permission and coroner's approval were obtained to use the samples in the studies described here. The hepatic microsomal fraction was isolated following homogenisation of the samples in 0.25 M potassium phosphate buffer, pH 7.25 containing 1 mM EDTA and 0.15 M KCl, by differential ultracentrifugation [21]. The microsomal pellet was resuspended and stored at −80° in 0.25 M potassium phosphate buffer, pH 7.25 containing 30% v/v glycerol. Assays were performed in a buffer containing 75 mM Tris–hydrochloride buffer pH 7.4, 3 mM magnesium chloride, and 500 μg of microsomal protein and the drug. Samples were preincubated for 5 min at 37° and the reaction started by the addition of NADPH (final concentration 1.2 mM). The reaction was stopped by the addition of 1 mL cold acetonitrile. Controls were incubated either without NADPH or without microsomes. Samples were analysed by HPLC as described below. Inhibition studies were performed using a pool of human liver microsomes and several well-characterised specific inhibitors and substrates of cytochrome P450, namely furafylline [22], coumarin [23], sulfaphenazole [24], quinidine [25], chlorzoxazone [26], and troleandomycin [27]. Inhibitors were prepared as stock solutions in methanol and diluted 1:100 in incubation mixtures. In the case of furafylline and troleandomycin, microsomes were preincubated for 15 min with NADPH and the reaction was started by the addition of diclofenac.

HPLC Analysis and Identification of Diclofenac Metabolites in Incubation Samples

Two HPLC methods were developed to analyse diclofenac metabolites. In method A, samples from microsome incubations were precipitated with acetonitrile and centrifuged for 10 min at 8,000 *g*. After dilution with 20 mM phosphate buffer pH 7.4 to reach 25% (v/v) acetonitrile, samples were HPLC analysed using a C-18 reverse-phase Kromasil 5 μm 20 × 0.46-cm column. The mobile phase (75% triethanolamine 0.02% in 20 mM phosphate buffer pH 7.4, 25% acetonitrile) was delivered at 1 mL/min, and the effluents were monitored at 282 nm. Carprofen (8 μM) was used as an internal standard during the analysis. Metabolites were identified on the basis of their retention times and UV spectra, and compared with chemically synthesised metabolites. A second method was developed to fit the criteria for MS analysis. Aliquots of the culture media were enzymatically deconjugated with 50 mU/mL β-glucuronidase and 30 mU/mL arylsulphatase in 0.1 M acetate buffer pH 4.5 for 4 hr at 37° and extracted several times with ethyl acetate. The organic phase was dried under nitrogen atmosphere, dissolved in methanol and injected into a 150 × 2.1 mm C-18 reverse-phase column (Symmetry). The mobile phase (20 mM ammonia acetate buffer pH 6.5: methanol; 63:37, v/v) was delivered at 0.3 mL/min. The column effluent was monitored at 282 nm using a photodiode detector (M996, Waters) and a mass spectrometer coupled on line to the HPLC (Therma-Beam® detector, Waters). The UV and MS spectra of the major metabolites were compared with those of chemically synthesised samples.

Data Analysis

Experiments were done at least in triplicate, and the statistical significance of the data was analysed. Enzyme kinetic parameters were calculated by Lineweaver–Burk transformation. Correlation matrix and stepwise regression analysis were performed using a commercial statistical program (Statgraphics).

RESULTS

Metabolism of Diclofenac by Human Hepatocytes

Human hepatocytes were incubated with a non-cytotoxic concentration of diclofenac (100 μM), and aliquots of

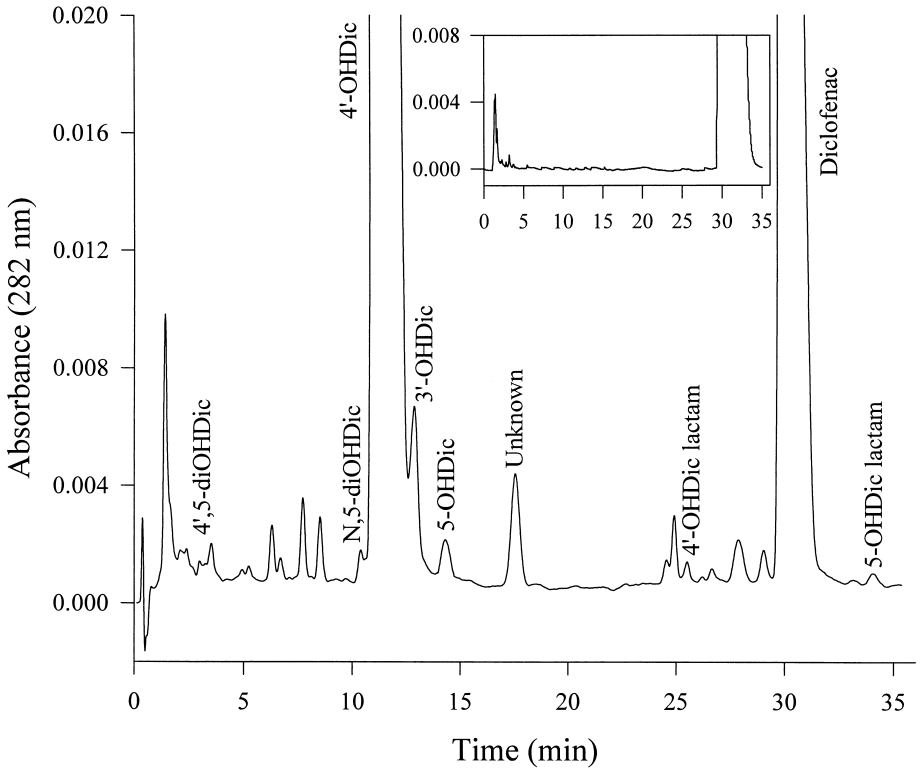

FIG. 2. HPLC chromatogram of diclofenac and metabolites formed by human hepatocytes. Diclofenac (100 μM) was incubated with cultured hepatocytes for 24 hr and the supernatant analysed by HPLC/UV/MS (method B; Materials and Methods). The column effluent was monitored at 282 nm with a photodiode detector and the identification achieved by UV and mass spectra. In addition, a chromatogram at t = 0 is shown in the inset.

culture medium were withdrawn at regular time intervals and analysed by HPLC/UV/MS. The chromatogram shown in Fig. 2 is an analysis of a representative sample showing the presence of several diclofenac-related metabolites in culture medium. The peaks were identified on the basis of their retention times, UV and MS, by comparison with chemically synthesised probes. The major metabolite found in the human hepatocyte culture was 4′-OHDic (R_T: 11.0 min). Two minor monohydroxylated metabolites 5-OHDic (R_T: 14.6 min) and 3′-OHDic (R_T: 12.9 min) were also found. The peak eluting at 3.6 min showed the UV spectra characteristics and the R_T of chemically synthesised 4′,5-diOHDic. Peaks at 25.5 and 34.0 min were identified as the lactam of 4′-OHDic and 5-OHDic. A minor metabolite found at R_T 17.6 min showed a mass spectrum compatible with a monohydroxylated derivative (m/z: 311 [mass peak], 293, 258, 230, and 194). The UV spectra and retention time did not fit any of the synthesised monohydroxylated derivatives of diclofenac. The peak at R_T 10.4 min showed a displacement of the UV absorption maximum to lower values (268 nm) and spectra comparison with chemically synthesised *N*,5-diOHDic confirmed the identity of the peak. A peak with R_T 24.9 min was detected in samples of culture supernatant showing the following m/z features: 309 (mass peak), 265, 229, 201, 195, and 166; this fragmentation pattern and the UV spectra are in agreement with the diclofenac iminoquinone described by Miyamoto *et al.* [11]. We did not observe the presence of 3′-OH-4′-OCH_3-Dic in the culture medium of human hepatocytes after 24-hr incubation with diclofenac.

Diclofenac Metabolism by Human Liver Microsomes

Incubation of diclofenac with human microsomes yielded only 4′-OHDic and 5-OHDic. The formation of both metabolites followed a Michaelis–Menten kinetics (Fig. 3). The following kinetic parameters were obtained: K_m 9 μM and V_{max} 432 pmol/min/mg for 4′-hydroxylation; K_m 43 μM and V_{max} 15.4 pmol/min/mg for 5-hydroxylation. Incubation of 5-OHDic with human liver microsomes showed that the formation of 4′,5-diOHDic observed in hepatocyte cultures was subsequent to CYP oxidation of 5-OHDic (K_m 15 μM and V_{max} 96 pmol/min/mg). This dihydroxylated metabolite was not formed when microsomes were incubated with 4′-OHDic. In microsome incubations with 5-OHDic, it was also possible to detect the formation of *N*,5-diOHDic. The rate of formation of the hydroxylamine did not follow typical Michaelis–Menten kinetics as it did not reach a plateau upon an increased concentration of substrate, suggesting that *N*,5-diOHDic could be partially formed non-enzymatically.

Identification of CYP Involved in the Oxidative Metabolism of Diclofenac

In order to identify the CYP involved in the formation of the oxidised metabolites, incubations were carried out with a pool of microsomes from a human liver bank in the presence of specific CYP inhibitors (Fig. 4). Incubation with furafylline, a potent inhibitor of CYP1A2, [22] did not show any effect on the reactions studied, suggesting that

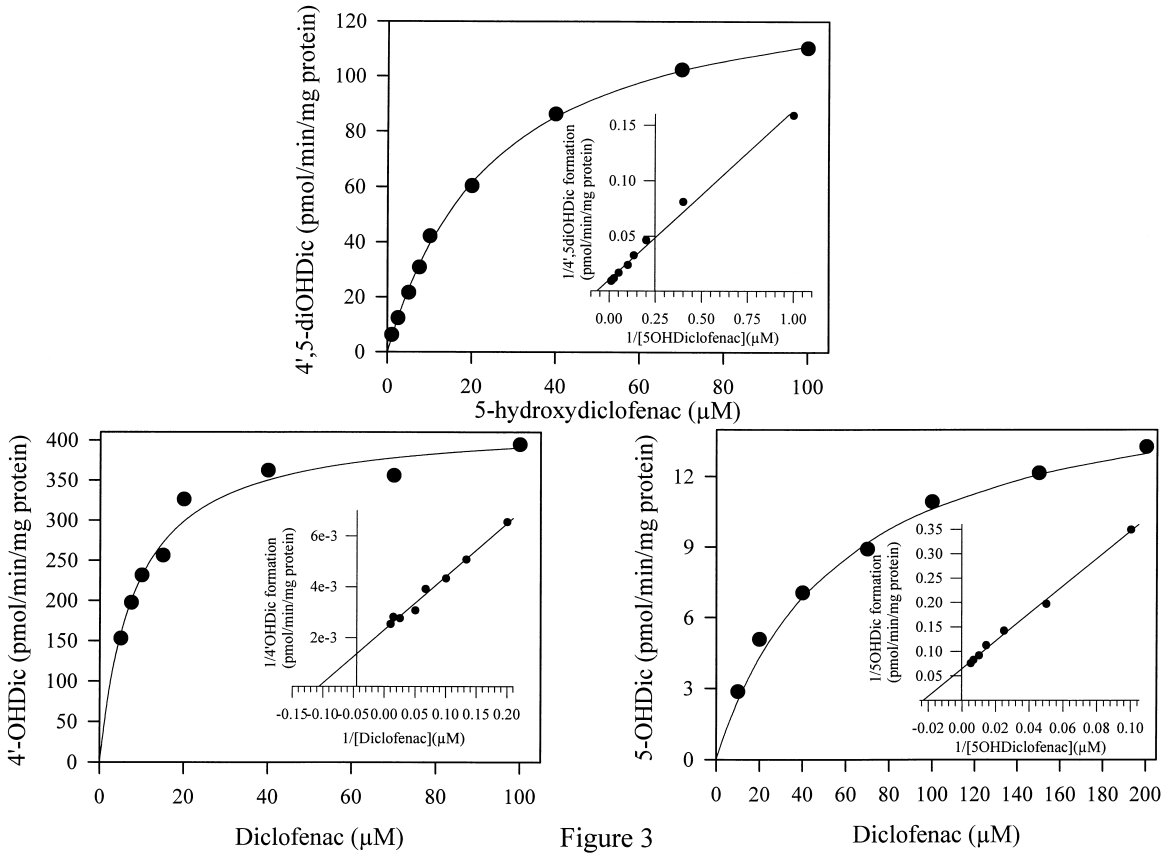

Figure 3

FIG. 3. Kinetic data of hydroxylation by human microsomes. 5-OHDic (1–100 μM) and diclofenac (5–200 μM) were incubated with a pool of human liver microsomes for 15 and 60 min, respectively. The reaction was stopped by addition of 1 mL acetonitrile and analysed by HPLC (method A; Materials and Methods).

CYP1A2 is not responsible for either 4′- or 5-hydroxylations. Similarly, an excess of coumarin, a well-known specific substrate of CYP2A6 [23], failed to inhibit diclofenac hydroxylation. The CYP2C9 specific inhibitor sulfaphenazole [24] clearly inhibited 4′hydroxylation of diclofenac and 5-OHDic, while 5-hydroxylation of diclofenac was not affected even at high concentrations of this inhibitor (no effect at 100 μM; data not shown). Interestingly, quinidine, a substrate for CYP3A4 but also a potent inhibitor of CYP2D6 [24], clearly failed to inhibit any hydroxylation, which indicates that CYP2D6 is not likely to be involved in the metabolism of diclofenac. Surprisingly, an increased formation of 5-OHDic was observed. Chlorzoxazone, a specific substrate for CYP2E1 [26], caused no inhibition of diclofenac hydroxylation. Finally, the CYP3A4/5 inhibitor troleandomycin [27] slightly inhibited 5-hydroxylation.

Incubation experiments were also conducted with microsomes from a well-characterised liver bank. The rates of metabolite formation were determined in 16 liver microsome preparations and correlated to specific P450 activities, namely CYP1A2, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4. As shown in Table 1 and Fig. 5, there was a good correlation between tolbutamide methylhydroxylase (CYP2C9) activity and the formation of 4′-OH and 4′,5-

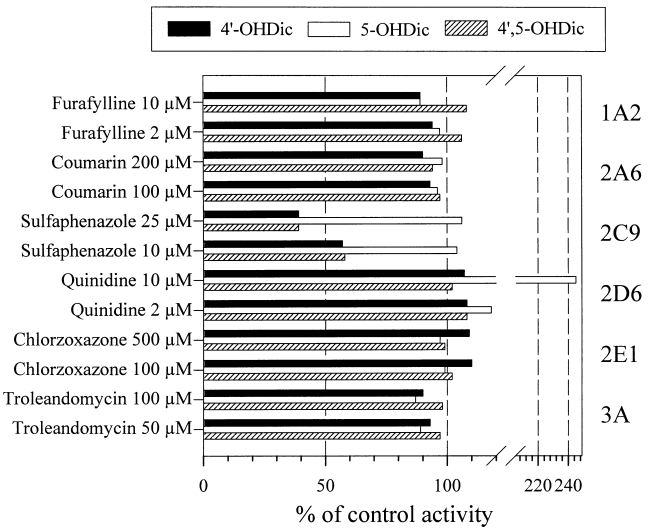

FIG. 4. Effects of various substrates and inhibitors of cytochrome P450 on diclofenac and 5-OHDic hydroxylations. Diclofenac (200 μM) and 5-OHDic (50 μM) were incubated with a pool of five different human liver microsomes in the presence or absence of two concentrations of various chemicals. Each bar represents the mean of duplicate incubations with less than 10% deviation. Forming rates of uninhibited samples for 4′-OHDic (black bar), 5-OHDic (white bar) and 4,5-diOHDic (dashed bar) were 372, 12.2, and 101.4 pmol/min/mg protein, respectively.

TABLE 1. Regression analysis between specific CYP activities and diclofenac metabolism

Simple regression analysis of metabolite formation

Specific reaction	CYP	4′-hydroxylation of diclofenac	5-hydroxylation of diclofenac	4′-hydroxylation of 5-OHDic
Ethoxyresorufin-O-deethylation	1A2	0.079	0.507	0.146
Tolbutamide methylhydroxylation	2C9	0.960*	0.673†	0.932*
S-Mephenytoin 4′-hydroxylation	2C19	0.327	0.749*	0.288
Debrisoquine-4-hydroxylation	2D6	−0.025	0.193	0.040
Chlorzoxazone-6-hydroxylation	2E1	0.189	0.127	0.185
Midazolam-1-hydroxylation	3A	0.378	0.624†	0.384

Stepwise regression analysis of 5-OHDic formation

Specific reaction	Coefficient	Std. Error	t‡	*P*	F‡	VIF‡
S-Mephenytoin 4′-hydroxylation	0.083	0.009	4.590	0.0004	9.37	1.187
Tolbutamide methylhydroxylation	0.040	0.017	4.990	0.0002	7.29	1.259

*$P < 0.001$.
†$P < 0.01$.
‡t, Student's *t*-test; F, F-statistic; VIF, variance inflation factor.

diOHDic (R = 0.960 and 0.932, respectively). A very poor correlation was obtained in sample-to-sample regression in the case of debrisoquine-4-hydroxylase (CYP2D6, R = −0.025 and 0.040). In accordance with the above-mentioned results with CYP inhibitors, there was a poor correlation (R = 0.378 and 0.384) with midazolam-1-hydroxylation (CYP3A4/5) and CYP1A2 catalysed ethoxyresorufin-O-dethylation (R = 0.079 and 0.146). In the case of 5-OHDic, there was not a clear outcome when simple correlation analysis was performed with each CYP activity assayed. The data from simple regression analysis (Table 1) indicated, in order of decreasing regression coefficient, that CYP2C19 (S-mephenytoin 4′-hydroxylation; R = 0.749), CYP2C9 (tolbutamide methylhydroxylation; R = 0.673), CYP3A4 (midazolam-1-hydroxylation; R = 0.624), and CYP1A2 (ethoxyresorufin-O-deethylation; R = 0.507) could be partially involved in 5-OHDic formation. Data was further analysed by stepwise multiple regression analysis to better accommodate the experimental results obtained for 5-OHDic formation. Previously, the Kolmogorov–Smirnov test was performed to ensure the normality of all the data sets; also, a variance inflation factor below 1.5 guaranteed the absence of multicollinearity. The stepwise regression analysis (Table 1) selected only two activities corresponding to CYP2C19 and CYP2C9, while the other two activities were rejected even under less stringent conditions (F-to-enter < 4.0). The results of this mathematical statistical analysis were highly significant ($P < 0.0001$), with a multiple correlation coefficient (R = 0.969). The resulting algorithm: "diclofenac-5-hydroxylation = 0.040 × S-mephenytoin 4′-hydroxylation + 0.083 × tolbutamide methylhydroxylation", accounted for the activity observed in the individual microsome preparations with an accuracy of 93.8%.

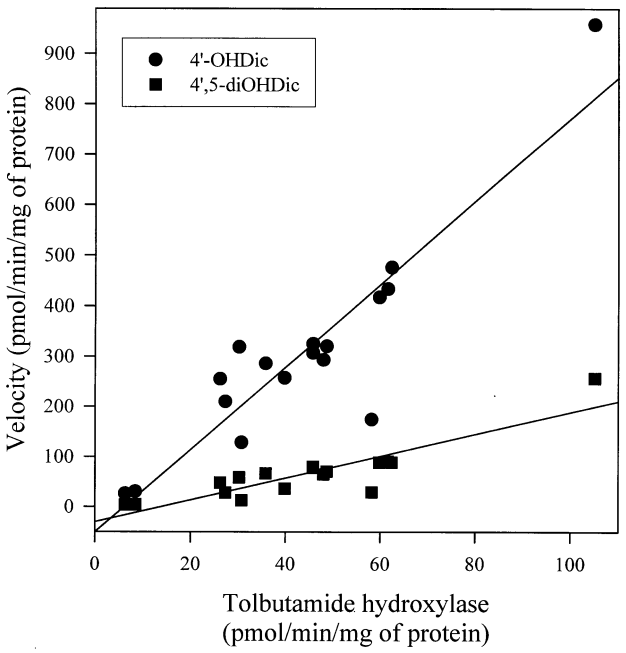

FIG. 5. Simple regression analysis of tolbutamide methylhydroxylation and the rates of formation of 4′-OHDic and 4′,5-diOHDic. Diclofenac (200 μM) and 5-OHDic (50 μM) were incubated with different human liver microsome samples. Forming rates of 4′-OHDic (black circles) and 4,5-diOHDic (black squares) were determined by HPLC (method A; Materials and Methods). Data are expressed as the means of duplicate incubations with less than 10% deviation.

Metabolism of Diclofenac by CYP-Expressing Cells

Diclofenac and 5-OHDic were incubated with genetically engineered cells expressing different human cytochromes (CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, and CYP3A4), and the formation of diclofenac metabolites was measured by HPLC. The results clearly confirmed that CYP2C9 was responsible for the 4′-hydroxylation of diclofenac and

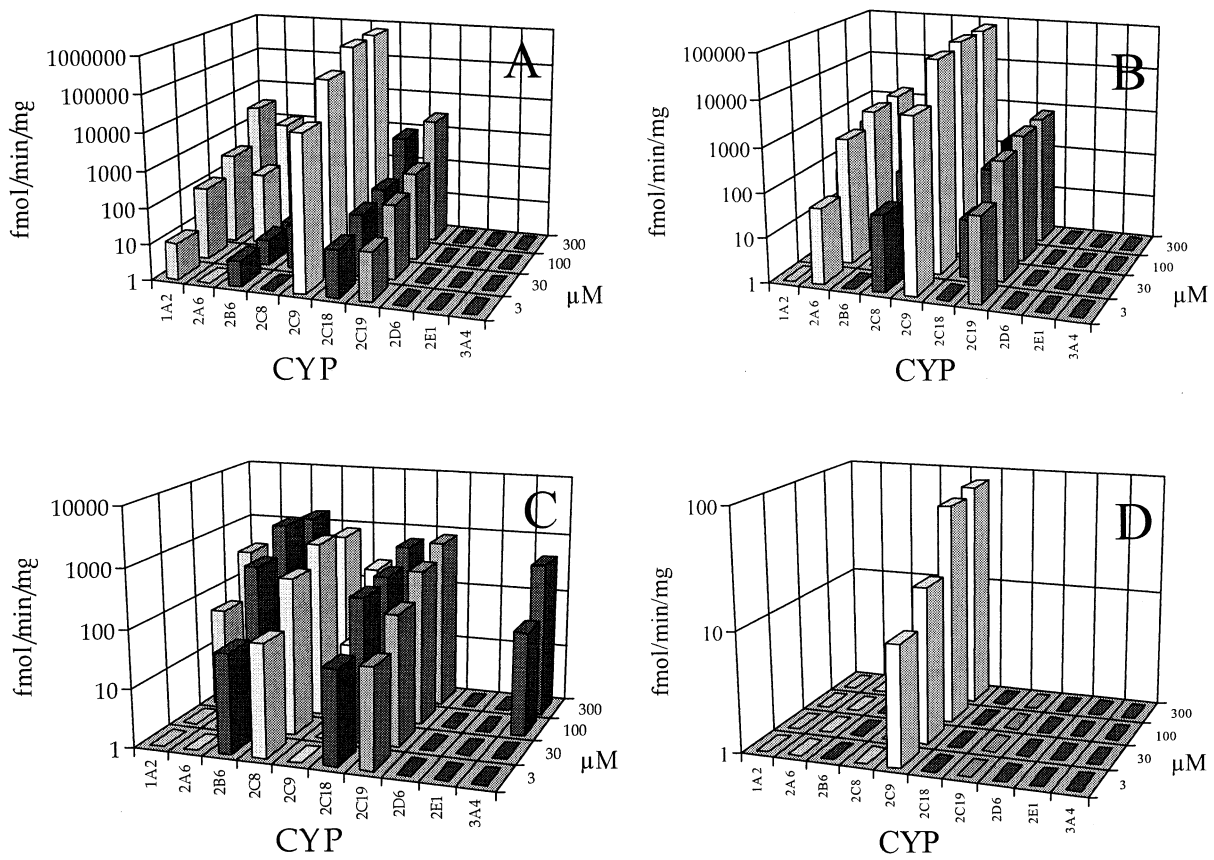

FIG. 6. Rate of diclofenac metabolism by CYP**-engineered cell lines. Diclofenac or 5-hidroxydiclofenac were incubated at 300, 100, 30, and 3 μM with *CYP*-engineered cells. After 24-hr incubation, cell supernatant was withdrawn and the content of 4′-OHDic (A), 4′,5-diOHDic (B), 5-OHDic (C), and 3′-OHDic (D) analysed by HPLC (method A; Materials and Methods).**

5-OHDic (Fig. 6, A and B, respectively). Preliminary experiments on the 5-hydroxylation of diclofenac revealed that as many as seven cytochromes were potentially involved (Fig. 6C). To get a better estimation of the relevance of each CYP in these hydroxylation pathways, diclofenac was incubated at various concentrations (100, 30, and 3 μM). Analysis of the 5-hydroxylation rate indicated that, despite the fact that CYP1A2, CYP2C9, and CYP3A4 can 5-hydroxylate diclofenac at 300 μM, CYP2B6, CYP2C8, CYP2C18, and CYP2C19 were the isoenzymes able to do it more efficiently at a pharmacokinetically relevant concentration (3 μM) [28]. In the case of 3′-hydroxylation, only CYP2C9 was able to catalyse its formation at any concentration of diclofenac (Fig. 6D).

To estimate the participation of the various CYP in the 5-hydroxylation *in vivo*, we compared the rates of formation of the different diclofenac metabolites in *CYP*-engineered cells and in 24-hrs cultured human hepatocytes. In parallel, individual CYP activities were measured with specific substrates in both cell systems, and the ratio activity in cell line/activity in hepatocyte was determined (Table 2). Based on this factor, we estimated the participation of each of the individual CYP in the metabolism of diclofenac in the human hepatocyte (Fig. 7). The results strongly suggest that

TABLE 2. Cytochrome P450 activities in 24-hr cultured human hepatocytes versus **CYP-expressing THLE cells**

	CYP	Hepatocytes	CYP-expressing THLE cells	Hepatocyte/CYP cell ratio
Methoxyresorufin-O-deethylase*	1A2	5.5 ± 3.1	28.5 ± 5.5	0.134
Benzoxyresorufin-O-deethylase*	2B6	2.4 ± 1.8	58.9 ± 3.5	0.041
Taxol-6-hydroxylase†	2C8	3.4 ± 1.2	24 ± 1	0.142
Diclofenac-4′-hydroxylase†	2C9	149 ± 35	669 ± 55	0.223
S-Mephenytoin-4′-hydroxylase†	2C19	11.9 ± 3.8	78 ± 12	0.153
Testosterone-6β-hydroxylase*	3A4	177 ± 98	260.4 ± 90.6	0.680

Activities are expressed in pmol/min/mg cellular protein.
*See Ref. 17.
†See Ref. 20.

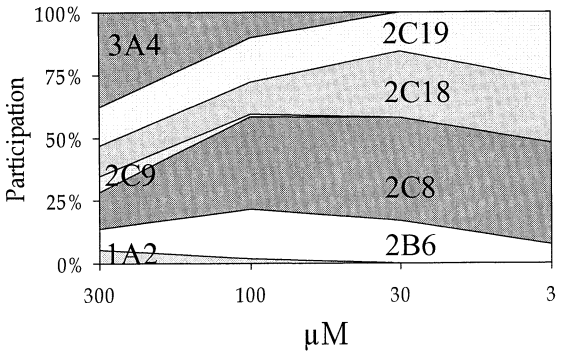

FIG. 7. Estimation of the contribution of the different CYP to the metabolism of diclofenac in human hepatocytes. The percentage of contribution of each CYP to the *in vivo* formation of 5-OHDic was estimated by applying a factor that relates the levels of individual CYP in the cell lines and hepatocytes to the data shown in Fig. 6.

in the human liver CYP2C9 would be exclusively responsible for the 4′-hydroxylation of diclofenac (participation greater than 99.5%) as well as 5-OHDic (>97%), while the isoforms CYP2C8, CYP2C19, CYP2C18, and CYP2B6 are those expected to catalyse the formation of 5-OHDic. CYP2C9 is the only isoform involved in the 3′-hydroxylation of diclofenac.

DISCUSSION

Diclofenac is metabolised in man by CYP-mediated oxidation as well as conjugation with glucuronic acid. The major metabolite found in plasma and urine of human volunteers is 4′-OHDic [3]. In addition, several minor metabolites are also present, whose origin could also be hepatic. Analysis by HPLC/UV/MS of culture media of human hepatocytes incubated with diclofenac revealed the existence of several monohydroxylated metabolites, 4′-OHDic, 3′-OHDic and 5-OHDic, as well as the dihydroxylated derivatives 4′,5-diOHDic and *N*,5-diOHDic (Fig. 1). Based on the experiments with human liver microsomes (Fig. 3), it became evident that the reduced formation of 5-OHDic by human hepatocytes (and hence its reduced presence in human urine) has a kinetic basis: the formation of 5-OHDic by human microsomes has much less favourable kinetic parameters than that of 4′-OHDic (V_{max}/K_m relative ratio 48 vs 0.38). The formation of 3′-OHDic could not be detected in microsome experiments but was detected in cultured hepatocytes in relative abundance, similar to what is observed *in vivo*. On the contrary, we could not detect the formation of 3′-OH-4′-OCH_3-Dic described by Faigle *et al.* [4] in culture medium of human hepatocytes, even using an MS-selected ion-monitoring mode. This would suggest a non-hepatocyte formation of the metabolite, for instance by the ubiquitous cathecol-*O*-methyltransferase. In culture medium of hepatocytes, we could detect the presence of *N*,5-diOHDic. This metabolite seems to be involved in the toxicity exerted by diclofenac to hepatocytes [15].

The cytochromes involved in the different oxidative pathways of diclofenac were identified by the combined use of human liver microsomes and *CYP*-engineered human-derived cells. The inhibition and regression experiments (Figs. 4 and 5 and Table 1) using human liver microsomes suggested a predominant role of CYP2C9 in the hydroxylation of diclofenac at the 4′ position, clearly sustained by the observations made with the *CYP*-expressing cell lines. In the case of 4′-OHDic formation, the small contribution of other members of the CYP2C subfamily (i.e. CYP2C8 and CYP2C19) as well as other CYP (CYP1A2 and CYP2A6) was negligible compared to that of CYP2C9, suggesting this isozyme plays the major role *in vivo*. The experiments concerning the formation of 4′,5-diOHDic clearly demonstrated that this metabolite was formed by the CYP2C9-catalysed 4′-hydroxylation of 5-OHDic, and not by 5-hydroxylation of 4′-OHDic (Fig. 8). The kinetics of the 4′-hydroxylation of diclofenac, 5-OHDic, and diclofenac analogues [16] are very similar, thus supporting a key role of CYP2C9 in this reaction. The 3′-hydroxylation pathway could not be properly investigated in microsomes because of the negligible formation of this metabolite. However, the studies with *CYP*-expressing cell lines brought to light the involvement of CYP2C9 in its formation. Studies on the 5-hydroxylation of diclofenac by microsomes were less convincing. Microsome studies using chemical inhibitors were not clear; however, simple correlation analysis with specific CYP activities identified CYP2C9, CYP2C19, CYP3A4, and CYP1A2 as potentially involved in 5-OHDic formation. The stepwise regression analysis partially confirmed this hypothesis: it revealed that CYP2C9 and CYP2C19 were involved, while the role of CYP3A4 and CYP1A2 was not significant. Indeed, the correlation coefficient value did not significantly improve when these two activities were forced to enter the mathematical model. The formation of 5-OHDic could be accurately predicted by a linear combination of the two CYP activities, *S*-mephenytoin 4′-hydroxylase and tolbutamide methylhydroxylase.

The results were further expanded by the *CYP*-expressing cell line experiments. Although some showed a certain capacity to metabolise diclofenac at 300 μM, only CYP2C8, CYP2C18, CYP2C19, and CYP2B6 were able to 5-hydroxylate at pharmacokinetically relevant concentrations of the drug (3 μM). The involvement of the tolbutamide methylhydroxylase activity predicted by the stepwise regression analysis was further differentiated from the results obtained with the *CYP*-engineered cell lines. In agreement with inhibition studies using human liver microsomes in which sulfaphenazole (specific inhibitor of CYP2C9) did not cause any significant change in the production of 5-OHDic, *CYP*-expressing cell lines confirmed the significant contribution of CYP2C8 at high or low concentration of the drug, while CYP2C9 only participated at concentrations between 100–300 μM. The ability of CYP2C8, in addition to CYP2C9, to methylhydroxylate tolbutamide [29] may explain this fact.

By comparing the data on metabolic rates obtained with

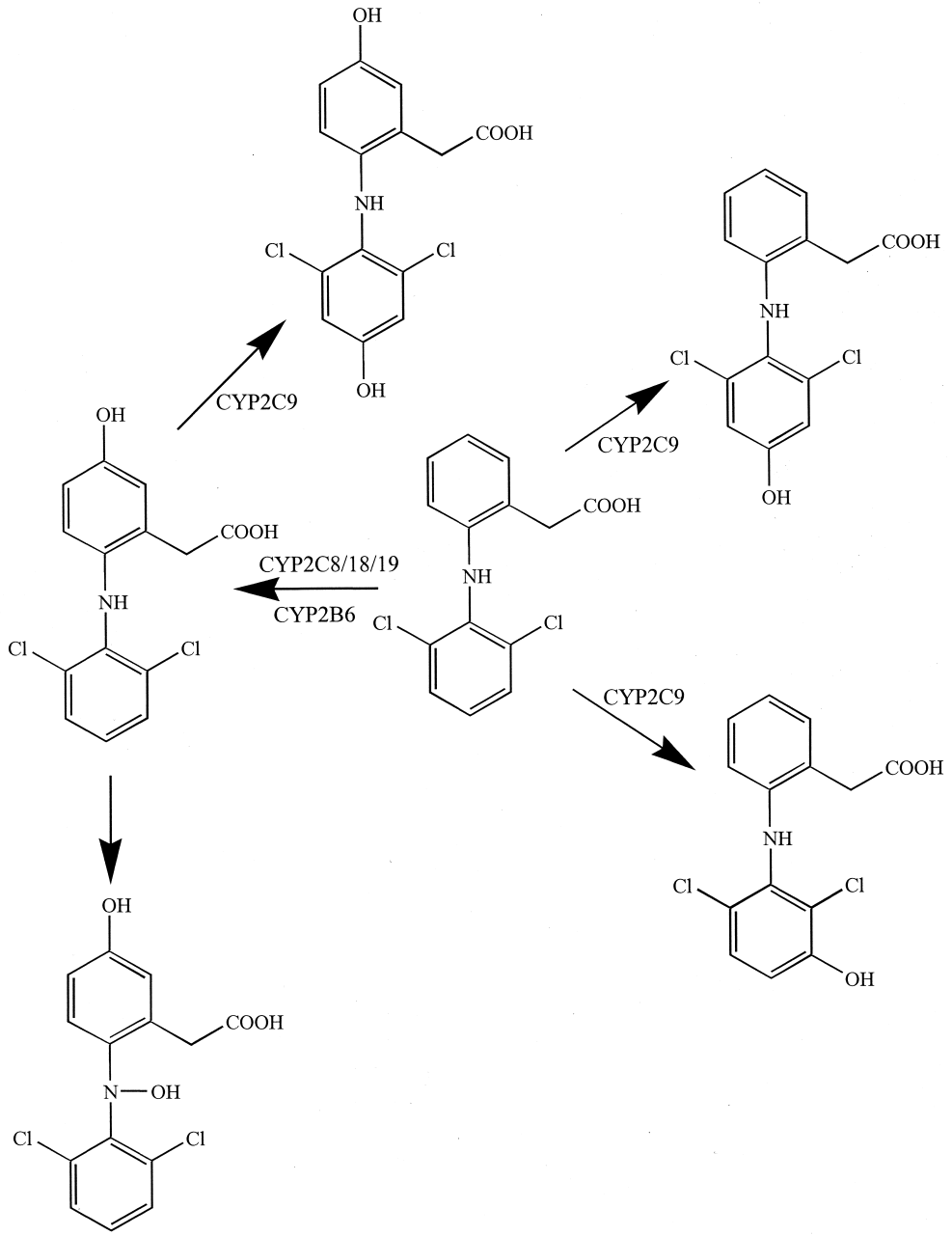

FIG. 8. Metabolic pathways of diclofenac in human hepatocytes.

CYP-expressing cells and human hepatocytes, an estimation of the degree of participation of each CYP in the metabolism of diclofenac in the human liver can be made. As suggested by Remmel *et al.* [30], one possible approach is to determine the activity of each CYP using a specific substrate in the cell line and hepatocytes (Table 2). Knowing the rate of formation of a given metabolite by cell line and the specific CYP activity, the rate of production by the CYP present in the human hepatocyte can be inferred. This parallelism was carried out for the various CYP implicated in the formation of the different diclofenac metabolites. Based on these estimations, CYP2C8 > CYP2C19 ≅ CYP2C18 were identified as the enzymes that would contribute most actively to the *in vivo* 5-hydroxylation of diclofenac (Figs. 7 and 8). Apart from the predominant role of CYP2C9 in the metabolisation of diclofenac to 4′-OHDic and 3′-OHDic, and indirectly to 4′,5-diOHDic, the participation of the polymorphic CYP2C19 in the formation of 5-OHDic and, indirectly, *N*,5-diOHDic confers CYP2C19 relevance in terms of explaining the variability of the hepatotoxicity exerted by diclofenac in man.

The authors wish to thank J. Hidalgo for his help in the statistical analysis and to Miss E. Belenchón for her technical help in cell cultures. This research was supported by the European Union (BMH4-CT96-0254) and the Fondo de Investigaciones Sanitarias (97/1961). R. B. was holder of a research fellowship from the Consellería de Cultura de la Generalitat Valenciana.

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