Extrapolation of Diclofenac Clearance from in Vitro Microsomal Metabolism Data: Role of Acyl Glucuronidation and Sequential Oxidative Metabolism of the Acyl Glucuronide

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ABSTRACT

Diclofenac is eliminated predominantly (~50%) as its 4'-hydroxylated metabolite in humans, whereas the acyl glucuronide (AG) pathway appears more important in rats (~50%) and dogs (>80–90%). However, previous studies of diclofenac oxidative metabolism in human liver microsomes (HLMs) have yielded pronounced underprediction of human in vivo clearance. We determined the relative quantitative importance of 4'-hydroxy and AG pathways of diclofenac metabolism in rat, dog, and human liver microsomes. Microsomal intrinsic clearance values (CLint = Vmax/Km) were determined and used to extrapolate the in vivo blood clearance of diclofenac in these species. Clearance of diclofenac was accurately predicted from microsomal data only when both the AG and the 4'-hydroxy pathways were considered. However, the fact that the AG pathway in HLMs accounted for ~75% of the estimated hepatic CLint of diclofenac is apparently inconsistent with the 4'-hydroxy diclofenac excretion data in humans. Interestingly, upon incubation with HLMs, significant oxidative metabolism of diclofenac AG, directly to 4'-hydroxy diclofenac AG, was observed. The estimated hepatic CLint of this pathway suggested that a significant fraction of the intrahepatically formed diclofenac AG may be converted to its 4'-hydroxy derivative in vivo. Further experiments indicated that this novel oxidative reaction was catalyzed by CYP2C8, as opposed to CYP2C9-catalyzed 4'-hydroxylation of diclofenac. These findings may have general implications in the use of total (free + conjugated) oxidative metabolism excretion for determining primary routes of drug clearance and may question the utility of diclofenac as a probe for phenotyping human CYP2C9 activity in vivo via measurement of its pharmacokinetics and total 4'-hydroxy diclofenac urinary excretion.

In vitro drug metabolism systems, especially liver microsomes, offer tremendous promise as a tool in drug discovery and development to make human pharmacokinetic projections for potential drug candidates (Obach et al., 1997; Obach, 1999). These systems allow for lead selection based on metabolism data in human tissue that seem more relevant to the human in vivo situation than the in vitro animal models. The popularity of liver microsomes, in comparison with other in vitro systems such as hepatocytes and liver slices, stems from the ease of their preparation, use, and long-term storage and viability. However, the use of liver microsomes for extrapolation of in vivo clearance suffers from a number of limitations such as nonspecific binding of compounds to microsomal components, reduced rates of metabolism because of potential product inhibition kinetics, and the difficulties in examining conjugative metabolism (e.g., glucuronidation) in microsomal incubations. These limitations lead to frequent underprediction of in vivo clearance from microsomal metabolism data (Houston and Carlile, 1997; Obach, 1999). There have been only a few attempts to extrapolate in vivo clearance from microsomal metabolism data for compounds that have a significant glucuronidation component in their elimination. This is primarily related to the fact that in contrast to P450s and flavin-containing monooxygenases, UGTs demonstrate a “latency” in their activity in microsomes (Burchell and Coughtrie, 1989). This latency arises from the location of ABBREVIATIONS: P450, cytochrome P450; UGT, UDP glucuronosyltransferase; ER, endoplasmic reticulum; AG, acyl glucuronide; UDPGA, uridine diphosphoglucuronic acid; LC-MS/MS, liquid chromatography with tandem mass spectrometry; HPLC, high-performance liquid chromatography; HLM, human liver microsome; RT, retention time; CID, collision-induced dissociation; MS, mass spectrometer; B/P, blood-to-plasma concentration ratio; CLint, intrinsic clearance; CLmic, microsomal intrinsic clearance; CLint, whole liver intrinsic clearance; CLmic,AG, microsomal intrinsic clearance of oxidative metabolism of diclofenac acyl glucuronide; CLmax, whole liver intrinsic clearance of oxidative metabolism of diclofenac acyl glucuronide; f u, unbound fraction of the drug in plasma; K m, Michaelis constant or substrate concentration required for half-maximal enzyme activity; Q h, hepatic blood flow; Vmax, maximal velocity of the enzymatic reaction.
the active site of UGTs within the lumen of the endoplasmic reticulum (ER), such that the ER membrane presents a diffusional barrier for the access of substrates and cofactors to the enzyme. A disruption of this barrier is required to overcome enzyme latency and obtain maximal glucuronidation activity in microsomal incubations. This has been achieved most commonly by the use of detergents in microsomal incubations, with the choice of detergent and its concentration determined empirically to achieve maximal enzyme activity for the glucuronidation reaction in question (Lett et al., 1992; Fulceri et al., 1994). This approach for the extrapolation of in vivo clearance of UGT substrates in a few published reports has yielded mixed results, with in vivo clearance significantly underpredicted in most instances (Mistry and Houston, 1987; Izumi et al., 1997; Furlan et al., 1999; Andersson et al., 2001). More recently, a membrane pore-forming peptide, alamethicin, has been suggested as a more universal alternative to detergents for overcoming the latency of all UGT isoforms and achieving maximal glucuronidation activity in liver microsomes (Fisher et al., 2000).

Diclofenac (Fig. 1) is a nonsteroidal anti-inflammatory drug that is widely used for the treatment of a variety of inflammatory conditions such as rheumatoid arthritis, osteoarthritis, and acute muscle aches (Insel, 1991). Diclofenac has previously been used as a model compound to examine the utility of human liver microsomes for the prediction of in vivo clearance in human (Carlile et al., 1999; Obach, 1999). This is because oxidative metabolism appears to account for nearly all of in vivo clearance of diclofenac in humans, and only 10 to 20% of the total administered dose is excreted as the acyl glucuronide (AG) of the parent drug itself (Reiss et al., 1978; Stierlin et al., 1979; Stierlin and Faigle, 1979). Among oxidative pathways, as much as 50% of the total dose of diclofenac is excreted in human urine and bile as its 4’-hydroxy diclofenac oxidative metabolite and a glucuronide conjugate thereof (Fig. 1); other oxidative metabolites, such as 5-hydroxy-, 4’-5-dihydroxy-, and 3-hydroxy diclofenac and their conjugates, each account for <5 to 10% of the administered dose. In spite of the apparent predominance of oxidative pathways in diclofenac elimination, the in vivo clearance of this drug is underpredicted by as much as 70 to 90% if human liver microsomal metabolism data are used for the scale-up (Carlile et al., 1999; Obach, 1999). The objective of this study was to examine the reasons for this discrepancy between the rates of in vitro microsomal metabolism and in vivo clearance of diclofenac. As part of these studies, we have reevaluated the role of acyl glucuronidation of diclofenac in its overall metabolism and clearance in humans. The quantitative role of acyl glucuronidation in diclofenac clearance appears to be species-dependent, with this pathway accounting for 80 to 90% and 50 to 60% of total clearance in dogs and rats, respectively; the remainder of the clearance in these species is contributed by oxidative metabolism, primarily via the 4’-hydroxy pathway (Reiss et al., 1978; Stierlin et al., 1979; Stierlin and Faigle, 1979). Thus, we have also included in vitro-in vivo correlations of diclofenac clearance in these two species in our studies.

Materials and Methods

Diclofenac sodium and uridine-diphosphoglucuronic acid (UDPGA) were purchased from Sigma-Aldrich (St. Louis, MO). Diclofenac acyl glucuronide was synthesized using the Mitsunobu coupling technique (Juteau et al., 1997). The 4’-hydroxy diclofenac metabolite standard was obtained from BD Gentest (Woburn, MA). Microsomes containing individual recombinant human P450 isoforms and monoclonal antibodies against human P450 isoforms were obtained from Dr. Thomas H. Rushmore (Department of Drug Metabolism, Merck Research Laboratories, West Point, PA). Recombinant P450 microsomes were prepared from Sf21 insect cells infected with recombinant baculoviruses encoding individual P450 cDNAs and cytochrome P450 reductase (Mei et al., 1999). Monoclonal antibodies against human CYP3A4, 2D6, 2C8/9, and 2C19 were prepared in mice after immunization with individual recombinant isozymes as described previously (Mei et al., 1999). Protein assay reagent kit was purchased from Pierce Chemical (Rockford, IL). All other chemicals were purchased from Sigma-Aldrich and were of reagent grade.

Preparation of Liver Microsomes. Liver microsomes from male Sprague-Dawley rats, Beagle dogs, and humans were prepared by differential centrifugation of the homogenized liver tissue (Raucy and Lasker, 1991). Livers from 40 naive male rats were pooled for microsome preparation, whereas dog and human liver microsomes...
were prepared separately from individual livers. Before experimentation, equal amounts of microsomal protein from five individual dog liver microsomal preparations were pooled to provide a representative average preparation; a microsomal pool from five individual human livers was similarly prepared. Microsomal protein concentrations were measured with bicinchoninic acid according to manufacturer’s instructions for the use of protein assay kit.

Kinetics of 4'-Hydroxy Diclofenac Formation in Rat, Dog, and Human Liver Microsomes. All microsomal incubations (0.2-ml total volume) were conducted at 37°C in 100 mM potassium phosphate buffer (pH 7.4) containing 10 mM MgCl₂ and with or without (controls) an NADPH-regenerating system consisting of 10 mM glucose 6-phosphate, 1 mM NADP, and 0.15 units of glucose-6-phosphate dehydrogenase. Linearity of product (4'-hydroxy diclofenac) formation with respect to incubation time and microsomal protein concentration was established in initial studies. Final rat and human liver microsomal incubations to determine Michaelis-Menten kinetic parameters for the formation of 4'-hydroxy diclofenac were conducted at 100 μg/ml microsomal protein concentration with a 10-min incubation time. Formation of 4'-hydroxy diclofenac was relatively slower in dog liver microsomes, and thus incubations were conducted at 500 μg/ml microsomal protein concentration with a 30-min incubation time. Diclofenac substrate concentrations used for these kinetic studies were 0.5, 1, 2.5, 5, 10, 25, 50, 100, 150, and 200 μM. Substrate stocks were prepared in acetonitrile at appropriate concentrations so as to keep final organic solvent concentration in the incubation below 1%. At the end of the incubation period, the reactions were stopped by adding 400 μl of acetonitrile containing 3% formic acid and 1 μg/ml indomethacin (internal standard). Samples were centrifuged and the supernatant was separated from precipitated protein for analysis by LC-MS/MS.

Kinetics of Acyl Glucuronidation of Diclofenac in Rat, Dog, and Human Liver Microsomes. Michaelis-Menten kinetic parameters for the acyl glucuronidation of diclofenac in liver microsomes was determined in the presence or absence of alamethicin. All incubations were conducted at 37°C in 100 mM potassium phosphate buffer, pH 7.4, containing 10 mM MgCl₂ and 5 mM saccharic acid lactone (an inhibitor of β-glucuronidase). Reactions were started by the addition of UDPGA in water (2 mM final concentration). For incubations in the presence of alamethicin, microsomes were preincubated with alamethicin for 15 min on ice before initiation of the reaction. Initial studies demonstrated that maximal activation of diclofenac glucuronidation was achieved at an alamethicin concentration of 25 μg/mg microsomal protein. All subsequent studies with alamethicin were conducted at this concentration. Similar to 4'-hydroxy diclofenac studies mentioned above, linearity of product formation with respect to protein concentration and incubation time was established in initial studies. Final kinetic studies were conducted with a 50 μg/ml (HLMs) or 100 μg/ml (rat liver microsomes and dog liver microsomes) microsomal protein concentration and a 5-min incubation time at the substrate concentrations described above. Incubation time was kept short so as to minimize errors resulting from the hydrolytic cleavage of diclofenac AG at pH 7.4 (t½ of ~30 min; see below). Reactions were quenched and samples processed as described previously for 4'-hydroxy diclofenac studies.

Calculation of Michaelis-Menten Kinetic Parameters for the Formation of 4'-Hydroxy Diclofenac and Diclofenac Acyl Glucuronide in Liver Microsomes. Formation rates (v) of diclofenac AG and 4'-hydroxy diclofenac (as picomoles per minute per milligram of microsomal protein) were calculated at each of the substrate concentrations ([S]) specified above. Eadie-Hofstee plots (v/[S] versus v) were then constructed to determine whether a single- or a multiple enzyme Michaelis-Menten model should be fitted to these data. All Eadie-Hofstee plots appeared to be monophasic, suggesting that the two metabolic reactions follow single-enzyme kinetics. Thus, a single enzyme Michaelis-Menten model (eq. 1) was fitted to the metabolism formation rate versus substrate concentration data using the nonlinear regression software Sigma Plot (Sigma Plot for Windows version 5.00; SPSS Inc., Chicago, IL):

\[
v = \frac{V_{\text{max}} \cdot [S]}{K_m + [S]}
\]

Maximal formation rate (V_max) and Michaelis constant (K_m, substrate concentration required for half-maximal reaction velocity) parameters for the formation of 4'-hydroxy diclofenac and diclofenac AG in liver microsomes were obtained for each species from these analyses.

Determination of Nonspecific Diclofenac Binding in Liver Microsomal Incubations. Nonspecific binding of diclofenac to microsomal membrane components in all species was determined using the ultrafiltration method. Incubations were prepared as described above, with all substrate concentrations used for kinetic studies, except that the cofactors (NADPH or UDPGA) were omitted. A 0.5-ml aliquot of the incubation was filtered through ultrafiltration membranes with molecular weight cutoff of 12,000 Da (Millipore Corporation, Bedford, MA) by centrifugation at 10,000g. The ultrafilter, thus obtained, was analyzed by unbound diclofenac concentration using LC-MS/MS as described below. Unbound fraction of diclofenac in incubations was determined as the ratio of drug concentration in the ultrafiltrate to the total drug concentration in the incubation.

Extrapolation of in Vivo Clearance of Diclofenac from In Vitro Microsomal Metabolism Data. In vitro intrinsic clearance (CLint) for the metabolism of diclofenac to AG and 4'-hydroxy diclofenac in liver microsomes was calculated as the V_max/K_m ratio for these individual metabolic pathways. In agreement with a previous investigation, our studies also demonstrated negligible nonspecific binding of diclofenac to microsomal components (Obach, 1999). Hence, no correction was required to convert the above CLint to unbound CLint in liver microsomal incubations. Extrapolation of CLint to whole liver intrinsic clearance (CLint) for each pathway was performed using the following equation (Obach, 1999):

\[
\text{CL}_{\text{int}} = \frac{45 \text{ mg microsomes}}{g \text{ liver}} \cdot \frac{0.45 \text{ g liver}}{kg \text{ body weight}}
\]

Liver weight values used for rat, dog, and human were 40, 32, and 26 g of liver/kg of body weight, respectively (Davies and Morris, 1993).

CLint values obtained were then used to extrapolate the in vivo hepatic blood clearance of diclofenac via these metabolic pathways using the well stirred model of hepatic clearance:

\[
\text{CL}_{\text{blood}} = \frac{Q_h \cdot \text{CL}_{\text{int}}}{B/P} + \frac{Q_h \cdot \text{CL}_{\text{int}}}{B/P}
\]

where CLblood is the hepatic blood clearance of diclofenac via a particular metabolic pathway and Q_h is hepatic blood flow. f_u is the unbound fraction in plasma, and B/P is the blood-to-plasma concentration ratio of diclofenac. Average values of 0.005 and 0.55 were used for the f_u and B/P parameters for extrapolation; these values are reasonably similar in at least the rat and the human (Reiss et al., 1978; Obach, 1999). Q_h values used for these calculations were 55, 31, and 20 ml/min/kg for the rat, dog, and human, respectively (Davies and Morris, 1993).

Oxidative Metabolism of Diclofenac Acyl Glucuronide in Rat, Dog, and Human Liver Microsomes. Diclofenac AG (2 μM) was incubated with rat, dog, and human liver microsomes (1 mg/ml microsomal protein concentration) either with or without (controls) the NADPH-regenerating system in a 100 mM potassium phosphate buffer (pH 7.4) containing 10 mM MgCl₂ and 5 mM saccharic acid lactone in a total volume of 0.7 ml. Reactions were started by addition of the substrate, and 100-μl samples were withdrawn from these
incubations at time 0, 5, 15, 30, and 45 min postsubstrate addition. Reactions were quenched with 200 μl of acetonitrile containing 3% formic acid and 1 μg/ml indomethacin (the internal standard). Because diclofenac AG undergoes spontaneous hydrolysis in liver microsomal incubations, the rate of its oxidative metabolism was represented by the difference in the rate of its disappearance in incubations with and without the NADPH-regenerating system. In vitro $t_{1/2}$ of diclofenac AG because of oxidative metabolism in liver microsomes was calculated from the percentage remaining versus incubation time plots that were constructed after correction for its spontaneous hydrolysis in incubations lacking the NADPH-regenerating system. Intrinsic clearance for the oxidative metabolism of diclofenac AG in liver microsomes ($CL_{int,AG}$) was calculated as indicated below (Obach, 1999):

$$CL_{int,AG}^{mic} = \frac{V_{max}}{K_m} \cdot \frac{1}{mg/ml \text{ microsomal protein in incubation}}$$

Equation (4)

$CL_{int,AG}^{mic}$ was then extrapolated to the whole liver ($CL_{int,AG}^{liver}$) using eq. 2 and the values for biological constants as described above (Obach, 1999).

The in vitro $t_{1/2}$ approach was used for the extrapolation of oxidative intrinsic clearance of diclofenac AG because $V_{max}$ and $K_m$ parameters could not be determined due to significant "nonoxidative" degradation (hydrolysis) of the glucuronide in incubations.

Identification of P450 Isoforms Involved in Oxidative Metabolism of Diclofenac Acyl Glucuronide. Diclofenac AG (2 μM) was incubated with HLMs in the presence or absence of monoclonal antibodies against CYP2C8/9, CYP2C19, CYP2D6, and CYP3A4 isozymes. Each incubation contained 1 mg/ml microsomal protein and 2 μl of the antibody preparation, along with the buffer described above and NADPH-regenerating system, in a total volume of 200 μl. The disappearance of diclofenac glucuronide and the formation of metabolites in the incubation were determined by LC-MS/MS.

Further confirmation of the P450 isoform(s) responsible for the oxidative metabolism of diclofenac AG was obtained from incubations with microsomes prepared from baculovirus-infected cells containing individually expressed human P450 isoforms (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, and 3A4) and cytochrome P450 reductase. Each incubation contained 2 μM diclofenac AG, 250 μmol/ml P450 protein, 5 mM saccharic acid lactone, an NADPH-regenerating system, and 10 mM MgCl₂ in 100 mM potassium phosphate buffer (pH 7.4). Incubations were carried out for 30 min at 37°C, after which time the reaction was halted by the addition of an equal volume of acetonitrile containing 3% formic acid and 1 μg/ml indomethacin (internal standard). After centrifugation, the supernatant was analyzed by LC-MS/MS.

LC-MS/MS Analysis. Chromatographic separation of compounds of interest was achieved on a Fluro Sep-RP Phenyl HS HPLC column (5 cm × 2 mm, 5 μm) using two PE series 200 micropumps and a PE series 200 Autosampler (PerkinElmer Life Sciences, Boston, MA). Mobile phase A consisted of 5 mM ammonium acetate and mobile phase B of a 50:50 acetonitrile/methanol mixture, both containing 0.1% formic acid. Metabolites of interest were eluted using a gradient profile where the HPLC run began with 30% solvent B for the first 0.5 min, which was then increased to 95% at 1.5 min using a linear gradient and held at this solvent composition for 2 min before restoration of initial solvent conditions for column re-equilibration. HPLC flow rate was 0.5 ml/min and the column effluent was split 1:5 between mass spectrometer and waste, respectively. Analytes were detected using a Sciex API 2000 triple quad mass-spectrometer that was operated in multiple reaction monitoring mode. Ion spray voltage was 5000 V and source temperature was set at 300°C. The characteristics used for monitoring diclofenac, diclofenac AG, 4-hydroxy diclofenac, 4'-hydroxy diclofenac AG, and indomethacin (the internal standard) were $m/z$ 296 → 214, 472 → 296, 312 → 230, 488 → 312, and 358 → 139, respectively. Standard curves were constructed by spiking known amounts of analytes into microsomal incubations prepared without the cofactors; standard curve samples were also processed in the same way as the other incubation samples. Concentrations of metabolites formed in the incubations were determined by comparison of the peak area ratios of the analyte to the internal standard to those in the standard curve samples using a linear power fit model. The lower limits of quantitation for each of the analytes were 0.02 μM or lower.

Results

Figure 2 shows data from studies on the kinetics of 4'-hydroxy diclofenac formation in rat, dog, and human liver microsomes. $V_{max}$ and $K_m$ parameters obtained after fitting of the single-enzyme Michaelis-Menten model to these data are presented in Table 1. Liver microsomal intrinsic clearance (CL$_{int}$) values for the formation of 4'-hydroxy diclofenac were calculated as the $V_{max}$/$K_m$ ratio for each species and are also presented in Table 1. The kinetic parameters for the 4'-hydroxylation of diclofenac that we determined in HLMs were similar to those determined in previous studies (Carli et al., 1999). The CL$_{int}$ values of diclofenac 4'-hydroxylation in rat and dog liver microsomes was ~2 and 40-fold lower, respectively, compared with that in HLM, largely as a result of higher $K_m$ values for this pathway in the former two species (Table 1).

Figure 3 shows data on the kinetics of diclofenac acyl glucuronidation in liver microsomes from different species in the presence or absence of the membrane pore-forming peptide alamethicin. Kinetic parameters ($V_{max}$, $K_m$, and CL$_{int}^{mic}$) for this metabolic pathway, calculated as described above for
the 4'-hydroxy metabolite, are presented in Table 2. Alami-
ethicin enhanced the $V_{\text{max}}$ of diclofenac acyl glucuronidation by $\sim$3- to 7-fold in liver microsomes from different species, with minimal effects on the $K_{m}$ of this conjugation reaction; thus, the net result of this stimulation was an increase of nearly equal magnitude in $\text{CL}_{\text{int}}^\text{mic}$ of this pathway (Table 2).

The $\text{CL}_{\text{int}}^\text{mic}$ parameters for the 4'-hydroxy- and AG pathways were determined separately using the $V_{\text{max}}$ and $K_{m}$ parameters as opposed to the in vitro parent disappearance or $t_{1/2}$ approach (Obach, 1999) because different optimal reaction conditions were needed for these two pathways.

$\text{CL}_{\text{int}}^\text{mic}$ values were scaled-up to the whole liver intrinsic clearances ($\text{CL}_{\text{int}}^\text{mic}$) for the 4'-hydroxy, as well as the AG pathway using the scaling factors described under Materials and Methods and are presented in Table 3. Whole liver intrinsic clearance values were then used to extrapolate the in vivo systemic blood clearance using the well stirred model of hepatic clearance; these extrapolated systemic blood clearance values of diclofenac using the 4'-hydroxy diclofenac pathway alone or in combination with the AG pathway are also presented in Table 3.

Figure 4 shows the disappearance plots of diclofenac AG in human and dog liver microsomal incubations in the presence or absence of an NADPH-regenerating system; this plot in rat liver microsomal incubations was similar to that shown for the dog liver microsomes. In HLMs, the percentage of diclofenac AG remaining at 15-, 30-, and 45-min postincubation was significantly lower in incubations conducted in the presence of an NADPH-regenerating system relative to the corresponding values in incubations without NADPH (Fig. 4A). In rat and dog liver microsomal incubations, however, the percent remaining plots for diclofenac AG in the presence or absence of NADPH-regenerating system were nearly superimposable (Fig. 4B).

Figure 5 shows representative extracted ion chromatograms of diclofenac, diclofenac AG, 4'-hydroxy diclofenac, and 4'-hydroxy diclofenac AG in human liver microsomal incubations of diclofenac AG (2 $\mu$M) either with or without an NADPH-regenerating system. When diclofenac AG was incubated with HLM in the presence of an NADPH-regenerating system, significant amounts of 4'-hydroxy diclofenac (Fig. 5A, peak at RT 2.5 min) and 4'-hydroxy diclofenac AG (Fig. 5A, peak at RT 2.1 min) were detected; the peak at 2.1 min in the 4'-hydroxy diclofenac channel most likely arises from the CID of 4'-hydroxy diclofenac formed via hydrolysis of 4'-hydroxy diclofenac AG in the MS source. The identity of 4'-hydroxy diclofenac metabolite was confirmed by comparison of its retention time and MS fragmentation with those of the synthetic standard. The metabolite peak at 2.1 min was concluded to correspond to 4'-hydroxy diclofenac AG because its molecular weight (488 Da) and MS fragmentation (loss of 176 Da) were similar to what would be expected for a glucuronide conjugate of a hydroxy diclofenac metabolite, and the fact that upon NaOH-catalyzed hydrolysis, this peak converted to 4'-hydroxy diclofenac (RT 2.5 min). Since in these incubations glucuronidation of 4'-hydroxy diclofenac cannot

**Table 1**

<table>
<thead>
<tr>
<th>Species</th>
<th>$V_{\text{max}}$ (pmol/min/mg protein)</th>
<th>$K_{m}$ (µM)</th>
<th>$\text{CL}_{\text{int}}^\text{mic}$ (µl/min/mg protein)</th>
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<tbody>
<tr>
<td>Rat</td>
<td>1400</td>
<td>14</td>
<td>100</td>
</tr>
<tr>
<td>Dog</td>
<td>440</td>
<td>90</td>
<td>19.9</td>
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<tr>
<td>Human</td>
<td>1160</td>
<td>5.4</td>
<td>215</td>
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**Table 2**

<table>
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<th>$K_{m}$ (µM)</th>
<th>$\text{CL}_{\text{int}}^\text{mic}$ (µl/min/mg protein)</th>
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</thead>
<tbody>
<tr>
<td>Without Alamethicin</td>
<td>Rat</td>
<td>606</td>
<td>14</td>
</tr>
<tr>
<td>Dog</td>
<td>4,480</td>
<td>48</td>
<td>93.3</td>
</tr>
<tr>
<td>Human</td>
<td>2,660</td>
<td>18</td>
<td>148</td>
</tr>
<tr>
<td>With Alamethicin</td>
<td>Rat</td>
<td>4,160</td>
<td>20</td>
</tr>
<tr>
<td>Dog</td>
<td>13,900</td>
<td>60</td>
<td>232</td>
</tr>
<tr>
<td>Human</td>
<td>11,500</td>
<td>18</td>
<td>639</td>
</tr>
</tbody>
</table>

**Fig. 3.** Kinetics of acyl glucuronidation of diclofenac in rat (A), dog (B), and human (C) liver microsomes in the presence or absence of the membrane pore-forming peptide alamethicin (at a concentration of 25 µg/mg microsomal protein). Actual data points and the Michaelis-Menten model fitted curves are shown. Each data point is an average of triplicate determinations. †, with alamethicin; ○, without alamethicin.
and systemic blood clearance of diclofenac in these species
hydrolysis of diclofenac AG; the peak at 2.3 min in the di-
oxidative metabolism of any amounts that are formed via
diclofenac itself was not detected, possibly because of rapid
NADPH-enriched incubations of diclofenac AG with HLMs,
/H11032 -hydroxy diclofenac. In these
than an ether-, glucuronide of 4
P450-catalyzed oxidation of diclofenac AG; this further es-
/H11032 -hydroxy diclofenac glucuronide must arise from direct
occur because of a lack of the required cofactor (UDPGA),
4'-hydroxy diclofenac glucuronide must arise from spontaneous
hydrolysis of diclofenac AG in the incubation but is not fur-
ther metabolized.
Rates of disappearance of diclofenac AG as a result of
oxidative metabolism in HLM incubations were obtained af-
fter correcting for its spontaneous hydrolysis rate in incuba-
tions lacking the NADPH-regenerating system. An in vitro
t1/2 value of 148 min was thus calculated for the disappear-
ance of the diclofenac AG as a result of oxidative metabolism
in HLM incubations. From this in vitro t1/2 value, whole liver
intrinsic clearance for the oxidative metabolism of diclofenac
AG (CLint,AG) was extrapolated using eqs. 2 and 4, and scal-
ing factors described previously; a value of 5.5 ml/min/kg was
thus estimated for this parameter.
Figure 6 shows data on the effect of monoclonal antibodies
against various P450 isoforms on the metabolism of diclofe-
nac AG in HLMs. The amounts of 4'-hydroxy diclofenac and
its acyl glucuronide formed in the presence of antibodies
against individual P450 isoforms relative to those in a con-
control incubation are depicted. The data clearly demonstrate
that the formation of both 4'-hydroxy diclofenac and its AG in
incubations of diclofenac AG with HLMs is catalyzed by the
CYP2C isofoms. However, based on these data, it is difficult
to make a distinction between the contribution of individual
members of the CYP2C family to these metabolic transfor-
mations because of cross-reactivity of monoclonal antibodies
among the CYP2C8, 2C9, and 2C19 isoforms. This distinc-
tion was achieved by experiments in which diclofenac AG
was incubated with microsomes containing individual recom-
binant human CYP isozymes in the presence of an NADPH-
regenerating system. In incubations of diclofenac AG with
microsomes containing recombinant CYP1A2, 2A6, 2B6,
2C19, 2D6, 2E1, and 3A4, 4'-hydroxy diclofenac and its AG
were not detected, and only diclofenac, which is likely formed
via spontaneous hydrolysis of diclofenac AG, was detectable
(data not shown). When diclofenac AG was incubated with
CYP2C9 containing microsomes, only 4'-hydroxy diclofenac

Fig. 4. Disappearance (mean ± S.D.) of diclofenac acyl glucuronide (2 µM) in human (A) and dog (B) liver microsomal incubations in the presence or absence of NADPH-regenerating system.

<table>
<thead>
<tr>
<th>Species</th>
<th>4'-Hydroxy Diclofenac AG Formation Only</th>
<th>4'-Hydroxy Diclofenac AG + AG Formation</th>
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<tbody>
<tr>
<td>Rat</td>
<td>180</td>
<td>77.9</td>
</tr>
<tr>
<td>Dog</td>
<td>7.04</td>
<td>134</td>
</tr>
<tr>
<td>Human</td>
<td>252</td>
<td>173</td>
</tr>
</tbody>
</table>

Whole Liver Intrinsic Clearance of Diclofenac Extrapulated from in Vitro Microsomal Metabolism Data

Systemic Blood Clearance of Diclofenac Extrapulated from in Vitro Microsomal Metabolism Data

In Vivo Blood Clearance of Diclofenac from Literature Data

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* Blood clearances were calculated from plasma clearance values reported in literature as \( CL_{blood} = CL_{plasma}/B/P \), where B/P is blood-to-plasma concentration ratio of diclofenac; a value of 0.55 was used for B/P in all species.


c Estimated hepatic blood clearance of diclofenac in isolated perfused rat liver, with physiological concentrations of protein in the perfusate (Hussein et al., 1993; Weiss et al., 2000).

d Estimated from data in Biess et al., 1978 and Tsuchiya et al., 1980

e From Willis et al., 1979, 1980.
(RT 2.5 min) was detected (Fig. 7A); it likely arises from hydroxylation of diclofenac that is formed via hydrolysis of diclofenac AG in the incubation. In contrast, 4′/H11032-hydroxy diclofenac AG was detected only in incubations of diclofenac AG with microsomes containing the CYP2C8 isozyme (Fig. 7B); the small peak at 2.1 min in the 4′-hydroxy diclofenac channel likely arises from CID of the 4′-hydroxy diclofenac formed from hydrolysis of its acyl glucuronide in the MS source. These data strongly suggest that the conversion of diclofenac AG to its 4′-hydroxy derivative is exclusively catalyzed by the CYP2C8 isozyme.

**Discussion**

As pointed out in the Introduction, previous studies on the extrapolation of oxidative metabolism of diclofenac in HLMs have yielded pronounced underprediction of human in vivo diclofenac clearance (Carlile et al., 1999; Obach, 1999). This is in spite of the fact that a major portion (70–80%) of an oral diclofenac dose is excreted in human urine and bile as its hydroxylated metabolites and their glucuronide conjugates (Reiss et al., 1978; Stierlin et al., 1979; Stierlin and Faigle, 1979). In our studies, excellent correlations of extrapolated and actual in vivo clearance were obtained for the dog and the human when glucuronidation data in the presence of alamethicin, along with the 4′-hydroxy diclofenac data, were included for extrapolation (Table 3). In rats, the extrapolated blood clearance appears to be lower than both the observed in vivo systemic clearance, and the hepatic clearance of diclofenac in isolated perfused rat liver, although the difference in the latter case seems to be smaller (Table 3). The exact reasons for this discrepancy remain unclear; it may be related to possible errors in the assumed values for fu and B/P parameters or additional unaccounted for hepatic or extrahepatic pathways of diclofenac clearance in the rat. Similar to previous studies, the human in vivo clearance of diclofenac was significantly underpredicted when only microsomal 4′-hydroxy diclofenac data were used for extrapolation. These data suggest that glucuronidation may be a significant component of the overall hepatic clearance of diclofenac in the three species and highlight the potential utility of alamethicin for extrapolating the in vivo clearance of UGT substrates from their conjugative metabolism data in liver microsomes. The estimated CL\text{int} values in Table 3 suggest that acyl glucuronidation accounts for ~70, 98, and 75% of the total clearance of diclofenac in the rat, dog, and human, respec-
with the 4’-hydroxy-pathway accounting for the remainder (assuming that the 4’-hydroxy- and acyl glucurononide pathways account for all of diclofenac clearance). This is in good agreement with the in vivo disposition data of diclofenac in rats and dogs, where ~50% and 80 to 90% of the total dose of diclofenac, respectively, is recovered in bile and urine as its AG (Reiss et al., 1978; Seitz et al., 1998). Similarly, the contribution of the 4’-hydroxy pathway to diclofenac clearance in rats and dogs is also predicted very well from the in vitro microsomal metabolism data, because ~30 and <5% of the total diclofenac dose, respectively, is eliminated via this pathway in the two species.

In contrast to rats and dogs, the CLmic data in HLMs appear to be at odds with the known disposition of diclofenac in humans in vivo, where the AG and 4’-hydroxy pathways account for ~10 to 20 and ~50% of the total diclofenac metabolites, respectively. A possible explanation for this discrepancy is provided by our observation of the P450-catalyzed oxidative metabolism of diclofenac AG in HLMs. A more rapid rate of disappearance of diclofenac AG in HLM incubations supplemented with the NADPH-regenerating system, relative to those without, provides strong evidence for significant oxidative metabolism of diclofenac AG. Further confirmation of this is provided by the detection of 4’-hydroxy diclofenac AG in HLM incubations containing NADPH. It is difficult to conduct kinetic studies to estimate Vmax and Km parameters for the oxidation of diclofenac AG in HLM because the rate of hydrolysis of the formed 4’-hydroxy diclofenac AG to 4’-hydroxy diclofenac is significant relative to the rate of its formation; this contrast to the formation of diclofenac AG from diclofenac where the rate of metabolite formation far exceeded the rate of its hydrolysis. It is also not possible to estimate the total amount of 4’-hydroxy diclofenac AG formed by measuring 4’-hydroxy diclofenac after complete hydrolysis of samples because a portion of the 4’-hydroxy diclofenac metabolite is most certainly formed via metabolism of diclofenac AG that results from hydrolysis of diclofenac AG in the incubation. A reasonable approximation of the quantitative significance of the oxidative metabolism of diclofenac AG can be made via the in vitro fuv2 approach after correction for the rate of its spontaneous hydrolysis in incubations. Using this approach, a value of 5.5 ml/min/kg was estimated for the hepatic intrinsic clearance of diclofenac AG oxidation (CLint,AG). The magnitude of this estimated CLint,AG is <1% relative to the estimated intrinsic clearance of diclofenac AG formation from diclofenac in the presence of alamethicin (Table 3). However, it is important to consider that the intrinsic clearance of diclofenac AG formation in vivo is limited by the unbound fraction of diclofenac in blood flowing into the liver, i.e.,

$$\text{Effective } CL_{\text{int}} \text{ liver} = \frac{f_u \cdot CL_{\text{int}} \text{ liver}}{B/P} = \frac{0.005}{0.55} \cdot 748 = 6.8 \text{ ml/min/kg}$$

where values of 0.005 and 0.55 for the $f_u$ and B/P parameters are from literature data, as described under Materials and Methods.

In contrast to the metabolism of diclofenac to diclofenac AG, the sequential oxidation of diclofenac AG to 4’-hydroxy diclofenac AG would occur on a metabolite that is generated intrahepatically. For a number of reasons, it is likely that this secondary metabolic reaction will not be as significantly affected by the substrate binding to blood components. First, a significant amount of diclofenac AG may be oxidized to 4’-hydroxy diclofenac AG via the so-called “metabolite first-pass effect” before its egress from the hepatocytes into the bloodstream (Pang and Gillette, 1979). Second, acyl glucuronides, in general, exhibit a significantly reduced degree of plasma protein binding relative to the parent drug because of their greater hydrophilicity (Ojingwa et al., 1994; Bischer et al., 1995). Third, it is possible that because of rapid metabolism of diclofenac via this pathway, high local concentrations of diclofenac AG are achieved in the liver, which may saturate any binding to blood components. Thus, from the estimated effective intrinsic clearances for the formation of diclofenac AG from diclofenac (6.8 ml/min/kg), and for the sequential oxidative metabolism of diclofenac AG (5.5 ml/min/kg), it appears that a significant fraction of the intrahepatically formed diclofenac AG may be converted to 4’-hydroxy diclofenac AG via the metabolite first-pass effect. Subsequently, additional amounts of diclofenac AG that circulate systemically after egress from the liver may be metabolized to its 4’-hydroxy derivative upon recirculation to the liver. These data suggest that acyl glucuronidation may be a primary determinant of diclofenac clearance in humans. Excretion of 4’-hydroxy diclofenac (free or glucuronidated) as a major excretory product after a diclofenac dose in humans.

Fig. 7. Representative extracted ion chromatograms of 4’-hydroxy diclofenac and its acyl glucuronide in 15-min incubations of diclofenac acyl glucuronide with microsomes containing human recombinant CYP2C9 (A) and CYP2C8 (B) isozymes in the presence of an NADPH-regenerating system. The data demonstrate that only CYP2C8, and not CYP2C9, is capable of catalyzing the direct hydroxylation of diclofenac acyl glucuronide to 4’-hydroxy diclofenac acyl glucuronide, and only CYP2C9, and not CYP2C8, can covert diclofenac (formed via the hydrolysis of diclofenac acyl glucuronide in incubations) to 4’-hydroxy diclofenac.
may in fact result from a combination of the direct formation of this metabolite from diclofenac, and also from the secondary oxidation of diclofenac AG to its 4′-hydroxy derivative.

A further interesting observation from our studies was that the conversion of diclofenac AG to its 4′-hydroxy derivative was catalyzed by the CYP2C8 isozyme. Metabolism of diclofenac to 4′-hydroxy diclofenac in vitro in HLMs has been widely used as a specific probe for measuring CYP2C9 activity. Some recent studies have demonstrated that the in vitro diclofenac 4′-hydroxylation activity tends to differ among liver microsomal samples obtained from individuals who have been genotyped as heterozygous allelic variants in the CYP2C9 gene such as CYP2C9*1/*1 (wild type), CYP2C9*1/*2, CYP2C9*1/*3, and CYP2C9*1/*5 (Aithal et al., 2000; Takanaishi et al., 2000; Dickmann et al., 2001). Because diclofenac is widely used in the clinic, its single doses are well tolerated, and it is excreted as one major metabolite (i.e., 4′-hydroxy diclofenac glucuronide), it has been proposed as an in vivo probe for phenotyping CYP2C9 activity in humans.

However, some initial clinical studies have suggested that there is little difference in overall diclofenac oral clearance, 4′-hydroxy diclofenac plasma concentrations, or the urinary excretion of total 4′-hydroxy diclofenac (free + conjugated) among different CYP2C9 genotypes (Shimamoto et al., 2000; Morin et al., 2001; Yasser et al., 2001). These clinical data on diclofenac do not seem to correlate with what has been observed for other CYP2C9 substrates such as warfarin, losartan, and tolbutamide where the pharmacokinetic (and as a result pharmacodynamic) characteristics appear closely linked to the CYP2C9 genotype (Aithal et al., 1999; McCrea et al., 1999). Our data on the metabolism of diclofenac in HLMs may provide a possible explanation for these discrepancies. It would appear from these data that the overall pharmacokinetics of diclofenac in humans are determined by the kinetics of acyl glucuronidation because it accounts for nearly 75% of the total estimated hepatic CLint for this drug.

CYP2C9-catalyzed 4′-hydroxylation of diclofenac may have a smaller contribution (<25%) to the overall elimination of this drug than previously assumed. Also, the total amount of 4′-hydroxy diclofenac formed and excreted (free + conjugated) in humans may be dependent on the activities of not only CYP2C9 but also on those of CYP2C8 and UGT2B7 enzymes (enzyme that primarily catalyzes diclofenac acetyl glucuronidation; King et al., 2001) because of the potential direct oxidation of diclofenac AG to its 4′-hydroxy derivative via the activity of these isozymes. Thus, it is possible that diclofenac oral clearance or 4′-hydroxy diclofenac urinary recoveries in these studies are not good indicators of CYP2C9 activity.

To our knowledge, this is the first reported instance of P450-mediated oxidation of a glucuronide conjugate. Although this is most certainly an interesting phenomenon that blurs the traditional definitions of phase I and phase II metabolism, it may also be an important determinant of the final metabolite excretion profile and may confound the identification of primary clearance mechanisms for xenobiotics.

Summary. In summary, we have demonstrated that the in vivo diclofenac clearance can be extrapolated fairly accurately from a combination of its oxidative metabolism and acetyl glucuronidation kinetics in liver microsomes in the rat, dog, and human. In all the three species studied, acetyl glucuronidation was determined to be an important component (~70 to >90%) of diclofenac elimination. For the rat and the dog, the relative magnitudes of CLint of diclofenac 4′-hydroxylation and acetyl glucuronidation also accurately predicted the quantitative contribution of these pathways to in vivo metabolite excretion profile in these species. In HLM, however, a unique CYP2C8-catalyzed conversion of diclofenac AG to its 4′-hydroxy derivative was identified. Thus, it is possible that although the overall human pharmacokinetics of diclofenac are determined primarily by the acetyl glucuronidation pathway, yet a large fraction of the dose is eventually excreted as 4′-hydroxy diclofenac glucuronide as a result of this metabolic reaction. Also, the total urinary excretion of 4′-hydroxy diclofenac may be dependent on the activities of CYP2C8 and UGT2B7 enzymes in addition to that of CYP2C9. These findings have implications in the use of diclofenac as a phenotypic probe for measuring CYP2C9 activity in humans in vivo.

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References


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