BIOACTIVATION OF DICLOFENAC VIA BENZOQUINONE IMINE INTERMEDIATES—IDENTIFICATION OF URINARY MERCAPTURIC ACID DERIVATIVES IN RATS AND HUMANS

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ABSTRACT:

The metabolism of diclofenac has been reported to produce reactive benzoquinone imine intermediates. We describe the identification of mercapturic acid derivatives of diclofenac in rats and humans. Three male Sprague-Dawley rats were administered diclofenac in aqueous solution (pH 7) at 50 mg/kg by intraperitoneal injection, and urine was collected for 24 h. Human urine specimens were obtained, and samples were pooled from 50 individuals. Urine samples were analyzed by liquid chromatography-tandem mass spectrometry (LC/MS/MS). Two metabolites with MH + ions at m/z 473 were detected in rat urine and identified tentatively as N-acetylglutathione adducts of monohydroxydiclofenac. Based upon collision-induced fragmentation of the MH + ions, accurate mass measurements of product ions, and comparison of LC/MS/MS properties of the metabolites with those of synthetic reference compounds, one metabolite was assigned as 5-hydroxy-4-[(N-acetylcytstein-S-yldiclofenac and the other as 4'-hydroxy-3'-[(N-acetylcytstein-S-yldiclofenac. The former conjugate also was detected in the pooled human urine sample by multiple reaction-monitoring LC/MS/MS analysis. It is likely that these mercapturic acid derivatives represent degradation products of the corresponding glutathione adducts derived from diclofenac-2,5-quinone imine and 1',4'-quinone imine, respectively. Our data are consistent with previous findings, which suggest that oxidative bioactivation of diclofenac in humans proceeds via benzoquinone imine intermediates.

Diclofenac is a nonsteroidal anti-inflammatory drug frequently prescribed for treatment of arthritis, ankylosing spondylitis, and acute muscle pain (Davies and Anderson, 1997). It causes a rare, but potentially severe, liver injury (Breen et al., 1986; Helfgott et al., 1990; Banks et al., 1995). The hepatotoxicity resembles either acute viral hepatitis or chronic hepatitis and has been described as idiosyncratic in nature due to the delayed onset of symptoms and lack of a clear dose-response relationship (Banks et al., 1995; Boelsterli et al., 1995). Elderly and female patients appear to be most susceptible to the toxicity of diclofenac (Banks et al., 1995; Boelsterli et al., 1995).

Although the mechanism of diclofenac hepatotoxicity remains elusive, two hypotheses have been developed, and both focus on metabolic activation of the drug. The first implicates the reactive acyl glucuronide of diclofenac as a potential causative diclofenac in the drug-associated toxicity (Pumford et al., 1993; Hargus et al., 1994). In this regard, it has been shown that the glucuronidation of diclofenac is catalyzed by uridine 5'-diphosphoglucuronosyl transferase 2B1 and 2B7, respectively, in rats and humans (King et al., 2001) and that the resulting acyl glucuronide is capable of covalent modification of cellular proteins (Kretz-Rommel and Boelsterli, 1993, 1994). The second proposed mechanism for diclofenac toxicity is based upon observations of NADPH-dependent covalent modification of proteins in incubations of the drug with rat or human liver microsomes and the formation of glutathione adducts in rats treated with the drug (Shen et al., 1999; Tang et al., 1999a). In light of these findings, it has been postulated that cytochrome P450 (CYP)-catalyzed oxidation of diclofenac results in the formation of benzoquinone imine intermediates that react further with glutathione or microsomal proteins (Shen et al., 1999; Tang et al., 1999a). The CYP enzymes involved in diclofenac metabolism were identified as CYP2B, 2C, and 3A in rats and CYP2C9 and 3A4 in humans (Leemann et al., 1993; Shen et al., 1999; Tang et al., 1999a,b).

The formation of glutathione adducts of diclofenac also was observed in incubations of the drug with human hepatocytes (Tang et al., 1999a). Glutathione adducts are known to undergo sequential hydrolysis by γ-glutamyltranspeptidase and dipeptidases to form the corresponding cysteinylglycine and cysteine derivatives. N-Acetylation of the latter species leads to the formation of mercapturic acids, which can be detected in the urine. Therefore, characterization of urinary mercapturic acid derivatives represents a classic approach for investigation of the metabolic activation of xenobiotics in vivo. In this
In this article, we describe the detection of mercapturic acid derivatives of diclofenac in the urine of rats and humans treated with diclofenac. Urine samples were analyzed by liquid chromatography-tandem mass spectrometry (LC/MS/MS), and metabolites were identified based on comparison with synthetic reference compounds.

**Experimental Procedures**

**Materials.** Diclofenac, N-acetylcysteine, NADPH, and trifluoroacetic acid were purchased from Sigma Chemical Co. (St. Louis, MO). BondElut C_{18} solid-phase extraction cartridge columns were obtained from Varian Chromatography Systems (Walnut Creek, CA). All other chemicals were obtained from Fisher Scientific (Fair Lawn, NJ). Recombinant CYP 3A4 and 2C9 were purchased from GENTEST (Woburn, MA).

**Instrumentation.** LC/MS/MS analyses were carried out on a PerkinElmer Sciex API3000 triple quadrupole mass spectrometer (Toronto, ON, Canada) interfaced via a turbo ion spray source to a PerkinElmer Series 200 microquaternary HPLC pump and a Series 200 autosampler (PerkinElmer Instruments, Norwalk, CT). The source temperature was set at 400°C, ionization voltage at 5 kV, and orifice voltage at 60 V. Nitrogen was used as the collision gas for collision-induced dissociation (CID) with collision energy set at 35 eV. The instrument was operated in the positive ion mode. Chromatography was performed on a Zorbax Rx C_{18} narrow-bore column (150 × 2.1 mm, 5 μm).

The fragmentation of the metabolites of interest upon CID also was investigated on a Finnigan LCQ mass spectrometer (Thermo Finnigan, San Jose, CA) coupled to a Shimadzu Scientific LC-10AD liquid chromatograph (Shimadzu, Kyoto, Japan). Electrospray LC/MS was performed in the positive ion mode.

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**Fig. 1.** Product ion mass spectra of (A) 5-OH-4-NAC-diclofenac and (B) 4’-OH-3’-NAC-diclofenac found in the urine of rats treated with diclofenac.

The spectra were obtained by CID of the MH\(^+\) ions at m/z 473. Fragmentation patterns are discussed in the text.
mode, with the spray voltage maintained at 4.1 kV, collision energy at 32 eV, and capillary temperature at 200°C. Samples were introduced via a Zorbax Rx C_18 narrow-bore column (150 × 2.1 mm, 5 μm). The mobile phase consisted of 70% aqueous acetonitrile containing 0.1% formic acid. The flow rate was set at 0.2 ml/min.

Accurate mass measurements were performed on a Micromass QTOF tandem quadrupole time-of-flight mass spectrometer (Beverly, MA). The instrument was operated in the positive ion mode. The cone voltage and collision energy were maintained at 20 V and 25 eV, respectively, and the collision gas was argon. Mass measurements were based on a lock mass of 556.2771 (the MH⁺ ion of leucine enkephalin). Samples were introduced by a Hewlett Packard Series 1100 HPLC (Palo Alto, CA) via a Betasil C_18 column (100 × 2 mm, 3 μm). The mobile phase consisted of 80% aqueous methanol containing 0.2% formic acid at a flow rate of 0.2 ml/min.

HPLC purification of synthetic products was performed on a Shimadzu Scientific LC-10AD liquid chromatograph equipped with a Zorbax RX-C_8 column (4.6 × 250 mm, 5 μm). The mobile phase consisted of aqueous acetonitrile containing 10% methanol and 0.05% trifluoroacetic acid and was programmed by a linear increase from 10 to 70% acetonitrile during a 30-min period. The flow rate was 1 ml/min, and UV detection was carried out at 210 nm. NMR spectra of synthetic products were obtained on a Varian Inova 600 spectrometer operated at 600 MHz. Chemical shifts are expressed relative to tetramethylsilane.

**Synthesis of Diclofenac Metabolites.** Synthesis of 5-hydroxy-4-(N-acetylcystein-5-yl)diclofenac (5-OH-4-NAC-diclofenac, M1). 5-Hydroxydiclofenac was synthesized via conversion of 5-iododiclofenac to the corresponding arylboronic ester followed by oxidation with oxone in aqueous acetone (Tang et al., 1999a).

5-Hydroxydiclofenac (3 mg) and N-acetylcysteine (3 mg) were mixed in 60% aqueous acetonitrile (2 ml, pH 8). The mixture was stirred at 37°C for 7 h, and the product, 5-OH-4-NAC-diclofenac, was isolated by HPLC. LC/MS, m/z 473 (MH⁺). 1H NMR (CD₃OD), δ 1.82 (s, CH₃CONH), 2.96 to 3.24 (m, SCH₂), 3.67 (s, CH₂COOH), 4.38 (m, SCH₂CH₂), 5.72 (s, 3-CH), 6.54 (s, 3-C₆H₅), 6.77 (s, 6-C₆H₅), 6.97 (t, 4'-CH), 7.34 (d, 3'-CH). 3-OH-4-NAC-diclofenac also was generated in incubations with recombinant CYP3A4. Briefly, diclofenac, N-acetylcysteine, and the enzyme (0.5 mg
of protein/ml) were mixed in phosphate buffer (0.1 M, pH 7.4). The final concentrations of reactants were 0.1 mM diclofenac, 5 mM N-acetylcysteine, and 1 mg/ml NADPH. The mixture was incubated at 37 °C for 4 h. Samples from incubations were applied to a C18 extraction cartridge column, which had been prewashed with methanol and water. The column was washed consecutively with water and methanol. The methanol eluate was evaporated to dryness under a stream of nitrogen to afford the product.

Synthesis of 4'-/H11032-hydroxy-3'-/H11032-(N-acetylcystein-S-yl)diclofenac (4'-/H11032-OH-3'-/H11032-NAC-diclofenac, M2).

Diclofenac, N-acetylcysteine, and NADPH in phosphate buffer (pH 7.4) were added to recombinant CYP2C9 (0.5 mg of protein/ml) suspended in phosphate buffer (0.1 M, pH 7.4). The final concentrations of reactants were 0.1 mM diclofenac, 5 mM N-acetylcysteine, and 1 mg/ml NADPH. The mixture was incubated at 37 °C for 4 h, and the product was isolated by HPLC. LC/MS, m/z 473 (MH+/H11001). 1 H NMR (CD3OD), δ 1.82 (s, C3H3CONH), 3.02–3.22 (m, SC2H2), 3.54 (s, CH2COOH), 4.35 (m, CH2CH2), 6.15 (d, 3-CH), 6.61 (t, 5-CH), 6.70 (s, 5-CH), 6.85 (t, 4-CH), 7.08 (d, 6-CH).

Animal Experiments. Experiments were performed according to procedures approved by the Merck Institutional Animal Care and Use Committee.

**TABLE 1**

<table>
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<tr>
<th>Ions</th>
<th>Calculated</th>
<th>Observed</th>
<th>Δ Da (×10³)</th>
<th>ppm</th>
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<td>473.0341</td>
<td>473.0337</td>
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<tr>
<td>m/z 368, C18H10NO3SCl2</td>
<td>367.9915</td>
<td>367.9923</td>
<td>0.8</td>
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<tr>
<td>m/z 350, C18H10NO3SCl2</td>
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<td>349.9815</td>
<td>0.5</td>
<td>1.6</td>
</tr>
<tr>
<td>m/z 342, C18H10NO3SCl2</td>
<td>341.9758</td>
<td>341.9770</td>
<td>1.1</td>
<td>3.3</td>
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<tr>
<td>m/z 324, C18H10NO3SCl2</td>
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<td>323.9658</td>
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<td>1.6</td>
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<tr>
<td>m/z 292, C17H8NO3S2</td>
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<tr>
<td>m/z 162, C13H11NO2S</td>
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<td>162.0221</td>
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<tr>
<td>m/z 130, C10H13NO2</td>
<td>130.0504</td>
<td>130.0500</td>
<td>-0.4</td>
<td>-2.8</td>
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</tbody>
</table>

Δ Da, the difference between observed and calculated values.

**FIG. 3.** Detection of 5-OH-4-NAC-diclofenac in human urine by multiple reaction monitoring LC/MS/MS analysis.

Responses for three characteristic mass transitions, namely m/z 473.1 → 162.1, 473.1 → 324.2, and 473.1 → 342.2, coincided with the HPLC retention time (13.7 min) of a synthetic sample of 5-OH-4-NAC-diclofenac.
Three male Sprague-Dawley rats (350 g) were allowed free access to commercial rat chow and water and treated with diclofenac (50 mg/kg) in aqueous solution (pH 7) by intraperitoneal injection. Urine samples were collected for 24 h. Aliquots of rat urine were acidified to pH 2 with 10% aqueous trifluoroacetic acid and centrifuged at 13,600 g for 5 min. The resulting supernatant was injected onto the Zorbax C18 narrow-bore column and analyzed by LC/MS/MS.

**Human Experiments.** Human urine samples were obtained from patients who were enrolled in a program designed for comparative treatment of osteoarthritis (Cannon et al., 1998). Briefly, patients were administered 50 mg of diclofenac sodium orally three times a day for 26 weeks, with no other medication. Urine was collected in the morning following the last evening dose and was pooled from 50 individuals. Aliquots of the sample (100 ml) were acidified to pH 2 and extracted with ethyl acetate (2 × 200 ml). The organic extracts were combined and concentrated under nitrogen, reconstituted in 70% aqueous trifluoroacetic acid and centrifuged at 13,600 g for 5 min. The resulting supernatant was injected onto the Zorbax C18 narrow-bore column and analyzed by LC/MS/MS.

**Results and Discussion**

Two metabolites of diclofenac with MH$^+$ at m/z 473 and characteristic chlorine isotope clusters (m/z 473/475) were detected in rat urine samples and arbitrarily assigned as M1 and M2. The protonated parent ion of these metabolites was consistent with that of mercapturic acid derivatives of hydroxyclofenac. Subsequent CID of the MH$^+$ ion at m/z 473 resulted in the formation of product ions at m/z 162 and 130, which suggested the presence of an N-acetylcyesteine moiety in the metabolites (Fig. 1). The fragment ion at m/z 162 was assigned as the protonated form of dehydro-N-acetylcyesteine, whereas the ion at m/z 130 most likely derived from cleavage of the thioether bond with attachment of a proton to the resulting dehydroalanine moiety (Tang and Abbott, 1996).

Further characterization of the mercapturic acid derivatives of diclofenac was carried out by CID analysis on an LCQ instrument, the results of which are summarized in Fig. 2. For M1, combined neutral losses of ammonium acetate and formic acid appeared to lead to the fragment ion at m/z 368, which underwent a further loss of water to give the ion m/z 350. The fragment ion at m/z 342 may derive from cleavage of the thioether bond with charge retention on the aromatic moiety, and the ion at m/z 324 from a further loss of water from the species at m/z 342. The fragment ion at m/z 292 may be accounted for by the combined neutral losses of N-acetylcyesteine and water from the MH$^+$ ion (Fig. 2A). For M2, the fragment ion at m/z 413 appeared to arise from the combined neutral losses of water and ketene. Further losses of water and carbon monoxide would produce the ion at m/z 367. The fragment ions at m/z 350 and 294 derived from the ion m/z 367 (loss of ammonia and loss of S=CH—CH=NH, respectively), whereas the ion at m/z 324 originated from m/z 413 (cleavage of the thioether bond with charge retention on the aromatic moiety plus a further loss of water) (Fig. 2B).

The above interpretation of CID data was in good agreement with the elemental composition information derived from the accurate mass measurements (Table 1). Thus, the MH$^+$ ions were observed at m/z 473.0337 for M1 and m/z 473.0353 for M2, consistent with the calculated value of m/z 473.0341. With the exception of the ion at m/z 367 in the CID spectrum of M2, major fragment ions of the isomeric mercapturic acid derivatives also exhibited deviations within an arbitrary limit of ±5 ppm from theoretical values (Table 1).

The precise structural assignment of M1 and M2 was based on comparison of the LC/MS/MS properties of the metabolites with those of the corresponding synthetic reference compounds. On this basis, M1 was identified as 5-OH-4-NAC-diclofenac, and M2 was identified as 4'-OH-3'-NAC-diclofenac (Fig. 1, A and B).

The detection of mercapturic acid derivative(s) of diclofenac in the urine of human subjects was achieved via multiple reaction-monitoring LC/MS/MS analysis using three characteristic mass transitions: m/z 473.1 → 162.1, 473.1 → 324.2, and 473.1 → 342.2. By this approach, coincident responses were observed in each ion current chromatogram at a retention time of 13.7 min, corresponding to the
retention time of synthetic 5-OH-4-NAC-diclofenac (Fig. 3). In contrast, no evidence was obtained for the presence of 4′-OH-3′-NAC-diclofenac in human urine (retention time, 14.2 min).

Previously, it has been reported that the metabolism of diclofenac in rats affords 5-hydroxy-4-(glutathion-S-yl)diclofenac, 4′-hydroxy-3′-(glutathion-S-yl)diclofenac, and 5-hydroxy-6-(glutathion-S-yl)diclofenac, the precursors of which possibly are diclofenac-1′,4′-quinone imine and diclofenac-2,5-quinone imine (Fig. 4; Tang et al., 1999a). In the present study, 5-OH-4-NAC-diclofenac and 4′-OH-3′-NAC-diclofenac were identified in the urine of rats treated with diclofenac. These mercapturic acid derivatives most likely are derived from the corresponding glutathione adducts, thereby providing a link between mercapturic acids, glutathione adducts, and reactive benzoquinone imine intermediates during the biotransformation of diclofenac in the rat (Fig. 4).

With respect to diclofenac metabolism in humans, one mercapturic acid derivative, namely 5-OH-4-NAC-diclofenac, was identified in pooled urine of treated subjects. It should be noted that the human specimens were obtained from an experiment that was not designed for metabolic studies, and urine was collected at least 16 h after dosing. This lengthy collection period might have contributed to the inability to detect 4′-OH-3′-NAC-diclofenac. Nevertheless, our data are consistent with the previous report in which 5-hydroxy-4-(glutathion-S-yl)diclofenac was observed in incubations of diclofenac with human hepatocytes (Tang et al., 1999a). On this basis, identification of the urinary mercapturic acid derivative of diclofenac is taken as a reflection of the formation in vivo of a benzoquinone imine intermediate in humans, namely diclofenac-2,5-quinone imine, derived from oxidation of 5-hydroxydiclofenac (Fig. 4).

The formation of 5-hydroxydiclofenac in humans has been shown to be catalyzed by CYP3A4 (Shen et al., 1999; Tang et al., 1999b). In fact, 5-OH-4-NAC-diclofenac was made in this study by incubations of diclofenac with recombinant CYP3A4 in the presence of N-acetylcysteine (see Experimental Procedures). The enzyme is the most abundant CYP in human liver, with its activity varying 10- to 20-fold among individuals (Wrighton and Stevens, 1992; Guengerich, 1997). The same enzyme also is inducible upon chronic or subchronic administration of therapeutic agents, such as rifampin or carbamazepine (Wrighton and Stevens, 1992; Guengerich, 1997). Most recently, the 5-hydroxylation of diclofenac in incubations with human liver microsomes or hepatocytes has been demonstrated to be enhanced in the presence of quinidine by a mechanism yet to be identified (Ngui et al., 2000). It is tempting to speculate, therefore, that individuals with higher CYP3A4 activity, possibly in combination with compromised “detoxification” mechanisms such as reduced hepatic glutathione status, could be predisposed to the idiosyncratic hepatotoxicity of diclofenac. In this regard, the excretion of a mercapturic acid derivative in the urine of human subjects treated with diclofenac may be of some practical use in future clinical studies of the mechanism of diclofenac-mediated liver injury.

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References


