CONJUGATION OF DESMETHYLNAPROXEN IN THE RAT—A NOVEL ACYL GLUCURONIDE-SULFATE DICONJUGATE AS A MAJOR BILIARY METABOLITE

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ABSTRACT:
The nonsteroidal anti-inflammatory drug naproxen is primarily metabolized in humans by acyl glucuronidation to form naproxen acyl glucuronide and by O-dealkylation to form 6-O-desmethyl-naproxen (DMN). DMN contains both carboxy and phenolic groups and has been shown to form acyl glucuronide and sulfate conjugates. This project aimed to investigate whether DMN formed a phenolic glucuronide and diglucuronide(s) (with both the carboxy and phenolic groups glucuronidated). Male Sprague-Dawley rats (300–350 g) with exteriorized bile flow were dosed i.v. with DMN at 50 mg/kg. Four major DMN-related peaks were detected in bile by high-performance liquid chromatography (HPLC) analysis at 225 nm, including the known acyl glucuronide and sulfate conjugates. Selective hydrolyses using acidic and alkaline conditions and digestion with β-glucuronidase allowed tentative identification of the two unknown peaks as the phenolic glucuronide of DMN and a novel acyl glucuronide-sulfate diconjugate of DMN (i.e., formed by sulfonation of the phenolic group and glucuronidation of the carboxy group). The identities were confirmed by liquid chromatography-tandem mass spectrometry analysis of individual HPLC fractions. Total recovery of the DMN dose was approximately 80%, with the sulfate conjugate (50%) and unchanged DMN (10%) being excreted predominantly in urine and the acyl glucuronide (10%), phenolic glucuronide (6%), and acyl glucuronide-sulfate diconjugate (4%) being excreted predominantly or exclusively in bile. No evidence for a diglucuronide metabolite of DMN was found in either bile or urine of the DMN-dosed rats.

The salicylate derivative diflunisal, a nonsteroidal anti-inflammatory drug (NSAID), has both carboxy and phenolic functional groups and forms acyl glucuronide, phenolic glucuronide, and sulfate mono-conjugates as major metabolites in humans and animals (Tocco et al., 1975; Lin et al., 1985; Loewen et al., 1986; Dickinson et al., 1989). In previous work, we have reported the additional formation of small quantities of quasi “diglucuronides” of diflunisal in the rat and the perfused rat liver (King and Dickinson, 1991; Wang and Dickinson, 1998). The yield of diglucuronides was greater when the preformed biosynthetic acyl glucuronide was administered and much greater again when the acyl migration rearrangement isoforms were administered. By contrast, no diglucuronides were formed after administration of the phenolic glucuronide of diflunisal. Interestingly, the mixture of diglucuronides appeared to comprise the phenolic glucuronides of the 2-, 3-, and possibly 4-O-positional isomers of the acyl glucuronides (King and Dickinson, 1991). Whether any “real” diglucuronide (i.e., the phenolic glucuronide of the biosynthetic acyl glucuronide) was formed was equivocal. These results brought forth interesting questions about the presence of recognition/transport/metabolism processes pertaining to real (i.e., biosynthetic) acyl glucuronides versus “look-alikes” (i.e., nonbiosynthetic acyl migration rearrangement isoforms).

The NSAID naproxen (6-methoxy-a-methyl-2-naphthaleneacetic acid) is primarily metabolized in humans and rats by direct acyl glucuronidation to form naproxen acyl glucuronide and by O-dealkylation to produce 6-O-desmethylnaproxen (DMN; Fig. 1). DMN possesses both a carboxy and a phenolic functional group and has been shown to form acyl glucuronide (DMN-AG) and sulfate (DMN-S) conjugates (Kiang et al., 1989; Vanggard-Andersen and Hansen, 1992; Vree et al., 1993; Fig. 1). However, the phenolic glucuronide of DMN (DMN-PG) has not been reported to date. DMN, possessing both carboxy and phenolic functions (like diflunisal), was thus an interesting substrate to investigate the formation of novel diglucuronides. The present study reports on the disposition of DMN administered to bile-exteriorized rats, with particular emphasis on the identification of the phenolic glucuronide and a novel acyl glucuronide-sulfate diconjugate in the bile of rats.

Materials and Methods

Chemicals and Reagents. Naproxen (as the S-enantiomer) and β-glucuronidase (from Escherichia coli) were purchased from Sigma Chemical Co. (St. Louis, MO). Probenecid was obtained from BDH Chemicals (Sydney, Queensland, Australia, 3–6 December 2000, page 105.)

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Australia), and isoflurane was supplied by Abbott Australasia Pty Ltd (Sydney, Australia). Methanol and acetonitrile (HPLC grade) were purchased from Mallinckrodt (Melbourne, Australia). All other reagents used were of analytical reagent-grade purity.

DMN was prepared by refluxing naproxen (0.5% w/v) in HCl (4 M) for 5 h. The solution was cooled on ice, and the resulting precipitate was recrystallized in boiling water, filtered, and dried. The structure of DMN was confirmed by LC-MS/MS analysis. DMN formed with a yield of 80%, and its purity was found to be >99% when tested by HPLC analysis.

**Animal Experiments.** Male Sprague-Dawley rats (300–350 g) were obtained from the Herston Medical Research Center (Brisbane, Australia). Rats were prepared, under anesthesia with isoflurane, with a specially constructed indwelling catheter inserted into the right external jugular vein and exteriorized between the scapulae, as described earlier (King and Dickinson, 1996). The common bile duct was catheterized and also exteriorized between the scapulae in addition to the i.v. catheter. The rats were allowed to recover from surgery for ca. 2 h before dosing and were held unrestrained in metabolism cages for the duration of the experiment, with free access to water, but food was withheld. The rats were dosed i.v. via the jugular catheter with a single dose of DMN (50 mg/kg), prepared as a 10 mg/ml solution in NaHCO3 (0.05 M) and administered over 2 min. Blood samples (200 l) were collected predose and at 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, and 360 min postdose via the jugular catheter. Samples were immediately centrifuged at 3000 g; the plasma was snap frozen on dry ice and stored at −80°C for analysis. Heparinized saline (100 l) was infused into the jugular vein after each sampling. Bile samples were collected over ice predose and over the periods 0 to 1, 1 to 2, 2 to 4, and 4 to 6 h postdose. The bile samples were frequently adjusted with acetic acid (1 M) to maintain a pH of 4 to 5. Urine samples were collected over ice predose and over a time period of 0 to 6 h postdose. Both bile and urine samples were snap frozen over dry ice and stored at −80°C for further analysis. At completion of the experiment (6 h), the rats were anesthetized with pentobarbitone (60 mg/kg) via the i.v. catheter and exsanguinated via the aorta. The contents of the bladder were aspirated and added to the 0- to 6-h voidings. The Animal Experimentation Ethics Committee of The University of Queensland approved animal experiments.

**Sample Preparation and HPLC Analysis.** Alkaline hydrolysis of acyl glucuronide conjugates in bile samples was carried out in NaOH solution (2 M) at 90°C for 1 h. Acid hydrolysis of sulfate conjugates in bile samples was carried out in HCl solution (2 M) at 90°C for 1 h. Enzymatic hydrolysis of phenolic glucuronide conjugates in bile samples was achieved by incubation with β-glucuronidase (250 U/ml) at pH 7.0 at 37°C for 2 h.

HPLC analysis was carried out with a system consisting of a model 510 HPLC pump, a model 481 ultraviolet detector (Waters, Milford, MA), and a K65B autosampler (ETP Kortec, Sydney, Australia). The analytical column was a Platinum EPS C18 (Alltech Associates, Sydney, Australia) preceded by a µBondapak C18 Corasil guard column (Waters). The mobile phase consisted of an aqueous solution containing NaH2PO4 (0.01 M) and Na2SO4 (4% w/v), adjusted to pH 4.0, and MeOH (35% v/v) and was used at a flow rate of 1 ml/min. Peaks were detected at 225 nm, and quantitative analysis was carried out against standard curves constructed from blank samples of appropriate matrix, spiked with increasing concentrations of DMN. All standard curves had a coefficient of determination of 0.999 or greater.

Plasma samples were analyzed for DMN and DMN-S as follows. Aliquots of plasma samples (45 l) were added to phosphate buffer (5 l, 0.1 M, pH 4.0) and internal standard (probenecid; 100 l, 0.375 mg/ml) in acetonitrile.

![Fig. 2](image-url). HPLC profiles of DMN and its metabolites in rat bile after i.v. administration of DMN (50 mg/kg).

A, control bile; B, DMN and metabolites in untreated bile; C, bile after base hydrolysis with NaOH (2 M) at 90°C for 2 h; D, bile after base hydrolysis and β-glucuronidase digestion at 37°C for 4 h.
After vortexing and centrifugation (5000 g) for 2 min, 100 μl of the supernatant was dried to completeness under a gentle stream of air. The residue was reconstituted in 100 μl of mobile phase, and 10 μl was injected into the HPLC system. Bile and urine samples were diluted in phosphate buffer (0.1 M, pH 4.0) 10- and 50-fold, respectively, and mixed with equal volumes of internal standard solution (probenecid; 500 μg/ml in MeOH). The mixtures were centrifuged (5000 g) for 2 min, and 10 μl was injected into the HPLC system.

DMN-AG and DMN-S concentrations in bile and urine samples were estimated from the difference in the DMN content before and after alkaline or acid hydrolysis, respectively. The DMN-PG concentration was estimated from the difference in the DMN content of a base hydrolyzed sample before and after digestion with β-glucuronidase, and the DMN-AG-S concentration was estimated from the difference in DMN-S content before and after alkaline hydrolysis.

**LC-MS/MS Analysis.** LC-MS/MS analysis was conducted by infusion of fractions separately collected from the HPLC system described above at an infusion rate of 10 μl/min. The analysis was carried out in the negative ion mode on an API 2000 system (Applied Biosystems, Foster City, CA) with the following system parameters: turbo spray tip, −4000 V; orifice plate, 0 V; ring voltage, −350 V; and collision energy, 19 eV. The collision-activated dissociation gas was set to 3 psi.

**Data Analysis.** The apparent plasma half-lives \( t_{1/2} \) of DMN and DMN-S were determined from the slope of the log concentration-time profile using linear regression analysis. AUC\(_{0-t} \) was calculated using the trapezoidal method.

**Results**

Selective Hydrolysis and HPLC Analysis of Biliary Metabolites. Figure 2 shows the HPLC chromatograms of bile from male Sprague-Dawley rats dosed i.v. with DMN at 50 mg/kg (B), after alkaline hydrolysis (C), and after sequential alkaline and β-glucuronidase.
hydrolysis (D). Five drug-related peaks are evident in untreated bile (B) based on comparison with control bile (A), with the parent DMN present only as a minor peak at a retention time of approximately 15 min (peak 5).

HPLC analysis of urine from rats dosed with DMN revealed one major peak with a retention time corresponding to that of peak 3 in control bile (Fig. 2B). Peak 3 showed resistance to alkaline hydrolysis (Fig. 2C) but was substantially reduced after incubation with acid [particularly in the presence of diethyl ether (data not shown)], suggesting it was a sulfate conjugate [as shown previously for the major urinary metabolite of rats dosed with naproxen (Sugawara et al., 1978)]. These combined observations allowed the tentative identification of peak 3 as the sulfate conjugate DMN-S.

The metabolites corresponding to peaks 1 and 4 in Fig. 2B were labile under alkaline conditions (Fig. 2C), suggesting acyl glucuronides. Peak 1 also displayed lability when exposed to acidic conditions. This was accentuated in the presence of diethyl ether (data not shown). DMN has only one carboxy group available for conjugation to form an acyl glucuronide; however, both peaks 1 and 4 were hydrolyzed under alkaline conditions, with corresponding increases in the areas of both peak 3 (tentatively identified above as DMN-S) and peak 5 (DMN). Based on their relative elutions in reverse-phase HPLC and their acid/base labilities, peaks 1 and 4 were tentatively assigned as 1) the acyl glucuronide-sulfate diconjugate DMN-AG-S and 2) the acyl glucuronide DMN-AG, respectively.

Figure 2D shows a chromatogram of bile after sequential exposure to alkaline conditions and incubation with β-glucuronidase. Peak 2 was completely removed from the chromatogram. Peak 5 (DMN) showed a corresponding increase, whereas peak 3 remained unchanged. Peak 2 had already been shown to be resistant to alkaline hydrolysis but did display lability when exposed to conditions of strong acid and heat. This peak was therefore tentatively assigned as the phenolic glucuronide DMN-PG.

**LC-MS/MS Identification of Biliary Metabolites.** The tentative identities assigned above to the metabolites corresponding to peaks 1 to 4 in Fig. 2B were confirmed by LC-MS/MS analysis. Individual preparative HPLC fractions were processed and infused into the LC-MS/MS system. Peak 1 was confirmed as the acyl glucuronide-sulfate diconjugate DMN-AG-S, indicated by a parent ion at m/z 391 and an ion at m/z 295, which corresponds to a fragment ion produced by the cleavage of the ester bond and the subsequent loss of the glucurononide moiety (Fig. 3). Figure 4 shows a molecular ion at m/z 391, which corresponds to a (phenolic) glucuronide of DMN (Fig. 2B, peak 2). This was confirmed by the presence of two more fragment ions in which the first ion at m/z 347 corresponds to that produced after the loss of CO₂; the second fragment ion at m/z 215 corresponds to the loss of the glucurononide moiety. This and the hydrolytic data confirmed the identity as DMN-PG. Figure 5 shows a molecular ion at m/z 295, corresponding to the sulfate conjugate DMN-S (Fig. 2B, peak 3). The fragment ions at m/z 250.5, 215, and 171 correspond to the fragments remaining after 1) the loss of CO₂, 2) the cleavage of the sulfate group and loss of SO₃, and 3) the loss of both the CO₂ and sulfate groups, respectively. Peak 4 was confirmed (Fig. 6) as the acyl glucuronide DMN-AG, with a molecular ion at m/z 391 and fragment ions at m/z 215, 193, and 171, which is indicative of the loss of 1) the glucurononide moiety, 2) the cleaved glucurononide moiety, and 3) the glucurononide moiety and CO₂, respectively.

**Plasma Pharmacokinetics.** Figure 7 shows that the concentration of DMN in plasma of conscious, bile-exteriorized rats declined rapidly after i.v. administration [half-life of 9.6 ± 2.0 min (n = 4; r² of regression data, 0.947–0.997); Table 1]. In contrast, the concentration...

**Fig. 5.** LC-MS/MS fragment ion spectra of DMN-S corresponding to peak 3 in Fig. 2.

**Fig. 6.** LC-MS/MS fragment ion spectra of DMN-AG corresponding to peak 4 in Fig. 2.
Both DMN and DMN-S had similar values for AUC₀–plateau between 20 and 45 min before declining in concentration. Of DMN-S, the only metabolite measurable in plasma, reached a peak by acyl glucuronidation to its acyl glucuronide conjugate and by glucuronidation to its glucuronide conjugate DMN-AG-S were recovered almost entirely in bile [with only very small amounts (below the detection limit of the assay) present in urine] and accounted for 10.3, 6.2, and 3.8% of the original dose, respectively (Table 2).

Recovery of DMN and Its Metabolites. The majority (56%) of the dose was recovered in urine as a combination of DMN-S (46%) and the parent drug DMN (9.5%; Table 2). A small amount of DMN-S was also excreted in bile and accounted for 2.9% of the dose. The glucuronidated conjugates DMN-AG, DMN-PG, and the novel diconjugate DMN-AG-S were recovered almost entirely in bile [with only very small amounts (below the detection limit of the assay) present in urine] and accounted for 10.3, 6.2, and 3.8% of the original dose, respectively (Table 2).

**Discussion**

The NSAID naproxen has been shown to be metabolized primarily by acyl glucuronidation to its acyl glucuronide conjugate and by O-dealkylation to DMN (Vanggard-Andersen and Hansen, 1992; Vree et al., 1993). DMN contains a carboxy and a phenolic group and, thus, forms an intermediate for further conjugation pathways (e.g., to the documented acyl glucuronide and sulfate conjugates; Fig. 1). A previous study from this laboratory with diflunisal (an NSAID already containing both carboxy and phenolic functional groups) revealed that, in addition to acyl glucuronide, phenolic glucuronide, and sulfate conjugates, novel diglucuronides (acyl glucuronide-phenolic glucuronide diconjugates) were formed (King and Dickinson, 1991). To explore further those previous observations, the present study investigated in detail the conjugation of DMN in rats, with particular reference to the potential formation of novel diglucuronides.

Bile samples from DMN-dosed rats revealed four major DMN-related peaks, two of which were identified as the acyl glucuronide (DMN-AG) and the sulfate (DMN-S) conjugates (identified previously as metabolites of DMN; Kiang et al., 1989; Vanggard-Anderson and Hansen, 1992; Vree et al., 1993). Another peak was shown to correspond to the phenolic glucuronide of DMN (DMN-PG). Although DMN-PG has been shown to be formed in enzymatic synthesis catalyzed by rabbit microsomes (Vanggard-Anderson and Hansen, 1992), this is the first time DMN-PG has been detected in an in vivo experiment. A highly polar diconjugate of DMN was indeed found in rat bile. However, this was identified as an acyl glucuronide-sulfate diconjugate of DMN (DMN-AG-S). No evidence was found for the formation of significant amounts of a diglucuronide metabolite of DMN.

The formation of the DMN-AG-S diconjugate raises questions about the metabolic necessity for diconjugation. Both initial sulfonation and subsequent glucuronidation, or alternatively initial glucuronidation followed by further phase II metabolism of compounds, have been reported. In a recent publication, Mutlib and colleagues (1999) showed that a metabolite of the human immunodeficiency virus reverse transcriptase inhibitor efavirenz was first sulfonated and subsequently glucuronidated to a glucuronide-sulfate diconjugate. Evidence that sulfate conjugates can serve as substrates for further metabolism has also been previously reported for the sulfonated steroid estradiol, which is further hydroxylated (Watanabe and Yoshizawa, 1982; Watanabe et al., 1991). Glucuronidated compounds have also been shown to undergo further metabolic modifications; for example, Tang and Abbott (1996) demonstrated that the glucuronide of the 2,4-diene metabolite of valproic acid undergoes further conjugation with glutathione to form a glucuronide-glutathione diconjugate. The detailed pathway(s) of formation of DMN-AG-S will be the subject of further investigations.

The intrahepatic disposition of DMN is particularly interesting since the acyl glucuronide and sulfate moieties of DMN are being conjugated in different compartments of the hepatocyte. Although

**Table 1**

**Pharmacokinetic parameters for DMN and DMN-S after i.v. administration of DMN to conscious bile-exteriorized rats**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DMN</th>
<th>DMN-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>t₁/₂ (min)</td>
<td>9.6 ± 2.0</td>
<td>N.D.</td>
</tr>
<tr>
<td>AUC₀–₅₅ (μg ⋅ h/ml)</td>
<td>17.1 ± 4.7</td>
<td>15.3 ± 1.9</td>
</tr>
</tbody>
</table>

N.D., not determined.

**Table 2**

**Biliary and urinary recoveries of DMN and metabolites in conscious bile-exteriorised rats after i.v. administration of DMN**

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Recovery of the Administered Dose as:</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMN-AG-S</td>
<td>DMN-PG</td>
</tr>
<tr>
<td>Bile</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–1 h</td>
<td>2.57 ± 2.4</td>
<td>5.47 ± 2.2</td>
</tr>
<tr>
<td>1–2 h</td>
<td>1.22 ± 0.7</td>
<td>0.71 ± 0.4</td>
</tr>
<tr>
<td>2–6 h</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>0–6 h</td>
<td>3.80 ± 3.1</td>
<td>6.19 ± 2.4</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–6 h</td>
<td>&lt;</td>
<td>&lt;</td>
</tr>
<tr>
<td>Total Bile + Urine</td>
<td>3.80 ± 3.1</td>
<td>6.19 ± 2.4</td>
</tr>
</tbody>
</table>

<, below the detection limit of the assay.
glucuronides are formed by membrane-bound UDP glucuronosyl transferases in the lumen of the hepatic endoplasmic reticulum, sulfonation takes place in the cytosol, catalyzed by soluble sulfotransferases (Fulceri et al., 1994; Logusch et al., 1999). The discovery of the novel DMN-AG-S diconjugate will allow further exploration of the mechanisms and intrahepatic transport processes involved in the formation of diconjugates.

As stated earlier, a previous study on diflunisal in this laboratory identified a mixture of diglucuronides comprised of the phenolic glucuronides of the 2-, 3-, and possibly 4-O-positional isomers of diflunisal acyl glucuronide (called diglucuronides of diflunisal). However, no strong evidence was found in those studies for formation of the real diglucuronide of diflunisal (i.e., phenolic glucuronidation of the biosynthetic 1-O-acyl glucuronide itself). Likewise, the phenolic glucuronide of diflunisal did not undergo acyl glucuronidation (King and Dickinson, 1991) when dosed to rats. In contrast, the acyl glucuronide moiety of the DMN-AG-S diconjugate identified in the present study appears to be predominantly in the form of the biosynthetic 1-O-β-linked glucuronide rather than in the form of rearrangement isomers. Evidence supporting this conclusion comes from the observations that 1) no rearrangement isomers of DMN-AG or DMN-AG-S metabolites were detected in rat bile samples [in a separate experiment the retention times of the peaks of rearrangement isomers were determined (data not shown)] and 2) when a bile sample from a DMN-dosed rat was exposed to mild alkaline conditions at 37 °C, both DMN-AG and DMN-AG-S could be rearranged into their respective isomers, as indicated by the disappearance of the parent peaks and the concurrent appearance of peak multiplets [corresponding to their rearrangement isomers (data not shown)]. The latter observation is significant since it is known that acyl migration between the 2-, 3-, and 4-O-positional isomers is reversible; however, the parent acyl glucuronide is not reformed.

Approximately 80% of the DMN dose was recovered in urine and bile after i.v. administration, with urinary excretion of DMN-S and unchanged DMN accounting for 46 and 10% of the dose, respectively. The total biliary excretion of the DMN metabolites accounted for approximately 24% of the dose, which is comparable to our earlier findings for diflunisal (Dickinson et al., 1989). The high urinary recovery of DMN-S compares with the approximate 87% recovery as DMN-S in rats dosed with naproxen (Sugawara et al., 1978).

DMN-S was the only metabolite measurable in plasma. It was present in appreciable concentrations at 90 min, long after the parent DMN had declined to nonmeasurable concentrations. Slow renal clearance of sulfonated metabolites has been reported previously for the sulfate metabolite of hydroxytriamterene, which achieved plasma concentrations in humans approximately 10 times greater than those of the parent drug (Hasegawa et al., 1982; Jacob et al., 2000). The reason for the decreased renal clearance may be the high plasma protein binding of the sulfate conjugate (Hasegawa et al., 1982).

The identification of a diconjugate of DMN, taken together with (limited) earlier work documenting diconjugation of small drug metabolites, points to the possibility of metabolic processes little explored in the past. In particular, such studies may offer insights into intrahepatic processes of recognition, transport, and metabolism of polar species.

**References**


