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STUDIES ON THE REACTIVITY OF ACYL GLUCURONIDES—I

PHENOLIC GLUCURONIDATION OF ISOMERS OF DIFLUNISAL ACYL GLUCURONIDE IN THE RAT

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Abstract—Diflunisal (DF) is metabolized primarily to its acyl glucuronide (DAG), phenolic glucuronide (DPG) and sulphate (DS) conjugates. Whereas DPG and DS are stable at physiological pH, DAG is unstable, undergoing hydrolysis (regeneration of DF) and rearrangement (intramolecular acyl migration to the 2-, 3- and 4-*O*-acyl-positional isomers). We have compared the *in vivo* disposition of DAG with that of an equimolar mixture of its three isomers after i.v. administration at 10 mg DF equivalents/kg to conscious, bile-exteriorized rats. After dosing with DAG, excretion in urine and bile (46% as DAG), hydrolysis (as assessed by recovery of 9% DPG and 8% DS resulting from reconjugation of liberated DF) and rearrangement (17% recovery as isomers of DAG) were important pathways. Highly polar metabolites excreted almost exclusively in bile and accounting for 13% of the dose were identified as an approximate 4:1 mixture of the 2- and 3-*O*-isomers of DAG which had been glucuronidated at the phenolic function of the salicylate ring i.e. "diglucuronides" of DF. Evidence for trace quantities only of the phenolic glucuronides of the 4-*O*-isomer of DAG, and of DAG itself, was found. After dosing rats with an equimolar mixture of the isomers, 52% was recovered (as the isomers) in urine and bile in 6 hr. Hydrolysis was less important — <3% (total) of the dose was recovered as DPG and DS. The phenolic glucuronides of the 2- and 3-*O*-isomers (ratio *ca.* 3:7) accounted for 37%. Evidence for appreciable formation of the phenolic glucuronide of the 4-*O*-isomer was not found. In one rat dosed with DPG, there was no evidence for further glucuronidation of the salicylate ring at its carboxy function. The data suggest that the 2- and 3-*O*-isomers of DAG, but not the 4-*O*-isomer, DAG itself or DPG, are good substrates for further glucuronidation.

Diflunisal (DF†, Fig. 1) is a difluorophenyl derivative of salicylic acid which is metabolized in humans and rats primarily by direct conjugation to its acyl glucuronide (DAG), phenolic glucuronide (DPG) and sulphate (DS) conjugates [1–4]. In rats, the glucuronides are excreted both in bile and urine, whereas DS is excreted almost exclusively in urine [4]. These original studies in rats also revealed unidentified, highly polar metabolites of DF — a single product excreted in urine and group of products excreted in bile [5]. We have subsequently identified the urinary product as a conjugate, probably a sulphate, of the 3-hydroxy derivative of DF [6]. This Phase 1 hydroxylation of DF occurs as a minor pathway in both humans and rats, though it is considerably enhanced in the homozygous Gunn rat [7].

Our special interest in DF disposition, however, has been in elucidating the *in vivo* fate of its reactive acyl glucuronide metabolite, DAG. As a class, acyl glucuronides have been shown over the past 10 years to be potentially reactive conjugates, capable of undergoing hydrolysis (regeneration of aglycone), intramolecular rearrangement (isomerization via acyl migration) and intermolecular reactions with small nucleophiles and proteins [8–12]. In the rearrangement reaction (Fig. 2), acyl group migration to an adjacent hydroxy group on the glucuronic acid ring forms the 2-, 3- and 4-*O*-acyl-positional isomers, which, unlike the parent acyl glucuronide itself, are resistant to hydrolysis by β-gluicuronidases. The migrations between the three positional isomers (which are not products of biosynthesis and therefore technically not glucuronides) are reversible, but the parent acyl glucuronide appears not to be reformed.

DAG has been shown to undergo hydrolysis, rearrangement and covalent bonding reactions to plasma protein *in vitro* [5, 13–15]. We have also

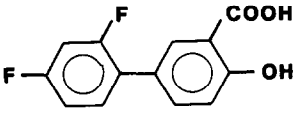

Fig. 1. Chemical structure of DF.

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† Abbreviations: DF, diflunisal; DAG, diflunisal acyl glucuronide; DPG, diflunisal phenolic glucuronide; DS, diflunisal sulphate; *iso*-DAG, mixture of 2-, 3- and 4-*O*-acyl isomers of diflunisal acyl glucuronide formed by acyl migration; D-2G, mixture of the phenolic glucuronides of diflunisal acyl glucuronide and its isomers; CA, clofibric acid; CL, total plasma clearance.

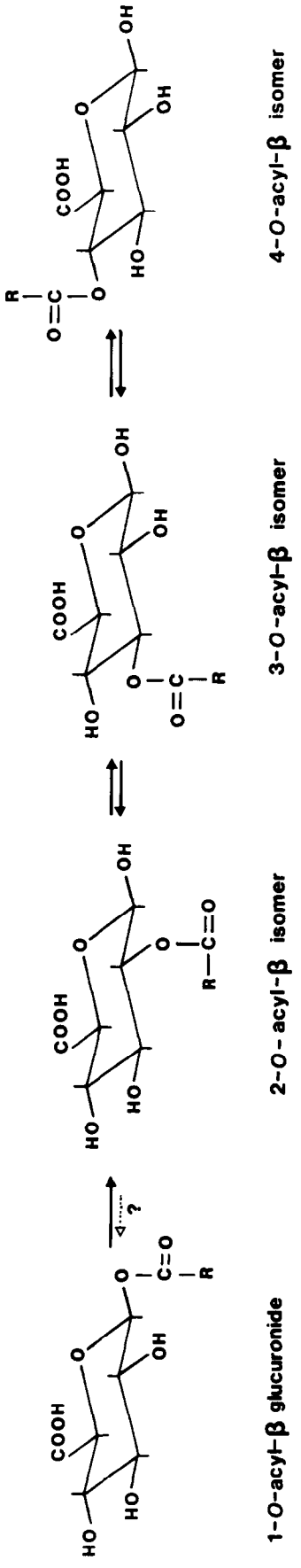

Fig. 2. Scheme showing rearrangement of biosynthetic, β-glucuronidase-susceptible 1-*O*-acyl-β glucuronides to the β-glucuronidase-resistant 2-, 3- and 4-*O*-acyl positional isomers.

demonstrated the occurrence of these reactions in rats *in vivo* [15–17], though the covalent bonding reaction appears to be a quantitatively minor pathway (both *in vitro* and *in vivo*). Rearrangement of DAG to yield the 2-, 3- and 4-*O*-acyl positional isomers (*iso*-DAG), each of which always presents chromatographically as the C-1 α- and β-anomer pair [5, 18]) is the predominant primary reaction *in vitro* [5, 15]. By contrast, systemic hydrolysis, presumably mediated by esterases, is the predominant initial pathway of DAG disposition *in vivo* [16, 17]. Rearrangement to *iso*-DAG also occurs *in vivo*, with this pathway of DAG disposition becoming more apparent when biliary and/or urinary excretion routes are blocked [16, 17].

The present investigation was undertaken to determine the *in vivo* disposition of *iso*-DAG by i.v. administration of both DAG and *iso*-DAG (as an equimolar mixture of the 2-, 3- and 4-*O*-isomers) to rats. The study shows that the hitherto unidentified, highly polar DF metabolites found earlier in bile from DF-dosed rats are produced ultimately from *iso*-DAG by metabolic attachment of a second glucuronic acid group to the phenolic function of the salicylate ring.

MATERIALS AND METHODS

Materials and animals. DF was a gift from Merck, Sharp and Dohme (Australia) Pty Ltd (Sydney, Australia). Authentic samples of DAG, DPG and DS used as analytical standards were kindly provided by Dr R. K. Verbeeck, Université Catholique de Louvain (Brussels, Belgium). CA was a gift from ICI Pharmaceuticals Division (Macclesfield, U.K.). β-Glucuronidases (EC 3.2.1.31, type H2 from *Helix pomatia* and type VII from *Escherichia coli*) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Methanol and acetonitrile (HPLC grade) and diethyl ether and ethyl acetate (AR grade) were purchased from Mallinckrodt (Melbourne, Australia). 1-Chlorobutane (glass-distilled) was purchased from Burdick and Jackson (Muskegon, MI, U.S.A.). Other chemicals and solvents were AR grade. Pentobarbitone sodium solution (Nembutal®) was purchased from Ceva Chemicals (Sydney, Australia). Male Sprague–Dawley-derived rats (300–350 g) were obtained from The University of Queensland Medical Faculty Animal House. Experiments were approved by the University's Animal Experimentation Ethics Committee.

Preparation of doses. DAG and DPG were isolated, as described previously [17], from the urine of a male volunteer who ingested four 500-mg doses of DF (Dolobid®, Merck Sharp and Dohme) over 2 days (approved by The University of Queensland Human Experimentation Ethics Committee). Dosing solutions at *ca.* 2 mg DF equivalents/mL saline (adjusted to pH 6) were calibrated using the analytical methodology described elsewhere [5]. The DAG dosing solution contained < 2% as isomers, < 1% as DF and no measurable DPG. The DPG solution contained no other measurable DF-related species.

Iso-DAG was obtained by isomerization of DAG

under slightly alkaline conditions prior to purification. Thus, urine was collected for 48 hr from two male volunteers who ingested four 500-mg doses of DF (Dolobid®) over 2 days, and evaporated at 40° under reduced pressure (rotary evaporator) to *ca.* 10% of volume. The pH of the concentrate was adjusted to 8.5 with 2 M NaOH, followed by incubation at 40° for 4 hr. The course of rearrangement and hydrolysis of DAG during incubation was monitored by analytical HPLC [5]. The pH was then adjusted to 1.5 using 10 M HCl and three extractions with two volumes of diethyl ether were carried out. The combined extracts were evaporated at room temperature and the residue was taken up in water (*ca.* 4 mL) and washed three times with 1-chlorobutane (10 mL). Aliquots (100 μL) were injected into the HPLC system using the equipment and conditions described previously [5], except that a μBondapak C_{18} semipreparative column (Waters Associates, Milford, MA, U.S.A.) was used at a flow rate of 3.5 mL/min with UV monitoring at 280 nm. Fractions corresponding to the α and β anomers of the 2-*O*-acyl, 3-*O*-acyl and 4-*O*-acyl isomers of DAG (i.e. six fractions) were collected over ice and stored at −20°. Na_2SO_4 present in the mobile phase precipitated during this cooling. The supernatants were decanted and the methanol removed at 40° under reduced pressure on a rotary evaporator. Evaporation was continued until the isomers began to precipitate. The suspensions were then cooled on ice. The isomers precipitated as slightly pink powders, which were filtered, washed with the minimum quantity of ice-water, and dried over anhydrous $CaCl_2$ in a vacuum desiccator (*ca.* 30-mg yield of each isomer). Solutions of each isomer were prepared at *ca.* 2 mg DF equivalents/mL saline (adjusted to pH 6) and calibrated as DF after alkaline hydrolysis, as described previously for DAG itself [5]. The *iso*-DAG dosing solution was prepared by mixing the individual solutions prepared above to give an approximately equimolar mixture of the 2-, 3- and 4-*O*-acyl isomers of DAG at a final concentration of 2-mg DF equivalents/mL. The solution contained no measurable DAG or DPG, and <3% DF.

pH-Dependent anomerization and acyl migration. Volumes of 0.1 M H_3PO_4, 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 solutions were mixed as required to give solutions at pH 3, 5, 7.4 and 8.5. Small quantities (100–400 μg) of the later-eluting anomer (putatively the α-anomer [5, 12]) of 3-*O*-acyl isomer of DAG were added to 200-μL aliquots of the prepared phosphate buffers, dissolved by vortex mixing (20 sec) and incubated at room temperature (22–24°). Samples (50 μL) drawn after 1 and 50 min were immediately injected into the analytical HPLC [5].

i.v. Administration of DAG, iso-DAG and DPG at 10 mg DF equivalents/kg. Rats were prepared, under ether anaesthesia, with catheters in the right external jugular vein and bile duct as described previously [19], and then placed unrestrained in metabolism cages and allowed to recover for 1–3 hr before administration of the dose. No food was provided. Water was available *ad lib.* but, in addition, volumes of saline equivalent to the volume of bile secreted were infused i.v. at regular intervals to prevent dehydration. The prepared dosing solutions of DAG, *iso*-DAG and DPG were given as a bolus (10 mg DF equivalents/kg) over 1 min, with the midpoint being designated time zero. Blood samples (150 μL) were drawn pre-dose and at 2.5, 5, 10, 15, 20, 30 and 45 min, and 1, 1.5, 2, 2.5, 3, 4, 5 and 6 hr after dosing. Heparinized saline (150 μL) was infused into the jugular vein after each sampling. Blood samples were immediately centrifuged and 50 μL aliquots of plasma transferred to 1-mL vials and snap frozen over dry ice. Bile was collected over ice into prepared vessels containing 1 M acetic acid (500 μL) with frequent swirling to achieve mild acidification (pH 3.5–5). Bile samples (pre-dose and 0–2, 2–4 and 4–6 hr post-dose) were frozen until analysis. Urine samples (pre-dose and 0–6 hr post-dose) were collected over dry ice and stored frozen. On thawing for analysis, 1 M acetic acid (500–1000 μL) was added to give a pH of 3.5–5. At completion of the experiments at 6 hr, the rats were killed by overdose with i.v. pentobarbitone. The contents of the bladder were aspirated and added to the 0–6-hr voidings. Doses of DAG and *iso*-DAG were each given to five rats; a dose of DPG was given to one rat only.

Isolation and identification of D-2G. Portions (0.6 mL) of the 0–2-hr bile collections from two rats dosed with *iso*-DAG were combined. Similar 1.2-mL samples of the 0–2-hr bile from two DAG-dosed rats and of pre-dose (control) bile from two rats were prepared. To each was added 1 M H_3PO_4 (0.6 mL) and the mixture was extracted three times with ethyl acetate (3 mL). The combined extracts were evaporated under a stream of air at room temperature and the residue reconstituted in 300 μL of mobile phase. Aliquots (50 μL) were injected into the analytical HPLC system [5] using a flow rate of 1 mL/min. Fractions eluting at the D-2G retention time were collected and stored on ice whereupon Na_2SO_4 present in the mobile phase precipitated. After centrifugation and decantation, the supernatant was evaporated at room temperature under a stream of air to *ca.* 25% of volume. The resultant aqueous samples (*ca.* 0.6 mL, pH 3–4) were stored at −20°.

For the following tests, 40-μL samples of the above solutions were incubated at 37° for 10 min: (a) in 40 μL 0.1 M NaH_2PO_4/Na_2HPO_4, final pH *ca.* 5; (b) in 40 μL of 0.1 M NaH_2PO_4/Na_2HPO_4 containing 400 units of β-glucuronidase (Type H2, from *H. pomatia*, also contains sulphatase activity), final pH *ca.* 5; or (c) with 40 μL of 0.1 M NaH_2PO_4/Na_2HPO_4 containing 250 units of sulphatase-free β-glucuronidase (type VII, from *E. coli*), final pH *ca.* 7. On completion of incubation, internal standard solution (100 μg clofibric acid/mL acetonitrile, 80 μL) was added with vortex mixing. After centrifugation, a 50-μL aliquot of the supernatant was injected into the analytical HPLC system. Aliquots (40 μL) of the bile extracts were also subjected to hydrolysis by heating at 65° for 2 hr with: (a) 5 μL of 10 M NaOH, final pH *ca.* 12; or (b) 10 μL 10 M HCl, final pH < 1. The pH was then adjusted to *ca.* 5 using 10 M HCl or NaOH solutions as appropriate, and the final volume brought to 80 μL using 0.1 M NaH_2PO_4/Na_2HPO_4, pH 5. Internal standard solution (80 μL) was added with vortex mixing. Following centrifugation, a 50-μL aliquot was injected into the HPLC system.

Analyses. DF and its conjugates DAG, DPG and

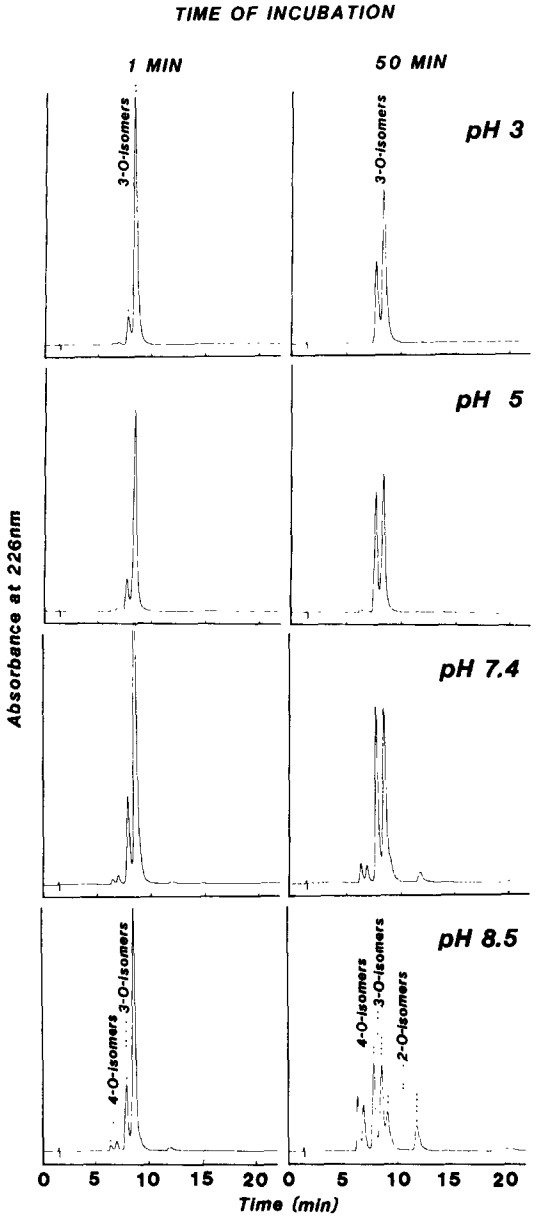

Fig. 3. Reversed-phase HPLC chromatograms of the α-anomer of the 3-*O*-acyl positional isomer of DAG after incubation at pH 3, 5, 7.4 and 8.5 for 1 and 50 min.

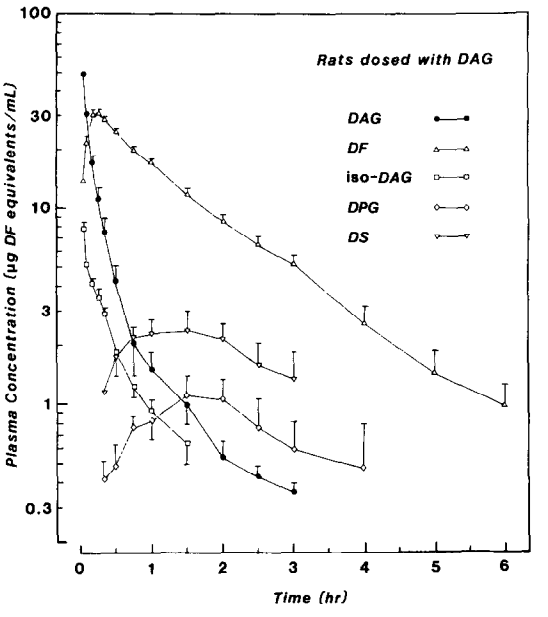

Fig. 4. Plasma concentration–time profiles for DAG (●), *iso*-DAG (□), DF (△), DPG (◇) and DS (▽) after i.v. administration of DAG at 10 mg DF equivalents/kg to conscious, bile-exteriorized rats. Results are means ± SE for five rats.

DS, in plasma, urine and bile samples were analysed using a direct, isocratic HPLC procedure described in detail elsewhere [5]. This procedure also separates the α- and β-anomers of the 2-, 3- and 4-*O*-acyl isomers of DAG. Using the solutions of the purified individual isomers (above) and calibrating as DF after alkaline hydrolysis, it was shown that the isomers had the same molar extinction at 226 nm (the analytical wavelength) as DAG itself. Thus, they could be assayed directly by applying the DAG standard curve.

The new metabolites D-2G in bile and urine samples were quantified collectively as DPG after alkaline hydrolysis, i.e. as the difference in DPG content of the samples before [5] and after alkaline hydrolysis. (This approach also gives a second quantification of *iso*-DAG in the samples, i.e. as the difference between the sum of directly measured DF and DAG versus total DF arising from the sum of DF, DAG and *iso*-DAG after alkaline hydrolysis.) For the hydrolysis procedure, bile or urine (80 µL) was mixed with NaOH (20 µL) and incubated at 65° for 2 hr. After cooling, 2 M HCl (20 µL), 0.1 M NaH_2PO_4/Na_2HPO_4 pH 5 (40 µL) and internal standard solution (200 µL of 100 µg clofibric acid/mL acetonitrile) were added. The samples were vortex mixed (1 min), centrifuged and 20-µL aliquots of supernatant applied to the HPLC.

The isomer composition of D-2G was estimated as the difference in the content of the 2-, 3- and 4-*O*-acyl isomers of DAG in the bile samples before and after incubation with β-glucuronidase. For the enzyme hydrolysis procedure, 0–2-hr bile samples (50 µL) from DAG- and *iso*-DAG-dosed rats were incubated with 150 µL 0.1 M NaH_2PO_4/Na_2HPO_4 pH 5 containing 400 units of β-glucuronidase (Type H-2, *H. pomatia*) at 37° for 30 min. Internal standard solution (200 µL of 100 µg clofibric acid/mL acetonitrile) was then added, followed by vortex mixing and centrifugation. A 20-µL aliquot was applied to the HPLC.

Data analysis. Apparent plasma half-life ($T_\frac{1}{2}$) was determined from the slope (k_e) of the terminal linear portion of the log plasma concentration–time profile. CL was calculated as the quotient of dose and area under the plasma concentration–time curve from 0 to ∞ ($AUC_{0-\infty} = AUC_{0-last}$ by trapezoidal rule

Table 1. Pharmacokinetic parameters for DF, DAG and *iso*-DAG after i.v. administration of DAG or *iso*-DAG to conscious bile-exteriorized rats*

	Rats dosed with DAG (N = 5)	Rats dosed with *iso*-DAG (N = 4)
$AUC_{0-\infty}$ (μg.hr/mL)		
DAG	13.2 ± 1.0	—
iso-DAG	3.8 ± 0.4	16.4 ± 1.3
DF	50.9 ± 3.8	10.8 ± 0.8
CL (mL/min per kg)		
DAG	12.9 ± 0.9	—
iso-DAG	—	10.4 ± 0.8
$T_{\frac{1}{2}}$† (hr)		
DF	1.22 ± 0.10	1.22 ± 0.17

* The dose was 10 mg DF equivalents/kg; results are means ± SE.
† Calculated from the measured concentrations after 1 hr.

Table 2. Biliary and urinary recovery of DF and its metabolites in conscious bile-exteriorized rats after i.v. administration of DAG or *iso*-DAG*

	% Recovery of the administered dose as:						
Sample	DAG	*iso*-DAG	D-2G	DPG	DS	DF	TOTAL
Rats dosed with DAG							
Bile 0–2 hr	31.4 ± 3.7	10.1 ± 1.9	9.0 ± 0.9	3.0 ± 0.4	0.2 ± 0.1	0.7 ± 0.2	54.4 ± 5.3
Bile 2–4 hr	2.3 ± 0.3	1.5 ± 0.5	1.9 ± 0.4	1.0 ± 0.1	0.1 ± 0.1	<†	6.8 ± 0.8
Bile 4–6 hr	0.5 ± 0.1	0.6 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	<	<	2.2 ± 0.3
Bile 0–6 hr	34.2 ± 4.0	12.2 ± 2.5	11.5 ± 1.0	4.5 ± 0.4	0.3 ± 0.1	0.7 ± 0.2	63.4 ± 6.1
Urine 0–6 hr	11.6 ± 2.6	4.5 ± 1.5	1.0 ± 0.5	4.9 ± 0.9	8.1 ± 1.5	4.1 ± 1.2	34.1 ± 3.6
Total bile + urine	45.8 ± 3.0	16.7 ± 1.1	12.5 ± 1.5	9.4 ± 0.8	8.4 ± 1.5	4.8 ± 1.3	97.5 ± 4.5
Rats dosed with *iso*-DAG							
Bile 0–2 hr	<	36.8 ± 4.6	35.2 ± 3.0	0.6 ± 0.2	<	0.1 ± 0.1	72.7 ± 1.7
Bile 2–4 hr	<	0.6 ± 0.5	0.7 ± 0.4	0.2 ± 0.1	<	<	1.5 ± 0.9
Bile 4–6 hr	<	<	<	<	<	<	0.1 ± 0.1
Bile 0–6 hr	<	37.4 ± 4.5	36.0 ± 3.4	0.7 ± 0.2	<	0.1 ± 0.1	74.2 ± 1.5
Urine 0–6 hr	<	14.8 ± 1.8	0.6 ± 0.4	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.2	18.5 ± 2.1
Total bile + urine	<	52.3 ± 4.7	36.5 ± 3.7	1.7 ± 0.3	1.1 ± 0.1	1.1 ± 0.2	92.7 ± 2.3

* The dose was 10 mg DF equivalents/kg; results are means ± SE, N = 5.
† Below the limit of the assay; in rats dosed with *iso*-DAG, the small amounts of DAG were not resolved from the relatively large amounts of *iso*-DAG.

integration + $AUC_{last-\infty}$ as the quotient of the last measured plasma concentration and k_e). Apparent volume of distribution (V_d) was calculated as the quotient of CL and k_e.

RESULTS

pH-Dependent anomerization and acyl migration

The effect of pH on both C-1 anomerization and isomerization via acyl group migration, using the later-eluting anomer (putatively the α anomer [5]) of the 3-*O*-acyl isomer of DAG as starting material, is shown in Fig. 3. Anomerization occurred at all pH values studied (3–8.5), with the rate increasing with pH. By contrast, acyl migration was inhibited under acidic conditions but was able to occur under slightly alkaline conditions as shown by formation of the α- and β-anomers of the 2- and 4-*O*-acyl isomers. Some hydrolysis at pH 8.5 was indicated by the appearance of a small DF peak at 21 min (Fig. 3).

Intravenous administration of DAG, iso-DAG and DPG to rats

After i.v. administration of DAG at 10 mg DF equivalents/kg to conscious bile-exteriorized rats, DAG concentrations in plasma declined rapidly (Fig. 4). CL was calculated as 12.9 mL/min/kg (Table 1), using linear regression of the last three or four measured plasma concentrations to estimate the terminal phase. DF, generated by systemic hydrolysis, achieved peak plasma concentrations of *ca.* 30 μg/mL at 10–15 min; its elimination from plasma was essentially linear thereafter ($T_{\frac{1}{2}}$ 1.22 hr, Table 1). DPG and DS, formed by conjugation of DF, were easily measurable in plasma. Isomerization of DAG by systemic acyl migration gave plasma profiles of *iso*-DAG (measured as the sum of the 2-, 3- and 4-*O*-isomers) which were roughly parallel to those of DAG. Recovery of DAG and its metabolites in 6-hr bile and urine samples was almost complete (Table 2). Of the dose, 46% was recovered as DAG itself,

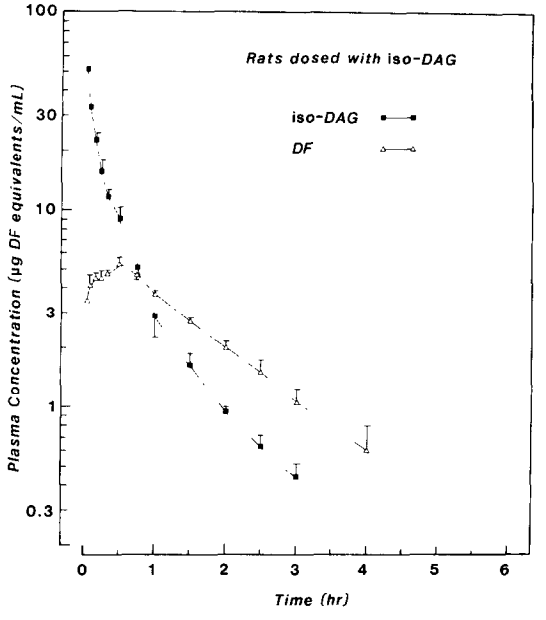

Fig. 5. Plasma concentration–time profiles for *iso*-DAG (■) and DF (△) after i.v. administration of *iso*-DAG at 10 mg DF equivalents/kg to conscious, bile-exteriorized rats. Results are means ± SE for four rats.

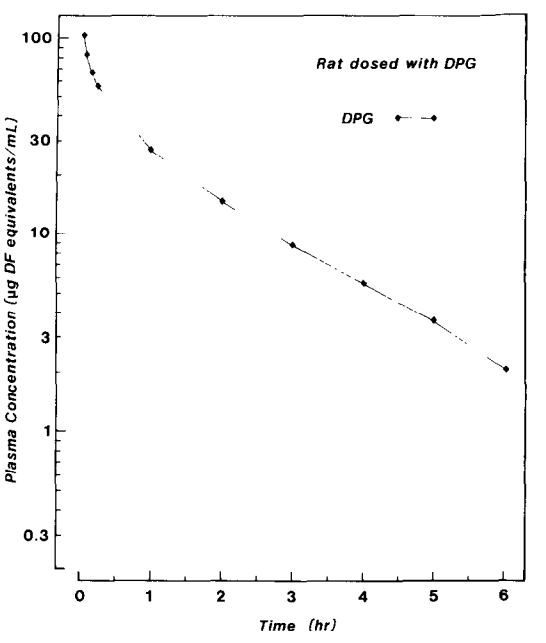

Fig. 6. Plasma concentration–time profile for DPG (◆) after i.v. administration of DPG at 10 mg DF equivalents/kg. Results are from one rat only.

mainly in bile. This figure comprises a portion resulting from regeneration of DAG via systemic cycling (DAG hydrolysis → DF; DF conjugation → DAG, DPG and DS) and that portion of the DAG dose which survived systemic hydrolysis and was excreted intact. DPG and DS, systemically stable conjugates of DF [17, 20], accounted for 9.4 and 8.4% of the dose whilst *iso*-DAG accounted for 16.7%. A hitherto unidentified group of highly polar "diglucuronides" (D-2G, discussed later), accounting for 12.5% of the dose, was excreted almost exclusively in bile (Table 2).

After dosing another group of rats with *iso*-DAG (as an approximately equimolar mixture of the 2-, 3- and 4-*O*-acyl isomers) at 10 mg DF equivalents/kg, *iso*-DAG was eliminated rapidly from plasma (Fig. 5, CL 10.4 mL/min/kg, Table 1). Systemic hydrolysis to DF also occurred, although the peak concentrations (5 μg DF/mL plasma) were much lower than after dosing with DAG and the concentrations of resultant DAG, DPG and DS in plasma unmeasurable. Of the *iso*-DAG dose, 52% was recovered as such in 6 hr: 37% in bile and 15% in urine (Table 2). The relative quantitative unimportance of systemic hydrolysis of *iso*-DAG was underlined by recovery of only 1.7 and 1.1% of the dose as DPG and DS, respectively. The small amounts of DAG present in urine and bile could not be measured separately in the presence of large amounts of *iso*-DAG. Recovery of the new metabolites, D-2G (36.5%, Table 2), was much greater than after dosing with DAG.

After dosing one rat with DPG i.v. (Fig. 6), no other DF-related species were observed in plasma, urine or bile. Pharmacokinetic parameters for this conjugate are shown in Table 3. Only 80% of the dose was recovered in 6 hr: 59% in urine and 21% in bile.

Table 3. Pharmacokinetic parameters for and recovery of DPG after its i.v. administration to a conscious, bile-exteriorized rat at 10 mg DF equivalents/kg

$AUC_{0-\infty}$ (μg.hr/mL)	104
CL (mL/min per kg)	1.6
$T_{\frac{1}{2}}$* (hr)	1.4
V_d (L/kg)	0.20
Recovery (% as DPG)	
Bile 0–2 hr	17.9
Bile 2–4 hr	2.5
Bile 4–6 hr	0.8
Bile 0–6 hr	21.2
Urine 0–6 hr	59.4
Total bile + urine	80.6

* Calculated from the measured concentrations after 2 hr.

Isolation, identification and quantification of D-2G

A group of non-resolved peaks, eluting very early on reversed-phase HPLC, was observed in chromatograms of bile from rats dosed with DAG and *iso*-DAG, but not DPG. Fig. 7A shows chromatograms of pre-dose and 0–2-hr bile from an *iso*-DAG-dosed rat. In the latter chromatogram are peaks corresponding to the pairs of anomers of the 2-, 3- and 4-*O*-isomers (part of the *iso*-DAG dose excreted unchanged in bile), DPG (from conjugation of DF liberated from *iso*-DAG by systemic hydrolysis) and the new metabolic products, D-2G. Pilot

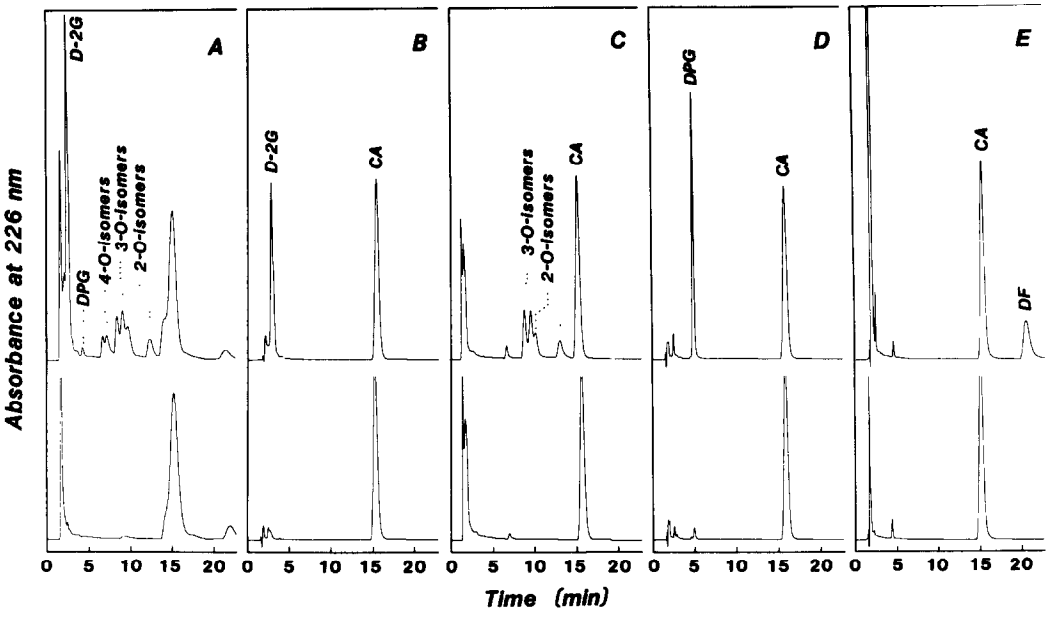

Fig. 7. Reversed-phase HPLC chromatograms of: (A) upper panel, unpurified bile from a rat dosed i.v. with *iso*-DAG as an equimolar mixture of the 2-, 3- and 4-*O*-acyl positional isomers showing D-2G and DPG, as well as the isomers; lower panel, unpurified pre-dose bile; (B) D-2G partially purified by extraction and preparative HPLC with CA internal standard; (C) partially purified D-2G after incubation with β-glucuronidase from *H. pomatia* at pH 5 at 37° for 10 min; (D) partially purified D-2G after hydrolysis at pH 12 at 65° for 2 hr; (E) partially purified D-2G after hydrolysis at pH <1 at 65° for 2 hr. The lower panels of B–E are equivalent incubations of an equivalent extract of pre-dose bile.

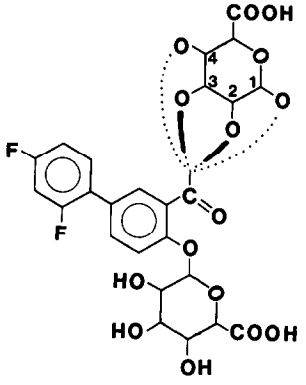

Fig. 8. Proposed structures of the group of highly polar metabolites found in bile. The mixture comprises predominantly the phenolic glucuronides of the 2- and 3-*O*-acyl isomers of DAG; only trace quantities of the phenolic glucuronides of the 4-*O*-isomer and of DAG itself were found.

experiments on the identity of D-2G by various hydrolyses were carried out directly on bile samples. Subsequently, D-2G was partially purified by acidification and extraction of the bile, followed by preparative HPLC using the analytical column. Chromatograms of the sample of D-2G thus prepared from the 0–2-hr bile of *iso*-DAG-dosed rats, and of an equivalent preparation from pre-dose bile, are shown in Fig. 7B.

Incubation of this partially purified D-2G with β-glucuronidase/sulphatase (from *H. pomatia*) at pH 5 for 10 min produced primarily the 2- and 3-*O*-isomers of DAG (Fig. 7C); trace quantities of the 4-*O*-isomer and DF were apparent on signal attenuation. Similar results were obtained using sulphatase-free β-glucuronidase (from *E. coli*) at pH 7 (not shown). These products of enzyme hydrolysis could all be converted to DF by subsequent alkaline hydrolysis (not shown). Direct alkaline hydrolysis of the D-2G preparation yielded DPG (Fig. 7D), which could be converted to DF by subsequent hydrolysis with β-glucuronidase or acid catalysts (not shown). Finally, D-2G was converted directly to DF by heating in acid (Fig. 7E). Thus, the partially purified D-2G from *iso*-DAG-dosed rats was shown to be a mixture of the phenolic glucuronides of the 2- and 3-*O*-isomers of DAG, and perhaps of the 4-*O*-isomer and DAG itself; i.e. "diglucuronides" of DF (Fig. 8).

Fig. 7C suggests that this sample of D-2G, partially purified from the bile of *iso*-DAG-dosed rats by extraction and semi-preparative HPLC, is composed primarily of the phenolic glucuronides of the 2- and 3-*O*-isomers of DAG. A similar result was obtained after β-glucuronidase treatment of partially purified D-2G obtained from dosing rats with DAG. The isomer composition of D-2G in the *unpurified* 0–2-hr bile samples, estimated as the difference in the 2-, 3- and 4-*O*-isomer content before and after hydrolysis with β-glucuronidase, was *ca.* 80:20:0, respectively, from DAG-dosed rats and *ca.* 28:70:2, respectively, from *iso*-DAG-dosed rats. Concurrent

estimates of the phenolic glucuronide of DAG itself as a component of D-2G in unpurified bile samples were not possible, because the bile samples already contained DPG and DAG which were also converted to DF with β-glucuronidase.

Whereas the foregoing hydrolyses of bile samples with β-glucuronidase provide information on the composition of D-2G, quantification was best achieved via alkaline hydrolysis to DPG. Thus, D-2G was reliably measured as the difference in DPG content of the samples before and after alkaline hydrolysis.

DISCUSSION

In previous studies on the disposition of DF in rats and humans [4, 16, 21–23], the analysis of plasma, bile and urine samples revealed DAG always to be accompanied by various amounts of its isomers formed by intramolecular acyl migration (Fig. 2). In such biological samples, stabilized as soon as possible after collection by mild acidification to pH 3.5–5, the 2-*O*-isomer was most abundant with the 3-*O*-isomer and sometimes the 4-*O*-isomer also apparent (i.e. abundance decreasing with the required number of migrations between adjacent hydroxyl groups). The isomers always presented as pairs of the C1 α- and β-anomers, and our earlier experiments [5] showed that anomerization occurred under acidic conditions inhibitory to acyl migration. However, Bradow *et al.* [12] reported that anomerization of the isomers of flufenamic acid acyl glucuronide did not occur if the samples were not exposed to pH <5. Thus, the spectre was raised that the α-anomers of isomers of DAG were artifacts arising from the acidic conditions used for stabilization, purification and analysis *in vitro*. As the primary goal of the present study was to trace the *in vivo* disposition of isomers of DAG, formed *in vivo* by rearrangement of DAG, it was mandatory to clarify the pH-dependency of the anomerization of isomers of DAG. Using the purified later-eluting anomer (putatively the α-anomer [5, 18]) of the 3-*O*-isomer as a model, it was established (Fig. 3) that both anomerization and acyl migration were facile reactions at physiological pH.

After dosing conscious, bile-exteriorized rats i.v. with DAG or *iso*-DAG (as an equimolar mixture of the 2-, 3- and 4-*O*-isomers, each as an anomer pair), direct excretion in urine and bile was, not surprisingly, a major pathway of disposition of these polar, acidic molecules. Thus 46 and 52% of the DAG and *iso*-DAG doses (respectively) were recovered as such in 6 hr, about $\frac{1}{3}$ in urine and $\frac{2}{3}$ in bile (Table 2). However, for DAG-dosed rats, this figure represents a substantial overestimate of the DAG actually excreted unchanged, because of systemic hydrolysis of DAG to DF (Fig. 4) and of the latter molecule's reconjugation to DAG, DPG and DS (i.e. systemic cycling). An earlier study targeted at this specific point [17] estimated that about 55% of DAG dosed i.v. to anaesthetized rats was hydrolysed to DF. Similar estimates for the present work were made: (a) by comparison of the $AUC_{0-\infty}$ values for plasma DF after dosage with DAG at 10 mg DF equivalents/kg with those after dosage with DF itself at 10 mg/kg [4]; and (b) by comparison of the values for total recovery of systemically stable DPG and DS after dosing with DAG and with DF. Values obtained for systemic hydrolysis of i.v. administered DAG were 49% (AUC method) and 47% (stable metabolite method). Applying the same methods to the plasma and recovery data after dosing with *iso*-DAG gave estimates of 8 and 11% (respectively) for systemic hydrolysis of *iso*-DAG to DF. Thus, the isomers were much more resistant to hydrolysis than DAG, a result in agreement with earlier work comparing the *in vivo* dispositions of valproic acid acyl glucuronide and its isomers in rats [24]. This result could suggest the participation of β-glucuronidase catalysts, as DAG is susceptible to these enzymes whereas the isomers are resistant [5, 14, 18]. However, DPG, also susceptible to β-glucuronidases, was quite stable after i.v. administration (Fig. 6 and Table 3). Esterases, rather than β-glucuronidases, have been implicated as the primary catalysts causing systemic hydrolysis of acyl glucuronides of clofibric acid [25, 26], diphenylacetic acid [27] and zomepirac [28].

After i.v. administration of DAG, an appreciable portion (*ca.* 17%) of the dose was recovered in bile and urine as *iso*-DAG. The relative abundance was always in the order 2-*O*-isomer > 3-*O*-isomer > 4-*O*-isomer and this accords with kinetic expectations of sequential acyl migration as the samples were stabilized by acidification as soon as possible after collection. After dosing with *iso*-DAG as an approximate equimolar measure of the 2-, 3- and 4-isomers, 52% was recovered as the isomers over 6 hr in urine and bile. In these samples, the 3-isomer was usually most abundant. It should be recalled that the migrations between adjacent hydroxy groups of the glucuronic acid ring are reversible (Fig. 2) except that the thermodynamically unfavourable 1-*O*-β acyl glucuronide itself is not reformed [12]. The only reported exception is the work of Hansen-Møller *et al.* [18], who presented evidence suggesting very minor reformation of DAG from the 2-isomer at pH 8.5 *in vitro*.

The identification of the highly polar metabolites (D-2G), found in the bile of rats after dosage with either DAG or *iso*-DAG, as the 2- and 3-*O*-isomers of DAG which had been glucuronidated at the phenolic function of the salicylate ring is based on selective and sequential hydrolyses using enzyme, acid and alkali catalysts. Evidence for only trace quantities of the phenolic glucuronides of DAG and its 4-*O*-acyl isomer was found in partially purified samples of D-2G from either DAG- or *iso*-DAG-dosed rats. It was therefore conceivable, albeit highly unlikely, that these particular "diglucuronides" were indeed present in the bile samples but did not survive the purification steps, i.e. were either not extracted into ethyl acetate after acidification of the bile or not collected during fraction collection from preparative HPLC. Evidence confirming that only trace amounts of the phenolic glucuronide of the 4-isomer were present in the bile samples from either DAG- or *iso*-DAG-dosed rats was obtained by β-glucuronidase treatment of unpurified bile samples. Simultaneous confirmation of the essential absence of the phenolic glucuronide of DAG (a true diglucuronide) was not

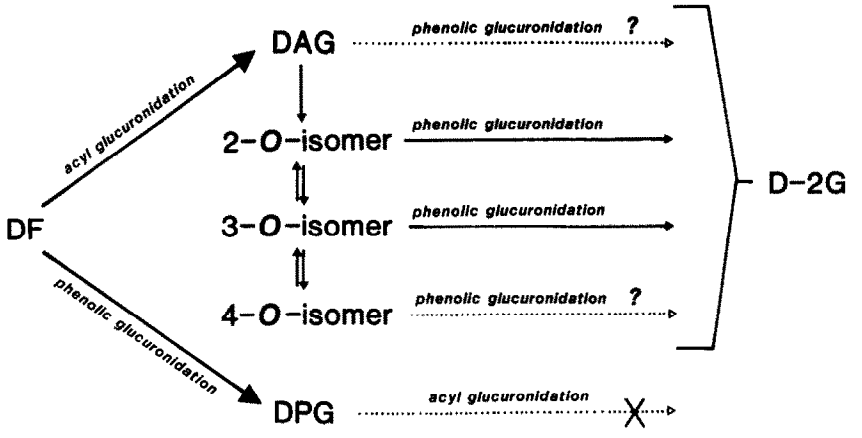

Fig. 9. Scheme illustrating the *in vivo* formation of "diglucuronides" (D-2G) of DF via metabolism to DAG, non-enzymic rearrangement to the 2-, 3- and 4-*O*-isomers of DAG, and phenolic glucuronidation of predominantly the 2- and 3-*O*-isomers. Further glucuronidation of DPG does not occur.

possible for analytical reasons. However, there was no evidence for formation of this diglucuronide after dosing one rat with DPG (Fig. 6 and Table 3). Samples re-analysed from an earlier study where DPG was dosed i.v. to anaesthetized rats [17] also failed to reveal the presence of this diglucuronide. It is noteworthy that there was no evidence for formation of "diglucuronides" of DF when the bile of homozygous Gunn rats dosed with DF [7] was re-analysed in the light of the present results. These mutant rats, as well as lacking the UDP glucuronosyltransferase isozyme coupling bilirubin, were shown not to form DPG. However, DAG (and its isomers) was formed in Gunn rats, though at reduced rates compared to those in Wistar and Sprague–Dawley rats. Thus DAG and its 4-*O*-isomer, as well as DPG, appear not to be substrates for further glucuronidation of the salicylate ring (Fig. 9). Whether this simply reflects that they are poor substrates for any of the UDP-glucuronosyltransferase isozymes in rat liver [29, 30] or that intrahepatic transport processes are different from those applicable to the 2- and 3-isomers of DAG is unknown. There can be no doubt, however, that these polar, acidic molecules are readily taken up by hepatocytes, as demonstrated by the extent of their biliary excretion after i.v. administration (Tables 2 and 3).

The almost exclusive biliary excretion of the "diglucuronides" (D-2G) and their consequent essential absence from plasma and urine, is in accord with earlier studies reporting the influence of molecular weight/size and chemical structure/lipophilicity on preferential biliary or urinary excretion [31–33]. Thus, in rats, compounds with molecular weight: (a) > *ca.* 450 daltons (e.g. D-2G, 602 daltons) are excreted predominantly in bile; (b) *ca.* 350–450 daltons (e.g. DAG, *iso*-DAG and DPG, 426 daltons) are excreted both in bile and urine; and (c) < *ca.* 350 daltons (e.g. DS, 330 daltons) are excreted predominantly in urine.

Isomers of acyl glucuronides found in biological samples were originally regarded only as analytical artifacts arising from the improper storage of samples (i.e. the absence of mild acidification). However, evidence has steadily accumulated that isomers of labile acyl glucuronides *must* be formed *in vivo*, particularly when their excretion pathways are blocked [34–36]. With the exception of the present study and an earlier investigation of valproate acyl glucuronide [24], the *in vivo* fate of i.v.-administered isomers of acyl glucuronides has not been explored, to our knowledge. In our earlier work on DF disposition in rats [4, 5], highly polar metabolites of DF were observed in chromatograms of bile samples. These products are herein identified as "diglucuronides" of DF, arising by phenolic glucuronidation of the 2- and 3-*O*-acyl isomers of DAG which themselves arise from (putatively non-enzymic) intramolecular acyl migrations of DAG *in vivo*. We regard these "diglucuronides" as novel metabolites, in that: (a) they are ultimately metabolic products subsequent to Phase II conjugation which usually ends the metabolic sequence (at least for xenobiotics) and (b) they appear not to be formed from the biosynthetic Phase II conjugate itself. Indeed, similar results were obtained in our earlier study comparing valproate acyl glucuronide and its isomers administered i.v. to rats [24] where the isomers were found to be better substrates for Phase I hydroxylation at the 4- position of the valproate chain.

Clearly, the *in vivo* disposition of acidic drugs forming acyl glucuronides can be quite complex, with both the inherent lability of the 1-*O*-β ester linkage to glucuronic acid as well as the physicochemical properties of the drug moiety itself being important factors. Thus, in general, conjugation of a carboxylic drug to its acyl glucuronide competes with other metabolic pathways and direct excretion of the drug in urine and bile. Major competing pathways for the biosynthesized acyl glucuronide include direct excretion, hydrolysis to regenerate the parent drug (i.e. systemic cycling) and intramolecular acyl migration to the 2-, 3- and 4-*O*-acyl positional iso-

mers (i.e. systemic rearrangement). The isomers so formed may be excreted in urine or bile, and may also hydrolyse systemically to regenerate the parent drug. Catalysts here exclude the β-glucuronidases. Both the acyl glucuronide and its isomers may react with proteins to yield covalent drug–protein adducts. The mechanisms of these reactions are uncertain, but both transacylation [9, 37] and Schiff's base/Amadori rearrangement [11, 38] reactions have been postulated. The possibility of such protein adducts initiating toxicity/hypersensitivity responses has been raised [11, 37, 39]. Finally, results obtained in the present and an earlier [24] study suggest that rearrangement isomers of the acyl glucuronide may undergo novel metabolic pathways which are not, or are less, applicable to the acyl glucuronide itself.

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