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STUDIES ON THE REACTIVITY OF ACYL GLUCURONIDES—V. GLUCURONIDE-DERIVED COVALENT BINDING OF DIFLUNISAL TO BLADDER TISSUE OF RATS AND ITS MODULATION BY URINARY pH AND β-GLUCURONIDASE*

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Abstract—Acyl glucuronide conjugates of acidic drugs have been shown to be reactive metabolites capable of undergoing non-enzymic hydrolysis, rearrangement (isomerization via acyl migration) and covalent binding reactions with plasma protein. In an earlier study (King and Dickinson, *Biochem Pharmacol* **45**: 1043–1047, 1993), we documented formation of covalent adducts of diflunisal (DF), a salicylate derivative which is metabolized in part to a reactive acyl glucuronide (DAG), with liver, kidney, skeletal muscle and small and large intestine (in addition to plasma protein) of rats given the drug i.v. twice daily at 50 mg DF/kg for 7 days. The present study shows that covalent adducts of DF were also formed with urinary bladder tissue of these rats, achieving concentrations (*ca.* 5 μg DF equivalents/g tissue) higher than those found in the other tissues noted above. After cessation of dosing, the adduct concentrations declined with an apparent $T_{1/2}$ value of *ca.* 20 hr. Adducts were also formed *ex vivo* in excised rat bladders in which DAG or a prepared mixture of its acyl migration isomers (*iso*-DAG) were incubated at pH 5.0, 6.5 and 8.0. After 8 hr incubation, the highest concentrations (*ca.* 11 μg DF equivalents/g) were produced with *iso*-DAG at pH 5.0, and the lowest (*ca.* 2.3 μg DF equivalents/g) with DAG at pH 5.0. However, a major competing reaction for DAG (at least at pH 5.0) was hydrolysis by β-glucuronidases originating from bladder tissue. By contrast, *iso*-DAG was quite resistant to such hydrolysis. The phenolic glucuronide conjugate, another important metabolite of DF, was hydrolysed only slowly. Similar results were obtained in fresh rat urine adjusted to pH 5.0. The results support covalent DF adduct formation in rat bladder originating from both DAG and *iso*-DAG as ultimate reactants, though the extent of binding is modulated by both urinary pH and β-glucuronidases.

Acyl glucuronide conjugates are the metabolic products of coupling endogenous glucuronic acid with an acceptor carboxylic acid group of endogenous substrates such as bilirubin or exogenous substrates such as many acidic drugs. However, unlike most other classes of metabolic conjugates, the acyl glucuronides are not necessarily stable end-products *in vivo* [1, 2]. Manifestation of their intrinsic chemical reactivity includes hydroxide ion-catalysed acyl migration to yield the 2-, 3- and 4-*O*-acyl positional isomers (Fig. 1) and hydrolysis to regenerate the aglycone. The migration isomers of the acyl glucuronide retain the ester linkage and thus also undergo chemical hydrolysis. However, unlike their parent, the isomers are *not* glucuronides, as they are not themselves products of biosynthesis, and they are not susceptible to β-glucuronidase-mediated hydrolysis. As well, since the 1-*O*-position of the isomers is no longer substituted, they can exist as mixtures of the C-1 β- and α-anomers, through the intermediacy of the transient open-chain form of the sugar ring [2, 3].

A third manifestation of the reactivity of acyl glucuronides is their ability to cause the covalent binding of the aglycone moiety, directly or indirectly, to biological macromolecules, notably serum albumin. Two mechanisms have been advanced to account for this binding, and evidence has been variously presented to support both. The transacylation mechanism involves nucleophilic attack by —SH, —OH or —NH_2 groups of protein on the acyl carbon of the glucuronide itself, resulting in displacement of the glucuronic acid moiety and direct covalent attachment of the aglycone moiety to protein via a thioester, ester or amide linkage, respectively [1, 4, 5]. The alternative rearrangement/glycation mechanism [6, 7] *requires* prior acyl migration before interaction with the protein: upon transient ring-opening of the isomers so formed, the reactive aldehyde group generated at C-1 condenses with an amino group of protein to yield reversibly an imine (Schiff's base), which could then undergo Amadori rearrangement to a more stable ketoamine derivative. In this mechanism, the aglycone moiety becomes covalently bound to the protein indirectly

* For Part IV in the series "Studies on the reactivity of acyl glucuronides" see A. R. King and R. G. Dickinson, *Biochem Pharmacol* **45**: 1043–1047, 1993.

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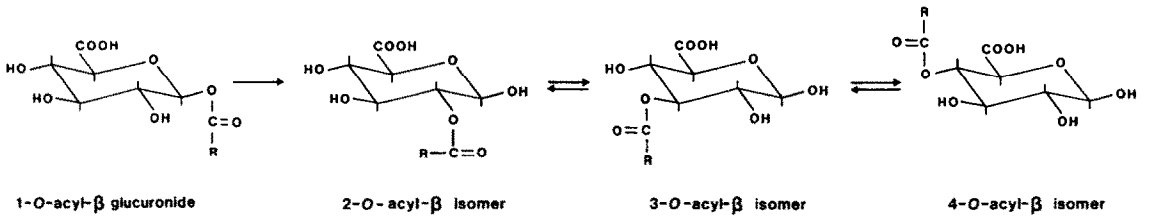

Fig. 1. Scheme showing rearrangement of biosynthetic, β-glucuronidase-susceptible 1-*O*-acyl-β glucuronides to the β-glucuronidase-resistant 2-, 3- and 4-*O*-acyl positional isomers.

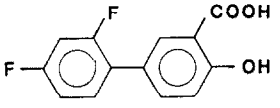

Fig. 2. Chemical structure of diflunisal.

through the glucuronic acid moiety.* Formation of covalent adducts with plasma protein has now been demonstrated for many acidic drugs *in vitro* and for a more limited number *in vivo* [2]. As the net result is covalent modification of native protein by foreign molecules, this property of acyl glucuronides has been hypothesized to be toxic [1, 2, 4], e.g. in the induction of immune responses via the hapten hypothesis [8, 9]. To date, however, the only study of glucuronide-derived immune responses has concerned the anti-epileptic agent valproic acid, where anti-adduct antibodies were found in some patients on chronic therapy with the drug, but the titres were very low [10].

Studies of adduct formation have been limited to plasma protein, until recently, when adducts of clofibric acid [11] and diclofenac [12, 13] with rodent liver protein were described. We recently showed [14] that diflunisal (DF,† Fig. 2), a salicylate derivative which forms a reactive acyl glucuronide (DAG) [3, 15] as well as stable phenolic glucuronide (DPG) [3, 16] and sulphate (DS) [17] conjugates, forms covalent adducts with plasma protein, liver, kidney, skeletal muscle and small and large intestine of rats dosed with the drug. The present study examines the *in vivo* formation of adducts of DF with the urinary bladder of these rats, and additionally as a function of pH in excised bladders "dosed" with DAG or a prepared mixture of its acyl migration isomers (*iso*-DAG). We hypothesized that an acidic urinary pH would have a protective effect against adduct formation in the urinary bladder, as acyl glucuronide reactivity is hydroxide ion catalysed.

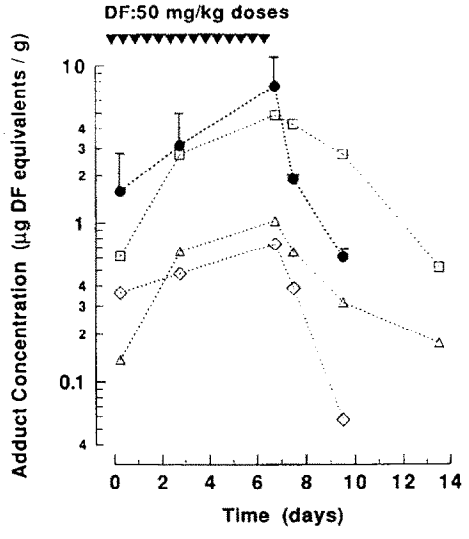

Fig. 3. Concentration–time profiles for DF covalently bound to bladder tissue (●) of rats during and after dosage at 50 mg DF/kg i.v. twice daily for 7 days. Results are means ± SD, N = 3 or 4. The mean concentrations of DF covalently bound to liver (□), kidney (△) and plasma protein (◇), determined earlier in the same animals [14], are reproduced for comparison.

If so, this dispositional scenario would starkly contrast with that of another class of reactive glucuronides, i.e. the glucuronides of pro-carcinogenic *N*-hydroxy metabolites of aromatic amines. These glucuronides, biosynthesized in the liver, can act as stable transport forms in blood until excretion in urine, where the slightly acidic pH decomposes the glucuronide to release the ultimate bladder carcinogen [18, 19].

MATERIALS AND METHODS

Materials and animals. DF and D-saccharic acid 1,4-lactone were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Authentic samples of DAG, its 2-, 3- and 4-*O*-acyl migration isomers (*iso*-DAG), DPG and DS were prepared as described previously [3, 16, 17]. Clofibric acid was a gift from ICI Pharmaceuticals Division (Macclesfield, U.K.). Methanol and acetonitrile (HPLC grade), diethyl

* For simplicity of presentation in this report, terms such as "covalent binding of drug to protein" or "covalent drug–protein adducts" are used in an overall descriptive sense without implying either postulated mechanism of formation.

† Abbreviations: DF, diflunisal; DAG, diflunisal acyl glucuronide; DPG, diflunisal phenolic glucuronide; DS, diflunisal sulphate; *iso*-DAG, mixture of 2-, 3- and 4-*O*-isomers of diflunisal acyl glucuronide formed by acyl migration; $T_{1/2}$, half-life.

ether (AR grade) and hexane (nanograde) were purchased from Mallinckrodt (Melbourne, Australia). Reagents were AR grade. Methoxyflurane anaesthetic (Penthrane®) was purchased from Abbott Australasia (Sydney, Australia). Male Sprague–Dawley-derived rats (290–350 g) were obtained from The University of Queensland Medical Faculty Animal House, and were maintained on standard rat pellets (Norco Co-operative, Lismore, Australia) and water. Experiments were approved by the University's Animal Experimentation Ethics Committee.

Incubation of DAG and iso-*DAG in excised rat bladders.* Rats were anaesthetized with methoxyflurane and exsanguinated via the aorta. The urinary bladder was exposed and catheterized with polyethylene tubing (1.2 mm o.d., 0.8 mm i.d.; Dural Plastics, Sydney, Australia) which had been heat moulded into a bulb on one end to prevent withdrawal from the bladder. The ureters and urethra were ligated and the bladder excised. Via the catheter, the bladder contents were aspirated, and the lumen rinsed once with 1 mL of 0.1 M phosphate buffer at pH 5.0, 6.5 or 8.0, as required. A solution (1 mL) of freshly prepared DAG or *iso*-DAG (as an approximately equimolar mixture of the individual isomers) at 200 μg DF equivalents/mL of 0.1 M phosphate buffer at final pH 5.0, 6.5 or 8.0 was then injected, along with an air bubble (*ca.* 100 μL), to facilitate mixing. The bladder was then sealed in a 5 mL Reacti-Vial® (Pierce Chemical Co., Rockford, IL, U.S.A.) containing 100 μL normal saline, and rotated in an incubator at 37° for 8 hr. The contents of the bladder were then removed and the tissue analysed for covalently bound DF, as described below.

Three bladders were studied for each of the DAG and *iso*-DAG substrates at each of the 5.0, 6.5 and 8.0 pH values. In addition, three bladders were studied using DF itself as substrate at pH 6.5, and another three where DAG was incubated at pH 5.0 in the presence of 2 mg/mL (10 mM) of the β-glucuronidase inhibitor, D-saccharic acid 1,4-lactone. (In this, as in all experiments using the acidic inhibitor D-saccharic acid 1,4-lactone, the stated incubation pH is the pH in the presence of the inhibitor, not that before its addition.) In one experiment for each substrate incubation at pH 5.0, 50 μL samples of bladder contents were taken before and after 2, 4 and 8 hr incubation for analysis of non-covalently bound DF species as described below.

Incubation of DAG, iso-*DAG, DPG and DS in buffer exposed to bladder lumen.* Bladders were catheterized and excised from rats as described above, and the lumen rinsed once with 1 mL 0.1 M phosphate buffer pH 5.0. A further 1 mL portion of the buffer, plus an air bubble, were injected into the bladder, which was then rotated in the incubator at 37° as described above for 1 or 4 hr. The bladder contents were then removed, and used in the following incubations. To 200 μL samples of blank pH 5.0 buffer and the buffer exposed to rat bladder lumen for 1 or 4 hr were added 200 μL samples of DAG, *iso*-DAG, DPG or DS at 200 μg DF equivalents/mL 0.1 M phosphate buffer (final pH 5.0), and the solutions incubated at 37° for 6 hr. Aliquots (50 μL) were withdrawn before and after 1, 2, 4 and 6 hr incubation for analysis of DF species as described below.

In an additional experiment using buffer exposed to bladder for 4 hr, the DAG incubations were repeated in the presence and absence of 10 mM D-saccharic acid 1,4-lactone, at final pH 5.0 throughout.

Incubation of DAG and DPG in rat and human urine. Urine from two rats was collected under methoxyflurane anaesthesia by direct syringe aspiration of the bladder, and from another two animals immediately after spontaneous voiding when placed on a clean tray (care being taken to avoid any faecal contamination). The pH values were recorded and the urine then adjusted to pH 5.0 with 1 M H_3PO_4. To 150 μL aliquots were added 150 μL of either DAG or DPG solutions (with or without D-saccharic acid 1,4-lactone at 20 mM) in 0.1 M phosphate buffer (final pH 5.0) to give initial concentrations of 100 μg DF equivalents/mL. The solutions were then incubated at 37° for 6 hr. Samples (50 μL) were drawn at 2, 4 and 6 hr, and analysed for DF species as described below.

Urine from six healthy adult volunteers was freshly collected, the pH recorded and adjusted to pH 5.0, and then incubated with DAG, in an analogous fashion to that used for rat urine above. A single sample was taken at 6 hr for measurement of DF species.

Analysis of reversibly bound DF and metabolites. DF, DAG, *iso*-DAG, DPG and DS in 50 μL aliquots of buffer, bladder contents or urine were analysed using the direct isocratic HPLC method described previously [20]. Isomers of DAG arising from intramolecular acyl migration were measured using the same molar extinction as DAG itself, an approach that has been verified experimentally [3].

Analysis of DF covalently bound to bladder tissue. Bladders (each *ca.* 0.1 g) from the present *ex vivo* study and an earlier *in vivo* study ([14], where rats were administered 50 mg DF/kg i.v. twice daily for 7 days, and which had been excised at the time of sacrifice, their contents removed, and stored at −20°) were cut into thin strips with scissors and then finely chopped using a scalpel (attempted homogenization of this elastic tissue using various tissue grinders proved to be more problematic). To each were added 200 μL 0.01 M phosphate buffer pH 4.5, 300 μL ice-cold methanol, 600 μL acetonitrile and 3 μL H_3PO_4, with vigorous vortex mixing after each addition. After centrifugation, the supernatant was discarded, and the pellet exhaustively washed (10 times) sequentially by resuspension in 2.5 mL methanol–diethyl ether (3:1 v/v), agitation for 20 min, followed by centrifugation. The pellet was then dried at 40° under a stream of air and digested in 400 μL 2 M NaOH at 75° for 3 hr. After cooling and acidification (160 μL 10 M HCl), the HPLC internal standard (5 μg clofibric acid in 50 μL water) was added and the mixture extracted with 3 mL hexane–diethyl ether (1:1 v/v). The organic layer was removed and evaporated at 50° under a stream of air. The residue was reconstituted in 200 μL mobile phase, and a 60 μL sample analysed for DF as described previously [20].

Standard curves were prepared from bladders of

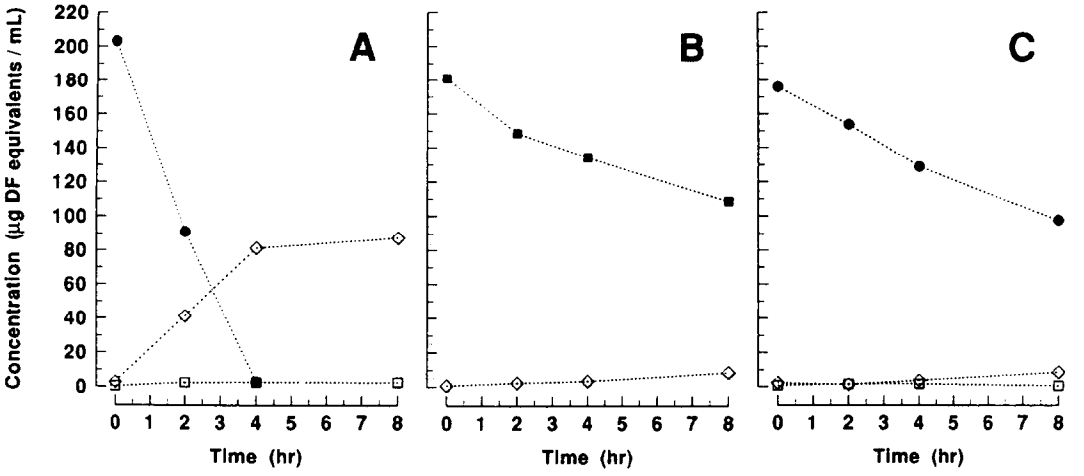

Fig. 4. Concentration–time profiles for DAG (●) incubated at pH 5.0 and 37° in an excised rat bladder in the absence (panel A) and presence (panel C) of D-saccharic acid 1,4-lactone at 10 mM, showing formation of *iso*-DAG (□) and DF (◇). Panel B shows incubation of *iso*-DAG (■) and formation of DF (◇) over the same time period.

untreated rats by spiking precipitated and washed pellets (prior to alkaline digestion) with the calculated amounts of DF. Standard curves were derived from spiked concentrations of 0, 0.1, 0.3, 1 and 3 μg DF/bladder. Coefficients of determination (r^2) were about 0.99. The adequacy of the washing procedure to remove non-covalently bound DF was verified by analysis of chopped bladders (from untreated rats) which had been spiked with DF by incubation for 2 hr in phosphate buffer pH 6.5 containing DF at 100 μg/mL.

Data analysis. The apparent half-life ($T_{1/2}$) of DF covalently bound to bladder tissue in the *in vivo* study was calculated from the slope of the log concentration–time profile after the last dose, using linear regression analysis of the mean concentrations at each time point. Similarly, the $T_{1/2}$ values of DAG or DPG degradation (almost exclusively hydrolysis) in diluted rat and human urine were calculated by linear regression analysis.

RESULTS

Figure 3 shows the profiles of DF covalently bound to bladder tissue of rats during and after 7 days of twice daily i.v. dosing at 50 mg DF/kg; the profiles for liver, kidney and plasma protein determined earlier in the same animals [14] are reproduced for comparison. Covalent DF adduct concentrations increased steadily over the dosing period, and were considerably higher in bladder than in other tissues examined, with the exception of liver. The peak concentration, measured 6 hr after the last dose on day 7, was 7.4 ± 3.8 μg DF equivalents/g tissue (compare 4.8 ± 1.2 μg/g for liver). After cessation of dosing however, adduct concentrations in bladder tissue (as in plasma) declined more rapidly (apparent $T_{1/2}$ values: 20 hr bladder, 18 hr plasma, compare 38–67 hr [14] for the other tissues), becoming unmeasurable by 7 days.

In vitro covalent binding of DF after 8 hr incubation of excised bladders containing DAG or *iso*-DAG in buffer at pH 5.0, 6.5 and 8.0 is shown in Table 1. Incubation of bladders with DF itself was assessed only at pH 6.5, and only low amounts of DF were recovered (Table 1). These values are probably the result of incomplete removal of non-covalently bound DF during analytical workup rather than actual covalent binding mediated by DF itself as reactant. Adduct formation appeared to increase with pH for DAG and to decrease with pH for *iso*-DAG, though there was considerable inter-bladder variability in binding. Both the highest (11.0 μg DF/g) and lowest (2.3 μg DF/g) adduct concentrations were found after incubation at pH 5.0, with *iso*-DAG and DAG, respectively (Table 1). At this pH, hydroxide ion-catalysed acyl migration and hydrolysis of DAG and *iso*-DAG are known to be minimal [3, 20]; thus the adduct concentrations found after 8 hr incubation were thought to reflect the capacities of DAG and *iso*-DAG as reactants for covalent binding at this pH, in the essential absence of other competing pathways. To confirm this disposition, samples of the pH 5.0 buffer solutions containing DAG and *iso*-DAG were withdrawn at various times after commencement of incubation (one bladder each substrate) for analysis of DF species. Surprisingly, DAG concentrations declined very rapidly with appearance of considerable free DF and negligible *iso*-DAG (Fig. 4). On the other hand, incubation of *iso*-DAG resulted in its modest decrease with time, without appearance of appreciable free DF. These results suggested β-glucuronidase-mediated hydrolysis of DAG, as *iso*-DAG is known to be a resistant to the enzyme [3, 20]. When DAG was incubated in pH 5.0 buffer in a bladder in the presence of the β-glucuronidase inhibitor D-saccharic acid 1,4-lactone, hydrolysis was indeed inhibited (Fig. 4).

This finding was explored further by incubating

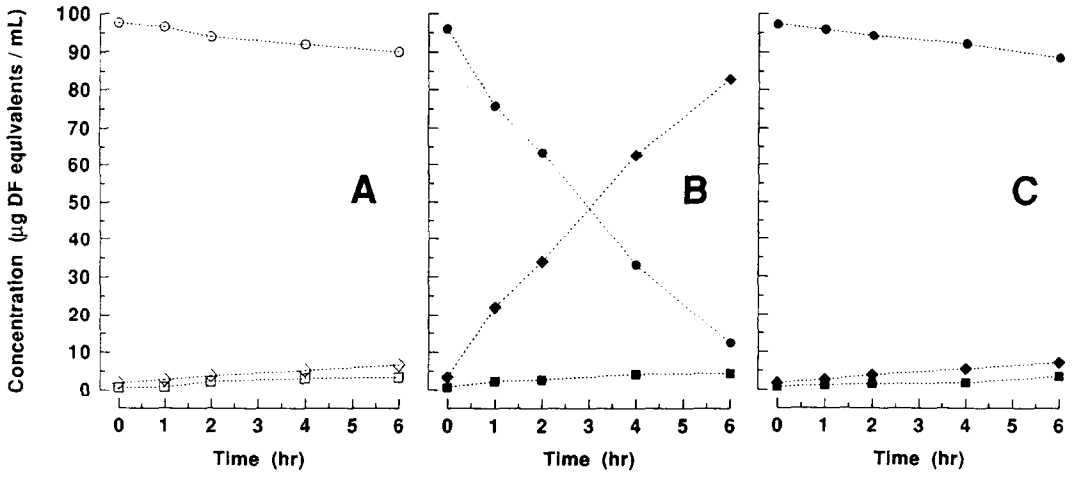

Fig. 5. Concentration–time profile for DAG (○) incubated at 37° in "blank" pH 5.0 buffer, showing formation of *iso*-DAG (□) and DF (◇) (panel A). The analogous profiles, using pH 5.0 buffer previously exposed to rat bladder lumen for 4 hr, and obtained in the absence and presence of D-saccharic acid 1,4-lactone at 10 mM, are shown with closed symbols in panels B and C, respectively.

Table 1. DF covalently bound to tissue after incubation of bladders containing DAG, *iso*-DAG or DF for 8 hr at 37°

Incubation substrate*	pH	Covalently bound DF (μg DF/g bladder)†
DAG	5.0	2.3 ± 1.2
DAG	6.5	4.2 ± 1.6
DAG	8.0	7.2 ± 1.1
iso-DAG	5.0	11.0 ± 3.2
iso-DAG	6.5	7.5 ± 4.4
iso-DAG	8.0	4.6 ± 2.9
DF	6.5	0.7 ± 0.06
DAG + D-saccharic acid 1,4-lactone	5.0	1.3 ± 0.2

* Initial concentrations of DAG, *iso*-DAG and DF were 200 μg DF equivalents/mL; D-saccharic acid 1,4-lactone was at 10 mM.

† Results are means ± SD, N = 3.

DAG and *iso*-DAG, as well as the other DF conjugates, DPG and DS, in pH 5.0 buffer which had been previously exposed to bladder lumen by incubation in a bladder for 1 or 4 hr. Figure 5 shows that over 6 hr incubation in such buffer, DAG underwent extensive hydrolysis to DF and negligible rearrangement to *iso*-DAG. The hydrolysis was inhibited in the presence of D-saccharic acid 1,4-lactone, becoming comparable to that found in buffer not exposed to bladder. In parallel experiments, hydrolysis of DPG (the phenolic glucuronide of DF), was discernible in buffer exposed to bladder, but its extent was very modest in comparison to that of DAG (Fig. 6). By contrast, *iso*-DAG and DS were unaffected by pre-exposure of the incubating buffer to bladder lumen for 4 hr. In parallel experiments using buffer pre-exposed to bladder for 1 hr, the same net result was obtained, through the rates of hydrolysis of DAG and DPG were considerably reduced (data not shown).

Finally, hydrolysis of DAG and DPG was assessed in fresh rat urine (pH 7.2 ± 0.5, N = 4) buffered to pH 5.0, with and without D-saccharic acid 1,4-lactone (Table 2). Urine was obtained immediately after spontaneous voiding (rats 3 and 4) and also by direct aspiration from the bladder (rats 1 and 2) to prevent any possibility of contamination from β-glucuronidases originating from the gastrointestinal tract. Facile β-glucuronidase-mediated hydrolysis of DAG was confirmed in urine: $T_{1/2}$ values were < 0.4–1.3 hr (compared with *ca.* 50 hr in buffer) and substantially increased in the presence of D-saccharic acid 1,4-lactone. DPG was much more resistant to the enzyme than DAG (Table 2). For comparison, β-glucuronidase-mediated hydrolysis of DAG was also assessed in fresh human urine (pH 6.2 ± 0.5, N = 6) buffered to pH 5.0. Hydrolysis of DAG was much slower: after 6 hr incubation, only 10 ± 6% (range 0–17%) of substrate DAG had undergone hydrolysis attributable to β-glucuronidase.

DISCUSSION

The present study documents the *in vivo* formation of covalent adducts of DF with bladder tissue of rats dosed with the drug, and complements our earlier work [14] showing adduct formation with liver, kidney, intestine, skeletal muscle and plasma protein in the same animals. For bladder, as for other tissues, DF adduct concentrations increased steadily over the dosing period, and declined slowly ($T_{1/2}$ *ca.* 20 hr) upon cessation of dosing. With the exception of skeletal muscle, the tissues were selected because they are involved in the biosynthesis, transport and excretion of glucuronides, and are thus likely to encounter relatively high glucuronide concentrations. From the available evidence, it seems likely that covalent adducts will form, at least to some extent,

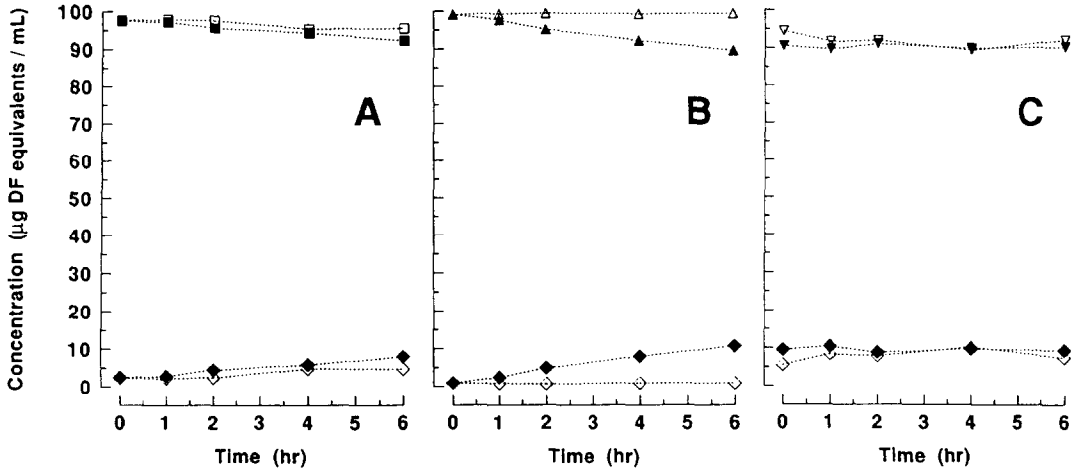

Fig. 6. Concentration–time profiles for *iso*-DAG (□), DPG (△) and DS (▽) incubated at 37° in "blank" pH 5.0 buffer, showing formation of DF (◇) (panels A, B and C, respectively). The analogous profiles obtained using pH 5.0 buffer previously exposed to rat bladder lumen for 4 hr are shown with closed symbols.

Table 2. Hydrolysis of DAG and DPG at 37° in buffer and rat urine adjusted to pH 5.0*

	$T_{1/2}$ (hr)‡			
Medium†	DAG	DAG + D-saccharic acid 1,4-lactone	DPG	DPG + D-saccharic acid 1,4-lactone
Buffer	50 (0.85)	—	>‖	—
Rat 1 urine	0.8 (0.99)	—	—	—
Rat 2 urine	1.3 (0.98)	—	—	—
Rat 3 urine	<0.4§	15 (0.99)	2.5 (0.99)	>‖
Rat 4 urine	<0.4§	11 (0.99)	1.4 (0.99)	220 (0.63)

* Initial concentrations of DAG and DPG were 100 μg DF equivalents/mL in urine diluted with an equal volume of DAG or DPG solution; D-saccharic acid 1,4-lactone was at 10 mM.

† Urine from rats 1 and 2 was aspirated from the bladder under anaesthesia; urine from rats 3 and 4 was collected immediately after spontaneous voiding.

‡ Results in parentheses are r^2 values.

§ Minimum value calculated on residual DAG at 2 hr being <3%.

‖ No detectable decline in concentration.

with all tissues which come into contact with reactive acyl glucuronides such as DAG. The previous [14] and present studies, however, are only very coarse screens, and point to the need for further work to identify subcellular sites of adduct formation, mechanisms of binding and adduct structures, as well as to determine the occurrence and extent of any consequent perturbations of biological function, toxic sequelae or immune responses. However, it should be clearly stated that there is, as yet, no *direct* evidence linking glucuronide-derived covalent binding of acidic drugs with any toxicity. Nonetheless, the possibility should not be understated.

In the *in vivo* DF studies, the intermediacy of DAG in formation of drug–tissue adducts must be presumed, since it is the parent drug which is administered, and it is this same chemical species which is used to measure covalent adducts (i.e. following base digestion of washed tissue pellets). However, confirmation of the essential roles of metabolic activation of DF via formation of DAG, and of non-metabolic further activation by rearrangement of DAG to its isomers, comes from the *ex vivo* incubation studies in excised bladders (Table 1). Thus, incubation of DF itself gave little or no covalent binding to bladder tissue in comparison to DAG and *iso*-DAG as substrates.

Further interpretation of these covalent binding results however needs to be made in the light of the modulating effects of pH and β-glucuronidases. The experiments conducted (Figs 4–6) unequivocally demonstrated that, at least at pH 5.0, DAG was capable of being very rapidly hydrolysed by β-glucuronidases of bladder origin. By contrast, its isomers formed by acyl migration (*iso*-DAG) were quite resistant to such hydrolysis. Mammalian β-

glucuronidases display pH optima at about 4.5 and 5.2 [21, 22], and in one study of human urinary β-glucuronidase, the activity at pH 6.5 was 36% of that at its optimum pH of 5.0 [23]. Thus β-glucuronidase-mediated hydrolysis would be expected to be a progressively less effective competitor for acyl glucuronide disposition in the urinary bladder the more the urinary pH differs from 5.0. Formation of covalent DF–bladder adducts from substrate DAG did increase with pH over the range 5.0–6.5–8.0 (Table 1), and this is most likely attributable to increasing intrinsic chemical reactivity in concert with declining competition from β-glucuronidase hydrolysis. The former of course includes acyl migration, and admits the probability of contributions to adduct formation from the isomers formed at higher pH values. However, at pH 5.0, hydroxide ion-catalysed hydrolysis and acyl migration of DAG are minimal [20]; thus the adducts found at this pH appear to be attributable to DAG itself as the ultimate reactant (presumably via a transacylation mechanism). At this pH, we expected an increase in the formation of adducts when DAG was protected from β-glucuronidase by incubation in the presence of the inhibitor D-saccharic acid 1,4-lactone (Fig. 4). Adduct concentrations were lower however (Table 1), and perhaps this is due to blockage of tissue binding sites by the β-glucuronidase inhibitor, which was present at a concentration (10 mM) about 12 times higher than that of substrate DAG.

Incubation of bladders with *iso*-DAG as substrate should be considerably easier to interpret because (1) the isomers are resistant to β-glucuronidase hydrolysis and (2) they do not reform DAG during rearrangement. Interestingly, concentrations of adducts formed from *iso*-DAG (presumably via a glycation mechanism), declined with increasing pH (Table 1). This could be taken to reflect an unexpected catalysis of adduct formation at slightly acidic pH, or alternatively an enhanced hydrolysis of adducts at slightly alkaline pH. As only single point (8 hr incubation) data were obtained in the present study, kinetic profiles were not available to assist with interpretation. No systematic studies of the effects of pH on adduct formation via both acyl glucuronide and isomers have been reported, to our knowledge, although Volland *et al.* [24] found a 3–4-fold increase in formation of adducts during incubation of fenoprofen acyl glucuronide with human serum albumin as the pH increased from 5.5 to 7.4.

Facile β-glucuronidase-mediated hydrolysis of DAG was observed both in pH 5.0 buffer exposed to rat bladder lumen and in (physiological) rat urine adjusted to pH 5.0. The enzyme activity in fresh human urine adjusted to pH 5.0 was very modest by comparison. It should be noted in this context that the bladder itself need not be the only source of urinary β-glucuronidases [21, 22]. DAG was much more susceptible to the urinary enzyme than DPG, an observation made also for hydrolysis of these two glucuronides by rat liver microsomal β-glucuronidase [25]. Hydrolysis of DF conjugates in the bladder *in vivo* might be expected to be followed by significant reabsorption of lipophilic DF into the blood circulation. Indeed, salicylate has been recently shown to be readily absorbed into the bloodstream after its administration to the bladders of rats [26], and there are many other examples of this often-ignored aspect of drug disposition [27]. In the case of DF disposition in rats given the drug i.v., DAG and DPG (biosynthesized primarily in the liver) were excreted preferentially by the biliary route rather than the urinary route [28]. Nonetheless, the recovery of DAG relative to DPG in urine was much less than that found in bile, and major contributors to this phenomenon are clearly the systemic instability of DAG and the systemic stability of DPG [3, 16]. The present study extends this differential stability scenario to the urinary bladder, where, dependent upon pH, substantial β-glucuronidase-mediated hydrolysis of DAG may occur and could lead to a significant resorption of DF. Thus there may well be a vesico-hepato-renal cycling of DF via DAG, in addition to systemic cycling [3, 16] and entero-hepatic cycling [28].

In summary, the present study has documented the formation of covalent adducts of DF with bladder tissue of rats. The adducts can originate from both DAG and *iso*-DAG as reactants, and their formation be modulated by both urinary pH and β-glucuronidases. Facile hydrolysis of DAG in the bladder could result in a significant vesico-hepato-renal recirculation of DF via DAG.

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