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REACTIVITY OF DIFLUNISAL ACYL GLUCURONIDE IN HUMAN AND RAT PLASMA AND ALBUMIN SOLUTIONS

JULIA A. WATT and RONALD G. DICKINSON*
Department of Medicine of the University of Queensland, Royal Brisbane Hospital, Brisbane, Qld 4029, Australia

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Abstract—Diflunisal acyl glucuronide (DAG) is a major metabolite of diflunisal (DF) in rats and humans. We have investigated the reactivity of DAG, in purified albumin solutions and plasma from both rat and human sources, along three interrelated pathways: rearrangement via acyl migration to yield positional isomers of DAG, hydrolysis of DAG and/or its isomers to liberate DF, and formation of covalent adducts of DF (via DAG and/or its isomers) with plasma protein. Two initial concentrations of DAG (*ca.* 50 and 10 μg DF equivalents/mL) were used throughout. In all incubations, the order of quantitative importance of the reactions was: rearrangement > hydrolysis > covalent binding. At pH 7.4 and 37°, degradation of DAG in albumin solutions (e.g. half-life *ca.* 95 min in fatty acid-free human serum albumin) was retarded in comparison to that found in buffer alone (half-life *ca.* 35 min). Degradation in unbuffered rat and human plasma containing heparin was comparable to that found in buffer. Maximal covalent binding to protein was achieved after 4–8 hr incubation, and was greatest for fatty acid-free human serum albumin (165 ng DF/mg albumin). Thereafter, slow degradation of the adducts was observed. Formation of DF-plasma protein adducts *in vivo* was also found in rats and humans dosed with DF.

Diflunisal (DF†), a difluorophenyl derivative of salicylic acid, is metabolized in man and rat primarily by formation of its acyl glucuronide (DAG), phenolic glucuronide and sulphate conjugates [1–4]. Whereas the phenolic glucuronide and sulphate conjugates are chemically stable at physiological pH [5], DAG undergoes hydroxide ion-catalysed intramolecular rearrangement wherein the aglycone moiety migrates from the biosynthetic 1-*O*-β-position to the 2-, 3- and 4-*O*-positions of the glucuronic acid ring [5–8]. The isomers so formed retain the ester linkage and, like DAG itself, can liberate DF by hydrolysis at physiological pH. We have recently shown‡ that DAG, unlike the phenolic glucuronide of DF, is highly unstable after i.v. administration to rats. The major fate of DAG in that study was hydrolysis to regenerate parent DF, which was then reconjugated with both glucuronic acid and sulphate. Rearrangement of DAG *in vivo* seemed to be a relatively minor pathway.

Hydrolysis and rearrangement reactions of acyl glucuronide metabolites of carboxylic drugs and endogenous bilirubin have been extensively documented in recent years (e.g. Refs 9–18). A third reaction manifesting the inherent chemical instability of acyl glucuronides [19] involves their capacity to act as substrates for the covalent binding of the aglycone to plasma protein, notably albumin. van Breeman and Fenselau [20] reported covalent binding of flufenamic acid, indomethacin, clofibric acid and benoxaprofen to bovine serum albumin *in vitro* when the acyl glucuronides were incubated with the protein, and suggested that the mechanism involved transacylation with the free sulphydryl group of cysteine residues. Ruelius and co-workers [21, 22] documented covalent binding of oxaprozin to human serum albumin (HSA) *in vitro*, and concluded, on the basis of extensive inhibition studies, that the site of covalent binding to HSA was an active tyrosine residue located within the benzodiazepine binding site i.e. transacylation with the hydroxy group of tyrosine. Benet and colleagues [23, 24] found "irreversible" binding of zomepirac and tolmetin to plasma protein of humans given single doses of the drugs. They suggested that the binding was initiated by formation of an imine between the 1-position of glucuronic acid and a lysine residue of albumin, i.e. the acyl-migrated isomers, but not the biosynthetic acyl glucuronides themselves, were precursors of the covalent adducts. Covalent binding to protein via the acyl glucuronides has also been reported for fenofibric acid [25] and carprofen [26] though to our knowledge, its occurrence *in vivo* has been limited to zomepirac [23], tolmetin [24] and carprofen [26] and as well to endogenous bilirubin (formation of "biliprotein") [27–30].

Clearly, covalent modification of circulating albumin *in vivo* by acyl glucuronides of xenobiotic acids serves no obvious therapeutic purpose. Indeed, such protein adducts have been suggested as *potential* mediators of toxic responses [19, 23, 24, 26], including the hypersensitivity reactions which have sometimes been associated with carboxylic non-steroidal anti-inflammatory agents.

The present study was carried out to extend our investigations of DAG reactivity by determining its capacity to act as a donor leading to covalent binding

* Address all correspondence to: Ronald G. Dickinson, Ph.D. Department of Medicine, Clinical Sciences Building, Royal Brisbane Hospital, Herston Qld 4029, Australia.

† Abbreviations: DF, diflunisal; DAG, diflunisal acyl glucuronide; RSA, rat serum albumin; HSA, human serum albumin; HPLC, high performance liquid chromatography.

‡ Watt JA, King AR and Dickinson RG, submitted.

of DF to plasma protein. DF therapy has been associated with mild hypersensitivity reactions such as dermatitis, although one recent report [31] describes three cases of severe generalized hypersensitivity.

MATERIALS AND METHODS

Materials. DF was a gift from Merck, Sharp & Dohme (Australia) Pty. Ltd (Sydney, Australia). DAG was isolated from the urine of a healthy volunteer who ingested 500 mg DF daily for several days, and purified by preparative HPLC and crystallization.* Clofibric acid was a gift from ICI Pharmaceuticals Division (Macclesfield, U.K.). Rat serum albumin (RSA) and human serum albumin (HSA) were purchased from the Sigma Chemical Co. (St Louis, MO). Specifications provided by the supplier were: RSA Product A4538, globulin-free and <0.005% fatty acids; HSA Product A8763, essentially globulin-free (0.5% Alpha 1 globulin and 0.4% Alpha 2 globulin) and <0.005% fatty acids; HSA Product A1887, 3% Alpha 1 globulin and <0.005% fatty acids. Plasma was obtained from heparinized blood (125 I.U. lithium heparin per 10 mL) from a male Sprague–Dawley-derived rat exsanguinated under ether anaesthesia, and from a healthy human volunteer not on any medication. Solvents (HPLC grade) and reagents (analytical grade) were obtained from commercial sources.

Incubation of DAG in buffer, albumin solutions and plasma. DAG was incubated at two concentrations (*ca.* 50 and 10 μg DF equivalents/mL) at 37° for 24 hr in solutions of phosphate buffer pH 7.4, globulin- and fatty acid-free RSA, fatty acid-free HSA, globulin- and fatty acid-free HSA (all buffered to pH 7.4), and in unbuffered rat and human plasma. The buffer solution used was 0.1 M Na_2HPO_4 adjusted to pH 7.4 with concentrated H_3PO_4; albumin concentrations were 30 mg/mL (RSA) and 46 mg/mL (HSA). The DAG stock solution (750 μg DF equivalents/mL) was prepared in 0.1 M $NaHCO_3$ solution adjusted to pH 6.0 with 2 M HCl. For the 50 μg DF equivalents/mL concentration, incubation was initiated by adding 250 μL DAG stock to 3.5 mL of the buffer, albumin or plasma solutions (prewarmed to 37°). For the 10 μg DF equivalents/mL concentration, 50 μL of DAG stock and 200 μL of the phosphate buffer pH 7.4 were added. Two samples (50 and 500 μL) of each incubation mixture were withdrawn immediately and at 2 min, 1, 4, 8 and 24 hr after mixing for analysis of DAG, its rearrangement isomers and reversibly-bound DF (50-μL aliquot) and of covalently-bound DF (500-μL aliquot).

Analysis of DAG, its isomers and reversibly-bound DF. Intramolecular rearrangement of DAG to its isomers and hydrolysis of DAG and/or its isomers in the incubation mixtures were monitored using a direct, isocratic HPLC procedure [5]. To the 50-μL aliquots (above) were immediately added 75-μL aliquots of internal standard solution (100 μg clofibric acid per mL of 4% v/v acetic acid in acetonitrile), the mixtures vortexed, centrifuged and 20-μL aliquots of supernatant injected into the HPLC within 1 hr. The method permits simultaneous analysis of DF and its conjugates. The acyl-migrated isomers of DAG appear as three pairs of peaks corresponding to the β- and α-anomers of the 2-, 3- and 4-*O*-positional isomers [5, 8]. Pure standards of the isomers were not available for the present work. Consequently, they have been measured as DAG itself (assuming the same molar extinction at 226 nm) and are presented as the individual positional isomers by summation of the contributions from each α- and β-anomer pair.

Analysis of covalently-bound DF. For analysis of DF-protein adducts in the incubation mixtures, the timed 0.5-mL aliquots were immediately precipitated with 1.0 mL of a solution of 4% v/v acetic acid in acetonitrile, and the mixtures vigorously vortexed and centrifuged. The supernatant was then discarded and the pellet resuspended in 1.5 mL of a mixture of 4% v/v acetic acid in acetonitrile (2 vol.) and 0.01 M NaH_2PO_4/Na_2HPO_4 pH 4.5 (1 vol.), vigorously vortexed and again centrifuged. This washing procedure was repeated nine times. The pellet was then gently dried at 30° under a stream of air and digested in 0.5 mL of 1 M NaOH at 65° overnight. The digest was then cooled and acidified with 10 M HCl (100 μL). Internal standard solution (100 μL of 10 μg clofibric acid per mL 0.01 M NaH_2PO_4/Na_2HPO_4 pH 4.5) was added, and the mixture gently equilibrated with 3.5 mL of ether:hexane (1:1 v/v). After centrifugation, the organic layer was transferred to a tapered glass tube and evaporated to dryness at 30° under a stream of air. The sample was reconstituted in 100 μL of HPLC mobile phase and 20 μL injected within 1 hr.

The HPLC system comprised a model 510 pump, RCM-100 radial compression module containing a 4 micron Novapak C-18 cartridge and preceded by a guard column containing Bondapak C-18 Corasil, a model 481 LC spectrophotometer (all from Waters Associates, Milford, MA), a model 7125 injector (Rheodyne Inc., Cotati, CA) and a model C-R3A Chromatopac integrator with FDD-1A disk drive and CRT display (Shimadzu Corp., Kyoto, Japan). The mobile phase consisted of 560 mL of methanol made up to 1:1 with an aqueous phosphate buffer (0.05 M NaH_2PO_4 pH 4.5). The flow rate was 1.8 mL/min in the recycling mode. Column eluant was monitored at 226 nm. Standard curves were prepared by adding DF itself to blank protein pellets followed by digestion and extraction, and were linear over the range 0.1 to 10 μg DF/mL protein solution or plasma.

The protein pellet washing procedure was verified as satisfactory for removal of non-covalently bound DF-related species (originating from DAG) by analysis of each of the 10 washes of a blank human plasma sample spiked to 50 μg DF/mL. (At this concentration, DF is known to be >99.9% reversibly-bound to plasma protein [32].) Each wash was *ca.* 80% efficient, and no traces of DF were found after seven washes. The adequacy of the overnight (*ca.* 18 hr) digestion step for complete base hydrolysis was verified using a human plasma sample incubated with DAG. Digestion for 1 hr gave *ca.* 90%

* Watt JA, King AR and Dickinson RG, submitted.

of the DF released overnight; extended digestion for 36 hr failed to liberate further DF.

Plasma protein analysis. Albumin and total protein concentrations in human and rat plasma samples were measured by the Department of Pathology, Royal Brisbane Hospital, using an automated multiple biochemical analyser.

Samples from rats dosed with DF. Two male Sprague–Dawley-derived rats (*ca.* 330 g) were obtained from the University of Queensland Medical Faculty Animal House, and fitted, under ether anaesthesia, with a catheter in the jugular vein [33]. After recovery from the anaesthesia, the rats were maintained unrestrained in metabolism cages and given bolus i.v. doses of 30 mg DF/kg (10 mg DF/mL 0.1 M $NaHCO_3$ solution) according to the following regimen: day 1, 3:00 p.m. (surgery commenced at 9:00 a.m.); days 2–5, 9:00 a.m. and 3:00 p.m. Blood samples (1 mL) were withdrawn at *ca.* 2:45 p.m. on day 1 and on 6 of the following 8 days. Plasma (0.5 mL) was assayed for covalently-bound DF.

Samples from renal failure patients given DF. The elimination of DF and its conjugates in patients with renal failure had been investigated in a separate study (unpublished) which involved oral administration of a single 250 mg DF dose to a panel of volunteers. Blood samples were collected over 5 days, and the plasma immediately separated and snap frozen. Plasma samples from two patients were not required for that study (because of incomplete urine collections), and were analysed for covalently-bound DF. Patient 1 (a 32-year-old female) and patient 2 (a 56-year-old male) had the following plasma chemistry (respectively): urea 33.1, 28.7 mmol/L; creatinine 0.97, 0.62 mmol/L; albumin 39, 45 g/L, total protein 66, 73 g/L. Both were on multiple medication.

RESULTS

The profiles of rearrangement of DAG (initial concentration 52.5 μg DF equivalents/mL) to its acyl-migrated isomers and of concurrent liberation of DF by hydrolysis of DAG and/or its isomers in pH 7.4 buffer at 37° are shown in Fig. 1. Under these specified conditions, the half-life of DAG was *ca.* 37 min, with rearrangement clearly predominating over hydrolysis in the early stages of reaction (Table 1 and Fig. 1). At 1 hr, 68% of the DAG had disappeared, but only 10% had been hydrolysed to DF. The same experiment carried out at an initial DAG concentration of 12 μg DF equivalents/mL (not shown) gave profiles for DAG, its isomers and DF which were comparable to those in Fig. 1, but at *ca.* 1/5 concentrations. It should be noted that in this and previous work [5], the attachments of the aglycone moiety of the isomers at the 2-, 3- and 4-*O*-positions of the glucuronic acid ring have been identified by their order of appearance during acyl migration of DAG. Because of the absence of pure reference standards, the isomers have been quantified as DAG itself. Consequently, the values should strictly be regarded as semiquantitative, though only minor differences would be expected between the chromophores of DAG and its isomers. Indeed, the recovery

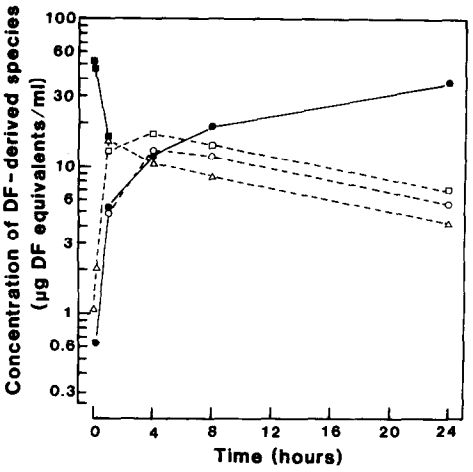

Fig. 1. Profiles for rearrangement and hydrolysis reactions of diflunisal acyl glucuronide (DAG, ■—■), initial concentration 52.5 μg DF equivalents/mL, at 37° in phosphate buffer pH 7.4. The 2-*O*-acyl (△---△), 3-*O*-acyl (□---□) and 4-*O*-acyl (○---○) isomers arising from rearrangement of DAG via acyl migration also liberate diflunisal (DF, ●—●) by hydrolysis.

data in Table 1 are in broad agreement with this belief.

The profiles of rearrangement and hydrolysis of DAG (initial concentration 55.6 μg DF equivalents/mL) during incubation in a solution of RSA (essentially globulin- and fatty acid-free) at pH 7.4 and 37° are shown in Fig. 2A. DAG disappearance was slightly slower (half-life 48 min) in comparison to incubation in buffer alone (Table 1), and concentrations of the rearrangement isomers were considerably lower throughout the 24 hr incubation period. Formation of the DF-albumin adduct (Fig. 2A) was presumed to occur via a covalent binding mechanism since alkaline digestion, but not exhaustive solvent washing, of the precipitated protein pellet liberated this DF fraction. That DAG and/or its isomers were the precursors of the DF-protein adduct was verified when incubation of DF itself with RSA (and subsequently HSA) under the same conditions failed to yield detectable covalent binding of DF to the protein. It was not apparent from the concentration–time profiles (Fig. 2A) which of DAG and its isomers was the primary precursor of the DF-protein adduct. The peak concentration of the adduct was found at 8 hr: further incubation resulted in substantial reduction in the adduct concentration, presumably by hydrolysis to free DF. Analogous profiles (not shown) were obtained using an initial DAG concentration of 10.8 μg DF equivalents/mL, with the peak adduct concentration again achieved at 8 hr (Table 1).

Initial concentrations of *ca.* 50 and 10 μg DF equivalents/mL were also used for incubation of DAG with unbuffered, heparinized rat plasma (Fig. 2B and Table 1). Without buffer control, the pH steadily increased to *ca.* 8.5 over the incubation period. In this milieu, DAG disappeared more rapidly: half-lives (24 and 30 min, Table 1) were

Table 1. Kinetic constants and recovery data during incubation of diflunisal acyl glucuronide at 37° in pH 7.4 buffer, in purified rat and human serum albumin solutions pH 7.4, and in unbuffered rat and human plasma

Incubation mixture	Initial DAG concentration (μg DF equiv./mL)	DAG half-life (min)	Percentage recovery after 1 hr incubation					Maximum concentration of DF–protein adduct*	
			DAG	Sum of DAG isomers	Free DF	DF–protein adduct	Total	ng/mg albumin	ng/mg protein
Buffer (pH 7.4)	52.5	37	32	62	10	—	104	—	—
	12.0	34	29	52	9	—	90	—	—
RSA (globulin and fatty acid-free, pH 7.4)	55.6	48	43	34	13	1.2	91	87	87
	10.8	46	40	35	12	3.5	91	30	30
Rat plasma (unbuffered)	48.0	28	22	58	21	2.0	103	85	46
	9.7	34	29	54	17	3.1	103	13	7
HSA (fatty acid-free, pH 7.4)	54.9	86	61	27	6	5.8	100	165	165
	10.9	101	69	17	5	7.6	99	39	39
HSA (globulin and fatty acid-free, pH 7.4)	48.8	51	53	25	20	2.5	101	152	152
	9.9	53	45	21	20	3.4	89	36	36
Human plasma (unbuffered)	49.4	36	31	56	11	5.8	104	73	43
	11.6	41	36	35	6	5.5	83	22	13

* Maximum concentrations of the adduct occurred at 8 hr except human plasma 4 hr.

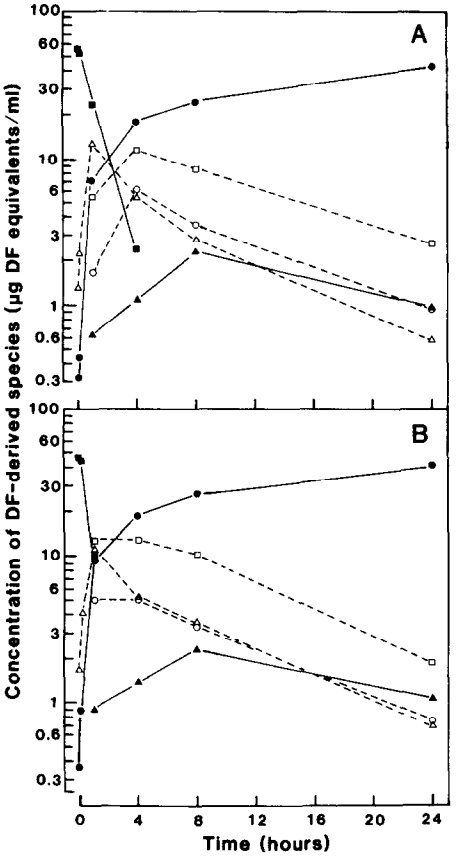

Fig. 2. Profiles for rearrangement, hydrolysis and covalent binding reactions of diflunisal acyl glucuronide (DAG, ■—■), initial concentration *ca.* 50 μg DF equivalents/mL, at 37° in solutions of globulin- and fatty acid-free rat serum albumin buffered to pH 7.4 (panel A) and in unbuffered rat plasma (panel B). The 2-*O*-acyl (△---△, 3-*O*-acyl (□---□) and 4-*O*-acyl (○---○) isomers arising from rearrangement of DAG by acyl migration also liberate diflunisal (DF, ●—●) by hydrolysis, and may further act as precursors for covalent binding of DF to protein (▲—▲).

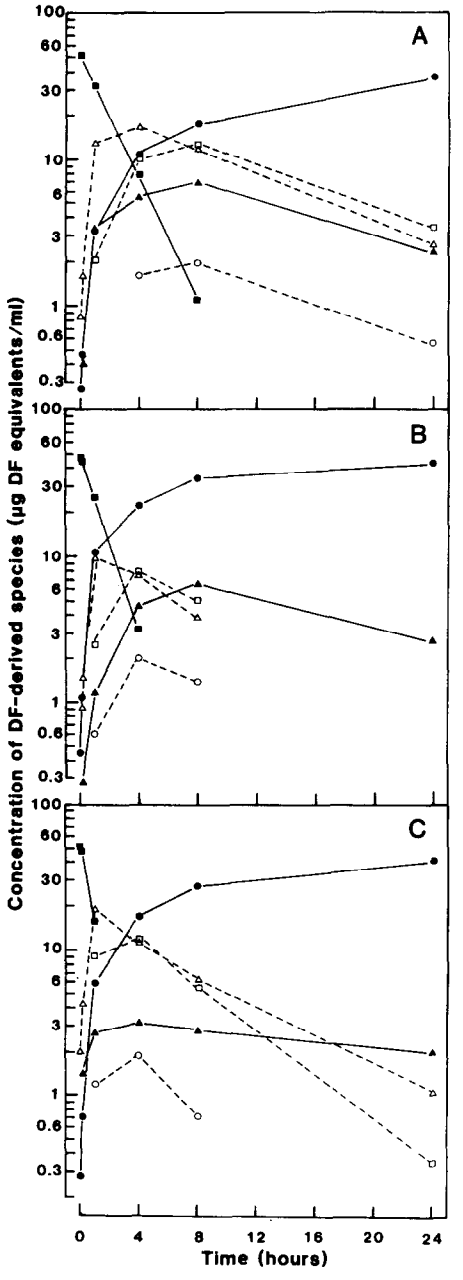

Fig. 3. Profiles for rearrangement, hydrolysis and covalent binding reactions of diflunisal acyl glucuronide (DAG, ■—■), initial concentration *ca.* 50 μg DF equivalents/mL, at 37° in solutions of fatty acid-free human serum albumin buffered to pH 7.4 (panel A), globulin- and fatty acid-free human serum albumin buffered to pH 7.4 (panel B) and in unbuffered human plasma (panel C). The 2-*O*-acyl (△---△), 3-*O*-acyl (□---□) and 4-*O*-acyl (○---○) isomers arising from rearrangement of DAG by acyl migration also liberate diflunisal (DF, ●—●) by hydrolysis, and may further act as precursors for covalent binding of DF to protein (▲—▲).

lower than those found in the purified albumin solution (48 and 46 min) and in buffer alone (37 and 34 min). This acceleration can be attributed, at least in part, to the higher pH values, and perhaps as well to the presence of other plasma constituents (e.g. esterases?) capable of participating in the reactions. Nonetheless, the profiles of covalent binding of DF to plasma protein (Fig. 2B) were similar to those found in purified RSA solutions. As rat plasma contained 30 mg/mL albumin and 56 mg/mL total protein, it could not be assumed that DF became covalently bound only to albumin. Consequently, Table 1 shows the peak adduct concentrations (at 8 hr) related to both albumin itself and to total plasma protein.

DAG was also incubated at 37° with two different purified HSA fractions (essentially fatty acid-free, and essentially globulin- and fatty acid-free, see Materials and Methods) prepared in pH 7.4 buffer, and with unbuffered, heparinized human plasma. The concentration profiles of DAG, its isomers, free DF and the DF-protein adduct resulting from 24 hr incubation of DAG (initial concentration *ca.* 50 μg DF equivalents/hr) with each of the above preparations are shown in Fig. 3A, B and C, respectively.

An initial DAG concentration of *ca.* 10 μg DF equivalents/mL was also investigated for each preparation. Profiles of the DF-related species were qualitatively similar in each case to their counterparts in Fig. 3, and are not shown.

Incubation in fatty acid-free HSA caused a marked reduction in the rate of DAG disappearance (half-lives 101 and 86 min, Table 1). Although the profile of the 2-isomer during the first 4 hr of incubation (Fig. 3A) was practically superimposable on that obtained in buffer alone (Fig. 1), concentrations of the 3- and 4-isomers, and of free DF, were considerably reduced. The concentration of the DF-protein adduct was highest at 8 hr, where it accounted for *ca.* 14% of reactant DAG. Disappearance of DAG was also retarded in the presence of globulin- and fatty acid-free HSA (half-lives 51 and 53 min, Table 1), though not to the same extent found for the (less pure) fatty acid-free HSA fraction. Comparison of the concentration–time profiles in Fig. 3B with those in Figs 3A and 1 reveals that isomer concentrations were reduced and appearance of free DF vastly accelerated, suggesting promotion of direct hydrolysis of DAG by this albumin preparation. Whereas covalent binding of DF to protein was substantially reduced during the first 4 hr of incubation, subsequent concentrations were quite comparable to those found for the fatty acid-free HSA. It seems clear from these results that both DAG and its isomers act as precursors for the DF-albumin adduct. However, it was surprising that such major differences in the profiles were obtained from the two highly-purified HSA fractions, particularly as both were specified as essentially free of fatty acids. In unbuffered heparinized human plasma (Fig. 3C), acceleration of DAG disappearance (half-lives 36 and 41 min) was associated with lower concentrations of free DF than those found in globulin- and fatty acid-free HSA. Although covalent binding of DF to protein peaked earlier and at a lower concentration, the adduct appeared to be more stable during further incubation (even in the absence of pH control of the plasma by buffering).

In broad terms, there was a greater degree of formation of DF adducts to purified HSA and human plasma proteins than to purified RSA and rat plasma proteins (Table 1). This covalent binding of DF via DAG and/or its isomers appeared to be slowly reversible under the controlled conditions of these incubations. Thus it was of considerable interest to determine whether detectable comparable reactions could occur *in vivo* in rats and humans after administration of the parent drug itself.

In an exploratory experiment, two rats were dosed i.v. with DF at 30 mg/kg once on the first day and twice daily thereafter for 4 days. Figure 4 shows the profile of covalent binding of DF to plasma protein during dosing, and partial elimination of the adduct over the following days. Concentrations of free DF and DAG were not measured in this study. Other work [4] would suggest that peak concentrations of DAG would not have exceeded 10 μg DF equivalents/mL plasma.

Human renal failure patients given DF orally have been shown to have impaired elimination of the drug and its glucuronides from plasma [34, 35]. In a recent

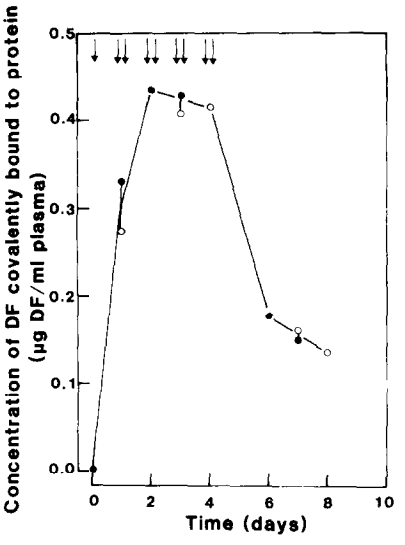

Fig. 4. Profile of covalent binding of diflunisal (DF) to plasma protein of two rats (● and ○) given nine 30 mg DF/kg doses i.v. over 5 days (marked by the arrows).

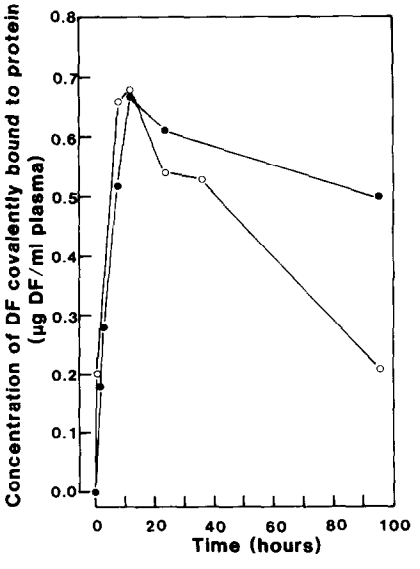

Fig. 5. Profile of covalent binding of diflunisal (DF) to plasma protein of two renal failure patients (● and ○) given a single oral 250 mg DF dose at time zero.

re-investigation of DF disposition in human renal failure, plasma samples from two patients given a single 250 mg oral dose of the drug were not required because of missed urine collections. These samples were therefore investigated to determine whether DF became covalently bound to plasma proteins of humans *in vivo*. Some details of the patients are given in Materials and Methods. Although DAG concentration in plasma of the patients never exceeded 3 μg DF equivalents/mL and was not measurable 48 hr after ingestion of the dose, covalent binding of DF to protein was indeed found (Fig. 5).

Peak levels were achieved *ca.* 12 hr after dosing, with measurable concentrations persisting for at least 4 days.

DISCUSSION

This study documents the reactivity of DAG in solutions of albumin and plasma along three interrelated pathways namely rearrangement via acyl migration to yield positional isomers of DAG, hydrolysis of DAG and/or its isomers to yield DF, and interaction of DAG and/or its isomers with protein to yield covalent DF-protein adducts. For the purified rat and human albumin solutions (buffered to pH 7.4), albumin concentrations were selected to match those found in rat and human plasma (*ca.* 30 and 46 mg/mL, respectively). The plasma samples themselves were not buffered (i.e. their pH values were allowed to increase over the incubation period) and contained lithium heparin (at *ca.* 125 I.U./10 mL whole blood) in order to mimic the conditions under which such samples would have been treated had they been taken as routine specimens from rats or humans dosed with DF. Inspection of the profiles in Figs 1–3 and the recovery data in Table 1 reveals that, in all solutions (including buffer alone) in the early stages of reaction whilst DAG was still measurable, the order of quantitative importance of the competing alternative reactions was: rearrangement > hydrolysis > covalent binding. Nonetheless, there were clear differences in the various concentration–time profiles e.g. the isomers of DAG tended to persist longer in RSA and rat plasma than in HSA and human plasma. These differences may be considered as relatively minor given the different animal origins of the samples or their state of purification. It was surprising, however, to find relatively major differences in the profiles of rearrangement and hydrolysis for the two highly-purified HSA fractions (Fig. 3A and B). Both fractions were specified by the supplier as essentially free of fatty acids, though the globulin content differed. This finding suggests that seemingly trivial differences in purity can be important [22], and that caution should be exercised in interpretation of such comparative data.

Disappearance of DAG followed first-order kinetics over its measurable concentrations (Figs 1–3). Subsequent loss of the rearrangement isomers (formed as a result of this degradation) was much slower than that of DAG itself, with profiles of the individual 2-, 3- and 4-*O*-isomers being essentially parallel. These results are consistent with current perception of the reversibility of acyl migration i.e. reversibility exists between the 2-, 3- and 4-positions, but the parent 1-*O*-β acyl glucuronide itself is not reformed. Hansen-Moller *et al.* [8] presented evidence for minor regeneration of DAG from its isomers at pH 8.0, though all other work on rearrangement of acyl glucuronides has concluded that this particular migration seems not to occur. Whereas formation of DF by hydrolysis of DAG and its isomers is irreversible in albumin solutions and plasma, slow degradation of the covalent DF-protein adducts, presumably by spontaneous hydrolysis, was observed in all incubation mixtures.

Transacylation reactions of acyl glucuronides with simple chemical nucleophiles are well known [19, 36], but the mechanisms of the covalent binding reactions to protein, including the identities of the recipient nucleophilic groups, are uncertain. Transacylation of —SH [20], —OH [21] and —NH_2 [29] groups of protein residues have been postulated: such thioester, ester and amide linkages would be expected to have different stabilities under physiological conditions [17, 19]. Benet *et al.* proposed a non-transacylation mechanism for covalent binding of zomepirac [23], tolmetin [24] and carprofen [26] to plasma albumin, via the acyl-migrated isomers but not the parent biosynthetic glucuronides themselves. They suggested that the binding reaction was initiated by formation of an imine between a lysine residue of albumin and the 1-position of glucuronic acid. Such a mechanism apparently does not apply to covalent binding of oxaprozin to HSA [21]. This work found substantial incorporation of radioactivity into HSA when the label was located in the oxaprozin moiety, but not when it was located in the glucuronic acid moiety. They also found the 2-isomer to be far less reactive than oxaprozin glucuronide itself.

Degradation of zomepirac glucuronide in blood and plasma was found to be faster than in buffer [37]. Ruelius *et al.* [21] also found accelerated degradation of oxaprozin glucuronide in HSA solutions and plasma: indeed, they showed that albumin was catalytic for all three reactions (rearrangement, hydrolysis and covalent binding). They hypothesized that the first step in the reaction sequence was reversible binding of oxaprozin glucuronide to the benzodiazepine binding site of albumin (site II according to the classification of Sudlow *et al.* [38]). Within this site, which contains an active tyrosine residue, all the reactions were catalysed. Support for this hypothesis was obtained when other agents, including oxaprozin itself, which strongly bind to the site, inhibited the three reactions [21, 22]. In the present study, degradation of DAG was retarded, rather than accelerated, in purified albumin solutions, and the rates of degradation in unbuffered rat and human plasma were comparable to those found in buffer pH 7.4 (Table 1). In fatty acid-free HSA, the DAG half-life was *ca.* three times that found in buffer. These results can be readily explained if the primary site of reversible binding of DAG to albumin is different to the primary site catalysing degradation of DAG including covalent attachment of (at least) the DF moiety. In other work from this laboratory (unpublished), we have shown that DAG is highly protein-bound (reversibly) in both rat plasma (97–98%) and human plasma (*ca.* 99%) at concentrations of 10–20 μg DF equivalents/mL. The site of this binding is not known, but DF itself is known to bind (reversibly) even more strongly (*ca.* 99.7% in rat plasma [2] and 99.9% in human plasma [32]) at these concentrations. DF has been classified as binding primarily to site I (the warfarin binding site) of HSA [39]. Thus, if e.g. the primary site of reversible binding of DAG to albumin is site I, and covalent binding occurs primarily at e.g. site II, then the high affinity of DAG for the former site may indeed protect it from catalytic degradation at the latter site. Further study on such speculation is clearly required.

The peak concentrations of covalently-bound adducts achieved in the purified albumin solutions were higher than those achieved in whole plasma (Table 1). This no doubt reflects the multiple effects of other plasma constituents e.g. other proteins, esterases and fatty acids capable of participating in the reactions e.g. by competing for binding sites or catalysing hydrolysis of the ester linkages of DAG and its isomers. Heparin contained in the plasma may also play some role by inducing displacement of non-esterified fatty acids from their binding sites [40]. Since the plasma samples were not buffered, the higher pH values will certainly accelerate the reactions, perhaps in a differential manner. Thus these plasma experiments reveal the extent to which the three reactions will occur *ex vivo* if normal precautions are not taken to stabilize DAG (i.e. acidification to pH 3–5 and cooling).

The covalent binding reaction is currently attracting interest because of its potential to mediate toxic responses i.e. the possibility exists that such protein chemically modified *in vivo* may be immunogenic. This possibility has been raised particularly with zomepirac [23] and tolmetin [24], where covalent adducts were found in plasma of human volunteers ingesting single doses of the drugs. On the other hand, Ruelius *et al.* [21] concluded that there was little or no potential for biological significance of covalent binding of oxaprozin to protein via its glucuronide, because detectable concentrations of the glucuronide had never been found in plasma of any species. Furthermore, their *in vitro* studies showed that oxaprozin adduct formation was strongly inhibited by low concentrations of oxaprozin itself. Similar results and conclusions were reached by Weil *et al.* [25] in regard to fenofibric acid. In the present study, both an exploratory experiment giving multiple doses of DF to rats (Fig. 4) and an opportunistic examination of surplus plasma samples from two renal failure patients given a single dose of 250 mg DF (as part of a different investigation) revealed covalent attachment of DF to plasma protein *in vivo*, presumably via DAG. Although these experiments also revealed *in vivo* degradation of the covalent adducts, they did indeed persist in plasma for several days following cessation of dosing. Further work is obviously needed to document the occurrence and extent of such binding in patients taking the drug chronically, but it is premature to suggest that toxic sequelae may be linked to DF–protein adduct formation. For example, immunological consequences of prolonged circulation of covalent bilirubin–albumin adducts [30] in man *in vivo* have not been reported, to our knowledge. With the exception of one recent report [31], only mild hypersensitivity reactions have been associated with DF intake.

The existence of covalent drug–protein adducts adds a new dimension of complexity to true analysis of acidic drugs in the presence of their acyl glucuronide conjugates. With recognition of the potential of acyl glucuronides to undergo acyl migration reactions, routine use of β-glucuronidase enzymes in such analyses was questioned, because the biosynthetic glucuronides themselves, but not their rearrangement isomers, are substrates for this family of enzymes. Thus isomers arising from acyl migration *in vivo* and in biological samples *ex vivo* would not be measured. Alkaline hydrolysis was therefore promoted as a better alternative. Such a step applied to plasma samples will (most likely) also cleave any covalent drug–protein adduct. Thus cautious assessment must be made of the true origin of parent drug liberated by alkaline hydrolysis.

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