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STUDIES ON THE REACTIVITY OF ACYL GLUCURONIDES—VI

MODULATION OF REVERSIBLE AND COVALENT INTERACTION OF DIFLUNISAL ACYL GLUCURONIDE AND ITS ISOMERS WITH HUMAN PLASMA PROTEIN *IN VITRO**

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Abstract—Acyl glucuronide conjugates are chemically reactive metabolites which can undergo hydrolysis, rearrangement (isomerization via acyl migration) and covalent binding reactions with protein. The present study was undertaken to identify factors modulating the reactivity of diflunisal acyl glucuronide (DAG) with human serum albumin (HSA) *in vitro*, by comprehensively evaluating the interplay of the three pathways above when DAG and a mixture of its 2-, 3- and 4-isomers (*iso*-DAG) were incubated with protein. Buffer, plasma, fraction V HSA, fatty acid-free HSA, globulin-free HSA and fatty acid- and globulin-free HSA were investigated at pH 7.4 and 37°, each in the absence and presence of warfarin, diazepam and diflunisal (DF) as reversible binding competitors. DAG and *iso*-DAG were highly reversibly bound (*ca.* 98–99.5%) in plasma and HSA solutions. The binding was primarily at the benzodiazepine site, since displacement occurred in the presence of diazepam and fatty acids but not warfarin. DAG degradation, via rearrangement, hydrolysis and covalent adduct formation (in that order of quantitative importance), was retarded in plasma and HSA solutions compared to buffer. The protective effect of protein was afforded by the high reversible binding to the (non-catalytic) benzodiazepine site. The warfarin site appeared to be catalytic for DAG hydrolysis, whereas rearrangement appeared to be hydroxide ion-catalysed only. In contrast to DAG, *iso*-DAG degradation was greatly accelerated in the presence of protein, through both covalent binding and catalysis of hydrolysis. Covalent binding via DAG was increased in the presence of warfarin but decreased in the presence of diazepam, DF and fatty acids. The opposite effects were found for covalent binding via *iso*-DAG. The data suggest that covalent binding of DF to HSA via DAG and *iso*-DAG occurs by different mechanisms (presumably transacylation and glycation, respectively) at different sites (benzodiazepine and warfarin, respectively) whereas reversible binding occurs primarily at the same site (benzodiazepine).

Acyl glucuronides are frequently the major metabolites of drugs bearing carboxylic acid groups, and have been shown to be potentially reactive conjugates capable of undergoing hydrolysis, intramolecular rearrangement and intermolecular covalent binding reactions with protein [1, 2]. This reactivity is due to the inherent susceptibility of the ester group linking the drug and glucuronic acid moieties to nucleophilic attack. The mechanism of intramolecular rearreangement is well established [3] and occurs via base-catalysed acyl-migration of the drug moiety to adjacent 2-, 3- and 4-hydroxy groups on the glucuronic acid ring (Fig. 1). Migration between the three positional isomers is reversible but the parent 1-*O*-acyl glucuronide is not reformed. The isomers (but not the 1-*O*-acyl-β glucuronide itself) can exist in both β- and α-anomeric forms (at C-1) through the intermediacy of the open-chain form of the glucuronic acid ring [3, 4].

Formation of covalent drug–protein adducts has been demonstrated for a number of carboxylic drugs [2]. Two distinct mechanisms—direct transacylation [5, 6] and rearrangement/glycation [7, 8]—have been proposed. Transacylation involves direct attack on the ester group by a nucleophilic group such as -SH, -OH and -NH_2 on the protein, resulting in displacement of the glucuronic acid moiety and direct linkage of the drug to protein via a thioester, ester or amide bond. The glycation (or Schiff's base/Amadori rearrangement) mechanism requires prior acyl rearrangement to permit ring-opening of the glucuronic acid moiety. The aldehyde group so produced at the C-1 position can then form an imine by condensation with a lysine $\in$-amino group. Subsequent Amadori rearrangement could then yield a stable ketoamine derivative. In this mechanism, the drug remains linked via an ester group to the glucuronic acid moiety which in turn is linked to the protein. Evidence has been presented variously in support of each mechanism, though recently mass spectrometric studies have confirmed formation of tolmetin–albumin adducts via the glycation mechanism [9].

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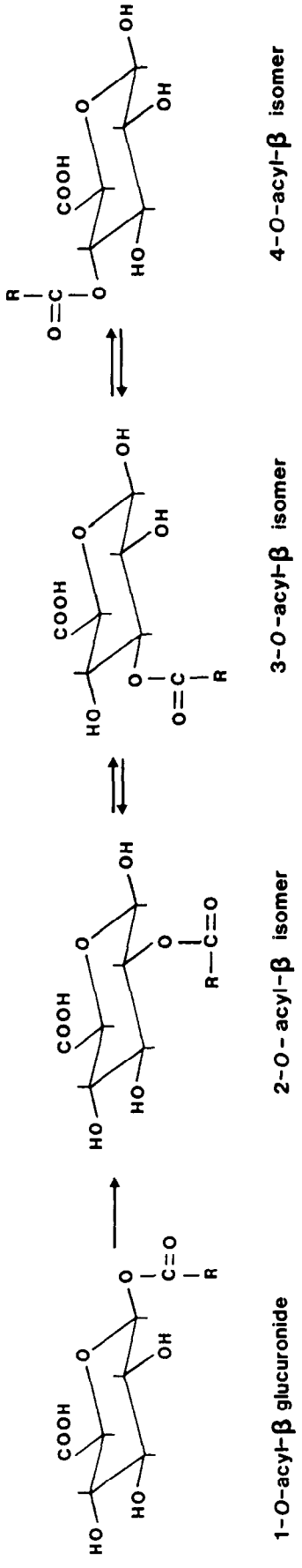

Fig. 1. Scheme showing rearrangement of biosynthetic, β-glucuronidase-susceptible 1-*O*-acyl-β glucuronides to the β-glucuronidase-resistant 2-, 3- and 4-*O*-acyl positional isomers.

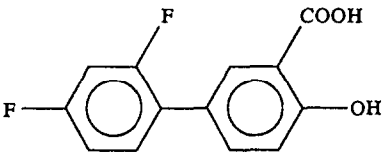

Fig. 2. Chemical structure of diflunisal.

Human serum albumin (HSA‡) is known to bind numerous endogenous and exogenous ligands at specific and non-specific binding sites. The location of these binding sites has been reviewed in detail [10–12], but there is no complete agreement about their number and nature. Perhaps the best characterized binding sites on albumin are the warfarin and benzodiazepine sites, designated also as sites I and II, respectively [13, 14]. Drugs binding at site I include aspirin, warfarin and phenylbutazone analogues, while drugs binding to site II include diazepam, flurbiprofen and related arylpropionic acids [11]. An active§ tyrosine residue and an active lysine have been implicated in binding at the benzodiazepine site, whereas a number of active lysine residues have been identified within the warfarin binding region [12]. Identification of the sites of interaction of acyl glucuronide metabolites with HSA *in vitro* should assist in determination of the factors modulating acyl glucuronide reactivity in plasma *in vivo*.

We have shown previously that the anti-inflammatory drug diflunisal (DF, Fig. 2) is metabolized in humans and rats to a reactive acyl glucuronide (DAG), a stable phenolic glucuronide and a stable sulphate conjugate [4, 15, 16]. DAG has been shown to undergo hydrolysis and rearrangement and to form covalent protein adducts *in vitro* [17, 18]. We have also demonstrated the occurrence of these reactions in rats and humans, *in vivo* [4, 15, 18, 19]. In an earlier study investigating the reactivity of DAG in human and rat albumin solutions, major differences in the effects of two highly purified HSA fractions were observed [18]. The present study was undertaken to identify factors modulating acyl glucuronide reactivity, by evaluating comprehensively the reactivity of both DAG and a mixture of its rearrangement isomers (*iso*-DAG) in plasma and various HSA preparations, both in the absence and presence of reversible binding competitors.

‡ Abbreviations: DF, diflunisal; DAG, diflunisal acyl glucuronide; *iso*-DAG, mixture of 2-, 3- and 4-*O*-acyl isomers of diflunisal acyl glucuronide formed by acyl migration; HSA, human serum albumin; FAF-HSA, essentially fatty acid-free human serum albumin; GF-HSA, essentially globulin-free human serum albumin; FA&GF-HSA, essentially fatty acid- and globulin-free human serum albumin; FrV-HSA, fraction V human serum albumin; $T_{1/2}$, half-life; k, slope of log concentration–time profile.

§ In this context, an "active" residue is one known or presumed to be directly involved in binding/catalytic interactions within the binding site.

MATERIALS AND METHODS

Materials. DF and warfarin were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Authentic samples of DAG and its 2-, 3- and 4-*O*-acyl migration isomers were prepared as described previously [4, 15]. Diazepam was a gift from Roche Products Pty Ltd (Sydney, Austalia). Also gifts were clofibric acid (ICI Pharmaceuticals, Macclesfield, U.K.) and ketoprofen (May & Baker Pty Ltd, Melbourne, Australia). Solvents were HPLC grade and obtained from Mallinckrodt Pty Ltd (Melbourne, Australia). Reagents were of AR grade purity.

HSA fractions purchased from the Sigma Chemical Co. were supplied with following specifications: (1) fraction V (FrV-HSA), Product A1653, 96–99% albumin, remainder mostly globulins; (2) essentially fatty acid-free (FAF-HSA), Product A1887, <0.005% fatty acids, <3% α-1 and α-2 globulins, no β-globulin; (3) essentially globulin-free (GF-HSA), Product A8763, 0.5% α-1 globulin, 0.4% α-2 globulin, no β-globulin, <0.005% fatty acids (but see Results); (4) essentially fatty acid- and globulin-free (FA&GF-HSA), Product A3782, <0.005% fatty acids, <1% α-1 globulin, no α-2 or β-globulin. Plasma was obtained from heparinized blood (125 IU lithium heparin/10 mL) from a healthy human volunteer not on any medication.

Reversible protein binding studies with DAG and iso-*DAG.* The unbound fractions of DAG and *iso*-DAG in plasma and HSA solutions were determined by ultrafiltration using Ultrafree-MC units, 10,000 nominal molecular weight limit, regenerated cellulose membrane (Millipore, Bedford, MA, U.S.A.). Stock solutions of DAG and *iso*-DAG were prepared at 270–280 μg DF equivalents/mL of 0.01 M sodium phosphate buffer pH 5.0 and stored frozen until required. HSA solutions (FrV-HSA, FAF-HSA, GF-HSA and FA&GF-HSA) were prepared at 43 mg/mL of 0.1 M sodium phosphate buffer pH 7.4. Plasma was freshly collected and buffered to pH 7.4 (8.0 mL plasma, 0.80 mL 1 M sodium phosphate buffer, pH 7.1).

DAG or *iso*-DAG stock solution (0.33 mL) was added to 4.17 mL of HSA solution or plasma (final concentrations of 20 μg DF equivalents/mL and 40 mg HSA/mL) immediately prior to ultrafiltration. Due to the low capacity of the filtration units, duplicate samples (0.4 mL) were centrifuged at 10,000 *g* for 15 min at room temperature. Filtrates from the duplicates were pooled and a 200 μL aliquot frozen until analysis. The time from mixing of DAG or *iso*-DAG with protein until freezing of the filtrate was less than 40 min.

The effect of reversible binding competitors was determined by ultrafiltration using Ultrafree-CL units, 10,000 nominal molecular weight limit, regenerated cellulose membrane (Millipore). Stock solutions of DAG and *iso*-DAG were prepared at 380 μg DF equivalents/mL of 0.01 M sodium phosphate buffer pH 5.0 and stored frozen until required. HSA solutions were prepared at 21.6 mg/mL of 0.1 M sodium phosphate buffer, pH 7.4. Plasma was collected fresh, diluted to obtain *ca.* 20 mg albumin/mL (20 mL plasma, 30 mL 0.1 M sodium phosphate buffer, pH 7.4) and then buffered to pH 7.4 (50 mL diluted plasma, 5 mL 1 M sodium phosphate buffer, pH 7.1). Warfarin and diazepam stock solutions were prepared freshly (52.8 mM in 0.1 M NaOH and 0.5 M HCl, respectively) and 100 μL samples added to the HSA and buffered plasma solutions (9.8 mL) along with 100 μL of 0.1 M HCl (for warfarin) or 0.5 M NaOH (for diazepam) to maintain the pH at 7.4. Water (200 μL) was added to HSA and buffered plasma solutions (9.8 mL) in the case of controls and all solutions were mixed gently at room temperature for 15 min. Immediately prior to ultrafiltration DAG or *iso*-DAG stock solution (240 μL) was added to 4.36 mL of the prepared protein solutions, yielding final concentrations of DAG or *iso*-DAG at 20 μg DF equivalents/mL (80 μM), HSA at 20 mg/mL (300 μM) and warfarin or diazepam at 500 μM. Samples (0.8 mL) were centrifuged as described above. Duplicate samples were not required in these larger capacity ultrafiltration units.

Incubation of DAG and iso-*DAG in buffer, HSA solutions and plasma.* DAG and *iso*-DAG were incubated at 20 μg DF equivalents/mL at 37° for 4 hr in solutions of sodium phosphate buffer (0.1 M, pH 7.4), the four HSA solutions (40 mg/mL of 0.1 M sodium phosphate buffer, pH 7.4) and fresh human plasma buffered to pH 7.4 (*vide supra*). Buffer and protein solutions were prewarmed to 37° and incubation initiated by addition of DAG or *iso*-DAG stock. Two aliquots (50 and 500 μL) were withdrawn after 1, 10 and 30 min and 1, 2 and 4 hr incubation. The 50 μL aliquots were used to measure reversibly bound DAG, its isomers and DF, while DF bound covalently to protein was measured using the 500 μL samples.

To assess the effects of reversible binding competitors on DAG and *iso*-DAG reactivity in the presence of protein, solutions of HSA and plasma were prepared in the absence and presence of warfarin and diazepam as described above for the reversible protein binding experiments. In addition, the protein solutions were prepared in the presence of DF itself as competitor, by mixing 100 μL of a stock solution of DF (52.8 mM in 0.1 M NaOH) with HSA solution or plasma (9.8 mL) and 0.1 M HCl (100 μL). The prepared protein solutions were prewarmed to 37° and incubations initiated by addition of DAG or *iso*DAG stock solutions, yielding final concentrations of DAG and *iso*-DAG at 20 μg DF equivalents/mL (80 μM), HSA at 20 mg/mL (300 μM) and warfarin, diazepam and DF at 500 μM.

Analysis of DAG, iso-*DAG and DF.* The unbound fractions of DAG and *iso*-DAG in the ultrafiltrate were determined following alkaline hydrolysis to DF [20]. Aliquots of filtrate (200 μL) were heated with 2 M NaOH (100 μL) for 30 min at 90°. After cooling and acidification (150 μL of 10 M HCl), internal standard (50 μL of 100 μg ketoprofen/mL methanol) was added, and the mixture equilibrated with 3 mL of ether:hexane (1:1 v/v). After centrifugation, the organic layer was removed and evaporated to dryness under a stream of air. The sample was reconstituted in 200 μL of HPLC mobile phase and 100 μL injected. The HPLC system and mobile phase were as described previously [17, 20].

Reversibly bound DAG, *iso*-DAG and DF in the

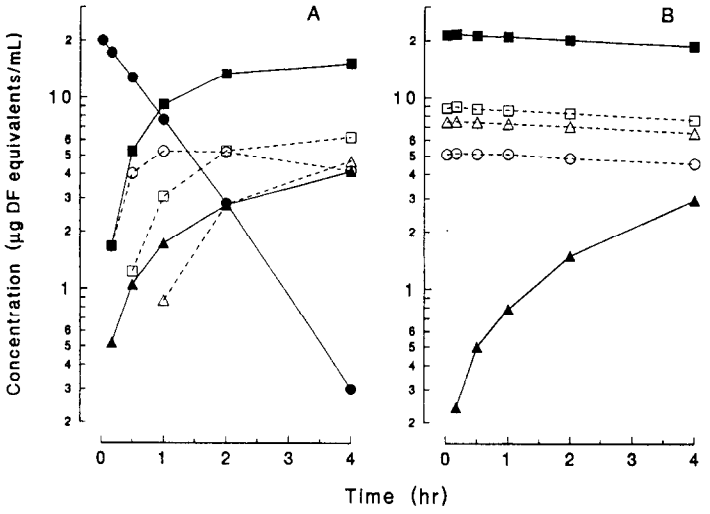

Fig. 3. Profiles for rearrangement and hydrolysis following incubation of DAG (●) (panel A) and a 5:8:7 mixture of its 2-isomer (○), 3-isomer (□) and 4-isomer (△) (collectively ■) (panel B) in buffer at pH 7.4 and 37°. Hydrolysis is represented by liberation of DF (▲).

50 μL aliquots of incubation media were quantified using the direct, isocratic HPLC procedure described previously [17]. Quantification of covalently bound DF in the 500 μL samples of incubation media was achieved after exhaustive solvent washing of the precipitated protein followed by base digestion, as described previously [18], excepting that ketoprofen was used as the internal standard.

Data analysis. The half-life ($T_{1/2}$) of DAG was calculated from the slope (k) of the log concentration–time profile, using linear regression analysis of all DAG concentrations over the 4 hr incubation period. For *iso*-DAG (calculated as the sum of the individually measured 2-, 3- and 4-isomers, each as the α- and β-anomers [4]), linear regression analysis was carried out using the concentrations over 0–2 hr incubation. Coefficients of determination (r^2) are given in the text. Estimates of the individual pathways (rearrangement, hydrolysis and covalent binding) initially contributing to DAG and *iso*-DAG degradation were obtained by apportionment of the overall degradation rate constant (k) using the mean composition of DF species measured at 0.5 and 1.0 hr.

Table 1. Reversible protein binding of DAG and *iso*-DAG in plasma and HSA solutions*

Medium	Fraction unbound (%) DAG	*iso*-DAG
Plasma	2.0 ± 0.1 (3)	2.4 ± 0.4 (4)
FrV-HSA	1.9 ± 0.2 (2)	1.6 ± 0.4 (2)
FAF-HSA	0.5 ± 0.2 (2)	1.2 ± 0.3 (2)
GF-HSA	5.9 ± 0.1 (2)	4.6 (1)
FA&GF-HSA	0.6 (1)	0.4 ± 0.0 (2)

* Determined at pH 7.4 and room temperature at initial DAG and *iso*-DAG concentrations of 20 μg DF equivalents/mL of plasma or HSA solution at 40 mg/mL, means ± SD (N).

RESULTS

The profile for DAG degradation ($T_{1/2}$ 0.67 hr) in buffer at pH 7.4 and 37° is shown in Fig. 3A. The major initial pathway is rearrangement via acyl migration. Although DAG is not reformed in this process (Fig. 1), the migrations amongst the 2-, 3- and 4-isomers are reversible. An equilibrium ratio of 5:8:7 (2-isomer:3-isomer:4-isomer) in buffer alone is achieved by about 4 hr. The *iso*-DAG used throughout the present protein-interaction studies was prepared by mixing the individual isomers in this ratio. Slow degradation ($T_{1/2}$ 22.7 hr) of *iso*-DAG by hydrolysis to DF is shown in Fig. 3B.

Against this background of intrinsic chemical reactivity, reversible protein binding of DAG and *iso*-DAG was studied using rapid ultrafiltration (total experimental time <40 min) to minimize artifactual contributions from other DF species being formed. Both DAG and *iso*-DAG were highly bound to protein (Table 1), although the extent of binding varied considerably with the "purity" of the protein preparation used. The unbound fractions of DAG and *iso*-DAG were lowest with FAF-HSA and FA&GF-HSA, and several-fold higher with the (less pure) FrV-HSA and plasma itself. However, the unbound fractions of DAG and *iso*-DAG were substantially increased with GF-HSA (approximately 10-fold when compared with FA&GF-HSA).

DAG and *iso*-DAG degradation in the presence of each of the five different protein preparations at 40 mg/mL was monitored over 4 hr incubation at pH 7.4 and 37°. Concentration–time profiles of the type shown for plasma (Fig. 4) were produced. In

all cases, excellent linearity of DAG degradation was obtained (Table 2). The presence of protein in the incubation medium retarded degradation of DAG, as compared to incubation in buffer alone (Table 2). DAG was most stable in FAF-HSA and FA&GF-HSA solutions (these proteins showed the greatest reversible binding of DAG, see Table 1), and least stable in plasma. The contributions of the competing pathways of DAG degradation, (i.e. rearrangement, hydrolysis and covalent binding) were assessed as soon as practicable in order to minimize contributions (to hydrolysis and covalent binding) from the isomers being formed. The estimates of these pathways are presented as rate constants in Table 2. Quantitatively, rearrangement was the most important pathway of DAG degradation, except in the presence of GF-HSA, where hydrolysis to DF occurred at approximately the same rate as rearrangement. The rate of rearrangement of DAG in plasma was about 2-fold higher than in the HSA solutions but still less than that found in buffer alone. Hydrolysis occurred 2–3 times more rapidly with GF-HSA than with the other HSA preparations, plasma and, indeed, buffer. The initial rate of formation of covalent DF–protein adducts was greatest with plasma and least (being unmeasurable) with GF-HSA (Table 2). The overall profiles of adduct formation from 4 hr incubation of DAG with the five different protein preparations are shown in Fig. 5A. This reveals almost identical profiles for plasma, FAF-HSA and FA&GF-HSA: that for FrV-HSA was parallel but at roughly half the adduct concentrations. Covalent adduct formation with GF-HSA was not measurable until 2 hr i.e. not until considerable rearrangement had taken place.

Following incubation of *iso*-DAG (as a 5:8:7 mixture of the 2-, 3- and 4-isomers) with the five different protein solutions, the concentration–time profiles (illustrated by that for plasma, Fig. 4B) for *iso*-DAG degradation were, in general, less linear over 4 hr than those obtained for DAG degradation. This is presumably a consequence of greater analytical error (quantification of *iso*-DAG requires the summation of six individual HPLC peaks—three isomers each as α- and β-anomers) and real non-linearity (e.g. see the biphasic profile for the 4-isomer in Fig. 4B). For these reasons, kinetic parameters for degradation of *iso*-DAG were calculated only over the first 2 hr incubation (Table 2). In contrast to that observed for DAG as substrate, the presence of protein in the incubation medium grossly accelerated degradation of *iso*-DAG (Table 2). *Iso*-DAG was least stable in plasma and GF-HSA and most stable in FAF-HSA and FA&GF-HSA, with the result for FrV-HSA being intermediate. In terms of the initial competing pathways of *iso*-DAG degradation, hydrolysis was accelerated in the protein solutions as compared to buffer. Hydrolysis and adduct formation were 2–3 times greater with plasma and GF-HSA than with the other protein preparations. The overall profiles for adduct formation over 4 hr (Fig. 5B) were similar for plasma, GF-HSA and FrV-HSA, and yielded adduct concentrations about 3-fold higher than from FAF-HSA and FA&GF-HSA, which were almost identical.

The results for reversible protein binding (Table 1) and degradation (Table 2) of DAG and *iso*-DAG for the various HSA preparations were puzzling when taken in the context of the purity specifications provided by the supplier (see Materials and Methods). HSA treated to be globulin-free (i.e. GF-HSA) differed greatly from HSA treated to be fatty acid-free (i.e. FAF-HSA). However, GF-HSA which had been treated to remove fatty acids (i.e. FA&GF-HSA) behaved almost identically to FAF-HSA. This denies a significant role for globulin contaminants and points to fatty acid contaminants. However,

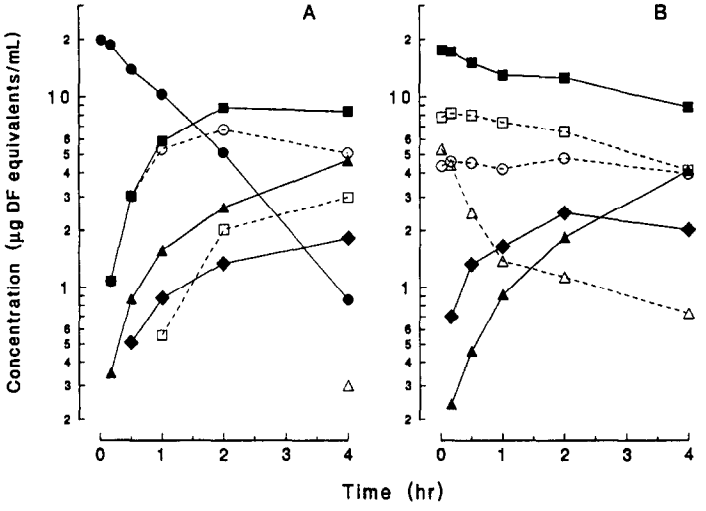

Fig. 4. Profiles for rearrangement, hydrolysis and covalent binding reactions following incubation of DAG (●) (panel A) and a 5:8:7 mixture of its 2-isomer (○), 3-isomer (□) and 4-isomer (△) (collectively ■) (panel B) at 37° in human plasma buffered to pH 7.4. Hydrolysis is represented by liberation of DF (▲) and covalent binding by formation of DF–protein adducts (◆).

Table 2. Kinetic parameters for degradation of DAG and *iso*-DAG in buffer, plasma and HSA solutions*

	Substrate degradation†			Initial pathways‡ k (hr^{-1})		
Medium	$T_{1/2}$ (hr)	r^2	k (hr^{-1})	Rearrangement	Hydrolysis	Adduct formation
DAG as substrate						
Buffer	0.67	0.999	1.05	0.87	0.18	—
Plasma	0.88	0.995	0.78	0.55	0.15	0.09
FrV-HSA	1.60	0.999	0.47	0.28	0.15	0.04
FAF-HSA	1.88	0.999	0.37	0.22	0.09	0.06
GF-HSA	1.05	0.999	0.66	0.31	0.34	<
FA&GF-HSA	1.70	0.999	0.41	0.25	0.09	0.06
iso-DAG as substrate						
Buffer	22.67	0.979	0.03	—	0.03	—
Plasma	2.20	0.988	0.32	—	0.10	0.22
FrV-HSA	4.45	0.964	0.16	—	0.06	0.10
FAF-HSA	6.64	0.887	0.11	—	0.05	0.06
GF-HSA	2.34	0.967	0.31	—	0.12	0.19
FA&GF-HSA	5.23	0.925	0.13	—	0.08	0.05

* Determined at pH 7.4 and 37° at initial DAG and *iso*-DAG concentrations of 20 μg DF equivalents/mL of buffer, plasma or HSA solution at 40 mg/mL.

† Calculated from 0 to 4 hr for DAG as substrate and from 0 to 2 hr for *iso*-DAG as substrate.

‡ Calculated from the substrate degradation rate constant by apportionment of the mean individual pathways measured at 0.5 and 1.0 hr.

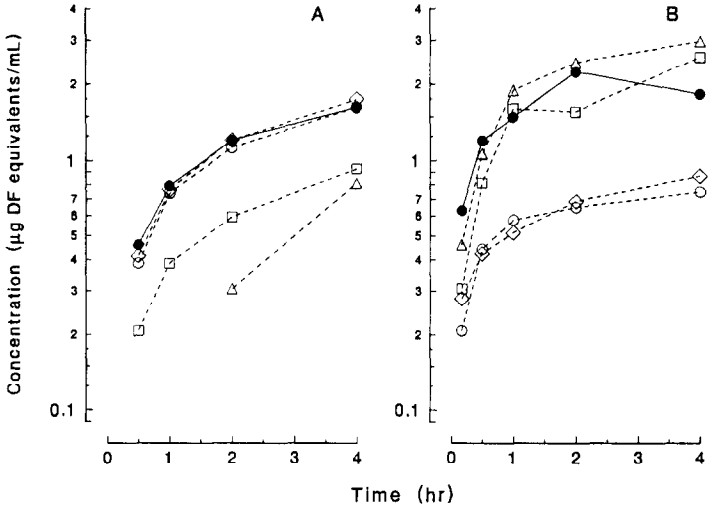

Fig. 5. Profiles for formation of covalent DF–protein adducts following incubation of DAG (panel A) and a mixture of its rearrangement isomers (panel B) with human plasma (●), FrV-HSA (□), FAF-HSA (◇), GF-HSA (△) and FA&GF-HSA (○) at pH 7.4 and 37°.

these had been specified twice by the supplier (upon personal enquiry) to be <0.005% fatty acids for all three HSA preparations. Continuing contact with the supplier ultimately revealed that the fatty acid specifications of <0.005% for GF-HSA (provided earlier) were incorrect. Indeed, octanoate (a C_8 fatty acid) was *added* during production of GF-HSA,and the fatty acid content, although never assayed, was believed to be >0.05%.* (This knowledge has implications for appropriate interpretation of the present and an earlier study [21] on reactivity of DAG and its individual isomers with GF-HSA).

To identify sites of interaction of DAG and *iso*-

* For simplicity of presentation in the remainder of this report, GF-HSA is regarded as being contaminated with unspecified amounts of unspecified fatty acids. It should be noted, however, that the results obtained with GF-HSA need not be representative of the effects of specified fatty acids at specified concentrations.

Table 3. Effects of warfarin, diazepam and unspecified fatty acids on reversible protein binding of DAG and *iso*-DAG in HSA solutions*

Medium	Competitor†	Fraction unbound (%) DAG	*iso*-DAG
FAF-HSA	—	1.5 ± 0.5 (5)	1.4 ± 0.6 (5)
FAF-HSA	Warfarin	1.4 ± 0.3 (3)	1.6 ± 0.2 (3)
FAF-HSA	Diazepam	7.1 ± 1.0 (3)	6.5 ± 0.8 (3)
GF-HSA	Fatty acids (unspecified)	12.6 ± 0.1 (2)	10.7 ± 0.7 (2)

* Determined at pH 7.4 and room temperature at initial DAG and *iso*-DAG concentrations of 20 μg DF equivalents/mL (i.e. 80 μM) of HSA solution at 20 mg/mL (i.e. 300 μM), means ± SD (N).

† Concentrations of warfarin and diazepam were 500 μM; the exact composition of fatty acids in GF-HSA was unspecified.

DAG with HSA, the effects of reversible protein binding competitors were investigated. Experiments were conducted with all five protein preparations i.e. plasma, FrV-HSA, FAF-HSA, GF-HSA and FA&GF-HSA each in the absence and presence of warfarin, diazepam and DF itself. The results for FA&GF-HSA were again almost identical to those for FAF-HSA. Results for FrV-HSA were qualitatively similar to those for GF-HSA. For clarity and brevity, only those results obtained for FAF-HSA in the absence and presence of the competitors warfarin, diazepam and DF, and for GF-HSA alone (which may give some insight into the effect of unspecified fatty acid competitors, particularly added octanoate) are presented.

Warfarin had no significant displacement effect on DAG or *iso*-DAG (Table 3), whereas diazepam displaced both DAG and *iso*-DAG, with the unbound fraction increasing more than 4-fold. Displacement however was still greater with the (unspecified) fatty acids contained in GF-HSA. Experiments with plasma yielded results (not shown) broadly similar to those for FAF-HSA, with warfarin causing no displacement and diazepam causing a 2-fold increase in unbound fractions of DAG and of *iso*-DAG.

In terms of the profiles of DAG degradation upon incubation with FAF-HSA, the presence of warfarin slowed the rate of DAG degradation, whereas diazepam, DF and (unspecified) fatty acids enhanced degradation (Table 4). In the absence of reversible binding competitors, the order of quantitative importance of the competing pathways of DAG degradation was rearrangement > hydrolysis > covalent binding (see also Table 2). The presence of warfarin altered this profile by reducing the rates of rearrangement and hydrolysis of DAG. Conversely, diazepam increased the initial rates of rearrangement (*ca.* 4-fold) and hydrolysis (*ca.* 2-fold), but reduced the rate of adduct formation (*ca.* 5-fold). The individual pathways could not be calculated (for analytical reasons) when DF was added as competitor. Incubation of DAG with GF-HSA gave a DAG degradation rate similar to that observed for FAF-HSA in the presence of DF. Compared to FAF-HSA in the presence of diazepam, the rate of rearrangement was lower but the rate of

Table 4. Effect of reversible protein binding competitors on kinetic parameters for degradation of DAG and *iso*-DAG in HSA solutions*

Medium	Reversible binding competitor	Substrate degradation† $T_{1/2}$ (hr)	r^2	k (hr^{-1})	Initial pathways‡ k (hr^{-1}) Rearrangement	Hydrolysis	Adduct formation
DAG as substrate							
FAF-HSA	—	2.32	0.988	0.30	0.19	0.07	0.05
FAF-HSA	Warfarin	4.13	0.990	0.17	0.07	0.04	0.06
FAF-HSA	Diazepam	0.75	0.999	0.92	0.75	0.17	0.01
FAF-HSA	DF	1.28	0.994	0.54	—	—	—
GF-HSA	Fatty acids	1.13	0.999	0.61	0.34	0.27	0.01
iso-DAG as substrate							
FAF-HSA	—	4.75	0.984	0.15	—	0.07	0.07
FAF-HSA	Warfarin	9.35	0.756	0.07	—	0.04	0.03
FAF-HSA	Diazepam	2.27	0.976	0.31	—	0.11	0.20
FAF-HSA	DF	2.99	0.999	0.23	—	—	—
GF-HSA	Fatty acids	3.50	0.978	0.20	—	0.07	0.13

* Determined at pH 7.4 and 37° at initial DAG and *iso*-DAG concentrations of 20 μg DF equivalents/mL (80 μM) in HSA solutions at 20 mg/mL (300 μM); warfarin, diazepam and DF were at 500 μM; fatty acids (including added octanoate) in GF-HSA were unspecified.

† Calculated from 0–4 hr for DAG as substrate and from 0–2 hr for *iso*-DAG as substrate.

‡ Calculated from the substrate degradation rate constant by apportionment of the mean individual pathways measured at 0.5 and 1.0 hr.

hydrolysis higher. The initial rate of adduct formation with GF-HSA was very low and similar to that found with FAF-HSA in the presence of diazepam. The profiles of adduct formation over 4 hr (Fig. 6A) show that diazepam and diflunisal had comparable inhibitory effects on formation of covalent adducts and that the (unspecified) fatty acids present in GF-HSA created a very different profile for adduct formation. Warfarin, diazepam and DF caused similar changes to the rates of rearrangement, hydrolysis and adduct formation when DAG was incubated with buffered human plasma (results not shown).

Incubation of *iso*-DAG with FAF-HSA in the presence of warfarin, diazepam and DF resulted in loss of *iso*-DAG which was first order only in the early stages of the incubation. An appreciable contribution appears to be the very rapid initial loss of 4-isomer, compared to the 2- and 3-isomers (e.g. see profiles for incubation of *iso*-DAG with plasma, Fig. 4B) presumably via adduct formation. This may indicate that this particular route of adduct formation is saturable. Nonetheless, the effects of the reversible binding competitors on the reactivity of *iso*-DAG were clear-cut. Warfarin greatly retarded overall *iso*-DAG degradation through both hydrolysis and adduct formation pathways (Table 4). Conversely, diazepam increased both hydrolysis and adduct formation. The presence of the fatty acids in GF-HSA had intermediate effects on the rate of loss of *iso*-DAG. Figure 6B shows that, over the 4 hr incubation, covalent adduct formation was enhanced in the presence of diazepam and DF, and in the presence of the fatty acids in GF-HSA. The inhibitory effect of warfarin on adduct formation was sustained for the whole 4 hr period. Experiments with plasma showed that warfarin, diazepam and DF had effects on reactivity of *iso*-DAG which were similar to those found in FAF-HSA (results not shown).

DISCUSSION

This study was carried out to characterize further the reactivity of DAG and *iso*-DAG in the presence of human plasma protein *in vitro*. The initial impetus came from an earlier finding of markedly different reactivity profiles of DAG itself when incubated with two highly purified preparations of HSA i.e. FAF-HSA and GF-HSA [18], a finding which appeared to offer an unexpected opportunity to gain insight into covalent binding mechanisms. The fatty acid specifications (originally supplied on request on two separate occasions) were <0.005% for both FAF-HSA and GF-HSA, with the latter having a lower globulin content. Indeed, GF-HSA was selected for a subsequent *in vitro* study [21] because it appeared to be the more pure preparation. However, as noted in Results, the fatty acid specification of <0.005% supplied for GF-HSA was erroneous: indeed, the C_8 fatty acid, octanoate, had been added during preparation of GF-HSA, and the actual fatty acid content, although unspecified, was believed to be >0.05%. The results of the present study, as well as those earlier [18, 21], therefore need to be interpreted in this light.

DF is known to be very highly bound (~99.9%) to plasma protein [22], and we have recently reported [20] that its three major metabolites (i.e. DAG, DF phenolic glucuronide and DF sulphate) retain relatively high binding (~99, 98 and 99.5%, respectively) in unbuffered human plasma. The present results in buffered plasma and HSA solutions (Table 1) confirm and extend these observations by showing that the acyl migration isomers of DAG i.e. *iso*-DAG show reversible protein binding equivalent to that of DAG itself. The reversible binding of DAG and *iso*-DAG reported herein is higher than previously reported for drug conjugates, with the exception of diastereomeric carprofen acyl

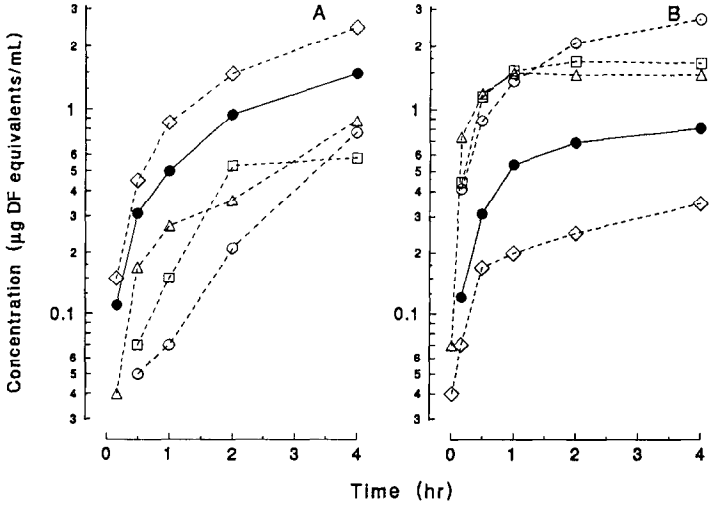

Fig. 6. Profiles for formation of covalent DF–protein adducts following incubation of DAG (panel A) and a mixture of its rearrangement isomers (panel B) with FAF-HSA in the absence (●) and presence of warfarin (◇), diazepam (□) and DF (△), and with GF-HSA (○).

glucuronides [23],where binding to HSA was calculated at >99%. DAG and *iso*-DAG were most highly bound to FAF-HSA and FA&GF-HSA preparations. The fraction unbound was higher with FrV-HSA and buffered human plasma, possibly due to conformational changes in the albumin caused by the presence of fatty acids or other impurities. Reduced binding of acyl glucuronides in the presence of FrV-HSA compared with FAF-HSA was also observed with carprofen glucuronides [23]. However, the unbound fractions of DAG and *iso*-DAG were up to 10-fold higher in the presence of the (unspecified) fatty acids in GF-HSA as compared with FAF-HSA.

DAG was more stable in protein solutions than in buffer (Table 2), although stability varied greatly with the purity of the protein. Rearrangement was the dominant competing pathway, except for GF-HSA where hydrolysis and rearrangement were comparable. In all cases, rearrangement in the presence of protein was less than found in buffer under the same conditions (see also Table 4), and this appears to deny a net catalytic role for HSA in the acyl migration process for DAG. The same is clearly not true of hydrolysis, given the result with GF-HSA. Of the three pathways, adduct formation was least important quantitatively. By contrast to DAG, *iso*-DAG degradation was vastly accelerated in protein solutions compared to buffer (Table 2), through both adduct formation and catalysis of hydrolysis. Literature reports of the effect of protein on stability of acyl glucuronides vary with the particular drugs studied. Tolmetin glucuronide was stabilized in the presence of HSA [24], whereas glucuronides of oxaprozin [6], ketoprofen [25], fenoprofen [26] and zomepirac [27] were degraded more rapidly. Similar rates of rearrangement and hydrolysis of fenofibric acid glucuronide were reported in buffer and HSA solutions [28], as found also for valproic acid glucuronide [29]. However, hydrolysis of acyl migration isomers of valproic acid glucuronide was accelerated in the presence of HSA and plasma [29].

The extent of adduct formation varied greatly depending on the particular protein preparation used (Table 2). However, the effects of protein purity on adduct formation from DAG and *iso*-DAG as substrates were opposite, whereas the effects on reversible binding for DAG and *iso*-DAG as ligands had been the same (Table 1). The 4 hr profiles show that adduct formation was greatest with plasma, FAF-HSA and FA&GF-HSA with DAG as substrate (Fig. 5A) but greatest with plasma, FrV-HSA and GF-HSA with *iso*-DAG as substrate (Fig. 5B). These differences suggest that adduct formation via DAG and *iso*-DAG occurs at different sites on the albumin molecule. The effect of the fatty acids (specifically added octanoate) in GF-HSA may be instructive here. Medium chain (C_6 to C_{11}) fatty acids displace markers from site II (benzodiazepine site) but not site I (warfarin site) on HSA [30, 31]. Direct competition between octanoate and diazepam for a common high affinity binding site on defatted HSA has also been reported [32]. Therefore, it seemed likely that competition for reversible binding to site II by octanoate (and perhaps other fatty acids) present in GF-HSA was ultimately responsible for the observed major differences in reactivity of DAG and *iso*-DAG beween GF-HSA and FAF-HSA or FA&GF-HSA.

To test such hypotheses, the experiments were repeated in the presence of diazepam, warfarin and DF as reversible binding competitors. Diazepam caused displacement of DAG and *iso*-DAG (Table 3), whereas warfarin had no effect. However, the fatty acids present in GF-HSA caused greater displacement, perhaps due to the combination of direct competition for binding sites and fatty acid-induced conformational changes in HSA. The effect of DF itself on reversible binding of DAG and *iso*-DAG could not be determined in the present study. DF has been reported [33] to reversibly bind to three discrete sites on HSA—warfarin, digitoxin and diazepam. However, it has been proposed that the sites for digitoxin and warfarin binding are situated quite close to each other in a large and flexible binding region which is relatively susceptible to conformational changes of the protein [34, 35]. Addition of fatty acids to defatted albumin increased the binding of warfarin at site I [36]. Therefore, it seems possible that, in the absence of fatty acids, the preferred site of DF binding is the benzodiazepine site. For other drugs and their acyl glucuronide conjugates, differential binding studies have shown that oxazepam and its *S*(+)-glucuronide bind preferentially at the same site on HSA [37], whereas carprofen and its glucuronides may not [23].

Hydrolysis of DAG and *iso*-DAG was diminished in the presence of warfarin and accelerated in the presence of diazepam (Table 4). As DAG and *iso*-DAG bind extensively (reversibly) at the benzodiazepine binding site, this suggests that the warfarin binding region may be catalytic for their hydrolysis. The effects of the fatty acids in GF-HSA, expected to displace ligands from the benzodiazepine site, were generally supportive of this premise (Tables 2 and 4). As noted earlier, rearrangement of DAG in all protein solutions, either in the absence or presence of competitors, was less than in buffer, so there is no strong evidence for site-specific catalysis of rearrangement. This contrasts with oxaprozin glucuronide [6, 28], which was found to bind reversibly at the benzodiazepine site where hydrolysis and rearrangement were catalysed.

The effects of warfarin and diazepam/DF on adduct formation were very different for DAG and *iso*-DAG, in contrast to their effects on reversible binding. Adduct formation via DAG was increased by warfarin and decreased by diazepam, DF and the fatty acids of GF-HSA (Fig. 6A). The opposite effects were found for adduct formation via *iso*-DAG (Fig. 6B). The effects of competitors on adduct formation with buffered plasma were similar (data not shown). The available evidence is thus consistent with adduct formation via DAG occurring predominantly at the benzodiazepine site, and that via *iso*-DAG occurring predominantly at the warfarin site. However, it should be kept in mind that strict interpretation of these interaction studies between protein, glucuronide substrate and competitor ligand is complicated by the effects of glucuronide degradation occurring during the studies (e.g. effects

on binding of increasing concentrations of drug due to hydrolysis).

Of the two proposed mechanisms of adduct formation, transacylation should be strongly favoured by the acyl glucuronide itself [21] whereas the glycation mechanism requires prior rearrangement of the glucuronide to its isomers before interaction with the protein [7]. We previously showed (using GF-HSA) that the individual isomers of DAG, but in particular the 4-isomer, were much better substrates for adduct formation than DAG itself [21]. (It is worth noting in this context that the 4-isomer was also more reactive with all HSA preparations in the present study e.g. see Fig. 4B.) However, we concluded from that study [21] that the data did not support transacylation as a mechanism of formation of DF–HSA adducts. That conclusion should now be qualified by recognition that GF-HSA is contaminated with fatty acids (at least octanoate) and thus it is representative of HSA only in the presence of these fatty acids. This highlights the need for circumspection in interpretation of protein–acyl glucuronide interaction studies, particlarly if only a single protein preparation is used.

It has been demonstrated that a reactive tyrosine residue (tyr-411) is located within the benzodiazepine binding site [38–40]. Modification of this residue diminished covalent binding of oxaprozin via its glucuronide metabolite [41]. Ligands known to bind at this site (including oxaprozin) also inhibited covalent binding while ligands known to bind at other sites on HSA did not. It seems likely that adduct formation via DAG, occurring preferentially at the benzodiazepine binding site, may be via transacylation with the active tyrosine residue. An active lysine residue (lys-414) has also been reported at this site [12], and may also participate in adduct formation. Adduct formation via *iso*-DAG seems to occur preferentially in the warfarin binding region, presumably by the glycation mechanism, which requires the involvement of lysine residues. The reported availability of active lysine residues in this region [12] is consistent with this hypothesis. Covalent binding of zomepirac with HSA was observed via zomepirac glucuronide and its positional isomers [7] whereas covalent binding of oxaprozin to protein occurred to a significant extent with oxaprozin glucuronide but not its 2-isomer [6]. Munafo *et al.* [24] concluded that both tolmetin glucuronide and its isomers could react with protein to form covalent adducts and that at least two mechanisms existed. In a later mass spectrometric study of tolmetin–HSA adducts produced *in vitro* [9], tolmetin was found to be attached covalently, via the glucuronic acid moiety, primarily to lysines 195, 199 and 525, with minor attachment at lysines 137, 351 and 541. This study provides direct structural evidence in support of the glycation mechanism.

Covalent binding of carboxylic drugs to protein is attracting interest because of its toxification potential *in vivo* e.g. such modified proteins may be immunogenic [2, 5]. Formation of covalent adducts is not limited to plasma protein. We have recently reported formation of covalent adducts of DF with liver, kidney, intestine [19] and urinary bladder [42] of rats dosed with the drug. DF has been associated with hypersensitivity responses [43], and, as a class, the non-steroidal anti-inflammatory drugs are associated with a relatively high incidence of toxic and/or immune responses [2]. Whether or not covalent adduct formation via glucuronide metabolites plays a role is yet to be directly established. In other work, however, we have demonstrated the presence of antibodies (albeit at very low and clinically insignificant titres) against valproic acid–protein adducts in the plasma of some patients taking the drug chronically for control of epilepsy [29]. The origin of the adducts *in vivo* cannot be ascertained. However, a major pathway of valproic acid metabolism is formation of its acyl glucuronide conjugate, which has been shown *in vitro* to be a precursor or covalent adducts with plasma protein.

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