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STUDIES ON THE REACTIVITY OF ACYL GLUCURONIDES—II

INTERACTION OF DIFLUNISAL ACYL GLUCURONIDE AND ITS ISOMERS WITH HUMAN SERUM ALBUMIN *IN VITRO*

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Abstract—A major metabolite of diflunisal (DF) is its reactive acyl glucuronide conjugate (DAG) which can undergo hydrolysis (regeneration of DF), intramolecular rearrangement (isomerization via acyl migration) and intermolecular reactions with nucleophiles. We have compared the fate of DAG and its individual 2-, 3- and 4-*O*-acyl positional isomers (at *ca.* 55 μg DF equivalents/mL) after incubation with human serum albumin (HSA, 40 mg/mL) at pH 7.4 and 37°. Initial half-lives ($T_{\frac{1}{2}}$) for DAG and its 2-, 3- and 4-isomers were 53, 75, 61 and 26 min, respectively. DAG was more labile to hydrolysis than any of its isomers but the latter, in particular the 4-isomer, were much better substrates for formation of covalent DF–HSA adducts. After a 2-hr incubation, 2.4, 8.2, 13.7 and 36.6% of substrate DAG and its 2-, 3- and 4-isomers (respectively) were present as DF–HSA adducts. With long term incubation, the concentrations of adducts so generated *in situ* declined in a biphasic manner, with apparent terminal $T_{\frac{1}{2}}$ values of *ca.* 28 days. DAG was much more labile to transacylation with methanol (i.e. formation of DF methyl ester) than an equimolar mixture of its isomers after incubation in a 1:1 methanol:pH 7.4 buffer solution at 37° ($T_{\frac{1}{2}}$ values of 5 and 70 min, respectively). The data do not support direct transacylation with nucleophilic groups on protein as the predominant mechanism of formation of covalent DF–HSA adducts *in vitro*.

Acyl glucuronides frequently occur as major metabolites of drugs bearing carboxylic acid groups and have been shown to be potentially reactive metabolites, capable of undergoing hydrolysis, intramolecular rearrangement (isomerization via acyl migration) and intermolecular reactions with small nucleophiles and proteins, both *in vitro* and *in vivo* [1–6]. The mechanism of the rearrangement reaction has been well established as involving hydroxide ion catalysed migration of the drug moiety from the 1-*O*-acyl-β position to neighbouring 2-, 3- and 4-hydroxy groups on the glucuronic acid ring (Ref. 7 and references therein). The biosynthetic 1-*O*-β glucuronide itself, but not the isomers, is a substrate for β-glucuronidases. The acyl migrations are reversible with the exception of reformation of the parent 1-*O*-β glucuronide.

By contrast, the mechanism(s) of formation of covalent drug–protein adducts has not been established, though two types have been proposed. The first is analogous to nucleophilic displacement of glucuronic acid from the ester group by small molecules such as methanol [8] and 4-(*p*-nitrobenzyl)pyridine [9] (i.e. a transacylation mechanism). Thus, evidence for the involvement of nucleophilic groups such as -SH of cysteine residues [2], -OH of tyrosine residues [3, 10] and $-NH_2$ of lysine residues [11, 12] in the formation of covalent adducts between protein and various acyl glucuronides has been presented. The alternative mechanism is analogous to the non-enzymic glycosylation of albumin [13–16] and requires prior acyl migration of the drug moiety away from the biosynthetic 1-*O*-β position to permit ring-opening of the sugar. The reactive aldehyde group so exposed can then reversibly form an imine (Schiff's base) with an amine group on protein. Subsequent Amadori rearrangement could then yield a stable ketoamine derivative. Thus, in contrast to the transacylation mechanism, both the drug and glucuronic acid moieties (still linked together by an ester group) become bonded to the protein. This mechanism was first proposed for the "irreversible" binding of zomepirac to plasma protein [5, 16]; further evidence has recently been presented [17]. Both mechanisms could operate, as suggested for tolmetin glucuronide [18]. Irrespective of their mechanism of formation, the drug–protein adducts have been hypothesized as potential mediators of toxic and/or hypersensitivity responses to acidic drugs forming reactive acyl glucuronides *in vivo* [1, 2, 5].

We have been investigating the anti-inflammatory drug diflunisal (DF†), a difluorophenyl derivative of salicylic acid, mainly in order to probe the *in vivo* disposition of its reactive acyl glucuronide conjugate (DAG). DAG is a major metabolite of DF in both humans and rats [19–23] and has been shown to be quite labile to hydrolysis and acyl migration, and to form covalent adducts with albumin *in vitro* [24–28].

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† Abbreviations: DF, diflunisal; DAG, diflunisal acyl glucuronide; HSA, human serum albumin.

We have also demonstrated the occurrence of such reactions in both humans and rats *in vivo* [28–32]. An earlier study [28] investigated the formation of DF–protein adducts by incubation of DAG itself with HSA and rat serum albumin. The present investigation was undertaken to explore the origins of these adducts by incubation of the purified, individual 2-, 3- and 4-isomers of DAG, as well as DAG itself, with HSA *in vitro*.

MATERIALS AND METHODS

Materials. DF was purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Authentic samples of DAG and its 2-, 3- and 4-*O*-acyl isomers were obtained as described previously [33]. Clofibric acid was a gift from ICI Pharmaceuticals Division (Macclesfield, U.K.). Flurbiprofen was a gift from The Boots Company (Nottingham, U.K.). HSA (product A8763) purchased from Sigma was specified as ≤1% globulin and <0.005% fatty acids. Methanol and acetonitrile (HPLC grade) were purchased from Mallinckrodt (Melbourne, Australia). Other chemicals and solvents were AR grade.

Incubation of DAG and its isomers with HSA. Stock solutions of DAG and each of its 2-, 3- and 4-*O*-acyl positional isomers, as well as an equimolar mixture of the three isomers, were prepared at 750–800 μg DF equivalents/mL of 0.01 M NaH_2PO_4/Na_2HPO_4 (final pH 6.0) and stored frozen until required. HSA was prepared at 43 mg/mL of 0.1 M NaH_2PO_4/Na_2HPO_4, pH 7.4.

Incubations were initiated by adding *ca.* 330 μL of glucuronide/isomer stock solution to 4.5 mL HSA solution pre-warmed to 37° (giving concentrations of the glucuronide/isomers at *ca.* 55 μg DF equivalents/mL and of HSA at *ca.* 40 mg/mL). Two aliquots (50 and 500 μL) were withdrawn immediately and after 10 and 30 min, and 1, 2, 4, 8 and 24 hr incubation at 37°. The 50-μL aliquots were used to measure DAG, its isomers and (reversibly bound) DF, whilst DF bonded covalently to HSA was measured using the 500-μL aliquots.

Long term incubations of DAG and an equimolar mixture of its 2-, 3- and 4-isomers with HSA were carried out analogously, except that: (a) the HSA stock solution was prepared with 0.05% (w/v) sodium azide (to inhibit bacterial growth) and (b) volumes were doubled to enable additional 500-μL aliquots to be drawn after *ca.* 2, 3, 5, 10, 23 and 35 days incubation at 37°, for measurement of covalently bonded DF only.

Incubation of DAG and its isomers with methanol. Solutions of methanol (770 μL) and 0.1 M NaH_2PO_4/Na_2HPO_4 pH 7.4 (720 μL) were pre-warmed to 37°. Incubations were initiated by addition of stock solution (25 μL) of DAG or an equimolar mixture of its isomers (prepared above). Two aliquots (50 μL each) were withdrawn immediately and after 5, 10, 15, 20, 30 and 60 min, and snap frozen over dry ice until analysis (as soon as practicable thereafter). One aliquot was used for analysis of DAG, its isomers and DF, and the other for the methyl ester of DF.

Analysis of DAG, its 2-, 3- and 4- isomers and DF. Quantification of DAG, its isomers and reversibly bound DF in 50-μL aliquots of incubation media was achieved using the direct, isocratic HPLC procedure described previously [27]. The individual 2-, 3- and 4-*O*-acyl positional isomers of DAG, each of which presents chromatographically as a pair of peaks corresponding to the C-1 α- and β-anomers, were quantified using the DAG standard curve. Equivalence of the molar extinction at the analytical wavelength (226 nm) of DAG and its individual isomers has been verified [33].

Quantification of DF covalently bonded to HSA in the 500-μL aliquots of incubation media was achieved after exhaustive solvent washing of the precipitated protein followed by its digestion in alkali, as described earlier [28].

Preparation and analysis of DF methyl ester. DF (20 mg) was heated overnight at 50° in a mixture of methanol (1 mL) and concentrated H_2SO_4 (3 mL). After extraction of the solution with 2 volumes of ether–hexane (1:3 v/v), the organic layer was separated, back-extracted with 0.1 M Na_2HPO_4/Na_3PO_4, pH 9.0 (1 mL) and evaporated to dryness under a stream of air. A portion of the product methyl ester (*ca.* 2 mg) was dissolved in acetonitrile (8 mL) and shown to have chromatographic properties identical to an earlier sample whose structure was confirmed by mass spectrometry [27]. DF methyl ester concentration in the stock solution was determined by alkaline hydrolysis to DF. Thus, each of four aliquots (50 μL) was evaporated to dryness and the residue heated at 90° overnight with 150 μL of 0.2 M NaOH. After cooling, 1 M HCl (50 μL) and internal standard solution (100 μg clofibric acid/mL acetonitrile, 200 μL) were added. After vortex mixing and centrifugation, a 20-μL sample was injected into the analytical HPLC for quantification of DF [27].

DF methyl ester in samples from the incubation experiments was determined directly by HPLC using standard curves constructed from the calibrated DF methyl ester solution prepared above. Thus, samples (50 μL) of incubation media were mixed with internal standard solution (100 μg flurbiprofen/mL of 4% v/v acetic acid in acetonitrile, 75 μL), and a 20-μL aliquot was injected into the HPLC. The equipment and operating conditions were as described previously [27], except that the mobile phase was 69% v/v methanol in 0.05 M NaH_2PO_4/Na_2HPO_4 buffer, pH 4.5. DF methyl ester and flurbiprofen eluted at 24 and 6 min, respectively. Standard curves constructed over the range of 0.3 to 30 μg DF equivalents/mL were linear with correlation coefficients exceeding 0.99.

RESULTS

DAG, its individual 2-, 3- and 4-*O*-acyl positional isomers and an equimolar mixture of the three isomers (all solutions at *ca.* 55 μg DF equivalents/mL) were incubated at pH 7.4 and 37° with HSA (40 mg/mL) for 24 hr. The resultant concentrations of the various DF species in the incubation solutions from 0–4 hr are shown in Fig. 1. Hydrolysis to DF and rearrangement by acyl migration to the 2-isomer were the major pathways of DAG disposition discernible at early times (0–30 min, Fig. 1A). DF–HSA covalent adducts were not measurable until 1

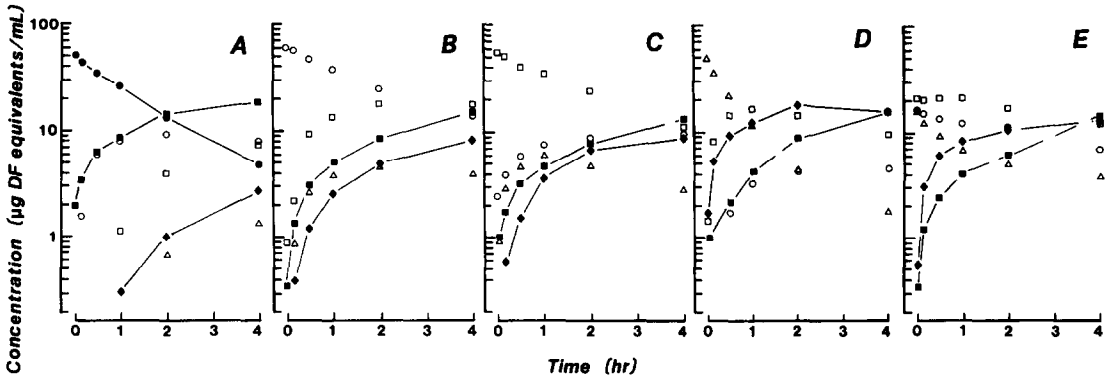

Fig. 1. Profiles for hydrolysis, acyl migration and covalent binding following incubation of DAG (●, panel A), its 2-isomer (○, panel B), its 3-isomer (□, panel C), its 4-isomer (△, panel D) and an equimolar mixture of the three isomers (dotted line, panel E) with human serum albumin (HSA, 40 mg/mL) at pH 7.4 and 37°. The initial substrate concentration was *ca.* 55 μg diflunisal DF equivalents/mL in each case. Hydrolysis is represented by liberation of DF (■) and covalent binding by formation of DF–HSA adducts (◆).

Table 1. Composition of DF-derived species after 2-hr incubation of DAG, its individual 2-, 3- and 4-isomers, and an equimolar mixture of the three isomers with HSA at pH 7.4 and 37°

Dosing species	$T_{\frac{1}{2}}$ of dosing species* (min)	% Composition after 2-hr incubation						
		DAG	2-Isomer	3-Isomer	4-Isomer	Sum of the isomers	DF	DF–HSA adducts
DAG	53	30.4	22.3	9.0	1.6	33.0	34.2	2.4
2-Isomer	75	<	39.7	30.0	7.0	77.7	14.1	8.2
3-Isomer	61	<	17.3	44.6	9.1	71.1	15.2	13.7
4-Isomer	26	<	8.7	27.6	9.0	45.4	18.0	36.6
Equimolar mixture of the isomers	101	<	22.5	32.9	10.0	65.4	12.4	22.2

Incubation solutions contained HSA at 40 mg/mL and DF-derived species at *ca.* 55 μg DF equivalents/mL.
* Determined by linear regression of concentrations from 0–30 min.

hr, i.e. after considerable rearrangement had taken place. DAG concentrations had fallen to *ca.* 10% of initial values by 4 hr, with an apparent initial half-life ($T_{\frac{1}{2}}$) of 53 min (Table 1). After incubation of HSA with the individual 2-, 3- and 4-isomers (Fig. 1B–D), hydrolysis to DF was clearly less important. Reversible acyl migration between the isomers, without reformation of DAG itself, was most important quantitatively. DF–HSA adduct formation paralleled hydrolysis, though at lower concentrations, during incubation with the 2- and 3-isomers (Fig. 1B and C). By contrast, adduct formation from the 4-isomer was considerably greater than its hydrolysis at early incubation times and became comparable to rearrangement as manifested by acyl migration to the 3-isomer (Fig. 1D). Incubation of HSA with an equimolar mixture of the three isomers gave profiles for hydrolysis and adduct formation essentially intermediate to those observed for the individual isomers. Apparent $T_{\frac{1}{2}}$ values for the individual isomers, calculated at early times (0–30 min) before reformation of the substrate isomer by reversible acyl migration could be considered appreciable, are shown in Table 1. The considerably shorter $T_{\frac{1}{2}}$ for the 4-isomer (26 min) reflected more facile adduct formation. Thus, after 2-hr incubation, 2.4, 8.2, 13.7 and 36.6% of substrate DAG and its 2-, 3- and 4-isomers (respectively) were present as DF–HSA adducts (Table 1). Overall, the data in Fig. 1 and Table 1 show that DAG is more prone to hydrolysis than any of its isomers, whereas the isomers, in particular the 4-isomer, are better substrates for DF–HSA adduct formation.

The time profiles for adduct concentrations over the complete 24-hr incubation period are shown in Fig. 2. Thus, irrespective of their source, the adduct concentrations declined after 8 hr. The long term stabilities of adducts generated *in situ* by incubation of DAG and an equimolar mixture of its isomers with HSA at pH 7.4 and 37°, and in the presence of 0.05% sodium azide to inhibit bacterial growth, are shown in Fig. 3. Biphasic profiles for decline in adduct concentrations were obtained in both cases, with apparent terminal $T_{\frac{1}{2}}$ values of *ca.* 28 days. It

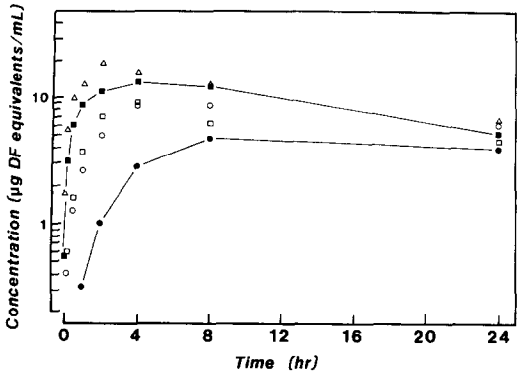

Fig. 2. Profiles for DF–HSA adducts (●, ○, □, △, ■) obtained by incubation of (respectively) DAG, its 2-isomer, its 3-isomer, its 4-isomer and an equimolar mixture of the three isomers with HSA (40 mg/mL) at pH 7.4 and 37°. The initial substrate concentration was *ca.* 55 μg DF equivalents/mL in each case.

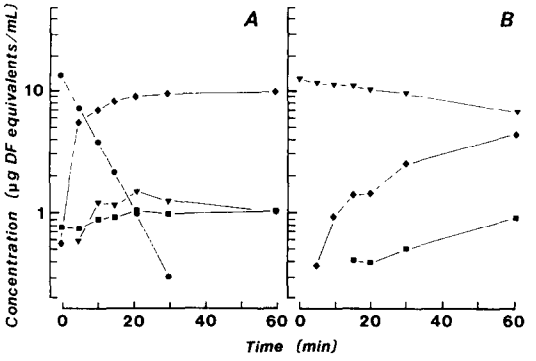

Fig. 4. Profiles for methanolysis and hydrolysis of DAG (panel A) and an equimolar mixture of its three isomers (panel B) after incubation in 1:1 (v/v) methanol:phosphate buffer pH 7.4 at 37°. (●) Represents DAG and (▼) represents the sum of its isomers. Methanolysis is represented by formation of DF methyl ester (◆) and hydrolysis by liberation of DF (■).

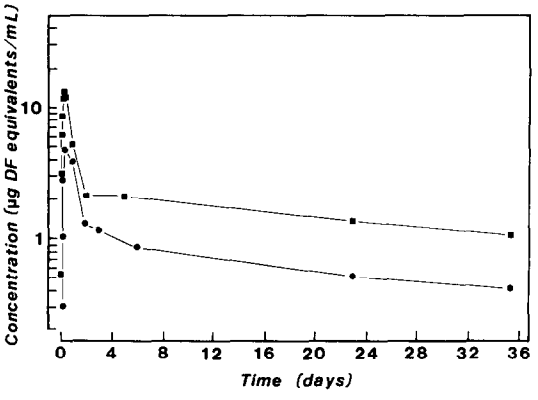

Fig. 3. Profiles for DF–HSA adducts (●, ■) obtained by incubation of (respectively) DAG and an equimolar mixture of its three isomers with HSA (40 mg/mL) at pH 7.4 and 37° in the presence of 0.05% sodium azide to inhibit bacterial growth. The initial substrate concentration was *ca.* 55 μg DF equivalents/mL in each case.

should be noted that the presence of sodium azide in the incubation medium had no obvious effect on the disposition of DAG or the mixture of its isomers, since the 24-hr profiles for DF–HSA adducts (and the various other DF species measured) were very similar to those obtained in the absence of this bacteriostat.

Transacylation with methanol was assessed by incubation of DAG and an equimolar mixture of its three isomers in a 1:1 (v/v) methanol:phosphate buffer pH 7.4 solution at 37° (Fig. 4). Formation of DF methyl ester was much more rapid from DAG than from its isomers and in both incubations vastly exceeded hydrolysis to DF. Under these conditions, the $T_{\frac{1}{2}}$ values for DAG and the mixture of its isomers were 5 and 70 min, respectively.

DISCUSSION

The covalent binding of acidic drugs, via their reactive acyl glucuronides, to proteins such as HSA has been well documented in recent years. The formation of such adducts *in vivo* serves no obvious therapeutic purpose. On the contrary, toxic and/or hypersensitivity responses have been hypothesized [1, 2, 5], as found for other xenobiotics ultimately yielding covalent adducts to tissue macromolecules *in vivo* [1, 34, 35]. However, the mechanism of formation of the adducts (*in vitro* or *in vivo*) has not yet been established; nor have the resultant toxic responses *in vivo*.

The two mechanisms so far proposed for adduct formation are quite contrasting. The simpler transacylation alternative [1–4] involves nucleophilic attack at the ester group by -SH, -OH or $-NH_2$ groups on protein. The drug moiety itself thus becomes directly linked to the protein via a thioester, ester or amide band, and glucuronic acid is lost. Under physiological pH conditions, relatively facile transacylation reactions might be expected of the acyl glucuronide itself but not of its 2-, 3- and 4-isomers (only in the 1-*O*-β glucuronide itself is the carboxyl group of the drug linked to the glucuronic acid moiety via an acetal). Conversely, the Schiff's base mechanism for adduct formation [5, 16, 17] *requires* prior migration of the drug moiety away from the 1-position of the glucuronic acid ring and thus is operative from the isomers but not the acyl glucuronide itself. According to this mechanism, the glucuronic acid moiety, still bearing the ester-linked drug, becomes bound to an amine group on protein via an imine (Schiff's base).

Because the reversibility of acyl migration does not include reformation of the parent acyl glucuronide (i.e. 1-*O*-β glucuronide → 2-isomer ⇆ 3-isomer ⇆ 4-isomer), the two mechanisms of adduct formation are theoretically distinguishable on the basis of which of the glucuronide and its isomers is the better substrate. However, little definitive work has been reported on this aspect. Smith *et al.* [16] presented data showing that zomepirac–HSA adduct formation from zomepirac acyl glucuronide was

roughly comparable to that from its purified 2-isomer and greater than that from its purified 4- and 3-isomers (in order), over 45-min incubation with HSA at pH 7.4 and 37°. Other data concurrently reported showed 60% greater adduct formation from zomepirac glucuronide itself than from an unpurified mixture of its isomers. Nonetheless, these authors proposed the Schiff's base/Amadori rearrangement mechanism and recently presented additional supporting evidence also pertaining to covalent binding of zomepirac glucuronide [17]. Ruelius *et al.* [3] presented strong evidence favouring a transacylation mechanism for covalent binding of oxaprozin to HSA. After incubation of radiolabelled oxaprozin glucuronide with HSA at pH 7 for 1 hr, 22% of the radioactivity became attached to HSA when the ^{14}C-label was in the oxaprozin moiety but only 0.6% when the label was in the glucuronic acid moiety. Furthermore, only 2.1% attachment of label to HSA occurred after incubation with the 2-isomer of [^{14}C]-oxaprozin glucuronide. Munafo *et al.* [18] found the rate of covalent binding of tolmetin to HSA was 10 times greater for tolmetin glucuronide than for a mixture of its isomers (predominantly the 3-isomer, generated *in situ* from tolmetin glucuronide by pre-incubation in albumin-free buffer). These authors suggested that more than one binding mechanism was operative.

In the present HSA incubation study, DAG was more labile to hydrolysis than any of its purified individual isomers but the isomers were much better substrates (in order, 4- > 3- > 2-isomer) for covalent binding to HSA (Fig. 1 and Table 1). In particular, adduct formation from DAG was not measurable until 1 hr, i.e. after considerable isomer formation by acyl migration had occurred. It thus seems possible that DAG itself did not contribute at all to adduct formation. By contrast, DAG was much more labile to transacylation, at least with the small nucleophile methanol (Fig. 4). Nonetheless, some methanolysis of the isomers did occur. Taken together, these results clearly do not point to transacylation as an appreciable mechanism of formation of DF–HSA adducts *in vitro* and provide indirect support for a mechanism of the Schiff's base type. Further indirect support comes from our earlier studies [27, 33] documenting the facile anomerization of the 2-, 3- and 4-isomers of DAG. Thus each isomer exists as an approximately equimolar mixture of the C-1 α- and β-anomers, and pH-dependent anomerization (via reversible ring-opening of the sugar) occurs even under acidic conditions inhibitory to acyl migration. Ring-opening to expose the reactive aldehyde group is also a prerequisite for formation of an imine (Schiff's base) with amino groups on protein.

Previous studies investigating formation of drug–HSA adducts by incubation with oxaprozin [10], zomepirac [16], tolmetin [18] and DF [28] glucuronides have shown that adduct concentrations peaked at 2–6 hr and subsequently declined slowly. This decline presumably corresponds to hydrolysis of the adducts (irrespective of their structure) but has not been investigated. In the present study, long term (36-day) incubation of DF–HSA adducts generated *in situ* (in the presence of the bacteriostat sodium azide) revealed a biphasic decline with an apparent terminal half-life of about 28 days (Fig. 3). Assuming that no degradation of the protein itself had occurred (for which there was no evidence), the profile suggests the presence of at least two types of adducts with quite different stabilities. This result is not in disagreement with the Schiff's base mechanism [16, 17] in which the (relatively unstable) imine first formed could undergo an Amadori rearrangement to yield a (relatively stable) ketoamine derivative. It should be noted, however, that sequential involvement of two adduct types is not excluded from the transacylation mechanism. For example, transacylation of a glucuronide with a thiol group on protein would yield a (relatively reactive) thioester, which could then transacylate amino groups on the protein [1, 2].

The biphasic profile obtained in the present *in vitro* study is reminiscent of that found earlier for DF–plasma protein adducts *in vivo* [29]. In that study five volunteers ingested DF for 6 days with attendant increasing concentrations of DF–adducts in plasma. Following cessation of dosage, the adduct concentrations declined in a biphasic manner with an apparent terminal half-life of about 10 days. In the same study, probenecid (also an acidic drug forming a reactive acyl glucuronide [36]) was co-administered during the last 2 days. Adducts to plasma protein were also found but their elimination following cessation of dosage was monophasic (apparent half-life about 13.5 days). These results thus underline emerging recognition both of the generality of acyl glucuronide reactivity towards hydrolysis, acyl migration and covalent binding reactions and of the diversity of the manifestation of this reactivity, attributable to inherent differences in the chemical makeup of the drug moiety itself.

In summary, this work extends our earlier study [28] reporting formation of DF–HSA adducts from DAG *in vitro* by revealing the intermediacy of acyl migration. The data thus do not support transacylation as the operative mechanism for adduct formation. DAG and its individual 2-, 3- and 4-isomers show considerable differences in their capacity to act as substrates for adduct formation *in vitro*, thus complementing our companion study [33] which shows major differences in their capacity to act as substrates for further glucuronidation *in vivo*.

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