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STUDIES ON THE REACTIVITY OF ACYL GLUCURONIDES—VII

SALICYL ACYL GLUCURONIDE REACTIVITY *IN VITRO* AND COVALENT BINDING OF SALICYLIC ACID TO PLASMA PROTEIN OF HUMANS TAKING ASPIRIN*

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Abstract—Salicyl acyl glucuronide (SAG) is a significant metabolite of salicylic acid (SA) and aspirin. We have shown that, under physiological conditions *in vitro*, SAG undergoes rearrangement in a manner consistent with acyl migration to its 2-, 3- and 4-*O*-acyl positional isomers as the predominant pathway ($T_{1/2}$ values were 1.4–1.7 hr in buffer at pH 7.4 and 37°). Incubation of SAG or a mixture of its rearrangement isomers (*iso*-SAG) (each at ~50 μg SA equivalents/mL) with human serum albumin (HSA, at ~40 mg/mL) revealed the formation of covalent adducts with the protein, with peak concentrations of 1–2 μg SA equivalents/mL. The data support a role for the rearrangement/glycation mechanism of adduct formation. Covalent adducts of SA were also detected in the plasma of humans taking aspirin (at ≥1200 mg/day), but the concentrations were low (≪100 ng SA equivalents/mL). Reactivity of SAG thus provides a mechanism (though of uncertain quantitative importance) of covalent attachment of the salicyl moiety of aspirin to tissue macromolecules, which is in addition to its well-known acetylating capacity.

It is now well established that acyl glucuronides are (at least potentially) reactive metabolites *in vitro* and *in vivo*, capable of undergoing hydrolysis, intramolecular rearrangement and intermolecular covalent binding reactions with protein [1, 2]. Rearrangement occurs by hydroxide ion-catalysed migration of the drug moiety from the biosynthetic 1-*O*-β position to the neighbouring 2-, 3- and 4-positions on the glucuronic acid ring (Fig. 1). The migrations are reversible, with the exception of any appreciable reformation of the parent glucuronide [3]. The isomers can exist in both C-1 β- and α-anomeric forms through the intermediacy of the open-chain form of the glucuronic acid moiety [4, 5].

Two mechanisms have been proposed to account for the covalent binding of acidic drugs to protein via their acyl glucuronide metabolites. The transacylation mechanism [6, 7] involves direct displacement of the glucuronic acid moiety from the glucuronide by attack of a suitable nucleophilic group such as -SH, -OH or $-NH_2$ located on the protein. In this mechanism, the drug moiety becomes directly linked to the protein via a thioester, ester or amide bond. The alternative rearrangement/glycation mechanism [8, 9] has a prerequisite for acyl migration which permits, as noted above, transient ring-opening of the glucuronic acid moiety with consequent production of an aldehyde group at C-1. Condensation of the aldehyde with an amino group on protein would yield an imine: subsequent Amadori rearrangement could then yield a more stable ketoamine. In this mechanism, the drug remains linked to the glucuronic acid moiety which itself becomes linked to the protein. As noted earlier, this mechanism *requires* formation of the acyl migration isomers before interaction with the protein. By contrast, the transacylation mechanism should be strongly, if not exclusively, favoured by the glucuronide itself rather than its isomers [10]. Irrespective of the specific pathways involved, formation of such covalent drug–[glucuronic acid]–protein adducts has been suggested as mediating toxic and/or immune responses [1, 2, 6, 8]. Although most work on covalent binding has focused on plasma protein, notably human serum albumin (HSA‡), adduct formation is not limited to this protein. We have recently documented formation of covalent adducts of the salicylate derivative diflunisal with liver, kidney, intestine [11] and urinary bladder [12] tissue of rats dosed with this drug.

The acyl glucuronides of many carboxylic drugs have been demonstrated to undergo rearrangement via acyl migration and to form covalent adducts with protein [1, 2]. Not included in this list, surprisingly, is salicyl acyl glucuronide (SAG), a significant metabolite of salicylic acid (SA, Fig. 2), which in turn is the primary metabolite of the commonly used

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‡ Abbreviations: SA, salicylic acid; SAG, salicyl acyl glucuronide; *iso*-SAG, mixture of rearrangement isomers of salicyl acyl glucuronide; HSA, human serum albumin; $T_{1/2}$, half-life.

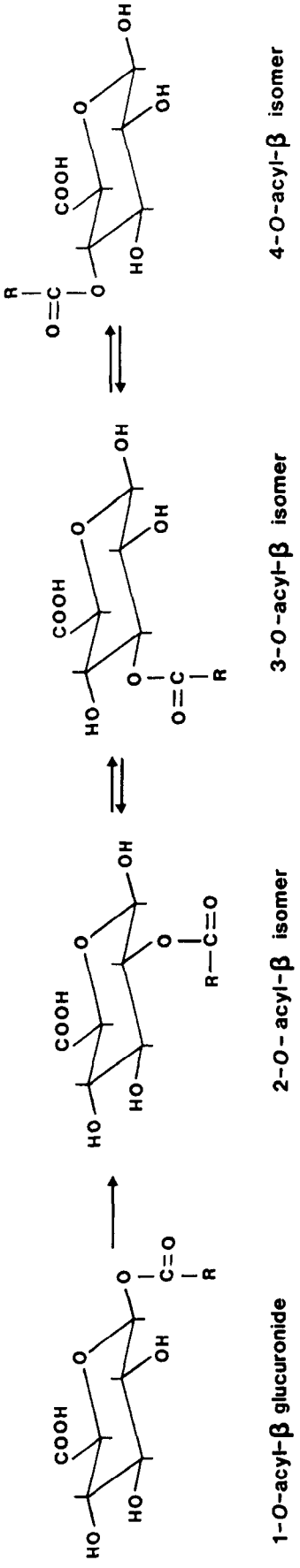

Fig. 1. Scheme showing rearrangement of biosynthetic, β-glucuronide-susceptible 1-*O*-acyl-β glucuronides to the β-glucuronidase-resistant 2-, 3- and 4-*O*-acyl positional isomers.

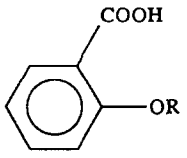

Fig. 2. Chemical structure of aspirin (R = $-COCH_3$) and its metabolite salicylic acid (R = H).

"over-the-counter" non-steroidal anti-inflammatory and analgesic agent, aspirin [13, 14]. SAG has been cited as the only example of an acyl glucuronide known not to undergo rearrangement [15]. A later study reported that SAG did rearrange, though by a different mechanism involving the neighbouring phenolic group of the SA ring, to yield a single isomer which had relatively high stability [3]. It seemed to us then, that if SAG showed the ability to yield covalent adducts with protein, in the absence of an ability to undergo rearrangement via acyl migration, this would clearly favour the transacylation mechanism. The present study was carried out to address this question.

MATERIALS AND METHODS

Materials and animals. SA was purchased from BDH Chemicals (Poole, U.K.). Also purchased were NaSA (E. Merck, Darmstadt, Germany), phenoxyacetic acid (BDH Chemicals) and 4-methylsalicylic acid (Tokyo Kasei, Tokyo, Japan). An authentic sample of salicyl phenolic glucuronide was a gift from Dr A. Somogyi, University of Adelaide (Adelaide, Australia). Fatty acid-free HSA (product A1887) and β-glucuronidase (Type H-2, from *Helix pomatia*) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Methanol and acetonitrile (HPLC grade), diethyl ether (AR grade) and hexane (nanograde) were purchased from Mallinckrodt (Melbourne, Australia). Reagents were AR grade. Male Sprague–Dawley-derived rats (~330 g) were obtained from The University of Queensland Medical Faculty Animal House. Experiments were approved by the University's Animal Experimentation Ethics Committee.

Isolation of SAG from rat urine. A male Sprague–Dawley-derived rat was injected, via a catheter in the jugular vein [16], three times with 100 mg SA/kg (as 20 mg NaSA/mL water) at 8 hr intervals. The 0–24 hr urine (~7 mL) was collected over ice into a tube containing 0.5 mL of 1 M acetic acid solution to achieve a final pH value of ~3.5. Following centrifugation, 200 μL aliquots were injected into HPLC system 1, with operating conditions as described, except that the flow rate was 2.0 mL/min. Under these conditions, the retention times for salicyl phenolic glucuronide, SAG and SA were ~11, 23 and 48 min, respectively. The SAG fractions were collected, and further clean-up and desalting achieved with Sep-Pak cartridges (Waters, Milford, MA, U.S.A.). The cartridges were prepared by wetting with 10 mL of 1% (v/v) acetic acid in

acetonitrile and flushing with 5 mL of 0.01 M phosphate buffer pH 4.5. A 5 mL sample of the combined SAG fractions (above) was loaded onto the cartridge and rinsed with 1 mL 0.01 M phosphate buffer pH 4.5. The SAG was eluted with 5 mL of 1% acetic acid in acetonitrile. The eluted fractions were combined and concentrated to *ca.* 5 mL. Quantification of the SAG content was achieved by alkaline hydrolysis and measurement of liberated SA. Thus, 25 μL aliquots were evaporated to dryness, hydrolysed in 50 μL of 1 M NaOH at 80° for 2 hr, and neutralized with 50 μL of 1 M HCl. After mixing with 100 μL of a solution of acetic acid in acetonitrile (4% v/v) containing phenoxyacetic acid (1 mg/mL) as internal standard, a 40 μL sample was analysed for SA using HPLC system 1. The stock solution contained SAG at ~0.6 mg SA equivalents/mL, was free of other SA-related species, and was stored at −20°. When required for incubation experiments, appropriate aliquots were removed and evaporated to dryness.

Rearrangement of SAG in buffer. Solutions of SAG at ~150 μg SA equivalents/mL in various buffers at various pH values (detailed in Results) were incubated at 37°. Aliquots (30 μL) were taken at appropriate times for injection into HPLC system 1. An internal standard was not used. Instead, accurate 20 μL volumes were applied to the column by use of a 20 μL loop on the injector. First order SAG degradation was analysed by linear regression of its peak areas, using data from at least five time-points.

Isolation of iso-*SAG.* A second batch of SAG, isolated from rat urine as described above, was evaporated to dryness, reconstituted in 2 mL of water adjusted to pH 7.4 (NaOH) and rerranged by incubation at 37° for 8 hr. The pH was then adjusted to ~3.5 (H_3PO_4). A mixture of the rearrangement isomers (*iso*-SAG) was then isolated by injection into HPLC system 1 of aliquots, collection of fractions containing the six rearrangement peaks (see Fig. 3A), and desalting using Sep-Pak cartridges as described above for SAG itself. After concentration of the combined fractions to ~3 mL, the *iso*-SAG content was quantified by alkaline hydrolysis to SA and measurement on HPLC system 1 (as detailed above for SAG stock solutions). The stock solution contained *iso*-SAG at ~0.42 mg SA equivalents/mL, was free of SAG and SA, and was stored at −20°.

Incubation of SAG and iso-*SAG with HSA.* Solutions (*ca.* 5 mL) of SAG and *iso*-SAG were prepared at ~50 μg SA equivalents/mL of pre-warmed 0.1 M phosphate buffer pH 7.4 containing HSA at 40 mg/mL, and incubated at 37°. Aliquots (duplicate 20 μL and single 0.5 mL) were taken immediately and after 0.5, 1, 2, 4, 8 and 24 hr incubation. The 20 μL samples were either stored on dry ice until assay, or immediately assayed for non-covalently bound SA, SAG and *iso*-SAG using HPLC system 1. The 0.5 mL samples were stored on dry ice until assayed or covalently bound SA using HPLC system 2.

Human studies. Two healthy human volunteers took 1200 mg aspirin (600 mg at ~8 a.m. and 8 p.m.) per day for 14 days. Blood samples (10 mL) were drawn pre-dose, at 1–2 day intervals (at ~2 p.m.) during the dosing period, and at 2, 4, 7 and 10 days after completion of the dosing period. Another three volunteers took the same daily dose for 3 days, with blood samples collected pre-dose and ~6 hr after the last dose had been taken. In addition, blood samples were collected pre-dose and every second day from a subject who took high doses of aspirin (3000–3600 mg/day) for 8 days to treat musculoskeletal pain. The blood samples were collected into tubes containing lithium heparin, centrifuged immediately, and the plasma snap-frozen on dry ice and stored at −20° until aliquots (0.5 mL) were required for analysis of covalently bound SA.

Analysis of (non-covalently bound) SAG, iso-*SAG and SA (HPLC system 1).* Samples (20 μL) from incubations of SAG and *iso*-SAG with HSA solutions were mixed with 40 μL of a solution of acetic acid in acetonitrile (4% v/v) containing phenoxyacetic acid (1 mg/mL) as internal standard. After centrifugation, the supernatant was transferred and carefully evaporated to the point of dryness at room temperature under a stream of air. The residue was reconstituted in 100 μL of HPLC mobile phase, and a 40 μL sample applied to the column.

HPLC system 1 comprised a model 510 pump, a RCM-100 radial compression module containing a 4 μM Novapak C_{18} cartridge and preceded by a guard column containing Bondapak C_{18} Corasil, a model 481 LC spectrophotometer (all from Waters, Milford, MA, U.S.A.), a model 7125 injector (Rheodyne, Cotati, CA, U.S.A.) and a model C-R3A Chromatopac integrator (Shimadzu, Kyoto, Japan). The mobile phase was prepared by mixing 0.1 M NaH_2PO_4 adjusted to pH 2.7 with H_3PO_4 and methanol in a v/v ratio of 9:1. The flow was 2.5 mL/min with column eluent being monitored at 240 nm. Under these conditions, the retention times for the six peaks comprising *iso*-SAG were ~7.7 and 9.5 min (presumed anomers of the 4-isomer), 8.6 and 11.0 min (presumed anomers of the 3-isomer) and 12.5 and 14.8 min (presumed anomers of the 2-isomer), and for SAG, phenoxyacetic acid internal standard and SA were ~16.5, 25 and 35 min, respectively. The relative molar absorbances of SAG, *iso*-SAG and SA under these specified HPLC conditions at the analytical wavelength of 240 nm were determined to be 1:1:0.6, verified by (a) monitoring peak areas during complete conversion of SAG to SA via *iso*-SAG by incubation at pH 7.4 and 8.0 and (b) direct alkaline hydrolysis of SAG and *iso*-SAG to SA. Calibration curves for SA were prepared over the concentration range 1–100 μg SA/mL, and were linear with correlation coefficients about 0.999. Precision and accuracy, respectively, were found to be 3.0 and 2.7% at 10 μg SA/mL, 5.9 and 5.1% at 20 μg SA/mL, and 2.9 and 2.4% at 50 μg SA/mL.

Analysis of covalently bound SA (HPLC system 2). To a sample (0.5 mL) of HSA solution or plasma was immediately added 1 mL of a 4% (v/v) solution of acetic acid in acetonitrile, followed by vortex agitation and sonication (10 min). After centrifugation, the supernatant was discarded and the pellet resuspended in 0.75 mL of 0.1 M phosphate buffer pH 4.7 and vortex agitated. The protein was then

precipitated with 3.75 mL of acetonitrile, with vortex agitation. Following centrifugation, the supernatant was discarded. This washing procedure was repeated nine times. (The adequacy of the washing procedure to remove non-covalently bound SA was verified using blank plasma spiked to ~100 μg SA/mL). The pellet was then carefully dried at room temperature under a stream of air, and digested in 0.5 mL of 1 M NaOH solution by heating overnight at 65°. Following cooling and acidification (200 μL of 5 M HCl solution), internal standard (100 μL of 25 μg 4-methylsalicylic acid/mL 0.01 M phosphate buffer pH 4.7) was added, and the mixture extracted with 3.5 mL of 1:1 (v/v) ether–hexane. After centrifugation, the organic layer was transferred and equilibrated with 100 μL of 0.5 M NaOH solution. Following centrifugation, the organic layer was aspirated to waste and its residue evaporated under a stream of air. The residual aqueous layer was neutralized by addition of 20 μL of 2.5 M HCl solution and diluted by addition of 100 μL of HPLC mobile phase. A sample (40 μL) was injected.

HPLC system 2 comprised the same hardware components as system 1 except that a model 470 fluorescence detector (Waters) was used. The mobile phase was prepared by adding an aqueous solution (0.1% w/v tetra-*n*-butylammonium bromide in water with pH adjusted to 7.4 by addition of 2 M phosphate buffer pH 7.4) to 250 mL acetonitrile to a final volume of 1 L. The flow was 0.8 mL/min with column eluent being monitored at excitation and emission wavelengths of 300 and 490 nm, respectively. Under these conditions, SA and 4-methylsalicylic acid were eluted at retention times of ~9 and 14 min, respectively. Calibration curves were prepared over the concentration ranges 10–100 ng SA/mL plasma and 0.1–5 μg SA/mL HSA solution, and were linear with correlation coefficients exceeding 0.99. Precision and accuracy, respectively, were found to be 19 and 14% at 10 ng SA/mL, 5.9 and 5.2% at 20 ng SA/mL, and 4.7 and 6.1% at 50 ng SA/mL for plasma, and 1.2 and 1.0% at 0.1 μg SA/mL, 4.3 and 3.0% at 0.5 μg SA/mL, and 1.6 and 1.7% at 2 μg SA/mL for HSA solutions. The limits of detection and quantification of SA in plasma were ~5 and 10 ng SA/mL, respectively.

RESULTS

SAG was isolated by preparative HPLC from the urine of rats dosed i.v. with NaSA and from the urine of human volunteers taking aspirin. Details of the latter are not presented: isolation from rat urine was selected from preliminary experiments as the method of choice since (a) rats do not form salicyluric acid (which is a major metabolite in humans), (b) rat urine contained fewer endogenous components capable of interfering with the HPLC separations, and (c) because of higher dosages, SAG in rat urine could be obtained at concentrations well in excess of 10 times those found in human urine. The identity of SAG was confirmed by both β-glucuronidase- and alkali-mediated hydrolysis to SA, using two different HPLC systems and authentic SA and salicyl phenolic glucuronide (β-glucuronidase-susceptible but alkali-resistant) as reference materials (data not shown).

Degradation of SAG during incubation in 0.1 M borate buffer at pH 7.4 and 37° is illustrated in Fig. 3A. Decline of the SAG peak (~16.5 min) was accompanied by sequential appearance of pairs of earlier-eluting peaks (~12.5 and 14.8 min, 8.6 and 11.0 min, 7.7 and 9.5 min, in order of appearance) and by appearance of the SA peak (~35 min). This behaviour was consistent with classical acyl migration of the biosynthetic 1-*O*-β glucuronide (to the 2-, 3- and 4-*O*- positional isomers, each existing in the C1 β- and α-anomeric forms) [4, 5], and concurrent hydrolysis of the glucuronide and/or its isomers. This interpretation was supported by collection of fractions containing the three first-eluting peaks (i.e. 7.7, 8.6 and 9.5 min) from a 4 hr incubation. After reconstitution at pH 7.4, additional peaks corresponding to the three last-eluting rearrangement isomers/anomers (at ~11.0, 12.5 and 14.8 min), SA itself (at ~35 min), but not SAG, were formed with time. This behaviour was consistent with reversibility of migration amongst isomers without significant reformation of the acyl glucuronide, and with hydrolysis of isomers to the aglycone.

There was little chromatographic evidence for the existence of rearrangement pathways other than classical acyl migration: specifically that of SAG rearrangement by involvement of the neighbouring phenolic group of the SA ring to yield a relatively stable isomer [3]. The small peak at retention time ~18.7 min in Fig. 3A was clearly a contaminant present from the outset, though the origin of another small peak at retention time ~15.0 min, visible in the chromatogram from 96 hr incubation, was uncertain. Attempts to divert rearrangement of SAG from the apparent acyl migration pathway to that involving the phenolic group [3] were made by reproducing the specified incubation conditions (i.e. SAG in 0.1 M phosphate buffer pH 8.0 for 72 hr at room temperature), as well as using buffers of different ionic strengths at different pH values at 37°. Degradation half-lives for SAG are summarized in Table 1. These studies always supported classical acyl migration as the predominant rearrangement pathway for SAG, though there was chromatographic evidence for a more stable rearrangement product at higher pH, best illustrated by incubation in 0.1 M borate buffer at pH 8.6 (Fig. 3B). Thus, a relatively broad peak (more than one compound?) at retention time ~15.0 min was formed in greater proportion to that observed in other incubations, though it ultimately disappeared on continued incubation, albeit at a slower rate than the presumed acyl migration isomers.

The profiles for rearrangement, hydrolysis and covalent binding when SAG was incubated with fatty acid-free HSA at pH 7.4 and 37° are shown in Fig. 4A. There was a slight protective effect of the presence of the protein on SAG degradation ($T_{1/2}$ of 2.2 hr, $r^2 = 0.999$) compared with incubation in buffer alone under the same conditions (Table 1). Rearrangement was the predominant pathway, with formation of covalent adducts of SA with HSA least important quantitatively. Steady production of SA obviously corresponded to hydrolysis of SAG and its isomers, with presumably a small contribution from hydrolysis of the adducts. The corresponding

profiles when a prepared mixture of the rearrangement isomers (*iso*-SAG) was incubated with the proteins are shown in Fig. 4B. *Iso*-SAG declined with an overall $T_{1/2}$ of 7.9 hr ($r^2 = 0.990$), similar to the terminal $T_{1/2}$ of *iso*-SAG generated *in situ* (8.2 hr between 8 and 24 hr, Fig. 4A). The initial rates of hydrolysis and adduct formation from *iso*-SAG were greater than those found for SAG, though the final adduct concentrations (measured at 24 hr) were comparable.

To ascertain whether covalent adducts of SA with plasma protein were formed *in vivo*, blood samples were obtained from subjects taking aspirin at 1200 mg/day. The same exhaustive protein washing procedure and base digestion method successfully used for quantification of adducts from the *in vitro* HSA studies was applied to the plasma samples. However, the results were somewhat equivocal: peaks corresponding to SA released from base digestion of the washed protein pellets were found, though at concentrations (≤20 ng SA equivalents/mL) close to the limit of quantification of the assay (i.e. ~10 ng SA/mL plasma). In a single subject who took high doses of aspirin (3000–3600 mg/day) for 8 days, adduct concentrations of 50–70 ng SA/mL plasma were achieved.

Table 1. Degradation half-lives of SAG in buffer at 37°

Buffer	pH	$T_{1/2}$* (hr)
0.2 M phosphate	4.5	>70
0.2 M phosphate	6.0	18
0.1, 0.5 and 2.0 M phosphate, 0.1 M borate	7.4	1.4–1.7
1.0 M phosphate, 0.1 M borate	8.0	0.6
0.1 M borate	8.6	0.12

* Coefficients of determination (r^2) were about 0.999.

DISCUSSION

Rearrangement of acyl glucuronides via acyl migration at physiological pH (Fig. 1) is now well-established, having been first observed for bilirubin glucuronide in the late seventies [17] and subsequently for the glucuronides of many acidic drugs [1–3]. Such acyl migration between neighbouring hydroxy groups of polyhydroxy compounds is well-known in carbohydrate chemistry, as is the existence of reducing sugars as β- and α-anomers through the intermediacy of the open-chain form of the sugar ring. In earlier work with the salicylate derivative diflunisal, we and others have documented "classical" rearrangement of its acyl glucuronide metabolite (i.e. acyl migration and anomerization of the isomers) [4, 5, 18, 19]. It was therefore surprising to us that the acyl glucuronide of salicylic acid itself was reported either not to undergo rearrangement [15] or to rearrange to a single relatively stable isomer by involvement of the phenolic group neighbouring the carboxy group of the salicylate ring [3]. In the context of involvement of the *ortho*-phenolic group of the salicylate ring, it should be noted that evidence for a novel (albeit quantitatively very minor) pathway of rearrangement of diflunisal acyl glucuronide (i.e. formation of diflunisal phenolic glucuronide postulated via the 2-*O*-α isomer of the acyl glucuronide) has been presented [19]. However, as we have previously reported [4], we found no evidence for this reaction of diflunisal acyl glucuronide under the same experimental conditions.

Incubation of SAG under the experimental conditions found elsewhere to yield a single stable isomer [3] gave, in our hands, results consistent with classical rearrangement (i.e. acyl migration and anomerization of the isomers so formed) as the predominant pathway (Fig. 3). Indeed, this result was obtained under a multitude of incubation conditions (variations in pH, temperature, buffer type and ionic strength) which was tested to ascertain whether the rearrangement pathway could be driven in a different direction. However, there was chromatographic evidence (see Fig. 3 and Results) for involvement of an additional pathway(s) at higher pH values, notably in borate buffer at pH 8.6. It seems to us then, that there may well be incubation conditions which catalyse rearrangement by interaction with the *ortho*-phenolic group, but we are unable to identify them clearly.

The results for incubation of SAG with HSA clearly show that covalent adducts of SA are formed with this protein *in vitro*. A role for the rearrangement/glycation mechanism of adduct formation seems assured from the *iso*-SAG data (Fig. 4B), since (a) adducts formed more rapidly than with SAG (Fig. 4A) and (b) reversibility of acyl migration does not include reformation of the acyl glucuronide itself in any significant amount. Whether adducts were formed directly by interaction of SAG itself with the protein (e.g. via a transacylation mechanism) could not be ascertained from the present data (Fig. 4A), as the adducts formed could have arisen from *iso*-SAG generated by rearrangement of SAG *in situ*. From this and earlier studies on reactivity of isomers of the acyl glucuronides of diflunisal [10, 20] and valproate [21] with HSA, it seems that the glycation mechanism is a general one which may be expected of all acyl glucuronides, with the extent of adduct formation depending on the ease with which rearrangement occurs. Recent structural studies on adducts formed by incubation of tolmetin acyl glucuronide with HSA have confirmed the operation of this mechanism *in vitro* [22]. However, it should be stressed that a role for the transacylation mechanism is not excluded, though chemical structural evidence has yet to be presented.

Only very low concentrations of adducts (<0.1 μg SA equivalents/mL) were found in the plasma of subjects taking moderate to high doses of aspirin. Such concentrations are some 20 times lower than those observed in plasma of subjects taking short courses of diflunisal and probenecid [23], and presumably reflect that (a) SAG formation is a quantitatively less important pathway for aspirin metabolism than acyl glucuronidation of the other drugs and (b) SAG is less reactive ($T_{1/2}$ *in vitro* ~1.4–2.0 hr compared to ~0.4–1.0 for diflunisal acyl glucuronide [10, 20]). Covalent attachment of foreign compounds to native proteins *in vivo* has been

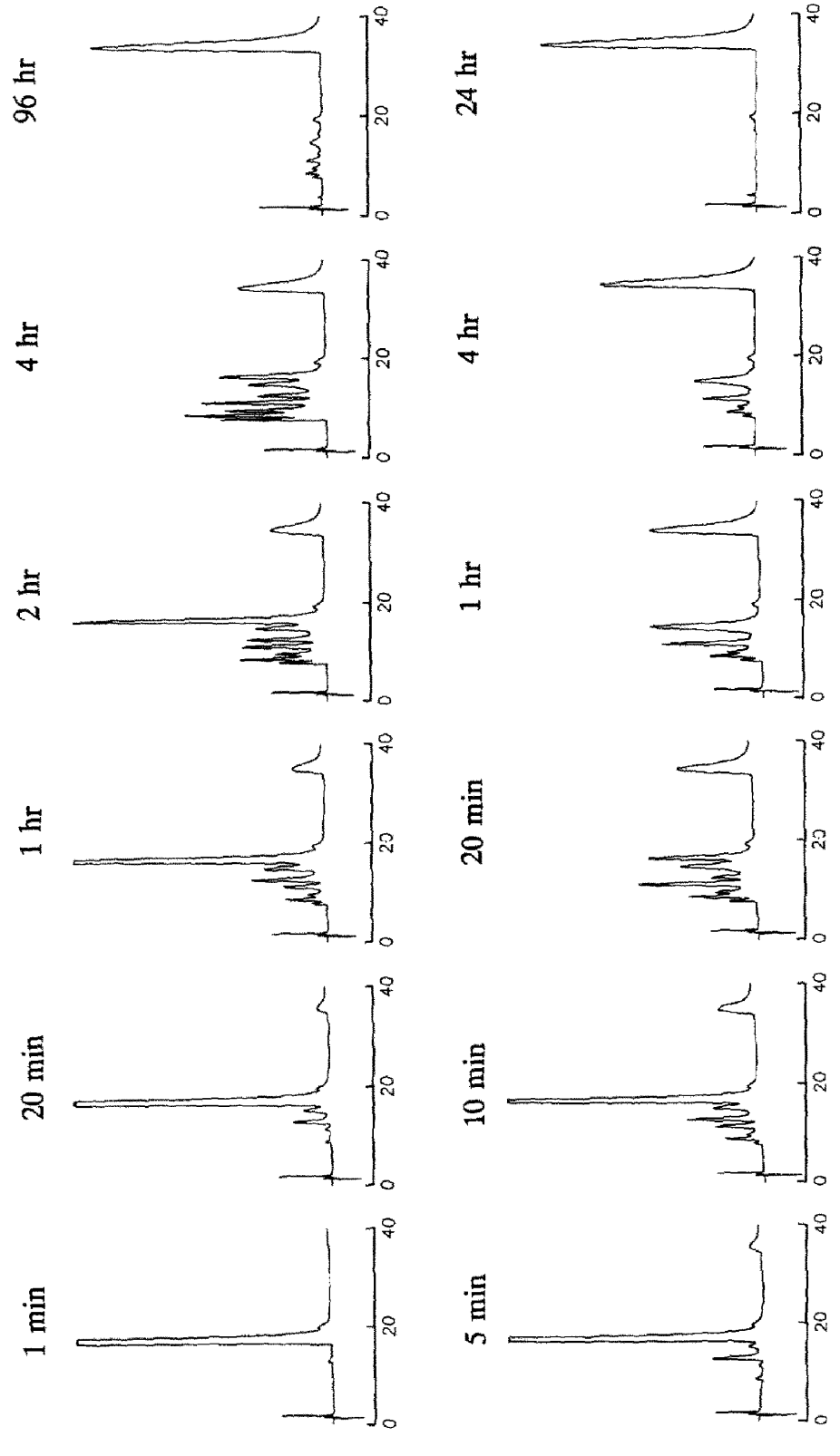

Fig. 3. Chromatograms showing degradation of SAG (retention time 16.5 min) via rearrangement (sequential appearance of pairs of earlier-eluting peaks at 12.5 and 14.8 min, 8.6 and 11.0 min, and 7.7 and 9.5 min) and hydrolysis to SA (retention time 35 min) at 37° in 0.1 M borate buffer pH 7.4 (panel A) and 8.6 (panel B). A broader peak at retention time 15.0 min was more visible at later incubation times in panel B.

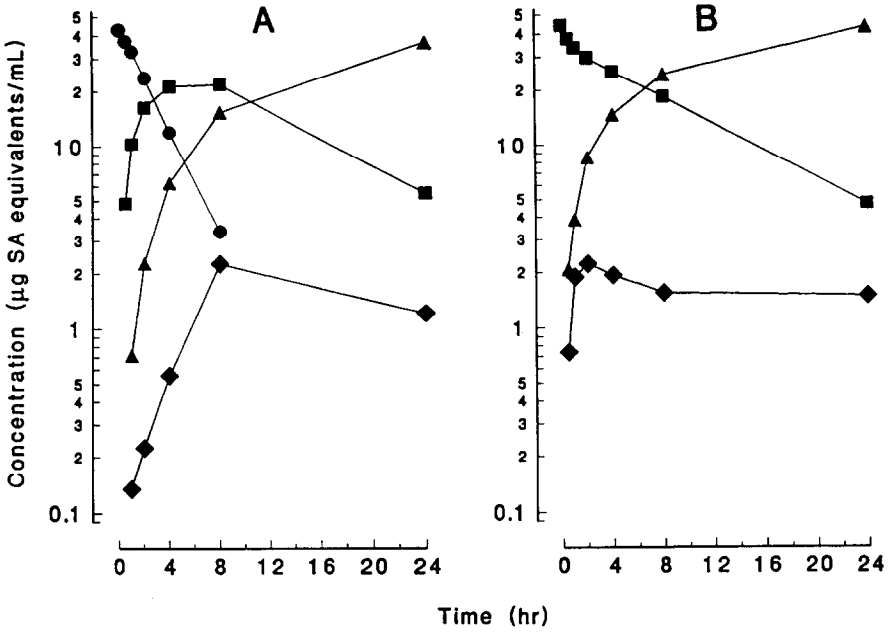

Fig. 4. Profiles for degradation of SAG (●) (panel A) and a mixture of its rearrangement isomers (■) (panel B) upon incubation of initial concentrations of *ca.* 50 μg SA equivalents/mL with fatty acid-free HSA (*ca.* 40 mg/mL) in 0.1 M phosphate buffer pH 7.4 and 37°. Hydrolysis is represented by liberation of SA (▲) and covalent binding by formation of drug–protein adducts (◆).

associated with immune and/or toxic responses [24, 25]. Aspirin is well-known to cause hypersensitivity and other adverse reactions, though the incidence must be regarded as relatively rare given its widespread usage [26, 27]. Many such side effects can be attributed to the acetylation capacity of aspirin [28]. The present study shows that the salicyl moiety of aspirin may also become covalently bound to plasma protein (albeit at very low concentrations). Whether any biological responses may result is yet to be established.

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