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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE QUANTITATION OF SALICYLAMIDE AND ITS METABOLITES IN BIOLOGICAL FLUIDS

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SUMMARY

A high-performance liquid chromatographic (HPLC) assay is described for the determination of salicylamide (SAM) and its metabolites in biological fluids obtained from an in situ rat liver preparation and rat in vivo. SAM and its six metabolites, salicylamide sulphate, salicylamide glucuronide, gentisamide (GAM), gentisamide 2-sulphate, gentisamide 5-sulphate and gentisamide 5-glucuronide, were separated on a μ Bondapak C_{18} column with 0.085 *M* potassium dihydrogenphosphate pH 3.35, as mobile phase and by using a flow gradient. Enriched recovery of SAM and GAM from blood was achieved by extraction followed by a second HPLC procedure with a mobile phase consisting of acetic acid and methanol. Furthermore, the reproducibility and the stability of the samples under assay conditions were investigated.

INTRODUCTION

Salicylamide (SAM) is employed not only for the study of drug metabolism in the liver [1–3], but also for that in extrahepatic organs such as the intestine [4–7] and the lung [8], because of its high first-pass effect [9–13]. The biotransformation of SAM includes sulphation and glucuronidation at the 2-position and hydroxylation at the 5-position to form gentisamide (GAM). GAM further undergoes conjugation to form both sulphates and glucuronides. Recently, two different GAM monosulphate conjugates (2- and 5-positions) have been identified in our laboratory whereas glucuronidation occurs exclusively at the 5-position [14]. A scheme of the metabolic pathways of SAM is shown in Fig. 1.

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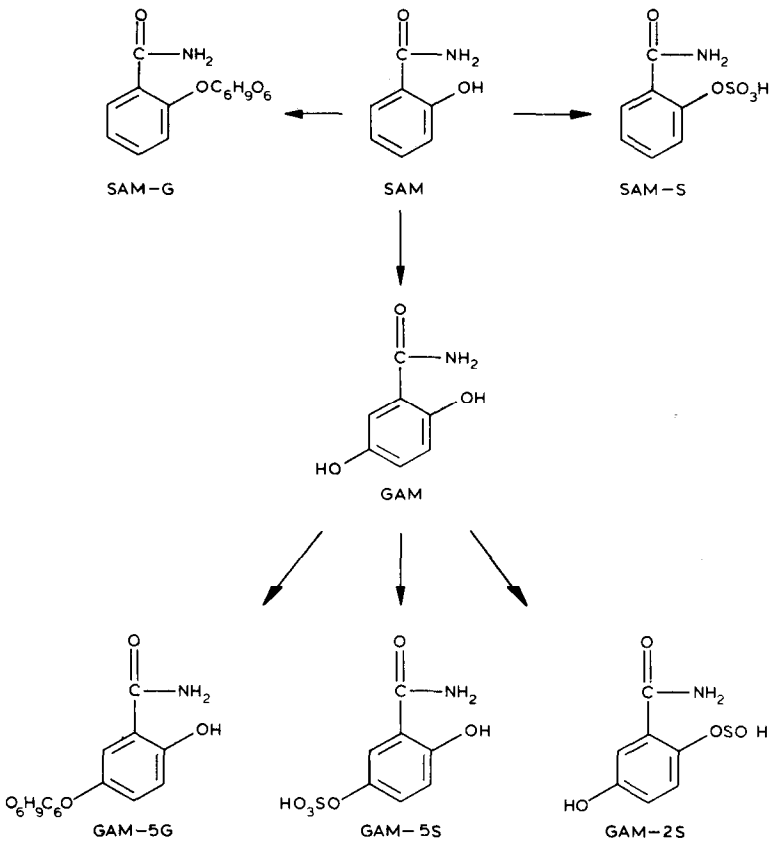

Fig. 1. Metabolic pathways of salicylamide (SAM).

Determination of SAM metabolites in biological fluids is usually performed by enzymatic hydrolysis [4–6,9]. However, this indirect method has several disadvantages. First, it is not suitable for aglycones which are unstable upon incubation. Unpublished results from our laboratory showed that GAM is unstable during incubation at 37 °C, with a decomposition rate of 2.4%/h. Therefore, if this indirect method is used to estimate GAM formation from SAM, total hydroxylation products will be underestimated. Second, with the hydrolytic procedure one may not be able to distinguish between the mono- and diconjugates or discriminate positional isomers of the monosulfate or monoglucuronide conjugates. Therefore, a method allowing direct measurement by chromatography is desirable.

Recently, several liquid chromatographic assays have been published for the determination of SAM and its metabolites in blood, plasma and urine [15,16]. However, there has been no report on the separation of all SAM and GAM conjugates in a single chromatographic procedure nor on the identification of different GAM conjugates. In this study, a high-performance liquid chromatographic (HPLC) method is described which separates SAM and all of its six metabolites in plasma, bile and urine. This assay utilizes a simple mobile phase with a flow

gradient program to achieve optimal separation conditions. Verification of the identity of SAM and GAM conjugates is performed by incorporation of sodium [^{35}S]sulphate or [^{14}C]glucose, precursors of sulphates and glucuronides of SAM and GAM, by a perfused rat liver preparation, followed by isolation and identification. Quantification of unconjugated SAM and GAM in blood is also described.

EXPERIMENTAL

Materials

Male Sprague-Dawley rats were purchased from Charles River (St. Constant, Canada). Unlabelled SAM was obtained from Sigma (St. Louis, MO, U.S.A.). [^{14}C]SAM (specific activity 6.82 mCi/mmol) and unlabelled GAM were synthesized according to Mendel et al. [17] and Foye et al. [18], respectively, with modifications. [^{3}H]GAM was obtained from catalytic exchange with tritiated water from Dupont (specific activity 39 mCi/mmol). The purity of synthesized compounds was checked by thin-layer chromatography (TLC) on silica gel GF, 250 μm (Analtech, Newark, DE, U.S.A.). Gentisic acid was obtained from Aldrich (Milwaukee, WI, U.S.A.). Sodium [^{35}S]sulphate (447.35 mCi/mmol) and [^{14}C]glucose (57 mCi/mmol) were purchased from Dupont (NEN) Products (Boston, MA, U.S.A.). Glusulase (*Helix pomatia*) was obtained from Boehringer Mannheim (Darmstadt, F.R.G.); β-glucuronidase (bovine liver) and arylsulphatase (*Aerobactor aerogenes*) were purchased from Sigma. All other reagents and solvents (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) were of HPLC grade.

Isolation and identification of SAM and GAM conjugates

Because of a lack of authentic standards for SAM and GAM conjugates, the conjugates were biosynthesized by a recirculating in situ perfused rat liver preparation with cell-free and protein-free perfusate. Krebs Henseleit bicarbonate (KHB) buffer (100 ml), adjusted to pH 7.4, was used as the perfusion medium at a flow-rate of 40 ml/min. The rat liver was perfused in a recirculating fashion for 2 h at 37°C. Two groups of experiments were performed. In the first group, [^{14}C]SAM (30 μCi) and unlabelled SAM (150 μmol) (study A) or [^{3}H]GAM (50 μCi) and unlabelled GAM (100 μmol) (study B) were recirculated through the rat liver. In the second group, radiolabelled precursors of essential cofactors for sulphation and glucuronidation, sodium [^{35}S]sulphate (0.6 mCi) or [^{14}C]glucose (50 μCi), were recirculated through the rat liver with unlabelled SAM (20 μmol) or GAM (10 μmol): study C, sodium [^{35}S]sulphate and unlabelled SAM; study D, sodium [^{35}S]sulphate and unlabelled GAM; study E, [^{14}C]glucose and unlabelled SAM; study F, [^{14}C]glucose and unlabelled GAM. Bile was collected in toto throughout each experiment.

For isolation of SAM and GAM conjugates, perfusate (KHB) from all studies was filtered through 0.45-μm membranes (Nihon Millipore Kogyo, Yonezawa, Japan) at the end of the study. Aliquots of perfusate (100 μl) and bile (50 μl) were injected into the HPLC system, and eluates were collected at 0.5- or 1-min intervals with a fraction collector (Model 202, Gilson International, Middleton, WI, U.S.A.). After the addition of 8 ml of scintillation fluor (Ready-Solv HP/b, Beckman Instruments, Palo Alto, CA, U.S.A.), the samples were counted in a

liquid scintillation counter (Model LS 6800, Beckman). Perfusates were then concentrated via freeze-drying with a lyophilizer (Labconco, Kansas City, MO, U.S.A.) and the residues stored at −20°C until further analysis. In order to obtain purified conjugates, preparative HPLC was performed by repeated injections of 100 μl of reconstituted lyophilized sample (2 ml of water) or 50 μl of diluted bile. Radioactive peaks, as identified earlier, were again collected and concentrated by lyophilization. The identity of the conjugates in each fraction obtained by preparative HPLC was established by a comparison of the chromatographic patterns before and after hydrolysis with either acid or enzyme.

Acid hydrolysis was performed on the isolated, radiolabelled fractions from studies C–F. An aliquot (100 μl) of the reconstituted, purified fraction was incubated with 100 μl of 10 *M* hydrochloric acid (5 *M* in the final solution) at 60°C. After 0.5, 3, and 6 h, 100 μl of this solution were injected into the HPLC system, and the elution profiles were compared with those for sodium [^{35}S]sulphate, [^{14}C]glucose and unconjugated (unlabelled) GAM and SAM standards. For quantitative recovery of SAM and GAM due to acid hydrolysis of the conjugates, authentic standards of SAM (0.3–6 μg) and GAM (0.5–7.5 μg) were treated under identical conditions to provide calibration curves at 0.5 and 6 h. The stability of SAM and GAM (5 μg) under acidic conditions at 60°C was further evaluated at 0.5, 1, 2, 3, 5 and 6 h by comparing the UV absorbances (peak heights) with those found for SAM and GAM (5 μg) at 0 h (without acid).

For enzymatic hydrolysis, fractions isolated by preparative HPLC originating from studies A and B were used. After reconstitution of the lyophilized residues with water (1–2 ml), aliquots (100 μl) of these fractions were incubated with (a) 0.2 ml of Glusulase and 0.7 ml of 0.1 *M* acetate buffer, pH 5.5, (b) 0.2 ml of β-glucuronidase and 0.7 ml of 0.2 *M* acetate buffer, pH 5.0 and (c) 0.2 ml of arylsulphatase, 0.2 ml of 100 m*M* D-saccharo-1,4-lactone (an inhibitor for β-glucuronidase) and 0.5 ml of 0.2 *M* Tris buffer, pH 7.1, at 37°C for 24 h. The extent of hydrolysis at the end of incubation was determined by TLC separation of the aglycones, using radiolabelled SAM and GAM. Aliquots of the sample (100 μl) were spotted directly onto TLC plates (silica gel GF, 250 μm), which had been prespotted with unlabelled authentic standards of GAM and SAM at the origin for UV visualization after development. The plates were developed with chloroform–methanol–16.5 *M* ammonium hydroxide (80:20:1, v/v/v), giving zones with R_F values of 0.86 and 0.63 for unlabelled SAM and GAM, respectively. In order to verify the comigration of radiolabelled and unlabelled SAM and GAM, radiolabelled SAM and GAM were also spotted with unlabelled standards, and segments of 0.5-cm strips were scraped off into mini-counting vials for determination of the radioactivity. The R_F values for [^{14}C]SAM and [^{3}H]GAM were found to be identical to those for unlabelled SAM and GAM standards. Calibration curves for [^{14}C]SAM (500–10 000 dpm) and [^{3}H]GAM (8750–43 750 dpm) were also prepared under the same conditions. Radiolabelled SAM and GAM were incubated with enzyme and buffer, as described above, and separated by TLC. The corresponding zones for [^{14}C]SAM and [^{3}H]GAM on TLC were scraped off and the radioactivity was counted. By plotting the radioactivity spiked versus the radioactivity recovered from the TLC plate calibration curves were obtained.

Furthermore, the stability of SAM and its metabolites in buffers at 37°C for 24 h (without added enzyme) was investigated by comparison of the UV absorbances (peak heights) from HPLC before and after the incubation procedure.

Preparation of plasma, bile, blood and urine samples for HPLC

Blood, plasma and bile from an in situ rat liver perfusion and urine from the rat in vivo (male Sprague-Dawley, 325–378 g) were used as the biological media. The method of liver perfusion was identical to that described previously [19]. Perfusate consisted of 20% washed out-dated human red blood cell (Red Cross, Toronto, Canada), 1% bovine serum albumin (Sigma) and 300 mg/100 ml glucose (Travenol Canada, Ontario, Canada) in KHB solution buffered to pH 7.4.

An aliquot of plasma (0.2 ml) was precipitated with 0.1 ml of 1 *M* perchloric acid. After centrifugation at 12 000 *g* for 3 min, the supernatant was carefully transferred and 100 μl of this solution were injected into the HPLC system. Recovery of the radioactivity was checked by a comparison of the total counts collected in the eluate with the value obtained from direct counting. Correction for volume changes due to protein precipitation was also made. The stability of the conjugates in "plasma water" (supernatant) in the presence of acid was investigated at room temperature and at 4°C at hourly intervals, for 24 h by comparison of the UV absorbance for each compound with that found at time 0 h.

Bile was diluted with water (1:10) before injection into the HPLC system. An aliquot of diluted sample (20 μl) was used for the chromatographic assay. Again, loss of sample on the column and changes in volume due to dilution were corrected for.

Blood samples were made up to a constant volume (3 ml) with blank blood and extracted with 6 ml of ethyl acetate, with *p*-nitrophenol (0.1 ml of 10 μg/ml) as the internal standard. After shaking and centrifugation (10 min at 3000 *g*), the organic phase was transferred and dried under a stream of nitrogen. The residue was reconstituted with 100 μl of methanol and 20 μl of the reconstituted sample were injected into the HPLC system. Authentic standards of SAM (0.5–10 μg) and GAM (0.5–10 μg) in blood (3 ml) were prepared and processed simultaneously in the same manner as for the determination of the calibration curves. Standards for the calibration curves were repeatedly injected on three consecutive days, and the reproducibility of the assay was evaluated by a comparison of the regression parameters (slope and intercept) of the calibration curves. Furthermore, ten samples (low and high concentrations of unlabelled SAM and GAM) were prepared by the same procedure in order to estimate the coefficient of variation (C.V.) for the assay.

Urine in vivo was obtained after an intraperitoneal (i.p.) injection of SAM (140 mg/kg) and [^{14}C]SAM (3×10^7 dpm) to the rat. Urine was collected at 8, 16, 24 h, and daily thereafter for three days. The volume of urine collected during each interval was estimated by the weight of urine, assuming a density of 1. Total recovery of SAM and its metabolites in urine was determined by the amount of radioactivity in each sample divided by that in the injected dose (3×10^7 dpm). To illustrate the application of the present HPLC method, blank (0 h) and 8-h urine samples from the same rat were filtered through 0.45-μm membranes and

diluted with water (1:4). Aliquots (30 μl) were then injected into the HPLC system. HPLC elution was monitored at the wavelengths of 280 and 313 nm. The corresponding radioactivity elution profile was obtained by collecting the eluate at 0.5-min intervals for 60 min, and plotting the radioactivity measured in each fraction versus the midpoint time of the collection interval.

HPLC conditions

A Waters high-performance liquid chromatograph (Waters Canada, Mississauga, Canada) was used. The instrument consisted of a 6000A solvent delivery system, a WISP 710B automatic sampler, a 680 automated gradient controller, and a 441 spectrophotometric detector (wavelengths for the detection of SAM and GAM conjugates were set at 280 and 313 nm, respectively). Chromatograms were recorded on a Hewlett-Packard 5880A Series chart recorder (Level IV, Palo Alto, CA, U.S.A.). Separation was achieved by using a Waters μBondapak C_{18} column (10 μm, 25 cm $\times$ 4.6 mm I.D.) and a precolumn (C_{18} μBondapak, 40 μm).

Assay for plasma, bile and urine (HPLC system I). For the separation of SAM and its metabolites in plasma, bile and urine, the mobile phase (0.085 *M* potassium dihydrogen phosphate, pH adjusted to 3.35 by glacial acetic acid) was delivered at varying flow-rates with a flow gradient program (Fig. 2A). An initial flow-rate at 0.8 ml/min was maintained for 34 min. After elution of all SAM and GAM conjugates (run time 34 min), the flow-rate was increased at 0.2 ml/min to 1.8 ml/min from 34 to 39 min, and was maintained constant at this flow-rate for the next 11 min, during which GAM and SAM were eluted from the column (Fig. 2A).

The sensitivities of the UV detection at 280 and 313 nm for SAM and its metabolites were determined at a signal-to-noise ratio of three. For SAM and GAM, authentic unlabelled standards were injected. However, the sensitivities for salicylamide 2-sulphate (SAM-S), salicylamide 2-glucuronide (SAM-G), gentisamide 2-sulphate (GAM-2S), and gentisamide 5-sulphate (GAM-5S) were assessed by initially estimating the concentration of each purified fraction obtained from studies C–E by acid hydrolysis and liberation of unconjugated SAM and GAM (see *Isolation and identification of SAM and GAM conjugates*). This was followed by injection of varying amounts of each compound into the HPLC system. Owing to the difficulty in quantifying gentisamide 5-glucuronide (GAM-5G) by acid hydrolysis (GAM-5G was resistant to acid during short-term incubations or decomposed to gentisic acid after 6 h), the sensitivity of the method for GAM-5G was investigated by using bile samples of known specific activity for [^{14}C]SAM (74.2 μCi/mmol) from a once-through rat liver perfusion study [3]. After the injection of 20 μl bile, the radioactive fraction associated with GAM-5G was collected from the column and the radioactivity counted. The amount of GAM-5G (nmol) in bile was calculated from the specific activity of SAM, assuming that it remained the same for the metabolite. The sensitivity of the assay for GAM-5G was then found by injection of varying aliquots of this diluted bile.

Assay for unconjugated SAM and GAM in blood (HPLC system II). Separation of unconjugated SAM and GAM after extraction of blood was carried out with a reversed-phase column (μBondapak C_{18}) and a second mobile phase consisting

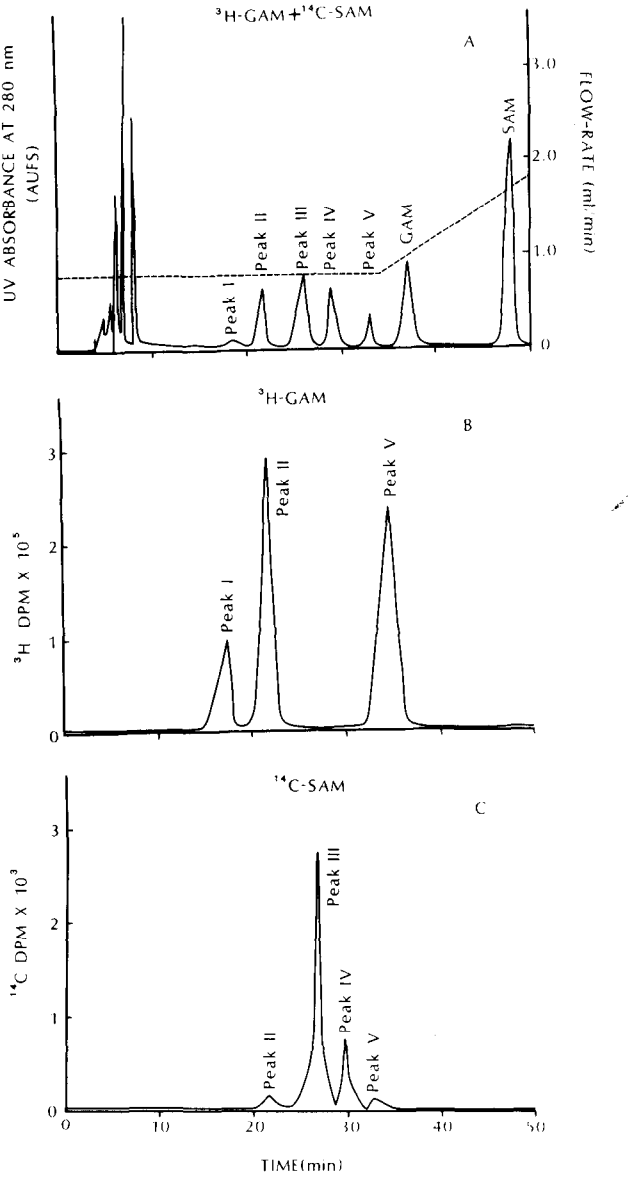

Fig. 2. HPLC elution profiles after the injection of 100 μl of (A) combined perfusate (equal mixture of KHB from biosyntheses with [^{14}C]SAM and [^{3}H]GAM; studies A and B), and authentic SAM and GAM standards; (B) KHB from biosynthesis with [^{3}H]GAM (study B) and (C) perfusate from biosynthesis with [^{14}C]SAM (study A).

of 0.5% acetic acid–methanol (60:40) (system II). A constant flow-rate of 0.6 ml/min was maintained, and the UV absorbance was monitored at 313 nm for detection of both unconjugated GAM and SAM [15].

RESULTS

Stability of SAM and its metabolites

SAM and GAM in 5 M hydrochloric acid at 60° C. It was found that SAM was stable for up to 6 h under acidic conditions (5 *M* hydrochloric acid) at 60° C. Significant decomposition of GAM occurred after 0.5 h and the formation of gentisic acid and two other unknown peaks was observed. At 6 h, only 75% GAM remained.

SAM and its metabolites in buffers at 37° C. During the incubation with buffers (without enzyme) at 37° C, SAM, SAM-G and GAM-5G were stable at 37° C for 24 h. However, only 93.3, 68.8, 76.5 and 83.9% of SAM-S, GAM, GAM-2S and GAM-5S, respectively, remained at 24 h. Other stability tests from previous experiments in our laboratory have shown that all GAM conjugates (GAM-2S, GAM-5S and GAM-5G) were stable for up to 6 h under similar conditions [14].

SAM and its metabolites in 1 M perchloric acid. All SAM and GAM conjugates in the supernatant were stable for only up to 3 h at room temperature, and for 6 h at 4° C after protein precipitation with 1 *M* perchloric acid.

Separation of SAM and its metabolites by flow gradient HPLC

Separation of SAM and its metabolites was successfully achieved with system I. The UV absorbance and HPLC elution profiles from injections of 100 μl of perfusate containing [^{3}H]GAM or [^{14}C]SAM metabolites (studies A and B) are shown in Fig. 2. The unknown metabolites were assigned in the order of their retention times: peak I, 18 min; peak II, 21 min; peak III, 26 min; peak IV, 29 min; peak V, 34 min; GAM and SAM appeared at 38 and 48 min, respectively (Fig. 2A). Radioactive peaks associated with [^{3}H]GAM (peaks II and V) also appeared with [^{14}C]SAM, but peak I was only associated with GAM. Peaks III and IV (possibly SAM sulphate and glucuronide conjugates of SAM) were present only with [^{14}C]SAM but not with [^{3}H]GAM (cf. Figs. 2B and 2C). Correspondence between UV absorbance and radioactivity profiles was good, except for a short delay due to sample collection (Fig. 2). Recovery of the radioactivity from the column was 95% for the plasma samples and 90% for the bile samples.

Identification of SAM and GAM conjugates

For samples labelled with [^{35}S]sulphate (studies C and D), two GAM metabolites retained radioactivity and appeared at 18 and 34 min, corresponding to peaks I and V, respectively (cf. Figs. 2B and 3B): this suggests that peaks I and V are sulphate conjugates of GAM. Two SAM metabolites also retained radioactivity and correspond to peaks III and V with retention times of 26 and 34 min, respectively (cf. Figs. 2C and 3C). The data suggest that peak III is the sulphate conjugate of SAM and peak V is a common metabolite of SAM and GAM. Analogous experiments with [^{14}C]glucose (studies E and F) further suggest that peak II is a GAM glucuronide and peak IV is a SAM glucuronide conjugate (Fig. 4). Because SAM has only one phenolic group (at the 2-position), peaks III and IV are SAM-S and SAM-G, respectively. The sulphate conjugates of GAM (peaks I

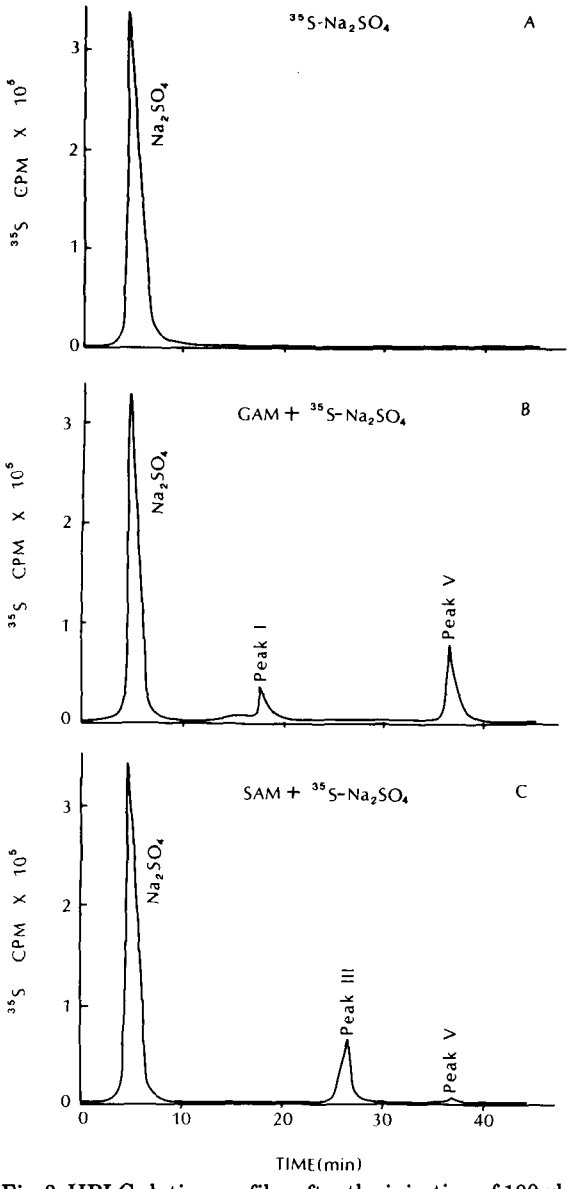

Fig. 3. HPLC elution profiles after the injection of 100 μl of (A) sodium [^{35}S]sulphate standard; (B) KHB from biosynthesis with unlabelled GAM and sodium [^{35}S]sulfate (study D) and (C) KHB from biosynthesis with unlabelled SAM and sodium [^{35}S]sulphate (study C).

and V) were further found to be monosulphate conjugates: GAM-2S and GAM-5S, respectively, and peak II is GAM-5G, as suggested by NMR studies [14].

Similar results were provided by acid/enzymatic hydrolysis. Only 0.5 h was required for complete acid hydrolysis of the sulphate conjugates, whereas 6 h were required for the glucuronides. Upon hydrolysis, peaks I and V, which contained ^{35}S, furnished a constant ratio of unlabelled GAM and [^{35}S]sulphate in as much

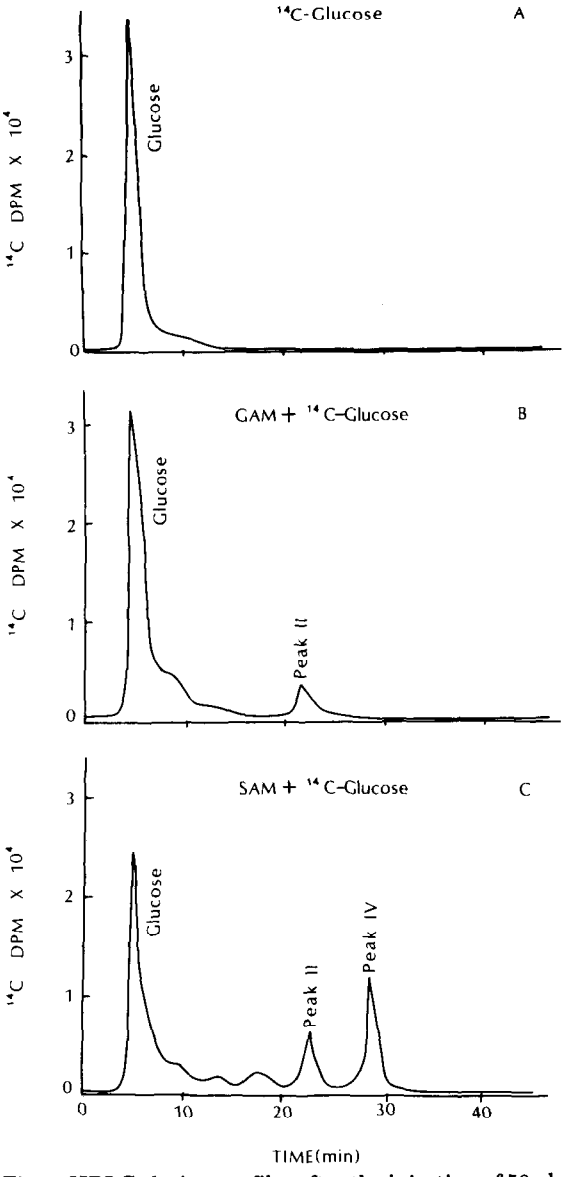

Fig. 4. HPLC elution profiles after the injection of 50 μl of (A) [^{14}C]glucose standard; (B) bile from biosynthesis with unlabelled GAM and [^{14}C]glucose (study F) and (C) bile from biosynthesis with unlabelled SAM and [^{14}C]glucose (study E).

as there are monosulphate conjugates of GAM; hydrolysis of peaks III and V yielded unlabelled SAM and [^{35}S]sulphate and unlabelled GAM and [^{35}S]sulphate, respectively. Analogous treatment of GAM and SAM glucuronides, which had incorporated ^{14}C from labelled glucose, furnished unlabelled GAM and its decomposition product, gentisic acid, for peak II, and unlabelled SAM for peak IV, respectively, and a radiolabelled peak with retention time similar to that of glucose.

Data from enzymatic hydrolysis with [^{14}C]SAM agreed with results from the

TABLE I

SENSITIVITY OF UV DETECTION FOR SAM AND ITS METABOLITES AT 280 AND 313 nm

Signal-to-noise ratio is 3. Concentration of conjugates was found by acid hydrolysis.

Wavelength (nm)	Sensitivity (nmol)						
	SAM	SAM-S	SAM-G	GAM	GAM-2S	GAM-5S	GAM-5G★
280	1.46	2.35	0.97	3.25	3.55	7.64	0.82
313	1.09	22.5	29.2	0.649	7.09	3.82	0.21

★Concentration of conjugate found from liver perfusion study (see text for details).

incorporation studies. Peak III was susceptible to hydrolysis with sulphatase and Glusulase and peak IV with β-glucuronidase and Glusulase, refurnishing [^{14}C]SAM quantitatively. Complementary results for [^{3}H]GAM metabolites, however, were only semi-quantitative because recovery of [^{3}H]GAM was less than complete owing to the instability of GAM during the incubation.

Sensitivity, stability and reproducibility of HPLC assay for SAM and its metabolites in plasma, bile and blood

The sensitivities of UV detection for SAM and its six metabolites at 280 and 313 nm in bile and perfusate are summarized in Table I. Detection at 280 nm was preferable for SAM-S, SAM-G and GAM-2S, whereas SAM, GAM, GAM-5S and GAM-5G showed optimal absorbances at 313 nm.

The quantitation of unconjugated SAM and GAM in blood after extraction with ethyl acetate was carried out by a separate HPLC procedure (system II), which was considerably faster. Detector response was linear over the range of 0.25–10 μg for both SAM and GAM. The sensitivity of the assay was 0.083 μg/ml. Upon repeated injections of the same reconstituted samples on three consecutive days, linearity was preserved, and the calibration curves remained relatively constant. The slopes and intercepts of the calibration curves for SAM and GAM over this period were 0.091 ± 0.0006 and 0.029 ± 0.00012, and 0.098 ± 0.00058 and -0.018 ± 0.0052, respectively. The C.V. for the assay was less than 6% (Table II).

TABLE II

INTRA-ASSAY VARIABILITY FOR UNCONJUGATED SAM AND GAM EXTRACTED FROM PERFUSATE BY THE HPLC PROCEDURE — SYSTEM II ($n=10$)

	Amount spiked (μg)	Amount recovered (mean ± S.D.) (μg)	C.V. (%)
SAM	5.0	0.48 ± 0.02	4.2
	10.0	10.2 ± 0.34	3.3
GAM	0.5	0.52 ± 0.02	3.8
	10.0	10.5 ± 0.65	6.2

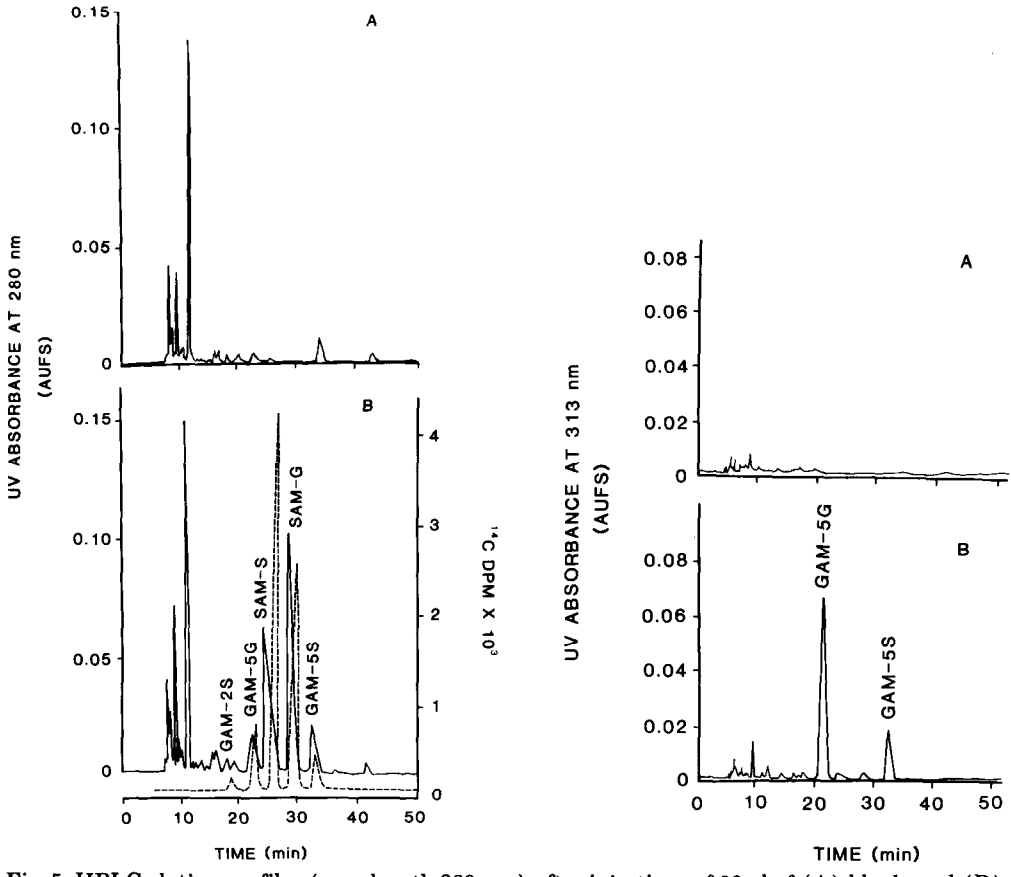

Fig. 5. HPLC elution profiles (wavelength 280 nm) after injections of 30 μl of (A) blank, and (B) 8-h rat urine samples obtained after an i.p. injection of SAM (140 mg/kg) and [^{14}C]SAM ($3.0 \cdot 10^7$ dpm) to the rat. Urine was diluted with water (1:4) before injection into the HPLC system. The dotted lines indicate the radioelution profile of the sample.

Fig. 6. HPLC elution profiles (wavelength at 313 nm) after injections of 30 μl of (A) blank, and (B) 8-h rat urine samples obtained after an i.p. injection of SAM (140 mg/kg) and [^{14}C]SAM ($3.0 \cdot 10^7$ dpm) to the rat. Urine was diluted with water (1:4) before injection into the HPLC system. The samples were the same as those in Fig. 5.

Application of the HPLC method

The applications of systems I and II to the quantitation of SAM (unlabelled and radiolabelled) and its metabolites in plasma, bile and blood samples from once-through rat liver perfusions have been demonstrated [3]. We also employed system I for the quantitation of SAM metabolites in urine after i.p. injections of SAM (140 mg/kg and $3 \cdot 10^7$ dpm) to two rats. The 8-h urine sample accounted for 80.3% dose and was found to contain two SAM and three GAM conjugates (Figs. 5 and 6) when detected at wavelengths of 280 and 313 nm, and from elution profiles of radioactivity (Fig. 5B). SAM and GAM were not found. SAM-S (48.2% dose) and SAM-G (28.5% dose) were the major components, and GAM-2S, GAM-

5S and GAM-5G were present as 0.6, 3.0 and 6.2% dose, respectively, at the end of 8 h. At 24 h, total radioactivity accounted for > 90% dose. The proportions of metabolites, normalized to the injected dose, were: SAM-S, 41.4%; SAM-G, 24.9%; GAM-2S, 2.1%; GAM-5S, 5.9%; GAM-5G, 15.7%. At a wavelength of 280 nm, most of the peaks were devoid of interference excepting GAM-5S (retention time 34 min) as shown by the chromatogram of a blank urine (Fig. 5). This problem could be circumvented by changing the detection wavelength to 313 nm, whereby the interfering peak disappeared (Fig. 6). Moreover, an improvement in sensitivity for the GAM conjugates was seen at this wavelength (cf. Figs. 5B and 6B), as demonstrated earlier (Table I).

DISCUSSION

Stability of SAM and its metabolites

GAM was found to be unstable during hydrolysis by either acid or enzyme. Therefore, quantitation of GAM conjugates by indirect means, namely hydrolytic methods, would provide underestimates. Hydrolytic methods, in contrast to direct quantitation of the conjugates by HPLC, also fail to separate the two GAM monosulphates (GAM-2S and GAM-5S).

After protein precipitation with 1 *M* perchloric acid, the sulphate conjugates of SAM and GAM were stable for only 3 h at room temperature. Therefore, fresh samples should be prepared for accurate quantitation of the conjugates in plasma. We have also employed methanol for protein precipitation (plasma–methanol, 2:5, v/v) in lieu of perchloric acid. However, this caused peak broadening and resulted in poor resolution and separation.

Isolation and identification of SAM metabolites

Because authentic SAM and GAM conjugates were unavailable, biosynthesis of the metabolites through an in situ rat liver perfusion was performed in two groups of experiments. In the first group (studies A and B), [^{14}C]SAM or [^{3}H]GAM was perfused through rat livers, generating labelled metabolites of SAM or GAM. HPLC with monitoring of the radioactivity of the eluate, therefore, provided the total metabolic profiles for SAM and GAM. However, no differentiation between the sulphate and glucuronide conjugates could be made from these radio-chromatograms. Acid or enzymatic hydrolysis is required to identify the conjugates.

In the second group of experiments (studies C–F), the radiolabelled precursors for sulphation and glucuronidation allowed us to establish the identities of both the sulphate and glucuronide conjugates, merely by the retention of labels, without evoking any hydrolytic procedures. A comparison of HPLC profiles obtained by direct injections of KHB and bile from the first and second group of experiments (Figs. 2–4) established the identity of the conjugates. Further confirmation was provided by acid hydrolysis of isolated/purified conjugate fractions from studies C–F, liberating the aglycones, SAM and GAM, which were subsequently quantified by using the calibration curves of SAM and GAM generated under identical conditions.

Evaluation of assays for SAM and its metabolites

An HPLC assay has been developed for the separation of SAM and its six metabolites. In this system (HPLC system I), the pH of the solvent was very critical for the separation, and required adjustments daily. Small shifts in the retention times were often noted, possibly due to changes in the pH of the mobile phase on standing or to deteriorating column conditions. We have also tried solvent gradients. However, a flow gradient appears to be more reproducible and expedient inasmuch as the equilibration time for a solvent gradient is longer, rendering analysis times even longer.

The sensitivity of the UV detection was very different for SAM and GAM metabolites at the wavelengths of 280 and 313 nm. For example, more than a tenfold difference was observed for SAM-S and SAM-G at 280 and 313 nm. A wavelength of 280 nm appears optimal for SAM conjugates, whereas it is 313 nm for GAM conjugates (cf. Figs. 5B and 6B and Table I). UV monitoring at either dual (280 and 313 nm) or multiple wavelengths (at the λ_{max} of each metabolite) is expected to provide better results. While this method (system I) may not be very sensitive for the quantitation of SAM and GAM metabolites, the method is suitable for studies performed with radiolabelled precursors, as illustrated above.

A TLC system was also developed to separate SAM and GAM. Although this method was utilized for [^{14}C]SAM and [^{3}H]GAM quantitation after enzymatic hydrolysis, since impurities due to enzymes were present, this TLC procedure may by used as an alternative to HPLC in the quantitation of radiolabelled SAM and GAM, when calibration curves are run simultaneously.

Application of HPLC method (system I)

The utility of the HPLC method (system I) was shown for rat urine after i.p. injections of SAM (140 mg/kg) and [^{14}C]SAM (3.0×10^{7} dpm). The results showed that SAM was metabolized to its sulphate (41% dose) and glucuronide (25% dose) conjugates and hydroxylated metabolite, GAM (24% dose), found in urine after 24 h. System I effectively separated these conjugates (Figs. 5 and 6) for quantitation either by radioelution or by UV detection. The interfering endogenous peak present at 34 min in the chromatogram for urine may be avoided by dual-wavelength monitoring: 280 nm for 0–30 min and 313 for 30–50 min.

CONCLUSION

In this study, an HPLC assay has been described for the separation and quantitation of SAM and its metabolites in biological fluids. This assay, contrary to other published methods [15,16], allowed separation of SAM and all its metabolites in a single chromatographic system with a constant mobile phase and a flow gradient. Although the run time was rather long (50 min), six metabolites could be separated reproducibly. Faster separation of unconjugated SAM and GAM may be obtained by a second HPLC system with a mobile phase consisting of acetic acid and methanol. These methods provide analytical tools for the study of SAM metabolism in first-pass organs.

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