pitfall when extrapolating the data from small animal species to humans.

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Determination of Salicylamide and Five Metabolites in Biological Fluids by High-Performance Liquid Chromatography

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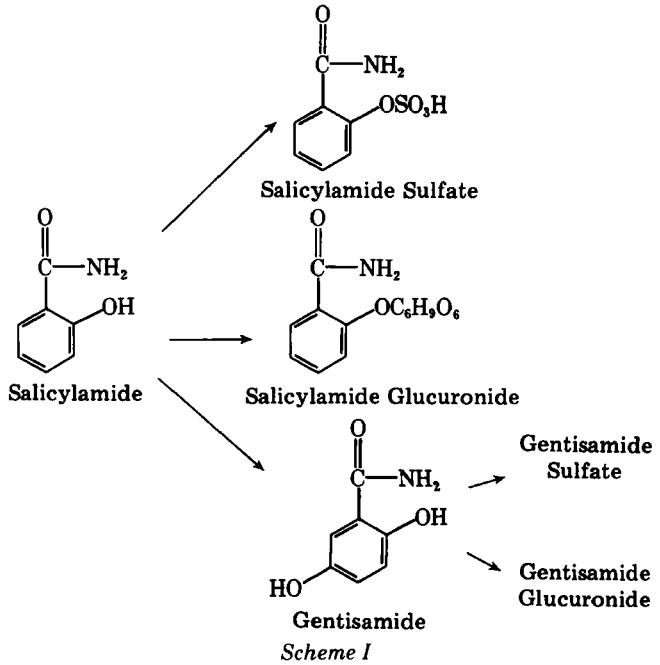
Abstract □ Two high-performance liquid chromatographic (HPLC) assay procedures were developed for the determination of salicylamide and its metabolites in serum, urine, and saliva. One method involves reverse-phase ion-pair chromatography and UV detection, and is used to determine salicylamide, salicylamide glucuronide, and salicylamide sulfate. The other method, with a different mobile phase and without the *ion-pairing reagent, is used to* determine gentisamide (the hydroxylated metabolite of salicylamide), gentisamide glucuronide, and gentisamide sulfate. The assays are performed by direct injection of the sample after protein precipitation with ethanol containing the internal standard. Increased sensitivity for the determination of low concentrations of salicylamide is obtained by organic extraction of this drug from serum or saliva. Calibration curves for the conjugates of salicylamide and gentisamide were obtained, in the absence of authentic standards, by partial enzymatic hydrolysis, using the decrease of the conjugate peaks and the concomitant increase of free salicylamide or gentisamide concentrations to determine peak area ratio–concentration relationships. Application of the HPLC assay procedures to the determination of salicylamide excretion products in the urine of three normal human subjects resulted in 98.6% (range: 97.1–100.1%) recovery of a 1-g oral dose of the drug. All five metabolites of salicylamide were found in urine, but only salicylamide glucuronide, salicylamide sulfate, and gentisamide glucuronide were found consistently and in appreciable quantities. Salicylamide and all of its metabolites except gentisamide sulfate were found in human and rat serum, and unconjugated salicylamide as well as gentisamide were found in human saliva.

Keyphrases □ High-performance liquid chromatography—assay for salicylamide and metabolites in biological fluids, drug conjugate calibration curves in the absence of authentic standards □ Salicylamide—determination in biological fluids, high-performance liquid chromatography □ Metabolites—salicylamide, determination in biological fluids, high-performance liquid chromatography

Salicylamide has analgesic, antipyretic, and hypnotic activities (1–3) but its clinical effectiveness is limited (4–6) due to extensive presystemic biotransformation after oral administration (7, 8). The drug is, however, a valuable research tool for the exploration of drug conjugation reactions (9, 10), drug absorption (11–14) and metabolism interactions (15, 16), route of administration effects on drug disposition (17, 18), effects of disease on drug disposition (19–21), drug concentration–effect relationships (22), product inhibition (23), fetal development (24), and the clinical assessment of metabolic immaturity and disorders (25–27).

Salicylamide is eliminated almost entirely by biotransformation (Scheme I). Many assay methods are available for the determination of this drug in biological fluids (9, 14, 22, 28–36), but none of these provide for the direct determinations (*i.e.*, without prior hydrolysis) of salicylamide conjugates. Apparently only an indirect colorimetric method (9) and a qualitative TLC method (37) have been used for the determination of gentisamide, the hydroxylated metabolite of salicylamide. To facilitate future pharmacokinetic studies with salicylamide, we have developed high-performance liquid chromatographic (HPLC) procedures for the direct determination of salicylamide, gentisamide, and their glucuronide and sulfate



Scheme I

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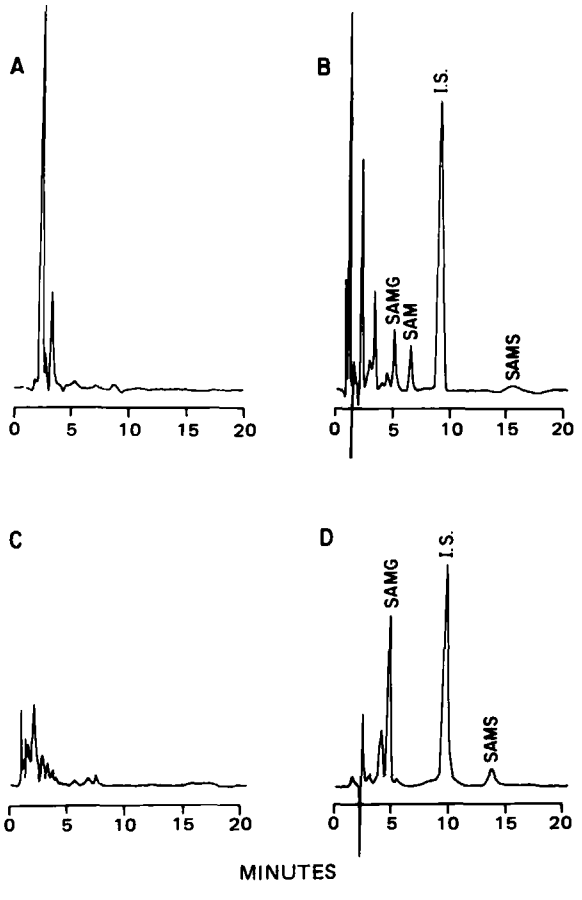

Figure 1—*Chromatograms of human serum and urine samples obtained before and after ingestion of 1 g of salicylamide by a healthy human subject. Key: (A) serum before drug ingestion; (B) serum obtained 1 hr after drug ingestion; (C) control urine diluted 1:25 with water; (D) urine obtained between 1.5 and 2.5 hr after drug ingestion and diluted 1:25 with water. Abbreviations: (SAM) salicylamide, (SAMS) salicylamide sulfate, (SAMG) salicylamide glucuronide, (I.S.) internal standard.*

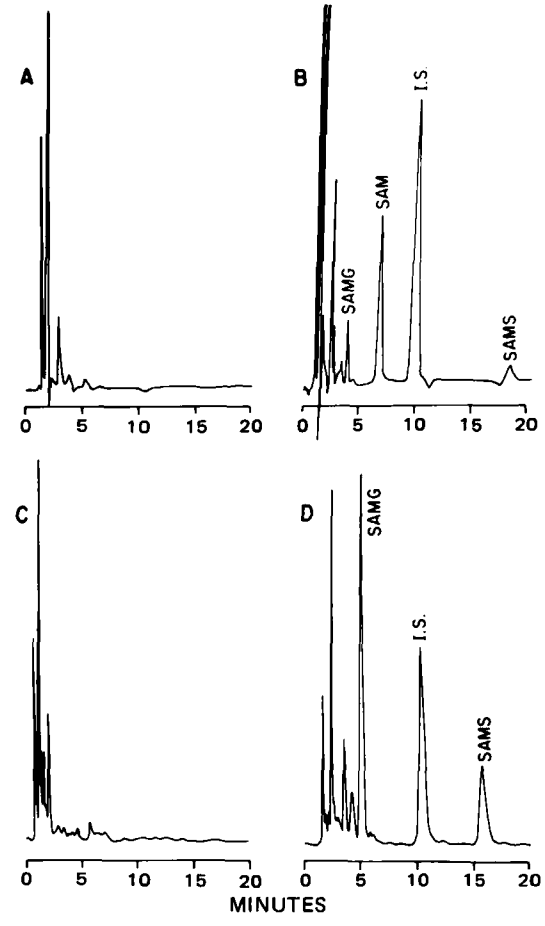

Figure 2—*Chromatograms of rat serum and urine samples obtained before and after intravenous administration of salicylamide. Key: (A) serum before drug administration; (B) serum obtained 30 min after administration of salicylamide, 62.5 mg/kg; (C) control urine diluted 1:25 with water; (D) urine collected ~4 hr after administration of salicylamide, 125 mg/kg, and diluted 1:25 with water. Abbreviations: (SAM) salicylamide, (SAMS) salicylamide sulfate, (SAMG) salicylamide glucuronide, (I.S.) internal standard.*

conjugates in human and rat serum and urine, and human saliva. The assay development for the conjugated metabolites was carried out without authentic standards and exemplifies a general approach that can be used when conjugated drug metabolites are not available in pure form.

EXPERIMENTAL

A high-performance liquid chromatograph[1] equipped with a UV detector[2], automatic injector[3], integrator[4], and an octadecyltrichlorosilane-bonded column[5] was used in this investigation. The column temperature was ambient and the flow rate of the mobile phase varied between 1.6 and 2.0 ml/min in the different assays. The injection volume was 25 μl.

Urine samples were assayed after dilution with distilled water and filtration through a membrane filter[6]. One milliliter of the diluted sample was added to 0.5 ml of a 100-μg/ml aqueous solution of the internal standard, *o*-methoxybenzoic acid[7]. Serum samples were assayed after protein precipitation. One part by volume of serum was added to 2 parts absolute ethanol containing the internal standard (concentration ~40 μg/ml).

An extraction procedure was developed to increase the sensitivity of the assay for unconjugated salicylamide in human serum or saliva. One milliliter of sodium acetate buffer (2 *M*, pH 5.0) plus 1 g of sodium chloride were added to 1 ml of saliva or serum. This mixture was shaken with 6 ml of ether for 10 min and centrifuged. A 4-ml aliquot of the ether phase was removed and the extraction procedure was repeated. The two 4-ml aliquots (one from each extraction) were combined, evaporated to dryness under nitrogen, and redissolved in 0.2 ml of methanol containing the internal standard, *o*-methoxybenzoic acid (concentration ~25 μg/ml).

Salicylamide and its conjugated metabolites were assayed by reverse-phase ion-pair chromatography with UV detection at 254 nm. The mobile phase consisted of 3 m*M* tetrabutylammonium hydroxide[8] in a mixture of 8 parts methanol and 92 parts (v/v) acetic acid 7%.

Low concentrations of unconjugated salicylamide, such as found in human serum or saliva, were assayed after extraction with UV detection at 313 nm. The mobile phase consisted of 1% acetic acid in 25% (v/v) methanol–water solution. Gentisamide and its conjugates were assayed by a separate procedure. The sample preparation and internal standard were the same as described for salicylamide and its conjugates; only one sample preparation was necessary. UV detection was at 313 nm and the mobile phase consisted of 1% acetic acid in 15% (v/v) methanol–water solution. Unconjugated salicylamide is also separated and detected by this procedure.

The intraday reproducibility of the serum salicylamide and gentisamide assay procedures at various concentrations was determined using blank human serum with added salicylamide or gentisamide or pooled rat serum samples obtained after administration of salicylamide.

Gentisamide was synthesized by a previously described procedure (38).

[1] Model M6000A, Waters Associates, Milford, Mass.
[2] Model 440, Waters Associates, Milford, Mass.
[3] WISP 710B, Waters Associates, Milford, Mass.
[4] Data Module, Waters Associates, Milford, Mass.
[5] μBondapak C_{18}, Waters Associates, Milford, Mass.
[6] Metricel, Lot 3056031, Gelman Sciences Inc., Ann Arbor, Mich.
[7] Lot 95C-0165, Sigma Chemical Co., St. Louis, Mo.
[8] Lot 081797, Aldrich Chemical Co., Milwaukee, Wis.

Table I—Reproducibility of Calibration Curves for the Conjugates of Salicylamide and Gentisamide

Metabolite	Number of Samples[a]	Slope Ratio[b]	r^{2c}
Salicylamide	14	0.772	0.982
glucuronide	6	0.772	0.998
Salicylamide	12	0.497	0.984
sulfate	6	0.505	0.992
Gentisamide	13	1.17	0.998
glucuronide	29	1.17	0.992
Gentisamide	5	0.754	0.994
sulfate	3	0.744	0.994
	9	0.750	0.974

[a] Samples assayed in duplicate. [b] Ratio of the slope of the calibration curve for the metabolite to the slope of the calibration curve for the parent compound (salicylamide or gentisamide). Concentration of the metabolites are expressed in terms of the parent compound. [c] Coefficient of determination for the linear regression of the peak area ratio of the metabolite and the concentration of the metabolite (expressed in terms of salicylamide or gentisamide).

It was characterized by NMR, mass spectroscopy, melting point, TLC, HPLC, and elemental analysis[9].

For assay development, the conjugates of salicylamide and gentisamide were quantitated after partial or complete enzymatic hydrolysis of biological samples or sample fractions collected from the HPLC which contain these conjugates. HPLC fractions containing the individual conjugates were lyophilized and reconstituted to various volumes; these solutions were analyzed before and after enzymatic hydrolysis.

Hydrolysis of the glucuronide conjugates was carried out with a β-glucuronidase[10] preparation which, in preliminary studies, produced negligible hydrolysis of the sulfate conjugates. Equal volumes of sample, β-glucuronidase (typically 1250 Fishman U/ml) dissolved in sodium acetate buffer (pH 4.5), and 2.0 or 0.1 *M* sodium acetate buffer pH 4.5

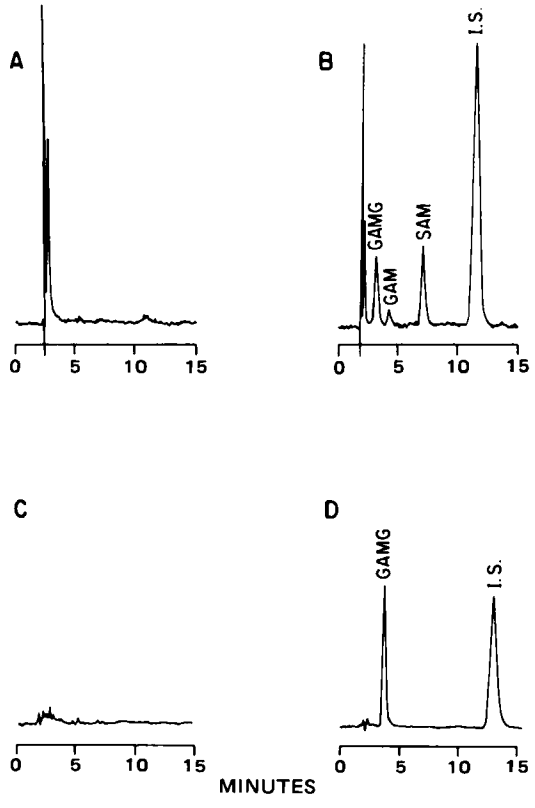

Figure 3—*Chromatograms of human serum and urine samples obtained before and after ingestion of 1 g of salicylamide by a healthy human subject. Key: (A) serum before drug ingestion; (B) serum obtained 1 hr after drug ingestion; (C) control urine diluted 1:25 with water; (D) urine obtained between 1.5 and 2.5 hr after drug ingestion and diluted 1:25 with water. Abbreviations: (GAM) gentisamide, (SAM) salicylamide, (GAMG) gentisamide glucuronide, (I.S.) internal standard.*

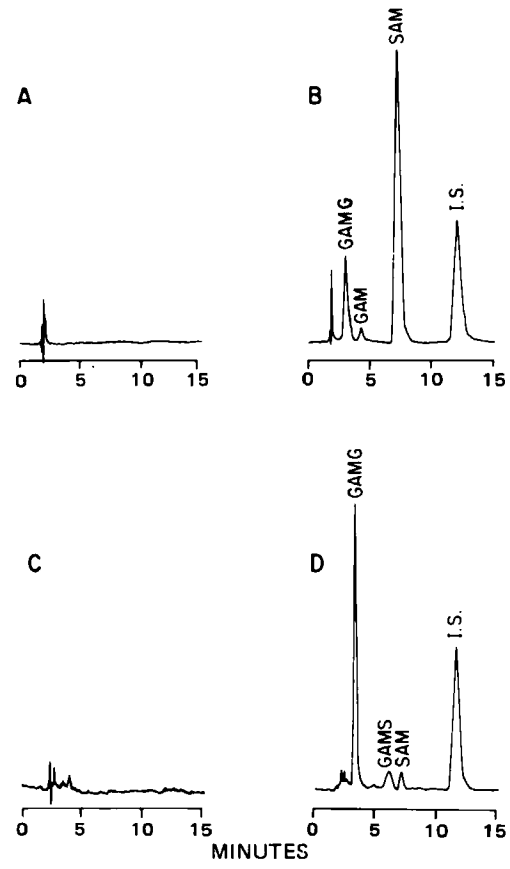

Figure 4—*Chromatograms of rat serum and urine obtained before and after intravenous administration of salicylamide. Key: (A) serum before drug administration; (B) serum obtained 30 min after administration of salicylamide, 125 mg/kg; (C) control urine diluted 1:10 with water; (D) urine collected ~5 hr after administration of salicylamide, 62.5 mg/kg, and diluted 1:10 with water. Abbreviations: (GAMS) gentisamide sulfate, (GAM) gentisamide, (SAM) salicylamide, (GAMG) gentisamide glucuronide, (I.S.) internal standard.*

were mixed and incubated at 37° for varying periods of time. Since only partial hydrolysis of the conjugates was desired, the hydrolysis times were kept short (0.5–4 hr). The sulfate conjugates were hydrolyzed with a β-glucuronidase–sulfatase[11] mixture, with and without the addition of the β-glucuronidase inhibitor, saccharo-1,4-lactone[12], at a final concentration of 0.33 mg/ml. Hydrolysis was carried out similarly to that described for the glucuronide conjugates except that a 2.0 or 0.1 *M* sodium acetate buffer (pH 5.2) was used with the β-glucuronidase–sulfatase preparation (which was usually diluted six- to tenfold with buffer). A limpet sulfatase preparation[13] with low β-glucuronidase activity was tried for the quantitation of salicylamide sulfate, but was found to be unsuitable due to assay interference.

The enzymatic hydrolyses of the gentisamide conjugates were carried out with 0.1 *M* buffer solutions, since there was some loss of gentisamide when added to the 2.0 *M* acetate buffer. No such problem was noted with salicylamide, and, therefore, most hydrolysis experiments with salicylamide conjugates were carried out using the 2.0 *M* buffer.

Assay calibration curves were obtained from the results of the hydrolysis of biological samples or HPLC fractions containing the conjugates. The peak area ratio of the conjugate (ratio of the area of the conjugate peak–area of the internal standard peak) was plotted against the concentration of the conjugate, measured and expressed in terms of the parent compound salicylamide or gentisamide. The slope of this plot, calculated by linear regression, and the slope of the plot of the parent compound were determined on the same day. The ratio of the two slopes was calculated. The hydrolysis experiments and slope value determinations for each conjugate were repeated several times within a 6-month period to check the reproducibility of the slope ratios.

The serum, saliva, and urine samples used in the assay development

[9] M. E. Morris and G. Levy, manuscript in preparation.

[10] Lot 76C-7370, Sigma Chemical Co., St. Louis, Mo.

[11] Glusulase, Lot 00550A, Endo Laboratories, Garden City, N.Y.

[12] Lot 410104, Calbiochem-Behring Corp., LaJolla, Calif.

[13] Lot 47C-9540, S-8629, Sigma Chemical Co., St. Louis, Mo.

Table II—Recovery of Salicylamide and Metabolites in Urine of Normal Human Subjects Determined by HPLC and Colorimetric Methods [a]

Subject	HPLC			Colorimetric [b]		
	Total Salicylamide, mg	Total Gentisamide [c], mg	Recovery, % of dose	Total Salicylamide, mg	Total Gentisamide [c], mg	Recovery, % of dose
A-1 (M, 86) [d]	821	150	97.1	820	156	97.6
A-2	792	181	97.3	796	185	98.1
B (F, 46)	867	140	100.1	872	140	101.2
C (M, 57)	852	147	99.9	824	151	97.5

[a] Volunteers ingested 1 g of salicylamide in aqueous solution and urine was collected for 24 hr. [b] Total salicylamide and total gentisamide were determined using previous methods (refs. 9 and 37, respectively). [c] Expressed in terms of salicylamide. [d] Gender and body weight in kilograms in parentheses.

Table III—Composition of Salicylamide Metabolites in Urine of Human Subjects Determined by HPLC Assay [a]

Subject	Salicylamide Glucuronide	Salicylamide Sulfate	Salicylamide	Gentisamide Glucuronide	Gentisamide Sulfate	Gentisamide
A-1	47.8	34.3	ND [b]	14.9	ND	0.39
A-2	45.5	33.1	0.68	16.3	1.76	ND
B	59.5	26.3	0.88	13.1	0.79	ND
C	51.5	33.7	0.39	14.7	ND	ND

[a] Salicylamide (1 g) in aqueous solution was ingested and urine collected for 24 hr. Results are expressed as percent of dose. [b] Not detected.

were obtained from healthy human volunteers who ingested salicylamide orally (1 g in aqueous solution), or from rats following intravenous administration of salicylamide or gentisamide (62.5–125 mg/kg) *via* a right jugular cannula.

The results of the HPLC assay were compared with results obtained by colorimetric techniques in terms of urinary recovery of metabolites from healthy volunteers and rats. Three volunteers (one on two occasions) ingested 1 g of salicylamide in aqueous solution and collected urine over a 24-hr period. Urinary recovery of total salicylamide (free and conjugates) determined by HPLC was compared with that determined by the colorimetric method of Levy and Matsuzawa (9). Total gentisamide was determined by HPLC and colorimetrically by the method of Becher *et al.* (37). This method involves the acid hydrolysis of gentisamide and its conjugates to gentisic acid and determination using Folin–Ciocalteu reagent[14]. Two rats were administered gentisamide (100 mg/kg iv) *via* a right jugular cannula and urine was collected for 48 hr. All samples were stored at −20° until analyzed. They were assayed by HPLC and colorimetrically (37).

RESULTS AND DISCUSSION

Salicylamide and its glucuronide and sulfate conjugates in human or rat serum and urine are readily separated and detected by HPLC (Figs. 1 and 2). There are no interferences by endogenous substances. Use of the ion-pairing reagent tetrabutylammonium hydroxide and the low pH of the mobile phase (~2.6) cause a selective increase in the retention time of salicylamide sulfate with only a small effect on the retention time of the glucuronide. Gentisamide and its conjugates are also detected by this assay, but their retention times are inconvenient: gentisamide and its glucuronide are eluted before salicylamide glucuronide, while gentisamide sulfate elutes after salicylamide sulfate.

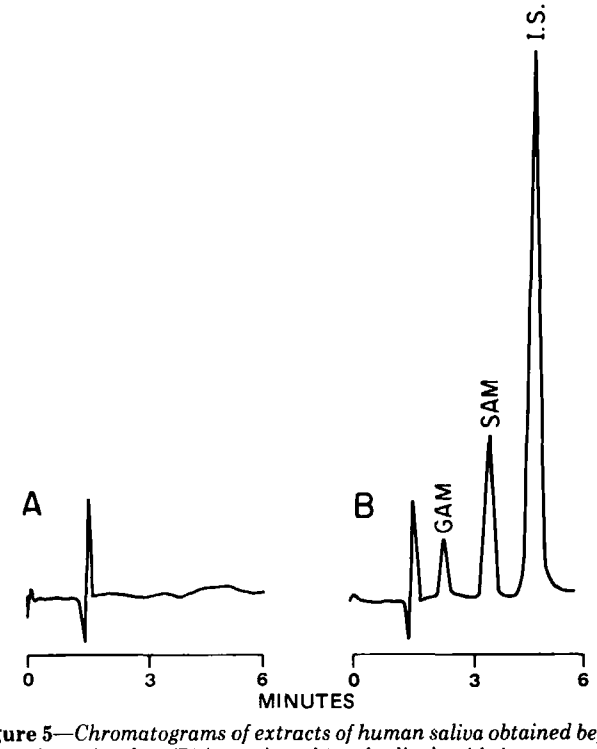

Figure 5—*Chromatograms of extracts of human saliva obtained before (A) and 45 min after (B) ingestion of 1 g of salicylamide in aqueous solution by a healthy human subject. Abbreviations: (SAM) salicylamide, (GAM) gentisamide, (I.S.) internal standard.*

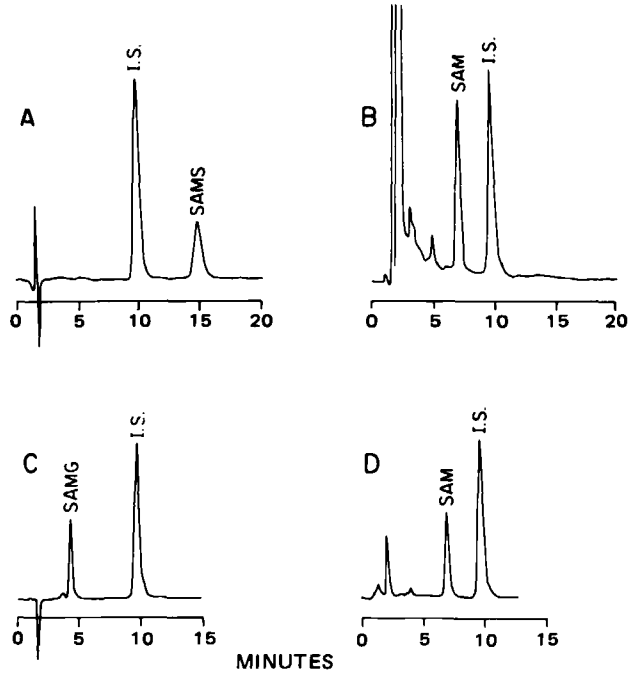

Figure 6—*Chromatograms of chromatographic fractions of human urine containing either salicylamide sulfate (SAMS) or salicylamide glucuronide (SAMG), before and after enzymatic hydrolysis. Key: (A) fraction containing salicylamide sulfate (diluted 1:2 with water); (B) salicylamide sulfate fraction after enzymatic hydrolysis with β-glucuronidase–sulfatase mixture (diluted 1:2 with water); (C) fraction containing salicylamide glucuronide (diluted 1:10 with water); (D) salicylamide glucuronide fraction after enzymatic hydrolysis with β-glucuronidase (diluted 1:10 with water); (I.S.) internal standard; (SAM) salicylamide.*

[14] Lot 707013, Fisher Scientific Co., Fair Lawn, N.J.

Table IV—Urinary Excretion Products of Gentisamide in Rats After Administration of Gentisamide [a]

Rat	HPLC				Colorimetric
	Gentisamide Glucuronide[b], mg	Gentisamide Sulfate[b], mg	Free Gentisamide, mg	Total Gentisamide, mg	Total Gentisamide, mg
N	16.5	14.7	1.98	33.2	32.2
O	17.6	16.7	1.51	35.9	33.9

[a] Dose of gentisamide was 100 mg/kg iv; N and O received 40.1 and 43.7 mg, respectively. Urine was collected for 48 hr. [b] Expressed in terms of gentisamide.

A separate HPLC assay procedure was developed for gentisamide and its glucuronide and sulfate conjugates. The internal standard used in this procedure, *o*-methoxybenzoic acid, is the same as that used in the assay of salicylamide and its metabolites. This affords the convenience of only one sample preparation for both assays. The gentisamide assay involves use of a mobile phase without an ion-pairing reagent and UV detection at 313 nm rather than at 254 nm. At this wavelength, gentisamide and its conjugates and salicylamide absorb more strongly, thereby increasing the sensitivity of the assay. On the other hand, the conjugates of salicylamide have little absorptivity at 313 nm and do not appear in the chromatograms. Gentisamide and its glucuronide and sulfate conjugates are well separated and detected in serum and urine (Figs. 3 and 4). Gentisamide sulfate has not been reported previously as a metabolite of salicylamide. In these experiments, it was found regularly in rat urine but only in some human urine samples after salicylamide administration. It is a quantitatively minor metabolite.

Gentisamide sulfate is relatively stable, showing negligible hydrolysis in rat urine at room temperature over 24 hr. Gentisamide glucuronide is less stable; rat urine samples containing this metabolite should not be keep at room temperature for >4 hr. The glucuronide is more stable in human urine and serum.

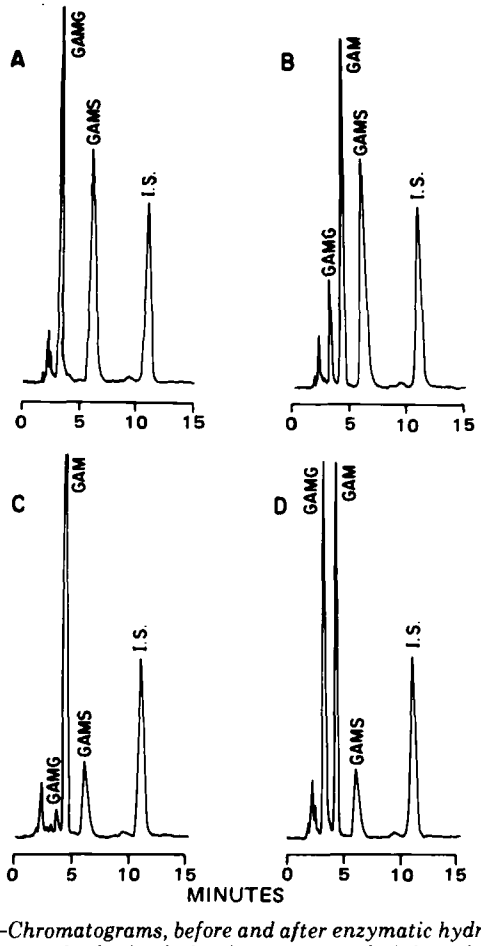

Figure 7—*Chromatograms, before and after enzymatic hydrolysis, of a rat urine sample obtained after intravenous administration of gentisamide (GAM), 100 mg/kg. Key: (A) before hydrolysis; (B) after partial hydrolysis with β-glucuronidase. [There was no hydrolysis of gentisamide sulfate (GAMS) since the peak height ratio of this metabolite to the internal standard is the same as that in A.] (C) after partial hydrolysis with β-glucuronidase–sulfatase mixture; (D) after partial hydrolysis with β-glucuronidase–sulfatase mixture plus saccharo-1,4-lactone, 0.33 mg/ml. [There was no hydrolysis of gentisamide glucuronide (GAMG) in D since the peak height ratio of this metabolite to the internal standard is the same as that in A.] (I.S.) internal standard.*

The relationship between detector response (peak area ratio relative to the internal standard) and concentration of salicylamide and gentisamide is linear over a wide range (at least 4–200 μg/ml for salicylamide and 2–100 μg/ml for gentisamide). The intraday variability of the assay for serum salicylamide in the 10–200 μg/ml concentration range was independent of concentration (n = 5–10 at each of six concentrations), with a coefficient of variation from 1.7 to 3.4%. The coefficient increased to 5.5% at a serum salicylamide concentration of 4 μg/ml. The coefficient of variation for gentisamide in serum (n = 10 per concentration) ranged from 7.0 to 3.3% at concentrations from 2 to 28 μg/ml. The lowest of these concentrations is approximately twice the minimum detectable concentration in these assays.

To increase the sensitivity of the assay for salicylamide, particularly for determinations in serum and saliva, an organic extraction procedure was used and the wavelength for detection was changed from 254 to 313 nm. Use of sodium chloride in the extraction procedure increased extraction efficiency from ~75 to 95%. The coefficient of variation of this more sensitive assay at concentrations of 1–3 μg/ml is <3%. Using the extraction procedure, a chromatogram of saliva obtained from a human subject 45 min after an oral dose of salicylamide indicated the presence of both salicylamide and gentisamide (Fig. 5).

During the assay development for gentisamide conjugates, some loss of gentisamide was observed at high (1–2 M) acetate buffer concentrations. This was not a function of the pH or ionic strength and was not accompanied by formation of gentisic acid. Use of 0.1 M acetate (but not citrate) buffer prevented the loss of gentisamide which may have been due, at least in part, to precipitation.

Calibration (reference) curves for the HPLC assay of salicylamide and gentisamide conjugates were obtained by partial selective hydrolysis of urine samples containing these metabolites (Figs. 6 and 7). The decrease in peak area ratio of a conjugate and the corresponding increase in peak area ratio of the unconjugated compound provided the necessary data for the construction of the calibration curves. The curves were linear over the concentration range examined (~2–60 μg/ml in terms of the parent compound). The slope values of these curves were calculated as a ratio relative to the slope for the unconjugated compound, which was determined on the same day under the same conditions. The slope ratio values for the conjugated metabolites of salicylamide were determined on several occasions over 6 months and were found to be highly reproducible (Table I). Peak area rather than height ratios were used for the calibration curves, since peak height ratios are sensitive to changing column conditions (retention times). This was found to be a problem particularly with salicylamide sulfate, the metabolite with the longest retention time.

The suitability of the HPLC procedures and calibration curves for metabolic studies was tested and confirmed by determining the excretion products of salicylamide in the urine of three human subjects by the HPLC method and by a previously used colorimetric method which requires hydrolysis of the conjugated metabolites. The recovery of salicylamide excretion products was excellent and the results obtained by the two methods were in good agreement (Table II). The HPLC assay revealed the presence of salicylamide and five of its metabolites in human urine, including gentisamide sulfate, a metabolite not previously reported (Table III). The amounts of salicylamide glucuronide, salicylamide sulfate, and total gentisamide recovered are similar to those found in other studies (9, 16, 21).

The results for total gentisamide determined by colorimetry are slightly higher than those obtained by HPLC (Table II). This may be because the colorimetric analysis requires correction for a relatively high (~1 mg/hr) blank value for excretion rate. The colorimetric method detects 2,3,4-trihydroxybenzamide and 2,3-dihydroxybenzoic acid as gentisic acid (the form in which gentisamide is assayed by colorimetry) and may therefore be affected by as yet unidentified minor metabolites of salicylamide and gentisamide.

A limited study of the disposition of gentisamide in rats revealed extensive biotransformation of this compound, including substantial for-

mation of gentisamide sulfate (Table IV). The good agreement between the HPLC and colorimetric assay results for total gentisamide demonstrate the suitability of the HPLC assay for gentisamide and its metabolites.

In conclusion, a combination of two HPLC procedures with the same sample preparation (deproteinization and addition of internal standard) were developed for the direct determination of salicylamide and its metabolites in biological fluids. One of these metabolites, gentisamide sulfate, is reported for the first time. The excretion products of salicylamide in human urine can account for essentially the total amount of administered drug. The HPLC assays for the conjugated metabolites were developed without synthetic or isolated pure standards by a procedure that should be useful also for conjugates of other drugs.

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Synthesis, Isolation, and Characterization of Two Stereoisomeric Ring Sulfoxides of Thioridazine

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Abstract □ A selective oxidation of thioridazine to give exclusively its ring sulfoxides and a separation of the resulting products as diastereoisomeric pairs of enantiomers (DL, LD and DD, LL) are reported. These pairs were characterized by TLC, high-performance liquid chromatographic, IR, UV, ^{1}H-NMR, ^{13}C-NMR, GC–MS, and elemental analyses, and by reduction to thioridazine by lithium aluminum hydride. Structural data for the separated diastereoisomeric pairs or their nitric acid salts were obtained from NMR and IR studies. Gram quantities of each of the two diastereoisomeric pairs of enantiomers were isolated in better than 99% purity.

Keyphrases □ Thioridazine—oxidation to ring sulfoxides, separation of the diastereoisomeric pairs of the ring sulfoxides by crystallization of the nitric acid salts, ^{13}C-NMR analysis of thioridazine ring sulfoxides

A single ring sulfoxide of thioridazine has been detected by TLC (1–5), high-performance liquid chromatography (HPLC) (6, 7), and GC (8–10). Also, EKG abnormalities have been attributed "to a single ring sulfoxide" in plasma (8). Recently "two very similar ring sulfoxides" of thioridazine were identified (11) as urinary metabolites and prepared (with other products) by chemical oxidation.

Evidence is presented here that these ring sulfoxides are

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