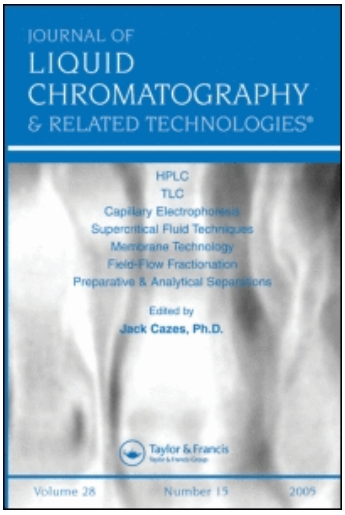


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# Analysis of Salicylamide and Its Metabolites in Blood and Urine by HPLC

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# ANALYSIS OF SALICYLAMIDE AND ITS METABOLITES IN BLOOD AND URINE BY HPLC

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## ABSTRACT

Sensitive liquid chromatographic assays for salicylamide and its metabolites in urine and plasma were developed to facilitate pharmacokinetic studies of the drug's metabolism. The drug and its hydroxylated metabolite, gentisamide, were extracted and concentrated prior to separation on a small-bore reverse-phase column. Conjugated metabolites were assayed separately using reverse-phase ion-pair chromatography. An accurate method of assay calibration in the absence of pure metabolite standards was developed using radioactively-labelled parent drug. In addition one of the metabolites, salicylamide sulfate, was isolated by ion-pair extraction and purified. A significant species difference in salicylamide metabolism was observed. In the dog the drug is almost exclusively (90%) metabolized to its sulfate conjugate, while in humans the glucuronide conjugates of salicylamide (50%) and gentisamide (15%) predominate over salicylamide sulfate (30%).

## INTRODUCTION

Because of its dose-dependent oral bioavailability, salicylamide (SAM) has been a useful drug with which to study first-pass metabolism (1-3). SAM is metabolized by at least three pathways:



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hydroxylation, sulfation and glucuronidation (4,5). We have studied the kinetics and mechanisms of nonlinear SAM metabolism in dogs after oral and i.v. doses of the drug (6,7). Other reported assays (8-12) lacked the sensitivity and specificity required to measure plasma concentrations of SAM and its metabolites in the dog after the administration of small oral doses with low bioavailabilities. We have developed sensitive HPLC assays for SAM and its metabolites in blood and urine which are applicable to dog or human studies.



## MATERIALS

### Reagents

Methanol, ethyl acetate, acetonitrile and dichloromethane (all HPLC grade) were supplied by Burdick and Jackson Laboratories (Muskegon, MI). Salicylamide and tetrapentylammonium chloride were obtained from Eastman Kodak Co. (Rochester, N.Y.). Salicylic acid methyl amide (N-methylsalicylamide) was purchased from ICN Pharmaceuticals (Plainview, N.Y.). Gentisamide (GAM) (m.p. 214-215°C) was synthesized from gentisic acid (Sigma Chemical Co., St. Louis, MO) using methods described by Bray et al. (13) and Raistrick and Simonart (14). Tetrabutylammonium sulfate (TBA-SO4) was made by mixing tetrabutylammonium hydroxide (40 wt % in water) and tetrabutylammonium hydrogen sulfate (both from Aldrich Chemical Co., Milwaukee, WI) to give a pH of 6.0 in aqueous solution. Phenol reagent solution (2N) was from Fisher Scientific Co (Pittsburg, PA). Carboxyl-$^{14}$C-salicylamide, 50 mCi/mmole, was custom synthe-

sized by ICN Pharmaceuticals (Irvine, CA). Bacterial β-glucuronidase (Type VII), sulfatase (arylsulfatase Type VI) and myristic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Aquasol® was purchased from New England Nuclear (Boston, MA) and ethyl aminobenzoate (Benzocaine, U.S.P.) was supplied by Merck & Co., Inc. (Rahway, N.J.).

**Liquid Chromatography Instrumentation**

Mobile phase was delivered by a model A-60-S pump (Eldex Laboratories, Menlo Park, CA) or a model 6000A pump (Waters Assoc., Milford, MA). The injector was a WISP 710B (Waters Assoc.). The column effluent was monitored with a model SF770 detector (Kratos Analytical Instruments, Westwood, N.J.) and its output signal recorded and integrated by a model 3390A integrator (Hewlett-Packard, Avondale, PA).



## METHODS

**Assay of Salicylamide and Gentisamide**

Sample preparation. Frozen plasma samples were thawed at room temperature and gently mixed. A volume of 1.0, 1.5 or 2.0 ml of each sample was added to a 16 mm x 100 mm test tube and the total volume brought to 2.0 ml with water as necessary. Each sample was extracted twice with 6 ml of ethyl acetate and the pooled organic layers were evaporated to dryness under a gentle stream of nitrogen at 40°C. Methanol (20 µl) was added to redissolve the residue in each tube followed by 0.2 to 2.0 ml of mobile phase which contained 8 µg/ml of the internal standard, salicylic acid methylamide

(SAMA). The relative concentration of samples (sample volume/ reconstututed volume) during the extraction was varied from 0.5 to 10 in order to keep peak heights within the range of the calibration curve. Each sample was filtered before injection through a 0.45 micron centrifugal microfilter (#SS009, Schleicher & Schuell, Keene N.H.). Duplicate 40 microliter injections of each sample were made and the plasma concentrations of salicylamide and gentisamide calculated by comparing their average peak height ratios (compound peak height/internal standard peak height) to a calibration curve. The calibration curve was prepared by spiking blank plasma with salicylamide and gentisamide at several concentrations between 0.25 and 5 µg/ml and extracting in the same manner as with unknown samples.



Chromatographic conditions. For the assay of SAM and GAM a 150 mm x 2.1 mm I.D. column packed with RoSil® Phenyl, 5 micron (Alltech Assoc., Deerfield, IL) was employed. The mobile phase was 10 mM pH 2.2 phosphate buffer in water pumped at a rate of 0.3 ml/min. Column effluent was monitored at 296 nm.

## Assay of Sulfate and Glucuronide Conjugates

Sample preparation. Urine samples frozen at -20°C for up to six weeks were thawed and gently mixed. One ml of each urine sample was mixed with 3 ml of methanol, centrifuged for 5 min at 1000 x g and the supernate filtered through a 0.2 micron disposable syringe filter assembly (#4192, Gelman Sciences, Ann Arbor, MI). Duplicate 15 µl injections of each prepared urine sample were made

and the amount of each metabolite was determined by comparing its peak height with a calibration curve prepared from urine samples of known metabolite concentration (see Results and Discussion).

Plasma samples to be assayed for SAM metabolites were concentrated in the following manner. To each 1.0 ml plasma sample was added 0.1 ml of 0.2 M phosphate buffer (pH 5.2) and this aqueous mixture extracted twice with 5 ml of 1:1 hexane/ethyl acetate to remove SAM. After a brief evaporation to remove any residual organic phase, 3.0 ml of acetonitrile containing 8 µg/ml of the internal standard, ethyl aminobenzoate, were added and the samples centrifuged for 5 min at 1000 x g. The supernate was evaporated to dryness under a gentle stream of nitrogen in a water bath maintained at 40°C and the residue was redissolved in 0.3 ml of mobile phase 'A'(see below). After filtration through a 0.45 micron centrifugal microfilter, duplicate 30 µl injections were made of each sample and the concentration of each metabolite determined by comparing its average peak height ratio (compound/internal standard) with a standard curve prepared from urine samples of known metabolite concentration (see Results and Discussion).



Chromatographic conditions. For the conjugates assay a modified Alltech 605 RP column (Alltech Assoc., Deerfield, IL) was employed. The column, packed with 5 micron C18 particles, with an I.D. of 4.6 mm, was cut to a length of 120 mm. A 40 mm x 2.1 mm I.D. precolumn packed with CO:PELL ODS (Whatman, Inc., Clifton, N.J.) was attached directly to the main column. Mobile phase 'A'

was 40% methanol, 12 mM tetrapentylammonium chloride and 10 mM pH 6.2 phosphate buffer. Mobile phase 'B' contained 50% methanol but was otherwise identical to 'A'. The flow rate was 1.0 ml/min. A step gradient from mobile phase 'A' to 'B' 5.0 min after sample injection and a return to 'A' 12.5 min after injection was achieved by a solid state timing device attached to an in-line solvent switching valve (Model 5300 and 5302, Rheodyne, Inc., Cotati, CA). Column effluent was monitored at 230 nm.

**Purification of Salicylamide Sulfate (SAMS)**

A modification of the method of Mattox (15,16) for the ion-pair extraction of steroid conjugates from aqueous solution was employed in the extraction and purification of salicylamide sulfate from urine. Following a 2-g oral dose of SAM, 200 ml of urine from an adult human male subject was collected and washed with an equal volume of dichloromethane. To the washed urine was added 3.5 g of TBA-SO4 in 25 ml water. The urine was then extracted with 175 ml of dichloromethane. The organic layer was removed, treated with 2.85 g of myristic acid and back extracted with 200 ml of 0.5 M sodium bicarbonate. The aqueous phase was lyophilized and then dissolved in 40 ml of water. Four ml of this solution was injected in aliquots onto the chromatograph described above for assay of SAM conjugates. The effluents under the SAMS peak were collected, pooled and extracted into an equal volume of dichloromethane. This organic phase was washed with water, dried with anhydrous sodium sulfate and evaporated. The resulting crystals of the

tetrabutylammonium salt of salicylamide sulfate had a melting point of 105-110°C. The identity of the purified salt was confirmed by secondary ion mass spectrometry.

## RESULTS AND DISCUSSION

Five peaks appeared in chromatograms of human urine and plasma following the oral administration of a 1-g dose of SAM (Figures 1 & 2). Two of these peaks, which appeared only in the plasma, were identified as SAM and GAM by comparison with chromatograms of the pure compounds. To identify the remaining peaks as SAM metabolites, chromatographic fractions containing each of the peaks were collected and subjected to the following treatments: acid hydrolysis (in 2N HCl for 20 hours at 65°C), incubation with bacterial β-glucuronidase (from 1 to 20 hours at 37°C), incubation with sulfatase (from 1 to 20 hrs at 37°C and pH 7.0) and colorimetric determination of gentisic acid derivatives (17). The earliest fraction, identified as salicylamide glucuronide (SAMG), was converted to SAM by glucuronidase or acid hydrolysis, gave no color in the gentisic acid test and was unaffected by treatment with sulfatase. The next fraction was identified as gentisamide glucuronide (GAMG) because it was converted to GAM by glucuronidase or acid hydrolysis, gave a positive gentisic acid test and was unaffected by treatment with sulfatase. The final eluting fraction, identified as salicylamide sulfate (SAMS), was converted to SAM by either sulfatase or acid hydrolysis but not glucuronidase and gave a negative gentisic acid test. Although SAM and GAM were

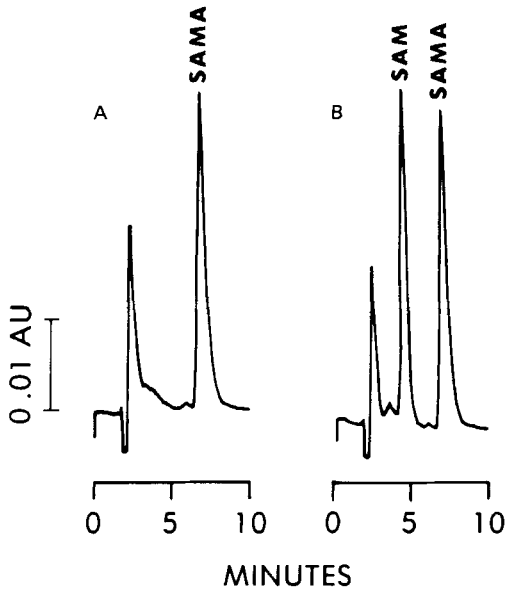


FIGURE 1 - SALICYLAMIDE ASSAY. Chromatograms of dog plasma extract before (A) and 9 min after (B) a 5 mg/kg oral dose of salicylamide (SAM concentration=1.0 µg/ml).



separated under the conditions used to assay the conjugated metabolites, they were assayed separately under conditions which provided greater sensitivity. Using a phenyl rather than a C-18 bonded phase column greatly improved the peak shape of salicylamide and therefore the sensitivity of the assay. Measuring the absorbance of SAM at the secondary maximum of 296 nm rather than at a lower wavelength significantly reduced the level of background interference and thus further increased assay sensitivity.

Calibration curves for SAM and GAM were derived from the pure compounds but the difficulty in obtaining metabolites of known purity and stability led to the development of an alternative

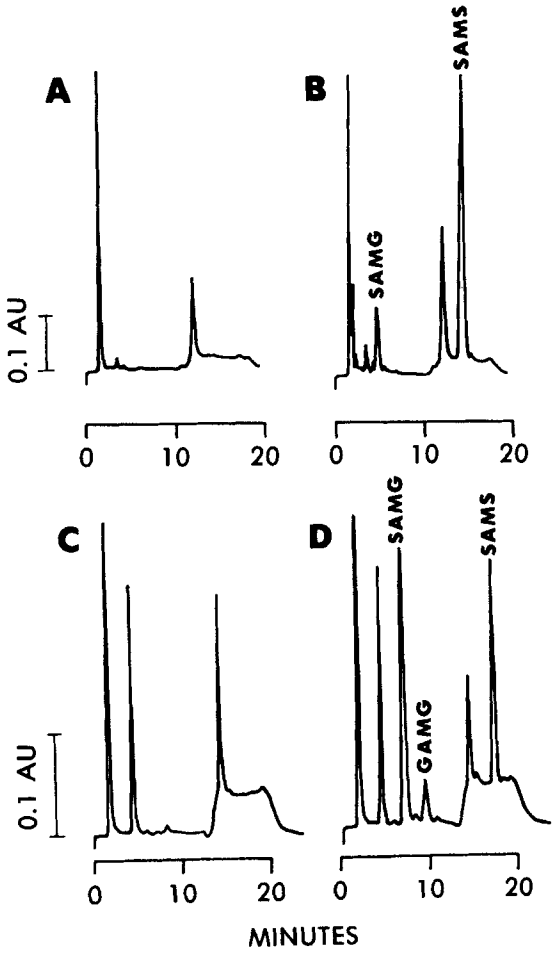


FIGURE 2 - METABOLITE ASSAY. Chromatographs of dog urine collected before (A) and for 320 min after (B) a 40 mg/kg oral dose of salicylamide and human urine collected before (C) and for 240 min after (D) a 10 mg/kg oral dose of salicylamide.

method for quantitation of GAMG, SAMG and SAMS. An adult male mongrel dog was given an oral dose of 200 mg SAM labelled with 40 microcuries of $^{14}$C-salicylamide. Urine collected from this animal during the next 2 hrs was subjected to the assay for SAM metabolites and fractions of the HPLC eluent were collected. The amount of radioactivity under each metabolite peak was determined by counting each 1 ml fraction in 10 ml of Aquasol on a Beckman LS 9800 scintillation counter. The amount of each metabolite in this urine sample was calculated by dividing the amount of radioactivity collected under each peak by the known specific radioactivity of the administered drug. A calibration curve for the metabolite assay was prepared by diluting this urine sample with blank urine. Standard urine samples were stored at -20°C with no loss of metabolites for 3 months at which time the calibration procedure was repeated. The accuracy of this method was confirmed by using the assay to measure a 0.1 mg/ml solution of the purified tetrabutylammonium salt of SAMS. The urine-calibrated metabolite assay gave an average value of 0.104 mg/ml (± 0.01, n=4) for this solution.

Although the lowest point on the calibration curve for the SAM/GAM assay corresponds to a plasma concentration of 75 ng/ml, the assay is capable of measuring plasma levels of 10 ng/ml if the calibration curve is extended. Within-run coefficients of variation for this assay were ⩽ 3% (n=10) over the range of the calibration curve (0.075 to 16 μg/ml) and inter-assay variation was less than 10% (n=6). For the conjugated metabolite assay the

**TABLE 1**

Salicylamide metabolite recovery in urine of dog and man

| Metabolite | Species and Dose[a] | | | |
|---|---|---|---|---|
| | Dog, 10 mg/kg n=5 | Dog, 20 mg/kg n=13 | Human, 10 mg/kg n=2 | Human, 10-20 mg/kg[b] n=4 |
| SAMG | 12 ± 7[c] | 10 ± 7 | 51 | 51 ± 6 |
| GAMG | 0 | 0 | 14 | 15 ± 1.3 |
| SAMSO4 | 88 ± 24 | 90 ± 15 | 34 | 32 ± 4 |

[a] Data from seven adult male mongrel dogs, weight 21-27 kg, and one adult male human subject, weight 100 kg. Dogs received dose via gastric tube in a vehicle of 8 ml propylene glycol and 2 ml ethanol, followed by 100 ml water. Human subject ingested the powdered drug followed by 250 ml water.

[b] Data of Morris and Levy (12) from three human subjects.

[c] Values shown (mean ± S.D.) are expressed as % of recovered dose in urine collected for at least 4 hours after the indicated oral dose of SAM.



coefficients of variation over the range of the calibration curve (0.07 to 0.3 mg/ml for SAMG and 1.0 to 4.0 mg/ml for SAMS) were 5% (SAMG, n=6) amd 7% (SAMS, n=6) within runs and 6.5% (both conjugates, n=7) between runs. Calibration curves for both assays were linear with intercepts near zero.

Table 1 compares the urinary recovery of SAM metabolites found in dogs and humans after the oral administration of SAM. These

data indicate that at the dose levels studied there are significant differences in metabolism between species. Most striking is the absence of hydroxylation in the dog. Neither gentisamide nor its conjugates are present in dog plasma or urine even at doses of 40 mg/kg SAM. In contrast, humans metabolize a significant portion of orally administered SAM by hydroxylation to GAM. It is also apparent from our data that the conjugated metabolites are formed to a different extent in the two species. In humans, salicylamide glucuronide is the major metabolite, accounting for just over half the urinary metabolites while in the dog salicylamide sulfate accounts for about 90% of the recovered metabolites.



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