# Elimination of salicylic acid in goats and cattle

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## SUMMARY

Sodium salicylate was administered to cattle and goats IV and PO according to a crossover design. Total urinary excretion of SA and its metabolites was measured for 3 days after dosing. Salicyluric acid (SUA) was the only metabolite detected in urine of either species. Recovery of sodium salicylate and SUA in goats amounted to 67.9 and 34.6% of the dose, respectively, after IV administration. After oral dosing, total recoveries were 30.2% (sodium salicylate) and 71.7% (SUA) of dose. By comparison, cattle excreted significantly ($P < 0.05$) less sodium salicylate (54.0%) and more SUA (49.9%) after IV dosing. The same pattern was observed after oral administration, wherein cattle excreted <12% as sodium salicylate and more than 99% as SUA. In both species, almost 90% of the drug excreted as sodium salicylate was found in urine within the first 12 hours after an IV dose and within 24 hours after oral dosing. The excretion of SUA was somewhat slower in both species, especially after oral administration. The data suggested that there were only quantitative differences in the metabolism and elimination of sodium salicylate between the 2 species, with cattle excreting a higher proportion of the drug as the glycine conjugate SUA.

The Consortium for Research on Minor Use Animal Drugs was established through funding from the FDA to compare drug disposition between major and minor species. Drugs were selected for study largely on the basis of their routes of metabolism and excretion. Salicylic acid (SA) was chosen as a model for drugs eliminated mainly as a function of conjugative metabolism and excretion in urine.

Salicylic acid is found in many plants and has been used for more than 2,000 years in the treatment of various conditions. The salicylates still play a major role in the treatment of rheumatic diseases and in the prophylaxis of cerebrovascular disease, coronary artery disease, and arterial thrombosis.[1] Salicylic acid is metabolized through glucuronide formation (to produce salicylacyl glucuronide and salicylphenolic glucuronide), conjugation with glycine (to produce salicyluric acid, SUA), and oxidation to 2,5-dihydroxybenzoic acid (gentisic acid, GA; Fig 1). Salicyluric acid has been shown to be the major urinary metabolite in most species.[2-4] Minor metabolites that have been identified in carnivous and omnivous (eg, man, dogs, cats) include the glycine conjugate of GA (gentisuric acid), 2,3-dihydroxybenzoic acid (DHBA), and 2,3,5-trihydroxybenzoic acid.[2-8] The purpose of the study reported here was to compare the disposition and elimination of SA in cattle (a major species) and goats (a minor species). The urinary elimination profile of SA and its metabolites after IV or PO administration of sodium salicylate is discussed.

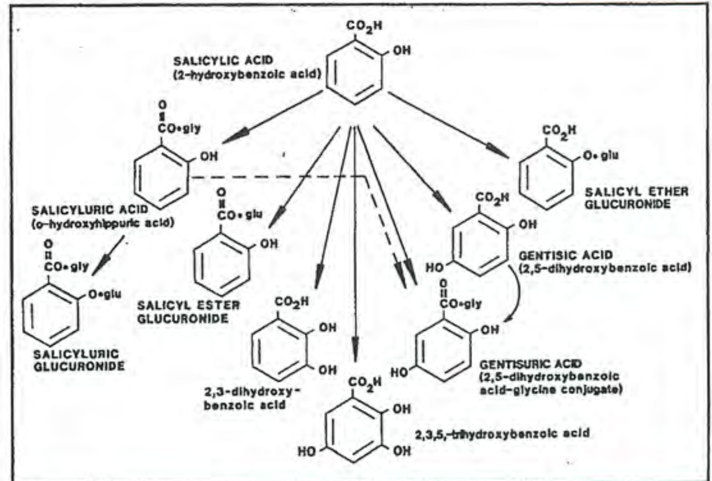


Figure 1—Known pathways of metabolism of salicylic acid.

## Materials and Methods

*Animals*—The animal studies were performed at the Veterinary Medical Research Farm at the University of Illinois. Six female Toggenburg crossbred goats weighing 51.8 to 64.5 kg and 6 Angus heifers weighing 248.6 to 307.7 kg were used in a randomized crossover study. Before and after experiments, all animals were housed in box stalls and allowed free access to hay and water.

*Experimental procedures*—Food was withheld from goats and cattle for 24 hours prior to drug administration; however, hay, grain, and water were provided during the 72 hours of the experiment. During the experiments, animals were confined to steel metabolism crates fitted with mangers. On the day before drug administration, a Foley catheter was inserted into the urinary bladder via the urethra, and the cuff was inflated with water. Ascorbic acid and sodium azide were added to the urinary collection containers as preservatives. Samples of urine were collected before drug administration for use as analytic blanks.

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Sodium salicylate was administered PO or IV at a rate of 44 mg/kg of body weight, and urine samples were collected at 12-hour intervals for 72 hours. (Results of a preliminary study using $^{14}C$-ring-labeled SA in 2 subjects of each species indicated that all radioactivity could be accounted for in urine within 72 hours). Volumes of urine were measured and recorded for each interval. After sample collections were complete, specimens were packed in dry ice and shipped by express mail to Louisiana State University for analysis. Eight to 14 days were allowed before the second drug administration in the crossover design.

A second experiment was conducted on an additional 3 goats to determine the effect of freezing and storage of urine samples containing preservatives on the analysis of glucuronide conjugates of SA and SUA. In these 3 goats, sodium salicylate was administered IV (44 mg/kg) and ·ine was collected for 24 hours without the addition of ·ium azide or ascorbic acid. Urine samples were analyzed immediately following a 24-hour collection period.

*Chemicals*—Sodium salicylate, SA, GA, DHBA, SUA, and the internal standard, o-methoxybenzoic acid, were purchased from one source[a] and were all 99% pure. Solvents of high-performance liquid chromatography (HPLC) grade were obtained from commercial sources and were used without further purification. Triple-distilled and deionized water was purified by passage through a modular polisher.[b]

*Salicylic acid and metabolite analysis*—Salicylic acid and its metabolites were analyzed by HPLC, using a method adopted from those reported by Cham et al[9] and Ogunbona.[10] Urine samples were diluted from 9 to 90 (for 12-hour samples) times with water. Half a milliliter of acetonitrile containing 20 mg of o-methoxybenzoic acid (internal standard)/L was added to 0.5 ml of the diluted urine. This mixture was centrifuged at 13,600 × g for 5 min·s, and the supernatant was filtered through a 0.45-µm ·ter.[c] A 10-µl aliquot was removed for analysis by HPLC.

Urine samples were also incubated with either glucuronidase or sulfatase to determine, by difference, the degree of conjugation with glucuronic acid or sulfate. For glucuronidase treatment, incubates consisted of 0.5 ml of unfiltered urine, 0.5 ml of 0.2*M* $KH_2PO_4$ buffer (adjusted to pH 3.8 with $H_3PO_4$), and 500 U of β-glucuronidase.[d] Incubation was conducted at 45 C for 1 hour in a shaking water bath. The effectiveness of these incubation conditions in releasing the glucuronic acid of phenolphthalein glucuronide was confirmed. After incubation, 3.4 ml of water was added, and 0.5 ml of the diluted sample was added to 0.5 ml of acetonitrile containing internal standard. Incubates subjected to sulfatase treatment contained 0.5 ml of unfiltered urine, 0.5 ml of a solution containing 0.5*M* sodium acetate, 2.0 m*M* ascorbic acid, 0.19 mg of glucuronolactone (D-saccharic acid 1,4-lactone), and 106.5 U of arylsulfatase. The final pH of the incubate was 5.0, and conditions of incubation were identical to those for incubates containing glucuronidase. Standard curves for glucuronidase-treated, sulfatase-treated, and untreated urine samples containing salicylic acid and each of the aforementioned metabolites were prepared from pooled urine collected from animals before drug administration.

Samples were analyzed by use of a liquid chromatograph[e] equipped with a variable wavelength UV detector[f] set at 313 nm. Reverse-phase chromatography was conducted using an octadecylsilane column[g] with a 3-µm average particle size. Chromatographic development was achieved by use of a solvent system containing water:acetic acid:methanol (78:4:18) and an isocratic flow rate of 1 ml/min to achieve efficient separation of SA, its metabolites, and the internal standard. Peak height ratios of SA (or metabolites) to internal standard were used to calculate concentrations of analyte, using standard curves obtained in the same manner. Concentrations and peak height ratios were linearly related from 0.625 to 100 mg/L for each compound.

*Mass spectral confirmation of metabolite identification*—The identity of the metabolite SUA and of SA was confirmed by extracting two 1-ml urine samples (pH 3.5, adjusted with 1.0*N* $H_3PO_4$) at 12-hour intervals with 2 ml of ethyl acetate. The combined extracts were evaporated with dry nitrogen gas, and the residue was redissolved in 100 µl of methanol. Five milliliters of an ethereal diazomethane solution was added to this solution, and the mixture was allowed to stand for 10 minutes. Two milliliters of water was added to the ether solution. This mixture was vortexed and centrifuged, and the ether layer was transferred to a clean tube and evaporated. The residue was dissolved in 100 µl of methylene chloride prior to analysis by mass spectrometry. Urine blanks, 12-hour urine samples, and a urine blank spiked with the aforementioned standards, at 1.0 µg/ml, were prepared in the same manner.

Mass spectral analyses were conducted, using a gas chromatograph coupled to a triple-stage quadrupole mass spectrometer.[h] Gas chromatography was conducted by use of a 25-m fused silica glass capillary column[i] (0.25-mm ID, 0.20-mm coating) operated at a head pressure of 15 psi of helium and with a temperature program of 50 C for 1 minute, increasing 30 C/min to 300 C, and holding for 10 minutes. Injections were made in the splitless mode at 220 C (1 µl) with the purge function being initiated 0.5 minutes after injection.

Electron impact mass spectra (70-cV positive ion) of pure standards for SUA and SA were identical to those observed for major peaks detected at the same retention time for the 12-hour urine samples obtained from goats and cattle. Screening of the samples for other metabolites for which standards were available did not indicate whether they were in either goat or cattle samples.

*Statistical analysis*—Data were analyzed for significant differences in mean values by a least-squares analysis of variance in a split-plot design.[11] Differences were considered significant at the $P < 0.05$ level.

[a] Aldrich Chemical Co Inc, Milwaukee, Wisc.
[b] Modulab Polisher, Continental Water Systems Inc, San Antonio, Tex.
[c] Micro Prep-Disc, Bio-Rad Inc, Richmond, Calif.
[d] Type L-II, Sigma Chemical Co, St Louis, Mo.
[e] HP-1090, Hewlett-Packard Co Inc, Palo Alto, Calif.
[f] Spectro-Monitor III, Laboratory Data Control, Riviera Beach, Fla.
[g] LC-18 Supelco Inc, Bellefonte, Pa.
[h] TSQ-45, Finnigan-MAT, Palo Alto, Calif.
[i] DB-5, JW Scientific, Folsom, Calif.

## Results

The major metabolite of SA found in urine was SUA. Neither sulfatase nor β-glucuronidase treatment increased the concentration of SA in preserved and frozen urine of goats or cattle. Additionally, the oxidative metabolites of SA (ie, GA, 2,3-dihydroxybenzoic acid, 2,3,5-trihydroxybenzoic acid) could not be identified.

Salicylic acid and SUA were rapidly eliminated in the urine of both species (Fig 2) after IV administration. Nearly 90% of the dose of salicylate was excreted within 12 hours, although SA and SUA could be identified in urine collected up to posttreatment hour 72. Cattle excreted significantly more of the drug as the glycine conjugate SUA and a proportionally smaller amount of the dose as the parent compound. This difference was even more pronounced when the drug was administered orally (Fig 3). Oral dosing slowed the elimination of SA as both parent drug and glycine conjugate, requiring approximately 24 hours for excretion of 90% of the dose. The total 72-hour recovery of SA and SUA was 102.6% in goats and 103.9% in cattle after IV dosing (Table 1). Cattle excreted less of the dose as SA than did goats and more of the dose as SUA. After oral dosing, the total 72-hour recovery was 101.9% in goats and 111.9% in cattle (Table 2); cattle eliminated substantially less of the free drug than did goats. The ratio of SUA to SA at posttreatment hour 72 was 8.35 for cattle and 2.37 for goats.

Results of a study conducted to determine the effect of freezing and storage of urine containing sodium azide and ascorbic acid on the detection of glucuronide conjugates indicated that only 3.6% of the dose could be attributed indirectly (by difference) to glucuronide conjugates of SA (Table 3). Virtually none of the dose could be attributed to the glucuronide conjugate of SUA.

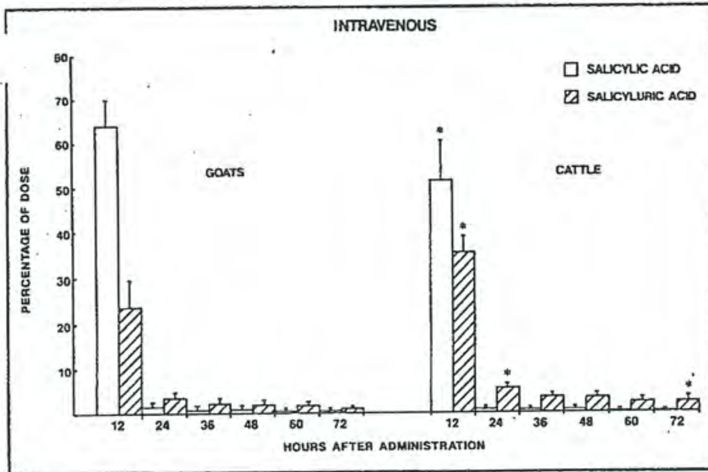


Figure 2—Mean ± SD percent recoveries of salicylic acid and salicyluric acid in urine as a function of time after an IV dose of 44 mg of sodium salicylate/kg of body weight. * Significantly different from time matched values in goats ($P < 0.05$).

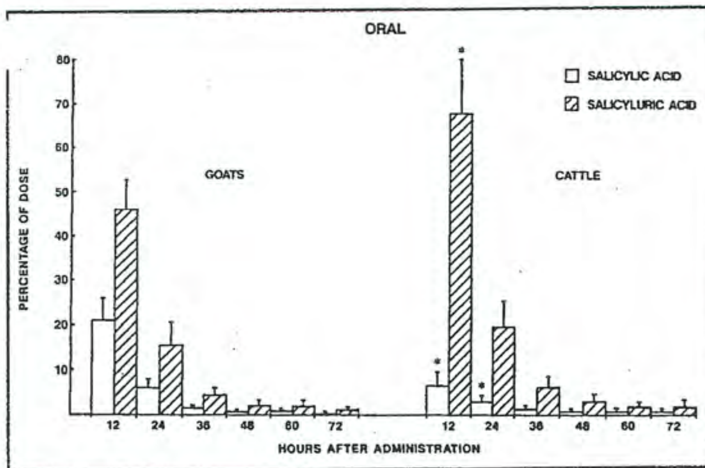


Figure 3—Mean ± SD percent recoveries of salicylic acid and salicyluric acid in urine as a function of time after an oral dose of 44 mg of sodium salicylate/kg. * Significantly different from time matched values in goats ($P < 0.05$).

Table 1—Urinary excretion of salicylic acid (SA) and salicyluric acid (SUA) after IV administration of sodium salicylate (44 mg/kg of body weight)

| PTH | Goats SA | Goats SUA | Cattle SA | Cattle SUA |
|---|---|---|---|---|
| 12 | 63.84±5.73† | 23.62±6.14 | 51.77±9.54* | 32.84±4.42* |
| 24 | 1.64±1.07 | 3.49±1.49 | 0.77±0.50 | 5.47±1.19* |
| 36 | 0.87±0.47 | 2.40±1.05 | 0.48±0.24 | 3.46±0.82 |
| 48 | 0.75±0.47 | 2.19±1.24 | 0.46±0.16 | 3.34±1.03 |
| 60 | 0.53±0.43 | 1.79±0.61 | 0.22±0.20 | 2.28±1.11 |
| 72 | 0.30±0.31 | 1.13±0.66 | 0.29±0.23 | 2.49±1.10* |
| Total | 67.93±7.61 | 34.62±7.19 | 53.99±9.95* | 49.88±6.70* |
| Species total | 102.55±12.74 | | 103.87±11.48 | |

* Significantly different ($P < 0.05$) from comparable mean value for goats; † Percentage recovery ± SD.
PTH = posttreatment hour.

Table 2—Urinary excretion of salicylic acid (SA) and salicyluric acid (SUA) after oral administration of sodium salicylate (44 mg/kg)

| PTH | Goats SA | Goats SUA | Cattle SA | Cattle SUA |
|---|---|---|---|---|
| 12 | 20.76±5.65† | 46.15±6.88 | 6.72±2.82* | 67.87±12.70* |
| 24 | 6.18±1.89 | 15.46±5.03 | 2.62±1.33* | 19.31± 5.92 |
| 36 | 1.35±0.30 | 4.34±1.41 | 1.14±0.55 | 6.22± 2.01 |
| 48 | 0.83±0.35 | 2.42±0.91 | 0.76±0.41 | 2.97± 1.61 |
| 60 | 0.74±0.46 | 2.10±1.32 | 0.37±0.37 | 1.76± 1.07 |
| 72 | 0.38±0.25 | 1.22±0.45 | 0.36±0.41 | 1.82± 1.35 |
| Total | 30.24±6.23 | 71.69±7.61 | 11.97±2.83* | 99.95± 9.52* |
| Species total | 101.93±4.91 | | 111.92±7.75 | |

* Significantly different ($P < 0.05$) from mean comparable value for goats; † Percentage recovery ± SD.
PTH = posttreatment hour.

Table 3—Excretion of salicylic acid (SA) and salicyluric acid (SUA) in unfrozen goat urine analyzed immediately after collection*

| Goat No. | SA A† | SA B‡ | SA Difference | SUA A† | SUA B‡ | SUA Difference |
|---|---|---|---|---|---|---|
| 1 | 72.72 | 70.07 | 2.35 | 29.78 | 29.36 | 0.42 |
| 2 | 69.38 | 66.32 | 3.06 | 22.38 | 21.82 | 0.56 |
| 3 | 64.77 | 59.38 | 5.39 | 21.32 | 19.71 | 1.61 |
| Mean | 68.86 | 65.26 | 3.60 | 24.49 | 23.63 | 0.86 |
| (± SD) | (±2.85) | (±5.43) | (±1.59) | (±4.60) | (±5.07) | (±0.65) |

Percentage recovery.
* Sodium salicylate administered IV (44 mg/kg) and urine collected for 24 hours without preservatives; † Urine samples incubated in duplicate with β-glucuronidase; ‡ Urine samples not incubated with β-glucuronidase.

## Discussion

The lack of evidence for sulfate conjugates of SA is consistent with previous reports on its metabolism in various species; however, the sulfate conjugate of diflunisal, a derivative of salicylate (5-[2',4'-difluorophenyl] salicylic acid), has been described.[12] In addition, studies with fenben-

dazole have shown that cattle[13] and goats[14] are efficient in forming sulfate conjugates of p-hydroxyfenbendazole. Nevertheless, sulfate conjugates of SA or its hydroxylated metabolites were not detected in urine of either species.

The virtual lack of glucuronide conjugates, either acyl or phenolic, was unexpected. Hutt et al[7] reported that the excretion of the 2 salicyl glucuronides was highly variable, ranging from 0.8 to 42% of a dose of aspirin within 12 hours in a group of 12 human volunteers. The mean percentage of dose excreted was 5.6% for the acyl glucuronide and 7.2% for the phenolic glucuronide. Similar results were reported by Montgomery et al[15] in a group of 44 people of various ages and both genders. However, Marsh et al[16] reported the salicyl glucuronides to be minor metabolites of SA in horses, accounting for only about 2% of a dose of SA. Nevertheless, salicyl glucuronides have been identified after an IV dose of sodium salicylate in pups, pigs, a foal, and a kid ranging from 1 to 30 days old.[17] Furthermore, the percentage of dose excreted in urine by the 30-day-old kid was approximately 30%.

The acyl glucuronide has been shown to be more labile than the phenolic conjugate.[18] The incubation conditions used in our study, however, should have released both types of conjugate if they were in the urine. These same conditions were rigorous enough to release the phenolic glucuronide of fenbendazole in previous studies,[13,14] and phenolphthalein glucuronide in the present study. Additionally, the calculated recovery was greater than 100% of the dose in both ruminant species by either route of administration, making it impossible for all but trace amounts of unaccounted for glucuronide conjugate to remain in incubated urine samples. It is possible that some salicyl acyl glucuronide could have been hydrolyzed spontaneously in frozen samples during storage. The study in which samples of goat urine were analyzed immediately after collection indicated that this may have been the case to some extent. Still, it was shown that only a small amount of SA glucuronide was in the urine.

Salicyluric acid has been reported as the major metabolite of SA in all species studied, except in dogs (pups).[7,8,15-17] Studies in people taking aspirin orally have shown that 77.9 to 84% of the dose is excreted within 72 hours as SUA.[15,19] Dixon et al[20] reported that phenylacetic acid was conjugated primarily with glycine in rat-liver preparations, whereas $\alpha$-substituted phenylacetic acid derivatives interacted primarily with endoplasmic reticulum and were conjugated preferentially with glucuronic acid. They concluded that mitochondria contained receptors moderately specific for phenylacetic acid and that $\alpha$-substituted analogs bound poorly to these receptors and were therefore not subject to any appreciable degree of amino acid conjugation. Salicylic acid, however, is an $\alpha$-substituted phenylacetic acid, and the results of this study and many others have indicated that the conclusion of Dixon et al does not have universal applicability.

The reason for the > 100% recovery of a dose of SA was not clear, but may relate to the method of analysis. Dilutions of up to 90-fold were required to obtain samples dilute enough for HPLC analysis. Small errors in measurement would have been magnified manyfold in calculation of the total amount excreted, and only 1 ml of urine was used for analysis, although several liters were excreted daily. This was particularly noticeable for SUA in cattle given the drug orally. Nevertheless, it was clear that cattle conjugated more SA with glycine than goats, regardless of the route of administration. From a comparative point of view, however, this was the only difference between the 2 species. Thus, we concluded that there is only a small difference in the manner in which cattle and goats metabolize and eliminate SA.

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