3-Hydroxylation of Salicylamide in Mice

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Received March 13, 1987, from the *Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS 66103.* Accepted for publication November 18, 1987.

Abstract □ Salicylamide is an important model compound for use in investigations concerning drug disposition. In this study the metabolic fate of salicylamide at high doses was evaluated in male mice using HPLC methodology. The concentrations of salicylamide and its metabolites were determined in urine and in blood at various times after the administration of 2 or 4 mmol kg^{-1} salicylamide. Salicylamide, gentisamide, and their glucuronide and sulfate conjugates were detected. 2,3-Dihydroxybenzamide, the 3-hydroxy metabolite of salicylamide, as well as its glucuronide and sulfate conjugates, were identified and quantitated for the first time by HPLC. 2,3-Dihydroxybenzamide had previously been detected only as a minor metabolite of salicylamide by paper chromatography. However, in the present study, 18% of the salicylamide metabolites appearing in urine after either dosage of salicylamide were 3-hydroxylation products. When a previously published HPLC method for salicylamide analysis was used, 2,3-dihydroxybenzamide glucuronide coeluted with salicylamide glucuronide. The possible formation of 3-hydroxy metabolites must be evaluated in any study of drug metabolism using salicylamide as a model compound.

The use of salicylamide for its analgesic, antipyretic, and anti-inflammatory properties is limited[1] due to its rapid metabolism and limited bioavailability.[2] However, salicylamide is frequently employed as a model compound for investigation of drug absorption,[3,4] elimination,[5] metabolism,[6,7] and conjugation,[8,9] among other effects. Within this context, an understanding of the metabolic fate of this compound is of interest.

Biotransformation accounts for virtually all of the elimination of salicylamide from the body (Figure 1). Gentisamide, its glucuronide and sulfate conjugates, and the glucuronide and sulfate conjugates of salicylamide have been considered to be the only significant products of salicylamide metabolism, with salicylamide glucuronide being the major urinary metabolite.[9-12] While developing an HPLC method to investigate the metabolic disposition of salicylamide, we detected 2,3-dihydroxybenzamide (2,3-DBA) and its glucuronide and sulfate conjugates in the urine of mice treated with salicylamide. In this communication, we demonstrate that 3-hydroxylation is a significant route of biotransformation after administration of high doses of salicylamide to mice.

Experimental Section

Chemicals—Tetrahydrofuran, tetrabutylammonium hydroxide, and 2,5-dihydroxybenzoic acid were of HPLC grade and obtained from Aldrich Chemical Co. (Milwaukee, WI). Salicylamide and bovine liver β-glucuronidase (Type B-1) were purchased from Sigma Chemical Co. (St. Louis, MO). Glusulase (sulfatase/β-glucuronidase) was obtained from DuPont Pharmaceuticals (Wilmington, DE). Methanol (HPLC grade) was purchased from Fisher Scientific (Fair Lawn, NJ). Methyl 2,3-dihydroxybenzoate was obtained from ICN K&K Laboratories (Plainview, NY). Carrier-free $Na_2{}^{35}SO_4$ was obtained from ICN Radiochemicals (Irvine, CA) and complete scintillation cocktail 3a70 was purchased from Research Products International (Elk Grove, IL). Water was purified by a Milli-Q system (Milford, MA).

Instrumentation—For HPLC, an M6000 solvent delivery system, μBondapak C-18 column (30 cm × 3.9 mm, 10 μm) and model 450 variable wavelength detector (Waters Associates, Milford, MA) were employed. The sample injector used was a Rheodyne 7125 (Alltech Associates, Deerfield, IL) fitted with a 20-μL injection loop. The analytical column was protected by a precolumn (2.5 cm × 3.9 mm) packed with Bondapak C-18/Corasil (37–50 μm). The mobile phase consisted of 1.5 mM tetrabutylammonium hydroxide in a 95:5 mixture of 1% (v/v) acetic acid and tetrahydrofuran. The flow rate was 1.6 mL min^{-1}, and the column effluent was monitored at 240 nm. The detector signal was recorded and integrated by an IBM 9000 computer operating under a Chromatography Applications System (Version 1.3, IBM Instruments, Danbury, CT).

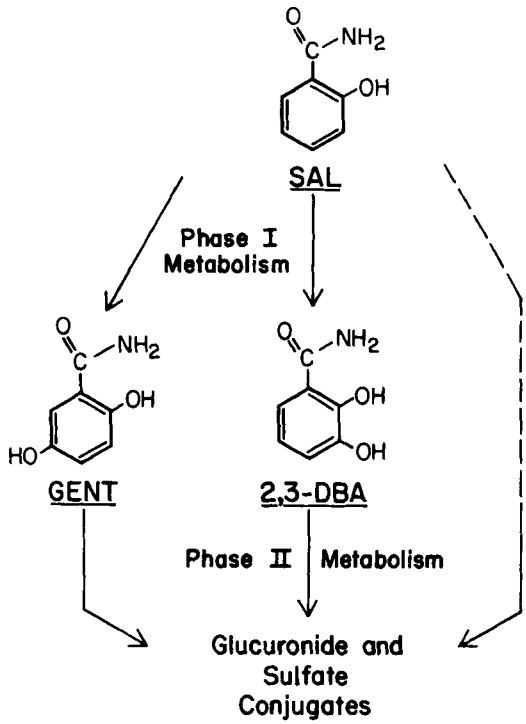

Figure 1—*Pathways of salicylamide metabolism.*

Electron-impact mass spectra were recorded on a Nermag R1010 quadrapole spectrometer using a direct insertion probe. Proton NMR spectra were recorded using a Bruker WP200 fourier transform spectrometer at 200 MHz.

Synthetic Procedures—Methyl 2,5-dihydroxybenzoate, mp 84–85 °C (lit. 85–86 °C),[13] was synthesized by the method of Raistrick and Simonart.[13] This and commercially obtained methyl 2,3-dihydroxybenzoate were used to synthesize gentisamide and 2,3-dihydroxybenzamide (2,3-DBA), respectively, by a modification of the method of Bray et al.[14] One gram of methyl ester was added to 10 mL of deaerated ammonium hydroxide along with 0.5 g of activated zinc powder. The mixture was refluxed under nitrogen for 2 h to form the amide. Gentisamide was purified by treatment with activated charcoal and recrystallization from water (50% yield, green crystals, mp 215–216 °C; lit. 217–218 °C).[14] 2,3-Dihydroxybenzamide was purified by preparatory HPLC, using an Alltech C-18 column (25 cm × 10 mm, 10 μm) and a tetrahydrofuran:1% (v/v) acetic acid (1:9) mobile phase at a flow rate of 2.5 mL min^{-1}. We were not able to crystallize this material.

0022-3549/88/0400-0309$01.00/0

Animal Experiments and Sample Preparation—Male CF1 mice (Sasco, Omaha, NE), 25–30 g, were housed in a temperature-controlled room with a 12-h (6 am–6 pm) light/dark cycle, and had free access to Purina Laboratory Rodent Chow (Ralston Purina Co., St. Louis, MO) and water. The mice were injected with salicylamide (2 or 4 mmol kg^{-1}, ip) in isotonic saline (pH 8.7) between 7 and 8 am. The injection volume was 20 mL kg^{-1}. At various times after injection, blood was collected by retro-orbital sinus puncture. One part of blood was mixed with two parts of cold methanol and centrifuged prior to HPLC analysis. Urine was collected by the procedure of Plaa and Larson[15] without the use of filter paper. During urine collection, mice were housed in a temperature-controlled chamber; food and water were withheld, but the mice were gavaged with 20 mL kg^{-1} of saline 4 h after salicylamide administration. At the end of 24 h, urine was taken up in 5 mL of water and centrifuged. The supernatant was used for HPLC analysis.

Metabolite Identification and Quantification—In order to identify the glucuronide conjugate peaks in the HPLC chromatograms, urine was incubated with β-glucuronidase [one part sample plus two parts 1.4% (w/v) β-glucuronidase in 0.2 M acetate buffer, pH 4.5, 37 °C]. This treatment decreased the size of the glucuronide conjugate peaks. Column effluant fractions containing these peaks were collected and treated individually with β-glucuronidase. The resultant hydrolysis products were identified by chromatographic comparison with authentic standards.

To identify the sulfate conjugate peaks, radiolabeled conjugates were formed in vivo. Mice were injected with $Na_2{}^{35}SO_4$ (100 μCi in 0.1 mL saline, ip) and 5 min later, either salicylamide (3 mmol kg^{-1}), gentisamide (1 mmol kg^{-1}), 2,3-DBA (1 mmol kg^{-1}), or saline was injected. Twenty-four-hour urine samples from these animals were injected onto the HPLC and the eluate was collected in 30-s fractions. These fractions were mixed with 5 mL of counting solution, and their radioactivity was measured in a liquid scintillation spectrometer.

Separate calibration curves were generated for the HPLC analysis of salicylamide, gentisamide, and 2,3-DBA. To quantitate the conjugates of these three compounds, the glucuronide or sulfate conjugates were partially hydrolyzed with β-glucuronidase:sulfatase (one part sample plus two parts Glusulase diluted 1:16 with 0.2 M acetate buffer, pH 5.2, 37 °C). The ratio of the decrease in conjugate peak area to the increase in unconjugated compound peak area was determined for each conjugate. These ratios express the relationships of the extinction coefficients of the conjugate and the parent compound and were used in conjunction with the calibration curves for the parent compounds to quantitate the conjugates.[10]

Results

Chromatographic Metabolite Pattern—The HPLC analyses of blood (Figure 2) and urine (Figure 3) samples from salicylamide-treated mice revealed the presence of several peaks that were not present in blood or urine from control mice. Salicylamide and gentisamide were identified by matching their retention times with those of authentic samples. β-Glucuronidase treatment of the compounds eluting at 5.1 and 6.6 min produced salicylamide and gentisamide, indicating that these peaks were due to salicylamide glucuronide and gentisamide glucuronide, respectively. The peak at 4.2 min hydrolyzed to a compound that was tentatively identified by its retention time as 2,3-DBA. Comparison of the mass spectra of this material and synthesized 2,3-DBA (Figure 4) supported this identification. Mass fragments were assigned as follows: *m/e* 153 (37%), M$^+$; *m/e* 136 (100%), M$^+$-NH_3; *m/e* 108 (26%), M$^+$-$CONH_3$; and *m/e* 80 (26%), M$^+$-$(CO_2)NH_3$. The identity of the biological material was confirmed by comparison of its NMR spectrum and that of synthesized 2,3-DBA (Figure 5). In these spectra, the doublets at 7.06 and 6.89 ppm were due to the aromatic protons at the six and four positions, respectively, and the triplet at 6.76 ppm was due to the aromatic proton at the five position. Thus, the compound eluting at 4.2 min was 2,3-DBA glucuronide.

The radiochromatograms from urine of mice treated with saline, salicylamide, gentisamide, or 2,3-DBA after administration of $Na_2{}^{35}SO_4$ are presented in Figure 6. The urine of salicylamide-treated mice contained three radioactive peaks, with retention times of 13.2, 19.0, and 24.7 min, which were not present in saline-treated mice. The urine of mice treated with 2,3-DBA and gentisamide contained radioactive peaks which eluted at 19.0 and 24.7 min, respectively. Therefore, the corresponding peaks in the urine of salicylamide-treated mice were concluded to be 2,3-DBA sulfate (retention time: 19.0 min) and gentisamide sulfate (retention time: 24.7 min).

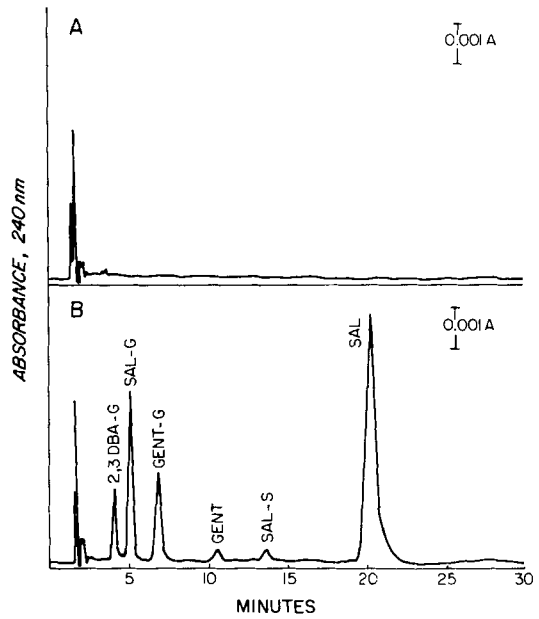

Figure 2—*The HPLC separation of salicylamide and salicylamide metabolites in blood. (A) Chromatogram of methanol extract of blood from an untreated mouse. (B) Chromatogram of methanol extract of blood collected 30 min after administration of a 4-mmol kg^{-1} dose of salicylamide. Identity of the peaks is as indicated, with G denoting glucuronide and S denoting sulfate.*

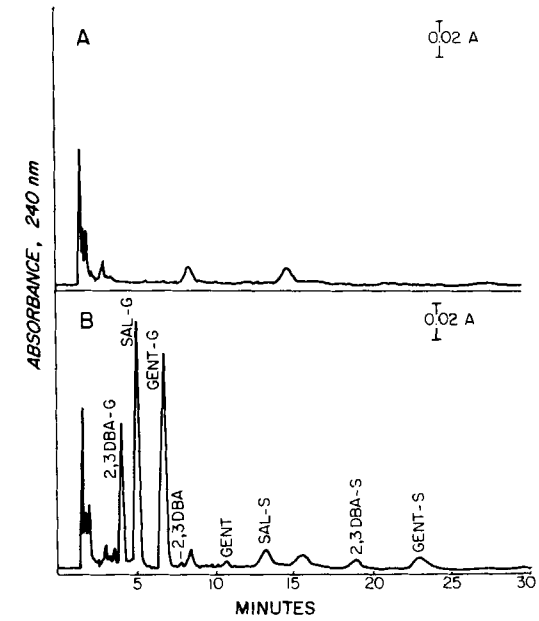

Figure 3—*The HPLC separation of urinary metabolites of salicylamide. (A) Chromatogram of diluted 24-h urine from an untreated mouse. (B) Chromatogram of diluted 24-h urine from a mouse treated with a 4-mmol kg^{-1} dose of salicylamide. Identity of the peaks is as indicated, with G denoting glucuronide and S denoting sulfate.*

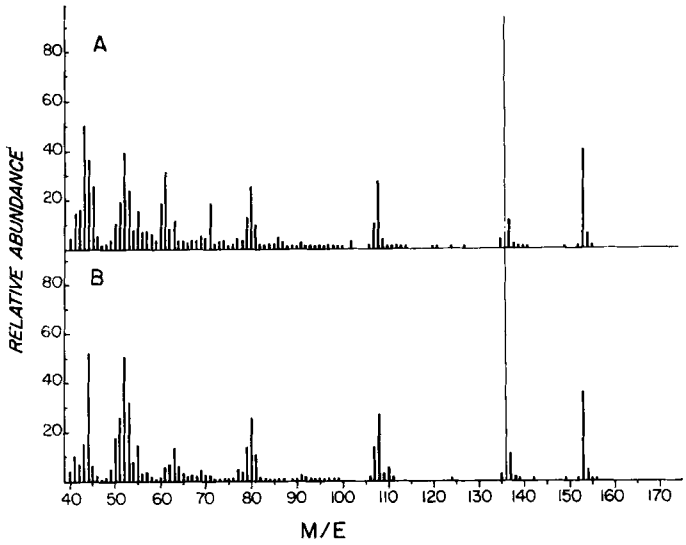

Figure 4—*Mass spectra of 2,3-dihydroxybenzamide (2,3-DBA).* (A) *Isolated from urine of mouse treated with salicylamide.* (B) *Synthesized.*

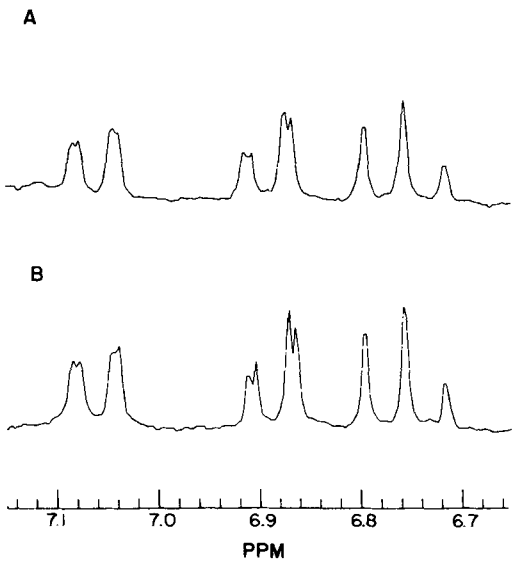

Figure 5—*Aromatic proton region of ^1H NMR spectra of 2,3-dihydroxybenzamide.* (A) *Isolated from urine of mouse treated with salicylamide.* (B) *Synthesized.*

The remaining peak was concluded to be salicylamide sulfate (retention time: 13.2 min).

Quantitation in Biological Samples—The concentration-to-peak area ratios for salicylamide, gentisamide, and 2,3-DBA were linear from 30 to 5000 nmol mL^{-1} ($r^2 > 0.99$). The peak area ratios (conjugated-to-unconjugated compound), used in conjunction with the calibration curves for the unconjugated compounds to quantitate conjugates, are presented in Table I.

The decrease in blood salicylamide concentration over time after administration of either a 2- or a 4-mmol kg^{-1} dosage of salicylamide exhibited a biphasic pattern (Figure 7). The disposition rate constants for the initial phase of elimination were 0.028 and 0.020 min^{-1}, respectively. For the later phases, the values were 0.083 and 0.053 min^{-1}, respectively.

The concentrations of the major salicylamide metabolites appearing in the blood at various times after administration of 2 or 4 mmol kg^{-1} of salicylamide are shown in Figure 8. Gentisamide glucuronide and salicylamide glucuronide were the major metabolites present in the blood after salicylamide

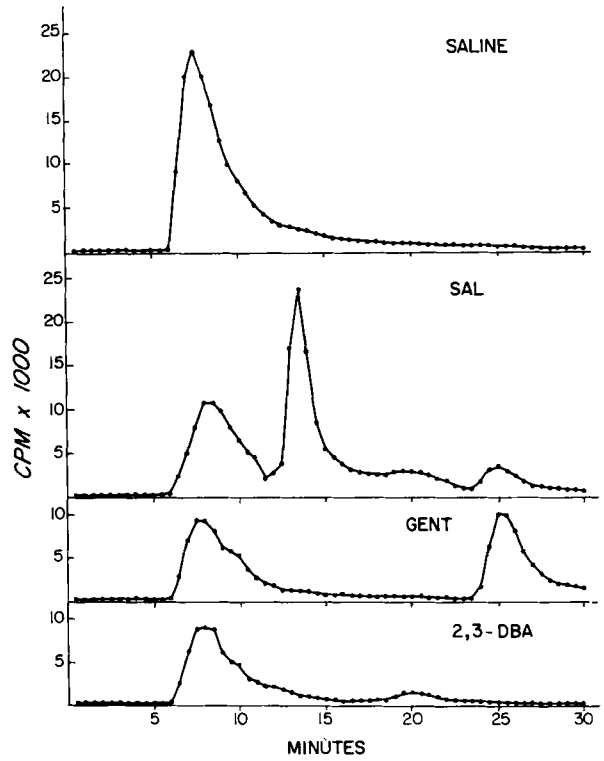

Figure 6—*Radiochromatograms of urine from mice given $Na_2{}^{35}SO_4$ and treated with saline, gentisamide, or 2,3-dihydroxybenzamide.*

Table I—Ratios of the Absorbance of Conjugates to That of Their Unconjugated Form[a]

Conjugate	n	Absorbance Ratio (conjugated:unconjugated)[b]
Salicylamide glucuronide	13	0.659 ± 0.013
Salicylamide sulfate	6	0.432 ± 0.023
Gentisamide glucuronide	13	1.011 ± 0.026
Gentisamide sulfate	5	0.891 ± 0.025
2,3-Dihydroxybenzamide glucuronide	13	1.229 ± 0.032
2,3-Dihydroxybenzamide sulfate	5	0.757 ± 0.030

[a] Ratios were obtained as outlined in the *Experimental Section;* determinations were made on two or three different occasions and the results were pooled; data are expressed as means ± SE.
[b] Determined at 240 nm.

administration. Peak concentrations of 710 and 620 nmol mL^{-1}, respectively, were attained after 4 mmol kg^{-1} of salicylamide. After 2 mmol kg^{-1} of salicylamide, the peak concentrations were 315 and 265 nmol mL^{-1}, respectively. The peak blood concentrations of 2,3-DBA glucuronide were 300 and 180 nmol ml^{-1} after 4 and 2 mmol kg^{-1} of salicylamide, respectively. Blood concentrations of salicylamide sulfate also approached 300 nmol mL^{-1} after 4 mmol kg^{-1} of salicylamide, but this compound was not detected after 2 mmol kg^{-1} of salicylamide. Gentisamide and gentisamide sulfate were detected in trace amounts 30–75 min after the administration of 4 mmol kg^{-1} of salicylamide (data not shown), but neither 2,3-DBA nor 2,3-DBA sulfate were detected in blood.

Eight metabolites of salicylamide were detected in urine (Table II). The relative amount of each metabolite in urine generally reflected the maximum concentration of the metabolite in blood. Gentisamide glucuronide was the most abundant metabolite, and the amounts of 2,3-DBA glucuronide and salicylamide sulfate were similar (both were about half of the concentration of salicylamide glucuronide). Un-

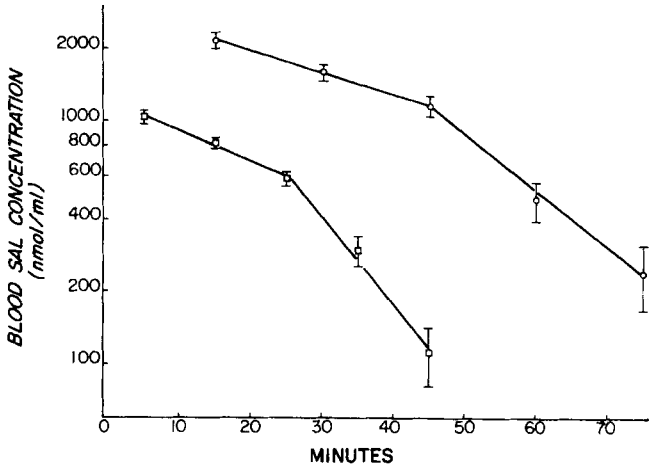

Figure 7—*Blood concentration of salicylamide versus time. Key: (□) after 2 mmol kg $^{-1}$ of salicylamide, ip; (○) after 4 mmol kg^{-1} of salicylamide, ip. Each point represents the mean ± SE of four determinations.*

conjugated gentisamide and 2,3-DBA each accounted for ~1% of the metabolites in urine, but salicylamide was not detected.

Discussion

The use of salicylamide as a tool in drug metabolism research is longstanding, but only in recent years has HPLC been used to analyze salicylamide and its metabolites.[10,16] The method employed in the present study allowed the quantitation of all previously observed metabolites of salicylamide, including gentisamide sulfate, which was first reported in 1983.[10] In addition, three metabolites of salicylamide, not previously detected by HPLC, were quantitated. These were 2,3-DBA and its glucuronide and sulfate conjugates. Only two previous reports have mentioned the existence of a 3-hydroxy metabolite of salicylamide. In 1950, Bray et al., using paper chromatography, found that <1% of a dose of salicylamide administered to rabbits appeared in urine as conjugated 2,3-DBA.[17] Also using paper chromatography, Bekemeier found a substance "tentatively identified as 2,3-dihydroxybenzamide" in the urine of cats given salicylamide.[18] Understandably, the role of 2,3-DBA as a metabolite of salicylamide has generally been neglected in research concerning salicylamide metabolism.

In the present study in mice, we demonstrated that hydroxylation of salicylamide at the three position is a significant metabolic pathway after administration of high doses. 2,3-Dihydroxybenzamide and its conjugates constitut-

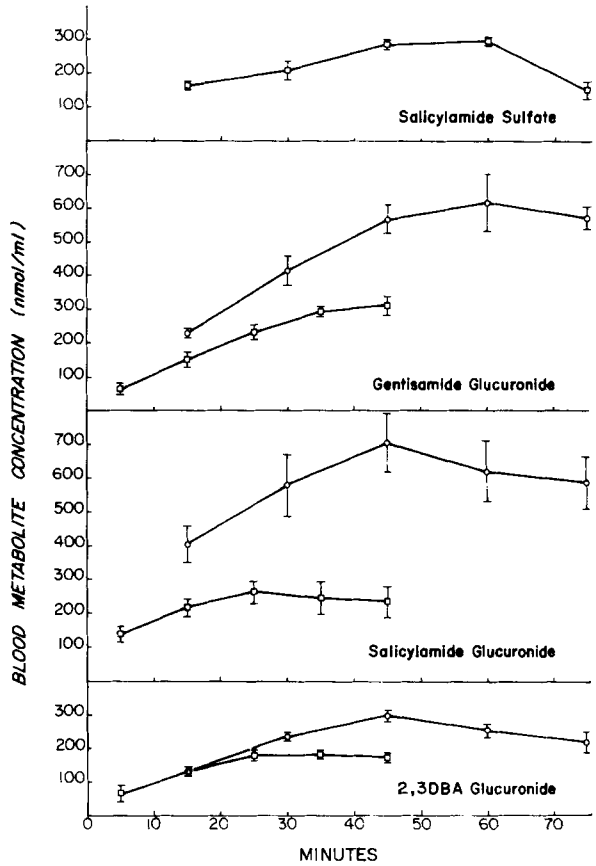

Figure 8—*Blood concentrations of salicylamide metabolites versus time. Key: (□) after 2 mmol kg $^{-1}$ of salicylamide, ip; (○) after 4 mmol kg^{-1} of salicylamide, ip. Each point represents the mean ± SE of four determinations.*

ed 18% of urinary salicylamide metabolites. We have also detected small amounts of 2,3-DBA glucuronide in the bile and urine of rats treated with salicylamide (4 mmol kg^{-1}, ip, data not shown), indicating that three hydroxylation occurs in species other than the mouse. Using the HPLC method of Morris and Levy,[10] we found 2,3-DBA glucuronide to coelute with salicylamide glucuronide (data not shown), suggesting that the use of this method may result in erroneous estimation if salicylamide glucuronide is present. In addition, the extent of the phase I metabolism of salicylamide would be underestimated by this method. While the significance of 3-hydroxylation at lower doses of salicylamide and in other species was not studied, our results suggest that failure to analyze the 3-hydroxy metabolites of salicylamide separately

Table II—Urinary Metabolites of Salicylamide[a]

Metabolite	Dosage, mmol·kg^{-1}	
	2	4
Salicylamide glucuronide	20.8 ± 2.3[b]	27.0 ± 1.0[c]
Salicylamide sulfate	13.9 ± 2.8	12.5 ± 0.8
Gentisamide	1.3 ± 0.2	1.1 ± 0.2
Gentisamide glucuronide	37.2 ± 1.1	34.7 ± 0.6[c]
Gentisamide sulfate	8.9 ± 0.7	6.6 ± 0.5[c]
2,3-Dihydroxybenzamide	0.9 ± 0.1	0.6 ± 0.1
2,3-Dihydroxybenzamide glucuronide	14.3 ± 0.7	14.0 ± 0.7
2,3-Dihydroxybenzamide sulfate	2.7 ± 0.2	3.5 ± 0.3

[a] Urine was collected for 24 h after salicylamide administration; results are expressed as percentages of the total metabolites appearing in the urine; percent of injected dose recovered in urine was 66.0 ± 6.4 and 78.0 ± 4.3 after 2 and 4 mmol kg $^{-1}$, respectively ($p < 0.05$). [b] Each value represents the mean ± SE of four (2 mmol kg^{-1}) or eight (4 mmol kg $^{-1}$) mice. [c] Significantly different from 2-mmol kg^{-1} dosage value ($p < 0.05$).

could confuse the results of experiments using salicylamide as a model compound for the study of drug metabolism.

Blood levels of salicylamide declined in two phases, the first having a less rapid rate of decline than the second. This was probably due to continued absorption of salicylamide from the peritoneum during the first phase, although a concentration-dependent elimination of salicylamide could also account for this effect. However, the rate of decline in blood salicylamide concentration during the second phase probably reflects the true rate of blood clearance. This rate was lower after the 4- than the 2-mmol kg^{-1} salicylamide dosage. A similar dose-dependent salicylamide elimination has been observed in dogs[16] and humans,[9] and was interpreted as saturation of the salicylamide metabolizing system. The present data indicate that a similar phenomenon occurs in mice.

In xenobiotic metabolism, glucuronidation and sulfation are often competing processes, with glucuronidation exceeding sulfation as substrate load increases.[19] This effect has been observed with salicylamide metabolism in dogs,[16] humans,[9] and isolated rat hepatocytes.[8] Similarly, in the present study, the fraction of urinary salicylamide metabolites appearing as salicylamide glucuronide increased with higher dosages of salicylamide.

In summary, it was determined that 3-hydroxylation is a significant route of salicylamide biotransformation in mice after high doses. It is important that metabolism by this route be evaluated in any experiment using salicylamide as a tool for the study of drug metabolism.

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Acknowledgments

The authors wish to thank Jeanene Hanley for her technical assistance, Dr. Khalifah of the V.A. Hospital, Kansas City, MO, for performing NMR analyses, and Dr. Tozer of the University of California, San Francisco, for supplying authentic samples of salicylamide sulfate and gentisamide. Mass spectroscopic analyses were performed by Mass Spectroscopy Laboratory in the Chemistry Department of the University of Kansas, Lawrence, KS.

This work was supported by USPHS grant ES-03192, SRH was supported by USPHS training grant ES-07079, RLD was supported by USPHS training grant ES-07079 and a Stauffer Fellowship, and CDK was a Burroughs Wellcome Toxicology Scholar.