Renal Contribution to Overall Metabolism of Drugs IV: Biotransformation of Salicylic Acid to Salicyluric Acid in Man

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Abstract □ Salicylic acid was infused at three different steady-state levels to study its possible metabolism to salicyluric acid within the kidney in man. Concomitant with the salicylic acid infusion, radiolabeled salicyluric acid was infused at a constant rate to define its intrinsic renal clearance characteristics. Thereby, the rate of formation of salicyluric acid from salicylic acid, within the kidney, was calculated to be approximately 60–70% of the total amount formed.

Keyphrases □ Salicylic acid—renal metabolism to salicyluric acid in man □ Salicyluric acid—biotransformation after salicylic acid administration in man, renal clearance characteristics determined using salicyluric acid-^{14}C □ Renal metabolism—biotransformation of salicylic acid to salicyluric acid, man

The liver is usually considered the primary organ involved in the metabolism of drugs. Nevertheless, conjugation of aromatic acids with glycine in the kidney has been shown to take place both *in vitro* (1–7) and *in vivo* (8–10). Wan and coworkers (11–13) showed extensive *in vivo* conjugation in animals and reviewed the earlier studies in this field. In some species studied, the specific enzyme activity in the kidney has been found to be higher than that of the liver (1, 5–7, 10). However, Schachter and Manis (14) found no evidence for the renal synthesis of salicyluric acid from salicylic acid in man. Their statement is based on the observation that the clearance of salicyluric acid did not exceed the approximate renal plasma flow. The latter was calculated from the endogenous creatinine clearance. This conclusion is not in agreement with the results reported here.

Previous studies from these laboratories on the conversion of benzoic acid to hippuric acid in the rabbit (11), aminobenzoic acid to *p*-acetamidobenzoic acid and *p*-aminohippuric acid in the rabbit (12), and salicylic acid to salicyluric acid in the monkey (13) showed clearly that the kidney plays a major role as a metabolizing organ for these compounds. A summary of these findings is shown in Table I. These results encouraged the investigation of the renal rate of formation of salicyluric acid in man. A survey of the literature revealed that the apparent half-lives for the plasma disappearance of salicylic acid in man increased with dose (15–17), with values ranging from 2.4 to 19 hr. in adults. Similar results are evident from studies of the urinary excretion of salicylates as a function of dose (18), which show that the percentage of the dose excreted at a given time decreases with increasing dose. Practically all of an administered dose can be recovered in the urine as free, unaltered salicylic acid and as four major metabolites. At low doses, the main metabolite is salicyluric acid. The percent of salicyluric acid excreted in the urine has been observed to range from 0 to 91% of the dose of salicylic acid administered (14, 18, 19), decreasing as the total dose of salicylic acid increases. The remainder of the dose is excreted as salicyl phenolic glucuronide (12–30%), salicyl acyl glucuronide (0–10%), and gentisic acid (1%) (19) and its conjugates. The dose-dependent kinetics of salicylic acid have been attributed to the saturation of the metabolic process, which leads to the formation of salicyluric acid. This has been shown to be the reason for the observed wide variation in the plasma half-lives (18, 20–23). Since the rhesus monkey has been shown (12) to metabolize salicylic acid to salicyluric acid in similar amounts as man, it became increasingly interesting to examine if the human kidney functions similarly.

Table I—Renal Contribution to Metabolism of Benzoic Acid Derivatives and Salicylic Acid in Animals

Compound	Animal	Percent of Metabolite Formed in— Kidney	Liver[a]
Benzoic acid–hippuric acid	Rabbit	79	21
	Rabbit	28	72
	Rabbit	55	45
Aminobenzoic acid–*p*-acetamidobenzoic acid	Rabbit	52	48
Aminobenzoic acid–*p*-aminohippuric acid	Rabbit	70	30
Salicylic acid–salicyluric acid	Monkey	100	0

[a] Metabolism within the body as a whole, other than that which takes place in the kidney, is referred to in this table as "liver" metabolism.

EXPERIMENTAL

Subject One healthy male adult with no history of liver or kidney dysfunction or salicylate idiosyncrasy was studied.

Materials—Salicyluric acid-carboxy-^{14}C was synthesized, m.p. 170°, from salicylic acid-carboxy-^{14}C[1], following the method of Froemming and Vollenberg (24). The specific activity, as determined by liquid scintillation counting, was 34.9 μc./mmole. The efficiency was calculated using the internal standard method. A purity of >99% was established by radioscan of the TLC silica gel plate. The solvent system was butanol–acetic acid–water (4:1:1). Salicylic acid and salicyluric acid have R_f values of 0.49 and 0.62, respectively. No trace of radioactive starting material was observed in the radioscan. The absence of glycine was confirmed by NMR, which is capable of detecting low concentrations of glycine (2% or more).

The salicylic acid infusion solution was prepared by dissolving varying amounts of sodium salicylate in normal saline, whereas salicyluric acid was only soluble in a 5% sterile solution of sodium bicarbonate. All intravenously administered solutions were prepared under aseptic techniques and were bacteriologically filtered shortly before use.

[1] Tracerlab.

Infusion Procedures—A 10-μc. sample of salicyluric acid-carboxy-^{14}C, isotopically diluted in 300 mg. total salicyluric acid and dissolved in 5% sodium bicarbonate solution, was infused at a rate of 27.4 mg./hr. throughout the experiment. An intravenous priming dose of 200 mg. (8 μc.) salicyluric acid-carboxy-^{14}C was administered 20 min. prior to the start of the infusion. This initial infusion was done to define the renal clearance characteristics of salicyluric acid in man. The salicyluric acid infusion was continued through the experiment to ascertain the constancy of the salicyluric acid renal clearance. Two hours later, an intravenous priming dose of 250 mg. salicylic acid was given, followed by infusion of salicylic acid at a dose level of 14.2 mg./hr. (level 1). This procedure was repeated with an intravenous priming dose of 50 mg. salicylic acid and an infusion rate of 28.4 mg./hr. (level 2) and another intravenous priming dose of 100 mg. salicylic acid and an infusion rate of 56.8 mg./hr. (level 3). The infusion was maintained at each level for 2 hr. At these doses, the calculated plasma levels of salicylic acid were 5–20 mcg./ml. All infusions were made into a fast flowing intravenous drip of normal saline–5% dextrose.

Sampling Time—Five-milliliter blood samples were obtained at each sampling period. Blood and urine blanks were taken before the start of the experiment. Four blood samples were taken at 15-min. intervals during the 2nd hr. of the infusion at each level. (A total of 16 blood samples was taken.) Urine was voided in 15-min. intervals and collected between the blood sampling times for a total of 20 urine samples. Due to the amount of fluids infused into the test subject, the amount of urine voided in 15 min. averaged 111 ml.

ANALYTICAL METHODS

Plasma and urine samples were assayed for salicylic acid and salicyluric acid spectrophotometrically after elution from a column[2] according to the method described by Wan and Riegelman (12). Amounts of salicylic acid and salicyluric acid greater than 0.5 mcg./ml. of plasma or urine could be detected. The calibration curve showed linearity to at least 20 mcg./ml. and allowed accurate analysis even if one substance was in a 20-fold excess. None of the other metabolites of salicylic acid interfered with the assay. For the detection of the lowest levels of salicyluric acid, 0.5 ml. plasma was treated with 0.5 ml. isopropyl alcohol to precipitate the protein. A 0.6-ml. sample of the supernate was placed on the column and the salicyluric acid and salicylic acid fractions were collected as described previously (12). The effluents were directly measured in a spectrophotofluorometer[3]. A 50–100-μl. sample of urine was placed on the column and eluted with buffer (0.7 *M* phosphate buffer, pH 7.0). The fractions were measured at an activation wavelength and emission wavelength of 305 and 400 nm., respectively. The slit width was 6 mm.

The radioactivity in the urine samples was determined in a liquid scintillation counter[4]. A 0.5-ml. sample of urine was mixed in a polyethylene vial with 10 ml. modified Bray's cocktail having a composition of 8 g. 2,5-diphenyloxazole, 0.6 g. 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (or 6 g. of an alternate cocktail[5]), and 150 g. reagent grade naphthalene, dissolved in 100 ml. ethoxyethanol and 20 ml. ethylene glycol, and was made up to 1 l. with scintillation grade dioxane. The window was set on 50–1000 and the gain was set on 50 ± 1%. The efficiency was determined by the internal standard method (25) using a toluene-^{14}C standard with an activity of 3.48×10^6 d.p.m./ml. The efficiency ranged between 94 and 97% with a maximum standard deviation of 0.7%.

RESULTS AND DISCUSSION

Studies by Rowland and Riegelman (26) elucidated some pharmacokinetic parameters. When a 250-mg. i.v. dose of salicylic acid was administered, the half-life for the slow disposition phase was found to range from 120 to 170 min. and the volume of distribution at steady state ($V_{d_{ss}}$) was 9.6 ± 0.8 l. From these parameters, the priming dose as well as the rate of infusion was calculated for each level. During the first infusion period (level 1), the concentration of salicylic acid did not reach steady state. The rate of excretion of ^{14}C-labeled salicyluric acid stayed constant throughout the experiment except for the first level (Fig. 1). This indicates that no changes in the clearance took place for levels 2 and 3, in spite of the increasing doses of salicylic acid.

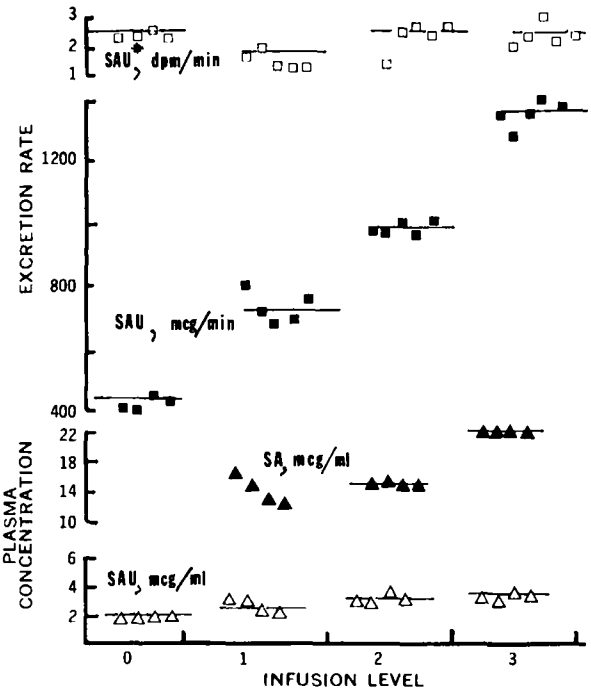

Figure 1—*Plasma concentration and urinary excretion rates during salicyluric and salicylic acid infusion in man. Each horizontal line represents 1-hr. infusion interval. (SAU = salicyluric acid, and SA = salicylic acid.)*

The renal clearance of salicyluric acid remained essentially constant at 227 ml./min., uncorrected for protein binding and body surface. Since the assessment of this value is critical to the remaining calculation of renal metabolism, comparison of this value with literature values is warranted. Corrected renal clearance values of salicyluric acid were reported by Schachter *et al.* (14) to be 444 ± 112 ml./min./1.73 m.[2] body surface. Unfortunately, the data of Schachter *et al.* (14) did not permit an estimate of the free fraction of salicyluric acid. Knoefel *et al.* (27), in 1962, reported data for salicyluric acid binding; the data indicate a rather large change in binding of the drug with changes in total plasma concentration[6]. Schachter *et al.* (14) used a complex assay procedure[7]. Although there are potential errors in the procedure due to the number of steps, the assay is essentially based on the separation of salicylic acid from the insoluble salicyluric acid when carbon tetrachloride is used. In our studies of this system, salicyluric acid was found to be slightly, but significantly, soluble in carbon tetrachloride. For example, 2- and 4-mcg./ml. solutions of salicyluric acid in 0.1 *N* hydrochloric acid were extracted with 15 volumes of carbon tetrachloride. The organic phase was reextracted with 1 *N* sodium hydroxide and as-

[2] Sephadex G-10.
[3] Hitachi MPF-2A fluorescence spectrophotometer.
[4] Packard model 3375.
[5] Omnifluor, New England Nuclear.

[6] When using the Freundlich absorption isotherm, log-log plots result in the following data. They include two data points of Knoefel *et al.* (27) and two data points obtained by extrapolation to lower total plasma concentration of salicyluric acid.

Total Plasma Concentration, mcg./ml.	Free Concentration, mcg./ml.	Bound Concentration, mcg./ml.	Percent Free
82.4 [experimental (27)]	71	11.4	86
5.0 [experimental (27)]	32	18	64
1.9 (extrapolated)	10	9	53
0.92 (extrapolated)	4	5.2	44

[7] The assay included a formation of salicylic hydroxamate from the acylglucuronide, precipitation of plasma protein, extraction of salicylic acid and salicyluric acid with ether, and evaporation to dryness. The residue, equivalent to 1.6 ml. plasma, was then extracted with 18 ml. carbon tetrachloride to remove salicylic acid, presumably leaving the salicyluric acid for the final fluorometric assay.

Table II—Steady-State Plasma Concentrations and Excretion Rates of Salicylic Acid and Salicyluric Acid at Different Infusion Levels

Level of Infusion	Cp, mcg./ml.		dA/dt, mcg./min.[a]	
	Salicylic Acid	Salicyluric Acid	Salicylic Acid	Salicyluric Acid
0	0	1.9 ± 0.15	0	431 ± 44
1	14.4 ± 1.8[b]	2.3 ± 0.1	0	717 ± 56
2	13.4 ± 0.9	2.8 ± 0.4	0	981 ± 32
3	21.7 ± 0.2	3.1 ± 0.3	54	1352 ± 108

[a] This refers to the urinary excretion of salicylic acid or salicyluric acid in micrograms per minute. [b] This plasma salicylic acid level was still declining from a high of 16.2 to a low of 12.6 mcg./ml. during the experimental period of infusion.

sayed on a spectrophotofluorometer (excitation 320 nm., emission 420 nm.). The results indicate that 25% of the salicyluric acid was extracted into carbon tetrachloride. Additional studies of salicylic acid in the presence of salicyluric acid indicate incomplete extraction of the salicylic acid by the carbon tetrachloride, leading to further potential error.

Smith *et al.* (28), in 1945, estimated the corrected clearance of salicyluric acid in two subjects. Their results for the two subjects were 269 and 487 ml./min./1.73 m.^{2} body surface. They infused salicylic acid and estimated the values using a reaction of the phenolic group of salicyluric acid with *N*-2,6-trichlorobenzoquinonimine. Our studies indicated that the Smith *et al.* reaction leads to high and variable plasma blank values, and once again there may be some question as to the method of correction for protein binding since the results are not specified.

Levy and coworkers (29, 30) also utilized the carbon tetrachloride method for the separation of salicylic acid and salicyluric acid. The exact method is not clearly identified in the reference; however, it undoubtedly included a solvent extraction and fluorometric analysis. They (29) reported the partitioning characteristics of salicyluric acid at 250 mcg./ml. in the presence of 500 or 1000 mcg./ml. of salicylic acid. These concentrations in the extraction procedure are probably appropriate for urine, so, their data are probably valid for urine studies. However, they do not verify separation of the two compounds at plasma levels, which usually are 1–3 mcg./ml. of salicyluric acid in the presence of 1–40 mcg./ml. of salicylic acid.

One can attempt to estimate the renal clearance of salicyluric acid from the data of Levy *et al.* (30) by estimating the excretion rate of salicyluric acid at the appropriate equivalent plasma concentrations. The resultant (corrected) value appeared to possibly exceed the renal plasma flow rate.

It is suggested, therefore, that the available literature data on renal clearance of salicyluric acid are ambiguous. The data of the present study were derived with concomitant infusions of the salicyluric acid-^{14}C. The renal excretion of this compound remained fairly constant throughout the study, which indicated that the excretion remained virtually constant. The excretion rate determined from the amount of salicyluric acid-^{14}C in the urine was found to be 426 mcg./min. This value agrees well with the value of 431 mcg./min. found from the cold assay. At an infusion rate of 456 mcg./min., the value of 431 mcg./min. represents a 94.5% urinary recovery of salicyluric acid. Unfortunately, the plasma radioactivity levels were in the range of background and, therefore, could not be used to estimate clearance. The plasma concentrations and rates of excretion found for salicylic acid and salicyluric acid at each infusion level are included in Table II and Fig. 1. At level 1, the plasma concentration of salicylic acid did not reach steady state. The same problem was observed with the plasma level of salicyluric acid, where only the last two data points of the first level could be considered to be at steady state. In the urine, salicylic acid could only be detected in the terminal infusion level, indicating that only negligible amounts of salicylic acid were excreted unchanged over this range of infusion.

The true and apparent clearance values for salicyluric acid (Table III) were calculated from the observed plasma levels and excretion rates of salicyluric acid. In spite of the declining plasma concentration of salicylic acid during the first infusion period, one can calculate an apparent clearance of salicyluric acid since the salicyluric acid plasma data do not reflect the magnitude of difference seen in the salicylic acid data. However, the apparent clearance for the first infusion period (Table III) was calculated only from the last two data point times (Fig. 1). With increasing amount of salicylic acid being infused, the percent of the dose excreted as salicyluric acid decreased (column 2 of Table IV). The mass balance for the initial infusion of salicyluric acid (level 0) shows that 94.5% of the input was recovered in the urine. This value agrees well with the value reported by Levy *et al.* (30). The method of calculating the contribution of kidney metabolism to overall metabolism was recently published (1). Equation 1 describes the rate of formation of salicyluric acid by the kidney:

$$\left(\frac{dM}{dt}\right)_{\text{kidney}} = \left(\frac{dM}{dt}\right)_{\text{total}} - \left(\frac{dM}{dt}\right)_{\text{liver}} \qquad \text{(Eq. 1)}$$

At steady state, (dM/dt) liver[a] is given by the equation:

$$\left(\frac{dM}{dt}\right)_{\text{liver}} = (\dot{V}_{cl})_{ex} \times Cp(\text{SAU}) \qquad \text{(Eq. 2)}$$

where $(\dot{V}_{cl})_{ex}$ is the renal clearance of salicyluric acid (SAU) as calculated from the level 0 data (Table III). By multiplying this value by the plasma concentration of salicyluric acid at each infusion level, one obtains the rate of formation of metabolite at any time. At steady state, the total rate of metabolism, (dM/dt) total, equals the rate of excretion. This definition is valid only if no portion of metabolite synthesized by the kidney is reabsorbed back into the blood.

Table IV includes an estimate of the rate of formation of salicyluric acid by the kidney and extrarenal processes (liver) and also as a percent of the total rate of metabolism. The resultant data indicate that an average of 67 ± 4% of salicyluric acid was formed in the kidney. To our knowledge, no similar study of the contribution of renal metabolism has been reported for man. While this study was done in only one subject, the results indicate clearly the importance of the kidney as a site of salicylate metabolism in man.

Table III—True and Apparent Renal Clearances of Salicyluric Acid in Man

Compound $(\dot{V}_{cl})_{ex}$	$(\dot{V}_{cl})_{app}$ at Levels		
	1	2	3
Salicyluric acid 227	312	350	436

Table IV—Contribution of Renal Metabolism to Overall Metabolism of Salicylic Acid in Man

Level of Infusion	Percent of Dose Excreted as Salicyluric Acid[a]	Excretion Rate (dM/dt), mcg./min.		Percent Salicyluric Acid Formed	
		Kidney	Liver	Kidney	Liver
1	85	195	91	70	30
2	81.5	346	204	63	37
3	68.3	649	272	71	29

[a] The values were estimated by calculating the excretion rate of salicyluric acid resulting from salicylic acid metabolism (by correcting for the steady-state excretion rate for salicyluric acid of 431 mcg./min.), multiplying by the time interval, and correcting for the molecular weight.

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[a] It should be emphasized that (dM/dt)liver is the sum of the rates of metabolism of all sites in the body other than the kidney.

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Animal Models for Investigating Intestinal Drug Absorption: Various Antibiotics

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Abstract □ Among the most commonly used methods for studying intestinal drug absorption are the *in vitro* everted rat gut and the *in situ* rat intestinal loop. The methods were compared employing various penicillins, cephalosporins, and tetracyclines. Steady-state and, particularly, initial drug clearances across the everted rat gut and the *in situ* absorption data were not always in agreement. A comparison of these results with antibiotic absorption data in man demonstrated rank-order agreement between absorption from the *in situ* intestinal loop and human GI absorption. Based on present observations, the *in situ* intestinal loop of the rat appears suitable as an animal model for predicting human drug absorption.

Keyphrases [ ] Absorption, intestinal- *in vitro* everted rat gut and *in situ* rat intestinal loop drug permeability data, compared to antibiotic absorption data in man □ Antibiotic (penicillins, cephalosporins, and tetracyclines) intestinal absorption *in vitro* everted rat gut and *in situ* rat intestinal loop data compared to absorption data in man □ Drug permeability across intestinal membrane data from *in vitro* everted rat gut and *in situ* rat intestinal loop techniques compared to antibiotic absorption in man

A variety of *in vitro* and *in situ* animal techniques have been employed to study drug permeability across the intestinal membrane. Many of these techniques were recently discussed by Bates and Gibaldi (1). The present investigation was undertaken to compare the data obtained from two such techniques, the *in vitro* everted gut and the *in situ* intestinal loop, and to evaluate these techniques as possible models for human drug absorption. Several groups of antibiotics were studied, since this class of compounds offers a wide range in the degree to which absorption occurs in man.

METHODS

Male, Sprague–Dawley strain rats[1], weighing approximately 250 g., were fasted 14–20 hr. prior to the experiment. Water was allowed *ad libitum*.

Drug Transfer across Isolated Everted Rat Intestine—Intestinal transfer rates were determined using a modification of the method of Crane and Wilson (2). The method for preparing the everted intestine preparation was described previously (3). After severing the intestine at the pyloric junction, the first 15 cm. of intestine was discarded, the gut was everted, and the proximal portion was divided into two 10-cm. segments. The initial, proximal segment was designated Segment 1 and the distal portion was designated Segment 2.

Both segments were placed into test tubes containing approximately 100 ml. of mucosal drug solution, previously equilibrated at 37° and continually gassed with oxygen–carbon dioxide (95:5 v/v). Two milliliters of a modified physiological Krebs bicarbonate

[1] Blue Spruce Farms, Altamont, N. Y.