Renal Contribution to Overall Metabolism of Drugs I: Conversion of Benzoic Acid to Hippuric Acid

SUK HAN WAN* and SIDNEY RIEGELMAN▲

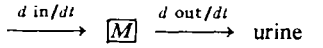
Abstract □ The kinetics of biotransformation of benzoic acid to hippuric acid during infusion in animals were studied at steady state. The contributions to the glycine conjugation of benzoic acid made by the kidney in contrast to the body as a whole were quantified by the utilization of apparent clearances, $(\dot{V}_{cl})_{app.}$, and true renal clearances, $(\dot{V}_{cl})_{ex.}$. Michaelis–Menten kinetics appear to describe the rate of biotransformation of both metabolic processes. The data result in estimation of the *in vivo* K_m and $V_{max.}$ values.

Keyphrases □ Benzoic acid biotransformation to hippuric acid—kidney metabolism contribution contrasted to whole body, renal and apparent clearances □ Hippuric acid from benzoic acid biotransformation—kidney metabolism contrasted to whole body, renal and apparent clearances □ Kidney metabolism—contribution to biotransformation of benzoic acid to hippuric acid, contrasted with whole body, renal and apparent clearances □ Biotransformation kinetics—benzoic acid to hippuric acid, kidney metabolism contribution

In pharmacokinetics, one attempts to define the rates of absorption, distribution, metabolism, and excretion of drugs on which the rationale for drug dosage and maintenance therapy is based. Metabolism and excretion are usually considered to take place primarily from the central pool or compartment, since the major metabolic organs have high blood perfusion rates and are assumed to equilibrate almost instantaneously with the vascular pool. Biochemical studies have shown that the liver is a particularly rich site of metabolizing enzymes, and this finding has led to the general belief that drug metabolism is primarily a liver function. However, GI tissues, blood cells, kidneys, lungs, and brain tissue, among others, also have been shown to contain appreciable amounts of such enzymes (1, 2). The contribution to total drug metabolism of these extrahepatic systems is usually considered to be minor. Noted authorities in the field of drug metabolism have repeatedly stated that the liver is clearly the major metabolizing organ in the body (3). In fact, it is sometimes considered to be the only organ involved in drug metabolism (4).

THEORETICAL CONSIDERATIONS

During the absorption phase, all of the drug molecules are transported to the liver *via* the hepatic portal system at a relatively high concentration. A larger percentage of the drug molecules traverse the liver and are metabolized during this first pass than after the drug has distributed throughout the body when only a small fraction of the molecules traverse the liver during each pass. In some instances, a drug may be so rapidly metabolized during the first pass that the contribution of other metabolic sites in the body may become insignificant. When liver metabolism is not rapid, the contribution of extrahepatic sites can become significant. Of the extrahepatic tissues examined for enzymic activity, those of the kidney and GI tract appeared most promising. Studies such as those by Barr and Riegelman (5, 6) on salicylamide and by Hülsmann and Statius van Eps (7) on *p*-aminohippuric acid have contributed to the realization that the GI tract can contribute appreciably to metabolism of drugs, especially when they are orally administered. Studies such as those by Quick (8) on benzoic acid and by Knoefel *et al.* (9) on the amino and acetamidobenzoic acids have indicated the importance of the kidney as another source of metabolizing enzymes.

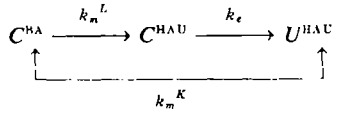


Scheme I

The conversion of benzoic acid to hippuric acid has been found to occur in the kidney in most animals and exclusively in this organ in the dog (8). The classic experiments of Bunge and Schmiedeberg (10) showed that hippuric acid is formed when blood containing benzoic acid and glycine is perfused through the dog kidney. Their work was corroborated by the perfusion experiments of Kochs (11) and Snapper *et al.* (12). Snapper *et al.* (12) also found that the same reaction occurs in the perfused human kidney. It is interesting to note that the study of Bunge and Schmiedeberg (10) dated back to 1876. In the rabbit, 80–100% of an oral dose is metabolized to hippuric acid. At sufficiently low doses, only negligible amounts (0–3%) of the drug are excreted unchanged and another 0–20% may be excreted as the glucuronide. At high doses, formation of hippuric acid proceeds at a constant rate, which varies considerably between species with an average rate of formation of 147 mg./hr. in the rabbit (13).

This article reports a study of the overall metabolic conversion of benzoic acid to hippuric acid in the whole animal. An attempt was made to quantify the contributions made by the kidney in contrast to the body as a whole. It was assumed that the blood level of a metabolite is a measure of liver metabolism, although other organs may have contributed to the metabolite concentration. For several reasons the experiments were carried to steady-state conditions before biological systems were obtained:

1. It is well known that when a drug is administered, there is a "real" lag time before the rate of excretion reflects the rate of decay of the plasma concentration curve. This lag time exists regardless of occurrence of metabolism and is due to physiological events such as the effects of distribution within the body, as well as within the kidney, and to what might be referred to as mechanical events such as frictional forces which retard the flow of fluid through narrow kidney tubules. The contribution of each factor is not easily defined, but the lag time is real and has been estimated to be as long as 5 min. (14). This is a short time period, but if a compound has a rapidly falling blood level, a calculation error is introduced.
2. The interpretation of data involves some sort of correlation between substrate concentration in the central compartment and the rate of metabolism, which is calculated from plasma and urinary excretion data. The correlations that are made when the kinetics of a compound is in a state of flux can be affected by distribution and equilibration processes (15) and the type of model used.
3. Supposing that all the products of a metabolic pathway contribute to a metabolite pool, M, from which the metabolite is excreted into urine, under steady-state conditions the rate of input into M, d in/dt, equals output, d out/dt; *i.e.*, the rate of metabolism equals the rate of excretion (Scheme I). The effects of reabsorption of metabolite, if any, would also be negated. From analysis of urine data, one can then conveniently estimate the rate of metabolism.



Scheme II

The first step is to estimate the clearance of the metabolite, hippuric acid. Hippuric acid (HAU) was infused until a steady state was reached and was maintained throughout the entire experiment. The rate of excretion is given by the equation:

$$\left(\frac{dA}{dt}\right)^{HAU} = k_e V_p C_p^{HAU} \qquad \text{(Eq. 1)}$$

Clearance is obtained by rearrangement of Eq. 1:

$$(\dot{V}_{cl})_{ex.}^{HAU} = \frac{(dA/dt)^{HAU}}{C_p^{HAU}} = k_e V_p \qquad \text{(Eq. 2)}$$

At sufficiently low plasma concentrations, when T_m is not exceeded, the clearance value is presumed to be constant. The clearance so defined will be referred to as the true renal clearance, $(\dot{V}_{cl})_{ex.}$, to differentiate it from the apparent renal clearance, $(\dot{V}_{cl})_{app.}$, which is later defined mathematically.

If the parent compound, benzoic acid (BA), is now infused into the system, metabolism occurs which adds hippuric acid to the system. If it is postulated that metabolism to hippuric acid occurs in both the liver and the kidney, then a model can be set up to illustrate the fate of benzoic acid in the system (Scheme II), where C^{BA} and C^{HAU} denote amounts of the respective compounds in the central pool, U denotes the amount excreted in the urine, and $k_m{}^L$ and $k_m{}^K$ are rate constants for the metabolism of benzoic acid in the liver and kidney, respectively. The rate constant for excretion of hippuric acid from the central pool is k_e. In this model, benzoic acid introduced intravenously into the systemic circulation is assumed to be metabolized by the liver, with a rate constant equal to $k_m{}^L$. The metabolite is then reintroduced *via* the hepatic vein into the systemic circulation before being transported to the kidneys and excreted into urine. Metabolites formed in the kidney are introduced directly into the urine. It is possible that the metabolite is reabsorbed into the vascular system. From considerations of the location of mitochondria in the tubules, blood flow to this region, partition characteristics of hippuric acid, and clearance data, it is likely that hippuric acid is excreted directly into urine with practically none of it appearing in the blood.

If kidney metabolism takes place during administration of benzoic acid, hippuric acid clearance is affected. By considering Eq. 2, the rate of excretion of hippuric acid, $(dA/dt)^{HAU}$, is increased due to formation of hippuric acid in the liver and kidney and subsequent excretion; $C_p{}^{HAU}$ may also be increased but not to a proportionate extent. Most of the hippuric acid that is formed in the kidney tubules is directly excreted into urine. The ratio of rate of excretion to plasma concentration of hippuric acid or $(\dot{V}_{cl})_{app.}^{HAU}$, therefore, increases if metabolism in the kidney takes place. To differentiate this value from true renal clearance, this term will be called apparent renal clearance. The proof of drug metabolism in the kidney therefore rests in the change in clearance after administration of the precursor as compared to the estimate during administration of the metabolite itself. The apparent renal clearance exceeds the true renal clearance, the difference being due to renal metabolism:

$$(\dot{V}_{cl})_{app.}^{HAU} = (\dot{V}_{cl})_{ex.}^{HAU} + (\dot{V}_{cl})_{met.}^{HAU} \qquad \text{(Eq. 3)}$$

Clearances, however, can be affected by other factors. An increase in clearance can be brought about by changes in protein binding and volumes of distribution of hippuric acid in the presence of benzoic acid. Benzoic acid can compete with tubular secretion or the T_m for secretion can be exceeded, in which case erroneous values for rate of metabolism would be obtained. To monitor the effects of changing plasma concentrations of benzoic acid and hippuric acid on the excretory process, the hippuric acid that was infused throughout the experiment was labeled at the carboxyl carbon with ^{14}C. In this way, by comparing the rate of excretion of radiotagged compound to total metabolite, one can detect changes in plasma clearance. If T_m for secretion is not exceeded and there is no competition for secretion, then the rate of excretion of radiotagged metabolite should be a constant during the entire experiment.

The contribution of the kidney and presumably the liver can be calculated in the following way. During the simultaneous infusion of benzoic and hippuric acids, the total rate of excretion of hippuric acid is the sum of excretion from infused hippuric acid or ^{14}C-labeled hippuric acid and excretion of the nonlabeled metabolite as it is metabolized from benzoic acid:

$$\left(\frac{dA}{dt}\right)^{\text{total HAU}} = \left(\frac{dM}{dt}\right)^{T} + \left(\frac{dA}{dt}\right)^{HAU*} \qquad \text{(Eq. 4)}$$

where $(dM/dt)^T$ refers to the amount of hippuric acid per unit time formed by all sites of metabolism. The metabolites are supplied from two sources, the liver and the kidney:

$$\left(\frac{dM}{dt}\right)^{T} = \left(\frac{dM}{dt}\right)^{liver} + \left(\frac{dM}{dt}\right)^{kidney} \qquad \text{(Eq. 5)}$$

and at steady state:

$$\left(\frac{dM}{dt}\right)^{liver} = (\dot{V}_{cl})_{ex.} \cdot C_p^{HAU} \qquad \text{(Eq. 6}a\text{)}$$

$$= (\dot{V}_{cl})_{ex.}^{HAU} \qquad \text{(Eq. 6}b\text{)}$$

calculated from infusion of ^{14}C-labeled hippuric acid × plasma concentration of hippuric acid at any data time point under consideration, and:

$$\left(\frac{dM}{dt}\right)^{kidney} = \left(\frac{dM}{dt}\right)^{T} - \left(\frac{dM}{dt}\right)^{liver} \qquad \text{(Eq. 7)}$$

These reactions are obviously enzymic transformations and are expected to exhibit Michaelis–Menten kinetics. To study further the kinetics of metabolism, the infusion of benzoic acid was administered at different rates, attempting to reach steady state at each level of infusion.

As far as is known, this study is the first of its kind to attempt to utilize magnitudes of true renal clearance as an index for the determination of renal drug metabolism and metabolism in other organs, presumed in this instance to be the liver.

EXPERIMENTAL

Animal Preparation—Male, New Zealand, white rabbits and a Rhesus monkey were the test animals. Initially, intravenous doses of benzoic acid and hippuric acid were administered to the animals to determine pharmacokinetic parameters. The results of these intravenous bolus studies were used as the basis for the estimation of priming doses, infusion rates, and times for subsequent infusion experiments. Before the infusion experiments, the animals had to be surgically prepared to allow for collection of blood and accurate urine samples. Most of the surgical maneuvers, with the exception of ear vein cannulation in rabbits, were performed under barbiturate anesthesia. The infusion experiments were started after a recovery period of at least 16 hr. For the infusion experiments, animals were kept in restraining chairs or cages. In the first step of the infusion experiment, radiolabeled metabolite was infused, carried to steady state, and maintained throughout the experiment. Varying doses of parent drug were then infused, and each rate of infusion was again carried to steady state. Blood and urine samples were obtained at each infusion level. Aliquot portions of plasma and urine were analyzed for benzoic acid and hippuric acid and for radioactivity.

Infusion Procedure—A priming dose of 50 mg. radiotagged hippuric acid was followed 20 min. later by infusion of ^{14}C-labeled hippuric acid at 500 mcg./min. From the intravenous bolus data, the steady-state plasma concentration expected with a 500-mcg./min. infusion is 4.4–11 mcg./ml. The following is the sequence used in the administration of benzoic acid.

Benzoic Acid Infusion Period 1—At 140 min., a 50-mg. priming dose of benzoic acid was administered, followed at 160 min. by infusion of benzoic acid at 500 mcg./min. The expected $C_{P_{ss}}$ from this rate of infusion is 1.5–2.4 mcg./ml. Samples were obtained from 220 to 280 min.

Benzoic Acid Infusion Period 2—At 280 min., a bolus dose of benzoic acid of 10 mg. was administered and the rate of benzoic acid infusion was increased from 550 to 1100 mcg./min., with an expected $C_{P_{ss}}$ of 3–4.8 mcg./ml. Samples were obtained from 360 to 420 min.

Benzoic Acid Infusion Period 3—At 420 min., a bolus dose of benzoic acid of 10 mg. was administered and the rate of benzoic acid infusion was again increased to 1500 mcg./min. The expected $C_{P_{ss}}$ is 4.6–6.7 mcg./ml. Samples were obtained from 480 to 540 min.

Benzoic Acid Infusion Period 4—At 540 min., a bolus dose of 35 mg. was administered and the rate of infusion was increased to 2950 mcg./min., with an expected $C_{P_{ss}}$ of 9.1–13.2 mcg./ml. At the higher infusion rates, the observed $C_{P_{ss}}$ values exceeded the predicted values. Samples were obtained from 600 to 660 min.

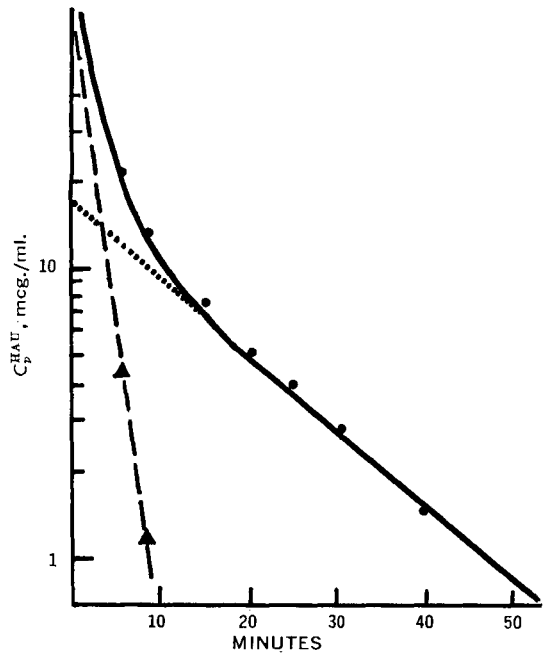


Figure 1—*Plasma concentration–time curve obtained after intravenous administration of 50 mg. hippuric acid to Rabbit 1.*

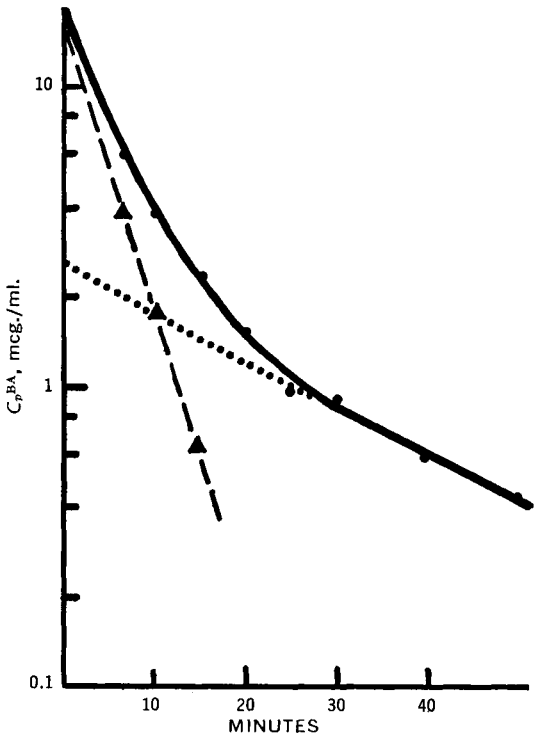


Figure 2—*Plasma concentration–time curve obtained after intravenous administration of 50 mg. benzoic acid to Rabbit 1.*

The mentioned infusion rates apply to Rabbits S3 and S7. The infusion rates in Rabbit S2 were lower and were at 285, 560, 1097, and 1535 mcg./min. at Periods 1–4, respectively, preceded by priming doses in the same order of magnitude as those discussed previously. It should be noted that at the higher rates of infusion where saturation is approached, the prediction of a steady-state plasma concentration is difficult and a longer time may be required to reach steady state.

Materials—Catheters for ear vein cannulation in rabbits consisted of polyvinyl tubing[1] [0.038 cm. (0.015 in.) i.d.]. Catheters used for ureteral implantation in both the monkey and rabbits were polyvinyl tubing[2] [0.05 cm. (0.022 in.) o.d., 0.031 cm. (0.012 in.) i.d.]. For chronic implantation of intravascular catheters in the monkey, a No. 20 size tubing was used [0.08 cm. (0.034 in.) o.d., 0.041 cm. (0.016 in.) i.d.].

Analysis of Benzoic Acid in Plasma—Benzoic acid was quantitatively converted to the more volatile trimethylsilyl derivative and assayed by a GLC method. Plasma was acidified to a pH of 2.8 ± 0.2 and extracted with ether. The pKa of benzoic acid is 4.2. The ratio of ether to aqueous volumes was 1:20. Under these conditions, all of the benzoic acid was quantitatively transferred into the ether fraction. The procedure for extraction is as follows. A 25-μl. solution of saturated potassium bisulfate was added to 0.5 ml. plasma and extracted with 10 ml. distilled ether. A 9-ml. aliquot of the ether fraction was pipeted off and evaporated down to a volume of about 100 μl. at 45–50°. To the residual solution was added 1 ml. of a carbon disulfide solution containing 2 mcg. diethyl diethyl malonate[3] and 5 μl. bis(trimethylsilyl)trifluoroacetamide[4]. The sides of the test tube were thoroughly rinsed with the carbon disulfide mixture. The solution was again evaporated in a hood at 45–50° to a volume of 50 μl. About 1–2 μl. was injected into a gas chromatograph[5] fitted with a 1.82 m. (6 ft.) × 0.32 cm. (0.125 in.) o.d. stainless steel column and a flame-ionization detector (FID). The packing material consisted of 3% OV-1 coated on Gas-Chrom Q, 100–120 mesh. The operating conditions were as follows: injection port temperature, 200°; oven temperature, 110°; and detector block temperature, 160°. Nitrogen, air, and hydrogen flows were at 30, 300, and 25 ml./min., respectively. The range was 1, and attenuation was 4–32. The retention times of benzoic acid and diethyl diethyl malonate were 3.2 and 3.8 min., respectively.

Quantitation of benzoic acid was by comparison of peak height ratios of benzoic acid to the internal standard, diethyl diethyl malonate. An OV-1 column was found to give the cleanest chromatograms with plasma samples. This GLC method is accurate to concentrations of 0.5 mcg./ml., with a coefficient of variation of 3% at this concentration. Plasma blanks invariably had very small peaks with the same retention times as benzoic acid on both OV-17 and OV-1 columns. The peak could possibly be endogenous benzoic acid.

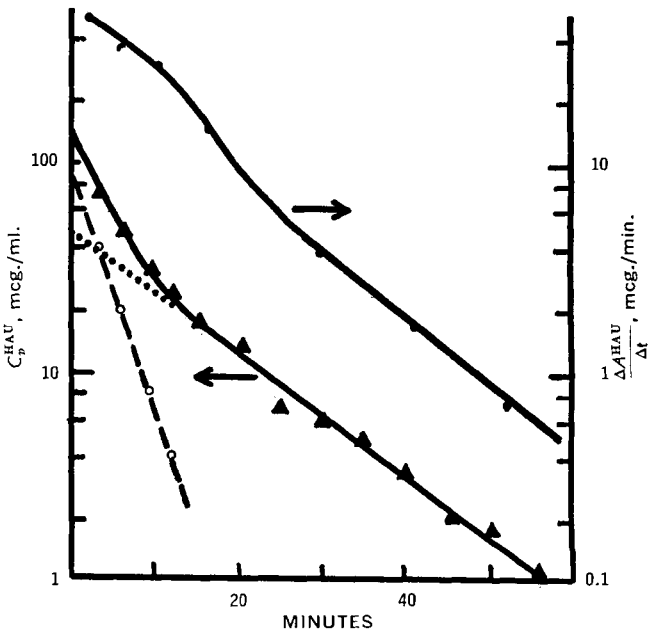


Figure 3—*Plasma concentration–time curve and rate of excretion–time curve obtained after intravenous administration of 100 mg. hippuric acid to Rabbit 3. Key: ●, C_p^{HAU} curve; and ▲, $\Delta A^{HAU}/\Delta t$ curve.*

[1] Bardic Intracath.
[2] Resinite Hi Heat 105C Vinyl Insulation Sleeving No. 24, Borden Chemical Co.
[3] Eastman.
[4] Regisil.
[5] F & M 700.

Table I—Kinetic Parameters Calculated from Data Obtained after Intravenous Injection of Hippuric Acid to Rabbits

Rabbit Number	Dose, mg.	A, mcg./ml.	B, mcg./ml.	C_p°, mcg./ml.	α, min.$^{-1}$	β, min.$^{-1}$	$(\dot{V}_{cl})_{pl}$[a], ml./min.	V_p, ml.
1 (4.5 kg.)	50	75	17	92	0.50	0.06	115	543
2 (3.6 kg.)	50	65	25.5	91	0.23	0.03	44	550
3 (3.7 kg.)	100	80	47.6	127.6	0.41	0.07	114	784

[a] Corrected for body weight, the values are 26, 12, and 31 ml./min./kg. for Rabbits 1, 2, and 3, respectively.

Table II—Kinetic Parameters Calculated from Data Obtained after Intravenous Injection of Benzoic Acid to Rabbits

Rabbit Number	Dose, mg.	A, mcg./ml.	B, mcg./ml.	C_p°, mcg./ml.	α, min.$^{-1}$	β, min.$^{-1}$	$(\dot{V}_{cl})_{pl}$[a], ml./min.	V_p, ml.
1 (4.5 kg.)	50	16	2.6	18.6	0.20	0.04	323	2695
2 (3.6 kg.)	50	18.5	3.8	22.3	0.23	0.03	224	2242
3 (3.7 kg.)	100	55	4.6	59.6	0.17	0.03	201	1679

[a] Corrected for body weight, the values are 72, 62, and 54 ml./min./kg. for Rabbits 1, 2, and 3, respectively.

The peak heights were too small to be measured with accuracy and can only be estimated to be about 0.05 mcg./ml. plasma.

Analysis of Hippuric Acid in Plasma—Hippuric acid was first hydrolyzed to benzoic acid, which was then extracted and quantitated by the GLC method. Since the ratio of hippuric acid to benzoic acid in plasma was always greater than 1.5, the error involved in a difference method is small. A suitable volume of plasma (0.05–0.25 ml.) was diluted to 0.25 ml. with distilled water, and 0.25 ml. of a 6 *N* hydrochloric acid solution was added. The test tube was securely capped and heated at 100° in a heating block for 16 hr. After cooling, the sample was extracted with 10 ml. ether.

Analysis of Benzoic Acid in Urine—Benzoic acid in urine was quantitated by the GLC method since the amounts present were very low; 0.5 ml. of a 10–20-fold dilution of urine was used. The procedure for extraction and quantitation were as described for benzoic acid in plasma.

Analysis of Hippuric Acid in Urine—The amounts of hippuric acid in urine are high and therefore do not require a sensitive GLC method for determination. A colorimetric method based on the formation of the colored azolactone when hippuric acid is reacted with benzenesulfonyl chloride in a basic solution was used. The procedure and the reaction were discussed by Umberger and Fiorese (16). The red-orange color that is formed had a stable absorbance at 380 nm., which follows the Beer-Lambert law over a wide range of concentrations. Salicyluric acid and other amino acids are also reactive, but benzoic acid, amino acids, and other related acids do not give the same reaction.

The method used was as follows. Water was removed from a quantity of urine (5–100 μl.), estimated to contain from 20 to 150 mcg. hippuric acid, by passing a stream of nitrogen over the samples kept at 50–60°. The time required for complete removal of water did not exceed 30 min. To the residue was added 0.5 ml. pyridine and 0.2 ml. of benzenesulfonyl chloride. After standing 20 min. or longer, the mixture was diluted to 5 ml. with chloroform and the absorbance was read against a urine blank on a spectrophotometer[6]. The calibration curve had a slope of 225.4 and a coefficient of variation of 3.2% at a concentration of 20 mcg./5 ml. solution.

Analysis of Radioactive Hippuric Acid in Urine—Radioactive hippuric acid in the urine was measured by liquid scintillation counting in a scintillation spectrometer[7]. An aliquot of 0.2–0.5 ml. urine was pipeted into a 20-ml. volume, low potassium glass or polyethylene vial, and 10 ml. of a modified Bray's cocktail was added. The cocktail had a composition of 6 g. Omnifluor[8] and 150 g. reagent grade naphthalene, dissolved in 100 ml. ethoxyethanol and 20 ml. ethylene glycol, made up to 1 l. with scintillation grade *p*-dioxane. The cocktail was kept in the dark until required. Settings on the scintillation counter were: window, 50–1000; and gain, 12 ± 1%. Quench determination was made by the internal standard method (17), using a ^{14}C-toluene standard with an activity of 3.48×10^5 d.p.m./ml. The efficiency of counting ranged from 70 to 80% but did not vary by more than 2% on individual runs.

RESULTS AND DISCUSSION

The plasma decay curves after an intravenous bolus of 50 mg. hippuric acid and benzoic acid from one animal are shown in Figs. 1 and 2, respectively. Both compounds exhibited a biexponential plasma decay curve. The simplest empirical model which corresponds to this type of decay is a two-compartment open system. The general solution for such a model is:

$$Cp = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 8)}$$

The semilogarithmic plots of the plasma concentration–time curves were solved graphically to give an estimate of the exponents α and

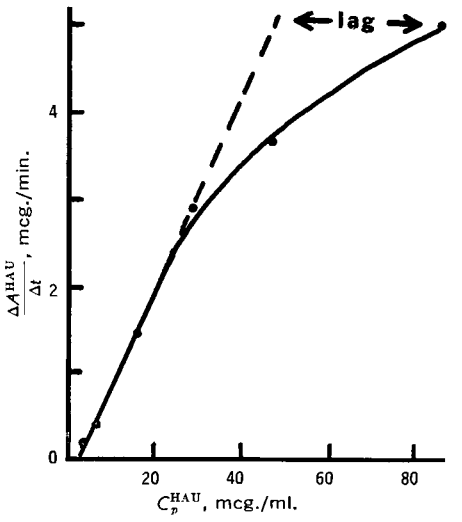


Figure 4—*Plot of excretion rate* versus *plasma concentration to obtain clearance after intravenous administration of 100 mg. hippuric acid to Rabbit 3. Slope* = $(\dot{V}_{cl})_{ex.}$ = *107 ml./min.*

[6] Beckman DB.
[7] Packard Tri-Carb.
[8] New England Nuclear.

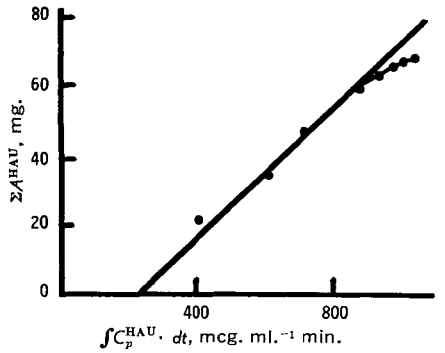


Figure 5—*Plot of* ΣA^{HAU} *against* $\int C_p^{HAU} \cdot dt$ *to obtain the clearance of hippuric acid from urinary excretion data after intravenous administration of 100 mg. hippurate to Rabbit 3. Slope* = $(\dot{V}_{cl})_{ex.}$ = *95 ml. min.*$^{-1}$.

β, the coefficients A and B, and other pharmacokinetic parameters (Tables I and II).

With reference to Table I, it is noteworthy to recall that hippuric acid is excreted without further metabolism. Therefore, k_{el} represents the excretion rate constant. Whether expressed in terms of $(\dot{V}_{cl})_{pl}$ or k_{el}, it is clear that the difference observed in these animals is in the difference in the ability of the kidney to handle the excretion. Serial urine samples were obtained from Rabbit 3, which had an indwelling Foley catheter in the bladder. Large amounts of hippuric acid were observed in the urine as early as 5 min. after administration of the compound. The rate of urinary excretion paralleled plasma concentrations after an initial lag period (Fig. 3). The clearance was calculated from plasma data by the area method. Clearance was also obtained from urinary data, from the plot of rate of excretion of hippuric acid against plasma concentrations of hippuric acid with $(\dot{V}_{cl})_{ex.}$ being the slope (Fig. 4). Another computation of $(\dot{V}_{cl})_{pl}$ from excretion data is from the plot of the integrals of rate of excretion and plasma concentration, ΣA^{HAU} and $\int_0^t C_p^{HAU} \cdot dt$, respectively (Fig. 5). This method is less sensitive to errors from incomplete urine collection, and there is no correction for midpoint plasma concentrations. The clearances calculated by the different methods are shown in Table III. It can be seen that $(\dot{V}_{cl})_{pl}$ of 114 ml./min. is higher than $(\dot{V}_{cl})_{ex.}$ calculated from either the rate plot (107 ml./min.) or the integrals plot (95 ml./min.). The values obtained by the latter two methods of calculation have an inherent error due to the lag time which exists before urine excretion parallels plasma concentration. The clearance values for hippuric acid are much higher than inulin clearance but approach those of ^{14}C-labeled *p*-aminohippuric acid, which averages 105 ml./min. in rabbits (18). Therefore, the clearance of hippuric acid approximates renal plasma flow at high plasma concentrations of greater than 5 mcg./ml. At low plasma concentrations, there is a flection in the curve in both the rate plot and the integrals plot (Figs. 5 and 6) which is possibly due to tubular reabsorption of the compound.

Table III—Comparison of Hippurate Clearances Obtained by Different Methods of Calculation[a]

Method of Calculation	Area Method: Intravenous Data	Urinary Excretion Data *a*	Urinary Excretion Data *b*
Equation	$(\dot{V}_{cl})_{pl}$ = dose/area	$(\dot{V}_{cl})_{ex.} - \frac{dA}{dt} \Big/ C_p$	$(V_{cl})_{ex.} = \frac{(\Sigma A)t}{\int_0^t C_p dt}$
Clearance, ml./min.	114	107	95

[a] Using Rabbit 3.

The results of benzoic acid and ^{14}C-labeled hippuric acid infusion into one rabbit are shown in Figs. 6–8. The rate of excretion of ^{14}C-labeled hippuric acid was ostensibly constant throughout the experiment (Fig. 6). Fluctuations of ^{14}C-labeled hippuric acid excretion paralleled those of total excretion of hippuric acid and was probably due to fluctuations in renal plasma flow or urine flow. The urine pH was relatively constant, ranging from 5.0 to 5.65. Urine flow rate was fairly constant. Slight increases were observed during the higher infusion rates and could have been due to either the increased volume of infusion or high amounts of salt in the urine. The renal clearance of hippuric acid in these animals ranged from 20 to 58 ml./min. These values are generally lower than the clearances obtained after intravenous bolus injections but were constant throughout the infusion period.

As part of the surgical procedure, ureteral catheters 3–4 times the length of the ureter were placed in the animals. The internal diameter of the synthetic catheter was smaller than its natural counterpart. The increased length and smaller diameter supply a substantial resistance to flow which builds up in the catheter and can result in low clearance values. The rabbit is also very responsive to hemodynamic changes caused by surgical trauma. This did not affect the primary purpose of this investigation, which was to observe the metabolism of the kidney. At the higher infusion rates of benzoic acid, the renal clearance of hippuric acid was appar-

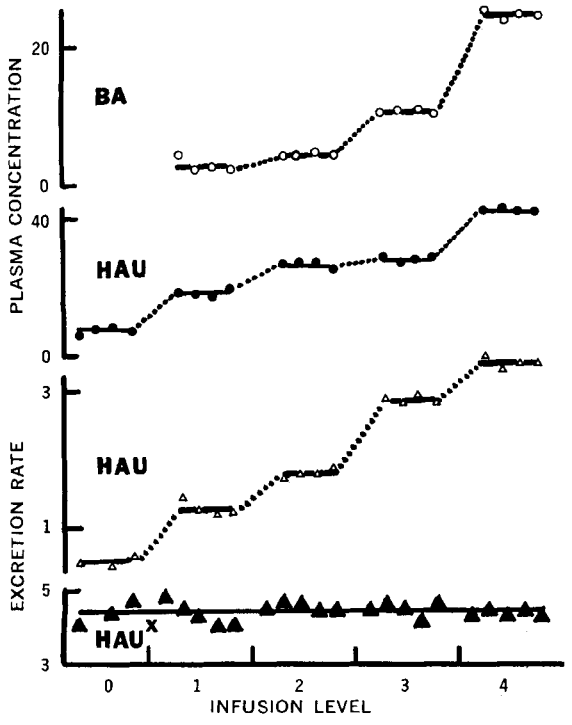


Figure 6—*Plasma concentrations and urinary excretion rates during sequential hippurate and benzoate infusion in Rabbit S3. Key:* ●, C_p^{HAU} *(mcg. ml.*$^{-1}$*);* ○, C_p^{BA} *(mcg. ml.*$^{-1}$*);* Δ, $\Delta A^{HAU}/\Delta t$ *(mg. min.*$^{-1}$*); and* ▲, $\Delta A^{HAU*}/\Delta t$ *(d.p.m.* × 10^4 *min.*$^{-1}$*). Each solid line and each broken line represent a 1-hr. interval.*

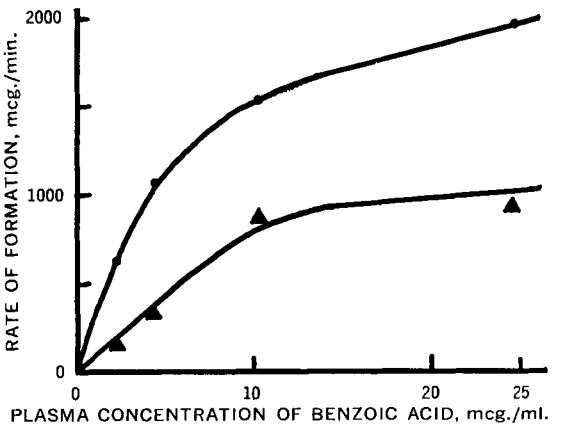


Figure 7—*Rate of hippurate formation at varying plasma concentrations of benzoic acid in Rabbit S3. Key:* ●, *liver; and* ▲, *kidney.*

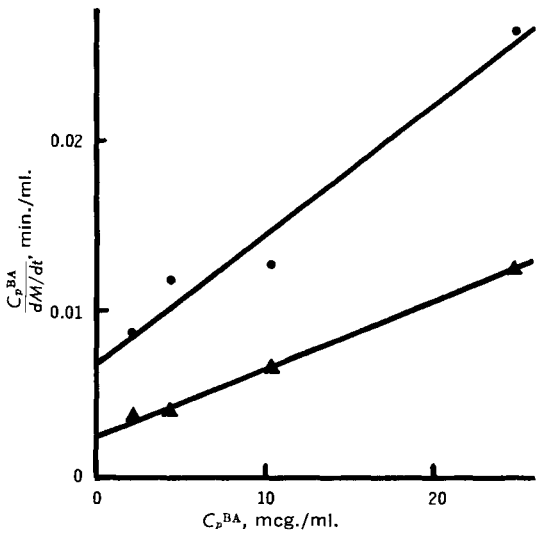


Figure 8—*Woolf plot of* S/V versus S *for liver and kidney enzyme systems during infusion of benzoic acid in Rabbit S3. Key:* ▲, *liver; and* ●, *kidney.*

ently constant over the infusion period, indicating the constant rate of metabolism in the kidney. The rate of renal metabolism and total body metabolism appears to be capacity limited when plasma concentrations of benzoic acid are high (Fig. 7). The curves for a plot of rate of metabolism against plasma concentration of benzoic acid resemble those of typical enzyme kinetics. Such data may be plotted according to one of the linearized forms of the Michaelis–Menten equation. The data were plotted according to the method of Woolf (19) and resulted in the regression lines noted in Fig. 8. The tabulated regression coefficients are listed in Table IV, where the K_m and $V_{max.}$ values are also shown. There is considerable variation in the K_m and $V_{max.}$ for both enzyme systems: of the liver and of the kidney. The fraction of the dose metabolized by the liver or the kidney also showed large variations. The fractional metabolism in the kidney ranged from 21 to 88% of the total body metabolism. The fraction of the dose metabolized as hippuric acid by all systems decreased as the dose was increased. The appropriate data are tabulated in Table V. The maximum rates of kidney metabolism and of all participating systems were calulated for the three animals reported previously (Table VI). The range was 93–300 mg./hr. Bray *et al.* (13) also found a large variation in the maximum rate of metabolism within animals of the same species, with an average maximum rate of 147 mg./hr. The monkey, on the other hand, was found to metabolize benzoic acid only in the liver. The ^{14}C-labeled hippuric acid clearance of 150 ml./min. did not increase when benzoic acid was administered. Renal plasma flow in the Rhesus monkey is between 93 and 143 ml./min. (20), so clearance of hippuric acid approaches renal plasma flow in this species of animal.

Metabolism can be capacity limited by one of several events. In an enzyme system, a specific enzyme combines with a substrate to give an end-product. In some conjugations, *e.g.*, glycine conjugation, a secondary substrate is required. If substrate concentration is sufficiently high that all the enzyme is bound as ES complex, *i.e.*, $S \gg K_m$, then the limiting factor is the concentration of free enzyme itself. In the conjugation of benzoic acid with glycine, the rate-limiting factor is the limited availability of the secondary substrate, glycine (21). The rate of conjugation of benzoic acid with glycine is very rapid and reaches a limiting rate only at high concentrations of benzoic acid. Krüger-Thiemer and Levine (22) showed, by analog computer simulation, that a limitation by any of the above events would result in Michaelis–Menten kinetics indistinguishable kinetically from one another.

The reported experiments were done in the whole animal, and the kinetics of metabolism appear to follow the Michaelis–Menten equation. The observed kinetics are undoubtedly the net result of more than one enzyme system, including the effects of enzyme systems involved in transport through cell membranes. These studies were also performed using the intravenous route of administration, and the effect of passage through the GI wall with the subsequent first passage through the liver was therefore eliminated. Different results may be expected when drugs are given *via* the oral route. Metabolism during passage through the intestinal mucosa or during passage through the liver may result in a lower fractional metabolism by the kidney. Furthermore, no attempt was made in these studies to isolate the rate-limiting event. As noted before, the metabolism of benzoic acid requires a quick turnover of glycine which may be the rate-limiting event in benzoic acid metabolism.

These studies showed clearly that the kidney makes an appreciable contribution to the total body metabolism of benzoic acid in the rabbit. Where kidney malfunction exists, it is possible that the liver or other metabolizing enzymes undergo rapid induction, thereby minimizing the potential for toxic response. The authors are not aware of detailed studies of this phenomenon, which warrants further examination. Frequently, pharmacokinetic analyses are attempted from urinary excretion data, on the presumption that the excretion pattern of the drug and its metabolites essentially mirror their time course in the blood. If kidney metabolism takes place, this assumption would not hold for the metabolite nor for the parent drug. The rate of disappearance of the metabolite from the blood and its rate of appearance in the urine will differ significantly from one another. Estimation of the volume of distribution of the metabolite or its plasma clearance from plasma and urinary excretion data assuming no kidney metabolism occurs will

Table IV—Data Obtained from the Woolf Plot of the Michaelis–Menten Equation for Formation of Hippuric Acid by the Liver and Kidney in Rabbits[a]

Rabbit Number	Metabolizing Organ	K_m, mcg./ml.	$V_{max.}$, mcg. min.$^{-1}$	r^b
S2	Liver	16.0	625	0.99
	Kidney	2.9	917	0.99
S3	Liver	6.0	2500	0.99
	Kidney	8.8	1299	0.97
S7	Liver	25.6	2564	0.95
	Kidney	17.2	2439	0.91

[a] There is no completely satisfactory method of weighting data treated by the Woolf procedure since there are two variables, *S* and *V*. Therefore, no attempt was made to weight the data. [b] Correlation coefficient from the linear regression line.

Table V—Fractional Metabolism of Benzoic Acid by Specific Organs and by Total Body Metabolic Sites at Various Plasma Concentrations

Rabbit Number	C_p Benzoic Acid[a]	Percent Hippuric Acid —Formed in— Kidney	Liver	Percent Dose Excreted as Hippuric Acid
S2	3.6	80.6	19.4	100
	2.6	78.5	21.5	100
	13.0	87.9	12.1	56.5
	20.5	69.2	30.8	55.8
S3	2.24	21.1	79.9	96.8
	4.3	25.2	74.8	86.7
	10.3	35.6	64.4	79.5
	24.7	31.9	68.1	70.0
S7	4.0	63.6	36.4	107.9
	10.2	49.0	51.0	98.0
	13.0	53.5	46.5	63.7
	23.6	55.2	44.8	61.1

[a] Steady-state plasma concentrations of benzoic acid.

Table VI—Maximum Rates of Conversion of Benzoic Acid to Hippuric Acid by the Kidney and Liver in Rabbits

Rabbit Number	$(V_{max.})^{kidney}$, mg. hr.$^{-1}$	$(V_{max.})^{liver}$, mg. hr.$^{-1}$
S2	55	93
S3	80	228
S7	146	300

be seriously in error. *p*-Aminohippuric acid is used routinely for the evaluation of kidney function and effective renal blood flow. It was noticed that in some species consistently low clearance values were being obtained, and this finding was traced to the metabolism occurring in the kidney (23).

In the clinical situation, it may be important to know what types of drugs are metabolized in the kidney and the extent to which this occurs. One should be aware of the possible effects of administering these drugs to anephric patients or to patients suffering from any form of renal failure. These patients often have other accompanying ailments and may be treated with a large variety of drugs. Accumulation of drugs and toxic reactions may occur if the kidneys no longer contribute to metabolism.

REFERENCES

(1) M. Dixon and E. C. Webb, "Enzymes," Longmans, Green, London, England, 1958.

(2) P. D. Boyer, H. Landy, and K. Myrbäck, "The Enzymes," vol. 4, 2nd ed., Academic, New York, N. Y., and London, England, 1960.

(3) R. T. Williams, "Detoxication Mechanisms," 2nd ed., Wiley, New York, N. Y., 1959.

(4) H. Remmer, *Ann. Rev. Pharmacol.*, **5**, 405(1965).

(5) W. H. Barr and S. Riegelman, *J. Pharm. Sci.*, **59**, 154(1970).

(6) *Ibid.*, **59**, 164(1970).

(7) W. C. Hülsmann and L. W. Statius van Eps, *Clin. Chim. Acta*, **15**, 233(1967).

(8) A. J. Quick, *J. Biol. Chem.*, **96**, 73(1932).

(9) P. K. Knoefel, K. C. Huang, and A. Despopoulos, *Amer. J. Physiol.*, **196**, 1224(1959).

(10) G. Bunge and O. Schmiedeberg, *Arch. Exp. Pathol. Pharmakol.*, **6**, 233(1876–1877).

(11) W. Kochs, *Arch. Ges. Physiol.*, **20**, 64(1874).

(12) I. Snapper, A. Grünbaum, and J. Neuberg, *Biochemistry*, **145**, 10(1924).

(13) H. G. Bray, W. V. Thorpe, and K. White, *Biochem. J.*, **48**, 88(1951).

(14) N. G. Heatley, *Ant. Med. Clin. Ther.*, **2**, 33(1956).

(15) T. R. Tephly, R. E. Parks, Jr., and G. J. Mannering, *J. Pharmacol. Exp. Ther.*, **143**, 292(1964).

(16) C. J. Umberger and F. F. Fiorese, *Clin. Chem.*, **9**, 91(1963).

(17) "Liquid Scintillation Counting," C. G. Bell, Jr., and F. N. Hayes, Eds., Pergamon, New York, N. Y., 1958, p. 88.

(18) B. Josephson, A. Grieg, G. Kakossaios, and J. Kallas, *Acta Physiol. Scand.*, **30**, 11(1953).

(19) B. Woolf, quoted in "Allgemeine Chemie der Enzyme," by J. B. S. Haldane and K. G. Stern, Steinkopf-Verlag, Dresden, Germany, 1932, p. 119.

(20) R. P. Forsyth, A. S. Nies, F. Wyler, J. Neutze, and K. L. Melmon, *J. Appl. Physiol.*, **25**, 736(1968).

(21) A. J. Quick, *J. Biol. Chem.*, **92**, 65(1931).

(22) E. Krüger-Thiemer and R. R. Levine, *Arzneim.-Forsch.*, **18**, 1575(1968).

(23) B. P. Setchell and E. Blanch, *Nature*, **189**, 230(1961).

ACKNOWLEDGMENTS AND ADDRESSES

Received July 14, 1971, from the *Department of Pharmacy, School of Pharmacy, University of California, San Francisco, CA 94122*

Accepted for publication November 18, 1971.

Abstracted from a dissertation submitted by Suk Han Wan to the Graduate Division, University of California, San Francisco, in partial fulfillment of the Doctor of Philosophy degree requirements.

Supported in part by a grant-in-aid from the Academic Senate Committee on Research, San Francisco Division, University of California; a grant-in-aid from the Patent Board, University of California; and Grant NIGMS 16496 from the National Institutes of Health, U. S. Public Health Service, Bethesda, MD 20014

The authors thank Dr. Ralph P. Forsyth for advice and assistance with the monkey used in the study.

* Present address: Department of Clinical Pharmacology, University of Kansas Medical Center, Kansas City, KS 66103

▲ To whom inquiries should be directed.

Renal Contribution to Overall Metabolism of Drugs II: Biotransformation of Salicylic Acid to Salicyluric Acid

SUK HAN WAN* and SIDNEY RIEGELMAN▲

Abstract □ The biotransformation of salicylic acid to salicyluric acid in the Rhesus monkey was studied, and the contributions made by the kidney to the metabolic process were estimated from analysis of clearance data.

Keyphrases □ Salicylic acid biotransformation to salicyluric acid—renal metabolism contribution, renal and apparent clearances, Rhesus monkey □ Salicyluric acid from salicylic acid biotransformation—renal metabolism contribution, renal and apparent clearances, Rhesus monkey □ Renal metabolism—biotransformation of salicylic acid to salicyluric acid, renal and apparent clearances, Rhesus monkey □ Biotransformation kinetics—salicylic acid to salicyluric acid, renal metabolism contribution, Rhesus monkey

It was shown in a previous paper (1) that the conversion of benzoic acid to hippuric acid occurs in the kidney of the rabbit. This paper presents a similar study of salicylic acid metabolism to salicyluric acid in the monkey. After reviewing the literature, it was found that none of the laboratory animals have been reported to metabolize salicylic acid (SA) to salicyluric acid (SAU) in quantities similar to those of man. No information was available on the metabolism of salicylic acid in the Rhesus monkey, a useful experimental animal. A preliminary study was undertaken to determine the fate of salicylic acid in this animal. At a dose equivalent to a 625-mg. dose/70-kg. man, it was found that the Rhesus monkey excreted high amounts of salicyluric