Species Differences in the Urinary Excretion of the Novel Primary Amine Conjugate: Tocainide Carbamoyl *O*-β-D-Glucuronide

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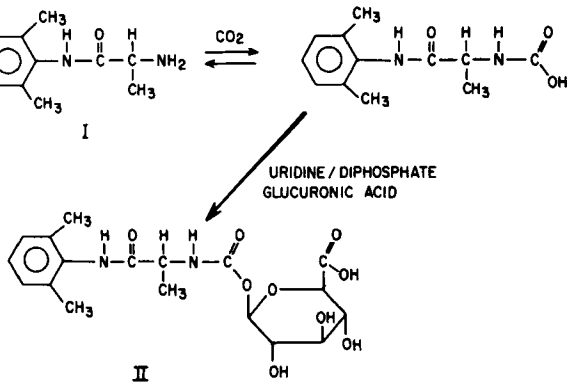
Abstract □ The metabolism of the antiarrhythmic drug tocainide (I) has been shown previously to occur *via* a novel pathway involving the addition of carbon dioxide to the primary amine nitrogen of I followed by conjugation with glucuronic acid. The product of this reaction, tocainide carbamoyl *O*-β-D-glucuronide (II), the principal metabolite of I in humans, has been found to cyclize under strongly basic conditions to form 3-(2,6-xylyl)-5-methylhydantoin (III). Thus, evidence for the existence of II can be obtained by two different procedures: conversion of II to III in the presence of strong base and by hydrolysis of II with β-glucuronidase. The principal purpose of the present investigation was to identify suitable species for studies of the mechanism involved in the formation of II, as well as to find an animal model suitable for toxicological evaluation of tocainide and structurally related compounds. Eight animal species were examined to identify those capable of metabolizing I into II. The fraction of an intraperitoneal dose excreted in urine as II was estimated by measurement of tocainide released by β-glucuronidase mediated hydrolysis of urine and by the quantitation of III formed after alkalinization of urine samples. Urinary recovery of unchanged drug ranged from 9.5% of the dose in the gerbil to 48.7% in the cat. The percent of the dose excreted in urine as acid hydrolyzable conjugates ranged from <1% in the gerbil to a mean of 13% in the rabbit. Guinea pigs, dogs, cats, rabbits, and pigtail monkeys excreted amounts of II ranging from 0.2 to 2.4% of the dose. Thus, none of the species appeared to be a suitable model for the study of the mechanism of formation of II because of the quantitative insignificance of this pathway.

Keyphrases □ Excretion—urinary, novel primary amine conjugate, tocainide carbamoyl *O*-β-D-glucuronide, species differences □ Tocainide carbamoyl *O*-β-D-glucuronide—species differences in urinary excretion, novel primary amine conjugate □ Metabolism—tocainide carbamoyl *O*-β-D-glucuronide, species differences in urinary excretion

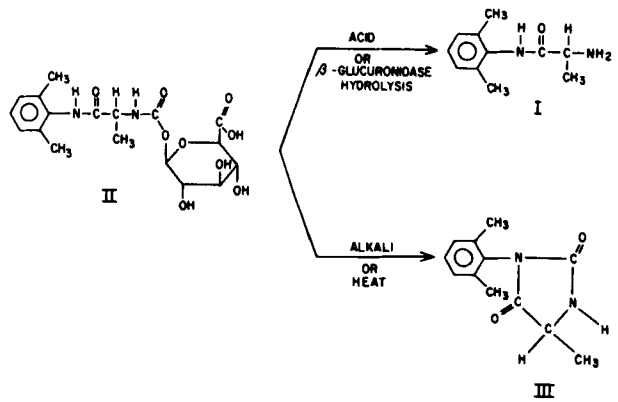
Tocainide (I) is an antiarrhythmic drug undergoing extensive clinical evaluation (1, 2). Substantial evidence suggests that the major pathway for the metabolism of I in humans is formation of a carbaminic acid from tocainide and carbon dioxide followed by conjugation with glucuronic acid (Scheme I). In normal healthy humans ~25–40% of a dose of I is excreted in urine as tocainide carbamoyl *O*-β-D-glucuronide (II) and another 25–40% is excreted as unchanged I (3). In the Wistar rat, urinary excretion of administered I was found to be dose-dependent with 15–50% of a dose excreted as I (4) and <10% as II (5, 6). Other mixed function oxidase products may account for the remainder of the dose in both species (5–7).



Scheme I

Formation of a carbaminic acid from an amine and carbon dioxide followed by conjugation with glucuronic acid is a metabolic pathway that has been reported only for tocainide (8). However, precedent exists for the formation of a glucuronide conjugate of a carbaminic acid in that formation of a carbamoyl glucuronide after administration of *p*-chlorophenyl cyanamide to rabbits has been reported (9). Furthermore, there is overwhelming evidence that covalent bonding of carbon dioxide to amine functions of hemoglobin (forming a carbaminic acid) is the principal mechanism of carbon dioxide transport in blood (10). Carbaminic acid formation followed by glucuronide conjugation may be an important metabolic pathway for primary amine drugs other than tocainide. However, substantial difficulties in isolation and detection may have prevented previous discovery of this pathway because acids of this type readily hydrolyze to parent drug and carbon dioxide (8). Compound II was discovered because the unique structural characteristics of I allowed facile cyclization of II to form 3-(2,6-xylyl)-5-methylhydantoin (III) under alkaline conditions (Scheme II). Since acid or β-glucuronidase mediated hydrolysis of II yields I (8), estimation of hydantoin (III) formed by alkalinization and the quantitation of free drug released by β-glucuronidase provide reliable independent methods to determine the concentration of this novel glucuronide in biological fluids[1].

Animal toxicological studies intended for extrapolation



Scheme II

[1] R. A. Ronfeld and coworkers have established that II is quantitatively converted to III; unpublished observation.

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Figure 1—*Urinary recovery of unchanged tocainide (□), total acid hydrolyzable conjugates (▨) and tocainide carbamoyl O-β-D-glucuronide (■). The figure presented for tocainide carbamoyl O-β-D-glucuronide is the estimate of III formed from II.*

to humans must be conducted in species that have qualitative (and preferably quantitative) metabolic profiles similar to that observed in humans. Furthermore, the identification of interspecies differences in the capacity to carry out specific types of drug biotransformation (*i.e.*, acetylation, formation of ester glucuronides, *etc.*) has been of fundamental concern to pharmacologists for decades, since such information can sometimes explain interspecies differences in the toxicology and pharmacology of drugs (11, 12). Thus, after the discovery of the novel metabolic pathway which results in the conversion of tocainide to II, it was appropriate to determine which common laboratory animal species could also form this type of glucuronide. In all, eight animal species received intraperitoneal doses of I to determine if any of them could be used effectively to study this metabolic process.

EXPERIMENTAL

Chemicals—Tocainide (2-amino-2′,6′-propionoxylidide), glycine xylidide, bupivacaine (prepared as the hydrochloride salts), and 3-(2,6-xylyl)-5-methylhydantoin were provided by the same source[2]. Heptafluorobutyrylimidazole[3], mollusk β-glucuronidase[4] (lyophilized; 0.7% arylsulfatase), and saccharo-1,4-lactone[4] were obtained from commercial sources. All solvents were spectral or high-pressure liquid chromatographic (HPLC) grade.

Instrumentation—GLC was performed on dual column gas chromatographs[5] equipped with flame ionization detectors (FID). The GLC conditions were: glass columns, 1.8 m × 2-mm i.d. packed with 3% OV-17, 10% OV-17, or 3% OV-101 on 80–100 mesh chromosorb W-HP[6]. Helium, hydrogen, and air flow rates were 30, 30, and 300 ml/min, respectively. Injector and detector temperatures were 250°. Column temperatures for the various assays will be described.

Extraction and Preextraction Treatment of Urine Samples—The extraction procedures used to quantitate unchanged tocainide and tocainide released from conjugates in urine have been described previously. The following is a summary of the information provided by each procedure (8).

The quantitative estimation of I extracted from alkalinized urine allows the determination of the concentration of unchanged I. Acid hydrolysis followed by this standard extraction procedure yields the concentration of unchanged I plus acid labile conjugates.

Incubation with β-glucuronidase followed by the standard extraction procedure yields the sum of unchanged I, glucuronide conjugates, and other conjugates released by commercial mollusk β-glucuronidase preparations.

Incubation of urine samples with the mollusk β-glucuronidase preparation described above in the presence of saccharo-1,4-lactone (a selective β-glucuronidase inhibitor) followed by the standard extraction procedure yields an estimate of the sum of unchanged I plus conjugates of I other than glucuronides (presumably sulfates).

Quantitative Estimation of I in Urine—After the urine samples were extracted using the procedures described above, tocainide concentrations were determined using the GLC method described previously (8).

Quantitative Estimation of III in Urine—The following extraction procedures were used to quantitate tocainide carbamoyl *O*-β-D-glucuronide by measuring III formed by base catalyzed cyclization.

One milliliter of 1 *N* NaOH, 0.25 ml of urine, 0.25 ml of bupivacaine solution (10 μg/ml in deionized water, used as an internal standard), and 3.0 ml of methylene chloride were added to 15-ml centrifuge tubes. The tubes were shaken, centrifuged, and the aqueous layer removed and discarded. The organic phase was evaporated at 50°, reconstituted with 20 μl of methylene chloride, and 1–2 μl was injected into a gas chromatograph (3% OV-17) at a column temperature of 250°. When a peak with retention time and shape identical to III was found, the same samples were reassayed on a 3% OV-101 column (210°) to confirm the observations made in the standard assay. This procedure quantitated III formed *in vitro* from II plus any III which may have formed *in vivo*. A second extraction procedure quantitated III formed *in vivo*, *i.e.*, III that existed prior to alkalinization. This procedure was identical to the above method, except that 1.0 ml of 0.2 *M* acetate buffer (pH 5) was substituted for 1.0 ml of 1.0 *N* NaOH.

Additional Experimental Control Procedures—Predose urine samples were assayed before and after acid or enzymatic hydrolysis to ensure the absence of interfering compounds in urine.

Table I—Physical Characteristics of Animal Species Studied

No. of Animals Studied	Species	Strain	Sex	Weight Range, kg
3	Rat	Sprague-Dawley	male	0.350–0.360
3	Rat	Gunn	male	0.190–0.210
3	Rat	Wistar, inbred	male	0.190–0.210
4	Mouse	Brown Bal	female	0.022–0.032
3	Guinea Pig	Mixed breed	male	0.740–0.850
2	Gerbil	Mixed breed	male	0.034–0.041
4	Dog	Mixed breed	males	8.6–12.7
1	Cat	Siamese	male	
2		Mixed breed	1 female; 1 male	3.4–5.4
3	Rabbit	New Zealand White	male	3.6–3.8
1	Monkey	African Green	female	2.7
2	Monkey	Pigtail	female	5.0–8.6

[2] Astra Pharmaceutical Products, Inc., Framingham, Mass.
[3] Pierce Chemical Co., Rockford, Ill.
[4] Calbiochem, La Jolla, Calif.
[5] Varian model 2400 Aerograph, Palo Alto, Calif.
[6] Alltech Associates, Arlington Heights, Ill.

Table II—Urinary Recovery of Tocainide and its Metabolites in Various Species[a]

Species (strain)	Unchanged Tocainide	Tocainide Carbamoyl O-β-D-Glucuronide: Measured as I[b], Released Enzymatically	Tocainide Carbamoyl O-β-D-Glucuronide: Measured as III, Formed upon Alkalinization	Tocainide Carbamoyl O-β-D-Glucuronide: Total Acid Hydrolyzable Conjugates
Rat				
Sprague-Dawley	20.3(17–22)	ND[c]	ND[d]	4.7(3–6)
Gunn	18.3(10–27)	ND[c]	ND[d]	4.8(2–9)
Wistar	30.3(24–34)	ND[c]	ND[d,e]	10.0(7–15)
Mouse	38.5(33–44)	—[f]	ND[d]	1.0(0–3)
Guinea Pig	36.3(30–42)	2.3(2–3)	1.0(0.9–1.1)	5.4(4–8)
Gerbil	9.5(7–12)	—[f]	ND[d]	0.55(0.3–1)
Dog	21.2(11–32)	1.5(1–2)	1.4(0.4–2.2)	4.9(2–8)
Cat	48.7(41–54)	0.3(0–1)	0.2(0.0–0.3)	11(5–20)
Rabbit	17.6(10–27)	1.4(0–3)	1.2(1.0–1.3)	13(9–29)
Monkey				
African Green	47.4	1.4	ND[d]	7.9 NA
Pigtail	44.6(39–50)	0.1(0–0.1)	2.4(0.8–4.0)	3.9(2–6)

[a] Expressed as percent of the dose excreted in urine; on a molar basis. Numbers in parentheses give the range of observed values. [b] Glucuronide conjugates were quantitated by measuring the difference between tocainide released by β-glucuronidase and β-glucuronidase plus saccharo-1,4-lactone. [c] Not detected. Based on the limited sensitivity of the GLC procedure utilized, the minimum percentage dose which could have been detected was between 1.0 and 1.5%. [d] Not detected. The minimum percentage of dose detectable was 0.02%. [e] The presence of III in urine of tocainide treated (intraperitoneally and orally in these species) Wistar-Vancouver rats has been noted (14, 15) using a highly sensitive qualitative electron-capture GC assay with confirmation by GLC–mass spectrometry. The quantitative study described here using GLC with FID failed to confirm the presence of III, due either to limitations in detection or because of differences in the animals studied. [f] Enzymatic hydrolysis was not performed because acid hydrolysis studies indicated that ≤1% of the dose was excreted as conjugated tocainide.

Three coded unknown urine samples, prepared to contain concentrations of tocainide from 0 to 135 μg/ml, were assayed every experimental day by an analyst blinded to the tocainide concentration. An error of ≥10% caused data to be rejected and mandated the reassay of all samples from that day. Since urine pH has previously been shown to influence the urinary excretion of tocainide in humans (13), the pH of all urine samples was measured.

A check for bacterial breakdown of II was performed by diluting urine known to contain approximately equal amounts of I and II (obtained from humans who had received tocainide) with blank animal urine in a 1:1 ratio. Both this sample and the undiluted sample were left at room temperature for 24 hr and were then assayed. If the 1:1 dilution contained <55% of the tocainide concentration seen in the undiluted sample, no significant bacterial breakdown of II to I was considered to have occurred. However, if >55% of the undiluted sample's concentration was in the 1:1 dilution, the extra tocainide was considered to have been liberated by the breakdown of II. In such cases the data obtained for the urinary recovery of II were considered to be an underestimate and were not to be used for further data analysis.

If acid hydrolysis failed to show significant generation (<10% increase in the concentration of I in the urine) of I from conjugates, urine was not subject to treatment with β-glucuronidase. However, these urines were assayed for the presence of III before and after treatment with NaOH to rule out the presence of II.

Animal Study Design—Animals were given an injection of 10 mg/kg ip of tocainide hydrochloride after fasting for 24 hr. Fasting was continued for at least 24 hr following injection. Water was allowed *ad libitum* throughout the experiment. Animals were housed in plexiglass or stainless steel metabolism cages. Predose urine specimens were obtained and urine was also collected until at least 24 hr following dosing. Urine specimens were frozen at −20° until assayed. The weight, sex, and strain of the animals used in these studies are detailed in Table I.

RESULTS

The principal observations made in the various species are summarized in Fig. 1 and Table II. The dogs, rabbits, guinea pigs, pigtail monkeys, and cats synthesized and excreted tocainide carbamoyl O-β-D-glucuronide (Table II). The amounts were small but were readily detectable as III formed upon alkalinization of the urine sample. No evidence was obtained to suggest the presence of II in the urine of mice, gerbils, the African green monkeys, or in any of the three strains of rats (*i.e.*, III was not observed upon alkalinization of the urine samples, and little or no I was released by β-glucuronidase mediated hydrolysis). All available evidence suggests that the III which was observed in the other species was formed from II; *i.e.*, no III was found in samples that had not been alkalinized.

The details of the urinary recovery studies are presented in Table II. The percent of the dose excreted as unchanged tocainide ranged from 9.5% in gerbils to 48.7% in cats. The percent of the dose recovered as II was ≤2.4% in all species studied (Table II). Total acid hydrolyzable conjugates accounted for ≤13% of the dose in all species. No evidence of bacterial breakdown of II to I by human or animal urine was observed. Furthermore, no trend in the urinary excretion of I or II was seen as a function of urine pH (data not presented).

DISCUSSION

A principal purpose of the present investigation was to identify a common laboratory animal that could biotransform tocainide into II. In an earlier investigation of tocainide metabolism (using GLC–mass spectrometry and electron capture GLC analysis), the presence of trace amounts of III in the urine of Wistar rats was reported prior to alkalinization (14, 15). Thus, it seemed reasonable to examine a number of rodents for their capacity to form II. However, the results from the present study suggested that among common rodents, only the guinea pig could form amounts of II measurable using FID detection methods. It was also observed that rabbits, cats, pigtail monkeys, and dogs excreted small amounts of II (~1% of the dose), but none approached the amount found in human urine (25–40% of dose). These results indicate that the enzyme(s) needed to produce II may be widespread in the animal kingdom, but the capacity to produce substantial amounts of II has only been documented in humans, following oral administration (3, 7, 8). Alternative explanations have not been ruled out. For example, the various animal species studied may have synthesized large amounts of II but eliminated it *via* an alternative pathway such as biliary excretion, or II may be formed by gut flora, and hence (in the absence of biliary excretion) could only be formed following oral administration. At present, no evidence exists suggesting the biliary excretion of II or metabolism of I to II by gut flora. Thus, it is reasonable to assume that none of the animals studied is an adequate model of humans. It is important to find a species that produces substantial amounts of II, both for toxicological studies and for investigations into the fundamental nature of this potentially widespread biochemical transformation. The toxicity, mechanism of formation, and factors that influence the rate of formation of II remain unknown. Such investigations will be greatly facilitated when an appropriate model (whole animal) study system has been identified.

From a toxicologic standpoint, the demonstrated lability of II, plus the large amount formed in humans raise questions as to whether II may play a role in the toxicity of tocainide. A recent publication noted that some patients exhibit allergic reactions (*i.e.*, rashes) when treated with tocainide (16). These reactions may be due to II. Though toxic glucuronide metabolites have only been recently recognized, two have been identified that irreversibly bind (covalently bond) to proteins (17) and nucleic acids (18). These reactive compounds share some structural analogy with tocainide carbamoyl O-β-D-glucuronide. However, it would be premature to attribute any of tocainide's toxicity to II or any of its other metabolites (5, 7).

Independent of the toxicological considerations, it is of fundamental interest to identify species producing carbamate glucuronides from amines, since such conjugates represent a category of drug metabolite which has been discovered only recently (8).

While precedents exist for enzymatic carbamylation of amine functional groups (10) and glucuronidation of carbamates (9), little is known of the mechanisms involved in the sequential carbamylation and glucuronidation of primary amine drugs. After an appropriate animal model has been identified, studies to ascertain these mechanisms will be possible. The elucidation of these mechanisms may substantially expand understanding of the glucuronidation process.

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In Vitro Skin Evaporation and Penetration Characteristics of Mosquito Repellents

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Abstract □ An *in vitro* apparatus was used to study mosquito repellent evaporation and penetration characteristics with skin. The mosquito repellents 2-ethyl-1,3-hexanediol, *N,N*-diethyl-*m*-toluamide, *N,N*-diethyl-*p*-toluamide, 1-(butylsulfonyl)hexahydro-1*H*-azepine, and *N,N'*-dicyclohexamethyleneurea were studied. *In vitro* repellent duration, calculated from repellent evaporation rates, was compared to *in vivo* duration at the same dose (0.3 mg/cm^2) to assess the validity of the model. *In vitro* durations for 2-ethyl-1,3-hexanediol, *N,N*-diethyl-*m*-toluamide, *N,N*-diethyl-*p*-toluamide, and *N,N'*-dicyclohexamethyleneurea correlated with *in vivo* durations ($r^2 = 0.94$), although *in vitro* duration was longer than *in vivo* duration. 1-(Butylsulfonyl)hexahydro-1*H*-azepine, which had the longest *in vivo* duration, had an *in vitro* duration that exceeded the test period (12 hr). The 0–12-hr *in vitro* percutaneous penetration correlated with corresponding data available from *in vivo* studies.

Keyphrases □ Mosquito repellents—*in vitro* skin evaporation, penetration □ Evaporation—*in vitro* skin penetration, mosquito repellents □ Penetration—*in vitro* skin evaporation, mosquito repellents

Evaporation of mosquito repellents from the skin surface and percutaneous penetration represent important modes of loss of mosquito repellents from the skin surface. Various estimates of the percutaneous penetration of mosquito repellents have been made (1–4). However, only one repellent (*N,N*-diethyl-*m*-toluamide) whose loss from the skin surface by evaporation and skin penetration has been quantified (5). The percentages of *in vitro* skin evaporation and percutaneous penetration of the following five mosquito repellents are reported in this paper: 2-ethyl-1,3-hexanediol (I), *N,N*-diethyl-*m*-toluamide (II), *N,N*-diethyl-*p*-toluamide (III), 1-(butylsulfonyl)hexahydro-1*H*-azepine (IV), and *N,N'*-dicyclohexamethyleneurea (IV). Two dose levels were used: a dose corresponding to a repellent's minimum effective dose against *Aedes aegypti* mosquitoes (6) and a dose of 0.3 mg/cm^2, which has been used to determine the effective duration of the repellents on the skin of humans (6).

The duration of steady-state evaporation rate of repellents from aluminum planchets has been compared with the duration of effectiveness of several mosquito repellents on the skin of humans (7). The findings suggest a possible relationship between evaporation rate from skin and repellent duration. In this report, this possible relationship was examined by computing the *in vitro* durations for each repellent from *in vitro* evaporation rates and comparing them to previously reported values for *in vivo* duration (6).

EXPERIMENTAL

Labeled Compounds—The following radiolabeled mosquito repellents were used: [1,3-^{14}C]2-ethyl-1,3-hexanediol(Ia)[1], specific activity, 6.06×10^4 dpm/μg; [carbonyl-^{14}C]*N,N*-diethyl-*m*-toluamide(IIIa) (8), specific activity, 1.15×10^4 dpm/μg; [carbonyl-^{14}C]*N,N*-diethyl-*m*-toluamide(IIa) (8), specific activity, 2.47×10^4 dpm/μg; 1-(butylsulfonyl)-[2,2'-^{14}C] hexahydro-1*H*-azepine(IVa), specific activity, 332 dpm/μg; and *N,N'*-[2,2'-^{14}C]dicyclohexamethyleneurea(Va), specific activity, 174 dpm/μg. For skin applications of I at the minimum effective dose and the 0.3 mg/cm^2 dose, cold I[3] was used to dilute the radiolabeled samples to give total radioactive doses of 0.02 and 0.14 μCi, respectively. For one replicate (skin No. A8478) of skin application of III at 0.3 mg/cm^2, cold III[4] was used to dilute the radiolabeled sample to give a final ra-

[1] New England Nuclear Corp., Boston, Mass.
[2] SRI International, Menlo Park, Calif.
[3] Niagara Chemical Division, FMC, Middleport, N.Y.
[4] Hercules, Inc., Wilmington, Del.

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