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Identification and Gas Chromatographic Determination of Some Carboxylic Acid Metabolites of *N,N*-Diethyl-*m*-toluamide in Rat Urine†

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Adult Wistar rats dosed intraperitoneally and topically (50 mg/kg) with *N,N*-diethyl-*m*-toluamide (**1**) excreted *m*-[(diethylamino)carbonyl]benzoic acid (**3**), *m*-[(ethylamino)carbonyl]benzoic acid (**6**), *m*-(aminocarbonyl)benzoic acid (**9**), and *m*-toluic acid (**10**) in their urine. These carboxylic acids were isolated by extraction of acidified urine samples with ethyl acetate and were detected as their methyl esters by GC and GC–MS. A quantitative analytical procedure, based on GC with a nitrogen–phosphorus detector, a capillary column of DB-1, and an internal standard of *m*-[(dipropylamino)carbonyl]benzoic acid (**13**), was developed to determine **3** and **6** in urine samples from treated rats. After 24 h, these major metabolites together accounted for 77% and 82% of the intraperitoneally administered dose in separate experiments with four male rats per experiment. In trials with **1** applied topically, **3** and **6** collectively represented 47% (males) and 49% (females) of the dose in 0–48-h urine collections. In the topical experiments, 5–6% of **1** was excreted unchanged.

N,N-Diethyl-*m*-toluamide (**1**), commonly known as deet, is used extensively as a topical insect repellent for protection against mosquitoes and other blood-sucking flies. It is applied ad libitum to the skin and clothing either neat or in alcohol-based sprays and other formulations. Despite a long history of use, published reports are scarce on the metabolism of **1** in man or animals [see the review by Robbins and Cherniack (1986)].

In experiments with rodents, the distribution and urinary excretion of radiolabeled **1** has been studied in mice (Blomquist et al., 1975; Blomquist and Thorsell, 1977; Lur'e et al., 1978) and rats (Lur'e et al., 1978; Moody et

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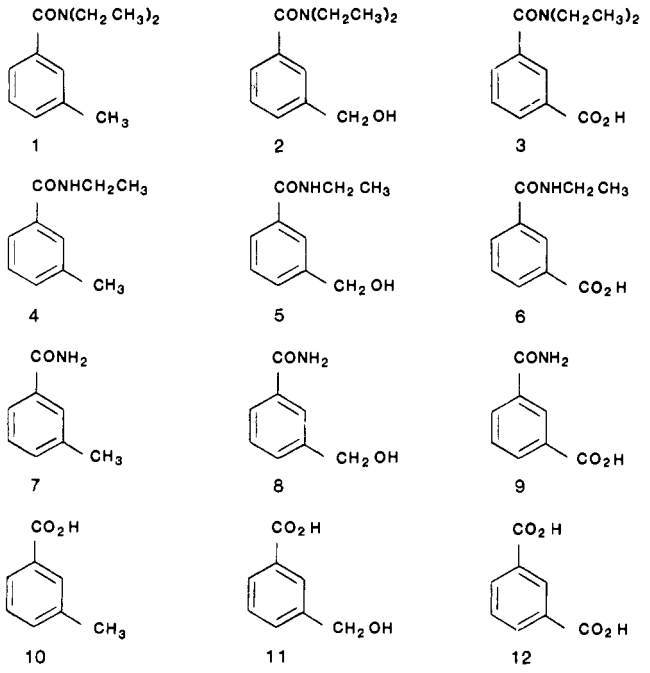

Figure 1. Structures of 1 and metabolites or potential metabolites of 1 that were characterized and utilized in this work.

al., 1989) but without metabolite identification. The purpose of this study was to identify and specifically determine some of the metabolites of 1 that were excreted in the urine of rats following intraperitoneal (ip) and topical dose administration.

During the course of this and earlier work, reference standards of 11 potential metabolites were obtained (Figure 1). Compounds **2–4** were proposed as urinary metabolites of 1 in man (Wu et al., 1979), whereas **2**, **4**, **5**, and **7** were identified as in vitro metabolites in fortified liver microsomes from phenobarbital-pretreated Wistar rats (Taylor, 1986).

EXPERIMENTAL PROCEDURES

Gas Chromatography (GC). Instrumentation and conditions used for flame ionization detection (FID) and nitrogen–phosphorus detection (NPD) have already been described (Taylor, 1983, 1986). For GC–FID (Hewlett-Packard 5830), a 30 m × 0.26 mm DB-1 fused silica column (0.25 μm film thickness; J and W Scientific) was used with helium as the carrier gas (20 psi). Splitless injections (30 s) were done with the initial oven temperature at 50 °C. After 1 min, the temperature rose at 25 °C/min to 125 °C, at 1 °C/min to 137 °C, at 3 °C/min to 224 °C, and then at 5 °C/min to 250 °C.

For GC–NPD (Hewlett-Packard 5890), a 30 m × 0.25 mm or a 30 m × 0.24 mm column of DB-1 was used, with 30-s splitless injections. After 1 min, the temperature rose from 70 °C at 25 °C/min to 150 °C, at 2 °C/min to 180 °C, and then at 4 °C/min to 250 °C. This instrument, equipped with a Hewlett-Packard 7673A automatic sampler, was used for all quantitative determinations.

Selected extracts from experiments 2–4 were also examined on a Hewlett-Packard 5890 instrument equipped with a FID and a SPB-5 column (30 m × 0.32 mm) from Supelco. The helium pressure (carrier gas) was 10.8 psi. Temperature programming was the same as for NPD. Retention times of authentic methyl esters were recorded on a column of DB-WAX (30 m × 0.24 mm), under the same conditions as for SPB-5 (Table I).

Capillary Gas Chromatography–Mass Spectrometry (GC–MS). A Hewlett-Packard 5985B instrument was used with a 30 m × 0.25 mm column of SPB-1. Samples of the standards and urinary extracts were injected splitless (30 s) with column temperature programming from 50 °C at 25 °C/min to 125 °C, at 1 °C/min to 137 °C, at 3 °C/min to 224 °C, and then at 5 °C/min to 240 °C. By use of a similar temperature program, several analyses were also done with another SPB-1 column (30 m × 0.32 mm) and with a 30 m × 0.25 mm column of DB-5.

Table I. Gas Chromatographic Retention Times of Methyl Ester Derivatives on Three Capillary Columns Obtained during GC–FID Analyses

compd	DB-1, min	SPB-5, min	DB-WAX, min
3	29.4	19.1	36.4
6	27.2	17.5	45.5
9	23.3	14.8	34.7
10	6.2	5.1	8.9
11	12.3	8.2	32.2
12	13.8	8.7	22.5
13	34.6	23.7	38.5

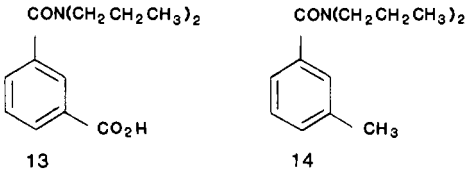

Figure 2. Structures of the internal standards that were synthesized and employed for gas chromatography.

Reference Standards. Samples of **2–6**, **8**, **9**, and **11** (Figure 1) and the internal standards (**13**, **14**; Figure 2) were synthesized from commercial starting materials. Details of their preparation and purification as well as elemental analysis, IR, ^{1}H NMR, and positive (isobutane) chemical ionization mass spectral data are given in the supplementary material. Compounds **7**, **10**, and **12** were purchased from Aldrich (Milwaukee, WI). Complete electron-impact (70 eV) mass spectral ions from GC–MS analysis of 1–14 are reported in Table 1 of the supplementary material.

Solvents. Methanol, methyl *tert*-butyl ether (MBE), and ethyl acetate were distilled in glass grade (OmniSolv, supplied by BDH Chemicals, Edmonton, AB, Canada). Water was distilled and further purified by a Barnstead NANOpure II system.

Analytical Solutions. Authentic samples of reference compounds were dissolved in methanol at a concentration of 100 μmol/10 mL. A more concentrated stock solution of **3** (500 μmol/10 mL) was used for spiking control urine at the 2.0- and 4.0-μmol level. Analytical solutions of the methyl esters of **3**, **6**, and **9–13**, dimethyl terephthalate, and dimethyl phthalate were also prepared as reference standards. All methyl esters were obtained from the corresponding carboxylic acids by using diazomethane (from Diazald).

Treatment Solutions. The sample of 1 obtained from MCB Manufacturing Chemists, Norwood, OH, was redistilled before use (bp 103–105 °C at 0.05 mmHg). For ip injections, a 2.5% w/v solution of 1 in water containing 0.9% sodium chloride and 1% Tween 80 was administered at a dose of 2.0 mL/kg. For topical applications, a 15% solution of 1 in absolute ethanol was applied at a dose of 0.333 mL/kg.

Animals and Treatments. In experiments 1 and 2, two groups of four male Wistar rats (8–10 weeks old, 300–350 g each) from Charles River Canada Inc. (St. Constant, Quebec) were individually held for 48 h before treatment in glass metabolism cages (Stanford Glassblowing Laboratories, Inc., Palo Alto, CA) with free access to food (Purina Laboratory Chow) and tap water. The room was held at 23–26 °C and 60–70% relative humidity with a 12-h light cycle. Each rat was weighed and then injected ip with the treatment solution at a dose of 50 mg/kg. Urine from each animal was collected in a flask for 24 h immediately before and after dosing, using 0.1 mL of a 2% solution of disodium ethylenediaminetetraacetic acid as preservative. Each urine sample was measured, transferred to two sterilized containers, and frozen (−40 °C) until analysis.

In experiments 3 and 4, a group of four male (8–10 weeks of age) and a group of four female (10–12 weeks of age) Wistar rats were carefully shaved with electrical hair clippers (Oster Model A2 with size 40 head) on the base of the neck above the shoulders (upper back) 24 h before treatment. The solution of 1 was applied at a dose of 50 mg/kg to an area of skin (1.5–2

cm²) with an electronic digital pipet (Rainin EDP-2500). After evaporation of the ethanol, the rats were placed in glass metabolism cages and urine was collected as above but in intervals of 0–24, 24–48, and 48–72 h (females only) after treatment.

Urine Extractions for Metabolite Identification. A 5-mL sample of 0–24-h urine was centrifuged (2000 rpm) for 15 min and transferred without sediment to a small beaker equipped with a magnetic stir bar. The pH was adjusted to 12 with 1 N NaOH (pH meter). After 5 min, 2.5 mL of 0.1 M phosphate buffer (NaH_2PO_4–Na_2HPO_4, pH 7) was added. The pH was adjusted to 8 with 5 N HCl and extracted twice with 5 mL of MBE. These neutral extracts were combined before GC–FID and GC–MS. The aqueous layer remaining was adjusted to pH 2 with 5 N HCl and extracted once with 5 mL of ethyl acetate. Extractions of individual urines were done in test tubes equipped with screw caps (Teflon lined); gentle shaking on an Ames aliquot mixer and centrifuging were used as required. In experiment 1, pretreatment control urine and posttreatment urine samples from each rat were extracted. In experiments 2–4, 0–24-h urine samples from 6 of the 12 rats were extracted by this procedure.

Derivatization of Urinary Extracts. Ethyl acetate fractions were concentrated (rotary evaporator), cooled (ice bath), and treated in a well-ventilated fumehood with 1–2 mL of a cold, freshly prepared solution of diazomethane. After 1 h, the reaction was gradually allowed to come to room temperature and then transferred by using ethyl acetate to a 5-mL volumetric flask. A 1–2-μL portion of this solution was used for GC–FID and GC–MS.

Stability Experiment. Phosphate buffer (pH 7, 5 mL) was spiked in separate experiments with 1 μmol (from 100 μL of stock solution) of **1**, **3**, **4**, **6**, **7**, and **9**. The pH was adjusted to 12 (5 N NaOH), and the samples were extracted at pH 8 and pH 2 exactly as described for urine. The ethyl acetate fractions were derivatized (diazomethane) and analyzed by GC–FID and GC–MS for the presence of methyl esters of **10** and **12**.

Quantitative Procedure. Rat urine of a designated time interval from each treated animal was mixed by vortexing and a 1-mL subsample (in duplicate) in a screw-cap test tube was diluted with 1.5 mL of distilled water. The internal standard of **13** (1.0 μmol in 100 μL of methanol) was added. After 1 min of vortexing, 5 N NaOH (50 μL) was added, and stirring was continued for 10 min. The sample was transferred to another test tube, centrifuged at 1800 rpm for 5 min, and transferred back to the original test tube. Water (2.5 mL) was added to the sediment, which was vortexed and recentrifuged, and the aqueous phase was combined with the original urine sample. After 5 N HCl (30 μL) was added, the mixture was extracted twice with MBE (4–5 mL). The acidic extract was obtained by acidification of the aqueous phase with 5 N HCl (50 μL) and one extraction with ethyl acetate (2 mL). Before derivatization, the samples were evaporated to dryness in 13 × 100 mm glass tubes by using a Savant evaporator (Model SVC-100H, Emerston Instruments Inc., Scarborough, Ontario). Ethyl acetate (2 mL) was added, the tubes were briefly vortexed (Scientific Industries Model K-550-D), and the organosoluble portion was decanted into test tubes. After derivatization, the extracts were made up to 5 mL with ethyl acetate. A portion (100 μL) of this extract was transferred to an autosampler vial and diluted with 400 μL of ethyl acetate. One or two microliters of each extract was injected splitless, using GC–NPD and the automatic sampler.

For experiments 1–4, drug-free rat urine samples (1 mL) that had been diluted with water (1.5 mL) were spiked from stock solutions with varying quantities of **3** (0.1, 0.25, 0.5, 0.75, 1.0, 2.0, and 4.0 μmol in 10–100 μL of methanol) and **6** (0.01, 0.05, 0.1, 0.15, 0.25, 0.5, and 1.0 μmol in 1–100 μL of methanol) and a fixed quantity (1.0 μmol) of **13**. These samples, plus one that was unspiked and another that was only spiked with **13**, were extracted, derivatized, and analyzed exactly as described above. GC–NPD peak area ratios of **3** to **13** and **6** to **13** were obtained and used to construct the calibration curves. Typical correlation coefficients were 0.995–0.998.

Calculations and Statistics. The concentration (micromoles per milliliter) of **3** and **6** from specified urine samples was obtained by least-squares linear regression analysis. A dose of 50 mg of **1** is equivalent to 261.4 μmol, so the theoretical amount of **1** or metabolites that could be excreted is readily obtained from animal weights at the time of treatment. That amount (micromoles) is divided into the amount of **3** and **6** found in the 24-h urine samples, which gives the percent of the applied dose that was excreted as these metabolites in the specified time interval (see Table II). Statistical comparisons were performed by analysis of variance. Comparisons between experiments were made by using interanimal variation, whereas, in experiment 2, variation due to the animals and subsamples was removed and the hydrolysis methods were regarded as a further subplot factor (Steel and Torrie, 1980).

Recoveries. Diluted rat urine (5 × 2.5 mL) collected before treatment was spiked with **3** (2.0 μmol in 40 μL of methanol) and **6** (0.5 μmol in 25 μL of methanol). These samples were carried through the entire quantitative procedure. One milliliter of the ethyl acetate extract was saved and evaporated. Just before derivatization, the internal standard of **13** (1.0 μmol in 100 μL of methanol) was added to each sample in ethyl acetate (2 mL). Recoveries of **3** and **6** were estimated by reference to standard curve samples, prepared by adding 0.1–4.0 μmol of **3**, 0.01–1.0 μmol of **6**, and 1.0 μmol of **13** to tubes for derivatization. The recovery extracts were analyzed by GC–NPD.

Quantitation of 1 in Urine. Urine (1 mL) collected from topically treated rats in experiments 3 and 4 was diluted with water (1.5 mL) and spiked with an internal standard of **14** (1.0 μmol in 100 μL of methanol), and a MBE extract was obtained exactly as described before. A calibration curve was obtained by spiking the control urine with varying quantities of **1** (0.1, 0.25, 0.5, 0.75, and 1.0 μmol in 10–100 μL of methanol) and a fixed quantity (1.0 μmol) of **14**. These samples were analyzed by GC–NPD as described for the carboxylic acid metabolites except that the dried ($MgSO_4$) MBE extract (10 mL) was not further diluted.

RESULTS AND DISCUSSION

Metabolite Synthesis. The diethyl alcohol **2** was synthesized from *m*-formylbenzoic acid. Reaction of this acid with dicyclohexylcarbodiimide and diethylamine in tetrahydrofuran (Sheehan and Hess, 1955) gave *N*,*N*-diethyl-*m*-formylbenzamide in 30–40% yields after preparative HPLC. Reduction with sodium borohydride gave **2**, which was purified by HPLC and fully characterized. Diethyl acid **3** was prepared from isophthalic acid (**12**) by reaction of this diacid with 1 equiv of dicyclohexylcarbodiimide and diethylamine. The low yield (18.5%) was probably due to competing formation of the diamide of **12**.

N-Ethyl-*m*-toluamide (**4**) (Farlow and Moodie, 1970) was prepared by a standard conversion with *m*-toluoyl chloride and ethylamine, whereas alcohol **5** and acid **6** were obtained by the same coupling techniques utilized in the synthesis of **2** and **3**. The acid **9** (Gorog et al., 1982) was obtained in 78% yield by oxidation of **7** with potassium permanganate and sodium carbonate. Use of a strong base (10% NaOH) also resulted in hydrolysis of the amide group, and **12** was isolated. The ethyl ester of **9** (Bromilow et al., 1981) gave alcohol **8** by reduction with lithium borohydride. An authentic sample of *m*-(hydroxymethyl)benzoic acid (**11**) was obtained by reduction of *m*-formylbenzoic acid with sodium borohydride. The internal standards **13** and **14** (Naumov et al., 1975) were synthesized by the same procedures utilized in the preparation of **3** and **4**. Methyl ester derivatives of **3**, **6**, **9**–**12**, and **13** were obtained from these acids (1-mmol scale) by using diazomethane (see supplementary material).

Metabolite Identification. As previously reported (Taylor, 1986), **1**, **2**, **4**, **5**, and **7** showed good GC properties on various capillary columns, and these compounds could be extracted from microsomal suspensions with MBE. Rat urine from experiment 1, adjusted to pH 8, was therefore extracted with MBE and examined by GC–FID with DB-1 or SPB-5 capillary columns. Although

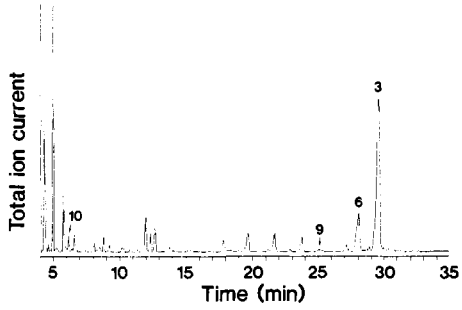

Figure 3. Total ion mass chromatogram from GC–MS analysis (70 eV) of a derivatized urinary extract from a male rat (experiment 1) dosed intraperitoneally with 50 mg of 1/kg of body weight. The labeled peaks represent methyl esters of the indicated carboxylic acids. Unlabeled components were also observed in the control mass chromatogram from a 5-mL urine sample collected prior to treatment. The column was SPB-1.

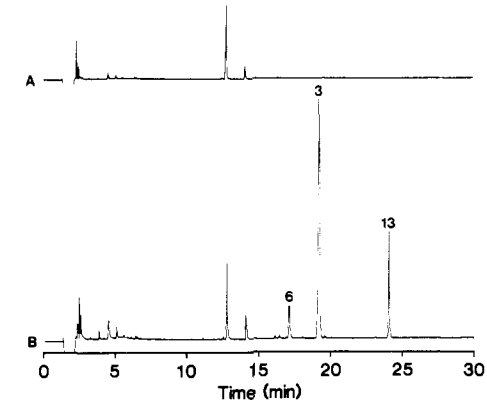

Figure 4. Capillary gas chromatograms from GC–NPD determination of the methyl esters of **3** and **6** in urinary extracts from experiment 2. (A) Trace from pretreatment control urine. (B) Trace from a 0–24-h urine collection to which **13** (1 μmol) was added prior to extraction and derivatization. Conditions are given under Experimental Procedures. The column was DB-1. Attenuation ×5.

many endogenous components were evident, the chromatograms from treated rats showed a small peak with the same retention time as **1** that was absent in chromatograms from pretreatment control urine. Using GC–MS, we did not positively identify any neutral metabolites of **1** in these rat urine extracts.

It should be noted that conjugation of the benzylic alcohols might occur in vivo, which could account for the failure to detect **2**, **5**, and **8** in the neutral extracts. Thus, urine from experiment 1 was first treated with hydrochloric acid (5 N, 100 °C for 1 h) before extraction at pH 8 with MBE. Under these conditions, conjugates such as glucuronides and sulfates should be hydrolyzed and, if the metabolites were stable in strongly acidic solution, **2**, **5**, and **8** should be detected as their acetyl derivatives following treatment of the extracts with acetic anhydride (Taylor, 1986). The chromatograms from GC–FID analysis of acetylated extracts were very similar to those obtained before treatment with hydrochloric acid, except that a small peak (approximately equal in area to that of **1**) corresponding to **4** was present. This observation suggested that **4**, or possibly an α-hydroxyamide claimed to be an intermediate in the formation of **4** (Wu et al., 1979), was conjugated, likely with glucuronic acid. Moody et al. (1989) found that **4** was extracted from human urine following topical application of **1** and acid (HCl) or enzyme (β-glucuronidase) hydrolysis.

To check for acidic metabolites, urine samples washed with MBE were adjusted to pH 2, extracted with ethyl acetate, and derivatized with diazomethane. Spiking experiments with **3**, **6**, and **9** showed that these potential metabolites were recovered from phosphate buffer or pretreatment control urine and, with the exception of **9**, were detected as their methyl esters in low nanogram amounts by GC–FID with a DB-1 column. Analysis of derivatized urinary extracts from treated and untreated rats indicated that **3**, **6**, **9**, and **10** were in vivo metabolites of **1**. This was confirmed in all experiments by GC–FID with different capillary columns and by GC–MS (Figure 3).

Confirmation of **9** in urinary extracts was done by GC–MS in the chemical ionization mode by finding the quasimolecular ion at m/z 180 in the small component eluting at 25 min, which corresponded to the retention time of this compound under the reported GC–MS conditions. Identification of **9** was not surprising because *m*-toluamide (**7**) was identified as a minor metabolite of **1** in vitro (Taylor, 1986). Didemethylation of the amide herbicide diphenamid also occurs to a minor extent in the rat (McMahon and Sullivan, 1965).

Although **10** could possibly arise by acid- or base-catalyzed hydrolysis of the amide bond of **1**, **4**, or **7**, no evidence was obtained for that by spiking phosphate buffer with these compounds, adjusting the pH, and analyzing the acidic extracts for the methyl ester of **10** by GC–FID and GC–MS. We conclude, therefore, that **10** is a minor metabolite of **1** but **11** and **12** were not positively identified in the metabolic extracts.

It is interesting to compare the major metabolites from this study to those that were identified in incubations of **1** with liver microsomes from normal (untreated) Wistar rats (Yeung and Taylor, 1988). The alcohols **2** and **5**, likely precursors to **3** and **6**, were formed in vitro, which illustrates once again the predictive value of microsomal oxidations in establishing in vivo metabolic pathways. These findings are reminiscent of rat studies on the metabolism of toluene. Benzyl alcohol (and cresols) are produced in vitro (Pathiratne et al., 1986), whereas benzoic acid (and hippuric acid) are the predominant in vivo metabolites (Pyykko, 1984).

Metabolite Quantitation. Approaches to measure **3** and **6** in urinary extracts were based on the use of **13** as an internal standard and the use of GC–NPD to determine these metabolites as their methyl esters. Subsamples of urine were first adjusted to pH 12 with sodium hydroxide. Addition of alkali might hydrolyze the glucuronides of **3** and **6** since this approach has recently been used to effectively hydrolyze the glucuronides of other carboxylic acids excreted in urine (Jamali et al., 1988). Basification also facilitated the removal of neutral and basic impurities by washing the urine (at pH 8) with MBE. Adjustment of the pH to 2 allowed the extraction of **3**, **6**, and **13**. These extracts were treated with diazomethane and analyzed by GC–NPD. With a column of DB-1, it was found (Figure 4) that chromatograms from pretreatment control urine, when compared with chromatograms from treated rats, were free of interferences at the retention times for **3**, **6**, and **13**. At 14.5 min, a coextractive from the urine with the same retention time as **9** interfered with the determination of this minor metabolite. Furthermore, injection of authentic samples of these methyl esters showed that the sensitivity to **9** was relatively poor. With splitless injections, the NPD was capable of detecting about 20 pmol of **9** (3.6 ng), 5 pmol of **6** (1 ng), and 1 pmol of **3** (235 pg) and **13** (263 pg). Of course, the methyl ester of **10** did not respond to NPD.

By spiking control urine (Experimental Procedures), the mean recovery (±SD) was found to be 65.5 ± 4.3%

Table II. Percent of Intraperitoneally and Topically Administered 1 Excreted as 3 and 6 in Rat Urine

expt[a]	sex	route of administration	collection period postdose, h	% (mean ± SE, $N = 4$) of 50 mg/kg dose excreted as 3	6
1	male	ip	0–24	55.4 ± 3.1	21.6 ± 0.7[b]
2	male	ip	0–24	65.9 ± 2.4	16.2 ± 0.4[b]
			0–24[c]	61.2 ± 2.1	16.7 ± 0.8
			0–24[d]	58.3 ± 4.0	18.5 ± 1.1
3	male	topical	0–24	26.2 ± 1.2	7.7 ± 0.2
			24–48	10.2 ± 0.7[e]	2.8 ± 0.2[f]
4	female	topical	0–24	31.6 ± 3.0	10.4 ± 1.0
			24–48	5.1 ± 0.4[e]	1.7 ± 0.1[f]
			48–72	2.1 ± 0.4	0.5 ± 0.2

[a] Four rats were used for each experiment. The 1-mL subsamples (in duplicate) were assayed by the quantitative procedure described under Experimental Procedures. [b] These values in experiments 1 and 2 are significantly different at the 1% level. [c] These subsamples were not adjusted to pH 12 before extraction at pH 8 and pH 2. [d] These subsamples were first diluted with 1.5 mL of 0.1 M sodium acetate buffer (pH 4.5) and placed in a Dubnoff shaking incubator at 37 °C. β-Glucuronidase (type H-1, Sigma) was added (30 mg) in four equal portions at 15-min intervals, and the mixture was incubated for 22 h before extraction at pH 8 and pH 2. [e,f] These values in experiments 3 and 4, for the 24–48-h period, are significantly different at the 1% level.

for **3** and 34.8 ± 0.9% for **6**. These recoveries could likely be improved by multiple extractions with ethyl acetate but at the expense of more interferences appearing from urinary components.

In experiment 1, **3** and **6** were determined in duplicate subsamples of 0–24-h urine from a group of four ip-dosed male rats. Expressed as a mean value, these two metabolites accounted for 77% of the administered dose of **1**. Of this total, **3** represented 55.4% (range 45.7–62.8%) and **6** represented 21.6% (range 18.8–27.9%). Most of the observed variability was between animals because the coefficient of variation between subsamples was usually less than 5%. Experiment 2, a repeat of experiment 1, gave similar results (82% excreted as **3** and **6**) except that the proportion of each metabolite was slightly different (Table II).

To study the possibility that **3** and **6** were excreted as ester glucuronides, urine from experiment 2 was also processed by avoiding the alkaline treatment and by incubating the subsamples in the presence of β-glucuronidase. The percentage of the dose excreted as **3** and **6** did not differ significantly at the 1% level among the three treatments. This indicated that glucuronidation was probably an unimportant biosynthetic reaction for the excretion of these metabolites from Wistar rats.

Reference samples of potential glycine conjugates (hippurates) of these metabolites were unavailable, but no evidence was obtained to support their presence in derivatized metabolic extracts by searching the chemical ionization GC–MS data for quasimolecular ions corresponding to the methyl esters of the glycine conjugates of **3**, **6**, and **9–12**.

In experiments 3 and 4, **1** was applied to the skin on the upper backs of male and female Wistar rats and the urine collected at 24-h intervals was quantitatively analyzed (after adjustment to pH 12) by GC–NPD. It was found that **3** and **6** were major excretion products, accounting for 45–50% of the applied dose in the 48-h posttreatment. Female Wistar rats appeared to excrete these metabolites faster than males (Table II). This was unexpected because liver microsomes from male Wistar rats metabolized **1** at a faster rate than microsomes from females (Yeung and Taylor, 1988).

It was possible to quantitate some **1** in urine samples from topically treated rats. With an internal standard of **14** and GC–NPD, the amount (mean ± SE) of **1** found in 0–24-h urine samples was 2.7 ± 0.25% and 2.8 ± 0.4% of the applied dose for males and females, respectively. The levels of **1** did not change dramatically in 24–48-h samples (2.8 ± 0.2% for males; 1.9 ± 0.35% for females), and about 1% of the applied dose was detected as **1** in 48–72-h collections from female rats. The observation that **1** is excreted in larger amounts in urine following topical treatments follows the reasoning that ip-administered compounds are expected to undergo first-pass liver metabolism to a greater extent before renal excretion.

Although **1** is partially absorbed through human skin (Feldman and Maibach, 1970; Blomquist and Thorsell, 1977), it is not yet known if these acidic metabolites from the rat can be used to monitor percutaneous exposure in man during normal use (Robbins and Cherniack, 1986). Percutaneous absorption of organic chemicals is usually much higher in rats than in humans (Wester and Noonan, 1980), and many factors can influence the extent and rate of absorption in man (Wester and Maibach, 1985) and experimental animals (Bartek et al., 1972; Shaw and Guthrie, 1983). Since the discovery that **1** is a percutaneous absorption enhancer (Windheuser et al., 1982), interest has increased in potential interactions of **1** with other chemicals, for example, fenitrothion insecticide (Moody et al., 1987). The procedures described here should be helpful in other toxicological and pharmacokinetic studies with experimental and domestic animals.

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Supplementary Material Available: Synthetic methods, purification details, and mass spectral ions for **1–14** (10 pages). Ordering information is given on any current masthead page.

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Registry No. 1, 134-62-3; 2, 72236-22-7; 2 (acetyl derivative), 126926-37-2; 3, 72236-23-8; 3 (methyl ester), 126926-38-3; 4, 26819-07-8; 5, 105394-84-1; 5 (aldehyde), 126926-39-4; 6, 126926-33-8; 6 (methyl ester), 126926-40-7; 7, 618-47-3; 8, 126926-34-9; 8 (acetyl derivative), 126926-41-8; 9, 4481-28-1; 9 (methyl ester), 106748-24-7; 9 (ethyl ester), 78950-33-1; 10, 99-04-7; 11, 28286-79-5; 11 (methyl ester), 67853-03-6; 12, 121-91-5; 13, 126926-35-0; 13 (methyl ester), 126926-42-9; 14, 5448-35-1; *m*-formylbenzoic acid, 619-21-6; diethylamine, 109-89-7; *N,N*-diethyl-*m*-formylbenzamide, 126926-36-1; ethylamine, 75-04-7; *m*-toluoyl chloride, 1711-06-4; *m*-formylbenzoic acid, 619-21-6; dipropylamine, 142-84-7.

Flavonoids with Mosquito Larval Toxicity

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Culture broths of an unidentified species of *Streptomyces*, designated 85–88, showed toxicity to mosquito larvae that was traced to be due to three crystalline compounds, identified as tangeretin (**1**) (5,6,7,8,4′-pentamethoxyflavone), genistein (**2**) (5,7,4′-trihydroxyisoflavone), and daidzein (**3**) (7,4′-dihydroxyisoflavone). Activity was produced only when soybean meal was included in the culture medium, and at least compounds **2** and **3** appear to arise as a result of the hydrolysis of the corresponding glycosides known to be present in soybean. The acetates of **2** and **3** also showed activity, actually somewhat greater than that shown by the corresponding isoflavones.

INTRODUCTION

During the past several decades, limitations observed with many synthetic pesticides such as slow biodegradability and mammalian toxicity, including carcinogenic potential, have prompted an active search for pesticides from alternative sources such as plants and microorganisms. The rationale behind this search is based on the general expectation that natural products are at least readily biodegradable and that other factors such as

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