Table II—Plasma Concentrations in Colonies of Fasted Mice following Ingestion of Caffeine

Minutes	Plasma Caffeine Concentration, μg/ml
0	<0.1
15	5.9
30	1.9
60	1.2

feine more rapidly than humans. Table II indicates some typical plasma level data for a colony of mice fasted overnight and then given approximately 1 mg of caffeine as a single dose in the morning. This dose is equivalent to about 10 cups of coffee for a 70-kg human. Peak absorption occurred within 15 min, and concentrations declined rapidly thereafter. In colonies of nonfasted mice allowed to drink standard brews of coffee (350 μg/ml) *ad libitum*, typical plasma concentrations between drinks were 0.3–1.5 μg/ml throughout the day.

In general, the assay appears to be well suited to the rapid and routine measurement of human or animal plasma caffeine concentrations following administration of caffeine-containing beverages or medications. It is sensitive and highly selective and has permitted a variety of human and animal studies aimed at quantitating plasma concentration–time profiles following varying amounts of caffeine ingestion and correlating them to the possible incidence of adverse effects. The high sensitivity attainable also allows this method to be useful in assessing plasma concentration of caffeine in toxic situations from small volumes of blood.

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GLC Determination of Ticrynafen and Its Metabolites in Urine, Serum, and Plasma of Humans and Animals

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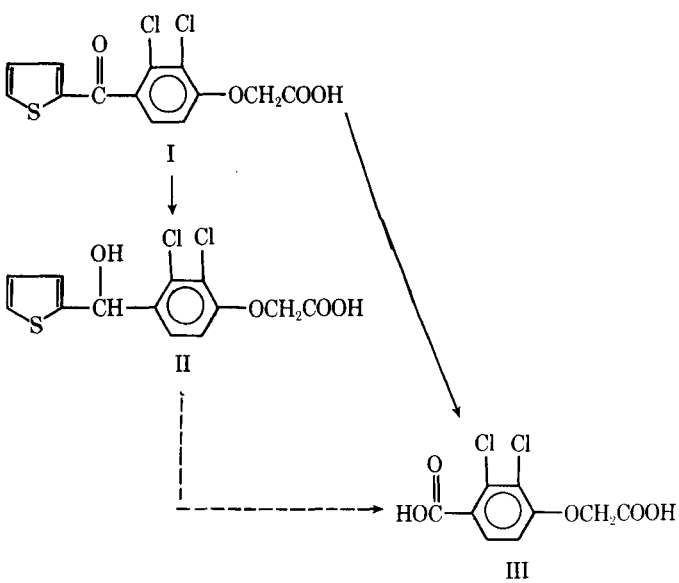
Abstract □ A sensitive GLC assay for ticrynafen, a diuretic agent with uricosuric properties, and its two metabolites in urine, serum, and plasma is described. The method employs methylation of carboxylic acid groups and trimethylsilylation of the hydroxyl group on one metabolite that cannot otherwise be separated readily from ticrynafen as a simple methyl ester. Urinary output and serum or plasma levels of ticrynafen and its two metabolites were measured in specimens from human volunteers receiving one 250-mg tablet.

Keyphrases □ Ticrynafen—and metabolites, GLC analyses in biological fluids □ GLC—analysis, ticrynafen and metabolites in biological fluids □ Diuretics—ticrynafen and metabolites, GLC analyses in biological fluids

Ticrynafen [tienilic acid[1] or [2,3-dichloro-4-(2-thienylcarbonyl)phenoxy]acetic acid] (I) is a new potent diuretic agent with uricosuric properties (1–4). A pharmacokinetic study with the ^{14}C-labeled drug in animals (5) and a GLC method (6) for the drug were reported recently. However, the reported GLC method does not distinguish the parent drug from one of its metabolites, [2,3-dichloro-4-(α-hydroxy-2-thienyl)phenoxy]acetic acid (II). The drug is metabolized by two pathways (5): reduction of the ketone function to the corresponding alcohol (II) and oxidation of the thienylcarbonyl moiety to form [2,3-dichloro-4-carboxyphenoxy]acetic acid (III) as shown in Scheme I.

This report describes a procedure capable of measuring



Scheme I

[1] WHO approved name.

Table I—Reproducibility and Recovery from Urine Based on Peak Height Ratios

Sample	Extracted			Unextracted		
	I, 10 μg/ml	II, 10 μg/ml	III, 1 μg/ml	I, 10 μg	II, 10 μg	III, 1 μg
Blank	0	0	0	0	0	0
1	1.67	1.15	0.23	2.19	2.64	0.33
2	1.65	1.61	0.23	2.23	2.69	0.31
3	1.51	1.58	0.23	2.03	2.72	0.31
4	1.65	1.66	0.24	2.10	2.78	0.30
5	1.64	1.67	0.22	2.23	2.58	0.33
$\bar{x}$	1.62	1.62	0.23	2.16	2.68	0.32
Sx	0.06	0.04	0.006	0.08	0.06	0.01
$S\bar{x}$	0.03	0.02	0.003	0.04	0.03	0.005
Recovery[a], %	100	84	104			

[a] Recovery = ($\bar{x}$ extracted/$\bar{x}$ unextracted) × 1.39. The factor 1.39 = 9.7/7.0; a 7-ml aliquot was taken from 9.7 ml of the organic layer, which resulted from mixing 10 ml of ether and 3 ml of the aqueous mixture.

I–III simultaneously in urine, serum, or plasma at very low concentrations. Analyses of specimens from human volunteers receiving a single dose of 250 mg of I also are reported.

EXPERIMENTAL

Reagents—The following reagents were used: 1.0 *N* HCl[2], chloroform[3], ether[3], methanol[2], *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide[4], bis-(trimethylsilyl)trifluoroacetamide plus 10% trimethylchlorosilane[5], acetonitrile[6] (dried over molecular sieve 3A), pyridine[7], and boron trifluoride–methanol complex[4].

Preparation of Diazomethane—Diazomethane ethereal solution (~1 mmole/ml) was prepared by mixing 2 g of *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide in 10 ml of ether and 0.2 g of potassium hydroxide in 5 ml of 90% ethanol (10% water) at 0° or below. The mixture was then heated in a water bath (70°), and 6 ml of diazomethane ethereal solution distillate was collected in an ice-cooled test tube. The solution was stored in a freezer until used.

Preparation of Internal Standard Solution—The methyl ester of ethacrynic acid, used as an internal standard for this assay, was prepared by reacting ethacrynic acid with methanol in the presence of boron trifluoride. A 500-μg sample of ethacrynic acid was dissolved in 0.5 ml of methanol and mixed with 25 μl of boron trifluoride–methanol complex. The mixture was kept at 60° for 30 min and evaporated under nitrogen.

To the residue, 1 ml of 5% Na_2CO_3 was added; the product, methyl ethacrynate, was extracted with 2 ml of ether. The organic layer was evaporated to dryness, and the residue was dissolved in 200 ml of acetonitrile (predried over molecular sieves) to give an approximate concentration of 2.5 μg/ml. The structure of the methyl ester was verified by its NMR and mass spectra.

Diazomethane esterification of ethacrynic acid did not provide a suitable standard because diazomethane reacted with the activated double bond in ethacrynic acid to give two products, one of which interfered with the analysis.

Instrumentation—The gas chromatograph[8] was equipped with an on-column injection port, an electron-capture detector, and a 180-cm long, 2-mm i.d. glass column packed with 3% OV-17 on 100–120-mesh

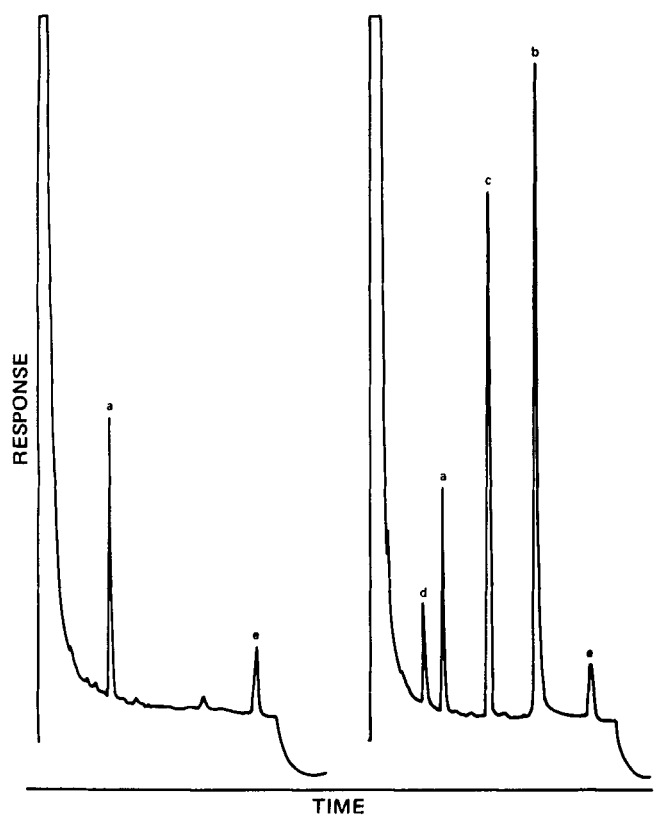


Figure 1—*Typical chromatograms of human urine with internal standard (a). Key: left, control urine; and right, urine containing 10 μg of I/ml (b), 10 μg of II/ml (c), and 1 μg of III/ml (d). Peak e is due to endogenous material in human urine.*

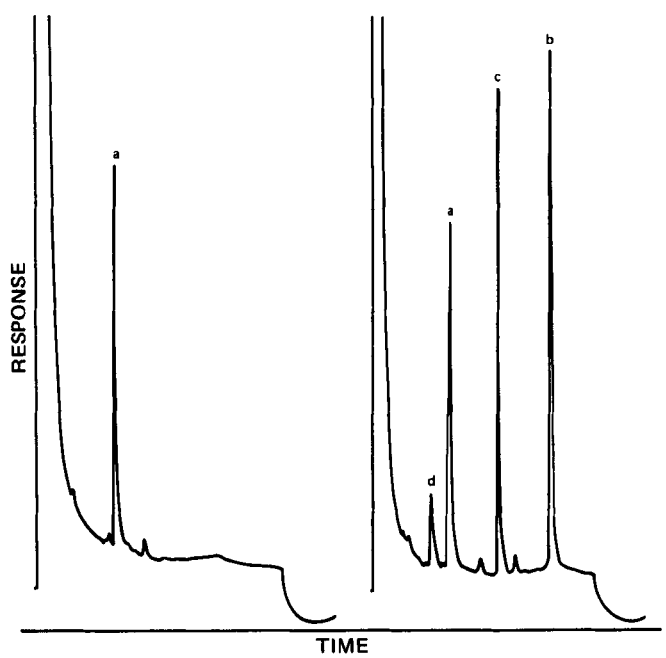


Figure 2—*Typical chromatograms of human plasma with internal standard (a). Key: left, control plasma; and right, plasma containing 5 μg of I/ml (b), 5 μg of II/ml (c), and 0.5 μg of III/ml (d).*

[2] Target electronic grade, Lehigh Valley Chemical Co., Easton, Pa.
[3] Analytical reagent, Mallinckrodt, St. Louis, Mo.
[4] Aldrich Chemical Co., Milwaukee, Wis.
[5] Regisil No. 3, Regis Chemical Co., Morton Grove, Ill.
[6] Fisher Scientific Co., Pittsburgh, Pa.
[7] Burdick & Jackson Laboratories, Muskegon, Mich.
[8] Model 3920B, Perkin-Elmer Corp., Norwalk, Conn.

Table II—Reproducibility and Recovery from Plasma Based on Peak Height Ratios

Sample	Extracted			Unextracted		
	I, 5 μg/ml	II, 5 μg/ml	III, 0.5 μg/ml	I, 5 μg	II, 5 μg	III, 0.5 μg
Blank	0	0	0	0	0	0
1	1.55	1.58	0.20	2.17	2.17	0.28
2	1.51	1.57	0.18	2.10	2.20	0.27
3	1.44	1.57	0.19	2.14	2.14	0.27
4	1.46	1.54	0.19	2.20	2.16	0.27
5	1.54	1.53	0.18	2.12	2.14	0.27
$\bar{x}$	1.50	1.56	0.19	2.15	2.16	0.27
Sx	0.04	0.02	0.007	0.04	0.02	0.004
$S\bar{x}$	0.02	0.01	0.003	0.02	0.01	0.002
Recovery[a], %	97	100	98			

[a] Recovery = ($\bar{x}$ extracted/$\bar{x}$ unextracted) × 1.39. The factor 1.39 = 9.7/7.0; a 7-ml aliquot was taken from 9.7 ml of the organic layer, which resulted from mixing 10 ml of ether and 3 ml of the aqueous mixture.

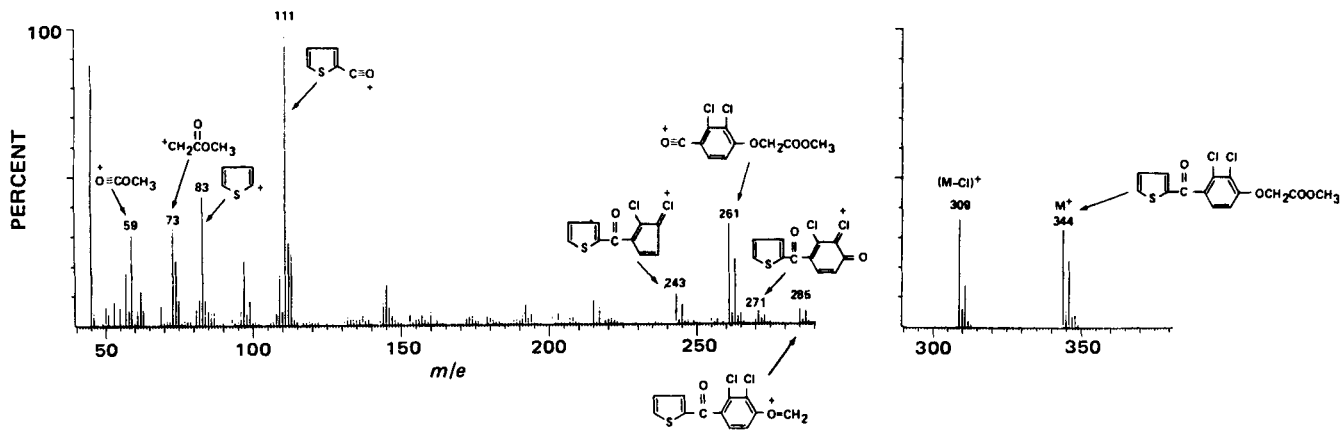


Figure 3—*Mass spectra of ticrynafen methyl ester.*

Gas Chrom Q. The oven temperature was programmed from 230° after 2 min initial temperature time and heated to 282° at the rate of 8°/min. The analysis ended 4 min after the final temperature was reached. Injection port, interface, and detector temperatures were 270, 310, and 300°, respectively. Argon–methane (95:5) was used as a carrier gas at 4.2 kg/cm^2. The detector standing current was 1.0 amp, and attenuation was at 512.

Under these conditions, retention times of the derivatized compounds were: diacid (III), 4 min; internal standard, 5 min; reduced metabolite (II), 8 min; and ticrynafen (I), 10.5 min. Since a urinary component had a retention time of about 15 min, the final temperature had to be maintained until this substance was eluted off the column before the oven was returned to the initial temperature for the next analysis. Temperature settings may have to be adjusted to suit individual columns to obtain good chromatograms.

Extraction and Preparation of Samples for Chromatography—Each 1-ml sample (urine, plasma, or serum) was diluted with 2 ml of 1.0 *N* HCl in a 40-ml centrifuge tube. Ether, 10 ml, was added to each tube, and the tubes were capped and shaken on a rocking mechanical shaker for 45 min. After centrifugation at 2000 rpm for 15 min, 7 ml of the organic layer from each tube was transferred to 12-ml centrifuge tubes. Each extract was cooled in an ice bath for 5 min, 0.5 ml of the diazomethane solution was added, and the tubes were mixed and kept at ice bath temperature for 1 hr.

The tubes were removed from the ice bath to a 40° water bath, and the solvent was evaporated under a constant flow of nitrogen. Aliquots of 1 ml of methanol were used to wash the residue from the wall of each tube, and the methanol also was evaporated to dryness under nitrogen at 40°. Finally, 1 ml of a solution consisting of 75 parts of acetonitrile containing a suitable concentration of the internal standard, 10 parts of pyridine, and 15 parts of the silylating reagent was added to the tube; then the tube was mixed well and sealed with a cap. The samples were ready for analysis after 3 hr. Samples can be kept overnight (or several days) at room temperature.

Method of Quantitation—Suitable ranges of standard solutions of I–III in urine, plasma, or serum were prepared for each group of unknown samples and treated similarly and simultaneously. The standard curves, based on peak height ratio and concentration, were used to quantitate each component. For analysis of samples from subjects receiving 250 mg of I, the appropriate concentration ranges of standard solutions for the standard curves were 1.0–10.0 μg/ml in urine for I and II, 0.25–5.0 μg/ml in urine for III, 0.5–10.0 μg/ml in plasma or serum for I and II, and 0.1–2.0 μg/ml in plasma or serum for III.

GLC–Mass Spectrometry—Mass spectra were obtained on two in-

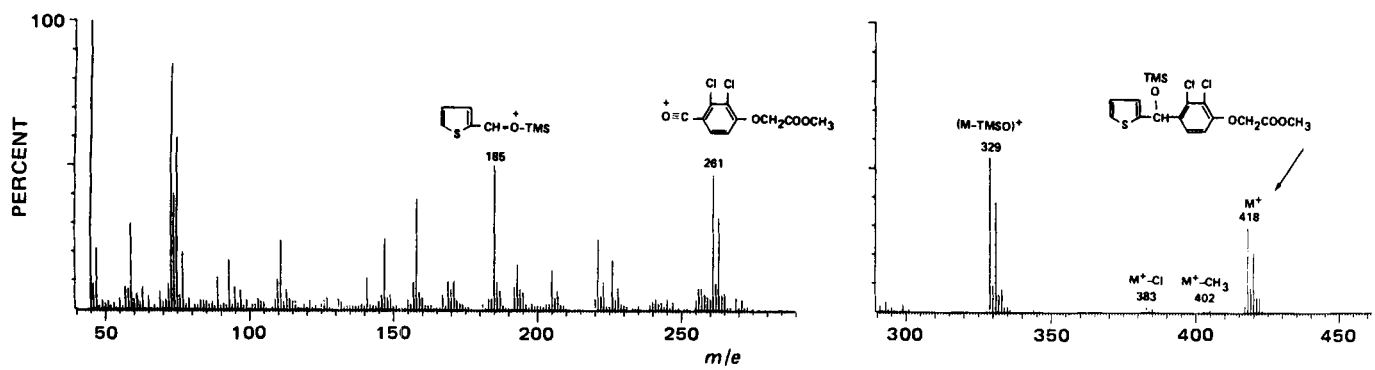


Figure 4—*Mass spectra of methyl ester–trimethylsilyl derivative of II.*

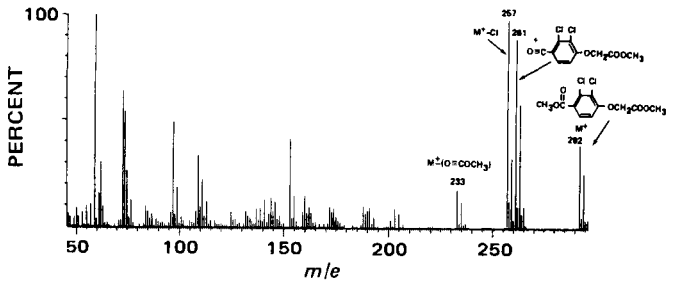


Figure 5—*Mass spectrum of dimethyl ester of III.*

struments using GLC inlets. A 180-cm × 2-mm (i.d.) glass column packed with 3% OV-17 was used on one instrument[9]. The column temperature was programmed from 220 to 275° at 10°/min with a carrier gas helium flow rate of 30 ml/min. The mass spectrometer was operated by autotune software. A 180-cm × 2-mm (i.d.) glass column packed with 3% OV-101 was used on the other instrument[10]. The column temperature was programmed from 210 to 280° at 10°/min with the helium flow rate at 20 ml/min. The mass spectrometer was operated under the following conditions: emission voltage, 70 v; extractor voltage, 5 v; and multiplier, 2200 v. Similar results were obtained from the two instruments.

RESULTS AND DISCUSSION

Linear standard curves were obtained for all compounds. The assay sensitivity was 0.01 μg/ml for each compound in biological fluids using the method described. Peak height of 1% or more of full-scale deflection at an attenuation of 512 was attainable by injecting 1 μl of the final solutions prepared from 1 ml of biological fluids containing 0.01 μg of compounds. Figures 1 and 2 show some typical chromatograms of control urine and plasma containing first the internal standard alone and then all three compounds and the internal standard. Chromatograms of serum samples were similar to those of plasma samples.

Recovery was calculated from the peak height ratio of each component to the internal standard in extracted samples and solutions spiked with each component. The loss of samples during transfer of extracts was considered. As shown in Tables I and II, reproducibility and recovery from urine and plasma were very good. Quantitative recovery and good reproducibility also were observed in every case with serum.

The identity of the three compounds being measured and confirmation of the specificity of analysis were shown by analyzing representative urine

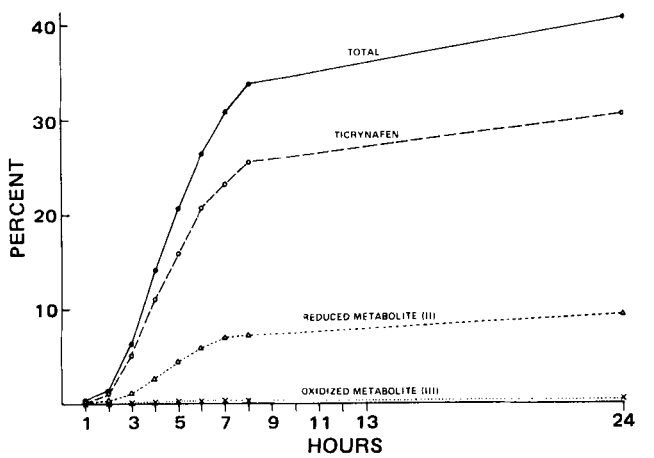


Figure 6—*Average urinary excretion of I–III of eight volunteers receiving 250 mg of ticrynafen (percent of dose excreted, cumulative).*

[9] HP 5992A GC/MS system.
[10] Finnigan model 4000 GC/MS automated system.

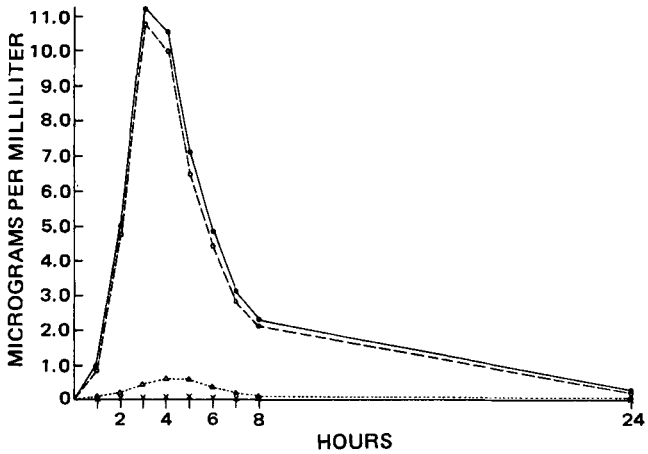


Figure 7—*Average plasma levels of I–III in eight volunteers receiving 250 mg of ticrynafen (data are reported in micrograms of ticrynafen per milliliter or equivalent in the case of the metabolites). Key: ●, total; ○, I; △, II; and ×, III.*

samples by combined GLC–mass spectrometry using the technique described. The spectra obtained from the specimen were identical to those obtained from authentic compounds derivatized by the same procedure. All three compounds gave molecular ions showing two chlorine atoms. Strong fragment ions are in agreement with the structures of derivatized I–III (Figs. 3–5).

The present method was used for the determination of I–III in urine and plasma of eight normal volunteers. Volunteers received a standard waterload of 20 ml/kg, followed by a 250-mg ticrynafen tablet at 9:00 am; water was given hourly (300 ml/hr during the first 15 min of the hour) for the next 7 hr. Results of analysis are shown in Figs. 6 and 7. Average plasma I–III levels peaked between 3 and 4 hr at about 11, 0.7, and 0.07 μg/ml, respectively. Urinary excretions in 24 hr averaged about 40% of the dose. No increase in urinary I–III after enzymatic hydrolysis indicated the absence of glucuronides.

Analyses of two sets of plasma and serum samples from the same blood of two volunteers gave similar results. The method was also suitable for animal urine and serum (or plasma). Analysis of urine and serum samples of a dog administered I, 5 mg/kg iv, showed that the ratios of the levels of II to I in the dog serum and urine were much higher than in humans. The dog excreted 12 and 53% of the dose as I and II, respectively, in the 0–140-min urine collection. No oxidized metabolite (III) was detected in the dog urine or serum samples.

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