GLC Determination of Free and Conjugated Triclosan in Human Plasma and Urine

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Abstract ☐ A method for the determination of free and conjugated triclosan at concentrations as low as 2 ng/ml in human plasma or urine is described. Conjugated metabolites are split by enzyme hydrolysis. After addition of an internal standard, triclosan is extracted at acid pH into petroleum ether, transferred to an alkaline aqueous solution, and back-extracted into petroleum ether after acidification. Both compounds are acetylated with acetic anhydride in the presence of pyridine. The acetyl derivatives are determined by GLC using a ^{63}Ni-electron-capture detector.

Keyphrases ☐ Triclosan—GLC analysis, free and conjugated, human plasma and urine ☐ GLC—analysis, free and conjugated triclosan, human plasma and urine ☐ Disinfectants—triclosan, free and conjugated, GLC analysis, human plasma and urine

Triclosan[1] [5-chloro-2-(2,4-dichlorophenoxy)phenol] (I) is a bacteriostatic agent. Radiolabeled triclosan was used to study its percutaneous absorption and metabolic fate in humans and animals (1). A GLC technique quantitatively determined free and conjugated triclosan in whole blood without an internal standard (2).

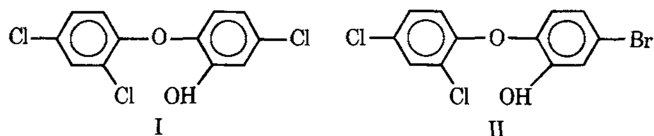
This paper describes an improved procedure for the GLC determination of free and conjugated triclosan in plasma and urine, at concentrations as low as 2 ng/ml, using 5-bromo-2-(2,4-dichlorophenoxy)phenol (II) as an internal standard. β-Glucuronidase is used for the specific determination of the glucuronide; enzymatic hydrolysis with a mixture of β-glucuronidase and sulfatase is preferred to acid hydrolysis for the determination of total (free plus conjugated) triclosan.

EXPERIMENTAL

Reagents—The acetate buffer was prepared by adding 4.8 ml of 0.2 *M* acetic acid to 35.2 ml of 0.2 *M* sodium acetate. Acetic anhydride was purified before use by adding 20 g of sodium acetate to 100 ml of acetic anhydride and boiling under reflux for 15 min. Then acetic anhydride was distilled and collected between 135 and 140°.

Pyridine was distilled at 115–116° with potassium hydroxide pellets and stored over potassium hydroxide pellets. β-Glucuronidase was bacterial β-glucuronidase[2] containing about 47,000 units/g. The mixture of β-glucuronidase and aryl sulfatase[3] contained about 5 and 8 units/ml, respectively. All other reagents were analytical grade[4,5].

Test solutions of acetyl derivatives of both triclosan and internal standard were prepared according to the procedure described under *Acetylation and GLC*. The petroleum ether solution, containing 200 μg of each derivative, was applied as a stripe on a TLC plate (silica gel with fluorescent indicator) to separate and eliminate underivatized triclosan and the internal standard from their acetyl derivatives. The plate was developed with benzene–toluene–formic acid–methanol (50:50:5:10 v/v).



[1] Irgasan DP 300, Ciba-Geigy.
[2] Sigma Type 2.
[3] Calbiochem.
[4] Mallinckrodt.
[5] Merck A.G.

Table I—Precision and Accuracy of the Assay Applied to Spiked Human Plasma Samples

Amount Added, ng/ml	Amount Found, Average of Five Assays, ng/ml	95% Confidence Interval	*SE*, %
2	2.6	2.3–3.0	4.9
5	4.7	3.7–5.6	6.5
20	19	16–22	5.0
50	50	48–52	1.4
200	200[a]	194–205	1.0
500	500	488–512	0.8
1000	998	970–1026	1.0
3000	3087	2870–3303	2.5
6000	5961	5729–6192	1.4

[a] Four assays only.

Table II—Free Triclosan in Urine (Percent of Administered Dose)

Hours	Isotope Dilution Analysis	GLC
0–8	0.08	0.05
8–24	0.60	0.56
24–48	0.09	0.10
48–72	0.03	0.02

After the addition of 2 *N* HCl, the derivative was eluted into petroleum ether.

The 1 *N* sodium hydroxide internal standard solution contained 500 ng of II/ml.

Materials—The glassware was dried at 100° and immersed for 0.5 hr in an ultrasonic bath, first in water and then in methanol.

The gas chromatograph[6] was equipped with a linear 15-mCi ^{63}Ni-electron-capture detector[7]. The peak areas were given by an electronic integrator[8]. The operating temperatures were: column, 200°; injector, 230°; and detector, 290°. The argon–methane (90:10) flow rate was 60 ml/min.

Glass columns were washed with 1 *N* hydrochloric acid, distilled water, acetone, and benzene and then silanized with a 1% (v/v) solution of hexamethyldisilazane in benzene. After this treatment, the columns were washed again with benzene and dried at 100°.

The column packing was 2% OV 1[9]–2% QF 1[9] on 80–100-mesh Chromosorb W HP[9]. The filled column was flushed with the carrier gas at 50° for 30 min, then gradually heated to 240° at a rate of 1°/min with a flow rate of 30 ml/min, and kept at 245° for 3 days.

Triclosan Glucuronide Enzyme Hydrolysis—Aliquots of 1 ml of plasma or urine, 1 ml of pH 5.5 acetate buffer, and 10 mg of β-glucuronidase were incubated for 1 hr at 38°. Distilled water, 1 ml, was then added, and extraction was performed as described under *Extraction*.

Triclosan Glucuronide and Sulfate Enzyme Hydrolysis in Plasma—A 1-ml aliquot of plasma, 1 ml of distilled water, and 0.5 ml of β-glucuronidase–sulfatase mixture, diluted with pH 5.5 acetate buffer (1:10 v/v), were incubated for 15 hr at 38°. Distilled water, 0.5 ml, was then added, and extraction was performed as described under *Extraction*.

Triclosan Conjugates Acid Hydrolysis—Aliquots of 1 ml of plasma or urine and 3 ml of concentrated hydrochloric acid were heated for 1 hr at 100°. Then extraction was performed as described under *Extraction*.

Extraction—A 1-ml aliquot of the internal standard solution was

[6] Hewlett-Packard 5710 A.
[7] Hewlett-Packard 18713 A.
[8] Infotronics.
[9] Applied Science Laboratories.

Table III—Free and Conjugated Triclosan in Urine (Percent of Administered Dose)

Hours	Total Radioactivity	Hydrolysis with β-Glucuronidase: Isotope Dilution Analysis	Hydrolysis with β-Glucuronidase: GLC	Hydrolysis with Hydrochloric Acid: Isotope Dilution Analysis	Hydrolysis with Hydrochloric Acid: GLC
0–8	23.78	23.75	29.80	24.02	24.92
8–24	15.82	14.18	12.82	14.30	12.96
24–48	11.24	9.67	9.66	9.92	9.68
48–72	3.57	2.69	2.78	2.35	3.03

measured into a stoppered glass tube. Then 1 ml of the sample, 2 ml of distilled water, 500 mg of anhydrous sodium sulfate, and 1 ml of concentrated formic acid were added. This solution was mixed thoroughly[10], and the pH was checked (it must be 1–2).

Petroleum ether, 3 ml, was added, and the tube was shaken mechanically for 5 min and centrifuged at 5000 rpm for 5 min.

An aliquot volume of the petroleum ether extract was transferred to another 10-ml tube, and 3 ml of 1 *N* sodium hydroxide was added. The tube was shaken for 5 min and centrifuged for 3 min at 4000 rpm. The petroleum ether phase was separated and discarded. In the same tube, 2 ml of 2 *N* hydrochloric acid and 3 ml of petroleum ether were added to the sodium hydroxide phase. The tube was shaken for 5 min and centrifuged for 3 min at 4000 rpm.

An aliquot volume of the petroleum ether phase was transferred to another tube and taken to dryness under a nitrogen stream in a water bath at 37°.

Acetylation and GLC—To the dry residue were added 100 μl of acetic anhydride and 10 μl of pyridine. After mixing, the tube was allowed to stand for 15 min at room temperature. Then excess reagent was removed by evaporation to dryness at 37° under a gentle nitrogen stream; 500 μl of petroleum ether was added, and the tube was shaken on a mixer.

A 3-μl portion of the petroleum ether solution was injected into the gas chromatograph using the solvent-flush technique. The triclosan content was calculated from the peak area ratio by reference to a calibration curve, prepared from a series of 1 *N* sodium hydroxide–triclosan solutions added to plasma to yield concentrations between 2 and 50, 50 and 500, or 500 and 6000 ng/ml, depending on the concentration range studied.

Human Experiments—*Experiment 1*—One healthy volunteer was given a single oral dose of 203.57 mg of ^{14}C-triclosan (100.15 μCi) in a gelatin capsule. Heparinized blood samples were drawn 2, 4, 8, and 24 hr after treatment and centrifuged immediately. Plasma was collected and stored at −20° until analysis. Urine was collected after treatment as follows: 0–8, 8–24, 24–48, and 48–72 hr.

Experiment 2—Two healthy volunteers were each given a single oral dose of 25 mg of triclosan in a cachet. Blood was collected 2, 4, 6, and 8 hr after administration.

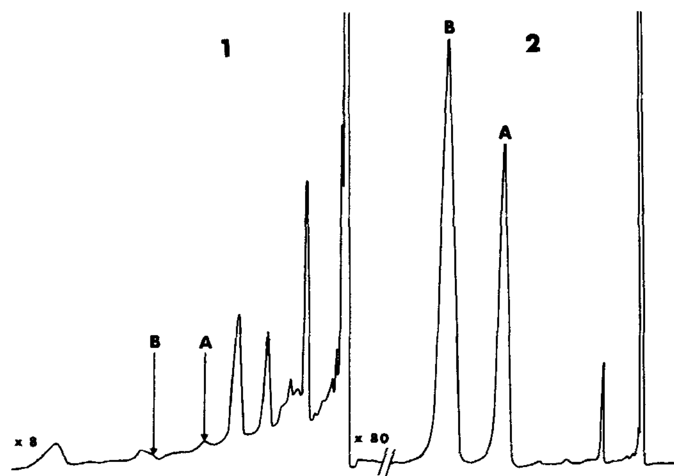


Figure 1—*Examples of chromatograms of human plasma blank (1) and 500 ng/ml of triclosan (A) and internal standard (B) in human plasma (2).*

[10] Vortex mixer.

RESULTS AND DISCUSSION

Analytical Yield—The duration of the acetylation reaction was varied from 5 to 30 min. A maximum was reached after 15 min.

The overall analytical yield was determined by comparing samples spiked with known amounts of triclosan and internal standard with corresponding extracts to which known amounts of the two acetyl derivatives had been added just before injection. The average yields (±*SE*) of five assays were 59.6 ± 6.0% for triclosan and 77.0 ± 4.5% for the internal standard in plasma and 65.2 ± 4.0% for triclosan and 77.2 ± 4.7% for the internal standard in urine.

Sensitivity and Accuracy—Table I gives the results obtained with spiked plasma samples in the concentration range of 2–6000 ng/ml. The 95% confidence intervals were calculated for five replicate analyses on each sample. The lower concentration may be taken as the sensitivity limit of the assay, although even lower concentrations could be detected.

Plasma or Urine Interference—Figure 1 shows the chromatograms of a blank human plasma extract and of plasma spiked with 500 ng of both triclosan and internal standard. No interference of the normal plasma components was recorded. Urine contained fewer detectable substances. Enzyme or acid hydrolysis did not change the chromatograms.

Specificity—Since the metabolism of triclosan has not been fully elucidated, the specificity of the assay was tested by comparing GLC with isotope dilution analysis after administration of the ^{14}C-labeled drug.

In urine, there was a satisfactory agreement between the two techniques for free (Table II) and free plus conjugated triclosan (Table III). The conjugate present in urine was entirely a glucuronide, as shown by the similar results obtained by acid and β-glucuronidase hydrolysis.

In plasma, a first experiment (Table IV) showed a large difference between these two types of hydrolysis. After β-glucuronidase hydrolysis,

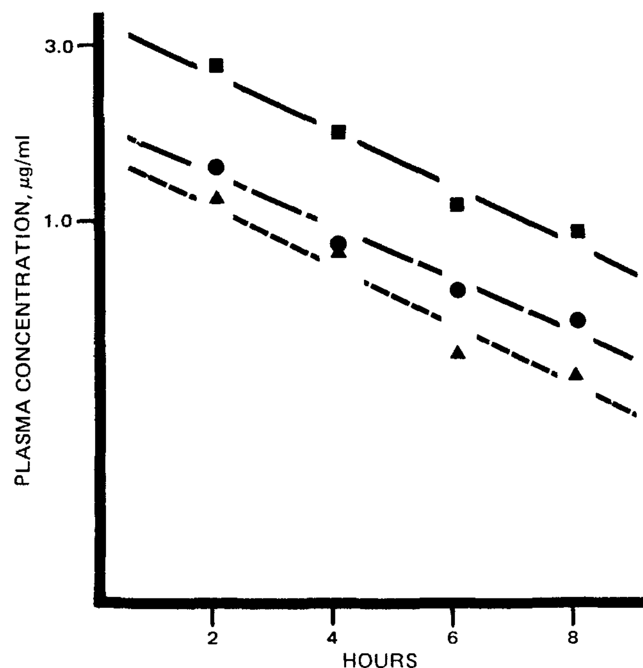


Figure 2—*Plasma concentrations of triclosan conjugated metabolites after oral administration of 25 mg of triclosan. Key: ▲, glucuronide; ■, glucuronide plus sulfate; and ●, sulfate (by difference).*

Table IV—Free and Conjugated Triclosan (Micrograms per Milliliter) in Plasma after Human Experiment 1

Hours	Total Radioactivity	Hydrolysis with β-Glucuronidase Isotope Dilution Analysis	Hydrolysis with β-Glucuronidase GLC	Hydrolysis with Hydrochloric Acid, Isotope Dilution Analysis
2	1.11	0.56	0.56	1.00
4	10.54	4.70	6.02	8.32
8	8.28	2.73	3.85	7.05
24	2.45	1.13	1.32	1.97

however, GLC and isotope dilution analysis gave comparable results. The conjugate present in plasma was only partly a glucuronide.

A second experiment (Table V) demonstrated that the same results were obtained by acid hydrolysis and by treatment with β-glucuronidase and sulfatase. The second conjugate thus appeared to be a sulfate.

Table V—Free and Conjugated Triclosan (Micrograms per Milliliter) in Plasma after Human Experiment 2

Volunteer	Hours	Hydrolysis by β-Glucuronidase Alone	Hydrolysis by β-Glucuronidase With Added Sulfatase	Hydrolysis by Hydrochloric Acid
1	2	1.10	2.72	2.56
	6	0.36	1.36	1.34
	8	0.28	1.14	1.12
2	2	1.18	2.62	2.46
	4	0.86	1.74	1.60
	6	0.46	1.15	1.08
	8	0.41	0.98	0.96

Hydrolysis Reproducibility—Glucuronidase-sulfatase enzyme hydrolysis gives reproducible results. Acid hydrolysis may create problems, which seem to be due to partial degradation of triclosan and the internal standard, to differing extents, during acid treatment. Therefore, enzyme hydrolysis is preferable.

Application—The technique was applied to a study of the elimination of triclosan after oral administration to human subjects (Experiment 2). The concentrations of free triclosan were extremely low. The two conjugated metabolites were eliminated at the same rate, corresponding to a half-life of about 4 hr (Fig. 2).

Conclusion—The proposed technique permits the quantitative assay of triclosan and its conjugated metabolites in human plasma and urine. It is specific, reproducible, and sufficiently sensitive for the determination of the absorption of triclosan in humans.

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(2) J. Schulze, F. H. Marcquardt, F. Lyman, and C. Spitzer, *J. Am. Oil Chem. Soc.,* **52,** 215 (1975).

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Fluorometric Determination of Nadolol in Human Serum and Urine

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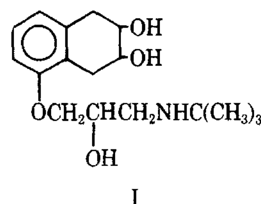
Abstract □ To determine nadolol, a new β-adrenergic blocking agent, in serum and urine, the drug is extracted into *n*-butyl acetate or ether from alkaline potassium chloride saturated samples. After back-extraction into 0.1 *N* HCl, the drug is oxidized with periodic acid; the resulting aldehyde is coupled with *o*-phenylenediamine to produce a fluorescent compound. The method can measure as little as 0.01 μg of nadolol/sample.

Keyphrases □ Nadolol—fluorometric analysis, human serum and urine □ Fluorometry—analysis, nadolol in human serum and urine □ Antiadrenergics—nadolol, fluorometric analysis in human serum and urine

Nadolol (I), a new β-adrenergic blocking agent, is designated chemically as *cis*-5-[3-[(1,1-dimethylethyl)amino]-2-hydroxypropoxy] - 1,2,3,4-tetrahydro-2,3-naphthalenediol. Its pharmacology (1–4), antiarrhythmic action (5–8), and specificity of action (9) were discussed previously.



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For measuring microgram or nanogram levels of nadolol in human urine or serum, a sensitive method is needed. This paper describes a fluorometric method based on the oxidation of the drug with periodic acid and coupling of the resulting aldehyde with *o*-phenylenediamine.

EXPERIMENTAL

Apparatus—Fluorometric measurements were made in a spectrofluorometer[1] equipped with a colored filter[2] and round microcells. Samples were shaken on a heavy-duty shaker[3]. Screw-capped, 150-mm test tubes and plastic caps with polypropylene linings[4] were washed by shaking with 20 ml of 1 *N* HCl for 1 hr, rinsed three times with tap water and three times with distilled water, and then dried.

[1] Perkin-Elmer model 204.
[2] Corning No. 3-73.
[3] Fisher Scientific.
[4] Polyseal Corp.