Determination of Free and Conjugated Triclosan[1] in Blood by Electron Capture Gas Liquid Chromatography[2]

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ABSTRACT

Unconjugated triclosan in blood can be determined by extraction with hexane, followed by its conversion to an acetyl derivative and analysis by electron capture gas liquid chromatography. The conjugated triclosan can be analyzed by an identical procedure, after the complexes have been hydrolyzed with hydrochloric acid. The method was validated by recovery studies using spiking and radioactive techniques and can be used for the determination of triclosan in blood in the concentration range of 1 ppb (ng/ml) to 100 ppm (μg/ml). The precision and accuracy of the method are discussed, and examples of its application are presented.

INTRODUCTION

Triclosan is a novel broad spectrum antibacterial agent (1,2), which was determined in the past in our laboratory, both qualitatively and quantitatively, in a variety of substrates utilizing several analytical techniques. Of these techniques, the simplest is the UV spectrophotometric determination at the 282 nm absorption maximum of the compound. This method can be used to detect concentrations as low as 5 μg/ml, but, due to its low selectivity, it is limited in its applications. Of much broader applicability are methods which are not only quantitative but, in addition, are also highly selective. Thus, thin layer chromatography (TLC), when coupled with a scanning densitometer, can detect and measure 1 μg triclosan (Fig. 1), and high pressure liquid chromatography gives valid results with as little as 20 ng substance (Fig. 2). None of these techniques is sensitive enough to carry out the analyses required for toxicological investigations. Only gas liquid chromatography (GLC), with an electron capture detector, gave us the tool which satisfied the requirements of blood analysis at the picogram level.

In addition to the choice of the actual measuring technique, the pre-treatment of the sample was also a matter of concern. In blood level studies, such a pre-treatment usually includes an extraction with a solvent of the lowest possible polarity, since such a low polarity reduces the chance of extracting the generally less lipophylic metabolites and extraneous materials. Thus, a simple hydrocarbon, such as hexane, which was reportedly used with success in similar areas of work (3,4) became our choice as the extracting solvent. Another potential problem for this analysis was the fact that phenolic compounds tend to adsorb on GLC columns, which leads to tailing and erratic results (5,6). This problem can be avoided by derivatization, and, since acetylation is known to be the best for similar compounds (7), an acetylation step also was incorporated into our method.

This paper describes an analytical procedure for the determination of conjugated and unconjugated triclosan in blood, which is based upon all the above mentioned considerations.

[1]Triclosan is the generic name of 2',4,4'-trichloro-2-hydroxydiphenyl ether, and is produced by CIBA-GEIGY Corporation under the tradename IRGASAN DP-300.

[2]Presented at the AOCS Spring Meeting, Mexico City, April 1974.

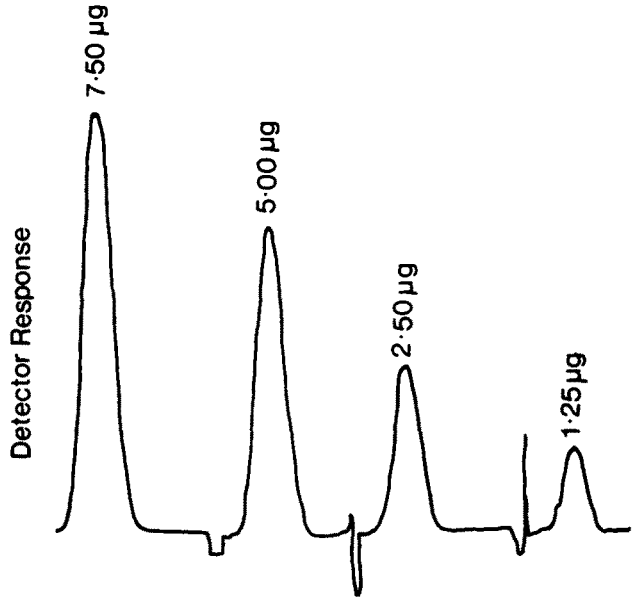

FIG. 1. Thin layer chromatography (TLC) analysis: Fluorodensitometer curve of the TLC spots from four standard solutions of triclosan. Plate: Silica Gel GF; eluent: benzene/methanol = 90/10; scanner: Hitachi model MPF-2A fluorescence spectrophotometer with model 018-0057 TLC accessory.

EXPERIMENTAL PROCEDURE

Materials and Equipment

Hydrochloric acid, pyridine, acetic anhydride, citric acid

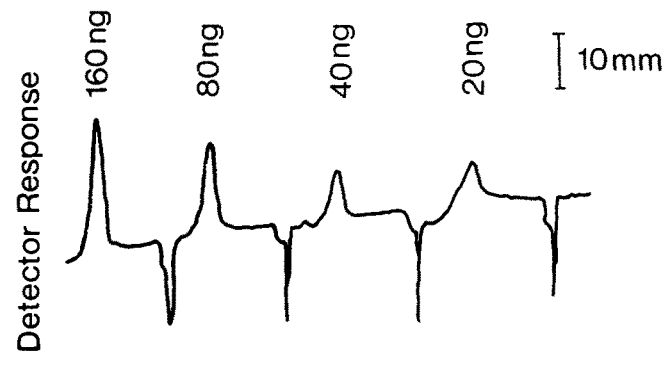

FIG. 2. High pressure liquid chromatogram of four standard solutions of triclosan. Instrument: Nester Faust model 2100; column: 0.5 meter – 2.1 mm ϕ – stainless steel – Vydac reverse phase; eluent: water/CH_3CN = 60/40, 0.82 ml/min; detector: UV – 254 nm.

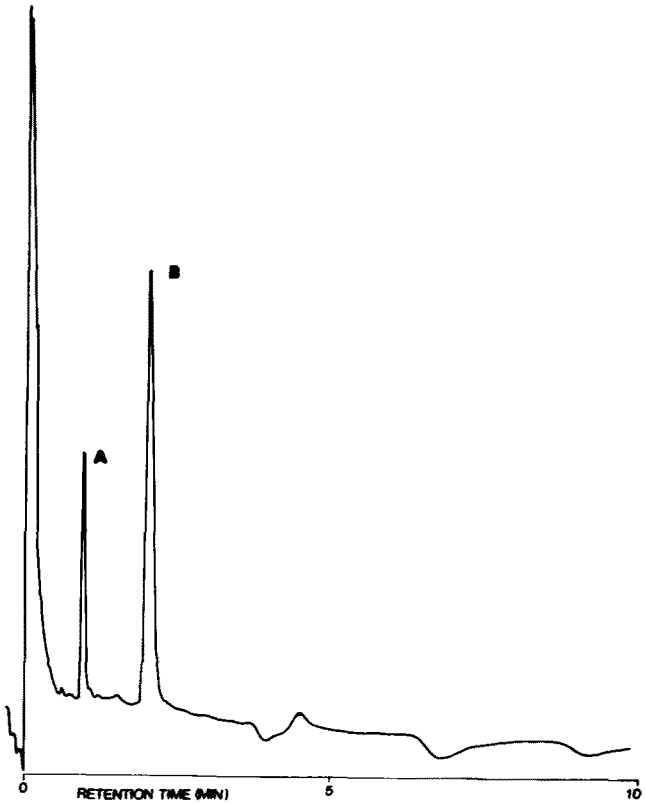

FIG. 3. Electron capture gas liquid chromatogram of an acetylated extract of blood containing 487 ng/ml triclosan. A = internal standard Aldrin (70 pg) and B = triclosan (146 pg).

(anhydrous), and disodium hydrogen phosphate (heptahydrate) were all Baker analyzed reagents. Hexane (Pesticidequality) was obtained from Matheson, Coleman & Bell, East Rutherford, N.J.; sodium heparin injection USP 5000 from Spencer-Mead, Valley Stream, N.Y.; and Aldrin from K&K Laboratories, Plainview, N.Y.

Buffer solution pH 2.2: Citric acid (20.6 g) and disodium hydrogen phosphate (1.07 g) were dissolved in 1 liter deionized water.

Dilute heparin solution: Sodium heparin injection USP 5000 was diluted 10-fold with deionized water.

Acetylating reagent: Equal volumes of acetic anhydride and pyridine were mixed.

Internal standard solution: Aldrin (17.5 mg) was dissolved in 100 ml absolute ethyl alcohol, and this stock solution brought to a concentration of 17.5 ng/ml by a series of dilutions with hexane.

Gas chromatograph: GLC analysis was accomplished with Hewlett-Packard 5750 and 7610 gas chromatographs equipped with 4 ft x 1/4 in. glass columns packed with 3% OV-17 on 80-100 mesh Supelcoport and ^{63}Ni detectors which worked in a pulsed mode with a 50 micro second pulse interval. The column was conditioned prior to use at 260 C for 24 hr under argon with 5% methane at 54 ml/min. These same gas and flow rates also were used for chromatography, with a column temperature of 230 C, injection port temperature of 240 C, and detector temperature of 290 C. The range and attenuation settings were 10^2 and 4, respectively, and the signal intensity was measured by peak ht.

TABLE I

Recovery of Free Triclosan from Fortified Whole Blood (Duplicate Determinations)

Triclosan added (ng/ml)	Recovery (%)		
	First determination	Second determination	Average
100	92.4	95.2	93.8
50	98.6	97.5	98.1
12.5	96.0	96.0	96.0

Other equipment: Other equipment used included: nitrogen evaporator (N-Evap) model 106, Organomation Associates, Shrewsbury, Mass.; Super-Mixer, Lab-Line Instruments, Melrose Park, Ill.; International centrifuge size 2 model K with no. 267 rotor, International Equipment Co., 45 ml glass centrifuge tubes with Teflon lined screw caps; 5 ml graduated glass centrifuge tubes with glass stoppers; and serological glass disposable pipettes (assorted sizes), Corning Glass Works, Corning, N.Y.

Analytical Method

For the determination of free triclosan, a 3.0 ml sample of blood was treated in a 45 ml centrifuge tube with 1 ml diluted heparin solution and 1 ml pH 2.2 buffer. The mixture was agitated vigorously for 3 min with 10 ml hexane, frozen at -20 C, and left to thaw at room temperature, and the phases were separated by 5 min of centrifugation. The clear supernatant then was diluted or concentrated in such a way that its triclosan concentration was between 20-200 ng/ml (the response of the electron capture detector is linear with the concentration only up to 200 ng/ml). A 1.0 ml sample of this adjusted solution was transferred to a 5 ml centrifuge tube, 1.0 ml internal standard solution added, and the volume reduced to ca. 1/2 ml under a stream of nitrogen at room temperature. The residue was mixed thoroughly with 0.1 ml of the acetylating reagent and the tube stoppered and heated for 10 min in a 60 C water bath. After cooling to room temperature, the volume was made up to 1.0 ml with hexane, and the resulting solution was washed by mixing with 1 ml water. A 4 μliter sample of the clear organic supernatant then was analyzed by electron capture gas chromatography. The triclosan content was calculated either by using the aldrin signal as internal standard or by comparing the intensity of the triclosan signal to a calibration curve, which had been prepared from a series of triclosan solutions by acetylation and GLC measurement in the above described way.

For the determination of total (free and conjugated) triclosan content of blood, a 2 ml sample of blood was mixed in a closed centrifuge tube with 3 ml hydrochloric

TABLE II

Recovery of Total Triclosan from Whole Blood (Triplicate Determinations)

Concentration by LSC[a] (μg/ml)	Recovery (%)			
	First determination	Second determination	Third determination	Average
7.28	96.3	97.7	98.6	97.5
113.93	95.0	88.4	96.9	93.4

[a]LSC = liquid scintillation counting.

TABLE III

Precision of the Determination of Free Triclosan in Blood

Sample	Peak ht			Peak area		
	Aldrin	Triclosan	Ratio	Aldrin	Triclosan	Ratio
1	51	108	2.12	712	3180	4.47
	52.5	109.5	2.09	741	3293	4.44
		108.8	2.105		3237	4.46
2	50	109	2.18	727	3136	4.31
	48	105	2.19	688	3036	4.44
		107.0	2.185		3086	4.38
3	49	105	2.14	724	3343	4.62
	49	106	2.16	715	3415	4.77
		105.5	2.150		3379	4.70
Average		107.1	2.148		3234	4.51
Standard deviation		1.5%	1.9%		4.5%	3.7%

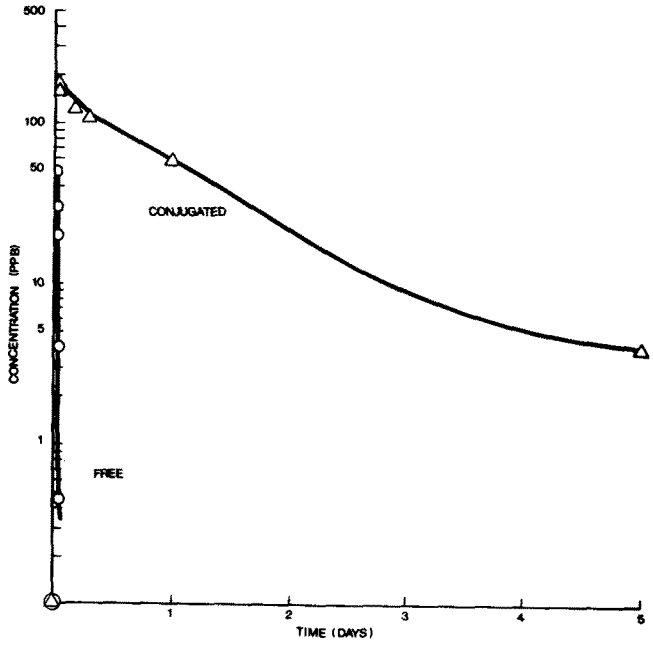

FIG. 4. Change of blood level of triclosan in beagle dogs after single intravenous application of a 1 mg dose (semilog plot).

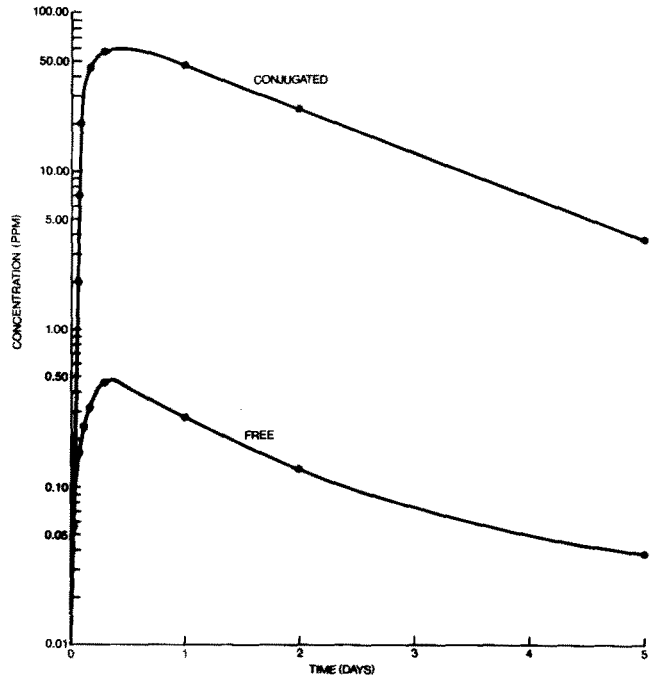

FIG. 5. Change of blood level of triclosan in beagle dogs after single per oral application of a 100 mg/Kg dose (semilog plot).

acid and heated for 1 hr in a 100 C oil bath. After this hydrolysis step, the sample was extracted with 10 ml hexane, and the above procedure was followed for derivatization and analysis.

RESULTS AND DISCUSSIONS

The chromatogram obtained by this analytical procedure (Fig. 3) shows well resolved signals for the solvent, the aldrin (internal standard), and the triclosan acetate, with retention times of 1 and 2 min, respectively, for these last two signals. Quantitative interpretations of these chromatograms can, therefore, be done easily, and a 5% full-scale deflection was observed for 100 picograms of triclosan. The sensitivity of the method is, therefore, such that it can detect the presence of 1 ng/ml triclosan in blood.

Figure 3 also shows that the gas chromatogram has negative signals at 4, 7, and 9 min. These negative signals were reproducible. In case of repetitive analysis, care must be taken that these negative signals do not overlap important analytical peaks.

Typical recovery values of free triclosan from whole blood were determined by analyzing samples which had been fortified at levels of 12.5-100 ng/ml. In this range, it was found that the recovery was 96 ± 2% as shown in Table I.

The investigation of the recovery of total, i.e. conjugated and unconjugated, triclosan from whole blood involved obtaining blood samples from dogs which had been fed with appropriate doses of radiolabeled triclosan (1-^{14}C-triclosan with a specific activity of 0.0652 μCi/mg), so that the triclosan could go through its normal metabolic transformations prior to sampling of the blood. The recovery then was determined by comparing the EC-GLC results with those obtained by liquid scintillation counting of combusted blood. Table II shows that, for the concentration range of 10-100 μg/ml, recoveries of 93-98% were obtained.

The precision of the method was determined by repetitive analysis of whole blood samples which had been fortified with triclosan to a concentration of 487 ng/ml. Table III summarizes the results, showing that standard deviations of 1.5-1.9% characterize this analytical method. It was surprising that lower precision (standard deviation = 3.7-4.5%) was observed when peak areas were used instead of peak ht for these analyses.

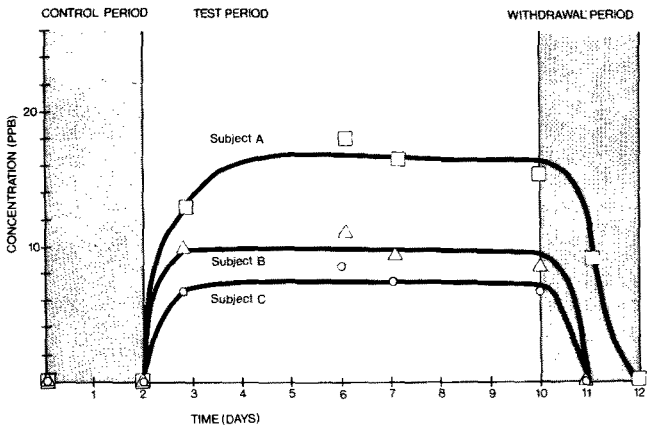

FIG. 6. Change in blood level of free triclosan in the course of a handwash study. □ = Subject A, △ = subject B, ○ = subject C.

TABLE IV

Recovery of Free Triclosan from Fortified Urine

Triclosan concentration		Recoveries
Added (ng/ml)	Found (ng/ml)	(%)
120	109.0	91
60	52.9	89

TYPICAL APPLICATIONS OF PROCEDURE

The method was applied successfully to analyze large numbers of rabbit, dog, cat, monkey, and human blood samples to establish the safety of triclosan. Three examples of the application of this method are shown below.

Dose Kinetic Study in Dogs

The concentration of free and total triclosan was determined in whole dog blood at fixed time intervals after a single intravenous injection of 1 mg substance or after a single oral application of 100 mg/kg dose. Figures 4 and 5 show the results of these studies. After intravenous injection, the disappearance of unconjugated triclosan from the bloodstream is very fast, its kinetic time scale being measured in min, while the disappearance of the conjugated compound is somewhat slower, its kinetic time scale being measured in days. This last time scale corresponds to that found in excretion studies carried out with radiolabeled triclosan.

The measurements for this investigation were carried out on blood samples which contained triclosan in the range of concentrations of 5 ng/ml (ppb)-60 μg/ml (ppm).

Human Handwashing Studies

Free triclosan was monitored for this study in the blood of three subjects who regularly used triclosan containing soap. The blood levels found in this investigation were all in the range of 5-18 ng/ml. In all the subjects, a constant level was reached after ca. 1 day of usage, and all the triclosan again was eliminated from the blood in the course of 1-2 days after the use of triclosan ceased (Fig. 6).

Triclosan Determination in Human Urine

The analytical procedure also could be applied to the determination of triclosan in urine. Typical recoveries of 89-91% were obtained from samples which had been fortified at levels of 60-120 ng/ml (Table IV).

ACKNOWLEDGMENT

The radiolabeled triclosan was prepared by D. Ryskiewich, and part of the analytical work was carried out with the assistance of D. Smith.

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