also be applicable to urine and plasma samples obtained after administration of conventional doses of the drug.

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Quantitative GLC Determination of Cyclophosphamide and Isophosphamide in Biological Specimens

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Abstract □ A method for measuring the antitumor agent cyclophosphamide was developed and applied to the determination of the drug in biological specimens. After extraction with ether, cyclophosphamide and the internal standard isophosphamide are converted to their trifluoroacetyl derivatives and assayed by GLC, using either an electron-capture or a flame-ionization detector. The minimum detectable amount is 25 pg/injection using the electron-capture detector. Linearity was found up to microgram amounts of substance, without any interference of endogenous substrates. Values from serum, urine, and liver in mice treated with a single dose of cyclophosphamide (85 mg/kg) are also reported.

Keyphrases □ Cyclophosphamide—quantitative GLC determination in biological specimens □ Isophosphamide—quantitative GLC determination in biological specimens □ GLC—cyclophosphamide and isophosphamide, quantitative determination in biological specimens

Cyclophosphamide {2-[bis(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide} (I) is a well-known anticancer drug utilized both in animal studies and in clinical practice (1–3).

Colorimetric, titrimetric, and IR methods have been used for estimating I (4–8), although they suffer from a lack of sensitivity and specificity, particularly when applied to biological specimens. Labeled I has been also utilized, but the results require validation for specificity because I undergoes a complicated metabolic pathway (9–12).

More recently, a GLC method for measuring I was described (13), but its usefulness in quantitative determination was limited by the presence of decomposition products. Compound I was also measured by direct injection into the ion source of a mass spectrometer, but the specificity of the determination was not accompanied by sufficient sensitivity (14).

The method described here does not suffer from some of these disadvantages. It is based on the formation of a stable trifluoroacetyl derivative of I suitable for GLC analysis of biological specimens (Scheme I). Isophosphamide [3-(2-chloroethyl)-2-(2-chloroethylamino)tetrahydro-1,3,2-oxazaphosphorine-2-oxide] (II), an analog of I with similar antitumor activity (15–17), also forms a stable trifluoroacetyl derivative (Scheme I) and was chosen as the internal standard for quantitation of I.

EXPERIMENTAL

Standards and Reagents—The following reagents were used: trifluoroacetic anhydride[1], sodium hydroxide, ether, and ethyl acetate[2].

Cyclophosphamide[3] and isophosphamide[3] were used as the hydrate salts, with all concentrations expressed in terms of the free base. Drugs were dissolved in double-distilled water; methylaminochlorobenzophenone[4] (internal marker) was dissolved in ethyl acetate.

GLC—GLC was carried out on a gas chromatograph[5] equipped with a flame-ionization detector[6] or a ^{63}Ni electron-capture detec-

[1] Fluka AG, Buchs, Switzerland.
[2] Carlo Erba, Milan, Italy.
[3] Supplied by Asta Werke AG, Brackwede, West Germany.
[4] Supplied by Ravizza, Milan, Italy.
[5] Carlo Erba Fractovap model Gl.
[6] Carlo Erba model 20.

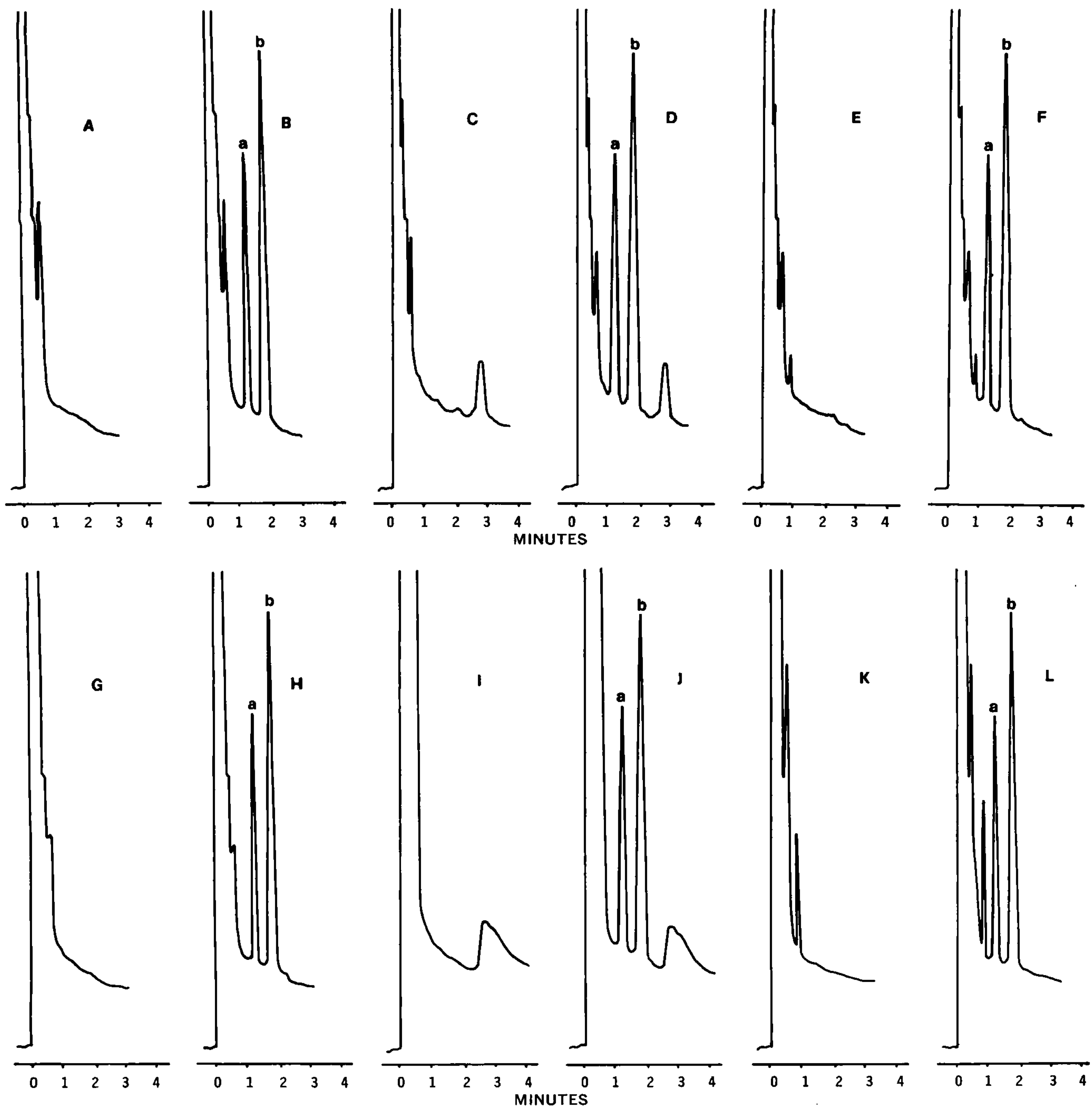

Figure 1—*Gas chromatograms of extracts by using an electron-capture detector: A, serum blank; B, I (peak a) and II (peak b) extracted from serum; C, liver blank; D, I (peak a) and II (peak b) extracted from liver; E, urine blank; and F, I (peak a) and II (peak b) extracted from urine. Gas chromatograms of extracts by using a flame-ionization detector: G, serum blank; H, I (peak a) and II (peak b) extracted from serum; I, liver blank; J, I (peak a) and II (peak b) extracted from liver; K, urine blank; and L, I (peak a) and II (peak b) extracted from urine. All other peaks present in the chromatograms are due to endogenous substances present in the final residue.*

tor[7]. In both cases the column was a glass tube, 1.5 m long and 4 mm i.d., packed with 100–120-mesh Chromosorb Q coated with 3% SE-30[8].

All newly prepared columns were conditioned at 280° for 1 hr without carrier gas flow and then for 12 hr with a carrier gas flow rate of 15 ml/min. During analysis, nitrogen was used as the carrier gas at a flow rate of 35 ml/min for both electron-capture and flame-ionization detection. Air and hydrogen flow rates were adjusted to give maximum response when operating with the flame-ionization detector. The operating conditions were: column oven temperature, 200°; injection port heater temperature, 250°; flame-ionization detector temperature, 250°; and electron-capture detector temperature, 280°. The electron-capture detector was used under the following conditions: pulse current; excitation voltage, 10 v; pulse width, 3 μsec; period, 30 μsec; and scavenger gas (nitrogen), 35 ml/min.

Mass Spectrometry—A mass spectrometer[9] coupled with a gas chromatograph was used under the following conditions: energy of the ionization beam, 70 ev; ion source temperature, 290°; acceler-

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[8] Applied Science Laboratories, State College, Pa.
[9] LKB model 9000.

Table I—Extraction Recoveries of Cyclophosphamide and Isophosphamide from Water, Serum, Liver, and Urine

Drug	Percent Extraction Recovery from			
	Water	Serum	Liver	Urine
Cyclophosphamide	77.4 ± 0.8	77.0 ± 1.5	78.3 ± 1.2	77.1 ± 1.3
Isophosphamide	76.2 ± 1.3	77.8 ± 1.9	80.7 ± 2.1	80.3 ± 1.8

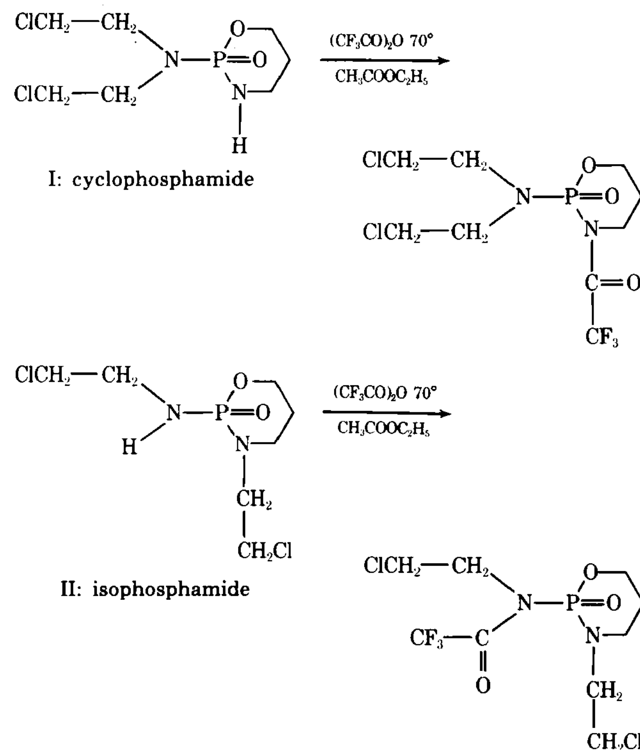
ating voltage, 3.5 kv; and trap current, 60 μmp. The introduction of the sample was carried out by a GLC procedure on a glass column, 1.5 m long and 4 mm i.d., packed with 3% SE-30 on Chromosorb Q (100–120 mesh) under the following conditions: injector temperature, 240°; oven temperature, 200°; helium (carrier gas) flow rate, 35 ml/min; and detector, total ion monitor.

Extraction Procedure from Water, Serum, Liver, and Urine—The procedure was designed to be suitable for the measurement of I in the range of picograms up to micrograms. To 0.1 ml of water, serum, or urine or to 100 mg of mouse liver (after homogenization) were added (depending on the anticipated concentration range for quantitation and the detector utilized) either 100 ng or 5 μg of II (internal standard), 0.9 ml of 0.1 *N* NaOH, and 5 ml of ether. The appropriate amount of internal standard to be added to 0.1 ml of specimen was determined from a preliminary experiment to find a suitable ratio of peak areas with respect to I (Fig. 1).

The test tubes were shaken mechanically for 10 min and, after centrifugation for 5 min at 4°, 4.5 ml of the organic phase was transferred to a second test tube. Then it was dried under a gentle stream of nitrogen at 35° in a water bath. The alkaline aqueous layer was extracted again with another 5 ml of ether and, after shaking and successive centrifugation, 5 ml of the organic phase was transferred into the corresponding test tubes and then dried.

The extraction recoveries of I and II from water, serum, liver, and urine are reported in Table I.

Trifluoroacetylation Reaction—The dry residue was redissolved in 150 μl of a mixture of trifluoroacetic anhydride and ethyl acetate (1:2 v/v), and the stoppered tubes were heated at 70° in a sand bath for 20 min. The samples were dried under nitrogen, 100 μl of ethyl acetate was added, and a 1-μl aliquot was injected into



Scheme I

Table II—Urinary Excretion of Cyclophosphamide Expressed in Micrograms and Percentage of the Administered Drug

Time[a]	Micrograms ± *SE*	Percent Administered Dose ± *SE*
0–1 hr	114.91 ± 8.54	5.46 ± 0.31
1–4 hr	17.15 ± 9.53	0.81 ± 0.45
4–24 hr	1.98 ± 0.84	0.09 ± 0.04
Total	124.23 ± 7.20	5.91 ± 0.34

[a] Determinations at 1 and 4 hr were performed by using a flame-ionization detector; determinations at 24 hr were performed by using an electron-capture detector.

the gas chromatograph. Reaction kinetics of the trifluoroacetyl derivative formation of I and II at room temperature (25°) and at 70° and the stability of the trifluoroacetyl derivatives in relation to time are reported in Fig. 2. Those measurements were performed using methylaminochlorobenzophenone as an internal marker added after the reaction.

Biological Measurements of I in Serum, Liver, and Urine—The validity of this procedure for the *in vivo* determination of I was demonstrated by studying the elimination of the drug in mice. Male C57 Bl/6J inbred mice, 25 ± 2 g, were injected with a single dose of 85 mg/kg iv of I, prepared immediately prior to administration by dissolving the compound in double-distilled water. Groups of five animals were sacrificed, and biological samples (serum and liver) were collected at 1, 5, 15, 30, 60, and 120 min after drug injection.

The method of "feathering" was applied for calculating pharmacokinetic parameters (18, 19). Urines were collected from mice housed in glass metabolic cages, and collections were made 1, 4, and 24 hr after I administration.

RESULTS AND DISCUSSION

Figure 1 shows some typical chromatograms of extracts from urine, serum, and liver. No interfering peaks from endogenous substrates were found. The calibration curves for I in serum are illustrated in Fig. 3; the linearity of the method ranges from concentra-

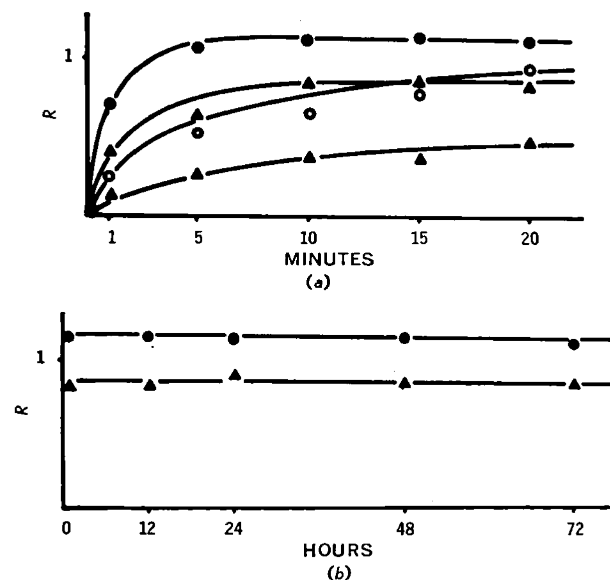


Figure 2—(a) *Reaction kinetics of I and II at room temperature and at 70°.* (b) *Stability of the trifluoroacetyl derivatives.* R = *the peak area ratio of I or II to the internal marker (methylaminochlorobenzophenone). Key:* △, *cyclophosphamide (25°);* ○, *isophosphamide (25°);* ▲, *cyclophosphamide (70°); and* ●, *isophosphamide (70°). These data were obtained from a concentration of 100 ng of I or II. Quantitation was performed using 0.5 μg of methylaminochlorobenzophenone as an internal marker added after the reaction.*

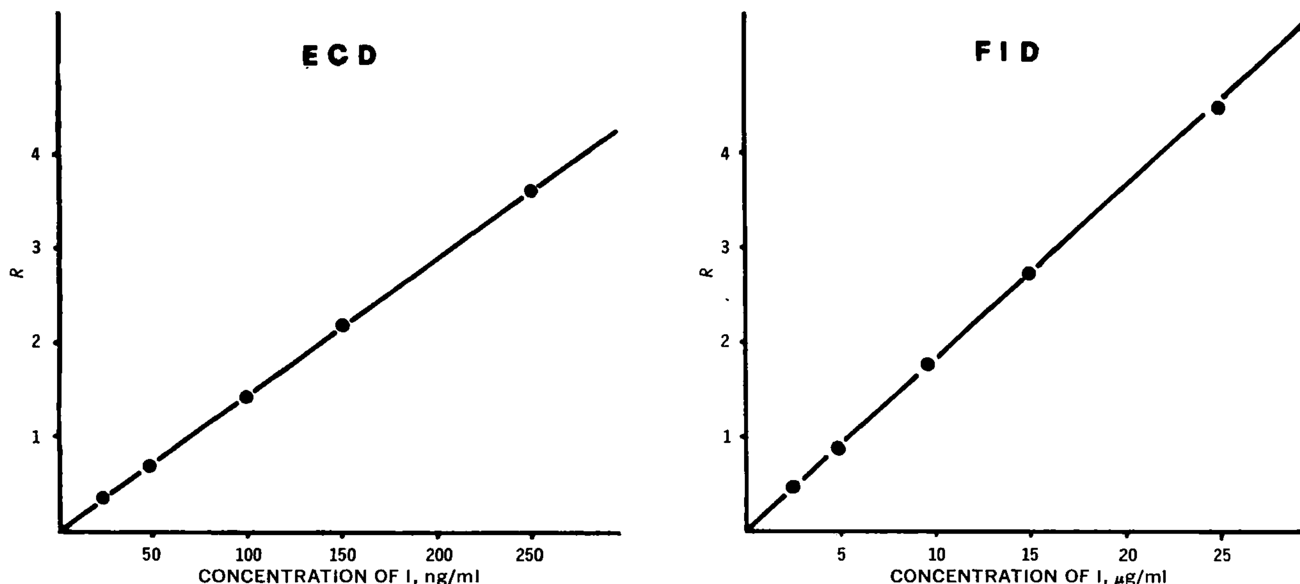


Figure 3—*Standard calibration curves of I to the internal standard II.* R = *the peak area ratio of I to II.*

tions of 25 to 250 ng/ml of specimen with the electron-capture detector and from 2.5 to 25 μg/ml of specimen with the flame-ionization detector.

Calibration curves from urine and liver are not reported because they were identical to those of serum, due to the same recovery after extraction (Table I). All these results are also valid when II is determined by utilizing I as an internal standard.

The chemical identity of the peaks obtained in the GLC analysis of I and II after reaction with trifluoroacetic anhydride was established by means of GLC–mass spectroscopy. The mass spectra (Fig. 4) show molecular ions at *m/e* 356, which means that only one trifluoroacetyl group is present in the molecules.

The results of the distribution in serum and liver of I are reported in Fig. 5. The uptake of the drug by the liver seems to occur very rapidly, since liver levels of the unchanged I are always higher than in serum. Indeed, at 5 min the drug concentration in liver is more than double that in serum. However, the half-life calculated within 30 and 120 min by the regression line of the logarithms of drug concentration *versus* time is not statistically different for serum and liver.

In the urine, I excretion proceeds very rapidly within the 1st hr, but the excretion of the unchanged drug is complete at 24 hr after

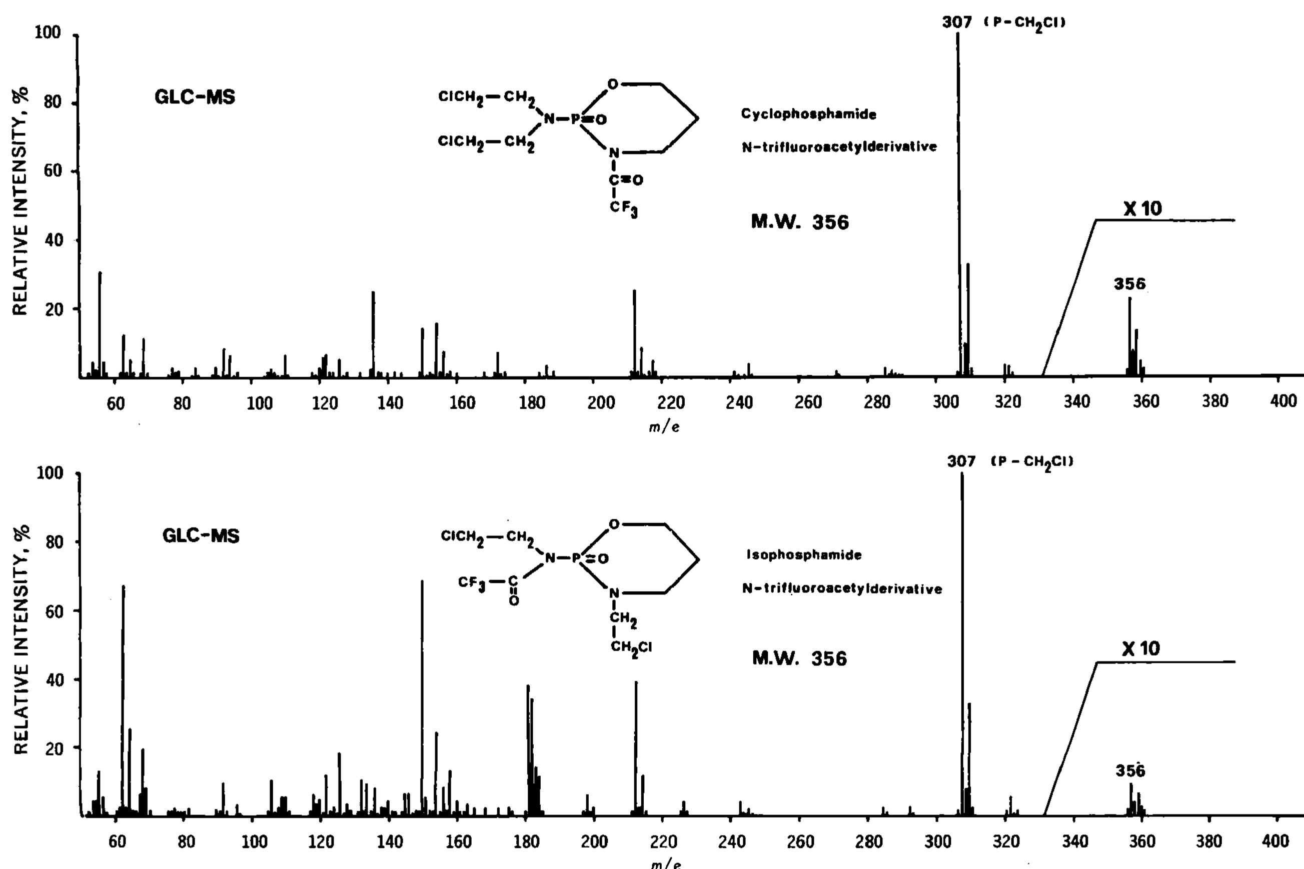


Figure 4—*Mass spectra of the trifluoroacetyl derivatives of I and II obtained by GLC–mass spectroscopy analysis.*

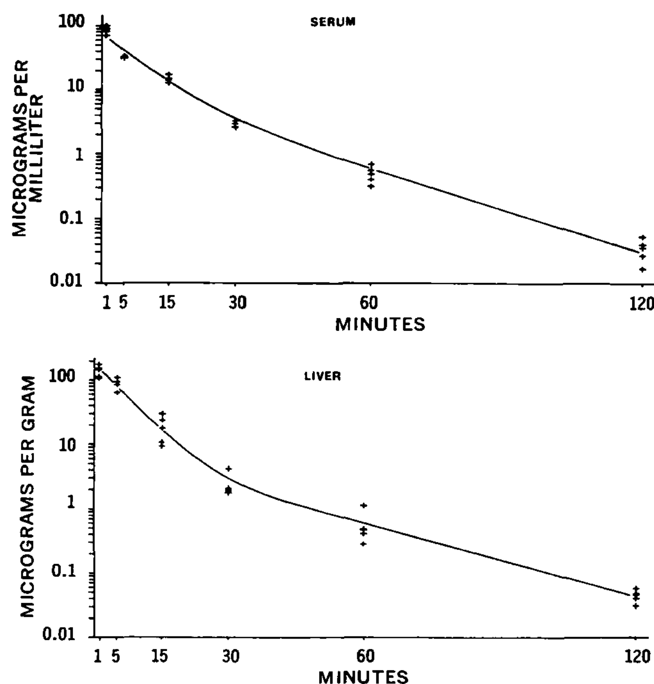


Figure 5—*Levels of I in serum and liver at different times after intravenous injection. Each point is the average of five determinations. Measurements at 1, 5, and 15 min were performed by using a flame-ionization detector, while measurements at 30, 60, and 120 min were performed by using an electron-capture detector. The pharmacokinetic parameters for serum were: half-life β,* $t_{1/2}$ *(min), 95% confidence limits 13 min 55 sec (12 min 45 sec–15 min 25 sec); volume of distribution,* Vd *(liter/kg), 95% confidence limits 0.076 (0.053–0.111); area under the curve,* $\int_0^\infty$ Cp dt *(µg/ml × min), 663.07; and total body clearance (liter/min/kg), 0.128. The pharmacokinetic parameters for liver were: half-life β,* $t_{1/2}$ *(min), 95% confidence limits 15 min 55 sec (14 min 10 sec–18 min 20 sec); and area under the curve,* $\int_0^\infty$ Cp dt *(µg/ml × min), 1255.31.*

the administration (Table II); a very low percentage (5%) of the unchanged I is excreted in the urine. Experiments are in progress to apply this technique to pharmacokinetic studies of I in tumor-bearing animals.

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