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ANALYSIS OF SOMAN AND SARIN IN BLOOD UTILIZING A SENSITIVE GAS CHROMATOGRAPHY–MASS SPECTROMETRY METHOD

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SUMMARY

Gas chromatography with electron impact mass spectrometry and selected ion monitoring provided a simple and sensitive method for measuring organophosphorus compounds sarin and the two isomers of soman (isomer I and isomer II) in blood. These compounds were extracted from blood or isotonic saline using a modification of the method developed by Sass *et al.* Blood was deproteinized with perchloric acid before extraction. The acid-induced degradation of the organophosphorus compounds could be minimized by neutralizing the acid immediately after deproteinizing. In saline and blood, 81% of the extractable soman and 74% of the extractable sarin was recovered with a single extraction. The overall recovery of added organophosphorus was less in blood than in saline because of the binding of organophosphorus to blood constituents, probably various enzymes and proteins. A time-dependent decrease in extractable organophosphorus was found in whole blood but not in saline. Although soman isomer II was degraded in blood faster than soman isomer I, no significant difference in the affinities of these two isomers to acetylcholinesterase was observed.

INTRODUCTION

Sarin and soman are two closely related members of the group of chemicals known as the organophosphates (OPs). OPs are highly toxic because they effectively inhibit the acetylcholinesterase (AChE) of blood and nervous tissues.

Other esterases present in blood and nervous tissues of mammals, including butyrylcholinesterase, serine esterase, and carboxylesterase, are also inhibited by the OPs[1,2]. Although the toxicological consequences of inhibition of these esterases are not known, the reaction of OPs with them may serve to reduce the amount of OP available for inactivation of AChE. Rodent plasma is known to have a high capacity for binding with soman[3].

Along with esterases, mammalian tissues also contain phosphorylphosphatase activity which hydrolyzes and irreversibly inactivates OP compounds[4,5]. The amount of free OP in tissues is further reduced by these enzymes, and, therefore, if organisms

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are exposed to OPs, the amount of free OP present in blood will depend upon the binding capacity of the various blood esterases and the activities of phosphoryl-phosphatases.

To determine the levels of free OPs in biological samples such as blood, sensitive, reliable, and simple methods for extraction and quantification of these OPs are essential. Gas chromatographic and gas chromatographic–mass spectrometric (GC–MS) methods were developed earlier for the analysis of sarin and soman in aqueous solutions and brine samples[6,7]. The purpose of this investigation was to develop a simple and rapid assay for determining the levels of sarin and soman in canine blood. We have used this technique to determine the amounts and stabilities of OPs added to the canine blood and to study the binding of these OPs to blood AChE and other proteins.

EXPERIMENTAL

Materials

The GC–MS system used was a Hewlett-Packard Model 5993C-OP-95 with an electron impact ionizer and an integral gas chromatograph. The column used in all analyses of OP compounds was a 15-m HP-SE-54 bonded methylsilicon column. Sarin and soman were provided by the U.S. Army Medical Research Development Command. Physostigmine and AChE were obtained from Sigma. Perchloric acid, chloroform (GC grade), and other reagents were purchased from Fisher Scientific.

GC–MS conditions

GC–MS conditions were selected to yield a short run time with clean separation of sarin and soman and with no interfering ions. The following GC conditions were found to be optimal for analysis of sarin or soman independently: inlet temperature, 180°C; oven temperatures, isothermal at 60°C for sarin and 75°C for soman; run times, 5 min. For the simultaneous analysis of sarin and soman, the GC was programmed as follows: inlet temperatures, 180°C; initial oven temperatures, 60°C; oven temperature program, isothermal at 60°C for 4 min, then increasing at 25°C/min to 250°C; run time, 13 min.

After analysis of ten blood samples, it was necessary to raise the oven temperature to 270°C to remove from the column impurities introduced from the chloroform extract.

The mass detector was set for an ion source chamber pressure of $6 \cdot 10^{-6}$ Torr and an electron impact voltage of 70 eV. Mass detection was initiated 1.5 min after sample injection to allow elution of solvent and to protect the ion detector. Characteristic ions of m/z 99 and 125 were monitored for sarin and m/z 99 and 126 for soman. When sarin and soman both were present in a sample, m/z 99 and 125 were monitored for the first 3.5 min to detect sarin and m/z 99 and 126 for the next 6 min to detect soman.

Extraction procedure and analysis

To establish a standard curve, known amounts of sarin or soman (ranging from 1 to 5 nmol/ml) were added to 4.0 ml of chloroform and 1.0 μl of the various concentrations injected into the GC–MS system. The integrated areas of the peaks

at ions of m/z 99 and 125 were directly proportional to the amount of soman or sarin, respectively, injected up to a concentration of 5 nmol/ml.

To determine recovery from extracted saline and blood samples, known amounts of sarin or soman (ranging from 1 to 5 nmol/ml) were added to 1.0 ml of blood or saline. Freshly drawn, heparinized canine blood and isotonic saline were used. After OP had been added, the samples were mixed with 4.0 ml of 0.3 *N* perchloric acid. The samples were then centrifuged at 1500 *g* for 5 min at 4°C. The supernatants were rapidly filtered through a Whatman GB filter into tubes containing 1.0 g of sodium chloride. The salt was dissolved, 100 μl of chloroform were added, and the tubes were vortexed for 2 min. The chloroform layer was collected, and exactly 3.0 μl were injected directly into the GC–MS system. A small amount of saline was layered on the chloroform extract to prevent evaporation. In initial experiments the perchloric acid supernatant was extracted a second time with 100 μl of chloroform and the OP content was analyzed. It was consistently found that the second extraction contained an additional 16% of the free, extractable soman or sarin. Therefore, subsequent experiments were conducted with single extractions and are based on an extraction efficiency of 81% for soman and 74% for sarin (see Fig. 4).

Determining the binding of OP to blood ChE and purified AChE

To determine the affinity of OP to blood ChE, fresh blood was incubated with physostigmine (36 nmol/ml) for 30 min at room temperature prior to the addition of OP. The OP was extracted as described above, and the levels of sarin or soman were determined. The amount of sarin or soman (sum of two isomer peaks) bound to ChE was calculated by taking the difference between the measured OP levels in physostigmine-treated and untreated blood samples.

To determine the binding of soman to AChE, 2.75 nmol of soman were added to 1 ml of AChE solution purified from electric eel (in normal saline, 185 ng of protein and 250 U total activity). The mixture was incubated for 30 min at room temperature. The amount of soman left unbound was determined by GC–MS and subtracted from the amount added initially.

Determining the stability of OP in perchloric acid

Sarin (3.6 nmol) or soman (2.75 nmol) was added to 1 ml of isotonic saline, and 4 ml of 0.3 *N* perchloric acid were added to each sample. The OP was extracted at 0, 5, 10, 15, 20, 30, 45, and 60 min after the addition of the acid by the method described above. Then 3 μl of the chloroform phase were injected directly into the GC–MS system to determine the OP levels.

To determine whether the neutralization of perchloric acid immediately after deproteinization would prevent acid-induced degradation, sarin (3.6 nmol) or soman (5.5 nmol) was added to three groups of tubes (each group prepared in triplicate) containing 1 ml of isotonic saline, and 4 ml of 0.3 *N* perchloric acid were added to each tube. Immediately after the addition of perchloric acid, the samples were divided into three groups ($n = 3$) and were treated as follows:

Group 1, OPs were extracted 5 min after the addition of acid.

Group 2, OPs were extracted 60 min after the addition of acid.

Group 3, The acid was neutralized with 3 *M* sodium hydrogen carbonate immediately after addition to OP samples, and OPs were extracted after 60 min.

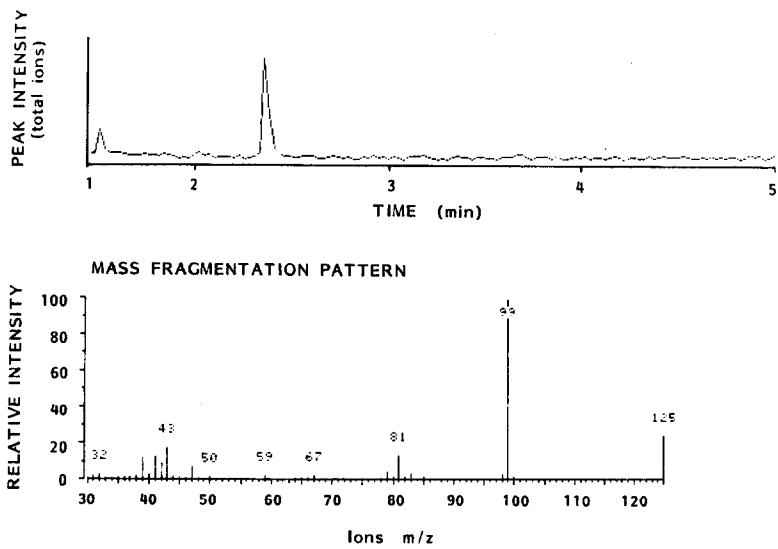

Fig. 1. GC–MS analysis of sarin. A total ion chromatogram of the analysis of sarin is shown at the left. The mass fragmentation pattern of the sarin peak at 2.4 min is shown below.

Determining the stability of OP in the blood

Sarin (3.6 nmol) or soman (2.75 nmol) was added to blood or isotonic saline at room temperature, and 1-ml aliquots were removed at 0, 5, 10, 15, 20, and 30 min and extracted for OP. The levels of sarin and individual isomers of soman were determined.

RESULTS AND DISCUSSION

Selection of ions

The fragmentation pattern for sarin shows that the highest ion observed was *m/z* 125 $(M-15)^+$, whereas the ion present in greatest abundance was *m/z* 99. No molecular ion was observed for sarin (Fig. 1). Sass and Fisher[8] have reported that major ions produced by soman were at *m/z* 99 and 126 (Fig. 2). Since soman is reported to have two pairs of isomers[9,10], these two peaks of soman were labeled soman isomer I and soman isomer II (Fig. 2). Benschop *et al.*[11] proposed that the presence of a center of asymmetry in the pinacolyl moiety (C) and the phosphorus atom (P) are responsible for levorotation (−) and dextrorotation (+) of the soman molecule, which form the two pairs of the isomers. Based on the results of Benschop, we believe that soman isomer I peak contains $C_{(-)}P_{(-)}$ and $C_{(-)}P_{(+)}$ stereoisomers and that soman isomer II contains $C_{(+)}P_{(+)}$ and $C_{(+)}P_{(-)}$ stereoisomers.

Sass and Fisher[8] proposed that ions at *m/z* 99 are characteristic ions for alkyl-methylphosphonofluoridates such as sarin and soman. The ion at *m/z* 125 for sarin is the result of methyl cleavage, and the ion at *m/z* 126 for soman $(M-56)^+$ is formed from the loss of isobutane from molecular ions[8]. Based on these observations, we chose ions *m/z* 99 and 126 for monitoring soman. Other ions were not monitored since the sensitivity of this assay decreased by increasing the number of low abundance ions monitored.

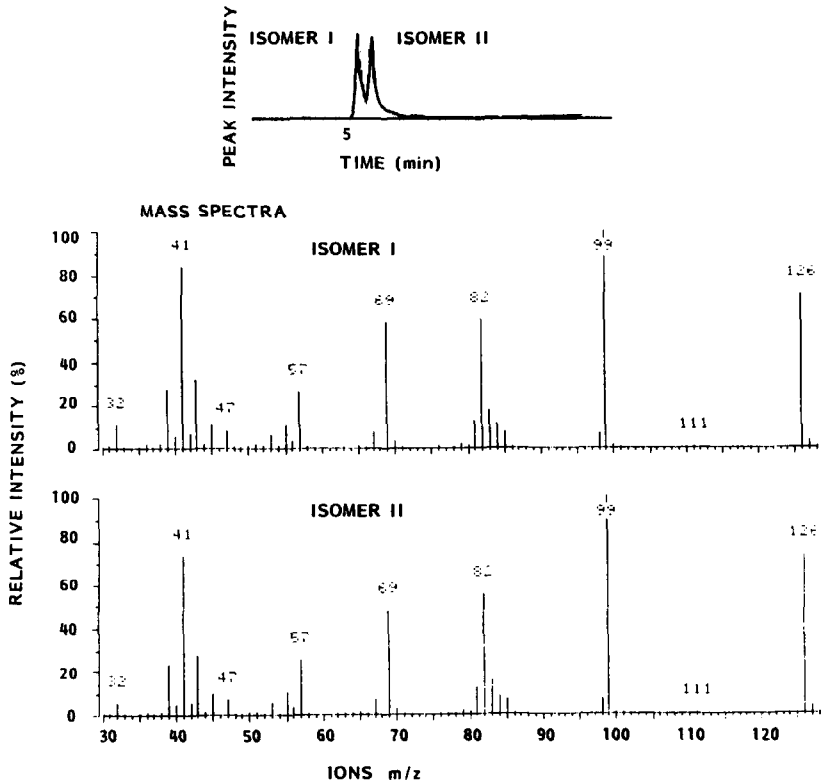

Fig. 2. Chromatographic separation and mass fragmentation patterns of the two soman isomers.

When we analyzed sarin and soman separately under isothermal conditions, sarin eluted at 2.4 min and soman at 5.12 min. If sarin and soman were analyzed simultaneously, a clean separation of sarin and soman was obtained with sarin eluting at 1.80 min and soman at 5.57 min (Fig. 3).

Quantitative analysis

A proper internal standard is not known for these highly reactive OP compounds. Sterri *et al.*[12] used *n*-decane as an internal standard in the GC–MS analysis of soman. *n*-Decane is insoluble in aqueous solution and could not be used in this or any other study using physiological solutions. Beck *et al.*[10] used deuterated soman as an internal standard. However, deuterated OP will react with cell components in

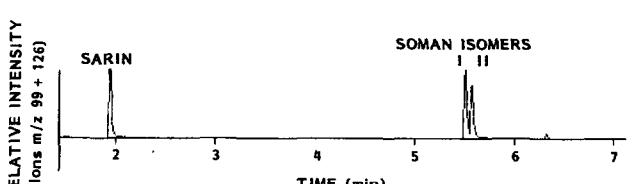

Fig. 3. Chromatographic separation of a mixture containing sarin and the two soman isomers.

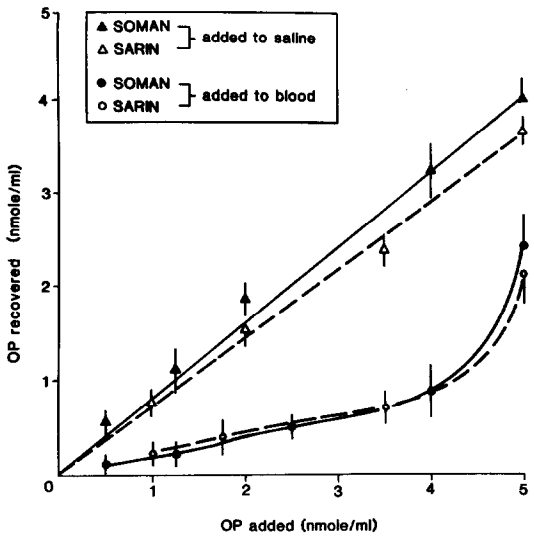

Fig. 4. Recovery of free sarin or soman from blood. Various amounts of sarin or soman (1–5 nmol) were added to 1 ml of blood. Immediately after addition, sarin or soman was extracted in 0.1 ml of chloroform, and 3 μl of the chloroform phase were injected into the GC–MS system. The amount of OP present in each sample was determined. Values are mean ± S.E. (n = 4).

the same manner as non-deuterated OP and therefore will be unsuitable as a true internal standard. We determined the extraction efficiency by using a double extraction method.

Recovery of OPs from saline and blood

Approximately 74% of sarin and 81% of soman added to the saline was extracted by the procedure described above (Fig. 4). Although a similar procedure was utilized for the extraction of OPs added to the blood or saline, the overall recovery of added OP was less in blood than in saline. We did not detect any extractable OP when 0.25 nmol was added to 1 ml of blood (Fig. 4) probably because the amount of extracted OP fell below the detection limit of the method. No significant accumulation of extractable OP occurred up to a 1.25 nmol/ml dose. Increasing the dose thereafter resulted in significant accumulation of extractable OP in the blood (Fig. 4). These observations suggest that at lower doses most of the OP added to blood was present in an enzyme- or protein-bound form since sarin and soman are known to bind with esterases in the blood[13]. Sterri *et al.*[1] and Fonnum and Sterri[2] have reported that rodent blood contains certain enzymes which bind with free OP and reduce the level. At higher doses the amounts of free, extractable OPs increased in the blood, which may be the result of the saturation of the OP-binding enzymes (or proteins). Preincubating the blood with physostigmine increased the level of extractable OP 10–15%. Since physostigmine is known to inhibit ChE by reversibly binding with its active sites[14], it is proposed that only 10–15% of the total OP which was not recovered from whole blood was specifically bound to ChE. The remaining OP may bound to proteins other than ChE.

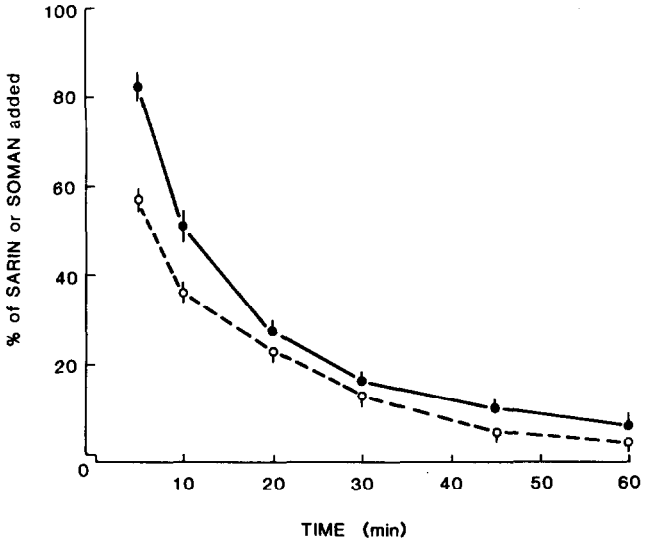

Fig. 5. Stability of sarin (○) or soman (●) in perchloric acid. Soman (5.5 nmol/ml) or sarin (7.2 nmol/ml) was added to perchloric acid. The amounts of these OPs extractable from the acid at 5, 10, 20, 30, 45, and 60 min were determined. Values are mean ± S.E. (n = 4).

Stability of OPs in perchloric acid

We used 0.3 N perchloric acid to deproteinize the blood before extracting the OPs. Sterri *et al.*[12] extracted soman directly from liver tissue and liver perfusate with an organic solvent. In our experience organic solvent extraction of blood samples without deproteinization resulted in rapid deterioration of the capillary column. This presumably was because of the introduction of soluble, non-volatile components with the injectate. Acid deproteinization of the blood samples, although solving the problem of column deterioration, resulted in acid-induced degradation. Sarin was more sensitive to acid degradation than soman (Fig. 5).

We also observed that neutralization of perchloric acid with sodium hydrogen

TABLE I

STABILITY OF SOMAN AND SARIN IN PERCHLORIC ACID (PCA) AND NEUTRALIZED PERCHLORIC ACID (N-PCA)

Values (mean ± S.E.) are percent of total sarin and soman added. The amounts of OP added were 2.75 nmol/ml soman and 3.6 nmol/ml sarin.

	Incubation time (min)	
	10	*60*
Soman PCA	83.2 ± 4.0	10.3 ± 0.47*
N-PCA	87.0 ± 2.5	79.5 ± 2.1
Sarin PCA	57.6 ± 5.5	2.2 ± 0.25*
N-PCA	55.8 ± 3.8	51.5 ± 1.8

* $p < 0.05$ when compared with 10-min values.

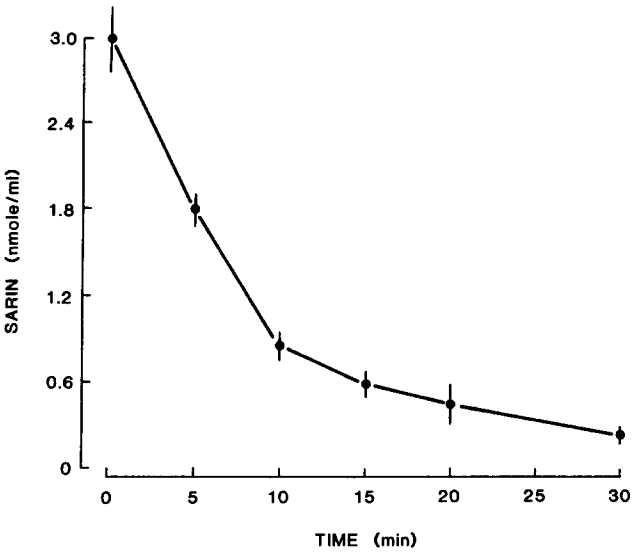

Fig. 6. Stability of sarin in blood. Sarin (3.6 nmol) was added to 1.0 ml of blood. The concentration of sarin in each sample was determined at 0 (immediately after addition), 5, 10, 15, 20, and 30 min after addition. Values are mean ± S.E. (n = 4).

carbonate immediately after deproteinization prevented the acid-degradation of OPs without affecting the extraction efficiency (Table I).

It is therefore proposed that the acid-induced degradation of OP could be prevented by neutralizing the acid immediately after blood deproteinization.

Time-dependent decay of extractable OP in the blood

When in isotonic saline, the amounts of extractable sarin and soman were unchanged after 30 min at room temperature. Amounts of the two soman isomers also were not significantly different. In blood, however, a gradual decrease in the levels of sarin and soman was noted (Figs. 6 and 7). The amount of the soman isomer II decreased faster than did that of the soman isomer I (Fig. 5). After 15 min the amounts of sarin and soman isomer I decreased by more than 60%, whereas soman isomer II had completely decayed (Figs. 7 and 8). These decreases might be due to the presence in the blood of phosphorylphosphatase enzymes which are known to degrade OPs[4,5]. Benschop *et al.*[9] have reported that the soman isomers which are hydrolyzed preferentially by phosphorylphosphatase have low anticholinesterase activity. When soman was incubated with a pure AChE suspension for 30 min, the levels of the two soman isomers decreased significantly. However, no significant difference appeared between the levels of the two isomers. Although soman isomer II was degraded in blood faster than soman isomer I, these results suggest also that there were no significant differences in the affinities of these two soman isomers to AChE. Factors other than OP–ChE binding may be responsible for the observed preferential decay of soman isomer II in blood.

From the results of this study it is concluded that (1) selected ion monitoring provided a simple and sensitive method for measuring OPs sarin and soman, (2) the

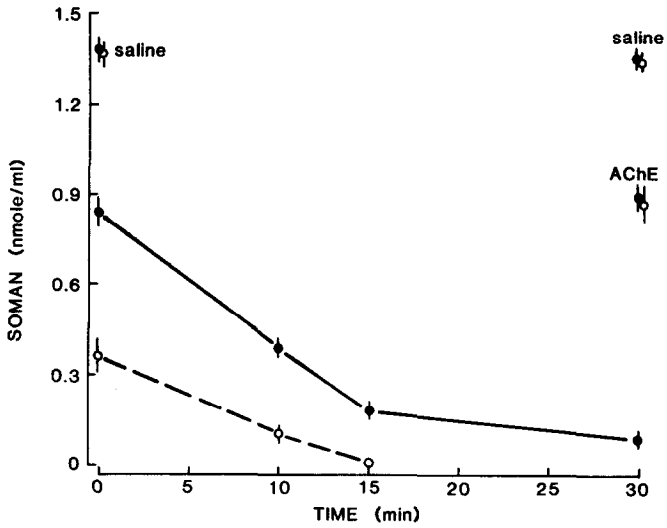

Fig. 7. Stability of the two soman isomers in blood, saline, and AChE solution. Soman was added to 1 ml of blood, saline, and AChE (44 U/ml). From blood the concentration of soman isomers were determined at 0, 10, 15, and 30 min after addition. From saline the concentration of soman was determined at 0 and 30 min and from AChE it was determined at 30 min after addition of soman. Values are mean ± S.E. (n = 4). ●, Soman isomer I; ○, soman isomer II.

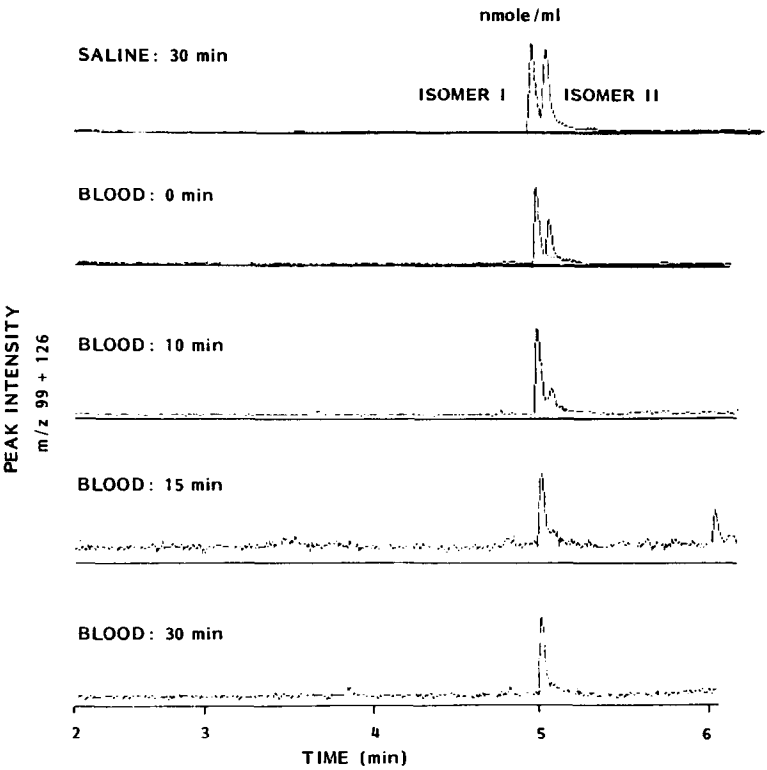

Fig. 8. Chromatographic illustration of the decay of soman isomer peaks in the blood. Isomer II decayed faster than isomer I.

recovery of added OP was less in whole blood than in saline because of the binding of OP to blood enzymes (or proteins), (3) a time-dependent decrease in extractable OP was found in blood but not in saline, and (4) in blood, soman isomer II decayed faster than isomer I, although their affinities to AChE-binding were the same.

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