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Bioanalysis of the Enantiomers of (±)-Sarin using Automated Thermal Cold-Trap Injection Combined with Two-Dimensional Gas Chromatography

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Abstract

A fully automated multidimensional gas chromatographic system with thermal desorption injection and alkali flame detection was developed for analysis of the enantiomers of the nerve agent (±)-sarin. The chiral stationary phase was CP Cyclodex B on which the sarin enantiomers were completely resolved. The absolute detection limit was 2.5 pg per enantiomer. The method is intended to be used for the analysis of the sarin enantiomers in biological samples. For this purpose, sarin was isolated from guinea pig blood via solid-phase extraction. Deuterated sarin was used as internal standard. Stabilization of sarin in the blood sample by acidification and addition of an excess of a competitive organophosphorus compound (neopentyl sarin) appeared to be essential. The absolute recovery of the extraction procedure was 60%, whereas the recovery relative to the internal standard was 100%.

Introduction

Previously, we have studied the toxicokinetics of the nerve agent C(±)P(±)-soman[1] (1–4). (±)-Sarin is now included in our studies because it is, like C(±)P(±)-soman, a volatile nerve agent designed for intoxication via inhalation and is stockpiled by major military powers in chemical weapons and as a bulk chemical. Recently, a new type of proliferation has emerged with the use of sarin by the Aum Shinrikyo sect in large-scale terrorist attacks in the Tokyo subway and several months earlier at Matsumoto (5) and VX in Osaka, Japan (6). Alleged exposures to sarin were confirmed by analysis of sarin covalently bound to butyryl-cholinesterase and its major metabolite in blood samples (7,8). This extension of our studies to sarin demanded the development of stabilization and analysis procedures for (±)-sarin in biological samples. It was expected that the stabilization procedures would be analogous to those for C(±)P(±)-soman (9).

As in the case of C(±)P(±)-soman, it was considered to be essential to differentiate between the enantiomers of (±)-sarin. Previous investigations in our laboratory have shown that the bimolecular rate constant for inhibition of electric eel AChE by (–)-sarin is approximately four orders of magnitude higher than that of (+)-sarin (9). Consequently, we expect that (+)-sarin, by analogy with the C(±)P(+)-isomers of soman, will be almost nontoxic in comparison with (–)-sarin. This assumption is strengthened by our observation that the LD_{50} (i.v., mouse) of (–)-sarin is approximately half that of (±)-sarin (1).

For the analysis, we developed a two-dimensional gas chromatographic (GC) system with a chiral analytical column in combination with thermal desorption injection and nitrogen phosphorus detection (NPD).

In order to maximize the daily sample throughput, the thermal cold-trap injection was automated.

Experimental

Materials and chemicals

(±)-Isopropyl methylphosphonofluoridate [(±)-sarin][2] and O-2,2-dimethylpropyl methylphosphonofluoridate [(±)-neopentyl sarin, NPS] were prepared at TNO Prins Maurits Laboratory from reaction of the appropriate alcohol with methylphosphonic difluoride and methylphosphonic dichloride according to the procedure of Bryant et al. (10). The compounds were distilled over a Spaltrohr column until a purity of > 99% was obtained by GC.

The synthesis of (±)-sarin-d_7 was analogous to that of C(±)P(±)-soman-d_{13} (9), using isopropanol-d_7 (Merck, Darmstadt, Germany) instead of perdeuteropinacolyl alcohol. The synthesis was performed on a gram scale with a reaction yield of 86%. GC analysis did not indicate any impurities. The most prominent fragment in the mass spectrum (EI) was *m/z* 101, identified as $DO(CH_3)P(O{+}D)F$. Other major fragments were *m/z* 129 $[CH_3(CD_3CDO)P(O)F]$ and 81 $[(CH_3)P(O)F]$. The

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[1] The stereoisomers of soman (pinacolyl methylphosphonofluoridate) are denoted as C(–)P(+), C(–)P(–), C(+)P(+), and C(+)P(–), in which C is the asymmetric carbon atom in the pinacolyl moiety and P is the asymmetric phosphorus atom. Likewise, the enantiomers of sarin are denoted as (+) and (–) with respect to the configuration of the phosphorus atom.

[2] WARNING: In view of its extreme toxicity, sarin and related organophosphates should be handled only in specialized laboratories where trained medical personnel are continuously present.

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^1H-NMR spectrum ($CDCl_3$, 400 MHz) was in accordance with the proposed structure: δ 1.62 [dd, 3 H, J_{PH} = 18.7 Hz, J_{FH} = 5.7 Hz, F-P-CH_3]; as is the ^{13}C-NMR spectrum ($CDCl_3$, 100.6 MHz): δ 72.2 [CD]; 22.8 [2 × CD_3]; and 10.5 [dd, 1 C, J_{PC} = 150.6 Hz, J_{CF} = 27.6 Hz, CH_3-P-F].

The internal standard, (–)-sarin-d_7, was isolated with an optical purity > 98% by incubating the d_7 racemate in rabbit serum by analogy with the method used to isolate the P(–)-isomers of C(±)P(±)-soman (11).

The synthesis of Chirasil-(D)-valine was analogous to that of Chirasil-(L)-valine, using D-valine *tert*-butylamide instead of the L-isomer (2) The reaction yield was approximately 70%. The identity of the product was confirmed by ^1H-NMR, IR, and elemental analysis. The nitrogen content of the product was 3.1% (theoretically 3.2%).

Tenax TA, 60–80 mesh, and the CP Cyclodex B column were obtained from Chrompack (Middelburg, The Netherlands). SepPak C18 cartridges were obtained from Millipore (Bedford, MA). Ethyl acetate (zur Rückstandanalyse) was procured from Merck and distilled over a column packed with Dixon rings (plate number 80, NGW, Wertheim, Germany) before use. Isopropanol (Brocacef, Rijswijk, The Netherlands) was purified by means of the same procedure (purity > 99.7%). Aqueous dilutions were prepared with high-performance liquid chromatography-grade water from Fisons Ltd. (Loughborough, U.K.).

The following products were obtained commercially and were used without further purification: saponine (BDH Analar, Poole, U.K.), aluminum sulfate (BDH, > 98%), sodium bicarbonate (Lamens en Indemans, 's-Hertogenbosch, the Netherlands, > 99.5%), acetic acid (Lamens en Indemans, > 99%), sodium acetate (Merck, zur Analyse, > 99.5%).

Apparatus and procedures

The chromatographic system consisted of a Carlo Erba HRGC 5300 Mega series (Milan, Italy), equipped with an alkali flame detector (NPD, Carlo Erba), a thermodesorption autosampler (TDAS, Carlo Erba), and a Chrompack MUSIC system (Multiple Switchable Intelligent Controller) for two-dimensional GC.

The desorption tubes (10-cm length, 3-mm i.d.) were partly (about 80%) filled with Tenax. A glass-wool plug was firmly pushed on top of the Tenax material and was fixed with a metal clamp. The tubes were preconditioned by heating under a stream of helium at 300°C for at least 4 h. The autosampler tray was thermostatted at 10°C via a cooled circulator (TLC 3, Tamson, Zoetermeer, The Netherlands) filled with ethylene glycol. The maximum capacity of the autosampler tray was 30 desorption tubes. The sample was desorbed from Tenax by heating for 3 min at 190°C. The cold trap of the injection system was a deactivated CP-Sil 8 CB column (~ 1-m length, 0.53-mm i.d., 5.25-μm film thickness). The cold trap was kept at –60°C with liquid nitrogen. Cooling was started before the tube to be desorbed was positioned. The analytes were reinjected from the cold trap via a temperature increase from –60 to 180°C at a rate of 21°C/s. This flash-heating signal started the program of the GC and the MUSIC system. The precolumn of the MUSIC system was a chemically bonded CP-Sil 8 CB column (10-m length, 0.53-mm i.d., 5.25-μm film thickness). The precolumn was programmed at 87°C isothermally for 2 min. The cut containing the compounds of interest was trapped in the cold trap of the MUSIC system (uncoated deactivated fused silica, ~ 30-cm length, 0.25-mm i.d.), which was cooled at –70°C with liquid carbon dioxide. Meanwhile the GC oven was cooled from 87 to 70°C. The trapped cut was injected onto the analytical column by flashheating the trap to 180°C. The analytical column (CP Cyclodex B, 50-m length, 0.25-mm i.d., 0.25-μm film thickness) was programmed at 70°C for 13 min, after which it was heated to 87°C at 'infinite' rate and kept at this temperature for 4 min. Helium flow-rate was 12.4 mL/min, and the inlet pressure was constantly 1.48 kPa. The retention times of the sarin enantiomers and (–)-sarin-d_7 were in the range of 13 to 15 min. The detector base was kept at 250°C. Make-up gas for the detector was helium at a flow-rate of 40 mL/min. Flow-rates of air and hydrogen through the detector were 350 and 35 mL/min, respectively.

For work-up of blood samples for toxicokinetic measurements, (±)-sarin isomers were stabilized by the addition of acetate buffer containing aluminum sulfate, NPS, and the internal standard, (–)-sarin-d_7, prior to extraction over SepPak C_{18} cartridges as described previously for soman (1). Analytes in ethyl acetate were concentrated at reduced pressure with a Rotavapor-M (Buchi, Switzerland).

Results and Discussion

Chiral gas–liquid chromatography of (±)-sarin enantiomers

The gas chromatographic configuration using two-dimensional chromatography which we developed for analysis of the four stereoisomers of soman, should be very promising for toxicokinetic investigations of nerve agents more volatile than C(±)P(±)-soman, such as (±)-sarin, fulfilling the superior selectivity requirements for analysis in biological material, containing many components with a volatility comparable to that of (±)-sarin.

First, the chromatographic resolution of the (±)-sarin enantiomers had to be accomplished. Several years ago, we succeeded in resolving the enantiomers of (±)-sarin by means of GC on an optically active nickel camphorate stationary phase (12). However, the peaks obtained for the enantiomers were quite broad, especially at lower (±)-sarin concentrations, which obviously influenced the sensitivity unfavorably. Furthermore, the long-term stability of the stationary phase was problematic. This led, for example, to variable retention times of the enantiomers. As an alternative, the chiral resolution of (±)-sarin was studied with the Chirasil-L Val stationary phase, which is used for the analysis of the four C(±)P(±)-soman stereoisomers (13). The enantiomers of (±)-sarin were adequately resolved on this phase, which was also the case for the enantiomers of (±)-sarin-d_7. The elution order of the stereoisomers on this stationary phase is the same as for C(±)P(±)-soman, that is, the P(+)-isomer is eluted before the P(–)-isomer. Unfortunately, the (±)-sarin enantiomers were hardly resolved from the deuterated enantiomers. The (+)-enantiomer-d_7 was resolved from

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the (±)-sarin enantiomers, whereas (–)-sarin-d_7 co-eluted with (+)-sarin. As a result, this stationary phase can only be used for (±)-sarin analysis if optically pure (+)-sarin-d_7 is available as the internal standard. Unfortunately, we did not succeed in isolating (+)-sarin-d_7 from the racemic mixture. On the other hand, the (–)-enantiomer of the deuterated compound could be isolated in a relatively simple way.

We anticipated that reversing the elution order by using a Chirasil-D Val stationary phase might solve the problem. This phase was prepared in house (see Experimental). The phase was custom-coated by Chrompack onto a fused-silica capillary column. Indeed, on this stationary phase the (+)-enantiomer of the deuterated sarin co-eluted with the (–)-enantiomer of sarin, and (–)-sarin-d_7 was completely resolved. This column can be used for the analysis of (±)-sarin enantiomers, using (–)-sarin-d_7 as the internal standard. Meanwhile, a glass capillary column was coated in house with the Chirasil-D Val stationary phase, with a slightly thinner coating than on the custom-coated column, with a method described by Degenhardt et al. (13). This column proved to be even capable of resolving the four peaks of interest (see Figure 1). For practical purposes, however, we prefer to use fused-silica columns, which are much easier to handle than glass columns. Furthermore, we prefer to use commercially available columns because the synthesis of the stationary phase and its coating onto a capillary column are tedious operations that are difficult to reproduce.

During the past several years the number of commercially available chiral stationary phases has increased. Among these, the cyclodextrin phases seemed promising with respect to the resolution of chiral organophosphorus compounds because in earlier studies in our laboratory, the inclusion of (±)-sarin and several other organophosphorus compounds in the cavity of α-cyclodextrin appeared to be stereoselective (14).

With the commercially available CP Cyclodex B column, a β-cyclodextrin phase coated on fused silica, baseline resolution of (–)-sarin-d_7, (–)-sarin, and (+)-sarin was accomplished, as shown in Figure 2. On this column (+)-sarin-d_7 is not resolved from (–)-sarin. In view of the aforementioned considerations, we chose to use this column for the bioanalysis of (±)-sarin enantiomers, using the optically pure (–)-sarin-d_7 as the internal standard.

The absolute limit of detection (LOD) with an NP detector (s/n = 3) is approximately 2.5 pg per sarin enantiomer.

Two-dimensional GC (MUSIC) with automated thermal cold-trap injection

In previous toxicokinetic studies (3), we used a thermal cold-trap injector. The benefit of this type of injector is the potential to inject a large volume onto the GC system. However, the hand-operated exchange of the Tenax sample tubes can be considered as a drawback because it limits the daily sample throughput.

In view of the expected large numbers of samples to be analyzed in our future toxicokinetic study, we constructed a system equipped with a thermodesorption autosampler (TDAS). A schematic representation of the injection system is shown in Figure 3.

The autosampler tray has a capacity of 30 samples. Because each analysis takes about 20 min, this means that the final sample will remain in the autosampler for about 10-h. Problems anticipated with this procedure were (1) degradation of the analytes absorbed on Tenax within the 10-h period and (2) cross-contamination between the tubes. To minimize the risks of degradation of the absorbed analytes, the tray was thermostated at 10°C. Absence of degradation and cross-contamination was checked by analysis of known concentrations of (±)-sarin and analysis of blank tubes at various time points after placing the tubes in the autosampler. The results are presented in Table I. The within-day variation of the analysis was about 4% for (–)-sarin and about 5% for (+)-sarin, for 11 samples that were analyzed in a 4.5-h time period after filling of the autosampler.

The Tenax material is introduced into the desorption tubes by using reduced pressure. The glass wool has to be pushed firmly on top of the Tenax material and secured with a metal clamp. Otherwise, some Tenax material may be blown out of the tube as a result of the pressure originating from the MUSIC system. If some of these Tenax particles adhere onto the rim of

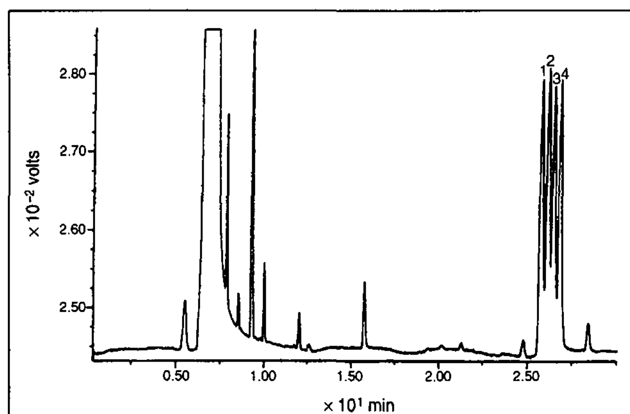

Figure 1. Chromatogram of racemic (±)-sarin-d_7 and (±)-sarin on Chirasil-D Val (50-m length, 0.24-mm i.d., glass capillary). Carrier gas was helium; oven temperature was 60°C. Peak identification: 1, (–)-sarin-d_7; 2, (+)-sarin-d_7; 3, (–)-sarin; and 4, (+)-sarin.

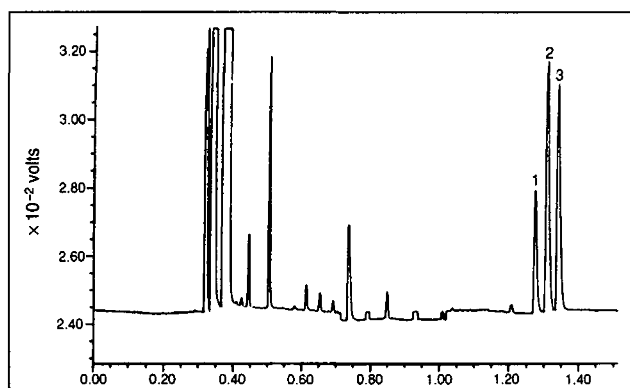

Figure 2. Chromatogram of (–)-sarin-d_7 and (±)-sarin on CP Cyclodex B, coated onto WCOT fused silica (50-m length, 0.25-mm i.d.). Carrier gas was helium, oven temperature was 70°C, and injector/detector (FID) temperature was 180°C. Peak identification: 1, (–)-sarin-d_7; 2, (–)-sarin; and 3, (+)-sarin.

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the tube, a gastight fit of the desorption tube in the injector is not possible, resulting in erroneous injections.

A potential problem was also anticipated with respect to the connection with the MUSIC, which proceeds via a valve with a metallic interior, in view of previous experiences with degradation of C(±)P(±)-soman on hot metal surfaces. The temperature of the valve was varied from 100 up to 190°C; no degradation of (±)-sarin was observed. The valve temperature was set at 190°C, which is equal to the desorption temperature.

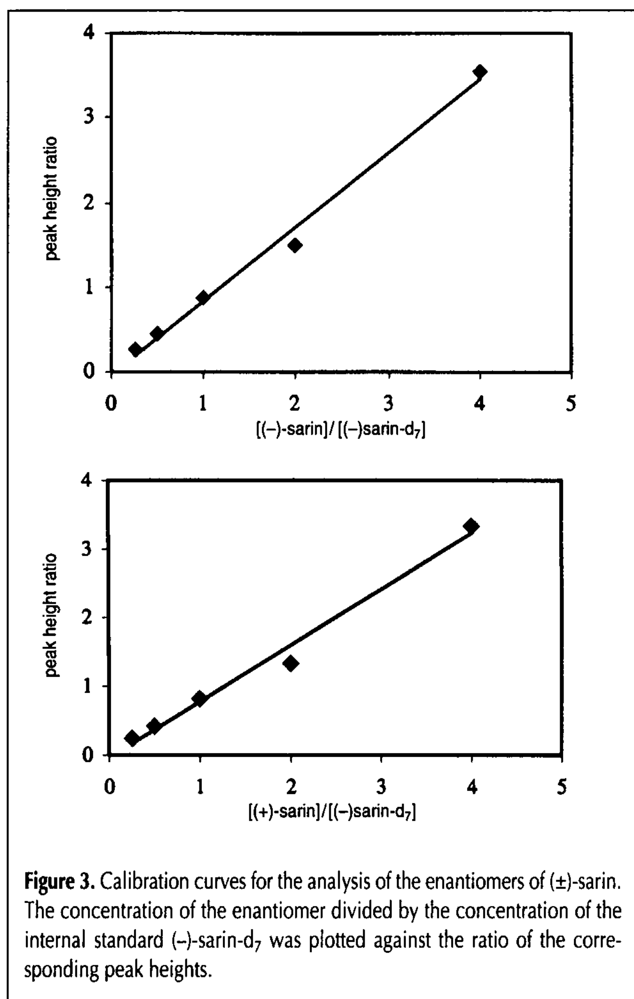

Figure 3. Calibration curves for the analysis of the enantiomers of (±)-sarin. The concentration of the enantiomer divided by the concentration of the internal standard (–)-sarin-d_7 was plotted against the ratio of the corresponding peak heights.

Table I. Analysis of (±)-Sarin in Order to Establish Possible Degradation of the Analytes or Cross-Contamination during Storage in the GLC Autosampler

Sample	(±)-Sarin (pg)	Storage time (min)	Peak height (μVs)
1	250	0	184432
2	250	37	193388
3	250	81	161228
4	0	111	n.d.*
5	0	152	n.d.
6	250	81	190464
7	0	177	n.d.

* n.d. = not detectable.

Validation of the extraction of (±)-sarin enantiomers from blood samples

The extraction procedure with SepPak C_{18} cartridges, used for the isolation of C(±)P(±)-soman stereoisomers from biological samples appeared to be applicable as such to the isolation of (±)-sarin. The absolute recovery of the extraction procedure of sarin appeared to be approximately 60%, which is comparable to that of C(±)P(±)-soman. The absolute recovery was determined by comparing the chromatograms of a worked-up blood sample which was spiked with the same amount of (±)-sarin as an analyzed standard solution of (±)-sarin. The recovery compared to the internal standard (–)-sarin-d_7 is 100%.

The extraction procedure for (±)-sarin from guinea pig blood was validated as follows: blank blood samples were obtained from a naïve guinea pig and treated with an excess of C(±)P(±)-soman for 30 min in order to occupy all possible binding sites for sarin; next, the stabilization buffer and known amounts of (±)-sarin and (–)-sarin-d_7 were added, and the samples were subjected to solid-phase extraction on the SepPak C_{18} cartridges; the blank showed no peaks on the retention time of (±)-sarin and (–)-sarin-d_7. The results are listed in Table II.

In order to examine the most suitable solvent to extract (±)-sarin from the SepPak cartridge, different solvents such as diethyl ether, dichloromethane, pentane/methanol (95:5, v/v), butyl acetate, and methyl acetate were tested for extraction recovery and reproducibility. Ethyl acetate proved to give the best results.

The limit of quantitation appeared to be 8.3 pg per sarin enantiomer (s/n = 10), and the precision of the method for (–)-sarin was 1.5% and (+)-sarin was 0.9% (n = 6) at a level of 10 ng/mL.

For the analysis of blood samples, a calibration curve (see Figure 3) of each enantiomer was used. For this purpose, known amounts of (±)-sarin in ethyl acetate were mixed with

Table II. Validation of the Analysis of Low Concentrations of (±)-Sarin in Guinea Pig Blood (n = 2)

Added (±)-sarin (ng/mL)	Measured (–)-sarin ± s.d. (ng/mL)	Measured (–)-sarin ± s.d. (ng/mL)
0.1	0.053 ± 0.001	0.050 ± 0.001
0.5	0.027 ± 0.001	0.025 ± 0.001
0	0	0

Table III. Accuracy of the Verification Test of the Solid-Phase Extraction of (±)-Sarin with SepPak C_{18}-Cartridges from Blood Obtained from a Guinea Pig 2 h after Administration of Two LD_{50} of (±)-Sarin

Added (±)-sarin (ng/mL)	Measured (±)-sarin ± s.d. (ng/mL) (n = 5)	Accuracy (%)
10	10.0 ± 0.3	100
1	1.02 ± 0.01	102
0.1	0.098 ± 0.009	98

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known amounts of (–)-sarin-d_7 in ethyl acetate and analyzed as described before for soman (2,3). The amounts of the individually enantiomers were divided by the amount of (–)-sarin-d_7 and were plotted against the resulting peak heights.

The accuracy of the method was determined by analyzing blood samples spiked with a known amount of (±)-sarin (see Table III) and appeared to be 100, 102, and 98% at (±)-sarin levels of 10, 1, and 0.1 ng/mL, respectively.

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