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Development and application of procedures for the highly sensitive quantification of cyclosarin enantiomers in hemolysed swine blood samples

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This paper is dedicated to Professor Dr. Peter Eyer, Walther-Straub-Institute of Pharmacology and Toxicology, Ludwig-Maximilians-University, Munich, Germany, in celebration of his 65th birthday.

Abstract

The present study was initiated to develop a sensitive method for the analysis of cyclosarin (*O*-cyclohexyl methylphosphonofluoridate, GF) enantiomers in biological samples utilizing classical configurations of GC–MS and automated solid phase extraction. To achieve this goal, a specific procedure had to be developed to extract cyclosarin from swine blood samples thereby stabilising and minimising the racemisation/deracemisation of its enantiomers. The chiral stationary phase was GAMMA DEX^{TM} (gamma cyclodextrin), on which GF and deuterated GF enantiomers were baseline-resolved. The limit of detection was 1 pg for (−)-GF with GC–EI-MS and 5 pg for (+)-GF with GC–NCI-MS. The absolute recovery of the overall procedure for sample preparation was 85%. After an intravenous infusion of a supralethal dose of GF in anaesthetised swine only (−)-GF could be quantified, (+)-GF was not detected.

Keywords: Gas chromatography–mass spectrometry; *O*-Cyclohexyl methylphosphonofluoridate; Cyclosarin; GF; Enantiomers; Solid phase extraction; Cyclodextrin; Poisoning; Nerve agents; Toxicokinetics; Swine; Oxime; Reactivator; Acetylcholinesterase

1. Introduction

Poisoning due to highly toxic organophosphorus (OP) nerve agents (e.g. sarin, cyclosarin, soman) requires immediate medical aid. The presently available treatment with a muscarinic antagonist (e.g. atropine) and some of the clinically used acetylcholinesterase (AChE) reactivators (obidoxime, pralidoxime) is considered to be insufficient with certain nerve agents, including cyclosarin (GF, *O*-cyclohexyl methylphosphonofluoridate). In order to develop an efficient treatment, toxicological and toxicokinetic parameters of an agent must be determined [1]. A common feature of organophosphonate nerve agents is the presence of a stereogenic phosphorus atom, resulting in the presence of enantiomers in a racemic product. Nerve agent isomers exhibit very different biochemical and toxicological characteristics. P(−) isomers of sarin, soman, tabun and VX are much more toxic compared to P(+) isomers [2]. (−)-GF inhibits AChE more strongly than racemic GF and high-probably represents the more toxic isomer [3]. Therefore, to assess toxicokinetic and toxicodynamic properties reliable analytic methods are required for the determination of individual isomers of free OP in the blood.

The nerve agent cyclosarin (GF) has been examined to a minor extent compared to other nerve agents, e.g. sarin and soman. One reason is that this agent was not regarded as a high priority chemical warfare agent until it was found to be stockpiled by Iraq in the early 1990s [4,5]. The toxicity of individual

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GF enantiomers has not been reported in the open literature and toxicokinetic and quantitative parameters of metabolism of GF enantiomers are virtually unknown. To this end, an adequate sample preparation and a baseline separation of GF isomers is a prerequisite for a specific and sensitive analysis of this agent in biological material. Published data indicate that the separation of GF enantiomers is a challenging task [3,6]. Gas chromatographic (GC) separation of the GF isomers with a partial resolution on a Chirasil-Val phase was carried out by research groups at the TNO Prins Maurits Laboratory and the U.S. Army Edgewood Chemical Biological Center [3,6,7]. The separation of GF isomers by cyclodextrin GC capillary columns was reported by a research group at the U.S. Army Medical Research Institute of Chemical Defense [8]. Unfortunately, in this paper no details on the successful chiral separation of GF enantiomers were described. The estimated retention times for GF isomers were approximately 80 min.

The present study was initiated to develop a procedure for the quantitative determination of GF isomers in biological matrices. GF enantiomers were separated by GC and analysed by mass spectrometric (MS) detection. The procedure was applied to the detection of cyclosarin in hemolysed swine blood samples after an intravenous infusion of 86.5 μg/kg GF in anaesthetised swine that underwent sequences of resuscitation.

2. Experimental

2.1. Materials

The following materials and GC columns were obtained commercially:

- GAMMA DEX^{TM} 225 (Supelco USA, 30 m length, 0.25 mm i.d., 0.25 μm film thickness) from SIGMA–ALDRICH Chemie GmbH (Taufkirchen, Germany);
- C18(EC) 100 mg/1 ml cartridges (octadecyl end-capped, IST Limited, UK) from Separtis GmbH (Grenzach-Wyhlen, Germany);
- deionised water (Tracepur, for organic trace analysis), *n*-hexane (UniSolv, for organic trace analysis), 2-propanol and methanol (SupraSolv, for gas chromatography) from Merck KGaA (Darmstadt, Germany);
- ethylenediaminetetraacetic acid, tripotassium salt dihydrate (99%, Sigma), sodium chloride (99.99%, Aldrich) from SIGMA–ALDRICH Chemie GmbH (Taufkirchen, Germany).

GF and the internal standard (IS) d_{11}-cyclosarin (>98% by GC–MS, ^{1}H NMR and ^{31}P NMR) were made available by the German Ministry of Defence.

2.2. Standards and spiking solutions

Stock solutions of GF and IS (10% and 1%, w/v) were prepared in hexane and stored for 1 month at −20 °C in glass vials. Spiking solutions were prepared in 2-propanol in volumetric flasks daily or on the day of the experiment. In the blood samples, the volumes of spiking solutions did not exceed 1% (v/v).

2.3. Instrumentation

Two gas chromatographic systems 6890N with a 5973N MS detector (GC A, GC–EI-MS) and a 5975N MS detector with negative ion chemical ionisation using methane (GC B, GC–NCI-MS) were used (Agilent Technologies, Waldbronn, Germany). Both systems were equipped with a cold injection system CIS 4^{Plus} (Gerstel, Mülheim an der Ruhr, Germany).

An automatic system for solid phase extraction (AS SPE) RapidTrace SPE workstation, a Turbo Vap LV workstation (both from Caliper Life Sciences, Rüsselsheim, Germany) and a centrifuge Rotina 35R (Hettich, Tuttlingen, Germany) were used for sample preparation.

2.4. Laboratory animals and blood sampling

Swine experiments were carried out at Defence Research & Development Canada, Suffield, Canada, in accordance with the Canadian Council on Animal Care (CCAC). The protocol was approved by DRDC Suffield Animal Care Committee. A detailed description of the experiment has been described by Dorandeu et al. [9].

Briefly, 9–10-week-old castrated juvenile male Yorkshire-Landrace cross swine, weighing approximately 20 kg, were used. Spontaneously breathing, chloralose anaesthetised swine were challenged with a supralethal dose of GF (86.5 μg/kg), administered by i.v. route over 5 min. Atropine sulphate (0.05 mg/kg) was injected i.m. 3 min after the start of the GF infusion. Further pharmacological treatment comprised another i.m. injection of 0.1 mg/kg atropine ca. 8 min after the initiation of the challenge and an i.v. bolus of the oxime HI 6 dimethanesulfonate (12.7 mg/kg) combined with atropine (0.1 mg/kg) between 16 and 18 min after the start of GF infusion. Further atropine was injected i.v. based on changes from normal heart rate values. Arterial blood samples (2 ml per time point) were taken before challenge (*in vivo* blank) and 2, 5, 7, 10, 15, 20, 45, 90, 120, 180, 240, 300 and 360 min after the start of GF infusion using EDTA as an anti-coagulant. The blood samples were instantly hemolysed, frozen and stored at −80 °C until analysis (cf. Section 2.5). Blood samples from 12 animals were investigated. For experiments with blank and spiked blood samples individual and pooled hemolysed blood from 12 healthy swine were used. The sample processing for hemolysed blood samples and for *in vivo* samples was identical.

2.5. Sample preparation (hemolysis, extraction, SPE)

Stabilization of GF was achieved by successive treatment of the blood samples with two formate buffers (Fig. 1): the first (50 mM) served for hemolysis and the second (100 mM) for reduction of the pH value. At low pH values enzymatic activity of A-esterases in the mammalian blood, spontaneous hydrolysis of some highly toxic *O*-alkyl methylphosphonofluoridates, inhibition of BuChE and AChE by OP and spontaneous reac-

tivation of inhibited cholinesterases are all strongly reduced [10–14]. The racemisation and deracemisation of GF were excluded by relatively low total salt concentration and absence of the potential exogenous catalysts (e.g. fluoride anion) [6,14,15].

Stabilised hemolysed blood samples were kept frozen (−80 °C) until analysis. Different liquid and solid phase extraction procedures were tested in pilot experiments and the described procedure combining liquid/liquid and SPE proved to be most effective in removing endogenous contaminants. The samples were thawed and kept in an ice bath until the extraction with hexane (less than 30 min). Nine millilitres of each sample were used for further treatment while the remainder was frozen again to serve as control. A flow diagram for the sample preparation of swine blood for quantitative determination of GF enantiomers is shown in Fig. 1. Immediately before the start of the preparation the samples were spiked with IS (d_{11}-cyclosarin) at a final concentration of 0.5 ng/ml. After the addition of aqueous 1 M NaCl (1:1), extraction was performed using hexane (1:5) (hexane and aqueous sodium chloride solution were ice-cooled). After centrifugation (4000 × *g*, 4 °C, 15 min), the organic phase was transferred to the Turbo Vap LV workstation. The hexane phase was evaporated at 30 °C with nitrogen (15 psi). Then, the solid phase extraction on C18 cartridges was carried out by AS SPE as follows:

- conditioning of C18(EC) cartridges (3 ml methanol at 5 ml/min, 2 ml deionised water at 20 ml/min, 3 ml deionised water at 5 ml/min);
- loading of the hexane extract at 5 ml/min;

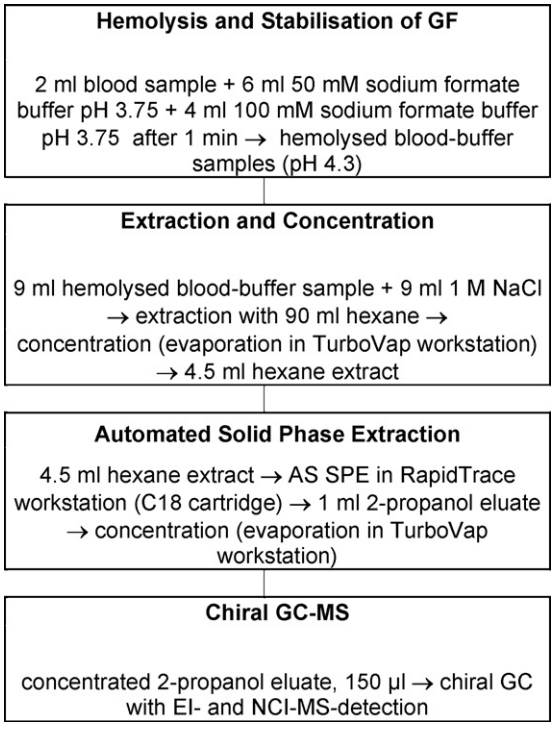

Fig. 1. Flow diagram for the sample preparation of swine blood samples for the quantitative determination of GF enantiomers by GC–MS.

- washing cycles (4 ml × 6 ml deionised water at 40 ml/min, hexane 2 ml × 6 ml at 40 ml/min);
- elution (1 ml 2-propanol at 2.5 ml/min).

A column air push volume of 6 ml at 10 arbitrary RapidTrace units after each step in the RapidTrace program was used throughout. The 2-propanol eluate was evaporated to an end volume of 150 µl in the Turbo Vap LV at 30 °C under nitrogen (20 psi) and was transferred to an autosampler vial with a glass insert for analysis.

2.6. GC–MS analysis

The GF enantiomers were base line resolved on a GAMMA DEX™ 225 GC column. GC A and B were used for quantification of GF enantiomers in biological samples. The elution order and identity of the GF isomers was performed as described previously [15].

GC conditions for GC A and B were as follows:

- helium as carrier gas at a constant flow of 1.3 ml/min;
- solvent vent stop-flow injection mode was used. A volume of 5 µl was injected into unpacked deactivated baffled glass liner within 1 min at a pre-column pressure of 0 bar. The injector initial temperature was 50 °C. The final temperature of 260 °C was reached at a rate of 720 °C/min and was kept constant for 2 min. The initial time was 1.1 min, vent time was 1.01 min, vent flow was 10 ml/min, purge flow was 50 ml/min and purge time was 3 min;
- the column oven temperature program consisted of 50 °C for 2 min, increased to 100 °C at 40 °C/min and to 135 °C at 3 °C/min, maintained for 2 min and then further increased to 170 °C at 40 °C/min.

MS conditions for GC A were as follows:

- electron impact ionisation (EI) at 70 eV, transfer line temperature 220 °C, ion source temperature 230 °C, solvent delay 13 min;
- selected ion monitoring (SIM) mode, dwell-time 100 ms for *m/z* 99 (GF) for $[MeP(=OH)(OH)F]^+$ and *m/z* 101 (IS) for $[MeP(=OD)(OD)F]^+$ [16].

MS conditions for GC B were as follows:

- negative chemical ionisation (NCI) with methane at 170 eV, methane flow 2.5 ml/min, transfer line temperature 220 °C, ion source temperature 150 °C, solvent delay 13 min;
- selected ion monitoring (SIM) mode, dwell-time 100 ms for *m/z* 179 (GF) for $[CH_2P(=O)(OC_6H_{11})F]^-$ and *m/z* 190 (IS) for $[CH_2P(=O)(OC_6D_{11})F]^-$ (proposed ion formulae).

2.7. Study of specificity

The specificity and stereospecificity of the assay was checked by analysing six blank hemolysed blood samples and six hemolysed blood samples spiked with GF. All chromatograms were

compared with chromatograms from GF standard solutions with GC–EI-MS and GC–NCI-MS. *In vivo* samples were also used for additional comparison.

2.8. Determination of recovery rates

The absolute recovery of the extraction was calculated by comparing the chromatogram peak areas of GF in hemolysed blood after the analytical procedure to those of a standard solution of GF prepared in 2-propanol from racemic GF. The recovery was determined at three concentrations (0.2, 2, 20 ng/ml; $n = 5$). The individual recovery of evaporation and SPE were determined for samples after addition of GF to final concentrations of 2 and 20 ng/ml to hexane extracts of blank hemolysed blood samples or to 2-propanol eluates after SPE of the hexane extracts (Fig. 1). Five parallel determinations were made at each concentration. The recovery compared to IS (d_{11}-GF) was determined by comparing peak area ratios obtained from analyte to IS versus the peak area ratios of the same concentration of GF standards and IS spiked in extracted blood blank samples. Identical concentrations of GF as for the determination of the absolute recovery were used.

2.9. Study of linearity, sensitivity and stability

Linearity test calibration curves were generated within a GF concentration range of 0.02–20 ng/ml by using spiked pooled hemolysed blood samples ($n = 5$). The linearity of each calibration curve was determined by plotting the peak area ratio $A_{(-)\text{-GF}}/A_{(-)\text{-}d_{11}\text{GF}}$ versus $C_{(-)\text{-GF}}/C_{(-)\text{-}d_{11}\text{GF}}$ by means of standard linear regression analysis. The limit of detection was determined for mass signals exceeding a signal-to-noise ratio of at least 3:1. The stability of GF in frozen hemolysed blood samples was investigated by using 10 samples of pooled swine hemolysed blood spiked with GF to a final concentration of 100 ng/ml. These samples were frozen and kept at −80 °C. Five samples at a time were thawed after 3 and 18 months, spiked with IS and analysed (*ut supra*).

2.10. Accuracy and precision

Accuracy and precision of the analytical procedure were tested at three GF concentrations (20, 2 and 0.2 ng/ml) which were added to pooled hemolysed swine blood samples. Spiked and blank samples ($n = 8$) were analysed daily for 8 days.

2.11. GF quantification in in vivo hemolysed blood samples

GF concentrations in *in vivo* swine blood samples were determined by GC–EI-MS (GC A) and were referred to calibration curves of pooled swine blood samples spiked with GF (0.02, 0.2, 1, 2, 5, 10, 20 ng/ml) and the internal standard (d_{11}-GF). Calibration curves were generated for each analytical run.

3. Results and discussion

3.1. Resolution of enantiomers of cyclosarin and d_{11}-cyclosarin by gas chromatography

The enantiomers of GF were completely (baseline) separated on a GAMMA DEX^{TM} GC column. The enantiomers of d_{11}-GF were completely (baseline) separated, both from each other and from the GF enantiomers (Fig. 2). The relation of the peak areas of a pair of individual isomers (GF and d_{11}-GF) remained equal to 1 and was not affected by changes in injection temperature (75–260 °C), by organic solvents in the injection solution (hexane, 2-propanol) and by endogenous impurities from hemolysed blood after sample preparation. This was valid for a concentration range of GF from 0.3 to 2000 ng/ml in the injection solution.

3.2. Specificity and stereospecificity

Interference with endogenous compounds and potential impurities derived from sample processing (e.g. SPE cartridges, solvents) is shown in Figs. 3 and 4. The peak of (−)-GF isomer was clearly detectable in all spiked and *in vivo* hemolysed blood samples. No interference of this peak with peaks from other impurities from spiked and *in vivo* blood samples was found in comparison to GF standard samples. The retention times and the resolution of the isomers did not vary in standard, spiked and *in vivo* samples, minimal differences in retention time were due to slightly different column lengths. Neither racemisation nor deracemisation of GF occurred in organic media (standard solution in 2-propanol and 2-propanol eluate produced on the basis of the spiked and *in vivo* blood samples). Neither racemisation nor deracemisation of GF were observed during storage of the concentrated eluate at 4–25 °C up to 1.5 months. The relationship between the peak areas of the both enantiomers of GF did not vary during the injection of the eluate into the GC–MS system at injection temperatures between 75 and 260 °C.

The peak of (+)-GF in the EI mode was partially overlapped by endogenous impurities (Fig. 3). Therefore, only the quantita-

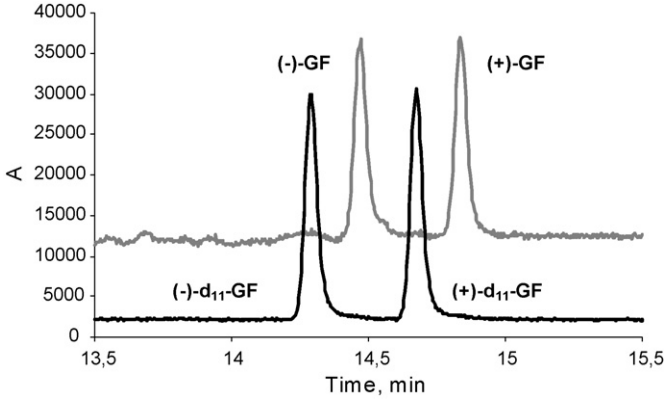

Fig. 2. Chromatogram of racemic cyclosarin on gamma cyclodextrin, GAMMA DEX^{TM} 225 column (details of the experimental conditions are given in Section 2.6).

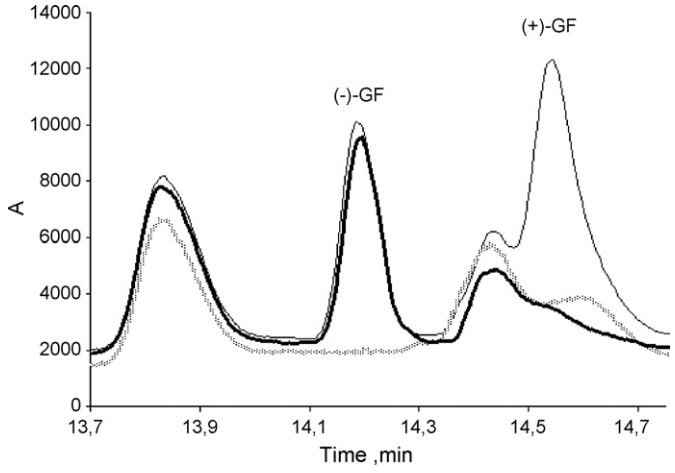

Fig. 3. GC–EI-MS chromatograms with *m/z* 99 of a blank hemolysed blood sample (grey line), a spiked hemolysed blood sample (thin black line) and an *in vivo* hemolysed blood sample (thick black line) using a column GAMMA DEX^{TM} 225 (cf. Section 2.1). Differences to retention times in comparison to Figs. 2 and 4 resulted from a reduced column length.

tive analysis of (−)-GF was possible in the EI mode. Chemical ionisation in the negative ion mode (NCI-MS) using methane as the reagent gas [17] was applied additionally to detect (+)-GF in hemolysed blood samples with the system GC B. (+)-GF was not detected in any of the *in vivo* samples examined after i.v. infusion of 86.5 μg/kg of GF (Fig. 4). No deracemisation of GF in the spiked blood samples or racemisation of (−)-GF in *in vivo* samples was observed, therefore, solutions of GF and d_{11}-GF were used as standard solutions of individual isomers.

3.3. Recoveries

All recoveries were evaluated for racemic GF (no deracemisation was determined for GF during the sample processing, cf. Section 2.8). A summary of the results is given in Table 1.

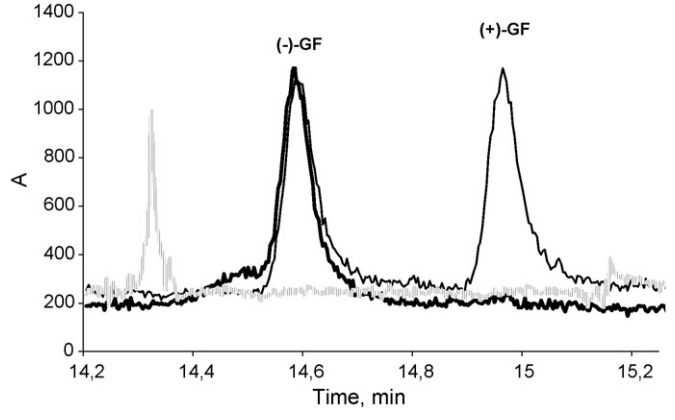

Fig. 4. GC–NCI-MS chromatograms with *m/z* 179 of a blank hemolysed blood sample (grey line), a spiked hemolysed blood sample (thin black line) and an *in vivo* hemolysed blood sample (thick black line) using a column GAMMA DEX^{TM} 225 (cf. Section 2.1).

Table 1
Recoveries of GF ($n = 5$)

C_{GF} (ng/ml)	0.2	2	20
Absolute recovery in hemolysed blood samples	80 ± 15	88 ± 12	85 ± 11
Recovery compared to the IS	101 ± 2	100 ± 3.5	102 ± 4
Recovery of SPE	n.t.	93.5 ± 8.5	95 ± 7
Recovery of the evaporation from hexane	n.t.	98 ± 3	99 ± 3
Recovery of the evaporation from 2-propanol	n.t.	98 ± 2	98 ± 2

n. t. = not tested.

Table 2
Summary of precision and accuracy of the analytical method for cyclosarin in the hemolysed and stabilized blood samples of swine ($n = 8$)

$C_{(-)\text{-GF}}$ added (ng/ml)	$C_{(-)\text{-GF}}$ measured (ng/ml)	Relative standard deviation (%)	Accuracy (%)
0.0	0.0		
0.20	0.18	7.2	90
2.0	2.0	5.5	100
20	20	5.6	100

3.4. Linearity, sensivity and stability

All calibration curves for the analysis of (−)-GF in hemolysed blood samples $A_{(-)\text{-GF}}/A_{(-)\text{-}d_{11}\text{GF}}$ versus $C_{(-)\text{-GF}}/C_{(-)\text{-}d_{11}\text{GF}}$ were linear within a GF concentration range of 0.02–20 ng/ml. Representative calibration equation was $(A_{(-)\text{-GF}}/A_{(-)\text{-}d_{11}\text{GF}}) = 0.799(C_{(-)\text{-GF}}/C_{(-)\text{-}d_{11}\text{GF}}) + 0.065$ ($R^2 = 0.999$). The limit of detection of (−)-GF in EI mode and of (+)- and (−)-GF in NCI mode amounted to approximately 1 and 5 pg, respectively. The lowest concentration of the calibration graph for (−)-GF converted into mass was 4.8 pg, which therefore corresponded to the lower limit of quantification. The measured concentrations of GF in spiked (100 ng/ml) hemolysed blood samples after storage (−80 °C) for 3 and 18 months amounted to 96 ± 6 and 98 ± 7 ng/ml. No deracemisation of GF enantiomers in all these samples was detected. Therefore, GF appeared to be stable in stabilized formate buffer systems at

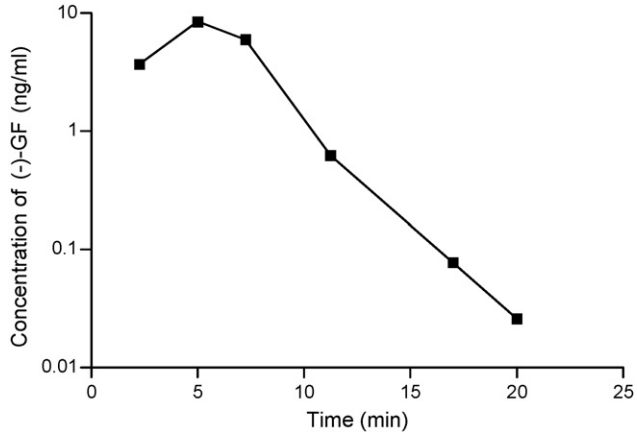

Fig. 5. (−)-GF concentration in hemolysed blood from a swine poisoned by an i.v. infusion of 86.5 μg/kg GF.

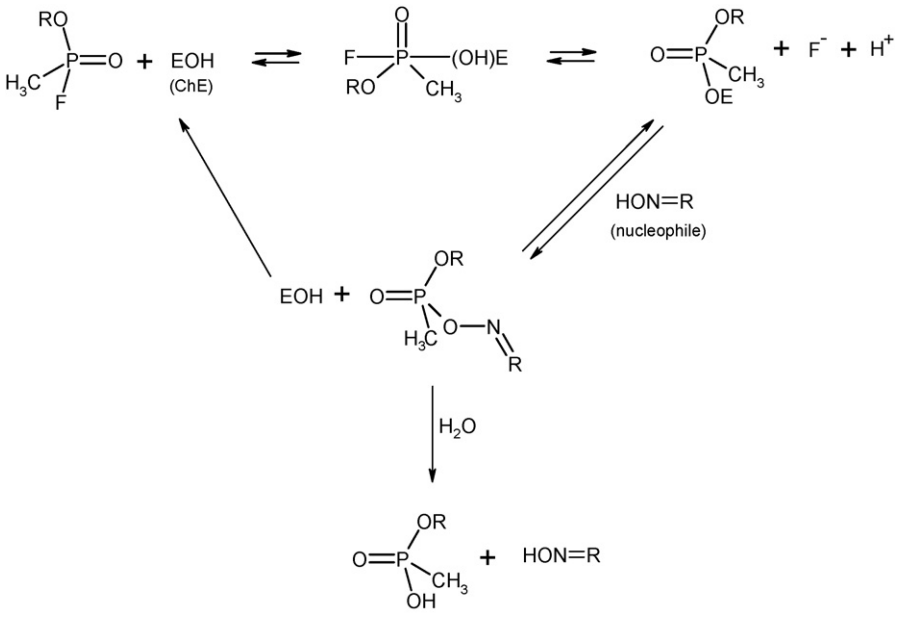

Fig. 6. Hydrolysis of GF by means of reactivation–inhibition cycles of AChE and BuChE in the presence of the nucleophile HI 6.

−80 °C for at least 18 months. GF was also stable in samples after two freeze–thaw cycles and incubation in an ice bath for at least 1 h.

3.5. Accuracy and precision

A summary of the results on precision and accuracy as derived from the measured concentrations of GF in the spiked blood samples is given in Table 2.

3.6. Determination of (−)-GF concentration in swine blood after an i.v. supralethal infusion of cyclosarin

The analytical procedure for the quantitative determination of GF isomers was applied to hemolysed blood samples from swine poisoned by a GF infusion. Fig. 5 gives the data for the concentration of (−)-GF in hemolysed blood samples from a swine poisoned by GF and treated by i.v. HI 6 and atropine. The mean infusion time was estimated graphically from the experimental data of all animals and amounted to 4.8 ± 0.3 min which corresponded to the infusion time of 5 min described in the experimental protocol (Section 2.5). In general (−)-GF could be determined only for a limited period, up to 20 min after start of GF infusion (Fig. 5). (+)-GF could not be determined in any blood sample from GF poisoned swine even by using the GC–NCI-MS method which masked out overlapping contaminants (Section 3.2, Fig. 4). The short residence time of (−)-GF, compared to other nerve agents, e.g. soman [18], may be due to a relatively rapid hydrolysis (enzymatic and non-enzymatic) of GF in blood *in vivo* [19] and to consumption of GF by repeated reactivation–inhibition cycles of GF-inhibited acetylcholinesterase and butyrylcholinesterase in the presence of the reactivator HI 6 (Fig. 6). Previous studies with sarin and soman showed that P(+) isomers were hydrolyzed extremely fast by endogenous enzymes *in vivo* [2,20] and (+)-GF by human plasma *in vitro* [15]. Hence, it may be assumed that (+)-GF undergoes rapid hydrolysis *in vivo* in swine (much more rapid than (−)-GF) being the reason for the failure to detect this isomer in swine blood samples.

4. Conclusions

The described combined method on the basis of the extraction, automated solid phase extraction and GC–EI(NCI)-MS allowed complete chiral separation of the GF enantiomers and accurate quantification of (−)-GF in hemolysed swine blood. This method is characterised by its relative simplicity, high reproducibility, high selectivity and sensitivity. The application of this method was demonstrated by quantifying GF in blood samples obtained from GF poisoned swine.

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