Forensic Science International 323 (2021) 110818

Contents lists available at ScienceDirect

Forensic Science International

journal homepage: www.elsevier.com/locate/forsciint

Evidence of exposure to organophosphorus toxicants by detection of the propionylated butyrylcholinesterase-derived nonapeptide-adduct as a novel biomarker

Harald John [a,*], Annika Richter [b], Markus Siegert [a,b], Florian Eyer [c], Horst Thiermann [a]

[a] Bundeswehr Institute of Pharmacology and Toxicology, Neuherbergstrasse 11, 80937 Munich, Germany
[b] Department of Chemistry, Humboldt-Universität zu Berlin, Brook-Taylor-Strasse 2, 12489 Berlin, Germany
[c] Department of Clinical Toxicology, TUM School of Medicine, Technical University of Munich, Ismaninger Strasse 22, 81675 Munich, Germany

A R T I C L E I N F O

Article history:
Received 10 December 2020
Received in revised form 26 April 2021
Accepted 26 April 2021
Available online 5 May 2021

Keywords:
BChE-adducts
Derivatization
LC MS/MS
Nerve agents
Pesticides
Propionic anhydride
Verification

A B S T R A C T

Organophosphorus (OP) nerve agents represent a class of highly toxic chemical warfare agents banned by the Chemical Weapons Convention. Nevertheless, in the past few years they have been used repeatedly for warfare, assassination and attempted murder. In addition, the chemically related OP pesticides were frequently used for suicide and may be deployed for terroristic attacks. Therefore, sensitive and selective bioanalytical methods are indispensable to investigate biological specimens as pieces of evidence to prove poisoning. OP agents form long-lived covalent reaction products (adducts) with endogenous proteins like human serum albumin (HSA) and butyrylcholinesterase (BChE). The adducted nonapeptide (NP) obtained by proteolysis of the BChE-adduct is one of the most sensitive and important biomarkers. We herein present a novel class of NP-adducts propionylated at its N-terminal phenylalanine residue (F^{195}). The biomarker derivative is produced by addition of propionic anhydride to the NP-adduct inducing its quantitative conversion in aqueous buffer within 5 min at room temperature. Afterwards the mixture is directly analyzed by micro-liquid chromatography-electrospray ionization tandem-mass spectrometry (µLC-ESI MS/MS). The sensitivity of the method is comparable to that of the non-derivatized NP-adduct. These characteristics make the method highly beneficial for forensic analysis especially in cases in which the OP agent does not form adducts with HSA that are typically targeted as a second biomarker of exposure. This novel procedure was successfully applied to nerve agent-spiked samples sent by the Organisation for the Prohibition of Chemical Weapons (OPCW) as well as to plasma samples of real cases of pesticide poisoning.

© 2021 The Author(s). Published by Elsevier B.V.
CC_BY_NC_ND_4.0

1. Introduction

According to the Chemical Weapons Convention the development, storage, transport and deployment of toxic organophosphorus (OP) nerve agents (NA) and chemical warfare agents in general are forbidden [1]. Abidance by that rules is observed and controlled by the Organisation for the Prohibition of Chemical Weapons (OPCW) [2]. Nevertheless, several recent incidents in the Syrian Arab Republic [3–8], at the airport of Kuala Lumpur (Malaysia) in 2017 [9,10], in Salisbury (UK) in 2018 [11–13] as well as the current case of Alexei Navalny document that chemical agents are still used for warfare, attempted murder and assassination. In addition, OP pesticides, that are structurally closely related to OP-NA, also cause several hundred thousand's death per year worldwide due to accidental or intentional incorporation and also represent potential poisons for terroristic attacks [14–17]. Therefore, forensic bioanalytical procedures are indispensable to verify poisoning by OP toxicants and are thus required for criminal prosecution. In surviving individuals lowest concentrations of OP-NA may result from the short half-lives of the agents and their polar enzymatic and non-enzymatic degradation products, which in vivo often not exceed a couple of hours or days [18]. Therefore, protein-adducts that represent covalent reaction

Abbreviations: ACN, acetonitrile; BChE, butyrylcholinesterase; BioPT, bioproficiency test of the OPCW; CID, collision-induced dissociation; CVX, Chinese VX; d3-Atr, triple deuterated atropine; DEP, diethyl phosphate; DMP, dimethyl phosphate; ESI, electrospray ionization; FA, formic acid; GA, tabun; GB, sarin; GD, soman; GF, cyclosarin; HR, high-resolution; HSA, human serum albumin; ICU, intensive care unit; IMS, immunomagnetic separation; IMPA, isopropyl methyl phosphonic acid; µLC, micro liquid chromatography; LOD, lower limit of detection; LOI, lower limit of identification; MS, mass spectrometry; MS/MS, tandem-mass spectrometry; NA, nerve agent; NP, nonapeptide; OP, organophosphorus; OPCW, Organisation for the Prohibition of Chemical Weapons; PA-NP, propionylated nonapeptide; Q1 – Q4, qualifying product ions 1–4; rf, molecular response factor; RT, room temperature; RVX, Russian VX; SRM, selected reaction monitoring; UF, ultrafiltration

* Corresponding author.
E-mail address: haraldjohn@bundeswehr.org (H. John).

https://doi.org/10.1016/j.forsciint.2021.110818
0379-0738/© 2021 The Author(s). Published by Elsevier B.V.
CC_BY_NC_ND_4.0

products of the agent with endogenous proteins from e.g. plasma are very beneficial biomarkers for post-exposure analysis. Adducts with the highly reactive but low abundant butyrylcholinesterase (BChE, approximately 4 μg/mL, UniProtKB No. P06276, EC 3.1.1.8) and with the less reactive but high abundant human serum albumin (HSA, 40 mg/mL, UniProtKB No. P02768) may be traceable up to 4 weeks after exposure [3,5,18,19]. After proteolysis biomarker peptides that still bear the covalently bound poison-derived phosphyl-moiety are selectively detected by e.g. micro liquid chromatography tandem-mass spectrometry (μLC MS/MS) [18–21]. Following this procedure the presence of BChE-adducts is proved by the detection of the nonapeptide (NP) biomarker FGES*AGAAS containing the adducted serine residue Ser[198] (marked with an asterisk; numbering of amino acids of BChE used herein does not include the signal peptide) [20–22].

However, based on OPCW guidelines at least two biomarkers have to be detected for the verification of poisoning. Peptide-adducts derived from the less reactive HSA may thus represent the only long-term biomarkers required for analysis in addition to NP-OP-adducts. Nevertheless, detection of HSA-derived biomarkers will fail, if only a smallest amount of an OP-NA or if an OP-NA of highly selective reactivity not forming adducts with HSA per se was incorporated. In such cases the generation and detection of derivatized NP-NA-adducts may be beneficial and essential to provide additional evidence of poisoning. Therefore, we elaborated the suitability of propionic anhydride for derivatization prior to μLC MS/MS analysis and applied this procedure to real cases of poisoning.

2. Materials and methods

2.1. Chemicals

Acetonitrile (ACN, gradient grade), isopropanol (iPrOH, p.A) and water (LiChrosolv) were purchased from Merck (Darmstadt, Germany) and propionic anhydride (≥99%) from Sigma-Aldrich (Steinheim, Germany). Formic acid (FA ≥ 98%) and NaOCl solution for decontamination (12% Cl_2) were from Carl Roth (Karlsruhe, Germany), pepsin from gastric mucosa was from Roche Diagnostics (Mannheim, Germany), NH_4HCO_3 (ultra-grade, ≥ 99.5%) from Fluka (Buchs, Switzerland) and three-fold deuterated atropine (d3–Atr) from CDN Isotopes (Pointe Claire, Quebec, Canada). OP-NA comprizing tabun (GA, CAS No 77–81–6), sarin (GB, CAS No 107–44–8), soman (GD, CAS No 96–64–0), cyclosarin (GF, CAS No 329–99–7), VX (CAS No 50782–69–9), Chinese VX (CVX, CAS No 468712–10–9) and Russian VX (RVX, CAS No 159939–87–4) were made available by the German Ministry of Defence and tested for integrity and purity in-house by nuclear magnetic resonance spectroscopy. Working solutions of these OP-NA in iPrOH (4 mM) were used for mixing with plasma. The diethyl phosphate (DEP) pesticide paraoxon as well as the dimethyl phosphate (DMP) pesticide malaoxon were from Dr. Ehrenstorfer (Augsburg, Germany) in purities >97%. Working solutions of the pesticides in iPrOH (200 mM) were used for plasma spiking. Human EDTA plasma from different individuals was purchased from in.vent Diagnostica (Hennigsdorf, Germany) and plasma pooled from numerous donors was from Dunn Labortechnik (Asbach, Germany). Magnetic beads pre-coated with protein G (Dynabeads) were from ThermoFisher Scientific (Waltham, MA, USA) and mouse monoclonal anti-human BChE antibodies (3E8) from Antibody Shop (Utrecht, The Netherlands).

2.2. Incubation of plasma with OP toxicants

Human EDTA plasma was separately mixed with the OP toxicant (OP-NA and pesticides) working solutions listed above resulting in concentrations of 40 μM for OP-NA and 2 mM for pesticides. Blank plasma was mixed with respective volumes of neat iPrOH, exclusively. Incubation under gentle shaking was allowed for 1 h at 37 °C followed by overnight storage at 4 °C. Complete inhibition of the BChE activity in plasma was tested by a modified Ellman assay. If necessary, plasma samples were stored at −80 °C prior to use.

2.3. Ellman assay for BChE activity

For determination of the BChE activity a modified Ellman assay was used as described before [23,24].

2.4. Plasma sample preparation for BChE-adduct analysis

As addressed below, the sample preparation procedure included BChE extraction from plasma, its proteolysis, derivatization of the resulting NP-adduct and final μLC MS/MS analysis in the selected reaction monitoring (SRM) mode after positive electrospray ionization (ESI).

2.4.1. Extraction of BChE and its adducts from plasma

BChE was extracted from plasma (200 μL) applying immunomagnetic separation (IMS) as described by Sporty et al. [22] using magnetic beads labeled with mouse monoclonal antibodies (mAb) directed against human BChE.

2.4.2. Proteolysis and ultrafiltration

The preparation of extracted BChE was based on the procedure described by Fidder et al. [21]. In brief, the proteolysis of BChE bound to the beads was carried out with pepsin (235 μg/mL in 0.6% v/v FA, 85 μL in total) for 30 min at 37 °C followed by ultrafiltration (UF, 10 min at 10,270×g, 15 °C) using 0.5 mL Amicon ultrafiltration units (molecular weight cut-off 10 kDa, Merck-Millipore, Darmstadt, Germany) and two-times washing of the retentate with 0.5% v/v FA yielding about 120 μL ultrafiltrate in total containing the BChE-derived NP.

2.4.3. Derivatization of BChE-derived NP

A portion (30 μL) of the ultrafiltrate obtained after proteolysis and UF was evaporated to dryness and re-dissolved in 50 μL 50 mM NH_4HCO_3 (pH 8.0). An aliquot (20 μL) was mixed with 4 μL propionic anhydride followed by a 5 min incubation at room temperature (RT) to produce the propionylated derivatives of NP (PA-NP) and its adducts (PA-NP-adduct). Afterwards, 20 μL of this solution were mixed with 70 μL d3-Atr solution (3 ng/mL in 0.5% v/v FA) and 1.8 μL 8 M NaOH prior to MS-based analysis either as μLC-ESI MS/MS (SRM) or as μLC-ESI high-resolution tandem-mass spectrometry (MS/HR MS).

2.5. μLC-ESI MS/HR MS analysis

For MS/HR MS detection of PA-NP-adducts produced from GA, GB, GD, GF, VX, malaoxon and paraoxon as well as the non-adducted PA-NP initial μLC-ESI MS/HR MS runs were carried out. Spectra of product ions of the single and double protonated precursor ions were monitored to measure their exact masses and for structural assignment.

For chromatographic separation a microLC 200 system (Eksigent Technologies, Dublin, CA, USA) equipped with a HTC-xt DLW autosampler (CTC Analytics, Zwingen, Switzerland) and a 20 μL sample loop (Sunchrom, Friedrichsdorf, Germany) was used. The separation was carried out on an Acquity UPLC HSS T3 column (50 × 1.0 mm I.D., 1.8 μm, 100 Å; Waters, Eschborn, Germany) protected by a pre-column (Security Guard Ultra cartridge C18 peptide; Phenomenex, Aschaffenburg, Germany). Gradient elution was applied using solvent A (0.05% v/v FA) and solvent B (ACN/H_2O 80:20 v/v, 0.05% v/v FA) with a flow of 30 μL/min at 40 °C: t [min]/B [%]: 0/2, 5/25, 14/35, 15/95, 18/95, 19/2, 20/2. Chromatography was controlled using the

Eksigent control software (version 4.2) as an extension of the Analyst TF 1.7.1 software (both ABSciex, Darmstadt, Germany).

The µLC system was on-line coupled to a TT5600+ TripleTOF mass spectrometer (ABSciex) by positive ESI to record product ion spectra of the protonated NP as well as PA-NP-adducts in the range from *m/z* 50 to *m/z* 1200 with an accumulation time of 50 ms. The following MS parameters were applied: ion spray voltage floating (ISVF) + 5500 V, declustering potential (DP) 60 V, collision energy (CE) 45 V, collision energy spread (CES) 5 V, ion release delay (IRD) 67 ms, ion release width (IRW) 25 ms, source gas 1 (GS1) 50 psi (3.45×10^5 Pa), source gas 2 (GS2) 50 psi (3.45×10^5 Pa), curtain gas (CUR) 30 psi (2.07×10^5 Pa) and temperature 300 °C. Data acquisition, processing and data analysis were performed using the Analyst TF 1.7.1, PeakView 2.1 and MultiQuant 2.1.1 software package (all ABSciex).

2.6. µLC-ESI MS/MS (SRM) analysis

A M5 microLC system with an integrated autosampler (kept at 15 °C, ABSciex) equipped with a 20 µL sample loop and a column oven as an integral part of the on-line coupled mass spectrometer (QTrap 6500+, ABSciex) was used for chromatography applying the same conditions of mobile and stationary phase as described above.

MS/MS measurements of the single protonated precursor ions of the derivatized and non-derivatized NP-OP-adducts were performed in SRM mode after positive ionization (5 kV) and collision-induced dissociation (CID) using nitrogen as collision gas. The following MS parameters were generically applied to all analytes: DP 60 V, CUR 30 psi (2.07×10^5 Pa), temperature 200 °C, GS1 50 psi (3.45×10^5 Pa), GS2 60 psi (4.14×10^5 Pa), entrance potential (EP) and cell exit potential (CXP) 10 V each, and dwell time 30 ms. d3-Atr was monitored by the following transitions: *m/z* 293.3 > *m/z* 127.1 and *m/z* 93.1 using a CE of 42 V. The CE values were individually set between 25 V and 40 V for each transition as indicated in Table 1 allowing monitoring of four qualifying ions (Q1-Q4) each. The entire system was controlled by the Analyst 1.7.1 and the Eksigent control (version 4.3) software (both ABSciex).

2.7. Selectivity

Blank plasma obtained from 6 human individuals was prepared and analyzed by µLC-ESI MS/MS (SRM) to monitor any interference for any of the transitions used for monitoring of PA-NP and the diverse PA-NP-adducts listed in Table 1.

2.8. Stability of PA-NP and PA-NP-adducts in the autosampler

Prepared plasma samples containing PA-NP-adducts derived from GB, GD, GF, VX as well as paraoxon and malaoxon were stored in the autosampler at 15 °C and frequently analyzed ten-times within a 24 h period. Peak areas obtained from the individual Q1 were determined to monitor the respective relative concentration profiles.

2.9. Determination of the derivatization yield and calculation of the molecular response factor

BChE-adducts were extracted from plasma containing completely inhibited BChE (100% inhibition) and subsequently subjected to proteolysis and UF as described above. The ultrafiltrate thus contained a maximum concentration of NP-adducts. To produce samples corresponding to only 50% inhibition of BChE, ultrafiltrates produced from completely inhibited plasma were mixed with those of the blank plasma (0% inhibition) with equal volumes thus adjusting only half of the maximum NP-adduct concentration. All filtrates –those corresponding to 100% inhibition as well as to 50% inhibition- were split (2 aliquots of 30 µL, each), evaporated to dryness and redisolved in 50 µL 50 mM NH_4HCO_3. A portion of 20 µL of one aliquot was mixed with 4 µL water (set 1) and a portion of 20 µL of the other aliquot with 4 µL propionic anhydride (set 2) followed by a 5 min incubation period at RT each. The described procedure was carried out in triplicate for the blank and each adduct. Subsequently, 70 µL d3-Atr solution (3 ng/mL in 0.5% v/v FA) and 1.8 µL 8 M NaOH were added to a portion of 20 µL of both mixtures each. The resulting solutions were injected for µLC-ESI MS/MS (SRM) analysis to monitor the derivatized and non-derivatized NP and NP-adduct.

2.9.1. Determination of the derivatization yield

The peak area (A) obtained for Q 1 of the non-derivatized NP-adduct, that remained after the derivatization step (set 2), was measured by µLC-ESI MS/MS (SRM) to calculate the ratio to the corresponding peak area obtained without derivatization (set 1). The

Table 1

Chromatographic and mass spectrometric properties of the propionylated nonapeptide-adduct-derivatives. [a]: Q1 is the most intense product ion (qualifying ion), Q2 the second most, Q3 the third most and Q4 the fourth most intense product ion; [b]: the ion ratio was calculated as the ratio of peak areas obtained from the extracted ion chromatograms of the respective product ions (Q1 - Q4); [c]: rf is the ratio of the peak area of the PA-NP-adduct to the peak area of non-derivatized NP-adduct based on the individual Q 1 ions considering the yield of derivatization, where PA-NP-adduct is the propionylated nonapeptide-adduct. The listed values were calculated as the mean ± SD obtained from samples with two NP-adduct concentrations corresponding to 50% and 100% inhibition of BChE in plasma; [d]: blank plasma not exposed to any OP was analyzed to monitor the non-phosphylated NP; [e]: CE 25 V; [f]: CE 35 V; [g]: CE 30 V; [h]: CE 40 V.

OP toxicant	t_R [min]	Precursor ion [*m/z*]	Q1[a] [*m/z*]	Q2[a] [*m/z*]	Q3[a] [*m/z*]	Q4[a] [*m/z*]	Ion ratio[b] Q2/Q1 [%]	Ion ratio[b] Q3/Q1 [%]	Ion ratio[b] Q4/Q1 [%]	rf[c] [-]
none[d]	7.9	852.4	747.3[e]	676.3[f]	605.3[f]	n.d.	70.7	39.9	n.d.	4.68 ± 0.19
GA	12.2	987.4	834.4[g]	658.3[h]	882.4[g]	811.3[g]	72.3	69.2	60.4	0.21 ± 0.13
GA (aged)	8.2	960.4	855.3[g]	784.3[g]	834.4[h]	n.d.	87.2	27.0	n.d.	0.38 ± 0.12
GB	11.6	972.4	834.4[g]	867.4[g]	658.3[h]	796.3[g]	72.8	71.8	58.8	0.41 ± 0.04
GD	16.2	1014.5	658.3[h]	834.4[g]	909.4[g]	838.4[g]	97.3	72.1	67.0	2.14 ± 0.26
GF	16.1	1012.4	834.4[g]	907.4[g]	658.3[h]	836.4[g]	72.0	65.5	50.5	1.66 ± 0.26
GB (aged)	7.5	930.4	754.3[g]	825.3[g]	658.3[g]	834.4[h]	94.4	26.3	9.3	0.44 ± 0.06
VX	10.0	958.4	834.4[g]	853.3[g]	658.3[h]	782.3[g]	78.2	76.9	67.2	0.51 ± 0.04
CVX	14.8	986.4	834.4[g]	881.4[g]	658.3[h]	810.3[g]	76.3	71.2	59.0	0.50 ± 0.06
RVX	14.6	986.4	834.4[g]	881.4[g]	658.3[h]	810.3[g]	78.5	74.4	61.9	0.42 ± 0.04
DMP	9.5	960.4	855.3[g]	784.3[g]	834.4[h]	658.3[g]	88.4	30.7	20.9	0.58 ± 0.01
DMP (aged)	7.6	946.3	841.3[g]	770.3[g]	834.4[g]	658.3[h]	89.0	47.9	34.2	0.40 ± 0.03
DEP	12.8	988.4	834.4[g]	883.4[g]	658.3[g]	812.3[g]	89.3	82.3	73.9	0.39 ± 0.05
DEP (aged)	8.1	960.4	855.3[g]	784.3[g]	834.4[g]	n.d.	85.4	26.7	n.d.	0.36 ± 0.08

CVX: Chinese VX; DEP: diethyl pesticide; DEP (aged): aged variant of DEP adduct; DMP: dimethyl pesticide; DMP (aged): aged variant of DMP-adduct; GA: tabun; GA aged: aged variant of the GA-adduct (loss of $HNMe_2$); GB: sarin; GB aged: aged variant of the GB-adduct (loss of alkoxy-group, identical for all G-and V-type nerve agents except GA); GD: soman; GF: cyclosarin; n.d.: not determined; none: non-adducted NP; NP: nonapeptide derived from BChE after pepsin-mediated hydrolysis; OP: organophosphorus; rf: molecular response factor; RVX: Russian VX; t_R: retention time; VX: nerve agent VX

yield (κ) after the derivatization process was calculated according to Eq. (1):

$$\kappa = 1 - A(\text{NP-adduct})_{set\ 2}/A(\text{NP-adduct})_{set\ 1} \quad (1)$$

2.9.2. Calculation of the molecular response factor

The molecular response factor (rf) introduced herein is a measure of the relative signal response (peak area in µLC-ESI MS/MS SRM analysis) of the derivatized NP and the derivatized NP-adduct in comparison to their corresponding non-derivatized molecules. To compare these peak areas, solutions of equal concentrations of NP or NP-adduct were analyzed either with or without derivatization. These measurements were carried out for two concentrations of the NP-adduct (correlating to 50% and 100% inhibition of the BChE activity in plasma). The rf was calculated as the ratio of the peak area obtained for Q 1 of PA-NP and PA-NP-adduct (set 2) to the peak area obtained for Q 1 of the respective non-derivatized NP and NP-adduct (set 1) corrected by the derivatization yield following Eq. (2):

$$rf = A(\text{PA-NP-adduct})_{set\ 2}/[\kappa * A(\text{NP-adduct})_{set\ 1}] \quad (2)$$

These rf-values were calculated for both concentrations of each PA-NP-adduct corresponding to 100% and 50% inhibition of BChE. Due to their low deviations the mean of both values was calculated yielding the averaged rf-value.

2.10. Samples from real cases of pesticide poisoning and of OPCW bioproficiency test

Plasma samples of real cases of poisoning were obtained from two patients intoxicated after swallowing of diverse pesticides. Detailed clinical information on these cases are provided in our earlier reports [15,16].

In brief, Case 1: A 79 years old man had ingested an unknown amount of the DEP-pesticide parathion-ethyl {*O*,*O*-diethyl *O*-(4-nitrophenyl) phosphorothioate, CAS No. 56–38–2} trying to commit suicide. After transfer to the intensive care unit (ICU) plasma samples were taken documenting severely inhibited BChE activity and therapeutic treatment started. Twenty-one days after poisoning the patient died due to multi-organ failure as a consequence of the cholinergic crisis and septic complications [15]. Informed consent for plasma investigations was obtained from close relatives of the anesthetized and artificially ventilated patient [25].

Case 2: A 87 years old man had accidentally ingested two sips of a commercial pesticide solution containing the DMP-pesticide dimethoate {O,O-dimethyl S-(2-(methylamino)-2-oxoethyl) phosphorodithioate, CAS-No. 60–51–5}. Feeling sick, suffering from stomach pain, vomiting and being disorientated he called the ambulance for medical treatment. After first care he was admitted to the toxicological department of the local hospital where plasma samples were taken that showed considerably inhibited BChE activity. Nevertheless, typical signs and symptoms of cholinergic crisis were missing allowing the release of the patient the next day [16]. Informed consent was obtained from the patient [25].

Within the course of the 4th (2019) and 5th (2020) bioproficiency test (BioPT) plasma samples spiked in vitro with OP-NA not known to the participating laboratories were provided by the OPCW. Subsequent analyses in-house targeting adducts of BChE and HSA revealed the presence of e.g. GB and GF (4th BioPT) as well as CVX (5th BioPT). After storage at −80 °C these samples were analyzed again by the herein presented method now targeting the propionylated NP-adducts.

3. Results and discussion

Forensic analysis of poisoning with OP toxicants is most promising by the detection of biomarkers derived from long-term protein-adducts. Therefore, novel methods targeting peptide biomarkers are important to extend bioanalytical possibilities. Following this idea, derivatives of the NP-adduct biomarkers should also be considered.

In the past decades the derivatization of analytes was often required to make polar molecules more hydrophobic and thus suitable for gas chromatography (GC) analysis. In addition, derivatization should also improve the properties of the analytes in LC analysis in terms of selectivity and sensitivity when using e.g. ultraviolet (UV)-absorbance or fluorescence detection. Multiple reagents and derivatization procedures exist as summarized by e.g. Blau and Halket [26]. In contrast, LC methods making use of highly selective and sensitive MS-based detection most often do not need any derivatization. Nevertheless, peptide and protein derivatizations with e.g. stable isotope-labeled reagents are often the method of choice in proteomics applications especially when intending relative quantification of differently expressed proteins [27–31]. In most cases nucleophilic sites like the nitrogen-atom of the N-terminal amine-function are effectively targeted. Recently, van Faassen et al. reported on a simple and highly effective procedure to derivatize serotonin, 5-hydroxyindoleacetic acid (5-HIAA) and 5-hydroxytryptophan (5-HTP) at the only nucleophilic OH-group of these molecules by adding propionic anhydride (25% v/v in ACN) directly to plasma for a 15 min reaction at ambient temperature [32]. Adducts of the carboxylic acid function of tryptophan, 5-HTP and 5-HIAA were not found. Propionic anhydride has also been used before to derivatize e.g. nucleotides [33], phenolic compounds [34] and cytokines [35] improving chromatographic separation and ESI responses. Therefore, we decided to transfer this rapid and simple procedure to the NP-adducts and to elaborate their applicability for forensic analysis. Accordingly, BChE-adducts were produced by incubation of plasma with OP toxicants and extracted by IMS followed by derivatization and µLC-ESI MS/MS (SRM) analysis.

3.1. Incubation of plasma with OP toxicants and BChE extraction by IMS

BChE-adducts were produced in plasma and extracted by IMS following established protocols yielding highly purified and concentrated BChE immobilized on the beads [22,36]. Plasma samples incubated with diverse OP-NA exhibited complete inhibition (100% inhibition) of the BChE-activity as proven by Ellman assay (data not shown). Therefore, IMS of these samples resulted in a maximum amount of adducted BChE bound to the beads. In contrast, extraction of blank plasma (0% inhibition) yielded the respective maximum amount of the non-inhibited BChE. Pepsin-mediated proteolysis of the immobilized BChE [22] thus yielded either the adducted peptide FGES*AGAAS (NP-adduct) or its non-adducted variant FGESAGAAS (NP) present in the ultrafiltrate after UF (Fig. 1).

3.2. Derivatization of NP and NP-adducts

Dried ultrafiltrates were redisolved in 50 mM NH_4HCO_3 and mixed with propionic anhydride for short-term derivatization of the NP and NP-adduct in aqueous buffer under mildest conditions in terms of pH and temperature. Subsequent addition of NaOH allowed the increase of the pH to only slightly acidic conditions (pH 5) thus optimizing the chromatographic peak shape especially of the derivatized aged NP-adducts possessing a phosphonic or phosphoric acid hydroxyl-group (Fig. 1). µLC-ESI MS/HR MS analysis revealed that NP and NP-adducts were quantitatively converted into one single derivative eluting as one single peak each and bearing only one propionyl-moiety each (Fig. 1). Accordingly, derivatives were characterized by a mass increase of 56 Da and occurred as single, $[M+H]^+$, and double protonated species, $[M+2H]^{2+}$. Under CID the $[M+H]^+$ ions underwent a common scheme of fragmentation independent of the adducted OP toxicant. Fig. 2A exemplarily

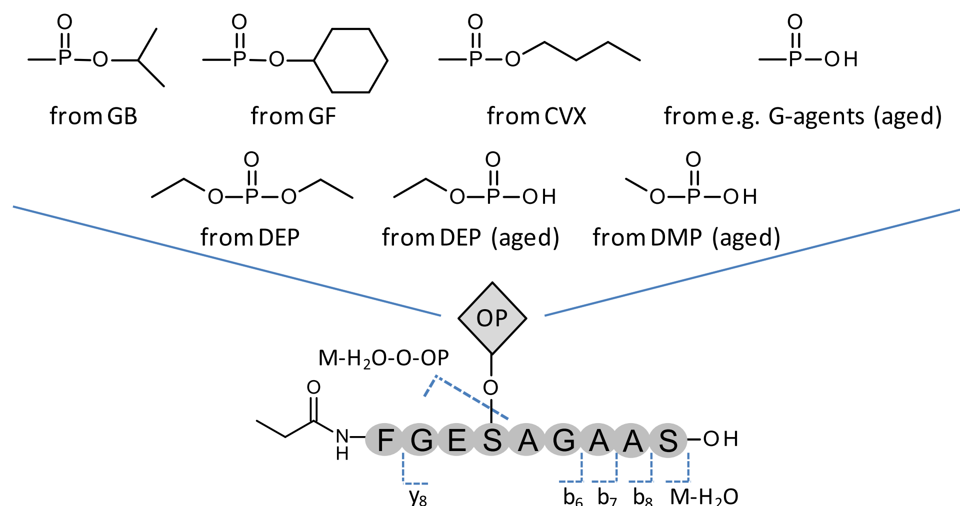

Fig. 1. Chemical structures of the N-terminally propionylated nonapeptide-adduct (PA-NP-adduct) derived from BChE adducted by diverse organophosphorus (OP) toxicants. BChE is inhibited after intoxication by diverse OP nerve agents like sarin (GB), cylosarin (GF) and Chinese VX (CVX) or by pesticides of the diethyl phosphate (DEP) and dimethyl phosphate group (DMP) covalently binding to the S^{198} residue (phosphylation). The phosphyl-moieties of these adducts (depicted as the gray diamond labeled with OP) might undergo partial hydrolysis forming their aged variants. The chemical structures of some phosphyl-moieties, that were detected in plasma samples obtained from real cases of pesticide poisoning as well as from the OPCW BioPT, are exemplarily shown in the upper part of the figure. BChE-adducts were proteolyzed yielding the respective NP-adducts followed by derivatization with propionic acid anhydride to be detected by µLC-ESI MS/MS. Generic product ions of PA-NP-adducts resulting from tandem-mass spectrometric fragmentation are marked by the dashed lines.

illustrates the MS/HR MS spectrum of the propionylated NP adducted with an isopropyl methyl phosphonic acid-moiety (IMPA) derived from GB treatment ($[M+H]^+$ *m/z* 972.410). The signals at *m/z* 834.361 ($[M\text{-}IMPA+H]^+$), *m/z* 729.3169 (b_8-IMPA), *m/z* 658.2824 (b_7-IMPA), *m/z* 587.2453 (b_6-IMPA) and *m/z* 530.2248 (b_5-IMPA) represent product ions of the b-series after the loss of the IMPA-moiety. The presence of the y_8 ion (*m/z* 769.3116, loss of PA-F) clearly proved that the derivatization by propionic anhydride had occurred at the N-terminal nitrogen atom of the peptide. The derivatization of the OH-groups of the side chains of the Ser^{203} residue in the adducted NP and of Ser^{198} and Ser^{203} in the non-adducted NP potentially yielding propionic acid esters were not observed. In addition to the b-IMPA product ion series (Fig. 2A) discussed above, signals were detected that were assigned to ions of the b-ion series that still born the intact phosphonyl-moiety ($[M\text{-}H_2O+H]^+$: *m/z* 954.3947, b_8: *m/z* 867.3642, b_7: *m/z* 796.3267, and b_6: *m/z* 725.2885). The presence of such phosphonylated ions was quite unexpected as similar fragmentation patterns of the diverse non-derivatized NP-adducts have not been described before [20–22]. This phenomenon was most likely due to the decreased basicity of the N-terminal nitrogen-atom after derivatization to an amide bond thus affecting the most likely sites of protonation within the PA-NP-adduct. Accordingly, the relative binding strength of the phosphyl-moiety seemed to be stabilized and those of the amide bonds of the peptide backbone were weakened due to a higher relative probability of protonation. However, this kind of product ions was found with high signal intensities (Fig. 2A) and therefore also considered for the µLC-ESI MS/MS (SRM) method, e.g. *m/z* 867.4 and *m/z* 796.3 for the derivatized NP-adduct of GB (Table 1).

The double protonated precursor ion of the propionylated NP-adduct of GB underwent similar fragmentation but also showed product ions corresponding to the cleaved phosphyl-moiety, e.g. $[IMPA+H]^+$ *m/z* 139.052 (Fig. 2B). The same phenomenon was also observed for the non-derivatized NP-adducts (data not shown).

3.3. Selectivity

None of the blank plasma samples derived from 6 individuals showed any interference for Q1–Q4 obtained from single protonated PA-NP and PA-NP-adducts (Table 1). Examples for the adduct of a DEP- and a DMP-pesticide are shown in Fig. 3 and examples for adducts with GB, GF and CVX are illustrated in Fig. 4. In contrast, interferences were detected for the most intense product ions of the double protonated precursors thus hindering from sensitive and selective monitoring. Therefore, product ions of $[M+H]^+$ were monitored exclusively (Table 1). In conclusion, the presented method and PA-NP derivatives in principle are well suited for investigations of unknown samples providing optimum selectivity.

3.4. Derivatization yield

A yield of about 98% ($\kappa = 0.98$) was observed for the derivatization of all NP-adducts and the non-adducted NP in all samples independent of the OP toxicant and the concentration (50% or 100% inhibition) indicating a highly effective procedure under mild conditions.

3.5. Molecular response factor

For forensic analysis the traceability of lowest concentrations of the respective biomarkers is typically requested. The lower limit of detection (LOD) and the lower limit of identification (LOI) are often provided in published reports as relevant measures to characterize the sensitivity of the method used [21,22,37–39]. However, LOD and LOI are highly dependent on the method-specific sample preparation procedure in terms of e.g. concentrating or diluting working steps. Accordingly, values obtained by the use of different procedures do not necessarily allow comparison of the suitability of different biomarkers. Therefore, we herein introduce the rf-values that only address the molecular mass spectrometric response as an independent measure that allows the estimation of sensitivity for any procedure. As the rf-values of the diverse adducts obtained from samples corresponding to 100% and 50% inhibition were quite similar documenting no concentration-dependent effects the averaged rf-values were calculated (Table 1). The rf-value for most of the analytes tested was about 0.4 indicating a somewhat lower signal response of the derivatives when compared to their non-derivatized NP-adducts. The lowest rf-value found for the GA-adduct

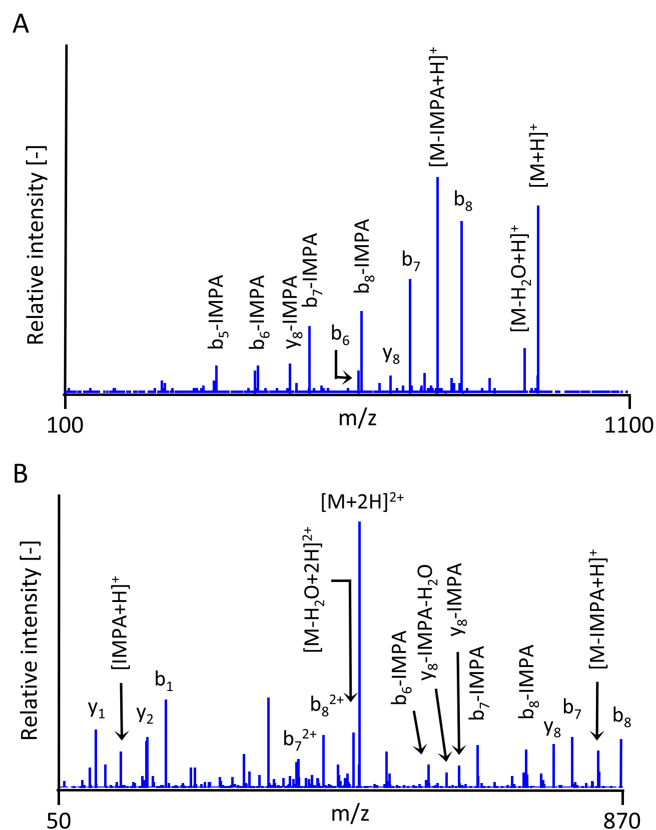

Fig. 2. Product ion spectrum of the propionylated nonapeptide-adduct (PA-NP-adduct) derived from BChE after its inhibition with the nerve agent sarin. The spectrum was extracted from a μLC-ESI MS/HR MS run monitoring product ions of the A) single protonated and B) double protonated PA-NP-adduct. IMPA represents the loss of the sarin-specific phosphyl moiety isopropyl methyl phosphonic acid as illustrated in Fig. 1. A) Signals are sorted by decreasing mass: $[M+H]^+$: *m/z* 972.410, $[M-H_2O+H]^+$: *m/z* 954.395, b_8: *m/z* 867.364, $[M-IMPA+H]^+$: *m/z* 834.361, b_7: *m/z* 796.327, y_8: *m/z* 769.312, b_8-IMPA: *m/z* 729.317, b_6: *m/z* 725.289, b_7-IMPA: *m/z* 658.282, y_8-IMPA: *m/z* 631.268, b_6-IMPA: *m/z* 587.245, b_5-IMPA: *m/z* 530.225. CID was performed with a CE of 45 V. Product ions of the b-series either still comprised the IMPA-moiety (b_9 – b_6) or had also lost this group (b_8-IMPA – b_6-IMPA). B) Signals are sorted by decreasing mass: b_8: *m/z* 867.360, $[M-IMPA+H]^+$: *m/z* 834.361, b_7: *m/z* 796.322, y_8: *m/z* 769.308, b_8-IMPA: *m/z* 729.317, b_7-IMPA: *m/z* 658.281, y_8-IMPA: *m/z* 631.264, y_8-IMPA-H_2O: *m/z* 613.257; b_6-IMPA: *m/z* 587.243, $[M+2H]^{2+}$: *m/z* 486.705, $[M-H_2O+2H]^{2+}$: *m/z* 477.699, b_8^{2+}: *m/z* 434.184, b_7^{2+}: *m/z* 398.668, b_1: *m/z* 204.101, y_2: *m/z* 177.086, $[IMPA+H]^+$: *m/z* 139.052, y_1: *m/z* 106.050. CID was performed with a CE of 15 V. The mass error calculated for the respective theoretical masses was always <5 ppm.

(0.21 ± 0.13) was most presumably due to the highly limited stability of the adduct that undergoes rapid ageing thus causing low concentrations and deterioration of quantitative evaluation. However, higher signal responses were also observed as listed for adducts of GF (1.66 ± 0.26), GD (2.14 ± 0.26) and especially the non-adducted NP (4.68 ± 0.19) documenting an improved detectability after derivatization.

Accordingly, the traceability of PA-NP-adducts was quite similar to the non-derivatized NP-adducts thus pointing out to the applicability of these novel derivatives and making them valuable additional biomarkers for OP poisoning.

3.6. Stability of PA-NP-adducts in the autosampler

All derivatized NP-adducts tested were stable for at least 24 h showing no trend of decomposition (data not shown). Accordingly, derivatized analytes were of excellent stability thus allowing analysis of larger sets of samples.

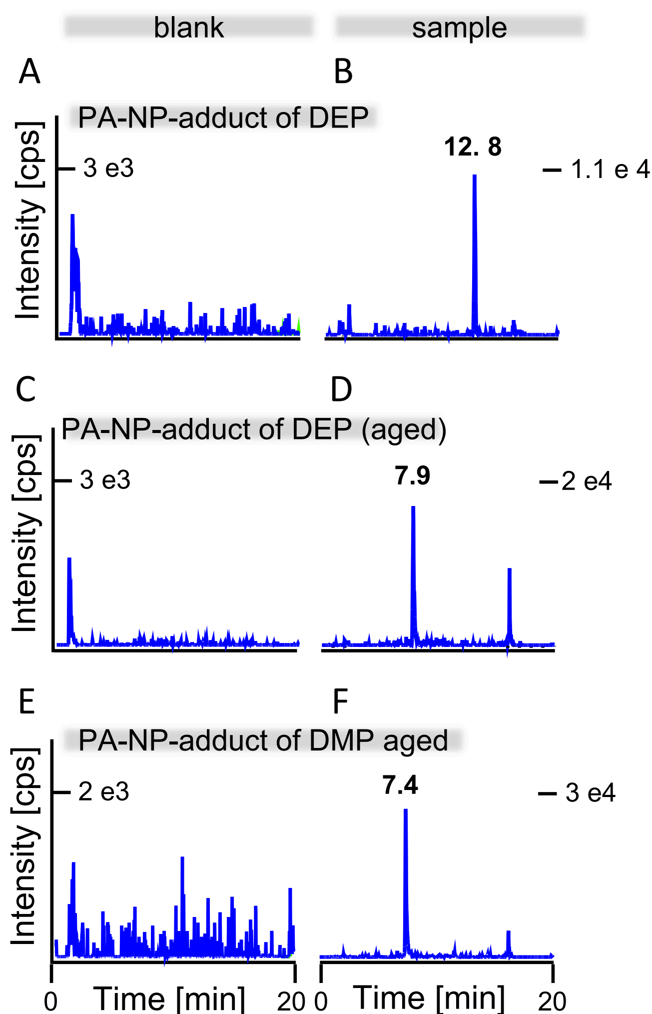

Fig. 3. Derivatized nonapeptide adducts (PA-NP-adducts) derived from BChE as biomarkers of poisoning by organophosphorus (OP) pesticides. BChE adducted with the phosphyl-moieties of OP pesticides was extracted from patient plasma and subjected to pepsin-mediated hydrolysis to quantitatively derivatize the resulting nonapeptide-adduct afterwards with propionic acid anhydride yielding its N-terminally propionylated derivative (PA-NP-adduct). Adduct derivatives confirmed poisoning by: A,B) a diethyl-pesticide (DEP, from paraoxon), C,D) a diethyl pesticide present in its aged form in the same sample (DEP aged), E,F) a dimethyl pesticide present in its aged form (DMP aged, from omethoate). Chemical structures of the adduct-derivatives are shown in Fig. 1. PA-NP-adducts were detected by μLC– ESI MS/MS in SRM mode. Plasma blanks (A,C,E) document the absence of interferences for each biomarker. For reasons of clarity, chromatograms represent the extracted ion chromatograms (XIC) only of the individual most intense product ions: A,B: *m/z* 988.4 > *m/z* 834.4, C,D: *m/z* 960.4 > *m/z* 855.3 and E,F: *m/z* 946.4 > *m/z* 770.3.

3.7. Application of the novel method to samples of real cases of poisoning and of the BioPTs

The applicability of the presented derivatization was documented by the analyses of real samples of human OP pesticide poisoning [15,16] and of human plasma samples spiked with OP-NA sent by the OPCW for the 4th and 5th BioPT. All the samples mentioned have been analyzed successfully by our working group before applying methods targeting HSA-adducts (real cases) [15,16] as well as non-derivatized BChE-adducts (BioPT samples). Therefore, this is the first report on the bioanalytical evidence of BChE-adducts in these real cases of pesticide poisoning and on NP-derivatives in the BioPT samples. The ion ratios of individual product ions found in the samples were similar to those of the references (Table 1) and thus fulfilled the quality criteria defined by the OPCW for biomedical verification and documented excellent selectivity of the method.

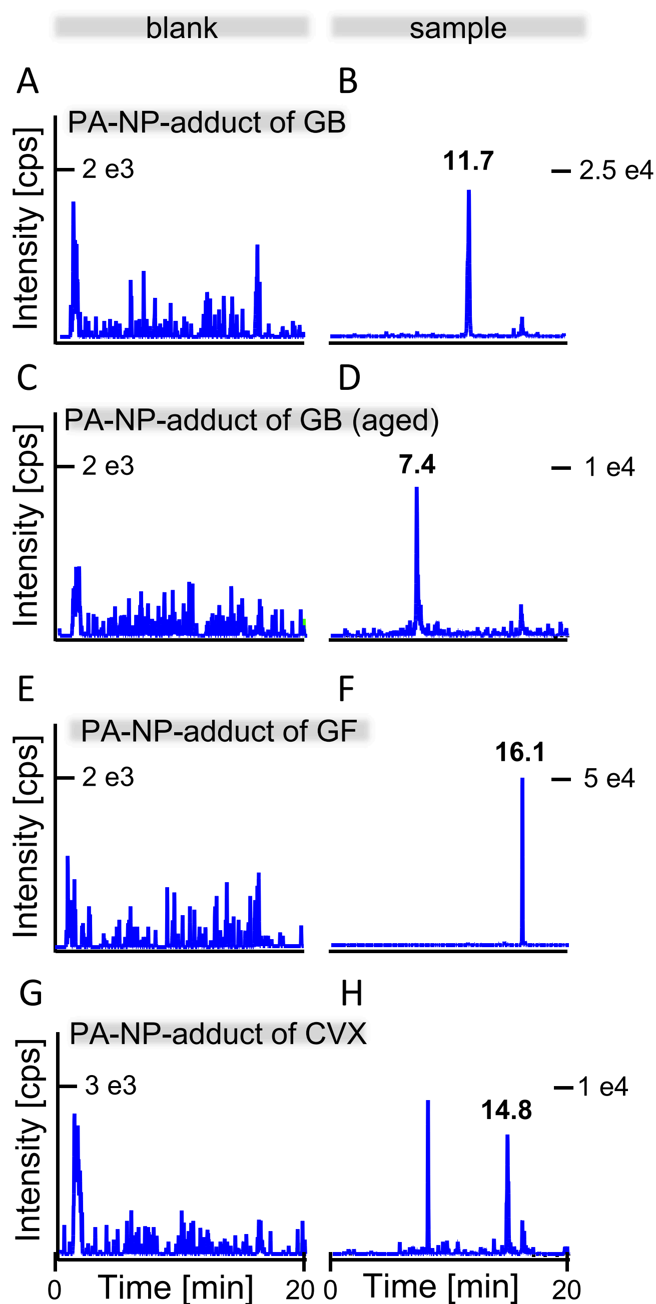

Fig. 4. Derivatized nonapeptide adducts (PA-NP-adducts) derived from BChE as biomarkers of exposure to organophosphorus (OP) nerve agents. BChE adducted with the phosphonyl-moieties of OP nerve agents was extracted from plasma samples sent by the OPCW within the course of the 4th (2019) and 5th (2020) bioproficiency tests (BioPT). Afterwards adducted BChE was enzymatically cleaved with pepsin followed by quantitative derivatization of the resulting nonapeptide-adducts with propionic acid anhydride generating the N-terminally propionylated peptide (PA-NP-adduct). Detected adduct derivatives confirmed the exposure to: A,B) sarin (GB), C,D) sarin, present in its aged adduct form (GB aged), E,F) cyclosarin (GF) and G,H) Chinese VX (CVX). Chemical structures of the adduct derivatives are shown in Fig. 1. PA-NP-adducts were detected by μLC– ESI MS/MS in SRM mode. Plasma blanks (A,C,E,G) document the absence of interferences for each biomarker. For reasons of clarity, chromatograms represent the extracted ion chromatograms (XIC) only of the individual most intense product ions: A,B: *m/z* 972.4 > *m/z* 834.4, C,D: *m/z* 930.4 > *m/z* 754.3, E,F: *m/z* 1012.4 > *m/z* 834.4 and G,H: *m/z* 986.4 > *m/z* 834.4.

Case 1. The PA-NP-adduct bearing a DEP-moiety (Figs. 1 and 3B, t_R 12.8 min) as well as its aged variant bearing a monoethyl-phosphate-moiety (Figs. 1 and 3D, t_R 7.9 min) were detected. The aged variant of a BChE-adduct is formed by hydrolysis of one alkoxy-group of the phosphyl-moiety thus causing a desalkytion process and producing a free hydroxyl-group [18]. Therefore, in congruency to our previous forensic analyses [15] the intoxication by a DEP-pesticide was confirmed supporting the assumption that parathion-ethyl has been ingested by the patient. This pesticide was biotransformed in the liver to the more toxic paraoxon-ethyl that formed adducts with BChE.

Case 2. Poisoning by a DMP-pesticide was documented by the detection of the PA-NP-adduct comprising a monomethyl-phosphate-moiety (aged variant of the DMP-adduct, Figs. 1 and 3F, t_R 7.4 min). The non-aged adduct was not detected documenting quantitative ageing of the BChE-adduct. In accordance to our previous analyses confirming the phosphorylation of HSA as well as its adduct formation with the leaving group of the pesticide [16] the conjecture of incorporation of dimethoate and its subsequent biotransformation into omethoate was supported.

Furthermore, in the BioPT plasma samples the presence of GB, GF and CVX was confirmed as illustrated in Fig. 4B,D,F,H. In addition to adducts bearing the intact phosphonyl-moiety (Figs. 1 and 4B,F,H) their aged variants bearing a methyl phosphonyl-group (Fig. 1) were also found in all samples as exemplarily shown in Fig. 4D for the GB-containing sample.

These examples provide evidence that our method is applicable to verify real exposure scenarios covering a broad range of common OP toxicants with similar sensitivity and selectivity as the conventional method applied to BChE-adducts.

4. Conclusions

We herein present for the first time a rapid and highly effective procedure for the derivatization of NP-adducts quantitatively forming propionylated analogs within 5 min at ambient temperature in an aqueous buffer. As the signal response of the derivatives was typically somewhat lower than that of the un-derivatized NP-adducts (rf ≈ 0.4 – 0.5) the method was selective and allowed biomarker detection with similar sensitivity. The analysis of derivatized adducts should be carried out as a complementary approach in combination with the analysis of the non-derivatized peptide-adducts. Accordingly, the procedure described herein provides sufficient sample material for both methods only with the need of one common IMS extraction step. This procedure might therefore be highly beneficial especially in cases of poisoning with very low amounts of OP-NA or with those agents that do not form HSA-adducts.

Furthermore, the product ion that is generated by CID of the double charged PA-NP-adducts and represents the protonated phosphyl-moiety formerly bound to the NP will provide highly important information on the structure of the OP poison accessible by e.g. MS^3 fragmentation. This phenomenon is favored by the derivatization of the NP-adduct and provides an additional benefit of the presented procedure potentially being essential for the forensic analysis of poisoning by so far unknown OP agents.

Funding

Part of the work was supported by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG, Grant/Award Number Research Training Group GRK 2338).

CRediT authorship contribution statement

Harald John: Conceptualization, Methodology, Writing - original draft, Supervision; **Annika Richter:** Methodology, Investigation; **Markus Siegert:** Investigation, Validation; **Florian Eyer:** Resources; **Horst Thiermann:** Resources, Supervision.

Acknowledgements

We like to thank Marina Dentzel (Bundeswehr Institute of Pharmacology and Toxicology) for her excellent technical assistance.

References

[1] Organisation for the Prohibition of Chemical Weapons, Chemical Weapons Convention: Convention on the prohibition of the development, production, stockpiling and use of chemical weapons and on their destruction. ⟨https://www.opcw.org/sites/default/files/documents/CWC/CWC_en.pdf⟩, 2005 (Accessed 14 November 2020).
[2] Organisation for the Prohibition of Chemical Weapons, homepage ⟨https://www.opcw.org⟩, 2020 (Assessed 14 November 2020).
[3] H. John, M.J. van der Schans, M. Koller, H.E.T. Spruit, F. Worek, H. Thiermann, D. Noort, Fatal sarin poisoning in Syria 2013: forensic verification within an international laboratory network, Forensic Toxicol. 36 (2018) 61–71, https://doi.org/10.1007/s11419-017-0376-7
[4] H. John, M. Koller, F. Worek, H. Thiermann, M. Siegert, Forensic evidence of sulfur mustard exposure in real cases of human poisoning by detection of diverse albumin-derived protein-adducts, Arch. Toxicol. 93 (2019) 1881–1891, https://doi.org/10.1007/s00204-019-02461-2
[5] D. Steinritz, E. Striepling, K.D. Rudolf, C. Schröder-Kraft, K. Püschel, A. Hullard-Pulstinger, M. Koller, H. Thiermann, F. Gandor, M. Gawlik, H. John, Medical documentation, bioanalytical evidence of an accidental human exposure to sulfur mustard and general therapy recommendations, Toxicol. Lett. 244 (2016) 112–120, https://doi.org/10.1016/j.toxlet.2015.08.1105
[6] S. Sezigen, K. Ivelik, M. Ortatatli, M. Almacioglu, M. Demirkasimoglu, R.K. Eyison, Z.I. Kunak, L. Kenar, Victims of chemical terrorism, a family of four who were exposed to sulfur mustard, Toxicol. Lett. 303 (2019) 9–15, https://doi.org/10.1016/j.toxlet.2018.12.006
[7] S. Sezigen, L. Kenar, Recent sulfur mustard attacks in Middle East and experience of health professionals, Toxicol. Lett. 320 (2020) 52–57, https://doi.org/10.1016/j.toxlet.2019.12.001
[8] S. Sezigen, R.K. Eyison, E. Kilic, L. Kenar, Evidence of sulfur mustard exposure in victims of chemical terrorism by detection of urinary β-lyase metabolites, Clin. Toxicol. 58 (2020) 36–44, https://doi.org/10.1080/15563650.2019.1614190
[9] T. Nakagawa, A.T. Tu, Murders with VX: Aum Shinrikyo in Japan and the assassination of Kim Jong-Nam in Malaysia, Forensic Toxicol. 36 (2018) 542–544, https://doi.org/10.1007/s11419-018-0426-9
[10] Organisation for the Prohibition of Chemical Weapons, Statement by H.E. Ambassador Kenneth D. ward permanent representative of the United States of America to the OPCW in response to the director-general´s presentation of the report of the OPCW investigation and identification team for Syria. ⟨https://www.opcw.org/sites/default/files/documents/2020/04/IIT%20Report%20USA%20National%20Statement%2004082020.pdf⟩, 2020 (accessed 13 November 2020).
[11] T.C.C. Franca, D.A.S. Kitagawa, S.F. de A. Cavalcante, J.A.V. da Silva, E. Nepovimova, K. Kuca, Novichoks: the dangerous fourth generation of chemical weapons, Int. J. Mol. Sci. 20 (2019) (2019) 1222, https://doi.org/10.3390/ijms20051222
[12] J.A. Vale, T.C. Marrs, R.L. Maynard, Novichok: a murderous nerve agent attack in the UK, Clin. Toxicol. 56 (2018) 1093–1097, https://doi.org/10.1080/15563650.2018.1469759
[13] Organisation for the Prohibition of Chemical Weapons, Summary of the report on activities carried out in support of a request for technical assistance by the United Kingdom of Great Britain and Northern Ireland (Technical Assistance Visit TAV/02/18) S/1612/2018, ⟨https://www.opcw.org/sites/default/files/documents/S_series/2018/en/s-1612–2018_e___1_.pdf⟩, 2018 (Accessed 13 November 2020).
[14] E.J. Mew, P. Padmanathan, F. Konradsen, M. Eddleston, S. Chang, M.L. Philips, D. Gunnell, The global burden of fatal self-poisoning with pesticides 2006-15: systematic review, J. Affect. Disord. 219 (2017) 93–104, https://doi.org/10.1016/j.jad.2017.05.002
[15] J. von der Wellen, P. Winterhalter, M. Siegert, F. Eyer, H. Thiermann, H. John, A toolbox for liquid chromatography-tandem-mass spectrometry analysis of albumin-adducts as biomarkers of organophosphorus pesticide poisoning, Toxicol. Lett. 292 (2018) 46–54, https://doi.org/10.1016/j.toxlet.2018.04.025
[16] A. Kranawetvogl, H. Thiermann, M. Siegert, F. Eyer, H. John, Verification of organophosphorus pesticide poisoning: detection of phosphorylated tyrosines and a cysteine-proline disulfide-adduct from human serum albumin after intoxication with dimethoate/omethoate, Toxicol. Lett. 299 (2018) 11–20, https://doi.org/10.1016/j.toxlet.2018.08.013
[17] H. John, M. Siegert, F. Eyer, F. Worek, H. Thiermann, A. Kranawetvogl, Novel cysteine- and albumin-adduct biomarkers to prove human poisoning with the pesticide oxydemeton-S-methyl, Toxicol. Lett. 294 (2018) 122–134, https://doi.org/10.1016/j.toxlet.2018.05.023
[18] H. John, F. Balszuweit, D. Steinritz, K. Kehe, F. Worek, H. Thiermann, Toxicokinetic aspects of nerve agents and vesicants, in: R. Gupta (Ed.), Handbook of Toxicology of Chemical Warfare Agents, 3rd edn.,, Elsevier Inc./Academic Press, San Diego, 2020, pp. 875–929.
[19] H. John, F. Worek, H. Thiermann, LC-MS-based procedures for monitoring of toxic organophosphorus compounds and verification of pesticide and nerve agent poisoning, Anal. Bioanal. Chem. 391 (2008) 97–116, https://doi.org/10.1007/s00216-008-1925-z
[20] H. John, F. Breyer, C. Schmidt, B. Mizaikoff, F. Worek, H. Thiermann, Small scale purification of butyrylcholinesterase from human plasma and implementation of a µLC-UV/ESI MS/MS method to detect its organophosphorus adducts, Drug Test. Anal. 7 (2015) 947–956, https://doi.org/10.1002/dta.1792
[21] A. Fidder, A.G. Hulst, D. Noort, R. de Ruiter, M.J. van der Schans, H.P. Benschop, J.P. Langenberg, Retrospective detection of exposure to organophosphorus anticholinesterases: mass spectrometric analysis of phosphylated human butyrylcholinesterase, Chem. Res. Toxicol. 15 (2002) 582–590, https://doi.org/10.1021/tx0101806
[22] J.L.S. Sporty, S.W. Lemire, E.M. Jakubowski, J.A. Renner, R.A. Evans, R.F. Williams, J.G. Schmidt, M.J. van der Schans, D. Noort, R.C. Johnson, Immunomagnetic separation and quantification of butyrylcholinesterase nerve agent adducts in human serum, Anal. Chem. 82 (2010) 6593–6600, https://doi.org/10.1021/ac101024z
[23] G.L. Ellman, K.D. Courtney, V. Andres, R.M. Featherstone, A new and rapid colorimetric determination of acetylcholinesterase activity, Biochem. Pharmacol. 7 (1961) 88–95.
[24] F. Worek, P. Eyer, H. Thiermann, Determination of acetylcholinesterase activity by the Ellman assay: a versatile tool for in vitro research on medical countermeasures against organophosphate poisoning, Drug Test. Anal. 3/4 (2012) 282–291, https://doi.org/10.1002/dta.337
[25] F. Eyer, F. Worek, P. Eyer, N. Felgenhauer, M. Haberkorn, T. Zilker, H. Thiermann, Obidoxime in acute organophosphate poisoning: 1 – clinical effectiveness, Clin. Toxicol. 47 (2009) 798–806, https://doi.org/10.1080/15563650903206828
[26] K. Blau, J. Halket, Handbook of Derivatives for Chromatography, 2nd ed., John Wiley & Sons, Chichester, UK, 1993.
[27] W. Yan, H. Lee, E.W. Deutsch, C.A. Lazaro, W. Tang, E. Chen, N. Fausto, M.G. Katze, R. Aebersold, A dataset of human liver proteins identified by protein profiling via isotope-coded affinity tag (ICAT) and tandem mass spectrometry, Mol. Cell. Proteom. 3 (2004) 1039–1041, https://doi.org/10.1074/mcp.D400001-MCP200
[28] P.L. Ross, Y.N. Huang, J.N. Marchese, B. Williamson, K. Parker, S. Hattan, N. Khainovski, S. Pillai, S. Dey, S. Daniels, S. Purkayastha, P. Juhasz, S. Martin, M. Bartlet-Jones, F. He, A. Jacobson, D.J. Pappin, Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents, Mol. Cell. Proteom. 12 (2004) 1154–1169, https://doi.org/10.1074/mcp.M400129-MCP200
[29] L. Choe, M. D'Ascenzo, N.R. Relkin, D. Pappin, P. Ross, B. Williamson, S. Guertin, P. Pribil, K.H. Lee, 8-plex Quantitation of changes in cerebrospinal fluid protein expression in subjects undergoing intravenous immunoglobulin treatment for Alzheimer's disease, Proteomics 7 (2007) 3651–3660, https://doi.org/10.1002/pmic.200700316
[30] A. Hoffmann, M.L. Meade, M.K. Makley, J.J. Schlager, T.H. Snider, J.M. Gearhart, Quantitative proteomic analysis of the guinea pig brainstem following exposure to sarin, Brain Res. 2015 (1611) 101–113, https://doi.org/10.1016/j.brainres.2015.03.041
[31] G. Schwarz, S. Beck, M.G. Weller, M.W. Linscheid, MeCAT—new iodoacetamide reagents for metal labeling of proteins and peptides, Anal. Bioanal. Chem. 401 (2011) 1203–1209, https://doi.org/10.1007/s00216-011-5189-7
[32] M. van Faassen, G. Bouma, L.D. de Hosson, M.A.M. Peters, G. Kats-Ugurlu, E.G.E. de Vries, A.M.E. Walenkamp, I.P. Kema, Quantitative profiling of platelet-rich plasma indole markers by direct-matrix derivatization combined with LC-MS/MS in patients with neuroendocrine tumors, Clin. Chem. 65 (2019) 1388–1396, https://doi.org/10.1373/clinchem.2019.305359
[33] A. Nordström, P. Tarkowski, D. Tarkowska, K. Dolezal, C. Åstot, G. Sandberg, T. Moritz, Derivatization for LC-electrospray ionization-MS: a tool for improving reversed-phase separation and ESI responses of bases, ribosides, and intact nucleotides, Anal. Chem. 76 (2004) 2869–2877, https://doi.org/10.1021/ac0499017
[34] W.M. Pierce Jr., D.E. Nerland, Qualitative and quantitative analyses of phenol, phenylglucuronide, and phenylsulfate in urine and plasma by gas chromatography/mass spectrometry, J. Anal. Toxicol. 12 (1988) 344–347.
[35] A. Escrig-Doménech, E.F. Simó-Alfonso, J.M. Herrero-Martínez, G. Ramis-Ramos, Derivatization of hydroxyl functional groups for liquid chromatography and capillary electroseparation, J. Chromatogr. A 1296 (2013) 140–156, https://doi.org/10.1016/j.chroma.2013.04.027
[36] H. John, F. Breyer, J.O. Thumfart, H. Höchstetter, H. Thiermann, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for detection and identification of albumin phosphylation by organophosphorus pesticides and G- and V-type nerve agents, Anal. Bioanal. Chem. 398 (2010) 2677–2691, https://doi.org/10.1007/s00216-010-4076-y
[37] F. Gandor, M. Gawlik, H. Thiermann, H. John, Evidence of sulfur mustard exposure in human plasma by LC-ESI MS/MS detection of the albumin-derived alkylated HETE-CP dipeptide and chromatographic investigation of its cis/trans isomerism, J. Anal. Toxicol. 39 (2015) 270–279, https://doi.org/10.1093/jat/bkv010
[38] H. John, M. Siegert, F. Eyer, F. Worek, H. Thiermann, A. Kranawetvogl, Novel cysteine- and albumin-adduct biomarkers to prove human poisoning with the pesticide oxydemeton-S-methyl, Toxicol. Lett. 294 (2018) 122–134, https://doi.org/10.1016/j.toxlet.2018.05.023
[39] A. Kranawetvogl, J. Küppers, M. Siegert, M. Gütschow, F. Worek, H. Thiermann, P.W. Elsinghorst, H. John, Bioanalytical verification of V-type nerve agent exposure: simultaneous detection of phosphonylated tyrosines and cysteine-containing disulfide-adducts derived from human albumin, Anal. Bioanal. Chem. 410 (2018) 1463–1474, https://doi.org/10.1007/s00216-017-0787-7