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On-line coupling of immunoextraction, digestion and micro liquid chromatography-tandem mass spectrometry for the analysis of sarin and soman-butyrylcholinesterase adducts in human plasma

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Abstract

Organophosphorus nerve agent (OPNA) adducts formed with human butyrylcholinesterase (HuBuChE) can be used as biomarker of OPNAs exposure. Indeed, intoxication by OPNAs can be confirmed by the LC/MS^2 analysis of a specific HuBuChE nonapeptide on which OPNAs covalently bind. A fast, selective and highly sensitive on-line method was developed to detect sarin and soman-adducts in plasma, including immunoextraction by anti-HuBuChE antibodies, pepsin digestion on Immobilized Enzyme Reactors (IMER) and microLC-MS^2 analysis of the OPNA-adducts. The potential of three different monoclonal antibodies, covalently grafted on sepharose, were compared for the extraction of HuBuChE. The on-line method developed with the most promising antibodies allowed to extract up to 100% of HuBuChE contained in plasma and to digest 45% of it in less than 40 min. Moreover, OPNA-HuBuChE adducts, aged OPNA-adducts and unadducted HuBuChE could be detected (with S/N> 2000), even in plasma spiked with low concentration of OPNA (10 ng.mL^{-1}). Finally, the potential of this method was compared to approaches involving other affinity sorbents, already described for HuBuChE extraction.

Keywords

Human butyrylcholinesterase; Immunosorbents; Huprine; Procainamide; Sarin; Soman

1. Introduction

Organophosphorus nerve agents (OPNAs) are highly toxic chemical warfare agents that can bind to serine esterases (acetylcholinesterase, butyrylcholinesterase, carboxylesterase) [1,2] or albumin but also to several proteins that have not been identified or applied for analytical use yet [3]. The acute toxicity of OPNAs results from their binding on the active site of acetylcholinesterase (AChE) [4]. Inhibition of AChE activity leads to accumulation of acetylcholine on synaptic receptors and to a loss of nerve impulses transmission [5]. Clinical symptoms appear as early as 50% inhibition of AChE activity and include myosis, twitching, seizures, hypersalivation and respiratory depression. Levels of inhibition greater than 90% induce severe poisoning that can cause death in a few minutes [6].

The upsurge of chemical attacks over the world stressed the importance of implementing sensitive and specific analytical methods for the detection of OPNAs and their metabolic degradation products in blood or urine.

The monitoring of cholinesterase activity in the blood of individuals by the Ellman assay is a simple, affordable and rapid approach for the measurement of OPNA exposure. However, it implies to have a baseline of the enzyme activity level, which is sometimes difficult because of the intra- and inter-individual enzyme activities variations due to age or health status [7]. Furthermore, this method does not distinguish OPNAs from organophosphorus pesticides and is only limited to cholinesterase inhibition levels higher than 20%, which restricts the diagnosis of chronic intoxication [4,8].

Identification of intact OPNAs is also problematic, because of their short lifetime in blood, covalent binding to proteins and rapid hydrolysis [9]. Detection of OPNAs metabolites in blood and urine can be carried out, after a preliminary sample treatment, by the use of gas chromatography after a derivatization step [10,11] or liquid chromatography [12–16] coupled with high resolution tandem mass spectrometry. Although this method is more specific and sensitive than the Ellman assay, sampling has to be performed within 48 h after exposure to get significant levels of hydrolysis products. Indeed, 90% of metabolites are eliminated after this time span and their concentration become too low to be measured.

Fluoride reactivation can regenerate OPNA from cholinesterase, which allows the identification of the OPNA by GC/MS [17]. However, fluoride reactivation cannot measure aged OPNA adducts. Thus, the total amount of inhibited HuBuChE cannot be estimated [18]. Besides, fluoride reactivation methods are limited by denaturing of the enzyme and ageing of OPNA adducts.

Detection of OPNA adducts is currently the most promising method. Indeed, OPNAs form very stable adducts with HuBuChE, AChE and albumin, which allows their detection and identification by LC/MS for several months after exposure. Albumin has been used as a biomarker of exposure because of its high concentration in plasma and the good stability of the OPNA-albumin adducts [3,19]. However, AChE and HuBuChE are more often used than albumin because of their higher reactivity with OPNAs that allows detecting lower levels of exposure [18]. Since HuBuChE is ten times more abundant than AChE, located in plasma and more sensitive to inhibition by some OPNAs than AChE, analysis of OPNA-HuBuChE adducts has been preferred [20–22]. In most of the studies, HuBuChE is purified and digested by pepsin into the nonapeptide

^{195}FGESAGAAS^{203} which includes the OPNA moiety fixed on the 198 serine residue and the adducted nonapeptide is further identified by LC/MS^2 [7,18,23,24]. After a few minutes for soman to a couple of days for VX, the alkoxy-group of the adducted OPNA moiety is cleaved and replaced by a hydroxyl function during a phenomenon called ageing [25]. The measurement of these aged OPNA-adducts is essential to avoid underestimating or missing the exposure in case of total aging of OPNA before sampling or during storage [23]. The critical step of these methods is the extraction of HuBuChE from plasma. Indeed, HuBuChE is a low abundant protein in plasma (4 µg.mL^{-1}) which implies to perform a purification step to extract and isolate HuBuChE from the most concentrated proteins (e.g. albumin).

On-column procainamide gel extractions have been frequently used to purify HuBuChE from plasma [25–34]. However, this method is considered as poorly sensitive, time-consuming and requires large volume of sample or additional purification steps. Another support was recently developed by Brazzolotto *et al*. for the extraction of HuBuChE using huprine-sepharose affinity gel [35]. This support has a higher binding capacity and specificity than procainamide gels and would lead to HuBuChE purity between 54% to 90%, which could not be achieved in a single step using procainamide-based affinity, according to Brazzolotto *et al*. Alternatively, the purification by immunomagnetic beads [7,18,23,24,36,37] is more sensitive and specific, requires lower amount of plasma and is faster than the procainamide approach. Nevertheless, the antibodies fixed on the magnetic beads are digested in most of the protocols, which excludes the reusability of the beads and increases the cost of the procedure. Besides, apart from the recent work of Mathews *et al.* [38], the immunomagnetic methods were not applied to the simultaneous detection of OPNAs and OPNA aged adducts.

Very recently we developed an on-line system based on immunosorbents (IS) and immobilized enzyme reactors (IMER) coupled to microLC/MS^2, for the total analysis of native HuBuChE from plasma [publication in process]. Using the set-up described in **Fig. 1**, HuBuChE was extracted from 50 µL of plasma by B2 18-5 monoclonal antibodies in only 14 min, before being digested for 20 min on a pepsin-based IMER. After digestion, HuBuChE peptides were concentrated on a trap column and directly transferred to the analytical system. The target nonapeptide was detected in about 20 min. In the present work, we took advantage of this optimized on-line method to study the potential of three monoclonal antibodies (mAb2, 3E8 and B2 18-5), grafted on sepharose, to extract HuBuChE from plasma. The repeatability of the extraction was evaluated for each IS in triplicate as well as the repeatability of IS synthesis. The procedure was then applied to plasma samples spiked with sarin and soman, for the identification of OPNA-HuBuChE adducts and their aged forms. Finally, ISs were compared to the huprine and procainamide approaches by evaluating the sensitivity of the three methods for the detection of unadducted and OPNA-HuBuChE adducts in plasma.

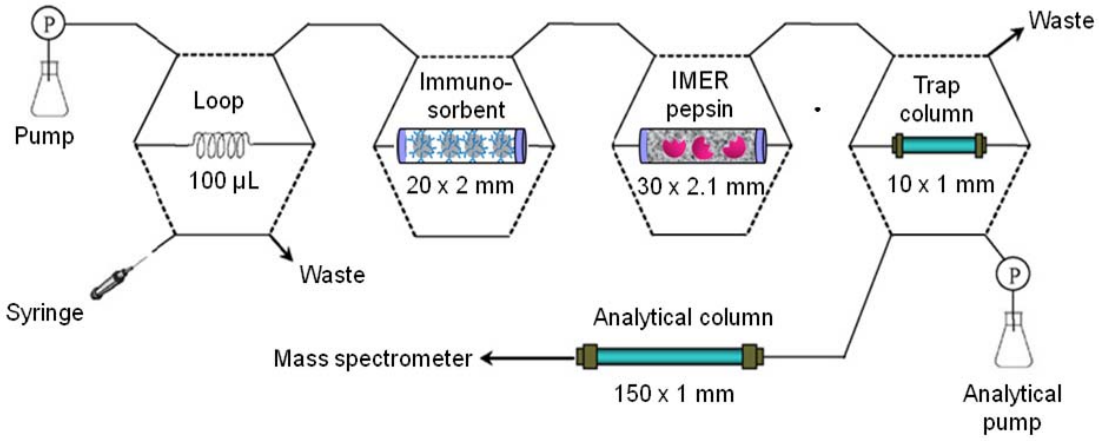

Fig. 1 Set-up used for the on-line immunoextraction of HuBuChE in 50 µL of plasma, followed by pepsin digestion on IMER and analysis of HuBuChE peptides by microLC/MS^2

2. Experimental

2.1. Chemicals

Pepsin from porcine gastric mucosa, sodium azide (NaN_3), sodium chloride (NaCl), Trizma hydrochloride ($NH_2C(CH_2OH)_3$, HCl), cyanogen bromide-activated Sepharose 4B (90 µm, 700 Å) and tetramethylammonium chloride were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Potassium dihydrogen phosphate (KH_2PO_4), di-sodium hydrogen phosphate (Na_2HPO_4), sodium bicarbonate ($NaHCO_3$) sodium acetate (CH_3CO_2Na), acetic acid (CH_3COOH), formic acid (HCOOH), glycine and hydrochloric acid were purchased from VWR (Fontenay-sous-Bois, France). Sodium hydroxide was obtained from Merck (Darmstadt, Germany). HPLC-grade acetonitrile was ordered from Carlo Erba (Val de Reuil, France). High purity water was obtained from a Milli-Q purification system (Millipore, Saint Quentin en Yvelines, France). Amicon Ultra-4 mL (100 kDa) and 0.5 mL centrifugal filters (10 kDa) were obtained from Merck Millipore (Darmstadt, Germany). BCA protein assay reagents were from Thermo Fisher Scientific (Illkirch, France). Synthetic nonapeptide (FGESAGAAS, MW: 795.3 $g.mol^{-1}$) was obtained from Proteogenix (Schiltigheim, France). Procainamide sepharose 4 fast flow (24 µmol procainamide/mL of gel) was purchased from GE Healthcare (Uppsala, Sweden). Huprine-sepharose was a gift from Dr. Florian Nachon (French Defence Health Service, Bretigny, France).

The phosphate buffer saline solution (pH = 7.4) consisted of 0.01 $mol.L^{-1}$ of both Na_2HPO_4 and KH_2PO_4 and 0.15 $mol.L^{-1}$ of NaCl.

Monoclonal antibodies against human BuChE mAb2 (KJ141199 and KH141200) and B2 18-5 (KT189143 and KT189144) [39] were kindly provided by Prof. Oksana Lockridge (University of Nebraska, Medical Center) and monoclonal anti-HuBuChE antibody 3E8 was purchased from Thermo Fisher Scientific (HAH 002-01-02).

Human plasmas from two healthy individuals without known exposure to OPNA were obtained from the Centre de Transfusion Sanguine des Armées (Clamart, France). In these samples, the couple sodium citrate/citric acid was used as anticoagulant. Human plasma samples spiked with sarin and soman were provided by DGA/CBRN Defence (Vert-le-Petit, France). Two

plasma-sarin samples were obtained after addition of 50 µL of a sarin solution at 1 or 10 µg.mL^{-1} in isopropanol to 5 mL of plasma. The resulting sarin-plasma sample spiked at 10 ng.mL^{-1} was incubated at 25 °C for 2 h while the sample spiked at 100 ng.mL^{-1} was incubated for 17 h at 37 °C to ensure that most of HuBuChE was adducted and that aged sarin adducts were formed. The plasma-soman sample at 10 ng.mL^{-1} was obtained by incubating 50 µL of a soman solution at 1 µg.mL^{-1} in isopropanol to 5 mL of plasma, for only 30 min at 25°C to avoid excessive ageing of soman. The plasma samples were aliquot in 500 µL and stored at -20°C or -80°C (plasma spiked with sarin at 37°C) until further use.

2.2. MicroLC/MS analysis

The chromatographic system was equipped with a micro-pump (Dionex Ultimate 3000, controlled by Chromeleon 6.8 SR11) and with a Acclaim PepMap 100 column (C18, 150 x 1 mm I.D., 3 µm, 100 Å, Thermo Scientific), preceded by a Hypersil Gold trap column (C18, 10 x 1 mm I.D., 5 µm, Thermo Scientific). Mobile phase A was water acidified with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. The separation of OPNA-adducted and unadducted HuBuChE nonapeptides was achieved at a flow rate of 50 µL.min^{-1} with a gradient varying from 2% to 90% B in 20 min. Detection was achieved with a triple quadripole mass spectrometer (TSQ Quantum Access Max^{TM}, Thermo Scientific) assembled with an ESI source (IonMax source probe HESI-II). Ionization was performed in positive mode with a source voltage set at 3000 V, capillary temperature at 250 °C, sheet gas and auxiliary gas pressures at 10, vaporizer temperature at 80 °C, tube lens offset at 70 V and skimmer offset at 0 V. The three most intense b-fragments of the HuBuChE nonapeptide, b_8, $b_{8\text{-}H2O}$ and $b_{7\text{-}H2O,}$ fragmented with a 30 eV collision energy, were followed in MRM mode (*m/z* 796.3→691.3; 796.3→673.3; 796.3→602.3 respectively). Calibration curves were performed for these three MRM transitions by injecting 5 µL of different concentrations of a synthetic nonapeptide (6.8, 33.8, 67.5, 338, 675 ng.mL^{-1}) into the microLC-MS^2 to quantify the nonapeptide resulting from HuBuChE immunoextraction and digestion by microLC-MS^2. The linear regression equations and coefficient values of the regression curves obtained for the transitions *m/z* 796.3→602.3, 796.3→673.3 and 796.3→691.3 were respectively y = 45372 x + 876.04, R^2 = 0.999; y = 42509 x + 2232.1, R^2 = 0.996; y = 53669 x – 264.52, R^2 = 0. 999. The calibration curves are shown in supplementary material.

The monitoring of the sarin-nonapeptide adduct (m/z = 916.4) was performed by following the transitions 916.4→602.3 and 916.4→673.3 and the soman-nonapeptide adduct (m/z = 958.4) was detected with the transitions 958.3→602.3 and 958.3→673.3. The aged sarin and soman-nonapeptide adduct (m/z = 874.3) were detected with the transitions 874.3→602.3 and 874.3→673.3. These transitions were chosen according to previous studies on these adducts [23,25,38,40].

2.3. Synthesis of pepsin-based IMERs

The pepsin-based IMER was prepared following the protocol previously developed by our group [41]. Briefly, 1.7 mL of a 4 mg.mL^{-1} pepsin solution in 0.1 mol.L^{-1} sodium acetate (pH = 5.8) was put in contact with 58 mg of CNBr activated sepharose for 16 h at 4 °C. Then, 50 mg from the 58 mg sorbent, were packed in a 30 x 2.1 mm I.D. precolumn. A glycine solution (pH = 2) was

percolated through the precolumn at a flow rate of 400 µL.min^{-1} for 200 min to block the remaining uncoupled sites. The gel was washed three times with sodium acetate (pH = 5.8) and HCl (1 mmol.L^{-1}) for 16 min at 400 µL.min^{-1} to remove unbound enzymes. Finally, IMER was stored for months at 4 °C in a formic acid solution (pH = 2.2). As previously described [41], bicinchoninic acid (BCA) assay was used to evaluate the amount of pepsin immobilized on sepharose. Two calibration curves were made by adding 10 µL of pepsin at a concentration range varying from 100 µg.mL^{-1} to 4 mg.mL^{-1} in a 96 wells microplate. Then, 10 µL of the working reagent were added in each microwell and the microplate was incubated at 37 °C for 30 min. The absorbance was measured at 562 nm. Pepsin grafting yields were calculated by dividing the amount of pepsin remaining in the supernatant, evaluated thanks to the calibration curves, by the initial amount of pepsin introduced in the coupling solution. The IMER was reused 18 times and no loss of efficiency was observed.

2.4. Synthesis of sepharose-based immunosorbents

The anti-HuBuChE antibodies mAb2, B2 18-5 and 3E8 were grafted on sepharose similarly to the procedure already developed by our team for other antibodies [42]. The CNBr activated sepharose (35 mg) was swollen for 15 min in 1 mL of HCl (1 mmol.L^{-1}) and washed two times with $NaHCO_3$ (0.1 mol.L^{-1}, pH = 8.3) containing NaCl 0.5 mol.L^{-1}. Then, 100 µg of anti-HuBuChE antibodies diluted in 100 µL of PBS (0.01 mol.L^{-1}, pH = 7.4,) were added to sepharose. After incubation under stirring at 4 rpm, for 24 h at 4 °C, the sorbent was packed into a precolumn (20 x 2 mm I.D.). The precolumn was connected to a pump and, in order to block the remaining uncoupled sites of sepharose, a Tris buffer solution (0.1 mol.L^{-1}, pH = 8) was percolated for 2 h at 400 µL.min^{-1}, at room temperature. The gel was washed 3 times to remove unbound antibodies with 4 mL of acetate solution (0.1 mol.L^{-1} + NaCl 0.5 mol.L^{-1}, pH = 4) and 4 mL of $NaHCO_3$ (pH = 8.3). The immunosorbents were stored at 4 °C in a PBS-azide solution and were reused for months. They were called IS mAb2$_{SP}$, B2 18-5$_{SP}$ and 3E8$_{SP}$. Similarly to IMER, a BCA assay was performed to quantify the antibodies remaining in the supernatant after grafting and thus to evaluate the amount of antibodies immobilized on sepharose. The grafting yields were calculated by dividing the absorbance of antibodies remaining in the supernatant by the absorbance of the solution used for the grafting.

2.5. Purification of HuBuChE and OPNA-HuBuChE adducts from plasma

2.5.1. Immunosorbents and IMER coupled on-line to microLC/MS^2

The complete analysis of HuBuChE from plasma (P06276) was performed on the set-up described on **Fig. 1**. The immunosorbent (mAb2$_{SP}$, 3E8$_{SP}$ or B2 18-5$_{SP}$), coupled on-line to the IMER and to the analytical system, was conditioned for 30 min with PBS buffer (pH = 7.4) at 50 µL.min^{-1}. Then, 100 µL of a diluted plasma sample (plasma/PBS 1/1 v/v) were percolated through the immunosorbent that was further washed with 700 µL of PBS (0.1 mol.L^{-1}, pH = 7.4) to remove non-specifically retained plasmatic proteins. To avoid exceeding the volume of IMER (62 µL) and to digest a maximum of HuBuChE, the protein was eluted in two times from the IS through the IMER by 62 µL of formic acid (pH = 2.2), at 50 µL.min^{-1}. A digestion by stop flow was performed

for 10 min after each elution step. The resulting HuBuChE peptides were transferred from the IMER to the trap column by 250 µL of formic acid (pH = 2.2) before being eluted by the LC mobile phase into the analytical system. Immunosorbents were regenerated after each immunoextraction by a solution of PBS (pH = 7.4) for at least 24 h at 4 °C before being re-used. The immunosorbents were reused 12 times with no loss of efficiency. The same procedure was applied for the analysis of plasma samples spiked with OPNAs.

2.5.2. Procainamide gel

Procainamide-sepharose gel from GE Healthcare (2.5 mL) was preconditioned with 15 mL of buffer A (15 mmol.L^{-1} Na_2HPO_4, 5 mmol.L^{-1} NaH_2PO_4, pH = 6.7) and 100 µL of plasma was added and gently mixed with the gel, following Liu *et al.* protocol [27]. The mixture was incubated for 1 hour at room temperature and shaken at a speed of 60 rpm. The mixture was poured in a 6 mL SPE column (between two PTFE frits) and washed with 10 mL of Buffer A before being eluted with 4.5 mL of buffer B (15 mmol.L^{-1} Na_2HPO_4, 5 mmol.L^{-1} NaH_2PO_4, 2 mol.L^{-1} NaCl, pH = 6.7). The 4.5 mL eluate was collected and concentrated about 110 µL using a 4.0 mL 100-kDa cut-off filter by two successive centrifugation steps at 4500 rpm for 20 min. Then, 400 µL of solution C (0.2 mol.L^{-1} formic acid) were added and the solution was filtered with a 100-kDa cut-off filter at 4500 rpm for 7 min. The resulting proteins on the filter were diluted to 110 µL with solution C. Two different digestions by pepsin were applied to digest HuBuChE obtained after this filtration. First, a digestion in solution was performed at 37°C for 1 h by adding 16.0 µL of a 2.0 mg.mL^{-1} pepsin solution in solution C to the 110 µL protein extract. The digested solution was size fractioned with a prewashed 10 kDa cut-off filter to remove pepsin or proteins and 5 µL of the peptides solution were injected into the microLC-MS^2 system. Alternatively, the protein solution recovered on the filter was diluted by 2 into 125 µL of formic acid (pH = 2.2) to be digested on IMER, similarly to the digestion applied after immunoextraction. The 125 µL were collected into a syringe, connected to a syringe-pump and the solution was pushed through the IMER at 50 µL.min^{-1} by 62 µL of formic acid pH = 2.2 and digested by stop flow for 10 min. This step was repeated twice. The peptides were then transferred with 250 µL of formic acid at 50 µL.min^{-1} to the trap column to be analyzed by microLC-MS^2.

2.5.3. Huprine gel

Huprine-sepharose gel (1 mL), synthesized by the company Chemforase under the name “hupresin”, was put in a 6 mL SPE column and equilibrated with 5 mL of a Tris buffer (0.02 mol.L^{-1}, NaCl 0.1 mol.L^{-1}, pH 7.4) [35]. Then, 50 µL of plasma or plasma spiked with 10 ng.mL^{-1} of sarin was diluted by two in a PBS buffer (pH = 7.4, 0.1 mol.L^{-1}) and loaded onto the huprine gel column. After extensive washing with the same buffer (5 mL) and with 5 mL of Tris 0.02 mol.L^{-1} containing NaCl 0.25 mol.L^{-1} (pH 7.4), HuBuChE was eluted with 2.5 mL of Tris (0.02 mol.L^{-1}, NaCl 0.25 mol.L^{-1}, pH 7.4) containing 0.5 mol.L^{-1} tetramethylammonium chloride. The eluate was concentrate about 110 µL using a prewashed 100-kDa cut-off filter by centrifugation at 4500 rpm for 10 min. Then, 400 µL of formic acid (pH = 2.2) were added and the solution was filtered with a 100-kDa cut-off filter at 4500 rpm for 5 min. The resulting solution was diluted to 125 µL with formic acid pH = 2.2 and digested on IMER before being analyzed as previously mentioned.

3. Results and discussion

3.1. Analysis of plasma without OPNA by the on-line coupling of IS and IMER to microLC/MS^2

The aim of this study was to compare the potential of three anti-HuBuChE monoclonal antibodies (3E8, mAb2 and B2 18-5) grafted on sepharose, to extract HuBuChE and OPNA-adducted HuBuChE from plasma. Antibodies 3E8 are commercially available, and were mainly used for the extraction of HuBuChE from plasma, using the immunomagnetic approach [7,18,23,24,38–40,43]. The use of mAb2 and B2 18-5 antibodies for HuBuChE purification is more recent and was developed by Oksana Lockridge, using different immobilization supports like Pansorbin cells [43], Dynabeads-Protein G [39,43,44] or CNBr-activated sepharose [45,46]. In these works [39,43], the potential of the three antibodies to extract unadducted HuBuChE from plasma was compared, but only in a semi-quantitative way. Indeed, the amount of HuBuChE bound on antibodies was evaluated by comparing the HuBuChE activity in plasma that may vary before and after extraction, without taking into account the elution step. Moreover, the comparison of the potential of these three antibodies to extract OPNA-adducted HuBuChE has never been done before and will be discussed in the present work.

For this, immunosorbents (3E8$_{SP}$, mAb2$_{SP}$ and B2 18-5$_{SP}$) were synthesized by the covalent grafting of 100 µg of anti-HuBuChE antibodies on 35 mg of CNBr-activated sepharose, packed in 20 x 2 mm I.D. precolumns. To compare the affinity of the antibodies towards HuBuChE, it was necessary to ensure that similar amounts of antibodies were grafted on the ISs by controlling the amount of antibodies grafted on sepharose. Antibody grafting yields on sepharose were evaluated by BCA assay for the three ISs and similar values, between 95% and 97% were obtained (**Table 1)**, attesting the efficient grafting of antibodies. To evaluate their ability to extract HuBuChE, each IS was coupled on-line to the IMER and the microLC/MS^2 analysis as described in **Fig. 1**. The coupling of immunosorbent to the on-line digestion set-up had already been optimized in a previous study [publication in process], thus the same conditions were applied for the coupling with IS B2 18-5$_{SP,}$ IS mAb2$_{SP}$ and IS 3E8$_{SP}$. Briefly, 700 µL of PBS (0.1 mol.L^{-1}, pH = 7.4) were used to transfer 50 µL of plasma to the IS and to wash it, this volume allowing the removal of more than 99.9% of albumin, the most concentrated protein in plasma. HuBuChE was eluted from the IS to the IMER, to be digested by two consecutive stop flow digestions, for 20 min. The peptides were transferred and concentrated into a trap column and analysis was performed by microLC/MS^2. This procedure was applied in triplicate for each IS to evaluate their respective affinity towards HuBuChE.

One example of chromatograms obtained after immunoextraction on IS mAb2$_{SP}$ is shown in **Fig. 2**. The intact nonapeptide was observed at 19.0 min with a signal/noise (S/N) higher than 10000, equivalent to a limit of quantification (LOQ) of 2 femtomoles (S/N = 10). Thanks to the nonapeptide calibration curves depicted **Section 2.2.**, the amount of nonapeptide obtained after immunoextraction and digestion was quantified for each IS to compare their performances. Transitions 796.3→602.3 and 796.3→673.3 were used to quantify while 796.3→691.3 was used as qualitative ion.

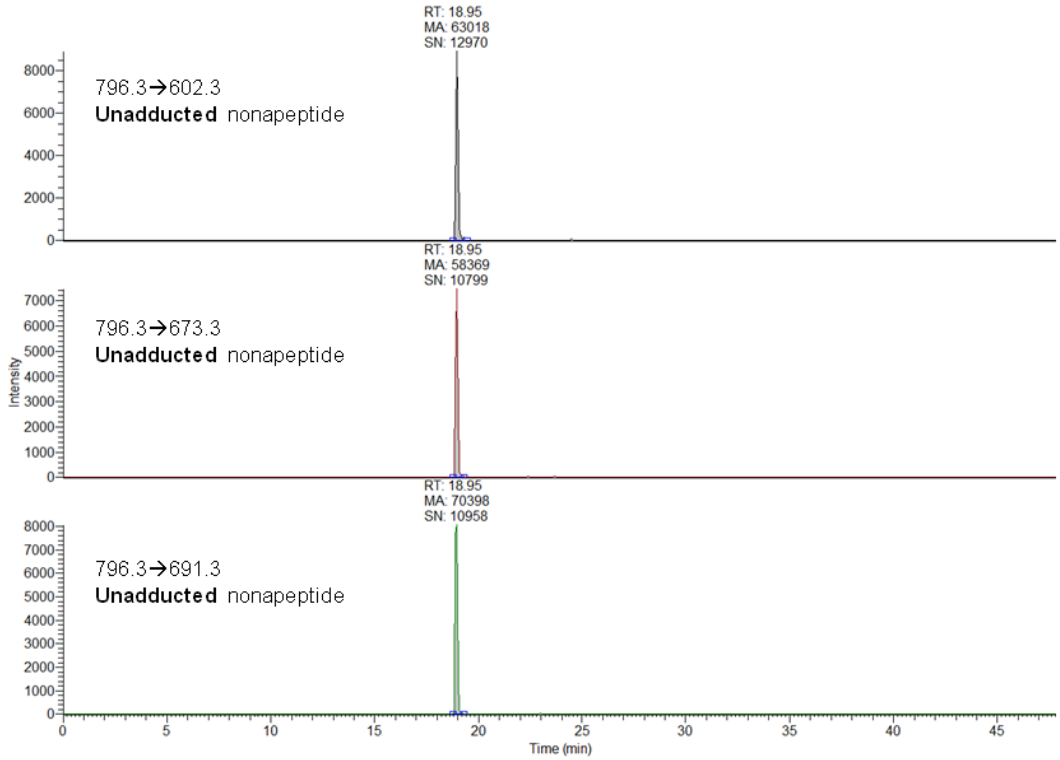

Fig. 2 Chromatograms of the nonapeptide obtained after immunoextraction of 50 µL of HuBuChE from plasma on IS mAb2$_{SP}$ and its digestion on IMER followed by microLC/MS^2 analysis (MRM mode)

The total recovery of HuBuChE is shown on **Table 1** and was calculated by dividing the amount of nonapeptide obtained after immunoextraction and digestion by the theoretical amount of nonapeptide corresponding to a 100% extraction and digestion yield. HuBuChE total recovery yield was 47 ± 7% for IS mAb2$_{SP}$ and was 42 ± 3% for IS B2 18-5$_{SP,}$ IS 3E8$_{SP}$ was clearly less efficient (28 ± 4%). The total HuBuChE recovery could not dissociate HuBuChE extraction yields from the HuBuChE digestion yields. However, we demonstrated in a previous work [publication in process] that HuBuChE digestion on IMER was repeatable (45 ± 4%) for amounts of HuBuChE similar to those present in 50 µL of plasma (0.2 µg). Considering a digestion yield of 45%, it was estimated that IS mAb2$_{SP}$ and B2 18-5$_{SP}$ extract between 90% and 100% of HuBuChE and IS 3E8$_{SP}$ about 60% of HuBuChE. Although the methods used to estimate the amount of HuBuChE bound on antibodies were different, these results were consistent with those described by Peng *et al.* for the extraction of HuBuChE from 500 µL of plasma incubated overnight with B2 18-5, mAb2 and 3E8 antibodies, immobilized on Dynabeads-Protein G [39]. In this work, HuBuChE activity in plasma was measured before and after extraction and showed that B2 18-5 extracted about 97 ± 1% of HuBuChE, mAb2 about 72 ± 16% and 3E8 about 70 ± 14%. Even, if mAb2$_{SP}$ gave higher extraction yields compared to Peng *et al.*, this value was similar to the 95-98% extraction yields evaluated by Schopfer *et al*. for the extraction of HuBuChE from 500 µL of human plasma incubated overnight with mAb2 grafted on sepharose beads [45].

Table 1. Grafting yields of 100 µg of monoclonal antibodies B2 18-5, 3E8 and mAb2 on sepharose and HuBuChE recovery yields obtained after immunoextraction of 50 µL of plasma on the resulting immunosorbents, pepsin digestion on IMER and microLC/MS^2 analysis.

Antibodies	Immunosorbents	Antibody grafting yields	Global HuBuChE recovery yields (n = 3)
B2 18-5	B2 18-5_{SP}	95%	42 ± 3 %
3E8	$3E8_{SP}$	95%	28 ± 4 %
mAb2	$mAb2_{SP}$	97%	47 ± 7 %

Therefore, immunosorbents allowed, in less than 20 min, to obtain similar HuBuChE extraction yields to those obtained in one night for the same antibodies, using binding by partition. Finally, low RSD values were obtained using this online set-up (<15%), despite the variations due to this 4 steps procedure (immunoextraction, digestion, trapping of peptides and LC/MS^2 analysis), which confirmed the reliability of this on-line set-up.

3.2. Repeatability of the IS mAb2 synthesis

Even if the grafting yields of the three different anti-HuBuChE antibodies were similar, it was interesting to study the repeatability of the grafting for the same antibody. For that, the synthesis of IS $mAb2_{SP}$ was performed in triplicate. This antibody was chosen for its high grafting yields on sepharose and its high HuBuChE extraction yields. After each grafting using 100 µg of mAb2 antibodies on CNBr-sepharose and packing of the resulting gel into a precolumn (20 x 2 mm I.D.), the amount of antibodies grafted on sepharose was evaluated thanks to a BCA assay (**Section 2.4.**). Antibodies grafting yields of 94 ± 3% were obtained, attesting the high repeatability of the IS synthesis. As the synthesis of the first IS mAb2 was performed 12 months before the other two and by a different operator, it was concluded that a robust method was developed for the synthesis of immunosorbents. HuBuChE extraction yields were evaluated the same day on the three ISs coupled on-line to IMER and microLC-MS^2, by percolating 50 µL of a plasma sample (diluted 1/1 in PBS). An average HuBuChE recovery of 47 ± 6% was obtained, which was similar to the recovery obtained after three extractions on one IS mAb2 (47 ± 7%). Consequently, the high repeatability of the immunosorbents in terms of extraction yields and grafting yields was confirmed.

3.3. Analysis of plasma spiked with sarin and soman by the on-line coupling of IS and IMER to microLC/MS^2

The same procedure was then applied to the analysis of sarin and soman-HuBuChE adducts. A plasma spiked with 100 µg.mL^{-1} of sarin for 17 h at 37°C was diluted 1/1 (v/v) in PBS (pH = 7.4) and analyzed using the on-line set-up previously described. Each immunosorbent was

tested in triplicate to assess its potential to extract sarin-adducted HuBuChE, aged sarin-adducted HuBuChE as well as unadducted HuBuChE. As displayed in **Fig. 3**, in a qualitative way, the MRM chromatograms showed that IS mAb2 could capture adducted and non-adducted HuBuChE, allowing the detection by microLC/MS^2 of the sarin-nonapeptide adduct at 20.5 min with a S/N of 26000 and the detection of the unadducted nonapeptide at 19.0 min. The aged-sarin adduct was also detected at a retention time of 20.2 min, with a relatively high signal intensity (S/N > 7000). This showed that despite the high concentration of sarin in the plasma sample, a small amount of HuBuChE was unadducted. It may be due to a kinetic competition between hydrolysis of sarin and its addition to HuBuChE and other proteins such as albumin [47]. For reasons of clarity, only MRM chromatograms with daughter ions m/z = 673.3 were displayed on **Fig. 3**, although the transitions 796.3→602.3; 916.4→602.3 and 874.3→602.3 were acquired and showed the same phenomenon.

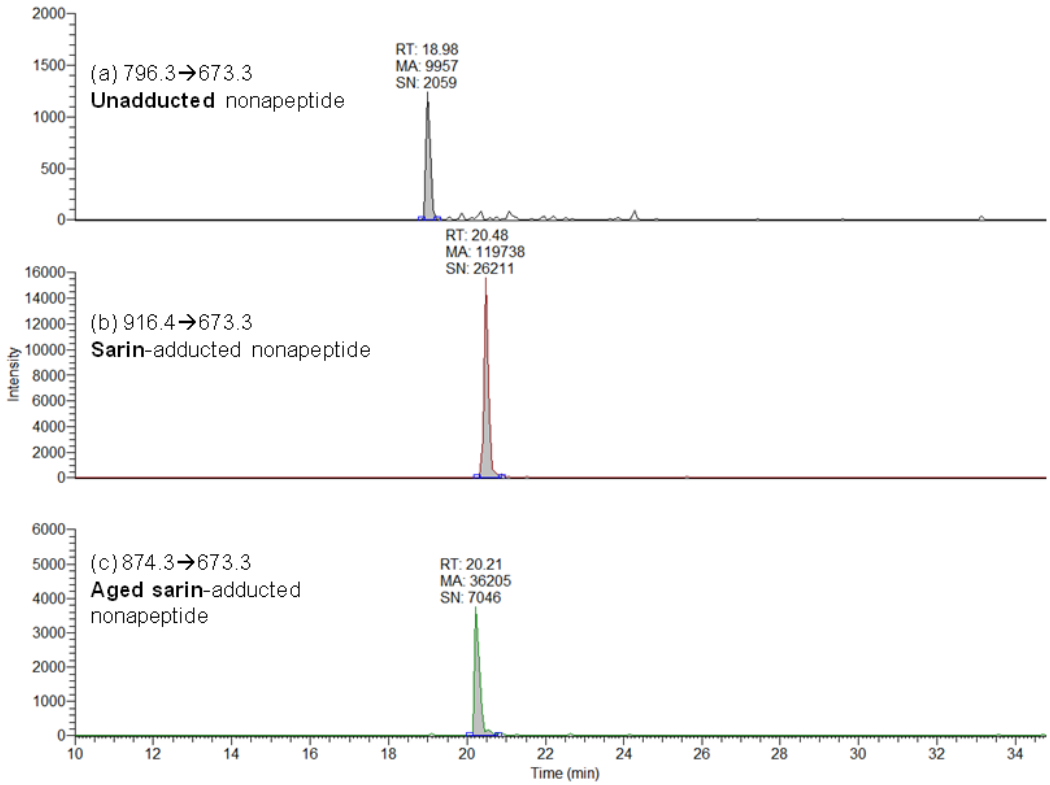

Fig. 3 MRM chromatograms of the intact nonapeptide (a), sarin-nonapeptide adduct (b) and aged sarin-nonapeptide adduct (c) obtained after immunoextraction on IS $mAb2_{SP}$ of 50 µL of HuBuChE from plasma spiked with 100 $ng.mL^{-1}$ of sarin, and digestion on IMER, followed by microLC/MS^2 analysis

As shown in **Fig. 4,** the three antibodies can bind sarin-adducted and aged sarin-adducted HuBuChE as well as unadducted HuBuChE. By measuring the area of the peak of both OPNA-adducted and unadducted HuBuChE, the level of OPNA-adducted HuBuChE can be estimated, without the need of baseline HuBuChE activity measurement. As shown in **Fig. 4**, close

unadducted/sarin-adducted nonapeptide ratios were obtained for the 3 ISs. The proportion of HuBuChE adducted with sarin was evaluated to 94 ± 2%, by dividing the amount of sarin and aged-sarin adducted-nonapeptide to the total amount of nonapeptide (sum of unadducted, sarin and aged-sarin adducted nonapeptides). The largest amounts of sarin-adducts were extracted with ISs $mAb2_{SP}$ and B2 $18\text{-}5_{SP}$.

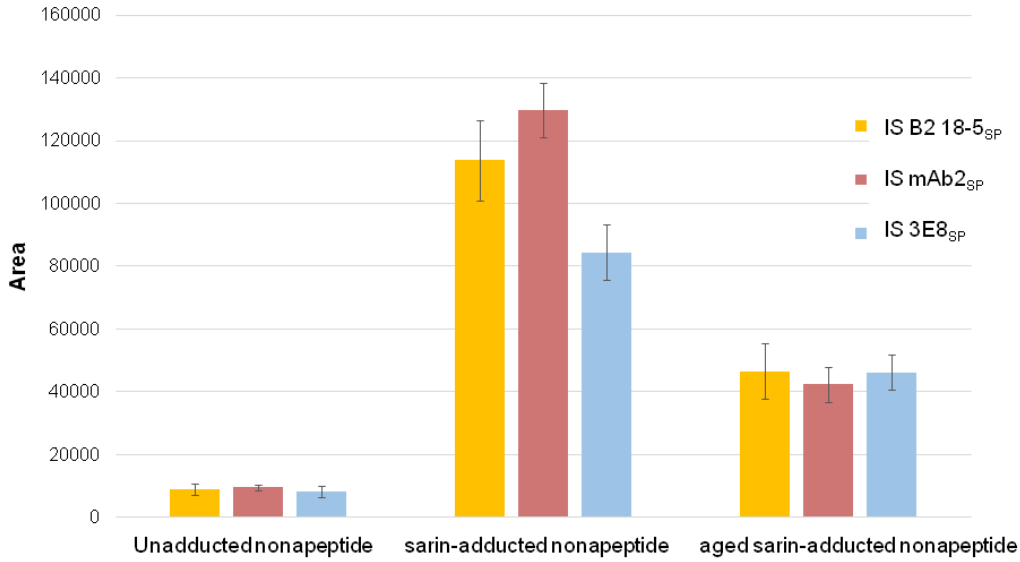

Fig. 4 Peak area of the intact nonapeptide, sarin-nonapeptide adduct and aged sarin-nonapeptide adduct, obtained after immunoextraction of 50 µL of plasma spiked with sarin (100 $ng.mL^{-1}$) on immunosorbents B2 $18\text{-}5_{SP}$, $mAb2_{SP}$ and $3E8_{SP}$, followed by digestion on IMER and analysis by microLC/MS^2 (n = 3)

To ensure that no memory effect was observed on ISs, after the immunoextraction of plasma spiked with sarin, a blank was performed on the three ISs by injecting a plasma sample that was not spiked with OPNAs. The **Fig. 5** shows that only intact nonapeptide was distinguishable after immunoextraction and thus, that IS could be reused without the risk of false positive results.

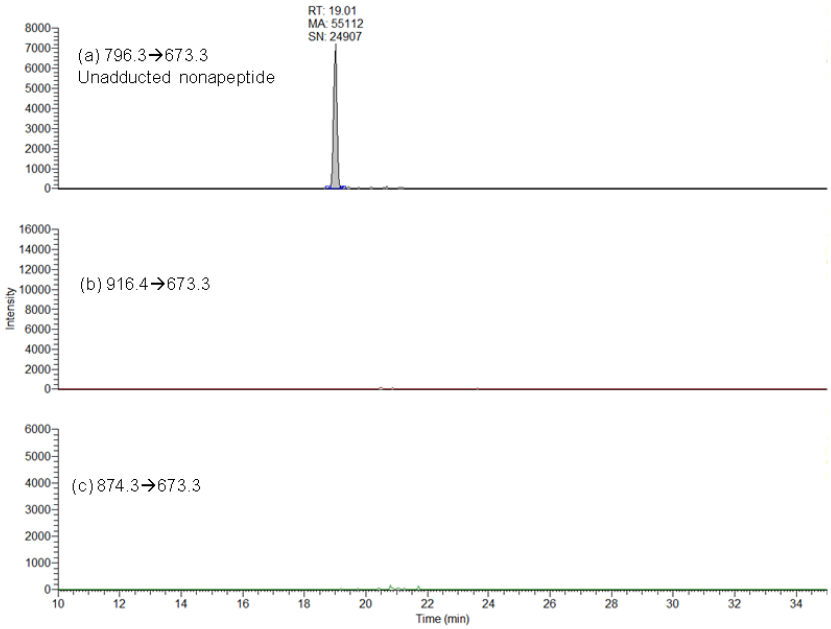

Fig. 5 MRM chromatograms of the intact nonapeptide (a), obtained after immunoextraction of 50 µL of BuChE from plasma without OPNA, and its digestion on IMER, followed by microLC/MS^2 analysis. Used immunosorbent gives no false positive results (b and c).

To evaluate the ability of the on-line method to detect lower amounts of OPNAs in plasma, two plasma samples were incubated with only 10 µg.mL^{-1} of sarin and soman at 25 °C. To be able to detect both the aged and un-aged OPNA adducts, sarin was incubated for 2 h and soman was incubated for only 30 min, because of its high ageing rate. The OPNA-spiked plasmas were analyzed using IS $mAb2_{SP}$. As depicted in **Fig. 6**, MRM chromatograms clearly showed sarin-adduct, soman-adduct, and their aged adducts. As soman ages faster than sarin, the predominant form of soman-HuBuChE adduct in plasma after 30 min of incubation was the aged soman-adducted nonapeptide while the aged sarin-HuBuChE adduct was minority in plasma after 2 h of incubation. Unadducted nonapeptide was also observed in the two samples and was most intense in plasma spiked with soman (S/N = 10000) than the one spiked with sarin (S/N = 2500, data not shown). It was estimated that 94% of HuBuChE was adducted after 2 h of incubation with sarin and 84% of HuBuChE was adducted with soman after 30 min of incubation. This was explained by the different incubation times but also by the lower reactivity of soman to HuBuChE because of its higher steric hindrance. Compared to the standards approaches that have been used for the analysis of sarin in plasma [48], this on-line method allows a fast, sensitive and specific analysis. The extraction step of OPNA-HuBuChE adducts by anti-HuBuChE antibodies, that is usually performed in 2 h with the use of immunomagnetic beads (7) was here performed in less than 20 min, leading to high recovery and high specificity. The use of IMER digestion allowed to reduce the digestion time to 20 min instead of hours in solution and to get higher amount of nonapeptide available for the analysis [41]. Besides, thanks to the evaluation of the extraction and digestion yields, the use of OPNA-adducted standards of nonapeptide will allow the quantitation of the OPNA-nonapeptide adducts in plasma.

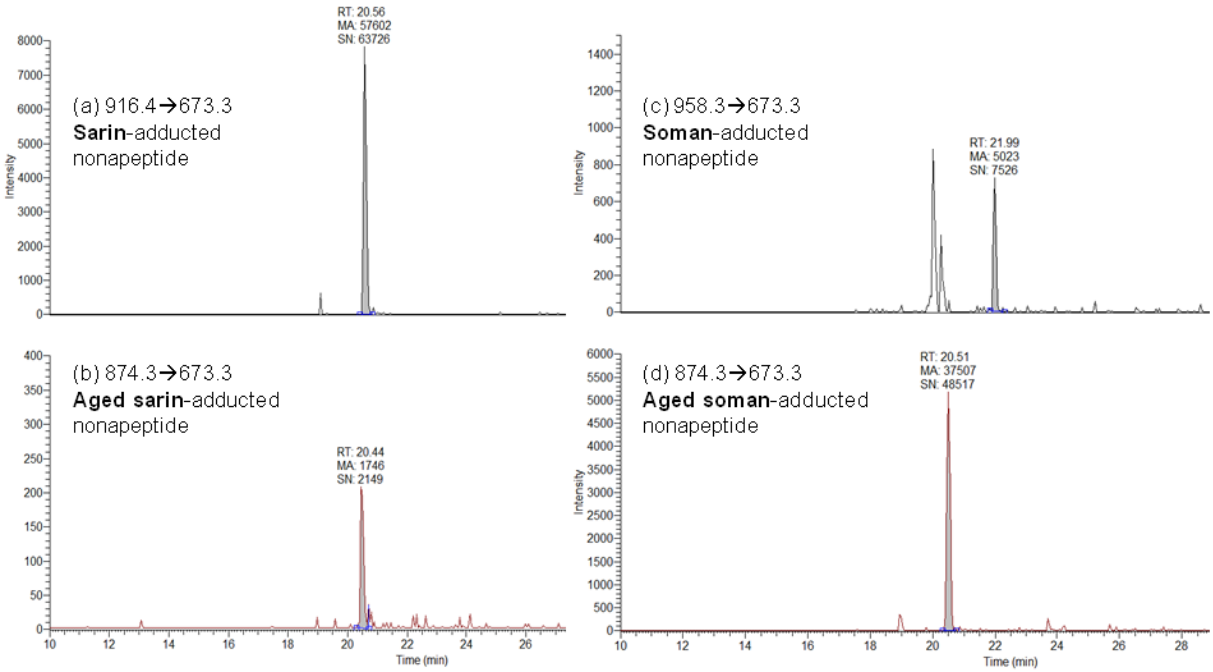

Fig. 6 MRM chromatograms of the sarin-nonapeptide adduct (a), aged sarin-nonapeptide adduct (b), soman-nonapeptide adduct (c) and aged soman-nonapeptide adduct (d) obtained after immunoextraction of 50 µL of HuBuChE from plasma spiked with 10 ng.mL^{-1} of sarin or soman and its digestion on IMER, followed by microLC/MS^2 analysis

To conclude, sarin-adduct, soman-adduct and their aged adduct can be easily identified by this on-line method, which would allow one to detect very low level of HuBuChE inhibition by OPNAs in plasma, or to detect it in volumes of plasma much more lower than 50 µL.

3.4. Comparison of the on-line set-up with the procainamide and huprine approaches

The efficiency of immunosorbents to selectively extract HuBuChE from plasma was compared to the most frequently used or efficient approaches found in literature to purify HuBuChE. Procainamide gel, has been widely used to extract HuBuChE, combined or not with additional purification steps [25–27,29–31,49]. The method depicted by Liu *et al.* [27] put forward that more than 90% HuBuChE could be extracted with only one purification step with off-column procainamide gel. Consequently, this method was chosen and reproduced in this work closely as possible to compare HuBuChE recovery obtained after purification by procainamide to that obtained after on-line immunoextraction. For this, 100 µL of plasma was incubated with procainamide gel for 1 h and loaded on a SPE column for washing. HuBuChE was eluted and digested in solution for 1 h with a pepsin solution before being analyzed by microLC/MS^2. Extraction of HuBuChE from plasma and its digestion took more than three hours and the nonapeptide could not be observed by microLC/MS^2 by this method (data not shown). To avoid the presence of peptides resulting from pepsin digestion [41], the digestion step was alternatively performed on IMER for 2 x 10 min, by diluting the HuBuChE solution in 125 µL of formic acid, to

mimic the digestion step applied after immunoextraction. However, HuBuChE digestion on IMER did not allow quantifying the nonapeptide, even if lower background noise was noticed (S/N <10). Liu *et al.* mentioned good extraction recovery of HuBuChE by procainamide gel but no information was given concerning the purity of the extract. The low nonapeptide intensity observed in our chromatograms could be explained by the difference of procainamide gels used in the two studies. Contrary to other teams [25,26,29–31,49] and to our protocol, Liu *et al.* synthesized their own procainamide gel, which results in higher procainamide binding rate on sepharose (38 µmol/mL of gel) and to higher capacity than procainamide gels commercially available (24 µmol/mL of gel). However, even with this home-made procainamide gel, chromatograms shown by Liu *et al*. were less intense and clean than those obtained with on-line immunoextraction described in this work. Indeed, their MRM chromatograms, obtained after purification with procainamide gel and digestion of plasmas spiked with 0.1 $ng.mL^{-1}$ of VX or with 0.5 $ng.mL^{-1}$ of soman, were not intense, with S/N ratios close to 10.

Brazzolotto *et al.* developed a novel and fast protocol to purify recombinant HuBuChE using huprine-based affinity chromatography [35]. Huprine is known as a cholinesterase inhibitor and thus to have high affinity towards HuBuChE [50]. Huprine was immobilized on NHS-sepharose by Brazzolotto *et al.* and recombinant HuBuChE produced by CHO cells was purified on the huprine gel leading to purity up to 90%. To compare the potential of huprine to immunoextraction for the extraction of HuBuChE from plasma, the procedure mentioned by Brazzolotto *et al.* [35] was reproduced and was applied to 50 µL of plasma sample. Briefly, after percolation of the plasma sample and washing of the sorbent to remove contaminants, HuBuChE was eluted from the huprine column and digested on IMER for 2 x 10 min to be analyzed by microLC/MS^2 similarly to the digestion procedure following immunoextraction and procainamide gel purification. Contrary to the chromatograms obtained after HuBuChE purification by procainamide gel, the unadducted nonapeptide was observed at 19.0 min (S/N >1200, data not shown), supporting the efficient extraction of HuBuChE from plasma by huprine. Besides, huprine purification was easier and faster (30 min) than the procainamide approach (2 h). Considering this results, huprine extraction protocol was applied to the analysis of 50 µL of plasma spiked with 10 $ng.mL^{-1}$ of sarin and the resulting chromatograms are shown in **Fig.7**. The sarin-adducted nonapeptide was detected at 20.4 min with a S/N above 3200 but the aged sarin adduct could not be detected, although it was clearly observed on the chromatograms shown in **Fig. 6b**, obtained after on-line immunoextraction and digestion of the same plasma sample. The area of the sarin-adducted nonapeptide obtained after extraction by huprine (**Fig. 7**) was 10 times inferior to the one obtained after immunoextraction (**Fig. 6a**). Thus, it was estimated that the purification of HuBuChE by huprine was 10 times less efficient than the on-line immunoextraction.

Finally, huprine-gel should be preferred to procainamide gel for the one-step purification of HuBuChE from plasma because of its higher selectivity for HuBuChE. However, the best results in terms of HuBuChE recoveries and sensitivity were obtained using the immunosorbent coupled on-line with IMER and microLC/MS^2, with extraction yields of HuBuChE up to 100% and high purity of the extract.

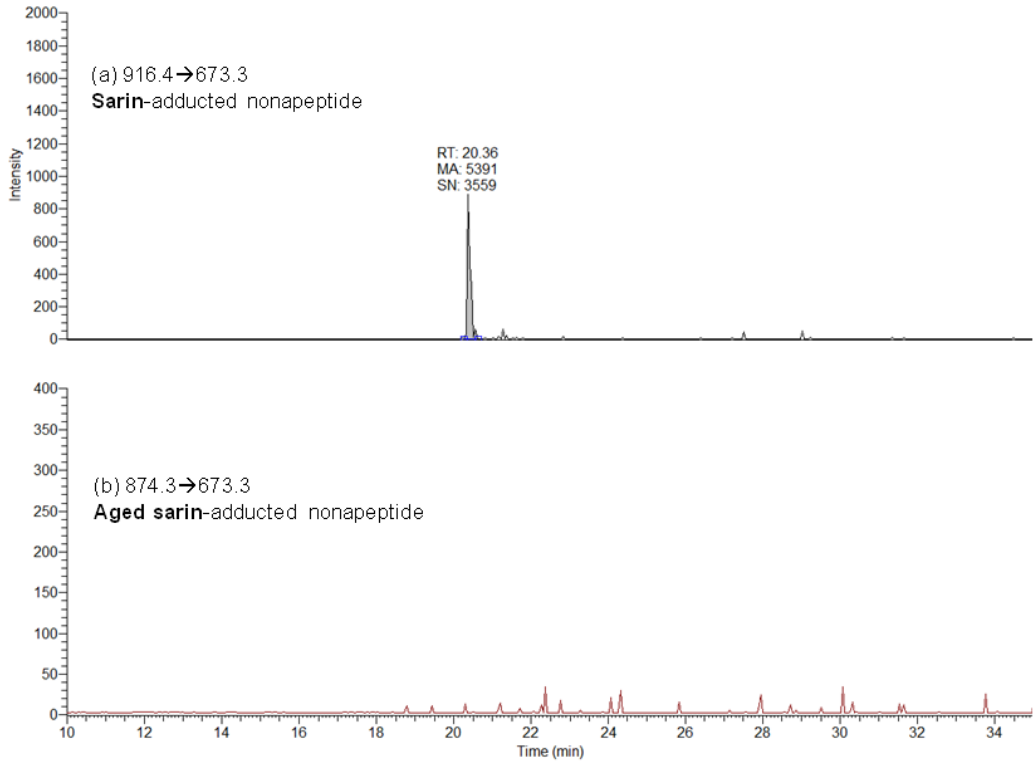

Fig. 7 MRM chromatograms of the sarin-nonapeptide adduct (a) and aged sarin-nonapeptide adduct (b) obtained after purification on huprine of 50 µL of HuBuChE from plasma spiked with 10 ng.mL^{-1} of sarin and its digestion on IMER for 20 min, followed by microLC/MS^2 analysis

4. Conclusion

Three monoclonal anti-HuBuChE antibodies, mAb2, 3E8 and B2 18-5, were compared for the extraction of HuBuChE and OPNA-adducted HuBuChE from plasma. Antibodies were efficiently grafted on sepharose with grafting yields between 94% and 97% and with high repeatability (RSD < 3% for IS mAb2 synthesized in triplicate). The resulting immunosorbents were coupled on-line to the digestion step on IMER and to microLC/MS^2 and the HuBuChE nonapeptide was detected in less than 1 hour, with a LOQ of 2 fmol. The total HuBuChE recovery using this on-line set-up was estimated to 47 ± 7%, with a HuBuChE extraction yield close to 100% for IS mAb2$_{SP}$. The measurement of both adducted and unadducted HuBuChE allowed estimating the percentage of OPNA-adducted HuBuChE in plasma. Aged-OPNAs adducts, OPNAs adducts and unadducted HuBuChE were easily detected in 50 µL of plasma spiked with 10 ng.mL^{-1} of sarin or soman. No memory effect was observed after percolating a plasma sample spiked with OPNAs, showing that immunosorbents could be reused without false positive results.

Finally, extraction on immunosorbent was compared to extraction with procainamide or huprine for the one-step purification of HuBuChE and it was concluded that IS was 10 times more efficient than huprine to extract HuBuChE from plasma and that procainamide gel was less efficient.

In the future, this powerful method will be automated and apply to the fast analysis of simultaneous detection of several OPNA-adducts and aged-OPNAs in human plasma.

Compliance with Ethical Standards:
This is not a clinical study on humans with an ethics committee. Voluntary healthy donors (who signed a questionnaire authorizing the use of their blood bag for research purposes) donate blood to the Army Blood Transfusion Center (CTSA). DGA Maîtrise NRBC has a "Memorandum of Understanding for the transfer of products from blood or its non-therapeutic components" with the CTSA for the supply of healthy donor blood bags (number 2013-063575, March 2013). The CTSA, as a sampling agency authorized to do so, ensures donor-product-laboratory traceability and donor consent (compliance with articles R1221-22 to R1221-48 of the French public health code). Following receipt, Maîtrise NRBC polluted the blood samples in vitro that were subjected to the treatment and the analysis shown in this publication. As DGA MN is not allowed to transfer the delivered blood samples to another institution, the experiments were carried out at the DGA.

Conflict of Interest:
The authors declare that they have no conflict of interest.

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Supplementary material

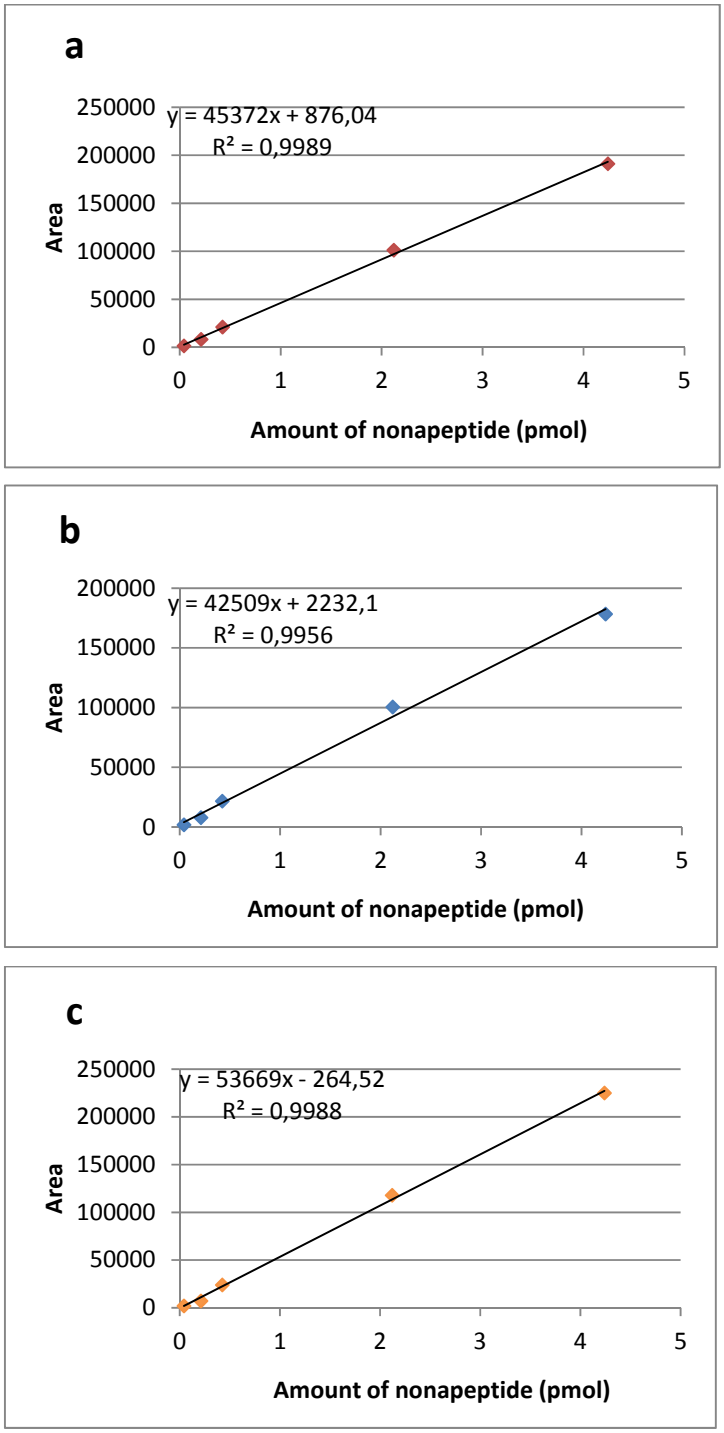

Fig. S1. Calibration curves obtained after the injection of various amounts of synthetic nonapeptide varying from 0.04 pmol (6.75 ng.mL^{-1}) to 4.24 pmol (675 ng.mL^{-1}) for the transitions *m/z* 796.3→602.3 (a), 796.3→673.3 (b) and 796.3→691.3 (c)