Development of immunosorbents coupled on-line to immobilized pepsin reactor and micro liquid chromatography–tandem mass spectrometry for analysis of butyrylcholinesterase in human plasma

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Development of immunosorbents coupled on-line to immobilized pepsin reactor and micro liquid chromatography-tandem mass spectrometry for analysis of butyrylcholinesterase in human plasma

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Abstract

Human butyrylcholinesterase (HuBuChE) has been widely used as a biomarker of exposure to organophosphorus (OPs) warfare agents. Indeed, intoxication by OPs can be proven by LC-MS/MS analysis of a specific HuBuChE nonapeptide on which OPs covalently bind. Therefore, we developed a fast, selective and sensitive on-line set-up for the analysis of HuBuChE from plasma that combines immunoextraction by anti-HuBuChE antibodies, pepsin digestion on Immobilized Enzyme Reactors (IMER) and microLC-MS/MS analysis of the target nonapeptide, FGESAGAAS.

Two pepsin-based IMERs were prepared and characterized in terms of grafting and digestion yields and were coupled on-line to microLC-MS/MS analysis. In addition, immunosorbents were prepared by covalent grafting of three anti-HuBuChE antibodies on CNBr-sepharose and epoxy-polymethacrylate supports and packed in precolumns. The best antibody grafting yields were obtained with sepharose-based supports, with grafting yields up to 98%. B2 18-5 monoclonal antibody grafted on sepharose led to the best immunosorbent, with HuBuChE recovery close to 100%. The immunosorbent was introduced upstream of the on-line digestion set-up and immunoextraction of HuBuChE was achieved in 14 min while digestion was performed in 20 min, allowing detection of the target nonapeptide in less than 1 hour. The global recovery of the nonapeptide was higher than 42% using the best immunosorbent with a RSD value lower than 7% (n = 3). Finally, the limit of quantification evaluated in plasma sample was 2 fmol of nonapeptide. This value, corresponding to 0.5 fmol of HuBuChE tetramer, is well below the average amount of HuBuChE tetramer in 50 µL of plasma (590 fmol).

Keywords

Human butyrylcholinesterase; MicroLC-MS/MS; FGESAGAAS, Immunosorbents; Immobilized Enzyme Reactor; On-line coupling.

1. Introduction

Human butyrylcholinesterase (HuBuChE, UniProt accession P06276) is a serine hydrolase present in plasma [1,2]. It is a tetrameric glycoprotein composed of four identical subunits (85 kDa) with a molecular weight of 340 kDa. The tetrameric form, that is made of two dimers, is the predominant form in plasma (95%) [3,4]. HuBuChE is known as a scavenger protein that protects the cholinergic system against acetylcholinesterase poisons [5]. HuBuChE can covalently bind to organophosphorus compounds (OPs) including pesticides and chemical warfare agents such as sarin [6–9], soman [7,8,10], tabun [8,10,11] or VX [7,12]. The high reactivity of HuBuChE with OPs makes HuBuChE a good biomarker of OP exposure. Indeed, the binding of OP nerve agents to serine 198 of the HuBuChE active site allows retrospective detection of HuBuChE-OP adducts in blood for as long as 16 days after exposure when OP urinary metabolites are no longer detectable [13]. The detection of these adducts is usually performed after digestion by pepsin, commonly over 2 hours. Pepsin digestion produces small peptides, including the nonapeptide FGESAGAAS, covalently bound to OP, that is further analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) [14,15]. However, as the pepsin is free in solution, autoproteolysis can occur, generating peptides that can interfere during LC/MS analysis of the nonapeptide. To overcome this drawback, we recently developed pepsin-based immobilized enzyme reactors (IMERs) [16]. These supports were prepared by grafting pepsin on a CNBr-activated sepharose phase, and were packed in precolumns (20 x 2 mm i.d.). Optimization of the HuBuChE digestion parameters on IMER enabled us to get lower digestion times and higher digestion yields than those obtained in solution [14,15,17] or using commercial pepsin-IMER [9]. Indeed, IMERs allowed efficient digestion of HuBuChE in only 20 min, without pretreatment, while minimizing sample contamination during handling and avoiding pepsin autoproteolysis. Moreover, these supports can be reused [16,18] and can also be directly coupled to LC/MS [9,19].

Human plasma contains about 4 $\mu g.mL^{-1}$ of HuBuChE, while other proteins such as albumin can be 10000 times more concentrated [5,20]. Hence, it is essential to extract HuBuChE from highly concentrated proteins in plasma before digestion and analysis by LC/MS to avoid ion suppression. Current purification methods are based on HuBuChE extraction from plasma by affinity chromatography on procainamide gels [4,8,21,22] or by antibodies immobilized on magnetic beads activated with streptavidin or protein G [23–27]. Procainamide gels are relatively cheap but not selective enough, implying the need of additional purification steps, which extends the extraction time and reduces the total recovery of the method [4,28]. Purification of HuBuChE by immunomagnetic beads is more specific than the procainamide gels approach and requires smaller amounts of plasma. In most protocols, HuBuChE is directly digested on the immunomagnetic beads in contact with the protease, which involves the use of a new batch of antibodies for each sample since they are also digested [12,13,25–27]. The digestion step leads to peptides derived from HuBuChE but also from antibodies, protein G, protease by self-digestion and thus to numerous peptides that could affect the mass spectrometry response of the target peptide adducts. To reduce the presence of unwanted peptides, an elution step can be applied to release HuBuChE from the beads by acetic acid prior to digestion [23,24,29]. However, these extraction methods are based on a partitioning equilibrium between HuBuChE and antibodies, which induces long extraction times, varying from 2 h to 18 h because of the long diffusion time of the protein. Besides, none of these studies mentioned the regeneration of the antibodies and thus the reusability of the beads, nor the repeatability of the grafting and the extraction processes. In the current work, we developed an alternative approach that consists in packing

covalently grafted antibodies into a precolumn to extract HuBuChE by solid phase extraction (SPE). SPE is faster than adsorption by partition, and can be easily automated. Moreover, the washing step is easier to optimize as it can be directly monitored by UV detection by on-line coupling of the immunosorbent (IS) to the UV detection system [19]. After immunoextraction, regeneration of the antibodies allows to reuse the IS for months and thus to reduce the cost of the extraction procedure. In addition, IS can be directly coupled on-line to digestion on IMER and to LC/MS analysis, which avoids sample loss and contamination and allows automation of the whole analytical procedure. Among different grafting supports available for antibody immobilization, like silica [30,31], polystyrene divinyl benzene [32], glycidyl methacrylate-co-ethylene glycol dimetacrylate (GMA-EDTA)[33,34] or magnetic beads [13,25,26,35], two supports, i.e. epoxy activated polymethacrylate resin [36] and CNBr activated sepharose [19,37] were chosen. Sepharose is an agarose gel that has been widely used for antibody immobilization [19,29,37,38]. It displays several advantages such as good chemical stability covering a wide range of pH (2-9) and a relative hydrophilicity that minimizes non specific interactions between sepharose and hydrophobic proteins. Its 700 Å pore size provides high capacity and relatively good accessibility of target molecules to the pores. Polymethacrylate resin was also chosen as grafting support for its high pore size (> 1000 Å), which allows proteins with high molecular weight and hydrodynamic diameter like HuBuChE (200 Å [38]) to easily access the antibodies bound inside the pores. It also has low cost and good stability at low pH, which is crucial regarding the acidic condition applied on IS during the elution step.

Until now, no research group reported the use of an immunosorbent coupled on-line to an IMER for the total on-line analysis of HuBuChE-OP adducts from plasma. Three anti-HuBuChE antibodies directed against three different epitopes of HuBuChE were immobilized on CNBr sepharose or on epoxy resin supports and packed in precolumns. The resulting IS were first characterized in terms of antibody grafting yields. HuBuChE extraction yields were then evaluated for each support by LC/UV. Similar IMERs to those developed and optimized in our previous study were synthesized and packed in precolumns of different sizes, by immobilizing pepsin on CNBr sepharose supports [16] and were coupled on-line to microLC/MS analysis. Finally, the best immunosorbent was integrated to the analytical system including the IMER coupled on-line with the microLC/MS analysis and the set-up was applied for analysis of HuBuChE in plasma.

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), human serum albumin, cyanogen bromide-activated Sepharose 4B (90 µm, 700 Å), Toyopearl® AF-Epoxy-650M Bulk Media (65 µm, 1000 Å) were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). Potassium dihydrogen phosphate (KH_2PO_4), di-sodium hydrogen phosphate dihydrate ($Na_2HPO_4(2H_2O)$), sodium bicarbonate ($NaHCO_3$) sodium acetate (CH_3CO_2Na), acetic acid (CH_3COOH), formic acid (HCOOH), glycine, hydrochloric acid and acetonitrile (ACN) were purchased from VWR (Fontenay-sous-Bois, France). Sodium hydroxide was purchased 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). Human plasma was obtained from the Centre de Transfusion Sanguine des Armées (Clamart, France).

Polyclonal antibodies N-15 (sc46803 goat polyclonal to the N-terminal of HuBuChE) and C-18 (sc46801 goat polyclonal to the C-terminal of HuBuChE) were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Mouse monoclonal B2 18-5 [20] was provided by Prof. Oksana Lockridge (University of Nebraska Medical Center, Omaha). Mouse IgG from serum (I5381) was purchased from Sigma-Aldrich.

HuBuChE, (670 µg of lyophilized HuBuChE with 0.02 mol.L^{-1} ammonium bicarbonate, 200 U.mg^{-1}, MBS173030) obtained by the purification of human serum, was purchased from Lee Biosolutions (Maryland heights, Missouri, USA). Synthetic nonapeptide (FGESAGAAS, MW: 795.3 g.mol^{-1}) was obtained from Proteogenix (Schiltigheim, France). Bicinchoninic acid (BCA) assay reagents and tryptic cytochrome C digest (16 nmol.mL^{-1} in water) were from Thermo Fisher Scientific (Illkirch, France).

The phosphate buffer saline solution (PBS, 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. The PBS-azide solution is a solution of PBS with 0.1% (w/w) of NaN_3. Pepsin solutions were prepared in saline sodium acetate solution (CH_3CO_2Na, 0.1 mol.L^{-1}, pH 5.8, NaCl 0.5 mol.L^{-1}).

2.2. MicroLC/MS analysis

The microLC/MS analytical system was composed of a micro-pump (Dionex Ultimate 3000, controlled by Chromeleon 6.8 SR11) interfaced with a triple quadrupole mass spectrometer (TSQ Quantum Access Max™, Thermo Scientific) assembled with an ESI source (IonMax source, probe HESI-II). The analytical column was an Acclaim PepMap 100 (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). The synthetic nonapeptide at 8.6 nmol.mL^{-1} in 0.5 mol.L^{-1} formic acid (5 µL) was mixed with 10 µL of a tryptic digest of cytochrome C at 16 nmol.mL^{-1} and this solution was used to optimize the separation conditions in scan mode (m/z from 300 to 1500). The mobile phase was water and acetonitrile both acidified with 0.1% formic acid and delivered at a flow rate of 50 µL.min^{-1}. The separation of peptides was achieved by increasing the amount of acidified acetonitrile from 2% to 90% in 20 min. The dwell time was set to 500 ms and ionization was performed in positive mode with the following source conditions: source voltage was set at 3000 V, capillary temperature at 250 °C, vaporizer temperature at 80 °C, sheet gas and auxiliary gas pressures at 10, tube lens offset at 70 V and skimmer offset at 0 V. Peptides resulting from HuBuChE digestion on IMER were analyzed by microLC-MS/MS. The three most intense b-fragments of the nonapeptide, b_8, $b_{8\text{-}H2O}$ and $b_{7\text{-}H2O,}$ were followed in MRM mode (*m/z* 796.3→691.3; 796.3→673.3; 796.3→602.3 respectively) between 0 min and 19.3 min using a fragmentation voltage of 30 eV. These transitions were used to quantify the amount of nonapeptide resulting from HuBuChE digestion with or without a previous immunoextraction step. For this purpose, calibration curves were established for each MRM transition by injecting 5 µL of different concentrations of a synthetic nonapeptide (6.8, 33.8, 67.5, 338, 675, 1688 and 3375 ng.mL^{-1}) into the microLC-MS/MS system. The concentration range was adjusted depending on the amount of HuBuChE digested and new calibration curves were regularly made to take into account mass spectrometry variations. To evaluate non specific interactions resulting from the

percolation of plasma on immunosorbents, the most intense albumin peptide (LGMF m/z = 468) was followed by Single Ion Monitoring (SIM) between 19.3 min and 21.7 min. This peptide was chosen after the digestion on IMER of a commercial human serum albumin solution and it was analyzed by microLC/MS in scan mode (m/z between 300 and 1500), using the same gradient and mass spectrometry source parameters described above.

2.3. Preparation of the immunosorbents

2.3.1. Sepharose-based immunosorbents

The grafting of the N-15, C-18 and B2 18-5 antibodies on sepharose was achieved according to the procedure already developed in our laboratory [19]. Briefly, 35 mg of CNBr activated sepharose was swollen in 1 mL of HCl (1 mmol.L^{-1}) for 15 min and washed twice with a solution containing $NaHCO_3$ (pH = 8.3, 0.1 mol.L^{-1}) and NaCl (0.5 mol.L^{-1}). Then, 100 µg of anti-HuBuChE antibodies diluted in 100 µL of PBS (pH = 7.4, 0.1 mol.L^{-1}) were incubated with sepharose for 24 h at 4 °C, while stirring at 4 rpm. The resulting sorbent was packed into a precolumn (20 x 2 mm I.D.). The precolumn was connected to a pump (LC-10 AS, Shimadzu) and the remaining uncoupled sites of sepharose were blocked by percolating a Tris buffer solution (pH = 8, 0.1 mol.L^{-1}) at 400 µL min^{-1} for 2 h at room temperature. Then, the sorbent was alternatively washed 3 times at 400 µL min^{-1} with 4 mL of acetate buffer (0.1 mol.L^{-1} + NaCl 0.5 mol.L^{-1}, pH = 4) and $NaHCO_3$ buffer (0.1 mol.L^{-1} + NaCl 0.5 M, pH = 8.3) to remove unbound antibodies. The immunosorbents were stored at 4 °C in a PBS-azide solution and were called N-15_{SP}, C-18_{SP} and B2 18-5_{SP}.

2.3.2. Epoxy resin-based immunosorbents

To determine the optimal conditions of grafting on the epoxy resin, twelve vials containing 30 mg of epoxy resin were each swollen in 1 mL of H_2O and stirred at 4 rpm for 15 min. The supernatant was removed and 1 mL of PBS buffer (pH = 7.4) or borate buffer (pH = 8, 8.5 or 9) was added. This step was repeated 3 times. Then, 100 µg of mouse IgG from serum (Sigma-Aldrich, I5381) diluted in 100 µL of PBS buffer (pH = 7.4) or borate (pH = 8, 8.5 or 9) were added to the epoxy resin. For each value of pH, three samples were prepared. One sample was stirred at 4 °C, a second at 25 °C and the last one at 37 °C, for 72 h. To evaluate the amount of antibodies grafted on the epoxy resin, 10 µL of supernatant were collected from the 12 vials every 24 h and quantified by BCA assay.

After optimization of the grafting conditions with mouse IgG from serum, B2 18-5 antibodies were grafted on the swollen epoxy resin by introducing 100 µg of B2 18-5 antibody diluted in 100 µL of PBS buffer (pH = 7.4). After 24 h of stirring at 25 °C, the grafted resin was filled in a precolumn (20 x 2 mm I.D.) and washed with ethanolamine for 6 h at 100 µL.min^{-1} to block the remaining uncoupled epoxy sites. PBS buffer (pH = 7.4), followed by a solution of NaCl (1 mol.L^{-1}) and PBS buffer (pH = 7.4) were percolated through the precolumn at 400 µL.min^{-1} for 20 min to remove unbound antibodies. The resulting immunosorbent, called B2 18-5_{ER}, was stored at 4 °C in a PBS-azide solution.

2.3.3. Quantification of antibodies grafted on sepharose and epoxy resin

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 and epoxy resin. Antibodies were available in small amount because of their high cost. It was therefore not possible to make calibration curves to quantify the amount of grafted antibodies. Consequently, the absorbance corresponding to 10 µL of the initial solution of antibodies was used as reference value and was compared to the absorbance of 10 µL of supernatant containing the unbounded antibodies remaining after grafting (in triplicate). Then, 10 µL of supernatant was added in triplicate and 100 µL of working reagent (mix of bicinchoninic acid and Cu^{2+}) was added in each microwell. The microplate was incubated at 37 °C for 30 min with stirring at 300 rpm. Absorbance was measured at 562 nm with a spectrophotometer (SpectraMax M2, Molecular Devices, St Gregoire, France). 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.

2.4. Immunosorbents coupled to UV detection

The immunoextraction procedure was first carried out on a set-up composed of two connected switching valves: one carries a 5 µL injection loop and is linked to the pump while the other carries the IS, connected to the UV detector (**Fig. 1A**). After conditioning of the IS for 30 min with PBS, 5 µL of HuBuChE solution (300 $\mu g.mL^{-1}$ in PBS buffer, pH = 7.4) was percolated through the IS that was further washed for 10 min with the PBS solution at 100 $\mu L.min^{-1}$. Afterwards, HuBuChE was eluted from the IS by percolating a formic acid solution (pH = 2.2, 0.5 $mol.L^{-1}$) at 100 $\mu L.min^{-1}$ for 10 min. Detection of HuBuChE during the percolation and washing with PBS and elution steps was followed at 280 nm. It was previously checked that HuBuChE had the same UV response in PBS as in formic acid. The extraction yield was calculated by dividing the area of the peak observed during the elution step by the sum of the area of the peaks corresponding to the percolation/washing step with PBS and to the elution step with formic acid.

2.5. Preparation of pepsin-based IMERs

Two pepsin-based IMERs were prepared according to the protocol previously developed by our group for pepsin immobilization [16]. Briefly, 1.7 mL of a 4 $mg.mL^{-1}$ pepsin solution in 0.1 $mol.L^{-1}$ sodium acetate was incubated with 35 mg or 58 mg of CNBr activated sepharose for 16 h at 4 °C. Then, 30 mg from the 35 mg of sorbent, were packed in a 20 x 2 mm I.D. precolumn (IMER 1) while 50 mg, from the 58 mg of sorbent, were packed in a 30 x 2.1 mm I.D. precolumn (IMER 2). The remaining uncoupled sites were blocked by percolating a glycine solution through the precolumn at a flow rate of 400 $\mu L.min^{-1}$ for 120 min for IMER 1 and 200 min for IMER 2. The gel was washed three times at 400 $\mu L.min^{-1}$ for 10 min (IMER 1) or 16 min (IMER 2), alternating between sodium acetate and HCl solutions to remove unbound enzymes. Finally, IMERs were stored at 4 °C in a formic acid solution (pH = 3) when not used.

2.6. Quantification of pepsin grafted on IMERs

A bicinchoninic acid (BCA) assay was used to evaluate the amount of pepsin immobilized on sepharose. Two calibration curves (six levels of concentration) were made by adding 10 µL of pepsin at a concentration range from 100 µg.mL^{-1} to 4 mg.mL^{-1} in the microwells of a 96 microplate. After addition of 10 µL of the working reagent in each microwell and incubation of the microplate at 37 °C for 30 min, the absorbance was measured at 562 nm. Pepsin grafting yields were calculated by comparing the amount of pepsin remaining in the supernatant to the initial amount of pepsin in the coupling solution.

2.7. HuBuChE digestion procedure on IMERs

The digestion was performed using a set-up composed of three serial six-port switching valves (**Fig. 1B**). One carries a 5 µL injection loop and is connected to a syringe-pump, the second carries an IMER and the third the trap column (Hypersil Gold C18, 10 x 1 mm I.D., 5 µm) connected to the IMER and to microLC/MS. Briefly, 2.4 µg of a commercial HuBuChE solution was loaded into the 5 µL injection loop and was transferred by 30 µL (IMER 1) or 40 µL (IMER 2) of formic acid (pH = 2.2) to the IMER at 50 µL.min^{-1}. After digestion by *stop flow* for 10 or 20 min, peptides were transferred with 150 µL (IMER 1) or 250 µL (IMER 2) of formic acid at 50 µL.min^{-1} to the trap column for concentration. Finally, the third valve was switched and the peptides were transferred from the trap column to the analytical column to be analyzed by microLC-MS/MS as described in **Section 2.2**. IMER could be directly reused after a 30 min washing step with formic acid at pH = 2.2 (0.5 mol.L^{-1}).

Digestion yields were evaluated by dividing the amount of nonapeptide resulting from pepsin digestion by the theoretical amount of nonapeptide corresponding to a 100% digestion yield. This amount was calculated taking into account that 1 mole of HuBuChE (Mw = 340 kDa) gives 4 moles of nonapeptide.

2.8. Immunoextraction and digestion steps coupled on-line to microLC-MS/MS

For total analysis of HuBuChE from plasma, IS B2 18-5$_{SP}$ and IS B2 18-5$_{ER}$ were coupled on-line to IMER 2 according to the set-up described in **Fig. 1C.** After conditioning the immunosorbent for 30 min with PBS buffer (pH = 7.4) at 50 µL.min^{-1}, immunoextraction was achieved in two steps. The first step consisted in transfer of 100 µL of a diluted plasma sample (plasma/PBS 1/1 v/v) through the immunosorbent with 700 µL of PBS buffer at 50 µL.min^{-1} to remove non-specifically retained plasma proteins. At that time, the IS is not linked to the IMER but only to the waste. Afterwards, the valve containing the IMER was switched and HuBuChE retained by the IS was eluted and transferred to the IMER with 62 µL of formic acid at pH = 2.2 (0.5 mol.L^{-1}) at 50µL.min^{-1} and a *stop flow* digestion was performed for 10 min. The elution and the stop flow digestion were repeated twice. Then, HuBuChE peptides were transferred from the IMER to the trap column with 250 µL of the same acid solution at 50 µL.min^{-1}. Finally, the valve connected to the trap column was switched and the peptides were transferred by the LC mobile phase to the analytical column to be analyzed as mentioned in **Section 2.2.** After immunoextraction, immunosorbents were stored in a PBS-azide solution for at least 24 h to allow the regeneration of antibodies before their re-use.

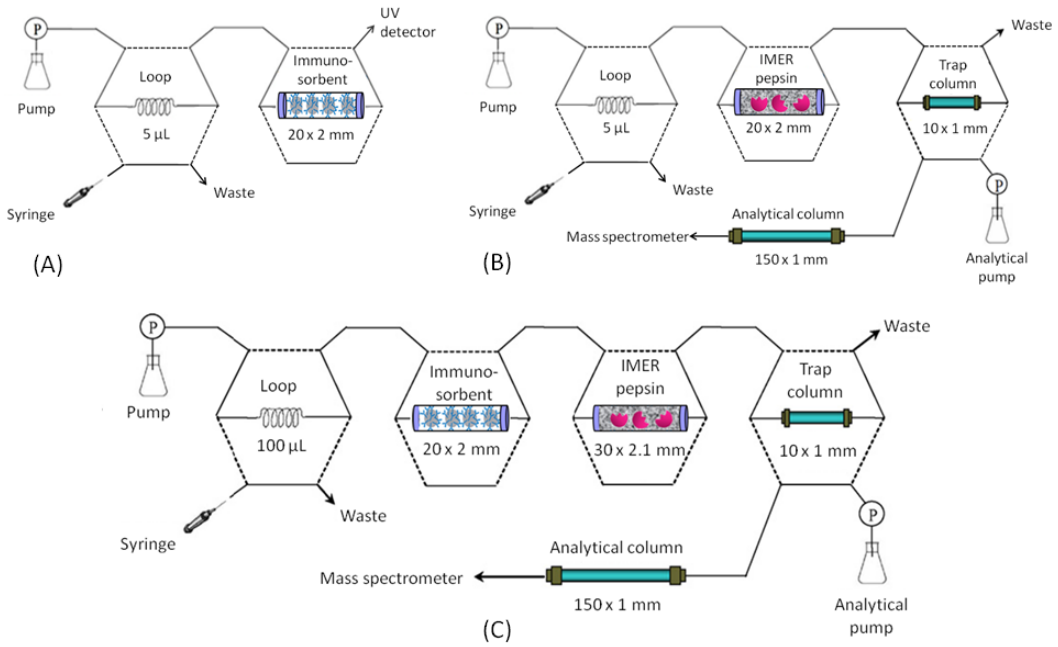

Fig. 1. Set-up used for the immunoextraction of 5 µL of HuBuChE solution on the immunosorbents (A). Set-up used for the on-line pepsin digestion of 5 µL of HuBuChE solution on IMER and its analysis by microLC/MS (B). Set-up used for the on-line immunoextraction, followed by pepsin digestion and analysis of HuBuChE in 50 µL of plasma (C). The trap column concentrated the peptides.

3. Results and discussion

3.1. Preparation of immunosorbents and evaluation of their grafting yields

According to the results found in the literature in terms of affinity for HuBuChE, three antibodies directed against three epitopes were chosen for the preparation of immunosorbents: two polyclonal antibodies whose epitopes where respectively located near the N-terminal (N-15) [24] and C-terminal (C-18) amino acid sequence of HuBuChE [39] and one monoclonal antibody B2 18-5 whose epitope was not mentioned but whose affinity for HuBuChE was demonstrated by Peng *et al*. [20,38,40]. The choice of the immobilization support is a key parameter for antibody grafting since it conditioned the chemical and mechanical properties of the immunosorbent. The support must be stable, chemically inert and have good mechanical resistance. It also needs to be easily functionalized and should not be hydrophobic to avoid developing non specific interactions with molecules present in the sample. Two materials, matching these conditions, were chosen as grafting supports. Sepharose, that has been widely used for antibody immobilization was selected for its low hydrophobicity and for its high capacity. Because sepharose has relatively low pore size (700 Å), an epoxy resin support, with higher porosity (1000 Å) but never described for antibody grafting, was chosen to ensure optimal access of HuBuChE (140 Å) to the antibodies bound inside the pores.

3.1.1. Sepharose-based immunosorbents

To evaluate the potential of this support, 100 µg of polyclonal or monoclonal anti-HuBuChE antibodies were covalently bound to 35 mg of CNBr activated sepharose and packed in a precolumn (20 x 2 mm I.D.). At the end of the grafting step, the supernatant containing unbound antibodies was analyzed by BCA assay to quantify the amount of antibodies grafted on sepharose. Monoclonal B2 18-5 antibodies (100 µg, 1 $mg.mL^{-1}$ in PBS with 0.1% sodium azide) were easily bound to sepharose (35 mg) with 95% grafting yields, close to the 99% yields described by Peng *et al*. for the immobilization of 5 mg of B2 18-5 antibodies on 1 g of CNBr activated sepharose, for 20 h [38]. Polyclonal antibodies N-15 and C-18 (100 µg, 200 $\mu g.mL^{-1}$ in PBS with 0.1% sodium azide and 0.1% gelatin) were grafted on sepharose and the grafting yield was estimated to be 35% for N-15 and 38% for C-18 antibodies. By comparison with the results obtained with the B2 18-5 monoclonal antibodies, the grafting yields of N-15 and C-18 polyclonal antibodies were quite low. Considering that identical antibody/sepharose ratios were used, it was assumed that the low grafting yields were due to the antibody concentration or to the 0.1% gelatin added by the supplier in the PBS solution that could bind to CNBr sepharose grafting sites. Consequently, the same grafting procedure was applied with N-15 and C-18 antibodies solutions that were ordered without gelatin (1 $mg.mL^{-1}$ in PBS with 0.1% sodium azide) and excellent grafting yields were obtained for N-15 and C-18 antibodies (94% and 98% respectively). The resulting immunosorbents were called $N\text{-}15_{SP}$, $C\text{-}18_{SP}$ and $B2\ 18\text{-}5_{SP}$.

3.1.2. Epoxy resin-based immunosorbent

Several reactive groups were available for this resin: tresyl, formyl, amino, carboxy and epoxy groups. Among them, the epoxy group was chosen for its gentle and easy reaction condition, the stability of the covalent linkage and the grafting versatility. Indeed ligands can be bound by their amino or thiol groups, depending on the pH and the temperature used during the grafting step. Epoxy groups have already been used for antibody immobilization on magnetic beads or sepharose [30,32–34] but antibodies have not been grafted on this epoxy resin so far. Therefore, the grafting of antibodies on the resin needed to be optimized.

Based on the literature [41], the use of a grafting solution with a pH between 7 and 8 at 25 °C must favor protein grafting on epoxy resin by thiol group while solutions with a pH close to 9 at 40 °C favor grafting by amino group. Because of the high cost of anti-HuBuChE antibodies, the grafting yield of antibodies on this resin was studied with mouse IgG. To evaluate the best grafting conditions, 100 µg of mouse IgG were grafted on 30 mg epoxy resin at different pH (7.4, 8, 8.5 and 9), temperature (4 °C, 25 °C or 37 °C) and grafting time (24 h, 48 h or 72 h). BCA assays were performed every 24 h to evaluate the grafting yields for each conditions. As shown in **Fig. 2**, increase of the grafting time, whatever the pH or the temperature, did not enhance the grafting of IgG mouse antibodies on this resin. This can be explained by saturation of the epoxy sites by 24 h. Even though no significant difference in terms of grafting yields was observed when increasing the pH or the temperature, two trends may be discerned. For pH 7.4 and 8, favoring the grafting of antibodies by thiol groups, the grafting seemed less efficient at 4 °C than 25 °C or 37 °C. On the contrary, temperature did not influence the grafting of antibodies by amino groups at pH 8.5 and 9. Considering the low impact of pH on grafting yields, it was chosen to graft the antibodies by thiol group at 25 °C and pH = 7.4, which corresponds to the optimal pH for antibody stability. The optimized procedure was then applied to B2 18-5 anti-HuBuChE antibodies. As

depicted in **Section 2.3.2**, 100 µg of B2 18-5 antibodies were grafted on the epoxy resin for 24 h at 25 °C and pH 7.4. After evaluation of the amount of antibody remaining in the supernatant by BCA assay, a grafting yield of 59% was obtained and was superior to those observed for mouse antibodies at pH 7.4 and 25 °C (45%). However, this value was almost 2-fold lower than that obtained on sepharose (95%). This can be explained by a weaker specific surface area of the epoxy resin due to its larger pore size (1000 Å) compared to sepharose (700 Å). Despite this low grafting yield of antibodies on the epoxy resin, access of HuBuChE to antibodies could be favored by the high pore size of the resin. Consequently, retention of HuBuChE was studied on both IS B2 18-5_{SP} and B2 18-5_{ER} to compare them in terms of extraction recoveries and selectivity.

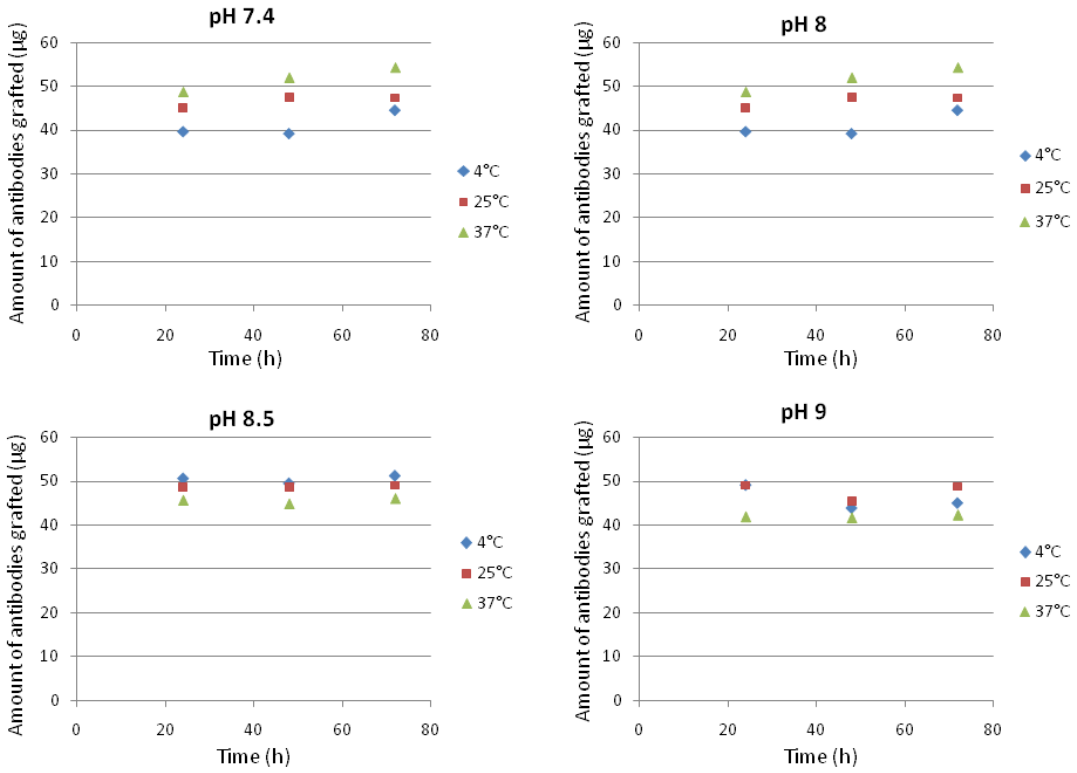

Fig. 2. Amount of mouse IgG grafted on epoxy resin (30 mg) depending on the pH, the temperature and the grafting time.

3.2. Immunoextraction of HuBuChE

Immunoextraction involves three basic steps. The first step is called percolation and consists in loading of the sample onto the immunosorbent. It occurs in an aqueous buffer like PBS, under conditions close to physiological medium to ensure the best retention of the target analyte on antibodies. Then, a washing step is applied to remove the interfering compounds slightly retained on the support without disturbing the interaction between the target analyte and the antibodies. Finally, the last step is the elution of the analyte from the immunosorbent by an acidic or chaotropic solution that disrupts analyte-antibody interactions [42]. In this study, the immunoextraction procedure was carried out with a set-up including a 5 µL injection loop, an IS

(20 x 2 mm I.D.) and a UV detector (**Fig. 1A**). After conditioning of the IS, 5 µL of HuBuChE solution was percolated and the IS was washed with 1 mL of PBS before elution of HuBuChE from the IS at 100 µL.min^{-1} by a formic acid solution for 10 min. The presence of HuBuChE during the percolation, the washing and the elution steps was followed at 280 nm by UV detection. During the development of the immunoextraction procedure, we noticed that whatever the antibodies, no retention of HuBuChE was observed when percolating a HuBuChE solution containing 0.1% sodium azide. Consequently, all of the immunoextractions were performed with azide-free HuBuChE solutions. As shown on the UV chromatogram obtained after percolation and elution of HuBuChE on the IS B2 18-5$_{ER}$ (**Fig. 3**), a small peak was observed at 280 nm during percolation and washing with PBS. It indicates that a low amount of protein was lost during this step and could correspond to HuBuChE, or to interfering proteins present in the commercial HuBuChE preparation. Elution of HuBuChE was achieved in 2 min, with an elution volume of 200 µL (**Fig. 3**). Even in the case that a small amount of HuBuChE was lost during percolation, it was estimated that more than 80% of HuBuChE was retained on the IS B2 18-5$_{ER}$ by comparison of the area of the percolation and the elution peaks (**Section 2.4)**. This value was similar to the 97% extraction yield obtained by Peng *et al* for the immunoextraction of 500 µL of plasma on B2 18-5 antibodies (20 µg) immobilized on Dynabeads Protein G [20]. The slight difference between these two values was explained by the different origins of HuBuChE (commercial or plasmatic) and by the different methods applied to evaluate the binding of HuBuChE on antibodies. In our case, HuBuChE extraction was followed by UV measurement at 280 nm while Peng *et al* evaluated it by measuring the residual HuBuChE activity in plasma. The immunoextraction procedure was then applied to the sepharose-based immunosorbents B2 18-5$_{SP}$, N-15$_{SP}$ and C-18$_{SP}$. About 85% of HuBuChE was retained on the monoclonal IS B2 18-5$_{SP}$ with an elution volume of 150 µL. However, no retention was observed for polyclonal-based immunosorbents N-15$_{SP}$ and C-18$_{SP}$. Indeed, HuBuChE was totally released during the percolation and washing steps and was missing on the elution chromatogram. No information was found in the literature concerning the retention of HuBuChE on C-18 antibodies. However, Arial *et al.* showed that N-15 antibodies, immobilized on magnetic beads, could effectively capture HuBuChE from plasma after a 15 h incubation time [24]. The absence of retention of HuBuChE on the IS N-15 was due to a weak affinity of the N-15 antibodies for HuBuChE. Contrary to the washing step by partition, the washing step by SPE requires high affinity antibodies to maintain retention of HuBuChE on the IS. Thus, only B2 18-5$_{SP}$ and B2 18-5$_{ER}$ immunosorbents were kept for on-line coupling to IMER for digestion by pepsin.

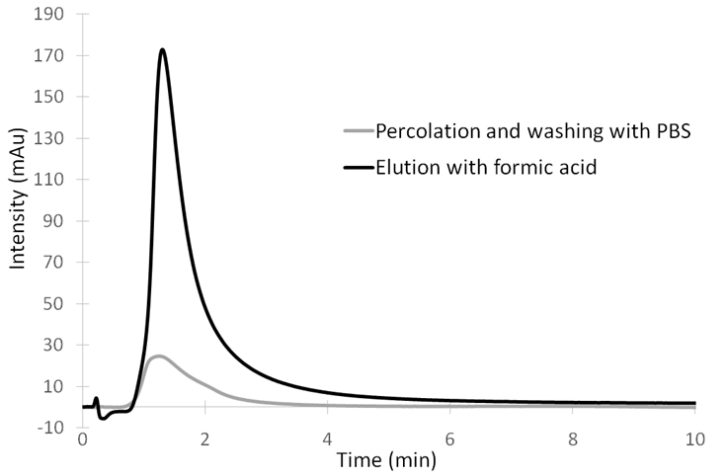

Fig. 3. UV analysis (λ= 280 nm) of the effluent of B2 18-5_{ER} immunosorbent (20 x 2 mm I.D.) after percolation of 5 µL of a HuBuChE solution (1.5 µg). The grey chromatogram corresponds to the percolation and washing steps (PBS solution pH = 7.4 at 100 µL.min^{-1}) and the black chromatogram to the elution step (formic acid solution pH = 2.2 at 100 µL.min^{-1}).

3.3. On-line coupling of IMER to microLC-MS/MS

3.3.1. Analysis of HuBuChE peptides by microLC-MS/MS

The goal of this study was to couple on-line a selective immunoextraction step with ~~a~~ rapid and efficient HuBuChE digestion on IMER to LC/MS for analysis of the target nonapeptide FGESAGAAS. In a preliminary study, nanoLC/MS was used for the analysis of HuBuChE peptides [16]. Nano-chromatography brought high sensitivity while only requiring a low amount of sample (250 nL), which allowed minimization of the cost of development of the method. However, the on-line coupling of the IMER (20 x 2 mm I.D.) to the nano-column (EASY-Spray PepMap® C18, 150 x 0.75 mm I.D., 3 µm, 100 Å) was tricky because of the incompatibility of their volumes. Indeed, 100 µL of formic acid were required to recover the nonapeptide from the IMER while injected volumes higher than 250 nL would cause a loss of efficiency in nanoLC/MS. Consequently, a trap column was needed to concentrate the 100 µL peptide solution collected from the IMER before its injection on the analytical column. Current nano-trap columns can only be applied to low volumes of sample (1 to 5 µL) which was not compatible with the 100 µL peptide solution. To analyze the totality of the sample by nanoLC/MS, it was necessary to reduce the size of the IMER. However, low diameter devices are hard to pack with sepharose beads. Thus, we decided to move from nanoLC/MS to microLC/MS analysis, to couple the previously developed IMERs to a micro trap column (10 x 1 mm I.D.), which was compatible with the 100 µL peptide solution.

To carry out the separation by microLC-MS/MS, a solution of synthetic nonapeptide (5 µL) was mixed with a solution of tryptic cytochrome C digest to simulate peptides resulting from HuBuChE digestion. The peptides were separated at 50 µL.min^{-1} on an Acclaim PepMap 100 analytical column (C18, 150 x 1 mm, 3 µm, 100 Å) with a water (A)/ ACN (B) gradient varying from 2% B to 90% B in 20 min. Peptides were detected by mass spectrometry in SCAN mode. The total ion chromatogram resulting from analysis of the peptides is shown in **Fig. 4A**. An extracted ion chromatogram corresponding to the nonapeptide (m/z = 796.3) was performed and the

nonapeptide was observed at a retention time of 18.9 min (**Fig. 4B)**. Calibration curves were established for the MRM transitions *m/z* 796.3→602.3, 796.3→673.3 and 796.3→691.3 to further identify and quantify by microLC-MS/MS the nonapeptide resulting from HuBuChE digestion with or without immunoextraction upstream. The linear regression equations and coefficient values of the regression curves obtained for the concentration range 6.8 to 675 $ng.mL^{-1}$ were equal to y = 33584 x – 26.856, R^2 = 0.999; y = 34237 x – 218.65, R^2 = 0.999; y = 34804 x – 36.013, R^2 = 0.998 for the transitions *m/z* 796.3→602.3, 796.3→673.3 and 796.3→691.3 respectively (see calibration curves in supplementary material **S.1**). For the transition m/z 796.3→602.3, the limit of detection (LOD, S/N = 3) and of quantification (LOQ, S/N = 10) of the nonapeptide were estimated to be 0.08 $ng.mL^{-1}$ (0.4 pg injected) and 0.28 $ng.mL^{-1}$ (1.4 pg) respectively. The LOD and LOQ values were respectively 0.10 $ng.mL^{-1}$ (0.5 pg) and 0.35 $ng.mL^{-1}$ (1.7 pg) for the transitions m/z 796.3→673.3 and 796.3→691.3. These LOD values were 9 to 36 times below the LOD of the nonapeptide reported by Pantazides *et al.* (1 $ng.mL^{-1}$ for 10 µL injected) by microLC-MS/MS [27], Sporty *et al.* (3 $ng.mL^{-1}$ for 5 µL injected) by microLC-MS/MS, Carter *et al.* (0.96 $ng.mL^{-1}$ for 3 µL injected) by LC-MS/MS [26] and similar to those resulting from analysis of the nonapeptide by nanoLC-MS/MS in our previous work (0.15 $ng.mL^{-1}$ for 250 nL injected).

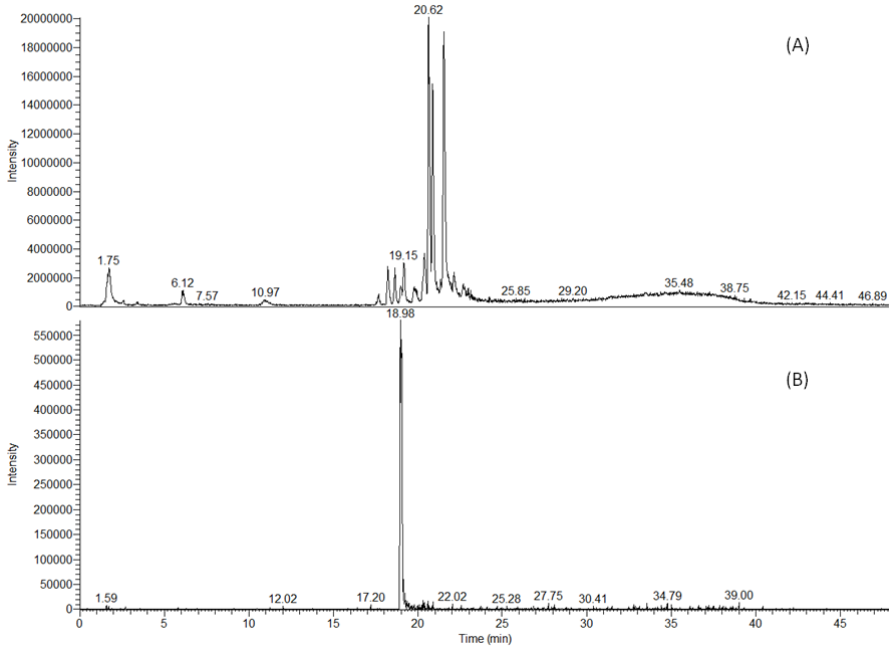

Fig. 4. Total ion chromatogram of the nonapeptide/cytochrome C digest mixture (A) and the extracted ion chromatogram of the nonapeptide FGESAGAAS (m/z 796.3) (B).

As previously mentioned, a trap column was needed to couple on-line the IMER (20 x 2 mm I.D) to the microLC/MS analysis. However, it was crucial to evaluate the breakthrough volume of the trap column (Hypersil Gold) to ensure that the totality of the nonapeptide resulting from the digestion on IMER could be retained on this support. The setup used to estimate the breakthrough volume was made of a six-port switching valve including a syringe pump containing the synthetic nonapeptide solution (5 $µg.mL^{-1}$ in formic acid, pH = 2.2) linked to the trap column

and to the mass spectrometer. The solution was injected at 50 µL.min^{-1} on the trap column and in order to avoid exceeding the column capacity, the nonapeptide solution was replaced after 16 min by a formic acid solution (pH = 2.2) for 40 min. In these conditions, the breakthrough volume was reached after 30 min, corresponding to 1.5 mL of formic acid. This volume is significantly higher than the 100 µL usually applied to recover peptides from off-line digestion of HuBuChE on IMER [16]. Thus, the target nonapeptide resulting from HuBuChE digestion on IMER could be retained on the trap column.

To conclude, a sensitive method was developed to trap, separate and detect the target nonapeptide by microLC-MS/MS and was applied to the detection of the nonapeptide resulting from the on-line digestion of HuBuChE on IMER.

3.3.2. Digestion of HuBuChE on IMER coupled on-line to microLC-MS/MS

In our previous studies, we showed that sepharose was the best performing support for enzyme immobilization and that among different amounts of pepsin grafted on sepharose, the highest off-line digestion yields of HuBuChE on IMER (20 x 2 mm I.D.) were obtained with IMERs grafted with 6.8 mg of pepsin, with digestion yields between 20% and 24% [16,19]. Thus, a similar IMER called IMER 1 (20 x 2 mm I.D.), synthesized by the grafting of 6.8 mg of pepsin on 35 mg of CNBr-activated sepharose, was chosen for on-line digestion on IMER, to allow comparison with off-line digestion. The grafting yield of pepsin on CNBr-sepharose, was evaluated to be 24% by BCA assay and was thus similar to previous results (22%) [16,19]. Hence, the good repeatability of the grafting process of pepsin on CNBr-activated sepharose was confirmed. A standard HuBuChE solution at 480 µg/mL (5 µL) was digested on-line on IMER 1 using the set-up depicted in **Fig. 1B**. After digestion by *stop flow* for 20 min, HuBuChE peptides were trapped in a precolumn and analyzed by microLC-MS/MS. The amount of nonapeptide obtained after digestion was quantified by following the MRM transitions *m/z* 796.3→691.3; 796.3→673.3 and 796.3→602.3. As shown in **Fig. 5**, the nonapeptide was observed with a retention time of 18.9 min, stating an efficient digestion of HuBuChE. The on-line digestion was repeated 3 times and the digestion yield of HuBuChE on IMER was 24 ± 2% which was similar to that obtained off-line: 24 ± 3% [16]. IMER synthesis and digestion were repeatable over time and high retention of HuBuChE peptides on the trap column was achieved. Therefore, IS could be coupled on-line to the IMER.

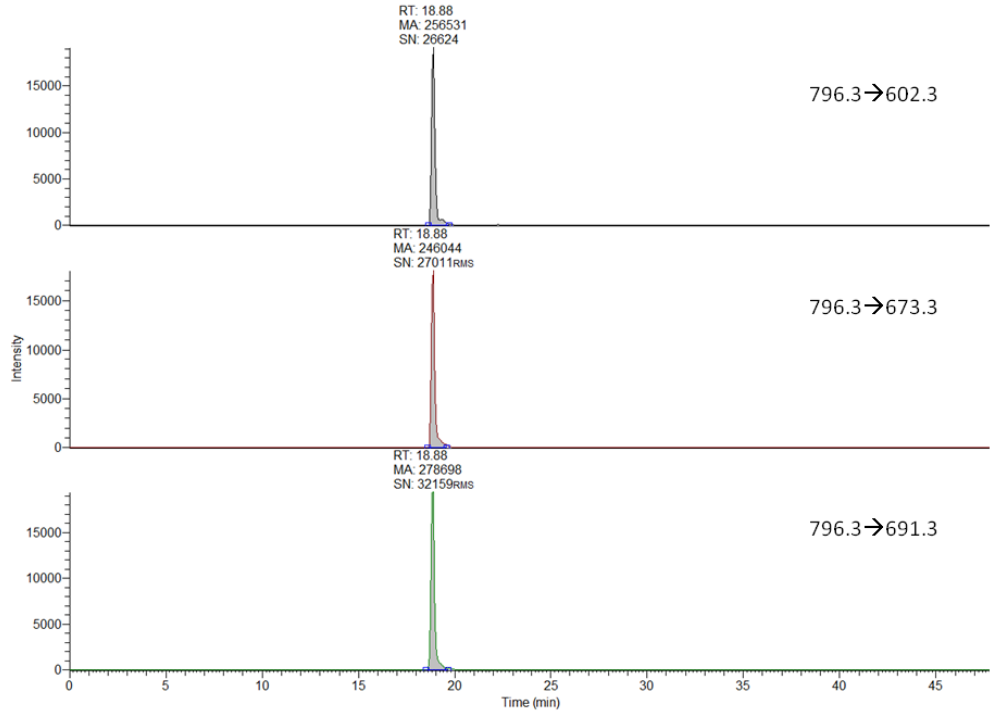

Fig. 5. MRM chromatograms (transitions 796→602, 796→673 and 796→691) of the target nonapeptide obtained after on-line digestion of 2.4 µg of HuBuChE on IMER 1 for 20 min and its analysis by microLC-MS/MS.

3.4. On-line coupling of immunosorbents to IMER and microLC-MS/MS

As with any coupling, many constraints had to be taken into account to realize efficient coupling of the immunoextraction step with enzymatic digestion on IMER. Contrary to the percolation and the washing steps, the elution step was crucial for this coupling. Indeed, the elution solution applied on IS had to be compatible with the solution used for the digestion on IMER, in terms of pH and volumes. The pH of the solution was not problematic in this coupling, as the solution chosen for HuBuChE elution and digestion was a formic acid solution pH = 2.2. Nevertheless, the elution volume of HuBuChE on IS was important to assess its compatibility with the IMER dwell volume (37 µL). The elution volume was previously estimated around 150 µL for the IS B2 18-5_{SP} and B2 18-5_{ER}. To digest the majority of HuBuChE eluted from the IS on IMER, many ways can be considered. HuBuChE can be eluted at a low flow rate through the IMER, or eluted by *stop flow* with a succession of small volumes, equal or lower than the dwell volume of the IMER. Because the dwell volume of IMER 1 (20 x 2 mm I.D) was equal to 37 µL, at least 4 digestions on IMER 1 were necessary to digest HuBuChE resulting from the elution step. To preserve a reasonable digestion step on IMER, it was essential to reduce the digestion time. For this, 2.4 µg of HuBuChE were diluted in 150 µL of formic acid to mimic the elution conditions in term of pH and volume, and digested on-line on IMER 1 by three different methods. The HuBuChE solution was consecutively digested by *stop flow* either for 4 x 5 min or 4 x 10 min or by continuous flow at 7.5 µL.min^{-1} for 20 min. The best digestion yields were obtained for the 4 x 10 min digestion, with digestion yields 2-fold higher than continuous flow digestion and 1.5 times

higher than the 4 x 5 min digestion. Considering that a 40 min digestion time was too long, a bigger size IMER, called IMER 2 (30 x 2.1 mm I.D) was synthesized by grafting 6.8 mg of pepsin on 58 mg of CNBr-activated sepharose to reduce the number of digestions. The volume to transfer HuBuChE from the loop to the IMER was adapted to the dimensions of the IMER 2 and set to 40 µL while 250 µL of formic acid pH 2.2 were used to transfer the peptides from IMER 2 to the trap column. Thanks to its higher dwell volume (62 µL), digestion of the HuBuChE solution on IMER 2 was completed by only two *stop flow* digestions of 10 min. The linearity of the HuBuChE digestion on IMER 2 was checked by digesting increasing amount of HuBuChE between 0.15 µg and 2.4 µg **(Fig. 6)**. The digestion yield of HuBuChE on IMER 2 was evaluated as 18% (RSD = 8%) and good linearity was observed. Considering that the amount of HuBuChE digested on IMER 2 was compatible with the amount of HuBuChE in 100 µL of plasma (0.4 µg), the IMER 2 was coupled on-line to B2 18-5_{SP} or B2 18-5_{ER} immunosorbents for the on-line immunoextraction and digestion of HuBuChE in plasma.

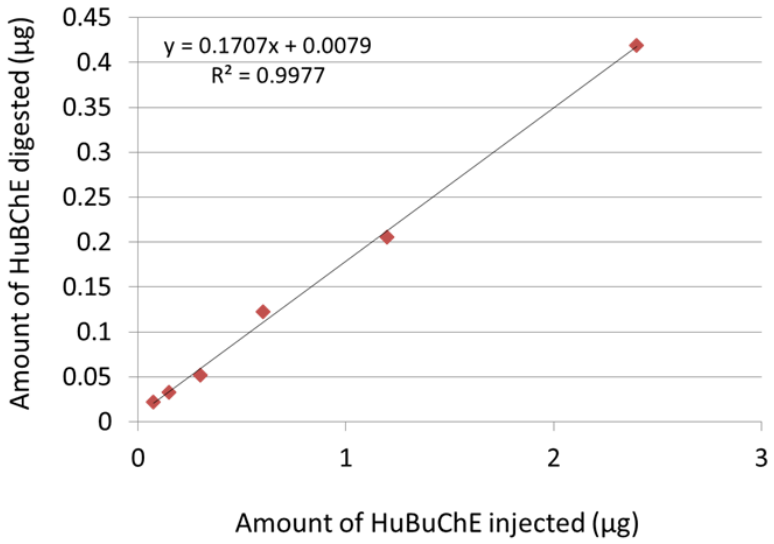

Fig. 6. Amount of HuBuChE digested on IMER 2 in 10 min depending on the amount of HuBuChE introduced.

3.5. Optimization of the immunoextraction procedure

First, selectivity of the whole analytical system was estimated by LC/MS, by following the presence of human albumin (UniProt accession P02768) after the immunoextraction and digestion steps. For that, 5 µL of a 200 µg.mL^{-1} commercial albumin solution was digested on IMER 2 for 10 min and the resulting peptides were analyzed by LC/MS in scan mode**.** Albumin peptides were recognized by MASCOT and the most intense peptide $[M+H]^+$ = 467.6 at 19.6 min, corresponding to the sequence of amino acids ^{351}LGMF^{354} of human albumin was chosen as qualifying ion. Then, the presence of this peptide after percolation of plasma on B2 18-5_{SP} and B2 18-5_{ER} immunosorbents was followed by SIM mode from 19.3 min to 21.7 min, while the MRM method was used to quantify HuBuChE between 0 min and 19.3 min. The albumin peak was observed at 19.6 min and it was estimated that more than 99.9% of albumin was removed after

immunoextraction using a 700 µL PBS buffer washing step, which confirmed the selectivity of the whole procedure. In order to decrease the duration of the immunoextraction step, washing steps with 500 µL and 300 µL of PBS buffer were tested. Nevertheless, an increase of non specific interactions by a factor of 2 was observed on each immunosorbent when using a 300 µL washing volume. Accordingly, the washing volume was kept at 700 µL.

3.6. On-line analysis of HuBuChE from plasma

A human plasma sample was diluted (1/1 v/v plasma/PBS) and 100 µL of this sample was directly percolated and washed through the immunosorbent B2 18-5_{SP} or B2 18-5_{ER} with 700 µL of PBS buffer pH = 7.4. HuBuChE was eluted from the immunosorbent with formic acid and digested on IMER 2 by *stop flow* for 2 x 10 min. HuBuChE peptides were transferred from the IMER to the precolumn with 250 µL of formic acid and analyzed by microLC-MS/MS. This procedure was carried out in triplicate for both immunosorbents. The MRM chromatograms of the nonapeptide resulting from the coupling of IS B2 18-5_{SP} to IMER 2 are shown in **Fig. 7.** The target nonapeptide was observed at 18.9 min with a S/N ratio higher than 10000 for the first two transitions and the LOQ was estimated to a few femtomoles. It confirmed the efficiency of the immunoextraction and digestion steps and the high sensitivity of the method. The amount of nonapeptide obtained after these two steps was evaluated thanks to the nonapeptide calibration curves described in **Section 3.3.1.** The total yield of the method, corresponding to this amount divided by the amount of nonapeptide expected for 100% extraction and digestion was evaluated considering a 4 µg.mL^{-1} medium concentration of HuBuChE in plasma. The coupling of IS B2 18-5_{ER} to IMER 2 led to a total yield of 8 ± 3% while a 42 ± 3% total yield was obtained for IS B2 18-5_{SP}. Thus, it appeared that sepharose was the most appropriate support for the coupling of the immunoextraction procedure to IMER 2. However the 42% yield was quite high compared to the theoretical expectations. Considering an 18% digestion yield of HuBuChE on IMER 2, it was not expected to get a total yield higher than this value, even with a 100% extraction yield. These variations between theoretical and experimental results can be explained by three phenomena: (i) as the concentration of HuBuChE in plasma is person dependent, the real concentration in plasma was somewhat higher than the 4 µg.mL^{-1} value used for quantification, (ii) matrix effect during LC-MS/MS analysis could have an impact on evaluation of the digestion yields (iii) the amount of commercial HuBuChE estimated by the supplier was overestimated because of contaminant proteins in the sample and it lowered the digestion yields on IMER 2. This third explanation was explored and confirmed by Oksana Lockridge with the evaluation of the purity of LeeBiosolution HuBuChE by SDS gel. According to O. Lockridge, 100 % pure HuBuChE, purified from Cohn paste by anion exchange chromatography and procainamide gel, has a specific activity of 500 U.mg^{-1}. HuBuChE used in this work having a specific activity of 200 U.mg^{-1} it was estimated that the protein was only 40 % pure. SDS gel experiment confirmed that and showed that LeeBiosolutions HuBuChE was contaminated with albumin and other proteins (data not shown). Taking into account that HuBuChE is only 42% pure, digestion yield of HuBuChE on IMER2 was re-evaluated to 45 ± 4 %. The total yields of the method being 42 ± 3% for IS B2 18-5_{SP}, it was estimated that B2 18-5 allowed to extract about 100 % of HuBuChE from plasma.

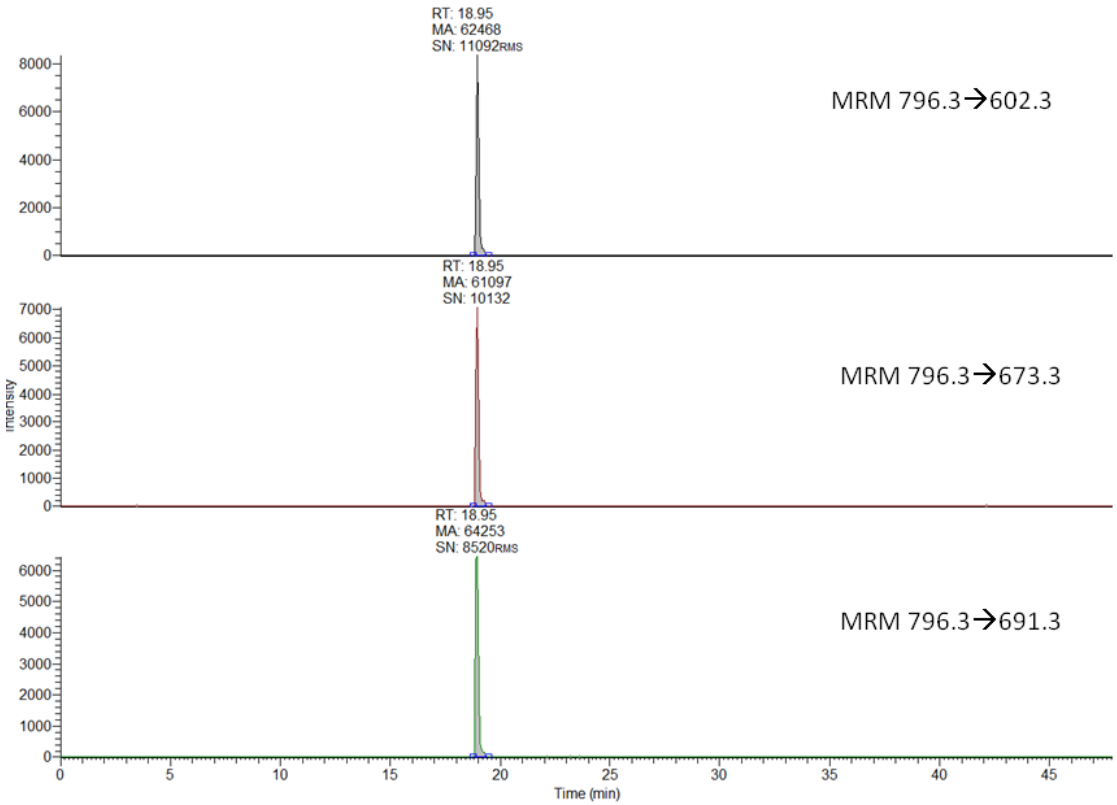

Fig. 7. MRM chromatograms of the nonapeptide FGESAGAAS obtained after on-line immunoextraction of HuBuChE from 50 µL plasma and digestion on IMER 2, followed by microLC-MS/MS analysis. The immunoextraction was performed in 14 min on IS B2 18-5$_{SP}$, the digestion on IMER 2 occurred in 20 min and the peptides were analyzed by mass spectrometry in MRM mode (transitions 796→602, 796→673 and 796→691).

Conclusions

An on-line analytical system including an immunoextraction step, a digestion step and a microLC-MS/MS analysis was developed for the detection of the target HuBuChE nonapeptide in plasma in less than 1 h. The quantification limit of the nonapeptide on this system was evaluated to a few femtomoles for the analysis of 50 µL of plasma (S/N > 10000) which would allow one to achieve quantification of the nonapeptide in a further reduced volume of plasma (< 10 µL). HuBuChE digestions were carried out on-line on reusable IMERs and were quantified by microLC-MS/MS. HuBuChE digestion yields of 45% were obtained on IMER (30 x 2.1 mm I.D.) after only 10 min digestion by stop flow and were repeatable over different HuBuChE amounts. Three immunosorbents were successfully prepared by covalent grafting of three anti-HuBuChE antibodies on CNBr-sepharose with grafting yields up to 98%. HuBuChE recovery was close to 100% for the best immunosorbent B2 18-5$_{SP}$ grafted with B2 18-5 antibodies. This immunosorbent was coupled on-line to an IMER and immunoextraction of HuBuChE was achieved in 14 min while digestion was performed in 20 min. The nonapeptide global recovery was higher than 42% for the B2 18-5$_{SP}$ immunosorbent with a RSD value lower than 7% (n = 3). Non-specific interactions on this set-up were evaluated by following the presence of the most intense albumin peptide

obtained after the percolation of plasma and it was estimated that more than 99.9% of albumin was removed during percolation and washing.

To conclude, the coupling of a selective immunoextraction step to a brief and repeatable digestion on IMER has allowed a rapid and sensitive on-line analysis of HuBuChE in a low volume of plasma and will be applied to the analysis of HuBuChE-OP adducts.

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Supplementary material

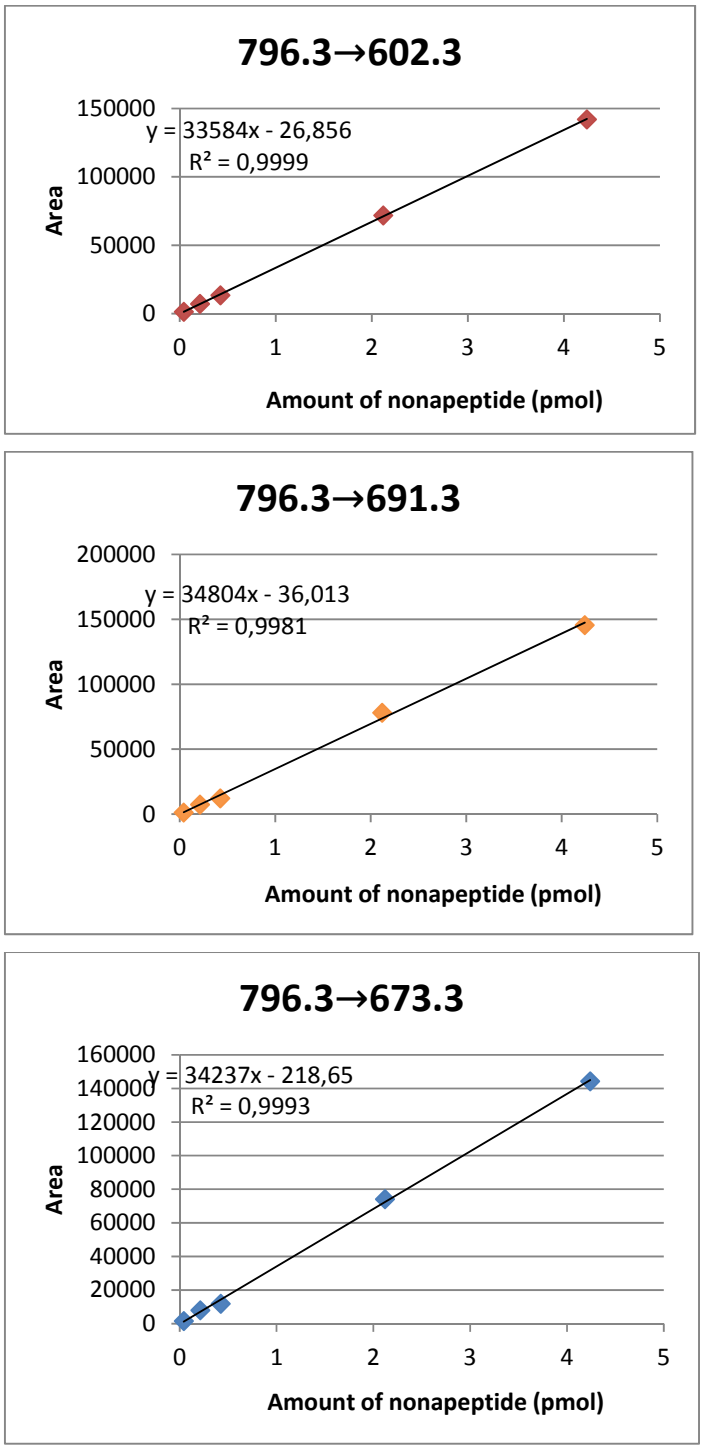

Fig. S1. Calibration curves obtained after the injection of various amounts of synthetic nonapeptide varying from 0.04 pmol (6.75 ng.mL^{-1}) to 4.24 pmol (675 ng.mL^{-1}) for the transitions *m/z* 796.3→673.3; 796.3→602.3 and 796.3→691.3.