Journal of Chromatography B, 878 (2010) 213–221

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Total on-line analysis of a target protein from plasma by immunoextraction, digestion and liquid chromatography–mass spectrometry☆

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ARTICLE INFO

Article history:
Received 25 February 2009
Accepted 27 July 2009
Available online 3 August 2009

Keywords:
Immunoextraction
Immobilized pepsin
On-line analysis
Protein analysis
LC–MS/MS

ABSTRACT

A total on-line analysis of a target protein from a plasma sample was made using a selective immunoextraction step coupled on-line to an immobilized enzymatic reactor (IMER) for the protein digestion followed by LC–MS/MS analysis. For the development of this device, cytochrome *c* was chosen as model protein due to its well-known sequence. An immunosorbent (IS) based on the covalent immobilization of anti-cytochrome *c* antibodies on a solid support was made and an immunoextraction procedure was carefully developed to assess a selective extraction of the target protein from plasma. For the first time, IS was easily coupled on-line with a laboratory-made IMER based on pepsin. The whole on-line device (IS-IMER-LC-MS/MS) allowed the quantification of cytochrome *c* from 8.5 pmol to 1.7 nmol in buffer medium. Finally, this device was applied to the analysis of only 85 pmol of cytochrome *c* from plasma with a RSD value lower than 10% ($n = 3$).

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1. Introduction

The proteomic community has paid increasing attention to the need for protein biomarkers discovery [1]. Biomarkers are used in a wide range of applications that range from diagnosis and prognosis of diseases to the monitoring of a biological response or therapeutic intervention. So, the analysis of a target protein acting as biomarker from biological samples is nowadays a real challenge for human health.

The proteins identification is commonly carried out by using liquid chromatography coupled with mass spectrometry (LC–MS) [2]. However, ionization by electrospray of large molecules produces a very complex envelope of multiply charged ions that requires sophisticated deconvolution analysis. So, a bottom-up approach is favoured by digesting the proteins to produce smaller peptide fragments, which are then separated and easily identified by LC–MS/MS [2,3]. Moreover, proteins of interest are usually part of a very complex mixture of other proteins and molecules that co-exist in the biological medium. It is estimated that the protein concentrations in biological samples span over 12 orders of magnitude. In plasma, only 22 proteins account for 99% of the plasma protein content and proteins of interest as biomarkers belong to the remaining 1% [4]. Unfortunately, by ionization of a such mixture by electrospray, the identification of the low-abundant proteins is generally hampered by the presence of the more abundant proteins such as albumin, transferrin, haptoglobulin, immunoglobulins, and lipoproteins [5]. Therefore, the aim of this study is the development of an automated method for the analysis of a target protein in a biological sample as plasma that integrates the possibility to remove other proteins which may interfere during the analysis. Immuno-based sample preparation methods are becoming increasingly popular biological tools by exploiting the high affinity of the antigen–antibody interaction. Indeed, immunoaffinity depletion columns containing immobilized antibodies against the high-abundant proteins are commercially available. However, they are very expensive and interfering proteins still remains after this pretreatment [6,7]. Another alternative is the use of immunosorbents (IS) based on the immobilization on a solid support of antibodies developed against a target analyte. Immunoextraction has been largely developed in our department, particularly for the selective trapping of small-size molecules [8–10]. Many examples have been also described for the immunoextraction of larges molecules as antibodies, enzymes, proteins or hormones with antibodies immobilized on various solid supports (agarose, synthetic organic supports, derivatized silica and glass) as reviewed by Hage [11] and Lee and Lee [12]. In most of the reported works, an enzymatic digestion is performed in solution after the selective immunoextraction step. However, the digestion in solution is time consuming [13,14], autoproteolysis of enzymes can occur and manual sample handling can cause a risk of sample contamination. To overcome these drawbacks, the proteolytic enzymes can be immobilized on a solid support and be integrated to the analytical system thus allowing the total automation of the analysis. As recently reviewed, immobilized enzymatic reactors (IMERs) were integrated into the analytical system and dedicated to proteins

☆ This paper is part of the special issue "Immunoaffinity Techniques in Analysis", T.M. Phillips (Guest Editor).

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doi:10.1016/j.jchromb.2009.07.032

analyses [15,16]. Trypsin is the most widely used enzyme in proteomic field because of its high selectivity of cleavage. Trypsin based IMERs were already successfully developed under various formats, i.e. pre-columns [17–23], membranes [24], capillaries [25–29] and disks [30–32]. Recently, our group highlighted the important effect of the immobilization support on the performances of the resulting IMER [33]. By this work, CNBr activated sepharose showed the best performances as immobilization support for a trypsin based IMER coupled on-line to LC/MS. Indeed, this hydrophilic support prevents non specific hydrophobic interactions with peptides and presents a good chemical stability thus allowing the reusability of this sorbent [33].

The aim of this present study was to combine an immunosorbent for the selective extraction of a target protein with an IMER and to integrate both devices on-line with LC–MS analysis. Until now, few research groups reported the use of an immunosorbent coupled on-line with an enzyme based IMER to ensure a total automated analysis of human hemoglobin proteins from mixture containing several different human plasma proteins [23] and of bovine serum albumin from undiluted human urine samples [34]. All these studies used a commercial trypsin immobilized reactor, Poroszyme® (Applied Biosystems). In addition, the on-line coupling between IS and IMER involved problems of solvent compatibility because protein are eluted at low pH from IS and trypsin cleaves at a slightly basic pH. Consequently, a decrease of the immobilized enzyme activity was unfortunately observed [34]. To overcome this drawback, the same group developed an on-line solution phase digestion [35,36]. The digestion took place in a reaction capillary serving as reaction coil by mixing a proteolytic solution with the LC effluent. However, a decrease of sensitivity of the mass spectrometer can be observed due to the presence of the enzyme and its autodigestion products. More recently, an application for the automated analysis of matrix metalloproteases in urine samples was developed [37]. The on-line procedure consisted of a selective enrichment step of only active form of enzymes by inhibitor affinity chromatography coupled to a digestion on trypsin IMER taking care that the elution buffer does not affect the digestion efficiency and followed by a preconcentration on a trap column and analysis by nano-LC–MS/MS. Taking into account our knowledge concerning the immobilization of biomolecules on solid supports [9], a digestion on immobilized enzymatic reactor was preferred over flow enzyme cleavage to overcome the autodigestion products. In addition, proteolytic enzyme acting under acidic conditions, i.e. pepsin, was chosen. Pepsin cleaves proteins at C-end of hydrophobic amino acids, such as phenylalanine and leucine residues [38]. In contrast to trypsin, only a few studies deal with the use of pepsin based IMER. To our knowledge, no coupling with IS was reported. Most of developed pepsin-based IMERs were followed by a capillary electrophoresis (CE) analysis [39,40]. Pepsin based IMERs were also associated with LC–MS analysis but the study was dedicated to protein dynamics [41,42]. More recently, proteins such as hemoglobin, casein, albumin and myoglobin [43,44] were digested on-line by pepsin IMER followed of a LC–MS/MS analysis. However, proteins were only analyzed in pure samples without selective pretreatment.

For the first time, an immunosorbent was in this study coupled on-line with a pepsin based IMER to provide a total automated analysis of a target protein from a spiked plasma. A carefully attention was given for the solvent compatibility between these various steps to keep a simple device. For this, all the development was made with a model protein which its sequence is well known, cytochrome *c*. The aim of this work was to show the feasibility of such a method. By the previous results obtained for the development of the trypsin based IMER [33], CNBr activated sepharose was chosen as immobilization support. The resulting IMER was then packed in a pre-column and coupled on-line to the chromatographic system.

Concerning the IS, CNBr activated sepharose was also chosen for the immobilization of the anti-cytochrome *c* antibodies. Firstly, immunosorbent was characterized and a selective extraction procedure was developed. In a second part, IS was integrated to the analytical system including the pepsin based IMER on-line coupled with the chromatographic separation. This automated procedure was evaluated in real medium with a plasma sample.

To provide a detailed evaluation of the IMER and to optimize the on-line coupling, a tool of quantification was used. Here two specific peptide fragments of cytochrome *c* were monitored. The relationship between the peak areas of selected peptides and the amount of the protein was used to quantify the protein and then to measure the recovery yield of the whole procedure including the selective pretreatment.

2. Material and methods

2.1. Reagents

Pepsin from porcine gastric mucosa (EC 3.4.23), myoglobin, bovine serum albumin and horse heart cytochrome *c* were purchased from Sigma (Sigma–Aldrich, Saint Quentin Fallavier, France). Mouse monoclonal anti-horse cytochrome *c* antibodies were purchased from Interchim (Montluçon, France). Human plasma was provided by EFS (Strasbourg, France).

Sodium acetate (CH_3CO_2Na), sodium hydrogen phosphate (Na_2HPO_4), potassium hydrogen phosphate (K_2HPO_4), Trizma hydrochloride ($NH_2C(CH_2OH)_3$, HCl), Trizma base ($NH_2C(CH_2OH)_3$), sodium azide (NaN_3) and sodium chloride (NaCl) were also purchased from Sigma. Potassium dihydrogen phosphate (KH_2PO_4), sodium bicarbonate ($NaHCO_3$), calcium chloride ($CaCl_2$), acetic acid (CH_3COOH), formic acid (HCOOH), glycine and acetonitrile (MeCN) were purchased from VWR (Fontenay-sous-Bois, France). Ultrapure water was obtained from a MilliQ water purification system and Millex-HV 0.45 μm filters were used for plasma sample preparation (Millipore, St Quentin en Yvelines, France). Cyanogen bromide-activated-Sepharose 4B (Seph-CNBr) used to immobilize pepsin and antibodies was purchased from Sigma.

The phosphate-buffer solution (PBS) consisted of a 0.01 mol L^{-1} phosphate buffer (pH 7.4) containing 0.15 mol L^{-1} of NaCl. The PBS-azide solution is a solution of PBS with 0.02% (w/w) of NaN_3. Myoglobin, albumin and cytochrome *c* solutions were prepared in a saline Tris buffer (Trizma hydrochloride and Trizma base, 50 mmol L^{-1} pH 8 and 10 mmol L^{-1} of $CaCl_2$). The anti-cytochrome *c* antibodies solution was prepared in PBS.

2.2. Apparatus and HPLC-MS analysis

The cytochrome *c* analysis was performed on-line with the system set-up depicted on Fig. 1. This device was composed of four six-port switching valves. The first one was connected to the injection loop (20 μL) and to an isocratic pre-concentration pump (LC-10AS, Shimadzu, Champs sur Marne, France). The second one was connected with the IS anti-cytochrome *c* packed in a pre-column (20 × 2 mm I.D. or 1 mm I.D., CIL, Ste Foy la Grande, France). The third one was connected with the pepsin IMER packed in a pre-column (20 × 2 mm I.D., CIL) and placed in an oven set at 37 °C (Crococil oven, CIL). As ISs and pepsin IMERs are no pressure resistant sorbents, the on-line coupling to the LC analytical column was ensured by an intermediary trapping pre-column containing a polymeric reversed phase (PRP-1, 20 × 2.3 mm I.D., 2 μm, Hamilton, Switzerland) connected with the fourth switching valve. The backpressure generated by this system was not over 5 bars. To perform the chromatographic analysis, this column switching set-up

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