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Selective quantitative bioanalysis of proteins in biological fluids by on-line immunoaffinity chromatography–protein digestion–liquid chromatography–mass spectrometry

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Abstract

A quantitative method for the determination of proteins in complex biological matrices has been developed based on the selectivity of antibodies for sample purification followed by proteolytic digestion and quantitative mass spectrometry. An immunosorbent of polyclonal anti-bovine serum albumin (BSA) antibodies immobilized on CNBR agarose is used in the on-line mode for selective sample pretreatment. Next, the purified sample is trypsin digested to obtain protein specific peptide markers. Subsequent analysis of the peptide mixture using a desalination procedure and a separation step coupled, on-line to an ion-trap mass spectrometer, reveals that this method enables selective determination of proteins in biological matrices like diluted human plasma. This approach enhances substantially the selectivity compared to common quantitative analysis executed with immunoassays and colorimetry, fluorimetry or luminescence detection. Hyphenation of the immunoaffinity chromatography with on-line digestion and chromatography–mass spectrometry is performed and a completely on-line quantification of the model protein BSA in bovine and human urine was established. A detection limit of 170 nmol/l and a quantification limit of 280 nmol/l is obtained using 50 μl of either standard or spiked biological matrix. The model system allows fully automated absolute quantitative mass spectrometric analysis of intact proteins in biological matrices without time-consuming labeling procedures.

Keywords: Immunoaffinity; On-line analysis; Trypsin digestion; Protein; Peptide; Electrospray; Quantification; LC–MS

1. Introduction

The quantitative bioanalysis of protein drugs in biological matrices is an important issue in early stage drug development. Different approaches for the absolute quantitative analysis of proteins in biological fluids have been reviewed by Hopfgartner and Varesio [1]. They emphasize that mass spectrometry (MS) has great potential for quantification of peptides and proteins in the off-line mode. Other groups used a stable isotope-incorporated peptide as internal standard for the absolute quantification of proteins and peptides [2,3]. Barnidge et al. [4] evaluated the use of tryptic cleavage sites incorporated into an internal standard synthetic peptide. Storme et al. [5] performed absolute quantification of Cystatin C by analysis of tryptic peptide markers. Application of this approach is limited by laborious sample pre-treatment to remove biological matrices which has not been described in this work. Nelson et al. [6] used MALDI-MS for the quantification of proteins comprising an isotopically labeled internal standard. ESI-MS for the quantification of relatively small protein comprising a similar protein as internal standard derived from a different species that is mass shifted has been performed by Ji et al. [7]. Garbis et al. [8] amplify in their review the drawbacks of protein quantification procedures using MS. Generally, the procedures applied in the quantification of proteins are significantly different from those applied for the bioanalysis of small drug molecules. In fact, enzyme immunoassays are frequently used for the quantification of proteins in biological matrices [9,10], while combined liquid

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chromatography tandem mass spectrometry (LC–MS/MS) and selected-reaction monitoring (SRM) is the method-of-choice for small molecules [11]. In enzyme immunoassays, which are considered to be selective, detection of protein drugs in, e.g., serum or plasma is usually performed using colorimetry, fluorescence or luminescence. However, these detection methods do not supply additional information on the target measured, and therefore strongly rely on the selectivity of the immunoassay. In this report, the development of an alternative approach for the quantitative bioanalysis of proteins is described. The fully automated method is based on an on-line coupling of immunoaffinity chromatography, proteolytic digestion, and LC–MS. In this way, absolute quantitation of the protein drug can be achieved with improved selectivity and certainty of identity.

Immunoaffinity chromatography (IAC) has frequently been used for selective sample purification, both off-line and on-line to the subsequent LC [12–15] separation. This approach was reviewed by Hennion and Pichon [16]. While in off-line enzyme immunoassays for protein drugs, often two or more antibody–antigen interactions are needed for capturing and detection [10], in IAC only one type of immobilized antibody is needed. The applicability of IAC as a selective sample-pretreatment method for protein drugs strongly depends on the ability to immobilize appropriate antibodies against the target protein drugs onto a chromatographic support and to pack IAC columns with sufficient efficiency. The IAC procedure comprises two separate steps: capturing of the analyte from the biological matrix and washing interferences away, and desorption of the captured analytes towards the next stage of analysis.

The on-line coupling of IAC to LC–MS was pioneered by Rule et al. [17,18] for the determination of small molecules, such as carbofuran in environmental samples. On-line IAC–LC and IAC–LC–MS are frequently performed via a short solid-phase extraction (SPE) trapping column to concentrate the eluate from the IAC column prior to injection into the LC. An eluent composition switch can be achieved in this stage as well, enabling the use of optimal eluent in both IAC and LC. The latter is especially important in LC–MS which poses stringent restrictions to the mobile-phase composition. In this case, SPE is used for the pre-concentration of peptides from the protein digest step (see below).

Performing quantitative bioanalysis of proteins by LC–MS results in additional challenges. First of all it should be emphasized that the procedure developed aims at absolute protein quantitation, thereby excluding the use of the elegant isotope-coded affinity-tag labeling (ICAT) currently frequently applied for relative quantitation in proteomics studies [19,20]. Challenges in the absolute quantitation of protein drugs are related to the need to achieve an efficient LC analysis of proteins and problems due to the formation of an ion envelope of multiply-charged ions in electrospray ionization-MS (ESI-MS). Although quantitative LC–MS analysis of a 10 kDa peptide using an internal standard was described by Ji et al. [21], this procedure seems to show various limitations. The relative abundance of the peaks in the ion envelope may be a delicate function of experimental parameters, which may hamper reliable quantitation [22]. In order to avoid such problems, an on-line proteolytic digestion on an immobilized trypsin column has been applied in this study [23,24]. For the resulting peptide mixture, simpler and more efficient chromatographic procedures can be applied and the peptide mass spectra are significantly less complex than the protein mass spectra.

In order to enable the use of the on-line trypsin column, two issues have to be addressed. First, the pH of the solution used to elute the analytes from the IAC column must be modified to comply with the conditions required for the protein digestion [25]. Secondly, after the digestion which is performed in a phosphate buffer, an SPE trapping column has to be applied to concentrate the eluate from the trypsin column, to desalinate the sample, and to switch from a phosphate to a formic acid containing eluent which is compatible with LC–MS [26].

Finally, LC–MS is applied to separate and detect the peptides from the mixture. The peptide map acquired in this way can in principle be used to confirm the identity of the protein, e.g., via database searching using Mascot software (Matrix Science Inc.), and/or via additional MS–MS or MS^n experiments [27]. More importantly, the peak areas of selected peptide fragments can, after adequate validation, be applied to quantify the amount of protein initially introduced into the system.

This paper describes the development of this hyphenated method. Initially, various steps of the procedure were separately developed and optimized. Preliminary evaluation and validation was performed for a system comprising IAC, off-line protein digestion, and SPE–LC–MS analysis of the digested protein samples, enabling the overnight analysis of different batches of samples, running IAC, protein digestion, and SPE–LC–MS in parallel. However, especially the off-line digestion step is a very time-consuming procedure requiring some manual sample and reagent manipulation. Finally, hyphenation of IAC and SPE–LC–MS via the on-column trypsin digestion was achieved.

2. Experimental

2.1. Reagents

Sodium di-hydrogen phosphate and glycine hydrochloride were purchased from Sigma (St. Louis, MO, USA). Disodium hydrogenphosphate and bovine serum albumin (purity 97%, HPCE) were obtained from Fluka (Buchs, Switzerland) and sodium chloride and formic acid came from Riedel-de-Haën (Seelze, Germany). Acetonitrile and hydrochloric acid (36–38%) were supplied by J.T. Baker (Deventer, The Netherlands). Water was produced by a Millipore (Bedford, MA, USA) Milli-Q unit. Sodium hydroxide was obtained from Merck (Darmstadt, Germany). Proteins were digested using sequencing-grade modified trypsin (Promega, Madison, WI). A 2 l stock of a 10-fold concentrated PBS buffer was made by dissolving 57.30 g of $Na_2HPO_4{\cdot}12H_2O$, 137.99 g of NaH_2PO_4, 175.30 g NaCl and 4.03 g KCl in 2 l of water. This stock was used to prepare the PBS by diluting 100 ml of the concentrate with 900 ml of water. The pH was brought to the required value with 8 M sodium hydroxide or hydrochoric acid.