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Evaluation of affinity-based serum clean-up in mass spectrometric analysis: Plastic vs monoclonal antibodies

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ABSTRACT

Mass spectrometric assays are now of great relevance for trace compound analysis in complex matrices such as serum and plasma samples. Especially in the quantification of low abundant protein-biomarkers, the choice of the sample preparation is crucial. In the present paper immunocapture and Molecular Imprinted Polymers (MIPs) have been applied in the determination of pro-gastrin-releasing peptide, a Small Cell Lung Cancer marker. These affinity-based techniques were compared in terms of matrix effect, limits of detection, repeatability and extraction specificity. In addition, protein precipitation was included for comparison as it is a typical sample preparation method of biological matrices. The results highlighted differences in the methods' performance and specificity, strongly affecting the outcome of the mass spectrometric determination. Plastic and monoclonal antibodies confirmed to be sensitive and specific sample preparations able to determine ProGRP at clinical relevant concentration, although only the use of monoclonal antibodies allowed the reliable quantification of ProGRP at reference levels (8 pM). In addition better insight in the specificity of the three sample preparation techniques was gained. This might also be of interest for other biological applications.

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1. Introduction

Serum and plasma are the foremost studied matrices in clinical proteomics for the identification and quantification of protein biomarkers as results of cells secretions and tissues outflows [1]. For clinical quantification of protein biomarkers as alternative to immunoassays for serum and plasma samples more and more tandem mass spectrometry-based methods have been developed [2]. However, the bottom-up approach, which provides tryptic digestion and peptide determination in the mass detector, encounters many challenges for the quantification of low abundant protein biomarkers, due to the large dynamic range of plasma protein concentration [3]. Depletion of the most abundant proteins such as serum albumin and enrichment of the proteins expressed in very low concentration are thus required for sensitive and reliable biomarker quantification.

Among many techniques, immunoaffinity enrichment coupled to tandem mass spectrometry has been demonstrated to be a successful strategy for the quantification of several low-abundant proteins [4]. The use of monoclonal antibodies for targeting the protein of interest assures indeed the required specificity to capture the biomarker to be quantified, allowing the development of high-throughput and sensitive assays. Among different criticisms about appropriate specificity and robustness, the cost of monoclonal antibodies is the main drawback to be faced when developing a sensitive, robust and cost-sustainable method for the clinical routine [5].

A recent alternative to antibodies has been established by Molecularly Imprinted Polymers (MIPs) [8,9]. These polymers behave as receptors by recognizing, binding and releasing, under determined conditions, targeted molecules which they are developed for [10]. Widely employed for the extraction of small molecules in solid phase extraction (SPE) of food, environmental and biological matrices [11], MIPs selective for peptides have lately been developed for their implementation in proteomic workflows [12–15].

In contrast to the affinity-based sample preparations, simple and inexpensive protein precipitation is widely applied for the depletion of high-abundant proteins in biological matrices [6]. Despite being roughly unspecific and thus leading to extract of variable protein composition, protein precipitation offers the advantage to be easily tunable by using a wide range of solvents [7].

All the aforementioned techniques have been integrated in tandem mass spectrometry assays and employed for the target

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determination of a very low abundant protein biomarker for Small Cell Lung Cancer, pro-gastrin-releasing peptide (ProGRP) [16–18]. Immunocapture enrichment of the entire ProGRP followed by trypsination and injection of the sample into the chromatographic system coupled to the triple quadrupole (LC–MS/MS) allowed a sensitive assay which could differentiate between ProGRP isoforms and could discriminate between samples from healthy and patient donors, achieving limit of detection below the reference concentration of ProGRP in serum samples (7.6 pM) [16]. MIP selective for the signature peptide of ProGRP (NLLGLIEAK) were employed for the off-line extraction of digested spiked serum samples [18]. Extracts were analyzed by the LC–MS/MS assay previously developed [16]. The method resulted in a fast sample preparation with the achievement of a detection limit in the higher pM range (625 pM). This insufficient detection limit was overcome by using MIP selective for NLLGLIEAK in an on-line setting which notably improved limit of detection (17 pM) and reduced sample volume consumption [manuscript submitted]. The protein precipitation method provided the simple depletion of abundant proteins by cold acetonitrile [17]. The extracts were digested and injected into the MS via a restricted access material column for on-line clean-up and although the method was not able to determine concentrations close to the reference level of ProGRP, it showed limit of detection ranging between 130 and 190 pM.

In this paper we wanted to benchmark affinity-based sample preparations such as immunocapture and on-line MIP extraction, and compare it to the traditional protein precipitation by the analysis of 6 different lots of serum spiked with ProGRP for the comparison of the methods' performances in terms of ion suppression, sensitivity and specificity of the enrichment. The applicability of these techniques to real clinical settings has also been discussed by considering cost- and time-effectiveness of the methods together with sample consumption required in each technique and the availability of the chemicals employed.

2. Materials and methods

2.1. Reagents and standards

Recombinant short ProGRP (AA 31–98) was cloned from human cDNA (OriGene Technologies, Rockville, MD, US), expressed in *Escherichia coli* (Promega, Madison, WI, US) using pGEX-6P-3 constructs (GE Healthcare, Oslo, Norway) and purified as described elsewhere [19]. ProGRP concentration was assessed by absorbance at 280 nm and then diluted to the desired concentration with 50 mM ammonium bicarbonate (ABC) buffer solution and stored at −20 °C. Internal standard (IS) NLLGLIEA[K_$^{13}C_6{}^{15}N_2$], AQUA Peptide with purity above 95% (Sigma-Aldrich) was diluted according to the Custom AQUA Peptides Storage and Handling Guidelines by Sigma-Aldrich and stored at −20 °C. TPCK-treated lyophilized trypsin from bovine pancreas was sequencing grade (Sigma-Aldrich). All other chemicals used were of analytical grade.

2.2. Serum samples and spiked serum samples

Six different lots of human serum were obtained from Ullevål Hospital (Oslo, Norway), and stored at −30 °C. Spiked serum samples were obtained by the addition of short ProGRP (AA 31–98) to serum for a final concentration of 2 and 20 nM and then vortexed for 30 s before the sample preparation.

2.3. Sample preparation

2.3.1. Sample preparation by immunocapture

Spiked serum samples (1000 μL or 50 μL diluted to 500 μL with ABC 50 mM) were treated as described by Torsetnes et al. [16] by the addition of 20 μL of prewashed antibody-coated magnetic beads (Dynabeads M280 Tosylactivated, Life Technology, Invitrogen Dynal, Oslo, Norway), prepared as described elsewhere [20,21]. The samples were then rotated and shaken for 1 h on a HulaMixer (Invitrogen) to facilitate the antigen-antibody interaction. The tubes were then placed in the magnetic rack (DynaMag-2 from Invitrogen) for the focalization of the beads and removal of the solution. The beads were then washed by 500 μL of Phosphate-buffered saline (PBS) containing 0.05% Tween 20, followed by 500 μL of PBS, 300 μL of Tris(hydroxymethyl)aminomethane chlorohydrate (Tris-HCl) (pH 7.4) and by 300 μL of 50 mM ABC-buffer. A volume of 90 μL freshly prepared 50 mM ABC buffer was added to the tubes containing the beads before the digestion was initiated by adding 10 μL freshly prepared trypsin to give an enzyme-to-antibody ratio of 1:5 (w/w). Samples were incubated over night at 800 rpm at 37 °C. Next morning the eppendorf tubes were briefly centrifuged (Centrifuge model 5804, Eppendorf) and placed in the magnetic rack. The solution of tryptic peptides was transferred to new Protein LoBind tubes, centrifuged at 5000 rpm for 2 min to sediment remaining trace particles. 50 μL of the supernatant was then transferred to vials for LC–MS/MS analysis.

2.3.2. Sample preparation by molecularly imprinted polymers

50 μL of serum sample were protein precipitated and digested as described below. The MIP extraction was performed on-line to the LC–MS/MS system by using *N*-(2-aminoethyl)methacrylamide hydrochloride and DVB-based MIP imprinted with the template Z-NLLGLIEA[Nle] (University of Strathclyde) and packed into trap column (1.4 × 5 mm G & T Septech, Norway) [manuscript submitted]. 20 μL of digested sample was loaded by an ISO-3100-A loading pump into the MIP trap column, using 20 mM formic acid (FA) with an isocratic flow (30 μL/min) directed to the waste via the MIP cartridge. After 10 min, the system was switched in order to forward-flush the content of the MIP cartridge to the analytical column and subsequently to the MS detector as following described.

2.3.3. Sample preparation by protein precipitation

The protocol used was previously described by Winther et al. [17] and provided the addition of cold acetonitrile (MeCN) (−32 °C) to the serum samples in a ratio of 1:1 (v/v) with subsequent vortexing for 1 min 1000 μL or 50 μL of spiked samples were then centrifuged at 10000 rpm and the supernatant was evaporated under nitrogen stream at 50 °C to dryness. 50 μL of ABC 50 mM was used to reconstitute the samples and digestion was consequently performed at 37 °C overnight by adding trypsin using an enzyme-to-protein ratio of 1:40 (w/w). Initial serum volumes considered were 1000 μL and 50 μL.

2.4. LC–MS analysis

2.4.1. LC-TSQ method for analysis of spiked serum samples

The LC–MS/MS analysis was performed using a triple quadrupole mass spectrometer according to the established MS assay for ProGRP analysis [16]. The chromatographic system consisted of LPG–3400 M pump with degasser, WPS-3000TRS autosampler, and FLM-3300, MIC flow-manager (all Dionex, Sunnyvale, CA, USA). The LC system was controlled by Chromeleon v. 6.80 SR6 (Dionex). The chromatographic separation was carried out by using Hypersil GOLD aQ, analytical column (Thermo Scientific, 100 Å, 3 μm, 1 × 50 mm) preceded by a Hypersil GOLD aQ Drop-In Guard Cartridge (Thermo Scientific, 100 Å, 3 μm, 1 × 10 mm)

The chromatographic separation for samples pretreated with immunocapture and protein precipitation was performed by loading 20 μL of sample with mobile phase A (20 mM FA: MeCN 95:5, v/v) and eluting with a 30 min linear gradient from 1 to 85% of mobile phase B (20 mM FA: MeCN 5:95, v/v). After gradient run the

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