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Introducing plasma/serum glycodepletion for the targeted proteomics analysis of cytolysis biomarkers

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ABSTRACT

A major class of clinical biomarkers is constituted of intracellular proteins which are leaking into the blood following ischemia, exposure to toxic xenobiotics or mechanical aggression. Their ectopic presence in plasma/serum is an indicator of tissue damage and raises a warning signal. These proteins, referred to as cytolysis biomarkers, are generally of cytoplasmic origin and as such, are devoid of glycosylation. In contrast, most plasma/serum proteins originate from the hepatic secretory pathway and are heavily glycosylated (at the exception of albumin). Recent advances in targeted proteomics have supported the parallelized evaluation of new blood biomarkers. However, these analytical methods must be combined with prefractionation strategies that reduce the complexity of plasma/serum matrix. In this article, we present the glycodepletion method, which reverses the hydrazide-based glycocapture concept to remove plasma/serum glycoproteins from plasma/serum matrix and facilitates the detection of cytolysis biomarkers. Glycodepletion was integrated to a targeted proteomics pipeline to evaluate 4 liver cytolysis biomarker candidates in the context of acetaminophen-induced acute hepatitis.

1. Introduction

Biomarkers are biological parameters which are measured to characterize a physiological or pathological state, the evolution of a disease, or a response to treatment. Several types of biomarkers are routinely exploited in clinical laboratories: small molecules (vitamins, hormones, etc.), nucleic acids and proteins. In the category of protein biomarkers, a study performed by Anderson in 2010 [1] listed over 200 protein analytes used in clinical biology, 109 of which have been cleared or approved by the American Food and Drug Administration (FDA). These biomarkers can be divided between the following classes: immunoglobulins, ligands, proteins with a function in plasma, abnormal secretions, and cytolysis biomarkers. Cytolysis biomarkers are defined as intracellular proteins which are released into the extracellular medium following cellular necrosis and an inflammation, which increases vascular permeability. The presence of these proteins in the blood is indicative of tissue damage due to ischemia, exposure to xenobiotics or mechanical trauma [2].

The vast majority of protein biomarkers are assayed in plasma or serum samples. In biochemical terms, these two biological matrices are immensely complex and have a very wide dynamic range. Thus, plasma (and serum) contains more than 2000 proteins, but the 20 major proteins represent over 99% of the total protein mass [3]. Among these major proteins, albumin (50 mg/mL) and immunoglobulins (17 mg/mL) alone represent 90% of the total proteins. Because these proteins are so highly represented, the plasma concentrations of all the proteins in plasma/serum varies by over ten orders of magnitude (10^{10}) [4]. This extremely broad dynamic range is a real challenge when analyzing biomarkers, as these proteins are generally present in medium- or low-abundance in the matrix.

Abbreviations: ADH4, alcohol dehydrogenase 4; ALT1, alanine aminotransferase 1; BHMT, betaine-homocysteine S-methyltransferase; FABP1, fatty acid binding protein 1; PRM, Parallel Reaction Monitoring; SRM, Selected Reaction Monitoring; PSAQ, Protein Standard Absolute Quantification

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In routine clinical applications, protein biomarkers are generally assayed by immunological methods based on antigen/antibody interactions or enzymatic tests. These assays have several advantages, including their sensitivity, simplicity of use, rapidity, low cost, and they are easily automated. However, during the process of developing new biomarkers, multiple candidates must be assessed, this can be done most effectively using liquid chromatography (LC) analysis and mass spectrometry in targeted mode, by Selected Reaction Monitoring (SRM) [5] or Parallel Reaction Monitoring (PRM) [6]. These analytical methods have an unparalleled multiplexing capacity, and thus allow several tens of target proteins to be simultaneously assayed in clinical samples without systematically requiring the development of antibodies. In addition, because peptides produced by digestion of target proteins can be selectively analyzed, these approaches are highly specific and can distinguish between protein isoforms [7,8]. However, the concentrations of most biomarker candidates in plasma/serum (from µg/mL to pg/mL) are beyond the dynamic range of these analysis methods (10^5). Therefore, these approaches must be combined with upstream biochemical steps to prepare samples for analysis [9]. This pre-analytical step aims to accomplish the following two objectives: (i) to make it possible to detect candidate biomarkers by enriching them or by reducing the dynamic range and complexity of the matrix and (ii) to digest the proteins into peptides, some of which (signature peptides) will be analyzed as "surrogate proteins".

Various biochemical strategies are currently employed to reduce serum/plasma protein complexity and dynamic range [9–11]. Among these strategies, a very popular and straightforward approach consists in depleting highly abundant proteins using immunoaffinity devices (resin, spin or HPLC columns) [12]. This method significantly reduces the dynamic range of protein concentrations, thus improving detection of medium- and low-abundance proteins. However, this approach only eliminates a limited number of proteins from plasma or serum (in general between 1 and 20 proteins) and it is expensive when processing numerous sample or volumes exceeding 20 µL [9]. Another limitation of this method is that proteins which have formed complexes with the captured abundant proteins will also be eliminated [13]. This scenario becomes problematic if a candidate biomarker interacts with the major proteins in serum or plasma, as its bound fraction will be retained on the depletion device resulting in reduced amounts available for MS analysis.

In this study, we have developed a novel method for the preparation of serum and plasma samples. This method is specifically adapted for proteomics analysis of cytolysis biomarkers (candidate or validated). The protocol is based on the depletion of plasma or serum glycoproteins (glycodepletion) to facilitate access to non-glycosylated proteins such as cytolysis biomarkers. Following optimization of the plasma glycodepletion procedure, a proteomics analysis pipeline involving LC-SRM analysis was optimized and applied for the assessment of three novel candidate biomarkers of liver cytolysis.

2. Material and methods

2.1. Biological samples

Plasma and serum glycodepletion was optimized using human plasma and human serum purchased from Sigma Aldrich (Saint Quentin Fallavier, France). Serum samples from patients with acetaminophen-induced acute hepatitis were collected at the Hepatology Department at Paul-Brousse Hospital (Villejuif, France) within the first 48 h after hospital admission. Samples were supplied by the Biological Resource Centre of the Paris-Sud Faculty of Medicine, France (approval number: 2011/39938). Research using blood samples was approved by the institutional review board of Paul-Brousse Hospital (Villejuif, France). All the patients provided written informed consent for participation. The French Blood Service (Etablissement Français du Sang, La Tronche, France) provided anonymous serum samples from healthy donors. Serum samples were collected in non-treated tubes (BD Biosciences, Le Pont de Claix, France) and were centrifuged at 1000*g* for 15 min to obtain serum supernatant. Serum samples were aliquoted and frozen at −80 °C for proteomics analysis.

2.2. Recombinant proteins

Recombinant ADH4, ALT1, BHMT and FABP1 proteins were obtained from Abcam (references ab132569, ab113862, ab51280 and ab82994 respectively). Isotopically-labeled recombinant protein analogs (PSAQ standards) for these four proteins were synthesized using cell-free expression (RTS 500 Proteomaster E. coli HY kit, 5 Prime, Hamburg, Germany) in the presence of [$^{13}C_6$, $^{15}N_2$] L-lysine and [$^{13}C_6$, $^{15}N_4$] L-arginine (Eurisotop, Saint-Aubin, France) as previously described [14]. Production was scaled-up at Promise Advanced Proteomics (Grenoble, France). PSAQ standards were checked for isotope incorporation (> 99%) and were quantified by amino acid analysis [15].

2.3. Glycodepletion

Plasma or serum samples (18 µL) were prepared using the GlycoLink Immobilization Kit (Life Technologies, Villebon-sur-Yvette, France) according to the manufacturer's instructions. Plasma/serum glycoproteins were oxidized by 0.1 M meta-periodate. Then, samples were loaded on spin columns filled with hydrazide-activated resin. Unbound proteins were eluted by centrifugation using 400 µL of urea 8 M. Eluates were concentrated to 50 µL using 3000 Da cut-off ultrafiltration devices before digestion.

2.4. Depletion of abundant proteins

Plasma or serum samples (14 µL) were depleted of the six most abundant proteins using the Affinity Removal System (MARS) Hu-6 spin cartridge (Agilent Technologies, Les Ulis, France) according to the manufacturer's instructions. Depleted samples were concentrated to 50 µL and buffer was exchanged using a 3000 Da cut-off ultrafiltration device (Merck Millipore, Molsheim, France) in 4 M urea and 50 mM NH4HCO3. The resulting concentrates were then submitted to digestion.

2.5. Protein digestion

In-solution digestion was performed using an endoLysC/Trypsin mix (Promega, Charbonnières les Bains, France) at a protein/enzyme ratio of 1:30 (w/w) in 4 M urea and 50 mM NH_4HCO_3 at 37 °C. After 3 h of incubation, samples were diluted (4X) and incubated overnight at 37 °C. Digestion was stopped by adding formic acid (0.1% final concentration). Samples were purified on C18 Macro SpinColumns (Harvard Apparatus, Les Ulis, France) and dried by vacuum centrifugation. Digests were resolubilized in 15 µL of 2% acetonitrile, 0.1% formic acid.

2.6. LC-MS/MS analysis

Peptides were analyzed by nanoliquid chromatography coupled to tandem mass spectrometry (Ultimate 3000 coupled to LTQ-Orbitrap Velos Pro, Thermo Scientific). Peptides were sampled on a 300 µm×5 mm PepMap C18 precolumn and separated on a 75 µm×250 mm C18 column (3 µm beads, PepMap, Thermo Scientific) using a 120 min gradient ranging from 5% to 37% acetonitrile in 0.1% formic acid during 114 min before reaching 72% acetonitrile in 0.1% formic acid for the last 6 min. Spray voltage and heated capillary were set at 1.4 kV and 200 °C, respectively. MS and MS/MS data were acquired using Xcalibur (Thermo Scientific). Survey full-scan MS spectra (m/z=400–1600) were acquired in the Orbitrap