Journal of Chromatography B 1060 (2017) 272–280

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Sample preparation for detection of low abundance proteins in human plasma using ultra-high performance liquid chromatography coupled with highly accurate mass spectrometry

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ARTICLE INFO

Keywords:
Biomarkers
Human plasma
Low abundance proteins
LTQ orbitrap
Sample preparation

ABSTRACT

Proteomics is a valuable approach to discover biomarkers in human plasma for early diagnosis. However, detection of biomarkers in the plasma is still challenging because of its large protein content. In our study, we established albumin/IgG depletion methods for identification of low abundance proteins using two commercial kits with additional buffer conditions and various concentrations of cold acetone. Trypsin digestion, desalting, and data-dependent acquisition were also optimized. More than 80% depletion of albumin/IgG was achieved with two commercial kits and 98% depletion of albumin was obtained with 70% cold acetone. Recovery of four reference proteins, BNP (47–76), insulin, cytochrome c, and ubiquitin was obtained in all optimized methods. The best recovery of reference proteins was obtained using the ProteoExtract albumin/IgG removal kit with buffer A (61%–106%). After cold acetone precipitation, three reference proteins were recovered more than 48% except ubiquitin (12%). The number of identified proteins by Mascot was 28, 35, 17, and 34 for ProteoExtract, ProteoPrep, 70%, and 50% cold acetone, respectively. Furthermore, optimized methods detected MS/MS fragmentation patterns of elevated BNP in patient samples with cardiac disease. Our study provides the conditions for efficient biomarker discovery by minimal removing of high abundant proteins.

1. Introduction

Over several decades, knowledge of disease biomarkers has increased, and various biomarkers have been discovered for diagnosis or prognosis of disease [1–3]. In cancer, human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor, prostate specific antigen (PSA), and carcinoembryonic antigen have been used for diagnosis of breast, lung, prostate, and colon cancers, respectively [4–7]. However, few cancer biomarkers have shown clinical utility [8,9]. Even though PSA is commonly used to screen for prostate cancer, it is insufficient evidence for diagnosis [10], and HER2 screening results in many false positives or false negatives [11].

Acute myocardial infarction (AMI) is still a major cause of mortality and morbidity worldwide [12,13], leading to over 2.4 million deaths in the USA and over 4 million deaths in Europe and Northern Asia [14]. For diagnosis of AMI, several biomarkers have been discovered, such as aspartate aminotransferase, lactate dehydrogenase, creatine kinase, creatine kinase MB isoenzyme, and cardiac troponin I and T. Among these biomarkers, cardiac troponins are the most popular due to having the highest sensitivity and specificity for myocyte necrosis [12,15]. However, cardiac troponins as biomarkers have two limitations: a low sensitivity in the early phase of AMI, and the need for repetitive measurements between 8 and 12 h after the event [16,17].

The difficulty in detecting biomarker proteins is due to the low concentration of meaningful biomarkers in plasma proteins. Therefore, the major challenge of plasma analysis is depletion of highly abundant proteins, which include albumin, total IgG, transferrin, fibrinogen, total IgA, alpha-2-macroglobulin, total IgM, alpha-1-antitrypsin, C3 complement, and haptoglobin. Because these 10 proteins account for 90% of the plasma constituents, they are saturated and provide the strongest signal. In contrast, potential biomarker candidates are found in the 10% remaining proteins [18]. Therefore, removal of highly abundant proteins is crucial.

For depletion of highly abundant proteins, many researchers have used 2DE, polyclonal HPLC column, multiple affinity removal system (MARS), or various commercial albumin/IgG removal kits before

Abbreviations: AMI, acute myocardial infarction; DDA, data-dependent acquisition; HER2, human epidermal growth factor receptor 2; IAA, iodoacetamide; MARS, multiple affinity removal system; Pro-BNP, pro-brain natriuretic peptide; PSA, prostate specific antigen

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http://dx.doi.org/10.1016/j.jchromb.2017.06.023
Received 14 February 2017; Received in revised form 31 May 2017; Accepted 1 June 2017
Available online 16 June 2017
1570-0232/

analysis using LC–MS/MS [19–21]. Although 2DE is frequently used for separation and visualization of proteins, it is very time-consuming and labor-intensive, and has poor reproducibility [22,23]. Bead-conjugated antibodies against highly abundant proteins, MARS, are very expensive despite providing the highest removal efficiency of the top 14 major plasma proteins. Therefore, commercial albumin/IgG removal kits are frequently used to deplete highly abundant plasma proteins [19–21,24–26].

Proteomics is a still promising technique for biomarker studies even though genomics and metabolomics have become increasingly important [27]. Our aim was to establish the conditions for the detection of low abundance proteins in human plasma for biomarker discovery. Normally, biomarkers exist at low concentration in plasma. In addition, they have an extremely wide range of pKa values and hydrophobicity indexes. In this study, we selected four reference proteins with pKa values, ranging from 5.30 to 12, and low molecular weight, ranging from 3.5 kDa to 12.4 kDa. Clinically, BNP is a diagnostic biomarker of heart failure [28], soluble cytochrome c is a biomarker of apoptosis in liver disease [29], ubiquitin is related to the diagnosis of traumatic brain injury [30], and insulin is implicated in diabetes.

In this study, we tested various concentrations of organic solvents and two commercial kits for sample preparation. And protein identification by the Mascot search engine in the mass spectrometry data was optimized for both low abundance protein detection and low molecular weight.

2. Materials and methods

2.1. Materials

Pro-brain natriuretic peptide (Pro-BNP) (47–76) was obtained from Phoenix Pharmaceuticals (Burlingame, CA, USA), cytochrome c from Acros Organics (Morris Plains, NJ, USA), insulin and ubiquitin-FLAG (sequence: DYKDDDDK) from Sigma-Aldrich (St. Louis, MO, USA), ProteoExtract albumin/IgG removal kit from EMD Bioscience (Darmstadt, Germany), ProteoPrep immunoaffinity albumin and IgG depletion kit from Sigma-Aldrich (St. Louis, MO, USA), acetone from Duksan Pure Chemicals (Ansan, Korea), Pierce Trypsin Protease MS Grade for trypsin digestion from Thermo Scientific (Rockford, IL, USA), and Tris-tricine gel (16.5%) from Bio-Rad Laboratories (Hercules, CA, USA). Oasis HLB 1 cc Extraction Cartridge (Waters, Milford, MA, USA), DK-Tip C18 (Diatech, Seoul, Korea), and Pierce Detergent Removal Spin Columns, 2 mL (Thermo Scientific, Rockford, IL, USA) were obtained for desalting of trypsin-digested samples. Commercial plasma was purchased from Biochemed (Gwangju, Korea). Plasma samples of healthy controls and AMI patients were obtained from the Korea University Medical Center (approved by the Institutional Review Board KUGH12118-005). All plasma samples were collected with the individual's informed consent.

2.2. Sample preparation

Plasma from 20 to 45-year-old women and 20–30-year old men were pooled at a 1:1 ratio. For spiking of reference proteins, BNP (47–76), cytochrome c, insulin, and ubiquitin (7 μL each) were added into 35 μL of human plasma. The final concentration of BNP, cytochrome c, and insulin was 400 μg/mL, and of ubiquitin was 200 μg/mL in 63 μL of human plasma. For depletion of albumin/IgG, buffers supplied with the ProteoExtract kit and the ProteoPrep kit were used to dilute the spiked human plasma samples 10-fold and to 100 μL, respectively. Aqueous acetone at 50% to 90% was prepared and stored at −20 °C for 3 h. Four volumes of cold aqueous acetone (−10 °C to −5 °C) were added into human plasma spiked with the four reference proteins and the samples were incubated at −20 °C for 2 h.

The pH and concentration of buffer components were optimized for higher recovery (%) of the four reference proteins from human plasma. Three different buffers were prepared: Buffer A corresponded to distilled water (DW) containing 10% formic acid (FA) (pH 1.8), Buffer B to 0.2 M sodium acetate buffer (pH 5.6), and Buffer C to 0.1 M Tris (pH 7.4).

2.3. Albumin/IgG depletion

Albumin/IgG depletion was conducted according to the manufacturer's instructions at 20 °C. The buffers supplied with the ProteoExtract and ProteoPrep kits were used for first elution step and additional buffer A, B, and C were used for second elution step. Albumin/IgG-depleted human plasma was dried using a speed-vacuum centrifuge connected to a freeze-dryer. The dried sample was stored at −20 °C until analysis. Diluted human plasma precipitated with the cold acetone (as described in Section 2.2) was centrifuged at 13000*g* for 10 min at 4 °C and the supernatant was collected. The pellet was washed twice with four volumes of cold acetone and the centrifugation step was repeated twice. All supernatants were collected to the same tube and acetone was evaporated using nitrogen gas. The samples were dried further using a speed vacuum centrifuge connected to a freeze-dryer overnight. The pellets were dried at room temperature for 10 min. Powdered supernatant and pellet were stored at −20 °C until analysis.

2.4. SDS-PAGE

Human plasma samples were reconstituted in 50 mM ammonium bicarbonate (pH 7.8) and analyzed by SDS-PAGE using Tris-glycine (12.5%, 20 μg of sample in 15 μL) and Tris-tricine (16.5%, 30 μg of sample in 30 μL) gels. Separation voltage was 150 V and 100 V for Tris-glycine and Tris-tricine gels, respectively. Separation time was 100 min. Depleted protein concentration was determined using a BCA protein assay kit. The depletion rate of albumin and IgG was calculated by densitometric quantitation analysis using a CS Analyzer (Atto, Tokyo, Japan).

2.5. In-solution digestion

Samples were reduced by incubation in 20 mM DTT at 60 °C for 1 h, and alkylated in 40 mM iodoacetamide (IAA). The alkylation reaction was quenched with the addition of 10 mM DTT. Trypsin was added to the sample to a final protease:protein ratio of 1:30 (w/w) and the mixture incubated at 37 °C for 24 h. The digestion reaction was stopped by storing the sample at −20 °C.

2.6. Desalting

Oasis HLB 1 cc Extraction Cartridge, DK-Tip C18, and Pierce detergent removal spin columns were tested for desalting efficiency. For Oasis HLB cartridges, the digested sample was diluted 10-fold with 10% ACN in DW and centrifuged at 14000*g* for 5 min at 4 °C to remove the pellet. The cartridge was pretreated with 1 mL of methanol and 1 mL of DW using a pump, and the supernatant was loaded slowly and then washed twice with 1 mL of DW. The sample was eluted using 1 mL of 50% methanol aqueous solution and the eluate was dried with a freeze-dryer. The powder obtained was dissolved in 0.2% FA in DW for UHPLC–MS/MS analysis. DK-Tip C18 and Pierce detergent removal spin columns were used according to the manufacturer's instructions at room temperature.

2.7. Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS) analysis

The desalted sample was analyzed on a Dionex Ultimate 3000 RSLC systems connected to an LTQ Orbitrap Velos Pro (Thermo Fisher Scientific, Sunnyvale, CA, USA). The sample (5 μL) was injected and separated using an Aeris Widepore 3.6 μm XB-C18, 100 × 4.6 mm