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Mass spectrometry characterization of circulating human serum albumin microheterogeneity in patients with alcoholic hepatitis

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ABSTRACT

Human serum albumin (HSA) is the most abundant plasma protein, endowed with several biological properties unrelated to its oncotic power, such as antioxidant and free-radicals scavenging activities, binding and transport of many endogenous and exogenous substances, and regulation of endothelial function and inflammatory response. These non-oncotic activities are closely connected to the peculiarly dynamic structure of the albumin molecule. HSA undergoes spontaneous structural modifications, mainly by reaction with oxidants and saccharides; however, patients with cirrhosis show extensive post-transcriptional changes at several molecular sites of HSA, the degree of which parallels the severity of the disease. The present work reports the development and application of an innovative LC–MS analytical method for a rapid and reproducible determination of the relative abundance of HSA isoforms in plasma samples from alcoholic hepatitis (AH) patients. A condition of severe oxidative stress, similar to that observed in AH patients, is associated with profound changes in circulating HSA microheterogeneity. More interestingly, the high resolution provided by the analytical platform allowed the monitoring of novel oxidative products of HSA never reported before.

1. Introduction

Human serum albumin (HSA) has a fundamental role in the modulation of fluid distribution because of its intravascular concentration and charge distribution along the molecule. HSA presents other biological properties, such as regulation of the endothelial function, detoxification activity, and binding and transport of many endogenous and exogenous substances [1]. Furthermore, HSA has been employed clinically for the treatment of hypoalbuminemia in different clinical settings, including chronic liver disease [2,3].

HSA is a monomeric multi-domain macromolecule consisting of 585 amino acids, with a molecular mass, based on the amino acid sequence, of 66,438 Da. HSA contains 35 cysteine (Cys) residues forming 17 disulfide bridges, which significantly contribute to the stability and long half-life of the protein. The only free Cys residue is located at position 34 (Cys34) and represents the largest fraction of free thiol in human plasma. Cys34 is characterized by a strong nucleophilicity and, because of the high HSA plasma concentration and relatively long half-life, it represents the most important scavenger group of reactive oxygen species (ROS) in the extracellular compartment [4]. In healthy adults, about 70–80% of HSA molecules have the Cys34 residue with the free sulfhydryl group whereas about 20–30% of HSA molecules show Cys34 involved in a mixed disulfide with free Cys, homocysteine or glutathione. Finally, a fractional yield of ~5% may be found with Cys34 in its sulfinic or sulfonic acid form [5].

Recently, the interest of researchers has focused on the characterization of the structural and functional alterations of HSA, particularly those affecting Cys34: actually, the isoforms arising from such modifications are now considered promising and innovative biomarkers for several pathological conditions, such as acute liver failure and chronic liver disease [4,6,7]. The aim of the present work is to investigate how an exacerbated oxidative stress

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condition affects the structural integrity of HSA. Oxidative stress has been detected in almost all clinical and experimental conditions of alcoholic liver disease [8,9]. Therefore, a preliminary study was performed on a population of patients with alcoholic hepatitis (AH).

Mass spectrometry (MS)-based methodologies are the techniques of choice for the determination of HSA isoforms in terms of their identification and quantitation [10–12]. The chromatographic separation of HSA from other plasma proteins is, however, a time-consuming step, particularly for the screening of large populations of patients in the field of biomarker discovery.

For this reason the chromatographic approach was here modified, adopting a monolithic chromatographic column, in order to reduce analysis times. Moreover, an enzymatic digestion followed by a nanoLC–nanoESI-MS/MS approach was employed for the identification of new HSA isoforms.

2. Materials and methods

2.1. Materials

The monolithic CIMac C4 Analytical column was provided by BIA Separations (Ljubljana, Slovenia). Acetonitrile, formic acid (FA), trifluoroacetic acid (TFA), sinapinic acid (SA), α-cyano-4-hydroxycinnamic acid (CHCA), ammonium bicarbonate, urea, dithiothreitol (DTT), iodoacetamide (IA) and trypsin were purchased from Sigma-Aldrich (Milan, Italy). Water was purified by means of a Milli-RX system (Millipore, Milford, MA, USA) and used to prepare buffers and standard solutions. All solvents used for the preparation of mobile phases were filtered through 0.22-μm membrane filters.

2.2. Patients

Peripheral blood samples were obtained from 10 patients admitted to the Aarhus University Hospital (Denmark) with acute AH in the period 2009–2014. AH patients were selected using the following inclusion criteria: (A) first-time diagnosis of AH by a combination of physical and laboratory criteria; (B) history of excessive alcohol ingestion (10 units or more per day) until at least three weeks before admission; (C) acute jaundice (developed over at most 2 weeks, serum bilirubin >80 μmol/L). The diagnosis was verified by liver biopsy; in all cases, histological analysis confirmed the clinical diagnosis. Exclusion criteria were: viral hepatitis, autoimmune liver disease, bile duct obstruction, liver tumor or any other cancer, presence of an infectious focus (either clinically assessed or based on chest X-ray, urine samples or ascites puncture), age below 18 or above 75 years, ongoing gastrointestinal bleeding or bleeding within the previous 3 months, or any prior immune-modulating therapy. Moreover, 10 healthy volunteers were enrolled at the Sant'Orsola-Malpighi University Hospital (Bologna, Italy) as a reference population.

The procedure for sample collection was highly standardized: 9 mL of peripheral blood were withdrawn from the brachial vein of participants to the study, collected in sterile tubes containing ethylenediaminetetraacetic acid (EDTA) and centrifuged at 3000 rpm for 10 min to obtain plasma samples, which were immediately frozen as single aliquots and maintained at −80 °C until analysis. Informed written consent was obtained from both patients and healthy volunteers. The study protocol was approved by the Central Denmark Region Committees on Health Research Ethics for patients and at Sant'Orsola-Malpighi University Hospital for healthy volunteers, according to the 1975 Declaration of Helsinki.

2.3. LC–MS analysis of HSA

Plasma samples were diluted 1:100 (v/v) with a mixture of water–acetonitrile (98:2, v/v) and filtered through a 0.22-μm filter (Merck KGaA, Darmstadt, Germany).

The chromatographic separation of HSA from other plasma proteins was performed on an Agilent 1200HPLC system (Walbronn, Germany) using a monolithic CIMac C4 Analytical column (5 mm × 5.3 mm I.D.). Mobile phases A [water–acetonitrile-FA (99:1:0.1, v/v/v)] and B [water–acetonitrile-FA (2:98:0.1, v/v/v)] were used to develop a gradient. The optimized mobile phase gradient program was: A–B from (70:30, v/v) to (30:70, v/v) in 10 min; from (30:70, v/v) to (20:80, v/v) in 1 min; (20:80, v/v) for 1 min. The column was equilibrated with the mobile phase composition of the starting conditions for 3 min before the next injection. The flow rate was set at 0.8 mL/min and a T valve was used to split part of the mobile phase directly to the waste. The injection volume was 3 μL.

The carry-over effect of the protein was investigated on diluted plasma samples. Only traces of the protein were revealed after each analysis; for this reason, one blank injection was performed between two different LC–ESI-MS analyses.

MS analysis was carried out on a Q-ToF Micro quadrupole time-of-flight (Q-TOF) hybrid analyser (Micromass, Manchester, UK) with a Z-spray electrospray ion source (ESI). The ESI-Q-TOF source temperature was set at 100 °C, the capillary voltage at 3.5 kV and the cone voltage at 42 V. The scan time was set at 2.4 s and the inter scan time at 0.1 s. The cone gas flow was set at 120 L/h and the desolvation gas at 500 L/h. Mass chromatograms were recorded in total ion current (TIC), within 1000 m/z and 1700 m/z. The HSA baseline-subtracted spectrum (m/z 1000–1600) was deconvoluted onto a true mass scale using the maximum entropy (MaxEnt1)-based software supplied with MassLynx 4.1 software. Output parameters were set as follows: mass range 61,500–71,500 Da; resolution 2 Da/channel. The uniform Gaussian model was used, with 0.5 Da width at half height.

The abundance of single isoforms was calculated as the ratio between their fractional intensity and the sum of the intensities of all isoforms, expressed as percentage. Data were analyzed by Microsoft Excel software. In order to evaluate reproducibility, a plasma sample was analyzed 5 times and the relative standard deviations (RSD%) for the abundance of each HSA isoform was calculated.

2.4. LC–MS method validation

The chromatographic method was validated in terms of reproducibility of HSA retention times and peak areas. Since the MS analytical methodology was slightly modified with respect to the published protocol [12], intra-day and inter-day variabilities were investigated and determined for both the isoform molecular weight determination and the isoform relative abundance. To this purpose, a sample of plasma was analyzed six times on the same day and five times over three consecutive days ($n = 15$), and the corresponding relative standard deviations (RSD%) were calculated.

2.5. LC–UV purification of HSA

Plasma samples were diluted 1:10 (v/v) with a water–acetonitrile (98:2, v/v) mixture, filtered with a 0.22-μm filter and purified by a chromatographic approach. In details, HSA was purified from other plasma proteins by using a Jasco PU-1585HPLC system (Jasco Corporation, Tokyo, Japan) equipped with a Rheodyne 7281 injection valve (20 μL sample loop). The detection was performed with a Jasco UV-1575 detector set at 215 nm. The LC preparative purification of HSA was performed on