Analytica Chimica Acta 800 (2013) 103–110

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

Capillary zone electrophoresis and capillary electrophoresis-mass spectrometry for analyzing qualitative and quantitative variations in therapeutic albumin

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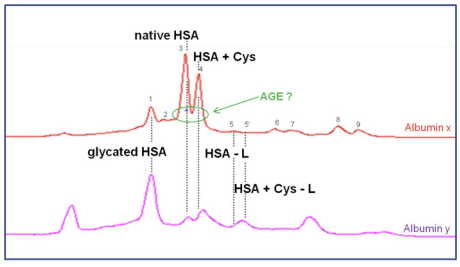
HIGHLIGHTS

- Development of a reproducible CZE method allowing the separation of nine HSA forms.
- Qualitative and quantitative CZE method pointing out important differences between competitive HSA preparations.
- CE-MS coupling reveals new truncated forms and Advanced Glycation End products in the HSA preparation.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 16 May 2013
Received in revised form 29 July 2013
Accepted 11 September 2013
Available online 14 September 2013

Keywords:
Human serum albumin
Capillary zone electrophoresis
Mass spectrometry
Advanced Glycation End products

ABSTRACT

The present study describes a reproducible and quantitative capillary zone electrophoresis (CZE) method, which leads to the separation of nine forms (native, oxidized and glycated) of human serum albumin (HSA). In an attempt to identify the different species separated by this CZE method, the capillary electrophoresis was coupled to mass spectrometry using a sheath liquid interface, an optimized capillary coating and a suitable CE running buffer. CE-MS analyses confirmed the heterogeneity of albumin preparation and revealed new truncated and modified forms such as Advanced Glycation End products (AGEs). Assignment of the CZE peaks was carried out using specific antibodies, carboxypeptidase A or sample reduction before or during the CE separation. Thus, five HSA forms were unambiguously identified. Using this CZE method several albumin batches produced by slightly different fractionation ways could be discriminated. Furthermore, analyses of HSA preparations marketed by five pharmaceutical industries revealed that two therapeutic albumins, including that marketed by LFB, contained the highest proportion of native form and lower levels of oxidized forms.

Abbreviations: AGE, Advanced Glycation End product; BGE, background electrolyte; CE, capillary electrophoresis; CEL, N_{ε}-(1-carboxyethyl)-lysine; CE-MS, capillary electrophoresis-mass spectrometry; CML, N_{ε}-carboxymethyl-lysine; CPA, carboxypeptidase A; CZE, capillary zone electrophoresis; DGH, N_{δ}-[5-(2,3,4-trihydroxybutyl)-5-hydro-4-imidazolon-2-yl]-ornithine; DOLD, 3-deoxyglucosone-derived lysine dimer 1,3-di(N_{ε}-lysino)-4-(2,3,4-trihydroxybutyl)-imidazolium salt; EOF, electroosmotic flow; ESI, electrospray ionization; GH, N_{δ}-(5-hydro-4-imidazolon-2-yl)-ornithine; GOLD, glyoxal-derived lysine dimer, 1,3-di(N_{ε}-lysino)-imidazolium salt; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid; HPC, hydroxypropyl cellulose; HSA, human serum albumin; HSA + Cys, cysteinylated HSA; HSA + HCys, homocysteinylated HSA; HSA + G, glutathionylated HSA; HSA-SNO, nitrosylated HSA; HSA-SOH, HSA-sulfenic acid; HSA-SO_2H, HSA-sulfinic acid; HSA-SO_3H, HSA-sulfonic acid; HSA-DA, HSA minus aspartic acid and alanine from the N-terminus; HSA-L, HSA minus leucine from the C-terminus; IT, ion trap; MGH, N_{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; MOLD, methylglyoxal-derived lysine dimer, 1,3-di(N_{ε}-lysino)-4-methyl-imidazolium salt; MS, mass spectrometry; PEO, polyethylene oxide; PMMA, polymethylmethacrylate; SDS, sodium dodecyl sulftate; TBS, tris-buffered saline; THP, N_{δ}-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidin-2-yl)-ornithine; TIC, total ion current.

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http://dx.doi.org/10.1016/j.aca.2013.09.023

1. Introduction

Human serum albumin (HSA), obtained by fractionation of donor plasma, is used as a therapeutic molecule to correct hypovolemia and hypoalbuminemia, which are symptoms associated with liver failure, renal diseases or sepsis [1,2]. Apart from maintaining oncotic pressure, HSA exhibits many other important physiological functions such as transport of endogen and exogen ligands (fatty acids, hormones, bilirubine and drugs) and powerful antioxidant activity owing to its single free cysteine (Cys34), acting as a free radical scavenger, and its capacity to bind metal ions (Cu^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+}, ...) known as oxidation catalysts [3–5].

For intrinsic (natural source) and extrinsic reasons (bioprocess, storage conditions) HSA preparations are very heterogeneous [6]. Indeed native HSA can undergo physical (aggregation, dimerization) [7,8] or chemical (truncation, oxidation, nitrosylation, glycation) modifications [4,9]. The free sulfhydryl residue (SH) of the native albumin is implicated in a great number of oxidation reactions [10]. For instance it can bind other thiol-containing compounds like cysteine, homocysteine or glutathione (leading to the so-called HSA + Cys, HSA + HCys and HSA + G species, respectively) [11], interact with nitric oxide (NO) and thereby form S-nitrosothiols (HSA-SNO) [12] or be oxidized into sulfenic (HSA-SOH), sulfinic (HSA-SO_2H) or sulfonic (HSA-SO_3H) acids [13].

Furthermore, serum albumin is a plasma protein highly sensitive to glycation, because of its long half-time (21 days) and its high concentration. The glycation process, also known as the Maillard reaction, is a slow non-enzymatic reaction that initially involves attachment of a hexose (glucose, galactose, fructose) or derivatives such as glyoxal and methylglyoxal with free amine groups of albumin. This reaction forms a reversible Schiff base product, which leads to stable fructosamine residue (ketoamine) following Amadori rearrangement. The Schiff base and fructosamine, called early glycation adducts, can subsequently be oxidized, polymerized or cleaved to give irreversible conjugates, called Advanced Glycation End products (AGEs). Numerous studies have identified the main sites on HSA molecule modified by glycation in vivo [14]. Because of their high nucleophile properties, lysine, arginine and cysteine are the sole residues prone to glycation. Fig. 1 describes the different structures of AGEs reported in the literature [15,16].

It has been shown that massive albumin oxidation occurs in vivo in different biological fluids and, to some extent, that this process is correlated to organ dysfunction [4]. For instance, albumin can undergo increased glycation in diabetic subjects [17] and liver diseases are accompanied by high levels of cysteinylated or sulfonic forms [18]. Depending on donor population but also on the fractionation process, commercial albumin preparations may vary in their composition regarding the different degraded or modified forms of HSA and this heterogeneity is suspected to impact their therapeutic effect. Recently, the quality of administrated HSA in critically ill patients has been discussed. It appears that preference should be given to preparations with a higher reduced HSA percentage since an increased percentage of oxidized HSA is responsible for impaired HSA functions [19]. It is therefore of paramount importance to detect, characterize and quantify the different forms of HSA. Several methods, mainly based on liquid chromatography, have been developed but most of them still cannot discriminate native HSA from all its related forms. Due to its high separation efficiency, capillary electrophoresis (CE) has emerged as a powerful method for the analysis of protein isoforms. However, one of the major difficulties in CE is to prevent the adsorption of proteins on the inner capillary wall [20]. In 1995, Denton and his coworkers succeeded in separating eight HSA forms using a neutral polyacrylamide coating but no reproducibility was demonstrated and only four peaks were identified [21]. Recently our laboratory has developed a reproducible CZE method that allows to separate nine isoforms of HSA and two of which were identified [22]. The present paper reports the improvement of the above-mentioned strategy with the aim of a comprehensive identification of the nine HSA species by using the innovative CE-MS coupling [23]. Until now, only few studies have reported the analysis of human serum albumin by CE-MS [24,25]. The CE-MS experiments we have carried out revealed many unexpected forms of HSA despite the loss of resolution upon the CZE separation when coupled to MS. To attribute the nine peaks of our CZE separation, we used chemical and enzymatic reactions during or before the CE separation, without MS coupling. The CZE method was evaluated in terms of reproducibility and efficiency by analyzing albumin preparations obtained from slightly modified fractionation ways. Finally we have applied the CZE method to the comparison of albumin preparations produced by five competitive pharmaceutical industries.

2. Materials and methods

2.1. Chemicals

Sodium hydroxide, hydrochloric acid, ammonium hydrogenocarbonate, polyethylene oxide (PEO, average Mw 200,000) were obtained from ProLabo (Fontenay-sous-Bois, France). Ammonium acetate, acetonitrile, formic acid, hydroxypropyl cellulose (HPC, average Mw 100,000) were purchased from Sigma–Aldrich (Saint-Quentin Fallavier, France). 4-(2-Hydroxyethyl)-1-piperazine-ethane-sulfonic acid (HEPES), sodium dodecyl sulfate (SDS), copper nitrate ($Cu(NO_3)_2$), n-dodecyl-β-D-maltoside, dithiothreitol (DTT), carboxypeptidase A (from Bovine Pancreas) and glycated albumin (2 mol of hexose per mol of albumin) were obtained from Sigma (St. Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris) was from Merck (Darmstadt, Germany). Anti-AGE antibody was obtained from Acris Antibodies (ref. BP8039, Herford, Germany). The HSA samples (marketed by LFB, Sanquin, Baxter, CSL Behring, Octapharma) were provided by LFB (Les Ulis, France). All buffers and samples used for CE and CE-MS were prepared with Milli-Q water using a Direct-Q 3 UV purification system (Millipore, Bedford, MA, USA) and were filtered through a 0.2 μm Millex membrane (Millipore) before use.

2.2. MS infusion

For MS direct infusion albumin samples were desalted by centrifugation on a Vivaspin® 500 filter with a 10 kDa cut-off (Sartorius Stedim Biotech) by applying 6 concentration/dilution steps in ammonium acetate 50 mM. The desalted samples were then diluted to a concentration of 1 μM in water/acetonitrile 50/50 with formic acid 0.1%. The MS apparatus used was a Synapt G2S (Waters, Milford, MA, USA) calibrated using sodium iodide (NaI, Waters, Milford, MA, USA) as recommended by the manufacturer. The mass spectrometer was operated in the positive ion mode using the TOF analyzer recording from *m/z* 400 to 4000 and the quadrupole in RF only mode. Samples were infused in nanospray at an estimated flow rate of 0.2 μL min^{-1}, using a TriVersa NanoMate system operating in Chip-Based Infusion mode. Scans were registered every 4 s and mass spectra were obtained from the combination of 30 scans.

2.3. CE system

2.3.1. CE apparatus and method

The CE experiments were carried out on a P/ACE 5500 capillary electrophoresis instrument equipped with a UV detector (Beckman Coulter, Fullerton, CA, USA). Bare fused-silica capillaries with an internal and external diameter of 50 and 375 μm, respectively, were purchased from Phymep (Paris, France). The capillaries were