Journal of Chromatography B, 981–982 (2015) 57–64

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Simultaneous determination of albumin and low-molecular-mass thiols in plasma by HPLC with UV detection

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ARTICLE INFO

Article history:
Received 24 October 2014
Accepted 29 December 2014
Available online 12 January 2015

Keywords:
Albumin
Endogenic plasma thiols
High performance liquid chromatography
Derivatization
Ultraviolet detection

ABSTRACT

In this paper, we describe a simple and robust HPLC based method for determination of total low- and high-molecular-mass thiols, protein S-linked thiols and reduced albumin in plasma. The method is based on derivatization of analytes with 2-chloro-1-methylquinolinium tetrafluoroborate, separation and quantification by reversed-phase liquid chromatography followed by UV detection. Disulfides were converted to their thiol counterparts by reductive cleavage with tris(2-carboxyethyl)phosphine. Linearity in detector response for total thiols was observed over the range of 1–40 μmol L^{-1} for Hcy and glutathione (GSH), 5–100 μmol L^{-1} for Cys–Gly, 20–300 μmol L^{-1} for Cys and 3.1–37.5 μmol L^{-1} (0.2–2.4 g L^{-1}) for human serum albumin (HSA). For the protein S-bound forms these values were as follows: 0.5–30 μmol L^{-1} for Hcy and GSH, 2.5–60 μmol L^{-1} for Cys–Gly and 5–200 μmol L^{-1} for Cys. The LOQs for total HSA, Cys, Hcy, Cys–Gly and GSH were 0.5, 0.2, 0.4, 0.3 and 0.4 μmol L^{-1}, respectively. The estimated validation parameters for all analytes are more than sufficient to allow the analytical method to be used for monitoring of the total and protein bound thiols as well as redox status of HSA in plasma.

1. Introduction

Human serum albumin (HSA) is the most abundant plasma protein and accounts for 50% of the total plasma proteins [1]. It plays an important role in regulation of osmotic pressure, buffering of plasma pH, distribution of fluid between different compartments, transporting of long chain fatty acids, bilirubin, carbon dioxide and acts as an efficient extracellular antioxidant [2]. Moreover, HSA is believed to be linked to interactions with a broad spectrum of inorganic compounds, such as calcium, magnesium, zinc and copper [3]. Antioxidant properties of HSA arise from the presence of the thiol group derived from cysteine 34 (Cys34), the only one uninvolved in intrachain disulfide bonding. Although —SH groups of HSA, in chemical terms, behave like low-molecular-mass thiols, the biochemical reactivity of HSA is much more complicated due to steric hindrance, charge distribution and affinity of nucleophilic groups in biomolecule to the solvent. In contrast to most of the low-molecular-mass plasma thiols, the *p*Ka of the thiol group of Cys34 in HSA is surprisingly low (~5) [4], thus, at physiological conditions, HSA exists in reduced (HSA-SH) and oxidized forms (HSA-S-R) [2]. Typical plasma concentrations of HSA range from 0.6 to 0.75 mmol L^{-1}, therefore Cys34 provides a source of 80% of the total thiols in plasma [5]. It has been shown that —SH group of Cys34 plays the crucial role in the defense against oxidative damage [2]. Oxidative stress, which is widely believed to be an important factor in the pathogenesis of several diseases such as liver and renal failure [6], uremia [7], diabetes mellitus [8], alcaptonuria [9], and obstructive sleep apnea [10], was proven to be associated with a decrease of the HSA thiol group content. Additionally, the increasing impact of reactive dicarbonyl compounds which occur during carbonyl stress leads to the cysteine (Cys) side chain carbonylation and, therefore, to the decrease of the HSA-SH content [11]. It has been established that protein carbonyls accumulate on tissue proteins during aging and disease development [12–16]. Sulfur-containing amino acids play an important role in the human metabolism. Cysteine, metabolically related to homocysteine (Hcy) and glutathione (GSH), is involved in a variety of important cellular functions, among others protein synthesis, detoxification and metabolism. However, due to its high toxicity in reduced form Cys may cause neurodegenerative changes such as excitotoxic brain damage, stroke or certain neurodegenerative disorders [17]. GSH functions as a major endogenous antioxidant and redox buffer and plays an important role in cellular defence including detoxification of xenobiotics and peroxides and the maintenance of immune function [18]. Many clinical studies have indicated that plasma total Hcy

Abbreviations: CMQT, 2-chloro-l-methylquinolinium tetrafluoroborate; Cys, cysteine; Cys–Gly, cysteinylglycine; GSH, glutathione; Hcy, homocysteine; HSA, human serum albumin; TCEP, tris(2-carboxyethyl)phosphine.

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http://dx.doi.org/10.1016/j.jchromb.2014.12.032
1570-0232/

is a risk factor for coronary vascular diseases and stroke and can act as a predictor of mortality in cardiovascular patients [19,20]. Genetic and environmental determinants of total Hcy are well-known as well as the possibility of reduction of plasma total Hcy by B-vitamin supplementation [21,22]. Increased protein S-thiolation is considered an in vivo marker of oxidative injury [23].

The dynamic interactions of sulfhydryl compounds result in a variety of disulfide forms in vivo. Thus, about 60% of plasma Cys is protein bound while 8–10% exist in reduced form [24]. In the case of Hcy the redox species percentages are greatly different, being usually more than 95% and less than 1%, respectively [24]. In normal human plasma reduced and oxidized forms of albumin amounts to ~25% and ~75%, respectively [25]. Reduced, free oxidized and protein-bound forms of Cys, cysteinylglycine (Cys–Gly), GSH, Hcy as well as HSA-SH and HSA-S-R, comprise the plasma redox thiol status [26]. Altered redox status of aminothiols has been observed in a number of diseases [27], thus the knowledge of concentration of particular redox forms is desirable. It has been proposed that the levels of S-glutathionylated plasma proteins as well as other forms of GSH may serve as a useful marker for oxidative stress [28]. Ratio of cysteinyl albumin was also considered as a possible biomarker of oxidative stress [29].

Several methods have been developed for the determination of HSA [25,30,31] or protein sulfhydryls [32]. Ellman's method is the most frequently used procedure for plasma total sulfhydryls estimation [32], but determination of plasma albumin sulfhydryls requires its isolation prior to measurement, which is usually time consuming and technically demanding [33,34]. Many assays have been also proposed for low-molecular-mass thiols determination in plasma or urine [35,36]. To the best of our knowledge the problem concerning simultaneous determination of HSA and main plasma aminothiols has received no attention as yet.

Here, we describe a new HPLC-UV based method for simultaneous determination of HSA, total and protein bound thiols in human plasma that relies on derivatization of analytes with CMQT and direct injection of not deproteinized sample into chromatographic column.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used throughout this study were of analytical-reagent grade except for the derivatization reagent – 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT), that was synthesized in this laboratory [37]. Human serum albumin (HSA) as well as reduced thiols cysteine (Cys), homocysteine (Hcy), glutathione (GSH), cysteinylglycine (Cys–Gly) and its oxidized forms were received from Sigma (St. Louis, MO, USA). Lyophilized plasma for calibration was obtained from Siemens (Siemens Healthcare, Marburg, Germany). Hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium hydrogen phosphate heptahydrate ($Na_2HPO_4{\cdot}7H_2O$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$) and HPLC-grade acetonitrile were from J.T. Baker (Deventer, The Netherlands). Trichloroacetic acid (TCA) and tris-(2-carboxyethyl)phosphine (TCEP) were from Merck (Darmstadt, Germany).

2.2. Instrumentation

The analyses were performed on a 1220 Infinity LC system from Agilent equipped with binary pump integrated with two-channel degasser, autosampler, column oven and DAD detector. A 2 μL of the samples were injected with the aid of an autosampler and chromatographic separation was achieved on an Aeris WIDEPORE XB-C18 (150 × 4.6 mm) column from Phenomenex, packed with 3.6 μm particles. For instrument control, data acquisition and analysis, OpenLAB CDS ChemStation Edition was used. Water was purified using Milli-QRG system (Millipore, Vienna, Austria).

2.3. Human plasma samples

Blood was collected into evacuated tubes containing EDTA by venipuncture, immediately placed on ice, and centrifuged at 800 × *g* for 15 min at room temperature. Plasma was then used for the determination of low- and high-molecular-mass thiols without delay or stored at −80 °C. The investigation was performed after approval by the Ethical Committee of the University of Łódź.

2.4. Stock solutions of TCEP and CMQT

Stock solutions of 0.25 mol L^{-1} TCEP and 0.1 mol L^{-1} CMQT were prepared by dissolving appropriate amount of the compound in 1 mL of 0.2 mol L^{-1} pH 7.4 phosphate buffer. TCEP was prepared freshly each day.

2.5. Stock solutions of HSA and low-molecular-mass thiols

Stock solution of albumin was prepared by dissolving 50 mg of the protein in 1 mL of 0.1 mol L^{-1} pH 7.4 phosphate buffer.

Stock solutions of 10 mmol L^{-1} Cys, Hcy, GSH, Cys–Gly or their disulfides needed in the method development procedure were prepared by dissolving appropriate amount of the compound in 1 mL of 0.05 mol L^{-1} HCl and diluting to the volume of 2 mL.

These solutions were kept at 4 °C for several days without noticeable change of the analyte content. The working solutions were prepared by appropriate dilutions with water as needed, and processed without delay.

2.6. Preparation of HSA-SH and HSA-S-Cys

HSA-SH as well as HSA-S-Cys were prepared according to the modified previously published procedure [38].

Briefly, HSA (50 g L^{-1}) was converted to HSA-SH by treatment with 2 mmol L^{-1} DTT in 0.1 mol L^{-1} potassium phosphate buffer, pH 7.4, 0.2 mmol L^{-1} EDTA for 25 min at room temperature and diluted 10-fold with 0.01 mol L^{-1} potassium phosphate buffer, pH 5.8. Low-molecular-mass thiols were removed from reaction mixture by ultrafiltration through a Vivaspin 500 30-kDa cut-off membrane (Sartorius) at 4 °C. Under these conditions none of intrachain disulfide bonds of HSA were reduced.

HSA (50 g L^{-1}) was treated with 2-fold molar excess of Cys overnight at 37 °C. Excess Cys/Cystine was removed from mixture by ultrafiltration through a Vivaspin 500 30-kDa cut-off membrane (Sartorius) at 4 °C.

The conversion of HSA to HSA-SH and HSA-S-Cys was monitored by anion exchange chromatography [38]. Both forms of HSA were then used as the calibrators for total and reduced plasma albumin determination.

2.6.1. Total thiols assay (procedure 1)

The assay is based on the procedure used previously for the determination of plasma total aminothiols [39]. 25 μL of plasma was diluted with 90 μL of 0.2 mol L^{-1} pH 7.4 phosphate buffer. Disulfide bonds were reduced by treatment with 10 μL of TCEP solution for 10 min and derivatized with 10 μL of CMQT for 3 min at room temperature. Next, the reaction mixture was treated with 15 μL of 3 mol L^{-1} HCl, and 2 μL of the sample was injected onto an HPLC column.