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Development of a new column switching method for simultaneous speciation of selenometabolites and selenoproteins in human serum

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ABSTRACT

A method for the simultaneous speciation of selenoproteins and selenometabolites in human serum has been developed on the basis of in series three dimensional chromatography: size exclusion, affinity and anion exchange high performance liquid chromatography (3D/SE-AF-AEC-HPLC), using different columns of each type and hyphenation to inductively coupled plasma-(quadrupole) mass spectrometry (ICP-qMS). The method allows the quantitative simultaneous analysis of selenoprotein P (SeP), extracellular glutathione peroxidase (eGPx), selenoalbumin (SeAlb), selenite and selenate in human serum using species-unspecific isotope dilution (SUID). The 3D chromatographic separation is proposed to remove typical spectral interferences in this matrix from chloride and bromide on ^{77}Se ($^{40}Ar^{37}Cl$), ^{80}Se ($^{79}Br^{1}H$) and ^{82}Se ($^{81}Br^{1}H$). In addition, a previous method based on 2D/SE-AF-HPLC is proposed as a simple alternative when low molecular mass selenium species are absent in the samples. The method is robust, reliable and fast with typical chromatographic runtime less than 35 min. Detection limits are in the range of 0.2–1.3 ng of Se g^{-1}. Method accuracy for determination of total protein-bound to Se was assessed by analyzing an human serum reference material (BCR-637) certified for total Se content and method reliability checked in samples of human serum providing results in good agreement with the total selenium concentration. In addition, the application of the method to commercial human serum and plasma reference materials for quality control analysis, certified for total Se, has provided, for the first time, indicative levels of selenium containing proteins in these samples.

1. Introduction

It is well known that selenium (Se) is an essential element in humans, and the importance of its biochemistry has been widely reported. This element is presents into various proteins, which have many biological functions [1,2], but the exact mechanism used by Se to play its essential role in human health is still unclear. Nevertheless, it has been checked that most biological functions attributed to Se are mediated by selenoproteins [2] such as extracellular glutathione peroxidase (eGPx) and selenoprotein P (SeP), which contain selenocystein (SeCys) specifically incorporated into their active sites, and selenoalbumin (SeAlb), not considered as a selenoprotein because the element is not specifically incorporated into the albumin moiety as selenomethionine (SeMet) [3,4].

The selenium species most abundant in the bloodstream is the SeP, and its concentration is a good indicator of Se status in humans [3,4], while eGPx activity in human serum is a complementary marker of selenium status in several clinical studies [5–8]. Both Se-proteins are interrelated, because Se bound to albumin is assumed to be transported to the liver for new synthesis of SeP that is then released into the bloodstream [9] (Fig. 1). Although the antioxidant activity of purified SeP has been demonstrated as well as the reduction of phospholipid hydroperoxides in an eGPx-like manner, its biological action mechanisms are still unclear [10]. In addition, selenometabolites also play important roles in plasma, but information on their presence in this fluid is scarce and not convincingly documented. Recently, low concentration of inorganic selenium metabolites and not detectable concentrations in free selenium aminoacids such as SeMet and SeCys have been reported by Nikolay Solovyev et al. [11]. In addition, Michalke and Schramel [12] proposed the use of capillary electrophoresis-inductively coupled plasma mass spectrometry (ICP-MS) for the identification of free SeMet and SeCys, but failed to quantify them in an human serum sample, because the concentrations of these species are around their detection limits [12]. For these reasons, a feasible alternative for the determination of intact selenium-containing proteins is

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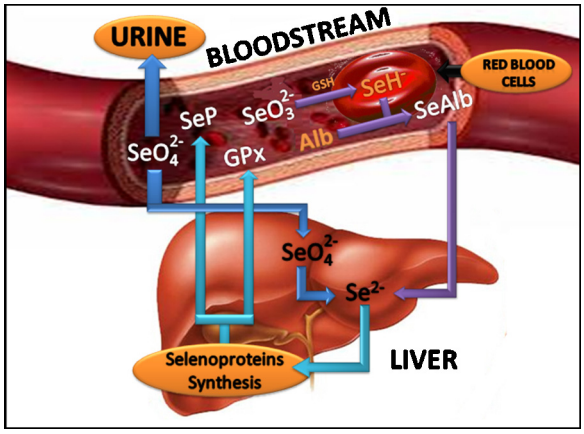

Fig. 1. Metabolic pathway and traffic of selenium in the bloodstream and liver of humans.

based on enzymatic hydrolysis followed by determination of selenoaminoacids (SeCys and SeMet) in the enzymatic extract [13]. In contrast, the presence of selenite and selenate in human serum is well known [11,12]. The selenite in the bloodstream is readily taken up by red blood cells (RBCs) [14] and reduced to selenide (SeH^-), for latter effluxion into the bloodstream in the presence of albumin and transferred to the liver in the form of SeAlb for the synthesis of selenoproteins [15] (Fig. 1). On the other hand, selenate is not taken up by RBCs, but it interacts with hepatocytes and is used for the synthesis of selenoprotein P and extracellular eGPx, which are released into the bloodstream for final partial excretion into urine [16] (Fig. 1). For these reasons, a good approximation of the selenium balance can be obtained by the quantification of eGPx, SeAlb, SeP, selenite and selenate in human serum.

For trace analysis the use of inductively coupled plasma mass spectrometry (ICP-MS) allows multielemental capabilities, low detection limits, tolerance to matrix and large linearity range [17,18]. Additionally, this technique permits element isotope ratios measurement and consequently, isotopic dilution analysis (IDA) for accurate and precise quantification of multi-isotopic elements, particularly selenium containing proteins, which is well documented in the literature [19]. Species-unspecific isotope dilution mode (SUID) is especially useful either when the structure and composition of analyzed species is not exactly known or the corresponding isotopically labelled compounds are not commercially available [19]. Due to the polyatomic interferences of $^{40}Ar_2^+$ and $^{79}Br^1H^+$ on selenium ICP-MS signal, hydrogen is usually recommended as reaction gas in an octopole reaction system (ORS) for accurate quantification of selenium, especially in human serum samples, which contains high levels of bromide [20,21]. However, the determination of different selenium-containing species in serum, in a broad molecular mass range, requires a suitable speciation method combining Se-species separation by orthogonal chromatographic techniques with ICP-MS detection.

Several chromatographic methods have been proposed in the literature for the separation of selenoproteins in human plasma or serum, based on size exclusion chromatography [22] (SEC), anion exchange chromatography [20–23] (AEC) and affinity chromatography [20] (AFC). Although, SEC does not provide precise quantitative results for selenoproteins because of its low chromatographic resolution that provokes overlapping between high abundance selenium containing proteins [22,24,25]. On the other hand, AEC provides good recoveries of analytes but chromatographic resolution is not acceptable either [20]. Finally, when AFC is used, the weakly-retained eGPx is eluted beside non-target matrix components, which cause difficulties for the accurate quantification of the different selenium species that usually are quantified together [20]. Moreover, it should be considered the high concentration of Cl^- and Br^- in biological fluids, such as plasma and serum (~3.5 g L^{-1} and ~3.5 mg L^{-1}, respectively) [26], which coelute with eGPx and Se metabolites in the cited procedures. For this purpose, AEC has been recently on line coupled to AFC for the analysis of selenium containing proteins alleviating the spectral interferences of Cl^- and Br^-, and making possible the use of ICP-ORS-MS [27,28].

In this work, a method for the quantification of selenium-tagged proteins and inorganic selenium species in human serum has been developed using species-unspecific isotope dilution (SUID)-ICP-ORS-qMS online coupled to 3D/SE-AF-AEC-HPLC involving five columns in one run for the analysis of 100 mg of sample. Using this chromatographic arrangement, the spectral interference produced by bromide and chloride are removed and the total chromatographic runtime is less than 35 min. Consequently, a reliable speciation method for the analysis of eGPx, SeP, SeAlb and inorganic selenium compounds is obtained for the first time. The analytical approach was validated using a human serum reference material (BCR-637) certified for total Se content. In addition, it has been obtained for the first time indicative levels of selenium containing proteins in a commercially available human serum, namely the ClinChek CRM serum and plasma control with certified levels for total Se.

2. Experimental

2.1. Reagents and samples

All reagents used for sample preparation were of the highest available purity. phenylmethanesulfonyl fluoride (PMSF) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (BioUltra grade, >98%) were obtained from Sigma–Aldrich (Steinheim, Germany). Hydrogen used as reaction gas in SEC-AF-AEC-ICP-ORC-MS system was of high-purity grade (>99.999%).

The mobile phase solution used in SEC and AFC was ammonium acetate (Bioultra grade) purchased from Merck (Darmstadt, Germany), which was prepared daily with ultrapure water (18 MΩ cm) obtained from a Milli-Q system (Millipore, Watford, UK) and adjusted to pH 7.4 with ammonia solution, this later prepared by dilution of 20% (v/v) ammonia solution (Suprapur, Merck) with ultrapure water. On the other hand, the mobile phase solution used in AEC was sodium phosphate (for analysis grade) purchased from Merck (Darmstadt, Germany), which was prepared daily with ultrapure water and adjusted to pH 8 with a 50% (v/v) aqueous phosphoric acid solution obtained from Sigma Aldrich (Steinheim, Germany).

The human serum certified reference material (CRM) BCR-637 was purchased from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). Others reference materials were also used for lyophilized plasma (Clinchek of plasma control, Level II) and serum (Level I) (Recipe Chemicals, Munich, Germany). Standard solutions containing 1000 mg L^{-1} of Se and 1000 mg L^{-1} of Br, both stabilized in 5% (v/v) Suprapur nitric acid were purchased from Merck (Darmstadt, Germany). Enriched ^{74}Se was obtained from Cambridge Isotope Laboratories (Andover, MA, USA) as elemental powder and it was dissolved in the minimum volume of nitric acid (Suprapur grade) and diluted to the appropriate volume with ultrapure water. The concentration of this solution was established by reverse isotope dilution analysis as described elsewhere [20].

2.2. Instrumentation

A microwave oven (CEM Matthews, NC, USA, model MARS) was used for the mineralization of plasma and serum samples. Selenium trace levels and selenium-linked biomolecules were analyzed