Talanta 189 (2018) 332–338

Contents lists available at ScienceDirect

Talanta

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

Quantification and isotopic analysis of bulk and of exchangeable and ultrafiltrable serum copper in healthy and alcoholic cirrhosis subjects

Sara Lauwens[a], Marta Costas-Rodríguez[a], Joris Delanghe[b], Hans Van Vlierberghe[c], Frank Vanhaecke[a,*]

[a] Ghent University, Department of Chemistry, Atomic and Mass Spectrometry (A&MS) Research Unit, Campus Sterre, Krijgslaan 281-S12, 9000 Ghent, Belgium
[b] Ghent University, Department of Clinical Chemistry, Microbiology and Immunology, Campus UZ Gent, De Pintelaan 185-P8, 9000 Ghent, Belgium
[c] Ghent University Hospital, Department of Gastroenterology and Hepatology, Campus UZ Gent, De Pintelaan 185-1K12IE, 9000 Ghent, Belgium

ARTICLE INFO

Keywords:
Exchangeable and ultrafiltrable fraction
Copper
Isotope ratio
Multi-collector ICP-MS
Alcoholic liver cirrhosis

ABSTRACT

Information on the Cu speciation in blood serum can be valuable for a better understanding of the metabolism of this essential transition metal, but Cu speciation analysis and, to an even larger extent, compound-specific high-precision Cu isotopic analysis are challenging. In this work, quantification and isotopic analysis of Cu were carried out in bulk serum and in both its exchangeable + ultrafiltrable (EXCH + UF) Cu fraction and its non-exchangeable + non-ultrafiltrable (NEXCH + NUF) fraction using quadrupole and multi-collector ICP-mass spectrometry, respectively. The EXCH + UF serum Cu represents the labile Cu pool, *i.e.* Cu loosely bound to proteins, such as albumin, alpha-2 macroglobulin and other low molecular weight compounds, while the NEXCH + NUF serum Cu contains the Cu firmly bound to ceruloplasmin (Cp). The method was evaluated using human, goat and fetal bovine serum and applied to serum samples from assumed healthy subjects and from patients with alcoholic liver cirrhosis (AC). The healthy subjects showed an isotopic composition of EXCH + UF serum Cu heavier (by on average + 0.4‰) than that of their total serum Cu. In general, patients with AC showed higher EXCH + UF serum Cu concentrations and significantly lower $\delta^{65}Cu_{EXCH+UF}$ and $\delta^{65}Cu_{serum}$ values than did healthy subjects. Within the AC population, $\delta^{65}Cu_{EXCH+UF}$ values were comparable to or lower than the corresponding $\delta^{65}Cu_{serum}$ values, potentially reflecting the extent of labile Cu deregulation. As to be expected, the NEXCH + NUF serum Cu isotopic composition was similar to that of the total serum Cu, as most of the serum Cu is firmly bound to Cp.

1. Introduction

Alcohol-related liver disease encompasses a spectrum of injuries, ranging from fatty liver (steatosis) to hepatitis and cirrhosis, which are not necessarily distinct stages of evolution of the disease [1–3]. Alcohol is metabolized through the alcohol dehydrogenase (ADH) pathway to acetaldehyde, which is further converted into acetate. Both reactions involve the reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH. Hence, excessive alcohol consumption can lead to the accumulation of NADH, which can affect the lipid metabolism by inhibition of fatty acid oxidation, which favors steatosis [4]. Steatosis is therefore the most frequent primary change associated with chronic alcoholic consumption [2,3]. Progression of the disease can be induced by the toxic effects of acetaldehyde and by oxidative stress generated by the ethanol-inducible CYP2E1, a key enzyme of the microsomal ethanol-oxidizing system (MEOS) [2,4].

Chronic liver disease has a profound effect on Cu status [5], as the liver controls the whole body Cu homeostasis [6]. Dietary Cu is absorbed through the intestinal mucosa and enters into the blood stream as albumin- or amino acid-bound Cu [6,7]. The majority of Cu present in the circulation is taken up by hepatocytes for incorporation into Cu-transporting P-type ATPase (ATP7B), Cu/Zn superoxide dismutase and cytochrome C oxidase [8]. Within hepatocytes, ATP7B is responsible for the Cu incorporation into ceruloplasmin (Cp) and the excretion of excess of this metal through the bile [6,9]. Cu can induce cellular toxicity due to its participation in the formation of reactive oxygen species (*via* Fenton-like reactions), which are known to contribute to the pathogenesis of liver cirrhosis. Liver and plasma concentration levels of metallothioneins (MT) are increased in liver disease associated with increased hepatic Cu concentrations [10,11] for detoxification of the accumulated Cu. MT are cysteine-rich proteins that also act as chaperone for Cu incorporation into several metalloenzymes [12].

* Corresponding author.
E-mail address: frank.vanhaecke@UGent.be (F. Vanhaecke).

https://doi.org/10.1016/j.talanta.2018.07.011
Received 2 April 2018; Received in revised form 1 July 2018; Accepted 5 July 2018
Available online 06 July 2018

It has been shown using multi-collector ICP-mass spectrometry (MC-ICP-MS) that liver disease affects the isotopic composition of serum Cu [13]. The lighter serum Cu isotopic composition observed in end-stage liver disease (ESLD) patients (compared to the reference population), was hypothetically attributed to biliary obstruction, non-efficient incorporation of Cu into Cp and/or redox condition changes [14,15]. Long-term monitoring of liver-transplant recipients revealed a normalization of the serum Cu isotopic composition (shift towards the reference range) when the outcome was successful, while it remained light in case of recurrence of liver failure or hepatocellular carcinoma (HCC) [15]. Trends towards lighter serum Cu isotopic composition were also observed in patients with cancer [16,17]. Also patients with Wilson's Disease (WD) show a lighter serum Cu isotopic composition than healthy individuals, probably caused by a low efficiency of Cu incorporation into Cp due to mutations in the ATP7B gene [18].

Isotopic analysis of Cu bound to specific proteins, contributing to the isotopic composition of the bulk serum Cu, could be valuable for unraveling the sources of the established isotope ratio shifts [19]. Most of the serum Cu is firmly bound to Cp (~ 90%), while the remaining Cu is loosely bound to albumin (Alb), alpha-2 macroglobulin (α2M) and amino acids. However, compound-specific isotopic analysis *via* MC-ICP-MS is not straightforward due to low analyte concentrations, high procedural blanks and/or high content of major elements introduced during protein separation strategies. One of the primary strategies used for separating Alb from Cp is based on Cibacron-blue affinity chromatography, but for this approach, a relatively large volume of a NaCl-containing buffer is required for elution of Alb [20]. Under such circumstances, the accuracy of Cu isotope ratio determination with MC-ICP-MS is jeopardized as the concomitant matrix will affect the extent of instrumental mass discrimination and will give rise to spectral interference, mainly caused by Na- and Mg-based molecular ions. Gel filtration chromatography strategies for speciation of protein-bound Cu are prone to trace metal contamination present in the gel and buffer, thus, requiring exhaustive cleaning steps before and after each separation process [21,22].

The exchangeable Cu (also called labile pool) in serum contains the Cu just absorbed from the gut and is considered as the form in which Cu^{2+} in blood is bioavailable for tissues. The significance of the exchangeable Cu under diseased conditions is not well understood [23]. The exchangeable + ultrafiltrable (EXCH + UF) serum Cu fraction represents the labile Cu fraction of serum and, thus, contains Cu loosely bound to proteins, mostly Alb and, to a lower extent, α2M and low molecular weight compounds, such as amino acids. In contrast, the non-exchangeable + non-ultrafiltrable (NEXCH + NUF) serum Cu fraction consists of Cu bound to Cp, and, thus, it forms the largest fraction of serum Cu. Determination of the EXCH + UF serum Cu concentration has been suggested useful for diagnosis of WD [24–26], but its concentration may also be elevated in acute liver failure (ALF) and chronic cholestasis [27]. Elevated EXCH + UF serum Cu can be indicative of abnormally high levels of toxic extrahepatic Cu [28]. For the determination of EXCH + UF serum Cu, a chelating agent such as EDTA is used for complexation of the loosely-bound Cu and the complexes thus formed are separated from the firmly-bound copper by ultrafiltration [25,29].

The aim of this study was to investigate the Cu concentration and the Cu isotopic composition of bulk serum, EXCH + UF serum and NEXCH + NUF serum Cu in healthy and alcoholic liver cirrhosis subjects. The protocol for separation of the EXCH + UF serum Cu fraction from the NEXCH + NUF was evaluated. Cu concentrations were determined by quadrupole-based ICP-mass spectrometry (Q-ICP-MS) and Cu isotope ratio measurements were accomplished using multi-collector ICP-mass spectrometry (MC-ICP-MS). Both concentrations and isotopic compositions of bulk serum and EXCH + UF serum Cu obtained for AC patients were compared to the corresponding data for healthy subjects.

2. Experimental

2.1. Materials and reagents

Amicon® Ultra-4 centrifugal filter devices with a 30-kDa cut-off Ultracel® regenerated cellulose membrane purchased from Merck Millipore (Cork, Ireland) were used for sample ultrafiltration. TraceSELECT ethylenediaminetetraacetic acid (EDTA) was acquired from Honeywell-Fluka (Seelze, Germany). Poly-Prep® polypropylene chromatographic columns and AG MP-1 strong anion exchange resin (100–200 mesh, chloride form) purchased from Bio-Rad (Temse, Belgium) were used for chromatographic Cu isolation.

Single-element standard solutions of various elements (1000 mg L^{-1}), acquired from Inorganic Ventures (Nieuwegein, The Netherlands), were used for quantification purposes. Ga was used as an admixed internal standard for (i) internal mass bias correction in Cu isotopic analysis and for (ii) correction for instrument instability and potential matrix effects in Cu quantification. The Cu isotopic reference material NIST SRM 976, purchased from National Institute for Standards and Technology (NIST, Gaithersburg, USA), was used for external mass bias correction in a standard-sample bracketing approach. The Cu standard solution (Inorganic Ventures, lot C2-Cu02116) used for quantification, was also used as an in-house isotopic standard for measurement quality control [30].

Ultrapure water (resistivity ≥ 18.2 MΩ cm) from a Milli-Q Element water purification system (Merck Millipore, Molsheim, France) was used throughout the study. *Trace analysis* grade 14 M nitric acid (PrimarPlus, Fisher Scientific, Loughborough, UK) was further purified in-house by sub-boiling distillation. *Optima* grade 12 M hydrochloric acid (Fisher Scientific) and ultra-pure 9.8 M hydrogen peroxide (Fluka) were used as such.

2.2. Samples

In this study, 5 samples were used for the evaluation of the method: 2 commercially available serum samples, *i.e.* goat serum (lot SLB0738V, Sigma-Aldrich, Overijse, Belgium) and fetal bovine serum (FBS, Greiner Bio One, Vilvoorde, Belgium), and 3 serum samples from patients with hemochromatosis (HH) under phlebotomy (thus making relatively large amounts of sample from each patient available). In addition, 21 middle-aged male volunteers were participating in the study: 14 patients with alcoholic liver cirrhosis (AC population) and 7 assumed healthy individuals (reference population).

Blood was collected in a Venosafe™ blood tube, containing a gel and clot activator. After collection, the blood samples were centrifuged at 3000 rpm for 10 min and aliquots of about 1.5 mL serum were subsequently transferred to pre-cleaned Eppendorf tubes and stored at −80 °C until sample preparation.

Ethical approval for this study was obtained from an independent commission for medical ethics connected to the Ghent University Hospital (UZGent, Belgium). The study was performed in accordance with the guidelines for good clinical practice and the statement of Helsinki, emplaced to protect volunteers participating in experiments. All patients and individuals forming the reference population signed an informed consent form.

2.3. Sample preparation

The serum fractions were split in three aliquots; one of these was used for determination of the total serum Cu concentration and bulk Cu isotopic composition, another one for separation of EXCH + UF and NEXCH + NUF serum, and the last one for the determination of Cp and Alb levels (using a BN II nephelometer, Siemens).

The protocol applied for separation of EXCH + UF and NEXCH + NUF serum Cu was based on those described by Favier et al. [25] and by El Balkhi et al. [29]. The serum samples were thawed at room