Journal of Chromatography B, 957 (2014) 41–45

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Short Communication

Measurement of concentrations of whole blood levels of choline, betaine, and dimethylglycine and their relations to plasma levels

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ARTICLE INFO

Article history:
Received 31 October 2013
Accepted 20 February 2014
Available online 28 February 2014

Keywords:
Choline
Betaine
Dimethylglycine
Whole blood
Homocysteine

ABSTRACT

We aimed at developing a method for the measurement of choline and its metabolites in whole blood (WB). After an extraction step, quantification of choline, betaine, and dimethylglycine (DMG) was performed using ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS). Plasma and WB metabolites were evaluated in a group of 61 elderly people. The calibration curves were linear ($r^2 > 0.997$) for all compounds. The inter- and intra-assay coefficients of variation for all analytes were <10%. The recoveries were >90% and the relative matrix effect were ≤4.0%. The median concentrations of choline, betaine, and DMG were 11.3, 27.8, and 5.9 μmol/L in plasma and 66.6, 165, and 13.7 μmol/L in WB, respectively. There were positive correlations between WB and plasma markers; for choline ($r = 0.42$), betaine ($r = 0.61$), and DMG ($r = 0.56$) (all $p \leq 0.001$). The concentrations of betaine in WB and plasma were significantly higher in men than in women. The concentrations of WB choline and DMG did not differ significantly according to sex. In conclusion, we have established a reliable method for measuring choline metabolites in WB. The concentrations of WB choline, betaine, and DMG seem to reflect intracellular concentrations of these metabolites.

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1. Introduction

Choline is an essential nutrient that is required for the biosynthesis of methyl groups, acetylcholine, phosphatidylcholine, and sphingomyelin [1]. Red blood cells (RBCs) contain significant amounts of choline [2]. Disturbed choline uptake and content have been observed in a number of physiological or pathological conditions [3–5]. For example, increased intracellular choline levels were reported in malignancies [6] and other diseases [7].

Choline is irreversibly oxidized into betaine, the methyl donor that converts homocysteine (Hcy) into methionine via betaine-homocysteine methyltransferase (BHMT). Betaine demethylation produces dimethylglycine (DMG) that indicates the flow of the methyl group from betaine to Hcy [8]. Betaine serves as a methyl donor and osmolyte [9]. A large variation in the relationship between plasma and tissue betaine has been reported [10].

Concentrations of WB choline, betaine, and DMG require further testing, since they have the potential to better reflect the stores or the metabolic condition in tissues. The aims of this study were: first, to extend and adapt an already existing ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) method for the quantification of WB choline, betaine, and DMG [11]; second, to test plasma and WB markers in a group of 61 elderly people.

2. Materials and methods

2.1. Standards and chemicals

The quantification of WB choline, betaine, and DMG was based on an earlier method by our group [11]. Choline chloride, betaine chloride, and *N*,*N*-dimethylglycine (Sigma–Aldrich, Munich, Germany) were used for preparation of standard solutions. d_9-Choline chloride, d_9-betaine chloride (Isotec, Sigma–Aldrich, Munich, Germany), d_6-dimethylglycine HCl (CDN isotopes, Quebec, Canada) were used as internal standards. Other chemicals used were: methanol, acetonitrile (UPLC/MS grade; Biosolve, Valkenswaard, The Netherlands), chloroform (Merck, Darmstadt,

Abbreviations: BHMT, betaine-homocysteine methyltransferase; CV, coefficient of variation; DMG, dimethylglycine; Hcy, homocysteine; IDL, instrumental detection limit; LOD, limit of detection; MRM, multiple reaction monitoring; RBC, red blood cell; tHcy, total homocysteine; UPLC–MS/MS, ultra performance liquid chromatography tandem mass spectrometry; WB, whole blood.

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http://dx.doi.org/10.1016/j.jchromb.2014.02.030
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Germany), and ultrapure water (18.2 MΩ) from a Milli-Q water purification system (Millipore, Molsheim, France).

2.2. Subjects and blood samples

The study included 61 elderly individuals (median age: 82 years, 11 males), which were not supplemented with choline or B-vitamins. The study was approved by the medical ethics commission of the Saarland and informed consents were obtained from all participants. The original clinical trial was conducted in accordance with the Declaration of Helsinki and was registered [ClincalTrial Number: NCT01105351].

Fasting blood samples were collected from all participants. Blood was collected both in potassium EDTA-containing tubes and in tubes without anticoagulant. The specimens were processed and stored as WB, EDTA plasma, and serum. Blood count was available from all participants. Blood samples were centrifuged within 30 min at 5500 × g and 4 °C for 10 min, plasma and serum were separated. All samples were immediately stored at −70 °C until analyses.

The plasma concentrations of choline, betaine, and DMG were measured as mentioned before [11]. Serum total Hcy (tHcy) was determined by gas chromatography–mass spectrometry (GC/MS) [12]. Serum creatinine concentrations were determined by Cobas System (Roche Diagnostics, Mannheim, Germany).

In house pools of WB and plasma for quality control purposes were prepared and stored as aliquots at −70 °C. A certain volume of WB pool was dialyzed against 100 volumes of phosphate-buffered saline containing 4 mmol/L EDTA (Sigma–Aldrich, Munich, Germany) using a Spectra/Por Float-A-Lyzer CE (MWCO: 8000 Dalton; Carl Roth GmbH, Karlsruhe, Germany). The dialyzed pool was used for the determination of the limit of detection (LOD).

2.3. Sample preparation and measurement

The preparation of stock solutions, calibrators, and quality control samples was described earlier [11]. Calibrators, quality control, WB, and plasma pool samples were included in each batch. The extraction of WB choline, betaine, and DMG was based on the method of Shryock et al. [13] with some modifications. Briefly, RBC was lyzed by freezing and thawing, 100 μL of WB hemolysate was added to 300 μL ice-cold methanol and mixed in an Eppendorf tube to precipitate the proteins. This was followed by 1 h incubation at 70 °C in a water bath to extract the analytes of interest [14]. Later 300 μL of chloroform was added to the mix at room temperature to improve the yield of the analytes by dissolving the lipid components. Finally, the resulting extract mixture was vacuum evaporated to dryness (Eppendorf-Concentrator plus) at room temperature. The residues were resuspended in 300 μL water, since the analytes of interest are soluble in water. Using water reduces any possible interference of lipids with subsequent mass spectrometry analysis [15]. After centrifugation for 5 min at 10,000 × g at room temperature, the aqueous supernatant was slightly red because of hemoglobin. 100 μL of the supernatant was added to 300 μL internal standard mix that has been prepared in acetonitrile according to the method described by Kirsch et al. [11]. Acetonitrile precipitates the unwanted proteins (e.g. hemoglobin) and is compatible with the mobile phase (87.5–80% acetonitrile). Samples were then centrifuged to pellet precipitated proteins. The supernatant was transferred to a final vial and 1 μL of this solution was injected into the Acquity ultra performance liquid chromatography system coupled to a MicroMass Quattro Premier XE mass spectrometer (Waters Corporation), using the same conditions mentioned before [11].

2.4. Data analyses

The data acquisition was performed using MassLynx version 4.1 and QuanLynx. The concentrations of the WB analytes were calculated as (measured concentration × dilution factor × 100)/hematocrit. The recovery (%) was calculated as [measured concentration/(expected concentration + concentration added)] × 100.

The LOD was determined in dialyzed WB pool by the $t_{99}s_{LLMV}$ method [16], using the following equation: LOD = $t_{99(n-1)} \times$ SD; where $t_{99(n-1)}$ is the one-tailed t statistic for $n-1$ observations at the 99% confidence level ($t_{99(n-1)} = 2.602$ for 16 aliquots or 15 degrees of freedom), and SD is the standard deviation.

The correlations between analytes in serum, plasma, and WB were determined by Pearson's test that was applied on the log-transformed data. Paired t-test was used to investigate differences between plasma and WB levels. Differences between groups were tested with the Mann–Whitney test. P values <0.05 were considered significant. Statistical analyses were performed using SPSS (version 18.0; SPSS, Chicago, IL, USA).

3. Results

3.1. Method validation

Optimal multiple reaction monitoring (MRM) transitions for the analytes were obtained in the positive electrospray ionization mode (Supplementary Fig. S1). The peaks of the analytes were well separated and free from evidence of ion suppression or enhancement. The validation results are shown in Table 1. The linearity of the assay was established earlier [11]. The correlation coefficient (r^2) was greater than 0.997 for all curves.

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2014.02.030.

To calculate the LOD, we utilized a two-step approach suggested by the US Environmental Protection Agency [16,17]. To estimate LOD, we used sixteen dialyzed WB pools and added concentrations between 1–5 times the IDL. Mean instrumental detection limits (IDLs) [11] and WB LODs of the analytes are presented in Table 1. The recovery of the assay was investigated by spiking the WB pool with two different concentrations of the analytes in the physiological range (Table 1).

The relative matrix effects were determined by calculating the standard line slopes of five different lots [18] of WB samples. After extraction, WB samples were spiked with all concentrations utilized for the construction of calibration curves, then 300 μL of internal standard mix was added. The addition of the stable isotope-labeled analytes effectively eliminated the relative matrix effect in WB extracts (relative matrix effect 4.0% or less) (Table 1).

The precision of the method was determined by measuring three control materials in each run: in-house prepared WB pool and quality control samples at two defined concentrations (low and high). The intra- and inter-assay coefficients of variation (CVs) are presented in Table 1. Intra-assay CVs for all analytes of WB pool ranged between 3.4% and 8.7% and the inter-assay CVs ranged between 6.1% and 9.3%. The analytes in WB samples were stable over at least 5 months at - 7070 °C and showed no remarkable changes over 4 freeze/thaw cycles (data not shown).

3.2. Metabolites in plasma and WB

The main characteristics of the study population and the concentrations of the metabolites are presented in Table 2. The concentrations of DMG, betaine, and choline were higher in WB

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