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A multiplexed targeted assay for high-throughput quantitative analysis of serum methylamines by ultra performance liquid chromatography coupled to high resolution mass spectrometry

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ABSTRACT

Methylamines are biologically-active metabolites present in serum and urine samples, which play complex roles in metabolic diseases. Methylamines can be detected by proton nuclear magnetic resonance (NMR), but specific methods remain to be developed for their routine assay in human serum in clinical settings. Here we developed and validated a novel reliable "methylamine panel" method for simultaneous quantitative analysis of trimethylamine (TMA), its major detoxification metabolite trimethylamine-*N*-oxide (TMAO), and precursors choline, betaine and L-carnitine in human serum using Ultra Performance Liquid Chromatography (UPLC) coupled to High Resolution Mass Spectrometry (HRMS). Metabolite separation was carried out on a HILIC stationary phase. For all metabolites, the assay was linear in the range of 0.25–12.5 μmol/L and enabled to reach limit of detection of about 0.10 μmol/L. Relative standard deviations were below 16% for the three levels of concentrations. We demonstrated the strong reliability and robustness of the method, which was applied to serum samples from healthy individuals to establish the range of concentrations of the metabolites and their correlation relationships and detect gender differences. Our data provide original information for implementing in a clinical environment a MS-based diagnostic method with potential for targeted metabolic screening of patients at risk of cardiometabolic diseases.

1. Introduction

Metabolic profiling technologies enable detection and quantification of low molecular weight compounds in biological samples to enhance our understanding of gene function, disease mechanisms and drug treatments [1]. They represent powerful high-throughput and high-density molecular phenotyping tools to uncover diagnostic and prognostic metabolic biomarkers [2]. Technological and methodological advances in the field provide opportunities in clinical settings to profile patient metabolism [3] and in genetic research to identify metabolites associated with complex diseases [4,5].

There is increasing interest in methylamines in clinical and fundamental research. Variations in trimethylamine (TMA), the product of microbial metabolism of choline, and its detoxification

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metabolite, trimethylamine-*N*-oxide (TMAO), have been associated with nephrotoxin treatments [6], microbial colonization of germ-free rats [7], insulin resistance in mice [8] and cardiovascular disease in humans [9–11]. Butyrobetaine, another metabolite in this pathway derived from dietary L-carnitine, has also been proposed as a marker for atherosclerosis [12]. These findings suggest that the search for disease-associated metabolite biomarkers and gut microbial-mammalian co-metabolites should be extended beyond TMAO to other metabolites in the methylamine pathway.

Untargeted high field proton nuclear magnetic resonance (NMR) spectroscopy is widely used for TMAO and choline analysis [13,14] but detection limits (around 0.5×10^{-5} M) make TMA quantification difficult in blood compared to mass spectrometry (MS). LC-MS/MS methods have been developed to individually assay TMAO [15], choline and betaine [16] and L-carnitine and acylcarnitines compounds [17]. In addition, methods for quantitative analysis of TMA/TMAO and L-carnitine (Fast Atom Bombardment mass spectrometry (FAB-MS) [18,19], Metastable Atom Bombardment (MAB-MS) [20,21], Matrix-Assisted Laser Desorption-Time-of-Flight (MALDI-TOF) [22]) use analytical instruments uncommon in clinical laboratories. GC-MS has also been used for indirect quantification of TMAO [23–25], but it requires TMA reduction and derivatization and is therefore time-consuming. Finally, flow injection electrospray ionization-mass spectrometry (FIA-MS) [26,27] prevents addition of other compounds in the method without prior chromatographic technique.

Here we developed a multiplexed MS-based method, which can be applied in both research and clinical settings for simultaneous quantitative analysis of TMA and four quaternary amine compounds (betaine, choline, TMAO and L-carnitine). It is based on HILIC ultra performance liquid chromatography coupled with high-resolution mass spectrometry (UPLC-HRMS). We assessed the sensitivity and reliability of the method, and tested its applicability in a group of healthy individuals.

2. Material and methods

2.1. Chemicals and reagents

Certified pure trimethylamine hydrochloride (TMA), trimethylamine-*N*-oxide dihydrate (TMAO), betaine hydrochloride, L-carnitine hydrochloride, and choline chloride were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The corresponding stable isotopes used as internal standards of trimethylamine-^{13}C, ^{15}N, choline-d_4, betaine-d_3 and L-carnitine-methyl-d_3 were also obtained from Sigma-Aldrich. d_3 labelled L-carnitine was purchased from Cluzeau Info Labo (Sainte Foy La Grande, France) and d_9 labelled TMAO from Euriso-tope (Saint-Aubin, France). Formic acid and ammonium formate were LC-MS Chromasolv® Fluka, HPLC quality and purchased from Sigma-Aldrich. Ultra pure water (resistivity: 18 mΩ) was obtained with a Milli-Q Integral purification system (Millipore, Molsheim, France) with a 0.22 μm filter. Human and bovine sera were obtained from Life technologies (Saint-Aubin, France).

2.2. Preparation of standard and calibration solutions

Stock standard solutions were prepared in acetonitrile (CH_3CN) for TMA, TMAO, choline and betaine at a concentration of 20mmoL/L. L-carnitine was dissolved in a mixture of acetonitrile/water (95:5) at a concentration of 20 mmol/L. Working solutions of the reference compounds mixture were prepared at concentrations of 100, 50 and 25 μmol/L. Individual solutions for each stable isotope, associated to each native compound were prepared in the same conditions. A working solution mixture of stable isotope standards was set at a concentration of 100 μmol/L. All standard solutions were stored at −20 °C.

2.3. Serum sample collection and preparation

Human serum samples were prepared from blood of 67 healthy individuals (39 males and 28 females aged 24–59 years) who were recruited from Boston area clinics and community health care centers. This study was approved by the institutional review board of Massachusetts General Hospital and all individuals involved provided informed consent to participate. Work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Samples were stored at −80 °C until analysis. Sample preparation was based on protein precipitation and liquid-liquid extraction with acetonitrile (1:9, *v:v*). Proteins were eliminated by centrifugation and the supernatant was injected for analysis. Experiments were carried out with 50 μL serum aliquots which were spiked with 100 μL of a mixture of internal standards before completing to 500 μL with acetonitrile. Samples were vortexed at 2500 rpm during 3 min with an automatic shaker (Heidolph©, VWR, Fontenay-sous-bois, France) and centrifuged at 4100 g. Sample extracts were then transferred into vials for injection on the analytical system.

2.4. Liquid chromatography

Liquid chromatography was carried out on a Waters Acquity UPLC® (Waters Corp, Saint-Quentin en Yvelines, France) equipped with a sample manager, a binary solvent delivery system and a PDA detector. The flow rate was 750 μL/min and the injection volume was 5 μL. The autosampler vial tray and the column temperatures were set at 5 °C and 50 °C, respectively. The needle was washed with a mixture of acetonitrile, isopropanol and water (1:2:1 *v:v:v*). The system was fitted with an Acquity BEH HILIC column (2.1 × 150 mm, 1.7 μm) and a corresponding guard column (ACQUITY BEH HILIC 1.7 μm) purchased from Waters®. The mobile phase consisted of 10 mM ammonium formate and 0.6% of formic acid (v/v) in water (A) or in 95:5 (v/v) CH_3CN:Water (B). Mobile phase for HILIC chromatography was prepared by dissolving the appropriate amount of ammonium formate in water before mixing with acetonitrile. The HILIC gradient started at an initial composition of 100% solvent A for 2 min, followed by a 4 min linear gradient up to 30% of solvent A, which was held for 1 min before returning to initial conditions in 1 min. The column was thoroughly conditioned during 6 min until the next injection, for a total chromatographic run time of 14 min.

2.5. Mass spectrometry

The chromatographic system was coupled with a Q-Exactive™ hydrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Illkirch, France). Instrument calibration was performed by infusing a calibration mixture (caffeine, MRFA and Ultramark® 1621). A heated-electrospray HESI-II interface was used with the following parameters: S-Lens 80 V, Sheath gas: 50, Auxiliary gas: 20 arbitrary units, capillary voltage 3.5 kV, capillary temperature 255 °C and vaporization temperature 320 °C. The maximum target capacity of the C-trap (AGC) target was defined as 3e6 and the maximum injection time was set to 200 ms. Full scan was acquired in positive ion mode with a resolution of 70 000 FWHM, in the scan range of *m/z* 50–400. The Xcalibur Quanbrowser software (Thermo Fisher Scientific, Illkirch, France) was used for quantification. Targeted analyte signals were extracted with a mass window accuracy <0.5 ppm.