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Simultaneous determination of trimethylamine *N*-oxide, choline, betaine by UPLC–MS/MS in human plasma: An application in acute stroke patients

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ABSTRACT

Trimethylamine-*N*-oxide (TMAO) is derived from the gut microbiome and tissues metabolism of dietary choline and betaine. These molecules are closely related to the development of cardiovascular and cerebrovascular diseases. A rapid, sensitive and accurate method has been developed and validated for the simultaneous determination of trimethylamine *N*-oxide (TMAO), choline and betaine in human plasma using d9-trimethylamine *N*-oxide (TMAO), d9-choline, d9-betaine as the internal standard (IS). After methanol precipitation with 10 μL plasma samples, the analytes were extracted and then separated on Amide column (2.1 × 100 mm, 1.7 μm, waters) with an isocratic elution program consisting of acetonitrile-water (containing 10 mM ammonium formate pH = 3.0) at a flow of 400 μL/min. The detection was achieved under the selected reaction monitoring (SRM) scan using positive electrospray ionization (ESI+) in 3 min. The mass transitions monitored were as follows: *m/z* 76.3 → 58.4 for TMAO, *m/z* 104.2 → 60.3 for choline, *m/z* 118.1 → 58.3 for betaine, *m/z* 85.1 → 66.3 for d9-TMAO, *m/z* 113.2 → 69.3 for d9-choline, and *m/z* 127.1 → 67.2 for d9-betaine, respectively. The method has been fully validated for specificity, lower limit of quantification, linearity, stability, intra- and inter-day accuracy and precision. This assay combines simple sample processing with a short run time and small plasma volumes, making it well suited for high-throughput routine clinical or research purposes. The newly developed method was successfully applied to the patients (n = 220) suffered from acute stroke, and the concentration of choline was firstly found to be closely related with the prognosis of these patients.

1. Introduction

Choline and betaine are important human nutrients that can be obtained from daily diet involving fish, meat and eggs which are rich in phospholipids. In the gut, choline and betaine can be metabolized into trimethylamine (TMA) by intestinal flora. Trimethylamine (TMA) is then transferred into the liver and further oxidized by flavin containing monooxygenase 3 (FMO3) to the proatherogenic form TMAO [1–4]. Interestingly, it was found that the plasma concentrations of TMAO, choline and betaine were closely related with the development of cardiovascular disease such as stroke and myocardial infarction [1]. The similar results were also found in recent years [4–6]. It was reported that TMAO could promote platelet hyperresponsiveness and increased the risk of thrombosis [7]. And inhibition of plasma TMAO levels with the analog 3, 3-dimethyl-1-butanol (DMB) could reduce the risk of atherosclerosis in mice with high-choline diet [8]. However, the association between the gut microbiota-generated metabolites and the prognosis in acute stroke patients has not been investigated yet until recently. In view of the study of the role of TMAO, choline and betaine in the pathological processes of cardiovascular and cerebrovascular diseases, it is of great importance to develop a rapid and robust method for simultaneous quantification of these potential markers for clinical or research purpose.

Up till now, several methods have been used to determine TMAO, choline or betaine, mainly including fast atom bombardment mass spectrometry (FAB-MS) [9], flow injection electrospray ionization tandem mass spectrometry [10], direct infusion electrospray quadrupole time-of-flight mass spectrometry and stable isotope dilution liquid chromatography tandem mass spectrometry

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(HPLC–MS/MS) [11–15]. Only few ultra-performance liquid chromatography tandem mass-spectrometry (UPLC–MS/MS) methods have been developed to determine TMAO and the related metabolites until recently [16–19]. These LC–MS methods have been used for the measurement of the metabolites in different matrices including plasma, urine and seawater. Chromatographic separation is often performed by Silica- [12], HILIC- [13,16–18], Amide columns [15,19]. However, these methods have some limitations in terms of processing complexity, running times, or detection range.

In this study, a simple, sensitive and specific method was established and validated for the simultaneous quantification of TMAO, choline and betaine in human plasma from acute stroke patients. The volume of the plasma samples was small (10 μL) and matrix effects were minimized. In addition, the detection range was widened and the running time was shortened as well. The newly developed method was successfully applied to detect the plasma from acute stroke patients. Furthermore, it was firstly found that the concentration of choline was closely related with the prognosis of patients suffered from acute stroke.

2. Materials and methods

2.1. Chemicals and reagents

Trimethylamine N-oxide (TMAO), choline, and betaine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deuterated internal standards (d9-trimethylamine N-oxide, d9-choline, d9-betaine) were also purchased from Sigma-Aldrich. Methanol, formic acid and acetonitrile were purchased from Tedia Company Inc. Ultrapure water was prepared with a Milli-Q Advantage A10 system (Millipore, MA, USA). All other chemicals were of analytical grade. Blank plasma was from healthy volunteers with heparin as anticoagulant.

2.2. Instrument and chromatographic conditions

The UPLC (Thermo Fisher Scientific Inc., Boston, USA) system was made up with Ultimate 3000 RSLC system equipped with binary pumps and S Surveyor autosampler (Thermo Fisher Scientific Inc., Boston, USA). The treated sample (10 μL) was injected to the system on a Waters Acquity BEH Amide (2.1 × 100 mm, 1.7 μm) column. The flow rate was set as 0.4 mL/min and composed of Water (A) (containing 10 mM ammonium formate, pH = 3.0) and acetonitrile (B) as the mobile phase. The condition of isocratic elution was set to 60% B and the total running time was 3 min. All the samples were kept in the auto-sampler at 10 °C.

The triple-quadrupole tandem mass spectrometric detection was performed on a TSQ Quantum Acess Max API mass spectrometer (Thermo Fisher Scientific Inc, Boston, USA) furnished with an electrospray ionization (ESI) interface. Under selected reactions monitoring (SRM), quantification was obtained using positive ion pairs at m/z transitions of m/z 76.3 → 58.4 for TMAO, m/z 104.2 → 60.3 for choline, m/z 118.1 → 58.3 for betaine, m/z 85.1 → 66.3 for d9-TMAO, m/z 113.2 → 69.3 for d9-choline, and m/z 127.1 → 67.2 for d9-betaine, respectively (Fig. 1). The optimized parameters were as follows: heated capillary temperature: 350 °C; spray voltage: 3500 v; sheath gas (nitrogen): 30 psi; the auxiliary gas (nitrogen): 5 psi; the collision gas (argon): 1.5 mm Torr; the collision energy: 13 eV for TMAO; 14 eV for d9-TMAO; 21 eV for choline; 21 eV for d9-choline; 21 eV for betaine; 22 eV for d9-betaine. The data acquisition and procession was performed with Thermo Scientific Xcalibur 2.0.7 SP1 data system.

2.3. Preparation of standard and quality control (QC) samples

The stock solutions of TMAO, choline, and betaine were precisely weighed and dissolved with methanol to obtain the final concentration of 1 mg/mL, respectively. The stock solution was then serially diluted with phosphate buffer saline (PBS) to obtain a series of standard mixture working solutions; the concentrations were as follows: 5–10000 ng/mL for TMAO; 10–10000 ng/mL for choline; 50–10000 ng/mL for betaine. D9-choline, d9-betaine, d9-TMAO were also prepared in methanol with a final concentration of 1 mg/mL, respectively. The mix IS working solution (500 ng/mL) was diluted with methanol.

Furthermore, the standard calibration curves were prepared by adding 10 μL of the working solution and 10 μL of IS working solution to 200 μL methanol. QC samples were prepared in the same way at low, middle, and high concentrations (15, 500, 8000 ng/mL for TMAO; 30, 500, 8000 ng/mL for choline; 150, 1000, 8000 ng/mL for betaine) with the same operation listed above. All the solutions were kept at −80 °C.

2.4. Sample preparation

Plasma samples (10 μL) from acute stroke patients and 10 μL of IS solution (500 ng/mL) were put into a 1.5 mL Eppendorf tube. Then 200 μL of methanol was added and the mixture was vortex-mixed for 2 min, followed by centrifugated at 15,000 rpm at 4 °C for 5 min. The supernatant was transferred to another tube and evaporated to dryness using a vacuum drying concentrator at 25 °C. Finally, the drying residue was dissolved in100 μL of methanol-acetonitrile (25:75, v: v), followed by vortex-mixed for 2 min, and then centrifuged at 15000 rpm for 5 min. The corresponding supernatant was transferred to an auto-sampler vial with an insert (LVI, 150 μL, Waters) and 20 μL was injected into the UPLC–MS/MS system for analysis.

2.5. Method validation

2.5.1. Linearity of calibration curves and LLOQ

Calibration curves were determined by assaying standard PBS samples at different concentration levels. For each curve, the peak area ratio (Y) of three different compounds to corresponding stable isotope labeled IS versus the nominal concentration (χ) of analytes were calculated with weighted ($1/\chi^2$) least square linear regression. The LLOQ for different analytes were determined by the lowest concentrations with signal to noise ratio of 10:1 which were repeated triplicates, and the acceptable levels were accuracy (RE ± 20%) and precision (RSD < 20%).

2.5.2. Precision and accuracy

To evaluate the precision and accuracy, low, middle and high concentrations of QC samples were analyzed in 6 replicates on the same day and another two consecutive days. Precision was determined as relative standard deviation (RSD) of the measured concentration and the accuracy as the relative error (RE) of the mean measured concentration that were deviated from the nominal value.

2.5.3. Recovery and matrix effect

The recoveries of TMAO, choline and betaine were determined by analyzing the plasma samples from a healthy volunteer spiking at the three QC concentrations (low, middle and high). The spiking samples and baseline (unspiking) were analyzed by the calibration standard curves. The recoveries were calculated by comparing the norminal concentrations (baseline concentration + spiking QC concentrations) to the measured concentrations. The carryover was evaluated by injecting a blank of methanol without analytes after