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Measurement of trimethylamine-*N*-oxide by stable isotope dilution liquid chromatography tandem mass spectrometry

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ABSTRACT

Trimethylamine-*N*-oxide (TMAO) levels in blood predict future risk for major adverse cardiac events including myocardial infarction, stroke, and death. Thus, the rapid determination of circulating TMAO concentration is of clinical interest. Here we report a method to measure TMAO in biological matrices by stable isotope dilution liquid chromatography tandem mass spectrometry (LC/MS/MS) with lower and upper limits of quantification of 0.05 and >200 μM, respectively. Spike and recovery studies demonstrate an accuracy at low (0.5 μM), mid (5 μM), and high (100 μM) levels of 98.2, 97.3, and 101.6%, respectively. Additional assay performance metrics include intraday and interday coefficients of variance of <6.4 and <9.9%, respectively, across the range of TMAO levels. Stability studies reveal that TMAO in plasma is stable both during storage at −80 °C for 5 years and to multiple freeze thaw cycles. Fasting plasma normal range studies among apparently healthy subjects ($n = 349$) show a range of 0.73–126 μM, median (interquartile range) levels of 3.45 (2.25–5.79) μM, and increasing values with age. The LC/MS/MS-based assay reported should be of value for further studies evaluating TMAO as a risk marker and for examining the effect of dietary, pharmacologic, and environmental factors on TMAO levels.

Trimethylamine-*N*-oxide (TMAO)[1] is a gut microbiota -dependent-generated metabolite of certain dietary trimethylamine containing compounds, including choline, phosphatidylcholine (lecithin), and carnitine [1,2]. Several recent clinical studies demonstrate that systemic levels of trimethylamine-*N*-oxide are independently associated with cardiovascular risks, including incident risks for myocardial infarction, stroke, and death [1–3]. Of interest, TMAO appears to be more than simply a biomarker of risk since it is mechanistically linked to the development of atherosclerosis and cardiovascular disease through changes elicited in cholesterol and bile acid metabolism in multiple compartments [1,2]. Thus, the development of a rapid and accurate method for the quantification of circulating levels of TMAO is of clinical interest.

Several methods for quantifying TMAO in biological matrices have been reported. Early gas chromatography–mass spectrometry (GC/MS) studies employed a complicated multistep derivatization protocol that first reduced TMAO to the gas trimethylamine (TMA), which was then derivatized with 2,2,2-trichloroethyl chloroformate and detected as *N,N*-dimethyl-2,2,2-trichloroethyl carbamate [4]. In addition to being labor intensive, this method has multiple limitations, including the high volatility of TMA complicating its quantification, and the fact that the method does not easily discriminate among TMA, dimethylamine, and TMAO since all give the same product. Thus, TMAO levels with this common assay method are calculated as the difference in product formed in samples run twice before and then following reduction. More recent studies have reported TMAO analyses in urine samples employing fast atom bombardment mass spectrometry or electrospray quadrupole time-of-flight mass spectrometry [5,6]. While specific, critical assay performance metrics were not reported, and the mass spectrometry platforms employed are not widely used as routine analytical instruments in diagnostic laboratories. Additional reports have used either nonsuppressed ion chromatography with determination of organic nitrogen or NMR for the determination of blood TMAO [7,8]. The utility of these platforms for the high-throughput demands of a clinical diagnostic TMAO test has not yet been assessed. We and others have reported TMAO quantification in human and animal plasma, serum, or urine by LC/MS/MS without derivatization [1–3,9]. However, detailed methodology amenable for implantation of an assay as a high-throughput

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[1] *Abbreviations used:* CID, collision-induced dissociation; MRM, multiple reaction monitoring; TMA, trimethylamine; TMAO, trimethylamine-*N*-oxide.

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clinical diagnostic test has not yet been reported. In the present report we describe a rapid, sensitive and accurate approach for the quantification of TMAO in biological matrices using stable isotope dilution liquid chromatography with on-line electrospray ionization tandem mass spectrometry (LC/MS/MS).

Materials and methods

Reagents

Deuterated trimethylamine-*N*-oxide (d_9-TMAO) was purchased from Cambridge Isotope Laboratories (Cat. No. DLM-4779-1, Andover, MA). All other reagents were purchased from either Sigma-Aldrich (St. Louis, MO) or Fisher Scientific Chemicals (Pittsburg, PA) unless otherwise stated.

Research subjects

Samples and associated clinical data were collected from fasting subjects undergoing community health screens. All subjects gave written informed consent and the Institutional Review Board of the Cleveland Clinic approved all study protocols.

Sample processing

Samples (20 μl plasma or serum) were aliquoted to a 1.5 ml Eppendorf tube and mixed with 80 μl of 10 μM internal standard comprised of d_9-TMAO in methanol. Protein in the samples was precipitated by vortexing for 1 min and then the supernatant was recovered following centrifugation at 20,000 *g* at 4 °C for 10 min. In general, when analyzing TMAO levels, we run 3 different quality control (QC) samples with TMAO concentrations ranging between 0.25 and 20 μM in duplicate before each sample batch of less than 30 samples. TMAO concentrations from the batch were acceptable when the accuracy of the values determined from each QC sample were within 100 ± 10% of their expected values and the intrabatch CVs for the same QC samples were all less than 10%.

LC/MS/MS

Supernatants (10 μl) were analyzed by injection onto a silica column (4.6 × 250 mm, 5 μm Luna silica; Cat. No. 00G-4274-E0, Phenomenex, Torrance, CA) at a flow rate of 0.8 ml min^{-1} using a 4 LC-20AD Shimadazu pump system, SIL-HTC autosampler, and dual column switching valve system (2 Rheodyne 6 port automated valves, Cat. No. MXP7900, IDEX Health & Science, Oak Harbor, WA) [10] interfaced with an API 4000 Q-TRAP mass spectrometer (AB SCIEX, Framingham, MA). A discontinuous gradient was generated to resolve the analytes by mixing solvent A (0.1% propanoic acid in water) with solvent B (0.1% acetic acid in methanol) at different ratios starting from 2% B linearly to 15% B over 10 min, then linearly to 100% B over 2.5 min, then hold for 3 min, and then back to 2% B. TMAO and d_9-TMAO were monitored using electrospray ionization in positive-ion mode with multiple reaction monitoring (MRM) of precursor and characteristic product-ion transitions of *m/z* 76 → 58 and 85 → 66 amu, respectively. The parameters for the ion monitoring were as follows: spray voltage, 4.5 kV; curtain gas, 15; GS1, 60; GS2, 50; CAD gas, medium; DP, 60; CE, 25.0 V for TMAO and 28.3 V for d_9-TMAO; CXP, 10; EP, 10. Nitrogen (99.95% purity) was used as the source and collision gas. Various concentrations of nonisotopically labeled TMAO standard were spiked into control plasma to prepare the calibration curves for quantification of TMAO. The internal standard d_9-TMAO was used for quantification as well as to calculate recovery rate of TMAO (which was 99 ± 1% based on separate control studies).

Precision, accuracy, limit of quantitation, and linearity

Four replicates were performed on a single day to establish the intraday coefficient of variation (CV) for 6 different pooled plasma samples with different concentrations of TMAO. The interday CV was determined by assaying aliquots of these pooled samples daily over a span of more than 20 days. Carryover between injections was not observed. Accuracy is expressed as the ratio of the TMAO concentration measured to the TMAO concentration added to a plasma sample that was dialyzed against 1000 vol of 0.89% NaCl for 3 times. The lower limit of detection (LLOD) was defined as the lowest concentration of TMAO spiked into the dialyzed plasma on-column generating a signal-to-noise ratio of 3. The lower limit of quantification (LLOQ) was determined by reducing the concentration of standard solution gradually to the dialyzed plasma and is expressed as the lowest concentration yielding a signal-to-noise ratio of 10. No upper limit of quantification (ULOQ) was found, with linear responses noted in serially diluted samples spiked with increasing the concentration of standard solution as long as the diluted sample (concentration, peak area ratio of TMAO/d_9-TMAO) was still situated on the standard curve. To determine assay linearity, a standard curve over the 0.01–200 μM concentration range was checked for linearity by linear regression fit. The linear range was defined as the region of the standard curve where the difference between calculated TMAO concentration and standard TMAO concentration was less than 15%.

Results

Optimization of parameters for mass spectrometric assay of TMAO and d_9-TMAO

In order to determine the TMAO concentration in human blood, we first optimized the parameters for TMAO and its isotope labeled internal standard, d_9-TMAO, by direct infusion of these two standards. Shown in Fig. 1A and B are the collision-induced dissociation (CID) mass spectra of TMAO and d_9-TMAO, which are fragmented with collision energy of 25 eV. The product ions are 58 and 59 amu for TMAO, which are due to loss of water and hydroxyl group, respectively, as are the product ions for d_9-TMAO at 66

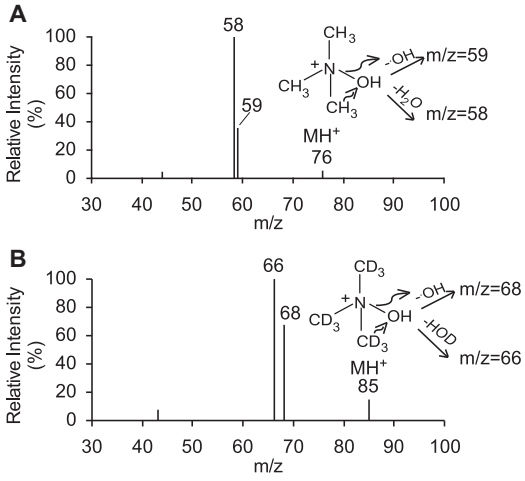

Fig.1. Collision-induced dissociation (CID) mass spectra of trimethylamine-*N*-oxide (TMAO, Panel A) and trimethylamine-*N*-oxide-d_9 (d_9-TMAO, Panel B). Proposed pathways for formation of the fragment ions are also shown.