A validated simple LC-MS/MS method for quantifying trimethylamine N-oxide (TMAO) using a surrogate matrix and its clinical application

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ABSTRACT

Trimethylamine N-oxide (TMAO) is a small molecular amine oxide generated from dietary choline and carnitine through intestinal microbial metabolism. Recently, TMAO has attracted much public attention as its role in disease progression has been proven in many clinical studies. The plasma concentration of TMAO in humans was found to be positively associated with the increased risk of many diseases including cardiovascular diseases and chronic kidney diseases. To achieve accurate and sensitive quantitation of TMAO for clinical applications, we established and validated a simple quantitative method using a liquid chromatography tandem mass spectrometry (LC-MS/MS) system. We constructed an eight-point calibration curve in an artificial surrogate matrix instead of the commonly used biological matrices to avoid interference from the endogenous TMAO. The calibration curve showed excellent linearity in the range of 1 to 5,000 ng/mL, with a correlation coefficient ($R^2$) higher than 0.996 in each validation batch. Moreover, both the intra-day and inter-day assays achieved satisfactory precision and accuracy results ranging from 1.65– 7.15% and 96.36– 111.43%, respectively. Further, this method was cross-validated using a human plasma matrix and applied to a clinical pharmacology study. Overall, these results demonstrate that the developed quantitation method is applicable in clinical research for monitoring disease progression and evaluating drug effects.

Keywords: TMAO; Biomarkers; Liquid Chromatography; Tandem Mass Spectrometry

INTRODUCTION

Currently, trimethylamine N-oxide (TMAO) has attracted public attention because of its potential as a clinical biomarker and therapeutic target for many diseases [1-3]. TMAO is a downstream oxidative product of trimethylamine (TMA), a gut microbial metabolite generated from dietary choline, betaine, and carnitine [4]. TMAO levels are positively correlated with disease progression in humans from the perspectives of immunity, inflammation, and cholesterol metabolism [5,6]. High levels of TMAO in human blood have been reported to elevate the risk of coronary heart disease (CHD) [7], chronic kidney diseases...
Conflict of Interest
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Author Contributions
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TMAO is mostly measured in blood and urine samples using mass spectrometry. Generally, a large sample volume and time-consuming derivatization procedures are needed when using gas chromatography-mass spectrometry (GC-MS). However, direct detection of TMAO is not possible with GC-MS as TMAO needs to be reduced to TMA, which has high volatility during derivatization [15, 16]. In this study, we used a liquid chromatography tandem mass spectrometry (LC-MS/MS) system for TMAO quantitation because of its effective sample preparation steps as well as its high accuracy and sensitivity.

However, one of the considerable challenges of TMAO quantitation is the high concentration of endogenous TMAO in typical biological matrices such as plasma or serum [17]. In this case, to avoid endogenous interference, a surrogate matrix is more suitable for sensitive quantitation of low-concentration samples [18]. Therefore, to quantify the biomarker TMAO more sensitively and accurately for clinical applications, we sought to generate calibration standard curves in a surrogate matrix instead of human plasma. Thus, in this study, we established and validated an LC-MS/MS method for TMAO quantitation, which is time-saving and accurate.

METHODS

Chemicals and reagents
Trimethylamine N-oxide (TMAO, 95%), internal standard Trimethylamine-d<sub>9</sub> N-oxide (TMAO-d<sub>9</sub>, 98%), 1.0 M phosphate buffer solution (PBS, pH 7), bovine serum albumin (BSA, 98%), and ammonium acetate (98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile (ACN, 99.9%) and water in HPLC grade were purchased from J.T. Baker (Phillipsburg, NJ, USA). Human blood plasma was procured from Seoul National University Hospital, Seoul, South Korea.

Preparation of solutions and surrogate matrix
Stock solutions of TMAO and TMAO-d<sub>9</sub> were accurately weighed and dissolved at a concentration of 1 mg/mL in HPLC grade water. The stock solution of TMAO-d<sub>9</sub> was further diluted into 500 ng/mL by water as the internal standard solution. 50% ACN was used to serially dilute the stock solution of TMAO to prepare various concentrations of working solution of TMAO. BSA dissolved in PBS solution was proved as a relatively ideal substitute for plasma or serum matrix. Therefore, the surrogate matrix used in this study was prepared by dissolving BSA in PBS solution at an approximate concentration of 40 mg/mL.

Preparation of calibration standard and quality control solutions
For calibration standard (STD), TMAO was spiked at final concentrations of 1, 10, 50, 250, 750, 1,500, 3,000, and 5,000 ng/mL by adding 20 µL of TMAO working solution into 380 µL surrogate matrix. For quality control (QC), TMAO was spiked at final concentrations of

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1 ng/mL (LLOQ, lower limit of quantification), 3 ng/mL (LQC, low quality control), 600 ng/mL (MQC, medium quality control) and 4,000 ng/mL (HQC, high quality control) by adding 20 µL of TMAO working solution into 380 µL surrogate matrix with 5 replications in each concentration. To ensure that the addition of TMAO exceeded the endogenous amounts, the QC samples in plasma (PQC) were prepared at concentration of 1,000 ng/mL (PLQC, low quality control in plasma), 2,500 ng/mL (PMQC, medium quality control in plasma) and 5,000 ng/mL (PHQC, high quality control in plasma) by adding 20 µL of TMAO working solution into 380 µL five random plasma from different volunteers in each concentration.

Sample preparation
Sample preparation was achieved by simple protein precipitation. 10 µL of TMAO-d9 (IS, 500 ng/mL) was added to 50 µL of each sample and the mixture was then extracted by 200 µL ACN. Next, the new mixture was vortexed at room temperature for 10 min, and then centrifuged at 14,000 rpm at 4 °C for 5 minutes. After then, 100 µL supernatant was transferred into a new tube and mixed with 100 µL 30% ACN solution. Finally, 100 µL of the new mixture was taken into an HPLC vial and 5 µL was injected into analyze system.

Instrumental conditions
To identify and quantify TMAO, samples were analyzed by an LC-MS/MS system which is equipped with an Agilent 1260 Infinity LC system and an Agilent 6490 triple quadrupole mass spectrometer (Santa Clara, CA, USA). A Gemini-NX C18 column (100 × 3 mm, 3 µm) purchased in Phenomenex (Torrance, CA, USA) was connected in the LC system for separation of the analytes. Mobile phases were prepared for two pumps. Pump A was a 5 mM ammonium acetate solution, pump B was acetonitrile. The gradient elution started with 70% A at 0.00 min, linearly reducing to 20% at 1.50 minutes and returning to 70% at 2.7 min until 6.00 min. The column oven temperature was maintained at 40°C and the mobile phase flow is 0.3 mL/min.

Multiple reaction monitoring (MRM) mode was performed under a positive electrospray ionization (ESI). The capillary voltage was set to +3000 V and the nozzle voltage is +1500 V. Nitrogen was applied as a nebulizer gas of 20 psi, a carrier gas of 14 L/min at 200 °C, and a sheath gas of 10 L/min at 350 °C. The monitored transitions (corresponding collision energy) were as follows: TMAO [76.2 → 58.2 (20 V)], TMAO-d9 [85.3 → 66.2 (20 V)] (Fig. 1).

Validation
According to the FDA guideline for the validation of bioanalytical methods, partial validations for this method consisted of linearity, sensitivity, selectivity, carry-over, extraction recovery, accuracy, and precision were carried out [19]. An eight-point calibration curve was constructed by plotting the relative response of each calibration standard to the internal standard using 1/x^2 as a weight factor. The linearity of the calibration curve in each batch was evaluated by calculating the correlation coefficient (R^2). The mean response of TMAO in five LLOQ and a zero sample (processed surrogate matrix with internal standard) was compared to assess sensitivity in each inter-day batch.

Selectivity was evaluated by comparing the mean response of six individual blank samples (processed surrogate matrix) and six individual LLOQ samples. To evaluate the carry-over of this method, the deviation of the responses of a zero sample before and after the detection of the highest calibration standard was calculated. Carry-over was evaluated in five replicates.

For calculation of extraction recovery, responses of pre-extracted and post-extracted three
different levels of QC samples were compared with six replications. The extraction recovery in the same levels of PQC samples was also calculated, and the results were compared with the QC data to obtain the extraction recovery ratio of this extraction method.

The precision and accuracy of this quantitation method were assessed by analyzing the four different levels of QC samples in the surrogate matrix. To prove that this surrogate matrix is applicable for human plasma samples, the precision and accuracy of three different levels of PQC were also evaluated for cross validation. The precision and accuracy of PQC samples were evaluated in the same validation batch. In addition, both intra-day and inter-day of precision and accuracy were evaluated in this study. The intra-day results were obtained by evaluating five replicates of each QC and PQC sample described above in the surrogate matrix. The inter-day assay was performed by repeating the intra-day validation procedure on three different days.

Clinical application

A total of 36 human plasma samples obtained from 9 healthy male adults were analyzed in this assay. This study was a part of an IRB approved phase I clinical trial (IRB No. B-1809-492-003), which was carried out in Seoul National University Bundang Hospital (ClinicalTrials.gov identifier: NCT03809260) [20]. This clinical trial was conducted to demonstrate the relationship between the antihyperglycemic effect of metformin and the gut microbiome. In the clinical trial, metformin and vancomycin were given orally in different periods and the clinical samples were collected. The plasma samples at baseline and after a 4-day metformin oral administration (1,000 mg, twice daily) were collected first. After washout for five days, a 7-day vancomycin oral administration (500 mg, twice daily) was given to the subjects followed by a 4-day metformin oral administration (1,000 mg, twice daily), which started on the 6th day of vancomycin treatment. To ensure the safety of the subjects, the drug dose was reduced to half on the first day of each treatment. Before and after the twice metformin administration, totally four different time points of plasma samples were collected and analyzed, which were baseline, post-metformin, post-vancomycin, and post-metformin+vancomycin, respectively.
Statistical analyses
Statistical analyses were carried out in IBM SPSS Statistics (v26; Armonk, NY, USA). The raw concentration data were log transformed and tested by Shapiro-Wilk test to ensure each time point dataset is in a normal distribution. Further, paired t-test was performed to determine the mean difference between each two time point datasets for the matched subjects.

RESULTS
Linearity and sensitivity
An eight-point calibration curve for TMAO was constructed on each validation day. Good linearity in the range of 1–5,000 ng/mL was achieved with a correlation coefficient ($R^2$) ranging from 0.9962–0.9979. The average slope was 0.0082, with a relative standard deviation of 9%. A representative calibration curve is shown in Fig. 2. In this method, the validated LLOQ was 1 ng/mL using 50 µL of the calibrator sample. The precision and accuracy of the inter-run LLOQ were 7.15% and 111.43%, respectively (Table 1). Further, the TMAO responses of the LLOQ compared to the zero samples define the sensitivity of the calibration curve. The response of the zero samples was 11.87–16.41% (less than 20%, acceptable) of the LLOQ after normalization to the IS responses (Fig. 3).

Selectivity, carry-over and extraction recovery
No significant interference from any endogenous compound was found at the retention time of TMAO, indicating the good selectivity of this method (Fig. 3). Further, no significant carry-over effect was detected for either TMAO or the internal standard throughout this experiment. The extraction recovery of QC samples in the surrogate matrix ranged from 81.50–90.42% with precision results lower than 2.32%. As the extraction recovery of human plasma QC samples was 84.12–88.92%, the extraction recovery ratio of this method in the

![Figure 2](https://tcpharm.org)

**Figure 2.** STD and QC for trimethylamine N-oxide.
STD (black dots), calibration standards in the surrogate matrix at concentrations of 1, 10, 50, 250, 750, 1,500, 3,000, and 5,000 ng/mL; QC (blue triangles), quality control samples in the surrogate matrix at concentrations of 1, 3, 600, and 4,000 ng/mL with 5 replicates each; PQC (red triangles), quality samples in human plasma at concentrations of 1,000, 2,500, and 5,000 ng/mL with 5 replicates each. All samples were run in the same batch. STD, calibration standard; QC, quality control in surrogate matrix; PQC, quality control spiked in plasma.
surrogate matrix and human plasma was calculated to be in the range of 94.97–104.77% (data not shown).

**Precision and accuracy**

The precision and accuracy data for the partial validation of intra-day and inter-day data are summarized in Table 1. The intra-day mean precision and accuracy of the four levels of QC were 1.40–7.62% and 91.99–105.52%, respectively. Likewise, the inter-day mean precision and accuracy of the four levels of QC were 1.65–7.15% and 96.36–111.43%, respectively.

**Cross validation**

To prove that this surrogate matrix is a good substitute for other biological matrices such as plasma, cross validation was also carried out. The precision and accuracy of three different levels of PQC were evaluated for cross validation (Table 1). The intra-day mean precision and accuracy of the three levels of PQC were 0.37–1.98% and 99.61–103.36%, respectively. Likewise, the inter-day mean precision and accuracy of the three levels of PQC were 0.53–1.62% and 99.91–104.33%, respectively.

| QC types | Levels | Nominal concentration (ng/mL) | Observed concentration (ng/mL) | Precision (%) | Mean accuracy (%) | Observed concentration (ng/mL) | Precision (%) | Mean accuracy (%) |
|----------|--------|-------------------------------|--------------------------------|---------------|------------------|--------------------------------|---------------|------------------|
| QC       | LLOQ   | 1 1.02 ± 0.08                 | 7.62                           | 102.34        | 1.11 ± 0.08      | 7.15                           | 111.43        |
|          | LQC    | 3 2.76 ± 0.08                 | 2.90                           | 91.99         | 2.89 ± 0.12      | 3.93                           | 96.36         |
|          | MQC    | 600 588.48 ± 11.27            | 1.92                           | 98.08         | 579.77 ± 11.82   | 2.04                           | 96.63         |
|          | HQC    | 4,000 4,220.93 ± 58.97        | 1.40                           | 105.52        | 4,160.31 ± 68.65 | 1.65                           | 104.01        |
| PQC      | PLQC   | 1,000 1,033.56 ± 20.45        | 1.98                           | 103.36        | 1,043.32 ± 16.90 | 1.62                           | 104.33        |
|          | PMQC   | 2,500 2,490.21 ± 27.25        | 1.09                           | 99.61         | 2,497.81 ± 13.15 | 0.53                           | 99.91         |
|          | PHQC   | 5,000 5,000.39 ± 18.40        | 0.37                           | 100.01        | 5,036.34 ± 62.27 | 1.24                           | 100.73        |

QC, quality control in surrogate matrix; LLOQ, lower limit of quantification; LQC, low quality control; MQC, mid quality control; HQC, high quality control; PQC, quality control spiked in plasma; PLQC, low quality control in plasma; PMQC, medium quality control in plasma; PHQC, high quality control in plasma.

**Figure 3.** Selectivity of TMAO in this quantification method. Blank (black): processed surrogate matrix without spiking; zero (green): 500 ng/mL TMAO-d9 spiked in surrogate matrix; LLOQ (red, low limit of quantification): 1 ng/mL TMAO together with 500 ng/mL TMAO-d9 spiked in surrogate matrix. TMAO, trimethylamine N-oxide; LLOQ, lower limit of quantification.
Clinical plasma samples
To describe the applicability of this validated method in clinical studies, a total of 36 plasma samples from nine healthy male adults were analyzed. The TMAO in human plasma samples was successfully detected and quantified within the curve range. TMAO concentrations in these subjects ranged from 12.38–292.27 ng/mL (Fig. 4). No significant difference in plasma TMAO levels was found between the baseline and post-metformin or post-vancomycin periods (Fig. 4A and B). However, the plasma TMAO levels of the subjects were decreased dramatically after treatment with both vancomycin and metformin. The mean difference in TMAO concentration between two time points is statistically significant determined by paired t-test ($p = 0.002$, Fig. 4C).

DISCUSSION
Herein, we present a simple and sensitive LC-MS/MS method for TMAO quantitation. Comparing to a quantitation method that was recently published [21], we only need simple ACN protein precipitation for sample preparation here. The dryness and reconstitution procedures in sample preparation step, which are much time-costing usually, can be omitted. It suggested the superiority of simple and fast of this described method. Further, the LLOQ in this study, which is 1 ng/mL, was much lower than that of other LC-MS/MS quantitation assays [21-23]. All intra-day and inter-day LLOQs showed satisfactory precision and accuracy, revealed the good sensitivity of the method [19]. Meanwhile, we proved that the constructed eight-point calibration curve generated in the surrogate matrix achieved good linearity and reproducibility in the range of 1–5,000 ng/mL with $R^2$ ranged between 0.9962 and 0.9979. In addition, the high extraction recovery of QC and PQC, which were higher than 80%,
signified that the simple and fast protein precipitation procedure extracted TMAO efficiently. The extraction recovery ratio between QC and PQC, which ranged from 94.97–104.77%, demonstrated extraction properties similar to those of the surrogate matrix and human plasma [24]. Further, the acceptable precision and accuracy results of the QC samples indicated the suitability of the surrogate matrix for TMAO quantitation. Acceptable PQC results implied the applicability of the surrogate matrix as a substitute for other biological matrices, such as plasma. We also successfully detected TMAO in human plasma within our curve range for clinical applications, which denoted the clinical applicability of this method.

In the clinical study, we applied this quantitation method to a selected set of plasma samples to evaluate the variations in TMAO levels caused by drug administration. In the post-metformin and post-vancomycin groups, the change of TMAO concentration in the subjects were not statistically significant compared to the baseline. Only simultaneous dosing of metformin and vancomycin significantly reduced the plasma concentrations of TMAO in healthy subjects. Compared to post-vancomycin group, the total administration period of vancomycin is three days longer in post-metformin+vancomycin group (one week). Therefore, the long-term administration of vancomycin may be one possible reason for the significant change of TMAO in post-metformin+vancomycin group. Another possible reason is that metformin has synergistic effect with vancomycin on reducing TMAO production by change the gut microbial communities [25]. The decreased relative abundances of three gut microbiome genera Eubacterium, Blautia, and Alistips, which were presented in a previous article, may be responsible for this result [20]. Eubacterium is one of the TMA-producing human commensal strains and may play a significant role in reduced TMAO synthesis [26]. Decreased relative abundance of Blautia [6] and Alistips [27] may also account for decreased TMAO production due to inadequate nutrient absorption. Due to the data limitation, our interpretations for the results of the clinical application are slightly insufficient in this study. Nevertheless, the clinical application still substantially proved the new prospect of the described quantitation method for monitoring gut microbial communities in clinical researches.

In summary, we provide a simple LC-MS/MS analytical method for TMAO quantitation with high accuracy and sensitivity by using a surrogate matrix. This method might improve the ability to monitor disease progression, evaluate drug effects, and infer changes in the gut microbiome.

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