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Determination of Urinary Trimethylamine and Trimethylamine *N*-oxide by Liquid Chromatography-Tandem Mass Spectrometry Using Mixed-Mode Stationary Phases

Sang Kyu Lee, Dong-Hyun Kim,[†] Changbae Jin, and Hye Hyun Yoo[*]

Doping control center, Korea Institute of Science and Technology, Seoul 130-650, Korea. [*]E-mail: behappy@kist.re.kr
[†]Yuyu Pharma, Inc., Seoul 100-828, Korea
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Trimethylamine (TMA), a volatile short-chain aliphatic amine, is derived from the diet either directly by the consumption of food high in TMA or by enterobacterial metabolism of dietary precursors such as choline, lecithin and possibly carnitine and other betaines.[1,2] TMA is malodorous but normally oxidized to odorless trimethylamine *N*-oxide (TMANO) by flavin containing monooxygenase 3 (FMO3) (EC 1.14.13.8) in the liver and excreted from the body. However, when FMO3 is not working correctly or if not enough enzyme is produced, TMA from precursor compounds in food digestion is not properly broken down into TMANO. TMA then builds up and is excreted in the person's sweat, urine, breath, and other body secretions with a strong fishy odor or strong body odor. Trimethylaminuria or 'fish odour syndrome' (McKusick 602079) is known to be a metabolic syndrome caused by mutations of the FMO3 gene.

The majority of publications have used the ratio of the urinary TMA and TMANO (or total TMA) to diagnosis this disorder.[1-5] The absolute cut-offs vary, but generally, for normal subjects on a free diet the ratio (TMANO/TMA) is greater than 20. In patients with trimethylaminuria, the ratio is less than 2.[3,5] Many analytical methods have been reported to determine TMANO/TMA ratio for clinical diagnosis. They include proton nuclear magnetic resonance spectroscopy (H-NMR),[3] fast atom bombardment mass spectrometry (FAB-MS),[4] matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS),[5] gas chromatography (GC),[6] fourier transform infrared spectroscopy (FT-IR),[7] flow injection analysis with mass spectrometry (FIA-MS),[8] and GC-MS methods.[9] However, liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) has not been developed yet although an LC-MS/MS system has been widely used for qualitative and quantitative analysis in many areas, especially in the fields of clinical chemistry. This may be because it is difficult to analyze TMANO and TMA using a general reverse phase LC-MS/MS system due to the low stability of product ions of TMA and TMANO in collision-induced dissociation condition and their poor retention on the general reverse stationary phases such as C_{18}, C_8, CN, and phenyl columns.

In the present study, TMA was derivatized with ethyl bromoacetate in the simple and fast one-step procedure to increase the ionization efficacy and to improve the reproducibility of fragmentation of TMA in electrospray ionization (ESI). Subsequently, a mixed mode phase column containing strong cation exchange and C_{18} phases was adopted to apply the derivatized TMA and TMANO. The mixed-mode column improved the retention of TMA and TMANO to allow the analysis using LC-MS/MS system. The resulting chromatogram showed sharp peaks of TMA and TMANO with good resolution at 0.45 and 1.5 min, respectively (Fig. 1). Ion suppression by mobile phases was shown to have a minimal effect on the analyte peaks (Fig. 1A) and matrix effect was considered negligible as satisfactory recovery of spiked analytes was achieved as shown in Table 1.

Acceptable linearity was observed over the concentration range 25 - 500 ng/mL of TMA (r^2 = 0.9998) and 1 - 25 μg/mL of TMANO (r^2 = 0.9990) (Fig. 2). The limits of detection for TMA and TMANO were 2 and 10 ng/mL (S/N ratio = 3), res-

Table 1. Intra- and inter-day accuracy and precision for the determination of trimethylamine (TMA) and trimethylamine *N*-oxide (TMANO) in human urine (*n* = 5)

Analytes	Conc. added	Intra-day			Inter-day		
		Conc. found[a]	% RSD	Accuracy (%)	Conc. found[a]	% RSD	Accuracy (%)
TMA (ng/mL)	25	26.4 ± 1.3	5.2	105.7	26.4 ± 0.9	3.7	105.8
	100	99.3 ± 3.0	3.0	99.3	106.9 ± 3.9	3.9	106.9
	500	500.1 ± 26.5	5.3	100.0	509.0 ± 12.8	2.6	101.8
TMANO (μg/mL)	1	0.9 ± 0.1	10.6	89.1	0.9 ± 0.1	9.2	91.7
	5	4.6 ± 0.4	7.7	92.5	4.5 ± 0.5	9.5	89.6
	25	25.6 ± 1.4	5.6	102.6	24.3 ± 1.3	5.1	97.1

[a]Display as Mean ± SD.

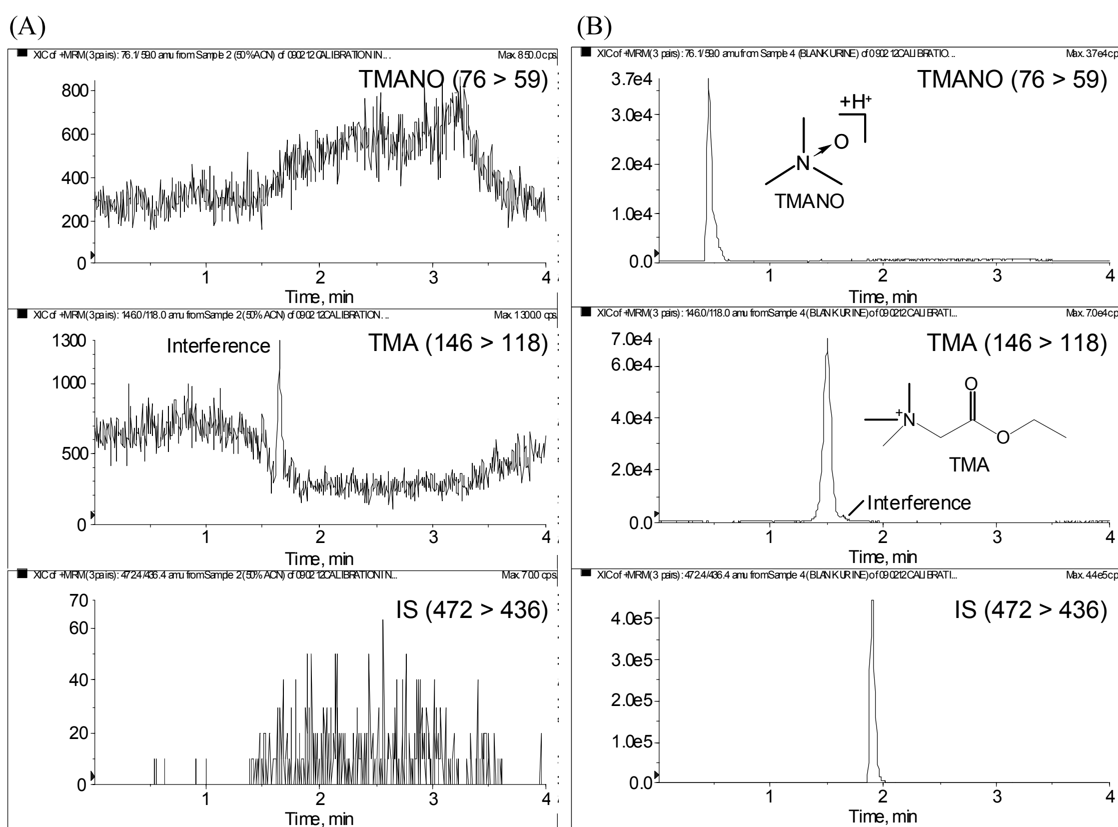

Figure 1. MRM chromatograms of 50% acetonitrile solution (A) and blank human urine (B). IS, terfenadine.

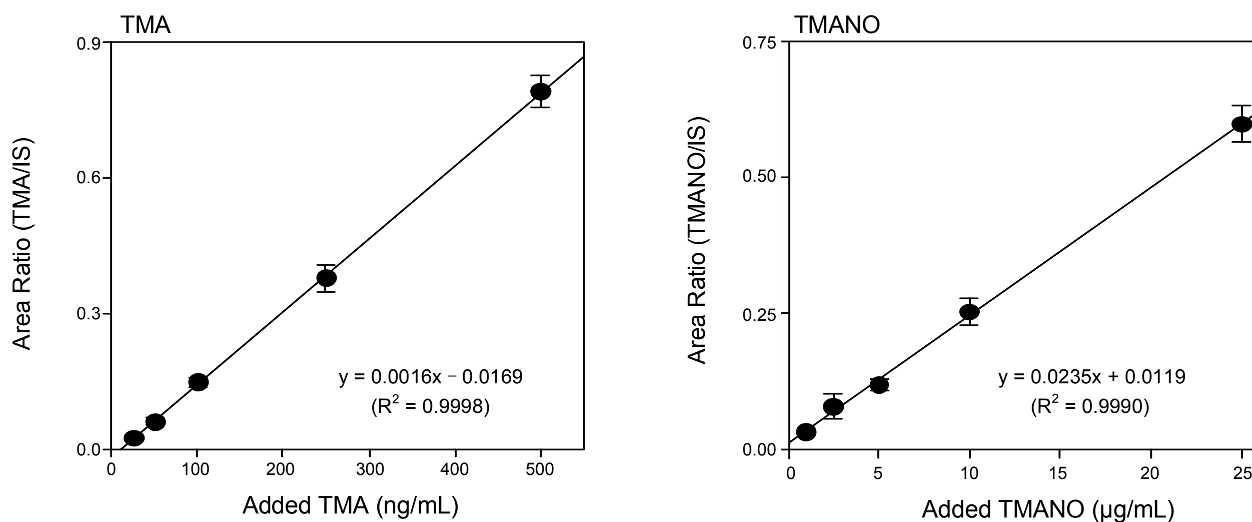

Figure 2. Calibration curves for trimethylamine (TMA) and trimethylamine *N*-oxide (TMANO) by LC-MS/MS.

pectively. The limits of quantitation were determined to be 25 ng/mL for TMA and 1 μg/mL for TMANO (S/N ratio > 10) which were the lowest concentration reproducible with a precision of less than 20% and accuracy of 80 - 120% in the linear range of the present assay (Fig. 2). Compared with the previous reported methods using H-NMR, MALDI-TOF-MS, and FT-IR[3,5,7] the sensitivity of the analytical method was increased more than 10 times. The intra- and inter-day accuracies and precisions for the spiked human urine samples are presented in Table 1. For TMA and TMANO, the intra-day precisions were 3.0 - 5.3% and 5.6 - 10.6%, and accuracies ranged 99.3 - 105.7% and 89.1 - 102.6%, respectively. The inter-day precisions were 2.6 - 3.9% and 5.1 - 9.5%, and accuracies ranged 101.8 - 106.9% and 89.6 - 97.1%, respectively. The method was sensitive enough to quantify the low concentrations of TMA in normal urine and the precision was acceptable for a volatile compound.

The developed method was successfully applied to analyze TMA and TMANO in human urine samples (n = 20) (Table 2).

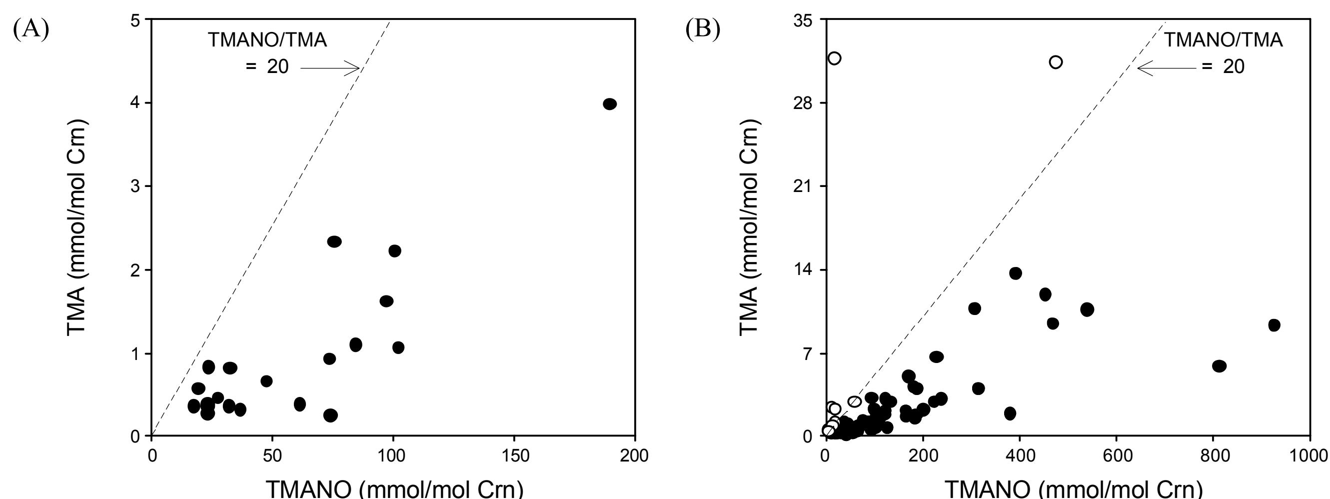

Figure 3. Trimethylamine (TMA) and trimethylamine *N*-oxide (TMANO) concentrations in control (A) and test samples (B). ●, Samples within the normal range; ○, samples suspected of trimethylaminuria. The dashed line denotes a TMANO: TMA ratio of 20.

Table 2. Urinary trimethylamine (TMA) and trimethylamine *N*-oxide (TMANO) concentrations in controls and suspected trimethylaminuria patients

Subject	TMA[a]	TMANO[a]	TMANO/TMA ratio
Controls (n = 20)	0.24 - 2.33	17.6 - 189	28.4 - 162
J91	31.4	475	15.2
J125	31.7	19.1	0.60
J142	1.09	20.2	18.6
J156	0.42	5.12	12.1
J170	0.82	14.0	17.0
J176	0.41	7.72	18.9
J178	2.42	12.7	5.23
J218	2.21	21.5	9.72

[a]mmol/mol creatinine.

The resulting values were expressed as mmoles TMA or TMANO per mole creatinine (mmol/mol creatinine). The urinary TMA and TMANO were determined to be 0.24 - 2.3 mmol/mol creatinine and 17.6 - 189 mmol/mol creatinine, respectively. The range of TMANO/TMA ratio was 28.4 - 162, which was comparable to the literature values.[3,5,9] Subsequently, 127 urine samples were analyzed as test samples (Fig. 3). Among test samples, 8 samples were suspected of trimethylaminuria which showed TMANO/TMA ratios less than 20 and especially, sample J125 with a TMANO/TMA ratio of 2 was supposed to be severe trimethylaminuria (Table 2),[1-5] although positive urine samples could not be co-analyzed due to unavailability of samples from patients with trimethylaminuria.

In conclusion, the LC-MS/MS method was developed to measure urinary TMA and TMANO in this study. The derivatization and mixed-mode stationary phase improved the sensitivity, reproducibility, and column retention of TMA and TMANO to allow a quantitative analysis using an LC-MS/MS system. The method could offer a better means for diagnosis of inherited trimethylaminuria in clinical metabolic laboratories.

Experimental Section

Materials. TMA, TMANO, terfenadine, creatinine, ethyl bromoacetate and acetaminophen were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Human urine samples were provided by Doping Control Center, Korea Institute of Science and Technology (Seoul, Korea). Among them, 20 samples from healthy adults were used as control samples and unidentified 127 samples were used as test samples for the method application. The urine sample was stored at −70 °C until analysis. All other chemicals were of analytical grade and used as received.

Sample preparation. Derivatization of TMA was carried out according to the method by Johnson *et al*. using ethyl bromoacetate as a derivative reagent.[8] To 25 μL of blank human urine samples, 1 μL of concentrated ammonia solution and 30 μL of ethyl bromoacetate (20 mg/mL in acetonitrile) were added in room temperature. The reaction was stopped after 30 min by the addition of 1 mL 50% acetonitrile (ACN) in 0.025% formic acid and 20 μL of IS (terfenadine, 8 μM in ACN) was added. The mixture was centrifuged for 3 min at 13000 rpm, and the 1 μL of supernatant was directly injected into the LC-MS/MS system.

Instruments. For quantification of TMANO and TMA, HPLC was performed using a Nanospace SI-2 inert pump system, HTS autosampler Z and column oven with cooling function (Shiseido Co. Ltd., Kyoto, Japan). The analytical column was a Capcell Pak CR 1:50 (50 mm × 2.0 mm i.d., 5 um, Shiseido, Japan). The mobile phases used were 90% ACN in 5 mM ammonium formate buffer of pH 6.0 (A) and 5 mM ammonium formate buffer of pH 6.0 (B). Separation was conducted using a linear gradient system from 5:95 (A:B) to 90:10 (A:B) for 0.5 min and held for 1.5 min with a flow rate of 0.3 mL/min at 40 °C. The HPLC was couple to an API 4000 triple-quadruple mass spectrometer (Applied Biosystems SCIEX, Concorde, Canada) equipped with a TurboIonSpray source. Electrospray ionization (ESI) was performed in the positive mode. The precursor-product ion pairs used in MRM mode were: m/z 76 → 59 for TMANO, m/z 146 → 118 for TMA and m/z 472 → 436

for IS (terfenadine). The data acquisition was controlled by Analyst 1.42 software.

Measurement of calibration curves, accuracy and precision. For calibration standards, 25 μL of each stock solution was prepared using the reconstitution solution (50% ACN in 0.025% formic acid). The concentrations of the calibration standards were 25 - 500 ng/mL of TMA and 1 - 25 μg/mL of TMANO. The detailed preparation protocols were as described above. The calibration curves were constructed by plotting the peak-area ratio of TMA or TMANO to the internal standard versus its concentration. Spiked human urine samples of three concentrations (25, 100 and 500 ng/mL for TMA; 1, 5 and 25 μg/mL for TMANO, $n = 5$) were prepared and assayed to determine the intra- and inter-day accuracy and precision. Accuracies were expressed as a percentage of the nominal concentration and precisions as a relative standard deviation (%RSD). The analysis was repeated over 5 days for the inter-day assay.

Creatinine measurement. The amount of urinary creatinine was determined using the LC-MS/MS method by Park *et al.*.[10] Briefly, 1 μL of urine samples were added to 1 mL of 50% ACN in 0.025% formic acid containing 20 μg/mL of acetoaminophen as an internal standard. After centrifugation, the 1 μL of supernatant was directly injected into the LC-MS/MS system. The calibration curve was constructed with a range of 10 to 5000 ng/mL.

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