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Determination of trimethylamine-*N*-oxide in combination with L-carnitine and γ-butyrobetaine in human plasma by UPLC/MS/MS

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ABSTRACT: An ultra-high-performance liquid chromatography–mass spectrometry (UPLC/MS/MS) method was developed and validated for the quantification of trimethylamine-*N*-oxide (TMAO) simultaneously with TMAO-related molecules L-carnitine and γ-butyrobetaine (GBB) in human blood plasma. The separation of analytes was achieved using a Hydrophilic interaction liquid chromatography (HILIC)-type column with ammonium acetate–acetonitrile as the mobile phase. TMAO determination was validated according to valid US Food and Drug Administration guidelines. The developed method was successfully applied to plasma samples from healthy volunteers. Copyright © 2015 John Wiley & Sons, Ltd.

Keywords: trimethylamine *N*-oxide; l-carnitine; γ-butyrobetaine; UPLC/MS/MS; human plasma

Introduction

Trimethylamine-*N*-oxide (TMAO) is a metabolite generated from trimethylamine (TMA) and is associated with cardiovascular risks (Wang *et al.*, 2011; Ussher *et al.*, 2013). Dietary L-carnitine is metabolized to TMA by gut microbiota, and concomitant elevated circulating L-carnitine and TMAO levels have been predicted to contribute to an increased risk of cardiovascular disease (Koeth *et al.*, 2013). Plasma concentrations of L-carnitine and its bio-precursor γ-butyrobetaine (GBB) have been studied in association with diabetes, cardioprotection and drug discovery (Dambrova and Liepinsh, 2015; DiNicolantonio *et al.*, 2013; Liepinsh *et al.*, 2014). Additionally, a recent study reported a role for GBB as a proatherogenic intermediate in the gut microbial metabolism of L-carnitine to TMAO (Koeth *et al.*, 2014). Taking into account the number of studies addressing various aspects of the involvement of TMAO and related metabolites in physiological and pathological processes, the aim of the present study was to develop a robust assay for the simultaneous determination of TMAO in combination with L-carnitine and its bio-precursor GBB in human blood plasma.

Several methods have been developed to quantify TMAO in biological samples, including methods ranging from analyte reduction in the human blood plasma matrix to free TMA followed by GC/MS analysis (Bain *et al.*, 2006) to LC/MS applications for urine samples in either the multiple reaction monitoring (MRM) mode (Lee *et al.*, 2010) or direct infusion combined with time-of-flight detection (Mamer *et al.*, 2010). To the best of our knowledge, the majority of TMAO assays in blood plasma are based on LC/MS in the MRM mode. Tandem LC/MS/MS analysis of human plasma samples was applied by Wang *et al.* (2011, 2014). Similarly, both plasma and urine samples obtained from healthy volunteers were analyzed by the UPLC/MS/MS method for TMAO content (Dambrova *et al.*, 2013). The application of stable isotope dilution LC/MS/MS was described for TMAO analysis with high precision and high throughput (Wang *et al.*, 2014). A number of other metabolites were analyzed along with TMAO using the LC/MS/MS procedure (McEntyre *et al.*, 2014). However, neither L-carnitine nor GBB levels were measured by any of these procedures. In the present study, we report the development of a procedure for the simultaneous determination of TMAO, L-carnitine and GBB levels in human blood plasma samples.

Materials and methods

Chemicals

TMAO was purchased from Alfa Aesar (Heysham, UK), L-carnitine, acetonitrile (gradient grade), formic acid and ammonium acetate from Sigma Aldrich (St Louis, MO, USA) and methanol (HPLC grade) from Merck KGaA (Darmstadt, Germany). GBB was obtained

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Abbreviations used: GBB, γ-butyrobetaine; MRM, multiple reaction monitoring; TMA, trimethylamine; TMAO, trimethylamine-*N*-oxide.

from JSC Olainfarm (Olaine, Latvia). The internal standard, 3-(2,2-dimethyl-2-prop-1-yl-hydrazinium)propionate (IS), was synthesized in-house (Goldberg *et al.*, 1990).

Preparation of calibration standards and quality control samples

A TMAO stock solution (440 μM) was prepared in water and stored at 4°C. A fresh solution was used for each sequence of samples. All calibrators were prepared in water and subjected to serial dilutions to prepare a standard curve ranging from 0.17 to 21.85 μM. For clinical sample analysis, five calibrator solutions containing L-carnitine (0.6–64 μM), GBB (0.3–35 μM) and TMAO (0.3–20 μM) were prepared. The quality control samples (QC) were prepared at four concentrations as follows: LLOQ (lower limit of quantification, diluted pooled plasma, 1:10), LQC (lowest quality control, pooled plasma), MQC (medium quality control, spiked pooled plasma) and HQC (highest quality control, spiked pooled plasma).

Sample preparation

A total of 900 μL of internal standard solution (200 ng/mL) in an acetonitrile–methanol mixture (3:1, v/v) was added to a 40 μL volume of each sample or calibrator solution. The samples were thoroughly mixed and centrifuged for 10 min at 13,000***g***, and the supernatants were subjected to UPLC/MS/MS analysis.

UPLC/MS/MS analysis

The separation of analytes was performed using an Acquity UPLC system (Waters Corp., Milford, MA, USA) on an Acquity HILIC BEH column (2.1 × 50 mm, 1.7 μm, Waters Corp., Milford, MA, USA) with a gradient elution from 75 to 55% acetonitrile in 10 mM aqueous ammonium acetate (pH 4) at a flow rate of 0.25 mL/min. The analytes were ionized by electrospray ionization in positive ion mode on a Quattro Micro triple-quadrupole mass spectrometer (Waters Corp, Milford, MA, USA). The mass spectrometer was set up as follows: capillary voltage of 3.3 kV; source and desolvation temperatures of 120 and 350°C, respectively; and desolvation gas (nitrogen) flow of 500 L/h. Cone voltage (V) and collision energy (eV) values were specified for each compound: 26 and 14 for TMAO; 20 and 16 for L-carnitine; 20 and 12 for GBB; and 25 and 14 for IS. The TMAO, L-carnitine and GBB analysis was performed in MRM mode. Four precursor to product ion transitions (*m/z* 75.8 → 58.3 for TMAO, *m/z* 162.0 → 103.0 for L-carnitine, *m/z* 146.1 → 87.1 for GBB and *m/z* 175.4 → 86.0 for the internal standard) were monitored. Data acquisition and processing were performed using the MassLynx V4.1 and QuanLynx V4.1 software (Waters Corp, Milford, MA, USA). The plasma extracts were kept at 10°C in the autosampler. The calibrators were run in triplicate and the samples in duplicate.

Method application to clinical use

The method described in this study was used to analyze plasma samples from six healthy volunteers. Plasma samples were obtained from venous blood by venipuncture and stored at –20°C prior to the analysis of TMAO, L-carnitine and GBB levels. This study was performed with the approval of the Central Medical Ethics Committee (Riga, Latvia), and informed consent was obtained from all subjects.

Validation procedure

Method validation was performed according to the US Department of Health and Human Services *et al.* (2001) guidelines for bioanalytical method validation, and the accepted recommendations for matrix evaluation were followed (Annesley, 2003).

Selectivity. The selectivity of the method was evaluated based on the presence or absence of undesirable peaks at the retention time of the analytes in all used MRM channels.

Linearity. To assess the linearity, calibration standards (six non-zero standards) were prepared in water and analyzed in duplicate in three analytical runs. The LLOQ was set as the lowest concentration in the linear range where deviations from the nominal concentration values were within the US Food and Drug Administration validation guidelines (US Department of Health and Human Services *et al.*, 2001) (accepted range ±20%).

Precision and accuracy. Accuracy was defined as the difference between the measured and native amount of TMAO in the QC samples and was evaluated with three QC samples (LQC, MQC and HQC). The relative standard deviation (RSD) of the repetitive quantification results for each analysis was calculated as the measurement of precision. Precision was evaluated with four quality control samples (LLOQ, LQC, MQC and HQC).

The intra-day precision and accuracy were evaluated by analysis of six aliquots of each sample on the same day. For inter-day precision and accuracy, the same experiment was repeated on three consecutive days.

Stability. The QC samples were assessed for short-term and long-term stability. The short-term stability was evaluated using pooled plasma samples stored at room temperature for 4 h and processed samples in an autosampler at 10°C for 20 h. The freeze–thaw stability was determined using pooled plasma samples after three freeze–thaw cycles from room temperature to −20°C on three consecutive days.

Results

In the current study, we aimed to develop a UPLC/MS/MS method for the determination of metabolite TMAO simultaneously with its precursors GBB and L-carnitine in one run. Therefore, the existing UPLC/MS/MS method for GBB and L-carnitine determination in biological matrices (Liepinsh *et al.*, 2011) was adopted for the TMAO assay.

A synthetic analog of both GBB and L-carnitine [3-(2,2-dimethyl-2-prop-1-yl-hydrazinium)-propionate] was selected as the IS for all analytes. This compound can be easily prepared using the protocol described in a published reference (Goldberg *et al.*, 1990) and is easy to characterize using common analytical techniques. This compound has a fully synthetic origin and cannot be found in any biological matrix. The chemical structures of the internal standard and all analytes are provided in Fig. 1.

Method validation

Typical chromatograms of the TMAO, L-carnitine, GBB and IS MRM traces of human blood plasma samples are presented in Fig. 2; these results clearly demonstrate the selectivity of the method. The peaks owing to the analytes under study were fairly separated, and no extra peaks at the expected retention time on the used MRM channels were observed.

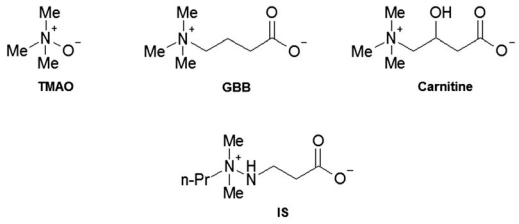

Figure 1. The chemical structures of trimethylamine-*N*-oxide (TMAO), γ-butyrobetaine (GBB), carnitine and internal standard 3-(2,2-dimethyl-2-prop-1-ylhydrazinium) propionate (IS).

The analytical response of the assay was linear over a concentration range from 0.17 to 21.85 μM. The r^2 value was >0.99. The linear regression equations and the coefficients of regression are presented in Table 1. Representative chromatograms from the UPLC/MS/MS analysis are shown in Fig. 2. The working concentration range of the TMAO determination method (0.17–21.85 μM) was sufficiently broad for the present study population.

The lower limit of quantification was determined by analysis of a diluted pooled plasma sample (1:10, v/v). At a concentration of 0.15 μM TMAO, the obtained values of imprecision (RSD 7.4%) and accuracy (relative error 13.3%) were within the acceptable limits (<20%; Table 2).

The imprecision of the assay was evaluated by replicate analysis of the four quality control samples. The intra-assay imprecision (RSD) for all QC samples was <7% (n = 6), while the inter-assay imprecision was <11% (n = 3) (Table 3). The method accuracy was calculated by comparing the known amount of added TMAO and the difference between the measured and native amount of TMAO. This recovery value varied from 97 to 99% for the MQC and HQC samples (Table 4).

The plasma samples were stable after three freeze–thaw cycles, for at least 4 h at room temperature and 20 h in an autosampler at 10°C as judged by the analysis of TMAO in the quality control samples (Table 5).

Table 1. Statistical parameters of trimethylamine-*N*-oxide (TMAO) calibration curves (range from 0.17 to 21.85 μM)

Curve	Slope	Intercept	r^2
1	0.0425	0.00115	0.9994
2	0.0433	0.00117	0.998
3	0.0406	0.00057	0.9991

Table 2. Determination of LLOQ

Dilution (pooled plasma)	TMAO, μM		Precision, RSD, % (n = 6)	Relative error, %
	Calculated	Found		
1:5	0.27	0.25	5.6	7.4
1:10	0.15	0.13	7.4	13.3

Table 3. Imprecision of the TMAO assay

Level	Intra-assay (n = 6), RSD, %	Inter-assay (n = 3), RSD, %
LLOQ	6.42	3.30
LQC	3.41	10.62
MQC	5.90	5.65
HQC	3.76	2.27

Ion suppression was evaluated using a T-infusion experiment (Fig. 3). A constant electrospray response was obtained in the region of retention time for all of the compounds of interest (from 1.7 to 2.6 min, Fig. 2), indicating that ionization interference (suppression) does not exist.

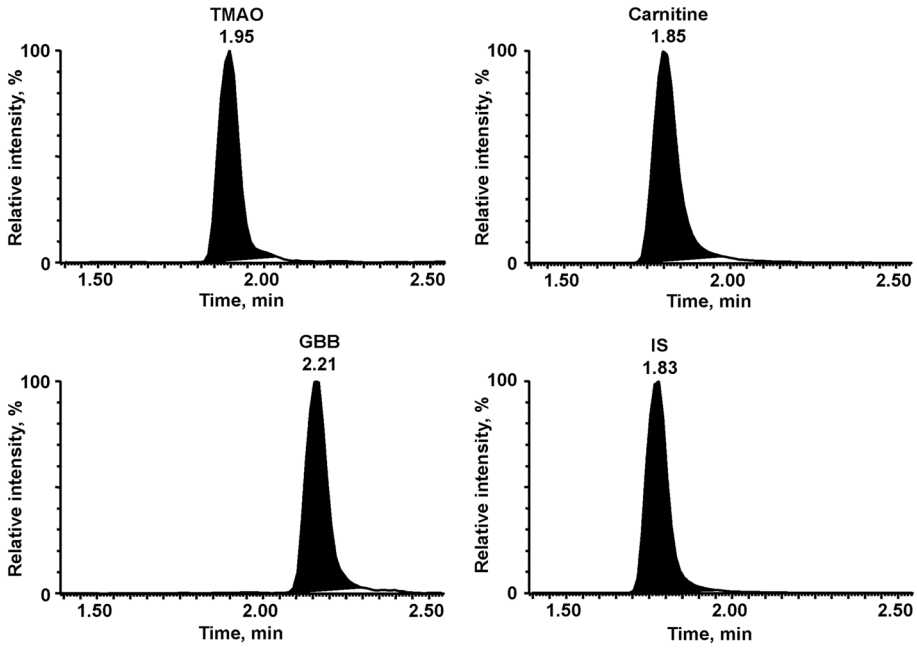

Figure 2. Representative chromatograms of TMAO, L-carnitine, GBB and IS multiple reaction monitoring (MRM) traces of plasma samples from healthy volunteers (1.62 μM of TMAO, 29.46 μM of L-carnitine and 0.73 μM of GBB were detected).

Table 4. Accuracy of TMAO analysis

TMAO level	Spike, μM	Mean measured amount of TMAO, μM (SD)	Spike recovery, %
LQC	0	1.77 (0.19)	—
MQC	7.28	8.85 (0.50)	97.25
HQC	14.57	16.21 (0.37)	99.11

Table 5. Stability of TMAO in plasma (pooled plasma) and processed samples

	Recovery, % (n = 6)
Freeze–thaw (three cycles)	90.5
Room temperature, 4 h	95.3
Autosampler, 10°C for 20 h	94.0

Method application

The developed method was tested on six blood plasma samples from healthy volunteers. The results are presented in Table 6. The measured level of TMAO was slightly lower than the range

Table 6. Endogenous plasma TMAO, L-carnitine and γ-butyrobetaine (GBB) concentrations in six individual healthy volunteers

Volunteer	TMAO, μM	L-Carnitine, μM	GBB, μM
1	1.66	37.46	1.10
2	1.26	45.64	0.98
3	1.62	29.46	0.73
4	1.63	43.23	0.88
5	3.34	47.47	1.18
6	3.88	41.63	1.02

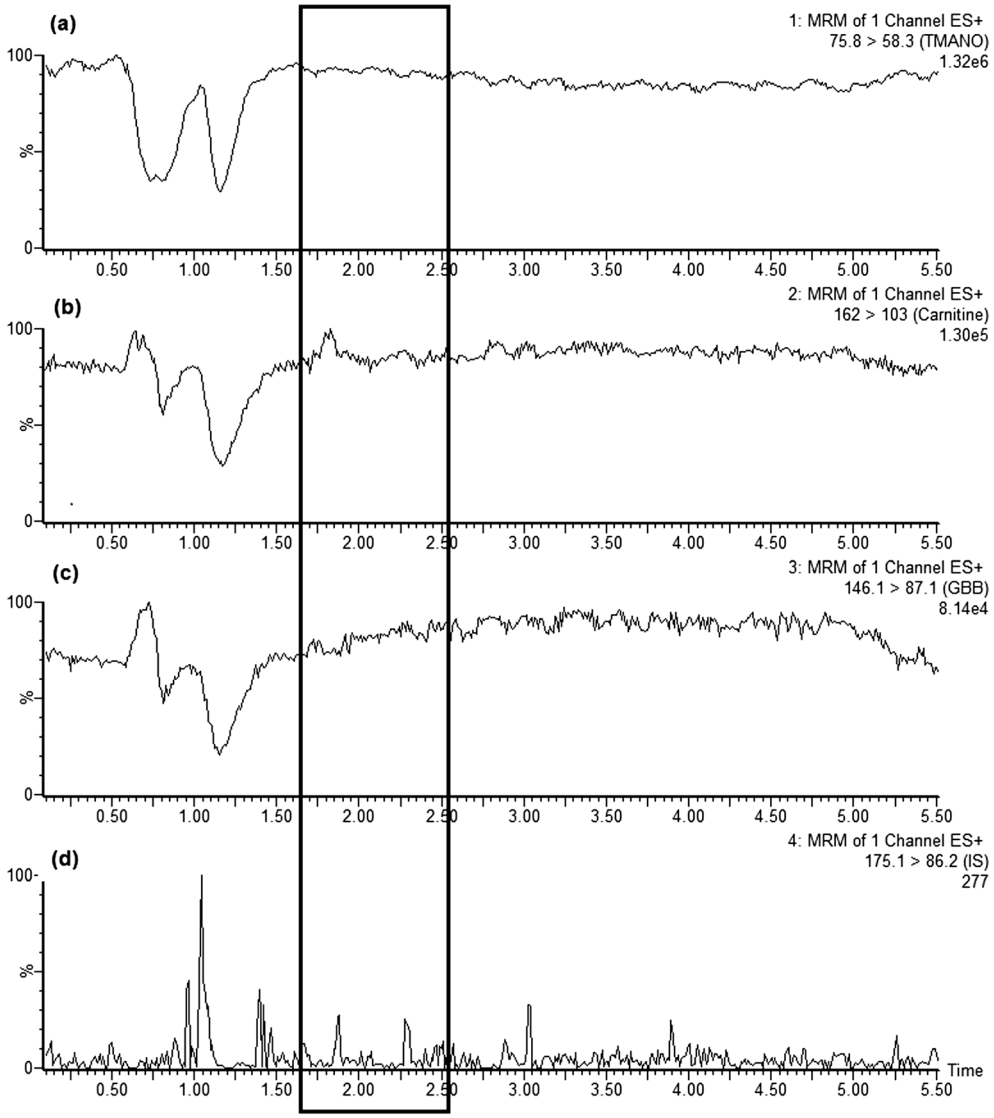

Figure 3. Ion suppression experiment. An acetonitrile-precipitated human plasma sample was subjected to LC/MS/MS analysis. A calibration standard solution containing TMAO, L-carnitine, GBB and IS was infused into the eluate using a T-piece after the UPLC column. MRM channels corresponding to (a) TMAO, (b) carnitine, (c) GBB and (d) IS were recorded. Significant ion suppression was observed at retention times of 0.5–1.5 min. The compounds of interest were eluted in a marked (retention time > 1.5 min) region where no ion suppression was observed.

from 2.3 to 5.8 μM found by Wang *et al.* (2014), which might be explained by differences in the health status of the study participants. The L-carnitine concentration was the same as the levels determined previously (Primassin and Spiekerkoetter, 2010). The GBB level was in agreement with previously published data (Hirche *et al.*, 2009; Terada *et al.,* 1999).

The developed assay allows the simultaneous quantification of TMAO, L-carnitine and γ-butyrobetaine from human blood plasma. TMAO is reported to be a significant biomarker for the prediction of cardiovascular diseases (Wang *et al.*, 2011), whereas L-carnitine (Koeth *et al.*, 2013) and GBB (Koeth *et al.*, 2014) are involved in the metabolic production of TMAO through a microbiota-dependent mechanism. Thus, analysis of these endogenous compounds is of great importance for pharmacological research related to TMAO homeostasis. The method is easy to use and does not require the application of expensive materials. The total runtime of the assay is short (6 min), making it suitable for the analysis of a large number of samples.

Conclusions

The described assay is robust, sensitive, accurate, and reproducible for measuring TMAO, free L-carnitine and GBB concentrations in plasma samples. The assay can be applied for clinical and experimental studies addressing the importance of these metabolites in health and disease states.

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