Journal of Chromatography B, 1008 (2016) 206–211

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Simultaneous determination of phosphatidylcholine-derived quaternary ammonium compounds by a LC–MS/MS method in human blood plasma, serum and urine samples

Christian Steuer[a,*], Philipp Schütz[b], Luca Bernasconi[a], Andreas R. Huber[a]

[a] Kantonsspital Aarau, Centre of Laboratory Medicine, Tellstrasse 1, CH-5001 Aarau, Switzerland
[b] Kantonsspital Aarau, Medical University Department, Tellstrasse 1, CH-5001 Aarau, Switzerland

ARTICLE INFO

Article history:
Received 13 August 2015
Received in revised form
23 November 2015
Accepted 1 December 2015
Available online 2 December 2015

Keywords:
Isotope dilution assay
HILIC-chromatography
LC–MS/MS analysis
TMAO

ABSTRACT

The determination of circulating trimethylamine-*N*-oxide (TMAO), choline, betaine, L-carnitine and *O*-acetyl-L-carnitine concentration in different human matrices is of great clinical interest. Recent results highlighted the prognostic value of TMAO and quaternary ammonium containing metabolites in the field of cardiovascular and kidney diseases. Herein, we report a method for the rapid and simultaneous measurement of closely related phosphatidylcholine-derived metabolites in three different biological matrices by stable isotope dilution assay. Plasma, serum and urine samples were simply deproteinized and separated by HILIC-chromatography. Detection and quantification were performed using LC–MS/MS with electrospray ionization in positive mode. For accuracy and precision, full calibration was performed covering more than the full reference range. Assay performance metrics include intra- and interday imprecision were below 10% for all analytes. To exclude matrix effects standard addition methods were applied for all matrices. It was shown that calibration standards and quality control prepared in water can be used instead of matrix-matched calibration and controls. The LC/MS/MS-based assay described in this article may improve future clinical studies evaluating TMAO and related substances as prognostic markers for cardiovascular risk and all-cause mortality in different patient populations.

1. Introduction

Choline, betaine, L-carnitine and its acetylated derivative *O*-acetyl-L-carnitine are used for a wide range of biological activities, including maintaining the structural integrity of cell membranes, energy metabolism, and donating methyl groups in a number of biosynthetic reactions [1–3]. Although choline and its oxidation product betaine are synthesized endogenously, to fulfill the necessary requirements, the main part of both is derived by dietary intake [2,4]. The non-proteinogenic amino acid L-carnitine is synthesized endogenously from lysine and methionine but also supplied by dietary intake. Choline, betaine and L-carnitine are main ingredients found in red meat, fish and sea-products and especially L-carnitine is found in many life-style and sports nutrition products [5,6].

Many *in vitro* and animal studies have shown the protective effect of L-carnitine as a free radical scavenger to prevent oxidative damage to antioxidant enzymes [7]. Additionally, some clinical trials found a protective effect on cardiac metabolism and performance at higher doses of L-carnitine [8]. Also choline and betaine are highly discussed in the scientific community because of beneficial health effects found in some studies [3,4,9]. However, not all studies were able to reproduce these findings [10–12]. Recent studies described the intestinal breakdown of choline, betaine and L-carnitine to trimethylamine (TMA) by gut flora microorganism [13–15]. Trimethylamine is well absorbed and following transformed in the liver to trimethylamine-*N*-oxide (TMAO) by flavin monooxygenases 1 and 3 (FMO1 and FMO3) [16]. Briefly, the metabolic pathway for dietary phosphatidylcholine (PC) is as follows: dietary PC/choline/betaine/L-carnitine → gut flora-produced TMA → hepatic FMO-formed TMAO. Several research groups highlighted the importance of plasma concentrations of TMAO, choline, betaine, and L-carnitine in predicting cardiovascular events, long term mortality for chronic kidney disease (CKD)-patients and the influence in diabetes mellitus type 2 [10,14,17–19]. As a precursor of TMAO, it is furthermore assumed that L-carnitine, betaine and choline influence the development of atherosclerotic plaques formation [12,13,20].

* Corresponding author. Fax: + 41 628385399.
E-mail address: christian.steuer@ksa.ch (C. Steuer).

http://dx.doi.org/10.1016/j.jchromb.2015.12.002
1570-0232/

Particularly a combined method for the highly polar analytes like TMAO and its precursors are scarce in literature. Due to their physico-chemical properties and therefore low volatility, analysis of quaternary ammonia containing compounds is mainly done by LC–MS/MS techniques. Chromatographic separation is often performed by HILIC-[1,20,21], C4-[15], Phenyl-[14] or Silica columns [22]. To the best of our knowledge no methods for simultaneous detection and quantification in blood plasma, serum and urine of the five metabolites mentioned above are currently available. Because there is an increase in urinary concentration after oral intake of L-carnitine, betaine, choline and TMAO, it is also interesting to extend the method to urine samples.

Here we report a detailed isotope dilution LC–MS/MS method for the rapid, sensitive and accurate simultaneous quantification of five quaternary ammonium containing compounds that can be applied to blood plasma and serum samples as well as urine samples.

2. Material and methods

2.1. Ethics

A total of 24 urine, plasma and serum samples were randomly collected from people hospitalized in the Kantonsspital Aarau (Aarau, Switzerland). All biological specimens were obtained during routine measurements in the Kantonsspital Aarau. The ethic committee of the local authorities approved the use of leftover samples for method development and evaluation.

2.2. Chemicals and reagents

Hydrochlorides of choline, betaine, *O*-acetyl-L-carnitine, L-carnitine and TMAO were obtained from Sigma–Aldrich (Buchs, Switzerland) choline-d9 and TMAO-d9 were purchased from Cambridge Isotopes (Andover, Massachusetts, USA). Water, acetonitrile (ACN) and methanol (MeOH) were of LC–MS/MS grade and obtained from Sigma (Buchs, Switzerland). All other chemicals used were from Sigma (Buchs, Switzerland) and of the highest grade available, otherwise indicated.

2.3. LC–MS/MS analysis

Different mobile phases were tested on a Phenomenex (Aschaffenburg, Germany) Luna-HILIC column (150 × 4.6 mm, 3.0 μm) for the separation of the permanently charged analytes. The analysis was performed using a Thermo Fischer Ultimate 3000 UHPLC (Thermo Fisher, San Jose, California, USA) system coupled to an ABSciex 5500 QTtrap linear ion trap (LIT) quadrupole mass spectrometer (ABSciex, Darmstadt, Germany). Experiments were performed using gradient elution with 10 mM ammonium acetate in 90% ACN/water (A1) and 10 mM ammonium acetate buffer pH 4 (B1) at a flow rate of 0.75 mL/min. Secondly a mobile phase composition of 10 mM ammonium formate in 90% ACN (A2) and 10 mM ammonium formate in water pH 3 (B2) at a flow rate of 0.75 mL/min were tested. All chromatographic separations were done at 35 °C. The final LC gradient was as follows: 0–1.5 min 0% B, 1.5–7 min to 90% B, 7–9 min hold at 90% B, 9–9.2 min to 0% B, reequilibrating for 1.8 min. The MS was operated in the multiple reaction monitoring (MRM) mode using 2 transitions for each analyte. The MS settings for each analyte are given in Table 1. The autosampler was set to 10 °C. The Turbo V ion source was operated in positive ESI mode with the following MS conditions: gas 1, nitrogen (40 psi); gas 2, nitrogen (40 psi); ion spray voltage, 3000 V; ion-source temperature, 300 °C; curtain gas, nitrogen (35 psi), collision gas, high.

2.4. Biological samples

Blood samples and urine samples were obtained from 24 different patients of the Kantonsspital Aarau. Blood plasma and serum were obtained after centrifugation at 5000 × *g* for 10 min and used immediately. In case of storage supernatant of the samples was separated and stored at −20 °C until further usage. Urine was always used freshly.

Human blood samples for controlling the performance of the method in respect of the reference range were obtained from 13 different apparently healthy volunteers of the Center of Laboratory Medicine and the Medical University Department of the Kantonsspital Aarau as K_2-EDTA- blood. All blood samples were collected after an overnight fastening period. Blood plasma was obtained as described above.

2.5. Sample preparation

The plasma and serum samples were prepared by simple protein precipitation; urine was analyzed after simple dilution with water (5-fold). Briefly, to 25 μL EDTA-plasma or serum 225 μL of MeOH containing deuterated internal standard (TMAO-d9, choline-d9; concentration: 25 μM each) were added. To 25 μL of diluted urine 225 μL of deuterated internal standard mix in methanol was added. Deproteinization was performed by addition of methanol because of better peak shape and greater column lifespan [1]. Further procedure was the same for the different matrices. The mixture was vortexed for 5 s and centrifuged (15,000 × *g*, 10 min). The supernatant (100 μL) was transferred into an autosampler vial and 5 μL of this solution was injected into the LC–MS/MS system.

2.6. Method validation

Method validation was performed according to international guidelines in respect of selectivity, carry over, limit of quantification (LOQ), linearity, accuracy, precision, recovery and processed sample stability [23].

Separate stock solutions for calibration and QC sample of each metabolite were prepared in water. Concentrations of the stock solutions were 100 mM. Working solutions were prepared by dilution from each stock solution. Spiking solutions for calibration standards and QC samples were prepared by mixing appropriate amounts of the corresponding stock or working solution to obtain concentrations two to ten times higher than the corresponding blood plasma reference range. All solutions were stored in aliquots at −80 °C. The final calibration and QC concentrations are given in Table 2 for each metabolite.

Replicates ($n = 6$ on 6 different days) at each concentration level were analyzed as described above. The regression lines were calculated using, a weighted [1/*X*] least-squares regression models. Daily calibration curves (single measurement per level) were prepared with each batch of validation samples. Concentrations of each calibration level were back-calculated using the selected regression lines. The back-calculated concentrations of all calibration samples were compared to their respective nominal values and quantitative accuracy was required within 20% of target. As for all other metabolites no deuterated standards were commercially available, TMAO-d9 was successfully used for betaine, *O*-acetyl-L-carnitine and L-carnitine. In this work, the use of isotopically labeled internal standards corrected for any apparent loss of analyte during the extraction [24].

QC samples (LOW, MED, HIGH) were prepared and analyzed according to the procedures described above in duplicate on each of eight days. Accuracy was calculated in terms of bias as the percent deviation of the mean calculated concentration at each concentration level from the corresponding theoretical concentra-

Table 1
MS settings (DP declustering potential, EP entrance potential, CE collision energy, CXP collision cell exit potential) and retention times of the measured analytes sorted by elution order. Quantifiers are given in bold print.

Analyte	Q1 (mass)	Q3 (mass)	RT (min)	DP (V)	EP (V)	CE (eV)	CXP (V)
Choline	104	60	5.73	221	10	29	8
	104	58	5.73	221	10	39	8
Choline *d9*	114.0	69	5.73	1	10	29	8
	114.0	67	5.73	1	10	39	8
Betaine	118.0	59	6.52	211	10	29	8
	118.0	58	6.52	211	10	45	12
O-Acetyl-L-carnitine	205.0	85.0	6.66	80	10	15	12
	205.0	144.0	6.66	80	10	15	8
L-Carnitine	162.0	102	6.98	221	10	33	12
	162.0	85	6.98	216	10	27	8
TMAO	75.9	57.9	7.27	1	10	27	12
	75.9	59.1	7.27	1	10	17	10
TMAO *d9*	85.1	68	7.27	1	10	23	8
	85.1	66	7.27	1	10	35	10

Table 2
Concentrations for calibrator (Cal.) and quality control (QC) samples used for method the validation.

Analyte	Concentrations in μM (mg/L)						
	Cal. 1	Cal. 2	Cal. 3	Cal. 4	QC low	QC med	QC high
Choline	2.00	20.0	100	200	2.40	40.0	160
	(0.21)	(2.08)	(10.4)	(20.8)	(0.25)	(4.16)	(16.3)
Betaine	1.00	10.0	50.0	100	1.20	20.0	80.0
	(0.12)	(1.18)	(5.9)	(11.8)	(0.14)	(2.36)	(9.44)
O-Acetyl-L-carnitine	0.50	5.00	25.0	50.0	0.60	10.0	40.0
	(0.10)	(1.02)	(5.10)	(10.2)	(0.12)	(2.04)	(8.16)
L-Carnitine	1.00	10.0	50.0	100	1.20	20.0	80.0
	(0.16)	(1.62)	(8.10)	(16.2)	(0.19)	(3.24)	(12.9)
TMAO	0.50	5.00	25.0	50.0	0.60	10.0	40.0
	(0.38)	(3.76)	(18.77)	(37.6)	(0.45)	(7.52)	(30.1)

tion. Intra-day and inter-day precision were calculated as relative standard deviation (RSD) according to Reference [23].

Recovery (RE) was determined at QC MED and HIGH concentration respectively using 5 different blood plasma sources according to the simplified approach described by Matuszewski et al. [25]. Briefly, 2 sets of samples were prepared. Samples set 1 representing matrix spiked after extraction and samples set 2 consisting of matrix spiked before extraction. The two sets were either worked up as described under sample preparation. RE results were obtained by comparison of the peak areas of sample set 2 with those of the corresponding peaks in sample set 1. Matrix effects were determined according to the standard addition method [26]. By adding calibrator 1–3 to individual aliquots of the sample of interest, a calibration curve was created. The endogenous concentration in the sample is back-calculated from the intercept of this calibration curve with the *x*-axis. Stability of processed sample in the autosampler (10 °C) were investigated at QC MED and HIGH concentration ($n = 8$ each) according to References [23,27]. Stability data were collected only for processed samples because freeze–thaw experiments and benchtop-stability experiments are method independent and described elsewhere. Freeze–thaw stability and bench-top stability of choline and betaine in EDTA-plasma was evaluated by Yue et al. [21]. Stability data for TMAO, L-carnitine and *O*-acetyl-L-carnitine were evaluated by different research groups [28,29].

3. Results and discussion

3.1. Method development and optimization

While C18 chromatography is the most applied chromatographic mode in biomarker and drug analysis it is not well suited for retention of highly polar compounds. After intense literature search HILIC separation seems to be the method of choice for these permanently charged metabolites. The final choice of solvent for HILIC-chromatography with addition of 10 mM ammonia formate was made because of significant lower background noise in comparison to solvent containing ammonia acetate. An example chromatogram of QC HIGH is given in Fig. 1. No analyte free matrix was available and therefore calibration and QC samples were prepared in water as surrogate matrix [26]. For control standard addition experiments were performed.

3.2. Method validation

Calibration curves using four concentration levels with six replicates each were constructed to evaluate the calibration model. The limits for the calibration curve were assessed based on the reference range for the five metabolites. Calibration ranges for all analytes are given in Table 2 and should allow quantification in plasma and serum without further dilution. The calibration ranges were adapted to the commonly accepted reference intervals [1,10,28,30]. A weighted ($1/X$) calibration model was used to

Table 3
Calibration equations and correlation coefficients (R^2) found for a linear weighted $1/X$ regression model.

Analyte	Regression blank
Choline	$y = 0.0813x + 0.0006$, $R^2 = 0.994$
Betaine	$y = 0.0107x + 0.0030$, $R^2 = 0.989$
O-Acetyl-L-carnitine	$y = 0.1070\,x + 0.0050$, $R^2 = 0.986$
L-Carnitine	$y = 0.0034\,x + 0.0006$, $R^2 = 0.997$
TMAO	$y = 0.2633x + 0.0150$, $R^2 = 0.998$

Table 4
Method validation data: bias, intra-day precision (RSD_R), interday-precision (RSD_T).

	QC low			QC med			QC high		
Analyte	Bias (%)	RSD_R (%)	RSD_T (%)	Bias (%)	RSD_R (%)	RSD_T (%)	Bias (%)	RSD_R (%)	RSD_T (%)
Choline	−8.1	4.8	5.7	−9.2	2.3	7.2	−3.8	2.6	3.8
Betaine	−0.6	7.7	9.7	4.5	5.9	7.0	7.4	4.6	5.9
O -Acetyl-L-carnitine	−7.6	3.3	7.0	10.8	2.8	8.3	7.8	2.5	5.3
L-Carnitine	2.9	3.4	7.7	8.2	2.2	6.7	11.1	2.6	4.2
TMAO	−8.8	3.6	6.8	−12.7	2.8	3.9	−1.3	1.9	1.8

Table 5
Standard addition calculation against standards in MeOH. For urine samples a 5-fold predilution with water is recommended.

		Blank calibration [μM]					Standard addition [μM]					CV [%]				
		Choline	Betaine	Ac-Carn	Carn	TMAO	Choline	Betaine	Ac-Carn	Carn	TMAO	Choline	Betaine	Ac-Carn	Carn	TMAO
Plasma	1	48.7	55.9	10.7	94.3	9.4	46.9	58.2	8.4	100	8.7	−1.9	2.0	−11.9	2.9	−3.7
	2	50.2	75.3	6.7	54.1	2.4	53	79	5.8	57.4	2.4	2.7	2.4	−7.0	3.0	-0.3
	3	61.7	65.3	13.0	66.3	7.6	66.4	64.5	12.2	59.4	7.8	3.7	−0.6	−3.3	−5.5	1.4
	4	51.9	24.3	12.9	53.5	11.0	48.1	21.2	11.8	53.1	10.8	-3.8	−6.9	−4.5	−0.4	−1.1
	5	21.3	45.6	5.0	30.7	2.5	23.3	46.5	4.9	37.9	2.4	4.5	1.0	−0.6	10.5	−2.5
	6	58.3	44.9	7.6	68.4	7.6	57	37.7	6.2	63.6	7.6	−1.1	-8.7	−10.3	−3.6	0.1
Urine	7	15.2	12.9	26.6	80.5	101.0	19.6	13.7	27.8	104.1	162.1	12.6	3.1	2.1	12.8	23.2
	8	13.5	32.1	27.9	42.5	154.7	12.4	26.0	25.5	44.7	160.8	−4.2	−10.6	−4.4	2.6	1.9
	9	26.2	19.3	56.4	147.3	247.7	28.8	15.9	46.7	163.5	261.4	4.6	−9.7	−9.4	5.2	2.7
	10	20.5	11.7	12.3	50.1	45.4	24.4	11.1	11.7	62.1	74.9	8.8	−2.5	−2.2	10.7	24.5
	11	12.8	7.3	5.8	11.7	74.8	9.4	5.5	3.5	13.1	81.0	−15.2	−13.5	924.6	5.4	4.0
	12	13.2	5.1	1.4	5.8	35.0	9.5	5.2	1.1	5.7	43.0	−16.4	0.6	−11.3	−0.4	10.3
Serum	13	12.8	36.0	10.6	47.0	1.5	14.4	32.5	10.3	51.4	1.5	6.0	−5.2	−1.4	4.4	1.0
	14	14.6	46.3	16.5	44.8	2.3	18.1	42	16.3	47.4	2.3	11.0	−4.9	−0.7	2.7	−1.0
	15	7.8	55.6	3.9	42.2	1.2	11.6	55.1	4.2	45.9	1.5	19.6	−0.5	3.7	4.2	9.8
	16	8.9	69.4	8.3	51.1	4.5	13.6	68.5	8.2	59.5	4.7	13.3	-10.0	−2.3	−2.9	−0.1
	17	7.1	35.1	3.9	37.0	1.8	9.3	28.7	3.7	34.9	1.8	18.0	0.5	3.5	6.3	3.5
	18	15.6	52.1	11.0	34.8	3.4	22.5	52.6	11.8	39.5	3.7	13.8	-5.3	−4.5	0.8	0.9

Table 6
Metabolite concentrations from plasma samples of 13 apparently healthy volunteers. Reference ranges were adapted from literature.

	Choline [μM]	Betaine [μM]	*O*-Acetyl-L-carnitine [μM]	L-Carnitine [μM]	TMAO [μM]
Ref. range	7.6–12.2 [10]9.8–18.5 [1]	15.2–66.3 [1]	4.5–8.8 [30]	34.7–51.9 [30]	2.3–5.8 [28]
1	24.5	61.4	8.8	40.5	2.2
2	14.8	32.9	5.5	44.4	1.8
3	21.7	88.2	2.7	38.6	2.3
4	13.4	13.5	2.1	16.4	1.7
5	20.0	56.6	4.5	38.1	3.5
6	18.0	43.5	3.8	40.4	5.0
7	15.9	63.5	9.6	63.3	1.6
8	14.5	58.5	5.9	59.8	0.9
9	12.5	60.2	4.2	44.2	3.4
10	13.9	21.5	4.3	43.8	0.7
11	12.1	38.8	7.0	37.7	1.5
12	13.3	29.7	4.5	34.7	2.2
13	16.2	69.1	4.3	68.2	2.5

account for unequal variances (heteroscedasticity) across the calibration range. As there were several possibilities with different ISs and calibration models, the final decision was made after evaluation of the accuracy and precision data. The final calibration model was linear, $1/X$ weighting for all analytes. Regression equations and correlation coefficients (R^2) are given in Table 3, indicating sufficient correlation with R^2 above 0.98 for all analytes. However, these data were determined using calibration curves measured on 6 different days. The daily back-calculated concentrations of all calibration samples compared to their respective nominal values were obtained with a quantitative accuracy within 20% of target for each day. The lowest point of the calibration curve was defined as the limit of quantification (LOQ) of the method and fulfilled the requirement of LOQ, signal to noise ratio of 10:1 determined *via* peak heights. The limit of detection (LOD) was not systematically investigated and was set as equal to LOQ.

QC samples (LOW, MED, and HIGH) were analyzed in duplicate on each of eight days as was proposed by Peters and co-workers [23]. QC concentrations were calculated from daily calibration curves. Calibrator concentrations were within 20% of target based on the full calibration curve. Accuracy, intra-day and inter-day precision were calculated as described above (Table 4). Carryover between injections was not observed. All analytes fulfilled the validation parameters. Addition of choline, betaine, and TMAO, L-carnitine an *O*-aceytl-L-carnitine to authentic plasma serum and urine samples caused a comparable increase in absolute abundance of the respective product ions. As shown by standard addition experiments (Table 5) calibration can be done in Water/MeOH solution. For plasma samples there is a high correlation between the values calculated by standard addition method and calibration done in Water/MeOH. The same correlation is observed for blood serum samples. Only for choline observed values are slightly lower when calibration is done in water/methanol. In case of urine sam-

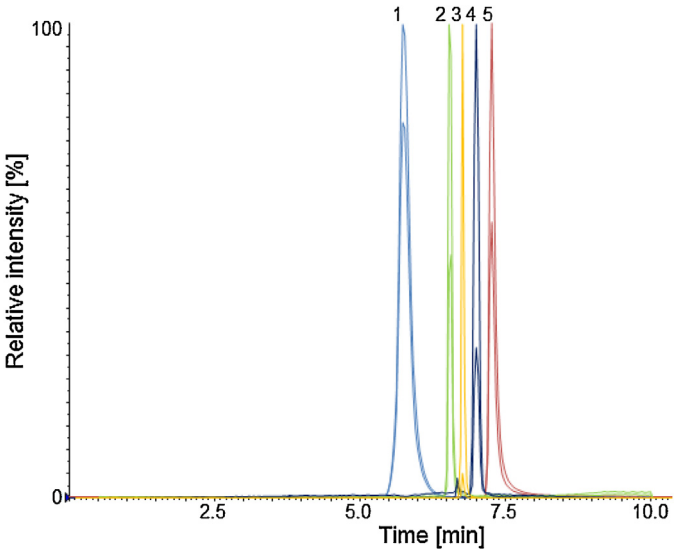

Fig. 1. Extracted ion chromatogram (TIC) of QC HIGH level showing choline (1), betaine (2), *O*-acetyl-L-carnitine (3), L-carnitine (4) and TMAO (5).

ples, 5-fold pre-dilution with water is recommended for choline, betaine, L-carnitine, *O*-acetyl-L-carnitine and TMAO. Because of urinary concentration effects, especially higher values of TMAO are detected and classified above the blank calibration curve. Therefore a 5-fold pre-dilution was applied and was sufficient for TMAO determination. As shown in Table 5 correlation between standard addition and calibration is high. These data also indicate that matrix effects in EDTA plasma, serum and urine are negligible. Regression equations and coefficients of standard addition experiment are given in Supporting information. The LOQs of all analytes were consistent with the lowest calibrator with less than 20% bias as compared to the target concentration (Table 4). Those limits were comparable to those published by other authors for TMAO, betaine, choline, L-carnitine and *O*-acetyl-L-carnitine.

All analytes could be extracted with REs over 60% with acceptable CVs (Supporting information). Although protein precipitation is not an actual extraction procedure and theoretically should result in 100% recovery, it is known, that co-precipitation can occur, decreasing final recoveries [31]. No degradation was observed for any analyte in processed samples stored on the autosampler for 18 h at 10 °C (data not shown). There is no difference observed between human plasma, serum and urine. Only for TMAO and TMAO-d9 in urine a decrease of more than 20% is observed after 10 h. One may speculate if lower pH or bacterial contamination of the urine cause decrease in TMAO and TMAO-d9 concentration. Therefore processing time for urine samples should not exceed 10 h.

The described method was successfully applied for TMAO and related compound determination in human plasma from 13 healthy volunteers. Plasma samples were obtained after an overnight fastened period. As shown in Table 6 determined values correspond to the reference range reported in literature for all five metabolites.

4. Conclusion

This LC–MS/MS multi-analyte approach allowed the simultaneous accurate and precise quantification of TMAO and its relevant precursors in EDTA-plasma, serum and urine using the same scheduled MRM method for each compound. Standard addition method for all matrices showed good correlation with calibrators prepared in MeOH. All together the presented method was successfully applied to 13 samples of healthy volunteers. Based on a literature review and contact of experts in the field we aim to measure the following markers in an upcoming clinical study to evaluate the predictive qualities of these metabolic biomarkers in case of community acquired pneumonia (CAP) and exacerbated chronic obstructive pulmonary disease (ECOPD). The results of this study will be presented elsewhere.

Conflict of interests

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors would like to thank Christoph Saxer and Manuela Nickler for their helpful support and discussion.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2015.12.002.

References

[1] S.J. Bruce, P.A. Guy, S. Rezzi, A.B. Ross, Quantitative measurement of betaine and free choline in plasma, cereals and cereal products by isotope dilution LC–MS/MS, J. Agric. Food Chem. 58 (2010) 2055–2061.
[2] M. Gossell-Williams, H. Fletcher, N. McFarlane-Anderson, A. Jacob, J. Patel, S. Zeisel, Dietary intake of choline and plasma choline concentrations in pregnant women in Jamaica, West Indian Med. J. 54 (2005) 355–359.
[3] S.A. Craig, Betaine in human nutrition, Am. J. Clin. Nutr. 80 (2004) 539–549.
[4] S.H. Zeisel, K.A. da Costa, Choline: an essential nutrient for public health, Nutr. Rev. 67 (2009) 615–623.
[5] A.E. Jeukendrup, R. Randell, Fat burners: nutrition supplements that increase fat metabolism, Obes. Rev. 12 (2011) 841–851.
[6] J.T. Gonzalez, E.J. Stevenson, New perspectives on nutritional interventions to augment lipid utilisation during exercise, Br. J. Nutr. 107 (2012) 339–349.
[7] B.J. Lee, J.S. Lin, Y.C. Lin, P.T. Lin, Effects of L-carnitine supplementation on oxidative stress and antioxidant enzymes activities in patients with coronary artery disease: a randomized, placebo-controlled trial, Nutr. J. 13 (2014) 79.
[8] J.J. DiNicolantonio, C.J. Lavie, H. Fares, A.R. Menezes, J.H. O'Keefe, l-Carnitine in the secondary prevention of cardiovascular disease: systematic review and meta-analysis, Mayo Clin. Proc. 88 (2013) 544–551.
[9] G.R. Steenge, P. Verhoef, M.B. Katan, Betaine supplementation lowers plasma homocysteine in healthy men and women, J. Nutr. 133 (2003) 1291–1295.
[10] O. Danne, M. Mockel, Choline in acute coronary syndrome: an emerging biomarker with implications for the integrated assessment of plaque vulnerability, Expert Rev. Mol. Diagn. 10 (2010) 159–171.
[11] G.W. Dalmeijer, M.R. Olthof, P. Verhoef, M.L. Bots, Y.T. van der Schouw, Prospective study on dietary intakes of folate, betaine, and choline and cardiovascular disease risk in women, Eur. J. Clin. Nutr. 62 (2008) 386–394.
[12] W.H. Tang, Z. Wang, K. Shrestha, A.G. Borowski, Y. Wu, R.W. Troughton, A.L. Klein, S.L. Hazen, Intestinal microbiota-dependent phosphatidylcholine metabolites, diastolic dysfunction, and adverse clinical outcomes in chronic systolic heart failure, J. Card. Fail. 21 (2015) 91–96.
[13] R.A. Koeth, Z. Wang, B.S. Levison, J.A. Buffa, E. Org, B.T. Sheehy, E.B. Britt, X. Fu, Y. Wu, L. Li, J.D. Smith, J.A. DiDonato, J. Chen, H. Li, G.D. Wu, J.D. Lewis, M. Warrier, J.M. Brown, R.M. Krauss, W.H. Tang, F.D. Bushman, A.J. Lusis, S.L. Hazen, Intestinal microbiota metabolism of L-carnitine, a nutrient in red meat, promotes atherosclerosis, Nat. Med. 19 (2013) 576–585.
[14] W.H. Tang, Z. Wang, B.S. Levison, R.A. Koeth, E.B. Britt, X. Fu, Y. Wu, S.L. Hazen, Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk, N. Engl. J. Med. 368 (2013) 1575–1584.
[15] K.A. Romano, E.I. Vivas, D. Amador-Noguez, F.E. Rey, Intestinal microbiota composition modulates choline bioavailability from diet and accumulation of the proatherogenic metabolite trimethylamine-*N*-oxide, mBio 6 (2015) e02481.
[16] J. Miao, A.V. Ling, P.V. Manthena, M.E. Gearing, M.J. Graham, R.M. Crooke, K.J. Croce, R.M. Esquejo, C.B. Clish, Morbid Obesity Study Group, D. Vicent, S.B. Biddinger, Flavin-containing monooxygenase 3 as a potential player in diabetes-associated atherosclerosis, Nat. Commun. 6 (2015) 1–10.
[17] M. Lever, P.M. George, S. Slow, D. Bellamy, J.M. Young, M. Ho, C.J. McEntyre, J.L. Elmslie, W. Atkinson, S.L. Molyneux, R.W. Troughton, C.M. Frampton, A.M. Richards, S.T. Chambers, Betaine and trimethylamine-*N*-oxide as predictors of cardiovascular outcomes show different patterns in diabetes mellitus: an observational study, PLoS One 9 (2014) e114969.
[18] W.H. Tang, Z. Wang, D.J. Kennedy, Y. Wu, J.A. Buffa, B. Agatisa-Boyle, X.S. Li, B.S. Levison, S.L. Hazen, Gut microbiota-dependent trimethylamine-*N*-oxide (TMAO) pathway contributes to both development of renal insufficiency and mortality risk in chronic kidney disease, Circ. Res. 116 (2015) 448–455.

[19] G.D. Lewis, R. Wei, E. Liu, E. Yang, X. Shi, M. Martinovic, L. Farrell, A. Asnani, M. Cyrille, A. Ramanathan, O. Shaham, G. Berriz, P.A. Lowry, I.F. Palacios, M. Tasan, F.P. Roth, J. Min, C. Baumgartner, H. Keshishian, T. Addona, V.K. Mootha, A. Rosenzweig, S.A. Carr, M.A. Fifer, M.S. Sabatine, R.E. Gerszten, Metabolite profiling of blood from individuals undergoing planned myocardial infarction reveals early markers of myocardial injury, J. Clin. Invest. 118 (2008) 3503–3512.
[20] M. Dambrova, E. Skapare-Makarova, I. Konrade, O. Pugovics, S. Grinberga, D. Tirzite, R. Petrovska, I. Kalvins, E. Liepins, Meldonium decreases the diet-increased plasma levels of trimethylamine-*N*-oxide, a metabolite associated with atherosclerosis, J. Clin. Pharmacol. 53 (2013) 1095–1098.
[21] B. Yue, E. Pattison, W.L. Roberts, A.L. Rockwood, O. Danne, C. Lueders, M. Mockel, Choline in whole blood and plasma: sample preparation and stability, Clin. Chem. 54 (2008) 590–593.
[22] P.I. Holm, P.M. Ueland, G. Kvalheim, E.A. Lien, Determination of choline, betaine, and dimethylglycine in plasma by a high-throughput method based on normal-phase chromatography-tandem mass spectrometry, Clin. Chem. 49 (2003) 286–294.
[23] F.T. Peters, M. Hartung, M. Herbold, G. Schmitt, T. Daldrup, F. Mußhoff, Anhang B zur Richtlinien der GTFCh zur Qualitätssicherung bei forensisch-toxikologischen Untersuchungen, Anforderungen an die Validierung von Analysenmethoden (GTFCh validation guidelines for forensic toxicological analysis), Toxichem Krimtech 76 (2009) 185–208.
[24] E. Ciccimaro, I.A. Blair, Stable-isotope dilution LC–MS for quantitative biomarker analysis, Bioanalysis 2 (2010) 311–341.
[25] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC–MS/MS, Anal. Chem. 75 (2003) 3019–3030.
[26] N.C. van der Merbel, Quantitative determination of endogenous compounds in biological samples using chromatographic techniques, Trends Anal. Chem. 27 (2008) 924–932.
[27] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies, Pharm. Res. 9 (1992) 5.
[28] Z. Wang, B.S. Levison, J.E. Hazen, L. Donahue, X.M. Li, S.L. Hazen, Measurement of trimethylamine-*N*-oxide by stable isotope dilution liquid chromatography tandem mass spectrometry, Anal. Biochem. 455 (2014) 35–40.
[29] J. Sowell, M. Fuqua, T. Wood, Quantification of total and free carnitine in human plasma by hydrophilic interaction liquid chromatography tandem mass spectrometry, J. Chromatogr. Sci. 49 (2011) 463–468.
[30] A.M. Evans, R.J. Faull, R.L. Nation, S. Prasad, T. Elias, S.E. Reuter, G. Fornasini, Impact of hemodialysis on endogenous plasma and muscle carnitine levels in patients with end-stage renal disease, Kidney Int. 66 (2004) 1527–1534.
[31] G. Sedgwick, T. Fenton, J. Thompson, Effect of protein precipitating agents on the recovery of plasma-free amino-acids, Can. J. Anim. Sci. 71 (1991) 953–957.