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Quantification of acetylcholine, choline, betaine, and dimethylglycine in human plasma and urine using stable-isotope dilution ultra performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Disorders in choline metabolism are related to disease conditions. We developed a stable-isotope dilution ultra performance liquid chromatography–mass spectrometry (UPLC–MS/MS) method for the simultaneous quantification of acetylcholine (ACh), betaine, choline, and dimethylglycine (DMG). We used this method to measure concentrations of the analytes in plasma and urine in addition to other biological fluids after a protein precipitation by acetonitrile. The detection limits were between 0.35 nmol/L (for ACh in urine) and 0.34 μmol/L (for betaine in urine). ACh concentrations were not detectable in plasma. Intraassay and interassay coefficient of variation (CVs) were all <10.0% in biological fluids, except for DMG in cerebrospinal fluid (CV = 12.44%). Mean recoveries in urine pool samples were between 99.2% and 103.9%. The urinary excretion of betaine, choline, and DMG was low, with approximately 50.0% higher excretion of choline in females compared to males. Median urinary excretion of ACh were 3.44 and 3.92 μmol/mol creatinine in males and females, respectively ($p = 0.689$). Plasma betaine concentrations correlated significantly with urinary excretions of betaine ($r = 0.495$, $p = 0.027$) and choline ($r = 0.502$, $p = 0.024$) in females. Plasma choline concentrations correlated significantly with urinary excretion of ACh in males ($r = 0.419$, $p = 0.041$) and females ($r = 0.621$, $p = 0.003$). The new method for the simultaneous determination of ACh, betaine, choline, and DMG is sensitive, precise, and fast enough to be used in clinical investigations related to the methylation pathway.

1. Introduction

Choline is an essential micronutrient that is involved in several biochemical pathways. First, choline is a precursor of acetylcholine (ACh) that is formed by choline acetyltransferase (EC 2.3.1.6) [1]. Second, choline is utilized for synthesis of phosphatidylcholine and sphingomyelin thus having a functional role in cellular membrane composition and functions [1]. Third, the enzymatic oxidation of choline (mainly in the liver) delivers betaine, a methyl donor for the remethylation of homocysteine (Hcy) into methionine [2]. This reaction is catalyzed by the enzyme betaine:homocysteine methyltransferase (BHMT; EC 2.1.1.5) [3]. Betaine can be alternatively obtained from the diet [1] and it is also considered an important osmolyte. The product of betaine-demethylation is dimethylglycine (DMG) which can be further converted to sarcosine, and later on to glycine.

Deficiency of choline can cause developmental disorders, fetal brain damage, fatty liver [4], or muscle damage [5]. Moreover, deficiencies of choline, betaine, and folate cause Hcy accumulation, which is a risk factor for several diseases [6]. In addition, reduced plasma concentration of betaine has been related to lipid disorders [7], the metabolic syndrome [7], and diabetes [8]. The metabolisms of choline and folate are interrelated thus explaining the Hcy-lowering effect of betaine [9] and choline [10–12].

ACh is an important neurotransmitter and an endothelium-dependent vasodilator in the central and peripheral nervous systems [13]. In animal models, choline deficient diet caused renal necrosis [14,15] and hepatic dysfunction which could be partly related to phospholipid dysbalance or to low ACh production. Cholinesterase inhibitors that allow high concentrations of ACh for a longer time have been developed and tested for dementia treat-

Abbreviations: ACh, acetylcholine; AD, Alzheimer's disease; AF, amniotic fluid; BHMT, betaine:homocysteine methyltransferase; CSF, cerebrospinal fluid; DMG, dimethylglycine; Hcy, homocysteine; HILIC, hydrophilic interaction liquid chromatography; HPLC, high-performance liquid chromatography; IDL, instrumental detection limit; IQL, instrumental quantification limit; LC–MS/MS, liquid chromatography with tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MDL, method detection limit; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; MQL, method quantification limit; RMSE, root mean square error; SAM, S-adenosyl methionine; tHcy, total homocysteine; TIC, total ion chromatogram; UPLC, ultra performance liquid chromatography.

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ment [16], and for protection against ischemic acute renal failure [17]. Recent data suggests that the activity of acetylcholinesterase is induced during ischemia reperfusion thus causing apoptosis in vivo [17] and probably connecting acute renal failure to ACh. Ye et al. have shown that inhibition of acetylcholinesterase lowered serum creatinine and urea nitrogen in animals subjected to ischemia reperfusion [17]. Therefore, ACh might be causally related to renal dysfunction.

The quantification of ACh, betaine, choline, and DMG in biological material is of interest. Choline is a small polar molecule that lacks a chromophore and thus cannot be detected by immunoassays. Chemiluminescent enzyme assays [18], as well as several UV-detection based high-performance liquid chromatography (HPLC) methods [19] have been described. Recently, liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods for plasma samples have been developed [20–22]. We used a hydrophilic interaction liquid chromatography (HILIC) which might offer an advantage, because the metabolites are weakly retained on reversed phase liquid chromatographic columns.

The physiological concentrations of ACh are in the nanomolar range and that of betaine, choline, and DMG are in the micromolar range. The ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) offers higher sensitivity and allows for all 4 metabolites to be measured in one run. We describe an improved, stable-isotope dilution UPLC–MS/MS HILIC method for the simultaneous quantification of ACh, betaine, choline, and DMG in several biological materials, including EDTA plasma, urine, amniotic fluid (AF), and cerebrospinal fluid (CSF). In addition, we utilized the new method for investigating concentrations of the metabolites in plasma and urine samples.

2. Materials and methods

2.1. Standards and chemicals

Acetylcholine iodide (Merck Chemicals, Darmstadt, Germany), betaine chloride, choline chloride, and *N*,*N*-dimethylglycine (Sigma–Aldrich, Munich, Germany) were used for preparation of standard solutions. d_9-Betaine chloride, d_9-choline chloride (Isotec, Sigma–Aldrich, Munich, Germany), d_9-acetylcholine chloride, and d_6-dimethylglycine HCl (CDN isotopes, Quebec, Canada) were used as internal standards. Other chemicals used were: ammonium formate, formic acid, acetonitrile (ULC/MS grade; Biosolve, Valkenswaard, The Netherlands), and ultrapure water (18.2 MΩ) from a Milli-Q water purification system (Millipore, Molsheim, France).

Stock solutions of ACh and d_9-ACh (each 1000 nmol/L) and betaine, d_9-betaine, choline, d_9-choline, DMG, and d_6-DMG (each 1000 μmol/L) were prepared in acetonitrile/H_2O (1:1; v/v). Aliquots of each stock solution were stored at −70 °C, used within 6 months, and thawed only once.

2.2. Subjects and sample collection

EDTA plasma samples from a total of 146 adults (34 males) were available. Serum samples were available from a subset of 74 females. The concentrations of choline and betaine were compared between serum and plasma samples from a subset of 74 females (mean age 35 years). Additionally, we investigated the effect of fasting and non-fasting conditions on the concentrations of the analytes. To this end, plasma samples from 28 subjects (mean age 52 years; 10 males) were available after overnight fasting and on the same day 4 h after lunch. Finally, we tested concentrations of ACh, betaine, choline, and DMG in plasma and urine samples from 44 older adults (>50 years; 24 males). For this purpose, fasting plasma and urine samples were collected on the same morning.

Blood was collected both in potassium EDTA-containing tubes and in tubes without anticoagulant. Blood samples were centrifuged within 30 min at 2000 × *g* and 4 °C for 10 min. The EDTA plasma and serum were then separated and immediately stored at -70 °C until analysis. Samples of cerebrospinal fluid (CSF) and amniotic fluid (AF) were collected for diagnostic purposes. Aliquots of these samples were immediately separated by centrifugation and stored at −70 °C. Pooled samples of EDTA plasma, CSF, AF, and urine were prepared, stored at −70 °C, and one aliquot was measured in each run. We used in-house prepared pools of EDTA plasma, CSF, AF, and urine for determination of detection and quantification limits of the assay. Pool of each biological material (3 mL) was dialyzed using a Spectra/Por Float-A-Lyzer CE (MWCO: 8,000 Dalton; Carl Roth GmbH, Karlsruhe, Germany) against 150 volumes of phosphate-buffered saline containing 4 mmol/L sodium EDTA (Sigma–Aldrich, Munich, Germany). Samples were collected for the determination of the detection and quantification limits when the concentrations of the analyte were in the ranges of 1–5 times of the instrumental detection limit (IDL). Urinary creatinine concentrations were determined by COBAS INTEGRA System (Roche Diagnostics, Mannheim, Germany). The study was approved by the local ethics committee and each participant signed an informed consent.

2.3. Sample preparation

Sample preparation was carried out on ice and vials were immediately sealed to avoid evaporation of acetonitrile at room temperature. Six calibrators of different concentrations were included in each batch of samples (Table 1). Quality control samples at two different concentrations (high and low) were included in each batch (Table 1). Calibrators and quality control samples were prepared from stock solutions in acetonitrile and processed as described below.

Plasma, serum, urine, CSF, and AF samples were thawed and centrifuged at 2000 × *g* for 5 min at 4 °C. The urine samples were diluted with H_2O (1:5) prior to analysis because of the high salt concentrations which can damage or shorten the life of the UPLC column and tubings. The clear sample, calibrator, and quality control (100 μL) were each added to a 1.5 mL tube containing 300 μL internal standard mix [5 nmol/L d_9-ACh and d_9-betaine, d_9-choline, d_6-DMG (each 10 μmol/L)] in acetonitrile. The addition of acetonitrile caused protein precipitation and the analytes were extracted by vigorous vortexing of the sample for 30 s. The samples were centrifuged for 5 min at 10,000 × *g* at room temperature; the super-

Table 1
Concentration of calibrators and quality control samples.

	ACh (nmol/L)	Betaine (μmol/L)	Choline (μmol/L)	DMG (μmol/L)
Calibration	0, 0.2, 0.4, 1, 5, 10, 40	0, 0.5, 1, 2.5, 12.5, 25, 100	0, 0.3, 0.6, 1.5, 7.5, 15, 60	0, 0.2, 0.4, 1, 5, 10, 40
Calibration for instrumental detection limit	0, 0.4, 0.8, 1.6, 3.2, 6.4	0, 0.2, 0.4, 0.8, 1.6, 3.2	0, 0.2, 0.4, 0.8, 1.6, 3.2	0, 0.2, 0.4, 0.8, 1.6, 3.2
Calibration for linearity range determination	0, 0.2, 0.4, 1, 10, 50, 150	0, 0.2, 0.4, 1, 10, 50, 150	0, 0.2, 0.4, 1, 10, 50, 150	0, 0.2, 0.4, 1, 10, 50, 150
High control	30	75	45	30
Low control	3	7.5	4.5	3

ACh: acetylcholine, DMG: dimethylglycine.

Table 2
Instrument parameters for LC–MS/MS analysis of ACh, betaine, choline, DMG, and their internal standards.

Analyte	Cone voltage (V)	Collision energy (eV)	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Retention time (min)
ACh	21.0	15.0	146.13	86.93	1.75
Betaine	31.0	17.0	118.08	59.22	3.20
Choline	34.0	16.0	104.08	60.05	2.19
DMG	21.0	12.0	104.08	58.18	3.00
d_9-ACh	22.0	14.0	154.92	86.86	1.75
d_9-betaine	26.0	18.0	127.15	68.16	3.20
d_9-choline	29.0	18.0	113.23	69.13	2.18
d_6-DMG	21.0	12.0	109.97	64.17	3.00

ACh: acetylcholine, DMG: dimethylglycine.

natant was transferred to glass vials. Sealed vials were either immediately measured or stored at −70 °C for no longer than one week.

2.4. UPLC–MS/MS conditions

UPLC–MS/MS analyses were carried out using an Acquity Ultra Performance LC system coupled to a MicroMass Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA). The samples were separated on an Acquity UPLC BEH HILIC column (100 mm × 2.1 mm (i.d.); 1.7 μm particle size) with an Acquity HILIC VanGuard pre-column (5 mm × 2.1 mm (i.d.); 1.7 μm particle size) and a 0.2 μm in-line filter (Waters Corporation). The column temperature was set to 30 °C. The flow rate was 0.6 mL/min. The solvents were 15 mmol/L ammonium formate (solvent A, pH 3.5) and acetonitrile (solvent B) with a gradient over the 6.5 min run time as follow: 0.0–3.5 min (12.5% A), 3.5–3.6 min (20% A), and 4.5–4.6 min (12.5% A). All gradient steps were linear. The sample injection volume was 1 μL.

The target analytes and their internal standards were identified by a triple quadrupole mass spectrometer (MicroMass Quattro Premier XE) using positive electrospray ionization mode. Source temperature was 150 °C, capillary voltage was 0.40 kV, desolvation gas (N_2) temperature was 350 °C at a flow rate of 700 L/h, and the cone gas (N_2) flow rate was 50 L/h. Collision gas (Ar) flow was maintained at 0.30 L/h. Inter-scan and inter-channel delay were set to 0.02 s, dwell times were 0.05 s. Individual cone and collision energy voltages, as well as multiple reaction monitoring (MRM) mass transitions are summarized in Table 2.

2.5. Data analyses

Data was acquired and processed using MassLynx V4.1 and QuanLynx software. Calibrators were used for the construction of a standard curve by plotting the response (y = area analyte/area internal standard) against the corresponding concentrations (c) of the calibrators. The slope (m) and the intercept (i) of the standard curve were used for calculating the concentration of the unknown sample (x) as $y = m \times x + i$.

Recovery (%) was calculated as: (Measured concentration/(Expected concentration + Concentration added)) × 100.

Root mean square error (RMSE) was calculated as follows:

$$\mathrm{RMSE} = \left[\frac{\sum_{j=1}^{n} E_j^2}{n-2}\right]^{\frac{1}{2}}$$

where n is the number of standards and E is the error associated with each measurement.

IDL was calculated as 3 × RMSE/m, and instrumental quantification limit (IQL) was calculated as 10 × RMSE/m. Method detection limit (MDL) was determined by $t_{99}s_{\mathrm{LLMV}}$ method, using the following equation:

$$\mathrm{MDL} = t_{99(n-1)} \times \mathrm{SD}$$

where $t_{99(n-1)}$ is the one-tailed t-statistic for $n-1$ observations at the 99% confidence level ($t_{99(n-1)}$ = 2.821 for 10 aliquots or 9 degrees of freedom) and SD is the standard deviation. Method quantification limit (MQL) = 3 × MDL.

Urinary excretion was calculated as the ratio of concentration of the analyte:creatinine concentration. Linear regression analysis was used to verify the linearity of the calibration curves. Results are shown as median (10–90th percentile) or mean (standard deviation; SD).

Paired t-test was used for comparison of sample material and fasting conditions on the analytes. Correlation analyses were performed by using Spearman-Rho test. Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences, version 17.0).

3. Results

3.1. Chromatography and tandem mass spectrometry

Optimal MRM conditions for the quantification of the analytes and their stable-isotope labeled internal standards were obtained in the positive electrospray ionization mode of the Quattro Premier XE mass spectrometer. MRM chromatograms from quality control and urine pool samples are shown in Fig. 1. ACh and d_9-ACh coeluted at 1.79 and 1.78 min, choline and d_9-choline at 2.22 min, DMG and d_6-DMG at 3.03 and 3.00 min, and betaine and d_9-betaine at 3.14 and 3.16 min (Table 1). No peaks above the detection limit for ACh were found in plasma, serum, AF, and CSF samples. In urine, interfering peaks for ACh appeared approximately at 0.87 min, 2.56 min, 3.66 min, and 5.67 min. Because these peaks did not appear in calibration and quality control samples, they might be caused by sample matrix. Due to similar mass transitions, an additional choline peak (m/z 104.08 → 60.05) could be detected in the DMG trace (m/z 104.08 → 58.15) at 2.23 min. All interfering peaks were chromatographically separated from the compounds and were excluded from the data analyses. Compared to calibration and quality control samples, ACh showed minor ion suppression effects in pool samples, whereas there was a slight ion enhancement for choline, betaine, and DMG. Using stable-isotope labeled analogs of the analytes eliminated matrix effects.

3.2. Stability

Determining ACh concentration in biological samples requires the inhibition of the naturally occurring cholinesterases, for instance by using carbamates, Ca^+ chelators (e.g. EDTA), or organophosphate compounds, or by protein precipitation. EDTA plasma pool samples were measured immediately after collection and again after freezing over several months at −70 °C. Concentra-

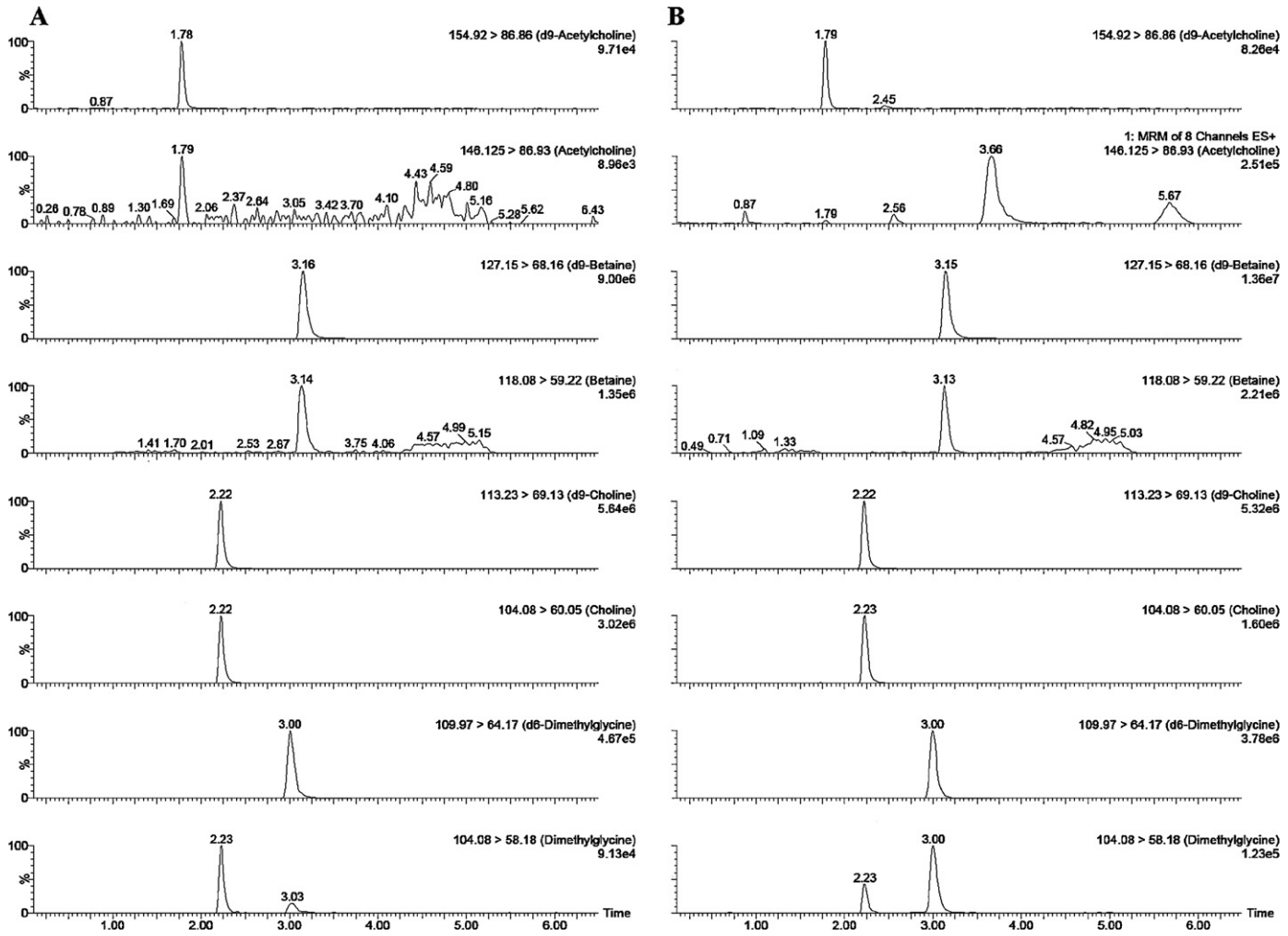

Fig. 1. MRM chromatograms of acetylcholine (ACh), betaine, choline, dimethylglycine (DMG), and their internal standards obtained by UPLC–MS/MS in (A) a quality control sample, containing 3 nmol/L ACh, 7.5 μmol/L betaine, 4.5 μmol/L choline, and 3 μmol/L DMG, and (B) one urine pool sample (1:5 diluted with H_2O) containing 3.62 nmol/L ACh, 7.63 μmol/L betaine, 2.45 μmol/L choline, and 3.03 μmol/L DMG. *m*/*z* transitions and peak intensities are shown in the upper right.

tions of betaine, choline, and DMG in frozen CSF, AF, and plasma samples were stable for at least 6 months. Concentrations of ACh, betaine, choline, and DMG were also stable in frozen urine pool for at least 6 months. No remarkable changes in concentrations of betaine, choline, or DMG were observed after at least three freeze/thaw cycles (data not shown).

3.3. Linearity and sensitivity

The assay was linear over the physiological ranges (Table 1). Concentrations of the standard solutions correlated strongly to the peak areas. The coefficients of linear regression for 5 independent experiments were: $r^2 > 0.999$ (mean (SD) linear equation $y = 0.0318$ (0.0028) $x + 0.0239$ (0.0075)) for ACh, $r^2 > 0.999$ ($y = 0.0189$ (0.0023) $x + 0.0030$ (0.0038)) for betaine, $r^2 > 0.999$ ($y = 0.1232$ (0.0084) $x + 0.0274$ (0.0065)) for choline, and $r^2 > 0.999$ ($y = 0.0095$ (0.0016) $x + 0.0035$ (0.0022)) for DMG (Supplemental Data Fig. 1).

The limits of detection (LOD) and quantification (LOQ) were calculated in a two-step approach, proposed by the U.S. Environmental Protection Agency. This consists of (1) the determination of the IDL and IQL (defined as the smallest amount of an analyte that can be reliably detected/quantified from the background on an instrument), and using these values to estimate MDL and MQL and (2) the calculation of the MDL and MQL (defined as the smallest amount of an analyte that can be reliably detected/quantified from the background for a particular matrix) using 10 aliquots of the dialyzed pool sample [23]. IDL and IQL were estimated by calculating the RMSE of five 5-point calibration curves (concentrations see Table 1) [24]. Mean (SD) IDL was 0.32 (0.24) nmol/L for ACh, 0.18 (0.08) μmol/L for betaine, 0.09 (0.02) μmol/L for choline, and 0.20 (0.12) μmol/L for DMG. MDLs were estimated by first preparing EDTA plasma, CSF, AF, and urine (1:5 diluted with H_2O) samples with concentrations between 1–5 times the IDL. In addition, 10 blank calibrators containing internal standard were prepared. Mean concentration of blank calibrators was subtracted from each sample concentration. Mean (SD) concentrations for plasma and urine samples and their LOD are presented in Table 3. LODs [mean (SD) concentrations] for AF were 0.25 μmol/L [0.61 (0.09) μmol/L] for betaine, 0.03 μmol/L [0.29 (0.01) μmol/L] for choline, and 0.07 μmol/L [0.42 (0.02) μmol/L] for DMG and LODs [mean (SD) concentrations] for CSF were 0.11 μmol/L [0.19 (0.04) μmol/L] for betaine, 0.04 μmol/L [0.33 (0.01) μmol/L] for choline, and 0.13 μmol/L [0.43 (0.05) μmol/L] for DMG.

3.4. Recovery and precision

Recovery experiments were performed by spiking the urine (1:5 diluted) or plasma pools with two different concentrations of the analytes in the physiological range (Table 3). Mean recoveries in the diluted urine pool samples were 102.6% for ACh, 103.9% for

Table 3
Limit of detection (LOD) and recovery of ACh, betaine, choline, and DMG.

Analyte	LOD		Recovery			
	Mean (SD)[a]	LOD	Measured mean (SD)	Added	Measured in spiked samples mean (SD)[b]	Recovery[b] (SD)(%)
Analyte concentrations in diluted urine pool[c]						
ACh (nmol/L)	0.56 (0.12)	0.35	3.97 (0.19)	20.0	23.58 (2.27)	98.4 (9.5)
				10.0	14.93 (0.46)	106.9 (3.3)
Betaine (μmol/L)	0.90 (0.12)	0.34	6.40 (0.36)	20.0	27.03 (1.11)	102.4 (4.2)
				10.0	17.29 (0.15)	105.4 (0.9)
Choline (μmol/L)	0.37 (0.06)	0.16	2.50 (0.10)	20.0	22.19 (0.18)	98.6 (0.8)
				10.0	12.48 (0.30)	99.8 (2.4)
DMG (μmol/L)	0.45 (0.04)	0.12	2.19 (0.15)	4.0	5.87 (0.38)	94.8 (6.1)
				2.0	4.52 (0.27)	107.9 (6.4)
Analyte concentrations in plasma pool						
Betaine (μmol/L)	0.16 (0.06)	0.18	32.55 (0.92)	20.0	49.72 (1.27)	94.6 (2.4)
				10.0	38.92 (1.81)	91.5 (4.3)
Choline (μmol/L)	0.20 (0.04)	0.13	12.59 (0.66)	20.0	33.19 (1.43)	101.8 (4.4)
				10.0	22.35 (0.94)	98.9 (4.2)
DMG (μmol/L)	0.40 (0.04)	0.13	3.83 (0.38)	4.0	8.08 (0.80)	103.2 (10.2)
				2.0	5.93 (0.66)	101.8 (11.3)

ACh: acetylcholine, DMG: dimethylglycine, SD: standard deviation. For LOD determination, pool samples were dialyzed prior experiment until they reached concentrations of 1–5 times the instrumental detection limit (IDL).

[a] $n = 10$ samples.

[b] Mean of 3 independent experiments.

[c] Urine samples were 1:5 diluted with H_2O prior experiments.

betaine, 99.2% for choline, and 101.4% for DMG. Mean recoveries in the plasma pool samples were: 93.0% for betaine, 100.4% for choline, and 102.5% for DMG.

Precision of the method was determined by quantification of ACh, betaine, choline, and DMG in the in-house prepared pools of EDTA plasma, CSF, AF, and urine, as well as quality control samples at two defined concentrations (low and high). Coefficients of variation (CVs) for different sample matrixes are presented in Table 4. Intraassay CVs ($n = 10$) ranged between 2.59–3.36% for ACh, 1.56–5.15% for betaine, 1.84–4.90% for choline, and 4.48–9.27% for DMG.

Interassay CVs for different biological samples ($n = 10$ each) ranged between 2.18–4.19% for ACh, 2.65–6.50% for betaine, 3.61–8.76% for choline, and 4.88–12.44% for DMG. The highest CVs for DMG in CSF pool of 12.44% were probably related to the low concentrations of this analyte there. A summary and a comparison with available methods are shown in Table 5.

3.5. ACh, betaine, choline, and DMG concentrations in blood and urine

Concentrations of choline-related metabolites were measured in plasma, serum, and urine samples. In 74 female subjects, plasma and serum concentrations of betaine were significantly correlated (Spearman correlation coefficient $r = 0.792$, $p < 0.001$) (AppendixB-Supplemental Data Fig. 2). The mean (SD) betaine concentration was 11.65 (3.06) μmol/L in plasma and 12.75 (3.36) μmol/L in serum ($p < 0.001$; paired t-test). Mean (SD) choline concentration in serum (13.54 (5.86) μmol/L) was significantly higher than the plasma concentration (6.68 (1.51) μmol/L) ($p < 0.001$), probably reflecting increased choline release in serum from phospholipids by means of cholinesterases. Because EDTA is a calcium chelator, the activity of cholinesterase might be inhibited in EDTA plasma.

The median (10–90th percentile) plasma betaine concentration of 28 subjects in fasting condition was 46.98 (30.95–63.44) μmol/L, which was not significantly different from that in non-fasting condition [42.67 (24.88–74.61) μmol/L ($p = 0.414$; paired t-test)]. We observed significantly higher plasma choline concentrations under non-fasting compared to fasting conditions [median 14.05 (10.61–18.90) μmol/L vs. 7.58 (4.42–9.78) μmol/L ($p < 0.001$; paired t-test)]. This might be related to enhanced homocysteine remethylation in the liver in non-fasting conditions. The fasting and non-fasting concentrations of choline were strongly correlated ($r = 0.779$, $p < 0.001$) (AppendixBSupplemental Data Fig. 3).

From 44 older adults (age > 50 years) the ranges of choline-related analytes as well as gender-related analyte concentrations were assessed (Table 6). The concentrations of betaine in plasma and those in urine showed wide inter-individual variations. Males showed significantly higher concentrations of plasma DMG (44.4%) than females which might be explained by a larger liver size in males compared to females. Females showed higher urinary excretion of choline (Table 6). In females, the concentration of plasma betaine correlated significantly to urinary excretion of betaine ($r = 0.495$, $p = 0.027$) and that of choline ($r = 0.502$, $p = 0.024$). This was not the case in male subjects (AppendixBSupplemental Data Table 1). In male subjects, a significant positive correlation of plasma DMG and urinary DMG excretion ($r = 0.418$, $p = 0.042$) was found. In addition, the concentration of plasma choline and urinary ACh:creatinine ratio showed a strong direct correlation in female subjects ($r = 0.621$, $p = 0.003$), which was lower in male subjects ($r = 0.419$, $p = 0.041$). Significant positive correlations between betaine and betaine excretions in urine, as well as choline excretion were observed in male and female subjects. Positive correlations between urinary betaine and DMG excretion were found in males ($r = 0.768$, $p < 0.001$) and females ($r = 0.543$, $p = 0.013$). In male subjects, significant correlations between urinary choline and betaine excretion, as well as DMG excretion were found. In the total group ($n = 44$) significant correlations of plasma betaine and DMG ($r = 0.380$, $p = 0.011$) were found. Plasma choline correlated significantly with urinary ACh excretion ($r = 0.455$, $p = 0.002$). Positive correlations between urinary betaine and choline excretion ($r = 0.587$, $p < 0.001$) and DMG excretion ($r = 0.700$, $p < 0.001$), as well as urinary choline and DMG excretion ($r = 0.514$, $p < 0.001$) were found.

4. Discussion

Choline is utilized for synthesis of ACh or phosphatidylcholine. Choline metabolism has been related to Hcy accumulation, fatty liver, renal and corneal disorders [1,6], or cognitive dysfunction

Table 4
Precision of ACh, betaine, choline, and DMG in quality control and pool samples ($n = 10$).

Analyte	High control		Low control		EDTA plasma pool		Urine pool		AF pool		CSF pool	
	Mean (SD)	CV (%)	Mean (SD)	CV (%)	Mean (SD)	CV (%)	Mean (SD)	CV (%)	Mean (SD)	CV (%)	Mean (SD)	CV (%)
Intraassay CV												
ACh (nmol/L)	30.61 (0.79)	2.59	3.22 (0.11)	3.34	ND	–	16.93 (0.57)	3.36	ND	–	ND	–
Betaine (μmol/L)	75.32 (1.17)	1.56	7.72 (0.16)	2.02	34.27 (0.69)	2.01	34.76 (0.82)	2.35	39.97 (2.06)	5.15	6.11 (0.15)	2.52
Choline (μmol/L)	44.22 (1.26)	2.86	4.60 (0.14)	3.04	14.02 (0.34)	2.42	12.23 (0.39)	3.23	26.28 (1.29)	4.90	4.65 (0.09)	1.84
DMG (μmol/L)	30.93 (1.39)	4.48	3.24 (0.17)	5.35	3.91 (0.27)	6.97	15.43 (1.17)	7.61	4.57 (0.42)	9.27	0.22 (0.01)	6.58
Interassay CV												
ACh (nmol/L)	30.21 (0.88)	2.90	3.08 (0.13)	4.19	ND	–	17.95 (0.39)	2.18	ND	–	ND	–
Betaine (μmol/L)	75.50 (2.00)	2.65	7.68 (0.26)	3.40	33.91 (1.95)	5.75	35.16 (1.70)	4.84	38.38 (2.09)	5.43	6.30 (0.41)	6.50
Choline (μmol/L)	45.44 (1.69)	3.72	4.65 (0.17)	3.61	13.77 (1.15)	8.37	13.62 (1.07)	7.85	25.00 (1.53)	6.11	4.89 (0.43)	8.76
DMG (μmol/L)	30.16 (1.57)	5.20	3.05 (0.15)	4.88	4.20 (0.40)	9.59	13.90 (1.17)	8.42	4.58 (0.40)	8.84	0.25 (0.03)	12.44

ACh: acetylcholine, AF: amniotic fluid, CSF: cerebrospinal fluid, CV: coefficient of variation, DMG: dimethylglycine, ND: not detected, SD: standard deviation.
High control samples contained 30 nmol/L ACh, 75 μmol/L betaine, 45 μmol/L choline, and 30 μmol/L DMG.
Low control samples contained 3 nmol/L ACh, 7.5 μmol/L betaine, 4.5 μmol/L choline, and 3 μmol/L DMG. Urine pool samples were diluted 1:5 with H_2O prior experiment; shown results have been multiplied by the dilution factor.

Table 5
Comparison of LC–MS/MS methods.

Analyte	Material	Run time; sample preparation	LOD (μmol/L)	Recovery	Intraassay CV in EDTA plasma	Linearity range	Reference
ACh Betaine Choline DMG	EDTA plasma Serum Urine AF CSF	6.5 min; Protein precipitation	ACh: 0.35 nmol/L Betaine: 0.11–0.34 Choline: 0.03–0.16 DMG: 0.07–0.13 (Various materials)	93.0–102.5% (EDTA plasma) 99.2–103.9% (urine)	Betaine: 2.01% Choline: 2.42% DMG: 6.97%	ACh: 0.2–150 nmol/L Betaine, choline, DMG: 0.2–150 μmol/L	Present study
Betaine Choline DMG	EDTA plasma Serum	6 min; Protein precipitation	Betaine: 0.3 Choline: 0.1 DMG: 0.2 (EDTA plasma)	90.2–104.5% (EDTA plasma[a])	Betaine: 2.1% Choline: 4.3% DMG: 4.9%	LOD–400 μmol/L	[20]
ACh Betaine Choline[b]	Plasma Tissues Foods	>30 min; Complex sample preparation	1–40 pmol (pure standards)	91.6–104% (rat liver)	ACh: 2.1% Betaine: 2.4% Choline: 8.5% (rat liver)	~10–200 nmol	[22]
Choline	EDTA plasma Whole blood	5 min; Protein precipitation	–	–	Choline: 4.7%	0.1–100 μmol/L	[21]

ACh: acetylcholine, AF: amniotic fluid, CSF: cerebrospinal fluid, CV: coefficient of variation, DMG: dimethylglycine, LOD: limit of detection.
[a] Mean of low and high concentration.
[b] In addition, following choline metabolites were analyzed: phosphocholine, glycerophosphocholine, cytidine diphosphocholine, phosphatidylcholine, and sphingomyelin.

Table 6
Median (10–90th percentiles) concentrations in fasting older subjects.

	Females	Males	p^a
n	20	24	
Age, years	59 (50–73)	66 (57–75)	0.062
EDTA plasma			
Betaine (μmol/L)	28.99 (18.67–37.49)	29.71 (25.21–46.78)	0.150
Choline (μmol/L)	8.96 (6.99–11.74)	9.19 (7.43–12.35)	0.465
DMG (μmol/L)	2.14 (1.47–3.65)	3.09 (1.97–4.26)	0.001
Urine			
ACh (μmol/mol creatinine)	3.92 (2.57–5.81)	3.44 (1.78–6.57)	0.689
Betaine (mmol/mol creatinine)	5.57 (3.28–11.42)	4.06 (2.40–25.30)	0.322
Choline (mmol/mol creatinine)	2.58 (1.14–4.04)	1.72 (1.01–4.70)	0.027
DMG (mmol/mol creatinine)	2.26 (1.30–4.65)	2.07 (0.57–7.02)	0.494

ACh: acetylcholine, DMG: dimethylglycine.
[a] *p*-values according to Mann–Whitney test.

[25]. Cholinesterase and inhibitors of this enzyme have been used to maintain concentrations of ACh in dementia [16]. Therefore, quantification of choline metabolites seems promising for epidemiological studies.

We described a new method for the simultaneous measurement of ACh, betaine, choline, and DMG in various biological fluids using stable-isotope dilution UPLC–MS/MS. The linearity over a broad concentration range, the short time for sample preparation (2 h/48 samples) and measurement (6.5 min/sample), and the small sample volume (100 μL) required make the method optimal for use in large-scale clinical studies. Coefficients of variation were <10% in intra- and interassay experiments. A higher CV for DMG assay in CSF (12.44%) is probably related to the low CSF concentrations of DMG (mean 0.25 μmol/L; LOQ = 0.26 μmol/L).

Our results on the performance of the betaine, choline, and DMG assay in plasma are comparable to that by Holm et al. (Table 5) [20]. However, our method also enables the measurement of ACh and it can be applied to different biological samples like urine, AF, and CSF. The sample preparation procedure and the assay time is more convenient than that of Koc et al. (Table 5) [22]. The method described by Yue et al. enables the measurement of only choline in plasma and whole blood [21].

In line with an earlier report [20] concentrations of betaine and choline were significantly different between serum and plasma. Moreover, the fasting concentrations of choline were lower than the non-fasting ones (AppendixBSupplemental Data Fig. 3) [20,26,27], but in contrast to an earlier study [20] plasma betaine concentrations did not differ according to fasting status. Different dietary intakes between the two populations might explain the effect of fasting status on betaine concentration [28,29]. Additionally, differences in choline metabolites according to gender are consistent with earlier studies [7,30–32]. The urine choline concentrations found in our study [median (10–90th) 22.41 (10.19–49.69) μmol/L] are in agreement with earlier results [33,34]. Urinary excretion of ACh correlated stronger to plasma choline in females than in males suggesting that plasma choline might be related to a higher production of ACh. The regulation (possibly hormonal) and the biological significance of these results have to be established.

In conclusion, we developed a fast, precise, and reliable method for the quantification of choline-related metabolites in various sample materials. Our method using UPLC–MS/MS provides high efficiency separation and enables for large-scale clinical studies. In comparison with other LC–MS/MS methods our method allows the simultaneous quantification of ACh, betaine, choline, and DMG.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2010.10.016.

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