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Nutrition

Determination of Choline, Betaine, and Dimethylglycine in Plasma by a High-Throughput Method Based on Normal-Phase Chromatography–Tandem Mass Spectrometry

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Background: **The quaternary ammonium compounds, choline and betaine, and dimethylglycine (DMG) reside along a metabolic pathway linked to the synthesis of neurotransmitters and membrane phospholipids and to homocysteine remethylation and, therefore, folate status. Lack of a convenient, high-throughput method for the determination of these compounds has prevented population-based studies of their possible associations with lifestyle, nutrition, and chronic diseases.**

Methods: **Serum or plasma samples were deproteinized by mixing with three volumes of acetonitrile that contained d_9-choline and d_9-betaine as internal standards. We used a normal-phase silica column for the separation of choline (retention time, 2.8 min), betaine (1.3 min), DMG (1.15 min), and internal standards, which were detected as positive ions by tandem mass spectroscopy in the multiple-reaction monitoring mode, using the molecular transitions *m*/*z* 104→60 (choline), *m*/*z* 113→69 (d_9-choline), *m*/*z* 118→59 (betaine), *m*/*z* 127→68 (d_9-betaine), and *m*/*z* 104→58 (DMG).**

Results: **For all three metabolites, the assay was linear in the range 0.4–400 μmol/L, and the lower limit of the detection (signal-to-noise ratio = 5) was ≤0.3 μmol/L. The within- and between-day imprecision (CVs) was 2.1–7.2% and 3.5–8.8%, respectively. The analytical recovery was 87–105%. The fasting plasma concentrations (median, 25th–75th percentiles) were 8.0 (7.0–9.3) μmol/L for choline, 31.7 (27.0–41.1) μmol/L for betaine, and 1.66 (1.30–2.02) μmol/L for DMG in 60 healthy blood donors. In individuals who had eaten a light breakfast, plasma concentrations of all three metabolites were significantly (25–30%) higher than in fasting individuals.**

Conclusion: **This is the first method for the combined measurement of choline, betaine, and DMG in human plasma or serum. The assay is characterized by simple sample preparation, no derivatization, high throughput, imprecision (CV) <10%, detection limits below the values seen in volunteers, and the high specificity provided by tandem mass spectroscopy.**

The quaternary ammonium compounds, choline and betaine, and *N*,*N*-dimethylglycine (DMG),[1] have a variety of biological effects and are closely connected metabolically. Choline is obtained from the diet or by sequential methylation of phosphatidylethanolamine. It is a precursor of membrane and lipoprotein phospholipids and the neurotransmitter acetylcholine; it thus is important for the integrity of cell membranes, lipid metabolism, and cholinergic nerve function *(1, 2)*. An alternative metabolic route for choline is oxidation to betaine, catalyzed by the sequential action of choline oxidase (EC 1.1.3.17) and betaine aldehyde dehydrogenase (EC 1.2.1.8). Betaine, in turn, is the methyl donor and DMG a product of the enzyme betaine-homocysteine methyltransferase (EC 2.1.1.5). This enzyme catalyzes the remethylation of homocysteine to methionine, a metabolic conversion that is also carried out by the folate-dependent enzyme, methionine synthase (EC 2.1.1.13) *(1, 3)* (Fig. 1). The betaine-dependent homocysteine remethylation in this way connects betaine to the metabolism and function of folates, homocysteine, and methionine. It also explains the homocysteine-lowering and therapeutic effect of betaine in patients with homocystinuria *(4)* and the moderate reduc-

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[1] Nonstandard abbreviations: DMG, *N*,*N*-dimethylglycine; MS, mass spectrometry; and LC, liquid chromatography.

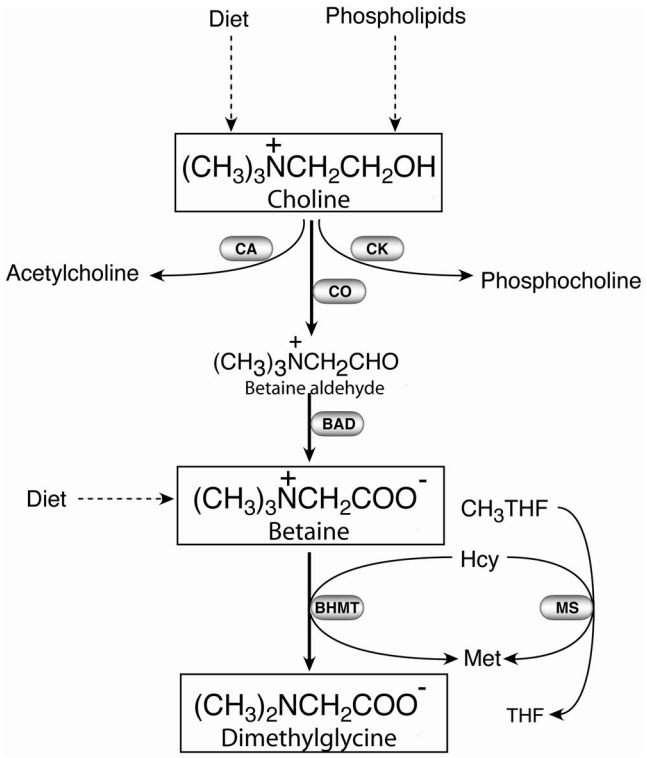

Fig. 1. Metabolism of choline, betaine, and DMG.

BAD, betaine aldehyde dehydrogenase; *BHMT*, betaine-homocysteine methyltransferase; *CA*, choline acetyltransferase; *CH_3THF*, methyltetrahydrofolate; *CK*, choline kinase; *CO*, choline oxidase; *Hcy*, homocysteine; *Met*, methionine; *MS*, methionine synthase; *THF*, tetrahydrofolate.

tion in plasma total homocysteine in healthy individuals *(5)* and renal patients *(6)*.

Plasma choline has been determined in several small human studies *(2)*. The concentration in healthy fasting humans is 7–10 μmol/L *(2)*, is remarkably high in neonates *(7, 8)*, is influenced by diet *(2)* and exercise *(9)*, and is increased in renal patients *(10)*. Data on betaine and DMG in human plasma/serum in health and disease are sparse. Betaine and DMG concentrations have been reported in small numbers of healthy individuals *(11)* and in patients with vitamin B deficiencies *(11)* or renal disease *(12)*. Notably, in HIV-1-infected patients, markedly increased DMG, which is normalized after antiviral therapy, has recently been demonstrated *(13)*. This metabolic abnormality may reflect tissue wasting, including membrane degradation, which generates choline, which is further metabolized to DMG.

The published data would appear to strongly motivate larger epidemiologic and clinical studies on plasma choline, betaine, and DMG and their relationships with nutritional status and disease risk, but no such investigations have been conducted. This is probably explained by the inconvenient methods for the determination of the quaternary ammonium compounds choline and betaine. Various methods have been reported for the measurement of plasma free choline, including radioenzymatic assays *(14, 15)*, gas chromatography *(8, 16)*, HPLC with electrochemical detection *(17)*, and very recently, HPLC–mass spectrometry (MS) *(18)*. The radioenzymatic assay is cumbersome with variable recovery *(8)*, whereas the chromatographic assays require complex extraction or derivatization procedures. A novel HPLC method for DMG and betaine in blood requires derivatization and is characterized by low sensitivity and limited throughput *(19)*. Betaine in serum has been assayed by gas chromatography with MS, but betaine has to be enzymatically converted to DMG to be detected by this method *(11)*.

In this report, we describe a sensitive method based on liquid chromatography–tandem MS (LC-MS/MS) for the simultaneous determination of choline, betaine, and DMG in human plasma and serum. The sample processing is a simple protein precipitation step carried out by a robotic workstation, followed by separation by a fast HPLC procedure. This method is suitable for large-scale epidemiologic studies.

Materials and Methods

REAGENTS

Choline chloride, betaine glycine, and DMG were obtained from Sigma Chemicals. d_9-Choline chloride was from Aldrich Chemical, and d_9-betaine was from Isotec. Methanol, acetonitrile, and formic acid were HPLC grade and purchased from Merck Chemicals. Other reagents were of the highest purity available. Pooled human EDTA plasma for assay calibration and linearity experiments was dialyzed three times (i.e., until the concentrations of choline, betaine, and DMG were below the detection limit) against 10 volumes of phosphate-buffered saline containing 4 mmol/L EDTA. We used phosphate-buffered saline to prevent substantial changes in the salt content of the plasma samples.

SAMPLE COLLECTION AND PROCESSING

EDTA plasma was obtained by collecting blood into Vacutainer Tubes (Becton Dickinson); the final EDTA concentration in the samples was 4 mmol/L. The EDTA-blood samples were centrifuged within 60 min. Serum was obtained by collecting blood into Vacutainer Tubes with no additive. Blood was allowed to clot at room temperature for 30 min before the serum fraction was transferred to an empty glass vial. Plasma and serum were either processed immediately or stored at −20 °C until use.

Plasma or serum samples (30 μL) were mixed with three volumes of acetonitrile containing 10 μmol/L each of d_9-choline and d_9-betaine. Samples were then centrifuged for 2 min at 5800*g*. The supernatant was then transferred to sealed polypropylene microtiter plates (Costar) or sealed autosampler glass vials (Chromacol). The mixing of plasma/serum with acetonitrile and the transfer of supernatant were carried out by a robotic device (Plato 7; RoSyst Anthos).

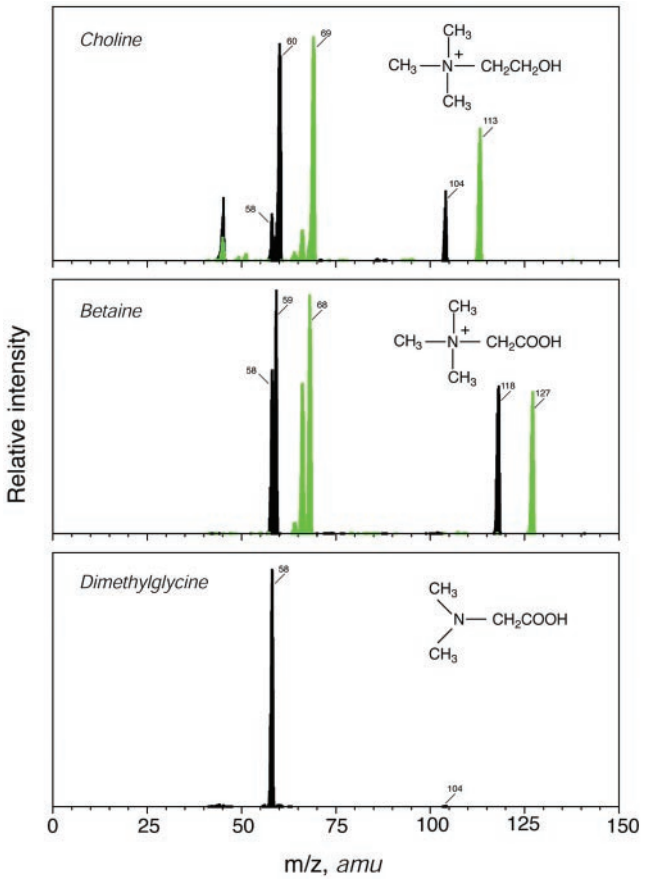

Fig. 2. Collision-induced MS/MS product ion spectra of protonated molecular ions ($[M+H]^+$) of analytes and stable-isotope internal standards.

The *top panel* shows the products for choline ($[M+H]^+$ ion, *m/z* 104) and d_9-choline (*m/z* 113), the *middle panel* shows the products for betaine (*m/z* 118) and d_9-betaine (*m/z* 127), and the *bottom panel* shows the products for DMG (*m/z* 104). The spectra of the authentic standards are shown in *black*, and the spectra for the deuterated internal standards are in *green*. *amu*, atomic mass units.

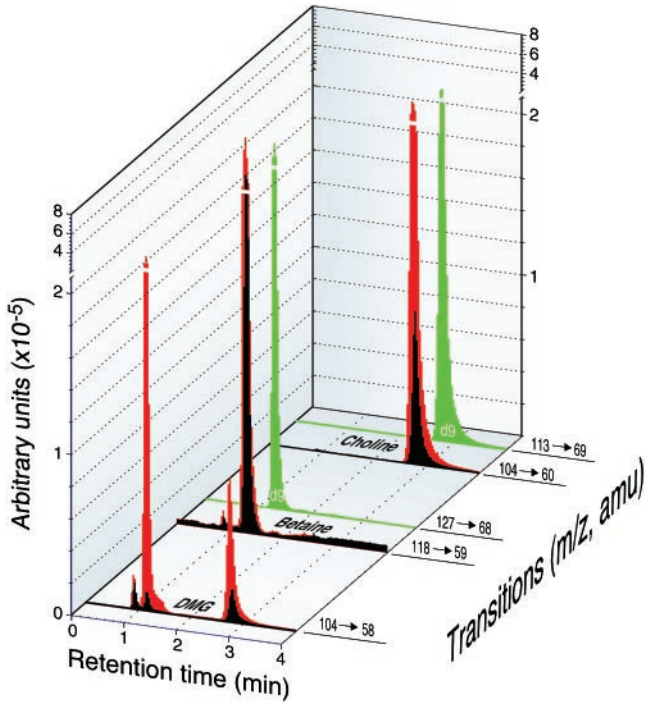

Fig. 3. LC-MS/MS multiple-reaction monitoring chromatograms of human plasma and the same plasma supplemented with 50 μmol/L choline, betaine, and DMG.

The traces for the molecular transitions of choline, betaine, and DMG in plasma without added analytes are shown in *black*, the traces for plasma with added analytes are in *red*, and the transitions of deuterated internal standards are shown in *green*. *amu*, atomic mass units.

LC-MS/MS

An Agilent series HPLC system (Agilent Technologies) equipped with a thermostated autosampler and a degasser were used for solvent delivery and sample introduction. Serum samples deproteinized with acetonitrile were placed in a cooled (4 °C) sample tray and injected (2 μL) into a normal-phase column (10 × 2.1 cm) packed with 5-μm diameter particles of Hypersil silica (Agilent Technologies) and equilibrated with 25% solution A (15 mmol/L ammonium formate, pH 3.5) and 75% solution B (acetonitrile). The column was eluted at ambient temperature at a flow rate of 0.6 mL/min and developed with gradient elution as follows: 0–0.1 min, 25% A/75% B; 3.5 min, 80% A/20% B; 3.6 min, 25% A/75% B; and 5.6 min, 25% A/75% B. All gradient steps were linear. The column effluent was split at a ratio of 1:4, delivering the eluate at a flow rate of 150 μL/min into the mass spectrometer. The injection interval was 6 min.

We used an API 3000 (Applied Biosystems/MDS SCIEX) triple-quadrupole tandem mass spectrometer with Turbo Ion Spray™ interface in the positive-ion mode. Nitrogen was used as the drying gas at a flow rate of 6 L/min and for collision-activated dissociation. The collision energy was 28 eV, the declustering potential was 31 V, and the ion source temperature was 350 °C. For development work, the product-ion spectra for choline, d_9-choline, betaine, d_9-betaine, and DMG were acquired in the continuous flow injection mode, with use of a Harvard Model 11 syringe pump connected directly to the ion source by PEEK tubing. For signal optimization, we dissolved each compound at a concentration of 10 μmol/L in a mixture of 15 mmol/L ammonium formate and acetonitrile (75:25 by volume), infused at a rate of 10 μL/min.

The quaternary ammonium compounds and DMG were detected in the multiple-reaction monitoring mode of the tandem mass spectrometer with the following transitions: choline, *m/z* 104→60; d_9-choline, *m/z* 113→69; betaine, *m/z* 118→59; d_9-betaine, *m/z* 127→68; and DMG, *m/z*104→58.

Analyst software (Applied Biosystems/MDS SCIEX) was used for HPLC system control, data acquisition, and data processing.

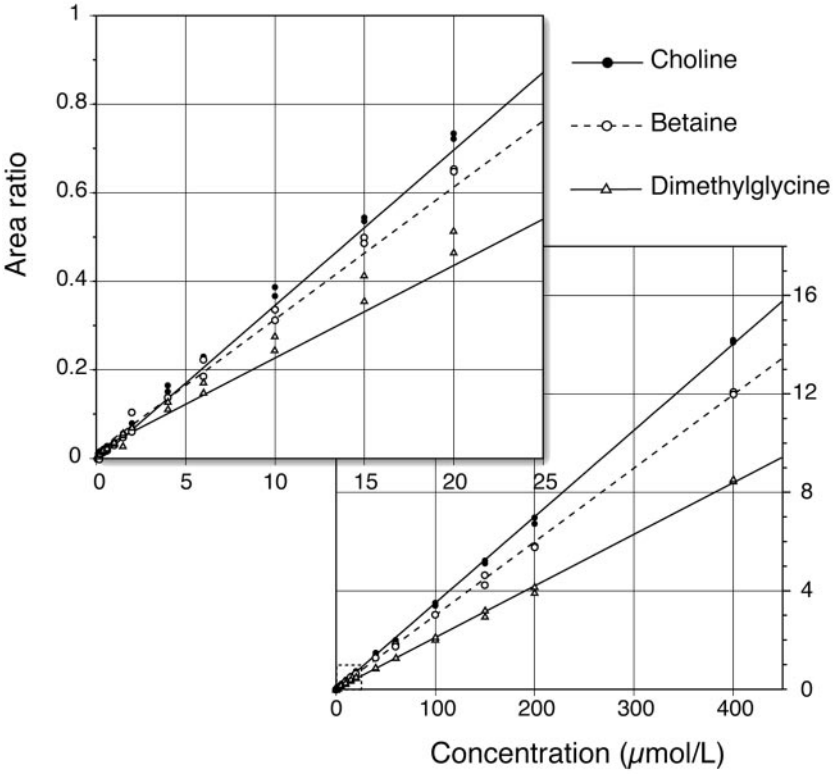

Fig. 4. Linear dynamic range for choline, betaine, and DMG.

LINEARITY AND ASSAY CALIBRATION

We assessed the linear range and sensitivity of the assay by adding 0.01–400 μmol/L choline, betaine, and DMG to dialyzed plasma. The peak-area ratios of choline to d_9-choline, betaine to d_9-betaine, and DMG to d_9-betaine were determined and plotted against the concentrations of choline, betaine, and DMG.

The assay was routinely calibrated by analyzing four samples containing added choline (3, 7.5, 15, and 30 μmol/L), betaine (6, 15, 30, and 60 μmol/L) and DMG (0.6, 1.5, 3, and 6 μmol/L) in dialyzed EDTA plasma.

RECOVERY AND ANALYTICAL VARIATION

Pooled EDTA plasma was dialyzed against two volumes of phosphate-buffered saline containing 4 mmol/L EDTA. This was done to obtain a sample with low-normal concentrations of choline (2.7 μmol/L), betaine (11.4 μmol/L), and DMG (0.91 μmol/L). The samples were divided into three portions, and medium or high concentrations of choline (10 or 30 μmol/L), betaine (20 or 60 μmol/L), and DMG (2 or 6 μmol/L) were added to two of the portions. At each analyte concentration, 18 replicates were analyzed in one analytical run. Recovery (%) was calculated as:

$$\frac{\text{Measured concentration} - \text{Endogenous concentration}}{\text{Concentration added}} \times 100$$

To determine within-day precision, we used the data from the recovery experiments. Between-day precision was determined by assaying the same samples on 20 different days over a period of 3 weeks.

STABILITY IN EDTA PLASMA AND SERUM

EDTA-plasma and serum samples were collected from the same individual. Serum and plasma samples were incu-

Table 1. Analytical recovery of the assay.[a]

	Concentration, μmol/L								
		Added[b]		Expected[b]		Measured[b]		Recovery,[b] %	
Analyte	Endogenous	Low	High	Low	High	Low	High	Low	High
Choline	2.7	10	30	12.7	32.7	13.2 (0.3)	33.8 (1.0)	105.2 (2.9)	103.7 (3.3)
Betaine	11.4	20	60	31.4	71.4	32.0 (1.4)	67.6 (2.7)	102.9 (6.8)	93.7 (4.4)
DMG	0.91	2	6	2.9	6.9	2.8 (0.14)	6.2 (0.3)	92.9 (6.8)	87.4 (4.3)

[a] n = 18 for all concentrations.
[b] Concentrations are given as the mean (SD).

Table 2. Precision of the assay.[a]

	Within-day		Between-day	
Analyte	Concentration,[b] μmol/L	CV, %	Concentration,[b] μmol/L	CV, %
Choline	2.7 (0.2)	6.2	2.7 (0.2)	8.8
	13.2 (0.3)	2.1	12.7 (0.5)	3.5
	33.8 (1.0)	2.9	31.2 (1.4)	4.3
Betaine	11.4 (0.5)	4.3	11.1 (0.6)	5.5
	32.0 (1.4)	4.3	32.0 (1.3)	4.1
	67.6 (2.7)	3.9	63.9 (3.3)	5.2
DMG	0.91 (0.07)	7.2	0.91 (0.08)	8.8
	2.8 (0.1)	4.9	2.7 (0.2)	8.6
	6.2 (0.3)	4.2	5.9 (0.5)	8.0

[a] n = 18 for within-day precision and 20 for between-day precision for all concentrations.
[b] Concentrations are given as the mean (SD).

bated at 0 or 25 °C for up to 72 h. The incubation times were 10, 20, and 60 min and 2, 6, 12, 24, 48, and 72 h. Each sample was run in triplicate.

CHOLINE, BETAINE, AND DMG IN PLASMA AND SERUM FROM HEALTHY INDIVIDUALS

Plasma and serum samples were collected from 120 healthy blood donors (mean age, 43.3 years; range, 20–65 years). One-half of the donors, 22 males and 38 females, were fasting. The other half, 42 males and 18 females, had eaten a light breakfast 2–3 h before blood sampling.

Results

MS/MS ANALYSIS

The protonated molecular $[M+H]^+$ ions for choline (*m/z* 104), d_9-choline (*m/z* 113), betaine (*m/z* 118), d_9-betaine (*m/z* 127), and DMG (*m/z* 104) were the predominant Q1 ions obtained by electrospray MS in the positive-ion mode by infusion of the respective compounds (data not shown). The product ion spectra obtained by Q3 transmissions of the protonated molecular $[M+H]^+$ ions are shown in Fig. 2. Under relatively mild collision-activated dissociation conditions, each compound broke down to give essentially one major fragment (*m/z* 60, 69, 59, 68, and 58 for choline, d_9-choline, betaine, d_9-betaine, and DMG, respectively).

LC-MS/MS

Choline, d_9-choline, betaine, d_9-betaine, and DMG were analyzed separately by normal-phase chromatography on a silica gel column equilibrated with a mixture of 15 mmol/L ammonium formate and acetonitrile (25:75 by volume), and eluted with a gradient of increasing portion of ammonium formate, as detailed in the *Materials and Methods*. Choline and d_9-choline coeluted at 2.8 min, betaine and d_9-betaine coeluted at 1.3 min, and DMG eluted at 1.15 min. Each compound showed a single peak (data not shown).

Shown in Fig. 3 is an extracted multiple-reaction monitoring chromatogram for choline, betaine, and DMG and the two deuterated internal standards in EDTA plasma and the same EDTA plasma to which each analyte had been added at a concentration of 50 μmol/L. Addition of authentic choline, betaine, and DMG to plasma caused a comparable increase in absolute abundance of the respective product ions. The chromatograms for the mass ion transitions of choline, d_9-choline, betaine, and d_9-betaine each showed one main peak, whereas three peaks were detected for the mass transitions of DMG. The DMG peak (retention time, 1.15 min) was increased 30-fold in the plasma to which analyte had been added, and a second peak coeluting with choline (retention time, 2.8 min) increased moderately (Fig. 3).

Total run time, which included column equilibration, was 6 min.

PERFORMANCE OF THE ASSAY

The sensitivity and linearity of the assay were assessed by analyzing a dialyzed plasma to which choline, betaine, and DMG had been added at concentrations of 0.01–400 μmol/L. The lower limits of detection, defined as a signal-to-noise ratio of 5, were 0.1 μmol/L for choline, 0.3 μmol/L for betaine, and 0.2 μmol/L for DMG. A linear dynamic range was demonstrated from the lower limit of detection up to 400 μmol/L for choline ($y = 0.035x - 0.0053$ μmol/L; $R^2 = 0.9996$), betaine ($y = 0.029x + 0.014$ μmol/L; $R^2 = 0.9935$), and DMG ($y = 0.0021x + 0.017$ μmol/L; $R^2 = 0.9989$; Fig. 4).

The precision and recovery of the assay were investigated at low- to high-normal concentrations of choline (2.7, 12.7, and 32.7 μmol/L), betaine (11.4, 31.4, and 71.4 μmol/L), and DMG (0.9, 2.9, and 6.9 μmol/L) in dialyzed plasma. The analytical recovery was 87–105% for all compounds and concentrations. The within- and between-day imprecision (CVs) was 2.1–8.8%, with the highest imprecision for DMG (Tables 1 and 2). Notably, similar values for recovery were obtained in nondialyzed plasma (data not shown).

Preparation of samples for LC-MS/MS analysis, which includes mixing samples with acetonitrile containing deuterated internal standard, and removal of the supernatant after centrifugation are carried out by a robotic liquid

handler that can process >200 samples in 60 min. The chromatographic run time of the current assay format is 6 min and limits the capacity of the assay, which is ~200 samples per day. The required sample volume is 30 μL.

STABILITY OF METABOLITES

Serum and EDTA-plasma samples were incubated at 0 or 25 °C for up to 72 h (Fig. 5). Choline, betaine, and DMG were essentially stable in EDTA plasma at both temperatures and in serum at the lower temperature. In serum incubated at 25 °C, DMG increased moderately and choline increased linearly as a function of time, reaching a sevenfold increase after 72 h (Fig. 5).

In serum and EDTA plasma mixed with three volumes of acetonitrile, all three metabolites were completely stable for at least 72 h at both temperatures (data not shown).

METABOLITES IN PLASMA AND SERUM IN FASTING AND NONFASTING INDIVIDUALS

We measured choline, betaine, and DMG in both serum and EDTA plasma from 60 fasting and 60 nonfasting blood donors (Table 3). The median (25th–75th percentiles) plasma concentrations in fasting donors were 8.0 (7.0–9.3) μmol/L for choline, 31.7 (27.0–41.1) μmol/L for betaine, and 1.66 (1.30–2.02) μmol/L for DMG. In donors who had eaten a light breakfast, plasma concentrations of all three metabolites were significantly (25–30%) higher than in fasting donors (Table 3). The prandial status influenced the whole distribution curve for all metabolites, which were displaced to the right in nonfasting individuals, demonstrating that food intake increased both low- and high-normal metabolite concentrations (Fig. 6).

For betaine and DMG, concentrations were not significantly different in plasma vs serum, whereas choline was (13–22%) higher in serum than in plasma (Table 3). The fasting plasma concentrations of choline, betaine, and DMG were significantly correlated (Fig. 7; Pearson r = 0.5–0.6; P <0.001).

Discussion

We have developed a LC-MS/MS method for the determination of free choline, betaine, and DMG in human plasma or serum. The sample processing involves mixing with acetonitrile, carried out by a robotic workstation, followed by centrifugation of the samples. The sample processing is fast and simple, requiring no derivatization, and all steps except for centrifugation are fully automated. This is the first published method that allows the simultaneous analysis of these three metabolites with use of the same preparation procedure and detection method.

The samples were deproteinized and metabolites were extracted by mixing with three volumes of acetonitrile. Preconditions for this convenient sample preparation are no covalent binding of analytes to plasma proteins (requiring steps such as reduction or acid hydrolysis), solubility of analytes at high percentages of acetonitrile, and no adsorption of analytes to precipitated proteins or protein pellet. The metabolites are all stable under these mild conditions, but the extracted samples must be stored in sealed or cooled tubes to avoid evaporation. Further-

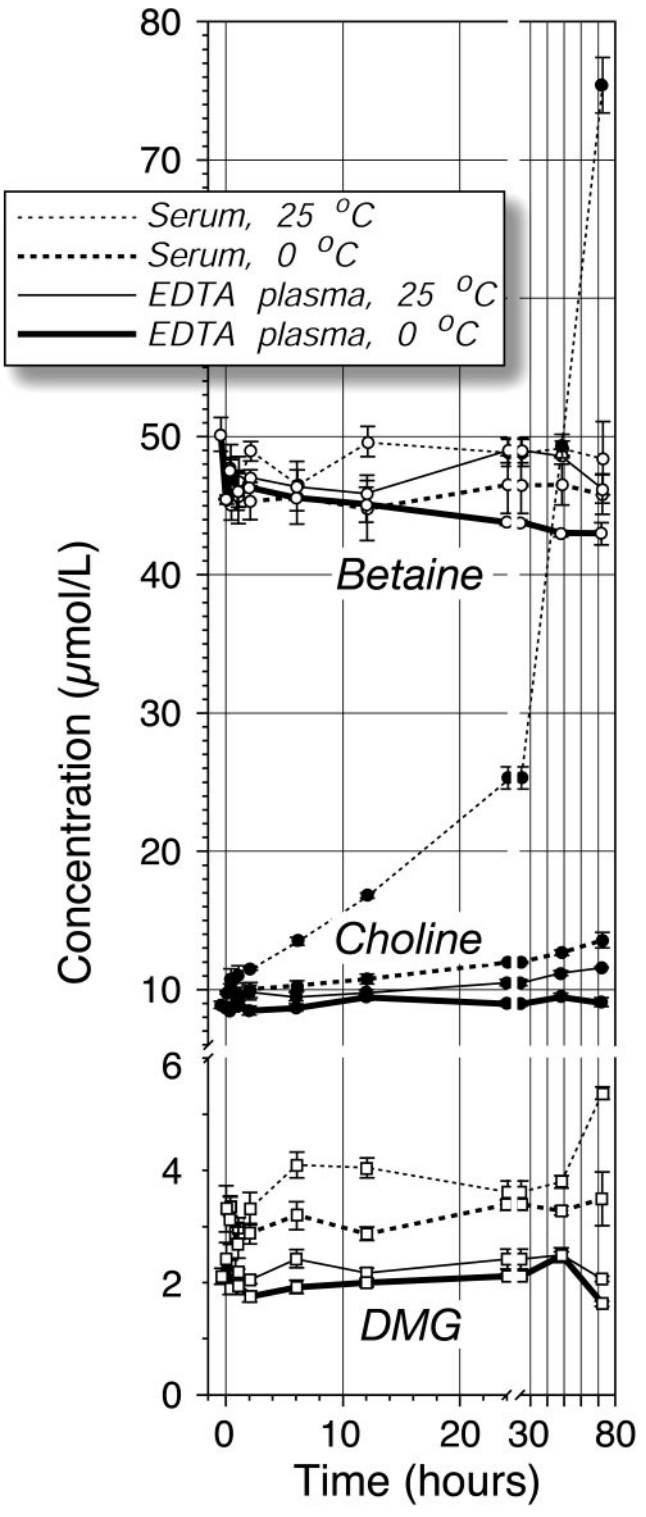

Fig. 5. Stability of choline, betaine, and DMG in EDTA plasma and serum at high and low temperatures.

Table 3. Concentrations of choline, betaine, and DMG in plasma and serum from healthy donors.[a]

Analyte	Fasting			Nonfasting			*P*, fasting vs nonfasting donors[c]	
	Plasma (n = 60)	Serum (n = 59)	*P*[b]	Plasma (n = 60)	Serum (n = 46)	*P*[b]	Plasma	Serum
Choline, μmol/L	8.0 (7.0–9.3)	9.8 (8.8–10.8)	<0.001	10.5 (9.0–12.3)	11.9 (10.7–13.3)	0.003	<0.0001	<0.0001
Betaine, μmol/L	31.7 (27.0–41.1)	32.8 (28.3–42.1)	NS[d]	41.7 (35.9–46.8)	41.5 (37.4–47.9)	NS	<0.0001	<0.0001
DMG, μmol/L	1.66 (1.30–2.02)	1.88 (1.39–2.19)	NS	2.07 (1.60–2.45)	1.93 (1.64–2.52)	NS	0.003	NS

[a]Concentrations are given as the median (25th–75th percentiles).
[b]Concentrations in plasma and serum compared by unpaired *t*-test.
[c]Concentrations in fasting and nonfasting donors compared by unpaired *t*-test.
[d]NS, not significant.

more, acetonitrile has been recommended for the extraction of choline because it is highly polar and a poor lipid solvent that does not support the hydrolysis of choline esters *(20)*. The analytes in 750 mL/L acetonitrile are retained on the silica stationary phase and eluted by a short gradient of ammonium formate. Thus, the acetonitrile step combines five components of the assay procedure: protein precipitation, analyte extraction, inhibition of choline ester hydrolysis, addition of deuterated internal standard, and an injection matrix that enables analyte retention.

The sample throughput of the present version of the assay is ~200 samples per 24 h, which matches the capacity of two 96-well microtiter plates. If required, the capacity can be substantially increased by reduction in column dimensions or by increasing the flow rate of the mobile phase. However, this may increase predisposition to high back-pressures and column clogging, which will decrease the ruggedness of the method.

The electrospray ionization mass spectra of choline, betaine, and DMG all produced prominent base peaks corresponding to their protonated molecular $[M+H]^+$ ions. The product ion spectra (Fig. 2) were obtained by exciting the protonated molecules with an equivalent collision energy, which fragments the ions into still smaller pieces. For choline (*m/z* 104), the main product ion at *m/z* 60 corresponds to $(CH_3)_3NH^+$. This was confirmed by the Q3 spectrum of the d_9-analog (*m/z* 113), which produced a base peak at *m/z* 69, i.e., 9 mass units higher than the unlabeled compound. Similarly, for betaine (*m/z* 118) and d_9-betaine (*m/z* 127), the main product ions of *m/z* 59 and 68 are consistent with the structures $(CH_3)_3N^+$ and $(CD_3)_3N^+$, respectively. DMG (*m/z* 104) produced one base peak at *m/z* 58. Thus, DMG and choline have one common molecular transition of *m/z* 104→58 (Fig. 2).

Detection of the analytes by MS/MS affords sufficient sensitivity. Specificity, on the other hand, is related to the selectivity of the molecular transitions and the chromatographic separation. In plasma and serum, at least two compounds exist with the same transition as DMG (Fig. 3). One coelutes with choline, probably arises from the common transition of *m/z* 104→58, and is easily separated from DMG. Some chromatographic optimization, however, was required to obtained resolution of DMG and the material eluting immediately before DMG (Fig. 3).

Stable-isotope-labeled internal standards are preferable in MS/MS, but deuterated analogs were available only for choline and betaine. We therefore quantified DMG by

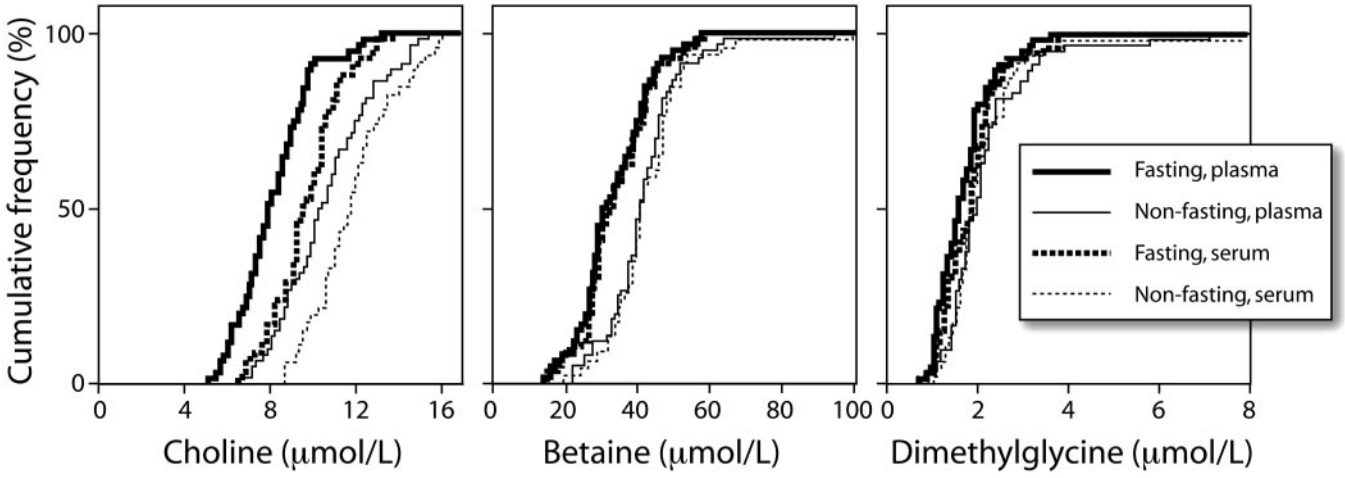

Fig. 6. Cumulative distribution of choline, betaine, and DMG in serum and plasma from 60 fasting and 60 nonfasting healthy blood donors. Serum values for 1 fasting donor and 14 nonfasting donors are missing.

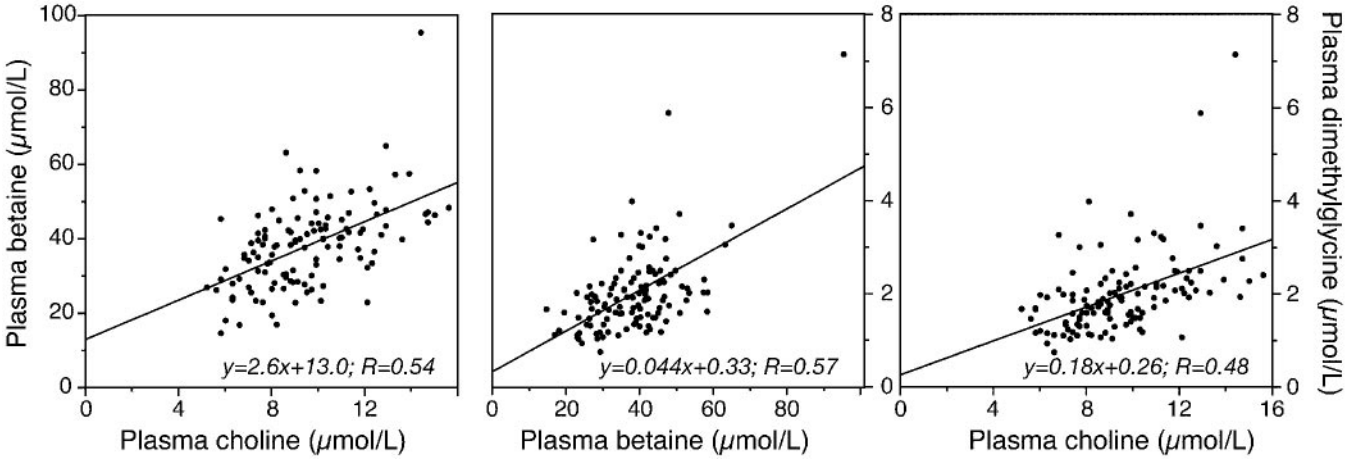

Fig. 7. Simple correlations between choline, betaine, and DMG concentrations in plasma from 120 healthy blood donors.

measurement of the peak-area ratios of DMG to d_9-betaine. Using d_9-betaine as internal standard may not correct for the possible matrix effect on DMG ionization. This may partly explain the somewhat higher between- and within-day CVs for DMG compared with choline and betaine (Tables 1 and 2).

The median (25th–75th percentiles) plasma concentrations of choline [8.0 (7.0–9.3) μmol/L], betaine [31.7 (27.0–41.1) μmol/L], and DMG [1.66 (1.30–2.02) μmol/L] determined by the present LC-MS/MS method are the same as reported by several other authors *(11, 12, 15, 21–23)*. There is one report on higher plasma DMG in the range 4–13 μmol/L, but this result was based on an assay with a detection limit of 2 μmol/L *(19)*.

The plasma concentrations of choline, betaine, and DMG were 25–30% higher in donors 2–3 h after a small meal (breakfast) compared with donors who had fasted overnight (Table 3). There are no data in the literature on postprandial effects on betaine or DMG concentrations in plasma or serum. The effect on choline, however, is in agreement with published data demonstrating a moderate (10%) increase in plasma 1 h after a typical meal *(15)*. A diet low in choline has no effect on plasma concentrations *(24)*. Marked increases (two- to fourfold increase) have been reported after the intake of a diet with high choline content or supplemented with lecithin *(21, 24)*, whereas fasting *(23)* or a choline-deficient diet *(25)* cause a moderate (30%) reduction in plasma choline.

The concentration of free choline increased markedly (~1 μmol/L per hour) in serum incubated at room temperature (Fig. 5). Others have demonstrated that on incubation at room temperature, choline content increases in plasma but not in heated plasma and ultrafiltrate *(8, 14, 15)*. Such in vitro formation of choline may account for the higher choline concentrations in serum than in plasma (Table 3) and could be explained by enzymatic cleavage of choline esters. Conceivably, enzymatic formation of choline may occur during the time interval when serum is left at room temperature and may be inhibited in plasma by the EDTA in the collections tubes.

In conclusion, the postprandial effect on all of the metabolites, the pronounced in vitro formation of choline but not betaine and DMG, and the lower choline concentration in plasma than in serum (Table 3 and Fig. 6) have implications for determination of these metabolites in clinical and epidemiologic research. On the basis of the observations listed above, we recommend the use of fasting EDTA-plasma samples for the collective measurement of choline, betaine, and DMG. Our results also indicate that variable storage of samples at room temperature and nonfasting samples may cause bias, leading to underestimation of "true" associations between metabolite concentrations and biological traits. Finally, we believe that the strong correlations between plasma concentrations of choline, betaine, and DMG (Fig. 7), probably related to their close metabolic connection (Fig. 1), indicate a need for future investigation of the biological and clinical relevance of metabolite ratios.

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