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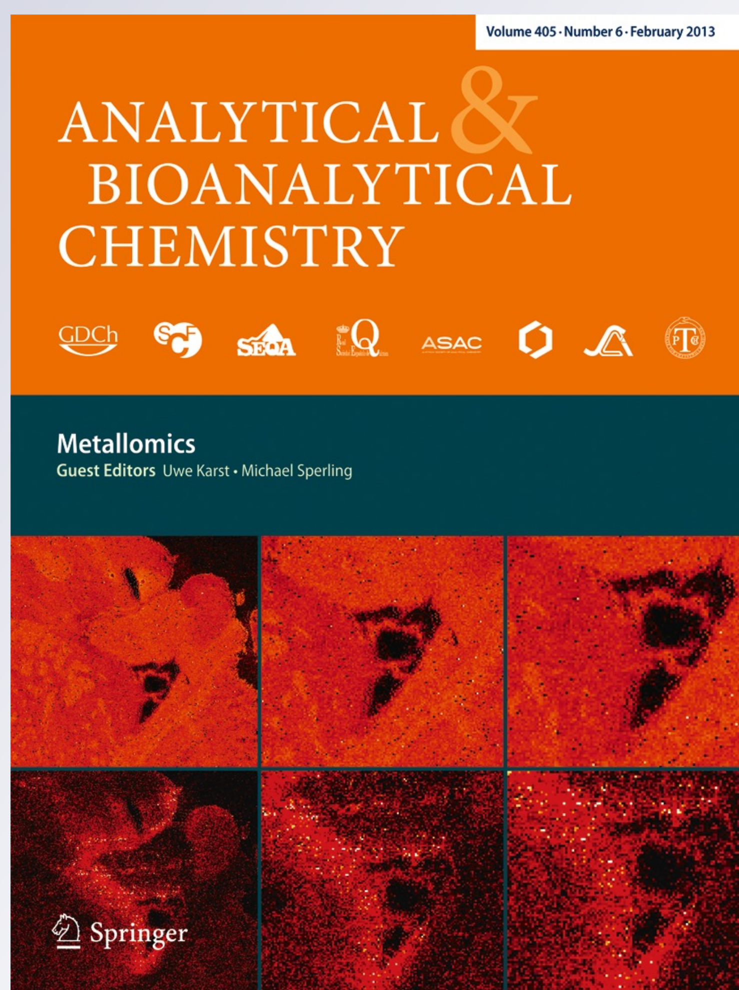
Analytical and Bioanalytical Chemistry

ISSN 1618-2642
Volume 405
Number 6

Anal Bioanal Chem (2013)
405:2009-2017
DOI 10.1007/s00216-012-6602-6



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ORIGINAL PAPER

High-throughput, low-volume, multianalyte quantification of plasma metabolites related to one-carbon metabolism using HPLC-MS/MS

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Received: 30 August 2012 / Revised: 20 November 2012 / Accepted: 23 November 2012 / Published online: 13 December 2012
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Abstract Risk of chronic diseases, like cardiovascular disease and cancer, has been associated with biomarkers related to one-carbon metabolism, which comprises a metabolic network of cross-talking pathways. To address this complexity in epidemiological studies, we have established an isotope dilution HPLC-MS/MS method for quantification of 12 biomarkers and metabolites. All sample handling is performed by a robotic workstation. The assay uses 45 μL of plasma, and sample treatment consists of protein precipitation by trichloroacetic acid. The analytes were separated on a Fortis Phenyl column using an isocratic mobile phase that contained water, methanol and acetic acid. Methionine, methionine sulfoxide, choline, betaine, dimethylglycine, arginine, asymmetric dimethylarginine, symmetric dimethylarginine, homoarginine, creatinine, cystathionine and trimethyllysine all showed limits of detection well below the 5th percentile of plasma distributions in healthy humans, coefficients of variation were in the range 2.2–12.3 %, and recoveries were 80–131 %. Simple sample processing, low-volume consumption, multiplexing and high capacity/short run time of this method make it suitable for large-scale metabolic profiling of precious biobank samples.

Electronic supplementary material The online version of this article (doi:10.1007/s00216-012-6602-6) contains supplementary material, which is available to authorized users.

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Keywords One-carbon metabolism · HPLC-MS/MS · High throughput · Epidemiology

Abbreviations

ADMA	Asymmetric dimethylarginine
BHMT	Betaine–homocysteine methyltransferase
CV	Coefficient of variation
DMG	Dimethylglycine
ESI	Electrospray ionization
EDTA	Ethylenediaminetetraacetic acid
HPLC-MS/MS	High-performance liquid chromatography tandem mass spectrometry
HSID	Hot source-induced desolvation
LOD	Limit of detection
MeOH	Methanol
MetSO4	Methionine sulfoxide
MRM	Multiple reaction mode
NO	Nitric oxide
NOS	Nitric oxide synthase
OCM	One-carbon metabolism
Q	Quadropole
S/N	Signal-to-noise ratio
SDMA	Symmetric dimethylarginine
TCA	Trichloroacetic acid
TML	Trimethyllysine

Introduction

Biomarkers related to one-carbon metabolism (OCM) have been associated with risk of chronic diseases, including cardiovascular disease [1] and cancer [2–4]. Methionine, a key component in OCM, is a precursor of the universal methyl donor *S*-adenosylmethionine [5]; it is obtained through diet or formed by remethylation of homocysteine. Alternatively, homocysteine is directed to cystathionine through the transsulfuration pathway. Homocysteine

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remethylation is catalyzed by ubiquitous methionine synthase or (in the liver and kidney) by betaine–homocysteine methyltransferase (BHMT) requiring betaine as methyl donor [5]. Betaine is formed by oxidation of choline and is demethylated to dimethylglycine (DMG) during the BHMT reaction [2]. OCM also has ramifications to asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), creatinine and trimethyllysine (TML) since the syntheses of these metabolites all involve *S*-adenosylmethionine-dependent transmethylation [6–8].

Several metabolites of the outlined network have been assigned important roles in normal physiology, in the pathogenesis of human diseases or serve as useful biomarkers of disease risk. Choline and choline derivatives are components of structural lipoproteins, blood and membrane lipids. Choline is a precursor of the neurotransmitter acetylcholine, and its perinatal availability is important for neurodevelopment in rodents; betaine functions as an intracellular osmoregulator [2]. Recently, plasma choline and betaine have been shown to have opposite relations with key components of the metabolic syndrome [9]. Arginine [10], but also homoarginine [10] is a substrate of the enzyme nitric oxide synthase (NOS) forming nitric oxide (NO), a regulator of endothelial function; ADMA is an inhibitor and potential regulator of NOS activity, whereas SDMA may decrease arginine availability for NO synthesis [11]. Interference with NO synthesis may partly explain the observed associations of ADMA [12], SDMA [13–15] and homoarginine [16] with cardiovascular risk. In addition, SDMA is a marker of renal function, and may even outperform creatinine in this respect [17].

Most evidence on metabolite and disease risk referred to above has been obtained from epidemiological or clinical studies based on targeted plasma measurement of one or a few structurally related analytes. Methods used measure methionine and its oxidation product methionine sulfoxide ($MetSO_4$) by GC-MS [18], choline, betaine and DMG [19, 20] by LC-MS/MS or various combinations of arginine, ADMA, SDMA and homoarginine using either HPLC coupled with fluorescence detection [21–27] or mass spectrometry-based methods [28–31].

To save precious biobank material, we have established complementary platforms/methods which each simultaneously measures a panel of biomarkers or metabolites in a low volume of plasma. These analytes have been selected based on function and the actual biochemical pathway, rather than chemical class, i.e. quantitative metabolic profiling [32]. The present report describes a high-throughput, low-volume, multiplexing assay for profiling of 12 metabolites (methionine, $MetSO_4$, choline, betaine, dimethylglycine, arginine, ADMA, SDMA, homoarginine, creatinine, cystathionine and trimethyllysine) related to OCM.

Materials and methods

Chemicals

$^2H_4$-Cystathionine (purity >95 %) was from C/D/N isotopes. Cystathionine (>95 %) and creatinine (>99 %) were purchased from Fluka. $^2H_7$-Arginine (98 %), $^2H_7$-ADMA (98 %), $^{13}C_7{}^{15}N_4$-homoarginine (>98 %) and $^2H_4$-methionine (>99 %) were from Cambridge Isotope Laboratories. Arginine (98 %), $MetSO_4$ (>99 %), $^2H_6$-DMG (99 %), DMG (99 %), homoarginine (99.5 %), betaine (>99 %), methionine (>99 %), SDMA (>99 %), ADMA (98 %), $^2H_9$-choline (98 %), choline (>99 %), $^2H_3$-creatinine (98 %) and TML (>97 %) were from Sigma-Aldrich. $^2H_2$-SDMA (purity not given by producer) and $^9H_2$-TML (>95 %) were from PharmAgra Labs. $^2H_9$-betaine (>99 %) was from Isotec.

Individual stock solutions of all analytes and internal standards were prepared in water and stored at −80 °C. Unlabelled compounds were not detected in any of the isotope-labelled internal standards. Using mass spectrometry, we investigated if isotopologues other than $^2H_2$-labelled was present in $^2H_2$-SDMA, and none were found.

A fraction of a plasma was spiked with all analytes, the unspiked and spiked plasma were analysed and the analyte concentrations in the unspiked plasma were estimated by using the standard addition method. Thus, the concentrations in the unspiked plasma were calculated as 0.330 μmol/L cystathionine, 73.5 μmol/L arginine, 2.30 μmol/L $MetSO_4$, 4.46 μmol/L DMG, 1.95 μmol/L homoarginine, 37.1 μmol/L betaine, 0.540 μmol/L SDMA, 32.0 μmol/L methionine, 0.550 μmol/L ADMA, 11.2 μmol/L choline, 70.2 μmol/L creatinine and 0.740 μmol/L TML. The unspiked and spiked portions of this plasma were aliquoted and stored at −80 °C, and the unspiked version was used as calibrator in routine use of the assay.

Instruments

A series 1100 HPLC system (Agilent Technologies) equipped with thermostated autosampler and degasser was used for solvent delivery and sample introduction. The HPLC system was coupled to an API 3000 triple-quadrupole tandem mass spectrometer (AB Sciex) equipped with an electrospray ion source and fitted with a hot source-induced desolvation (HSID) from IONICS. A column switcher from Valco (type VMHA) was used to divert the flow to waste during the first 1.2 min of each run and between each sample injection. Analyst (Ver. 1.5.2; AB Sciex) was used for data acquisition and analysis.

Sample collection and processing

Ethylenediaminetetraacetic acid (EDTA) plasma was obtained by collecting blood into Vacutainer Tubes (Becton Dickinson)

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giving a final EDTA concentration of 4 mmol/L. The samples were immediately put on ice and centrifuged (at 2,000×*g* for 10 min at 4 °C) within 60 min. The plasma was collected and processed further, or stored at −80 °C until use.

Sample processing was performed by a robotic workstation (MikrolabAT Plus) fitted with disposable tips with liquid detection. In 96-well microtiter plates, 45 μL of plasma was mixed with 45 μL of trichloroacetic acid (TCA, 6 %) containing the internal standards (1.50 μmol/L $^2H_4$-cystathionine, 30.0 μmol/L $^2H_7$-arginine, 5.00 μmol/L $^2H_6$-DMG, 1.25 μmol/L $^{13}C_7{}^{15}N_4$-homoarginine, 20.0 μmol/L $^2H_9$-betaine, 70.0 μmol/L $^2H_4$-methionine, 2.00 μmol/L $^2H_2$-SDMA, 1.00 μmol/L $^2H_7$-ADMA, 10.0 μmol/L $^2H_9$-choline, 94.0 μmol/L $^2H_3$-creatinine, 1 μmol/L $^9H_2$-TML) were added and the solution mixed by the robot. The mixture was then centrifuged at 6,000×*g* for 5 min at 5 °C, and the supernatant was transferred to a new plate which was placed in the autosampler compartment.

HPLC-MS/MS

Processed samples were kept in an autosampler at +6 °C in subdued light. Samples of 10 μL of deproteinized plasma were injected onto a Fortis Phenyl column (150×4.6 mm; particle size, 3 μm) from Fortis Technologies guarded by a Phenomenex Polar-RP SecurityGuard Cartridge (4×3.0 mm). The temperature in the column compartment was +20 °C.

The mobile phase consisted of 2.5 % methanol (MeOH) in 150 mmol/L acetic acid (pH=5.2). Chromatography was isocratic, with a flow rate of 1.2 mL/min and run time of 3.4 min, and samples were injected every 4.2 min.

The mass spectrometer was operated in the positive ESI mode, and analytes and internal standards were detected in MRM with unit resolution at quadropole 1 (Q1) and low resolution at Q3 (in order to increase the sensitivity for low-abundance analytes). Other common instrument parameters were nebulizer gas=7 psig, curtain gas=12 psig, collision-activated dissociation gas=3 psig, ion spray=5,000 V, ion source temperature=500 °C, declustering potential=23 V, focusing potential=30 V and HSID temperature=200 °C. For each compound and internal standard, the ion pair used consisted of the singly protonated molecular ion and its most abundant product ion. Table 1 lists the ion pairs, together with retention times and ion-pair specific instrument parameters for all analytes and internal standards. We used isotopologues as internal standards for all analytes when available ($^2H_6$-DMG was used for $MetSO_4$), and quantitation was performed by dividing

Table 1 Retention times and instrument settings

Analyte	t_r, min	Transitions, *m/z*		EP, V	CE, V	CXP, V
		Precursor ion	Product ion			
$^4H_2$-Cystathionine	1.48	226.9	137.9	6	20	8
Cystathionine	1.48	222.9	133.9	6	20	8
$^7H_2$-Arginine	1.69	181.9	77.2	10	27	5.6
Arginine	1.70	174.9	70.2	10	27	5.6
$MetSO_4$	1.75	166.1	73.9	10	20	5
$^6H_2$-DMG	1.81	110.1	64.1	10	20	10
DMG	1.82	104.1	58.1	10	20	10
$^{13}C_7{}^{15}N_4$-Homoarginine	1.89	200.2	90.2	10	30	6
Homoarginine	1.90	189.2	84.1	10	30	6
$^9H_2$-Betaine	2.18	127.1	68.1	8	28	4
Betaine	2.20	118.1	59.1	8	28	4
$^4H_2$-Methionine	2.34	154.2	108.0	8	15	6
Methionine	2.35	150.2	104.0	8	15	6
SDMA	2.39	203.2	172.1	10	30	7
$^2H_2$-SDMA	2.51	205.2	72.2	10	35	7
$^7H_2$-ADMA	2.60	210.2	77.2	10	24	7
ADMA	2.63	203.2	46.1	10	24	7
$^9H_2$-Choline	2.66	113.1	69.1	12	28	4
Choline	2.73	104.1	60.1	12	28	4
$^3H_2$-Creatinine	3.15	117.2	47.2	14	28	4
Creatinine	3.19	114.2	44.2	14	28	4
$^9H_2$-TML	2.05	198.1	84.2	10	30	6
TML	2.05	189.2	84.1	10	30	6

t_r retention time, *EP* entrance potential, *CE* collision energy, *CXP* collision cell exit potential.

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the analyte peak area by the area of the corresponding internal standard.

Method validation

Linearity was investigated in solutions of 4 % albumin in phosphate-buffered saline. Limit of detection (LOD) was determined from the linearity experiments as signal-to-noise ratio (S/N, determined using the script supplied with the Analyst software by Applied Biosystems) of 3.

Within-day and between-day coefficients of variation (CV) were determined by analysing a plasma pooled from three healthy individuals at three concentrations (low=endogenous, medium and high spiking concentrations) of each analyte. For the within-day experiments, 19 replicates of the same samples were analysed on the same day, while for the between-day experiments the same samples were analysed twice on 19 different days. From the within-day CV experiments, recoveries were determined. The spiking levels for the CV and recovery experiments are given in Table 2.

Matrix effects were studied by analysing plasma from ten persons and water. Both plasma and water were spiked after sample treatment with the high concentrations used in the CV and recovery experiments (Table 2), and calculated as

$$\text{Matrix effect} = \frac{[\text{analyte peak area}]_{\text{spiked}} - [\text{analyte peak area}]_{\text{endogenous}}}{[\text{analyte peak area}]_{\text{water}}} \times 100\%$$

and also by replacing the analyte peak areas in the formula by the analyte peak area divided by the internal standard peak area. The relative matrix effect, which describes the spread of matrix effects, was calculated as the CV of the matrix effects of the ten plasma samples. $^2H_6$-DMG was selected as internal standard for $MetSO_4$ based on the method validation results and similar retention times.

In order to establish the analyte concentration in healthy subjects, we analysed plasma concentrations in 171 healthy Norwegian adult blood donors [39.8 % female, average age 44 (range, 21–68) years].

Results and discussion

The presented assay analyses 12 plasma biomarkers (Figure S1, Electronic supplementary material) related to OCM. Sample treatment is very quick and simple, and involves protein precipitation only. We exploited the specificity and sensitivity of combining HPLC with tandem mass spectrometry to develop an assay which requires only 45 µL of plasma and has a chromatographic run time of 3.8 min.

Sample treatment, stability of analytes and internal standards

Sample preparation consisted of precipitation of proteins by mixing the plasma with TCA (performed by a robotic workstation) followed by centrifugation, a simple and quick procedure, enabling high-throughput analyses. The final concentration of TCA was such that more than 99.5 % of the plasma proteins removed from the plasma [33], giving a clean supernatant ensuring long column lifetimes and reducing the need for ion-source cleaning. On average, columns were replaced every 1,000 injections.

Table 2 Analytical recovery of the assay

	Concentration (µM)[a]			Recovery[b], %	
	Endogenous	Endogenous+added		Added	
Analyte	Low	Medium	High	Medium	High
Cystathionine	0.22	1.13	2.13	92	96
Arginine	73.0	137.9	206.3	89	90
$MetSO_4$	1.80	3.31	4.98	98	106
DMG	3.9	7.7	11.8	125	131
Homoarginine	1.73	4.62	7.37	118	114
Betaine	38	61	83	91	90
Methionine	18.9	50.4	83.0	105	107
SDMA	0.47	1.32	2.16	84	84
ADMA	0.45	1.72	3.02	102	103
Choline	8.4	16.6	24.8	80	82
Creatinine	79.3	141.1	205.5	91	91
TML	0.52	1.52	2.51	66	66

[a] n=19 for all concentrations

[b] Data are given as mean values of the 19 runs (same as for the within-day CV experiments)

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Published information about analyte stability was used as a guide in establishing sample treatment and storage conditions. Plasma methionine is stable during short-term storage at room temperature, but may be oxidized to $MetSO_4$ when stored for years at −25 °C [34]. Thus, the sum of methionine plus $MetSO_4$ represents plasma total methionine [34]. Choline concentrations in EDTA plasma increase during storage at room temperature [34, 35], but are stable for at least 72 h at 0 °C and for years at −80 °C [19]. Betaine and DMG are stable in EDTA plasma at 0 °C for at least 72 h [19, 34] and for at least 29 years at −25 °C [34]. Arginine, ADMA and SDMA are stable in EDTA plasma for several years at −20 °C [21] and for at least 8 days at room temperature [21, 27, 34]. Homoarginine is stable in EDTA plasma for at least 8 days at room temperature [27]. Cystathionine is stable in plasma at room temperature for up to 8 days, but degrades if stored for several years at −25 °C [34]. Creatinine is stable [34].

No changes in signal intensities for analytes or internal standards were observed for treated plasma samples stored for up to 48 h in the autosampler compartment in subdued light at +6 °C, demonstrating that the analytes and internal standards were stable under these conditions.

HPLC-MS/MS

Selection of mobile phase and column was optimized to give the best combination of signal intensities and method performance for the included analytes. Optimization of the amount of MeOH in the mobile phase ensured adequate peak shapes for all analytes, and all analytes eluted within 3.2 min (Table 1 and Fig. 1). The high concentration of acetic acid in the mobile phase provides good ionization of the analytes [36].

Selection of ion-pairs for analytes and internal standards were based on peak intensities and S/N ratios, and the selected ion-pairs are listed in Table 1. We avoided using mass transitions that corresponds to loss of water or ammonia to avoid interference from coeluting isobaric compounds that may also lose such fragments. The combined use of chromatographic separation and tandem mass spectrometry thus provided chromatograms that were usually without interfering peaks (Fig. 1). Homoarginine and TML, which have identical masses and primary product ions, were separated by carefully optimizing the chromatographic conditions. TML eluted between two interfering peaks, but sufficient chromatographic resolution was obtained to give adequate assay performance (see below) also for this analyte. Aquisition parameters were optimized for each compound, and are listed in Table 1.

Matrix effects

Matrix effects were investigated in plasma from ten persons and calculated from analyte peak areas only and also for the analyte/ISTD peak are ratios (Table 3). The range of matrix effects was 32–99 %, and relative matrix effect was below 6 % for all analytes except MetSO4 (12 %) and DMG (11 %). After adjustment for internal standards, the matrix effects for all compounds (except $MetSO_4$) were in the range 90–104 %, and the relative matrix effects were reduced for 7 of the 11 analytes. For $MetSO_4$, the matrix effect

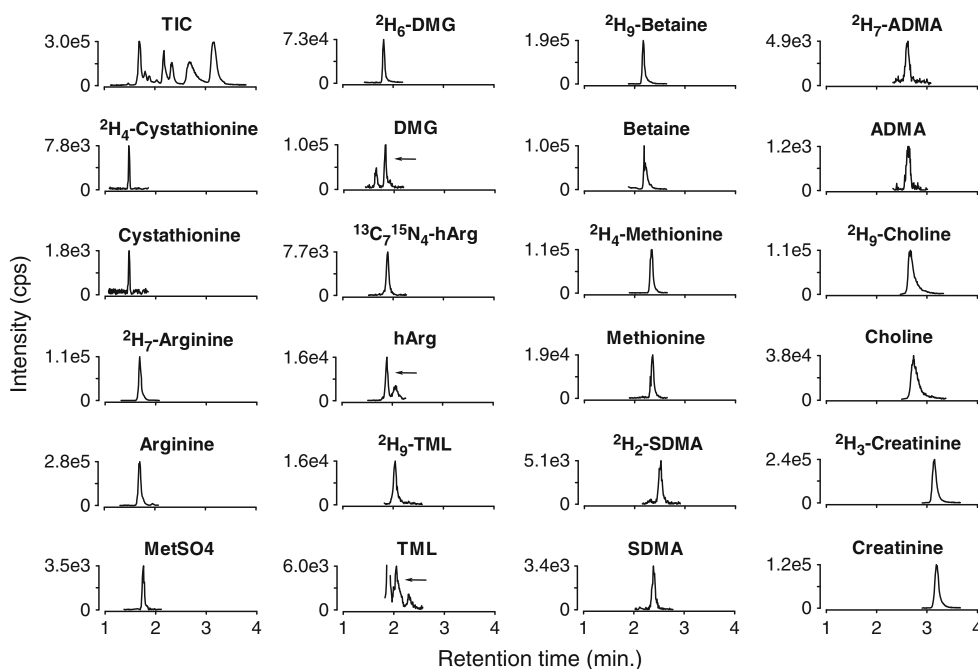

Fig. 1 Normalized HPLC-MS/MS chromatogram of the calibrator plasma (spiked with $MetSO_4$) containing 1.50 μM $^2H_4$-cystathionine, 0.33 μM cystathionine, 30.0 μM $^2H_7$-arginine, 73.5 μM arginine, 6.59 μM $MetSO_4$, 5.00 μM $^2H_6$-DMG, 4.5 μM DMG, 1.25 μM $^{13}C_7{}^{15}N_4$-homoarginine, 1.95 μM homoarginine, 20.0 μM $^2H_9$-betaine, 37 μM betaine, 70.0 μM $^2H_4$-methionine, 32.0 μM methionine, 2.00 μM $^2H_2$-SDMA, 0.54 μM SDMA, 1.00 μM $^2H_7$-ADMA, 0.55 μM ADMA, 10.0 μM $^2H_9$-choline, 11.2 μM choline, 94.0 μM $^2H_3$-creatinine and 70.2 μM creatinine

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Table 3 Matrix effects

Analyte	Based on analyte area	Based on analyte/ISTD area ratio
Cystathionine	39 (5)	101 (2)
Arginine	57 (4)	92 (3)
$MetSO_4$	32 (11)	45 (10)
DMG	72 (12)	99 (12)
Homoarginine	96 (5)	93 (7)
Betaine	71 (5)	96 (3)
Methionine	94 (4)	99 (2)
SDMA	88 (4)	90 (7)
ADMA	99 (6)	104 (7)
Choline	78 (6)	99 (4)
Creatinine	75 (5)	92 (3)
TML	69 (7)	89 (6)

The matrix effects are based on ten different plasma samples. All values (percentage) are given as mean (CV)

ISTD internal standard

was generally somewhat reduced, while the relative matrix effect was generally not improved, by adjustment with different internal standards (data shown using only $^2H_6$-DMG as internal standard, Table 3).

CV, recovery, LOD and linearity

CVs were 2.2–12.3 % (Table 4), and recoveries were in the range 66–131 % (Table 2). LODs (Table 5) were all below the 5th percentile of the concentrations found in 171 healthy subjects (Table 6). The linear ranges (Table 5) spanned the endogenous concentrations observed in plasma/serum from healthy humans (Table 6).

Method validation characteristics were generally similar to those reported for other methods for arginine [27, 30], $MetSO_4$ [18], DMG [19, 20], homoarginine [27], betaine [19, 20], SDMA [27, 30], methionine [18], ADMA [27, 30], choline [19, 20] and creatinine [37].

Recoveries that deviate significantly from 100 % are common [38], and we observed high recovery for DMG (125–131 %) and low recovery for TML (66 %). Such marked deviations from 100 % may reflect uneven distribution of analytes between the supernatant and pellet, and may affect the accuracy of an assay.

Assays using much larger sample volumes have shown better method characteristics for cystathionine and methionine [39, 40] than the current method. Better method performance has also been demonstrated for cystathionine by using a mass spectrometer with higher sensitivity combined with a higher concentration of acetic acid in the mobile phase [36], and for homoarginine by a more time-consuming, single analyte assay [41]. For methods including different combinations of arginine, homoarginine, SDMA and ADMA, similar or better validation characteristics have been published [22–31]; however, all but one of these methods require higher sample volume (100–1000 μL) [22–29, 31] and/or longer chromatographic separation time (up to 52 min) [22–28, 31] than the current assay. The combination of low volume consumption, multiplexing, high throughput and good method performance makes the presented assay useful for metabolic profiling in large-scale studies.

Plasma concentrations in healthy subjects

Plasma concentrations were investigated in 171 nonfasting healthy adult blood donors (Table 6). Median concentrations

Table 4 Imprecision of the assay

Analyte	Within-day CV (N=19)[a], %			Between-day CV (N=19), %		
	Low	Medium	High	Low	Medium	High
Cystathionine	12.0	8.0	5.9	10.6	10.3	7.0
Arginine	5.6	4.5	3.6	2.6	5.5	2.2
$MetSO_4$	12.3	8.8	9.0	7.9	7.6	7.6
DMG	11.7	8.9	6.7	7.7	9.4	5.0
Homoarginine	6.9	5.9	6.4	5.5	8.0	5.1
Betaine	7.2	7.3	5.5	8.3	9.5	7.8
Methionine	6.5	5.4	4.3	5.1	7.0	3.2
SDMA	7.7	7.9	8.6	7.7	9.1	6.4
ADMA	7.0	5.2	6.6	7.8	8.5	6.5
Choline	5.4	7.6	5.9	5.4	6.8	3.8
Creatinine	4.6	4.6	3.4	2.2	5.8	2.7
TML	7.3	6.0	5.6	8.7	9.8	12.2

[a] Data from the recovery experiments

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Table 5 Limit of detection and linearity

Analyte	Linear range (µM)	LOD (µM)	Regression parameters Slope	Intercept	r^2
Cystathionine	0.025–40	0.025	0.5895	0.1247	0.999
Arginine	0.25–200	0.25	0.0403	0.0771	0.999
$MetSO_4$	0.14–229	0.14	0.0144	0.0225	0.987
DMG	1.25–80	1.25	0.0227	−0.0020	0.999
Homoarginine	0.10–40	0.10	1.0123	0.0143	0.998
Betaine	0.50–400	0.50	0.0174	0.0738	0.998
Methionine	1.0–100	1.0	0.0102	0.0009	0.999
SDMA	0.080–2.5	0.080	0.7875	0.0504	0.995
ADMA	0.080–5.0	0.080	0.5084	−0.0018	0.985
Choline	0.50–100	0.50	0.0166	0.0109	0.997
Creatinine	0.25–800	0.25	0.00065	0.0423	0.998
TML	0.20–20	0.20	0.3987	0.2581	0.983

Linearity performed in PBS

LOD limit of detection (defined as S/N >3), *LLQ* lower limit of quantification (LLQ=2×LOD)

(µmol/L) were 0.26 for cystathionine, 115.0 for arginine, 3.65 for $MetSO_4$, 4.0 for DMG, 1.93 for homoarginine, 39 for betaine, 0.49 for SDMA, 30.4 for methionine, 0.50 for ADMA, 11.3 for choline, 79.2 for creatinine and 0.68 for TML. These concentrations were similar to those reported previously for cystathionine [42, 43], arginine [26, 27, 29, 30], $MetSO_4$ [18], homoarginine [24, 27, 41], betaine [43, 44], SDMA [24, 27, 29, 30], methionine [18, 43, 45], ADMA [24, 27, 29, 30], choline [44], creatinine [42, 44] and TML [46] (Table 6).

Defining the lower limit of quantification (LLQ) as two times LOD (Table 5), Table 6 shows that the 5th percentile of all analytes except DMG and TML are well above LLQ. For quantification of DMG and TML a lower precision would be expected at the lower end of normal plasma concentrations.

Plasma concentrations of $MetSO_4$ were calculated by dividing the area of $MetSO_4$ by the areas of several of the available internal standards. Comparing the resulting concentrations by Passing–Bablok regression showed that there were essentially no differences between the concentrations obtained by using different internal standards (not shown).

Conclusions

We have developed a low-volume, high-throughput HPLC-MS/MS assay that determines 12 plasma biomarkers related to OCM. The assay is presently being used for metabolic profiling in large-scale epidemiologic studies of chronic diseases.

Table 6 Analyte concentrations (µM) in plasma or serum

	This work[a]		Literature	
Analyte	Median	5–95 percentile	Median or mean	Reference
Cystathionine	0.26	(0.13–0.64)	0.19, 0.11	42, 43
Arginine	115.0	(81.3–163.5)	72.6, 94.2, 85, 85	26, 27, 29, 30
$MetSO_4$	3.65	(2.18–6.59)	4	18
DMG	4.0	(2.4–6.4)	2.5, 3.1	43, 44
Homoarginine	1.93	(1.17–3.31)	2.15, 2.5, 2.5	24, 27, 41
Betaine	39	(25–58)	35, 30.3	43, 44
Methionine	30.4	(21.0–43.5)	28.3, 20, 26.5	18, 43, 45
SDMA	0.49	(0.38–0.65)	0.40, 0.41, 0.6, 0.5	24, 27, 29, 30
ADMA	0.50	(0.39–0.65)	0.45, 0.48, 0.6, 0.5	24, 27, 29, 30
Choline	11.3	(8.6–15.0)	7.8	44
Creatinine	79.2	(58.9–99.3)	68.9, 74	42, 44
TML	0.68	(0.40–1.31)	0.33	46

[a] Plasma from 171 nonfasting individuals

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Acknowledgments This study was funded by the non-profit "Foundation to Promote Research into Functional Vitamin B12 Deficiency", the Western Norway Regional Health Authority and the Norwegian Cancer Society. We thank Saleh Rayyan and Marit Krokeide for technical assistance.

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