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Review

Quantitative profiling of folate and one-carbon metabolism in large-scale epidemiological studies by mass spectrometry

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Abstract

Background: Derangements of one-carbon metabolism have been related to the development of chronic diseases. Metabolic profiling as part of epidemiological studies in this area should include intermediates involved in the transfer of one-carbon units, cofactors for the relevant enzymes and markers of inflammation, kidney function and smoking.
Methods: We established five platforms that measured 6–16 analytes each. Platforms A (gas chromatography-mass spectrometry; GC-MS) and B (gas chromatography-tandem mass spectrometry; GC-MS/MS) involved methylchloroformate derivatization of primary amines, thiols and carboxylic acids. Platform C determined basic compounds by liquid chromatography-tandem mass spectrometry (LC-MS/MS), using an ether-linked phenyl reversed-phase column. Platforms D and E (LC-MS/MS) exploited the efficient ionization and high sensitivity obtained for a wide range of analytes, using a mobile phase containing a high concentration of acetic acid. The chromatographic run times ranged from 3 to 8 min.
Results: The analyte concentrations ranged from 0.2 nmol/L to 400 μmol/L. Platforms A and B both measured methylmalonic acid, total homocysteine and related amino acids. Platform B also included sarcosine, cystathionine, tryptophan and kynurenine. Platform C was optimized for the measurement of choline and betaine, but also included arginine, asymmetric and symmetric dimethylarginine and creatinine. A diversity of low abundance compounds mainly occurring in the nanomolar range were measured on platform D. These were vitamin B_2 and B_6 species, neopterin, cotinine and tryptophan metabolites. Platform E measured folates and folate catabolites.
Conclusions: Approximately 40 analytes related to one-carbon metabolism were determined in less than 1 mL of plasma/serum using five complementary analytical platforms. As a method control, several metabolites were measured on two or more platforms. Logistics and data handling were carried out by specially designed software. This strategy allows profiling of one-carbon metabolism in large-scale epidemiological studies.
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Keywords: choline; folate; mass spectrometry; one-carbon metabolism; riboflavin; vitamin B_6.

Non-standard abbreviations: 10fTHF, 10-formylTHF; 5fTHF, 5-formylTHF; AA, anthranilic acid; ADMA, asymmetric dimethylarginine; AdoHcy, S-adenosyl-L-homocysteine; AdoMet, S-adenosyl-L-methionine; apABG, acetamidobenzoylglutamate; Arg, arginine; B12, vitamin B12 (cobalamin); B2, vitamin B2 (riboflavin); B6, vitamin B6 (pyridoxal phosphate); BD, betaine aldehyde dehydrogenase; Bet, betaine; BHMT, betaine-homocysteine methyltransferase; Chol, choline; CHTHF, methenyl THF; CL, cystathionine gamma-lyase; CO, choline oxidase; Cot, Cotinine; Cre, creatinine; CS, cystathionine beta-synthase; Cysta, cystathionine; DD, dimethylglycine dehydrogenase; DHF, dihydrofolate; DMG, dimethylglycine; DR, DHF reductase; ESI, electrospray ionization; FA, folic acid; GAA, guanidinoacetic acid; GC-MS, gas chromatography-mass spectrometry; Gly, glycine; GM, guanidinoacetate methyltransferase; GT, glycine N-methyltransferase; HAA, 3-hydroxyanthranilic acid; HK, 3-hydroxykynurenine; hmDHF, 4-alfa-hydroxy-5-methyl-DHF; KA, kynurenic acid; Kyn, kynurenine; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MA, methionine adenosyltransferase; Met, methionine; MM, methylmalonyl-CoA mutase; MMA, methylmalonic acid; MRM, multiple reaction mode; MS, methionine synthase; MT, methyltransferase; mTHF, 5-methyltetrahydrofolate; MTHF, 5,10-methylenetetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; Neo, neopterin; PA, pyridoxic acid; pABG, p-aminobenzoylglutamate; PL, pyridoxal; PLP, pyridoxal 5-phosphate; PM, pyridoxamine; PN, pyridoxine; Pr-L-Arg, arginine residue in proteins; Protein-L-Arg(CH3)2, dimethylated arginine residue in proteins; PRMT, protein-arginine methyltransferase; R, methyl acceptor; Sar, sarcosine; SD, sarcosine dehydrogenase; SDMA, symmetric dimethylarginine; Ser, serine; SH, S-adenosylhomocysteine hydrolase; ST, serine hydroxymethltransferase; SIM, selective-ion monitoring; Smt, scan segment; ST, serine hydroxymethyltransferase; tCys, total cysteine; tHcy, total homocysteine; THF, tetrahydrofolate; Trp, tryptophan; XA, Xanthurenic acid.

Introduction

Folate is a class of water-soluble B-vitamins that are present in mammalian cells in several interconvert-

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ible forms. Their chemical structure consists of a reduced pteridine moiety coupled through a methylene group to p-aminobenzoic acid, to which glutamic acid is attached via an amide bond. Most intracellular folates are polyglutamates with 2–7 glutamate residues. Reduced tetrahydrofolate (THF) may have a one-carbon substituent, such as methyl, formyl or formino group, at position N-5, or a methylene or methenyl bridge connecting this position to position N-10. These substituents serve as a pool of activated one-carbon units that are donated to target molecules in a variety of biosynthetic reactions (1).

The term folate-mediated one-carbon metabolism refers to a network of several interrelated reactions that use THF to activate single carbons (2). Folate cofactors are involved in the synthesis of purines and thymidylate and in remethylation of homocysteine to methionine. The latter is converted to the universal methyldonor, S-adenosylmethionine (AdoMet). Most labile carbon units are probably provided from dietary methionine and choline (via betaine) or synthesized de novo (methylneogenesis) as 5-methyltetrahydrofolate (mTHF) by methylenetetrahydrofolate reductase (MTHFR) (3).

Para-aminobenzoylglutamate and its acetylated derivative, acetamidobenzoylglutamate, are products of folate catabolism, and are mainly excreted in the urine. Increased folate catabolism is believed to take place during chronic intake of excess folate, during the last trimester of pregnancy, and possibly in cancer patients and subjects taking oral contraceptives or anticonvulsant drugs (4). Concentrations of para-aminobenzoylglutamate and acetamidobenzoylglutamate in serum/plasma should be evaluated as markers of folate catabolism or turnover in human pathologies.

One-carbon metabolism is dependent on several water-soluble B-vitamins in addition to folate (5). Homocysteine metabolism represents a point of intersection between these B-vitamins. The ubiquitous methionine synthase, which catalyzes homocysteine remethylation to methionine, requires cobalamin (vitamin B_{12}) as cofactor and mTHF as substrate; mTHF in turn is formed by MTHFR, which requires flavin-adenine dinucleotide (FAD, vitamin B_2) as cofactor. Two vitamin B_6-dependent enzymes, cystathionine β-synthase and cystathionine γ-lyase, catalyze the conversion of homocysteine to cystathionine and further to cysteine. Vitamin B_6 is also involved in the serine hydroxymethyltransferase reaction that converts serine and THF to glycine and 5,10-methylenetetrahydrofolate (MTHF) (5).

Choline metabolism is connected to the methionine cycle by serving as a precursor for betaine, which functions as a substrate for betaine-homocysteine methyltransferase (BHMT). This enzyme, confined to the liver and kidney, catalyzes an alternative route of homocysteine remethylation to methionine, and thereby delivers one-carbon units from choline into the labile one-carbon pool (6). Experimental and human studies have demonstrated that folate metabolism and choline metabolism are interrelated. During choline deprivation leading to low betaine content, more mTHF is used for homocysteine remethylation, and thereby folate requirements are increased. Conversely, during folate deficiency, methyl groups from choline and betaine are used, and thereby choline requirements are increased. Thus, mTHF and choline/betaine have been regarded as fungible sources of methyl groups (7).

Dimethylglycine is the product of the BHMT reaction and is demethylated to sarcosine and further to glycine by the sequential action of two mitochondrial enzymes, dimethylglycine dehydrogenase and sarcosine dehydrogenase. Both enzymes contain FAD (vitamin B_2) and both use THF to scavenge one-carbon groups as MTHF (8, 9).

In addition to the metabolites and vitamins summarized above, some metabolic markers have ramifications to one-carbon metabolism and/or are often

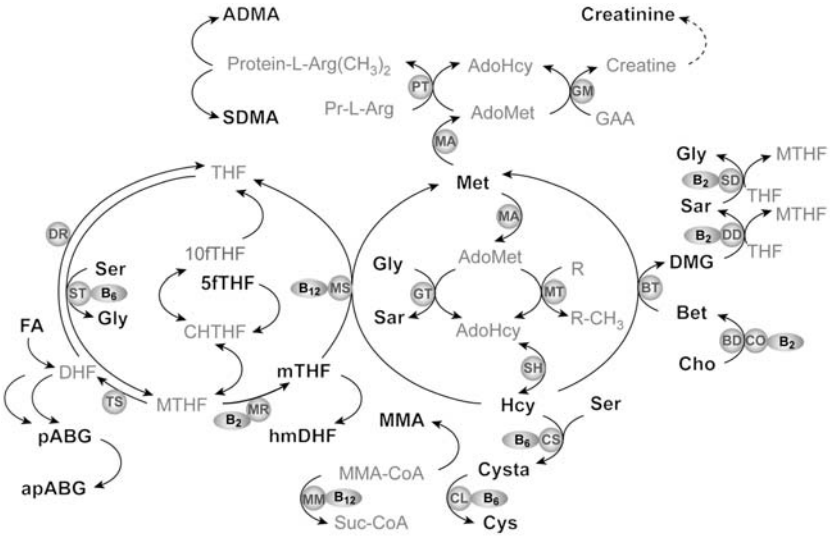

Figure 1 Vitamins, metabolites and enzymes involved in one-carbon metabolism.
Analytes determined by methods (platforms A–E) described in this paper are highlighted in bold. BT, BHMT, betaine-homocysteine methyltransferase; MR, MTHFR, methylenetetrahydrofolate reductase; PT, PRMT, protein-arginine methyltransferase.

measured in clinical studies of one-carbon metabolism and disease. Among these, creatinine is a convenient marker of renal function and a product of AdoMet-dependent transmethylation (10), which may explain the often-encountered correlation with plasma total homocysteine (tHcy) (11). Methylmalonic acid (MMA) in serum/plasma complements and is a more specific marker of cobalamin (vitamin B_{12}) status than plasma tHcy (11, 12). Both asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are turnover products of proteins subjected to posttranslational modification by the AdoMet-dependent enzymes, protein arginine methyltransferases (PRMTs) (13). The recent interest in ADMA in particular stems from its role as an inhibitor of nitric oxide production, and thereby endothelial function (14), and ADMA has been suggested to be a link between homocysteine and endothelial dysfunction (15). Finally, neopterin and the kynurenine/tryptophan ratio are markers of Th1-type immune activation and have recently been demonstrated to be strong predictors of plasma tHcy, independent of B-vitamin status (16–18).

The metabolic relations between key vitamins and metabolites are depicted in Figure 1.

One-carbon metabolism, folate and disease

There has been a growing interest in one-carbon metabolism and related B-vitamins, because of the relations to numerous diseases and medical conditions from birth to senescence. Folate deficiency has been assigned a role in the etiology of neural tube defects, and folate status has been associated with other birth defects and adverse pregnancy outcomes (19, 20). Deficiencies of folate or cobalamin are important causes of megaloblastic anemia (21). Impaired folate status or elevated homocysteine are risk factors for colorectal cancer and other malignancies (2, 22, 23), cardiovascular diseases (24), impaired cognitive function (25), depression (26) and osteoporosis (27). In vitro experiments suggest that high homocysteine may cause vascular lesion, and some but not all studies indicate that vascular dysfunction is mediated partly at least via accumulation of ADMA (28, 29). The concept of homocysteine as a causative agent has not been supported by most intervention trials with folic acid, which have demonstrated no reduction in risk of coronary heart disease (30–32), but a reduction in risk of stroke (33). Likewise, supplementation with folic acid or vitamin B_{12} does improve cognition in the elderly in one study (34), but not all studies (35, 36). The negative results may be related to inability of short-term (usually less than 4 years) therapy (37) with a single vitamin to reverse established vascular pathology or central nervous system lesions and do not preclude beneficial effect from adequate intake of B-vitamins in healthy subjects (38). However, these negative results will influence the direction of future research, which may focus on the long-term effect of more physiological and balanced doses of multiple B-vitamins in healthy subjects (39), a strategy that gains support from recent large-scale epidemiological studies, demonstrating that the B-vitamins, folate, cobalamin, riboflavin, vitamin B_6 (40) and betaine (41) form a functional network. Furthermore, future research should also address the influence of immune activation on homocysteine and B-vitamin status, a direction that is inspired by the strong correlation between plasma tHcy and markers, such as neopterin and the kynurenine/tryptophan ratio (16–18). These perspectives for future research will demand powerful analytical technology that enables the determination of multiple components of one-carbon metabolism in large-scale studies. This analytical approach can be referred to as profiling of one-carbon metabolism.

Strategies for metabolic profiling and selection of candidate metabolites

The present paper describes the methods developed in our laboratory for the profiling of one-carbon metabolism. Metabolite profiling means monitoring and quantification of a selected number of predefined low molecular weight compounds, generally related to (a) specific metabolic pathway(s), in specific tissues or compartments (42). The term is often confused with, but should be distinguished from metabolomics, which involves the non-targeted, comprehensive determination of changes in the complete set of metabolites in a biological system, i.e., the metabolome (42). This task is a demanding challenge, which is not yet technically feasible, and will require extremely selective and sensitive technologies.

When selecting compounds to be included in the repertoire, we targeted vitamins and metabolites involved in one-carbon metabolism, related markers, such as MMA, but also markers of immune activation (neopterin, the kynurenine/tryptophan ratio) and smoking (cotinine), which profoundly affect B-vitamin status (43). The extracellular concentration should be sufficiently high relative to the intracellular concentration to preclude a substantial artificial increase in plasma/serum concentration upon limited hemolysis (e.g., glutathione does not fulfill this criterion), and the compounds must be stable in serum/plasma for hours at room temperature and for years when stored below $-20°C$. The latter condition precludes the inclusion of AdoMet and S-adenosylhomocysteine (44).

Additional prerequisite for including an analyte is availability of standard material of high purity. We also obtained isotope labeled internal standard for most analytes or groups of analytes.

Analytical methods

The targeted vitamins and metabolites cover a 100,000-fold concentration range from a few nmol/L (riboflavin and cotinine) to several hundred μmol/L (total cysteine and glycine). They have different chemical structure and stability and variable polarity. To

measure, accurately and precisely, such a spectrum of compounds using a single method seems impossible with the currently available technology.

In general, gas chromatography-mass spectrometry (GC-MS and GC-MS/MS) is used to separate compounds on the basis of their relative vapor pressures and affinities for the material in the chromatography column and is regarded as the preferred method for measuring non-polar compounds as well as some polar compounds after derivatization, which increases volatility (45). GC-MS is characterized by ruggedness and offers high resolution, high sensitivity and high analytical precision (46). However, derivatization and extraction of analytes are required before chromatography, and the analytes must be heat-stable (42).

In liquid chromatography coupled to mass spectrometry (LC-MS), analytes are separated by elution from a stationary phase by a mobile phase composed of a mixture of water and an organic solvent, followed by soft ionization techniques, such as electrospray ionization or, less often, atmospheric pressure chemical ionization (47). This technique differs from GC-MS by simpler sample preparation and lower analysis temperature and is better suited for the analysis of labile, non-volatile, polar compounds in their native form. Compared with GC-MS, disadvantages are lower resolution and separation efficiency, more variable retention time, adduct formation and matrix effect causing ion suppression. Ion suppression may impede quantification (48), but variable ionization can often be corrected for by including an isotope-labeled internal standard (47).

Analytical platforms

MMA, tHcy, glycine, serine, methionine and total cysteine (tCys) were analyzed by a published method (49), which involves quantitative reduction of the disulfides, and derivatization and extraction in a single step by the addition of methylchloroformate and toluene. The *N*(*S*)-methoxycarbonyl ethyl ester derivatives are analyzed by GC-MS in the selected-ion monitoring mode (Platform A). The within-day coefficient of variations (CVs) ranged from 1% to 3.6%. This method has been further developed by monitoring the column effluent by MS/MS instead of MS. GC-MS/MS affords better selectivity and allows quantification of cystathionine and kynurenine. We also included sarcosine and tryptophan, the latter to obtain both components of the kynurenine/tryptophan ratio (Platform B, Figure 2). Other amino acids could easily be added to platforms A and B.

The quaternary ammonium compounds, choline and betaine, and the product of the BHMT reaction, dimethylglycine, are determined by a modification of a method based on LC-MS/MS (50). By replacing the silica with an ether-linked phenyl reversed-phase column, we were able to include basic compounds, such as arginine, ADMA and SDMA, neutral amino acids (homocysteine, methionine, methionine sulfoxide and cysteine) and creatinine and cystathionine in the same platform (Platform C, Figure 3). The within-day CVs ranged from 4% to 6%.

The concentrations of many analyzed compounds were in the nanomolar range. These included vita-

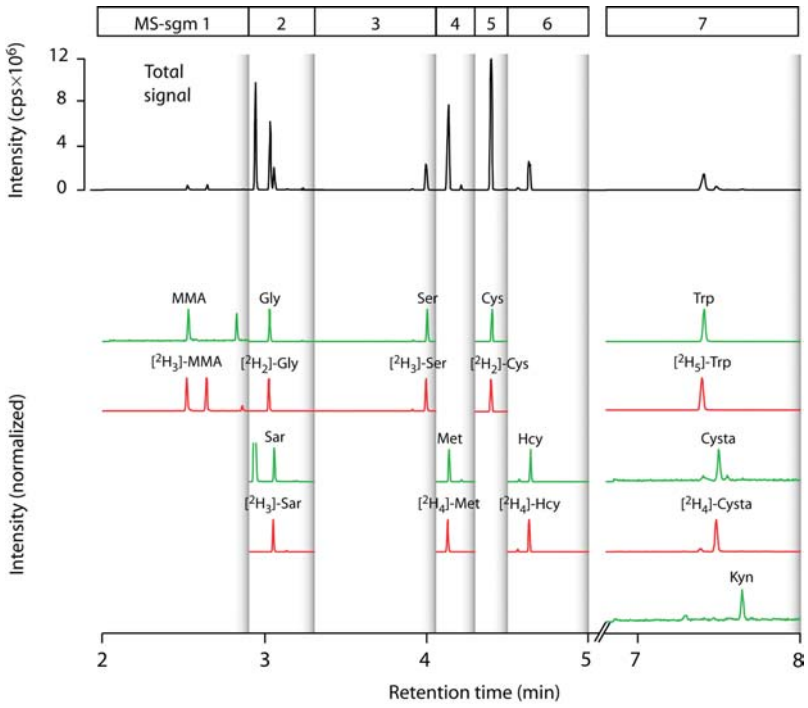

Figure 2 GC-MS/MS MRM chromatograms for MMA, homocysteine, and other amino acids in human plasma, derivatized with methylchloroformate (Platform B).
The analysis was divided into seven scan segments which each contained 1–3 analytes and the corresponding isotope-labeled internal standard(s). The upper trace (in black) shows the total ion count. The selected ion traces for the analytes are shown in green; the traces for the isotope-labeled internal standards are shown in red. sgm, scan segment.

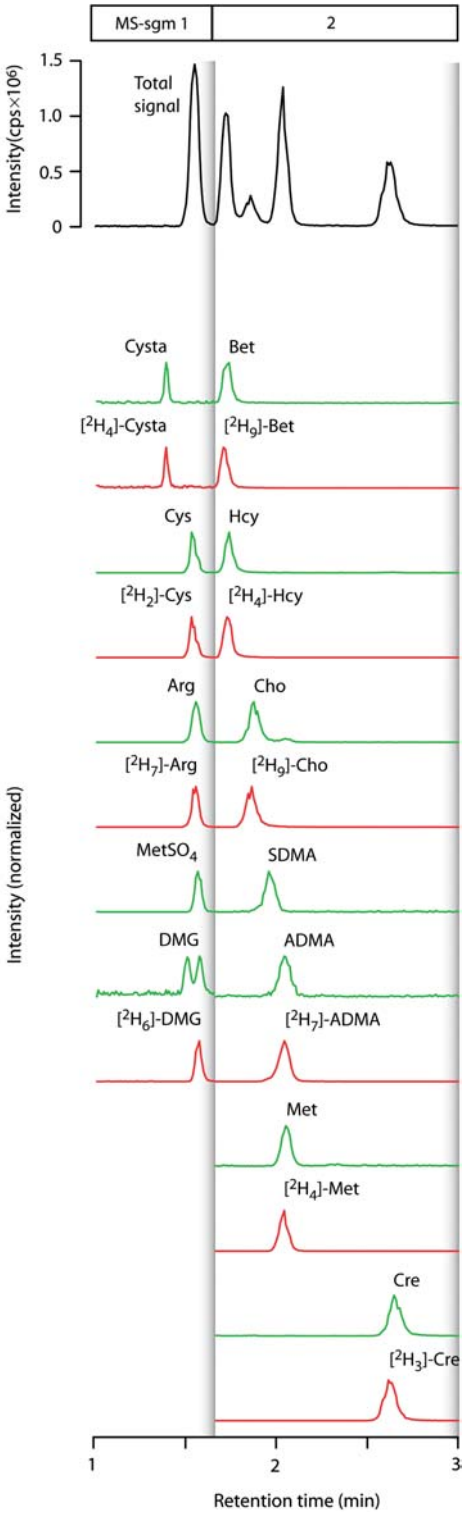

Figure 3 LC-MS/MS MRM chromatograms for choline, betaine, other basic compounds and neutral amino acids in human plasma (Platform C).
The analysis was divided into two scan segments which each contained 5–7 analytes and the corresponding isotope-labeled internal standard(s). The upper trace (in black) shows the total ion count. The selected ion traces for the analytes are shown in green; the traces for the isotope-labeled internal standards are shown in red. This is a modification of a published method (50). sgm, scan segment.

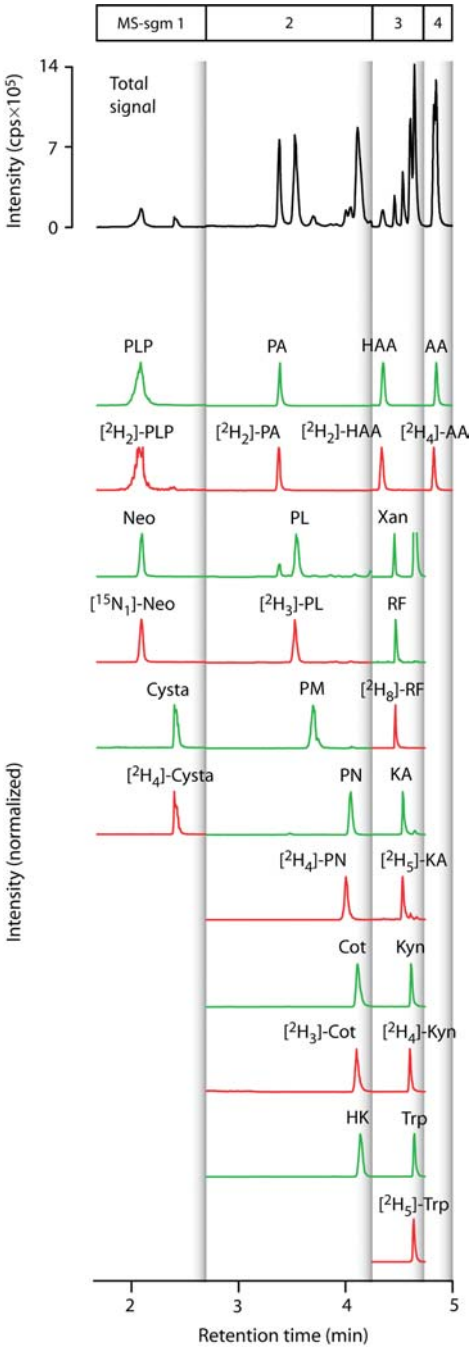

Figure 4 LC-MS/MS MRM chromatograms for B_6 and B_2 vitamins, tryptophan metabolites and other biomarkers in human plasma (Platform D).
The analysis was divided into four scan segments which each contained 2–9 analytes and the corresponding isotope-labeled internal standard(s). The upper trace (in black) shows the total ion count. The selected ion traces for the analytes are shown in green; the traces for the isotope-labeled internal standards are shown in red. This is a modification of a published method (51). sgm, scan segment.

mins B_6 species (pyridoxal phosphate, pyridoxal and pyridoxic acid), riboflavin, neopterin, a marker of inflammation, and cotinine, a nicotine metabolite. Sample handling involved only a simple protein precipitation step. To obtain sufficient sensitivity to

Table 1 Vitamins and metabolites related to one-carbon metabolism according to analytical platform based on mass spectrometry.

Analyte	Category	Function and role	Method
Methylmalonic acid	Carboxylic acid	Marker of B_{12} status	Platform A,
Homocysteine, total	Aminothiol	Marker of folate and B_{12} status	GC-MS
Cysteine, total	Aminothiol	Product of transsulfuration	
Methionine	Amino acid	Product of remethylation	
Serine	Amino acid	One-carbon donor	
Glycine	Amino acid		
Sarcosine	Amino acid	Intermediate in choline metabolism	
Tryptophan (Trp)	Amino acid		
Methylmalonic acid	Carboxylic acid	Marker of B_{12} status	Platform B,
Homocysteine, total	Aminothiol	Marker of folate and B_{12} status	GC-MS/MS
Cysteine, total	Aminothiol	Product of transsulfuration	
Methionine	Amino acid	Product of remethylation	
Serine	Amino acid	One-carbon donor	
Glycine	Amino acid		
Cystathionine	Thioether	Transsulfuration intermediate	
Sarcosine	Amino acid	Intermediate in choline metabolism	
Kynurenine (Kyn)	Trp metabolite		
Tryptophan (Trp)	Amino acid		
Kyn/Trp ratio		Marker of immune activation	
Choline, free	Quaternary ammonium	Betaine precursor	Platform C,
Choline, total	Choline esters		LC-MS/MS
Betaine	Quaternary ammonium	One-carbon donor	
Dimethylglycine	Amino acid	Product of betaine	
Homocysteine, total	Aminothiol	Marker of folate and B_{12} status	
Cysteine, total	Aminothiol	Product of transsulfuration	
Methionine	Amino acid	Product of remethylation	
Methionine sulfoxide	Amino acid	Oxidation product of methionine	
Cystathionine	Thioether	Transsulfuration intermediate	
Creatinine		Marker of renal function	
Arginine	Amino acid	Precursor of nitric oxide (NO)	
Asymmetric dimethylarginine	Amino acid	Inhibitor of NO synthesis	
Symmetric dimethylarginine	Amino acid		
Pyridoxal phosphate	B_6 vitamer	Cofactor	Platform D,
Pyridoxal	B_6 vitamer	B_6 intermediate	LC-MS/MS
Pyridoxic acid	B_6 vitamer	B_6 catabolite	
Pyridoxamine	B_6 vitamer	B_6 intermediate	
Pyridoxine	B_6 vitamer	In supplements	
Riboflavin	B_2 vitamer	Precursor of FMN and FAD	
Flavin mononucleotide (FMN)	B_2 vitamer	Cofactor	
Cystathionine	Thioether	Transsulfuration intermediate	
Neopterin	Pterin	Marker of immune activation	
Tryptophan (Trp)	Amino acid		
Kynurenine (Kyn)	Trp metabolite		
Kyn/Trp ratio		Marker of immune activation	
Kynurenic acid	Trp metabolite		
Anthranilic acid	Trp metabolite		
3-Hydroxykynurenine	Trp metabolite		
Xanthurenic acid	Trp metabolite		
3-Hydroxyanthranilic acid	Trp metabolite		
Cotinine	Nicotine metabolite	Marker of smoking	
5-Methyltetrahydrofolate	Folate vitamer	Prevailing folate in serum/plasma	Platform E,
5-Formyltetrahydrofolate	Folate vitamer		LC-MS/MS
Folic acid	Folate vitamer	In supplements	
4-α-Hydroxy-5-methyl-dihydrofolate	Folate oxidation product		
Para-aminobenzoyl-glutamate	Folate catabolite		
Acetamidobenzoylglutamate	Folate catabolite		

detect these compounds, we used a LC-MS/MS method optimized to analyze vitamin B_2 and B_6 species (51), which involved an unconventional mobile phase containing a high concentration (150–600 mmol/L) of acetic acid. The mass spectrometer was operated in multiple reaction mode (MRM) and positive ion mode. There is one previous report emphasizing the high sensitivity in the positive ion mode and efficient separation for a variety of compounds obtained with a mobile phase containing acetic acid concentration $>1\%$ (52). A modified version of this method also included cystathionine, neopterin, cotinine, trypto-

Table 2 Analytical platforms.

Platform	Analytes	Scan segments	Retention time, min[a]	Run time, min	Volume requirement, μL		Capacity[b], samples/24 h
					Min	Max	
A	8	7	7.5	14	120	400	96
B	10	7	7.5	14	120	400	96
C	12	2	2.6	4	40	120	350
D	16	4	4.8	6.5	60	200	182
E	6	2	2.7	5	60	200	182

[a]Retention time of last eluting analyte. [b]Capacity for one instrument dedicated to the analysis.

phan, kynurenine and other tryptophan metabolites (Platform D, Figure 4). The within-day CVs ranged from 4% to 7%.

We used a similar LC-MS/MS method exploiting the strong positive ion signal in the presence of a high concentration of acetic acid to measure folate species and catabolites in human plasma/serum. The substances included in this assay were mTHF, 5-formyltetrahydrofolate, folic acid, 4-α-hydroxy-5-methyldihydrofolate and the catabolites, para-aminobenzoylglutamate and acetamidobenzoylglutamate (Platform E). We included 4-α-hydroxy-5-methyldihydrofolate, because in serum/plasma samples left at room temperature for days, the decline in mTHF and folate detected by microbiological technique is recovered as 4-α-hydroxy-5-methyldihydrofolate. The within-day CVs are approximately 5% (at 30 nmol/L).

The vitamins and metabolites measured on the separate platforms and their role in one-carbon metabolism are summarized in Table 1.

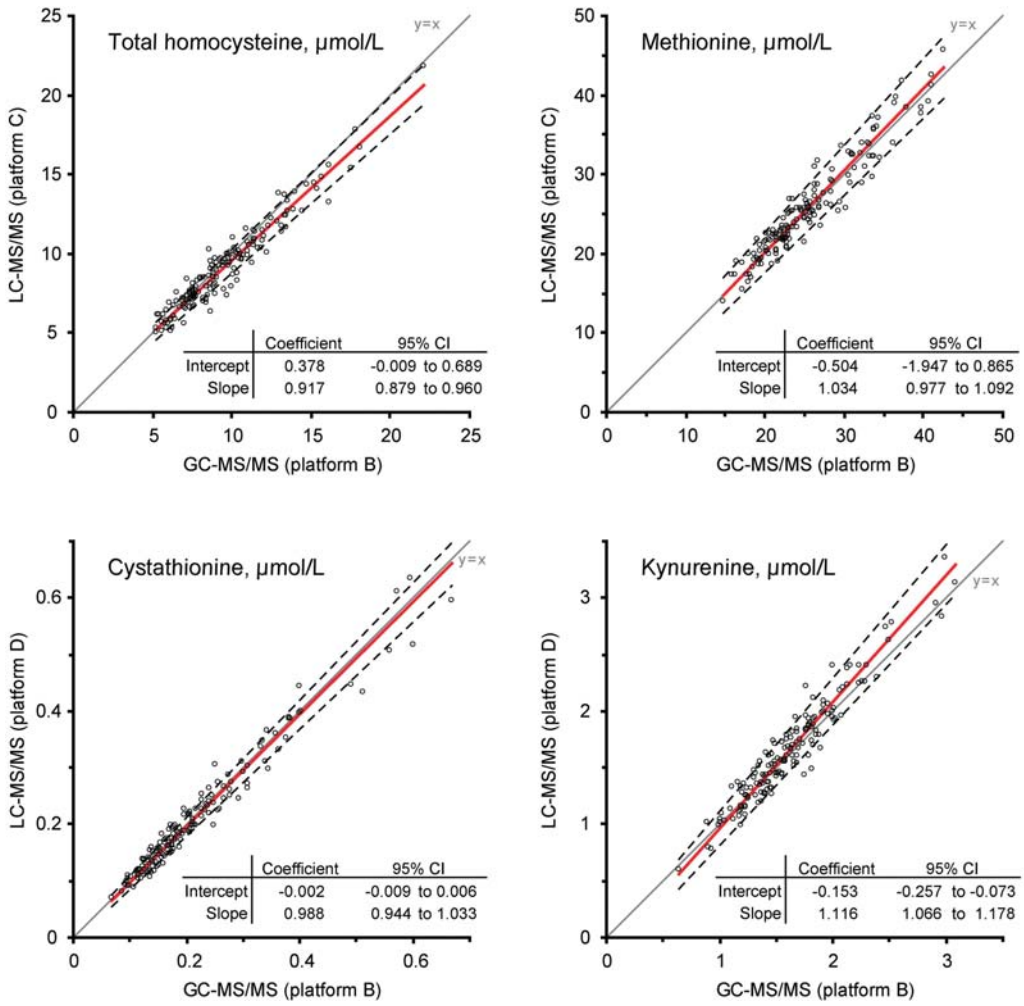

Figure 5 Method comparison.
The correlations between values for tHcy, methionine, cystathionine and kynurenine, as obtained by GC-MS/MS and LC-MS/MS, were analyzed using Passing-Bablok regression (53). n = 160.

The chromatographic run times were 4–14 min for platforms A–E (Figures 2–4, Table 2), and for all platforms, the chromatographic cycles were divided into 2–7 scan segments, which encompass a maximum of 13 analytes and internal standards.

Logistics, sample handling and data collection

The logistics for the determination of approximately 40 vitamins and metabolites related to one-carbon metabolism have been established. The vitamin B_2, B_6, folate species and neopterin included on platforms D and E are least stable and are measured during the first thawing-freezing cycle, together with cobalamin and folate by a microbiological method. The carboxylic acid, sulfur amino acids, amino acids (except serine) and quaternary, tertiary and secondary amines measured by platforms A, B and C are essentially stable at 4°C for days and during freezing/thawing and can be analyzed in any order.

We use a robotic workstation for sample handling, protein precipitation and extraction. For all platforms A–E, such sample handling consumes a total of 920 μL. Manual sample handling consumes less sample volume (320 μL) and could be used if sample volume is limited. The analytical capacity per instrument is determined by the retention time of the last analyte and the additional time required for column equilibration (LC) or stabilization of column oven temperature (GC), giving the total time between each sample injection, i.e., run time. The analytical capacity (samples/24 h) for platforms A and B (run time of 12–14 min) is 96, for platform C (4 min) 350, for platform D (6.5 min) 182 and for platform E (5 min) 182 (Table 2).

The raw data from the separate platforms and analysis sets are merged, handled and controlled by a specially designed software. Five stable metabolites (tHcy, tCys, cystathionine, kynurenine and tryptophan) are measured by two or more platforms. Correlations of values obtained by different methods are high, as demonstrated in Figure 5. Outliers are identified by ratio between values from different platforms. Unlikely combinations of values are flagged by macros. The system also generates summary statistics and printouts of selected samples in a format that matches the geometry of the sample trays of the autosamplers. These routines for data handling ensure efficiency and reduce the chance of logistic problems and human errors.

Summary and conclusions

We have established a procedure for the determination of approximately 40 vitamins and metabolites related to one-carbon metabolism in less than 1 mL of serum/plasma, using five analytical platforms based on GC-MS, GC-MS/MS and LC-MS/MS. Procedures have been established for sample handling and data collection to ensure efficiency and to minimize the chance of human errors. This procedure has enabled us to carry out extensive profiling of one-carbon metabolism in large-scale epidemiological studies (40, 41, 54–56).

References

1. Gregory JF. Chemical and nutritional aspects of folate research – analytical procedures, methods of folate synthesis, stability, and bioavailability of dietary folates. Adv Food Nutr Res 1990;33:1–101.
2. Stover PJ. Physiology of folate and vitamin B12 in health and disease. Nutr Rev 2004;62:S3–12;discussion:S3.
3. Stead LM, Brosnan JT, Brosnan ME, Vance DE, Jacobs RL. Is it time to reevaluate methyl balance in humans? Am J Clin Nutr 2006;83:5–10.
4. Suh JR, Herbig AK, Stover PJ. New perspectives on folate catabolism. Annu Rev Nutr 2001;21:255–82.
5. Finkelstein JD. Methionine metabolism in mammals. J Nutr Biochem 1990;1:228–37.
6. Ueland PM, Holm PI, Hustad S. Betaine: a key modulator of one-carbon metabolism and homocysteine status. Clin Chem Lab Med 2005;43:1069–75.
7. Zeisel SH. Choline: critical role during fetal development and dietary requirements in adults. Annu Rev Nutr 2006; 26:229–50.
8. Wittwer AJ, Wagner C. Identification of the folate-binding proteins of rat liver mitochondria as dimethylglycine dehydrogenase and sarcosine dehydrogenase. Flavoprotein nature and enzymatic properties of the purified proteins. J Biol Chem 1981;256:4109–15.
9. Steenkamp DJ, Husain M. The effect of tetrahydrofolate on the reduction of electron transfer flavoprotein by sarcosine and dimethylglycine dehydrogenases. Biochem J 1982;203:707–15.
10. Wyss M, Kaddurah-Daouk R. Creatine and creatinine metabolism. Physiol Rev 2000;80:1107–213.
11. Refsum H, Smith AD, Ueland PM, Nexo E, Clarke R, McPartlin J, et al. Facts and recommendations about total homocysteine determinations: an expert opinion. Clin Chem 2004;50:3–32.
12. Allen RH, Stabler SP, Savage DG, Lindenbaum J. Diagnosis of cobalamin deficiency. 1. Usefulness of serum methylmalonic acid and total homocysteine concentrations. Am J Hematol 1990;34:90–8.
13. Pahlich S, Zakaryan RP, Gehring H. Protein arginine methylation: cellular functions and methods of analysis. Biochim Biophys Acta 2006;1764:1890–903.
14. Siroen MP, Teerlink T, Nijveldt RJ, Prins HA, Richir MC, van Leeuwen PA. The clinical significance of asymmetric dimethylarginine. Annu Rev Nutr 2006;26:203–28.
15. Stuhlinger MC, Stanger O. Asymmetric dimethyl-L-arginine (ADMA): a possible link between homocyst(e)ine and endothelial dysfunction. Curr Drug Metab 2005;6: 3–14.
16. Schroecksnadel K, Frick B, Winkler C, Leblhuber F, Wirleitner B, Fuchs D. Hyperhomocysteinemia and immune activation. Clin Chem Lab Med 2003;41:1438–43.
17. Schrocksnadel K, Wirleitner B, Winkler C, Fuchs D. Monitoring tryptophan metabolism in chronic immune activation. Clin Chim Acta 2006;364:82–90.
18. Frick B, Gruber B, Schroecksnadel K, Leblhuber F, Fuchs D. Homocysteine but not neopterin declines in demented patients on B vitamins. J Neural Transm 2006;113: 1815–9.
19. Ueland PM, Vollset SE. Homocysteine and folate in pregnancy. Clin Chem 2004;50:1293–5.
20. Tamura T, Picciano MF. Folate and human reproduction. Am J Clin Nutr 2006;83:993–1016.

21. Carmel R. Anemia and aging: an overview of clinical, diagnostic and biological issues. Blood Rev 2001;15: 9–18.
22. Ulrich CM. Nutrigenetics in cancer research – folate metabolism and colorectal cancer. J Nutr 2005;135: 2698–702.
23. Powers HJ. Interaction among folate, riboflavin, genotype, and cancer, with reference to colorectal and cervical cancer. J Nutr 2005;135: S2960–6.
24. Clarke R, Collins R, Lewington S, Donald A, Alfthan G, Tuomilehto J. Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. J Am Med Assoc 2002;288:2015–22.
25. Stabler SP. Vitamins, homocysteine, and cognition. Am J Clin Nutr 2003;78:359–60.
26. Bottiglieri T. Homocysteine and folate metabolism in depression. Prog Neuropsychopharmacol Biol Psychiatry 2005;29:1103–12.
27. Cashman KD. Homocysteine and osteoporotic fracture risk: a potential role for B vitamins. Nutr Rev 2005;63: 29–36.
28. Lentz SR, Rodionov RN, Dayal S. Hyperhomocysteinemia, endothelial dysfunction, and cardiovascular risk: the potential role of ADMA. Atheroscler Suppl 2003; 4:61–5.
29. Endemann DH, Schiffrin EL. Endothelial dysfunction. J Am Soc Nephrol 2004;15:1983–92.
30. Bønaa KH, Njølstad I, Ueland PM, Schirmer H, Tverdal A, Steigen T, et al. Homocysteine lowering and cardiovascular events after acute myocardial infarction. N Engl J Med 2006;354:1578–88.
31. Bazzano LA, Reynolds K, Holder KN, He J. Effect of folic acid supplementation on risk of cardiovascular diseases: a meta-analysis of randomized controlled trials. J Am Med Assoc 2006;296:2720–6.
32. Ueland PM, Clarke R. Homocysteine and cardiovascular risk: considering the evidence in the context of study design, folate fortification, and statistical power. Clin Chem 2007;53:807–9.
33. Wang X, Qin X, Demirtas H, Li J, Mao G, Huo Y, et al. Efficacy of folic acid supplementation in stroke prevention: a meta-analysis. Lancet 2007;369:1876–82.
34. Durga J, van Boxtel MP, Schouten EG, Kok FJ, Jolles J, Katan MB, et al. Effect of 3-year folic acid supplementation on cognitive function in older adults in the FACIT trial: a randomised, double blind, controlled trial. Lancet 2007;369:208–16.
35. Vollset SE, Ueland PM. B vitamins and cognitive function: do we need more and larger trials? Am J Clin Nutr 2005;81:951–2.
36. Eussen SJ, de Groot LC, Joosten LW, Bloo RJ, Clarke R, Ueland PM, et al. Effect of oral vitamin B-12 with or without folic acid on cognitive function in older people with mild vitamin B-12 deficiency: a randomized, placebo-controlled trial. Am J Clin Nutr 2006;84:361–70.
37. Carlsson CM. Lowering homocysteine for stroke prevention. Lancet 2007;369:1841–2.
38. Wald DS, Wald NJ, Morris JK, Law M. Folic acid, homocysteine, and cardiovascular disease: judging causality in the face of inconclusive trial evidence. Br Med J 2006; 333:1114–7.
39. Lichtenstein AH, Russell RM. Essential nutrients: food or supplements? Where should the emphasis be? J Am Med Assoc 2005;294:351–8.
40. Hustad S, Midttun Ø, Schneede J, Vollset SE, Grotmol T, Ueland PM. The methylenetetrahydrofolate reductase 677C→T polymorphism as a modulator of a B-vitamin network with major effects on homocysteine metabolism. Am J Hum Genet 2007;80:846–55.
41. Holm PI, Hustad S, Ueland PM, Vollset SE, Grotmol T, Schneede J. Modulation of the homocysteine-betaine relationship by methylenetetrahydrofolate reductase 677 C→T genotypes and B-vitamin status in a large-scale epidemiological study. J Clin Endocrinol Metab 2007;92: 1535–41.
42. Dunn WB, Ellis DI. Metabolomics: current analytical platforms and methodologies. Trends Analyt Chem 2005; 24:285–94.
43. Piyathilake CJ, Macaluso M, Hine RJ, Richards EW, Krumdieck CL. Local and systemic effects of cigarette smoking on folate and vitamin B-12. Am J Clin Nutr 1994;60:559–66.
44. Gellekink H, van Oppenraaij-Emmerzaal D, van Rooij A, Struys EA, den Heijer M, Blom HJ. Stable-isotope dilution liquid chromatography-electrospray injection tandem mass spectrometry method for fast, selective measurement of S-adenosylmethionine and S-adenosylhomocysteine in plasma. Clin Chem 2005;51:1487–92.
45. Mastovska K, Lehotay SJ. Practical approaches to fast gas chromatography-mass spectrometry. J Chromatogr A 2003;1000:153–80.
46. Maurer HH. Role of gas chromatography-mass spectrometry with negative ion chemical ionization in clinical and forensic toxicology, doping control, and biomonitoring. Ther Drug Monit 2002;24:247–54.
47. Vogeser M. Liquid chromatography-tandem mass spectrometry – application in the clinical laboratory. Clin Chem Lab Med 2003;41:117–26.
48. Taylor PJ. Matrix effects: the Achilles heel of quantitative high-performance liquid chromatography-electrospray-tandem mass spectrometry. Clin Biochem 2005;38:328–34.
49. Windelberg A, Arseth O, Kvalheim G, Ueland PM. Automated assay for the determination of methylmalonic acid, total homocysteine, and related amino acids in human serum or plasma by means of methylchloroformate derivatization and gas chromatography-mass spectrometry. Clin Chem 2005;51:2103–9.
50. Holm PI, Ueland PM, Kvalheim G, Lien EA. Determination of choline, betaine, and dimethylglycine in plasma by a high-throughput method based on normal-phase chromatography-tandem mass spectrometry. Clin Chem 2003;49:286–94.
51. Midttun Ø, Hustad S, Solheim E, Schneede J, Ueland PM. Multianalyte quantification of vitamin B6 and B2 species in the nanomolar range in human plasma by liquid chromatography-tandem mass spectrometry. Clin Chem 2005;51:1206–16.
52. Kamel AM, Brown PR, Munson B. Effects of mobile-phase additives, solution pH, ionization constant, and analyte concentration on the sensitivities and electrospray ionization mass spectra of nucleoside antiviral agents. Anal Chem 1999;71:5481–92.
53. Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods. Application of linear regression procedures for method comparison studies in clinical chemistry, Part I. J Clin Chem Clin Biochem 1983;21:709–20.
54. Ulvik A, Ueland PM, Fredriksen A, Meyer K, Vollset SE, Hoff G, et al. Functional inference of the methylenetetrahydrofolate reductase 677 C→T and 1298A→C polymorphisms from a large-scale epidemiological study. Hum Genet 2007;121:57–64.
55. Midttun Ø, Hustad S, Vollset SE, Schneede J, Ueland PM. Plasma vitamin B6 forms and their relations to transsulfuration metabolites in a large-scale population-based study. Am J Clin Nutr 2007;86:131–8.
56. Fredriksen A, Meyer K, Ueland PM, Vollset SE, Grotmol T, Schneede J. Large-scale population-based metabolic phenotyping of thirteen genetic polymorphisms related to one-carbon metabolism. Hum Mutat 2007;28:856–65.

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