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Quantification of key folate forms in serum using stable-isotope dilution ultra performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Folates act as essential coenzymes in many biological pathways. Alteration in folate form distribution might have biological significance, especially in relation to certain genetic polymorphisms. We developed a stable-isotope dilution ultra performance liquid chromatography–mass spectrometry (UPLC–MS/MS) method for quantification of the folate forms 5-methyltetrahydrofolate (5-methylTHF), 5-formylTHF, 5,10-methenylTHF, THF, and folic acid in serum. After extraction using an ion exchange and mixed mode solid-phase, samples were separated and detected using an UPLC–MS/MS system. The quantification limits were between 0.17 nmol/L (5-formylTHF) and 1.79 nmol/L (THF), and the assay was linear up to 100 nmol/L (5-methylTHF) and 10 nmol/L (5-formylTHF, 5,10-methenylTHF, THF, and folic acid). The intraassay CVs for 5-methylTHF and 5-formylTHF were 2.0% and 7.2%, respectively. Mean recoveries were between 82.3% for THF and 110.8% for 5,10-methenylTHF. Concentrations of total folate measured by the new method showed a strong correlation with those measured by an immunologic assay ($r = 0.939$; $p < 0.001$). The mean total folate from 32 apparently healthy subjects was 18.09 nmol/L, of which 87.23% was 5-methylTHF. Concentrations of homocysteine showed a better correlation to the total folate measured by the new method compared to that obtained by an immunologic assay. We also confirmed that *MTHFR* polymorphism has a significant effect on folate distribution in this small population of non-supplemented subjects.

1. Introduction

Folates serve as essential cofactors in several metabolic pathways including biosynthesis of nucleotides and amino acids, as well as methylation reactions [1,2]. Over the last decades folate has gained considerable importance because of its putative role in health and disease [3–5]. Folic acid, a synthetic form of the vitamin, has been detected in serum of subjects consuming fortified foods or supplements [6]. 5-Methyltetrahydrofolate (5-methylTHF) is the predominant folate form, comprising 82–93% of the total folate (TFOL) in human serum [7]. The polyglutamate chains of the food folates must be deconjugated by the enzyme folylpoly-γ-glutamate carboxypeptidase in the jejunum of the small intestine before it can be absorbed. Because of the dual role of folate in purine and thymidylate synthesis and in homocysteine metabolism, a shift in folate form distribution might be of biological significance. Methods routinely used for quantifying TFOL show large disagreements [8] and are unable to detect various forms of the vitamins. Several high performance liquid chromatography (HPLC) [9,10] and gas chromatography [11,12] methods have been described for detection of folate forms. In recent years, liquid chromatography coupled with mass spectrometry (LC–MS) methods and liquid chromatography tandem mass spectrometry (LC–MS/MS) methods have been developed to quantify folate monoglutamates [7,13,14]. However, most of these methods require complex and time-consuming sample preparation procedures which might cause folate degradation.

We describe a fast, stable-isotope dilution ultra performance liquid chromatography tandem mass spectrometry (UPLC–MS/MS) method for the simultaneous quantification of 5-methylTHF, 5-formylTHF, 5,10-methenylTHF, THF, and folic acid in human serum. Due to the short time required for sample preparation (40 samples in 120 min) and measurement (2.5 min/sample), our assay is suitable for the determination of folate distribution in large-scale clinical studies. We compare concentrations of TFOL measured with our method with that from the traditional chemiluminescent method. In addition, we aim to study folate forms in relation

Abbreviations: DHF, dihydrofolate; GC/MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; LC–MS/MS, liquid chromatography with tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; MS/MS, tandem mass spectrometry; MTHFR, 5,10-methylenetetrahydrofolate reductase; pABA, *p*-aminobenzoic acid; PCR, polymerase chain reaction; SPE, solid-phase extraction; TFOL, total folate; THF, tetrahydrofolate; tHcy, total homocysteine; TIC, total ion chromatogram; UPLC, ultra performance liquid chromatography.

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to the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) *C677T* genotype (E.C. 1.5.1.20).

2. Materials and methods

2.1. Standards and chemicals

(6S)-5-FormylTHF, (6R)-10-formylTHF, (6R)-5,10-methenylTHF, (6S)-5-methylTHF, (6R)-5,10-methyleneTHF, (6S)-THF, 7,8-DHF, and folic acid (Merck Eprova AG, Switzerland) were used for preparation of standard stock solutions. Internal standards were [$^{13}C_5$]-5-formylTHF, [$^{13}C_5$]-5-methylTHF, and [$^{13}C_5$]-folic acid (isotopic purity >99%, Merck Eprova AG). Both, THF and 5,10-methenylTHF use [$^{13}C_5$]-5-formylTHF as internal standard. Other chemicals used were: acetic acid (glacial: >99.99%; Sigma–Aldrich); ascorbic acid (Riedel-de-Haën); L-cysteine (Fluka); ammonium acetate, formic acid, and methanol (ULC/MS grade; Biosolve, The Netherlands). Ultrapure water (18.2 MΩ) was prepared with a Milli-Q water purification system (Millipore, France).

2.2. Preparation of stock solutions

Stock solutions I (440 μmol/L) were prepared according to Pfeiffer et al. in 20 mmol/L di-ammonium hydrogen phosphate, pH 7.2 (Merck) [7]. Concentrations were photometrically verified for 5-methylTHF and [$^{13}C_5$]-5-methylTHF (λ = 290 nm, ε = 31,700), 5-formylTHF and [$^{13}C_5$]-5-formylTHF (λ = 285 nm, ε = 37,200), 10-formylTHF (λ = 253 nm, ε = 15,300), 5,10-methyleneTHF (λ = 295 nm, ε = 25,000), 5,10-methenylTHF (λ = 352 nm, ε = 25,000), THF (λ = 297 nm, ε = 29,100), DHF (λ = 282 nm, ε = 22,400), folic acid and [$^{13}C_5$]-folic acid (λ = 282 nm, ε = 27,600). Ascorbic acid (10 g/L) was added to all solutions except to that of folic acid. 20 μL/mL (v/v) NH_4OH was added to the standard solution of DHF to prevent precipitation at low pH. Stock solutions II (220 μmol/L) were prepared. Aliquots of stock solution II were stored at −70 °C for not longer than one year. Working stock solutions III (22 μmol/L) were prepared in 1 g/L aqueous acetic acid solution and were stored at −70 °C for not longer than one month.

2.3. Sample collection

70 serum samples were available for method comparison of the newly developed UPLC–MS/MS method with the chemiluminescent immunoassay. Data from a subset of 32 apparently healthy subjects (8 males, age range: 17–55 years) was used for the study of normal range of folate forms and mutation analysis. Blood was collected and centrifuged within 30 min at 2000 × *g* for 10 min at 4 °C, serum was separated, and ascorbic acid was added to obtain a final concentration of 1 g/L. The samples were immediately stored at −70 °C until analysis. A serum pool was prepared and used in each run for quality control. All forms of folate measured here were stable in serum for at least 48 h at 4 °C and at least six months at −70 °C. The study was approved by the local ethics commission, participants signed informed consents.

2.4. Sample preparation

Serum samples were thawed and centrifuged at 2000 × *g* for 5 min at 4 °C. 250 μL of the clear sample or calibrator was incubated with 700 μL ammonium acetate buffer (200 mmol/L, pH 10). 50 μL internal standard solution mix (1 μmol/L [$^{13}C_5$]-5-methylTHF, 0.5 μmol/L [$^{13}C_5$]-folic acid, and 0.2 μmol/L [$^{13}C_5$]-5-formylTHF) was added.

Sample cleanup was performed with Oasis MAX solid-phase extraction (SPE) columns (Waters Corporation). Columns were preconditioned with 2 × 1 mL methanol following 1 mL of 200 mmol/L ammonium acetate buffer (pH 10), containing 10 g/L ascorbic acid. The samples were loaded and impurities were removed by washing the columns with 1 mL 5% aqueous NH_4OH and 1 mL methanol. The elution of the folate was performed by 6 × 250 μL elution solution (methanol containing 1% formic acid). The eluates (1500 μL) were taken to dryness in an Eppendorf Concentrator 5301 at 45 °C and then dissolved in 100 μL H_2O/methanol (60:40, v/v), containing 0.1% formic acid. Concentrated eluates were immediately measured.

Calibrators were included in each batch of samples at concentrations of 0, 8, 20, 50, and 100 nmol/L for 5-methylTHF and 0, 0.8, 2, 5, and 10 nmol/L for 5-formylTHF, 5,10-methenylTHF, THF, and folic acid, respectively. Additionally, to each batch of samples control samples at two different concentrations from each folate form were added. The high control contained 80 nmol/L 5-methylTHF, and 8 nmol/L of 5-formylTHF, 5,10-methenylTHF, THF, and folic acid. The low control consisted of 25 nmol/L 5-methylTHF, and 2.5 nmol/L of 5-formylTHF, 5,10-methenylTHF, THF, and folic acid. Calibrators and control samples were processed together with serum samples as described above.

Concentrations of TFOL from 70 serum samples were measured by using a chemiluminescent immunoassay (ADVIA Centaur System, Bayer Diagnostics, Germany). This method consists of a competitive assay using folate-binding protein. Total serum homocysteine (tHcy) from 32 subjects was determined by gas chromatography–mass spectrometry (GC/MS). The *MTHFR C677T* polymorphism of 32 samples was assessed by polymerase chain reaction (PCR) of genomic DNA and pyrosequencing (PSQ 96MA instrument; Biotage AB, Uppsala, Sweden) using primers as previously described [15,16].

2.5. UPLC–MS/MS conditions

UPLC–MS/MS analyses were carried out using an Acquity Ultra Performance LC system (Waters Corporation, Milford, MA, USA) coupled to a MicroMass Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corporation). The samples were separated on an Acquity UPLC HSS T3 column (50 mm × 2.1 mm (i.d.); 1.8 μm particle size) with an Acquity BEH C_{18} VanGuard precolumn (5 mm × 2.1 mm (i.d.); 1.7 μm particle size) and a 0.2 μm in-line filter (Waters Corporation). The column temperature was maintained at 30 °C. The flow rate was 0.5 mL/min, the solvents were A: aqueous acetic acid (glacial), pH 2.636 and B: methanol with a step-wise gradient over a total run time of 2.5 min: 0.0 min, 10% B; 0.4 min, 25% B (convex curve 2); 0.6 min, 45% B (convex curve 2); 0.8 min, 85% B (linear gradient); 1.0 min, 85% B (concave curve 11); 1.1 min, 10% B (linear gradient). The sample injection volume was 10 μL.

The folate compounds as well as their internal standards were identified by a MicroMass Quattro Premier XE mass spectrometer using positive electrospray ionization mode. Capillary voltage was 3.26 kV, the source temperature was 110 °C, desolvation gas (N_2) temperature was 460 °C at a flow rate of 1100 L/h, and the cone gas (N_2) flow rate was 50 L/h. Collision gas (Ar) flow was maintained at 0.26 L/h. Inter-scan and inter-channel delay were set to 0.05 s. Cone and collision energy voltages, dwell times and multiple reaction monitoring (MRM) mass transitions were summarized in Table 1.

2.6. Calculations and statistics

Data acquisition was performed by MassLynx V4.1 and QuanLynx software. The concentration of each analyte in serum was calculated by interpolation of the observed analyte/internal standard peak-area ratio. THF and 5,10-methenylTHF were quantified using [$^{13}C_5$]-5-formylTHF as internal standard. Linear regression

Table 1
Multiple reaction monitoring in ESI$^+$ of folate compounds and internal standards.

Compound	Cone voltage [V]	Collision energy [eV]	Precursor ion (*m/z*)	Product ion (*m/z*)	Dwell time [s]	Retention time [min]
Folic acid	22	16	442.086	295.139	0.040	1.03
THF	25	22	446.314	299.287	0.035	0.67
5,10-MethenylTHF	55	27	456.128	412.124	0.040	0.70
5-MethylTHF	23	19	460.294	313.266	0.040	0.69
5-FormylTHF	27	20	474.275	327.244	0.080	0.97
[$^{13}C_5$]-folic acid	22	16	447.086	295.139	0.040	1.00
[$^{13}C_5$]-5-methylTHF	23	19	465.294	313.266	0.040	0.69
[$^{13}C_5$]-5-formylTHF	25	20	479.275	327.244	0.040	0.96

analysis was used to verify the linearity of the calibration curves. Correlation analyses (Spearman-Rho) were performed by using SPSS (Statistical Package for the Social Sciences, version 17.0). Agreement between methods (UPLC–MS/MS method vs. immunological method performed by ADVIA Centaur) was assessed by Bland-Altman difference plots [17].

3. Results

3.1. Chromatography and tandem mass spectrometry

Optimal MRM conditions were obtained in the positive electrospray ionization mode, typical *m/z* transitions of the folate forms are shown in Table 1. The mass loss from precursor to product ion can be explained by the neutral loss of the glutamic acid residue from the protonated molecule to produce the major product ions [M+H$^+$−147] for unlabeled and [M+H$^+$−152] for [$^{13}C_5$]-labeled compounds. However, 10-formylTHF, 5,10-methenylTHF, and DHF produced only small amounts of this ion. 10-FormylTHF mostly converted to 5,10-methenylTHF (*m/z* 456) and in the case of 5,10-methenylTHF a non-specific fragment loss of CO_2 [M+H$^+$−44] occurred. As reported by other groups, DHF showed mainly a cleavage between the pteridine and the *p*-aminobenzoic acid (pABA) moiety [M+H$^+$−266] [18].

MRM chromatograms from serum are shown in Fig. 1. The folate compounds are represented in the total ion chromatogram (TIC) as two base line separated peaks with peak-widths less than 0.20 min. 5-MethylTHF, [$^{13}C_5$]-5-methylTHF, 5,10-methenylTHF, and THF, coeluted at retention times between 0.67 and 0.70 min (Table 1). 5-FormylTHF and folic acid, including their [$^{13}C_5$]-labeled compounds, coeluted between 0.96 and 1.03 min. An additional peak appeared at 0.75 min for 5-formylTHF and [$^{13}C_5$]-5-formylTHF which might be caused by interconversion to 5,10-methenylTHF and/or 10-formylTHF or degradation. The peak with a retention time of 0.75 min was excluded from data analysis. As expected, 5-methylTHF, the predominant folate form, showed the highest peak intensity. Smaller but reproducible peaks were found for 5-formylTHF, whereas 5,10-methenylTHF, THF, and folic acid were barely quantifiable in serum pool samples that have been collected from non-supplemented subjects.

3.2. Stability of folate compounds in aqueous solutions

Reduced folate forms are known to be sensitive to heat, pH, oxidation, and ultraviolet light [19]. Several preanalytic conditions should be considered to avoid the degradation or interconversion of folates such as adding ascorbic acid, avoiding storage at room temperature and freeze/thaw cycles, as well as exclusion of direct sunlight [20–22]. We were interested in studying the stability of folate coenzymes at 4 °C over 24 h with or without ascorbic acid at different pH. For this purpose, solutions of each folate compound (100 nmol/L) were prepared in water, in 1 g/L aqueous solution of ascorbic acid without further pH adjustment (pH ≈ 3.4), in 1 g/L aqueous solution of ascorbic acid at pH 2.6, and 1 g/L aqueous solution of ascorbic acid at pH 7.0. Folate forms were then measured at start (time 0), 1, 5, and 24 h. Results of the assay are shown in Fig. 2 and Table 2. 5-MethylTHF and folic acid were stable over 24 h at 4 °C under all conditions with no evidence for interconversion to other forms (Table 2). DHF showed degradation at acidic conditions and 12.7–16.7% interconversion to folic acid. 5-FormylTHF, 10-formylTHF, and 5,10-methenylTHF are known to undergo complex interconversion reactions under acidic conditions [22]. Indeed, 5-formylTHF slowly converted under acidic conditions into 5,10-methenylTHF. In addition, small amounts of 10-formylTHF could be detected. Our sample preparation and chromatographic separation procedures depend on acidic conditions. Therefore, we expect that 5-formylTHF in serum samples may undergo interconversion thus causing some of the 5-formylTHF to be detected as 5,10-MethenylTHF. 5,10-methenylTHF was stable at acidic conditions and showed a slight interconversion to 5-formylTHF. 10-FormylTHF and 5,10-methyleneTHF were unstable under acidic and neutral conditions and were below the detection limit of the assay. Furthermore, 10-formylTHF immediately converted into 5,10-methenylTHF and small amounts of 5-formylTHF. Moreover, 5,10-methyleneTHF completely converted to THF and formaldehyde, with a slight interconversion to 5,10-methenylTHF. This unusual nonenzymatic oxidation reaction could not be prevented by the addition of the antioxidant ascorbic acid or flushing the H_2O with N_2 prior experiments. Impurities of the standards used could be an explanation. THF showed complete degradation within 24 h of incubation at 4 °C in H_2O without antioxidant. By addition of ascorbic acid, this process could be strongly decelerated (Fig. 2). Apart from degradation, THF showed minor interconversions into folic acid and DHF. Interestingly, under pH 7.0 conditions small amounts of 5,10-methyleneTHF could be detected. This seems unusual due to the lack of formaldehyde as one-carbon donor. Under the acidic conditions of our method, we were able to quantify 5-methylTHF, 5-formylTHF, 5,10-methenylTHF, THF, and folic acid. Due to interconversions of the folate forms, the measured THF concentrations are the sum of concentrations of THF and 5,10-methyleneTHF, whereas 5,10-methenylTHF concentrations represent the sum of 5,10-methenylTHF and 10-formylTHF.

3.3. Linearity and sensitivity

The calibration curves were linear over the ranges of 8–100 nmol/L for 5-methylTHF and 0.8–10 nmol/L for 5-formylTHF, 5,10-methenylTHF, and folic acid. Folate concentrations correlated strongly to peak areas. The coefficients of linear regression were: $r^2 > 0.999$ ($y = 0.096x - 0.0015$) for 5-methylTHF, $r^2 > 0.999$ ($y = 0.0025x - 0.0041$) for 5-formylTHF, $r^2 > 0.999$ ($y = 0.0011x - 0.0001$) for 5,10-methenylTHF, $r^2 > 0.999$ ($y = 0.0034x$) for folic acid, and $r^2 > 0.995$ ($y = 0.001x + 0.0002$) for THF.

The limit of detection (LOD) and limit of quantification (LOQ) of the method are defined as signal-to-noise ratio ≥5 and 10, respectively. The LODs and LOQs for the folate compounds were

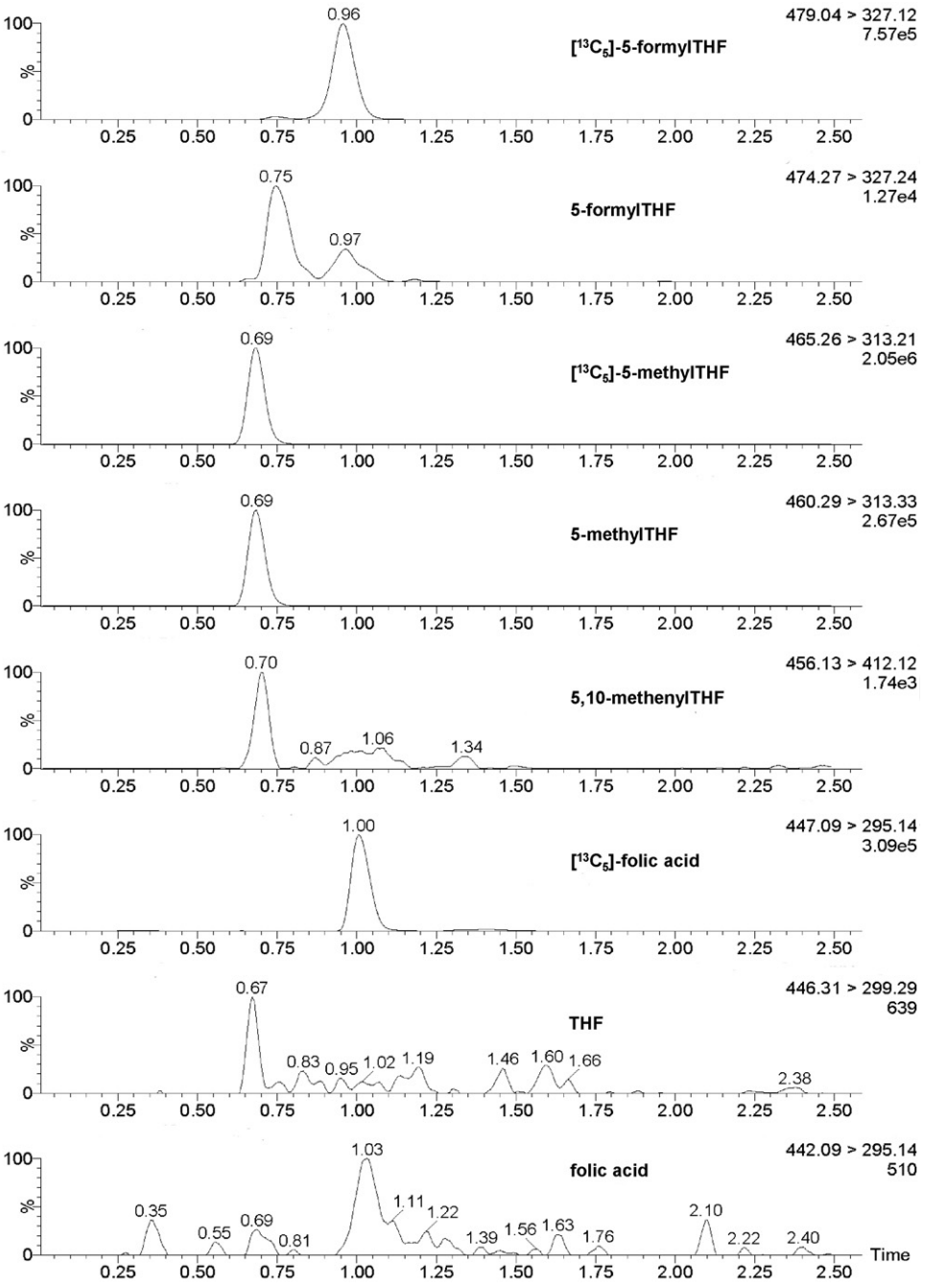

Fig. 1. MRM-traces of the target compounds in a serum sample obtained by UPLC–MS/MS. *m/z* transitions and peak intensities are shown in the upper right.

estimated by verifying the peak height of the analyte in comparison to the height of the noise in the calibrators. LODs were 0.09 nmol/L for 5-formylTHF, 0.10 nmol/L for 5-methylTHF, 0.15 nmol/L for 5,10-methenylTHF, 0.20 nmol/L for folic acid, and 0.90 nmol/L for THF. Corresponding, LOQs were 0.17 nmol/L for 5-formylTHF, 0.19 nmol/L for 5-methylTHF, 0.31 nmol/L for 5,10-methenylTHF, 0.40 nmol/L for folic acid, and 1.79 nmol/L for THF.

3.4. Precision and recovery

The precision of the method was assessed by quantifying folate in an in-house prepared serum pool. Intraassay and interassay coefficients of variations (CVs) are shown in Table 3. The concentrations of folic acid, THF, and 5,10-methenylTHF in serum pool were below the LOQs, but 5-formylTHF concentrations were slightly higher than LOQ. This caused higher CVs in serum samples for these compounds, compared to that for 5-methylTHF (intraassay CV = 2.0%). Interassay CV was assessed by the quantification of folate forms in quality control samples and serum pool samples over a period of 10 days. 5-MethylTHF had an interassay CV of 2.8% in serum samples.

Recovery was performed by spiking serum samples with two different levels over a period of three days (Table 4). Mean recoveries were 82.3% (THF), 98.2% (folic acid and 5-methylTHF), 102.3% (5-formylTHF), and 110.8% (5,10-methenylTHF).

3.5. Method comparison

Concentrations of TFOL were measured by the newly developed UPLC–MS/MS method and the routinely used immunological assay (ADVIA Centaur System) in 70 serum samples. UPLC–MS/MS

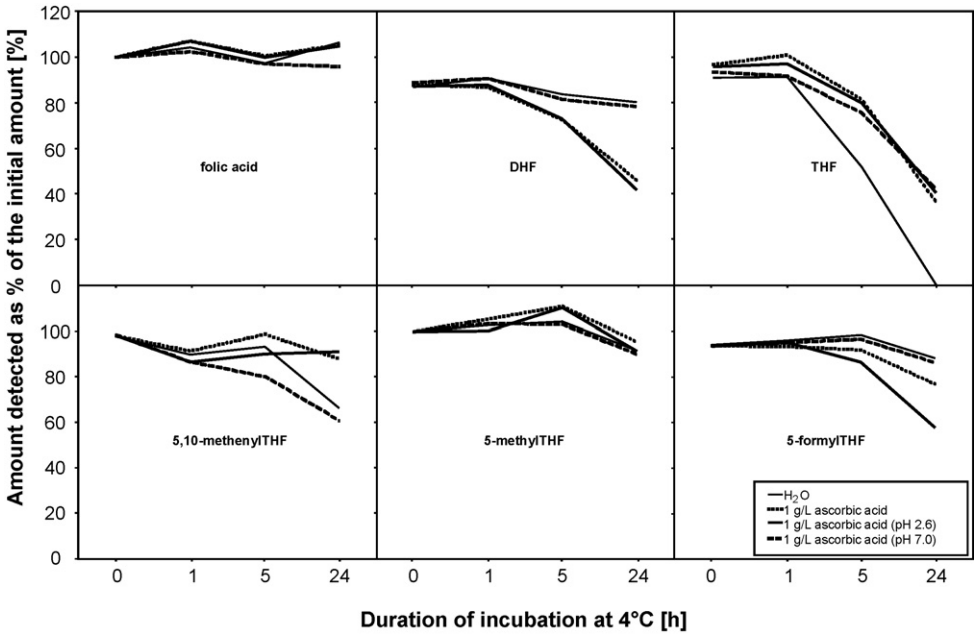

Fig. 2. Amount of folate compounds as percentage of the initial concentration (100 nmol/L) in H_2O, ascorbic acid solution (1 g/L), ascorbic acid solution pH 2.6, and ascorbic acid solution pH 7.0. Samples were incubated at 4 °C for 24 h and measured immediately (time 0), after 1, 5, and 24 h. 10-FormylTHF and 5,10-methyleneTHF were unstable under the selected conditions and were therefore not possible to detect.

Table 2
Interconversions of folate compounds after 24 h of incubation at 4 °C.

Compound	Main interconversion products	H_2O			
		Antioxidant pH	Ascorbic acid 3.4	Ascorbic acid 2.6	Ascorbic acid 7.0
Folic acid	None	–	–	–	–
5-MethylTHF	None	–	–	–	–
DHF	Folic acid	15.0	16.7	16.2	12.7
5,10-MethenylTHF	5-FormylTHF	4.8	4.7	4.3	3.4
5-FormylTHF	5,10-MethenylTHF	1.2	24.1	51.2	1.4
	10-FormylTHF	4.1	3.6	2.9	4.7
10-FormylTHF[a]	5,10-MethenylTHF	75.2	102.0	104.1	70.6
	5-FormylTHF	3.6	3.6	3.3	2.7
5,10-MethyleneTHF[b]	THF	0.1	34.2	28.1	45.9
	5,10-MethenylTHF	2.0	1.3	2.2	1.6
THF	DHF	4.4	1.2	1.5	4.2
	Folic acid	2.8	0.9	1.0	1.1
	5,10-MethyleneTHF	0.1	0.2	0.3	2.7

Concentration of ascorbic acid was 1 g/L.
Results are percentages of the initial concentration at time 0.
Interconversions below limit of detection were not included in this table.
[a] Immediate interconversion to 5,10-methenylTHF at pH ≤7.0.
[b] Immediate interconversion to THF and formaldehyde at pH ≤7.0.

Table 3
Precision of folate form assay.

Compound	High control		Low control		Serum pool	
	Mean (SD) [nmol/L]	CV [%]	Mean (SD) [nmol/L]	CV [%]	Mean (SD) [nmol/L]	CV [%]
Precision within day (n = 10)						
5-MethylTHF	76.88 (1.64)	2.1	24.87 (0.84)	3.4	12.59 (0.25)	2.0
5-FormylTHF	7.76 (0.25)	2.9	2.50 (0.10)	4.1	0.17 (0.01)	7.2
5,10-MethenylTHF[a]	7.80 (0.35)	4.5	2.39 (0.12)	5.0	*	*
THF[b]	8.09 (0.97)	12.0	2.63 (0.44)	16.7	*	*
Folic acid	7.76 (0.25)	3.2	2.41 (0.09)	3.8	*	*
Precision day to day (n = 10)						
5-MethylTHF	82.32 (2.18)	2.6	25.75 (0.56)	2.2	13.53 (0.37)	2.8
5-FormylTHF	7.84 (0.34)	4.4	2.56 (0.09)	3.6	*	*
5,10-MethenylTHF[a]	7.76 (0.55)	7.1	2.61 (0.27)	10.4	*	*
THF[b]	9.33 (1.87)	20.1	2.41 (0.37)	15.6	*	*
Folic acid	7.59 (0.46)	6.1	2.58 (0.12)	4.7	*	*

*Between limit of detection and quantification.
[a] Sum of 5,10-methenylTHF and 10-formylTHF.
[b] Sum of THF and 5,10-methyleneTHF.

Table 4
Limit of detection (LOD) and recovery of folate forms in spiked serum samples ($n = 3$).

Compound	LOD [nmol/L]	Spike high		Spike low		Mean recovery [%]
		Added [nmol/L]	Measured [nmol/L]	Added [nmol/L]	Measured [nmol/L]	
5-MethylTHF	0.10	47.32	46.93	12.03	11.71	98.2
5-FormylTHF	0.09	4.20	4.15	0.64	0.67	102.3
5,10-MethenylTHF[a]	0.15	3.98	4.46	0.49	0.54	110.8
THF[b]	0.90	5.28	3.88	0.80	0.73	82.3
Folic acid	0.20	3.67	3.97	0.56	0.54	98.2

[a] Sum of 5,10-methenylTHF and 10-formylTHF.
[b] Sum of THF and 5,10-methyleneTHF.

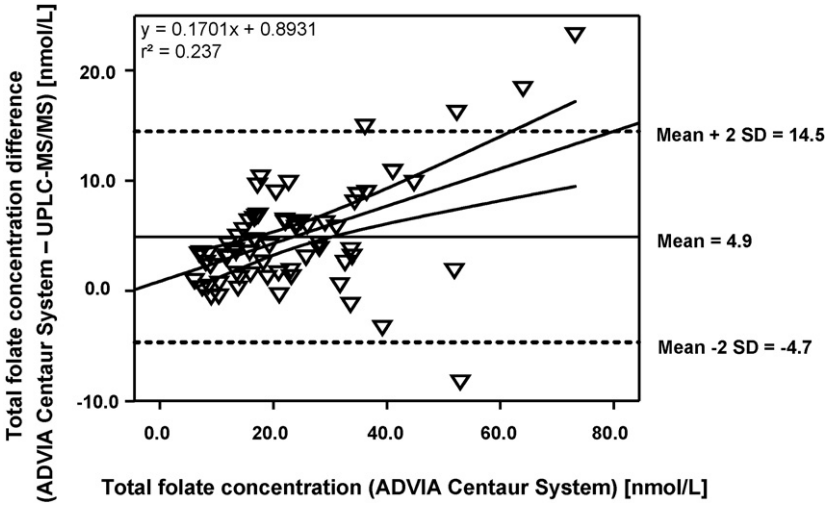

Fig. 3. Bland–Altman difference plot between of TFOL concentration obtained by immunological assay (ADVIA Centaur System) and UPLC–MS/MS of 70 serum samples. The *horizontal solid line* represents the mean difference between the two methods. The immunological assay is used as the comparison method. *Dashed lines* represent the 95% limits of agreement of the differences between the two methods (mean difference ±2 SD).

TFOL was the sum of the folate forms. Concentrations of TFOL measured by UPLC–MS/MS correlated strongly with that measured by immunological method ($r = 0.939$; $p < 0.001$). The mean (SD) TFOL concentration in serum samples was 18.5 (12.1) nmol/L for the UPLC–MS/MS method and 23.4 (13.7) nmol/L for the ADVIA Centaur platform.

TFOL levels measured by the newly developed method were generally lower than those measured by ADVIA Centaur. The Bland–Altman difference plot showed that the difference between the two methods positively correlated to TFOL measured by the immunoassay (Fig. 3). At folate concentrations below 35 nmol/L, the two methods showed a mean (SD) difference of 4.9 (4.8) nmol/L.

3.6. Folate concentrations in healthy individuals

Using the new method, concentrations of folate forms were measured in serum samples from 32 apparently healthy non-vitamin users. Median (10th–90th percentile) 5-formylTHF (0.16 (0.06–0.35) nmol/L), 5,10-methenylTHF (0.03 (0.00–0.12) nmol/L), and folic acid (0.10 (0.03–0.38) nmol/L) concentrations were above the LOD but could not reach the LOQ. Folate form distributions according to *MTHFR C677T* genotypes were summarized in Table 5. 5-MethylTHF constituted 87.23% of the concentrations of TFOL measured, THF 11.44%, 5-formylTHF 0.88%, folic acid 0.55%, and 5,10-methenylTHF 0.17%. Concentrations of TFOL measured by UPLC–MS/MS were significantly different between carriers and non-carriers of the mutated *MTHFR 677* T allele ($p = 0.036$), but concentrations of TFOL measured by Centaur did not differ significantly ($p = 0.062$). Compared to CC genotypes, carriers of the T allele showed lower concentrations of 5-methylTHF ($p = 0.043$), and THF ($p = 0.004$). Moreover, 5-methylTHF showed a strong correlation ($r = 0.729$; $p < 0.001$) with its demethylated product, THF, but not to any of the other folate forms. A negative correlation was detected between concentrations of 5-methylTHF and that

Table 5
Median (10–90)th percentiles in 32 serum samples of non-supplemented subjects, according to their *MTHFR C677T* genotype.

	All	[%] of TFOL	*MTHFR C677T* genotype				p (CC vs. CT + TT)*
			(CC) ($n = 20$)	[%] of TFOL	(CT + TT) ($n = 12$)	[%] of TFOL	
Age [years]	33 (20–51)		40 (20–51)		32 (19–54)		
tHcy [μmol/L]	10.3 (7.3–19.0)		10.3 (7.1–16.9)		10.0 (7.3–37.5)		0.967
TFOL (immunological assay) [nmol/L]	23.14 (9.95–36.33)		28.59 (9.08–40.57)		17.50 (9.29–35.03)		0.062
TFOL (UPLC–MS/MS) [nmol/L]	18.09 (7.37–30.39)		22.23 (8.00–30.55)		12.19 (5.97–29.90)		0.036
5-MethylTHF [nmol/L]	15.78 (5.62–26.68)	87.23	18.48 (6.63–27.16)	83.13	10.72 (4.73–26.25)	87.94	0.043
THF [nmol/L][a]	2.07 (0.45–4.05)	11.44	2.77 (1.18–4.43)	12.60	1.09 (0.20–3.50)	8.94	0.004

MTHFR: 5,10-methylenetetrahydrofolate reductase; tHcy: total homocysteine; TFOL: total folate; THF: tetrahydrofolic acid.
[a] Sum of THF and 5,10-methyleneTHF.
* Mann-Whitney-U-test.

of tHcy ($r = -0.481$; $p = 0.006$). Weaker correlations were noticed between concentrations of TFOL and that of tHcy. Concentrations of TFOL obtained by the ADVIA Centaur platform showed a weaker correlation to tHcy ($r = -0.372$; $p = 0.039$) than those obtained by UPLC–MS/MS ($r = -0.431$; $p = 0.015$).

4. Discussion

Little is known about the distribution of folate forms in serum or plasma. For instance the percentage of non-methyl folate in red blood cells has been shown to differ between the *MTHFR C677T* genotypes (0% for CC vs. 7–51% for TT) [18,23]. Reliable methods for quantitative estimation of folate subfractions are necessary, for example in subjects receiving folate supplements to test the fraction of unmetabolized folate.

We present a sensitive specific and high-throughput method for the simultaneous measurement of 5-methylTHF, 5-formylTHF, 5,10-methenylTHF, THF, and folic acid in serum using stable-isotope dilution UPLC-MS/MS. Because reduced folate is not stable, 10-formylTHF was quantified as 5,10-methenylTHF and 5,10-methyleneTHF was quantified as THF. DHF was partly included in the folic acid fraction. The short time required for sample preparation (40 samples in 120 min) and measurement (2.5 min/sample) enables folate measurements in large scale clinical studies. The high sensitivity (LOQs between 0.17 and 1.79 nmol/L) of our assay enables the accurate measurement of serum folate in the low or deficient ranges. Furthermore, the linearity of the method over a broad range enables also measuring sera from supplemented people without diluting the samples. High CVs for non-methyl folates can be explained by the low serum concentrations of those compounds.

Levels of TFOL obtained by the new UPLC–MS/MS method showed a strong correlation to those obtained by using the immunological assay, but they were lower especially in the upper range of serum folate (>35 nmol/L). We had preliminary results suggesting that the higher the THF fraction was, the bigger the difference between the two methods, suggesting that the immunological assay might not equally detect 5-methylTHF and other forms of folate. Another explanation could be related to the fact that the UPLC–MS/MS method uses the same analytes for preparing the calibration curve, whereas the immunological assay uses 5-methylTHF for quantifying TFOL.

In accordance with previous studies, we confirmed that 5-methylTHF constitute approximately 82-93% of TFOL in serum [7]. We detected however, less folic acid compared to a study by Pfeiffer et al. [7]. Nevertheless, this might be explained by differences in the population tested since the study by Pfeiffer et al. was conducted on US population after folate fortification [7].

The enzyme MTHFR converts 5,10-methyleneTHF to 5-methylTHF and thus makes this available for homocysteine remethylation. The common *MTHFR* C677T polymorphism results in an enzyme that is thermolabile and has less activity [24]. Accordingly, higher folate can stabilize the enzyme and retain its activity. [24]. Additionally, the TT variant has been reported to cause accumulation of formylated THF [23]. Our results obtained from 32 healthy non-supplemented people confirm that CC subjects have higher TFOL than the group of T allele carriers. Interestingly, CT and TT subjects who had less 5-methylTHF available for tHcy methylation, had also less THF. Furthermore, the 5-methylTHF available for tHcy remethylation seemed to be sufficient thus C and T allele carriers had comparable concentrations of plasma tHcy. The homocysteine increasing effect of the C to T mutation of the *MTHFR* gene is preferably seen in subjects having serum folate in the low normal to decreased concentration range. We recall however, that none of our tested subjects had folate concentrations in that range.

Few methods for the quantification of folate and folic acid have been described [7,13,14]. A low sensitivity or selectivity of those methods and the inability to detect some folate forms has limited their use in clinical studies. Our method using UPLC–MS/MS provided high efficiency separation and enables us to quantify main folate forms.

Our results regarding concentrations of TFOL and 5-methylTHF are in line with similar reports [13,25]. Wang et al. reported mean serum concentrations of TFOL of 20.32 nmol/L which comprises of 14.58 nmol/L 5-methylTHF, 4.35 nmol/L 5-formylTHF, and 1.39 nmol/L folic acid [13]. However, Wang et al. detected higher levels of 5-formylTHF and folic acid, but no THF and 5,10-methenylTHF. Another method for the simultaneous quantification of folate forms and their catabolites in serum [25] reported a median serum TFOL of 19.8 nmol/L which comprises of 16.4 nmol/L 5-methylTHF, 2.3 nmol/L 4-α-hydroxy-5-methylTHF, the oxidized form of 5-methylTHF, 0.0 nmol/L folic acid and no detectable 5-formylTHF. The measured TFOL, 5-methylTHF, and folic acid concentrations are similar to our results. Pfeiffer et al. found a mean TFOL concentration in serum of 35.5 nmol/L [7]. Samples with TFOL < 50 nmol/L contained 93.3% 5-methylTHF, 4.4% 5-formylTHF, and 2.3% folic acid. In comparison, in our study we found 87.2% 5-methylTHF, 1.1% 5-formylTHF, and 0.6% folic acid. However, we additionally found 11.4% THF and 0.2% 5,10-methenylTHF in serum. Differences between the studies might be related to the population tested and the use of vitamin supplements or fortified foods. Our results seemed however to reflect the expected biological relevance (correlation to tHcy and MTHFR effect) better than the widely used immunological assays.

In conclusion, we developed a fast, precise, and accurate method for quantification of 5-methylTHF, 5-formylTHF, 5,10-methenylTHF, THF, and folic acid in serum, which can be used in large-scale clinical studies. Compared to earlier LC–MS/MS procedures, the UPLC–MS/MS method demonstrates better sensitivity and selectivity for the quantification of folate.

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