Quantitative determination of erythrocyte folate vitamer distribution by liquid chromatography-tandem mass spectrometry.

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Abstract

Background
Given the role of folate in many disorders, intracellular distribution of folate vitamers is of potential clinical importance. In particular, accumulation of non-methyltetrahydrofolates due to altered partitioning of folate metabolism at the level of methylenetetrahydrofolate is of interest.

Methods
We describe a positive-electrospray liquid chromatography tandem mass spectrometry (LC-MS/MS) method that allows determination of erythrocyte folate vitamer distribution by accurately measuring both 5-methyltetrahydrofolate (5-methylTHF) and non-methyl folate vitamers. Whole blood lysates are deconjugated in ascorbic acid solutions, deproteinized, purified using folate-binding protein affinity columns, concentrated by solid-phase extraction (SPE) and evaporation, and separated on a C18 column within 6 min.

Results
The limit of quantification for both 5-methylTHF and non-methylTHF was 0.4 nmol/L (signal-to-noise >10). Intra- and inter-assay CVs for 5-methylTHF were 1.2% and 2.8%, respectively. Intra- and inter-assay CVs for non-methylTHF as a group were 1.6% and 1.5%, respectively. Recovery results were 97–107%. We measured 8–72% non-methyl folate vitamers in volunteers (n=5) with the methylenetetrahydrofolate reductase (MTHFR) 677 TT genotype. Concentrations ranged from 117 to 327 nmol/L and 23 to 363 nmol/L for 5-methylTHF and non-methylTHF vitamers, respectively. We measured 0–2% non-methylTHF vitamers in MTHFR 677 CC genotype volunteers. In addition, we found that storage of whole-blood samples in ascorbic acid at low pH resulted in 53–90% loss of the non-methylTHF fraction.

Conclusion
This LC-MS/MS method accurately determines erythrocyte 5-methylTHF and non-methyl folate vitamers.
Introduction

Disorders of folate metabolism have been implicated in the pathogenesis of a wide variety of diseases, including neural tube defects (1), anemia (2), cardiovascular disease (3), cognitive dysfunction (4), and cancer (5). Folate is the collective name for related molecules differing in the oxidation state of the pteridine ring, the one-carbon substitution at the N5 and N10 positions, and the number of conjugated glutamic acid moieties. Regulation of intracellular folate

Figure 1. Overview of the metabolic pathway of folates in humans. Folate has many more intracellular functions than just controlling homocysteine levels. Firstly, folate (in the form of 5-methylTHF) is the main provider of methyl groups for the regeneration of S-adenosylmethionine (SAM), the universal methyl donor. The transmethylation reactions nourished by S-adenosylmethionine are numerous, and include methylation of DNA, lipids, proteins, and neurotransmitters. After demethylation of 5-methylTHF, the only route open to metabolic recycling of this 5-methylTHF is the intracellular synthesis of 5,10-methyleneTHF from THF and the subsequent conversion to 5-methylTHF by the irreversible enzymatic reaction catalyzed by 5,10-methylenetetrahydrofolate reductase (MTHFR, EC 1.5.1.20). Alternatively, folate can donate a one-carbon group for the purpose of DNA and RNA synthesis. For example, 5,10-methyleneTHF supplies the pyrimidine synthesis pathway, and 10-formylTHF, the oxidation product of 5,10-methylenetetrahydrofolate, provides one-carbon units for purine synthesis (1, methionine synthase; 2, methylene tetrahydrofolate reductase; 3, serine hydroxymethyl transferase; 4, dihydrofolate reductase; 5, thymidylate synthase).
metabolism is complex (Figure 1) (6). Of particular interest is the point at which folate metabolism partitions to serve either the methylation cycle (by formation of 5-methyltetrahydrofolate, 5-methylTHF), which involves homocysteine as a crucial compound, or the DNA/RNA biosynthesis cycle (by channelling metabolism towards the formation of formylated THF).

Erythrocyte total folate is regarded as the best reflection of whole-body folate status. Given the role of folates in many diseases, the issue of which specific folate vitamers accumulate intracellularly is potentially of great additional importance. Recent studies have suggested that 5-methylTHF is the predominant intracellular folate vitamer in circulating erythrocytes. An important exception appears to be subjects homozygous for the C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene (substitution of an alanine residue by valine) (7). This mutation (prevalence of the homozygous variant is approximately 12–15% in Caucasian populations) renders the enzyme thermolabile and causes up to 50% loss of activity, thus impairing the reduction of 5,10-methyleneTHF to 5-methylTHF (8). Subjects with the 677 TT genotype, as compared to those with the CC genotype, have marked, but highly variable (7–51%) fractions of non-methylTHF stored in erythrocytes (9).

Recent progress in our understanding of the importance of folate in many diseases has sparked renewed interest in analytical methods. Today, a number of methods are available to measure folates in a variety of matrices. Each method has its own merits and pitfalls. Radioassays are fast and are widely used for the determination of plasma folate. However, they only provide total folate concentrations and can be very sensitive to assay conditions (10). Microbiological assays are relatively easy for the determination of total folate. However, when information on individual vitamers is required, complex multiple assays have to be performed (11). Coupling this technique to liquid chromatography (LC) provides a simple solution. However, precision is not optimal, since it lacks reliable internal standards (12). LC coupled to a fluorescence detector has also been used to determine various folate vitamers. However, for patients using folic acid, this technique would require an additional UV detector, since folic acid does not have natural fluorescence (13). The sensitivity of these LC systems can be boosted by replacing the fluorescence detector by a multi-channel electrochemical detector. In this way, information can be obtained on all separate folate vitamers, including their polyglutamate chain length, but for this technique reliable internal standards are also not available (14). Gas chromatography/mass spectrometry (GC/MS) methods use complex acid treatments to cleave the different vitamers to para-aminobenzoylglutamate.
Folate vitamers in erythrocytes by LC-MS/MS

(15). LC tandem MS (LC-MS/MS) methods offer the most reliable internal standards and the best specificity. Since sensitivity can be greatly influenced by matrix components, they usually require some type of sample clean-up (16).

Theoretically, very detailed information on cellular folate vitamer distribution could be obtained with some of the aforementioned methods. However, as several of the non-methylTHF are highly labile and interconvert during assays, the output usually generated is the sum of these interconverted non-methyl folates, previously referred to as “formylated folates” (9). In addition, although the polyglutamate chain length is of some biochemical relevance (17;18), maintaining polyglutamates results in complex method outputs and provides no additional information if the primary interest is in whether 5-methylTHF or non-methyl folates are present in cells.

We developed an LC-MS/MS method based on a highly selective clean-up using affinity chromatography. Although it is clearly more time-consuming than the partly automated solid-phase extraction (SPE) method published earlier (16), the sensitivity, precision, and accuracy may benefit from the affinity chromatography step. Like the previously published method, our method includes full deconjugation of the polyglutamate groups using naturally occurring plasma folate conjugase (g-Glu-X carboxypeptidase; EC 3.4.19.9). Although our method can detect separate non-methyl folate vitamers, the output generated also consists of 5-methylTHF (which is stable during the assay) and the sum of non-methyl folates (THF, 5,10-methyleneTHF, 5,10-methenylTHF, and 5- and 10-formylTHF), most of which (except 5-formylTHF, and to some extent THF) convert to 5,10-methenylTHF during the assay. In addition, the method allows for separate detection of unsubstituted (partly) oxidized folates (folic acid and dihydrofolate, DHF), which may be particularly relevant for people who use high-dose folic acid supplements (19). Data interpretation for this method is relatively straightforward, and because the detection limits are low, even minor amounts of non-methyl folates can be detected in relatively small sample volumes.

Materials and methods

Materials
Folic acid (purity 98%), DHF (purity 90%), THF (purity 70%) and 5-formylTHF (purity 90%) were obtained from Sigma (Deiselhofen, Germany). (6S)-5-MethylTHF (purity 90%), (6S)-5,10-methenylTHF (purity 90%), (6S)-5,10-
methyleneTHF (purity undetermined), $[^{13}\text{C}_5]$-5-formylTHF and $[^{13}\text{C}_5]$-5-methylTHF (isotopic purity >99%) were obtained from Eprova AG (Schaffhausen, Switzerland). 10-FormylTHF was prepared using previously described methods (20). $[^2\text{H}_4]$-Folic acid (isotopic purity >99%) was obtained from the Institute of Food Research (IFR, Norwich, UK). $[^{13}\text{C}_5]$-5,10-MethenylTHF was prepared by incubating $[^{13}\text{C}_5]$-5-formylTHF in 1% formic acid at 4ºC for 24 h, resulting in 99% purity.

**Preparation of calibrants**

Stock solutions were prepared for all individual labeled and unlabeled folates. Since several folate vitamers are reported to be unstable, not all folate vitamers produce reliable calibrants. The Results section describes which folate vitamers were used as calibrants under specific conditions. Concentrations of the stock solutions were determined by spectrophotometric analysis directly after preparation, and after 6 months. The following molar extinction coefficients (mol$^{-1}$ cm$^{-1}$ L) were used: 31,700 for 5-methylTHF ($\lambda=290$ nm); 27,600 for folic acid ($\lambda=282$ nm); 29,100 for THF ($\lambda=297$ nm); 25,000 for 5,10-methenylTHF ($\lambda=352$ nm); and 37,200 for 5-formylTHF ($\lambda=285$ nm) (21). Chemical and isotopic purity was determined by LC-MS/MS directly after preparation, and after 6 months. All water used for the preparation of stock solutions was degassed beforehand. The stock solutions were stored in small aliquots at -80ºC.

Each series was accompanied by a six-point calibration curve in 10 mmol/L formic acid (containing 5-methylTHF, 5,10-methenylTHF, 5-formylTHF, and folic acid, concentrations ranging from 0 to 30 nmol/L) and three quality control samples in duplicate (a standard containing 5-methylTHF, 5,10-methenylTHF, 5-formylTHF, and folic acid; whole blood from a CT genotype volunteer; and whole blood from a TT genotype volunteer). Stabile isotope dilution was used for quantification of the folate vitamers with a combined internal standard solution (containing 870 nmol/L $[^{13}\text{C}_5]$-5-methylTHF, $[^{13}\text{C}_5]$-5-formylTHF, $[^{13}\text{C}_5]$-5-methenylTHF, and $[D_4]$-folic acid). All standard solutions used were prepared fresh from frozen stock solutions.

**Subjects**

The validity of this LC-MS/MS method for determination of the erythrocyte folate distribution was tested by analyzing whole blood samples from 10 healthy volunteers (5 with the MTHFR 677 CC genotype and 5 with the TT genotype). Details on the sex and age of these volunteers are listed in Table 2. Informed consent was obtained from all volunteers. Fasting venous blood
was collected in EDTA Vacutainers (Becton Dickinson, Plymouth, UK) and frozen immediately. All samples were frozen at -80ºC until extraction.

**Sample stability**
Sample stability was estimated before (90 min at room temperature) and after freezing (3 months at -80ºC). Traditionally, samples used for folate vitamer analysis are stored in ascorbic acid solutions. We tested whether storage with or without ascorbic acid influenced the stability of different vitamers. To test the effects of ascorbic acid on the folate vitamer concentration, 100 µL of whole blood was diluted prior to freezing with 900 µL of 56.7 mmol/L (10 g/L) ascorbic acid solution (both with and without pH adjustment to 4 by adding 1 mol/L NaOH) (22).

**Method validation**
The sensitivity of the method was assessed by estimating the limit of quantification (signal-to-noise >10) for the different vitamers. A blood sample from an MTHFR 677 CC genotype individual was spiked directly after thawing (except for 5-methylTHF, in which case estimations were based on diluted standards and average noise of a whole blood sample). The precision of the method was determined by obtaining intra- (n=5) and inter-assay (n=10) variation for an MTHFR 677 TT genotype blood sample. The accuracy of the method was determined by estimating the recovery of the different folate vitamers added to an MTHFR 677 CC genotype blood sample (five different concentrations of 5-methylTHF, 5,10-methenylTHF, 5-formylTHF and folic acid were added). Linearity was assessed by constructing calibration curves of the different folate vitamers.

**Sample preparation**
The sample preparation consisted of five steps.

**Hemolysis of red blood cells** EDTA blood was thawed, and to 100 µL of whole blood, 20 µL of an internal standard solution (containing 870 nmol/L each of [13C5]-5-methylTHF, [13C5]-5-formylTHF, [13C5]-5-methenylTHF, and [D₄]-folic acid), and 900 µL of 56.7 mmol/L (10 g/L) ascorbic acid solution (adjusted to pH 4 with 1 mol/L NaOH) were added (22). The mixture was transferred to 50-mL polypropylene tubes and flushed with nitrogen.

**Deconjugation of folate polyglutamate groups** After hemolysis, the samples were placed in a 37ºC water bath for 90 min to undergo deconjugation by naturally occurring plasma folate conjugase (23).
**Protein denaturation** Subsequently, 10 mL of an extraction buffer (0.1 mol/L K₂HPO₄, pH 7, containing 50 mmol/L ascorbic acid and 10 mmol/L mercaptoethanol as anti-oxidants) was added and the proteins were denatured in a boiling water bath for 20 min. The samples were cooled on ice and subsequently centrifuged (2000Xg, 10 min).

**Extraction of folates by affinity chromatography** Folate affinity columns (bed volume 2 mL) were prepared by binding folate-binding protein (Scripps Laboratories, San Diego, CA, USA) to the hydroxysuccinimide spacers of Affigel 10 (BioRad, Hemel Hempstead, UK). This was done overnight in 0.1 mol/L sodium hydrogen carbonate at 4ºC. They were stored in 0.1 mol/L K₂HPO₄ buffer, pH 7 [containing 0.2% (w/v) sodium azide] at 4ºC. The capacity of these affinity columns was checked by running a standard with each series. Preceding the extraction, the columns were conditioned by rinsing three times with 5 mL of 0.1 mol/L K₂HPO₄ buffer, pH 7. The supernatant fractions were then applied to the columns and the columns were washed with 5 mL of 0.1 mol/L K₂HPO₄, 1 mol/L NaCl buffer, pH 7, 5 mL of 0.05 mol/L K₂HPO₄ buffer, pH 7, and 5 mL of 0.025 mol/L K₂HPO₄ buffer, pH 7. The folates were eluted with 5 mL of 0.1 mol/L HCl and the eluate was collected in glass tubes (containing 5 mg of ascorbic acid). To prevent carryover to future samples, the columns were washed with an additional 5 mL of 0.1 mol/L HCl. After this step, the affinity columns were reconditioned by rinsing three times with 5 mL of 0.1 mol/L K₂HPO₄ buffer, pH 7, and stored at 4ºC in 0.1 mol/L K₂HPO₄, pH 7, containing 0.2% (w/v) sodium azide until further use (24).

**Sample concentration** To concentrate the sample, 3-mL Hydrophilic-Lipophilic Balance (HLB) Oasis cartridges (Waters, Milford, MA, USA) were used. The cartridges were conditioned with 1 mL of methanol and 1 mL of water. Then the 0.1 mol/L HCl fraction was applied to the cartridges. The cartridges were washed with 2 mL of water and eluted with 500 µL of methanol into vials (containing 2 mg ascorbic acid). The methanol fraction was evaporated under nitrogen at 40ºC and redissolved in 500 µL of 10 mmol/L formic acid.

The samples were stored at -20ºC until analysis.

**Liquid chromatography-tandem mass spectrometry** All analyses were performed on an API 3000 triple quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a Perkin-Elmer Series 200 HPLC pump and a Perkin-Elmer Series 200 auto sampler (operated at 4ºC).
Using an Xterra MS C18 analytical column (3.9 X 100 mm; 3.5 µm; Waters) 25 µL of the sample was separated using 10 mmol/L formic acid/acetonitrile (90:10), pH 2.3 as mobile phase at a flow rate of 0.8 mL/min. The column was connected to the turbo ion electrospray source by a splitter with a split ratio of 1:5. The turbo ion electrospray was operated in positive ion mode, the cone temperature was set to 450ºC and the cone voltage was 5000 V. Nitrogen was used as the turbo ion gas at a flow rate of 8 L/min. Collision-induced dissociation was initiated using nitrogen as the collision gas at a pressure of 0.06 kPa. The collision energy was set to 31 V (41 V for 5,10-methenylTHF), the focusing potential to 260 V, and the declustering potential to 46 V. All MS/MS experiments were performed using unit resolution. For each precursor fragment transition, a dwell time of 50 ms was applied. The mass spectrometer was optimized for each folate vitamer using constant infusion of the analytes with a Harvard Apparatus Pump 11 infusion pump (Harvard Apparatus, Inc., Massachusetts, USA). The LC-MS/MS data were acquired and processed using Analyst for Windows NT software (Applied Biosystems).

**MTHFR genotype**
DNA was isolated from EDTA blood using a QIAamp DNA blood kit (Qiagen, Hilden, Germany). The C677T polymorphism of MTHFR was assessed as previously described (7).

**Calculations and statistics**
Folate vitamer concentrations were quantified using calibration curves (stable isotope dilution). The ratio analyte peak area/internal standard peak area was plotted against the concentration. Least-squares linear regression analysis was used to fit a line to the data points. Erythrocyte concentrations of folate vitamers were calculated by dividing whole blood results by the hematocrit value and multiplying by the dilution factor.

**Results**

**Tandem mass spectrometry**
All folate standards exhibited intense protonated molecular ions under positive turbo electrospray conditions. The collision-induced dissociation spectra of these protonated molecular ions of the folate vitamers are given in Figure 2. In general, the compounds showed neutral loss of the glutamate moiety.
Figure 2. Collision-induced product ion spectra of each individual folate vitamer (the spectra are scaled to 100% on the basis of the most abundant product ion). Spectra were generated by positive-electrospray ionization LC-MS/MS. For each vitamer, the precursor–product transition used for this method is listed directly above the appropriate spectrum. At the top, the general structure of folates is shown. The part of the molecule inside the box changes for the different folate vitamers and is depicted on each spectrum.
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(COOHCHNH₂CH₂CH₂COOH) to produce the major product ions [MH–147]+. This was confirmed by the [MH–152]+ product ion produced by the [¹³C₅]-glutamic acid-labeled compounds. However, 10-formylTHF and DHF produced this product ion in lesser abundance, while 5,10-methenylTHF did not produce this ion at all. Theoretically, the complete p-amino-benzoic part of the folate molecule could be important in resonance stabilization of these fragment ions. Substitution at the N₁₀ position in these folate molecules immobilizes and withdraws the free nitrogen electrons from the conjugated p-system, which is of importance for stabilization of the cation. This phenomenon probably resulted in strong or even complete inhibition of loss of glutamate. Although LC-MS seemed to be a better option for these N₁₀ substituted compounds, it drastically diminished the signal-to-noise ratio compared to LC-MS/MS. Thus, by choosing a non-specific fragment (loss of CO₂), enhanced results were obtained for 5,10-methenylTHF. For 10-formylTHF, conversion to 5,10-methenylTHF was more likely to occur than loss of the glutamate moiety. For DHF, cleavage between the pteridine and the aminobenzoic acid group (~266 amu) yielded a more intense fragment than loss of the glutamate moiety. The precursor-product transitions used are shown in Figure 2. In addition, the following transitions were used for the four internal standards: 446.2→299.1 for [D₄]folic acid; 461.2→416.2 for [¹³C₅],10-methenylTHF; 465.2→313.1 for [¹³C₅]5-methylTHF; and 479.2→327.1 for [¹³C₅]-5-formylTHF.

Stability of the folate vitamers in standards
Since it has been reported that not all folate vitamers are stable, we started by determining which stock solutions were sufficiently stable to produce reliable calibrants. Folic acid was stable in both water and a 56.7 mmol/L ascorbic acid solution (pH 4). 5-MethylTHF was most stable in a 56.7 mmol/L ascorbic acid solution (pH 4) (slight degradation if stored in only water). 5-FormylTHF was stable in both water and a 56.7 mmol/L ascorbic acid solution (pH 7) (slight conversion to 5,10-methenylTHF at pH 4). 5,10-MethenylTHF was stable in 25 mmol/L formic acid (pH ~3). These four folate vitamers were sufficiently chemically stable to be used as calibrants: when tested by UV absorption detection and LC-MS/MS after 6 months, no decay or conversion (both <2%) of these four vitamers was observed. For the remaining vitamers (THF, 10-formylTHF, 5,10-methyleneTHF and DHF) we did not find a solution that was stable, even if ascorbic acid was added as anti-oxidant. THF was most stable in 0.1 mol/L Tris/HCl buffer (pH 7) with 0.2% mercaptoethanol. Mercaptoethanol provided better THF stability than ascorbic acid. However, approximately 5% cleavage to pABG was still observed directly after preparation (no further decrease during 2 weeks of storage at -80ºC). Thus,
<table>
<thead>
<tr>
<th>Vitamer group</th>
<th>Folate vitamer</th>
<th>Stability during sample preparation</th>
<th>LOQ nmol/L (fmol) (^d)</th>
<th>Intra-assay variation (n=5)</th>
<th>Inter-assay variation (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean nmol/L</td>
<td>CV %</td>
<td>Mean nmol/L</td>
</tr>
<tr>
<td>Unsubstituted</td>
<td>Folic acid</td>
<td>Stable</td>
<td>2.0 (5.0)</td>
<td>5.8</td>
<td>3.2</td>
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<tr>
<td></td>
<td>DHF</td>
<td>Conversion to folic acid (^a)</td>
<td>4.0 (10.0)</td>
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<td>-</td>
</tr>
<tr>
<td>Non-methylTHF</td>
<td>5,10-MethenylTHF</td>
<td>Stable</td>
<td>0.4 (1.0)</td>
<td>232.7</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>5-FormylTHF</td>
<td>Stable</td>
<td>4.0 (10.0)</td>
<td>110.8</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>10-FormylTHF</td>
<td>Conversion to 5,10-methenylTHF</td>
<td>20.0 (50.0)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>5,10-MethyleneTHF</td>
<td>Conversion via THF to 5,10-methenylTHF (^b)</td>
<td>4.0 (10.0)</td>
<td>-</td>
<td>-</td>
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<tr>
<td></td>
<td>THF</td>
<td>Partly converts to 5,10-methenylTHF (^c)</td>
<td>0.4 (1.0)</td>
<td>-</td>
<td>-</td>
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<tr>
<td>5-methylTHF</td>
<td>5-MethylTHF</td>
<td>Stable</td>
<td>0.4 (1.0)</td>
<td>160.3</td>
<td>1.2</td>
</tr>
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</table>

LOQ, limit of quantification. \(^a\) 50% degradation to pABG occurs, \(^b\) 15% degradation to pABG occurs \(^c\) When THF was stabilized using mercaptoethanol, approximately 70% was recovered as THF, and 10% as 5,10-methenylTHF, \(^d\) amount injected on-column, \(^e\) estimated since standards are unstable, and therefore not used as calibrants, \(^f\) for non-methylTHF as a group: mean 343.5 nmol/L (CV 1.6%), \(^g\) for non-methylTHF as a group: mean 344.3 nmol/L (CV 1.5%). Although 5-formylTHF has a higher quantification limit than 5,10-methenylTHF, 5-formylTHF only appears in samples with a high 5,10-methenylTHF concentration. Thus, the effective quantification limit of non-methylfolates as a group can still be regarded to be 0.4 nmol/l.
this standard has to be checked regularly if it is to be used as a calibrant. 10-FormylTHF was stable enough in 0.1 mol/L Tris/HCl buffer. However, when acidified (as is necessary for protonation in the MS), it converted to 5,10-methenylTHF. The optimal solution for 5,10-methyleneTHF was 0.5 mol/L NaOH. However, when acidified, it converted via THF to 5,10-methenylTHF (with some cleavage to pABG). DHF was not stable in any of the solutions investigated, even if ascorbic acid or mercaptoethanol was added (50% cleavage to pABG and 50% oxidation to folic acid occurred). Stability of the folate vitamers during sample cleanup Table 1 summarizes the stability and conversion data during sample preparation. Since all one-carbon-substituted non-methyl folates (except 5-formyl-THF) and THF vitamers largely converted to 5,10-methenylTHF under the acidic conditions of our method, we simplified the output to a pooled result for non-methyl folate vitamers as a group, as reported by others (9,16). Thus, we divided the vitamers into three groups: 5-methylTHF; non-methylTHF (5,10-methyleneTHF, 5,10-methenylTHF, 5-formylTHF, 10-formylTHF and THF); and unsubstituted oxidized folates (DHF and folic acid). We checked for crossover between these different groups with isotopically labeled standards, and found none. Since mercaptoethanol contributed to a large extent to the stability of THF in standards in particular, we also investigated whether it would be beneficial to add it during the sample clean-up procedure. We added mercaptoethanol during deconjugation, during the extraction with HCl, and during storage of the LC-MS/MS sample. For spiked MTHFR 677 CT samples, mercaptoethanol added during deconjugation, but not during extraction or subsequent storage, had beneficial effects (50% increase) on THF recovery. The only other vitamer affected was 5,10-methenylTHF, which decreased in proportion to the increase in THF, reflecting less conversion of THF to 5,10-methenylTHF. In the ten whole blood samples measured, one of which contained a minor amount of THF when measured without addition of mercaptoethanol ethanol, adding mercaptoethanol during deconjugation did not change the concentrations of any of the folate vitamers (see below).

**Liquid chromatography**

All folates could be measured individually within 6 min. All peaks were separated from the void volume (0.8 min). The second isotope peak of folic acid did not interfere with the [M+2] peak of dihydrofolate. A typical chromatogram of a whole blood sample of a TT genotype volunteer is shown in Figure 3. As is evident from this Figure, the peak shape of 5-formylTHF is relatively wide. This suggests interaction with the C18 column, most probably with free silanol groups. However, the addition of amine groups to the mobile
phase or the use of gradients did not narrow the peak. Possibly, the peak shape was caused by rapid interconversion to 10-formylTHF during elution, which elutes prior to 5-formylTHF. Adding more organic modifier to the mobile phase and thus decreasing the time spent on the column improved the shape of this peak. However, this resulted in elution of other folate vitamers in the void volume. Other HPLC columns showed a somewhat better peak shape for 5-formylTHF, but proved unsuitable for other vitamers. Since 5,10-methenylTHF was the predominant non-methylTHF vitamer measured in our method, we chose this column because it offered the best peak shape for both 5-methylTHF and 5,10-methenylTHF, and also allowed for sufficiently accurate quantification of both 5-formylTHF and folic acid, in combination with a short analysis time. THF eluted near the void volume, which may preclude detection of minor amounts of THF (even though ion suppression experiments showed no suppression of the signal). However, since most of the THF is converted into 5,10-methenylTHF during the procedure, little THF is expected to be present.

**Deconjugation of the polyglutamate groups**
As previously reported (23), incubation at 37ºC for 90 min was sufficient to convert all polyglutamate forms of 5-methylTHF to the monoglutamate form of 5-methylTHF. We tested if this was also the case for other folate vitamers and observed no further increase in the measured monoglutamate form of the folate vitamers after prolongation of the incubation time from the proposed 90 min to 120 and 150 min. Recently, it was suggested that diglutamate forms could be detected in lysates incubated for only 90 min (25). However, we found none in our samples (the transitions used were m/z 589.2→313.2 for 5-methylTHF diglutamate and m/z 585.2→412.2 for 5,10-methenylTHF diglutamate).

**Stability of the folate vitamers in whole blood**
Whole blood samples from a TT genotype volunteer were tested for stability of the folate vitamers before (up to 90 min at room temperature) and during freezing (up to 3 months at -80ºC). Both experiments resulted in a minimal (<2%) decrease in the folate vitamers. However, storage in an ascorbic acid solution (even for 1 day) resulted in a decrease in the non-methylTHF concentration of 53–89% (n=5) at pH 4.0 (lysate pH 4.7) and 77–90% (n=5) at pH 2.8 (lysate pH 4.0). There was no difference in concentration between samples in ascorbic acid solution frozen for 1 day and 3 months. Freeze-thaw cycles had a minimal effect (<3%) on the folate vitamer concentrations.
Sensitivity, precision, and accuracy
The sensitivity results for the folate vitamers are shown in Table 1 (signal/noise >10). The precision results for a TT genotype blood sample are also listed in Table 1. The intra- (n=5) and inter-assay (n=10) CVs for all folate vitamers were <3.2% and <7.1%, respectively. Intra- and inter-assay CVs for non-methylTHF as a group were 1.6% and 1.5%, respectively. The average recovery (five different concentrations) in relation to the internal standard was 99.1±2.1% for 5-methylTHF, 100.7±4.1% for folic acid, and 96.8±1.8% for the total concentration of the non-methyl folates. We also tested the absolute recovery by comparing a standard that was run through the entire sample clean-up procedure to the standard before clean-up. The results were 104.8±2.0% for 5-methylTHF and 106.7±2.1% for the total concentration of the non-methyl folates.

Linearity
The calibration curves for 5-methylTHF, 5-formylTHF, 5,10-methenylTHF and folic acid were linear over a concentration range of 0.4–320 nmol/L. Intercept values for calibration curves of inter-assay analyses (over a period of 3 months) were 0.012±0.005, 0.017±0.008, 0.034±0.026, and 0.043±0.007 (n=9), respectively. Slope values for the calibration curves of the inter-assay analyses were 0.063±0.001, 0.0864±0.003, 0.0903±0.004, and 0.079±0.001 (n=9), respectively. Correlation coefficients for inter-assay analyses were 0.9997±0.0003, 0.9986±0.0021, 0.9994±0.0007 and 0.9988±0.0003 (n=9), respectively.

Concentration in MTHFR 677 TT and CC genotype volunteers
Results for the MTHFR 677 TT and CC genotype volunteers are listed in Table 2. All samples from the MTHFR 677 TT genotype volunteers contained measurable amounts of 5-formylTHF, 5,10-methenylTHF and 5-methylTHF. Only one MTHFR 677 TT sample contained minor amounts of THF (approx. 10 nmol/L). All samples were analyzed both without and with addition of mercaptoethanol during the deconjugation step, and no difference was observed in any of the results. We measured folates in whole blood lysates, and converted this result to erythrocyte values by dividing by the hematocrit value and multiplying by the dilution factor. This procedure does not compensate for the small amount of folates present in plasma, but since the concentration of folates in plasma is approximately 50-fold lower than in erythrocytes, the difference will be negligible. No folic acid was detected, which was consistent with the fact that none of the volunteers used folic
Figure 3. Typical multiple-reaction monitoring chromatogram of folate vitamers and the isotopically labeled internal standard for a MTHFR 677 TT genotype erythrocyte sample (generated by positive-electrospray ionization LC-MS/MS). THF (approximately 10 nmol/L), 5-methylTHF (145 nmol/L), 5,10-methenylTHF (289 nmol/L), and 5-formylTHF (74 nmol/L).
acid supplements and DHF is not, or only minimally, present in erythrocytes.

Table 2. Erythrocyte folate vitamer distribution in healthy subjects with MTHFR 677 CC or TT genotype.

<table>
<thead>
<tr>
<th>C677T genotype</th>
<th>Sex</th>
<th>Age, years</th>
<th>Non-methylTHF, nmol/L</th>
<th>5-MethylTHF, nmol/L</th>
<th>Non-methylTHF, %a</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>M</td>
<td>53</td>
<td>0</td>
<td>330.2</td>
<td>0.0</td>
</tr>
<tr>
<td>CC</td>
<td>M</td>
<td>41</td>
<td>1.1</td>
<td>300.4</td>
<td>0.4</td>
</tr>
<tr>
<td>CC</td>
<td>M</td>
<td>24</td>
<td>8.7</td>
<td>528.5</td>
<td>1.6</td>
</tr>
<tr>
<td>CC</td>
<td>F</td>
<td>30</td>
<td>5.2</td>
<td>261.0</td>
<td>1.9</td>
</tr>
<tr>
<td>CC</td>
<td>F</td>
<td>33</td>
<td>6.6</td>
<td>328.2</td>
<td>2.0</td>
</tr>
<tr>
<td>TT</td>
<td>M</td>
<td>36</td>
<td>26.7</td>
<td>326.9</td>
<td>7.6</td>
</tr>
<tr>
<td>TT</td>
<td>F</td>
<td>23</td>
<td>23.3</td>
<td>117.4</td>
<td>16.6</td>
</tr>
<tr>
<td>TT</td>
<td>M</td>
<td>47</td>
<td>125.4</td>
<td>239.6</td>
<td>34.4</td>
</tr>
<tr>
<td>TT</td>
<td>M</td>
<td>61</td>
<td>171.0</td>
<td>218.3</td>
<td>43.9</td>
</tr>
<tr>
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<td>M</td>
<td>38</td>
<td>363.1</td>
<td>145.0</td>
<td>71.5</td>
</tr>
</tbody>
</table>

aNon-methyl folates are the total sum of 5,10-methenylTHF, 5,10-methyleneTHF, 5-formylTHF, 10-formylTHF and THF.

Discussion

We describe a straightforward approach to determine intracellular folate vitamer distribution. By quantitatively deconjugating all polyglutamyl folates to their monoglutamyl form, the amount of (often superfluous) information generated is reduced. As for other published methods for the determination of erythrocyte folate distribution (9;12;15;16), this method provides information on 5-methylTHF, and nonmethylTHF as a group. The sensitivity and accuracy for these measurements are high. In addition, the method accurately quantifies unsubstituted (partly) oxidized folates, which seems particularly relevant for folic acid.

Owing to the availability of labelled internal standards, LC-MS/MS has become a very specific and reliable analytical technique. It has already been successfully used for the determination of folate vitamers in various matrices such as food, serum and whole blood (16;26;27). One LC-MS/MS method measures folate concentration in whole blood samples as para-aminobenzoic
acid derivatives (26). By measuring this compound, any loss of the original folate during storage due to oxidation or degradation is compensated for, thus providing an accurate method for measuring total folate. However, no information on the separate vitamers is obtained. Other LC-MS/MS methods are able to measure the different vitamers in foods and whole blood after SPE clean-up (16;27). The main difference between these SPE methods and our method is the sample clean-up. Although sample clean-up by affinity chromatography on folate-binding columns is more time-consuming, the selectivity of this approach ensures high sensitivity and precision for quantification of the various folate vitamer concentrations. This may be particularly important for the correct determination of 5,10-methenylTHF, for which collision-induced loss of CO$_2$ in the mass spectrometer does not provide optimal selectivity.

As was demonstrated by previous methods, quantification of all separate intracellular folate vitamers is hampered by several factors. One of them is the instability of several calibrants. We found several stock solutions of folates to be unstable, even after the addition of ascorbic acid or mercaptoethanol. However, all folate vitamers that were actually measured by our method had stable calibrants. Another issue is the interconversion of non-methyl folate vitamers during sample work-up. The degree of interconversion varies between methods. Other methods have a less extensive sample clean-up and a lesser degree of interconversion (12;14). However, even in those methods, the degree of interconversion is difficult to predict or control at the level of individual samples. We therefore think it remains preferable to report comprehensive results for interconverting folates, rather than individual vitamers. As long as interconversion is restricted to within folate groups between which distinction is important (5-methylTHF, non-methylTHF, and unsubstituted oxidized folates), the data can be usefully interpreted. A method that could fully control or even exclude all interconversion between the different folate vitamers is not yet available. Although some loss of information with respect to the distribution of individual non-methylTHF vitamers is thus inevitable, comparison of methyl- and non-methyl folate vitamers provides valuable information on partitioning of folate metabolism in different conditions. When we compared 5-methylTHF to non-methyl folate vitamers in volunteers with the MTHFR 677 CC or TT genotype, we found distinct differences for the percentage of non-methyl folate vitamers between the two genotypes (0–2% for CC and 8–72% for TT) that are quantitatively comparable to an earlier report (0% for CC and 7–51% for TT) (9). We have not yet systematically studied MTHFR 677 CT subjects, and thus cannot determine if such subjects show an intermediate phenotype with respect to
folate vitamer distribution. The fact that we found small amounts of non-methyl folate vitamers in MTHFR 677 CC genotype volunteers, whereas the previously reported study found 0%, is probably because of ten-fold lower detection limit of our method, and is consistent with data obtained with a more recently published SPE-LC-MS/MS method (16). In contrast to this SPE method, we did not measure significant amounts of THF in MTHFR 677 TT genotype volunteers. When we spiked blood from an MTHFR 677 CT genotype volunteer with THF and added mercaptethanol during the deconjugation step, approximately 70% was recovered as THF and 10% as 5,10-methenylTHF (approx. 80% total recovery). The remaining 17% was not recovered as another folate vitamer, and was possibly converted to pABG. Other methods reported low recovery (20–50%) of THF, especially under acidic conditions (28;29). A possible explanation for the fact that we and others (9) did not find substantial amounts of THF in MTHFR 677 TT samples and other methods did (16) might well be that THF can be an analytical artefact (resulting from endogenous 5,10-methyleneTHF). Thus, the practical relevance of the imperfect recovery of THF in our method remains to be determined. In samples in which THF is expected, for example during pharmacological manipulation of folate metabolism or in biological samples other than erythrocytes, the addition of mercaptoethanol is recommended, especially if THF concentrations need to be reported separately from other reduced non-methyl folates.

In addition, we observed a marked loss of non-methyl folate vitamer concentration when blood samples were stored after ten-fold dilution with 56.7 mmol/L ascorbic acid, which is commonly done (16;22;23;30). As was previously demonstrated, this dilution produced a hemolysate with a pH below the irreversible denaturation point for haemoglobin (22). We compared samples frozen in a dilution of 56.7 mmol/L ascorbic acid (pH 2.8, lysate pH 4.0), samples in a dilution of 56.7 mmol/L ascorbic acid (pH 4, lysate pH 4.7) and undiluted samples (lysate pH 7.4). For undiluted samples, no significant decrease was observed for the various folate vitamer concentrations. Even though dilution with ascorbic acid resulted in only a minor decrease (<2%) of the 5-methylTHF concentration, significant amounts of 5,10-methenylTHF were lost during storage at both pH 4.0 (53–89%, n=5) and pH 2.8 (77–90%, n=5). None of this was recovered as any of the other folate vitamers. Since this decrease was not observed in standards (at the same pH), pH-mediated enzymatic degradation is a possibility (31). Another possibility could be cleavage of the unstable vitamers 5,10-methenylTHF and THF to pABG. An even higher storage pH or the addition of mercaptoethanol may offer a solution, which we did not investigate. Thus, samples for analysis of folate
Folate vitamers in erythrocytes by LC-MS/MS

Vitamer distribution should not be stored in even moderately acidic ascorbic acid solutions, because this will reduce the non-methylTHF concentration significantly.

In conclusion, our LC-MS/MS method for measuring folate vitamer distribution provides straightforward interpretation of erythrocyte folate vitamer distribution by accurately measuring 5-methylTHF, non-methylTHF, and (partly) oxidized folate vitamers with low detection limits. We further conclude that for the correct quantification of non-methyl folates, whole blood samples should not be stored in low-pH ascorbic acid solutions.

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References


