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A validated ultra-high-performance liquid chromatography-tandem mass spectrometry method for the selective analysis of free and total folate in plasma and red blood cells

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Abstract

A stable isotope dilution LC-MS/MS method is the method of choice for the selective quantitative determination of several folate species in clinical samples. By implementing an integrated approach to determine both the plasma and red blood cell (RBC) folate status, the use of consumables and time remains limited.

Starting from a single 300 µl whole blood sample, the folate status in plasma and RBCs can be determined after separating plasma and RBCs and sequential washing of the latter with isotonic buffer, followed by reproducible lysis using an ammonium-based buffer. Acidification combines both liberation of protein bound folates and protein precipitation. Sample cleanup is performed using a 96-well reversed-phase solid-phase extraction procedure, similar for both plasma and RBC samples. Analyses are performed by UHPLC-MS/MS.

Method validation was successfully performed based on EMA-guidelines and encompassed selectivity, carry-over, linearity, accuracy, precision, recovery, matrix effect and stability. Plasma and RBC folates could be quantified in the range of 1 to 150 nmol/l and 5 to 1500 nmol/l, respectively.

This method allows for the determination of 6 folate monoglutamates in both plasma and RBCs. It can be used to determine short and long term folate status in both normal and severely deficient subjects in a single analytical sequence.

Keywords

Folate, plasma, red blood cell, polyglutamylation, chromatography, mass spectrometry

1. Introduction

Folates, a group of essential water-soluble B-vitamins (B9), are key cofactors in both the methylation cycle and DNA-synthesis. Folate deficiency has been implicated in several health disorders, with a proven link between periconceptional folate deficiency and the prevalence of neural tube defects [1]. Other health issues such as increased cancer risk due to a disruption of DNA methylation and misincorporation of uracil may be related to folate deficiency [2]. Also, an impaired or halted methylation cycle causes neural damage through the reduced myelination of the nervous system [3] and leads to increased plasma homocysteine concentrations [4,5]. Following several decades of research it is evident that knowledge of nutrient status, including folate concentrations, is of paramount importance for the evaluation of individual health.

Clinical folate status is traditionally determined in serum by a competitive folate binding protein (FBP) assay or a microbial assay. However, more recently developed liquid chromatographic tandem mass spectrometric (LC-MS/MS) methods allow to discriminate between different metabolically active folate species for which the microbial and ligand binding assays are not equally specific [6-8].

For LC-MS/MS analysis of folates in plasma or serum, several methods have been published, differing in certain steps. Nelson et al. showed that affinity extraction with FBP led to lower detection limits, compared to reversed-phase solid-phase extraction (SPE) [9]. However, Hannisdal et al. omitted the purification step and simply used protein precipitation and evaporation as sample cleanup technique, yielding similar results [10]. Also, Garbis et al. showed that hydrophilic interaction liquid chromatography can be used to separate four different folate species [11]. Using an automated phenyl-based SPE system, a sensitive and

high-throughput method for the measurement of serum folates was devised by Fazili et al. [12].

Lysis of whole blood samples for folate analysis is commonly performed using an L-ascorbic acid (AA) solution as a lysing agent [13-15]. However, this strategy was criticized due to differences in the assayable folate concentrations, attributable to differences in solution strength, which may lead to incomplete lysis, non-linear dilution curves and variable lysis times [16]. The addition of AA to whole blood lysates causes deoxygenation of hemoglobin due to the acidification of the sample. Since folates are trapped within the structure of oxyhemoglobin, deoxygenation is required for deconjugation [17]. Alternatively, lyophilized RBCs have also been used as a starting material to which an extraction buffer is added [18].

Since most LC-MS/MS procedures only measure folate monoglutamates, it is necessary to incubate the lysate at 37 °C for 2 to 12 hours to allow human γ-glutamylhydrolase (GGH), present in plasma, to deconjugate the folate polyglutamates present in the sample. Mostly heat inactivation is used to stop this reaction [13]. Samples can be purified using affinity extraction with FBP, followed by SPE to concentrate the sample [13]. However, most used is ion-exchange or reversed-phase SPE without affinity extraction [18,19]. Mixed-mode sorbents can be used, though this necessitates an evaporation-dissolution step to remove the organic solvent from the sample [14].

Given the high amount of folates present in RBCs, it is difficult to obtain a blank matrix. A surrogate matrix, consisting of lyophilized egg white and sunflower oil in an isotonic sodium chloride solution, is sometimes used [18]. Another approach is to serially dilute samples with water in order to estimate the detection limits of the method [19]. In both approaches, the obtained results may not be a true reflection of actual method performance. A better approach, as applied by e.g. Kirsch et al., may be to use a matrix treated with activated charcoal to

assess method performance, although it should be noted that this matrix also differs in many aspects from non-treated matrix [14].

To selectively quantify plasma, RBC free and RBC total folate, we developed a new LC-MS/MS method, with the focus on the sample preparation steps. This method was set up as such, that most of the treatment steps, consumables and solvents were shared for both plasma and RBC analyses, thereby optimizing the workflow and augmenting sample throughput. Also, reproducible RBC lysis, an obstacle troubling previously published methods, was achieved using a lysis buffer specific for RBCs, while addition of dithiothreitol (DTT) assures the release of folates from hemoglobin. To assess method performance, a full validation was performed, based upon European Medicines Agency (EMA) guidelines, including selectivity, carry-over, linearity, accuracy, precision, recovery, matrix effects and stability in whole blood, prepared extracts and while being in the autosampler [20]. Additionally, certified reference materials for both plasma and RBCs were analyzed to assure interlaboratory accuracy.

2. Materials and methods

2.1 Reagents and materials

The six folate monoglutamates studied are: tetrahydrofolate (THF), 5-methyltetrahydrofolate (5MeTHF), 5,10-methenyltetrahydrofolate (5,10CH^+THF), 10-formylfolic acid (10FoFA), 5-formyltetrahydrofolate (5FoTHF) and folic acid (FA). The folate reference standards were obtained from Schirck's Laboratories (Jona, Switzerland), while the labeled internal standards (ISs), with a labeling yield higher than 98%, were obtained from Merck Eprova (Glattbrugg, Switzerland). At the pH values reached during sample preparation and analysis, the metabolically active 10-formyltetrahydrofolate (10FoTHF) is detected as 5,10CH^+THF. Also, 5,10-methylenetetrahydrofolate (5,10CH_2THF) is unstable at acidic pH and is measured as

THF [21, 22]. All calibrators and $^{13}C_5$ labeled IS solutions were prepared in a final concentration of 200 nM in a 50mM sodium phosphate buffer, pH 7.5, containing 1% AA and 0.5% DTT / methanol (50/50 v/v%). $^{13}C_5$-FA was used as IS for FA, 5-FoTHF and 10-FoFA, whereas $^{13}C_5$-THF, $^{13}C_5$-5-MeTHF and $^{13}C_5$-5,10-CH^+THF were used as IS for their respective isotopologues. Stripped rat serum was obtained starting from non-sterile, non-hemolysed rat serum, obtained from Harlan (Horst, The Netherlands), which was stirred on ice for 1 hour with 100 mg/ml of activated charcoal (Sigma-Aldrich, Diegem, Belgium) to remove endogenous folates. Following removal of the activated charcoal by centrifugation at 4500 g for 15 minutes at 4 °C, the solution was filtered over a 0.45 µm syringe filter (GD/X CA 25/0.45, Whatman, GE Healthcare, Little Chalfont, UK), divided into aliquots and frozen at -80 °C. Acetonitrile (ACN) and methanol of LC-MS quality were purchased from Biosolve (Valkenswaard, The Netherlands). Formic acid, trisodium phosphate, AA and DTT were obtained from Sigma Aldrich. SPE was performed in a 96-well format with Bond Elut C_{18} 100 mg sorbent, purchased from Agilent (Palo Alto, CA, USA). Deionized water (H_2O-MQ) was produced in house by means of a Synergy UV water-purification system from Millipore (Billerica, MA,USA).

2.2 Preparation of stripped calibrator and QC-matrix

EDTA-anticoagulated blood samples were obtained from healthy volunteers, 4 male and 2 female, aged 25 to 37. To remove endogenous folates, plasma and RBC extracts were treated with activated charcoal as described for rat serum in paragraph 2.1.

2.3 Sample preparation

Folate analysis was performed starting from 300 µl EDTA-anticoagulated venous blood. Plasma extracts were prepared by adding 500 µl of phosphate buffered saline (PBS) to the whole blood sample. After centrifugation at 1000 g for 5 minutes at 4 °C, 500 µl of supernatant was withdrawn. To this aliquot 50 µl of a 1% AA, 0.5% DTT solution (containing all ISs in a final concentration of 3.0 nmol/l) was added to stabilize the folates. Following 15 minutes of incubation at room temperature, the samples were either analyzed immediately or frozen at -80°C. To the remainder of the samples, which contain the RBCs, 1 ml of PBS at room temperature was added, followed by resuspension, centrifugation at 1000 g for 5 minutes at 4 °C and removal of 1 ml supernatant. This washing procedure was repeated two more times. After the final removal of 1 ml supernatant, 800 µl of lysis buffer (168 mM NH_4Cl; 10 mM $KHCO_3$; 0.1 mM EDTA) was added [23]. Selective RBC lysis occurred during a 10-minute incubation at room temperature, after which cell residues were spun down by centrifugation at 2500 g for 10 minutes at 4 °C. One milliliter of supernatant was withdrawn and 100 µl of IS/AA/DTT solution was added. After incubation for 15 minutes at room temperature, these samples were analyzed further or frozen at -80°C. A flowchart describing the sample preparation procedure is shown in Supplementary Figure S1.

2.4 Enzyme treatment and protein precipitation

To measure the total folate content in RBCs, 100 µl of stripped rat serum was added to 500 µl of RBC lysate and incubated for 2 hours at 37 °C. This stripped rat serum serves as an exogenous source of GGH to deconjugate the folate polyglutamates present in RBC samples. The reaction was halted by addition of 300 µl of 10 vol% acetic acid in water. This acidification of the sample also liberates protein-bound folates and causes protein precipitation [24]. While no enzyme treatment is necessary to measure free monoglutamates

in plasma and RBCs, these samples were also acidified by adding 250 µl of 10% acetic acid. The acidified samples were incubated for 20 minutes at 4 °C prior to centrifugation at 11 000 g for 15 minutes at 4 °C.

2.5 Sample cleanup

SPE was performed using a Bond Elut 96-well plate containing 100 mg C_{18} sorbent per well. The sorbent was activated with 2 x 600 µl of methanol and conditioned with 2 x 600 µl of 1.67 / 3.03 / 30.30 / 65 v/v/v% CH_3COOH / 10% AA, 1% DTT-solution / PBS / H_2O-MQ. Next, plasma and RBC samples were diluted with an equal volume of water of which 1 ml for plasma extracts and 800 µl for RBC extracts was loaded onto the sorbent. The sorbent was then washed with 2 x 600 µl of conditioning buffer prior to elution with 450 µl of elution buffer (7.5 / 92.5 v/v% ACN/ 50 mM trisodium phosphate with 1% AA and 0.1% DTT; pH= 7.3). The SPE procedure is summarized in Supplementary Figure S1.

2.6 LC-MS/MS analysis

Samples were analyzed using a Waters Acquity UPLC® system coupled to an ABSciex API 4000™ triple quadrupole mass spectrometer equipped with a TurboIonSpray® probe. The autosampler was equipped with a sample loop of 10 µl and samples were kept at 4 °C prior to injection. After injection the needle was cleaned with 600 µl of 10/90 v/v% H_2O-MQ/ACN followed by 800 µl of 90/10 v/v% H_2O-MQ/ACN. Chromatographic separation was achieved using a mobile phase composed of H_2O-MQ (A) and ACN (B), each containing 0.1% of formic acid, with a flow rate of 0.6 ml/min on a Waters HSS T3-column (150 mm x 2.1 mm; 1.8 µm) held at 60 °C. The composition of the mobile phase starts with 100% solvent A and is kept constant for 1.6 minutes. Thereafter the amount of solvent B is increased to 10% in 1.2 minutes and to 16% in another 0.7 minutes after which the column is cleaned for 1.5 minutes

with 95% solvent B and equilibrated to initial solvent composition for 3 minutes. This amounts to a total time of approximately 9 minutes between injections. Mass spectrometric detection was performed in scheduled multiple-reaction monitoring mode. Electrospray ionization was used to ionize the analytes with the ion spray voltage set at 2500 V and a temperature of 600 °C. Nitrogen was used as curtain gas, gas 1 and gas 2, with respective pressure settings at 25, 75 and 90 psig, and as collision gas at setting 11. The interface heater was switched on. The transitions monitored per compound were the same as described before [25], settings can be found in Supplementary Table S1.

2.7 Validation

The developed method was validated based on the EMA guidelines for bioanalytical method validation [20]. Selectivity, carry-over, limit of detection (LOD) and lower limit of quantification (LLOQ), linearity, precision (including incurred sample reanalysis), accuracy, matrix effect, recovery and stability were evaluated.

2.7.1 Selectivity and carry-over

Selectivity was evaluated by analyzing plasma and RBC lysates from six individual donors (4 male and 2 female), both with and without treatment with activated charcoal. Cross-interferences were evaluated by adding the 6 folates and 4 labeled ISs individually to stripped matrix in a concentration of 100 nmol/l for plasma samples (150 nmol/l for 5FoTHF) and 300 (THF and 5,10CH+THF), 500 (5MeTHF, FA and 10FoFA) or 750 nmol/l (5FoTHF) for RBC extracts. For the analytes, the acceptance criterion for the selectivity assessment was set at 20% of the peak area corresponding to the LLOQ level, while for the labeled ISs only 5% was considered acceptable. Carry-over was evaluated by injecting blank samples after calibrators with identical concentrations and acceptance criteria as for the evaluation of selectivity.

2.7.2 Linearity, accuracy and precision

The actual LLOQ was verified as the concentration which could be measured with a %bias and relative standard deviation (RSD) below 20%. The LOD was calculated as one third of the LLOQ. Duplicate calibration curves of 7 calibrators and 1 zero sample for plasma or 9 calibrators (8 for THF and 5,10CH^+THF) and 1 zero sample for RBC were constructed on 4 non-consecutive days to evaluate linearity. Homoscedasticity was evaluated by plotting the residuals versus the nominal values. The sum of the residual error was used to evaluate the need for a weighted model ($1/x$, $1/x^2$, $1/y$, $1/y^2$, $\sqrt{x}$, $\ln(x)$, $\log(x)$). For the selected model to be acceptable, back-calculated mean concentrations should be within 15% of the nominal value (20% at LLOQ-level). Accuracy and precision were evaluated using 4 QCs (LLOQ to 3/4 upper limit of quantification (ULOQ), Table 1), prepared and analyzed on 4 non-consecutive days. A single-factor ANOVA was used to calculate inter- and intrabatch variability (%RSD). Accuracy (%bias) was calculated as the measured value divided by the nominal value. Acceptance criteria for %bias and %RSD are 15%, except at LLOQ level, where they are 20% [26]. An RBC sample of a healthy volunteer was analyzed in duplicate on 3 non-consecutive days to evaluate the precision of a sample requiring deconjugation of the folate polyglutamates. Also, the dilution integrity of RBC extracts exceeding the ULOQ was verified by two- or fourfold dilution of RBC matrix spiked at a concentration of 600 to 3000 nmol/l with SPE elution buffer prior to UHPLC-MS/MS analysis (n=4).

2.7.3 Recovery and Matrix effects

Matrix effect and recovery were evaluated at 3 concentration levels (3LLOQ to 3/4ULOQ, Table 1) in matrix obtained from 6 individual sources according to Matuszewski et al. [27]. At the 3LLOQ concentration level, stripped matrix was used. To prepare the MID and 3/4ULOQ concentration levels, untreated matrix was used. The analytes were spiked either before (A) or after (B) the extraction and SPE procedure. Also, analytes were spiked in SPE elution buffer to obtain a sample free of matrix (C). Absolute recovery was calculated as the percent ratio of

peak areas A over B, while absolute matrix effect was calculated as the percent ratio of the peak areas B over C. Relative recovery and matrix effect are expressed as %RSD of the absolute recovery and matrix effect values and should not exceed 15%.

2.7.4 Stability

The stability of folates in whole blood samples was verified by storing whole blood at 4 °C for 0, 1, 2, 7 and 10 days, while protected from light, before performing the entire sample preparation procedure in triplicate. Autosampler stability was assessed by reinjection of samples stored for 24 hours in the autosampler while concentrations were calculated based on fresh calibrators. Long term stability of plasma and RBC lysates at -80°C was evaluated by analysis of samples in duplo after 0, 1, 2 and 4 weeks including freshly prepared calibration curves. Also, the effect of up to three freeze-thaw cycles was evaluated by freezing and thawing the samples in cycles of 1 hour. Stability is acceptable if the concentration measured does not deviate more than 15% from that measured initially.

2.7.5 Application

The accuracy of the method was evaluated via the measurement of two certified reference materials, namely: Vitamin B12 and Serum Folate (03/178) and Folate in Whole Blood Haemolysate (95/528), both WHO International Standards issued by the National Institute for Biological Standards and Control (NIBSC). The reference material for serum has previously been measured by e.g. Fazili et al. [12].

3. Results and discussion

3.1 Method optimization

3.1.1 Sample treatment

The method starts from 300 µl of whole blood. Following removal of the plasma sample, the RBC pellet is washed to remove plasma residues. To evaluate the washing procedure of RBCs we monitored the removal of GGH activity by measuring the concentration of 5MeTHF monoglutamate in RBC lysates after 2 hours of incubation at 37 °C without addition of rat serum GGH. Whole blood lysate, sole plasma removal and 1 to 4 washing steps were evaluated. The amount of 5MeTHF monoglutamates remained stable after at least 2 washing steps, indicating that human GGH was sufficiently removed. One additional washing step was included to ensure reproducible removal of plasma proteins, plasma folates and other constituents.

Given the need for complete and reproducible RBC lysis, several solvent compositions described in literature were evaluated. These were either based on the selective absorption of ammonia by RBCs (0.15 M NH_4Cl, 10 mM $NaHCO_3$, 10 mM EDTA at pH 7.4), hyper- (1% AA) or hypotonicity (deionized water) or susceptibility to ethanol or saponin [28-32]. The addition of 1% AA, ethanol or saponin resulted in incomplete lysis and would require at least one freeze-thaw cycle for full lysis. In contrast, the ammonia lysis buffer and deionized water led to full lysis of RBCs. The former was selected because it has been reported to selectively lyse RBCs, leaving other cells unaffected [29]. However, this buffer showed limited shelf life, with prolonged storage resulting in increased lysis times. Therefore, it was used within fourteen days after preparation and was stored at 4 °C.

3.1.2 Folate deconjugation

Since folates trapped within the quaternary structure of oxyhemoglobin are not accessible to GGH, deoxygenation of hemoglobin is necessary to measure the total amount of folates in the sample. While other methods rely on the Bohr effect (decrease of pH) to decrease oxygen binding affinity [17], in our method DTT was added to RBC extracts, which gently

deoxygenates hemoglobin to produce a mixture of deoxy-, met- and sulfhemoglobin, leading to a release of folates [33].

To deconjugate the folate polyglutamates present in RBCs, the samples are incubated with stripped rat serum, which serves as a reliable source of the necessary GGH enzyme. Addition of 100 µl stripped rat serum to 500 µl of RBC lysate, containing AA, DTT and ISs, and incubation for 2 hours at 37 °C proved optimal to assure full deconjugation. However, the incubation of RBC extracts at 37 °C led to a minor conversion of THF to 5,10CH^+THF. From literature it could be concluded that this reaction, which is catalyzed by a formate-activating enzyme (or formyltetrahydrofolate synthetase) present in RBCs, can be prevented by denaturing the enzyme at 55 °C for 5 minutes or by addition of NaCl to a concentration of 50 mM [34]. Since the latter inhibited the deconjugation of folate polyglutamates, the heat treatment was preferred.

3.1.3 Protein precipitation

A significant part of folates is bound to FBP. These folates can be released by acidification to a pH below 5.5, which strongly diminishes the binding capacity of FBP [24]. Thus, 10% acetic acid was added as this combines protein precipitation and the liberation of protein-bound folates. Moreover, this renders an additional treatment with protease unnecessary.

3.1.4 Solid-phase extraction

Since both reversed-phase and ion-exchange SPE [9,18,19] have been used for the determination of folates in plasma and RBC extracts, the retention behavior of 6 folate monoglutamates on various sorbent types was evaluated. Strong-anion exchange showed inferior retention of 5,10CH^+THF, but gave less background noise. However, since 5,10CH^+THF was regularly detected in plasma and RBC samples, the superior recovery obtained using the reversed-phase sorbents was of greater importance. Comparable results

were found with phenyl and C_{18} sorbents. The C_{18} sorbent was selected for further optimization.

3.2 Method validation

3.2.1 Selectivity and carry-over

Method selectivity was evaluated using spiked stripped blood samples of 6 apparently healthy volunteers. Apart from a limited conversion of THF to 5,10CH$^+$THF in RBC extracts, the MS signals for none of the folates exceeded 20% of the LLOQ of the others. A 5 minute treatment at 55 °C restricted the conversion of THF to 5,10CH$^+$THF to on average 3.1% of the amount of THF present in the RBC sample. When the THF concentration exceeds 33 nmol/l, an apparent 5,10CH$^+$THF concentration will be observed which is higher than 20% of the LLOQ. In non-stripped samples, no interfering peaks were observed that would hamper folate analysis, based on an evaluation of peak shape and baseline resolution. No unacceptable carry-over was observed in either plasma or RBCs for any of the folates analyzed (Supplementary Table S2).

3.2.2 Linearity, accuracy and precision

The data were heteroscedastic; a linear unweighted model was preferred for all folates in both matrices, since weighting provided little benefit based on the evaluation of the sum relative error. Also, the back-calculated concentrations of the calibrators fell within the acceptance criteria using this linear model. The LLOQ and ULOQ range from 1 to 1.5 and 100 to 150 nmol/l, respectively, for plasma and from 5 to 7.5 and 300 to 1500 nmol/l, respectively, for RBCs (Table 1).

In plasma the interbatch variation for 5FoTHF at the LLOQ level slightly exceeded the 20% acceptance criterion (21.3%), which may be improved using the commercially available $^{13}C_5$-

5FoTHF as IS. All other accuracy and precision values fell within 15% bias resp. RSD (or 20% at the LLOQ level) (Table 2).

To assess the reproducibility of measurements in a true RBC extract, in which mainly folate polyglutamates are present, incurred samples were analyzed in duplicate during three different analytical runs. In these samples only 5MeTHF and 5,10CH^{+}THF could be quantified, with a respective intrabatch precision (%RSD) of 7.5 and 9.6% and a respective interbatch precision (%RSD) of 7.7 and 9.6%, at a respective average concentration of 124 and 22.8 nmol/l. A two- or fourfold dilution of samples exceeding the ULOQ with SPE elution buffer proved possible, with bias and RSD below 15%. (Supplementary Table S3)

To investigate whether the treatment of the matrix with activated charcoal, used to prepare calibrators, affected the calibration results, two calibration curves were compared. One was prepared in stripped matrix, the other in untreated matrix. As can be seen in Table 3, the slope of both the plasma and the RBC calibration curves deviated less than 7.3% and 6.8% between stripped and untreated matrix. Also, cross-calculated concentrations fell within the acceptance criteria, except for RBC folates at LLOQ level, which was likely due to the high background folate concentrations in untreated RBC samples. As such, the use of stripped matrix does not influence the analytical results.

3.2.3 Recovery and Matrix effects

Matrix effects and recoveries for both plasma and RBCs are shown in Table 4, both with and without compensation by the ISs. Overall, IS-compensated recoveries lay between 91.2 and 107.5% for plasma and 84.8 and 117.2 for RBCs, while matrix effects -with a single exception for 5FoTHF- did not exceed 20%. Both 10FoFA and 5FoTHF showed slightly different recoveries and matrix effects than $^{13}C_5$-FA, which is used as an IS for these compounds. Though some values for IS-compensated relative recovery and matrix effect exceeded the 15% acceptance criterion, none were larger than 20%. The relative recovery and

matrix effect values without IS-compensation demonstrate the necessity of using labeled ISs to compensate for the variability between samples.

3.2.4 Stability

Folates present in the plasma fraction of whole blood proved stable for 24 hours, while the folates present in RBCs proved stable up to 10 days (Supplementary Figures S2 A and B). As observed by Hannisdal et al., the stability of plasma folates can be improved through the use of heparin or citrate instead of EDTA as anticoagulant.[35] Therefore, if sample stability is an issue, it is unlikely that the use of heparin as an alternative anticoagulant would negatively influence method performance. Long term storage of plasma samples at -80 °C was possible up to 4 weeks (Supplementary Figure S3 A). RBC folates showed a steady decrease upon storage at -80 °C, where after storage for 4 weeks a loss of approximately 20% was observed for all folates (Supplementary Figure S3 B). Processed samples were stable for at least 24 hours in the refrigerated autosampler at 4 °C (Supplementary Table S4 A and B). Although samples with a low folate concentration were more susceptible to the effects of freeze-thaw cycles, up to three freeze-thaw cycles did not have an unacceptable effect on the folate levels measured in both plasma and RBC samples at higher concentration levels (Supplementary Tables S5 A and B).

3.3 Application

In the plasma certified reference material (NIBSC 03/178), analyzed in sevenfold, only 5-MeTHF could be detected and quantified in a concentration of 11.1 nmol/l (SD: 0.233 nmol/l). While this concentration is slightly higher than that reported by the NIBSC and by Fazili et al. [12] (resp. 13.8 and 7.8%), the total folate concentration measured is slightly lower than that reported by the NIBSC (8.3%). Also, Fazili et al. [12] measured higher total folate (15.9%), reporting on the presence of FA and MeFox (an oxidation product of

5MeTHF) as well (Table 5). There is no real discrepancy between our results and those of Fazili et al. First, although the FA concentration reported by these authors is low, lying between the LOD and LLOQ of our method, we did not detect FA. We cannot exclude that slight differences in FA formation during sample processing may account for this. Second, MeFox was not included in our method given the lack of commercially available reference standards. Also, MeFox was not detected in samples using the mass transitions described for this compound.

For RBCs, peak areas are back-calculated to the folate concentration in the reconstituted reference material. THF, 5MeTHF and 5,10CH^{+}THF could be quantified in the reference material. Also FA and 10FoFA were present, with peak areas between the LOD and LLOQ, while no 5FoTHF was detected. The sum of the individual folates only exceeds the total folate measurement reference value set by the NIBSC by 6.0%. All values can be found in Table 5.

The method was tested on blood samples obtained from 6 healthy volunteers (4 male and 2 female) after overnight fasting. Results are depicted in Figure 1 and Supplementary Figure S4. The total plasma folate concentration ranged from 7.05 to 17.0 nmol/l. The main folate form was 5-MeTHF, minor folate forms exceeding the LLOQ were 10FoFA, THF and FA.

In only four out of six subjects, RBC folate monoglutamate exceeded the LLOQ, 5MeTHF being the major folate species and THF being quantifiable in only 1 sample. Total folate concentrations in RBCs ranged from 222 to 504 nmol/l. Similarly to plasma, 5MeTHF is the major folate species in RBCs, while THF and 5,10CH^{+}THF are present in minor amounts. FA, 10FoFA and 5FoTHF could also be quantified in some samples. Based on the total folate concentration and the concentration of folate monoglutamates, the degree of polyglutamylation of RBC folates was calculated, which appeared to be fairly constant in samples obtained from these volunteers, ranging from 96.9 to 98.4% (n=4).

4. Conclusion

Due to the similarity between the plasma and RBC analyses, these can be performed simultaneously, thus optimizing workload and sample throughput while minimizing the consumption of solvents and consumables.

Starting from a single sample of 300 µl EDTA-anti-coagulated whole blood, the fully validated analytical method presented here allows to quantitatively determine 6 folate species in plasma and RBC extracts. The validation data showed linearity of the method from 1 to 150 nmol/l for plasma samples and 5 to 1500 nmol/l for RBC samples, encompassing the clinically relevant ranges. Our method includes selective and reproducible ammonia-based RBC lysis, the addition of DTT to all solvents and acidic protein precipitation. This offers an advantage over previously published methods which may suffer from poor reproducibility due to incomplete lysis and uncontrolled protein binding [17]. In RBCs, both free folate monoglutamates and total folate (monoglutamates + polyglutamates) can be quantified. From this, the degree of polyglutamylation can be calculated. This may provide information on the efficacy of the FPGS enzyme responsible for the intracellular accumulation of folate polyglutamates. Since plasma GGH is removed prior to RBC lysis, the presence of different folate polyglutamates may be visualized using the chromatographic method developed by Wang et al. [36]. Our method can also be used to identify individuals with a polymorphism in the gene encoding the methylenetetrahydrofolate reductase enzyme. A significant difference in folate speciation has been observed in blood samples obtained from these individuals [37].

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Figure captions

Figure 1: UPLC®-MS/MS chromatogram (A) of a stripped plasma sample, (B) a stripped plasma sample spiked at LLOQ level, (C) a sample of a healthy volunteer, (D) a stripped RBC sample, (E) a stripped RBC sample spiked at LLOQ level, (F) a RBC sample of a healthy volunteer