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Clinical determination of folates: recent analytical strategies and challenges

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Abstract

Since the introduction of liquid chromatography tandem mass spectrometry in clinical laboratories, folate analysis has shifted from microbiological or protein binding assays to chromatographic methods. Now it is possible to sensitively and selectively determine several folate species in clinical samples where only a total folate content could be quantified using a microbiological or a binding assay. Although several chromatographic methods have been developed, validated and published, interlaboratory variability limits the comparability of the results. In this review we provide an overview of the latest strategies for sampling, sample treatment and analysis and how these may influence the final analytical result. Amongst the variables covered are the effect of pH, temperature, storage and the use of antioxidants and anticoagulants on analyte stability. In addition, we highlight the importance of correct assay calibration and the use of (labeled) certified reference materials in order to obtain correct and comparable results among different laboratories.

Keywords

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

1. Introduction

Within the water soluble vitamins, the group of folates is essential for the maintenance of adequate methylation potential in an individual and is involved in the synthesis of purines and pyrimidines.

Currently, clinical folate determination primarily depends on the use of binding assays for the quantification of total folate in serum samples. However, the introduction of liquid chromatography tandem mass spectrometry (LC-MS/MS) as a higher order method in the clinical lab allows to study the folate status and speciation more into depth than was previously possible using microbiological assays (MA) or protein-binding assays (PBA). This is relevant since more information is needed for a complete understanding of the effect of genetic polymorphisms on nutritional status and folate distributions.

The multitude of sample treatment procedures preceding these LC-MS/MS methods hinders comparability of clinical data across laboratories. This has proven to be the Achilles' heel of current folate determinations in clinical samples. In addition, the application of cut-off values, derived from MA or PBA, on results obtained via these higher order methods causes another major challenge.

In this review, we focus on the use of LC-MS/MS for folate quantitation in blood, plasma and serum, by providing a non-exhaustive overview of methods improving the existing analytical procedures since the year 2000. The strengths and weaknesses of these methods are evaluated and alternative procedures are discussed to obtain an understanding of the measurement process and related results.

Furthermore, this review elaborates on method comparability and on the risk of misinterpreting folate status due to mismatching of cut-off values and population-based data.

1.1. Chemical properties

Folates consist of three distinct parts: a pterin moiety with a varying oxidation state, a para-aminobenzoic acid (pABA) entity and a tail of repeating glutamic acid molecules bound at the γ-position. As shown in Figure 1, different functional groups may be bound at either or both the 5 and 10 position [1]. Folates can be present in 3 oxidation states, i.e. a fully oxidized form, known as folic acid (FA), a fully reduced form, known as tetrahydrofolate (THF), and an intermediate form, known as dihydrofolate (DHF). Stability of folates depends on both the oxidation state and the substitution with a methyl- or formyl group at the 5 and/or 10 –position. In addition, folate polyglutamylation, an important factor for both intracellular folate retention and enzyme binding, indirectly results in folate stabilization. Since folate polyglutamylation favors enzyme binding, folates are protected from oxidative degradation [2].

Although FA is the commonly used folate in supplementation and food fortification, it is 5-methyltetrahydrofolate (5MTHF) that is the predominant species in both natural food items and in clinical samples.

1.2. Folate metabolism

In human cells, folates take part in two different metabolic cycles, as depicted in Figure 2. In the methylation cycle, the vitamin B_{12} dependent methionine synthase transfers the methyl group of 5MTHF to homocysteine, resulting in the formation of THF and methionine. The latter can be further converted to S-adenosyl methionine (SAM), which is involved in many different metabolic processes, including methylation of lipids and DNA and the formation of DOPA and myelin, the insulating material of neural tissue [3]. Disruption of the above-mentioned cycle, as a result of a vitamin B_{12} and/or folate deficiency, can be clinically measured as an elevated plasma homocysteine concentration, known as hyperhomocysteinemia. This is associated with an increased risk for cardiovascular diseases (CVDs) [3,4]. Folate supplementation is the method of choice to treat hyperhomocysteinemia. Hyperhomocysteinemia has also been

associated with an increased risk for neural diseases including dementia and Alzheimer's disease [5]. Best established is undoubtedly the profound influence of folate status on the prevalence of neural tube defects (NTDs). During early pregnancy, a low folate status can lead to failure of the neural tube to close in the developing fetus, resulting -depending on the severity- in disability or even death [6].

Secondly, 5,10-methylenetetrahydrofolate and 10-formyltetrahydrofolate (5,10CH_2THF and 10FoTHF) take part in the *de novo* synthesis of pyrimidines and purines, respectively. Especially the methylation of uracil to thymidine is strongly influenced by folate status. Folate deficiency has for instance been implicated in uracil incorporation in DNA, leading to chromosomal damage [7]. In addition, severe folate deficiency might lead to megaloblastic anemia, the presence of enlarged red blood cells in the bloodstream, since cell replication is inhibited due to the impairment of DNA synthesis. Megaloblastic anemia is also used as an indicator for vitamin B_{12} deficiency. The use of FA as a folate supplement is problematic, since the presence of FA in the bloodstream leads to a bypass of the methionine synthase enzyme. Although this allows DNA replication to be maintained, alleviating megaloblastic anemia, the formation of methionine is still blocked, potentially leading to neural damage [8].

In general, the assessment of the clinical folate status does not involve determination of folate polyglutamate derivatives, since only folate monoglutamates are present in plasma and serum. However, in cells (including RBCs), glutamate chain elongation is responsible for folate accumulation [2,9-11]. Although methods do exist for distinguishing individual folate polyglutamate species [12], the lack of pure reference standards limits the use of these methods for true quantitative folate determination in clinical matrices.

1.3. Folate status

Poor folate status is primarily caused by an inadequate intake of sources rich in folates such as legumes, green leafy vegetables, fruits and fortified cereals [13]. In addition, food preparation, such as boiling, might cause a further decrease of the nutritional value of food products. The recommended daily allowance (RDA) for adults is 400 μg/day. However, folate requirement increases during periods of growth, development and reproduction, such as pregnancy and lactation [13]. Therefore, supplementation with folic acid is recommended. Several diseases, such as chronic disease and some cancers, may also affect folate status due to the disturbance of folate absorption. The use of certain medicines may have an impact as well. Methotrexate, used for the treatment of autoimmune and inflammatory conditions, and trimethoprim, used for bacterial infections, are folate antagonists. Inducers of metabolic enzyme activity, such as phenytoin and phenobarbital, may cause folate depletion due to the enhancement of folate degradation [14]. Another important condition, influencing folate status, is chronic alcoholism. Alcohol acts via several mechanisms; malnutrition, inhibition of folate transporters, increase of folate degradation due to enzyme induction, and increase of urinary excretion, resulting in folate depletion [15]. Last, given the complexity of the folate pathway and the numerous enzymes involved, it is not surprising that a link has been found between several genetic polymorphisms and folate status. The most studied is a single nucleotide polymorphism (SNP) in the methylene tetrahydrofolate reductase gene at nucleotide 677 (MTHFR 677 C>T), changing an alanine to a valine, which results in a thermolabile and less efficient enzyme [16]. This SNP is very rare in the African population but approximately 10% of Caucasians and 20% of Asians are homozygous for this functional polymorphism [17]. While intracellular folates in 677C homozygotes and in heterozygotes mostly consist of 5MTHF, 677T homozygotes have a much lower fraction of 5MTHF and are at higher risk for low folate status, leading to hyperhomocysteinemia, which is associated with an increased risk of CVDs, and posing a higher risk for NTDs. Additionally, other polymorphisms have been described that may

influence folate status. As described by DeVos et al., SNPs can occur in nearly all metabolically relevant enzymes such as folylpolyglutamate synthase (FPGS) and gamma-glutamylhydrolase (GGH), enzymes governing polyglutamylation and hydrolysis, respectively, methionine synthase (MS), responsible for demethylation of 5MTHF in order to increase affinity for FPGS and folate hydrolase (FOLH1), which hydrolyses polyglutamated food folates in the brush border membrane of the jejunum before absorption [18]. Folate carriers such as proton coupled folate transporter (PCFT), which absorbs folate monoglutamates from the gut, and reduced folate carrier (RFC), responsible for the uptake of circulating folates into tissues, are subject to polymorphic variations as well [18]. However, not all polymorphisms have a significant influence on the intracellular folate profile and/or concentration. Ideally, a method for the analysis of folates in plasma or serum should be able to discern differences in concentrations of all relevant folate species, including the degree of polyglutamylation in RBCs.'

1.4. Cut-off values for folate status

In 2015, the World Health Organization (WHO) released an updated guideline on the use of serum (or plasma) folate and red blood cell folate concentrations to assess the folate status in different populations [19]. Such an assessment is useful to monitor trends in folate status and to evaluate the impact of public health interventions. A quantitative value for an adequate folate status is not easily determined since this depends on the metabolic indicator used to define the cut-off value. Since the 1970's, the prevalence of megaloblastic anemia, a hematological indicator of folate deficiency, was used to categorize patients with various folate concentrations. Patients with serum or plasma folate concentrations below 6.8, between 6.8 and 13.4, between 13.5 and 45.3, or above 45.3 nmol/l were respectively categorized as deficient, possibly deficient, normal and elevated. To assess long-term folate status, a cut-off value of 226.5 nmol/l (derived via a microbiological assay) in RBCs was used to indicate folate deficient levels [20,21]. In 2005, the elevation of plasma homocysteine concentrations was chosen as a marker

for folate deficiency, based on the National Health and Nutrition Examination Survey (NHANES) III data. This population survey investigated the effect of dietary intake on folate status and related clinical parameters and health outcomes. The cut-off levels indicating folate deficiency (i.e. the folate concentrations below which homocysteine concentrations started to rise) were set at 10 and 340 nmol/l (values derived via a radioprotein-binding assay) for serum (plasma) and RBCs, respectively [22,23]. As folate requirements are increased during pregnancy and lactation, a 2015 WHO guideline for women of reproductive age recommends in this population a RBC folate concentration above 906 nmol/l (derived with the use of a microbiological assay) to achieve the greatest reduction of NTD risk [24].

The use of convenient cut-offs is essential to correctly assess folate status. Recently, Pfeiffer *et al.* made clear that the application of inappropriate cut-offs leads to misinterpretation of folate status in the U.S. [25]. In their study, they demonstrated that the mismatching of cut-offs derived from one assay (e.g. microbiological assay) with population-based data from another assay (e.g. radioprotein-binding assay) risks misjudgment of the nutritional status. When the comparability between different assays is known, assay-adjusted cut-offs can be calculated. However, this information is often missing. Therefore, the authors encourage to invest in comprehensive method comparison studies for the derivation of intermethod conversion factors in order to establish assay-adjusted cut-offs.

2. The measurement of folates in clinical matrices

Plasma or serum folate measurement is widely applied in clinical laboratories and, to this day, provides clinicians with an indication of the folate status. However, these measurements are affected by recent intake changes. Therefore, this method relies on an adequate (overnight) fasting period to assure that steady-state folate concentrations are measured, since only these correlate with folate stores elsewhere in the body. Absorption following ingestion of chemically pure 5MTHF is rapid, with peak plasma concentrations readily occurring after one hour. Food

5MTHF is absorbed more slowly, with peak plasma concentrations after only 2 hours [26]. Following consumption of oxidized folate, i.e. FA, peak plasma concentrations are reached after only 3 hours because a reduction by DHF reductase (DHFR) is required prior to release in the portal circulation. Alternatively, RBC folate concentrations provide an average folate concentration over the lifetime of these cells as folates accumulate in these cells during erythropoiesis and remain present there as polyglutamate derivatives [2].

The relevant folate species are 5MTHF, THF, DHF, 5,10CH_2THF and 10FoTHF [27]. However, as a result of folate instability and interconversions, not all of these can be quantified directly. Moreover, oxidation of 5MTHF leads to the formation of 4'-hydroxy-5-methyltetrahydrofolate (hmTHF), which structurally rearranges to a pyrazino-s-triazine derivative known as MeFox. This folate species, of which the chemical structure is presented in Figure 1B, is metabolically inactive. However, since it may be formed during sample treatment or storage and may interfere with the determination of other folates, it is of interest to measure this compound as well when aiming at an accurate folate profiling of a sample [28]. As a summary, Figure 3 depicts the degradation and interconversion pathways of the different folate vitamers under different conditions.

2.1. Sampling strategy

Samples for clinical folate analysis are typically blood, plasma or serum derived thereof, obtained following venipuncture. Table 1 shows that recent methodologies aim to limit the burden on the patient by the collection of microsamples, obtained following a finger or heel prick. Already in 1999, O'Broin and Gunter reported on the use of a MA for screening the folate status in neonates using dried blood spots [29]. In 2015, Kopp and Rychlik published a stable isotope dilution assay using LC-MS/MS to determine 5MTHF in dried blood and serum spots [30]. Hereby, aliquots of blood, plasma or serum were spotted on paper pretreated with ascorbic acid. The folates were extracted from 3-mm punches by sonication in a buffer containing 2-(N-

morpholino)-ethanesulfonic acid, Triton X-100 and dithiothreitol (DTT). To measure total folate, whole blood spot extracts were treated with rat serum and chicken pancreas GGH. Plasma folates could be determined from whole blood spots by first performing heat denaturation to abolish endogenous GGH activity. These authors found that 5MTHF could be determined at LOD's of 1.5, 2.2 and 9.1 nM for serum, plasma and whole blood, respectively, and that spotted samples were stable for 11 days when kept desiccated at -20°C. During storage and extraction, the presence of labeled internal standards, added after spotting, compensated for the degradation of the folates present. Pretreating the paper with ascorbic acid did not provide adequate stabilization of THF, which consequently could not be quantified [30]. For longer storage, lower storage temperatures (-80°C) are required, as shown by Zimmerman *et al.* [31].

More recently, the same research group published the development and application of volumetric absorptive microsampling (VAMS) as an alternative microsampling strategy for whole blood 5MTHF assessment [32]. With this technique a fixed volume of blood is collected on an absorbent tip rather than on filter paper. This approach can offer a solution to the well-known hematocrit (Hct) effect in DBS analysis. Nevertheless, we and others observed that Hct may still influence analyte recovery from VAMS [33-35]. Although the authors didn't assess the impact of Hct on extraction efficiency, they did acknowledge the necessity of evaluating this parameter. Despite a fivefold reduction in sample volume compared to DBS (10 µl vs. 50 µl), a comparable LOD of 9 nM could be achieved when using VAMS. Remarkably, in contrast to their DBS methodology, where filter paper was pretreated with ascorbic acid, VAMS were used without pretreatment. The authors hypothesize that this may be owing to the influence of the chemical composition of the absorptive matrix on the degree of hemolysis. Samples were stable during 2.5 h drying at RT. Once dried, samples could be kept for 3 weeks at -20°C without any significant analyte degradation.

In conclusion, the above mentioned dried matrix sampling techniques have been reported to offer an important advantage for follow-up by increasing folate stability [30-32]. Furthermore, the possibility for automation, the easier sample storage and handling, and no need for medical surveillance during sampling are important improvements in the field of clinical folate determination, especially in remote regions.

2.2. Sample preparation

Sample preparation is often laborious and time-consuming, whereby analytes are exposed to elevated temperatures, light, oxidants, pH etc. Although isotope labeled internal standards are included in LC-MS/MS methods, accounting for folate losses, their utility may be lost when too little internal standard is left following sample preparation. Therefore, it is crucial to consider conditions that support the stabilization of folates during each step of the analysis. Figure 3 represents the effects of different conditions, applied during sample preparation, on folate stability.

2.2.1. Anticoagulants

Ethylenediamine tetraacetic acid (EDTA) is the most used anticoagulant to generate plasma as well as for whole blood folate analysis. However, Hannisdal *et al.* investigated the influence of anticoagulants on folate stability during storage at room temperature and, strikingly, found a significant impact of EDTA-treatment on the stability of 5MTHF [36]. During the first hours of storage, a significant oxidation of this folate to MeFox was observed. Given its biological inactivity, this compound is not measured using a MA. However, the total folate content (including MeFox), either measured via LC-MS/MS or GC-MS analysis of pABA-glutamate(s), is not influenced by this oxidation reaction. In a study investigating the stability of fat- and water-soluble vitamins during long term storage at -20°C, a similar pattern was observed for EDTA-plasma samples analyzed by a competitive binding assay [37]. Based on

the data obtained, storage was considered acceptable up to 6 months, provided that samples were kept at -20°C. Serum samples as well as citrate or heparin treated plasma samples showed less oxidation and should therefore be preferred when long term storage is foreseen [36]. However, depending on the anticoagulant used, measured plasma folate concentrations may differ to a limited extent [38]. Also the folate concentration in erythrocytes was found to be influenced by the anticoagulant. O'Broin *et al.* found substantial losses of folate activity (assessed by MA) in the presence of EDTA during storage at room temperature, which were observed to a lesser extent in heparinized samples [39]. While heparinization may improve sample stability upon storage, fresh heparinized samples readily had a slightly lower (3.61%) folate activity as compared to fresh EDTA-treated samples [39].

2.2.2. Antioxidants

Erythrocyte folate analysis is commonly performed by 10-fold dilution of whole blood with a 1% L-ascorbic acid solution in deionized water (pH=±3). The use of an antioxidant during cell lysis ensures immediate protection against oxidative damage of intracellular folates. Also when an alternative lysis procedure is performed, for instance using a selective ammonia based buffer, the addition of L-ascorbic acid is required to protect the intracellular folates against degradation [11]. Since the utilized lysis procedure might differ between labs, it can be a possible source of variability between methods, due to incomplete lysis. This influence of different lysis procedures was demonstrated by O'Broin *et al.*, whereby the pH of the 1% L-ascorbic acid solution affected hemolysis and therefore the final analytical result [40].

Addition of a thiol such as DTT or 2-mercaptoethanol is required to capture the formaldehyde released by degradation of ascorbic acid at elevated temperature. This is relevant since free formaldehyde causes the methylation of THF to 5,10-methenyltetrahydrofolate (5,10CH^{+}THF) and, as such, influences the measured folate distribution [1].

Though performed in a food matrix, Patring *et al.* investigated the effect of different reducing agents on the stability of folates during sample preparation [41]. The addition of 2-mercaptoethanol, dithiothreitol, 2,3-dimercapto-1-propanol and 2-thiobarbituric acid in combination with sodium ascorbate was evaluated for different experimental conditions. The choice of antioxidant had an effect on the measurement of THF following various sample treatment procedures including heat treatment, freeze-thawing and frozen storage. While 2,3-dimercapto-1-propanol was most effective, these authors suggested to evaluate the stability of all folates for each specific sample treatment procedure, a suggestion we concur.

2.2.3. pH

Folates are ionogenic and amphoteric compounds. They undergo changes in ionic forms as a function of the pH, which explains the influence of pH on the stability during analysis (Figure 3). De Brouwer *et al.* investigated the influence of pH on folate recovery following 2 hours of incubation in a phosphate buffer with a pH ranging between 4 and 8 [1]. While most folates proved stable at the different pH levels, some degradation was observed for THF at acidic pH. Both DHF and 5,10CH_2THF are extremely sensitive to incubation at a pH lower than 8. Though 5,10CH_2THF is an essential intermediate for folate metabolism in living cells, this compound itself is difficult to determine due to its limited stability at physiological pH [42,43]. This property implies that 5,10CH_2THF, as well as DHF, cannot be measured accurately using the commonly applied sample preparation techniques [1,43,44]. Horne showed that extraction of liver tissue at high pH (> 9.5) results in stability of 5,10CH_2THF, despite the absence of antioxidants [45]. However, other folates (e.g. THF) require antioxidants (e.g. ascorbic acid) for stabilization at this high pH. 5,10CH_2THF can then be determined indirectly via subsequent borohydride reduction to 5MTHF, allowing the differential measurement of 5,10CH_2THF. Likewise, 10-FoTHF, a possible intermediary during the purine biosynthesis, cannot be detected when acidic conditions are employed, since it converts to 5,10CH^+THF [1]. However,

very recently, Schittmayer *et al.* did develop a method for the determination of all naturally occuring cellular folate species [46]. Derivatization with heavy isotope labelled reagents resulted in the stabilization of folate derivatives. Therefore, the method allows discrimination of the structural isomers 5-FoTHF and 10-FoTHF. In addition, this method permits the use of unlabeled standards, derivatised with unlabeled reagents, as internal standard rather than the costly isotope labeled compounds. Both mono-and polyglutamylation state of folates are quantified, hereby avoiding lengthy incubation steps and gaining valuable information about the cellular regulation of the folate pathway.

Akhtar *et al.* studied the influence of light on the stability of FA in aqueous solutions and found that a high pH, exceeding 10.0, was optimal to minimize degradation (Figure 3) [47]. However, since most sample treatment steps are performed at or below neutral pH, sample treatment is best performed under subdued light.

2.2.4. Enzyme treatment

Theoretically, no enzyme treatment is necessary for the analysis of folates in serum or plasma samples: while intracellular folate retention depends on polyglutamylation, only mono-glutamates have been observed in plasma. This is owing to the presence of GGH in plasma, an exopeptidase that deconjugates any polyglutamate that would be released in plasma following cell lysis [28]. When measuring folates in whole blood lysate, monoglutamates are recovered by the endogenous GGH, present within the plasma portion of whole blood. However, this requires incubation times for up to 4h at 37°C prior to analysis [48]. These lengthy incubation periods risk degradation of the labile folates. When the RBC folate concentration is directly determined, the addition of exogenous GGH (rat serum or chicken pancreas) is required. Recently, Stamm *et al*. demonstrated that the additional use of a commercially available recombinant exogenous GGH would lead to a minimization of the incubation time, reducing degradative losses of folate vitamers [49].

Large quantities of proteins capable of binding folates, most importantly folate binding proteins (FBP), are found in clinical samples, rendering folates unavailable for analysis. Total folate measurement requires release of these bound folates via protease treatment or via gentle acidification, since both the specific (FBP) and non-specific binding capacity is negligible below pH 4 [50].

2.3. Sample storage

The optimal storage of a clinical sample is critical to obtain a trustworthy result. Since folates are known to be relatively unstable, different conditions, to ensure both short-and long-term stability, need to be evaluated. First, short-term storage includes time of transport (whole blood), serum-clot contact time and the time until samples are stored for a longer period (serum/plasma). As samples may be transported from distant clinics for analysis, whole blood folate stability should be evaluated. Van Eijsden *et al.* reported 24 h folate stability in EDTA-anticoagulated blood at room temperature [51]. Next, Zhang *et al.* were the first to investigate the effect of delayed whole blood processing (or serum-clot contact time) on folate stability [52]. They found acceptable stability for total folate after 1 day of contact time at 32°C. However, this was not confirmed by Drammeh *et al.*, who reported a significant decrease in total folate level after 1 day at 32 °C [53]. Fazili *et al.* evaluated this effect of processing delay during 3 days at 32°C, whereby they differentiated between the effect on total folate and on the different folate forms [54]. Similar to Drammeh *et al.* significant decreases were observed in total folate level, which was mainly caused by a decrease in 5MTHF, while FA concentrations remained stable. Those same two research groups also investigated the influence of delayed freezing of serum samples on folate stability. Both observed significant decreases in total folate levels after longer delays in freezing (7-14 days) [53,54].

Second, epidemiological studies often involve long storage times between sample collection and laboratory analysis. Therefore, several research groups investigated long-term folate

stability in serum samples. Hannisdal *et al.* measured folate levels in serum samples stored at -25°C for up to 29 years [55]. Concentrations were derived using 3 different assays: 1) an LC-MS/MS assay, measuring the different folate species; 2) a microbiological assay, measuring microbiologically active folate; and 3) an LC-MS/MS assay, assessing folate status as p-aminobenzoylglutamate equivalents. The authors observed that folate is substantially degraded in serum frozen for years. However, they also noted that most of the folate loss was recovered as p-aminobenzoylglutamate equivalents. Therefore, this latter assay is suggested as the method of choice for the analysis of long-term stored samples. Both Jansen *et al.* and Fazili *et al.* confirmed this folate instability in serum samples stored at -20 °C [54,56]. They observed that storage at -70°C is necessary to obtain reliable results from samples stored for 1 year.

On the other hand, sometimes samples have to be reanalyzed due to an analysis which didn't meet the predetermined criteria. Therefore, the evaluation of folate stability during repeated freeze/thaw cycles is of great importance. Fazili *et al.* found that folates (total, 5MTHF and FA) are fairly stable when exposed to a limited number of freeze/thaw cycles. However, stability of the most labile folate form THF was not discussed [54].

Last, the group of Pfeiffer recently investigated the influence of ascorbic acid (5 g/L), added to serum, during repeated freeze/thaw cycles (within 8 months) and long-term storage (≥4 years at -70°C), on serum folate stability [57]. The authors showed that the presence of the antioxidant may even protect the most labile folate form THF during the repeat analysis from a same vial within an 8-month period. In addition, all serum folate forms showed acceptable stability during at least 4 years of storage at -70°C. Therefore, the addition of ascorbic acid to serum is highly recommended to ensure long-term folate stability.

2.4. LC-MS/MS analysis

Multiple chromatographic methods have been developed for the measurement of 5MTHF and other folate species. The availability of LC-MS(/MS) equipment has fueled the development of higher-order assays to measure folates in both serum or plasma and RBCs. Below, we focus on the most recent developments for clinical folate analysis (represented in Table 1). For an in-depth overview of other methods, we refer to Pfeiffer *et al.*, 2010 [58].

GC-MS based methods rely on the cleavage of the 9-10 bond between the pterin and the pABA-Glu moiety followed by derivatization (Figure 1). Though these methods allow for a total folate measurement, they do not allow for individual folate speciation and rely on a quantitative (i.e. complete and selective) folate cleavage.

While LC-MS/MS methods attempt to quantify the different folate species as they occur at the time of phlebotomy, some methods explicitly interconvert some folate species. Both van Haandel *et al.* and Huang *et al.* performed acidification of the final sample to determine 10FoTHF and 5FoTHF as 5,10CH^{+}THF (Figure 3) [10,59]. While at a pH below 10, almost instantaneous conversion of 10FoTHF to 5,10CH^{+}THF will occur, it is possible to determine 5FoTHF and 5,10CH^{+}THF separately (Table 1) (cfr. Kiekens *et al*., Fazili and Pfeiffer, Nandania *et al.*, Smith *et al*., Kirsch *et al*., and Fazili *et al*.) [11,38,44,60-62].

2.4.1. Sample Clean-up

To minimize the presence of matrix compounds and assure method selectivity, sample clean-up is performed prior to LC-MS/MS analysis. This can range from simple protein precipitation to combined affinity and solid phase extraction (SPE) (Table 1). As with any sample clean-up procedure, simplicity and throughput need to be balanced against the sensitivity and robustness that will eventually be required.

While extensive sample treatment is often preferred to obtain purified extracts, some methods simply use protein precipitation with an organic solvent, like methanol or acetonitrile, followed

by evaporation to dryness and reconstitution in mobile phase (Table 1). For serum or plasma, this approach seems feasible since these are relatively clean matrices. For RBCs, however, either SPE alone or SPE combined with affinity extraction is needed.

Given its selectivity, FBP is ideally suited to extract folates from complex matrices. While FBP is now commercially available in purified form, it can also be obtained from bovine milk. For affinity extraction procedures, FBP is bound to agarose beads and stored refrigerated in the presence of sodium azide as a preservative [63,64]. Sample preparation is performed in a column configuration, with elution under acidic conditions since FBP shows little folate retention at a pH below 4. The fact that FBP does not have equal affinity for all folate species necessitates a substantial excess of binding sites to quantitatively retain all folates [65]. The number of samples which can be purified using one column is limited due to degradation of and irreversible binding to the protein. As such, there is a gradual decrease of binding capacity, which has to be controlled to avoid erroneous sample handling [66].

Recently developed methods have shown the value of SPE. Especially for the analysis of numerous samples, SPE is preferred over FBP columns. For low-throughputs, manual column-format SPE can be used, while for higher throughputs, the different SPE steps can be automated and/or 96-well formats can be used [62]. Hence, samples can be run in parallel, limiting total analysis time, and therefore improving folate stability during mass screenings. Depending on the charge state of folates in solution, both reversed phase and ion exchange sorbents can be used to separate folates from matrix constituents. The former being used more frequently, since serum samples are commonly diluted in 1% L-ascorbic acid solution, which promotes the protonation of α and γ glutamate carboxyl groups. Phenyl and octadecyl sorbents are most frequently used and elution is often performed using a small percentage of organic solvent, either methanol or acetonitrile, acidified to correspond to the mobile phase. In contrast, Monch *et al.* published on the extraction of blood folates using 2-(N-morpholino)ethanesulfonic acid

buffer at pH 5 [67]. At these conditions, the carboxyl groups are ionized, necessitating the use of an anion exchange sorbent for sample clean-up. Additionally, this set-up enabled the authors to quantify folates as well as their catabolites, para-aminobenzoylglutamate and acetyl-para-aminobenzoyglutamate. Further, as already discussed before, the acidic pH of both elution solvent and mobile phase may influence folate stability. In addition, it must be considered that high concentrations of salt, used for elution, may interfere with compound ionization when using LC-MS/MS. Finally, samples can be concentrated prior to injection by evaporation or by using a vacuum system, followed by reconstitution in the initial mobile phase or in another appropriate solvent [11,36,60,61,63,64,67-73].

2.4.2. Chromatographic separation

Both standard HPLC and UHPLC have been applied for the separation of folate species in clinical samples. Using traditional reversed phase columns, typically C_8 or C_{18}, folate species can relatively easily be separated. MeFox, despite being an oxidation product of 5MTHF, is also worth measuring as it allows insight into possible oxidative stress the sample was subjected to during e.g. sample storage or treatment. However, care should be taken to differentiate MeFox from its isobar 5FoTHF, which requires optimization of the chromatography [71,74]. Alternatively, when using MS/MS-based detection, it is possible to distinguish both folate species by selecting certain product ions (see 2.3.3), although maximal sensitivity may not be achieved this way [71].

Separation is mostly achieved by applying gradient elution with acidic mobile phases containing either acetic or formic acid in ultrapure water and methanol, acetonitrile or a combination of both. The use of salt buffers is not common and mainly reserved for hydrophilic interaction liquid chromatography (HILIC) [68]. In 2006, Patring *et al.* investigated the elution pattern of folates when varying the stationary phase and the mobile phase constituents [75]. The ionization of the α- (pKa= 3.1 - 3.5) and γ- (pKa= 4.6 - 4.8) carboxyl groups of the glutamate

part of the folate molecule was found to influence the retention behavior. A pH<3 is needed to completely suppress the ionization of both carboxyl groups. At higher pH (>3), the retention of the folates decreases to a varying extent depending on the column chemistry. This also negatively impacts peak symmetry and peak width. This effect is also noticed when the amount of organic solvent at the time of injection is increased. High retention of folates was achieved on columns with either polar endcapping or with a high carbon content. When volatile modifiers are used, i.e. either acetic or formic acid, retention is generally increased while peak shape deteriorates, though this effect was most pronounced using polar endcapped columns.

2.4.3. Detection methodology

Both ultraviolet, fluorescence [76,77] and electrochemical [78] detection have been applied for folate analysis in food items. Reduced folates show strong fluorescence (290-295 nm→ 356 nm), making this type of detection highly efficient and specific for the detection of THF, 5MTHF and 5FoTHF in food matrices and for quality control purposes. Typical methods using fluorescence detection for 5MTHF in food items have an LOD of 20 to 40 fmol on column [76,77]. Oxidized folates do not fluoresce but all folates can be detected using a UV detector (±280 nm), albeit at the expense of sensitivity, which drops with a factor 50 to 100 compared to fluorescence detection [78]. With detection limits down to 0.2 to 0.4 fmol on column, recent LC-MS/MS methods for the determination of folates in plasma or serum are 50 to 100 times more sensitive than fluorescence detection, allowing quantitation in ever decreasing volumes (microsamples *cfr*. [30,32]). As such, the use of fluorescence detection for clinical folate analysis would be limited to the quantification of RBC or whole blood folates due to the higher folate concentration in these samples. UV and electrochemical detection are not able to match the sensitivity of fluorescence detection and cannot be used for clinical samples as they would require vast sample volumes [78].

For clinical analyses, MS (and more particularly MS/MS) is most used as detection technique hyphenated with liquid chromatography. Folates, containing both alkaline (pterin moiety) and acidic (glutamate moiety) sites, can be positively and negatively charged in the pH range of mobile phases containing volatile modifiers such as acetic and formic acid. As such, MS systems can be operated both in positive and negative mode. Electrospray ionization (ESI) is most commonly used as an interface system to vaporize the mobile phase. To achieve high ionization efficiencies, a modifier (*i.e.* formic or acetic acid) is commonly used and is either included in the mobile phase (*cfr. 2.4.2*) or infused post column [72]. In general, folates show predominant single ionization in either negative or positive mode, with the latter being used most. However, double ionization can occur, though more in positive than in negative mode. As such, singly charged ions are typically used as Q1 ions (MW+1 or MW-1) during MS/MS-analysis. Generally, 2 fragment ions are monitored, as this allows to add the ion ratio as an extra identification criterion. In positive mode, the most abundant fragment ion, used as quantifier, generally consists of the ionized remainder (pterin-pABA$^{+\bullet}$), following neutral loss of the glutamic acid$^{\bullet}$ moiety [60,79]. 5,10CH^{+}THF differs from the other folates in that the glutamate moiety is not split off due to the withdrawal of electrons from the nitrogen atom at the 10 position and the resulting influence on ion stabilization [60]. During fragmentation, the formyl groups bound to the nitrogen at the 5 or 10 position have a tendency to split off. This phenomenon, the expulsion of CO, does not influence the charge of the remaining ion and has little influence on the signal intensity of the ion which is monitored [80]. The second fragment ion is typically formed after cleavage of the bond between position 6 and 9 or 9 and 10, yielding ions of 166 (representing the reduced pterin cation), 176 or 194 Da (the latter two resulting from loss of both Glu and pABA). For 5,10CH^{+}THF, the second fragment ion that is typically followed is formed after the loss of glutamate-C=O [81]. The quantitative LC-MS/MS determination of 5FoTHF and MeFox is complicated by their identical molecular mass of 474

amu and the fact that both are predominantly fragmented to a fragment ion of 327 amu. Both compounds also have a tendency to coelute. Though baseline resolution is achievable, the less abundant ions of 284 and 299 amu, specific for MeFox and 5FoTHF, respectively, can be used as quantifiers [71].

2.5. Method comparability

Method comparability has remained a substantial issue hindering folate analysis because of the influence of several procedural aspects such as the use of calibrators, strains of micro-organisms and antioxidants. Indeed, even when the same analytical technique is applied, folate results may show poor comparability across laboratories [82,83]. E.g., a review of the performance of different assays in relation to a comparison assay (the CDC MA, calibrated with 5MTHF) showed that different methods within the same assay type can generate very different results relative to a comparison assay. To this day, the MA, despite its limitations, is still considered the gold standard for folate measurement. As such, the performance of alternative methods such as PBA and LC-MS/MS assays is measured against this MA [84]. For serum or plasma, the total folate results obtained using the MA (calibrated with 5MTHF) and LC-MS/MS analysis were found to be in good agreement, although slightly higher concentrations were found with LC-MS/MS. Results obtained using a PBA (calibrated with FA) were much (i.e. close to 30%) lower than those of either the MA or LC-MS/MS assay. This is likely due to a lower recovery of certain folate species, amongst which 5MTHF, in the PBA. As such, results depend on whether 5MTHF or FA is used as a calibrator. For RBC folates, a good agreement was found between results obtained by MA and those obtained by LC-MS/MS, although here total folates determined by LC-MS/MS were 10% lower than those scored by MA. Again, a substantial difference was seen between the results obtained by MA and PBA, the results in the latter being 45% lower. Importantly, this difference was genotype dependent, with as an underlying cause

a differential recovery of different folate species [85]. As such, the MA was maintained as reference method for whole blood or RBC folate measurement [84].

2.5.1. Assay calibration

One of the major issues complicating folate analysis is the comparability of analytical results between labs throughout the world. Therefore, correct calibration of an assay is of paramount importance to obtain correct results.

Perhaps the most important part of an assay is its calibration using pure reference standards. These can be obtained from various sources, such as Merck Eprova (Schaffenhausen, Switzerland), Schircks Laboratories (Jona, Switzerland) or Sigma-Aldrich (Saint-Louis, MO, USA). As mentioned above, several authors have indicated that the calibrator choice can strongly influence analytical results [69,86]. The actual panel of folates that is measured will determine the total folate concentration measured by LC-MS/MS, as the latter is derived from the sum of the individual folate species. On the other hand, the composition of the panel of folates that can be reliably quantified, depends on the availability of pure reference standards.

While the use of MS allows to differentiate individual folate species, the technique does suffer from matrix-associated effects, including differences between individual samples influencing the measured signal. For this reason, isotopically labeled internal standards are commonly used for LC-MS/MS folate analysis, so-called stable isotope dilution assays, to compensate for the influence of the matrix on recovery and ionization efficiency. For this purpose, isotopologues of the parent compounds are used in which either carbon or hydrogen atoms are replaced with ^{13}C or ^{2}H atoms. A mass difference of 4 to 5 atomic mass units (amu) ensures that the analyte and the IS can be monitored separately, given the presence of natural isotopologues in samples (up to +2 amu) [87]. While deuterated ISs are easier to manufacture, deuterium ions from the IS and hydrogen atoms from the aqueous solvent can interchange, which impacts the isotopic

purity of the IS and may influence the analytical result. Also, given their location at the outside edge of the molecule, deuteration can change the charge density of the molecule, thereby slightly influencing its retention time [88]. For this reason, ^{13}C-labeled ISs are preferred over deuterium labeled ISs. Unfortunately, not every folate form has its own labeled IS commercially available. In that case, the nearest folate species in terms of stability and elution time is used as an IS. However, it should be evaluated on a case-by-case basis which IS is most appropriate for a specific folate.

Recently, the group of Pfeiffer showed that errors in method calibration are an important source for inaccurate results [89]. They experienced problems with folic acid solubility at certain pH and concentration conditions. As a result, an incorrect value was assigned to the folic acid calibrator, leading to an overestimation of serum folic acid concentrations.

The limited stability of folates also requires careful storage of prepared calibrators and, if these are gravimetrically prepared, likewise careful storage of the powder form. Reduced folates are susceptible to oxidative degradation while some, like 5FoTHF, are very hygroscopic. Therefore, it is advised to verify the purity of the standard material as part of the preparation of stock solutions. This can be performed spectrophotometrically based on the molar extinction measured by Blakley [90]. In addition, it is worth noting that L-ascorbic acid, commonly used to stabilize folates in solutions, absorbs light at the same wavelengths as folates, making the spectrophotometric verification of folate concentrations in the presence of this antioxidant impossible [91].

2.5.2. Possible certified reference materials and the values measured

Key to the evaluation of method performance is the use of reference materials with a known folate content. These are commercially available materials that allow the comparison of results

obtained in different laboratories. Ideally, the concentration obtained should match, although for sensitive compounds, such as folates, this may be a tall order.

To accurately assess the performance of an entire method, a certified reference material (CRM) should resemble a native sample as close as possible. The spiking of folates to samples can be problematic due to differences in protein binding and may lead to divergent extraction behavior. It is therefore preferred to mix different blood pools to obtain a certain folate concentration. However, this is not always possible due to the limited and unpredictable presence of non-methyl-folates.

Two WHO-approved international standards are available for folates through The National Institute for Biological Standards and Control (NIBSC), one consisting of lyophilized serum (03/178) and the other of a lyophilized whole blood lysate (95/528). The NIBSC serum reference material has been assigned reference values for total folate, as well as for 5MTHF, 5FoTHF and FA. The application of the international standard was evaluated following the analysis of the standard material by 24 laboratories in 7 different countries, published by Thorpe *et al.* in 2007 [70]. These included 19 protein binding assays, 3 MA and 2 LC-MS/MS assays. Though significant variability was observed between the results obtained using the different assays, this study showed the potential of such a reference material. When the reference standard 03/178 for folates in serum was used to correct for the systematic error between the laboratories, significant gains in comparability were obtained [70]. Also, the National Institute of Standards and Technology (NIST) has issued a standard for folates in serum, the standard reference material (SRM) 1955 [92]. Concentration values were assigned in 2004 by four independent NIST methods and CDC method. To replace the SRM 1955 when stocks were depleted, a new standard reference material was prepared, i.e. the SRM 3949. This set of three serum samples was devised to contain, apart from 5MTHF and FA, the minor folate species THF, 5FoTHF, MeFox and 5,10CH$^+$THF. Given the limited availability of analytical results,

consensus concentrations for the different folate species using a variety of methods have not been reached yet [93].

Recently, Fazili *et al*. conducted, for the first time, two international round-robin studies for the systematical assessment of comparability, precision and accuracy of serum folate LC-MS/MS methods [89]. Next to 6 serum pools and 6 calibrators form the CDC, the two NIST SRMs (1955 and 3949) were analyzed by the CDC laboratory, 7 laboratories with independently developed methods and 6 laboratories with an adapted CDC method. Comparability was good for 5MTHF but poor for folic acid. Interpretation of the results for minor folate forms (FA, 5FoTHF and 5,10CH^{+}THF) was limited because 1) for most serum samples concentrations were below LOD and 2) fewer laboratories included these compounds in their method. This study demonstrated the key role of certified reference materials in order to compare results among different laboratories. In addition, this work showed the need for reference materials with certified concentrations for minor folate forms in order to improve method accuracy.

For whole blood lysate (95/528), a consensus value was assigned following a study in which 13 laboratories participated for a total of 34 assay results. Unfortunately, only MA and PBAs were used [94]. However, recently we published an LC-MS/MS method for the analysis of folates in RBC samples which includes data obtained using this reference material [11]. Table 2 lists the consensus concentrations for the reference materials (serum and whole blood) for the different analytical techniques, where available.

3. Conclusion

Given the ability of higher-order LC-MS/MS methods to differentiate between different folate forms and degradation products (e.g. MeFox), folate analysis has shifted from microbiological or protein binding assays to chromatographic methods. However, the relative instability of folates poses some challenges, whereby degradation and interconversions between folate

species may impact the final analytical result. Therefore, care must be taken during sampling, sample preparation and analysis in order to obtain accurate and comparable results.

Beside the classical sampling methods, it has been demonstrated recently that alternative strategies, such as dried blood spot sampling and volumetric absorptive microsampling, are suitable alternative techniques for the clinical determination of 5MTHF. So in future, it may be investigated whether such microsampling techniques can be applied to other folate species.

Given the problematic storage stability of EDTA-anticoagulated samples, heparin or citrate may be preferred over EDTA to limit the degradation and interconversion during sample storage. While whole blood lysis using a diluted ascorbic acid solution is commonly performed, small variations in the procedure can significantly influence the analytical outcome. The use of a thiol in combination with L-ascorbic acid has proven to be required for the stabilization of THF in food samples. This approach may prove beneficial to stabilize this folate species in clinical samples as well. Also the pH (extraction, elution solvent and mobile phase) has been proven to strongly influence folate stability, given the ionogenic nature of the compounds of interest. Overall, it is highly recommended to evaluate the stability of all folates for each specific procedure of analysis.

Inherent to MS detection is the influence of matrix-associated effects on the measured signal. Therefore, isotopically labeled internal standards should be included to compensate for the influence of the matrix on recovery and ionization efficiency. Which IS is most appropriate for a specific folate should be evaluated on a case-by-case basis, where, if available, ^{13}C-labeled ISs are preferred.

Lastly, given the problematic analytical calibration, a harmonization of folate calibrator preparation is recommended to compare results obtained using different procedures. In this respect, the use of certified reference materials, analyzed using a variety of methods, including

MA, PBA and, importantly, also LC-MS/MS assays, would prove beneficial to compare analytical results.

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plasma/serum							
Method	Year	Sample source	Folate species monitored	sample preparation	LOD-range [nmol/l]	Start volume [µl] (*whole blood)	Ref.
Garbis *et al.*	2001	plasma	5MTHF, THF, FA, 5FoTHF	PP-EV	0.04 - 0.43	2000	[68]
Hart *et al.*	2002	plasma	5MTHF	PP-AE	0.2	5000	[64]
Rychlik *et al.*	2003	plasma	5MTHF	PP-SPE	3.5	1000	[95]
Kok *et al.*	2004	EDTA plasma	5MTHF, FA	PP-AE-SPE-EV	0.012, 0.05	2000	[96]
Pfeiffer *et al.*	2004	serum	5MTHF, 5FoTHF, FA	SPE	0.05-0.13	275	[69]
Nelson *et al.*	2004	serum and citrate plasma	5MTHF	PP-AE or AE-SPE-EV	0.07 (affinity) or 0.87 (SPE)	500	[63]
Huang *et al.*	2008	EDTA plasma	5MTHF, 5,10CH$^+$THF, THF, FA	SPE-EV	1.5	300	[59]
Liu *et al.*	2009	plasma	6S-5MTHF, 6S-5FoTHF, 6R-5FoTHF	PP-EV-SPE	54.41, 105.7*	200	[97]
Hannisdal *et al.*	2009	serum	5MTHF, FA, 5FoTHF, hmTHF (now known to be MeFox)	PP-EV	0.07 - 0.52	60	[74]
Mönch *et al.*	2010	EDTA plasma	5MTHF, THF, FA, 5FoTHF, 10FoFA	SPE	0.05 - 5	400	[67]
Kirsch *et al.*	2010	serum	5MTHF, 5,10CH$^+$THF, THF, FA, 5FoTHF	SPE-EV	0.09 - 0.9	250	[61]
van Haandel *et al.*	2012	EDTA plasma	5MTHF, 5,10CH$^+$THF, THF, FA	PP	0.1 - 0.5	50	[10]
Fazili *et al.*	2013	serum	5MTHF, 5,10CH$^+$THF, THF, FA, 5FoTHF, MeFox	SPE	0.06 - 0.31	150	[62]
Wang *et al.*	2014	serum	5MTHF, FA, 5FoTHF	EV	0.11	100	[98]
Kiekens *et al.*	2015	EDTA plasma	5MTHF, 5,10CH$^+$THF, THF, FA, 5FoTHF, 10FoFA	PP-SPE	0.33-0.5	300*	[11]
Zheng *et al.*	2015	plasma	5MTHF, FA	PP-EV	11.0, 0.56	500	[99]
Kopp and Rychlik	2015	plasma	5MTHF	SPE	1.5	30	[30]
Guiraud *et al.*	2017	plasma	5MTHF	PP	6	50	[100]
RBC							
Method	Year	Sample source	Folate species monitored	sample preparation	LOD-range [nmol/l]	Start volume [µl]	Ref.
Fazili and Pfeiffer	2004	EDTA whole blood	5MTHF, 5,10CH$^+$THF, THF, FA, 5FoTHF, 10FoFA	SPE	0.05 - 2.5	100	[38]
Smith *et al.*	2006	EDTA whole blood	5MTHF, 5,10CH$^+$THF, 5,10CH$_2$THF, DHF, THF, FA, 5FoTHF, 10FoTHF	PP-AE-SPE-EV	0.4 - 20*	100	[60]
Huang *et al.*	2008	EDTA whole blood	5MTHF, 5,10CH$^+$THF, THF, FA	SPE-EV	1.5	500	[59]
Mönch *et al.*	2010	lyophilized RBCs	5MTHF, THF, FA, 5FoTHF, 10FoFA	SPE	4 - 54	100	[67]
Kirsch *et al.*	2012	EDTA whole blood	5MTHF, THF, FA, 5FoTHF, 10FoFA	SPE-EV	0.12 - 0.40	200	[73]
van Haandel *et al.*	2012	EDTA whole blood	5MTHF, 5,10CH$^+$THF, THF, FA	PP	0.2 - 1.0	50	[10]
Kiekens *et al.*	2015	EDTA whole blood	5MTHF, 5,10CH$^+$THF, THF, FA, 5FoTHF, 10FoFA	PP-SPE	0.33-0.5	300	[11]
Kopp and Rychlik	2015	EDTA whole blood	5MTHF	SPE	9.1	50	[30]

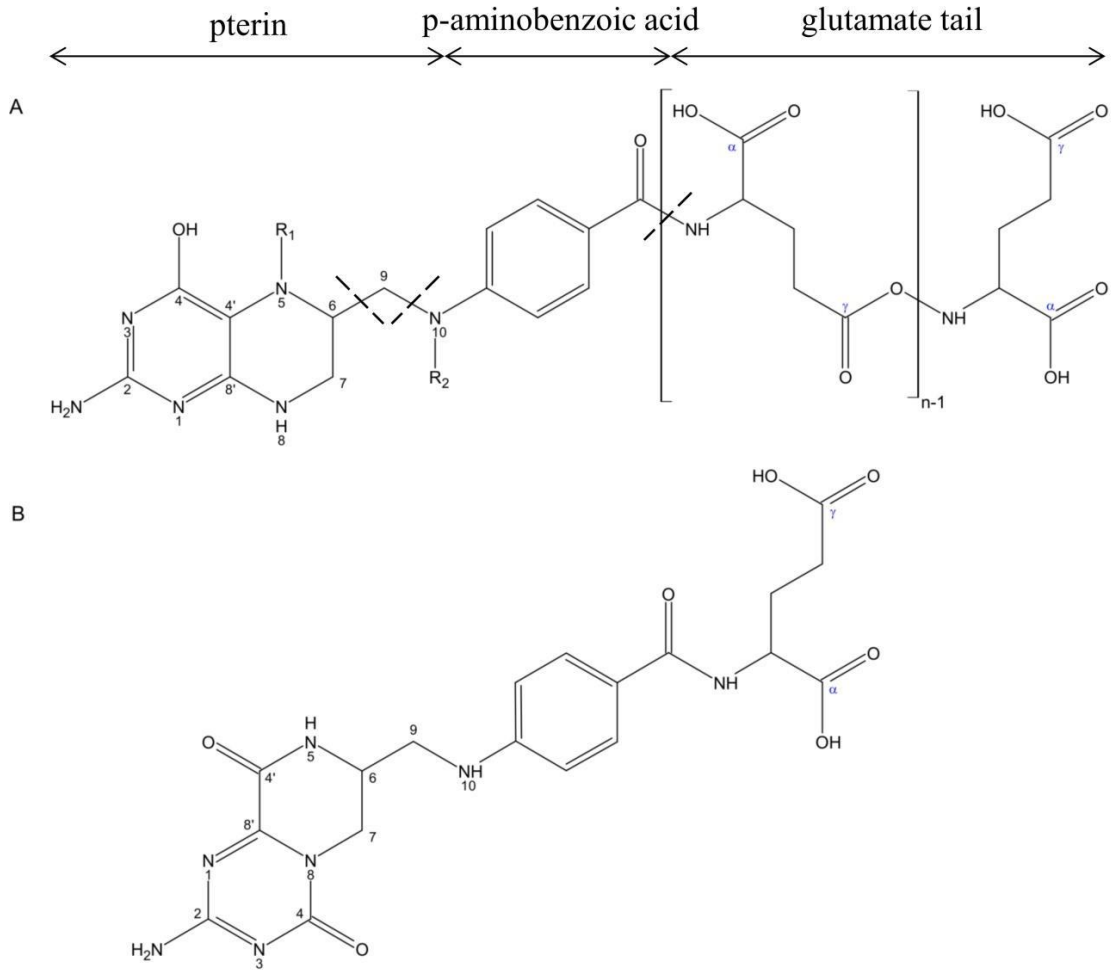
Kopp and Rychlik	2017	EDTA whole blood	5MTHF	PP	9	10.8	[32]
Nandania *et al*.	2018	Whole blood	5MTHF, 5,10CH_2THF, THF, 5FoTHF, 5,10CH^+THF	SPE	0.218-0.656	40	[44]

Table 1: overview of recent LC-MS/MS methods for folate measurement. *LLOQ as no LOD was reported; AE, affinity extraction; 5,10CH^+THF, 5,10-methenyltetrahydrofolate; 5,10CH_2THF, 5,10-methylene tetrahydrofolate; EV, evaporation to dryness; FA, folic acid; 10FoFA, 10-formylfolic acid; 5FoTHF, 5-formyltetrahydrofolate; hmTHF, 4α-hydroxy-5-MTHF; LLOQ, lower limit of quantification; LOD, limit of detection; MeFox, pyrazino-s-triazine derivative of hmTHF; 5MTHF, 5-methyltetrahydrofolate; PP, protein precipitation; RBC, red blood cell; SPE, solid-phase extraction; THF, tetrahydrofolate.

			MA	PBA	LC-MS/MS			
reference material	matrix	level	total folate [nM]	total folate [nM]	FA [nM]	5MTHF [nM]	5FoTHF [nM]	sum [nM]
NIST SRM 1955	serum	I	5.6±1.2	4.5±0.4	0.49±0.17	4.26±0.25	1.3±0.4	6.0±0.4
		II	14±3	10±1	1.05±0.16	9.73±0.24	2.3±0.8	13±1
		III	44±11	25±3	1.07±0.24	37.1±1.4	3.6±1.3	41±2
NIBSC 03/178	serum		12.6±3.3	8.75-19.2[a]	0.74±0.47	9.75±1.1	1.59±0.13	12.1±3
NIBSC 95/528	whole blood		9.45-19.33[b]	6.75-16.68[b]	n.a.	n.a.	n.a.	n.a.

Table 2: consensus concentrations for NIST and NIBSC reference materials. Values obtained from Thorpe *et al*
a: for serum [70] and b: for whole blood [94]; FA, folic acid; 5FoTHF, 5-formyltetrahydrofolate; MA, microbiologic assay; 5MTHF, 5-methyltetrahydrofolate, PBA, protein-binding assay.

Figure 1: chemical structure and typical MS/MS fragmentation (indicated by dotted lines) of a number of relevant folates (A) and chemical structure of MeFox, the metabolically inactive degradation product of 5MTHF (B).



folate (A)	**5 − 6**	**7 − 8**	**R_1**	**R_2**
tetrahydrofolate	−	−	-H	-H
dihydrofolate	=	−	-H	-H
folic acid (pteroyl glutamate)	=	=	-H	-H
5-methyltetrahydrofolate	−	−	$-CH_3$	-H
5-formiminotetrahydrofolate	−	−	-CHNH	-H
5-formyltetrahydrofolate	−	−	-CHO	-H
10-formyltetrahydrofolate	−	−	-H	-CHO
10-formylfolic acid	=	=	-H	-CHO
5,10-methylenetetrahydrofolate	−	−	$-CH_2-$	
5,10-methenyltetrahydrofolate	−	−	$-CH^+-$	

Figure 2: cytosolic folate metabolism; 5,10CH^{+}THF, 5,10-methenylTHF; cSHMT, cytoplasmic serine hydroxymethyltransferase; DHFR, dihydrofolate reductase; FA, folic acid; 5FiTHF, 5-formiminoTHF; 10FoFA, 10-formylFA; 5FoTHF, 5-formylTHF; FTCT, glycine formiminotransferase/ formimidoyltetrahydrofolate cyclodeaminase and glutamate formiminotransferase/ formimidoyltetrahydrofolate cyclodeaminase; FTHFS, 10-formyltetrahydrofolate synthetase; hmTHF, 4α-hydroxy-5-methylTHF; MeFox, pyrazino-s-triazine derivative of hmTHF; 5MTHF, 5-methylTHF; MS, vitamin B_{12} dependent methionine synthase; MTHFC, methenyltetrahydrofolate cyclohydrolase; MTHFD, methenyltetrahydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; SAHH, S-adenosyl-L-homocysteine hydrolase; SMAT, methionine adenosyltransferase; THF, tetrahydrofolate; TS, thymidylate synthase [101,102].

1

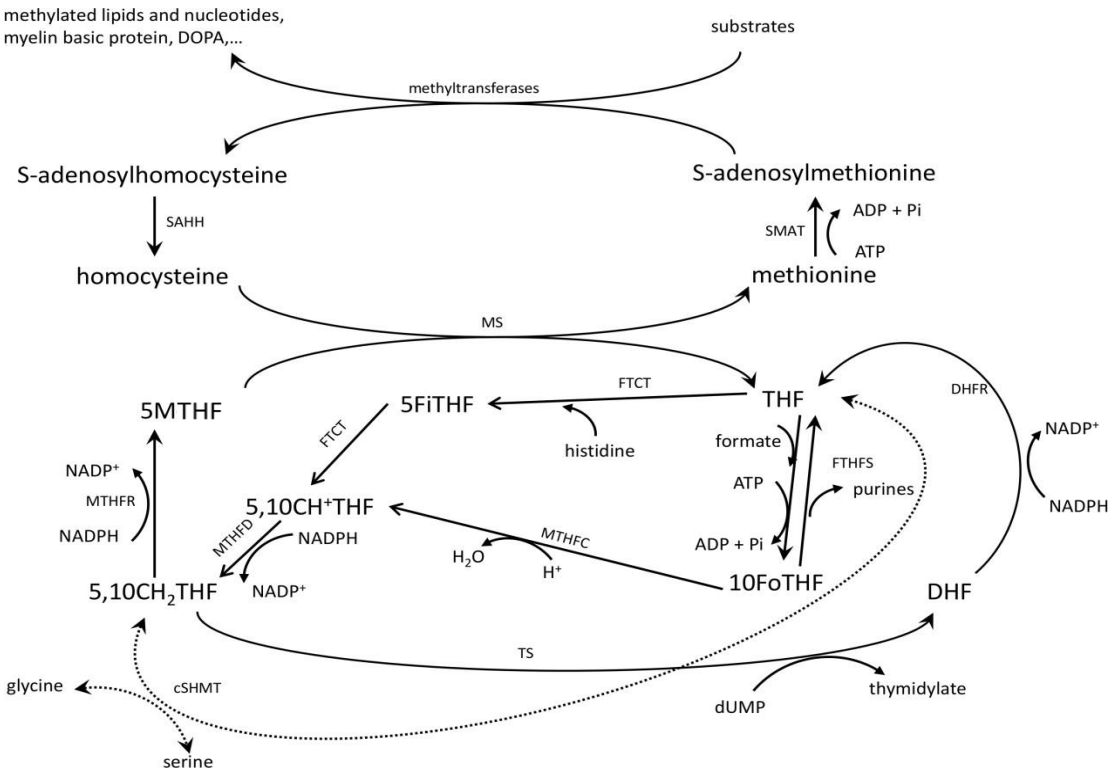


Figure 3: folate degradation and interconversion pathways under different conditions; 5MTHF, 5-methylTHF; 5MDHF, 5-methylDHF 5FoTHF,

5-formylTHF; 10FoFA, 10-formylFA; 10FoTHF, 10-formylTHF; 10FoDHF, 10-formylDHF; 5,10CH^{+}THF, 5,10-methenylTHF; 5,10CH_2THF,

5,10-methyleneTHF; DHF, dihydrofolate; FA, folic acid; hmTHF, 4α-hydroxy-5-methylTHF; MeFox, pyrazino-s-triazine derivative of hmTHF;

pABG, para-aminobenzoylglutamate; THF, tetrahydrofolate [1,42,47,103].