Journal of Chromatography A, 1398 (2015) 20–28

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

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

Filip Kiekens[a], Jeroen Van Daele[a], Dieter Blancquaert[b], Dominique Van Der Straeten[b], Willy E. Lambert[a], Christophe P. Stove[a,*]

[a] Laboratory of Toxicology, Department of Bioanalysis, Ghent University, Ottergemsesteenweg 460, B-9000 Gent, Belgium
[b] Laboratory of Functional Plant Biology, Department of Physiology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium

ARTICLE INFO

Article history:
Received 21 February 2015
Received in revised form 13 April 2015
Accepted 14 April 2015
Available online 20 April 2015

Keywords:
Folate
Plasma
Red blood cell
Polyglutamylation
Chromatography
Mass spectrometry

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 1–150 nmol/l and 5–1500 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.

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

* Corresponding author at: Laboratory of Toxicology, Faculty of Pharmaceutical Sciences, Ghent University, Ottergemsesteenweg 460, B-9000 Gent, Belgium. Tel.: +32 9 264 81 21; fax: +32 9 264 81 83.
E-mail addresses: filip.kiekens@UGent.be (F. Kiekens), jeroen.vandaele@UGent.be (J. Van Daele), dieter.blancquaert@UGent.be (D. Blancquaert), dominique.vanderstraeten@UGent.be (D. Van Der Straeten), willy.lambert@UGent.be (W.E. Lambert), christophe.stove@UGent.be (C.P. Stove).

http://dx.doi.org/10.1016/j.chroma.2015.04.025
0021-9673/

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 2–12 h 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$_2$THF) 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 50 mM 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 h 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 min 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 25–37. To remove endogenous folates, plasma and RBC extracts were treated with activated charcoal as described for rat serum in Section 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 min 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 min 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 min 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-min incubation at room temperature, after which cell residues were spun down by centrifugation at 2500 × *g* for 10 min at 4 °C. One milliliter of supernatant was withdrawn and 100 μl of IS/AA/DTT solution was added. After incubation for 15 min at room temperature, these samples were analyzed further or frozen at −80 °C. A flowchart describing the sample preparation procedure is shown in Supplementary Fig. 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 h at 37 °C. This stripped rat serum serves as an exogenous source of GGH to deconjugate the folate polyglutamates present in RBC