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Simultaneous measurement of folate cycle intermediates in different biological matrices using liquid chromatography–tandem mass spectrometry

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ABSTRACT

The folate cycle is an essential metabolic pathway in the cell, involved in nucleotide synthesis, maintenance of the redox balance in the cell, methionine metabolism and re-methylation reactions. Standardised methods for the measurement of folate cycle intermediates in different biological matrices are in great demand. Here we describe a rapid, sensitive, precise and accurate liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method with a wide calibration curve range and a short run time for the simultaneous determination of folate cycle metabolites, including tetrahydrofolic acid (THF), 5-methyl THF, 5-formyl THF, 5,10-methenyl THF, 5,10-methylene THF, dihydrofolic acid (DHF) and folic acid in different biological matrices. Extraction of folate derivatives from soft and hard tissue samples as well as from adherent cells was achieved using homogenisation in buffer, while extraction from the whole blood and plasma relied on the anion exchange solid-phase extraction (SPE) method. Chromatographic separation was completed using a Waters Atlantis dC_{18} 2.0 × 100 mm, 3-μ column with a gradient elution using formic acid in water (0.1% v/v) and acetonitrile as the mobile phases. LC gradient started with 95% of the aqueous phase which was gradually changed to 95% of the organic phase during 2.70 min in order to separate the selected metabolites. The analytes were separated with a run time of 5 min at a flow rate of 0.300 mL/min and detected using a Waters Xevo-TQS triple quadrupole mass spectrometer in the multiple reaction monitoring mode (MRM) at positive polarity. The instrument response was linear over a calibration range of 0.5 to 2500 ng/mL ($r^2 > 0.980$). The developed bioanalytical method was thoroughly validated in terms of accuracy, precision, linearity, recovery, sensitivity and stability for tissue and blood samples. The matrix effect was compensated by using structurally similar isotope labelled internal standard (IS), $^{13}C_5$-methyl THF, for all folate metabolites. However, not all folate metabolites can be accurately quantified using this method due to their high interconversion rates especially at low pH. This applies to 5,10-methylene THF which interconverts into THF, and 5,10-methenyl-THF interconverting into 5-formyl-THF. Using this method, we measured folate cycle intermediates in mouse bone marrow cells and plasma, in human whole blood; in mouse muscle, liver, heart and brain samples.

1. Introduction

Folic acid, or vitamin B_9, is a water-soluble compound essential for normal tissue growth and development [1]. It consists of a pteridine ring, *p*-aminobenzoate and a glutamyl moiety. Animal cells are not able to synthesise folic acid; therefore, diet and microbiota are the sources of this vitamin. After being transported into the cell, folic acid undergoes poly-γ-glutamylation to retain within the cell, followed by stepwise reduction by dihydrofolate reductase using NADPH to its active form of tetrahydrofolate (THF), which can carry a one-carbon unit conjugated to nitrogen at position 5 or 10 in the pteridine ring [2,3]. The most common one-carbon donor for THF is serine [4]. THF with a bound carbon unit can exist in several intermediates distinguished by different oxidation states of the shuttled carbon. The transformation of one form of THF to the other takes place during the folate cycle. Each THF intermediate fuels a certain metabolic pathway linked to the folate cycle. As such, 5-methyl THF donates the methyl group for methionine regeneration in the methionine cycle and, therefore, for all re-methylation reactions in the cell; 5,10-methylene THF is used in thymidylate synthesis; and 10-formyl THF is the source of formate and substrate in

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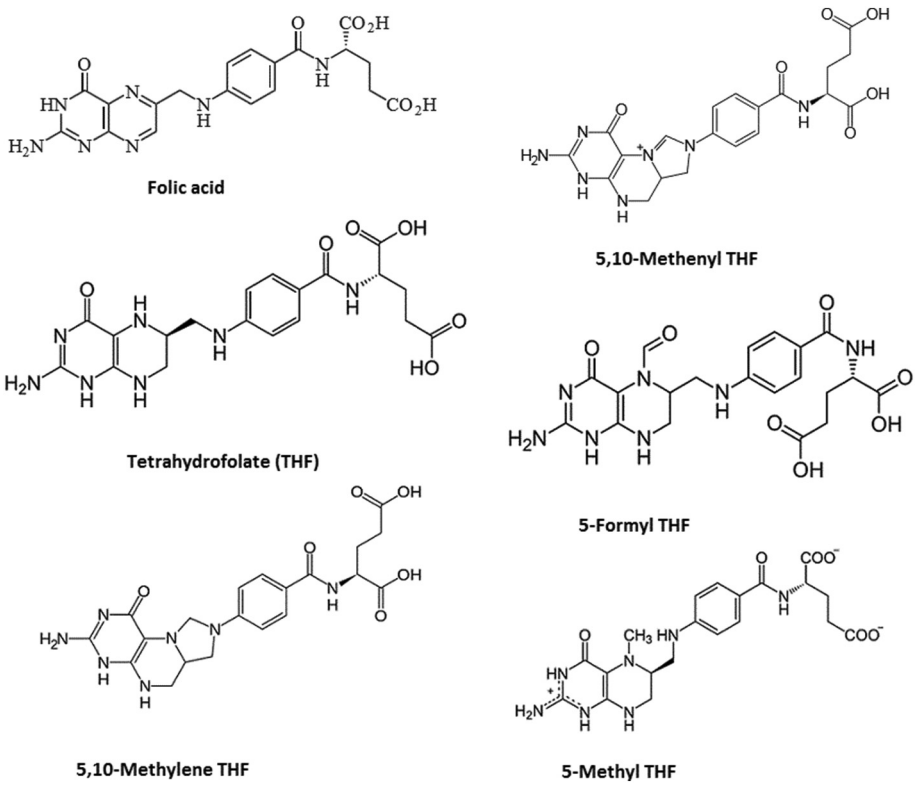

Fig. 1. Chemical structures of the folate metabolites.

purine synthesis [5]. Chemical structures of the folate metabolites are shown in Fig. 1. The oxidation of a shuttled carbon atom in the folate cycle is accompanied by the production of NADPH, critical for defence against oxidative stress and reductive biosynthesis in the cell [6]. The folate cycle is especially important for highly proliferative cells [7]. However, not much is known about the tissue- and condition-specific modulation of the folate cycle, including whether all THF intermediates are present in equal amounts or the production of one form of THF can overweigh production of the others. Progress in this area is hampered by the absence of a sensitive quantitative method for the simultaneous measurement of all THF intermediates from a single biological sample. The main challenges in the analysis of these compounds are their instability, pH-dependent interconversion and the varying length of the poly-γ-glutamyl tail. Furthermore, endogenous folate metabolites are present in very low amounts within biological matrices. Although a number of publications described analytical methods for the determination of selected folate cycle metabolites [8–13], there is no standardised method which could be used for the simultaneous determination of all THF derivatives in different biological matrices. For instance, Jia et al. quantitatively measured all folate metabolites except 5,10-methylene THF from human colonic mucosa samples using labelled isotope compounds as the internal standard [9]. In addition, Odin et al. measured 5,10-methylene THF and 5-methyl THF from colorectal mucosa and tumour tissues [10]. Fazili et al. quantitatively determined folate metabolites such as folic acid, 5-methyl THF and 5-formyl THF from blood and serum samples using a solid-phase extraction technique [11,12]. By contrast, Filip et al. analysed free and total folate in plasma and red blood cells using a stable isotope dilution liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method in clinical samples [13].

The primary objective of the present work, therefore, was to develop a rapid, simple, accurate, precise and sensitive method for the measurement of core folate cycle metabolites—folic acid, tetrahydrofolic acid, 5,10-methenyl THF, 5,10-methylene THF, 5-formyl THF, 5-methyl THF and dihydrofolic acid—from different biological matrices. To do so, we used tandem mass spectrometry, which emerged as an established analytical technique for the analysis of metabolites from complex biological samples. The key advantages of the LC–MS/MS technology include its ability to accurately identify and quantify several coenzyme species simultaneously.

2. Materials and methods

2.1. Chemicals, reagents and samples

Folate metabolites, including folic acid (purity > 98.5%), tetrahydrofolic acid (purity 95–98%), 5-methyl THF (purity > 95%), 5-formyl THF (purity > 99%), 5,10-methenyl THF (purity > 97.5%), 5,10-methylene THF (purity > 93%), dihydrofolic acid (purity 90–95%) and labelled internal standard $^{13}C_5$-methyl THF, were purchased from Schricks Laboratory (Jona, Switzerland). LC–MS grade acetonitrile and methanol (HiPerSolv) were obtained from VWR International (Helsinki, Finland). Other chemicals, such as formic acid, β-mercaptoethanol, ascorbic acid, potassium hydroxide and a HEPES buffer, were of high-grade purity and purchased from Sigma Aldrich (St. Louis, MO, USA). Deionised water (18 MΩ·cm at 25 °C) used for solution preparation was made using a Milli-Q water purification system procured from Merck Millipore (Billerica, MA, USA). Rat plasma with heparin as the anticoagulant was purchased from Innovative Research Laboratory (Novi, MI, USA). Whole human blood was procured from the Finnish Red Cross blood service (Helsinki, Finland). Mouse heart, liver, brain and muscle tissue samples from CD5 mice 7- to 12-weeks old maintained on standard small rodent chow, were obtained from Innovative Research Laboratory (Novi, MI, USA). Finally, mouse bone marrow and mouse plasma samples were kindly donated by Professor Anu Wartiovaara's laboratory (Biomedicum, University of Helsinki, Finland). Each bone marrow sample was obtained from two femur bones from a single animal. All biological samples were stored at −80 °C until used.