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Development and validation of an LC-MS/MS bioanalytical method for simultaneous determination of five main metabolites from the folate-methionine cycle in biological samples: Implications for anti-folate treatment

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ABSTRACT

The folate-methionine cycle metabolites are essential for methylation reactions and cell growth, with their imbalances implicated in cardiovascular disease, neural tube defects, cancer, and psychiatric disorders. Reliable quantification of these metabolites is important for disease prevention and monitoring of antifolate therapies. However, existing analytical methods are often limited by a low number of analytes, lengthy sample preparation, high reagent consumption, derivatisation requirements or lack of efficiency for simultaneous analysis. In this study, we report the development and validation of a liquid chromatography-tandem mass spectrometry method for the simultaneous quantification of five key metabolites: 5-methyltetrahydrofolate, S-adenosylmethionine, S-adenosylhomocysteine, folic acid, and homocysteine in biological samples utilising simple and fast sample preparation by protein precipitation. Method validation followed the guideline M10 on bioanalytical method validation, demonstrating selectivity, concentration-response relationship, precision, accuracy and stability across short- and long-term storage. The method covered wide dynamic ranges between the lower and upper limits of quantification, namely 50–4000 μg/L for homocysteine, 500–10,000 μg/L for S-adenosylmethionine, 2.5–50 μg/L for S-adenosylhomocysteine, 2.5–100 μg/L for folic acid and 2.5–50 μg/L for 5-methyltetrahydrofolate. In addition, the method's robustness was shown in endogenous matrices of six different cell lines. Applicability was demonstrated on human lymphoblastoid cells treated with methotrexate, a known antifolate, revealing expected metabolic changes; increase in intracellular levels of folic acid and homocysteine, and decreased levels of 5-methyltetrahydrofolate and S-adenosylmethionine. These results highlight the ability of the method to detect metabolic changes and its potential as a robust tool for assessing methylation and folate status in biomedical research.

1. Introduction

Folic acid (FA) is an essential nutrient that plays a crucial role in cell growth and development [1]. The metabolic pathway involving FA and its derivatives – among them 5-methyltetrahydrofolate (MTHF), S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), and homocysteine (HCY) – is important for fundamental physiological processes, including purine and thymidine synthesis, DNA repair, and methylation. Disruptions in these pathways can result in health issues, such as anemia [1], cardiovascular diseases [2,3] and cancers, like acute lymphoblastic leukemia [4], cervical intraepithelial neoplasia and cervical cancer [5]. FA is particularly important during pregnancy, as

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adequate levels are associated with a lower risk of congenital malformations, including neural tube defects and congenital heart defects [3,6,7]. Consequently, several food supplementation programmes have been established [6,8] and increased FA intake is strongly recommended prior and during pregnancy [9,10].

The metabolic pathway of folate is complex and is tightly intertwined with the methionine cycle (Fig. 1). Folate enters the cell via active transport by folate transporter 1 in its monoglutamate form. Within the cell, folylpolyglutamate synthase attaches several glutamate residues, forming folate polyglutamate, to prevent excretion of folate by transporter proteins. Subsequently, dihydrofolate reductase reduces folate to dihydrofolate and tetrahydrofolate (THF). A triple-activity enzyme – methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1 reversibly transforms THF to 10-formyltetrahydrofolate, 5,10-methenyltetrahydrofolate and, finally, 5,10-methylenetetrahydrofolate (5,10-methylen-THF). The pathway continues with the reduction of 5,10-methylen-THF by methylenetetrahydrofolate reductase to the biologically active form of folate, MTHF. MTHF can then be converted back to THF during methyl group transfer by the remethylation cycle in which methionine (Met) is formed from HCY. The reaction is catalysed by methionine synthase and methionine synthase reductase with vitamin B12 as a cofactor. In the remethylation cycle, methionine adenosyltransferase activates Met by attaching an adenosyl group to the sulphur atom, producing SAM, the main methyl donor for methyltransferases in the cell. The transfer of the methyl group from SAM to methylation substrates yields SAH, which can then be converted to HCY by adenosylhomocysteinase, completing the remethylation cycle.

The interplay between 5 key metabolites in the folate pathway (MTHF, SAM, SAH, FA and HCY) is essential to maintain methylation processes and overall metabolic function [11]. A major methyl donor, SAM, plays a fundamental role in epigenetics, biosynthetic processes and the metabolism of sulphur compounds [12–14]. A decrease in MTHF and methionine levels can reduce SAM to SAH ratio, thereby impairing methylation reactions. They can also lead to elevated HCY levels, which are associated with an increased risk of cardiovascular diseases [15,16], non-alcoholic fatty liver disease [17], neural tube defects, impaired childhood cognition, macular degeneration [15], and cognitive impairment in the elderly [14]. However, not only an increase in HCY levels is associated with disease. Low HCY levels have been found in

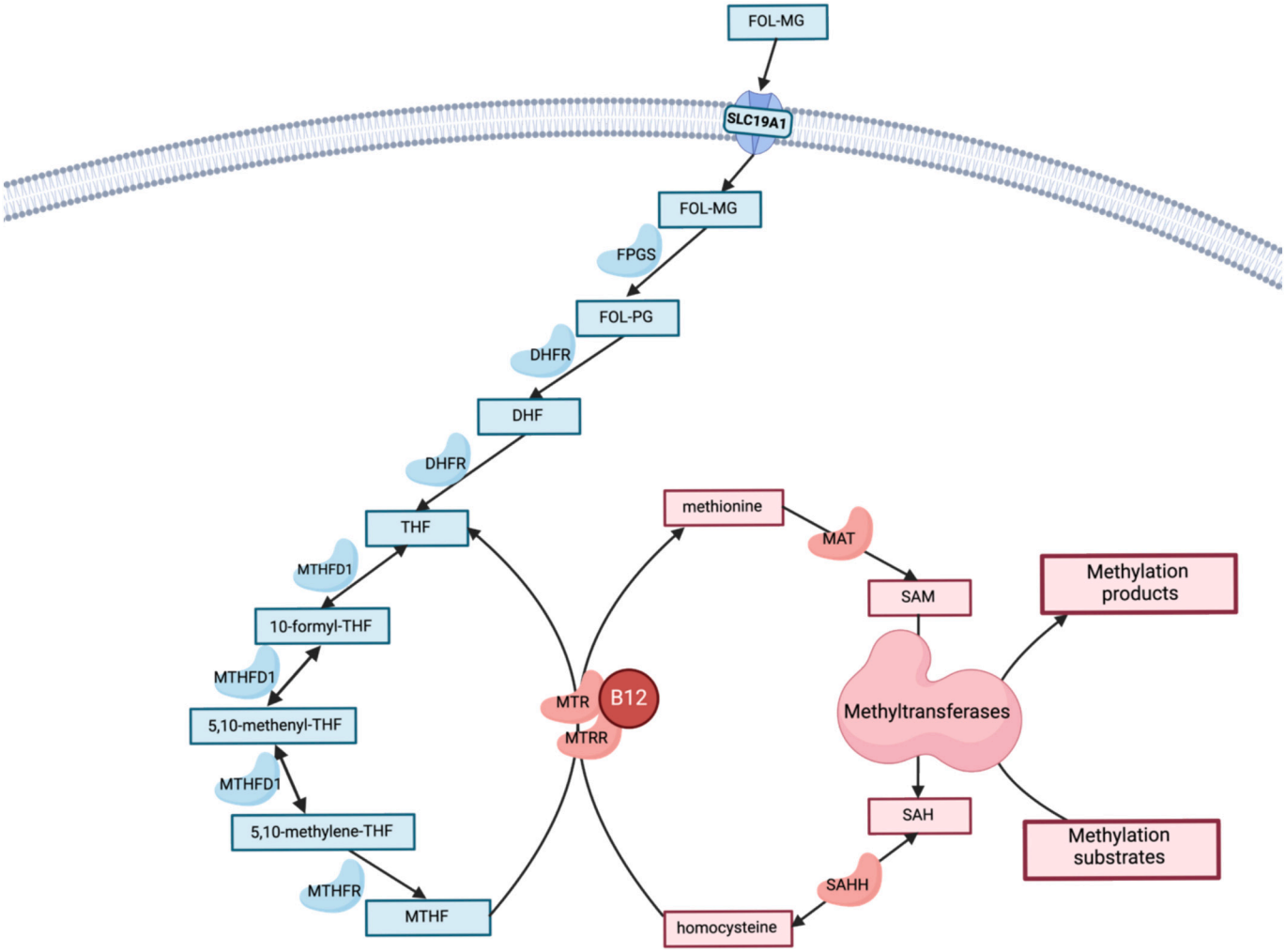

Fig. 1. The intertwined folate and methionine cycle. SLC19A1: folate transporter 1. FOL-MG: folate monoglutamate. FGPS: folylpolyglutamate synthase. FOL-PG: folate polyglutamate. DHFR: dihydrofolate reductase. DHF: dihydrofolate. THF: tetrahydrofolate. MTHFD1: methylenetetrahydrofolate dehydrogenase, cyclohydrolase and formyltetrahydrofolate synthetase 1. 10-formyl-THF: 10-formyltetrahydrofolate. 5,10-methenyl-THF: 5,10-methenyltetrahydrofolate. 5,10-methylen-THF: 5,10-methylenetetrahydrofolate. MTHFR: methylenetetrahydrofolate reductase. MTHF: 5-methyltetrahydrofolate. MTR: methionine synthase. MTRR: methionine synthase reductase. B12: vitamin B12 as a cofactor. MAT: methionine adenosyltransferase. SAM: S-adenosylmethionine. SAH: S-adenosylhomocysteine. SAHH: adenosylhomocysteinase.

patients with idiopathic peripheral neuropathy [18]. Low HCY may also impair the *de novo* synthesis of glutathione and thus increase susceptibility to oxidative stress [19].

When identifying biomarkers in the folate pathway, it is important not to focus solely on measuring individual metabolites in the folate metabolism but to consider the relations between them. However, measuring folate metabolites in biological samples by liquid chromatography-tandem mass spectrometry (LC-MS/MS) presents several challenges, such as the instability of several folate species, interconversion of metabolites, co-elution of isomers and similar metabolites, and the use of expensive internal standards (IS) – also thoroughly described in a recently published review paper [20]. Previously reported methods for the determination of metabolites in the folate pathway have several disadvantages (Supplementary material (SM) Table S1). They usually measure only 2–3 metabolites of the folate–methionine cycle simultaneously [21–24]. They are limited by lengthy sample preparation times, resulting from the need for derivatisation (e.g., for HCY), solid-phase extraction and lyophilisation [25]. Those methods that measure several metabolites in folate-interconnecting pathways do not quantify SAM, an actual methyl donor and the effector molecule, which is of paramount importance for most methylation processes in cells [26]. Therefore, developing a method that simultaneously measures 5 metabolites would provide valuable insights into their interconnected roles, offering a more comprehensive understanding of folate implications.

Due to the limitations of current methods, reliable analytical approaches are needed to accurately measure multiple key metabolites from the folate and methionine cycles simultaneously. In this study, we aimed to address these analytical challenges by developing and validating an LC-MS/MS method for the simultaneous determination of five biologically relevant metabolites: MTHF, SAM, SAH, FA and HCY. Furthermore, we evaluated the applicability of the method in a cellular context, employing an *in vitro* cell model of human lymphoblastoid cell lines, treated with methotrexate, a known antifolate agent.

2. Experimental

2.1. Chemicals and reagents

The external standards DL-HCY (>95.0 %), L-SAM p-toluenesulfonate salt (91 %), L-SAH (99.03 %), calcium DL-MTHF (>98.0 %) and FA (97 %) were purchased from Sigma Aldrich (Germany). Stable isotope-labelled L-SAM-$^{13}C_5$ (99.1 % isotopic, 89.7 % compound), L-SAH-$^{13}C_5$ (98.8 % isotopic, 96 % compound) and FA-$^{13}C_5$ (98.6 % isotopic, 92.2 % compound) were purchased from LGC Standards (Germany). The isotope-labelled MTHF-$^{13}C_5$ (99 % isotopic, 96 % compound) was purchased from Sigma Aldrich (Germany). d_4-L-HCY was obtained by reduction from d_8-labelled L-homocystine (>95 % isotopic, 100 % compound), which was also purchased from LGC Standards (Germany). Acetonitrile (ACN, >99.8 %), methanol (MeOH, >99.8 %), dimethyl sulfoxide (DMSO, >99.7 %), methotrexate (>98 %) and ascorbic acid (>98 %), used for sample and standard preparation, were purchased from Sigma Aldrich (Germany). Ultrapure water was generated by an ELGA PureLab Flex LabWater water purification system (UK). LC-MS grade methanol (≥99.9 %) and acetonitrile (≥99.9 %) were obtained from Honeywell (USA). Acetic acid (99.8 %) was obtained from Merck (Germany). Bovine serum albumin (BSA), phosphate buffered saline (PBS), L-glutamine, penicillin/streptomycin and RPMI 1640 media were purchased from Sigma Aldrich (Germany). Fetal bovine serum (FBS) was purchased from Gibco (USA).

2.2. Instrumentation and chromatographic conditions

The samples were analysed by an Agilent 1290 Infinity liquid chromatograph coupled with a 6460 Triple Quadrupole detector (Agilent Technologies, Santa Clara, USA) equipped with a Jetstream electrospray ionisation interface. Chromatographic separation was performed on a Kinetex PFP column (100 × 3.0 mm, 2.6 μm) (Phenomenex, Torrance, USA) maintained at 30 °C. A gradient elution method was applied using 0.1 % acetic acid in ultrapure water as mobile phase A and 99.8 % ACN with 0.2 % ultrapure water as mobile phase B, with the following gradient profile (time in min, % B, flow rate in mL/min): (0.0, 0, 0.4), (1.5, 0, 0.4), (3.0, 10, 0.4), (6.0, 20, 0.4), (7.0, 55, 0.4), (8.0, 99, 0.6), and (9.1, 0, 0.6). The run time was 11.5 min and the injection volume was 1 μL. The time segments for analyte and corresponding IS data acquisition at the MS were: 1.15–1.42 min for HCY and SAM, 3.6–6.5 min for SAH, MTHF and FA. At all other times, the sample from the column was routed to waste. The mass spectrometer was operated in positive ionisation mode, with the following ion source parameters: gas flow and temperature at 5 L/min and 320 °C; nebulizer pressure set at 310 kPa; sheath gas flow rate and temperature at 11 L/min and 380 °C; and the capillary voltage at 4000 V. Multiple reaction monitoring (MRM) transitions applying quantifier and qualifier ions were used for analyte quantification. The quadrupoles were set to operate in wide (1.2 amu) or widest (2.5 amu) mass resolution (Table 1). The widest mass resolution was employed when necessary to maximize analytical sensitivity. Instrument control, data acquisition and data processing were performed with Mass Hunter Workstation software (Agilent Technologies, Santa Clara, USA).

2.3. Preparation of calibration standards and quality control samples

Primary stock solutions of IS were prepared at concentrations 0.5 mg/mL for FA, SAH and SAM, or 1 mg/mL for HCY and MTHF, and external standards (ES) at concentrations 5 mg/mL by dissolving appropriate amounts of FA, MTHF or SAH in DMSO, while HCY and SAM were dissolved in a 50:50 MeOH/H_2O mixture containing 10 mg/mL ascorbic acid. Isotopically labelled compounds (FA-$^{13}C_5$, MTHF-$^{13}C_5$, SAM-$^{13}C_5$, SAH-$^{13}C_5$, and HCY-d_4) were used for the preparation of ISs.

From primary ES solutions, two working solutions (WS) of each ES were prepared by dilution in 50:50 MeOH/H_2O, containing 10 mg/mL ascorbic acid and 2 mg/mL BSA to simulate a surrogate matrix. The concentrations of the "high WS" were as follows: SAM^{WS}_{HIGH} = 50 mg/L, HCY^{WS}_{HIGH} = 10 mg/L, SAH^{WS}_{HIGH} = 1 mg/L, FA^{WS}_{HIGH} = 1 mg/L and $MTHF^{WS}_{HIGH}$ = 1 mg/L. Corresponding "low WS" were obtained by a 1:10 dilution from each "high WS".

Calibration standards were prepared from these WS covering the following concentration ranges: HCY = 50–4000 μg/L, SAM = 500–10,000 μg/L, SAH = 2.5–50 μg/L, FA = 2.5–100 μg/L and MTHF = 2.5–50 μg/L (SM Table S2). The concentration ranges were selected on the basis of physiological concentrations of the metabolites. Quality control (QC) samples were prepared at four concentration levels following the protocol used for calibration standards, using freshly prepared primary stock solutions of ES and the surrogate matrix (50:50 MeOH/H_2O, with 10 mg/mL ascorbic acid and 2 mg/mL BSA). The QC concentration levels were: HCY at 50, 150, 1500 and 3200 μg/L; SAM at 500, 1500, 4500 and 7500 μg/L; SAH at 2.5, 6, 20 and 40 μg/L; FA at 2.5, 6, 40 and 75 μg/L; and MTHF at 2.5, 6, 20 and 40 μg/L (SM Table S3).

Working solutions of IS were obtained by diluting the primary stock solutions of IS in MeOH/H_2O (50:50) containing 10 mg/mL ascorbic acid, achieving the following dilutions: SAM^{WS}_{IS} (1:100), HCY^{WS}_{IS} (1:1000), SAH^{WS}_{IS} (1:5000), FA^{WS}_{IS} (1:5000) and $MTHF^{WS}_{IS}$ (1:10,000).

2.4. Extraction

Working solutions of ISs were added to each calibration standard and QC samples to obtain a 155 μL mixture with final IS concentrations of 100 μg/L for HCY, 500 μg/L for SAM, 10 μg/L for SAH, 10 μg/L for FA and 10 μg/L for MTHF. The mixture was sonicated for 15 s at 40 mA, followed by the addition of 750 μL ice-cold ACN. Samples were then vortexed vigorously and left on ice for 20 min to complete the

Table 1
Multiple reaction monitoring (MRM) transitions and retention times for folate metabolites. The qualifier/quantifier ratio provided for each analyte is the average of the three days of validation.

Analyte	MRM transitions	Fragmentor [V]	Collision energy [eV]	Qualifier/ Quantifier ratio [%]	Retention times [min]
HCY	136.0*⟶ 90.1* (56.1*)	66	9 (19)	58.6	1.24
HCY-d_4	140.0* ⟶ 60.1*	66	19 (9)	/	1.24
SAM	399.2 ⟶ 250.2* (136.2*)	110	13 (33)	43.4	1.18
SAM-$^{13}C_5$	404.2 ⟶ 255.2*	110	13 (33)	/	1.18
SAH	385.1 ⟶ 136.2* (88.2*)	108	17 (49)	77.3	4.10
SAH-$^{13}C_5$	390.1 ⟶ 136.2*	108	17	/	4.10
MTHF	460.2 ⟶ 313.3* (180.1*)	100	20 (22)	6.4	5.02
MTHF-$^{13}C_5$	465.2 ⟶ 313.3*	100	20	/	5.02
FA	442.2 ⟶ 295.1* (176.1*)	102	13 (41)	30.3	6.24
FA-$^{13}C_5$	447.2 ⟶ 295.1*	102	13	/	6.24

*Note: Qualifier ions are provided in brackets. *: mass-to-charge ratio (m/z) of an analyte at the widest mass resolution (2.5 amu), no *: m/z of an analyte at wide mass resolution (1.2 amu). HCY: homocysteine. HCY-d_4: homocysteine-d_4. SAM: S-adenosylmethionine. SAM-$^{13}C_5$: S-adenosylmethionine-$^{13}C_5$. SAH: S-adenosylhomocysteine. SAH-$^{13}C_5$: S-adenosylhomocysteine-$^{13}C_5$. MTHF: 5-methyltetrahydrofolate. MTHF-$^{13}C_5$: 5-methyltetrahydrofolate-$^{13}C_5$. FA: folic acid. FA-$^{13}C_5$: folic acid-$^{13}C_5$.*

precipitation process. Subsequently, the samples were centrifuged at 10,000 ×*g* for 10 min at 4 °C, and 850 μL of the resulting supernatants were transferred to fresh 1.5 mL tubes. The supernatants were dried under a gentle stream of nitrogen at 35 °C for 60 min. The dried residues were reconstituted in 150 μL of a 50:50 MeOH/H_2O containing 10 mg/mL ascorbic acid and then transferred to the autosampler for LC-MS/MS analysis. Analyte concentrations were calculated based on the relative response to the IS. Additionally, 10 μL of each calibration standard was aliquoted for the determination of protein content. Total protein concentration was measured using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules/CA, USA), in accordance with the manufacturer's instructions.

2.5. Cell culture, treatment and sample preparation

Human lymphoblastoid cell lines (LCLs) are B-lymphocytes that were immortalized through transformation with Epstein-Barr virus. They are prepared from peripheral blood cells obtained from individual donors and preserve the donor's genetic background. Because of immortalization, LCLs proliferate continuously in the culture and are suitable source of B-cells for *in vitro* studies [27]. In this study, LCLs from six different individuals were cultured in a RPMI 1640 medium, supplemented with 10 % FBS, 4 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. For experimental treatments, LCLs (500,000 cells/mL) were seeded in 6-well plates containing 2 mL of complete growth medium and treated with either 25 nM methotrexate, a known antifolate, or PBS as a negative control at 37 °C and 5 % CO_2. After 72 h, the cells were washed with PBS and resuspended in 150 μL of ultrapure water containing 10 mg/mL ascorbic acid. The cell suspension was sonicated for 15 s at 40 mA. A 10 μL aliquot of the lysed cell suspension was reserved for the protein concentration analysis. Working solutions of ISs were then added to the remaining cell lysate to yield 155 μL with final IS concentrations of 100 μg/L for HCY, 500 μg/L for SAM, 10 μg/L for SAH, 10 μg/L for FA and 10 μg/L for MTHF. Sample preparation then followed the extraction protocol established for calibration standards and QC samples (Section 2.4). The procedure was repeated in at least two independent experiments.

2.6. Method validation

The method developed for the simultaneous determination of HCY, SAM, SAH, FA and MTHF was validated according to European Medicines Agency (EMA) guidelines for bioanalytical method validation [28].

Method selectivity was assessed by analysing a blank surrogate matrix composed of a MeOH and H_2O mixture (50:50) containing 10 mg/mL ascorbic acid and 2 mg/mL BSA. A zero sample, containing only isotopically labelled ISs, was also used each day of the validation to assess selectivity. Potential interferences in the blank surrogate matrix were determined by comparing the peak areas of analytes at the lower limit of quantification (LLOQ) level in the surrogate matrix to those of the blank surrogate matrix.

Analyte identity was confirmed by ensuring the qualifier-to-quantifier ion ratio deviated by less than 20 % and that the retention time of the analyte did not differ from the retention time of the IS. The LLOQ was determined as the lowest calibration standard that achieved acceptable accuracy (100 ± 20 %) and precision (coefficient of variation (CV) below 20 %). Additionally, the analyte signals at LLOQ were checked to be at least 5-fold greater compared to the response from the blank surrogate matrix.

The method concentration-response relationship was evaluated using seven non-zero calibration standards (see Section 2.3) over three consecutive days of validation. For the concentration-response curves, the nominal concentration of the calibration standards was plotted against the ratio of peak areas between the analytes and their corresponding ISs. The acceptance criterion for the regression model of each analyte was a correlation coefficient (r^2) of more than 0.99.

Carry-over was assessed by injecting a blank sample after analysis of the highest calibration standard of each analyte. The acceptance criteria were carry-over of less than 20 % of the LLOQ for each analyte and less than 5 % for the corresponding IS.

Accuracy and precision of the method were assessed on QC samples at four concentration levels (LLOQ, low, medium and high) within the calibration range, prepared fresh for each of the three consecutive days of validation. Within-run accuracy and precision were evaluated using five replicates of QC samples analysed in a single run. Between-run accuracy and precision were evaluated on QC samples ($n = 5$) prepared and analysed across the three consecutive days of validation. Accuracy was acceptable when the mean concentration was within ±15 % of the nominal value for all QC samples, except at the LLOQ, where ±20 % deviation was accepted. Precision, expressed as CV, was acceptable within ±15 % for all QC levels, except at the LLOQ (±20 %).

Recovery (RE) and matrix effect (ME) were evaluated in the surrogate matrix using BSA and using cells, which served as the endogenous matrix. ME in the surrogate matrix used for the preparation of calibration standards was determined by comparing the peak area vs. IS of analytes for all seven calibration standards prepared without the surrogate matrix (BSA), with the peak area vs. IS of calibration standards with the surrogate matrix. ME in the surrogate matrix was expressed as a percentage of all analyte responses at each concentration level. RE in the surrogate matrix was expressed as a percentage of responses vs. IS from calibration standards with surrogate matrix undergoing the extraction protocol (see Section 2.4), compared to the response vs. IS of calibration standards without surrogate matrix that did not undergo extraction and were just diluted in a MeOH and H_2O mixture (50:50) containing 10 mg/mL ascorbic acid to the same nominal analyte and IS concentration. The suitability of the surrogate matrix used to prepare calibration standards

and QCs was investigated in an experiment using endogenous matrix spiked with QCs (prepared without surrogate matrix), the endogenous matrix only and QCs prepared in the surrogate matrix. Endogenous matrix was obtained from 6 different cell lines. Accuracy in these experiments was determined as a percentage of the difference between the measured concentration of spiked endogenous samples and endogenous concentrations, divided by the measured concentrations of QCs in the surrogate matrix.

Analyte stability was tested at all four QC levels, with each level prepared in quintuplicate. Stability studies included freeze and thaw stability (three cycles), short-term stability (6 h at room temperature) and long-term stability (1 and 2 weeks at −80 °C) of analytes in MeOH/H_2O (50:50) containing 10 mg/mL ascorbic acid and 2 mg/mL BSA, and autosampler stability of analytes in samples (reanalysed after 24 and 48 h at 4 °C). Stock solution stability at −80 °C was assessed based on the responses vs. IS from calibration standards prepared at the beginning of validation and 2 months later without the surrogate matrix that did not undergo extraction and were just diluted in a MeOH/H_2O mixture (50:50) containing 10 mg/mL ascorbic acid. Deviations in mean analyte concentrations at each QC level of ±15 % were considered acceptable, except at the LLOQ, where up to ±20 % deviation was accepted.

3. Results and discussion

Reliable monitoring of 5 key metabolites in the folate pathway, namely MTHF, SAM, SAH, FA and HCY is crucial both for early disease detection and for evaluating the effects of antifolate therapies. In this study, we aimed to develop a rapid, accurate, sensitive, and selective LC-MS/MS method for the simultaneous quantification of these five metabolites in biological samples. The method was optimised and validated in accordance with EMA guidelines [28] for the quantification of HCY, SAM, SAH, FA and MTHF in biological samples.

3.1. Method optimisation

Quantifying metabolites in the folate and methionine cycles presents several analytical challenges that must be carefully addressed during method development [20]. These include the physico-chemical instability of folate derivatives, demethylation of SAM, particularly under neutral or alkaline conditions, and the susceptibility of HCY to oxidation and subsequent formation of dimers [29,30]. Establishing suitable chromatographic and detection conditions is further complicated by the co-elution of structurally related metabolites and the influence of matrix effects due to a very hydrophilic nature of some metabolites [20,26]. In addition, sample preparation can be time-consuming, with a risk of analyte loss during processing steps [31]. The mass spectrometry ion source parameters were adjusted to maximize the responses for each analyte and their isotopically labelled ISs. The MRM transitions (listed in Table 1) were determined using MassHunter Optimizer method development software. For each analyte, quantifier and qualifier ions were selected based on the most and second-most abundant fragment ions, respectively, to ensure specificity. The ratio of qualifier-to-quantifier ions was used to confirm the identity of all five analytes. To increase the sensitivity of the method for the analytes, only one mass transition was used for ISs. The selection of the chromatographic column was based on achieving optimal signal responses across all five analytes. After testing multiple columns with different stationary phases (Kinetex C18, Poroshell CS-C18, Kinetex PFP, Synergi Fusion, Acquity HSS and Kinetex Biphenyl), the Kinetex PFP proved the best, due to the sufficient retention and separation of the most hydrophilic HCY and SAM. Several chromatographic conditions including pH, mobile phase composition, column temperature, and flow rate were then systematically tested to optimise detection sensitivity and achieve satisfactory separation of analytes with a focus on HCY and SAM. Acidic pH (3.4) proved the most important in ensuring good signal responses and symmetrical peaks for HCY, while too acidic (pH < 2.8) mobile phase significantly lowered the signal responses of SAH, MTHF and FA. Increasing the percentage of acetic acid in mobile phase A to 0.1 % (v/v) also improved the detection of HCY and SAM with regard to ME. Better ionisation of all analytes was achieved in acetic acid in comparison to formic acid. The use of ammonium formate as a buffer in different concentrations (1, 2.5 and 5 mM) and ammonium fluoride (0.2 mM) was also tested at different pH values (2.8, 3.4 and 4.0) to achieve better peak shapes, especially for SAM, however, this proved unfavourable for HCY determination and was therefore abandoned. Column temperature also improved SAM detection, as a lower column temperature (30 °C vs. 35 °C or 40 °C) increased the signal response and improved peak shape of SAM, while also retaining good signals and peak separations of the other analytes. After achieving sufficient separation of HCY and SAM, we further optimised the method for the elution for the other 3 less hydrophilic analytes, namely SAH, MTHF and FA by using a gradient elution (see Section 2.2). Sample preparation followed an adapted protocol based on Vidmar Golja et al. [24], further refined to enable the additional detection of SAM and SAH. Dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP) are both strong reducing agents widely used in HCY detection in order to prevent the oxidation and formation of dimers by HCY [20,24,25,32,33]. In our experiments, sample preparation with DTT or TCEP proved incompatible with SAM detection, drastically reducing the signal response; therefore, we instead used ascorbic acid at a concentration of 10 mg/mL. This provided sufficient sample stability for all analytes (see 3.2.6 Stability), adequately preventing HCY oxidation, while still allowing the detection of SAM.

3.2. Method validation

3.2.1. Selectivity and carry-over

Since the analytes measured are endogenously present in biological systems, the use of a blank cell lysate as a matrix was unsuitable. Instead, we used a surrogate matrix, consisting of BSA, which is also recommended in EMA guidelines [28]. The optimised method enables selective detection of HCY, SAM, SAH, FA and MTHF as no interferences at the retention times of the analytes at the LLOQ were observed in the surrogate matrix (Fig. 2). The concentration of BSA was 2 mg/mL, corresponding to the total protein concentration in the biological samples used during method development. The blank solution injected immediately after the highest calibration standard did not reveal any quantifiable carry-over (SM Fig. S1).

3.2.2. Regression models

The concentration-response relationship of the method was evaluated across a broad concentration range for each analyte: HCY = 50–4000 μg/L, SAM = 500–10,000 μg/L, SAH = 2.5–50 μg/L, FA = 2.5–100 μg/L and MTHF = 2.5–50 μg/L (SM Table S2). These ranges were selected based on previously reported intracellular and plasma concentrations and were intended to quantify variations in analyte levels under different conditions. The relationship between the response vs. IS response (y) and the concentration (x) for three analytes, namely HCY, FA and MTHF, followed a linear regression model, while quadratic curve fitting was more appropriate for the calibration data for SAM and SAH. The mean slope and intercept values (for HCY, FA and MTHF) and quadratic function parameters (for SAM and SAH) of the calibration curves, plotted as response ratios of analytes to their respective ISs versus analyte concentrations, are presented in Table 2, along with the lowest determination coefficients observed over the three days of validation. The method demonstrates a good concentration-response relationship for each analyte within its specified concentration range and is compliant with EMA requirements for bioanalytical method validation [28]. The wide concentration range enables quantification of analytes in various samples without the need for additional sample processing (e.g., dilution).

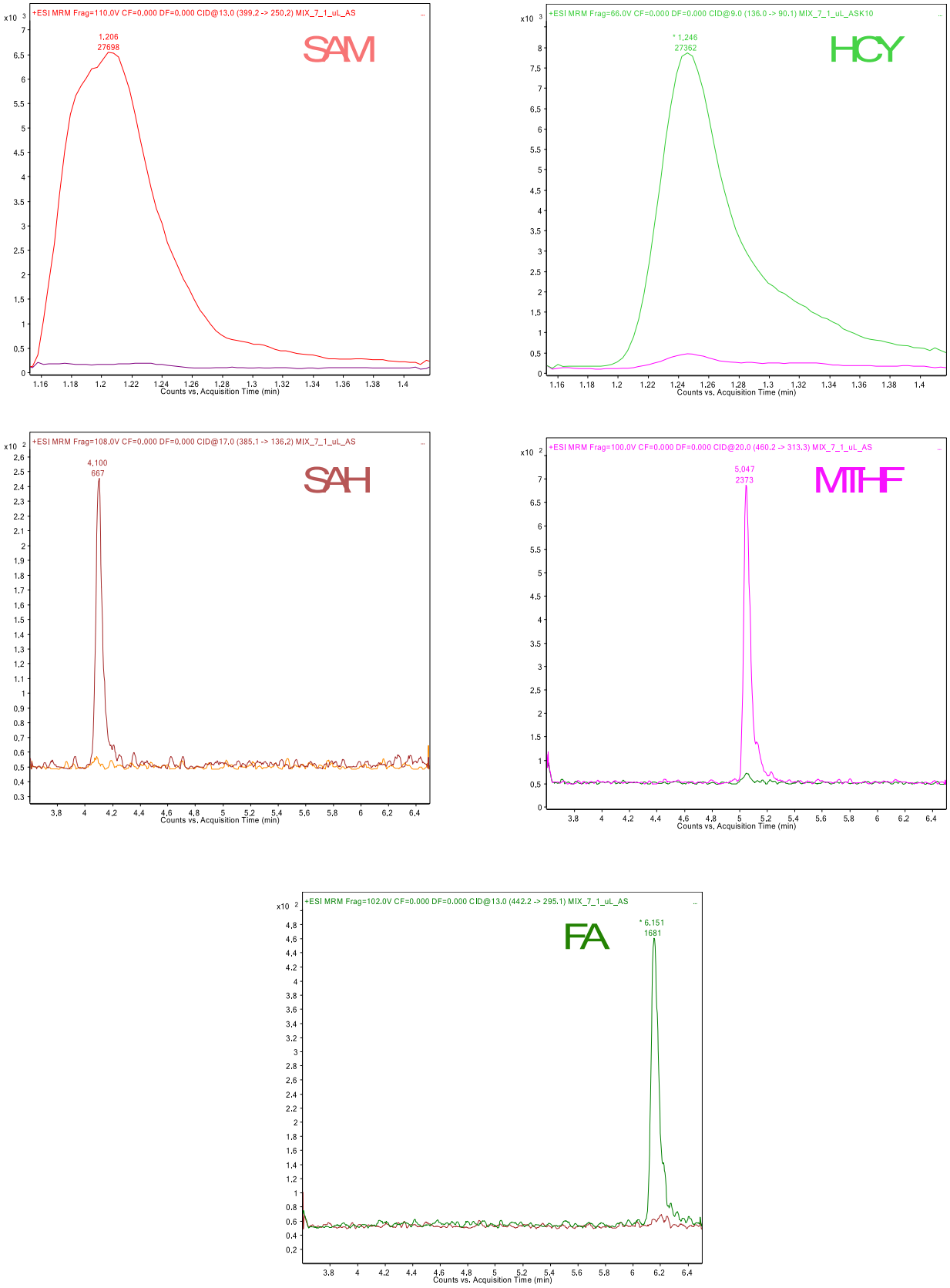

Fig. 2. Mass spectrometry chromatograms at the lower limit of quantification (LLOQ) of SAM, HCY, SAH, MTHF and FA showing acceptable signal-to-noise ratios and the absence of potential interferences from the blank surrogate matrix. The responses of the blank surrogate matrix are shown overlayed in a different colour than the LLOQ responses. HCY: homocysteine. FA: folic acid. MTHF: 5-methyltetrahydrofolate. SAM: S-adenosylmethionine. SAH: S-adenosylhomocysteine.

Table 2
Calibration curve parameters. Mean slope and intercept values (for HCY, MTHF and FA) and quadratic function parameters (for SAH and SAM) are given. The correlation coefficient (r^2) provided is the lowest of the three days of validation for each analyte.

Analytes following linear regression model	Slope	Intercept	r^2
HCY	0.0817	0.0565	0.9975
MTHF	0.0647	−0.0241	0.9954
FA	0.0483	0.0363	0.9990

Analytes following quadratic regression model	*a*	*b*	*c*	r^2
SAM	1.4667E-08	0.00073333	−0.0189	0.9989
SAH	−0.0009	0.14676667	−0.2045	0.9968

HCY: homocysteine. SAM: S-adenosylmethionine. SAH: S-adenosylhomocysteine. MTHF: 5-methyltetrahydrofolate. FA: folic acid.

Table 3
Precision and accuracy of the developed method for HCY, FA, MTHF, SAM and SAH.

HCY				
	Accuracy (%)		Precision (CV %)	
QC level	Within-run	Between-run	Within-run	Between-run
LLOQ	95.2	97.0	8.5	12.1
Low	104.4	107.0	4.9	2.1
Medium	105.5	107.9	3.9	2.2
High	103.9	105.2	4.8	2.8
FA				
	Accuracy (%)		Precision (CV %)	
QC level	Within-run	Between-run	Within-run	Between-run
LLOQ	91.4	103.3	5.5	12.3
Low	107.8	102.0	5.1	5.7
Medium	103.4	103.7	5.3	2.1
High	108.1	99.2	4.4	7.9
MTHF				
	Accuracy (%)		Precision (CV %)	
QC level	Within-run	Between-run	Within-run	Between-run
LLOQ	116.0	113.4	3.6	3.7
Low	102.8	102.2	4.5	7.8
Medium	107.8	108.4	3.4	2.3
High	109.1	106.8	4.1	2.1
SAM				
	Accuracy (%)		Precision (CV %)	
QC level	Within-run	Between-run	Within-run	Between-run
LLOQ	101.8	106.6	7.8	5.0
Low	109.0	105.7	3.8	6.8
Medium	107.8	104.8	5.1	2.5
High	100.8	105.6	3.4	4.1
SAH				
	Accuracy (%)		Precision (CV %)	
QC level	Within-run	Between-run	Within-run	Between-run
LLOQ	117.5	114.9	3.6	2.1
Low	103.2	98.6	4.6	6.3
Medium	93.8	89.6	2.1	4.1
High	96.6	92.8	5.3	3.7

HCY: homocysteine. FA: folic acid. MTHF: 5-methyltetrahydrofolate. SAM: S-adenosylmethionine. SAH: S-adenosylhomocysteine. QC level: Quality control concentration level. LLOQ: Lower limit of quantification.

3.2.3. *Lower limit of quantification (LLOQ)*

Based on criteria for precision (CV below 20 %), accuracy (100 ± 20 %), and an LLOQ-to-blank ratio (at least 5-fold), the method demonstrated sufficient sensitivity for HCY, SAM, SAH, FA and MTHF (Table 3 and SM Table S4). The method's LLOQs, determined as the lowest calibration standards meeting these criteria, were: 50 μg/L for HCY, 500 μg/L for SAM, 2.5 μg/L for SAH, 2.5 μg/L for FA, and 2.5 μg/L for MTHF. The low LLOQs achieved with this method are in line with several published methods (SM Table S1) and allow sensitive quantification of these metabolites in cell lysates and other biological samples. A discrepancy with some methods can be observed in the 10- to 100-fold higher LLOQ of SAM, as our method focused on intracellular quantification of folate metabolites, where reported SAM levels are 100-fold higher than in plasma [34–37].

3.2.4. *Accuracy and precision*

All results obtained for the determination of within-run and between-run accuracy and precision are within the acceptance criterion (100 ± 15/20 %), as presented in Table 3.

3.2.5. *Extraction recovery and matrix effect*

The ME and RE in the surrogate matrix were assessed across a wide concentration range for each analyte (SM Table S5 and SM Table S6). Additionally, we evaluated ME and RE in endogenous matrices from 6 different cell lines for all analytes at QC_{low} and QC_{high}. The results show good robustness of the developed method (CV values predominantly below 5 %, with only one case reaching 10.6 %, and average accuracy ranging from 88 % to 113 %; Table 4). Moreover, the data indicate no meaningful impact of endogenous matrices compared with surrogate matrix on accuracy and precision. This confirms that the surrogate matrix served as an appropriate substitute for the real endogenous matrix.

3.2.6. *Stability*

The results for short-term stability (6 h at room temperature, "benchtop"), autosampler stability (24 and 48 h at 4 °C), long-term stability (1 and 2 weeks at −80 °C), and freeze-thaw stability (3 cycles), expressed

Table 4
Matrix effect of analytes determined on 6 different sources of endogenous matrix for low (QC_{low}) and high spiked (QC_{high}) concentrations. Matrix effect was determined as a percentage (%) of the difference between the measured concentration of spiked endogenous samples and endogenous concentrations, divided by the measured concentrations of QCs in the surrogate matrix.

Matrix effect (%) in endogenous matrix for spiking with QC low					
Matrix source	**FA**	**HCY**	**MTHF**	**SAM**	**SAH**
1	108.6	86.6	105.1	88.9	94.6
2	111.7	92.1	96.6	94.5	91.3
3	113.8	111.3	113.9	92.0	100.9
4	111.9	95.3	100.5	86.3	96.6
5	106.0	102.3	97.1	87.9	103.1
6	98.8	112.8	88.6	86.7	94.8
Average	108.5	100.1	100.3	89.4	96.9
CV	5.0	10.6	8.6	3.6	4.5
Matrix effect (%) in endogenous matrix for spiking with QC high					
Matrix source	**FA**	**HCY**	**MTHF**	**SAM**	**SAH**
1	112.0	93.5	114.6	88.6	99.4
2	111.1	110.5	112.9	88.7	99.4
3	114.4	107.3	107.2	87.7	109.5
4	112.7	110.7	114.3	85.2	108.1
5	114.1	110.7	114.7	86.8	101.4
6	113.6	102.7	108.2	89.3	102.1
Average	113.0	105.9	112.0	87.7	103.3
CV	1.1	6.4	3.0	1.7	4.3

FA: folic acid. HCY: homocysteine. MTHF: 5-methyltetrahydrofolate. SAM: S-adenosylmethionine. SAH: S-adenosylhomocysteine. QC: Quality control concentration level.

as % of change from the initial sample, are summarised in SM Table S7. Stability of all analytes across all QC samples was acceptable and within ±15 % change or ±20 % change for LLOQ, under all tested conditions, including the 7-day long-term stability assessment. However, 14-day long-term stability for SAH and SAM did not meet guideline criteria, indicating that samples should be processed within 7 days or less to ensure accurate determination of all five metabolites.

The stock solutions were stored at −80 °C during the entire validation. The stock solution stability for 2 months of all analytes across the dilution range was: 94.9 % to 110.2 % for HCY, 98.9 % to 114.9 % for FA, 92.9 % to 101.2 % for MTHF, 85.0 % to 113.8 % for SAM and 102.5 % to 111.7 % for SAH (SM Table S8), indicating that analytes in stock solutions are stable.

3.2.7. *Method applicability*

Maintaining adequate folate levels is of high importance for several physiological processes, particularly those involving one-carbon metabolism [13,38,39]. Insufficient folate consumption is associated with several pathologies [6,40,41]. Although FA is the most commonly supplemented form of folates [10,42], its metabolites, such as MTHF and SAM play key roles in methylation reactions with the methylation potential often expressed as the SAM to SAH ratio [7,43,44]. Additionally, elevated levels of HCY have been strongly associated with the occurrence of neural tube defects, cardiovascular disorders, and liver disease [3,14–16].

In order to test the applicability of our method on biological samples, we measured the intracellular levels of folate metabolites (FA, MTHF, SAH, SAM, HCY) in immortalized B lymphocytes (LCLs) from 6 different individuals. We aimed to investigate whether our newly established method could detect changes in metabolite levels following disruption of folate metabolism by the antifolate methotrexate [45]. LCLs are a relevant cell model for *in vitro* studies of the folate cycle, as they express all enzymes of folate and methionine pathway [27,46,47]. Cells were exposed to 25 nM methotrexate for 72 h and the intracellular folate metabolites were quantified and normalised to protein content in cell lysates. Upon methotrexate treatment, we observed a significant increase in FA (64.3 ng/mg protein vs. 23.0 ng/mg protein) and HCY (429.8 ng/mg protein vs. 121.8 ng/mg protein) levels, while MTHF (9.0 ng/mg protein vs. 20.0 ng/mg protein) and SAM (494.1 ng/mg protein vs. 1565 ng/mg protein) levels decreased significantly (unpaired *t*-test, Fig. 3).

These results are in accordance with methotrexate's mode of action. Methotrexate, a folate antimetabolite, primarily inhibits DHFR, which in turn impedes MTHF synthesis [45,48,49]. Methotrexate-treated cells exhibit a reduced capacity for converting inactive forms of folate into the biologically active form, MTHF. This reduction disrupts the downstream folate pathway, leading to depleted SAM, which is directly synthesised from HCY and MTHF [13], and accumulation of HCY [15,19], which cannot be remethylated with MTHF. Additionally, elevated HCY levels also lead to increased SAH levels when cells are exposed to methotrexate, which is in line with our previous recent findings [24].

The method demonstrates its applicability by enabling the quantification of key folate metabolites at LLOQ levels that are lower than reported physiological concentrations for plasma and intracellular

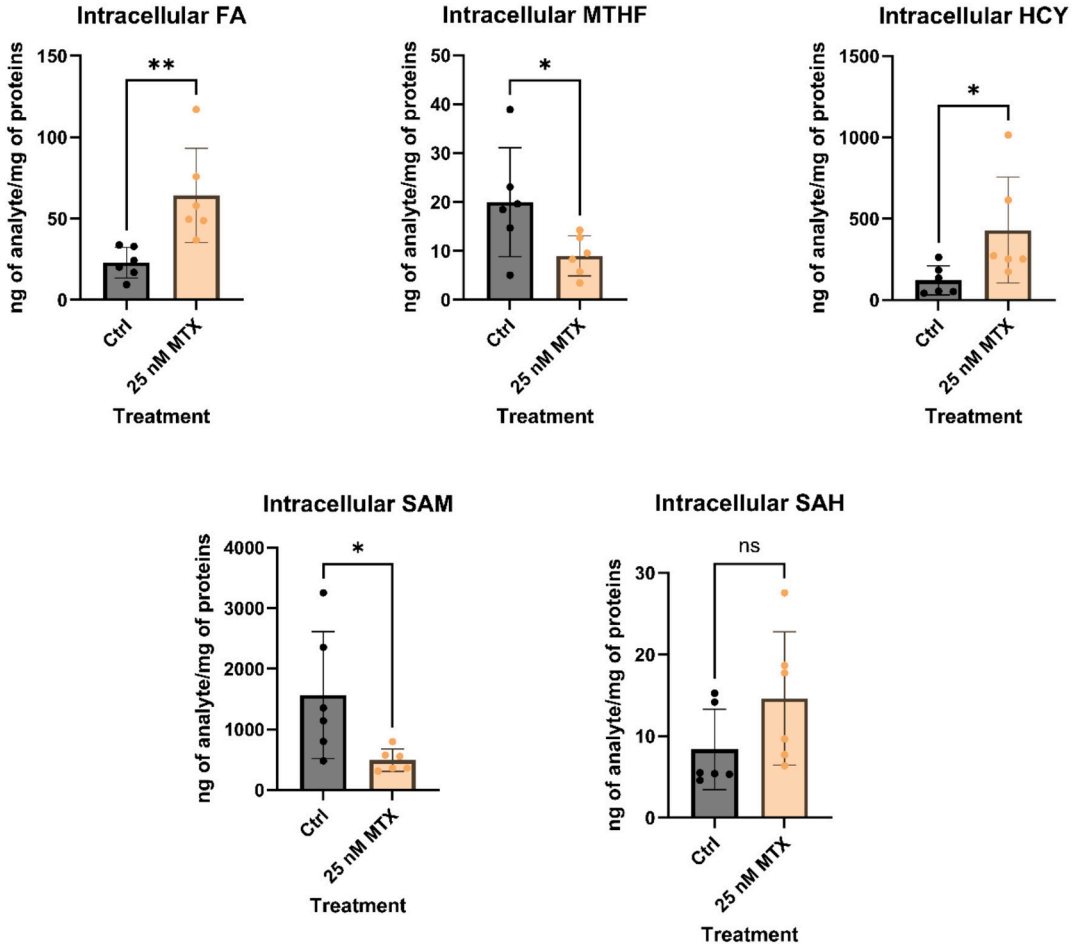

Fig. 3. Intracellular concentrations of folate metabolites in lymphoblastoid cells from 6 different individuals after a 72-h treatment with 25 nM methotrexate or control (PBS). HCY: homocysteine. FA: folic acid. MTHF: 5-methyltetrahydrofolate. SAM: S-adenosylmethionine. SAH: S-adenosylhomocysteine. MTX: methotrexate. Ctrl: control.

matrices [21,25,26,32,33]. The exception is SAM, for which plasma concentrations are approximately 100-fold lower than intracellular levels [34–37]. Subsequently, the method has a potential for diagnostic use in assessing folate status, particularly in patients with increased risks for folate-depletion related complications, mainly pregnant women and women of childbearing age [3,7,9,10,43]. Given the limitations associated with SAM quantification in plasma, peripheral blood mononuclear cells or hemolysates, both obtained from whole blood, represent suitable biological samples that would require minimal adaptation of the method, as previously reported [34–37]. The method is also suitable for mechanistic *in vitro* studies and (drug) screenings, enabling monitoring of intracellular methylation potential [50], drug-induced metabolic changes [51], or studying genotype-phenotype correlations in folate-methionine pathway [52].

4. Conclusion

We successfully developed and validated an LC-MS/MS method for the simultaneous quantification of five key metabolites in the folate and methionine cycle, namely FA, HCY, MTFH, SAM and SAH. To the best of our knowledge, this is the first LC-MS/MS method that enables simple sample preparation and simultaneous quantification of five major metabolites of the folate–methionine cycle. Most previously reported methods quantify no more than three key metabolites and/or often rely on derivatization or labor-intensive extraction. The method demonstrated selectivity, a concentration-response relationship, good accuracy, precision, absence of carry-over, both short- and long-term stability, and adequate sensitivity for determining all five analytes in biological samples. The robustness of the method was additionally proved on 6 LCLs from different donors in an experiment evaluating extraction recovery in different matrices. Our method is the first to employ ascorbic acid as the only antioxidant or mild reducing agent in the sample, avoiding the use of stronger reducing agents. To evaluate its applicability, the method was tested on an *in vitro* model of LCL cells, treated with the known antifolate methotrexate, revealing expected and consistent changes in the profile of folate and methionine metabolites. Due to its sensitivity, reliability, and efficiency, the method represents a valuable tool for profiling methylation-related metabolites in biological samples and may support future studies in disease monitoring and therapeutic response assessment.

CRediT authorship contribution statement

Jaka Rotman Primec: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Ksenija Geršak:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Irena Mlinarič-Raščan:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Dunja Urbančič:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Investigation, Conceptualization. **Robert Roškar:** Writing – review & editing, Validation, Methodology, Investigation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2025.116399.

Data availability

Data will be made available on request.

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