Original Communication

Quantification of Isotope-Labeled and Unlabeled Folates and Folate Catabolites in Urine Samples by Stable Isotope Dilution Assay

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Abstract: Dual-label stable isotope dilution assays for the simultaneous quantification of isotopologic folates in clinical samples offer the perspective for differentiating between unlabeled folates from endogenous body pools and administered [$^{13}C_5$]-labeled folates from a test dose when performing bioavailability trials. In contrast to intact folates, this methodology could hitherto not be applied to the quantification of the folate catabolites, p-aminobenzoyl glutamate and p-acetamidobenzoyl glutamate. In this study, [$^{2}H_4$]-p-aminobenzoyl glutamate, [$^{2}H_4$]-p-acetamidobenzoyl glutamate, and unlabeled p-acetamidobenzoyl glutamate were synthesized. The synthesis of the [$^{2}H_4$]-labeled compounds started at unlabeled p-aminobenzoic acid. For the formation of p-acetamidobenzoyl glutamate, p-aminobenzoyl glutamate was acetylated. The new substances were applied successfully in stable isotope dilution assays for the simultaneous quantification of the [$^{13}C_5$]-labeled and unlabeled folate catabolites, p-aminobenzoyl glutamate and p-acetamidobenzoyl glutamate, along with the predominant folate vitamers in urine. The assays were based on clean-up by strong anion exchange followed by liquid chromatography-tandem mass spectrometry detection. Assay sensitivity was sufficient to detect the folate catabolites in physiologic concentrations. The limit of detection was below 0.4 and 0.3 nmol/100 g for p-aminobenzoyl glutamate isotopologues and p-acetamidobenzoyl glutamate isotopologues in urine, respectively. The successful synthesis of [$^{2}H_4$]-p-aminobenzoyl glutamate, [$^{2}H_4$]-p-acetamidobenzoyl glutamate, and unlabeled p-acetamidobenzoyl glutamate and the implementation of these substances in stable isotope dilution assays allows dual-label designs that provide a more detailed insight into human folate metabolism.

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Introduction

Folate intake in Germany has been shown to be below the dietary recommendations (400 µg Dietary Folate Equivalents/day) [1]. Low folate status and folate deficiency are associated with elevated plasma homocysteine levels [2], a risk factor for vascular and brain diseases [3]. Additionally, folate deficiency is well known to increase the risk of neural tube defects [3]. Therefore, monitoring of dietary folate intake and individual folate status as well as knowledge of the organism's response to folate uptake, dietary requirements, and folate bioavailability are needed to protect human health.

Accurate methods for folate analysis in food and clinical samples are essential to study folate intake, absorption kinetics, metabolism, and bioavailability as a means of substantiating dietary recommendations. Folate determination is demanding due to the high number of vitamers, their occurrence in trace amounts, and their susceptibility to light, elevated temperature, and oxygen. Stable isotope dilution assays (SIDA) using stable isotope-labeled folates as internal standards (IS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) detection have proven their superiority over conventional methods in the analysis of food and clinical samples [4–6]. Further elaboration of the principle of SIDA has made it possible to simultaneously quantify [$^{13}C_5$]-labeled and unlabeled intact folates by using the respective [$^2H_4$]-labeled isotopologues as IS [7]. Studies on folate absorption kinetics, metabolism, and bioavailability profit from the new dual-label SIDA, as unlabeled endogenous folates can be differentiated from labeled dose folates [7, 8].

[^{2}H]-labeled folates are not available commercially. Several pathways have been published for the synthesis of [^{2}H]-labeled intact folates [9, 10]. In contrast, reports on the synthesis of [^{2}H]-labeled folate catabolites, p-aminobenzoyl glutamate (p-ABG), and p-acetamidobenzoyl glutamate (Ap-ABG) are rare. Thus, the dual-label SIDA is limited to the determination of intact folates.

Besides a small proportion of intact vitamers, folates are excreted as inactive catabolites, mainly Ap-ABG and p-ABG [5, 11]. The response between the amount of absorbed monoglutamate and excreted catabolites has been shown to be proportionally linear [12]. Thus, quantification of urine folate catabolites (amongst other parameters) contributes essentially to the determination of folate absorption and bioavailability.

The objective of the study was to extend the field of application for the dual-label SIDA to the simultaneous quantification of [$^{13}C_5$]-labeled and unlabeled folate catabolites. For this purpose, previous SIDA for the simultaneous quantification of (i) intact folates and folate catabolites in urine [5] and (ii) [$^{13}C_5$]-labeled and unlabeled intact folates relative to [$^2H_4$]-labeled IS [7] were combined. [$^2H_4$]-labeled IS for the urine folate catabolites, p-ABG and Ap-ABG, were synthesized, and the SIDA for p-ABG and Ap-ABG using deuterated analogues as IS was validated.

Materials and Methods

Chemicals

The following chemicals were obtained from the sources given in parentheses: Acetonitrile LiChrosolv, formic acid p.a. (98–100 %), n-hexane LiChrosolv, hydrochloric acid fuming 37 % LiChrosolv, 2-mercaptoethanol p.a. (MCE), methanol LiChrosolv, potassium dihydrogen phosphate, potassium hydroxide, sodium acetate trihydrate p.a., disodium hydrogen phosphate p.a., sodium hydroxide p.a., water for chromatography (Merck KGaA, Darmstadt, Germany), acetic anhydride, p-aminobenzoic acid potassium salt (p-ABA), p-aminobenzoyl glutamate, deuterium oxide (D_2O), *N,N'*-dicyclohexylcarbodiimide (DCC), *N,N'*-diisopropylethylamine (DIPEA), folic acid (PteGlu), L-glutamic acid dimethyl ester hydrochloride (L-Glu-diME), 1-hydroxybenzotriazole hydrate (HOBt), 2-(*N*-morpholino)ethanesulfonic acid (MES) monohydrate minimum 99.5 % titration, palladium on activated charcoal (30 %), sodium sulfate, tetrahydrofuran water-free (THF), trifluoracetic anhydride (TFA; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), L(+)-ascorbic acid (VWR International GmbH, Darmstadt, Germany), diethyl ether GPR Rectapur, ethyl acetate technical (VWR Prolabo, Leuven, Belgium), sodium chloride (Mallinckrodt Baker

B.V., AA Deventer, The Netherlands), dichlormethane picograde (Promochem, LGC Standards GmbH, Wesel, Germany), tetrahydrofolate (H_4folate), 5-methyltetrahydrofolate (5-CH_3-H_4folate), 10-formylfolic acid (10-HCO-PteGlu), 5-formyltetrahydrofolate (5-HCO-H_4folate; Schircks, Jona, Switzerland), [$^{13}C_5$]-5-CH_3-H_4folate, [$^{13}C_5$]-5-HCO-H_4folate, [$^{13}C_5$]-PteGlu (Merck Eprova AG, Schaffhausen, Switzerland), [$^2H_4$]-H_4folate, [$^2H_4$]-5-CH_3-H_4folate, [$^2H_4$]-10-HCO-PteGlu, [$^2H_4$]-5-HCO-H_4folate, [$^2H_4$]-PteGlu [10], [$^{13}C_5$]-p-ABG, [$^{13}C_5$]-Ap-ABG [5].

The synthesis of unlabeled Ap-ABG, [$^2H_4$]-labeled p-ABG, and Ap-ABG is described in the Supplemental Material. The formation of unlabeled and [$^2H_4$]-labeled p-ABG and Ap-ABG was based on two previously published pathways for the synthesis of (i) [$^2H_4$]-labeled intact folate [11] and (ii) [$^{13}C_5$]-labeled p-ABG and Ap-ABG [10].

Solutions and standards

MES buffer (Extraction buffer for urine samples) contained 0.2 mol/L MES monohydrate, 2 % ascorbic acid, and 1.4% MCE at pH 5.0.

Equilibration buffer [for clean-up of urine samples by solid phase extraction (SPE)] contained 3 mmol/L KH_2PO_4, 6 mmol/L Na_2HPO_4, and 0.2 % MCE at pH 7.0.

Phosphate buffer (0.1 mol/L) was prepared by adjusting an aqueous Na_2HPO_4*$2H_2O$ solution (0.1 mol/L) to pH 7.0 with an aqueous KH_2PO_4 solution (0.1 mol/L) [14].

Phosphate buffer (0.1 mol/L; 0.2 mol/L MCE) was prepared adding MCE (0.2 mol/L) to the phosphate buffer (0.1 mol/L). Above mentioned buffers were prepared on day of use [5].

Elution buffer (for clean-up of urine samples by SPE) contained 5 % sodium chloride, 1 % ascorbic acid, 0.1 mol/L sodium acetate trihydrate, and 0.1 % MCE. The buffer was kept chilled and used for a maximum of 4 weeks [15].

[$^2H_4$]-labeled folate vitamers (H_4folate, 5-CH_3-H_4folate, 10-HCO-PteGlu, 5-HCO-H_4folate, PteGlu [10]) and folate catabolites (p-ABG, Ap-ABG) were used as IS.

IS stock solutions contained 0.01 – 0.95 µmol [$^2H_4$]-labeled folates/mL MES buffer.

IS working solutions were prepared by dilution of the stock solutions with MES buffer. IS working solutions for folate quantification contained 0.5 – 4.5 nmol [$^2H_4$]-labeled folates/mL. All IS solutions were stored in amber glass bottles at –20 °C.

To determine the exact concentrations of the IS working solutions used, a "response mixture" consisting of the IS working solutions and solutions of the corresponding unlabeled folate derivatives in phosphate buffer (0.1 mol/L; 0.2 mol/L MCE) or phosphate buffer (0.1 mol/L) [6] for folate catabolites was analyzed with every batch of samples. The concentrations of the solutions of unlabeled folates were determined by spectroscopy using a SPECORD 50 photometer (Analytik Jena, Jena, Germany; [5, 6]) prior to the preparation of the "response mixture." Following LC-MS/MS analysis, the exact concentrations of the IS working solutions were calculated based on the corresponding response curves (see equations below) and the amount of unlabeled folates in the "response mixture."

Calculations

The calculation of the concentrations of the unlabeled folate solutions based on the absorption measured in the spectrophotometer used the following equation:

$$c\ [\mu g/mL] = (\text{Absorption} * MW)/(\varepsilon * d),$$

with: MW: Molecular weight
ε: Coefficient of absorption
d: Length of the cell (here: 1 cm)

For the calculation of the exact concentration of the IS working solution, the amount of IS in the "response mixture" n(IS) was determined based on the following equation:

$$n(IS) = ((y - b) * n(Ana))/R_f,$$

with: y: A(IS):A(Ana), Peak area ratio in the LC-MS/MS run
b: y-axis intercept (Table I)
n(Ana): amount of unlabeled folates in the "response mixture" sample based on the concentration determined by spectroscopy
R_f: Response factor (Table I)

Subsequently, n(IS) was set in relation to the volume of IS working solution added to the "response mixture."

For the calculation of the folate content in a sample, the amount of the respective folate vitamer n(Ana) was determined based on the following equation:

$$n(Ana) = (n(IS) * R_f)/(y - b),$$

with: y: A(IS):A(Ana), Peak area ratio in the LC-MS/MS run
b: y-axis intercept (Table I)
n(IS): amount of [$^2H_4$]-labeled IS added to the sample prior to extraction
R_f: Response factor (Table I)

Subsequently, n(Ana) was set in relation to the sample amount extracted.

Table I: Response curve parameters for intact folates and folate catabolites in urine (R^2 = 0.999 for all curves).

Vitamer	Linearity range of the response for A(IS):A(Analyte)	Response equation A(IS) : A(Analyte) = R_f * n(IS):n(Analyte) + b	
		Slope R_f	Intercept b
H_4folate	0.09 – 6.0	0.310	0.030
5-CH_3-H_4folate	0.2 – 14.1	1.076	0.043
[$^{13}C_5$]-5-CH_3-H_4folate	0.2 – 14.9	1.054	0.028
10-HCO-PteGlu	0.09 – 8.2	0.142	0.026
5-HCO-H_4folate	0.06 – 5.8	0.573	–0.001
[$^{13}C_5$]-5-HCO-H_4folate	0.1 – 3.9	0.556	0.038
PteGlu	0.08 – 7.4	0.362	0.014
[$^{13}C_5$]-PteGlu	0.09 – 7.9	0.403	0.002
p-ABG	0.04 – 3.5	0.653	0.002
[$^{13}C_5$]-p-ABG	0.09 – 8.6	0.642	–0.006
Ap-ABG	0.03 – 2.7	0.709	0.004
[$^{13}C_5$]-Ap-ABG	0.05 – 4.3	0.935	0.031

Clinical samples

Urine samples were obtained from one apparently healthy volunteer in a bioavailability trial similar to that reported recently [8]. After an overnight fast, the volunteer received a single test dose of 200 µg [$^{13}C_5$]-5-CH_3-H_4folate in apple juice. The subject had unlimited access to water and received lunch and a snack during a 12-hour post-dose period under standardized conditions. Thereafter, the volunteer had free access to dinner. Urine was collected during spontaneous bladder emptying into individual plastic bottles containing 1 % (w/v) ascorbic acid during 0–12 hours post-dose. Pooled urine samples were stored at –20 °C until analysis.

The study protocol was approved by the Regional Ethical Review Board in Uppsala and conducted according to the World Medical Association Declaration of Helsinki.

Extraction procedure for urine samples

By adapting our previously reported procedure [5], urine was thawed in a refrigerator overnight. Ten grams of urine, [$^2H_4$]-labeled IS (0.17 nmol H_4folate, 0.06 nmol 5-CH_3-H_4folate, 0.08 nmol PteGlu, 0.6 nmol p-ABG and 0.3 nmol Ap-ABG; targeted peak area ratio A(IS)/A(Analyte) covered by the respective response curves, i. e. between 0.5 and 3, Table I), and 4 mL extraction buffer were stirred in a Pyrex glass flask at room temperature (RT) in the dark to equilibrate analytes and standards. After an equilibration period of 30 minutes that has been reported to be sufficient [15], the sample was cleaned up by SPE, poured into a glass vial, and kept at –20 °C until LC/MS-MS analysis.

SPE clean up

Similarly to our recently reported procedure [5], urine samples were purified on a 12-port vacuum manifold (Visiprep, Supelco, Sigma Aldrich, Steinheim, Germany) equipped with strong anion exchange (SAX) SPE cartridges (Strata SAX 500 mg 3 mL, Phenomenex, Aschaffenburg, Germany). Columns were activated by 2 cartridge volumes of n-hexane, 2 volumes of methanol, and 3 volumes of equilibration buffer. Sample application was followed by washing with 5 cartridge volumes of equilibration buffer and running dry. Urine folates were eluted with 1.0 mL elution buffer, which proved to be the compromise to sufficiently elute all vitamers.

Liquid chromatography and mass spectrometry

Separation and detection of intact folates and folate catabolites followed a published procedure [4, 5]. MS detection was based on the transitions presented in Table II.

Calibration (Linearity range of response)

Separate response curves were established for each investigated folate derivative (H_4folate, 5-CH_3-H_4folate, [$^{13}C_5$]-5-CH_3-H_4folate, 10-HCO-PteGlu, 5-HCO-H_4folate, [$^{13}C_5$]-5-HCO-H_4folate, PteGlu, [$^{13}C_5$]-PteGlu, p-ABG, [$^{13}C_5$]-p-ABG, Ap-ABG, [$^{13}C_5$]-Ap-ABG) to convert peak area ratios [A(IS):A(Analyte)] into molar ratios [n(IS):n(Analyte)] for each respective IS ([$^2H_4$]-H_4folate, [$^2H_4$]-5-CH_3-H_4folate, [$^2H_4$]-10-HCO-PteGlu, [$^2H_4$]-5-HCO-H_4folate, [$^2H_4$]-PteGlu, [$^2H_4$]-p-ABG and [$^2H_4$]-Ap-ABG). Solutions of the [$^2H_4$]-labeled IS in MES buffer of known concentrations (1.2 – 8.0 nmol/mL) were mixed with solutions of known concentrations of the unlabeled and [$^{13}C_5$]-labeled analytes in phosphate buffer (0.1 mol/L; 0.2 – 1.3 nmol/mL) to different molar ratios (n = 8) ranging from 1 : 0.1 (0.12 : 0.01 nmol; standard surplus) to 1 : 25 (0.12 : 3.10 nmol; analyte surplus). The final concentrations in the eight different mixtures ranged from 0.02 to 3.2 nmol/mL and 0.07 to 16.3 nmol/mL for IS and analytes, respectively. The mixtures were analyzed by LC-MS/MS. Response curves were determined by plotting the molar ratios against the peak area ratios and application of simple linear regression.

Precision [Inter- and Intra-assay coefficient of variation (CV) for urine catabolites]

For investigating repeatability of folate determination in urine samples, intra-assay CV was determined. A urine sample was extracted five times according to the described method.

For determining reproducibility of the urine assay, inter-assay CV was determined by extraction of a urine sample once a week for three weeks according to the described method.

Limit of Detection (LOD) for urine catabolites

The LOD for determining folate catabolites in urine samples was estimated. A folate-free surrogate matrix [5] was spiked with unlabeled and [$^{13}C_5$]-labeled folate catabolites (p-ABG: 13.2 nmol/100 g, [$^{13}C_5$]-p-ABG: 1.1 nmol/100 g, Ap-ABG: 24.9 nmol/100 g, [$^{13}C_5$]-Ap-ABG: 2.2 nmol/100 g) and extracted in triplicate as detailed above. Subsequently, a proportion of the extracts was diluted in two steps with elution buffer (1 : 10 and 1 : 50) and all samples were analyzed by LC-MS/MS. LODs were estimated based on the peak heights observed in the LC-MS/MS chromatograms of the diluted extracts. A ratio of analyte signal height to noise signal height of 3 : 1 was considered as the LOD. For [$^{13}C_5$]-p-ABG, this ratio was found in the LC-MS/MS chromatograms of the 1 : 10-dilution for p-ABG, Ap-ABG, and [$^{13}C_5$]-Ap-ABG, this ratio was found in the LC-MS/MS chromatograms of the 1 : 50-dilution.

Table II: Investigated folate derivatives in urine samples and MS/MS transitions for their detection, adopted partly from previous reports [4, 5].

Vitamer	Precursor ion $[M+H]^+$, m/z	Product ion [M+H-γ-glutamyl]$^+$, m/z
H_4folate	446.1	299.2
[$^2H_4$]-H_4folate	450.1	303.2
5-CH_3-H_4folate	460.0	313.2
[$^2H_4$]-5-CH_3-H_4folate	464.0	317.2
[$^{13}C_5$]-5-CH_3-H_4folate	465.0	313.2
10-HCO-PteGlu	470.1	295.2
[$^2H_4$]-10-HCO-PteGlu	474.1	299.2
5-HCO-H_4folate	474.1	327.2
[$^2H_4$]-5-HCO-H_4folate	478.1	331.2
[$^{13}C_5$]-5-HCO-H_4folate	479.1	327.2
PteGlu	442.1	295.2
[$^2H_4$]-PteGlu	446.1	299.2
[$^{13}C_5$]-PteGlu	447.1	295.2
p-ABG	267.1	120.2
[$^2H_4$]-p-ABG	271.1	124.2
[$^{13}C_5$]-p-ABG	272.1	120.2
Ap-ABG	309.1	162.2
[$^2H_4$]-Ap-ABG	313.1	166.2
[$^{13}C_5$]-Ap-ABG	314.1	162.2

Recovery (for urine catabolites)

A matrix devoid of folates [5] was spiked with known amounts of unlabeled and [$^{13}C_5$]-labeled folate catabolites (ranging from 3.3 to 61.8 nmol/100 g, n = 3) and subsequently extracted according to the described method.

Data analysis

Analysis of NMR data was performed by ACD/1D NMR Processor Academic Edition (Advanced Chemistry Development Inc., Toronto, ON, Canada), ver-

sion 12.01. Analysis of LC-MS/MS data was performed using Xcalibur software (Thermo Electron Corporation, San Jose, CA, USA), version 2.0.

Simple statistical analysis was performed by Microsoft Office Excel 2007 (Microsoft Corporation, 2006).

Results and Discussion

Synthesis of unlabeled Ap-ABG and [$^2H_4$]-labeled p-ABG and Ap-ABG

For the development of dual-label SIDA, all target analytes along with their respective internal standards must be available. Of the analytes, [$^{13}C_5$]-labeled isotopologues were in our laboratory [5] and p-ABG is available commercially. In contrast, Ap-ABG was synthesised by a modification of a recently reported procedure [15]. Acetylation of p-ABG by acetic anhydride was simple to realize, and the yield was satisfactory (70 %) if temperature and pH value of the suspension were adjusted carefully. Addition of acetic anhydride to a suspension warmer than ~ 15 °C gave a brown, oily, undefined product. The yield was substantially increased by adjustment of the pH value from pH 2.3 to pH 4.5. At pH 2.3, the amino group of p-ABG (pK_s value 4.8) was mainly protonated ($-NH_3^+$); at pH 4.5 a higher proportion was deprotonated ($-NH_2$), thus favoring the nucleophilic attack at the carboxylic C-atom of the acetic anhydride molecule.

[$^2H_4$]-p-ABG and [$^2H_4$]-Ap-ABG were synthesized as IS for unlabeled and [$^{13}C_5$]-labeled folate catabolites in urine. The mass spectra of p-ABA before and after deuteration (Figure 1) showed clearly the incorporation of four [^{2}H]-atoms, as the mass of the molecular ion had been shifted from m/z = 138.1 to m/z = 142.2. Compared to previous results [10], the extent of the incorporation of [^{2}H]-atoms was lower although two additional reaction cycles were applied. To improve the deuteration extent of one reaction cycle, it was important to increase the reaction time. Neither D_2O surplus nor higher amounts of the catalyst led to a similar effect as did the prolonged reaction time. TFA and acetic anhydride were suitable reagents to acetylate the amino-moiety of [$^2H_4$]-p-ABA if the conditions of the reaction were controlled carefully, as discussed for unlabeled Ap-ABA. The coupling of [$^2H_4$]-*N*-TFA-p-ABA and [$^2H_4$]-Ap-ABA to L-Glu-diME, respectively, was accomplished at a low temperature to decrease the extent of side-product formation analoguously to a

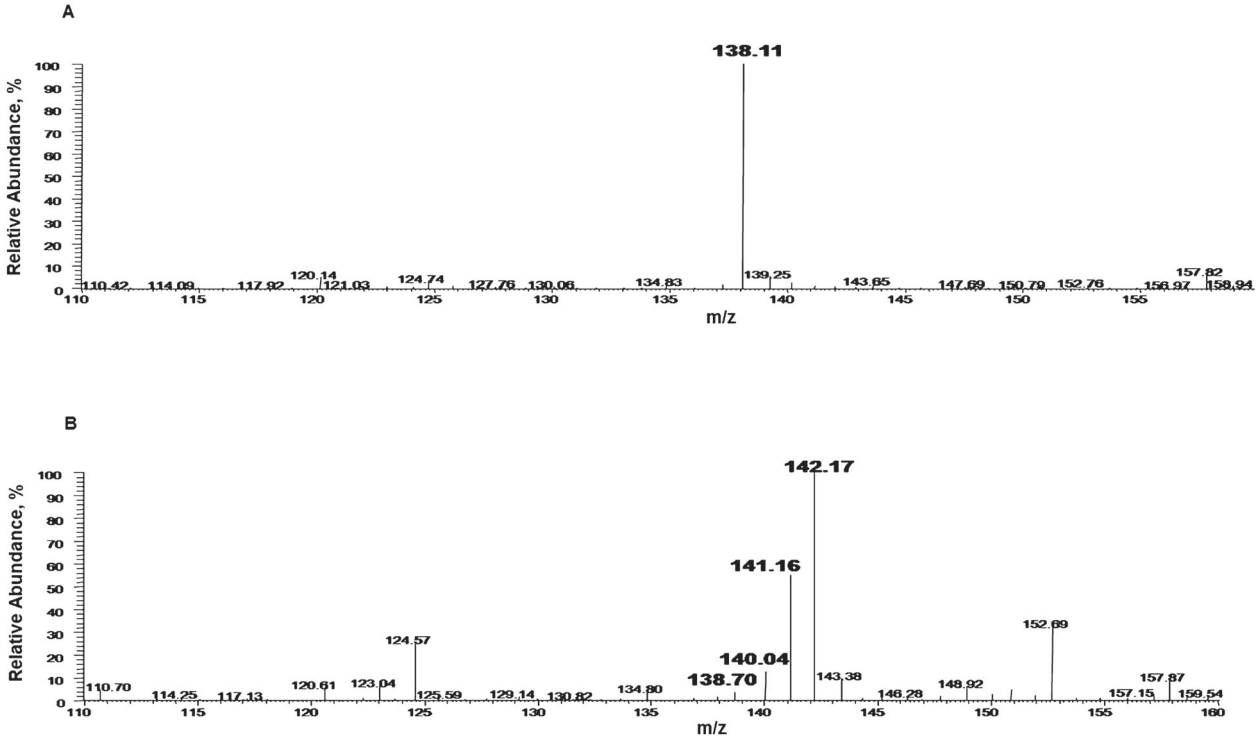

Figure 1: P-aminobenzoic acid before (A) and after (B; m/z 142.1 fourfold labeled) five deuteration cycles. Pathways of the synthesis of [$^2H_4$]-p-aminobenzoyl glutamate and [$^2H_4$]-p-acetamidobenzoyl glutamate.

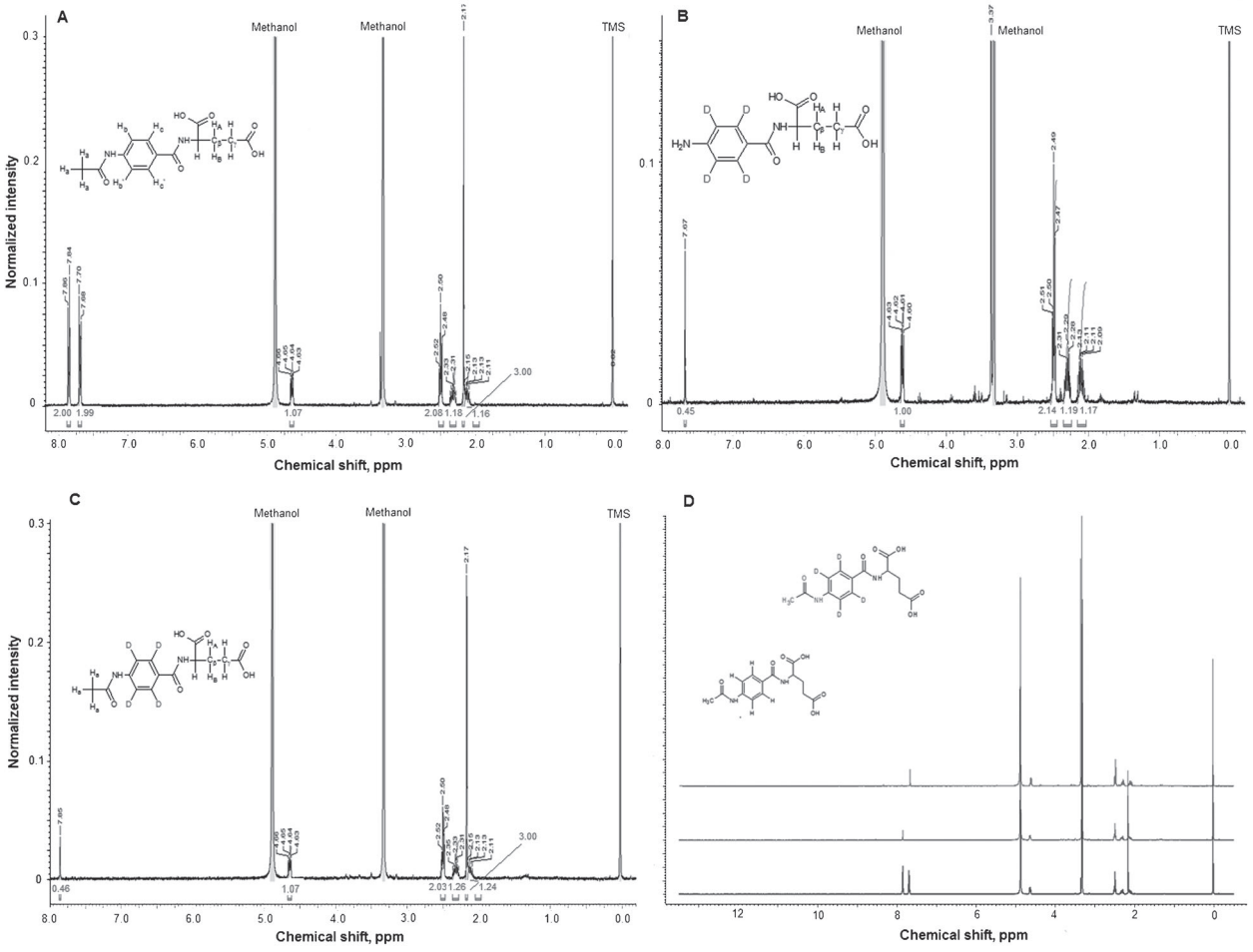

Figure 2: NMR spectra of the synthesized folate catabolites: unlabeled p-aminobenzoyl glutamate (A), [$^2H_4$]-p-aminobenzoyl glutamate (B), [$^2H_4$]-p-acetamidobenzoyl glutamate (C), overlay of the spectra A-C (D).

previously reported procedure [9]. L-Glu-diME * HCl was not completely soluble in anhydrous THF. The addition of DIPEA helped to neutralize and to completely dissolve the hydrochloride and HOBt activated the carboxyl group of the differently protected [$^2H_4$]-p-ABA forms. The reaction time of 48 hours was sufficient as no L-Glu-diME was detectable in the mass spectrum of the reaction suspension after that time.

Cleavage of the protection groups was simple to achieve. However, the total yield for both coupling and deprotection was low. Losses were possibly caused by entrapment of the synthesis product in the DCU precipitate or by incomplete extraction after cleavage of the protection groups. Additionally, losses were observed during preparative HPLC.

Despite its limitations, the presented pathway was suitable for synthesizing the [$^2H_4$]-labeled folate catabolites, p-ABG and Ap-ABG.

NMR and MS studies of Ap-ABG and [$^2H_4$]-labeled p-ABG and Ap-ABG

The identity of the synthesized substances was confirmed by NMR spectroscopy. The ^{1}H NMR spectrum of unlabeled Ap-ABG (Figure 2A) showed the signals of the glutamyl protons, a singlet at 2.17 ppm (integral of 3) corresponding to the protons of the acetyl-moiety, and two doublet signals (integral of 2 each) at 7.69 and 7.85 ppm, corresponding to the four aromatic protons. The ^{1}H NMR spectra of [$^2H_4$]-p-ABG and [$^2H_4$]-Ap-ABG (Figures 2B, 2C) showed identical signals apart from the two doublet signals of the aromatic protons. As deuteration was not complete, only one singlet at 7.69 or 7.85 ppm, respectively, was observed. Further, the singlet signal of the acetyl protons at 2.17 ppm was absent in the ^{1}H NMR spectrum of [$^2H_4$]-p-ABG (Figure 2B). Comparing these three spectra (Figure 2D), it was interesting to observe that [$^2H_4$]-p-ABG

lacked deuteration at $H_{b,b'}$, whereas [$^2H_4$]-Ap-ABG lacked deuteration at $H_{c,c'}$, both to an extent of 25 %. The cause for this effect remains unclear.

The extent of the fourfold incorporation of [^{2}H]-atoms was determined precisely for both [$^2H_4$]-labeled substances by a comparison of the integrals of the signals for the remaining aromatic protons and the α-proton. The integral of the α-proton was set to one, and the integral of the aromatic protons was calculated to 0.5. In the unlabeled molecule, this integral was 2. Thus, the extent of complete deuteration was 75 % in both [$^2H_4$]-labeled synthesis products. The remaining 25 % consisted of the one- to threefold labeled isotopologues. The unlabeled form was not detected, as indicated by the absence of the doublet characteristic for four aromatic protons.

In positive electrospray ionization (ESI) mode, all synthesized folate catabolites revealed the protonated molecule $[M+H]^+$ as the base peak in one-dimensional spectra. In two-dimensional spectra, some fragmentations were observed, which resulted mainly from the loss of the glutamyl moiety $[M+H-147]^+$ in collision-induced dissociation (CID). In accordance with the deuteration, precursor and product ion of both [$^2H_4$]-p-ABG and [$^2H_4$]-Ap-ABG, contained the [$^2H_4$]-label constantly.

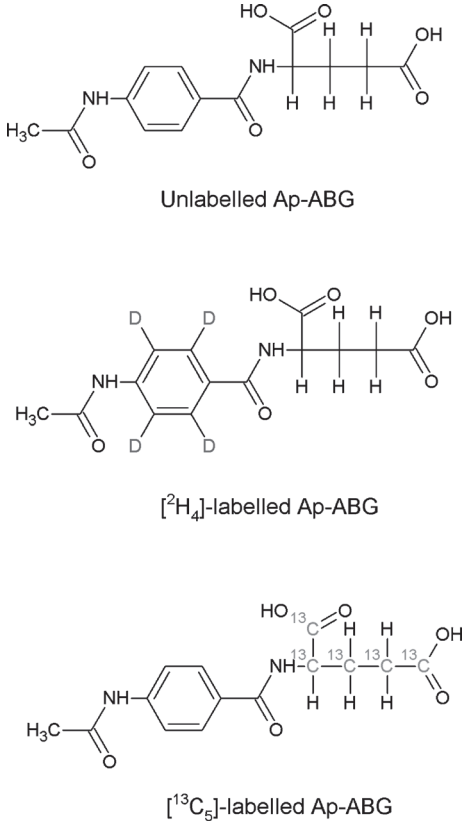

Figure 3: Structures of the p-acetamidobenzoyl glutamate isotopologues of studied in this trial.

Figure 3 presents the Ap-ABG isotopologues of interest for dual-label SIDA. The mass increment of four between the unlabeled and respective [$^2H_4$]-labeled precursor ion, as well as between the unlabeled and respective [$^2H_4$]-labeled product ion $[M+H-147]^+$, ensured unequivocal MS separation between unlabeled analyte and IS. Due to the natural abundance of stable isotopes, the mass increment of one between the precursor ion of the [$^2H_4$]-labeled IS and the [$^{13}C_5$]-labeled tracer was not sufficient for unequivocal MS separation of the protonated molecules. Yet, the mass increment of four between the respective product ions $[M+H-147]^+$ was sufficient. Hence, [$^2H_4$]-p-ABG and [$^2H_4$]-Ap-ABG were suitable IS for the determination of both unlabeled and [$^{13}C_5$]-labeled folate catabolites by LC-MS/MS.

Extraction of urine samples

Our recent methods did not allow analysis of [$^{13}C_5$]-labeled folate catabolites in human samples from trials using [$^{13}C_5$]-labeled dose folates by SIDA, as no differently labeled IS were available. Based on the successful synthesis of the [$^2H_4$]-labeled folate catabolites, it was possible to establish SIDA for the simultaneous determination of unlabeled and [$^{13}C_5$]-labeled p-ABG and Ap-ABG in urine.

For the concurrent analysis of differently labeled stable isotopologues, three prerequisites have to be fulfilled: a) complete equilibration between IS and analyte(s), b) stability of the label(s), and c) unequivocal spectral differentiation (for details see the review on SIDAs for mycotoxins [13]). Previous method validation studies confirmed compliance with prerequisites a) and b) [4, 11, 14], as well as with the prerequisite c) for intact folates [5]. In this study, the absence of spectral overlaps had to be verified for [$^{13}C_5$]-labeled, [$^2H_4$]-labeled, and unlabeled p-ABG and Ap-ABG. The MS studies had already proven the unequivocal MS separation of [$^2H_4$]-p-ABG and [$^2H_4$]-Ap-ABG from the unlabeled and [$^{13}C_5$]-labeled forms. Unlabeled p-ABG and Ap-ABG showed the same abundant product ions as [$^{13}C_5$]-p-ABG and [$^{13}C_5$]-Ap-ABG, since the label of the latter is lost during CID. However, both can be differentiated by the mass increment of five between the precursor ions.

Unequivocal differentiation of unlabeled, [$^2H_4$]-labeled and [$^{13}C_5$]-labeled isotopologues was proven

by response curve linearity and good agreement of response factors R_f for unlabeled and [$^{13}C_5$]-labeled folates (Table I). The variation between the R_f of an unlabeled folate vitamer and the R_f of the respective [$^{13}C_5$]-labeled vitamer was between 2 % and 10 %. Except for Ap-ABG, this variation was 24 %, resulting from a higher response of unlabeled Ap-ABG in comparison to [$^{13}C_5$]-Ap-ABG. Multiple measurements confirmed this value, which could not be explained by the isotopologic distribution, and, therefore, the reason for this variation remains open.

Intra-assay and inter-assay precision studies revealed relative standard deviations ranging from 0.8 to 3.3 and from 15.0 to 17.4 %, respectively. An estimation of the dimensions of the LOD from signal-to-noise ratios confirmed the sufficient sensitivity of the new assays. LOD was 0.3, 0.4, 0.3, and 0.06 nmol/100 g for p-ABG, [$^{13}C_5$]-p-ABG, Ap-ABG, and [$^{13}C_5$]-Ap-ABG, respectively. In a spiking range from 5.1 to 61.8 nmol/100 g, the recoveries (n = 3) in a surrogate urine matrix ranged from 90 % to 104 %. The quality control parameters for intact folate vitamers were published previously [5].

Application of the investigated quantification procedure to urine samples

Unlabeled and [$^{13}C_5$]-labeled folate vitamers and catabolites were quantified in a urine sample derived from one subject after ingestion of 200 µg [$^{13}C_5$]-5-CH_3-H_4folate in apple juice using the procedures presented to test the new SIDA's applicability in human trials. The 0-12 hour urine sample (220 mL) contained 0.8 nmol/100 g 5-CH_3-H_4folate, 0.5 nmol/100 g [$^{13}C_5$]-5-CH_3-H_4folate, 1.8 nmol/100 g H_4folate, 2.1 nmol/100 g p-ABG, 0.5 nmol/100 g [$^{13}C_5$]-p-ABG, 13.4 nmol/100 g Ap-ABG, and 0.8 nmol/100 g [$^{13}C_5$]-Ap-ABG. [$^{13}C_5$]-p-ABG content was close to the estimated LOD. As the [$^{13}C_5$]-p-ABG peaks were clearly detectable and the estimated LOD indicates only the dimension of sensitivity, the determined [$^{13}C_5$]-p-ABG content was still considered to be reliable.

The detection of both intact [$^{13}C_5$]-5-CH_3-H_4folate and [$^{13}C_5$]-labeled catabolites suggested fractional conversion of the ingested dose besides excretion, without metabolic transformation of the ingested vitamer. These findings cannot reveal whether the ingested [$^{13}C_5$]-5-CH_3-H_4folate was excreted directly or involved in metabolic reactions prior to excretion. Further information can be obtained from non-pooled urine samples. A study on these kinetics is under way.

Conclusion

[$^2H_4$]-labeled p-ABG and Ap-ABG and unlabeled Ap-ABG were generated by adoption of known procedures [9, 10, 15].

[$^2H_4$]-p-ABG and [$^2H_4$]-Ap-ABG were applied effectively as IS for the simultaneous determination by SIDA of the respective unlabeled and [$^{13}C_5$]-labeled isotopologues in urine.

The simultaneous quantification of [$^{13}C_5$]-labeled and unlabeled folate catabolites in urine provides the option of a more detailed insight into human folate metabolism in future studies. In combination with previously published SIDA for the quantification of folates in plasma, ileostomal effluent, and food [7], a tracer can be followed from ingestion to excretion in human trials on folate metabolism and bioavailability. The quantification of [$^{13}C_5$]-p-ABG and [$^{13}C_5$]-Ap-ABG in urine allows the assessment of folate turnover for a better understanding of human folate metabolism and bioavailability.

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