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Application of Stable Isotope Dilution Assays based on Liquid Chromatography – Tandem Mass Spectrometry for the Assessment of Folate Bioavailability

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Key words: bioavailability, folates, LC-MS-MS, spinach, stable isotope dilution assay

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ABSTRACT

A pilot study was performed to prove the suitability of stable isotope dilution assays for assessing the bioavailability of endogenous folates in foods. By using [$^{2}H_4$]-folic acid, [$^{2}H_4$]-tetrahydrofolate, [$^{2}H_4$]-5-methyltetrahydrofolate, [$^{2}H_4$]-5-formyltetrahydrofolate and [$^{2}H_4$]-10-formylfolic acid as internal standards, folates in spinach, apple juice and blood plasma were quantified by liquid chromatography coupled to tandem mass spectrometry. To liberate the pteroyl monoglutamates, sample extracts of foods were treated by rat plasma. Sample clean-up was achieved by solid phase extraction on anion exchange cartridges, which proved to be sufficient to obtain mass chromatograms devoid of matrix interferences. The bioavailability study was designed as a short-time protocol with three meals, the first consisting of 600 g spinach (meal A), the second consisting of 600 g apple sauce with additionally 400 µg synthetic folic acid (meal B) and the third consisting solely of 600 g apple sauce (meal C). Prior to the meals, the participating volunteer's tissue was saturated with folates to achieve a significant response of plasma folate to the meals. After consumption of meal A and B a significant rise in folate plasma level compared to meal C (mean level at 28 µg/ mL) was observed. The relative bioavailability of folate following meal A exceeded significantly the suggested value of 50 % for food folates by taking the dose-normalised area under the curve (AUC) following ingestion of meal B as reference.

ABBREVIATION USED

AC: affinity chromatography; AUC: Area under the curve; CHES: 2-[N-cyclohexylamino]ethanesulfonic acid; DFE: Dietary folate equivalents; FDA: Food and Drug Administration of the USA; DRI: Daily recommended intake; H_4folate: tetrahydrofolate or tetrahydrofolic acid, respectively; HEPES: N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; LC: liquid chromatography; meal A: meal consisting of spinach (600 g); meal B: meal consisting of apple sauce (600 g) and one tablet containing 400 µg of folic acid; meal C: meal consisting of apple sauce (600 g); MS: mass spectrometry; Pte Glu_3: pteroyltriglutamate; SAX: strong anion exchange; SIDA: stable isotope dilution assays; SPE: solid phase extraction; SRM: selected reaction monitoring

INTRODUCTION

In the past years it has become increasingly evident that folate deficiency may be associated with the risk of neural tube defects during pregnancies [1], coronary heart disease [2], certain forms of tumors [3] and Alzheimer's disease [4]. To lower the risk of these disorders, the Food and Drug Administration of the USA (FDA) has stipulated for adults a Daily Recommended Intake (DRI) of 400 µg Dietary Folate Equivalents (DFE) per day [5], which has been adopted by some national nutrition societies [6] in Europe. As several nutrition consumption surveys indicate the mean folate intake from foods to meet only about 50 % of the DRI [7; 8] or lower, fortification of grain products with folic acid is mandatory in the USA since 1998 [9]. In Europe, however, the authorities recommend to increase the folate intake by supplementation only on a voluntary basis. For this reason the knowledge of endogenous folate contents in foods and folate bioavailability is crucial for further health policy.

However, these data are still controversely discussed as the frequently employed methods of folate quantitation show several drawbacks.

The reference method is a microbiological assay which cannot distinguish between the derivatives of folic acid (henceforth referred to as folate vitamers). However, the lack of differentiation is a severe limitation, as the vitamers show different recoveries during sample preparation [10; 11] and the used microorganisms response differently to the single folate derivatives [12]. Due to these constraints, several data on folate contents and on bioavailability have to be questioned.

For this reason we recently developed stable isotope dilution assays (SIDAs) by using [$^2H_4$]-folic acid, [$^2H_4$]-tetrahydrofolate, [$^2H_4$]-5-methyltetrahydrofolate, [$^2H_4$]-5-formyltetrahydrofolate and [$^2H_4$]-10-formylfolic acid [13] as internal standards to correct for losses during sample preparation and mass spectrometry.

This method exhibits excellent sensitivity as well as recovery and the application to food samples confirmed many food folate data in several data bases. However, the contents of broccoli and bread, i. e., proved to be significantly lower when quantified by SIDA [14].

As this method is also applicable to blood folate analysis, it offers the perspective to attain accurate bioavailability data by combined quantification of food and blood folates.

Therefore, the aim of this study was to prove the benefits of SIDA in bioavailability research by performing a pilot short-time study with one volunteer, who consumed three meals consisting of spinach, apple sauce and apple sauce accompanied by one tablet containing synthetic folic acid. By following the blood folate level in combination with folate data from the consumed food, it was intended to calculate the relative bioavailability of spinach folates compared to synthetic folic acid.

MATERIALS AND METHODS

Materials

The following chemicals were obtained commercially from the sources given in parentheses: bacterial protease, formic acid, hexane, hydrogen peroxide, 2-

mercapto ethanol, methanol, *di*-potassium hydrogen phosphate, sodium dihydrogen phosphate, sodium chloride (Merck, Darmstadt, Germany), 2-[N-cyclohexylamino]ethanesulfonic acid (CHES), N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), sodium ascorbate (Sigma, Deisenhofen, Germany), acetonitrile (Baker, Gross-Gerau, Germany). Unlabeled folate vitamers (6*S*)-tetrahydrofolic acid (H_4folate), (6*S*)-5-methyltetrahydrofolic acid (5-methyl-H_4folate), 10-formylfolic acid and Pteroyl triglutamate were obtained from Dr. Schircks Laboratories (Jona, Switzerland), catalase and (6*S*)-5-formyltetrahydrofolic acid (5-formyl-H_4folate) were purchased from Sigma (Deisenhofen, Germany) and folic acid was from Fluka (Neu-Ulm, Germany). Rat serum was obtained from Biozol (Eching, Germany).

[$^2H_4$]-labeled folate standards were synthesized as reported recently [13].

Sample Preparation

Extraction buffer

A mixture of aqueous HEPES (50 mmol/L) and aqueous CHES (50 mmol/L) containing sodium ascorbate (2%) and 2-mercapto ethanol (0.2 mol/L) was adjusted to pH 7.85. The buffer was prepared on day of use.

Foods

Apple sauce and frozen spinach were purchased at local stores in the city of Giessen, Germany.

Spinach was frozen with liquid nitrogen before mincing with a blender (Privileg, Quelle, Fürth). Aliquots (1-2 g) were taken from the resulting powder-like homogenate and overlaid with 10 ml of extraction buffer. In case of apple sauce, aliquots (3 g) were slurried with extraction buffer. [$^{2}H_{4}$]-labeled internal standards were added to the suspensions in an amount to adjust a mass ratio of standard to analyte ranging between 1 and 5.

Sample suspensions were then purged with argon, capped tightly and placed in a boiling water bath for 10 min. Subsequently the extracts were rapidly cooled in an ice-bath and digested with protease (3 mg/g sample) for 6 h at 37°C. After the enzyme digestion, the samples were heated at 100°C for 10 min, cooled on ice and spiked with 100 µL of rat serum. The deconjugation was performed at 37°C overnight. At the end of the conjugase treatment, the samples were again heated at 100°C for 10 min, then cooled on ice and centrifuged at 6000 g for 20 min. After passing the residue through a syringe filter (0.4 µm, Millipore, Bedford, MA), Extracts were subjected to solid phase extraction clean-up as described below.

In order to assure the completeness of deconjugation, pteroyltriglutamate was added to a sample of spinach which was quantified by SIDA after treatment with protease and rat serum as detailed above.

Plasma

Plasma samples (1 mL) were spiked with [$^2H_4$]-5-methyl-H_4folate (20 ng) and eqilibrated for 30 min at room temperature. The solution was then centrifuged (10 min; 2000 *g*) and diluted with extraction buffer (2 mL) before being subjected to solid phase extraction as described below.

Sample Clean-up by Solid Phase Extraction (SPE)

Extracts were purified by SPE according to the method described recently [14], using an 12-port vacuum manifold (Alltech, Bad Segeberg, Germany) equipped with Bakerbond SAX cartridges (quaternary amine, 500 mg, No. 7091-3, Baker, Gross-Gerau, Germany). The cartridges were successively activated with 2 volumes of hexane, methanol and water, and then conditioned with 7 to 8 volumes of phosphate buffer (pH 7.5, 0.01 mol/L, containing 0.2 % 2-mercapto ethanol).

After applying the sample extracts, the columns were washed with 6 volumes of conditioning buffer, and the folates were eluted with 2 mL of aqueous sodium chloride (5 %, containing 1 % sodium ascorbate and 0.1 mol/L sodium acetate). 100 µL 2-mercapto ethanol was added to each eluate and the purified extracts were stored under argon at –30°C until analysis.

LC-MS-MS

Liquid chromatography was performed on a spectra series HPLC system (Thermo Separation Products, San Jose, CA) equipped with an Aqua C-18 reversed phase column (250 x 4.6 mm; 5 µm, Phenomenex, Aschaffenburg, Germany). The mobile phase consisted of variable mixtures of aqueous formic acid (0.1 %) and acetonitrile, at a flow of 0.8 mL/min. In case of food extracts, injection of samples (100 µL) was followed by gradient elution starting at 7 % acetonitrile maintained for 9 min. Then, the acetonitrile concentration was raised linearly to 13 % within 13 min and to 25 % within further 4 min. Subsequently, the mobile phase was programmed to 100 % acetonitrile over 4 min before equilibrating the column for 5 min with the initial mixture.

For MS-MS analysis the eluent was diverted to an LCQ-ion trap mass spectrometer (Finnigan MAT, Bremen, Germany) set at MS-MS conditions reported recently [14]. The UV-detector was operated at 280 nm.

As plasma samples contained 5-methyl H_4folate as the only vitamer and showed lower matrix interferences than the food extracts, gradient elution was shortened. Elution started at 10 % acetonitrile and was raised to 20 % acetonitrile within 6 min. Then, the concentration was sharply raised to 80 % acetonitrile within 1 min and to 100 % within another minute. This concentration was maintained for 2 min before lowering the acetonitrile concentration back to the initial value and allowing the column to equilibrate for another 3 min. Each plasma extract was analysed in triplicate. Amounts of the single vitamers were calculated from the peak areas in the respective mass traces using the calibration functions as detailed recently [14].

Performance Data

Detection and quantitation limits were determined using plasma samples from a volunteer who was not saturated with folic acid. In order to degrade endogenous 5-methyl-H_4folate in

the plasma sample, the following procedure was applied: plasma samples (10 mL) were heated for 10 min in a boiling water bath and cooled on ice. 5-Methyl-H_4folate was then oxidated by adding 750 µL H_2O_2 (30 %) and stirring it at room temperature for 60 min. The peroxide was then destroyed by addition of 0.3 mg catalase and stirring for 15 min at room temperature. To the folate-free plasma (10 mL) 10, 25, 50 and 100 ng 5-methyl-H_4folate were added. Extraction, addition of labeled 5-methyl-H_4folate, and sample clean-up was performed as described before. Then, LC-MS-MS analysis was conducted as outlined above. Subsequently, each addition assay was performed in triplicate and detection limits as well as quantification limits were calculated according to Hädrich and Vogelgesang [15].
Inter-assay precision was determined by analyzing blood plasma, which was taken during the bioavailability study and homogenized after sampling. Of this homogenate, four samples (1 mL each) were analyzed as detailed before.
Recovery data were obtained by adding 5-methyl-H_4folate (10 ng) to four plasma samples (1 mL each) treated by H_2O_2 as outlined above. The spiked samples were then analyzed as described before.

Study design

The bioavailability study was designed as a short-time protocol including the consumption of three different test meals by one non-smoking female volunteer (25 years old with a body mass index of 25.9 kg/m^2). Two weeks before consumption of the first test meal, tissue of the volunteer was saturated by supplementation with folic acid (800 µg/d) in order to attain a maximum plasma response after the consumption trials by preventing losses of plasma folates into tissue. Two days before the test the gavage of folic acid was stopped to allow for plasma level stabilization.

Meal A, which consisted of cooked spinach (600g), was consumed by the volunteer at 8 o'clock in the morning of the first trial day, and consumption was followed by blood sampling for each hour within 6 h. One week after the first trial, the volunteer ate meal B consisting of apple sauce (600 g) and a tablet containing folic acid (400 µg). Meal C was consumed one week later and consisted solely of apple sauce (600 g). Blood sampling was performed analogously to meal A. The plasma obtained for all trials as well as samples of spinach and apple sauce were stored at – 60 ° C until analysis.

Biokinetic calculations

Model-independent biokinetic evaluation was performed according to standard methods using the WinNonlin Professional software (version 3.3, Pharsight Co., Mountain View, CA, USA). The following target parameters for folate biokinetics were evaluated in plasma using the baseline subtracted folate data: peak plasma concentration (C_{max}), time to reach peak concentration (t_{max}), and area under the concentration-time curve from time zero up to six hours post intake (AUC(0-6)). AUC(0-6) was calculated according to the linear trapezoidal rule.

RESULTS AND DISCUSSION

Analysis of foods

To obtain accurate food folate data, the consumed apple sauce and spinach were analysed by the recently developed stable isotope dilution assay [16]. Prior to extraction, folate isotopomers [$^{2}H_4$]-folic acid, [$^{2}H_4$]-tetrahydrofolate, [$^{2}H_4$]-5-

methyltetrahydrofolate, [$^2H_4$]-5-formyltetrahydrofolate and [$^2H_4$]-10-formylfolic acid (structures shown in fig. 1) were added as internal standards to correct for losses during sample preparation and mass spectrometry. As foods are known to contain significant amounts of pteroyl polyglutamates, the cleavage to monoglutamates was achieved by treatment with rat plasma. To assure sufficient deconjugation activity, the treatment was tested by addition of pteroyltriglutamate ($PteGlu_3$) to spinach followed by SIDA of folic acid therein. As $PteGlu_3$ was converted to folic acid at a percentage of 100 ± 10 %, the enzyme treatment proved to be adequate. Sample clean-up was performed by solid phase extraction on strong anion exchange cartridges which gave sufficiently clean extract to allow unequivocal identification of folate vitamers by LC-MS. As the mass spectrometer was operated in the **s**elected **r**eaction **m**onitoring (SRM) mode, more sophisticated clean-up procedures such as affinity chromatography (AC) on folate binding protein could be omitted [10]. By using LC-Tandem MS, mass chromatograms revealed clearly separated peaks for all folate vitamers as shown in fig.2 for spinach.

SIDA was performed in triplicates and gave the results listed in table 1. The commercial spinach sample contained 47 µg / 100g total folates which was about 9 % higher than labelled on the package (40 µg/100g). Of all vitamers, only 5-methyltetrahydrofolate and 5-formyltetrahydrofolate were detected. As fresh spinach contains additionally tetrahydrofolate [14], the latter vitamer obviously had been degraded during processing, storage, and heating.

In comparison to spinach, the apple sauce contained only 2.4 µg/100g which confirmed its suitability to serve as basis diet without supplying significant amounts of folates.

Analysis of blood plasma

As expected, the analysis of blood plasma proved to be more easier than that of foods. As no pteroyl polyglutamates occur, the deconjugation treatment could be omitted. Additionally the sample extracts contained only 5-methyl-H_4folate, which was also in agreement with earlier findings [17]. Due to the lack of other vitamers, the LC gradient could be shortened to give a total run time of 15 min. The mass chromatograms were totally devoid of matrix interferences and enabled accurate quantification. Performance data of the SIDA developed for blood plasma shown in table 2 proved the method suitable to obtain accurate data.

Recently, there were two other applications of LC-MS to blood folate analysis with the difference that only the single stage MS mode was used. Although the performance data given by Pawlosky et al. [18] were quite similar to those reported in the present study (table 2), the chromatograms shown by the latter authors revealed incomplete resolution from interferences. These drawbacks were overcome by Hart et al. [19], who used AC for sample clean-up. As our chromatograms reveal in fig. 3, application of tandem MS made AC evitable.

To allow correct comparison of AUC, plasma folate levels determined prior to consumption of meal A and B were subtracted from the levels determined after consumption. To control for circadian rhythm of folate, plasma levels were also determined following intake of the basis diet (meal C) which was poor in folate. In addition, to correct for the different folate doses administered with the test meals AUC and C_{max} values were divided by dose.

Pilot study for assessing bioavailability

Due to the folate saturation protocol, plasma folate of the volunteer was elevated at a high level of 28 ng/mL. This concentration was more than twice as high as the base levels of 11 ng/ml measured in a comparable study reported by Prinz-Langenohl et al. [20] without folate saturation. After consumption of the virtual folate-free meal C the baseline level shown in figure 4 was not significantly changed over the observed sampling period of 6 h. Thus it seemed justified regarding AUC comparison to subtract folate levels determined prior to ingestion from those determined after ingestion of the respective test meals. In contrast to this, the consumption of spinach (meal A) resulted in a significant increase in plasma folate with a maximum level of 43.9 ng/mL after 2 h. This behaviour was in good agreement with other studies giving t_{max} ranging between 1 and 3 h [20; 21]. Contrary, meal B consisting of folic acid and apple sauce revealed a plasma level that peaked after 5 h, which did not agree with comparable studies showing t_{max} that ranged between 1 and 3 h [22; 23]. We suppose that the tablet containing folic acid revealed worse absorption properties than usual products. A similar finding was reported by Pietrzik and Rehmer [24], who observed a negligible plasma response after application of a tablet containing folic acid and other vitamins.

As some studies reported the appearance of folic acid in plasma after oral application, we analysed plasma samples of meal B for folic acid by application of the LC gradient for food samples. However, we could not detect the unmetabolized compound, which indicated that all folic acid had been metabolized during passage through intestinal mucosa or during first-pass in the liver.

As can be seen from table 3, the relative bioavailability of folate from spinach was 194% in the subject under study by taking the dose-normalised AUC following ingestion of apple sauce with synthetic folic acid as reference. Because t_{max} of meal B was unexpectedly high, only AUC(0-6) (area under the curve covered by measuring points) could be calculated without extrapolating to the time when the plasma curve again reaches the base plasma level. Taking AUC of meal B as a reference value of 100 % bioavailability, the bioavailability of spinach folates would result in 194%. As synthetic folic acid has been proven to show the highest folate bioavailability [25], this value appears too high and should be interpreted cautiously. As the evaluation of bioavailability is normally based on AUC extrapolated to infinity the value observed in the volunteer under study awaits further investigation. In the case of apple sauce with the tablet it was not possible to extrapolate AUC up to infinity due to the lack of sampling points after t_{max}. In the case of spinach the extrapolated portion of AUC would have exceeded the AUC(0-6) by more than 20% which is regarded as unreliable extrapolation. This may be the reason that the bioavailability estimated in our study exceeds that of a similar study by Prinz-Langenohl et al. [20] obtaining bioavailabiliy ranging between 89 % and 118 % following meals consisting of 600 g and 300 g spinach, respectively.

However, the results may be different from those obtained in this pilot study, if the number of test persons and the duration of the blood sampling period is increased.

CONCLUSION

The SIDA presented here proved to be very accurate and precise and, therefore, appears to be a remarkable improvement for bioavailability studies. Given a longer sampling period and a standard cross-over design using a suitable sample size, accurate data for bioavailability can be obtained. Regardless of the constraints of the study presented, the folates of spinach appear to exhibit higher bioavailability than the generally supposed degree of 50 % [26].

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Table 1. Folate contents in spinach and applesauce used for the consumption trials. The contents were measured by SIDA and are expressed in µg / 100 g fresh / frozen weight ± standard deviation (n=4).

Food	Total folate	5-methyl-H_4folate	H_4folate	5-formyl-H_4folate
Spinach, frozen, commercial product	47 ± 2	39 ± 1.4	n.d.	11 ± 0.8
Apple sauce, commercial product	2.4 ± 0.4	2.1 ± 0.3	n.d.	0.4 ± 0.2

n.d. not detectable, limit of detection for H_4folate: 1.5 µg/100g

Table 2. Performance data of the stable isotope dilution assay (SIDA) for 5-methyltetrahydrofolate in blood plasma presented in this study in comparison to that reported by Pawlosky et al. [18].

Performance criterion	SIDA presented in this study	SIDA reported by Pawlosky et al. [18]
Detection limit	1.6 ng/mL	n. d.
Quantification limit	4.9 ng/mL	0.9 ng/mL
Intra-assay precision: Coefficient of variation at a level of	 4.7 % (n=4) 20 ng/mL	 5.3 % (n=4) 11.5 ng/mL
Recovery at a level of 10 ng/mL	93 ± 5 % (n=4)	n. d.

n. d. not determined

Table 3. Biokinetic data obtained following consumption of test meals

Meals	D (µg)	t_{max} (h)	C_{max} [a] (ng/mL)	C_{max}/D (ng/mL/µg)	AUC(0-6) [a] (h*ng/mL)	AUC(0-6) /D (h*ng/mL/µg)
A: Spinach	284	2	20.4	0.072	93.4	0.33
B: Apple sauce with folic acid	414	5	17.6	0.042	71.1	0.17

D: administered amount of total folate; AUC: area under the curve
[a] relative values (corrected for base-line folate concentration determined at t=0h)

LEGENDS TO THE FIGURES

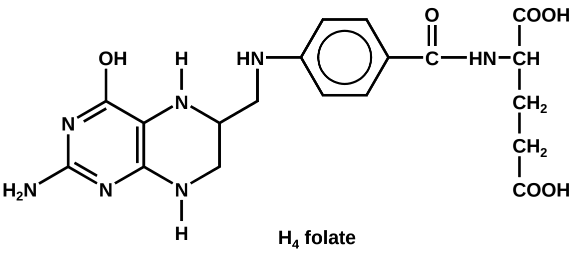
Figure 1. Structures of folates to be analyzed and the corresponding [$^{2}H_{4}$]-labelled internal standards.

Figure 2. LC-MS-MS chromatogram of an extract from spinach showing the mass ranges of folate vitamers and corresponding internal standards. The content of H_4folate was below the detection limit. SRM: selected reaction monitoring *m/z* precursor ion / *m/z* product ion .

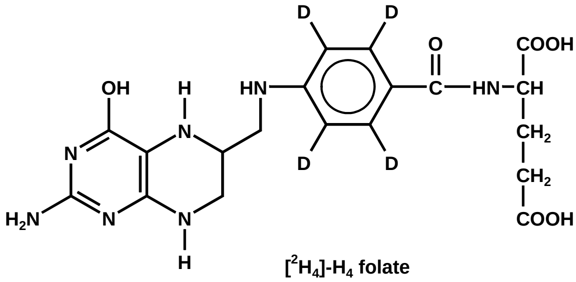
Figure 3. LC-MS-MS chromatogram of a blood plasma extract showing the mass ranges of 5-methyl-H_4folate and the corresponding internal standard [$^{2}H_{4}$]-5-methyl-H_4folate.

Figure 4. Plasma folate level after consumption of 600 g apple sauce (meal C, virtual folate-free).

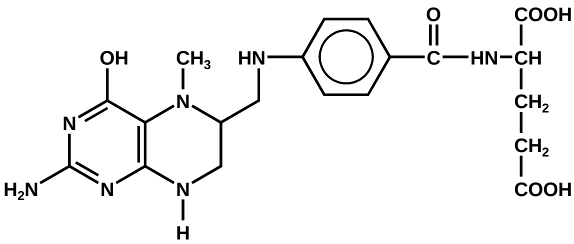
Figure 5. Relative plasma folate levels after consumption of 600 g spinach (meal A) or 600 g apple sauce with 400 µg folic acid (meal B), corrected for base-line folate concentration determined at t=0h.



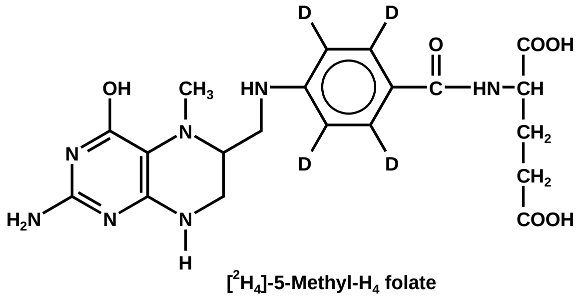
H_4 folate



$[^2H_4]$-H_4 folate



5-Methyl-H_4 folate



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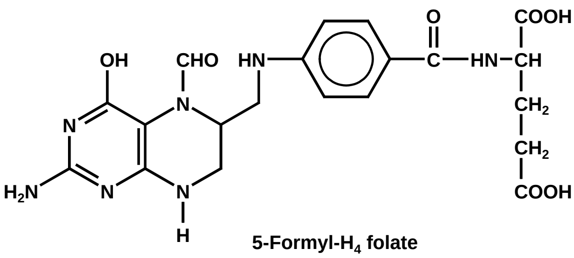

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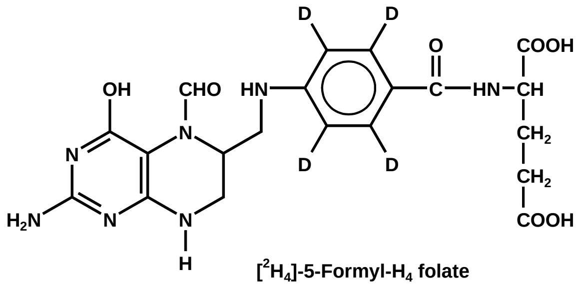
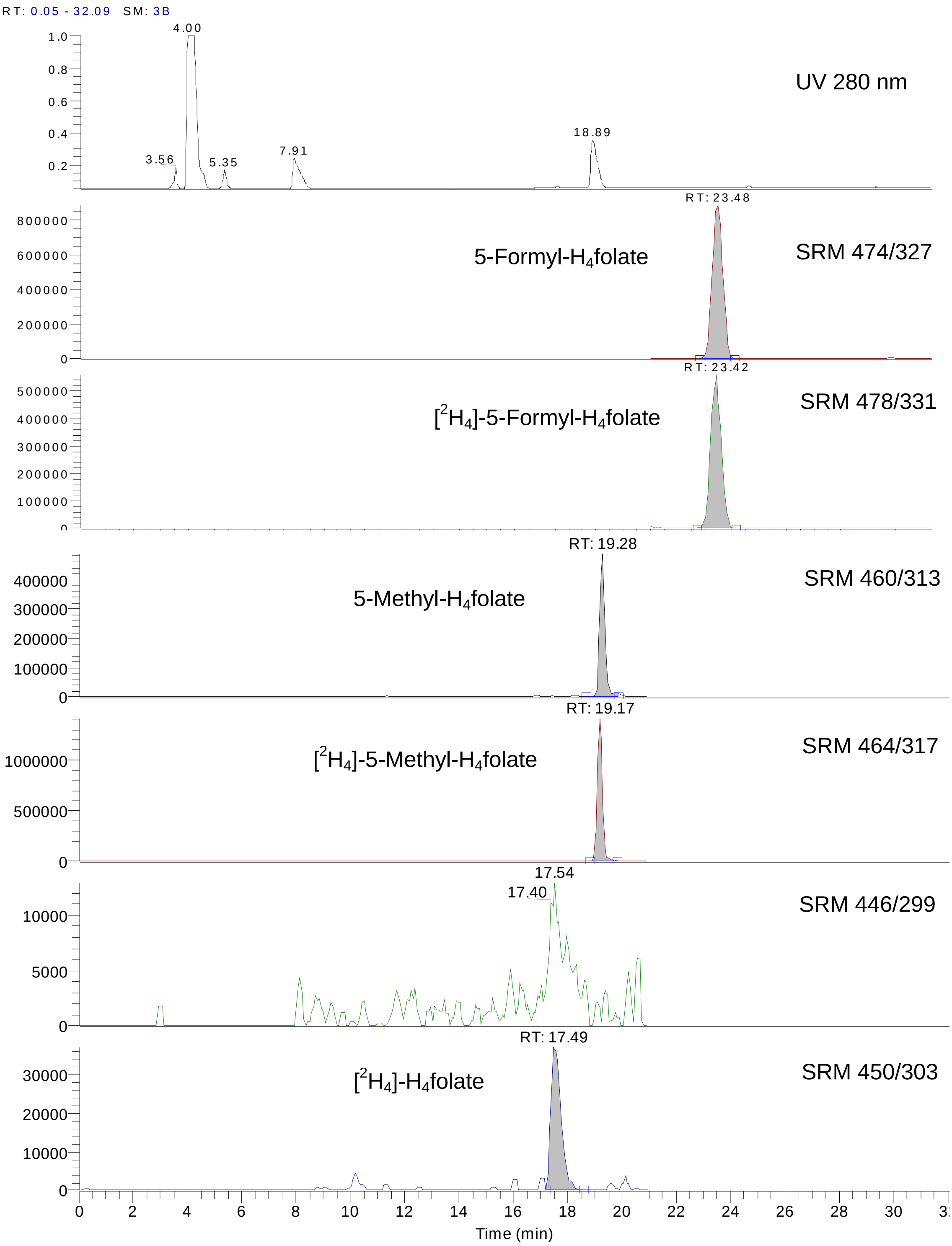


$[^2H_4]$-5-Formyl-H_4 folate

Figure 1



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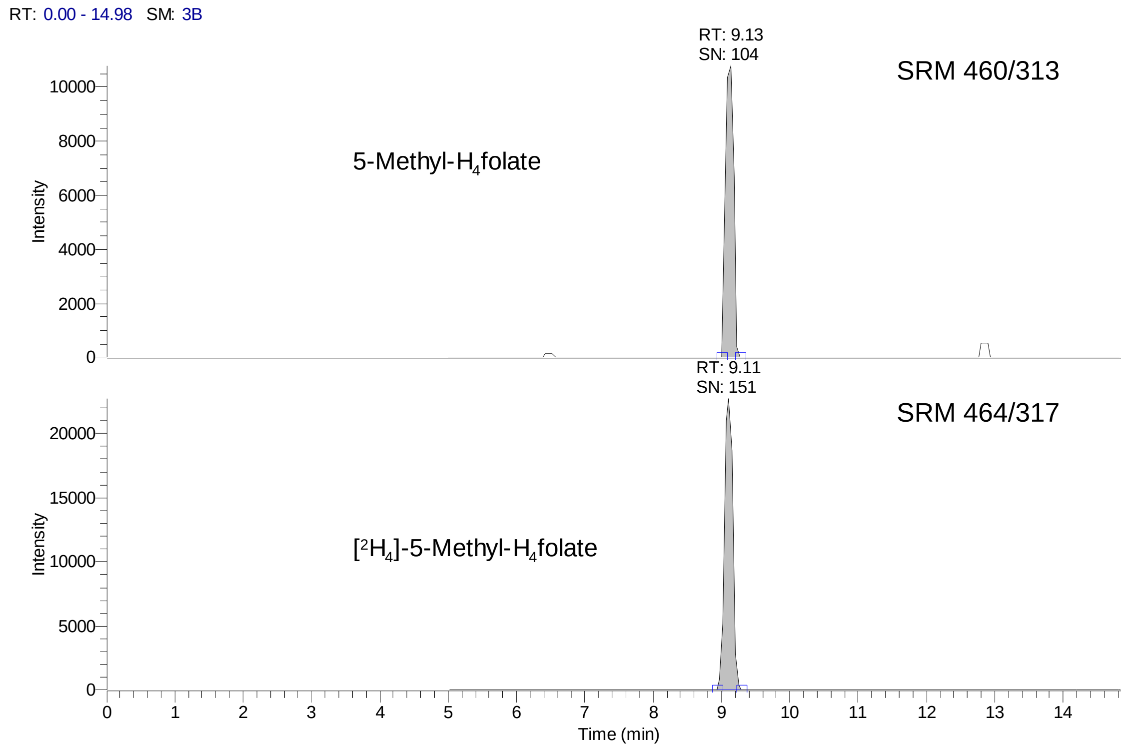
UV 280 nm
4.00
3.56
5.35
7.91
18.89
RT: 23.48
5-Formyl-H4folate
SRM 474/327
RT: 23.42
[2H4]-5-Formyl-H4folate
SRM 478/331
RT: 19.28
5-Methyl-H4folate
SRM 460/313
RT: 19.17
[2H4]-5-Methyl-H4folate
SRM 464/317
17.54
17.40
SRM 446/299
RT: 17.49
[2H4]-H4folate
SRM 450/303
Time (min)

Figure 2



RT: 0.00 - 14.98 SM: 3B
RT: 9.13
SN: 104
SRM 460/313
5-Methyl-H4folate
Intensity
RT: 9.11
SN: 151
SRM 464/317
[2H4]-5-Methyl-H4folate
Intensity
Time (min)

Figure 3

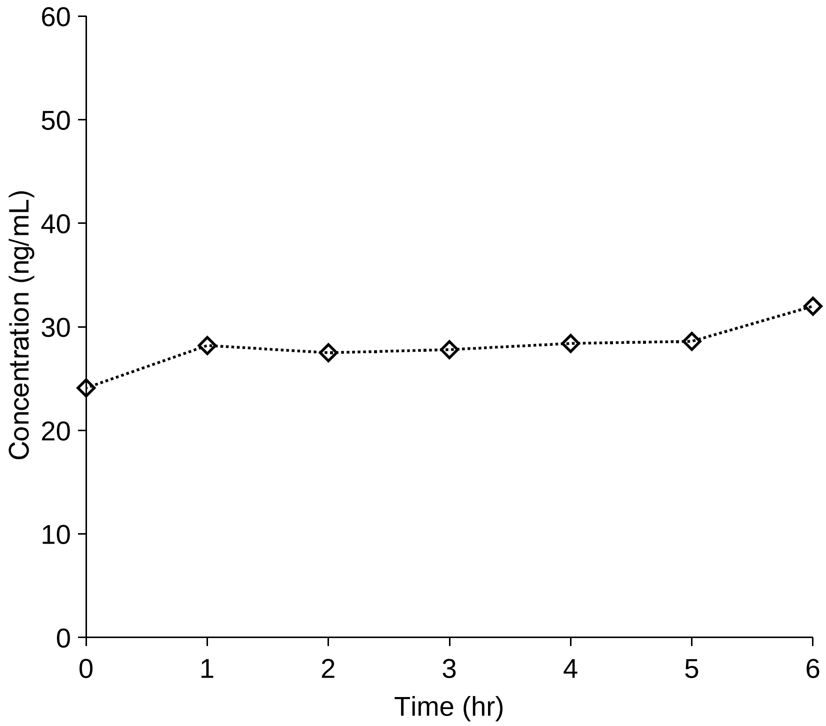


Figure 4

17.40

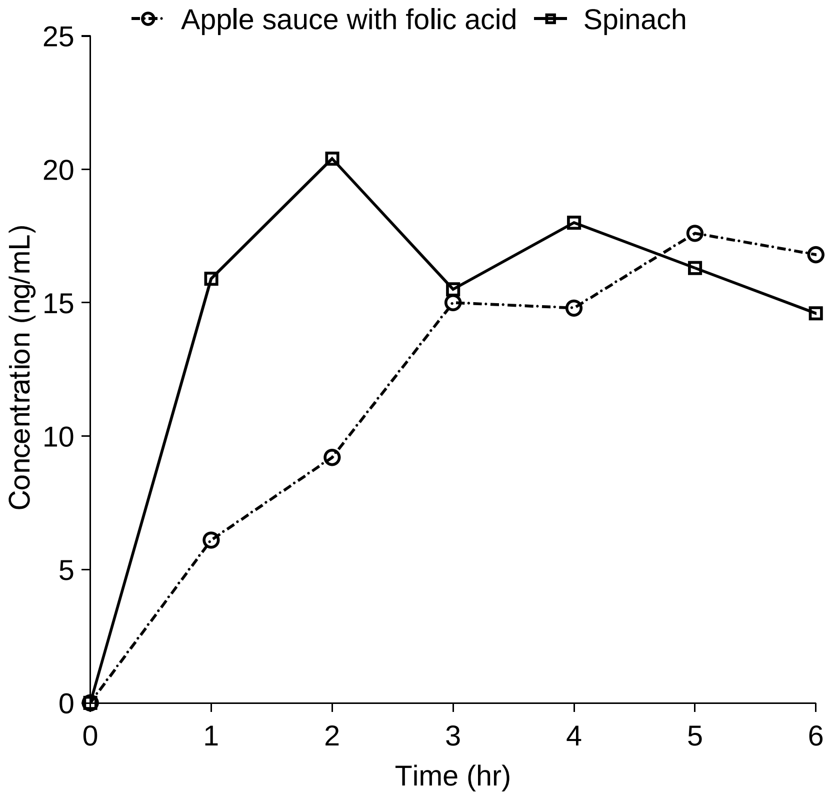


Figure 5