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Synthesis and Physicochemical Characterization of the One-Carbon Carrier 10-Formyltetrahydrofolate; A Reference Standard for Metabolomics

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Abstract

Introduction—Metabolomics analysis depends on the identification and validation of specific metabolites. This task is significantly hampered by the absence of well-characterized reference standards. The one-carbon carrier 10-formyltetrahydrofolate acts as a donor of formyl groups in anabolism where it is a substrate in formyltransferase reactions in purine biosynthesis. It has been reported as an unstable substance and is currently unavailable as a reference standard for metabolomics analysis.

Objectives—The current study was undertaken to provide the metabolomics community thoroughly characterized 10-formyltetrahydrofolate along with analytical methodology and guidelines for its storage and handling.

Methods—Anaerobic base treatment of 5,10-methenyltetrahydrofolate chloride in the presence of anti-oxidant was utilized to prepare 10-formyltetrahydrofolate.

Results—Pure 10-formyltetrahydrofolate has been prepared and physicochemically characterized. Conditions toward maintaining the stability of a solution of the dipotassium salt of 10-formyltetrahydrofolate in solution have been determined.

Conclusion—This study describes the facile preparation of pure (>90%) 10-formyltetrahydrofolate, its qualitative physicochemical characterization, as well as conditions to enable its use as a reference standard in physiologic samples.

Keywords

One-carbon carrier; formate; tetrahydrofolate; formyl; stability; characterization

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Compliance with ethical standards

Conflict of interest

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Research involving human participants and/or animals

No human participants or animals were involved in this study.

Informed consent

No human participants were involved in this study.

1.0 Introduction

Tetrahydrofolate is the principal one-carbon carrier in biological systems with derivatives that serve as donors of single carbons in any one of three oxidation states. At the oxidation level of methanol is 5-methyl-tetrahydrofolate, at the oxidation level of formaldehyde is 5,10-methylenetetrahydrofolate, while 5,10-methenyl-, 5-formimino-, 10-formyl-, and 5-formyltetrahydrofolates are at the formate oxidation level. Of the latter group, it has been stated that only 5-formyltetrahydrofolate is stable in solution over the physiological pH range (Stover and Schirch 1992). However, both 5-formyltetrahydrofolate and 5,10-methenyltetrahydrofolate have been isolated and characterized as stable salts and both are commercially available. In their role as one-carbon carriers, tetrahydrofolates are responsible for the synthesis of purine and thymidine nucleotides and for homocysteine remethylation to methionine (Fox and Stover 2008) and their levels throughout the biological system are crucial for normal and malignant cells to synthesize and repair DNA.(Anderson et al. 2012) As such, folates are the target of extensive research, including metabolomics analysis. (Danenberg et al. 2016) In the context of the NIH Common Fund Metabolomics Program, we were interested in providing the research community with reference standards of additional tetrahydrofolate derivatives at the oxidation of formate, including 10-formyltetrahydrofolate “Natural” 10-formyltetrahydrofolate, derived from (6*S*)-tetrahydrofolate, is named as (6*R*)-10-formyltetrahydrofolate, due to the Cahn-Ingold rules for substituent prioritization around a stereocenter; the 5-formyl isomer, has the same stereochemistry but is named (6*S*)-5-formyltetrahydrofolate, based on these rules.

The literature describes enzymatically derived (6*R*)-10-formyltetrahydrofolate (**1**) as highly labile (Tabor and Wyngarden 1959); the molecule rearranges to the thermodynamically favored 5-formyl analog **3** (May et al. 1951);(Allison et al. 1967); readily undergoes oxidation, particularly to the corresponding dihydrofolate (**4**); and cyclizes to 5,10-methenyltetrahydrofolate (**2**), even under neutral conditions (Tabor and Wyngarden 1959) (Scheme 1). On the other hand, solutions of **1** are reported to be stable for several months at −70 °C if purged with argon and overlaid with mineral oil (Stover and Schirch 1992). This report describes the successful preparation and physicochemical characterization of 10-formyltetrahydrofolate (**1**) in a solution suitable for metabolomics studies.

2.0 Materials and Methods

2.1 Instrumentation

¹HNMR spectra were recorded on a Bruker Avance 300 MHz spectrometer. High-resolution LC/MS analysis was performed on an Agilent system consisting of a 1290 Infinity UPLC coupled to a 6230 accurate-mass time-of-flight mass spectrometer with a Dual AJS ESI source. Mass spectral data were acquired in positive-ion mode over the range of 100–1700 m/z using a gas temperature of 350 °C, a nozzle potential of 1000 V, and a capillary potential of 3500 V.

2.2 Materials

The chemicals, reagents and solvents used in this synthesis were inspected and released for use based on the manufacturer's Certificates of Analysis. (6*RS*)-5,10-Methenyltetrahydrofolate Chloride and (6*RS*)-5-formyltetrahydrofolate were from Schircks Laboratories. Perdeuterated BME was from Cambridge Isotopes Laboratories. All other chemicals and solvents were from Sigma Aldrich. UPLC analyses used HPLC-grade acetonitrile (Fisher), HPLC-grade ammonium acetate (Fisher), and de-ionized water. For high-resolution LC-MS, water and acetonitrile were from Fisher (Optima LC/MS grade); ammonium acetate was from Fluka (LC-MS Ultra grade). All buffers and reagents were reagent grade or better.

2.3 10-Formyltetrahydrofolate (1)

All solutions were purged with argon gas immediately before use. Working in a glove bag under argon, a solution of 2N KOH (15 drops, 0.35 mL, 0.7 mmol) was added to a solution of 5,10-methenyltetrahydrofolate (2) hydrochloride (11 mg, 0.022 mmol) in 1M BME (11 mL) to achieve pH 8. The resulting yellow tinted solution with solid yellow particles was swirled while cooling in an ice bath; over ~10 min it became clear, homogenous, and colorless. This solution was pipetted into 20×2-mL vials (0.5 mL each). Each vial was sealed with aluminum crimp caps (PTFE/silicone). All vials were stored at –80 °C immediately after removing from the glove bag.

2.4 UPLC Analysis

The UPLC/UV method developed for impurity profile analysis of the Master Batch of 10-formyl-tetrahydrofolate utilized a Waters Acquity UPLC system, a photodiode array detector, and chromatographic separation was achieved using a Waters Acquity HSS T3 column (2.1×100 mm, 1.8 μ particle size) at 25 °C. The method employed mobile phase A (10 mM NH₄OAc pH 6.8) with a linear gradient of 0–15% mobile phase B (methanol) from 0–8 min followed by 15–90% B from 8–10 min, a hold at 90% B from 10–12 min, and re-equilibration to 0% B from 12.1–15 min. at a flow rate of 0.2 mL/min. The auto-sampler temperature was 5 °C to minimize sample degradation. The nominal injection parameters were 2 μL of a 0.1 mg/mL solution. UV detection was at 280 nm, and the complete UV spectrum (190–400 nm) was included to support peak identification.

2.5 UPLC Method Qualification

Linearity—To verify that analyzed sample concentrations were well within the linear range of the detector response for the analyte, method linearity was determined by single analysis of three test concentrations of 10-formyltetrahydrofolate (1) over the nominal concentration range of 0.05 mg/mL to 0.15 mg/mL (i.e., 50%–150% of the target analyte concentration of 0.10 mg/mL). A test solution was prepared by 10-fold dilution of the stock sample solution (nominally at 1 mg/mL in 1M BME containing KOH to pH 8) into 100 mM Tris HCl (pH 7.4) to produce a test solution at 0.1 mg/mL (target concentration). To simulate sample concentrations in the range of 0.05–0.15 mg/mL (50%, 100% and 150% of target concentration) three separate direct injections of the diluted stock solution of 1, 2 and 3-μL were made. Note that 10-formyltetrahydrofolate (1) was observed to elute as two peaks,

likely representing the two diastereomers (6*R* and 6*S*); the elution order of the two isomers has not been determined. For this qualification, the *total* peak area was used. Analyte response (peak area) was plotted versus concentration for the three injections of the 10-fold diluted solution of **1**. The data were fit using linear regression techniques to give a best-fit line with $r^2 = 0.9927$ and % error within 10.0% at each concentration, establishing a linear range (peak area) for 10-formyltetrahydrofolate from 0.05–0.15 mg/mL.

Limit of Detection (LOD)—The LOD for the impurity method was visually confirmed at 0.2% of the target assay concentration (0.1 mg/mL), corresponding to 0.2 µg/mL. Based on the analyte peak height at 0.2% of the target assay concentration the signal-to-noise level was determined to be 3:1 (from 1st peak) and 5:1 (from 2nd peak) confirming that the method is suitable for detection of related organic impurities at 0.2% of the target assay concentration level for **1**.

Specificity—To specifically confirm detection and separation of potential impurities control solutions of authentic (6*R,S*)-5,10-methenyltetrahydrofolate (**2**) chloride (starting material) (6*R,S*)-5-formyltetrahydrofolate (**3**) calcium (degradation product) each at ~ 0.1 mg/mL in methanol were analyzed (single injection) to determine the retention times relative to 10-formyltetrahydrofolate (**1**). Fig. 1 is a representative chromatogram of **1**, **2**, and **3** where both potential impurities (**2** and **3**) elute after **1** (RRT 1.53 and 1.26, respectively). By visual inspection, all were baseline-resolved from each other.

2.6 Stability

Samples stored at –20 °C and –80 °C were analyzed at specific time points over 7 weeks. At each time point, samples were removed from storage, prepared neat (no dilution) or diluted 10-fold into 100 mM Tris-HCl under inert atmosphere, and analyzed by UPLC/UV and/or LC-MS. The method was a variation of the qualified method (section 2.4), using pH 5.8 in mobile phase A, a 0.3-mL/min flow rate, and a linear gradient of 0–20% mobile phase B over 5 min. The 72-hr dilution stability study performed on the 7-week aged samples was performed in a similar manner. The 48-hr dilution stability study performed on the 6-month aged samples was performed using careful sample preparation under an inert atmosphere, and purity analysis was performed using the UPLC/UV according to the parameters used for the final qualified purity method.

3.0 Results

3.1 Synthesis

The chemical synthesis of 10-formyltetrahydrofolate (**1**) has been reported as having been carried out following two separate pathways. The first synthetic pathway (Scheme 2) consists of treatment of 5,10- methenyltetrahydrofolate (**2**) chloride with base in the presence of an oxidation inhibitor (Rabinowitz 1963); Stover and Schirch 1992). The second synthetic pathway (Scheme 3) involves formylation of folate (**5**), recrystallization of the product to isolate pure 10-formylfolate (**6**), and hydrogenation of **6** to give **1** (Temple et al. 1979).

To examine the feasibility of preparing 10-formyltetrahydrofolate (**1**) following the latter approach (Temple et al. 1979) (Scheme 3), a solution of commercial folate (**5**) in formic acid was heated at 50 °C for one hour. After cooling to room temperature, half the solution was treated with ether to effect precipitation. The precipitated solid was recrystallized from water to give a yellow solid with mass spectral and ¹HNMR features consistent with expected values for 10-formylfolate (**6**). The remaining solution was evaporated and the residual solid was likewise recrystallized from water to give a tan solid with MS and ¹HNMR results identical to those of the yellow solid. Catalytic hydrogenation (Pt) of each solid in either trifluoroacetic acid (TFA) or formic acid following the literature (Temple et al. 1979) gave the same new product. Isolation of the product of hydrogenation in TFA as a solid TFA salt, followed by analysis of a methanolic solution of this salt by HPLC and UPLC/MS, showed the solution to contain only 5,10-methenyltetrahydrofolate (**2**); the ¹HNMR spectrum, recorded in DMSO solution, was also identical with the spectrum of authentic **2**. Since the presence of **2** was likely to have resulted from TFA-promoted cyclization of 10-formyltetrahydrofolate (**1**), the isolated TFA salt was dissolved in a buffer in which 5-formyl-tetrahydrofolate (**3**) had been found to be stable. Analysis of this solution by UPLC/MS (positive ion) revealed the presence of a two-component (1:1) mixture consisting of a more polar component with [M+H]⁺ *m/z* 474.17, and a less polar compound with [M+H]⁺ *m/z* 472.16. Control experiments confirmed that the compound with *m/z* 474.17 did not co-elute with 5-formyltetrahydrofolate (**3**), suggesting that the observed peak with *m/z* 474.17 was 10-formyltetrahydrofolate (**1**) (*m/z* [M+H]⁺ calcd for C₂₀H₂₃N₇O₇: 474.14). The less polar material with *m/z* 472.16 implied the presence of the dihydrofolate analog **4** (*m/z* [M+H]⁺ calcd for C₂₀H₂₁N₇O₇: 472.14), which may have been formed by oxidation either in the reaction buffer or during UPLC/MS analysis. These results suggested that it would be difficult to obtain pure 10-formyltetrahydrofolate (**1**) following this synthetic approach without additional measures to prevent oxidation to the dihydrofolate analogue.

To investigate the approach described in Scheme 2, probe reactions, following the literature, (Rabinowitz 1963) were conducted by treating an aqueous solution of commercially-procured 5,10-methenyltetrahydrofolate (**2**) chloride with potassium hydroxide (to pH 8) in the presence of β-mercaptoethanol (BME). Monitoring the reaction showed the disappearance of **2** occurring concurrently with the appearance of slightly more polar material that exhibited mass and UV spectra consistent with 10-formyltetrahydrofolate (**1**). Analysis by LC/UV (Fig. 2, top panel) and LC/MS (data not shown) indicated the formation of a compound with [M+H]⁺ *m/z* 474.17 (as expected for **1**), and with retention time and UV spectrum (λ_{max} 287 nm) different from those of the 5-formyl analog **3**; (Tabor and Wyngarden 1959) small amounts of **3** and **2** were also detected. The major product was no longer detectable after 72 hours at room temperature, consistent with the reported instability of **1** (Tabor and Wyngarden 1959). Additional probe experiments under strictly anaerobic conditions were conducted using several different bases and varying the concentration of BME, and the optimum conditions for product formation were identified as potassium hydroxide (pH 8) and 1M BME. To further confirm the identity of the sample, the reaction was repeated using deuterated water and deuterated BME, allowing for the determination of the ¹HNMR spectrum (Suppl. Fig. 1). Comparison with the ¹HNMR spectra of 5,10-methenyltetrahydrofolate (**2**) and 5-formyltetrahydrofolate (**3**) (Suppl. Fig. 2 and 3,

respectively) showed distinct differences, particularly in the low-field region. Specifically, addition of base to **2**, led to shifting of the resonance at 6.7 ppm to 7.3 ppm. Allowing the solution to stand at room temperature for three days produced a proton NMR spectrum suggesting that the sample had converted to 5-formyltetrahydrofolate (**3**). Thus, the low-field resonances at 7.3 and 7.7 ppm (Suppl. Fig. 1) shifted to 6.8 and 7.6 ppm in agreement with reported (Li et al. 2014) chemical shift values for **3** and with the ^1H NMR spectrum recorded for standard commercially procured calcium 5-formyltetrahydrofolate (Suppl. Fig. 3).

Attempted isolation of **1** as a solid by lyophilization of a portion of the reaction solution under strictly anaerobic conditions gave inconsistent results in that a solid was not always formed. However, regardless of whether the residue was solid or not, the isolated material was found to be rapidly converted to 5-formyltetrahydrofolate (**3**) and to 10-formyldihydrofolate (**4**) (Fig. 2, bottom panel). Since lyophilization was not a viable option for product storage, aliquots of the reaction solution were transferred to ampoules under an inert atmosphere and stored at -20 and -80 °C.

3.2 Physicochemical Characterization

The chromatographic purity of ampouled 10-formyltetrahydrofolate (**1**) (in 1M BME containing potassium hydroxide to pH 8), was determined using a RP-UPLC/UV method that was developed and qualified. The UPLC/UV impurity method was demonstrated to be acceptable for analysis of **1** for the presence of the related organic impurities **2** and **3** and revealed a chromatographic purity of 91.9% (280 nm). The presence of residual 5,10-methenyltetrahydrofolate (**2**) (relative retention time (RRT) 1.40, 1.8 %) was confirmed, and a dimer related to BME was also observed as a late-eluting peak in the chromatogram (BME (m/z $[\text{M}+\text{NH}_4]^+$ and $[\text{M}+\text{Na}]^+$ calcd for $\text{C}_4\text{H}_{10}\text{O}_2\text{S}_2$: 172 and 177; found: 172, 177). Analysis (Fig. 1) revealed that **1** eluted as two partially-resolved peaks. The UV/vis spectrum (Fig. 3, Panel A) of each of the two main peaks exhibited three maxima at 203 nm, 258 nm, and 304 nm, consistent with the reported spectrum for **1** (Tabor and Wyngarden 1959). The high-resolution ESI+ mass spectrum (Fig. 1, inset) for the two major components exhibited the $(\text{M}+\text{H})^+$ ion (m/z 474.1744) as the base peak, which is within 2.5 ppm of the theoretical value.

3.3 Stability Studies

Monitoring the stability of **1** in the reaction solution (1M aqueous solution of BME containing potassium hydroxide at pH 8) by LC/MS demonstrated that the sample purity remained relatively constant (Suppl. Fig. 4) after seven-week low temperature storage (-20 °C, -80 °C). At each time point, samples were diluted 10-fold into 100 mM Tris HCl (pH 7.4) prior to analysis. Most of the decrease in purity in the 7-day and 14-day samples was accounted for by the formation of the major oxidation product **4**; since analysis after 49 days showed higher purity, the oxidation product was likely formed due to inadvertent air exposure during sample preparation. Modest amounts of 5-formyltetrahydrofolate (**3**) and 5,10-methylenetetrahydrofolate (**2**) were also observed at each time point.

To explore the stability of 10-formyltetrahydrofolate (**1**) during sample handling, the effects of dilution of the reaction solution with aqueous buffers was investigated (Suppl. Fig. 5).

Aliquots of the reaction solution that had been stored for 7 weeks at -20°C and -80°C were analyzed neat. In addition, the sample stored at -80°C was diluted 10-fold into 100 mM Tris-HCl (pH 7.4) buffer and, separately, into 100 mM phosphate (pH 6.8) buffer. All sample handling (dilution) was performed using buffers that had been purged with nitrogen, and the sample solutions were transferred to purged HPLC vials using gas-tight syringes to limit exposure to oxygen. All 4 samples were analyzed initially, then stored at 5°C and analyzed daily over a 72-hr period. Strikingly, whereas the stock (undiluted) solutions, and the sample diluted 10-fold into 100 mM Tris-HCl (pH 7.4), were essentially unchanged over the 72-hour monitoring period, dilution with 100 mM phosphate (pH 6.8) buffer led to sample degradation within the first 18-hour monitoring period, and continued over the 72-hour monitoring period (Suppl. Fig. 5). Based on RRT and LC-MS analysis, the primary degradation products for the phosphate-diluted sample were identified as 5-formyltetrahydrofolate (**3**) and 5,10-methenyltetrahydrofolate (**2**). A similar experiment was conducted after 6-months of storage at -80°C , this time investigating the effect of buffer and buffer pH on short-term storage at 5°C (Suppl. Fig. 6). Over 48 hours of storage at 5°C , the ampouled (undiluted) solutions of **1** showed a 5–7% decrease in purity, and samples diluted 10-fold into 100 mM Tris-HCl (pH 7–9) showed a 3–5% decrease in purity. By contrast, samples diluted into 100 mM phosphate at pH 7 showed a 25% decrease in purity over 48 hours, while samples diluted into phosphate at pH 6 and 8 showed >50% decrease in purity over 48 hr.

4.0 Discussion

The results described above confirm that the chemical preparation of 10-formyltetrahydrofolate (**1**) from 5,10-methenyltetrahydrofolate (**2**) following the approach described by Rabinowitz and, separately, by Stover, provides high quality 10-formyltetrahydrofolate (**1**) that is quite stable when stored in argon-purged 1M BME containing potassium hydroxide (pH 8) at -20°C and -80°C . Moreover, dilution of the sample solution with a physiologically compatible buffer such as Tris-HCl does not affect the stability of **1**. Specifically, dilution with Tris buffer at pH 7.4, which had been used to measure the UV spectrum of **1**, (Baggott et al. 1998) did not affect sample stability. In fact, dilution in 0.1M Tris-HCl buffer over pH 7–9 range maintains reasonable sample stability even at 5°C for up to 48 hr. By contrast, dilution with 0.1M sodium phosphate buffer at pH 6.79, which had been used to record the ^1H NMR spectrum, (Poe and Benkovic 1980) led to rapid degradation, particularly at 5°C , even under nitrogen. Rapid degradation in 0.1M phosphate buffer occurred over the physiologic pH (6–8) range for samples stored at 5°C . Noteworthy is the ease of oxidation of 10-formyltetrahydrofolate (**1**) to the dihydro analog **4** upon minimal exposure to air, as seen in the 7-day and 14-day timepoints in Suppl. Fig. 4, which highlights the need to handle the material under strictly anaerobic conditions (inert atmosphere). A summary of recommended handling and storage conditions is provided in the supplementary material.

The conversion of **1** to its thermodynamically more stable isomer **3** (May et al. 1951) at pH 8, which had not been previously reported, must proceed through the cyclic intermediate **2**, which is interesting in that it indicates that **1** undergoes cyclization to **2** under these

conditions. While it is well known that **1** readily cyclizes to **2** with acid and in neutral media, cyclization of **1** in base has not been previously reported.

The most likely explanation for the detection of equal amounts of two isomers of **1** in the UPLC/UV and LC-MS experiments is that they are (6*R*)- and (6*S*)-10-formyltetrahydrofolate (elution order unknown), derived from (6*R,S*)-5,10-methenyltetrahydrofolate (**2**) chloride, which was the starting material for the preparation of **1**. However, base-promoted racemization at the α -carbon of glutamate is also possible; this would give the diastereomeric (6*R*, α *S*)/(6*S*, α *S*)- and (6*R*, α *R*)/(6*S*, α *R*)-10-formyltetrahydrofolates. At present, we cannot distinguish these two possibilities.

While the studies described above were performed using (6*R,S*)-5,10-methenyltetrahydrofolate (**2**) chloride, inevitably resulting in a diastereomeric mixture of (6*R*)- and (6*S*)-10-formyltetrahydrofolate, the (6*R*)- diastereomer (the “natural” diastereomer derived from (6*S*)-tetrahydrofolate) could be readily prepared from (6*R*)-5,10-methenyltetrahydrofolate chloride, assuming that, base-promoted racemization at the α -carbon of glutamate does not take place. Thus, cyclization of commercially available (6*S*)-5-formyltetrahydrofolate will afford (6*R*)-5,10-methenyltetrahydrofolate chloride, that would afford (6*R*)-10-formyltetrahydrofolate upon base treatment as described above. Similarly, 10-[¹³C]formyltetrahydrofolate, useful in on-carbon metabolomics studies, may be prepared by using 5-[¹³C]formyltetrahydrofolate (obtained by formylation of tetrahydrofolic acid with carbon-13 labeled formate) as starting material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Analysis of Ampouled 1 by UPLC/UV and LC-MS

UPLC/UV results were obtained using method parameters from the qualified method (see section 2.4). ESI-MS results (inset) are shown for the two main peaks

Fig. 2. UPLC/UV Analysis of the Product from Base Treatment of 2 (Scheme 2)
Top panel: before lyophilization; bottom panel: after lyophilization

Fig. 3. UV Spectra from UPLC/UV Analysis of Ampouled 1

(A) Spectra of the 2 major products that eluted near 5.6 min (dotted line) and 5.9 min (solid line). (B) Representative spectrum of 5,10-methenyltetrahydrofolate (**2**). (C) Representative spectrum of 5-formyltetrahydrofolate (**3**)

Scheme 1. Instability of 10-Formyltetrahydrofolate (1)

Scheme 2. Synthesis of 10-Formyltetrahydrofolate (1) From 5,10- methenyltetrahydrofolate (2) Chloride

Scheme 3. Synthesis of 10-Formyltetrahydrofolate (1) From Folate (5)