Synthesis of ^{14}C- labeled 6-(S)-5-methyltetrahydrofolic acid for *in vivo* human metabolic studies

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ABSTRACT

Determination of the *in vivo* human metabolism of 6-(S)- 5-methyltetrahydrofolic acid (5MTHF) would allow proper determination of the bioavailability of this most abundant natural form of folate found from vegetables and helps suggest proper recommended dietary requirements for folate. A quantitative metabolic study on 5MTHF is lacking. Using a ^{14}C label on the stable glutamate position of 5MTHF as a metabolic tracer would allow one to quantify the total folate metabolism as a coenzyme and with ^{14}C labelling facilitate true physiological dosing. Likewise, a comparison of the quantitative metabolism in healthy and diseased individuals like those with leukemia would allow one to quantify differences in folate utilization. It would also be interesting to see how one-carbon metabolism varies in healthy people and those with cancer considering that epigenetic changes contribute to carcinogenesis. The study aimed to develop a practical and efficient synthesis of ^{14}C- labeled 6-(S)- 5-methyltetrahydrofolic acid (^{14}C-5MTHF) suitable for metabolic studies. To develop a practical method for the purification and characterization of ^{14}C-5MTHF.To synthesize ^{14}C labeled 6-(S)-5-methyl tetrahydrofolic acid (^{14}C-5MTHF), [^{14}C-U]-glutamate was conjugated to pteroyl azide to produce folic acid. The resulting folic acid was reduced to dihydrofolic acid using dithionite and further reduced to (6S)-tetrahydrofolic acid using dihydrofolate reductase (DHFR) from chicken liver. Addition of formaldehyde solution followed by reduction with $NaBH_4$ produced ^{14}C- 5MTHF. High Pressure Liquid Chromatography (HPLC) using a semi-preparative C-18 stationary phase and a 6-25 % acetonitrile gradient in 30 mM phosphate buffer pH 2.2 as mobile phase was done to purify the final product. Liquid scintillation counting (LSC) and mass spectrometry (MS) analysis were conducted to assess the radiochemical purity of synthesized ^{14}C-5MTHF. A practical synthesis of ^{14}C-5MTHF was accomplished through a one-pot procedure of chemo-enzymatic synthesis – from coupling of radiolabel up to the chemo-enzymatic reduction (five synthetic steps) to the final product. Over-all yields of 58% through this one-pot reaction procedure was accomplished. Identity was confirmed by HPLC co-elution with a standard. The amount of C-14 incorporated was estimated to be 2.8 ± 0.4 % by MS analysis comparable to results of LSC. A practical one-pot chemo-enzymatic synthesis of ^{14}C-5MTHF was reported. Purification was accomplished through semi-preparative HPLC on a C-18 column. The radiochemical purity was determined through LSC and MS analysis.

KEYWORDS: ^{14}c- Labeleling, Recommended Dietary Allowance (Rda), 5-Methyltetrahydrofolic Acid, Metabolism, Vitamins

1. INTRODUCTION

Folate constitutes an important and essential class of vitamins, the deficiency of which causes megaloblastic anemia [1]. Folic acid (FA) intervention studies [2, 3] demonstrated a reduction in the incidence of neural tube defects (NTD). This resulted in the mandatory fortification of grains in the US since 1998. FA supplementation was also found to lower plasma total homocysteine levels [4]. Hyperhomocysteinemia, with more than 15 µg homocysteine per dL plasma is a risk factor for cardiovascular disease. Epidemiological evidence also shows close relation between low folate intake and increased risk of developing colorectal and cervical cancer [5].

While folate is an important nutrient, there is controversy regarding the level of fortification which would be optimal in preventing neural tube defects, lowering plasma homocysteine levels, among other health benefits [6,7]. There are concerns fortification levels elevate FA level to one that could prevent

detection of vitamin B12 deficiency. The mechanism of FA - masking B12 deficiency detection is known as methylfolate trap [8] which could be avoided if the natural folates are utilized instead. Likewise, there is epidemiological evidence relating FA supplementation and risk of developing breast cancer. Thus, there is a growing interest in using natural folate like 5-methyltetrahydrofolic acid (5MTHF) instead of the synthetic form - FA.

The optimal amount of folate necessary for maintaining good health is still in debate. A quantitative study of folate metabolism would enable to estimation proper vitamin intake. The advent of Accelerator Mass Spectrometry (AMS) makes it possible to quantify trace amounts of C-14 in biological samples. Thus, C-14 labeled folic acid derivatives are very important tools in such metabolic studies.

Folate Forms

Folates (vitamin B9) are water-soluble vitamins that are important in the proper maintenance of human health. The parent compound (folic acid) is made up of a pteridine ring conjugated with p-amino benzoate (pABA) linked with L-glutamate (L-Glu). The synthetic form is the most oxidized and most chemically stable form also known as folate or folic acid (FA) and is further reduced in the body to yield the biologically active form. Natural forms exist in two reduced forms – dihydrofolic acid (DHF) and 6-(S)-tetrahydrofolic acid (THF) which are often methylated as in 5-methyl tetrahydrofolic acid (5MTHF) or 5-formyl tetrahydrofolic acid (5fTHF). In addition, the methine and methylene forms exist bridging N5 and N10 making folates versatile one-carbon carrying units able to contain different oxidation states of carbon as in 5,10-methenyl tetrahydrofolic acid (CH+THF) and 5,10-methylene tetrahydrofolic acid (CH_2-THF). 5-formimino-THF (HN=CH-THF) also exists. It could also contain up to 11 glutamate units linked uniquely through γ-peptide (amide) linkages giving different extents of γ-glutamylation. The penta- and hexa-glutamylated pteroates are common in humans [9].

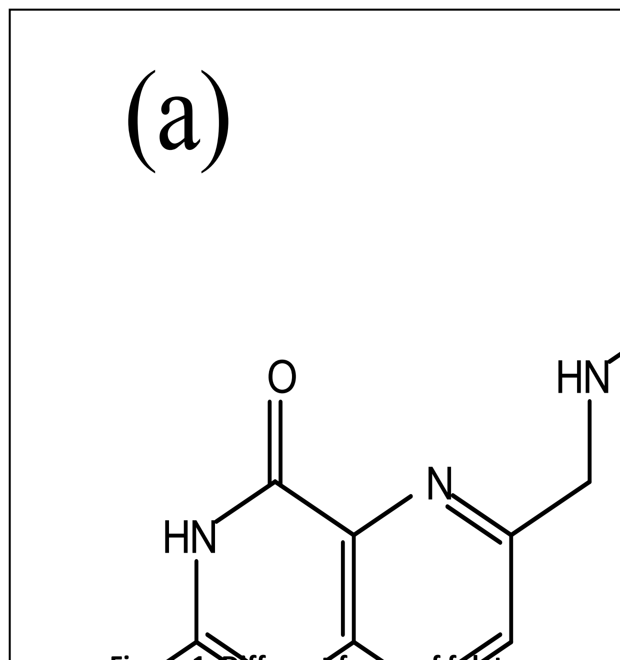

Figure 1. Different forms of folate.

(a)folic acid, FA
(b)dihydrofolic acid, DHF
(c)tetrahydrofolic acid, THF
(d)5-methyltetrahydrofolic acid, 5MTHF
(e)methylenetetrahydrofolic acid, CH2THF
(f)5-formyltetrahydrofolic acid, 5fTHF
(g)10-formyltetrahydrofolic acid, 10fTHF
(h)methenyltetrahydrofolic acid, CHTHF
(i)5-formiminotetrahydrofolic acid, NHCHTHF
(j) pteroyl polyglutamate.

Human metabolism

One-carbon metabolism in eukaryotes is often dependent on folate coenzymes. It takes place in three distinct but interdependent compartments - cytosol, mitochondria and nucleus. Polyglutamylation is essential for cellular retention of folate and the extent of glutamylation affects the binding of these folate coenzymes to the enzymes catalyzing one-carbon transformations. In the mitochondria, metabolism of serine, glycine and choline generates formate which is utilized in the cytosol for *de novo* purine biosynthesis and thymine biosynthesis, methionine and S-adenosyl-methionine (the universal methyl donor) synthesis. In the nucleus, folate coenzymes are involved in thymidylate synthesis during DNA replication and salvage pathways during repair. While most of the biochemical one-carbon transformations have already established, a quantitative understanding of

the metabolic flux is missing. Likewise, coordination and regulation of these pathways as they relate to homeostasis and disease are current areas of investigation [10, 11, 12].

Nutritional Importance

The nutritional importance of folate was not known until Lucy Wills (1931) demonstrated that yeast and liver extracts reversed “pernicious anemia of pregnancy” or megaloblastic anemia in monkeys [13]. Studies on the antianemia factor in chicks [14] and on the essential growth factor for certain strains of *Lactobacilli* and *Streptococci* [15] and on the extract from spinach leaves revealed folate as the active component in all of these. FA intervention studies [3, 16] also demonstrated the reduction in the incidence of neural tube defects, though exact mechanism of the activity in preventing this birth defect was not understood. Likewise, epidemiological studies clearly support the chemo-preventive role of dietary folate in preventing some cancers [17].

Nutritional Requirements

Recommended Nutrient Intake (RNI) is the average daily dietary intake sufficient to meet the nutrient requirements of 98% of healthy individuals in a particular life-stage and gender group. For folate, the FAO/WHO recommend 400 µg/d dietary folate equivalent (DFE) for adults and 600 µg/d DFE for pregnant women. The tolerable upper limit for adults was set at 1000 µg/d for FA- the synthetic form DFE= Dietary Folate Intake= 1.7 x µg FA in fortified food+µg of naturally occurring food folate [18].

The US FDA in 1998 mandated the fortification of flour and enriched cereal grains with 1.4 mg FA/kg and recommended the fortification of ready-to-eat products like breakfast cereals with 400 µg FA/serving. This was done following studies confirming that peri-conceptual intake of FA reduced risks of neural tube defects NTD by 50-70% - the most common birth defect, occurring in 0.1% of the US population [18].

There was a significant improvement in serum folate levels in the US after FA fortification according to a recent National Health and Nutrition Examination Survey (NHANES) conducted from 2005-2006. The median serum folate levels increased to 12.2 ng/mL from 5.5 ng/mL in 1988-94 and RBC folate increased to 266 ng/mL from 174 ng/mL in 1988-1994 in US population 4 yrs and older. Likewise, the percentage of women of child-bearing age (15-45 yrs) with low serum folate (<3 ng/mL) was reduced to 0.3% from 15.5% and percentage for those with low RBC folate (<140 ng/mL) reduced to 4.5% from 37.6%. White women of child-bearing age had significantly higher serum folate levels than black and hispanic women [19].

Bioavailability

Folate bioavailability is the proportion of ingested folate absorbed and available for metabolic processes. Estimates of bioavailability of natural folate vary from <50%, to 60-98% relative to FA. In a recent article review, 5MTHF is reported to have the same bioavailability as FA based on single large-dose studies, which may not be extrapolatable to physiological dosage studies [20]. Assessing natural folate bioavailabilities presents challenges associated with the instability and lability of natural folate especially to cooking. Other factors influencing natural folate bioavailability are food matrix, hydrolysis of pteroyl polyglutamates and their absorption in the small intestine and genetic factors affecting folate transporters and enzymes [21].

Metabolic Studies

Numerous metabolic studies using unlabeled and labeled folates have been carried out [22]. Most involved high-dosage short-term monitoring of folate [23]. A long-term metabolic study carried out for more than 40 days used ^{14}C labelled folate. They dosed 0.5 nmol of folate (100 nCi radioactivity) together with 79.5 nmol folate and monitored for ^{14}C in plasma, erythrocytes, urine and feces [24]. A kinetic model was built for folate but no such kinetic model exists for the natural folate form.

Objectives of the Study

This study aimed to develop a practical method to synthesize C-14 labeled 6-(S)-5-methyltetrahydrofolic acid. Synthetic material was used in *in vivo* studies to determine the kinetics of 6-(S) -5-methyltetrahydrofolic acid metabolism in humans. Through this study, the bioavailability of the most abundant natural form of folate can be assessed for the first time considering physiological doses.

2. MATERIALS AND METHODS

Pteroyl L-Glutamate

Pteroyl azide (3.7 mg, 0.0109 mmol) and L-glutamate (6.0 mg, 0.041 mmol) were dissolved in dimethyl sulfoxide (200 uL) and N,N,N′,N′-tetramethyleneguanidine (5uL). The resulting solution was stirred at room temperature for 9 hrs to allow complete conjugation. The solution was then passed through celite and the product was recovered by ex-tracting with acetonitrile (20 mL) and precipitating with diethyl ether (50 mL). Centrifugation followed by vacuum drying yields FA as a yellow solid [25].

Pteroyl [^{14}C-U]-L-Glutamate

[^{14}C-U]-L-Glutamate (50 µCi, American Radiolabeled Chemicals, Inc.) solution was lyophilized to recover the solid [^{14}C-U]-L-Glutamate. This was added to pteroyl azide, dimethyl sulfoxide and N,N,N′,N′ - tetramethyleneguanidine. Reaction conditions and product recovery were carried out as described above.

Dihydrofolic acid

Folic acid was reduced to dihydrofolic acid by modifying a literature procedure [26]. Folic acid (20 mg, 0.045 mmol) was dissolved in 0.80 mL 1N NaOH, the 6mL of freshly prepared aqueous ascorbate solution (10% w/v) brought to pH 6 with 1N NaOH previously flushed with N_2 gas was added to the solution. Sodium dithionite (300 mg, 1.723 mmol) was added. The resulting solution was allowed to stand at room temperature with stirring for 30 minutes. Acidification to pH 2.8 was done by adding 0.10 mL of 1N HCl per minute with stirring while cooling in an ice-water bath. Stirring was continued for another 5 mins in ice-water bath. The suspension was centrifuged for 2 mins. The precipitate was redissolved in 5mL ice cold ascorbate solution, pH 6 (10% w/v) and brought to pH 6 with 1N NaOH. It was stirred in ice-water bath for 5 mins. Acidification was done as previously described. After stirring for 5 minutes in ice-water bath, the suspension was centrifuged for 2 mins. The precipitate was washed twice with 10 mL ice cold 0.001N HCl, frozen in dry ice and lyophilized.

Dihydrofolate Reductase from chicken liver

Fresh liver from jungle fowl rooster UCD-1 (Department of Animal Science Poultry, University of California, Davis) was cooled in ice and homogenized in 0.1M potassium phosphate buffer, pH 7.5 with 1 mM EDTA for 1 min in a blender. The suspension was centrifuged at 10 000 rpm for one hour at 4 $^{\circ}$C to remove cell/tissue debris. The supernatant was filtered through cheesecloth and mixed with 0.3 vol of 2% protamine solution and stirred for 30 mins in ice-water bath. The suspension was then centrifuged at 10 000 rpm at 4 $^{\circ}$C for 1 hr to remove the nucleic acid. Ethanol and chloroform were added to the supernatant and shaken for a minute. The suspension was centrifuged at 10 000 rpm for 1 hr at 4 $^{\circ}$C to re-move the lipids. One micromole of NADPH was added to the supernatant and then dialyzed against 6L cold water overnight. The dialyzate was brought to 60% ammonium sulfate saturation and then stirred for an 1 hr in ice-water bath. The resulting supernatant was made 85% with ammonium sulfate and stirred again for an hour in ice-water bath. Centrifugation at 10 000 rpm for 1 hr 4 $^{\circ}$C allowed recovery of the precipitate which was dissolved in minimum potassium phosphate buffer (50 mM, pH 6.5). The resulting solution was dialyzed against 6 L of the same buffer overnight. The dialyzate was then made 20% in glycerol prior to storage in the freezer. This procedure involved several modifications from the literature procedure [27].

Tetrahydrofolic acid

NADPH (2.4 mg, 0.00288 mmol) and dihydrofolate reductase were added to a solution of dihydrofolate (0.5 mg, 0.00112 mmol) in 50 mM potassium phosphate buffer (80 uL) containing 0.1% ascorbic acid, pH 7.24 β-mercaptoethanol (5 uL) was added and the solution was allowed to stand at room temperature for 1 hr. The reaction was monitored using reversed phase HPLC. The reaction mixture was placed in ice-water bath and used for the next reaction without further purification. This procedure involved several modifications from the literature procedure [28].

5-Methyl tetrahydrofolic acid

Formaldehyde solution (37%, 8 µL) was added in the prepared tetrahydrofolic acid solution. It was stirred under nitrogen in an ice-water bath for 30 mins. $NaBH_4$ (8 mg, 0.2115 mmol) was then dissolved in minimum amount of phosphate buffer (50 mM) containing 0.1% ascorbic acid. The solution was stirred for 1 hr in ice-water bath. Aqueous acetic acid solution (1M, 2 mL) was added to the reaction mixture to neutralize excess borohydride and then brought to pH 7. The product was recovered from the supernatant after centrifugation and purified using preparative re-versed phase HPLC. The product fraction was lyophilized and then stored in the freezer.

Reversed phase high pressure liquid chromatography

Detection of the folates was done on an analytical C18 column with gradient elution from 6% to 25% acetonitrile in 30 mM potassium phosphate buffer, pH 2.2 at a flow rate of 0.8 mL/minute. Purification was done on a C18 semi-prep column at a flow rate of 4 mL/min using a gradient of 6% to 25% acetonitrile in 30 mM potassium phosphate buffer, pH 2.2.

Liquid scintillation counting

Aliquots of HPLC fractions collected were analysed for ^{14}C using a Wallac 1410 liquid scintillation counter. Radioactivity was measured in disintegration per minute against a set of ^{14}C calibration standards.

Mass Spectrometry

Mass spectrometric analysis of the cold and hot synthesized 5MTHF were carried out in MSU, East Lansing, MI with Professor A Daniel Jones. The mass spectrometer was run under positive ion mode using electro-spray ionization.

3. RESULTS AND DISCUSSION

Considerations for isotope labeling of compounds for metabolic studies using ^{14}C Accelerator Mass Spectrometry (AMS)

Labeling of compounds for metabolic studies allows one to trace the fate of the parent compounds – their absorption, storage, excretion, transport, distribution, utilization and transformations. A quantification of the rate of all of these processes allows one to model the *in vivo* kinetic metabolism of the compound.

Tritium (^{3}H) and Carbon-14 (^{14}C) are commonly used radioactive labels. Stable isotope labels like Deuterium (^{2}H), Carbon-13 (^{13}C) are more frequently used because of their safety. But ^{14}C labeling offers advantages by being more sensitively detectable because of their very low natural abundance (10^{-12}) and therefore would be amenable to physiological dosing. Taking advantage of the recent developments in Accelerator Mass Spectrometry (AMS), very small amounts of ^{14}C can be detected unambiguously allowing dosing of minute amounts of labeled material, making radioactive exposure really below allowable limits and therefore safe [29]. In the study, a greater understanding of the graphitization process to improvement in the sample preparation that allows analysis of mg to sub-mg quantitites of total carbon, making this technique practical for bioanalytical and even environmental analyses [30, 31]. These make possible long-term kinetic studies, which are often not feasible when unlabeled or even stable isotope labeled nutrients is utilized.

Labeling of compounds can be specific or nonspecific. Specifically labeled compounds have a defined atom position for the stable/radio-isotope and are often produced through chemical synthesis. Non-specifically labeled compounds are often biologically-derived products generated from utilizing labeled precursors which are incorporated randomly during biosynthesis. Specifically labeled compounds are preferred in metabolic studies because it allows tracing in a clear-cut way the metabolic fate of the label. Deductions regarding possible degradation/catabolic steps as well as chemical transformations of the labeled compound are straight-forward. This approach simplifies kinetic modeling, which frees it from ambiguities that may arise from using randomly labeled compounds.

Efficient labeling of compounds for metabolic studies entails:

1. using minimum number of chemical reaction steps;
2. using high yielding isotope labeling steps;
3. minimum number of purification steps; and
4. introducing label at a position with slower expected turn-over rate, to allow long-term monitoring;

It is also important to consider the availability and cost of the isotopic material and the technical capabilities that may be required for the synthesis [32].

The kinetic modelling of nutrient metabolism allows one to summarize and present quantified parameters (nutrient amount changes in time) into reasonable platform of source/sinks/compartments in which nutrient flux from one site to another describes their interconnection, over-all describing their *in vivo* PK/PD behavior. Likewise, nutrient status among human subjects, being phenotypic characteristics can be very well correlated with genetic variations among individuals, allowing identification of mutations compromising nutrient availability. Over-all, this presents a more holistic view - a whole systems approach towards understanding nutrient metabolism.

Synthesis of ^{14}C-5MTHF

Starting with easily available FA converted to an activated pteroate in the form of pteroyl azide following the literature procedure [33] and [^{14}C-U]-L-Glutamate, as the radioisotope source, a ^{14}C-labeled FA as pteroyl [^{14}C-U]-L-glutamate was synthesized using N,N,N′,N′-tetramethyleneguanidine as superbase. The resulting folic acid with the radiolabel was then reduced efficiently with dithionite to DHF. An in-house prepared DHF reductase was then used to carry out enantioselective reduction to 6-(S)-tetrahydrofolic acid. Addition of formaldehyde solution produces CH_2THF which upon addition of $NaBH_4$ in aqueous ascorbate solution produced 5MTHF.

Other reducing agents for FA– $NaBH_4$, $NaCNBH_3$ and hypophosphorus acid- did work but produced both DHF and THF even when the reactions were carried out at 4° C as detected by HPLC. This demonstrates the greater reduction potential of DHF compared to FA.

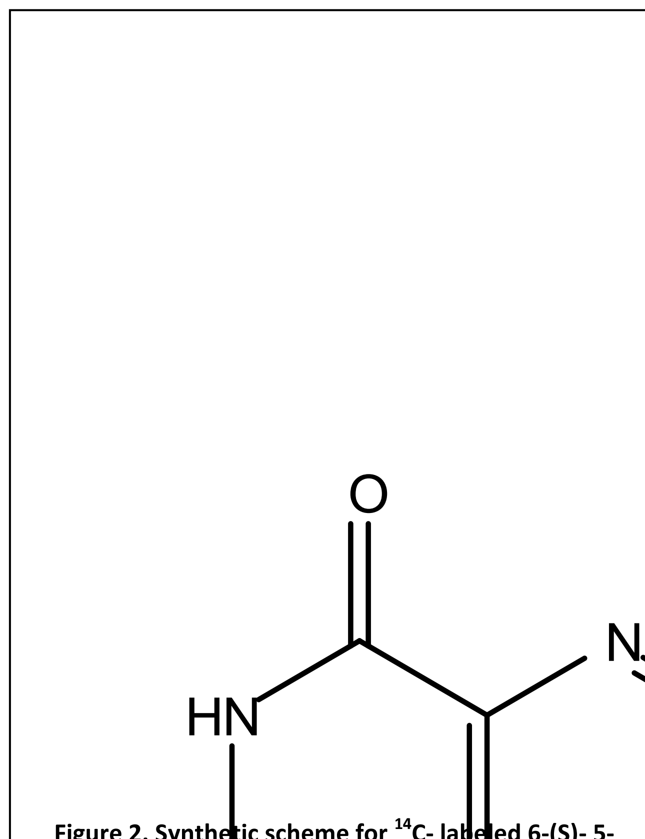

Figure 2. Synthetic scheme for ^{14}C- labeled 6-(S)- 5-methyltetrahydrofolic acid.

The reactions producing FA, DHF, THF, CH_2THF and 5MTHF were all monitored using HPLC by comparison against folate standards. Proper timing for conversion was essential as prolonged reaction time would lead to degradation even when all the reactions

reactions were carried out with 0.1 % (w/v) ascorbic acid, 2 % (v/v) β-mercatoethanol and under nitrogen atmosphere. Over-all yield of ^{14}C- labeled 6-(S)- 5-methyltetrahydrofolic acid was 58%.

Purification of ^{14}C-5MTHF

Purification of the synthesized ^{14}C-5MTHF was done through reversed phase HPLC using a C-18 semi-prep column as stationary phase and a gradient of 6% to 25% acetonitrile in 30 mM potassium phosphate buffer, pH 2.2. Fractions corresponding to the 5MTF peak were pooled together and stabilized by adding ascorbic acid at a final concentration of 0.1% (w/v). Samples were freeze-dried and stored at -80 ˚ C prior to use.

Characterization of ^{14}C-5MTHF

Analysis of the 40 µL aliquot of this ^{14}C-5MTHF pooled solution gave a radioactivity of 2.8 nCi. Specific activity of the solution was calculated as 9.45 Ci/mol based on a calibration plot comparison with standard 5MTHF.

The amount of ^{14}C-labeled 6-(S)- 5-methyltetrahydrofolic acid was 2.8 ± 0.4 % based on the percentage of species with [M+2] in the labeled relative to the unlabeled sample. Panels A and B (Figure 3) show the mass spectrum for the standard 5MTHF and the ^{14}C-5MTHF, respectively.

To confirm the identity of the synthesized C-14 5-MTF, the sample was run in two different HPLC conditions: the synthesized ^{14}C-5MTHF co-elutes with standard 5MTHF (Panels C and D and Panels E and F). Furthermore, the radioactivity profile of the HPLC fraction was shown to co-elute with the HPLC peak for 5-MTHF and demonstrated the true identity of ^{14}C-5MTHF (Panel F).

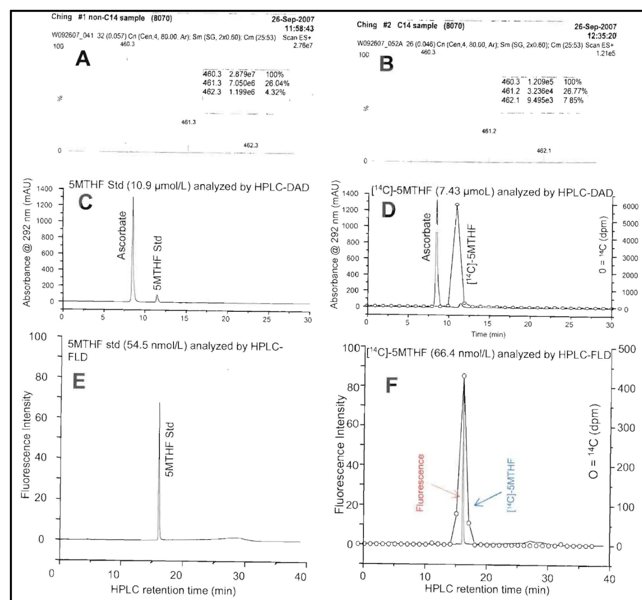

Figure 3. Mass spectrum of standard 5MTHF [A],
Mass spectrum of ^{14}C-5MTHF [B],
HPLC chromatogram for the standard 5-MTHF [C]
^{14}C-5MTHF with absorbance detection at 292 nm [D]
HPLC chromatogram for the standard 5MTHF [E]
^{14}C-5MTHF with fluorescence detection superimposed to the radioactivity counts of the aliquots of the HPLC fractions [F]

The concentration of the ^{14}C-5MTHF was determined to be 3.41µg/mL. This value was obtained through a linear regression analysis of the area of the peak for 5MTF on the HPLC chromatogram for the sample up against those for a set of standard solutions of 5MTF.

4. CONCLUSIONS

Practical synthesis of ^{14}C- labeled 6-(S)- 5-methyltetrahydrofolic acid is achieved by efficient conjugation of [^{14}C-U]-L-Glutamate with activated pteroate - pteroyl azide and efficient chemo-enzymatic approach of synthesizing 5MTHF. Having any one of the carbons of glutamyl as ^{14}C would allow one to trace the fate of 5MTHF and evaluate the metabolism of the most abundant natural folate as a coenzyme.

5. RECOMMENDATIONS

Investigation on the probable utility of (carboxy) peptidases for the facile hydrolysis of folic acid to pteroic acid and glutamate and the direct coupling of pteroic acid and L-glutamate in organic medium would greatly simplify the radiolabeling step.

Likewise, synthesis and evaluation of chiral hypophosphite as reducing agent for DHF may provide alternative chemical synthesis route towards making 6-(S)-tetrahydrofolic acid and 6-(S)- 5-methyltetrahydrofolic acid.

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