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Preliminary application of liquid chromatography–electrospray-ionization mass spectrometry to the detection of 5-methyltetrahydrofolic acid monoglutamate in human plasma

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Abstract

Liquid chromatography (LC) in direct combination with mass spectrometry (MS) has been shown to be a good analytical technique for the selective separation and detection of labile folate monoglutamates. Reversed-phase LC and electrospray-ionization MS conditions were developed and optimized for the separation and detection of 5-methyltetrahydrofolic acid, 5-formyl tetrahydrofolic acid, tetrahydrofolic acid, dihydrofolic acid and folic acid in aqueous samples. Representative and reproducible positive ion mass spectra were generated for each folate under mild MS conditions. The selective MS detection and identification of endogenous 5-methyltetrahydrofolic acid in human plasma was accomplished through the development of a straightforward C_{18}-based solid-phase extraction procedure. This procedure allows for the qualitative assessment of 5-methyltetrahydrofolic acid in plasma. Based upon an isotope-dilution internal standard calibration study with standards, the LC–MS limit of quantitation for 5M-THF was estimated to be 0.39 ng/ml.

Keywords: Folates; Methyltetrahydrofolic acid

1. Introduction

Deficiencies of folate result in impaired biosynthesis of nucleic acids and reduced cell viability. Clinical folate deficiency is not a well-defined disease state even though it is widely recognized that deficiency can lead to neural-tube defects, megaloblastic anemia, accelerated atherosclerosis, hyperhomocysteinemia and carcinogenesis [1]. More than 100 folate analogues based on the parent molecule, folic acid, exist in nature [2], but few analogues are stable enough to be isolated for full chemical analysis. Structures for five of the most biologically relevant folate monoglutamates, i.e., folic acid (F), dihydrofolic acid (DHF), tetrahydrofolic acid (THF), 5-methyltetrahydrofolic acid (5M-THF) and 5-formyl tetrahydrofolic acid (5F-THF) are shown in Fig. 1. The predominant circulating folate monoglutamate in human plasma/serum (>90%), and thus the most significant folate for accurately diagnosing folate deficiency, is 5M-THF [3–9]. The qualitative/quantitative determination of folates in plasma/serum is, in general, challenged by their low levels (pg/ml–ng/ml), their instability and their

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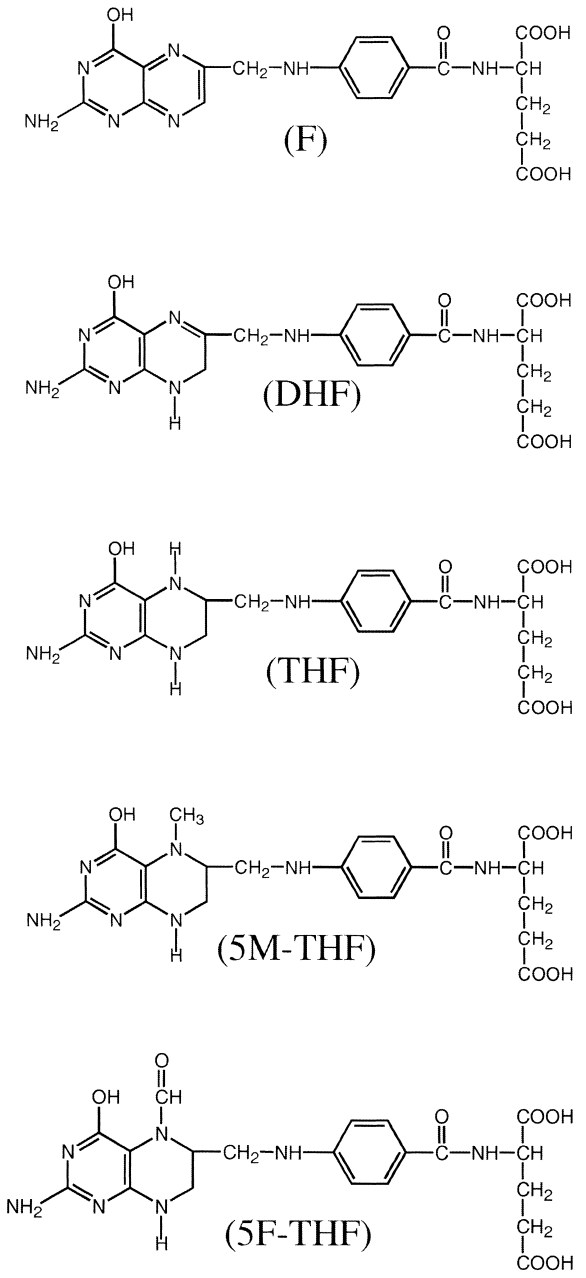

Fig. 1. Structures of five biologically relevant folate monoglutamates. F, folic acid (441.4 g/mol); DHF, dihydrofolic acid (443.4 g/mol); THF, tetrahydrofolic acid (445.4 g/mol); 5M-THF, 5-methyltetrahydrofolic acid (459.5 g/mol); 5F-THF, 5-formyl tetrahydrofolic acid (473.4 g/mol).

tendency to interconvert. The normal range of endogenous 5M-THF lies between ca. 9 and 21 ng/ml [10]. Mass spectrometry (MS) is an instrumental technique that has the ability to provide absolute identification (structure confirmation) and accurate quantitation of both low and high-molecular mass biomolecules. The MS analysis of non-derivatized folates has generally been hindered by their low volatility and their rapid oxidative degradation [11–14]. Initial studies of folates using secondary ion, desorption chemical-ionization or electron impact MS resulted in the formation of weak folate molecular ion signals due to extensive fragmentation [11,13]. Only the application of field desorption MS led to formation of stable molecular ions $[M]^+$ or protonated molecular ions $[M+H]^+$ [13,15,16]. Recent advances in the areas of MS sample introduction and sample ionization have led to the development of softer ionization techniques such as electrospray-ionization (ESI) and matrix-assisted laser desorption-ionization (MALDI) [17–19]. These ionization techniques cause less molecular fragmentation, which results in remarkably enhanced folate detection sensitivity [20,21]. Additionally, gas chromatographs and liquid chromatographs have been linked to mass spectrometers (GC–MS, LC–MS) in order to effect the selective separation and on-line identification of folates [22–26].

The research presented herein describes the preliminary results from the application of liquid chromatography–electrospray-ionization mass spectrometry (LC–ESI-MS) to folate analysis, and in particular, to the qualitative detection of 5M-THF in human plasma. Specifically, it is shown that five biologically relevant folate standards can be baseline separated by LC in less than 15 min and that reproducible and stable, positive-ion mass spectra can be generated. Next, it is shown that endogenous 5M-THF can be extracted from human plasma using C_{18} solid-phase extraction cartridges. The extracted 5M-THF can then be detected and unambiguously identified using LC–MS with selected ion monitoring (SIM). The feasibility of using the LC–MS method for quantitation is demonstrated by assessing the response linearity of 5M-THF in both aqueous standards and plasma extracts in relation to an isotopically labeled form of 5M-THF that functions as an internal standard.

2. Materials and methods[1]

2.1. Materials

All chemical reagents were ACS reagent grade unless stated otherwise. Disodium EDTA dihydrate, L-ascorbic acid, L-cysteine, folic acid (F), dihydrofolic acid (DHF), tetrahydrofolic acid (THF), 5-methyltetrahydrofolic acid disodium salt (5M-THF), 5-formyl tetrahydrofolic acid (5F-THF), formic acid, *p*-aminobenzoylglutamic acid (PABG), potassium phosphate monobasic and potassium phosphate dibasic were obtained from Sigma (St. Louis, MO, USA). $^{13}C_5$ 5-methyltetrahydrofolic acid calcium salt (^{13C5}5M-THF) was obtained from Eprova (Schaffhausen, Switzerland). ^{13C5}5M-THF contains five stable ^{13}C isotopes on its glutamic acid residue. Citrate-stabilized human plasma was obtained from Interstate Blood Bank (Memphis, TN, USA). Sep-Pak C_{18} solid-phase extraction cartridges were obtained from Waters (Milford, MA, USA). Ammonium formate was obtained from Aldrich (Milwaukee, WI, USA). HPLC-grade methanol, water, hydrochloric acid and trifluoroacetic acid (TFA) were obtained from JT Baker (Phillipsburg, NJ, USA). Purified water (18 MΩ), prepared using a Millipore Milli-Q purification system (Bedford, MA, USA), was used to prepare all samples and standards.

2.2. Special note

As folate derivatives are easily degraded by light and contact with oxygen, all folate stock solutions and standards were prepared under subdued laboratory lighting conditions with helium-degassed buffers. Additionally, all buffers and LC mobile phases were filtered through 0.45-μm pore nylon filters and degassed prior to use. When not in use, solid folate powders were stored at −20°C and folate stock–standard solutions were stored at −80°C. Reagent concentrations given in terms of percent (%) are to be considered as mass fractions (g/g) in all listed procedures.

2.3. Preparation of qualitative folate stocks and standards

Folate stock solutions (≈100 μg/ml) were prepared by weighing the selected folate powder (mg) into an amber glass vial containing a known amount (g) of 20 m*M* potassium phosphate–1% cysteine, pH 7.4, buffer. The stocks were subdivided into 0.5-ml aliquots and stored in 2-ml amber Eppendorf sample tubes at −80°C until use. Single folate standards and mixed standards were prepared by weighing an appropriate amount of folate stock into weighed amounts of phosphate–cysteine buffer.

2.4. Preparation of quantitative 5M-THF and ^{13C5}5M-THF stocks and standards

5M-THF and ^{13C5}5M-THF stocks were prepared as described above. The exact concentrations for 5M-THF and ^{13C5}5M-THF stock solutions were determined by measuring the UV absorbance of 100× dilutions of each stock and using the literature absorptivity value of 32.0×10^3 l mol^{-1} cm^{-1} at 290 nm for 5M-THF [9,27]. The concentrations were then corrected for impurities that contributed to the absorbance readings by performing an LC analysis on each stock solution using absorbance detection at the same wavelength used for the absorptivity determination [28]. The 5M-THF concentrations were calculated by multiplying the spectrophotometric concentrations by the peak ratio (peak area/total peak area) determined from the LC analysis. The initial stock concentrations (final corrected concentrations) for 5M-THF and ^{13C5}5M-THF were 111.0 μg/ml (75.6 μg/ml) and 121.2 μg/ml (101.7 μg/ml), respectively. The 5M-THF stocks were subdivided into 0.5-ml aliquots and stored in 2-ml amber Eppendorf sample tubes at −80°C until use. 5M-THF and ^{13C5}5M-THF standards (ng/ml) were prepared by weighing known amounts of the appro-

[1]Certain commercial equipment, instruments and materials are identified in order to specify experimental procedures as completely as possible. In no case does such identification imply a recommendation or endorsement by the National Institute of Standards and Technology (NIST) nor does it imply that any of the materials, instruments or equipment identified are necessarily the best available for the purpose.

priate stock solution into weighed amounts of 1% ascorbic acid solution.

2.5. LC analyses of folates

LC separations were performed on a HP 1100 series LC system. The system was outfitted with a variable wavelength UV detector and on-line mobile phase vacuum degasser. Samples were analyzed using a Waters Nova-Pak phenyl analytical column (3.9×150 mm, 4-μm particle size) with an attached Nova-Pak phenyl guard column (3.9×20 mm, 4-μm particle size). In all instances, the column temperature was thermostatted at 35°C and the detection wavelength was 280 nm. The LC elution conditions for the analysis of individual and mixed folate standards were as follows: Buffer A, 25 m*M* ammonium formate, pH 3.5 (the pH was adjusted with concentrated formic acid); Buffer B, 25 m*M* ammonium formate in methanol; time program, 0 min, 90% A/10% B; 30 min, 50% A/50% B; 31 min, 0% A/100% B; 36 min, 0% A/100% B; 37 min, 90% A/10% B; 42 min, 90% A/10% B; flow-rate, 1000 μl/min. The LC elution conditions for the analysis of aqueous standards and plasma extracts for 5M-THF were as follows: Buffer A and B same as stated above; time program, 0 min, 80% A/20% B; 9.0 min, 80% A/20% B; 9.5 min, 0% A/100% B; 11.5 min, 0% A/100% B; 12 min, 80% A/20% B; 17 min, 80% A/20% B; flow-rate, 350 μl/min.

2.6. LC–MS analyses of folates

LC–MS analyses were performed on a HP 1100 series LC directly connected to a single quadrupole mass selective detector (MSD) with an installed ESI source. The LC instrumental parameters have been described previously. Scanning and SIM mode mass spectra of the eluted folates were obtained and optimized via positive ion ESI. The following instrumental parameters were used for the ESI detection of folates in scanning mode: scan range, 50–600 *m/z*; s/scan cycle, 0.77; threshold, 20; step size, 10; ion mode, positive; gain, 15; fragmentor voltage, 50 or 70 V; capillary voltage, +3000 V; drying gas flow-rate, 11.0 l/min; drying gas temperature, 350°C; nebulizer pressure, 345 kPa. The following instrumental parameters were used for the ESI detection of 5M-THF and ^{13C5}5M-THF in SIM mode: threshold, 20; step size, 10; ion mode, positive; gain, 15; fragmentor, 70 V; capillary voltage, +3000 V; drying gas flow-rate, 11.0 l/min; drying gas temperature, 350°C; nebulizer pressure, 345 kPa; SIM ions (*m/z* 460.00 for 5M-THF and *m/z* 465.00 for ^{13C5}5M-THF).

2.7. Solid-phase extraction of 5M-THF from human plasma with LC–MS detection

5M-THF was extracted from unspiked human plasma or plasma spiked with ^{13C5}5M-THF internal standard using Sep-Pak C_{18} solid-phase extraction cartridges as follows: (1) 500 μl of plasma in a 2-ml microcentrifuge tube were combined 1:1 with a solution of 1% EDTA–1% ascorbic acid–1% cysteine in water (solution A). The 1-ml sample was vortex mixed and kept on ice until applied to the cartridge; (2) the cartridge was preconditioned by rinsing with 2 ml of each of the following in sequence: methanol, water, solution A; (3) the sample was applied to the cartridge and allowed to equilibrate for 2 min; (4) the cartridge was washed with 1 ml of each of the following: 50 m*M* potassium phosphate buffer, pH 7.4, and water–methanol (95:5, v/v); (5) and, finally, 5M-THF was eluted directly from the cartridge into a 2-ml microcentrifuge tube by rinsing the cartridge with 1 ml of 10% ascorbic acid in water–methanol (50:50, v/v, solution B). The plasma extract containing the 5M-THF was evaporated to approximately 1/2 initial volume, transferred to a sample vial and injected (50 μl) onto the LC–MS system.

2.8. Response linearity, LOD and LOQ for 5M-THF in aqueous standards by isotope-dilution LC–MS

An internal standard stock solution (10 ml) containing exactly 17 ng/ml ^{13C5}5M-THF in 10% ascorbic acid was prepared in an amber glass vial. A calibration stock solution (1 ml) containing exactly 20 ng/ml 5M-THF and 17 ng/ml ^{13C5}5M-THF in 10% ascorbic acid was prepared by combining the appropriate amount of 5M-THF stock solution with the ^{13C5}5M-THF internal standard stock solution. A

set of 10 volumetric serial dilutions was prepared from the calibration stock solution covering the range from 20 to 0.04 ng/ml 5M-THF. Each calibration standard, containing 17 ng/ml ^{13C5}5M-THF internal standard, was injected (50 μl) in duplicate and analyzed by the developed LC–MS method to estimate the method's linear range, LOD and LOQ.

2.9. Response linearity for 5M-THF in plasma extracts by isotope-dilution LC–MS

Note: The plasma standards were kept cold (on ice) between all preparation steps. Five hundred-μl aliquots of freshly thawed plasma were weighed into six 2-ml microcentrifuge tubes. Each tube was spiked, by weight, with a stock solution of 50 ng/ml ^{13C5}5M-THF so that the final concentration of ^{13C5}5M-THF in the plasma was 17 ng/ml. Next, each of the plasma standards was spiked, by weight, with a stock solution of 500 ng/ml 5M-THF so that the final concentration of added 5M-THF in the plasma standards was either 0 (blank plasma), 5, 10, 15, 20 or 25 ng/ml 5M-THF. The six standards were then extracted using the previously described C_{18} extraction procedures. The resulting plasma extracts, containing ^{13C5}5M-THF internal standard, were injected (50 μl) in triplicate and analyzed by the developed LC–MS method to demonstrate response linearity for 5M-THF.

3. Results and discussion

3.1. LC separation of biologically relevant folates

The solid-phase extraction and LC–MS detection of 5M-THF in human plasma was systematically approached by initially developing a LC method that could separate all five biologically relevant folates. A 10-μg/ml mixed standard of the five folate monoglutamates was prepared as described in Section 2, and analyzed by reversed-phase LC (Fig. 2). During the development of the separation, PABG was identified as an impurity in both DHF and THF by MS analysis. It was noted that commercial DHF was nominally contaminated with ≈14% F, while commercial THF was contaminated with DHF (≈22%), as well as with F (≈8%). These levels were determined based on the use of LC/UV peak area ratio calculations. These impurities (degradation products) are known to form due to the oxidation of the parent compounds.

3.2. MS and LC–MS analysis of folates

Before interfacing the LC separation to the MS system, flow injection analyses on 10 μg/ml folate standards were conducted using the atmospheric pressure chemical ionization (APCI) and the ESI ion sources in both negative and positive ion modes. A

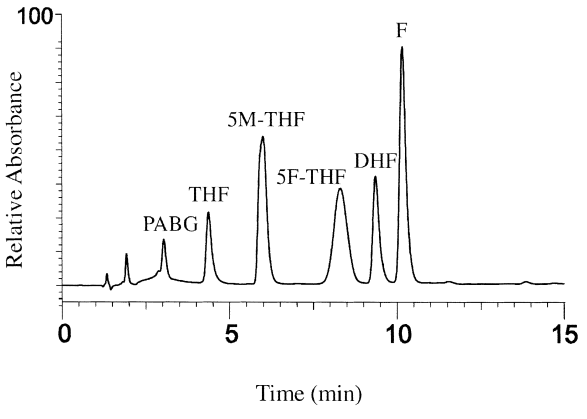

Fig. 2. Optimized LC separation of a standard mixture of folates (≈10 μg/ml each) prepared in 20 m*M* potassium phosphate–1% cysteine, pH 7.4. LC conditions: injection volume, 25 μl. All other LC parameters described in Section 2.

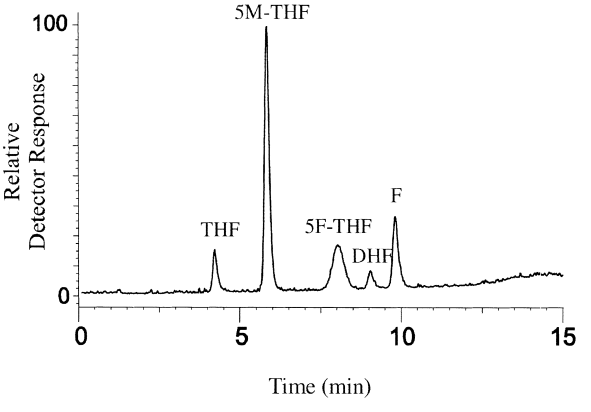

Fig. 3. Total ion chromatogram from the LC–MS analysis of a standard folate mixture (≈10 μg/ml each) prepared in 20 m*M* potassium phosphate–1% cysteine, pH 7.4. LC conditions: injection volume, 25 μl. All other LC parameters and MS conditions described in Section 2.

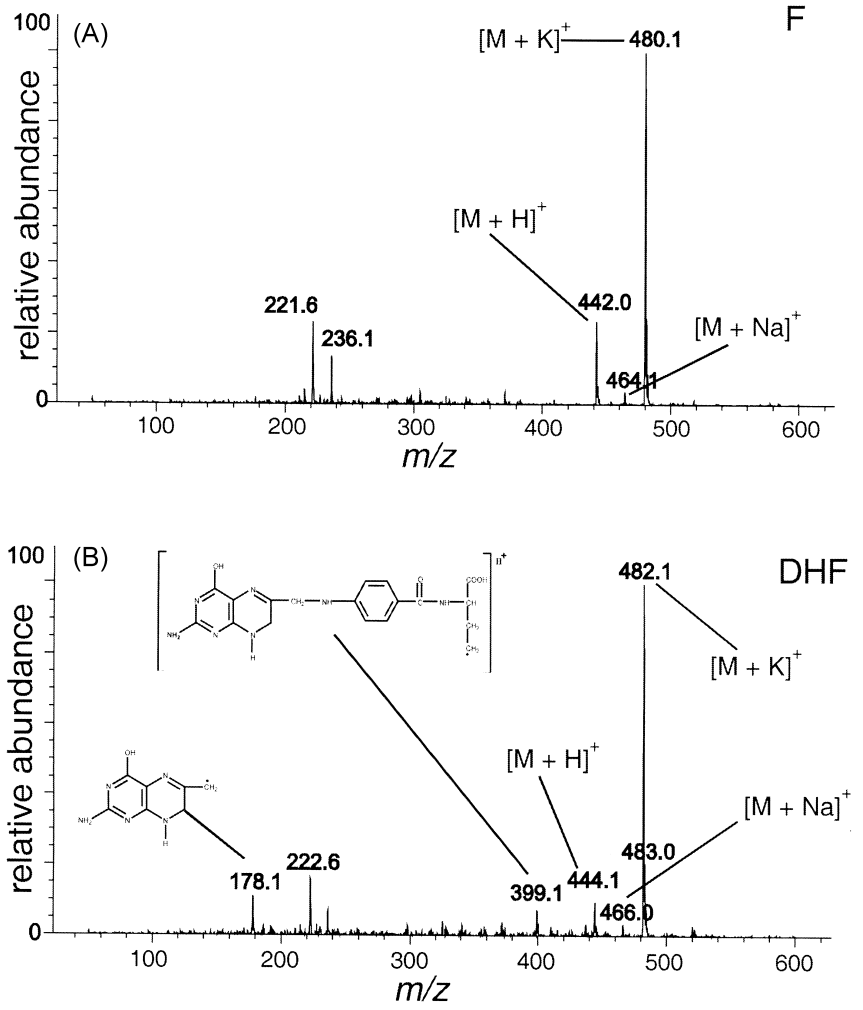

Fig. 4. Averaged positive-ion mass spectra and suggested fragmentation products for biologically relevant folates (≈100 μg/ml each). Mass spectra: (A) F; (B) DHF; (C) THF; (D) 5M-THF; (E) 5F-THF. LC conditions: injection volume, 25 μl. All other LC parameters and MS conditions described in Section 2.

one-variable screening analysis was performed in which the fragmentor voltage was varied between 0 and 200 V (by units of 25 V) to determine the ionization efficiency of each folate. The results from the APCI ion source screening were insubstantial. None of the folates produced a detectable signal in either positive or negative ion mode. This was as expected since APCI ionization usually requires high desolvation temperatures (=400°C) which probably cause the labile folates to degrade within the source. Detectable ion signals were realized for all of the folates during the ESI ion source screening in both positive and negative ion modes. Strong positive ion $[M+H]^+$ signals were seen for all folates, with 5M-THF producing the most intense response. The optimum fragmentor voltages were 50 V for F and DHF (ca. 35 000 and 17 500 counts, respectively) and 75 V for 5M-THF, 5F-THF and THF (ca. 175 000, 45 000 and 45 000 counts, respectively). The negative ion $[M-H]^-$ signals were also strong for all folates. F produced the most intense ion signal in this mode. The optimum fragmentor voltages were 125 V for F, 5F-THF and 5M-THF (ca. 250 000, 120 000 and 60 000 counts, respectively) and 100 V

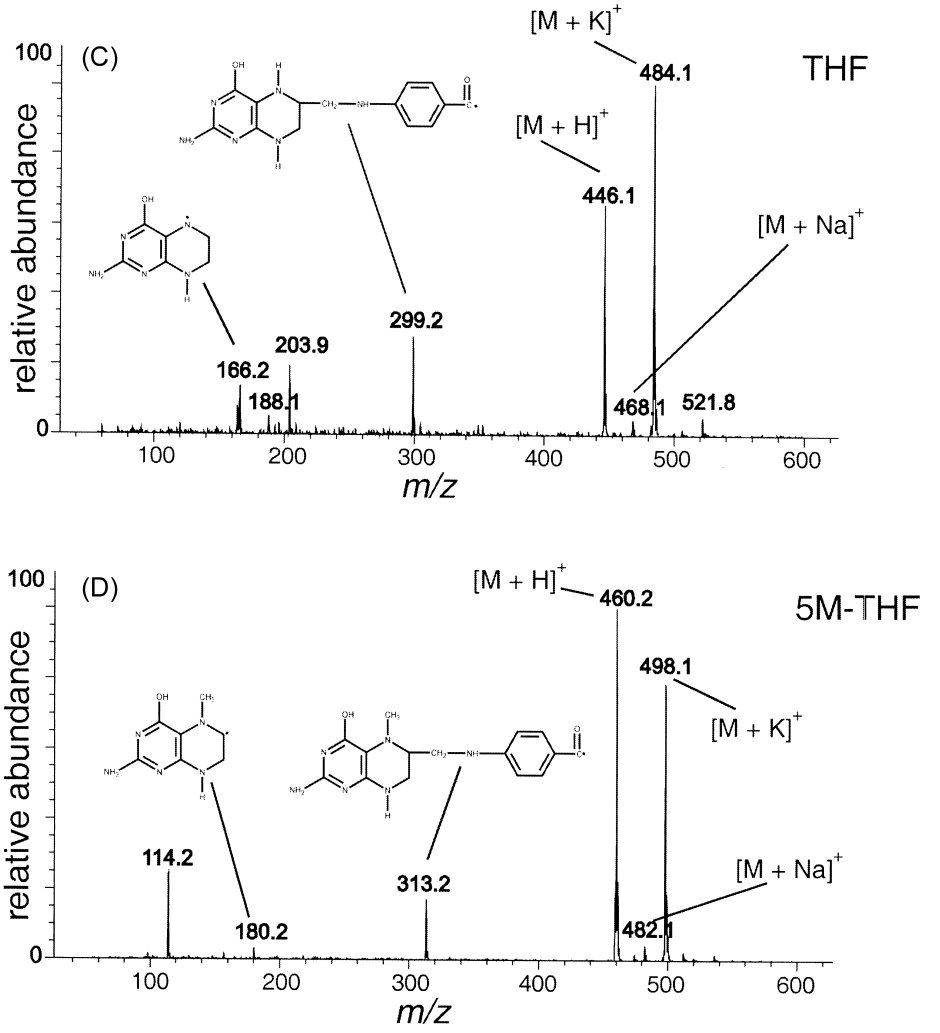

Fig. 4. (*continued*)

for DHF and THF (ca. 50 000 and 20 000 counts, respectively). In summary, THF and 5M-THF produced stronger signals in positive ion mode while 5F-THF, DHF and F produced stronger signals in negative ion mode. After determining that the positive ion ESI source was the optimum mode/source for the detection of 5M-THF, other ESI source parameters such as the capillary voltage, drying gas flow-rate, drying gas temperature and nebulizer pressure were screened and optimized using a one-variable-at-a-time approach. The resulting total ion chromatogram (TIC) from an actual LC–MS analysis of a mixture of the five folate standards is shown in Fig. 3. It should be noted that the intensity of the 5M-THF response significantly exceeds the intensity of all other folates.

Individual folate standards were analyzed using the optimized LC–MS conditions to generate the characteristic mass spectra depicted in Fig. 4A–E. Each mass spectrum allows the straightforward identification of the relevant folate due to the presence of a protonated molecular ion and the absence of extensive fragmentation. Tentative assignments of fragment ion structures are shown for selected folates. It should be noted that the spectra show extensive levels of molecular ion cationization with

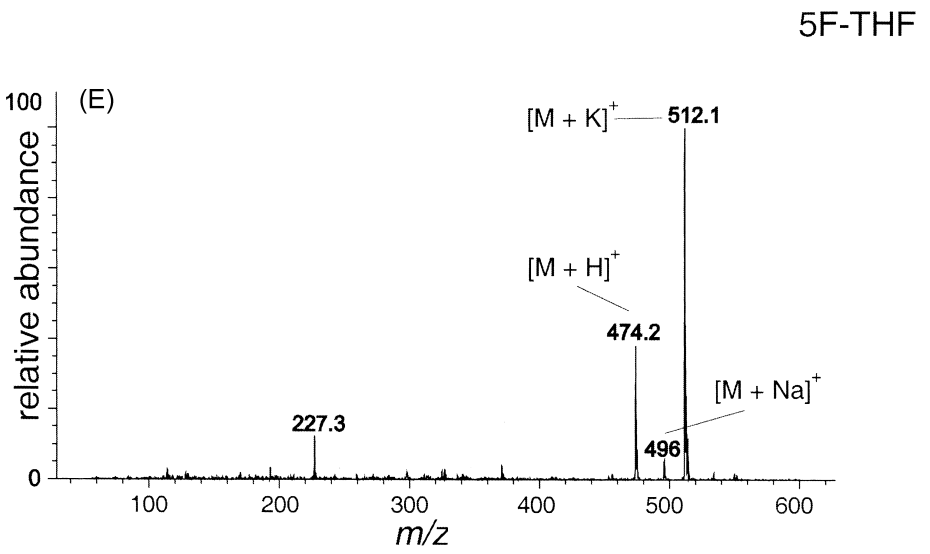

Fig. 4. (*continued*)

sodium and/or potassium. The utility of the potassium ion adduct $[M+K]^+$ for the positive identification of 5M-THF (Fig. 4D) at various concentrations was tested by preparing 10 μg/ml, 1 μg/ml and 20 ng/ml standards of 5M-THF and analyzing each of these standards using scanning LC–MS. Each scan produced a representative mass spectra for 5M-THF showing the presence of the protonated molecular ion, *m/z* 460, and the potassium adduct ion, *m/z* 498. Selected-ion chromatograms of *m/z* 460 and *m/z* 498 for 5M-THF indicated that these two ions were produced simultaneously by 5M-THF. The simultaneous detection of *m/z* 460 and *m/z* 498 can therefore be used to positively identify 5M-THF.

3.3. Solid phase extraction of 5M-THF from human plasma with LC–MS detection

Before attempting to extract and identify endogenous 5M-THF in human plasma, the LC method was modified to reduce the overall analysis time from 42 to 17 min (see Section 2). Normal plasma contains ca. 9–21 ng/ml 5M-THF [10], thus 15 ng/ml aqueous standards of 5M-THF were used to optimize Sep-Pak extraction conditions. The extraction and LC–MS detection procedures were developed on aqueous standards and then tested on plasma samples. Extraction of 5M-THF from plasma both with and without including a protein precipitation and centrifugation step yielded similar results in terms of total MS area counts. Thus, it was deemed simpler to extract the folate without incorporating a protein precipitation and centrifugation step. A typical LC–MS analysis of a plasma extract spiked with ^{13C5}5M-THF internal standard is shown in Fig. 5. Positive identification of 5M-THF was verified by simultaneously matching its retention time, selected ion chromatogram and its mass spectrum against aqueous standards of 5M-THF. The retention times (average±standard deviation) for both 5M-THF and ^{13C5}5M-THF in plasma extracts were 9.59±0.04 and 9.62±0.04 min (n=18), respectively.

3.4. LC–MS response linearity for 5M-THF in aqueous standards

The response linearity and detection sensitivity of the LC–MS method for 5M-THF was assessed by performing an isotope-dilution LC–MS calibration study. The results from duplicate injections of each calibration standard were plotted and analyzed by linear least-squares analysis. A plot of the area response ratio (5M-THF/^{13C5}5M-THF) versus 5M-THF concentration (ng/ml) resulted in a calibration equation and r^2 value of $y=0.22314x-0.04654$ and

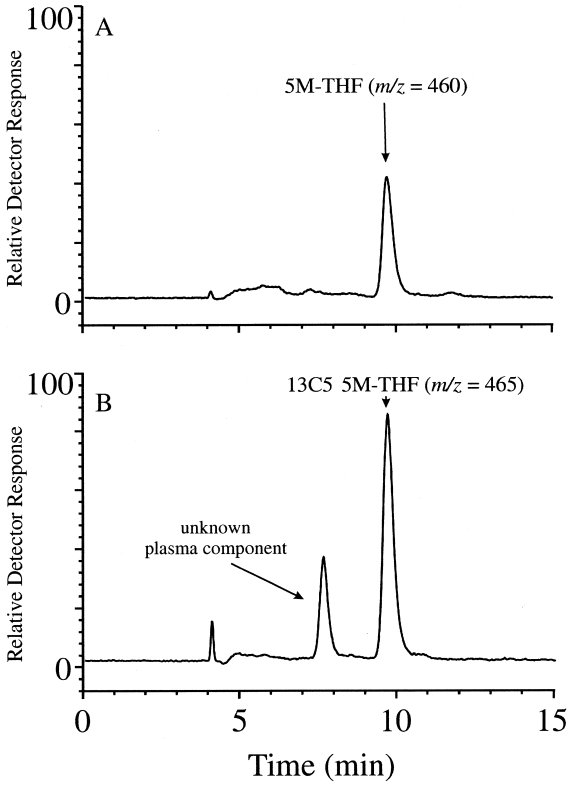

Fig. 5. Simultaneous selected ion chromatograms of endogenous 5M-THF and ^{13C5}5M-THF internal standard extracted from human plasma. Both chromatograms are on the same scale. (A) SIM-MS chromatogram for endogenous 5M-THF. (B) SIM-MS chromatogram for spiked ^{13C5}5M-THF internal standard (17 ng/ml). LC conditions: injection volume 50 μl. All other LC parameters and MS conditions described in Section 2.

0.997, respectively. The error in the slope and *y*-intercept were 0.00428 and 0.03127, respectively. The calculated r^2 value and visual inspection of the plot indicated that good response linearity from 0.04 to 20 ng/ml 5M-THF was achieved. Using the plot and a noise threshold of ca. 2000 ion counts, the estimated limits of detection (LOD) and quantitation (LOQ) for 5M-THF were 0.04 and 0.39 ng/ml, respectively, under the described LC–MS conditions.

3.5. LC–MS response linearity for 5M-THF in plasma extracts

The response linearity of 5M-THF extracted from plasma standards was also assessed by conducting an isotope-dilution LC–MS calibration study. The results from triplicate injections of six extracts (see Section 2) were plotted and analyzed by linear least-squares analysis. A plot of the area response ratios (5M-THF/^{13C5}5M-THF) versus analyte mass (mg) ratios (5M-THF/^{13C5}5M-THF) resulted in a linear plot with a r^2 value of 0.998. The equation of the line was $1.04635x+0.44442$ and the error in the slope and *y*-intercept were 0.02304 and 0.04122, respectively. Good response linearity was demonstrated for 5M-THF spiked into plasma over the working range of 0–25 ng/ml 5M-THF. Based upon the equation of the line, the extrapolated amount (average±standard deviation) of endogenous 5M-THF in this pool of plasma was calculated to be 7.2±0.3 ng/ml ($n=3$). The percent recovery of 5M-THF was assessed by spiking 5 ng/ml 5M-THF into plasma samples before and after solid-phase extraction. The estimated percent recovery (average %±%RSD) was 94±10% ($n=3$).

4. Conclusions

LC directly combined with ESI-MS can be used to selectively separate and detect five biologically relevant folates (F, DHF, THF, 5F-THF, 5M-THF) in standard solutions. Stable and reproducible positive ion mass spectra for each folate can be generated which should allow for the detection and identification of these folate species in other aqueous environments (plasma, serum, blood, urine, erythrocytes, etc.). An important example of this possibility is illustrated by the solid-phase extraction of endogenous 5M-THF from human plasma and its specific detection by LC–MS. 5M-THF can be extracted using reversed-phase extraction cartridges and qualitatively detected using mild MS conditions. The LC–MS detection sensitivity for 5M-THF is excellent, with a calculated LOQ of 0.39 ng/ml based on isotope-dilution internal standard calibration. The combination of the 5M-THF extraction procedure with the LC–MS detection conditions could eventually lead to the development of a quantitative method for the selective identification and sensitive quantification of 5M-THF in plasma, as well as in other biological matrices.

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