Anal. Chem. **2005,** *77,* 3586−3593

Simultaneous Quantification of Homocysteine and Folate in Human Serum or Plasma Using Liquid Chromatography/Tandem Mass Spectrometry

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A unified extraction and quantification procedure based on stable isotope-dilution liquid chromatography/tandem mass spectrometry (LC/MS/MS) has been developed for the simultaneous determination of total homocysteine and folate (5-methyltetrahydrofolic acid and folic acid) levels in human serum and plasma. This is the first report documenting the simultaneous extraction and quantification of these structurally dissimilar analytes. Analytes are quantitatively isolated from samples (500 μL) prior to LC/MS/MS analysis using a two-step stabilization process combined with C_{18} solid-phase extraction. The method exhibits excellent linearity over 4 orders of magnitude for each analyte. Measurement repeatability (RSD, $N = 2$) ranged from 0.3% to 3% for all analytes over 1 day of analysis. Total method variability (RSD, $N = 6$) ranged from 0.7% to 10% for all analytes over three independent days of analysis. The accuracy and practical applicability of the method were demonstrated by applying the method to the quantitative determination of each analyte in a new NIST serum Standard Reference Material (NIST SRM 1955 Homocysteine and Folate in Frozen Human Serum) and in a small subset of normal donor plasma samples.

Cardiovascular disease (CVD), which includes arteriosclerosis, stroke, angina, and high blood pressure, is the leading cause of adult mortality in the United States.[1,2] Sensitive biological markers of CVD (serum risk factors) and specific analytical methods for identifying, characterizing, and quantifying the aforementioned markers are needed for reliable disease prevention and risk assessment.[3] Established markers such as cardiac troponin I,[4,5] C-reactive protein,[6,7] cholesterol,[8,9] and fibrinogen[10,11] are frequently used to clinically assess CVD, CVD risk status, or both. Epidemiological studies have shown that increased serum levels of B-type natriuretic peptide[12,13] and total homocysteine (tHcy)[14−16] are also strongly correlated with increased risk of cardiovascular events. Recently, case control and prospective studies have suggested that folate, or rather folate deficiency, could be an emerging risk factor for CVD.[17,18]

The connection between tHcy and folate in CVD risk assessment is particularly intriguing because of the fact that serum/plasma tHcy status is inversely associated with folate status. tHcy and folate vitamers (5-methyltetrahydrolate (5MT), folic acid (FA), etc.) are intertwined within the cycle of human one-carbon metabolism, and it has been repeatedly demonstrated that the intake of folate (supplements, fortified foods) can significantly reduce tHcy levels.[18,19] Further, both tHcy and folate strongly influence the status of the vascular endothelium, a surrogate indicator of cardiovascular health.[18,20] Recent experimental evidence has shown that endothelial cell dysfunction, the preclinical manifestation of CVD, is directly correlated with high serum/plasma tHcy levels (tHcy ≥ 10 μmol/L).[21,22] The oral or intravenous intake of high levels of either FA or 5MT supplements can improve endothelial cell function, but it is not known whether this effect is due to the reduction of tHcy levels or through independent action of the folates.[18,23] Intravenous folate can improve endothelial function, and thus CVD risk status, regardless of the effect of folate on endogenous tHcy levels.

Hcy exists as a multiplicity of reduced (free homocysteine monomer) and oxidized (homocysteine−homocysteine dimer,

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10.1021/ac050235z
Published on Web 04/26/2005

homocysteine−protein complex, etc.) forms while folates exist as a variety of methyl, formyl, and formimino derivatives commonly bound to endogenous folate binding proteins and to albumin. Quantitative determination of tHcy is difficult because of the instability of its reduced form and the diversity of its oxidized forms. Quantitative determination of folates is challenged by their susceptibility to interconvert and to degrade during sample preparation. Arguably, the most specific and accurate methods for the determination of either tHcy[24−30] or folate[29−38] in plasma or serum are based on chromatography (LC) coupled to isotope-dilution mass spectrometry (MS). Most, but not all, of the reported LC/MS methods depend on the selective extraction of the pertinent analyte using either reversed-phase, anion exchange or affinity solid-phase extraction (SPE) protocols. The SPE protocols are inherently geared toward extraction of either Hcy or folate, but not both types of analytes simultaneously; this limitation is due to the distinct differences in analyte stabilization requirements and in analyte solution polarity (Hcy is strongly polar while folates are only moderately polar in solution). The vastly dissimilar structural features (Figure 1) of Hcy and folate compounds have also hindered the development of chromatographic methods for the simultaneous retention and separation of the analytes. To date, published methods have shown good results for the singular quantification of either tHcy or folate in serum/plasma samples on the basis of independent sample preparation, extraction, and analysis procedures. The practical relationship between tHcy and folate in the clinical assessment, i.e., clinical trials, of CVD could be better and more rapidly understood by the detection and measurement of both risk factors in the same sample using a single accurate analytical method.

We now report the development of a reversed-phase SPE procedure for the simultaneous isolation of tHcy and folate from human serum/plasma. In addition, the extraction procedure has been combined with LC/isotope-dilution/tandem MS (LC/MS/MS) to create a unified method for the simultaneous quantification of tHcy and folate. We have adapted certain analytical steps from our previously reported methods[26,33] in the development of this unified method. The method is only applicable to the specific folate forms (5MT, FA) described herein and, as such, cannot be used to assess "total" serum/plasma folate levels. The analytical development, characteristics, and applicability of this new LC/MS/MS method are reported.

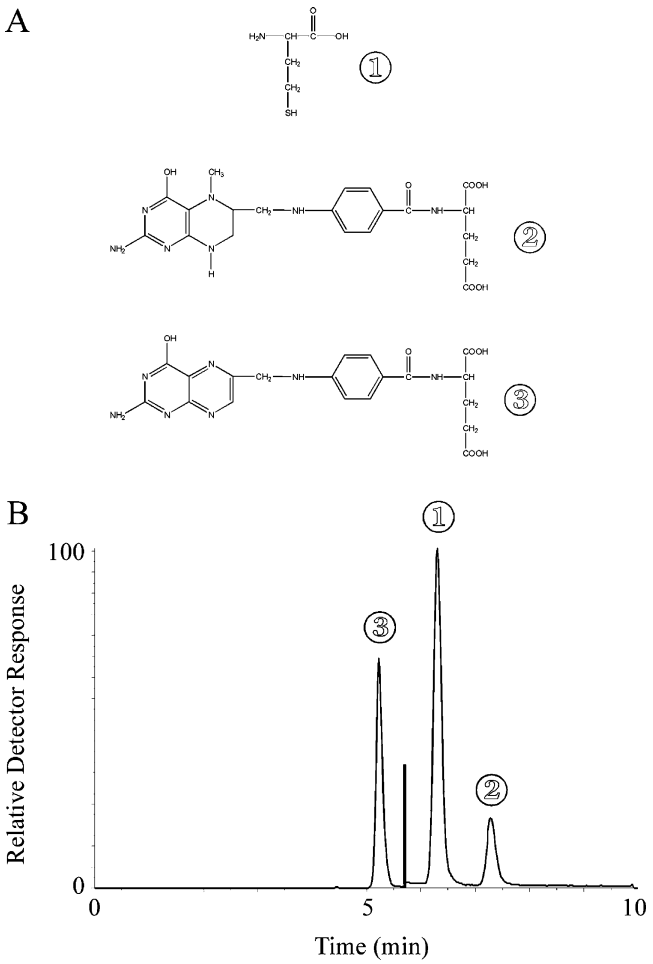

Figure 1. (A) Chemical structures for (1) homocysteine (Hcy), (2) 5-methyltetrahydrofolic acid (5MT), and (3) folic acid (FA). The relative masses of Hcy, 5MT, and FA are 135.2, 459.5, and 441.4 g/mol, respectively. (B) Representative total ion current chromatogram of the analytes extracted from NIST SRM 1955 (level 1). Profiles were collected using the LC/MS/MS conditions described in the Experimental Section. MRM mode MS/MS data were collected only during the chromatographic elution period (4.5−10 min) of the analytes from the chromatographic column. At all other times, the column effluent was diverted to waste. The vertical bar between peak 3 and peak 1 signifies the end of the first MS/MS operating period (period 1) and the beginning of the second MS/MS operating period (period 2). Peak identities correspond to peak numbers: (1) Hcy (4.1 μmol/L); (2) 5MT (4.2 nmol/L); (3) FA (0.6 nmol/L).

EXPERIMENTAL SECTION

Safety Considerations. The handling of biological source material (tissue, whole blood, serum, etc.) should be regarded as potentially hazardous to human health. Safe working (use of safety goggles and disposable protective gloves), disposal, and disinfection procedures should be clearly established before initiating work. The serum and plasma pools and samples utilized in this report were tested and found negative by the commercial

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vendors for human immunodeficiency virus, hepatitis B virus, and hepatitis C virus.

Reagents and Materials. Dithiothreitol (DTT) and L-ascorbic acid (AA) were obtained from Sigma Chemical Co. (St. Louis, MO). Pooled human serum, pooled human plasma (acid citrate-stabilized), and anonymous male/female normal donor plasma samples (acid citrate-stabilized) were obtained from Interstate Blood Bank (Memphis, TN). Pooled serum was utilized for all method optimization experiments. NIST serum Standard Reference Material (SRM 1955: Homocysteine and Folate in Frozen Human Serum) samples were prepared by Aalto Scientific, Ltd. (Carlsbad, CA) under the direction of NIST. SRM 1955 was prepared in bulk at three different levels (low, level 1; medium, level 2; high, level 3), each level containing increasing amounts of 5MT and Hcy. Exogenous FA was not a specified component of the SRM preparation protocol; thus, each SRM level contained only native FA. The specific preparation details of each level of SRM 1955 will be reported in a separate publication.[39] Serum quality control material (serum QC1) was prepared at NIST for internal use by fortifying pooled human serum with 5MT and FA. Sep-Pak Vac 3-cm^3 SPE cartridges (C_{18}, 500 mg) were obtained from Waters Corp. (Milford, MA). Purified water (18 MΩ), prepared using a Millipore Milli-Q purification system, was used to prepare all samples and standards. All other chemical reagents and solvents were ACS reagent grade unless stated otherwise. Reagent concentrations given in terms of percent (%) are to be considered as mass fractions (g/g) in all listed procedures. Preparation of analyte stocks/standards and biological samples was performed gravimetrically in all listed procedures, except where noted otherwise.

(1) Folates. [$^{13}C_5$]-5-Methyltetrahydrofolic acid calcium salt ((6*S*)-5-CH$_3$-H$_4$Pte[$^{13}C_5$]Glu-Ca), 5-methyltetrahydrofolic acid calcium salt ((6*S*)-5-CH$_3$-H$_4$PteGlu-Ca), and [$^{13}C_5$]-pteroylmonoglutamic acid (PteGlu, folic acid) were obtained from Merck Eprova AG (Schaffhausen, Switzerland); unlabeled pteroylmonoglutamic acid was obtained from Sigma Chemical Co. These compounds are designated as [$^{13}C_5$]-5MT, 5MT, [$^{13}C_5$]-FA, and FA respectively, throughout the article. The purity of [$^{13}C_5$]-5MT, 5MT, [$^{13}C_5$]-FA, and FA was established by both flow injection MS/MS and LC/MS/MS analyses. [$^{13}C_5$]-5MT contained 0.3% 5MT and 0.1% FA, [$^{13}C_5$]-FA contained 0.4% FA, and 5MT contained 0.3% FA. FA contained no detectable impurities, ≥0.02%.

(2) Homocysteine. Homocysteine reagents were gravimetrically prepared by the chemical reduction of commercial homocystine as described previously.[26] Reduced homocysteine reagents were utilized in all measurement procedures. DL-[$^2H_8$]-Homocystine was obtained from C.D.N. Isotopes (Pointe-Claire, PQ, Canada). DL-Homocystine was obtained from Sigma Chemical Co. The purity of DL-[$^2H_8$]-homocystine and DL-homocystine, as established by the suppliers, was ≥97.9 atom % deuterium (NMR) and >99% (TLC), respectively. The purity of DL-[$^2H_8$]-homocystine and DL-homocystine was confirmed by full-scan LC/MS, flow injection MS/MS, and LC/MS/MS analyses. The purity of the reduced reagents, DL-[$^2H_4$]-homocysteine and DL-homocysteine, was also assessed by full-scan LC/MS, flow injection MS/MS, and LC/MS/MS analyses; the reagents contained no detectable impurities, ≥0.02%. DL-[$^2H_4$]-Homocysteine and DL-homocysteine are designated as [$^2H_4$]-Hcy and Hcy respectively, throughout the article.

Methods. (1) Preparation of Hcy Stock Solutions and Calibration Standards. Hcy and [$^2H_4$]-Hcy stock solutions were prepared as described previously.[26] Hcy and [$^2H_4$]-Hcy standards were prepared by weighing known amounts of the appropriate stock solution into weighed amounts of 1.5% aqueous DTT.

(2) Preparation of Folate Stock Solutions and Calibration Standards. 5MT, [$^{13}C_5$]-5MT, FA, and [$^{13}C_5$]-FA stock solutions were prepared by weighing the appropriate folate powder into an amber glass vial containing a weighed amount of 50 mmol/L potassium phosphate/1% β-mercaptoethanol buffer (pH 7.4). The concentrations for the folate stock solutions were assigned using previously published procedures.[33,36] An appropriate amount of solid AA was added to each stock solution so that the concentration of AA in solution was 1% and the final pH of the solution was 4.8. The stabilized stock solutions were aliquoted and stored at −80 °C until use. 5MT and [$^{13}C_5$]-5MT standards were prepared by weighing known amounts of the appropriate stock solution into weighed amounts of 1% AA. FA and [$^{13}C_5$]-FA standards were prepared by weighing known amounts of the appropriate stock solution into weighed amounts of 0.1% aqueous DTT.

(3) Preparation of Linearity Standards. An internal standard stock solution (20 mL) containing 10 μmol/L [$^2H_4$]-Hcy, 22 nmol/L [$^{13}C_5$]-5MT, and 22 nmol/L [$^{13}C_5$]-FA in eluent A (1% AA + 0.4% formic acid in 50:50 water/methanol) was prepared in an amber glass vial. A linearity stock solution (1 mL) containing 400 μmol/L Hcy, 871 nmol/L 5MT, and 906 nmol/L FA was prepared using the internal standard stock solution as diluent. A set of 18 volumetric serial dilutions was prepared from the linearity stock solution using the internal standard stock solution as diluent. Each linearity standard was injected (50 μL) in duplicate onto the LC/MS/MS to estimate the method's linear dynamic range, limit of detection (LOD) and limit of quantification (LOQ).

(4) Preparation of Samples and Calibrants. A plasma or serum sample (500 μL) was added into a 2-mL microcentrifuge tube and spiked with 25 μL of [$^2H_4$]-Hcy stock standard (192 μmol/L), 48 μL of [$^{13}C_5$]-5MT stock standard (224 nmol/L), and 25 μL of [$^{13}C_5$]-FA stock standard (455 nmol/L). The nominal concentrations of [$^2H_4$]-Hcy, [$^{13}C_5$]-5MT, and [$^{13}C_5$]-FA in the sample were 10 μmol/L, 22 nmol/L, and 22 nmol/L, respectively. The sample (pH 7.2) was vortex mixed and then spiked with 77 μL of 10% aqueous DTT. The sample was allowed to equilibrate at room temperature (25 ± 2 °C) in the dark for 15 min. Following the initial incubation period, the sample was spiked with 500 μL of 10% aqueous AA, vortex mixed, and allowed to equilibrate on ice (0 °C) for an additional 15 min (sample pH 3.5), prior to SPE.

Matrix calibrants (serum or plasma) were prepared by adding sequentially increasing amounts of Hcy (194 μmol/L), 5MT (555 nmol/L), and FA (222 nmol/L) stock solutions and constant amounts of [$^2H_4$]-Hcy, [$^{13}C_5$]-5MT, and [$^{13}C_5$]-FA stock solutions to 500-μL aliquots of pooled serum or pooled plasma. The spiked analyte concentrations in the matrix calibrants corresponded to 0 (calibrant blank), 5, 10, 15, 20, and 25 μmol/L for Hcy, 0 (calibrant blank), 11, 22, 33, 44, and 54 nmol/L for 5MT, and 0 (calibrant blank), 11, 23, 34, 45, and 57 nmol/L for FA. The nominal concentrations of [$^2H_4$]-Hcy, [$^{13}C_5$]-5MT, and [$^{13}C_5$]-FA

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in the matrix calibrants were 10 μmol/L, 22 nmol/L, and 22 nmol/L, respectively. The matrix calibrants were processed in an identical manner to the samples and allowed to equilibrate on ice prior to SPE.

Eluent A calibrants were prepared by adding sequentially increasing amounts of Hcy, 5MT, and FA stock solutions and constant amounts of [$^2H_4$]-Hcy, [$^{13}C_5$]-5MT, and [$^{13}C_5$]-FA stock solutions to 1-mL aliquots of eluent A. The nominal analyte concentrations corresponded to 0 (calibrant blank), 1, 5, 10, 15, and 25 μmol/L for Hcy, 0 (calibrant blank), 2, 11, 22, 33, and 54 nmol/L for 5MT, and 0 (calibrant blank), 0.6, 2, 11, 23, and 57 nmol/L for FA. The nominal concentrations of [$^2H_4$]-Hcy, [$^{13}C_5$]-5MT, and [$^{13}C_5$]-FA in the calibrants were 10 μmol/L, 22 nmol/L, and 22 nmol/L, respectively. The calibrants were stored at −20 °C until needed.

(5) Extraction of Samples and Calibrants. Analytes were extracted from room-temperature samples and matrix calibrants using disposable C_{18} extraction cartridges as follows (all extractions were conducted with a manual vacuum-manifold system): (1) the cartridge was preconditioned by rinsing with 2 mL of each of the following in sequence, methanol, water, 1% aqueous AA; (2) the sample was applied to the cartridge and pulled through under a light vacuum (2 min); (3) the cartridge was washed with 2 mL of 1% AA in 95:5 water/methanol (volume fractions) solution; and finally, (4) the analytes were eluted by rinsing the cartridge with 1 mL of eluent A. Each sample extract, calibrant extract, or eluent A calibrant was injected (50 μL) in duplicate onto the LC/MS/MS system. Analyte/internal standard peak area ratios (area/area) and mass ratios (mg/mg) were subjected to linear least-squares regression analysis to produce calibration curves and calibration equations for each set of calibrants. Analytes in the sample extracts were quantified on the basis of the relevant calibration equation and the analyte/internal standard peak area response ratio detected in the sample extract.

(6) LC/MS/MS Instrumentation and Methods. LC/MS/MS experiments were conducted on a HP1100 LC system (Agilent Technologies) coupled to a Sciex API 4000 triple quadrupole MS/MS system (Applied Biosystems) operating in electrospray ionization (ESI) mode. The LC system was outfitted with a binary pump, a variable-wavelength UV absorbance detector, a temperature-controlled (15 °C) autosampler, and an in-line mobile-phase vacuum degasser. Samples were analyzed using a Supelcosil LC-CN cyano analytical column (4.6 mm × 250 mm, 5-μm particle size) with an attached Supelcosil LC−CN guard column (3 mm × 20 mm, 5-μm particle size) held at 40 ± 1 °C. The LC elution conditions were as follows (all solvent percentages are volume fractions): mobile phase A, 0.4% formic acid in water; mobile phase B, 0.4% formic acid in methanol; time program, 0 min, 50% A/50% B; 3.0 min, 50% A/50% B; 6.0 min, 0% A/100% B; 8.0 min, 0% A/100% B; 8.1 min, 50% A/50% B; 14.0 min, 50% A/50% B; flow rate, 750 μL/min. The MS/MS operating parameters for all analytes were obtained and optimized via positive ion mode ESI. Multiple reaction monitoring (MRM) transitions for each analyte were individually optimized for protonated analyte molecules [M + H]$^+$ and stable, protonated fragments. Specific compound-dependent and instrument-dependent MS/MS parameters for each analyte/internal standard are given in Table 1 and Table 2, respectively.

Table 1. Compound-Dependent MS Parameters for Homocysteine and Folate Analytes[a]

analyte	MRM transition (m/z)	DP (eV)[b]	CE (eV)[c]
Hcy	136 → 90	46	27
[$^2H_4$]-Hcy	140 → 94	46	27
FA	442 → 295	51	19
[$^{13}C_5$]-FA	447 → 295	51	19
5MT	460 → 313	46	27
[$^{13}C_5$]−5MT	465 → 313	46	27

[a] The collision exit potential (10 eV) and the entrance potential (10 eV) were identical for all analytes. [b] DP (declustering potential). [c] CE (collision energy).

(7) Confirmational Analytical Methods and Measurements. The values for tHcy, 5MT, and FA were measured and confirmed in NIST SRM 1955 (levels 1, 2, and 3) using a variety of analytical methods (Table 3). Table 3 gives a brief description of each method. A full report giving complete, detailed descriptions of each method and the specific application of each method to the assignment of certified, reference, and informational values to the analytes in SRM 1955 is in preparation.[39]

RESULTS AND DISCUSSION

Simultaneous analysis of tHcy, 5MT, and FA (Figure 1A) via isotope-dilution LC/MS/MS requires complete equilibration of the isotopically labeled internal standards and reproducible extraction of the analytes from the biological matrix. Folates must be released from noncovalent attachment to endogenous proteins and prevented from interconverting/degrading while the Hcy disulfides (and mixed disulfides) must be released from covalent attachment to endogenous proteins and reduced to monomeric form for accurate quantitation. Our group,[32,33] as well as other folate researchers,[40,41] has shown that certain folate compounds (5MT, FA) can be effectively stabilized in situ via use of aqueous AA at the level of 1% or higher. AA is most effective from acidic to neutral pHs. However, AA does not possess sufficient reductive capacity to break the S−S bonds of Hcy disulfides. Aqueous DTT, on the other hand, is extremely effective at reducing Hcy disulfides to Hcy monomers and stabilizing the monomers once they are formed. DTT is most effective from neutral to basic pHs. Based upon this information, sample preparation can be divided into two discrete steps. In step one, the slightly basic pH (pH 7.2−7.8) of plasma or serum, in combination with the chemically reducing power of DTT, is utilized to release protein-bound Hcy and to reduce Hcy disulfides to Hcy monomers. The use of DTT in this step has the additional advantage of providing a moderate stabilizing effect on endogenous folates. In step two, the sample is spiked with 10% aqueous AA, which stabilizes and releases the folates from endogenous proteins and lowers the sample pH to pH 3.5. The incorporation of AA in this step also ensures that any 5-methyldihydrofolic acid that may have formed during sample incubation with DTT is efficiently converted back to 5MT.[41]

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Table 2. Instrument-Dependent MS/MS Parameters for Homocysteine and Folate Analytes[a,b]

analyte	dwell time (ms/ion)	ion spray voltage (V)	curtain gas flow (kPa)	gas 1 flow (kPa)	gas 2 flow (kPa)	CAD gas (kPa)[c]
Hcy	250	5500	69	414	414	41
$[^2H_4]$-Hcy	250	5500	69	414	414	41
FA	500	4500	138	483	345	55
$[^{13}C_5]$-FA	500	4500	138	483	345	55
5MT	250	5500	69	414	414	41
$[^{13}C_5]-$5MT	250	5500	69	414	414	41

[a] FA and $[^{13}C_5]$-FA were monitored during MS/MS period 1 (0−5.7 min). Hcy, $[^2H_4]$-Hcy, and 5MT, $[^{13}C_5]$-5MT were monitored during MS/MS period 2 (5.7−14.0 min). Quadrupoles 1 and 3 were operated at unit resolution. [b] The electrospray source temperature (450 °C) was identical for all analytes. [c] Collisionally activated dissociation gas. The CAD gas was nitrogen.

Table 3. Other Methods Used in the Confirmation and Assignment of Values to tHcy, 5MT, and FA in NIST SRM 1955

analytical method	analyte	method modifications	ref
SPE LC/MS/MS[a]	5MT, FA	n/a[i]	36
SPE LC/MS/MS[b]	5MT	Analyte detection mode changed from selected ion monitoring (SIM) mode to MRM mode to enhance detection sensitivity.	33
SPAE LC/MS/MS[c]	5MT	Isocratic mobile phase changed from 80:20 A/B to 70:30 A/B to improve analyte peak shape.	33
SPE LC/MS/MS[d]	5MT, FA	Analyte detection mode changed from SIM mode to MRM mode to enhance detection sensitivity.	33
GC/MS[e]	tHcy	GC column changed from DB-5 stationary phase to DB-17 stationary phase for enhanced retention of Hcy.	26, 27
SPE LC/MS/MS[f]	tHcy	LC column changed from cyanopropyl stationary phase to pentafluorophenylpropyl stationary phase for enhanced retention of Hcy	27
FPIA[g]	tHcy	n/a	48, 49
LC/FD[h]	tHcy	n/a	50

[a] Extraction of analytes based on the use of C_{18} SPE. [b] Extraction of analyte based on the use of C_{18} SPE. SPE elution solvent changed to 1% ascorbic acid/1% formic acid in 50:50 water/methanol. Utilized an isocratic mobile phase consisting of 70:30 A/B. [c] Solid-phase affinity extraction; extraction based on the use of bovine folate binding protein. [d] Extraction of analytes based on the use of C_{18} SPE. Utilized a C_{18} LC column and an isocratic mobile phase consisting of 70:30 A/B for simultaneous analysis of 5MT and FA. [e] Gas chromatography/mass spectrometry; extraction of Hcy based on the use of anion-exchange resins; Hcy monitored using SIM mode MS of m/z 420. [f] Extraction of Hcy based on the use of anion-exchange resins; Hcy monitored using MRM mode MS of m/z 136 → 90. [g] Fluorescence polarization immunoassay. [h] Liquid chromatography/fluorescence detection. [i] na, not applicable.

Optimum incubation conditions for stabilizing 5MT with aqueous AA, and by logical extension, FA, were previously established and reported in a recent publication.[33] The release of folates from endogenous proteins and stabilization of the released folates has also been established,[31−34] and thus, the majority of the current experimental work relates to step one, the reduction of Hcy disulfides to free Hcy. The rate-limiting step in the preparation of samples for extraction is the conversion of all unstable Hcy disulfides into free Hcy.

Optimal DTT-to-Serum Ratio. The optimal DTT-to-serum ratio (mol of DTT/100 μL of serum) was determined by testing a series of 12 samples containing increasing amounts (moles) of DTT. Reduction and release of Hcy disulfides via the chemical action of DTT in serum was deemed complete when the Hcy/$[^2H_4]$-Hcy area response ratio was stable and maximized. Serum samples containing 3.6×10^{-7} total mol of DTT/100 μL of serum to 2.1×10^{-5} total mol of DTT/100 μL of serum were prepared, processed, and analyzed as described in the Experimental Section. The 3.6×10^{-7} mol of DTT/100 μL of serum sample corresponds to the control sample, which only contains DTT contributed from the $[^{13}C_5]$-FA and $[^2H_4]$-Hcy internal standards. The Hcy/$[^2H_4]$-Hcy area response ratios were stable and maximal from 9.3×10^{-6} mol of DTT/100 μL of serum to 2.1×10^{-5} mol of DTT/100 μL of serum. DTT/serum ratios less than 9.3×10^{-6} (for example 5.4×10^{-6}) produced Hcy/$[^2H_4]$-Hcy area response ratios that were reduced by 10% on average. On the basis of these results, the optimal DTT/serum ratio was selected as 1×10^{-5}, which corresponds to an addition of 77 μL of 10% aqueous DTT to 500 μL of serum. The use of 1×10^{-5} mol of DTT/100 μL of serum ratio for reduction of Hcy disulfides was also found to be effective by Magera et al. and Gempel et al.;[24,25] however, these researchers did not report on the effects of using lower or higher ratios. The area response ratios for both 5MT/$[^{13}C_5]$-5MT and FA/$[^{13}C_5]$-FA were essentially constant over the range of DTT/serum ratios. The relative standard deviation (RSD) in the area response ratios for 5MT/$[^{13}C_5]$-5MT and FA/$[^{13}C_5]$-FA were 2% and 14%, respectively. The higher variability in the FA/$[^{13}C_5]$-FA area response ratio was due to the exceedingly low level (1.1 nmol/L) of FA present in the serum pool; the calculated levels of 5MT and tHcy in the pool were 41.6 nmol/L and 7.2 μmol/L, respectively.

Optimal DTT Incubation Time. The optimal DTT incubation time was determined by incubating serum samples for increasing periods of time (1, 10, 20, and 30 min). Samples were processed and analyzed as described in the Experimental Section. The study indicated that complete reduction of Hcy disulfides was achieved almost instantaneously; the Hcy/$[^2H_4]$-Hcy area response ratios were constant (within analytical error) from the 1-min time point through the 30-min time point. The RSD in the Hcy/$[^2H_4]$-Hcy area response ratio did not vary by more than 4% for all four time points. For practical convenience, the default incubation time of

15 min was chosen to allow efficient manual processing of samples. A critical aspect of the DTT incubation step is that the sample pH must be ≥7 to allow the DTT to react efficiently and the sample must be at room temperature (see next section). The area response ratios for both 5MT/[$^{13}C_5$]-5MT and FA/[$^{13}C_5$]-FA were essentially constant over the tested time periods. The RSDs in the area response ratios for 5MT/[$^{13}C_5$]-5MT and FA/[$^{13}C_5$]-FA were 5% and 13%, respectively.

Optimal Sample Incubation Temperature. The activity of DTT on disulfide bond disruption is influenced by the temperature of the sample matrix. The optimal temperature for Hcy disulfide reduction was determined by incubating serum samples at either 25 or 0 °C. Samples were processed and analyzed as described in the Experimental Section. The resulting data showed that the Hcy/[$^2H_4$]-Hcy area response ratio decreased significantly as the incubation temperature decreased; the Hcy/[$^2H_4$]-Hcy ratio decreased 9% from 25 to 0 °C. The decreased ratio indicates that low-temperature incubation results in less free Hcy formation per unit time in comparison to room-temperature incubation. Interestingly, if serum is equilibrated at 0 °C before and during the DTT incubation step, the activity of DTT is suppressed even further; the Hcy/[$^2H_4$]-Hcy ratio was reduced by 22% in this sample in comparison to the room-temperature incubation sample. The data suggest that, for proper reduction and release of Hcy disulfides, the sample must be at room temperature both before and during the DTT reduction step. No effect on the area response ratios for either 5MT/[$^{13}C_5$]-5MT or FA/[$^{13}C_5$]-FA was observed during the temperature study. The current procedures for the simultaneous determination of tHcy and folate in serum are not readily amenable to sample incubation temperatures greater than approximately room temperature (25 °C) because of intractable gel formation in the sample matrix.

Extraction and LC/MS/MS Analyses. Screening of anionic, cationic, and reversed-phase SPE cartridges indicated that the analytes could be extracted from processed samples using either anionic or reversed-phase (C_{18}) adsorbents. However, the folates, in particular FA, could not be easily eluted from anionic adsorbents without the inclusion of high levels of sodium chloride in the eluent; high salt concentrations are not compatible with stable ESI MS. On the other hand, both folate analytes and Hcy could be readily trapped and released from C_{18} adsorbents using salt-free, ESI MS-compatible eluents. Folate ($pK_{a1,2}$ = 3.5, 4.8)[41] and Hcy molecules ($pK_{a1,2,3}$ = 2.2, 8.9, 10.9)[42] exist predominantly as protonated, uncharged species in the stabilized samples (pH 3.5) prior to extraction. During the extraction process, the analytes and internal standards are simultaneously captured by the C_{18} SPE cartridges on the basis of hydrophobic interactions. Analytes were subsequently eluted from the cartridges, and the resulting extracts were analyzed by LC/MS/MS or stored at −20 °C and analyzed at a later time. The stability of sample extracts was demonstrated to be ≥3 months when stored at −20 °C in eluent A. Good separation of the three analytes was achieved via the LC-CN chromatographic column with the mobile-phase composition optimized to yield the most sensitive detection of the analytes in MRM MS/MS mode. Each analyte was baseline separated by ∼1 min with mean retention times (mean ± SD) of 5.2 ± 0.01, 6.3 ± 0.01, and 7.3 ± 0.01 min for FA, tHcy, and 5MT, respectively. A total ion current chromatogram from analysis of a representative serum extract is presented in Figure 1B. The analysis was divided into two discrete MS/MS operating periods (period 1, FA; period 2, tHcy and 5MT) to permit increased sensitivity for the detection of FA. FA is unstable, in comparison to Hcy and 5MT, during the positive ion mode ESI process and requires substantially lower collision energies (Table 1) to produce the relevant protonated molecules/protonated fragments. FA, in fact, is more stable during negative ion mode ESI and produces more intense product ion fragments during negative ion mode collisionally activated dissociation.[36] However, analytical conditions for negative ion mode ESI are not compatible with the current extraction and LC separation conditions for Hcy or 5MT. On the other hand, Hcy and 5MT are both stable during positive ion mode ESI and both analytes can be sensitively detected under identical collision energy conditions (Table 1) within the same MS/MS operating period.

Following optimization of the extraction and LC/MS/MS detection of the analytes, we evaluated the effect of ion suppression on both the analyte and internal standard responses in serum and plasma via the technique of post-extraction matrix spiking.[43,44] Full-scan MS analyses of unspiked sample extracts were also conducted.[45] No significant ion suppression of either the analytes or internal standards was observed over the analytical calibration range.

Method Performance Characteristics. Matrix-based (serum or plasma) calibration curves do not produce results that are significantly (> 6%) different from eluent A-based calibration curves for the three analytes. Analytical slopes for serum-based and plasma-based calibration curves were 0.543, 1.043, 1.157 and 0.547, 1.045, 1.159 for tHcy, 5MT and FA, respectively. While analytical slopes for eluent A-based calibration curves were, 0.569, 1.037, and 1.117 for tHcy, 5MT, and FA, respectively. Calculated analyte concentrations in a serum QC sample (serum QC1) were also not significantly different if based on serum versus eluent A calibration curves (7.6 vs 7.2 μmol/L for tHcy; 63.1 vs 63.3 nmol/L for 5MT; 29.9 vs 30.6 nmol/L for FA) or plasma versus eluent A calibration curves (7.5 vs 7.2 μmol/L for tHcy; 63.1 vs 63.3 nmol/L for 5MT; 29.7 vs 30.6 nmol/L for FA). Overall recoveries (mean ± RSD) of tHcy, 5MT, and FA added to serum at five different concentrations (5−25 μmol/L for tHcy, 11−54 nmol/L for 5MT, and 11−57 nmol/L for FA) were 100.8% ± 5.4%, 98.6% ± 6.6%, and 99.5% ± 4.9% for tHcy, 5MT, and FA, respectively. Overall recoveries of tHcy, 5MT, and FA added to plasma (same five concentration ranges as serum) were 97.6% ± 5.2%, 96.7% ± 3.7%, and 103.4% ± 1.6% for tHcy, 5MT, and FA, respectively. On the basis of these results, method characterization and analyte measurements in serum and plasma samples were conducted with standards or calibrants prepared in eluent A. The instrument response for each analyte was linear over more than 4 orders of magnitude under the optimized MS/MS conditions. Detailed information regarding the analytical linear dynamic range and LOD and LOQ sensitivity for each analyte is given in Table 4.

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Table 4. Method Characteristics

analytical parameter	Hcy (μmol/L)	5MT (nmol/L)	FA (nmol/L)
linear dynamic range[a]	0.003−51[b]	0.02−829[c]	0.05−913[d]
LOD[a,e]	0.003	0.02	0.05
LOQ[a,f]	0.01	0.07	0.14
LC/MS/MS mass sensitivity[g] (pg)	68	1.5	3

[a] Determined via duplicate analysis of serially diluted calibrants as described in the Experimental Section. [b] Calculated $r^2 = 0.998$, slope 0.0508 (0.0006), y-intercept = 0.0150 (0.0095), SE estimate for regression line 0.0330. Values in ( ) signify the standard error (SE). [c] Calculated $r^2 = 1.000$, slope 0.0354 (0.0001), y-intercept = −0.0381 (0.0248), SE estimate for regression line 0.0895. [d] Calculated $r^2 = 1.000$, slope 0.0381 (0.0001), y-intercept = −0.0236 (0.0213), SE estimate for regression line 0.0738. [e] The LOD is the minimum detectable analyte signal that is at least three times the noise signal. [f] The LOQ is calculated by multiplying the LOD by a factor of 3. The S:N ratio for each analyte at the listed LOQ is 64:1, 35:1 and 12:1 for Hcy, 5MT and FA, respectively. All S:N ratios are based on peak-to-peak calculations. [g] Mass of analyte in a sample extract required to achieve a quantifiable signal with the reported method conditions. Calculated by multiplying the LOQ by the sample injection volume (50 μL).

Table 5. Simultaneous Determination of tHcy, 5MT, and FA in NIST SRM 1955[a]

	new method				other methods[b]			
NIST SRM 1955	mean (μmol/L)	N	SD	95% CI	mean (μmol/L)[c]	N	SD	95% CI
				tHcy				
level 1	4.11	6	0.30	3.80−4.43	3.89	48	0.27	3.81−3.97
level 2	8.78	6	0.69	8.06−9.50	8.55	47	0.34	8.45−8.65
level 3	16.46	6	0.47	15.97−16.95	17.08	48	0.56	16.92−17.24
				5MT				
	mean (nmol/L)				mean (nmol/L)[d]			
level 1	4.21	6	0.07	4.13−4.29	4.34	36	0.47	4.26−4.42
level 2	9.79	6	0.07	9.72−9.86	9.69	36	0.22	9.53−9.85
level 3	36.47	6	0.52	35.92−37.02	37.27	36	2.18	36.53−38.00
				FA				
	mean (nmol/L)				mean (nmol/L)[e]			
level 1	0.62	6	0.03	0.59−0.65	0.44	18	0.07	0.41−0.48
level 2	1.17	6	0.12	1.04−1.30	1.01	18	0.11	0.95−1.07
level 3	1.25	6	0.08	1.17−1.33	1.01	18	0.13	0.94−1.07

[a] The 95% confidence intervals (CIs) of the mean analyte values were calculated using t-values based on $N - 1$ degrees of freedom. [b] For a brief description of each of the other analytical methods, refer to Table 3. [c] tHcy determinations were performed using four other analytical methods. [d] 5MT determinations were performed using four other analytical methods. [e] FA determinations were performed using two other analytical methods.

The method's quantitative range for tHcy (0.01 μmol/L ≤ tHcy ≤ 50 μmol/L) exceeds the currently recommended tHcy range (∼3−40 μmol/L) for routine clinical methods.[46] Most (∼99%) patient samples from the general population[46] lie within the range of routine clinical methods; however, for individual samples that exceed 50 μmol/L tHcy, i.e., individuals suffering from severe hyperhomocysteinemia (homocystinuria), the samples will have to be diluted to within the method's linear range and reanalyzed. The quantitative range (0.1 nmol/L ≤ folate ≤ 900 nmol/L) established for the folate analytes is broad enough to allow detection and quantification of FA and 5MT in samples from individuals suspected of folate deficiency (folate <7 nmol/L), in normal population samples (7 nmol/L ≤ folate < 76 nmol/L) and in individual samples in which there exists a large excess of unmetabolized FA (folate ≥76 nmol/L).[36]

Quantification of Analytes in NIST Serum SRM 1955 and in Normal Donor Plasma. The new simultaneous LC/MS/MS method, along with a variety of other well-established analytical methods (Table 3), was applied to the quantification of tHcy, 5MT, and FA in three levels of NIST serum SRM 1955. Two samples/vial per day were processed and analyzed over 3 days ($n = 6$) for each SRM level (at a minimum) by each analytical method. The data in Table 5 present a comparison of the results determined by the new method versus the combined results determined by the other analytical methods. The mean analyte values from the new method were calculated based on a simple statistical average while a Bayesian statistical model[47] was utilized to calculate the overall mean for the other method analyte values. In general, the new method results were in good accord with the other method results for the three analytes at each SRM level.

The new method tHcy and 5MT results were both in good agreement with the other method tHcy and 5MT results for all SRM levels. Statistical analysis of the data did not indicate the presence of any bias associated with either the tHcy or 5MT new method measurements. Sufficient overlap of the 95% confidence intervals (95% CIs) around the mean values for all tHcy and 5MT measurements was demonstrated. In terms of quantification accuracy, the FA new method results were in reasonable agreement with the other methods' FA results. The slight discrepancy (0.2 nmol/L FA on average) in measured values was mostly due to the increased measurement imprecision that naturally arises during the measurement of low-level analytes. Another factor that must be considered when comparing the new method FA values to the other methods' FA values is the new method's analytical sensitivity (∼0.2 nmol/L, estimated using LOQ = 0.14 nmol/L) for FA. The new method is pushed to its sensitivity limit in trying to discriminate at the 95% confidence level between 0.1 and 0.3 nmol/L FA. For most practical purposes, an offset of ±0.2 nmol/L for mean FA values is not likely to be clinically significant on an individual sample basis.

In terms of quantification precision (Table 6), both the within-day measurement repeatability and total method variability were good. Measurement repeatability ranged from 0.3% to 3% for all analytes over 1 day of analysis. Total method variability ranged from 0.7% to 10% for all analytes over 3 days of analysis.

The practical utility of the new method was also tested on a small subset of human plasma samples. Anonymous normal donor male ($N = 4$) and female ($N = 4$) plasma samples were obtained from a commercial source and subsequently processed and analyzed as described in the Experimental Section. Summary ranges for each analyte were as follows: tHcy, 5.4−10.1 μmol/L (RSDs = 0.3%−5.4%); 5MT, 10.6−46.4 nmol/L (RSDs = 0.2%−1.7%); FA, 0.5−12.6 nmol/L (RSDs = 0.4%−13.5%). The measured

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Table 6. Precision Data for the Determination of tHcy, 5MT, and FA in NIST SRM 1955[a]

NIST SRM 1955	within-day repeatability (% RSD)	total method variability (% RSD)
	tHcy	
level 1	3.0	7.3
level 2	3.0	7.8
level 3	0.3	2.9
	5MT	
level 1	1.7	1.8
level 2	0.8	0.7
level 3	2.0	1.4
	FA	
level 1	0.9	4.8
level 2	2.3	10.4
level 3	0.7	6.2

[a] $N = 2$ for within-day repeatability (2 preparations × 1 analysis of each preparation; $N = 6$ for total method variability (2 preparations × 1 analysis of each preparation × 3 days).

tHcy values lie within the expected concentration range for general population tHcy values (tHcy, 2−71 μmol/L) as determined and reported via the use of a dedicated tHcy LC/MS/MS method.[24] The measured folate values lie within the expected concentration range for normal donor folate values (5MT, 7−61 nmol/L; FA, <0.6−25 nmol/L) as recently determined and reported via the use of a dedicated folate LC/MS/MS method.[36] Overall, the measurement repeatability of the plasma analyses was similar to the serum analyses; imprecision in the low-level FA values was greater, as expected, than the imprecision in either the tHcy or 5MT values.

At the conclusion of the method testing exercises, it was determined that the required sample size could be effectively reduced to 200 μL (combined with a 200-mg C_{18} SPE cartridge) to achieve good detection and good quantification of tHcy and 5MT in the serum or plasma pools and in all levels of SRM 1955 (data not shown). Sample preparation was identical to the 500-μL sample preparation scheme, except that reagent volumes (masses) were reduced by 60%. Extraction of analytes was identical to the 500-μL extraction scheme, except that elution of the analytes from the SPE cartridges was performed using an elution volume of 400 μL of eluent A. The limited amount of FA in the samples could be detected but not adequately quantified (signal RSD > 20%, $N = 3$) using the reduced sample size. Studies are on going to further reduce the required sample size and to improve the quantification of FA in these low-volume samples.

CONCLUSIONS

The newly developed LC/MS/MS method has demonstrated good, quantitative accuracy and precision for the simultaneous determination of tHcy and 5MT/FA in serum or plasma. This is the first report documenting the concurrent quantitative determination of homocysteine and folates in a single plasma or serum sample using a single extraction and analysis protocol. The method has analyte LOQs that are as good as or dramatically better than the current single-analyte methods for homocysteine or folates. The method has a wider dynamic range than most single-analyte methods, and the total sample preparation time (<1 h) and analysis time (<15 min) are on the same time scale as the single-analyte methods. The analyte extraction component of the method can be easily converted to an automated high-throughput format (96 well plate) for clinical use, especially for the quantification of tHcy and 5MT. The method has many potential applications including, but not limited to, the detection of folate deficiency and the detection of hyperhomocysteinemia within the general population. Further, the method should prove extremely useful to researchers involved in quantitatively clarifying the clinical relationship between hyperhomocysteinemia, folate deficiency, and CVD risk.

ACKNOWLEDGMENT

We thank Zia Fazili (folate LC/MS/MS), Mindy Zhang (folate microbiologic assay, Hcy LC/FD), and Sonya Strider (Hcy FPIA) of the Centers for Disease Control and Prevention (Atlanta, GA) for performing folate and homocysteine measurements on NIST SRM 1955. We are extremely grateful to the following: Véronique Ducros (CHU de Grenoble, Grenoble, France); Donald Jacobsen and Pat DiBello (Cleveland Clinic Foundation, Cleveland, OH); Stuart Moat (University Hospital of Wales, Cardiff, Wales); Christine Pfeiffer (Centers for Disease Control and Prevention, Atlanta, GA), and Helga Refsum (Oxford University, Oxford, U.K.) for their thorough and critical review of the manuscript during its preparation. We also thank Nien-Fan Zhang and Adriana Hornikova (NIST, Gaithersburg, MD) for their statistical assessment of the data. 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.

Received for review February 7, 2005. Accepted April 1, 2005.

AC050235Z