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Development of a new standard reference material: SRM 1955 (homocysteine and folate in human serum)

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Abstract Total homocysteine (tHCY) and folate are interrelated biomarkers for arteriosclerosis and coronary heart disease. Although many different methods for both tHCY and folate are clinically available, the intermethod and interlaboratory results are often poor, resulting in the need for a matrix reference material and reference methods. The National Institute of Standards and Technology (NIST) has developed isotope dilution liquid chromatography/mass spectrometry (LC/MS) and liquid chromatography/tandem mass spectrometry (LC/MS/MS) methods for determination of tHCY and several folate forms including 5-methyltetrahydrofolic acid (5MT) and folic acid (FA). Additionally, a method for simultaneous measurement of tHCY, 5MT, and FA has been developed and validated. In collaboration with the Centers for Disease Control and Prevention (CDC), mass spectrometric methods and methods used in clinical laboratories have been applied to characterize a new Standard Reference Material (SRM), SRM 1955, "Homocysteine and Folate in Human Serum," containing low, medium, and high levels of tHCY and 5MT. Additionally, FA, 5-formyltetrahydrofolic acid (5FT), vitamin B_{12}, and total folate values are provided. Use of the new SRM should improve clinical measurements and will permit traceability to internationally recognized certified reference materials, as described by European Directive 98/79/EC on in vitro diagnostic medical devices.

Keywords Isotope dilution mass spectrometry · Standard reference material · Homocysteine · Folate · 5-Methyltetrahydrofolic acid · 5-Formyltetrahydrofolic acid · Folic acid

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Introduction

Total homocysteine (tHCY) and folate are markers related to cardiovascular health that have been targeted by the National Institute of Standards and Technology (NIST) for measurement and inclusion in a human serum-based Standard Reference Material (SRM). The process of selecting these analytes for inclusion in an SRM, choosing the material to be used, developing the measurement techniques necessary, making the pertinent measurements, and the expected effect on clinical measurement of tHCY and folate is detailed herein.

Homocysteine (HCY), a sulfur-containing amino acid, is a product of methionine degradation that has come under scrutiny due to the positive correlation between elevated levels of tHCY and cardiovascular disease (CVD) [1, 2]. Most clinical assays measure homocysteine as tHCY, a composite of forms found in circulation. At this time it has not been determined if high levels of tHCY are a causative factor in CVD or if the relationship is due to other factors, such as low levels of folate, vitamin B_{12}, and vitamin B_6 [3, 4].

The dominant form of folate, the general term for a series of vitamers, is 5-methyltetrahydrofolic acid (5MT), comprising 80–95% of the total folate in serum [5]. The health effects of folate are well known in terms of their relationship to neurological defects in neonates; low levels of maternal folate result in an increased incidence of offspring with spina bifida and other related birth defects [4]. To improve folate status in the population, addition of folic acid to cereal grains in the USA began in 1998, resulting in decreased numbers of neurological defects in

newborns [6]. Low levels of folate are also related to the incidence of CVD as well as being correlated with high homocysteine levels [7], and associated with increased cancer incidence [8].

Numerous methods of measurement of tHCY are available including enzyme immunoassay (EIA), fluorescence polarization immunoassay (FPIA), liquid chromatography with fluorescence or electrochemical detection (LC/FD or LC/ED), and gas chromatography/mass spectrometry (GC/MS) [9–13]. Newer liquid chromatography/mass spectrometry (LC/MS) or liquid chromatography/tandem mass spectrometry (LC/MS/MS) methods have been reported [14–16]. Some comparison studies using the older methods found that measurements for homocysteine were not congruent, and that most methods were not comparable. In contrast, two studies found good correlation among laboratories and methods under certain conditions, i.e., when plasma calibrants were supplied as part of the study [17, 18]. In these cases, the plasma calibrants acted as quality control samples, providing validation as well as traceability. As shown by these two interlaboratory studies, the poor agreement among laboratories and among most methods can be improved by the use of a matrix standard with known values.

The measurement of folate in serum or plasma is complicated by the fact that the traditional tests, microbiological assays [19, 20] and radioassays [21], measure total folate as a combination of FA, 5MT, and 5-formyltetrahydrofolic acid (5FT), as well as other less stable folate forms. Although both of these traditional methods purport to measure total folate, comparison studies have shown a distinct lack of agreement between the methods [22]. Methods for measurement of FA, 5MT, and/or 5FT in serum and plasma independently include LC/ED [23, 24] and LC/MS [25–29]. A recently developed method by Pfeiffer et al. at the Centers for Disease Control and Prevention (CDC) was able to measure 5MT, FA, and 5FT in serum using isotope dilution LC/MS/MS [5]. Recent work at NIST has focused on measurement of 5MT by isotope dilution LC/MS and LC/MS/MS using different extraction and separation techniques [26–28]. The latest method for measurement of 5MT developed at NIST involves the detection of tHCY and folate in the same analysis [30].

To bring greater concordance to the measurements of tHCY, 5MT, FA, 5FT, vitamin B_{12}, and total folate in serum, NIST has developed and produced a Standard Reference Material, SRM 1955, Homocysteine and Folate in Frozen Human Serum. NIST has been involved in the development of health-related SRMs since the late 1960s, beginning with pure crystalline standards such as cholesterol and glucose, and continuing with matrix standards including cholesterol in human serum and electrolytes in human serum (see http://www.nist.gov). SRMs are designed for use in evaluating the accuracy of clinical procedures, and in validating calibrators and controls. After interest by the clinical community established the need for a serum-based SRM for folate and tHCY, the CDC collaborated with NIST in setting the proposed analyte levels. Scientists at both CDC and NIST performed measurements of the folate and tHCY.

Three levels of SRM 1955 have been prepared and certification measurements made: one with low levels of the analytes, one with medium levels, and the third with high levels. The molecular structures of the analytes measured are shown in Fig. 1. Measurements using highest-order methods which produced values with the greatest certainty (certification measurements) were made at NIST for tHCY and 5MT using isotope dilution GC/MS, LC/MS, and LC/MS/MS, and at the CDC for 5MT using LC/MS/MS. Measurements whose sources of uncertainties were not known well enough to assign certified values were made for FA using three different LC/MS/MS methods, two at NIST and one at the CDC, and reference values were assigned. Clinically acceptable methods used at the CDC provided measurements with the least certainty (information values) [31]. All measurements were subjected to statistical analysis using a weighted Bayesian approach, and include associated uncertainties [32, 33].

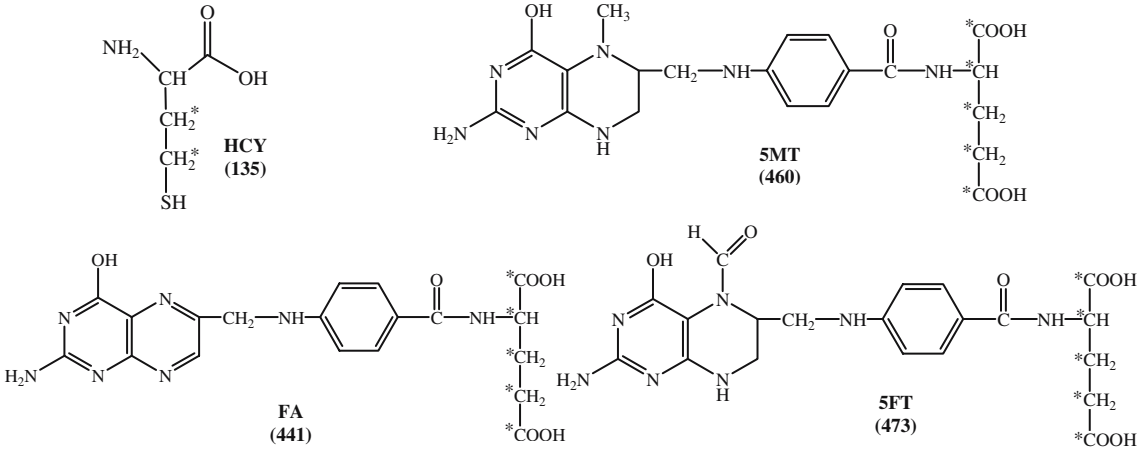

Fig. 1 Molecular structures of homocysteine (tHCY), folic acid (FA), 5-methyltetrahydrofolic acid (5MT), and 5-formyltetrahydrofolic acid (5FT). Relative molecular masses are given in *parentheses*, and isotopically labeled sites are identified by *asterisks*

Experimental [1]

Reagents and materials

The SRM was prepared by Aalto Scientific, Ltd. (Carlsbad, CA). Each donor unit of serum or plasma used in the preparation of this product was tested by Aalto using an FDA-approved method and was found to be nonreactive for hepatitis B surface antigen (HbsAG), hepatitis C virus (HCV), and human immunodeficiency virus 1 (HIV-1) antibodies. However, since no known test method can offer complete assurance that infectious agents are absent from this material, this human blood-based product should be handled at the Biosafety Level 2 or higher as recommended for any potentially infectious human serum or blood specimen. Disposal of this material should follow guidelines as recommended for potentially infectious material.

Dithiothreitol (DTT), ascorbic acid, pteroylmonoglutamic acid (FA), and DL-homocystine (>99%) were purchased from Sigma (St. Louis, MO). The internal standard, homocystine-d_8 (DL- homocystine-3,3,3′,3′,4,4,4′,4′-d_8) (97.9% (atom fraction) deuterium at the labeled sites), was purchased from CDN Isotopes (Quebec, Canada).

5-Methyltetrahydrofolic acid calcium salt ((6*S*)-5-CH_3-H_4PteGlu-Ca), [$^{13}C_5$]5-methyltetrahydrofolic acid calcium salt ((6*S*)-5-CH_3-H_4Pte[$^{13}C_5$]Glu-Ca), [$^{13}C_5$]-pteroylmonoglutamic acid (FA), 5-formyltetrahydrofolic acid calcium salt ((6*S*)-5-CHO-H_4PteGlu-Ca), and [$^{13}C_5$]5-formyltetrahydrofolic acid calcium salt ((6*S*)-5-CHO-H_4Pte[$^{13}C_5$]Glu-Ca) were obtained from Eprova AG (Schaffhausen, Switzerland). The purity of these compounds was confirmed by UV spectrophotometry, HPLC with UV and fluorescence detection, and MS analysis. *N*-Methyl-*N*-(*tert*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) was purchased from Pierce Chemical Company (Rockford, IL). All solvents used were HPLC grade from commercial suppliers. Chemicals and solvents were used without further purification.

SRM preparation, storage, handling, and stability

The SRM material was prepared from a master pool of approximately 5 L of human serum. For the level 3 material, homocystine was dissolved in base serum to a concentration of 20 mmol L^{-1}, and used to spike the serum. For 5MT spiking, the Eprova standard was dissolved in phosphate-buffered saline, pH 7.04, to produce a 10 mmol L^{-1} monobasic sodium phosphate solution with 150 mmol L^{-1} sodium chloride. The pH was adjusted with 8 mol L^{-1} sodium hydroxide. For the low-level SRM, the serum was diluted with phosphate-buffered saline solution, pH 7.04. Based on the sample sizes used in the measurements, the SRM was prepared in 1-mL amounts in clear glass vials designed to be impervious to temperatures down to −80 °C. Consideration was given to using amber vials for storage of the SRM serum to protect the photosensitive folate from degradation, but clear vials were chosen owing to the stability of the frozen folate and the possibility of metals leaching from the amber glass. The samples were sent to NIST on dry ice, and stored at −80 °C upon receipt.

To purchasers of the SRM, the serum is shipped on dry ice, and upon receipt should be stored frozen until use. A freezer temperature of −20 °C is acceptable for storage up to one week. If a longer storage time is anticipated, the material should be stored at or below −50 °C. The SRM should not be exposed to sunlight or ultraviolet radiation. Storage of thawed material at room or refrigerator temperatures may result in changes in the analyte concentrations.

Bottles of the SRM to be analyzed should be removed from the freezer and allowed to stand at room temperature until thawed. After the material is thawed to room temperature, it should be used immediately. The material should be swirled gently to mix before aliquots are withdrawn.

Due to the recent certification of this material, stability testing results are not available at this time. NIST will continue to monitor the stability of the analytes in this material and will notify purchasers of the material of any changes in the certified concentrations.

A Certificate of Analysis summarizing the methods used and with instructions for use of the SRM is available at http://www.nist.gov/srm.

Analytical methods (NIST)

All steps were performed gravimetrically. Percentages refer to mass fractions (g g^{-1}) unless denoted otherwise. In all cases, analyte concentrations were calculated by linear interpolation from calibration curves constructed independently for each set of samples.

tHCY (GC/MS)

tHCY was extracted from SRM 1955 and prepared for analysis as described [34], with the following alterations. An HP 5890 Series II GC/MS with an electron ionization (EI) source and a 30-m 17% mole fraction phenyl methyl polysiloxane (DB-17MS, J&W Scientific, Folsom, CA) column was used for analysis of the samples. The fragments of $[M-57]^+$ were monitored at *m*/*z* 420 and *m*/*z* 424, corresponding to the loss of a butyl group from the unlabeled and labeled derivatized tHCY. For confirmation, fragments of $[M-159]^+$, derivatized tHCY minus a *tert*-butyldimethylsilyl group and a carboxylic acid group at *m*/*z* 318 and *m*/*z* 322, were monitored.

[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 is necessarily the best for the purpose.

tHCY (LC/MS and LC/MS/MS)

Extraction and measurement of tHCY in SRM 1955 were carried out as described [34] with the following alterations. Spiked serum samples were prepared as described for the GC/MS method. The standards and samples were mixed with 1.5% DTT in 0.15 mol L^{-1} NaOH, and then heated at 40 °C for 15 min to fully reduce the multiple forms of homocysteine. Solid-phase extraction (SPE) was carried out as described but the final GC/MS conjugation step was replaced by reconstituting the extract with 1.5% DTT.

Using a Waters 2795 LC, injections of the tHCY extract were made onto a Supelco Discovery HS F5 column with a pentafluorophenylpropyl stationary phase (4.6 mm×250 mm, 5-μm particle diameter) at 30±2 °C, with a Discovery HS F5 (20 mm×4.0 mm, 5-μm particle diameter) guard column. Isocratic elution of 90:10 (volume fractions) 0.1% formic acid (aq.)/0.1% formic acid in methanol at 0.5 mL min^{-1} was used from 0 min to 15 min.

For LC/MS, selected ion monitoring of the protonated molecules of tHCY and tHCY-d_4, at *m/z* 136 and 140, respectively, was carried out. For LC/MS/MS, the selected reaction monitoring mode was used with detection of the dominant fragments at *m/z* 90 and 94 after collisionally induced dissociation of the protonated precursors at *m/z* 136 and 140. Additionally, secondary fragments at *m/z* 118 and 122 were also monitored.

tHCY/ folate (unified method)

As described in Nelson et al. [30], an aliquot of SRM 1955 was spiked with the internal standard solutions to yield nominal concentrations of tHCY-d_4, [$^{13}C_5$]-5MT, and [$^{13}C_5$]-FA of 10 μmol L^{-1}, 22 nmol L^{-1}, and 22 nmol L^{-1}, respectively. SPE extraction and LC/MS/MS instrumentation and methods were as described.

Folate (LC/MS/MS)

In methods 1, 2, and 3 an aliquot of SRM 1955 spiked with the [$^{13}C_5$]-5MT and/or [$^{13}C_5$]-FA stock standard was prepared and immediately diluted 1:1 (volumetrically) with 10% ascorbic acid/1% EDTA solution followed by equilibration on ice for ≥15 min. Changes in the published methods are as follows [27].

Method 1 (SPE1—LC/MS/MS) [28] 5MT was extracted from samples using C_{18} SPE cartridges: methanol, water, and 1% ascorbic acid/1% EDTA solution were used for conditioning, 95:5 water/methanol solution was used for washing, and 1% ascorbic acid/1% formic acid in 50:50 water/methanol was used for analyte elution. LC/MS/MS analyses were performed on a Waters 2795 LC separations module coupled to a Micromass Ultima triple quadrupole with an electrospray-ionization source. LC separations were carried out using a Waters NovaPak phenylpropyl LC column (3.9 mm×150 mm, 4-μm particle diameter) with an attached NovaPak phenyl guard column (3.9 mm×20 mm, 4-μm particle diameter) maintained at 35 °C. The mobile phase elution program, using a flow rate of 0.35 mL min^{-1} was: eluent A=1% formic acid in water; eluent B=1% formic acid in methanol; time program=0 min, 70% A/30% B; 5.0 min, 70% A/30% B; 5.1 min, 0% A/100% B; 7 min, 0% A/100% B; 7.1 min, 70% A/30% B; 10 min, 70% A/30% B. LC/MS/MS detection and quantification of 5MT and [$^{13}C_5$]-5MT were conducted in selected reaction monitoring mode.

Method 2 (solid-phase affinity extraction—LC/MS/MS) [28] 5MT was extracted from SRM 1955 samples using solid phase affinity extraction (SPAE) based on the use of folate binding protein. LC/MS/MS analyses were conducted on an HP1100 LC system (Agilent Technologies) coupled to a Sciex API 4000 triple quadrupole (Applied Biosystems) operating in electrospray-ionization (ESI) mode. LC separations were carried out using an Ace C_{18} LC column (4.6 mm×150 mm, 5-μm particle diameter) (Advanced Chromatography Technologies) with an attached C_{18} guard column (1 mm×10 mm, 5-μm particle diameter) maintained at 35 °C. The mobile phase elution program was (flow rate=0.35 mL min^{-1}): eluent A=1% formic acid in water; eluent B=1% formic acid in methanol; time program=0 min, 70% A/30% B; 7.0 min, 70% A/30% B; 7.1 min, 0% A/100% B; 9 min, 0% A/100% B; 9.1 min, 70% A/30% B; 13 min, 70% A/30% B. LC/MS/MS detection and quantification of 5MT and [$^{13}C_5$]-5MT were conducted in selected reaction monitoring mode.

Method 3 (SPE2—LC/MS/MS) [28] Using a method based upon previously published procedures utilizing SPE combined with LC/MS quantitation, both 5MT and FA in the three levels of SRM 1955 were measured with the following differences. Instead of LC/MS analysis after SPE, LC/MS/MS analyses were conducted. An HP1100 Series LC system coupled to an Applied Biosystems-Sciex API 4000 triple quadrupole MS/MS system operating in ESI mode was used. LC separations were carried out using an Ace C_{18} reversed-phase analytical column (4.6 mm×150 mm, 5-μm particle diameter) (Advanced Chromatography Technologies) with an attached C_{18} guard column (1 mm×10 mm, 5-μm particle diameter) maintained at 35 °C. The mobile phase elution program was (flow rate=0.35 mL min^{-1}): eluent A=1% formic acid in water; eluent B=1% formic acid in methanol; time program=0 min, 70% A/30% B; 9.0 min, 70% A/30% B; 9.1 min, 0% A/100% B; 11 min, 0% A/100% B; 11.1 min, 70% A/30% B; 15 min, 70% A/30% B. LC/MS/MS detection and quantification of 5MT/[$^{13}C_5$]-5MT and FA/[$^{13}C_5$]-FA were conducted in selected reaction monitoring mode. Selected reaction monitoring mass transitions were *m/z* 460→*m/z* 313 for 5MT, *m/z* 465→*m/z* 313 for [$^{13}C_5$]-5MT, *m/z* 442→*m/z* 295 for FA, and *m/z* 445→*m/z* 295 for [$^{13}C_5$]-FA.

Analytical methods (CDC)

tHCY (LC/FD)

Following a previously published method [12], tHCY was measured by isocratic HPLC with fluorometric detection at 385 nm excitation and 515 nm emission after reduction of protein-bound and oxidized thiols (disulfides and mixed disulfides) to free thiol with tris(2-carboxyethyl)phosphine, protein precipitation with trichloroacetic acid, and fluorescent derivatization with ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F). The separation of the obtained thiol derivatives was performed isocratically within 7 min, with the use of an acetate mobile phase at pH 5.5. Cystamine was used as an internal standard. Quantitation was determined by peak area ratio (analyte to internal standard) and based on a three-point standard curve in a serum matrix, used after linearity of the measurement region had been validated.

tHCY (fluorescence polarization immunoassay)

tHCY was measured in SRM 1955 by the Abbott Homocysteine Assay, a fully automated fluorescence polarization immunoassay (FPIA) from Abbott Diagnostics [35, 36] that utilizes DTT for reduction of tHCY and S-adenosyl-homocysteine (SAH) hydrolase for catalytic conversion of tHCY to SAH in the presence of added adenosine. In the subsequent steps, the specific monoclonal antibody and the fluoresceinated SAH analog tracer constituted the FPIA detection system. tHCY concentrations were calculated by the Abbott AxSym using a machine-stored calibration curve. The calibration curve is calculated by the Abbott AxSym based on the measurement of tHCY in calibrants and used to calculate tHCY concentrations.

5MT, FA, 5FT (LC/MS/MS)

An aliquot of SRM 1955 was spiked with ^{13}C-labeled folates, [$^{13}C_5$]-5MT, [$^{13}C_5$]-FA, and [$^{13}C_5$]-5FT as internal standards. 5MT, FA, and 5FT were quantitatively isolated from serum using a phenyl SPE cartridge. Recovery of the folates was investigated and described as part of a previous study [5]. Detection and quantification were carried out by positive ion electrospray ionization LC/MS/MS using an isocratic mobile phase of acetic acid in organic solvent on a C_8 analytical column [5].

Total folate (microbiological assay)

Using a 96-well plate microtiter method, diluted SRM 1955 was added to an assay medium containing all of the nutrients, except FA, necessary for growth of *Lactobacillus casei* (*L. casei*, NCIB 10463). The assay medium was then inoculated with *L. casei* and the microtiter plate incubated for 42 h at 37 °C. The growth of *L. casei* is proportional to the amount of total folate present in the serum sample, the folate concentration was quantified by measuring the turbidity of the inoculated assay medium at 590 nm in a micro plate reader [19, 20]. The assay was calibrated with 5MT.

Total folate (radioassay)

Total folate and vitamin B_{12} in SRM 1955 were measured by a radio protein binding assay [21]. An aliquot of SRM 1955 was combined with the tracers ^{125}I-folate and ^{57}Co-vitamin B_{12}, then boiled to inactivate endogenous folate-binding proteins and to convert the various forms of vitamin B_{12} to cyanocobalamin. After cooling, the solution was combined with immobilized affinity-purified porcine intrinsic factor and folate-binding proteins, incubated for 1 h at room temperature, centrifuged, decanted, and the radioactivity associated with the pellet counted. Standard curves prepared by using the pre-calibrated folate/vitamin B_{12} standards in a human serum albumin base were used to determine the concentration of the folate and vitamin B_{12} in the patient serum.

Statistical analysis

Measurements of tHCY, 5MT, 5FT, and FA were made using different analytical methods at NIST and CDC and the following factors were investigated: day (three days), vial (two vials), and preparation (two sample preparations). Measurement results had a small number of missing values and outliers.

Measurements of tHCY at NIST included two different GC/MS measurements of one set of samples, three different LC/MS and LC/MS/MS measurements of one set of samples, and LC/MS/MS measurements of a third set of samples. The two GC/MS measurements were treated as one method, the LC/MS and LC/MS/MS measurements from the second set of samples were treated as one method, and the independent LC/MS/MS measurements were treated as a separate method. Measurements of 5MT included four NIST methods and one CDC method, all of which were treated equally.

Multi-factor ANOVA tests for each different analyte and concentration level revealed that the factors of day, vial, and preparation are not statistically significant (α=0.05). Based on this, the results for each analyte at a given concentration level for a given method was then treated as a sub-series. For each sub-series, the mean value and its expanded uncertainty (expansion factor always k=2) were reported on the SRM certificate. Type B errors such as the one for the purity measurements were not included in the calculation of uncertainty due to the negligible contribution from purity measurements and other sources. To determine the certified values for 5MT and the reference values for FA, further statistical analysis was performed to combine the results from each method using a Bayesian approach

proposed elsewhere [33]. In the case for 5MT, equal weights were used. The certified values for the tHCY were obtained by the Bayesian approach with weights equal to the number of repeats.

The traditional approaches to combining results from multiple methods such as the t-interval have problems [37]. The Bayesian approach used for certification measurements is based on a measurement equation approach, in which a measurand is specified as a measurable function of the true unknown state of the nature of the methods and some ancillary variable. This method works well and is ISO Expression of Uncertainty in Measurements (ISO GUM) compliant since the measurement equation approach is the one used by ISO GUM [31].

All certified and reference values are listed using both micromole per liter (μmol L^{-1}) and microgram per milliliter (μg mL^{-1}) units.

Results and discussion

SRM development

Preparation of SRM 1955 involved decisions regarding which analytes to measure, suitable matrix, analyte levels, and sample storage. Initial choices were made in a planning meeting between the CDC and NIST at which decisions were made to prepare three levels of serum with certified values for tHCY and 5MT, and to produce a liquid frozen material to avoid the additional sample manipulation of lyophilization. Since tHCY and 5MT track inversely in clinical samples the initial choice for this SRM was to have 1) a high tHCY level/low 5MT level, 2) a medium tHCY/medium 5MT level, and 3) a low tHCY/high 5MT level. Upon discussions with the proposed supplier of the serum, the difficulties of preparing samples with inverse levels of tHCY and 5MT became apparent. While possible, additional manipulation of the samples such as stripping the serum to obtain low analyte levels and spiking the serum to increase the analyte to high levels was considered unacceptable. In following the Clinical and Laboratory Standards Institute (formerly known as the National Committee for Clinical Laboratory Standards (NCCLS)) guidelines, document EP14-A2, for commutability which call for as little manipulation of samples as possible, the decision was made to have three levels of SRM 1955, one with low levels of tHCY and 5MT, the next with medium levels of both analytes, and the third with high levels of tHCY and 5MT.

Initial analytical work on the SRM began by investigating current methods of measurement of the folate and tHCY. Where possible, based on the precision and comparability to other well-validated methods, the investigated methods were modified as necessary, or new methods were developed, with the goal of having several different well-characterized methods of measurement. For example, the GC/MS method used for certification of tHCY in this SRM is based on a method by Stabler et al. [13], but the LC/MS method for tHCY measurement was developed in-house [16]. Additionally, a novel method in which tHCY, FA, and 5MT are measured in the same trial has been developed at NIST and was used for certification measurements of this SRM [30]. Additional work was done comparing mass spectrometric methods of homocysteine measurement, measuring tHCY levels in existing SRMs, and also applying the methods to a pool of patient samples [34]. Excellent precision among the GC/MS, LC/MS, and LC/MS/MS methods was reported, and the correspondence of results produced by these reference techniques (using different sample preparation methods) was documented.

Determination of values as certified, reference, or information values

Prior to statistical analysis, decisions were made as to whether measured values of tHCY, 5MT, 5FT, FA, and vitamin B_{12} would be considered as certified, reference, or information values, based on the measurement methods. For calculation of certified values, methods nominated or accepted by the Joint Committee for Traceability in Laboratory Medicine (JCTLM) as approved higher-order measurement methods were used. In the present case, isotope dilution mass spectrometry-based methods were used to establish certified values for 5MT and tHCY in the SRM (Table 1). A complete list of approved reference materials and reference measurement procedures is available at http://www.bipm.fr/en/commit tees/jc/jctlm/jctlm-db/. The isotope dilution mass-spectrometry-based methods used at NIST and CDC for measurement of tHCY and 5MT are in the process of nomination for acceptance by the JCTLM.

Methods used for determination of analytes that did not meet the criteria for certification measurements, e.g., FA, are included as reference values, and are shown in Table 2. Additional measurements of 5FT, tHCY, vitamin B_{12}, and total folate were made by the CDC and are included as method-specific values in Table 3, which may be of interest for members of the clinical community that employ such methods. Since the methods often appeared to be measuring different entities, it was deemed most appropriate to list the analyte measurements correlated to the measurement method.

Statistical analysis of tHCY and folate measurements

To determine certified values for tHCY and 5MT from measurements using multiple methods at 2 different laboratories, statistical analysis using a Bayesian approach was performed [33, 37]. The consensus mean and associated uncertainty at an approximate 95% confidence level were calculated using data from multiple methods. Calculations for different analytes were accomplished using non-weighted means or a weighted means approach, with weights based on the number of measurements. The Bayesian approach uses the consensus mean as a measurable function of the method means and some ancillary

Table 1 Certified concentrations[a] and uncertainties for tHCY and 5MT in SRM 1955

Concentration levels for tHCY determined by GC/MS, LC/MS, and LC/MS/MS		
	μmol L^{-1}	μg mL^{-1}
Level I	3.98±0.18	0.538±0.024
Level II	8.85±0.60	1.196±0.082
Level III	17.7±1.1	2.39±0.15
Concentration levels for 5MT determined by LC/MS/MS and LC/MS/MS		
	nmol L^{-1}	ng mL^{-1}
Level I	4.26±0.25	1.96±0.12
Level II	9.73±0.24	4.47±0.11
Level III	37.1±1.4	17.03±0.64

[a]Each certified value is the weighted mean from multiple methods (see text for details). A Bayesian approach [32] was used to combine the data from the multiple methods to determine the combined standard uncertainties, u_c. The expanded uncertainties, U, equal k times u_c, with k=2, and conform to ISO guidelines for the expression of uncertainty [31]

variable. The Bayesian approach estimators are ISO GUM compliant [31, 32]. The certified concentrations and associated uncertainties for tHCY and 5MT shown in Table 1 should meet the traceability needs of the clinical community.

Measurements of tHCY, 5MT, FA, 5FT, total folate, and vitamin B_{12}

After receipt of the SRM serum, measurement of the analytes began as part of the certification process. The experimental plan was as follows: two randomly chosen bottles of each level were analyzed each day for three days. Two aliquots from each bottle were analyzed. Although

Table 2 Reference concentrations[a] for FA in SRM 1955 determined by LC/MS/MS and LC/MS/MS

	nmol L^{-1}	ng mL^{-1}
Level I	0.487±0.17	0.215±0.075
Level II	1.05±0.16	0.463±0.071
Level III	1.07±0.24	0.47±0.11

[a]Each reference value is the weighted mean from multiple methods. A Bayesian approach [32] was used to combine the data from the multiple methods to determine the combined standard uncertainties, u_c. The expanded uncertainties, U, equal k times u_c, with k=2, and conform to ISO guidelines for the expression of uncertainty [31]

Table 3 Method-specific concentrations (information values) for total folate, 5FT, tHCY, and vitamin B_{12} in SRM 1955[a]

	Analyte	Method	Concentration	Units
Level 1	Total folate	LC/MS/MS	6.0±0.4	nmol L^{-1}
	Total folate	Microbiological	5.6±1.2	nmol L^{-1}
	Total folate	Radioassay	4.5±0.4	nmol L^{-1}
	5FT	LC/MS/MS	0.6±0.2	ng mL^{-1}
	tHCY	FPIA	4.2±0.3	μmol L^{-1}
	tHCY	HPLC-FD	3.5±0.3	μmol L^{-1}
	Vitamin B_{12}	Radioassay	0.16±0.01	nmol L^{-1}
Level 2	Total folate	LC/MS/MS	13±1	nmol L^{-1}
	Total folate	Microbiological	14±3	nmol L^{-1}
	Total folate	Radioassay	10±1	nmol L^{-1}
	5FT	LC/MS/MS	1.1±0.4	ng mL^{-1}
	tHCY	FPIA	8.6±0.4	μmol L^{-1}
	tHCY	HPLC-FD	8.2±0.6	μmol L^{-1}
	Vitamin B_{12}	Radioassay	0.36±0.05	nmol L^{-1}
Level 3	Total folate	LC/MS/MS	41±2	nmol L^{-1}
	Total folate	Microbiological	44±11	nmol L^{-1}
	Total folate	Radioassay	25±3	nmol L^{-1}
	5FT	LC/MS/MS	1.7±0.6	ng mL^{-1}
	tHCY	FPIA	17±1	μmol L^{-1}
	tHCY	HPLC-FD	17±1	μmol L^{-1}
	Vitamin B_{12}	Radioassay	0.35±0.05	nmol L^{-1}

[a]Uncertainties are ±2 sd

initial plans had specified measurement of only tHCY and 5MT, subsequent development of methods for determination of additional analytes resulted in the ability to measure the less abundant folate forms, FA and 5FT. The CDC also made measurements of total folate and vitamin B_{12}. As shown in Tables 1, 2, and 3, tHCY, 5MT, FA, 5FT, total folate, and vitamin B_{12} were measured using a variety of methods, and the values designated as certified, reference, or information, based on the measurement methods used and the uncertainty of their analysis [31].

tHCY measurements at NIST

Measurement of tHCY at NIST employed isotope dilution mass spectrometry methods, including GC/MS, LC/MS, and LC/MS/MS, with the use of HCY-d_4 as the internal standard (reduced from homocystine-d_8). HCY was measured as tHCY which includes reduced forms of HCY from homocystine, cysteine–homocysteine, and HCY bound to cysteine in protein such as albumin.

In the GC/MS analysis two different ion channels were monitored, *m*/*z* 420/424 (corresponding to the tHCY/tHCY-d_4) (method 1) and *m*/*z* 318/322 (method 2). As shown in Fig. 2a in which *m*/*z* 420/424 were monitored, baseline separation of tHCY from all other sample components is evident. tHCY concentrations determined with method 2 were slightly higher than those determined with method 1 (Fig. 3); however both sets of values were within the expanded uncertainty at all levels. The LC/MS/MS method monitored two fragments of protonated tHCY, the dominant fragment at *m*/*z* 90 (Fig. 2b) and a less prevalent fragment at *m*/*z* 118. As expected, the dominant transition of 136→90 (method 3) resulted in more precise values than the less prevalent transition of 136→118 (method 4) (Fig. 3). tHCY values determined with method 4 were typically greater than those determined by method 3. Measurement of tHCY by LC/MS resulted in an increased baseline as compared to LC/MS/MS due to the decreased selectivity of the method; however, tHCY is clearly separated from other possible interferences in the sample (Fig. 3b). For all three SRM levels, the majority of tHCY concentrations determined using LC/MS (method 5) were within the expanded uncertainty range (Fig. 3).

An additional method employing LC/MS/MS, developed at NIST, was used for simultaneous detection and measurement of tHCY, 5MT and FA (Fig. 2d), referred to as the unified method [30]. The method used for detection of tHCY was similar to the single analyte methods in that SPE was used followed by separation and detection by LC/MS/MS. In addition to the fact that the unified method was able to extract and detect tHCY, 5MT, and FA in one trial, differences between the single analyte method and the unified method include the use of a different SPE chemistry, a different chromatography column chemistry, and the monitoring of a single transition at *m*/*z* 136→90. tHCY measurements obtained with the unified method (method 6) compare favorably to those methods in which only tHCY was extracted and detected (Fig. 3).

All of the measurements of tHCY made at NIST were used in the calculation of certified levels in the SRM. Overall, as seen in a previous study [34], measurement of tHCY using mass-spectrometry-based methods resulted in similar values. The chromatograms shown in Fig. 2 of homocysteine in SRM 1955 Level 1 illustrate the selectivity and sensitivity of mass spectrometry, as well as the effectiveness of the SPE cleanup.

tHCY measurements at CDC

The CDC determined tHCY concentrations in SRM 1955 with methods frequently used in the clinical setting, namely, FPIA [35, 36] and HPLC-FD [12]. Both methods involved initial reduction of the tHCY followed by protein precipitation and fluorescence derivatization in the FPIA method, and conversion of tHCY to a species extractable by a specific monoclonal antibody in the HPLC-FD method. The measurements of tHCY by FPIA and HPLC-FD were not included in the calculations of the

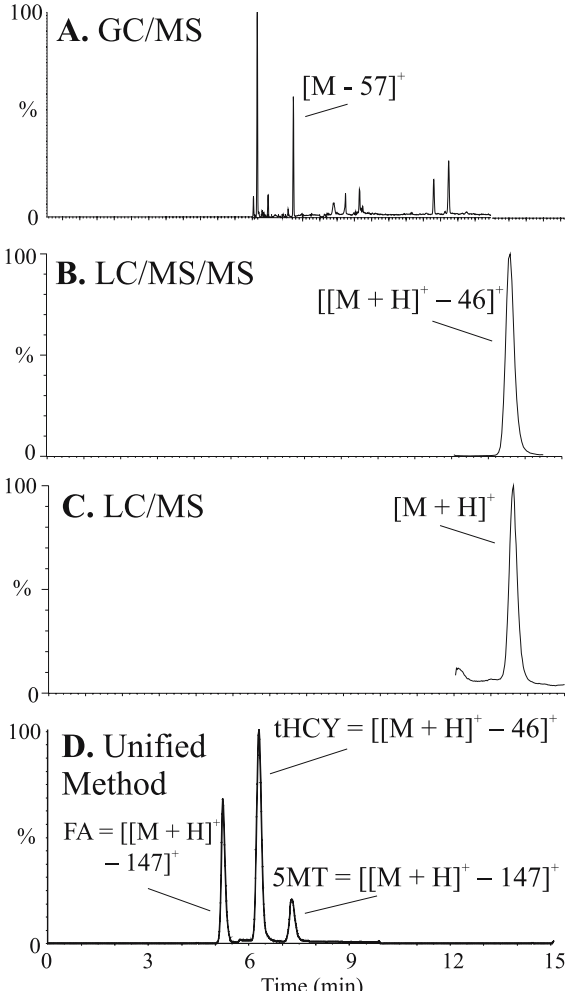

Fig. 2 Chromatograms of tHCY in SRM 1955 level 1 with extraction by SPAE and detection by **a** GC/MS, **b** LC/MS, **c** LC/MS/MS, and **d** LC/MS/MS (unified method of detection of tHCY, 5MT, and FA)

certified values, but were included as information values and are shown in Table 3. The information values shown in Table 3 are very similar to the certified concentrations shown in Table 1 and should be especially useful for clinical and research laboratories using these or similar methods; they also illustrate the commutability of this SRM for routine measurements.

Folate measurements at NIST

For measurements of 5MT and/or FA at NIST, four variations of LC/MS/MS-based methodology were used, all of which utilized $^{13}C_5$-5MT and/or $^{13}C_5$-FA as the internal standard. In the first method, 5MT was extracted from serum samples using C_{18} SPE followed by LC separation using a phenylpropyl LC column. Method 2 differed from Method 1 in that a solid-phase affinity extraction column based on folate binding protein was used for extraction of the 5MT, and a C_{18} LC column was used prior to MS/MS detection. The third method was used to detect both 5MT and FA using C_{18} SPE followed by use of a C_{18} LC column. The fourth method used at NIST for determination of 5MT and FA in SRM 1955 was the unified method, in which C_{18} SPE was used followed by separation using a cyano LC column prior to tandem MS detection [30]. Chromatograms of the separation of 5MT from the SRM serum are shown in Fig. 4. Measurements of 5MT from the four LC/MS/MS methods at NIST and one LC/MS/MS method at the CDC were used for assigning the certified 5MT values, as shown in Table 1. Comparison of the different methods used for measurement of 5MT is shown in Fig. 5 for each level. Overall, the 5MT measurements were not as precise as the tHCY measurements with several outliers evident in method 2 and 3. The

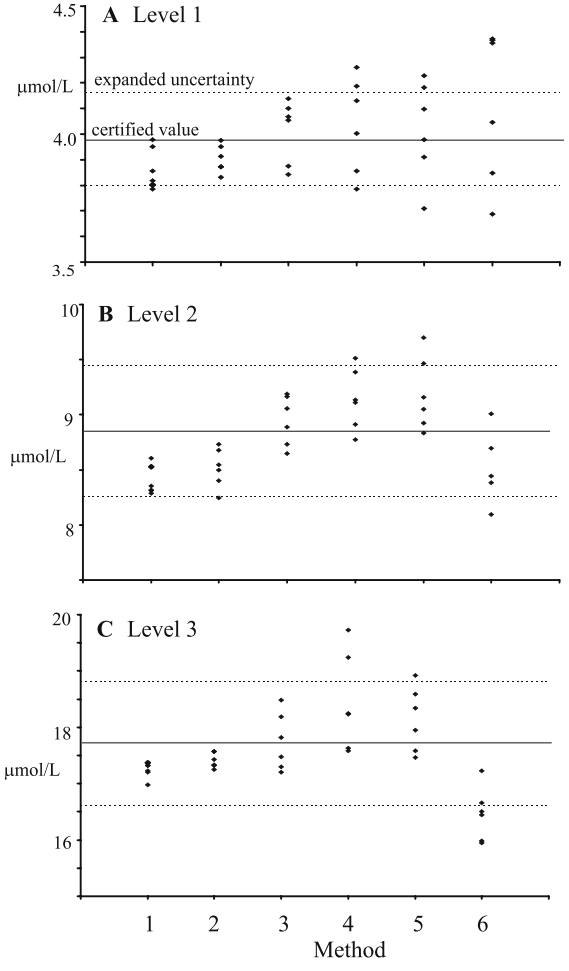

Fig. 3 Individual measurements of tHCY in SRM 1955 **a** level 1, **b** level 2, and **c** level 3. Method 1=GC/MS *m/z* 420; method 2=GC/MS *m/z* 318; method 3=LC/MS/MS 136→90; method 4=LC/MS/MS 136→118; method 5=LC/MS *m/z* 136; method 6=LC/MS/MS 136→90 (unified method)

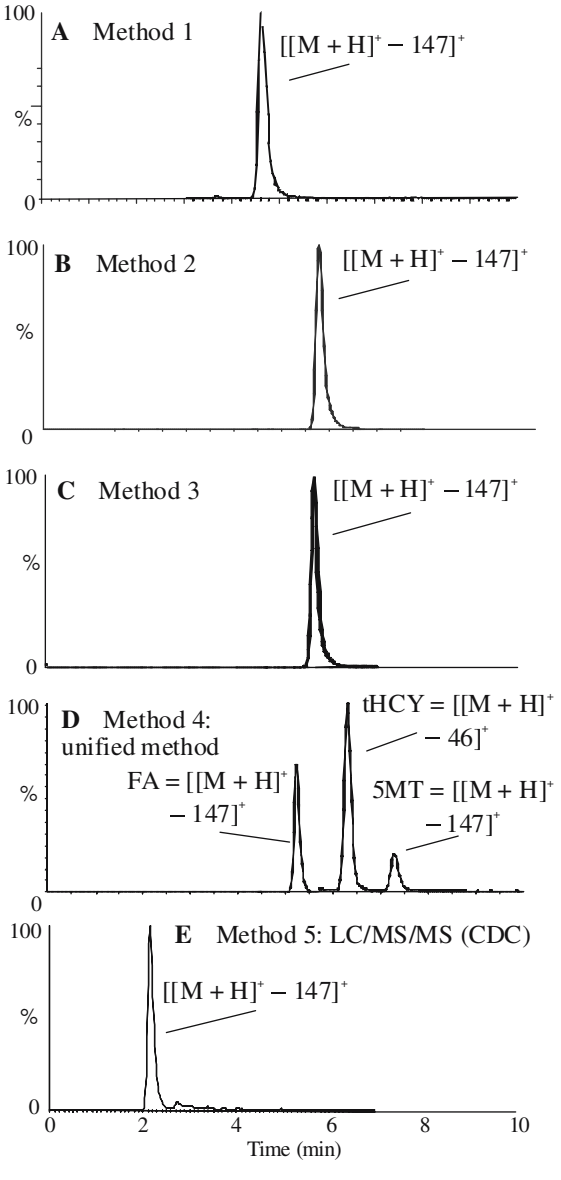

Fig. 4 Chromatograms of 5MT in SRM 1955 **a** method 1: LC/MS/MS (C_{18}, SPE, phenyl LC column; NIST), **b** method 2: LC/MS/MS (SPAE, C_{18} LC column; NIST), **c** method 3: LC/MS/MS (C_{18} SPE, C_{18} LC column; NIST), **d** method 4: LC/MS/MS (unified method; NIST), and **e** method 5: LC/MS/MS (CDC)

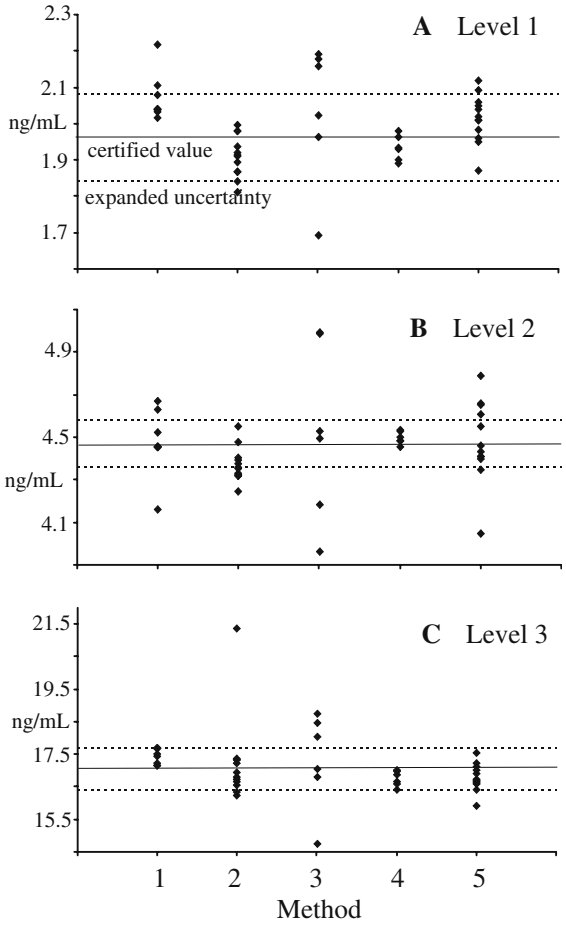

Fig. 5 Individual measurements of 5MT in SRM 1955 **a** level 1, **b** level 2, **c** level 3. Method 1=LC/MS/MS (C_{18}, SPE, phenyl LC column; NIST); method 2=LC/MS/MS (SPAE, C_{18} LC column; NIST); method 3=LC/MS/MS (C_{18} SPE, C_{18} LC column; NIST); method 4=LC/MS/MS (unified method; NIST); method 5=LC/MS/MS (CDC)

NIST unified method in which tHCY, 5MT, and FA were measured in the same trial showed the best fit within the expanded uncertainties for the certified range.

FA was also measured at NIST. However, in part due to the low levels of this analyte, the values did not meet the criteria for certification [31], so are included as reference values (Table 3).

Folate measurements at CDC

Three different methods of measurement of folate were used by CDC, namely, isotope dilution LC/MS/MS, microbiologic assay, and radioassay. In the isotope dilution LC/MS/MS method, 5MT, 5FT, and FA were extracted using phenyl SPE followed by LC separation on a C_8 column prior to detection. The 5MT values were included in the calculations of the certified values and comparison of the 5MT values determined by the CDC to NIST measurements is shown in Fig. 5. Total folate as calculated from the sum of 5MT, FA, and 5FT by LC/MS/MS corresponded well with total folate measured by microbiologic assay, but was higher than total folate measured by radioassay, especially for SRM levels 2 and 3 (Table 3). Interestingly, total folate measured by radioassay in SRM levels 1 and 2 corresponded well with certified 5MT concentrations. These information values for folate as well as the information value for vitamin B_{12} (Table 3) should be especially useful for clinical and research laboratories using these or similar methods.

Conclusion

The growing recognition of the importance of tHCY and 5MT as markers for a variety of health conditions has led to a closer examination of the clinical assays used to measure these analytes, which in turn has resulted in the call for a matrix-based SRM. NIST and the CDC have collaborated on production of this frozen serum SRM with certified levels of tHCY and 5MT, determined with measurements made using higher-order reference methods based on isotope dilution mass spectrometry. Other measurements using more common clinical methods have provided reference and information values for these analytes as well as for FA, 5FT, total folate, and vitamin B_{12}. Use of the SRM should improve interlaboratory and intermethod agreement.

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