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RESEARCH PAPER

Development of a matrix-based candidate reference material of total homocysteine in human serum

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Abstract We developed and evaluated a candidate serum reference material to help improve clinical routine measurement, and to provide traceability of the measurement results. D_8-Homocystine, dithiothreitol, and acetonitrile were used as an internal standard, the reducing agent, and the protein precipitating agent, respectively. A triple quadrupole mass spectrometer with an electrospray ionization source was used for monitoring the transitions (m/z 140.0 → 94.0, 136.0 → 90.0) in multiple-reaction-monitoring mode. We used a calibration model relying on bracketing and gravimetric measurements to give SI-traceability and higher accuracy to serum value assignments. The method was evaluated for accuracy using NIST Standard Reference Material SRM1955. The results of the three concentrations (1, 2, and 3) of total homocysteine in human serum samples were determined by an isotope-dilution liquid chromatography-tandem mass spectrometry method; tHcy 1 is 28.8 ± 1.1 μmol/L, tHcy 2 is 17.93 ± 0.57 μmol/L, and tHcy 3 is 14.38 ± 0.46 μmol/L.

Keywords Homocysteine · Human serum · Reference material · Isotope dilution mass spectrometry

Introduction

Homocysteine (Hcy) is a homologue of the amino acid cysteine, differing by an additional methylene bridge (-CH_2-). It is biosynthesized from methionine, an essential amino acid. Hcy is regarded as an independent risk factor for cardiovascular disease. Increased levels of Hcy in plasma are strongly correlated with coronary artery disease [1–4], cerebrovascular disease [5, 6], and venous thrombosis [7, 8].

Various methods have been developed to determine the concentration of total homocysteine (tHcy) in serum or plasma. Radioimmunoassay (RDA), fluorescence polarization immunoassay (FPIA), enzyme-linked immunosorbent assay (ELISA), enzymatic cycling assay (ECA), and liquid chromatography with either fluorescence detection (LC-FD) or electrochemical detection (LC-ED) are more traditional approaches and are widely used in routine clinical measurements [9–11]. However, the results obtained with different methods are often not comparable because of inter-method and inter-laboratory variability. Thus, establishing reference methods for value-assigning certified reference materials (CRMs) and external quality assessment is the best way to help verifying standardization effectiveness.

Most recently, isotope-dilution gas chromatography coupled with mass spectrometry (ID/GC/MS) and isotope-dilution liquid chromatography–tandem mass spectrometry (ID/LC/MS/MS) have been effectively used to measure plasma tHcy because of their specificity and accuracy [12–17]. There are currently three Reference Measurement Procedures for homocysteine in serum listed in the JCTLM database (GC/MS [14], LC/MS and LC/MS/MS [13] from NIST). The increased accuracy, provided by the use of stable-isotope-labeled internal standards, allows ID/MS to be an effective quantitative approach for the accurate determination of tHcy via minimizing the matrix effects in serum or plasma.

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Table 1 The main MS parameters for homocysteine determination

Compound	Parent ion (*m*/*z*)	Product ion (*m*/*z*)	Fragmentor (*V*)	Collision energy (*V*)
Hcy	136.0	90.0	80	7
[$^2H_4$]-Hcy	140.0	94.0	80	7

Experimental

Reagents and materials

The candidate reference material (RM) was prepared by Beijing Aerospace General Hospital (Beijing, China). Each donor unit of serum used in the preparation of this product was fresh and tested by the colloidal gold 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 Biosafety Level 2 or higher, as recommended for any potentially infectious human serum or blood specimen [18].

DL-Homocystine was obtained from Sigma Chemical Company (St. Louis, MO, USA), and the purity, determined by NMR, was calculated as 98.8%, the internal standard was acesulfame potassium (NIM CRM GBW(E)100065, the purity is 99.7%, and the uncertainty is 0.6%). DL-[$^2H_8$]-Homocystine was obtained from C.D.N. Isotopes (Pointe-Claire, Quebec, Canada). DL-[$^2H_8$]-Homocystine contains eight stable ^2H isotopes (3, 3, 3′, 3′, 4, 4, 4′, 4′-d_8) incorporated into its methylene groups, and the supplier's listed purity was >99.5 atom-% deuterium. Dithiothreitol (DTT, purity ≥ 99%) was obtained from INALCO SPA Company, Milano, Italy.

NIST serum Standard Reference Material (SRM 1955: Homocysteine and Folate in Frozen Human Serum) was used to evaluate our method. Purified water (18.2 MΩ), prepared using a Millipore Milli-Q purification system, was used to prepare all samples and standards. All other solvents were HPLC grade, and chemicals were reagent grade unless stated otherwise.

Preparation of candidate material

The candidate serum material was prepared from 1000 patients in Beijing Aerospace General Hospital. According to the concentration of Hcy determined by a routine method, the 1000 serum samples of approximately 1 L were divided into three concentration levels. The volume of each pool was approximately 300 mL after abandoning the infected samples. Each pool was mixed magnetically stirring at room temperature (20–25 °C) for 30 min and then sub-packed in amber vials and stored at −80 °C.

Homogeneity study

The homogeneity study was delegated to the Beijing Aerospace General Hospital Reference Laboratory. Seventeen samples at each concentration level were selected in a stratified random manner and measured by enzymatic cycling assay (S-adenosine homocysteine hydrolase based on small molecule capture technology) in a Hitachi 7170A automatic analyzer. The injection volume was 18 μL, the linear range was 0–50 μmol/L, and each sample was analyzed in triplicate. Statistical tests for homogeneity of the standard materials were performed by means of variance analysis according to ISO Guide 35: 2006.

Stability study

The stability of reference materials is defined as the property of the standard substance under a specified time interval and set of environmental conditions. Serum tHcy standard material was stored at −80 °C under the protection of dry ice during transport. The long-term stability test was performed using the isotope dilution mass spectrometry method. The sample preservation temperature was −80 °C and all experiments were completed at room temperature in 2 h. The short-term stability test was completed by ECA in the Beijing Aerospace General Hospital Reference Laboratory. We tested the stability of the candidate RM stored under the following different conditions: three freezing and thawing cycles at −20 °C, storage at −20 °C for 30 days, storage at 4 °C for 7 days, and storage at room temperature for 3 days. For each concentration level, the three vials were selected in a stratified random manner at regular

Table 2 Total homocysteine concentration 1

Source of difference	SS	df	MS	*F*	*P* value	*F* crit
Between groups	0.373851	16	0.023366	0.671503	0.800532	1.951566
Within groups	1.183067	34	0.034796			
Total	1.556918	50				

No significant difference was found among the aforementioned level of Hcy

Table 3 Total homocysteine concentration 2

Source of difference	SS	df	MS	*F*	*P* value	*F* crit
Between groups	0.458931	15	0.030595	1.03662	0.446609	1.99199
Within groups	0.944467	32	0.029515			
Total	1.403398	47				

No significant difference was found among the aforementioned level of Hcy

intervals and the average value was taken. Statistical tests for stability of the standard materials were performed according to ISO Guide 35: 2006.

Commutability study

Commutability with native clinical samples is a property that exists for a reference or trueness control material among different measurement procedures [19]. In this study, we chose the GCell kit (Beijing Strong Biotechnologies, Inc) coupled with a Hitachi 7170A automatic analyzer system used in Beijing Aerospace General Hospital Reference Laboratory. The principle of GCell kit is enzymatic cycling assay (ECA). Twenty fresh human serum samples covering the three concentrations of candidate reference material were selected as the object of investigation, and the commutability of this candidate RM was preliminarily evaluated in this conventional analysis system according to CLSI EP30-A [19].

Calibration preparation

Homocysteine (Hcy) and deuterated homocysteine ([$^2H_4$]-Hcy) stock solutions (~10 μg/g) were prepared by chemically reducing the gravimetrically prepared homocystine and deuterated homocystine ([$^2H_8$]-homocystine) stock solutions (~1000 μg/g), respectively. Briefly, dried Hcy or deuterated homocystine powder (~3 mg) was weighed into a glass vial containing a known amount (~3 g) of 0.1 mol/L HCl. The homocystine stock solution was then neutralized (pH ≈ 7) by the addition of a small portion (30 μL) of 10 mol/L NaOH. The aforementioned stock solutions were then reduced and diluted with 1.5% DTT. The final concentration of the Hcy stock solution was calculated on the basis of the relationship that 1 mole of homocystine is chemically reduced to 2 moles of Hcy by the action of DTT.

We prepared four calibration solutions by adding a series of different volumes of Hcy stock solutions to a constant volume of [$^2H_4$]-Hcy stock solution to achieve unlabeled-to-labeled Hcy mass ratios of 0.5, 0.8, 1.2, and 1.5.

Sample pre-treatment [13]

As with the preparation of the calibration solutions, all steps were performed gravimetrically. Serum samples were prepared by measuring 150 μL of serum and spiking it with sufficient [$^2H_4$]-Hcy stock solution to produce a final ratio of approximately 1:1 unlabeled-to-labeled Hcy. We calculated the amount of internal standard solution needed for this step from the approximate concentration of Hcy in the sample, as determined by a routine method. After 30 μL of 5% DTT was added, the solution was thoroughly mixed on a vortex mixer and allowed to stand at room temperature for at least 15 min. To precipitate the protein, 600 μL of 0.1% formic acid plus 0.05% trifluoroacetic acid in acetonitrile was added to the sample, and the solution was vortex-mixed for 2 min. After standing for another 15 min and being vortex-mixed again, the sample was centrifuged at 13,000*g* for 2 min, and the supernatant was filtrated through a 0.22-μm organic diameter filter for analysis.

At the same time, we treated SRM 1955 using the same procedures to complete the method validation.

LC-MS/MS conditions

We performed the analyses on an Agilent 6410 Triple Quad mass spectrometer equipped with an Agilent 1200 LC system. Samples were analyzed using a Supelcosil LC-CN analytical column (4.6 mm × 250 mm, 5 μm particle size) at a temperature of 30 °C and were eluted at 0.5 mL/min using a mobile phase of 10:90 acetonitrile/water with 0.1% formic acid (isocratic elution). The autosampler temperature was 4 °C and the injection volume was 10 μL. MS parameters were optimized by infusing the Hcy and [$^2H_4$]-Hcy stock solution. Full scan, selected-ion-monitoring (SIM), and multiple-

Table 4 Total homocysteine concentration 3

Source of difference	SS	df	MS	*F*	*P* value	*F* crit
Between groups	0.386008	16	0.024125	0.598618	0.862642	1.951566
Within groups	1.370267	34	0.040302			
Total	1.756275	50				

No significant difference was found among the aforementioned level of Hcy

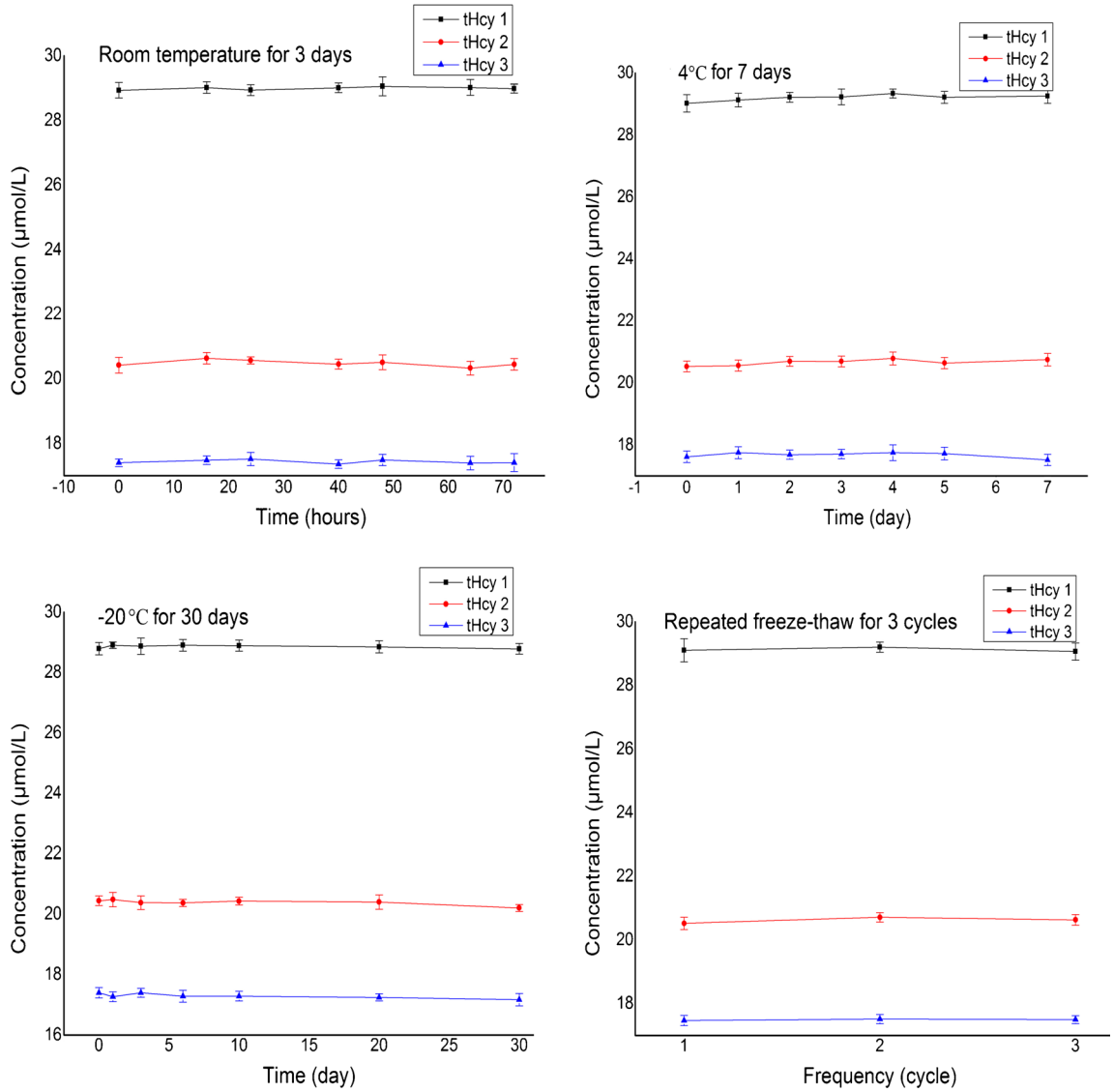

Fig. 1 Candidate serum RM stored under different conditions

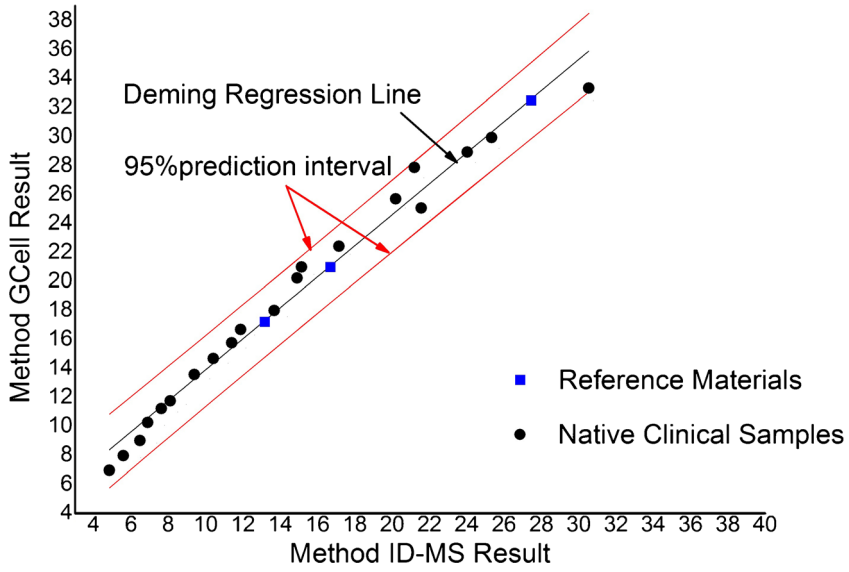

Fig. 2 Result of evaluating commutability between methods ID-MS and conventional analysis system

Table 5 Certified concentrations determined by LC-ID/MS/MS

Concentration level	Vial	Concentration (μmol/L)				Intra-RSD (%)	Overall average	Inter-RSD (%)
		1	2	3	Average			
tHcy 1	1	28.64	28.66	28.70	28.67	0.11	28.75	0. 44
	2	28.49	28.71	27.88	28.69	0.64		
	3	28.94	28.89	28.85	28.89	0.17		
tHcy 2	1	18.09	18.07	17.88	18.02	0.64	17.93	0.81
	2	18.11	18.03	17.91	18.02	0.54		
	3	17.80	17.63	17.86	17.76	0.66		
tHcy 3	1	14.47	14.45	14.46	14.46	0.07	14.38	0.58
	2	14.34	14.40	14.39	14.38	0.22		
	3	14.15	14.30	14.42	14.29	0.96		

reaction-monitoring (MRM) mode mass spectra of Hcy and [$^2H_4$]-Hcy were obtained and optimized via positive-ion ESI (Table 1).

Data analysis

We used Agilent MassHunter Workstation Software Qualitative Analysis (B.03.01) to integrate selected peaks. Quantification was performed with the peak area count ratio of the Hcy quantification ion over the [$^2H_4$]-Hcy quantification ion. We used the mean area count ratios from the three injections for data analysis. We plotted the area count ratios over the mass ratios of the calibration solutions and applied a linear regression model. The mass ratio of the sample was determined by applying the calibration curve to the area count ratio of the sample. We calculated the mass of Hcy in the sample with the mass ratio and the mass of [$^2H_4$]-Hcy added to the sample. The mass concentration of Hcy in the serum sample was determined from the mass of the serum analyzed.

Results and discussion

Homogeneity study

The single-factor variance analysis results are shown in Tables 2, 3, and 4.

Stability study

Because of the recent certification of this material, the three concentrations of total Hcy show good stability at −80 °C after 12 months. NIM will continue to monitor the long-term stability of the analyte in this material.

The short-term stability testing results are shown in Fig. 1.

In conclusion, a freezer temperature of −20 °C is acceptable for storage for up to 1 month or even longer, but continued monitoring is needed to determine the exact time limit. Storage of thawed material at room temperature for 3 days, 4 °C for 7 days, and repeated freeze-thaw for three cycles did not induce any significant changes in the analyte concentrations.

This candidate RM exhibits very good stability under different storage conditions; however, we still recommend using it under specified working conditions (room temperature or 4 °C placed in an airtight container within 1 day) to avoid the occurrence of unknown problems. If a longer storage time is anticipated, the material should be stored at −80 °C.

Commutability study

The result of the previously discussed commutability study indicates that the candidate RMs are commutable in the pairwise comparison involving IDMS and the GCell method using the prediction interval approach (Fig. 2).

Table 6 The determination results for SRM 1955, as obtained using the LC-ID/MS/MS method

	Reference concentrations (μmol/L)	Determined results (μmol/L)	Relative deviation
Level I	3.98 ± 0.18	3.95	−0.75%
Level II	8.85 ± 0.60	8.86	0.11%
Level III	17.7 ± 1.1	17.5	−1.13%

The results are all in agreement with the certified value in SRM 1955

Table 7 The combined uncertainty of the candidate RM of tHcy in human serum

RM	Value (μmol/L)	u_{char}	u_{bb}	u_{lts}	u_c	k	U
tHcy 1	28.8	0.438	0.053	0.323	0.547	2	1.1
tHcy 2	17.93	0.275	0.019	0.054	0.281	2	0.57
tHcy 3	14.38	0.220	0.057	0.018	0.228	2	0.46

Value assignment and the uncertainty evaluation

At the same time, NIST serum Standard Reference Material (SRM 1955: Homocysteine and Folate in Frozen Human Serum) was used to evaluate our method (Tables 5 and 6).

The combined standard uncertainty (u_c) of the tHcy concentration for the candidate RM was estimated by combining the contributions of the value assignment (u_{char}), the inhomogeneity of the material (u_{bb}), the uncertainty due to long-term instability (u_{lts}), and the uncertainty due to short-term instability (u_{sts}). The u_c was calculation of using the following equation:

$$u_c{}^2 = u_{char}{}^2 + u_{bb}{}^2 + u_{lts}{}^2 + u_{sts}{}^2$$

Because the transportation conditions (dry ice transport) are consistent with the long-term preservation conditions, we did not consider the uncertainty caused by transport instability. Under the guidance of ISO Guide 35: 2006, we performed T test on each slope of the four storage conditions in the short-term stability test, all t values are less than $t_{\alpha,\ \nu}$, indicating there is no significant change; thus, the u_{sts} can be ignored (Table 7).

From these values, the certified value of the candidate RM was determined as shown in Table 8.

Conclusion

The determination of total Hcy in human serum cannot only be used as diagnostic criterion for patients with cardio cerebral vascular disease but also is important for prognosis judgment. In the present study, we reported the development of a matrix-based candidate reference material of tHcy in human serum, the value of which was assigned by higher-order reference methods based on isotope dilution mass spectrometry, and the uncertainty was fully evaluated. The homogeneity and stability under different storage conditions were tested to provide users with more information. NIST has developed SRM 1955 (homocysteine and folate in human serum) to improve inter-laboratory and inter-method agreement [16], it ranges from 3.98 to 17.7 μmol/L, and this candidate RM can be complementary at the high concentration level. We also assessed the commutability on one routine clinical measurement method and intended to investigate with other more assays.

Table 8 Certified concentrations and uncertainties for tHCY in the candidate RM

RM	μmol/L
tHcy 1	28.8 ± 1.1
tHcy 2	17.93 ± 0.57
tHcy 3	14.38 ± 0.46

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Compliance with ethical standards All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committee of Beijing Aerospace General Hospital and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent was obtained from all individual participants included in the study.

This article does not contain any studies with animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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