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Method for the Determination of Total Homocysteine in Plasma and Urine by Stable Isotope Dilution and Electrospray Tandem Mass Spectrometry

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Background: **Total homocysteine (tHcy) has emerged as an important independent risk factor for cardiovascular disease. Analytical methods are needed to accommodate the high testing volumes for tHcy and provide rapid turnaround.**

Methods: **We developed liquid chromatography electrospray tandem mass spectrometry (LC-MS/MS) method based on the analysis of 100 μL of either plasma or urine with homocystine-d_8 (2 nmol) added as internal standard. After sample reduction and deproteinization, the analysis was performed in the multiple reaction monitoring mode in which tHcy and Hcy-d_4 were detected through the transition from the precursor to the product ion (*m/z* 136 to *m/z* 90 and *m/z* 140 to *m/z* 94, respectively). The retention time of tHcy and Hcy-d_4 was 1.5 min in a 2.5-min analysis.**

Results: **Daily calibrations between 2.5 and 60 μmol/L exhibited consistent linearity and reproducibility. At a plasma concentration of 0.8 μmol/L, the signal-to-noise ratio for tHcy was 17:1. The regression equation for the comparison between our previous HPLC method (y) and the LC-MS/MS method (x) was $y = 1.097x - 1.377$ ($r = 0.975$; $S_{y|x} = 1.595$ μmol/L; n = 367), and for comparison between a fluorescence polarization immunoassay (Abbott IMx; y) and LC-MS/MS (x) was $y = 1.039x + 0.025$ ($r = 0.969$; $S_{y|x} = 1.146$ μmol/L; n = 367). Inter- and intraassay CVs were 2.9–5.9% and 3.6–5.3%, respectively, at mean concentrations of 3.9, 22.7, and 52.8 μmol/L. Mean recovery of tHcy was 94.2% (20 μmol/L) and 97.8% (50 μmol/L).**

Conclusions: **The sensitivity and specificity of tandem mass spectrometry are well suited to perform high-volume analysis of tHcy. Reagents are inexpensive and sample preparation of a batch of 40 specimens is completed in less than 1 h and is amenable to automation.**

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The amino acid homocysteine (Hcy)[3] represents the main biochemical marker of several inborn errors of transsulfuration *(1)*. In addition to the diagnosis of these important but relatively rare disorders, the clinical significance of Hcy has expanded enormously in recent years after its recognition as an independent predictor of cardiovascular disease (atherosclerosis, heart disease, and thromboembolism) *(2)*, as an indicator of nutritional cofactor deficiency (folate and cobalamin) *(3, 4)*, and as a contributing factor in the pathogenesis of neural tube defects *(5)*. In plasma, total Hcy (tHcy) is the sum of free and protein-bound Hcy, homocystine, and several other mixed disulfides *(6)*. Although the mechanisms underlying the toxicity of tHcy have not been fully elucidated, its role in the pathogenesis of vascular occlusion is supported by an increasing number of epidemiological studies in at risk groups *(7)*. In addition to genetic traits *(5)*, additional factors influencing circulating tHcy concentrations include age and gender, renal function, nutrition, disease states, and use of selected medications *(6)*.

The increased demand in clinical practice for measuring plasma tHcy raises the issue of developing new methods better suited to accommodate high testing volumes and faster turnaround time. Before the development of this method, tHcy was measured in our laboratory with an automated sample processor for sequential prepara-

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[3] Nonstandard abbreviations: Hcy, homocysteine; tHcy, total Hcy; FPIA, fluorescence polarization immunoassay; ESI, electrospray ionization; LC-MS/MS, liquid chromatography-tandem mass spectrometry; and MRM, multiple reaction monitoring.

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tion of plasma/serum and urine specimens. The methodology involves reduction of disulfides by sodium borohydride and dithioerythritol, derivatization of reduced sulfhydryls with monobromobimane, HPLC separation, and fluorometric detection *(8, 9)*. This method is fully automated and relatively robust, but it requires the frequent analysis of quality-control samples to compensate for the lack of an internal standard and entails the use of five separate systems to meet the turnaround time needs of a high-volume clinical laboratory (150–200 samples/day). Although a fluorescence polarization immunoassay (FPIA) for plasma tHcy has been developed *(10, 11)* and is now widely used in clinical laboratories, we have sought the development of an alternative method for the determination of tHcy that takes advantage of the analytical versatility, specificity, and sensitivity unique to the combination of stable isotope dilution and electrospray ionization (ESI) tandem mass spectrometry (MS/MS) *(12)*.

Materials and Methods

MATERIALS

D,L-Hcy and D,L-homocystine-3,3,3′,3′,4,4,4′,4′-d_8 were purchased from Sigma and Cambridge Isotope Laboratories, respectively. Working solutions of Hcy (200 μmol/L) and homocystine-d_8 (100 μmol/L) were prepared in 500 mL/L acetonitrile-500 mL/L water with 1.0 mL/L formic acid. D,L-Dithiothreitol was purchased from Sigma. Bovine calf serum for recovery, precision, and stability studies was purchased from Pel Freez Biologicals. All other chemicals and solvents were of the highest purity available from commercial sources and used without further purification.

SAMPLE PREPARATION

A serum or plasma sample (100 μL) was mixed with 20 μL of internal standard solution (2 nmol homocystine-d_8). When reduced, homocystine-d_8 yields homocysteine-d_4 (Hcy-d_4) at double the initial concentration. Complete reduction of disulfides was accomplished by the addition of 20 μL of 500 mmol/L dithiothreitol, which was allowed to react at room temperature for 15 min. Proteins were precipitated by the addition of 200 μL of 1 mL/L formic acid and 0.5 mL/L trifluoroacetic acid in acetonitrile. After 1 min centrifugation at 13 400*g*, 100 μL of the clear supernatant was transferred to an autosampler vial. The preparation for urine differed from that for plasma/serum only in the adjustment of urine pH to >7.0 with 1 mg/L NaOH before disulfide reduction and deproteinization. Calibrators were prepared in plasma of known tHcy concentration (typically <5 μmol/L) by the addition of a 200 μmol/L working solution corresponding to tHcy concentrations of 0, 2.5, 5, 10, 20, 30, 40, 50, and 60 μmol/L. The endogenous concentration was derived from the blank specimen and subtracted from the calibration points after visual verification of results.

METHODS

A bench top triple quadrupole mass spectrometer API 2000 (Perkin-Elmer Sciex) operated in ion evaporation mode with the TurboIonSpray ionization probe source (operated at 5800 V) was used. Peripherals included a Perkin-Elmer Series 200 pump and an autosampler. To enhance the stability of the signal, separation of tHcy and Hcy-d_4 from the bulk of the specimen matrix was achieved by use of a short column (LC-CN, 3 cm × 4.6 mm; Supelco). Autosampler injections of 1 μL (corresponding to 0.3 μL of the original sample) were made using a mobile phase composed of acetonitrile in 1 mL/L aqueous formic acid (600 mL/L acetonitrile-400 mL/L aqueous formic acid) at a flow rate of 1.0 mL/min. The column was directly connected to the TurboIonSpray ionization probe operating with the turbo gas on (6 L/min; sensor temperature, 250 °C) with the LC column effluent flow-splitting set at 1:5. The retention times of tHcy and Hcy-d_4 were between 1.0 and 1.5 min in a 2.5-min chromatographic analysis. Total instrument acquisition cycle time was 3 min per sample.

All results were generated in positive ion mode with the orifice voltage set at 36 V, automatically optimized using the protonated Hcy ion. For all MS/MS experiments, mass calibration and resolution adjustments (at 0.7 amu full width at half height) on both the resolving quadrupoles were automatically optimized using a poly(propylene)glycol 1×10^{-4} mol/L solution introduced via the built-in infusion pump on the API 2000.

Collisionally activated decomposition MS/MS was performed through the closed-design Q_2 collision cell, operating with nitrogen at 0.06 kPa as collision gas. The 17 eV (lab frame) collision energy was adjusted automatically by the AutoTune algorithm.

MS/MS spectra were collected in continuous flow mode by connecting the built-in infusion pump directly to the TurboIonSpray probe. For MS/MS optimization, a 15 μmol/L Hcy solution was prepared in 500 mL/L acetonitrile-500 mL/L water containing 0.25 mL/L formic acid and infused at a flow rate of 10 μL/min. In the multiple reaction monitoring (MRM) mode, the instrument was optimized automatically by the built-in algorithm to monitor the 136.1 to 90 *m/z* and 140.1 to 94 *m/z* transitions for Hcy and Hcy-d_4, respectively.

Data were acquired and processed using the MassChrom software (Ver. 1.1.1; Perkin-Elmer Sciex) including Multiview, Ver. 1.4, for chromatographic and spectral interpretation and Turboquan for Windows NT (Ver. 1.0; Perkin-Elmer Sciex) for the quantitative processing.

For the purpose of method comparison, an automated sample processor (model 232XL; Gilson) was used for sequential preparation and injection of plasma/serum and urine specimens. The methodology was adapted from previously published procedures *(8, 9)* involving reduction of disulfides using sodium borohydride and dithioerythritol, derivatization of reduced sulfhydryls with monobromobimane, HPLC separation, and fluorometric

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detection. Reversed-phase column chromatography was performed using a LC-18 column (15 cm × 4.5 mm; Supelco) and gradient elution with acetonitrile vs aqueous ammonium formate/ammonium nitrate. HPLC components included a Waters 600 solvent controller and Waters 474 scanning fluorescence detector (excitation, 365 nm; emission, 475 nm; 5-μL flow cell). Injections of 10 μL were made using the built-in LC switching valve on the sample processor. The total time to process and chromatograph a single specimen was ~40 min. Data were acquired and processed using Waters Millennium, Ver. 3.15, chromatography workstation software.

FPIA analysis for tHcy was accomplished using an IMx system from Abbott Diagnostics *(10, 11)*. Instructions for use and analysis as well as reagents and supplies were obtained from the manufacturer.

Results

The MS/MS spectrum obtained by infusion of 15 μmol/L Hcy is shown in Fig. 1A. This spectrum was acquired by transmitting the protonated molecular ion via Q_1 and scanning the second resolving quadrupole (Q_3) for products resulting from fragmentation in the collision cell.

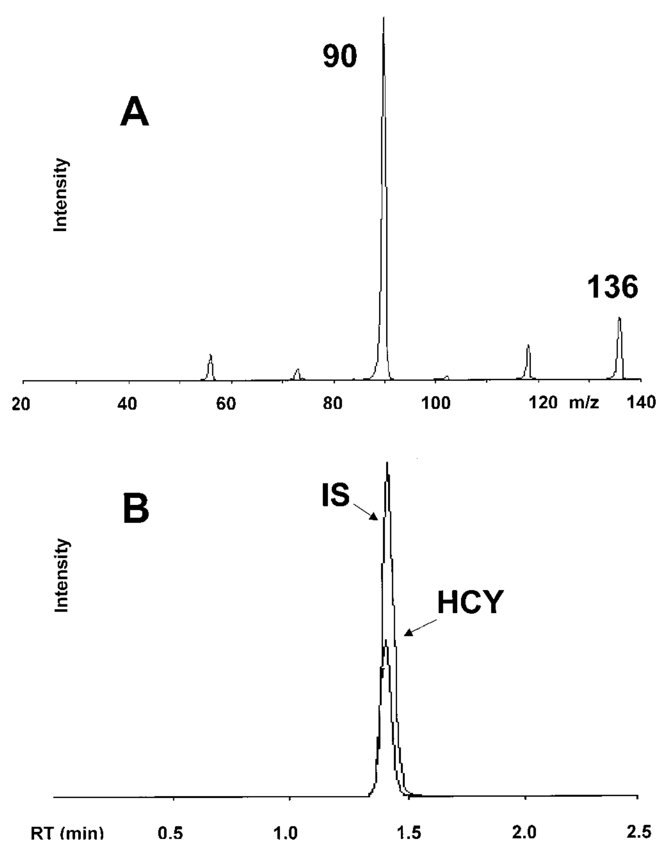

Fig. 1. MS/MS spectrum of tHcy (A) and overlay of MRM extracted ion chromatograms of tHcy and the internal standard Hcy-d_4.

(*A*), tHcy calibrator was infused at 15 μmol/L. Chromatogram shows the $[M+H]^+$ molecular ion at *m/z* 136.1, which produces the main fragment at *m/z* 90.0. (*B*), 40 μmol/L Hcy-d_4. Transition from *m/z* 140.1 to *m/z* 94.0 shown. *RT*, retention time.

Loss of the carboxy moiety from the protonated molecular ion of Hcy (*m/z* 136) yielded the fragment at *m/z* 90. Using the autotune algorithm provided in the system software, the instrument was optimized for transmission of the protonated molecular ion and for maximum intensity of the selected fragment. These results were used to design the MRM experiment to sequentially transmit the *m/z* 136 protonated molecular ion and the *m/z* 90 fragment via Q_1 and Q_3, respectively. Fig. 1B shows an extracted MRM chromatogram obtained by analysis of a mixture of tHcy (15 μmol/L) and Hcy-d_4 (40 μmol/L). The retention time of tHcy/Hcy-d_4 was slightly influenced by mobile phase composition and pH (a lower pH decreased the retention time).

The extracted MRM chromatograms obtained from a specimen in which the calculated tHcy concentration was 0.8 μmol/L is shown in Fig. 2. The signal-to-noise ratio for the tHcy extracted MRM signal was 17:1 (injected amount equal to 0.25 pmol); the ratio for the Hcy-d_4 (40 μmol/L) extracted MRM was 877:1.

LINEARITY

The interassay variability of calibration data obtained for concentrations from 2.5 to 60 μmol/L on 6 consecutive days is shown in Fig. 3. The mean slope, intercept, and coefficient of linear regression (r^2) were 0.9925 (95% confidence interval, 0.9697–1.0154), 0.0220 (95% confidence interval, 0.0110–0.0330), and 0.9971 (95% confidence interval, 0.9960–0.9982), respectively.

RECOVERY, PRECISION, AND STABILITY

The recovery and precision data are summarized in Table 1. These experiments were conducted using as matrix

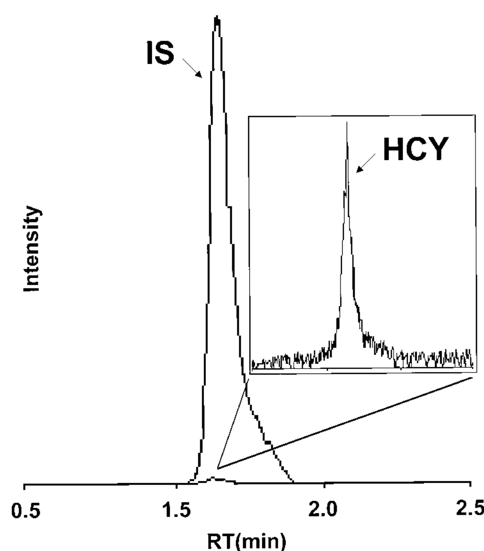

Fig. 2. Overlay of MRM extracted ion chromatograms of tHcy and the internal standard Hcy-d_4 in a sample with a calculated tHcy concentration of 0.8 μmol/L.

The *inset* shows a magnification of tHcy signal (17:1 signal-to-noise ratio for *m/z* 136 to *m/z* 90). *RT*, retention time.

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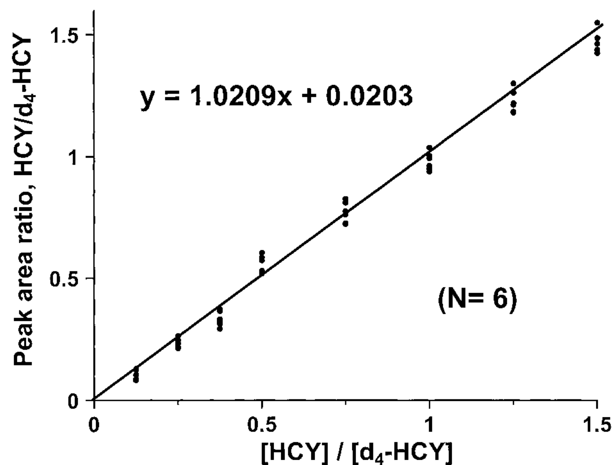

Fig. 3. Calibrations curves of peak area ratio vs tHcy/Hcy-d_4 concentration ratio (n = 6).

Hcy data points correspond to 2.5, 5, 10, 20, 30, 40, 50, and 60 μmol/L. The amount of internal standard added to each sample was 2 nmol of homocystine-d_8.

bovine calf serum diluted 1:1 with distilled water. Hcy (0, 10, or 25 μL of a 200 μmol/L solution) was added to 108 aliquots of bovine calf serum (100 μL), corresponding to final tHcy concentrations of 4, 24, and 54 μmol/L. Six aliquots of each set were prepared as described above and analyzed in single determinations on each of 6 consecutive days. One of each set of final samples was analyzed six times to verify the instrumental precision of the method. Quantitative recovery and good precision were obtained, demonstrating the accuracy of the present method for the quantitative determination of tHcy. The stability of prepared specimens was investigated by repeat injection of one set of 7 calibrators and 10 plasma samples (selected over a concentration range of 5.5–36.1 μmol/L) with interim storage at 4 °C. The data obtained are shown in Table 2 and indicate stable reduction of sulfhydryls and stability of tHcy and Hcy-d_4 stored up to 72 h after sample preparation.

METHOD COMPARISON

Leftover specimens of 367 analyses routinely performed by HPLC over a 2-day period were reanalyzed on the same day by LC-MS/MS and FPIA (IMx) in our laboratory (Table 3). The correlation between the LC-MS/MS method and the HPLC assay was $y = 1.097x - 1.377$. The distribution of the mean vs the difference of paired tHcy values [Bland-Altman plot, *(13)*] is shown in Fig. 4. The correlation between the LC-MS/MS method and the FPIA assay was $y = 1.039x + 0.025$; the corresponding Bland-Altman plot is shown in Fig. 5. These comparative data indicate equivalence of tHcy results over the concentration range encountered in routine specimen analysis. Because urine is not a suitable specimen for the immunoassay method, a small group of urine specimens were analyzed by LC-MS/MS and HPLC methods, and correlations similar to those presented for plasma were obtained (n = 19; r^2= 0.994; $y = 1.016x - 1.427$).

Discussion

MS/MS techniques based on magnetic sector mass spectrometers have been available for decades *(14)*, but the prohibitive instrumentation cost and the complexity of operation have been major obstacles to their routine application to laboratory medicine. The commercial avail-

Table 1. Performance statistics of the LC-MS/MS assay.

	tHcy recovery			CV, % (n = 6)	
No. of aliquots	Added, μmol/L	Detected,[a] μmol/L	Recovery, %	Intraassay	Interassay
6	0	3.9 ± 0.2		4.0	5.9
6	20	22.7 ± 0.8	94.2	5.3	3.6
6	50	52.8 ± 1.5	97.8	3.6	2.9

[a] Mean ± SD.

Table 2. Stability of tHcy after reduction.

	tHcy measured,[a] μmol/L			
	0 h	24 h	48 h	72 h
tHcy added,[b] μmol/L				
5	5.9	5.6	5.8	5.5
10	10.4	10.2	10.6	10.6
20	19.8	19.7	20.9	20.8
30	29.0	29.5	29.7	28.9
40	38.8	39.6	39.3	39.1
50	49.9	49.6	49.8	49.5
60	61.4	60.9	61.4	61.7
Plasma sample[c]				
1	5.5	5.6	5.7	5.6
2	5.6	5.6	5.7	5.6
3	6.1	6.0	6.1	6.0
4	6.4	6.8	6.9	6.7
5	10.7	11.0	11.0	10.9
6	11.3	11.0	11.8	11.5
7	13.6	13.5	13.7	13.6
8	17.8	17.8	18.4	17.6
9	36.1	36.0	36.0	35.7

[a] Samples were analyzed immediately after preparation (time 0 h) and after the times indicated above. Storage was at 4 °C in the autosampler vials covered with parafilm.

[b] Matrix was bovine calf serum diluted 1:1 with distilled water.

[c] Samples selected from a batch of plasma specimens submitted for tHcy determination.

Table 3. Comparison between methods for the analysis of 367 plasma samples.

			Mean Δ[a]	
Method	Range, μmol/L	Median, μmol/L	vs HPLC	vs FPIA[b]
HPLC	1.0–84.0	8.6		
FPIA[b]	2.5–66.9	9.5	0.8	
LC-MS/MS	2.1–70.8	9.1	−0.3	0.4

[a] See Figs. 4 and 5 for additional information.

[b] Fluorescence polarization immunoassay (Abbott IMx).

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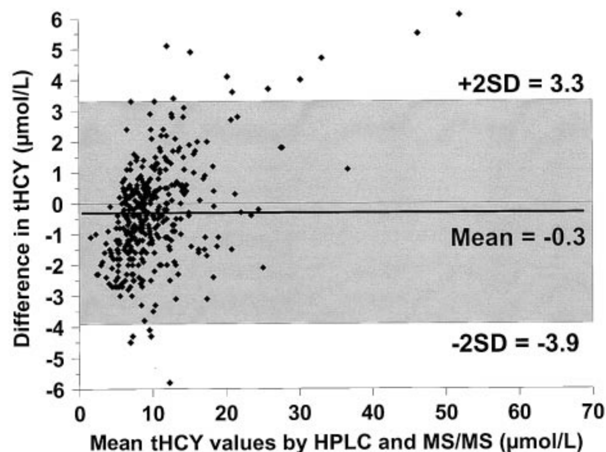

Fig. 4. Bland-Altman plot of the difference vs the mean of paired tHcy values between the LC-MS/MS assay and our previous HPLC method *(8, 9)*.

ability of more affordable bench top triple quadrupole mass spectrometers and especially the reliability of the LC-MS interface based on ESI *(15)* have been critical factors behind the increasing impact this technology is having in laboratory medicine. MS/MS has already emerged as a powerful analytical tool in clinical biochemical genetics *(16)*. Analyses of amino acids, acylcarnitines, and a growing number of other metabolites are now routinely performed by MS/MS to evaluate patients suspected of having inborn errors of metabolism, where it has been demonstrated as superior to conventional HPLC and gas chromatography–mass spectrometry methods *(17)*. Accordingly, MS/MS is gradually replacing previous methods used to perform newborn screening programs for inborn errors of metabolism *(18)*.

Briefly, the principle of operation of an ESI-MS/MS system can be summarized as follows: ESI generates charged ions at near-atmospheric pressure from a solution nebulized as a fine spray of droplets by a high voltage electric field and/or pneumatically *(19)*. Ion desorption from the droplets is induced by a counter flow of gas, and a fraction of the charged molecules is drawn in to the high vacuum of the quadrupole through a narrow opening. The first (Q_1) or third (Q_3) quadrupoles can be set either to scan a mass range or to select one or more individual ions. The second quadrupole (Q_2) is used as a collision cell. When nitrogen is introduced into the Q_2 region, fragmentation of ions passed or scanned through Q_1 is enhanced by collisional activation, and the resulting fragment ions are then resolved by Q_3. In the MRM mode, Q_1 is set to transmit only the parent ions of interest (*m/z* 136 and *m/z* 140 for Hcy and Hcy-d_4, respectively), Q_3 scans only in the mass range of the daughter ions (*m/z* 90 and *m/z* 94) arising from the primary, collisionally activated fragmentation in Q_2.

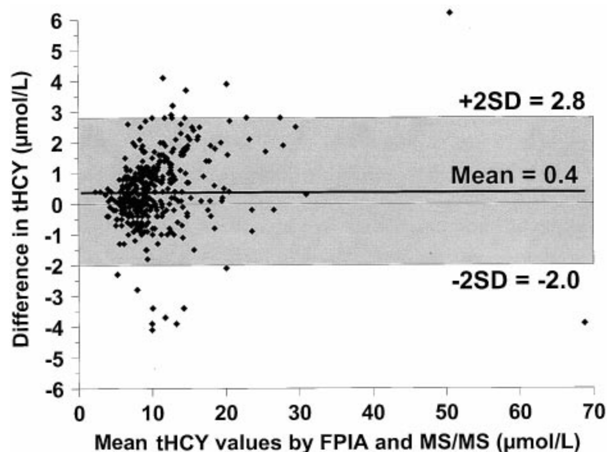

Fig. 5. Bland-Altman plot of the difference vs the mean of paired tHcy values between the LC-MS/MS assay and the Abbott IMx FPIA *(10, 11)*.

A variety of different procedures are currently available for the determination of tHcy *(20, 21)*. Currently, HPLC-based procedures and a FPIA are the two methods most widely applied in clinical practice *(11)*. Despite automation of sample preparation, our previous HPLC method *(8, 9)* required a visual verification of each profile by the operator and frequent manual corrections of baseline points at low concentrations. Inaccurate quantification because of interfering peaks or incomplete separation was a constant concern. The lack of internal standard and the relatively long instrument cycle time (17 min) were additional negatives. Although the FPIA is fully automated and faster than most HPLC methods, it could be affected by reagent batch variability and conceivably by other factors *(22)*, and is not applicable to urine. Because urinary tHcy excretion has been reported to mirror plasma concentrations *(2, 23–25)*, noninvasive determination of urinary tHcy could be regarded as the method of choice not only to monitor patients with homocystinuria but also as a tool to establish reliable pediatric reference ranges and to longitudinally study the impact of abnormal tHcy concentrations on cardiovascular disease from birth to adult life *(26, 27)*.

Our results indicate that ESI-MS/MS provides a novel approach to the determination of tHcy in plasma and urine. Sample preparation is based on a simplified manual procedure (40 samples are prepared in <1 h including incubation time) that can be automated, requires inexpensive reagents and no derivatization, and takes advantage of a stable isotope-labeled internal standard with identical chromatographic behavior, which also serves as internal control of the reduction step. No interference from other compounds present in either serum/plasma or urine samples were noted in the analysis of >4500 samples.

The relatively high initial investment needed to acquire a tandem mass spectrometer has to be considered in the proper perspective: In our laboratory, for example, a single MS/MS instrument has replaced five sample processor/HPLC systems that were used to run 150–200 samples per day, with reductions of supply costs, space, personnel, and turnaround time of 35%, 80%, 29%, and

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81%, respectively, whereas the equipment cost per sample increased only 25 cents per sample. The total (supplies plus equipment) cost per test has dropped 14%. Because the analysis of 200 samples requires no more than 10 h of instrument time, which could be conveniently set up overnight, one instrument can be very efficiently utilized for other applications or for development and research purposes.

We thank George G. Klee (Mayo Clinic, Rochester, MN) for critical review of the manuscript and Wendy L. Hagan for expert technical assistance.

Note added in proof: A similar method has been developed and applied to the measurement of homocysteine in blood samples applied to filter paper (Gempel K, Gerbitz K-D, Casetta B, Bauer MF. Rapid determination of total homocysteine in blood spots by liquid chromatography-electrospray ionization-mass spectrometry. Clin Chem 1999;45:submitted).

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