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Improved and simplified LC–ESI-MS/MS method for homocysteine determination in human plasma: Application to the study of cardiovascular diseases

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ABSTRACT

A liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) method was developed and validated for the determination of human plasma homocysteine (Hcy), an important independent risk factor for cardiovascular disease, with a simplified sample pretreatment procedure and a zero blank free of endogenous Hcy for calibrator/QC preparation. Following protein precipitation, chromatographic separation was performed on Hypersil Aquasil C_{18} column (50 mm × 2.1 mm, 5 μm, Thermo) using mobile phase of aqueous 10% methanol containing 0.02% formic acid at 0.25 mL/min. Hcy and deuterated internal standard were detected in the multiple reaction monitoring mode with precursor to product ion transitions of m/z 136.1/90.0 and 140.1/94.0, respectively. The retention time was 1.2 min, and the total run time was 2 min per injection. A streamlined three-point calibration curve and one-point QC was used. Excellent linearity was observed with correlation coefficient (r) > 0.99. The intra- and inter-batch were ≤3.24% and ≤4.04%, and accuracy was within ±10%. Method comparison between the proposed method (y) and FPIA assay (x) demonstrated a correlation equation of $y = 1.003x + 0.4318$ ($r = 0.9589$). The developed method, improved for automation with cost-effective reagents, was proven to be suitable for high-throughput quantitative determination of Hcy in clinical practice by successfully applying it to the cardiovascular disease study.

1. Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid derived from methionine. Increased plasma homocysteine has been identified as a sensitive marker of folate and vitamin B_{12} deficiency [1] and an independent risk factor for cardiovascular disease (CVD) [2,3]. The measurement of plasma Hcy concentration has become an important technique for risk assessment and disease diagnosis in clinical practice and research setting.

A series of analytical procedures based on enzyme immunoassay and chromatographic methods have been described to be effective for Hcy determination during recent years. Fluorescence polarization immunoassay (FPIA), which runs on Abbott IMx or Axsym platform, is one of the enzyme immunoassays widely used in both research and routine laboratory analysis [4–6]. This method was characterized by full automation of sample preparation and fast turnaround. However, the relative expensive reagent cost is the major obstacle to its application. Chromatographic methods including high performance liquid chromatography (HPLC) [7,8], capillary electrophoresis (CE) [9,10] and gas chromatography–mass spectrometry (GC–MS) [11,12] for Hcy quantification have been reported. However, these methods are either time consuming or labor intensive in sample derivatization or limited throughput. Compared with the above mentioned methods, LC–ESI-MS/MS demonstrated significant advantages of accuracy, sensitivity, specificity, high-throughput and cost-efficiency for Hcy analysis [13–18]. Hence, it was recommended as a reference method [5].

In a previously reported method, however, the inevitable problem was the endogenous background of Hcy presented in plasma. It could not directly mirror the true concentration of a sample by its fortified calibration curve. In this work, we developed an improved LC–ESI-MS/MS method by optimizing the chromatographic conditions. This was achieved by simplifying the sample preparation procedures and producing zero blank free of endogenous background of Hcy. This method, based on the simplified procedure of sample preparation which is amenable to automation,

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high-throughput turnaround with 2 min per injection, and sensitive electrospray MS/MS detection, can be efficiently applied to Hcy routine clinical practice and research study.

2. Experimental

2.1. Reagents and chemicals

DL-homocysteine (purity 98%) and trichloroacetic acid were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA), DL-homocystine-3,3,3′,3′,4,4,4′,4′-d8 (internal standard, purity 98%) from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA), dithiothreitol from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), and HPLC-grade acetonitrile, methanol, and formic acid from Tedia Company Inc. (Fairfield, OH, USA). All other reagents were of analytical grade. Double distilled water was used throughout the study.

2.2. LC–ESI-MS/MS analysis

The Shimadzu HPLC system (Kyoto, Japan) that consisted of two LC-20AD pumps, a SIL-HTC autosampler, and an online DGU-20A3 vacuum degasser was used. Chromatographic separation was achieved on the analytical column Thermo Hypersil Aquasil C_{18} column (50 mm × 2.1 mm i.d., 5 μm) coupled with a guard column Phenomenex C_{18} (4.0 mm × 3.0 mm i.d., 5 μm) used at room temperature. The mobile phase was composed of aqueous 10% methanol containing 0.02% formic acid at 0.25 mL/min. The injection volume was 3 μL with a total run time of 2 min per injection.

A triple quadrupole tandem mass spectrometer API 3000 (Applied Biosystems/MDS Sciex, Toronto, Canada) equipped with Turbo Ionspray source was operated in positive ionization mode. Multiple reaction monitoring (MRM) was used to detect Hcy and Hcy-d4 with precursor to product ion transitions of m/z 136.1/90.0 and m/z 140.1/94.0. The ion spray voltage was set at 2.5 kV and the source temperature at 450 °C. The collision activated dissociation (CAD) was set at 12 and nitrogen was used as the collision gas. Analyst 1.4 software was used for instrument control and data acquisition.

2.3. Stock solutions, calibrators, and quality control (QC)

Standard stock solutions of Hcy and IS were separately prepared at 1 mg/mL in water. Three calibrators were prepared at concentrations of 5, 15, and 45 μmol/L by spiking appropriate Hcy stock solution to the solution of zero blank. Quality control was prepared in the same way at a concentration of 20 μmol/L. All the solutions were stored at 4 °C.

2.4. Zero blank preparation

Pooled human plasma was deproteinated by mixing two volumes of acetonitrile and centrifuged at 16,000 × g for 3 min. Moreover, the supernatant was diluted three times with water to obtain the zero blank, a solution free of endogenous homocysteine, to be used for preparation of calibrators and QC sample.

2.5. Sample preparation

To a 1.5 mL polypropylene centrifuge tube, each 50 μL of plasma, deuterated IS solution (5 μmol/L), and reduce agent (300 mmol/L dithiothreitol) was added and allowed to stand at room temperature for 10 min. After adding 50 μL of protein precipitation agent (15% trichloroacetic acid), the mixture was briefly vortexed for 10 s then centrifuged at 16,000 × g for 3 min. The supernatant was transferred to clean vials for sample injection.

2.6. Study subjects and sample collection

The method was applied to determine the plasma concentrations of Hcy from 193 subjects for physical examination and 171 hospital patients who were treated at the Department of Cardiology, Shanghai Xuhui Central Hospital from June 2006 to September 2007. In the physical examination group, 149 subjects were diagnosed with hypertriglyceridemia, 24 subjects with diabetes, and 20 subjects were observed for health control. In the hospital patient group, 40 patients were diagnosed with cerebral infarction (CI), 45 patients with coronary artery disease (CAD), 48 patients with hypertension (HT), and a control group of 38 patients with various diseases other than CI, CAD, and HT was selected.

Overnight fasting blood samples were collected from all subjects. Plasma collected for Hcy determination was immediately centrifuged (3000 × g for 10 min) and stored at −20 °C until analysis. The study was approved by the Ethics Committee at the Shanghai Xuhui Central Hospital, and all the patients gave a written informed consent.

2.7. Method comparison

The developed LC–ESI-MS/MS method was compared with Abbott FPIA Axsym Hcy assay, a fully automated method based on fluorescence polarization immunoassay. One hundred seventy-three plasma samples collected from patients for clinical diagnosis were stored at −20 °C for no more than 1 month. Method comparison was carried out at three participating laboratories with 57, 60, and 56 plasma samples determined. Hcy concentrations were analyzed simultaneously with the validated method and FPIA method. Pearson correlation analysis was conducted by using SPSS 11.5.

2.8. Statistics analysis

Measurement data were expressed as mean ± S.D. Analysis of variance (ANOVA) was performed to assess Hcy concentration mean differences between disease groups, and Pearson correlation analysis was used to compare the proposed method to the Abbott Axsym assay by using SPSS 11.5 software for windows. Statistical significance was set at $p < 0.05$.

3. Results and discussion

3.1. LC–ESI-MS/MS condition optimization

High intensity was observed in the positive ion mode for Q1 full scan and product ion scan when Hcy standard solution was injected with flow injection analysis. The full scan mass spectra showed predominately protonated molecular ion $[M+H]^+$ at m/z 136.1 for Hcy. Hcy produced a major product ion at m/z 90 when scanned with product ion scan mode at 20 eV of collision energy. Fig. 1 indicated the CID-MS/MS breakdown of the protonated molecule $[M+H]^+$ at m/z 136, by the loss of a molecule of formic acid, into the product ion $[M+H–HCOOH]^+$ at m/z 90 (loss of 46 Da). The parent ion was further fragmented when higher collision energy was used, into product ions at m/z 73, 56, and 46. In order to obtain higher intensity and to reduce background noise, ion transitions of m/z 136.1/90.0 and 140.1/94.0 were selected for MRM acquisition of Hcy and d4-Hcy, a reduced form of IS. Compound dependant parameters including declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) were tuned to produce the most intense ion signal.

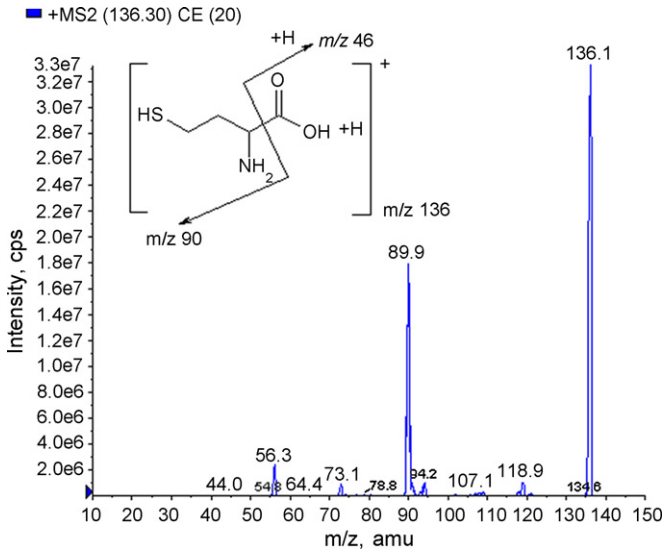

Fig. 1. Fragmentation and product ion spectra of homocysteine.

Hcy is a highly polar amino acid, which makes it difficult to retain in reversed-phase chromatographic separation. Therefore liquid chromatographic conditions such as analytical column and mobile phase optimization remained a very important task in achieving adequate retention on column and desired separation with endogenous matrix. In a previous report on Hcy determination with LC–ESI-MS/MS, Bodamer and co-workers used acetonitrile/water/formic acid (5000/5000/2.5) as mobile phase. However, it did not describe which analytical column was used [15]. Kuhn and co-workers performed chromatographic separation on Phenomenex C_8 precolumn eluted with aqueous 50% methanol containing 0.1% formic acid [16]. However, neither of them presented the chromatogram for Hcy. Pencharz and co-workers selected the LC conditions of Waters Symmetry C_8 column and aqueous 30% methanol/0.1% formic acid mobile phase, but <1 min retention time may cause severe ion inhibition [18]. In the experiment, we tested several types of analytical columns and mobile phases. Satisfactory retention time (1.2 min) and relatively short run time (2 min) (Fig. 2B) were obtained when chromatographic separation was performed on Hypersil Aquasil C_{18} column using a mobile phase of 10% methanol and 90% water (both containing 0.02% formic acid) with flow rate of 0.25 mL/min. This column contains a polar C_{18} phase which gives greater retention for polar compounds and exhibits high stability under 100% aqueous conditions.

In previous reports on the method of Hcy assay, an unavoidable problem surfaced as a result of its endogenous Hcy background in blank sample. The endogenous concentration had to be calculated from an unspiked sample, and subtracted from the calibrators when a standard curve to be constructed [13,15,18]. Therefore, preparation of a zero blank "free of homocysteine" to substitute the blank plasma would help to resolve the troublesome problem. In this study, we described a novel approach to prepare such a zero blank with pooled plasma, which avoided interference from endogenous background. Fig. 2A showed the typical chromatogram of a zero calibrator prepared with zero blank. No significant background of Hcy and other endogenous interference with analyte and IS was detected.

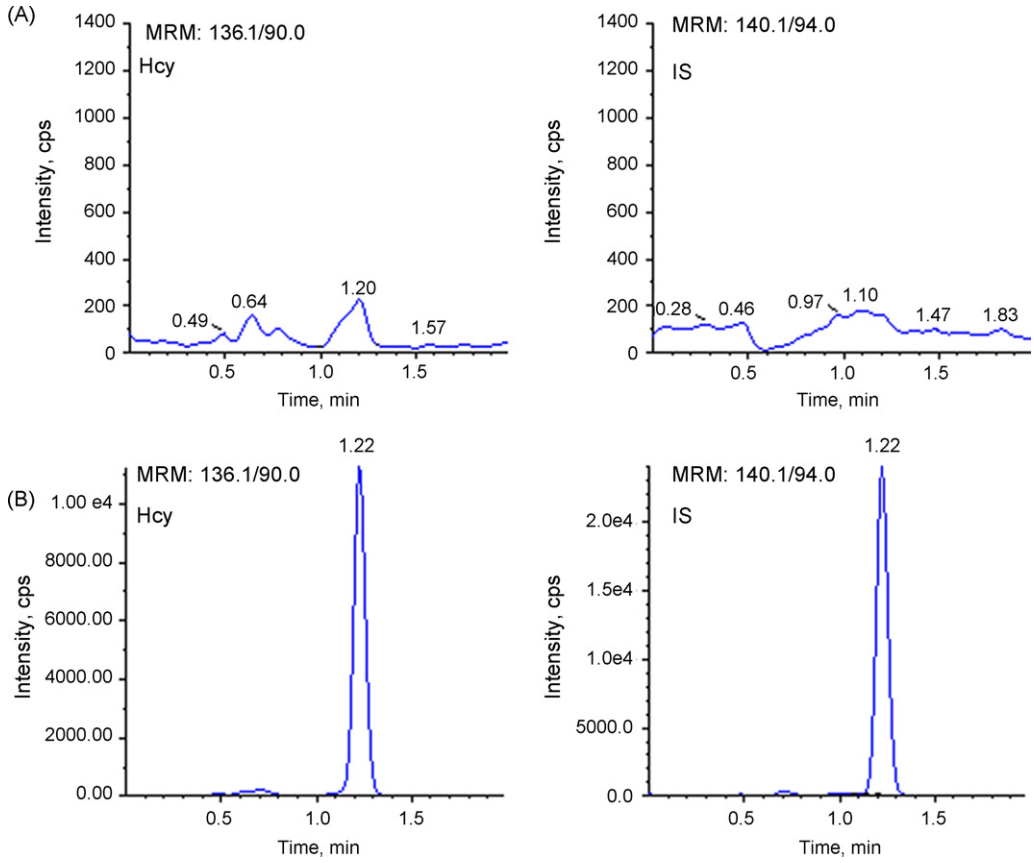

Fig. 2. Typical MRM chromatograms with ion transitions of *m*/*z* 136.1/90.0 (homocysteine) and *m*/*z* 140.1/94.0 (internal standard) acquired from (A) zero blank and (B) 5 μmol/L calibrator.

Table 1
Inter-batch calibration curves and linear regression of homocysteine

	Calibrator 1 5 μmol/L	Calibrator 2 15 μmol/L	Calibrator 3 45 μmol/L	Slope	Intercept	r
Run 1	5.03	14.88	45.09	0.114	−0.0968	0.9998
Run 2	4.98	15.09	44.93	0.108	−0.0968	0.9976
Run 3	5.03	14.90	45.08	0.106	−0.0656	1.0000
Run 4	4.96	15.17	44.87	0.103	−0.0527	0.9998
Run 5	5.07	14.72	45.21	0.106	−0.1100	0.9991
Run 6	5.07	14.73	45.20	0.113	−0.0153	0.9990
n	6	6	6	6	6	6
Mean	5.02	14.92	45.06	0.108	−0.073	0.9992
S.D.	0.05	0.18	0.14	0.004	0.035	0.0009
%R.S.D.	0.92	1.24	0.31	3.99	NA	NA

3.2. Linearity and LLOQ

In the process of method validation, we constructed a simplified three-point calibration curve for Hcy at 5, 15, and 45 μmol/L in dilution solution. Peak area ratio versus concentration was fitted at a linear regression with $1/x$ weighting. Over six continuous analytical runs for Hcy assay, the %R.S.D. at three levels of calibrator ranged from 0.31% to 1.24%, while the slope was at 3.99%. The equation of calibration curves, which is $y = (0.108 \pm 0.004)x - (0.073 \pm 0.035)$, and good linearity $r = (0.9992 \pm 0.00089)$, was obtained. The lower limit of quantification (LLOQ) was 5 μmol/L for Hcy, at which sufficient precision and accuracy were achieved from the data listed in Table 1. Actually, the limit of detection (LOD) was around 0.5 μmol/L. This figure showed enough sensitivity to determine Hcy in human plasma.

3.3. Precision, accuracy and recovery

Intra- and inter-batch precision and accuracy were evaluated by assaying the 20 μmol/L QC samples from six continuous analytical runs. In this assay, the intra-batch %R.S.D. was 3.24% or less ($n = 6$), and the inter-batch %R.S.D. was 4.04% or less ($n = 36$). The accuracy ranged from 90.2% to 104.0% (average 98.6%) at QC level of Hcy. The results demonstrated that the values were within the acceptable range, and the method was sufficiently accurate and precise. The recoveries of Hcy and IS were assessed by comparing the peak areas of QC standard spiked before and after extraction in six replicate of plasma. Mean recoveries of Hcy and IS were 96.4% and 98.3% ($n = 6$), respectively.

3.4. Stability

The stock solutions of Hcy in water were stored at 4 °C for 30 days, and results indicated its stability under storage condition. Three pooled human plasma samples collected from clinical practice were used to investigate Hcy stabilities after short-term storage, and long-term storage, in autosampler, after three freeze-thaw cycles. After exposure to room temperature for 6 h, the plasma samples remained stable with 95.4–105.5% accuracy. Long-term stability was obtained by comparing the Hcy concentration in plasma at −20 °C for 30 days of storage with those obtained before the storage period. No difference between concentrations was found with the samples that had 92.7–108.7% accuracy. The stability results for processed samples showed that the analytes were stable in an autosampler at 4 °C for at least 24 h with 96.3–103.6% accuracy. Hcy in plasma was stable after three freeze-thaw cycles, and the accuracy ranged from 94.5% to 102.8%. Although there was good stability, Hcy increased by 5–15% in whole blood at room temperature because of continuous production of Hcy from red blood cells [19]. Hence, the blood samples collected for Hcy assay were suggested to be kept on ice until centrifugation or until blood cells will be removed by immediate centrifugation [3].

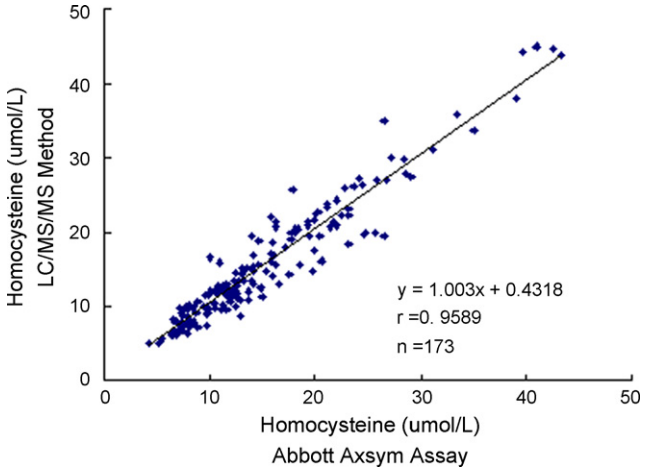

Fig. 3. Linear regression comparing the LC–ESI-MS/MS homocysteine method vs the Abbott Axsym homocysteine assay.

3.5. Method comparison

Hcy concentrations determined for 173 different plasma samples from patients for clinical diagnosis gave good agreement between Abbott Axsym homocysteine assay (x) and the validated procedure (y). The correlation equation, $y = 1.003x + 0.4318$, $r = 0.9589$ (Fig. 3), was comparable to the results obtained from previous studies on method comparison of Hcy measurements [5,13]. The described LC–ESI-MS/MS method was shown to be reproducible and robust when used in three different laboratories despite being less automated than the Abbott FPIA homocysteine assay. Significant method advantages in terms of specificity, regent cost, and throughput may be suitable for large numbers of samples assay.

3.6. Clinical application

The developed LC–ESI-MS/MS method has been successfully applied to determine Hcy concentration in human plasma from a clinical study in which 193 subjects for physical examination and 171 hospital patients were included. Disease category, age, and homocysteine concentration are shown in Table 2. A marked age difference between the physical examination group and hospital patient group was observed. There was significant difference in the Hcy concentration of patients with hypertriglyceridemia,

Table 2
Plasma homocysteine concentrations of physical examination subjects and hospital patients

Group	*n*	Age (year)	Homocysteine (μmol/L)
Physical examination subjects			
Healthy control	20	39.9 ± 9.9	13.1 ± 3.4
Hypertriglyceridemia[a]	149	45.0 ± 7.9	20.2 ± 12.7
Diabetes[a]	24	47.2 ± 5.9	21.3 ± 12.3
Hospital patients			
Patients control	38	69.2 ± 12.3	13.9 ± 6.6
Cerebral infarction[b]	40	75.0 ± 9.4	22.0 ± 14.9
Coronary artery disease[c]	45	79.3 ± 5.8	18.3 ± 7.9
Hypertension[c]	48	71.1 ± 10.3	16.8 ± 7.8

[a] $p < 0.01$ vs healthy control.
[b] $p < 0.01$ vs patients control.
[c] $p < 0.05$ vs patients control.

diabetes, cerebral infarction, coronary artery disease and hypertension compared to those of respective control individuals. The results of the clinical study confirmed that increase in Hcy level in plasma strongly correlated with the incidence of cardiovascular diseases.

4. Conclusion

The metabolism of homocysteine involves several B vitamins including folate, B_{12}, B_6, and B_2. Homocysteine has generally been accepted as an independent predictor of cardiovascular disease and as an indicator of folate and B_{12} deficiency in recent years. Consequently, the determination of Hcy has played an important role for clinical diagnosis and risk assessment of cardiovascular disease. Compared to previous reported methods, the described LC–ESI-MS/MS method presented an improved and simplified total Hcy assay in human plasma. A zero blank was prepared for calibrator and quality control, and sample preparation was simplified to suit for automation procedure and for further assay kit development. By optimizing chromatographic conditions, high-throughput analysis was achieved with 2 min per injection and 400 samples per day. Furthermore, good correlation was observed between the current LC–ESI-MS/MS method and the Abbott FPIA assay. The method has been successfully applied to a clinical study in which 193 subjects for physical examination and 171 hospital patients were included. More than 5000 samples have been determined in our laboratory from routine clinical practice since 2002–2007.

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