Rapid micro-scale assay for homocysteine by liquid chromatography-tandem mass spectrometry

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Abstract

Objectives: Increased total homocysteine (tHcy) level is an independent risk factor for atherosclerosis and cardiovascular disease. Here, we describe a rapid tHcy micro-scale assay.

Methods: We developed an easy sample preparation and rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the measurement of tHcy in human serum and plasma, respectively.

Results: The tHcy assay was linear up to 61.6 μmol/L, the detection limit was 1.0 μmol/L, the lower limit of quantification was 1.8 μmol/L, the imprecision in the range of 9.30 – 25.9 μmol/L was less than 5.9% and the analytical recovery was 94.7 ± 6.3%.

Conclusions: Our novel tHcy LC-MS/MS assay is a quick, precise and robust method for tHcy determination in routine diagnostics.

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Keywords: Homocysteine; Mass spectrometry; Atherosclerosis; Cardiovascular disease

Introduction

Homocysteine (2-amino-4-mercaptobutyric acid) was first described in 1932 by Butz and du Vigneaud [1]. The clinical relevance of homocysteine became apparent about 30 years later. In 1969, McCully discovered a relationship between elevated plasma levels of homocysteine and accelerated vascular disease. Within the past several years, researchers have found that increased total homocysteine (tHcy) level is an important parameter of different diseases. It is an independent risk factor for premature atherosclerosis and cardiovascular disease [2] as well as a sensitive marker of folate and cobalamin (vitamin B12) deficiency [3]. In addition, plasma tHcy concentrations are associated with birth defects and pregnancy complications [4]. The precise measurement of tHcy concentrations in human plasma or serum is, therefore, often useful. Different analytical methods, including high performance liquid chromatography [5], immunoassay [5,6] and gas chromatography mass spectrometry [5] to determine tHcy in plasma, were developed in recent years. However, most methods for the determination of tHcy levels are time-consuming or need expensive reagents. Here, we report the development and validation of a rapid, inexpensive LC-MS/MS assay for tHcy.

Methods

Sample collection

Venous blood samples were collected in serum separation tubes or EDTA tubes from KABE Labortecnik GmbH (Nümbrecht-Elsenroth, Germany). After clotting and centrifugation, the serum was stored at −20°C. Plasma was obtained immediately by centrifugation of the EDTA blood at 2000 × g for 5 min. It was stored at −20°C.

Sample preparation

The samples were prepared by mixing 50 μL blood plasma or blood serum with 5.0 μL of 140 μM d8-homocystine ([3, 3′, 3′′, 4′, 4′′]-2H8-homocystine, Cambridge Isotope Laboratories, Andover, MA, DLM-3619) as an internal standard and 5.0 μL of 1.0 M dithiothreitol solution as a reducing agent. Proteins were precipitated using 10 μL of 90%
trichloroacetic acid. After centrifugation at 10,000 × g for 10 min, 5 μL of the clear colorless supernatant was added to 45 μL 55% methanol containing 0.1% formic acid, vigorously mixed and used for the LC-MS/MS measurement. Plasma-based calibrators (Recipe, Munich, Germany) and controls (Recipe, Munich, Germany and Chromsystems, Munich, Germany) were used to generate the calibration curve and were handled in the same manner as the blood donor specimen.

Liquid chromatography-MS/MS homocysteine assay

For the measurement of tHcy, 5.0 μL of the prepared sample was injected into the LC-MS/MS system. The 4.0 mm (length) × 3.0 mm (inner diameter) C8 column (Phenomenex, Part No: AJ0-4290-S, Germany) was isocratically eluted with aqueous 50% methanol containing 0.1% formic acid at 100 μL/min. The cycle time (injection-to-injection) was 3 min. The samples were cooled to 6°C in the autosampler, and chromatography was performed at 40°C. The samples were measured using a Quattro micro-tandem mass spectrometer (Micromass) operated in the multiple reaction mode with a dwell time of 0.25 s per channel using the following transitions: m/z 136.1 for homocysteine and m/z 140.0 for d4-homocysteine. The capillary voltage of the mass spectrometer was set to 3000 V, the source temperature to 115°C, the desolvation temperature to 340°C and the sample cone voltage energy to 15 V for homocysteine and to 13 V for d4-homocysteine. Collision energy was set to 13 V for homocysteine and to 12 V for d4-homocysteine. The collision gas pressure was 3.0 × 10⁻³ mbar.

System control and data acquisition were performed with MassLynx NT 4.0 software provided with the mass spectrometer. The raw ESI-MS spectra were processed using the Quantify program incorporated as part of the Micromass MassLynx software package (Micromass, Eschborn, Germany). Calibration curves were constructed with the MassLynx Quantify program using a linear least-square regression with 1/x weighting.

Total homocysteine immunoassay

Total homocysteine in plasma was measured on the Axsym instrument (Abbott Diagnostics, Wiesbaden, Germany) using the commercially available “Abbott Homocysteine (HCY) assay”, a fluorescence polarization immunoassay from Abbott Diagnostics according to the manufacturer’s instructions.

Results

To achieve maximum sensitivity for homocysteine, the product ions from the fragmentation of the protonated molecule [M + H]+ were measured (Fig. 1). A solution of 10 μmol/L homocysteine was infused into the mass spectrometer, and the cone voltage was optimized to maximize the intensity of the [M + H]+ precursor ion (m/z 136.1). Thereafter, collision energy was adjusted to optimize the signal for the most abundant product ion (m/z 90.0), using argon as a collision gas at a pressure of 3.0 × 10⁻³ mbar. The process was repeated for d4-homocysteine, which was used as an internal standard.

In the experiments performed, both components, homocysteine and the internal standard d4-homocysteine, were eluted at 1.0 min, allowing an injection-to-injection cycle time of 3.0 min. Quantitation was performed by integrating the area under the product ions from the fragmentation of the protonated molecule [M + H]+ were measured (Fig. 1). A solution of 10 μmol/L homocysteine was infused into the mass spectrometer, and the cone voltage was optimized to maximize the intensity of the [M + H]+ precursor ion (m/z 136.1). Thereafter, collision energy was adjusted to optimize the signal for the most abundant product ion (m/z 90.0), using argon as a collision gas at a pressure of 3.0 × 10⁻³ mbar. The process was repeated for d4-homocysteine, which was used as an internal standard.

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the extracted ion chromatograms for homocysteine and d₄-homocysteine for a series of calibrators. A calibration curve was constructed by plotting the homocysteine/external standard peak area ratio against homocysteine concentration. The calibration curve of the tHcy assay was linear over the calibration range up to 61.6 μmol/L (Fig. 2A). The limit of detection (LOD) was 1.0 μmol/L (2.5 × SD of zero calibrator), the lower limit of quantification (LLOQ, concentration where the quantification CV/bias hits ≤20%) was 1.8 μmol/L, the upper limit of quantification (ULOQ, highest standard) was 61.6 μmol/L and the imprecision investigated for 9.30 μmol/L, 14.1 μmol/L and 25.9 μmol/L homocysteine was less than 5.9%. Analytical recovery was 94.7 ± 6.3%, with only little difference between plasma and serum samples. The mean homocysteine concentration of 10 blood donors was determined to be 13.6 μmol/L (SD 3.6) in serum. Plasma homocysteine of the same blood donors was on average 8% lower than the corresponding serum level. No interferences from other compounds present in serum or plasma were detected in the analysis of the samples. Method comparison between our novel tHcy LC-MS/MS assay (γ) and a commercially available fluorescence immunoassay (Abbott, AxSYM; x) shows a good correlation (y = 1.18x + 0.76, r = 0.990; n = 43 clinical samples; Fig. 2B). A mean difference of 2.9 μg/L was seen between the two methods.

Discussion

Here, we report a quick, precise and cost-effective LC-MS/MS method for the measurement of total homocysteine in human serum and plasma, respectively, using d₄-homocysteine as an internal standard. The sample preparation was rapid and simple, and the recovery, imprecision and sensitivity of the LC-MS/MS assay were comparable to other analytical techniques. Our novel method showed a good correlation to established fluorescence immunoassay methods. Moreover, the assay characteristics, such as imprecision or sensitivity, were comparable to those of high performance liquid chromatography (HPLC) methods. In contrast to HPLC, no time-consuming derivatization protocols are necessary, and, unlike with immunoassays, no expensive antibodies were used [5,6]. The LC-MS/MS methods described in the literature often use a large HPLC column which results in a time-consuming measurement of total homocysteine [7] or they measure the homocysteine without any preanalytical chromatography which may be fouling the sample cone of the mass spectrometer [8]. Other LC-MS/MS methods work without protein precipitation but with high sample dilutions which requires a high sensitive mass spectrometer [9]. In addition, most of the methods described used only plasma as sample material.

We found that a short C8 column used in the LC-MS/MS assay on the one hand allowed a quick chromatography and on the other hand reduced fouling of the sample cone of the mass spectrometer, which was observed in methods without any column. In addition, sensitivity was increased when the method was performed with the column (data not shown), suggesting that the small column removes ion suppressive components. The use of the short column resulted in injection-to-injection times of 3 min and enabled the processing of 20 samples per hour, including result generation. The application of a small sample size should prove useful when monitoring pediatric patients and will, in addition, spare expensive calibrators and quality control materials. Furthermore, it might be possible to validate tHcy determination in capillary blood by finger prick collection. The method is well suited to measure homocysteine in EDTA plasma and in serum, respectively. However, the use of EDTA plasma as sample material is recommended because the use of anticoagulant allows immediate sample processing. Serum homocysteine was on average 8% higher than plasma homocysteine, suggesting that this is attributable to an ongoing release of homocysteine from erythrocytes as serum is prepared from blood samples left at room temperature for about 30 min to allow coagulation. Increased homocysteine concentrations were also measured in plasma samples where the blood cells were not removed immediately after sample collection (data not shown). In conclusion, this LC-MS/MS assay for serum and plasma homocysteine is sensitive and precise and provides a high sample throughput which is well suitable for clinical application.

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References
