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Journal of Chromatography B, 842 (2006) 64–69
JOURNAL OF CHROMATOGRAPHY B
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Short communication

# A new method for determination of plasma homocystine by isotope dilution and electrospray tandem mass spectrometry

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Received 1 December 2005; accepted 4 May 2006
Available online 19 June 2006

**Abstract**

A new analytical determination method of homocystine in human plasma has been developed. The method utilises liquid chromatography coupled to ionspray tandem mass spectrometry. Quantitative analysis was achieved using as an internal standard homocystine-$d_8$. Mass spectrometer operated in the multiple reaction mode: homocystine and homocystine-$d_8$ were detected through the transition from the precursor to the product ion (from *m/z* 269.3 to 90.0, and *m/z* 277.3 to 94.0, respectively). The method is extremely sensitive, with limit of detection in the range of 6 fmol/L. The interassay and intraassay coefficients of variation for homocystine were 6.22% and 3.4%, respectively. The accuracy for the added homocystine ranged from 85% to 110%. High specificity of tandem mass spectrometry coupled with a fast chromatographic process is suitable for a rapid and reliable assay of homocystine.


*Keywords:* Homocystine; Electrospray tandem mass spectrometry

## 1. Introduction

Homocysteine (Hcy) is a thiol amino acid resulting from a methylation of methionine, an essential amino derived acid from dietary proteins (Fig. 1). Homocysteine is metabolised through two pathways: remethylation and transulfuration, which use as cofactor folic acid, Vitamin $B_6$ and Vitamin $B_{12}$.

The genetic defects of enzyme involved in homocysteine metabolism [1,2] and acquired factors inducing a reduction of folic acid's levels [3,4], Vitamin $B_6$ and Vitamin $B_{12}$, cause an increase of homocysteine's plasma levels.

In addition, age, gender, renal function, nutrition, disease states, and use of selected medications can regulate levels of Hcy in human plasma [5].

In plasma, total Hcy is the sum of free and protein-bound Hcy, homocystine (Fig. 2) (derived from autooxidation of homocysteine), and several other mixed disulphides [5].

Perturbation of plasma's redox status and of tissue's aminothiol is an important indicator of chronic oxidative stress [6]. A prooxidant state may accompany hyperhomocysteinemia in cardiovascular disease [7–10], in antioxidant deficiencies, and renal failure [11,12].

High homocystine's levels could warrant an antioxidant intervention [13,14]. One of the mechanism invoked for homocysteine-induced vascular dysfunction involves generation of reactive oxygen species, as $H_2O_2$, during the presumed transition of circulating homocysteine to homocystine [10,15–19]. Although there are many currently available methods for measuring plasma homocysteine [20–22], few analytical ones the quantitation in biological fluids of this oxidised aminothiol have been reported. Some of them include high-performance liquid chromatography (HPLC), coupled with either ultraviolet or fluorescent detection [23,24]. Gas chromatography–mass spectrometry (GS–MS) has also been used for detection and quantification of homocystine [25].

Here, we present a new validated accurate, reliable and rapid LC/MS/MS method for the quantitation of homocystine in human plasma.

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doi:10.1016/j.jchromb.2006.05.015

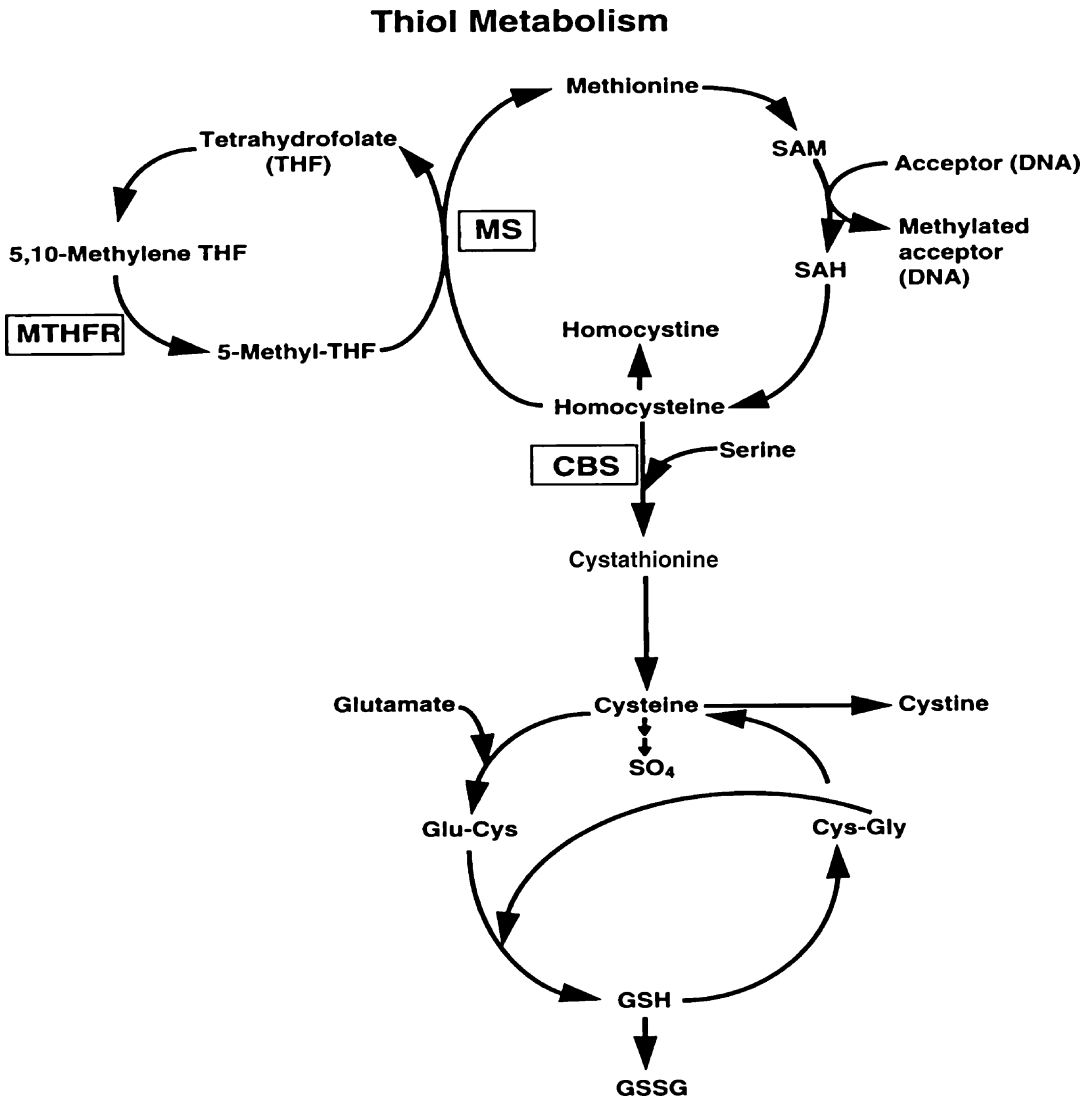


Fig. 1. Flow diagram of aminothiol metabolism including the anabolic homocysteine remethylation pathway and the catabolic homocysteine transulfuration pathway. SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; MTHFR, methlyenetetrahydrofolate reductase; CBS, cystathionine beta synthase; Glu-Cys, glutamylcysteine; Cys-Glu, cysteinylglycine; MS, methionine synthase; GSH, reduced glutathione; GSSG, oxidized glutathione.

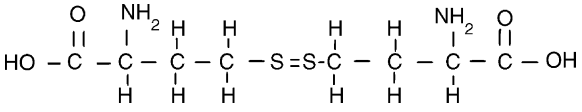

Fig. 2. Chemical structure of homocystine.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Unlabelled and labelled (D,L-homocystine-3,3,3′,3′,4,4,4′,4′-$d_8$) D,L-homocystine were obtained from Sigma–Aldrich Corporation (St. Louis, MO 63178, USA) and Cambridge Isotope Laboratories (50 Frontage Road, Andover, MA), respectively.

Acetonitrile and formic acid were obtained from Merck (642271 Darmstadt, Germany). 5-Sulfosalicylic acid hydrate was purchased from Sigma–Aldrich.

Double-distilled water from an in-house distillation system was used for chromatography and preparation of solutions.

### 2.2. Samples preparation

Samples were prepared by adding to 100 μl of plasma, 20 μl of an internal standard solution (2 nmol homocystine-$d_8$). To precipitate proteins, 10 μl of 5-sulfosalicylic acid (35%) were added. Then, samples were mixed. After centrifugation at 14,000 rpm for 5 min at r.t., 100 μl of the clear supernatant was transferred to an autosampler vial without further treatment and 1 μl was injected into the HPLC.

Linear calibrators curve consisting of 5 points for homocystine were prepared in plasma by addition of 200 μmol/L working solution corresponding to a concentration of 0, 10, 20, 30, 40 and 50 μmol/l. Working solution of homocystine and homocystine-$d_8$ at 200 μmol/l were obtained by adding to 500 ml/l acetonitrile–500 ml/l water–1 ml/l formic acid. Endogenous concen-

trations were derived from the blank specimens and subtracted from the calibrator points after visual verification of results.

### 2.3. Instrumentation

An Integral (Applied Biosystems) multi-dimensional LC system with an AB-Sciex API 3000 (Perkin-Elmer Sciex) benchtop triple quadrupole mass spectrometer was employed. To enhance the stability of signal, separation of homocystine and homocystine-$d_8$ from the bulk of the specimen matrix was achieved by use of a short column (LC-CN, 3 cm × 4.6 mm; Supelco). Data were acquired and processed using Applied Biosystems Analyst software package, revision 1.4.

Autosampler injections of 1 μl into a 5 μl sample loop were made using an isocratic elution phase that consisted of acetonitrile–$H_2O$–formic acid (600:400:1) at a flow rate of 1 ml/l. The column was directly connected to the TurboIon Spray ionisation probe, operating with the turbo gas on (6 l/min, sensor temperature, 250 °C) with the LC column effluent flow-splitting set 1:5. In the multiple reaction monitoring (MRM) mode, the instrument was optimised automatically by the built-in algorithm to monitor the *m/z* 269.3 to 90 and *m/z* 277.3 to 94 transitions for homocystine and homocystine-$d_8$, respectively.

All data were acquired in positive ion mode with the orifice voltage set at 36 V, automatically optimised using the homocystine ion. This was done by direct infusion of a standard solution containing homocystine with a syringe pump at 10 μl/min.

Collisionally activated decomposition MS/MS was performed through the closed-design Q2 collision cell operating with nitrogen at 0.06 kPa as collision gas. The 30 eV collision energy was adjusted automatically by the AutoTune algorithm. The methodology was adapted from a previously published procedure by Magera et al. [26].

## 3. Results

The full scan spectra obtained by infusion of homocystine and homocystine-$d_8$ are shown in Fig. 3. The MS/MS spectra were acquired by transmitting the protonated molecular ion via $Q_1$ and scanning the second resolving quadrupole ($Q_3$) for product resulting from fragmentation in the collision cell (Fig. 4).

Loss of the carboxy moiety from the protonated molecular ion of homocystine (*m/z* 269.3) yielded the fragment at *m/z* 90. Using the autotune algorithm provided in the system software, the instrument was optimised for the transmission of the protonated molecular ion and for maximum intensity of the selected fragment. These result were used to design the MRM experiment to sequentially transmit the *m/z* 269.3 protonated molecular ion and *m/z* 90 fragment via $Q_1$ and $Q_3$, respectively. Fig. 5 shows an extracted MRM chromatogram obtained by analysis of a mixture of homocystine (50 μmol/l) and homocystine-$d_8$ (100 μmol/l).

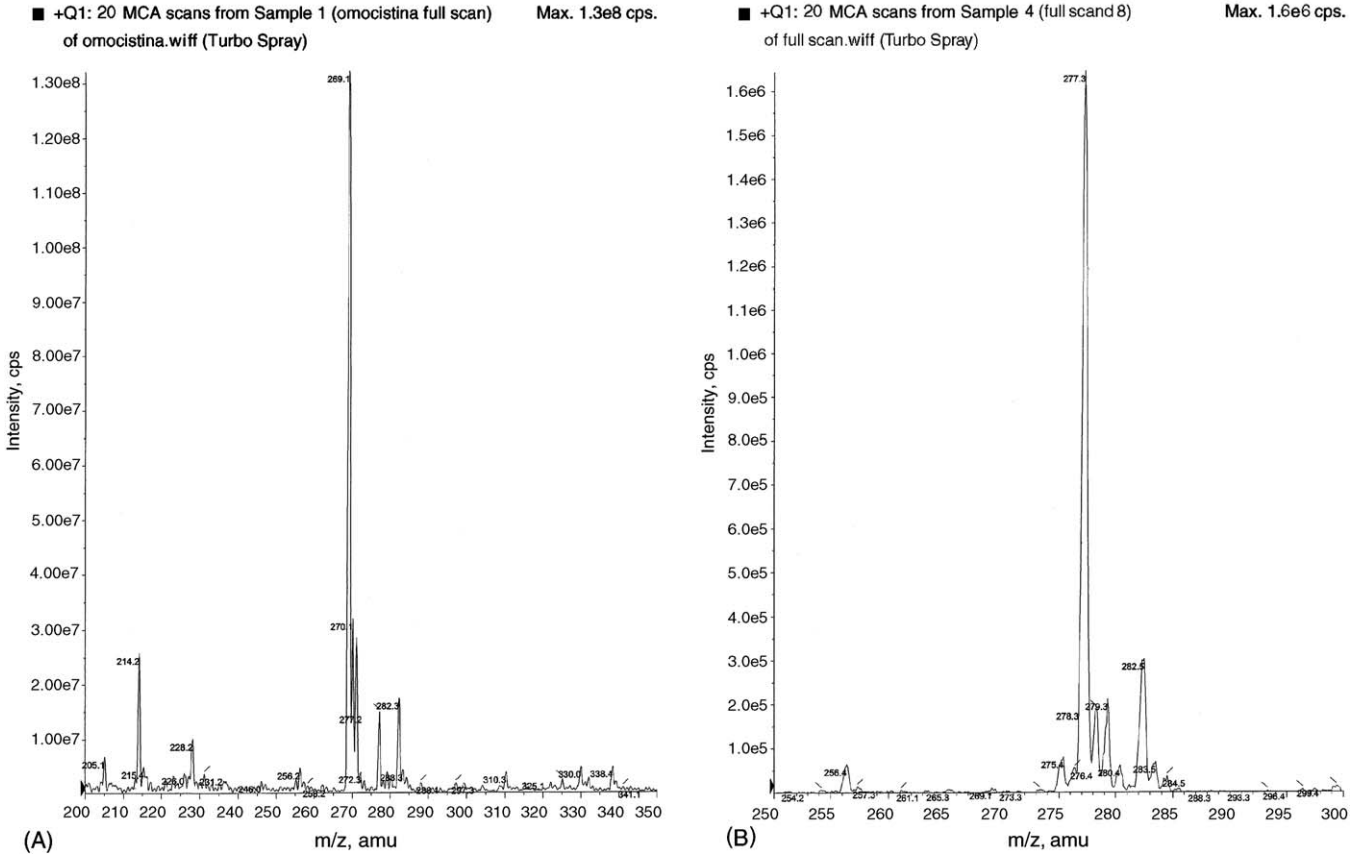


Fig. 3. Full-scan mass spectra of homocystine (A) and homocystine-d8(B).

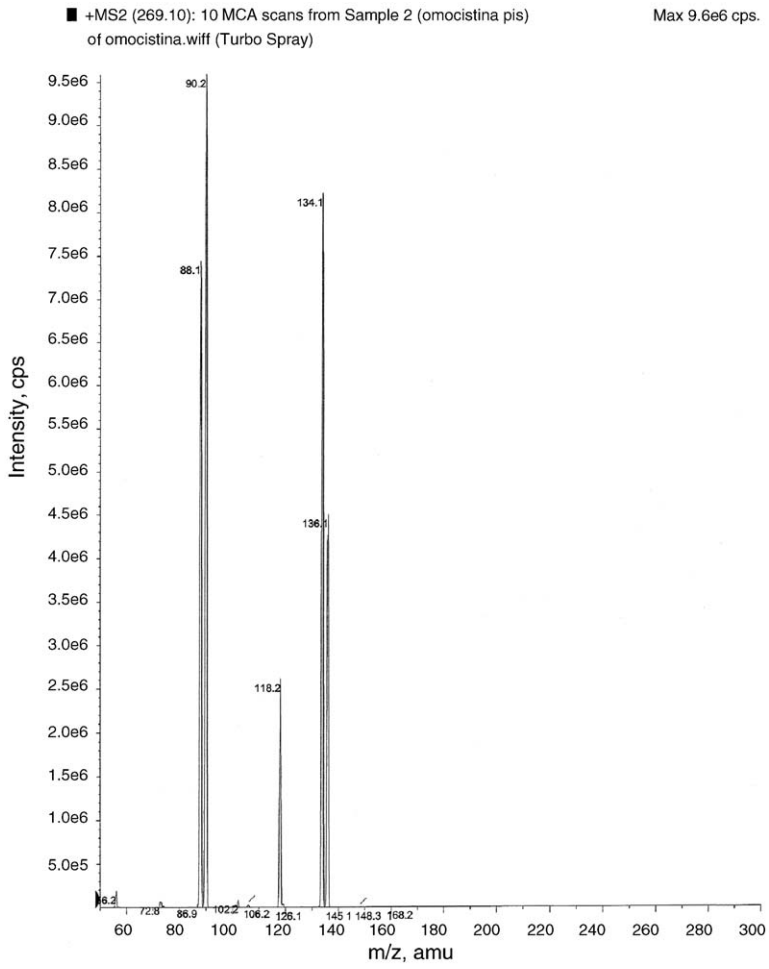


Fig. 4. MS/MS spectrum of homocystine.

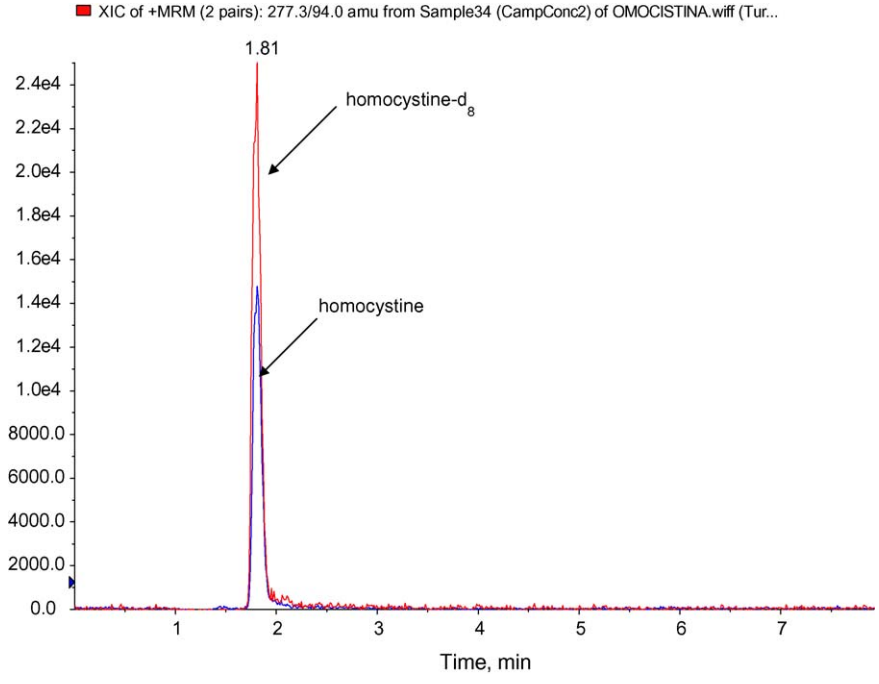


Fig. 5. MRM extracted ion chromatograms of homocystine and the internal standard.

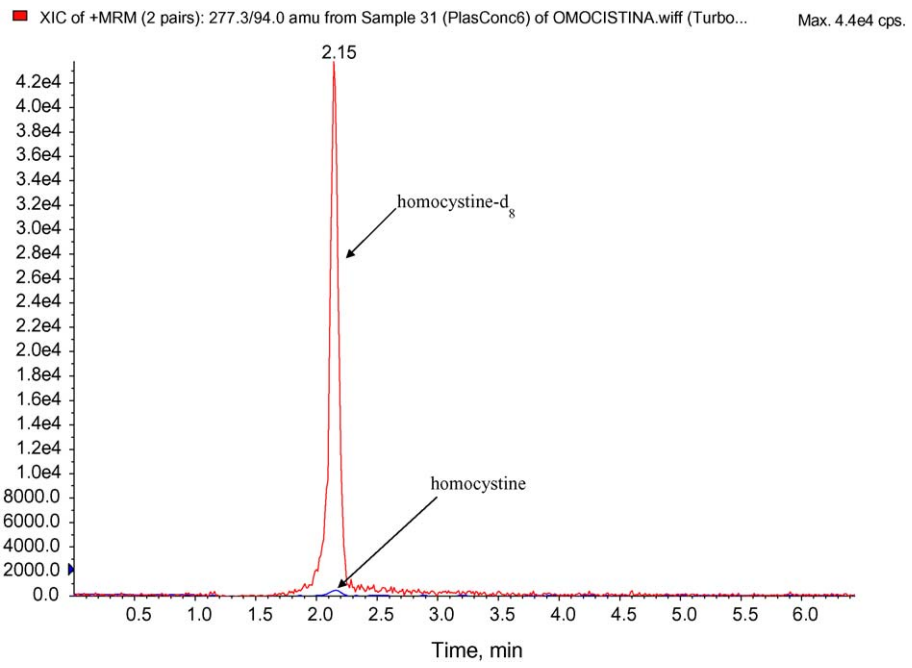


Fig. 6. MRM extracted ion chromatograms of homocystine and the internal standard in human plasma.

Fig. 6 shows the extracted MRM chromatograms obtained from a specimen. Retention time of homocystine and homocystine-$d_8$ was 1.2 min; it was slightly influenced by mobile phase composition and pH (a lower pH decreased the retention time).

### 3.1. Linearity

The inter- and intra-assay variability of the calibration data obtained for concentration from 10 to 50 μmol/l on 5 consecutive days were 6.22% and 3.4%, respectively. The response of the detector was linear. A linear regression analysis yielded $y = 1.02 \times 10^3 x + -2.5 \times 10^5$ with a coefficient of $r = 0.9982$, where $y$ is the peak area and $x$ is homocystine/homocystine-$d_8$ concentration ratio.

### 3.2. Recovery and precision

Recovery experiments were carried out by adding known amounts of homocystine to whole plasma sample before protein precipitation. A linear relation was observed between the peak area of homocystine-$d_8$ and the concentration of homocystine (μmol/l) added. Recoveries ranged from 85 to 110%. This recovery indicates that the processing and the analytical methods were accurate for the quantitative determination of plasma homocystine.

## 4. Discussion

Mild elevation of Hcy is associated with an increased risk for thrombosis, stroke and occlusive vascular disease. Cellular toxicity could indirectly result from auto-oxidation of homocysteine to homocystine. In fact, only traces of homocysteine circulate in plasma as the free thiol; the remainder is present as oxidation products. Fast oxidation of high Hcy levels may lead to precipitation of homocystine, if the aumont of this symmetric disulfide exceeds its saturation concentration. Homocystine could therefore form insoluble crystals in the bloodsteam, causing damage endothelial tissue. For these reasons, homocystine is an important indicator of oxidative stress: high levels of homocystine would be consistent with a prooxidant state and an indication that antioxidant intervention is warranted [11–13].

Although there are few methods currently available for measuring plasma homocystine, none combines liquid chromatography electrospray with mass spectrometry presented in this report. The method shows an immediate clinical application. Determination of homocystine can give additional informative leading to a best definition of the etiology and severity of abnormalities in homocysteine metabolism.

The present report describes a simple, rapid and accurate mass spectrometry coupled to liquid chromatography method for determining the concentration of homocystine in human plasma, and so quantifying oxidative stress in vivo. LC–MS–MS is a powerful technique for the specific and quantitative measurement of extremely low levels of biological molecules, since combines separation of the analytes on a HPLC specific column and detection of ions by mass spectrometer. For accurate determination of oxidised status of homocystine, sample handling is of critical importance: blood must be immediately centrifuged at 4 °C, and stored at −20 °C to prevent spontaneous oxidation of aminothiols. Sample preparation is based on a simplified manual procedure that can be automated, requires cheap reagents, and takes advantage of a stable isotope-labelled internal standard with identical chromatographic behaviour, which also serves as an internal standard control of reaction. High sensitivity and selectivity of the LC–MS–MS method will allow accurate deter-

mination of plasma homocystine concentration. At variance with other described methods [27,28], the advantages of this method are elimination of time-consuming precolumn derivatization step. No interference from other compounds in plasma was observed.

In addition to plasma homocystine, this method could be probably applied to other oxidised and reduced plasma aminothiols, involved in both the anabolic (remethylation) and catabolic (transulfuration) patways of homocysteine metabolism.

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