GENERAL PATHOLOGY AND PATHOPHYSIOLOGY

The Use of N-Ethylmaleimide for Mass Spectrometric Detection of Homocysteine Fractions in Blood Plasma

A. V. Ivanov, B. P. Luzyanin, and A. A. Kubatiev

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Fraction analysis of homocysteine in biological fluids is important for the diagnosis and studies of cardiovascular, nervous, urological, and other diseases. Measurements of total, free, and reduced homocysteine by mass spectrometry with HPLC by means of its modification with N-ethylmaleimide are proposed. The linearity and detection threshold were 0.025-10 and 0.001 μM, respectively, for reduced homocysteine fraction and 0.2-100 and 0.1 μM, respectively, for the rest fractions. The accuracy and reproducibility of the method were within 12%.

Key Words: *homocysteine; fractions; N-ethylmaleimide; high pressure liquid chromatography; mass spectrometry*

Homocysteine (Hcy) is an active metabolite of the methionine demethylation cycle, the key component of methyl groups metabolism. The main mechanism of excessive Hcy elimination is transsulfuration, transforming Hcy into cystein [6]. Animal experiments [8,15] and clinical studies [7] of hereditary disorders in expression of enzymes involved in Hcy transformation showed that the increase in its plasma concentrations was associated with numerous pathological processes in the cardiovascular, nervous, and other systems. Numerous clinical studies led to a conclusion that Hcy was an independent risk factor for cardiovascular diseases, such as coronary disease, atherosclerosis, *etc.* [1]. A relationship between Hcy level and Alzheimer's disease was shown [14]. The contribution of Hcy to the pathogenesis of various diseases was studied [4]. However, it remains unknown whether elevation of Hcy concentration induces or results from pathological processes.

Institute of General Pathology and Pathophysiology, Russian Academy of Medical Sciences, Moscow, Russia. ***Address for correspondence:*** b_luzyanin@niiopp.ru. B. P. Luzyanin

Normally, the concentration of total Hcy (Hcy_T) in human plasma is ~10-15 μM [13]. The bound (through -S-S-bonds with plasma proteins) form of Hcy (Hcy_B) constitutes 70-90% of Hcy_T, while free Hcy (Hcy_F) is present in the oxidized (Hcy_{OX}) or reduced (Hcy_R) forms. Oxidized Hcy includes mainly Hcy and cystein (Cys) disulfides. Hcy_{OX} and Hcy_R constitute 10-30% and <1% of Hcy_T, respectively [13]. Analysis of total Hcy is used in clinical practice. However, measurements of its fractions have certain advantages, as provide information about the Hcy redox status and the bioactive pool of its molecules.

Study of Hcy fraction composition is a more difficult problem than measurements of Hcy_T, because it implies the use of highly sensitive analytical methods; one more difficulty is rapid oxidation of Hcy molecule in isolated plasma [9].

The methods for Hcy measurements most widely used at present are based on its chemical modification and separation of the components by HPLC [3,9,13] or by capillary electrophoresis [5,16].

Mass spectrometry (MS) in complex with HPLC or capillary electrophoresis, more and more often used in

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biochemistry, can serve as an alternative. The sensitivity of MS is largely determined by the efficiency of ionization of the analyzed specimen. It can be stimulated by introduction of nucleophilic groups and/or hydrophobic residues into the specimen, which is particularly important for Hcy with poor ionization capacity in comparison with other amino acids. It is essential to render oxidation resistance to the thiol group by its modification. This problem has been solved by using N-ethylmaleimide (NEM), meeting all these requirements (a rapid and specific blocker of SH groups) which increases MS sensitivity to amino thiols by several times [10]. Some authors suggest direct measurements of Hcy and other thiols by HPLC–MS [11,12] and capillary electrophoresis–MS [2,10], by fraction analysis of thiols by means of capillary electrophoresis–MS using NEM and closely related compounds [10].

We evaluated total, free, and reduced Hcy in the plasma by using an approach including NEM modification of Hcy, separation of the components by HPLC, and their MS detection with electrospraying interface.

MATERIALS AND METHODS

The following reagents were used: Hcy, cysteamine (CA), dithiothreitol, NEM (Sigma), NaOH, NaCl, sodium citrate (Dia-M), and heparin (Synthesis).

Perkin Elmer Series 200 HPLC system with Dr. Maisch Reprosil-Pur C18-AQ column (3 μ, 150×2 mm) was connected to MS Agilent MSD Trap VL through standard electrospraying interface. Experimental data were recorded using MSD Trap Control software, primary processing was carried out by Agilent Data Analysis.

Calibration curves were plotted using fresh solutions of 150 μM Hcy and 500 μM CA (internal standard) in deionized water, to which NEM was added to a concentration of 10 mM and the solution was incubated for 10 min at 4°C. The reaction completeness was verified by MS: after direct addition of 10-fold diluted solution, disappearance of the peaks corresponding to Hcy and CA and appearance of Hcy-NEM and CA-NEM peaks indicated complete reaction. Concentrated solutions were stored at -80°C. Specimens with Hcy concentrations of 0-10.7 μM and CA concentrations of 5 μM were prepared and stored at 4°C.

The blood was collected from the marginal ear vein of a 1-year-old male Chinchilla rabbit. Blood was collected by free flow into a tube with anticoagulant 3.8% sodium citrate in 1:9 proportion and heparin (5000 U/ml blood). Directly after blood collection, NEM in NaCl solution (9 g/liter) was added for Hcy_R measurements to the concentration of 10 mM. Blood was directly cooled in on ice bath for at least 10 min. The plasma was separated by centrifugation at 3000*g* (10 min, 4°C) and centrifuged with Amicon Ultra 3K (Millipore) filters at 14,000*g* (15-25 min). Hcy_F concentration was measured out in the blood without preliminary addition of NEM; the plasma was separated as described above and its filtrate was reduced with 10 mM dithiothreitol at 37°C for 15 min, after which the sample was 10-fold diluted with deionized water with 10 mM NEM. The procedure for Hcy_T measurements differed from the above described by only one step: the plasma was reduced before filtration (Fig. 1). In order to minimize the level of endogenous Hcy, the plasma was additionally incubated for 30 min at ambient temperature and filtered as described previously. The specimens were stored at -80°C.

In order to plot the calibration curves, 0-10.7 μM Hcy-NEM solutions were added to deionized water and filtered pooled plasma. The internal CA-NEM reference sample in a concentration of 5 μM was added to the samples directly before injection. The samples were stored at 4°C. The Hcy-NEM/CA-NEM peaks proportion for each concentration was used for calculations of the calibration curve parameters (inclination and cutting across the ordinate) using the linear regression equations.

The analysis accuracy and error were evaluated by repeated injections of 0.15 μM Hcy-NEM sample in water and plasma, respectively. The reproducibility was evaluated by analyzing Hcy_T, Hcy_F, and Hcy_R at least 3 times a day for 3 days.

The HPLC–MS was carried out in an isocratic mode by 1.75% acetonitrilee solution (Kryochrome) with 0.1% formic acid (Fluka) at 0.3 ml/min flow for 20 min for plasma samples and 10 min for model solutions. A sample (20 μl) was injected through a loop dispenser. Regeneration of the column was carried out with 90% acetonitrile (10 min) with subsequent washing with the eluent for at least 20 min after every 10-12 injections of plasma samples. The parameters

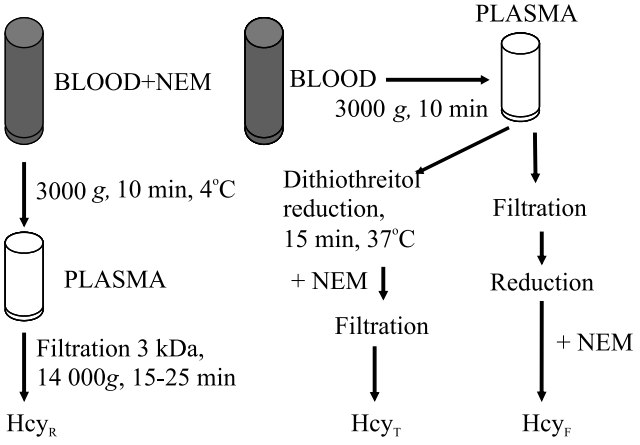

Fig. 1. Scheme of sample preparation.

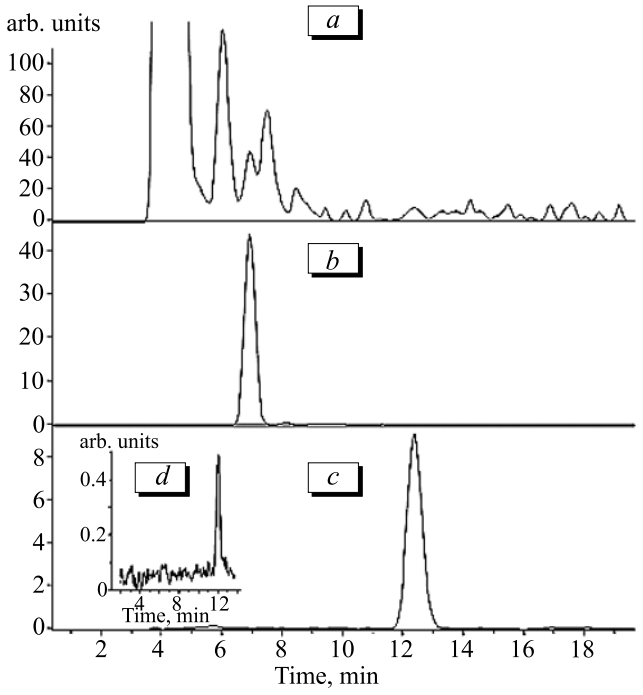

Fig. 2. Chromatogram of a plasma sample with internal standard (5 μM CA-NEM). *a*) total flow 200-270 m/z; *b*) CA-NEM, 5 μM; 203.3 m/z flow; *c*) Hcy-NEM, 0.64 μM; 261.1 m/z flow; *d*) Hcy-NEM, 0.01 μM. Ordinate: intensity.

of MS were as follows: drying gas flow 8 liters/min (320°C), nebulizer pressure 1.4 Bar, capillary potential 3500 V, target weight 261 m/z (proton-treated Hcy-NEM), scanning range 200-270 m/z.

RESULTS

Reaction of NEM with Hcy and CA yields products with molecular weights of 260 and 202 Da, respectively. The time of CA-NEM and Hcy-NEM release was 8 and 12.5 min, respectively (Fig. 2). Comparison of the intensities of these compounds in the water and plasma showed no significant differences (data not presented), which indicated sufficient separation of plasma components in chromatographic column for elimination of ionic suppression effect, often impeding analysis of biological samples.

In a water solution the Hcy-NEM signal demonstrated a linear relationship with Hcy concentration for 0.01-10 μM concentrations at threshold detection (s/n~3) 5 nM (Fig. 3). In the plasma because of chemical noise increase the linear range and threshold detection were 0.025-10 μM and 10 nM Hcy, respectively. Virtually identical inclination angles for the model system and plasma demonstrated that the choice of CA-NEM as the internal standard was adequate.

The accuracy and error were evaluated by repeated injections of 150 nM solution of Hcy-NEM in water and plasma with minimized level of endogenous reduced Hcy. Analysis of the model solution (14 injections) showed 151±7 nM Hcy, which corresponded to an error of 4.6% and accuracy (correctness) of 100.7%. In the plasma (8 injections), Hcy level was 152±6 nM (error 3.8%, accuracy (correctness) 101.3%.

The reproducibility was studied on plasma samples for all three fractions of Hcy (Table 1).

The intergroup (between days) of analysis increased from ~4 to 12% for the Hcy_R>Hcy_F>Hcy_T series, while the error within the group varied less markedly, despite the use of diluted plasma solutions (with low levels of impurities) for analysis of Hcy_F and Hcy_T. This probably suggests that more complex sample preparation procedure leads to significant deterioration of the reproducibility of the analysis results, because preparation of samples for Hcy_T analysis involved reduction and subsequent modification of a greater number of compounds than preparation of samples for measurements of Hcy_F and Hcy_R.

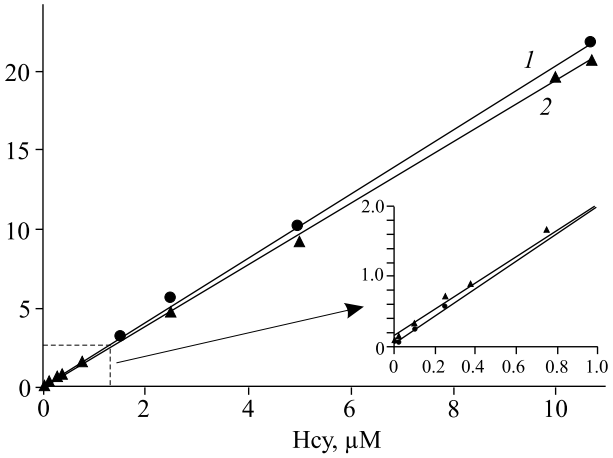

Fig. 3. Calibration curves, Hcy: model system (*1*) and plasma (*2*). Ordinate: Hcy-NEM/CA-NEM peaks. For water: y=2.0265x+0.0547, R^2=0.9995; for plasma: 1.9308x+0.0848, R^2=0.9995.

TABLE 1. Reproducibility of Rabbit Plasma Hcy Fraction Analysis

Fraction	Hcy, μM	±Error between groups (%)	±Mean error between groups (%)
Hcy_R	0.64	0.024 (3.8)	0.027 (4.2)
Hcy_F	3.9	0.3 (7.9)	0.2 (5.2)
Hcy_T	9.1	1.1 (12.1)	0.6 (6.6)

Evaluation of the fraction composition of plasma amino thiols is now little used primarily because of methodological problems. The use of MS detector solves some of them, for example, due to mass filtration and a wider choice of reagents for derivatization. For example, analysis of fractions of many of plasma amino thiols was carried out by capillary electrophoresis–MS [10]. However, the authors, using several maleimides, including NEM, preferred N-[2[(trimethylammonium) ethyl]maleimide chloride, despite good results of model experiments with NEM. Our previous experiments with capillary electrophoresis–MS showed ionic suppression in analysis of Hcy-NEM (data not published). The HPLC–MS platform allows measurements of Hcy-NEM without matrix effects of electrospray, the needed sensitivity is attained by increasing sample volume. Hence, the proposed analysis of Hcy fractions is highly sensitive, simple, specific, and economic and can be developed for the analysis of other amino thiol fractions.

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