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Liquid chromatographic determination of total homocysteine in blood plasma with photometric detection

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Abstract

A rapid and sensitive method for quantification of homocysteine total forms and glutathione levels in blood plasma via HPLC was developed. Dithiotreitol as a water soluble agent has been used as a reductant for both protein and nonprotein disulphides. Dithiotreitol reacts with the mixed disulphides under 60 °C treatment within 10 min. Reduced aminothiols and homocystein were easily derivated with 5,5′-dithiobis-(2-nitrobenzoic acid) and the resultant ultraviolet absorbance within 330 nm was detected by the HPLC method. The concentration of total plasma homocysteine was significantly higher in groups of patients: with the end stage of renal disease: 45.5 ± 40.9 μmol/l ($n = 79$), with cerebral vascular disorders 12.3 ± 7.0 μmol/l ($n = 65$), and with coronary atherosclerosis 15.4 ± 10.9 μmol/l ($n = 15$) than that in healthy subjects (6.2 ± 1.74 μmol/l, $n = 20$). Some major advantages of the method include: simultaneous measurement of both total homocysteine and total glutathione, no loss of oxidized form during processing of blood plasma for aminothiols measurement, use of protein-bound aminothiols solution as a calibrator.

Keywords: Homocysteine; Glutathione; Dithiothreitol

1. Introduction

Homocysteine is a sulphur-containing amino acid that is metabolized from methionine, an essential amino acid derived from dietary protein. More than 80% of homocysteine residues (Hcy) occur in plasma: most of them as conjugated to protein through disulphide bonding, as symmetrical disulphide homocysteine, as mixed disulphide homocysteine–cysteine, and as free thiol (less than 2%) [1–3]. Studies in the current literature show that formation of albumin-bound homocysteine proceeds through the generation of an albumin thiolate anion [4]. It is generally assumed that Hcy is exported from cells into circulation in the free reduced sulphydryl form due to the low redox potential of the intracellular milieu. Free homocysteine can undergo a reversible conversion to homocysteine thiolactone but it is present in very minor amounts in plasma, probably at nanomolar levels due to non-specific enzymatic hydrolysis [5].

One of the most critical steps in the sample-processing procedure is the reduction of disulphide bonds before derivatization [6]. In the HPLC method with photometric detection modification by Katrusiak et al., separation of homocysteine and other aminothiols including glutathione (Glt) is achieved after reduction with dithiothreitol and after pre-column derivatization with the thiol-specific reagent 5,5′-dithiobis-(2-nitrobenzoic acid) or Ellman's reagent (DTNB) [7]. These derivatives of aminothiols are detected during HPLC separation at 330 nm. DTNB was more commonly used for pre-column derivatization of Glt and cysteine [8,9]. In the present study, we investigate the action of DTT as a reducing agent under different temperature conditions and assess the method for its application in the serial analyses of Glt and Hcy derivatives from blood plasma.

2. Experimental

2.1. Chemicals

DL-Homocystine, L-cysteine, glutathione oxidized form, D-penicillamine (Pna), ethylenediaminetetraacetic acid (EDTA), 5,5′-dithiobis-(2-nitrobenzoic acid), and dithiothreitol, 5-sulfosalicylic acid were purchased from Sigma (St. Louis, MO, USA). Human serum albumin was obtained from

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Serva Chemicals (Heilderberg, Germany), HPLC grade acetonitrile was purchased from Criochrom (St. Petersburg, Russia) and Sephadex G 200 was from Pharmacia Fine Chemicals (Uppsala, Sweden). Potassium dihydrogen phosphate, *o*-phosphoric acid, and all other chemicals used in this study were of reagent grade.

2.2. Blood collection and processing

Twenty healthy adult volunteers of normal lifestyle (range 21–26 years, female:male = 1) were recruited as control subjects. The subjects were fasting and free from all medications, as determined by medical interview.

Sixty-five patients, 11 males and 54 females (range 30–55 years) with cerebral vascular disorders, were recruited from an out-patient neurology clinic. Each person gave written informed consent to participate in the study. Other patients were from clinics of St. Petersburg State I.P. Pavlov Medical University with confirmed diagnosis of coronary atherosclerosis ($n = 15$, range 35–60 years), and patients with the end stage of renal diseases ($n = 79$, range 40–60 years).

Blood was collected from healthy donors and patients by venipuncture into tubes with EDTA. The tubes were placed on ice and processed within 20 min. After centrifugation at 4000 × *g* at 4 °C for 7 min the clear plasma supernatant was collected and stored at −25 °C until analysis. The usual amount of plasma used for aminothiols determination was 0.3 ml.

2.3. Treatment of plasma samples and calibrator preparation

Powdered DL-homocystine was diluted with 20 ml of albumin solution (20 g/l in 0.15 mol/l sodium chloride with 4 mmol/l EDTA) to form 200 μmol/l stock Hcy preparation. The composed reaction mixture was incubated at 55 °C within 3 h in a shaking water bath. Then albumin solution was added to obtain the final Hcy concentration ranging from 0.1 to 200.0 μmol/l. Other aminothiols were prepared by dissolving Glt and Pna to the final concentration 400 μmol/l in EDTA 4 mmol/l. Only these preparations were used to improve derivatization procedure and to compose calibrator. The mixture of equal parts of the Hcy, Glt, and Pna solutions was applied as a calibrator at the final concentration of usually 20.0 μmol/l for each of them. The calibrator was mixed with the first and the final samples of the series. The first and the final samples of plasma were processed twice with and without calibrator as described below.

The derivatization procedure is a modification of the method originally published by Katrusiak et al. [7]. First of all we tried to avoid late mixing of the standard solution with the sample and the re-acidification step. Briefly, 100 μl of plasma sample was mixed with 50 μl of water or calibrator, incubated with 20 μl of DTT (10 mmol/l, dissolved in 0.5 M potassium phosphate buffer, pH 8.0) for 10 min at 60 °C to reduce the disulphides and release protein-bound Hcy. Then it was mixed with 100 μl of 0.5 mol/l DTNB dissolved in the same buffer and approximately 5 min later deproteinization was achieved by the addition of 150 μl of sulfosalicylic acid (90.0 g/l) containing 0.2 mmol/l EDTA. Precipitated proteins were removed by centrifugation at 8000 × *g* for 5 min, and the supernatant was filtered by 0.2 μm pore-sized, 3 mm filter, Aquilon manufacturing.

2.4. Chromatography conditions

Agilent 1100 HPLC system (Agilent Technologies, Germany) with a degasser, a quartery pump with low-pressure gradient flow control valve, a thermostat for the column, an autosampler, a VWD detector with a 14 μl cell and chemstation Leochem RUS verA08.03 were used. A reversed-phase Zorbax Eclipse XDB-C8 (150 × 4.6 mm i.d., 5 μm) column was placed in the thermostat at 30 °C. A mobile phase of 9% acetonitrile and 91% of 0.1 M pH 3.78 potassium phosphate buffer (filtered through 0.2 μm pore-sized filter) with the flow rate of 0.8 ml/min was used for isocratic elution of aminothiol derivatives which lasted for 4.7 min. Then, with the flow rate of 1.6 ml/min the buffer was substituted in the linear gradient mode for water by the 6.0 min, with the increase of acetonitrile amount up to 35%. From the 6.1 min up to the 7.0 min isocratic elution was going on with 80% acetonitrile and 20% of water and then by the 11.0 min a return to the initial amounts of the mobile phase components was achieved with the linear gradient mode and the flow rate of 1.6 ml/min. At the 12 min, the HPLC system was ready for the next separation in a programming sequence mode. The injection volume was 10 μl (or 2, or 50 μl). The Glt-, Hcy- and Pna-TNB peaks were detected at 330 nm.

2.5. Statistical analysis

The data were analyzed using the unpaired Student's *t*-test (two-tailed). Differences were considered significant when $P \leq 0.05$. the results are given as mean values with standard deviations (S.D.) or standard errors (S.E.). Statsoft® Statistica, release: 6, and Microsoft® Excel: 2002 were used for all statistical analyses.

3. Results

Analysis of blood plasma aminothiols using DTNB for derivatization was modified in our laboratory as the experiment required the condition under which disulphide forms of Glt and Hcy could be equally converted into the reduced ones. Besides, it was important to find a calibrator for modelling of blood plasma aminothiol condition.

3.1. Absorbtion spectra of derivatives

Glt-TNB, Pna-TNB and Hcy-TNB (TNB, thionitrobenzoate) had similar absorption spectra with the absorbtion

maximum between 320 and 330 nm, and correspondingly, the identical areas under the curve of chromatographic peaks at equal of the above-mentioned aminothiols concentrations. It was found, that complete derivatization of tGlt and tHcy with the DTT participation as reductant was achieved by treatment at 60–65 °C.

3.2. Optimization of protein-bound Hcy reduction

A substantial amount of tHcy is bound to plasma proteins especially to albumin [4,10]. This study of the reduction step of the Glt and Hcy mixture in the medium of albumin led to the optimization of derivatization, resulting in the complete detection of aminothiols in the chromatographic step.

As shown in Fig. 1, about 20% decrease in the added Glt concentration (20 μmol/l) was observed when the albumin solution (matrix) to which it had been added was incubated at 45 °C for 40 min. For practical use, it was important that both aminothiols derivated equally at 65 °C.

These clear up the situation with reduction of Hcy and Glt in the albumin matrices (Fig. 2). The concentrations of tGlt and tHcy (total forms) determined after reduction with DTT at 25 °C temperature were lower, on average, by 50 and 75%, respectively, than when the reduction was performed at 60 °C. Improvement of the previously described method was necessary, because in the present investigation different sensitivity of analysis with respect to the Glt-TNB and Hcy-TNB was not revealed.

3.3. Calibration procedure improvement

Stock preparation of albumin–Hcy was equilibrated with 0.05 M potassium phosphate buffer pH 7.4 with 0.15 M NaCl. Human plasma albumin with homocystine was stored at −4 °C within 20 h and applied (1 ml) to the column Sephadex G 200 (35.0; 2.0 cm). Albumin–Hcy disulphides and free forms of tHcy were then eluted from the column with the same buffer. The hold-up volume was withdrawn and the fractions (2 ml) were collected until the absorbance (A_{280}) was less than 0.1. Both the Hcy-deficient fraction and the Hcy-rich fraction were collected and tHcy was measured. About 80% of all tHcy was conjugated with matrix of 6–15 fractions. Protein concentrations in the fractions were determined according to Lowry.

In the proposed version of the procedure calibration solutions were added into a blood sample and recovery of the three aminithiols: Hcy, Glt and Pna (the latter being in the reduced form) was studied (Table 1). We calculated the of ratio of each area under the curve to the corresponding aminothiol concentration added to plasma. The ratio for Pna at 10.0 μmol/l was accepted as 100%.

Lower and upper limits of linearity for total aminothiols were confirmed at the following concentrations: Hcy 0.1–100 μmol/l; Pna 0.1–200 μmol/l; Glt 0.1–200 μmol/l. Linear regression of a typical six-point calibration curve used for total aminothiols generated the following equations and correlation coefficients: for tHcy, $y\,(\mathrm{mAU}) = 1.209x + 1.071$ ($r = 0.9992$); for tGlt, $y\,(\mathrm{mAU}) = 1.384x -$

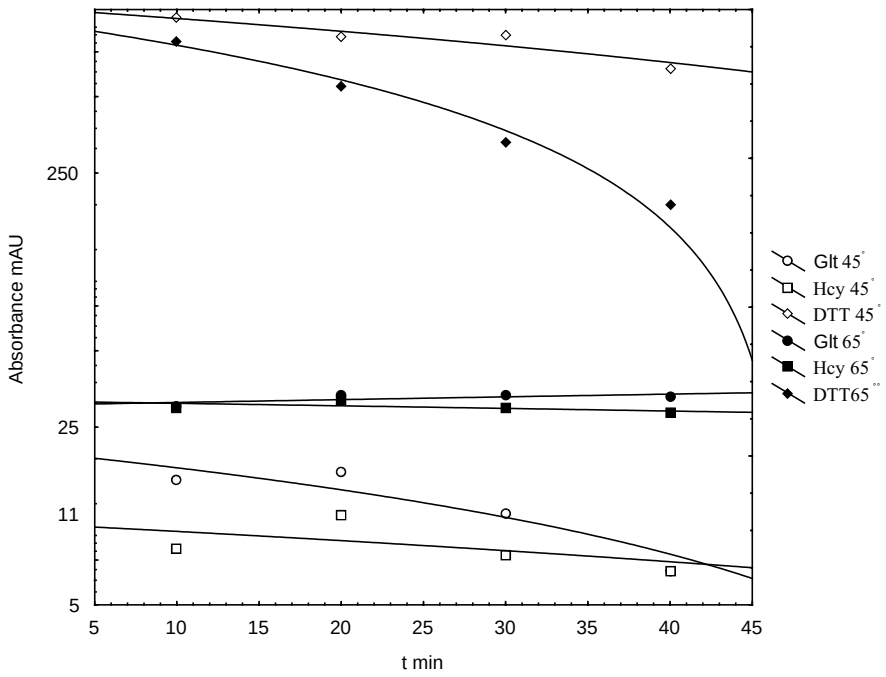

Fig. 1. Reduction of aminothiols and oxidizability DTT in 2% solution of human serum albumin depending on the time of incubation at different temperatures.

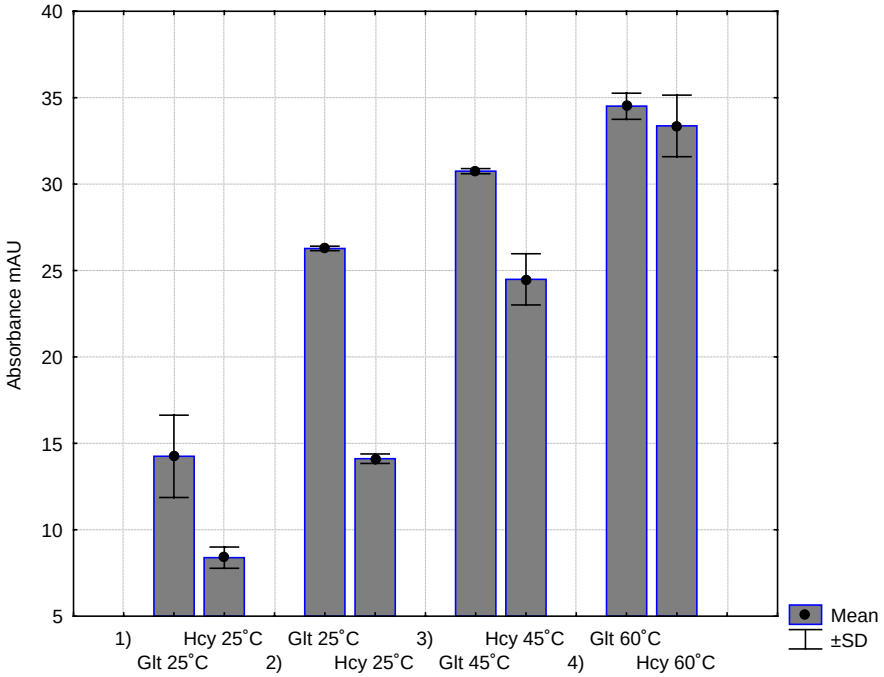

Fig. 2. Reduction of the oxidized aminothiols Glt 21 mkmol/l and Hcy 20 mkmol/l in the medium 1.76 mmol/l DTT, after incubation during 10 min: (1) aqueous solutions of the aminothiols; (2)–(4) solutions of the aminothiols in the 2% serum human albumin. Data are expressed as mean ± S.D., of four separate experiments.

Table 1
Recovery of aminothiols in plasma mixed with solutions of Gtt, Hcy, and Pna

Analyte	Recovery (%) aminothiols added to plasma in concentrations[a]		
	0.1; 0.5; 1.0; 2.0; 5.0 μmol/l ($n = 10$)	10.0; 20.0 μmol/l ($n = 18$)	50.0; 100.0 μmol/l ($n = 6$)
Gtt	100.1 ± 2.41	99.4 ± 1.97	102.2 ± 2.98
Hcy	99.2 ± 3.52	98.2 ± 1.84	98.8 ± 1.98
Pna	101.1 ± 1.48	100.0 ± 0.95	100.2 ± 1.23

[a] Result presented as mean ± S.E.

0.334 ($r = 0.9999$); for tPna, $y\,(\text{mAU}) = 1.376x + 0.342$ ($r = 0.9999$). The analyte concentration that produced a signal-to-noise ratio of 3:1 was accepted as the limit of detection. The detection limits for Glt and Hcy were approximately 0.02 and 0.028 μmol/l, respectively.

A typical chromatogram along with the corresponding retention time for each aminothiol in the calibrator is shown in Fig. 3. The intra-day retention time of Glt-TNB, Hcy-TNB, and Pna-TNB in 34 different samples was 3.80±0.20; 4.22± 0.15; 4.52 ± 0.12 min, respectively, and in case of 4 months ($n = 208$) that parameter was 3.85 ± 0.07; 4.29 ± 0.06; 4.52 ± 0.06 min, respectively. Parameter α for separation of Glt-TNB and Hcy-TNB in the course of analysis of 208 samples was 1.23 ± 0.019 (S.D.) For its calculation we used hold-up time value from the manufacturer column specification. Varying the pH of the mobile phase from 3.6 to 3.9 had not influence significantly on the separation between Hcy and Glt.

Table 2
Plasma levels of tHcy in a patients with the end stage of renal disease ($n = 79$), the cerebral vascular disorders ($n = 25$), the coronary atherosclerosis ($n = 15$), and healthy persons ($n = 20$)

Classes	tHcy (μmol/l)	CV (%)	*P*-value
Healthy persons ($n = 20$)	6.2 ± 1.74	28.1	
Coronary atherosclerosis ($n = 15$)	15.4 ± 10.89	70.7	0.0059
Cerebral vascular disorders ($n = 65$)	12.3 ± 7.01	57.0	$1.14E-08$
End stage of renal disease ($n = 79$)	45.5 ± 40.95	90.0	$9.5E-13$

Values are given as means and S.D.; *P*, by unpaired *t*-test to estimate the differences between the healthy persons and groups of patients.

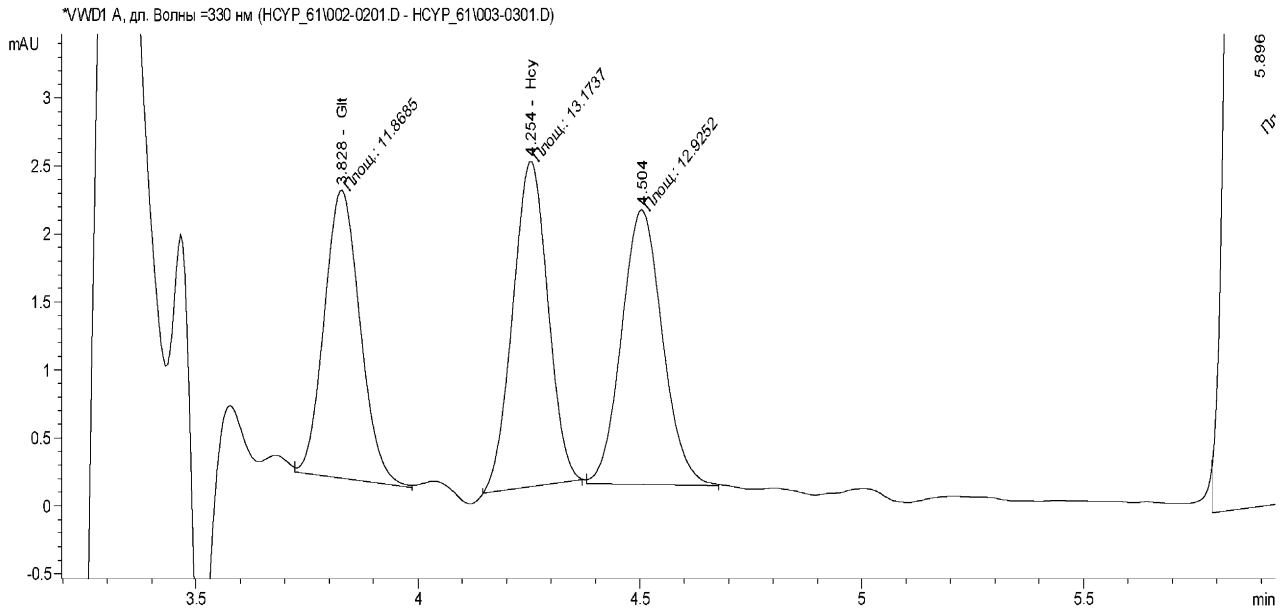

Fig. 3. Elution curve (deducting picture of two chromatograms) of three analyzed compounds (tGlt, tHcy, Pna) at the equal concentrations of 10 μmol/l, which was added to blood plasma, presented after Agilent chemstation autodeduct elution curve corresponding compounds presented in the initial plasma samples. TNB-anion, $t_R = 5.9$ min.

3.4. Serial blood plasma analysis

Observed mean tGlt indices in patients with cerebral vascular disorders were 4.8 ± 2.75 μmol/l (mean ± S.D.). No correlations between Glt and tHcy levels in the blood plasma of patients with cerebral vascular disorders were observed. Observed tHcy concentrations in the patients showed great variability (Table 2). But the concentrations of tHcy in plasma of the patients were significantly higher than those in healthy control subjects. As presented in Table 2 plasma tHcy concentrations in healthy donors (21–26 years) varied from 3.5 to 9.4 μmol/l, whereas those of tGlt showed large variability from 0.39 to 9.4 μmol/l (mean ± S.D. = 2.7 ± 2.69 μmol/l).

4. Discussion

In comparison to other aminothiols, most of Hcy in blood plasma exist in a protein-bound form, and according to Mansoor et al. its free/protein-bound ratio is approximately 0.2, for cysteine about 0.5–0.6, and for Glt 4–5 [1]. Later Sengupta et al. demonstrated that the Hcy binding velocity to protein was lower than that established for cysteine or Glt in the modelling reaction system with albumin or plasma, but finally in the aminothiol solution albumin-bound Hcy predominated [4]. Because of water solubility the reduced forms of Hcy are used as calibrators [11]. In our study we used albumin to solubilize homocystine, and to model protein-bound disulphides of plasma. Albumin solution with added oxidized Hcy and Glt was used as the calibrator in analytical procedure for serial plasmas performance. We used this calibration procedure because the equal yield of Hcy and Glt derivatives after heating for 5–10 min with DTT under 60–65 °C was observed (Figs. 1 and 2). Katrusiak et al. noted the difference between peak areas for Glt, Cisteine, on the one hand, and for Hcy on the other [7]. This chromogenic peculiarity of the Hcy derivative was not observed in our experiment. As described in Section 3, the temperature of the reduction step used in the assay may significantly influence the chromogenic product recovery. Furthermore, after 5–10 min of sample heating equal chromogenicy both for Glt and Hcy derivatives was observed. The procedure of derivatization with one step of acidification and centrifugation instead of the multistep procedure with two-fold acidifications, with the stage buffering in-between, and with twice repeated centrifugations was used.

We introduced the calibration procedure with two aminothiols to control the completeness of derivatization of both types of aminothiols. The use of water soluble Pna or Glt as the internal calibrator is required to check complete Hcy reduction and derivatization. In our series of probes the first and the last probes are analyzed twice with and without the addition of the calibrator.

DTNB derivatizaton tandeming to DTT reduction for aminothiol detection is a very simple and not expensive. Therefore, we evaluated DTT as a suitable reductant for tHcy determination in blood plasma. The sequence assay has a high capacity. About 100 specimens plus the appropriate calibrators were analyzed within 21 h.

Serial analysis with calibrator provides precise and accurate results for tHcy determination in plasma and for low

Hcy concentrations as well. The method is characterized its simplicity, speed and convenience in sample preparing during a short time. The authors suggest to use DTT with DTNB in HPLC method with spectrophotometric detectors both for research and routine tHcy determination in human plasma.

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