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ARTICLES

Determination of Nε-Homocysteinyl-Lysine and γ-Glutamylcysteine in Plasma by Liquid Chromatography with UV-Detection[1]

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Abstract—Homocysteine (**Hcy**) is a sulfur-containing nonprotein amino acid, a metabolite of methionine. Mechanisms by which Hcy is involved in the pathogenesis of vascular diseases remain unclear. One of the potential mechanisms underlying harmful effects of Hcy is the protein N-homocysteinylation induced by Hcy-thiolactone. Proteolytic degradation of N-homocysteinylated protein yields Nε-homocysteinyl-lysine, a novel and important component of Hcy metabolism. Here we describe new high-performance liquid chromatography assay for the determination of Nε-homocysteinyl-lysine and γ-glutamylcysteine in plasma, based on a derivatization with 2-chloro-1-methyllepidinium tetrafluoroborate and UV detection. Baseline separation was achieved on an analytical column from Phenomenex (Kinetex C18, 100 × 4.6 mm, 2.6 μm) using gradient elution, with a mobile phase consisting 0.1 M trichloroacetic acid (pH 2.3) – acetonitrile. The quantification limits for Nε-homocysteinyl-lysine and γ-glutamylcysteine in plasma were 0.1 and 0.2 μM, respectively. Other main endogenous thiols can also be measured during the same analytical run. The proposed method was applied for the analysis of 15 plasma samples for total form of Nε-homocysteinyl-lysine and γ-glutamylcysteine.

Keywords: Nε-homocysteinyl-lysine, γ-glutamylcysteine, main plasma thiols, 2-chloro-1-methyllepidinium tetrafluoroborate, HPLC, determination

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Despite advances in our understanding of cardiovascular diseases, coronary heart disease is still the major cause of mortality in industrial nations. Traditional risk factors such as hypertension, diabetes, hyperlipidemia, and smoking do not accurately predict cardiovascular events. Clinical studies have shown that plasma total homocysteine [1–3] and its metabolites: homocysteine thiolactone (**HTL**) [4] and N-homocysteinylated proteins (**N-Hcy-protein**) [5–8] are associated with cardiovascular disease and stroke and are strong predictors of mortality in cardiovascular patients.

tHcy comprises different Hcy species such as reduced, oxidized and protein-bound, each of which can exert a distinct biological effect, but does not encompass other Hcy metabolites, such as Nε-homocysteinyl-lysine isopeptide (**Nε-Hcy-Lys**). Because of this, a contribution of specific Hcy metabolites to cardiovascular risk or mortality may be overlooked by using tHcy as a marker.

Metabolism of Nε-Hcy-Lys is initiated by the conversion of Hcy to HTL catalyzed by methionyl-t-RNAsynthetase [9]. HTL spontaneously reacts with protein lysine residues generating N-Hcy-protein. Proteolytic degradation of N-Hcy-protein affords Nε-Hcy-Lys [10]. The isopeptide Nε-Hcy-Lys is present in humans and mice and its level increases in hyperhomocysteinemic subjects or mice [10]. Similarly to other Hcy metabolites (HTL, N-Hcy-protein), Nε-Hcy-Lys levels are elevated under pathological conditions, e.g., in human cystathionine-betha-synthase (**CBS**) deficiency as well as in mouse CBS or methylenetetrahydrofolate reductase deficiency. Recently it has been shown that Nε-Hcy-Lys is significantly increased in acute myocardial infarction (**AMI**) patients compared with controls and its formation is linked with the nitric oxide synthase inhibitor asymmetric dimethylarginine [11]. These findings have proved that Nε-Hcy-Lys can be used as a new marker of AMI independently of Hcy-related metabolites and cofactors.

γ-Glutamylcysteine (**γ-Glu-Cys**) is a precursor of glutathione (**GSH**). It is formed by γ-glutamylcysteine synthetase and used by glutathione synthetase to form GSH. GSH is a ubiquitous intracellular low-molecular-weight thiol that functions as a major endogenous

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antioxidant and redox buffer. It plays numerous roles in cellular defense including the detoxification of xenobiotics and peroxides and the maintenance of immune function.

Although there are numerous chromatographic methods for the determination of Hcy in biological samples [12–15], relatively few are available for the determination of γ-glutamylcysteine [16–19] and only one for Nε-Hcy-Lys [10].

Here, we describe a simple, fast, and sensitive HPLC method using derivatization with 2-chloro-1-methyllepidinium tetrafluoroborate (**CMLT**) and UV detection for the simultaneous determination of plasma total Nε-homocysteinyl-lysine (**tNε-Hcy-Lys**) and total γ-glutamylcysteine (**tγ-Glu-Cys**). Moreover, the other main endogenous plasma thiols cysteine, homocysteine, glutathione and cysteinylglycine can be also measured during the same analytical run if needed.

EXPERIMENTAL

Materials and Methods

Reagents. Nε-homocysteinyl-lysine was prepared in the laboratory according to the procedure described earlier [10]. D,L-Hcy-thiolactone hydrochloride was purchased from Lancaster (Eastgate, White Lund, Morecambe, England). D,L-homocysteine, γ-glutamylcysteine, cysteine (**Cys**), glutathione and cysteinylglycine (**Cys-Gly**) were from Sigma-Aldrich (St. Louis, MO, USA). Perchloric acid, sodium hydroxide, sodium hydrogen phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), HPLC-grade acetonitrile were from J.T. Baker (Deventer, Netherlands). *Tris*-(2-carboxyethyl)phosphine (**TCEP**) and trichloroacetic acid (**TCA**) were from Merck (Darmstadt, Germany).

Preparation of 2-chloro-1-methyllepidinium tetrafluoroborate. CMLT was synthesized in the laboratory according to the procedure elaborated earlier for 2-chloro-1-methylquinolinium tetrafluoroborate (**CMQT**) [14]. For this purpose 1 g of 2-chlorolepidine and 1 g of trimethyloxonium tetrafluoroborate were dissolved in 2 mL of nitromethane. After evolution of dimethyl ether has ceased (about 5 min), diethyl ether (4 mL) was added and the reaction mixture was put into refrigerator until appearance of crystals. White precipitate was filtered off, washed with diethyl ether (2 × 2 mL) and stored in a jar at 4°C. For thiol derivatization prior to HPLC, a 0.1 M aqueous solution of CMLT was used.

Instrumentation. For analytical separation a Hewlett–Packard 1100 Series HPLC instrument (Waldbronn, Germany), containing a quaternary pump, autosampler, thermostated column compartment, vacuum degasser, and diode array detector were used. For instrument control, data acquisition and analysis, Hewlett–Packard ChemStation for LC 3D system including single instrument ChemStation software and a Vectra computer were used.

Analytical procedure for determination of tNε-Hcy-Lys and tγ-Glu-Cys in plasma. To 50 μL of plasma 50 μL of phosphate buffer (pH 7.4, 0.2 M) and 5 μL of TCEP (0.25 M) in phosphate buffer solution (pH 7.4, 0.2 M) were added. The mixture was vortex-mixed followed by addition after 10 min 10 μL of 0.1 M CMLT. After 3 min 25 μL of 3 M perchloric acid was added and precipitated protein was separated by centrifugation for 10 min at 12000 g. The supernatant was transferred to a vial, and 10 μL was injected into the chromatographic system.

HPLC separation, detection, and quantification. Final analytical solution (10 μL) was injected onto a Kinetex C18 (100 × 4.6 mm i.d. packed with 2.6 μm particles) column from Phenomenex. A gradient elution was used with a flow rate of 1.0 mL/min. The elution profile was as follows: 0–10 min, 10–40% B; 10–12 min, 40–10% B (A : B, v/v). Elution solvent B was acetonitrile and solvent A was 0.1 M TCA water solution, adjusted to pH 2.3 with 1 M NaOH. The detection and quantification was by UV absorbance using 355 nm as analytical wavelength.

Method Validation Procedure

Linearity and specificity. Calibration curves for plasma tNε-Hcy-Lys and tγ-Glu-Cys were constructed by processing 50 μL of calibration standard samples of plasma, spiked with appropriate thiol and next analyzed in triplicate according to the procedure described in section "*Analytical procedure for determination of tNε-Hcy-Lys and tγ-Glu-Cys in plasma.*" The concentration ranges of Nε-Hcy-Lys and γ-Glu-Cys were 0.1–6.0 and 0.5–30 μM, respectively. Calibration curves were constructed by plotting the peak height against analyte concentration and fitted by least-squares linear regression analysis. Specificity was demonstrated by the ability to assess unequivocally the analytes in the presence of endogenous compounds of closely related structures Cys, Hcy, Cys-Gly and GSH.

Precision and accuracy. In order to judge the quality of the elaborated method, precision and accuracy were determined. They were measured in the conjunction with linearity studies. Three concentrations were studied: one at the lowest point on the standard curve, one in midrange, and one approaching the highest end of the calibration range. For intra-day precision and accuracy, samples of each concentration were analyzed in quintuple. Accuracy at each concentration levels was calculated by expressing the mean measured amount as percentage of added amount. Accuracy was calculated with the use of a formula:

$$\text{Recovery (\%)} = ((\text{measured amount} - \text{endogenous content})/\text{added amount}) \times 100\%.$$

Detection and quantification limits. The limit of detection (**LOD**) for calibrator samples was defined as

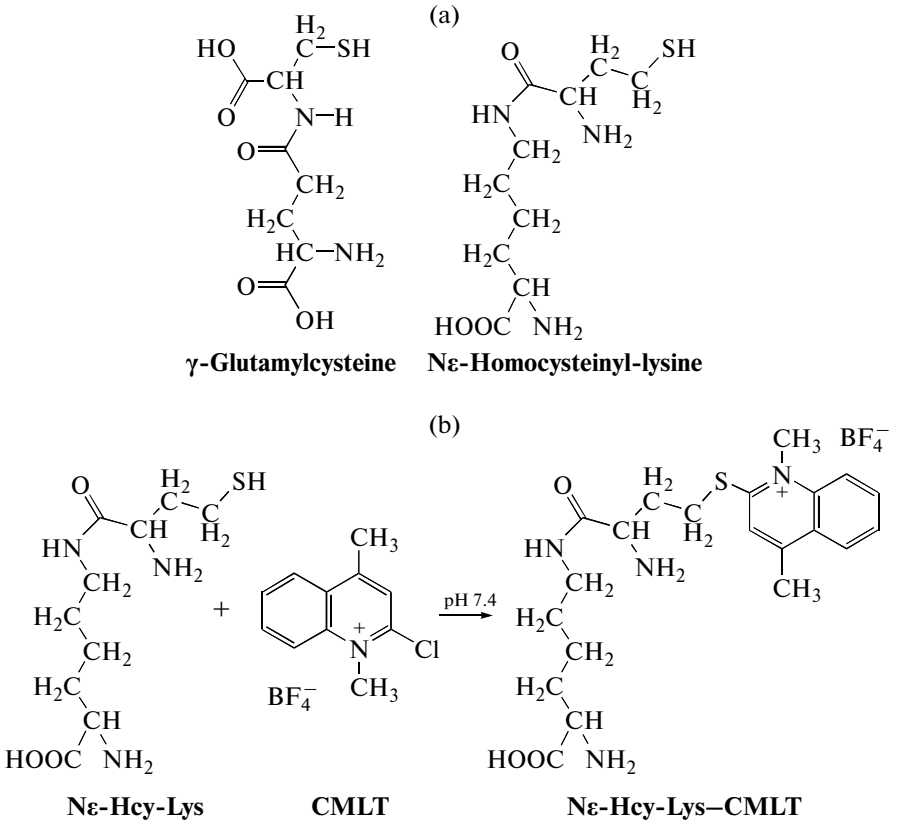

Fig. 1. Chemical structures of analytes (a) and equation for chemical derivatization reaction of plasma thiols, represented by Nε-Hcy-Lys, with CMLT (b).

the concentration that produces a signal-to-noise ratio of 3 : 1. The limit of quantification (**LOQ**) was the concentration that produces a signal-to-noise ratio of 6 : 1. These parameters were experimentally estimated by analysis of proxy matrix (0.1 M phosphate buffer, pH 7.4, containing 0.9% sodium chloride) spiked with the analytes.

RESULTS AND DISCUSSION

Cleavage of the disulfide bonds. A bulk of plasma thiols occur in the disulfide forms (symmetrical and mixed) and in order to render them accessible to the derivatization reagent disulfide bonds must be cleaved with a reducing reagent to liberate free thiols. A reducing agent is necessary both for the reduction of the sulfide bonds and to keep the thiol in a reduced form until start of derivatization. For this purpose TCEP was used [12].

Derivatization. Nε-Hcy-Lys and γ-Glu-Cys, similarly to majority of hydrophilic thiols, do not show the structural properties necessary for the production of signals compatible with the most common HPLC detectors, such as fluorescence or ultraviolet absorbance detector. Therefore, for signal enhancement and at the same time labile sulfhydryl group blocking several derivatization reagents were described in the literature [20, 21]. In this work for derivatization of Nε-Hcy-Lys and γ-Glu-Cys we have applied CMLT. The derivatization reaction equation of Nε-Hcy-Lys with CMLT as well as the chemical formula of both analytes are shown in Fig. 1**.** The derivatization reaction yield was optimized in terms of pH, reagent excess and time. Kinetic studies revealed that at pH 7.4 and with 10-fold reagent excess the reaction is virtually instantaneous with respect to thiol analytes (Fig. 2). Nε-Hcy-Lys and γ-Glu-Cys react quantitatively in plasma in the presence of slightly alkaline buffer to give corresponding 2-S-lepidinium derivatives. These stable thioethers show a well-defined absorption maximum at 350 nm (Fig. 3).

Chromatography. For optimum HPLC conditions for separation of CMLT derivatives of analytes from each other, reagent excess and other aminothiols or unidentified thiol matrix components, several mobile phase compositions and concentrations, as well as pH, temperature and flow rate were tested (Fig. 4). Preliminary experiments within method development were carried out on standard solutions. After a thorough study of the above mentioned chromatographic variables, the separation conditions chosen constitute a necessary compromise between maximum detectability, mobile phase pH stability and chromatographic

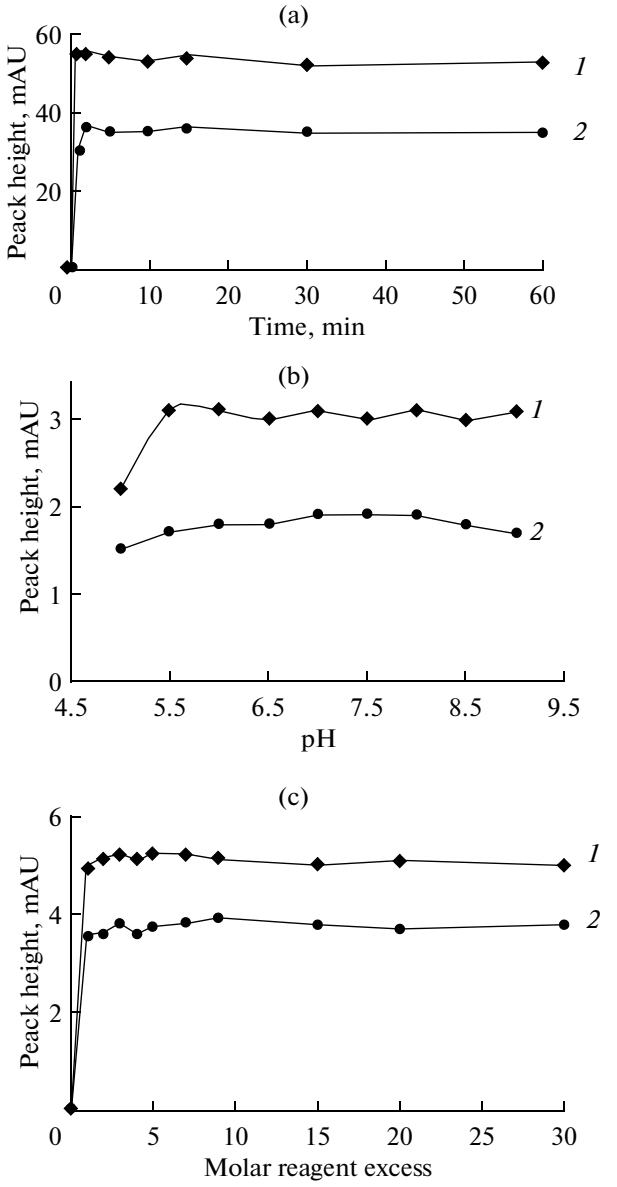

Fig. 2. Derivatization reaction yield as a function of time (a); pH (b); molar reagent excess (c). Other conditions: pH 7.4, ten-fold reagent excess (a); ten-fold reagent excess, time 1 min (b); pH 7.4, time 1 min (c); *1* – Nε-Hcy-Lys; *2* – γ-Glu-Cys.

resolution. Finally the mobile phase described in section "*HPLC separation, detection, and quantification*" was used. Typical chromatograms, obtained for plasma sample and standard solution of thiols are presented in Fig. 5. Under these conditions 2-S-lepidinium derivatives of Nε-Hcy-Lys and γ-Glu-Cys yield sharp peaks eluting at 4.8 and 6.4 min, respectively. Peaks of analytes were identified by comparison of their retention times and UV spectra with those of authentic standards. One of the most important factors limiting throughput of an HPLC analysis is column turnaround time. In our procedure is 8.5 min.

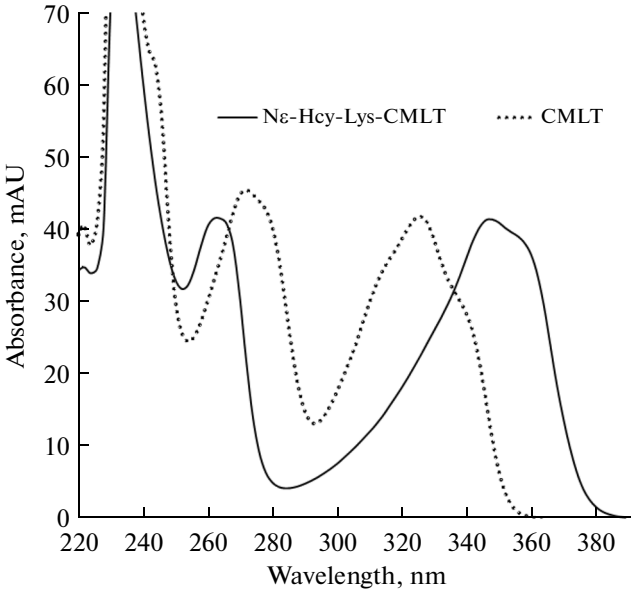

Fig. 3. Comparison of UV spectra of CMLT derivatization reagent and Nε-Hcy-Lys–CMLT derivative, taken at real time of analysis with DAD detector.

Validation. Validation procedure encompassed linearity, recovery, precision and limits of detection and quantification, which were tested according to the literature recommendations [22]. The peak heights of the thiol derivatives were plotted versus the analyte concentration and the curves were fitted by least-square linear regression analysis. The equations for the linear regression for tNε-Hcy-Lys and tγ-Glu-Cys were $y = 0.6675x + 0.4663$, $R^2 = 0.9997$ and $y = 0.5036x + 1.4154$, $R^2 = 0.9996$, respectively. Detailed calibration data are given in Table 1.

Intra-day precision and accuracy for the assay were determined for three concentrations representing the whole range of the calibration curve. The spiked plasma samples were processed according to the recommended analytical procedure for total thiols determination. Measured concentrations were assessed by application of calibration curve obtained on that occasion. The precision (RSD) for tNε-Hcy-Lys were within 4.2–7.4% and for tγ-Glu-Cys within 3.2–7.2%. Recovery, numerical value of accuracy, was 95.1–105.8% and 97.0–104.6%, respectively.

The LOD and LOQ values for Nε-Hcy-Lys and γ-Glu-Cys were 0.05 (0.1) and 0.1 (0.2) μM in plasma, respectively.

Stability of the 2-S-lepidinium derivatives. The 2-S-lepidinium derivatives resulted from the reaction are stable thioethers exhibiting a well defined absorption maximum at 350 nm. The plasma samples after derivatization and acidification with 3 M perchloric acid (final analytical solution) were kept at room temperature, and no significant changes in the peak heights were noted after 24 h (data not shown).

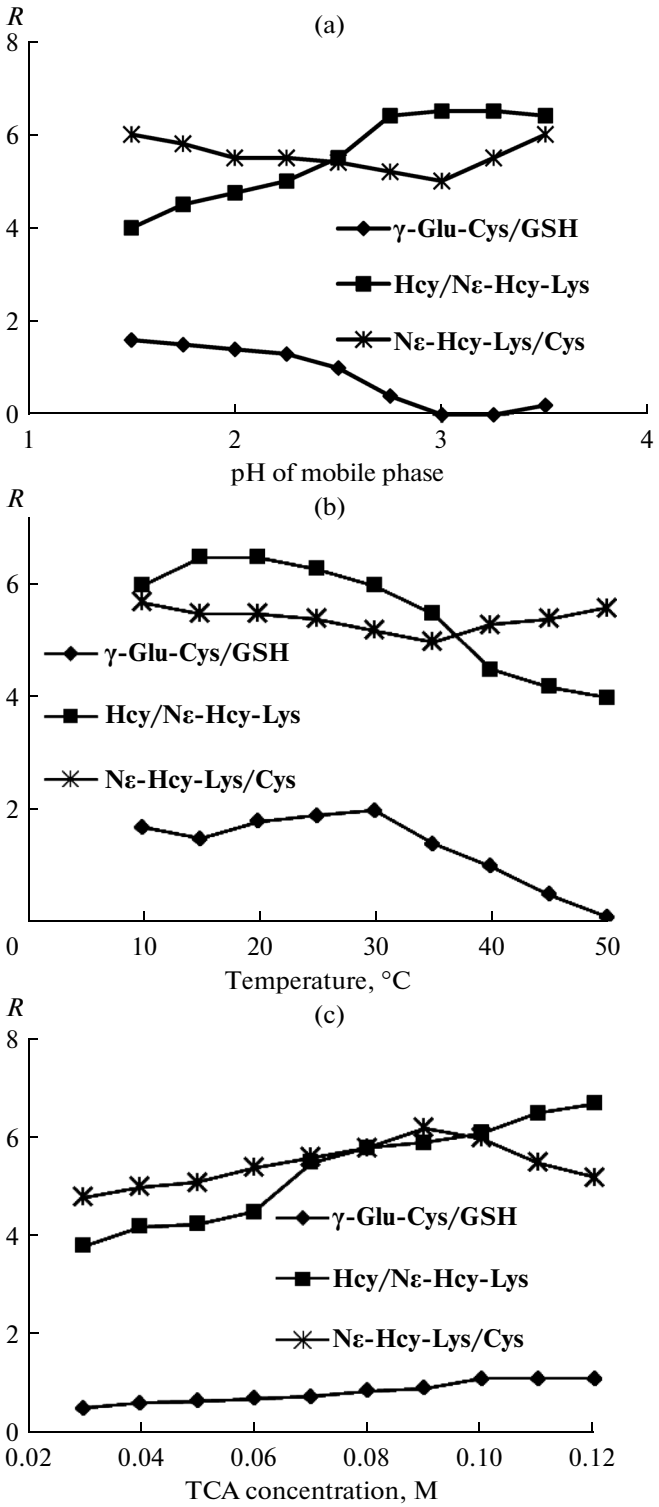

Fig. 4. Effect of mobile phase pH (a), column temperature (b) and mobile phase TCA concentration (c) on resolution. Other chromatographic conditions as described in section "*HPLC separation, detection, and quantification.*"

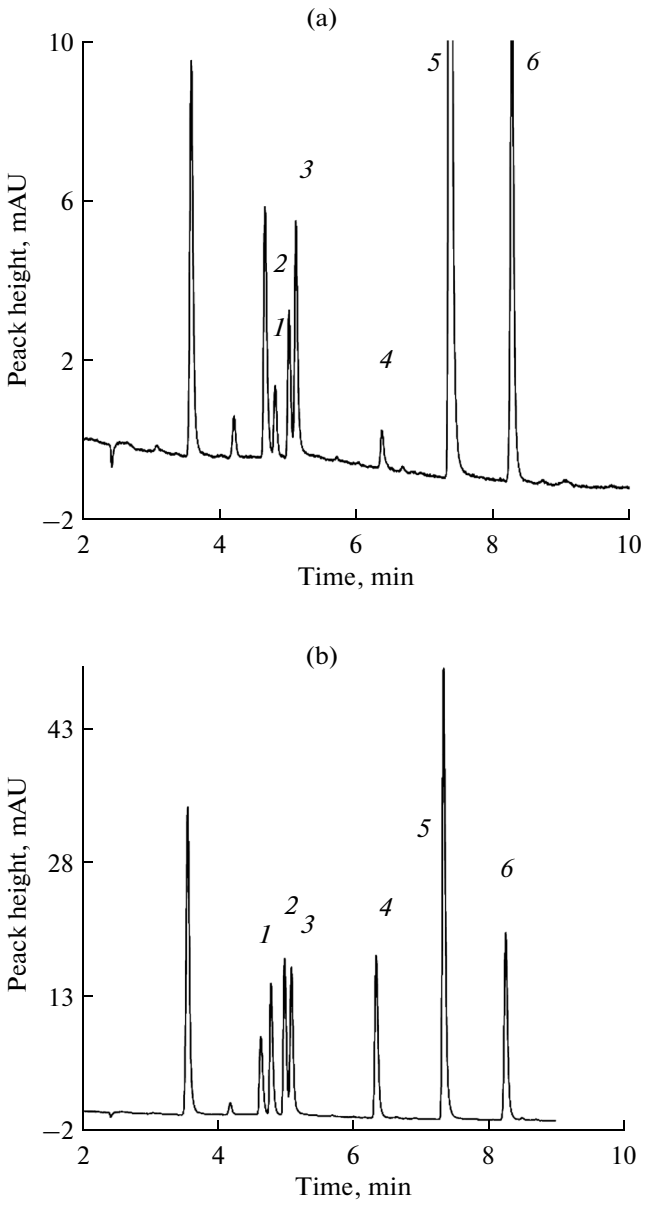

Fig. 5. A representative chromatogram of plasma sample after reduction with TCEP and derivatization with CMLT (a). Peaks: *1* (4.8 min) – γ-Glu-Cys (2.28 μM); *2* (5.0 min) – GSH; *3* (5.11 min) – Hcy; *4* (6.4 min) – Nε-Hcy-Lys (0.91 μM); *5* (7.3 min) – Cys; *6* – (8.3 min), Cys-Gly; chromatogram of water standard sample of thiols (10 nmol/mL each, except for Cys where it was 20 nmol/mL) derivatized with CMLT. Chromatographic conditions are described in "*Materials and methods*" section.

Application of the procedure to real samples. The developed method was applied to the analysis of 15 human plasma samples. Average total plasma Nε-Hcy-Lys and γ-Glu-Cys content was 0.35 ± 0.18 and 1.85 ± 0.58 μM, respectively. The maximum precision values (RSD, %, $n = 5$) for determination of Nε-Hcy-Lys and γ-Glu-Cys in plasma were 9.4 and 8.3%, respectively. For details see Table 2. Notably, such precision was obtained without including an internal standard and with no outliers excluded.

Table 1. Calibration data of the method

Added, mM	Found, μM	RSD, %	Recovery, %	Accuracy (E_{rel}), %
		Ne-Hcy-Lys		
0.10	0.10	6.8	102.2	2.2
0.20	0.21	3.3	103.9	3.9
0.40	0.37	2.8	93.6	–6.4
1.00	1.02	2.7	102.3	2.3
2.00	2.05	2.2	102.5	2.5
3.00	3.05	1.7	101.6	1.6
6.00	5.96	2.3	99.3	–0.7
		γ-Glu-Cys		
0.50	0.48	7.7	104.5	–0.9
1.00	0.98	6.2	98.1	–1.9
2.00	2.04	4.2	101.8	1.8
5.00	5.13	3.8	102.6	2.6
10.0	10.5	2.3	104.7	4.7
15.0	15.1	2.2	100.5	0.5
30.0	29.8	2.5	99.2	–0.8

Table 2. Application of the assay to the real samples ($n = 5$)

Ne-Hcy-Lys			γ-Glu-Cys		
found, mM	SD, μM	RSD, %	found, μM	SD, μM	RSD, %
0.19	0.01	5.66	1.94	0.07	3.63
0.46	0.03	6.96	1.53	0.08	5.51
0.22	0.02	9.43	1.14	0.04	3.69
0.36	0.02	5.89	1.60	0.04	2.64
0.37	0.02	5.66	1.09	0.03	2.57
0.50	0.03	6.33	1.19	0.06	4.71
0.36	0.02	5.89	1.91	0.11	5.89
0.26	0.01	4.04	2.48	0.14	5.66
0.30	0.02	7.07	1.69	0.14	8.32
0.17	0.01	6.15	1.39	0.03	2.02
0.31	0.02	6.73	2.48	0.14	5.66
0.30	0.02	7.07	3.18	0.00	0.88
0.91	0.01	1.17	2.28	0.14	6.15
0.26	0.01	4.04	1.86	0.13	6.81
0.31	0.01	3.45	1.94	0.07	3.63

* * *

The methodology described in the present communication allows facile and accurate simultaneous determination of plasma tNε-Hcy-Lys and tγ-Glu-Cys with the use of CMLT as derivatization reagent. The principal feature of the utility of CMLT is simplicity of the analytical procedure without compromising the sensitivity. The derivatization reaction mixture is ready to be chromatographed just after mixing of the substrates. The analytical figures of merit linearity, precision and recovery shown during the method validation procedure are well within the criteria for biological sample analysis.

There are numerous chromatographic methods for the determination of one or more thiols in plasma samples [12–15, 23]. To the best of our knowledge, the

HPLC method described herein is the first one that makes possible to determine of tNε-Hcy-Lys and tγ-Glu-Cys in plasma. Moreover, the other main endogenous plasma thiols Cys, Hcy, GSH and Cys-Gly can also be measured during the same analytical run (8.5 min HPLC time), if needed. Application of CMLT, a more hydrophobic derivatization reagent than CMQT, significantly improves separation. Obtained peaks are narrow and exhibit very good symmetry.

We believe that this method fulfils experimental and clinical requirements for routine determination of tNε-Hcy-Lys, tγ-Glu-Cys and main endogenous thiols in plasma. The new assay should help in elucidation of Hcy metabolism in health and disease.

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