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Journal of Chromatography A, 895 (2000) 157–166

JOURNAL OF CHROMATOGRAPHY A

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Determination of biologically active low-molecular-mass thiols in human blood
I. Fast qualitative and quantitative, gradient and isocratic reversed-phase high-performance liquid chromatography with photometric and fluorescence detection

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Abstract

The fast isocratic and gradient reversed-phase high-performance liquid chromatographic methods employing photometric and/or fluorescence detection are described for the precise reproducible simultaneous measurement of total homocysteine, cysteine, and glutathione in human blood. Sample preparation involves conversion of disulfides to free thiols with triphenylphosphine, precipitation of proteins with sulfosalicylic acid, and conjugation of thiols with monobromobimane. The aminothiol assay is optimized by reduction and derivatization step conditions (pH, temperature and time of reactions), as well as by chromatographic conditions to obtain reliable quantitative results within the concentration range corresponding to the levels of these thiols in human blood in norm and pathology. Its sensitivity allows the detection of aminothiol quantities >2 pmol. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Thiols; Homocysteine; Aminothiols; Monobromobimane; Cysteine; Glutathione; Amino acids

1. Introduction

Low-molecular-mass aminothiols (ATs) such as homocysteine (Hcy), cysteine (Cys) and glutathione (GSH) play a critical role in many biochemical pathways. Measurement of ATs in physiological liquids is important for diagnosing of several human diseases, especially premature arteriosclerosis, occlusive vascular and neurodegenerative disorders, leukemia, psoriasis, diabetes, cancer, acquired immunodeficiency syndrome, as well as for drug therapy monitoring [1–22].

Although a number of the techniques were employed to assay these thiols [1–31], including HPLC with different detection (fluorimetric [1,2,5–7,9,23,25–31], photometric [12–14], electrochemical [8,10,15,28], amperometric [24], chemiluminescence [30]), GC [16,25], GC–MS [25] and radioenzymatic methods [1,17], a well as the methods based on different ways of disulfide reduction and derivatization [1,2,5–9,23,26–31], some problems still arose due to an operation of sophisticated chromatographic

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PII: S0021-9673(00)00713-5

instrumentation or a complexity of the procedure and its quantitatation reproducibility, particularly in case of such multi-component samples as blood and urine so far. Simplification of the sample preparation, improving the sensitivity and reliability of the results is still actual. We developed a fast, simple, sensitive, and precise RP-HPLC method for identification and simultaneous quantitative determination of most important biologically active ATs, such as Hcy, Cys, and GSH. Besides, the version of our previously published method [23] based on disulfide reduction with triphenylphosphine (TP) and derivatization with monobromobimane (mBrB) was modified. Our modification of the sample preparation procedure and chromatographic conditions substantially improved the method. An on-line fluorescence detection was applied for determining biologically active ATs in complicated mixtures of the compounds, which hampered UV detection or for AT high sensitive assay. Higher selectivity and sensitivity of the fluorescence detection promoted to quantify AT–mB conjugates more precisely and reliably in a wider AT concentration range than UV detection. Due to the method simplicity and reliability, the developed procedure is suitable for routine analysis of tHcy and other biologically significant low-molecular-mass thiols in biological samples, required for clinical diagnostics and biochemical research.

2. Experimental

2.1. Chemicals and reagents

Ethylendiaminetetraacetic acid disodium salt (EDTA), sodium citrate, and dioxane were from Sigma (St. Louis, MO, USA). Monobromobimane (Thiolyte) was from Calbiochem (San Diego, CA, USA). L-Cysteine (Cys), L-cystine [$(Cys)_2$], Dl-*meso*-homocystine [$(Hcy)_2$], reduced glutathione (GSH), oxidized glutathione (GSSG), sulfosalicylic acid, triphenylphosphine, hydrochloric acid, 25% water ammonia, nitric acid (65%), formic acid were purchased from Merck (Frankfurt, Germany), and trifluoroacetic acid (TFA) was from Perkin-Elmer (Warrington, UK). Acetonitrile was from Criochrom (St.Petersburg, Russia). Purified water from a Milli-Q system, Millipore (Molsheim, France) was used throughout the experiments. The liquids used for HPLC systems were filtered through 0.22 μm membranes (Millipore) and degassed by helium for 3 min at 25°C. All solutions were mixed on a vibration mixer. Triphenylphosphine was prepared as 427.2 m*M* solution in water dioxane (80%, v/v) and 2 *M* hydrochloric acid. Monobromobimane was prepared as 8.72 m*M* solution in water acetonitrile (9%, v/v) containing 2 m*M* sodium EDTA. The working solutions of 20 μmol/ml Cys, GSH and 10 μmol/ml $(Cys)_2$, $(Hcy)_2$, GSSG in 0.2 *M* hydrochloric acid were prepared and kept at −20°C. Their aliquots were tested according to Section 2.4.

2.2. Instrumentation

HPLC analyses were performed on the system included two pumps Model 110 A (Beckman Instruments, San Ramon, CA, USA), spectrofluorometer Model 121 (Gilson, Villiers-le-Bel, France) with on-line spectrophotometer Model 100-40 (Hitachi, Yokohama, Japan), Controller Model 420 (Beckman Instruments), and an injector Model 210 (Beckman Instruments). The fluorescence detector output was recorded on Shimadzu Chromatopac C-R3A integrator (Shimadzu, Kyoto, Japan) and recorder Model 9176 (Varian AG, Zug, Switzerland), the photometer detector output was recorded on Shimadzu Chromatopac C-R3A integrator (Shimadzu) and Varian recorder Model 9176 (Varian AG). For pH measurement an Alkalit indicator (pH 6.0–10.0) (Merck, Darmstadt, Germany), a universal indicator (Lachema, Brno, Czechia) and Digital pH Meter pH 525 (Wissenschaflich-Technische Werkstatten, Germany) were applied. The centrifuge Model CLN 12 (Nauchpribor, Lvov, Ukraine), microcentrifuge Eppendorf 1540 (Eppendorf, Hamburg, Germany), vibrating mixer IKA Vibro Fix (IKA Werk, Hamburg, Germany) were used during sample preparation.

2.3. Preparation of plasma samples

The blood (4.5 ml) was obtained from overnight fasting healthy laboratory personnel and cardiology reanimation patients by venipuncture and then drawn into vacutubes containing 0.5 ml 3.8% (w/v) sodium citrate in water, immediately centrifuged for 10 min

at 3000 rpm (10 000 *g*) to obtain plasma, which was decanted, frozen, and stored at −70°C.

2.4. Determination of total homocysteine, cysteine and glutathione in plasma

0.062 ml volume of thawed plasma was pipetted into 1.4-ml snap-cap polypropylene centrifuge vial containing 0.005 ml 0.2 *M* hydrohloric acid, after addition of 0.025 ml of 427.2 m*M* triphenylphosphine, was capped, vigorously mixed and incubated for 15 min at room temperature. Sulfosalicylic acid (44%, w/v, 0.010 ml) was added, the snapped caps were mixed, incubated for 10 min at room temperature and centrifuged at 10 000 *g* for 5 min. Aliquots of the supernatant solutions (0.02 ml) were pipetted into clean 1.4 ml polypropylene snap-cap centrifuge vials. Then 0.03 ml of 8.72 m*M* monobromobimane was added to each vial, and after mixing 0.015 ml of 0.1 *M* sodium hydroxide was pipetted. The samples were mixed and incubated for 5 min in darkness at room temperature. The vials were centrifuged at 10 000 *g* for 1 min and 0.005–0.01 ml of each sample was applied to the reversed-phase HPLC system. Sample vials were stored at −20°C in darkness.

2.5. Preparation of calibration standards

Stock solutions of 50 μmol/ml $(Hcy)_2$, 600 μmol/ml Cys and 50 μmol/ml GSSG in 0.2 *M* hydrochloric acid were prepared. The working solutions were prepared by appropriate dilutions with 0.2 *M* hydrochloric acid as needed. For the preparation of calibration standards of human plasma, aliquots (0.062 ml) of plasma from a healthy donor were placed in a polypropylene microcentrifuge tubes (Eppendorf) containing 0.005 ml of the working standard solution. Concentrations of exogenous ATs were 0.5, 1, 2, 3.9, 7.8, 15.7, 31.3, 62.5, 125, 250, 375, 500 nmol/ml plasma for homocysteine and reduced glutathione; and 0.5, 1, 5.1, 10.2, 20.4, 40.7, 81.3, 122, 162.5, 244.3, 325, 497.5, 750.0 nmol/ml plasma for cysteine, assuming 100% disulfide reduction. Aliquots of the calibration standards were applied to determine the total homocysteine, cysteine, and glutathione contents as described in Section 2.4.

2.6. Chromatography

Samples were injected manually into the 20 μl Model 210 injector loop by Hamilton syringe (Hamilton, Reno, NV, USA). The column (75×4.6 mm) packed with 3-μm particles of Ultrasphere ODS (Beckman Instruments) equipped with in-line pre-column filter (2 μm diameter) (Upchurch Scientific, Oak Harbor, WA, USA) was used. The column temperature was 25°C; photometer detector wavelength was 250 nm. The fluorescence detector operated with an exiting wavelength of 300 nm, an emission wavelength 470 nm. The elution gradient profiles were as follows:

1. Profile 1: 0 min — 5% B; 0–5 min — 5–9% B; 5–9 min — 9–100% B; 10–11 min — 100–5% B. Elution solvent A was 30 m*M* ammonium nitrate and 40 m*M* ammonium formate, pH 3.6; solvent B was acetonitrile. The flow-rate was 1.5 ml/min. Sample volume was 7.5 μl.
2. Profile 2: (a) separation conditions: 0 min — 12.5% B; (b) column regeneration conditions: 7.5–8 min — 12.5–100% B, 9–10 min — 100–12.5% B. Elution solvent A was 0.1% TFA in water, pH 2.2, solvent B was acetonitrile. The flow-rate was 0.75 ml/min. Sample volume was 5 μl.

2.7. Statistics

Standard curves for plasma homocysteine, cysteine, and glutathione were constructed by adding the known amounts of $(Hcy)_2$, Cys, or GSSG, respectively, to normal plasma followed by the assay as described in Sections 2.5 and 2.6. The added $(Hcy)_2$ and GSSG ranged from 0.5 to 500 nmol/ml, Cys from 0.5 to 750 nmol/ml. The absolute fluorometer peak areas were plotted vs. the analyte concentration and the curves were fitted by least-square linear regression analysis. A within-run precision for the method was obtained by injecting the same sample for three times consequently to the HPLC system with the both eluent types and comparing the peak areas for cysteine, homocysteine, and glutathione derivatives obtained for every three injections. A between-run precision was determined by derivatizing the same sample for three different times and injecting the resulted three derivative mixtures in-

dependently to the HPLC system with the both eluent types. Data from recovery and precision studies are presented as mean±SD.

3. Results

The total plasma homocysteine, cysteine, and glutathione contents were determined in human plasma in a few steps: (i) reduction of disulfides with triphenylphosphine; (ii) deproteinization with sulfosalicylic acid; (iv) derivatization of reduced aminothiols with monobromobimane; (v) separation of aminothiol–monobimane conjugates by reversed-phase HPLC with photometric and fluorescence detection. Fast chromatographic solution with isocratic elution of a very simple and popular in daily use water–acetonitrile–trifluoroacetic acid elution buffer system for determination of homocysteine and glutathione in human blood as well as fast chromatographic solution with gradient elution an ammonium nitrate–ammonium formate–acetonitrile elution buffer system to determine of all mentioned low-molecular-mass aminothiols in human blood were developed. Resolution and detection of homocysteine, cysteine, and glutathione in human plasma on the RP-HPLC system with elution gradient profile 1 are shown in Figs. 1–5 and with profile 2 in isocratic conditions in Figs. 6–9. Under the conditions described, the homocysteine–monobimane conjugate has a retention time of 5.06 (±0.09) min in elution profile 1 and 4.70 (±0.06) min in isocratic con-

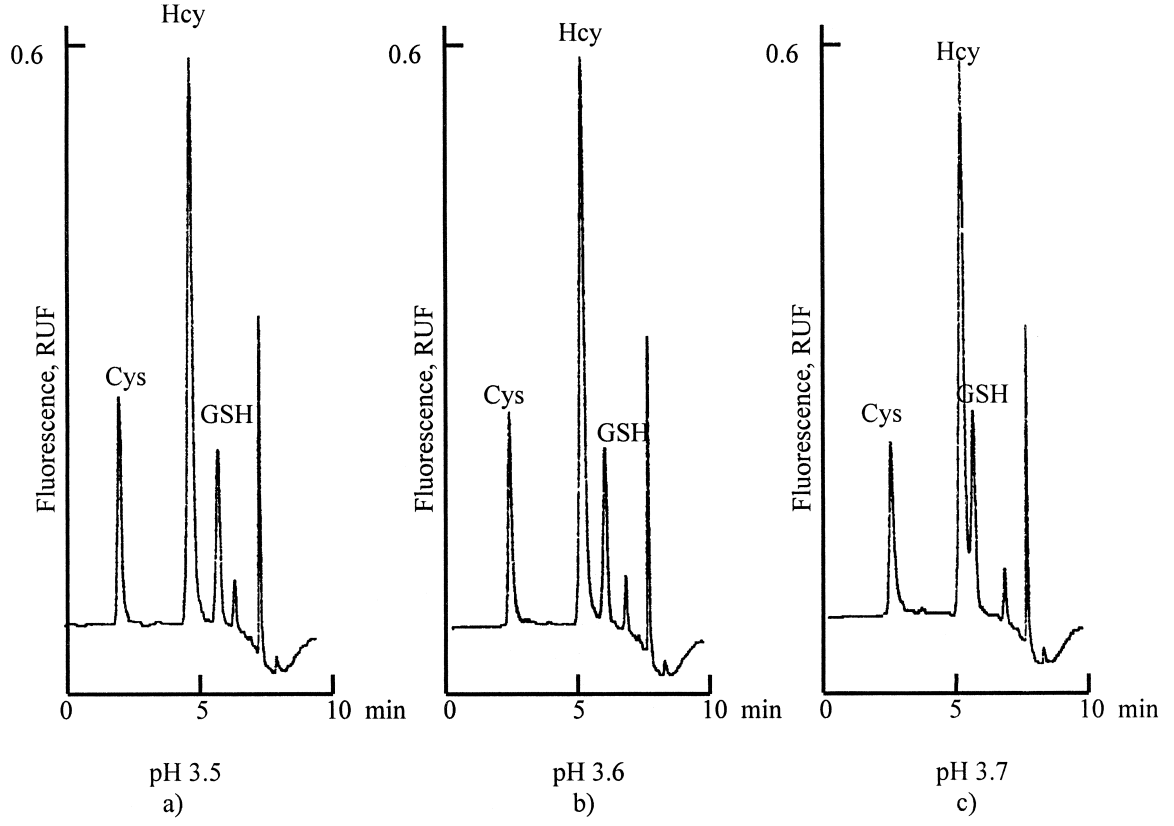

Fig. 1. RP-HPLC identification of monobimane conjugates of standard aminothiols with fluorescence detection. Buffer A pH influence on mB–aminothiol separation selectivity. The aminothiol content is 200.0 nmol/ml for cysteine (a) 2.94 min, (b) 2.97 min, (c) 2.93 min), 450.0 nmol/ml for homocysteine (a) 5.07 min, (b) 5.10 min, (c) 5.04 min and 180.0 nmol/ml for glutathione (a) 5.61 min, (b) 5.48 min, (c) 5.29 min. The eluent gradient profile was as follows: 0 min — 5% B; 0–5 min — 5–9% B; 5–9 min — 9–100% B. Elution solvent A was 30 m*M* ammonium nitrate and 40 m*M* ammonium formate (a) pH 3.5, (b) pH 3.6, (c) pH 3.7; solvent B was acetonitrile.

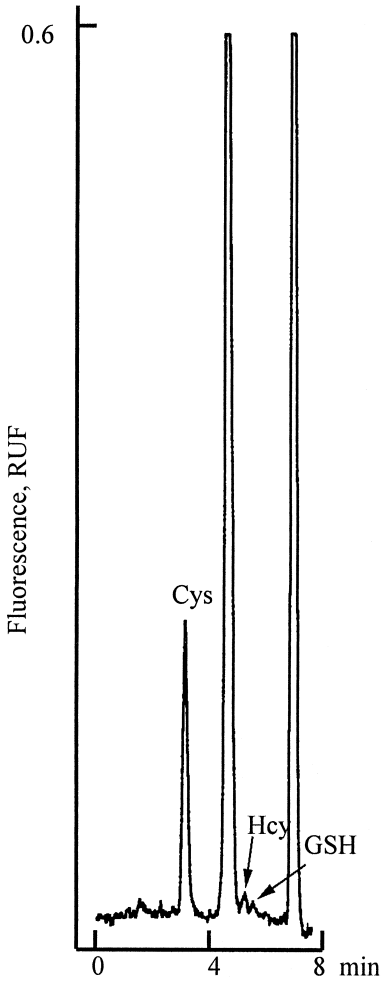

Fig. 2. RP-HPLC aminothiol identification in human plasma with fluorescence detection. The aminothiol content is 196.8 nmol/ml for cysteine (2.98 min), 8.4 nmol/ml (norm) for homocysteine (5.07 min) and 6.3 nmol/ml for glutathione (5.46 min). Elution solvent A was 30 m*M* ammonium nitrate and 40 m*M* ammonium formate, pH 3.6; solvent B was acetonitrile.

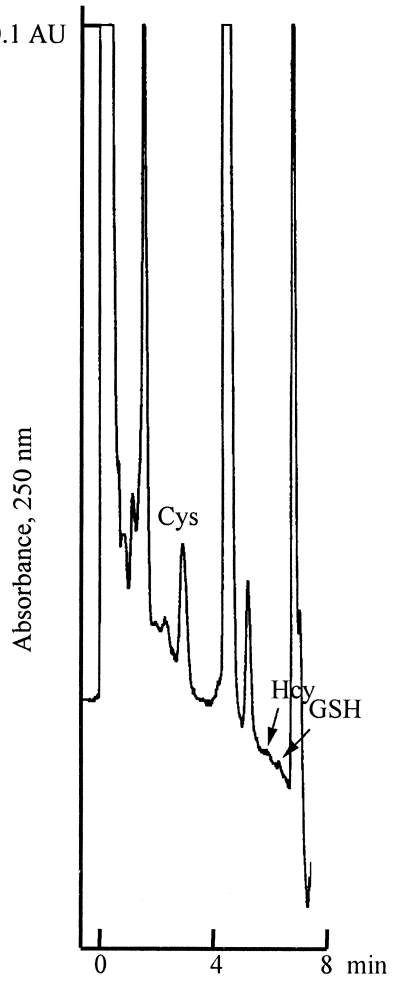

Fig. 3. RP-HPLC aminothiol identification in human plasma with photometric detection. Concentration and conditions as in Fig. 2. Baseline sites corresponding to derivatized homocysteine and glutathione elution time marked by arrows.

ditions of profile 2; the glutathione–monobimane 5.47 (±0.11) min and 5.27 (±0.12) min, respectively, and the cysteine–monobimane, mB-derivative of the major plasma low-molecular-mass aminothiol, eluting with a retention time of 2.96 (±0.06) min in elution gradient profile 1. We used two detection types for the method application in human plasma analysis. The detection wavelength of 250 nm was chosen as the local extinction maximum of thiol–monobimane conjugates for photometry detection. Higher sensitivity and selectivity of the fluorescence detection system promoted to quantify AT–mB conjugates more precisely and reliably in a wider AT concentration range than in the photometry detection system. Fluorescence detection was used for routine qualitative and quantitative plasma aminothiol assays. Photometry detection could be successfully applied for certain determination of pathological concentration levels of homocysteine as well as other aminothiols contents.

Standard curves for plasma homocysteine, cysteine, and glutathione were constructed by adding

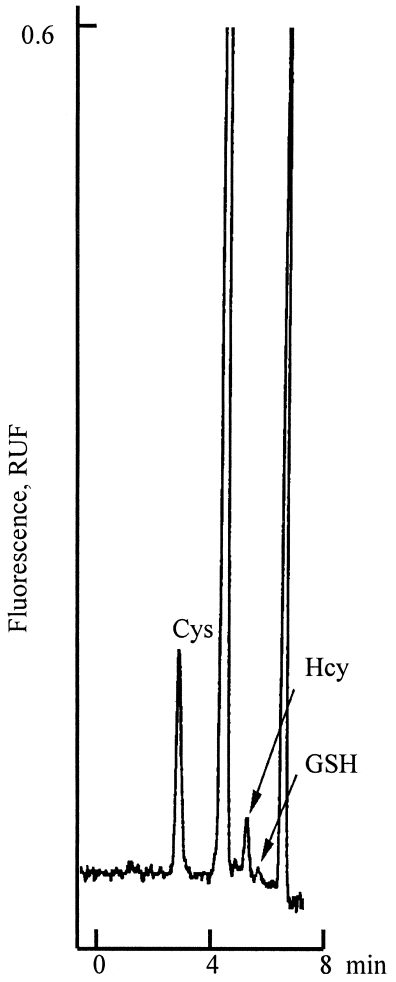

Fig. 4. RP-HPLC aminothiol identification in human plasma with fluorescence detection. The aminothiol content is 181.4 nmol/ml for cysteine (3.03 min), 67.5 nmol/ml (pathology) for homocysteine (5.09 min) and 5.7 nmol/ml for glutathione (5.51 min). Elution solvent A was 30 m*M* ammonium nitrate and 40 m*M* ammonium formate, pH 3.6; solvent B was acetonitrile.

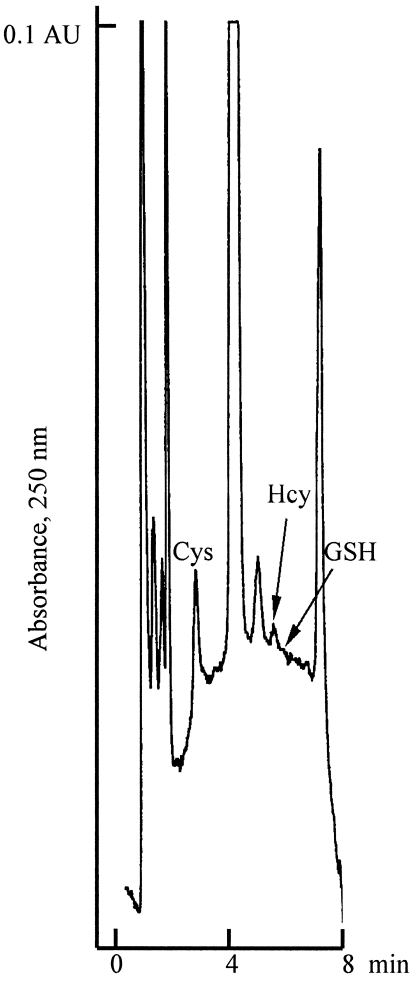

Fig. 5. RP-HPLC aminothiol identification in human plasma with photometric detection. Concentration and conditions as in Fig. 4. Baseline site corresponding to derivatized glutathione elution time marked by arrow.

the known amounts of authentic $(Hcy)_2$, Cys, or GSSG standard to normal plasma followed by immediate assay. The total concentration (μM) of three ATs in plasma ($\pm$SD) obtained by both the elution profile HPLC assays with fluorescence detection from 17 healthy overnight fasting males was 219.32 ($\pm$31.14) for Cys; 11.68 ($\pm$3.67) for Hcy, and 7.12 ($\pm$2.61) for GSH, and from 16 healthy overnight fasting females it was 215.28 ($\pm$34.57) for Cys, 9.75 ($\pm$2.91) for Hcy, and 6.63 ($\pm$2.49) for GSH. These results are similar to published data obtained by different techniques [1–3,5–10,12–22] and to our previous results [23]. The assay was applied to determine the ATs content in blood of cardiology reanimation patients and showed the tHcy pathological level in a number of cases (Figs. 4, 5, 8 and 9).

A within-run precision for the method was obtained by injecting the same sample for three times consequently to the HPLC system with both the elution profiles and comparing the peak areas for cysteine, homocysteine, and glutathione derivatives

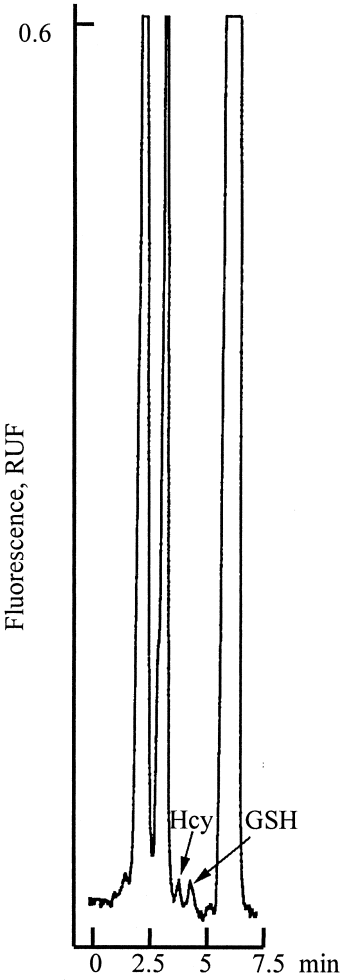

Fig. 6. Isocratic RP-HPLC aminothiol identification in human plasma with fluorescence detection. The aminothiol content is 9.8 nmol/ml (norm) for homocysteine (4.68 min) and 11.2 nmol/ml for glutathione (5.25 min). The eluent was 12.5% B. Elution solvent A was 0.1% TFA in water (pH 2.2), solvent B was 0.1% TFA in acetonitrile.

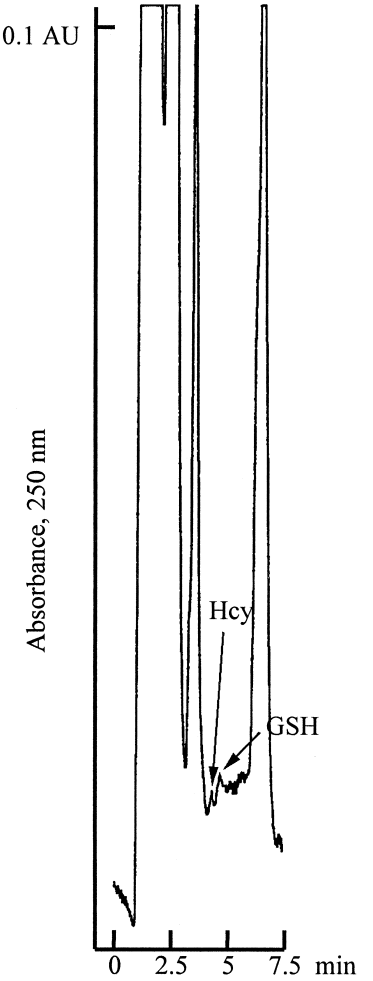

Fig. 7. Isocratic RP-HPLC aminothiol identification in human plasma with photometric detection. Concentration and conditions as in Fig. 6.

obtained for three injections. The variation coefficient obtained for the within-run precision was 4.73% for the Cys derivative peak, 4.59% for the Hcy derivative, and 4.81% for the GSH derivative. A between-run precision was determined by derivatizing the same sample for three different times and injecting three resulted mixtures independently to the HPLC system with both the elution systems. A satisfactory analytical precision (4.91% RSD) was obtained without inclusion of an internal standard. The sensitivity of the method allows the detection of AT quantities >2 pmol.

4. Discussion

This method was based on our previously published assay [23] with some changes in the sample preparation procedure and developing more preferable chromatographic conditions. We tried out to simplify a sample preparation step and to develop efficient and common HPLC conditions for the mB–

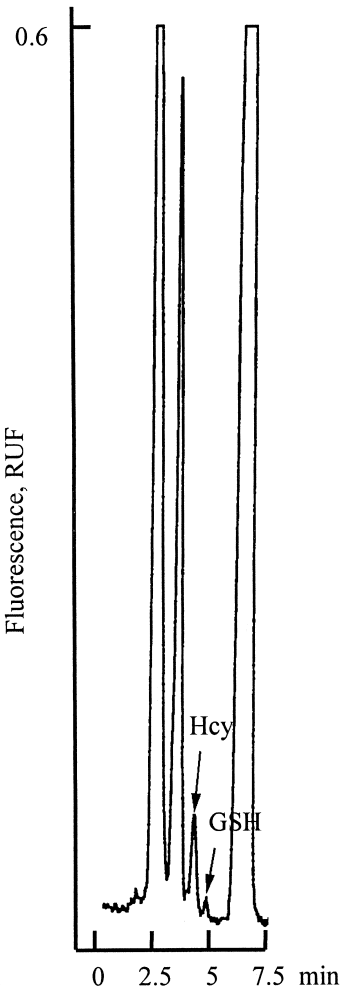

Fig. 8. Isocratic RP-HPLC aminothiol identification in human plasma with fluorescence detection. The aminothiol content is 97.3 nmol/ml (pathology) for homocysteine (4.65 min) and 10.8 nmol/ml for glutathione (5.22 min). Conditions as in Fig. 6.

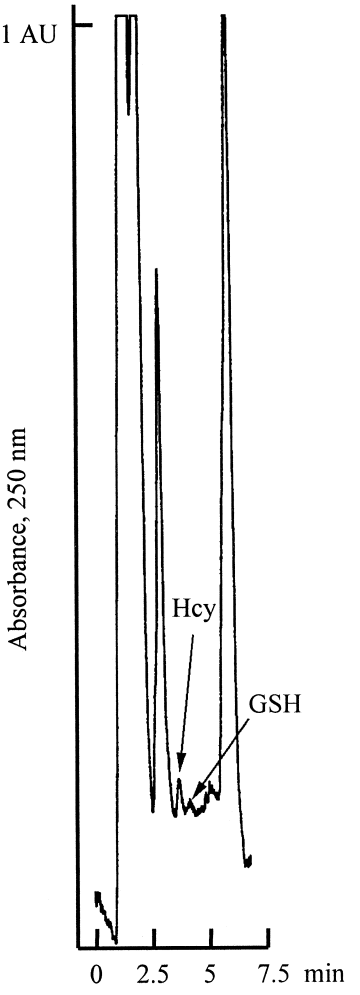

Fig. 9. Isocratic RP-HPLC aminothiol identification in human plasma with photometric detection. Concentration and conditions as in Fig. 8.

AT determination with photometry and fluorimetry detection. According to Figs. 1–9, these modifications resulted in an acceptable separation of AT–monobimane conjugates providing correlation of quantitative data with the assay results published. We omitted the heating steps during reduction and protein precipitation procedures that allowed us to minimize without method sensitivity loss the formation of fluorescent monobimane hydrolysis byproducts, interfering with the detection and separation of AT–monobimane conjugates. We replaced deproteinization agent trichloroacetic acid by sulfosalicylic acid to increase the AT contents in the samples with a decrease of the protein precipitation agent volume added. The sodium hydroxide (1 *M*) volume was also changed for optimal derivatization pH (~8.5) adjustment [23]. We developed fast chromatographic conditions with the earlier proposed [21,22] 30 m*M* ammonium nitrate–40 m*M* ammonium formate–acetonitrile elution system and showed the dependence of mB–ATs separation selectivity on the buffer pH (Section 2.6, Fig. 1). Another approach to separate the mB–ATs with a

common, easy-making, and prevailing water–acetonitrile–TFA elution system (Section 2.6) was developed for fast quantitative and qualitative determination of homocysteine and glutathione in isocratic elution conditions (Figs. 6–9). The main disadvantage of the isocratic system is co-elution of mB–Cys with one of the reaction byproducts (3.52 ± 0.13 min). But there are plenty of cheap, wide-spread, and common techniques for cysteine determination. Moreover, the proposed isocratic elution conditions let us cope with the main task, i.e. homocysteine content determination, in a very short time, favorably compared with our gradient elution conditions (profile 1) and the separation techniques published [1,2,5–11,21–23] with profitable difference (more than half) in eluent consumption.

The sensitivity of the developed method is favorably compared with that of other low-molecular-mass AT assays [1,2,7,8,21,22]. High chromatographic resolution, low noise, combination of dual detection types, linearity of the standard curves ($r^2 > 0.998$), as well as high fluorescence intensity of AT derivatives stipulated the assay accuracy. HPLC conditions are favorably compared with some assays in their simple performance, without use of several column types switching and complicated mobile phase [1,2,5,11].

In conclusion, the present assay includes three stages established to determine the reduced, oxidized, and protein-bound homocysteine, cysteine and glutathione as total amounts in human plasma. The oxidized and protein-bound ATs were converted to their reduced forms by employment of triphenylphosphine; following precolumn derivatisation of free sulfhydryls with mBrB; the aminothiol–monobimanes were separated and quantified by RP-HPLC with fluorescence and additional on-line photometry detection. Application of this approach with two detection types demonstrated possible Hcy determination at pathological concentrations in human plasma with high reliability by only use of common and widespread UV detection. The method was simplified and optimized in reduction and derivatization procedures to obtain the thiol-monobimane derivative maximum yield.

The method reported has several advantages: (1) simple sample preparation procedure; (2) low hydrolysis rate under optimized pH and temperature conditions of the derivatization reaction; (3) simultaneous determination of all mentioned low-molecular-mass aminothiols during the procedure; (4) fast chromatography procedure; (5) simple and prevalent mobile phases. Its final advantage is its easy use and high reliability.

Acknowledgements

We would like to express our sincere thanks to Dr. N. Fedorova, Dr. A. Ksenofontov and Dr. V. Merkulov for encouragement, assistance, and profitable discussions.

References

[1] H. Refsum, P.M. Ueland, A.M. Svardal, Clin.Chem. 35 (9) (1989) 1921.
[2] M.A. Mansoor, A.M. Svardal, P. Ueland, Anal. Biochem. 200 (1992) 218.
[3] R. Munday, Free Rad. Biol. Med. 7 (1989) 659.
[4] V.C. Wiley, N.P.B. Dudman, D.E.L. Wilcken, Metabolism 37 (1998) 191.
[5] D.J. Jacobsen, V.J. Gatautis, R. Green, Anal. Biochem. 178 (1989) 208.
[6] P.M. Ueland, H. Refsum, J. Lab. Clin. Med. 114 (1989) 473.
[7] R.A. Winters, J. Zukowski, N. Ercal, R.H. Matthews, D.P. Spitz, Anal. Biochem. 227 (1995) 14.
[8] D.L. Rabenstein, G.T. Yamashita, Anal. Biochem. 180 (1989) 259.
[9] N.K. Burton, G.W. Aherne, J. Chromatogr. 382 (1986) 253.
[10] E.G. Demaster, F.N. Shirota, B. Redfern, D.J.W. Goon, H.T. Nagasawa, J. Chromatogr. 308 (1984) 83.
[11] E. Kaniowska, G. Ghwatko, R. Glowacki, P. Kubalczuk, E. Bald, J. Chromatogr. A 798 (1998) 27.
[12] H. Kataoka, K. Takagi, M. Makita, J. Chromatogr. B 664 (2) (1995) 421.
[13] E. Jayatilleke, S. Shaw, Anal. Biochem. 214 (2) (1993) 452.
[14] A. Andersson, A. Isaksson, L. Brattstron, B. Hulberg, Clin. Chem. 39 (8) (1993) 1590.
[15] S.B. Thompson, D.J. Tucker, J. Chromatogr. 382 (1986) 247.
[16] J.O. Sass, W. Endres, J. Chromatogr. A 776 (2) (1997) 342.
[17] A.M. Svardal, M.A. Mansoor, P.M. Ueland, Anal. Biochem. 184 (1990) 338.
[18] A. Araki, Y. Sako, J. Chromatogr. 422 (1987) 43.
[19] L. Bratstrom, B. Israelsson, J.O. Jeppsson, B.L. Hulberg, Metabolism 37 (1988) 175.
[20] I. Daskalakis, M.D. Lucock, A. Anderson, J. Wild, C.J. Schorah, M.I. Levene, Biomed. Chromatogr. 10 (1996) 205.
[21] A. Pastore, R. Massoud, C. Motti, A.L. Russo, G. Fucci, C. Cortese, G. Federici, Clin. Chem. 44 (1998) 825.

[22] T. Fiskerstrand, H. Refsum, G. Kvalheim, P.M. Ueland, Clin. Chem. 39 (1993) 263.
[23] A.R. Ivanov, I.V. Nazimov, L.A. Baratova, J. Chromatogr. A 870 (2000) 433–442.
[24] S. Zhang, W. Sun, Y. Xian, W. Zhang, L. Jin, K. Yamamoto, S. Tao, J. Jin, Anal. Chim. Acta 399 (1999) 213.
[25] S. Myung, M. Kim, H. Min, E. Yoo, K. Kim, J. Chromatogr. B 727 (1999) 1.
[26] T. Oe, T. Ohyagi, A. Naganuma, J. Chromatogr. B 708 (1998) 285.
[27] V. Rizo, L. Montalbetti, M. Vally, T. Bosoni, E. Scoglio, R. Moratti, J. Chromatogr. B 706 (1998) 209.
[28] J. D'Eramo, A. Finkelstein, F. Boccazi, O. Fridman, J. Chromatogr. B 720 (1998) 205.
[29] G. Minniti, A. Piana, U. Armani, R. Cerone, J. Chromatogr. A 828 (1998) 401.
[30] K. Nakashima, C. Umekawa, S. Nakatsuji, S. Akiyama, R. Givens, Biomed. Chromatogr. 3 (1) (1989) 39.
[31] E.M. Kosower, N.S. Kosower, Methods Enzymol. 251 (1995) 133.