ELSEVIER

Journal of Chromatography A, 798 (1998) 27–35

JOURNAL OF CHROMATOGRAPHY A

Urinary excretion measurement of cysteine and homocysteine in the form of their S-pyridinium derivatives by high-performance liquid chromatography with ultraviolet detection

E. Kaniowska, G. Chwatko, R. Głowacki, P. Kubalczyk, E. Bald*

Department of Environmental Chemistry, University of Lodz, 163 Pomorska Str., 90-236 Lodz, Poland

Abstract

Several human diseases, in particular metabolic disorders, often lead to the accumulation of characteristic metabolites in plasma, urine and cells. The selected diseases of this type include cystinuria and homocystinuria. In the typical laboratory diagnosis of these two diseases, a positive nitroprusside test is followed by quantitative analysis of urine cysteine and homocysteine in order to differentiate between cystinuria and homocystinuria. A sensitive and reproducible assay for total urine cysteine and homocysteine has been developed. The essential steps in the assay include conversion of disulphides to free thiols with tributylphosphine, conjugation of the thiols with 2-chloro-1-methyl pyridinium iodide, separation of S-pyridinium derivatives of cysteine and homocysteine from other endogenous urine thiol derivatives by reversed-phase high-performance liquid chromatography, and detection and quantitation by spectrophotometry. The method has a sensitivity of 4 pmol and is reproducible, intra- and inter-day coefficients of variation are from 1.37 to 4.14% and from 2.38 to 5.01%, respectively. The mean concentration of total urine cysteine and homocysteine in healthy donors (7 men and 7 women) were for women, 92.0±45.8 and 16.4±4.8 respectively, and for men 120.9±46.6 and 21.5±7.4 nmol/ml, respectively. Total urine homocysteine represents approximately 17.7% of cysteine in the urine of normal individuals. © 1998 Elsevier Science B.V.

Keywords: Derivatization, LC; Cysteine; Homocysteine; Amino acids

1. Introduction

Homocysteine is formed from the endogenous transmethylase inhibitor S-adenosylhomocysteine [1] and exported to the extracellular space when its production exceeds the metabolic capacity [2]. Intracellularly, homocysteine is metabolized either to cystathionine or to methionine [3]. Cystathionine is further metabolized to cysteine. These thiols are thus metabolically related. Determination of cysteine and homocysteine in plasma and urine is useful both in the diagnosis of several human diseases other than cystinuria and homocystinuria [4,5], and in drug therapy monitoring. The latter includes investigation of renal metabolism of homocysteine in vivo [6] and monitoring of the response of tumor and normal cells to cytostatic drugs [7]. Notably, in some studies [8,9], urinary cysteine and homocysteine concentrations mirror the change in plasma. Determination of urinary content of these amino acids may be a valuable noninvasive method of diagnosis, especially in children.

Several procedures for the determination of cysteine and metabolically-related amino acids have been described in a large number of original papers and reviews [10]. All methods except those based on

*Corresponding author.

0021-9673/98/$19.00
PII S0021-9673(97)01193-X

electrochemical detection depend on derivatization, almost always via sulfhydryl function. Useful reagents must form thiol derivatives with sufficient absorption or fluorescent yield to measure thiols at picomolar concentrations. Furthermore, the ideal reagent should show no absorption and should be nonfluorescent and react rapidly and specifically with thiols to form stable products. No reagent meets all these requirements, but among precolumn derivatization reagents available, bimanes (mBrB) and halogenosulfonylbenzofurazans (SBD-F, ABD-F) have become increasingly popular [11].

In the present study we explore the usefulness of 2-chloro-1-methylpyridinium iodide (CMPI) as a thiol-derivatizing reagent in analysis of total cysteine and homocysteine in urine, examining its reactivity with the thiol function and characterising the chromatographic properties of the thiol derivatives under a variety of HPLC conditions. The S-pyridinium derivatives of the title thiols are shown to have high UV-absorption yields and to exhibit great stability during storage in urine matrix. This procedure, like the majority of the methods for detecting total thiols in physiological samples, requires the reduction of the disulphide bond. The performance of this analytical method was assessed in human urine.

2. Experimental

2.1. Chemicals and reagents

2-Chloro-1-methylpyridinium iodide (CMPI) was prepared as described previously [12]. For thiol derivatization prior to HPLC analysis, a 0.1 *M* water solution of CMPI was used. Ethylenediaminetetraacetic acid disodium salt (EDTA), Tris(hydroxymethyl)aminomethane (Tris), perchloric acid (PA) and HPLC-grade methanol were from J.T. Baker (Deventer, Netherlands). L-Cysteine hydrochloride (CSH), DL-cystine (CSSC) and trichloroacetic acid (TCA) were from Reanal (Budapest, Hungary). DL-Homocystine (HCSSCH) was purchased from Serva (Heidelberg, Germany). DL-Homocysteine (HCSH) from Sigma (St. Louis, MO, USA) and tri-*n*-butylphosphine (TNBP) from Fluka (Buchs, Switzerland). Purified water from Millipore Milli-QRG system was used throughout the experiments. All liquids used for HPLC system were filtered through 0.2-μm membranes.

Tris buffer (pH 8.2, 1 *M*) was prepared by potentiometric titration of a 1 *M* solution of Tris with 1 *M* hydrogen chloride solution. The system was calibrated with standard pH solutions.

2.2. Instrumentation

HPLC analyses were performed with a Hewlett–Packard 1100 Series system equipped with quaternary pump, an autosampler, thermostated column compartment, vacuum degasser and diode-array detector. For instrument control, data acquisition and data analysis a HP ChemStation for LC 3D system including single instrument HP ChemStation software and Vectra color computer was used. For pH measurement, a Hach One pH meter was used. Water was purified using a Millipore Milli-QRG system.

2.3. Chromatography

Samples were injected using an autosampler into a 125×3-mm column packed with 3-μm particles of BDS-Hypersil, equipped with a 20×3-mm precolumn containing 5-μm particles of ODS-Hypersil. The temperature was 40°C, the flow-rate 0.5 ml/min and detector wavelength 312 nm. Separation of the S-pyridinium derivatives of cysteine and homocysteine from other endogenous thiols and reagent excess was accomplished isocratically using mobile phase A. In order to remove late-eluting UV absorbing material, e.g. reagent excess, gradient elution started after 9 min. The elution profile was as follows: 0–9 min, 0% B; flow 0.5 ml/min; 9.1–14 min, 0–22% B, flow 0.5 ml/min; 14.1–19 min, 22% B, flow 0.5–0.7 ml/min, 19.1–24 min, 22–0% B, flow 0.7–0.5 ml/min. Allowing an additional 5 min for reequilibration with elution solvent A at flow-rate 0.5 ml/min, a sample could be injected every 30 min. Elution solvent A was 0.05 *M* trichloroacetic acid adjusted to pH 2.3 with lithium hydroxide solution–methanol (95:5, v/v). Solvent B was methanol.

2.4. Preparation of calibration standards

Stock solutions of 10 μmol/ml cysteine, cystine,

homocysteine and homocystine in 0.02 *M* hydrochloric acid (in the case of disulphides 0.2 *M* in order to increase solubility) were prepared. These solutions could be kept at 4°C for several days without noticeable change of the thiols content. The working solutions were prepared by appropriate dilutions with water as needed. For preparation of calibration standards of human urine, aliquots of 1 ml of urine from a healthy donor were placed each in a glass sample tube and spiked with the appropriate amount of working standard solution of cystine and homocystine to provide, assuming 100% of the future reduction of the disulphide bonds, concentration of exogenous cysteine and homocysteine of 20, 50, 100, 150 and 200 nmol/ml urine and 5, 7, 10, 20, 30, 50, 60, 80, 100 and 150 nmol/ml urine, respectively.

2.5. Cleavage of the disulphide bonds

For both cystine and homocystine, water and urine calibration standard samples of 100 nmol were introduced into the 10 ml volumetric flask followed by adding 1 ml of 1 *M* pH 8.2 Tris buffer, 1 ml of water, 0.5 ml of 0.1 *M* disodium EDTA and 50 μl of 1% tri-*n*-butylphosphine (10% solution for urine calibration standard). The samples were incubated at 55°C for 5, 10, 15, 20, 30, 45, 60 and 90 min, cooled and assayed further according to the recommended procedure.

2.6. Reproducibility

2.6.1. Autosampler injection

Calibration standards of cysteine and homocysteine in water of 20 nmol/ml were prepared and derivatized according to the recommended procedure and the autosampler was ordered to inject 5, 10, 15 and 20 μl in triplicate for each volume. The absolute mean peak height and peak areas were plotted verses volumes injected and the curves fitted by least square linear regression analysis.

2.6.2. Derivatization

Five sets of urine standards were prepared both for cysteine and homocysteine of 10, 20, 30, 40 and 50 nmol/ml and derivatized. Autosampler injected solution of each concentration in triplicate. The mean peak heights and peak areas were plotted against amino-acid concentration. The standard error and coefficient of variation of each point was calculated.

2.6.3. Within-day and inter-day variation

To determine within-day precision, ten replicates of the biological sample were assayed in one run. Between-day precision was obtained by determining the same urine samples on 10 different days over 20 days.

2.7. Stability of the S-pyridinium derivatives

To test the stability of the S-pyridinium derivatives of cysteine and homocysteine, a 20 nmol/ml solution of each analyte in water and in urine calibration standard was prepared, derivatized with CMPI and kept at room temperature and at +4°C. The samples were injected into the HPLC system in two series. The sample kept at room temperature was injected after 4 h and every hour till 26 h, and the other one after 1, 2 and 15 days.

2.8. Standard curves

Standard curves for urine cysteine and homocysteine were constructed by adding known amounts of cystine and homocystine to normal urine followed by assay according to the procedure described at 2.10. The ranges of cystine and homocystine added were from 10 to 100 nmol/ml and from 2.5 to 75 nmol/ml, respectively. The absolute peak heights were plotted verses analyte concentration and the curves were fitted by least-square linear regression analysis.

2.9. Detection and quantification limits

The detection and quantification limits were estimated for the analytes by analysis of standard solutions of decreasing concentrations. They were established as the concentration required to generate a signal-to-noise ratio of 3 and 10, respectively. The values obtained were confirmed by analysis of spiked urine samples at concentrations equivalent to the estimated limits.

2.10. Assay procedure

To 1 ml of urine was added 0.5 ml of 0.1 *M* disodium EDTA solution, 1 ml of pH 8.2 1 *M* Tris buffer and 50 μl of 10% tri-*n*-butylphosphine (TNBP) in methanol. The mixture was heated at 55°C for 30 min and after cooling 50 μl of 0.1 *M* 2-chloro-1-methylpyridinium iodide solution was added. After 15 min the reaction mixture was quenched with 300 μl of 3 *M* perchloric acid, centrifuged (12 000 *g*) for 10 min and made up to a volume of 5 ml with water followed by injection of an aliquot onto the chromatographic system.

3. Results

Total urine cysteine and homocysteine were determined in normal human urine by (1) reduction of disulphide bonds with tributylphosphine; (2) derivatization of free thiols with 2-chloro-1-methylpyridinium iodide; (3) separation of S-pyridinium derivatives of cysteine and homocysteine from other endogenous urine thiol derivatives by reversed-phase HPLC, and (4) detection and quantitation by spectrophotometry at 312 nm.

3.1. Cleavage of the disulphide bond

Determination of total cysteine and homocysteine in urine requires the reduction of the disulphide bond between each other and the rest of the endogenous thiols. Sulfhydryl-containing reductors such as dithioerythritol, dithiothreitol, and mercaptoethanol liberate homocysteine from various disulphides but they consume derivatization reagent as well. For this reason they could not be used. Sodium borohydride impose practical problems because of the formation of gas and foaming during the reaction. Among the reducing reagents available in our laboratory, tri-*n*-butylphosphine has been found to be practical. It does not react with CMPI nor does it form foam during reaction. In our experiments the reduction of disulphides was complete in 30 min at 55°C and with 20-fold molar excess of tri-*n*-butylphosphine as shown in Fig. 1.

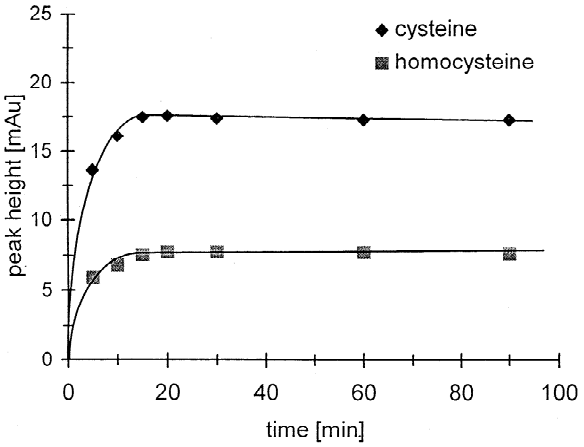

Fig. 1. Effect of incubation time on the reduction of cystine and homocystine with tri-*n*-butylphosphine at 55°C. Thiol to phosphine molar ratio 1:20. Analysis done by HPLC according to procedure in the Section 2.

3.2. Derivatization

The general conditions for derivatization of biological thiols in water solution using 2-chloro-1-methylpyridinium iodide (CMPI) were discussed elsewhere [13]. Briefly, CMPI reacts rapidly and specifically with thiols in slightly alkaline water solutions to produce stable thioethers — the S-pyridinium derivatives. These derivatives exhibit a well defined maximum at about 310 nm in the UV spectrum due to the bathochromic shift from the reagent maximum. As can be seen from the three-dimensional chromatogram (Fig. 2), absorption maximum of the S-pyridinium derivatives (analytical wavelength of the detector) falls far away from the derivatization reagent maximum enabling application of its large excess without interference with the chromatogram. In the case of a seven-fold excess of CMPI in pH 8.2 buffer, at room temperature the derivatization reaction came to an end within 10 min (Fig. 3). Experiments were carried out to determine the derivatization yield reproducibility within the calibration range. Good correlation was obtained for both thiols in question (for cysteine $y=0.943x-1.736$, $R^2=0.9972$; for homocysteine $y=0.385x-0.088$, $R^2=0.9896$).

The S-pyridinium derivatives of both cysteine and homocysteine were found to be stable at room

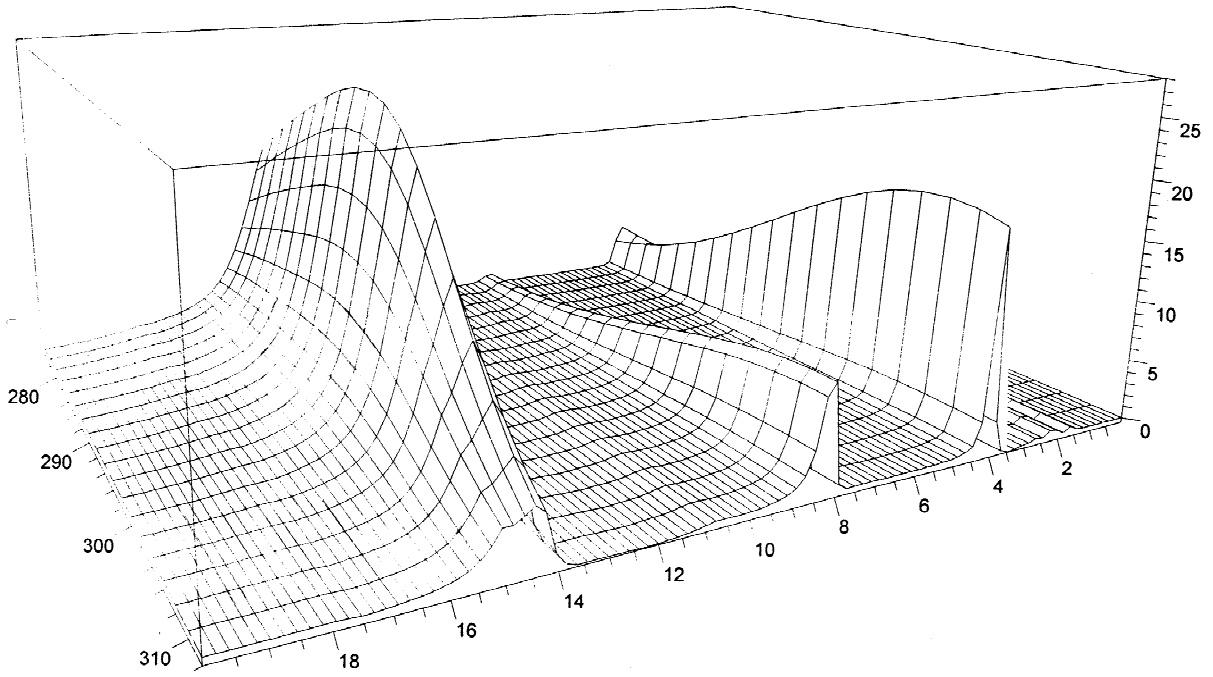

Fig. 2. Three-dimensional chromatogram of the derivatization mixture made with continuous spectral scanning during the elution. Peaks from right: cysteine derivative (400 pmol in peak), homocysteine (400 pmol in peak) and reagent (CMPI) excess. Initial molar reagent to analyte ratio 7:1. Axis titles: x=time, y=wavelength and z=absorbance.

temperature for a reasonable time which allows for long unattended runs. No significant change in chromatograms was noted when derivatized urine calibration standards were kept prior to HPLC analysis at room temperature for 26 h (Fig. 4). Samples kept at +4°C for 15 days lost approximately 15% of their initial S-pyridinium derivatives content (data not shown).

3.3. Separation of S-pyridinium derivatives

Fig. 5 shows the chromatogram of the two thiol components in urine from a healthy person. Cysteine and homocysteine eluted in this order, and their retention times were 3.95 and 8.22 min, respectively. Their content was estimated as 73.73 and 24.32 nmol/ml urine. Effect of pH on retention time was studied within the range of 2 to 7.5. As can be seen from Fig. 6, the capacity factor increases with decreasing pH throughout the range in the case of homocysteine and remains steady for cysteine. This result suggests that trichloroacetic acid might suppress ionization of the carboxylic acid residue of homocysteine. Elution based on this rationale did indeed provide sharper peaks and better resolution. Therefore we chose a mobile phase of pH 2.3 for routine HPLC target-thiol determination. Capacity factor of cysteine derivatives does not change either with the variation of methanol content in the mobile phase which is shown in Fig. 7.

3.4. Linearity, precision, and recovery

Absolute peak heights and area counts were used for the construction of the calibration curves. For routine urine analysis the absolute peak heights were used because in the case of cysteine the area counts gave slightly worse correlation. Calibration curves were linear for target thiols in the range of 20–200 for cysteine and 5–150 nmol/ml for homocysteine (for cysteine $y=0.8344x-1.8217$, $R^2=0.9894$ and for homocysteine $y=0.1849x-0.4974$, $R^2=0.9970$). The results from precision and recovery are shown in Table 1. Relative standard deviations ranged from

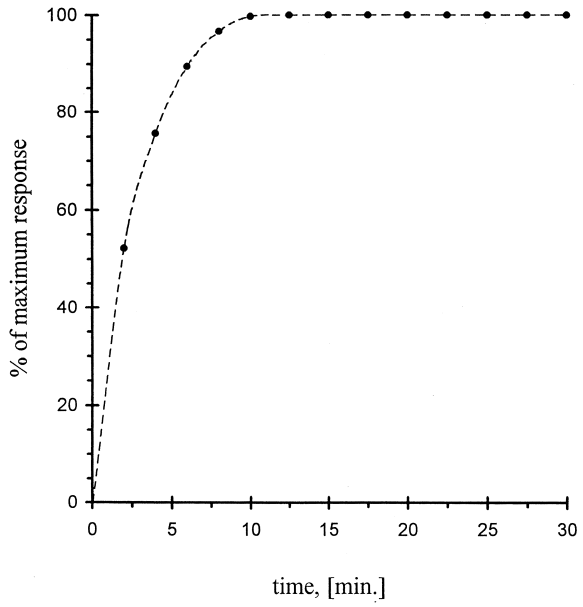

Fig. 3. Reaction-time profile of homocysteine and 2-chloro-1-methylpyridinium iodide (CMPI). The reaction mixture consisted of 50 nmol of homocysteine, 350 nmol of CMPI and 1 ml of 0.1 *M* pH 8.2 Tris made up with water in a 5-ml volumetric flask.

1.09 to 5.27% for homocysteine and from 1.29 to 4.14 for cysteine. The analytical recovery was 91–114% for both thiol components based on cystine and homocystine as primary calibrators. Disulphides were chosen as calibrators because they can be commercially obtained, its chemical stability and its ready conversion to thiols with a high nominal purity. It was reported [14] that commercial thiols mainly homocysteine contained up to 42% (w/w) of impurities and its purity decreased on storage even in the solid state.

The within-day R.S.D.s were for cysteine and homocysteine from 1.37 to 4.14% for urine samples enriched in corresponding disulphide with 10 and 50 nmol/ml. The between-day R.S.D.s were from 2.38 to 5.01% for the same concentrations.

3.5. Application of the method

The method was applied to determination of total cysteine and homocysteine urinary excretion in man. Urine was received from 14 laboratory staff members, 25–55 years old (7 men and 7 women). Total urinary cysteine and homocysteine concentrations were higher in men than in women (mean±S.D.:120.9±46.57 for CSH, 21.5±7.41 for HCSH, and 92.0±45.79 for CSH, 16.4±4.83 nmol/ml for HCSH, respectively. Urinary excretion per 24 h varied from 6 to 25 μmol for homocysteine and from 50 to 190 μmol for cysteine.

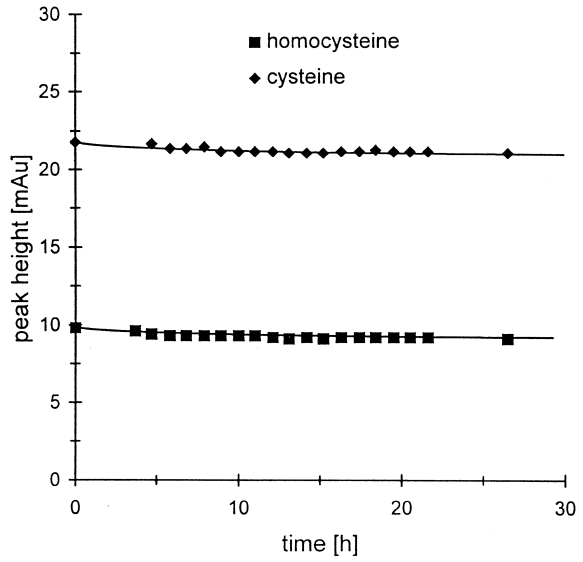

Fig. 4. Stability of the S-pyridinium derivatives in standard urine solution at room temperature.

4. Discussion

Practicality and low cost are central features of a routine laboratory test. The method described here can be practised in a laboratory equipped in standard HPLC instrumentation using a very simple and inexpensive mobile phase. The basic principle of the present technique was adopted from our previous work [13] dealing with the separation of cysteine and metabolically-related compounds in a water standard solution. The present procedure enables simultaneous determination of cysteine and homocysteine in urine by reversed-phase HPLC in isocratic mode. While investigating mobile phase buffers in the pH range of 2.5–7.5, we have found a significantly-enhanced retention of the cationic compounds when trichloroacetic acid was used without the addition of any other pairing reagent. Column equilibration time with this mobile phase was very short (5–10 min) in

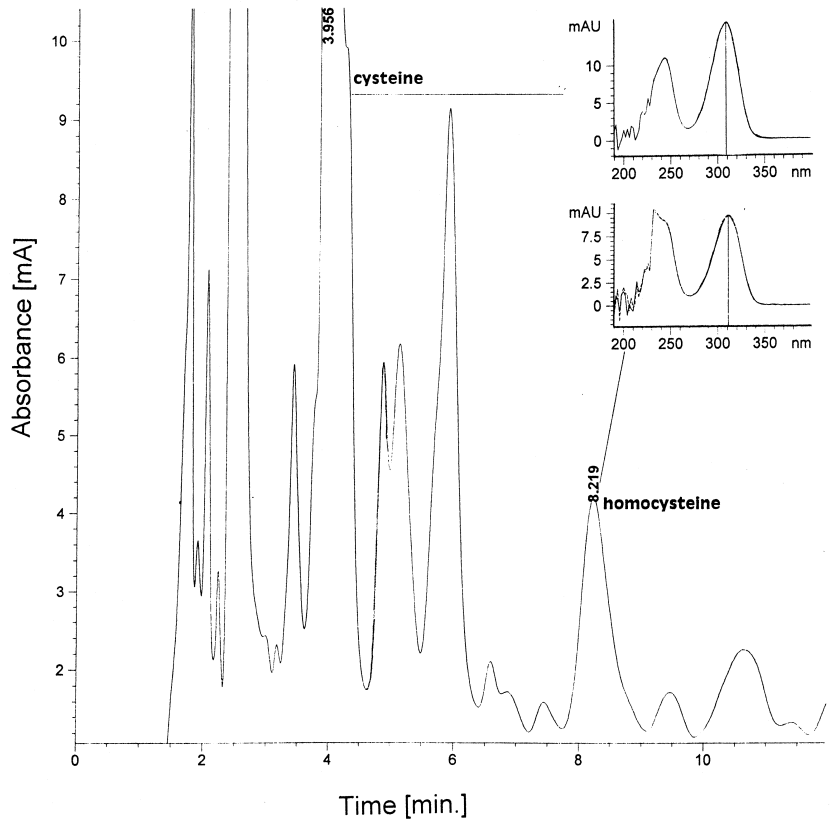

Fig. 5. HPLC profile of urine from healthy donor. Upper right: characteristic absorption spectra of the S-pyridinium derivatives of excreted total cysteine and homocysteine as recorded by diode-array detector. Cysteine and homocysteine content 73.7 nmol/ml and 24.3 nmol/ml, respectively. Elution conditions are described in Section 2.3.

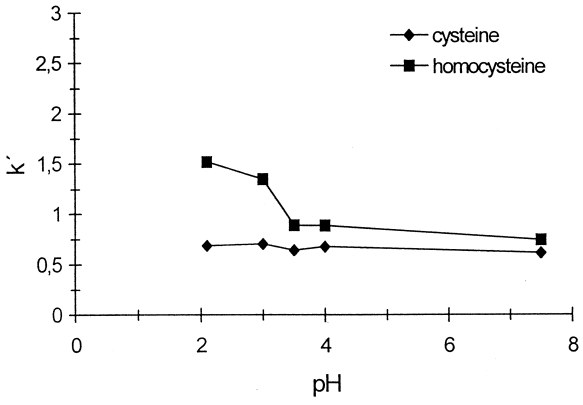

Fig. 6. Variation of the capacity factors of a mixture of the derivatives of cysteine and homocysteine as a function of the eluent pH. Mobile phase: 0.05 *M* trichloroacetic acid adjusted to desired pH with lithium hydroxide solution–methanol (95:5, v/v).

comparison with the phase containing expensive alkyl sulphonates (2–3 h) in our previous work [13].

The method has several advantages: (1) good solubility and stability of the derivatizing reagent in water, (2) high reactivity and selectivity toward thiols, (3) low absorption of the reagent at analytical wavelength and high optical yield of the derivatives, (4) low hydrolysis rate under slightly alkaline conditions of derivatization reaction, (5) simple and cheap mobile phase and short column equilibration time, (6) simultaneous determination of the two thiols with high precision, (7) high stability of all reagents enabling unattended runs.

Among precolumn derivatization reagents used for biological thiols analysis, fluorogenic reagents bimanes and halogenosulfonylbenzofurazans (ABD-F and SBD-F) are popular. mBrB reacts rapidly with thiols at room temperature to give a highly-fluores-

Table 1
Calibration data for the analysis of urine enriched in cysteine and homocystine

Homocysteine, $n=5$			Cysteine, $n=5$		
Added (nmol/ml)	Found±S.D. (nmol/ml)	R.S.D. (%)	Added (nmol/ml)	Found±S.D. (nmol/ml)	R.S.D. (%)
5	5.7±0.30	5.27	20	21.9±0.91	4.14
7	6.4±0.38	5.89	50	49.4±1.11	2.24
10	11.8±0.64	5.49	100	91.5±2.90	3.17
20	21.9±1.03	4.70	150	145.1±3.70	1.86
30	28.8±1.30	4.52	200	207.7±5.70	1.29
50	49.3±2.15	4.36			
60	60.5±2.88	4.77			
80	81.4±2.49	3.79			
100	101.8±3.09	1.31			
150	151.3±4.18	1.09			

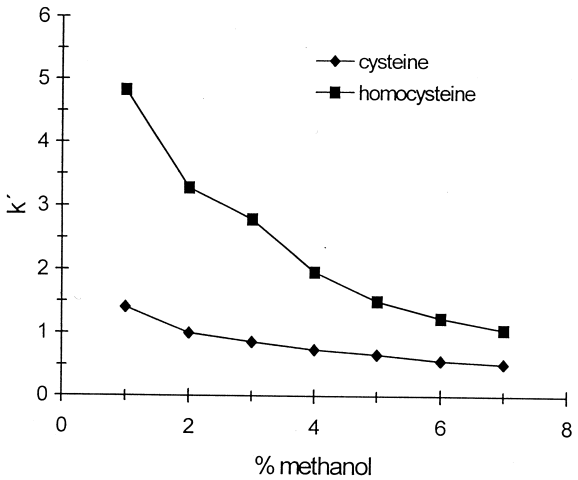

Fig. 7. Capacity factor of a mixture of the derivatives of cysteine and homocysteine as a function of eluent content of methanol at pH 2.3.

cent thioether [15]. One drawback is that the reagent itself, the hydrolysis products, and the impurities are fluorescent [16]. These materials give rise to several reagent peaks that may interfere with analysis [17]. Removal of excess reagent is included in some methods [15,18]. ABD-F requires heating with thiols at 50°C, whereas the less-reactive SBD-F requires more drastic conditions (pH 9 and 60°C for 1 h). Unreacted ABD-F and SBD-F are not fluorescent, the thiol derivatives are stable and no fluorescent hydrolysis products are formed [19].

5. Conclusion

We have developed a sensitive and reproducible assay for total urine cysteine and homocysteine determination by reduction of disulphide bonds with tributylphosphine, derivatization of resulted thiols with 2-chloro-1-methylpyridinium iodide and separation by reversed-phase high-performance liquid chromatography with ultraviolet detection. The method was applied to measurement of these thiols in normal urine. Although samples of urine from cystinuric and homocystinuric patients were not available, the method can be useful in routine diagnosis of these diseases. The work on automatization of the assay is now in progress.

Acknowledgements

This work was supported in part by Grant No 505/469 from the University of Lodz.

References

[1] P.M. Ueland, H. Refsum, J. Lab. Clin. Med. 114 (1989).
[2] B. Christensen, H. Refsum, O. Vintermyr, M. Ueland, J. Cell. Physiol. 146 (1991) 52.
[3] L. Brattstrom, B. Israelsson, B. Norrving, Atherosclerosis 81 (1990) 51.

[4] S.H. Mudd, H.L. Levy, in: C.R. Scriver, A.L. Beadet, W.S. Slay, D. Valle (Eds.), The Metabolic Basis for Inherited Diseases, McGraw-Hill, New York, 1989, pp. 693–734.
[5] J. Jellum, A.K. Thorsrud, E. Time, J. Chromatogr. 559 (1991) 455.
[6] J.T. Brosnan, B. Hall, J. Sulhub, M.R. Nadeau, A.G. Bostom, Biochem. Soc. Trans. 23 (1995) 470S.
[7] B.H. Lauterburg, T. Nguyen, B. Hartmann, E. Junker, A. Küand, T. Cerny, Cancer Chem. Pharm. 35 (1994) 132.
[8] H. Refsum, P.M. Ueland, S. Kvinnsland, Cancer Res. 46 (1986) 5385.
[9] A.A.M. Ermens, H. Refsum, J. Ruprecht, Clin. Pharmacol. Ther. 49 (1991) 385.
[10] K. Shimada, K. Mitamura, J. Chromatogr. B 659 (1994) 227.
[11] Y. Ohkura, H. Nohta, Adv. Chromatogr. 29 (1989) 221.
[12] E. Bald, Talanta 27 (1980) 281.
[13] S. Sypniewski, E. Bald, J. Chromatogr. A 676 (1994) 321.
[14] N.P.B. Dudman, X.W. Guo, R. Crooks, L. Xie, J.S. Silberberg, Clin. Chem. 42 (1996) 2028.
[15] R.C. Fahey, G.L. Newton, R. Dorian, E.M. Kosower, Anal. Biochem. 111 (1981) 357.
[16] W. Baeyens, G. van der Weken, B. Lin Ling, P. Moerloose, Anal. Lett. 21 (1988) 741.
[17] H. Refsum, P.M. Ueland, A.M. Svardal, Clin. Chem. 35 (1989) 1921.
[18] D.W. Jacobsen, V.J. Gatautis, R. Green, Anal. Chem. 178 (1989) 208.
[19] K. Imai, Adv. Chromatogr. 27 (1987) 215.