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Determination of biologically active low-molecular-mass thiols in human blood
II. High-performance capillary electrophoresis with photometric detection

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Abstract

A new high-performance capillary electrophoresis assay for aminothiols in human blood, including homocysteine, a marker of several human metabolism disorders, has been developed. Sample preparation involves conversion of disulfides to free thiols with triphenylphosphine, precipitation of proteins with sulfosalicylic acid, and conjugation of the thiols with monobromobimane. Derivatized thiols were separated in a sodium phosphate buffer using a fused-silica capillary (65 cm×50 μm I.D.) at 30°C. With the electric field of 250 V cm^{-1}, separation of homocysteine, glutathione and cysteine occurred at less than 10 min. Detection at 250 or 234 nm was used to confirm the monobimane–thiols peaks. The detection limit was ~5 nmol/ml for all labeled aminothiols. The proposed method for these compounds' analysis included simple sample preparation, high selectivity, good linearity (r^2>0.999), high reproducibility (within-run precision for derivatized aminothiol peaks area RSD<5% for three times consequently injected sample); high reliability and the small volumes required for analysis made it suitable for clinical studies. © 2000 Elsevier Science B.V. All rights reserved.

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

1. Introduction

Several human diseases, in particular, metabolic disorders, often lead to the accumulation of characteristic metabolites in blood, urine, and cells. Low-molecular-mass aminothiols (ATs) are important representatives of such metabolites. Therefore, separation and detection of these compounds are of keen interest having various medical applications. Here we report a system for monitoring total homocysteine (tHcy), cysteine (Cys), and glutathione (GSH) in human blood, based on the sample preparation procedure demonstrated in our previous publication [1] coupled with capillary zone electrophoresis (CZE) with common photometry detection. High-performance capillary electrophoresis (HPCE) is a potent tool for separation of a wide range of analytes, including both large and small molecules. Only a few studies deal with sulfur amino acids such as cysteine or homocysteine (Hcy) using HPCE [2–10].

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Usually, these ATs are determined either by high-performance liquid chromatography (HPLC) with different detection types [11–17]; GC [18,19]; GC–MS [19] and radioenzymatic methods [11,20]; or by the methods based on different disulfide reduction and derivatization ways [3,6–8,11,12,21]. Thiols and disulfides were separated using HPCE [2–10] prior to UV [10], diode-array [4], fluorescence [3,6–9], and electrochemical detection [2,5].

This paper discusses separation and detection of monobimane derivatized thiols by HPCE with UV detection, and quantification of plasma homocysteine. We developed and optimized a reproducible and linear method using CZE to determine the above low-molecular-mass thiols in human plasma. The method employed an easily prepared phosphate running buffer and the same simple sample preparation as for the HPLC assay (Part I). The sample preparation procedure involves conversion of disulfides to free thiols with triphenylphosphine, precipitation of proteins with trichloroacetic acid and conjugation of the thiols with monobromobimane (mBrB). A high relative recovery of the given facilitated quantification and improved detection limits.

Due to its simplicity and reliability, the developed procedure was convenient 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

Ethylenediaminetetraacetic 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 were purchased from Merck (Frankfurt, Germany). The sodium phosphate monobasic was purchased from Sigma. 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 HPCE buffer systems were filtered through 0.22 μm membranes (Millipore) and degassed by vacuum for 10 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 in [1]. Preparation of plasma samples and calibration standards, determination of total homocysteine, cysteine and glutathione in plasma were performed as described in [1].

2.2. Instrumentation

High-performance capillary electrophoresis analysis was performed on the HPCE system Model 270 A (ABI, Foster City, CA, USA). The chromatograms were recorded on Shimadzu Chromatopac C-R3A integrator (Shimadzu, Kyoto, Japan). 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), and water multi-block heater (Lab-Line Instruments, IL, USA) were used during sample preparation.

2.3. High-performance capillary electrophoresis

A running buffer of 80 m*M* sodium phosphate, pH 9.0 was used. The separation was performed on uncoated fused-silica capillaries (Perkin-Elmer) with the following dimensions: 65 cm (45 cm effective length)×50 μm I.D. Between injections, the capillary was conditioned for 5 min with 0.2 *M* NaOH and 5 min with running buffer. The separation voltage was +250 V/cm, with the capillary set to

30°C. The samples and standards were loaded on the capillary by hydrodynamic injection (7 s, 50 mbar).

2.4. Statistics

Standard curves for plasma homocysteine, cysteine, and glutathione were constructed by adding the known amounts of $(Hcy)_2$, $(Cys)_2$, or GSSG, respectively, to normal plasma followed by the assay as described in 2.4, 2.5 in [1], and 2.3 (see above). The added $(Hcy)_2$ and GSSG ranged from 0.5 to 500 nmol/ml, $(Cys)_2$ from 0.5 to 750 nmol/ml. The absolute 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 HPCE analysis 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 independently to the HPCE system. 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 HPCE with photometric detection. Detection wavelengths 234 and 250 nm were chosen as the local extinction maxima of thiol–monobimane conjugates for photometry detection [4]. As shown, photometry detection could be successfully applied to determine pathological concentration levels of homocysteine as well as other aminothiols contents.

3.1. Separation of standards

HPCE provides a baseline separation of mB-labelled homocysteine, cysteine, and glutathione (migration order) in less than 10 min (Fig. 1). Under the conditions described, the homocysteine–monobimane conjugate has the migration time of 6.01 (±0.19) min; the glutathione–monobimane 7.74 (±0.22) min, respectively, and the cysteine–monobimane, mB-derivative of the major plasma low-molecular-mass aminothiol, eluting with the migration time of 6.82 (±0.15) min. All mentioned derivatized ATs have approximately equal charge at pH 9.0, and an increase in the Stokes radius among mB–Hcy, mB–Cys, and mB–GSH molecules probably set conditions for increase in migration times of corresponding mB–ATs.

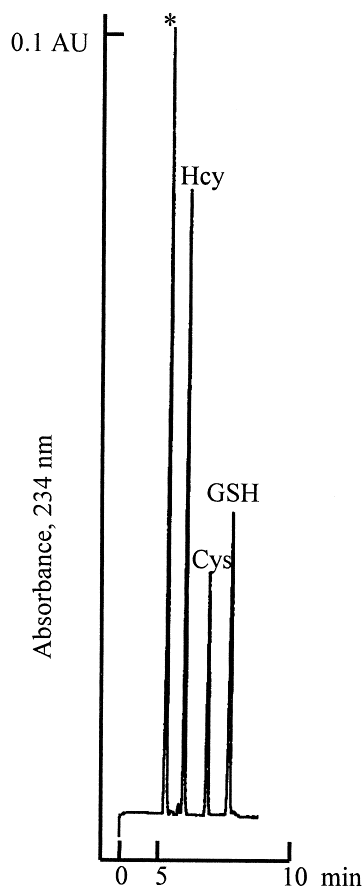

Fig. 1. HPCE identification of monobimane conjugates of standard aminothiols with photometry detection (234 nm). The aminothiol content is 1700.0 nmol/ml for homocysteine (6.15 min), 800.0 nmol/ml for cysteine (6.80 min), and 900.0 nmol/ml for glutathione (7.76 min). The peak marked by the asterisk corresponds to monobimane hydrolysis byproduct.

3.2. Plasma HPCE analysis

The plasma derivatized aminothiols ranged within the same migration time as the standards under the same conditions. Standard curves for plasma homocysteine, cysteine, and glutathione were constructed by adding the known amounts of authentic DL-*meso*-homocystine, L-cystine, or oxidized glutathione standard to normal plasma followed by immediate assay. The total concentration (μM) of three AT in plasma ($\pm$SD) obtained from 17 healthy overnight fasting males (23–58 years old) was 211.32 ($\pm$36.44) for Cys; 12.21 ($\pm$4.09) for Hcy, and 7.68 ($\pm$2.43) for GSH, and from 16 healthy overnight fasting females (21–46 years old) it was 209.83 ($\pm$31.48) for Cys, 9.51 ($\pm$2.90) for Hcy, and 7.16 ($\pm$2.94) for GSH.

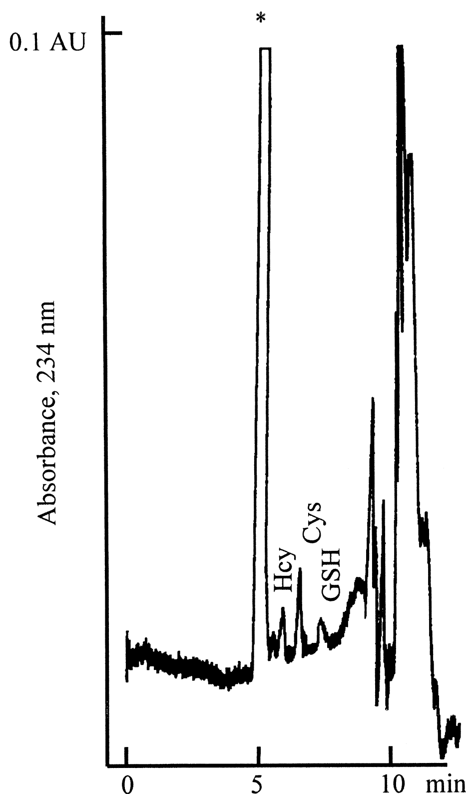

Fig. 2. HPCE identification of monobimane conjugates of aminothiols in human plasma with photometry detection (234 nm). The aminothiol content is 42.3 nmol/ml for homocysteine (pathology) (6.19 min), 171.4 nmol/ml for cysteine (6.78 min), and 10.1 nmol/ml for glutathione (7.81 min). The peak marked by the asterisk corresponds to monobimane hydrolysis byproduct.

These results are similar to our chromatographic assay data [1] and those obtained by different techniques [11–16]. 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 (Fig. 2).

A within-run precision for the method was obtained by three-time independent injections to the HPCE system and by comparing the peak areas for cysteine, homocysteine, and glutathione derivatives obtained for the three injections. The variation coefficient obtained for the within-run precision was 5.92% for the Cys derivative peak, 5.03% for the Hcy derivative, and 4.76% 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 HPCE system. A satisfactory analytical precision (4.97% RSD) was obtained without including an internal standard.

4. Discussion

This method was based on the same sample preparation procedure as described in Part I of this paper [1]. According to Figs. 1–3, the modifications resulted in an appropriate separation of AT-monobimane conjugates thus providing correlation of quantitative data and the assay results published.

Its sensitivity is favorably compared with that of other low-molecular mass AT photometry assays [10]. High electrophoretic resolution, low noise, linearity of the standard curves, as well as sufficient extinction of AT derivatives stipulated the assay accuracy.

In conclusion, the present assay includes three stages to determine the reduced, oxidized, and protein-bound homocysteine, cysteine and glutathione as the total amounts in human plasma. The oxidized and protein-bound ATs were converted to their reduced forms using triphenylphosphine and the following pre-assay derivatization of free sulfhydryls with mBrB; the aminothiol–monobimanes were separated and quantified by HPCE with photometry detection. Application of the HPCE approach with photometry detection demonstrated possible Hcy determination at pathological concentrations in

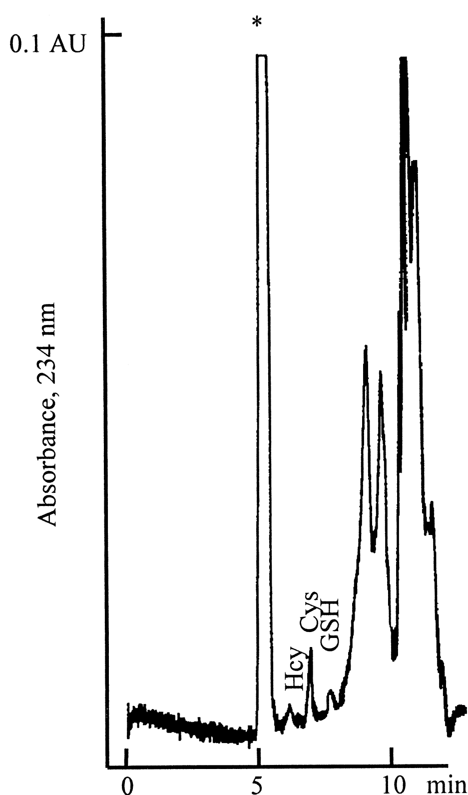

Fig. 3. HPCE identification of monobimane conjugates of aminothiols in human plasma with photometry detection (234 nm). The aminothiol content is 9.2 nmol/ml for homocysteine (norm) (6.20 min), 165.2 nmol/ml for cysteine (6.83 min), and 7.6 nmol/ml for glutathione (7.71 min). The peak marked by the asterisk corresponds to monobimane hydrolysis byproduct.

human plasma with high reliability only by use of common and widespread UV detection.

The method reported has several advantages: (1) very small analyte volume required for the assay; (2) simple sample preparation procedure; (3) sample preparation identical to chromatography AT determination; (4) simultaneous determination of the mentioned low-molecular-mass aminothiols; (5) rather inexpensive compared with chromatographic methods; (6) simple and prevalent separation buffers; (7) high reliability and reproducibility; and (8) short analysis time.

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References

[1] A.R. Ivanov, I.V. Nazimov, L.A. Baratova, J. Chromatogr. A. 895 (2000) 157.
[2] B.L. Lin, L.A. Colon, R.N. Zare, J. Chromatogr. A 680 (1994) 263.
[3] E. Causse, R. Terrier, S. Champagne, M. Nertz, P. Valdiguie, R. Salvayre, F. Couderc, J. Chromatogr. A 817 (1998) 181.
[4] K. Havel, K. Pritts, T. Wielgos, J. Chromatogr. A 853 (1999) 215.
[5] J. Zhou, T.J. O'Shea, S.M. Lunte, J. Chromatogr. A 680 (1994) 271.
[6] S.H. Kang, J.W. Kim, D.S. Chung, J. Pharm. Biomed. Anal. 15 (1997) 1435.
[7] M.W. Lada, R.T. Kennedy, J. Neurosci. Methods 72 (1997) 153.
[8] E. Jellum, A.K. Thorsrud, E. Time, J. Chromatogr. 559 (1991) 455.
[9] G. Vecchione, M. Magraglione, E. Grandone, D. Colaizzo, G. Cappucci, I. Fermo, A. D' Angelo, G. Di Minno, Electrophoresis 20 (1999) 569.
[10] J.S. Stamler, J. Loscalzo, Anal. Chem. 64 (1992) 779.
[11] H. Refsum, P.M. Ueland, A.M. Svardal, Clin. Chem. 35 (9) (1989) 1921.
[12] M.A. Mansoor, A.M. Svardal, P. Ueland, Anal. Biochem. 200 (1992) 218.
[13] D.J. Jacobsen, V.J. Gatautis, R. Green, Anal. Biochem. 178 (1989) 208.
[14] A. Andersson, A. Isaksson, L. Brattstron, B. Hulberg, Clin. Chem. 39 (8) (1993) 1590.
[15] D.L. Rabenstein, G.T. Yamashita, Anal. Biochem. 180 (1989) 259.
[16] S. Zhang, W. Sun, Y. Xian, W. Zhang, L. Jin, K. Yamamoto, S. Tao, J. Jin, Anal. Chim. Acta 399 (1999) 213.
[17] K. Nakashima, C. Umekawa, S. Nakatsuji, S. Akiyama, R. Givens, Biomed. Chromatogr. 3 (1) (1989) 39.
[18] J.O. Sass, W. Endres, J. Chromatogr. A 776 (2) (1997) 342.
[19] S. Myung, M. Kim, H. Min, E. Yoo, K. Kim, J. Chromatogr. B 727 (1999) 1.
[20] A.M. Svardal, M.A. Mansoor, P.M. Ueland, Anal. Biochem. 184 (1990) 338.
[21] T. Oe, T. Ohyagi, A. Naganuma, J. Chromatogr. B 708 (1998) 285.