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Short communication

Determination of biologically active low-molecular-mass thiols in human blood

III. Highly sensitive narrow-bore isocratic reversed-phase high-performance liquid chromatography with fluorescence detection

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Abstract

The fast isocratic narrow-bore reversed-phase high-performance liquid chromatographic method employing fluorescence detection is 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. Optimized sample preparation conditions as well as chromatographic conditions allowed to obtain reliable quantitative results within the concentration range corresponding to the levels of these thiols in human blood in norm and pathology. The detection limit was ~70 amol for all labeled aminothiols. The proposed method for these compounds analysis includes simple sample preparation, high selectivity, good linearity (r^2>0.998), high reproducibility (within-run precision for derivatized aminothiol peaks area RSD<1.8% for three times consequently injected sample); high reliability and the small sample volume (1 μl) required for analysis make it suitable for clinical studies.

Keywords: Thiols; Amino thiols; Sulphur compounds; Homocysteine

1. Introduction

New fast, simple, sensitive, and precise narrow-bore column RP-HPLC technique for qualitative and quantitative determination of total most important biologically active plasma low-molecular-mass aminothiols (ATs), such as homocysteine, cysteine (Cys) and glutathione (GSH) was developed. It is well-known, measurement of ATs in urine and blood is essential for diagnosing of several metabolic disorders [1–8]. This method based on previously published method [1] using disulfide reduction with triphenylphosphine, protein precipitation with sulfosalicylic acid, and derivatization with monobromobimane. Monobimane (mB) aminothiols were determined by HPLC with isocratic elution with the very popular water–acetonitrile–trifluoroacetic acid buffer system and use of fluorescence detection.

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Due to its simplicity, reliability, the developed procedure is convenient for routine analysis of total homocysteine (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

Chemicals, reagents, buffers, standard solutions, sample preparation procedures were the same as we described in our previous publication [1].

2.2. Instrumentation

HPLC analyses were performed on a narrow-bore column high-performance liquid chromatograph Milichrom A-02 (Envirochrom A-02) (Chromatography Institute Eko-Nova, Novosibirsk, Russia) equipped with syringe pumps, a thermostated column compartment, an autosampler, and fluorescence detector (Applied Systems, Minsk, Belarus) with 1 μl cell. The fluorescence detector output was recorded on Shimadzu Chromatopac C-R3A integrator (Shimadzu, Kyoto, Japan). The rest instrumentation was the same as published before [1].

2.3. Chromatography

Samples were injected using an autosampler into a narrow-bore 75×2 mm column packed with 5-μm particles of Nucleosil C_{18}, pore size 120 A (Machery–Nagel, Düren, Germany). The injection volume was 1 μl. The temperature was 50°C. The fluorescence detector operated with an exiting wavelength of 370 nm, an emission wavelength 470 nm. The elution conditions were as follows:

(a) isocratic separation conditions: 0 μl–5% B; 2000 μl–5% B; the flow-rate was 150 μl/min;
(b) column regeneration conditions: (1) 0 μl–100% B; 500 μl–100% B; the flow-rate was 250 μl/min; (2) 0 μl–5% B, 600 μl–5% B, the flow-rate was 200 μl/min.

Elution solvent A was 0.1% trifluoroacetic acid (TFA) (pH 2.2) in water, solvent B was acetonitrile. Statistical data evaluation was performed on chromatographic results by the procedures described in [1].

3. Results

The total plasma homocysteine, cysteine, and glutathione contents were determined in human plasma by use of sample preparation procedure published earlier [1] and isocratic reversed-phase narrow-bore column HPLC with fluorescence detection. Fast chromatographic solution with isocratic elution with the very simple and popular in daily use water–acetonitrile–trifluoroacetic acid 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 are shown in Figs. 1–3. Under the conditions described, the homocysteine-monobimane conjugate has a retention time of 10.23 (±0.12) mm; the glutathione-monobimane 12.09 (±0.10) min, and the cysteine-monobimane, mB derivative of the major plasma low-molecular-mass aminothiol, eluting with a retention time of 5.12 (±0.08) min. The total concentration (μM) of three ATs in plasma (±SD) obtained by HPLC assays from ten healthy overnight fasting males was 213.68 (±34.25) for Cys; 9.96 (±3.82) for Hcy, and 7.67 (±2.23) for GSH, and from ten healthy overnight fasting females it was 209.33 (±34.57) for Cys, 9.25 (±2.67) for Hcy, and 6.88 (±2.82) for GSH. These results are similar to published data obtained by different techniques [2–8] and to our previous results [1]. 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. 3).

The RSD obtained for the within-run precision was 1.86% for the Cys derivative peak, 2.08% for the Hcy derivative, and 2.16% for the GSH derivative for $n=3$. 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 (4.73% RSD) was obtained without inclusion of an internal standard. The sensitivity of the method

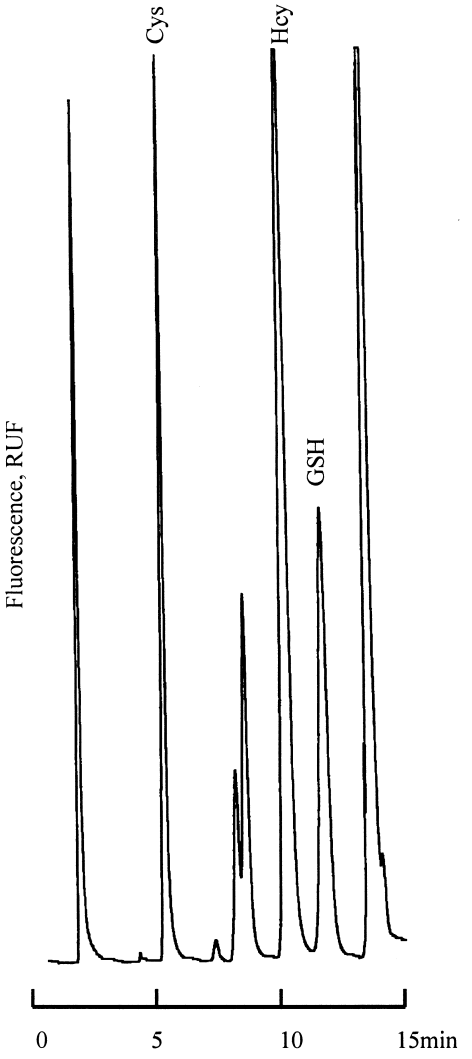

Fig. 1. RP-HPLC identification of monobimane conjugates of standard aminothiols. The aminothiol content is 180.0 nmol/ml for cysteine (5.24 min), 230.0 nmol/ml for homocysteine (10.09 min), and 100.0 nmol/ml for glutathione (12.15 min).

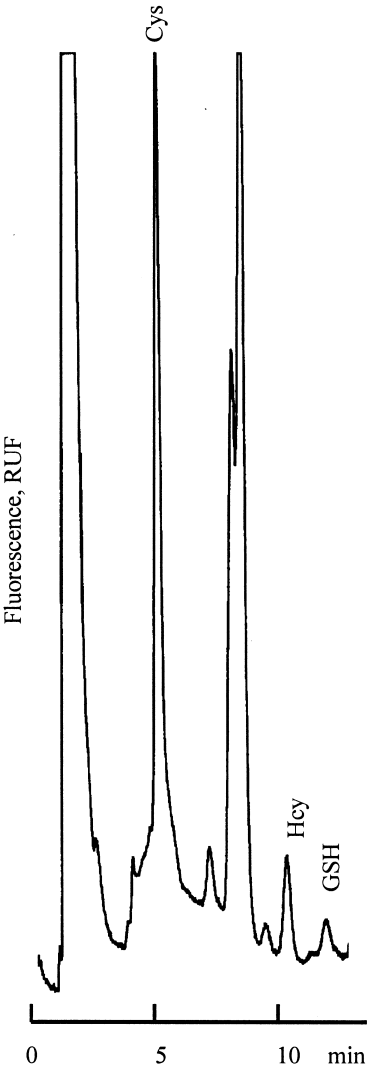

Fig. 2. RP-HPLC aminothiol identification in human plasma. The aminothiol content is 202.5 nmol/ml for cysteine (5.17 min), 8.7 nmol/ml (norm) for homocysteine (10.17 min) and 5.8 nmol/ml for glutathione (12.14 min).

allows the detection of AT quantities >70 amol after all chemical modifications.

4. Discussion

This method was based on our previously published assay [1] with developing more preferable chromatographic conditions. We tried out to develop efficient and common isocratic HPLC conditions for the mB-AT determination with fluorimetry detection. According to Figs. 1–3, these modifications resulted in an acceptable separation of AT-monobimane conjugates providing correlation of quantitative data with the assay results published. This approach to separate the mB-ATs with a common, easy-to-make and prevailing water–acetonitrile–TFA elution system was developed for fast quantitative and qualitative determination of homocysteine, cysteine, and

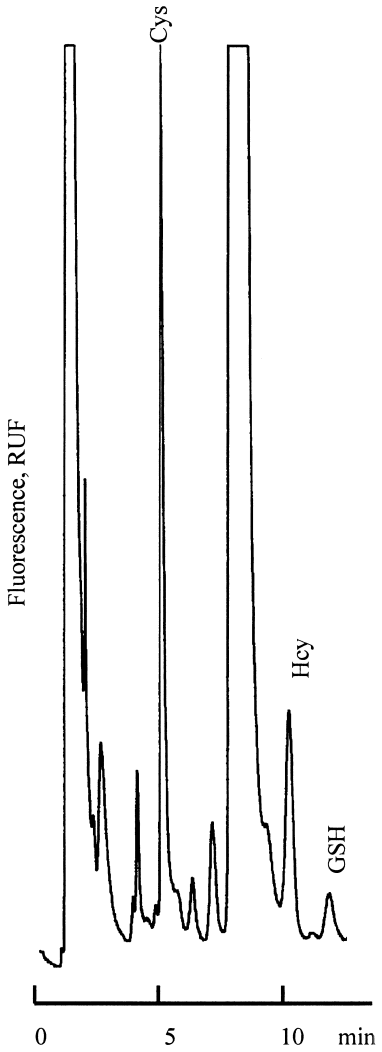

Fig. 3. RP-HPLC aminothiol identification in human plasma with fluorescence detection. The aminothiol content is 181.4 nmol/ml for cysteine (5.13 min), 26.5 nmol/ml (mild pathology) for homocysteine (10.12 min) and 6.3 nmol/ml for glutathione (12.11 min).

glutathione under isocratic elution conditions (Figs. 1–3).

The sensitivity of the developed method is very favorably compared with that of other low-molecular-mass AT assays [2–8]. High chromatographic resolution, low noise, 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 complicated mobile phase [2–8]. Moreover, this HPLC method require just 1 μl of sample, which means less than 200 nl of plasma. These narrow-bore HPLC conditions profitable differ from other published methods in eluent consumption. The optimal sample preparation conditions and the construction of the high-sensitive fluorescence detector equipped with l-μ1 cell allowed us to measure monobimane-aminothiol concentrations less than 100 fmol/ml.

The method reported has several advantages: (1) simple sample preparation procedure; (2) simultaneous determination of all mentioned low-molecular-mass aminothiols during the procedure; (3) high sensitivity of the assay; (4) low consumption of plasma, analytes, and elution buffers; (5) simple and prevalent mobile phases; (6) fast chromatography procedure. Its final advantage is its easy use and high reliability.

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References

[1] A.R. Ivanov, I.V. Nazimov, L.A. Baratova, J. Chromatogr. A 895 (2000) 157.
[2] H. Refsum, P.M. Ueland, A.M. Svardal, Clin. Chem. 35/9 (1989) 1921.
[3] M.A. Mansoor, A.M. Svardal, P. Ueland, Anal. Biochem. 200 (1992) 218.
[4] D.J. Jacobsen, V.J. Gatautis, R. Green, Anal. Biochem. 178 (1989) 208.
[5] J. D'Eramo, A. Finkelstein, F. Boccazi, O. Fridman, J. Chromatogr. B 720 (1998) 205.
[6] G. Minniti, A. Piana, U. Armani, R. Cerone, J. Chromatogr. A 828 (1998) 401.
[7] T. Oe, T. Ohyagi, A. Naganuma, J. Chromatogr. B 708 (1998) 285.
[8] V. Rizo, L. Montalbetti, M. Vally, T. Bosoni, E. Scoglio, R. Moratti, J. Chromatogr. B 706 (1998) 209.