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Determination of homocysteine and other thiols in human plasma by capillary electrophoresis[1]

Seong Ho Kang [a], Jong-Won Kim [b], Doo Soo Chung [a,*]

[a] *Department of Chemistry, Seoul National University, Seoul 151-742, South Korea*
[b] *Department of Clinical Pathology, Samsung Medical Center, Seoul 135-230, South Korea*

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Abstract

A new capillary electrophoresis (CE) assay for thiols in human plasma, including homocysteine, which is an indicator of several clinical states has been developed. The thiols were derivatized quantitatively at 50°C, pH 8.0 with a fluorogenic reagent, ABD-F (4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole), which is about 30 times faster compared to the other fluorogenic reagent, SBD-F (ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate). The separation of ABD-thiols was performed in a 50 mM sodium phosphate buffer (pH 2.1) using a bare fused silica capillary (27 cm × 50 μm i.d.) at 25°C. With the electric field of 560 V cm^{-1}, the time needed for the separation of homocysteine, glutathione and cysteine was less than 8 min. A filter-type ultraviolet detector and a 512-channel diode-array detector (DAD) were employed for ABD-thiol analysis. DAD was used to confirm the ABD-thiol peaks. The limits of detection (S/N = 3) for homocysteine, glutathione, and cysteine were 0.5, 1 and 2 μM at 220 nm, respectively. © 1997 Elsevier Science B.V.

Keywords: Homocysteine; Thiols; Capillary electrophoresis; Plasma

1. Introduction

Homocysteinemia has been identified as a risk factor for cerebrovascular, peripheral vascular, and coronary heart disease from clinical studies [1–3]. A speculative calculation implies that 10% of the population's risk for coronary artery disease appears to be attributed to elevated homocysteine level. A recent study showed that 9% of male and 54% of female coronary artery deaths could be prevented by fortification of food with folic acid [4]. Although the roles played by homocysteine in atherogenesis and thrombogenesis on cardiovascular disease have not been established well, various biological data and the epidemiologic evidence suggest that homocysteine itself be the cause, rather than an epiphenomenon, of atherosclerosis [4]. Accurate determination of the concentrations of homocysteine in serum and in plasma is essential to understanding the role of homocysteine in the pathogenesis of vascular dis-

* Corresponding author. Tel.: +82 2 8808130; fax: +82 2 8891568; e-mail: dschung@plaza.snu.ac.kr

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ease. Since plasma homocysteine concentrations can be lowered by administration of folic acid or cobalamin, the assessment of homocysteine status due to dietary modification or vitamin supplementation programs, as well as in cardiovascular disease patients at large, will require rapid and reproducible assays. Homocysteine exists in plasma as protein-bound and free forms [5]. The total homocysteine amount should be measured for the proper evaluation of risk of coronary artery disease. Various methods such as amino acid analyzer [6–10], radioenzymic assay [11], enzymatic assay [12], and high-performance liquid chromatography (HPLC) [13–21] have been developed for the analysis of homocysteine and other thiols.

Recently, capillary electrophoresis (CE) has made great progress as a complementary method to HPLC [22–24]. The former is a simple, rapid, and efficient separation method (theoretical plate number, $N \geq 10^5$) for the analysis of biomolecules. Automation and the use of extremely small volume (1–5 nl) are also advantageous.

In the case of both CE and HPLC, some fluorogenic reagents having a benzofurazan structure have been used for detection of thiols [25,26]. Benzofurazan reagents have much higher selectivities to thiols than other fluorogenic reagents such as I-AEDANS [27], dansylaziridine [28], bimane [29]. Among benzofurazan reagents, ABD-F (4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole, Fig. 1) and SBD-F (ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate) were used for the sensitive and specific detection of thiols in HPLC method because of their excellent reactivity, selectivity to thiol and good stability [25,30,31]. Especially, ABD-F reacts with thiols much faster than SBD-F at 50°C and pH 8.0. In this study, we developed a new CE method to measure the total homocysteine in human plasma derivatized with ABD-F which reacts with thiols much faster and at milder conditions than SBD-F.

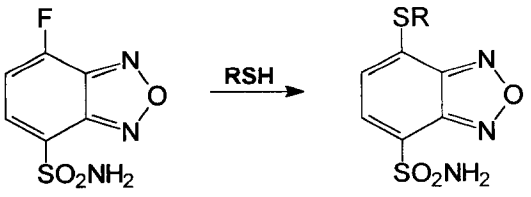

Fig. 1. Structure of ABD-F and its reaction with thiols.

2. Experimental

2.1. Chemicals

ABD-F and SBD-F were obtained from Dojindo Laboratories in Japan. The thiols (DL-homocysteine, L-cysteine, 2-mercaptoethanol, dithioerythritol, dithiothreitol and glutathione reduced) were purchased from Sigma Chemical (St. Louis, MO, USA) and Fluka (Buchs, Switzerland). Boric acid, sodium borate, EDTA × 2Na, and phosphoric acid were of analytical-reagent grade and used without further purification. Water used to prepare the solutions was obtained from a water purifier (Model NANOpure II purification system, Barnsted, USA).

2.2. Standard thiols derivatized with ABD-F and SBD-F

Standard thiols were derivatized with ABD-F using Toyo'oka and Imai's method [30,31]. 1.0 ml of a mixture consisting of thiols (200 μM of homocysteine, glutathione, and cysteine, respectively) in a 0.1 M sodium borate buffer (pH 8.0) containing 2 mM EDTA × 2Na was added to 1.0 ml of 1 mM ABD-F in a 0.1 M sodium borate buffer (pH 8.0). The reaction mixture was vortex-mixed and heated at 50°C for 5 min, and then cooled on ice-water. Then 500 μl of 0.1 M HCl was added to quench the reaction and the sample solution was injected into the CE system.

The thiols were also derivatized with SBD-F in a similar way. 1.0 ml of mixed thiols (150 μM of homocysteine, gluthione, cysteine, mercaptoethanol, dithiothreitol and dithioerythritol, respectively) in a 0.1 M sodium borate buffer (pH 9.5) containing 2 mM EDTA × 2Na was added to 1 ml of SBD-F (1 mM) in 0.1 M sodium borate buffer (pH 9.5). The reaction mixture was vortex-mixed and heated at 60°C for 60 min, and then it

was cooled. Then, 100 μl of 2 M HCl was added and the sample solution was injected into the CE system.

2.3. Sample preparation

Human plasma thiols were derivatized with ABD-F using the method of Araki and Sako [14]. About 3 ml of the fresh blood was collected in a vacutanier tube containing EDTA cooled on ice. The tube was immediately centrifuged at 2500 rpm for 10 min at 4°C. 0.5 ml of plasma was treated with 50 μl of 10% (v/v) tri-*n*-butylphosphine in dimethylformamide for 30 min at 4°C in order to reduce thiols and decouple them from plasma proteins. To the solution was added 0.5 ml of 10% trichloroacetic acid containing 1 mM EDTA × 2Na. Then the mixture was immediately vortex-mixed and then centrifuged at 12 500 rpm for 5 min at 4°C. 0.2 ml of the clear supernatant was vigorously mixed with 0.2 ml of a 0.1 M sodium borate buffer (pH 8.0) containing 2 mM EDTA × 2Na and 0.4 ml of 1 mM ABD-F in a 0.1 M sodium borate buffer (pH 8.0). The mixture was vigorously mixed and allowed to stand at 50°C for 5 min. The reaction mixture was cooled in an ice-water bath. The solution was filtered through a 0.45 μm puradisc 25 mm PP syringe filter (Whatman, UK) and injected into the CE system.

2.4. Capillary electrophoresis

We used a P/ACE 5000 series capillary electrophoresis system (Beckman Instruments) with a filter-type ultraviolet (UV) detector and a 512-channel diode-array detector (DAD). Various fused silica capillaries (Polymicro Technologies, USA) of different dimension (total capillary length = 27–57 cm and i.d. 25–75 μm) were tested in order to find the optimum CE condition. Also a capillary coated with cross-linked and surface bonded dimethyl-polysiloxane (DB-1, 50 μm i.d. J & W Scientific, USA) was used for comparison with the bare one. The 27 cm (effective length = 20 cm) × 50 μm i.d. capillary was employed for the most of the experiments. Beckman system Gold software (version 8.10) was used for system control, data collection, and data processing. HP 8453 UV-Visible spectrophotometer (Hewlett Packard, Germany) was used for the spectra of ABD-thiols. Mass spectrometry (VG ZAB-E, UK) and NMR (VXR-200S, Varian, USA) spectroscopy were used to check the purity of ABD-F.

The electrophoresis buffer was a 50 mM phosphate buffer adjusted to pH 2.1 with phosphoric acid. Various 20 mM borate buffers (pH 8.0–10.5) adjusted with 1 N NaOH were also used for monitoring the effect of pH. All solutions used for sample treatment and buffer systems were sonicated and filtered through a 0.45 μm membrane syringe filter. The separations were carried out at 25°C with detection at 220 nm. The DAD was used to confirm the ABD-thiol peaks and SBD-thiol peaks. After each run, the capillary was reconditioned prior to the next analysis by rinsing for 10 min with the sequence of water, 0.1 N NaOH, water, and the electrophoresis buffer.

Various stacking methods [32] were employed to enhance the concentration of sample plug. Derivatized thiols were dissolved in a 5 mM borate buffer (pH 8.0) which is significantly more dilute than the usual 100 mM borate buffer. Then they were injected hydrodynamically or electrokinetically after a water plug was injected hydrodynamically. The focusing and electrophoresis run was carried out at 15 kV constant voltage for 10 min using a 50 mM phosphate buffer (pH 2.1) as a running buffer.

3. Results and discussion

3.1. Optimization of capillary electrophoresis

The CE separation condition was first examined at the basic electrolyte condition (borate buffer, pH 8.0–10.5). Fig. 2(A) and (B) show electropherograms of SBD-thiols and ABD-thiols obtained at the basic condition, respectively. Six SBD-thiols and SBD-F were well separated within 10 min. When ABD-F was used, the simultaneous determination was not feasible since minor peaks denoted by (*) besides those of ABD-F and ABD-thiols were observed.

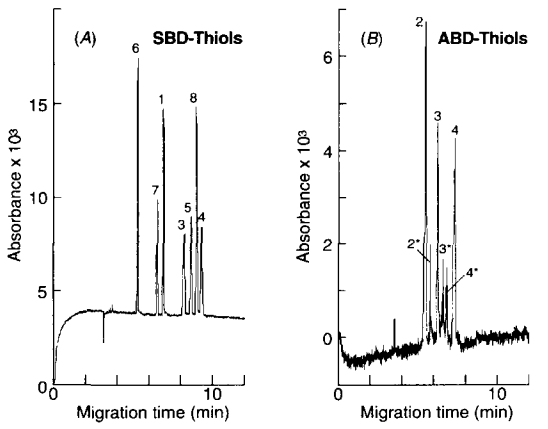

Fig. 2. Electropherograms of (A) SBD-thiols in a borate buffer (pH 9.8) and (B) ABD-thiols in a borate buffer (pH 9.5). Condition: 57 cm × 50 μm i.d. bare fused-silica capillary, 25 kV, 25°C, 5 s hydrodynamic injection at 0.5 psi, detection at 220 nm. Peaks: 1 = SBD-F, 2, 2* = ABD-F, 3, 3* = homocysteine, 4, 4* = cysteine, 5 = glutathione, 6 = mercaptoethanol, 7 = dithioerythritol, and 8 = dithiothreitol.

The acidic condition (phosphate buffer pH 1.8–5.0) was then investigated. The electropherograms in Fig. 3 show the effect of electrolyte pH. ABD-homocysteine, -glutathione, and -cysteine were eluted earlier than the electroosmotic flow (EOF). It indicates that the predominant forms of ABD-homocysteine, -glutathione, and -cysteine have positive charges in acidic buffers. ABD-mercaptoethanol, -dithioerythritol, and -dithiothreitol probably have a negatively-charged forms since these peaks were observed after the EOF. Above pH 2.3 (Fig. 3A and 3B), the peak of the ABD-glutathione was split into two, so the quantitative analysis of ABD-glutathione was not convenient. At pH 2.1 (Fig. 3C), ABD-homocysteine, -glutathione, and -cysteine peaks were well separated. However, as the pH was lowered below 2.1 (Fig. 3D), the peaks of ABD-gluthione and -cysteine were getting overlapped and the current was increased due to the increased electrolyte concentration. Thus, CE separation was done at pH 2.1, taking into account the sensitivity, resolution, and analysis time.

Fig. 4 compares the electropherograms obtained with bare fused silica and coated (DB-1) capillaries of the same length. The migration time with the coated capillary (Fig. 4B) was shorter than that with a bare fused silica capillary (Fig. 4A). But the peaks of ABD-glutathione and -cysteine were not well separated and the peak heights and areas of ABD-homocystein and -cysteine were decreased about 5% with the coated capillary. Although we could obtain a somewhat better result by using a longer DB-1 capillary, we chose to use the bare fused silica capillary, considering the expense of and difficulty of handling the coated capillaries. Optimum CE separation conditions for ABD-thiols are summarized in Table 1.

3.2. Reproducibility, accuracy, and detection limits

In order to determine the reproducibility of the migration time and peak area for ABD-thiols, the standard mixture of ABD-homocysteine, -glutathione, and -cysteine was injected repeatedly 10 times at the same condition. Relative standard deviations (R.S.D.) for the migration time and peak area were less than 0.27% and 1.70%, respectively. The detection limits of ABD-homocysteine, -glutathione, and -cysteine were 0.5, 1, and 2 μM, respectively, as summarized in Table 2.

The correlation between the peak area and the concentration of ABD-thiols in the range of 1–200 μM was examined for the quantitative analysis. The linear regression equations for ABD-homocysteine, -glutathione, and -cysteine were $y = 0.0021\ x + 0.0034$ ($R^2 = 0.9996$), $y = 0.0035\ x + 0.0063$ ($R^2 = 0.9986$), and $y = 0.0006\ x + 0.0034$ ($R^2 = 0.9994$), respectively (Fig. 5). This linearity guaranteed the determination of ABD-glutathione and ABD-cysteine up to 200 μM and ABD-homocysteine up to 100 μM.

3.3. Homocysteine in human plasma

Fig. 6A shows a typical electropherogram of the thiols in plasma of a normal person, derivatized with ABD-F. Fig. 6(B) and (C) show the identification of ABD-homocysteine by adding extra homocysteine into the plasma prior to the sample preparation. All the peaks were well separated and the migration times of ABD-homocysteine, -glutathione, and -cysteine were 5.42, 6.55,

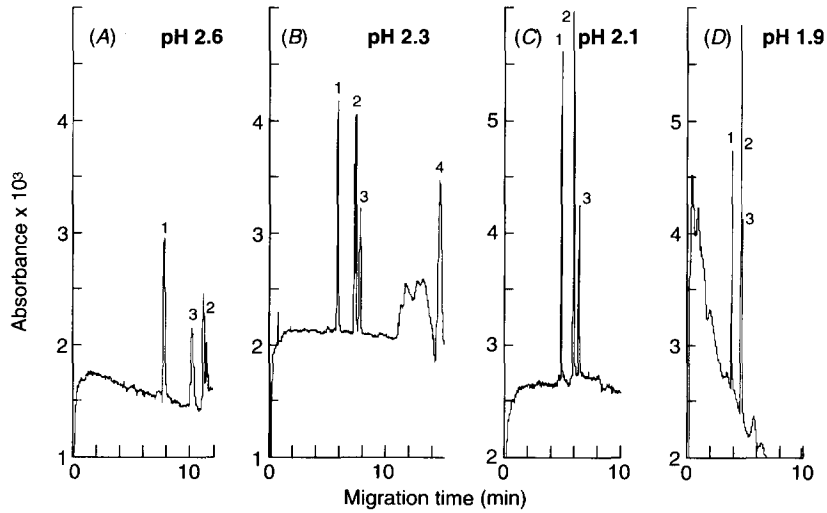

Fig. 3. Effects of electrolyte pH on the separation of ABD-thiols. pH = (A) 2.6, (B) 2.3, (C) 2.1, and (D) 1.9. Condition: see Table 1. Peaks: 1 = homocysteine, 2 = glutathione, 3 = cysteine, and 4 = ABD-F.

and 7.23 min, respectively. In particular, the peak of ABD-cysteine was larger than those of other thiols.

In general, moderate increase of plasma homocysteine has been reported in renal failure, some malignant states, and psoriasis. The concentration of homocysteine in plasma may be affected by various drugs [7,33,34]. Therefore, this method could be used for the determination of total homocysteine of patients. However, for monitoring the homocysteine level of a normal person, the sensitivity of the detector needs to be increased, for example, with a laser induced fluorescence detector (excitation at 380 nm and emission at 500–520 nm) [30], or the concentration of sample plug injected into the capillary needs to be enhanced by stacking [35].

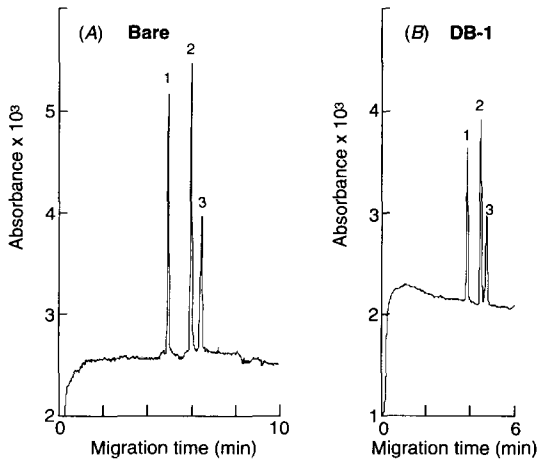

Fig. 4. (A) Bare fused silica capillary vs. (B) coated capillary (DB-1) on the separation of ABD-thiols. Condition: see Table 1. Peaks: 1 = Homocysteine, 2 = glutathione, and 3 = cysteine.

Table 3 compares the results obtained with various injection methods for ABD-homocysteine: Method I; hydrodynamic injection (0.5 psi for 5 s) of ABD-homocysteine dissolved in a 100 mM borate buffer (pH 8.0). Method II; hydrodynamic injection of a water plug (0.5 psi for 30 s) in front of the hydrodynamically injected (0.5 psi for 5 s) ABD-homocysteine dissolved in a 5 mM borate

Table 1
Optimum CE conditions for the separation of ABD-thiols

Electrolyte	50 mM sodium phosphate buffer (pH 2.1)
Applied voltage	15 kV
Capillary	27 cm × 50 μm i.d. bare fused silica capillary
Capillary temperature	25°C
Detection	UV 220 nm
Injection	Pressure (0.5 psi) 5 s

Table 2
Detection limits (S/N = 3) and reproducibility for ABD-thiols with UV detection

Compound	Detection limits (μM)	% R.S.D. (MT[a]/PA[b], $n = 10$)
Homocysteine	0.5	0.22/1.22
Glutathione	1	0.26/1.70
Cysteine	2	0.27/1.51

[a]MT = R.S.D. of the migration time.
[b]PA = R.S.D. of the peak area.

buffer (pH 8.0). Method III; hydrodynamic injection of a water plug (0.5 psi for 18 s) in front of the electrokinetically injected (20 kV for 18 s) ABD-homocysteine in a 5 mM borate buffer (pH 8.0). The peak heights of ABD-homocysteine with the method III increased about 2.5 times than with the method I. When a mixture of ABD-homocysteine, ABD-glutathione, and ABD-cysteine was injected by the method III, the peak height of ABD-homocysteine increased 2.3 times. However, the peak heights of ABD-glutathione and ABD-cysteine decreased more than 10%. Therefore, the stacking method III could be useful for determining homocysteine alone in the sample.

4. Conclusion

The described procedure is very simple, rapid, and reproducible for the analysis of homocysteine and other thiols in abnormal human plasma.

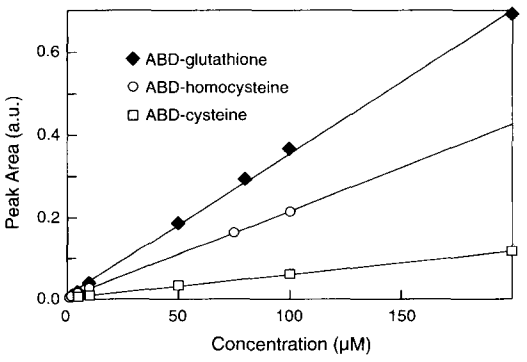

Fig. 5. Calibration curves of peak area vs. concentration ABD-thiols.

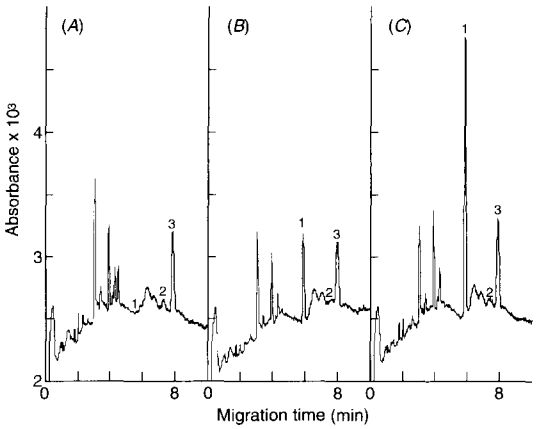

Fig. 6. Electropherogram of ABD-thiols in human plasma. (A) plasma sample from a normal person. (B) Mixture of the plasma sample and 32.3 μM homocysteine. (C0 Mixture of the plasma sample and 76.9 μM homocysteine. Condition: see Table 1. Peaks: 1 = homocysteine, 2 = glutathione, and 3 = cysteine.

Derivatization was finished in 5 min at 50°C, pH 8.0. Separation time was within 8 min for the ABD-homocysteine, -glutathione, and -cysteine. For the determination of homocysteine alone in plasma, the method III for the stacking will be useful. Determination of thiols in human plasma by using a CE with a laser induced fluorescence detector is also under consideration.

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Table 3
Comparison of relative peak heights and areas of ABD-homocysteine using various injection methods

Method	Relative peak heights	Relative peak areas
I	1	1
II	1.075	1.346
III	2.469	3.173

Concentration of ABD-homocysteine: 500 μm.

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