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On-column derivatization for the analysis of homocysteine and other thiols by capillary electrophoresis with laser-induced fluorescence detection

S.H. Kang, W. Wei, E.S. Yeung*

Ames Laboratory-USDOE and Department of Chemistry, Iowa State University, Ames, IA 50011, USA

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Abstract

On-column derivatization and capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection has been developed for the fully automated assay of homocysteine and other thiols. The unique feature of this CE technique comes from the direct injection of a sample including homocysteine, enabling the derivatization with 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadizole (ABD-F) to be accomplished in the capillary. After the derivatization for 10 min at 50°C, the homocysteine was analyzed within 7 min under an applied electric field of 333 V cm^{-1}. The detection limit obtained for homocysteine with on-column LIF detection was 5.0 n*M*, as compared to 2.5 n*M* with pre-column LIF detection. The method is a very simple, fast, and practical approach for the fully automated assay of homocysteine and other thiols contained in low-volume and low-concentration samples. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Laser induced flourcescence detection; Homocysteine; Thiols

1. Introduction

The development of a fully automated analytical method for the determination of homocysteine can serve an important role in the field of biochemical and clinical investigation. Homocysteinemia has been identified as a risk factor for peripheral, cerebrovascular, and coronary heart disease from clinical studies [1–5]. Increasing evidence indicates that high total homocysteine may be causally related to several clinical situations, such as cardiovascular disease, birth defects, and folate and vitamin B_{12} deficiency [6–9]. Since plasma homocysteine concentrations can be lowered by administration of folic acid, the assessment of homocysteine status due to dietary modification or vitamin supplement programs, as well as in cardiovascular disease patients at large, will require rapid and reproducible assays [10]. Therefore, accurate and fast determination of the concentrations of homocysteine in serum and plasma is essential to understanding the role of homocysteine in the pathogenesis of vascular disease. Various methods such as amino acid analysis [11–15], radioenzymatic assay [16], enzymatic assay [17], and high-performance liquid chromatography (HPLC) [18–27] have been developed for the determination of homocysteine and other thiols. These methods are

*Corresponding author. Tel.: +1-515-294-8062; fax: +1-515-294-0266.

E-mail address: yeung@ameslab.gov (E.S. Yeung)

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sensitive but time-consuming and require a relatively high volume of biological sample.

Recently, capillary electrophoresis (CE) associated with laser-induced fluorescence (LIF) detection has been successfully applied to detect and determine amino acids and amines in various samples such as protein [28,29], urine [30], single cell [31], wine [32] and cerebrospinal fluid [33,34]. To detect small amounts of amino acid and amines, various tags have been considered such as fluorescein isothiocyanate (FITC), naphthalene dicarboxaldehyde, 3-(4-carboxylbenzoyl)-2-quinoline carboxaldehyde, dansyl chloride, and carbocyanide [30–38]. In the case of thiols such as homocysteine and cysteine, some fluorogenic reagents having a benzofurazan structure [39,40], FITC [41], and 5-bromomethylfluorescein [42] have been used. Among the benzofuranzan reagents, ABD-F (4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole) and SBD-F (ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate) were used for the sensitive and specific detection of thiols using HPLC because of their excellent reactivity, selectivity to thiols, and good stability [43,44]. ABD-F reacts with thiols much faster and at milder conditions than SBD-F (Fig. 1). Kang et al. reported a CE method with UV absorbance to detect the homocysteine derivatized with ABD-F by pre-column derivatization in human plasma [45]. The method has enough sensitivity for the detection of homocysteine in the blood and urine of patients with disease (10-100 μM levels). However, in the case of a normal person, the total concentration of homocysteine is below μM (10-100 nM levels).

Ideally, one would like to determine low micromolar levels of homocysteine contained in sample volumes below 1 μl, so that clinical samples can be assayed without preconcentration. Therefore, there is the need to develop procedures for the rapid, sensitive, and specific determination of homocysteine in low sample volumes. Generally, most derivatization schemes are performed at a relatively high concentration of the analyte; once derivatized, the samples are diluted to demonstrate sensitive detection. The derivatization procedures are either relatively time-consuming or the side-products complicate the electropherograms. In addition, the possibility of tagging other compounds with similar functionality exists, which can create further complications. To overcome these drawbacks, we have developed a novel technique for the fully automated assay of homocysteine and other thiols using CE with on-column derivatization and LIF detection. LIF detection allows very sensitive and selective detection of the derivatized homocysteine and thiols with ABD-F.

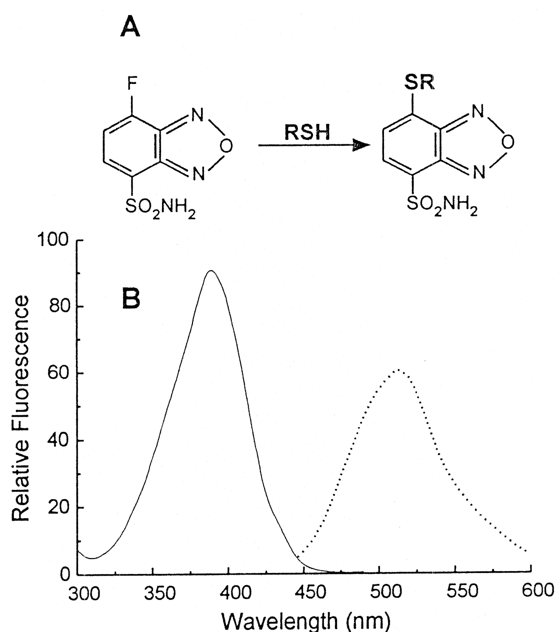

Fig. 1. Structure of ABD-F and its reaction with thiols including homocysteine (A) and fluorescence excitation (solid line) and emission (dotted line) spectra for ABD-homocysteine (B).

2. Experimental

2.1. Chemicals

4-aminosulfonyl-7-fluoro-2,1,3-benzoxadizole (ABD-F) was obtained from Dojindo Molecular Tech., Inc. (Gaithersburg, Maryland, USA). Boric acid, sodium borate, EDTA·2Na, Trizma®·Phosphate (mono[tris(hydroxymethyl)-aminomethane] phosphate), phosphoric acid and the thiols compounds such as D,L-homocysteine, L-cysteine and reduced glutathione were all purchased from Sigma Chemical Company (St. Louis, MO, USA) and used without further purification. Rhodamine B was purchased from Allied Chemical (Morristown, NJ, USA). The

water used to prepare solutions was deionized with a Milli-Q water purification system (Millipore, Worcester, MA).

2.2. Pre-column derivatization of thiols

In pre-column derivatization, homocysteine and other thiols were derivatized with ABD-F by Toyo'oka and Imai's method as shown in Fig. 1 [46,47]. 1.0 ml of 400 μ*M* ABD-F in 0.1 *M* sodium borate buffer (pH 8.0) was added to 1.0 ml of 100 μ*M* mixed standard thiols (homocysteine, glutathione and cysteine) in 0.1 *M* sodium borate buffer (pH 8.0) containing 2 m*M* EDTA·2Na. The reaction mixture was vortex mixed and heated at 50°C for 5 min. After the reaction, the mixture was cooled with ice-water, and 500 μl of 0.1 *M* HCl was added to the mixture to quench the reaction. The sample was either injected into the CE–UV system or the CE–LIF system, or the reaction mixture was directly injected without adding 0.1 *M* HCl.

2.3. CE with UV detection

All CE analyses with UV detection were performed at ambient temperature with an ISCO Model 3140 Capillary Eelectrophoresis System (Lincoln, NE). The detection wavelength was set at 220 nm for monitoring homocysteine and other thiols. A fused-silica capillary (Polymicro Technologies, Inc., Phoenix, AZ) with an inlet to detector length of 30 cm and a total length of 55 cm (50 μm I.D. and 361 μm O.D.) was used. The running buffer system used for all CE separations was composed of 20 m*M* Trizma® Phosphate (pH 2.1 with 0.1 *M* H_3PO_4). Sample injection was made by placing the inlet of the capillary into the sample vial and raising the sample vial 15 cm above the outlet vial, allowing the sample to siphon into the capillary for 10 s. The applied voltage was +15 kV. After each run, the capillary was rinsed by the following sequence with pressure; water, 0.1 *M* NaOH, water for 3 min, and running buffer for 4 min, respectively.

For on-column derivatization of homocysteine with ABD-F at CE–UV detection, the capillary was maintained at 38°C during the derivatization reaction.

2.4. CE with LIF detection

The experimental CE setup for on-column derivatization and LIF detection is similar to that described in Refs. [28,29] (Fig. 2). Briefly, a high-voltage power supply (Glassman High Voltage, Inc., Whitehorse Station, NJ) was used to drive the electrophoresis. The fused-silica capillary was 50 μm I.D., 360 μm O.D., and 45 cm long with a 30 cm length to the detector. The capillary inner surface was not treated or coated in any manner. Injection was performed hydrodynamically at a 15 cm height for both pre-column and on-column derivatization.

The reaction zone of the capillary for the derivatization was enclosed in a miniature heating system which was constructed by holding two frames (7.0 cm×3.5 cm×0.3 cm) together with four screws around the corners. The frame was made of thermally stable plastic. The base frame was affixed to one side of the heating tape (Omega, Stamford, CT) and a 25 μm thick brass sheet (Small Parts Inc, Miami Lakes, FL) was glued to the other side with thermally conductive epoxy. The cover frame was affixed to a second brass sheet also by thermally conductive epoxy. The front end (ca. 3 cm) of the capillary passed through the heating system. The entire electrophoresis and detection system was enclosed in a sheet-metal box with HV interlocks.

The 363.8 nm line from an argon-ion laser (Spectra Physics, Inc., Mountain View, CA; Model 2045-15) was isolated from other lines with prisms and focused with a lens of 1 cm focal length into the

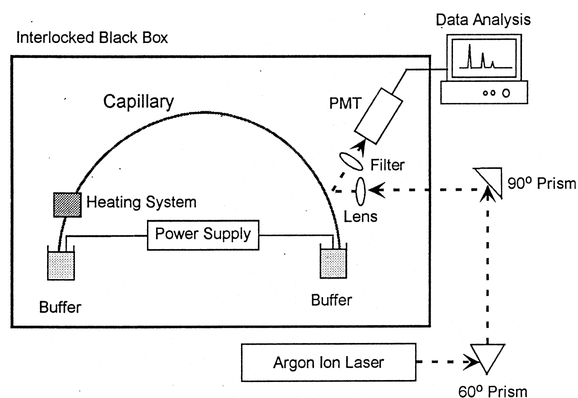

Fig. 2. Schematic diagram of on-column derivatization and CE–LIF detection system.

Table 1
Detailed timetable for on-column derivatization of homocysteine

Step	Operation	Value	Duration
1	Rinse-Water		3 min
2	Rinse-0.1 *M* NaOH		3 min
3	Rinse-Water		3 min
4	Rinse-Running buffer		4 min
5	Hydrodynamic injection of ABD-F	15 cm	30 s
6	Hydrodynamic injection of homocysteine	15 cm	10–15 s
7	Hydrodynamic injection of ABD-F	15 cm	20 s
8	Apply voltage	+1, −1, +1 kV	1 min, respectively
9	Incubation	38°C	10 min
10	Wait-Ambient temperature		
11	Separate-Voltage applied	+15 kV	20 min
12	End		

detection region of the capillary tubing. An interference filter (447–651 nm, Schott Glass Technologies, Inc., Duryea, PA) was used to isolate the emission signal and to prevent the excitation light from reaching the photomultiplier tube (PMT). The fluorescence signal from the PMT was transferred directly through a 10 kΩ resistor to a 24 bit A/D converter (Lawson Labs, Kalispell, MT) and stored in a 486/33 computer at 4 Hz.

2.5. On-column derivatization of homocysteine

For on-column derivatization, ABD-F, homocysteine and ABD-F were injected for 30, 10–15, and 20 s, respectively at a 15 cm height. The running buffer was injected for 3 min to transfer the reaction sample into the heating zone. +1, −1, and +1 kV of voltage were applied to achieve the mixing of homocysteine and ABD-F for 1 min, respectively. Then the sample was incubated for 10 min at 50°C. The detailed timetable for the on-column derivatization of homocysteine with ABD-F is shown in Table 1.

3. Results and discussion

3.1. CE–UV Detection

Fig. 3 shows the electropherogram of various thiols such homocysteine (Hcys), glutathione (Glu), and cysteine (Cys) analyzed by CE–UV detection after pre-column derivatization with ABD-F. All peaks were baseline separated within 11 min. Moreover, the remaining reagent peak (ABD-F) was far away from the derivatized thiols in the electropherogram. At pH 2.1, the ABD-thiols eluted earlier than the electroosmotic flow (EOF) peak because most of the silanol groups are protonated near pH 2.0 and the derivatized ABD-thiols have a

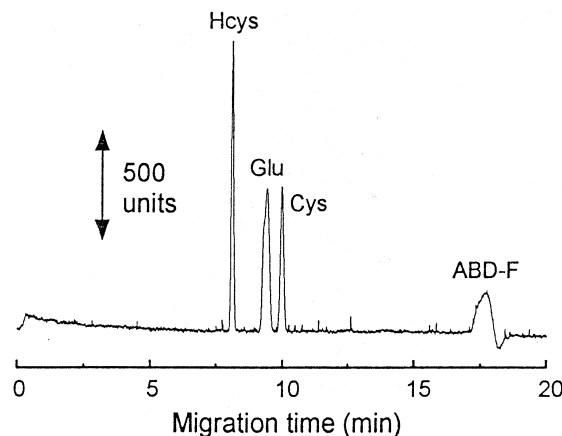

Fig. 3. Identification of various thiols analyzed by CE–UV detection after pre-column derivatization with ABD-F. CE conditions: running buffer, 20 m*M* Trizma®Phosphate (pH 2.1 with 0.1 *M* H_3PO_4); column, fused-silica capillary (55 cm×50 μm I.D., 30 cm effective length); hydrodynamic injection for 10 s at 15 cm height; applied voltage, +20 kV; 220 nm detection. Peak identification: Hcys: homocysteine. Glu: glutathione. Cys: cysteine. Sample concentration: 100 μ*M* each.

positive charge. As the applied field (cm V^{-1}) and the ionic strength increased, the analysis time decreased and the resolution increased. However, the current also increased.

Several approaches were attempted to derivatize ABD-Hcys on column. Fig. 4 shows the electropherogram of homocysteine obtained by various derivatization methods with UV detection. The excess reagent gives a shoulder after the main peak due to the presence of a contaminant. Although the optimum temperature for pre-column derivatization is 50°C [45–47], we could not maintain this temperature for on-column reaction because of the specifications of our commercial CE system. Even though we incubated the samples at 38°C for 60 and 20 min (Figs. 4B and 4C), the electropherograms showed adequate on-column reaction with ABD-F. Fig. 4B is an electropherogram obtained by hydrodynamic injection of a mixture of 1 m*M* of ABD-F and 1 m*M* of homocysteine (1:1, v/v), and Fig. 4C is obtained by consecutive hydrodynamic injection of 1 m*M* of ABD-F, homocysteine and ABD-F for 5, 5, and 3 s, respectively. As shown in the electropherograms, the reaction temperature and the effective mixing of homocysteine and ABD-F in the capillary were important factors for obtaining high yields of derivatized products.

3.2. CE–LIF detection

Fig. 5 shows a typical electropherogram of 100 n*M* thiols analyzed by pre-column ABD-F derivatization and LIF detection. The thiols were eluted within 16 min with baseline separation. This electropherogram is similar to the one obtained using UV detection.

Fig. 6 shows the on-column derivatization scheme for the determination of homocysteine. According to the Poiseulle equation [48], the volume of analyte injected per second, V_i, can be calculated by Eq. (1), where ρ is the density of buffer (0.9972 g ml^{-1}, for water at 20°C), g is the gravitational constant (980 cm s^{-2}), r is the capillary inner diameter in μm, ΔH is the difference in heights of the liquids in the sample and outlet vials, η is the viscosity of the sample, and L is the total capillary length.

$$V_i = \rho g r^4 \,\Delta H / 8\eta L \quad (1)$$

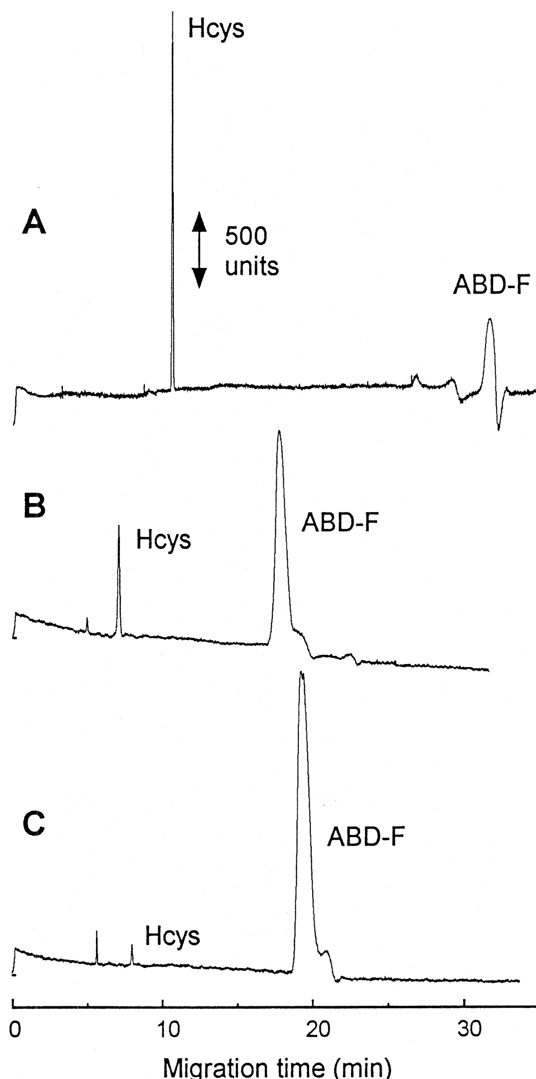

Fig. 4. Electropherograms of homocysteine obtained by various derivatization methods with UV detection. (A) Hydrodynamic injection for 5 s at 15 cm height with pre-column derivatized ABD-homocysteine (167 μ*M*) for 5 min at 50°C. (B) Hydrodynamic injection of a mixture of 1 m*M* ABD-F and 1 m*M* homocysteine (1:1, v/v) for 5 s followed by the running buffer for 3 s at 15 cm height. Voltage was applied (+1 kV) for 30 s, and the sample was incubated for 60 min at 38°C for on-column derivatization. (C) Hydrodynamic injection of 1 m*M* ABD-F, homocysteine, and ABD-F for 5, 5 and 3 s respectively at 15 cm height. Voltage was applied (+1, −1, and +1 kV) for 60, 60, and 30 s, respectively, and the sample was incubated for 20 min at 38°C for on-column derivatization. CE conditions: running buffer, 20 m*M* Trizma® Phosphate (pH 2.1 with 0.1 *M* H_3PO_4); column, fused-silica capillary (60 cm×50 μm I.D., 35 cm effective length); applied voltage, +25 kV, 220 nm detection.

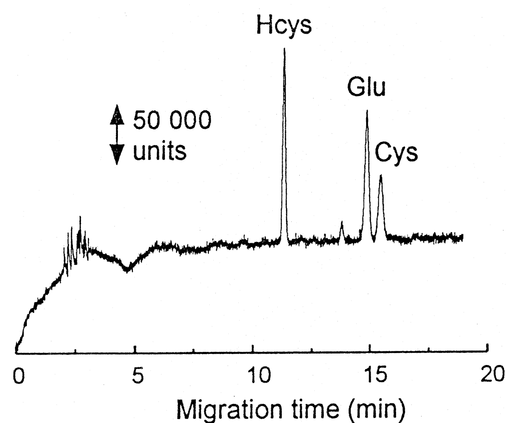

Fig. 5. Typical electropherogram of thiols analyzed by LIF detection after pre-column ABD-F derivatization. Sample concentration: 100 n*M*. CE conditions: column, fused-silica capillary 45 cm×50 μm I.D. (30 cm effective length); running buffer, 20 m*M* Trizma® Phosphate (pH 2.1 with 0.1 *M* H_3PO_4); applied voltage, +15 kV.

A more practical equation for injection volume in nL is

$$V_i = 2.84 \times 10^{-8}\, \Delta H\, tr^4/L \quad (2)$$

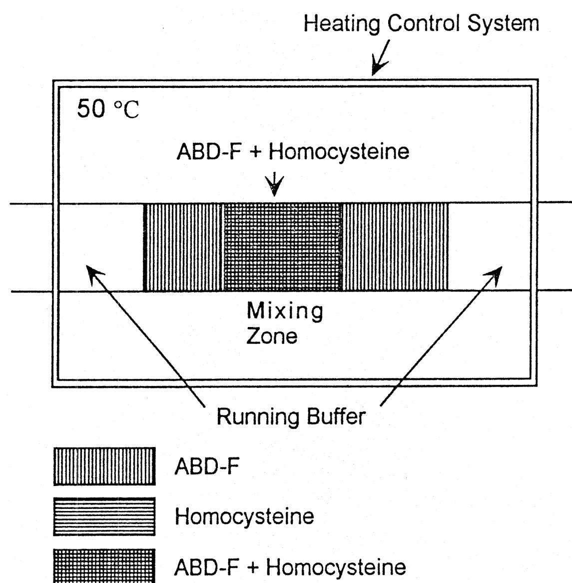

Fig. 6. Mixing zone of ABD-F and homocysteine inside the capillary.

where t is the time the sample is raised in seconds. In turn, the length of the sample plug, L_p, can be calculated from Eq. (3)

$$L_p = V_i/\pi r^2 = \Delta P r^2 t/8\eta L \quad (3)$$

The volume of homocysteine injected into the capillary for a 50 μm I.D. capillary are 5.9 and 8.8 nL, respectively, when the hydrodynamic injection times at 15 cm height are 10 and 15 s, respectively. By injection of 1 m*M* rhodamine B at a 15 cm height without applied voltage, we also confirmed that the velocity of homocysteine during injection was about 1.4 cm min^{-1}.

For on-column reaction, homocysteine required for the derivatization reaction was introduced between the ABD-F plugs. To move the homocysteine to the heating zone, running buffer was injected directly into the capillary by raising the sample vial 15 cm height difference for 3 min. The effective mixing of the homocysteine and ABD-F in the capillary was achieved with the electrokinetic movement induced by applying +1, −1, and +1 kV each for 1 min. Mixing was largely promoted by the difference in mobilities of these compounds under the electric field. The injection of the running buffer for 3 min at a 15 cm height further prevented the loss of the homocysteine sample toward the inlet vial when the voltage is reversed.

On-column derivatized ABD-homocysteine was detected by LIF. The homocysteine was injected at a concentration of 10 n*M*. Under the applied electric field of 333 V cm^{-1}, the determination of homocysteine was accomplished within 7 min. Compared to pre-column derivatization, on-column derivatization was superior in terms of simplicity and ease of automation. On the other hand, pre-column derivatization was better in terms of reproducibility and sensitivity. The overall reaction rate of the newly developed reaction scheme was faster than the typical pre-column derivatization procedure. Moreover, it can be performed on very small sample quantities (~nL level).

It should be noted that the entire operation in Table 1 can be fully automated by programming standard commercial CE instruments. Because the entire operation is performed inside a capillary tube, future implementation in multiplexed capillary arrays

Table 2
Detection limits ($S/N=3$) and reproducibility of pre-column derivatized thiols with ABD-F at UV and LIF detection

Compound	Detection limit (M)		% RSD[a] ($n=5$)	
	UV (10^{-6})	LIF (10^{-9})	UV	LIF
Homocysteine	1.0	2.5	0.22	2.46
Glutathione	2.0	4.0	0.26	2.06
Cysteine	2.5	5.0	0.27	1.63

[a] % RSD: Relative standard deviation of migration time (%).

[49–52] will provide the high throughput that is required for routine use in the clinical laboratory.

3.3. LOD and RSD by UV and LIF detection

The detection limit (LOD, $S/N=3$) of ABD-Hcys, -Glu, and -Cys by pre-column derivatization was in the range of 0.5–2.0 μM with UV detection and 2.5–5.0 nM with LIF detection. In order to determine the precision of the analysis, standard thiols were repeatedly injected 5 times under the same CE condition. The reproducibility was tested by injecting 100 μM thiols for CE–UV detection and 100 nM thiols for CE–LIF detection for 10 s at a 15 cm height. The RSD of migration times for UV detection and LIF detection were 0.22–0.27% and 1.63–2.46%, respectively, as shown in Table 2. The result of LIF detection showed almost 1000 times increased sensitivity than obtained with UV detection. This indicates it can be safely applied for the determination of thiols in clinical samples. The LOD of homocysteine on-column derivatization with LIF detection was 5.0 nM. Although the LOD was slightly poorer than that of the pre-column derivatization, the sensitivity of on-column derivatization is more than sufficient for the determination of homocysteine in normal human plasma.

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