Short Communication

Efficient Separation and Sensitive Detection of Biothiols by Hydrophilic Interaction Liquid Chromatography with Fluorescence Detection after Derivatization with 4-Aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole

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Abstract

Hydrophilic interaction liquid chromatographic (HILIC) separation of biologically important thiols derivatized with 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F) was performed for the first time. After optimization of mobile phase conditions, including acetonitrile content, pH, and buffer concentration, eight ABD-thiols (cysteamine, cysteine, N-acetylcysteine, glutathione, homocysteine, cysteinylglycine, γ-glutamylcysteine, and N-(2-mercaptopropionyl)glycine as an internal standard) were successfully separated in 16 min on ZIC-HILIC column with sulfoalkylbetaine groups. The optimum mobile phase was acetonitrile/50 mM ammonium formate buffer, pH 2.5 (82/18, v/v). The limits of quantitation for ABD-thiols, when signal-to-noise ratio was 10, ranged from 1–120 nM, lower than previous reports that used reversed-phase conditions. The increased sensitivity was partly due to enhancement of fluorescence intensity of ABD-thiols in the acetonitrile-rich mobile phase used in HILIC conditions.

Keywords: Cysteine; Glutathione; Homocysteine; ZIC-HILIC; Disulfide

1. Introduction

Low-molecular-weight thiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) play important roles in many biological processes, including the maintenance of extra- or intracellular redox homeostasis [1-3]. The reduced form of the thiol (R-SH) can act as a reducing agent, while the disulfide (R-S-S-R') form acts as an oxidizing agent. Imbalances in sulfur metabolism or the thiol/disulfide ratio are associated with cardiovascular diseases, several neurodegenerative diseases, cancer, and diabetes mellitus [4], making quantification of the thiols and disulfide form of each thiol imperative.

Typically, when analyzing the thiol content of biological samples, body fluids are extracted from tissue or whole blood, and the disulfides reduced to their thiol form for total thiol quantification. The mixture is then deproteinized prior to thiol-selective derivatization and separation, which is performed using fluorescence detection. The reduction step can be omitted to quantify the amount of reduced-form thiols, and the quantity of disulfides obtained by subtracting the amount of reduced-form thiols from the total thiol amount [4]. Each procedure is usually repeated twice to quantify the amount of thiols and disulfides in a biological sample. To simplify the procedure, Toyo’oka et al. proposed a method to quantify thiols and disulfides simultaneously [5-7]. In this method, both forms of the thiol were serially reacted with a fluorogenic reagent (4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F)), a reducing reagent, and a different fluorogenic reagent (ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F)) to generate ABD- and SBD-derivatives corresponding to reduced-form thiols and disulfides, respectively. Simultaneous separation of ABD-thiols and SBD-thiols was necessary, and it was performed using reversed-phase liquid chromatography (RPLC).

Recently, we developed an analytical method for separating total thiols derivatized with SBD-F using hydrophilic interaction liquid chromatography (HILIC) [8-10]. The method was faster and more sensitive than previous methods [4], as SBD-thiols are highly polar
compounds that can be easily separated under HILIC conditions [11-13]. Moreover, the organic solvent content of the mobile phase is higher under HILIC conditions [11-13], and the fluorescence intensity of SBD-thiols increases in organic solution [14]. Since ABD-thiols have similar properties, simultaneous determination of thiols and disulfides using HILIC should improve both the analysis time and sensitivity compared to RPLC. However, there is no report of the separation of ABD-thiols using HILIC, which is necessary for simultaneous determination of ABD- and SBD-thiols. Herein, we report that separation of eight thiols (N-acetylcysteine (NAC), cysteamine (CA), cysteinylglycine (CysGly), Cys, GSH, and γ-glutamylcysteine (γGluCys), and N-(2-mercaptopropionyl) glycine (MPG) as an internal standard) derivatized with ABD-F was successfully performed under HILIC conditions. The retention behavior of ABD-thiols under various HILIC conditions and the fluorescence properties of ABD-thiols were investigated.

2. Experimental

2.1. Chemicals and reagents

L-Cysteine (Cys), DL-homocysteine (Hcy), L-glutathione (GSH), cysteinylglycine (CysGly), γ-glutamylcysteine (γGluCys), and HPLC-grade acetonitrile were purchased from Sigma-Aldrich (St. Louis, MO, USA). N-Acetyl-L-cysteine (NAC), tiopronin (N-(2-mercaptopropionyl) glycine, MPG), and formic acid were obtained from Wako (Osaka, Japan). 4-Aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F) was purchased from Dojindo (Kumamoto, Japan). Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals were of analytical-reagent grade.

2.2. Preparation of standard sample

A 50 µl of thiol solution was added to the derivatization solution containing 125 µl of 100 mM borate buffer (pH 9.3), 5 mM disodium ethylenediamine-N,N,N',N'-tetraacetate solution, and 50 µl of 3.0 g/L ABD-F solution in the borate buffer. The resulting mixture was allowed to react for 5 min at 60°C. The derivatization reaction was quenched by adding 25 µl of 1 M hydrochloric acid, and the resulting solution was cooled on ice. The injection samples contained 90% (v/v) acetonitrile. Five microliters of the sample was injected into the HPLC system.

2.3. HPLC apparatus and chromatographic condition

The HPLC system consisted of a pump (PU-2080 Plus, JASCO, Tokyo, Japan), a degasser (DG-2080-53, JASCO), a column oven (860-CO, JASCO), and a fluorescence detector (RF-20A, Shimadzu, Kyoto, Japan). An analytical column used was ZIC-HILIC (150 mm × 2.1 mm i.d., 5 µm, Merck, Germany). Column temperature was set at 35°C, and the ABD-thiols were detected by fluorescence with excitation and emission wavelengths of 380 and 510 nm, respectively. Mixture of acetonitrile/ammonium formate buffer was used as the mobile phase. The flow rate was 0.2 mL/min. The chromatograms were analyzed using the software Chromato-Pro (Run Time Corporation, Sagamihara, Japan).

2.4. Fluorometry

ABD-thiol aqueous solutions were obtained by the derivatization method mentioned above. To examine the effects of dissolving solution on fluorescence intensity of ABD-thiols, the solution was diluted with 100 mM ammonium formate buffer (pH 2.5, 3.0, or 3.5) containing 0 or 80% (v/v) acetonitrile. The final concentration of ABD-thiol solution was set at 2 µM. The fluorescence spectra of ABD-thiols were measured by using a FP-6500 (JASCO) spectrofluorometer. The fluorescence intensity of ABD-thiols was defined as area under the excitation spectrum curve from 370 nm to 390 nm.

3. Results and discussion

3.1. Preliminary separation of ABD-thiols on ZIC-HILIC column

Using a ZIC-HILIC column with sulfoalkylbetaine groups, most ABD-thiols were not retained with a mobile phase of acetonitrile/10 mM ammonium formate buffer (pH 3.0) (75/25, v/v), which was the optimal mobile phase for the separation of SBD-thiols [8]. Therefore, more retentive mobile phases using 80% (v/v) acetonitrile were investigated. Although ABD-thiols were retained (retention times were between 2.2 and 13 min), several peaks co-eluted. The concentration of ammonium formate buffer
was changed to 100 mM, and several pH levels (2.5, 3.0, and 3.5) were investigated. All eight ABD-thiols were separated when pH was 2.5 or 3.5 (Fig. 1). While the resolution ($R_s$) between ABD-GSH and –γGluCys was lower at pH 2.5 ($R_s = 1.3$) than at pH 3.5 ($R_s = 1.7$), higher peaks were observed at lower pH. Specifically, the peak height of ABD–γGluCys was 3.8 times greater at pH 2.5 than at pH 3.5, meaning that the detection sensitivity of ABD–γGluCys was about 4 times higher at the lower pH. The detection sensitivity is especially important for ABD–γGluCys, as the concentration of γGluCys in human plasma is only 1 - ȝ0 much lower than other thiols such as Hcy, Cys, and CysGly [4,8,10].

3.2. Fluorescence properties of ABD-thiols

Fluorometric measurements were performed to investigate the effect of pH on the fluorescence of the ABD-thiols mentioned above. The fluorescence intensities of several ABD-thiols increased with decreasing pH, similar to the trend observed with ABD-Hcy in the previous study (Fig. 2) [15]. This result suggests that the increased peak heights associated with more acidic mobile phases was due in part to fluorescence enhancement of ABD-thiols at lower pH. The fluorescence intensities of derivatives with benzofurazan-type fluorogenic reagents, such as ABD-F and SBD-F, decrease in polar solvents, since probability of intersystem crossing, which compete fluorescence, would be increased in polar solvents [16]. This suggests that the detection sensitivity for most ABD-thiols is greater under HILIC conditions, which use an acetonitrile-rich mobile phase, than under RPLC conditions, which use a water-rich mobile phase.

3.3. Optimization of separation conditions of ABD-thiols

The concentration of the ammonium formate buffer (pH 2.5) used in the mobile phase was altered to achieve baseline separation of the adjacent peaks of ABD-GSH and –γGluCys. The $R_s$ values were 1.0, 1.4, 1.3, and 1.3 for 10, 30, 50, and 200 mM, respectively. To ensure full separation of ABD-CysGly and -CA from all other thiols such as Hey, Cys, and CysGly [4,8,10].

Fig. 2. Fluorescence intensity of ABD-thiols at various pH levels relative to pH 3.5. Symbols: ABD-derivatives of • MPG; ○ NAC; ▲ CA; □ Hcy; ● Cys; ○ CysGly; ■ GSH; and △ γGluCys.

Fig. 3. Effects of (A) concentration of ammonium formate buffer and (B) acetonitrile content on retention times of ABD-thiols. Mobile phase: (A) acetonitrile/ammonium formate buffer, pH 2.5 (80/20, v/v), and (B) acetonitrile/50 mM ammonium formate buffer, pH 2.5. Symbols: see caption for Fig. 2.
3.4. Detection sensitivity

The limits of detection (LOD) and quantitation (LOQ) of ABD-thiols (when signal-to-noise ratios are 3 and 10, respectively) were calculated using peak heights. The LODs for ABD-NAC, -CA, -Hcy, -Cys, -CysGly, -GSH, and -γGluCys were 2, 0.3, 3, 4, 4, 5, and 40 nM, respectively. The LOQs for ABD-NAC, -CA, -Hcy, -Cys, -CysGly, -GSH, and -γGluCys were 4, 1, 9, 15, 15, 20, and 120 nM, respectively. This detection sensitivity is approximately two times higher than that of a recently developed RPLC method [17] (LOQs for ABD-Cys, -CysGly, -Hcy, and -GSH were 20, 30, 30, and 50 nM, respectively.) This increase in detection sensitivity is likely due to the fluorescence intensity enhancement of ABD-thiols in the acetonitrile-rich mobile phase, as described above. The LODs achieved in this study were 2 - 20 times lower than those obtained using SBD-F and RPLC [18], while about ten times higher than those using SBD-F and HILIC [8].

4. Conclusion

Eight thiols derivatized with ABD-F were separated under HILIC conditions. As with SBD-thiols [8], the detection limit of ABD-thiols was improved under HILIC conditions as compared to RPLC conditions. The separation conditions for ABD-thiols were similar to those used to separate SBD-thiols under HILIC conditions, e.g., the mobile phase used for both separations was a mixture of acetonitrile/ammonium formate buffer, and both separations were performed using isocratic elution [8]. SBD-thiols are considered to be eluted after ABD-thiols on ZIC-HILIC column, the simultaneous separation of SBD- and ABD-thiols can be easily achieved. Thus, this study suggests that it is possible to simultaneously quantify the amount of thiols and disulfides derivatized with ABD-F and SBD-F under HILIC mode.

Acknowledgments

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References