Optimization of tris(2-carboxyethyl) phosphine reduction conditions for fast analysis of total biothiols in mouse serum samples

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ABSTRACT

In this study, we investigated suitable conditions for the reduction of disulfides in mouse serum samples by tris(2-carboxyethyl) phosphine (TCEP) for fast analysis of total biothiols. Disulfides were reduced with TCEP, and then, thiols were derivatized with the fluorogenic reagent, ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F). Interference peaks on chromatograms of mouse serum samples disappeared when the TCEP reaction was conducted on ice instead of at room temperature, which is used classically. Low-molecular-weight disulfides, such as cystine and glutathione disulfide, were nearly completely reduced by TCEP on ice. Six SBD-biothiols (homocysteine, cysteine, cysteinylglycine, glutathione, γ-glutamylcysteine, and N-acetylcysteine) were separated within 7.5 min on a sulfoalkylbetain-type column (ZIC-HILIC: 150 × 2.1 mm i.d., 3.5 μm), without interference peaks. The developed method showed good linearity and reproducibility, with inter- and intra-day precisions of less than 3%.

1. Introduction

Cysteine (Cys), homocysteine (Hcy), glutathione (GSH), cysteinylglycine (CysGly), and γ-glutamylcysteine (γGluCys) are low-molecular-weight thiols that play important roles in several biological processes, including cell signaling, metabolism, and detoxification [1, 2, 3, 4]. Numerous studies have shown that biothiols concentration is associated with various pathologies such as cardiovascular and neuro-degenerative diseases, and diabetes mellitus [5, 6, 7, 8, 9, 10]. Therefore, biothiols are useful biomarkers for thiol-related diseases [11], and accurate methods for determination and quantification are needed. Biothiols exist in the reduced form (free thiols) and in the oxidized form (disulfides). However, in clinical studies involving large numbers of subjects, total thiol concentrations (sum of reduced and oxidized forms) are generally determined [12]. The most common procedure to measure total thiols is based on the reduction of disulfides, followed by thiol-group selective derivatization and high-performance liquid chromatography (HPLC) analysis [13]. In this procedure, the reduction reaction is one of the most critical steps. Among several reducing reagents, tris(2-carboxyethyl) phosphine (TCEP) is widely used [14, 15]. To derivatize thiols for detection, fluorogenic reagents, such as 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F), which allows highly sensitive and specific fluorescence detection, are commonly used [16].

Recently, we developed analytical methods for total biothiols utilizing TCEP and SBD-F as a reductant and a derivatization reagent, respectively [17]. A hydrophilic interaction liquid chromatography (HILIC) column, which retains hydrophilic compounds [18], was used for separation. Six SBD-biothiols (N-acetylcysteine (NAC), Cys, Hcy, GSH, CysGly, and γGluCys) in human plasma could be simultaneously determined on a sulfoalkylbetain-type column within 10 min [17]. However, because of the presence of numerous interference peaks on the chromatograms, the same method failed when applied to serum samples of mice, which are frequently employed as animal models in preclinical research. As unknown peaks interfere with the quantification, it took long to separate all the target biothiols from the undesired peaks. For example, separation of five SBD-biothiols (Cys, Hcy, GSH, CysGly, and γGluCys) from the interference peaks for mouse serum samples took 16 min when an amide-type column was used. Two large interference peaks were from thiol-containing compounds with molecular weights of ca. 1000 [19].

Therefore, in this study, we aimed to develop a method that could avoid the production of unknown peaks on chromatograms of mouse serum samples by optimizing reaction parameters and testing different columns.

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2. Materials and methods

2.1. Chemicals and reagents

L-Cys, D,L-Hcy, L-GSH, CysGly, and γGluCys were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-Acetyl-L-cysteine (NAC), tiopronin (*N*-(2-mercaptopropionyl)glycine, MPG), and trichloroacetic acid (TCA) were obtained from Wako (Osaka, Japan). Ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBD-F) was purchased from Dojindo (Kumamoto, Japan). TCEP was from Tokyo Chemical Industry (Tokyo, Japan). Phosphate buffered saline (PBS) was purchased from Takara Bio (Shiga, Japan). HPLC-grade acetonitrile was used. Water was purified using a Milli-Q system (Millipore, Bedford, MA, USA). All other chemicals were of analytical grade.

2.2. Sample preparation for optimization of TCEP reduction conditions

Mouse serum and human plasma samples were obtained from Sigma-Aldrich. The sample pretreatment (Fig. 1) for quantification of total thiols was performed based on our previous study with little modification [19]. For the reduction of disulfides and protein-bound thiols, 10 μL of TCEP solution (120 g/L in PBS) was added to a mixture of 50 μL of mouse serum and 50 μL of MPG solution (8 μM in PBS) or NAC solution (80 μM in PBS). MPG and NAC were used as internal standards in analysis using an amide column and a sulfoalkylbetaine column, respectively. To investigate the reduction condition, the mixtures were incubated at room temperature or on ice, for 0, 5, 10, 15, 30, or 60 min. The samples were mixed with 50 μL of TCA solution (100 g/L) and disodium ethyl-enediamine-*N*, *N*, *N′*, *N′*-tetraacetate (EDTA) solution (1 mM) for deproteinization. After centrifugation at 15000 ×g for 10 min at 4 °C, 100 μL of the supernatant was derivatized with 100 μL of SBD-F solution (3.0 g/L in borate buffer) and 246 μL of borate buffer (125 mM, pH 9.5). To neutralize the solution, 4 μL of sodium hydroxide solution (5 M) was added. The mixture was incubated at 60 °C for 1 h, 50 μL of hydrochloric acid (1 M) was added, and the mixture was cooled on ice to quench the derivatization reaction. The solution was diluted 5 times with acetonitrile for injection to avoid deterioration of peak shape [20]. Five microliters of the sample was injected into the HPLC system for analysis.

2.3. HPLC conditions

The HPLC system consisted of a pump (PU-2080 Plus, JASCO, Tokyo, Japan), a column oven (CO-1560, JASCO), and a fluorescence detector (RF-20A, Shimadzu, Kyoto, Japan). An Inertsil Amide column (150 × 3.0 mm i.d., 5 μm, GL Sciences, Tokyo, Japan) was used to investigate TCEP reaction conditions. The mobile phase was acetonitrile-40 mM ammonium formate buffer (pH 3.0) (75/25, v/v), at a flow rate of 0.4 mL/min. For faster analysis of biothiols in mouse serum samples, a ZIC-HILIC column (150 × 2.1 mm i.d., 3.5 μm, Merck) was used. The mobile phase was acetonitrile-30 mM ammonium formate buffer (pH 3.4) (72/28, v/v) at a flow rate of 0.3 mL/min. The column temperature was set at 35 °C, and the SBD-thiols were detected based on fluorescence, with excitation and emission wavelengths of 375 and 510 nm, respectively. The chromatograms were analyzed using Chromato-Pro software (ver. 5.00, Run Time Corporation, Kanagawa, Japan).

2.4. Validation

To validate the analytical method for total biothiols, standard concentration ranges of 15–1500 nM, 60–6000 nM, 5–500 nM, and 50–5000 nM for SBD-Hcy and -GSH, SBD-Cys, SBD-CysGly, and SBD-γGluCys, respectively, were injected as calibration standards. Mouse serum samples were spiked with additional thiols at three concentrations to assess the recoveries of thiols. For assessment of intra- and inter-day precision, mouse serum samples of each concentration were injected in quintuplicate on the same day and on sequential days, respectively. The relative peak height to SBD-NAC (internal standard) was evaluated for quantification.

3. Results and discussion

3.1. Optimization of TCEP reduction conditions

TCEP is widely used as a reducing agent for detecting total thiols in biological samples. The TCEP reaction is generally considered temperature-insensitive [21], and most studies conducted the TCEP reaction at room temperature [13, 17, 19, 22]. However, our previous study [19] showed that interference peaks were produced on chromatograms of mouse serum samples, and that the TCEP reaction might be responsible for this phenomenon. Thus, we aimed to optimize the TCEP reaction conditions for disulfides in mouse serum samples.

We first investigated the effect of conducting the TCEP reaction on ice instead of at room temperature. The interference peaks observed in the chromatogram when the reaction was carried out at room temperature disappeared when the reaction was carried out on ice (Fig. 2). The discrepancy in terms of temperature dependence of the TCEP reaction between the previous [21] and this study is likely because different animal species were used in both studies. The previous study used human plasma as a sample matrix, which hardly produces interference peaks when disulfides are reduced with TCEP under both conditions, regardless of the reaction temperature.

Next, we explored several reaction times (0, 5, 10, 15, 30, and 60 min) at the two reaction temperatures. As shown in Fig. 3, reduction was largely completed after 5 min both at room temperature and on ice, and after 30 min of reaction, a plateau was reached, indicating this time was certainly sufficient. It should be noted that interference peaks appeared when the reaction was carried out at room temperature, and the peaks of SBD-CysGly and SBD-γGluCys (only at 60 min) could not be identified. From these results, optimal reaction conditions for TCEP reduction in mouse serum samples were found to be on ice for 30 min.

To confirm that the reduction reaction on ice was complete, we conducted further experiments. First, standard solutions of the oxidized form of glutathione (glutathione disulfide, GSSG) and cysteine (cystine) were reduced by the above procedure. As shown in Fig. 4A, peak height ratios (reduced on ice/at room temperature) of reduced GSSG and cystine were nearly 100%. Hence, disulfides were considered to be completely reduced on ice. Next, mouse serum and human plasma samples were evaluated using the same procedure. The peak height ratios of SBD-Hcy, -Cys, -GSH, and -γGluCys in mouse serum samples were 88.3%, 86.4%, 85.8%, and 98.2%, respectively (Fig. 4B). We reason that the peak height
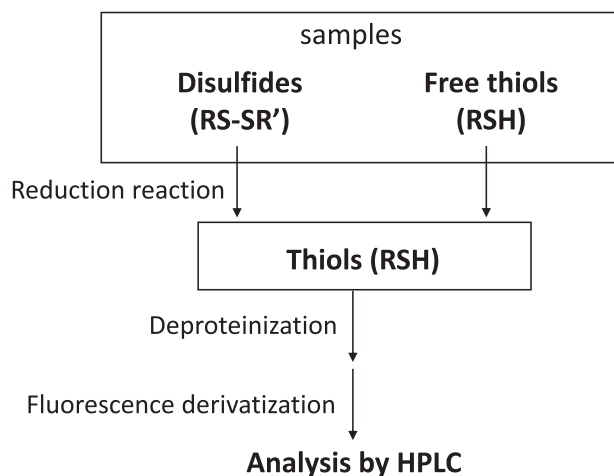

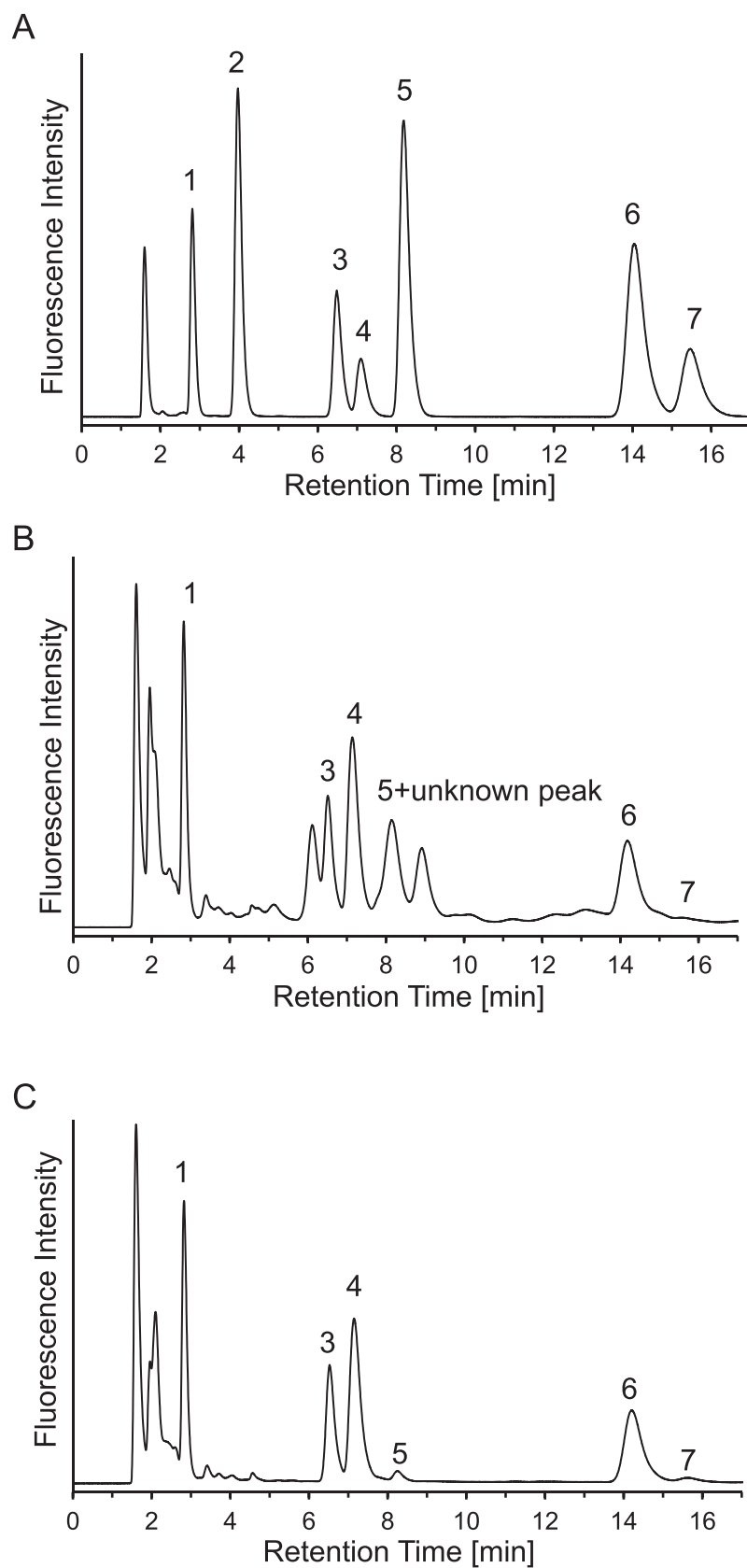**Fig. 1.** Scheme of the process of sample pretreatment for determining total thiols.

Fig. 2. Chromatogram of (A) 1 μM standard solution (0.1 μM MPG), (B) mouse serum samples with TCEP reaction at room temperature for 30 min, and (C) mouse serum samples with TCEP reaction on ice for 30 min. Peaks: 1, SBD-MPG; 2, SBD-NAC; 3, SBD-Hcy; 4, SBD-Cys; 5, SBD-CysGly; 6, SBD-GSH; 7, SBD-γGluCys. Column: Inertsil Amide (150 × 3.0 mm, 5 μm). Column temperature: 35 °C. Mobile phase: acetonitrile-40 mM ammonium formate buffer (pH 3.0) (75/25, v/v). Flow rate: 0.4 mL/min.
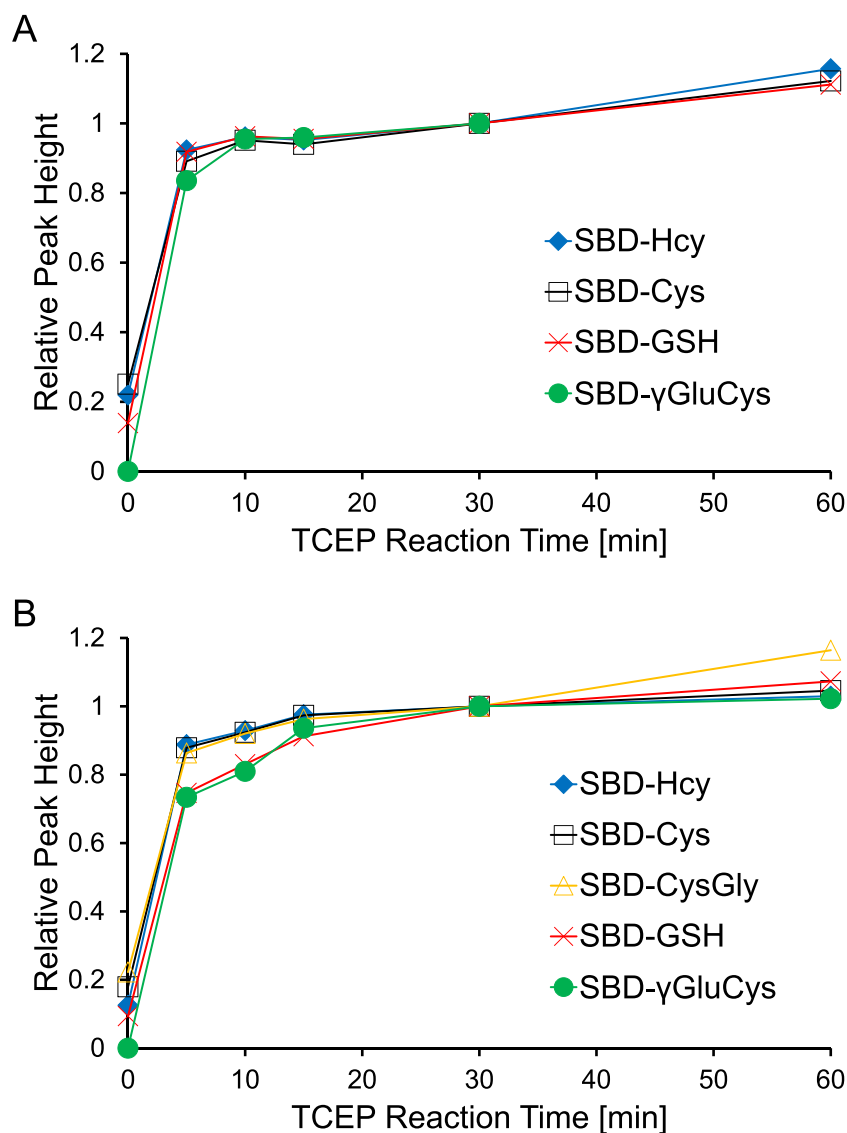
Fig. 3. Relative peak heights of SBD-thiols under TCEP reaction (A) at room temperature and (B) on ice. The relative peak height was obtained by setting the peak height of each SBD-thiol at 30 min as 1. Number of samples: at room temperature (RT), n = 3; on ice, n = 3. Symbols: ◆, SBD-Hcy; □, SBD-Cys; △, SBD-CysGly; ✕, SBD-GSH; ●, SBD-γGluCys.

ratios were relatively low because the peak heights of SBD-biothiols in the mouse serum samples reduced at room temperature were over-estimated because of the presence of interference peaks as shown in Fig. 2B. As for the human plasma samples, which produce less interference peaks than mouse serum samples, the peak height ratios of SBD-Hcy, -Cys, -CysGly, -GSH, and -γGluCys were 95.6%, 90.7%, 95.6%, 86.2%, and 97.1% (Fig. 4C). Although these peak height ratios were higher than those for mouse serum samples, those of SBD-Cys and -GSH were still low. As low-molecular-weight disulfides such as cystine and GSSG should be completely reduced as described above, it is possible that protein-bound Cys and GSH are difficult to reduce under low temperature. In other words, although the TCEP reduction reaction and the reduction of low-molecular-weight disulfides might be quite temperature-insensitive, the reduction of large-molecular-weight disulfides, such as protein-bound biothiols (and two large unknown peaks determined in our previous study [19]) might be hampered at low temperature. Further studies should be necessary to clarify the phenomena.

3.2. Development of a fast separation method for SBD-biothiols in mouse serum samples utilizing sulfoalkylbetain-type column

In our previous study on the determination of biothiols in mouse serum samples, an amide-type column (Inertsil Amide) was utilized for the separation of unknown peaks from the biothiols [19]. As the unknown peaks almost disappeared when the TCEP reduction was conducted on ice, a ZIC-HILIC column, which is the best for SBD-biothiol separation [17], was used to analyze SBD-thiols in mouse serum samples.

In our previous study [17], SBD-biothiols could be separated within 10 min on a ZIC-HILIC column with 5-μm-diameter particles. To achieve faster and more efficient separation, a ZIC-HILIC column with smaller particle size (3.5 μm) was used in this study. After optimization of mobile phase conditions such as acetonitrile content, pH of buffer, and salt concentration, six SBD-thiols (SBD-NAC, -Hcy, -Cys, -CysGly, -GSH, and -γGluCys) were separated within 7.5 min with a mobile phase of acetonitrile-30 mM ammonium formate buffer (pH 3.4) (72/28, v/v) (Fig. 5A). In mouse serum samples, five biothiols in mouse serum samples
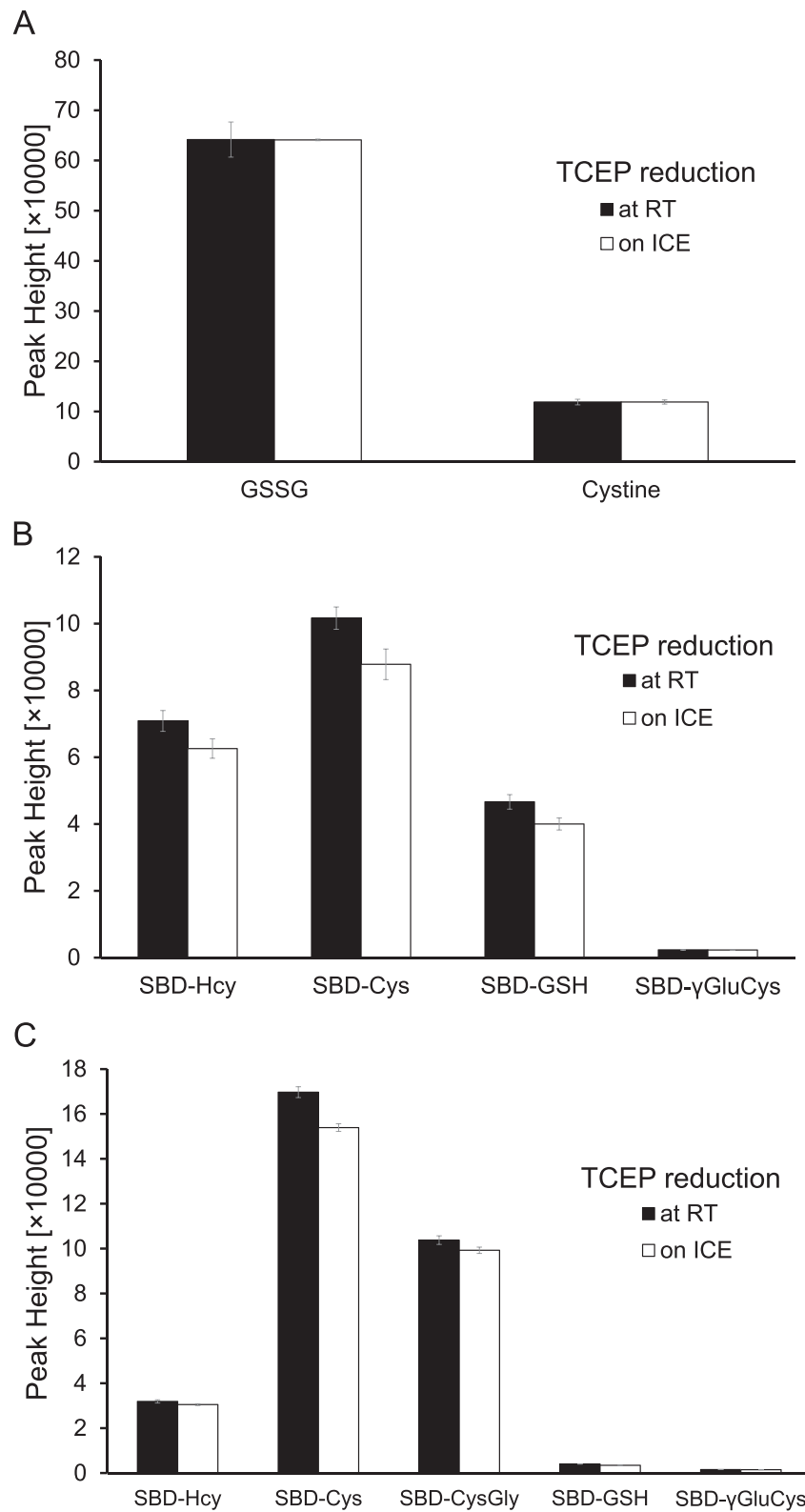
Fig. 4. Peak heights of SBD-biothiols in (A) standard solution of oxidized form of glutathione (glutathione disulfide, GSSG) and cysteine (cystine), (B) mouse serum samples and (C) human plasma samples under TCEP reduction reaction at room temperature and on ice. Number of samples: at RT, n = 3; on ice, n = 3. Error bars: standard deviation.
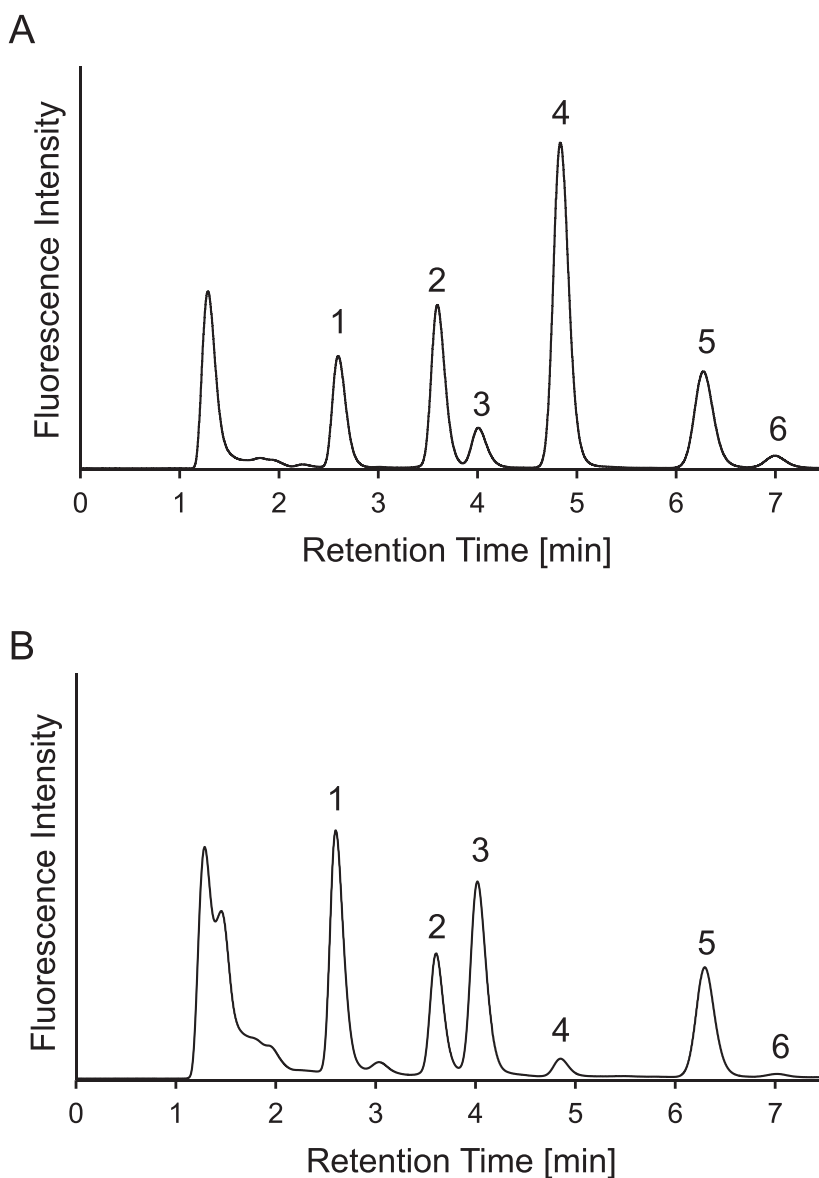
Fig. 5. Chromatograms of (A) 0.5 μM standard solution, and (B) mouse serum samples under the optimized conditions. Peaks: 1, SBD-NAC; 2, SBD-Hcy; 3, SBD-Cys; 4, SBD-CysGly; 5, SBD-GSH; 6, SBD-γGluCys. Column: ZIC-HILIC (150 × 2.1 mm, 3.5 μm). Column temperature: 35 °C. Mobile phase: acetonitrile-30 mM ammonium formate buffer (pH 3.4) (72/28, v/v). Flow rate: 0.3 mL/min.

were effectively separated (Fig. 5B), which was twice faster than when using an Inertsil Amide column.

Because disulfides in mouse serum samples were reduced on ice, few unknown peaks appeared after the elution of SBD-biothiols. Therefore, time-consuming column washing and further column equilibration were

not necessary. Thus, the ZIC-HILIC column with smaller particle size and simplified mobile phase allowed analyzing SBD-thiols in mouse serum samples in a shorter time.

3.3. Method validation

The method for analyzing thiols in mouse serum samples developed in this study was validated. Table 1 shows the linear range of the calibration curve, the limit of detection (LOD) at a signal-to-noise (S/N) of 3, and the limit of quantification (LOQ) at an S/N of 10. The LODs obtained in this study were 20–160 times lower than those under reversed-phase liquid chromatography conditions [23, 24, 25]. As described in our previous report [17], the use of an acetonitrile-rich mobile phase in HILIC enhances the fluorescence intensity of SBD-biothiols and thus improves sensitivity [26, 27].

Table 2 shows the intraday precision expressed as relative standard deviation (RSD), and the recoveries percentage for the thiols in mouse serum samples. The intraday precision was less than 2.3%, and recovery

Table 1
Linearity, limit of detection (LOD), and limit of quantification (LOQ) of SBD-thiols.

Thiols	LOD [nM]	LOQ [nM]	Linearity [nM]	Regression equation*
	S/N = 3	S/N = 10	$R^2 > 0.9999$	
Hcy	1.0	3.4	15–1500	y = 1.6E-3x + 2.0E-4
Cys	3.9	13	60–6000	y = 4.0E-4x – 1.2E-3
CysGly	0.5	1.7	5–500	y = 3.3E-3x – 2.5E-3
GSH	0.8	2.5	15–1500	y = 1.1E-3x + 8.6E-3
γGluCys	5.8	19	50–5000	y = 2.0E-4x + 3.2E-3

* y: relative height compared to SBD-NAC height, x: SBD-thiol concentration in injection sample [nM].

Table 2
Intraday precision and recovery of thiols in spiked mouse serum samples.

Thiols	Added [μM]	Measured (mean ± SD) [μM]	RSD [%]	Recovery [%]
Hcy	0	25.8 ± 0.2	0.8	-
	15	39.8 ± 0.2	0.6	93
	30	54.1 ± 0.2	0.4	94
	60	82.9 ± 0.2	0.2	95
Cys	0	178 ± 1	0.5	-
	75	241 ± 2	0.8	84
	150	306 ± 1	0.3	85
	300	435 ± 1	0.2	86
CysGly	0	2.00 ± 0.01	0.7	-
	1.5	3.31 ± 0.02	0.5	87
	3	4.64 ± 0.02	0.5	88
	6	7.31 ± 0.02	0.3	88
GSH	0	31.7 ± 0.4	1.4	-
	15	45.1 ± 0.3	0.7	89
	30	58.8 ± 0.3	0.6	90
	60	88.2 ± 0.5	0.5	94
γGluCys	0	8.6 ± 0.2	2.3	-
	7.5	15.6 ± 0.2	1.0	93
	15	22.6 ± 0.2	0.9	93
	30	37.6 ± 0.2	0.5	97

Table 3
Interday precision and recovery of thiols in spiked mouse serum samples.

Thiols	Added [μM]	Measured (mean ± SD) [μM]	RSD [%]	Recovery [%]
Hcy	0	25.9 ± 0.4	1.5	-
	15	39.6 ± 0.7	1.8	91
	30	53.7 ± 0.8	1.5	93
	60	81.7 ± 1.3	1.6	93
Cys	0	179 ± 3	1.8	-
	75	241 ± 5	2.1	83
	150	304 ± 6	2.0	83
	300	431 ± 9	2.0	84
CysGly	0	2.01 ± 0.06	2.8	-
	1.5	3.27 ± 0.08	2.6	84
	3	4.58 ± 0.09	2.0	86
	6	7.21 ± 0.17	2.4	87
GSH	0	31.5 ± 1.0	3.0	-
	15	44.3 ± 1.2	2.7	85
	30	57.7 ± 1.2	2.1	87
	60	85.8 ± 1.8	2.1	90
γGluCys	0	8.3 ± 0.2	2.0	-
	7.5	14.9 ± 0.3	2.0	88
	15	21.8 ± 0.4	1.8	90
	30	36.2 ± 0.9	2.6	93

ranged from 84% to 97% in spiked mouse serum samples. The interday precision was less than 3.0% (Table 3).

4. Conclusions

In this study, we developed a relatively fast analytical method for biothiols in mouse serum samples using a sulfoalkylbetaine-type column after optimizing TCEP reduction reaction conditions. The results indicate that the production of interference peaks from large-molecular-weight disulfides (unknown peaks determined in our previous study) in mouse serum samples can be avoided by conducting TCEP reduction at low temperature, while low-molecular-weight disulfides are nearly completely reduced under this condition. Owing to the absence of the interference peaks, faster separation of six SBD-thiols (SBD-NAC, -Hcy, -Cys, -CysGly, -GSH, -γGluCys) in mouse serum samples was achieved, with good validation results. This method could be applied to the analysis of other biological samples, including tissues, urine, or samples of other animals with large-molecular-weight disulfides.

Declarations

Author contribution statement

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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