Journal of Pharmaceutical and Biomedical Analysis 56 (2011) 103–113

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Quantification of reduced and oxidized thiols in mouse serum by column-switching hydrophilic interaction chromatography coupled with mass spectrometry

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ARTICLE INFO

Article history:
Received 17 March 2011
Received in revised form 24 April 2011
Accepted 27 April 2011
Available online 5 May 2011

Keywords:
Glutathione
Glutathione disulfide
Oxidative stress
Column-switching
HILIC–MS

ABSTRACT

An automated online solid-phase extraction method for the determination of the reduced and oxidized forms of thiols in mouse serum was developed and validated. Analysis was performed with column-switching hydrophilic interaction chromatography coupled with mass spectrometry (CS-HILIC–MS). The proposed CS-HILIC–MS method enabled the simultaneous determination of reduced and oxidized thiols in mouse serum samples. In addition, interference from endogenous compounds was removed by means of the column-switching technique. We also compared the effects of derivatization before and after preparing serum from blood samples and found that it was necessary to perform the derivatization immediately before preparing serum from blood samples. We investigated the role of thiol compounds in lipopolysaccharide (LPS)-induced acute inflammation *in vivo*. Serum glutathione disulfide and cystine levels were significantly decreased at 4 h after LPS treatment. Our method is expected to be useful for the assessment of the roles of reduced and oxidized glutathione in the oxidative state.

1. Introduction

Reduced glutathione (GSH) is a major intracellular nonprotein thiol that plays a vital role in protecting cells and tissues from oxidative injury. It is a tripeptide composed of cysteine (Cys), glutamic acid, and glycine. GSH is present in all organs, particularly the liver. It is present in virtually all mammalian tissues. Intracellular and blood GSH concentrations are in the millimolar range, whereas plasma GSH concentration is in the micromolar range and accounts for approximately 0.4% of total blood GSH level [1,2]. GSH plays an essential role in maintaining the intracellular redox environment that is critical for the function of cellular proteins. Under oxidative stress conditions, GSH is oxidized to glutathione disulfide (GSSG) and/or bound to a protein. Therefore, the GSH to GSSG (oxidized GSH and protein-bound GSH) ratio is altered. Many studies have indicated that GSH and GSSG concentrations are influenced by various diseases, with a tendency toward a decrease in the GSH/GSSG ratio [3,4].

GSH, other low-molecular-mass thiols, and disulfides are measured by several methods. For instance, high-performance liquid chromatography can simultaneously detect GSH, other low-molecular-mass thiols, and disulfides but requires sample derivatization because non-derivatized GSH and GSSG are highly polar compounds and thus difficult to determine by reversed-phase high-performance liquid chromatography. Studies have suggested that GSH and GSSG can be separated with a highly polar stationary phase, such as a hydrophilic interaction chromatography (HILIC) column [5,6]. HILIC, which combines bare silica or polar bound stationary phase with mobile phase having high organic solvent content, has been proven to be a valuable tool for the analysis of polar compounds in biological samples. However, in some cases, no analyte peak could be detected because HILIC coupled with mass spectrometry (HILIC–MS) was affected by ion suppression by a highly polar compound, such as sodium chloride.

High-performance liquid chromatography coupled with such detection techniques as ultraviolet (UV) [7] and fluorescence (FL) [8] is widely used because of its convenience, specificity, and satisfactory sensitivity. Liquid chromatography coupled with mass spectrometry (LC–MS) has been utilized for the determination of GSH and GSSG owing to its high detection sensitivity. However, most LC–MS methods require derivatization with

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doi:10.1016/j.jpba.2011.04.025

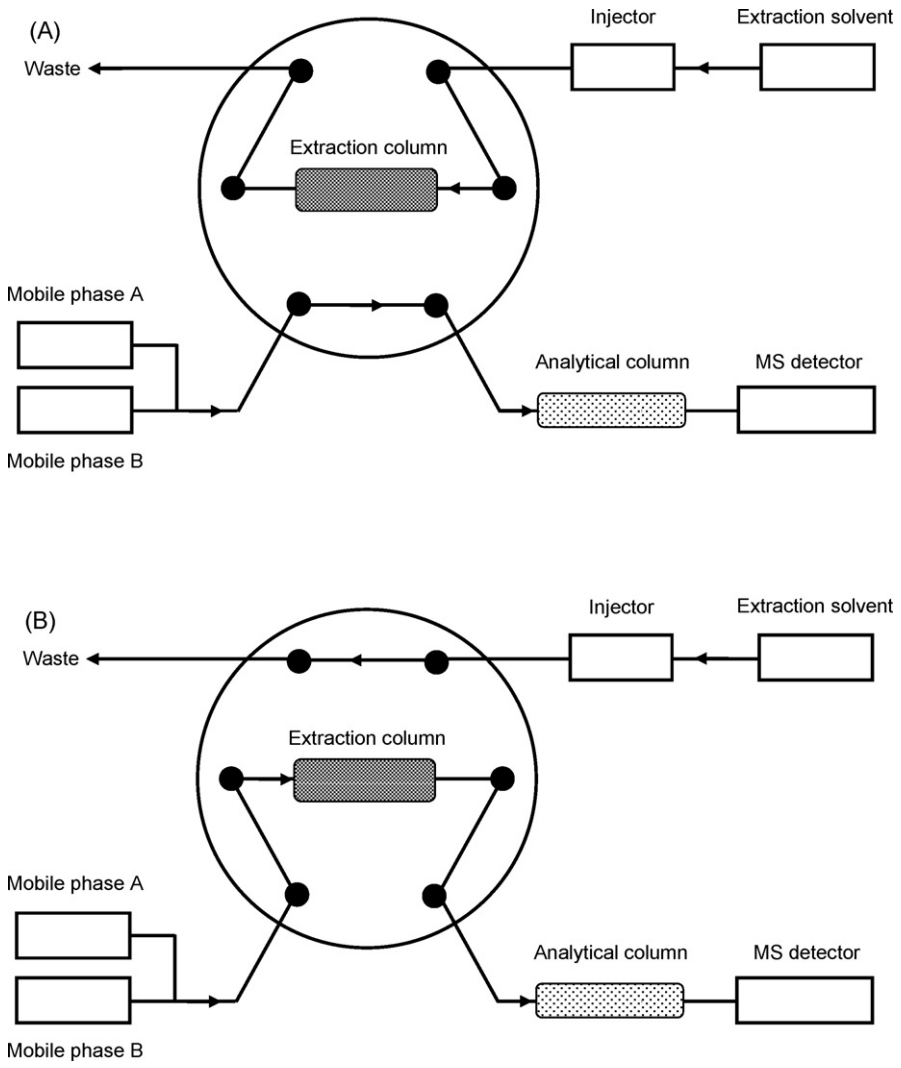

Fig. 1. Column-switching system for purification of serum samples. (A) System configuration for steps 1 (loading) and 3 (analysis). (B) System configuration for step 2 (transfer). Online solid-phase extraction was performed on an Oasis HLB column (25 μm, 2.1 mm × 20 mm; Waters, Japan) and the separation of analytes, on a ZIC-pHILIC column (5 μm, 2.1 mm × 100 mm; Merck, Japan). Mobile phase (A) was 10 mM aqueous acetic acid and (B) was 10 mM acetic acid in acetonitrile. Extraction solvent was water:methanol (90:10, v/v).

iodoacetic acid [9], *N*-ethylmaleimide (NEM) [10], or 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) [11] to prevent GSH auto-oxidation and γ-glutamyl glutamic acid [9], glutathione ethyl ester [10], or thiosalicylic acid [11] is added as the internal standard. It is necessary to use an internal standard for the precise quantification of analytes by MS, because of the effect of the sample matrix on the peak intensity. Some studies have given particular emphasis on the validation of the methods in terms of reproducibility, accuracy, recovery, and sensitivity. However, there is no published work on analytical validation, particularly sample stability, although whole blood samples are generally collected into plastic tubes and kept at room temperature for a few minutes when preparing serum samples.

In the present study, we have developed a highly sensitive and accurate method that employs column-switching HILIC–MS (CS-HILIC–MS) for the determination of reduced and oxidized thiols, using stable isotope labeled thiol as the internal standard. We also examined the chemical stability of the analytes in the target matrix under specific conditions. The developed method was validated and used to compare the effects of derivatization before and after preparing serum from blood samples. Finally, we applied the method to serum samples to assess the effect of oxidative stress induced by lipopolysaccharide (LPS) treatment on reduced and oxidized thiols.

2. Experimental

2.1. Chemicals and reagents

Reduced glutathione (GSH), glutathione disulfide (GSSG), *N*-benzylmaleimide (NBenzM), *N*-cyclohexylmaleimide (NCycloM), and dithiothreitol (DTT) were obtained from Wako Pure Chemical (Tokyo, Japan). DL-Homocysteine (Hcy), DL-homocystine (HcySS), DL-cysteine (Cys), DL-cystine (CysSS), and lipopolysaccharide (*Escherichia coli*, serotype 0111:B4) were obtained from Sigma (Tokyo, Japan). Glutathione ($^{13}C_2$, $^{15}N_1$) (GSH-$^{13}C_2$, $^{15}N_1$) was purchased from Toronto Research Chemicals (Ontario, Canada). L-Cysteine ($^{13}C_3$, $^{15}N_1$) (Cys-$^{13}C_3$, $^{15}N_1$) and DL-homocystine (3,3,3′,3′,4,4,4′,4′-d_8) (HcySS-d_8) were obtained from Cambridge Isotope Laboratories (Massachusetts, USA). Other chemicals and solvents were obtained from Wako Pure Chemical. DL-Homocysteine-d_4 (Hcy-d_4) was purified after reduction by DTT.

Table 1
Comparison of maleimide derivatization reagents for reduced glutathione.

Derivatization reagent	Relative intensity	Reaction rate (25 °C; M^{-1} s^{-1})
Malemide	11.3	0.144
N-Methylmaleimide	16.1	0.405
N-Ethylmaleimide	17.6	0.466
N-Propylmaleimide	18.1	0.180
N-*tert*-Butylmaleimide	18.7	0.070
N-Phenylmaleimide	16.4	0.248
N-Cyclohexylmaleimide	18.8	0.460
N-Benzylmaleimide	17.9	0.492
N-(*o*-Chlorophenyl)maleimide	12.2	0.352

2.2. Animals

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals at Hoshi University, as adopted by the Committee on Animal Research of Hoshi University. Six-week-old male ICR mice (Charles River Laboratories, Tokyo, Japan) weighing 20–25 g were housed at 23 ± 1 °C with a 12 h light/dark cycle. Food and water were available ad libitum and the mice were used after a one-week acclimation period. Six mice at the age of 7 weeks were sacrificed and blood was sampled immediately.

Six-week-old male ICR mice were randomly assigned to receive an intraperitoneal (i.p.) injection of the vehicle PBS (control), or 10 or 100 μg/kg LPS. Mice were sacrificed and blood was sampled after 1, 4, or 24 h.

All the blood samples were collected into plastic tubes containing NBenzM as the derivatization reagent of reduced thiols. Each blood sample that was immediately derivatized with NBenzM to protect the thiol group was centrifuged at 3000 rpm for 10 min. The serum samples were stored at −80 °C until analysis.

2.3. Preparation of calibration standards

A stock solution of an analytical compound (10 mM) was prepared by mixing with 0.1 mM deferoxamine mesylate. Working solutions of the analytical compound were prepared by serial dilution of the stock solution with water containing 0.1 mM deferoxamine mesylate. The concentrations of the calibration standards were: 10, 25, 50, 75, 100, 250, and 500 μM for GSH, GSSG, Cys, and CysSS; and 1, 2.5, 5, 7.5, 10, 25, and 50 μM for Hcy and HcySS. The analysis was conducted using stable isotope labeled thiol as the surrogate standard. Quality control (QC) samples for the determination of accuracy and precision at three concentrations for each calibration range were prepared by adding 25 μL of working solution into 25 μL of blank serum. All solutions were stored at 25 °C, 4 °C, and −80 °C and were stable under those conditions.

2.4. Sample preparation

Serum samples were derivatized in two stages. The sample was prepared by adding 25 μL of derivatized serum sample immediately to a 25 μL solution of the mixed internal standard (GSH-$^{13}C_2$, $^{15}N_1$, Cys-$^{13}C_3$, $^{15}N_1$, and Hcy-d_4), NBenzM (10 mM), and deferoxamine mesylate (0.1 mM). The mixed sample was vortexed for 15 s and allowed to react for 30 min at 40 °C to promote the derivatization of reduced thiol. After that, the sample was added to DTT (25 mM) and the reaction was allowed to proceed for 30 min at 40 °C to reduce oxidized thiol. Finally, the reduced sample was added to the mixed internal standard (GSH-$^{13}C_2$, $^{15}N_1$, Cys-$^{13}C_3$, $^{15}N_1$, and Hcy-d_4), NCycloM (50 mM), and deferoxamine mesylate (0.1 mM), and the reaction was allowed to proceed for 30 min at 40 °C. This was followed by centrifugation (10,000 × *g*, 5 min) after adding 25 μL of water containing 20% sulfosalicylic acid for deproteinization. The supernatant was injected into the CS-HILIC–MS instrument.

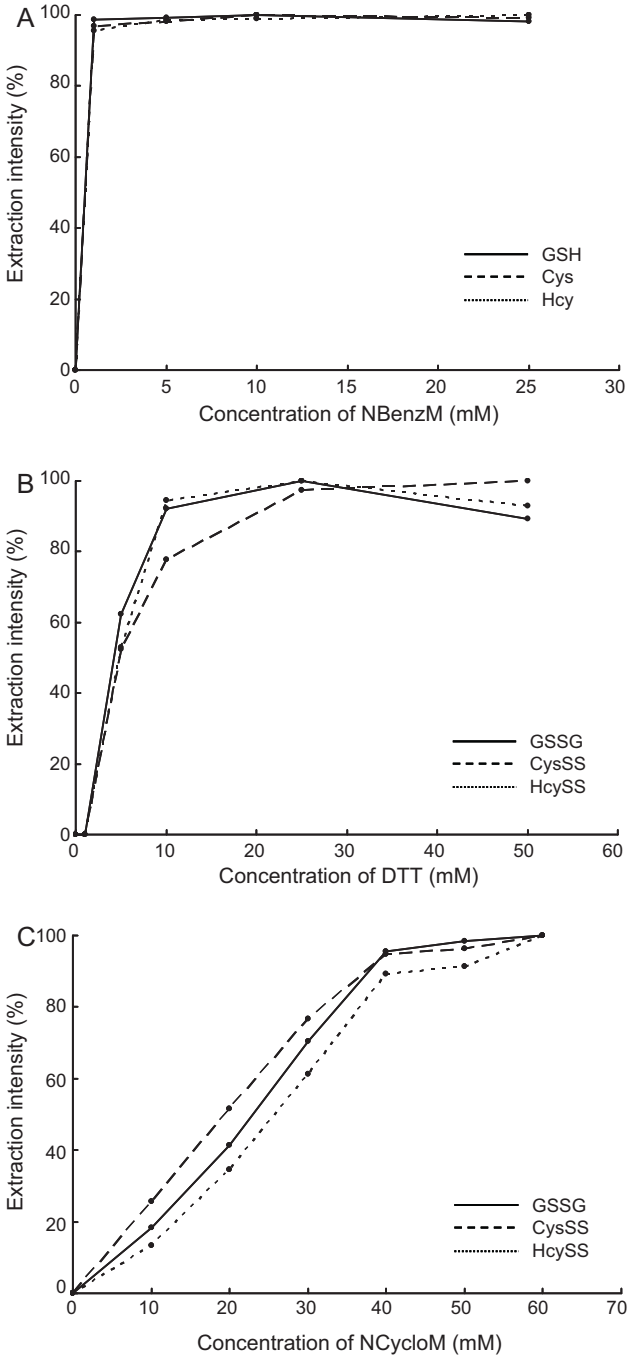

Fig. 2. Optimization of concentrations of (A) NBenzM, (B) DTT, and (C) NCycloM.

2.5. Chromatography and mass spectrometry conditions

High-performance liquid chromatography was performed with a SHIMADZU (Shimadzu, Tokyo, Japan) system that consisted of an LC-10AD_{VP} pump, an SIL-HT_C autosampler, a CTO-10A_{VP} thermostated column compartment, and a DGU-14AM vacuum degasser, and was connected to a SHIMADZU LCMS-2010 A mass spectrometer. Online solid-phase extraction was performed on an Oasis HLB column (25 μm, 2.1 mm × 20 mm; Waters, Japan) and analyte separation was achieved on a ZIC-pHILIC column

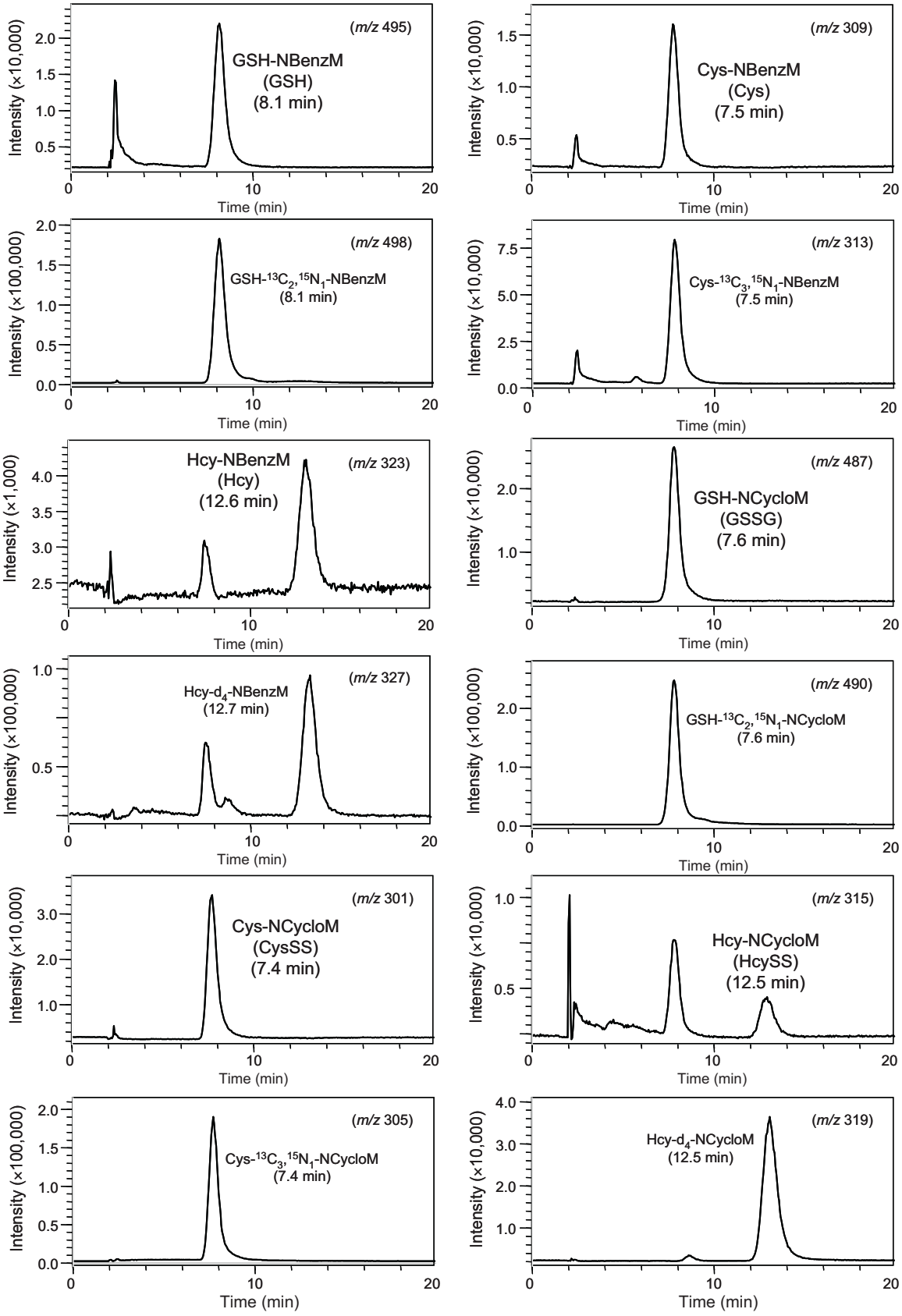

Fig. 3. SIM chromatograms of standard treated with NBenzM and NCycloM. The procedure for sample preparation is described in Section 2.

Table 2
Stability of reduced and oxidized thiols in serum samples under various conditions ($n = 6$).

Sample stability (mean ± S.D., %, $n = 6$)Before derivatization	25 °C		4 °C	−80 °C	
	15 min	30 min	24 h	1 wk	1 wk[a]
GSH	58.2 ± 17.6	31.7 ± 9.9	9.3 ± 1.6	26.2 ± 3.6	10.3 ± 0.7
GSSG	129.1 ± 12.3	116.1 ± 11.6	121.4 ± 18.1	91.8 ± 8.0	114.6 ± 17.4
Cys	49.4 ± 11.4	36.1 ± 5.8	21.5 ± 1.4	30.5 ± 3.2	22.6 ± 1.2
CysSS	133.3 ± 17.2	119.5 ± 9.9	121.1 ± 13.9	120.4 ± 4.5	134.8 ± 11.8
Hcy	51.6 ± 18.4	31.2 ± 8.5	10.0 ± 1.2	26.2 ± 4.7	14.5 ± 1.0
HcySS	137.4 ± 17.7	160.8 ± 8.0	128.9 ± 15.0	181.6 ± 15.9	179.5 ± 20.8

Sample stability (mean ± S.D., %, $n = 6$)After derivatization	25 °C 1 h[b]	4 °C (autosampler condition)	
		48 h[b]	120 h[b]
GSH	101.3 ± 2.2	103.5 ± 2.8	101.9 ± 5.0
GSSG	101.5 ± 3.2	100.9 ± 1.9	91.8 ± 0.8
Cys	99.1 ± 3.3	99.7 ± 2.2	101.1 ± 0.7
CysSS	97.3 ± 2.5	98.5 ± 2.8	101.9 ± 4.0
Hcy	96.3 ± 2.7	96.8 ± 1.4	100.9 ± 3.6
HcySS	97.8 ± 10.3	95.3 ± 9.8	95.6 ± 8.5

[a] Freeze–thaw stability of extract (three cycles).
[b] Sample stability after derivatization step.

(5 μm, 2.1 mm × 100 mm; Merck, Japan) fitted with a HILIC (5 μm, 2.1 mm × 10 mm; Waters, Japan) guard column. The column oven temperature was maintained at 40 °C. The solid-phase extraction column was previously conditioned with mobile phase (C) (water:methanol = 90:10, v/v) at 0.3 mL/min. After these cleanup and enrichment steps, the analytes were transferred to an Oasis HLB column in the backflush mode through a six-port valve time-controlled by the autosampler. Fig. 1 shows the backflush arrangement. Mobile phase (A) was 10 mM aqueous acetic acid, and (B) was 10 mM acetic acid in acetonitrile. Chromatography was performed using 30% mobile phase (A) and 70% mobile phase (B) at a constant flow rate of 0.4 mL/min. The sample solution was injected onto the extraction column and eluted with mobile phase (C) for 3.0 min (Fig. 1A). Analytes were transferred to the analytical column in backflush mode between 3.0 and 15.0 min (Fig. 1B). All steps were controlled by LCMS solution version 3.50 SP.

The analytes were detected in the electrospray positive ionization mode using the selected ion monitoring (SIM) mode. Curved desolvation line and heat block temperatures for the analysis were set at 250 °C and 200 °C, respectively. Nebulizer gas flow was set at 1.5 L/min and detector voltage was set at 1.3 eV. Settings for the target analytes were as follows: GSH-NBenzM m/z 495, GSH-NCycloM m/z 487, Cys-NBenzM m/z 309, Cys-NCycloM m/z 301, Hcy-NBenzM m/z 323, and Hcy-NCycloM m/z 315.

2.6. Validation

Prior to application to real samples, the method was tested in a validation protocol following the accepted criteria for bioanalytical method validation [12]. Linearity, accuracy, precision, limits of detection and lower limit of quantification (LOD and LLOQ), and stability were determined. Five replicates of pooled serum samples were used to calculate LOD and LLOQ. The standard deviation (S.D.) of the mean noise level of each analyte was used to determine LOD (S/N = 3) and LLOQ (S/N > 10). To be acceptable, the calculated LLOQ had to show precision and accuracy within 20% RSD and relative error, respectively.

The precision of the method was determined by replicate analysis of low (3 × LLOQ), intermediate, and high QC samples. Intra-day precision and accuracy were determined by replicate analysis of a group of standards in one day ($n = 6$), and inter-day precision and accuracy were determined by replicate analysis over three days ($n = 3$). The concentration of each sample was determined using calibration standards prepared on the same day. The carry-over effects of all the compounds were evaluated by injecting blank samples directly after injecting a sample that had the highest concentration in the calibration curve. The stability of the metabolites was evaluated at −80 °C, 4 °C, and 25 °C. Stability was tested by analyzing standard samples.

2.7. Measurement of cytokines

The concentration of each cytokine, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interferon-γ (IFN-γ), in serum was determined using commercially available ELISA kits (GE Healthcare, Japan) according to the manufacturer's manual. The amount of cytokine was calculated from the linear portion of the generated standard curve.

2.8. Statistical analysis

All results are expressed as means ± S.D. Statistics were analyzed using one-way analysis of variance (ANOVA), and if statistically significant, *post hoc* analysis using the Scheffe method was performed for multiple comparison among groups. Values with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Comparison of derivatization reagents for the determination of thiols

Reduced thiols are auto-oxidized by dissolved oxygen. Therefore, it is necessary to protect the thiol group with rapidly reacting maleimide derivatization reagents. Not only do the derivatization reagents protect the thiol group, they also significantly enhance ionization efficiency. Thus, we examined derivatization reagents that can potentially suppress auto-oxidation and enhance ionization efficiency. Table 1 shows the relative intensities and the reaction rates. NEM, NCycloM, and NBenzM showed high reaction rates with reduced thiol and the ionization efficiency of the mass spectrometer was enhanced. NEM-derivatized compounds could not be retained by RPLC because their polarities were too high. Meanwhile, NCycloM- and NBenzM-derivatized compounds were retained by RPLC and HILIC, respectively. NCycloM and NBenzM were the best derivatization reagents for reduced thiols because the products could be retained by chromatography, ionization efficiency was enhanced, and the reaction rate was high.

Table 3
Concentrations of reduced and oxidized thiols in mouse serum when derivatization reagent was added to blood sample before and after the preparation of serum.

Analyte	Derivatization step	
	After[a]	Before[b]
GSH (μM)	Trace (4.1 ± 1.2)	241.7 ± 54.9
GSSG (μM)	103.9 ± 23.5	43.1 ± 16.7
Cys (μM)	16.0 ± 2.9	40.7 ± 6.0
CysSS (μM)	75.7 ± 11.1	53.8 ± 7.8
Hcy (μM)	N.D.	Trace (0.6 ± 0.2)
HcySS (μM)	1.0 ± 0.5	1.1 ± 0.2

N.D.: not determined.
[a] Derivatization reagent was added after preparing serum sample.
[b] Derivatization reagent was added before preparing serum sample.

3.2. Optimization of two-stage derivatization conditions

Reduced and oxidized thiols are difficult to extract from biological samples because they are highly polar and soluble in water. Moreover, reduced thiols are auto-oxidized by dissolved oxygen. Therefore, it is necessary to protect the thiol group. Among the derivatization reagents investigated in this study, NBenzM and NCycloM were the best for reduced thiols because the products were retained by chromatography, ionization efficiency was enhanced, and the reaction rate was high. We examined the optimum concentration of the derivatization reagent for the simultaneous determination of reduced and oxidized thiols by the two-stage derivatization process. Fig. 2A shows that GSH, Cys, and Hcy were derivatized completely when 10 mM NBenzM, the reagent used in the first stage of the derivatization process, was added.

On the other hand, oxidized thiols could not be derivatized as they have no thiol groups. Therefore, it is necessary to reduce oxidized thiols. In the determination of total GSH (GSH, GSSG, and protein-bound GSH) in biological samples, the reduction of the disulfide bond between GSH and other thiol compounds, such as Cys or proteins, is required. Previous studies have revealed a variety of reductants, such as DTT [13], mercaptoethanol [14], and tris-(2-carboxyethyl)-phosphine (TCEP) [15]. TCEP has certain advantages over DTT, although the choice of reductant is dependent on the application [16]. Nevertheless, the analytes were affected by ion suppression when the oxidized thiols were determined using TCEP as the reductant. Because of this, we examined the optimum DTT concentration. When 10 mM NBenzM was added, the reduction by DTT was completed and the derivatization with NBenzM was stopped by adding 25 mM DTT (Fig. 2B).

Finally, we determined the optimum concentration of NCycloM for the derivatization of oxidized thiols. Maleimide compounds and reductants reacted with each other. Therefore, it was necessary to add a high concentration of NCycloM (Fig. 2C). The optimum concentrations of NBenzM, DTT, and NCycloM were set at 10 mM, 25 mM, and 50 mM, respectively.

3.3. Optimization of column-switching HILIC–MS conditions

There are several publications of the determination of reduced and oxidized GSH by LC/MS/MS [17–19]. MS/MS has higher specificity than MS. In our first series of experiments, we measured reduced and oxidized thiols by MS/MS. However, the largest fragment ion and the specific ion were not generated, and the high sensitivity of MS/MS could not be achieved. Thus, we tried to determine the optimal conditions for MS.

Mass spectrometry is affected by ion suppression by the HILIC column because HILIC can retain highly polar compounds, such as water, sodium chloride, and phosphoric acid. Therefore, it is recommended that other highly polar compounds be removed when determining a highly polar analyte. The Oasis HLB extraction cartridge has the characteristics of reversed-phase chromatography and thus can easily remove a highly polar matrix. We determined the optimum wash volume of the HLB cartridge. When the wash volume was increased, the peak area of GSH was decreased. On the other hand, a very small wash volume led to ion suppression, which affected MS and reduced the peak area of the analyte. The wash volume that gave the most intense and distinct peak was 0.9 mL.

Then, we examined whether or not the presence of trifluoroacetic acid, acetic acid, or formic acid in the mobile phase would increase both sensitivity and resolution of MS. Acetic acid gave better separation and sensitivity than trifluoroacetic acid or formic acid (data not shown). The mobile phase containing 10 mM acetic acid yielded the most intense peak.

Fig. 3 shows typical SIM chromatograms of the metabolites and the internal standard. Good separation and retention on the HILIC column were observed. It is important that the mass difference between the analyte and the stable isotope labeled internal standard be at least 3 mass units, in order to avoid contribution of the signal of the natural isotope to the signal of the internal standard [20]. All of the reduced and oxidized thiols could be detected under the optimum experimental conditions.

3.4. Sample stability of analytes under specific conditions

The chemical stability of the analytes was assessed in the target matrix under specific conditions and the results are shown in Table 2. Reduced thiols in mouse serum samples decomposed under several conditions (such as 25 °C, 4 °C, and −80 °C). It is said that maleimide adducts are unstable and converted into two ring-cleaved compounds at the N–C=O position of maleimide [21]. Nevertheless, we used maleimide compounds, such as NBenzM and NCycloM, and found that all analytes were stable in mouse serum extracts after the derivatization step using NBenzM and NCycloM, even when the extracts were kept at room temperature for 24 h or at least for 5 days at 4 °C (i.e., autosampler conditions). In general, when preparing serum samples, blood samples are kept at room temperature for a few minutes. This stability result indicated that it is necessary to derivatize and prepare the samples immediately.

Table 4
Validation results of reduced and oxidized thiols.

Analyte	Range (μM)	LOD[a] (μM)	LLOQ[b] (μM)	r	Matrix effect (%)		
					Low	Intermediate	High
GSH	10–500	0.10	10	0.9998	85.3 ± 3.9	66.2 ± 2.7	74.8 ± 2.3
GSSG	10–500	0.05	10	0.9999	89.0 ± 5.6	63.8 ± 3.6	57.2 ± 1.9
Cys	10–500	0.10	10	0.9999	76.3 ± 7.3	47.3 ± 2.0	48.8 ± 2.1
CysSS	10–500	0.05	10	0.9996	90.8 ± 4.5	64.9 ± 2.0	64.4 ± 2.7
Hcy	1–50	0.10	1	0.9998	91.0 ± 6.3	69.6 ± 3.3	84.1 ± 4.4
HcySS	1–50	0.05	1	0.9999	96.5 ± 5.5	65.5 ± 3.2	63.1 ± 3.9

[a] LOD:S/N = 3.
[b] LLOQ:S/N > 10, precision and accuracy <20%.

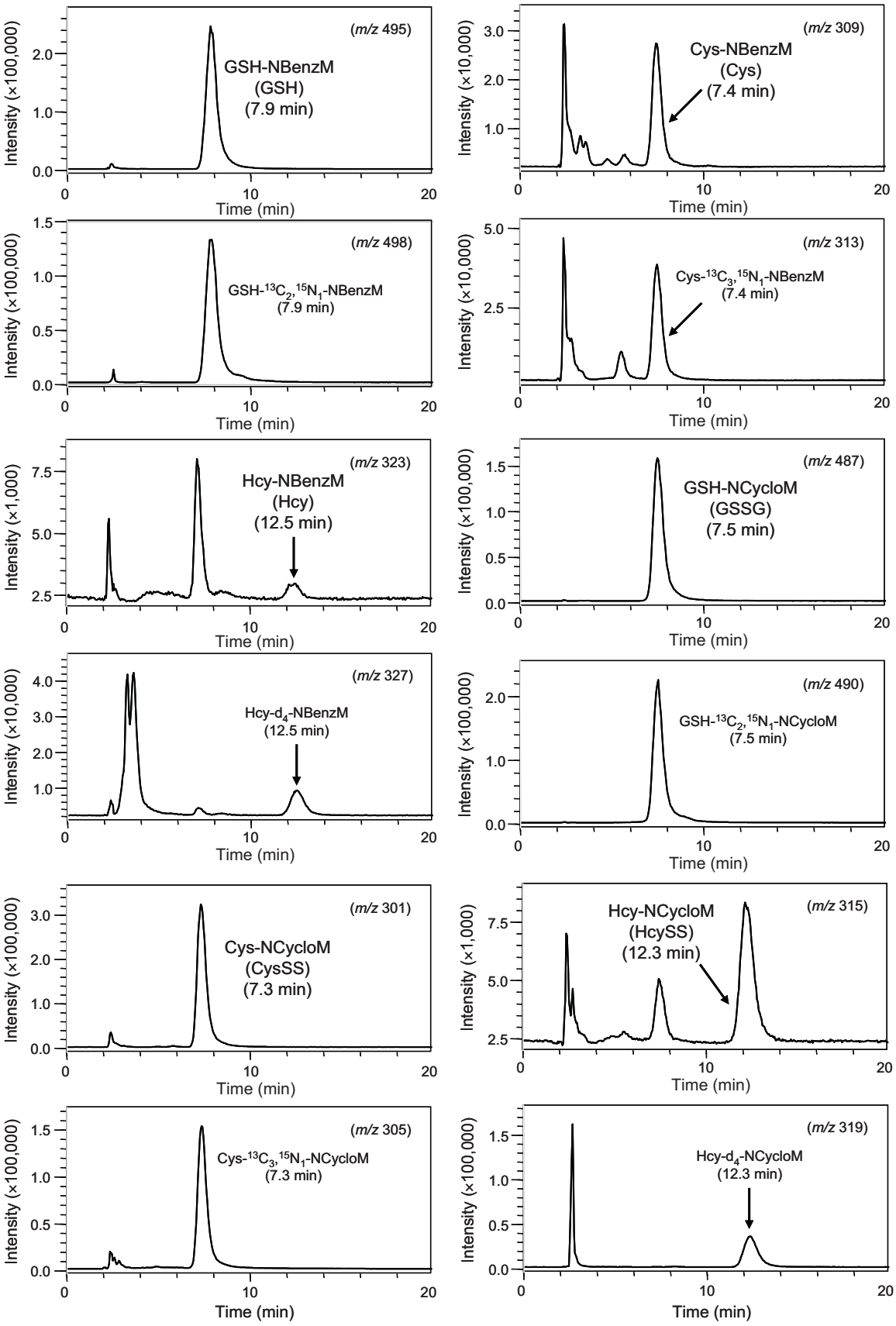

Fig. 4. Chromatograms of reduced and oxidized thiols in mouse serum sample. The procedure for sample preparation is described in Section 2.

Table 5
Intra- and inter-assay precision and accuracy for serum samples.

Analyte	Added conc. (HM)	I ntra-assay (n = 6)		I nter-assay (n = 9)	
		Precision (%RSD)	Accuracy (%)	Precision (%RSD)	Accuracy (%)
GSH	30	2.8	98.8	1.1	97.6
	50	3.2	97.5	2.4	96.7
	100	1.6	107.5	3.9	106.6
GSSG	30	2.9	107.4	1.7	105.5
	50	3.3	90.2	4.3	93.0
	100	1.5	90.2	4.0	93.2
Cys	30	3.9	99.3	0.4	99.0
	50	2.0	97.1	4.0	94.6
	100	1.5	102.8	3.1	100.3
CysSS	30	2.4	100.2	1.0	100.2
	50	2.5	97.9	3.7	93.6
	100	1.5	99.2	3.3	96.0
Hcy3	3	1.6	92.7	0.7	93.3
	5	2.6	87.6	7.5	92.5
	10	2.3	93.7	2.4	96.4
HcySS	3	1.3	109.6	1.0	108.5
	5	2.6	95.1	9.2	101.7
	10	3.7	94.9	11.4	106.9

The proposed method was applied to the analysis of serum samples from mice and the results are shown in Table 3. Serum GSH concentration was very high when the derivatization reagent was added before the preparation of serum sample. Reduced thiols were affected by auto-oxidation during the preparation of serum from blood samples. In our proposed method, the reduced thiols were derivatized immediately before preparing serum samples. As a result, GSH, Cys, and Hcy concentrations were markedly elevated when the derivatization reagent was added before the preparation of mouse serum samples. GSH concentration differed significantly in the ICR strain – it was 4.1 ± 1.2 when the derivatization reagent was added after the preparation of serum samples and 241.7 ± 54.9 when the same was added before serum sample preparation.

3.5. Validation of CS-HILIC–MS method

To validate the CS-HILIC–MS method, several experimental parameters, including LOD, LLOQ, and linearity, were examined. LOD is the concentration of analyte giving an S/N ratio of 3, whereas LLOQ refers to the lowest concentration in the calibration curve

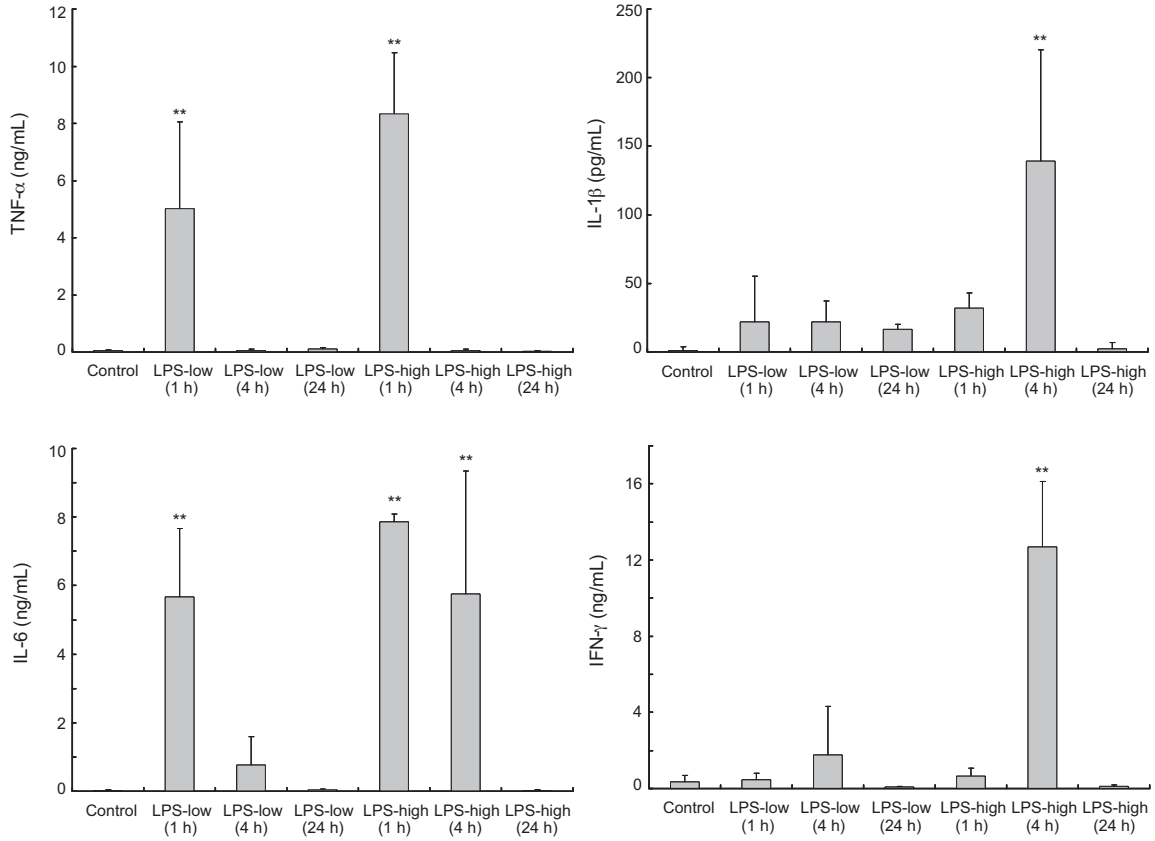

Fig. 5. Proinflammatory cytokine levels in serum sampled from LPS-treated mice. Serum was collected 1, 4, and 24 h after LPS treatment. Serum samples were subjected to cytokine measurements using ELISA kits. Data represent means ± S.D. (n = 10). * and ** statistically significant (P < 0.05 and P < 0.01 vs. control).

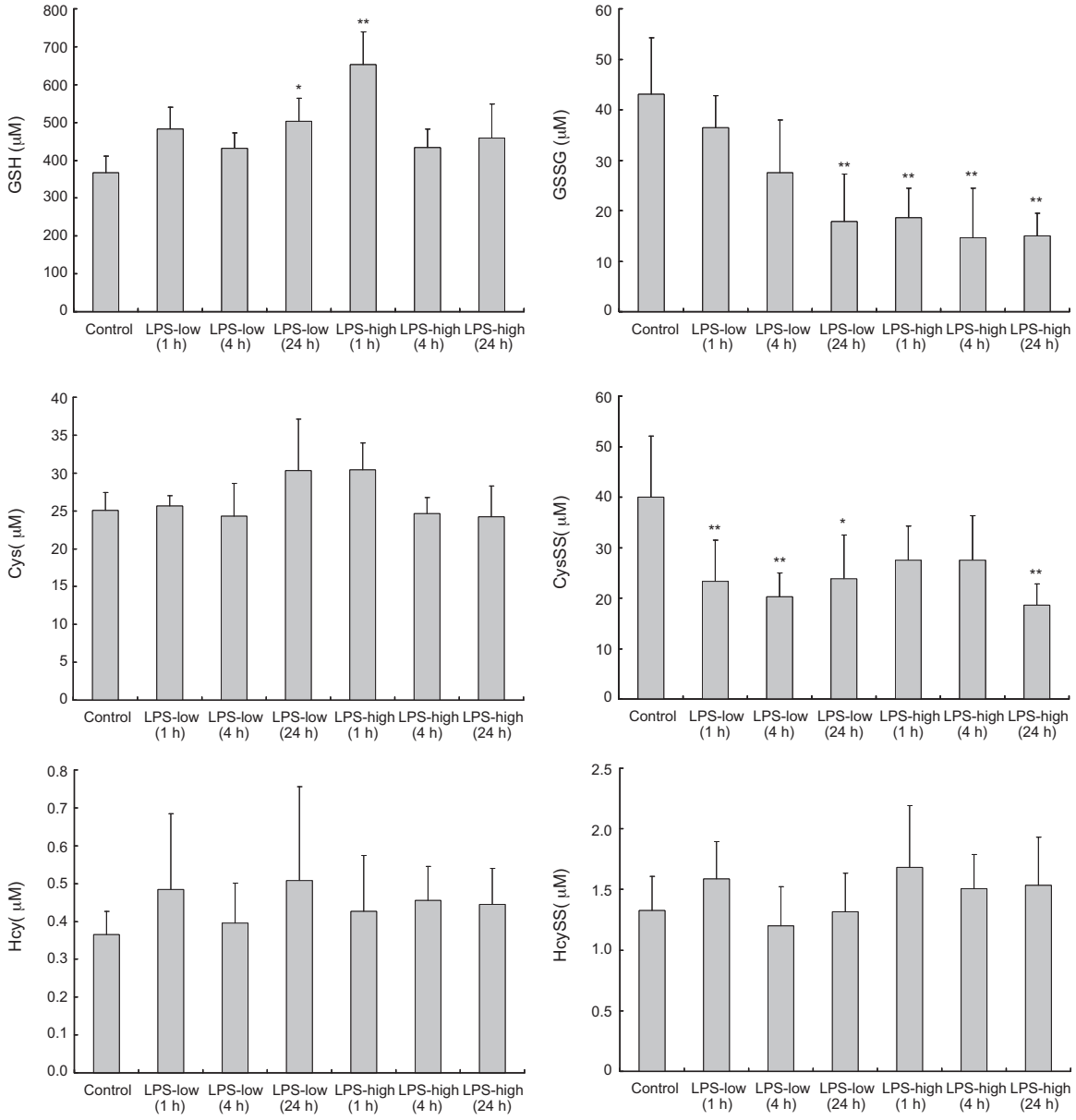

Fig. 6. Levels of reduced and oxidized thiols in serum of LPS-treated mice. Serum was collected 1, 4, and 24 h after LPS treatment. Serum samples were subjected to amino thiol measurements using column-switching hydrophilic interaction chromatography coupled with mass spectrometry. Data represent means ± S.D. ($n = 10$). * and ** statistically significant ($P < 0.05$ and $P < 0.01$ vs. control).

that can be measured with acceptable accuracy and precision (RSD below 20%) (Table 4).

Calibration curves were evaluated for concentrations ranging from LLOQ to 500 μM. As observed for all sulfur metabolites, the normalized areas of the chromatographic peaks were linear in the range of concentrations studied, with regression coefficients exceeding 0.99, thus allowing further quantification studies.

Matrix effects on the serum samples were evaluated by analyzing low, intermediate, and high QC samples. In the serum samples, the matrix effect was observed particularly for Cys, which had an average of 47.3%.

Precision and accuracy studies were performed by calculating RSD and the results are listed in Table 5. Inter-assay experiments were also performed by analyzing three independent extracts of the same serum sample. In the serum sample, inter- and intra-assay precision results were confirmed on the basis of the acceptance criteria at low, intermediate, and high QC levels, and were below 15% (RSD < 15% as required by the acceptance criteria).

3.6. Effect of lipopolysaccharide on thiol metabolites and cytokines

We investigated the role of thiol compounds in LPS-induced acute inflammation *in vivo*. Representative chromatograms of serum samples are shown in Fig. 4. LPS induces the expression of several kinds of cytokines, including TNF-α, IL-1β and IL-6, thus mediating the development of various inflammatory reactions. After the injection of LPS (10 or 100 μg/kg; i.p.), several cytokine levels were increased (Fig. 5). Meanwhile, serum GSSG and CysSS levels were significantly decreased at 4 h after LPS treatment. On the other hand, serum GSH was increased at 1 h after LPS treatment (Fig. 6). Thus, the reduced and oxidized thiol ratio correctly expresses the redox status *in vivo*.

4. Discussion

Oxidative stress is caused by an imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage [22]. Oxidative stress is manifested by the presence of an excessive amount of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), hydroxyl radical, and hypochlorous acid (HOCl), which are produced in the face of insufficient or defective antioxidant defense systems. Oxidative stress causes profound alterations of biological structures and molecules, including cell membranes, lipids, proteins, and nucleic acids. It is said that oxidative stress is involved in diabetes mellitus [23], Alzheimer's disease [24], and cancer [25]. There is increasing interest in identifying biomarkers for diseases in which oxidative stress is involved. Various markers of oxidative stress have been identified. Popular oxidative stress markers were designed for lipid peroxidation and DNA oxidation [26].

GSH plays an essential role in maintaining an intracellular redox environment that is critical for the function of cellular proteins. The antioxidant function of GSH is primarily due to its involvement in enzymatic pathways developed by cells to counter ROS. The most important pathway involves glutathione peroxidase and glutathione reductase. Glutathione peroxidase catalyzes the reduction of H_2O_2 that is produced by superoxide dismutase through the dismutation of superoxide anion or organic hydroperoxides. GSH and GSH-dependent enzymes act in cooperation to scavenge ROS and/or neutralize their toxic oxidative effect. These systems act cooperatively at the same time to protect the human body from ROS. For these reasons, many studies have indicated that the concentrations of GSH and GSSG are influenced by various diseases, where the GSH/GSSG ratio tends to decrease. Nevertheless, the measured concentrations of GSH and GSSG are highly divergent among research groups and so, those findings should be reconsidered once a general agreement on the physiological levels of these compounds is reached. Together, these results indicate that the determination of GSH is useful in the study of oxidative stress.

GSH and other thiols and their disulfides are measured by several methods. For instance, high-performance liquid chromatography can simultaneously detect GSH, other low-molecular-mass thiols, and disulfides, but requires sample derivatization. Of course, those methods can determine reduced and oxidized thiols. However, neutral-alkaline pH must be restored before derivatization. Rossi et al. [27] indicated that trichloroacetic acid seems to be the most useful deproteinization agent because only a small amount of GSH is oxidized with time. They observed that only 3–4% of GSH was oxidized within 20 h at 0 °C after the addition of trichloroacetic acid to the sample. The unnecessary oxidation of thiols during sample treatment may present a major problem because it may lead to an overestimation of disulfide concentration. There have been no attempts to prove this hypothesis, however. In this study, we found that reduced thiols in mouse serum decomposed under several temperature conditions (Table 2). Various studies have pointed out the importance of measuring serum GSH and GSSG for both pathologic and physiologic purposes. However, those studies showed large differences in the concentrations of GSH and GSSG. In general, when preparing serum samples, blood samples are kept at room temperature for a few minutes. Perhaps, reduced thiols are auto-oxidized by dissolved oxygen and/or the blood matrix. In our proposed method, reduced thiol levels were significantly elevated in serum when a derivatization reagent was added to mouse blood sample before serum preparation (Table 3).

LPS is a toxic component of the cell wall in Gram-negative bacteria and is widely present in the digestive tracts of humans and animals. The host defense response to LPS includes the expression of a variety of pro-inflammatory cytokines, such as TNF-α and IFN-γ [28]. On the other hand, it has been said that glutathione peroxidase and glutathione reductase activities were significantly inhibited by exposure to LPS [29]. Payabvash et al. [30] have reported a dose-dependent LPS-induced increment in GSH export from liver to plasma during the 1 h period after endotoxin exposure. Our results indicated that the levels of a variety of pro-inflammatory cytokines were increased by exposure to LPS (Fig. 5). Meanwhile, serum GSSG and CysSS levels were significantly decreased at 4 h after LPS treatment, whereas serum GSH level was increased at 1 h after LPS treatment (Fig. 6). Taken together, the results indicate that the reduced and oxidized forms of thiols are useful markers compared to cytokines, because they persisted even after oxidative and/or inflammation damage caused by LPS treatment.

5. Conclusion

The proposed CS-HILIC–MS method enabled the simultaneous determination of reduced and oxidized thiols in mouse serum samples. Interference from endogenous compounds was removed by means of the column-switching technique. We examined the potential of using maleimide compounds as derivatization reagents to suppress auto-oxidation and enhance ionization efficiency. Moreover, we compared the effects of derivatization before and after preparing serum from blood samples and found that it was necessary to perform the derivatization immediately before preparing serum from blood samples. Artifacts originating from sample collection may influence the results of GSH and GSSG assays in whole blood and serum samples. We were able to determine the real concentrations of reduced and oxidized thiols. The results obtained show that despite its simplicity, our developed method gives relatively accurate results and is sufficient for the assessment of the roles of GSH and GSSG in the oxidative state.

Acknowledgements

This work was supported in part by grants from the Ministry of Health, Labour and Welfare and the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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