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A new and versatile method for determination of thiolamines of biological importance

Pamela K. Dominick, Pamela B. Cassidy[1], Jeanette C. Roberts*

Department of Medicinal Chemistry, University of Utah, Salt Lake City, UT 84112, USA

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Abstract

A method for the separation and quantitation of several important biological thiolamines is described. The procedure employs a C_{18} reversed-phase HPLC system to separate the dinitrophenyl derivatives of reduced and oxidized glutathione and cysteine and relies on an internal standard, N^{ϵ}-methyllysine, to minimize experimental error. The method was validated in three matrices (water, HepG2 cell lysates, and mouse liver homogenates) using several criteria. The detector response was linear for the dinitrophenyl derivatives of glutathione, glutathione disulfide, cysteine, and cystine in the concentrations ranging from 10 to 50 nmol/ml. Inter- and intra-day variation, percent recovery in the biological matrices, and limits of detection and quantitation were determined. For the most accurate determination, it is essential that standard curves be produced daily and in the same matrix as that being analyzed.

Keywords: Thiolamines; Glutathione; Cystine; Cysteine; Glutathione disulfide

1. Introduction

The tripeptide glutathione (GSH) is the major nonprotein thiol in mammalian cells where it is present in millimolar concentrations. GSH serves many diverse physiological functions including redox homeostasis, amino acid transport, and protection against reactive oxygen species. Another major function of GSH is the detoxication of reactive metabolites generated during xenobiotic metabolism, which can lead to GSH depletion [1–3]. GSH biosynthesis is limited by the amount of available cysteine. The administration of cysteine in various forms has been shown to maintain GSH levels and protect against xenobiotic toxicity [3,4]. We are currently interested in the development of new cysteine prodrugs directed at elevating depleted GSH levels resultant of toxic insults. These studies would be facilitated by a suitable detection method for the measurement of biologically relevant thiolamines.

A variety of high-perfromance liquid chromatography (HPLC) methods are available for the detection and quantitation of thiol and disulfide containing compounds [5,6]. Many of these methods utilize thiol-specific reagents that attach a chromo-

*Corresponding author. 30 South 2000 East, Room 201, Department of Medicinal Chemistry, University of Utah, Salt Lake City, UT 84112, USA. Tel.: +1-801-581-3598; fax: +1-801-585-9119.

E-mail address: jroberts@deans.pharm.utah.edu (J.C. Roberts).

[1]Present address: Huntsman Cancer Institute, University of Utah, Salt Lake City, UT 84112, USA.

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phore to the thiol group thereby assisting in separation and detection. The more commonly used HPLC methods for the determination of thiols and disulfides are based on the formation of fluorescent derivatives of thiols using reagents such as monobromobimane, *o*-phthalaldehyde, or *N*-substituted maleimides. Fluorometric detection provides good sensitivity for thiol measurement but lacks the ability to simultaneously measure thiols and disulfides [5]. Disulfides are detected only as their free thiol form following a separate reduction step. Another commonly used HPLC method in which thiols and disulfides can be measured in the same sample uses electrochemical detection. However, this method suffers from a severe problem related to the sensitivity of the detector to interference from oxidizable impurities [5]. Another widely used HPLC method developed by Reed and coworkers is dependent on trapping free thiol groups with iodoacetic acid followed by the formation of *N*-dinitrophenylated (DNP) derivatives by reaction with Sanger's reagent [7,8]. The derivatives are then separated on an ion-exchange column and monitored at 365 nm. This procedure has been successful in the simultaneous measurement of GSH and GSH derivatives, as well as other thiols and disulfides, in the same sample [9–11]. Although UV–VIS detection is not as sensitive as other detection methods, the sensitivity of the Reed method has been reported in the nanomole range [7]. The primary disadvantage of this procedure is the inability to analyze thiol compounds of neutral charge (i.e., no free carboxyl group). Also, the high salt concentration necessary for the elution of the derivatives is damaging to the HPLC instrumentation, and the column itself becomes derivatized by Sanger's reagent leading to reduced performance over time.

We have developed a new method for determining concentrations of GSH, oxidized GSH (GSSG), cysteine, and cystine in a single analysis. In the method described here, both free thiols and amino groups are dinitrophenylated with Sanger's reagent and the derivatives are separated on a C_{18} reversed-phase column. This method allows the simultaneous determination of both reduced and oxidized thiol compounds. The development and validation of this method is presented.

2. Experimental

2.1. Chemicals

GSH, GSSG, L-cysteine, L-cystine, N^{ϵ}-methyl-L-lysine, bathophenanthrolinedisulfonic acid (BPDS), trifluoroacetic acid (TFA), 70% perchloric acid (PCA), 2,4-dinitrofluorobenzene (DNFB), Eagle's minimum essential medium (EMEM), antibiotic antimycotic solution (100×), Hank's balanced salt solution (without Ca^{2+} or Mg^{2+}), trypsin 1:250, EDTA, and phosphate-buffered saline (PBS), pH 7, were purchased from Sigma–Aldrich Chemical Company (St Louis, MO, USA). Fetal bovine serum (Fetal Clone I) was purchased from Hyclone Laboratories (Logan, UT, USA). Acetonitrile (HPLC grade), ethanol, potassium hydroxide, and potassium bicarbonate were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water was purified with an E-pure Barnstead purifier from Fisher Scientific. Acetonitrile was of HPLC grade and all other chemicals purchased were of analytical grade.

2.2. HPLC instrumentation and conditions

Samples were separated on a Rainin Dynamax 5 μm, 4.6×250 mm C_{18} column fitted with a Rainin Dynamax 5 μm 100 Å C_{18} guard module (Rainin, Emeryville, CA, USA). The chromatographic system consisted of a Hitachi Model L-6200A pump equipped with a 4250 UV–VIS detector and an AS-2000 autosampler with a Rheodyne Model 7010 injection valve and a 100 μl sample loop (Hitachi, San Jose, CA, USA). Hitachi Model D6000 version 2, revision 06 software was used to control system operation and facilitate data collection.

Samples were eluted with a mobile phase consisting of solvent A (water/0.1% TFA) and solvent B (acetonitrile/0.1% TFA). The samples were eluted with 20% B for an initial 5 min after injection followed with a 15 min linear gradient to 50% B and a 14 min isocratic period at 50% B, then a 3 min linear gradient to 100% B and a 3 min isocratic period. The column was then re-equilibrated to the initial conditions for 15 min. All HPLC solvents were filtered through a 0.45 μm nylon filter. Analy-

ses of 100 μl of sample were performed at a flow-rate of 1.0 ml/min at ambient temperature with UV–VIS detection at 365 nm.

2.3. Sample preparation

2.3.1. Cell culture

Tissue culture flasks (75 cm^2) containing 25 ml of EMEM (without nucleosides, supplemented with 10% fetal bovine serum and antibiotics) were seeded with 6×10^6 HepG2 cells (ATCC HB-8065, Manassas, VA, USA). The flasks were incubated at 37°C in a 5% CO_2 atmosphere for 7 days (media replaced every 3 days), at which time cells were confluent. Media was removed and the cells were rinsed with Hank's balanced salt solution. Cells were trypsinized with 5 ml of 0.25% (w/v) trypsin–0.03% (w/v) EDTA for 2 min at 37°C followed by further incubation in the absence of trypsin for 11 min at 37°C. The trypsin was quenched with 5 ml of EMEM. Cell clumps were broken up by striking the flask sharply against the palm of the hand 10 times followed by passage of the cell suspension four times through a 23-gauge needle. Cell suspensions were transferred to 50 ml centrifuge tubes and cell number was determined using a Coulter counter (Coulter Corporation, Miami, FL, USA). The cell suspension was centrifuged at 3400 *g* for 5 min, and the pellet was suspended in 5 ml of PBS, pH 7, and centrifuged again at 3400 *g* for 5 min. The pellet was resuspended in 2.14 ml of 0.9% NaCl/1 m*M* BPDS and 0.36 ml of 70% PCA was added. Samples were sonicated in a sonic bath for 5 min followed by centrifugation at 3400 *g* for 5 min. Supernatants were transferred to fresh centrifuge tubes and stored at −70°C until analysis.

2.3.2. Tissue samples

Samples of male Swiss Webster (Charles River Laboratories, Wilmington, MA, USA) murine liver tissue (50–100 mg wet weight) were flash frozen in liquid nitrogen and stored at −70°C. At the time of assay, frozen samples were homogenized in 3 ml of 10% w/v PCA/1 m*M* BPDS using a tissue homogenizer and centrifuged at 1400 *g* for 10 min at 4°C.

2.4. Stock solutions

Stock solutions were prepared for standard curves as follows: 0.48 mg/ml of N^{ϵ}-methyllysine (diluted 1:5), 0.15 mg/ml of cysteine, 0.29 mg/ml of cystine, 0.37 mg/ml of GSH, and 0.74 mg/ml of GSSG. All stock solutions were prepared in water except for L-cystine, which was prepared in 10% PCA/1 m*M* BPDS due to insolubility in water. A separate stock solution of the internal standard, N^{ϵ}-methyllysine, was prepared at a concentration of 0.05 mg/ml in water. All stock solutions were freshly prepared on the day of analysis.

2.5. Standard curves

Triplicate standard curves of N^{ϵ}-methyllysine, GSH/GSSG, and cysteine/cystine were generated in three matrices: water (10% PCA/1 m*M* BPDS), cell lysates, and mouse liver homogenates. Standard curves were prepared by taking a 0.5 ml aliquot of sample matrix and adding varying amounts (20–100 μl) of the stock solutions of N^{ϵ}-methyl-L-lysine, GSH/GSSG, and L-cysteine/L-cystine followed by 0.48 ml of 2 *M* KOH–2.4 *M* $KHCO_3$ (this made the solution basic to pH~9.0) and 1 ml of 1% DNFB in ethanol. The standard curves of GSH/GSSG and cysteine/cystine were spiked with 0.1 ml of the internal standard, N^{ϵ}-methyllysine. Samples were derivatized overnight at room temperature in the dark. Prior to injection, samples were acidified with 0.15 ml 70% PCA, clarified by centrifugation at 5600 *g* for 1 min, and filtered through a 0.45 μm PVDF syringe filter. Standard curves were generated by plotting the ratio of the peak area of analyte to internal standard versus analyte concentration. The standard curve of N^{ϵ}-methyllysine was generated by plotting the peak area versus analyte concentration.

2.6. Intra-day and inter-day variation

For intra-day variation, both the slope and the ratio of the peak area of analyte to internal standard were compared from the triplicate standard curves in all three matrices prepared above in Section 2.5. For inter-day variation, duplicate standard curves of GSH/GSSG and cysteine/cystine were generated for

three consecutive days at the same time each day. Standard curves were prepared in water as described above in Section 2.5. Inter-day variability of both the slopes and the ratio of peak area of analyte to internal standard from the standard curves was analyzed.

2.7. Recovery

Duplicate standard curves of GSH, GSSG, cysteine, and cystine were prepared individually in water as described above in Section 2.5. The three sample matrices were spiked with a known amount of GSH, GSSG, cysteine, and cystine. Blank samples were also prepared in the sample matrices by adding equivalent amounts of water. The recovery of analyte in each matrix was determined by measuring the ratio of the peak area of analyte to internal standard using the standard curves.

2.8. Limit of detection and quantitation

Triplicate standard curves of GSH and GSSG were prepared individually in water as described above in Section 2.5. Concentrations of GSH and GSSG for the standard curves were 0.01, 0.1, 1, and 10 nmol/ml. The limit of detection was defined as the lowest analyte concentration resulting in an observable peak. The limit of quantitation was defined as the analyte concentration resulting in the lowest measurable peak height with acceptable precision (coefficient of variation (C.V.)$\leq$15%).

2.9. Synthesis of DNP derivatives and mass spectral analysis

N,S-di-DNP–GSH, *N,N′*-di-DNP–GSSG, *N,S*-di-DNP–cysteine and *N,N′*-di-DNP–cystine were prepared by known procedures [7,12–15]. The following compounds were collected from an HPLC run and identified by mass spectrometry: 2,4-dinitrophenol LRMS (EI 80 eV) m/z 184 (M+); 2,4-dinitrophenyl ethyl ether LRMS (FAB) m/z 212 (M+); *N,S*-di-DNP–GSH LRMS (FAB) m/z 638 (M–H); *N,N′*-di-DNP–GSSG LRMS (FAB) m/z 943 (M–H); and *N,N′*-di-DNP–N^{ϵ}-methyllysine LRMS (ES) m/z 493 (M+H). FAB and EI data were collected from a Finnegan MAT 95 mass spectrometer (San Jose, CA, USA). ES data was collected from a Micromass Quattro II Triple Quadrupole mass spectrometer (Beverly, MA, USA).

2.10. Statistical analysis

Linear regression analysis was performed on all standard curves generated. Variability was expressed as mean$\pm$standard deviation (SD) and C.V. Tukey–Kramer multiple comparisons tests were performed where appropriate to compare variability between means. Means were considered significantly different if $P<0.05$.

3. Results

3.1. General HPLC procedure

3.1.1. Sample preparation

During sample preparation, cell and tissue samples were homogenized in 10% PCA in the presence of the metal ion chelator, BPDS, to prevent auto-oxidation of thiols and thiol–disulfide exchange. PCA was chosen for sample homogenization in this procedure in order to facilitate subsequent precipitation of the acid as the potassium salt. This prevented the separation of the reaction mixture into aqueous and organic phases. Previously, salicylic acid, which did not precipitate as a salt, was used, and a biphasic reaction mixture resulted preventing complete sample derivatization.

In the method described here, DNFB was used to derivatize both thiol and amino groups under basic conditions to give *N,S*-di-DNP derivatives of cysteine and GSH. N^{ϵ}-Methyllysine, GSSG, and cystine, which lack a thiol moiety, were derivatized with DNFB to yield *N,N′*-di-DNP derivatives.

3.1.2. HPLC analysis

A C_{18} reversed-phase column was used for separation of DNP analytes. Derivatized analytes were eluted with a water–acetonitrile gradient containing 0.1% TFA. The gradient conditions and time were optimized to allow for baseline separation of the DNP derivatives of cysteine, cystine, GSH, GSSG, and N^{ϵ}-methyllysine. The gradient conditions in the first 20 min were specifically designed to give

sufficient retention of *N,N′*-di-DNP–GSSG. Suboptimum gradients were particularly poor in their ability to separate *N,N′*-di-DNP–GSSG from neighboring peaks originating from the derivatization reaction. The overall time for analysis of the analytes examined was 55 min. This time included a 10 min column equilibration.

Typical chromatograms in each of the three matrices are shown in Figs. 1–3. The retention times of the DNP analytes and the major side products are shown in Table 1. Peaks were identified by mass spectrometry and comparison to retention times of synthesized standards. 2,4-Dinitrophenol and 2,4-dinitrophenyl ethyl ether were identified by mass spectrometry as by-products of the derivatization reaction. An internal standard was added to monitor the chemical derivatization process in each sample. Penicillamine (2-amino-3-mercapto-3-methylbutanoic acid) was initially chosen as the internal standard [7] but was shown to react with GSSG in a concentration dependent manner to form a mixed disulfide that interfered with quantitation (data not shown). Therefore, N^{ϵ}-methyllysine was chosen as the internal standard. N^{ϵ}-Methyllysine cannot participate in artifactual thiol–disulfide exchange, is not found in HepG2 cells or mouse livers, and possesses two functional groups that can be chemically modified by DNFB to allow attachment of two chromophores.

3.2. HPLC method validation

3.2.1. Linearity in different matrices

The standard curves of N^{ϵ}-methyllysine, GSH/GSSG, and cysteine/cystine in each matrix are defined in Table 2. The standard curve of N^{ϵ}-methyllysine was linear in the concentration range investigated (5–15 nmol/ml). From these results, a concentration of 10 nmol/ml was chosen for the internal standard. All standard curves generated for the analytes of interest in all three matrices were linear in the concentration range investigated (10–50 nmol/ml).

3.2.2. Variability

The intra-day variability analysis of the DNP derivatives of GSH, GSSG, cysteine, and cystine was investigated at three concentrations in all three matrices. Table 3 shows the intra-day variability in the peak area ratio for each analyte, which was reproducible within each matrix with C.V.≤17% in all but a few cases. The C.V. for the intra-day variability in the slopes of each analyte within each matrix was ≤12% (Table 4). Tukey–Kramer multiple comparisons tests were applied to compare the slopes between each matrix to evaluate if matrix effects were present (Table 4). The slopes were significantly different between the matrices for several experiments with $P<0.05$. These results indicate that matrix effects are present, and therefore, need to be considered when quantitating thiolamines in different matrices.

The inter-day variability of the DNP derivatives of GSH, GSSG, cysteine, and cystine was investigated at three concentrations in water. Table 5 shows that the inter-day variability in the peak area ratio for each analyte was C.V.≤12%. The C.V. for the inter-day variability in the slopes was ≤14% for each analyte and Tukey–Kramer multiple comparisons tests suggested that slopes were different between days in some cases with $P<0.05$ (Table 6). These results indicate the need to run standard curves on each day of analysis.

3.2.3. Recovery

The recoveries were determined in duplicate in three matrices. In water, the recoveries were 95% for GSH, 94% for GSSG, 103% for cysteine, and 102% for cystine. In HepG2 cells, the recoveries were 120% for GSH, 119% for cysteine, and 103% for cystine. The recovery of GSSG could not be measured reproducibly in the HepG2 cells due to an unidentified contaminating peak in certain (not all) samples. In mouse livers, the recoveries were 100% for GSH, 92% for GSSG, 104% for cysteine, and 107% for cystine.

3.2.4. Limit of detection and quantitation

The limit of detection and quantitation for GSH in water was 0.01 nmol/ml and 0.10 nmol/ml (C.V.= 13%), respectively. The limit of detection and quantitation for GSSG in water was 0.1 nmol/ml and 1 nmol/ml (C.V.=7%), respectively.

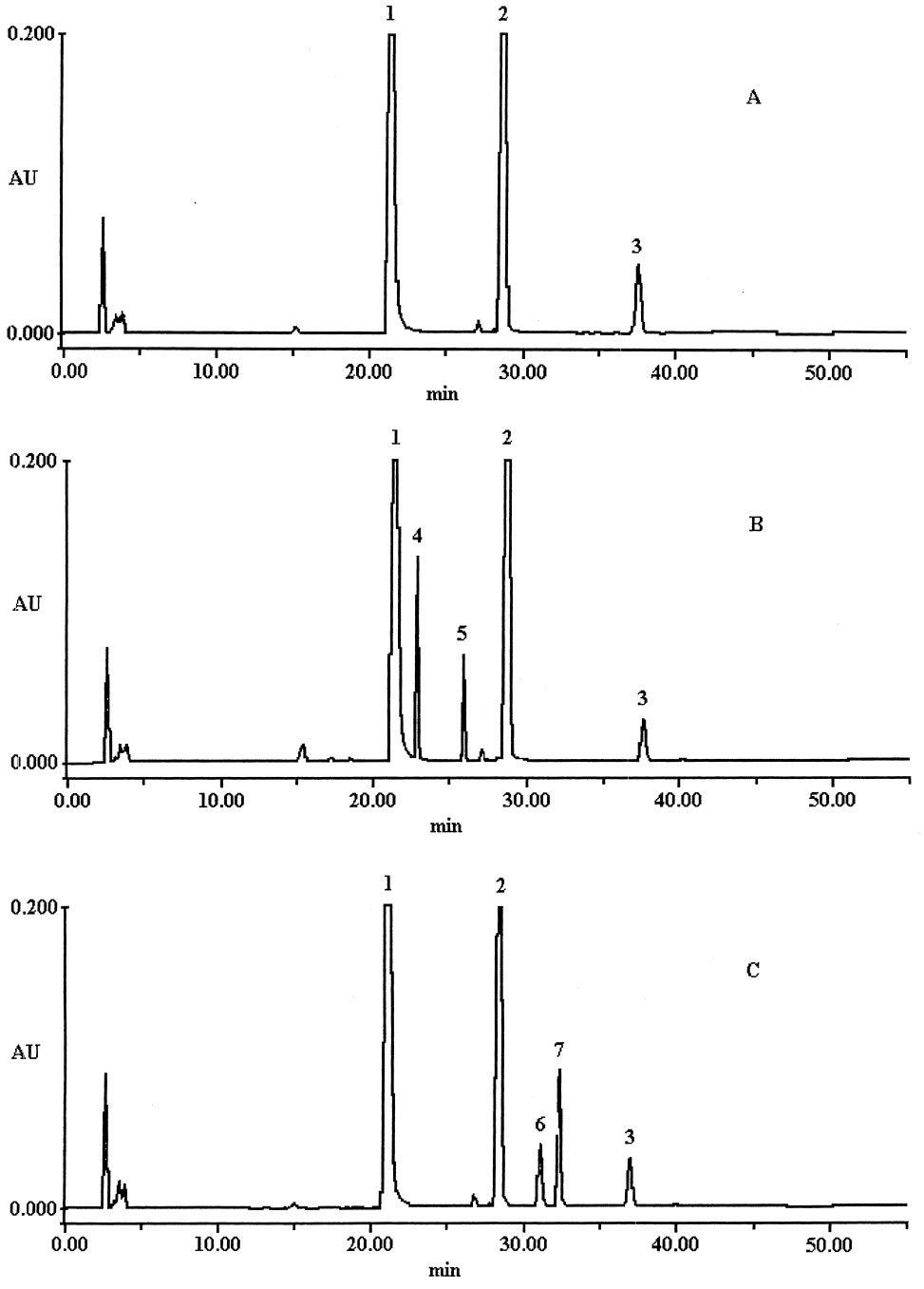

Fig. 1. Chromatograms of water spiked with (A) 15 nmol/ml of N^{ϵ}-methyllysine, (B) 10 nmol/ml of GSH, 30 nmol/ml of GSSG, and 10 nmol/ml of N^{ϵ}-methyllysine, and (C) 30 nmol/ml of cysteine, 10 nmol/ml of cystine, and 10 nmol/ml of N^{ϵ}-methyllysine. Peaks: 1=2,4-dinitrophenol; 2=2,4-dinitrophenyl ethyl ether; 3=*N,N′*-di-DNP–N^{ϵ}-methyllysine; 4=*N,N′*-di-DNP–GSSG; 5=*N,S*-di-DNP–GSH; 6=*N,N′*-di-DNP–cystine; and 7=*N,S*-di-DNP–cysteine. Chromatographic conditions are described in Section 2.2.

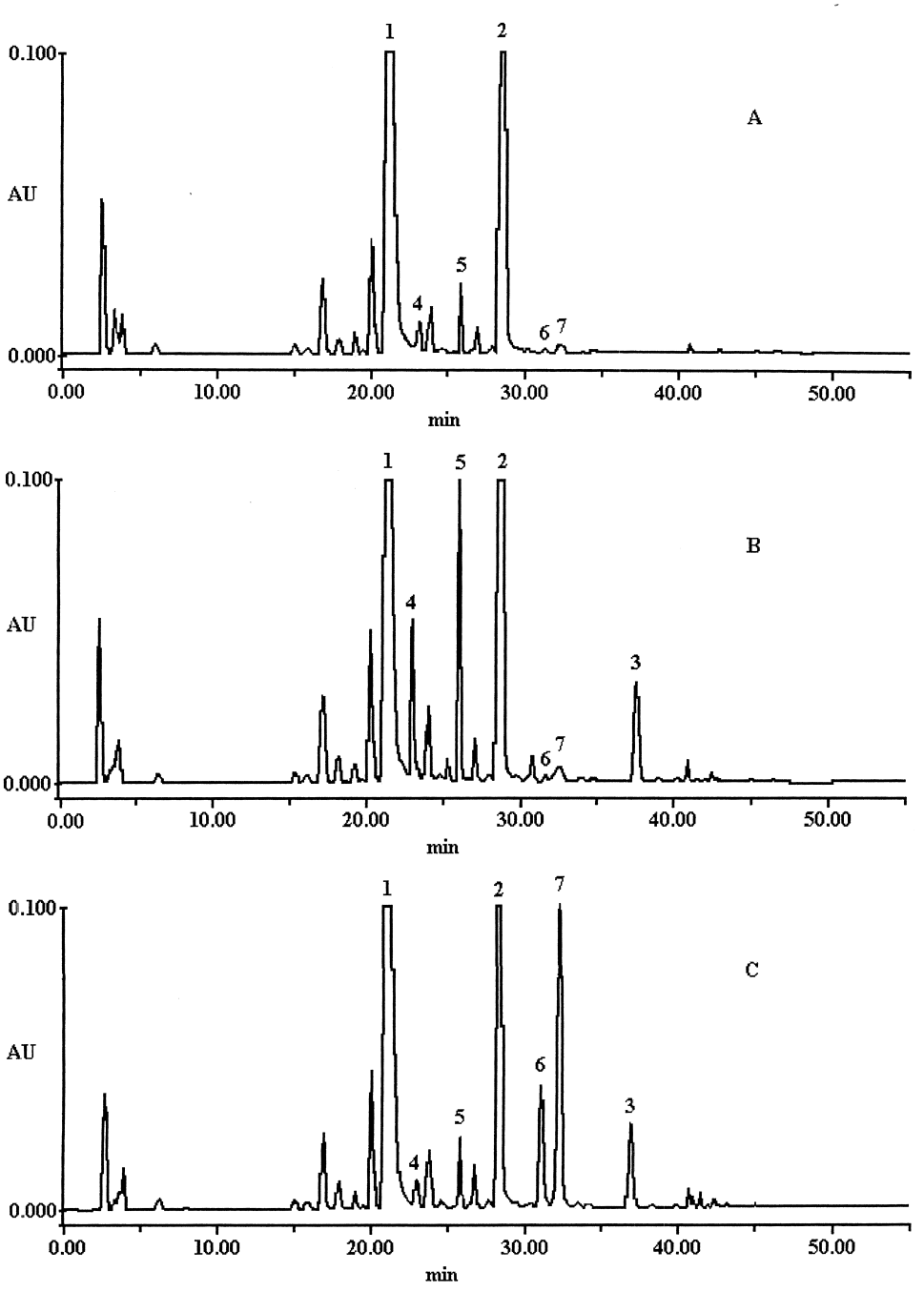

Fig. 2. Chromatograms of (A) blank HepG2 lysate, (B) HepG2 lysate spiked with 30 nmol/ml of GSH, 10 nmol/ml of GSSG, and 10 nmol/ml of N^{ϵ}-methyllysine, and (C) HepG2 cell lysate spiked with 30 nmol/ml of cysteine, 10 nmol/ml of cystine, and 10 nmol/ml of N^{ϵ}-methyllysine. Peaks: 1=2,4-dinitrophenol; 2=2,4-dinitrophenyl ethyl ether; 3=*N,N′*-di-DNP–N^{ϵ}-methyllysine; 4=*N,N′*-di-DNP–GSSG; 5=*N,S*-di-DNP–GSH; 6=*N,N′*-di-DNP–cystine; and 7=*N,S*-di-DNP–cysteine. Chromatographic conditions are described in Section 2.2.

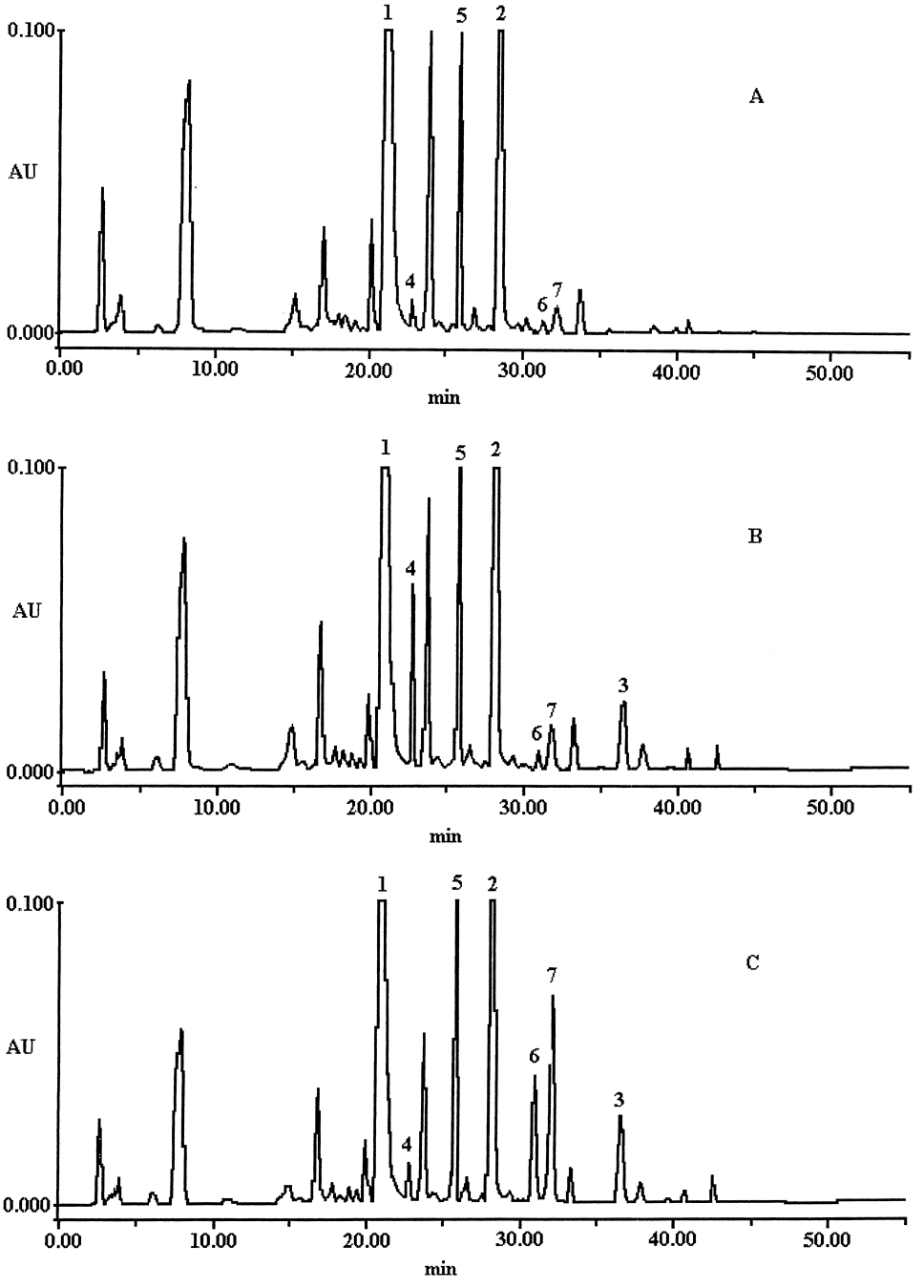

Fig. 3. Chromatograms of (A) blank mouse liver homogenate, (B) mouse liver homogenate spiked with 30 nmol/ml of GSH, 10 nmol/ml of GSSG, and 10 nmol/ml of N^{ϵ}-methyllysine, and (C) mouse liver homogenate spiked with 30 nmol/ml of cysteine, 10 nmol/ml of cystine, and 10 nmol/ml of N^{ϵ}-methyllysine. Peaks: 1=2,4-dinitrophenol; 2=2,4-dinitrophenyl ethyl ether; 3=*N,N′*-di-DNP–N^{ϵ}-methyllysine; 4=*N,N′*-di-DNP–GSSG; 5=*N,S*-di-DNP–GSH; 6=*N,N′*-di-DNP–cystine; and 7=*N,S*-di-DNP–cysteine. Chromatographic conditions are described in Section 2.2.

Table 1
Retention times of DNP derivatives and side products

Compound	Peak number	Retention time (min)[a]	*n*
2,4-Dinitrophenol	1	20.99±0.19 (1%)	108
GSSG	2	22.80±0.15 (1%)	27
GSH	4	25.80±0.16 (1%)	27
2,4-Dinitrophenyl ethyl ether	5	28.45±0.23 (1%)	108
Cystine	6	30.88±0.20 (1%)	27
Cysteine	7	32.13±0.16 (1%)	27
N^{ϵ}-methyllysine	3	36.94±0.44 (1%)	99

[a] Values are expressed as mean±SD (C.V.).

4. Discussion

We report a versatile method for the simultaneous measurement of biological thiols and disulfides including GSH, GSSG, cysteine, and cystine. This method is a modification of the techniques described by Mertens et al. [10] and Reed et al. [7,8]. We exploited the reactivity of DNFB with both thiol and amino groups [9] to produce *N,S*-di-DNP derivatives of GSH and cysteine. N^{ϵ}-Methyllysine, GSSG, and cystine were treated with DNFB to give *N,N′*-di-DNP derivatives. The derivatives were separated by HPLC using a C_{18} reversed-phase column. Many chromatographic methods for determination of aminothiol derivatives utilize C_{18} reversed-phase columns and we have realized several benefits in the use of C_{18} reversed-phase chromatography for this application. Reversed-phase columns are commercially available and chemically inert to the derivatization conditions. Columns containing a free amine, as previously employed, are susceptible to chemical modification, which decreases column life. Furthermore, the ion-exchange columns require high salt concentrations for the elution of the derivatives, which leads to undue wear on the HPLC system. The 0.1% TFA solutions used in our protocol are simple to prepare and easy to use.

Many of the published HPLC methods do not utilize an internal standard. It is important to monitor an HPLC system during use as changes can occur that cannot be readily detected without a proper internal control. The internal standard used here, N^{ϵ}-methyllysine, served to monitor both the derivatization reaction in each sample preparation and the properties of the HPLC system including injection volume, column performance, and detector response. Data analysis was then normalized to the internal standard.

To validate the method, we examined the linearity, variability, recovery, and limits of detection and quantitation of the HPLC system. A linear response was shown over the concentration range investigated

Table 2
Standard curves of DNP derivatives in water, HepG2 cell lysates, and mouse liver homogenates

Compound	Standard curves ($n=3$)		
	In water	In HepG2 cell lysates	In mouse liver homogenates
N^{ϵ}-methyllysine	$y=5.67\times10^{14}x+3.01\times10^{4}$ $r^2=1.000$	$y=5.22\times10^{14}x-2.03\times10^{4}$ $r^2=0.9987$	$y=4.58\times10^{14}x+2.15\times10^{3}$ $r^2=0.9950$
GSH	$y=5.62\times10^{8}x+4.48\times10^{-1}$ $r^2=0.9984$	$y=8.09\times10^{8}x+1.48\times10^{-1}$ $r^2=0.9953$	$y=6.62\times10^{8}x+7.97\times10^{-2}$ $r^2=0.9998$
GSSG	$y=5.78\times10^{8}x-1.97\times10^{-2}$ $r^2=0.9956$	$y=8.78\times10^{8}x-3.08\times10^{-1}$ $r^2=1.000$	$y=6.92\times10^{8}x+1.803\times10^{-1}$ $r^2=0.9791$
Cysteine	$y=6.63\times10^{8}x+4.28\times10^{-2}$ $r^2=0.9998$	$y=7.69\times10^{8}x+5.81\times10^{-2}$ $r^2=0.9986$	$y=6.14\times10^{8}x+1.40\times10^{-1}$ $r^2=0.9970$
Cystine	$y=8.73\times10^{8}x+1.24\times10^{-1}$ $r^2=0.9997$	$y=9.55\times10^{8}x+1.20\times10^{-1}$ $r^2=1.000$	$y=9.86\times10^{8}x+8.89\times10^{-3}$ $r^2=0.9948$

Table 3
Intra-day variation in the peak area ratio of DNP derivatives in water, HepG2 cell lysates, and mouse liver homogenates

Compound concentration (nmol/ml)	Peak area ratio (n=3)[a]		
	In water	In HepG2 cell lysates	In mouse liver homogenates
GSH			
10	1.03±0.10 (9%)	1.04±0.10 (10%)	0.71±0.11 (15%)
30	2.07±0.02 (1%)	2.49±0.24 (9%)	1.92±0.13 (7%)
50	3.26±0.10 (3%)	4.33±0.23 (5%)	3.19±0.21 (7%)
GSSG			
10	0.50±0.02 (3%)	0.60±0.24 (41%)	0.74±0.23 (31%)
30	1.74±0.13 (7%)	2.42±0.19 (8%)	2.41±0.34 (14%)
50	2.73±0.28 (10%)	4.23±0.34 (8%)	3.41±0.17 (5%)
Cysteine			
10	0.73±0.02 (2%)	1.09±0.06 (6%)	0.69±0.03 (4%)
30	2.04±0.06 (3%)	2.95±0.18 (6%)	1.96±0.30 (15%)
50	3.43±0.17 (5%)	5.06±0.34 (7%)	3.02±0.03 (1%)
Cystine			
10	1.00±0.03 (3%)	1.04±0.08 (8%)	1.11±0.19 (17%)
30	2.85±0.02 (1%)	2.87±0.19 (7%)	2.88±0.08 (3%)
50	4.59±0.03 (1%)	4.70±0.07 (1%)	5.16±0.21 (4%)

[a] Values are expressed as mean±SD (C.V.).

for all of the analytes. In HepG2 cells and mouse livers, GSSG gave a high C.V. for peak area ratios (41% and 31%, respectively). One possible explanation for this result in the HepG2 cells was the presence of an unidentified contaminating peak, which eluted at a similar retention time. The presence of this contaminating eluent also affected the ability to assess percent recovery of GSSG in HepG2 cells. Percent recoveries of the other analytes were comparable to other established protocols [10]. The

Table 4
Intra-day variation in the slope of DNP derivatives in water, HepG2 cell lysates, and mouse liver homogenates

Compound	Slope (n=3)[a]			Tukey–Kramer analysis[b]	
	In water	In HepG2 cell lysates	In mouse liver homogenates	P values	
GSH	(5.62±0.44)×10^8 (8%)	(8.09±0.32)×10^8 (4%)	(6.62±0.33)×10^8 (5%)	Water vs. cells	$P<0.001$
				Water vs. liver	$P<0.05$
				Cells vs. liver	$P<0.01$
GSSG	(5.78±0.70)×10^8 (12%)	(8.78±1.15)×10^8 (13%)	(6.92±0.62)×10^8 (9%)	Water vs. cells	$P<0.05$
				Water vs. liver	N.S.
				Cells vs. liver	N.S.
Cysteine	(6.63±0.29)×10^8 (4%)	(7.69±0.59)×10^8 (8%)	(6.14±0.02)×10^8 (0.3%)	Water vs. cells	$P<0.05$
				Water vs. liver	N.S.
				Cells vs. liver	$P<0.01$
Cystine	(8.73±0.01)×10^8 (0.1%)	(9.55±0.32)×10^8 (3%)	(9.86±0.82)×10^8 (8%)	Water vs. cells	N.S.
				Water vs. liver	N.S.
				Cells vs. liver	N.S.

[a] Values are expressed as mean±SD (C.V.).
[b] N.S., not significant.

Table 5
Inter-day variation in the peak area ratio of DNP derivatives in water

Compound concentration (nmol/ml)	Peak area ratio ($n=6$)[a]
	In water
GSH	
10	0.90±0.02 (2%)
30	2.00±0.06 (3%)
50	3.20±0.15 (5%)
GSSG	
10	0.54±0.02 (4%)
30	1.71±0.11 (6%)
50	2.81±0.11 (4%)
Cysteine	
10	0.76±0.09 (12%)
30	2.25±0.22 (10%)
50	3.74±0.40 (11%)
Cystine	
10	0.96±0.07 (7%)
30	2.60±0.18 (7%)
50	4.20±0.30 (7%)

[a] Values are expressed as mean±SD (C.V.).

limit of quantitation for GSH and GSSG was tested in water and found to be 0.01 nmol/ml and 0.1 nmol/ml, respectively. By comparison, the method of Reed allows detection in the nmol/ml range [7,8]. Similarly, Mertens et al. reported detection between 0.5 and 1.0 nmol/ml in cultured rat hepatocytes [10]. The 10-fold difference between the limit of quantitation for GSH and GSSG could be explained by their elution profiles. GSSG elutes in close proximity to 2,4-dinitrophenol, which could affect the accuracy of its measurement.

We observed that the matrix can affect the standard curves of the analytes. In the different matrices, such as cell lysates and mouse liver homogenates, the complex composition of the sample can affect various steps in the experimental procedure, including the derivatization reaction and HPLC analysis. We also noted that the standard curves varied to a small extent on a day-to-day basis. These results indicate the necessity to produce standard curves for each metabolite in the relevant matrix and on the day of analysis for the most accurate results.

Endogenous thiol levels in HepG2 cells and mouse livers were calculated. In HepG2 cells, GSSG could not be quantitated due to the inconsistent presence of an unidentified contaminant peak and cystine was not detected. It was apparent that the cystine levels were at or below the limit of quantitation for the HPLC method. In HepG2 cells, cysteine content ranged from 0.07 to 0.13 nmol/10^6 cells and GSH content ranged from 2 to 3 nmol/10^6 cells. The GSH content in HepG2 cells has been reported as high as 9–20 nmol/10^6 cells [16–18]. The values reported here are somewhat lower, but were consistent over time in our HepG2 cells. GSH content in HepG2 cells has been shown to be dependent on growth cycle and the

Table 6
Inter-day variation in the slope of DNP derivatives in water

Compound	Slope ($n=6$)[a]	Tukey–Kramer analysis[b]	
	In water	*P* values	
GSH	(5.85±0.13)×10^8 (2%)	Day 1 vs. day 2	N.S.
		Day 1 vs. day 3	N.S.
		Day 2 vs. day 3	N.S.
GSSG	(5.75±0.20)×10^8 (3%)	Day 1 vs. day 2	N.S.
		Day 1 vs. day 3	N.S.
		Day 2 vs. day 3	N.S.
Cysteine	(6.00±0.84)×10^8 (14%)	Day 1 vs. day 2	$P<0.01$
		Day 1 vs. day 3	$P<0.05$
		Day 2 vs. day 3	$P<0.01$
Cystine	(8.33±0.57)×10^8 (7%)	Day 1 vs. day 2	$P<0.05$
		Day 1 vs. day 3	N.S.
		Day 2 vs. day 3	N.S.

[a] Values are expressed as mean±SD (C.V.).
[b] N.S., not significant.

availability of sulfur amino acids for GSH biosynthesis [17,18]. Also, it has been shown that different HepG2 cell lines show differences in their plasma protein synthesis [19]. It is therefore reasonable that differing growth conditions, passage number, and cell growth history could contribute to the observed differences. The endogenous thiol levels in mouse liver ranged from 0.6–1 μmol/g liver for cysteine, 0.1–0.2 μmol/g liver for cystine, 0.1–0.4 μmol/g liver for GSSG, and 4–5 μmol/g liver for GSH, which is similar to numerous literature values (i.e., [20]).

We report and validate a new and versatile method for the determination of important biological thiolamines. The method is applicable for the simultaneous determination of both reduced and oxidized thiolamines as well as thiolamines that do not contain a carboxylate group. This technique may be useful for the routine determination of numerous thiolamines in a variety of biological systems.

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