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High-performance liquid chromatographic method to analyze picomole levels of glutathione, cysteine and cysteinylglycine and its application to pre-cancerous rat livers

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Abstract

A HPLC-based method for quantifying glutathione, cysteine and cysteine-containing peptide is described. *N*-(2,4-Dinitrophenylaminoethyl)maleimide was synthesized from ethylenediamine, 2,4-dinitrochlorobenzene and maleic anhydride. The maleimide was reacted at 40°C and pH 5.8 for 10 min with thiol compounds such as glutathione or cysteine. An aliquot of the reaction mixtures was applied to a reversed-phase column (4.6×150 mm) of HPLC. When glutathione and cysteine were simultaneously assayed, the column was eluted with a gradient of acetonitrile in potassium phosphate (pH 7.0). The derivatives were monitored at 350 nm. Good liner relationships existed between peak area and concentration of glutathione or cysteine from 10 pmol to 2 nmol. The recovery tests from rat liver homogenate were 99.7±2.2% for glutathione and 104.9±3.8% for cysteine. By this method γ-glutamylcysteine, cysteinylglycine, and homocysteine could be also quantified. The determination limits of glutathione, cysteine and other thiol compounds were 5 pmol. The method is simple: a sample solution is mixed with the labelling reagent and an aliquot of the reaction mixture is applied to a standard HPLC. The hepatic levels of cysteine and glutathione in pre-cancerous rats were determined by this method. The cysteine level in pre-cancerous livers was extremely elevated in comparison to that of the control groups, while no difference was observed in the glutathione contents between the pre-cancerous and control groups.

Keywords: Glutathione; Cysteine; Cysteinylglycine

1. Introduction

Various analytical methods have been established for quantifying glutathione and cysteine. These were reviewed in previous reports [1,2]. Current methods for the determination of glutathione and cysteine are mainly divided into HPLC and colorimetric methods. We reported the determination methods for cysteine and cystine [2] as well as glutathione and its disulfide [3] in biological samples. Both are colorimetric methods and have been applied to studies of thiol-compound levels in the liver during its regeneration in rats [4,5], during aging processes of rats and mice [6] and in *Saccharomyces cerevisiae* cultured under aerobic and anaerobic conditions [7]. Because of their simplicity, colorimetric methods are

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recommended for measuring cysteine [2,8] and glutathione [3,9] in biological samples such as the liver, especially if many sample numbers have to be tested. If, however, glutathione and cysteine in small amounts of samples have to be simultaneously measured, or when cysteine-containing peptides must be assayed, HPLC method must be used. Monobromobimane reacts easily with thiols at pH 8.0 to give fluorescent thioethers that can be determined by HPLC equipped with fluorimetric detection [10]. Toyooka et al. [11] used fluorogenic reagents, 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole for thiol analysis and ammonium 7-fluoro-2,1,3-benzodiazole-4-sulfonate for disulfides. The labelled compounds were analyzed by reversed-phase chromatography and detected by fluorimetry. Winters et al. [12] reacted thiols with *N*-(1-pyrenyl)maleimide and fluorescent derivatives were detected by fluorimetry after reversed-phase HPLC. Fifty to 100 fmol thiol can be quantified when fluorescence detection is used. Our goal was to design an easily synthesized labelling compound that could be detected by means other than fluorescence. The 2,4-dinitrophenyl and maleimide groups in the labelling reagent were chosen because of the relatively strong absorptivity in spectrum and the specific reactivity to thiol group, respectively. It is also an advantage that the new labelling compound can be easily synthesized in three steps from three simple industrial reagents.

2. Experimental

2.1. Chemicals

Ethylenediamine dihydrochloride, 2,4-dinitrochlorobenzene, maleic anhydride, bathophenanthroline disulfonic acid disodium salt (BAPS), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dioxane, perchloric acid, potassium dihydrogen citrate, potassium dihydrogen phosphate and acetonitrile (HPLC grade) were purchased from Wako Pure Chemicals (Osaka, Japan). L-Cysteine, cysteinylglycine and homocysteine were obtained from Sigma (St. Louis, MO, USA). Glutathione reductase and γ-glutamylcysteine were purchased from Oriental Yeast (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. Glutathione was kindly supplied by Senju Pharmaceutical (Osaka, Japan). 3′-Methyl-4-dimethylaminoazobenzene (3′-MDAB) was prepared in this laboratory according to the method of Giese et al. [13]. 4′-Methyl-4-dimethylaminoazobenzene (4′-MDAB) and 2-MDAB were purchased from Tokyo-Kasei (Tokyo, Japan).

2.2. Animals

Five-week-old Donryu/Crj (SPF/VAF) strain male albino rats were obtained from Charles River Japan (Yokohama, Japan) and kept for 1 week for adaptation. Forty rats were divided into four equal groups. One group was given rat cake ME (Oriental Yeast, Tokyo, Japan) and other three groups were given rat cake MF containing either 0.064% 3′-MDAB, 4′-MDAB or 2-MDAB, respectively. All rats received water ad libitum and were housed at 25°C. Animals were fed the DAB derivatives for 11 weeks and they were killed at the 11th week.

2.3. Synthesis of the labelling reagent, N-(2,4-dinitrophenylaminoethyl)maleimide

2.3.1. N-2,4-Dinitrophenylethylenediamine HCl (*I*)

Ethylenediamine dihydrochloride (15 g), sodium carbonate (19 g) and 100 ml water were placed in a 300-ml three-necked flask. 2,4-Dinitrochlorobenzene (10 g) was dissolved in 150 ml 99% ethanol. The alcoholic solution was added dropwise over 60 min to the flask under reflux. The mixture was refluxed a further 2 h. After cooling, sodium chloride was filtered off and washed once with 30 ml 99% ethanol. The filtrate and washing were combined and concentrated at about 30°C under reduced pressure to about 100 ml. Five grams sodium hydroxide were added to the concentrate and the concentrate was extracted twice with 50 ml of ethyl acetate. The water layer was discarded. The ethyl acetate layer was extracted with 50 ml 3 *M* HCl. Yellow crystals appeared in the extract and were filtered (dry weight 7 g). The filtrate was evaporated at about 40°C under reduced pressure. Additional crystals (dry weight 1 g) were obtained from the concentrate.

Both sets of crystals were judged pure by TLC: R_f value on silica gel, 0.76 (methanol–acetic acid, 10:1, v/v), 0.58 (*n*-butanol–acetic acid–water, 4:1:1 v/v).

For recrystallization, the crystals were dissolved in hot water and ethanol was added.

2.3.2. N-Maleinyl-N′-2,4-dinitrophenyl-ethylenediamine (II)

Compound **I** (1.3 g) was dissolved in 50 ml water and 1 g NaOH was added to the solution. The resulting suspension was extracted three times with 120 ml ethyl acetate. The ethyl acetate phases were combined, dried by sodium sulfate and filtered. When the filtrate was evaporated under reduced pressure, yellow crystals formed. These were dried over phosphorous pentoxide and KOH. One gram of crystals was dissolved in 50 ml dioxane and the solution was placed in a separatory funnel. Maleic anhydride (1 g) and 50 ml dioxane were placed in a 200-ml three-necked flask. The free amine solution was dropwise added over 30 min. The reaction mixture was stirred for a further 30 min at 50°C and then cooled in an ice bath. Crystals were collected by suction filtration and dried over phosphorous pentoxide and KOH under reduced pressure. The R_f value was 0.52 on silica gel (benzene–ethanol–conc. NH_3=5 ml: 5 ml: 3 drops).

2.3.3. N-(2,4-Dinitrophenylaminoethyl) maleimide (III)

The method of Cava et al. [14] was used for the maleimidation. One gram compound **II**, 100 ml acetic anhydride and 1 g anhydrous sodium acetate were placed in a 300-ml Erlenmyer flask. When the flask was heated, the solution became clear just before refluxing. The reaction was allowed to continue at the same temperature for 30 min. The mixture was cooled in an ice bath and 100 ml water was added. The acetic anhydride layer was decomposed by adding sodium hydrogen carbonate under stirring in an ice bath. The precipitate was filtered, washed with about 10 ml each water and 99% ethanol and dried over phosphorous pentoxide and KOH under reduced pressure. Crystals were judged pure by TLC on silica gel plate. The R_f value was 0.74. (solvent system: benzene–ethyl acetate=2:1, v/v). Yield was 0.9 g. The yellow crystals were recrystallized from ethyl acetate. m.p. 229–231°C. Practically insoluble in water, ethanol, acetone or ether. Calculated for C_{12} $H_{10}O_6$: C, 47.07; H, 3.29; N, 18.30. Found: C, 47.02, H, 3.57; N, 18.28%.

2.4. HPLC of thiol derivatives

A Shimazu LC-6A HPLC system (Kyoto, Japan) equipped with a SPD-6A detector, a SCL-6A system controller and a C-R6A data module was used. A Cosmosil-packed column 5 C_{18} AR-II (150×4.6 mm, 5 μ m) (Nacalai Tesque, Kyoto, Japan) was used. The chromatography was operated at 40°C and the detection was at 350 nm. Solution A was 10 m*M* potassium phosphate (pH 7.0) and B consisted of A and acetonitrile (1:1, v/v). For analysis of GSH and Cys, the proportion of B to A was increased linearly from 40 to 100% over 10 min (method I). The method was applied to the rat liver at pre-cancerous stage. For analysis of γ-GluCys, GSH, Cys, CysGly and homocysteine, the mobile phase system was programmed as follows: 0–5 min, 8% B; 5–25 min, linear gradient of B from 8–28%; 25–45 min, 28% B. The elution was performed at 1 ml/mm (method II). The separation of GSH, Cys and CysGly was performed on the same column using a linear gradient C–D (90:10, v/v) to 100% of D over 20 min at a flow-rate of 0.5 ml/min. Solutions C and D were mixtures of 10 m*M* of potassium phosphate (pH 7.0) and acetonitrile in ratios of 9:1 (v/v) and 1:1 (v/v), respectively (method III).

This method was used for the recovery test and for optimizing the labelling reaction conditions described below.

2.5. Sample preparation and labelling of thiol compounds

In order to avoid oxidation of the thiol groups, deproteinization was carried out in a manner similar to that described previously [3]. Tissue (0.5–1 g) was homogenized in 2 ml 0.02 *M* HC1 containing 1 m*M* BAPS with a Potter-Elvehjem homogenizer. To the homogenate, 360 μl perchloric acid (sp.gr. 1.54) was added, followed by centrifuge at 1700 *g* at 4°C for 15 min. One ml 3.8 *M* KOH was added to the supernatant. After standing at 0°C for 10 min, $KClO_4$ was removed by centrifugation at 5000 *g*. The supernatant was adjusted to pH 5.5 with 0.3 *M* potassium dihydrogen citrate and diluted with 0.1 *M* potassium phosphate (pH 5.5) so that the concentration of total thiols was not higher than 0.4 m*M*. Fifty μl each of the sample solution and 1 m*M*

solution of reagent **III** in dioxane were mixed in a 1-ml microtube and the mixture was heated at 40°C for 10 min. Ten μl of the reaction mixture were injected onto the HPLC column.

The maleimide derivatives of thiols such as cysteine, glutathione and cysteine-containing peptides all had a maximal absorption at 350–360 nm and were stable in aqueous solution at pH 5.5 and room temperature for at least 1 day.

3. Results

3.1. Reaction conditions for derivatization of thiol groups

The labelling reagent and test compounds (cysteine, glutathione and cysteinylglycine) were mixed and incubated at various pH from 4.5 to 8.5 at 40°C for 10 min. The peak area of derivatized glutathione was constant in this pH region, while the peak area of cysteine and cysteinylglycine derivatives were constant between pH 4.5 and 5.5, but gradually decreased at higher pH. We found that the Michael reaction also took place between amino group of cysteine and labelling reagent above pH 5.5. The peak area of derivatized glutathione was time-independent at pH 5.5 and 40°C (data not shown). Thus, thiol derivatization was carried out under at 40°C and pH 5.5 for 10 min.

3.2. High-performance liquid chromatograms of labelled SH compounds

Fig. 1a shows a chromatogram obtained from samples of labelled glutathione and cysteine and Fig. 1b shows a chromatogram of a sample of labelled rat liver homogenate.

Fig. 2a is a chromatogram of the derivatives of authentic γ-GluCys, glutathione, Cys, CysGly and homocysteine and Fig. 2b shows a chromatogram of the derivatized sample from rat liver homogenate. In Fig. 2, the derivatives of γ-gluCys and glutathione were eluted as two peaks due to a chirality of the C atom on the succinimide group attached to the sulfur atom [15–17]. The peak areas were calculated by Chromatopack (Shimazu SPD-R6A).

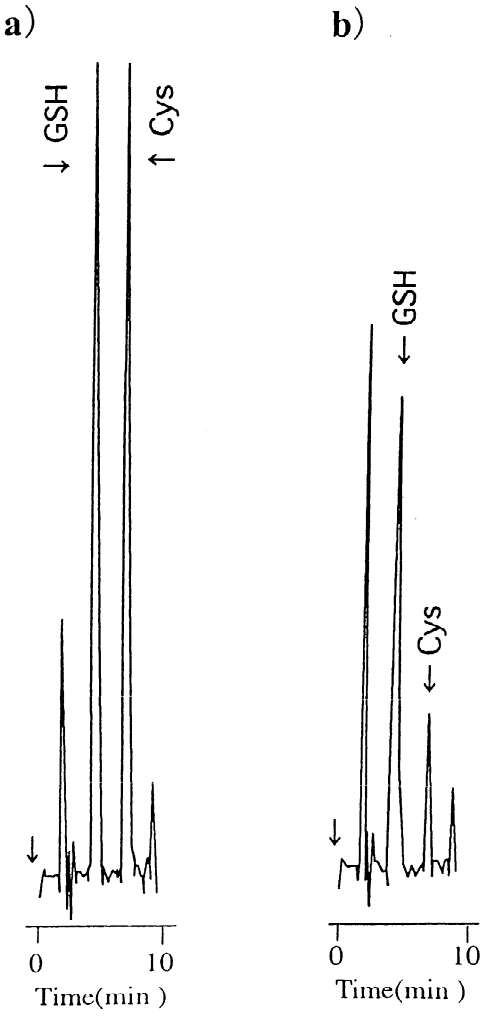


Fig. 1. High-performance liquid chromatograms. (a) Twenty μl of dioxane solution of labelling compound **III** (1 m*M*) and 20 μl of 50 μ*M* solution of cysteine and glutathione were mixed and reacted at 40°C for 10 min. Five μl of the reaction mixture was injected onto the column which was eluted by the mobile phase of solutions A and B. (b) As described in Section 2, after deproteinization of the rat liver homogenate, the supernatant was reacted with reagent **III**. The reaction mixture was analyzed by the method I.

3.3. Calibration curves

The peak area Y was exactly proportional to the glutathione concentration: $Y = 0.421X + 6.01$, $r^2 = 1.000$, when $X \leqq 300$ pmol and the unit of Y is mV/s. When unit of Y is V/s and the concentration range is 300 pmol$\leqq X \leqq$3 nmol, the equation is as follows:

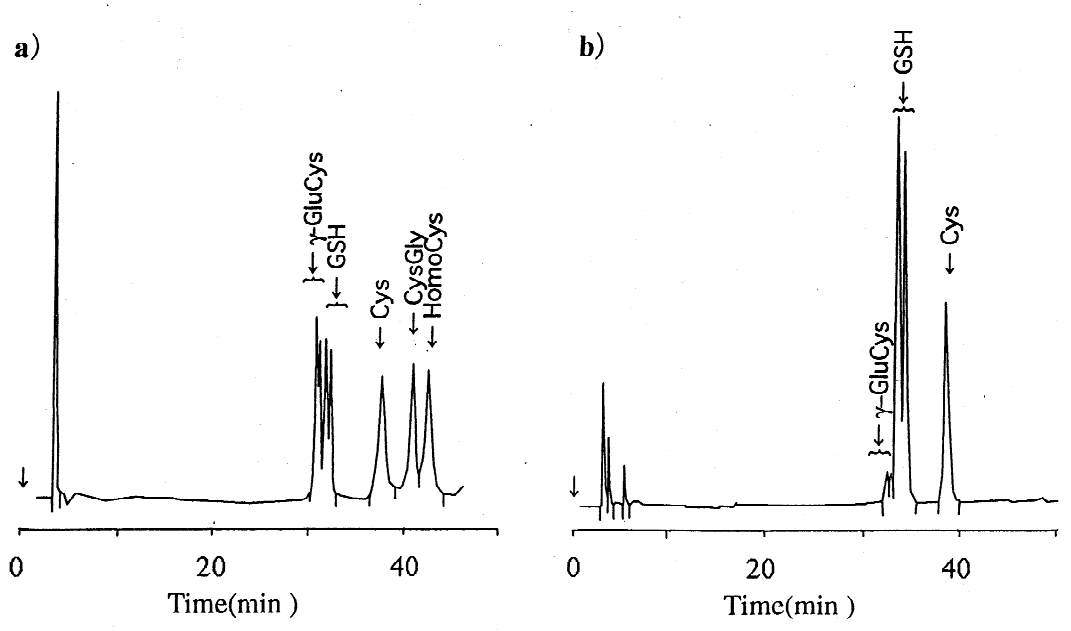


Fig. 2. High-performance liquid chromatograms. (a) Shows the derivatized authentic thiol mixture (γ-GluCys, glutathione, cysteine, CysGly and homocysteine) and (b) shows the derivatized supernatant of the deproteinized rat liver homogenate. The mobile system was programmed between A and B for three steps as described in Section 2.4., method II.

$Y = 0.366X + 0.023$, $r^2 = 1.000$. As for cysteine, the calibration curves gave the equation: $Y = 0.421X + 9.11$, $r^2 = 1.000$; here Y is expressed mV/s and $X <$ 300 pmol. If unit of Y is V/s and 300 pmol $< X <$ 3 nmol, the peak area is expressed as follows: $Y = 0.374X + 0.021$, $r^2 = 1.000$. As for other thiol compounds such as homocysteine, CysGly and γ-GluCys, which are present in very low amounts in usual biological samples, good linealities were also observed between the peak areas and the injected amounts. The determination limits were 0.5 pmol/μl of the reaction mixture for cysteine, glutathione and other thiol compounds.

3.4. Recovery test

For the purpose of applying this procedure to biological samples, a recovery test was carried out. After various amounts of glutathione, cysteine and cysteinylglycine were added to aliquots of a 6000 *g* rat liver homogenate, the thiol groups were derivatized and quantified. The recoveries were calculated from the calibration curves. The results are shown in Table 1. The recovery levels were very satisfactory.

3.5. Comparison of results obtained by the present method and by the methods published previously

In order to evaluate the yields obtained by the present method, glutathione in different rat tissues

Table 1
Recovery of thiol compounds from rat liver homogenate

	Added	Found	Recovery (%)
GSH (nmol)	0.00	0.375±0.013	
	0.50	0.872±0.019	100.5±3.8
	1.00	1.377±0.015	100.8±1.5
	1.50	1.840±0.017	97.7±1.2
Cys (nmol)	0.00	0.053±0.005	
	0.05	0.108±0.003	109.5±6.4
	0.10	0.158±0.002	105.0±2.2
	0.15	0.204±0.004	100.3±2.9
CysGly (pmol)	0.0	0.0±0.0	
	5.0	4.9±0.0	89.0±15.1
	10.0	10.2±0.0	101.8±10.0
	15.0	14.8±0.0	99.0±2.0

After various amounts of thiol compounds were added to the supernatant of rat liver homogenate (10%, from 1 g), they were derivatized and determined as described in Section 2.4., method III. Values are mean±SD ($n = 3$).

and cysteine added to the supernatant of rat liver homogenate were determined by two colorimetric methods [3,8]. The cysteine concentrations for the comparison test, however, had to be set in a higher range to be detected by the method of Gaitonde [8].

The results shown in Fig. 3 demonstrate that the values obtained by the present method are in good agreement with those obtained by two other methods.

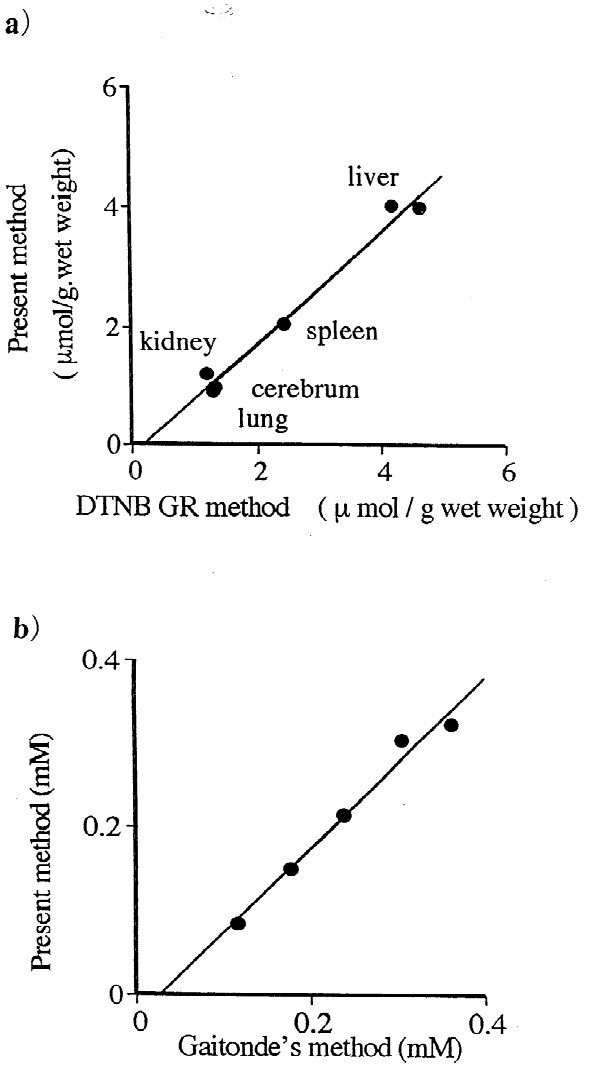
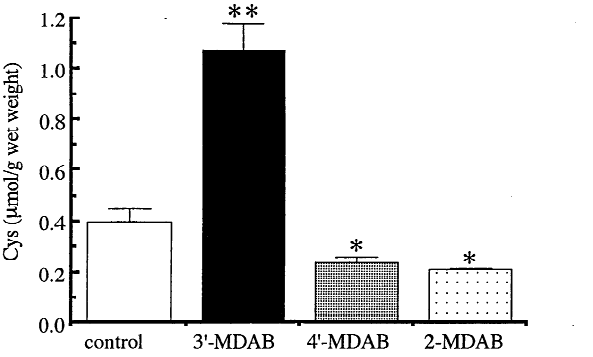


Fig. 3. Relationship between concentrations of glutathione in several organs from rat as determined by the present method I and by the enzymatic method (a), and the relationship between concentrations of cysteine determined by the present method I and by the method of Gaitonde [8] (b). Various amounts of cysteine were added to the supernatant of rat liver homogenate.



*,**show significant difference from those of control values.(*:P < 0.05, ** : P < 0.001)

Fig. 4. Cysteine levels in livers of rats fed a DAB dyes-rich diet. Three groups of rat were given rat cake containing 0.064% 3′-methyl-4-dimethylaminoazobenzene (3′-MDAB), its 4′-methyl isomer (4′-MDAB) or 2-methyl isomer (2-MDAB). Control group was fed with standard rat cake. All animals were killed at the 11th week after feeding azo dye and livers were removed and homogenized. The supernatants of homogenates were deproteinized and derivatized by labelling reagent. The derivatives were determined by method I.

3.6. Application to rat liver

3′-MDAB is a documented liver carcinogen, while its 4′-methyl isomer has low carcinogenic activity and the 2-methyl isomer is noncarcinogenic [18]. Rats whose diets were supplemented with 3′-MDAB for 11 weeks were considered to be precancerous [19]. The hepatic contents of glutathione and cysteine at the 11th week were measured by the present method. The hepatic cysteine level of 3′-MDAB-fed rats was very elevated in comparison to that of control group (Fig. 4). In contrast the cysteine level of the 4′-MDAB and 2-MDAB groups was lower than that the control group. No difference in the glutathione level was observed.

4. Discussion

Glutathione has important biological roles. It is the coenzyme of glyoxalase I and is the substrate of glutathione peroxidase that destroys peroxides and the substrate of glutathione *S*-transferase that detox-

ifies xenobiotic substances. The biological roles of glutathione have been reviewed previously [20–23]. However, physiological significance of glutathione and cysteine has not been elucidated. For example, an interesting experimental observation was made during liver regeneration in rat. The glutathione level increased during the first 24 h post-operation and reached a maximum (2.4-fold the sham-operated control level) on day 2. It decreased to the control level when liver regeneration was complete [4,5].

Glutathione is widely distributed in living cells, from microbes to higher organisms. It is present mainly in its reduced form and at about 1 mmol/l of whole blood [24,25] and 5.6 μmol/g fresh rat liver [3]. Its concentration is one of the highest of organic compounds in those cells.

Cysteine level is much lower than glutathione level in biological samples. For example its level is about 4% of glutathione level in mouse liver [6]. The molar ratio of cysteine to glutathione is more lower in cell lines [12]. The cysteine content is negligible small by comparison with glutathione in blood cells [26]. In *Saccharomyces cerevisiae*, the level of cysteine is 0.1 and 1% of glutathione in anaerobically and aerobically grown cells, respectively [7].

Homocysteine content is 5, 3 and 3% that of cysteine content in normal human serum, normal rat serum, and normal human urine, respectively [27]. Since cysteine is formed from methionine via homocysteine, it is natural that homocysteine should be found in larger quantities in biological samples, especially in liver. Needless to say, the homocysteine level in urine and blood sera of homocystinuric and cystathioninuric patients is elevated.

We have studied the analytical methods for glutathione and cysteine in biological samples and applied it to biochemical studies as mentioned above. We can confidently recommend the previous report [3] for analysis of glutathione in biological samples, if glutathione alone is measured in biological samples.

The present method was devised to analyze glutathione, cysteine and other thiol compounds in biological samples at the same time. However, it should not encounter in practice to measure all thiol compound in one run on HPLC because there are big differences in concentrations of thiol compounds in biological samples described above. For these reason, method I, II and III presented in this report should be used depending on the purpose of the study.

The HPLC method presented here has some advantages. The first is the specificity of the reaction, that is, maleimide group reacts with only thiol groups at pH 5.5. Second, there is no overlapping or excess peak on the chromatogram. We initially attempted to modify thiol groups with 2-vinylpyridine and to analyze the derivatives at 262 nm by HPLC. However, we had encountered some unnecessary peaks on HPLC chromatograms, which were attribute to UV-absorbing compounds in the biological samples. This problem could be overcome by the labelling compound which has an absorption maximum at 350–360 nm. Third, thiol compounds can be easily analysed by standard HPLC that is not equipped with a fluorescence spectrophotometer. Although the monobromobimane fluorescent labelling method is excellent for analysis of thiol compounds in terms of sensitivity and specificity, the bimane derivatives are photosensitive and should be protected from light during preparation and storage. In contrast, the labelling reagent **III** is stable. Fourth, the analytical procedure is simple: a sample solution is only mixed with the labelling reagent and an aliquot of the mixture is applied on HPLC. Fifth, the labelling reagent can be easily synthesized from simple chemicals. Finally, the method has satisfactory sensitivity and almost full recovery from rat liver homogenate.

We applied the present method to pre-cancerous rat liver. It was found that the cysteine level in the livers of rats fed with 3′-MDAB increased dramatically, while the cysteine level in rats fed with 4′-MDAB or 2-MDAB, which have weak or no carcinogenic activity, were lower than that of the control group. The hepatic levels of cysteine and glutathione were simultaneously determined by method I. The glutathione level in all four groups of rats was the same. Neish and co-workers [28,29] reported that the level of rat liver glutathione increased markedly after a single intraperitoneal injection of 3′-MDAB. The injection of 4′-MDAB, however, resulted in a small increase, while an injection of 2-MDAB caused a decrease in liver glutathione 24 h post-injection [28,29]. The results presented here can not be simply compared with the those of Neish and co-workers because the experiments differed in administration

and determination methods when the rats were tested. The experiments of the Neish team dealt with the acute toxicity of the azo dyes and not with carcinogenesis. The results that cysteine level of 3′-MDAB group was 2.9 times than that of the control group and that glutathione levels in all azo dye-fed groups and the control group were the same, can not be explained. The problem whether the results are due to production or availability of cysteine is the next investigation point. We wish also to know whether the phenomena are characteristic of all pre-carcinogenic organs.

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