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Short communication

Determination of γ-glutamylglutathione and other low-molecular-mass biological thiol compounds by isocratic high-performance liquid chromatography with fluorimetric detection

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Abstract

A method was developed for the simultaneous determination of γ-glutamylglutathione (γ-GluGSH) and other low-molecular-mass thiol compounds (cysteine, cysteamine, homocysteine, cysteinylglycine, γ-glutamylcysteine, glutathione and N-acetylcysteine) using high-performance liquid chromatography combined with precolumn fluorescence labeling with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F). These SBD-labeled thiol compounds were separated within 35 min on a Cosmosil 5C-18AR column with isocratic elution using 75 m*M* sodium citrate buffer (pH 2.90)–methanol (98:2) and detected fluorimetrically (ex. 386 nm, em. 516 nm). The calibration graphs using 2-mercaptoethanol as an internal standard showed good linearity in the range from 20 pmol to 10 nmol for all thiol compounds examined. The application of this method for the quantitative determination of thiol compounds in the urine from γ-glutamyl transpeptidase-deficient mice was also demonstrated. This method is sufficiently simple, rapid and sensitive for the determination of γ-GluGSH and other low-molecular-mass thiol compounds in biological samples. © 1998 Elsevier Science B.V.

Keywords: γ-Glutamylglutathione; Cysteine; Cysteamine; Homocysteine; Cysteinylglycine; γ-Glutamylcysteine; Glutathione; N-Acetylcysteine

1. Introduction

A variety of thiol compounds occurr widely in animal tissues and fluids, and most of them play various important biological roles [1]. Cysteine (Cys) participates in a number of biochemical process that depend directly on the particular reactivity of thiols [2]. Cys is synthesized from homocysteine (Homo-Cys) as a precursor and metabolized to other thiol compounds, such as glutathione (GSH; γ-GluCysGly), cysteamine and N-acetylcysteine (N-AcCys) derivatives [2]. GSH has been shown to play an important role in protection against oxidative stress, radiation damage and heavy metal intoxication, in the metabolism of various xenobiotics, and in resistance to anticancer drugs [3]. GSH is synthesized from γ-glutamylcysteine (γ-GluCys) and glycine by glutathione synthetase and decomposed to cysteinylglycine (CysGly) by the action of γ-glutamyl transpeptidase (γ-GTP) [3]. Concentrations of these biological thiol compounds in animals are influenced by various stress, such as drug administra-

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tion, oxidative stress, infection and disease. Determination of the concentrations of biological thiol compounds generally provides information about organ functions and stage of disease progression, etc. Recently, Cys and GSH deficiency has been observed in acquired immunodeficiency syndrome patients at all stages of the disease [4]. Associations of disorders of thiol metabolism with some diseases have also been observed. Hyperhomocysteinaemia has been reported to be associated with an increased risk of vascular artherosclerosis and thromboembolism [5]. An excessive excretion of GSH, γ-GluCys and γ-glutamylglutathione (γ-GluGSH) was observed in the urine from a patient with γ-GTP deficiency [6,7]. γ-GluGSH is formed by the reaction of γ-GTP with GSH as both γ-glutamyl donor and γ-glutamyl acceptor [7].

Many methods for the measurement of biological thiol compounds have been reported [8], but there are no reliable and simple methods for simultaneous determination of γ-GluGSH and other biological thiol compounds. Here, we report a novel method for the simultaneous determination of eight biologically important thiol compounds containing γ-GluGSH by isocratic high-performance liquid chromatography (HPLC) with precolumn fluorescence labeling. The method presented here is sufficiently simple, rapid and sensitive for the analysis of γ-GluGSH and other low-molecular-mass biological thiol compounds.

2. Experimental

2.1. Chemicals

Tri-*n*-butyl phosphine (TBP), N-AcCys, cysteamine and HomoCys were obtained from Nacalai Tesque (Kyoto, Japan). GSH, CysGly and L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (acivicin) were obtained from Sigma (St. Louis, MO, USA). L-Cys was obtained from Kyowa Hakko Kogyo (Tokyo, Japan). Ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F) and other chemicals were obtained from Wako (Osaka, Japan). γ-GluCys was prepared by the method of Anderson and Meister [9]. γ-GluGSH was prepared by a modification of the method of Abbot et al. [7]. Solvents for HPLC were purified by distillation and filtered through PTFE (0.5 μm, for organic solvents) or cellulose nitrate (0.45 μm, for aqueous solvents) membrane filters (Advantec Toyo, Tokyo, Japan) before use.

2.2. HPLC

HPLC was carried out using a Tosoh HPLC System (Tosoh, Tokyo, Japan) equipped with a SC-8020 control unit, an AS-8020 autosampler, a DS-8023 degassing unit, a CCPM-II multipump and a FS-8010 fluorescence detector. A Cosmosil 5C-18AR column (150 mm×4.6 mm I.D., 5 μm particles; Nakarai Tesque, Osaka, Japan) was used at a flow-rate of 1.0 ml/min at ambient temperature.

2.3. Collection of urine from γ-GTP-deficient mice

Acivicin, a γ-GTP inhibitor, dissolved in saline was administered i.p. (1 μmol/g) to male ICR mice weighing about 25–27 g. Urine was collected for 2 h after the administration of acivicin using a metabolic cage.

2.4. Derivatizations of low-molecular-mass thiol compounds

In the present study, SBD-F [10] was used as a fluorescence reagent because of its sensitivity, reactivity and water solubility. Derivatization of thiol compounds with SBD-F was performed by a modification of the method of Toyo'oka and Imai [11]. To the calibration solution (100 μl) or the urine samples (100 μl) obtained from mice were added 10 μl 2-mercaptoethanol (2-ME, 50 μ*M*) as an internal standard (I.S.) and 100 μl trichloroacetic acid (TCA, 10%) containing 10 m*M* EDTA. After centrifugation for 10 min (760 *g*, 4°C), 350 μl potassium borate buffer (1 *M*, pH 10.5), 100 μl TBP (1% in water) and 100 μl SBD-F (0.3% in water) were added to aliquots of the supernatant (150 μl) yielding a final pH of about 8.5. The resultant mixture was incubated at 60°C for 60 min. After the reaction, the mixture was put in an ice bath and 50 μl of 4 *M* HCl was added. Aliquots of this solution (10 μl) were injected into the HPLC system using 75 m*M* sodium citrate buffer (pH 2.90)–methanol (98:2) as the mobile phase. The eluate was monitored by fluorescence

detection (excitation wavelength, 386 nm; emission wavelength, 516 nm). In the present study, no significant changes in the fluorescence intensity of SBD-labeled thiol compounds were observed during storage of the reaction mixture for 2 weeks at 4°C.

3. Results and discussion

3.1. Separation of γ-GluGSH and other low-molecular-mass biological thiol compounds

Various combinations of buffer solution and organic solvent were examined to discover the most suitable mobile phase on a Cosmosil 5C-18AR, which is a polymeric type octadecylsilyl column. The use of sodium citrate buffer–methanol appeared promising. The chromatographic behavior of eight biologically important thiol compounds was investigated using this system. The retention factor (*k*) of each thiol compound as a function of the pH of 75 m*M* sodium citrate buffer–methanol (98:2) as the mobile phase is shown in Fig. 1. At acidic pH, the *k*-values of amino acids (Cys), dipeptides (γ-GluCys, CysGly), tripeptide (GSH) and tetrapeptide (γ-GluGSH) were increased with the number of amino acids. The *k*-values were markedly decreased with a change in pH from 2.75 to 4.00, depending on the number of carboxylic groups, which is attributed to the dissociation of the carboxyl moiety. In contrast, the *k*-values of components containing an excess of amino functions with respect to the carboxyl groups, i.e., being substantially basic, increase with increasing pH, e.g., in the range of pH 6 and 7, due to deprotonation of the amine moiety. In addition, the effects of ion-pair formation with citrate anions should play a substantial role in peptide retention.

From these findings, 75 m*M* sodium citrate buffer (pH 2.90)–methanol (98:2) was chosen as the suitable mobile phase. A typical chromatogram of the eight thiol compounds is shown in Fig. 2. Each thiol compound exhibited a sharp and symmetric peak and good separation from the other compounds.

3.2. Calibration graphs for thiol compounds

The calibration graphs for all thiol compounds examined in the range from 20 pmol to 10 nmol

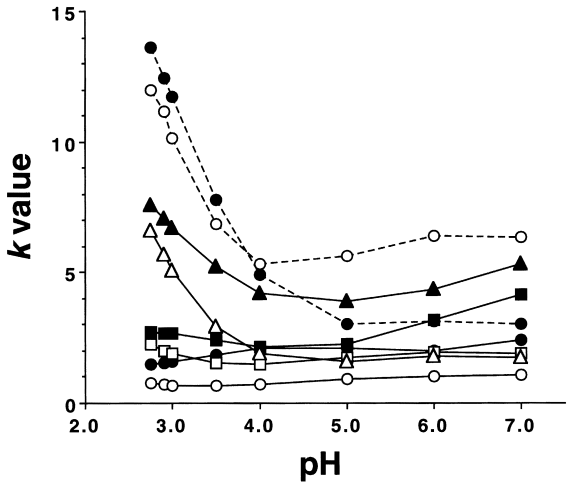

Fig. 1. Effects of pH of mobile phase on *k*-values of SBD-labeled thiol compounds. Each thiol compound was derivatized with SBD-F and eluted from a Cosmosil 5C-18AR column using 75 m*M* sodium citrate buffer (pH 2.90)–methanol (98:2) as the mobile phase. Fluorescence intensities of SBD derivatives were monitored with excitation at 386 nm and emission at 516 nm. -●-, γ-GluGSH; -○-, N-AcCys; –▲–, GSH; –△–, γ-GluCys; –■–, CysGly; –□–, HomoCys; –●–, cysteamine; –○–, Cys.

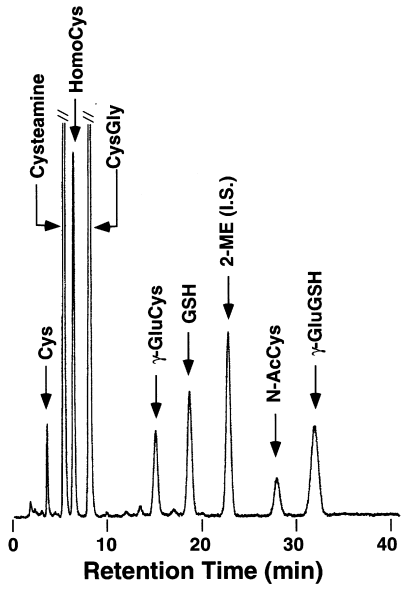

Fig. 2. Chromatogram of SBD-labeled thiol compounds. Standard thiol compounds were derivatized with SBD-F and eluted from a Cosmosil 5C-18AR column using 75 m*M* sodium citrate buffer (pH 2.90)–methanol (98:2) as the mobile phase.

using 500 pmol 2-ME as I.S. showed excellent linearities. The coefficients of correlation between amounts of these thiol compounds and observed peak heights were in the range from $r=0.989$ to 0.999.

3.3. Reduction of oxidized thiols by TBP

In the present study, TBP was added for reduction of oxidized thiols during the reaction of derivatization of thiol compounds with SBD-F, because thiol compounds are relatively unstable and easily converted to disulfides. Cys disulfide (CysSSCys) and GSH disulfide (GSSG) were added to 10% homogenate (100 μl) of mouse liver at the concentration equivalent to endogenous hepatic Cys and GSH, centrifuged after mixing with 100 μl of 10% TCA solution containing 10 m*M* EDTA, and then the supernatant was reacted with SBD-F in the presence of TBP. About 98% of CysSSCys and 99% of GSSG were recovered as Cys and GSH by the present method using HPLC (data not shown). However, the recoveries of CysSSCys and GSSG were only 5% and 2%, respectively, when the reaction was performed without TBP. Moreover, TBP did not affect the recovery of Cys and GSH when these thiol compounds were added to the liver homogenate. These results suggest that oxidized thiols do not react with SBD-F in the absence of TBP, and TBP efficiently reduces oxidized thiols under the present experimental conditions.

3.4. Determination of thiol compounds in mouse urine

On the basis of the above results, this analytical method was applied to the determination of thiol compounds in mouse urine. Chromatograms of urine obtained from normal mouse and acivicin (γ-GTP inhibitor [12])-treated mice are shown in Fig. 3(a and b), respectively. Excellent separation without any interfering peaks was achieved. In acivicin-treated mice, GSH, γ-GluCys and γ-GluGSH peaks, which are hardly found in normal urine, appeared at the μ*M* to m*M* level, as also reported by Meister and coworkers [6,7]. Increases in Cys and CysGly concentrations were also observed in the urine from

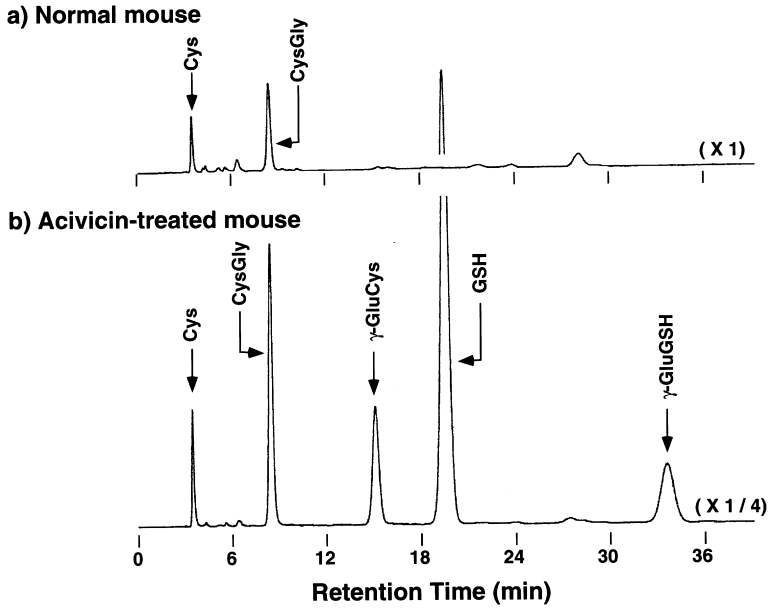

Fig. 3. Chromatograms of SBD-labeled thiol compounds excreted in mouse urine. Urine samples were collected for 2 h after administration of saline (a) or acivicin (b), derivatized with SBD-F and eluted from a Cosmosil 5C-18AR column using 75 m*M* sodium citrate buffer (pH 2.90)–methanol (98:2) as the mobile phase. Fluorescence intensities of SBD derivatives were monitored with excitation at 386 nm and emission at 516 nm.

acivicin-treated mice. Griffith and Meister [6] reported that most of γ-GluCys in the urine obtained from acivicin-treated mice was present in the oxidized form. However, in the present study, about 95% and 91% of γ-GluCys and 96% and 94% of γ-GluGSH in the urine of control and acivicin-treated mice, respectively, was detected even when the urine samples were reacted with SBD-F in the absence of TBP (data not shown). These observations indicated that most of the γ-GluCys and γ-GluGSH existed in the reduced form in the urine of both control and acivicin-treated mice.

The HPLC method established in this study was able to determine γ-GluGSH and additional seven biologically important thiol compounds simultaneously by isocratic HPLC. Compared with the gradient technique, isocratic elution provides great advantages, such as high reproducibility and sample throughput, particularly in cases where large numbers of samples are determined continuously because no column reequilibration is required. Moreover, sample pretreatment and derivatization are quite simple, and the resultant SBD-derivatized thiol compounds are very stable and can be stored for at least 2 weeks without any changes in fluorescence intensity. For this reason, the method is well suited for studies of the metabolism and the biological roles of γ-GluGSH and other thiol compounds, as well as monitoring of the toxicological profile of chemical substances, and evaluation of disease progression, etc.

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