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Note

Rapid and simple method for quantitative determination of non-protein sulphydryls in mouse liver by reversed-phase high-performance liquid chromatography

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Fractional estimations of the concentrations of endogenous sulphydryls in biological materials have been performed by means of reversed-phase high-performance liquid chromatography (HPLC) based on spectrophotometry with Ellman's reagent [1] and fluorometry with monobromobimane [2]. However, these methods are not convenient for the quantitative separation of specific endogenous cysteine, which is required for the study of glutathione turnover [3].

We have developed a rapid and simple method for the determination of endogenous sulphydryls including cysteine, γ-glutamylcysteine, and glutathione using Ellman's reagent and conventional reversed-phase HPLC. This method should be useful for the estimation of endogenous sulphydryls in the γ-glutamyl cycle [4], in which cysteine plays an important role [3].

MATERIALS AND METHODS

Apparatus

UV absorbance at 412 nm of a sample was recorded on a UVIDEC 610 spectrophotometer (Jasco, Tokyo, Japan), from which the total amount of non-protein sulphydryls was estimated.

High-performance liquid chromatography was performed with a Hitachi Model 655 solvent delivery system (Tokyo, Japan) equipped with a Rheodyne 7125 injection valve (20 μl) and a reversed-phase column (150 mm × 4.0 mm I.D. Hitachi-gel No. 3056 5C_{18}) containing C_{18} chemically bonded silica gel (5 μm particle size). The phosphate buffer solution (0.5 *M*, pH 3.0), con-

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taining 15% (v/v) of methanol, was delivered as the mobile phase at a flow-rate of 0.7 ml/min. The eluates were monitored by UV absorption at 330 nm using a Model 1638-41 variable-wavelength detector (Hitachi). Calculation of the eluate concentrations from the UV absorbances was carried out by a Model 833 data processor (Hitachi). All the analyses were performed at room temperature.

Materials

Reduced glutathione was obtained from Sigma (St. Louis, MO, U.S.A.). Ellman's reagent [5,5'-dithio-bis(2-nitrobenzoic acid), DTNB] and cysteine were purchased from Nakarai Chemicals (Kyoto, Japan). γ-Glutamylcysteine was prepared from oxidized glutathione [5]. All the other reagents were of the best available grades. All sample solutions were purged with oxygen-free nitrogen for 5 min prior to use.

Sample preparation

Female, 8-week-old C3H/He mice obtained from the animal centre of Kyoto University were used.

Homogenates (10%) of mouse liver were prepared in 0.02 *M* disodium ethylenediaminetetraacetic acid in an ice bath. Aliquots of 2.5 ml of the homogenates were diluted with 2 ml of water followed by addition of 0.5 ml of 50% trichloroacetic acid. The mixtures were shaken vigorously and permitted to stand for 15 min in the ice bath. The suspensions were centrifuged at 2000 *g* and 4°C for 10 min. To 4 ml of 0.4 *M* Tris buffer solution (hydrochloric acid, pH 8.9) were added 2.0 ml of the supernatant together with 0.1 ml of 10 m*M* DTNB. The sample solution thus obtained was shaken and then filtered through a 0.22-μm Millipore filter (Millipore, Bedford, MA, U.S.A.). After recording the absorbance at 412 nm [6], the sample was subjected to HPLC analysis.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of a standard solution of sulphydryls, consisting of 0.3 m*M* cysteine, 5.0 m*M* glutathione, and 0.1 m*M* γ-glutamylcysteine, after treatment with DTNB. Following our procedure, the separation of individual sulphydryl components as their disulphide forms and the baseline assay could be achieved within 20 min. The retention times were highly reproducible. The peak with the retention time of 15.6 min in Fig. 1 was assigned to 2-nitro-5-thiobenzoic acid as a common product from reactions of the sulphydryls with DTNB. The peak of unreacted DTNB failed to be detected under these conditions. The injected sample solution after treatment with DTNB did not contain any detectable amount of unreacted sulphydryls as confirmed by HPLC when eluted with 0.5 *M* phosphate buffer solution (pH 3.0) and UV absorption at 210 nm.

The HPLC peak heights for cysteine, glutathione and γ-glutamylcysteine showed good linear correlations with their concentrations in the physiological range: the correlation coefficient for each sulphydryl was very close to unity (see Table I).

Fig. 2 shows a representative chromatogram observed for endogenous

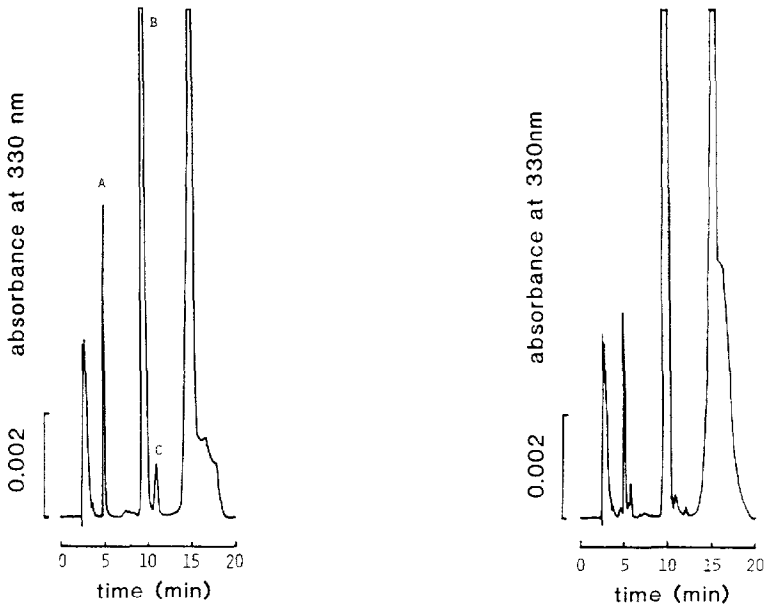

Fig. 1. Chromatogram of a standard solution containing 0.3 m*M* cysteine (A), 5.0 m*M* glutathione (B), and 0.1 m*M* γ-glutamylcysteine (C) after treatment with Ellman's reagent.

Fig. 2. Representative chromatogram of endogenous sulphydryls derived from mouse liver.

TABLE I

CALIBRATION FOR NON-PROTEIN SULPHYDRYLS

The relative HPLC peak height (*H*) calculated by a Model 833 Hitachi data processor was fitted to the sulphydryl concentrations (*C*, μ*M*), using a first-order regression: $C = a + bH$.

Compound	Parameter		Coefficient of correlation	Upper concentration range of the calibration experiment (m*M*)
	a	*b*		
Cysteine	5.895	0.2284	0.9998	1.0
γ-Glutamylcysteine	−4.676	0.8517	0.99997	1.0
Glutathione	−116.2	0.7103	0.9988	7.0

sulphydryls in mouse liver homogenate. By reference to the chromatogram for the standard sample in Fig. 1, we could confirm cysteine (retention time, t_R = 5.02 min), glutathione (t_R = 9.5), and γ-glutamylcysteine (t_R = 11.0). It is particularly important to note that the cysteine originated from the liver could be determined successfully by the present method, owing to the use of an eluent of suitable composition and UV monitoring at 330 nm.

Separate experiments showed that the concentrations of individual sulphydryls did not vary for at least 4 h when the acid extract prepared as described in the experimental section was sealed and cooled in the ice bath. Furthermore, the disulphides as the reaction products with DTNB in Tris buffer were stable for at least 3 h at room temperature, whereas 2-nitro-5-thiobenzoic acid oxidized gradually [6].

TABLE II

DETERMINATION OF ENDOGENOUS NON-PROTEIN SULPHYDRYLS (NPSH) IN MOUSE LIVER (mmol/kg)

Mouse No.	Cysteine	γ-Glutamyl cysteine	Glutathione	Total NPSH concentration	
				HPLC*	UV
1	0.262	0.027	6.37	6.66	7.02
2	0.209	0.025	6.40	6.64	6.90
3	0.258	0.031	6.09	6.38	6.84
4	0.275	0.033	6.06	6.37	6.78
5	0.265	0.030	6.01	6.31	6.92
6	0.216	0.038	6.38	6.64	7.23
7	0.243	0.027	5.94	6.21	6.94
8	0.246	N.D.**	5.60	5.83	6.57
Mean	0.247	0.030	6.11	6.38	6.91
± S.D.	±0.022	±0.004	±0.26	±0.27	±0.20
Coefficient of variation (%)	8.9	13.3	4.3	4.2	2.9

*Sum of three endogenous sulphydryls.
**N.D. = undetectable level.

As shown in Table I, the reproducibility of the present method for the analysis of endogenous sulphydryls was excellent. Table II shows that the total concentration of non-protein sulphydryls measured by UV absorbance was slightly greater than the sum of the individual component sulphydryls determined by HPLC. The γ-glutamylcysteine concentration in mouse liver was estimated as 0.030 mmol/kg. To our knowledge, this is the first determination of reduced γ-glutamylcysteine in mouse liver, though the total amount of γ-glutamylcysteine was reported as 0.70 μmol/g in rat liver [1].

Finally, the recoveries of cysteine (0.1 mmol/kg), γ-glutamylcysteine (0.01 mmol/kg), and glutathione (1.0 mmol/kg) from a liver sample, to which these sulphydryls were previously added, were found to be 96%, 103%, and 98%, respectively.

We have described a simple HPLC method to determine quantitatively endogenous sulphydryls without interference from the biological materials used as samples. This was achieved by the use of Ellman's reagent and a mobile phase of suitable composition.

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