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Note

Determination of natural thiols by liquid chromatography after derivatization with 3,5-di-*tert.*-butyl-1,2-benzoquinone

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Glutathione (GSH), the most abundant intracellular thiol, plays important roles in many biological processes, such as the transport of amino acids, the regulation of enzyme activities, protection against cell damage by active oxygen species and free radicals, and detoxicification of exogenous compounds [1]. Catechols and hydroxyindoles have frequently been determined using electrochemical detection (ED) because of the high sensitivity of the method [2]. Thiols have also reducing properties; however, their detection requires the use of mercury electrodes rather than the more common carbon electrode [3].

Some years ago, we developed a method for the detection of thiols on thin-layer plates [4]. The method was based on the reaction of thiols with 3,5-di-*tert.*-butyl-1,2-benzoquinone (DBBQ) to form 4,6-di-*tert.*-butyl-3-*SR*-catechols (Fig. 1). This paper reports the determination of these catechols by high-performance liquid chromatography (HPLC) with ED.

EXPERIMENTAL

Chemicals

Reduced glutathione (GSH), L-cysteine (Cys), DL-homocysteine, and L-ergothioneine were obtained from Sigma (St. Louis, MO, U.S.A.). DBBQ was from Aldrich (Milwaukee, WI, U.S.A.). Other chemicals were from Wako (Osaka, Japan).

4,6-Di-*tert.*-butyl-3-*S*-glutathionylcatechol (GS-catechol) was prepared as follows. A stirred solution of 1 mmol of GSH in water (10 ml) was mixed for 10 min with a solution of 0.5 mmol of DBBQ in ethanol (20 ml). Stirring was con-

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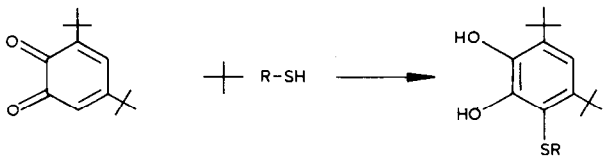

Fig. 1. Reaction of thiols (RSH) with 3,5-di-*tert.*-butyl-1,2-benzoquinone (DBBQ).

tinued for 10 min and then 100 ml of water were added. The resulting colourless precipitate was filtered, washed with water, and dried. Yield, 255 mg (93%); m.p., 215–216°C (dec.). Found: C, 53.26; H, 7.14; N, 7.67; S, 5.85%. Calculated for $C_{24}H_{37}N_3O_8S \cdot H_2O$: C, 52.83; H, 7.20; N, 7.70; S, 5.88%.

4,6-Di-*tert.*-butyl-3-*S*-cysteinylcatechol (Cys-catechol) was prepared analogously and was obtained as a pale-brown powder. Yield, 137 mg (78%); m.p. ca. 180°C (dec.). Found: C, 58.69; H, 8.00; N, 3.89; S, 8.63%. Calculated for $C_{17}H_{27}NO_4 \cdot \frac{1}{2}H_2O$: C, 58.26; H, 8.05; N, 4.00; S, 9.15%.

Chromatography

A Yanaco Model L-5000 liquid chromatograph (Yanagimoto, Kyoto, Japan) was used in combination with a Yanaco Model VMD-101A electrochemical detector (glassy carbon electrode). The working electrode was set at +1200 mV vs. a silver/silver chloride reference electrode. The column was a C_8 reversed-phase column (Develosil C_8, 5 μm particle size, 250×4.6 mm I.D., Nomura Chemical, Seto, Aichi, Japan). The mobile phase was methanol–water–1 *M* perchloric acid (70:30:1.5). The column temperature was maintained at 60°C, and the flow-rate was 0.8 ml/min.

Standard solutions

GS-catechol and Cys-catechol were dissolved in ethanol–0.1 *M* hydrochloric acid (8:2) at 1 μmol/ml. Thiols (10 μmol/ml) were dissolved in 0.4 *M* perchloric acid, and DBBQ (10 μmol/ml) in ethanol. These stock solutions were stored at −80°C and diluted with 0.4 *M* perchloric acid immediately before use. DBBQ was diluted with ethanol.

Sample preparation

Liver (1 g) or whole organs of lung or kidney from Wistar rats were homogenized in 9 vol. of cold 0.4 *M* perchloric acid with a Ten-Brocke glass homogenizer. The homogenates were centrifuged at 12 000 *g* and 4°C for 20 min. The clear supernatants were diluted ten-fold with 0.4 *M* perchloric acid.

Derivatization with DBBQ

To 0.1 ml of a perchloric acid tissue extract or a standard solution containing 10 nmol/ml of GSH and Cys, 0.1 ml of the solution of DBBQ (1 μmol/ml) was added and the mixture was shaken for 30 min at room temperature. Then 1 ml of hexane was added and shaking was continued for 2 min. After centrifugation (10 000 *g* for 30 s), the hexane layer was aspirated off and the hexane extraction

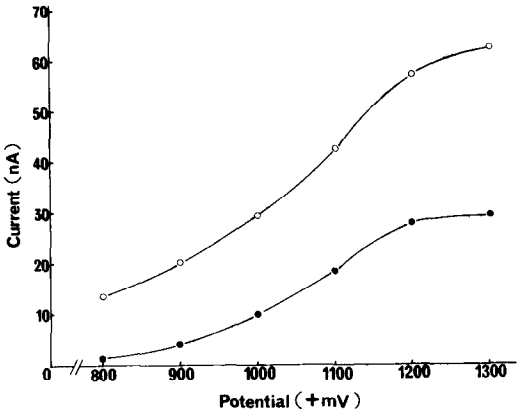

Fig. 2. Relationships between working potential and current: 10 μl of a standard solution containing 10 nmol/ml of GS-catechol (●) and Cys-catechol (○) were injected (average of two determinations).

was repeated twice. A 10-μl aliquot of the aqueous layer was analysed by HPLC. The concentrations of GSH and Cys in the extract were calculated by comparing peak heights of sample and standard solutions.

Spectrophotometric determination of total thiols

Total thiols were determined in 0.4 *M* perchloric acid tissue extracts using Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid)], according to the method of Buttar et al. [5].

RESULTS

Chromatographic conditions

A C_8 reversed-phase column was chosen for the separation of GS-catechol and Cys-catechol. Elution with methanol–water mixtures resulted in broad peaks. However, this problem was overcome by the addition of perchloric acid; elution with methanol–water–1 *M* perchloric acid (70:30:1.5) gave sharp peaks with retention times of 8.4 and 9.7 min for GS-catechol and Cys-catechol, respectively. Later on, we found that a C_{18} reversed-phase column (Develosil ODS-5) was also suitable for the separation of these catechols.

The column did not deteriorate in spite of the strongly acidic mobile phase and the high column temperature; more than 500 samples could be analysed without loss of column efficiency.

The relationships between oxidation potential and current were examined (Fig. 2). The currents reached plateau levels at +1200 mV. This potential was used during the subsequent work.

Reaction conditions

A standard solution of GSH and Cys (10 nmol/ml) was treated with an equal volume of DBBQ solution. At a concentration of 5 μmol/ml DBBQ, the reaction was complete within 10 min and the yields of catechols decreased thereafter. Fig. 3 shows the time course of the reaction of GSH and Cys with 1 μmol/ml DBBQ.

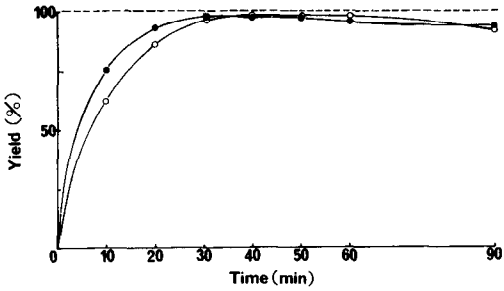

Fig. 3. Time course of the reaction of GSH and Cys with DBBQ. A standard solution containing GSH (●) and Cys (○) (10 nmol/ml each) was treated as described under Experimental. The results are expressed as percent yield (average of two determinations).

The yields of GS-catechol and Cys-catechol reached maxima at 30–40 min and then decreased slightly. Since the yields at a reaction time of 30 min were 96–98%, this time was used routinely. The extraction with hexane to remove unchanged DBBQ (and its degradation products) was indispensable; without this treatment a very large peak appeared at ca. 23 min.

Standard curves

The peak heights of GS-catechol and Cys-catechol standards were linearly related to concentration in the range 0.1–100 nmol/ml. Standard solutions of GSH and Cys were treated with 1 μmol/ml DBBQ. Linear relations were found for the reaction products in the same range. The lower limit of linearity corresponded to 10 pmol per 0.1 ml of sample solution.

Application to biological materials

Fig. 4B shows a typical chromatogram of the reaction products of tissue thiols with DBBQ, in comparison with that of a standard solution (Fig. 4A). GSH was the major thiol in the rat liver extract; Cys was detected only at low concentrations. The catechols derived from ergothioneine and homocysteine appeared at 6.3 and 10.4 min, respectively. Ergothioneine was found in liver extracts at low concentrations; homocysteine was below the detection limit (0.1 nmol/ml) of the method.

Evaluation of the method

Table I indicates that the coefficients of variation for determination of GSH and Cys in rat liver were 1.2–5.3%, depending on the concentration. Addition of GSH (3 μmol/g tissue) and Cys (0.4 μmol/g tissue) to the liver extract resulted in 96% recovery of both thiols. Because of the high precision and recovery, the use of an internal standard seems unnecessary.

The following compounds (100 nmol/ml) were found not to interfere with the analysis: cystine, methionine, tyrosine, tryptophan, lysine, arginine, uric acid, and ascorbic acid. Owing to the high methanol content of the mobile phase, most reducing compounds eluted with the front peak.

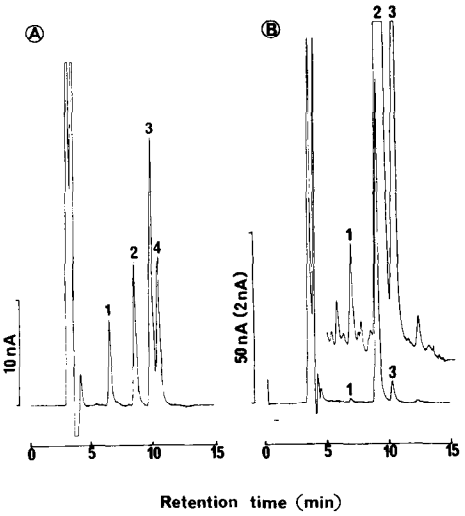

Fig. 4. Chromatograms of the reaction products with DBBQ. (A) Standard solution containing 10 nmol/ml of each thiol; full scale = 32 nA. (B) A sample prepared from rat liver extract (100-fold dilution); GSH, Cys, and ergothioneine content: 7.04, 0.26, and 0.15 μmol/g wet tissue, respectively; full scale = 100 and 4 nA. Volume injected, 10 μl. Peaks: 1 = ergothioneine; 2 = GSH; 3 = Cys; 4 = homocysteine.

Comparison of thiol concentrations in tissues as obtained by HPLC-ED and spectrophotometry

Table II shows the content of GSH and Cys in rat liver, lung, and kidney. GSH represents 96% of the sum of GSH and Cys in liver and lung, but only 63% in kidney. Our values are in agreement with the reported data [6]. The high content of Cys in the kidney may be due to the hydrolysis of GSH by γ-glutamyl transpeptidase [7]. Table II also shows that the sum of GSH and Cys as obtained by the present method agrees well with the total amount of thiols as obtained with Ellman's reagent.

TABLE I

PRECISION OF THE METHOD AND RECOVERY OF GLUTATHIONE AND CYSTEINE

A perchloric acid extract of rat liver (final dilution 100-fold) was analysed as described in Experimental. To 1 ml of the original tissue extract, 300 nmol of GSH and 40 nmol of Cys were added, and diluted ten-fold.

Thiol	n★	Amount added (nmol)	Amount measured (nmol)★★	Coefficient of variation (%)	Recovery (%)
GSH	9	None	5.88 ±0.09	1.5	—
	6	3.00	8.75 ±0.11	1.3	96
Cys	9	None	0.397 ± 0.021	5.3	—
	6	0.400	0.781 ± 0.021	2.7	96

★Number of analyses.
★★Mean ± S.D.

TABLE II

GLUTATHIONE, CYSTEINE, AND TOTAL THIOLS IN RAT LIVER, LUNG, AND KIDNEY

All values are in μmol per g wet tissue.

Tissue	n★	GSH★★	Cys★★	GSH + Cys (y)★★	Total thiols (x)★★★
Liver	6	6.37 ± 1.67	0.27 ± 0.06	6.64 ± 1.64	7.02 ± 1.72
Lung	6	1.76 ± 0.38	0.07 ± 0.08	1.83 ± 0.39	1.75 ± 0.43
Kidney	6	1.59 ± 0.41	0.93 ± 0.13	2.52 ± 0.40	2.59 ± 0.34

★Number of animals.

★★Present method; mean ± S.D.

★★★Spectrophotometric method using Ellman's reagent; mean ± S.D. $y = 0.918x + 0.188$; the correlation coefficient was 0.996.

DISCUSSION

The method described here for the specific determination of some thiols in biological materials takes advantage of the high nucleophilicity of thiols; they react rapidly with *o*-quinones to form thioether derivatives of catechols (Fig. 1) [8]. The method is simple and rapid: as many as 20 samples can be processed within 60–90 min, and HPLC analysis can be made every 12 min. The method is highly sensitive: the detection limit is 10 pmol per 0.1-ml extract. In addition to GSH and Cys, other biological thiols, such as ergothioneine and homocysteine, can also be determined in tissue extracts.

In recent years, the number of reports applying HPLC to analysis of biological thiols, especially of GSH, has increased. Demaster et al. [9] described the use of a mercury electrode for the detection of GSH, Cys, and homocysteine. A similar approach using ED with a chemically modified carbon electrode has been reported [10]. Precolumn derivatization followed by HPLC has been used with 5,5′-dithiobis(2-nitrobenzoic acid) [11]; however, absorption measurement at 280 nm is not specific owing to interference of other compounds. In a method developed by Reed et al. [12], thiols are converted into S-carboxymethyl derivatives followed by reaction with 1-fluoro-2,4-dinitrobenzene to yield 2,4-dinitrophenyl derivatives, which are separated and determined by absorption measurement at 365 nm. Since this method detects all compounds with an amino group, it is even less specific. Newton et al. [13] developed a sensitive and selective precolumn derivatization procedure using Kosower's monobromobimane reagent. Some minor drawbacks of this method appear to be the necessity for gradient elution and relatively long (more than 30 min) separation times. A modification of this method, which overcomes these problems, has recently been reported [6].

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