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Note

Separation of the oxidized and reduced forms of dithiothreitol and 2-mercaptoethanol by reversed-phase high-performance liquid chromatography

Application of the method to biological extracts and to the determination of disulphides

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Dithiothreitol and 2-mercaptoethanol are routinely used to maintain the sulphydryl groups of small molecular compounds and proteins in the reduced state[1–3]. Other readily oxidized compounds such as ascorbic acid are also stabilized by these compounds[4]. However, storage or freeze–thawing cycles often lead to oxidation of dithiothreitol and 2-mercaptoethanol, and thus loss of the protective activity. This especially occurs in the presence of biological materials. Consequently, it is not known how much dithiothreitol is needed to maintain the compound(s) of interest in the reduced state over a particular time. Therefore, a rapid and accurate reference method for determining the oxidized and the reduced forms of dithiothreitol and 2-mercaptoethanol is desirable.

The oxidized and reduced forms of dithiothreitol have been determined by ultraviolet spectroscopy. The oxidized form exhibits maximum absorbance at 295 nm, whereas the reduced form has no absorption at this wavelength[1]. This method is not always applicable, owing to the presence of other ultraviolet-absorbing compounds. Recently, spectroscopic methods have been introduced for the determination of the reduced and oxidized forms of 2-mercaptoethanol[5].

In this work the separation of the oxidized and reduced forms of dithiothreitol and 2-mercaptoethanol was achieved by reversed-phase high-performance liquid chromatography (HPLC). The chromatographic system finally adopted for this separation is identical with that recently developed for the determination of free coenzyme A in biological extracts[6]. The application of the method to the determination of disulphides was also assessed.

EXPERIMENTAL

Materials

2-Mercaptoethanol, the oxidized and reduced forms of DL-dithiothreitol, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), coenzyme A and oxidized CoA (CoA–S–S–CoA) were purchased from Sigma (St. Louis, MO, U.S.A.). 2-Hydroxyethyl disulphide (98%) was supplied by Aldrich Europe (Beerse, Belgium). All other reagents were of the highest purity commercially available.

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Preparation of biological extract

A 2500 rpm supernatant was prepared from rat liver in 5 m*M* HEPES (pH 7.4 at 25°C) containing 0.25 *M* sucrose, as recently described[6] except that tartrate and EDTA were omitted from the homogenization buffer. The supernatant contained about 30 mg/ml of protein. Dithiothreitol (final concentration 14 m*M*) was added to the 2500 rpm supernatant, and 300 μl of the 2500 rpm supernatant were mixed with 300 μl of 12% perchloric acid. After centrifugation in an Eppendorf centrifuge (Model 3200), the supernatant was neutralized to pH 5 with potassium hydroxide. The precipitated potassium perchlorate was removed by centrifugation and 20-μl samples of the supernatant were injected directly into the liquid chromatograph.

Reduction of the oxidized form of coenzyme A with dithiothreitol

An incubation mixture (total volume 300 μl) of 0.035 m*M* coenzyme A in the oxidized form, 20 m*M* Tris buffer (pH 9.0) and 3 m*M* dithiothreitol was incubated at room temperature for 90 min to ensure the complete reduction of oxidized coenzyme A[7]. Samples of the mixture (20 μl) were then injected directly into the liquid chromatograph. Parallel experiments were conducted with incubations in the absence of added coenzyme A in the oxidized form, in order to determine the disulphide-independent oxidation of dithiothreitol under these conditions.

Analysis of samples by high-performance liquid chromatography

The separations were achieved at room temperature using a microparticulate reversed-phase column (25 cm × 4.6 mm I.D.) packed with Spherisorb 5 ODS of particle size 5 μm (Phase Separations, Queensferry, Great Britain). A guard column (10 cm × 2 mm I.D.) was packed with HC Pellosil-ODS pellicular silica (Whatman, Clifton, NJ, U.S.A.).

A Model 6000A solvent delivery pump (Waters Assoc., Milford, MA, U.S.A.) was used. The eluate was monitored at 254 nm with a SpectroMonitor Model 1203 instrument (Laboratory Data Control, Riviera Beach, FL, U.S.A.). The samples were introduced into the chromatographic system with a Model 1725 injector (Rheodyne,Berkeley, CA, U.S.A.). The chromatograph was provided with a W + W Model 1107 recorder (W + W Electronic, Basle, Switzerland).

The solvent was 220 m*M* potassium phosphate (pH 4.0)–*ca.* 12% methanol–0.05% (v/v) thiodiglycol at a flow-rate of 1 ml/min (1700 p.s.i.). The phosphate buffer and methanol were degassed separately for 15 min before mixing and final adjustment of the pH.

Protein determination

The protein was measured using the Folin method with bovine serum albumin as a standard[8].

RESULTS AND DISCUSSION

Chromatography of standards

Fig. 1 shows the retention times of the oxidized and reduced forms of 2-mercaptoethanol and dithiothreitol.

The standards were dissolved in water immediately before analysis. Note that

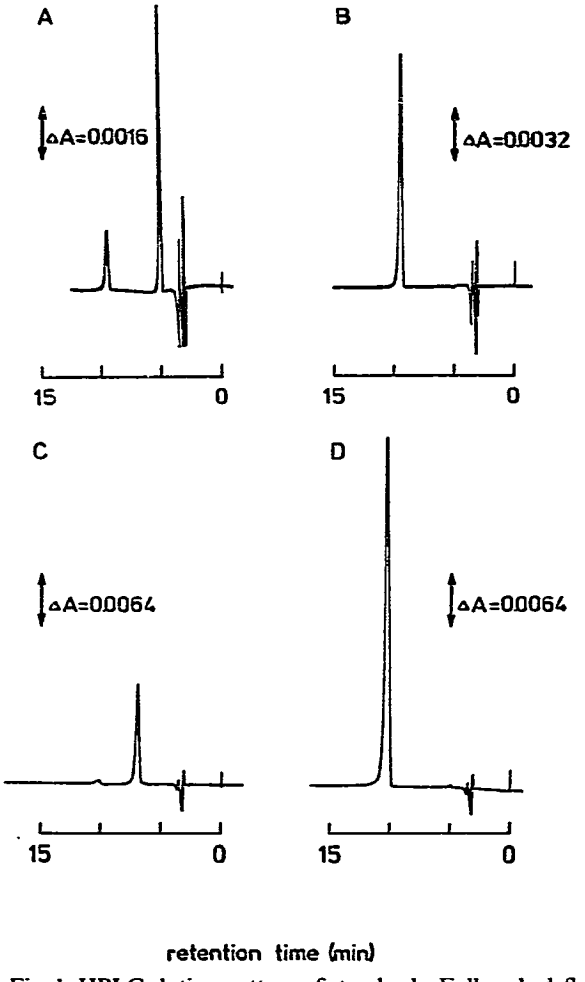

Fig. 1. HPLC elution pattern of standards. Full-scale deflection is 10 times the absorbance units indicated. Volumes of 20 μl of the standard solutions were injected into the chromatograph. A, 2-Mercaptoethanol (10 m*M*). Retention time 5.2 min. B, Oxidized form of 2-mercaptoethanol (2-hydroxyethyl disulphide) (0.5 m*M*). Retention time 9.6 min. C, Dithiothreitol (10 m*M*). Retention time 7 min. D, Oxidized form of dithiothreitol (10 m*M*). Retention time 10.4 min.

the stock of 2-mercaptoethanol contains some 2-hydroxyethyl disulphide (Fig. 1A), and the much higher molar absorptivity of 2-hydroxyethyl disulphide compared with 2-mercaptoethanol (Fig. 1A and B).

In accordance with previous experiments[9], complete oxidation of 2-mercaptoethanol and dithiothreitol after treatment with hydrogen peroxide was observed in this system (data not shown).

Application of the method to the determination of disulphides

Oxidized coenzyme A was used as a model compound. The conditions for the complete reduction of this compound were specified previously[7]. Whereas the free

coenzyme A is readily quantitated in the present chromatographic system[6], the oxidized form of coenzyme A is retained on the column (data not shown). It should be possible to determine the amount of disulphides present in a solution by measuring the increase in the peak corresponding to the oxidized form of dithiothreitol after appropriate incubation at alkaline pH. As the autoxidation of dithiothreitol is also significantly increased at alkaline pH, accurate timing of the blank measurement is essential, in order to determine the disulphide-dependent oxidation of dithiothreitol in the experiment.

This approach was tested with an incubation mixture of 0.035 m*M* coenzyme A in the oxidized form together with 20 m*M* Tris buffer (pH 9.0) and 3 m*M* dithiothreitol. After an incubation period of 90 min, the mixtures exhibited a chromatographic elution profile as shown in Fig. 2A. Free coenzyme A was formed, corresponding to a concentration of 0.068 m*M*, *i.e.*, 97% of the expected value. The disulphide-dependent oxidation of dithiothreitol was found to be slightly higher than expected in this experiment, *i.e.*, corresponding to a concentration of 0.05 m*M*. There was a linear relationship between the amount of oxidized dithiothreitol and the peak height in the chromatogram (data not shown). For three separate experiments, the disulphide-

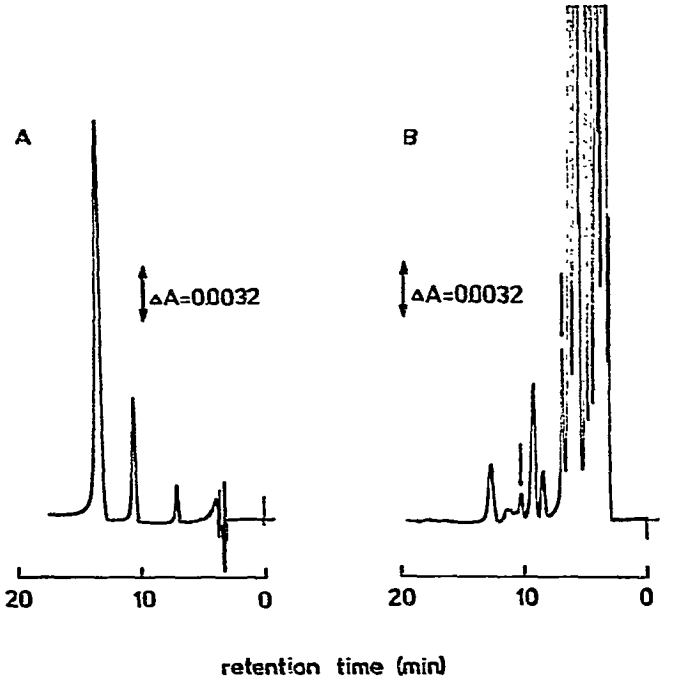

Fig. 2. A, HPLC elution pattern of a mixture of 0.035 m*M* oxidized coenzyme A, 20 m*M* Tris buffer (pH 9.0) and 3 m*M* dithiothreitol. The compounds were incubated for 90 min before analysis. Volumes of 20 μl of the mixture were injected into the chromatograph. Full-scale deflection was 0.032 absorbance unit. The retention times for coenzyme A, the oxidized and reduced forms of dithiothreitol were 13.3, 10.4 and 7 min, respectively. B, HPLC elution pattern of an extract of the 2500 rpm supernatant of rat liver. Dithiothreitol was added to a final concentration of 7 m*M*. Volumes of 20 μl of the extract were injected into the chromatograph. Full-scale deflection was 0.032 absorbance unit. The arrows indicate the oxidized and reduced form of dithiothreitol, with retention times of 10.4 and 7 min, respectively.

dependent oxidation of dithiothreitol occurred according to a concentration (mean) of 0.054 m*M* ($n = 3$), with a standard deviation of 0.01 m*M*. Long incubation times are needed the order to reduce oxidized coenzyme A, whereas 5–10 min are adequate for the reduction of other disulphides and the disulphide bonds in proteins[7]. The precision of the assay of disulphides is increased significantly when it is possible to perform the reduction of disulphides at lower pH, *e.g.*, 8, and in shorter time periods[10].

The incubation mixture used here for the determination of disulphides is similar to that published by Zahler and Cleland[7]. However, they determined the resulting monothiols in a reaction with DTNB in the presence of arsenite, which forms a tight complex with the remaining reduced dithiothreitol in the assay mixture, and thus inhibits the reaction of DTNB with the excess of dithiothreitol used as a reductant.

Application of the method to biological extracts

Fig. 2B shows the chromatogram of an extract prepared from the 2500 rpm supernatant with 7 m*M* dithiothreitol added to the extract. Both the reduced and oxidized forms of dithiothreitol are easily determined.

The identity and purity of the peak corresponding to the reduced form of dithiothreitol were confirmed by reaction with hydrogen peroxide[9]. After this treatment, all of the dithiothreitol was present in the oxidized form (data not shown).

The 2500 rpm supernatant was concentrated in this experiment, in order to indicate the capacity of the system. By appropriate dilution it is possible to obtain baseline separation of the reduced form of dithiothreitol.

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