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## Note

# Simplified monitoring of reduced and oxidized dithiothreitol using high-performance liquid chromatography

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Dithiothreitol (DTT) is a well known sulfhydryl reagent used to protect protein sulfhydryls in a variety of biochemical applications [1]. The radioprotective action of the compound has received significant attention for a number of years, and the radioprotective mechanisms of DTT have been examined in both chemical [2,3] and biological systems [4,5]. Recently, DTT has been used in studies on heat cell killing [6] in order to examine the role of non-protein sulfhydryl groups in the protection and/or sensitization of cells to hyperthermia treatment. The latter application is a direct result of recent interest in hyperthermia as a cancer treatment modality.

It has been shown that DTT undergoes autoxidation in solution [7] and that iron-catalyzed reactions enhance DTT oxidation in biological buffers [8]. Oxidation mechanisms producing active oxygen species have been proposed as being central to mechanisms of DTT cell toxicity in heated cells [9]. In our own work, we have shown significant yields of thymine glycol, a major product of OH and thymine reactions, result from the iron-catalyzed oxidation of DTT in solutions containing thymine [10]. Our interest in thymine–DTT reactions led to development of the chromatography assay presented here.

Since DTT readily undergoes autoxidation under conditions used in biological and biochemical studies [8], it is important to monitor the reduced and oxidized forms during the course of an experiment. Previous methods used to monitor DTT in solutions have included, for example, multi-wavelength UV spectrophotometry and colorimetry using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as a color reagent [11]. Each of these methods involves the monitoring of only one form of DTT and accounts for the remaining DTT by inference. At least one

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prior HPLC assay has been presented [12]; however, that procedure was somewhat more complicated than the procedure presented here.

The objective of the present report is to present a simplified HPLC assay for the simultaneous monitoring of reduced and oxidized DTT in solutions of biological interest. The method offers the distinct advantages of mass accountability (both reduced and oxidized DTT are monitored in the same analysis) and of simplicity, since a simple water flow gradient is used to elute both reduced and oxidized DTT.

## EXPERIMENTAL

### *Chemicals*

DTT, oxidized DTT (*trans*-4,5-dihydroxy-1,2-dithiane) and DTNB were obtained from Sigma (St. Louis, MO, U.S.A.). Potassium and sodium phosphates were either Mallinckrodt or Fisher reagent-grade chemicals and were used without further purification. High-purity water for high-performance liquid chromatography (HPLC) and the preparation of reagents was distilled, filtered through activated charcoal and two deionizing beds and then redistilled before use. Other solvents were of HPLC-grade purity. The tissue culture media used in this study were Ham's F12 medium (K.C. Biological, Lenexa, KS, U.S.A.) and Ham's F12 supplemented with 5% (v/v) fetal bovine serum (FBS) (K.C. Biological) where appropriate.

### *Chemical assays*

Two methods in addition to HPLC were used to measure DTT in solution. These methods were UV spectrophotometry and the DTNB colorimetric titration of sulfhydryls [11]. UV spectra were recorded with a Beckman Model 35 spectrophotometer. Data for the absorptivities of reduced and oxidized DTT were recorded by measuring the optical density at 210 nm of standard solutions containing 0.05–1.0 m*M* of the appropriate compound. These measurements were made in both 10 m*M* phosphate buffer (pH 7.0) and in high-purity water.

The colorimetric procedure was as described in Ellman [11], with the exceptions that the reagent volumes were proportionately reduced to a total volume of 1 ml and DTT was used in place of reduced glutathione.

### *Oxidation in phosphate buffer*

In order to monitor the oxidation of DTT in phosphate buffer, a solution of DTT was prepared by rapidly dissolving DTT in pre-equilibrated (45°C) and filter-sterilized phosphate buffer (sodium phosphates, 10 m*M*, pH 7.0). The reaction was allowed to proceed in the dark and in a 45°C water bath. At prescribed time intervals, 1-ml aliquots were withdrawn and immediately cooled in an ice bath. The samples were frozen (−20°C) before HPLC analysis.

### *Oxidation in tissue culture media*

Tissue culture media were mixed with DTT to produce a 2 m*M* solution before incubating the tubes at 37°C for up to 6 h. At each given time point, a sample of

medium was removed, and cold TCA was added to make a 5% solution. The resulting precipitate was centrifuged at 10 000 *g* for 10 min, and the supernatant was pipetted from the centrifuge tube. The supernatant was adjusted to pH 6.8–7.0 before injection into the HPLC system. When necessary, samples were stored frozen (−20°C) before analysis of 50-$\mu$l aliquots using HPLC.

*High-performance liquid chromatography*

The apparatus used included a binary gradient system (Beckman) coupled to a variable-wavelength UV monitor (Beckman Model 164) set at 210 nm. Chromatogram data analyses were routinely carried out using a data integrator (Spectra-Physics Model 4270). The columns used were reversed-phase, 5-$\mu$m spherical $C_{18}$ (ASI) and were 250 mm×4.8 mm O.D. An inlet filter was used; however, guard columns were not routinely used for the assay reported here.

Data were reported in relative area units or, where appropriate, the concentrations of oxidized and reduced DTT in the samples were calculated using multipoint (four to six points from 0.05 to 2 m*M*) standard calibration curves. Triplicate injections (50 $\mu$l) of the standards were made before the data were tabulated, and the least-squares regression fit was calculated. Minimum detection levels (MDL) were calculated using a linear calibration method assuming a 0.005 A.U. level for spectrophotometric data and 1000 area units for the HPLC data [13]. Assignment of chromatogram peaks were made using co-chromatography with authentic standards.

For elution of the compounds, gradient system A was a water flow gradient, consisting of 0.5 ml $min^{-1}$ from 0 to 10 min, 0.5–1.0 ml $min^{-1}$ on a linear ramp from 10 to 20 min and 1.0 ml $min^{-1}$ from 20 to 35 min.

## RESULTS

Molar absorptivities and relative sensitivities measured at 210 nm for oxidized and reduced forms of DTT are given in Table I. The results in Table I show that the MDLs for the HPLC assay are nearly as low as that for Ellman's procedure

TABLE I

CALIBRATION RESULTS FOR SPECTROPHOTOMETRIC AND HPLC ASSAYS OF DITHIOTHREITOL

| Method | Slope* (molar absorptivities) | Coefficient of variation (%) | Wavelength (nm) | Detection level** ($\mu M$) |
|---|---|---|---|---|
| HPLC (reduced DTT) | 5.973 | 7.32 | 210 | 19.2 (0.960 nmol) |
| HPLC (oxidized DTT) | 7.320 | 2.19 | 210 | 5.45 (0.273 nmol) |
| UV spectrophotometry | 1.452 | 1.45 | 210 | 86.0 |
| Ellman's assay [11] | 25.78 | 3.79 | 412 | 5.0 |

*Units are $\times 10^5$ area units $mM^{-1}$ for the HPLC assays and $cm^{-1}$ $mM^{-1}$ for the UV and Ellman's procedures.

**Detection levels were based on linear calibrations [12] and signal-to-noise ratios ≥6.

[11]. The fact that a greater MDL was found for the reduced versus the oxidized form of DTT reflects the greater noise in the reduced DTT samples: some oxidation can occur during analysis. Thus, optimum reliability of the assay arises from analysis of both oxidized and reduced DTT in a single analysis.

The UV absorption spectra showed absorption maxima near 200 nm for both forms of DTT and a second maximum at 195 nm for the oxidized form. The selection of 210 nm was made in order to optimally detect pyrimidine oxidation products; however, this wavelength has proven useful for monitoring DTT, also.

Fig. 1 shows a typical chromatogram of mixed oxidized and reduced DTT on gradient system A using UV detection at 210 nm. The ratio of the slopes (oxidized/reduced) is $1.30 \pm 0.117$, and this ratio is not statistically different ($t$-test, $p < 0.01$) from the ratio of the molar extinction coefficients measured at 210 nm using the spectrophotometer, $1.27 \pm 0.0900$. The absence of a statistically significant difference is an expected result since both systems, the spectrophotometer and the UV monitor, measure UV absorption at 210 nm.

Results of measurements on DTT oxidation in sodium phosphate buffer at 45 °C are shown in Fig. 2. Both forms of DTT were monitored and the sum of the peak areas were tabulated and plotted in order to show that the total mass of DTT in the sample remained constant. The lower panel of Fig. 2 shows that the monitoring of DTT can start with "old" DTT, i.e., a DTT solution that had been stored at refrigerator temperature for several weeks before the experiment. Thus, stocks of DTT, prepared in advance, can readily be checked for age before they are used in an experiment.

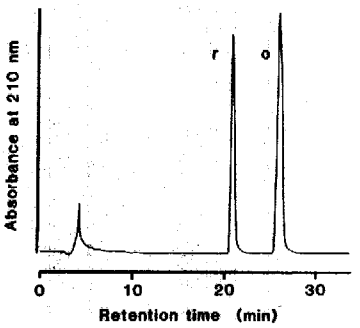



Fig. 1. Typical chromatogram of dithiothreitol in phosphate buffer (pH 7.0, 10 m$M$). The reduced (r) and oxidized forms (o) elute with capacity factors ($k'$) of 3.9 and 5.1, respectively.

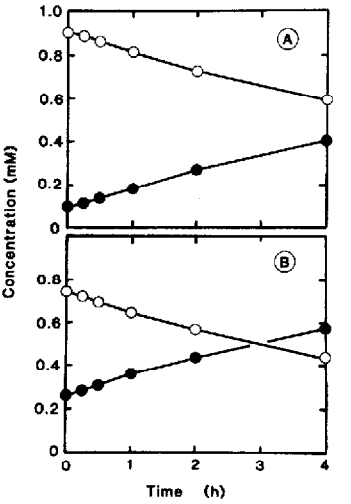
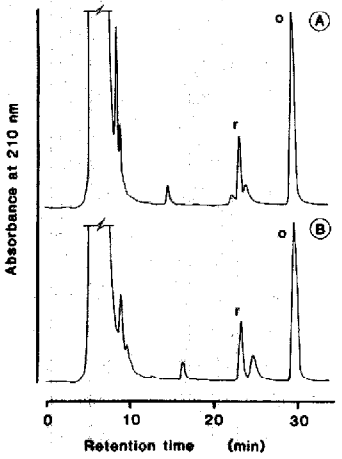



Fig. 2. Measurement of dithiothreitol oxidation in 10 m$M$ phosphate-buffered solution at 45 °C and pH 7.0. Oxidized DTT (●) increases and reduced DTT (○) decreases with time in the solution. Panel A shows the oxidation of a fresh solution of DTT; panel B shows DTT oxidation in an old (refrigerated for several weeks) solution. In either case, the sum of the two components (the total mass of DTT in the solution) remained constant.



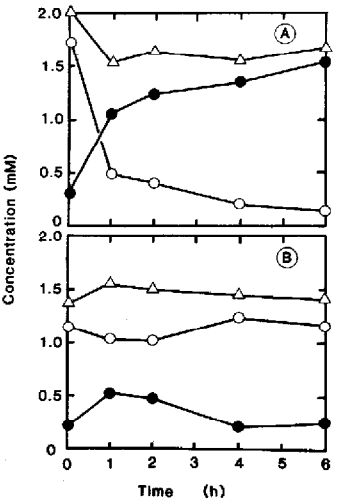




Fig. 3. Typical chromatograms of DTT from tissue culture medium with (A) and without (B) added fetal bovine serum. Both serum-containing and serum-free samples were treated with TCA precipitation and neutralized with sodium hydroxide before analysis.

Fig. 4. Typical measured oxidation of DTT in tissue culture medium at 37°C. A single experiment showing medium without serum (A) and medium containing serum (B). The increase in oxidized DTT (●) and corresponding decrease in reduced DTT (○) are shown. The total DTT in the sample (△) is also shown. The small fall in oxidized DTT (B) and the apparent drop in total DTT (A) are likely due to statistical variation.

The chromatographic system used here can be used to monitor the oxidation of DTT in complex biological mixtures such as tissue culture media. Chromatograms of tissue culture media containing DTT are shown in Fig. 3. Components of the media elute close to the reduced DTT peak; however, electronic data integration can be used to sort the peaks and produce quantitative data (Fig. 4). The technique was useful in both serum-free and serum-containing (5%, v/v) medium as shown in Fig. 4. The data in Fig. 4 also show that some of the DTT was lost in the serum-containing samples, presumably as a result of reaction with the protein–TCA precipitate. Both samples initially contained 2 m*M* DTT; however, the serum-containing samples have only 1.5 m*M* total DTT in the supernatant after precipitation with TCA. Since the loss of DTT in the precipitate was reproducible, it seems unlikely that it would present a significant analytical problem. The small, but apparent, rise in reduced DTT at 2 h could have resulted from normal statistical variation or from components in the complex media such as the amines and (other) thiol-containing compounds.

## DISCUSSION

The results presented here show that a simple and efficient HPLC assay can be used to monitor the oxidation of DTT in solutions of biological relevance. The system has been optimized for the simultaneous measurement of thymine oxi-

dation products, thymine and both forms of DTT in a single, 40-min chromatographic analysis. Although originally developed for biochemical studies, the system was immediately adaptable to measurements of DTT in both serum-supplemented and serum-free tissue culture media. Preliminary results show that the present assay can also be used to measure DTT oxidation in bacteria cultures (not shown). In this case, however, compounds appear in late log-phase cultures that tend to overlap with the reduced DTT peak. Although the overlapping peaks can be resolved using lower flow-rates, it is preferable to focus the analysis on oxidized DTT in complex biological mixtures. In some cases, secondary assays should be used to substantiate the procedure before it is adopted for routine use.

The HPLC system shown here differs from previous assays in several ways. Generalized thiol assays have used S-carboxymethyl derivatization prior to chromatography [14] or solid-phase derivatization of thiols using specially prepared columns [15]. Another, highly sensitive procedure using post-column reactions was used to measure 2-pmol levels of glutathione [16]. In reported literature concerning the HPLC of DTT, complicated mobile phases [12] and thiol-stabilizing reagents were used [17]. Whereas the present system has somewhat less sensitivity than some of the afore-mentioned assays, its advantage stems from the use of simple, commercially available columns and HPLC-grade water only for the mobile phase.

The present system has improved detectability over the previous DTT systems [12,17] because oxidized and reduced DTT are readily detectable in the 210–220 nm experiment. This feature would be particularly useful in cases where one form of the compound might be metabolized or disappear via reactions with components in the system tested.

It is likely that slight modifications of the present assay system will enable measurement of oxidized and reduced forms of other thiols. We have tested $\beta$-mercaptoethanol with equivalent results. Preliminary results have shown that glutathione can be monitored using this system.

## ACKNOWLEDGEMENTS

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