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Journal of Chromatography B, 770 (2002) 237–241

JOURNAL OF CHROMATOGRAPHY B

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Short communication

Monitoring of dithiothreitol clearance by means of micellar electrokinetic chromatography

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Abstract

A new method for specific determination of dithiothreitol (DTT) using micellar electrokinetic chromatography and on-column reaction with reactive disulfide–2,2′-dipyridyldisulfide is described. DTT in this reaction is quantitatively transformed into a mixed disulfide concomitantly with formation of equimolar amount of the 2-thiopyridone that is further separated by micellar electrokinetic chromatography and determined spectrophotometrically at 343 nm. The concentration of DTT is thus estimated indirectly from the result of 2-thiopyridone determination. The linear detection range for concentration versus peak area for the assay is from 0.05 to 2.5 m*M* (correlation coefficient 0.993) with a detection limit of 2.5 μ*M*. The inter-day reproducibility of the peak area was 1.35% and the inter-day reproducibility of the migration time 0.56%. The method can be applied for DTT monitoring both in chemical and biological systems.

Keywords: Dithiothreitol

1. Introduction

Dithiothreitol (DTT) synthesised in 1949 by Evans et al. [1] is a well known sulfhydryl reagent. Since 1964 when Cleland described its function [2] it was used to protect the sulfhydryl groups of small molecular compounds as well as proteins in a variety of biochemical applications. The radioprotective action of the compound has received significant attention for a number of years and its mechanisms have been examined in chemical [3–5] and biological [6–9] systems. Moreover DTT has been applied in studies on heat cell killing in hyperthermia as a cancer treatment modality [10]. DTT is also routinely used to solubilize recombinant proteins from inclusion bodies [11].

In view of the fact that DTT readily undergoes autooxidation in solution [12,13] the monitoring of the DTT state during biochemical and chemical experiments is an important task. Previous methods used to determine DTT in solutions have included spectrophotometry [14] and HPLC with UV spectrophotometric [15,16] and fluorescence [17] detection. Recently a method describing the usage of the on-column reaction of 2,2′-dipyridyldisulfide (DPDS) for the specific detection of thiols (RSH) during micellar electrokinetic chromatography (MEKC) (Fig. 1) has been published [18]. The purpose of this paper is to demonstrate its applicability for monitoring of DTT clearance during different types of

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1570-0232/02/$ – see front matter
PII: S0378-4347(01)00526-6

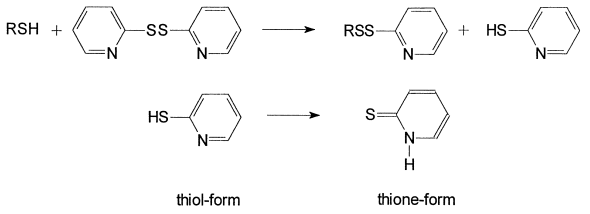

Fig. 1. Reaction of 2,2′-dipyridyldisulfide with thiol–RSH.

experiments. The whole procedure is simple and rapid and can be automated easily.

2. Experimental

2.1. Materials and reagents

DPDS, DTT, glutathione (GSH), homocysteine (HCy) and cysteine (Cys) were obtained from Sigma (St. Louis, MO, USA). All other chemicals and solvents were of analytical reagent grade, supplied from Fluka (Buchs, Switzerland). A 50 m*M* phosphate buffer (pH 7.5) was prepared by mixing of 50 m*M* solution of monosodium phosphate and 50 m*M* solution of disodium phosphate. Standards of thiols were prepared in 50 m*M* phosphate buffer (pH 7.5) containing 1 m*M* EDTA fresh each day. DPDS was prepared as 0.1 *M* stock solution in acetonitrile that was diluted twenty times with the background electrolyte (BGE) before the use. All solutions were prepared with Milli-Q Academic water (Millipore, Milford, WA, USA) and filtered through a 0.45 μm membrane filter.

2.2. Instrumentation

A Hewlett-Packard ^{3D}Capillary Electrophoresis System (Waldbronn, Germany) with a diode-array UV–Vis detector was used to carry out all separations. Data were collected on an HP Vectra VL 5 166 MHz personal computer using the Hewlett-Packard ^{3D}CE ChemStation software. A Hewlett-Packard extended light path capillary, 58.0 cm (49.5 cm effective length)×50 μm I.D., was used for all analyses. The capillary was washed with 0.1 *M* NaOH for 1 min, deionized water for 1 min and the BGE for 3 min before each run and washed with deionized water for 1 min after each run. The on-column detection reaction was performed by injection: 5 m*M* DPDS −50 mbar for 20 s; BGE −50 mbar for 360 s; sample −50 mbar for 2 s; BGE-50 mbar for 2 s in the described order. Separations were performed at 28 kV (positive polarity). Samples were detected by means of a diode-array detection system at 200 and 343 nm with a bandwidth of 10 nm. Spectra were also collected during the runs for peak identification. The purity of the peaks was checked by the spectral utility of the ChemStation software. In addition, spiking samples with the standard further supported the identity of the DTT peak.

2.3. Sample preparation

2.3.1. Liver homogenate

Pig liver (1 g) was partially thawed and homogenized with 10 ml of 50 m*M* phosphate buffer (pH 7.5) containing 1 m*M* EDTA. The homogenate was centrifuged at 13 500 rpm for 30 min. A 1.0 ml volume of 0.1 *M* trichloroacetic acid solution containing 1 m*M* EDTA was added to 1.0 ml of the liver homogenate spiked with 1 m*M* DTT. The sample was vortex-mixed for 5 min and centrifuged at 13 500 rpm for 5 min to remove protein precipitate. The supernatant was filtered through a 0.45-μm filter and used for analysis.

2.3.2. Iron-catalyzed oxidation

A 100 or 200 μl volume of 2.5 m*M* $K_3Fe(CN)_6$ was added to 0.9 ml or 0.8 ml, respectively, of 50 m*M* phosphate buffer (pH 7.5) containing 1 m*M* DTT. A 200 μl volume of reaction mixture was withdrawn after 5 min standing at laboratory temperature and used for MEKC analyses.

3. Results and discussion

The principle of on-column detection reaction as carried out in this study is schematically described in Fig. 2 [18]. DPDS and a sample of DTT were injected consecutively into the capillary as two discrete plugs separated with a short plug of BGE (Fig. 2A). Due to the differences in the mobilities of the DPDS and DTT, the zone of DTT approaches the zone of DPDS and on-column mixing and reaction

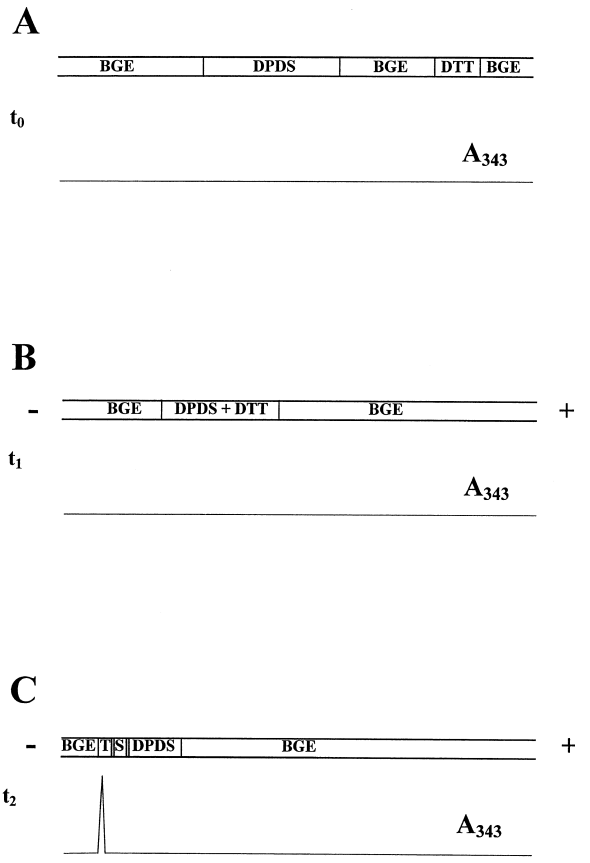

Fig. 2. Schematic representation of on-column detection procedure. BGE, background electrolyte; DPDS, 2,2′-dipyridyldisulfide; DTT, dithiothreitol; T, 2-thiopyridone; S, corresponding mixed disulfide.

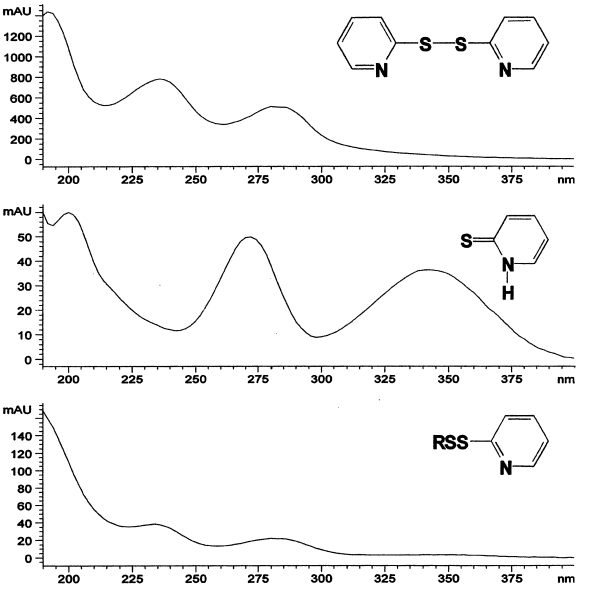

Fig. 3. Spectra of DPDS and its derivatives.

occur (Fig. 2B). DTT is thus quantitatively transformed into a mixed disulfide concomitantly with formation of the equimolar amount of 2-thiopyridone which is further separated by MEKC and detected spectrophotometrically at 343 nm (Fig. 2C). The reaction is rapid and quantitative at room temperature because the corresponding thiol form of 2-thiopyridone is stabilized by thiol–thione tautomerism. This fact also causes a large shift of the absorption maximum toward longer wavelengths resulting in higher specificity of detection (Fig. 3). Although this system has been demonstrated specifically for the determination of GSH, we proposed its application for monitoring of DTT clearance during chemical and biochemical experiments. Only slight modifications of sampling parameters were necessary to get suitable resolution between DTT and other thiols (see Experimental).

Fig. 4A shows an electropherogram of 1 m*M* DTT standard, the peak at 3.6 min is 2-thiopyridone with absorption maximum at 343 nm formed in reaction of DPDS with DTT. The identity of DTT was also confirmed by reaction with hydrogen peroxide [19]. After this treatment, all DTT was oxidized (Fig. 4B). A typical electropherogram of the mixture of common endogenous thiols is shown in Fig. 5. A good resolution of the peak corresponding to DTT from the peaks of the other endogenous thiols was achieved.

The quantitative parameters of the developed method were estimated with DTT standards. The calibration graph for the peak area was linear over the range of 0.05–2.5 m*M* of DTT with correlation coefficient better than 0.993. The detection limit was ~2.5 μ*M* at a signal-to-noise ratio of 3. The sensitivity of the method is approximately ten times higher than the HPLC method using direct UV detection at a low wavelength of 210 nm [16] where many of endogenous compounds interfere. Recently a HPLC method using precolumn fluorescent derivatization with a lower concentration detection limit has also been reported [17]; however, it needs a larger amount of the sample for derivatization.

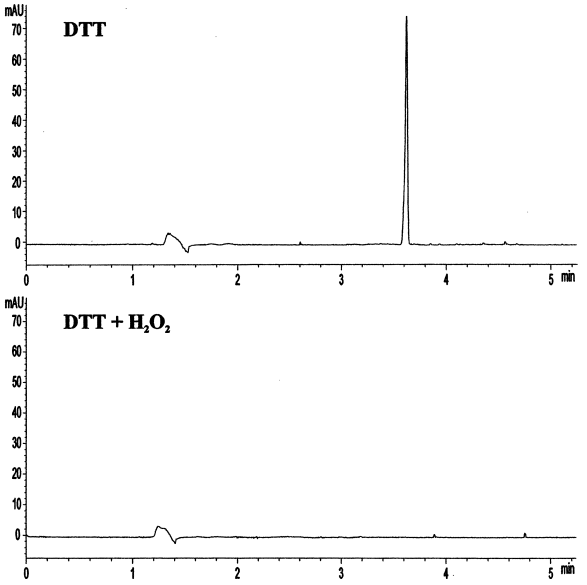

Fig. 4. Electropherogram of 1 m*M* DTT standard before (A) and after the treatment with hydrogen peroxide (B). A 10 μl volume of 30% hydrogen peroxide was added to 1 ml of 1 m*M* DTT in 50 m*M* phosphate buffer (pH 7.5). Separation conditions: BGE, 50 m*M* SDS in 50 m*M* phosphate buffer (pH 7.5); separation voltage, 28 kV (positive polarity); temperature of capillary, 20°C; detection, 343 nm; sampling, 5 m*M* DPDS −50 mbar for 20 s; BGE −50 mbar for 360 s; sample −50 mbar for 2 s; BGE −50 mbar for 2 s.

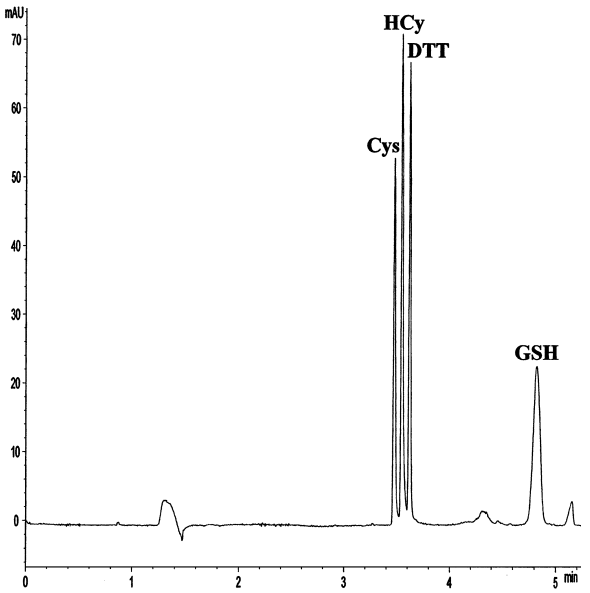

Fig. 5. Electropherogram of the mixture of thiols. Cys, cysteine (1 m*M*); HCy, homocysteine (1 m*M*); DTT, dithiothreitol (1 m*M*) and GSH, glutathione (2 m*M*). Other conditions as in Fig. 4.

Additionally a more expensive derivatization reagent (monobromobimane) was used — in contrast with the relatively low-priced DPDS. The results of replicated analyses ($n=10$) showed good reproducibility obtained for peak area (<1.4%) and excellent reproducibility obtained for migration time (<0.6%). The recoveries were tested by the analyses ($n=10$) of the samples spiked with known amounts of DTT. The recovery of DTT by the MEKC method described was estimated to be between 94 and 107%.

In order to demonstrate the application of the developed method for determination of DTT in a biological matrix, 1 m*M* DTT was added to a liver extract. Representative electropherograms are shown in Fig. 6. This figure also shows the specificity of the detection at 343 nm in comparison with the same electropherogram detected at 200 nm. As a second application we used this method for monitoring of iron-catalyzed oxidation of DTT. Although DTT is the most common protective agent for sulfhydryl

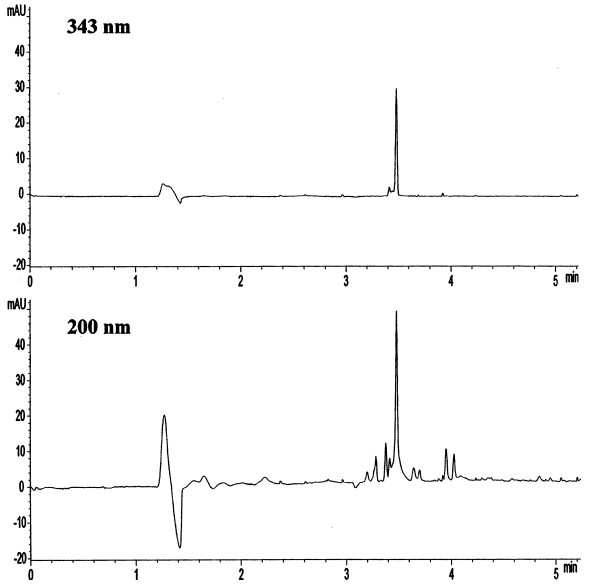

Fig. 6. Electropherograms of liver extract spiked with 1 m*M* DTT detected at 343 and 200 nm. The extract was prepared as described in Experimental; other conditions as in Fig. 4.

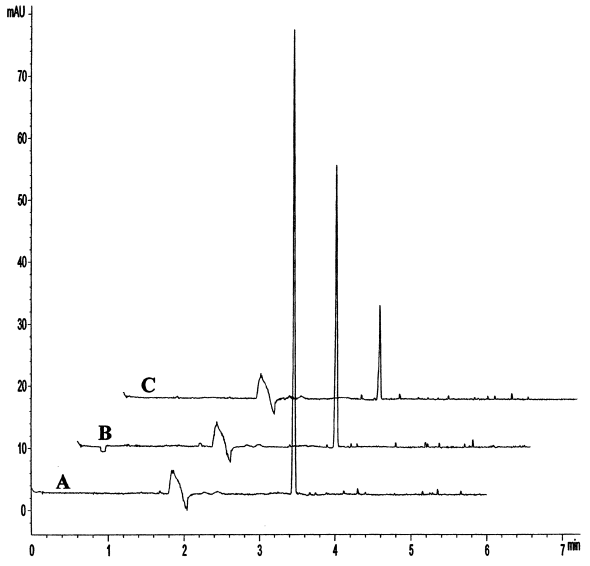

Fig. 7. Electropherograms from the oxidation of 1 m*M* DTT in presence of 0 (A), 250 μ*M* (B) and 500 μ*M* $K_3Fe(CN)_6$ as described in Experimental; other conditions as in Fig. 4.

group, it can induce oxidative damage of proteins [20], lipids [21] and DNA [22,23] in the presence of transition metals and O_2. The overlay electropherograms show the process of DTT oxidation (Fig. 7). These results were in agreement with previously published data [24].

4. Conclusion

A new method for specific determination of dithiothreitol using MEKC and an on-column reaction with 2,2′-dipyridyldisulfide has been developed. The method is relatively rapid and simple and offers a detection limit around 2.5 μ*M*. Moreover, the whole procedure can be easily automated.

Acknowledgements

This research was supported by grant 602/2001 from the Czech Ministry of Education.

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