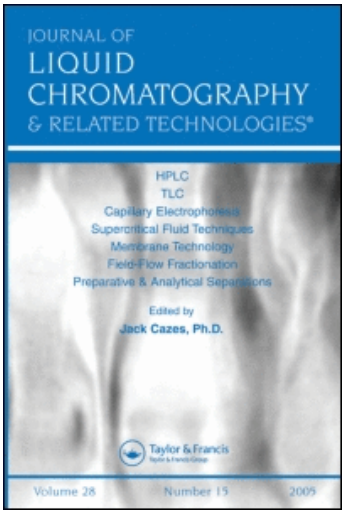

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Acidic Dithiothreitol Reduction on Disulfide Bonds using Cation-Exchange Chromatography

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Abstract: Dithiothreitol has been utilized to reduce the disulfide bonds between homocysteine and other thiols in total plasma homocysteine determination. The completeness of the reduction of disulfides into thiols is a prerequisite for accurate quantitation of total plasma homocysteine. In order to evaluate the effectiveness of dithiothreitol reduction on disulfide bonds, the acidic mixture of cystine and homocystine is reduced under controlled parameters including dithiothreitol concentration, reduction temperature, and reaction time. The reaction mixture is analyzed by cation-exchange chromatography. It is concluded that 1.0 M dithiothreitol concentration, 70°C reduction temperature, and 1 hour reaction time are required to convert more than 96% of the disulfides of cystine and homocystine to their thiols in acidic medium. This study also proves that dithiothreitol is able to keep cysteine and homocysteine from being reoxidized back to their disulfides.

Keywords: Dithiothreitol reduction, Disulfide bonds, Cation-exchange chromatography

INTRODUCTION

The total homocysteine content of plasma has been linked to the risk of occlusive vascular disease.[1–4] Homocysteine is present in plasma as non-protein-bound and protein bound forms. Non-protein bound homocysteines exist in multiple forms including homocysteine (monomer), homocysteine thiolactone (ring), homocystine (disulfide), and cysteine-homocysteine

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mixed disulfide.[5] Protein bound homocysteine is present via disulfide bonds between homocysteine and the thiols of proteins. It has been reported that as much as 85% of homocysteine is protein bound.[6] In order to determine total homocysteine, plasma must be reduced to break disulfide chains, and plasma proteins have to be precipitated and removed before analysis.

Various compounds have been used to reduce the disulfide bonds between homocysteine and other thiols in the determination of total plasma homocysteine. Potassium or sodium borohydride is a potent reductant; however, gas formation and foaming during reduction may impose practical difficulty in sample preparation.[7,8] Mercaptoethanol has been used to release homocysteine from disulfides, but it is an irritant with a very unpleasant odor.[9] Tri-n-butylphosphine does not form gas during reaction; however, it will consume a fluorogenic reagent.[10] Dithiothreitol (DTT), which is a compound used to reduce disulfide bonds and to retain the monothiols in the reduced state,[11] has been utilized to break disulfide linkage in total plasma homocysteine determination.[12,13] Dithiothreitol is also used for many other biochemical applications due to its high solubility in water. Therefore, DTT is chosen in this research.

The author has used DTT to reduce disulfide bonds in total plasma homocysteine determination.[14] The irregular results of measuring homocysteine in DTT-reduced plasma samples are experienced in cation-exchange chromatography analysis. This is mainly caused by the incomplete conversion of disulfides to thiols during DTT reduction. It is desired to study the effectiveness of DTT reduction on disulfide bonds in an acidic solution, which is a required condition for cation-exchange separation. Thus, the optimal parameters for completely reducing disulfides to thiols can be determined. Two available disulfides, cystine and homocystine, are used in this research to quantify the conversion of disulfides in DTT reduction.

An acidic mixture of cystine and homocystine with known concentrations is used to react with the DTT solutions of various concentrations at different temperatures and reaction times. Serine, which does not react with DTT, is added to the disulfide mixture as an internal standard. The reaction mixtures are separated and analyzed by a cation-exchange chromatograph with post-column ninhydrin derivatization and spectroscopic detection. The percentage of disulfide conversion (PDC) is obtained using the peak area ratio of the disulfide and the internal standard in chromatograms. The equation for calculating the PDC is illustrated in the section of results and discussion.

EXPERIMENTAL

Chemicals and Reagents

The chemicals, including DL-cystine, DL-homocystine, DL-serine, and dithiothreitol were obtained from Aldrich-Sigma (St. Louis, Missouri). A testing

solution of cystine, homocystine, and serine, with a concentration of 250 μM for each compound, was made by completely dissolving these compounds in 25 mL 2 M hydrochloric acid, and adding distilled water to a total volume of 250 mL. DTT solutions of 0.2 M, 0.5 M, and 1.0 M were prepared in distilled water.

The eluents for cation-exchange chromatography were lithium citrate buffers including Li280, Li750, and RG003 regenerant. These were purchased from Pickering Laboratories (Mountain View, California). The post-column derivatizing reagent, ninhydrin, was also obtained from Pickering Laboratories.

A series of reaction mixtures were prepared by mixing 0.250 mL testing solution with 0.250 mL DTT solutions of different concentrations (0.2 M, 0.5 M, and 1.0 M), respectively. After vortex mixing the reaction mixtures for 2 minutes, they were incubated at four different temperatures (25°C, 40°C, 55°C, and 70°C) for two different time durations (30 minutes and 1 hour), respectively. The reaction mixtures were cooled to room temperature. The pH of the reaction mixtures were monitored to be around 2.5, which was a proper pH for cation-exchange separation. The reaction mixtures were then injected into a liquid chromatograph for analysis.

Chromatographic System

The system used to analyze samples was a system, which combined a HP 1050 liquid chromatograph (Hewlett-Packard Corporation, Palo Alto, California) and a Pickering PCX3100 post-column derivatization unit (Pickering Laboratories). The detector was a HP 1050 diode array detector. The samples were injected into the HPLC through a Rheodyne 7125 injector with a sample loop of 25 μL. The system was controlled and run by the HP Chemstation (3.1 Version). The chromatographic parameters were as follows: A portion of 30 μL reduced sample was manually injected into the chromatograph. The sample was carried through a cation-exchange column (Pickering Laboratories), which was isothermally controlled at 40°C, by a 0.3 mL/min eluent flow. The gradient program is illustrated in Table 1. The samples were separated in the column and directed to a 130°C reactor where a 0.3 mL/min flow of ninhydrin was pumped through to react with the separated analytes. The formed Ruhemann's purple was spectrometrically detected at 570 nm.

RESULTS AND DISCUSSION

The chromatograms of unreduced (A) and DDT-reduced (B) mixtures of serine, cystine, and homocystine, which have a starting concentration of 125 μM for each compound, are shown in Figure 1. Chromatogram A is used as the reference in calculating the PDC. The peak appearing at retention time of 15.2 minutes is identified as serine, which is used as the

Table 1. HPLC gradient program

Eluent A (%) time (min)	Eluent B (%) (Li280)	Eluent C (%) (Li750)	(RG003)
0.00	100.00	0.00	0.00
5.50	100.00	0.00	0.00
6.50	92.00	8.00	0.00
26.00	76.00	24.00	0.00
30.00	40.00	60.00	0.00
34.00	0.00	100.00	0.00
36.00	0.00	100.00	0.00
37.00	0.00	90.00	10.00
38.00	0.00	90.00	10.00
39.00	0.00	100.00	0.00
40.00	100.00	0.00	0.00

internal standard. The peaks appearing at retention times of 20.9 minutes and 35.8 minutes are confirmed as cystine and homocystine, respectively. Chromatogram B is the representative chromatogram of DTT reduced disulfide mixtures. In addition to those peaks for serine, cystine, and homocystine, the peaks of cysteine (retention time at 17.7 minutes) and homocysteine (retention time at 24.5 minutes) emerge in chromatogram B. One more peak appearing at a retention time of 4.8 miuntes is determined to be DTT. The relative peak intensities of cystine and homocystine in chromatogram B have decreased due to the conversion of the disulfide to sulfhydryl group.

The PDC is defined in order to quantify the efficiency of DTT reduction. Serine is used as internal standard to ensure reliable results. The peak area ratio of disulfide and serine in the chromatogram of the DTT reduced mixture is divided by the same peak area ratio of the reference chromatogram to obtain the fraction of unreduced disulfide present in the reaction mixture. The fraction of disulfide which has converted to thiol can be calculated as PDC according to the following equation:

$$\mathrm{PDC}(\%) = \left(1 - \frac{(\mathrm{A_{disulfide}}/\mathrm{A_{serine}})_{\mathrm{reduced}}}{(\mathrm{A_{disulfide}}/\mathrm{A_{serine}})_{\mathrm{reference}}}\right) \times 100\%$$

The A terms in the above equation stand for the areas of chromatographic peaks.

The PDC's for cystine and homocystine at different reduction conditions are summarized in Table 2. The comparison of PDC's for cystine and homocystine in 0.5 M and 1.0 M DTT at different temperatures for 30 minutes, are plotted in Figure 2. The PDC's for both cystine and homocystine range from 1.3% to 46.3% at 55° and below. The PDC's of cystine are higher than those of homocystine when the reaction temperature is lower than 55°C.

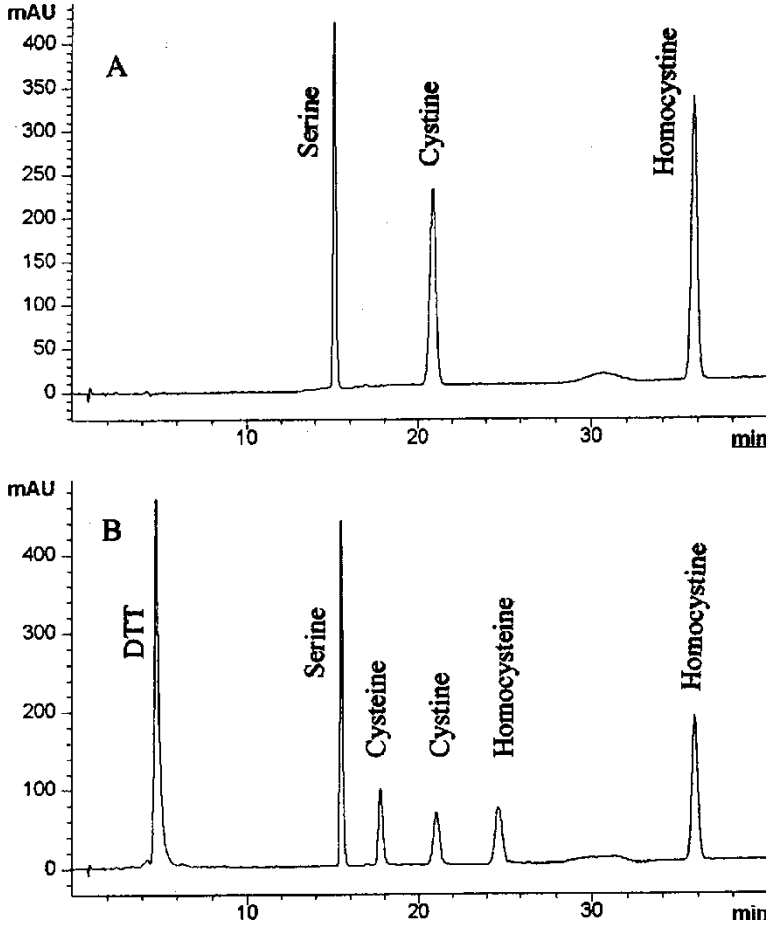

Figure 1. The chromatograms of (A) unreduced disulfide mixture and (B) DDT-reduced disulfide mixture.

At the reaction temperature greater than 55°C, the PDC's of both cystine and homocystine rise significantly and range from 62.2% to 97.8% when 0.5 M and 1.0 M DTT concentrations are used.

The comparison of PDC versus DTT concentration for cystine and homocystine at a reaction temperature of 70°C for 1 hour is shown in Figure 3. The concentration of DTT should be equal or greater than 0.5 M in order to achieve a PDC greater than 90% for both cystine and homocystine. The reaction time is also a factor. The results show that PDC's for cystine and homocystine in 1.0 M DTT at 70°C for 30 minutes are all below 84%. This indicates that a reaction time of 1 hour is needed to achieve a high conversion from disulfide to thiol.

Table 2. Percentage of disulfide conversion at various reduction conditions

PDC reduction conditions (%)	Cystine	Homocystine
0.5 M DTT, 30 minutes		
25°C	2.9	1.3
40°C	9.2	3.0
55°C	22.9	7.8
70°C	62.2	72.3
1.0 M DTT, 30 minutes		
25°C	6.1	2.2
40°C	19.5	4.8
55°C	46.3	8.8
75°C	78.8	83.8
70.0°C, 1 hour		
0.2 M DTT	39.1 (79.8)	7.5 (24.9)
0.5 M DTT	90.1 (100)	92.9 (100)
1.0 M DTT	96.8 (100)	97.8 (100)

The numbers in parentheses stand for the PDC's of stored samples.

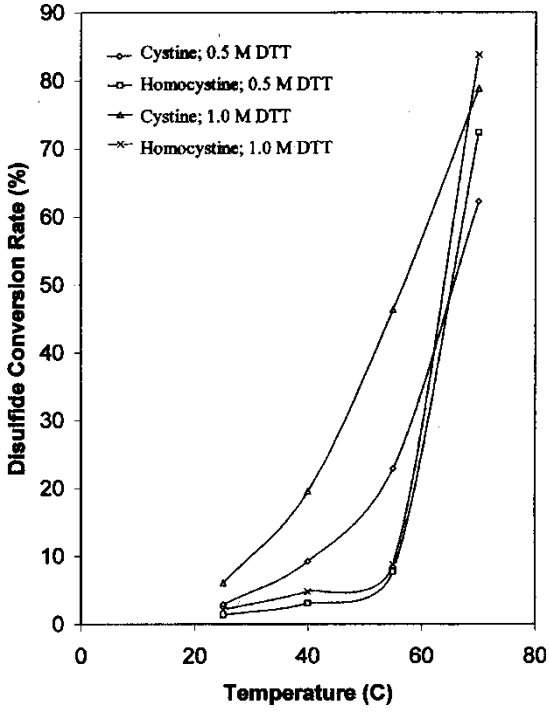

Figure 2. Comparison of PDC versus temperature for cystine and homocystine in 0.5 M and 1.0 M DTT for 30 minutes.

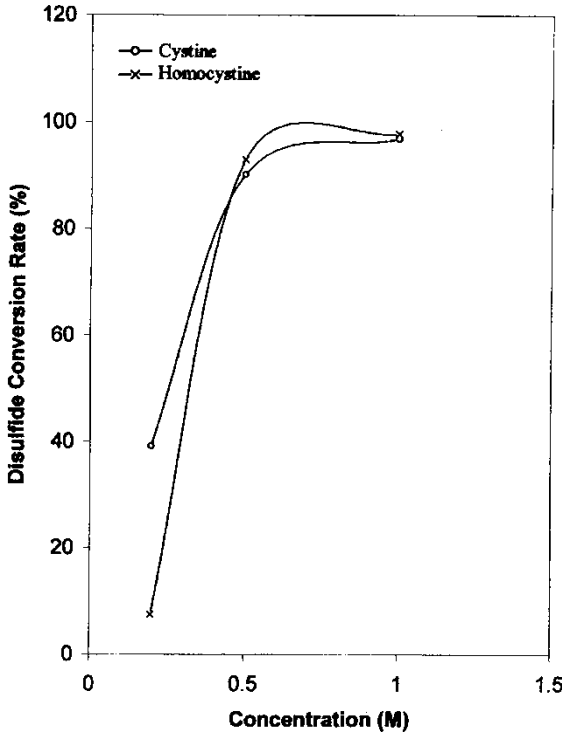

Figure 3. Comparison of PDC versus DTT concentration for cystine and homocystine at 70°C for 1 hour.

The disulfide mixtures that were reduced by DTT at 70°C for 1 hour were stored at room temperature for 8 days and reanalyzed to determine PDC. The PDC's of the stored samples are illustrated in the parentheses of Table 2. The higher PDC values of the stored samples demonstrate that DTT continues to reduce more disulfides and keeps the reduced thiols from being reoxidized back to disulfides.[11]

In conclusion, the effectiveness of DTT reduction on disulfide bonds in acidic medium depends on DTT concentration, reduction temperature, and reaction time. A combination of 1.0 M DTT concentration, 70°C reduction temperature, and 1 hour reaction time is required to convert 96% disulfide from both cystine and homocystine to their respective thiols. These conditions can be applied to the sample preparation for total plasma homocysteine determination to ensure a complete disulfide conversion. The efficiency of DTT reduction on protein bound homocysteine cannot be evaluated due to the unavailability of the protein bound sample. However, the use of cystine and homocystine is proven to be effective to evaluate the conversion of disulfide at different reduction conditions using cation-exchange chromatography.

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