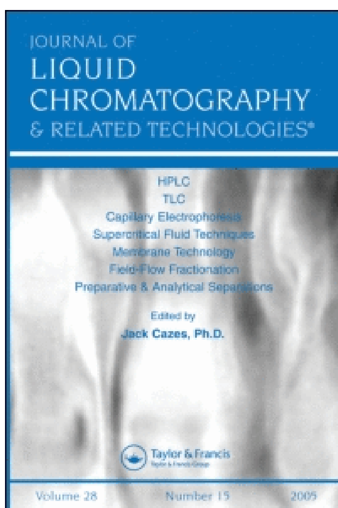
This article was downloaded by:
On: *24 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

EVALUATING THE FEASIBILITY OF DETERMINING TOTAL PLASMA HOMOCYSTEINE AS HOMOCYSTEIC ACID USING ION-EXCHANGE CHROMATOGRAPHY

Jinmo Huang[a]; Mark E. Arcaro[a]; Michael Lowry[a]
[a] Chemistry Department, The College of New Jersey, Ewing, NJ, U.S.A.

Online publication date: 23 April 2002

To cite this Article Huang, Jinmo , Arcaro, Mark E. and Lowry, Michael(2002) 'EVALUATING THE FEASIBILITY OF DETERMINING TOTAL PLASMA HOMOCYSTEINE AS HOMOCYSTEIC ACID USING ION-EXCHANGE CHROMATOGRAPHY', Journal of Liquid Chromatography & Related Technologies, 25: 5, 759 — 770
To link to this Article: DOI: 10.1081/JLC-120003033
URL: http://dx.doi.org/10.1081/JLC-120003033

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

J. LIQ. CHROM. & REL. TECHNOL., 25(5), 759–770 (2002)

EVALUATING THE FEASIBILITY OF DETERMINING TOTAL PLASMA HOMOCYSTEINE AS HOMOCYSTEIC ACID USING ION-EXCHANGE CHROMATOGRAPHY

Jinmo Huang,* Mark E. Arcaro, and Michael Lowry

Chemistry Department, The College of New Jersey, Ewing, NJ 08628, USA

Downloaded At: 09:08 24 January 2011

ABSTRACT

Total homocysteine content in plasma has been linked to the risk of occlusive vascular disease. Plasma homocysteine is present in multiple forms. Current methods for total plasma homocysteine analysis use reducing agents to convert other homocysteine-related compounds to homocysteine before analysis. Due to the incompleteness of reduction and the possibility of re-oxidation for homocysteine, accurate determination of total plasma homocysteine is not always achievable. In this research, performic acid is used to oxidize homocysteine-related compounds to homocysteic acid. The oxidized samples are separated and analyzed for homocysteic acid by ion-exchange chromatography with post-column ninhydrin derivatization and

*Corresponding author. E-mail: huangji@tcnj.edu

Copyright © 2002 by Marcel Dekker, Inc. www.dekker.com

spectrometric detection. The evaluation of performic acid oxidation on several homocysteine-related compounds and the feasibility of determining plasma homocysteine as homocysteic acid are studied.

Downloaded At: 09:08 24 January 2011

INTRODUCTION

The elevation of homocysteine level in plasma is an independent risk factor for arteriosclerosis.(1–6) Homocysteine is present in plasma as nonprotein-bound and protein-bound forms. Nonprotein-bound homocysteines exist in multiple forms including homocysteine (monomer), homocysteine thiolactone (ring), homocystine (disulfide dimer), and cysteine-homocysteine mixed disulfide. Protein-bound homocysteine is present through a disulfide bond between homocysteine and other thiols of plasma proteins. It has been reported that about 85% of homocysteine is protein-bound.(6) In order to obtain total homocysteine, the disulfide bonds of homocysteine and proteins have to be cleaved and homocysteine has to be released before analysis.

The current methods for determining total plasma homocysteine use reducing agents to convert all homocysteine-related compounds to homocysteine. The determination of total plasma homocysteine is then carried out by several approaches including radioenzymatic assay (7), gas chromatography/mass spectrometry (8,9), high performance liquid chromatography (HPLC) coupled with electrochemical detection (10), HPLC with precolumn derivatization and fluorescence detection (11), and HPLC with postcolumn derivatization and spectrometric detection.(12,13) Due to the incompleteness of reduction and the possibility of re-oxidation for homocysteine, irregular results have been experienced in the determination of total plasma homocysteine.

The authors have used ion-exchange chromatography to determine total plasma homocysteine in reduced plasma samples.(14) In addition to experienced irregularities caused by incomplete reduction, the required time of about 40 minutes to complete a chromatographic analysis is a drawback. In order to enhance the reliability and productivity in the plasma homocysteine analysis, the oxidation of homocysteine-related compounds is studied in this research. Performic acid, a powerful oxidizing agent (15,16), is used to oxidize several homocysteine-related compounds and plasma samples. The oxidized samples are separated and analyzed for homocysteic acid by ion-exchange chromatography with post-column ninhydrin derivatization and spectrometric detection.

EXPERIMENTAL

Chemicals and Reagents

The chemicals, including $_{DL}$-homocysteine, $_{DL}$-homocystine, $_{DL}$-homocysteine thiolactone, hydrogen peroxide (30%), and formic acid (88%), were obtained from Aldrich-Sigma (St. Louis, MO). The 1000 μM amino acid test solutions, including homocystine, homocysteine, and homocysteine thiolactone, were made by dissolving a specific amount of the respective amino acid in 20 mL 3 M hydrochloric acid, and then diluting with distilled water to 100 mL.

The eluents for ion-exchange chromatography were lithium citrate buffers including Li280, Li750, and RG003 regenerant. These were purchased from Pickering Laboratories (Mountain View, CA). The postcolumn derivatizing reagent (ninhydrin), precipitating reagent (Seraprep), and Li Diluent were also prepared by Pickering Laboratories.

The performic acid was prepared by vortex-mixing 1 mL 30% hydrogen peroxide and 9 mL 88% formic acid. The mixture was allowed to react at room temperature for 1 hour, then cooled and stored at 0°C before use.

Downloaded At: 09:08 24 January 2011

Oxidation Preparation

A portion of 0.100 mL 1000 μM amino acid test solution was vortex-mixed with 0.100 mL performic acid in a microcentrifuge for 1 minute. The reacting mixture was allowed to react for 20 minutes before 0.200 mL Li Diluent was added to adjust the pH of mixture to about 1.5. The reacting mixture was then injected for HPLC analysis. In order to compare the effectiveness of oxidation, two different reacting conditions, 25°C and 0°C, were used. The results were compared to those of the unoxidized amino acids.

The oxidation of plasma was prepared by vortex-mixing 0.200 mL plasma and 0.200 mL performic acid in a microcentrifuge for 1 minute. The mixture was allowed to react for 20 minutes. A portion of 0.200 mL Seraprep was then added to the microcentrifuge and mixed well to precipitate plasma proteins. The mixture was allowed to sit for 5 minutes, and thereafter, was centrifuged at 13,000 rpm for 5 minutes. After filtering through a 0.5 μm syringe filter, the supernatant was injected for HPLC analysis. Two oxidation conditions, 25.0°C and 0.0°C, were also used to compare the efficiency of plasma oxidation.

Chromatographic System

The system for analyzing plasma samples was a unit, which combined a HP 1050 liquid chromatograph (Hewlett-Packard Corporation, Palo Alto, CA) and a

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

Downloaded At: 09:08 24 January 2011

RESULTS AND DISCUSSION

Performic Acid Oxidation of Homocystine

The comparison of chromatograms for homocysteine, including the unoxidized (A), oxidized at 25°C (B), and oxidized at 0°C (C) by performic acid, is illustrated in Figure 1. The only significant peak in the chromatogram A at a retention time of 20.7 minutes is homocystine. Several peaks have emerged in the chromatogram B due to the oxidation of homocystine. The most intense peak at a retention time of 2.4 minutes is identified as homocysteic acid. Two shoulder peaks adjacent to the peak of homocysteic acid at retention times of 2.7 and 2.9 minutes are caused by performic acid present in the sample. Two broad peaks at retention times of 10.8 and 12.9 minutes are believed to be homolanthionine sulfoxide and homolanthionine sulfone, respectively. Due to the unavailability of

Table 1. HPLC Gradient Program

% Eluent A	% Eluent B	% Eluent C	
Time (min)	(Li280)	(Li750)	(RG003)
0.00	100.00	0.00	0.00
5.00	100.00	0.00	0.00
6.00	80.00	20.00	0.00
25.00	0.00	100.00	0.00
26.00	0.00	100.00	0.00
30.00	0.00	97.00	3.00
45.00	0.00	97.00	3.00
46.00	100.00	0.00	0.00

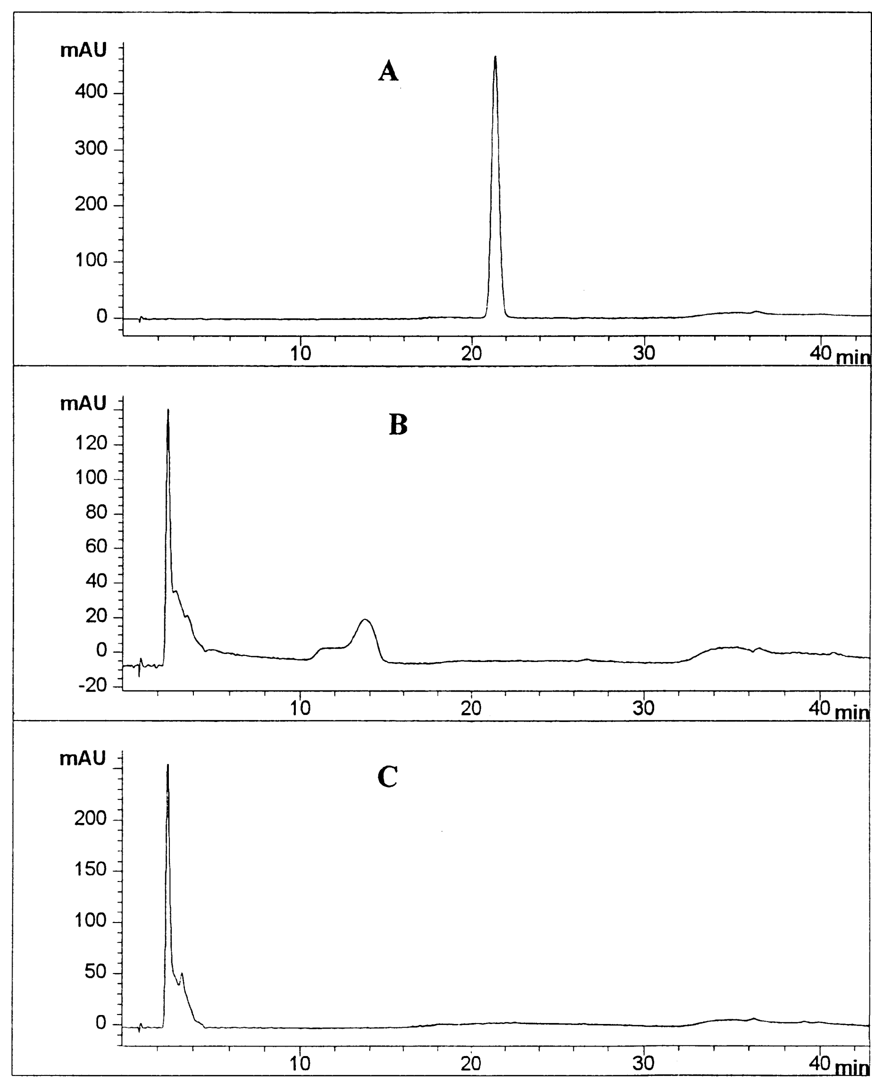


Figure 1. Comparison of chromatograms for homocystine. A: Unoxidized; B: Oxidized at 25°C; C: Oxidized at 0°C.

Downloaded At: 09:08 24 January 2011

these two compounds, the actual identification of these two peaks cannot be performed in our lab. However, they have been identified as the oxidation products of homocystine using hydrogen peroxide, which is a less powerful oxidizing agent than performic acid, in the Cleopath and McCulley's study (17). The humps around retention times between 32.0 and 42.0 minutes come from the scale augmentation of baseline.

The complete oxidation of homocystine to homocysteic acid is observed in the chromatogram C. The peak intensity of homocysteic acid increases significantly, and the peaks of homolanthionine sulfoxide and homothiolanthionine sulfone disappear entirely. The shoulder peak at a retention time of 2.8 minutes is due to performic acid left in the sample. The two shoulder peaks of performic acid observed in the chromatogram B may be due to the partial decomposition of performic acid. The incomplete conversion of homocystine to homocysteic acid may also be explained by the decomposition of performic acid at 25°C.

Downloaded At: 09:08 24 January 2011

Performic Acid Oxidation of Homocysteine

The comparison of chromatograms for homocysteine, including the unoxidized, oxidized at 25°C, and oxidized at 0°C by performic acid, is shown in Figure 2. The peak in the chromatogram A at a retention time of 17.8 minutes is apparently homocysteine. This peak is found as the most intense peak in the chromatogram B, because little quantity of homocysteine is oxidized by performic acid at 25°C. The emergence of peaks for homocysteic acid (2.4 minutes), homolanthionine sulfoxide (10.8 minutes), and homolanthionine sulfone (12.9 minutes) demonstrates the coexistence of the oxidation product and its intermediates. A small peak at retention time of 20.7 minutes is believed to be homocystine. This is due to the oxidation of homocyteine to the disulfide dimer, homocystine. The several humps around retention times of 32.0 and 42.0 minutes are again due to the scale expansion of baseline.

The complete oxidation of homocysteine to homocysteic acid is observed in the chromatogram C. This proves that a complete oxidation of homocysteine to homocysteic acid is achievable through controlling oxidation temperature at 0°C.

Performic Acid Oxidation of Homocysteine Thiolactone

The comparison of chromatograms for homocysteine thiolactone, including the unoxidized, oxidized at 25°C, and oxidized at 0°C by performic acid, is shown in Figure 3. The major peak at a retention time of 39.0 minutes in the chromatogram A clearly belongs to homocysteine thiolactone. A minor peak at a

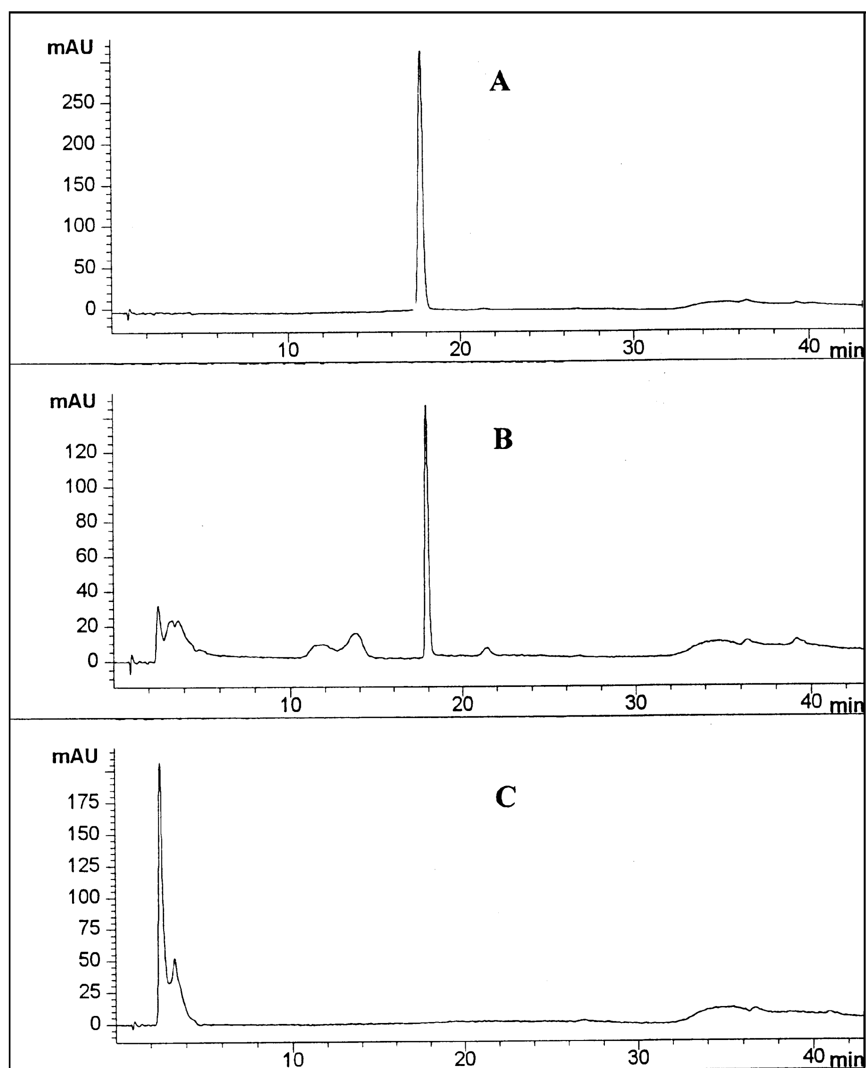


Figure 2. Comparison of chromatograms for homocysteine. A: Unoxidized; B: Oxidized at 25°C; C: Oxidized at 0°C.

Downloaded At: 09:08 24 January 2011

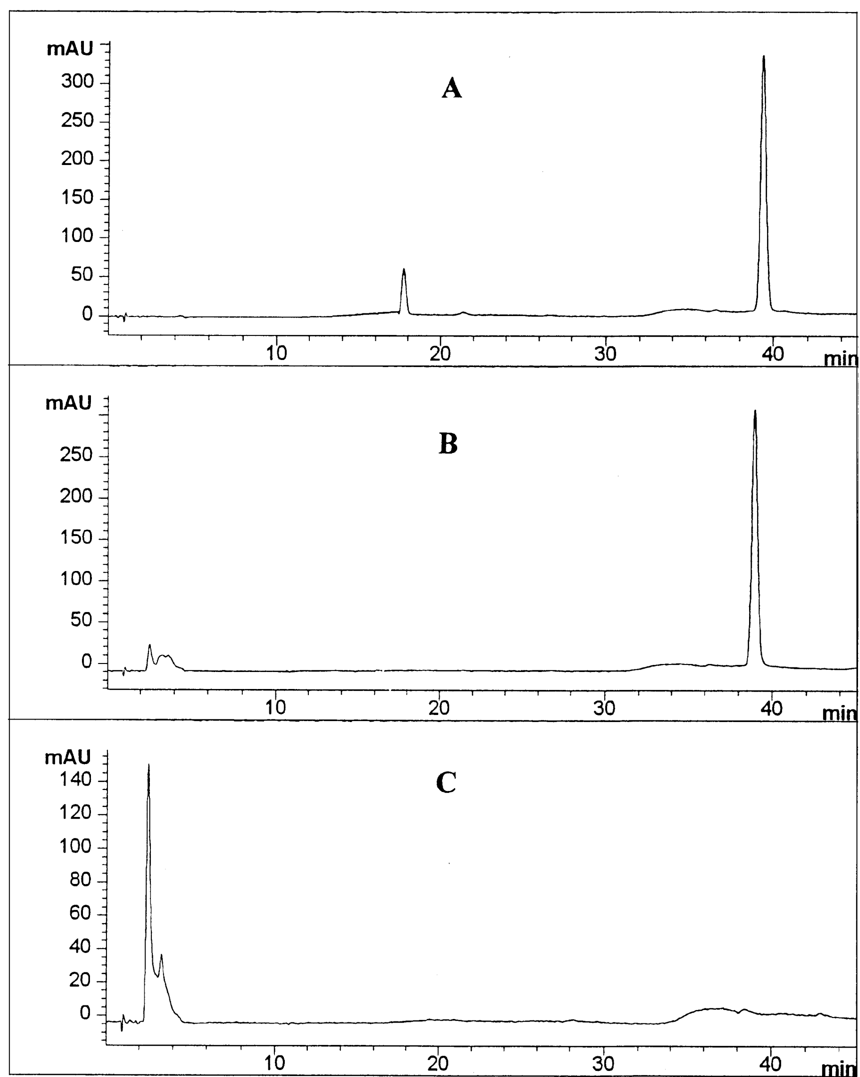


Figure 3. Comparison of chromatograms for homocysteine thiolactone. A: Unoxidized; B: Oxidized at 25°C; C: Oxidized at 0°C.

Downloaded At: 09:08 24 January 2011

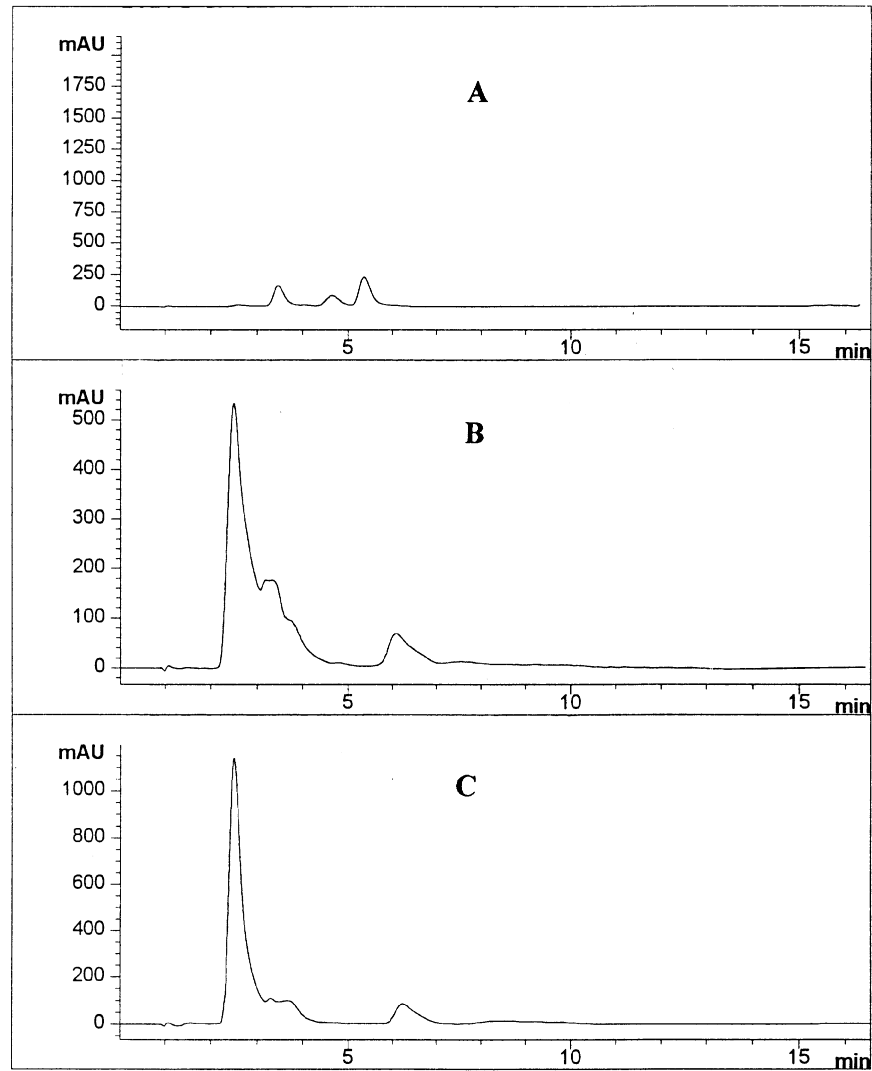


Figure 4. Comparison of chromatograms for plasma. A: Unoxidized; B: Oxidized at 25°C; C: Oxidized at 0°C.

Downloaded At: 09:08 24 January 2011

retention time of 17.8 minutes illustrates a small amount of homocysteine present in homocysteine thiolactone reagent. This homocysteine peak disappears in the chromatogram B due to oxidation. The weak emergence of homocysteic acid at a retention time of 2.4 minutes in the chromatogram B demonstrates an incomplete oxidation at 25°C. However, the complete conversion of homocysteine thiolactone to homocysteic acid is observed in the chromatogram C, when the oxidation is controlled at 0°C.

Performic Acid Oxidation of Plasma

The comparison of chromatograms for plasma samples, including the unoxidized, oxidized at 25°C, and oxidized at 0°C by performic acid is illustrated in Figure 4. The peak of homocysteic acid at a retention time of 2.4 minutes is not observed in the unoxidized plasma sample (chromatogram A). Several peaks in the chromatogram A represent other amino acids in the sample. The emergence of a chromatographic peak at a retention time of 2.4 minutes in the chromatograms B and C illustrates the presence of homocysteic acid in the oxidized plasma samples. The very intense peak of homocysteic acid in the chromatogram C illustrates a more complete oxidation of analytes at a reaction temperature of 0°C.

Downloaded At: 09:08 24 January 2011

CONCLUSION

Performic acid is proved to be a very powerful oxidizing agent for homocysteine-related compounds. Due to the unavailability of cysteine-homocysteine mixed disulfide and protein-bound homocysteine, only three homocysteine-related compounds are used in this study. Based on the results observed, the disulfide dimer (homocystine) is the easiest compound among these three to be completely oxidized by performic acid. It is sufficient to say that other disulfides, cysteine-homocysteine mixed disulfide and protein-bound homocysteine, will be completely oxidized by performic acid under a reaction temperature at 0°C. Thus, it is feasible to determine total plasma homocysteine as homocysteic acid. However, cysteic acid produced from the oxidation of cysteine-related compounds in plasma samples will interfere with the quantification of homocysteic acid. A study on the separation of homocysteic acid and cysteic acid using ion-exchange chromatography should be performed. When a successful separation technique for homocysteic acid and cysteic acid is developed, the chromatographic determination of total plasma homocysteine can be done in 5 minutes. As a result, the reliability and productivity of total plasma homocysteine analysis will be greatly enhanced.

ACKNOWLEDGMENTS

The support of this work by the College of New Jersey through the SOSA grant is gratefully acknowledged. Also, the authors would like to acknowledge Chambers Clinical Laboratories (Trenton, NJ) for providing the post-column derivatization unit and plasma samples used in this research.

Downloaded At: 09:08 24 January 2011

REFERENCES

1. McCully, K.S.; Wilson, R.B. Homocysteine Theory of Arteriosclerosis. Arteriosclerosis **1975**, *22*, 215–227.
2. Fortin, L.J.; Genest, J.J., Jr. Measurement of Homocysteine in the Prediction of Arteriosclerosis **1995**, *28*, 572–576.
3. McCully, K.S. Nature Med. **1996**, *2*, 386–394.
4. Tsai, M.Y. Clinical Chem. **1996**, *42*, 492–502.
5. Cramer, D.A. Homocystine vs. Cholesterol: Competing Views, or Unifying Explanation of Arteriosclerotic Cardiovascular Disease. Lab. Med. **1998**, *29*, 410–417.
6. Jacobsen, D.W. Homocysteine and Vitamins in Cardiovascular Disease. Clin. Chem. **1998**, *44*, 1833–1843.
7. Refsum, H.; Helland, S.; Ueland, P.M. Radioenzymic Determination of Homocysteine in Plasma and Urine. Clin. Chem. **1985**, *31*, 624–628.
8. Staber, S.P.; Marcell, P.D.; Podell, E.R.; Allen, R.H. Quantitation of Total Homocysteine, Total Cysteine, Methionine in Normal Serum and Urine using Capillary Gas Chromatography-Mass Spectrometry. Anal. Biochem. **1987**, *162*, 185–196.
9. Stabler, S.P.; Lindenbaum, J.; Savage, D.G.; Allen, R.H. Elevation of Serum Cystathionine Levels in Patients with Cobalamin and Folate Deficiency. Blood **1993**, *81*, 3403–3413.
10. Rabenstein, D.L.; Yamashita, G.T. Determination of Homocysteine, Penicillamine, and their Symmetrical and Mixed Disulfides by Liquid Chromatography with Electrochemical Detection. Anal. Biochem. **1989**, *180*, 259–263.
11. Araki, A.; Sako, Y. Determination of Free and Total Homocysteine in Human Plasma by High Performance Liquid Chromatography with Fluorescence Detection. J. Chromatogr. **1987**, *422*, 43–52.
12. Andersson, A.; Isakesson, A.; Brattstrom, L.; Hultberg, B. Homocysteine and other Thiols Determined in Plasma by HPLC and Thio-Specific Post-Column Derivatization. Clin. Chem. **1993**, *39*, 1590–1597.

13. Andersson, A.; Brattstrom, L.; Isaksson, A.; Israelsson, B.; Hultberg, B. Determination of Homocysteine in Plasma by Ion-Exchange Chromatography. Scand. J. Clin. Lab Invest. **1989**, *49*, 445–450.
14. Huang, J.; Williams, M. Total Plasma Homocysteine Determination Using Ion-Exchange Chromatography. J. Liq. Chrom. & Rel. Technol. **2000**, *23* (20), 3143–3153.
15. Moore, S. On the Determination of Cysteine as Cysteic Acid. J. Biol. Chem. **1963**, *238* (1), 235–237.
16. Mason, V.C.; Bech-Andersen, S.; Rudemo, M. Hydrolysate Precipitation for Amino Acid Determination in Feed Constituents. Verlag Paul Parey **1980**, *43*, 146–164.
17. Clopath, P.; McCully, K.S. Synthesis of Homolanthionine Sulfone and Homolanthionine Sulfoxide. Anal. Biochem. **1976**, *73*, 231–235.

Received September 18, 2001
Accepted October 25, 2001
Manuscript 5653

Downloaded At: 09:08 24 January 2011