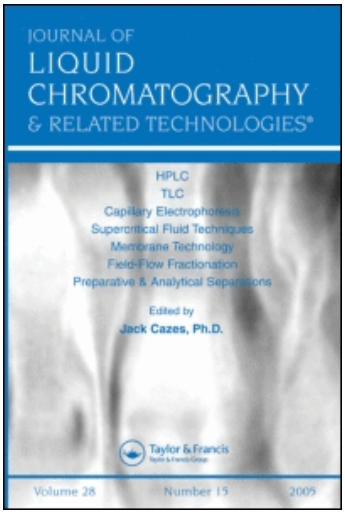

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TOTAL PLASMA HOMOCYSTEINE DETERMINATION USING ION-EXCHANGE CHROMATOGRAPHY

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ABSTRACT

Plasma homocysteine level has been linked with the risk of occlusive vascular disease. Due to the presence of multiple forms of homocysteine, with the majority found as protein-bound homocysteine, and the interchanges among non-protein-bound homocysteines, the concern of analytical reliability for total plasma homocysteine has been raised. In this research, plasma samples are reduced by dithiothreitol to break disulfide linkages.

The reduced samples are separated and analyzed for homocysteine and its related compounds by ion-exchange chromatography with post-column ninhydrin derivatization and spectrometric detection. The total homocysteine of plasma is determined as the sum of homocysteine, homocysteine thiolactone, homocystine, and cysteine-homocysteine disulfide.

INTRODUCTION

Homocysteine is a non-proteogenic amino acid produced by the biological metabolism of methionine and used in the enzymatic biosyntheses of cystathionine and methionine, which require vitamins B_6, B_{12}, and folate as cofactors.[1] When the concentration of homocysteine in human plasma becomes elevated, the resulting condition is known as hyperhomocysteinemia.[2, 3] This

condition results from the deficency of enzyme activity and/or the low quantity of cofactors in homocysteine metabolism.

Homocysteine is involved in virtually all of the pathogenic processes that result in arteriosclerosis.[4] Homocysteine in its reactive form, homocysteine thiolactone, affects the reactivity of platelets, and is extremely active in causing platelet aggregation, which can lead to clot formation.[5] Several clinical studies have shown that plasma homocysteine level correlates better than cholesterol levels with the risk of premature cardiovascular disease.[6]

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 (dimer), and cysteine-homocysteine disulfide. Also, it has been reported that as much as 85% of homocysteine is bound via disulfide linkage to plasma proteins.[7] In order to obtain total homocysteine, plasma must be reduced to break the disulfide linkages, and plasma proteins have to be precipitated and removed immediately.

Due to the fast oxidation of homocysteine into homocystine and cysteine-homocysteine disulfide in the presence of cysteine, and the imminent dehydration of homocysteine into homocysteine thiolactone, the reduced plasma sample must be separated and analyzed for all possible non-protein-bound homocysteines.

The determination of total plasma homocysteine is currently carried out by several approaches, including radioenzymatic assay,[8] gas chromatography/mass spectrometry,[9, 10] high performance liquid chromatography (HPLC) coupled with electrochemical detection,[11] HPLC with pre-column derivatization and fluorescence detection,[12] and HPLC with post-column derivatization and spectrometric detection.[13,14]

These methods require the use of reducing agents to break disulfide linkage to plasma proteins before analysis. Among them, the pre-column derivatization with fluorogenic reagents for thiols followed by HPLC analysis has become increasingly popular.[15] However, it has been reported that reducing agents might consume the pre-column reagent.[16] Furthermore, the stability of the products from pre-column derivatization would affect the reliability of results in automatic HPLC analysis.

In this research, plasma sample was reduced by dithiothreitol to release homocysteine. The plasma proteins were precipitated out and removed from the reduced sample. Due to the immediate conversion of homocysteine into its related compounds, the filtrate was separated and analyzed by ion-exchange

chromatography with post-column ninhydrin derivatization and spectrometric detection for all homocysteine-related compounds.

The total plasma homocysteine was determined as the sum of homocysteine, homocysteine thiolactone, homocystine, and cysteine-homocysteine disulfide.

EXPERIMENTAL

Chemicals and Reagents

The chemicals including $_{DL}$-homocysteine, $_{DL}$-homocysteine thiolactone hydrochloride, $_{DL}$-homocystine, $_{DL}$-cysteine hydrochloride monohydrate, and dithiothreitol were obtained from Aldrich-Sigma (St. Louis, Missouri). Freshly prepared standard solutions were made by dissolving specific quantities of these compounds in 20 mL 3 M hydrochloric acid to completely dissolve the reagents, then diluted by adding distilled water to a desired quantity. The dithiothreitol solutions were prepared in distilled water.

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

Sample Collection and Preparation

Blood samples for homocysteine analysis were drawn from patients after at least 8 hours of fasting. Blood samples were collected into EDTA-containing tubes. These tubes were mixed and centrifuged at 3,000 rpm for 5 minutes to separate blood cells from plasma.

A portion of 0.250 mL plasma, 0.250 mL 0.5 M dithiothreitol were mixed thoroughly in a microcentrifuge and reacted for 20 minutes. A portion of 0.250 mL of 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.

Chromatographic System

The system for analyzing plasma samples was a unit 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 variable wavelength detector. The plasma samples were injected into HPLC through a HP 1050 autosampler. The system was controlled and run by HP Chemstation (3.1 Version).

The chromatographic parameters were as follows: A portion of 10 μL reduced plasma sample was injected into chromatograph by an autosampler. 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. Non-protein-bound homocysteines 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 non-protein-bound homocysteines. The formed Ruhemann's purple was detected at 570 nm by a spectrometric detector.

RESULTS AND DISCUSSION

The chromatogram of a freshly prepared mixture of cysteine and homocysteine, with a concentration of 125 μM for each compound, is shown in Figure 1. The variation in intensity for the two major peaks, which are at retention times 18.5 and 30.0 minutes, is due to different reaction rates for these two compounds with the derivatizing agent, ninhydrin. Three weak peaks, one at retention time 27.6 minutes, the second one at 50.8 minutes, and the third one at 82.7 minutes are identified by spiking technique as cystine, homocystine, and

Table 1

HPLC Gradient Program

Time (min)	% Li280	% Li750	% RG003
0.00	100.00	0.00	0.00
17.00	90.00	9.00	0.00
44.00	66.00	34.00	0.00
54.00	40.00	60.00	0.00
65.00	0.00	100.00	0.00
75.00	0.00	99.50	0.50
85.00	0.00	996.00	4.00
87.00	0.00	94.00	6.00
88.00	0.00	92.00	8.00
89.00	50.00	42.00	8.00
90.00	100.00	0.00	0.00

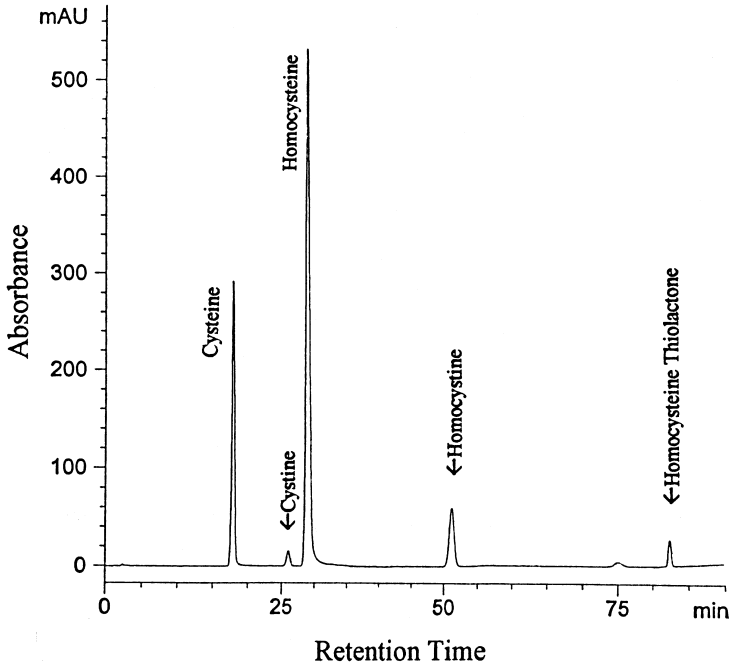

Figure 1. Chromatogram of fresh cysteine/homocysteine mixture.

homocysteine thiolactone, respectively. A tiny peak at retention time 75.0 minutes is confirmed as an unknown impurity, which is present in cysteine reagent.

When the mixture of cysteine and homocysteine is purged with air for 2 hours to enhance reaction and then run by HPLC, the peak intensities of homocysteine thiolactone and three disulfide compounds, which include cystine, homocystine, and cysteine-homocysteine disulfide, increase significantly. This has been confirmed by the chromatogram shown in Figure 2. The newly emerging peak at retention time 38.1 minutes is believed to be cysteine-homocysteine disulfide.

The identification of cysteine-homocysteine disulfide can not be performed by spiking technique due to the unavailability of this compound from chemical manufacturers. The emergence of these disulfide and dehydrated compounds via thiols has demonstrated the reactivity of thiol compounds, and attests to the necessity of analyzing homocysteine and its related compounds in order to obtain total plasma homocysteine.

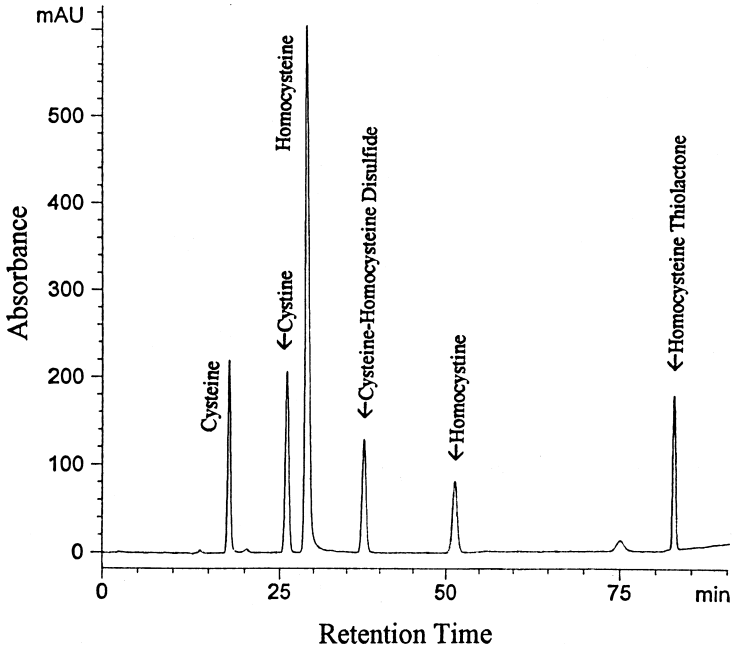

Figure 2. Chromatogram of air-purged cysteine/homocysteine mixture.

The chromatogram of non-protein-bound homocysteines and other amino acids in a typical plasma sample, without treatment with dithiothreitol, is illustrated in Figure 3. The homocysteine, homocysteine thiolactone, homocystine, and cysteine-homocysteine disulfide detected in the sample are minimal. This confirms that most homocysteine (about 85%) in plasma is bound to plasma proteins via disulfide.[7] It also proves the necessity of reducing plasma samples prior to the determination of total plasma homocysteine.

The chromatogram of non-protein-bound homocysteines and other amino acids in a dithiothreitol-treated plasma sample is shown in Figure 4. The emergence of homocysteine and cysteine in the reduced sample illustrates the reduction of disulfides into thiols. The peak intensities of homocysteine thiolactone, homocystine, and cysteine-homocysteine disulfide are still minimal. It is believed that the concentrations of these homocysteine-related compounds are dependent on the concentration of dithiothreitol present in the reduced sample and the time interval in which the reduced plasma samples are stored before

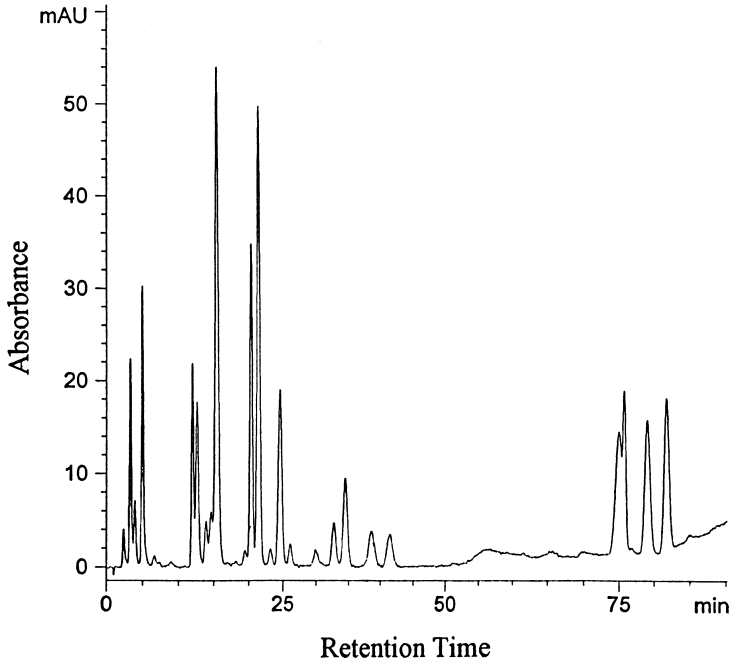

Figure 3. Chromatogram of typical plasma sample without dithiothreitol treatment.

analysis. This has reaffirmed the necessity of analyzing all non-protein-bound homocysteines in order to obtain total plasma homocysteine.

The treatment of plasma with different concentrations of dithiothreitol, including 0.25 M, 0.50 M, and 1.00 M, does not produce significant differences in total plasma homocysteine determination. The total plasma homocysteine contents of 16 analyzed plasma samples range from 9 to 16 μM. These experimental results are higher than the reported normal range, 8 - 12 μM, in the literature.[5]

The detection limits of homocysteine and its related compounds, excluding cysteine-homocysteine disulfide, range from 1 to 4 pmol for this method, with homocysteine thiolactone most detectable and cysteine least detectable. This has been demonstrated in the chromatogram (Figure 5) of a mixture of cysteine, homocysteine, homocysteine thiolactone, and homocystine, with a concentration of 125 μM for each compound.

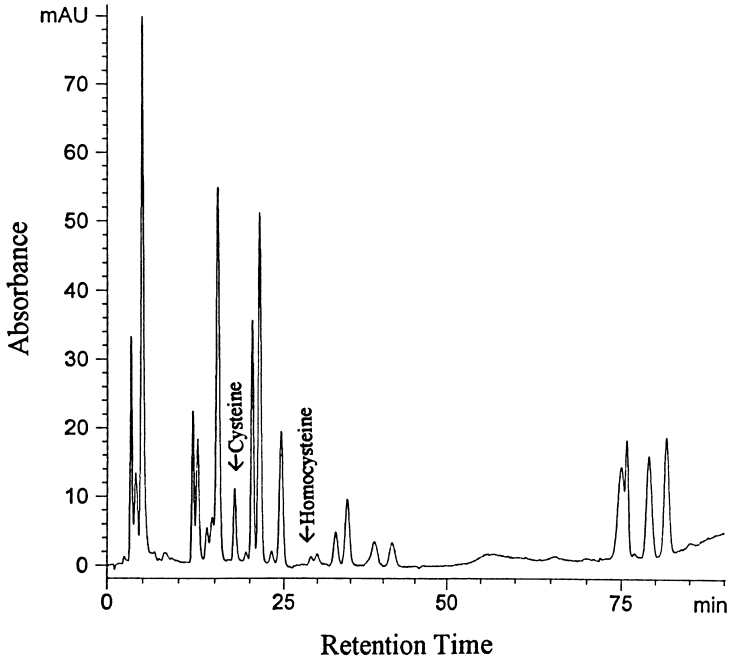

Figure 4. Chromatogram of dithiothreitol-treated plasma sample.

The peak area of homocystine in Figure 5 is greater than that of homocysteine thiolactone; however, one mole of homocystine is equivalent to two moles of homocysteine thiolactone. Therefore, homocysteine thiolactone is more detectable than homocystine. The linear range of calibration for these compounds, excluding cysteine-homocysteine disulfide, is determined at concentrations greater than 250 μM.

The detection limit and the linear range of calibration for cysteine-homocysteine disulfide are not determined due to the unavailability of this compound. The reproducibility is assessed as 6% coefficient of variation (CV) for intra-batch analysis, and 10% CV for inter-batch determination. The fluctuation in precision of this method is due to the instability of reagents and the fluctuation of instrument.

It is recommended that homocysteine standards should be freshly prepared, and calibration should be performed at least 2 times in each batch of analysis to compensate for the instability of reagents, including homocysteine

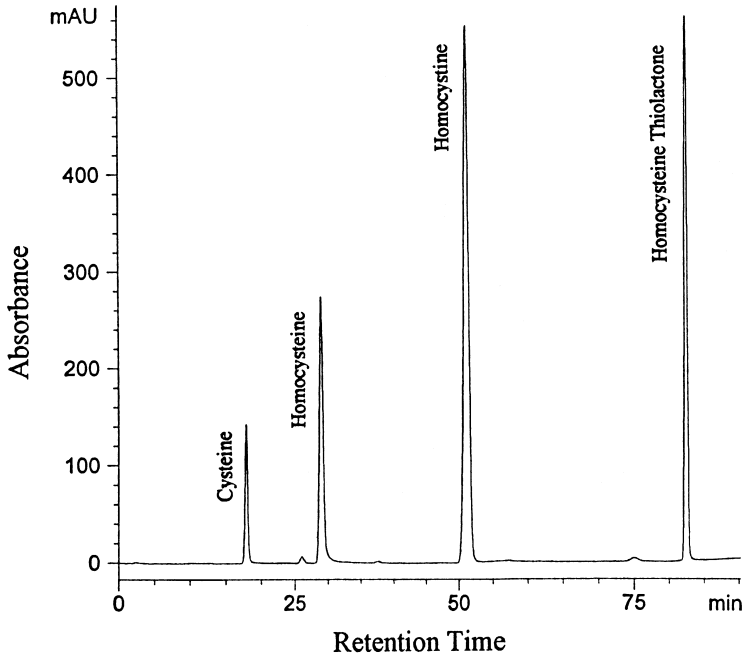

Figure 5. Chromatogram of fresh cysteine/homocysteine/homocysteine thiolactone/ homocystine mixture.

and ninhydrin. In addition, an internal standard is recommended in order to correct the fluctuation of the instrument.

The required time of a complete HPLC analysis for total plasma homocysteine and other amino acids is about 90 minutes. This is rather time-consuming. If the determination of other amino acids present in plasma is not required, the time needed for the analysis of total plasma homocysteine can be decreased to about 30 minutes. However, the co-elution of other amino acids with homocysteine and its related compounds will be unavoidable in a shortened analysis time. Thus, a shortened run time can result in an inaccurate quantification of total plasma homocysteine.

In conclusion, the total plasma homocysteine determination using ion-exchange chromatography with post-column ninhydrin derivatization and spectrometric detection is very suitable for automatic analysis. This technique provides for the separation and determination of homocysteine and its related

compounds in the same HPLC run. Thus, a more accurate total plasma homocysteine can be determined from the sum of homocysteine, homocyteine thiolactone, homocystine, and cysteine-homocysteine disulfide.

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