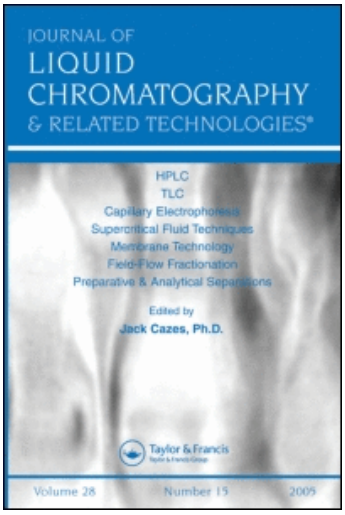

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A Rapid and Simple Assay to Determine Total Homocysteine and Other Thiols in Pediatric Samples by High Pressure Liquid Chromatography and Fluorescence Detection

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A RAPID AND SIMPLE ASSAY TO DETERMINE TOTAL HOMOCYSTEINE AND OTHER THIOLS IN PEDIATRIC SAMPLES BY HIGH PRESSURE LIQUID CHROMATOGRAPHY AND FLUORESCENCE DETECTION

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ABSTRACT

A rapid and simple method, using pre-column fluorescent labeling, followed by high pressure liquid chromatography (HPLC), for the determination of total concentration of thiols (homocysteine, cysteine, cystinylglycine, and glutathione) in small-volume, pediatric samples, is presented. Thiols in plasma were first reduced with tri-n-butylphosphine, and the resulting free —SH groups were labeled with the fluorescent compound 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F). The ABD-thiols were desorbed from plasma proteins by precipitating proteins with TCA, and then fractionated by reverse-phase chromatography. Peak areas were used to determine concentrations in the unknowns.

Baseline resolution of all four thiols was obtained in about 12 minutes. This method requires a maximum of 75 μL or less of the sample. It is specific to thiols, and chromatograms do not show any other peaks. It is sensitive to about 500 pmol/L, and is linear to at least 300 μmol/L of each of the thiols. Within- and between-day CV's were less than 4%. Reference ranges for children up to 18 years of age (mean ± SD), were: 8.9 ± 2.9, and 232 ± 56 umol/L for total homocysteine, and total cysteine, respectively. No sex- or age- dependent changes were observed in children up to 18 years of age of reference values for total homocysteine or total cysteine.

INTRODUCTION

Elevated plasma homocysteine is an independent risk factor for development of a variety of vascular occlusive diseases, including carotid, coronary, and peripheral arteries.[1-5] Increased plasma homocysteine can be due to either genetic defects or secondary to either drugs or nutritionally related from deficiency of vitamins B6, B12 or folate.[6] Atherosclerosis has its inception in childhood;[7] therefore, ability to prevent or delay this process depends on ability to identify and alter these factor(s) responsible early in life.[8] Thus, determination of homocysteine levels in pediatric populations may provide information for the role of this amino acid in pathogenesis of atherosclerosis.[6] Further, since difference between normal and clinically abnormal levels for homocysteine is at the most 2x above the upper normal range, and, most of the time, it is only 1.3 to 1.5 times of the normals,[1] a method with greater sensitivity and precision to suit pediatric samples is highly desired.

Several different methods are available to determine homocysteine in plasma.[9] Of these, use of pre-column fluorescent labeling agents specific to sulfhydryl groups, followed by high pressure liquid chromatography (HPLC) and fluorescence detection, offer high specificity and sensitivity. Fluorobenzoxadiazoles are specific fluorescent labeling agents for thiol groups and yield high fluorescence.[10] Two of these compounds, ammonium-7-fluoro-2.1.3,-benzoxadiazole-4-sulfonate (SBD-F) and 4-(aminosulfonyl)- 7-fluoro-2,1,3-benzoxadiazole (ABD-F) are particularly useful in the assay of thiols.[11,12] Both ABD-F and SBD-F are nonfluorogenic, provide stable and sensitive adducts, thereby allowing detection at picomolar levels.[10] Although SBD-F has been used for the determination of homocysteine and other thiols in plasma,[12-16] it's reaction with sulfhydril compounds is relatively slow and requires drastic conditions (pH 9.5, 60 minute incubation at 60°C) for

completion. These harsher conditions, in general, are not conducive and possible degradation of thiols occurs. Unlike SBD-F, ABD-F is 30X more reactive and requires milder conditions, (viz., pH 8.0, 5-10 minute incubation at 50°C) and, therefore, problems related to re-oxidation can be avoided.[11]

Araki and Sako[14] are the first to present a protocol to determine total thiols in plasma samples using tri-n-butylphosphine as the reducing agent, and the SBD-F as the labeling agent. They have separated and quantitated individual thiols by HPLC, using gradient elution, in about 20 minutes. Vester and Rasmussen[15] modified Araki and Sako's protocol, using an isocratic buffer system and smaller sample size. However, this procedure still required 20 minutes for completion. Ubbink et al.[16] further modified this method and obtained a complete elution of thiols in less than 10 minutes. However, this procedure requires an extremely acidic buffer of pH 2.1. Further, it is very sensitive to buffer pH and even a slight change in pH affected the resolution of cysteine, homocysteine and cystinylglycine.

In spite of several advantages associated with the use of ABD-F, the possibility of using this procedure routinely for the determination of sulfhydryl groups in clinical laboratories has not yet been explored. Hence, we report a simple procedure to quantitate various thiols in plasma, using ABD-F as a fluorescent labeling agent. Conditions for reduction and desorption of all thiols in plasma from the plasma proteins, labeling of the desorbed thiols with ABD-F, and fractionation of individual thiols by HPLC (i.e., columns, buffers, etc.) are also thoroughly evaluated. In addition, the use of this protocol in pediatric setting is explored to obtain a baseline separation of cysteine, homocysteine, cystinylglycine and glutathione in about 10 minutes, using a sample volume of less than 100 μL.

EXPERIMENTAL

Materials

4-(Aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) was purchased from Waco Chemicals (Richmond, VA, USA). D,L-homocystine, L-cystine, cystinylglycine (oxidized form), glutathione, tri-n-butylphosphine and other routine chemicals were obtained from Sigma Chemicals (St. Louis, MO, USA). HPLC grade methanol was from Scientific Products (Deerfield, IL, USA). A RP-18, 4.6 x 250 mm column, packed with 5 μm diameter particles, was

obtained from the Supelco Corp. (Bellefonte, PA, USA). Cartridges (3.2 x 40 mm size) with various packing materials were from Applied Biosystems (Foster City, CA, USA).

Apparatus

An HPLC unit from Thermo-Separation Products (Spectra-Physics Division, Freemont, CA) was used in this study. This unit (model #AS3000) was equipped with a ternary gradient pump, a refrigerated autosampler, and a fluorescence detector. Operation of the HPLC unit, as well as data collection and calculations, were accomplished through a PC unit. All solvents were filtered through a 0.45 μm filter (Type HA, Millipore, Bedford, MA, USA) before use. Solvents were degassed by continuous sparging with helium gas. Fractionation was carried out at room temperature (20 ± 2°C). Effluents were monitored for their fluorescence intensities at an excitation wavelength of 386 nm and an emission wavelength of 516 nm. Signal detection and quantitation of peak areas were accomplished using automated data acquisition software from the Thermo-Separation Products.

Specimens

Whole blood was collected into tubes containing either EDTA or heparin. Cells were separated within 60 minutes after collection to avoid influx of thiols from red blood cells[9] by centrifugation for 10 minutes at 3500 RPM (2500 x g). The resulting plasma was used for analysis, either immediately, or stored at -70°C.

To establish a pediatric reference range, samples were collected from 0.5- to 18-year-old male and female children. Fifteen samples each were collected from four different age groups, ranging from 0.1 to 4, 4.1 to 9.0, 9.1 to 14.0 and 14.1 to 18.0 years, with an equal number of samples each for males and females in each age group. To test for possible interference from different drugs, samples were obtained from patients undergoing therapy with a variety of drugs for infections, epilepsy, cardiac problems, cancer and other indicated conditions.

Sample Preparation

The general concepts to determine total thiols in plasma after reduction with tri-n-butylphosphine at pH 8.0, separation of the reduced forms from

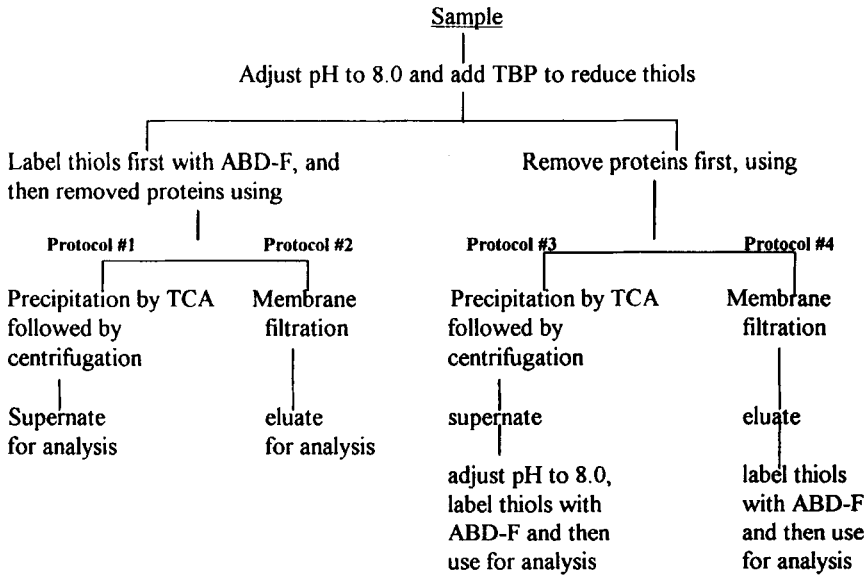

Figure 1. Summary of four different protocols that were used for sample preparation.

plasma proteins, and pre-column labeling with the ABD-F[10-13] were essentially followed. However, each individual step as well and it's sequence were evaluated to obtain results in a shorter time with high sensitivity using smaller sample volumes. For the separation of reduced thiols from plasma proteins and then labeling with ABD-F, four different protocols were used, and a summary of these different ones are given in Figure 1. Briefly, after reduction with tri-n-butylphosphine, the reduced thiols were derivatized, either before, or after removal of plasma proteins. Removal of proteins was accomplished either by acid precipitation using trichloroacetic acid (TCA) followed by centrifugation or by membrane filtration.

Calibration and Calculation

Individual stock solutions of cysteine (2 mM), homocysteine (0.5 mM), cystinylglycine (0.5 mM), (oxidized form), and glutathione (reduced form, 1 mM) were prepared by dissolving, first, in about 2 mL of 0.1 N HCl and then making up to volume with water. Stock solutions were stored at 4°C. A working standard mixture containing 100 μmol/L of homocysteine,

cystinylglycine, glutathione, and 400 μmol/L of L-cysteine was prepared using 0.2 M borate buffer, pH 8.0. For calibration, working standards, to have different levels in the range of 0 to 100 μmol/L, were prepared either in 0.2 M borate buffer, pH 8.0 containing 2 mM EDTA or in pooled plasma. Standards were reduced and derivatized similarly as the patient samples.

Each standard was analyzed in triplicate. From the resulting peak areas, a linear regression line was calculated by plotting concentrations on the abscissa and area on the ordinate. Concentration in the unknown sample was obtained using peak area of the sample from the slope of the calibration curve.

The detection limits were estimated, using aqueous standards, by injecting successively lower concentrations until a signal-to-noise ratio of 3:1 was obtained. Recoveries were determined by spiking plasma samples with known amounts from stock standards and by comparing the obtained values to the expected values after spiking. For this, standards at two different concentrations were used. Precision of the method was evaluated by calculating within-run and between-run coefficients of variation at three different concentrations.

Statistical Analysis

Data are presented as means ± SD. Linear regression analysis was performed using Spearman rank correlation. Student's t-test was used for comparisons, and a p value of < 0.05 was considered significant.

RESULTS AND DISCUSSION

Fluorescent Labeling

ABD-F was used as labeling agent for thiols, since it is highly reactive, even under mild conditions. It reacts faster with the —SH groups than with the other labeling agents, (e.g., SBD-F). As a result, the time required for completion was reduced to a maximum of 10 minutes. Further, ABD-F requires a reaction temperature of 50°C and, therefore, side reactions with other plasma compounds can be minimized. In this regard, this is the first report where ABD-F is used for a routine clinical work in a hospital setting.

Table 1

Comparison of Results Obtained after Using Four Different Sample Preparation Protocols

Protocol	Concentration (μmol/L,* Mean ± SD) Cysteine	Homocysteine
1	288 ± 11	10.8 ± 0.9
2	280 ± 8	10.9 ± 1.1
3	272 ± 16	10.1 ± 1.9
4	291 ± 13	11.1 ± 1.3

* Mean ± ISD for three different experiments.

Sample Preparation

The objectives here were to optimize conditions for reduction of the oxidized thiols, derivatization with ABD-F in the shortest possible time, and to use a minimum number of steps to minimize sample dilution. The pH for reduction of thiols with TBP, as well as the labeling of free —SH groups by the ABD-F reagent, is found to be optimal above pH 7.0.[10,12] However, ABD-adducts have maximum fluorescence at acidic pH.[10] To facilitate these two diametrically opposite optimal requirements, we have evaluated four derivatization protocols for labeling and removal of plasma proteins. A brief summary of these protocols and their ensuing results are given in Figure 1 and Table 1. Here, the following sample preparation protocols were evaluated:

1) Removal of proteins by precipitation with TCA, or

2) by membrane filtration; and

3) labeling of thiols before, or

4) after removal of plasma proteins.

Among these four different possibilities, labeling of thiols before the removal of plasma proteins, and removal of proteins by acid precipitation were found to be effective and convenient. This is due to the following reasons:

Both reduction and labeling require an optimum pH of above 7.0[10] and, therefore, reduction and labeling can be accomplished simultaneously without any need to manipulate the pH of these two reactions. Further, an added advantage here is that the normal plasma pH is around 7.4. By this, both reduction and labeling can be accomplished simultaneously at the same pH. Consecutive addition of TBP followed by ABD-F proved to be more convenient, since it minimized the number of steps. During the labeling step, any interference due to the presence of plasma proteins was not observed. However, it is possible that the ABD-F might also be reacting with the free —SH groups of these proteins, but it does not seem to be a problem here since the concentration of the ABD-F used here is 100 times higher than is required. Therefore, removal of proteins prior to the labeling of the —SH groups appears to be unnecessary.

Membrane filtration and acid precipitation, followed by centrifugation, were evaluated to remove proteins from the samples. Membrane filtration offered an advantage due to the absence of sample dilution. However, this procedure was found to be cumbersome for handling large numbers of samples, relatively expensive, and sample recovery was never more than 80%. This relatively poor recovery was found to be not ideal for small-volume, pediatric samples. Also, whatever advantage the membrane filtration procedure has offered, mainly in avoiding the sample dilution, at the end, it was nullified since it required pH adjustment to below 7.0 to obtain high fluorescence yield. Therefore, removal of proteins by acid precipitation using TCA was found to be convenient, easy and economical.

Based on these observations, the final optimized protocol for reduction and labeling, using smaller sample and reagent volumes, is as follows: To a 2.0 mL plastic centrifuge tube, 80 μL of sample or standards, 20 μL of 0.23 M borate buffer, pH 8.0, containing 4 mM Na_2 EDTA, were added, and the contents were mixed. To this, 10 μL of 10% (V/V) tri-n-butylphosphine in dimelthylformamide were added, mixed and incubated for 30 minutes at 4-6°C for reduction and decoupling of thiols from plasma proteins.

The —SH groups of these reduced and free thiols were labeled with ABD-F by adding 50 μL of ABD-F (5 mg/mL of 0.2 M borate buffer, pH 8.0 containing 2 mM Na_2 EDTA) and incubated for 10 minutes at 50°C in a circulating water bath. Proteins were precipitated by adding 40 μL of ice cold 20% trichloroacetic acid. Supernatants were obtained by centrifugation at 4°C for 10 minutes at 15,000 x g. About 120 μL of supernatants, containing the labeled homocysteine and other thiols, were carefully transferred to conical

autosampler vials, capped and analyzed immediately or stored at 4°C until analyzed, within 24-48 hours. Twenty-five microliters of each sample were used for the analysis.

The advantages of this protocol are: final dilution is only 2.5 X of the starting sample and, therefore, resulted in increased sensitivity, and combination of reduction and labeling steps. This procedure was simplified by avoiding the manipulation of sample pH. This, in turn, resulted in the use of small sample volumes for analysis.

Chromatography

The main objective, here, is to optimize the chromatographic conditions to obtain results in a shorter time, employing an isocratic elution system. A variety of parameters relating to columns, buffers, solvents, flow rates, sample-size, etc., were evaluated to obtain better resolution and to complete the analysis in a shorter time period.

1) Columns and Packing Material. Of the packing materials that were evaluated, a C_{18}, reverse phase, 5μ particle size was found to be superior to the others. In spite of the advantages offered by the cartridges (e.g., low cost, high flow rate, low back pressure, etc.), with cartridges peaks were never properly resolved and a "bunching" of the peaks was always seen. This is probably due to the weak binding of the ABD-adducts in presence of the solvents employed. Therefore, use of a long, standard size column was necessary for the proper resolution.

2) Buffers and Solvents. Several different buffers (e.g., biphthalate, pH 4.0; phosphate, pH 2.1 or pH 6.0; acetate, pH 4.0) and solvents of either methanol or acetonitrile were employed before for the isocratic resolution of ABD[11] and SBD[10,15,16] adducts of thiols in plasma. Phosphate buffer (0.1 M, pH 2.1) with 4% (v/v) ACN[16] did resolve peaks well, but resulted in poor precision. Under the acidic conditions, the column's effectiveness was poor beyond about 50 analyses. Acetate buffer, pH 4.0, required a longer time for completion.

Both ACN and methanol were effective, but methanol is preferred, due to its relatively low cost. Among the different concentrations, a methanol content of 8% gave a superior resolution. The concentration

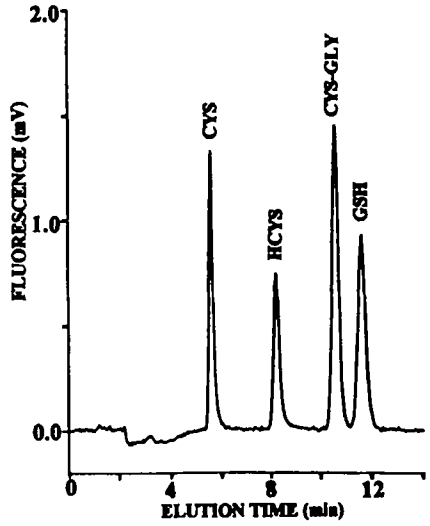

Figure 2. A typical elution pattern of a mixture of standards containing 200 μmol/L of cysteine (CYS), and 50 μmol/l each of homocysteine (HCYS), cystinylglycine (CYS-GLC) and glutalthione (GSH). Each peak represents 4 μmoles of cysteine and 1 μmole each of homocysteine, cystinylglycine and glutathione.

of methanol appears to be critical in terms of the time required for completion and for resolution. Thus, a phosphate buffer (0.1 M, pH 6.0) with 8% methanol (v/v) offered the best resolution and short period for completion of the analysis.

3) Flow Rate. A 2 mL/min flow rate was necessary to obtain sharper peaks. Although this high flow rate resulted in higher back pressure, it was necessary, since a flow rate of 1 mL/min resulted in peaks that were too broad and overlapping.

4) Sample Size. When different volumes of sample were injected, a 25 μL sample injection per analysis provided the best resolution and peak size which, in turn, gave a better precision in quantifying the peak areas.

Based on these observations, the following chromatographic conditions were found to be optimal: column size, 4.6 x 250 mm, with a packing of RP18 and 5 μm size particles; a 0.1 M phosphate, pH 6.0, buffer containing 8% (v/v) methanol; a 25 μL sample volume per injection; and an isocratic elution system with a 2.0 mL/min flow rate.

Under these conditions, the total analysis required only 12 minutes for completion, and it was able to analyze samples, in succession, every 12 to 14 minutes.

A typical chromatogram, showing an elution pattern of a standard mixture containing cysteine, homocysteine, cystinylglycine and glutathione, is presented in Figure 2. Using the above-described optimized chromatographic conditions, the elution times for these amino acids are: cysteine = 5.62 ± 0.16, homocysteine = 8.32 ± 0.23, cystinylglycine = 10.7 ± 0.5 and glutathione = 11.88 ± 0.47, minutes (mean ± SD, n = 20).

These elution times are relatively consistent and did not vary appreciably over a period of 6 to 8 weeks of continuous assays. Sometimes, an occasional drift in the elution time was seen with cystinylglycine and glutathione, but not with cysteine and homocysteine. This drift in elution time with cystinylglycine and glutathione was easily corrected by changing the methanol concentration in the elution buffer. During this work, it was observed that the elution, as well as clear separation of cystinylglycine from glutathione, are dependent on the methanol concentration in the buffer. Increase in percent of methanol resulted in faster elution times but poorer resolution; and an opposite effect (viz. better resolution and longer elution times) with the decreased percent of methanol. However, methanol concentration of the buffer has no significant influence on the resolution of cysteine and homocysteine.

Figure 3 represents a typical chromatogram showing different thiols that are commonly present in "normal" human plasma. Based on elution of standards, these were identified as cysteine, homocysteine, and cystinylglycine. Only three adducts of ABD-F (cysteine, homocysteine, and cystinylglycine) are seen in a typical plasma. However, ABD-glutathione is seen in samples with hemolysis only. Two additional unidentified peaks, labeled peak A and B in Figure 4, are always seen in samples from patients with renal failure. They are present at both pre- and post-dialysis and, therefore, dialysis did not specifically remove these ABD-reacting compounds.

Dialysis did decrease their concentrations, especially peak B. Although these two unidentified compounds are present in patients with renal failure, they did not interfere with resolution of homocysteine and cystinylglycine, since they are eluted between homocysteine and cystinylglycine. Except for these two, no other ABD-adducts were seen in over 400 samples analyzed, from patients with a variety of diseases and medications, indicating a high degree of specificity of the ABD-F.

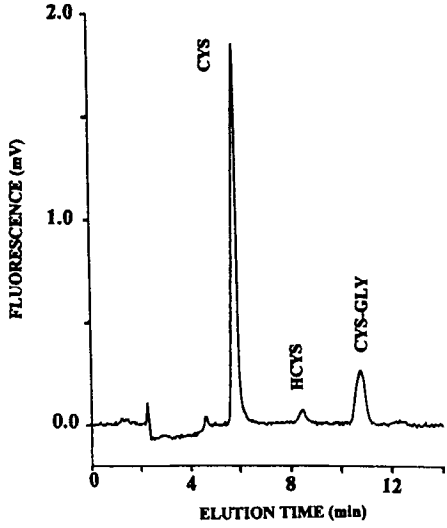

Figure 3. Elution pattern of various thiols that are seen in a "normal" human plasma sample, showing cysteine (CYS), homocysteine (HCYS) and cystinylglycine (CYS-GLY).

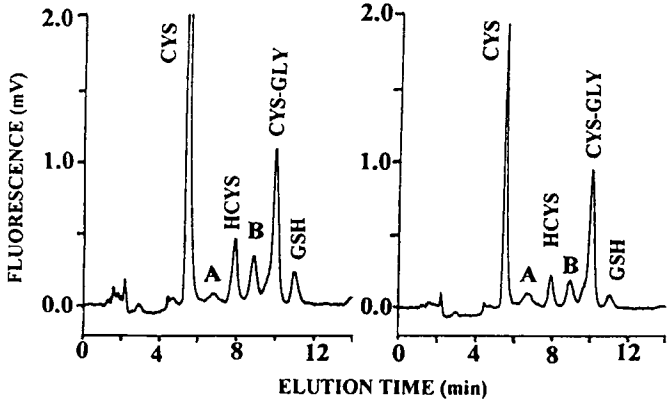

Figure 4. Pattern of various thiols that are seen in samples from patients with renal failure at pre- (Fig. 4A, left) and post- (Fig. 4B, right) dialysis, showing cysteine (CYS), homocysteine (HCYS), cystinylglycine (CYS-GLY), glutathione (GSH) and two unidentified compounds (peaks A and B).

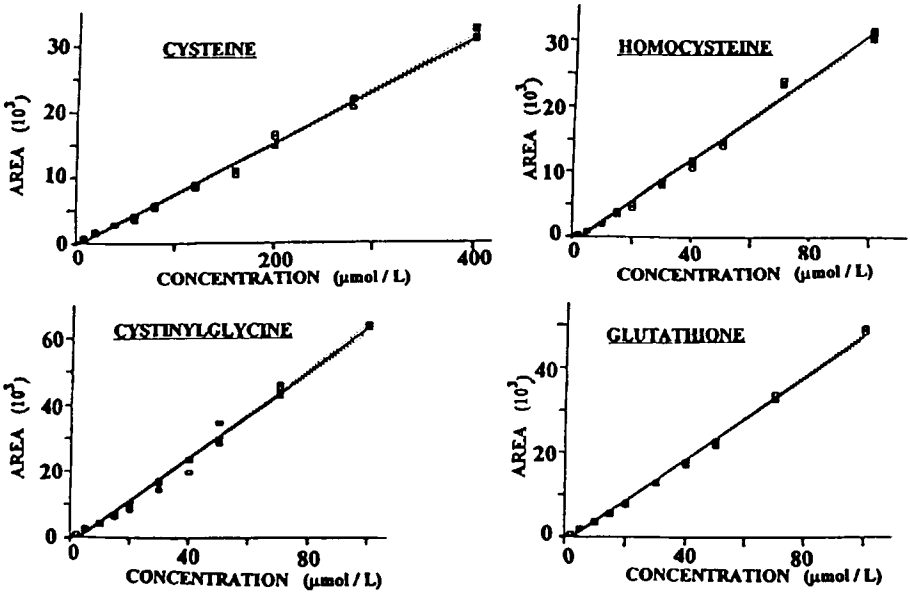

Figure 5. Calibration Curves for cysteine, homocysteine, cystinylglycine, and glutathione. The obtained regression equations were, cysteine: Y = -428 + 78 X, r^2 = 0.9970, X-intercept = 5.0; homocysteine: Y = -1061 + 326 X, r^2 = 0.9916, X-intercept = 3.3; cystinylglycine: Y = - 1860 + 647 X, r^2 = 0.9964, X-intercept = 2.9; glutathione: Y = - 1220+488 X, r^2 = 0.9979, X -intercept = 2.5 (X=concentration, μmol/L; Y= peak area).

Correlation

The present method, using ABD-F as the labeling agent, together with the modifications in sample preparation and fractionation protocol by the HPLC, was compared to the previously published procedure of Araki and Sako[13] as modified by Ubbink et al.[15] (see Table 2). It provided slightly lower values for cysteine and homocysteine. In spite of several modifications to the sample preparation and chromatography, reasonably good, comparable, data were obtained between these two methods.

Linear regression analysis of the data yielded good agreement between these two assays, and the obtained regression equations are: for cysteine, y = -4.2 + 1.012 X, r^2 = 0.9382; and for homocysteine, y = -0.18 + 1.001 X, r^2 = 0.9954.

Table 2

Validation of the Modified Derivatization Protocols to Determine Total Cysteine and Homocysteine

Amino Acid	n	Method	Concentration (μmol/L) Mean	SD	Range	p*
Cysteine	20	Ubbink et al.	291	68	152-380	---
		Present	280	72	141-364	0.0904
Homocysteine	20	Ubbink et al.	11.6	7.2	5.9-28.0	---
		Present	11.0	7.3	5.1-26.0	0.0325

* Paired t-test, two tailed. The regression analysis provided the following: Cysteine, $Y = -4.2 + 1.012X$, $r^2 = 0.9382$; Homocysteine, $Y = -0.18 + 1.001X$, $r^2 = 0.9954$. (Y = present method, and X = method of Ubbink et al.[15]

Table 3

Regression Analysis of Concentration of the Amino Acid and Obtained Peak Areas

Amino Acid	Regression Equation	r^2	X-intercept
Cysteine	y = -428 + 78X	0.9941	5.0
Homocysteine	y = -1061 + 326X	0.9893	3.3
Cystinylglycine	y = -1860 + 647X	0.9927	2.9
Gluthathione	y = -1220 + 488X	0.9958	2.5

Linearity

Excellent linearity to all the amino acids, when tested at 0 to 400 μmoles/L cysteine and 100 μmoles/L for others, was seen (see Fig. 5). We did not test these compounds at higher concentrations. Linear regression analysis was performed by plotting the observed peak area (Y) versus concentration in μmoles/L (X) of each thiol. The obtained linear regression equations showed a high degree of correlation between peak area and concentration (Table 3).

Table 4

Recoveries of Analysis

Compound	Concentration (μmol/L) Expected	Obtained	Recovery (%)
Cysteine	40.0	37.6	94
	200.0	210.0	105
Homocysteine	10.0	9.2	92
	50.0	52.0	104
Cystinylglycine	10.0	9.6	96
	50.0	52.4	105
Glutathione	10.0	9.6	96
	50.0	52.4	105

Recovery

Recoveries of analysis at two different concentrations are given in Table 4. They were obtained by analyzing samples with a known addition of amino acids to a freshly pooled human plasma. Excellent recoveries, with a maximum of ± 6% mean, were always obtained.

Detection Limits

Limits of quantitation (detection) were calculated from regression lines that were obtained with calibration curves from Table 3. The lowest quantifiable (detectable) limit, in μmoles/L, are: cysteine = 4.3, homocysteine = 3.3, for cystinylglycine = 2.9, and glutathione = 2.5.

In this assay, the minimal amount of detection, at a signal-to-noise ratio of 3:1, is about 5 pmol of each amino acid. With a starting plasma sample 80 μL and 25 μL for each analysis, the calculated sensitivity is at least 10 pmoles for each amino acid.

Table 5

Reproducibility at Within- and Between-Days, and at Three Different Concentrations

Compound	Conc'n	n	Reproducibility (% CV ± SD) Within-Day	Between-Days
Cysteine	20	5	5.4 ± 2.1	5.8 ± 2.8
	80	5	4.2 ± 1.8	5.8 ± 2.0
	200	5	3.1 ± 1.0	4.7 ± 1.8
Homocysteine	5	5	4.6 ± 2.0	4.9 ± 2.0
	20	5	3.7 ± 1.7	4.2 ± 1.8
	50	5	2.8 ± 0.8	3.6 ± 1.0
Cystinylglycine	5	5	3.8 ± 1.6	5.7 ± 2.0
	20	5	2.7 ± 1.0	4.5 ± 1.4
	50	5	1.9 ± 0.8	3.9 ± 1.0

Reproducibility

The method was tested at 200 μmol/L for cysteine and 50 μmol/L, each, for other amino acids. Each sample was tested at least 5 times on each day and on three different days. The CV's for reproducibility within-day was found to vary 1.9 to 3.8%, and between-days from 3.6 to 5.7%, (Table 5), indicating that this is a highly reproducible protocol.

Reference Range

Using the present protocol, reference ranges for total cysteine and homocysteine were calculated for pediatric populations ranging from 0.1 to 18.0 years of age, representing both sexes. The obtained ranges (mean ± 2SD) for total homocysteine and cysteine are: 3.3 to 14.5 and 122-346, μmoles/L, respectively. No significant sex- or age-dependent differences in reference values for total cysteine and homocysteine were observed in children up to 18 years of age. This is in contrast to the data obtained for adults,[1,17] where males tend to have higher homocysteine levels compared to females.

We have presented, here, a modified protocol to determine total homocysteine and total cysteine using ABD-F as the fluorescent labeling agent. Sample preparation and fractionation conditions of the ABD-adducts by the HPLC were optimized for small-volume, pediatric samples.

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