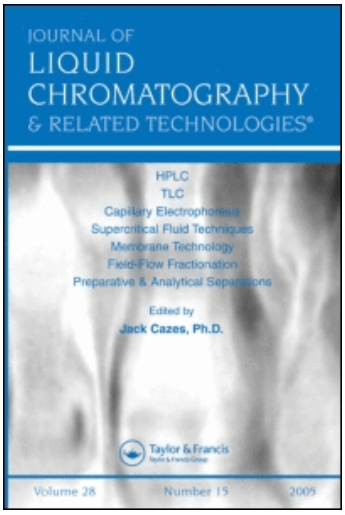

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Serum and Tissue Homocysteine Analysis by HPLC

Z. K. Shihabi[a]; M. E. Hinsdale[a]; P. E. Lantz[a]
[a] Pathology Department, Bowman Gray School of Medicine Wake Forest University, Winston-Salem, NC, USA

To cite this Article Shihabi, Z. K. , Hinsdale, M. E. and Lantz, P. E.(1998) 'Serum and Tissue Homocysteine Analysis by HPLC', Journal of Liquid Chromatography & Related Technologies, 21: 17, 2715 — 2723
To link to this Article: DOI: 10.1080/10826079808003418
URL: http://dx.doi.org/10.1080/10826079808003418

J. LIQ. CHROM. & REL. TECHNOL., 21(17), 2715–2723 (1998)

SERUM AND TISSUE HOMOCYSTEINE ANALYSIS BY HPLC

Z. K. Shihabi, M. E. Hinsdale, P. E. Lantz

Pathology Department
Bowman Gray School of Medicine
Wake Forest University
Winston-Salem, NC 27157, USA

ABSTRACT

A simplified HPLC method for analysis of homocysteine based on a two step procedure is described. In the first step, a simultaneous reduction, and derivatization of this amino acid in the presence of serum proteins takes place. In the second step proteins are removed by acid precipitation followed by an HPLC with fluorescence detection. The method is rapid and utilizes small volumes of serum. Both serum and tissues were analyzed by this method. The reference range for serum is 4-16 μmol/L, n=34. Patients with renal failure are shown to have elevated mean serum levels (18.5 μmol/L, n=20) compared to normal individuals (10.1 μmol/L, n=34).

INTRODUCTION

Elevated serum homocysteine levels occur in several disorders such as homocysteinuria, neural tube defects, renal failure, and folic acid and cobalamin deficiencies. Furthermore, these elevated levels are considered as an important risk factor for arterial occlusive disorders. [1-3] Thus, there is a great interest for a simple method suited for routine analysis of this amino acid.

Several difficulties are encountered with homocysteine analysis: The low concentration in serum (4-16 μmol/L) and the lack of a chromophoric group. In addition to that, most of the homocysteine in serum is bound to proteins and other thiol-containing compounds. Several HPLC and GC methods have been described for analysis of homocysteine. These methods involve basically the reduction of homocysteine, removal of the proteins, and multiple steps of pH adjustment followed by either direct electrochemical[3] or reaction for fluorescence [4-6] detection. Recently, Reddy and Behnke[7] described a method for direct analysis of homocysteine in which serum is reduced first then reacted with 4-(aminosulfonyl)-7-fluoro 2,1,3-benzoxadiazol (ABDF)[8]. We simplified the method further by adding the serum, the reducing agent (KBH4), and the ABDF; all at the same time. After 40-minutes of incubation at 47°C, trichloroacetic acid is added to precipitate serum proteins before injection on the column. Since data for the homocysteine level in tissues is lacking we applied this method to the analysis of this amino acid in both serum and tissues. Furthermore, this method is compared to a GC-MS procedure. [9]

EXPERIMENTAL

HPLC Instrument

We used a Model 112 pump (Beckman Instruments, Fullerton, CA) set at a flow rate of 1.5 mL/min; and a Model Spectra/Glo (Gilson Medical Instruments, Middleton, WI) with an excitation at 390 nm and an emission at 475 nm. The column was 250 x 4.6 mm (i.d.) CN Microsorb, 5 μm average particle size (Varian, Walnut Creek, CA). Every 50 samples the first few millimeters of the column bed is removed and replaced with the same but fresh packing material.

Tissue Homogenates

Tissues (0.2 g) from the human autopsy materials were homogenized in 1 mL water and centrifuged for 1 min at 14,000 rpm. The supernatant was used for analysis.

Reagents

EDTA-borate: Sodium EDTA 50 mg, isoamyl alcohol 1 mL, urea 5 g, and boric acid, 1 g were dissolved in 200 mL water. The pH was adjusted to 8.3 with NaOH (refrigerated). 2-Mercaptoethylamine (1 mg/L) can be added to this reagent as an internal standard for retention time only.

KBH_4 Reagent: KBH_4 (10 mg), immediately before use, was dissolved in 2 mL EDTA-Borate reagent. This reagent is mixed or tapped gently each time before introducing the pipette to eliminate the air bubbles in order to aspirate the correct volume.

Stock Homocysteine Standard: Equivalent to 1000 μM of homocysteine, refrigerated.

Working Homocysteine Standard: Equivalent to 24.4 μM of homocysteine. The stock reagent 500 μL is mixed with 20 mL water, refrigerated.

ABDF: 4-(aminosulfonyl)-7-fluoro 2,1,3-benzoxadiazol 2 mg/mL (Molecular Probes, Eugene, Oregon); a 1-mL aliquot was refrigerated while the other aliquots were kept frozen.

Pump solvent: 0.75 mL acetic acid, 1.2 g sodium acetate, and 60 mL acetonitrile were dissolved in 940 mL H_2O. For tissue analysis, the acetonitrile is reduced to 50 mL.

Check sample: ABDF 100 μL was incubated with 100 μL of 1 mM homocysteine for 40 min at 47°C and diluted to 5 mL with the pump solvent, refrigerated. This sample was injected daily to check on the column performance.

Procedure

In a 1.5 mL microfuge tube, 25 μL of serum (or tissue homogenates), and 25 μL ABDF were added. KBH_4 reagent was prepared fresh and immediately 100 μL was added to the tubes. The tubes were vortex mixed for 15 sec., and incubated at 47°C for 40 min. During the first ten minutes, the tubes were mixed and tapped occasionally to decrease the air bubbles. Finally, 25 μL of 50% trichloroacetic acid was added to the tubes, mixed, and centrifuged at 14,000 for 60 sec. An aliquot of 50 μL of the supernatant was injected on the column.

RESULTS AND DISCUSSION

The major advantage of this method is it avoids the multiple steps of pH adjustment used in other methods, which are difficult to perform accurately on small volumes. It is basically a two-step procedure. The first step is a simultaneous release of the SH group from the proteins and derivatization of the reduced homocysteine with ABDF for fluorescence detection. In the second

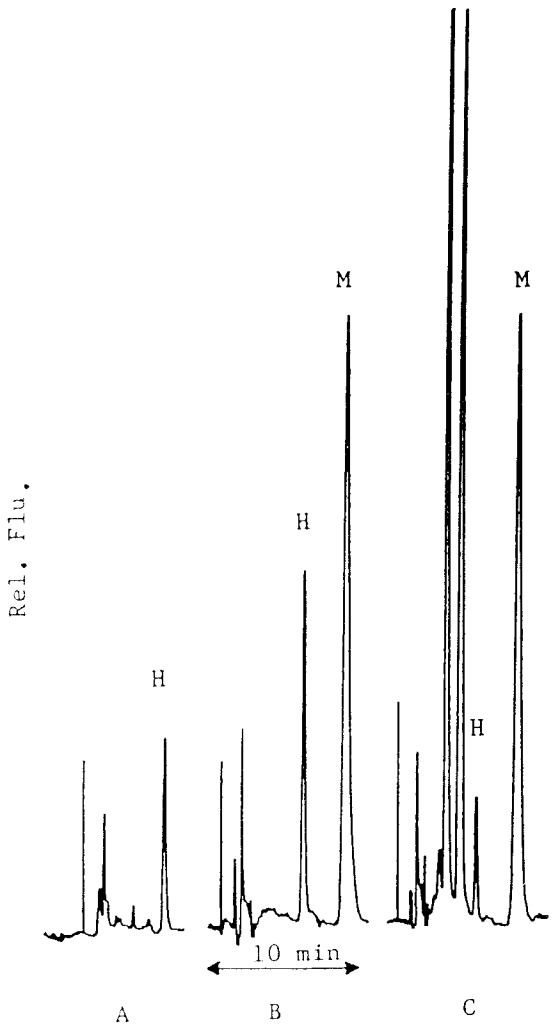

Figure 1. A chromatogram of: A- The check sample, B-Standard, 24 μM and C-Patient serum homocysteine (9.6 μM); (H, homocysteine, M = 2-mercaptoethylamine & others).

step serum proteins are removed by the acid deproteinization. Figure 1 shows a typical chromatogram for homocysteine analysis. The retention time for homocysteine is about 7 min and the chromatogram is completed in about 10 min. Other SH containing compounds such as cysteine and methionine, N-acetylcysteine do not interfere.

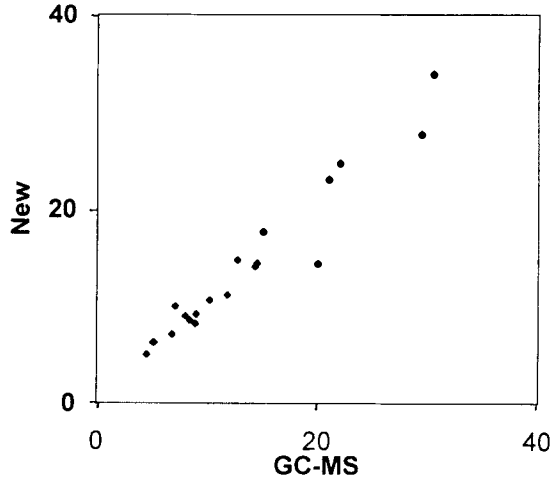

Figure 2. Comparison of homocysteine analysis by the new method and by GC-MS(9) (r=0.96).

The test is linear by peak height between 1-30 μmol/L. Under these conditions the within-run CV is 3.6 %(n=13, mean 14.2 μmol/L). Avoiding an excess of air bubbles is very important for obtaining a homogeneous mixture and for accurate volume delivery by the pipette. It is a key element for good precision. Replacing the top of the column with fresh packing routinely to prevent a decrease in the peak height is another important element for good precision. The check sample is used as a simple and direct method to monitor the column performance.

The KBH4 regent has to be prepared and used immediately to prevent a decrease in the peak height. The optimum concentration of the KBH_4 is about 5 g/mL. Increasing the concentration by 2 and 4 times reduced the peak height of homocysteine by 40 and 60 %; respectively. The average recovery of 7 μmol/L of homocysteine added as homocystine to a serum pool is 96.6 (n = 5). This method correlated well with a GC-MS method (r = 0.96, n = 20), Fig 2.

Homocysteine is increased in patients with primary homocysteinuria, renal failure [10,11] and those with folic acid deficiency. Fig 3 shows the distribution of homocysteine in patients with renal failure vs. normal individuals. The reference interval for normal individuals is 4-16 μmol/L (n=34) which is close that reported by other workers. [3,4,7] A great number of those patients with renal failure have an increased serum level, Fig. 3.

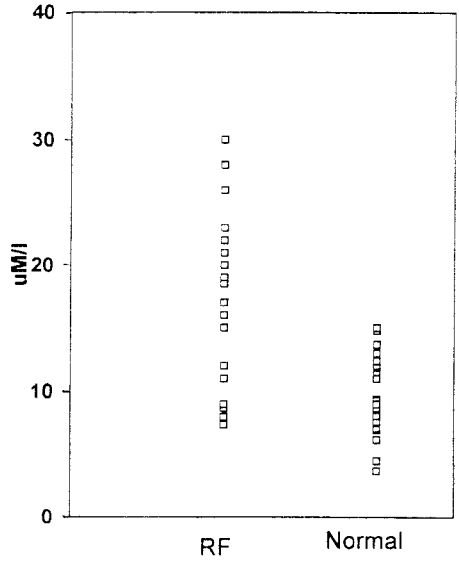

Figure 3. Homocysteine distribution in normal individuals and in patients with renal failure (RF).

Table 1

Homocysteine Levels in Human Tissues

	N	μM/Kg*	SD
Brain	8	4.3	2.7
Liver	5	10.2	5.4
Kidney	8	10.1	5.0
Heart	5	4.8	1.3

* Wet tissue.

Although the literature is abundant with values regarding serum level under normal and different pathological conditions [3,4,10,11]; there is almost no data concerning the level of this amino acid in tissues in normal or in pathological conditions. Thus, this method was extended to the analysis of homocysteine in tissues from autopsy materials. All the tissues were re-analyzed after spiking with 10 μmol/L of standard for verification. Surprisingly

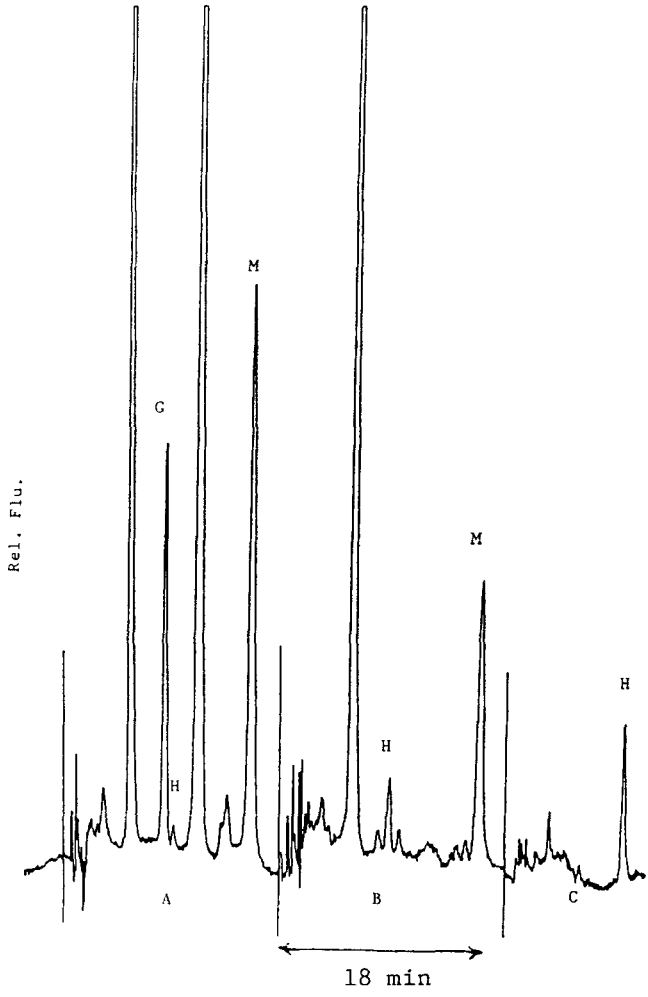

Figure 4. A chromatogram of homocysteine in: A- brain; B- Kidney; and the check sample (H = homocysteine, M = 2-mercaptoethylamine, and G = glutathione).

some tissues such as the brain have lower level than that of serum, Table 1. This low level could be due to the presence of enzymes in the tissues, which metabolize or convert this compound to other intermediates. Many of the tissues, including the red cells, contain high amounts of glutathione which elutes immediately before homocysteine (Fig 4), but it is almost absent in serum. Thus, the amount of acetonitrile in the elution buffer is reduced when these tissues are analyzed.

We attempted using membrane dialysis similar to what we described earlier for carnitine analysis[12] to increase the sensitivity for tissues; however, this also increased the analysis time and the number of steps. We used in some of this work 2-mercaptoethylamine and N-acetylcysteine as internal standards added to the EDTA-borate reagent. Since these compounds are not bound to proteins as the homocysteine they do not behave as true internal standards for quantification (only for retention time). Furthermore, since extraction is not involved here there is no great advantage to having an internal standard.

Before injection on the column we removed the proteins by acid precipitation. This step can be avoided by using "Restricted Access Media" columns for direct serum injection[13] rendering the method suitable for full automation. Unfortunately these columns are two expensive. The main advantage of this method is simplicity, which yields good precision and makes it suitable for routine work. As evident, this method is suitable for analysis of not only serum but also tissue samples.

ACKNOWLEDGMENT

The authors would like to thank Dr. Peter Wentz, LabCorp, Burlington, NC, for his help.

REFERENCES

1. M. R. Malinow, J. Intern. Med., **236**, 603 (1994).

2. S. S. Kang, P. W. K. Wong, M. R. Malinow, Ann. Rev. Nutr., **12**, 279 (1992).

3. M. R. Malinow, S. S. Kang, L. M. Taylor, P. W. K. Wong, B. Coull, T. Inahara, D. Mukerjee, G. Sexton, B. Upson, Circulation, **79**, 1180 (1989).

4. P. M. Ueland, H. Refsum, S. P. Stabler, M. R. Malinow, A. Andersson, R. H. Allen, Clin. Chem., **39**, 1764 (1993).

5. B. Vester, K. Rasmussen, Eur. J. Clin. Chem. Clin. Biochem., **29**, 549 (1991).

6. T. Fiskerstand, H. Refsum, G. Kvalheim, P. M. Ueland, Clin Chem., **39**, 263 (1993).

7. M. N. Reddy, C. A. Behnke, J. Liq. Chromatogr., **20**, 1391 (1997).

8. T. Toyo'oka, K. Imai, J. Chromatogr., **282**, 495 (1983).

9. S. P. Stabler, E. Podell, R. H. Allen, G. Savage, J. Lindenbaum, J. Clin. Invest., **81**, 466 (1988).

10. V. W. Dennis, K. Robinson, Kidney Intl. Supp., **57**, S11 (1996).

11. M. Arnadottir, B. Hultberg, P. Nilsson-Ehle, H. Thysell, Scand. J. Clin. Lab. Invest., **56**, 41 (1996).

12. Z. K. Shihabi, K. S. Oles, C. P. McCormick, J. K. Penry, Clin. Chem., **38**, 1414 (1992).

13. K. S. Boos, A. Rudolphi, LC-GC, **15**, 602 (1997).

Received January 8, 1998
Accepted February 15, 1998
Manuscript 4712