Homocysteine Determination in Plasma

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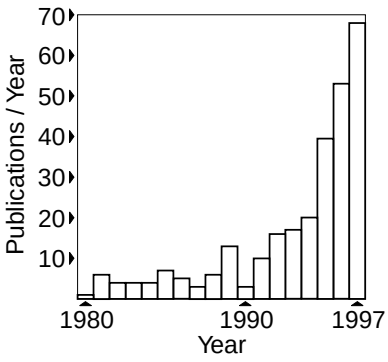
Recent publications suggest that high homocysteine levels are either 1) the cause of heart disease, 2) an independent risk factor for heart disease, 3) the result of heart disease, or 4) unrelated to heart disease. In any event, for both clinical and research purposes, a reliable method is needed for the determination of homocysteine in plasma. BAS launches its Total Plasma Homocysteine Kit to fill the need for collection of definitive data in larger populations. The kit is based on liquid chromatography with electrochemical detection (LCEC) and exhibits >97% recovery, with among-day RSD of 0.9 - 1.9% and within-day RSD of 0.3 - 0.4%.

Homocysteine (***F1***) is a naturally occurring amino acid, high levels of which have been associated with coronary artery disease (1-5). The link was first suggested by Kilmer McCully, who extrapolated the association from studies of patients with homocystinuria, a genetic defect leading to abnormally high levels of homocysteine (4). Children with this defect typically die at an early age from the complications of arteriosclerosis. If huge excesses of homocysteine can cause this, McCully reasoned, could moderately elevated levels cause the heart disease seen in middle-aged and older people? And will therapy to lower homocysteine levels reduce the incidence of cardiovascular disease? BAS hopes to contribute to the answers to these questions by making the determination of plasma homocysteine simple and reliable.

Much excitement has developed over the homocysteine theory of arteriosclerosis from articles that appeared in the popular press—the *New York Times*, *Time* magazine, *Newsweek*, television talk shows, and web sites. Basically, two features of the homocysteine theory have generated this excitement.



One is that it explains a number of observations of the disease that cannot be explained by the cholesterol/fat hypothesis. Second, the disease in many instances can be retarded by vitamin (B_6, B_{12}, folate) supplements and/or dietary changes. In fact, these changes early in life may prevent the disease or retard its onset.

Homocysteine Metabolism

Dietary factors that influence the concentration of blood homocysteine (HCys) are the amount of vitamins B_6, B_{12}, and folic acid in the diet and the total methionine content of dietary protein. Methionine is an essential amino acid and the only source of HCys in the body. The remethylation of HCys to methionine is catalyzed by a B_{12}-folate dependent enzyme (***F1***). HCys can also be metabolized to cysteine, via two B_6 dependent enzymes, for further breakdown and excretion in the urine. This pathway through transsulfuration is not reversible, while that through remethylation is reversible.

The role of B vitamins in HCys metabolism is supported by the observation of a negative relationship between vitamin status and HCys concentrations (6). Moreover, this relationship suggests a simple therapy for cases of moderately elevated HCys—reduced intake of animal protein and increased intake of B vitamins. Obviously, factors

other than diet are also important, such as genetic disorders (hereditary deficiency of cystathione β-synthase and enzymes involved in the synthesis of 5-methyltetrahydrofolate and CH_3-B_{12}), age, gender, toxins, lack of exercise, and elevated blood cholesterol (to list a few).

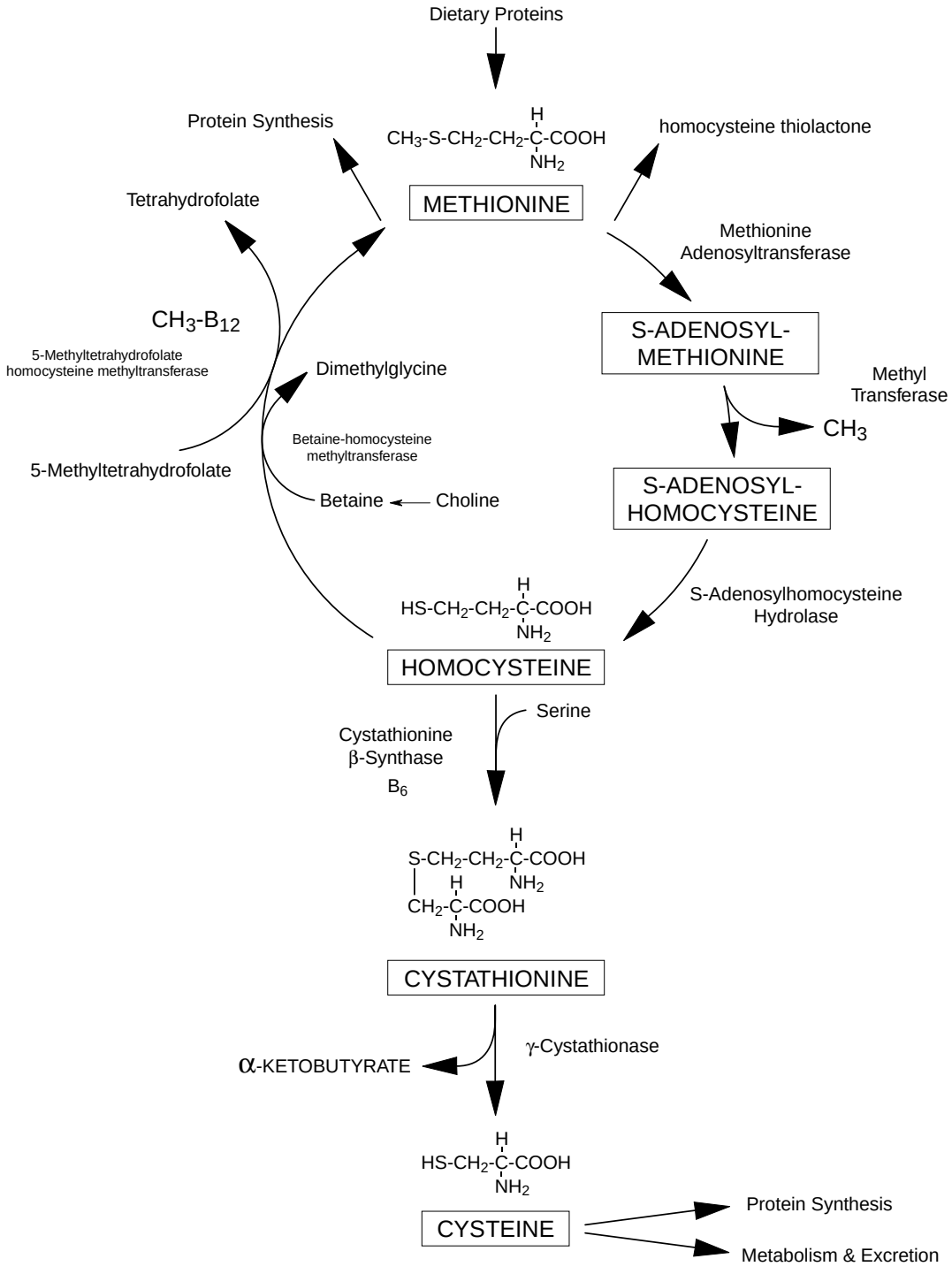
Elevated plasma HCys is associated with plaque formation from increased deposition of collagen and calcium, elastin degeneration, and endothelial cell damage. Theories have proposed that these effects occur by direct action of HCys or through a reactive form, homocysteine thiolactone. For an extensive and detailed presentation of the homocysteine theory of arteriosclerosis, we direct you to the recent book by Dr. McCully (3).

Determination of Homocysteine

Homocysteine occurs in plasma as the free thiol (trace amounts), its symmetrical disulfide

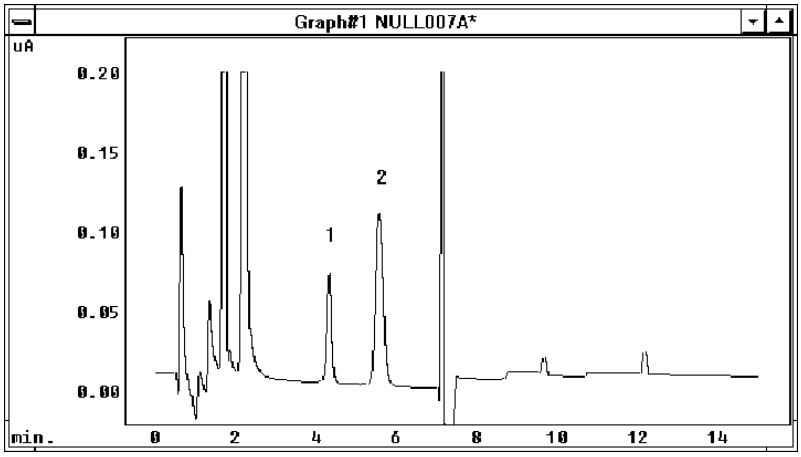
F1

Selected pathways of homocysteine biochemistry. Homocysteine, when taken as a branch point, is methylated to form methionine (remethylation) or condensed with serine to form cystathione that is then hydrolized to ketobutyrate and cysteine (transsulfuration).



F2

Separation of a typical plasma sample. Peak 1 = HCys. Peak 2 = cys-gly. The cleaning pulses are apparent at 7 minutes.



(homocystine) and asymmetrical disulfides (15-20%), and conjugated with protein (80-85%) through disulfide linkages. The bulk of plasma HCys thus occurs in conjugated form, making it inaccessible to common analytical techniques. Samples must therefore be treated with reducing agent before analysis to liberate HCys as the free thiol.

The commonly used reducing agents present problems, however. Sodium borohydride, for example, is most effective when the reduction process is performed at 50° C. And it tends to foam, so an antifoaming agent such as *n*-amyl alcohol (1) or methanol (5) must be added. Another reducing agent, tri-*n*-butylphosphine (7,8), is effective under a variety of conditions but presents the danger of explosion in its concentrated form. In contrast, the BAS procedure uses a safe reducing agent that does not cause foaming and is effective at room temperature (18° C and above). With our procedure, reduction takes place in just ten minutes on the bench—the time it takes to prepare the autosampler vials to receive the samples.

Previous assays for plasma HCys have used liquid chromatography (LC) with either an electrochemical detector (1,5) or a fluorescence detector (7,8). Fluorescence procedures have the disadvantage of requiring a derivatization step in addition to the required reduction step. And the derivatives are only marginally stable. An electrochemical detector oxidizes HCys directly, so no derivatization is needed. And in contrast to fluorescent derivatives, HCys is stable when the samples emerge from the reduction step of the BAS procedure.

The first LCEC determination of plasma HCys used a mercury pool electrode (12), a clever but not very user-friendly EC cell. Still using the thiol selectivity of mercury, in 1982 BAS commercialized a solid mercury film electrode in the form of a dual Hg/Au amalgam electrode. This electrode, when placed post-column in a series configuration, can be used to determine both thiols and disulfides (13). After LC separation, the eluate passes over the upstream electrode held at a reductive potential, to cleave disulfides to thiols. All thiols, whether present naturally or as upstream conversion products, are detected at the downstream oxidative electrode. A single oxidative Hg/Au electrode can be used if only thiols are to be determined. A variety of biological and synthetic thiols/disulfides have been determined by LCEC utilizing Hg/Au electrodes (14 and references therein). The first use of this technology to study plasma HCys concentration—and its relationship to B_6 deficiency—was reported in 1982 (15). The routine use of LCEC to determine basal concentrations of plasma HCys was instituted in Dr. R. Malinow's lab and resulted in a 1989 publication which confirmed that an elevated concentration of plasma HCys is an independent risk factor for occlusive atherosclerosis (1). This pioneer method has been used as a "reference method" for the development of LC/fluorescence methods for quantitation of plasma HCys.

A gold electrode has also been used successfully for LCEC determination of plasma HCys concentration (5). The gold electrode requires less preparation and maintenance, exhibits good selectivity for thiols, and does not require the use of toxic mercury. We have found, however, that a platinum electrode provides all these advantages and is more rugged than a gold electrode. We have therefore chosen a platinum electrode for the BAS kit and have developed a proprietary pulsing scheme to clean the electrode between sample injections.

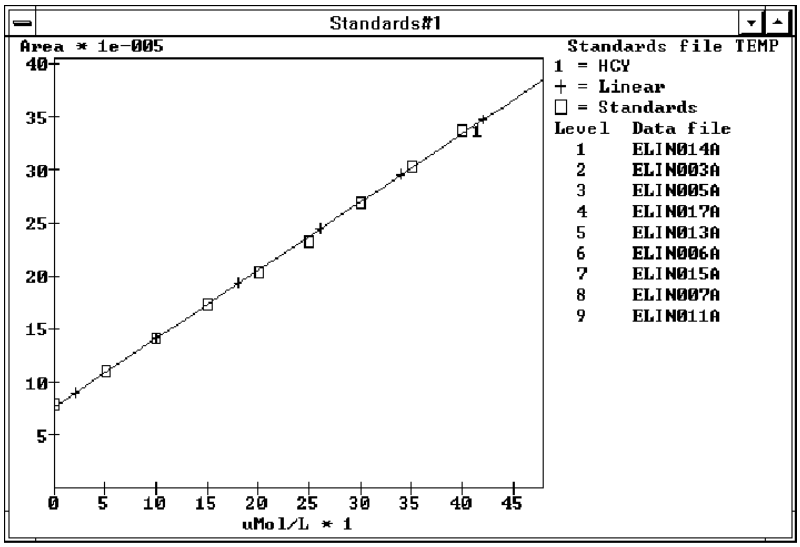
In addition to these advantages, the BAS Total Plasma Homocysteine Kit furnishes a notable separation (***F2***). Our proprietary chemistry provides a large, unambiguous HCys peak and a noise-free baseline, allowing rapid and accurate quantitation.

Specimen Collection, Preservation, and Storage

Blood samples should be collected in EDTA tubes. Separate the plasma from the blood cells by centrifugation at 2000 x g for 5 min at 0-2° C (9). This should be done as soon as possible after collection because erythrocytes continue to export HCys in collected whole blood (10). Addition of sodium fluoride to a final concentration of 4 g/L of blood has been proposed to inhibit this release (11). Store plasma at -70° C if it will not be analyzed immediately. Repeated freeze/thaw cycles may increase HCys levels and should be avoided.

F3

Linear regression of endogenous calibrators, from 0 - 40 µmol/L added to pooled plasma.



T1

Quantitation of pooled plasma samples spiked with known amounts of homocystine and determined by the BAS procedure.

Sample Type	Expected Value	Instrument 1 (BAS LC-44)	Instrument 2 (BAS 200B)
Plasma	—-	15.1	15.4
Plasma	—-	15.4	15.5
Plasma	—-	14.8	15.1
Plasma + 5	20.2	20.3	20.3
Plasma + 5	20.2	20.4	20.4
Plasma + 5	20.2	20.0	19.9
Plasma + 15	30.2	30.6	30.3
Plasma + 15	30.2	30.2	30.4
Plasma + 15	30.2	29.9	29.6
Plasma + 25	40.2	40.6	38.9
Plasma + 25	40.2	40.0	39.6
Plasma + 25	40.2	36.6	39.7

T2

Pooled plasma samples quantitated against external and endogenous standards.

Date	Homocysteine Endogenous	Homocysteine External	% Difference
31 Oct	12.7	12.8	0.5
	13.0	12.6	2.8
5 Nov	13.1	13.1	0.4
	12.9	13.2	1.9
18 Nov	12.5	12.3	1.4
	12.4	12.3	1.2
11 Dec	12.6	12.9	2.0
	13.1	12.4	5.8
Among-day RSD	0.9%	1.9%	
Within-day RSD	0.4%	0.3%	

Experimental Assay Procedure

Aliquots of plasma (200 µL) are combined with two other reagents in 1.8 mL microcentrifuge tubes and allowed to react at room temperature for ten minutes. A protein-precipitating reagent is then added and the tubes are centrifuged for five minutes. A 10 µL aliquot of the supernatant is injected into the chromatograph, resulting in a chromatogram similar to that in ***F2***.

Calibration

Either external aqueous calibrators or endogenous (using pooled plasma) calibrators may be used. We recommend the endogenous method because of greater stability of these samples (up to 24 hours at 4° C). ***F3*** shows a standard curve of endogenous calibrators.

Results

Accuracy

T1 shows results obtained when pooled plasma was spiked with known amounts of the disulfide homocystine, reduced with the kit reagents, and quantified against external standards on two LCEC systems. Results are in µmol/L. Expected value is the sum of the average unspiked plasma value plus the spike.

Precision

T2 shows the HCys content of the pooled plasma, determined over several days by the standard addition (endogenous) method and with external aqueous calibrators. Results are in µmol/L.

Conclusions

The BAS Total Plasma Homocysteine Kit provides accurate, precise determination of plasma HCys levels at reasonable cost. The kit features easy-to-use reagents, high sensitivity, and superb chromatography. We believe this is an excel-

lent tool to further explore the role of HCys as a diagnostic marker.

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