

**Total-Homocysteine Enzymatic Assay**, Yuying Tan, Li Tang, Xinghua Sun, Nan Zhang, Qinghong Han, Mingxu Xu, Eugene Baranov, Xuezhong Tan, Xiuying Tan, Babak Rashidi, Zili An, Andrew W. Perry, and Robert M. Hoffman\* (A/C Diagnostics, LLC, and AntiCancer, Inc., 7917 Ostrow St., San Diego, CA 92111; \* author for correspondence: fax 858-268-4175, e-mail all@anticancer.com)

McCully (1) in 1969 first observed that plasma hyperhomocysteinemia was involved in the pathogenesis of arteriosclerosis. More studies have reported that total serum or plasma homocysteine (tHCY) is an independent risk factor for cardiovascular diseases, including arteriosclerosis, coronary artery disease, cerebrovascular disease, and myocardial infarction (2–7). Furthermore, plasma tHCY is a strong predictor of mortality in patients with existing coronary artery disease (8).

A major basis for hyperhomocysteinemia is insufficient intake of vitamins B<sub>6</sub>, B<sub>12</sub>, and folic acid, which are necessary for homocysteine metabolism and which lower the risk for homocysteine-associated diseases (9–11). Homocystinuria can result from inborn errors, such as deficiencies in the enzymes cystathionine  $\beta$ -synthase, methionine synthase, and methylenetetrahydrofolate reductase (12). These patients have premature vascular disease, ~50% of untreated patients have thromboembolic events, and the mortality rate is ~20% before the age of 30 (12).

Many methods have been developed to measure tHCY, such as the tHCY enzyme conversion immunoassay designed for the Abbott IMx analyzer (13), the microtiterplate tHCY enzymatic immunoassay kit (14), and HPLC (15–17). However, these methods are relatively complex and require highly specialized equipment.

To meet the need for a widely available homocysteine assay, we have developed and describe here a simple single-enzyme tHCY assay based on a highly specific homocysteine  $\alpha,\gamma$ -lyase (rHCYase) (18).

L-Homocystine, L-cysteine (L-CYS), L-methionine (L-MET), dithiothreitol (DTT), Triton X-100, citric acid, potassium ferricyanide, and other common reagents for the tHCY enzymatic assay were obtained from Sigma. 4-Fluoro-7-sulfobenzofurazan ammonium salt was obtained from Wako Fine Chemicals, and *N,N*-dibutylaniline was obtained from Acros Organics. *N,N*-Dibutylphenylene diamine (DBPDA) was synthesized in our laboratory (L. Tang and Y. Tan, unpublished data). rHCYase was produced in our laboratory (18).

Blood was collected by venipuncture into Vacutainer Tubes (Becton Dickinson) containing EDTA. Samples were stored immediately at 0–4 °C. Plasma for tHCY measurements was separated within 1 h by centrifugation at 300g for 5 min at 4 °C. The tHCY is stable for 1 month at 4 °C and indefinitely at –20 or –80 °C (19, 20). To determine baseline homocysteine concentrations, blood donors fasted for 12 h.

The principle of the assay is as follows. In reaction I,

rHCYase specifically converts homocysteine to  $\alpha$ -ketobutyrate, ammonia, and H<sub>2</sub>S. In reaction II, the H<sub>2</sub>S combines with DBPDA to form 3,7-bis(dibutyl amino)phenothiazine-5-ium chloride, which is highly fluorescent.

For the reduction reaction, we used flat-bottomed 96-well cell culture cluster plates with low-evaporation lids (cat. no. 0720089; Corning). Five microliters of calibrators or plasma samples and 185  $\mu$ L of assay buffer [40 mmol/L sodium phosphate buffer (pH 8.4), containing 2 mL/L Triton X-100 and 0.25 mmol/L DTT] were added to the wells. Two wells were used for each sample. Reduction was carried out at 37 °C for 30 min. This reaction was necessary to break the disulfide linkages in plasma proteins and to reduce the low-molecular weight disulfides homocystine and homocysteine-cysteine mixed disulfide.

For each sample or calibrator, we added 30  $\mu$ L of rHCYase (0.05 g/L), the equivalent to 0.1 U, in 40 mmol/L potassium phosphate buffer containing 20  $\mu$ mol/L pyridoxal phosphate to one well. One unit of enzyme is defined as the amount that catalyzes 1  $\mu$ mol of H<sub>2</sub>S per minute from homocysteine (18). We added 30  $\mu$ L of enzyme buffer to the other well to serve as background. The enzymatic reaction was carried out at room temperature for 3 min. The lid was left on the dish to prevent possible loss of H<sub>2</sub>S. The enzymatic reaction was stopped by the addition of 50  $\mu$ L of chromophore reagent (20 mmol/L DBPDA in 3 mol/L H<sub>2</sub>SO<sub>4</sub>), followed by 30  $\mu$ L of oxidizing agent [5 mmol/L potassium ferricyanide in 10 mmol/L sodium phosphate buffer (pH 7.2)]. The total time for these additions, using a multichannel pipetter, was ~40 s, thus minimizing any H<sub>2</sub>S losses. The chromogenic reaction was carried out at room temperature for 10 min. The resulting fluorescence was measured at an excitation wavelength of 665 nm and an emission wavelength of 690 nm in a Spectra Gemini tunable fluorescence microtiter plate reader (Molecular Devices).

L-HCY was generated by the reduction of L-homocystine (Sigma) with 0.25 mmol/L DTT for 30 min at 37 °C. The resulting L-HCY was used as a calibrator at concentrations of 1–100  $\mu$ mol/L. Calibrators were always run in duplicate. Samples with 1–100  $\mu$ mol/L tHCY could be measured by the assay without additional dilution. Samples with tHCY >100  $\mu$ mol/L were diluted in normal saline. The calibration curve was linear and repeatable between 1 and 100  $\mu$ mol/L (Fig. 1A).

The HPLC tHCY assay used the ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) derivatization method as described by Ubbink et al. (21) and Dudman et al. (22) with some modifications. Plasma samples were reduced with tri-*n*-butylphosphine and were derivatized with SBD-F. An L-6200A Intelligent Pump HPLC (Hitachi) was used for the measurement of tHCY with a reversed-phase Supelcosil LC-18-DB column

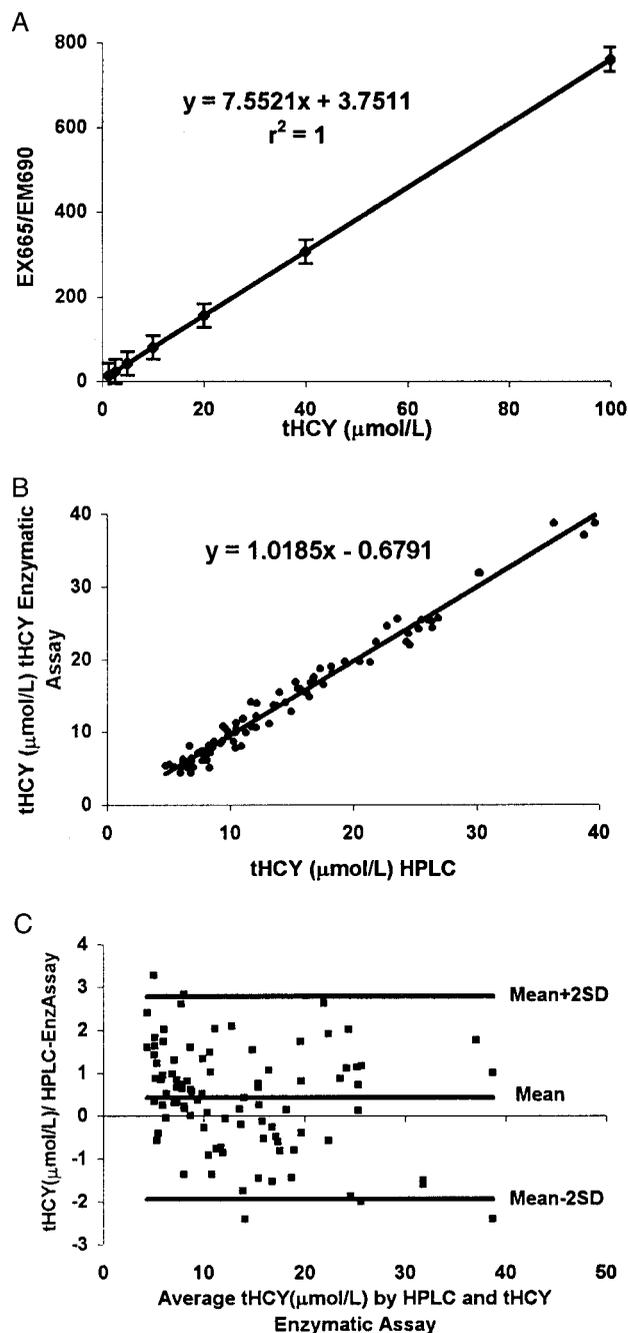


Fig. 1. Calibration curve for the tHCY enzymatic assay (A) and comparison of the tHCY enzymatic and HPLC methods by regression analysis (B) and Bland-Altman methods (C).

(A), the tHCY enzymatic assay was carried out as described in the text. L-HCY (1–100 μmol/L) in normal saline was used as the calibrator. EX, excitation; EM, emission. Bars, SD. (B), linear regression analysis data:  $r = 0.989$ ;  $S_{y/x} = 1.18$ . (C), plot shows the difference in tHCY results between the tHCY enzymatic assay and HPLC as function of their mean value and  $\pm 2$  SD.

(25 cm × 4.8 mm; particle size, 5 μm; Supelco) at room temperature.

Plasma samples from 100 subjects and patients were analyzed with the tHCY enzymatic assay and the HPLC

assay. Each sample was analyzed in duplicate, and the average homocysteine values and SDs were calculated. The correlation was calculated by regression analysis and Bland-Altman methods (23) (Fig. 1, B and C).

The precision of this assay was evaluated in a study based on NCCLS guideline EP5-T2 (24). Three plasma samples containing low, medium, and high tHCY were measured. Each analysis also included low, medium, and high L-homocystine calibrators (14). The within- and between-assay CVs were <5% and <10%, respectively (Table 1).

To study linearity, three plasma samples with homocysteine concentrations between 20 and 30 μmol/L were serially diluted one-, two-, four-, and eightfold with saline. The samples were analyzed in four replicates in a single analytical run. Mean concentrations and dilution recovery were calculated (14).

To determine the limit of quantification [defined as the lowest concentration having a CV <20% (14)], we diluted calibrators containing 25 μmol/L L-HCY with saline to obtain concentrations of 0.5, 1, 1.5, 2.0, and 2.5 μmol/L, and assayed four replicates. The limit of quantification was 1.0 μmol/L.

A series dilution of a sample with high tHCY (25 μmol/L as determined by HPLC), was measured by the tHCY enzymatic assay in duplicate. The observed homocysteine was 100% ± 20% (mean ± SD) of the expected concentration. Mean recovery of known amounts of L-HCY added to a plasma sample with a tHCY concentration of 8.24 μmol/L was 95%.

We tested for interference from L-CYS and L-MET by adding them to calibrator (20 μmol/L L-HCY solution) and plasma samples at concentrations of 0–200 μmol/L, and also by assaying the pure chemicals at the same concentrations. L-CYS had <5% interference in the concentration range tested, and L-MET had no interference. The time course of the HCY enzymatic assay was determined with L-HCY (20 μmol/L) alone, L-CYS (200 μmol/L) alone, and the mixture of L-HCY (20 μmol/L) plus L-CYS (200 μmol/L). The reaction rate for L-HCY was 21.5-fold faster than for L-CYS. There was almost

Table 1. Precision of the tHCY enzymatic assay.

	Sample		
	A	B	C
<b>A. Within-assay precision (n = 8)</b>			
tHCY, μmol/L			
Mean	7.45	10.51	26.49
SD	0.324	0.487	0.927
CV, %	4.3	4.6	3.5
<b>B. Between-assay precision (n = 10 days)</b>			
tHCY, μmol/L			
Mean	7.41	10.11	26.16
SD	0.694	0.377	1.365
CV, %	9.4	3.7	5.2

no interference from L-CYS to L-HCY after 3 min of enzymatic reaction. Lack of interference from L-CYS and L-MET was also confirmed by the high correlation between the tHCY enzymatic assay and the HPLC method.

The specificity of the homocysteine enzymatic assay for L-HCY was high. The ratios for L-CYS and L-MET in the assay were 0.78% and 0.067%, respectively, with L-HCY taken as 100%.

The increasing requirement for tHCY measurement has led to the development of various tHCY assays (13–16, 21, 22, 25), but none are ideal. HPLC methods (15, 16, 19–22, 25) have been used as the standard evaluation for tHCY, but the disadvantages of such methods for clinical application include the limited number of samples that can be processed by HPLC and the need for appropriate HPLC instruments and skilled operators.

Shipchandler and Moore (13) and Frantzen et al. (14) recently described an enzyme conversion immunoassay for tHCY. The assay involves conversion of homocysteine to S-adenosylhomocysteine. Detection is then based on use of a monoclonal antibody that recognizes S-adenosylhomocysteine, and a fluorescent analog. This assay has high precision but is limited to specialized equipment, has >10 steps, and processes only 20 samples in 60 min, which precludes high throughput.

The present tHCY enzymatic assay is a simple, high-throughput method with only three steps. This method can be performed with readily available instruments. The rHCYase developed in our laboratory (18) has high sensitivity and specificity for homocysteine. The enzyme is not inhibited by effective reducing agents, such as DTT, present in amounts sufficient to quantitatively reduce all homocysteine disulfides in plasma. Our laboratory has also developed a high-sensitivity fluorescent chromophore (DBPDA) to detect H<sub>2</sub>S that gives a signal of ~100 fluorescence units over background for 10 μmol/L plasma tHCY.

Only 5 μL of EDTA plasma is required for a microtiter plate tHCY enzymatic fluorescence assay, which is at least 10 times less than other assays. Plasma derived from a finger prick is sufficient for the tHCY enzymatic assay. Measurement of tHCY from 45 samples can be completed in less than 1 h in a single 96-well plate. Multiple plates can be analyzed simultaneously without much extra effort, enabling thousands of samples to be measured in 1 day in one laboratory.

This research was supported in part by NIH SBIR Grant 1R43HL63263-02.

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