Enzymatic Assay of Homocysteine on Microtiter Plates or a TECAN Analyzer Using Crude Lysate Containing Recombinant Methionine γ-Lyase

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Abstract. An enzymatic assay for plasma homocysteine was developed that uses a crude lysate of E. coli containing the recombinant enzyme, methionine γ-lyase. The assay uses a commercially available fluorophore and 96-well microtiter plates; it can be performed manually or with the TECAN automated analyzer. The CVs for within-run and between-run precision are <10%. Close correlation (r >0.9) was obtained between results by this enzymatic method vs a reference HPLC procedure. In a Chinese population, the concentration of plasma total homocysteine was found to be gender- and age-dependent. Mean concentrations of plasma total homocysteine increased with age and were higher in men than women. Serum homocysteine concentrations did not differ significantly from those in plasma, provided the whole blood specimens were kept at 4°C for 2 hr, or at room temperature for <45 min, between venepuncture and centrifugation.

Keywords: homocysteine, enzymatic assay, microtiter plate, TECAN analyzer, methionine γ-lyase

Introduction

In circulating blood, homocysteine exists in several forms (ie, homocysteine-homocysteine disulfide, cysteine-homocysteine disulfide, and a small amount of free homocysteine). The plasma total homocysteine concentration (tHcy) is well-established as an independent risk factor for cardio-vascular disease. Plasma homocysteine is increased in 15 to 40% of patients with coronary, cerebral, or peripheral arterial diseases [1-3]. Vitamin B₁₂ is a key component in the metabolic pathway of homocysteine; the plasma tHcy level is a sensitive marker for cobalamin (vitamin B₁₂) deficiency [4].

A review has recently summarized the methods for assay of plasma homocysteine, its clinical utility for risk assessment and for diagnosis of homocysteinuria, folate and cobalamin deficiencies, cardio-vascular disease, renal failure, psychiatric disorders, cognitive impairment, pregnancy complications, and birth defects [5]. We have reported that hyperhomocysteinemia may be a risk factor for cancer and could potentially serve as a tumor marker to monitor therapeutic response in cancer patients [6,7].

For more than 10 yr, plasma tHcy has generally been determined by high performance liquid chromatography (HPLC) [1,8]. New analytical methods (eg, immunoassay [9] and enzymatic assays [10,11]) have been developed to avoid the need for expensive HPLC equipment. The enzymatic procedure described by Tan et al [9] is a simple, practical method for tHcy determination, but it requires a purified, highly specific recombinant enzyme [12], homocysteine α,γ-lyase, and an in-house synthesized chromophore. In this report, we...
describe a similar enzymatic assay for tHcy that uses crude lysate of E. coli containing a different recombinant enzyme, methionine γ-lyase, and a chromophore that is commercially available. The assay can be performed manually using 96-well microtiter plates or by automation with the TECAN analyzer. The tHcy levels obtained by the enzymatic assay correlate closely with those assayed by HPLC.

Materials and Methods

Chemicals. Dithiothreitol (DTT) was purchased from Merck (Whitehouse Station, NJ). Homocysteine, N,N-diethyl-p-phenylenediamine, and other chemicals were purchased from Sigma (St Louis, MO).

Equipment. The Genesis RMP100 automated analyzer was from TECAN (Research Triangle Park, NC). The Molecular 374 fluorescence microplate reader was from Molecular Devices (Sunnyvale, CA). Black, flat-bottom, 96-well FluoroNunc microtiter plates were from Nalge Nunc International (Rochester, NY). These microtiter plates can be used for tHcy assays either by the manual procedure or the automated TECAN technique.

Preparation of recombinant methionine γ-lyase. Recombinant enzyme methionine γ-lyase was constructed using genomic DNA of the protozoan Trichomonas vaginalis, subcloned into a pET14b (Novagen) expression vector in E. coli BL21(DE3) pLysS. PCR amplification was performed with a DNA thermal cycler (Perkin-Elmer, GeneAmp 9600). The length of the PCR product was 1197-bp, as verified by DNA sequencing after digestion by restriction endonuclease. cDNA of the enzyme was expressed in E. coli. Isopropyl β-D-thiogalactopyranoside was used to induce enzyme expression [13]. The cells were harvested by centrifugation and then disrupted by sonication in lysis buffer (Tris-HCl, 0.2 mol/L, pH 7.5, 4°C, containing glycerol (20%, v/v) and phenylmethylsulfonyl fluoride (1 mmol/L). The lysate was used for homocysteine assays without further purification, and was lyophilized for storage.

Preparation of calibrators and controls. Plasma specimens from renal patients undergoing dialysis were pooled to contain graded levels of tHcy and were used as calibrators and controls for tHcy assays. After centrifugation to remove debris in the pooled plasma, tHcy concentrations were determined by HPLC. The calibrators and controls were stored at -70°C.

Specimen collection. EDTA plasma specimens were collected from 878 Chinese adults who visited Chang Gung Memorial Hospital for a health check-up or medical care. The subjects fasted >12 hr prior to blood drawing. Blood specimens remained at room temperature for <1.5 hr between venipuncture and centrifugation; plasma samples were then stored at 4°C and assayed within 2 weeks by the enzymatic and HPLC procedures. To compare tHcy concentrations in serum and plasma, paired samples of serum and plasma were derived from the same venepuncture.

Reagent compositions.

Buffers: 0.05 mol/L Tris-HCl, pH 7.5; 0.05 mol/L citrate buffer, pH 5.5.

Reducing agent: 0.01 mol/L dithiothreitol (DTT) in 0.05 mol/L citrate buffer (pH 5.5, 4°C).

Methionine γ-lyase: E. coli crude lysate containing this enzyme was used directly for the homocysteine assays without further purification. The enzyme was lyophilized in separate vials and was reconstituted before each assay by adding 1.5 ml of 0.05 mol/L Tri-HCl buffer to a vial. The reconstituted enzyme solution was stable for weeks at 4°C and for months at -80°C. The amount of enzyme in each batch was adjusted so that a reaction in the presence of excess homocysteine produced a fluorescence intensity >800 fluorimeter units.

Coenzyme: 70 mmol/L pyridoxal 5’-phosphate was dissolved in 0.05 mol/L Tris-HCl buffer, pH 7.5 and kept at 4°C with protection from light.

Chromophore: 4 mmol/L N,N-diethyl-p-phenylenediamine in 0.05 mol/L Tris-HCl buffer, pH 7.5, stored at 4°C.

Oxidizing reagent: 0.03 mol/L FeCl3 in 1 mol/L H2SO4, stored at 4°C.

Preparation of working reagents for ~50 tests.

Reagent I: For sample reduction, 1.5 ml of 0.01 mol/L DTT was mixed with 20 ml of 0.05 mol/L Tris-HCl buffer. (The same final concentration of reducing agent was used for the TECAN assay.)

Reagent II: Four ml of Reagent I was mixed with...
40 µl of coenzyme solution and 20 µl of reconstituted methionine γ-lyase.

Reagent III: Chromophore solution (0.2 ml) was mixed with 8 ml of oxidizing reagent immediately before the assay.

Manual microplate procedure.
1. Place duplicate 20 µl aliquots of each plasma or calibrator specimen into microwells. One of the duplicate microwells is for the enzymatic reaction sample and the other for the background control sample. After addition of 170 µl of Reagent I (reducing agent) to each well, the microtiter plate is incubated at 37°C for 20 min.
2. After the incubation, 30 µl of Reagent II (enzyme and coenzyme) is added to the enzymatic reaction sample well and 30 µl of Tris-HCl buffer to the background control sample well. The mixtures are kept at room temperature for 5 min.
3. Then 40 µl of Reagent III is added to each well. After 10 min at room temperature, the fluorescence of each well is measured by fluorometry at 710 nm with excitation at 656 nm.
4. The fluorometric reading of the background control sample well is subtracted from the reading of the corresponding enzymatic reaction sample well for all calibrators and samples.

Automated assay with the TECAN analyzer: Assay of homocysteine with the TECAN automated analyzer is performed on 96-well microtiter plates using the same proportions of working reagents (I, II and III) as the manual procedure.

HPLC assay. The HPLC procedure of Wu et al [8] was used for comparison studies and for determining the tHcy concentrations of calibrators and controls.

Results

Assay characteristics. Table 1 lists the CVs for within-day and day-to-day precision of assays performed with the TECAN automated analyzer. Assay CVs obtained by the manual procedure were slightly higher, but were all <10%. Fig. 1 shows a typical calibration curve for the enzymatic assay.

Comparison of enzymatic and HPLC procedures. Excellent correlation was observed between tHcy concentrations by the enzymatic vs the HPLC procedure, regardless of whether the enzymatic procedure was performed manually or by the TECAN automated analyzer (Fig. 2). Bias between the 2 methods was noted when specimens containing high homocysteine concentrations required dilution in order to be assayed by the enzymatic procedure.

Influence of gender and age. Influences of gender and age on plasma tHcy levels were reported earlier in Caucasians [14,16]. In this study, similar findings were observed in Chinese subjects (Table 2). In all of the age categories, mean plasma tHcy concent-

Table 1. Characteristics of the enzymatic tHcy assay on the TECAN automated analyzer.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Plasma tHcy level (µmol/L)</th>
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</thead>
<tbody>
<tr>
<td>Sensitivity</td>
<td>3.2</td>
</tr>
<tr>
<td>Concentration range of calibration curve</td>
<td>3.2 to 90</td>
</tr>
<tr>
<td>Within-day precision</td>
<td>11.2 ± 0.3 (CV 2.7%, n = 32)</td>
</tr>
<tr>
<td>Day-to-day precision</td>
<td>10.5 ± 0.7 (CV 6.6%, n = 22)</td>
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Assay sensitivity was determined by assaying serially diluted calibrators until there was no longer a decrease in fluorescence. The assay sensitivity was defined as the tHcy concentration that produced the least fluorescence at the highest dilution.

Fig. 1. A typical calibration curve for tHcy measured enzymatically.
The tHcy concentrations were higher in men than women; the tHcy concentrations increased with advancing age in men and women. The differences of mean plasma tHcy levels in men and women and in the different age groups are all statistically significant (p < 0.001).

Serum vs plasma. Plasma has been recommended for tHcy determinations [14]. Erythrocytes contain high concentrations of homocysteine, and false elevations may occur when serum is used, owing to homocysteine release from erythrocytes during blood coagulation. However, we found that if blood is left at room temperature 45 min before centrifugation, there was no significant difference in tHcy concentrations in paired serum and plasma specimens from the same venepuncture.

Moreover, no significant difference between tHcy concentrations in paired plasma and serum specimens was observed if blood was kept at 4ºC for 2 hr between venepuncture and centrifugation (Fig. 3). When serum specimens were prepared as described above, the serum tHcy levels were never more than 5% elevated, in comparison to plasma. Thus, with proper care, tHcy can be reliably measured in serum specimens.

Plasma tHcy concentrations did not change when whole blood specimens were kept at 4ºC for up to 8 hr before centrifugation (Fig. 4). Whole blood samples should not stay long at room temperature, since tHcy increased progressively [15,16].
Discussion

If pure homocysteine solution was used as the calibrator, the enzymatic assay for tHcy repeatedly produced slightly higher values. This was due to crossreaction of the enzyme with cysteine, which is present in plasma at much higher concentration than homocysteine. Under the conditions of the enzymatic assay, cysteine generates a small elevation of the final fluorescence, even though the enzyme used in this assay reacts more slowly with cysteine than with homocysteine.

That problem was avoided by using pooled plasma as the calibrator and preparing the calibration curve by plotting the homocysteine concentrations determined by HPLC against the fluorescence produced by the enzymatic assay. With this modification, correlation of tHcy levels by the HPLC and enzymatic methods became excellent (Fig 2).

However, the enzymatic method has pitfalls. The recombinant methionine γ-lyase is not specific for homocysteine. The enzyme also reacts with cysteine, although with less affinity. In plasma specimens, the concentration of cysteine is usually much higher than homocysteine. For this reason, pure homocysteine solution is unsuitable as a calibrator. Instead, pooled plasma containing the usual concentration of cysteine has to be used as the calibrator and the tHcy level of the calibrator needs to be determined by HPLC.

Usually during routine measurement of plasma tHcy, sample dilution is unnecessary since the tHcy concentration range in which we are interested is 5 to 50 µmol/L. However, if a plasma sample has a greatly elevated concentration of tHcy and dilution is needed for assay by the enzymatic procedure, reduced fluorescence intensity will be observed. The dilution reduces the plasma cysteine concentration in the sample below those in the calibrators, resulting in a lower value. Multiplying by the dilution factor amplifies the problem.

Analysts should be aware of the limitation of this method: that a lower value will be generated if sample dilution is performed. Thus in our correlation study (Fig. 2), bias was observed because specimen dilution was carried out for specimens with elevated tHcy concentrations.

Another limitation of the enzymatic method is the assay sensitivity. The enzymatic method can only detect tHcy levels 3.2 µmol/L. At low levels of plasma tHcy there is too much fluorescence background generated by the higher concentrations of cysteine in the calibrators. Fortunately, this limitation does not diminish the clinical value of the assay since the threshold limits of the reference ranges are considerably higher.

It proved to be unnecessary to purify methionine γ-lyase from the E. coli crude lysate for the assay. In fact, the enzyme may be more stable in the crude lysate. Apparently other proteins or substances in the lysate do not interfere with the assay but help to stabilize the enzyme. The commercial chromophore works well in the assay. The enzymatic assay is rapid (less than one hr), inexpensive, and easy to perform.

The enzymatic procedure is suitable for small laboratories without an expensive automated analyzer, but it can also be used in laboratories that process large numbers of specimens by performing the assay on the TECAN automated analyzer.

References


