·临床检验研究论著·

Effects of endogenous L-cystathionine on enzymatic assays of serum homocysteine in samples from renal dialysis patients

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Homocysteine (Hcy) is a thiol-containing amino acid produced by the intracellular demethylation of methionine. Approximately 80% of circulating Hcy in the blood is protein bound by disulfide linkage. The remaining unbound Hcy combines by oxidation either with itself to form the dimer homocystine or with cysteine to form the mixed disulfide cysteine-Hcy. Only a small amount circulates as free Hcy. Total homocysteine (tHcy) represents the sum of all forms of Hcy including forms of oxidized, protein bound and free. An elevated level of tHcy has emerged as an important risk factor in the assessment of cardiovascular disease and stroke[1-3]. Excess Hcy in the blood stream may cause injuries to arterial vessels due to its irritant nature, and result in inflammation and plaque formation, which may eventually cause blockage of blood flow to the heart. In 1999, a Science Advisory from the American Heart Association advised clinicians to begin screening high-risk patients with a personal or family history of heart disease for elevated homocysteine levels. Researchers believe that using homocysteine measurement as a preventive tool will play a central role in the risk assessment and management of cardiac patients in the coming preventive medicine era. Elevated levels of tHcy are also linked with Alzheimer's dis[4] and Osteoporosis[5].

Patients with chronic renal disease have a high morbidity and mortality rate due to arteriosclerotic CVD. An elevated concentration of tHcy is often found in the blood of these patients. Investigation suggested that the markedly elevated plasma tHcy found in end-stage renal disease patients contributes independently to their excess incidence of fatal and non-fatal CVD outcomes[6]. The increased levels of tHcy in these patients are mainly due to impaired removal of Hcy from the blood by the kidney[7-8]. It has been well documented that accompanying elevation of Hcy in the renal disease patients, serum cystathionine levels in these patients also significantly elevated[9]. Significant increase in serum cystathionine levels in patients with liver cirrhosis was also reported[10].

In the past decade, varies of methods have been developed for the measurement of human serum or plama Hcy including Abbott's tHcy enzyme conversion immunoassay[11] on IMx analyzer, Diazyme's Substrate-Trapped-Enzyme (STE) micro-titer plate enzymatic Hcy assay, HPLC method[12], Catch's enzymatic cycling Hcy assay and Diazyme's enzymatic cycling tHcy assay[13]. Among all the methods, both Diazyme's and Catch's Hcy assays involve an enzymatic cycling system with substantial amplification of detection signals and are applicable to all major automated clinical chemistry analyzers. Therefore, the enzymatic cycling based tHcy assays have become the mainstream methods for tHcy testing in clinical laboratories. Though both the Diazyme and Catch assays are enzyme cycling based, there are fundamental differences in assay principle and performance. This report will take the endogenous interfering substance L-cystathionine as an example to compare the performance between the two enzymatic cycling based tHcy assays and its clinical significance in diagnosis of homocysteinemia from renal dialysis patients.

1　Materials and Methods

Chemicals and reagents: Borate, EDTA, 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid (SBD-F), Tri-n-butyl phosphine (TBP), DMF, potassium dihydrogenphosphate, phosphoric acid, acetonitrile were purchased from Sigma. L-Cystathionine was purchased from Sigma. The Catch Enzymatic Homocysteine Assay reagents were purchased from a commercial source.

Specimens: Dialysis patient serum samples used for this study were from the patients of our hospital, which included an IRB (institutional review board) certification that the informed consent and procedures used to collect samples were IRB approved.

L-Cystathionine spiking experiment: 1mM stock solution of cystathionine was freshly prepared in distilled water.

1.0 mL of a serum sample containing 11.0 μM Hcy, L-cytathionine was spiked to final concentrations of 0, 10 μM, 30 μM, 40 μM and 100 μM respectively. The samples prepared were tested with the Diazyme and Catch tHcy enzymatic cycling assays on a Olympus AU 5400 analyzer.

Method comparison with dialysis patient samples: Diazyme and Catch tHcy assays: The dialysis patient samples were tested with the Diazyme Enzymatic cycling tHcy assay and Catch Enzymatic cycling tHcy assay on a Olympus AU 5400 analyzer.

HPLC method for tHcy assays: The HPLC tHcy assay was performed according to the protocol as described by Ub-

bink J. B. et al[14] using a Shimazu HPLC system and Bio-Rad tHcy calibrators.

Briefly, 30 μL of a 10% solution of tri-n-butyl-phosphine in dimethylformamide was added to 0.3 mL of serum or Bio-rad tHcy standard. After incubation at 4 ℃ for 30 minutes, 0.3 mL of 10% trichloroacetic acid containing 1 mmol/L EDTA was added to precipitate the protein. After centrifugation, 100 μL of the supernatant was added to a mixture of 20 μL of 1.55 mol/L sodium hydroxide, 250 μL of a 0.125 mol/L borate buffer pH 9.5 containing 0.4 mmol/L EDTA and 100 μL of ammonium 7-fluorobenzo-2-oxa-1, 3-diazole-4-sulphonate (SBD-F) solution (1 mg/mL dissolved in borate buffer). The mixture was incubated for one hour at 60 ℃ to derivatize the thiol group of the reduced homocysteine. A 20 μL of aliquot was submitted for Shimazu HPLC analysis using RP c-18 column eluted with 0.1 mol/L KH2PO4 buffer (pH 2.1), 4% acetonitrile at a flow rate of 2.0 mL/min. The fluorescence detection excitation wavelength used was 385 nm and emission was 515 nm.

2 Results

Comparison of assay principles between the Diazyme and Catch tHcy assays

Diazyme tHcy assay principle: Oxidized Hcy is first reduced to free Hcy which then reacts with S-adenosylmethionine (SAM) to form methionine (Met), catalyzed by the enzyme Hcy S-methyltransferase with concomitant generation of S-adenosylhomocysteine (SAH). SAH is then hydrolyzed into adenosine and Hcy by SAH hydrolase. The formed Hcy is cycled back to methionine formation by Hcy S-methyltransferase and adenosine accumulated in the cycling reactions is hydrolyzed to inosine and ammonia, which reacts with glutamate dehydrogenase with concomitant conversion of NADH to NAD^+. The concentration of Hcy in the sample is proportional to the amount of NADH converted to NAD^+ (A340 nm). The principle of the Diazyme enzymatic cycling based tHcy assay is depicted in the Figure 1.

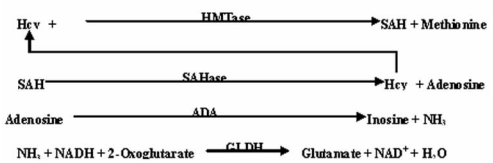


Figure 1　Depiction of the reaction scheme of Diazyme enzymatic cycling based tHcy assay

Catch tHcy assay principle: Oxidized Hcy is first reduced to free Hcy which is converted to cystathionine by the use of cystathionine beta-synthase (CBS) in the presence of excess serine. The beta-cystathionine is then broken down to Hcy, pyruvate, and ammonia by the enzyme cystathionine -lyase. Hcy is recycled back to the cystathionine beta-synthase reaction to form a Hcy cycling reaction, whereas the formed pyruvate is further converted to lactate by the enzyme lactate dehydrogenase with a concomitant conversion of NADH to NAD^+. The rate of NADH conversion to NAD^+, measured at 340 nm, is directly proportional to the concentration of Hcy in the sample. The principle of the Catch enzymatic cycling based tHcy assay is depicted in the Figure 2.

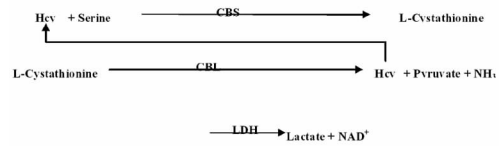


Figure 2　Depiction of the reaction scheme of Catch enzymatic cycling based tHcy assay

Effect of spiked L-cystathionine on the tHcy levels by Diazyme and Catch methods

As mentioned earlier, renal failure or dialysis patients have elevated levels of serum tHcy, which is considered as a new risk factor for developing cardiovascular complica-tions. It is also well documented that serum cystathionine levels in renal failure or dialysis patients are dramatically elevated. Based on the principle of the Catch method, reduced homocysteine in the patient samples is first converted to L-cystathionine, which is quantified through a further enzymatic reaction and a coupled cycling reaction. The method is, therefore, a method for determination of L-cystathionine. It is expected that endogenous cystathionine elevated in renal failure or dialysis patient samples will be co-measured as homocysteine in the Catch assay, and will give false positive results for tHcy levels in some renal dialysis patients. To confirm this L-cystathionine interference in Catch tHcy assay, the following experiments were performed:

2.1 L-cystathionine spiking and tHcy recovery experiment

A serum sample containing a normal level of tHcy was spiked with a stock solution of L-cystathionine to target concentrations of 0, 10, 30, 40, and 100 μM respectively. The samples prepared were tested for tHcy values with both Diazyme and Catch enzymatic cycling tHcy assays on a Olympus AU 5400 analyzer. The test results are shown in Figure 3.

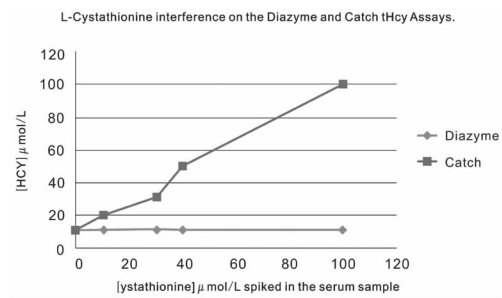


Figure 3　L-Cystathionine interference on the Diazyme and Catch enzymatic cycling tHcy assays

From Figure 3, it is obvious that the Diazyme enzymatic cycling tHcy Assay was not affected by L-cystathionine up to 100 μM. As expected, the Catch enzymatic cycling tHcy assay was significantly interfered by L-cystathionine at 10, 30, 40 and 100 μM L-cystathionine. In fact, the tHcy concentrations recovered by the Catch assay were almost exactly the sum of original tHcy concentration of 11 μmol/L and concentrations of μmol/L L-cystathionine spiked in the samples.

2.2 Determination of tHcy values in samples from dialysis

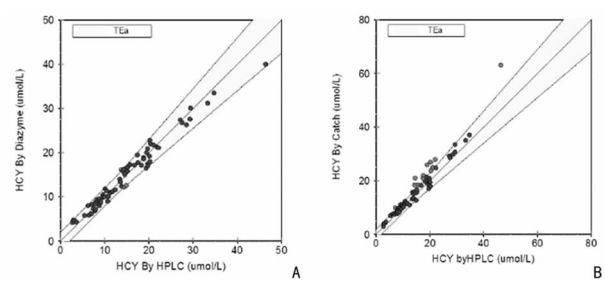
patients using the HPLC, Diazyme and Catch methods; Seventy one (71) dialysis patient samples were tested by HPLC, Diazyme, and Catch methods. The tHcy values obtained by the three methods are summarized in Table 1. Among the 71 samples tested, 13 samples (18.3% of total samples tested) gave significantly higher (minimum 2 μmol/L) tHcy results by the Catch method when compared to both the HPLC and Diazyme methods.

Table 1　Hcy results of Dialysis patient samples by HPLC, Diazyme, and Catch methods

Sample ID	[HCY](mol/L) By HPLC Method	[HCY](mol/L)By Diazyme tHcy Method	[HCY](mol/L)By Catch tHcy Method
1	20.2	18.0	18.0
2	14.5	15.0	21.0
3	14.4	12.2	16.0
4	5.4	5.8	7.0
5	17.4	19.5	21.0
6	34.7	33.5	37.0
7	21.1	21.7	25.0
8	19.7	17.4	20.0
9	19.7	20.9	21.0
10	17.5	17.7	22.0
11	12.4	11.6	11.0
12	19.5	20.0	17.0
13	8.2	9.3	8.0
14	22.2	21.1	24.8
15	16.7	17.2	18.2
16	33.3	31.2	35.1
17	20.4	22.0	23.8
18	8.7	9.5	8.5
19	3.1	4.7	4.1
20	2.7	4.2	3.0
21	14.3	16.3	18.4
22	20.1	19.2	19.4
23	46.4	40.0	63.1
24	20.2	22.8	26.9
25	6.2	8.0	7.6
26	29.3	27.6	30.7
27	9.6	10.5	10.7
28	6.4	5.9	7.8
29	3.6	4.3	4.7
30	10.4	11.3	11.6
31	9.1	8.9	11.1
32	13.4	14.0	13.2
33	5.4	5.8	7.1
34	10.1	11.8	10.2
35	13.7	16.1	15.4
36	18.8	18.9	20.1
37	11.3	11.0	11.7
38	7.9	8.8	9.6
39	14.1	15.8	15.3
40	7.9	8.8	8.8
41	2.9	4.8	4.1
42	7.1	8.3	9.9
43	7.0	6.2	7.5
44	8.7	8.0	10.1
45	10.8	10.1	12.4
46	9.0	8.6	9.9
47	7.4	7.3	8.1

Continued Table 1　Hcy results of Dialysis patient samples by HPLC, Diazyme, and Catch methods

Sample ID	[HCY](mol/L) By HPLC Method	[HCY](mol/L)By Diazyme tHcy Method	[HCY](mol/L)By Catch tHcy Method
48	7.8	6.9	7.7
49	7.9	8.4	8.7
50	9.4	9.8	9.6
51	7.9	7.2	8.4
52	15.1	15.8	15.6
53	14.9	12.6	12.7
54	13.8	12.4	13.6
55	10.0	10.0	10.5
56	9.7	10.0	10.7
57	11.7	11.2	11.4
58	13.5	13.4	15.8
59	10.7	9.0	11.3
60	18.3	17.2	19.5
61	19.8	17.1	20.1
62	19.4	16.5	18.4
63	15.1	16.7	16.6
64	15.7	17.3	18.4
65	18.9	18.6	25.9
66	18.8	18.6	21.4
67	21.9	21.2	27.9
68	28.5	26.3	29.8
69	27.5	26.8	28.6
70	29.4	30.1	33.5
71	27.1	27.4	29.2



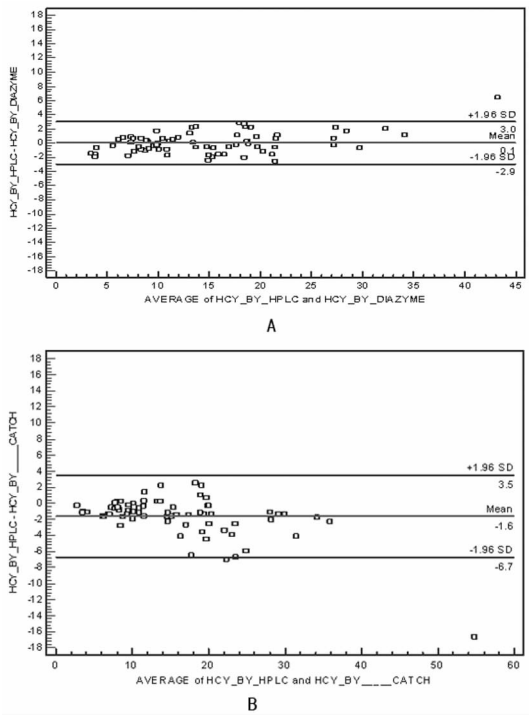
A. Deming regression analysis of Hcy measured by the Diazyme method in comparison to the HPLC method; B. Deming regression analysis of Hcy measured by the Catch method in comparison to the HPLC method

Figure 4　Deming plots between tHcy measured by the HPLC method and the Diazyme tHcy assay (Figure 4A), and the Catch tHcy (Figure 4B).

The Hcy results obtained by both the Diazyme and Catch methods were analyzed using the EP Evaluator software (Version 8.0) in comparison to the HPLC method. In the analysis, allowable total error of 2 μmol/L or 15% (Clinical Laboratory Improvement Amendments (CLIA) acceptance criteria[15]) were used in the Deming regression analysis. The results are shown in Figure 4. For the Diazyme method (Figure 4A), the difference with the HPLC results was within allowable error for 68 of 71 specimens tested (95.8% pass). Deming regression statistics showed the correlation coefficient (R) of 0.9852, slope of 0.919, intercept of 1.16 μmol/L HCY, and standard error of estimate of 1.32 μmol/L tHcy. Overall, the Diazyme assay passed the method comparison with HPLC.

However, for the Catch method (Figure 4B), the difference with the HPLC results was within allowable error for only 58 of 71 specimens tested (81.7% pass). 13 of 71 results failed to pass the CLIA acceptance criteria. Deming regression statistics showed the correlation coefficient (R) of 0.9724, slope of 1.19, intercept of −1.15 μmol/L tHcy, and standard error of estimate of 2.33 μmol/L tHcy. Overall, the Catch assay failed the method comparison with HPLC for serum samples from dialysis patients.

Bland-Altman plots comparing the Diazyme and Catch assays with HPLC are shown in Figure 5. Figure 5 also revealed that Catch method has significant positive bias when compared to the HPLC method. Presumably, this is caused by elevated cystathionine in these dialysis patient samples, which interferes the Catch method but not HPLC and Diazyme methods.



A, Bland and Altman Plot of Hcy measured by the Diazyme method in comparison to the HPLC method; B, Bland and Altman Plot of Hcy measured by the Catch method in comparison to the HPLC method

Figure 5　Bland and Altman plots: Difference (Mean and 95% confidence interval) between tHcy values measured by the HPLC method and Diazyme tHcy assay (Figure 5A), and Catch tHcy (Figure 5B).

3　Discussion

The assay principle of the Diazyme and Catch enzymatic cycling tHcy assays was compared. Both the Diazyme and Catch tHcy assays involve an enzymatic cycling system that is capable of substantial amplification of detection signals. The fundamental differences are that the Catch method detects L-Cystathionine while the Diazyme method detects S-adenosylhomocysteine. In the general population, normal levels of L-Cystathionine are 0.044 μmol/L to 0.342 μmol/L and normal levels of S-adenosylhomocysteine are 0.008 μmol/L to 0.026 μmol/L[16], while the normal range for tHcy is 5.0 to 15.0 μmol/L[12]. Thus presence of endogenous L-Cystathionine and S-adenosylhomocysteine in serum or plasma specimens from the normal population has a negligible effect on the detection of tHcy when measured by the Catch method and Diazyme method. However, it has been well reported that the levels of L-Cystathionine in dialysis renal patients can be elevated significantly and in some cases can reach up to 28 μmol/L[9,17]. In this case, the Catch method shall be affected significantly, and erroneous medical decisions could be made based on the the Catch tHcy results.

L-Cystathionine spiking experiments clearly demonstrated that the Catch assay recovered almost exactly the sum of original tHcy concentration and concentrations of μmol/L L-cystathionine spiked in the samples, while the Diazyme method was not affected by spiked L-Cystathionine in the serum sample up to 100 μmol/L.

The impact on patient results of a commonly found metabolite in End Stage Renal Disease was compared in both the Diazyme and Catch enzymatic cycling tHcy assays. From the method comparison with seventy one (71) dialysis patient samples, Deming regression demonstrated good correlation to the HPLC for both methods with correlation coefficient of 0.985 2 for the Diazyme method and 0.972 4 for the Catch method respectively. However, the slope showed differences with 0.919 for the Diazyme method and 1.19 for the Catch method. A more obvious difference is seen when looking at the bias as calculated by Bland-Altman plots. Not surprisingly, the Catch method gave significant positive bias when compared to the HPLC. It was noteworthy that three samples (Sample ID #2, Sample ID #3, and sample ID #58) were tested as normal with both the Diazyme and HPLC methods while they were tested as abnormal with the Catch method when using 15 μmol/L of tHcy as cutoff value. This cutoff value is used by most of the clinical laboratories and is also claimed in both the Diazyme and Catch tHcy assay package inserts. This represented 4.2% of the samples misclassified as homocysteinemia. In a separate laboratory, Stabler et al[17] tested levels of both cystathionine and and total homocysteine in the serum of patients with renal failure and revealed that levels of serum cystathionine were increased in all 15 patients tested (cystathionine ranging from 0.613 μmol/L to 28.4 μmol/L), although only 7 patients had elevated levels of total homocysteine measured by the HPLC method. However if cystathionine and tHcy are counted together as measured by the Catch assay, nine (9) patients will have elevated levels of total homocysteine, meaning two (2) more patients are misclassified as having homocysteinemia.

It was also reported that levels of S-adenosylhomocysteine in dialysis renal patients and cobalamin-deficient patients can be elevated significantly. However, it is very rare that the serum levels of S-adenosylhomocysteine are elevated above 0.3 μmol/L even among stage 5 renal patients[16-17]. Therefore, elevated S-adenosylhomocysteine among dialysis renal patients and cobalamin-deficient patients still has a negligible

effect on the detection of tHcy when measured by the Diazyme method. The good correlation and small bias between the Diazyme method and the HPLC method testing 71 dialysis patients is good evidence to support this hypothesis.

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PHC 的作用,而建立在预测变量 PRE 基础上为检验变量可以提高检测的灵敏度和特异度[8-10]。本研究结果提示在单项检测时,AFP 的 ROC 曲线下面积高于其他指标,说明 AFP 是这 6 项标志物中 PHC 诊断的最佳单项指标,联合检测时采用 Logistic 回归分析得出的预测变量 ROC 面积为 0.934,高于单存 AFP 检测,说明联合检测有助于提高诊断效能。同时还评价了 6 项肿瘤标志物及联合检测的相关诊断参数,AFP 的灵敏度、特异度、正确率在各单项检测指标中最高;然而联合检测时 Logistic 回归的预测变量 PRE 的灵敏度、特异度及正确率均高于 AFP,也进一步说明联合检测有助于 PHC 的检出。

综上所述,单项肿瘤标志物检测灵敏度、特异度有一定的局限性,多项指标联合检测能提高 PHC 的检出率且特异度高,其临床意义大于单项检测。通过血清中肿瘤标志物的联合检测、影像学分析并结合临床综合判断,对 PHC 患者早期诊断、治疗及预后有一定的临床价值。

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