Clinical Usefulness of a HPLC Method for Simultaneous Quantitation of Plasma Homocysteine and Cysteine

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Objective: *Homocysteine (Hcy) is a risk for vascular occlusion. It is metabolized via remethylation to methionine and transsulfuration to cysteine which has also been related to vascular occlusion. Simultaneous determination of Hcy and cysteine has additional clinical usefulness in providing a presumptive clue to the nature of hyperhomocysteinemia.*
Material and Method: *A manual HPLC method has been worked out for simultaneous determination of plasma Hcy and cysteine. Concentrations of Hcy were validated with the widely used automated Abbott AxSYM assay. Its usefulness was tested in 87 omnivores and 111 vegans.*
Results: *Excellent correlation between the values of Hcy was found between the manual HPLC method and the automated Abbott assay. The vegans had significantly higher levels of Hcy but lower levels of cysteine than the omnivores (mean $\pm$ SD, μmol/L 23.6 $\pm$ 18.0 vs. 8.8 $\pm$ 2.1 $p < 0.001$, 225 $\pm$ 30 vs. 245 $\pm$ 34 $p < 0.001$, respectively). In contrast, the vegans had significantly lower levels of serum vitamin B12 and plasma vitamin B6 than the omnivores (median values 186 vs 565 pg/ml, $p < 0.001$; 37.4 vs. 47.4 nmol/L, $p < 0.001$ respectively). These findings indicate that the hyperhomocysteinemia in the vegans results from impairment of both remethylation and transsulfuration pathways of Hcy secondary to inadequacy of vitamins B12 and B6 respectively. Thus simultaneous determination of Hcy and cysteine is more useful than determination of only Hcy in that it provides a clue to the nature of hyperhomocysteinemia.*
Conclusion: *The manual HPLC method and the Abbott assay gave comparable Hcy values, and thus can be used interchangeably. The HPLC method is economical, useful for hospitals with less demand for determination of Hcy, and capable of simultaneously determining cysteine which has implication in clinical practice.*

Keywords: *HPLC, Homocysteine, Cysteine, Abbott AxSYM*

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Evidences are accumulating which indicate that elevated plasma level of homocysteine (Hcy) is an independent risk factor for the development of cardiovascular disease, cerebral vascular disease, peripheral arterial occlusive disease and thrombosis[1].

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Homocysteine is a sulfur amino acid derived from methionine. It can be remethylated (vitamin B12 dependent) to methionine or transsulfurated (vitamin B6 dependent) to cysteine via pathways that are catalyzed by a series of enzymes and regulated by B-vitamins as co-factors or co-substrates[2]. Impairment of these metabolic pathways because of enzymatic defect and/or B-vitamin deficiency may result in accumulation of Hcy in plasma.

Recent studies have suggested that plasma cysteine, a catabolite of Hcy, may also be associated with cardiovascular disease[3-5]. The level of cysteine in hyperhomocysteinemic individuals may provide a clue to the nature of hyperhomocysteinemia, namely, defective remethylation pathway vs. defective transsulfuration pathway. Thus, simultaneous determination of Hcy and cysteine can have more clinical usefulness than determination of only Hcy.

Increasing interest in homocysteine has led to the development of different methods for measurement of homocysteine. They include gas chromatography-mass spectrometry[6], radioenzymatic assay[7], and high pressure liquid chromatography (HPLC). The last mentioned method has been widely used.

Several HPLC methods exist for the determination of plasma Hcy. They are based on differing chemical reactions and are performed with different detectors. Each HPLC method has both advantages and disadvantages. The choice depends on many factors such as availability of instruments, experience of personnel, utility and cost.

Most published HPLC methods for the determination of plasma Hcy were not set up to include determination of plasma cysteine. For this reason and because value of plasma cysteine may be of potential usefulness in clinical practice, the authors carried out the present study to work out a HPLC method capable of simultaneous determination of plasma Hcy and cysteine. The method was based on the information published by Fermo et al[8], and Hyland and Bottiglieri[9]. The results of plasma Hcy obtained with the present method were validated with those determined with the widely used commercial automated Abbott AxSYM system. The clinical usefulness of simultaneous determination of Hcy and cysteine was demonstrated in two distinct populations expected to have different levels of Hcy, namely, omnivores and vegans.

Material and Method

Healthy subjects comprised 92 men and 106 women, aged 30-50 years. Forty-one men and 46 women were omnivores while 51 men and 60 women were vegans. They contributed blood samples after an overnight fast. The blood was collected in EDTA vacutainer tubes, gently mixed, and then immediately placed on ice. The plasma was separated within 30 minutes by centrifugation at 3000 rpm for 10 minutes. It was stored in aliquots at -70°C until analysis. Plasma was determined for Hcy and cysteine with the present HPLC method and for Hcy with the Abbott AxSYM assay kit.

In-house prepared plasma pool (~7.5 □mol/L) was used as reference for quality control. Duplicate determinations were performed on every ten samples.

The plasma was simultaneously determined for Hcy and cysteine with a HPLC method based on the publication of Fermo et al[8], and Hyland and Bottiglieri[9].The same plasma was determined for Hcy with the popular commercial Abbott AxSYM homocysteine assay kit[10].

Statistical analysis

Statistical analysis was performed with SPSS software, version 11. Data were presented as mean ± SD. Differences in values obtained with the two methods were compared statistically using paired t-test and differences in values between omnivores and vegans were compared with unpaired t-test. For non-parametric data (vitamin B12 and vitamin B6) they were presented as medians and compared with the Mann-Whitney test. Agreement of plasma Hcy determined with both methods was evaluated with the Pearson correlation coefficients and by plotting the differences in Hcy values versus average concentrations according to the statistical method of Bland and Altman for assessment agreement[11]. P-values of < 0.05 denote statistical significance.

The present study was approved by the Ethics Committee of Ramathibodi Hospital. Informed consent was obtained prior to venipuncture.

Results

Two representative chromatograms show distinct peaks of cysteine and homocysteine, one (Fig. 1A) derived from cysteine and homocysteine standard solutions and the other (Fig. 1B) from plasma. Within-day coefficient of variation determined with 10 replicates of one plasma sample was 2.9% for Hcy and 2.7% for cysteine. The between-day coefficient of variation, evaluated with the same plasma sample every day for 10 days, was 5% for Hcy and 2.6% for cysteine.

The concentrations of plasma Hcy among the omnivores and vegans determined with the present manual HPLC method and the Abbott AxSYM system are shown in Table 1. When the concentrations of plasma Hcy were stratified in accordance with the classification of Kang et al[12] into 3 groups, normal (≤ 15 μmol/L), moderately high (> 15-30 μmol/L) and intermediately high (> 30-100 μmol/L), the concentrations of plasma Hcy determined with the two methods are comparable as shown in Table 2.

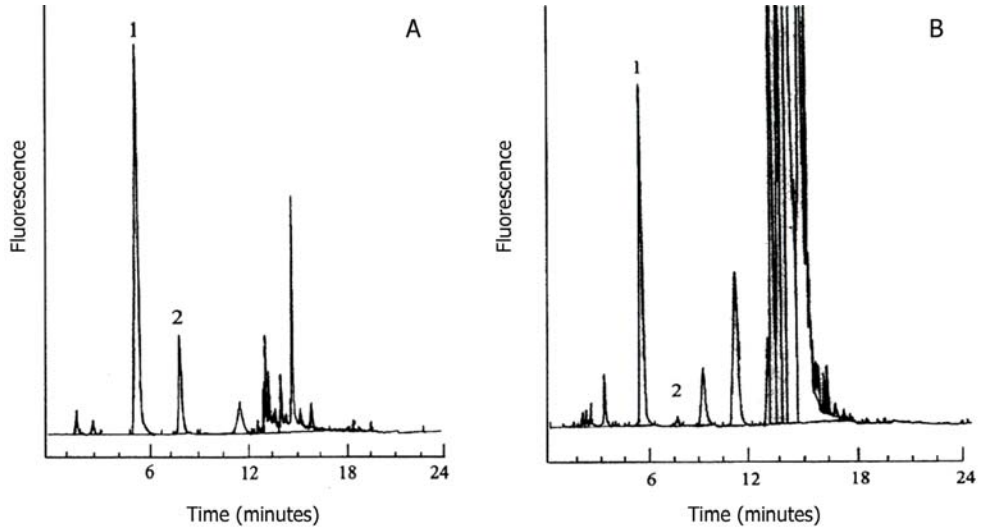

Fig. 1 Chromatograms showing distinct peaks of cysteine and homocysteine (A) from a standard solution containing cysteine (200 μmol/L) and homocysteine (50 μmol/L) and (B) from normal plasma 1 = cysteine, 2 = homocysteine

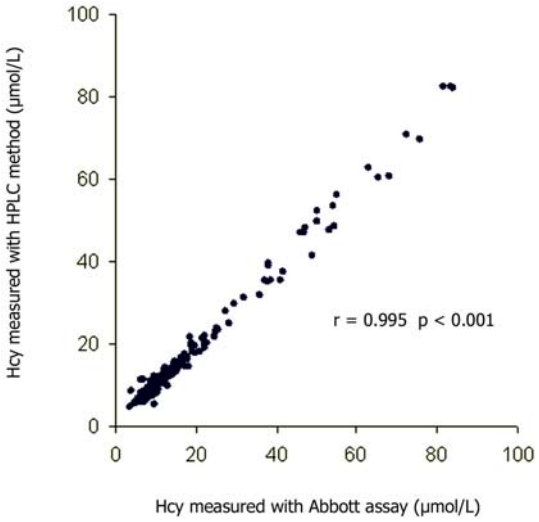

Fig. 2 Correlation between concentrations of plasma Hcy measured with the Abbott AxSYM assay and the present HPLC method (n = 198)

Excellent correlation between the values of plasma Hcy measured with the two methods (r = 0.995 and p-value < 0.001) is shown in Fig. 2. Bland and Altman[11] scatter plot of observed measurement differences between the two methods against their mean values is shown in Fig. 3A. Limits of agreement assessed by calculating the central 0.95 interval (mean ± 2SD) were 0.43 ± 3.51 (-3.08 to 3.94) μmol/L. After logarithmic transformation of the data (Fig. 3B) the differences of log Hcy values (mean ± 2SD) were 0.0036 ± 0.1114 (-0.108 to 0.115). The concentrations of plasma cysteine as shown in Table 1 were significantly lower in the vegans than in the omnivores.

Discussion

It is readily evident from Table 1 and 2 that the values of Hcy determined with the present HPLC method and the Abbott AxSYM immunoassay method are almost identical albeit some differences in the Hcy values are statistically significant between the two methods but the differences are trivial and of no clinical importance.

Correlation between the Abbott AxSYM assay and the present HPLC method (Fig. 2) was excellent but this correlation does not estimate the agreement or disagreement between the two methods. When the agreement between the two methods was assessed with the Bland and Altman analysis[11], the plot of the differences for Hcy measurements between the Abbott AxSYM assay and the present HPLC method against the averages of the two measurements resulted in mean differences ± 2SD (n = 198) of 0.43 ± 3.51 (-3.08 to 3.94) μmol/L and 0.0036 ± 0.1114 (-0.108 to 0.115) for log converted values of Hcy. The agreement in the present study agrees well with the results of previous studies of plasma Hcy comparing the Abbott IMx immunoassay with HPLC methods. One study by Mansoor in 1999 found mean differrences ± 2SD (n =

Table 1. Plasma concentrations of Hcy and cysteine among the omnivores and vegans (mean ± SD, μmol/L)

Amino acid	Omnivore (n = 87)	Vegan (n = 111)	p-value[b]
Homocysteine			
Present HPLC method	8.8 ± 2.1	23.6 ± 18.0	<0.001
Abbott AxSYM assay	8.6 ± 2.2	24.5 ± 18.7	<0.001
p-value[a]	0.259	<0.001	
Cysteine	245.0 ± 34.0	225.0 ± 30.0	<0.001

a, b = comparisons between HPLC method and Abbott AxSYM assay and between omnivores and vegans respectively

Table 2. Plasma Hcy values in the three ranges of Hcy measured with the present HPLC method and the Abbott AxSYM assay (mean ± SD, μmol/L)

Plasma Hcy range *	Plasma Hcy		p-value
	Present HPLC method	Abbott AxSYM assay	
Normohomocysteinemia:			
≤ 15 μmol/L (n = 131)	9.6 ± 2.5	9.5 ± 2.6	0.596
Moderate hyperhomocysteinemia:			
> 15-30 μmol/L (n = 40)	18.6 ± 3.9	19.6 ± 4.0	0.001
Intermediate hyperhomocysteinemia:			
> 30-100 μmol/L (n = 27)	51.3 ± 15.7	53.3 ± 15.6	0.002
Overall (n =198)	17.1 ± 15.4	17.5 ± 16.1	0.001

* According to Kang et al[12]

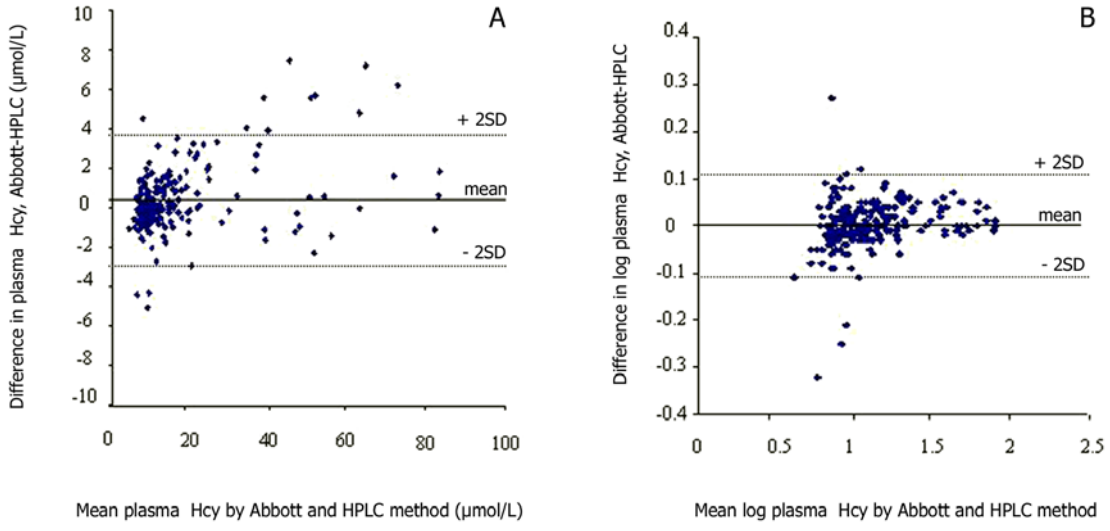

Fig. 3 Differences between Hcy measurements with the Abbott AxSYM assay and the present HPLC method; Mean ± 2SD = 0.43 ± 3.51 μmol/L on a linear scale (A) and 0.0036 ± 0.1114 on a log scale (B)

188) of 0.80 ± 6.66 (-5.86 to 7.46) μmol/L and 0.008 ± 0.126 (-0.118 to 0.134) for log transformed values between the Abbott IMx assay and the HPLC method[13]. In another study by Zighetti et al in 2002, the mean differences ± 2SD (n = 174) were 3.048 ± 6.78 (-3.732 to 9.828) μmol/L and 0.05 ± 0.1 (-0.05 to 0.15) on log transformed scale[14].

Since the present method and the Abbott AxSYM assay gave comparable values of Hcy in three different ranges, namely, ≤ 15 μmol/L, > 15-30 μmol/L and > 30-100 μmol/L, they can be used interchangeably.

In the present study the concentrations of plasma cysteine in the omnivores were 245 ± 34. These values agree with those published in the literature[15,16].

It appears that the vegans had higher plasma levels of Hcy and lower plasma levels of cysteine than the omnivores. This suggests that both the upstream and downstream metabolic pathways of Hcy in the vegans were less active than in the omnivores. The authors measured the concentrations of serum vitamin B12 and plasma pyridoxal 5'-phosphate; they were significantly lower in the vegans than in the omnivores (median values 186 vs. 565 pg/ml, $p < 0.001$ and 37.4 vs. 47.4 nmol/L, $p < 0.001$ respectively) which may explain the higher levels of Hcy and the lower levels of cysteine in the former than in the latter.

The present method has certain advantages, namely, economical where labor cost is low and commercial kits are expensive, and useful for hospitals which do not have large demand for the determination of plasma homocysteine. Besides, it can simultaneously determine cysteine which may have additional clinical usefulness in providing a presumptive clue to the nature of hyperhomocysteinemia, namely, impaired remethylation vs. impaired transsulfuration.

The results of homocysteine of the present method were validated with the widely used automated Abbott AxSYM immunoassay. Although methods for simultaneous determination of homocysteine and cysteine have been reported in the literature, the present method is another alternative; choice depends on availability of equipment and expertise of lab personnel.

In conclusion, the present study has documented a reliable manual HPLC method for simultaneous determination of Hcy and cysteine in human plasma. The results of Hcy were comparable between the automated Abbott AxSYM immunoassay and the present manual HPLC method. Thus, both methods can be used interchangeably for measurement of Hcy. The usefulness of simultaneous determination of Hcy and cysteine has been demonstrated in the vegans.

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ประโยชน์ทางคลินิกของการหาระดับโฮโมซิสเตอีนและซิสเตอีนพร้อม ๆ กันในพลาสมาด้วยวิธีเอชพีแอลซี

กัลยาณี อตมศิริกุล, เสาวณีย์ กาญจนชุมพล, ประพิณ วิไลรัตน์, เพียรวิทย์ ตันติแพทยางกูร

วัตถุประสงค์*: โฮโมซิสเตอีนเป็นปัจจัยเสี่ยงต่อการอุดตันของหลอดเลือด โฮโมซิสเตอีนถูกเปลี่ยนโดยรีเมทิเลชั่น เป็นเมไธโอนีนและโดยทรานซัลเฟอเรชั่นเป็นซิสเตอีน ซึ่งอาจเป็นสาเหตุของการอุดตันของหลอดเลือดได้เช่นกัน การหาระดับโฮโมซิสเตอีนและซิสเตอีนในพลาสมาพร้อม ๆ กัน จึงน่าจะมีประโยชน์ในทางคลินิกที่จะบอกถึงสาเหตุ ของภาวะโฮโมซิสเตอีนสูงในเลือดได้*

วัสดุและวิธีการ*: ทำการวัดระดับโฮโมซิสเตอีนและซิสเตอีนในพลาสมาได้พร้อม ๆ กันด้วยวิธีเอชพีแอลซีและเปรียบเทียบความถูกต้องของระดับโฮโมซิสเตอีนที่วัดได้กับระดับโฮโมซิสเตอีนที่วัดได้ด้วยวิธีAbbott AxSYM ที่ใช้กันและยอมรับกันทั่วไป และได้ทดสอบประโยชน์ในเวชปฏิบัติของการวัดระดับโฮโมซิสเตอีนและซิสเตอีนพร้อม ๆ กันในพลาสมา ในคนรับประทานอาหารปกติ87คนและคนรับประทานอาหารเจ 111คน*

ผลการศึกษา*: ระดับโฮโมซิสเตอีนที่วัดได้ด้วยเครื่องเอชพีแอลซีใกล้เคียงกันมาก กับระดับโฮโมซิสเตอีนที่วัดได้ด้วยวิธี Abbott AxSYM พบว่าคนที่รับประทานอาหารเจมีระดับโฮโมซิสเตอีนสูงกว่า และมีระดับซิสเตอีนต่ำกว่าคนที่รับประทานอาหารปกติอย่างมีนัยสำคัญทางสถิติ (23.6 ± 18.0 vs. 8.8 ± 2.1 $p < 0.001$ และ 225 ± 30 vs. 245 ± 34 $p < 0.001$ ไมโครโมล/ลิตร ตามลำดับ) ในทางตรงกันข้าม กลุ่มรับประทานอาหารเจ มีระดับซีรัมวิตามินบี12 และระดับพลาสมา วิตามินบี6 ต่ำกว่ากลุ่มรับประทานอาหารปกติ อย่างมีนัยสำคัญทางสถิติ (ค่ามัธยฐาน 186 vs. 565 พิโครกรัม/มิลลิลิตร, $p < 0.001$; 37.4 vs. 47.4 นาโนโมล/ลิตร, $p < 0.001$ตามลำดับ) ข้อมูลเหล่านี้บ่งว่าระดับโฮโมซิสเตอีนในพลาสมาที่สูงในคนรับประทานอาหารเจ เกิดจากความผิดปกติทั้งรีเมทิเลชั่น และทรานซัลเฟอเรชั่นของโฮโมซิสเตอีนซึ่งมีสาเหตุมาจากการได้วิตามินบี12 และวิตามินบี6 ไม่เพียงพอตามลำดับ ดังนั้นการหาทั้งระดับโฮโมซิสเตอีน และซิสเตอีนได้พร้อม ๆ กันจึงมีประโยชน์กว่าการหาระดับโฮโมซิสเตอีนเพียงอย่างเดียว โดยช่วยบ่งบอกถึงสาเหตุของภาวะโฮโมซิสเตอีนสูงในเลือดได้ทางอ้อม*

สรุป*: ระดับโฮโมซิสเตอีนในพลาสมาที่วัดได้ด้วยเครื่องเอชพีแอลซีและด้วยวิธี Abbott AxSYM มีค่าใกล้เคียงกันมาก ดังนั้นทั้งสองวิธีจึงทดแทนกันได้ วิธีเอชพีแอลซีมีราคาถูก เหมาะสำหรับโรงพยาบาลที่มีจำนวนตัวอย่างไม่มาก และสามารถวัดระดับซิสเตอีนได้พร้อม ๆ กันด้วย ซึ่งมีประโยชน์ในเวชปฏิบัติ*