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Measurement of Total Plasma Homocysteine in Patients with Chronic Renal Failure Using HPLC/FLD

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Abstract: Cardiovascular disease has been the leading cause of death among patients with chronic renal failure. Many reports have been described that homocysteine is one of the independent risk factor to the occulsive vascular disease. In this study, HPLC/FLD (high performance liquid chromatography-fluorescence detector) technique was used to measure homocysteine level in patients with chronic renal failure and normal control group. The detection limit and recovery of total plasma homocysteine using HPLC/FLD were 98.6±5.8% and 0.2 nmol/ml, respectively. The linearity of this method was established in concentration range of 2~50 nmol/ml (correlation coefficient=0.9997). The concentrations of total plasma homocysteine were 6.81±1.54 nmol/ml and 27.28±14.94 nmol/ml in normal control (n=20) and patient group (n=90), respectively ($p<0.05$). In this study, the HPLC/FLD method showed high sensitivity and reproducibility for a routine clinical laboratory testing. Moreover determination of homocysteine level in plasma might be useful for a biochemical marker for predicting the cardiovascular diseases and for monitoring of therapeutic effect of lowering homocysteine in patients with chronic renal failure.

Key Word: Homocysteine, Cardiovascular disease, Chronic renal failure

INTRODUCTION

Homocysteine is a thiol-containing amino acid metabolized by remethylation to methionine or by transsulfuration to cysteine (Fig. 1). Elevated homocysteine levels may occur as a result of inherited disorders that alter enzyme activity in the transsulfuration and remethylation pathways. Alternatively, nutritional deficiencies of cobalamin (vitamin B_{12}), folate, or pyridoxine (vitamin B_6), can result in blockade of homocysteine metabolic pathways[9]. Clinically, elevated homocysteine levels are significant because they may indicate cobalamin, pyridoxine, or folate deficiency. More importantly, elevated homocysteine levels can be an independent risk factor for thrombosis and occulsive vascular disease[4,11]. It is known that the elevated homocysteine level is related to the function of the kidney in maintaining the overall sulphur economy, and supports the view that the principal sulphur-containing amino acids, except methionine, accumulate because of reduced renal excretion[25]. Homocysteine in high concentration produces endothelial cell injury and enhances detachment of endothelial cells from their substrates, the changes that *in vivo* often lead to arterioscle-

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rosis[7]. During the past several years, evidences have been accumulating that cardiovascular disease is the major cause of death among patients undergoing chronic hemodialysis therapy[6,9,14,23]. Between 40% and 60% of all such deaths have been from cardiovascular causes, and by far the leading contributor has been heart disease[10]. Patients undergoing chronic hemodialysis therapy are subject to a number of well known cardiovascular risk factors such as hypertension, hyperuricemia and glucose intolerance[2,15]. The possibility of homocysteinemia might be an additional risk factor for vascular disease in chronic renal insufficiency has been supported by a number of recent studies[5,19].

A variety of methods including ion-exchange chromatography[28], radioenzymatic assays[17], gas chromatography-mass spectrometry (GC-MS)[21], HPLC/FLD (high performance liquid chromatography-fluorescence detector)[1] and a fully automated method using fluorescein[18] has been described to quantitate sulphur containing amino acid levels. Among several detection methods, in the present study, HPLC/FLD technique was used for the determination of plasma homocyteine level and elevated for the routine clinical laboratory test.

MATERIALS AND METHODS

Subjects

The subjects were 90 patients diagnosed with chronic renal failure from S-private hospital. Distribution of their age was 22~82 years old No patients have received folic acid supplements before entering the study. Control subjects (n=20) were normal healthy volunteer whose serum creatinine level was below 1.4 mg/dL.

Reagents

SBD-F (Ammonium-7-fluorobenzo-2-oxa-1, 3-diazole-4-sulphonate) was purchased from Wako (Kyoto, Japan). Homocysteine, tri-n-butylphosphine (TBP) and dimethylformamide (DMF) were purchased from Sigma (St. Louis, MO, U.S.A). All other chemicals were of analytical reagent grade. A C-18 reverse-phase column (15 cm × 4.6 mm I.D.: 5 μm) with fluorescence detector (exitation at 385 nm and emission at 515 nm) was used for HPLC system. Acetate buffer (pH 4.0, MeOH 2%) was used as mobile phase and buffer solution was filtered through 0.45 μm membrane and degassed just prior to use. The flow-rate of the eluent was 1.0 ml/min.

Sample preparation

Venous blood samples from patients and control subjects were collected into tubes containing EDTA (disodium salt) after fasting for overnight. The plasma was rapidly separated and stored at -20°C. Total homocysteine was measured using HPLC/FLD technique described by Vester and Rasmussen (1991)[24] and Ubbink et al (1991)[22]. For the determination of total homocysteine, 0.24 ml of plasma was treated with 30 μl of 10% (v/v) tri-n-butylphosphine (TBP) in dimethylformamide (DMF) to reduce thiols and to decouple protein-bounded thiols. The samples were incubated at 4°C for 30 min and 300 μl of 0.6 M $HClO_4$ containing 1 mM EDTA was added to precipitate proteins and then centrifuged at 3,000 xg for 10 min. A fifty microliter of the clear supernatant was mixed with 10 μl of NaOH (1.55 M), 125 μl of potassium tetraborate (0.125 M) pH 9.6 containing EDTA 4 mM and 50 μl of SBD-F (1.0 mg/ml) of potassium tetraborate. The mixture was incubated in a shaking waterbath for 60 min at 60°C. After terminating the reaction, the solution was cooled in crushed ice. The reaction mixture was filtered through a 0.45 μm filter. An aliquots of 25 μl was injected into the HPLC 1090 series II (Hewlett-Packard, San Fernando, CA, USA).

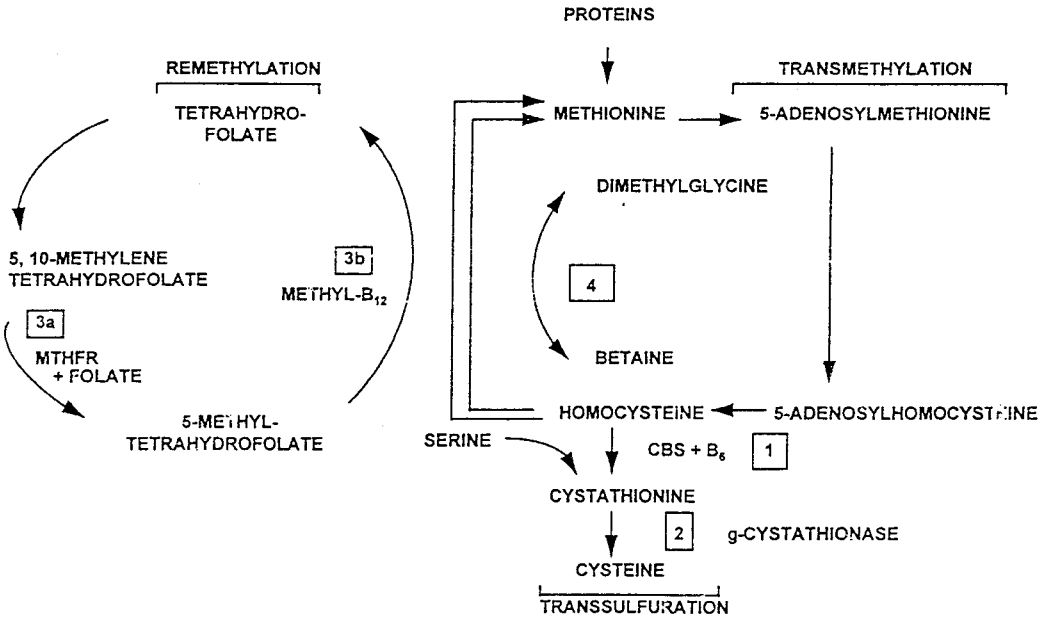

Fig. 1. Homocysteine metabolic pathways. Reaction 1 is catalyzed by choline oxidase; reaction 2, betaine-homocysteine methyltransferase; reaction 3, 5-methyltetrahydrofolate-homocysteine methyltransferase; reaction 4, phosphatidylethanolamine methyl transferase; reaction 6, glycine methyltransferase; reaction 7, cystathionine β synthase; reaction 8, γ-cystathionase.

Calibration & Statics

The calibration curve was obtained by adding five different concentrations of homocysteine (3, 6, 10, 20, 40, 80 nmol/ml) to a plasma pool. The plasma pool was used in each run. Results are given as the mean and standard deviation. All data analysis was done by using Microsoft Excel program. The probability (p) values are two-tailed and were considered significant when <0.05.

RESULTS

In the present experiments, total plasma homocysteine level was determined by using HPLC/FLD technique both in patients with chronic renal failure and normal control subjects to investigate the association with cardiovascular disease. The recovery of this method was 98.6±5.8% and its detection limit was 0.2 nmol/ml. The detector response in concentration range of 2~50 nmol/ml was linear (correlation coefficient = 0.9997). Plasma concentration of total homocysteine in both control subjects and patients were summarized in Table 1. The level of total homocysteine in control (n=20) and patients (n=90) were 6.81±1.54 nmol/ml and 27.28±14.94 nmol/ml, respectively. A significant difference was shown in the concentrations of homocysteine between patients and control subjects (p<0.05) (Table 1). Fig. 2 shows the HPLC chromatograms of SBD-F derivatized thiols in plasma. Homocysteine level in Figure (A) and

Table 1. Concentration of total homocysteine in plasma from patients with chronic renal failure and control subjects.

(nmol/mL)

	Total homocysteine (Mean±SD)	P value
Control subjects (n=20)	6.81±1.54	< 0.05
Patients (n=90)	27.28±14.94	

* SD: standard deviation.

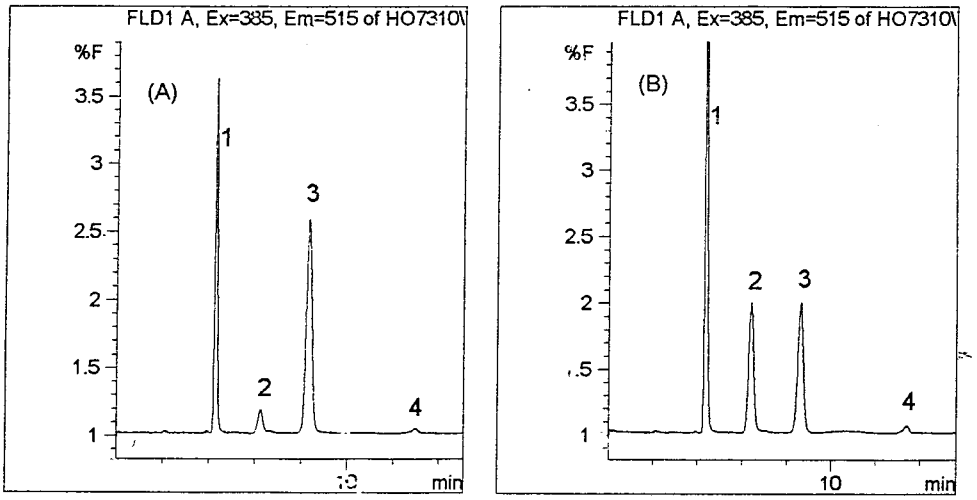

Fig. 2. HPLC chromatograms of SBD-F derivatized thiols in plasma. Homocysteine level in Figure (A) and (B) were 5.31 nmol/ml and 37.45 nmol/ml, respectively. Peak: 1=cysteine, 2=homocysteine, 3=cysteinylglycine, 4=glutathione. Eleuent: 0.1 M acetate buffer (pH 4.0) + MeOH 2%, FLD: excitation 385, emission 515 nm, Detection limit: 0.2 nmol/ml.

(B) were 5.31 nmol/ml and 37.45 nmol/ml, respectively.

DISCUSSION

Homocysteinemia is known to be associated with precocious vascular disease[8]. The study by Kang *et al.* (1979) was the first to demonstrate differences in basal levels of homocysteine in patients with cardiovascular disease when cases were diagnosed on the basis of coronary angiograpy[11]. The major cause of morbidity and early mortality among these patients, irrespective of the simultaneous occurrence of hyper- or hypomethioninemia, is the development of thrombosis and thromboembolism with resultant strokes, coronary occlusions, and other complications[12]. Pathological changes similar to those observed in arteriosclerosis have been described in homocystinuric patients and the accumulation of homocysteine and its derivatives in tissue fluids appears to be the primary factor associated with pathological changes in the intimal cells[13]. These findings suggest that the vascular disease is a homocysteine-induced response[16].

The measurement of plasma homocysteine which has usually been determined by ion exchange chromatography, has been replaced by HPLC and electrochemical or fluorometric detection or by mass spectroscopy[22]. In this study, homocysteine concentration in plasma was determined using HPLC/FLD in Korean; normal control (n=20) and chronic renal failure (n=90). The recovery and detection limit of total plasma homocysteine using HPLC/FLD was 98.6± 5.8% and 0.2 nmol/ml. The amounts of homocysteine in chronic renal failure was significantly hign in patient group ($p<0.05$) (Table 1). These results were consistent with those of Wilcken and Gupta (1970)[26]. Soria *et al.* (1990)[20] reported that concentrations of homocysteine in moderate (n=25) and severe renal failure group (n=28) and normal controls (n=45) were 18.2±1.7 nmol/ml vs 27.3±3.2 nmol/ml and 8.0±0.3 nmol/ml, respectively, in Caucacians. It also has been reported that total homocysteine was increased in the early stage of chronic renal failure, and that this concentration progresses with the worsening of renal failure[20]. Elevation of basal homocysteine levels has been defined as mild (15~24 nmol/ml), moderate (25~100 nmol/ml), or severe (>

100 nmol/ml)[27]. More recently, collaborative studies of vascular disease and homocysteine levels suggest that the upper limit of normal should be reduced to 12 nmol/ml[3].

From this study, this analytic method has sufficient sensitivity and reproducibility for the routine determination of plasma homocysteine. Further studies are needed for (i) the accurate reference ranges of sex and age group among Koreans (ii) whether there is prolonged accumulation of homocysteine in the development of increased vascular disease in chronic renal failure.

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REFERENCES

1. Araki A and Sako Y (1987): Determination of free and total homocysteine in human plasma by highperformance liquid chromatography with fluorescenece detection. *J Chromatography,* **422:** 43-52.
2. Bagdade JD and Albers JJ (1977): Plasma high-density lipoprotein concentrations in chronic hemodialysis and renal-transplant patients. *N Engl J Med,* **296:** 1436-1439.
3. Boushey CF, Bereford SAA, Omenn GS and Motulsky AG (1995): A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. *JAMA,* **274:** 1049-1057.
4. Brattstrom LE, Hardebs JE and Hultberg BL (1984): Moderate homocysteinemia - a possible risk for arteriosclerotic cerebrovascular disease. *Stroke,* **15:** 1012-1016.
5. Dieplinger H, Schoenfeld PY and Fielding CJ (1986): Plasma cholesterol metabolism in end-stage renal disease. Difference between treatment by hemodialysis or peritoneal dialysis. *J Clin Invest,* **77:** 1071-1083.
6. Green D, Stone NJ and Krumlovsky FA (1983): Putative atherogenic factors in patients with chronic renal failure. *Prog Cardiovasc Dis,* **26:** 133-144.
7. Harker LA, Harlan JM and Ross R (1983): Effect of sulfonpyrazone in homocysteine-induced endothelial injury and arteriosclerosis in baboons. *Circ Res,* **53:** 731-739.
8. Harker LA, Ross R and Slichter SJ (1976): Homocysteine-induced arteriosclerosis The role of endothelial cell injury and platelet response in its genesis. *J Clin Invest,* **58:** 731-741.
9. Ibels LS, Stewart JH and Mahoney JF (1977): Occlusive arterial disease in uremic and hemodialysis and renal transplant recipients. A study of the incidence of arterial disease and of the prevalence of risk factors implicated in the pathogenesis of arteriosclerosis. *Q J Med New Ser,* **46:** 197-214.
10. Ibels LS, Stewart JH, Mahony JF and sheil AGR (1974): Deaths from occulsive arterial disease in renal allograft recipients. *Br Med J,* **3:** 552-554.
11. Kang SS, Wong PWK and Becker N (1979): Protein-bound homocysteine in nomal subject and in patients with homocystinuria. *Pediatr Res,* **13:** 1141-1143.
12. Kang SS, Wong PWK, Cook HY, Norusis M and Messer JV (1986): Protein-bound homocysteine-A possible risk factor for coronary artery disease. *J Clin Invest,* **77:** 1482-1486.
13. Kang SS, Wong PWK and Malinow MR (1992): Hyperhomocyst(e)inemia as a risk factor for occlusive vascular disease. *Annu Rev Nutr,* **12:** 279-298.
14. Lowrie EG, Lazarus JM and Hampers CL (1974): Cardiovascular disease in hemodialysis patients. *N Engl J Med,* **290:** 737-738.

15. Rapaport J, Aviram M and Chaimovitz C (1978): Defective high density lipoprotein composition in patients on chronic hemodialysis. *N Engl J Med,* **29:** 1326-1329.
16. Rees MM and Rodgers GM (1993): Homocysteinemia: Association of a metabolic disorder with vascular disease and thrombosis. *Thrombosis Research*, **71:** 337-359.
17. Refsum H, Helland S and Ueland PM (1985): Radioenzyme determination of homocysteine in plasma and urine. *Clin Chem,* **31:** 624-628.
18. Refsum H, Unland PM and Svardal AM (1989): Fully automated fluorescence assay for determination total homocysteine in plasma. *Clin Chem,* **35:** 1921-1927.
19. Somer JB, Aiken JM and Abbott LK (1980): The influence of pre-transplant etiology of renal disease on lipoprotein lipids in female renal allograft recipients. *Atherosclerosis,* **37:** 1-9.
20. Soria C. Chadefaux B, Coude M, Gaillare O and Kamoun P (1990): Concentrations of total homocysteine in plasma in chronic renal failure. *Clin Chem*, **36:** 2137-2138.
21. Stabler SP, Marcell PD, Podell ER, Allen RH, Savage DG and Lindenbaum J (1988): Elevation of total homocysteine in the serum of patients with cobalamin or folate deficiency detected by capillary gas chromatography-mass spectrometry. *J Clin Invest,* **81:** 466-474.
22. Ubbink JB, Vermaak WJ and Bissbort S (1991): Rapid high performance liquid chromatographic assay for total homocysteine levels in human serum. *J Chromatogrphy*, **565:** 441-446.25.
23. Ueland PM and Refsum H (1989): Plasma homocysteine, a risk factor for vascular disease: Plasma level in health, disease, and drug therapy. *J Lab Clin Med*, **114:** 473-501.
24. Vester B and Rasmussen K (1991): High performance liquid chromatography method for rapid and accurate determination of homocysteine in plasma and serum. *Clin Chem,* **29:** 549-554.
25. Wilcken DEL and Gupta VJ (1980): Accumulation of sulphur-containing amino acids including cysteine-homocysteine in patients on maintenance hemodialysis. *Clin Sci*, **58:** 427-430
26. Wilcken DEL and Gupta VJ (1970): Cysteine-homocysteine mixed disulphide: Differing plasma concentrations in normal men and women. *Clin Sci*, **57:** 211-215.
27. Wilcken DEL and Gupta VJ (1981): Homocysteine in the plasma of renal transplant recipients: Effects of cofactors for methionine metabolism. *Clin Sci*, **61:** 743-749.
28. Wilcken DEL and Gupta VJ (1979): Sulphur containing amino acids in chronic renal failure with particular reference to homocysteine and cysteine-homocysteine mixed disulphide. *Eur J Clin Invest*, **9:** 301-307.

=국문초록=

만성신부전증환자에서 **HPLC/FLD**를 이용한 혈장 **Homocysteine**의 측정

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본 연구에서는 한국인 만성신부전증 환자에서 심혈관계 질환의 조기진단을 위한 생화학적 표지자로서 homocysteine의 임상적 유용성을 검토하기 위하여, 만성신부전증 환자와 건강인에서 HPLC/FLD (high performance liquid chromatography/fluorescence detector)를 이용하여 혈장 homocysteine 농도를 측정하고 그 결과를 비교하였다. 본 실험방법의 회수율은 98.6±5.8%를 나타내었으며, 0.2 nmol/L 보다 낮은 농도까지 측정이 가능하였고, 2~50 nmol/ml까지 직선성이 성립하였다 (correlation coefficient =0.9997). 한국인 중 건강한 정상인 (20명)과 만성신부전증 환자 (90명)에서 혈장 homocysteine은 각각 6.81±1.54 nmol/ml과 27.28±14.94 nmol/ml이었으며, 환자군에서는 정상인군에 비하여 약 4 배 정도 높은 수치를 나타내었다 ($p<0.05$). 본 실험의 결과로 볼 때 HPLC/FLD를 이용한 homocysteine 측정은 예민도와 재현성이 높아 routine 실험실 방법으로 유용성이 높을 것으로 생각되며, 또한 혈장 homocysteine의 측정은 만성신부전증 환자의 주요한 사망원인이 되는 폐쇄성 동맥질환을 조기에 진단하거나, 혹은 homocysteine 농도를 저하시키는 치료를 실시한 후 치료효과를 판단하기 위한 생화학적 marker로 활용될 수 있을 것으로 기대된다.

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