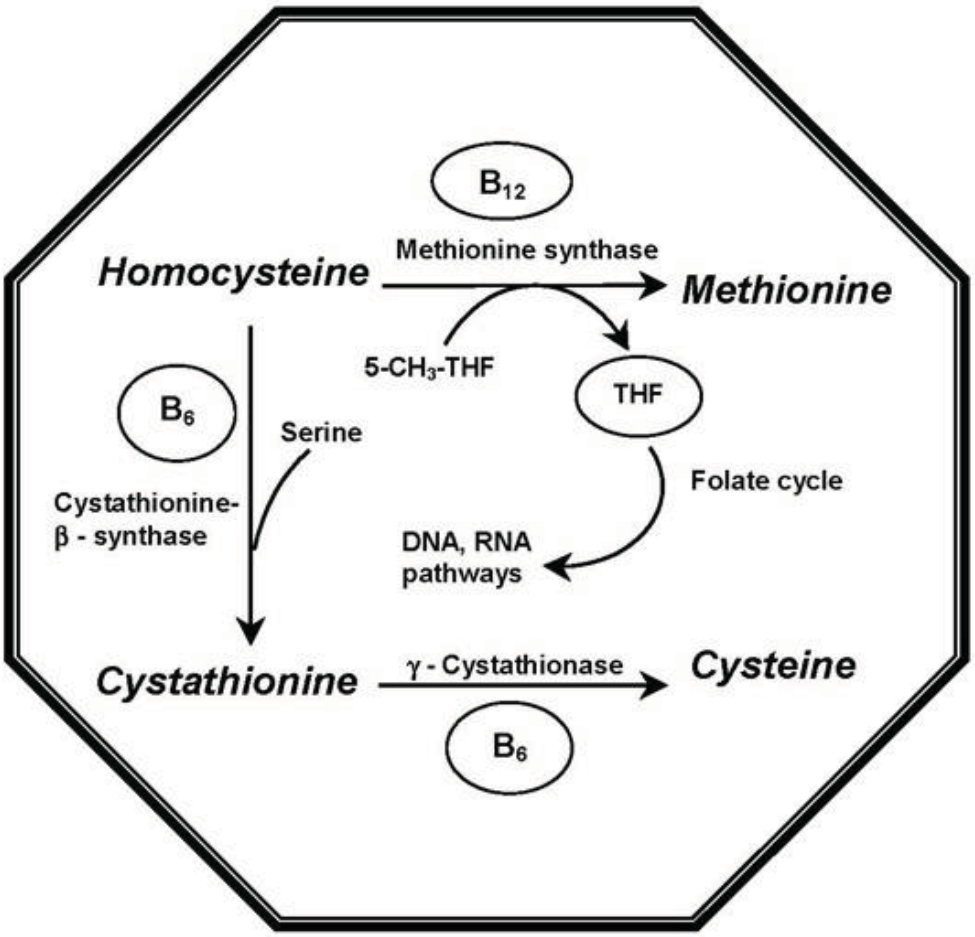
A New Gas Chromatographic-Mass Spectrometric Method for the Determination of Total Plasma Homocysteine and Related Amino Acids

Clinical Application in Studies with End-Stage Renal Disease Patients under Chronic Dialysis

Joseph I. Sigit



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A New Gas Chromatographic-Mass Spectrometric Method for the Determination of Total Plasma Homocysteine and Related Amino Acids

Clinical Application in Studies with End-Stage Renal Disease Patients under Chronic Dialysis

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dedicated to my wife Atiek and my son Boni

Zusammenfassung

Homocystein ist eine schwefelhaltige Aminosäure und stellt ein Intermediärprodukt im Methioninstoffwechsel dar. Unter Mitwirkung von Folsäure als Methylgruppendonor und Vitamin B_{12} als Kofaktor kann Homocystein zu Methionin remethyliert oder durch Transsulfurierung mittels Vitamin B_6 als Kofaktor über Cystathionin zu Cystein abgebaut werden (Abbildung 1). Die Hyperhomocysteinämie mit Plasmakonzentrationen grösser als 15 µmol/l für Gesamthomocystein (tHcy) wird als ein neuer unabhängiger Risikofaktor für Atherosklerose und vaskuläre Erkrankungen angesehen. Zusätzliche Gabe von Folsäure, Vitamin B_6 und B_{12} kann zu einer sicheren und deutlichen Senkung erhöhter Plasma tHcy-Konzentrationen in der Allgemeinbevölkerung und auch bei niereninsuffizienten Patienten unter Dialysebehandlung führen.

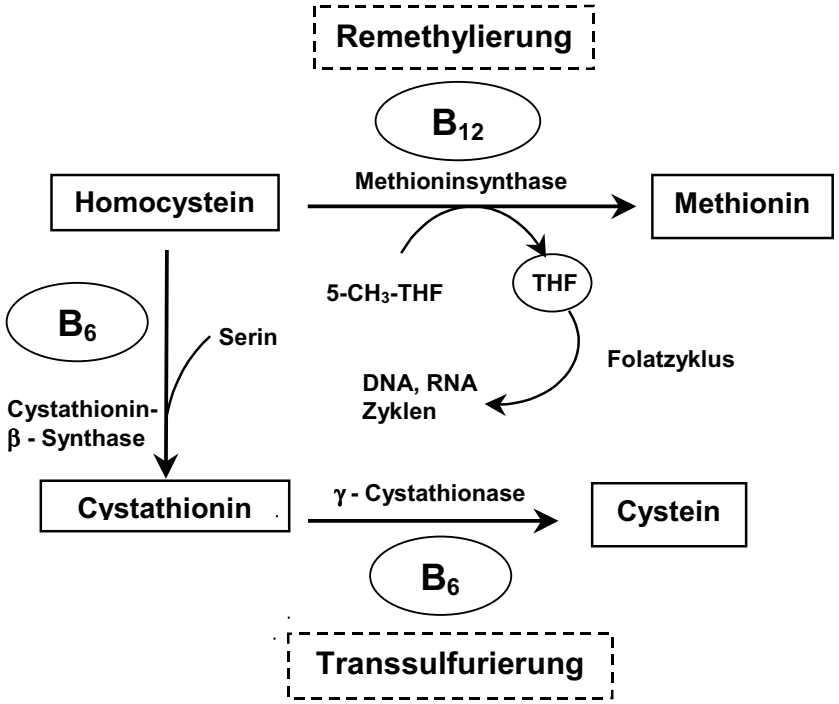

Abbildung 1 Der Methioninstoffwechsel

In dieser vorgelegten Dissertationsschrift wird die Entwicklung und praktische Anwendung eines sensitiven und spezifischen gaschromatographischen-massenspektrometrischen (GC-MS) Isotopen-Verdünnungsverfahrens unter Verwendung Deuteriummarkierter interner Standards zur Bestimmung von Homocystein neben Cystein, Methionin und Cystathionin nach Dekonjugation möglicher Disulfid- und Proteinbindungen und nach Derivatisierung zu N(O, S)-Ethoxycarbonyl-Aminosäureethylestern beschrieben.

Diese neue GC-MS Methode wurde für tHcy im Vergleich mit einer etablierten Hochdruckflüssigkeitschromatographie- (HPLC-) und einer IMx-Methode validiert, wobei gleichzeitig benachbarte Aminosäuren im Plasma von dialysepflichtigen niereninsuffizienten Patienten untersucht wurden. In einer weiteren vergleichenden Studie wurde der Einfluss von Leucovorin (N^5-Formyltetrahydrofolsäure) gegen Folsäure bei gleichen Dosierungen auf Änderungen des Plasmagehaltes an tHcy und benachbarter Aminosäuren bei niereninsuffizienten Patienten unter Dialyse analysiert.

Passing-Bablok Regressionsanalyse der Homocysteinkonzentrationen ergab im Vergleich der verschiedenen Methoden hochsignifikante positive Korrelationen, wobei jedoch die GC-MS-Analytik signifikant niedrigere tHcy-Plasmakonzentration gegenüber der HPLC- bzw. IMx-Messung ergab. Trotz Vitamin B_6-Substitution hatten Patienten mit chronischer Niereninsuffizienz weiterhin deutlich erhöhte tHcy-Plasmakonzentrationen. Eine leicht erhöhte mittlere Cysteinkonzentration zeigte eine signifikant positive Korrelation mit Homocystein. Methionin blieb hiervon unberührt. Dies weist bei diesen Patienten unter Vitamin B_6-Substitution auf einen parallelen Anstieg der Homocystein- und Cysteinkonzentration während der Transsulfurierung hin, während die Remethylierung zu Methionin unverändert bleibt (Abbildung 2).

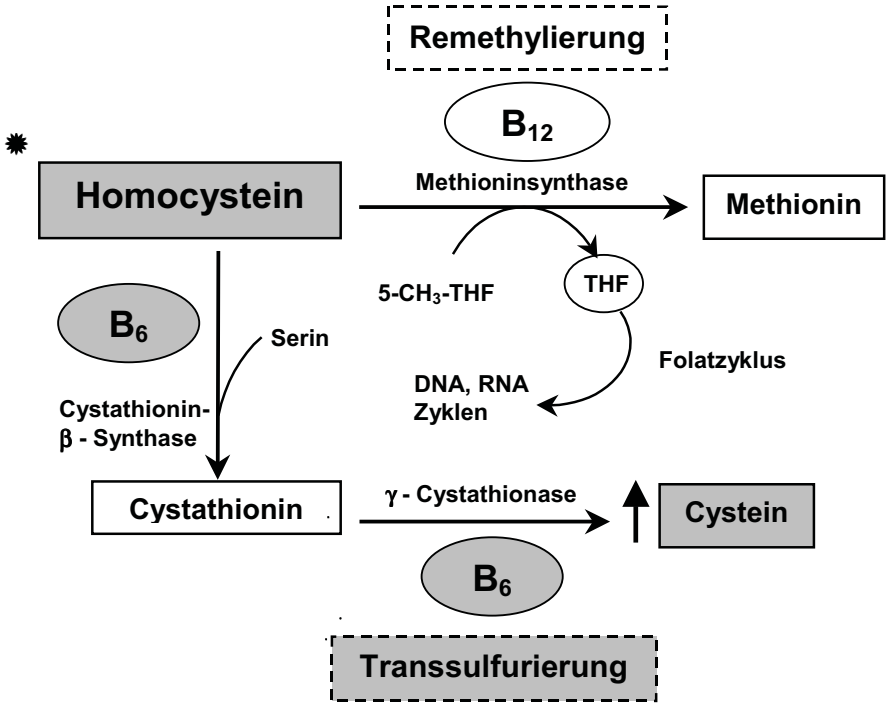

Abbildung 2 Pathobiochemie des Methioninstoffwechsels bei niereninsuffizienten Patienten unter Dialysebehandlung und Vitamin B_6-Substitution

✹ : deutliche Erhöhung der tHcy-Plasmakonzentration

↑ : leichte Erhöhung der Cystein-Plasmakonzentration

Die Ergebnisse der zweiten Untersuchung zeigten, dass es keine Unterschiede in der Senkung oder Normalisierung der Gesamthomocysteinplasmakonzentrationen bei dieser Dialyse-Patientengruppe und Vitamin B_6-Substitution nach intravenöser Gabe von Leucovorin im Vergleich mit Folsäure gab. Trotz zusätzlicher Leucovorin- oder Folsäuresubstitution blieb bei 71,2% unserer Patienten der Plasmahomocysteingehalt erhöht. Eine Normalisierung der Gesamtplasma-homocysteinkonzentration trat nur bei 23,1% der Patienten der Leucovorin- und bei nur 17,4% der Folsäuregruppe nach vier Wochen auf. Eine Gesamtsenkung um 27,6% konnte nach acht Wochen bei den Patienten der Leucovorin- und bei 30,3% der Folsäuregruppe festgestellt werden. Es wurde eine signifikant positive Korrelation zwischen der prozentualen Änderung von tHcy im Plasma und erythrozytärer Folsäure gefunden. Eine negative Korrelation zwischen den prozentualen Änderungen von tHcy und Methionin deuten an, dass der Reduktion von tHcy im Plasma unter Folsäuresubstitution eine Erhöhung des Methioningehaltes während des Folsäure- und Vitamin B_{12}-abhängigen Remethylierungsweges folgte (Abbildung 3). Trotz eines schwachen Effektes in der Senkung des Plasmahomocysteingehaltes durch die Folsäure und Vitamin B_6-Behandlung gab es keine signifikanten Korrelationen zwischen den prozentualen Änderungen von Plasma tHcy, Methionin, Cystein und erythrozytärer Folsäure.

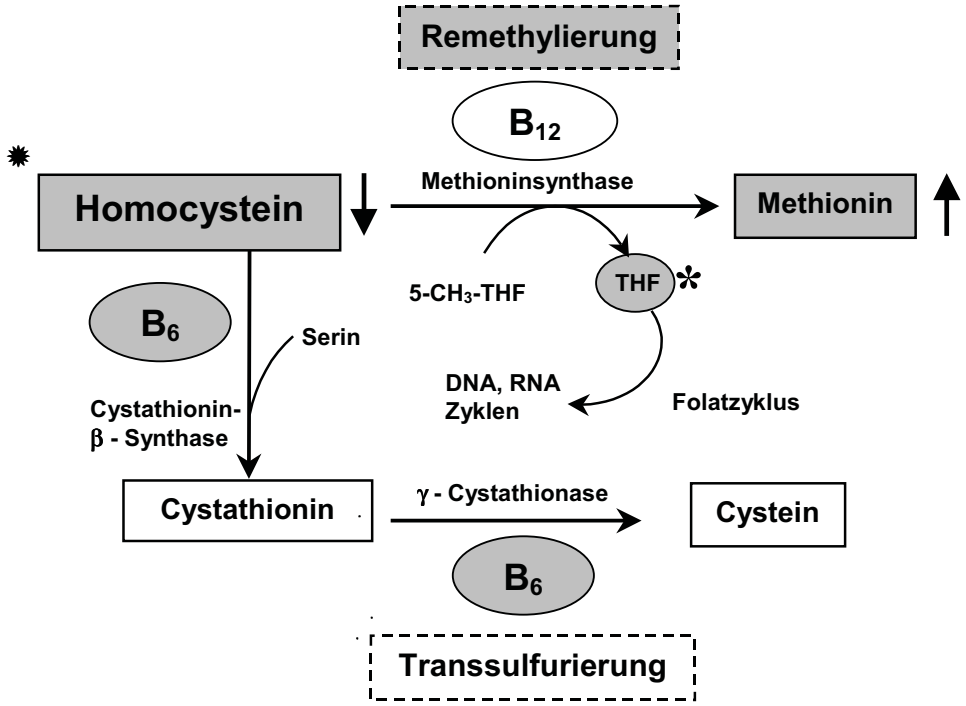

Abbildung 3 Pathobiochemie des Methioninstoffwechsels bei niereninsuffizienten Patienten unter Dialysebehandlung, Vitamin B_6-Substitution und zusätzlicher Gabe von Leucovorin oder Folsäure

↓ : leichte Senkung der deutlich erhöhten tHcy-Plasmakonzentration ✹

↑ : leichte Erhöhung der Methionin-Plasmakonzentration

* : zusätzliche Gabe von Leucovorin oder Folsäure

Summary

Homocysteine is an amino acid containing sulphur which is generated as an intermediate product in methionine metabolism. Homocysteine can be remethylated to methionine using folate as the methyl group donor and the co-factor vitamin $B_{12,}$ or is catabolized to cysteine by transsulfuration via cystathionine with vitamin B_6 as a cofactor (Figure 1). Hyperhomocysteinemia with plasma concentrations of total homocysteine (tHcy) greater than 15 µmol/l has been designated as a new independent risk factor for atherosclerosis and vascular disease. Additional administration of folic acid, vitamin B_{12} and B_6 can safely and effectively reduce the elevated plasma concentrations of tHcy in the general population and also in end-stage renal disease (ESRD) patients under maintenance dialysis.

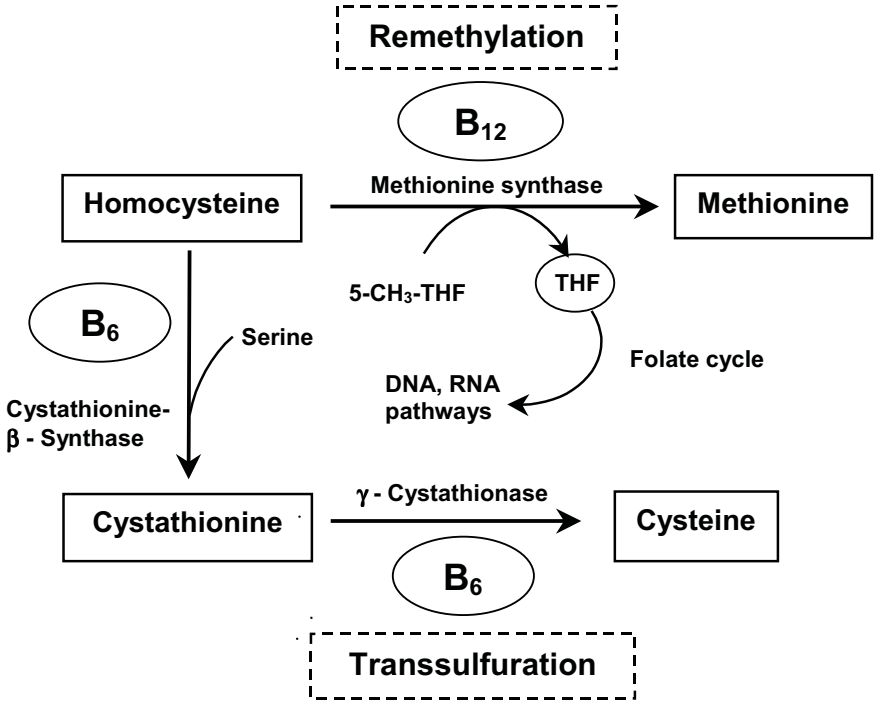

Figure 1 The methionine metabolism

The present dissertation describes the development and practical application of a new sensitive and specific gas chromatographic-mass spectrometric (GC-MS) isotope dilution assay using deuterated internal standards for determination of tHcy as well as cysteine, methionine, and cystathionine after deconjugation of disulfide and protein bindings and derivatization of free amino acids to N(O,S)-ethoxycarbonyl amino acid ethyl ester. This new

GC-MS method for tHcy determination was validated by comparison with an established high performance-liquid chromatographic (HPLC) method as well as an IMx homocysteine assay, in which we analyzed simultaneously other related amino acids in the plasma of ESRD patients under maintenance dialysis. In another comparative study, the effect of Leucovorin (5-formyltetrahydrofolate) versus folic acid, both applied in equimolar doses, on changes in plasma tHcy and related amino acids was investigated in ESRD patients under maintenance dialysis.

Passing-Bablok regression analysis of the tHcy concentrations revealed highly significant correlations between the different methods, whereas the mean plasma concentration of tHcy determined by GC-MS was significantly lower than that determined by HPLC or by IMx. Despite substitution with vitamin B_6 patients with chronic renal disease had markedly elevated plasma concentrations of tHcy. A slightly increased mean concentration of cysteine showed a significant positive correlation with homocysteine. Methionine remained unchanged. This indicates an apparent increase in homocysteine parallel to cysteine during transsulfuration in those patients undergoing high vitamin B_6-plasma substitution, while remethylation to methionine remains unchanged (Figure 2).

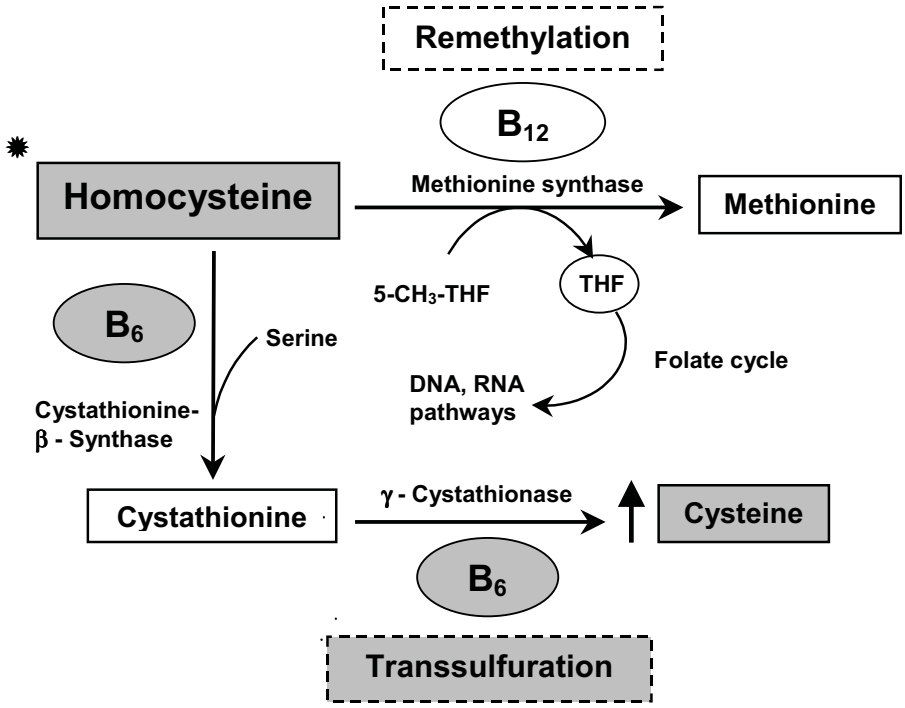

Figure 2 Pathobiochemistry of methionine metabolism in ESRD patients under maintenance dialysis and vitamin B_6 substitution

✹ : significantly elevated plasma concentration of tHcy

↑ : slightly elevated plasma concentration of cysteine

The results of the second study showed no difference in reduction or normalization of plasma tHcy in this patient group under maintenance dialysis and vitamin B_6 substitution, after intravenous administration of Leucovorin (N^5-formyltetrahydrofolate) in comparison to folic acid. Despite additional substitution with Leucovorin or folic acid, the plasma tHcy concentration remained elevated in 71.2% of our patients. Normalization of the plasma tHcy concentration was found in only 23.1% of the patients in the Leucovorin group and in 17.4% of the patients in the folic acid group after four weeks treatment. A total decrease after eight weeks could be observed in 27.6% of the patients in the Leucovorin group and in 30.3% of the patients in the folic acid group. A significant positive correlation was found between the percentage change of plasma tHcy and red blood cell folate. A negative correlation between the percentage change of tHcy and methionine indicates that the reduction of plasma tHcy under folate therapy was followed by an elevation of plasma methionine during folate- and vitamin B_{12}-dependent remethylation (Figure 3). Despite a weak effect on the decrease of plasma tHcy concentration by folate and vitamin B_6, there were no significant correlations between percentage changes of plasma tHcy, methionine, cysteine and red blood cell folate.

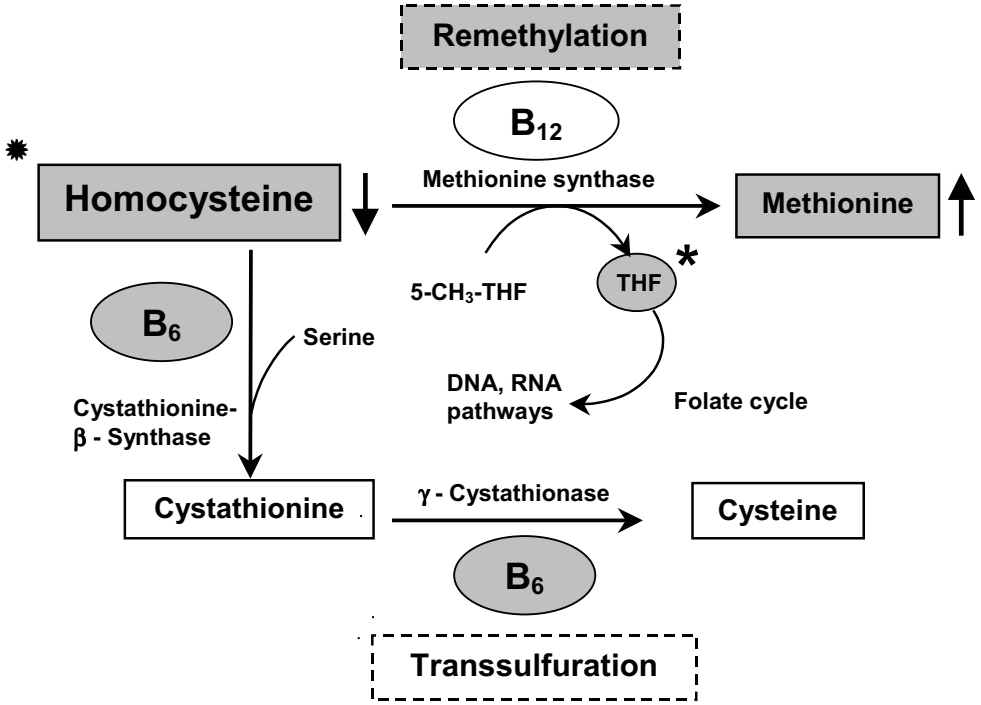

Figure 3 Pathobiochemistry of methionine metabolism in ESRD patients under maintenance dialysis, vitamin B_6 substitution and additional administration of Leucovorin or folic acid

↓ : slight decrease of significantly elevated plasma concentration of tHcy ✹
↑ : slight increase of plasma concentration of methionine
* : additional administration of Leucovorin or folic acid

C O N T E N T S

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List of Abbreviations

5-FU	5-fluorouracil
ApoB	apolipoprotein B
BMI	body mass index
CAD	coronary artery disease
CBS	cystathionine-ß-synthase
CI	confidence interval
CV	coefficient of variation
CVD	cardiovascular disease
Cys	cysteine
Cystat	cystathionine
DTT	dithiothreitol
ECF	ethylchloroformat
EDTA	ethylenediaminetetraacetic acid
ERNDIM	European research network for evaluation and improvement of screening, diagnosis and treatment of inherited disorders of metabolism
ESRD	end-stage renal disease
FA	folic acid
GC	gas chromatography
GC-MS	gas chromatography - mass spectrometry
GFR	glomerolus filtration rate
Hcy	homocysteine
HMG Co-A	3-hydroxy-3-methylglutaryl-coenzyme A
HPLC	high-performance liquid chromatography
IDDM	insulin independent diabetes mellitus
IMx	enzyme immunoassay
IS	internal standard
K_D	coefficient of distribution
LDL	low density lipoprotein
Lp(a)	lipoprotein (a)
M/Z	mass to charge ratio
Met	methionine

METHF	methylenetetrahydrofolate
MS	methionine synthase
MTHF	methyltetrahydrofolate
MTHFR	methyltetrahydrofolate reductase
NIDDM	non insulin dependent diabetes mellitus
PGA	pteroylmonoglutamic acid
PLP	pyridoxal-5-phosphate
RBC	red blood cell
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SBD-F	ammonium-7-fluorobenzo-2-oxa-1.3-diazole-4-sulfonate
SD	standard deviation
SIM	selected ion-monitoring
tHcy	total homocysteine, i.e. the sum of all homocysteine forms, whether reduced or oxidized, free or bound to proteins
THF	tetrahydrofolate
WHO	World Health Organization

1 Introduction

Within the general population and especially western population (Europe and American), infections and parasitic diseases and diseases of the cardiovascular system are the leading cause of death, followed by malignant tumors (WHO report 2000, Figure 1.1).

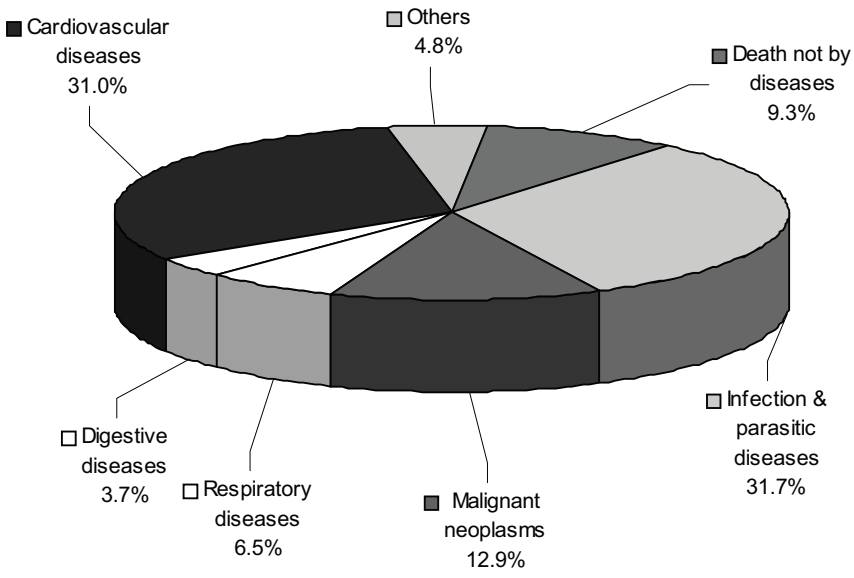

Figure 1.1 Cause of death in the general population in year 1999 (WHO report 2000)

Epidemiological research during the last twenty years emphasizes some risk factors for cardiovascular diseases: hyperlipidemia, hypercholesterolemia, smoking, high blood pressure, obesity and diabetes. Smoking cessation, reduction in LDL-cholesterol serum concentrations and normalization of blood pressure have been shown to be effective strategies in the prevention of cardiovascular disease (CVD). However, these major classic cardiovascular risk factors and such non-modifiable risk factors as age, sex and family history cannot fully explain why some persons develop myocardial infarction and/or stroke while others do not. Other factors may also increase the likelihood of developing cardiovascular diseases and may contribute to atherogenesis. Pathologic and epidemiological studies suggest that only about one half to two thirds of the variation in the anatomic extent of atherosclerosis and risk for atherosclerotic vascular disease can be explained by classic risk factors.

Therefore, many emerging risk factors have been found and among these, elevated plasma tHcy concentration (hyperhomocysteinemia) has been designated as a new risk factor for cardiovascular disease.

Homocysteine is a naturally sulfur containing amino acid which is generated as an intermediate product in the methionine metabolism, an essential dietary amino acid. Hyperhomocysteinemia is generally defined as a state when fasting plasma total homocysteine concentrations exceed concentrations of 15 μmol/l. This has been shown to be an independent risk factor for cardiovascular disease. However, there are many factors which directly or indirectly influence the concentration of tHcy such as genetic defects, vitamin deficiency, life style, disease and medication.

Since homocysteine is associated with the development of atherosclerosis and vascular disease, there is growing interest in highly specific and sensitive methods for the detection, quantification and progress of increased concentrations of tHcy in many clinical cases.

Generally, it is the aim of this dissertation to develop a sensitive and accurate new gas chromatographic-mass spectrometric (GC-MS) method for the determination of homocysteine, which also allows to determine simultaneously other related amino acids like methionine, cysteine and cystathionine. To validate this method we compared the results with those of an established high-performance liquid chromatography (HPLC) method and IMx homocysteine assay, and we applied this new GC-MS method in two clinical investigations. We targeted our study at patients with end-stage renal disease (ESRD), which is known to carry high risks for cardiovascular disease. In detail, the specific objectives were :

- To develop a GC-MS method for determination of tHcy and related amino acids like methionine, cysteine and cystathionine.
- To compare this new GC-MS method for determination of tHcy with an established HPLC method and an IMx homocysteine assay.
- To apply this GC-MS method on hemodialysis patients in order to observe the metabolism of homocysteine on ESRD patients and its relationship with related amino acids.
- To compare effects of Leucovorin (N^5-formyltetrahydrofolate) versus folic acid in reducing plasma tHcy in ESRD patients. Additionally, we observed the concentrations of the other related amino acids under this therapy.

In chapter 2, a short overview on the theme of homocysteine is presented describing a theoretical background of homocysteine including history, metabolism, determinants and its relationship with cardiovascular and renal disease.

In chapter 3, the development of the new GC-MS method using isotope dilution method is described, including the analytical performance of the method.

In chapter 4, the comparison of the new GC-MS method with an established HPLC method and IMx homocysteine assay is described.

In chapter 5, the application of this method in two clinical investigations is presented. In the first study we observed the homocyteine metabolism in ESRD patients, and in another study we compared the effects of Leucovorin versus folic acid in reducing plasma tHcy and the other metabolites in ESRD patients.

Finally, we discuss our findings in chapter 6 by comparing these with previous results from other research groups.

2 General Background

2.1 Historical Aspects

Homocysteine was discovered in 1932 by du Vigneaud. He described that treatment of methionine with concentrated acid yielded homocysteine. Having synthesized homocysteine and homocystine, du Vigneaud performed a series of classic studies designed to test the nutritional roles of methionine, cysteine and homocysteine (Finkelstein, 2000).

In 1962, homocystinuria was identified in mentally retarded children (Gerritsen *et al.*, 1962). Two years later the genetic defect of cystathionine-β-synthase (CBS) was presented by Mudd *et al.* (1964). It was reported that these patients had thromboembolic events and a high concentration of homocystine in urine could be found.

In 1969, McCully formulated the "homocysteine theory of atherosclerosis". He described the vascular pathology of homocystinuria and noted that thromboembolic disease was a characteristic feature of homocystinuria independent of the site of the metabolic defect, pointing to homocysteine as the causal agent. His theory implies that a moderately elevated homocysteine concentration may be a cardiovascular risk factor in the general population (McCully and Wilson, 1975).

In 1976, Wilcken and Wilcken studied patients without homocystinuria but with angiographically diagnosed coronary artery disease. Using the methionine loading test, in which 0.1 mg/kg body weight of this amino acid is administered orally, they found that the prevalence of high circulating homocysteine concentrations or hyperhomocysteinemia in these patients was higher than in healthy controls. They concluded that people with a genetic cystathionine-ß-synthase (CBS) deficiency might be at greater risk of coronary artery disease. It was the first report to state that coronary artery patients have abnormal homocysteine metabolism.

For the following 15 years there were only scattered reports on the relationship between homocysteine levels and cardiovascular disease. In the same period, the most important determinants of total homocysteine (tHcy) were identified, including age and sex, renal function, and vitamin status. However, since 1990, there has been an exponential increase in the publication rate on homocysteine and cardiovascular disease. This is related to the recognition of elevated tHcy as an independent cardiovascular risk factor. The first ever positive prospective studies on plasma homocysteine and coronary artery disease (CAD) were reported in 1992 by Stampfer *et al.* and Selhub *et al.* (1995). Both reported an association

between plasma homocysteine concentration and extracranial carotid artery stenosis in the elderly. Boushey *et al.* (1995) reviewed most studies on homocysteine and cardiovascular disease and a meta analysis of 27 studies, including approximately 4,000 patients, showed that elevated homocysteine was an independent graded risk factor for the incidence of atherosclerosis. Since then, there have been many additional studies on homocysteinemia as a risk factor for cardiovascular disease and its complications, the majority of them supporting the conclusion of the meta analysis by Boushey *et al.*

2.2 Biochemistry and Metabolism of Homocysteine

Homocysteine (2-amino 4-mercaptobutyric acid) is a sulfur-containing amino acid produced during the metabolism of the essential amino acid methionine. In plasma, the main part (around 70%) is bound to proteins, mainly to albumin by a disulfide bond. Another fraction exists as a free, oxidized (~30%) form, and a smaller fraction in a free reduced (sulfhydryl) form (1.5 – 4%). The free oxidized fraction occurs mainly as homocysteine-cysteine mixed disulfide and also as homocystine, a symmetric disulfide homocysteine form (Mansoor *et al.*,1992; Ueland *et al.*, 1996). The sum of all these forms, free or bound to proteins, is called total homocysteine (tHcy), or simply, homocyst(e)ine. The various common chemical structures of homocysteine, and its related amino acids methionine, cysteine, cystathionine are presented in Figure 2.1 and Figure 2.2.

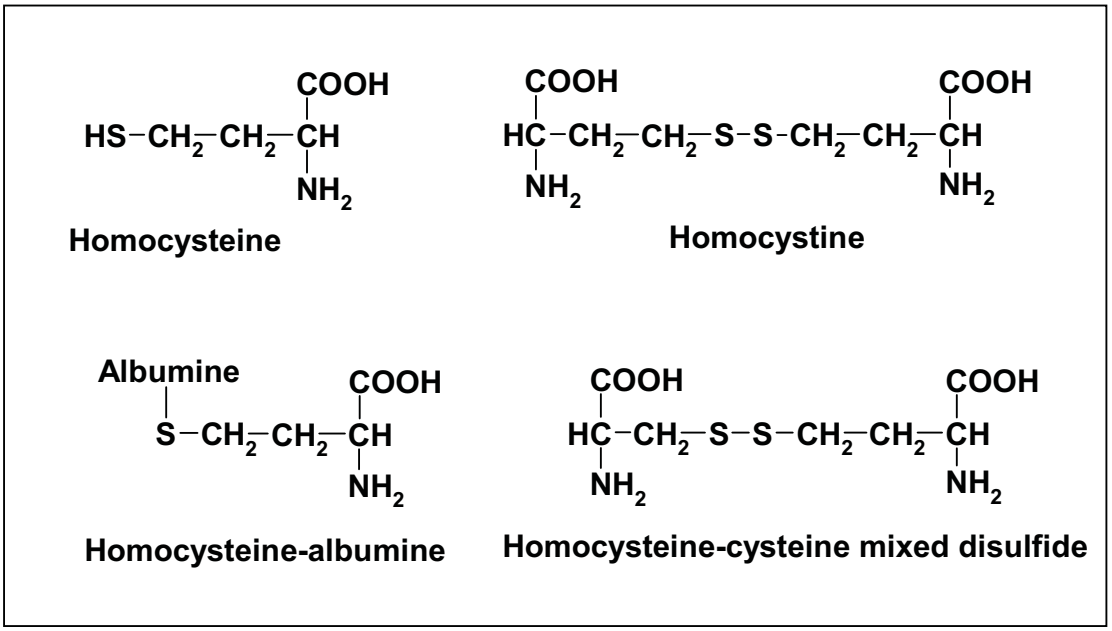

Figure 2.1 The various common structures of homocysteine

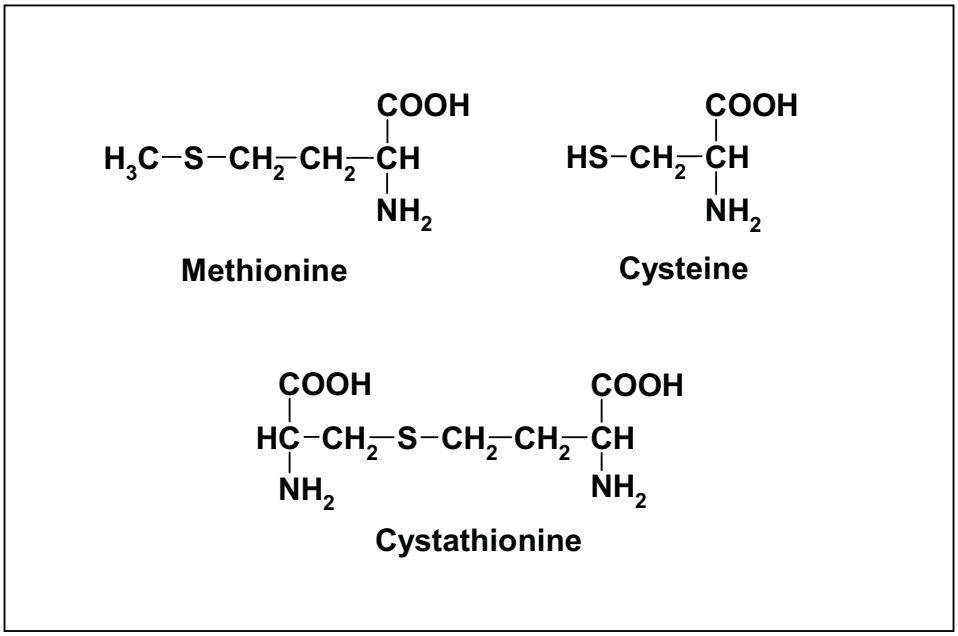

Figure 2.2 Chemical structure of methionine, cysteine, cystathionine.

Homocysteine is metabolized by two major pathways : it can be remethylated to methionine (remethylation pathway) or catabolized to cysteine via cystathionine (transsulfuration pathway). The scheme of methionine metabolism is shown in Figure 2.3.

2.2.1 Remethylation pathway

Within the remethylation pathway, homocysteine is converted to methionine, a reaction catalyzed by methyltetrahydrofolate homocysteine methyltransferase, also known as methionine synthase (MS). This enzymatic reaction requires methylenetetrahydrofolate (MTHF), which is generated by the enzyme MTHF-reductase (MTHFR), and is also dependent on the co-factor methylcobalamin, a form of vitamin B_{12}. Methionine is continuously converted into S-adenosylmethionine (SAM), which is the major source of methyl groups in many cellular methylation reactions (Chiang *et al.*, 1996). The methyl group donator SAM is then demethylated to form S-adenosylhomocysteine (SAH), and SAH is converted back to homocysteine by the enzyme SAH hydrolase (Minner *et al.*, 1997). An additional minor remethylation mechanism is catalyzed by betaine homocysteine methyltransferase and utilizes betaine as a methyl donor to form N,N-dimethylgycine and methionine. This reaction requires the action of the vitamin B_{12} and also the folate independent enzyme, betaine-homocysteine methyltransferase.

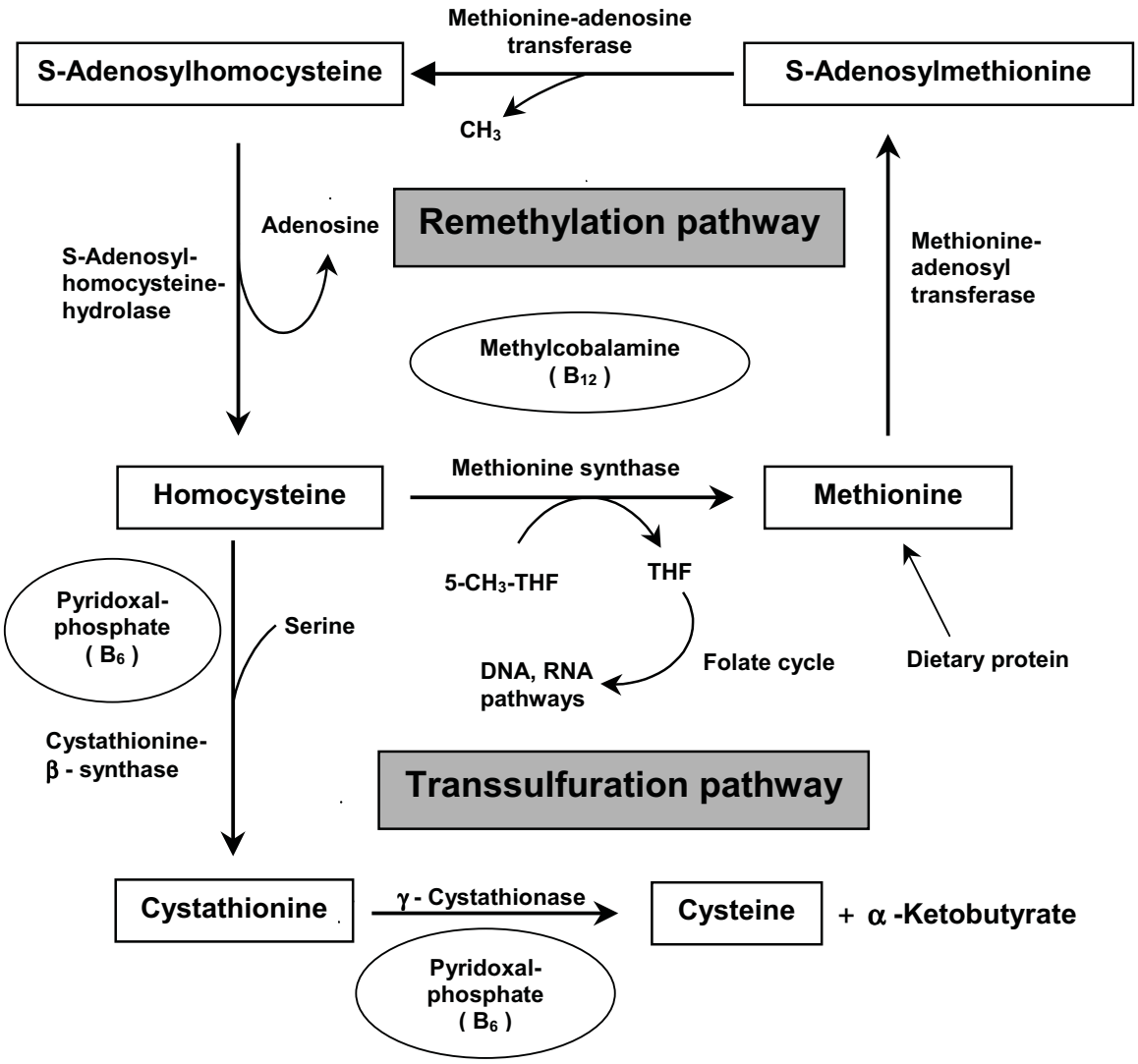

Figure 2.3 Scheme of methionine metabolism

2.2.2 Transsulfuration pathway

Within the transsulfuration pathway, homocysteine condenses with serine to form cystathionine. This irreversible reaction is catalyzed by the pyridoxal-5-phosphate (PLP, vitamin B_6) dependent enzyme, cystathionine-ß-synthase. Cysteine and α-ketobutyrate are formed by the action of γ-cystathionase, again requiring PLP as cofactor. Finally, cysteine is either converted into taurine, utilized in the synthesis of glutathione, or sulfated prior to urinary excretion.

The homocysteine metabolic pathways are regulated by the cellular concentration of methionine and SAM. When excessive methionine is present increased amounts of SAM are generated. SAM directly stimulates the activity of cystathionine ß-synthase and causes

inhibition of methyltetrahydrofolate reductase (Finkelstein *et al.*, 1998). This phenomena causes the increase of transsulfuration and the reduction of remethylation of homocysteine. Conversely, if methionine levels are low, SAM concentrations fall, resulting in reduced transsulfuration and an upgrading of remethylation of homocysteine to restore methionine levels.

2.3 Classification of Hyperhomocysteinemia

The normal fasting range of plasma tHcy is 5-15 µmol/l (Ueland *et al.*, 1993). Greater than 15 µmol/l of plasma tHcy is defined as hyperhomocysteinemia. Kang and co-worker classified several types of hyperhomocysteinemia in relation to plasma tHcy concentrations (Kang *et al.*, 1992). They defined hyperhomocysteinemia as a severe form for concentrations of plasma tHcy higher than 100 µmol/l, intermediate for concentrations between 30-100 µmol/l, and moderate for concentrations of 15-30 µmol/l, and a normal reference of plasma tHcy range of 5 to 15 µmol/l (mean, 10 µmol/l). Due to the influence of many factors, no consensus on what levels constitute hyperhomocysteinemia currently exists.

2.4 Determinants of Homocysteine

The determinants of plasma tHcy are complex and involve demographic, genetic and acquired factors. Gene-nutrient interactions are most likely important determinants in subjects who carry one or more mutations in genes that regulate homocysteine metabolism. Thus, genetic background, demographic, nutrition, lifestyle, medication and pathologic influence the homeostasis of homocysteine (Table 2.1).

2.4.1 Genetics

Cystathionine-ß-synthase (CBS) deficiency in the homozygous form causes homocystinuria and is associated with extremely elevated tHcy levels. This was first described by Mudd *et al.* who found a high concentration of homocystine in urine (Mudd *et al.*, 1964). However, this genetic enzyme defect is rare, with a frequency of 1:58,000 to 1:1,000,000 in newborns and a world-wide birth prevalence of 1:300,000. The prevalence of heterozygosity for this mutation in the general population is less than 1% (Mudd *et al.*, 1985). However, the CBS deficiency cannot be explained by the high incidence of homocystinuria alone, neither in healthy humans nor in the vascular disease population (Nygard *et al.*, 1995).

The common C677T polymorphism of the methyl tetrahydrofolate reductase (MTHFR) gene has been established as an important genetic determinant of elevated fasting tHcy (Engbersen *et al.*, 1995). Homozygotes for this polymorphism (TT genotype) are predisposed to intermediate hyperhomocysteinemia (Kang *et al.*, 1988). This genotype is strongly related to folate status. TT subjects with adequate folate levels usually have normal tHcy levels, whereas TT subjects with low folate status have high levels of tHcy. Guttormsen *et al.* have observed that in presumed healthy subjects with fasting tHcy concentrations > 40 µmol/l, the majority showed the TT genotype. When excluding subjects with cobalamin deficiency, 92% were found to be homozygous for T-allele compared with approximately 10% in the general population (Guttormsen *et al.*, 1996a).

2.4.2 Vitamin Deficiency

Folate and cobalamin deficiencies are the most common causes of moderate to severe fasting hyperhomocysteinemia (Allen *et al.*, 1993; Ubbink, 1997). The incidence of these deficiencies increases with age (Lewis, 1976). In most cases, cobalamin deficiency is the result of a malabsorptive disorder (Nilsson-Ehle, 1998), whereas the more frequently appearing folate deficiency is explained by poor diet, cooking method, use of certain drugs, and alcohol consumption. Impaired vitamin B_{12} status is often combined with folate deficiency (Lindenbaum, 1979; Russell, 1992; Matthews, 1995; Carmel, 1997; Saltzman and Russell, 1998). There is also a good metabolic interrelationship between B_{12} and folate, and deficiency of one vitamin may affect the status of the other (Chanarin *et al.*, 1985; Shane and Stokstad, 1985). Although vitamin B_6 is required in the two sequential reactions in which homocysteine is converted into cysteine, vitamin B_6 deficiency normally does not result in elevated fasting total homocysteine but increases the post-methionine load tHcy (Miller *et al.*, 1994). Therefore, an isolated nutritional vitamin B_6 deficiency is considered rare, and a low-dose vitamin B_6 supplementation is not believed to reduce fasting tHcy (Dierkes *et al.*, 1998; Pietrzik and Bronstrup, 1998). However, other reports indicate that fasting tHcy is negatively correlated with both vitamin intake and serum levels of not only folate and vitamin B_{12} but also vitamin B_6 (Selhub *et al.*, 1993; Shimakawa *et al.*, 1997).

Combined deficiencies of the previously mentioned vitamins are commonly found and display an interrelationship with other determinants of homocysteine status. A variety of drugs may impair vitamin absorption or function. The severity a of clinical vitamin deficiency may be modified by genetic predisposition, such as MTHFR polymorphism (Malinow *et al.*, 1997). Gastrointestinal disease will often result in impaired vitamin absorption (Lindenbaum,

1979). In pernicious anemia, there is a predisposition toward other autoimmune diseases, such as hypothyroidism, that may affect homocysteine status. Renal diseases may be associated with increased vitamin loss or demand (Gupta and Robinson, 1997) . Increasing demands have also been reported in chronic inflammatory diseases, cancer, and thyroid disease (Stokstad *et al.* 1980; Stene-Larsen *et al.*, 1988).

2.4.3 Age and Gender

Plasma tHcy increases throughout life in both sexes. However, especially in adults, tHcy concentration are higher in men than in women. In adults, the plasma tHcy levels in males are usually about 1 to 2 μmol/L higher than in females (Nygard *et al.*, 1995; Refsum *et al.*, 1998a). Sex-related differences are explained by the effects of sex steroid hormones on tHcy (Anker *et al.*, 1995; Wouters *et al.*, 1995). In addition, increased tHcy concentration in males may be the result of a comparatively higher homocysteine production possibly related to differences in the creatine-creatinine synthesis (Mudd *et al.*, 1985; Norlund *et al.*, 1998).

In the elderly, an increasing number of subjects exhibits hyperhomocysteinemia (Hermann *et al.*, 1999). The age-dependent increase may be attributed to a deterioration of the renal function (Arnadottir *et al.*, 1996) and impaired folate status (Tucker *et al.* 1996). Moreover, an increasing prevalence of cobalamin deficiency among the elderly may play an important role. Cobalamin deficiency often develops because of malabsorption related to the aging of the gut (Saltzman and Russell, 1998).

In conclusion, tHcy gradually increases with age, and higher tHcy concentrations in the elderly may be explained by an interaction of a variety of factors. The most important age related conditions are : suboptimal vitamin status, impaired renal function, dietary insufficiency and intestinal malabsorption.

2.4.4 Lifestyle

Several lifestyle factors are important determinants of homocysteine status in the general population. Lifestyle factors may essentially interact with any of the other determinants of homocysteine (Table 2.1).

Diet plays a significant role in homocysteine status. Circulating levels of homocysteine are directly related to methionine intake, and inversely related to vitamin intake (Guttormsen *et al.*, 1994; Ubbink, 1994). Diets rich in animal proteins have a significantly higher methionine content than those rich in plant-derived proteins. In addition, high intake of

fresh fruits and vegetables as well as vitamin supplements have been associated with decreased homocysteine levels (Ubbink, 1994; Kuschi *et al.*, 1995; Nygard *et al.*, 1995). Lipid status is only weakly associated with homocysteine levels (Alfthan *et al.*, 1994; Nygard *et al.*, 1995). However, nutritional factors influencing tHcy in normolipidemic subjects are also predictive in hyperlipidemic subjects (Glueck *et al.*, 1995).

In smokers, higher levels of homocysteine have been demonstrated than in non smokers (Bergmark *et al.*, 1993; Wu *et al.*, 1994; Mansoor *et al.*, 1995). There is a strong dose-response relationship between the number of cigarettes and tHcy levels, independent of age and sex (Nygard *et al.*, 1995). This relationship was even seen in subjects with high folate intake (Nygard *et al.*, 1998). Several explanations for this effect of smoking exist. Smokers have lower blood folate values compared to non-smokers (Mansoor *et al.*, 1997). Smokers generally consume less vegetables and more fat in their diet than non-smokers (Presston, 1991). Moreover, smokers have a reduced vitamin intake and low bloods levels of vitamin B_{12} (Piyathilake *et al.*, 1994) and B_6 (Vermaak *et al.*, 1990), which are involved in homocysteine metabolism. In addition, tobacco smoke contains abundant free radicals that cause oxidative stress and thereby may affect the redox status of thiols (Eiserich *et al.*, 1995), including homocysteine (Ueland, 1995).

Coffee consumption was among the strongest lifestyle determinants of homocysteine in the Hordaland homocysteine cohort (Nygard *et al.*, 1997a). Individuals, who drink more than six cups a day had 2-3 µmol higher mean tHcy levels than those who did not drink coffee. A recent study demonstrated tHcy elevation in the elderly consuming more than four cups a day (Stolzenberg-Solomon *et al.*, 1999). Coffee consumption is known to be associated with an unhealthy lifestyle and poor nutrition. However, as the consumption of decaffeinated coffee did not have an effect on tHcy, caffeine may play a mechanistic role (Nygard *et al.*, 1997a).

Higher alcohol intake increases plasma tHcy level. Plasma tHcy shows a transient increase during acute alcohol intoxication in alcoholics. A direct inhibition of methionine synthase by acetaldehyde could possibly explain this phenomenon (Kenyon *et al.*, 1998). Chronic alcoholism seems to be associated with hyperhomocysteinemia. This may be explained by impaired folate, vitamin B_{12}, or vitamin B_6 intake (Cravo *et al.*, 1996). Malabsorption may play an important role and is prominent in malnourished alcoholics (Lindenbaum, 1980 a and b).

2.4.5 Medication

There are many publications which indicate interactions between medication and homocysteine levels. Most drug effects due to interaction with absorption and metabolism of folate, cobalamine and vitamin $B_{6,}$ known as important factors in homocysteine metabolism.

Total homocysteine increases after high-dose infusions of methotrexate during cancer therapy. This effect is reversed by high doses of folic acid. Methotrexate as an antifolate substance, inhibits the dihydrofolate reductase and thereby depletes cells for reduced folates (Refsum *et al.*, 1986 and 1991).

Several anticonvulsive drugs may cause hyperhomocysteinemia through interference with the folate metabolism (James *et al.*, 1997; Ono *et al.*, 1997). These adverse effects are probably induced by depletion of liver folate stores through inhibition of the polyglutamate ion (Carl *et al.*, 1991) and may be modulated by the MTHFR genotype (Yoo *et al.*, 1999).

Homocysteine also increases during therapy with niacin in combination with the bile acid sequestrant colestipol (Blankenhorn *et al.*, 1991). The latter agent may interfere with folate absorption.

Plasma tHcy increases within hours in patients exposed to the anaesthetic gas nitrous oxide (Christensen *et al.*, 1994). The increase reflects irreversible oxidation of cob(I)alamin, which is formed as a transient intermediate of the methionine synthase reaction. In addition, the enzyme methionine synthase itself is irreversibly inactivated (Drummond *et al.*, 1994 a and b).

Beside cholestyramine and metformin, there are many drugs which interfere with cobalamin absorption such as the histamine H-2 antagonist and omeprazole. However, an increase in tHcy has been investigated only in patients using cholestyramine and metformin. These two drugs may also inhibit folate absorption.

Several drugs interfere with the function of vitamin B_6. A common mechanism involves the inhibition of pyridoxal kinase. Elevated leves of tHcy in plasma or urine have been reported following treatment with isoniazid, niacin and theophyllin.

The effect of sex steroid hormones on homocysteine is indicated by gender differences in tHcy levels and by the observation of low tHcy levels in premenopausal women and during pregnancy.

Several different classes of lipid lowering drugs (i.e. bile acid binding resins, niacin and fish oil) have been shown to modify homocysteine levels (Blankenhorn *et al.*, 1991; Olszewski and McCully, 1993). However, the evidence that dyslipidemic patients receiving

lipid lowering therapy with HMG-CoA reductase inhibitor (statins) display modification of homocysteine levels is still controversial. De Lorgeril *et al.* (1999) reported no effects of simvastatin (20 mg) on plasma tHcy. However, we found that high-dose administration of simvastatin (80 mg/day at night) significantly decreases the plasma concentration of tHcy in patients with hypercholesterolemia (Lütjohann *et al.*, 2001). These results were independent of the cholesterol-lowering potency of simvastatin and inhibition of the HMG-CoA reductase activity, as has been suggested from studies in human hepatoma cell lines (Karmin *et al.*, 1998). From the SEARCH-study (Study of Effectiveness of Additional Reductions in Cholesterol and Homocysteine), MacMahon *et al.* (2000) reported that simvastatin alone (80 mg/day) and the combination with homocysteine-lowering vitamin (folate and cobalamin) are effective at lowering tHcy concentration.

Dierkes *et al.* (1999) investigated the effect of fenofibrate and bezafibrate in hypertriglyceridemic patients. They found that tHcy increases after therapy with fenofibrate or bezafibrate. They postulate that impairment of the renal function by fenofibrate and bezafibrate is the underlying cause of raised tHcy. Recently, the same group investigated the effects of gemfibrozil, another fibrate with no effect on renal function, and found that gemfibrozil, in contrasts to fenofibrate, does not raise plasma tHcy (Westphal *et al.*, 2001). The same group described how vitamin supplementation can markedly reduce the homocysteine elevation induced by fenofibrate (Dierkes *et al.*, 2001).

2.4.6 Pathological Determinants

Beside folate and cobalamin deficiency, renal failure is the most frequent clinical cause of hyperhomocysteinemia. Although the pathogenesis of elevated tHcy in folate and cobalamin deficiency is well-described, little is known about the basis of hyperhomocysteinemia in chronic renal failure. Possible mechanisms are decreased renal homocysteine excretion, impaired renal metabolism, or generally reduced vitamin B status in renal failure (van Guldener *et al.*, 1998). This issues will be described in section 2.6.

In patients with non insulin-dependent diabetes mellitus (NIDDM) fasting and post-methionine loading levels of tHcy were found to be significantly higher than in non diabetic control groups (Munshi *et al.*, 1996; Chico *et al.*, 1998, Fiorina *et al.*, 1998). Significantly higher tHcy were also found in patients with insulin-dependent diabetes mellitus (IDDM), both fasting and post-methionine loading, compared with control subjects (Hofmann *et al.*, 1997 and 1998). These patients also had very significantly increased plasma levels of thrombomodulin.

Hyperhomocysteinemia is more common in diabetic patients with vascular and other complications than in patients without such complications. Araki *et al.* (1993) reported that in NIDDM patients with vascular complications, very significantly elevated tHcy levels were observed compared with patients without such complications. Thus, hyperhomocysteinemia may be an independent predictor of these complications.

A recent study involving 211 NIDDM patients under 70 years of age, followed for a median of 6.4 years, showed that plasma tHcy was a significant predictor of mortality in patients with or without albuminuria (Stehouwer *et al.*, 1999). In another study, tHcy values > 10 µmol/l were risk markers for death in middle-aged diabetics (Kark *et al.*, 1999). Ambrosch *et al.* (2001) also reported that an increase of one µmol/l in tHcy was associated with a 2.3 fold increased risk for diabetic neuropathy in IDDM patients.

Hypothyroid patients have been reported to have significantly higher plasma tHcy concentrations than healthy subjects and hyperthyroid patients. The study of Greene *et al.* (1995) showed that the mean plasma tHcy level in hypothyroid patients, 18.4 µmol/l, was very significantly elevated, compared with the values of hyperthyroid patients, 11.0 µmol/l. In another study, the mean tHcy level in the hypothyroid group was 16.2 µmol/l versus 10.5 µmol/l in healthy controls (Nedrebo *et al.*, 1998). These finding may be related to the influence of thyroid function. However, an impaired vitamin status reduced glomerular filtration rate (GFR), or creatinine synthesis could also be important. Elevated tHcy concentration may be normalized by L-tyroxine replacement therapy (Hussein *et al.*, 1999).

Elevated tHcy is frequently found in benign and malignant diseases associated with a large burden of proliferating cells such as acute lymphoblastic leukemia (Refsum *et al.*, 1991), psoriasis (Refsum *et al.*, 1989) and some chronic inflammatory diseases. In these conditions, there could be an increased export of homocysteine by rapidly dividing cells (Christensen *et al.*, 1991). The increased tHcy export might be explained by an intracellular redistribution of the folate pool in DNA synthesis and at the expense of homocysteine remethylation. In addition to folate, an impaired vitamin B_6 status may play a role (Bates *et al.*, 1999).

Recent findings indicate that hyperhomocysteinemia is common in rheumatoid arthritis patients. A combined influence of vitamin deficiency (B_{12}, B_6 and folate) and the MTHFR polymorphism may be responsible for tHcy elevations (Pettersson *et al.*, 1998; Haagsma *et al.*, 1999; Hernanz *et al.*, 1999). Elevated fasting tHcy levels were also reported

in patients with severe and long-standing rheumatoid arthritis combined with impaired cobalamin absorption and function (Pettersson *et al.*, 1998).

A number of gastrointestinal conditions and diseases may cause elevated tHcy concentrations. However, it is likely that a deficiency of vitamin B_{12} or folate, or both, is the predominating cause due to the malabsorption syndromes. Gastrointestinal surgery in general may cause malabsorption of vitamin B_{12} and folate may thereby increase tHcy levels. Sumner *et al.* (1996) and Borson-Chazot *et al.* (1999) could confirm elevated plasma tHcy concentrations after surgery. Elevated tHcy levels and thrombotic complication were also described in patients with chronic inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease (Cattaneo *et al.*, 1998; Mahmud *et al.*, 1999). Substitution of vitamin B_{12} is therefore strongly indicated in patients with gastrectomy.

Table 2.1 Determinants of the plasma total homocysteine

Genetic factors
CBS deficiency
MTHFR polymorphism
Methionine Synthase
Demographic
Age
Sex
Ethnic origin
Lifestyle
Diet
Smoking
Coffee consumption
Alcohol
Vitamin deficiency
Folate
Cobalamin (B_{12})
Pyridoxal (B_6)
Medication
Folate antagonists (Methotrexate)
Antiepileptic drugs
Vitamin B_{12} antagonists (nitrous oxide)
Vitamin B_6 antagonists (isoniazid, theophylline)
Steroid hormones, contraceptives
HMG-CoA reductase inhibitor (simvastatin)
Fibrates
Other (bile acid resins, niacin , fish oil, L-Dopa)
Pathologic
Renal failure
Diabetes
Hypothyroidism
Cancer
Rheumatoid arthritis
Gastrointestinal disease

2.5 Homocysteine and Cardiovascular Disease

2.5.1 Background and Retrospective Studies

Homocystinuria caused by congenital CBS and MTHFR defects or by genetic alteration of cobalamine deficiency is characterized by clinical problems including arterial and venous thrombosis and premature atherosclerosis (Mudd *et al.*, 1995; McCully *et al.*, 1996). Based on this observation, it is proposed that severe hyperhomocysteinemia plays a role in the etiology of cardiovascular disease. The pioneer work of Wilcken and Wilcken (1976) showed an abnormal increase in homocysteine after an oral methionine load in patients with coronary artery disease. A prevalence of partial CBS and MTHFR inherited defects is observed in patients suffering from cardiovascular disease (Clarke *et al.*, 1991; Kang *et al.*, 1993). Numerous retrospective case-controlled (Brattström *et al.*, 1992; Pancharuniti *et al.*, 1994; Verhoef *et al.*, 1994; Mansoor *et al.*, 1995) and some prospective nested case controlled studies (Stampfer *et al.*, 1992; Arnesen *et al.*, 1995; den Heijer *et al.*, 1996) established the link between hyperhomocysteinemia and occlusive coronary artery (Stampfer *et al.*, 1992; Pancharuniti *et al.*, 1994; Arnesen *et al.*, 1995), cerebrovascular (Brattström *et al.*, 1992; Verhoef *et al.*, 1994), and peripheral vascular (Mansoor *et al.*, 1995) diseases, as well as venous thrombosis (den Heijer *et al.*, 1996). All of this was also reviewed by Refsum *et al.* (1998a). As this relationship persists after statistical adjustments for conventional risk factors, hyperhomocysteinemia seems to be an independent risk factor for cardiovascular disease.

Using the data from 19 case-controlled studies, five cross-sectional studies, and three prospective studies published before 1995, the meta-analysis of Boushey *et al.* (1995), which relates basal hyperhomocysteinemia to atherosclerotic vascular disease, indicates that, above 10 μmol/l, a 5 μmol/l increase in circulating homoysteine is associated with a 50% and a 6.8-fold increase in the risk for cerebrovascular and peripheral arterial diseases, respectively. This study clearly documented that a 5 μmol/l increase in homocysteine is equivalent to a 0.5 mmol/l increase (20 mg/dl) in plasma cholesterol in calculating the odds for myocardial infarction.

The importance of moderate hyperhomocysteinemia, and of other conventional risk factors such as age and sex, for the incidence of vascular disease are described in a recent case-controlled study conducted in nine European centres (Graham *et al.*, 1997). It was estimated that the cardiovascular risk associated with hyperhomocysteinemia detected both under basal conditions (>12 μmol/l) and after methionine loading is similar to that of hyperlipidemia or smoking, although lower than that of hypertension. This study confirmed

the usefulness of the methionine-loading test to identify all of the subjects suffering from a hyperhomocysteinemia-dependent cardiovascular disease.

2.5.2 Prospective Studies

Despite the strong and independent relationship between homocysteine and cardiovascular disease that could be found in a number of retrospective case-controlled studies, prospective cohort studies, which generally provide the most rigorous evidence, have reported inconsistent results. Whereas several prospective studies provided evidence that elevated plasma tHcy concentrations are a major independent causative risk factor for cardiovascular disease (Stampfer *et al.*, 1992; Arnesen *et al*., 1995; Perry *et al.*, 1995; Petri *et al.*, 1996; Bostom *et al.*, 1997; Nygard *et al.*, 1997b; Moustapha *et al.*, 1998), some other prospective studies found no statistically significant association between elevated homocysteine levels and the incidence of cardiovascular disease, after adjustment for conventional risk factors (Alfthan *et al.*, 1994; Verhoef *et al.* 1994; Evans et al, 1997; Folsom *et al.*, 1998; Kuller and Evans, 1998). These ambiguous results might be explained by sample size, ethnic background and lifestyle of the subjects or by the selection of the population based cohort, including the choice of exclusion criteria.

2.5.3 Interaction between Homocysteine and the Usual Risk Factors for Cardiovascular Disease

In the Hordaland Homocysteine Study conducted in Norway (16,176 subjects), a significant correlation between plasma tHcy and plasma cholesterol, blood pressure, smoking and lack of physical activity was reported (Nygard *et al.*, 1995). The European multicenter study coordinated by Graham in agreement with this. Whereas basal hyperhomocysteinemia only shows an additive effect with hypercholesterolemia, it has synergistic effect with both hypertension and smoking on the incidence for atherosclerotic vascular disease (Graham *et al.*, 1997). Moreover, the hyperhomocysteinemia associated with an increased cardiovascular risk is stronger in type 2 diabetes mellitus/NIDDM than in normal subjects (Hoogeveen *et al.*, 1998). Thus, there are pronounced interactive effects with classic risk factors, suggesting that homocysteine might further increase the cardiovascular risk in patients with these basic factors.

However, circulating homocysteine levels are related to plasma creatinine and uric acid concentration as well as to sex and age (Brattstrom *et al.*, 1994; Lussier-Cacan *et al.*, 1996). The gender differences might be reversed when taking into account data obtained

during the oral methionine-loading test (Silberberg *et al.*, 1997). After adjustments for age, creatinine and serum vitamin (folate and cobalamin), the percentage increase of tHcy after the methionine load was greater in women than in men. A decreased response to oral methionine has been observed in men older than 45 years (Silberberg *et al.*, 1997). Although homocysteine levels increase with age, a reduction in the cardiovascular risk associated with hyperhomocysteinemia also has been reported in subjects older than 45 years (Reis *et al.*, 1995). This negative interaction between age and hyperhomocysteinemia for cardiovascular risk suggests that moderate hyperhomocysteinemia may play a major role in premature death from vascular pathologies.

2.6 Homocysteine and Renal Disease

2.6.1 Hyperhomocysteinemia in Renal Disease Patients

Elevated tHcy levels were initially suspected in end-stage renal disease (ESRD) patients in 1972 by Robins and coworkers and were later confirmed by others (Wilcken and Gupta, 1979; Smolin *et al.*, 1987). Numerous studies have documented increased plasma or serum levels of free, protein-bound, and tHcy among ESRD patients in the pre-dialysis phase, while undergoing maintenance dialysis, and following renal transplantation. Nevertheless, the exact mechanism of hyperhomocysteinemia induced by renal failure remains unclear.

Wilcken and Gupta (1979) reported an almost threefold increase in concentrations of fasting homocysteine-cysteine mixed disulfides in patients with renal failure. This correlated positively with serum creatinine. This finding is confirmed by another study from Chauveau *et al.* (1993) who determined the fasting plasma level of protein-bound homocysteine in chronic uremia patients according to their creatinine clearance. They found that mean plasma homocysteine concentrations increased and correlated respectively with the decreased of creatinine cleareance. The inverse relationship between plasma tHcy levels and glomerular filtration rate (GFR) is present in the range from normal renal function to dialysis dependency (Arnadottir *et al.*, 1996a).

The prevalence of hyperhomocysteinemia in patients with ESRD on standard multivitamin supplementation is about 80 to 85% (Bostom *et al.*, 1996; Robinson *et al.*, 1996), whereas it is 90 to 100 % in patients without vitamin supplement (Bachmann *et al.*, 1995; van Guldener *et al.*, 1998). In a study of more than 170 patients with ESRD (74% hemodialysis) an increased concentrations of plasma tHcy was reported (Robinson *et al.*, 1996). In these hemodialysis patients, the mean plasma tHcy concentrations is almost 30 µmol/l. The homocysteine concentration in patients with peritoneal dialysis are comparable to those undergoing hemodialysis (Hultberg *et al.*, 1993; Dierkes *et al.*, 1999). In another study, lower homocysteine levels were found in peritoneal dialysis patients (Robinson *et al.*, 1996).

The higher concentrations of homocysteine in renal transplant recipients compared to normal subjects was initially observed by Wilcken *et al.* (1981). This finding was also later confirmed by other research groups (Massy *et al.*, 1994; Arnadottir *et al.*, 1996b; Bostom *et al.*, 1997; Ducloux *et al.*, 1998). Arnadottir *et al.* (1996b) reported that transplant recipients on cyclosporine had higher plasma tHcy concentrations than those not on cyclosporine. They conclude that the hyperhomocysteinemia of renal transplant recipients not treated with

cysclosporine is due to renal insufficiency. In another study, Ducloux *et al.* (1998) did not find a significant difference in plasma tHcy between renal transplant recipients who were on azathioprine and prednisone treatment and who also received cyclosporine.

Results from two other studies described the decrease of tHcy plasma concentrations after renal transplantation. Van Guldener *et al.* (1998) studied the short-term effects of renal transplantation on hyperhomocysteinemia in eight dialysis patients after successful renal transplantation and demonstrated decreasing post-transplantation tHcy concentrations with improving renal function. Arnadottir *et al.* (1998) observed a mean reduction in serum tHcy concentration of 14% in 55 patients six months after renal transplantation. However, the post-transplantation tHcy concentrations were still higher compared to normal subjects.

2.6.2 Relation to Atherosclerosis

Both Cohen *et al.* (1977) and Wilcken and Gupta (1979) hypothesized that accumulation of homocysteine could lead to accelerated vascular disease in patients with chronic renal failure. In a cross-sectional analysis, Chaveau *et al.* (1993) reported higher homocysteine levels in 20 patients with prior histories of occlusive arterial disease.

Recently, Jungers *et al.* (1997) reported on risk factors and the incidence of atheriosclerotic vascular events in 147 predialysis chronic renal failure patients followed prospectively for nine years. They found that, beside LDL-cholesterol, triglycerides, apoB, Lp(a), plasma concentrations of homocysteine were also significantly higher in patients with vascular accidents. In the patients developing cardiovascular events, 83% had homocysteine plasma concentrations greater than 14 µmol/l. The relative risk for development of a cardiovascular events was 1.17 for each increase of 1 µmol/l homocysteine. In another prospective study in IDDM patients, where 19% of them had an impaired renal function, Stehouwer *et al.* (1999) also reported that plasma tHcy was a significant predictor of all-cause mortality with a relative risk of 1.09 per 1 µmol/l increase.

Several studies have shown that higher serum or plasma concentrations of homocysteine were found in the renal transplant recipients with cardiovascular events as well as a history of atherosclerotic complications compared to those without (Massy *et al.*, 1994; Arnadottir *et al.*, 1996; Ducloux *et al.*, 1998 and 2000a). However, Massy *et al.* (1998) found no association between homocysteine concentrations and cardiovascular events in 79 renal transplant recipients who were followed over a period of six years.

In another study with 50 patients on regular hemodialysis, Bachmann *et al.* (1995) found a significant association between homocysteine concentrations and occlusive arterial disease. In a larger study in ESRD patients, an increased concentrations of homocysteine greater than 27.8 μmol/l was associated with an almost threefold increase in risks for atherosclerotic and thrombotic complications, independent of other risk factors and length of time on dialysis. Moustapha *et al.* (1998) also reported that cardiovascular events and causes of mortality were related to tHcy values and other cardiovascular risk factors. The plasma concentrations of tHcy were higher in patients who had cardiovascular events or died of cardiovascular causes, and the relative risk for cardiovascular events, including death, increased by 1% per 1 μmol/l increase in tHcy plasma concentration. Theses studies confirm that hyperhomocysteinemia is an independent risk factor for cardiovascular morbidity and mortality in ESRD but do not establish that modification of this risk factors can improve mortality and morbidity.

3 Development of a Gas Chromatographic-Mass Spectrometric Method for the Determination of Total Homocysteine and Related Amino Acids by Stable Isotope Dilution

There is growing interest in highly specific and sensitive methods for the detection, quantification and progress of increased concentrations of total homocysteine (tHcy) in many clinical cases.

3.1 Methods for Determination of Total Homocysteine

The first study reporting homocystinuria including quantitative data was described in 1962 by Gerritsen *et al.*. Due to the extremely high concentration of homocysteine, the use of elementary chemical methods was sufficient. In the 1970s and early 1980s, the most common methodology was the use of amino acid analyzers (Wilcken and Wilcken, 1976; Ueland *et al.*, 1993). However, total homocysteine in human plasma was firstly determined with the radioenzymatic method described by Ueland *et al.* (1984), as modified by Refsum *et al.* (1985). After reduction of oxidized homocysteine with dithiothreitol, total homocysteine was converted to S-adenosyl-homocysteine in the presence of [^{14}C] adenosine and S-adenosyl-homocysteine hydrolase. The labelled S-adenosyl-homocysteine was identified by HPLC and its radioactivity determined by scintillation counting. This method, although sensitive, had the major drawback of being very time consuming.

3.1.1 High-Performance Liquid Chromatography (HPLC)

HPLC is the most commonly used analytical procedure for determination of tHcy. HPLC procedures for quantification of homocysteine can be classified based on their derivatization procedures.

Pre-column derivatization with fluorogenic reagents reacting with thiols has become very popular (Ohkura and Nohta, 1989). Three reagents have been extensively used for pre-column derivatization. Monobromobimane couples rapidly with thiols at pH 8 at room-temperature to yield a highly fluorescent thioester. However, an important drawback is that the reagent itself, the hydrolysis products, and the impurities are fluorescent, leading to peaks that may interfere with the homocysteine determination (Refsum *et al.*, 1989). Four-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate reacts quantitatively with thiols at

50^{o} C and pH 8-9.5 for 5-10 min. Ammonium-7-fluoro-2,1,3-benzoxadiazole-4-sulfonate is less reactive and requires more drastic condition : 60^{o} C at pH 9.5 for 1 h (Ling *et al.*, 1990).

The first fully automated assay for total plasma homocysteine was based on pre-column derivatization with monobromobimane, HPLC separation and fluorescent detector (Refsum *et al.*, 1989b). Recently, a fully automated assay has been described which enables quantification of total homocysteine and other thiols in plasma and urine (Pastore *et al.*, 1998). One method is described using postcolumn derivatization. This assay involves reduction of samples with dithiothreitol, separation by HPLC and post-column derivatization with 4,4'-dithiodipyridine and colorimetric detection at 324 nm (Anderson *et al.*, 1993).

Malinow *et al.* (1989) described an HPLC method for homocysteine determination using an electrochemical detector. However, a major drawback is a possible contamination of the flow cell and electrode fouling. Thus, amperometric detectors including gold/mercury amalgam electrodes were substituted by coulometric detectors using porous carbon electrodes and, more recently, pulse integrated amperometry, which offers a potential solution to these problems. The first HPLC method based on pulse integrated amperometry was recently used in a modified manner (Evrouski *et al.*, 1995 and Cole *et al.*, 1998)

3.1.2 Capillary Electrophoresis

Several procedures for an accurate determination of homocysteine by capillary electrophoresis were published recently. Within these procedures, thiols are derivatized quantitatively at 50^{o} C and pH 8 by the flourogenic reagent 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole and afterwards the derivatized thiols are separated in base fused-silica capillaries at room temperature with an electric field of 560 V/cm in less than 8 min, and they are detected by using a diode-array detector (Kang *et al.*, 1997). Causse and coworkers developed an alternative method. Here, thiols are derivatized by using fluorescent isothiocyanate, and homocysteine quantification is performed by using capillary electrophoresis and laser-induced fluorescence detector (Causse *et al.*, 1998). The workgroups from Causse *et al.* (1999) and Vecchione *et al.* (1999) described methods for the accurate determination of homocysteine using capillary electrophoresis and laser-induced fluorescence detector and compared this method with HPLC.

3.1.3 Enzyme Immunoassay

Another methodology used in most clinical chemistry laboratories for the determination of homocysteine is based on enzyme immunoassays. The first of these methods was published in 1995 by Schipchandler *et al.* and it consists of a fluorescence polarization immunoassay based on the highly selective enzymatic conversion of homocysteine to S-adenosylhomocysteine, which is then recognized by a monoclonal antibody. Recently, a combined methodology has been performed between this immunoassay method and an HPLC assay with internal standardization, yielding highly correlated results (Pfeiffer *et al.*, 1999a). The main advantage of this method is a very high throughput of samples by shortening the process time. The major drawback of this assay is its dependence on a specific Abbott IMx analyzer. Very recently, this drawback has been avoided by a modification which allows the use of the enzyme immunoassay technology in different formats and on various analytical platforms (Frantzen *et al.*, 1998).

3.1.4 Gas Chromatography - Mass Spectrometry

The first gas chromatographic-mass spectrometric method for determination of tHcy was developed by Stabler *et al.* (1987 and 1988), and later modified by the same group to render a much more simple method (Stabler *et al.*, 1993). The method involves reduction with dithiothreitol, derivatization with N-methyl-N-(tert.-butyldimethylsilyl) trifluoroacetamide and separation and quantification by capillary gas chromatography-mass spectrometry. Another group published a more rapid methodology, using a simple aqueous, one-step derivatization procedure at room temperature by ethyl chloroformate and gas chromatographic-mass spectrometric analysis (Pietzsch *et al.*, 1997a and b). The combination of a stable isotope dilution method and electrospray ionization-tandem mass spectrometry was published very recently (Magera *et al.*, 1999).

Here, we developed a new gas chromatographic-mass spectrometric method for the determination of tHcy and the related amino acids methionine, cysteine and cystathionine by stable isotope dilution. This method showes a good correlation with the HPLC method and the Abbott IMx immunoassay.

3.2 Gas Chromatographic-Mass Spectrometric Techniques

3.2.1 Gas Chromatography

The technique of gas chromatography (GC) or gas-liquid chromatography (GLC) is a form of partition chromatography in which the mobile phase is a gas and the stationary phase is a liquid. A sample is injected into the gas-phase where it is volatilized and passed onto the stationary phase, which is held in some form in a column; components spend different times in the mobile phase and the stationary phase, depending on their relative affinities for the latter, and emerge from the end of the column exhibiting peaks of concentration , ideally with a Gaussian distribution. These peaks are detected by equipment which converts the concentration of the component in the gas phase into an electrical signal, which is amplified and passed to a continuous recorder and integrator, so that the progress of the separation can be monitored and quantified.

Instrumentally, gas chromatography has three essential components, i.e. some form of inlet through which the samples is introduced onto the column, the column itself which contains the stationary phase and through which the mobile (gas) phase is passed continuously, and a detector.

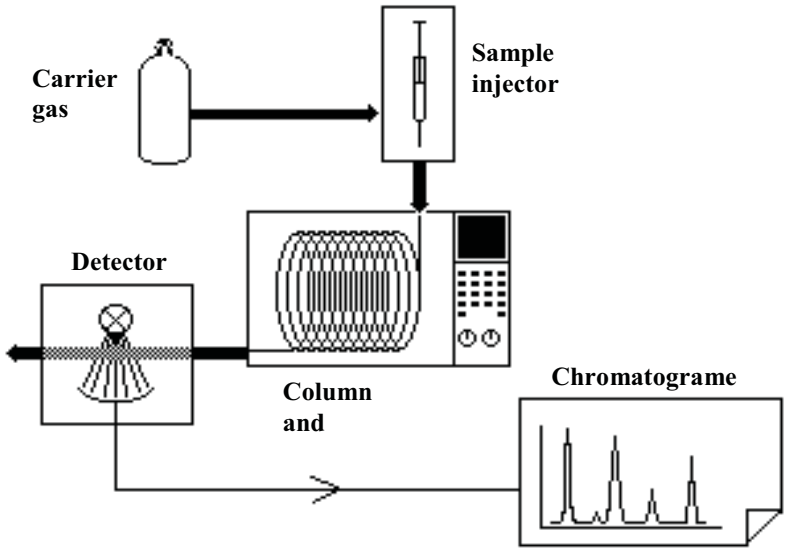

Figure 3.1 Scheme of gas chromatography

The function of the column is to allow partitioning of the constituents of the samples to be separated between the stationary and mobile phases, and this is aided by having the liquid phase as a thin film with a large surface area accessible to the flow of the gas phase. As the sample (or solute) passes through the column, the molecules of each component partition between the liquid and gas phase according to a distribution coefficients (K_D).

K_D is specific for a given solute and liquid phase at the temperature selected. As the gas phase is moving continuously, solute molecules continue to dissolve in the fresh liquid phase in relation to the K_D , while those molecules which have already dissolved overcome the various forces involved, reemerge into the gas phase and pass further down the column.

As long as a molecule is in the gas phase, it travels down the column at the same speed as the carrier gas. When mixture of components is present in the solute, they diffuse into liquid phase to varying degrees according to their K_D, and so travel down the column at different rates. In other words, the retention time on the column is different and the components tend to separate.

The efficiency of a given column is dependent on a number of factors, including the nature and flow-rate of the carrier gas, column dimensions, liquid-phase thickness and column temperature. By optimizing these factors, it may be possible to increase the resolution attainable quite considerably. However, this improved resolution may be obtained at the expense of increased analysis time. In practice, it may be desirable to compromise and select conditions for an analysis which offer adequate resolution in a reasonable time.

A large number of detectors operating on different principles have been developed for use in gas chromatography. But not all of these detectors are used to a significant extent. The flame ionisation detector is now almost universally used. This detector is simple to construct and operate, and it is highly reliable in prolonged use. The principle of the detector is that ions are generated by combustion of the organic compounds as they emerge from the column in a diffusion flame of hydrogen and air. The carrier gas from the column may be premixed with hydrogen, although with a WHOT column, the outlet of the column is usual positioned at the orifice of the combustion jet in a chamber through which an excess of air is passed. The collector electrode is cylindrical and is placed just above the flame, and the ion current is measured by establishing a potential between the collector and the jet tip. The signal current is passed to an amplifier and is then transmitted to a recorder.

In the electron-capture detector, a radioactive source is used to bombard the carrier gas with β particles as it passes through an ionization chamber. Each β particle can generate up to

a thousand thermal electrons, which are collected by applying a voltage potential. When solutes containing electron-capturing moieties enter the cell, they interact with thermal electrons and a diminution in the background current is seen and can be measured with high sensitivity. In addition to this sensitivity, the chief virtue of this detector is its specificity, as molecules containing halogen atoms, for example, give a very marked response.

Another detector which has recently been used in the identification and quantification is the mass spectrometry (MS) detector. It will be explained in section 3.2.2.

3.2.2 Mass Spectrometry

Mass spectrometry (MS) is being increasingly used as an analytical tool to solve complex problems in biochemistry and analytical chemistry such as :

(1) confirmation of structural identity
(2) identification of unknown compound
(3) elucidation of the structure of complex molecules
(4) quantification of selected constituents present in very small amounts in biological samples
(5) dynamic analysis of metabolic transformations both in vitro and in vivo

The principle of the technique in its simplest form is that organic molecules in the vapour phase are bombarded with electrons and form positively charged ions, which can fragment in a number of different ways to form smaller ionized entities. These ions are propelled through a magnetic or electrostatic field and are separated according to their mass to charge (*m/z*) ratio; they are collected in sequence as the ratio increases, the ion current is amplified and it is then displayed by some means. The largest (or base) peak is given an arbitrary intensity value of 100, and the intensities of all the other ions are normalized to this. The ion from the parent molecules is termed the molecular ion (M^+). With instruments of low resolution, peaks appear at unit mass numbers, but at high resolution the masses of individual ions can be measured with sufficient accuracy for the molecular formula of each ion to be determined.

Molecules do not fragment in an arbitrary manner but tend to split at weaker bonds, such as those adjacent to specific functional groups, or according to certain complex rules which have been formulated empirically from studies with model compounds. Frequently, it is possible to deduce the structure of the original compound from first principles from the nature of the fragment produced. With other compounds when the results are equivocal, the

spectrum can be compared with those of compounds with similar properties (nowadays with the aid of computer search facilities) until a good fit is obtained. The combination of mass spectral and GC retention data may also serve to eliminate an alternative structure. In the GC-MS application, the total ion current produced from the column effluent is recorded continuously, and a trace is obtained resembling that from another detector; spectra are also recorded continuously and can be related to specific peaks.

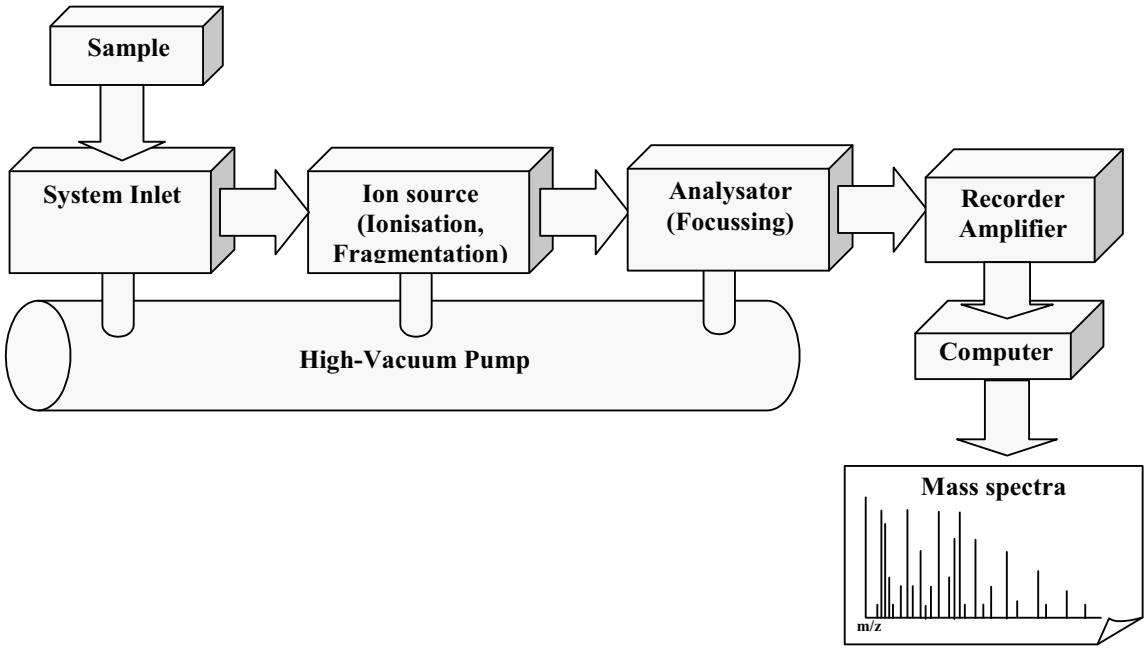

Figure 3.2 Scheme of mass spectrometric detector

3.2.3 Isotope Dilution Method

The general principle of isotope dilution was first described in 1913 by Hevesey and Paneth. In essence this technique utilizes a chemically authentic internal standard differing from the analyte only by its molecular weight. Thus the ratio of analyte/internal standard is an isotope ratio. The idea of an outstanding accuracy of such a technique is based on the assumption that the isotope ratio cannot be changed by chemical treatment or chromatographic procedures. In the final step, the ratio between the two isotopes can be determined with high precision and accuracy.

The most common way to measure the isotope ratio is the use of mass spectrometry. The combination of gas chromatography-mass spectrometry seems to be a more attractive technique in this connection. In this combination, the mass spectrometry functions as a detector for the effluent from the gas chromatographic column. One procedure involves

repetitive scanning of whole or partial mass spectra during analysis. In another procedure the mass spectrometer/selective detector only detects certain characteristic ions. This technique is called selected ion monitoring (SIM) or multiple ion detection (MID).

In selected ion monitoring, preselected prominent fragments in the mass spectra of the analyte are continuously recorded. In this way a compound can be characterized both by gas chromatographic retention time and the presence of one or several fragments. If the compound which is analyzed consists of a mixture of two different isotopes, the ratio between the two types of molecules can be calculated.

The repetitive scanning method is a more flexible method than selected ion monitoring and is the technique of choice in the analysis of complex mixtures of different compounds when a maximum of information is required. This method can also be used for quantitative work. Selected ion monitoring is far more sensitive than repetitive scanning and may be preferred in the detection and quantitation of small amounts of specific known compounds.

A quantitative determination of this method is made by comparing the measured isotope ratio of the analyte and the internal standard with their mass ratios in the unknown sample with calibration standard, a standard mixture with known concentrations of analyte and a fixed internal standard.

3.3 Experimental

3.3.1 Chemicals

All chemicals used were of analytical reagent grade. D,L-homocysteine, D,L-homocystine, L-methionine, L-cysteine, L-cystine and D,L-cystathionine were obtained from Sigma (Deisenhofen, Germany). All deuterium labeled internal standards D,L [3, 3, 3', 3', 4, 4, 4', 4' -D_8] homocystine, D,L [3, 3, 3', 3' -D_4] cystine, L [methyl – D_3] methionine, and D,L [3, 3, 4, 4 –D_4] (2-amino-2-carboxyethyl) homocystine were purchased from Cambridge Isotope Laboratories (Andover, USA). Ethylchloroformate as derivatization agent was purchased from Fluka (Deisenhofen, Germany). Dithiothreitol and sulfosalicyl acid were purchased from Sigma (Deisenhofen, Germany). Analytical-reagent grade NH_4OH, ethanol, pyridine, and chloroform were obtained from Merck (Darmstadt, Germany).

3.3.2 Sample Collection

All tests for the development of the methodology were based on pool plasma taken from healthy volunteers from the laboratory staff. Blood samples treated with EDTA were immediately placed on ice and centrifuged within 60 minutes at 1,600 x *g* for 10 minutes. The plasma samples were stored at -20° C until analysis.

3.3.3 Samples Preparation for GC-MS

A mixture of deuterated internal standard solutions containing 25 nmol D,L [3, 3, 3', 3', 4, 4, 4', 4' -$^{2}H_8$] homocystine, 125 nmol D,L [3, 3, 3', 3' –$^{2}H_4$] cystine, 50 nmol [methyl –$^{2}H_3$] methionine, and 1 nmol D,L [3, 3, 4, 4 –$^{2}H_4$] (2-amino-2-carboxyethyl) homocystine was prepared. Two-hundred-and-fifty µl of this internal standard solution was added to 500 µl human plasma. Fifty µl dithiothreitol (10 %) was added and, after generous Vortex mixing, the mixture was incubated at 40^{o} C for 30 min to reduce all compounds, yielding the free forms of the amino acids. After cooling to room temperature, a solution of 25 mg sulfosalicylic acid in 100 µl H_2O was added followed by vortexing and centrifugation for 10 min at 1600 x *g*. The pH of the supernatant was adjusted to 2-2.5 and was then applied to disposable water pre-equilibrated columns containing 200 µl of the cation exchange resin AG 50W-X8, hydrogen form (Bio-Rad Laboratories, Hercules, CA, USA) (Adams, 1974). After washing twice with 500 µl H_2O, the amino acids were eluted with 2 ml 2 N NH_4OH-solution (Adams, 1974). The eluates were collected and taken to dryness by a vacuum centrifuge (Speed-Vac, Fa. Thermoquest, Egelsbach, Germany).

3.3.4 Derivatization

The residual amino acids were treated with 60 µl water and 40 µl ethanol-pyridine (4:1). Ten µl ethyl chloroformate (ECF) were added and mixed by shaking the tube for 15 s. Then, 150 µl of chloroform containing 1 % ECF was added and the derivatives were extracted into the organic phase by mixing for about 15 s. An aliquot (100 µl) of the organic phase was transferred into glass vials with reduction inserts and 2 µl was injected into the column.

3.4 GC-MS Measurement

Separation of the different amino acid derivatives was performed on a dimethylpolysiloxane fused silica capillary column (25m x 0.2mm i.d x 0.33 µm film thickness; Ultra-1, Hewlett Packard (HP), Böblingen, Germany). The analysis was carried out

on a Model HP 5890 gas chromatograph equipped with a model HP 7673A autosampler. Helium was used as carrier gas with a column head pressure of 100 kPa. The injector temperature was set to 250° C and the transfer line at 280° C. The initial temperature of the GC oven was 120 ° C increasing to a final temperature of 320 ° C at 25 ° C min^{-1}. The compounds were detected by a mass selective detector (HP 5972) in the electron impact ionization mode at 70 eV. Electron multiplier voltage was set at 1600 eV and m/z were counted with 1.5 cycles/second.

3.4.1 Peak Identification

Homocysteine, the peak of interest, was identified by comparison with the authentic homocysteine standard which was derivatized directly with ethyl chloroformat. The other related amino acids cysteine, methionine and cystathionine were also derivatized by the same procedure. The complete derivatized amino acids were injected onto a column and monitored by GC-MS by a scan-mode. Figure 3.3a shows a typical mass spectrum of authentic homocysteine, Fig. 3.3b of cysteine, methionine and cystathionine in scan method. Figure 3.4 show a chromatogram of all measured amino acids.

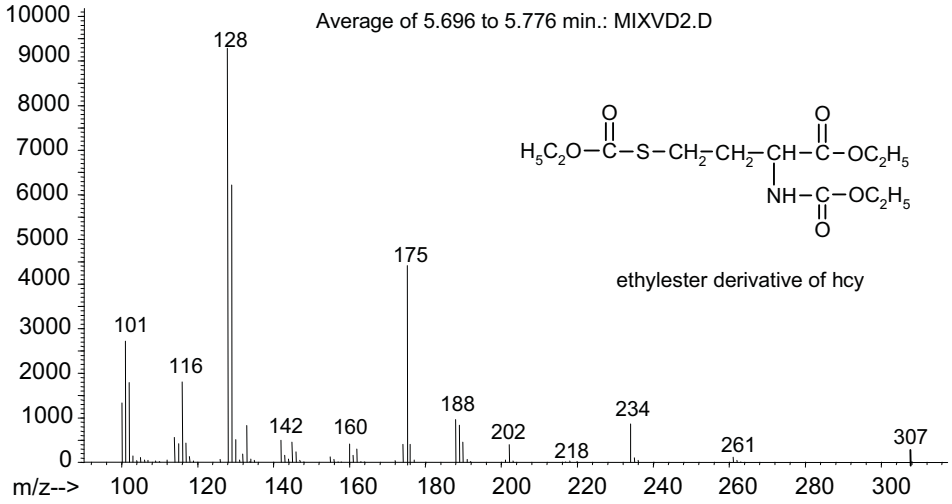

Figure 3.3a Mass spectrum of the N (O, S)-ethoxycarbonyl ethly ester derivative of natural homocysteine

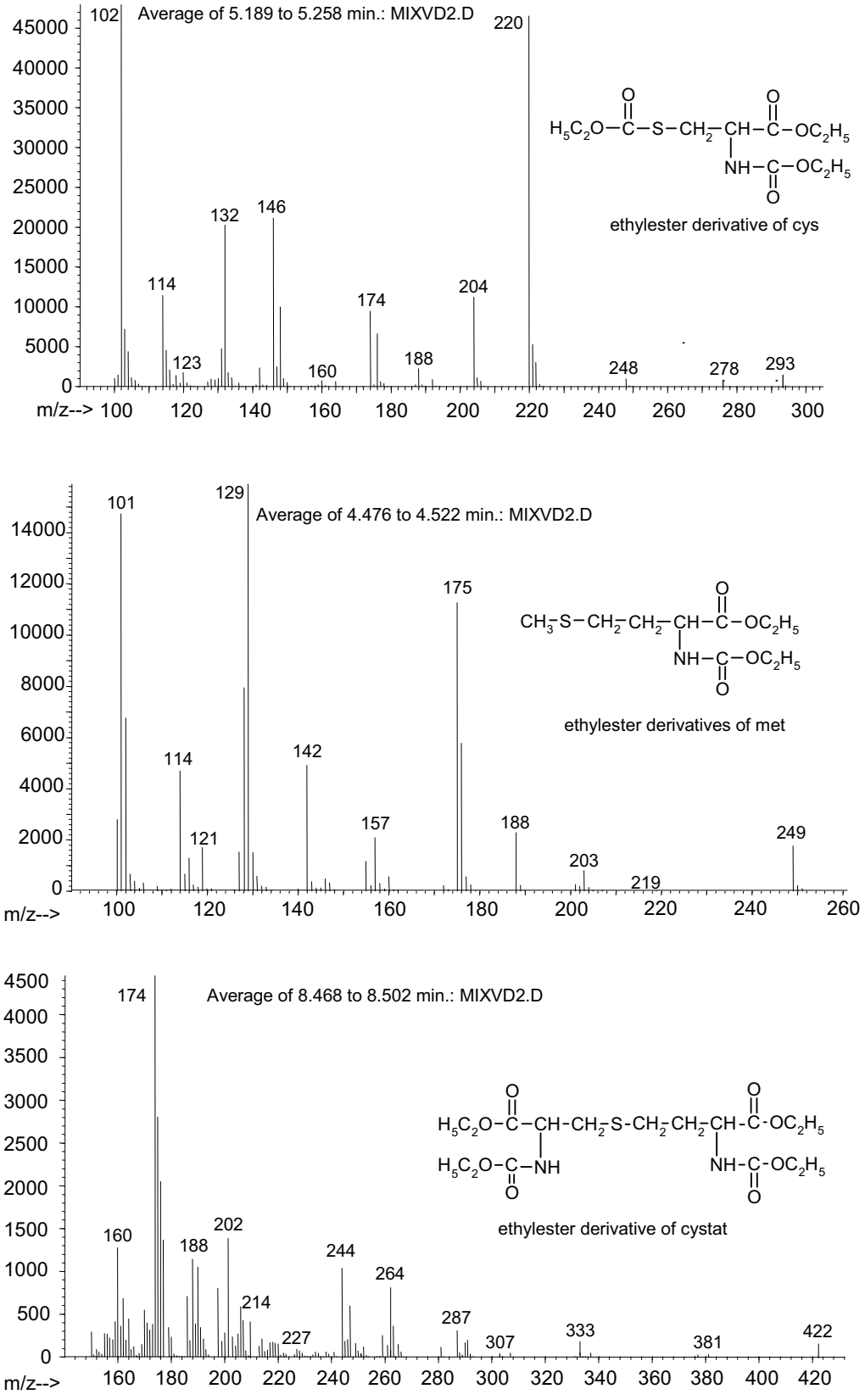

Figure 3.3b Mass spectrum of the N (O, S)-ethoxycarbonyl ethly ester derivatives of natural cysteine, methionine, and cystathionine

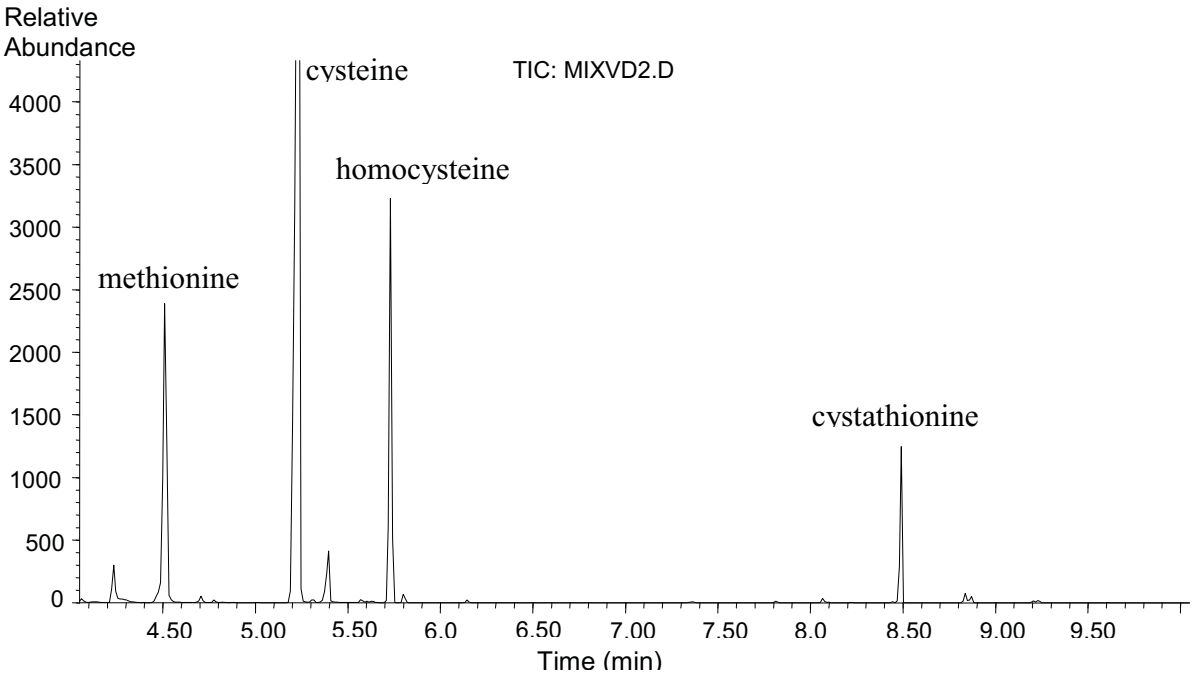

Figure 3.4 Chromatogram of a mixture of N(O,S)-ethoxycarbonyl ethyl ester derivates of natural methionine, cysteine, homocysteine, and cystathionine.

3.4.2 Selected Ion Monitoring

The unlabeled and labeled amino acid derivatives were analyzed by selected ion-monitoring using specific masses counting at a rate of 4 cycles/s. Characteristic ions for homocysteine (m/z 234), homocysteine d4 (m/z 238), methionine (m/z 249), methionine d3 (m/z 252), cysteine (m/z 220), cysteine d2 (m/z 222), cystathionine (m/z 260) and cystathionine d4 (m/z 264) were monitored. Chromatograms of selected ion from homocysteine and related amino acids with their corresponding stable isotope-labeled internal standards are shown in figure 3.5.

3.4.3 Quantification

Quantification of homocysteine and its related amino acids cysteine, methionine and cystathionine was based on the ratio between the calculated peak area from selected ions, from corresponding different amino acids, of the endogenous analyte and their corresponding labeled internal standards. The concentration of homocysteine, cysteine, methionine and cystathionine were then calculated using a calibration curve. This calibration curve was obtained by analysis of mixtures of known amounts of the unlabelled amino acids together

with the fixed amount of the labeled internal standard. It is of interest to note that standard curves were prepared by running through the same work-up procedures as the samples.

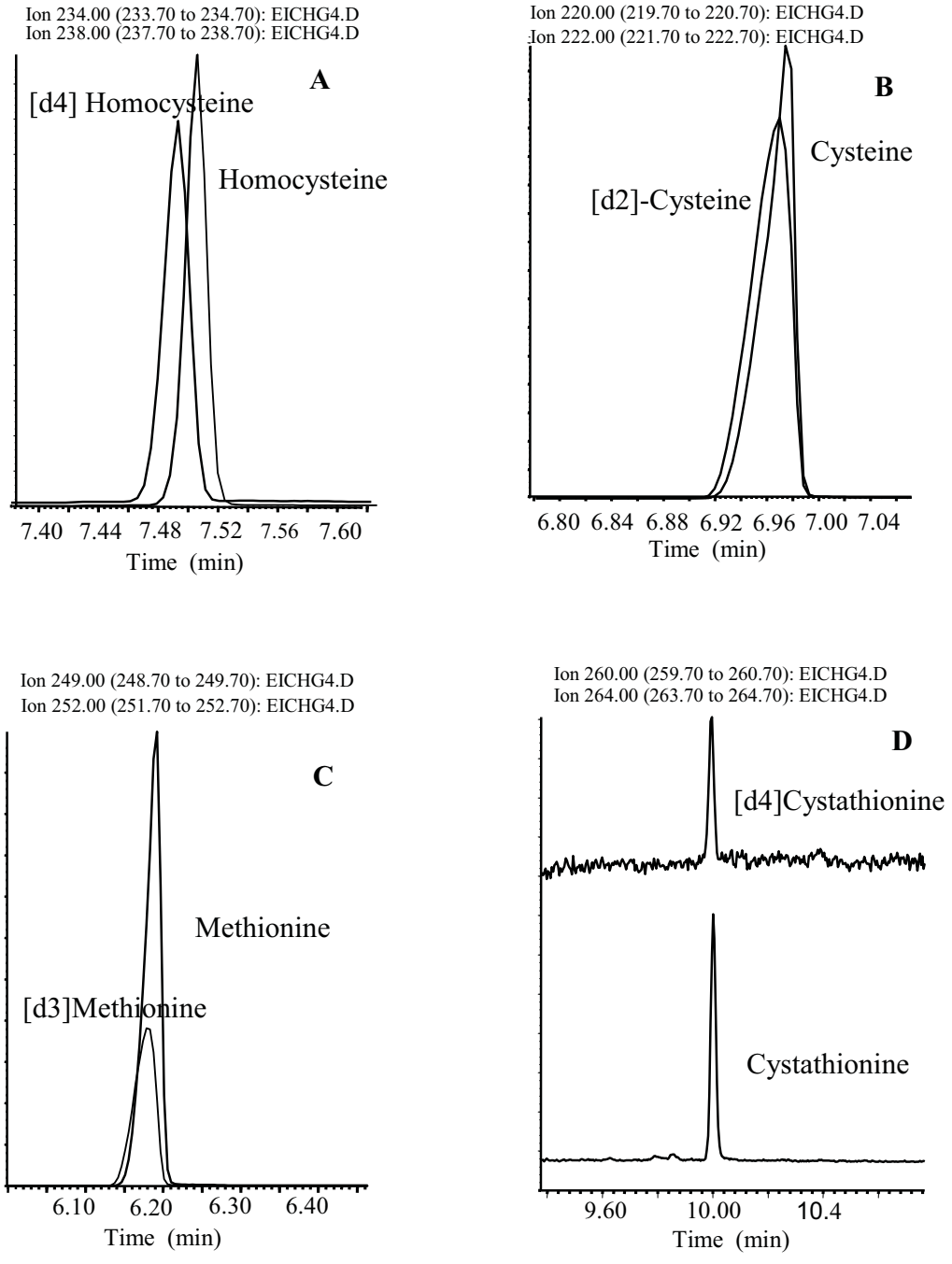

Figure 3.5 Chromatograms of specific masses of the different labeled and authentic amino acids homocysteine (A), cysteine (B), methionine (C) and cystathionine (D).

3.5 Analytical Performance

3.5.1 Specificity

Homocysteine was identified on the basis of retention time, the almost simultaneous appearance of d4-homocysteine and the specific m/z. The other related amino acids were identified on the same basis, having different retention times and fragmentation patterns. (Figure 3.3a and 3.3b and 3.4). Additionally, homocysteine and its related amino acids could be measured after a single work-up procedure and during the same run.

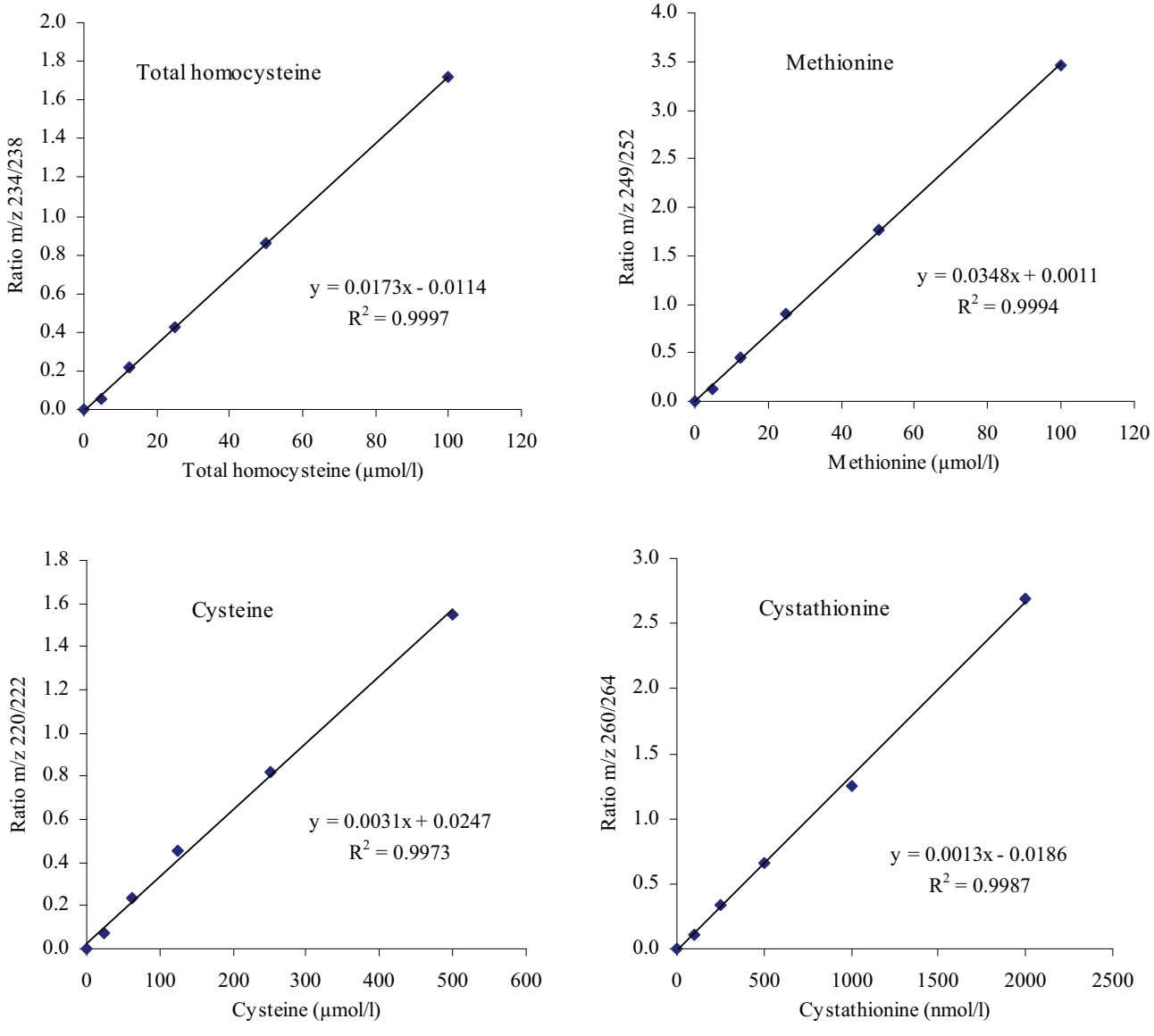

Figure 3.6 Calibration curves and equations of linear regression for tHcy and the related amino acids methionine, cysteine, cystathionine.

3.5.2 Linearity

Correlation coefficient, y-intercept, slope of the regression line and residual sum of squares are demonstrated in Figure 3.6. The specified range is derived from the linearity study and is within the range required for the intended application. A linear relationship was observed over the range 0-100 µmol/l for total homocysteine, 0-100 µmol/l for methionine, 0-500 µmol/l for cysteine, and 0-2000 nmol/l for cystathionine. Linear regression of the observed ratio authentic vs. labeled amino acid (y) *vs*. increasing concentrations gave the following equations:

Homocysteine: $y = 0.0173\ x - 0.0114$ ($r^2 = 0.9997$)
Methionine : $y = 0.0348\ x + 0.0011$ ($r^2 = 0.9994$)
Cysteine : $y = 0.0031\ x + 0.0247$ ($r^2 = 0.9973$)
Cystathionine : $y = 0.0013\ x - 0.0186$ ($r^2 = 0.9987$)

3.5.3 Precision

The repeatability of the method was evaluated from a pool serum sample which was determined six times following this procedure: extraction, derivatization and GC-MS analysis. The variation coefficients for these measurements were 1.63%, 1.40%, 1.37% and 2.28% for homocysteine, methionine, cysteine and cystathionine, respectively (see appendix, Table A1).

3.5.4 Analytical Recovery

Mean recoveries of homocysteine, introduced as homocystine, methionine, cysteine, introduced as cystine, and cystathionine, added to a plasma sample were 94.2% for homocysteine, 86.8% for methionine, 98.5% for cysteine, and 83.8% for cystathionine (see appendix, Table A2).

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3.5.5 Between-day Variation

During the different GC-MS measurements of the plasma samples of ESRD patients an additional pool plasma was measured within a period of one month (on seven occasions) and repeated after two months (on one occasion) to determine between-day variation. The between-day variations of homocysteine, methionine, cysteine, cystathionine were 2.98%, 4.39%, 6.99%, and 8.60%, respectively.

3.5.6 Limit of Detection and Quantification

Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analysate can be reliably detected. A signal-to-noise ratio between 3:1 is considered acceptable for estimating the detection limit. A signal-to-noise ratio of 10:1 is used as quantitation limit in biological samples.

	Detection limit	Quantitation limit
Homocysteine:	1 µmol/l	5 µmol/l
Methionine :	1 µmol/l	5 µmol/l
Cysteine :	5 µmol/l	10 µmol/l
Cystathionine :	20 nmol/l	100 nmol/l

4 Comparison of GC-MS Method with the Abbott IMx Homocysteine Assay and an Evaluated HPLC-Method for the Determination of Total Homocysteine

Increased plasma tHcy has been implicated as an independent risk factor for cardiovascular disease. Although the relationship between the reductions in plasma tHcy concentrations and a reduced incidence of premature vascular disease has not been established, some experts have recommended that plasma tHcy determination should be included in assessing individual risk profiles, particularly in patients with existing vascular disease or a with strong family history of premature vascular disorder.

Accordingly, the interest in tHcy determinations in plasma or serum has increased in routine and research laboratories. The increasing interest in the determination of tHcy in plasma led to the development of several different methods such as HPLC, GC-MS enzyme immunoassay and fluorescence polarization immunoassay (see chapter 3.1). Moreover, we developed a new GC-MS method for simultaneous determination of tHcy beside other related amino acids like methionine, cysteine and cystathionine.

In this study, we compared our new GC-MS method for determination of plasma tHcy with an HPLC method based on thiol derivatization with SBDF which has just been evaluated through ERDIM (European research network for evaluation and improvement of screening, diagnosis and treatment of inherited disorders of metabolism), and IMx enzyme immunoassay from Abbott in order to validate our new GC-MS method. This between–method comparison reveals the agreement of measurement.

4.1 Material and Methods

4.1.1 Sample Collection

For inter-method comparison between GC-MS *vs.* HPLC and GC-MS *vs.* IMx, blood samples had been drawn from 131 overnight fasting ESRD patients, collected in EDTA anticoagulant tubes and immediately placed on ice. Then, the supernatant plasma was removed from cells through centrifugation within 60 minutes at 1600 g for 10 minutes. The plasma samples were stored at -20° C until analysis. Intermethod comparison for GC-MS *vs.* IMx was additionally performed on 12 healthy subjects from the laboratory staff, the blood samples were drawn and processed as described above.

4.1.2 GC-MS Determination

Homocysteine determination was performed using a new GC-MS method (see chapter 3). Principally, tHcy was extracted and partially purified from plasma after reduction with dithiothreitol followed by cation-exchange chromatography. The addition of an internal deuterated homocystine standard at the early stage of sample analysis was used and included reduction of all disulfides binding.

The derivatization was based on the treatment of homocysteine and related amino acids with ethyl chloroformat to form N(O)-ethoxycarbonyl ethyl ester derivates and the derivates were analyzed using gas chromatography-mass spectrometry with selected ion monitoring.

4.1.3 HPLC Determination

Determination of tHcy in plasma was done with HPLC according to the methods described by Araki and Sako (1987), and Vester and Rasmussen (1991) with minor modifications.

Briefly, the first step comprises reduction and release of tHcy and other protein-bound amino acids by tri-n-butylphosphine. This is followed by precipitation of proteins with perchloric acid and derivatization of the reduced thiols with ammonium-7-fluorobenzo-2-oxa-1.3-diazole-4-sulfonate (SBD-F). The SBD-F derivatives of homocysteine and other thiols are then separated with reversed-phase HPLC and detected by fluorescence.

4.1.4 IMx Homocysteine Assay

The "Abbott Homocysteine (Hcy) assay" (Abbott Laboratories, Abbott Park, IL, USA) is an automated fluorescence polarization immunoassay based on the highly selective enzymatic conversion of homocysteine to S-adenosyl-L-homocysteine, which is then recognized by a monoclonal antibody (Shipschandler and Moore, 1995).

4.1.5 Statistical Methods

Correlation between all parameters was performed by Passing-Bablok (P/B) regression analysis (Passing and Bablok, 1983) using computer program EVAPAK 3.1 (Roche Diagnostics GmbH, Mannheim). Difference plots were used to assess the agreement between tHcy results obtained with GC-MS *vs.* HPLC and GCMS *vs.* IMx , respectively, as described by Bland and Altman (1986). The advantage of the Passing-Bablok regression analysis in

relation to normal correlation analysis is that P/B compares the line of regression from the results with the theoretically best-fit regression line.

Table 4.1 Characteristics of the three compared methods

Method	Reduction	Precipitation	Derivatization	Calibrator	Sample Volume (µl)
GC-MS	DTT	Sulfosalicylacid	ECF	Homocystine	500[a]
HPLC	Tri-n-buthylphosphine	Perchloric acid	SBD-F	Homocystine	150
IMx	DTT	None	None	SAH	50

[a]Sample is also used to determine simultaneously other related amino acid

4.2 Results

4.2.1 Comparison of the GC-MS Measurement with HPLC and IMx Technique in ESRD Patients

The mean plasma concentrations of tHcy in ESRD patients determined by GC-MS (28.7 + 11.9 µmol/l [mean ± SD]) was significantly lower than determined by HPLC (34.0 ± 14.5 µmol/l; $p<0.001$) or by IM_X (32.4 ± 13.9 µmol/l; $p<0.001$) (Table 4.2). There was a significant correlation between GC-MS and HPLC ($r = 0.931$; $p < 0.001$) and between GC-MS and IMx ($r = 0.896$; $p < 0.001$). P/B regression line was $y = 1.203\ x – 0.279$ for GC-MS *vs.* HPLC and $y = 1.105\ x + 0.766$ for GC-MS *vs.* IMx Abbott (Figure 4.1).

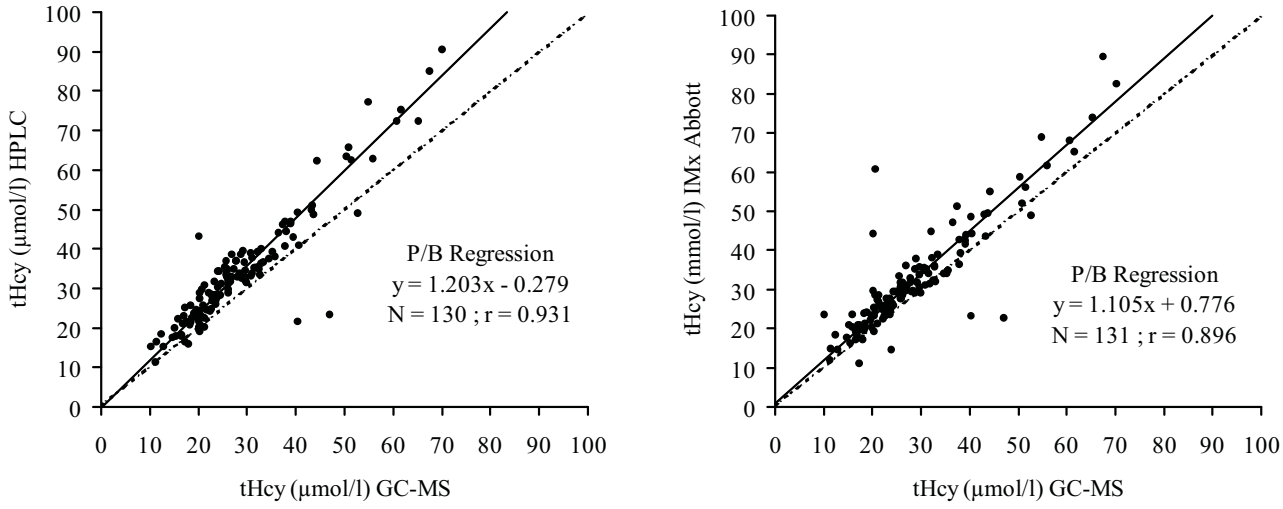

Figure 4.1 P/B regression analysis for the comparison of tHcy concentrations measured by GC-MS *vs.* HPLC (left) and GC-MS *vs.* IMx (right) in ESRD patients

Table 4.2 Comparison of results obtained for plasma tHcy in ESRD patients

	Fasting total plasma homocysteine concentration (µmol/l)			
Analytical method	**Mean**	**SD**	**Minimum**	**Maximum**
GC-MS (n = 131)	28.7	11.9	10.2	70.2
HPLC (n = 130)	34.0	14.5	11.2	90.6
IMx Abbott (n = 131)	32.4	13.9	11.1	89.5

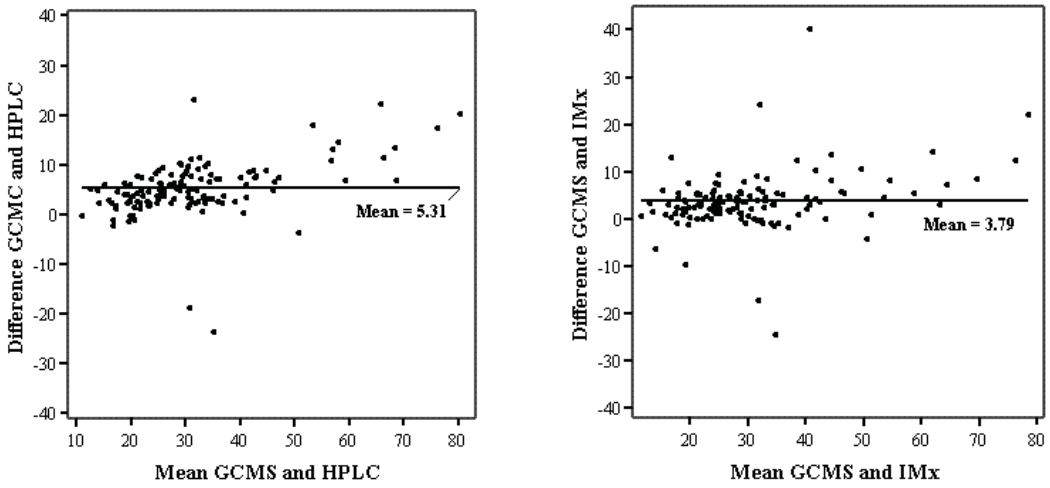

Figure 4.2 Bland-Altman plots showing the differences between the results for plasma tHcy as measured by GC-MS *vs.* HPLC and GC-MS *vs.* IMx method in ESRD patients. Compiled results for each method and tHcy concentrations are expressed in µmol/l.

Table 4.3 Difference plot analysis of results obtained from GC-MS *vs.* HPLC and GC-MS *vs.* IMx in ESRD patients

	Fasting total plasma homocysteine concentration (µmol/l)		
Variable[a]	**Mean differences**	**SD**	**Central 0.95 interval**
GC-MS vs HPLC (n = 130)	5.31	5.54	-5.77 to 16.39
GC-MS vs IMx (n = 131)	3.79	6.17	-16.13 to 16.13

[a] The mean (SD) differences between GC-MS and the other compared method as indicated above were calculated, and the central 0.95 interval was calculated as mean $\pm$ 2 SD

Scatter plots of observed measurement differences for fasting tHcy determinations in ESRD patients against the mean of GC-MS and the compared method are shown in Figure 4.2. The mean (SD) differences between GC-MS and HPLC, and GC-MS and IMx are reported in Table 4.3.

The central 0.95 interval (mean of the differences $\pm$ 2 SD) gives an indication of the agreement between GC-MS and the other compared methods in tHcy determination. Using this approach, HPLC agreed with GC-MS better than with IMx. The scatter plot of GC-MS *vs.* IMx (-16.13 to 16.13) displayed a relatively wider scatter of difference in data points compared to GC-MS *vs.* HPLC (-5.77 to 16.39).

4.2.2 Comparison of The GC-MS Measurement with IMx in Healthy Subjects

The mean plasma concentrations of tHcy in healthy subjects determined by GC-MS (9.5 ± 2.1 µmol/l) were lower than determined by IMx (10.4 ± 2.5 µmol/l). The coefficient of correlation between both methods was 0.988 ($p < 0.001$). The Passing-Bablok regression line was $y = 1.189\ x - 0.925$ (Figure 4.3). The Bland-Altman scatter plot of GC-MS *vs.* IMx (-2.03 to 0.09) in healthy subjects displayed a relatively small scatter of difference data points compared with ESRD patients (Figure 4.4).

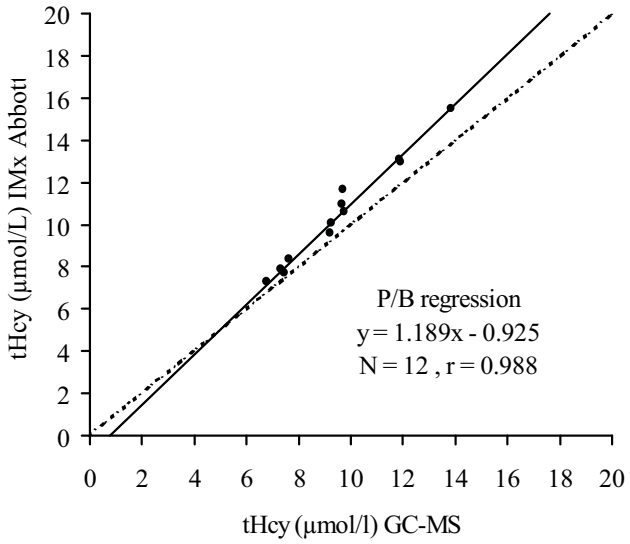

Figure 4.3 P/B regression analysis for the comparison of tHcy concentrations measured by GC-MS *vs.* IMx in healthy subjects

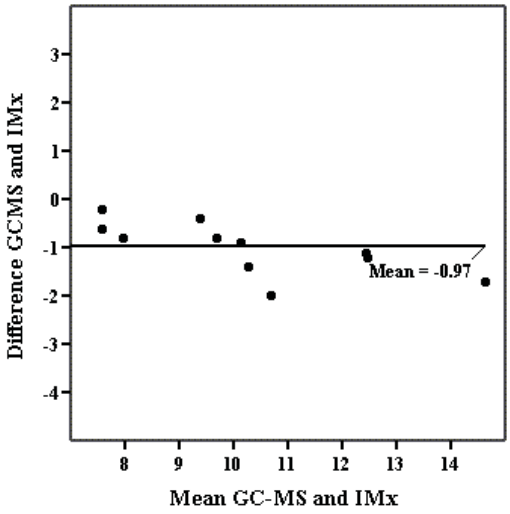

Figure 4.4 Bland-Altman plots showing the difference between the results for plasma tHcy as measured by GC-MS *vs.* IMx method. Mean difference of GC-MS and IMx is –0.97 $\pm$ 0.53 µmol/l.

5 Application of the GC-MS Method on Clinical Investigations

5.1 Total Plasma Homocysteine and Related Amino Acids in ESRD Patients

5.1.1 Background

The first link between the extreme elevation of homocysteine levels in homocystinuria and premature atherosclerosis was made in 1969 (McCully, 1969). More moderate elevations of homocysteine are now recognized as a risk factor for cardiovascular disease.

Homocysteine is mainly eliminated by the renal catabolism. Normally, only about 1% of the homocysteine filtered by the glomeruli is found in urine (Guttormsen *et al.*, 1997). The main part is reabsorbed and metabolized in proximal tubules. Thus, the kidneys are homocysteine-metabolizing rather than homocysteine-excreting (Bostom *et al.*, 1995a; Refsum *et al.*, 1998b; van Guldener *et al.*, 1998a). Patients with renal failure present excess morbidity and mortality in arteriosclerotic disease. The mortality in end-stage renal disease (ESRD) patients aged up to 50 years is five to ten times higher than in the general population (Bachmann *et al.*, 1995; Bostom *et al.*, 1995b; Robinson *et al.*, 1996). This seems mainly to be due to different risk factors for cardiovascular disease, which cannot be solely explained by hypertension, diabetes mellitus, hyperlipidemia and/or smoking. Hyperhomocyteinemia as a new independent cardiovascular risk factor has a high prevalence in ESRD patients and may play an important role in the development of vascular disease in this group of patients (Bachmann *et al.*, 1995; Gupta and Robinson, 1997; Manns *et al.*, 1999).

Because of the strong suggestions that homocysteine may play a causative role in cardiovascular disease in ESRD patients, the measurement of homocysteine and its metabolites in ESRD patients could explain this phenomenon. However, the elevation of tHcy concentration in ESRD patients is well known, but little is known about levels of related amino acids in this group. Here, we have determined total plasma homocysteine and related amino acids methionine, cysteine and cystathionine in order to observe the homocysteine and methionine metabolism in ESRD patients.

5.1.2 Methods

5.1.2.1 Sample Collection

Blood samples were obtained from 131 patients with ESRD, aged 20 to 85 years (mean age 56 ± 16 years; 47 women and 84 men) and undergoing hemodialysis therapy three times a week. All patients received daily vitamin B_6 (10-20 mg). Briefly, blood samples treated with EDTA were taken immediately before dialysis treatment, and were immediately placed on ice and centrifuged within 60 minutes at 1,600 x *g* for 10 minutes. The plasma samples were stored at -20° C until analysis.

5.1.2.2 Determination of Total Homocysteine, Methionine, Cysteine and Cystathionine

Total homocysteine, methionine, cysteine and cystathionine determination was performed using a new GC-MS method which had been developed previously (see chapter 3).

5.1.2.3 Determination of Vitamins

Folate and vitamin B_{12} concentrations in plasma were measured with commercial immunofluorescence IMX assay (IMx® Folic Acid Assay or IMx® B_{12} Assay; Abbott GmbH, Wiesbaden, Germany). Vitamin B_6 was determined by an HPLC analytical kit (No. 36000; Chromsystems, Martinsried, Germany). Briefly, 100 µl plasma was used for the assay. Fifty µl of the derivatized sample was injected onto a RP-18 column (No. 36100; Chromsystems, Martinsried, Germany) and the column was kept in an Shimadzu Cto-6A column oven at 25° C. The analysis was performed using a Waters™ 616 Pump at a flow-rate of 1.3 ml/min. Vitamin B_6 derivatives were detected at the wavelength of 470 nm by a Waters™ 474 Scanning Fluorescence Detector. Concentration of vitamin B_6 was calculated by comparison of the peak height of the samples with that of a calibration standard.

5.1.2.4 Statistical Analysis

All values were calculated as mean ± SD. Mean values of measurement were compared by student´s t-test, with homocysteine as the main paremeter. The threshold of significance was set at $p < 0.05$. Statistical analysis was performed using SPSS software (Version 9.0). Amino acids and vitamin levels were compared with reference ranges from literature.

5.1.3 Results

Table 5.1 shows the mean plasma concentrations of tHcy, methionine, cysteine, cystathionine, folate, vitamin B_{12}, vitamin B_6, and creatinine. There was a significantly higher concentration of methionine (13.8 ± 4.3 µmol/l *vs.* 11.9 ± 3.1 µmol/l; p = 0.005) and creatinine (709 ± 206 µmol/l *vs*. 626 ± 168 µmol/l; p = 0.037) and a lower concentration of folate (13.2 ± 6.7 nmol/l *vs*. 18.0 ± 7.4 nmol/l; p < 0.001) in male patients compared to female patients. Mean plasma concentrations of tHcy in all dialysis patients were markedly elevated. Ninety five per cent of the patients had higher than normal plasma concentrations of tHcy (> 15 µmol/l). Compared to normal reference, a higher mean plasma level of cysteine, cystathionine, creatinine and vitamin B_6 was found, while mean plasma levels of methionine, vitamin B_{12} and folate were within the normal range. Unlike healthy people, tHcy measured by GC-MS was not related to age (r = - 0.137; n.s.; male 56.8 ± 16.8 years., female 55.5 ± 13.7 years) or gender (male 28.8 ± 12.8 µmol/l, n = 84; female 28.31 ± 10.38 µmol/l, n = 47).

Table 5.1 Plasma concentrations of amino acids, vitamins, and creatinine in ESRD patients

	Plasma concentration				Normal Range (ref.)
	total	**male**	**female**	**p (t-test)**	
Homocysteine (µmol/l)	28.6 ± 11.9[a] (n=131)	28.8 ± 12.8[a] (n=84)	28.3 ± 10.4[a] (n=47)	0.809	5.0 – 15.0[b] (Stabler, 1988)
Methionine (µmol/l)	13.1 ± 4.0 (n = 131)	13.8 ± 4.3 (n=84)	11.8 ± 3.1 (n=47)	0.005*	13 – 42 (Stabler, 1988)
Cysteine (µmol/l)	412.1 ± 102.8 (n = 131)	407.3 ± 101.3 (n=84)	420.7 ± 106.1	0.481	200 – 361 (Stabler, 1988)
Cystathionine (nmol/l)	2261 ± 2593 (n = 131)	2515 ± 3126 (n=84)	1806 ± 1037 (n=47)	0.060	65 – 300 (Stabler, 1988)
Folate (nmol/l)	14.9 ± 7.3 (n = 131)	13.2 ± 6.7 (n=84)	18.0 ± 7.4 (n=47)	< 0.001*	6.8 – 56.6 (Stabler, 1986)
Vitamin B_{12} (pmol/l)	325.1 ± 178.9 (n = 130)	340.1 ± 195.8 (n=83)	298.8 ± 142.4 (n=47)	0.170	147.6 – 737.8 (Stabler, 1986)
Vitamin B_6 (nmol/l)	142.4 ± 123.1 (n = 131)	149.6 ± 131.0 (n=84)	126.8 ± 82.2 (n=47)	0.306	14.4 – 72.2 (Greiling, 1995)
Creatinine (µmol/l)	679 ± 191 (n = 94)	709 ± 206 (n=61)	626 ± 168 (n=33)	0.037*	< 107 (Krüch, 1994)

[a]mean ± SD , [b]determined by GC-MS
*significantly difference between male and female

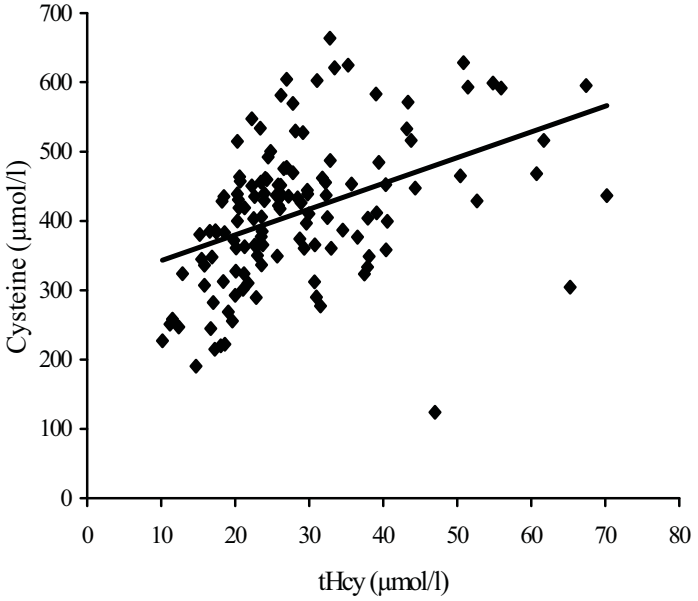

Figure 5.1 Relationship between plasma tHcy and cysteine concentration in ESRD patients (r = 0.434 ; p<0.001)

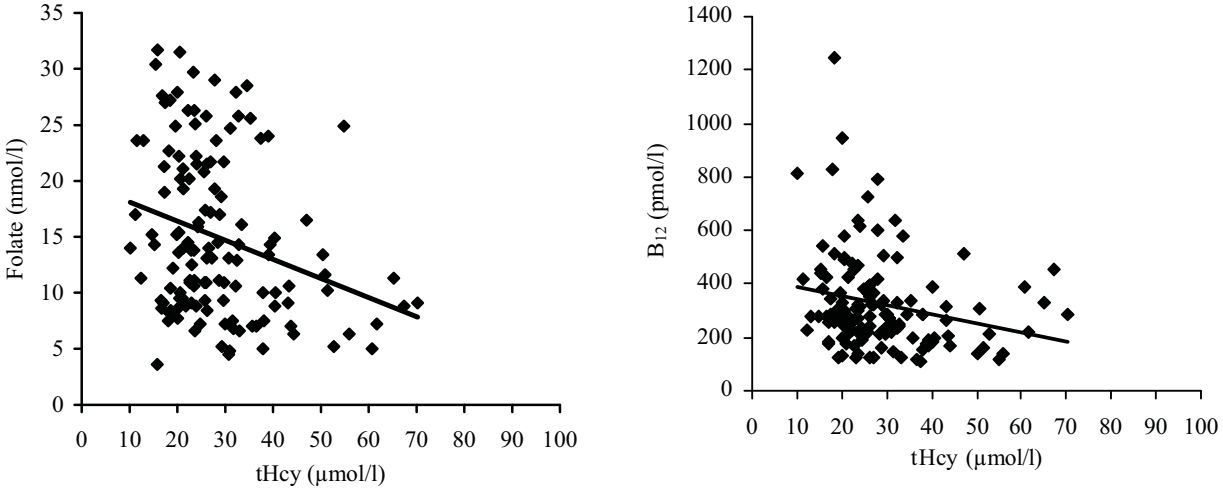

Figure 5.2 Relationship between plasma tHcy and folate or vitamin B_{12} concentration in ESRD patients (r = -0.281 ; p=0.001 and r = -0.229 ; p=0.009)

Linear regression analysis showed positive correlations between plasma concentrations of tHcy and cysteine (r = 0.434; p < 0.001) (Figure 5.1) and tHcy and cystathionine (r = 0.187; p = 0.032). Serum creatinine exhibited a moderate positive association with tHcy (0.314; p = 0.002), Met (r = 0.227; p = 0.028), and even stronger with cysteine (0.366; p < 0.001). Negative correlations were found between plasma concentrations

of tHcy and folate (r = - 0.281; p = 0.001), homocysteine and vitamin B_{12} (r = - 0.229; p = 0.009, respectively) (Figure 5.2), and cystathionine and folate (r = - 0.242; p = 0.005). No correlation could be found between vitamin B_6 and homocysteine.

5.2 Effects of Leucovorin (N^5-formyltetrahydrofolate) versus Folic Acid on Plasma Total Homocysteine and its Related Amino Acids in ESRD Patients

5.2.1 Background

The incidence of cardiovascular disease (CVD) is remarkably increased in dialysis patients compared to normal healthy subjects and accounts for the major cause of death in this group of subjects. This high cardiovascular morbidity and mortality in chronic hemodialyzed patients has led to the hypothesis of "accelerated atherosclerosis" since the excess burden of atherothrombotic cardiovascular risk factors including hypertension, diabetes, smoking and disorders of lipid metabolism is often present in these patients.

Chronic renal disease patients have a high prevalence of mild to moderate hyperhomocysteinemia, which has been independently linked to their development of CVD outcomes in recent prospective studies (Massy *et al.*, 1994; Bostom *et al.*, 1997; Jungers *et al.*, 1997; Moustapha *et al.*, 1998). Accordingly, the lowering of tHcy levels in patients with chronic renal disease may reduce the excess incidence of arteriosclerotic CVD outcomes.

The tHcy concentration can be reduced safely and effectively with combined folic acid, vitamin B_{12} and vitamin B_6 supplements. Reduction of elevated tHcy plasma levels may have beneficial effects on hospitalization and survival in patients with renal failure (Sunder-plassmann *et al.*, 1999). Homocysteine Lowering Trialist's Collaboration (1998) reported that in a majority of subjects without renal disease, elevated tHcy plasma concentration can be reduced by low-dose oral folic acid therapy. However, two randomized double-blind trials showed that oral folic acid, even in doses up to 60 mg/day, allowed normalization of hyperhomocysteinemia in only 30% of hemodialysis patients (Bostom *et al.*, 1996; Sunder-plassmann *et al.*, 2000). The reason for this poor response to folic acid treatment in patients with renal disease remains unclear.

Recently, in an uncontrolled retrospective study, administration of intravenous folinic acid (N^5-formyltetrahydrofolate, Leucovorin), which is a precursor of 5-methyltetrahydrofolate, with intravenous vitamin B_6 normalized hyperhomocysteinemia in 78% of hemodialysis patients (Touam *et al.*, 1999). This finding brings a new novel therapeutic concept for reducing plasma tHcy levels by treatment with Leucovorin.

Leucovorin (N^5-formyltetrahydrofolate) is a chemically reduced derivative of folic acid consisting of an equal mixture of the l-(6S) and the d-(6R) diastereoisomers. Only the naturally occurring L-diastereomer and its metabolites are associated with any significant

pharmacological activity. Biochemically, these reduced folates function as cofactors for single carbon transfers for nucleic acid, amino acid, protein, and lipid biosynthesis. D,L-Leucovorin is indicated for use in rescue from the toxicity of methotrexate, several mechanisms may be operative. Leucovorin competes with methotrexate for binding to dihydrofolate reductase, a key enzyme in intracellular folate metabolism. Competition also exists between Leucovorin and methotrexate for both active transport into cells and for polyglutamate synthase, an enzyme that converts folate and methotrexate to polyglutamate derivatives. Leucovorin is also indicated in the enhancement of the antitumor activity in 5-fluorouracil (5-FU) therapy and acts by increasing the stability of the covalent bond between 5-FU and thymidylate synthase (Goldin, 1978; Jolivet *et al.*, 1983; Matherly *et al.*, 1986).

In this study, we investigated whether intravenous Leucovorin is superior to intravenous folic acid in reducing plasma tHcy levels in hemodialysis patients in a randomized, double-blind intervention study. With simultaneous determination of the related amino acids methionine, and cysteine beside homocysteine, we obtained more insight into the homocysteine metabolism under administration of folate and Leucovorin. Before treatment, we allow the patients in a four weeks run-in-phase in order to prove the stability of the main parameter tHcy.

5.2.2 Methods and Patients

5.2.2.1 Design and Patients

This randomized, double-blind intervention study was conducted in the dialysis unit of the dialysis centres Ippendorf and Bad Godesberg, Bonn, Germany. Sixty hemodialysis patients, 36 men and 24 women with a mean age of 54.9 ± 14.9 years (mean ± SD), were selected and treated with 30 mg of the folate derivate Pteroylmonoglutamat (PGA) administered intravenously or with an equimolar amount of 30 mg of Leucovorin (N^5-formyltetrahydrofolate) administered intravenously once a week at the end of each hemodialysis session for eight weeks. All patients received daily vitamin B_6 (10-20 mg).

Pteroylmonoglutamat (PGA) (Folsäure Hevert forte®) was purchased from Hevert-Arzneimittel GmbH, Nußbaum, Germany and Leucovorin obtained from Lederle Arzneimittel GmbH, Münster, Germany.

The study was carried out in accordance with the Helsinki Declaration of 1975 (amended in 1996) and approved by the local ethical commission of the faculty of Medicine,

University of Bonn. Informed consent was obtained from each patient before enrollment in the study.

Predialysis blood samples were drawn four times weekly during a four weeks run-in phase for analysis of baseline characteristics and after four and eight weeks' treatment for measurement of plasma levels of tHcy and metabolites.

All patients were on regular standard care at dialysis unit. Adverse effects were documented on all patients. Side effects were assessed by interview and documented by the study physicians.

5.2.2.2 Biochemical Assay

Whole-blood counts and blood chemistry were analyzed by standard laboratory procedures. In order to analyze tHcy beside its related amino acids, blood was drawn before a dialysis session, immediately placed on ice and centrifuged within 60 minutes at 1,600 x *g* for 10 minutes. The plasma samples were stored at -20° C until analysis.

Plasma tHcy and related metabolites were determined using GC-MS method. Folate and vitamin B_{12} plasma levels were measured with Enzyme Immunoassay (IMx® Folic Acid Assay or IMx® B_{12} Assay; Abbott GmbH, Wiesbaden, Germany). Identification of MTHFR genotype was performed using a method described by Frosst *et al*., 1995.

5.2.2.3 Statistical Analysis

All values were calculated as mean ± SD. Student´s pair and unpaired t-test were used to evaluate the difference between groups. The Pearson test was used to evaluate the relationship between numeric parameter. The threshold of significance was set at $p < 0.05$. Statistical analysis was performed using SPSS software (Version 9.0).

5.2.3 Results

The demographic and biochemical parameters of the 60 study subjects are described in Table 5.2. The baseline levels of tHcy, methionine and cysteine were obtained from mean of measurement during a four weeks' run-in phase. There was no difference between tHcy concentration in both groups. Fifty one patients (85%) presented hyperhomocysteinemia (tHcy > 15 µmol/l). However, a slightly, but not significantly higher methionine and cysteine concentration of Leucovorin group was found compared with the results within the folic acid

group (2 µmol/l and 60 µmol/l). Generally, there was no relevant difference in baseline parameters between both treatment groups.

Table 5.2 Baseline characteristics of both study groups

	Plasma concentration		
	Leucovorin	**Folic Acid**	**p (t-test)**
N	30	30	-----
Sex, N (% Female)	30 (50%)	30 (87%)	-----
Age (years)	56 ± 15	53 ± 15	0.421
BMI (kg/m2)	24.6 ± 4.8	25.9 ± 4.8	0.382
Homocysteine[a] (µmol/l)	22.9 ± 6.7	22.1 ± 10.5	0.713
Methionine[a] (µmol/l)	19.8 ± 7.4	17.3 ± 5.7	0.152
Cysteine[a] (µmol/l)	466.2 ± 134.4	406.8 ± 81.5	0.043
RBC Folate[b] (µmol/l)	834.4 ± 475.3	981.9 ± 575.3	0.288
Vitamin B_{12}[a] (pmol/l)	357.0 ± 257.5	316.7 ± 135.5	0.451

Values are expressed as mean ± SD
[a]mean plasma concentration during run-in phase
[b]plasma concentration in week 0

5.2.3.1 Effect of Folic acid and Leucovorin on tHcy and Metabolites

There were significantly high positive correlations between plasma concentrations of tHcy, methionine and cysteine in pretreatment (baseline) as well as after four and eight weeks of treatment (Table 5.3).

Table 5.3 Correlations between plasma concentration of tHcy, methionine and cysteine before treatment and after four and eight weeks of treatment with Leucovorin and folic acid

	Pearson´s correlation test		
	week 0 (N = 60)	**week 4 (N = 58)**	**week 8 (N = 55)**
tHcy *vs*. met	r = 0.224; p = 0.085	r = 0.416; p = 0.001	r = 0.361; p = 0.007
tHcy *vs*. cys	r = 0.416; p = 0.001	r = 0.532; p < 0.001	r = 0.656; p < 0.001
met *vs*. cys	r = 0.598; p < 0.001	r = 0.481; p < 0.001	r = 0.480; p < 0.001

Abbreviations are : tHcy, total homocysteine; met, methionine; cys, cysteine.

Total plasma concentration of homocysteine decreased after four weeks and eight weeks of treatment in both groups. A slight increase of tHcy, but not a significant one, was found between four weeks and eight weeks. A significant difference of plasma tHcy concentration was found between pretreatment and after four and eight weeks treatment in the Leucovorin group and the folic acid group (Table 5.4).

Table 5.4 Effects of treatment with Leucovorin and folic acid on plasma concentration of tHcy, methionine and cysteine

	Plasma concentrations			Paired difference, p (t-test)		
	T0	**T4**	**T8**	**T 0-4**	**T 0-8**	**T4-8**
Homocysteine (µmol/l)						
Leucovorin	22.9 ± 6.7	17.9 ± 4.8	18.9 ± 5.4	< 0.001	< 0.001	0.417
Folic acid	22.1 ± 10.5	17.4 ± 6.9	18.5 ± 11.6	< 0.001	0.042	0.562
Total	22.5 ± 8.7	17.6 ± 5.9	18.7 ± 8.8	< 0.001	< 0.001	0.381
Methionine (µmol/l)						
Leucovorin	19.8 ± 7.4	18.6 ± 7.5	21.6 ± 9.6	0.442	0.105	0.083
Folic acid	17.3 ± 5.7	17.1 ± 7.0	18.3 ± 6.8	0.568	0.182	0.163
Total	18.6 ± 6.7	17.8 ± 7.2	20.0 ± 8.5	0.332	0.037	0.025
Cysteine (µmol/l)						
Leucovorin	466.2 ± 134.4	462.6 ± 153.0	487.8 ± 164.2	0.457	0.065	0.260
Folic acid	406.8 ± 81.5	406.9 ± 104.1	416.8 ± 88.2	0.848	0.568	0.562
Total	436.5 ± 144.2	434.8 ± 132.7	453.6 ± 136.7	0.636	0.046	0.227

Values are expressed as mean ± SD
Abbreviations are : T0, week 0/baseline; T4, after 4 week treatment; T8 after 8 week treatment.

A slight increase of plasma methionine was found after eight weeks of treatment. This was more distinct during treatment with Leucovorin than with folic acid. No significant change of plasma concentrations of methionine was found after four weeks of treatment for both groups. However, a significant increase of plasma concentrations of methionine was found in all patients (total) after eight weeks of treatment ($p=0.037$) and between four and eight weeks ($p=0.025$). There were no significant differences of plasma cysteine between pre-treatment and after four and eight weeks of treatment (Table 5.4).

The reduction of plasma tHcy concentration for the Leucovorin group and folic acid group were 18.1% and 17.5% after four weeks; 14.8% and 14.5% after eight weeks of treatment (Figure 5.3). There was also no significant difference between Leucovorin and folic acid treatment in percentage reduction of tHcy concentration in dialysis patients. Normalization of tHcy concentration (tHcy < 15 µmol/l) in hyperhomocysteinemia patients

after four weeks of treatment was achieved in six patients (23.1%) in the Leucovorin group and in four patients (17.4%) in the folic acid group. At the end of the study (after eight weeks), normalization of tHcy concentrations was found in eight patients (27.6%) in the Leucovorin group and in nine (30.3%) patients in the folic acid group.

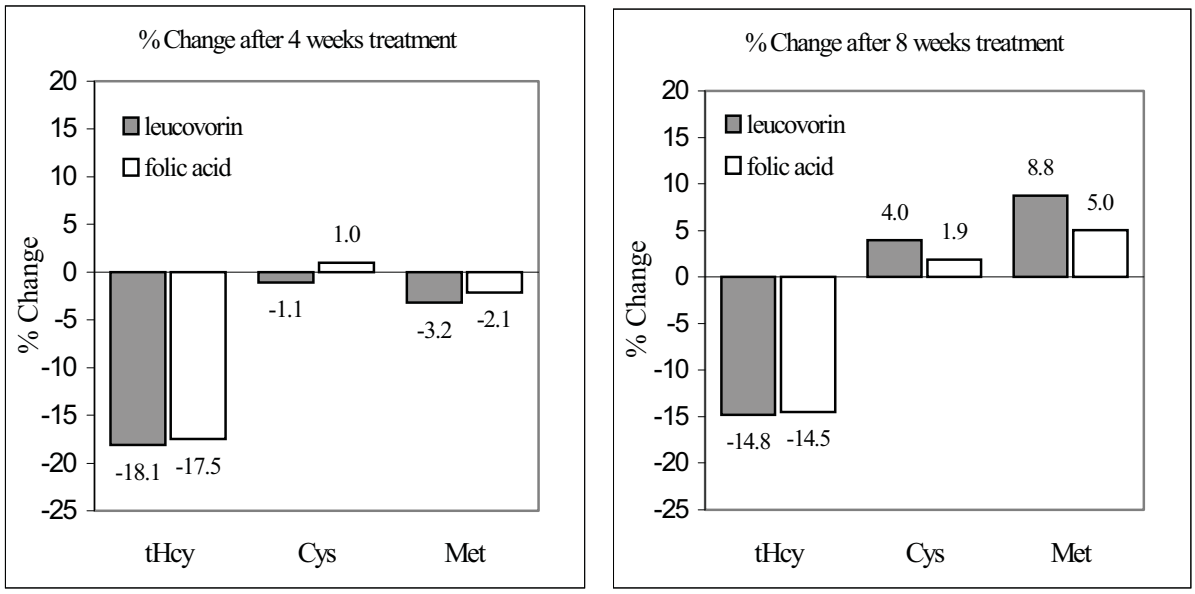

Figure 5.3 Percentage change of tHcy, methionine and cysteine after four and eight weeks of treatment with Leucovorin and folic acid

After four weeks of therapy plasma concentrations of methionine were slightly reduced by 3.2% and 2.1% for the Leucovorin and the Folic acid group, however after eight weeks of treatment elevated by 8.8% and 5.0% for the Leucovorin and the Folic acid group. No significant difference was found for the percentage methionine change between the Leucovorin and the folic acid group. There was no significant change in plasma concentrations of cysteine in both group (Figure 5.3).

5.2.3.2 Relative Response of Folate and Vitamin B_{12}

Red blood cell concentrations of folate were significantly increased ($p<0.001$) after treatment both in the Leucovorin and the Folic acid group as well as in all subjects (Table 5.5), while no significant difference was found in plasma concentration B_{12}. However, despite a clear increase of red blood cell folate concentrations, there was no significant difference between the Leucovorin and the folic acid group.

Table 5.5 Plasma concentration of vitamin B_{12} and red blood cell folate after treatment with Leucovorin and folic acid

	Concentrations		Difference, p (t-test)
	Tbf	Taf	
RBC fol (μmol/l) [a]			
Leucovorin	834.4 ± 475.3	2354.7 ± 413.7	< 0.001
Folic acid	981.9 ± 575.3	2665.7 ± 647.2	< 0.001
Total	864.6 ± 465.2	2496.1 ± 552.9	< 0.001
B_{12} (pmol/l)[b]			
Leucovorin	357.0 ± 257.5	318.2 ± 167.3	0.260
Folic acid	316.7 ± 135.5	323.5 ± 123.3	0.907
Total	339.6 ± 205.6	320.8 ± 146.1	0.323

Values are expressed as mean ± SD
Abbreviations are : Tbf, before treatment/baseline; Taf, after treatment; RBC fol (red blood cell folate).
[a]Taf was calculated from T8 (week 8)
[b]Taf was calculated from mean plasma after treatment (week 4 & 8)

As shown in Table 5.6, significantly positive correlations were found between the percentage change of red blood cell folate and plasma tHcy, and between tHcy and methionine, respectively. However, no correlations were found between the percentage change in B_{12} and tHcy, methionine and cysteine.

Table 5.6 Correlations between percentage changes of folate, vitamin $B_{12,}$ tHcy, methionine and cysteine

	Pearson Correlation test
RBCfol *vs*. tHcy	r = 0.557; p < 0.001*
RBCfol *vs*. met	r = -0.148; p = 0.284
RBCfol *vs*. cys	r = 0.058; p = 0.675
B_{12} *vs*. tHcy	r = -0.037; p = 0.782
B_{12} *vs*. met	r = -0.023; p = 0.864
B_{12} *vs*. cys	r = -0.124; p = 0.350
tHcy *vs*. met	r = -0.352; p = 0.006*
tHcy *vs*. cys	r = 0.025; p = 0.848
met *vs*. cys	r = -0.064; p = 0.630

Abbreviations are : tHcy, total homocysteine; met, methionine; cys, cysteine; RBCfol, red blood cell folate
Percentage of change was calculated between concentration before treatment (baseline) and after treatment (mean 4 & 8 week)
*Significant correlation

6 Discussion

6.1 Analysis of Total Plasma Homocysteine and Related Amino Acids by GC-MS

Many of the analytes which are currently assayed in biological samples by laboratories performing clinical chemistry or biochemistry have low physiological and/or pathological concentrations. Thus, their quantitative determination requires not only reproducible but also sensitive methods. It was the aim of this study to establish an accurate, specific and sensitive assay for the determination of homocysteine and its related amino acids methionine, cysteine and cystathionine in human plasma in order to observe the metabolism of homocysteine in clinical investigations.

Sample handling for homocysteine measurement is of critical importance. For the reduced homocysteine assay, plasma must be rapidly separated by refrigerated centrifugation and the proteins precipitated to prevent shifts in the ratio between free to bound forms at the very early beginning of bloodletting. To measure tHcy by specific assays, plasma must be separated from whole blood within one hour or, if refrigerated, within four hours. Otherwise erythrocytes and leukocytes continue to synthesize intracellulary appreciable amounts of homocysteine from methionine and contribute this additional amount to the extracellular free and bound homocysteine. At room temperature, the apparent tHcy in plasma can double within hours if no measures are taken to eliminate anaerobic glycolysis which allows conversion of methionine to homocysteine. This reaction can be blocked by fluoride which inhibits glycolysis and ATP synthesis, or by the addition of specific S-adenosylhomocysteine hydrolase inhibitors (Fiskerstrand *et al.*, 1993: Ueland *et al.*, 1993; Minner *et al.*, 1997).

For determination of compounds present in biological matrices, such as tissue homogenates, plasma or urine, very high selectivity and sensitivity can only be achieved with very sophisticated purification procedures. The compounds of interest must be extracted and purified from the biological sample. In our study, homocysteine and its related amino acids were separated from protein plasma by precipitation using sulfosalicyl acid. Prior to the precipitation process, homocysteine conjugates, such as disulfides are, first reduced with dithiothreitol. As described before (see chapter 2), homocysteine in human plasma is predominantly bound with itself, with cysteine and with protein sulfhydryl groups to form homocystine, homocysteine-cysteine and protein-bound homocysteine. All these forms together, free and bound to proteins, are called total homocysteine. As the free sulfhydryl form of homocysteine is unstable and highly variable, investigators have focused their

interests on methods which quantify tHcy, relying on sample pretreatment with specific reductants, such as dithiothreitol (DTT), sodium borohydride, mercaptoethanol, and other reagents to reduce all disulfide bonds.

Another, possibly important, step in homocysteine purification is ion-exchange chromatography. Ion-exchange columns such as a Dowex 50W X8 have been used as a simple method for extracting amino acids from biological fluids prior to GC-MS analysis (Stabler *et al.*, 1988; Pietzsch *et al.*, 1997). However, evaporation of the eluating solvent was laborious and time consuming. In our method, we used high-speed vacuum centrifugation (Speed vac.).

After extraction from biological matrices, the amino acids are usually metabolized by esterification or etherification into less polar and more volatile metabolites which can be easily separated in specific matrices by HPLC or GLC. Homocysteine and its neighbouring sulfuryl-amino acids have three reactive groups which can be conjugated: amino-, carboxylic- and sulfhydryl-groups. These three strongly polar groups must be modified before analysis by HPLC or GLC. Several derivatization methods have been used to measure sulfur amino acids. Stabler *et al.* (1988 and 1993) published the use of N-methyl-N(tert.-butyldimethylsilyl) trifluoracetamid as a derivatizing agent to form the corresponding t-butyl-dimethylsilyl derivatives. Another derivatization method for sulfur amino acids is the practical use of alkyl carbonate reagents in a medium of water-alcohol-pyridine as described by Husek and co-workers (Huang *et al.*, 1993; Husek, 1995 and 1998). A single step procedure allows the simultaneous acylation of the amino and thiol groups, as well as esterification of the carboxylic group. A mechanism was proposed based on the formation of an intermediate mixed carboxylic-carbonic acid anhydride followed by the exchange with an alcohol (Wang *et al.*, 1994). Various combinations of alkyl carbonate reagents and alcohol were used to generate a variety of N(O,S)-alkoxycarbonyl amino acid alkyl ester derivates for determination of homocysteine by GC-MS (Pietzsch *et al.*, 1997; Sass *et al.*, 1997; Myung *et al.*, 1999). In our method, we used etylchloroformate (ECF) as a derivatizing agent to form volatile N(O,S)-ethoxycarbonyl amino acid ethyl ester for enhanced GC-MS separation and detection. This method is an adaptation of a GC-MS method previously described by Pietzsch *et al.* (1997). The complete derivatization is performed in a few minutes in an aqueous solution at room temperature. It is simpler and requires less time than the method described by Stabler *et al.* (1988 and 1993).

Quantitative analysis depends on many pre-analytical factors, such as purification procedures, and on a number of instrumental variables. Therefore, the strategy used in GC-MS to overcome not only the yield of the purification and derivatization steps but also the instrumental variables, is the use of an internal standard added to the sample before the whole work-up procedure. The choice of the IS is of primary importance in the design of a new assay . In practice, the selection of an IS should be based on the consideration of its effects upon limiting errors at each stage of the analysis, with the greatest emphasis being given to the reduction of the largest errors. Internal standards are usually characterized by having appropriately similar chemical behavior and structure compared with the substance to be analyzed. We used a stable isotope-labeled eight-fold deuterated (D8-)homocystine for homocystine, d4-cystine, d3-methionine, d4-cystathionine for the related homocysteine metabolites, cysteine, methionine and cystathionine, respectively. These IS are chemical analogues of the analyte that contain a ^{2}H atom instead of a normal ^{1}H. Thus, their molecular weight is two to four mass units (m/z) higher than the analyte itself. They have (nearly) the same retention times as measured by GC but differ in their m/z values in comparison to the authentic analyte. The use of stable isotope-labeled internal standards, also called isotope dilution mass spectrometry, produces lowest variability factors due to instrumental stability and sample manipulation errors.

The addition of the corresponding, specific internal (deuterated) standards from the very beginning allows equilibration with endogenous homocysteine and its related amino acids during the reduction step and work-up procedure. The use of a deuterium labeled internal standard within this method increases the specificity for the individual amino acids compared to common HPLC methods. Due to its high sensitivity and specificity this method appears to be well suited for the reliable determination of low concentrations of tHcy and related amino acids in clinical investigations.

In the multiple-ion detection analysis (MIDA) or single-ion monitoring (SIM) method, the mass spectrometer is used as a mass selective detector (MSD) guaranteeing very specific and sensitive detection after gas chromatographic separation. Using the SIM technique, only one or few specific ions of the compound are monitored with subsequent mass selectivity, reduction of the chemical noise and increased sensitivity. Often, the selection of the ions to be monitored is important. As a general rule, monitoring of the molecular ion, if sufficiently intense, is preferable to the monitoring of fragment-ions, because of the greater specificity for the compound to be determined. Fragmentation of the molecule ion occurs predominantly during electrochemical ionization while poor fragmentation is obtained during chemical

ionization processes. Another possibility is the simultaneous monitoring of several characteristic fragment ions, which allow detection of unexpected interferences by a change in the peak ratio of different channels. We have chosen characteristic ions for homocysteine (m/z 234), homocysteine d4 (m/z 238), methionine (m/z 249), methionine d3 (m/z 252), cysteine (m/z 220), cysteine d2 (m/z 222), cystathionine (m/z 260) and cystathionine d4 (m/z 264), as they showed good intensity and no interference with other ions from other molecules when checked by comparison with mass spectra in the "Scan mode" (Figure 3.3).

We evaluated our GC-MS method and found good accuracy and precision. The variation coefficients for these measurements were 1.63%, 1.40%, 1.37% and 2.28% for homocysteine, methionine, cysteine and cystathionine, respectively. Between-day variations of homocysteine, methionine, cysteine, cystathionine were 2.98%, 4.39%, 6.99%, and 8.60%, respectively. Furthermore, mean recoveries of simultaneous determination of different amino acids by our GC-MS method were 94.2% for homocysteine, 86.8% for methionine, 98.5% for cysteine, and 83.8% for cystathionine. Limits of detection were found to be 5 µmol/l or less for tHcy, methionine and cysteine, and 20 nmol/l for cystathionine. Limits of quantitation were 10 µmol/l or less for tHcy, methionine and cysteine and 100 nmol/l for cystathionine. These limits are excellent for the determination and quantification of sulfhydryl amino acids from human serum or plasma samples.

The present method provides a sensitive and reliable quantification method for the simultaneous determination of tHcy and its related amino acids methionine, cysteine, and cystathionine from plasma or serum samples with high accuracy and precision. The advantage of this method is simultaneous determination of tHcy and other metabolites and can be applied in clinical investigations to study pharmacokinetic and metabolism of homocysteine with a particular interest in evaluating remethylation by measurement of methionine and transsulfuration by measurement of cysteine and cystathionine within the "methionine metabolism".

6.2 Comparison of the New GC-MS Methods with Other Methods

In analytical clinical chemistry comparison of a new methodological technique with established ones is obligate to validate whether these methodologies agree sufficiently in their results and can be used interchangeable. Many studies give the product-moment correlation coefficients (r) between the results of the two measurement methods as indicator for

agreement. However, this high correlation is not sufficient to conclude that two methods do agree.

Bland and Altman (1986) published a statistical method for assessing agreement between two analytical methods in determination of the same subject after they found some reasons why high correlation does not mean total agreement between two methods. First, the coefficient of correlation (r) only measures the strength of relation between two variables, not the agreement between them. Two different methods only show agreement if the points lie along the line of equality, but they will have perfect correlation if the points lie along any straight-line. Secondly, a change in scale of measurement does not affect the correlation, but it certainly affects the agreement. Thirdly, correlation depends on the range of the true quantity in the sample. If this is wide, the correlation will be higher than if it is narrow. Fourthly, the test of significance may show that the two methods are related, but it would be surprising for two methods designed to measure the same quantity to be not related. The test of significance is irrelevant for the question of agreement. At least, data which seem to be in poor agreement can produce quite high correlation. Thus, we compared our GC-MS method statistically using both correlation and statistic methods according to Passing Bablok regression and Bland and Altman statistics.

The accuracy of our newly established GC-MS methodology for the determination of plasma tHcy concentrations was compared with an HPLC technique (controlled by ERDIM) and an immunofluorescence IMx assay normally used in clinical laboratories. Both comparisons showed good agreement and highly significant correlations for GC-MS *vs.* HPLC and GC-MS *vs.* IMx, though tHcy concentrations determined by GC-MS were on average slightly lower compared to the other methods. Comparison of homocysteine concentrations in the normal range (healthy volunteers) between GC-MS and IMx also revealed lower concentrations measured by GC-MS. Discrepancies in the results using different methods can be explained by the use of different calibration standards (GC-MS/HPLC) and/or by interferences described for immunoassays (Nevo *et al.*, 2000). IMx analysis is an important issue in clinical studies of homocysteine and in routine clinical chemistry. The use of the GC-MS method should be preferred in studies investigating homocysteine metabolism, i.e. simultaneous measurement of homocysteine as well as related amino acids.

Comparison of HPLC and IMx with internal standardization was excellent for the range of the fully automated Abbott homocysteine assay as shown by Pfeiffer *et al.* (1999a).

Most of the HPLC methods used in different laboratories showed higher concentrations in comparison with GC-MS methods (Pfeiffer *et al.*, 1999b). However, it was found from this international study with 14 laboratories participating, that laboratories whose results did not agree well with the GC-MS results usually also show higher within-run imprecision and lower and more variable recoveries. Comparison of HPLC and GC-MS method by Ubbink *et al.* (1999) showed higher tHcy concentrations measured by GC-MS. Both chromatographic methods used independently prepared calibrators. The authors explain the lower tHcy concentrations observed with HPLC by light destruction of their homocysteine derivative.

6.3 Homocysteine Metabolism in ESRD Patients

We found that, despite substitution of vitamin B_6, patients on chronic renal disease have markedly elevated plasma concentrations of tHcy compared with normal values (< 15 µmol/l) from the literature. This finding supported the conclusions of Chauveau *et al.* (1996) who reported that pyridoxine does not appear to enhance the homocysteine lowering effect in dialysis patient. Homocysteine is mainly eliminated and metabolized by the renal system. Guttormsen *et al.* (1997) suggested that renal uptake and metabolism can account for approximately 70% of the daily tHcy elimination from plasma. In ESRD patients which have lost their renal function, the catabolism of homocysteine seems to be disturbed.

The concentration of cysteine in our study is slightly higher than normal and shows a significant positive correlation with homocysteine. However, normal concentrations of plasma methionine were found and showed no correlation with homocysteine.

In our investigation, we detected the strength of the relationship between tHcy and folate and vitamin B_{12} . This finding is similar to others for serum folate levels in the ESRD population (Bostom *et al.*, 1995a and 1995b; Robinson *et al.*, 1996; Moustapha *et al.*, 1998). The close association of high homocysteine levels and low folate stores is consistent with the route of metabolism. High homocysteine levels were found despite universal supplementation with folic acid at the recommended daily allowance. This suggests a requirement for a high-dose folic acid supplementation to reduce homocysteine levels. Recently, two randomized double-blind trials showed that oral folic acid, even in doses up to 60 mg/day, allowed normalization of hyperhomocysteinemia in only 30% of hemodialysis patients (Bostom *et al.*, 1996; Sunder-Plassmann *et al.*, 2000), however, with no clear dose-response relationship. The effect of cobalamin was also examined by den Heijer *et al.* (1998) who found no additional

lowering of homocysteine levels with a multivitamin preparation including cobalamin compared to folic acid alone.

The amino acid pattern indicated that in ESRD patients, substituted with high vitamin B_6-plasma levels, homocysteine transsulfuration appears to be increased parallel to increasing homocysteine concentrations and that most homocysteine is converted to cysteine (vitamin B_6-dependent), while remethylation and the transmethylation pathway to methionine (folate- and vitamin B_{12}-dependent) seem not to be migrated. The lack of correlation between homocysteine and methionine could be explained by the assumption that remethylation is not the only source of plasma methionine. Decreased homocysteine remethylation is possibly the main cause for a disturbed homocysteine metabolism in hemodialysis patients.

In ESRD-patients, age and male sex, as the usual risk factor for homocysteine-elevation, can be ignored. The correlation between tHcy and serum creatinine reflects the influence of renal (rest)-function and/or the dose and intensity of dialysis treatment on homocysteine concentration. Moreover, it can be a result of the association between homocysteine production and creatine-creatinine synthesis (Jacobsen, 1996).

Chronic dialysis patients have very high mortality rates, primarily due to excess vascular disease, while other traditional atherosclerotic risk factors are prevalent in this population. The high levels of homocysteine are also known as an independent risk factor for vascular disease in this population and might play an important role in the progress of atherosclerosis in patients with renal disease. In a retrospective study performed with patients suffering from renal failure, plasma levels of homocysteine were independently associated with vascular disease. However, another publication did not support this results (Bostom *et al.*, 1996b). Prospective studies are now appearing, confirming elevated homocysteine as a marker for cardiovascular morbidity and mortality in ESRD patients under dialysis treatment (Moustapha *et al.*, 1998). All these findings cannot easily explain the high mortality and relationship between hyperhomocysteinemia and the onset of atherosclerosis. So far, the mechanism remains unclear. Furthermore, many researcher investigated the effect of a homocysteine lowering therapy, such as B vitamin and folate, in reducing plasma tHcy in these patients and also in minimizing the progression of cardiovascular disease.

6.4 Effect of Leucovorin and Folic Acid on Total Plasma Homocysteine in ESRD Patients

Our study shows that intravenously applicated Leucovorin (N^5-formyltetrahydrofolate) is not superior to folic acid, given intravenously, in the reduction or normalization of hyperhomocysteinemia in hemodialysis patients. Despite substitution with Leucovorin and folic acid, 71.2% of our ESRD patients remained hyperhomocysteinemic (tHcy > 15 µmol/l).

The relatively poor response of folic acid supplementation in hyperhomocysteinemic patients with renal disease possibly results from abnormalities in the utilization of folate. Folic acid is a complex mixture of polyglutamate conjugate compouds with variable numbers of glutamate molecules covalently linked to the folate moiety. Folic acid is initially deconjugated in the cells of the intestinal wall to its monoglutamate form by γ-glutamyl-carboxypeptidase or conjugase. The monoglutamate forms are rapidly absorbed through an active transport system in the intestine, mediated by a membrane protein, the folic acid-binding protein, and taken-up into the portal circulation. In the liver, these compounds are reduced to dihydrofolate and subsequently to tetrahydrofolate (THF) via folate and dihydrofolate reductase. The next step is the conversion of THF to 5,10-methylenetetrahydrofolate (5,10-METHF) using serine as a major carbon source and pyridoxal phosphate (B_6) dependent serine hydroxymethyl-transferase as enzyme. A portion of 5,10-METHF thus produced undergoes irreversible enzymatic reduction to 5-MTHF by methylene-tetrahydrofolate reductase MTHFR. After 5-MTHF has been synthesized in the liver, this metabolically active compound is secreted into the small intestine with bile, where it is reabsorbed and distributed to tissues throughout the body. The cellular uptake of 5-MTHF is in part carried out by an active transport system, mediated by a membrane folate receptor. The 5-methyl group of 5-MTHF can be transferred to homocysteine via methionine synthase resulting in the regeneration of methionine and THF (Durand *et al.*, 1998) (see appendix, figure A5).

Up to now, the folate metabolism has not been well evaluated. However, some publications showed a reduction of intestinal absorption of 5-methyltetrahydrofolate in uremic rats (Said *et al.*, 1984) and humans (Retief *et al.*, 1977). Using uremic rats, Said *et al.* (1984) reported that predialysed serum inhibited the absorption of 5-methyltetrahydrofolate via jejunal sacs in rats. Other potential disturbances of folate metabolism are the impairment of transmembrane folate transport by anions retained in uremia and/or the existence of pathological folate-binding proteins in uremic patients with megaloblastic anemia,

presumably decreasing the free folate fraction. Livant *et al.* (1994) found that plasma folate conjugase activity is reduced in hemodialysis patients by the presence of a plasmatic inhibitor. Such inhibition of plasma folate conjugase activities might impair the cleavage of polyglutamate forms of folate into monoglutamate.

Because of abnormalities of folate metabolism, administration of reduced folate derivatives may improve hyperhomocysteinemia in renal disease more effectively than natural folic acid. Perna *et al.* (1997) reported the reduction of plasma tHcy levels in uremic patients after oral treatment with 5-methyltetrahydrofolate (5-MTHF). They stressed the importance of 5-MTHF in reducing tHcy improving the ratio S-adenosylmethionine to S-adenosylhomocysteine. However, another publication by Bostom *et al.* (2000) did not support this finding. In this controlled study shows that oral administration of 5-MTHF or folic acid has a similar effect on reducing tHcy levels in hemodialysis patient.

Another reduced folic acid derivative, N^5-formyltetrahydrofolate (Leucovorin), is an immediate precursor of 5,10-METHF. It is directly converted to 5,10-METHF by an ATP-dependent enzyme, 5,10-METHF synthetase. Oral administration of N^5-formyltetrahydrofolate bypasses the deconjugation and reduction steps required for the synthesis of folic acid. The major proportion of 5-formyltetrahydrofolate is metabolized to 5-MTHF during intestinal absorption. Recently, in an uncontrolled retrospective study, administration of intravenous N^5-formyltetrahydrofolate during intravenous vitamin B_6 substitution resulted in the normalization of hyperhomocysteinemia in 78% of hemodialysis patients (Touam *et al.*, 1999). This study supports the hypothesis described above.

This early finding by Touam *et al.* could not be confirmed by our intervention study using Leucovorin or folic acid. We found that plasma concentration of tHcy were reduced after four and eight weeks of intravenous administration of Leucovorin or folic acid. Concentrations of plasma tHcy (tHcy < 15 µmol/l) were normalized in hyper-homocysteinemic patients after four weeks of treatment following reduction by 23.1% in six patients and by 17.4% in four patients and after eight weeks in eight patients after reduction by 27.6% and in nine patients by 30.3% in the Leucovorin and folic acid group, respectively. The reason for this discordance between our and Touam's results could be the supportive effect of intravenous vitamin B_6 in the study of Touam *et al.* in normalizing hyperhomocysteinemia. Our hemodialysis patients also received oral vitamin B_6, though in a minor doses as standard therapy in patients under chronic dialysis. However, other studies showed that oral vitamin B_6 supplementation has no major effect on hyperhomocysteinemia

in hemodialysis patients (Arnadottir *et al.*, 1993) and subjects without renal failure (Homocysteine Lowering Trialist's Collaboration, 1998). The efficiency of intravenous folinic acid in combination with intravenous B_6 in normalizing tHcy levels could be related to the intravenous administration of both vitamins and may not be related to Leucovorin therapy alone.

Another possible cause for the limited response of tHcy concentrations on folic acid in uremic subjects is the accumulation of cysteinesulfinic acid (Suliman *et al.*, 1999) or S-adenosylhomocysteine (Perna *et al.*, 1995), which inhibits the conversion of homocysteine. S-adenosylhomocysteine is involved in the synthesis of 5-MTHF and in the activation of CBS; it may also have an inhibitory effect on methionine synthase (Finkelsteine *et al.*, 1974).

In our study, normalization of hyperhomocysteinemia appeared after intravenous application of folic acid (30 mg/week) for eight weeks in nine patients, 30.3% of all participating patients, may respond better to oral administration of folic acid which normalized tHcy levels in only two of 30 patients (van Guldener *et al.*, 1998). Another recent study reported that ora1 administration of folic acid (5 to 70 mg/week) normalized tHcy levels in only two of 14 patients (Arnadottir *et al.*, 2000). The similar 30 % normalization of tHcy levels in hemodialysis patients was reported using high dosages of folic acid (105 mg/week) (Bostom *et al.*, 1996; Sunder-Plassmann *et al.*, 2000).

Significantly high positive correlations were found between plasma concentrations of tHcy, methionine and cysteine both after four and eight weeks of treatment. In contrast, from our study with 131 ESRD patients (see section 5.1) a significant correlation was found only between tHcy and cysteine, and none with methionine. As discussed above (section 6.3), homocysteine transsulfuration in ESRD patients appears to be increased parallel to increasing homocysteine concentrations, while remethylation to methionine (folate- and vitamin B_{12}-dependent) seems to be disturbed. However, during treatment with Leucovorin or folic acid, for four and eight weeks, both, the transsulfuration and remethylation, seem to be increased parallel to increasing homocysteine concentrations. This indicates a response to the homocysteine change by folate supplementation, especially in the remethylation pathway which is folate- and vitamin B_{12}-dependent.

We also found a significant positive correlation between the percentage change of plasma tHcy and red blood cell folate, whilst a negative correlation between the percentage change of tHcy and methionine was found. This supports the above argument that reduction of plasma tHcy during folate therapy is followed by elevated plasma concentrations of

methionine. This reaction is folate- and vitamin B_{12}-dependent. A good response in tHcy reduction could be dependent on an increase of folate concentrations in red blood cells. The most probable mechanism by which folic acid supplementation reduces plasma tHcy concentration could be that folate enhances the remethylation of homocysteine to methionine after conversion to 5-MTHF. If folate leads to a decrease in tHcy levels via enhancement of remethylation, plasma methionine levels will be increased. Perna *et al.* (1997) also reported an increase in methionine and S-adenosylmethionine levels in hemodialysis patients, after supplementation with methyltetrahydrofolate, an active form of folate. However, rather weak effects were shown in the reduction of plasma tHcy by supplementation with vitamin B_{12}. No significant correlations with the percentage change of tHcy as well as methionine, cysteine and red blood cell folate could be observed.

Whether homocysteine reduction leads to reduced mortality or morbidity is not yet established in either ESRD or the general populations and many trials are in progress. Targeting subjects with a high incidence of hyperhomocysteinemia and with a high risk for cardiovascular events probably represent an important part of the strategy in the design of randomized clinical trials evaluating the reduction of tHcy. The renal population seems to be an “ideal population” to test the hypothesis that homocysteine-lowering therapies are effective in reducing the incidence of atherosclerotic events. Until the results of more definitive clinical trials are found to be consistent with those using surrogate markers of endothelial damage, a variety of approaches for vigorous treatment of high risk individuals has been suggested.

7 Conclusion

GC-MS proved to be a sensitive, specific and reliable method for the determination of total plasma homocysteine (tHcy) and its related amino acids such as methionine, cysteine and cystathionine, for the investigation of the homocysteine metabolism.

Despite vitamin B_6 substitution, ESRD patients requiring chronic maintenance hemodialysis have high plasma concentrations of tHcy which seems to be metabolized mainly within the transsulfuration pathway, while remethylation to methionine remains unchanged.

There was no difference in the reduction or normalization of plasma tHcy in ESRD patients under maintenance dialysis after intravenous administration of Leucovorin (N^5-formyltetrahydrofolate) compared to folic acid. The reduction of plasma tHcy under folate therapy was followed by elevation of the plasma methionine amount during folate- and vitamin B_{12}-dependent remethylation, while transsulfuration to cysteine, despite of B_6-substitution, was not effective.

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Appendix

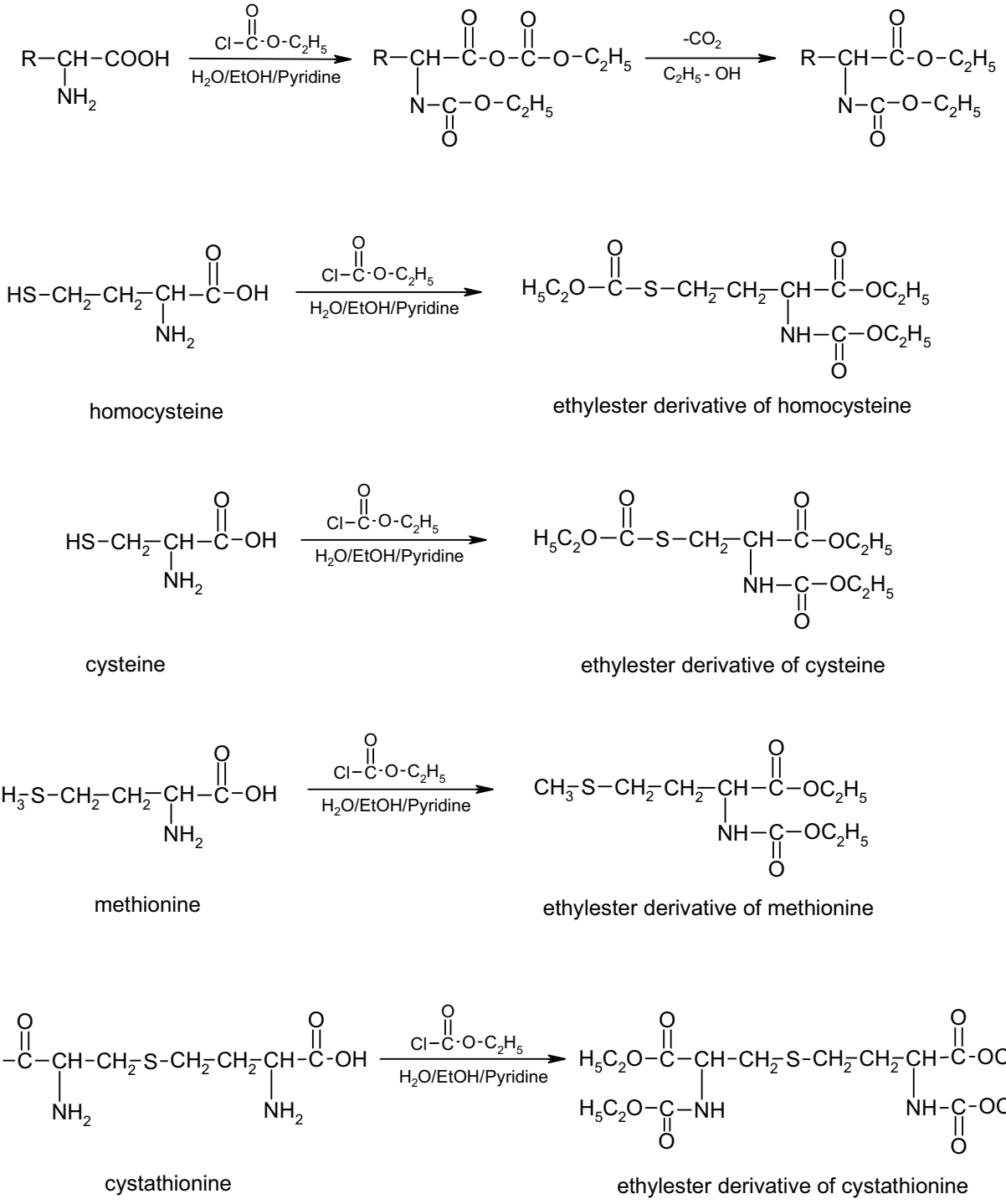


Figure A1 Mechanism of reaction for carboxyl group derivatization of homocysteine, cysteine, methionine and cystathionine reacting with ethylcholoroformate (ECF) in a medium of H_2O-Ethanol-Pyridine

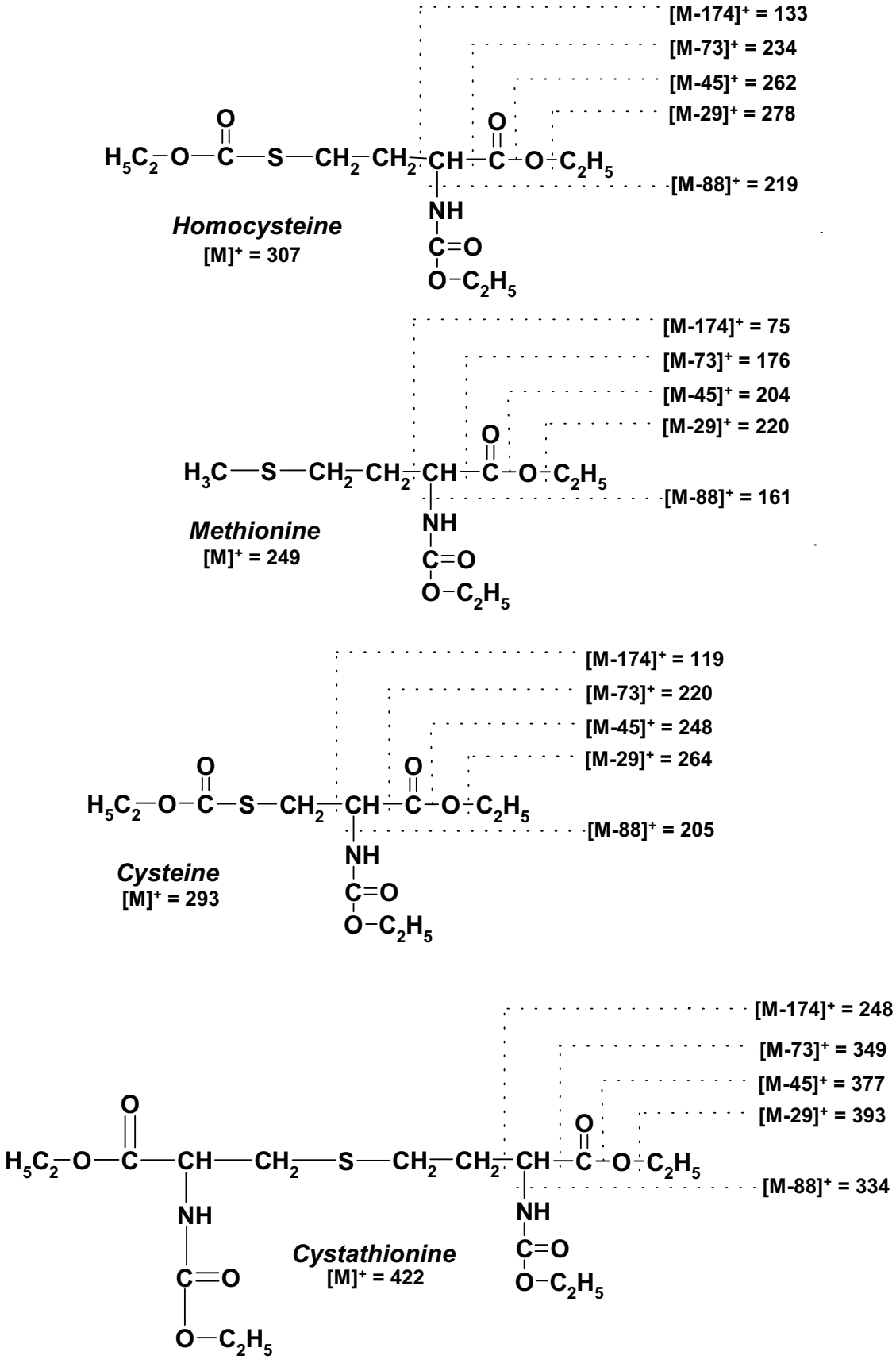


Figure A2 Mass fragmentation suggestions for ethylester derivatives of homocysteine, methionine, cysteine and cystathionine measured by electron impact mass-spectrometry

Table A1 Repeatability for the determination of total homocysteine, methionine, cysteine and cystathionine by GC-MS (N=6)

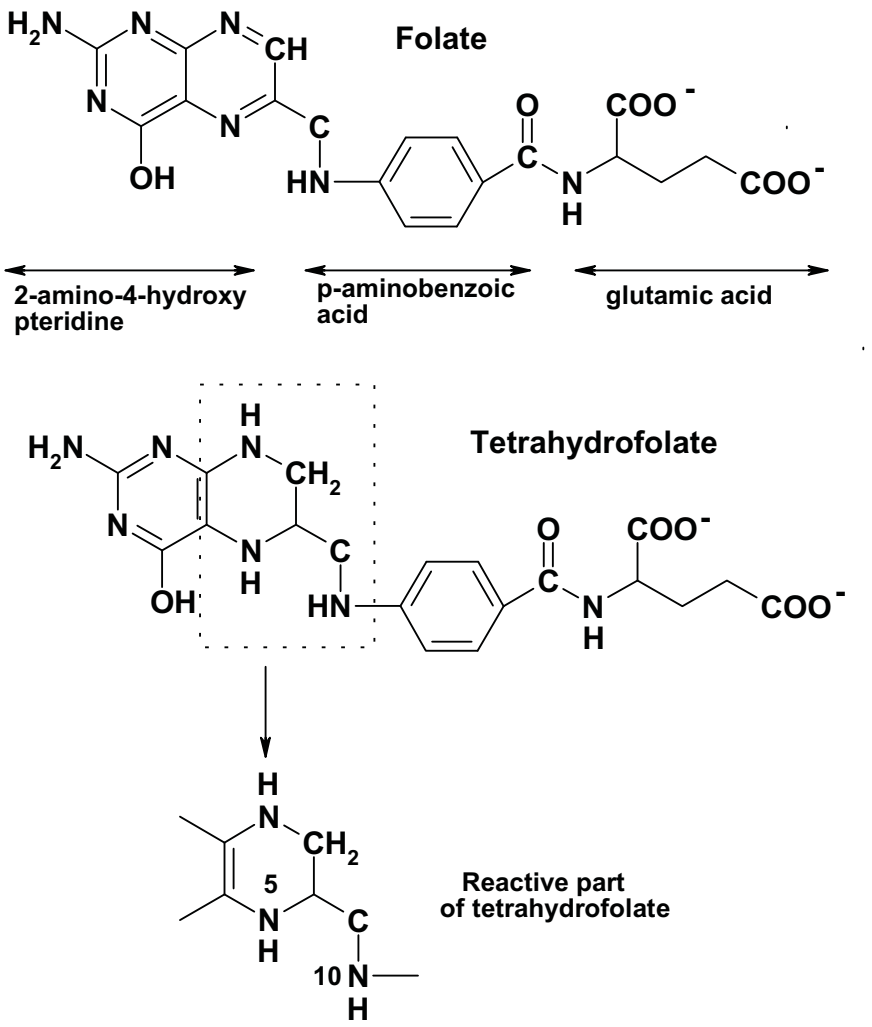
	Total Homocysteine (µmol/l)	**Methionine (µmol/l)**	**Cysteine (µmol/l)**	**Cystathionine (nmol/l)**
M1	13.51	17.73	384.88	344.97
M2	13.56	17.39	385.46	358.84
M3	13.44	17.25	384.23	363.94
M4	13.12	17.04	374.07	359.26
M5	13.50	17.58	386.34	365.16
M6	13.80	17.39	389.65	349.03
Mean	13.49	17.40	384.11	356.87
SD	0.22	0.24	5.27	8.14
CV	1.63 %	1.40 %	1.37 %	2.28 %

Abbreviations : M, measurement; SD, standard deviation; CV,coefficients of variation

Table A2 Recovery for homocysteine, methionine, cysteine and cystathionine by addition of homocystine, methionine, cystine and cystathionine to plasma

Added	Calculated	Observed	Recovery [%]
Homocysteine (nmol)			
None		5.80	
3.13	8.93	8.67	97.1
6.25	12.05	11.74	97.5
12.50	18.30	17.46	95.4
25.00	30.80	30.83	100.1
50.00	55.80	45.10	80.9
		Mean (SD)*	94.2 (7.6)*
Methionine (nmol)			
None		13.17	
3.13	16.29	15.36	94.3
6.25	19.42	17.02	87.6
12.50	25.67	19.79	77.1
25.00	38.17	35.89	94.0
50.00	63.17	51.07	80.8
		Mean (SD)*	86.8 (7.7)*
Cysteine (nmol)			
None		210.31	
15.63	225.93	222.79	98.6
31.25	241.56	239.94	99.3
62.50	272.81	267.46	98.0
125.00	335.31	371.73	110.9
250.00	460.31	393.77	85.5
		Mean (SD)*	98.5 (9.0)*
Cystathionine (nmol)			
None		0.63	
0.25	1.94	0.88	90.7
0.50	2.75	1.13	82.2
1.0	3.99	1.63	81.7
2.0	6.54	2.63	80.5
		Mean (SD)*	83.8 (4.7)*

* Mean (SD) : Mean (standard deviation)



Folate related compounds	Substituent at N-5	Substituent at N-10
Tetrahydrofolate	- H	- H
5 – methyltetrahydrofolate	- CH_3	- H
5, 10 – methylenetetrahydrofolate	- CH_2 -	- CH_2 -
5, 10 – methenyltetrahydrofolate	- CH = N^+ -	- CH = N^+ -
5 – formyltetrahydrofolate	- CHO	- H
10 – formyltetrahydrofolate	- H	- CHO
10 – formiminotetrahydrofolate	- H	- HCNH

Figure A3 Structure of folate, tetrahydrofolate and related compounds

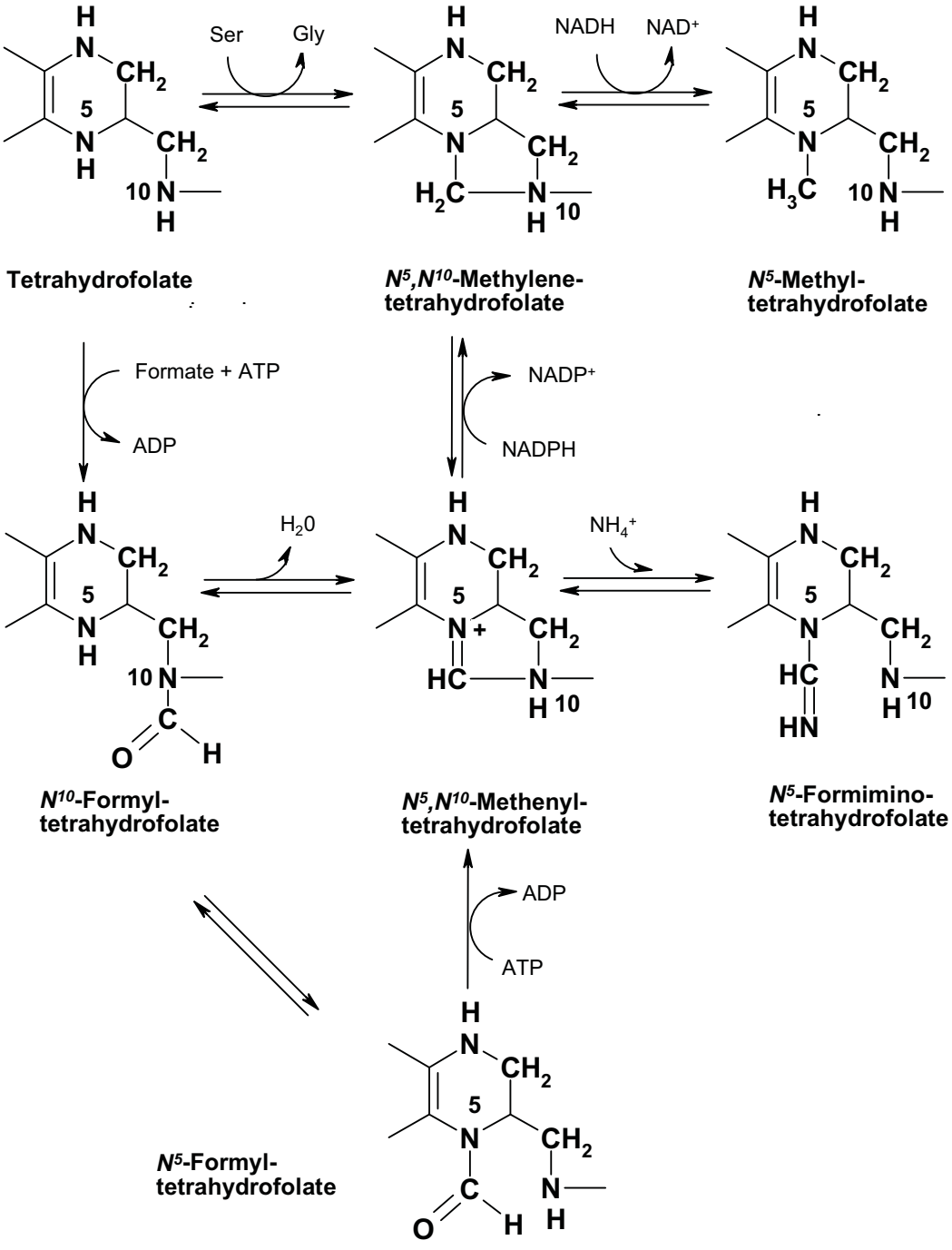


Figure A4 Conversions of one-carbon units attached to tetrahydrofolate

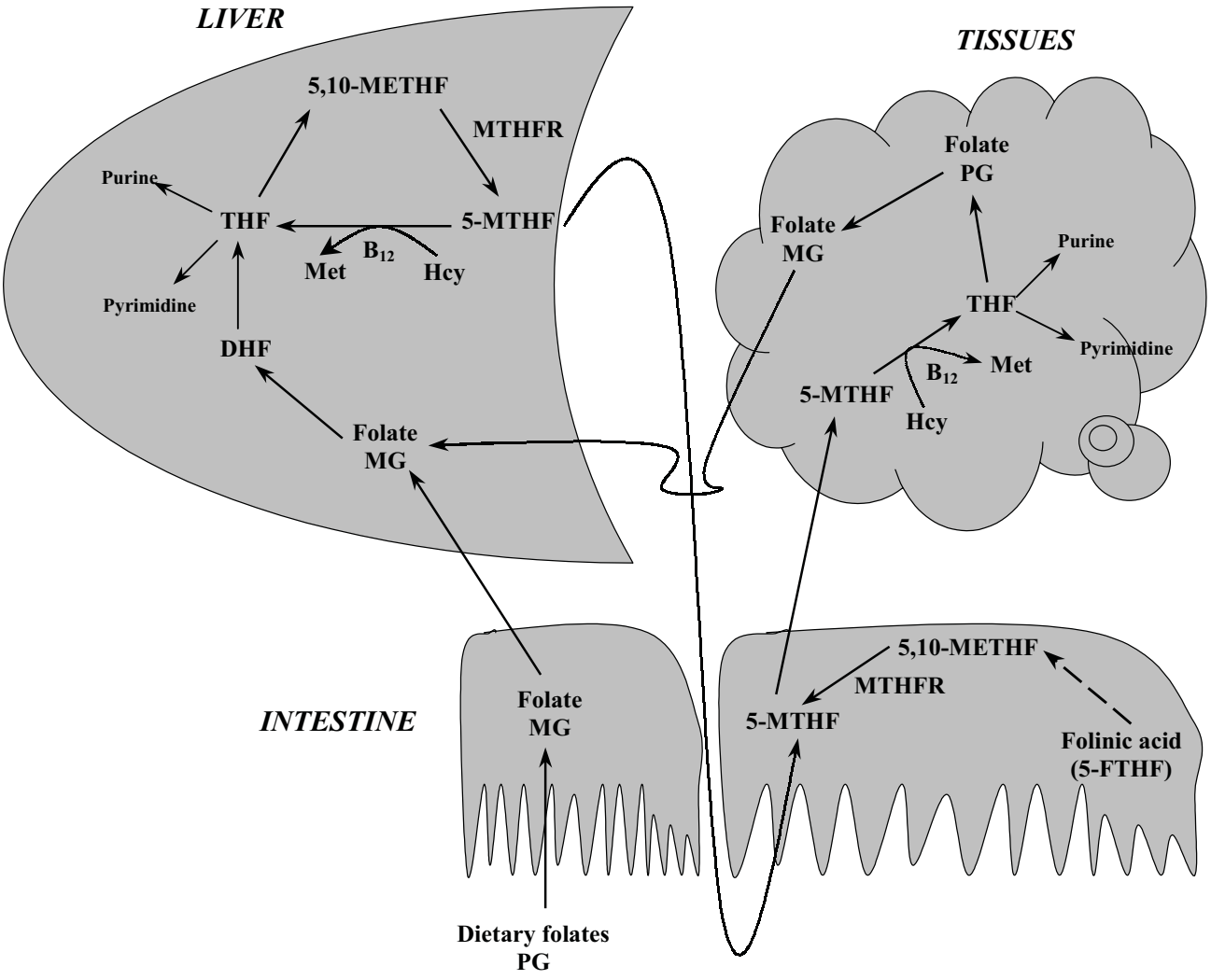


Figure A5 Scheme of folate metabolism

List of Publications and Presentations

Presentations

Brensing KA, Hages M, Lütjohann D, Sigit JI, Pietrzik K, von Bergmann K, Frotscher U. Homocysteine levels in hemodialysed patients assessed by immunofluorescence assay (IMx), HPLC and gas chromatography-mass spectrometry. Poster presented at Annual Congress of European Renal Association, European Dialysis and Transplant Association, Madrid, Spain, 5-8 September, 1999. Abstract : *Nephrol.Dial.Transplant.* 1999;14:(9), A160.

Sigit JI, Lütjohann D, Hages M, Brensing KA, Frotscher U, Pietrzik K, von Bergmann K. Homocysteine metabolism in patients with end-stage renal disease under maintenance dialysis. Poster presented at the XIIth International Symposium on Atherosclerosis, Stockholm, Sweden, 25-29 June, 2000. Abstract : *Atherosclerosis* 2000;151:(1), 253.

Lütjohann D, Sigit JI, von Bergmann K. Effect of high-dose simvastatin on plasma concentrations of homocyst(e)ine. Poster presented at the XIIth International Symposium on Atherosclerosis, Stockholm, Sweden, 25-29 June, 2000. Abstract : *Atherosclerosis* 2000;151:(1).

Sigit JI, Hages M, Brensing KA, Frotscher U, Pietrzik K, von Bergmann K, Lütjohann D. Homocysteine metabolism in patients with end-stage renal disease under maintenance dialysis. Poster presented at the 2nd Conference on Hyperhomocysteinemia, Saarbrücken, Germany, 30-31 March, 2001.

Publications

Lütjohann D, Sigit JI, Locatelli S, von Bergmann K, Schmidt HH. High-dose simvastatin (80 mg/day) decreases plasma concentrations of total homocyst(e)ine in patients with hypercholesteromia. *Atherosclerosis* 2001; 155:265-266.

Sigit JI, Hages M, Brensing KA, Frotscher U, Pietrzik K, von Bergmann K, Lütjohann D. Total plasma homocysteine and related amino acids in end-stage renal disease (ESRD) patients measured by gas chromatography-mass spectrometry--comparison with the Abbott IMx homocysteine assay and the HPLC method. *Clin Chem Lab Med* 2001; 39:681-690.

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Curriculum Vitae

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April 1993	"Sarjana –S1" degree (Drs., Doktorandus). Research title: The effect of the Decoctions of *Alstonia scholaris* Barks and *Allium sativum* on the Immune Response of Mice infected by *Ascaris suum* Worms
1993 – 1994	Professional school of Pharmacy at the Department of Pharmacy, Faculty of Mathematic and Natural Science, Institut Teknologi Bandung
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