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N-METHYLNICOTINAMIDE AS MARKER FOR BIOLOGICAL METHYLATION IN HUMANS

BY

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SUMMARY

N-METHYLNICOTINAMIDE AS MARKER FOR BIOLOGICAL METHYLATION IN HUMANS

The purpose of this study was to determine whether the methylation of nicotinamide to N-methylnicotinamide could discriminate between differences in methionine nutritional status, and by implication methylation capacity, in healthy humans.

As part of this thesis, a highly selective high performance liquid chromatography (HPLC) method for the determination of N-methylnicotinamide (NMN) in urine and plasma was developed and validated. Quantification was by fluorescence detection of the 1,6-naphthyridine derivatives, formed after incubation of NMN with acetophenone in alkaline conditions.

Seven volunteers participated in a trial to evaluate the ability of a nicotinamide load test to discriminate between changes in the methylation status of the individual. The methylation status was measured as the time dependent changes in plasma NMN concentrations after a nicotinamide load. A basal nicotinamide load test was performed on each individual. The methylation status was then changed, by means of a methionine load, and the nicotinamide load test was repeated during the enhanced methylation state.

The dynamic changes in N-methylnicotinamide levels indicated that the methionine load changed neither the plasma NMN concentrations, nor the rates of NMN formation.

The conclusion of this study was that nicotinamide loading could not be used as a dynamic function test to assess biological methylation in healthy humans.

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CHAPTER 1

BIOLOGICAL METHYLATION

1.1 INTRODUCTION

In the methylation pathway the amino acid methionine is metabolized, via the formation of S-adenosylmethionine, to homocysteine. Homocysteine, in turn, can be re-methylated back to methionine, completing the methionine cycle. The methylation pathway spans two principal areas of metabolism – firstly the utilization of the intermediate product S-adenosylmethionine as the single most important methyl donor group in the human body, and secondly, the re-methylation of homocysteine to form methionine. S-adenosylmethionine methylation reactions are mediated by numerous enzymes and are essential for the formation of many biologically active compounds such as hormones, neurotransmitters, proteins and phospholipids.

Various vitamins are used as co-substrates or co-factors in the methylation pathway, making this pathway susceptible to nutritional deficiencies. Heterozygous or homozygous mutations in genes coding for enzymes that participate directly or indirectly in methylation metabolism, can interact with inadequate dietary intake to exacerbate the metabolic pathology. Gene-nutrient interactions that compromise normal methylation are associated with increased risk of cardiovascular disease [1], certain cancers[2], birth defects[3], recurrent early pregnancy loss[4], central nervous system demyelinization[5] and neuropsychiatry disorders[6]. In many cases, appropriate nutritional intervention can normalize the metabolic imbalance, or retard the progression of the pathology.

S-Adenosylhomocysteine, the intermediate product formed after the transfer of the methyl group from S-adenosylmethionine, is a potent competitive inhibitor of all the methyltransferase enzymes. The relative changes in the *S*-adenosylmethionine/*S*-adenosylhomocysteine ratio may be an important predictor of the methylation potential of cells, and as such, may reflect various metabolic alterations associated with disease.

Due to the complexity of one-carbon metabolism and the multitude of enzymes involved, direct measurement of the methylation capacity may have advantages over the measurement of individual components of the pathway. Measurements of such a marker for methylation capacity may lead to critical insight into the origin of aberrations associated with impaired methylation, like hyperhomocysteinemia, neural tube defects and certain neurological disorders[7].

1.2 RESEARCH OBJECTIVES

As already mentioned, S-adenosylhomocystein is a potent competitive inhibitor of all the methyl transferase reactions, where a methyl group is transferred from S-adenosylmethionine. These transmethylation reactions are controlled through various mechanisms, including the adequate supply of methionine from the diet and re-methylation of homocysteine, to ensure sufficient S-adenosylmethionine for the maintenance of the methylation capacity. It also requires effective removal of homocysteine through transsulfuration and re-methylation to methionine to drive the reaction kinetics towards adenosylhomocysteine hydrolysis. An increase in homocysteine concentration will therefore lead to an accumulation of intracellular S-adenosylhomocysteine and a decrease in the methylation potential that, in turn, will inhibit transmethylation enzyme reactions.

Inhibition of the transmethylation reactions can lead to various deficiencies associated with disease. The extent to which environmental factors and genetic predisposition can interact to cause pathologies associated with decreased methylation capacity is still unclear, but the identification of biological markers that can measure the exposure directly, will probably lead to critical insight.

The aim of this study is to investigate a possible marker for biological methylation in healthy humans that can be used to study aberrations of methylation capacity. Such a marker must reflect changes in methylation capacity that can, for instance, be induced by an oral methionine load. N-methylnicotinamide is the product formed after the transfer of a methyl group from S-adenosylmethionine to nicotinamide. Results from other studies, which will be discussed in Chapter 2, indicated that the measurement of N-

methylnicotinamide, with or without an oral load of nicotinamide, shows promise as a parameter of methylation.

The main research question that will be addressed in this study is the following:

Can the measurement of N-methylnicotinamide, after an oral load of nicotinamide, be used as a marker for biological methylation capacity in healthy humans?

The main objective of this study is therefore to determine whether the methylation of nicotinamide to N-methylnicotinamide can discriminate between differences in methionine nutritional status, and by implication methylation capacity, in healthy humans. The approach used will be to measure the time dependent increase in N-methylnicotinamide after an oral load of nicotinamide in an analytical study, where each participant will serve as his/her own control. The methylation pathway will then be stressed by means of a methionine load before a second nicotinamide load, and the formation of N-methylnicotinamide will be compared to that during the first load.

The objectives of the study are the following:

1. To develop and establish an analytical method for the determination of N-methylnicotinamide.
2. To standardize the nicotinamide load test and sampling procedure for possible routine investigations.
3. To relate time dependent changes in N-methylnicotinamide concentrations, before and after a methionine load, to methylation capacity

CHAPTER 2

LITERATURE REVIEW

2.1. THE PHYSIOLOGICAL ROLE OF METHYLATION REACTIONS

The methyl group of methionine is utilized as a precursor for the formation of many different methylated bio-molecules, some of which are listed in Figure 1, together with their methylated products.

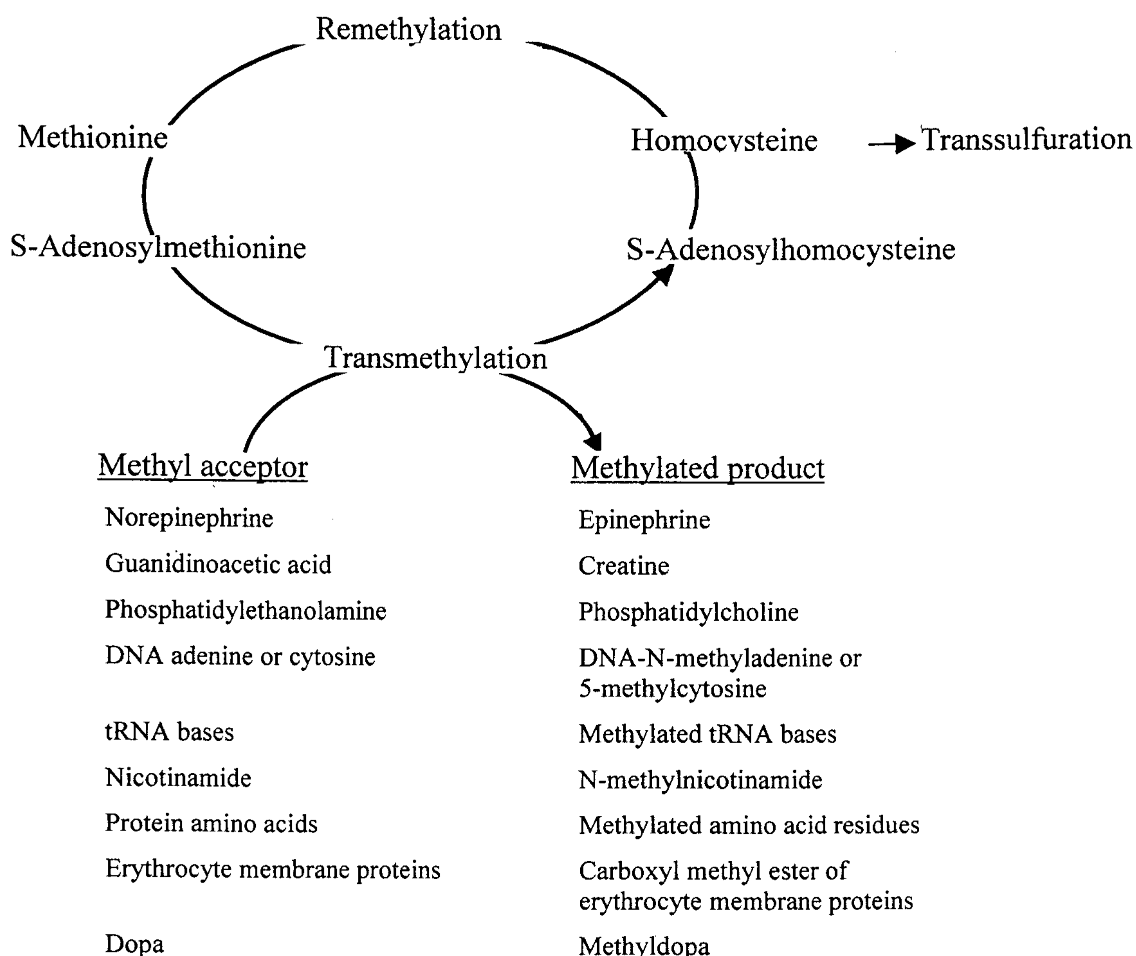

Figure.2.1 Biological important *S*-adenosylmethionine dependent transmethylation Reactions (Mathews and van Holde[7] 1990)

Although methylation does not always result in known physiological functions, these reactions are essential for normal physiological processes. The functions of some of the methylation reactions will briefly be discussed in this section.

The formation of creatine-creatinine utilizes most of the methyl groups derived from methionine, and uses more S-adenosylmethionine than all the other transmethylase reactions together[8]. Creatine and phosphocreatine play important roles in the storage and transmission of phosphate bond energy and is the major energy source for muscular contractions

Creatine is synthesized from arginine, glycine and methionine, after transmethylation of guanidinoacetic acid, an intermediate of the reaction [8]. Male subjects use more methyl groups than can be supplied as methionine in a normal diet and the short-fall in methyl groups are made up from two other sources: betaine and tetrahydrofolate. Betaine is derived from the diet and is converted to choline, which provides methyl groups for the conversion of homocysteine to methionine. The tetrahydrofolate pathway also supplies methyl groups for the conversion of homocysteine to methionine.

Norepinephrine and epinephrine are hormones secreted by the adrenal medulla and functions physiologically in the regulation of heart rate and blood pressure. Epinephrine is an activator of glycogen breakdown in liver and muscle via its stimulation of adenylate cyclase. Epinephrine is formed after transmethylation of norepinephrine, with the methyl group donated by S-adenosylmethionine.

Protein methylation is correlated with cell growth and differentiation, with hypo-methylation leading to structural abnormalities. Methylation of the amino- or carboxy-terminal of a protein can lead to alterations in steric orientation, charge or hydrophobicity and may lead to a global effect on molecules. Methylation of specific sites in the protein structure may therefore be essential for the functionality of the protein. For instance, proteins are methylated as part of the repair mechanisms after being exposed to environmental stresses. These repair functions have been ascribed to the carboxymethylation of D-aspartate or D-isoaspartate groups that arise from spontaneous degradation during protein aging[9].

S-adenosylmethionine is used in the production of putricine, which is subsequently involved in the manufacturing of polyamines[10]. The polyamines spermine and spermidine are ubiquitous structural components of all eukaryotic cells and serve to stabilize membrane structures and DNA molecules. It is also involved in immunological responses via the activation of macrophages and lymphocytes[11]. These compounds are incorporated into macrophages where it restrains the innate immunological response, thus activating the anti-inflammatory process. The precise role of phospholipid methylation in mammalian cells is still uncertain, but methylation takes place via three successive transmethylation steps in the conversion of phosphatidyl-N-monomethylethanolamine to phosphatidylcholine. S-adenosylmethionine acts as the methyl donor for all three these conversions[10].

Methylation of cytosine in DNA results in 5-methylcytosine (mC), and has been related to a large variety of mammalian gene activities, somatic inheritance and cellular differentiation[12]. Because cytosine is the most unstable base pair in DNA, any mCpG site can be a mutation hot spot. DNA-binding proteins can up- or down regulate transcription by binding to specific mCpG loci in genes, thereby influencing transcription events. Methylation reactions are therefore essential for post-translational protein modifications and repair mechanisms and it is well known that hypo-methylation of DNA can lead to modifications in gene expression[13]. In RNA, the methylation of the 5'-terminal cap plays an important role in mRNA export from the nucleus, in efficient translation of mRNA and also in protecting the integrity of the molecular structure [12].

The discussion above outlines some of the important functions associated with the transfer of single methyl groups, indicating that any abnormality in this pathway will have a profound effect on many biological processes.

2.2. OVERVIEW OF THE METABOLIC PATHWAY OF METHYLATION

An overview of methionine metabolism with emphasis on methylation reactions is represented in Figure 2.2. Methionine is activated by ATP and converted to S-adenosylmethionine (AdoMet) by the enzyme methyladenosinetransferase (EC 2.5.1.6). AdoMet is subsequently converted to S-adenosylhomocysteine (AdoHcy) by a variety of methyltransferase enzymes, present in all cells.

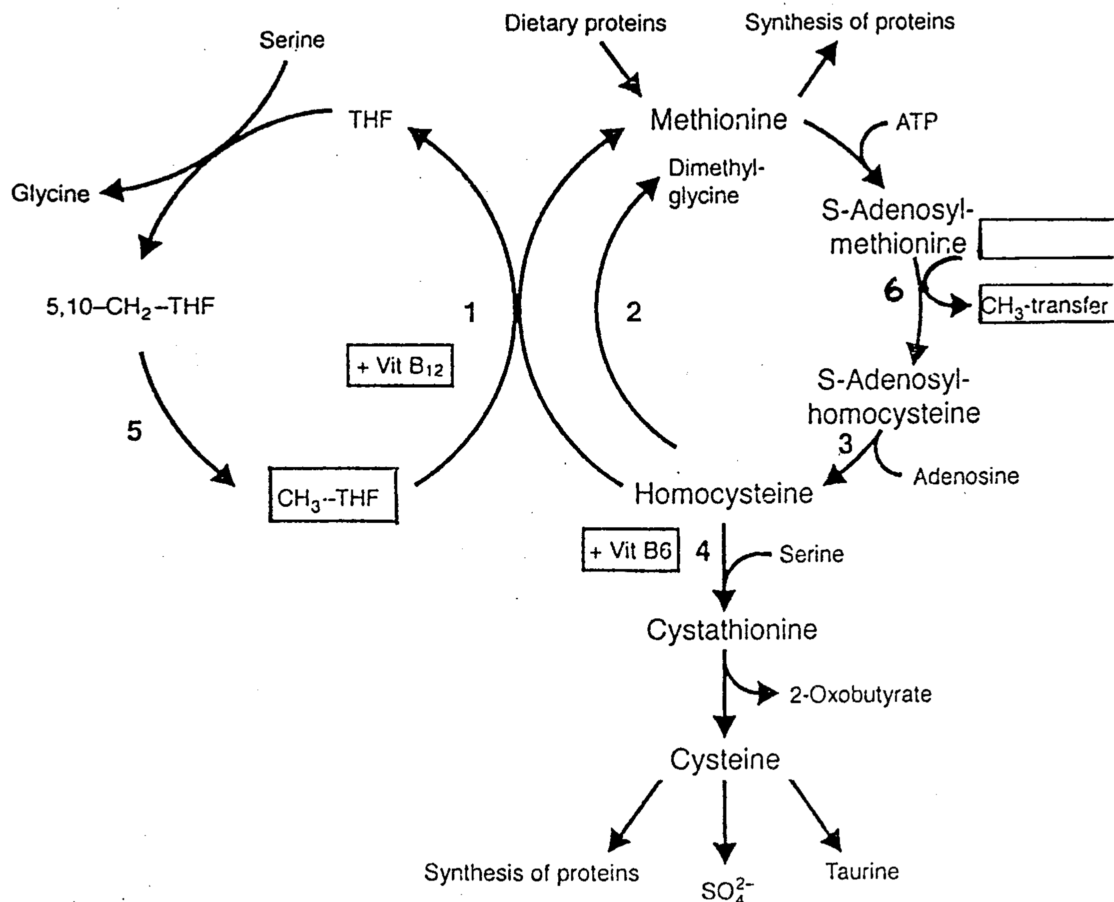

Figure 2.2: The metabolic pathway of methionine with emphasis on methyl group transfers (Adapted from Rasmussen and Moller[15])

S-Adenosylmethionine is the methyl donor in a wide range of transmethylation reactions. The transfer of the methyl group to an acceptor (**6**) *result in the formation of S-adenosylhomocysteine, which is subsequently, converted to homocysteine, by the enzyme* (**3**) *S-adenosylhomocysteine hydrolase (EC 3.3.1.1). In the transsulfuration pathway, homocysteine is condensed with serine to form cystathionine by the vitamin* B_6 *dependent enzyme* (**4**) *cystathionine B-synthase (EC 4.2.1.22). The enzyme* (**1**) *5-methyltetrahydrofolate:homocystein methyltransferase (EC 2.1.1.13), which uses vitamin* B_{12} *as a coenzyme, transfers a methyl group from methyltetrahydrofolate* (CH_3*-THF) to homocysteine to form methionine. 5-Methyltetrahydrofolate is formed by reduction of 5,10-methylenetetrahydrofoloate (5,10-*CH_2*-THF), by the enzyme* (**5**)*5,10-tetrahydrofolate reductase (EC 1.7.99.5) An alternative pathway for the methylation of homocysteine to methionine is mediated by the enzyme* (**2**) *betaine:homocysteine methyltransferase (EC 2.1.1.5) using betaine as a methyl donor.*

AdoMet as the major source of methyl groups transferred during biological cellular methylation is subject to competitive product inhibition by AdoHcy. The pathological accumulation of AdoHcy will lead to a decrease of the AdoMet/AdoHcy ratio, which will in turn inhibit most of the cellular methyltransferase reactions. AdoHcy is hydrolyzed to homocysteine and adenosine in a reversible reaction with thermodynamics that favors AdoHcy synthesis. The reaction kinetics is driven towards AdoHcy hydrolysis only by the effective removal of homocysteine and adenosine from the cell.

Homocysteine is the intersection between two metabolic pathways - remethylation to methionine and transsulfuration to cysteine. Although methionine serves as a general methyl donor to many methyl group acceptors, the remethylation of homocysteine to methionine occurs only via the two pathways in Figure 2.2. The one pathway is via the transfer of a methyl group from *N*-5-methylterahydrofolate (CH_3-THF). The CH_3-THF in turn derives from a limited number of metabolites, capable of donating one-carbon functional groups to tetrahydrofolate, particularly serine and glycine. Remethylation of homocysteine can also occur via betaine. Betaine is derived from choline that is partly supplemented by diet and is also synthesized through successive methylation of phosphotidylethanolamine. The methylation reaction of betaine is independent of vitamin B_{12} whereas the reaction with CH_3-THF is catabolized by the vitamin B_{12} containing enzyme CH_3-THF:homocysteine methyltransferase[15].

In the transsulfuration pathway, homocysteine condenses with serine to form cystathionine, in an irreversible reaction catabolized by the vitamin B_6 dependent enzyme cystathionine-*B*-synthase (CBS). Cystathionine is hydrolyzed to cysteine and excess cysteine is oxidized to taurine, and eventually to inorganic sulfates. The transsulfuration pathway effectively removes toxic homocysteine that is not needed for re-methylation.

2.3. REGULATION OF THE METHYLATION PATHWAY

Selhub et al[16] postulated a coordinate regulatory role for S-adenosylmethionine in the partitioning of homocysteine between re-methylation and transsulfuration. The kinetics of the reaction catalysed by S-adenosylhomocysteine hydrolase:
S-adenosylhomocysteine ↔ adenosine + homocysteine, favours the formation of S-adenosylhomocysteine and is driven to the right only by the continual removal of

homocysteine. When impaired homocysteine removal persists, the cell's export mechanisms leads to the disposition of homocysteine into the circulation to prevent toxicity to the cell, causing homcysteinemia and homocysteinuria. The ability of AdoMet to act as an enzymatic effector provides a means by which transsulfuration and re-methylation can be coordinated. When cellular AdoMet concentrations are low, CBS will be suppressed and the synthesis of CH_3-THF will proceed uninhibited and will lead to the production of methionine. Conversely, when cellular AdoMet is high, inhibition of CH_3-THF synthesis is accompanied by the diversion of homocysteine through the transsulfuration pathway because CBS is activated. The primary effect of this coordinate control is the regulation of cellular AdoMet concentrations, and also the maintenance of homocysteine to provide methyl groups for transmethylation as dietary sources alone cannot supply in the demand for methionine. Homocysteinemia is thus seen as a condition where the cell is no longer capable of coordination between the two pathways. According to Selhub's theory, the decreased availability of CH_3-THF in individuals with vitamin B_{12} deficiencies or defective CH_3-THF-reductase, will result in a decreased synthesis of methionine, and therefore also in decreased cellular AdoMet concentration. Cellular AdoMet concentrations will then be too low to stimulate CBS and this will lead to accumulation of homocysteine. This theory might explain the decrease in homocysteine levels in patients with homocysteinemia, after receiving vitamin supplementations.

Loerhrer et al (1997)[17] investigated the proposed inhibition of CH_3-THF by elevated AdoMet concentrations as suggested by Selhub. They measured levels of S-adenosylmethionine, S-adenosylhomocysteine, homocysteine and CH_3-THF in humans after administration of an oral load of AdoMet. Their observations suggested that the increased AdoMet obtained with the load administered did not inhibit the CH_3-THF activity, suggesting that the control mechanism is probably far more complex than described by Selhub. Little is known about the influence of enzymatic and vitamin deficiencies on intracellular AdoMet and homocysteine concentrations, and whether it is increased before the onset of homocysteinemia.

Another factor that contributes to the complexity of the control mechanism of this pathway is the structural composition of S-adenosylmethionine itself[10]. AdoMet exists as two sterioisomers, (S, S)-AdoMet and (R, S)-AdoMet, with the chirality's at the

sulfonium centre an important determinant of biological activity. While the (S, S) enantiomer is the biologically active form, (R, S)-AdoMet is a potent inhibitor of methylases and constitutes about 3% of the total AdoMet found in mouse liver. AdoMet formation from methionine is catalysed by AdoMet synthase, with multiple forms of AdoMet synthase, encoded by distinct genes and found in different tissues. The various AdoMet synthase isoenzymes differ in their physical and kinetic properties, and have non-identical subunits that can exist in aggregates, contributing to the complexity of its regulation. Unlike adenosylhomocysteine hydrolase, chemical agents do not easily inhibit AdoMet synthase. This complexity suggests an important role for AdoMet in the control mechanisms that exist in different tissues.

It is also likely that the regulatory importance of S-adenosylhomocysteine may be underestimated. AdoHcy hydrolase deactivation and AdoHcy accumulation are potent inhibitors of normal methylation reactions. The deletion of AdoHcy hydrolase gene in mouse embryos results in death, thus showing the indispensable importance of this gene[18]. AdoHcy hydrolase is also an indirect target for the inhibition of methylation by many nucleosides with potentially many therapeutic applications[19]. Under normal physiological conditions, AdoHcy concentrations are normally several times lower than AdoMet concentrations[20]. Alterations in cytosolic AdoHcy have tissue specific bio-regulatory functions and as already mentioned, have been reported to up-regulate CBS activity, decrease betaine-homocysteine methyltransferase activity, and decrease CH_3-THFR activity[21]. The inhibition of methyltransferase reactions by AdoHcy would thus be expected to spare the substrate AdoMet

It is clear from the results of these studies that the methylation capacity will be reduced by either a rise in AdoHcy or a decrease in AdoMet concentrations. Whether this capacity is influenced by, or exerts an influence on the availability of methyl group acceptors of this pathway, must still be investigated.

2.4. DISEASES OF IMPAIRED METHYLATION CAPACITY

Impaired methylation capacity can cause various neuro-psychiatric disorders and is associated with cognitive deficits, depression and cerebro-vascular disease[22]. AdoMet levels increase in cerebrospinal fluid after oral/intravenous dosage and it is therefore postulated that AdoMet can cross the blood-brain barrier[25]. Whether living cells take up AdoMet intact is however still controversial. Van Phi et al[26] found incorporation of [^{3}H]-methyl groups from exogenous AdoMet into the phospholipids of plasma membranes of rat hepatocytes, but not into intracellular RNA. AdoMet may actually exert a biological effect at membrane level without entering the target cell.

Sargent et al[23] reported that schizophrenia might involve aberrations in methylation capacity and found that the rate and total expiration of labelled CO_2 (derived from labelled methionine) are three times less in schizophrenic patients than in controls, with no overlap of data points between the two groups. AdoMet has also been used as a therapeutic agent for the treatment of psychiatric depression, although the exact antidepressant mechanism is unknown. Animal studies showed that the antidepressant effect might be due to activation of the central serotonergic system with an increased turnover of norepinephrine, serotonin and dopamine[24].

Parkinson's disease is also associated with disturbed methylation capacity. Cheng et al[27] reported a significant difference in AdoMet concentrations and methyladenosyl transferase (MAT) enzyme activity between Parkinson's patients and matched controls. AdoMet concentrations in whole blood from patients were 50% lower than in controls and the MAT catalytic activity about 30% higher in patients.

Elevation in plasma concentrations of homocysteine is also associated with impaired methylation and is a known risk factor for vascular disease. Genest et al[28] showed that patients with premature cardiovascular disease have significantly higher plasma homocysteine concentrations than matched controls, and found no correlation between plasma homocysteine and other indices of cardiovascular disease e.g. serum cholesterol, VLDL, HDL or trigliserides, indicating that hyperhomocysteinemia is an independent risk factor for cardiovascular disease. Brattström et al[29] reported that 28-36% of patients

with early onset cerebral or occlusive arterial disease had hyperhomocysteinemia or evidence of impaired homocysteine metabolism.

Capdevila and Wagner[35], however, reported that the evidence for homocysteine being the toxic agent in vascular disease is weak. They stated that most of the in vitro studies done on the effect of homocysteine on clotting and thrombolytic mechanisms have used homocysteine values that are much higher than the values associated with increased risk factors (> 20μM). There is also no known metabolic pathway that is sensitive to elevated levels of homocysteine. AdoHcy, on the other hand, is a potent inhibitor of all AdoMet mediated methyltransferase reactions. Lee et al[36] have shown that low levels of homocysteine (10-50μM) inhibit growth of vascular endothelial cells provided homocysteine can be converted to AdoHcy in the cell. Therefore, although hyperhomocysteinemia is traditionally associated with many of the diseases of impaired methylation, the increased levels may only be symptoms of aberrations that originated in impaired methylation capacity.

Increased homocysteine concentrations can be caused by a variety of factors, both genetic (mutations in the gene coding for CH_3-THF reductase, which renders the enzyme more thermo lable), and nutritional (deficiencies of vitamin B_6, folate or vitamin B_{12}). Menlyk et al[30] determined changes in plasma and intracellular AdoMet and AdoHcy concentrations with alterations in homocysteine and pyridoxal phosphate (vitamin B_6 derivate) in 58 women. They found that plasma AdoMet/AdoHcy ratios were inversely correlated to homocysteine concentrations. In women with increased homocysteine, plasma AdoHcy was increased, AdoMet levels were not affected and pyridoxal phosphate concentrations were lower. They concluded that increased serum homocysteine is associated with an increase in AdoHcy and a decrease in the methylation ratio that could negatively affect the methylation potential.

Loehrer et al (1996)[31] investigated methylation in 70 patients with coronary artery disease and in matched controls. Fasting plasma homocysteine levels were elevated in 17% of patients, who also had lower CH_3-THF levels. Of the normal-homocysteine patients, 37% also revealed low CH_3-THF levels, suggesting that the hyperhomocysteinemia was not necessarily caused by a decreased CH_3-THF concentration. The AdoMet levels in all the patients were however significantly lower than in the control group and this was

independent of homocysteine and CH_3-THF levels. Their findings suggest that decreased levels of AdoMet may also pose a risk for the development of coronary artery disease.

Impaired methylation is also observed in patients with end-stage renal failure. A number of studies investigated plasma total homocysteine levels in patients with varying degrees of renal impairment and revealed elevated homocysteine levels in the majority of these patients[32, 33]. Loehrer et al (1998)[34] reported very low methylation ratios in patients with end-stage renal failure, mainly caused by a significant increase in AdoHcy. Loehrer suggested that the impairment of methylation might contribute to the neurological syndromes observed in renal failure, as a disruption in monoamine neurotransmitter production can cause depression, dementia and degeneration of the spinal cord.

2.4.1. Methylation Capacity and Associated Congenital Disorders

Homocysteinuria can be caused by deficiencies of three distinct enzymes: 5,10-methylene tetrahydrofolate reductase, 5-methyltetrahydrofolate:L-homocysteine methyltransferase and most commonly, cystathionine ß-synthase (CBS).

CBS catalyses the condensation of serine and homocysteine to form cystathionine. The inherited metabolic defect in CBS activity is clinically characterized by abnormalities of the ocular, skeletal, nervous and vascular systems with dislocation of lenses occurring in almost all untreated cases[37]. Biochemically, homocysteine and methionine levels are increased in body fluids with reduced levels of cysteine. About half of the patients show improvement in their biochemical abnormalities when treated with oral pyridoxine in doses ranging from 50 to 1000 times the recommended daily intake (1mg/day), with the highest response in older patients with mild enzyme deficiencies[38].

Homocysteinuria can also be secondary to defects in the conversion of homocysteine to methionine. Two unrelated children with periodic rages, seizures and homocysteinuria were evaluated by Murphy et al[39]. Both patients improved dramatically when treated with folic acid. The patients did however deteriorate over time despite maintenance of folate therapy. The clinical picture in both patients (absence of ocular defects and osteoporosis, as well as normal U-methionine concentrations) indicated a defect in the conversion of homocysteine to methionine rather than a CBS deficiency. Both patients

lacked other signs of folate deficiency and it is therefore postulated that the biochemical defect was a deficiency of 5,10-CH_3-THF reductase activity. This disorder is an example of a correlation between impaired methylation and a behavioral disorder. In the one patient, behavioral deterioration was related to the reappearance of large amounts of homocysteine in the urine. An increase in the dose of folic acid and restricted protein intake resulted in improved symptoms.

Another congenital disorder associated with impaired methylation is heritable adenosinedeaminase (ADA) deficiency. In this case purine nucleoside accumulation leads to subsequent lymphoid toxicity[40]. ADA patients show severe combined immunodeficiency and several additional non-immunological abnormalities. Improvement of neurological abnormalities after enzyme replacement suggests that it may be an integral part of the ADA deficiency syndrome. Given the relationship between adenosine metabolism and sulfur amino acid metabolism, the amino acid patterns in biological fluids of ADA deficient patients with neurological symptoms were evaluated by Boronne et al[41]. An increase in adenosine, accompanied by a high level of AdoHcy, homocysteine and a degree of transsulfuration pathway inhibition was found. It is speculated that the central nervous system dysfunction associated with this disease, may be due to either ADA deficiency in neurons or to secondary derangements of the transsulfuration pathway.

2.4.2.Neural Tube Defects

In 1999 Ubbink et al[41] compared known risk factors for neural tube defects (NTD) between urban and rural black women with histories of NTD affected pregnancies. None of the known risk factors investigated could explain the etiology of NTD's observed. One of the motivations for the current study was to investigate a marker for methylation that could be used to test methylation capacity in these women. Neural tube defects are therefore discussed in more detail than the previous diseases of impaired methylation.

Each year spina bifida (SB) and anencephaly, the two most common forms of neural tube defects, occur in an estimated 300 000 newborns worldwide[43]. These defects are important factors in fetal and infant mortality, as all infants with anencephaly are stillborn or die shortly after birth. Many infants with SB now survive due to extensive medical

care, but are likely to have severe life-long disabilities. In the USA where the incidence of NTD is about 1 in 1000 pregnancies, the monetary cost of caring for the affected infants born in 1988 was estimated at $500 million during the first four years of life[44] In South Africa, the overall prevalence rate of NTDs, reported from various studies ranged from 0.99 to3.55 per 1000 births[45, 46]. The prevalence in the black population however, varies considerably with geographical location. Buccimazza et al[47] reported a NTD incidence of only 0.95 per 1000 live births in the urban populations of Cape Town, whereas Ncayiyana[48], reported an average incidence of 6.13 per 1000 births for the rural black population of Transkei. The dramatic decline in NTD incidence associated with urbanization is still unexplained, but the implied impact on the poor, rural communities highlights the need for primary prevention.

The clinical spectrum of NTD includes spina bifida, anencephaly, cranioracheiscisis, encephalocoele and inencephaly. Much is known about the anatomical progress of the developing brain [49]. The closure of the neural tube at the cephalic and caudal portions is the first critical period for organogenesis in humans and occurs between 18 and 21 days after conception. Closure is normally completed by the 28th day, although some studies suggests that the tube can re-open after closure[50], causing some of the defects seen in NTD. The closure of the neural tube occurs concurrently at several sites and the clinical manifestations of the defect depend on the site where closure failed. Incomplete closure of the cranial part of the neural tube will result in anencephaly and spina bifida will be caused by imperfect closure at the caudal part.

Chromosomal abnormalities, single gene mutations and teratogenic causes are identified in fewer than 10% of NTD cases[51]. The genetic controls of the cellular mechanism involved in neural tube closure have yet to be determined, although several possible genes have been identified in animals[52].

Curly-tail mouse occurs as a spontaneous mutation and was first described by Grunberg in 1954. The NTDs in these mice resemble those of humans and have therefore been used as a study model in humans. Exencephaly, SB or curly tails occur in about 60% of the offspring with the remainder phenotypically normal. Breeding experiments done by Embury[53] suggested that all the mice, regardless of their phenotype, was homozygous for a recessive gene, but the expression was affected by an unknown number of modifying

genes in the rest of the genome. Studies done by Seller et al[54] revealed that the administration of vitamin A to the curly-tail female, on the 8th day after conception, could increase the incidence of NTDs in the offspring in a dose responsive relationship. When the experiment was repeated, breeding curly-tail mice with an unrelated A strain, the actual incidence in the F_1 generation differed according to the maternal genotype. The incidence of NTD was significantly higher when the offspring was derived from an affected female showing that the maternal genotype influences the environmental component in the cause of NTD, possibly through different ways of metabolizing, storing, transporting or delivering the teratogens to the fetus. The 8th day after conception in the mouse is the time when closure of the folds proceeds from a point in the lower cervical region. Closure is essentially complete on day 9. When vitamin A was administered on day 9 instead of day 8, the incidence of NTD decreased to far below the expected incidence. In this case vitamin A acted as a teratogen on one day in gestation, and as a curative agent on another. This study showed that there is a critical time period and a dose responsive relationship in the development of NTD in mice with a genetic predisposition for the disease.

Other epidemiological studies done over the years revealed marked geographical and temporal variability in the occurrence of NTD, as well as strong associations with socioeconomic background[55]. These findings are generally indicative of nutritional differences and focused research efforts on the vitamin status of affected groups and individuals. In 1976 Smithells and colleagues[56] reported low levels of some vitamins in women who gave birth to babies with NTDs. Various studies since 1976, proved that preconception folic acid supplementation decreased the risk of NTD.
Smithells et al offered a multivitamin containing folic acid to women who had a previous pregnancy affected by NTD and planned to become pregnant again. They reported an 86% lower risk of having another affected pregnancy in the group that took the supplement than in the group of women who did not. The results of a trial sponsored by the British Medical Research Council released in 1991[57], also showed a significant reduction in risk of recurrent NTD in women who took 400µg folic acid daily. In 1992 the Hungarian study[58] found that women who took a multivitamin supplement containing 800µg folic acid had a significantly lower risk of a first occurrence of NTD than women who did not.

These findings generated much interest in the genetic and biochemical mechanisms of folate metabolism and possible role in the etiology of neural tube defects. The effect of folate supplementation observed in some mothers with NTD affected children may partly be explained by the high incidence of homozygosity for the C677T allele in the gene coding for the enzyme methylenetetrahydrofolate reductase[59]. This mutation renders an enzyme with reduced activity that can interact with the insufficient supply of folic acid, which then presents as a NTD. In some ethnic groups, e.g. Hispanics, the incidence of this mutation roughly correlates with that of NTD[60].

The beneficial effect of folic acid supplementation during early pregnancy is well established[61,62,63] but it is also known that at least 30% of NTDs will not be prevented by the consumption of folic acid supplements.[64].

In two recent studies both Mills [65] and Steegers-Theunissen[66] reported that fasting plasma homocysteine concentrations were significantly higher in woman who had given birth to infants affected by NTD. This was attributed in both studies to a reduction in re-methylation of homocysteine to methionine. The enzyme methionine synthase is vitamin B_{12} dependant and uses folate as a co-factor for the re-methylation of homocysteine to methionine. In a recent review, Ubbink[67] suggested that the failure of neural tube closure might be caused by a relative shortage of methionine during a crucial stage of fetal development. Vitamin B_{12} and folate supplementation under these circumstances may give rise to better enzyme activity, which, in turn will increase remethylation and subsequently the availability of methionine. This view is supported by studies with *axial defects* mutant mice[68], where it was found that methionine supplementation led to a marked reduction in the incidence of NTD's. This was also found with in vitro studies where neural tubes in rat embryos, cultured on methionine-deficient media, closed only with methionine supplementation[69]. An adequate supply of methionine ensures sufficient S-adenosylmethionine, the methyl group donor for the production of myelin basic protein, which is required for proper neural tube closure.

In summary it can be said that neural tube defects may be caused by environmental factors and/or teratogens, in individuals with genetic aberrations or nutritional insufficiencies, which renders them susceptible to these factors. The possibility exists

that these aberrations/insufficiencies will be revealed when the methylation capacity, rather than individual metabolites, can be measured.

CHAPTER 3

NICOTINAMIDE METABOLISM AS A MARKER OF METHYLATION

3.1. INTRODUCTION

The major urinary metabolites of nicotinamide have been correlated with various pathological states, with decreased excretion associated with schizophrenia[70], depression[71], and mental retardation[72]. Impaired methylation capacity, as seen in disturbed metabolism of monoamine neurotransmitters, as well as decreased cerebrospinal concentrations of AdoMet, is also observed in the same patient groups[2]. These findings suggest a possible correlation between impaired methylation capacity and nicotinamide metabolism. The methylation of nicotinamide was chosen as a possible marker for the investigation of methylation capacity.

The metabolism and biological functions of nicotinamide and its methylated product; N-methylnicotinamide is discussed in this chapter.

3.2. LITERATURE REVIEW

3.2.1.Background

Pellagra as a disease persisted in Southern Europe and the Southern United States until the early 1900`s and was associated with the consumption of a diet consisting mainly of corn. Pellagra is characterized by three main symptoms: dermatitis, diarrhea, and dementia, with a number of nonspecific symptoms preceding the actual clinical manifestation. In 1916 Goldberger[73] postulated that the disease was due to a lack of a specific nutrient, called the pellagra-preventing factor. In 1937 Elvehjem et al[74] isolated nicotinamide as the pellagra factor from liver and in 1938 Spies et al[75] showed that administration of nicotinic acid could cure pellagra. It was thereafter generally accepted that pellagra was caused by nicotinic acid or nicotinamide deficiency. Harden and Young discovered nicotinamide dinucleotide as coenzymes of alcohol fermentation and Warburg and Christian[76] nicotinamide dinucleotide phosphate as a hydrogen transporting

coenzyme. Subsequently, nicotinamide was found to be an integral part of nicotinamide dinucleotide and nicotinamide dinucleotide phosphate.

3.2.2. Biological Functions of Nicotinamide

Nicotinamide functions metabolically as an essential component of the enzyme co-substrates, nicotinamide adenine dinucleotide (NAD^+/H) and nicotinamide adenine dinucleotide phosphate ($NADP^+/H$). These co-substrates act as the most central electron transport carriers of cells, acting as the intermediate in most of the H^+ transfers in metabolism. More than 200 reactions in the metabolism of carbohydrates, fatty acids and amino acids are implicated. In the mitochondrial respiratory chain, ATP is generated by the oxidation of NADH to NAD^+. NAD^+ is also a substrate in non-oxidation/reduction reactions such as mono-ADP-ribosylation, poly-ADP-ribosylation, and the formation of cyclic ADP.

The physiological role of nicotinamide has led to various pharmacological studies where manipulations in different aspects of the metabolism have been attempted. Methotrexate is widely used in different diseases as a therapeutic agent, but is associated with various side effects, including liver toxicity. Kroger et al[77] reported that nicotinamide and methionine reduce the liver toxic effect of Methotrexate. They showed in a mouse model that the toxicity was related to an increase in glutamate-oxaloacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) activity. When nicotinamide or methionine is administered together with metotraxate, the associated GOT and GPT activity increase is markedly reduced, reducing the liver toxicity.

Sheng et al[78] studied the effect of nicotinamide on DNA repair mechanisms. They reported an enhancement of DNA repair by a combined supplement consisting of carotenoids, nicotinamide and zinc (nicoplex). This supplementation resulted in a statistically significant increase in resistance to DNA single-strand breaks, increased DNA repair 60 min after induction of damage, elevated poly (ADP-ribose) polymerase (PARP) activity and an increased proliferation response to phytohemaglutinin (PHA). This study indicated that nicotinamide plays a definite anti-oxidant role in the body.

As a possible mechanism Kolb et al[79] proposed that the key target for nicotinamide is PARP, and to a lesser extend (mono)ADP-ribosyltransferase. Suppression of PARP activity by nicotinamide not only decreases consumption of NAD, the substrate of PARP, but also has a regulatory effect on gene expression. In addition, PARP activity controls early steps of apoptosis. It is therefore suggested that high doses of nicotinamide primarily affects ADP-ribosylation reactions in beta cells, immune cells, and the endothelium. As a consequence, cell death pathway and gene expression patterns are modified, leading to improved beta-cell survival and altered immuno-regulatory balance.

Recent reports have suggested that both apoptosis and inflammation are regulated by the transcription necrosis factor NF-kappaB. Crowley et al[80] reported that nicotinic acid and nicotinamide protect cells against apotosis induced by sodium deoxycholate (NaDOC).). The bile salt NaDOC is a natural detergent that promotes digestion of fats. At high physiological levels it induces DNA damage and activates PARP, an enzyme that utilizes NAD as a substrate to repair DNA. NaDOC also induce oxidative stress, endoplasmic reticilum stress and protein mal-folding. Nicotinic acid and nicotinamide were found to protect cells against NaDOC-induced apoptosis. Nicotinamide thus has promise as a dietary supplement to help prevent disorders involving excessive apoptosis.

In a follow-up study, Pero et al[81] investigated the possible regulatory role that nicotinamide and benzamides may have on the process of DNA repair and apoptosis. They investigated whether benzamides and nicotinamide could inhibit the production of tumor necrosis factor alpha (TNFalpha) and the inflammatory response, as well as reduce apoptosis via inhibition of NF-kappaB. Results showed that nicotinamide and two of the N-substituted benzamides gave dose dependant inhibition of lipopolysacharide induced TNFalpha in the mouse. Their data supported the notion that nicotinamide have potent anti-inflammatory and anti-tumor properties and they speculate that the primary mechanism of action is regulation at the gene transcription level, by inhibition of NF-kappaB, which in turn inhibits TNFalpha.

The role of nicotinamide in the prevention of insulin dependent diabetes mellitus (IDDM) is still controversial with various long-term studies underway to prove or disprove the theory. Interest originated from a study that reported intraperitonial injection of streptozotocin to mouse, rat, monkey and dogs induced diabetes, but when a large dose of

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nicotinamide is administered before or within 2hours after streptozotocin, the induced diabetes is inhibited[82]. The study reported that the NAD content, in the B-cells of islands of Langerhans in the pancreas, is reduced by the administration of streptozotocin, but the decrease does not occur after prior administration of nicotinamide. The decrease in NAD in this study was attributed to the stimulation of poly-ADP-ribose synthase in the B-cell by nicotinamide administration.

In another short-term pilot study, this time in humans[83], nicotinamide supplementation prevented IDDM in 15 out of 16 high-risk patients while 8 out 8 non-treated patients developed the disease. The effect was contributed to the protective effect of nicotinamide on islet cells against in vivo cytotoxic actions associated with IDDM.

In contrast, the DENIS study[84] (1997) was terminated when no major reduction of IDDM risk was observed in the placebo versus the nicotinamide group. In this study the conclusion was that in the subgroup: diabetes prone individuals at very high risk and with assumed rapid disease progression (siblings age 3-12 years with IDDM), nicotinamide did not cause a major decrease in, or delay of IDDM development. However, the data do not exclude the possibility of a major clinical effect of nicotinamide in individuals with a lesser risk of progression to IDDM than in the group studied.

Another possible application of nicotinamide is the enhancement of brain choline levels as a possible therapeutic option in neuro-degenerative diseases. Brain choline levels are, however, kept within narrow limits by homeostatic mechanisms, including the rapid clearance of excess choline from the brain. Jenden and Vargas[85] demonstrated that high doses on nicotinamide given to rats led to a time dependent increase in brain choline levels and behavioral changes consistent with enhanced central cholinergic activity. They showed that the choline enhancing effect of nicotinamide was due to the formation of N-methylnicotinamide by the brain cytosolic enzyme, using AdoMet as methyl donor. N-methylnicotinamide inhibits choline transport at the choroids villus and reduces choline clearance through competitive inhibition of choline transport proteins.

A subsequent micro-dialysis study by Erb and Klein[86]confirmed the ability of nicotinamide to augment extra-cellular levels of choline in the hippocampus of free moving rats. In contrast, however, they reported that nicotinamide enhances brain

choline concentrations by mobilizing choline from choline containing phospholipids, presumably via activation of phospholipaseA2, while the formation of N-methylnicotinamide did not contribute to this effect.

As another possible therapeutic application of nicotinamide Stratford[87] et al showed that nicotinamide sensitises murine tumors to radiation. Little is known about the mechanism of action of nicotinamide in this instance. It is postulated that the active component is not the parent nicotinamide compound itself, but one of the metabolites and that N-methylnicotinamide might be the derivatives responsible for the sensitisation effect observed.

3.2.3. Nicotinamide Metabolism

Figure 3 is a summary of the major metabolic pathways of nicotinamide. Nicotinamide can be absorbed from the diet or from bacteriological production in the gut, or it can be produced from tryptophan. Nicotinamide is excreted in urine as N-methylnicotinamide (NMN), N-methyl-4- pyridone-3-carboxamide (4Py) or N-methyl-2-pyridone–5-carbox amide (2Py)[88].

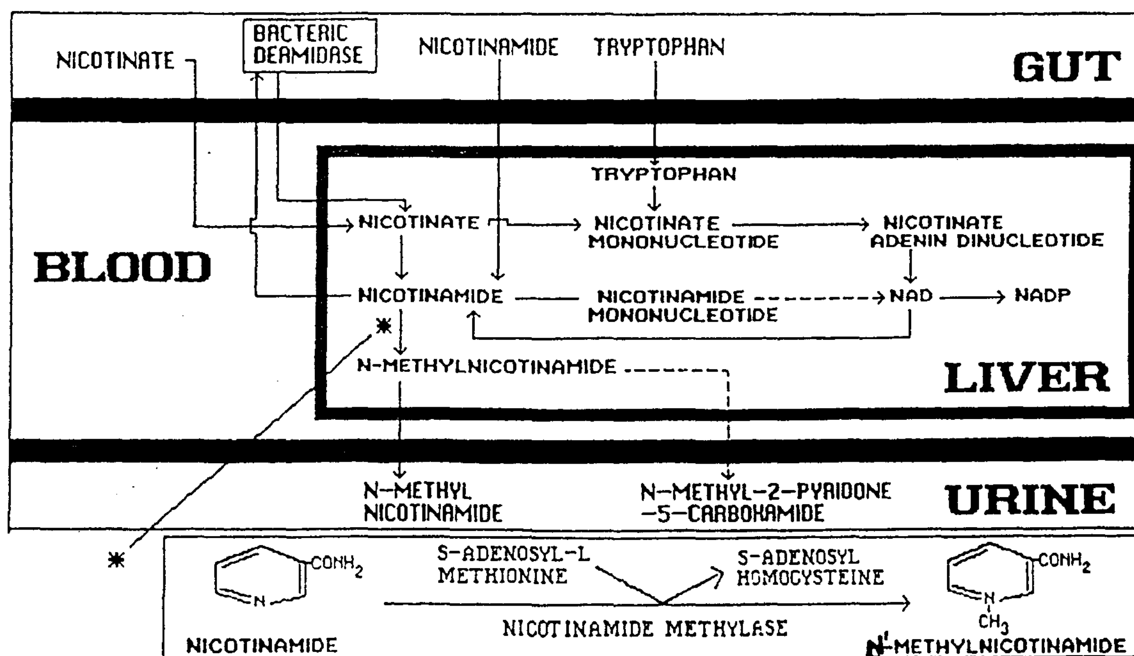

Figure 3.1: The metabolic pathway of Nicotinamide (from Cuomo (1994)[90])

All tissues can incorporate nicotinamide into NAD and prefers nicotinamide to nicotinic acid. Nicotinamide is the primary circulating form of the vitamin. Nicotinic acid is incorporated into NAD in the liver, which is then metabolized to nicotinamide. The primary regulatory substance of the homeostasis is nicotinamide itself [89]. The total NAD in the liver is greater than the functional NAD and this storage form of nicotinamide is used to buffer blood levels. Excess nicotinamide, formed by the hydrolysis of excess NAD, can be converted into various inert excretion products. In peripheral tissue nicotinamide is incorporated and metabolized to NAD with feedback inhibition through NAD levels. A major utilization and regeneration of nicotinamide is through the formation of adenosine diphosphate ribose derivatives, which are necessary for growth, differentiation and regulation. Excess nicotinamide inhibits this process and is toxic in tissue cultures at concentrations that increase NAD levels above normal

Exogenously supplied nicotinamide and nicotinic acid have different pathways to their incorporation into the pyridine nucleotide coenzymes. Furthermore, excretion of nicotinamide and nicotinic acid metabolites differs when nicotinic acid is administered in pharmacological and physiological doses. Shibate et al[91] reported that the catabolism of nicotinamide differs due to nicotinamide intake. For rats on a nicotinamide free diet the major urinary metabolite was N^1-methyl-4-pyridone-3-carboxamide. The percentage of 4Py decreased with increased intake of nicotinamide. In rats on a normal diet, NMN and 2Py are the major urinary excretion substances. Increasing the amount of nicotinamide to toxic levels resulted in urinary excretion of mainly N-oxide, as well as the excretion of nicotinic acid and its metabolites. The dietary effects were also evaluated in terms of the weight gained during the period on the specific diet. The growth retardation observed with the nicotinamide toxic diet indicated that the retardation was not simply due to a lack of the methyl donor AdoMet, but that other factors must be involved. When a large amount of nicotinamide is administered to rats, the activity of the 2Py forming NMN-oxidase is greatly reduced because it is inactivated during catabolism. This enzyme activity is related to dietary protein levels, with low protein intake causing low activity. The increase in NMN was therefore rather due to a decrease in the conversion of NMN to 2Py. Increased NMN, as an inhibitor of nicotinamide methyltransferase, will therefore lead to nicotinamide accumulation in the liver. As a result the reaction of nicotinamide-N-oxide occurs which is excreted in urine. Furthermore the reaction of nicotinamide to nicotinic acid can proceed because the concentration of nicotinamide exceeds the K_m

value of nicotinamidase. The accumulation of nicotinic acid leads to the next reaction, the conversion to nicotinuric acid, which needs Co-acetylA. This reaction is only observed when the body's capacity to methylate nicotinamide is exhausted, as seen with nicotinamide toxicity.

The metabolic pathway from tryptophan to nicotinamide is sensitive to a variety of nutritional, hormonal and pathological alterations, and has many steps dependent on vitamin B_6. The conversion of 3-hydroxykynurenine to 3-hydroxyanthranilic acid, catabolized by kynureninase is pyridoxal phosphate dependent. Leklem et al[92] examined the conversion of tryptophan to nicotinamide in women with vitamin B_6 deficiencies. All had elevated urinary NMN and NM2Py levels. After 4 weeks of vitamin B_6 supplementation these values decreased to half the initial values.

In a different study[93], five families were discovered with an autosomal dominant behavioral and psychiatric disorder, in which a number of affected family members showed intestinal tryptophan malabsorption. These abnormalities were treated with nicotinamide therapy and resulted in a marked improvement to normalization of their behavioral and psychiatric abnormalities.

2.2.4. N^1-Mehylnicotinamide

Under normal physiological conditions *N*-methylnicotinamide (NMN) is the major urinary excretion product of nicotinamide in mammals and its measurement in urine is still used for the diagnosis of pellagra.

Jansen and coworkers[96] reported an increase in urinary NMN in rats exposed to nitrite. They contributed the increase to either an indirect effect of nitrite on the adrenal gland, via corticosteroid induction of tryptophan pyrrolase (the 1st enzyme in the metabolism of tryptophan), or by direct inhibition of probably aldehyde dehydrogenase, involved in the metabolism of NMN. Experiments with labelled tryptophan proved that the increased NMN originated from tryptophan. Janssen proposed that NMN was a likely marker for nitrite/nitrate exposure in humans. Additional experiments with human volunteers showed that in certain individuals, nitrate consumption led to a marked increase in urinary NMN. This was true for only about 50% of the volunteers, with the rest showing

no effect what so ever. A probable reason for the difference in NMN induction is that a possible polymorphism exists for the enzymes involved in NMN metabolism.

The methylation of nicotinamide has also been used in other studies to assess various aspects of methylation capacity. Somogyi et al[94] determined the endogenous NMN plasma concentration of 37 subjects with various pathologies and found wide variations in the concentration (Table 3.1).

Table 3.1: The plasma NMN concentrations (nmol/L) in various pathologies (Somogyi (1990) [95])

Group	Plasma NMN concentration (nmol/L)		Number
	Mean	Range	
Elderly	117	51 - 420	11
Young	110	45 - 294	16
Renal disease	198	91 - 733	6
Cirrhotic	206	99 - 848	4

Somogyi suggested that the variation was likely to be explained by factors such as age, vitamin intake, metabolism and renal clearing of NMN. In general, young people had lower levels than older people.

Jenks et al[96] studied the ability of body tissues to synthesize methylated compounds, in the presence of a negative nitrogen balance, induced by a methionine deficient diet. They measured urinary excretion of NMN and 2Py after a nicotinamide dose, before and after a methionine deficient diet. They found that NMN and 2Py excretion values were not affected by the methionine status and proposed that NMN was not simply an excretion product of excess nicotinamide, but that the excretion rate was maintained even in the presence of a negative nitrogen balance. They concluded that the formation of NMN appear to have a higher metabolic priority than the maintenance of the net protein synthesis rate.

Hirayama et al[97] also determined the urine NMN concentration in 5 subjects and found that the concentration ranged between 30 and 90 μmol/L with the exception of one patient with cirrhosis who had a concentration of 370μmol/L. These findings suggested

that the methylation of nicotinamide was different in persons with cirrhosis and that it could therefore be used to assess liver function.

In a follow up study, Cuomo et al[90] compared nicotinamide methylation after an oral load of nicotinamide between cirrhotic patients and a control group. They found statistical significant differences between the two groups with both the urinary output and the plasma levels of NMN much higher in cirrhotic patients than controls. They postulated that the increase could be due to the activation of a dissipative metabolic pathway that would reduce the synthesis of NAD and NADP, which involves the consumption of ATP. These result are in accordance with the decrease in ATP availability and energy crisis observed in cirrhotic patients. Alternatively, it may be the result of increased nicotinamide methylases activity in regenerating hepatocytes of cirrhotic nodules. This view is supported by studies in rats[98] where it was found that nicotinamide methylase and NMN are involved in the control of hepatocellular DNA synthesis and cell proliferation.

Orlando et al[99] evaluated NMN renal clearance in patients with liver cirrhosis, of increasing severity, and compared it with the renal clearance in healthy subjects. They found that the urinary NMN clearance increased in relation to the severity of the liver dysfunction. NMN clearance, NMN-to-creatinine and NMN-to-inulin could all distinguish between the three groups investigated (with different degrees of liver cirrhosis), with sensitivities and specificities greater than 90%. They concluded that the potential of NMN as a diagnostic and prognostic test deserve further study

CHAPTER 4

ANALYTICAL DERMINATION OF N^1-METHYLNICOTINAMIDE BY HPLC.

4.1. INTRODUCTION

4.1.1 Overview of Analytical Methods

Established procedures that already exist for the determination of nicotinic acid and nicotinamide include microbiological assays based on the growth of *Lactobacillus plantarum*. These assays are sensitive and relatively easy to perform but often time consuming and difficult to standardize. Furthermore, microbiological methods lack specificity for single derivatives and are therefore less reliable. Another classic approach to nicotinamide determination is by König`s reaction. The resulting complex is then determined colorometrically. This technique also measures both nicotinamide and the different degradation products and thus lacks specificity for specific derivatives. These methods may also only be suitable for monitoring levels in pharmacological studies or after applying high doses but they are neither applicable for the measurement of endogenous amounts of nicotinic acid or nicotinamide, nor for studies on the metabolism of these vitamers.

Various methods exist for the simultaneous detection of several of the metabolites of nicotinamide. These metabolites can easily be separated using both TLC or ion-exchange chromatography. Detection of the compounds in these methods is by UV, which is not always sensitive enough for some of the biological samples [100,101]. Kutnink et al[102] reported an isocratic reverse phase HPLC-UV method for the determination of NMN in urine. An isocratic HPLC-UV method for the simultaneous detection of NMN and 4Py in urine after a simple anion exchange cleanup procedure has been described by Carter[103]. However, as mentioned above, UV detection suffers from a lack of sensitivity and cannot readily be used for plasma determinations.

N-methylnicotinamide in urine was traditionally measured after treatment with alkali and butanol, resulting in a fluorescent derivative with low intensity. Clark[104] replaced butanol with acetophenone, which resulted in a product with a 20-fold increase in molar

fluorescence. The derivatisation procedure used in this study for the determination of NMN is based on the method described by Clark, where N-alkylnicotinamides are condensed with acetophenone in alcoholic NaOH and the resulting product is then dehydrated with formic acid to form 1,6-naphthyridine derivatives.

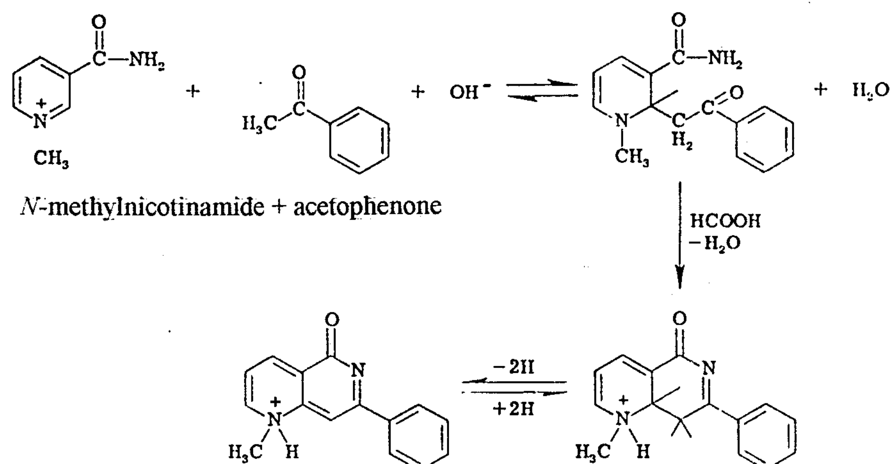

Figure 4.1: Proposed reaction mechanism for the formation of 1,6-naphthyridine derivatives (method by Clark[104])

The fluorescent product can then be measured using a fluorescence detector. This method has the disadvantage that both NADP and NAD yield fluorescent derivatives, which result in falsely elevated NMN levels in serum or plasma.

Tanaka et al[105] used micelle electrokinetic current chromatography (mECC) and separated mixtures of nicotinamide and related compounds. This technique allows for the simultaneous determination of both N^1- and N'-methylnicotinamide and all the other nicotinamide derivatives excreted by mammals. This technique yields high separation efficiency, easy operation and low running cost. It can also be coupled to a fluorescence detector and should, where available, be the technique of choice.

Another approach is to separate the various fluorescent derivatives of interest from interferences like NADP and NAD by HPLC. Somogiy et al[94] described a reverse phase ion-pair HPLC method for the separation of the 1,6-naphthyridine compounds. Quantification is by means of fluorescence detection at 366 emission- and 418 excitation

wavelengths. The excellent sensitivity obtained in this method makes it ideal for the determination of NMN in biological samples and after minor adjustments; the method was validated and used in this study for the determination of NMN in urine and plasma.

4.1.2 *N*-Methylated Nicotinamides

Some confusion was encountered in the literature on the N-substituted methylation products of nicotinamide. Figure 3.2 shows the structural formulas for nicotinamide, N^1- and N'-methylnicotinamide. The compound, N'-methylnicotinamide, was first identified in human urine by Holmen et al in 1981[106] using a gas chromatographic technique. However, it is not clarified whether N' -methylnicotinamide was a metabolite of nicotinic acid, nicotinamide or whether it was derived from dietary, drinking or smoking habits. For GC analysis, nicotinamide must be derivatized to volatile components that induce changes in the structures of the natural occurring molecules. In a subsequent study Shibata et al[107] tried tomeasured N'-methylnicotinamide in urine from healthy Japanese women, rats and mice by HPLC, but could not detect any N'-methylnicotinamide.

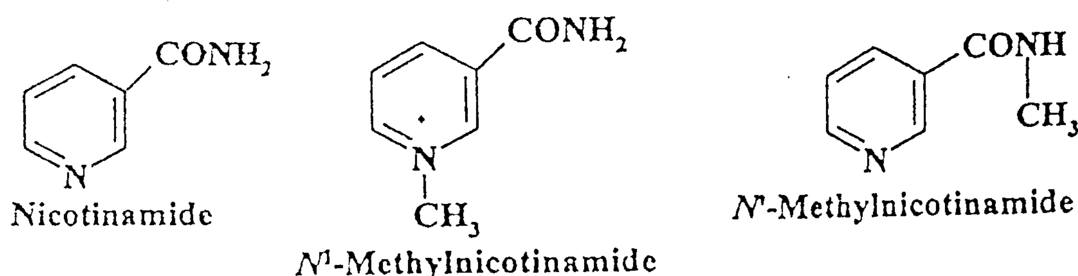

Figure 4.2: Structural formulas of nicotinamide, N^1-methylnicotinamide and N'-methylnicotinamide

N'-methylnicotinamide did not yield any fluorescent products under the conditions described in the analytical method used, and was thereby excluded from this study. The term N-methylnicotinamide in this study refers to the product N^1-methylnicotinamide.

4.1.2 *N*-Alkyl Nicotinamides

Hirayama[97] also investigated the formation of various N-alkyl nicotinamides in humans. The N^1-methylated nicotinamide was the only alkyl-substitution product excreted by humans. No N^1-substituted ethyl-, butyl-, propyl- or pentylnicotinamide were found and any of these compounds can therefore be used as an internal standard. The internal standard used in this method was N-propylnicotinamide iodide and was prepared according to the method described by Hirayama.

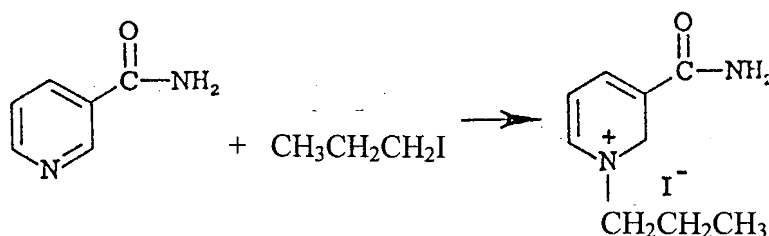

Figure 4.3: Reaction mechanism for the preparation of N-propylnicotinamide (method by Hirayama[95])

4.2. MATERIALS AND METHOD

4.2.1 Reagents

Acetophenone, triethylamine, Iodo-propane, 1-methylnicotinamide chloride and nicotinamide were supplied by Sigma. Heptanesulfonic acid sodium salt was obtained from Fluka. Hydrochloric acid, sodium hydroxide, ethanol, formic acid and acetonitrile were obtained from Merck Chemicals.

4.2.2 Chromatography

The liquid chromatograph consisted of a Hewlett Packard 1050 HPLC system with an automatic injector. The detector used was a HP1046 fluorescence detector set at an emission 366 and excitation 418. The analytical column used was a 4.6mm x 25cm Prodigy column supplied by Separations. The mobile phase used consisted of 77% buffer pH 3.2 containing 0.01M heptanesulfonic acid and 0.5% triethylamine and 22% acetonitrile. The flow speed was 1.5ml/min.

4.2.3 Preparation of the Internal Standard

N-propylnicotinamide iodide (NPN) was prepared by dissolving 1g nicotinamide in 5mL ethanol at 50°C. 1.5mL iodopropane was added and the mixture was left overnight at 55°C. The resulting N-propylnicotinamide iodide crystals were filtered and dried at 50°C. A stock internal standard solution was prepared in 0.1M HCl containing 1000µmol/L NPN. The NPN stock internal standard solution was diluted to 150µmol/L for urine and 1.5µmol/L for plasma determinations

4.2.4 Preparation of the Standards

A stock standard solution was prepared in 0.1M HCl containing 700µmol/L NMN. The NMN stock standard was diluted to concentrations ranging from 2 to 10 µmol/L for urine determinations and from 10 to 50nmol/L for plasma.

4.2.5 Procedure for Urine Determinations

600µL of standard, urine or control was transferred to 2mL Eppendorf tubes. 400µL of internal standard solution (150µmol/L) and 50µL of a 10% acetophenone in 80% ethanol solution was added to each tube. Tubes were mixed briefly on a vortex mixer and placed on ice for 10 minutes. 400µL of a 6N NaOH solution was added, the tubes were vortexed for another 30 seconds and incubated on ice for 60 minutes. 400µL of formic acid was added, the tubes were mixed and left on ice for a further 10 minutes. Samples were removed from the ice and placed in a boiling water bath for 5min. It was then centrifuged at 1500rpm for 5 minutes and 25µL of supernatant was injected on the HPLC system.

4.2.6 Procedure for Plasma Determinations

400µL of standard, plasma or control was transferred to 2mL Eppendorf tubes. 200µL of internal standard solution (1.5µmol/L) and 20µL of a 10%acetophenone in 80% ethanol solution was added to each tube. Tubes were mixed briefly on a vortex mixer and placed on ice for 10 minutes. 400µL of a 6N NaOH solution was added, the tubes were vortexed for another30 seconds and incubated on ice for 60 minutes. 400µL of formic acid was added, the tubes were mixed and left on ice for a further 10 minutes. Tubes were

removed from the ice and placed in a boiling water bath for 5min. Following centrifugation (1500rpm for 5 min), 100µl of supernatant was injected on the HPLC system.

4.3. VALIDATION

The first objective of this study was to develop and establish an analytical method for the determination of N-methylnicotinamide in biological fluids.
Validation can be defined as the process of proving that an analytical method is acceptable for its intended purpose. The method described here was extensively validated and the results will be discussed in this section.

N-Propylnicotinamide was selected as the internal standard because of its suitable retention time, sharpness of the peak and absence of interfering peaks in the blank preparation, at the corresponding retention time.

Figure 4.4 on page 34 is a typical chromatogram obtained with a standard solution prepared in 0.1N hydrochloric acid solution. The total amount injected was 0.3µmol NMN and 0.25µmol of the internal standard. The peaks are completely separated from each other, and from the peak at 14.9 minutes, which was also present in the blank preparation, and is possibly due to excess derivatizing reagents.
Figure 4.6 on page 36 is typical chromatogram obtained with a plasma sample, with the initial peaks from the plasma matrix, well separated from the peaks of interest. No interfering substances were found in any of the urine or plasma samples analysed.

4.3.1 Specificity

Specificity demonstrates the ability of the analytical method to accurately measure the analyte response in the presence of all potential sample components. The object of the specificity test is to ensure that the component of interest is free of interference of other components in the sample. Baseline resolution from other peaks is accepted as adequate separation, and the integrity of the analyte must be verified.

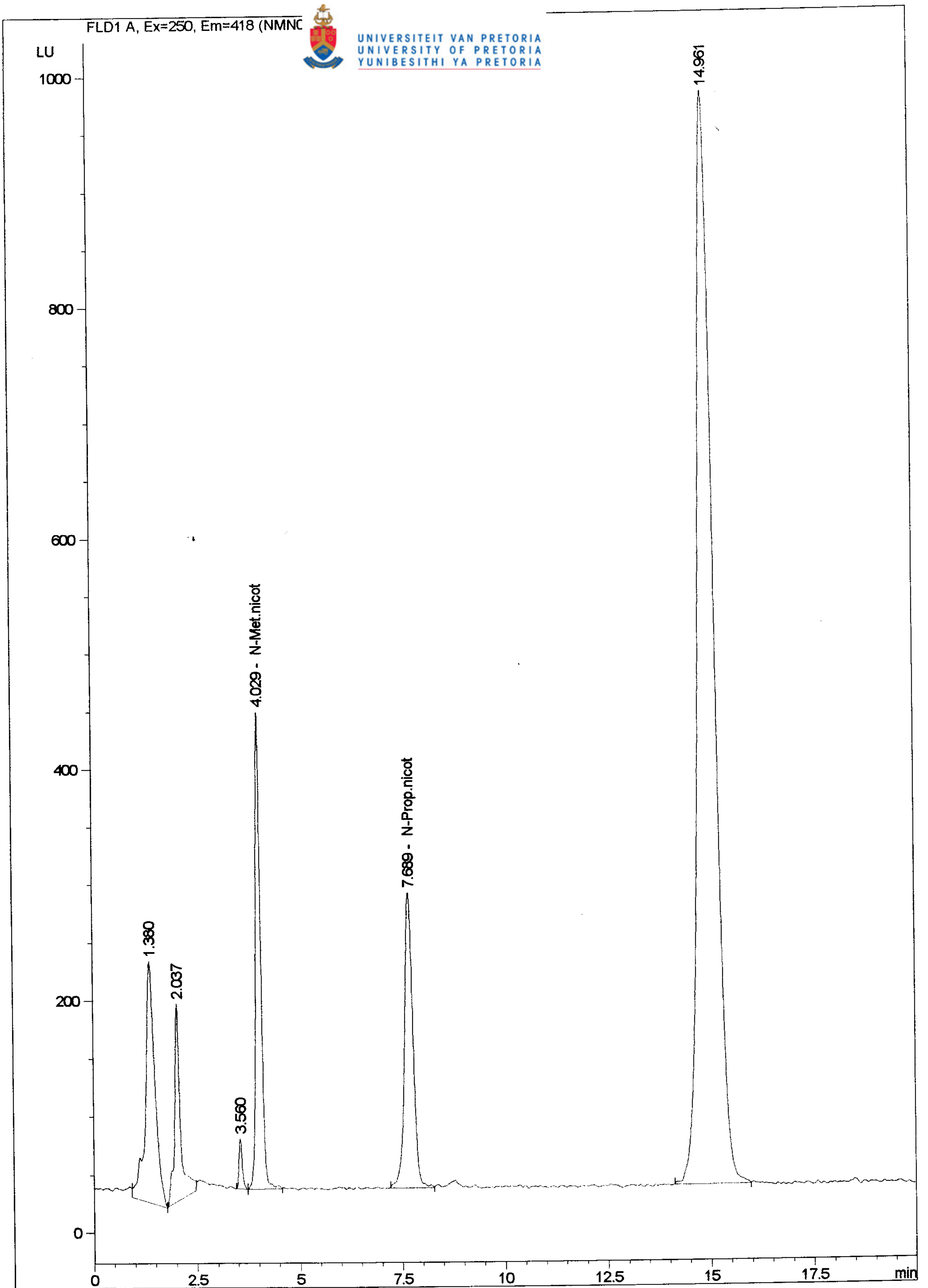

Figure 4.4: A typical chromatogram of a standard preparation. [*The total amount injected was 0.3µmol N-methylnicotinamide and 0.25µmolinternal standard]*

N-methylnicotinamide

N-propylnicotinamide

Figure 4.5: A three-dimensional excitation scan of the standard chromatogram (Fig 4.4)
[y-axis = absorbance, x-axis = retention time, z-axis =excitation wavelength]

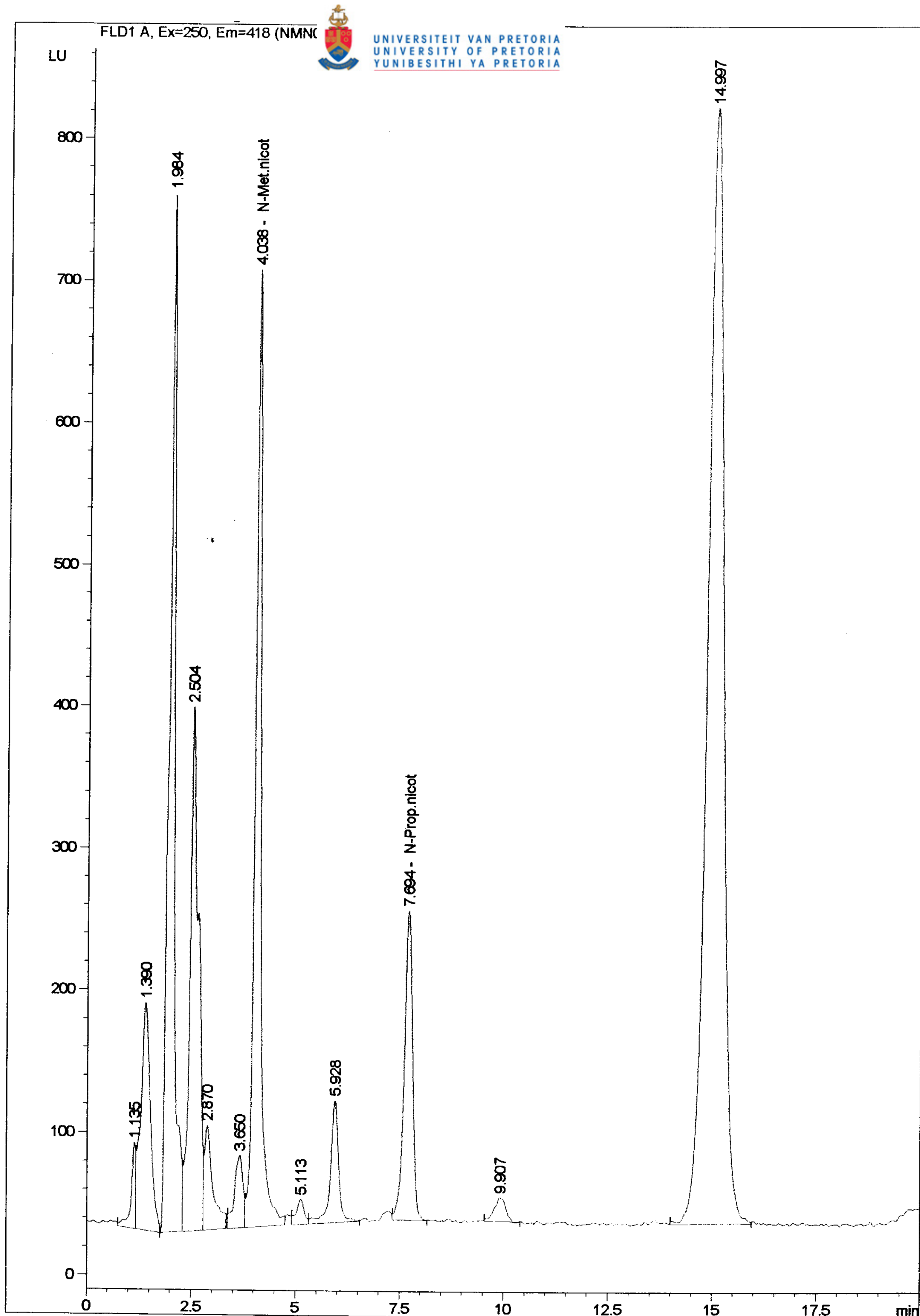

Figure 4.6: A typical chromatogram obtained with a plasma sample preparation

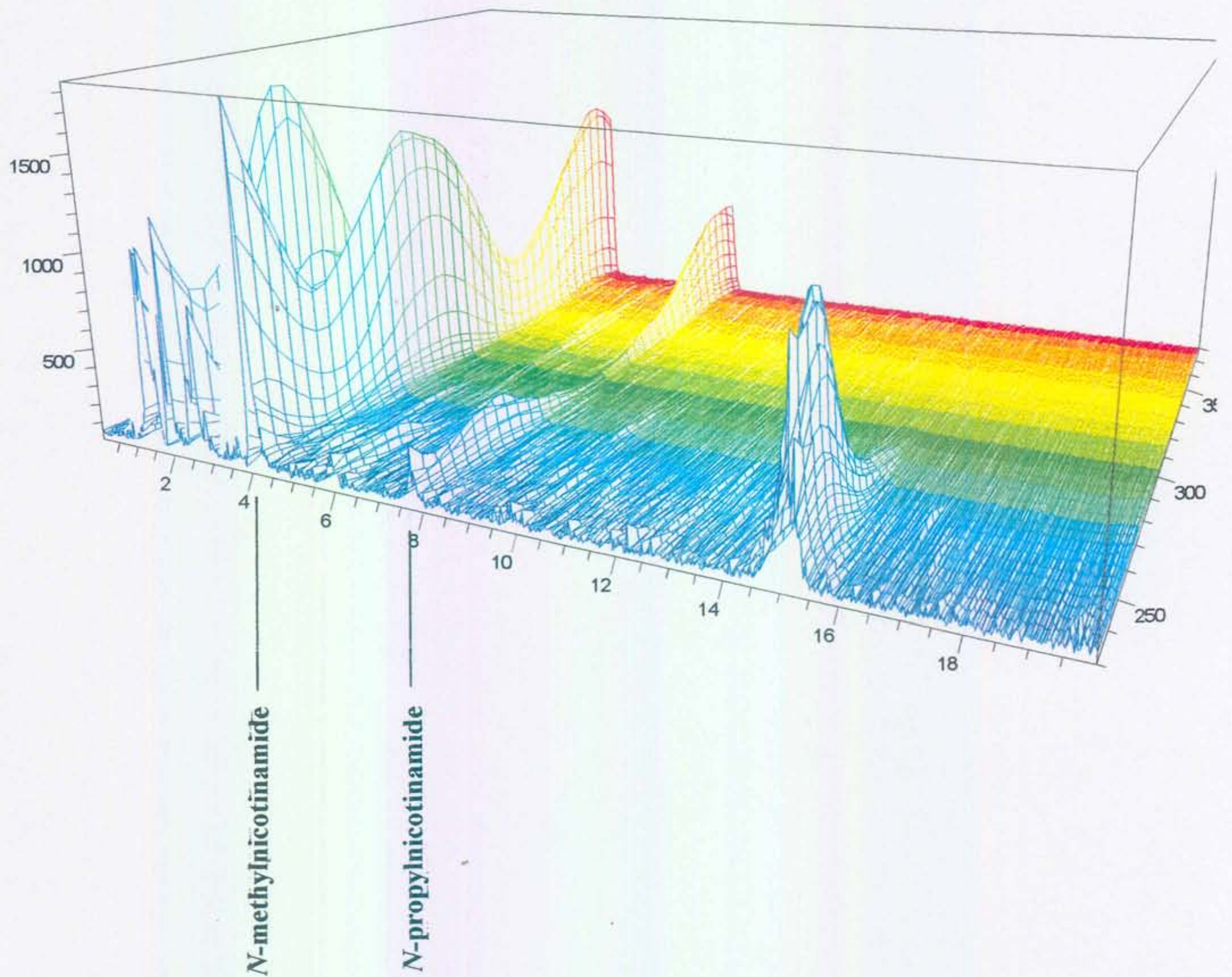

Figure 4.7: Three-dimensional excitation scan of the plasma sample (Figure 4.6).
[y-axis = absorbance, x-axis = retention time, z-axis= excitation wavelength]

Fluorescent detectors are hampered by their lack of specificity. In most cases the major criteria for peak identification has been the basis of comparing retention times with that obtained with known standards. Over the last few years increasing use has been made of more sophisticated detectors based upon the FLD scanning detector. Such an instrument is able to make repeated scans, over a preset range, of the chromatographic effluent passing through the detector cell in as little as 100msec or less. Using microcomputers and associated data storing devices, vast amounts of chromatographic data can be acquired in a single run. The scanning fluorescence detector adds another dimension, wavelength, to the conventional absorbance versus retention time plot. Another advantage is that these 3D plots supply valuable information on the purity of the compounds, as interfering substances will have different wavelength spectra.

The retention times of the NMN and PMN in the standard were used to identify the peaks in the samples. The peaks obtained in a standard chromatogram (Figure 4.4) and in a plasma sample preparation (Figure 4.6) were scanned using a HP 1100 fluorescence detector. The emission wavelength was kept at 418nm and the excitation was scanned from 230nm to 380nm. The three dimensional scans appear pages 34 and 36 and plots the absorbance (y-axis) as a function of the retention time (x-axis), with the excitation wavelengths on the z-axis. The spectral data for the peaks in the sample showed the same characteristic excitation patterns and maximum absorbance wavelengths (260 and 360nm) as the standard preparation, confirming the identity of both NMN and the internal standard, and showing that no other interfering substances were observed at these retention times.

Various unknown substances eluted before the N-methylnicotinamide peak in the sample preparations. These peaks were completely resolved from the NMN peak, but would have obscured the spectral scan of NMN in the sample. For this reason the scan of the sample preparation is presented in a different projection from that of the standard. This also serves as an example of how the computerized data can be manipulated to reveal different projections of the same 3D scans.

The components of interest, NMN and NPN, are baseline resolved from all the other components in the sample and the integrity has been verified by three-dimensional

spectral scans. No interference from the matrix was observed, and this method meets the general acceptance criteria for specificity in HPLC methods[108].

4.3.2 Linearity of Response

Linearity is usually expressed in terms of the variance around the slope of the regression line calculated according to an established mathematical relationship from the results obtained after analysis of standard solutions that contained various concentrations of the analyte. The slope of the regression line and its variance provide a mathematical measure of linearity with the intercept on the ordinate as a measure of the potential bias. The general acceptance criterion for linearity is that the value of n ($y = mx^n + b$) will fall between 0.9 and 1.1. For perfect linearity $n = 1$, and any deviation from 1 indicates a curvilinear relationship[109].

Separate linearity tests were done for urine and plasma, covering the expected ranges. The response at each concentration was calculated from the ratios of the NMN peak height divided by the peak height of the internal standard peak.

The linearity test for urine determinations was done by preparing six standard solutions, ranging from 15 – 40µmol/L in 0.1N HCl. The six solutions were then analysed according to the procedure described in paragraph 4.2. The data from the linearity test are listed in Table 4.1, and the regression results in Table 4.2. Figure 4.8 is the linearity plot obtained with the linearity test of urine determinations

Table 4.1: Linearity response over the concentration range tested for urine standards

NMN conc (µmol/L)	Response (pk/Istd)
1.64	0.13
3.28	0.26
6.56	0.57
13.10	1.19
26.21	2.33
39.31	3.43

Table 4.2: Regression data of the linearity test for urine NMN determinations

Constant	-5.3E-05
Std Err of Y Est	0.051
R Squared	**0.9995**
No. of Observations	6
Degrees of Freedom	4
X Coefficient(s)	1.562
Std Err of Coef.	0.017

From the regression data listed in Table 4.2, the response was almost totally linear with a correlation coefficient of 0.9995. The performance of the method for the determination of NMN in urine is within the general acceptance criteria for linearity.

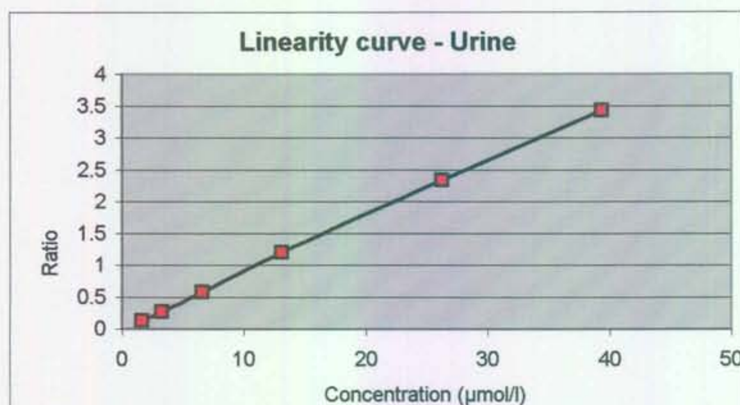

Figure 4.8. Linearity curve for urinary NMN determinations as concentration vs response ratio (peak/internal std)

A similar procedure was followed to determine the linearity of the response over the range expected for plasma determinations. Four standards were prepared in 0.1N HCl and analysed as described under materials and method - plasma determinations. The response was once again calculated as the peak height of the NMN peak divided by the peak height of the NPN (internal standard) peak. The results are listed in Table 4.3 and plotted in Figure 4.9. The results of the regression of the curve are presented in Table 4.4. The correlation coefficient is 0.99901 and the method for NMN determinations in plasma is within the general acceptance limits for a linear response.

Table 4.3: Linearity results over the concentration range tested for plasma standards

NMN conc (mol/L)	**Response** (pk/Istd)
71.95	0.58
143.90	0.98
287.79	1.85
431.69	2.80

Table 4.4: Regression data of the linearity test for plasma determinations

Constant	-2.305
Std Err of Y Est	0.842
R Squared	**0.9990**
No. of Observations	4
Degrees of Freedom	2
X Coefficient(s)	22.213
Std Err of Coef.	0.492

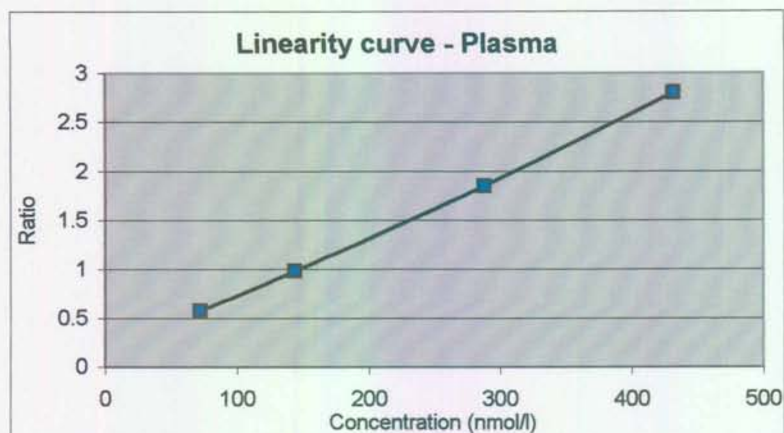

Figure 4.9: Linearity curve for plasma NMN determinations as concentration vs. response ratio (peak/internal std)

4.3.3 System Suitability Performance on the HPLC

Calculations used in determining system suitability performance appear in Appendix C. System suitability data is included as a measure of the performance of the system that was achieved during the validation process. Whenever these performance parameters can be achieved, the method is considered to be valid. This process allows other laboratories to apply the analytical method without having to duplicate the whole validation process. System suitability tests are included in most modern chromatographic software packages and the system performance can be measured over an entire batch. Table 4.5 represents the mean of the system suitability parameters achieved during the validation.

Table 4.5: Average values for system suitability performance, achieved during the validation process.

Performance parameter	Mean	
	NMN	NPN
Peak symmetry	0.90	1.03
Peak width (min)	0.03	0.11
Plate count (sigma)	34 641	6 544
Resolution (sigma)	13.63	12.16

4.3.4 Precision

Precision refers to the distribution of individual measurements within a batch, around their mean. Precision is expressed as the RSD(%) or the %CV. To validate precision, multiple analyses were performed on the same sample within the same batch.

For the precision test on urine determinations, a pooled urine sample was used. Ten individual samples were prepared from the pooled urine and analysed as described under Paragraph 4.2. The results from this test are listed in Table 4.6, together with the regression data. The CV for 10 urine determinations was 4.66%.

Table 4.6: Within-batch precision results for urine determinations

Analysis number	Result (µmol/L)
1	22.31
2	21.37
3	21.67
4	21.72
5	20.39
6	22.96
7	23.45
8	23.61
9	23.21
10	22.50

Regression data	
Mean	22.313
Standard error	0.116
Median	22.409
Standard deviation	1.04181
Variance	1.085
Minimum	20.39
Maximum	23.61
Sum	223.13
Confidence Level (0.9500)	0.193
CV%	4.66

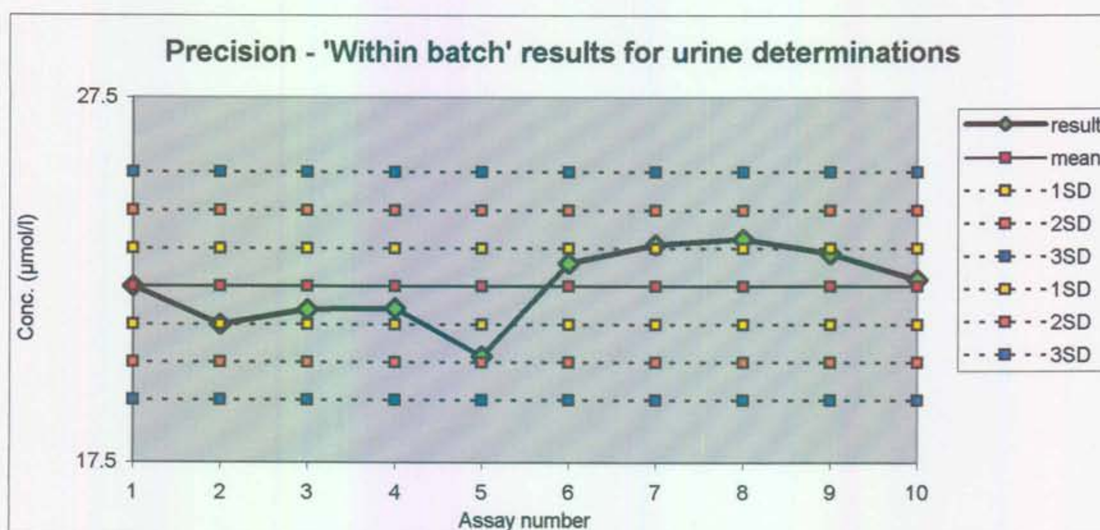

Figure 4.10: Within-batch distribution of NMN results after replicate analysis of a urine sample

The results from the precision test for urine are plotted in Figure 4.10. The data is presented as the result for each analysis, distributed around the mean of all 10 determinations. The other horizontal lines are the standard deviations (+/-1SD, +/-2SD and +/-3SD) calculated from the distribution around the mean.

For the precision test of plasma determinations, a pooled plasma sample was prepared. Twelve individual aliquots were prepared from the pooled plasma and analysed as described in Paragraph 4.2. The results are listed in Table 4.7 together with the regression data and plotted in Figure 4.11, using the same format as for the urine results. The CV for 12 plasma determinations was 4.26%.

Table 4.7: Within-batch precision results for plasma determinations

Assay number	Result (nmol/L)
1	85.61
2	87.64
3	81.82
4	78.77
5	78.92
6	82.05
7	78.63
8	78.12
9	79.43
10	77.54
11	85.46
12	78.42

Regression data	
Mean	81.03
Standard error	0.77
Median	79.18
Standard deviation	3.45
Variance	11.93
Minimum	77.54
Maximum	87.64
Sum	972.45
Confidence Level (0.9500)	0.194
CV%	4.26

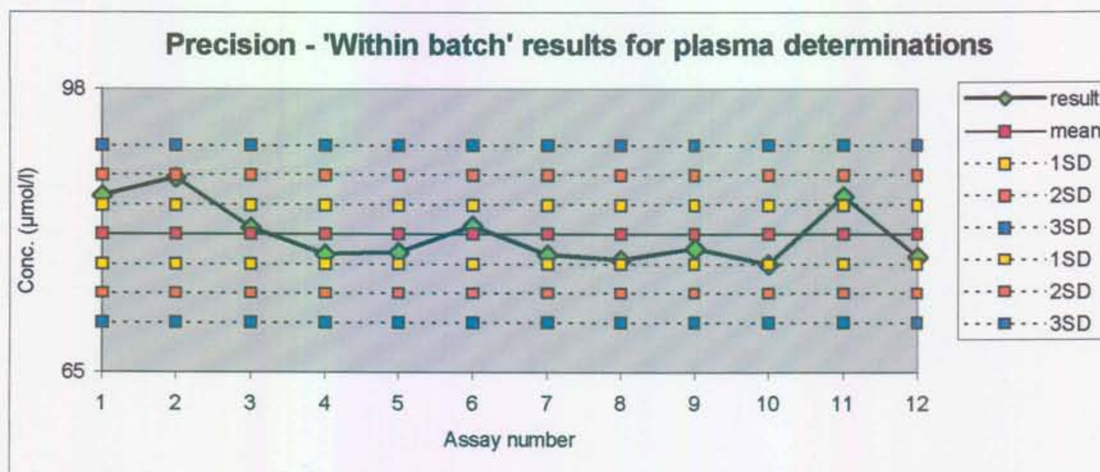

Figure 4.11: Within-batch distribution of NMN results after replicate analysis of plasma samples

For both the plasma and urine precision tests, the variations are within 2SD of the mean. The observed variations in results are acceptable when the complexity of the matrix, and the concentration of the analyte are taken into consideration. The intended application of

the method is also to measure substantial changes expected after a load test, which will not be influenced by the small variations observed.

4.3.5 Accuracy

Accuracy refers to the distance of the population mean from the true value (theoretical amount added to the sample). Accuracy measures the efficiency of the extraction of the analyte from the sample matrix.

A pooled sample was used for the determination of the accuracy of urine analysis. The N-methylnicotinamide content of the sample was determined and triplicate aliquots were spiked at three different concentrations. The spiked samples were analysed and the % recovery was determined. The results for the urine accuracy test are listed in Table 4.8.

Table 4.8: % Recovery of NMN from spiked urine samples

Analysis results of the pooled urine sample	
Results of triplicate analysis (μmol/L)	21.22
	20.49
	21.85
Mean	21.19

Analysis results of spiked samples						
Sample	1		2		3	
Added amount (μmol/L)	47.79		71.23		94.97	
Theoretical concentration	61.63		81.82		102.03	
Results obtained	μmol/L	% recov	μmol/L	% recov	μmol/L	% recov
1	62.79	101.2	84.37	103.1	104.36	102.3
2	63.15	102.5	84.45	103.27	105.16	103.08
3	61.34	99.53	81.47	99.56	101.74	99.72
Range = 99.5 – 103.3 % recovered						

The same procedure was used to determine the accuracy for plasma determinations. The NMN concentration of a pooled plasma sample was determined. Triplicate aliquots of the pooled sample was spiked at three different concentrations and analysed according to the procedure described under Paragraph 4.2. The results from this test are listed in Table 4.9.

Table 4.9: % Recovery of NMN from spiked plasma samples

Analysis results of the pooled plasma sample	
Results of triplicate analysis (nmol/L)	233.5
	230.5
	219.4
Mean	224.5

Analysis results of spiked samples						
Sample	1		2		3	
Added amount (nmol/L)	30.9		123.7		494.8	
Theoretical concentration	255.4		348.1		719.2	
Results obtained	nmol/L	% recov	nmol/L	% recov	nmol/L	% recov
1	234.3	91.7	350.3	100.63	687.1	95.54
2	236.6	92.7	319.3	91.71	752.9	104.13
3	233.9	91.5	366.6	105.3	662.7	92.14
Range = 91.5 – 103.5 % recovered						

The acceptance criteria in the accuracy test takes the SD of the method as determined in the precision test into account, since the deviation from the theory depends on the error inherent in the analytical method itself. The acceptance range is 4SD units and is intended to cover the additional error introduced in preparing the spiked samples[110].

The recoveries obtained with both the urine and the plasma samples are within the acceptance range and prove that the extraction method used can efficiently extract the added amounts from the matrix, and therefore accurately determine the NMN amounts in the matrix.

4.3.6 Reproducibility

Between-run precision of replicate analysis, over a set time period is influenced by random errors. For this reason the day-to-day reproducibility was evaluated for both plasma and urine by analysing spiked control samples with each batch. The reproducibility was calculated as the CV of the mean.

For both urine and plasma a pooled sample was used. The sample was divided into three aliquots, which were spiked with a standard solution to obtain three different concentration levels. These samples were numbered as control 1, 2 and 3 and aliquots

were stored frozen and analysed with each batch. Batches were prepared on 7 different days and the results obtained with the control samples on these different days are summarized in Table 4.10 for urine determinations and Table 4.11 for plasma. The general acceptance criteria for day-to-day reproducibility are that the RSD should not be more than twice the RSD obtained in the precision test. The day-to-day reproducibility results for urine and plasma are within the acceptable range.

Table 4.10: Day-to-day reproducibility results for urine determinations

Day number	Results (µmol/L)		
	C1	C2	C3
2	79.8	187.1	286.9
3	79.5	182.3	282.6
6	75.07	175.9	282.6
7	78.9	171.2	268.7

	Control 1	Control 2	Control 3
Mean	79.14	178.3	278.30
Standard Error	0.76	0.89	1.19
Median	79.28	177.62	282.2
Standard Deviation	4.67	5.54	7.39
Variance	3.01	4.23	7.51
Minimum	75.07	171.15	282.6
Maximum	79.79	187.10	286.9
Sum	553.9	1248.7	1948
Confidence Level	1.924	1.495	1.992
CV% =	**5.91**	**3.11**	**2.56**

Table 4.11: Day-to-day reproducibility results for plasma determinations

Day number	Results (nmol/L)		
	C1	C2	C3
1	369.3	1036.2	2350.9
2	363.2	985.0	2132.8
3	366.4	1047.5	2528.9
4	385.6	1028.5	2357.6
5	333.5	1014.7	2020.2
6	394.1	967.5	1941.1
7	373.0	998.3	2242.0

	Control 1	Control 2	Control 3
Mean	369.3	1011.0	2224.8
Standard Error	3.11	4.87	33.6
Median	223.9	1014.7	2242.0
Standard Deviation	19.2	28.9	206.9
Variance	50.9	114.9	5896.3
Minimum	333.5	967.5	1941.1
Maximum	394.1	1047.5	2528.9
Sum	2585.1	7077.7	15573.6
Confidence Level	5.191	7.747	55.827
CV% =	**5.21**	**2.86**	**9.30**

4.3.7 Limit of Detection and Quantitation:

A blank sample (0.1N HCl) was prepared and injected with each batch of plasma samples. The peak heights of the signal at the retention time for NMN in these chromatograms were used to determine the standard deviation (3.06×10^{-2}) and average blank signal (0.03135). These results were used to calculate the limit of detection and quantitation, using the equations described here[111].

The limit of detection is the lowest quantity that produces a response detectable above the noise of the system. This can be calculated:

$$\mathbf{Y_{LOD} = Y_B + 3(S_B)},$$

with Y_B the average blank signal and S_B the standard deviation

$$Y_{LOD} = 0.03135 + 3\,(3.06 \times 10^{-2})$$
$$= 0.1232 \text{ (peak height)}$$
$$= 11.2\text{nmol/L}$$

The limit of quantitation is the lowest level of the analyte that can accurately and precisely be measured with this method. It can be calculated with the formula:

$$\mathbf{Y_{LOQ} = Y_B + 10(S_B)}$$

$$Y_{LOQ} = 0.03135 + 10 \times (3.06 \times 10^{-2})$$
$$= 0.3374 \text{ (peak height)}$$
$$= 30.7\text{nmol/L}$$

The minimum concentration that can be detected in plasma with this method is 11nmol/L and a minimum concentration of 30nmol/L can be quantified. The analytical method is sensitive enough to measure NMN in plasma.

4.4 DISCUSSION

The first objective of this study was to establish an analytical method for the determination of N-methylnicotinamide in biological samples. A simple HPLC method was developed and validated. It is clear from the validation that the method can be used to produce valid and accurate results for determinations in both urine and plasma. The specificity test showed absence of interfering effects from the matrix, as well as the integrity of the component analysed (3D scans). The method is sensitive enough to accurately measure levels as low as 30nmol/L in a complex matrix like plasma, and is linear over range 70 – 430nmol/L (R>0.999). It can be used to determine plasma and urinary NMN concentrations accurately with recoveries of 91.5-103.5% and 99.5-103.3% respectively. The day-to-day variation (%CV) achieved with control samples was less than 6% for urine and less than 10% for plasma samples, which is within the acceptable range when the low concentrations and complexity of the matrix is taken into consideration. In conclusion it may be stated that the analytical method is suitable for the determination of N-methylnicotinamide in plasma and urine and that it can be used to evaluate clinical measurements and their possible applications.

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CHARACTERIZATION OF THE NICOTINAMIDE LOAD AS A DYNAMIC FUNCTION TEST.

5.1 INTRODUCTION

The aim of the study was to investigate NMN as a marker for methylation in humans. Certain aspects of the nicotinamide load test had to be investigated before a trial could be started. These pre-trial investigations are discussed in this chapter.

Both the nicotinamide, as well as the methionine metabolic pathways will be stressed during the course of this study. The rationale for using a dynamic load test is that patients with a mild disease may have biochemical abnormalities only during illness. Thus in order to demonstrate these biochemical abnormalities, it is be necessary to stress the specific pathway by administering excess amounts of the compound under investigation.

Meaningful comparisons between the responses to the loading test before and after manipulation cannot be made unless several conditions have been satisfied. Of these, the most important is that the volunteers be in a steady state prior to the loads and that it is performed under near identical metabolic conditions. To meet these criteria, volunteers were required to fast for at least ten hours before each of the load tests performed during the course of the trial.

5.1 1 Trueness of the Analytical Results

Different approaches can be used to test the trueness of analytical results obtained with a specific method. In this study, the method described in Chapter 4 was used to analyze a sample set, collected under conditions identical to that described by Cuomo[90]. The results obtained with the sample set from this study were then compared with the results for the normal subjects published in the Cuomo study.

5.1.2 Standardization of the Sampling Procedure

One of the objectives of the study was to standardize the sampling procedure used in a nicotinamide load test. This was investigated to ensure that the sampling intervals corresponded with critical changes in plasma concentrations. On the other hand, sampling intervals had to be kept at a minimum to simplify the procedure for possible routine applications. A pilot study was conducted to standardize the sampling intervals and to determine the time interval for the peak plasma NMN concentrations.

5.1.3 Investigation of the Reaction Kinetics

Cuomo showed that plasma NMN concentrations, after a nicotinamide load, could increase almost ten-fold under specific pathological conditions. High plasma NMN concentrations were expected in samples where the nicotinamide load was preceded by a methionine load, due to the increased availability of methyl groups. The possibility existed that a high load of nicotinamide could exceed the capacity of the enzyme S-Adenosylmethionine:nicotinamide-N-methyltransferase that converts nicotinamide to NMN. The reaction kinetics of this enzyme was therefore investigated to determine the load of nicotinamide to be used in the trial.

5.2 MATERIALS AND METHODS

For the validity test, a nicotinamide load test was performed on a single volunteer, according to the procedure described by Cuomo. The volunteer was given an oral load of nicotinamide (1.5mg/Kg) after a 12 hour fast and continued the fast for 5 hours after the oral load. Blood samples were taken at 0, 30, 60, 120, 180 and 300 minutes, and urine was collected and measured during the 24 hours preceding the load, as well as for 5 hours and 24 hours after the load. Samples were stored frozen until analysis.

Five volunteers, 2 males and 3 females participated in the pilot study that was conducted to standardize the sampling intervals. The volunteers took an oral load of nicotinamide (1.5mg/Kg) after a 10 hour fast and continued the fast for 5 hours while blood samples were collected. Blood samples (EDTA) were collected at 15, 30, 45, 60, 120, 210 and 300 minutes after the load. Plasma was separated, aliquoted and stored at –70°C until

analysis. Urine samples consisted of a 24 hour collection before the test, a second collection from the start of the load to 5 hours afterwards, followed by a third collection which ended 24hours after the load test. The volumes of each urine collection were measured and aliquots were stored frozen until analysis.

A single volunteer was used to investigate the reaction kinetics of the methyltransferase enzyme. This volunteer took different nicotinamide loads (0.05, 0.25, 0.5, 0.75, 1 and 1.5mg/kg body weight), on six consecutive days after a 10 hour overnight fast. On each day a basal blood sample was drawn, the oral nicotinamide load taken and a second blood sample drawn 60 minutes later. The plasma was separated and stored frozen until analysis.

The NMN concentrations in all these samples (plasma and urine) were determined using the HPLC method described in Paragraph 3.2. The urine volumes were used to calculate the urinary excretion as nmol NMN excreted during the corresponding collection period.

5.3 RESULTS

The NMN results for plasma and urine, found during the validity test (single volunteer), were compared with the mean values for the normal subject group (n=7), in the study by Cuomo et al. The data are listed in Table 5.1 and plotted in Figure 5.1.

Table 5.1: Comparison between the analytical and published results for urinary NMN excretion

	Time intervals (hour)			
Excretion mmol/interval	24h pre	0-5h	0-24h	19h post
Excretion mmol/interval	59.3	136.8	181.6	72.1
Cuomo: normal subjects	72.7	130.8	297.9	159.9

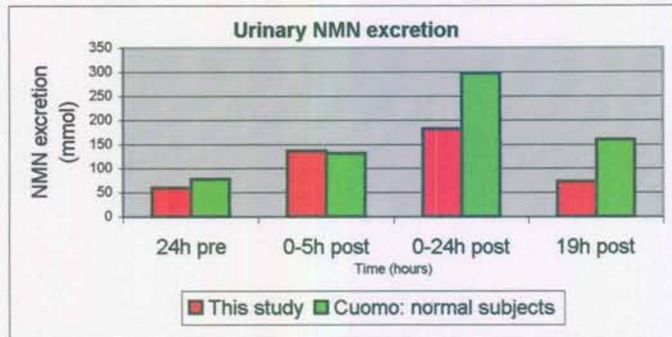

Figure 5.1: Graphic comparison of the urinary NMN excretion results in the two studies.

The plasma NMN results are listed in Table 5.2, together with the mean plasma concentrations from the study by Cuomo. These results are plotted in Figure 5.2 as the time dependent changes in NMN concentrations obtained during the two independent studies.

Table 5.2: Comparison between the analytical and published results for plasma NMN concentrations

Time interval (minutes)	0	30	60	120	180	300
Volunteer: NMN (nmol/L)	193	1300	1310	1218	1099	715
Cuomo normal subjects (nmol/L)	153	1017	1170	1090	1017	654

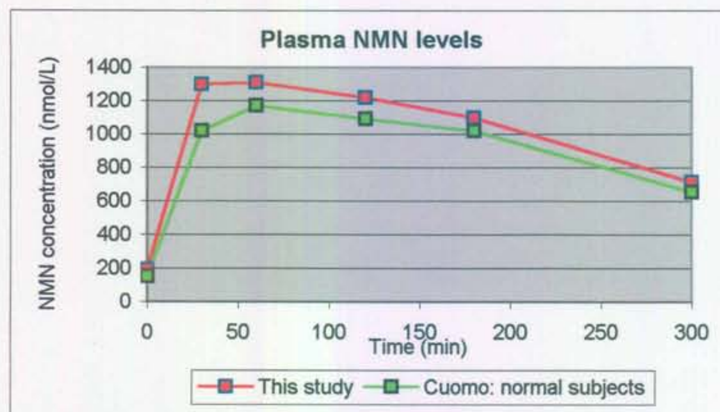

Figure 5.2: Graphic comparison between plasma NMN results in the two studies.

The urinary excretion results for each participant in the pilot study are listed in Table 5.3.

The total volume of each collection was used to calculate the urinary excretion of NMN (mmol) during each collection interval. These results are plotted in Figure 5.3.

Table 5.3: Pilot study: Urinary NMN excretion (mmol) for each time interval

Volunteer	Urinary excretion (mmol) during collection period		
	pre-24h	0-5h	0-24h
MR	47.5	114.0	195.1
AS	35.3	104.3	194.0
LS	42.5	109.0	264.2
JU	55.0	102.2	174.4
LB	50.7	119.3	214.5

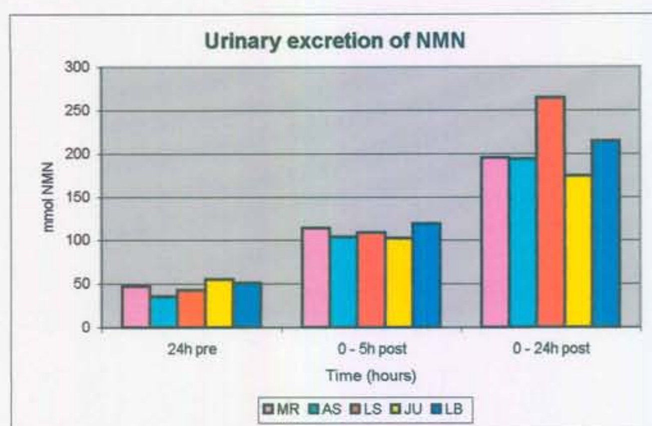

Figure 5.3: Pilot study: Urinary excretion of NMN for each time interval

The time dependant changes in the plasma NMN concentrations obtained during the pilot study, are listed in Table 5.4 plotted in Figure 5.4

Table 5.4: Pilot study: Dynamic changes in plasma NMN concentrations (nmol/L) for each time interval

Time	Plasma concentration (nmol/L)				
	MR	AS	LS	JU	LB
0	104.	175	76	67	228
15	740	1192	424	1025	702
30	1358	1404	800	1605	1302
45	1466	1584	1008	1633	1542
60	1490	1531	1385	1676	1808
120	1074	1640	1403	1145	1638
210	654	1092	875	897	1328
300	274	692	526	674	960

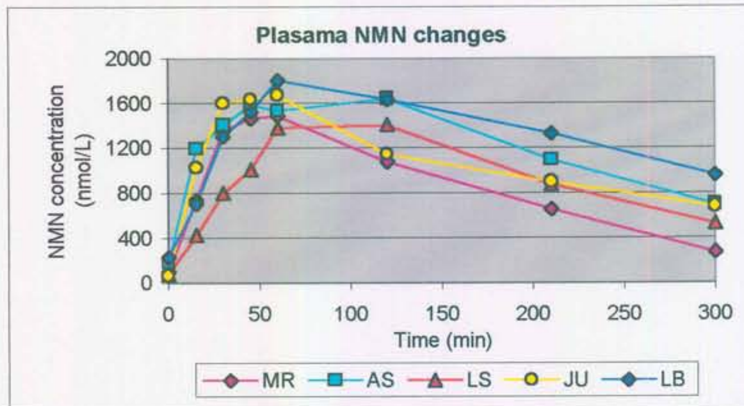

Figure 5.4: Dynamic changes in plasma NMN concentrations (nmol/L) for each participant of the pilot study.

The effect of different nicotinamide loads on the plasma NMN concentrations are listed in Table 5.5 and the results are plotted in Figure 5.5

Table 5.5: The effect of different nicotinamide loads on plasma NMN concentrations

Nicotinamide load (mg/kg)	Plasma NMN conc. (nmol/L)	
	Basal	60 min
0.05	44.3	140
0.25	53.8	212
0.5	74.8	464
0.75	50.1	1227
1	93.8	1412
1.5	76.7	1490

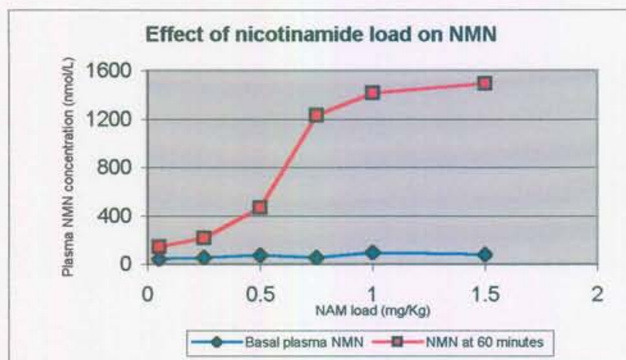

Figure 5.5: The effect of different nicotinamide loads on plasma NMN concentrations

5.4 DISCUSSION

In the validation experiment, the urinary excretion results over the initial collection periods are very similar. There was a substantial difference over the last 19hour collection period. This was most likely due to diet, as the fast ended after 5 hours. The lower excretion observed in this individual is however, still within the range of normal subjects published by Cuomo. The plasma results for both studies are nearly identical. From the similarities between the results from the two studies, it can be concluded that the method, as described in Paragraph 4.2, will produce valid results for the determination of NMN in both plasma and urine, comparable to published data..

The results from the pilot study were used to standardize the sampling procedure. The information obtained from urine analysis broadly reflected the changes in plasma NMN concentrations. Urine collections over long time periods are uncomfortable for participants in any trial situation, and the urine collection was omitted from the procedure, as it did not add any additional information to the plasma results. From the plasma results it became clear that the expected peak level was between 30 and 60 minutes for all the volunteers. It was expected that the increased availability in methyl groups caused by the methionine load would cause either an increase in NMN at the same interval, or at a slightly later interval. For the trial, the intervals for plasma collection were standardized to include the following times:

1. **T-60** : a blood sample taken one hour before nicotinamide load, to be used as a basal NMN value before methionine loading.
2. **T0** : a blood sample taken just before the nicotinamide load and one hour after the methionine load as a basal NMN sample for the nicotinamide load.
3. **T30** : a blood sample taken 30 minutes after the nicotinamide load, coinciding with the expected maximum NMN value.
4. **T90** : a blood sample taken 90 minutes after the nicotinamide load as the expected interval where the effect of the increased methylation might be seen.
5. **T180** : a sample taken 3 hours after the nicotinamide load test when NMN levels are returning to baseline levels. This interval also coincided with the expected maximum homocysteine levels.

The expected difference between the results of the loadings, with and without methionine, should be observed at these standardized intervals.

The results from the reaction kinetics study were used to determine the nicotinamide loads that would be used in the trial. It was decided to perform the nicotinamide trial at two different nicotinamide loads. A nicotinamide load of 1.5mg/Kg will be used in the first trial and 0.5mg/Kg in the second trial. From the graph in Figure 5.5 it became obvious that a nicotinamide load of 1.5mg/Kg body weight produced a response close to the V_{max} for the S-adenosylmethionine:nicotinamide –N-methyltransferase enzyme. It was decided to use a1.5mg/Kg load to achieve a level where the enzyme is functioning near maximum capacity. At this level, the enzyme capacity may be limited by the number of methyl donors available, and a methionine load under these conditions, adding an abundance of methyl groups, may lead to some insight into methylation capacity. The other nicotinamide load that will be investigated during the second trial is a load of 0.5mg/kg, which corresponds to approximately ½ V_{max}. This load was chosen, as a substantial increase in product formation (methylation capacity) was possible at this concentration.

The pre-trial investigations showed that measurements made by the analytical method were comparable with results from other studies. It established that critical changes in plasma concentrations would be observed when using the standardized sampling intervals. The pre-trial investigations also led to some insight into the reaction kinetics of a nicotinamide load test.

CHAPTER 6

AN EVALUATION OF NICOTINAMIDE LOADING TO ASSESS METHYLATION CAPACITY

6.1 INTRODUCTION

Nicotinamide is metabolized through various pathways, with the methylated product N-methylnicotinamide the major urinary excretion route. Methionine, via S-adenosylmethionine acts is the methyl donor and the reaction is mediated by the enzyme S-adenosyl-1-methionine:nicotinamide-N-methyltransferase.

A study was conducted to determine whether the methylation of nicotinamide to NMN could be used as a marker for biological methylation capacity in healthy humans. Methylation was measured as the time dependent changes in plasma NMN concentration after a nicotinamide load. The methylation status was then changed by means of a methionine load, and the nicotinamide load test was repeated. NMN as a prospective marker for methylation should reflect the induced change in methylation status.

It was clear from the pre-trial investigations in Chapter 5 that the nicotinamide load used would influence the reaction kinetics, and also the results. The study was therefore performed as two trials, identical in all aspects, except for the amount of nicotinamide load used.

Each trial consisted of two parts, which were performed one week apart. Part one of each trial consisted of a standard nicotinamide load test. In the second part of each trial a methionine load was consumed one hour before the standard nicotinamide load test.

6.2 NICOTINAMIDE LOADING TRIALS

6.2.1 Subjects and Specimen Collection

A total of seven volunteers, aged between 24 and 62 years, were recruited in the department for the study. Three of the volunteers participated in both trials. All the

volunteers gave informed consent for the study and refrained from taking any drugs or vitamin supplements in the seven days before and between the tests. The volunteers started to fast 10 hours before each nicotinamide load test, and continued the fast over the 3 hour specimen collection period.

For each load test, the nicotinamide was dissolved in 100mL water. The methionine (0.1mg/Kg) was first dissolved in water and an orange concentrate was then added to conceal the taste of the methionine. In the first part of each trial, the orange drink was consumed without the methionine added (placebo).

Blood specimens: About 5mL of venous blood was collected (EDTA as anticoagulant) at each standardized time interval as described in paragraph 3.2, the plasma was separated from the red blood cells within 30 min after collection. Plasma aliquots were stored frozen at –70°C until analysis.

6.2.2 Trial Procedure

The procedure followed during each trial was the following:

1st Trial: Part 1

- At T-60, one hour before the nicotinamide load, a basal sample was collected from each volunteer, who then consumed a placebo orange drink.
- At T 0, a second sample was collected and each volunteer then took a nicotinamide load (1.5mg/Kg) dissolved in water.
- Three more samples were collected 30, 90 and 180 minutes after the nicotinamide load.

1st Trial: Part 2. The second part of the trial took place one week after the first.

- At T-60, basal samples were collected, and the volunteers then took a methionine load (100mg/Kg) dissolved in the orange drink.
- At T 0, a second sample was once again collected and each volunteer took a nicotinamide load (1.5mg/Kg) dissolved in water.
- Three more samples were collected at 30, 90 and 180 minutes as before.

2nd Trial: The second trial was identical to the first, except for the nicotinamide load that changed to 0.5mg/Kg in both parts of the trial.

Plasma samples were analyzed according to the method described in paragraph 4.2

6.3.RESULTS

1st Trial

Two male, and three female volunteers took part in the first trial, and their ages ranged between 25 and 62 years. The time dependent changes in plasma NMN concentrations for each volunteer, with and without methionine, are listed in Table 6.1. The data for each part of the trial is plotted in Figure 6.1 as the mean-, high- and low plasma NMN concentrations after a 1.5mg/Kg nicotinamide load.

2nd Trial

Four males and one female, aged between 24 and 55 years, volunteered to participate in the second trial (three of the volunteers also participated in the first trial). The results from the 2nd trial, where a lower dose of nicotinamide (0.5mg/Kg) were consumed appear in Table 6.2. The mean-, high- and low plasma NMN levels for this trial are plotted in Figure 6.2

Table 6.1: The dynamic changes in plasma NMN concentrations (nmol/L) after a 1.5mg/kg nicotinamide load, with and without a methionine load.

NMN concentrations (nmol/L) after a nicotinamide load (1.5mg/Kg)						
Time	**MR**	**AS**	**LS**	**JU**	**LB**	**mean**
-60	122	89	156	193	88	130
0	79	86	116	144	78	100
30	1109	1056	1134	1115	1098	1102
90	1149	1584	871	1094	1068	1182
180	857	1394	817	1049	1002	1024
NMM concentrations (nmol/L) after a nicotinamide (1.5mg/Kg) and a methionine load (100mg/Kg)						
Time	**MR**	**AS**	**LS**	**JU**	**LB**	**mean**
-60	78	77	145	77	309	138
0	77	103	94	65	116	91
30	1138	456	1407	1029	976	1146
90	920	1369	1256	1097	1303	1149
180	609	813	1010	899	1099	886

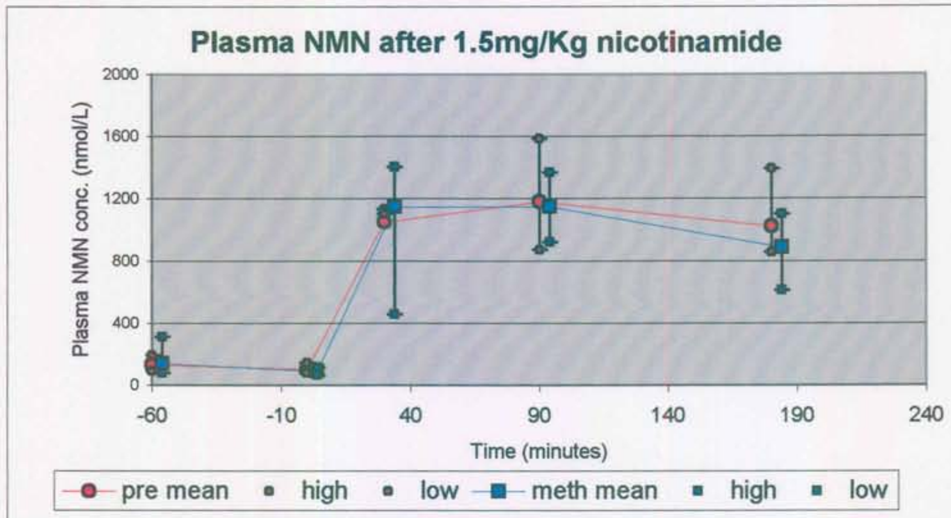

Figure 6.1: Post nicotinamide loading (1.5mg/Kg) mean, high and low plasma NMN concentrations with (pre) and without (Meth) a methionine load.

Table 6.2: The dynamic changes in plasma NMN concentrations (nmol/L) after a 0.5mg/kg nicotinamide load, with and without a methionine load.

	Time	MR	LS	JU	PP	AA	mean
NMN (0.5mg/Kg)	-60	89	116	110	89	71	95
	0	92	128	105	88	70	96
Pre-methionine	30	624	581	566	505	500	555
	90	389	391	478	436	378	414
	180	228	279	305	174	223	242
NMN (0.5mg/Kg)	-60	137	134	109	63	132	115
	0	140	120	100	83	132	115
Post-methionine	30	642	567	537	497	581	559
	90	414	365	469	391	512	430
	180	219	198	298	206	286	241

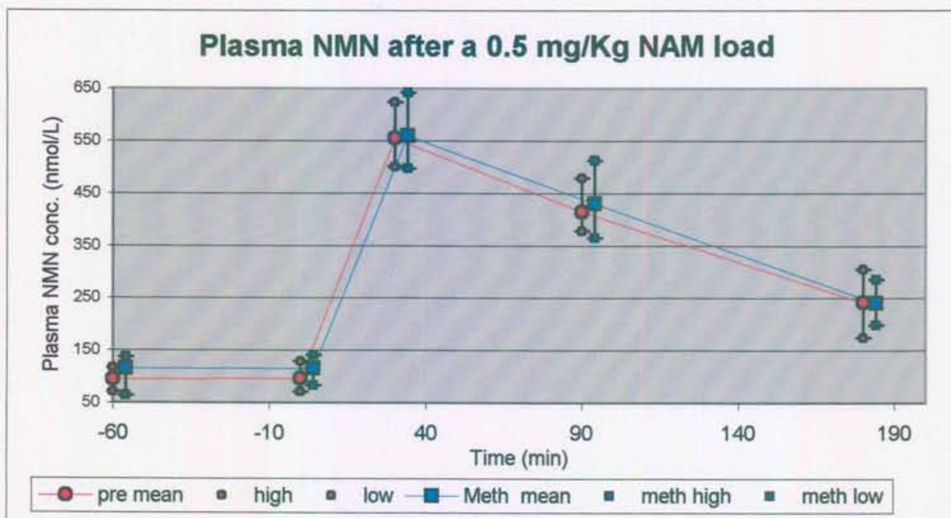

Figure 6.2: Post nicotinamide loading (0.5mg/Kg) mean, high and low plasma NMN concentrations with (pre) and without (Meth) a methionine load.

6.4 DISCUSSION

The average NMN concentrations in the two trials varied as expected, according to the load of nicotinamide consumed. The higher load of nicotinamide used in the first trial, caused higher maximum plasma NMN concentrations, with a delayed return to baseline levels when compared to the lower load in the second trial.

All the volunteers who participated in the trials were presumed healthy, from the information supplied in their health questionnaires (Addendum B), and no liver function, or other tests were performed prior to the trial. In the first trial, results of one of the volunteers (AS) differed considerably from the rest of the group. This person was the oldest volunteer at 62 years and experienced severe nausea after ingestion of the methionine. Even when the result for this volunteer was included, the differences in plasma NMN concentrations between the pre-and post-methionine loads in this trial were insignificant.

Large inter-individual variations were observed in the first trial, with and without methionine, but these inter-individual variations were much smaller than in the second trial. Inter-individual variations may be more pronounced when the metabolic pathway is stressed by a high nicotinamide load (1.5mg/Kg). The smaller inter-individual differences in the second trial may, however, simply be a result of to the smaller age difference in the group (none of the volunteers experienced any side effects from the methionine load in the second trial).

In the second part of both trials, the methylation status of the volunteers was changed by means of a methionine load. Peak homocysteine levels are reached around four hours after a methionine load[2] and the methionine load was consumed one hour before the nicotinamide load. This ensured an abundance of available methyl groups at the time when peak NMN levels are reached. It was expected that NMN, as a marker for methylation, would reflect the increased availability of methyl groups provided by the methionine load. From the results of the trials, it became clear that the methionine load changed neither the plasma NMN concentrations, nor the rates of NMN formation. Differences in results obtained before and after a methionine load, independent of the nicotinamide load used, were not significant and the conclusion of this study is therefore,

that nicotinamide cannot be used as a marker for biological methylation in healthy humans.

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CHAPTER 7

CONCLUDING DISCUSSION

Methionine, in addition to being a requirement for protein synthesis, has other essential functions in the body. It serves, via the active form, S-adenosylmethionine, as the primary methyl donor for the formation of numerous metabolically important methyl containing compounds such as choline, creatine and carnitine. In addition, some compounds, like the vitamin nicotinamide, are biologically inactivated via methylation.

As mentioned in Paragraph 2.4.2, the incidence of neural tube defects in rural, South African communities, are much higher than in their urban counterparts. None of the known risk factors for NTD that has been investigated could account for the observed differences[41]. The rational behind the methylation study was to search for a marker that could be used to investigate suspected impaired methylation in such groups, as it might reveal nutritional insufficiencies and/or genetic predispositions that are not yet known.

Nicotinamide was investigated in this study as the possible marker for methylation. Two trials were conducted where different loads of nicotinamide were given to healthy volunteers, with and without an additional methionine load. The dynamic changes in N-methylnicotinamide levels were measured in the plasma. The results obtained (Chapter 6) indicated that the methionine load changed neither the plasma NMN concentrations, nor the rates of NMN formation. The conclusion of this study was that nicotinamide loading could not be used as a dynamic function test to assess biological methylation in healthy humans.

These results, where the methylation capacity was unaffected by a change in methionine status, are in agreement with the results from a similar study by Jenks et al. They investigated the effect of methionine deficiency on nicotinamide methylation and found that the ability of the liver to methylate high doses of nicotinamide was not affected by a methionine deficient diet. They concluded that the urinary excretion of NMN and 2Py was not simply an excretion pathway of excess nicotinamide, but that it was kept within narrow limits, even when the supply of methionine from the diet was insufficient. The

methylation of nicotinamide therefore must have a higher metabolic priority than the maintenance of the overall protein-synthesis rate.

The first objective of this study was the development of an analytical method for the determination of N-methylnicotinamide in biological samples. The method developed and validated during the course of the study is sensitive, accurate, reproducible and simple to perform. This method can be used to investigate other aspects of nicotinamide metabolism and liver dysfunction.

Various authors [90,94,97] found increased levels of NMN in urine or plasma of patients with cirrhosis. The clinical application of this method, as a screening test for cirrhosis, warrants further investigation.

The cause of the increased excretion of NMN in cirrhosis is still uncertain. As discussed in Paragraph 3.2.4, it might be due to the activation of a dissipative metabolic pathway, or be an indication of increased hepato-cellular proliferation. Cuomo et al[90] concluded that NMN synthesis could serve a complex function rather than being a simple excretory pathway. The analytical method can also be used to investigate these unknown functions of NMN.

Nakagawa et al[112] found that in rats, increased levels of NMN was caused by a higher activity of the enzyme nicotinamide-N-methyltransferase in tumor cells when compared to the enzyme activity in the host liver. Unlike most other liver enzymes, this enzyme activity is unaffected by hepatic inflammation. NMN as a marker for nicotinamide-N-methyltransferase activity could therefore be used to distinguish cancer from other inflammatory diseases and also to detect low tumor budens.

Another possible future application of NMN measurement is in the evaluation of bioartificial liver (BAL) systems. The aim of bioartificial livers is to sustain liver-failure patients until a compatible organ becomes available for transplantation. Many researchers have reported BAL devices utilizing xenogenic hepatocytes or human hepatoblastomas as biological components to reproduce liver functions[113]. In the developing phase of most of these BAL systems, the cells are immobilized in a bioreactor, which is connected to a perfusion circuit that recycles a growth medium

through the reactor. Nicotinamide is one of the essential components contained in the growth medium. Determining the concentration of NMN in the growth medium can easily be used to assess the liver-specific trans-methylation reactions of the cells in the bioreactor. Together with other measurements this can be used to determine the viability of the BAL system to maintain liver functions.

APPENDIX A:

NICOTINAMIDE TOXICITY

Chronic administration of nicotinamide at 3g/day for an extended period (>3months) causes heartburn, nausea, headaches, hives, fatigue, sore throat, dry hair tautness of face and inability to focus eyes. The maximum amount of nicotinamide administered during the trail will be 1.5 mg/kg in a single dose. This implies that a man of average size (75kg) will consume 112.5mg of nicotinamide. This dose is small when compared to the dose of 3mg that was taken daily to produce the symptoms listed. It can be expected that the individuals participating in the trial will not experience any side effects from the nicotinamide loading.

APPENDIX B:

HEALTH QUESTIONNAIRE AND INFORMED CONSENT FORM TO BE COMPLETED BY PROSPECTIVE PARTICIPANTS.

1. Are you taking any vitamin supplements?
2. Are you currently taking any medication?
3. Do you have any kidney problems?
4. Have you ever had any liver disease including jaundice or hepatitis?
5. Do you have a family history of liver disease?

I________________________________, identity number__________________________

Agree to take part in a study at the Department of Chemical Pathology at the University of Pretoria titled: ***N1-methylnicotinamide as marker for biological methylation capacity in healthy humans (Research protocol # 87/97)***

The objectives as well as the procedure of the above mentioned study have been explained to me in full by ____

1. Purpose of the study: The purpose of this study is to determine if the nicotinamide load test will reflect changes in the methylation capacity of the individual that was induced by a methionine load test. If this test can reflect the methylation capacity in healthy humans, it can be used to investigate methylation in groups with associated risk factors e.g. hyperhomocysteinemia, depression and births affected by NTD`s.
2. Procedure of the study: Sampling will take place on four different occasions. On the trail dates, participants will report to the consulting rooms in a fasting state and continue to fast until the last sampling interval. The individuals will have blood drawn from a vein on the arm at five different time intervals. Participants will also supply three urine collections. Participants will take a nicotinamide load on each of the four trial days and a methionine load on two of the trial days.

3. Possible side effects during the study: It is expected that the participants will not experience any side effects due to the nicotinamide load. Methionine might cause slight nausea in some individuals.
4. Benefits: Participants will receive R50.00 for each of the four days.
5. Withdrawal from the study: Any participant may withdraw from the study at any time, without penalty. Written withdrawal should be addressed to Mrs M Rosemann, Department of Chemical Pathology
6. Confidentiality: This study has been approved by the Human Ethics Committee at the University of Pretoria, and is performed according to the guiodelines stated in the Declaration of Helsinki. This implies that the information disclosed by the participant in the completed questionnaire, in addition to all blood and urine test results will be treated as strictly confidential.
7. Participant declaration: I understood all the procedures and test that will be performed during the course of this study. I am participating strictly on a voluntary basis and I am not being influenced by any person to participate in this study. I understand that I may terminate my participation at any time without forfeiting any future or alternative medical treatment. I have the right to be informed, on request, of any information or results that may be obtained during the course of the study, regardless of whether I completed the study or not. I have had sufficient time to consider taking part in the trial and have been given the opportunity to ask any questions about the procedures that will be performed. I am aware that I may contact the person mentioned in section 5 at any time, should any questions concerning the study arise.

   I hereby give my informed consent for the procedures required for this study to be performed in the manner described in this document.

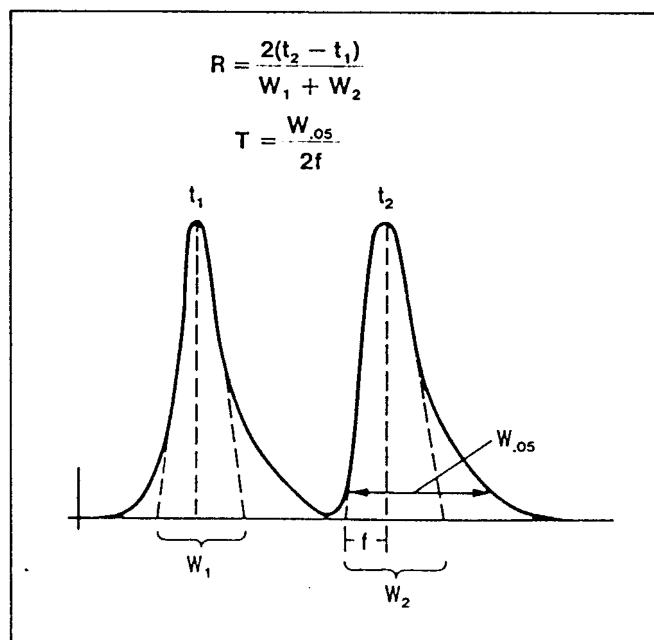
Signed ________________ at _________on this ___day of ____________

Signature of person who explained the project______________________

APPENDIX C

SYSTEM SUITABILITY PARAMETERS

The formulas used in calculating the performance parameters can be explained in the following theoretical chromatogram[110]:



Peak symmetry/tailing $T = W_{0.5}/2W$

It is preferable to avoid tailing through optimisation during the development stage, rather than attempt to control it later. This can be achieved by adjusting the pH or the ratio of organic/inorganic content of the mobile phase. When this value is close to 1, the peak is symmetrical and there is no tailing.

Theoretical plate count is a measure of the performance of the column and will decrease with column deterioration.

Resolution $R = 2(t_2 - t_1) / W_1 + W_2$ measures the ability of the system to separate the peaks of interest.

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PROVINSIALE ADMINISTRASIE

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GAUTENG

================

Enquires: Dr R Sommers

Reference:

Tel: (012) 354 1560

Fax: (012) 329 2256

Address: Ethics Committee
Ward 4 Room 19
Pretoria se
Akademiese Hospitale
Private Bag x 169
PRETORIA
0001

Date: 21-05-1997

Nommer : 87/97

TITEL : N-metielnikotienamied as 'n merker van metileringskapasiteit.

AANSOEKER : Mnr L Verhave; Dept. Chemiese Patologie; Pretoria se Akademiese Hospitale; PRETORIA.

This study has been considered by the Ethics Committee, Faculty of Medicine, Univ. of Pretoria and Pretoria Academic Hospital on 21-05-1997 and found to be acceptable.

Prof A.L. Coetzee	MA (Clin Psych); DSocSc (Leiden); MPA (Pret): Psychologist
Dr J.E.Davel (female)	MBChB: Hospital Superintendent
Prof A.P.du Toit	BA; Dipl Theo (Pret) BA (Hons) (Rhodes);MA;DPhil(Pret):Philosopher
Prof C.I. Falkson (female)	MBChB;M.Med(Int);MD;Med.Oncologist
Prof G. Falkson	MBChB;M.Med(Int);MD;OSG:Medical Oncologist
Dr A.G.S. Gous	MA; DPhil: Pastoral Psychologist
Prof S.V. Grey (female)	BSc (Hons)(Stell); MSc (PU vir CHO) DSc (Pret): Deputy Dean
Dr S.W. Johnson	MBChB: Hospital Superintendent
Dr V.O.L. Karusseit	MBChB; MFGP (SA); M.Med (Chir); FCS (SA): Surgeon
Ms B.C.F. Magardie(female)	B Cur: Matron/ Senior Nursing Sister
Senior Sr J. Moerane(female)	BCur (E et Al)Senior Nursing Sister
Prof T.R. Mokoena	MBChB; FRCS (Glasgow); DPhil (Oxford): Surgeon
Prof H.W. Pretorius	MBChB;M.Med (Psych)MD: Psychiatrist
Dr P. Rheeder	MBChB; MMed (Int); LKI (SA); MSc (KLIN.EPI): Specialist Physician
Prof J.R. Snyman	MBChB, MPharm Med: Pharmacologist
Prof De K Sommers	BChB; HDD; MBChB; MD: Pharmacologist
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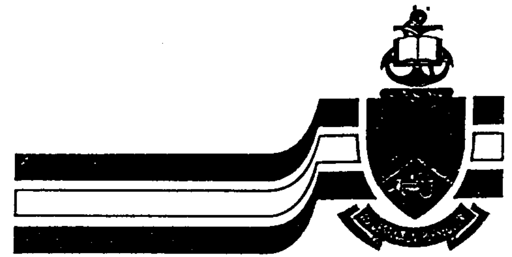
Prof F.W. van Oosten	BA; LLB; LLD (Pret); LLD (Unisa): Prof in Criminal and Medical Law

PROF G. FALKSON; MBChB; M.Med (Int); MD; OSG
CHAIRMAN



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Instituut vir Patologie

8 Desember 1998

Die Voorsitter
Navorsingsprotokol en Etiese Komitee
Saal 4 – Kamer 19
Pretoria Akademiese Hospitaal
PRETORIA
0002

Geagte Prof Falkson

NAVORSINGSPROTOKOL 87/97

Hierdie protokol is deur 'n nagraadse student onder leiding van Prof Ubbink aan die Etiese komitee voorgelê. Die student het egter nie gevorder nie en is na 'n paar maande weg. 'n Nuwe personeellid van die Departement wil nou met dieselfde protokol aangaan.

Die navorsing sal deur mev M Rösemann steeds onder leiding van Prof Ubbink uitgevoer word.

Dit sal waardeer word indien die komitee goedkeuring hiervoor sal verleen.

By voorbaat dankie

[signature]

ANNATJIE VAN DER MERWE
NAVORSINGSKOÖRDINEERDER

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