KINETIC STUDIES OF VITAMIN B_6 METABOLISM IN HUMANS

by

Christian Abraham van der Westhuizen

A thesis submitted in fulfilment of part of the requirements for the degree of **Master of Science** in the Faculty of Medicine (Department of Chemical Pathology), University of Pretoria.

January 2001

CONTENTS

CHAPTER 4 : VITAMER B_6 ENZYME KINETIC STUDIES

CHAPTER 5 : CONCLUDING DISCUSSION

ACKNOWLEDGEMENTS

The author wishes to thank :

Prof JB UBBINK, Department of Chemical Pathology, University of Pretoria, supervisor of this project, for his continuous guidance, advice, encouragement and *never-ending* patience.

The ***STAFF*** of the Department of Chemical Pathology, University of Pretoria, especially ***Mrs A SCHNELL*** for excellent technical assistance, ***Dr R DELPORT*** for statistical analysis of experimental data and ***Mrs L GODDARD*** for inspiration and advice.

Lt Col (Dr) J LUBBE, Head of the Department of Pathology, 1 Military Hospital, for his invaluable contribution regarding the theoretical aspects of Chemical Pathology included in this curriculum as well as the *opportunity* granted to undertake this study .

The ***STAFF*** of the Chemical Pathology and Haematology Departments, 1 Military Hospital, for their continuous support, understanding and encouragement.

His ***FRIENDS*** and late ***PARENTS*** for their persistent enthusiasm and inspiration.

LIST OF FIGURES

LIST OF TABLES

"Science is the captain
Practise is the soldier"
(Leonardo da Vinci)

CHAPTER 1 : GENERAL INTRODUCTION

1.1 STRUCTURE

Gÿorgy discovered a difference between rat pellagra preventive factor and vit B_2 in 1934. He called this vit B_6 (**1**). Animals fed purified diets supplemented with concentrates of thiamine and riboflavin did not show good growth and developed a dermatitis called acrodynia. This condition was prevented by the addition of yeast and was traced to the vit B-complex of the water soluble fraction (**2**). Vit B_6 is unique among the water soluble vitamins with respect to its chemistry, metabolism and the numerous functions it serves. Vit B_6 is the recommended term for the generic descriptor of all 3-hydroxy-2-methylpyridine derivates. **Figure 1.1** depicts the various forms of vit B_6, including the phosphorylated forms. The trivial names and abbreviations commonly used for the three principal forms of vit B_6, their phosphoric esters, and their analogues are as follows: pyridoxine, *PN* ; pyridoxal, *PL* ; pyridoxamine, *PM* ; pyridoxine -5'- phosphate, *PNP* ; pyridoxal -5'- phosphate, *PLP* ; pyridoxamine -5'- phosphate, *PMP* ; 4-pyridoxic acid, *4-PA*.

Generally, as a group, B_6 vitamers are considered labile. In solution the forms are light sensitive (**3, 4**), but this sensitivity is influenced by pH. *PN*, *PM* and *PL* are relatively heat stable in an acid medium, but are heat labile in an alkaline medium.

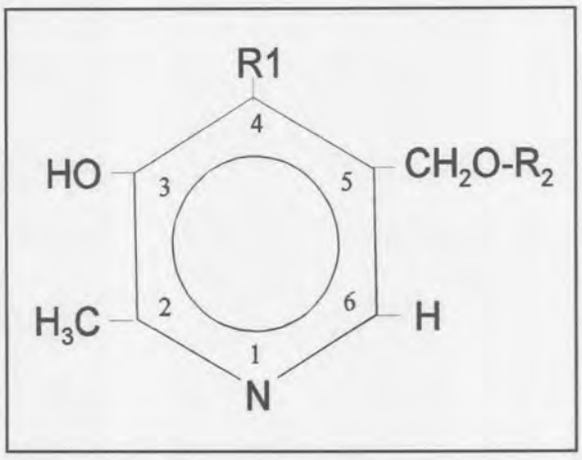


Figure 1.1 : Structure of B_6 vitamers and 4-pyridoxic acid.

PN : R_1=CH_2OH
R_2=H
PM : R_1=CH_2NH_2
R_2=H
PL : R_1=CHO
R_2=H
4-PA : R_1 = COOH

PNP : R_2=$PO_3^=$
R_1=CH_20H
PMP : R_1=CH_2NH_2
R_2=$PO_3^=$
PLP : R_1=CHO
R_2=$PO_3^=$

1.2 METABOLISM

Lumeng, Li and co-workers have shown that the liver is the primary organ responsible for metabolism of vit B_6 and supplies the active form of vit B_6, *PLP*, to the circulation and other tissues (**5, 6**). *PL, PM* and *PN* are converted to their respective phosphorylated forms by a kinase enzyme (pyridoxal kinase EC 2.7.1.35) which requires both zinc and ATP (as a source of the phosphate group). The two phosphorylated forms pyridoxamine -5'- phosphate and pyridoxine -5'- phosphate are converted to *PLP* via a flavin mononucleoticle (FMN) requiring oxidase (**7**). Dephosphorylation of the 5'- phosphate compounds occur by action of a phosphatase which occurs in the circulation and enzyme bound in the liver (**8**). *PLP* is the physiologically active form of vit B_6 and is largely albumin bound and can therefore not be transferred over cellular membranes (**9**). This binding prevents hydrolysis and allows for the delivery of *PLP* to other

tissues. Merrill *et al* suggested that the enzyme pyridoxal oxidase converts pyridoxal to 4-pyridoxic acid (**10**). *4-PA* is excreted by the kidneys. Accumulation is thus prevented by this mechanism. Within the circulating fluid, the erythrocyte also appears to play an important role in the metabolism and transport of vit B_6. Both *PN* and *PL* are rapidly taken up by simple diffusion (see **Figure 1.2**). In erythrocytes, *PN* and *PL* are converted to *PLP* since both kinase and oxidase activity are present. The *PLP* formed can then be converted to *PL* by the action of a phosphatase.

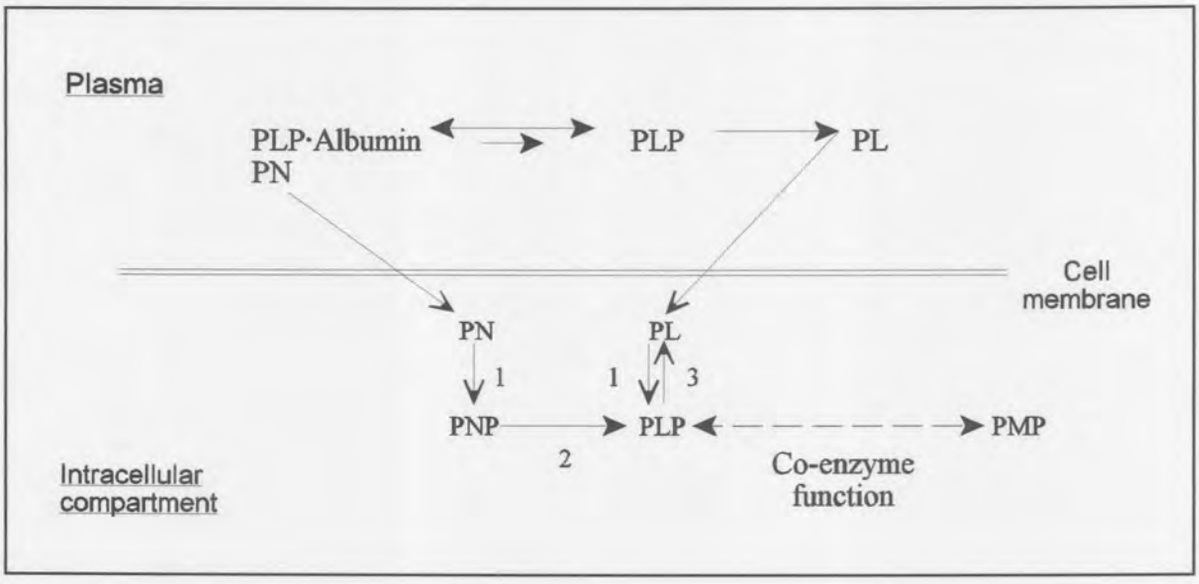


Figure 1.2 : Proposed relationship between plasma *PLP*, plasma *PL*, and erythrocyte vit B_6 metabolism.

Plasma *PLP* cannot cross cellular membranes, and the cell is dependant on circulating pyridoxal levels to obtain adequate amounts of vit B_6. Intracellularly, pyridoxal is phosphorylated to *PLP* by pyridoxal kinase. Reactions catalysed by : pyridoxal kinase (1), *PMP* (*PNP*) oxidase (2), and *PLP* phosphatase (3) [adapted from **11**].

1.3 HUMAN VIT B_6 REQUIREMENTS

The importance of vit B_6 in human metabolism is well established and summarized in **Table 1.1**:

Table 1.1 : Cellular processes affected by pyridoxal -5'- phosphate.

Function / System influenced	**Cellular process or enzyme**
Immune function	1-carbon metabolism, hormone modulation
Gluconeogenesis	Glycogen phosphorylase, transamination
Niacin formation	Tryptophan metabolism
Red cell metabolism and formation	Haem synthesis, transamination, O_2 affinity
Nervous system	Neurotransmitter synthesis, lipid metabolism
Hormone modulation.	Hormone modulation → binding of *PLP* to lysine on hormone receptor.

It is a well known fact that certain factors influence vit B_6 requirements and thus status in humans.

These are listed below [adapted from Machlin *et al* (**12**)] :

a. Dietary

1. Physical structure of a food and bioavailibility.
2. Binding forms of vit B_6.
3. Forms of vit B_6 due to processing.
4. Protein and carbohydrate intake.

b. Defect in delivery to tissues

1. Impaired gastrointestinal absorption.
2. Impaired transport-albumin, synthesis and binding, phosphatase activity.

c. Physiological / Biochemical

1. Physical activity → increased loss, gluconeogenesis.
2. Protein-enzyme induction.

3. Increased catabolism / turnover-phosphatase activity, illness.

4. Impaired phosphorylation and/or interconvention, competing pathways, nutrient deficiencies, drugs.

5. Pregnancy → demand of fetus.

6. Growth → increased cell mass, anabolism.

7. Lactation.

8. Excretion → urinary and menstrual loss.

9. Sex → differences in metabolism.

10. Age → differences in metabolism.

d. Genetic

1. Apoenzyme defects → altered binding to apoenzyme.

2. Altered apoenzyme levels → biochemical individuality.

e. Disease : Examples are discussed on pages **6** and **7**.

f. Drugs

1. Isoniazid.

2. Theophylline.

Evidence to date by Middleton, Henderson and Serebro *et al* indicates that pyridoxine and the other two major forms of vit B_6 are absorbed by a nonsaturable, passive process. (**13 - 16**). Absorption of the phosphorylated forms can occur but only to a very limited extent (**17, 18**). Dephosphorylation of the 5'- phosphate compounds occurs by action of a phosphatase which is considered to be alkaline phosphatase (**19**). Pyridoxal, arising from dephosphorylation, is converted to 4-pyridoxic acid by either an NAD-dependant dehydrogenase or a FAD-dependant aldehyde oxidase (**10**) .

Bioavailability of a nutrient from a given food is important to an organism in that it is the amount of a nutrient that is both absorbed and available to the cells. Vit B_6 that is not needed by the cell is simply excreted or metabolized to a non-utilizable form, e.g. *4-PA*.

A glycosylated form of vit B_6 has been identified and subsequently quantitated in several foods (**20**). Studies have shown that the bioavailability in certain foods correlated inversely with the level of glycosylated vit B_6. Kabir *et al* found that 50 % of the vit B_6 present in orange juice is pyridoxine 5' -ß glucoside (**21, 22**). Nelson *et al* found that the vit B_6 from orange juice was only 50 % as well absorbed as crystalline pyridoxine (**23**). The presence of other forms of vit B_6 and /or binding of specific forms of vit B_6 to other components in food as well as methods used in determining bioavailibility all play a major role in nutrient availability. Other important dietary factors influencing vit B_6 status, e.g. protein intake, may determine the daily requirement of vit B_6 (**24**). Amino acids resulting from a protein intake greater than need for growth and maintenance are catabolized, and this process requires increased transaminase levels and thus greater need for *PLP* in tissues. Excessive carbohydrates in the diet are also known to depress *PLP* concentrations (**25**).

Decreased plasma *PLP* levels with increasing age (**26**) and exercise (**27**) have been reported. Furthermore, sex differences in plasma *PLP* levels show a tendency to be lower in females compared to males. Numerous studies of pyridoxine ↔ drug interactions have been reported of which methylxanthines, penicillamine and isoniazid are known to depress plasma *PLP* (**11, 28 - 30**).

There are various diseases or pathological conditions in which vit B_6 metabolism is altered. The primary indicator of an alteration in the metabolism of vit B_6 has been in the evaluation of tryptophan metabolism or plasma pyridoxal -5'- phosphate (*PLP*) concentration. Disease states,

e.g. asthma, diabetes, certain cancers, pellagra and rheumatoid arthritis may alter tryptophan metabolism independent of a direct effect on vit B_6 metabolism. This also applies for certain hormonal influences **(31)**. Diseases and pathological conditions in which plasma *PLP* concentrations have been shown to be depressed include asthma **(32)**, diabetes **(33)**, renal disorders **(34)**, alcoholism **(35)**, heart disease **(36, 37)**, pregnancy **(38)**, breast cancer **(39)**, Hodgkins' disease **(40)** and sickle cell anaemia **(41)**. Hypophosphatasia is an example in which plasma *PLP* levels are markedly elevated in some individuals **(19)**. Vit B_6 in the form of *pyridoxine hydrochloride* has been used as a therapeutic agent to treat a variety of disorders including Downs' syndrome **(42)**, autism **(43)**, hyperoxaluria **(44)**, gestational diabetes **(45)**, premenstrual syndrome **(46)**, carpal tunnel syndrome **(47, 48)**, depression **(49)**, diabetic neuropathy **(50)** and epileptic seizures **(51)**. It must be emphasized that the extent to which pyridoxine was effective in treating these diseases or reducing symptoms has been variable. *Pyridoxine hydrochloride* is a safe therapeutic agent, but very high doses (>200 mg/day) may result in sensory neuropathy. **(52, 53)**.

1.4 ASSESSMENT OF VIT B_6 STATUS

The assessment of vit B_6 status is central to an understanding of vit B_6 nutrition in humans. A variety of methods have been utilized to assess vit B_6 status. Machlin *et al* **(12)** summarizes it as follows :

a. Direct

1. Plasma concentrations of :

- Plasma pyridoxal -5'-phosphate (*PLP*).

- Plasma pyridoxal (*PL*).

- Plasma total vit B_6.

2. Urine

- 4-pyridoxic acid (*4-PA*).

- Total vit B_6.

b. Indirect

1. Erythrocyte enzyme activities

- Erythrocyte *PLP* phosphatase, *PMP* (*PNP*) oxidase, *PL* kinase.

- Erythrocyte alanine aminotransferase.

- Erythrocyte aspartic aminotransferase

2. Dynamic function tests

- 2 gram Tryptophan load test (Xanthurenic acid).

- 3 gram Methionine load test (Cystathionine).

c. Assessment of dietary intake

- Vit B_6 intake → weekly average.

- Vit B_6 : protein ratio.

The measurement of vit B_6 in biological material is complicated by ; (a) the natural occurrence of vit B_6 in six different forms : Pyridoxine (*PN*), Pyridoxal (*PL*), Pyridoxamine (*PM*) and their phosphorylated derivatives ; (b) the relatively low levels of vit B_6 in most biological samples ; (c) the water solubility of vit B_6 and (d) the photosensitivity of vit B_6. Various methods in vit B_6 analysis have been developed, e.g. enzymic methods (**54**), use of microbiological assays (**55**), direct fluorometric quantification of *PLP* and *PL* (**56**) and several methods based on high performance liquid chromatography [HPLC] (**57 - 59**). Few, if any, are suitable for routine

analysis due to the lack of sensitivity, long analysis times and high costs involved for the simultaneous analysis of all six natural forms of vit B_6 at low concentrations encountered in biological material. To conduct this research, it was necessary to use available analytical methods that are suitable for analysis of B_6 vitamers at low concentrations encountered in biological material. The methodology should be simple enough to conduct large scale population studies on vit B_6 nutritional status, yet comprehensive enough to allow for detailed pharmacokinetic studies of *PN* supplementation in humans. Ubbink showed that *PLP* semicarbazone (*PLPSC*) and *PL* semicarbazone (*PLSC*) can be separated by reverse phase chromatography (**61**). Furthermore it has been shown that urinary *4-PA* concentrations may be used as an index of vit B_6 status in large scale population studies. *4-PA* lactone, which may be efficiently separated from interfering background components using cation exchange HPLC, combines the advantages of lactonization (high sensitivity) and HPLC (specificity) in urine *4-PA* analysis (**60 - 62**).

The plasma pyridoxal -5'- phosphate concentration is considered one of the better indicators of vit B_6 status (**63**). Studies that have indicated that the plasma *PLP* content is the better indicator of the body store of vit B_6 (**64, 65**), have recently been questioned, and determination of plasma pyridoxal (*PL*) in addition is also recommended (**66**). The fact that the physiologically active form of vit B_6 is intracellular *PLP*, questions the clinical significance of elevated plasma *PLP* that is largely albumin bound and not transferrable over cellular membranes (**9**). Plasma *PL,* on the other hand, being a less polar compound than *PLP,* is directly available for cellular metabolism. The urinary excretion product of vit B_6 metabolism, 4-pyridoxic acid (*4-PA*), is considered a short term indicator of vit B_6 status (**65**).

1.5 POPULATION DIFFERENCES IN VIT B_6 STATUS

Although Driskell *et al* found that vit B_6 status parameters of middle-aged black obese women were similar to those previously reported for non-obese white women (**67**), studies by Vermaak *et al* indicated that blacks have lower circulating *PLP* levels compared to whites (**68**). No satisfactory explanation for this phenomenon has yet been found. In this thesis, a systematic investigation of vit B_6 metabolism in blacks and whites is described in order to find possible explanations for the lower vit B_6 status in blacks. The **first phase** of this study was undertaken to confirm previous findings of observed differences in plasma *PLP* concentrations in humans. Furthermore, three enzymes that are involved in vit B_6 metabolism are expressed in the erythrocyte (**67, 69 - 71**). Human erythrocytes cannot oxidize *PL* to the end product of vit B_6 metabolism, *4-PA,* therefore erythrocytes release *PL* to the plasma and thus contribute to plasma *PL* levels, which presumably serve as a transport form of vit B_6 (**71 - 74**). This makes the erythrocyte a convenient cell system to study vit B_6 metabolism. Activities of the enzymes contained in the erythrocytes, pyridoxal kinase (EC 2.7.1.35), pyridoxamine -5'- phosphate (pyridoxine -5'-phosphate) oxidase (EC 1.4.3.5) and pyridoxal -5'- phosphate phosphatase were measured in blacks and whites and expressed as product formed / gram haemoglobin / hour. These findings are reported in chapter two.

Limited pharmacokinetic studies that have been performed show mono- and bi-exponential elimination rate constants for plasma *PLP* and *PL* respectively (**75, 76**). Wozenski *et al* reported that total plasma vitamer B_6 concentrations increased six fold more than pyridoxal -5'- phosphate after small doses of pyridoxine had been administered (**77**). Lumeng and Henderson *et al* demonstrated that plasma *PLP*, *PL* and *4-PA* excretion increased after a single oral dose of

pyridoxine hydrochloride (10-25 mg) had been administered, and that irrespective of whether the dose was administered orally or intravenously, the pyridoxine was cleared rapidly from the circulation (**64, 78**). During the **second phase**, I studied the pharmacokinetics of a crystalline *pyridoxine hydrochloride* supplementation in white and black young men. This was done to investigate whether racial differences in appearance rate, distribution, metabolism and / or excretion could predispose to the observed differences in plasma *PLP* concentration mentioned above. These results are reported in chapter three.

Finally as stated earlier, the erythrocyte contains the enzymes *PL* kinase, *PMP* (*PNP*) oxidase and *PLP* phosphatase that play an active role in vit B_6 metabolism. The **third phase** of this study investigates whether racial differences in vit B_6 status may be explained by differences in enzyme kinetics. Estimation of enzyme kinetic parameters, e.g. apparent Michaelis-Menten constants and maximum velocity rates were determined in erythrocytes from black and white male volunteers. These results are reported in chapter four.

CHAPTER 2 : DETERMINATION OF RANDOM PLASMA *PLP* CONCENTRATION AND B_6 VITAMER ENZYME ACTIVITIES IN ERYTHROCYTES FROM BLACKS AND WHITES

2.1 INTRODUCTION

To confirm previous observations that blacks have lower circulating plasma *PLP* levels than whites (**68**), the **first phase** of this study was undertaken. The *PLP* and *PL* in plasma as well as perhaps the *PL* in erythrocytes represent the major B_6 vitamers available to the tissues. A difference in plasma *PLP* between blacks and whites, which reflects long term vit B_6 nutritional status, warrants further exploration of B_6 metabolism regarding total enzyme activity. The simultaneous quantification of plasma *PLP* and *PL* by HPLC as described by Ubbink *et al* (**61, 62, 79**), makes the erythrocyte a convenient cell system to study vit B_6 metabolism. *PL* kinase and *PMP* (*PNP*) oxidase activities are monitored by *PLP* production from *PL* and *PMP* respectively. *PLP* phosphatase activity, on the other hand, is determined by release of *PL* after *PLP* dephosphorylation.

2.2 MATERIALS AND METHODS

2.2.1 EXPERIMENTAL PROTOCOL

2.2.1.1 Study design and participants

A group of 200 male volunteers between the ages of 18-28 years were recruited from the South African National Defence Force (SANDF) where they were exposed to the same socio-economic environment and same diet (basic mess diet). In this cross-sectional, non-randomized, single blind

(to the laboratory analyst) study, a single plasma- and serum blood-sample were collected, firstly to apply exclusion criteria and secondly to determine vit B_6 status (plasma *PLP* and total B_6 vitamer enzyme activities). After applying the exclusion criteria listed below, 71 white- and 54 black male subjects from this group were selected to participate in the **first phase** of this study.

2.2.1.2 Exclusion Criteria (See Laboratory request form - **APPENDIX 1**).

Exclusion criteria that were applied include: **(28, 81)**

a. Vit B_6 status.

Subjects with [*PLP*] > 150 and < 20 nmol/L were excluded.

b. Chemotherapy → under sections 'Treatment / Clinical diagnosis' on the laboratory request form.

c. Anaemic conditions.

Subjects with a haemoglobin concentration of < 11.0 g/L were excluded.

d. Infection : Exclusion according to 'Clinical diagnosis' on laboratory request form.

e. Liver-/ Renal impairment → activities of serum AST, ALP, urea & creatinine were determined.

Exclusion criteria applied : **(81)**

1. Aspartate aminotransferase : > 42 IU/L.

2. Alkaline phosphatase : > 128 IU/L.

3. Creatinine : > 115 μmol/L.

4. Urea : > 8.4 mmol/L.

f. Medication : exclusion according to laboratory request form under section 'Treatment':

(28) e.g. 1. Isoniazid.

2. Theophylline.

3. Penicillamine.

2.2.2 ANALYTICAL TECHNIQUES

2.2.2.1 Plasma *PLP* analysis

2.2.2.1.1 Sample collection

Venous blood-samples were collected with EDTA as anti-coagulant from the study participants. Plasma and blood cells were separated after centrifugation (2500 g, 5 minutes) [Clements, Sydney, Australia]. Approximately 2-3 mL of plasma were stored at -25 °C until HPLC analysis.

2.2.2.1.2 Standard and sample preparation

2.2.2.1.2.1 Reagents used in standard preparation

a. 10 % TCA (trichloroacetic acid).

b. 0.5 mol/L semicarbazide.

c. Standard Precipitation solution.

- 56 mL H_20.
- 28 mL 10 % TCA.
- 16 mL 0.5 mol/L semicarbazide.

d. Precipitation solution.

- 50 mL 10 % TCA.
- 30 mL 0.5 mol/L semicarbazide.

e. Standards : 10 mg *PL* - & 20 mg *PLP* in 200 mL H_2O, of which 100 μL were diluted to 25 mL in standard precipitation solution. This standard was incubated for 30 min at 37 ^{0}C to facilitate the formation of *PLP* - and *PL* semicarbazone.

2.2.2.1.2.2 Dilutions used to obtain standard (calibration) curve : See **Table 2.1**.

Table 2.1 : Standard curve concentrations for *PLP* and *PL* respectively (obtained from dilution of stock standard - see section 2.2.2.1.2.1.e)

DILUTION (mL)	*PLP* (nmol/L)	*PL* (nmol/L)
0.5 : 50	15.1	9.8
1.0 : 50	30.2	19.6
1.5 : 50	45.2	29.5
2.0 : 50	60.3	39.3
2.5 : 50	75.4	49.1

2.2.2.1.2.3 Standard preparation

1.0 mL of each standard respectively (**Table 2.1**) and 1.0 mL of precipitation reagent were mixed and a 500 μL aliquot was transferred to the autosampler vials used in HPLC analysis.

2.2.2.1.2.4 Sample preparation

1.0 mL of sample was added to 1.0 mL of precipitation solution, vortexed and incubated for 30 min at 37 °C. Samples were then centrifuged for 20 min at 2500 g [Beckman Model J-6B centrifuge] and carried over directly to the autosampler vials used in HPLC analysis. Throughout the assay procedures samples and substrate solutions were protected from light, as vit B_6 compounds are light sensitive (**3, 4**).

2.2.2.1.3 HPLC analysis

A Perkin Elmer [Norwalk, Conn, USA] series II liquid chromatograph was fitted with a Phenomonex [Torrano, Calif, USA] Prodigy 5 ODS 002 column (particle size : 10 μM). A 0.05 mol/L potassium dihydrogenphosphate buffer (pH adjusted to 2.9 with concentrated phosphoric acid) containing 7% acetonitrile was used as mobile phase at a flow rate of 1.1 mL/min. A

Beckman model 112 [Fullerton, Calif, USA] delivery pump was introduced for constant NaOH (3% w/v) post-column alkalinization at a flow rate of 0.1 mL/min. A Hitachi F2000 [Tokyo, Japan] fluorescence spectrometer was used for fluorescence detection (excitation wavelength 367 nm, emission wavelength 478 nm) with spectral slit widths chosen to give a bandpass of 10 nm. Maximum fluorescence was obtained in the pH range 11.5-12.0. To minimize pH fluctuations that would inevitably introduce considerable error in both *PLP* and *PL* quantification, pH was regularly monitored with Acilit pH strips [Merck, Darmstadt, Germany]. The HPLC procedure was fully automated by employing a Waters (TM) 717 autosampler [Millipore Waters, Minn, USA]. Finally a Windows 95 compatible computer programme [Chromspec CC, Peak Software, RSA] was used to process data. Before analysis of each batch of samples, 5 standards were used for calibration (see **Tables 2.1-2**) and quality control was assured by means of analysing a lypophilized human plasma control during each run.

2.2.2.2 Analysis of B_6 vitamer enzyme activity in erythrocytes

2.2.2.2.1 Sample collection

The red cells collected from venous blood with EDTA as anti-coagulant were washed twice with 0.9 % saline. A 100 μL volume of packed erythrocytes was added to 300 μL of CPP - Buffer (30 mmol/L NaH_2PO_4, 16.8 mmol/L Na_2HPO_4 and 109 mmol/L trisodiumcitrate in solution at pH 6-8 (mixed with glycerol in ratio 6:4), and stored at -25 °C until enzyme assays could be performed.

2.2.2.2.2 Sample and standard preparation

2.2.2.2.2.1 Reagents used in standard preparation

a. Reagents

As for plasma *PLP* and *PL* determination - see section 2.2.2.1.2.1.

b. Standards

30 mg *PL* - & 20 mg *PLP* in 200 mL H_2O, of which 500 μL were diluted to 25 mL. This standard was incubated for 30 min at 37 ^{0}C and protected from light as far as possible (77).

2.2.2.2.2.2 Standard preparation - see section 2.2.2.1.2.3

1.0 mL of each diluted standard (see **Table 2.2**) and 1.0 mL of precipitation reagent were mixed and carried over directly into the autosampler vials.

Table 2.2 : Standard curve concentrations for *PLP* and *PL* respectively (obtained from dilution of stock standard - see section 2.2.2.2.1.b).

Dilution (mL)	***PLP* (nmol/L)**	***PL* (nmol/L)**
0.5 : 50	75.4	147.3
1.0 : 50	150.8	294.6
1.5 : 50	226.2	441.9
2.0 : 50	301.6	589.2
2.5 : 50	377.0	736.5

2.2.2.2.2.3 Sample preparation

Stored patient samples (erythrocyte-CPP buffer) were thawed, centrifuged and CPP buffer removed. A 200 μL volume of packed erythrocytes was added to 1.0 mL of haemolysis buffer (10 mmol/L triethanolamine containing 0.1 % Triton X-100 : pH 7.4). For vit B_6 enzyme assays the haemolysates were diluted as follows :

a. *PMP (PNP)* oxidase : 500 μL haemolysate was diluted to 1.0 mL using 500 μL of **Buffer A** [10 mmol/L triethanolamine (TEA), 160 mmo/L dipotassium

hydrogen phosphate (K_2HPO_4) and 2 mmol/L magnesium chloride ($MgCl_2$) : pH 7.4].

b. *PL* kinase : 150 μL haemolysate was diluted to 1.0 mL using 850 μL of **Buffer B** [10 mmol/L triethanolamine (TEA), 90 mmol/L dipotassium hydrogen phosphate (K_2HPO_4), 2 mmol/L magnesium chloride ($MgCl_2$) and 2 mmol/L ATP : pH 7.4].

c. *PLP* Phosphatase : 25 μL haemolysate was diluted to 1.0 mL using 975 μL of **Buffer C** [50 mmol/L triethanolamine (TEA), and 5 mmol/L magnesium chloride ($MgCl_2$) : pH 7.4].

The diluted haemolysates were incubated for 10 min at 30 °C. The reactions were started by adding 50 μL of 2.1 mmol/L substrate → *PL* for *PLK*, *PMP* for *PMP* (*PNP*) oxidase and *PLP* for *PLP* phosphatase solutions. Incubation times were 20 min for the *PLP* phosphatase assay, and 60 and 120 min for *PL* kinase and *PMP* (*PNP*) oxidase assays, respectively. After the incubation period, the reactions were stopped by adding 1.0 mL of precipitation solution (10 % trichloroacetic acid and 0.5 mmol/L semicarbazide). The mixture was mixed and incubated for 30 min at 40 °C. The clear supernatant obtained after centrifugation was used for HPLC analysis. Throughout the assay procedure, samples and substrate solutions were protected from light because of the light sensitive characteristics of vitamin B_6 (**3**, **4**).

2.2.2.2.3 HPLC analysis

As described in section 2.2.2.1.3. for plasma *PLP* and *PL*. Longer retention times during HPLC analysis obtained, could be explained by a difference in acetonitrile concentration which was used as mobile phase - see **Figures 2.2-4.**

2.2.2.2.4 Measurement of haemoglobin concentration

Haemoglobin concentrations were determined in the haemolysates by using the spectrophotometric method of Drabkin & Austin (**80**). Analysis was carried out on a Perkin Elmer [Norwalk, Conn, USA] Lambda Series 2 PECSS spectrophotometer.

2.2.2.2.4.1 Ferricyanide reagent.

200 mg potassium ferricyanide [$K_3Fe(CN)_6$], 50 mg potassium cyanide (KCN), and 150 mg potassium dihydrogen phosphate (KH_2PO_4) were diluted to 1000 mL of H_20 at pH 7.0-7.4.

2.2.2.2.4.2 Haemoglobin standards

Human whole-blood with EDTA as preservative was obtained from the Department of Haematology [Pretoria Academic Hospital]. The Hb standards used in the curve were obtained by diluting whole blood with haemolysate buffer. See section 2.2.2.2.3. For a haemoglobin (Hb) concentration of 13.9 g/100 mL the following dilutions were performed :

Table 2.3 : Haemoglobin standard curve dilutions.

Standard	Whole blood (μL)	Haemolysate Buffer (μL)	Curve Hb (g/100 mL)
1	50.0	50.0	7.0
2	50.0	100.0	4.6
3	50.0	150.0	3.5
4	50.0	200.0	3.2
5	50.0	250.0	2.7

2.2.2.2.4.3 Method

50 μL haemolysate or 50 μL of standard was added to 2.5 mL of ferricyanide reagent. After vigorous mixing the samples were left for at least 4 minutes. Absorbance was determined at 540 nm within 30 minutes.

2.2.2.2.5 Calculation of enzyme activity in erythrocytes

Enzyme activity for the different enzymes were calculated as nmol product formed / g Hb / hour. Calculations based on the different dilutions performed were obtained from equations a-c.

a. **Pyridoxal kinase** :

$$\frac{[PLP]\ (\text{nmol/L})}{1.5 \times \text{Hb (g/dL)}}$$

b. **Pyridoxamine (pyridoxine -5'- phosphate) oxidase** :

$$\frac{[PLP]\ (\text{nmol/L})}{10 \times \text{Hb (g/dL)}}$$

c. **Pyridoxal -5'-phosphate phosphatase** :

$$\frac{12 \times [PLP]\ (\text{nmol/L})}{\text{Hb (g/dL)}}$$

2.2.3 STATISTICAL ANALYSIS

The Student t-test with a significance level of 0.05 was used to compare plasma *PLP* and the respective enzyme activities in blacks and whites by means of a computer software programme, Statistix version 4.1.

2.3 RESULTS

Figures 2.1-4 show examples of chromatograms obtained using HPLC in determination of *PLP* concentration and the respective total enzyme activities in 71 white- and 54 black male subjects. **APPENDIX 2** tables the plasma *PLP* concentrations and the respective vitamer B_6 enzyme activities as product formed / gram haemoglobin / hour obtained by HPLC in the respective

groups. Results summarized in **Table 2.4** and **Figures 2.5-8** (histograms), give the mean concentrations calculated during the first phase of this study. Two subjects from group 1 (whites) and one subject from group 2 (blacks) were omitted because they were found to use vitamin supplements on the basis of very high measured plasma *PLP* (> 150 nmol/L) concentration. They therefore did not comply with all the exclusion criteria as discussed in section 2.2.1.2.a.and were excluded from statistical analysis.

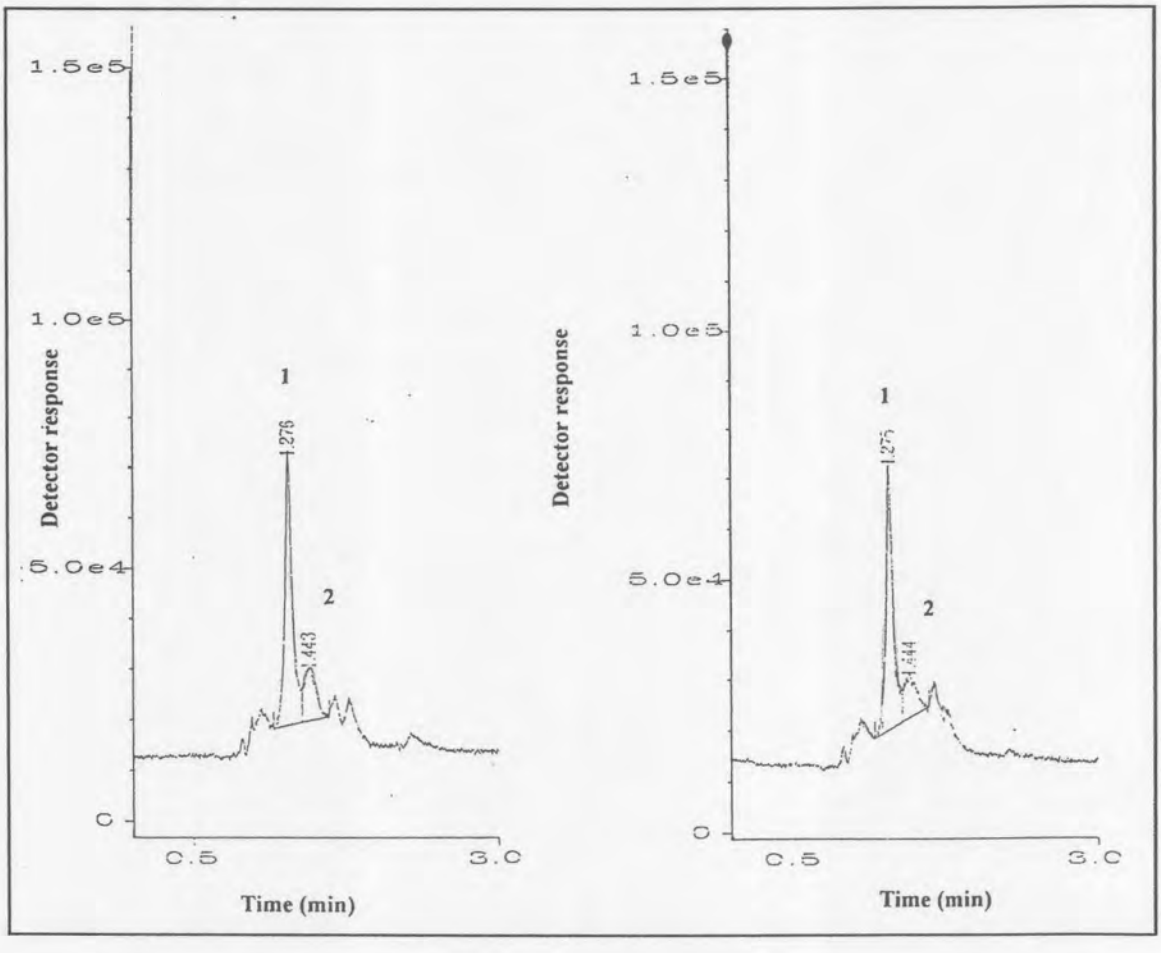


Figure 2.1 : Chromatogram shows the determination of plasma *PLP* and *PL* using HPLC. Example shows two subject analysis with retention times of 1.276 and 1.275 minutes respectively.

Peaks : 1 = *PLPSC* ; Peak 2 = *PLSC*

15938918
b15348465

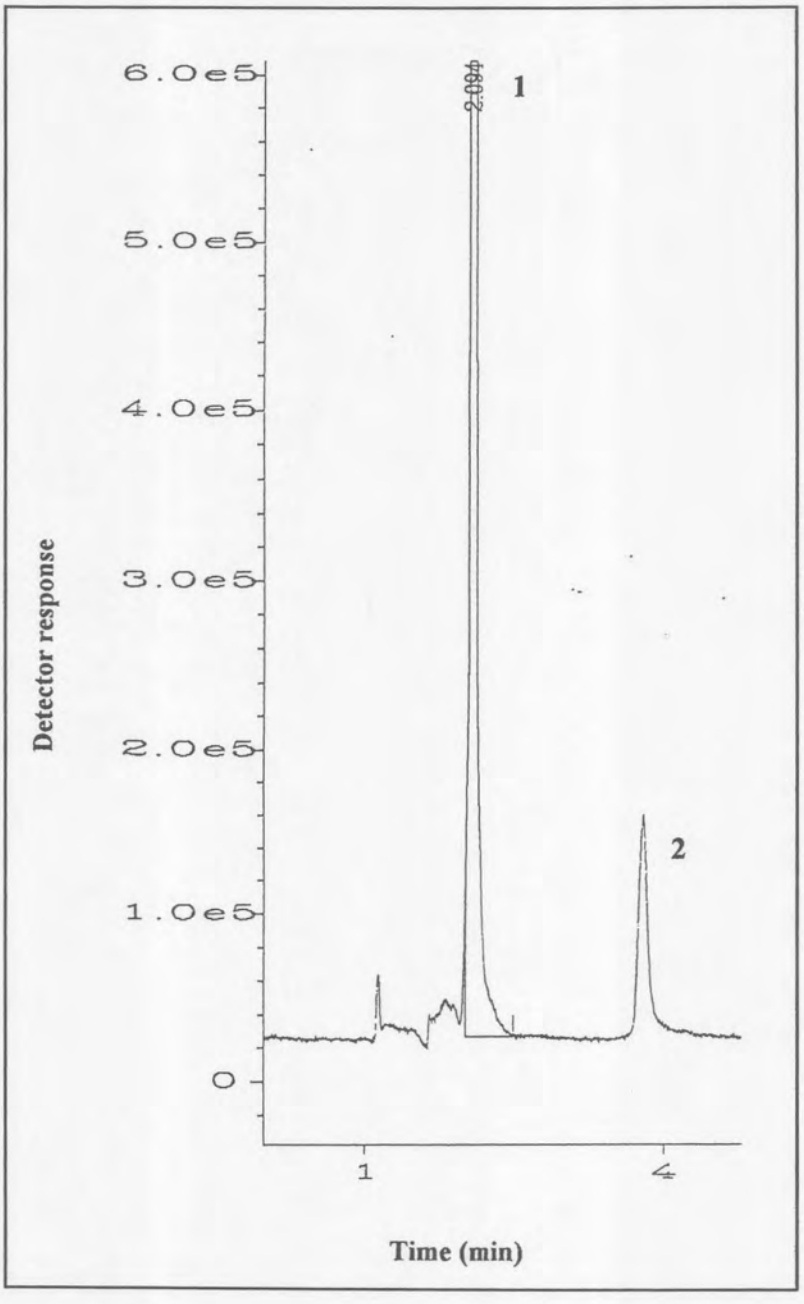


Figure 2.2 : Chromatogram → example shows HPLC assay of a haemaolysate to which *PMP* was added to measure *PMP (PNP)* oxidase.

Peaks : 1 = *PLPSC* ; 2 = *PLSC*

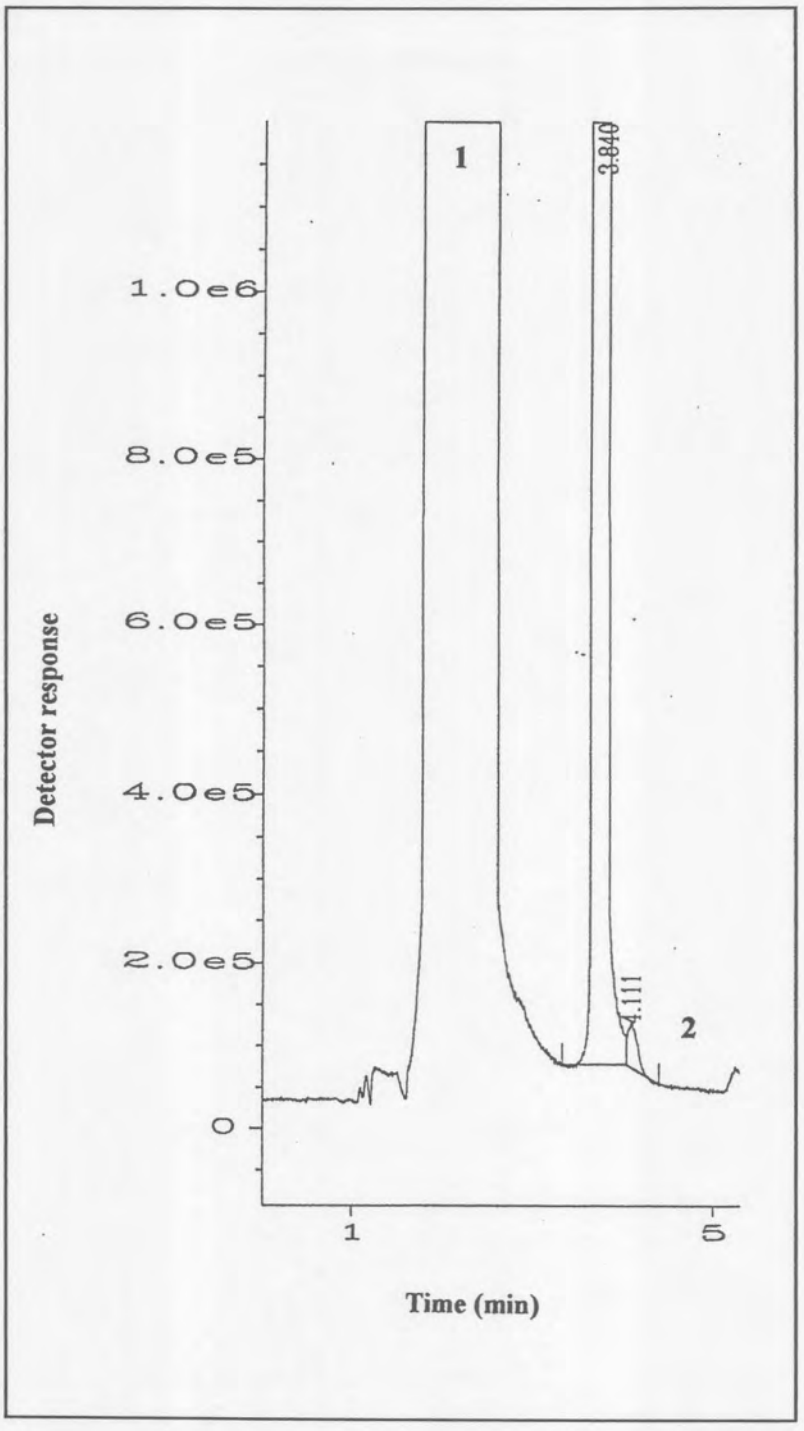


Figure 2.3 : Chromatogram → example shows HPLC assay of a haemaolysate to which *PLP* was added to measure *PLP* phosphatase.

Peaks : 1 = *PLPSC* ; 2 = *PLSC*

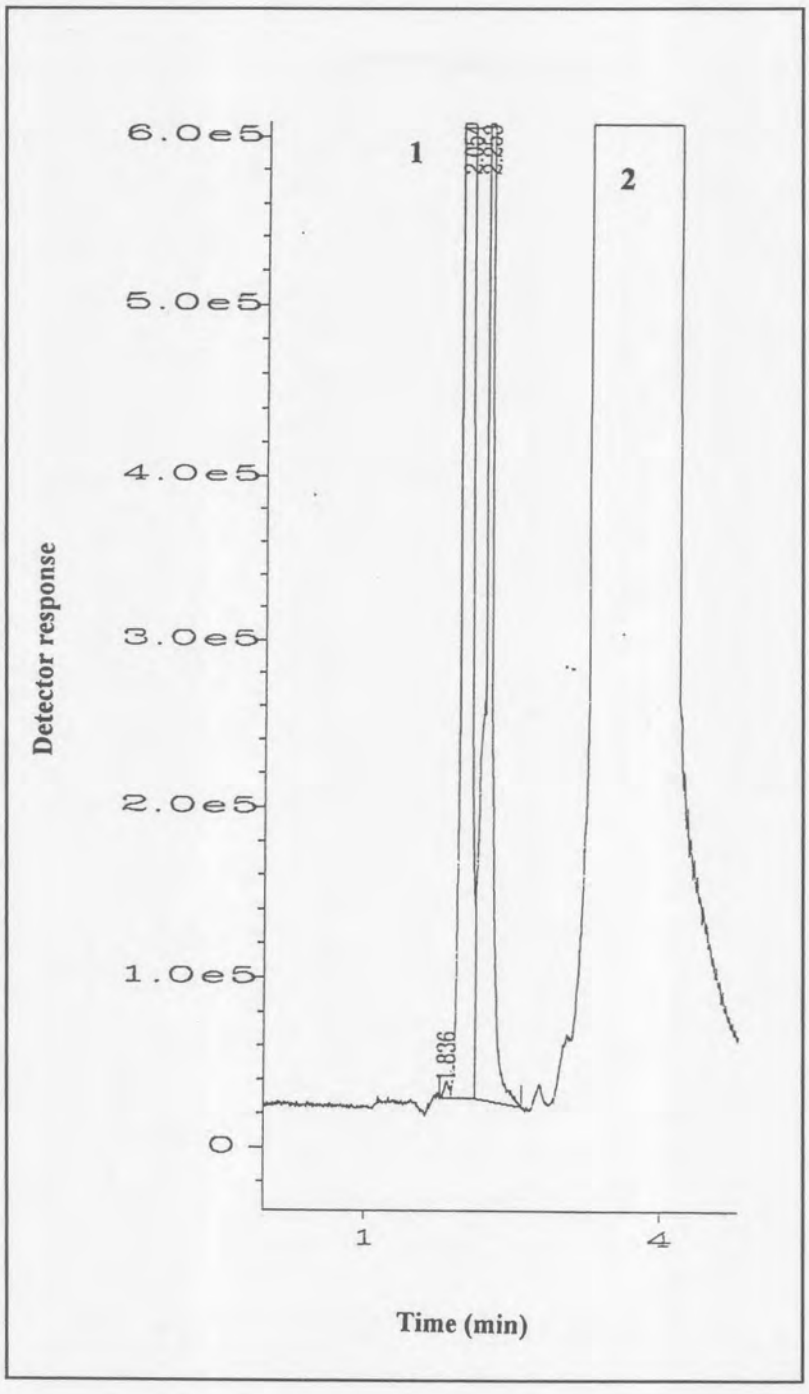


Figure 2.4 : Chromatogram → example shows HPLC assay of a haemaolysate to which *PL* was added to measure *PL* kinase. (Peak with a retension time of 2.253 min can be ascribed to an impurity in the Sigma *PL* preparation).

Peaks : 1 = *PLPSC* ; 2 = *PLSC*

Table 2.4 : Summary of plasma *PLP* concentrations and B_6 vitamer enzyme activities obtained using HPLC from whites and blacks.

	Plasma *PLP* (nmol/L)		***PL* Kinase (nmol/L/hr)**		***PMP* Oxidase (nmol/L/hr)**		***PLP* Phosphatase (nmol/L/hr)**	
	White	**Black**	**White**	**Black**	**White**	**Black**	**White**	**Black**
N	**69**	**53**	**69**	**53**	**69**	**53**	**69**	**53**
Mean	59.7	41.7	389.0	361.1	89.2	113.4	1009.8	987.8
Median	49.2	34.3	328.2	297.8	78.8	79.6	980.1	971.8
SD	43.5	27.0	209.0	298.4	62.4	139.4	236.5	241.5
p-Value	***0.005***		***NS***		***NS***		***NS***	

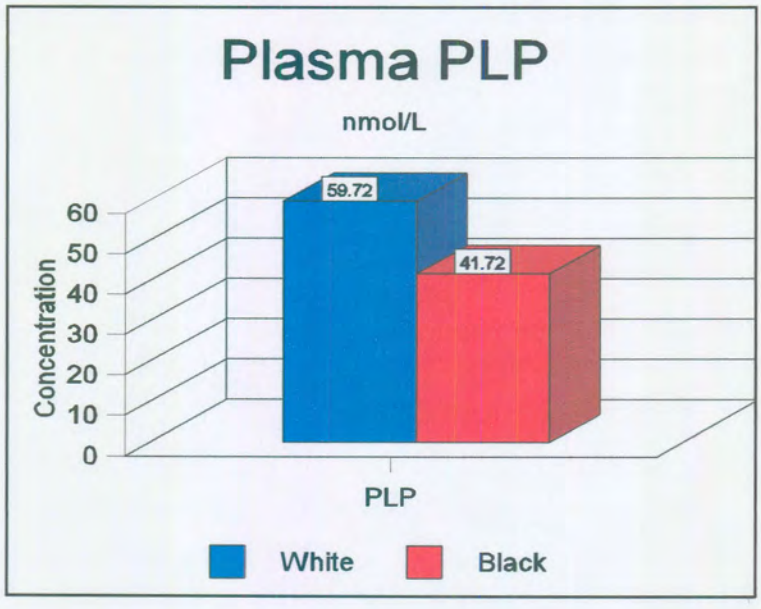


Figure 2.5 : Histogram shows mean plasma *PLP* concentrations obtained by HPLC for 69 white- and 53 black subjects respectively. Statistical analysis by means of the student t-test, showed a **significant difference** between the two groups. (***p = 0.005***).

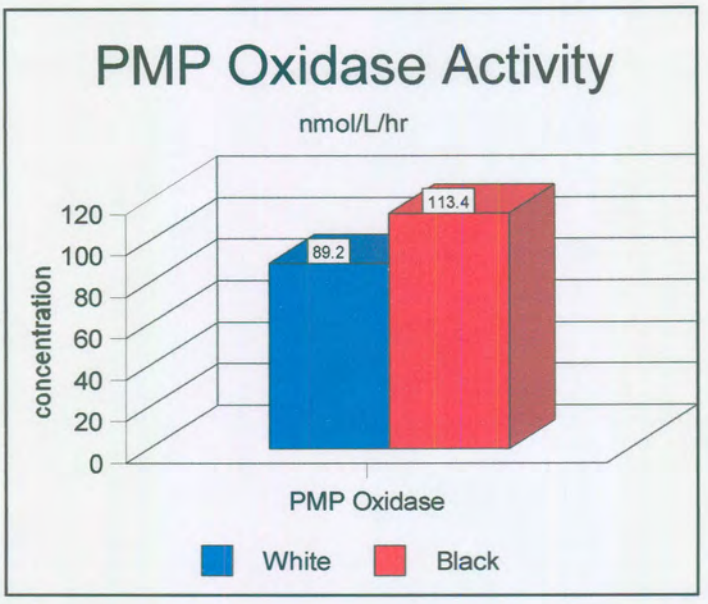


Figure 2.6 : Histogram shows *PMP (PNP)* oxidase enzyme activity obtained by HPLC in 69 white- and 53 black male subjects respectively. Statistical analysis (see **Table 2.4**) showed no significant difference between the two groups.

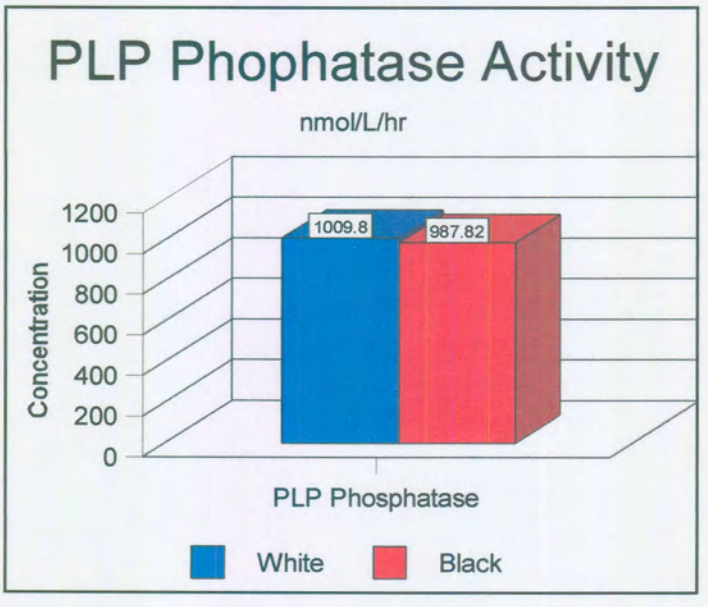


Figure 2.7 : Histogram shows *PLP* phosphatase enzyme activity obtained by HPLC in 69 white- and 53 black subjects respectively. Statistical analysis (see **Table 2.4**) showed no significant difference between the two groups.

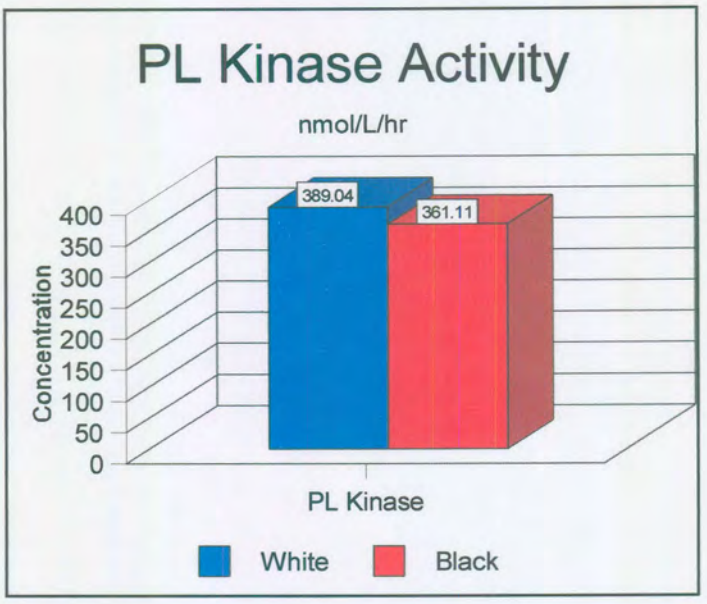


Figure 2.8 : Histogram shows *PL* kinase enzyme activity obtained by HPLC in 69 white- and 53 black male subjects respectively. No statistical difference in *PL* kinase activity was found between the two groups (See **Table 2.4**).

2.4 DISCUSSION

Evidence which suggested that blacks have lower circulating plasma *PLP* levels compared to whites (**68**), were confirmed by results obtained in 69 white and 53 black male subjects, using refined HPLC methodology as described by Ubbink *et al* (**60,61,79**). These findings showed a significant difference in plasma *PLP* levels, within the normal range, between the two groups (See **Table 2.4** and **Figure 2.5**). The main aim of this thesis was to find possible explanations to which these differences, regarding vit B_6 metabolism, could be ascribed to.

Vit B_6 interorgan metabolism has been described in chapter one. Plasma *PLP* is largely albumin bound (**15**) while plasma *PL* is directly available for intracellular metabolism (**19**). This suggests

that plasma *PL* is a better indicator of the body store content. The erythrocyte contains three enzymes that play an important role in vit B_6 metabolism (**72**). Phosphorylation of *PN*, *PM*, and *PL* are catalysed by *PL* kinase. *PMP* (*PNP*) oxidase oxidizes *PMP* and *PNP* to *PLP*. *PLP* is then converted to *PL* by *PLP* phophatase. As earlier stated, the erythrocyte cannot oxidize *PL* to *4-PA*, therefore *PL* is released into plasma where it binds to haemoglobin and serves as a transport form of vit B_6.

Measurement of these respective total enzyme activities within the erythrocyte in the same population study, failed to illustrate any significant difference between the whites and blacks (See **Table 2.4** and **Figures 2.6-8**).

This suggests similar vitamin enzyme activity involved in *PLP* production from *PMP* and *PL* as well as *PLP* dephosphorylation which result in the release of *PL* into the circulating fluid.

Factors influencing vit B_6 requirements and status are discussed in chapter one. In applying exclusion criteria, I tried to eliminate most biochemical-, physiological-, genetic- and disease related factors. An important factor which could have influenced vit B_6 status in this population study, is diet. Although the test subjects participating in this study were confined to the same basic mess diet, individual tastes and freedom of choice could explain the differences obtained between the two groups. This is an important consideration due to the fact that certain foods contribute in different ways to circulating plasma *PLP* concentration e.g. :

a. Glycosylated vit B_6 has an adverse effect on vit B_6 bioavailability (**22**).
b. Amino acids resulting from protein intake greater than the need for growth and maintenance requires increased transaminase levels and greater need for *PLP* in tissues (**24**).

c. Excessive carbohydrates in the diet are known to depress plasma *PLP* (**25**).

Although the aim of this thesis was not to assess vit B_6 status, it must be emphasized that the lower circulating plasma *PLP* concentrations obtained in blacks does not necessarily mean these subjects are vit B_6 deficient. Barnard *et al* (**82**) studied the vit B_6 status of pregnant females and non pregnant controls and found that plasma *PLP* was approximately 50 % lower in pregnant females. The concentrations of total *PLP* and *PL* was however only slightly lower. When concentrations were corrected for the serum albumin concentrations, there was no difference between the groups. In deficiency studies in males (**83**), the decrease in urinary *4-PA* excretion parallelled the decrease in plasma *PLP* concentration. In cases where excessive vit B_6 is present, the opposite is applicable → vitamin not needed by the cell is simply excreted or metabolized to a non-utilizable form such as *4-PA*. The assessment of total vit B_6 status in a population, must therefore include the measurement of plasma *PLP*, *PL* and urinary *4-PA* excretion.

Since this study was concerned with aspects regarding vit B_6 metabolism, the measurement of the active form of vit B_6 namely plasma *PLP*, was considered a good starting point to establish whether any racial differences existed. Whether dietary factors were responsible for lower plasma *PLP* concentrations obtained in blacks is still unclear, but the fact that a significant difference was found between the two groups, motivated the **second** and **third** phases of this study, concerned with the pharmacological and enzymatic aspects of vit B_6 metabolism.

CHAPTER 3 : PHARMACOKINETIC STUDIES AFTER A SINGLE ORAL SUPPLEMENT OF *PYRIDOXINE HYDROCHLORIDE*

3.1 INTRODUCTION

Although the **first phase** of this study supported previous findings and showed that whites do indeed have higher circulating plasma *PLP* levels compared to blacks (**68**), total enzyme activities measured in vit B_6 metabolism, did not seem to explain the observed differences found. The **second phase** of this study was conducted by means of calculating pharmacokinetic parameters to establish whether differences existed in the absorption, distribution, metabolism and excretion of a pyridoxine load between blacks and whites. Ubbink *et al* showed that plasma *PLP* increased moderately to maximum peak levels within 0.67-1.33 hours and then decreased mono-exponentially after a small (10 mg) oral *PN* dose. On the other hand, plasma *PL* peaked within 0.5-1.0 hour and then decreased bi-exponentially (**75**). Zempleni *et al* reported similar results after intravenous infusion of pyridoxine (**76**). These results suggest that different pharmacokinetic models are involved and could prove important in interpreting plasma *PLP* and *PL* concentrations in terms of vit B_6 status. A brief summary of the various pharmacokinetic terminology and theoretical aspects is required to interpret the results obtained in the different groups. ***It must be emphasized that pharmacokinetic studies are usually performed on drugs. For the purpose of this research, the metabolites of the pyridoxine supplement namely PLP and PL were treated as if they were 'drugs' in order to calculate pharmacokinetic parameters.*** As mentioned in chapter one, the end product of vitamin B_6, 4-pyridoxic acid, is considered to be a short term indicator of vit B_6 status (**65**). Differences in *4-PA* urinary output may also explain the earlier observed lower plasma *PLP* levels in blacks since urinary excretion is almost the sole route of vit

B_6 elimination and *4-PA* excretion parallel plasma *PLP* levels.

3.2 PHARMACOKINETICS (84, 85)

The specialized study of the mathematical relationships between a 'drug' dosage regimen and resulting 'drug' concentrations usually in serum or plasma, is known as pharmacokinetics. Clinical pharmacokinetics has been defined as the study of 'drug' liberation, absorption, distribution, metabolism and excretion. The time course of these events has been adequately described by various mathematical models that represent the body as a system of compartments. The serum concentration versus time data obtained from patients are fitted to various pharmacokinetic models and statistical methods are used to determine the best fit. Most often linear or first-order elimination and inter-compartmental transfer of the 'drug' is assumed.

3.2.1 ONE-COMPARTMENT MODEL

The one-compartment model with first-order elimination assumes that the 'drug' distributes immediately following administration. It represents the body as a single homogenous kinetic unit, and assumes distribution of the 'drug' from the vascular space to the tissues with instantaneous attainment of equilibrium. See **Figure 3.1.**

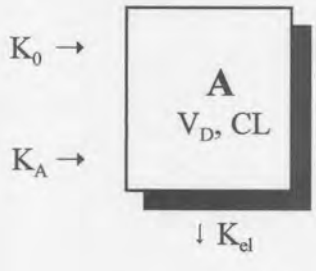


Figure 3.1 : One-Compartment Linear model.

where : K_A = first order absorption rate constant (hr^{-1})

K_0 = zero order input rate (mg/hr)

V_D = total distribution volume (L)

CL = total systemic 'drug' clearance (L/hr)

K_{el} = first order elimination rate constant (hr^{-1})

'Drug' concentration following input into a one compartment system is described by a single first order exponential term. See Equation :

$$\mathbf{C(t) = Cp_0 \,.\, e^{-k_{el} \cdot t}}$$

where : C(t) = 'drug' concentration(mg/L) at time (t)

Cp_0 = 'drug' concentration (mg/L) at completion of input.

t=0

K_{el} = first order elimination rate constant (hr^{-1})

t = time (hr) after completion of input

e = exponential term

According to this model, a plot of the log of 'drug' concentration (y-axis) versus time (x-axis) yields a straight line representing elimination from a single compartment, hence mono-exponential. A plot of the natural 'drug' concentration versus time produces a straight line with a slope equal to minus the elimination rate constant. See **Figure 3.2**.

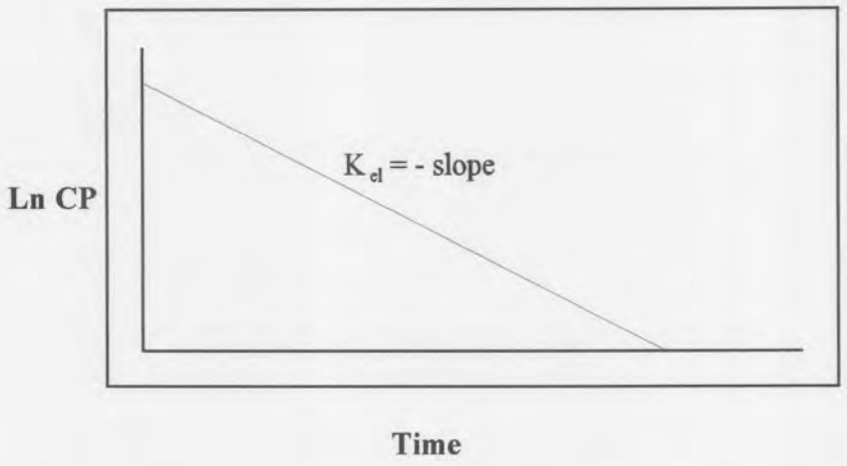


Figure 3.2 : Semilogarithmic plot of 'drug' concentration (CP) versus time for a one-compartment pharmacokinetic model.

3.2.2 TWO-COMPARTMENT MODEL

The two-compartment model consists of a central compartment, usually considered to be the vascular space and the highly perfused tissues, and a peripheral compartment made up of poorly perfused muscle, lean tissue and fat. Following 'drug' administration into a two-compartment system, the concentration in the central compartment declines during the distribution phase. Conversely, the concentration of 'drug' in poorly perfused tissues will initially rise to a plateau and then begin to decline. During continuous input an equilibrium is established between the central and peripheral compartment.

The duration of the distribution phase may be estimated by a time equal to five times the distribution half-life. ***This is of importance since concentrations obtained in plasma before distribution equilibrium has been attained, will not correlate with concentration at receptor sites, hence will not correlate with pharmacological response.***

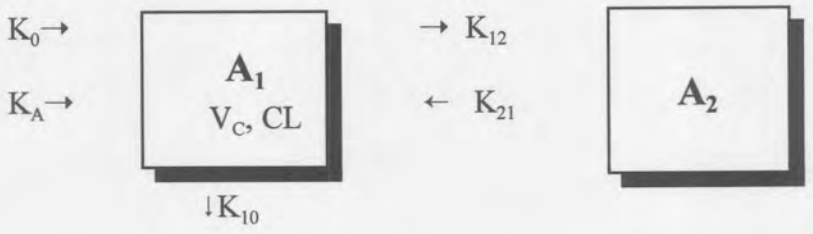


Figure 3.3 : Two-Compartment Linear model.

where : A_1 = compartment 1

A_2 = compartment 2

K_0 = zero order input rate (mg/hr)

K_A = first order absorption rate constant (hr^{-1})

V_C = central compartment volume

CL = total systemic 'drug' clearance (L/hr)

K_{12} = first order transfer rate constant (hr^{-1}) from the central compartment to the peripheral compartment

K_{21} = first order transfer rate constant (hr^{-1}) from the peripheral compartment to the central compartment

K_{10} = first order elimination rate constant (hr^{-1}) from the central compartment.

$K_{10} = CL / V_C$

'Drug' concentration following input into a two compartment system is described by the sum of two first order exponential terms. See equation :

$$\mathbf{C(t) = Cp_1 \cdot e^{-\alpha \cdot t} + Cp_2 \cdot e^{-\beta \cdot t}}$$

where : C(t) = 'drug' concentration (mg/L) at time (t)

Cp_1 = 'drug' concentration (mg/L) associated with phase 1 at completion of input

t=0

Cp_2 = 'drug' concentration (mg/L) associated with phase 2 at completion of input

t=0

α = first order elimination rate constant (hr^{-1}) associated with phase 1

ß = first order elimination rate constant (hr^{-1}) associated with phase 2

t = time (hr) after completion of input

e = exponential term

A plot of the natural log of 'drug' concentration versus time produces a two phase line. The slope of the terminal phase is equal minus the terminal elimination rate constant ß. The slope of the initial phase is equal to minus the sum of the terminal phase elimination rate constant $\propto$ and the terminal elimination rate constant ß. See **Figure 3.4.**

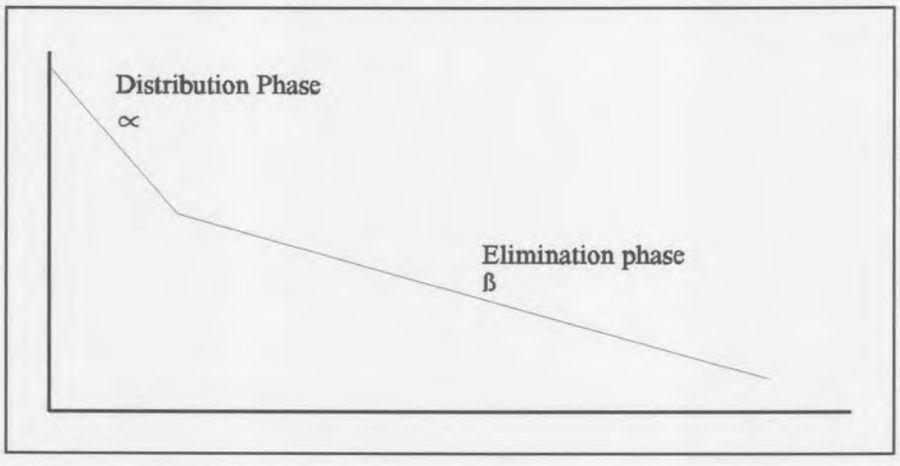


Figure 3.4 : Semilogarithmic plot of 'drug' concentration (CP) versus time for a two-compartment pharmacokinetic model.

3.2.3 DEFINITIONS OF PHARMACOKINETIC PARAMETERS UTILIZED IN PERFORMING AREA UNDER THE CURVE ANALYSIS (84,85)

3.2.3.1 Area Under the Curve (AUC)

The area under a '*drug*' *concentration versus time profile* with units that are the product of concentration and time. It can be calculated for any interval of time by the trapezoidal rule, and provides a measure of the amount of 'drug' entering the blood circulation and its persistence in the body. AUC is the sum of the individual areas between observations, from time = 0 to the last measured sample, time = n :

$$AUC_{(0\text{-}t\ n)} = \frac{C_1 + C_2\,(t_2 - t_1)}{2} + \frac{C_2 + C_3\,(t_3 - t_2)}{2} + \frac{C_{n-1} + C_n\,(t_n - t_{n-1})}{2}$$

where C is the serum/plasma concentration and t is the corresponding time of sample. The area under the curve is used for calculations of clearance, steady-state volume of distribution, drug half-life, mean residence time and elimination rate constant .

3.2.3.2 Clearance (CL)

A measure of the efficiency of 'drug' elimination, defined as the volume of plasma, serum, or blood that is totally cleared of 'drug' per unit time. Body or systemic clearance is the sum of clearances by individual eliminating organs e.g. renal / hepatic. Thus, clearance is the rate of 'drug' elimination normalized for 'drug' concentration in serum.Clearance may be altered in disease, or as a consequence of 'drug' interactions. Clearance can be calculated as the product of the apparent volume of distribution and the elimination rate constant, according to :

$$CL = V_D \,.\, K$$

3.2.3.3 Volume of distribution (V_D)

The only volume of distribution that is characterized by true physiologic volumes and the relative

binding of 'drug' to proteins in these spaces. The steady-state volume of distribution can be calculated from serum concentration versus time data.

3.2.3.4 Elimination half-life ($t_{½}$)

The time required for serum 'drug' concentration to decrease by 50 %. This pharmacokinetic parameter is a function of body clearance and volume of distribution, and therefore is not a pure measure of elimination efficiency.

$$t_{½} = 0.693 \cdot V_D / CL$$

3.2.3.5 Elimination rate constant (K)

A constant with units of inverse time that represents the fraction of 'drug' eliminated per unit time. The elimination rate constant is determined by measuring the slope of the terminal portion of a plot of log serum 'drug' concentration (Y-axis) versus time (X-axis). The elimination rate constant is related to elimination half-life by :

$$K = 0.693 / t_{½}$$

3.3 MATERIALS AND METHODS

3.3.1 EXPERIMENTAL PROTOCOL

3.3.1.1 Study participants

A group of 30 white and 30 black male volunteers were recruited from the South African Police Services (SAPS) where they were exposed to the same socio-economic environment and same diet for a period of at least six consecutive weeks. A single blood-sample was obtained to determine vit B_6 status of the participants. Age, body mass and smoking habits were recorded. The group was invited to participate in a *PN* load study. Nine white and seven black participants were enrolled into the trial. Exclusion criteria that were applied included poor vitamin B_6 status

(plasma *PLP* < 20 nmol/L) and the intake of any type of medication and/or vitamin supplements (plasma *PLP* > 150 nmol/L). The participants were requested to sign the appended conscent form. (See **APPENDIX 3**).

3.3.1.2 Study design

The study was designed to be a cross-sectional, non-randomized, single blind (to the laboratory analyst) study. A single dose of 10 mg *pyridoxine hydrochloride* [Pharma Natura, Wynberg, RSA] was administered orally after an eight hour period of fasting, and after a basal blood-sample was collected. A Butterfly needle was inserted into a peripheral vein from which serial blood-samples were collected over a 24 hour period. In between sampling, the needle was washed with 0.9% saline and heparin to maintain patency. Blood-samples were taken in accordance with a specific time schedule and the total volume of samples collected over the twenty-four hour period was approximately 60 mL (See **APPENDIX 4**). Marked changes were expected to occur in the plasma vitamer concentrations within the first two hours after administration of pyridoxine, therefore frequent samples were collected within this period. Participants were also requested to collect a 24 hour urine sample over the same 24 hours that the blood were collected, after initially emptying the bladder just before the first basal samples were collected.

3.3.2 ANALYTICAL TECHNIQUES

3.3.2.1 Plasma *PLP* and *PL* analysis

Plasma *PLP* and *PL* levels were determined using HPLC as described in chapter two.

3.3.2.2 Analysis of urinary 4-pyridoxic Acid

3.3.2.2.1 Sample collection

Urine samples were collected over a 24 hour period during phase two of the experimental stage and stored at -25 °C until HPLC analysis.

3.3.2.2.2 Standard and sample preparation

Standards containing 1-5 mg/L *4-PA* were prepared by dissolving *4-PA* in distilled H_2O. To 1.0 mL of standard or urine, 2.0 mL of 5 mol/L HCL was added, boiled for 20 min, cooled and diluted 1:20 in 0.025 mol/L ammoniumdihydrogenphosphate buffer (pH 2.8). 30 μL of this dilution was used directly for HPLC analysis. Concentrations of *4-PA* in μmol/L were expressed in terms of 24 hour urine volume.

3.3.2.2.3 Experimental HPLC analysis

The HPLC system as described in section 2.2.2.1.3 for plasma *PLP* and *PL* was modified as follows:

a. A Whatman Partisil 10 SCX analytical cation exchange column (25 cm x 4.6 mm id : particle size 10 μM) and a suitable guard column was fitted to the instrument.
b. The mobile phase was changed to 0.025 mol/L ammonium dihydrogenphosphate buffer (pH adjusted to 2.8 with concentrated phosphoric acid) and used at a flow rate of 1.1 mL/min.
c. The fluorescence spectrophotometer was changed to lowest sensitivity with excitation and emission wavelengths 360 and 430 nm respectively.

3.3.3 PHARMACOKINETIC PARAMETER CALCULATIONS

An "ABBOTTBASE" pharmacokinetic systems computer software programme [Abbott Laboratories - Diagnostics Division, Abbott Park, Illinois, USA, 1991](**84**) was used to compare the response of plasma *PLP* and *PL* in blacks and whites. The area under the curve analysis (AUC) is based on 20 concentration measurements following a 10 mg dose of *pyridoxine hydrochloride* determined at consecutive time intervals. Clearance (CL), volume of distribution (V_D), drug half-life ($t_{½}$), mean residence time (MRT), elimination rate constant (K), maximum peak obtained (Max P) and time to maximum peak [(X) t Post] were determined from the AUC.

Since basal plasma *PLP* and *PL* concentrations obtained were not zero, basal concentration was subtracted in each case from the consecutive observed concentrations. In order to calculate pharmacokinetic parameters, plasma *PLP* and *PL* concentrations in nmol/L were converted to ng/mL [conversion factor = 0.046](**86**) to ensure "ABBOTTBASE" computer programme compatibility. See **APPENDIX 5.1-2** for field descriptions entered to perform area under the curve analysis.

3.3.4 STATISTICAL ANALYSIS

The Kruskal-Wallis non-parametric analysis of variance for independent groups was used to compare the area under the curve for the different groups, while the Mann Whitney u-test, a non-parametric counterpart for the Student t-test for independent groups with a significance level of 0.05, was used to compare pharmacokinetic parameters and urinary *4-PA* excretion in blacks and whites respectively.

3.4 RESULTS

3.4.1 PLASMA *PLP* AND *PL* CONCENTRATIONS

Table 3.1-2 show the average plasma *PLP* and *PL* concentrations obtained using HPLC at different time intervals after a 10 mg *pyridoxine hydrochloride* supplement in whites and blacks respectively. These data are also displayed by **Figures 3.5-6**.

Table 3.1 : Plasma *PLP* concentrations after a 10 mg supplement of *pyridoxine hydrochloride*.

Time (hr)	Plasma *PLP* (nmol/L . 0.046 = ng/mL)					
	White (N=9)			Black (N=7)		
	Mean	Median	SD	Mean	Median	SD
0.08	2.4	1.3	2.8	0.4	0.3	0.3
0.16	4.3	2.9	3.2	1.4	1.0	1.2
0.25	6.2	5.8	4.3	4.0	3.0	1.8
0.33	8.2	9.6	5.0	7.6	6.4	4.1
0.42	10.4	7.6	7.1	8.9	8.3	3.5
0.58	12.7	11.6	7.8	15.2	14.1	5.2
0.83	14.8	12.8	8.4	18.2	18.5	4.9
1.08	15.9	14.5	8.3	18.1	17.2	4.8
1.33	15.0	13.3	6.9	18.8	18.9	4.9
1.58	16.4	14.3	7.4	18.0	16.8	4.6
2.0	15.9	13.4	7.1	17.6	17.8	4.3
2.5	15.9	15.0	7.3	16.2	14.9	3.4
3.0	15.5	14.2	6.4	16.6	16.3	3.3
4.0	14.8	12.1	6.2	15.6	15.0	3.1
6.0	15.5	13.7	7.0	15.9	15.0	4.2
8.0	16.2	13.9	7.1	17.0	15.6	5.1
12.0	19.6	16.1	8.3	19.6	17.8	4.6
24.0	13.1	9.9	6.7	11.4	11.3	2.9

Table 3.2 : Plasma *PL* concentrations after a 10 mg supplement of *pyridoxine hydrochloride*.

Time (hr)	Plasma *PL* (nmol/L . 0.046 = ng/mL)					
	White (N=9)			Black (N=7)		
	Mean	Median	SD	Mean	Median	SD
0.08	2.0	1.1	2.5	1.8	1.7	1.6
0.16	5.3	3.9	5.2	2.6	2.2	2.2
0.25	9.7	8.6	9.3	7.7	3.4	6.8
0.33	16.9	25.0	12.3	16.5	12.2	14.3
0.42	25.0	24.4	17.8	21.0	11.2	19.6
0.58	27.3	24.5	19.8	35.6	34.3	21.2
0.83	31.6	26.6	22.3	36.2	31.4	16.6
1.08	30.9	26.1	13.6	34.9	36.7	7.9
1.33	24.3	16.1	14.3	30.3	30.3	3.8
1.58	25.6	18.5	17.0	21.7	20.8	4.6
2.0	15.9	9.8	8.6	16.0	17.3	3.7
2.5	10.9	7.0	5.4	11.6	10.5	3.6
3.0	9.4	9.1	3.5	9.7	8.4	3.7
4.0	6.2	6.0	2.6	7.5	6.1	3.9
6.0	4.1	3.3	1.7	5.4	3.7	4.5
8.0	2.8	2.8	1.0	4.6	2.8	4.5
12.0	2.3	2.1	1.1	4.8	2.8	6.1
24.0	1.0	1.0	0.8	3.0	1.5	4.3

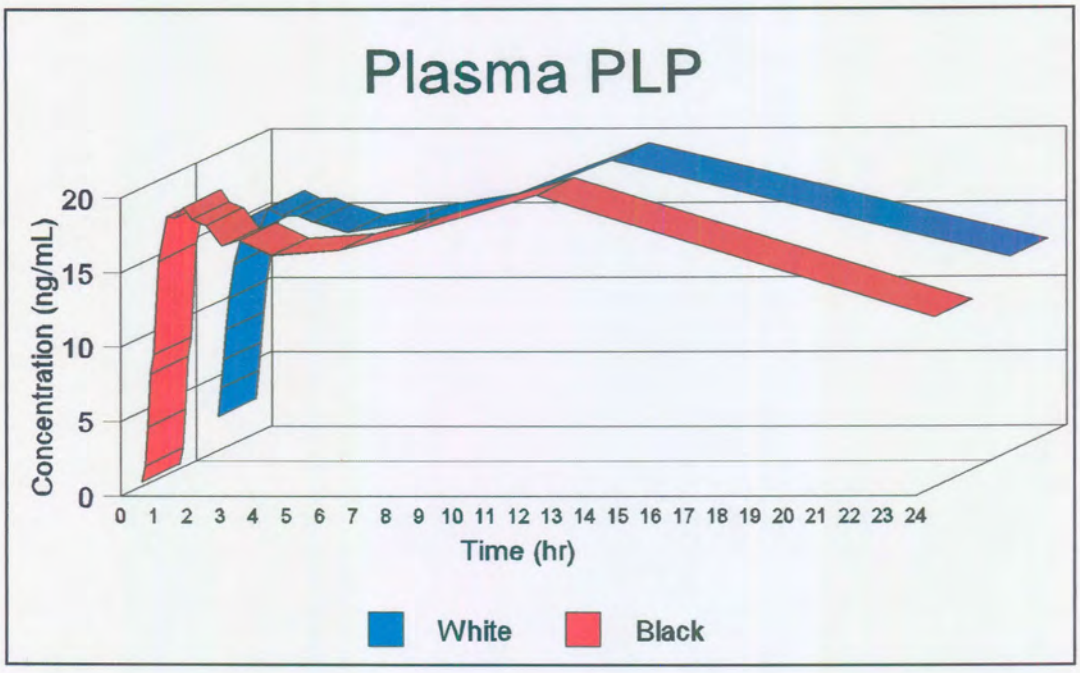


Figure 3.5 : Mean plasma *PLP* concentrations obtained for whites and blacks respectively after a 10 mg oral supplement of *pyridoxine hydrochloride*.

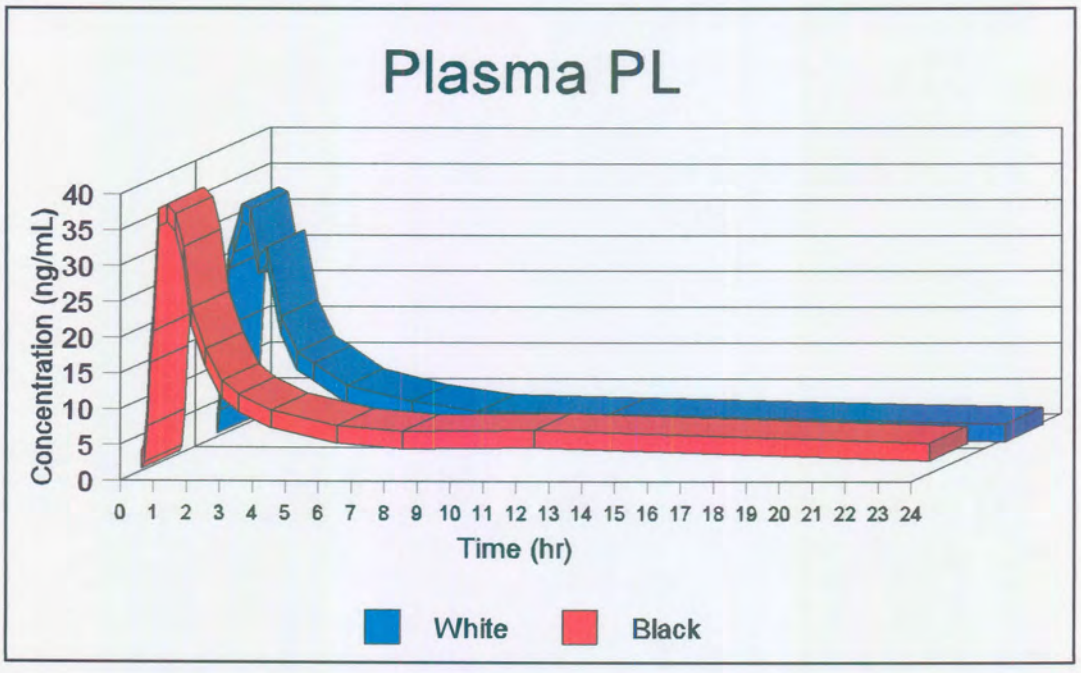


Figure 3.6 : Mean plasma *PL* concentrations obtained for whites and blacks respectively after a 10 mg oral supplement of *pyridoxine hydrochloride*.

3.4.2 AREA UNDER THE CURVE ANALYSIS IN BLACKS AND WHITES AFTER A 10 MG *PYRIDOXINE HYDROCHLORIDE* LOAD

An "ABBOTTBASE" computer software programme was used to perform area under the curve analysis from serial blood-samples entered at consecutive time intervals. **APPENDICES 6-7** are examples of these area under the curve reports generated by the "ABBOTTBASE" computer software programme. **Table 3.3** reflects the total sum of the area under the curve calculated for the different groups after a 10 mg *pyridoxine* load for *PLP* and *PL* respectively. **Table 3.4** reflects a 0.42 hour AUC cut-off point in the different groups for plasma *PLP* and *PL* respectively. Statistical analysis of these data failed to illustrate any significant differences between the groups.

Table 3.3 : AUC analysis in blacks and whites for plasma *PLP* and *PL* respectively.

Nr	AUC (*PLP*) (ng/mL . hr)		AUC (*PL*) (ng/mL . hr)	
	White	Black	White	Black
1	284.5	281.7	111.4	109.3
2	220.6	338.3	103.6	78.8
3	459.3	258.4	107.8	167.3
4	298.1	361.3	97.6	89.9
5	716.6	403.3	214.7	377.5
6	526.3	506.0	125.3	123.1
7	300.8	387.5	75.6	123.7
8	337.1		91.9	
9	268.5		79.5	
Mean	**379.1**	**362.4**	**111.9**	**152.8**
Median	**300.8**	**361.3**	**103.6**	**123.1**
SD	**159.2**	**82.5**	**41.6**	**103.1**
p-Value	***NS***	***NS***	***NS***	***NS***

Table 3.4 : 0.42 Hr AUC cut-off point in blacks and whites for plasma *PLP* and *PL* respectively.

Nr	AUC (*PLP*) 0.42 hr (ng/mL . hr)		AUC (*PL*) 0.42 hr (ng/mL . hr)	
	White	Black	White	Black
1	0.5	1.1	2.7	1.7
2	0.6	1.8	0.4	5.3
3	3.3	1.0	1.1	5.2
4	2.0	1.0	0.0	1.1
5	4.2	2.7	4.9	1.1
6	3.9	2.6	5.8	7.3
7	2.1	1.0	8.9	1.1
8	2.6		5.7	
9	2.5		4.1	
Mean	**2.4**	**1.6**	**3.8**	**3.3**
Median	**2.5**	**1.1**	**4.1**	**1.7**
SD	**1.3**	**0.8**	**2.9**	**2.6**
p-Value	***NS***	***NS***	***NS***	***NS***

3.4.3 PHARMACOKINETIC PARAMETERS

The plasma vitamer levels over time were used by "ABBOTTBASE" to calculate pharmacokinetic parameters in blacks and whites for plasma *PLP* and *PL* respectively. These included 'drug' half-life, elimination rate constant, clearance, volume of distribution, mean residence time, maximum peak concentration obtained and time to reach maximum peak concentration (See section 3.3.3). These findings are summarized in **Tables 3.5-6** and also displayed with **Figures 3.7-13.** See also **APPENDIX 8** for the individual parameters calculated. Statistical analysis of pharmacokinetic parameters failed to illustrate any significant differences between the groups.

Table 3.5 : Pharmacokinetic parameters calculated for plasma *PLP*.

PLP	**$t_{½}$ (hr)**		**K (1/hr)**		**CL (L/hr)**		**V_D (L)**		**MRT (hr)**		**Max P (ng/mL)**		**(X) t Post (hr)**	
	W	**B**	**W**	**B**	**W**	**B**	**W**	**B**	**W**	**B**	**W**	**B**	**W**	**B**
N	**9**	**7**	**9**	**7**	**9**	**7**	**9**	**7**	**9**	**7**	**9**	**7**	**9**	**7**
Mean	19.30	16.60	0.04	0.04	0.02	0.02	0.45	0.39	27.80	24.00	19.40	22.50	7.94	8.27
Median	15.60	16.50	0.04	0.04	0.02	0.02	0.46	0.38	22.90	23.80	16.10	24.00	12.00	12.00
SD	6.65	1.86	0.01	0.00	0.00	0.00	0.15	0.07	9.60	2.68	8.00	4.71	5.01	5.20
p-Value	***NS***		***NS***		***NS***		***NS***		***NS***		***NS***		***NS***	

where : Clearance (CL), volume of distribution (V_D), drug half-life ($t_{½}$), mean residence time (MRT), elimination rate constant (K), maximum peak obtained (Max P) and time to maximum peak [(X) t Post]

Table 3.6 : Pharmacokinetic parameters calculated for plasma *PL*.

PL	**$t_{½}$ (hr)**		**K (1/hr)**		**CL (L/hr)**		**V_D (L)**		**MRT (hr)**		**Max P (ng/mL)**		**(X) t Post (hr)**	
	W	**B**	**W**	**B**	**W**	**B**	**W**	**B**	**W**	**B**	**W**	**B**	**W**	**B**
N	**9**	**7**	**9**	**7**	**9**	**7**	**9**	**7**	**9**	**7**	**9**	**7**	**9**	**7**
Mean	8.23	8.31	0.12	0.12	0.08	0.07	0.90	0.61	11.90	12.00	41.90	44.70	0.88	0.80
Median	7.05	6.52	0.09	0.11	0.09	0.07	0.72	0.59	10.20	9.40	37.80	42.50	0.83	0.83
SD	5.69	6.17	0.10	0.07	0.02	0.04	0.58	0.18	8.20	8.90	16.30	15.10	0.40	0.19
p-Value	***NS***		***NS***		***NS***		***NS***		***NS***		***NS***		***NS***	

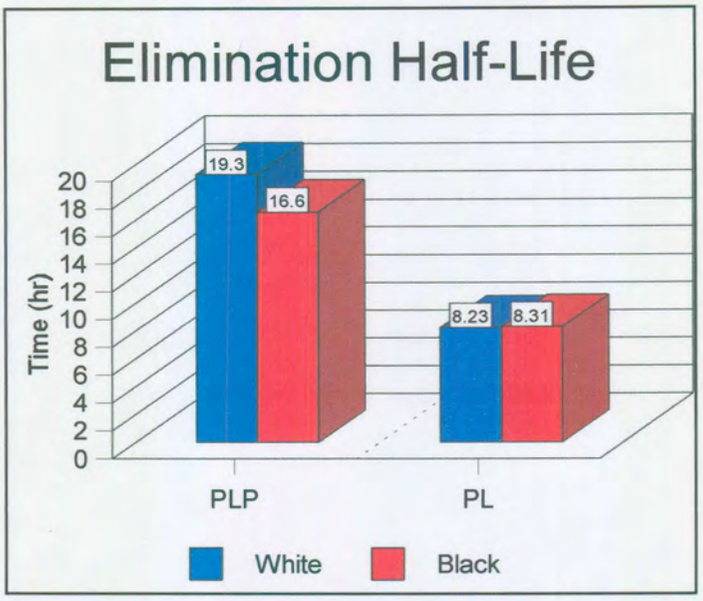


Figure 3.7 : Histogram shows the mean elimination half-lives calculated for plasma *PLP* and *PL* for whites and blacks respectively. Statistical analysis showed no significant difference in the two groups.

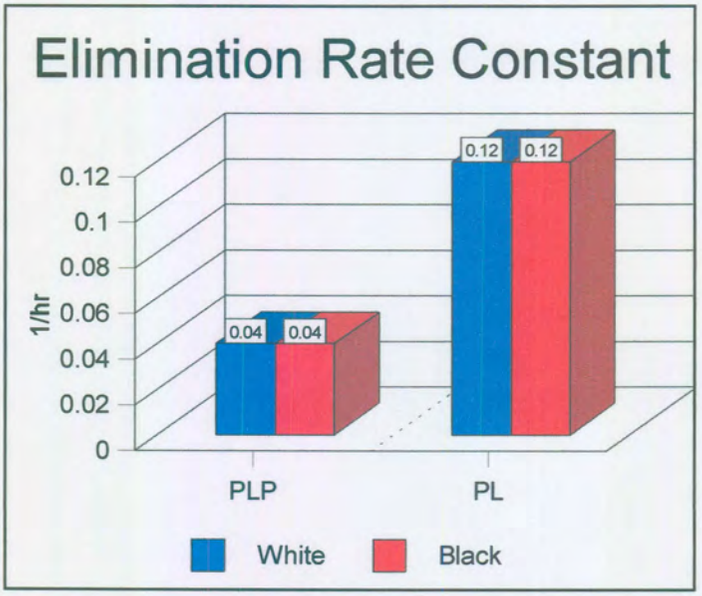


Figure 3.8 : Histogram shows the mean elimination rate constants calculated for plasma *PLP* and *PL* for whites and blacks respectively. No statistical difference could be found between whites and blacks.

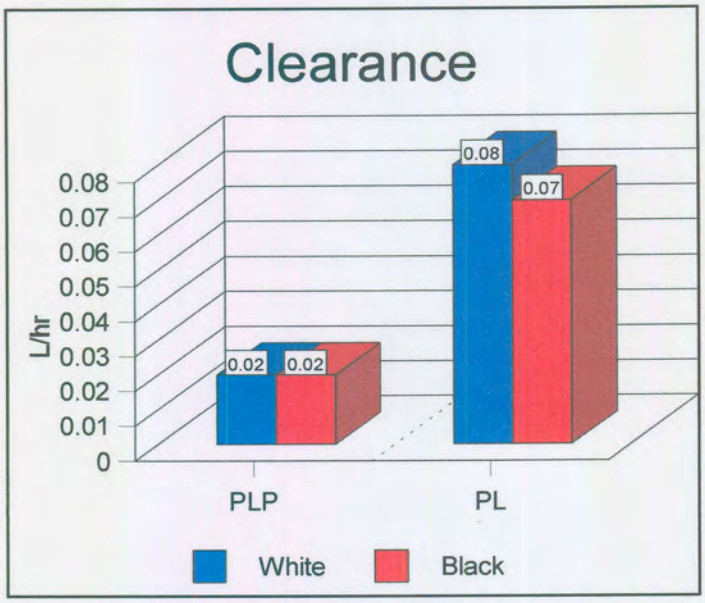


Figure 3.9 : Histogram shows the mean clearances calculated for plasma *PLP* and *PL* in whites and blacks respectively. Statistical analysis showed no significant difference.

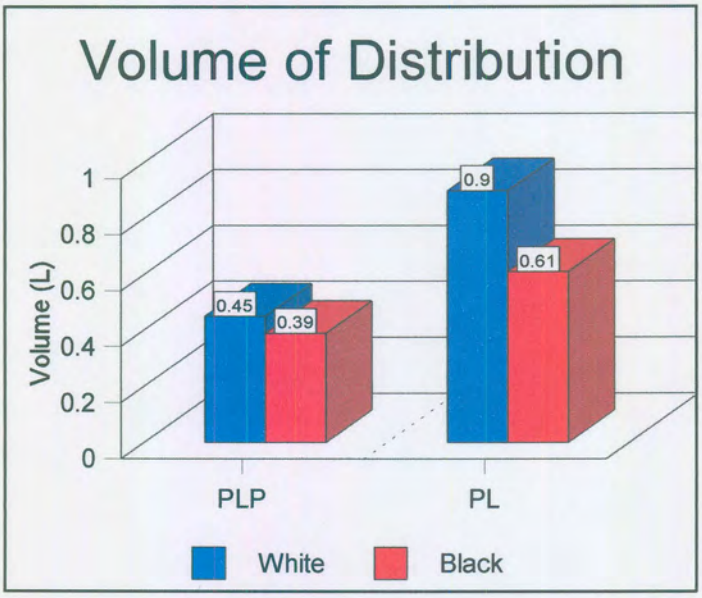


Figure 3.10 : Histogram shows the mean volumes of distribution calculated for plasma *PLP* and *PL* in whites and blacks respectively. No statistical difference was found.

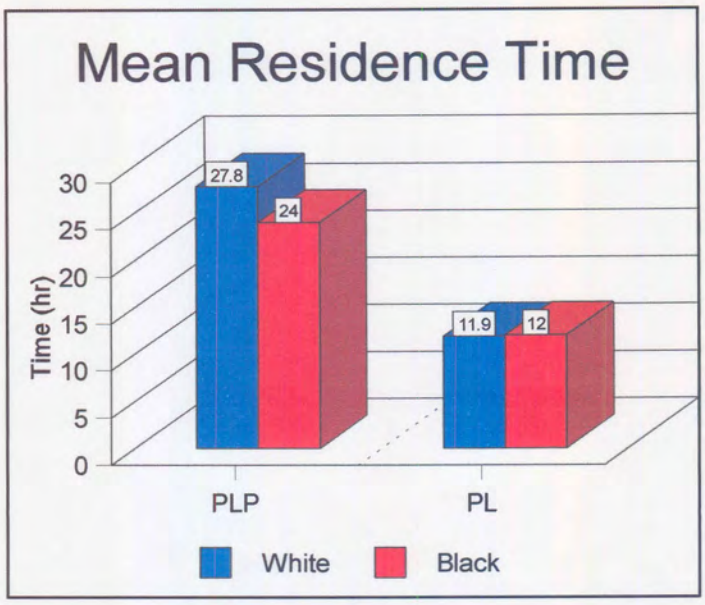


Figure 3.11 : Histogram shows the pharmacokinetic parameter mean residence time calculated for plasma *PLP* and *PL* for whites and blacks respectively. Statistical analysis showed no significant difference.

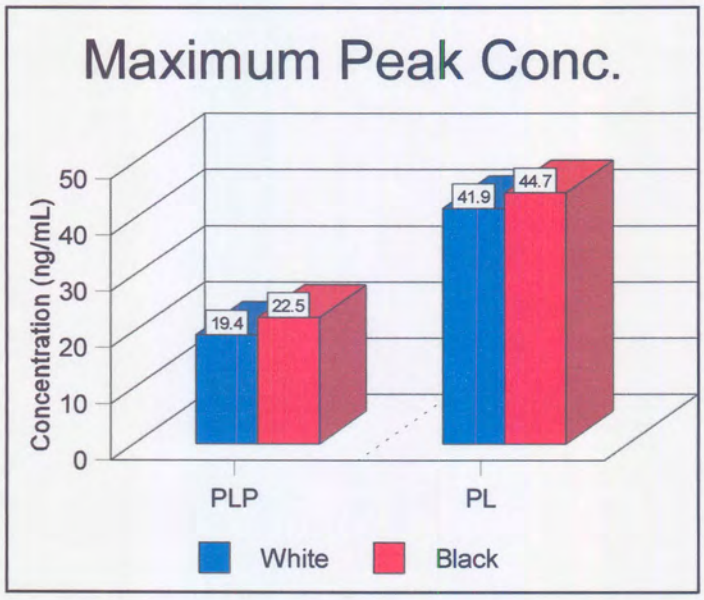


Figure 3.12 : Histogram shows the mean maximum peak concentrations obtained for plasma *PLP* and *PL* for whites and blacks respectively. No statistical difference could be obtained by statistical analysis.

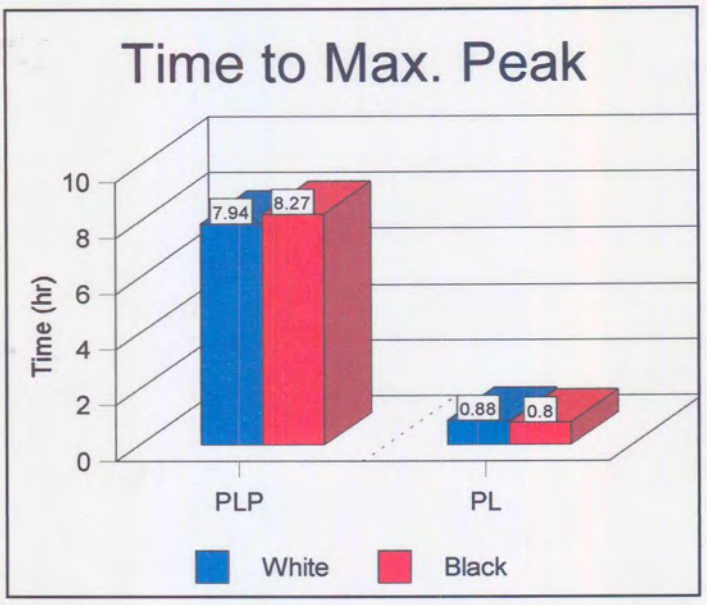


Figure 3.13 : Histogram shows the mean times to maximum peak concentration calculated for plasma *PLP* and *PL* for whites and blacks respectively. Statistical analysis showed no significant difference.

3.4.4 URINARY 4-PYRIDOXIC ACID (*4-PA*) EXCRETION

The end product of vit B_6 metabolism, *4-PA* was determined by HPLC as described in section 3.3.2.2.3. **Figure 3.14** shows the determination of urinary *4-PA* as *4-PA* lactone using HPLC. Concentrations of urinary *4-PA* in μmol/L, 24 hour urine volume and 24 hour urinary *4-PA* excretion are summarized in **APPENDIX 9**. **Table 3.7** and **Figure 3.15** express 24 hour urinary 4-PA excretion for whites and blacks respectively.

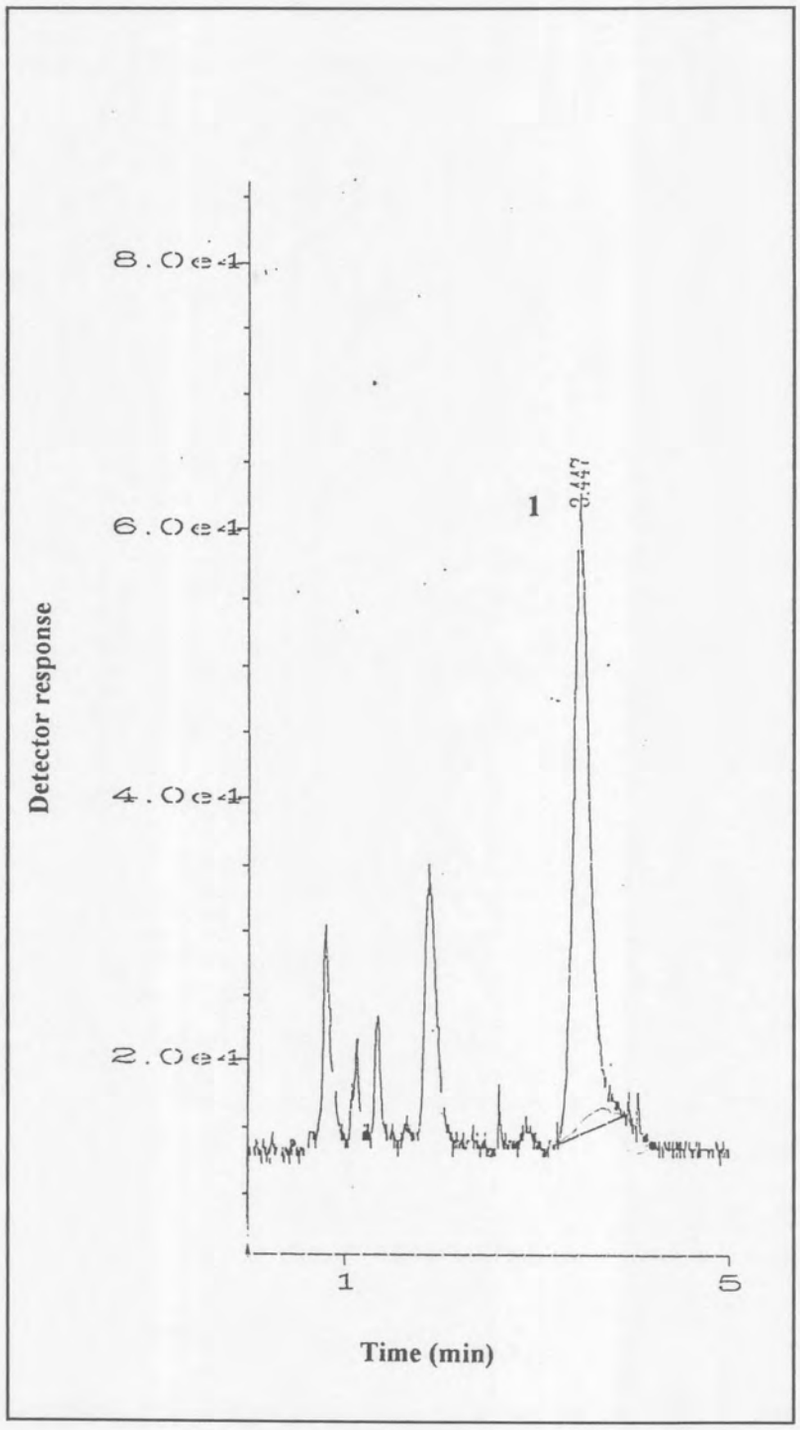


Figure 3.14 : The determination of urinary *4-PA* as *4-PA* lactone.

Retention time : 3.447 minutes.

Peaks : 1 = *4-PA* lactone - unmarked peaks were unidentified background components.

Table 3.7 : Urinary *4-PA* excretion expressed in terms of 24 hour urine volume.

4-PA	*4-PA* (24 hr) (μmol/24h)	
	White	**Black**
N	**9**	**7**
Mean	147.6	151.9
Median	144.4	151.4
SD	32.5	36.3
p-Value	***NS***	

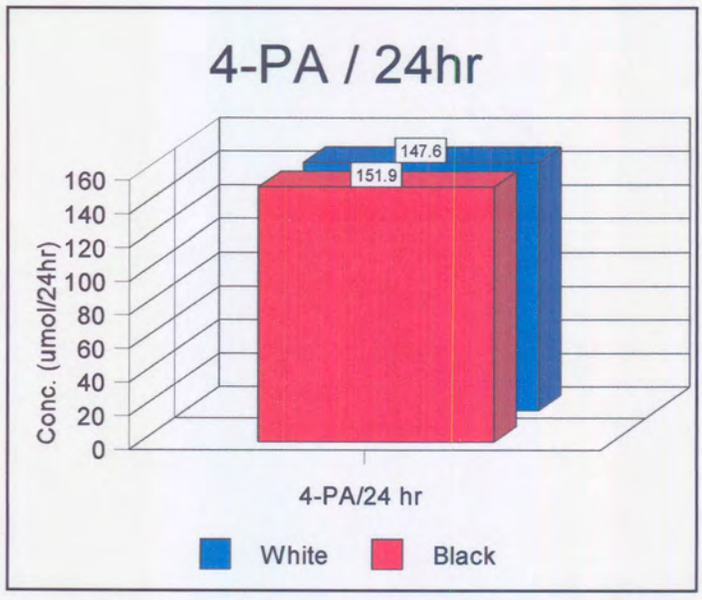


Figure 3.15 : Urine *4-PA* excretion as function of 24 hour urine volume. Statistical analysis showed no significant difference in whites and blacks.

3.5 DISCUSSION

It has been demonstrated that there are definite differences in pharmacological response to certain drugs between races (**87**). This, together with the lower plasma *PLP* concentration obtained in blacks, motivated the **second phase** of the study. Although the **first phase** of this study did not exclude the possible effect of absorption and bioavailability on lower circulating plasma *PLP* concentrations obtained in blacks, the **second phase** of this study concentrated solely on the pharmacokinetic response following a 10 mg crystalline *PN* supplementation.

Nelson *et al* (**23**) showed that glycosylated pyridoxine in certain foods is not as well absorbed as the crystalline form. Comparison of pharmacokinetic parameters after the administration of crystalline *PN* would therefore be considered theoretically ideal in assessing pharmacological response in the respective groups.

The fact that 'basal' plasma *PLP* and *PL* concentrations were not zero, complicated the comparison of calculated pharmacokinetic parameters in the different groups. Simple subtraction of 'basal' levels from the consecutive observed levels were performed in order to obtain data suitable for pharmacokinetic analysis.

Analysing the area under the curve data (see **Tables 3.3-6**) as well as the calculation of the respective pharmacokinetic parameters for plasma *PLP* and *PL* metabolites in 9 white and 7 male subjects, showed no statistical significant differences between the two groups (See **Tables 3.5-6** and **Figures 3.7-13**). These results suggest similar 'drug' appearance rate, distribution, and metabolism for the metabolites *PLP* and *PL* in this study population group after administration of crystalline pyridoxine. A tendency in slower appearance rates for both *PLP* and *PL* was however

observed in blacks and requires further investigation (See **Figure 3.16**).

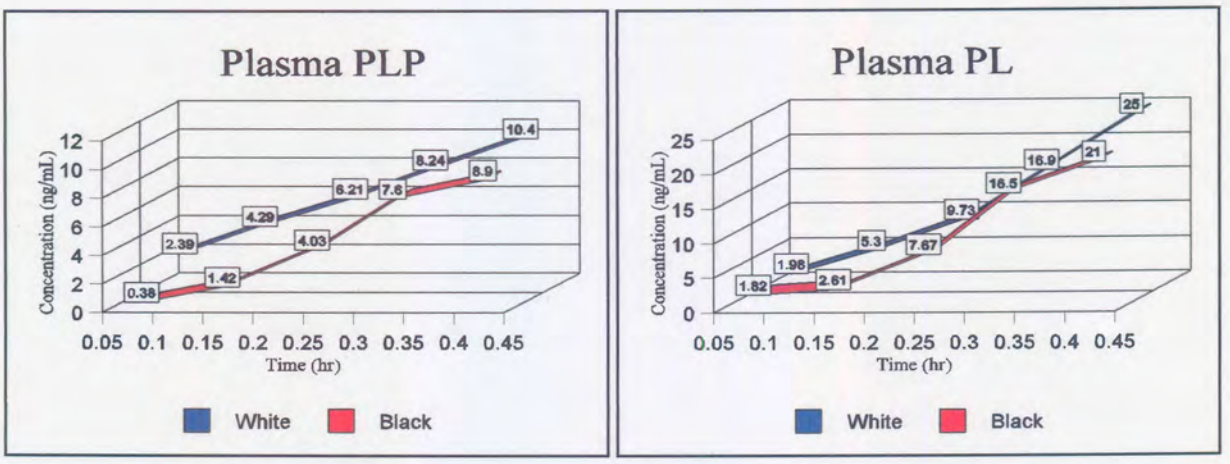


Figure 3.16 : 0.42 Hr AUC cut-off point in blacks and whites for plasma *PLP* and *PL* respectively

A 24 hour urine sample was collected from each of the study participants and the end product of vit B_6 metabolism, *4-PA*, was determined by HPLC as described by Ubbink *et al* (**62**). These results were expressed in terms of 24 hour urine volume. With no vit B_6 deficiency in the study participants (exclusion criteria applied), one would not expect significant variance in urinary *4-PA* excretion with corrected basal plasma *PLP* and *PL* concentrations and similar calculated pharmacokinetic parameters between the respective groups. This conclusion also supports the fact that urinary *4-PA* excretion is considered to be a better short term indicator of vit B_6 status (**64**). Urinary *4-PA* concentrations measured support this and no statistical difference was found in the respective groups, which also suggests similar 'drug' excretion (See **Table 3.7** and **Figure 3.15**)

The pharmacokinetic parameters calculated for plasma *PLP* and *PL* respectively, confirmed earlier observed mono- and bi exponential elimination characteristics respectively (**75, 76**). This suggests

a one- and two- compartment model for *PLP* and *PL* respectively (**Figures 3.1-4**). The different elimination characteristics could prove important during the assessment of vit B_6 status in an overall population study and especially under research conditions where an accurate indication of body stores of vit B_6 is required. In basic pharmacokinetics (**85**), the time difference between last 'dose" and a blood-sample is of extreme importance. If non-compliance or therapeutic failure is suspected, a trough blood-sample is preferred, especially in 'drugs' with a relatively short half-life. Samples obtained earlier in the case of a one compartment model are still of clinical significance as long as consecutive samples used in monitoring 'drug' response are obtained at approximately the same time since the last 'dose'. This is unfortunately not applicable on a two-compartment model. A two- compartment model consists of a distribution and elimination phase. Blood-samples obtained in serum / plasma before the completion of the distribution phase, will not correspond with the concentration at receptor sites, and will give falsely high serum / plasma concentrations. Examples of this include the cardiac glycoside, Digitalis [Digoxin] (**88**) and the anti-depressant, lithium carbonate (**85**). It is thus essential to allow at least five times the distribution half-life before pharmacological response is assessed in order to avoid false interpretation of concentration data. The distribution half-life can be calculated from the distribution phase (α) rate constant (see **Figure 3.4**). The computer software programme used to calculate pharmacokinetic parameters in this thesis (**84**), uses a technique called 'curve stripping' or method of residuals to determine estimates of macro rate constants for a two-compartment model. Using this method in conjunction with obtained experimental data (**Tables 3.1-2**) , the distribution $t_{½}$ for *PL* is estimated at 1.141 hours (see **APPENDIX 10**). One should therefore allow at least 5-6 hours after administering an oral dose / nutrient before blood-sampling in order to get a true reflection of body store *PL* concentration. Thus, to obtain true baseline values of body store *PL* under normal or experimental conditions, the collecting of a fasting blood-sample should be a prerequisite.

CHAPTER 4 : VITAMER B_6 ENZYME KINETIC STUDIES

4.1 INTRODUCTION

Pharmacokinetic studies performed during the **second phase** of this study did not demonstrate any significant differences in pharmacological response to a given dosage regimen of *pyridoxine hydrochloride* in humans. The final phase of the study was conducted to illustrate the metabolic interplay of enzymes that could predispose to the earlier observed racial differences found during the **first phase** of this study. As stated earlier, three enzymes involved in vit B_6 metabolism are expressed in the erythrocyte (see section 1.2). During the final phase of this study, I focused on the kinetic characteristics of the respective enzymes *PL* kinase and *PMP* (*PNP*) oxidase since their activities result in *PLP* production from *PL* and *PMP* respectively.

4.2 ENZYME KINETICS (adapted from **89**, **90**)

4.2.1 ENZYMES

Enzymes are protein catalysts of biological origin and virtually all the chemical reactions that take place in living matter are catalysed by specific enzymes. An enzyme changes the rate at which equilibrium is reached between reactants and products, it does not alter the equilibrium constant of the reaction. Enzymes act through an enzyme-substrate complex (ES), in which a molecule of a substrate is bound to the specialized substrate binding region (the active centre) of the enzyme molecule. The binding process transforms the substrate molecule to an activated state. The energy required for this transformation is provided by free energy of binding of substrate (S) and enzyme (E). Therefore, the energy barrier to the reaction is lowered and breakdown to products is accelerated. The ES complex breaks down to give the reaction products (P) and free enzyme.

See equation :

$$E + S \rightleftarrows ES \rightarrow P + E$$

4.2.2 FACTORS GOVERNING THE RATE OF ENZYME-CATALYSED REACTIONS

4.2.2.1 Enzyme concentration

Provided that an excess of free substrate molecules is maintained, addition of more enzyme molecules to the reaction system will increase the concentration of ES and thus the overall rate of the reaction. This accounts for the observation that the rate or reaction is generally proportional to the concentration of the enzyme present in the system.

4.2.2.2 Substrate concentration

If the enzyme concentration is fixed and the substrate concentration varied, the rate of reaction is almost directly proportional to substrate concentration at low values of the latter. The reaction is essentially first order (e.g. the reaction proceeds at a rate exactly proportional to the concentration of one reactant) with respect to substrate concentration. At low concentrations of substrate, only a fraction of the enzyme is associated with substrate, and the rate observed reflects the low concentration of the enzyme-substrate complex. At high substrate concentrations, variation in substrate concentration has no effect on rate, and the reaction is zero-order. Under these conditions, all the enzyme is bound to substrate and a much higher rate of reaction is obtained. Since all the enzyme is now present in the form of the complex, no further increase in complex concentration and no further increment in reaction rate is possible. The maximum possible velocity for the reaction has been reached. Under conditions in which excess substrate is present, the formation of an enzyme-substrate complex accounts for the typical hyperbolic relationship between reaction velocity and substrate concentration. These curves are usually known as Michaelis-Menten curves. The Michaelis-Menten theory assumes that the enzyme (E)

first combines with the substrate (S) to form the enzyme-substrate complex (ES), the latter then breaks down to form free enzyme and the product (P). The Michealis-Menten curve is described by the following equation :

$$V_0 = \frac{V_{max}\,[S]}{K_M + [S]}$$

V_{max} is the velocity that the observed value of initial velocity (V_0) approaches at high values of (S). It increases with increasing enzyme concentration. K_M, the Michealis-Menten constant, is the substrate concentration at which $V_0 = V_{max} / 2$, and it is constant for a given enzyme acting under given conditions. (The substrate concentration at which the reaction velocity is half maximal). If an equilibrium is set up between E and S, as is usually the case, K_M is the equilibrium constant of this reaction. See **Figure 4.1** :

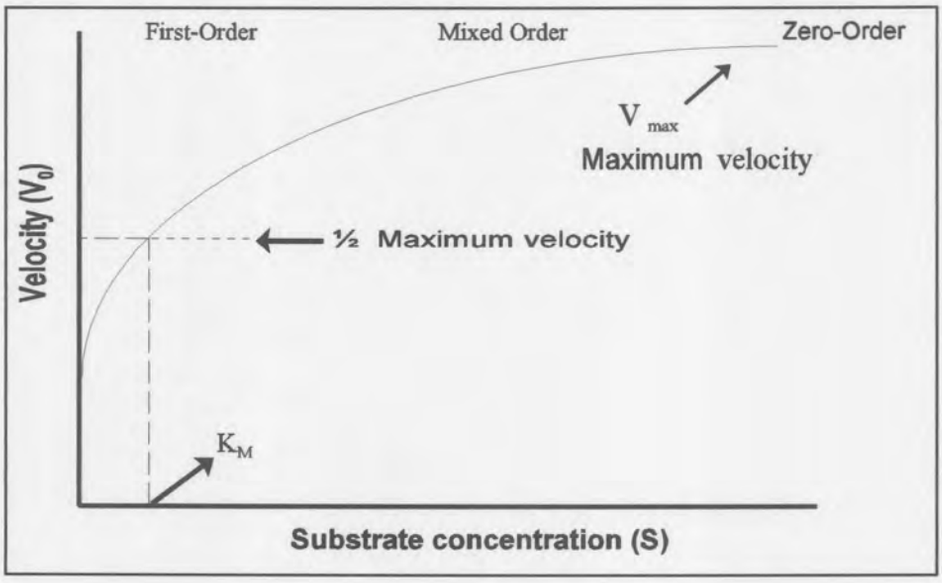


Figure 4.1 : Effect of substrate concentration on the rate of an enzyme-catalysed reaction.

4.2.2.3 Transformation of the Michaelis-Menten equation

One common transformation that is useful in plotting experimental data is simply by taking the reciprocal of the Michaelis-Menten equation :

$$\frac{1}{V_0} = \frac{K_M + [S]}{V_{max}\,[S]}$$

which reduces to :

$$\frac{1}{V_0} = \frac{K_M}{V_{max}}\,\frac{1}{[S]} + \frac{1}{V_{max}}$$

When $1/V_0$ is plotted against $1/[S]$, a straight line is obtained - the Lineweaver-Burk plot. This line will have a slope of K_M / V_{max}, an intercept of $1/V_{max}$ on the $1/V_0$ axis, and an intercept of $-1/K_M$ on the $1/[S]$ axis. See **Figure 4.2** :

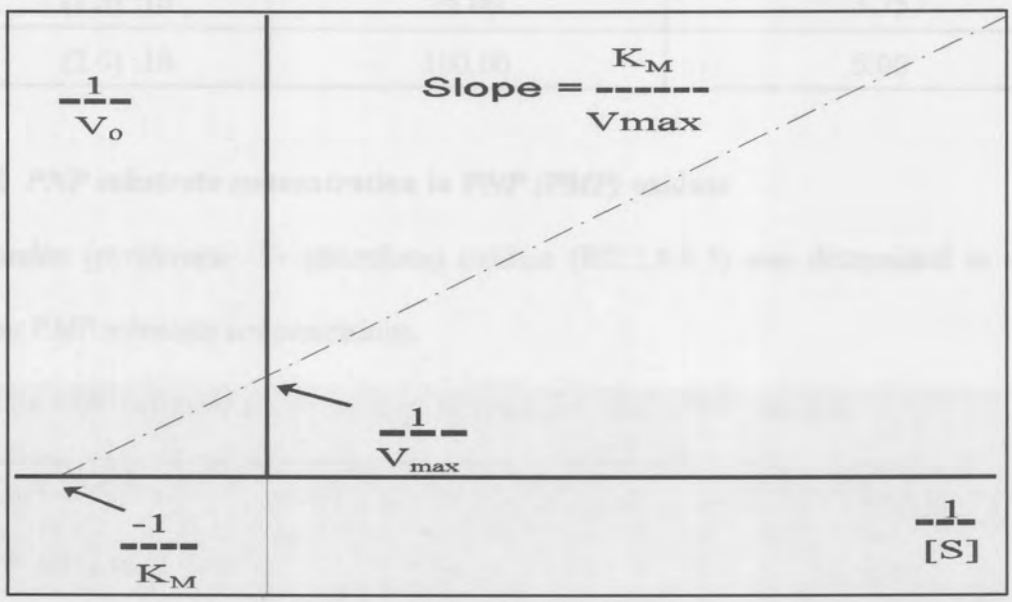


Figure 4.2 : A double reciprocal (Lineweaver-Burk) plot.

4.2.2.4 Effect of pH on enzymatic activity

The pH-activity relationship of any given enzyme, depends on the acid base behaviour of enzyme and substrate, as well as many other factors that are usually difficult to analyse quantitatively. The shape of the pH- activity profile usually varies with substrate concentration, since the K_M of most enzymes change with pH. In most studies of enzyme kinetics, the pH is held constant at or near optimum pH.

4.2.2.5 Effect of temperature on enzymatic reactions

The rate of enzyme-catalysed reactions generally increases with temperature within the temperature range in which the enzyme is stable and retains full activity. The rate of most enzymatic reactions approximately doubles for each 10 ^{0}C rise in temperature. Enzymes, being proteins, are denatured by heat and become inactive as the temperature is raised beyond a certain point. The apparent optimum temperature is thus the resultant of two processes :

a. The usual increase in reaction rate with temperature.

b. The increasing rate of thermal denaturation of the enzyme above a critical temperature.

4.3 MATERIALS AND METHODS

4.3.1 EXPERIMENTAL PROTOCOL

4.3.1.1 Study design and participants

A group of 40 male volunteers between the ages 18-28 years were recruited from the South African National Defence Force (SANDF) where they were exposed to the same socio-economic environment and same diet (basic mess diet). In this cross-sectional, non-randomized, single blind (to the laboratory analyst) study, a single plasma- and serum blood-sample was collected from a peripheral vein. From this group, 14 white and 14 black male subjects were selected after complying with exclusion criteria to participate in the final stage of this study.

4.3.1.2 Exclusion criteria

The exclusion criteria applied is discussed in more detail in chapter two under section 2.2.1.2.

These include :

a. Vit B_6 status. ([*PLP*] of <20 nmol/L and > 150 nmol/L).

b. Chemotherapy.

c. Anaemic conditions.([Hb] of < 11.0 g/L).

d. Vitamin supplements.

e. Infection.

f. Liver - / Renal impairment. (AST > 42 IU/L, ALP >128 IU/L, creatinine >115 μmol/L, urea > 8.4 mmol/L).

g. Medication :

1. Isoniazid.
2. Theophylline.
3. Penicillamine.

4.3.2 ANALYTICAL TECHNIQUES

4.3.2.1 Vitamer B_6 enzyme activities in erythrocytes

The activities of the enzymes *PL* kinase (EC: 2.7.1.35) and *PMP (PNP)* oxidase (EC: 1.4.3.5) were determined in 14 white- and 14 black male subjects using HPLC as described in chapter two. See section 2.2.2.2.

4.3.2.2 Substrate concentration

In order to calculate the Michaelis-Menten constants, substrate concentrations were varied . The different substrate concentrations used for *PL* kinase and *PMP (PNP)* oxidase are summarized in **Tables 4.1-4.2.**

4.3.2.2.1 *PL* substrate concentration in *PL* kinase

Pyridoxal kinase (EC: 2.7.1.35) was determined in vitro at increasing *PL* substrate concentrations:

Table 4.1 : *PL* substrate concentrations in assay for *PL* kinase.

N	DILUTION *(PL)* in 10mL H_2O	CONCENTRATION(vial) (umol/L)	CONCENTRATION (end) (mmol/L)
1	Distilled H_2O		
2	(0.2) :10	10.00	0.50
3	(0.3) :10	15.00	0.75
4	(0.5) :10	25.00	1.25
5	(0.8) :10	40.00	2.00
6	(1.0) :10	50.00	2.50
7	(1.5) :10	75.00	3.75
8	(2.0) :10	100.00	5.00

4.3.2.2.2 *PNP* substrate concentration in *PNP (PMP)* oxidase

Pyridoxamine (pyridoxine -5'- phosphate) oxidase (EC:1.4.3.5) was determined in vitro at increasing *PMP* substrate concentrations.

Table 4.2 : *PMP* substrate concentrations in assay for *PMP* (*PNP*) oxidase.

N	DILUTION *(PMP)* in 10 mL H_2O	CONCENTRATION(vial) umol/L	CONCENTRATION (end) (mmol/L)
1	Distilled H_20		
2	(0.08) :10	4.00	0.20
3	(0.16) :10	8.00	0.40
4	(0.24) :10	12.00	0.60

Table 4.2 (contd)

5	(0.32) :10	16.00	0.80
6	(0.4) :10	20.00	1.00
7	(0.48) :10	24.00	1.20
8	(0.56) :10	28.00	1.40

4.3.3 ENZYME KINETIC CALCULATIONS

A data analysis computer software program "ENZFITTER" [Biosoft, Cambridge, UK, 1987], which allows rapid analysis and plotting of experimental data using non-linear regression, was used to process experimental data (**91**). From the rate of enzyme-substrate formation at increasing substrate concentrations, data were transformed into Lineweaver-Burk plots to display experimental data and highlight outliers. A function called robust weighting was used to eliminate outliers. The program listed the changing parameter values calculated during successive iterations until it found the best fit values for K_M and V_{max} .(See **Figures 4.3-4**). Michaelis-Menten constant (K_M) and maximum velocity rate (V_{max}) were calculated for the enzymes *PL* kinase and *PMP (PNP)* oxidase respectively.

4.3.4 STATISTICAL ANALYSIS (92)

The non-parametric analysis for independent groups, the Mann Whitney u-test was used to compare calculated Michaelis-Menten constants and maximum velocity rates for the respective enzymes *PL* kinase and *PMP* (*PNP*) oxidase in whites and blacks. A 0.05 significance level was applied.

4.4 RESULTS

APPENDIX 11 shows the respective amount of product formed in nmol/L obtained by HPLC in the different groups for *PL* kinase and *PMP* (*PNP*) oxidase at increasing substrate concentrations. The best fit data were transformed into Lineweaver-Burk plots to highlight possible outliers and used to calculate Michaelis-Menten constants (K_M) and maximum velocity rates [V_{max}] (See **APPENDIX 12)**. Examples of these calculations and graphs generated by the "ENZFITTER" computer programme can be seen in **Figures 4.3-4**. Experimental data of two subjects in group 1 (white) were omitted in performing statistical analysis because of outliers identified in the Lineweaver-Burk plots.

Table 4.3 summarizes the Michaelis-Menten constants calculated for the respective vitamer B_6 enzymes kinase and oxidase in whites and blacks in section 4.4.1. These data are also displayed with **Figures 4.5-7**.

No statistical significant difference in K_M values was obtained between the different groups for the enzyme *PMP* (*PNP*) oxidase. Although not significant, a p-value of >0.05 ; <0.1 was obtained in the case of K_M values calculated for *PL* kinase in the different groups.

Maximum velocity rates calculated for the respective enzymes kinase and oxidase in the different groups are summarized in **Table 4.4** and displayed with **Figure 4.8** in section 4.4.2. No significant statistical differences were obtained for the respective enzymes *PL* kinase and *PMP* (*PNP*) oxidase between blacks and whites.

(PMP) Oxidase (Pat 14-white)

Michaelis Menten kinetics
Simple weighting
Robust weighting

Variable	Value	Std. Err.
k cat	2.98895E+02	1.84451E+01
Km	5.90722E-01	8.36180E-02

	[substrate]	Rate	Calculated
1	2.00000E-01	7.58000E+01	7.56007E+01
2	4.00000E-01	1.18000E+02	1.20678E+02
3	6.00000E-01	1.61900E+02	1.50612E+02
4	8.00000E-01	1.66400E+02	1.71937E+02
5	1.00000E+00	1.85800E+02	1.87899E+02
6	1.20000E+00	2.02800E+02	2.00296E+02

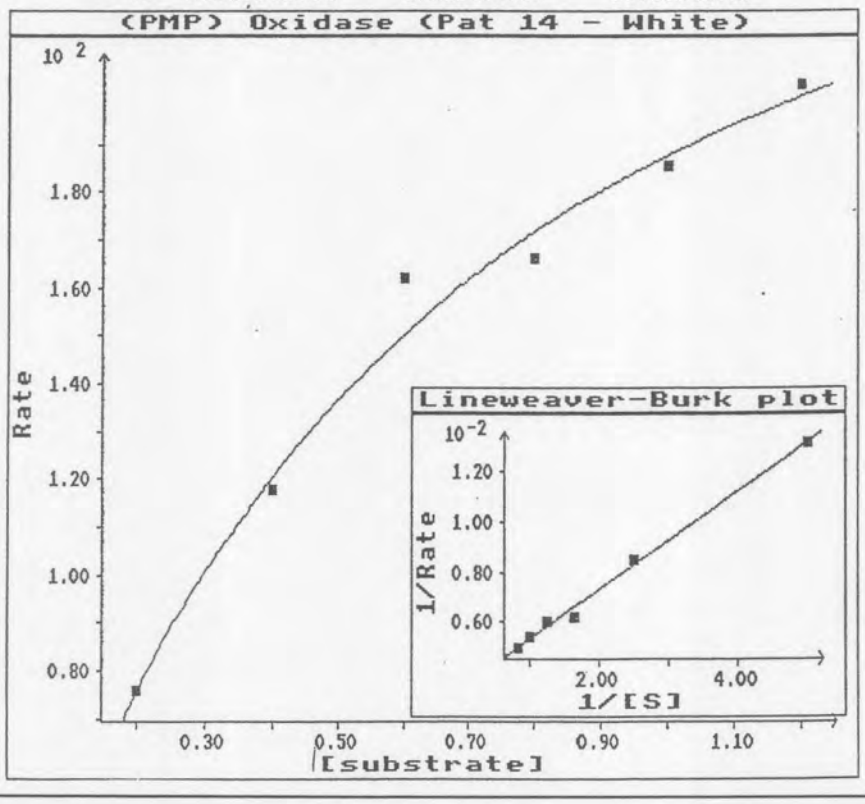


Figure 4.3 : Subject example shows the best fit data used to calculate Michaelis-Menten constant and maximum velocity rate for *PMP (PNP)* oxidase.

(Transformation of graph shows Lineweaver-Burk plot after extreme outliers were eliminated by means of robust weighting → in this case *PMP* substrate concentration of 1.4 mmol/L).

Kinase (Pat 14-white)

Michaelis Menten kinetics
Simple weighting
Robust weighting

Variable	Value	Std. Err.
k cat	3.84821E+02	1.10030E+01
Km	1.77139E+00	1.22557E-01

	[substrate]	Rate	Calculated
1	5.00000E-01	7.49000E+01	8.47104E+01
2	7.50000E-01	1.14600E+02	1.14467E+02
3	1.25000E+00	1.59700E+02	1.59207E+02
4	2.00000E+00	2.05500E+02	2.04074E+02
5	2.50000E+00	2.33100E+02	2.25232E+02
6	3.75000E+00	2.59300E+02	2.61361E+02
7	5.00000E+00	2.81800E+02	2.84152E+02

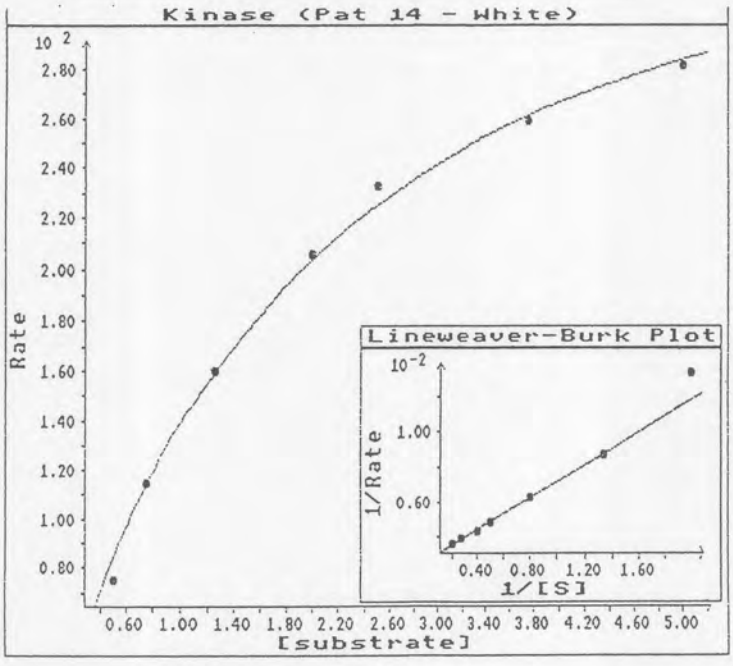


Figure 4.4 : Subject example shows best fit data used to calculate Michaelis-Menten constant and maximum velocity rate for *PL* kinase.

(Transformation of graph shows Lineweaver-Burk plot. (In this case no extreme error data points had to be eliminated by means of robust weighting and all 7 substrate concentrations could be utilized for calculation of K_M and V_{max}).

4.4.1 MICHAELIS-MENTEN CONSTANT

Table 4.3 : Michaelis-Menten constants (K_M) in nmol/L calculated at increasing substrate concentrations for the enzymes *PMP (PNP)* oxidase and *PL* kinase respectively.

Michaelis-Menten constant (K_M) (nmol/L)	*PMP (PNP)* Oxidase		*PL* Kinase	
	White	Black	White	Black
N	12	14	12	14
Mean	0.53	0.58	1.61	1.25
Median	0.40	0.53	1.71	1.28
SD	0.36	0.35	0.70	0.52
p-Value	*NS*		*0.05< p > 0.1*	

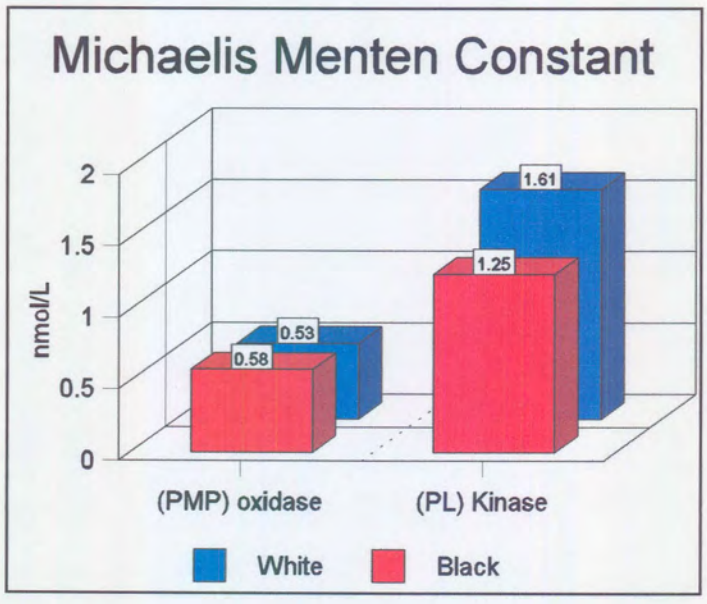


Figure 4.5 : Histogram shows Michaelis-Menten constants in nmol/L calculated for the enzymes *PMP (PNP)* oxidase and *PL* kinase in whites and blacks respectively.

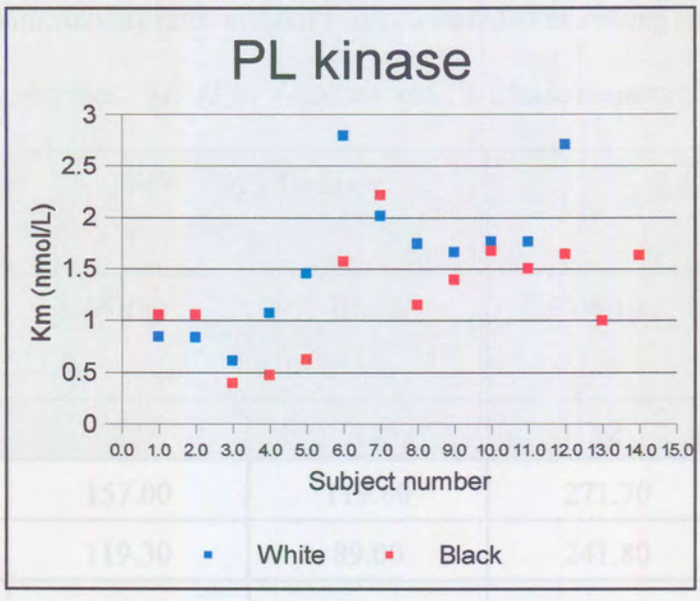


Figure 4.6 : Scatter plot displays the individual K_M values in nmol/L calculated for *PL* kinase in whites and blacks respectively.

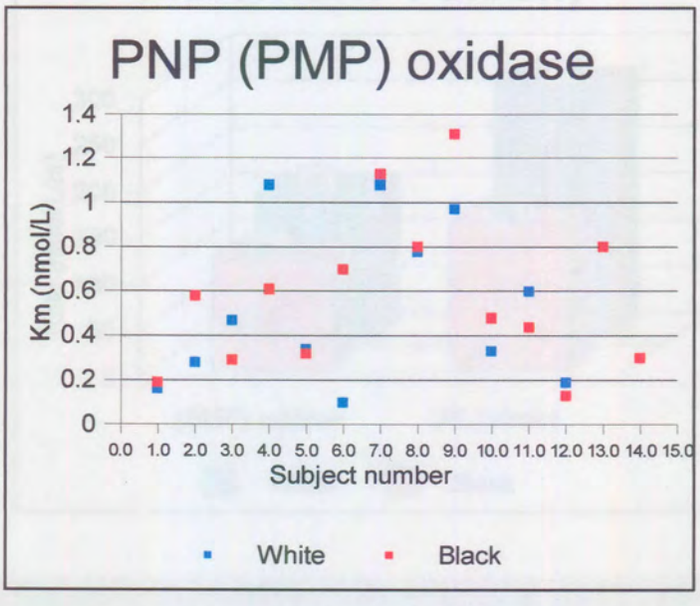


Figure 4.7 : Scatter plot displays the individual K_M values in nmol/L calculated for *PMP (PNP)* oxidase in whites and blacks respectively.

4.4.2 MAXIMUM VELOCITY RATE

Table 4.4 : Maximum velocity rates in nmol/L/hr calculated at increasing substrate concentrations for the enzymes *PMP (PNP)* oxidase and *PL* kinase respectively.

Maximum velocity (V_{max}) (nmol/L/hr)	*PMP (PNP)* Oxidase		*PL* Kinase	
	White	Black	White	Black
N	12	14	12	14
Mean	157.00	119.60	271.70	158.10
Median	119.30	89.00	241.80	123.80
SD	102.40	68.50	155.10	83.80
p-Value	*NS*		*NS*	

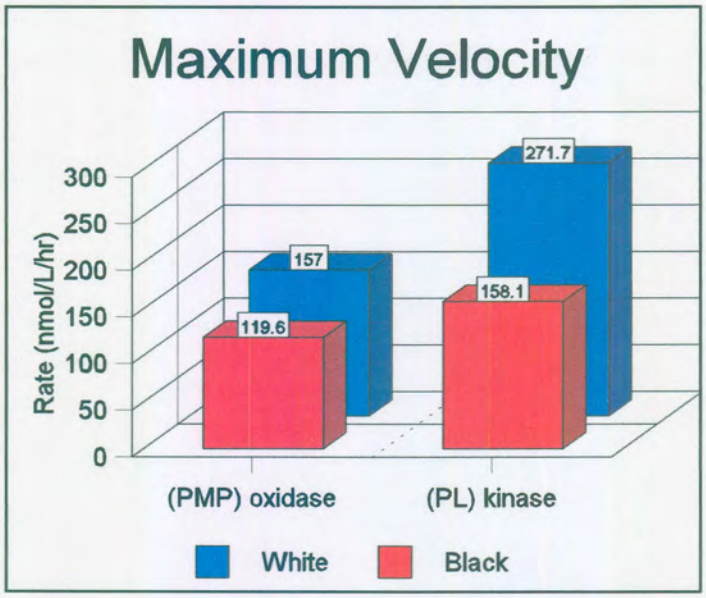


Figure 4.8 : Histogram shows the maximum velocity rates in nmol/L/hr calculated for the enzymes *PMP (PNP)* oxidase and *PL* kinase in whites and blacks respectively.

4.5 DISCUSSION

K_M - and V_{max} values obtained for the respective enzymes *PL* kinase and *PMP* (*PNP*) oxidase between blacks and whites can be summarized with **Figures 4.9-10**.

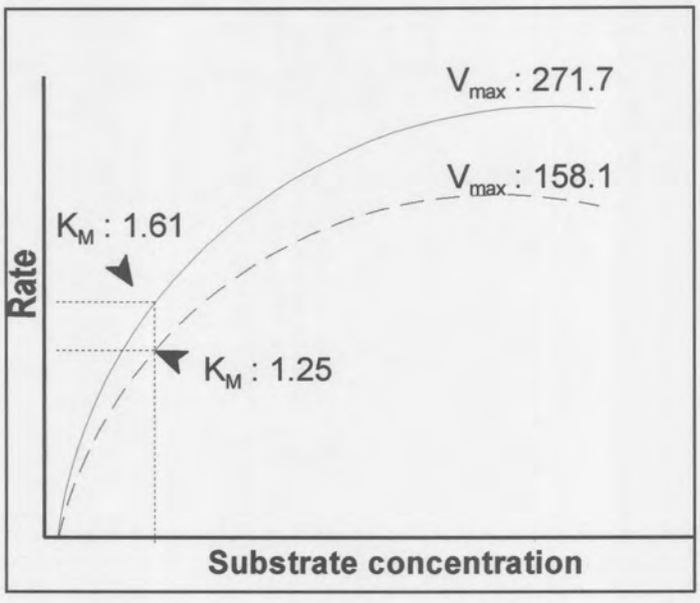


Figure 4.9 : K_M and V_{max} values for *PL* kinase in blacks ***(dotted line)*** and whites (***straight line***).

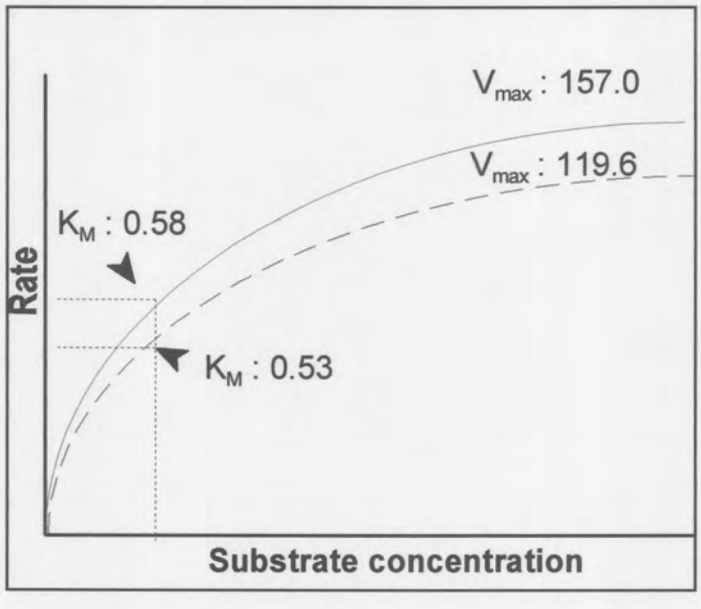


Figure 4.10 : K_M and V_{max} values for *PMP* oxidase in blacks ***(dotted -)*** and whites ***(straight line)***

PLP production from *PL* and *PMP* is dependant on both *PL* kinase and *PMP* (*PNP*) oxidase activity. Plasma *PLP* cannot cross cellular membranes, and the cell is dependant on circulating pyridoxal levels to obtain adequate amounts of vit B_6. Intracellularly, the enzyme *PL* kinase is primarily responsible for phosphorylation of pyridoxal to *PLP*. *PMP* (*PNP*) oxidase, on the other hand, is responsible for the oxidation of pyridoxamine to *PLP* (**60**).

Calculation of individual Michaelis-Menten constants in blacks and whites, for the respective enzymes *PL* kinase and *PMP* (*PNP*) oxidase, failed to illustrate any significant statistical differences. [Michaelis-Menten constants calculated for *PL* kinase were however higher in whites : $0.05 < p < 0.1$] (see **Table 4.3**).

Furthermore, no significant differences in maximum velocity between blacks and whites could be obtained for the respective enzymes *PL* kinase and *PMP* (*PNP*) oxidase. Maximum velocity calculated for *PL* kinase tended to be higher in whites, but this difference was not statistical significant.

Following the kinetic aspects of enzyme catalysed reactions, one could speculate that the higher maximum velocity (zero-order state) rates for the enzyme *PL* kinase, could explain the earlier higher observed *PLP* production from *PL* in whites (Chapter two). Calculation of K_M is dependant on substrate concentration and rate of reaction - thus the slope. At low substrate concentrations the initial reaction velocity (V_0) is nearly proportional to the substrate concentration (zero-order). When increasing the substrate concentration, the reaction becomes mixed order. Increasing substrate concentration even further, results in saturation of the enzyme with its substrate and the reaction is known as zero-order (**89,90**). If this criterion is applied to the experimental results obtained during the final stage in this study, the following conclusions can be made:

a. Despite the tendencies observed with the respective kinetic parameters calculated, high individual variation with respect to K_M and V_{max} in whites and blacks must also be taken into consideration. Red blood cell factors could explain the individual variation. High individual variation may be ruled out if a crystalline enzyme form was used in experimental studies. However, the question would then be : To what extent would this simulate in vivo conditions ?

b. The lower K_M and V_{max} obtained in blacks may be to some extent also reflected by the area under the curve data obtained after administering a *PN* load (Chapter three). Although not statistically significant, initial appearance rate tended to be slower in blacks compared to whites for both the respective metabolites *PLP* and *PL* (see **Table 3.4**)

This phenomenon in combination with the calculated enzymatic kinetic parameters could explain lower *PLP* production in blacks. Further studies regarding these aspects should be considered in the future.

CHAPTER 5 : CONCLUDING DISCUSSION

Since evidence suggested that there are racial differences in circulating plasma *PLP* concentration (**68**), I tried to establish with this research if these differences could be attributed to altered kinetics, whether by the metabolic interplay of total enzyme activity, pharmacological response or finally enzymatic reactions involved in vit B_6 metabolism.

Analytical methods using HPLC as described by Ubbink *et al* (**60, 61, 79**) proved comprehensive enough with precise quantification of plasma *PLP* and *PL*, and the respective enzymes expressed in the erythrocyte involved in vit B_6 metabolism, in large - and small population studies. Similar techniques that were developed by Ubbink *et al* (**62**) were used to quantify the end product of vit B_6 metabolism, *4-PA*, with great success.

This research comprised of three phases, each of which will be discussed in more detail hereafter:

5.1 DETERMINATION OF RANDOM PLASMA *PLP* CONCENTRATION AND B_6 VITAMER ENZYME ACTIVITIES IN ERYTHROCYTES FROM BLACKS AND WHITES

Results from the **first phase** of this study did show a significant statistical difference in random, mean plasma *PLP* concentrations obtained in the different groups. Analysis of the respective total enzyme activities in the erythrocyte namely *PL* kinase, *PMP* (*PNP*) oxidase and *PLP* phosphatase, did not demonstrate any significant differences. These findings suggest similar metabolic interplay of the enzymes involved in vit B_6 metabolism after absorption in the same population study.

It must be stressed again that although the test subjects were confined to the same basic mess diet, freedom of choice could have played a major role in the lower *PLP* concentration observed in blacks. The effects of protein intake, carbohydrates and glycosylated forms on vit B_6 bioavailability, absorption and metabolism are discussed in more detail in chapters one & two (**22, 24, 25**). These may have played an important role in the observed difference found in plasma *PLP* concentration.

Furthermore, it would be advisable to confine test subjects to exactly the same diet for a period of time whilst conducting research of this nature, in order to exclude personal preferences and choices regarding food intake.

As is the case here, virtually all publications on human vit B_6 nutritional status report only on plasma *PLP* levels. Do lower plasma *PLP* concentrations necessarily mean vit B_6 deficiency? It could be that lower plasma *PLP* concentration is in fact only associated with an altered *PLP* ⇌ *PL* equilibrium, resulting in higher or lower circulating *PL* levels (**82, 83**). It should be taken into account that vitamin not needed by the cell is simply metabolized to a non-utilizable form, in this case the end product, *4-PA*, and excreted. The opposite also applies. A higher *PL* level could be advantageous to the individual, since *PL* is directly available for cellular uptake whilst *PLP* is not (see Chapter one). Lower *PLP* concentrations obtained in blacks could just reflect increased mobilisation of *PLP* by hydrolysis to *PL* in order to maintain *PL* levels during periods of increased cellular demand for vit B_6.

5.2 PHARMACOKINETIC STUDIES AFTER A SINGLE ORAL SUPPLEMENT OF *PYRIDOXINE HYDROCHLORIDE*

The calculation of seven pharmacokinetic parameters following a 10 mg supplement of crystalline *pyridoxine hydrochloride* failed to illustrate any racial differences between the groups. Furthermore, no differences could be found in the amount of *4-PA* excreted, following the 24-hour period after supplementation. From analysing area under the curve data, blacks showed a tendency of slower appearance rates in plasma for *PLP* and *PL*. Although calculation of the respective pharmacokinetic parameters suggest similar 'drug' liberation, metabolism and excretion after the administering of a crystalline form of *pyridoxine hydrochloride,* further studies are suggested to confirm differences in appearance rates for *PLP* and *PL* respectively.

Other factors that must be taken into account before coming to a final conclusion are :

a. None of the test subjects with similar age, body mass index etc. was vit B_6 deficient - plasma *PLP* and *PL* were measured prior to the actual *pyridoxine hydrochloride* supplementation and deficient test subjects were excluded from participating.

b. A crystalline form was administered that excluded the possibility of dietary factors discussed in chapter one, influencing results.

c. Test subjects were not confined to the same diet during the test period.

d. Basal 'drug' concentrations were corrected in order to obtain more realistic values which made the comparison of pharmacokinetic parameters easier.

Thus, overall results from the **second phase** of this study support the idea that lower plasma *PLP* concentrations obtained during the **first phase** of this study could be diet related, hence further

investigation regarding appearance rates is recommended.

Comparing pharmacokinetic parameters in the first population study could generate totally different results since *PLP* ≠ *PL* equilibrium could be altered in this case. This could definitely influence pharmacokinetic parameters like half-life, elimination rate constant and mean residence time especially for plasma *PLP* which fluctuates, depending on vit B_6 body store requirements . Finally, pharmacokinetic parameters calculated for plasma *PLP* and *PL* confirmed mono- and bi-exponential elimination rates respectively (**75, 76**). As discussed in chapter three, this suggests a two-compartment pharmacokinetic model applicable to pyridoxal. This could prove useful when future research is conducted, to allow at least 5-6 hours after vit B_6 intake or supplementation before blood-sampling, in order to obtain equilibrium between cellular concentrations and circulating levels of *PL* .

5.3 VITAMER B_6 ENZYME KINETIC STUDIES

Since similar total vit B_6 enzyme activities and pharmacological response obtained during the first two phases of this study failed to explain the earlier observed racial differences, the last phase of this research concentrated on the kinetic characteristics of the respective enzyme ↔ substrate reactions of *PL* kinase and *PMP* (*PNP*) oxidase. Both these enzymes are actively involved in the formation of *PLP* from *PL* and *PMP* respectively. Results from this final stage however, were again inconclusive. Some differences obtained though, could prove useful and should be explored further in future research efforts:

a. The mean maximum rate of velocity, when the enzyme becomes saturated with its substrate, was higher in whites for both enzymes although these differences were not significantly different.

b. When the respective substrate concentrations were increased for the enzymes *PL* kinase and *PMP* (*PNP*) oxidase respectively, the resulting apparent Michaelis Menten constants did not show any significant differences between the two groups. K_M values were however higher in whites for the enzyme *PL* kinase.

The high individual variability obtained in the test subjects for both kinetic parameters K_M and V_{max} (see **Tables 4.3-4**), could possibly be ruled out if a crystalline enzyme form, prepared from each subject, is used. A bigger population study is suggested - results would probably prove more conclusive regarding enzyme kinetic characteristics.

Since the discovery of vit B_6 in 1934 (**1**), numerous research has been and is still carried out in order to establish its role in the context of health and disease. No conclusive evidence was found with this study to support the idea that lower plasma *PLP* concentration found in blacks is related to kinetic aspects of vit B_6 metabolism, whether pharmacological or enzymatic. The relationship between slower appearance rates for the metabolites *PLP* and *PL* respectively after a *PN* load and lower calculated apparent Michaelis-Menten constants calculated for *PL* kinase in blacks could be of some clinical importance and needs to be investigated in future research efforts. I trust that the findings, generated by conducting this research, may well be of some significance in further exploration, regarding the kinetic differences in vit B_6 metabolism in humans.

SUMMARY

The primal aim of this thesis was to establish whether kinetic aspects of vitamin B_6 metabolism predispose to earlier observed racial differences found in plasma pyridoxal -5'-phosphate (*PLP*).

The active forms of vitamin B_6 namely plasma *PLP* and pyridoxal (*PL*) as well as the three enzymes expressed in the erythrocyte involved in B_6 metabolism, *PL* kinase, *PLP* phosphatase and pyridoxamine -5'- phosphate (pyridoxine -5'- phosphate) [*PMP*(*PNP*)] oxidase were measured by high performance liquid chromatography.

Phase one supported earlier experimental evidence and lower plasma *PLP* concentrations were found in blacks in a group of 200 male volunteers recruited from the South African National Defence Force (SANDF). The respective enzyme activities involved in vitamin B_6 metabolism, from the same test subjects, suggested similar *PLP* production from *PMP* and *PL* as well as *PLP* dephosphorylation which result in the release of *PL* into the circulating fluid. Since applied exclusion criteria eliminated the majority of biochemical -, physiological -, genetical - and disease related factors that influence vit B_6 status, dietary factors and individual preferences regarding food intake, were most likely to be responsible for the significantly lower circulating plasma *PLP* encountered in blacks.

Phase two compared pharmacokinetic parameters between 7 black - and 9 white test subjects recruited from the South African Police Services after a single 10 mg oral

supplement of *pyridoxine hydrochloride*. Statistical analysis of the parameters elimination half-life, elimination rate constant, clearance, volume of distribution, mean residence time, maximum peak concentration and time to maximum peak concentration failed to demonstrate any significant differences between the two groups. These results suggest consistent appearance rate, distribution and metabolism for the metabolites *PLP* and *PL* in the study population. A tendency in slower appearance rate, for both the metabolites *PLP* and *PL*, were observed in blacks and needs to be investigated further.

The end product of vitamin B_6 metabolism, *4-pyridoxic acid*, which was expressed in terms of 24 hour urine volume, again failed to illustrate any significant differences between blacks and whites. These results suggested similar excretion properties in my population study. Furthermore, the pharmacokinetic parameters calculated for plasma *PLP* and *PL* respectively, were found to display one-compartment - and two-compartment pharmacokinetic model characteristics. This mono- and bi exponential elimination characteristics displayed by *PLP* and *PL* respectively could be of value in future research efforts in terms of sampling time. The distribution half-life can be determined by the calculation of two-compartment macro-rate constants. Fasting blood-samples should be collected when true baseline values are needed in the case of *PL*. Following vit B_6 supplementation, one should allow at least 5 times the distribution half-life (5-6 hr in the case of *PL*) before blood-sampling in order to achieve true pharmacological response.

Phase three of this study was conducted to illustrate the metabolic interplay of the enzymes *PL* kinase and *PMP* (*PNP*) oxidase involved in *PLP* production. The kinetic parameters, Michaelis-Menten constant and maximum velocity rate, at varying substrate concentrations,

for the enzymes *PL* kinase and *PMP (PNP)* oxidase, were compared in 14 white - and 14 black male test subjects recruited from the SANDF. Both the average Michaelis-Menten constant and maximum velocity rate were higher in whites, but these differences were not statistically significant. The high individual variability for both parameters calculated, can possibly be ruled out if a crystalline enzyme form is used and should be investigated further.

OPSOMMING

Die primêre oogmerk van hierdie verhandeling was om te bepaal of kinetiese aspekte van vitamien B_6 metabolisme aanleiding kan gee tot 'n verskil in plasma piridoksaal -5'- fosfaat (*PLP*) vlakke, soos voorheen gevind in verskillende rasse.

Die aktiewe vorms van vitamien B_6, naamlik plasma *PLP* en piridoksaal (*PL*), sowel as die drie ensieme *PL* kinase, *PLP* fosfatase en piridokamien -5'- fosfaat (piridoksien -5'- fosfaat) [*PMP*(*PNP*)] oksidase wat betrokke is by vitamien B_6 metabolisme in eritrosiete, is bepaal deur hoë-prestasie vloeistof chromatografie.

Fase 1 ondersteun vroeëre eksperimentele bevindings en laer plasma *PLP* konsentrasies is gevind in 'n groep van 200 manlike vrywilligers verbonde aan die Suid-Afrikaanse Nasionale Weermag (SANW). Die onderskeie ensiem aktiwiteite betrokke by vitamien B_6 metabolisme in dieselfde groep proefpersone, dui op soortgelyke *PLP* produksie vanaf *PMP* en *PL* asook *PLP* defosforilasie, wat aanleiding gee tot die vrystelling van *PL* in die bloedstroom. Aangesien toegepaste uitsluitingskriteria in die navorsingsmetodiek die meerderheid van biochemiese -, fisiologiese -, genetiese - en patologiese verwante faktore wat 'n invloed op vitamien B_6 status het, geëlimineer het, is die afleiding gemaak dat diëet faktore en individuele voorkeure t.o.v. voedsel inname, mees waarskynlik verantwoordelik is vir die betekenisvolle laer sirkulerende plasma *PLP* gevind in swartmense.

Tydens **Fase 2** is farmakokinetiese parameters tussen 7 swart - en 9 blanke proefpersone,

verkry vanaf die Suid-Afrikaanse Polisiediens (SAP), na 'n enkele orale dosis van 10 mg *piridoksien hidrochloried* vergelyk. Statistiese analisering van die parameters eliminasie half-leeftyd, eliminasiekoers - konstante, opruiming, volume van distribusie, gemiddelde residensiële tyd, maksimum piek konsentrasie en tyd tot maksimum piek konsentrasie, kon nie daarin slaag om enige betekenisvolle verskil tussen die twee groepe uit te wys nie. Hierdie resultate dui op 'n konsekwente verskyningskoers, distribusie en metabolisme van die metaboliete *PLP* en *PL* in die studiegroep.'n Neiging tot 'n laer verskyningskoers vir beide die metaboliete *PLP* en *PL* is waargeneem in swartmense en verg verdere navorsing. Die eindproduk van vitamien B_6 metabolisme, 4-piridoksien suur, soos bepaal in 24 uur urien uitskeidings monsters, het weer eens nie daarin geslaag om enige betekenisvolle verskille tussen swartmense en blankes uit te wys nie. Hierdie resultate suggereer gelyksoortige uitskeidings-eienskappe in my populasie studie. 'n Verdere bevinding was dat die farmakokinetiese parameters (soos bereken vir plasma *PLP* en *PL* onderskeidelik) eienskappe toon van een-kompartement - en twee-kompartement farmakokinetiese modelle. Hierdie mono- en bi-eksponensiële eliminasie eienskappe wat deur *PLP* en *PL* openbaar word, kan van waarde wees in toekomstige navorsing studies in terme van die tyd van bloedmonster versameling. Die distribusie half-leeftyd kan bepaal word deur die berekening van twee-kompartement makro-koers konstantes. Vastende volbloed monsters word benodig vir die bepaling van ware basislyn waardes in die geval van *PL*. Na toediening van vitamien B_6 behoort 'n wagperiode van minstens 5 maal die distribusie half-leeftyd te volg voordat bloedmonsters geneem word, ten einde 'n realistiese farmakologiese respons te verkry (5-6 ure in die geval van *PL*).

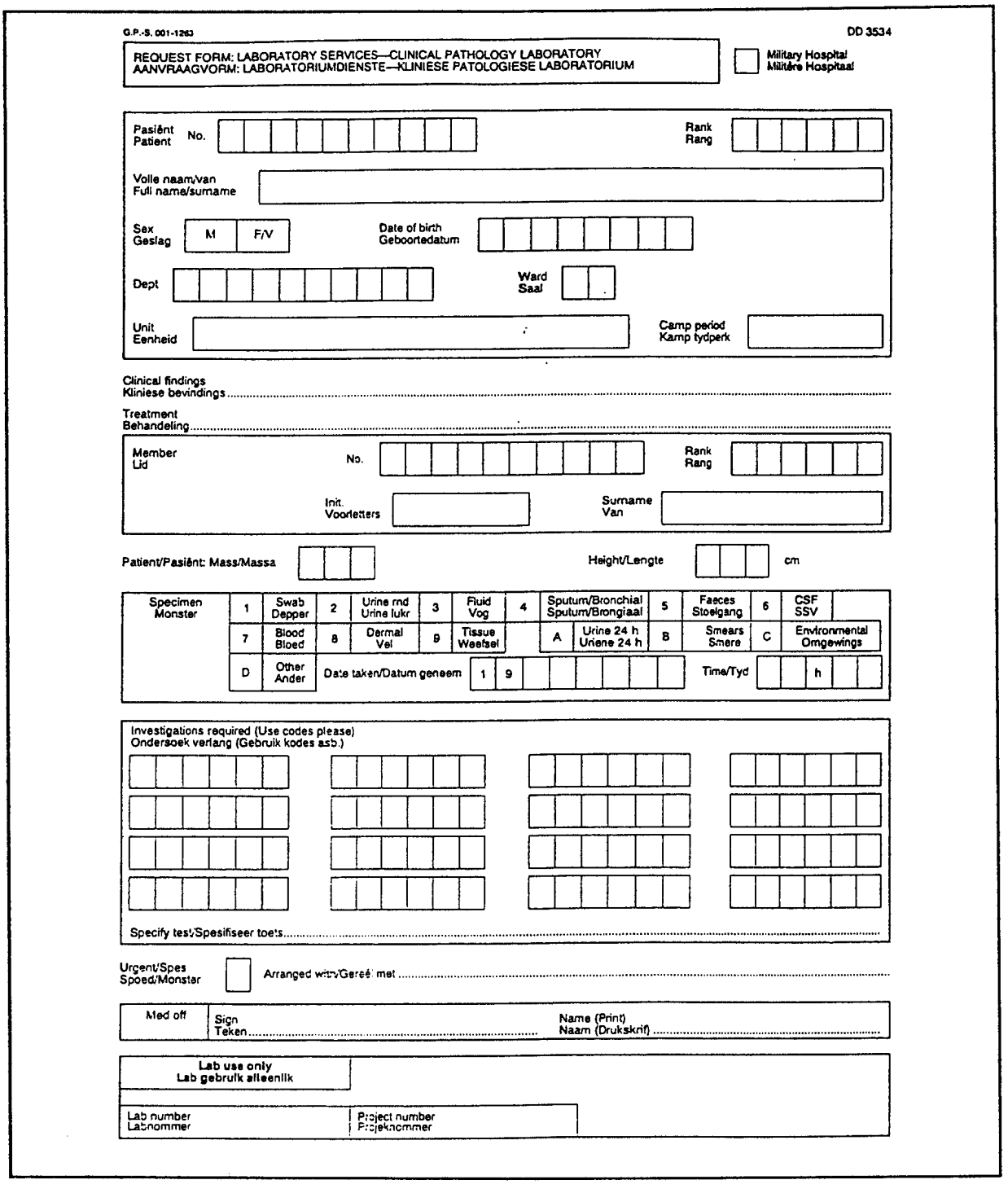
Fase 3 van hierdie navorsing is uitgevoer om die metaboliese wisselwerking tussen die

ensieme *PL* kinase en *PMP* (*PNP*) oksidase wat by *PLP* produksie betrokke is, te illustreer. Die kinetiese parameters ; Michaelis-Menten konstante en maksimum snelheid (teen verskillende substraat konsentrasies) vir die ensieme *PL* kinase en *PMP* (*PNP*) oksidase is vergelyk in 14 blanke - en 14 swart manlike proefpersone verbonde aan die SANW. Beide die gemiddelde Michaelis-Menten konstante en maksimum snelheid was hoër in blankes, alhoewel hierdie bevindings nie statisties betekenisvol verskil het nie. Die hoër individuele wiselvalligheid in beide berekende parameters kan waarskynlik uitgeskakel word indien 'n kristallyne vorm van die ensiem gebruik word, en verdien verdere aandag in toekomstige navorsing.

APPENDIX 1

LABORATORY REQUEST FORM USED IN SCREENING POTENTIAL TRIAL PARTICIPANTS

(By permission **1 Military Hospital** Clinical Pathology Laboratory).



G.P.-S. 001-1263 DD 3534

REQUEST FORM: LABORATORY SERVICES—CLINICAL PATHOLOGY LABORATORY
AANVRAAGVORM: LABORATORIUMDIENSTE—KLINIESE PATOLOGIESE LABORATORIUM

☐ Military Hospital / Militêre Hospitaal

Pasiênt No. / Patient No. | Rank / Rang

Volle naam/van / Full name/surname

Sex / Geslag: M | F/V

Date of birth / Geboortedatum

Dept | Ward / Saal

Unit / Eenheid | Camp period / Kamp tydperk

Clinical findings / Kliniese bevindings .....

Treatment / Behandeling .....

Member / Lid | No. | Rank / Rang

Init. / Voorletters | Surname / Van

Patient/Pasiênt: Mass/Massa | Height/Lengte | cm

Specimen / Monster												
	1 Swab / Dopper	2 Urine rnd / Urine lukr	3 Fluid / Vog	4 Sputum/Bronchial / Sputum/Brongiaal	5 Faeces / Stoelgang	6 CSF / SSV						
	7 Blood / Bloed	8 Dermal / Vel	9 Tissue / Weefsel	A Urine 24 h / Uriene 24 h	B Smears / Smere	C Environmental / Omgewings						
	D Other / Ander	Date taken/Datum geneem	1	9						Time/Tyd		h

Investigations required (Use codes please)
Ondersoek verlang (Gebruik kodes asb.)

Specify test/Spesifiseer toets .....

Urgent/Spes ☐ Arranged with/Gereël met .....

Med off | Sign / Teken ..... | Name (Print) / Naam (Drukskrif) .....

Lab use only / Lab gebruik alleenlik

Lab number / Labnommer | Project number / Projeknommer

APPENDIX 2

RANDOM PLASMA *PLP* CONCENTRATIONS AND B_6 VITAMER ENZYME ACTIVITIES AS PRODUCT FORMED / GRAM HAEMOGLOBIN / HOUR IN HUMANS

N	Plasma *PLP* (nmol/L)		*PMP (PNP)* Oxidase (nmol/L/hr)		*PLP* Phosphatase (nmol/L/hr)		*PL* Kinase (nmol/L/hr)	
	White	Black	White	Black	White	Black	White	Black
1	110.3	85.7	202.0	173.8	1,274.3	930.0	1,187.2	889.6
2	16.1	34.5	134.1	168.8	792.9	967.1	561.0	877.2
3	34.5	20.0	61.2	111.5	1,223.3	987.2	309.6	413.6
4	30.0	36.6	183.1	185.0	988.9	1,500.0	925.2	451.2
5	71.3	62.5	61.6	321.3	1,220.0	1,676.7	364.8	216.0
6	25.8	29.1	17.6	76.3	713.9	1,255.0	754.0	454.4
7	54.2	44.2	44.4	13.0	1,228.0	1,040.0	448.0	313.6
8	38.2	31.6	86.6	33.6	1,218.5	1,285.0	309.4	208.0
9	96.5	17.7	76.2	23.0	1,280.0	964.7	433.4	217.6
10	40.0	22.6	95.8	57.1	1,154.3	1,110.0	414.4	64.0
11	48.4	58.9	47.5	70.9	1,320.0	1,103.5	378.0	357.0
12	101.3	12.8	30.4	88.2	1,135.4	868.6	543.4	428.4
13	174.6	50.4	11.1	85.7	1,508.0	1,096.5	316.0	34.0
14	35.7	8.8	56.6	82.1	1,370.0	1,071.1	278.4	360.0
15	19.3	25.8	58.1	133.9	1,157.5	1,331.1	307.2	352.8
16	56.2	17.1	83.6	176.0	1,167.1	720.0	414.8	226.8
17	42.1	51.9	50.5	584.3	1,124.7	644.4	557.6	316.8
18	24.4	27.2	98.6	43.2	1,101.1	1,073.3	353.4	691.2
19	73.5	30.5	126.3	30.1	1,062.2	1,007.1	496.8	285.6
20	131.8	26.9	65.8	179.0	1,337.5	1,228.2	323.2	516.8
21	118.5	26.2	48.4	99.8	1,667.7	1,500.0	756.6	428.8

RANDOM PLASMA *PLP* CONCENTRATIONS AND B_6 VITAMER ENZYME ACTIVITIES IN HUMANS (CONTD.)

22	278.2	46.8	78.7	66.8	1025.3	976.5	672.6	17.0
23	42.8	15.5	25.0	166.3	1068.2	997.7	544.0	238.0
24	61.3	26.0	122.8	146.3	904.4	942.2	630.2	3.6
25	52.6	65.5	152.0	42.0	1,268.2	716.0	962.2	32.0
26	7.8	26.2	128.0	38.5	775.6	886.8	270.0	574.0
27	5.1	44.3	86.9	834.2	828.9	1,017.5	126.0	534.4
28	21.4	44.6	101.5	79.4	966.3	1,408.9	695.4	687.6
29	20.1	64.9	202.9	68.4	847.6	1,192.0	260.4	1,476.0
30	37.6	59.0	42.6	51.7	832.0	977.1	172.0	529.2
31	29.3	35.2	89.5	306.7	807.6	873.7	457.8	1,546.6
32	31.7	40.0	131.2	60.8	946.7	756.0	453.6	296.6
33	33.1	31.0	87.5	125.1	933.3	653.0	594.0	212.1
34	53.5	34.0	202.0	32.1	764.4	819.3	201.6	307.3
35	13.5	152.0	38.9	144.1	1,190.0	785.5	19.2	231.8
36	10.9	56.0	135.7	57.1	1,200.0	670.4	207.4	360.7
37	79.4	60.0	60.5	170.5	1,593.3	679.2	583.2	258.0
38	96.1	144.0	56.6	138.2	1,087.1	1,011.2	761.6	298.9
39	147.7	36.0	76.5	101.1	1,030.6	926.4	217.6	267.6
40	30.0	26.0	41.5	60.1	583.9	1,050.5	324.1	142.6
41	102.0	27.0	99.1	106.2	735.7	1,271.9	220.2	266.3
42	43.0	64.0	45.8	4.5	482.4	1,267.9	504.1	187.3
43	58.0	40.0	74.3	5.2	778.7	663.6	381.0	300.9
44	51.0	14.0	129.1	179.9	803.6	1,140.3	436.1	267.0.
45	40.0	56.0	258.2	1.8	899.4	808.9	338.5	148.4
46	27.0	54.0	57.3	5.2	979.7	862.6	194.6	216.6
47	38.0	30.0	4.9	3.1	882.7	928.7	162.3	115.1
48	113.0	22.0	90.3	6.6	797.3	786.6	226.2	135.6

RANDOM PLASMA *PLP* CONCENTRATIONS AND B_6 VITAMER ENZYME ACTIVITIES IN HUMANS (CONTD.)

49	50.0	38.0	96.0	38.5	1,584.2	846.7	128.6	273.6
50	72.0	72.0	144.3	79.8	980.5	545.5	301.8	417.6
51	28.0	26.0	90.2	1.7	1,072.5	821.5	356.3	68.8
52	36.0	27.0	43.1	87.4	973.9	1,097.7	96.6	404.4
53	48.0	24.0	192.8	73.5	938.8	681.8	323.0	431.2
54	46.0	31.0	79.0	105.7	800.0	928.4	317.5	147.4
55	61.0		3.0		878.1		301.7	
56	32.0		16.0		815.7		206.5	
57	96.0		246.2		517.9		291.3	
59	92.0		181.3		996.5		246.2	
60	62.0		180.6		934.5		378.4	
61	93.0		8.2		865.5		273.7	
62	75.0		175.0		909.1		306.2	
63	97.0		72.9		946.8		342.3	
64	72.0		46.2		808.2		238.0	
65	22.0		161.7		899.1		332.2	
66	52.0		94.8		1,006.7		266.2	
67	62.0		107.4		1,058.6		240.7	
68	91.0		7.2		925.9		160.4	
69	27.0		6.5		781.2		283.3	
70	35.0		187.4		1,009.8		410.0	
71	75.0		9.7		1,142.6		380.5	
72			7.2		1,060.0		306.6	
73			7.1		741.7		474.1	

APPENDIX 3

CONSENT FORM

The participants were requested to sign the appended consent form :

VITAMIN B_6 SUPPLEMENTATION STUDY

Department of Chemical Pathology, University of Pretoria, Pretoria

I, (full name) ....., the undersigned, hereby take note that this study is performed for scientific purposes only. A single vitamin B_6 (p*yridoxine hydrochloride 10 mg*) tablet will be administered to me and a needle will be placed into a vein in my arm, from which twenty 3 mL blood-samples will be collected (as stated in the table) over a 24 hour period.

I participate on my own free will and agree not to hold the University of Pretoria responsible, should I suffer from any ill-effects during the study period.

Signature of the participant :.....

Date : ..... Place :.....

Signature of witnesses :

1. .....
2. .....

APPENDIX 4

SAMPLE COLLECTION TABLE (*PYRIDOXINE HYDROCHLORIDE* SUPPLEMENT STUDY)

Sample no.	Sample time (min)	Sample time (hours)	Time interval	Comments
1	0		2.5 min	
2	2.5		2.5 min	
3	5		2.5 min	
4	7.5		2.5 min	
5	10		5 min	
6	15		5 min	
7	20		10 min	
8	30		15 min	
9	45		15 min	
10	60		15 min	
11	75		15 min	
12	90		15 min	
13	120	2	30 min	
14	150	2.5	30min	
15	180	3	30 min	
16		4	1 hr	
17		6	2 hr	
18		8	2 hr	
19		12	4 hr	
20		24	12 hr	

APPENDIX 5

1. FIELD DESCRIPTIONS USED TO PERFORM AUC ANALYSIS ("ABBOTTBASE" PHARMACOKINETIC SYSTEMS)

***PYRIDOXINE HYDROCHLORIDE* METABOLITE :**			
Patient ID :	**Height : (cm)**	**Age : (yr)**	
Sex :	**Weight : (kg)**		
Dose : (mg)		**Initial Cp : (ng/mL)**	
Route : oral		**Terminal phase Concentrations :**	
Duration : (hr)			
K_A : (L/hr)		**Cp C.V. : (*optional*)**	
f : (*optional*)		**Cp Exp : (*optional*)**	

No.	**Hours post dose**	***PLP* /*PL* Concentration**
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15-20		

2. DEFINITIONS IN FIELD DESCRIPTIONS USED TO PERFORM AUC ANALYSIS ("ABBOTTBASE" PHARMACOKINETIC SYSTEMS).

1. ***Pyridoxine hydrochloride*** **metabolite :** Plasma *PLP* and *PL*.

2. **Patient demographics** : ID, Height, Age, Sex, Weight.

3. **Dose** : 10 mg *pyridoxine hydrochloride* [Natura, Wynberg, RSA]

4. **Initial CP** : The 'drug' concentration in ng/mL just prior to administration of the dose. It was assumed that this measured concentration has been obtained during the terminal elimination phase (post absorption and post distribution) of any previously administered doses.

5. **Route** : Oral.

6. **Duration** : 24 hr.

7. **Terminal phase concentrations** : The estimated number of drug concentrations obtained during the terminal elimination phase. Linear regression analysis of log concentrations versus time for these terminal phase levels were used to determine the elimination rate constant and the estimated area under the curve from time of the last measured level to time equal infinity.

8. K_A : The estimated first-order absorption rate constant for the oral formulation administered.
   - ☞ *PLP* : 4.52
   - ☞ *PL* : 7.49

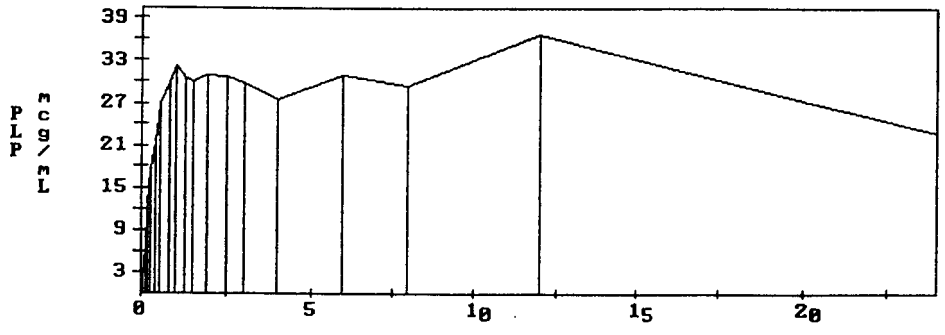
(75, 86)

APPENDIX 6

AREA UNDER THE CURVE EXAMPLE REPORT CALCULATED FOR PLASMA *PLP* GENERATED BY "ABBOTTBASE"

ID: Pat E (white)
Drug: PLP

Page: 1



Parameters

Half-life : 19.233 hr
Elimination rate constant : 0.03604 1/hr
Clearance : 0.01 L/hr
Volume of distribution : 0.22 L
Estimated Ka : 4.52
Estimated F : 1.0
Mean Residence Time : 27.75 hr

Dose

Amount (mg)	Duration (hr)
10.0	24.0

Drug Concentrations

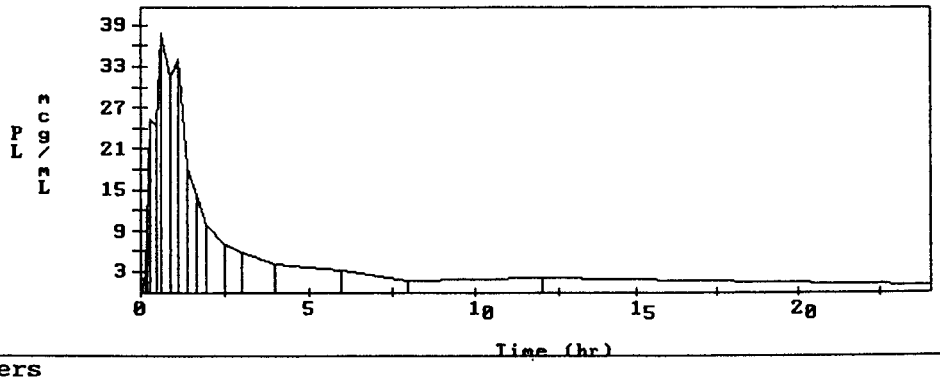
Time post (hr)	Cp mcg/mL	AUC	Accum.	AUMC	Accum.
0.08	2.19	0.088	0.088	0.007	0.007
0.17	6.32	0.383	0.471	0.0562	0.0632
0.25	12.26	0.743	1.214	0.1656	0.2288
0.33	17.64	1.196	2.41	0.3554	0.5842
0.42	21.24	1.75	4.16	0.6634	1.2476
0.58	26.97	3.857	8.017	1.9651	3.2127
0.83	29.79	7.095	15.112	5.046	8.2587
1.08	32.32	7.764	22.876	7.4539	15.7126
1.33	30.32	7.83	30.706	9.4039	25.1165
1.58	30.16	7.56	38.266	10.9973	36.1138
2.0	30.81	12.804	51.07	22.9473	59.0611
2.5	30.66	15.368	66.438	34.5675	93.6286
3.0	29.82	15.12	81.558	41.5275	135.1561
4.0	27.47	28.645	110.203	99.67	234.8261
6.0	30.78	58.25	168.453	294.56	529.3861
8.0	29.35	60.13	228.583	419.48	948.8661
12.0	36.67	132.04	360.623	1349.68	2298.546
24.0	22.66	355.98	716.603	5903.28	8201.826
	Tail :	564.902		27640.37	
	Sum :		1281.503		35842.2

APPENDIX 7

AREA UNDER THE CURVE EXAMPLE REPORT CALCULATED FOR PLASMA *PL* GENERATED BY "ABBOTTBASE"

ID: Pat M (white)
Drug: PL

Page: 1



Parameters

Half-life : 7.99 hr
Clearance : 0.09 L/hr
Estimated Ka : 7.49
Estimated F : 1.0
Elimination rate constant : 0.08675 1/hr
Volume of distribution : 1.04 L
Mean Residence Time : 11.53 hr

Dose

Amount (mg)	Duration (hr)
10.0	24.0

Drug Concentrations

Time post (hr)	Cp mcg/mL	AUC	Accum.	AUMC	Accum.
0.08	0.58	0.023	0.023	0.0019	0.0019
0.16	2.64	0.129	0.152	0.0188	0.0207
0.25	8.6	0.506	0.658	0.1158	0.1365
0.33	25.11	1.348	2.006	0.4175	0.554
0.5	24.5	4.217	6.223	1.7456	2.2996
0.67	37.79	5.295	11.518	3.1934	5.493
0.92	31.43	8.653	20.171	6.7794	12.2724
1.17	33.99	8.178	28.349	8.5855	20.8579
1.42	18.15	6.518	34.867	8.1927	29.0506
1.67	14.08	4.029	38.896	6.1608	35.2114
2.0	9.79	3.939	42.835	7.1104	42.3218
2.5	6.99	4.195	47.03	9.2638	51.5856
3.0	5.9	3.223	50.253	8.7938	60.3794
4.0	4.08	4.99	55.243	17.01	77.3894
6.0	3.11	7.19	62.433	34.98	112.3694
8.0	1.58	4.69	67.123	31.3	143.6694
12.0	1.94	7.04	74.163	71.84	215.5094
24.0	1.01	17.7	91.863	285.12	500.6294
	Tail :	18.568		786.986	
	Sum :		110.428		1287.615

APPENDIX 8

PHARMACOKINETIC PARAMETERS CALCULATED FROM THE AREA UNDER CURVE ANALYSIS FOR PLASMA *PLP* AND *PL* RESPECTIVELY

Table 1 : Plasma *PLP*

PLP	t ½ (hr)		K (1/hr)		CL (L/hr)		V_D (L)		K (hr)		Max.Peak (ng/mL)		(X) t Post (hr)	
N	W	B	W	B	W	B	W	B	W	B	W	B	W	B
1	30.60	13.70	0.02	0.05	0.01	0.02	0.63	0.49	44.10	19.80	16.40	16.00	2.00	12.00
2	13.40	17.80	0.05	0.04	0.03	0.02	0.62	0.45	19.30	25.70	12.90	16.50	0.83	12.00
3	27.70	16.70	0.03	0.04	0.01	2.00	0.38	0.37	40.10	22.60	12.90	26.50	6.17	8.00
4	13.90	19.20	0.05	0.04	0.02	0.02	0.46	0.43	20.10	27.60	16.00	24.00	2.50	0.83
5	19.20	15.40	0.04	0.05	0.01	0.02	0.22	0.36	27.80	22.20	36.70	24.60	12.00	1.08
6	15.90	18.00	0.04	0.04	0.01	0.01	0.28	0.30	22.90	26.00	28.40	28.20	12.00	12.00
7	14.70	16.50	0.05	0.04	0.02	0.02	0.42	0.38	21.20	23.80	19.90	21.90	12.00	12.00
8	13.70		0.05		0.02		0.46		19.80		16.10		12.00	
9	24.50		0.03		0.02		0.60		35.40		15.70		12.00	

Table 2 : Plasma *PL*

PL	t ½ (hr)		K (1/hr)		CL (L/hr)		V_D (L)		K (hr)		Max.Peak (ng/mL)		(X) t Post (hr)	
N	W	B	W	B	W	B	W	B	W	B	W	B	W	B
1	3.95	4.99	0.02	0.14	0.09	0.12	0.50	0.87	5.69	7.19	20.10	46.90	0.50	0.58
2	15.50	8.84	0.04	0.08	0.06	0.05	1.44	0.62	22.40	12.80	48.40	38.10	1.58	0.87
3	2.75	2.85	0.25	0.24	0.09	0.10	0.37	0.43	3.96	4.12	30.70	58.20	0.83	0.58
4	16.40	20.10	0.04	0.03	0.07	0.01	1.59	0.42	23.60	29.00	49.00	24.70	1.52	0.83
5	7.05	5.18	0.10	0.13	0.04	0.07	0.41	0.56	10.18	7.48	31.70	35.20	0.58	1.08
6	2.30	7.85	0.30	0.09	0.08	0.07	0.26	0.77	3.31	11.30	70.30	64.90	0.83	0.83
7	3.93	20.20	0.18	0.03	0.13	0.06	0.72	1.63	5.68	29.20	60.20	21.80	0.83	0.83
8	7.99		0.09		0.09		1.04		11.50		29.00		0.58	
9	14.2		0.1		0.1		1.8		20.5		37.8		0.7	

APPENDIX 9

EXCRETION OF URINARY 4-PYRIDOXIC ACID AFTER A 10 MG ORAL SUPPLEMENT OF *PYRIDOXINE HYDROCHLORIDE*

N	4-PA μmol/L		Urine Volume (L)		4-PA Excretion (μmol/L / 24 hr)	
	White	Black	White	Black	White	Black
1	3.9	6.6	2.7	1.6	107.2	105.6
2	6.9	10.7	1.7	2.1	115.9	221.5
3	16.3	10.2	1.2	1.5	197.4	151.4
4	6.1	6.5	1.8	2.0	110.9	131.7
5	8.6	8.6	1.9	1.6	167.2	133.5
6	11.7	9.4	1.4	1.8	162.0	165.4
7	13.4	10.3	1.1	1.5	144.4	154.1
8	9.6		1.9		186.7	
9	10.0		1.4		139.9	

APPENDIX 10

TWO-COMPARTMENT MACRO RATE CONSTANTS GENERATED BY "ABBOTTBASE" FOR PLASMA *PL*

ID:
Drug: PYRIDOXAL

Page: 1

Macro Rate Constants

Phase	A(i)	lambda(i)	half-life (hrs)
1	-43.2 +/- -2.44	3.99 +/- 0.386	0.174
2	44.4 +/- -1285.0	0.71 +/- 4.41	0.977
3	4.83 +/- 106.0	0.0663 +/- 0.238	10.5

Two Compartment Parameters

Vc =	0.049	+/-	0.362	L
CL =	0.0803	+/-	1.59	L/hr
ka =	0.71	+/-	4.41	1/hr
k12 =	2.26	+/-	29.1	1/hr
k21 =	0.161	+/-	3.17	1/hr

Time post (hr)	Cp mcg/mL	Fit Cp mcg/mL	Weight	Weighted Residual
0.08	1.98	15.4	1.69	514.0
0.16	5.3	21.6	0.856	195.0
0.25	9.73	26.0	0.591	92.6
0.33	16.9	28.3	0.5	32.4
0.42	25.0	29.6	0.457	4.38
0.58	27.3	29.8	0.45	1.27
0.83	31.6	27.6	0.524	4.33
1.08	30.9	24.5	0.664	17.8
1.33	24.3	21.5	0.867	5.97
1.58	25.6	18.7	1.14	61.2
2.0	15.9	15.0	1.79	2.89
2.5	10.9	11.6	2.96	4.53
3.0	9.44	9.24	4.69	0.9
4.0	6.24	6.3	10.1	0.373
6.0	4.09	3.87	26.7	33.8
8.0	2.75	2.99	44.6	118.0
12.0	2.33	2.19	83.5	139.0
24.0	0.98	0.984	413.0	2.97

AIC = 147.0 Sum of Squares = 1232.0

APPENDIX 11

1. *PL* KINASE ENZYME ACTIVITY IN nmol/L/hr AT INCREASING *PL* SUBSTRATE CONCENTRATIONS USING HPLC

Table 1 : Whites

(*PL*) Subst conc. (mmol /L)	*PL* kinase													
	Subjects (White)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	21.1	17.9	16.3	9.8	8.9	11.5	10.3	5.9	19.1	6.4	6.6	4.0	6.1	4.9
0.5	41.8	41.5	39.4	51.7	43.1	17.5	48.2	52.5	57.7	75.6	119.3	27.5	92.5	74.9
0.75	52.6	48.8	47.6	63.3	44.0	55.9	68.3	30.4	92.4	111.5	150.6	91.7	127.2	114.6
1.25	57.3	58.4	58.3	68.1	62.0	69.7	83.5	36.4	119.4	158.4	233.4	137.4	180.1	159.7
2	67.9	68.2	63.2	112.6	68.7	75.7	97.3	60.8	154.3	219.8	290.2	170.1	238.8	205.5
2.5	84.3	76.4	67.4	86.9	129.9	80.0	113.3	55.8	170.1	236.8	331.3	177.1	266.8	233.1
3.75	84.6	81.5	77.6	95.6	142.2	113.8	138.3	78.8	227.8	274.3	368.6	188.1	288.8	259.3
5	130.4	92.1	93.6	101.7	94.4	98.4	106.3	54.5	165.6	292.9	399.8	224.3	299.5	281.8

Table 2 : Blacks

(*PL*) Subst conc. (mmol /L)	*PL* kinase													
	Subjects (Black)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	21.8	15.7	16.8	11.1	13.3	10.3	7.6	5.5	8.3	7.3	3.6	4.8	5.1	4.5
0.5	42.6	26.1	100.9	43.9	66.4	38.1	35.5	23.4	34.3	29.5	44.5	83.1	77.2	48.4
0.75	44.4	34.1	107.8	43.9	75.0	42.1	35.1	27.6	40.1	44.1	63.8	105.2	112.3	67.2
1.25	58.5	36.6	114.8	50.7	98.7	46.0	51.6	36.5	57.4	46.7	87.3	148.5	143.8	91.3
2	69.6	42.6	183.0	58.2	92.4	70.8	55.4	50.0	66.0	53.3	113.0	203.1	164.7	128.4
2.5	80.4	47.7	184.5	63.3	108.8	64.6	77.3	58.0	108.6	88.1	118.6	223.7	188.2	135.7
3.75	98.2	55.0	177.9	67.7	113.0	67.5	79.0	71.1	76.8	73.3	147.3	242.3	196.6	152.8
5	106.9	66.1	220.9	69.4	180.4	67.5	95.1	73.9	91.3	68.7	149.8	251.4	57.8	160.1

2. *PMP (PNP)* OXIDASE ENZYME ACTIVITY IN nmol/L/hr AT INCREASING *PMP* SUBSTRATE CONCENTRATIONS USING HPLC

Table 3 : Whites

(*PMP*) Subst conc. (mmol/ L)	*PMP (PNP)* oxidase													
	Subjects (White)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	25.6	24.0	22.3	1.3	4.6	38.1	24.9	13.2	40.2	22.5	20.8	25.8	34.3	17.3
0.2	57.9	29.3	24.9	35.2	54.1	75.1	40.7	15.4	80.0	51.0	69.1	26.5	34.7	75.8
0.4	62.5	36.1	21.3	64.2	84.8	84.8	53.6	11.0	88.5	57.8	77.7	41.6	44.6	118.0
0.6	57.9	33.6	27.9	53.3	80.3	95.2	66.9	14.3	78.5	67.6	116.3	35.4	48.2	161.9
0.8	73.9	40.0	22.9	69.8	115.1	155.7	74.7	13.4	97.5	109.0	154.3	65.2	59.6	166.4
1	85.8	39.9	26.7	83.0	134.2	163.5	64.3	13.4	105.5	111.3	159.7	62.4	62.8	185.8
1.2	94.6	47.2	30.6	95.3	202.2	177.8	71.9	17.9	111.4	122.9	162.3	55.7	67.6	202.8
1.4	93.4	43.4	31.8	95.0	180.8	150.5	57.3	13.4	95.7	103.8	134.3	41.8	53.6	129.5

Table 4 : Blacks

(*PMP*) Subst conc. (mmol/ L]	*PMP (PNP)* oxidase													
	Subjects (Black)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
0	31.1	24.5	4.2	0.2	0.2	7.9	8.3	6.9	6.8	5.0	15.5	14.5	12.8	21.0
0.2	38.3	38.1	36.8	23.1	29.3	32.9	49.3	24.2	46.3	27.8	24.1	29.0	24.2	33.3
0.4	38.0	42.6	31.0	33.1	38.0	46.2	73.2	33.4	70.5	56.9	25.6	39.5	32.3	45.7
0.6	38.3	43.3	33.0	33.1	42.4	42.3	73.6	33.3	68.9	46.5	38.6	33.4	26.3	32.4
0.8	43.8	53.5	43.2	43.5	58.3	62.6	96.3	55.8	106.2	67.8	49.3	57.7	42.5	60.8
1	46.3	54.1	48.2	44.2	60.2	60.5	118.9	55.7	113.2	83.5	61.5	64.1	53.7	65.7
1.2	50.2	63.6	47.5	45.6	67.4	66.0	122.3	66.0	135.8	108.8	59.4	65.6	48.5	63.3
1.4	46.3	55.8	51.9	45.6	61.3	60.1	118.9	62.6	133.4	93.0	47.7	57.0	44.9	51.9

APPENDIX 12

MICHAELIS-MENTEN CONSTANTS (K_M) IN nmol/L AND MAXIMUM VELOCITY RATES (V_{max}) IN nmol/L/hr CALCULATED BY "ENZFITTER" AT INCREASING *PL* AND *PMP* SUBSTRATE CONCENTRATIONS FOR THE ENZYMES *PL* KINASE AND *PMP (PNP)* OXIDASE RESPECTIVELY

N	*PL* kinase				*PMP* (*PNP*) oxidase			
	White		Black		White		Black	
	K_M	V_{max}	K_M	V_{max}	K_M	V_{max}	K_M	V_{max}
1	2.71	189.70	1.65	138.60	0.19	100.80	0.13	49.80
2	0.85	102.20	1.06	72.10	0.16	50.90	0.19	66.30
3	0.84	96.70	1.06	266.00	0.28	32.30	0.58	72.70
4	0.62	111.00	0.40	73.10	0.47	124.60	0.29	57.20
5	1.08	129.20	0.48	129.20	1.08	338.10	0.61	98.60
6	1.46	183.30	0.63	78.20	0.34	105.20	0.32	84.70
7	2.80	383.40	1.58	118.40	0.10	115.00	0.70	190.60
8	2.02	420.00	2.22	108.60	1.08	233.30	1.13	127.10
9	1.75	544.10	1.16	107.00	0.78	278.00	0.80	218.00
10	1.67	293.80	1.40	105.20	0.97	123.50	1.31	207.80
11	1.77	384.80	1.68	204.40	0.33	83.80	0.48	93.20
12	1.77	422.00	1.51	339.70	0.60	298.90	0.44	70.10
13			1.01	254.50			0.80	255.50
14			1.64	218.60			0.30	82.30

APPENDIX 13

LIST OF ABBREVIATIONS

4-PA	4-pyridoxic acid
ATP	Adenosine tri-phosphate
AUC	Area under the curve
B	Black
BMI	Body Mass Index
CL	Clearance
CP	'drug' concentration
E	Enzyme
EDTA	Ethylene-diamine tetra-acetic acid
ES	Enzyme-substrate complex
Hb	Haemoglobin
HPLC	High performance liquid chromatography
hr	hour
K	Elimination rate constant
K_A	First order absorption rate constant
K_M	Michaelis-Menten constant
Ln	Log
Max P	Maximum peak
MRT	Mean residence time
N	Number of values / subjects (statistical)
Nr	Number
NS	Non significant
P	Product
PL	Pyridoxal
PLP	Pyridoxal -5'- phosphate
PLPSC	Pyridoxal -5'- phosphate semicarbazone

PLSC	Pyridoxal semicarbazone
PM	Pyridoxamine
PMP	Pyridoxamine -5'- phosphate
PN	Pyridoxine
PNP	Pyridoxine -5'- phosphate
S	Substrate
SANDF	South African National Defence Force
SAPS	South African Police Services
SD	Standard deviation
$t_{½}$	Elimination half-life
TCA	Trichloroacetic acid
V_0	Initial Velocity
vit	Vitamin
V_{max}	Maximum velocity
V_D	Volume of distribution
W	White
(X) t Post	Time to maximum peak

REFERENCES

1. Gÿorgy P. Vitamin B_2 and the pellagra - like dermatitis of rats. ***Nature 1934*** ; 133 : 448-449.

2. Foye WO. ***Principles of medicinal chemistry, third edition 1990*** ; Philadelphia, London, Lea & Febiger : 579-580.

3. Ang CYW. Stability of three forms of vitamin B_6 to laboratory light conditions. ***J Assoc Off Anal Chem 1979*** ; 62 : 1170-1173.

4. Schaltenbrand WE, Kennedy WS, Coburn SP. Low ultra-violet 'white' fluorescent lamps fail to protect pyridoxal phosphate from photolysis. ***Clin Chem 1987*** ; 33 : 61.

5. Lumeng L, Li TK. Mammalian vitamin B_6 metabolism : regulatory role of protein binding and hydrolysis of pyridoxal -5'- phosphate in storage and transport. In Tryfiates GP (eds.). ***Vitamin B6 metabolism and role in growth, Food and nutrition press 1980*** ; 27-51.

6. Lumeng L, Brashear RE, Li TK. Pyridoxal -5'- phosphate in plasma : source protein binding, and cellular transport. ***J Lab Clin Med 1974*** ; 84 : 334-343.

7. Wada H, Snell EE. The enzymatic oxidation of pyridoxine and pyridoxamine phosphates. ***J Biol Chem 1961*** ; 236 : 2089-2095.

8. Li TK, Lumeng L, Veitch RL. Regulation of pyridoxal -5'- phosphate metabolism in liver. ***Bioch Biophys Res Comm 1974*** ; 61 : 627-634.

9. Keresztesy JC, Stevens JR. Vitamin B_6. ***Proc Soc Exp Biol Med 1938*** ; 38 : 64-65.

10. Merrill AH, Henderson JM, Wang E, Mc Donald BW, Millikan WJ. Metabolism of vitamin B_6 by human liver. ***J Nutr 1984*** ; 114 : 1664-1674.

11. Ubbink JB, Bissbort S, Vermaak HJW, Delport R. Inhibition of pyridoxal kinase by

Methylxanthines. ***Enzyme 1990*** ; 43 : 72-79.

12. Machlin LJ ed. ***Handbook of Vitamins, second edition 1991*** ; New York & Basel, Marcel Dekker Inc. : 341-392.

13. Serebro HA, Solomon HM, Johnson JH, Henrix TR. The intestinal absorption of vitamin B_6 compounds by rat and hamster. ***Bull Johns Hopkins Hosp. 1966*** ; 119 : 166-171.

14. Middleton HM. Uptake of pyridoxine hydrochloride by the rat jejunal mucosa in vitro. ***J Nutr 1977*** ; 107 : 126-131.

15. Middleton HM. Characterization of pyridoxal -5'- phosphate disappearance from in vivo perfused segments of rat jejunum. ***J Nutr 1982*** ; 112 : 269-275.

16. Henderson LM. Intestinal absorption of vitamin B_6 vitamers. In : Reynolds RD, Leklem JE (eds.). ***Vitamin B_6 - Its role in health and disease 1985*** ; 22-33.

17. Mehansho H, Hamm MW, Henderson LM. Transport and metabolism of pyridoxal and pyridoxal phosphate in the small intestine of the rat. ***J Nutr 1979*** ; 109 : 1542-1551.

18. Hamm MW, Mehansho H, Henderson LM. Transport and metabolism of pyridoxamine and pyridoxamine phosphate in the small intestine of the rat. ***J Nutr 1979*** ; 109 : 1552-1559.

19. Coburn SP, Whyte MP. Role of phosphatases in the regulation of vitamin B_6 metabolism in hypophosphatasia and other disorders. In : Leklem JE, Reynolds RD (eds.). ***Clinical and physiological applications of vitamin B_6 1985*** ; 65-93.

20. Kabir H, Leklem JE, Miller T. Measurement of glycocylated vitamin B_6 in foods. ***J Food Sci 1983*** ; 48 : 1422-1425.

21. Kabir H, Leklem JE, Miller T. Comparative vitamin B_6 bioavailability from tuna, whole wheat bread and peanut butter in humans. ***J Nutr 1983*** ; 113 : 2412-2420.

22. Kabir H, Leklem JE, Miller LT. Relationship of the glycosylated vitamin B_6 content in foods of vitamin B_6 bioavailabilty in humans. ***Nutr Rept Int 1983*** ; 28 : 709-716.

23. Nelson EW, Lane HJR, Cerda JJ. Comparative human intestinal bioavailability of vitamin B_6 from a synthetic and natural source. ***J Nutr 1976*** ; 106 : 1433-1437.

24. Miller LT, Leklem JE, Schultz TD. The effect of dietary protein on the metabolism of vitamin B_6 in humans. ***J Nutr 1985*** ; 115 : 1663-1672.

25. Leklem JE. Physical activity and vitamin B_6 metabolism in men and women : interrelationship with fuel needs. In : Reynolds RD, Leklem (eds.). ***Vitamin B_6 : Its role in health and disease 1985*** ; 221-241.

26. Ferroli CE, Trumbo PR. Bioavailability of vitamin B_6 in young and older men. ***Am J Clin Nutr 1994*** ; 60 (1) : 68-71.

27. Leklem JE. Physical activity and vitamin B_6 metabolism in men and women : interrelationship with fuel needs. In : Reynolds RD, Leklem JE (eds.). ***Vitamin B 6 : Its role in health and disease 1985*** ; Liss AR, New York : 221-241.

28. Reynolds EF, Parfitt K (eds) : ***Martindale- The Extra Pharmacopoeia 1993 (30)*** ; 1054-1055.

29. Glen GM, Krober MS, Kelly P, Mc Carty J, Weir M. Pyridoxine as therapy in theophylline induced seizures. ***Vet Hum Toxicol 1995*** ; 37 (4) : 342-345.

30. Ebadi M, Gestured CF, Al-Sayegh A. Drug-pyridoxal phosphate interactions (review). ***Q Rev Drug Metab Drug Interact 1982*** ; 4 (4) : 289-331.

31. Bender DA. Oestrogens and vitamin B_6 actions and interactions. ***Wld Rev Nutr Diet 1987*** ; 51 : 140-188.

32. Reynolds RD, Natta CL. Depressed plasma pyridoxal phosphate concentrations in adults

asthmatics. ***Am J Clin Nutr 1985*** ; 41 : 684-688.

33. Hollenbeck CB, Leklem JE, Riddle MC, Connor WE. The composition and nutritional adequacy of subject selected high carbohydrate, low fat diets in insulin dependant diabetes mellitus. ***Am J Clin Nutr 1983*** ; 38 : 41-51.

34. Stone WJ, Warnock LG, Wagner C. Vitamin B_6 deficiency in uraemia. ***Am J Clin Nutr 1975*** ; 28 : 950-957.

35. Lumeng L, Li TK. Vitamin B_6 metabolism chronic alcohol abuse. Pyridoxal phosphate levels in plasma and the effects of acetaldehyde on pyridoxal phosphate synthesis and degradation in human erythrocytes. ***J Clin Invest 1974*** ; 53 : 693-704.

36. Serfontein WJ, Ubbink JB, De Villiers LS, Rapley CH, Becker PJ. Plasma pyridoxal -5'-phosphate level as risk index for coronary heart disease. ***Atherosclerosis 1985*** ; 55 : 357-361.

37. Malinow MR. Plasma homocysteine and arterial occlusive diseases : a mini review. ***Clin Chem 1995*** ; 41(1) : 173-176.

38. Hamfelt A, Tuvemo T. Pyridoxal phosphate and folic acid concentration in blood and erythrocyte aspartate amino transferase activity during pregnancy. ***Clin Chim Acta 1972*** ; 41 : 287-298.

39. Potera C, Rose P, Brown RR. Vitamin B_6 deficiency in cancer patients. ***Am J Clin Nutr 1977*** ; 30 : 1677-1679.

40. Devita VT, Chabner BA, Livingston DM, Oliverio VT. Energy and tryptophan metabolism in Hodgkins' disease. ***Am J Clin Nutr 1971*** ; 24 : 835-840.

41. Reynolds RD, Natta CL. Vitamin B_6 and sickle cell anaemia. In : Reynolds RD, Leklem JE (eds.). ***Vitamin B_6 : Its role in health and disease 1985*** ; 301-306.

42. Coleman M. Studies of the administration of pyridoxine to children with Downs' syndrome. In : Leklem JE, Reynolds RD eds. ***Clinical and physiological applications of vitamin B_6 1988*** ; 317-328.

43. Barthelemy C, Martineau J, Bruneau N, Muh JP, Lelord G, Callaway E. Clinical and biological effects of pyridoxine plus magnesium in autistic subjects. In : Leklem JE, Reynolds (eds.). ***Clinical and Physiological applications of Vitamin B_6 1988*** ; 329-356.

44. Watts RWE. Hyperoxaluria. In : Leklem JE , Reynolds RD (eds.). ***Clinical and physiological applications of vitamin B_6 1988*** ; 245-261.

45. Coelingh-Bennink HJT, Schreurs WHP. Improvement of oral glucose tolerance in gestational diabetes by pyridoxine. ***Brit Med J 1975*** ; 3 : 13-15.

46. Day JB. Clinical trials in the premenstrual syndrome. ***Curr Med Res Opin (6) 1979*** ; (suppl. 5) : 40-45.

47. Ellis J, Folkers T, Watanabe T, Kaji M, Saji S, Caldwell JW, Temple CA, Wood FS. Clinical results of a cross-over treatment with pyridoxine and placebo of the carpal tunnel syndrome. ***Am J Clin Nutr 1979*** ; 32 : 2040-2046.

48. Shizukuishi S, Nishii S, Folkers K. Distribution of vitamin B_6 deficiency in university students. ***J Nutr Sci Vitaminol (Tokyo) 1981*** ; 27 (3) : 193-197.

49. Adams PW, Wynn V, Seed M, Folkard J. Vitamin B_6 depression and oral conception. ***Lancet 1974*** ; 2 : 516-517.

50. Bernstein AL, Lobitz CS. A clinical and electrophysiological study of the treatment of painful diabetic neuropathies with pyridoxine. In : Leklem JE, Reynolds RD (eds.). ***Clinical and physiological application of vitamin B_6 1988*** ; 415-423.

51. Hansen KN, Ostergaard JR, Moller SM. Pyridoxine dependant seizures. ***Ugeskr Laeger***

1994 ; 156 (42) : 6222-6224.

52. Snodgrass SR. Vitamin neurotoxicity (review). ***Mol Neurobiol 1992*** ; 6 (1) : 41-73.

53. Dordain G, Deffond D. Pyridoxine neuropathies. Review of the literature. ***Therapie 1994***: 49 (4) : 333-337.

54. Chabner B, Livingston D. A simple enzymic assay for pyridoxal phosphate. ***Anal Biochem 1970*** ; 34 : 413-423.

55. Miller LT, Edwards M. Microbial assay of vitamin B_6 in blood and urine. In : Leklem JE, Reynolds RD (eds.). ***Methods in vitamin B6 nutrition 1981*** ; 45-55.

56. Chauhan MS, Dakshinamurti K. Fluorometric assay of pyridoxal and pyridoxal -5'-phosphate. ***Anal Biochem 1986*** ; 96 : 426-432.

57. Lim KL, Young RW, Driskell JA. Separation of vitamin B_6 components by high performance liquid chromatography. ***J Chromatogr 1980*** ; 188 : 285-288.

58. Mascher H. Determination of total pyridoxal in human plasma following oral administration of vitamin B_6 by high performance liquid chromatography with post-column derivatization. ***J Pharm Sci 1993*** ; 82 (9) : 972-974.

59. Kerry JA, Kwok F. Purification and characterization of pyridoxal kinase from human erythrocytes. ***Prep Biochem 1986*** ; 16 (3) : 199-216.

60. Ubbink JB, Schnell AM. High performance liquid chromatographic assay of erythrocyte enzyme activity levels involved in vitamin B_6 metabolism. ***J Chromatogr 1988*** ; 431 : 406-412.

61. Ubbink JB. Aspects of vitamin B_6 metabolism relating to myocardial infarction . ***Doctor of science thesis 1987*** ; 8-44.

62. Ubbink JB, Serfontein WJ, Becker PJ, De Villiers SD. Determination of urinary 4-

pyridoxic acid levels as 4-PA lactone using high performance liquid chromatography. ***Am J Clin Nutr 1896*** ; 44 : 608-613.

63. Leklem JE, Reynolds RD. Recommendations for status assessment of vitamin B_6. In : Leklem JE, Reynolds RD (eds.). ***Methods in Vitamin B_6 nutrition 1981*** ; 389-392.

64. Lumeng L, Li TK. The interorgan transport and metabolism of vitamin B_6. In : Reynolds RD, Leklem JE (eds.). ***Vitamin B_6 - Its role in health and disease 1985*** ; 35-54.

65. Lui L, Lumeng L, Aronoff GR, LI TK. Relationship between body store of vitamin B_6 and plasma pyridoxal-phosphate clearance : metabolic balance studies in humans. ***J Lab Clin Med 1985*** ; 106 (5) : 491-497.

66. Van den Berg H, De Kock JJ, Vermaak WJH, Potgieter GM. A new perspective in the assessment of vitamin B_6 nutritional status during pregnancy in humans. ***J Nutr 1987*** ; 117 : 303-1306.

67. Driskell JA, Chrisley BM, Reynolds LK, Moak SW. Plasma B_6 vitamer and plasma urinary 4-pyridoxic acid concentrations of middle-aged obese black women. ***J Chromatogr 1991*** ; 568 (2) : 333-340.

68. Vermaak WHJ, Barnard HC, Potgieter GM, Theron HD. Vitamin B_6 and coronary artery disease : epidemiological observations and case studies. ***Atherosclerosis 1987*** ; 63 : 235-238.

69. Hamfeldt A. Pyridoxal kinase activity in blood cells. ***Clin Chem Acta 1967*** ; 16 : 7-18.

70. Clements JE, Anderson BB. Pyridoxine (Pyridoxamine) phosphate activity in the red cell. ***Biochim Biophys Acta 1980*** ; 613 : 401-409.

71. Anderson BB, Fulford-Jones CE, Child JA, Beard MEJ, Bateman CJT. Conversion of vitamin B_6 compounds to active forms in the red blood cell. ***J Clin Invest 1971*** ; 50 : 1901-1909.

72. Anderson BB, in Tryfiates GP (Editor). Vitamin B_6 metabolism and role in growth. ***Food & Nutrition Press, Westport, CT 1980*** ; 53.

73. Lumeng L, Li TK, Lui A. In : Reynolds RD, Leklem JE (eds.), Current topics in nutrition and disease, vol 13. ***Vitamin B_6 : Its role in health and disease 1985*** ; 35.

74. Lumeng L, Li TK, in Tryfiates GP (Editor). Vitamin B_6 metabolism and role in growth. ***Food & Nutrition Press, Westport, CT 1980*** ; 27.

75. Ubbink JB, Serfontein WJ. The response of the plasma B_6 vitamers to a single, oral pyridoxine supplement. ***Clin & Phys Applic of Vit B_6 1988*** ; 29-34.

76. Zempleni J, Kubler W. The utilization of intravenously infused pyridoxine in humans. ***Clin Chim Acta 1994*** ; 229 (1-2) : 27-36.

77. Wozenski JR, Leklem JE, Miller LT. The metabolism of small doses of vitamin B_6 in men. ***J Nutr 1980*** ; 110 : 275-285.

78. Henderson JM, Codner MA, Hollins B, Kutner H, Merrill AH. The fasting B_6 vitamer profile and response to a pyridoxal load in normal and cirrhotic subjects. ***Hepatology 1986*** ; 6 : 464-471.

79. Ubbink JB, Serfontein WJ, De Villiers LS. ***J Chromatogr* 1985** ; 342 :277.

80. Drabkin DL, Austin JH. Spectrophotometric studies : Spectrophotometric constants for common haemoglobin derivates in human, dog and rabbit blood. ***J Biol Chem 1932*** ; 98 : 719-733.

81. Lubbe JC, Rossouw H, Ungerer K. ***Clinical Pathology Laboratory Manual , 1 Military Hospital 1996*** ; 1 : 1-50.

82. Barnard HC, De Kock JJ, Vermaak WJH, Potgieter GM. A new perspective in the assessment of vit B_6 nutritional status during pregnancy in humans. ***J Nutr 1987*** ; 117 :

1303-1306.

83. Kelsay J, Baysal A, Linkswiler H. Effect of vit B_6 depletion on the pyridoxal, pyridoxamine and pyridoxine content of the blood and urine of men. ***J Nutr 1968*** ; 94 : 490-494.

84. ABBOTTBASE Pharmacokinetic systems : Operations manual ; ***ABBOTTBASE Pharmacokinetic systems, version 1.00 1991*** ; Sec. 5.4, 8.1. (Abbott Laboratories, Diagnostics Division, Abbott Park, Illinois, USA).

85. Taylor WJ, Diers Caviness MH (eds.). ***A Textbook for the clinical application of therapeutic drug monitoring 1986*** ; 3-30. (Abbott Laboratories, Diagnostics Division, Abbott Park, Illinois, USA).

86. Tietz NW. ***Clinical Guide to Laboratory Tests, second edition 1990*** ; Philadelphia, Saunders WB Co. : 580.

87. Ziegler W, Biggs J. Tricyclic plasma levels : effect of age, race, sex, and smoking. ***JAMA 1977*** ; 238 : 2167-2169.

88. Gabriel Khan MI. ***Manual of cardiac drug therapy***. Saunders WB Co. **1985** ; 135.

89. Tietz NW ed. ***Fundamentals of Clinical Chemistry, third edition 1987*** ; Philadelphia, Saunders WB co. : 105-120 , 346-417 , 497-517.

90. Lehninger AL. ***Biochemistry - The molecular basis of cell structure and function , second edition 1975*** ; New York, Worth Publishers, Inc. : 183-213.

91. Leatherbarrow RJ. ***ENZFITTER - A Non-linear Regression Data Analysis Program for the IBM PC (and true compatibles) Manual 1987*** ; Biosoft, Cambridge,UK : 7-91.

92. Huysamen GK. ***Introductory statistics and research design 1976*** ; Johannesburg, Varsity Press : 225-251.