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Tryptophan and the kynurenine pathway in chronic renal failure patients on dialysis

by

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Dedicated to my parents and brothers

Summary

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Tryptophan is metabolised along the kynurenine pathway under the influence of tryptophan 2,3 dioxygenase and indoleamine 2,3 dioxygenase. Quinolinic acid and kynurenine, two neuroactive metabolites of the kynurenine pathway are, in chronic renal failure patients, considered as uraemic toxins. Related research is generally hampered by the non-availability of relevant analytical techniques. The primary aim of this study was, therefore, to develop and validate suitable methods for the determination of tryptophan, kynurenine and quinolinic acid. The second aim was to quantify the levels of these substances in the blood of chronic renal failure patients on renal replacement therapies and to compare the levels of haemodialysis patients to those on peritoneal dialysis. Patients' quality of life was investigated relative to disturbances in tryptophan metabolism.

Gas chromatography coupled to mass spectrometry (GC-MS) gave the best results for the analysis of tryptophan, kynurenine and quinolinic acid. A Hewlett Packard HP GC 6890 series gas chromatographer was coupled to a MS 5973 series mass spectrometer. Analytes were separated on a DB-5MS column with a nominal length of 30 metres, a diameter of 250.0 µm and film thickness of 0.10 µm. Helium was used as carrier gas, and the chromatographic analysis run time 12.5 minutes. The validation results were within the acceptance criteria for newly developed methods. The linear calibration curves constructed for all of the analytes gave r^2 correlation coefficients >0.99. Other validation data such as precision, bias, accuracy and stability all fell within acceptable validation limits.

In the study on chronic renal failure patients significant differences were seen between patients and controls. Tryptophan levels were 5.34 SD 5.04 µM for the haemodialysis group, 6.73 SD 3.18 µM for the peritoneal dialysis group and 28.4 SD 4.31 µM for the control group. Kynurenine levels were 4.7 SD 1.9 µM for the haemodialysis group, 2.9 SD 2.0 µM for the peritoneal dialysis group and 2.1 SD 0.6 µM for the control group. Quinolinic acid levels were 4.9 SD 2.0 µM for the haemodialysis group, 2.8 SD 2.0 µM for the peritoneal dialysis group and 0.3 SD 0.1 µM for the control group. Tryptophan was lower for the total patient group than for controls with significantly lower levels for haemodialysis *versus* control ($p<0.05$) and peritoneal dialysis *versus* control ($p<0.05$). Kynurenine levels were higher in the total patient group with significantly higher levels for the haemodialysis *versus* control group ($p=0.0001$). The patient groups had higher

quinolinic acid levels with significantly higher levels for the haemodialysis *versus* control ($p<0.05$) and peritoneal dialysis *versus* control ($p<0.05$) groups.

This study was the first to determine the three substances simultaneously in both haemodialysis and peritoneal dialysis patients. The study showed significant tryptophan depletion, as well as kynurenine and quinolinic acid accumulation for both groups. No significant differences were found between the patient groups other than higher kynurenine levels in the haemodialysis group. The quality of life (SF-36) was largely similar in the two patient groups. This decrease in the quality of life strongly correlated with the degree of tryptophan depletion.

Keywords
Tryptophan, kynurenine, quinolinic acid, chronic renal failure, haemodialysis, peritoneal dialysis, quality of life, gas chromatography – mass spectrometry, method validation

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5-HT	5-hydroxytryptamine/serotonin
µl	microlitre
µm	micrometer
µM	micromolar/micromoles per liter
5-HIAA	5-hydroxyindole acetic acid
BBB	blood brain barrier
CAPD	continuous ambulatory peritoneal dialysis
CI	chemical ionization
cm	centimeter
CNS	central nervous system
CRF	chronic renal failure
CRP	C-reactive-protein
CSF	cerebrospinal fluid
CV	co-efficient of variation
EI	electron impact ionization
g	gram
g/mol	grams per mole
GC	gas chromatography
GC-MS	gas chromatography – mass spectrometry
HD	haemodialysis
HPLC	High performance liquid chromatography
IDO	indoleamine 2,3-dioxygenase
IL-2	interleukin-2
INF-γ	interferon gamma
KYN	kynurenine
LOD	limit of detection
LOQ	limit of quantification
m/z	mass to charge ratio
mg/l	milligram per litre
MS	mass spectrometry
MSD	mass spectometric detector
MTBSTFA	N-methyl-N-t-butyldimethylsilyltrifluoroacetamide
NAD+	nicotinamide adenine dinucleotide
nCI	negative chemical ionization
NMDA	N-methyl-D-aspartate
PD	peritoneal dialysis
PFPA	pentafluoropropionic anhydride
PFPOH	pentafluoropropanol

ppm	parts per million
QA	quinolinic acid
QC	quality control
ROS	reactive oxygen species
RSD	relative standard deviation
SF-36	medical short form 36
SIM	selective ion monitoring
Std	standard
TCA	Tri-chloro acetic acid
TDO	tryptophan 2,3-dioxygenase
TNF α	tumor necrosis factor α
TPH	tryptophan-5-hydroxylase
TRP	Tryptophan
VC	volunteer control
WSS	working stock solution

Chapter 1

Introduction and Literature Review

1.1 Tryptophan and physiological metabolism

1.1.1 Background on tryptophan

Tryptophan (TRP) is classified as one of the essential amino acids. "Essential" meaning that it cannot be synthesized by the body and needs to be supplemented by the diet [1,2,3]. A daily intake of about 20mmol is usually required to sustain plasma reference ranges of between 45 and 60µmol/l [4]. The name tryptophan took origin in 1901 when Hopkins *et al* isolated it in the form of a chromogenic protein constituent [5]. Later it was established that tryptophan is an amino acid with the indole ring of its chemical structure (see figure 1.1) contributing to its chromogenic properties. From this pioneering work subsequent studies lead to the identification of the tryptophan metabolic pathways, functions and physiological importance, giving relevance to understanding a number of conditions and pathologies related to tryptophan metabolism [1,5].

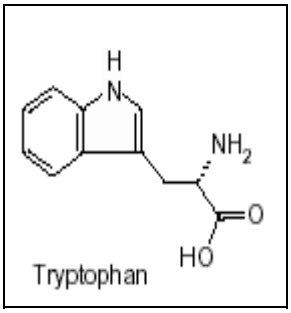

Figure 1.1 Chemical structure illustrating indole ring

Tryptophan is absorbed from the gut into the blood stream after normal dietary intake and digestion. The majority of tryptophan is transported in the blood in two forms, i.e., as a protein bound complex namely tryptophan bound to albumin [1,6] and unconjugated tryptophan which constitutes the free tryptophan [1,6,7]. Due to the instability of the binding of tryptophan to albumin, cerebral uptake of TRP across the blood-brain barrier is not hindered by the protein bound fraction [1,8]. Tryptophan is transported via the

blood to tissues where it may follow several metabolic pathways under specific enzymatic catabolism and it can eventually be converted to water and carbon dioxide which is the end result of TRP catabolism.

In order to understand the functions of TRP, as well as its physiological mechanisms in the body, it is relevant and important to briefly overview the metabolic pathways followed by tryptophan. One of the most important functions of TRP is its utilization for the synthesis of tissue proteins [1,9]. About 3,5grams of tryptophan contributes to the daily protein production. According to Peters [9] this tissue protein production accounts for calculably the most significant utilization of tryptophan. However, tryptophan does not only serve as a single nutritional entity but, as will be discussed later, forms an important substrate for alternative metabolic pathways.

1.1.2 Biochemical pathways of tryptophan metabolism

Tryptophan metabolism follows two major metabolic pathways. The first of the two pathways, of which 1% dietary tryptophan is used as the substrate, is the production of 5-hydroxytryptamine (serotonin/5-HT) in the nervous system and other body cells such as the enterochromaffin cells in the gut, blood platelets and in the pineal gland for the production of melatonin [1,4,10]. The second or alternative pathway for the metabolism of tryptophan is via the kynurenine pathway [1,11]. This pathway takes place in the liver, as well as in extra-hepatic tissue, is under the influence of the relevant enzymes, and occurs under specific induction criteria. The majority of tryptophan is metabolized along this pathway which results in the biosynthesis of nicotinamide adenine dinucleotide (NAD +) and complete oxidation of tryptophan to water and carbon dioxide. The main metabolite of the kynurenine pathway is kynurenine (KYN). This metabolite can be further metabolized via several pathways resulting in the production of specific metabolites such as kynurenic acid and quinolinic acid.

The serotonin pathway of tryptophan metabolism is under control of two enzymatic steps which control the production of serotonin from tryptophan. The initial and rate limiting step is the conversion of tryptophan to 5-hydroxytrytophan via the process of

hydroxylation. The vitamin B6 dependent enzyme tryptophan-5-hydroxylase is responsible for the afore mentioned step. Under the influence of the enzyme L-aromatic acid decarboxylase this hydroxylation is then followed by decarboxylation of 5-hydroxytryptophan to produce serotonin [1,10]. Serotonin is further broken down to form the end metabolite 5-hydroxyindole acetic acid (5-HIAA).

The alternative pathway to that of serotonin, as previously mentioned, is the kynurenine pathway of tryptophan metabolism. Most of the dietary tryptophan, approximating 99%, follows this metabolic pathway [1]. Kynurenine (KYN) is the main metabolite formed from the oxidative catabolism of tryptophan and two enzymes are specific for this process. The first enzyme responsible for this oxidation pathway is tryptophan 2,3-dioxygenase (TDO) [12]. TDO activity is limited mainly to hepatic tryptophan metabolism. The second enzyme is indoleamine 2,3-dioxygenase (IDO) which is specific for the oxidation of TRP and other indoleamines [13]. IDO activity is not confined to the liver and its omnipresence induces peripheral TRP oxidation to form KYN.

1.1.3 Factors influencing metabolic pathways and rate of enzyme activity

There are certain internal and external factors to consider with respect to the metabolic pathways of tryptophan metabolism. These factors influence the induction and rate of activity of the specific enzymes. A number of factors may alter or even completely inhibit the enzyme activity. This in turn may determine the levels of the particular metabolite, as well as its precursor.

Tryptophan metabolism via the two major pathways depends on the availability of tryptophan as a substrate, as well as the physiological requirements/demands for the metabolites of the relevant pathways. In other words, if there is an increased physiological requirement for serotonin, tryptophan metabolism will be shifted towards the serotonin pathway. Alternatively, if there is an increased physiological requirement for niacin then tryptophan metabolism will be shifted towards the kynurenine pathway. A tryptophan shift towards one pathway does not necessary totally exclude metabolism along the other pathway. This is not implied in a sense of causality because there are

internal and external factors influencing pathway shifts. Tryptophan metabolism is also influenced by the relative enzyme activity in the occurring pathways of tryptophan metabolism.

As will be discussed later, the enzymes that induce a specific pathway of tryptophan metabolism are in their own right under control of certain physiological and confounding factors. It is thus highly feasible that a specific increase in enzyme activity can cause a shift in tryptophan metabolism along one pathway which may result in a deficit of the metabolites of the alternative pathways. In other words tryptophan utilization for pathway A may for instance result in a substrate deficiency for pathway B. This can lead to a decrease in the end metabolite levels of pathway B and an increase in the metabolite levels of pathway A. This is the concept or argument from which the hypothesis and study objectives are derived - the substantiation which will be seconded on grounds of the literature to follow.

Serotonin availability is determined by the rate of activity of the enzymes tryptophan-5-hydroxylase (TPH) and L-amino acid decarboxylase, provided that there are sufficient substrates or precursors to ensure progression of the metabolic pathway. A depletion of tryptophan-5-hydroxylase will result in a decrease in the 5-hydroxytryptophan necessary for the synthesis of serotonin. Factors that are known to deplete tryptophan-5-hydroxylase include pyridoxine (vitamin B6) deficiency, stress and insulin resistance [1,14]. Certain catecholamines and related amino acids such as dopamine and L-tyrosine have also been shown to directly inhibit tryptophan-5-hydroxylase activity [15]. Ethanol demonstrates similar effects towards diminished enzyme activity [16]. Pyridoxine (vitamin B6) is a co-factor required for L-amino acid decarboxylase and therefore a vitamin B6 deficiency may result in reduced synthesis of serotonin from 5-hydroxytryptophan [14]. These factors can result in an increase in the conversion of tryptophan to kynurenine thus decreasing tryptophan availability for 5-hydroxytryptophan and serotonin production [17]. From a nutritional point of view, a dietary increase in tryptophan will increase tryptophan-5-hydroxylase phosphorylation activity and conversely malnutrition with associated tryptophan depletion may alter

tryptophan-5-hydroxylase phosphorylation activity, thus resulting in a decrease in serotonin synthesis [18].

Tryptophan 2,3-dioxygenase (TDO) is the enzyme specific for the normal oxidative catabolism of tryptophan along the kynurenine pathway. As with tryptophan-5-hydroxylase there are certain factors that influence the induction or inhibition of TDO as well as its rate of enzyme activity. The adrenal stress hormone cortisol is known to induce hepatic TDO activity and as a result reduces plasma tryptophan levels and increases the levels of kynurenine metabolites [19]. TDO activity is also induced by its substrate (tryptophan); hence an increase in tryptophan intake will cause an increase in the normal oxidative breakdown via the kynurenine pathway in conjunction with normal protein synthesis [20]. In contrast, there are certain agents that completely inhibit or alter the enzyme activity of TDO. Some non-steroidal anti-inflammatory agents such as tolmetin and sulindac have been demonstrated to be inhibitors of liver TDO activity [21]. These agents have been shown to be beneficial to patients with depression as they are also known to inadvertently increase brain serotonin levels. This may be attributed to increased tryptophan availability for the serotonin pathway as a result of TDO inhibition and redirection along the tryptophan metabolic pathways. Other inhibitors of TDO, the so-called novel 3-(2-pyridylethenyl) indoles [22], display similar effects as the mentioned non-steroidal anti-inflammatory agents and also increase brain tryptophan and 5-HT availability.

In addition to TDO, the enzyme indoleamine 2,3-dioxygenase (IDO) also contributes to the oxidative metabolism of tryptophan along the kynurenine pathway. The enzyme activity of IDO is relatively low during normal non-pathological conditions. Amongst one of the major factors that can induce IDO activity, in a number of tissues, is an inflammatory condition where pro-inflammatory cytokines are expressed to a significant extent [1,13]; some of these will be discussed later. IDO induction is demonstrated in vitro in cells containing pathogens such as *Chlamydia*, C. *pneumoniae*, cytomegalovirus and B *Streptococci* [23]. This induction is most likely due to the mediation of interferon gamma (INF-γ) - a cytokine known for its induction of IDO. A resulting systemic

induction of IDO also occurs during endotoxic shock [24]. IDO mediated tryptophan degradation was also demonstrated in clinical trials where direct intravenous injection of INF-γ was used for the treatment of cancer patients [25]. It is noteworthy that exposure to certain environmental factors are said to have the ability to influence IDO activity. Such environmental factors, including early exposure to microbial components (e.g. eating sand during childhood, exposure to bacteria and viruses results in immunity against future similar infections), which favour T helper cell-1 (Th-1) differentiation with subsequent enhanced INF-γ expression which thus upregulates IDO activity [26].

1.1.4 Kynurenine pathway metabolites

Kynurenine, as mentioned earlier, is the major metabolite of the kynurenine pathway and its production involves oxidative catabolism of tryptophan via either of the two enzymes, i.e., TDO or IDO. Kynurenine itself can be further metabolized along two distinctive pathways to form metabolites of the kynurenine pathway. These metabolites, include amongst others, kynurenic acid and quinolinic acid, and are excreted freely in the urine. They are also known to have certain physiological, as well as pathological, effects – depending on their concentrations.

Kynurenic acid and quinolinic acid are formed by different kynurenine pathways. Kynurenic acid is metabolized from kynurenine under influence of the enzyme kynurenine amino-transferase [27], while 3-hydroxykynurenine and quinolinic acid (QA) are metabolites of the other pathway of kynurenine metabolism. The latter metabolites constitute the precursors of NAD. The enzyme kynurenine 3-hydroxylase gives rise to the formation of 3-hydroxykynurenine. QA is formed from metabolites along the pathway. There are also a few minor metabolites of the kynurenine pathway that are formed directly from KYN or as a result of metabolism of the other metabolites in the pathway. The enzyme kynureninase is responsible for the direct conversion of KYN to anthranillic acid. This enzyme forms 3-hydroxyanthranillic acid from 3-hydroxykynurenine. Alternatively anthranillic acid is converted directly to 3-hydroxyanthranillic acid. Xanthurenic acid is another metabolite formed from 3-hydroxykynurenine via the enzyme kynurenine amino-transferase. Finally quinolinic acid is formed from 3-

hydroxyanthranillic acid and the enzyme responsible for this process is 3-hydroxyanthranillic acid oxygenase. QA can be metabolized via quinolinic acid phosphoribosyltransferase and follow further metabolic steps resulting in the formation of NAD along the kynurenine pathway [1,20,27].

It can be seen that the kynurenine pathway may be interpreted as being quite complex. It does, however, facilitate understanding if a metabolic pathway chart or diagram is scrutinized (See fig 2). From Figure 1.2, with elimination of the intermediate metabolites of the kynurenine pathway, it is clear that quinolinic acid and kynurenic acid are the foremost end metabolites. A further discussion on the physiological effects of these two metabolites will throw some light on the relationship between the kynurenine pathway and certain pathological processes.

Quinolinic acid is considered to be a neurotoxin and, under certain pathophysiological conditions, accumulates in the brain and cerebrospinal fluid (CSF) [27,28]. It is considered a neurotoxin because QA is an agonist of the N-methyl-D-aspartate (NMDA) receptor subgroup and when present in high concentrations, will exert excitatory effects on these receptors. QA is also known to cause lipid peroxidation; to increase the production of reactive oxygen species (ROS), and to an extent promote apoptosis [29,30,31]. Another reason for the metabolite's neurotoxicity is situated in its prevention of glutamate uptake into the astrocytes, thus causing an increase in glutamate and thereby overstimulating the receptors [27]. It is in this regard that QA is implicated in number of neurological and neurodegenerative diseases. The neurological diseases pertain to that of major inflammatory diseases such as amyotrophic lateral sclerosis (ALS); AIDS dementia encephalopathy and infections of the CNS [27,32]. These inflammatory conditions are generally associated with induction of IDO (IFN-γ) that promotes a shift towards the kynurenine pathway with a resultant increase in the concentration of QA. Apart from the neurological effects, certain other pathophysiological consequences were noted with peripheral accumulation of QA. Studies have shown the accumulation of QA to be involved in the inhibition of gluconeogenesis [33], erythropoiesis, drug binding and lymphocyte blast formation [34].

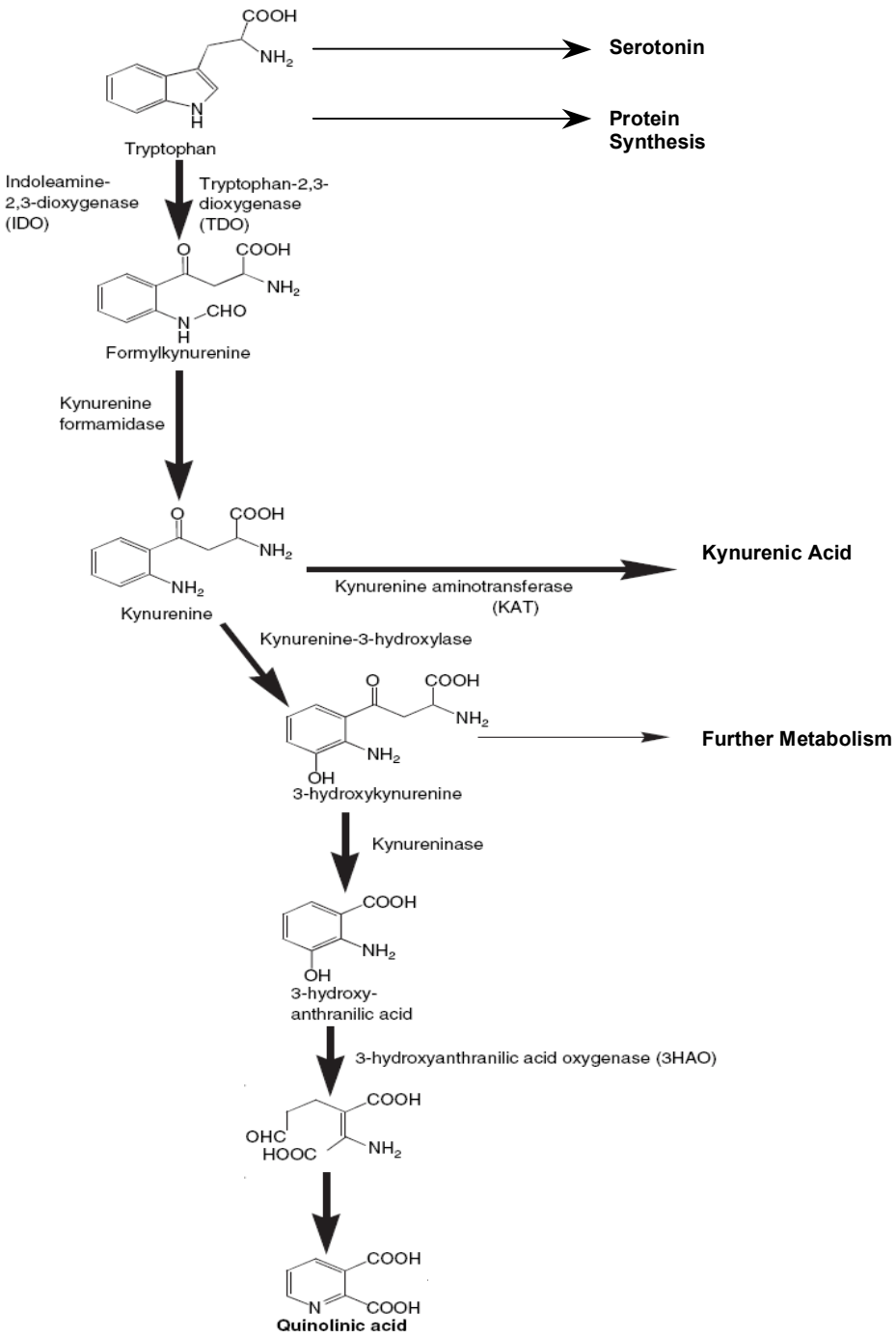

Figure 1.2 Illustration showing the tryptophan metabolic pathway. Adapted from [44]

Kynurenic acid exhibits effects that seem to contrast those of QA. High concentrations of KA depict anti-convulsant and neuroprotective characteristics [27]. Protection against excitotoxic injury is also displayed in the latter setting. An increase in the KA concentration is said to hinder the release of glutamate [35], which starkly opposes that reported for QA. In contrast to QA, a reduction in the KA concentration will thus increase the susceptibility to excitotoxic occurrences [36]. Hence glutamate receptor activation and excitotoxic neuronal damage can be reduced if kynurenine metabolism can be shifted away from QA towards the formation of KA [37]. Such an effect had in fact been obtained with a reduction in the production of QA. However, an increase in KA is also associated with negative consequences. These include exacerbation of inflammatory bowel disease and schizophrenia. An increase in serum KA is known to occur in chronic inflammatory bowel disease [38] and an increase in cerebral cortex KA has been reported in schizophrenia [39]. The first is to be expected in view of the pro-inflammatory nature of the disease. The same logic may perhaps also apply to schizophrenia as reports of overt pro-inflammatory cytokine production have been published [39,40].

As discussed above, it can be seen that the kynurenine pathway is more than a pathway or end route for the metabolism/catabolism of tryptophan. Tryptophan metabolism along the kynurenine pathways produces two major end metabolites which have significant involvement in a number of pathologies [35-40]. Although QA and KA seem to contrast each other in physiological effects, one metabolite cannot solely balance the effect of the other. The process is more complicated as there are confounding internal and external factors influencing the pathways and disease processes [27]. However, a shift in the metabolism of tryptophan along the kynurenine pathway resulting in abnormally high end metabolite concentrations, might give an indication of an increased induction of IDO and/or TDO. Factors influencing enzyme activity, as discussed earlier, should be further investigated in order to establish the underlying cause or causes. Hence, measurement of the metabolite concentrations can aid in understanding the role of the metabolic pathways and metabolites in certain disease processes. A further discussion on the role of tryptophan in immune activation and illness will follow at a later stage.

1.1.5 Tryptophan: substrate availability and regulation

The availability of tryptophan as a substrate depends initially on the dietary intake because TRP is an essential amino acid [1-3]. Thus decreased food intake or consumption of diets devoid of TRP [3] may result in a substrate deficiency for the TRP metabolic pathways and protein synthesis. This may also result in altered metabolite concentrations with specific consequences, as well as protein malnutrition. However despite an adequate intake of TRP [4], lack of substrate availability may be encountered in certain conditions. One of these relates to a poor intestinal absorption of TRP [41]. This can mostly be attributed to gastrointestinal disorders. One such disease found in a significant number of adults and children is coeliac disease [41,42]. Treatment with gluten-free diet over a time period seems to improve the metabolic pathways' functioning, as well as the clinical symptoms related to TRP deficiency. Enhancement of the intestinal absorption of TRP may be correcting the latter [41]. Crohn's disease is another gastrointestinal disease where reduced TRP levels were seen in the blood of a proportion of patients studied [43].

Once assimilated, the availability of TRP to the peripheral tissues and organs is determined by the transport process of TRP via the blood. Most of the TRP is transported conjugated to albumin and the remainder constitutes the free TRP [6,7]. The central availability of TRP to the brain depends on its transport across the BBB. Only the free unconjugated TRP can cross the BBB, however TRP binding to albumin may not affect this due the instability of the binding process [8]. However, large neutral amino acids (phenylalanine, tyrosine, threonine, leucine, isoleucine, and valine) can compete with TRP for transport across the BBB [1]. Substances, which compete with, or hinder this effective transport system, should be taken into account when identifying TRP substrate availability to cerebral structures.

The availability of tryptophan can also be seen as self-regulatory in context of TRP itself inducing the enzyme TDO along the kynurenine (or tryptophan-niacin pathway) [20]. Thus the substrate level of TRP is monitored by the liver enzyme activity under non-pathological situations. In addition the oxidative enzyme activity, as described earlier (TRP induces TDO enzyme activity), will result in an enhanced TRP catabolism thereby

reducing substrate availability for alternative pathways or utilization. In view of the previously mentioned fact, there are some of the end products of the kynurenine pathway that are used for NAD production, the synthesis of niacin, as well as nicotinamide derivatives. These nicotinamide derivatives may also inhibit TDO in a negative feedback mechanism [20] thereby also regulating substrate availability.

1.2 Tryptophan metabolism in context of immune responses and illness

As mentioned earlier, one of the key oxidative enzymes for the catabolism of tryptophan is IDO. IDO was initially noted to be induced during conditions of immune activation with the concomitant release of pro-inflammatory cytokines [1,13]. *In vitro* studies have in fact shown that interferons are strong stimuli of IDO activation. Certain immune mediators, such as tumor necrosis factor α (TNF α) and interleukin-2 (IL-2), increase interferon induction of IDO. INF-γ was reported to have a strong influence for the induction of IDO expression in the presence of a number of immune related conditions [23-25,45]. In summary it can thus be said that pro-inflammatory cytokines stimulate IDO activity and by implication tryptophan catabolism along the kynurenine pathway.

Tryptophan is essential for protein synthesis, not only for the host but also for the growth of pathogens, for uncontrolled cell proliferation (cancer cells) and for the intracellular replication of viruses [23,45]. Ideally, the latter processes may thus be inhibited by reducing tryptophan in the micro-environment. This may be accomplished by tryptophan degradation (with a resulting decrease in tryptophan levels) via INF-γ induction of IDO. Such a mechanism would form part of the antimicrobial and anti-proliferating characteristics of the immune response [45].

In contrast to the potential advantages of the interferon-mediated induction of IDO and the resulting tryptophan catabolism during immune activation, this mechanism may also cause suppression of the immune response. The latter process may contribute to a state of "immunodeficiency" as in the case with HIV infected patients [46]. The reason probably lies in the fact that tryptophan is also required for the proliferation, differentiation and maturation of T-cells. Thus, under conditions of chronic immune activation and T-cell

response (via release of pro-inflammatory cytokines; INF-γ), the resulting sustained IDO catabolism and subsequent depletion of tryptophan may prevent proliferation of Th1-type immune response. This is a classic profile of HIV, whereby an immune response occurs as a result of infection, but an immune suppression is evident. There are various other diseases that correspond to this, including autoimmune disorders and malignancies [45, 47] where the pathogenesis of these conditions is related to the production of pro-inflammatory cytokines (INF-γ, TNF-α). In fact, there frequently appears to be a co-existence of immune activation and immune deficiency. This potential immune suppression may give rise to the susceptibility and vulnerability of the host to contract various other infections, adding to the burden of the host's compromised immunity. This concept will be explored later for the case of chronic renal failure patients on dialysis where a state of longitudinal activation of the immune system is assumed to exist.

IDO activation can be determined directly by measurement of enzyme activity in the relevant tissue or matrix. However enzyme activity determination is invasive as tissue biopsies are required. A less invasive measure will be to determine the kynurenine to tryptophan ratio (measured from peripheral blood) as an expression of the IDO enzyme activity [48]. Markers of immune activation such as neopterin can be correlated with the kyn/trp ratio to substantiate that IDO activity occurs as a result of immune activation. These statements contribute to some of the objectives and major aims of this study.

There are a number of diseases for which tryptophan degradation with respect to activation of the immune system is known to occur. Activation of IDO has been noted in viral diseases (HIV infection); bacterial diseases (*Chlamydia*, C. *pneumoniae*, cytomegalovirus and B *Streptococci*, Lyme encephalopathy); autoimmune conditions (Systemic lupus erythematosus, rheumatoid arthritis,); malignancy (carcinoma of colon, T-cell leukaemia) and neurodegenerative disorders (Huntington's, Parkinson's and Alzheimer's disease) [28,35-40,44-46]. It is thus important to take these diseases and other conditions into consideration when a study evaluating tryptophan metabolism is to be designed. This was considered during the compilation of the inclusion and exclusion criteria for the selection of subjects and healthy controls for this study. As will be briefly

discussed in the later sections of this review, infectious complications and compromised host immunity is existent in chronic renal failure patients on maintenance dialysis treatment. This is important for substantiation of the hypothesis proposed for this study. Accordingly markers of immune activation have to be correlated with the levels of tryptophan and pathway metabolites.

1.3 Tryptophan depletion

Tryptophan depletion, if brought about by purposefully inducing a depletion in the tryptophan levels, illustrates that certain symptoms are evoked. In addition, it is also possible to selectively investigate the influence of cerebral tryptophan depletion. As mentioned in section 1.1.5 there are large neutral amino acids which compete with tryptophan for transport across the BBB [1]. Hence, intake of amino acid beverages rich in such substances and devoid of tryptophan will result in an increased competition for tryptophan transport across the BBB and thereby results in a central depletion of tryptophan [1,3,6].

Tryptophan depletion studies have been conducted in order to assess the resulting outcomes and effects in a number of psychiatric disorders [1,49,50]. These studies indicate that tryptophan depletion has a further negative impact on the mood of patients suffering from depression [51]. There has also been reports of slight influences of tryptophan depletion on the mood and degrees of irritability in normal healthy individuals [1,52] The latter can be explained in terms of tryptophan being the substrate for serotonin (see section 1.1.2 Biochemical pathways of tryptophan metabolism) and will be discussed in the following section, that is, the involvement of serotonin in mental health and well-being. Conditions of long-term tryptophan depletion may also cause other symptoms and these may or may not be detected – depending on the post-depletion observation of patients [1,3,6] and may also depend on the vulnerability of the individual. Such symptoms include irritability, frustration and depression of mood and mental awareness.

1.4 Serotonin, tryptophan and mental well-being

Different types of evidence confirm the role of serotonin on affective states. Three of the five serotonin terminal autoreceptors (5-HT1A, 5-HT1B and 5-HT1D) show involvement in depression. These autoreceptors control the release of serotonin into the serotonergic neurons' synapse [53]. Antidepressant activity can result if there is a short period blockade of the receptors with a resulting increase in the extracellular serotonin levels. Antidepressant medications, such as receptor antagonists and selective serotonin reuptake inhibitors (SSRI), have proven to be effective for the treatment of depression [54,55]. Serotonin depletion in general has implications for its involvement in depression according to the monoamine hypotheses of depression (low levels of one of more brain monoamine neurotransmitters) [53]. A decrease in brain serotonin function may cause and exacerbate the depressive sickness. In certain cases the primary defect may be substrate deficiency. Supporting evidence for this is a decreased level of tryptophan (serotonin precursor) in depressed patients [54], the antidepressant effects of tryptophan administration, as well as the previous section's discussion on tryptophan depletion.

It can thus be seen that if there is a shortage of tryptophan for the production of serotonin a depressive illness may prevail. Hence, factors decreasing tryptophan availability (diet etc.) or causing an increase in the tryptophan metabolism will have an influence on serotonin production and may result in depression in vulnerable individuals. Factors which may be instrumental in the link between serotonin, tryptophan metabolism and depression (and mental well-being) thus include: the involvement of pro-inflammatory cytokines, the kynurenine pathway metabolites and neurodegeneration. These factors can be seen as being interlinked because pro-inflammatory activity enhances the production of kynurenine pathway metabolites via induction of IDO (section 1.1).

An extensive review on cytokines and depression by Schiepers *et al*, 2005, [56] can be consulted for a detailed discussion on the involvement and contributions of the different pro-inflammatory cytokines towards depression. The participation of interferon *per se* towards depression is discussed by Fekkes *et al*, 2003. [57]. It is therefore not surprising that depression will also arise during the direct treatment of certain cancers with

interferon (INF-α) [23,25]. In fact, continuation of such treatment was, in certain countries, ruled out due to the resulting psychopathology.

In summary, it can be said that cytokine-induced tryptophan depletion can result via two mechanisms: by cytokines directly reducing food consumption (dietary tryptophan depletion) [58] and by cytokine-induced degradation of tryptophan along the kynurenine pathway via IDO [59]. For the latter mechanism tryptophan metabolism is shifted towards the kynurenine pathway and may thus result in decreased tryptophan levels for the synthesis of serotonin. This shift towards the kynurenine pathway also leads to an increase in the end metabolites (kynurenines) quinolinic acid and kynurenic acid. These metabolites have some implications for potential neurodegeneration [53]. Quinolinic acid is a neurotoxin (see section 1.1.4), whereas kynurenic acid is said to have neuroprotective properties. When the neurodegenerative effects of quinolinic acid is more pronounced than the neuroprotective effects of kynurenic acid, neurodegeneration may occur in stress coping regions of the brain (such as the hippocampus) [53]. This may cause disturbances of coping strategies in the brain and can thus result in depression.

1.5 Chronic Renal Failure

1.5.1 Background

One of the major functions of the kidney is the excretion of metabolic waste products. Inability or loss of adequacy of the kidneys to excrete these products will result in increased amounts of “toxins” in the blood. This is a characteristic of the uraemic syndrome. This subsection will attempt to briefly place chronic renal failure, tryptophan metabolism and immune activation into perspective for the purpose of this study.

1.5.2 Chronic renal failure and tryptophan metabolism.

Uraemia is a pathological condition that presents in patients with chronic renal failure (CRF) [60]. In the end stages of chronic renal failure (ECRF) endogenous metabolites which are normally excreted in the urine accumulate in the blood of such patients [60,61]. Metabolites of the kynurenine pathway are bound to constitute part of the latter. As

previously described, in the kynurenine pathway the essential amino acid tryptophan is enzymatically degraded as part of normal hepatic metabolism and this metabolic activity is partially regulated by immune activity which induces the relevant enzymes [60]. A small number of publications described tryptophan depletion and the levels of kynurenine and its major metabolites quinolinic acid and kynurenic acid to be high in the patient with end-stage renal failure. These metabolites were subsequently categorized as protein-bound uraemic toxins [62]. It has been suggested that they may contribute to certain uraemic symptoms such as anaemia [63], certain neurological disturbances and increased vulnerability to infections [60]. Disturbances in tryptophan metabolism and accumulation of the above mentioned endogenous metabolites are thus of concern in uraemic patients, particularly those undergoing dialysis.

There are, in fact, only a few full papers that describe tryptophan depletion and the accumulation of metabolites of the kynurenine pathway in end-stage renal failure patients, on renal function replacement modalities (dialysis treatment) [60,61,64]. This phenomenon is, however, supported by work done with animal experimentation [65,66,67,68]. This accumulation is not surprising as most metabolic end products usually excreted by the kidneys accumulate in the functionally anephric patient [1,61,64,69]. The accumulation of kynurenine metabolites in renal failure is thus generally thought to be due to the loss of renal clearance function [61,63]. However, other possibilities such as stimulation of the synthesis of these metabolites through inappropriate immune activation should also be considered. Immune activation in renal failure patients on renal replacement therapies is discussed in the following paragraphs.

1.5.3 Evidence for above normal immune activation in renal failure

Sufficient evidence exists to believe that end-stage renal failure patients on renal replacement therapies have enhanced immune activation. For instance, Fuchs *et al* [70], found increased neopterin levels in long term haemodialysis (HD) patients, Borazan *et al* [71] reported serum C-reactive-protein (CRP) and pro-inflammatory cytokine levels to be higher in haemodialysis patients, as well as in continuous ambulatory peritoneal dialysis (CAPD) and Viljoen *et al* [72] found neopterin to be significantly increased in chronic

renal failure patients. There are many factors that could potentially contribute to increased pro-inflammatory activity in end-stage renal failure patients – especially those on renal replacement therapies. It is, for instance, acknowledged that the haemodialysis procedure itself, partially as a result of the use of bio-incompatible membranes, and the high endotoxin levels could result in immune activation and in the release of pro-inflammatory cytokines [73,74]. However, increased plasma cytokines have also been reported in CRF patients who have not yet undergone dialysis and who are treated by conservative treatment [71,73].

1.5.4 Summary

In summary, it can be said that indications exist a) for the accumulation of metabolites of the kynurenine pathway in end-stage renal failure patients on renal replacement therapies b) for lower tryptophan and serotonin levels and c) for above normal pro-inflammatory activity in these patients. As a limited number of papers have this far addressed the accumulation of these metabolites in end stage renal failure patients on renal replacement therapies there is still uncertainty as to the extent to which tryptophan depletion and the accumulation of the metabolites occur and whether the degree of depletion and accumulation is dependent on the type of renal replacement therapy, e.g., whether haemodialysis clears these substances better than peritoneal dialysis. In fact, no paper could be found where the levels in the blood of haemodialysis and in those on peritoneal dialysis are compared. The one major obstacle in assessing the status of tryptophan and metabolites derived from tryptophan via the kynurenine pathway in renal failure patients is the fact that facilities for the analysis of these substances are not readily available.

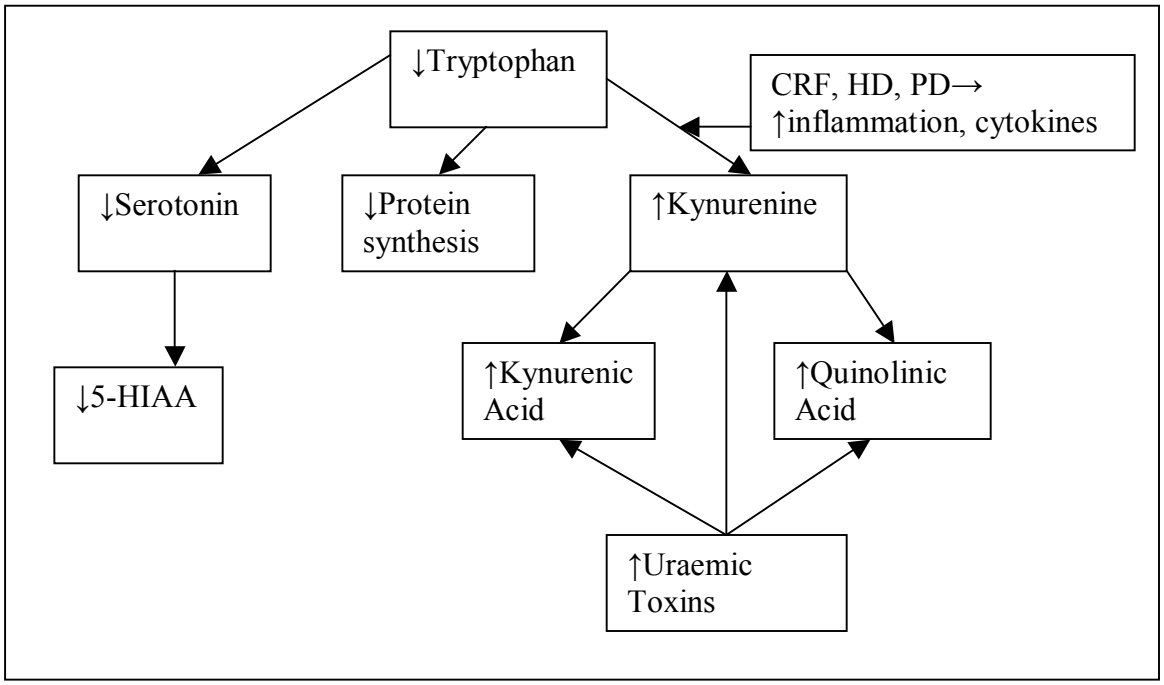

Figure 1.3 Proposed mechanistic pathway for tryptophan metabolism in chronic renal failure. (CRF = Chronic Renal Failure; HD = Haemodialysis; PD = Peritoneal Dialysis, 5HIAA = 5 hydroxyindoleacetic acid)

1.6 Aim

The primary aim of the study was to develop and validate suitable methods for the assessment of tryptophan and two of the metabolites of the kynurenine pathway, i.e., kynurenine and quinolinic acid in plasma.

The secondary aim was to confirm previous indications of tryptophan depletion and accumulation of the said metabolites in chronic renal failure patients on haemodialysis and peritoneal dialysis and to compare the levels of tryptophan and the said metabolites in the two groups of renal failure patients and to a matched control group.

The objectives of the study:

1. Development and validation of a sensitive, reliable and reproducible method for the analytical quantification of plasma tryptophan, serotonin, kynurenine and quinolinic acid.
2. Assessment of whether there are indeed tryptophan depletion and accumulation of the metabolites of the kynurenine pathway in renal failure on renal replacement treatment and, if so, whether there are significant differences in the levels between the renal replacement modalities of haemodialysis and peritoneal dialysis.
3. Testing for indications that stimulation of the kynurenine pathway activity may contribute to the disturbances in tryptophan metabolism by looking at the kynurenine/tryptophan ratios and indications for inflammation.
4. Examining possible correlations between the tryptophan and metabolite status on the one hand, and a) certain physiological parameters said to be influenced by the proposed disturbances in tryptophan metabolism and b) psychological status as represented by the quality of life profile, on the other.

1.7 References

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Chapter 2

Materials and Methods

This chapter deals with the methods used in this study. Assessment of tryptophan and its metabolites is riddled with technical problems – especially when not performed in a laboratory geared to do it on a routine basis. The methods used in this study had to be developed specifically for the present, and hopefully, subsequent studies. What is presented here are the final methods arrived at and do not include the work done to arrive at a reliable methodology.

2.1 Study Population Groups

The study groups entailed chronic renal failure patients on (a) haemodialysis, (b) peritoneal dialysis, and (c) a group of normal age, gender and race matched controls. The minimum numbers necessary for statistical validity was estimated with the assistance of Professor P Becker, Biostatistics, Medical Research Council (MRC), University of Pretoria. The matched control group was recruited from the general public as healthy volunteers. Ethical clearance for the study was obtained from the Faculty of Health Sciences, Research and Ethics Committee, University of Pretoria and the clearance number was S168/2006. All patients and controls signed informed consent forms (See Appendix).

The age group criteria for the study was limited to adults (18 years and older). CRF patients were sourced from the Pretoria Academic Hospital; Department of Nephrology; Haemodialysis unit and Peritoneal Dialysis ward. Major exclusion criteria were patients who developed chronic renal failure primarily as a result of immune related diseases.

2.2 Biological Specimens

Trained phlebotomy personnel collected blood samples in specified tubes (potassium EDTA, 4ml vacuum tubes: BD Vacutainer K2E 7.2mg purple top) from the control group. This was done by venous puncturing of the antecubital vein. The same applied for

the peritoneal dialysis group. Blood from haemodialysis patients was collected at the initiation of dialysis, from the arterial line, and with a transmembrane pressure set at 0mmHg to prevent ultrafiltration influencing the levels of the substances to be analysed. Care was taken not to dilute the blood by the priming saline. Blood from peritoneal dialysis patients was collected before initiation of the dialysis and the record of peritonitis was noted if any.

All blood specimens, collected for the analysis of tryptophan and tryptophan metabolites, for each group were collected after an overnight fast; immediately placed on ice before transportation to the desired location for processing and storage. Samples for each of the study groups were collected during the same time frame thereby eliminating circadian differences between the different samples. The samples were centrifuged at 3000 revolutions per minute at 4 degrees Celsius for ten minutes. The resulting plasma was distributed and stored in aliquots of 0.5 ml each. The samples were stored at -70^0C until analysis. Whole blood samples for determination of serotonin were appropriately distributed into aliquots of 0.5 ml each and stored at -70^0C until analysis.

2.3 Quantification of Tryptophan and Metabolites

To determine the levels of each analyte from the specimens, standard calibration curves for each analyte were drawn up (Relative response versus analyte concentration). For simulation of the matrix of the samples an appropriate plasma or whole blood pool was used for the spiking of standard solutions according to the standard addition method. The pools were generated from random blood samples donated by normal volunteers. The calibration curves consisted of standard measurements throughout the expected biological reference range for each analyte, with high and low points for out of range. External quality controls were analysed together with the samples so as to ensure compensatory stability, uniformity and acceptance/rejection of the chromatographic analysis for the individual analytes.

2.4 Experimental design

The quantification of tryptophan and metabolites in peripheral blood samples requires measuring of analyte concentrations from appropriate standard calibration curves (response directly proportional to concentration). This requires the need for specific analytical reagents identical to the chemical structure and form of these analytes as expressed in actual blood samples. It is thus important to obtain the identical and purest form of analytical reagents that can be analysed by an analytical method.

The following are details of the analytical reagents that were used to make up stock solutions for the drawing up standards:

- L-tryptophan: $C_{11}H_{12}N_2O_2$ (204.23g/mol)

Cas No. 72-22-3, reagent grade NT > 98% (Sigma-Aldrich)

- Serotonin-hydrochloride (5-hydroxytryptamine): $C_{10}H_{12}N_2O.HCl$ (212.68g/mol)

Cas No. 153-98-0

- L-Kynurenine: $C_{10}H_{12}N_2O_3$ (208.21 g/mol)

Cas No. 2922-83-0 (Sigma)

- Quinolinic Acid (2,3-pyridinecarboxylic acid): $C_7H_5NO_4$ (167.2g/mol) (FLUKA)

Cas No. 89-00-9

- 3-nitro-L-tyrosine: $O_2NC_6H_3$.4-$(OH)CH_2CH(NH_2)CO_2H$ (226.19g/mol)

Cas No. 621-44-3 (surrogate internal standard for kynurenine)

-2,6-pyridinecarboxylic acid: $C_7H_5NO_4$ (167.2g/mol) (FLUKA)

Cas No: 499-83-2 (surrogate internal standard for quinolinic acid)

The above reagents were of analytical reagent grade > 98% purity and were purchased from Sigma Aldrich packaged as dry weighed out powder.

The following stable isotope labelled internal standards were purchased from ISOTEC:

- 615862 L-Tryptophan-2',4',5',6',7'-D5 (INDOLE-D)
- 673455 Serotonin-α,α,β,β-d4, Creatinine SulfateMonohydrate, 98% CP

All other solvents and reagents used during experimental sample preparation were of analytical reagent grade as purchased from the relevant suppliers.

De-ionised water (double distilled water) was used for preparation of stock solutions and standards.

Preparation of stock solutions:

For preparation of stock solutions the appropriate masses of tryptophan, kynurenine, quinolinic acid and serotonin were weighed out. Stock solutions with concentrations of 1000ppm (1000mg/l) were prepared in separate vials. The internal standards tryptophan-d5, L-nitro-tyrosine, 2,6-pyridine dicarboxylic acid and serotonin-d4 for tryptophan, kynurenine, quinolinic acid and serotonin respectively were weighed out and dissolved to give 1000ppm and 100ppm stock solutions.

Preparation of working stock solution:

800µl of de-ionised water was added to 0.001g tryptophan and 100µl each of the kynurenine and the quinolinic acid 1000ppm stocks. The resulting working stock solution contained 1000ppm of tryptophan and 100ppm of kynurenine and quinolinic acid respectively. Internal standards were diluted to give 1000ppm tryptophan-d5 and 100ppm of L-nitro-tyrosine and 2,6-pyridine dicarboxylic acid.

Preparation of working standards

Diluted working stock (WSS) solution as follows:

Working Standard 1: 20µl of WSS + 980µl of water = 1000µl

Working Standard 2: 60µl of WSS + 940µl of water = 1000µl

Working Standard 3: 100µl of WSS + 900µl of water = 1000µl

Working Standard 4: 140µl of WSS + 860µl of water = 1000µl

Working Standard 5: 180µl of WSS + 820µl of water = 1000µl

Internal Standard: 100µl of Internal Std + 900µl of water = 1000µl

Procedure for sample preparation:

For the preparation of standard calibration curves the prepared working standards were spiked in pre-prepared plasma pool. The plasma pool was derived by pooling plasma

from normal volunteers. Tri-chloro acetic acid (TCA) was used as a deproteinising agent to precipitate proteins from the plasma samples and whole blood samples.

Table 2.1 Procedure for preparation of plasma samples

	Blank	**Level 1**	**Level 2**	**Level 3**	**Level 4**	**Level 5**
Plasma	200µl	200µl	200µl	200µl	200µl	200µl
Working Std	-	20µl	20µl	20µl	20µl	20µl
Trp-d5	20µl	20µl	20µl	20µl	20µl	20µl
L-nitro tyr	20µl	20µl	20µl	20µl	20µl	20µl
2,6 pyr dca	20µl	20µl	20µl	20µl	20µl	20µl
Water	20µl	-	-	-	-	-
TCA	100µl	100µl	100µl	100µl	100µl	100µl

Preparation of serotonin whole blood spikes were carried out similarly to those of the plasma samples.

2.5 Analytical Techniques

Many studies have reported methods for the quantification of tryptophan and its metabolites [1-5]. High performance liquid chromatography (HPLC); gas chromatography – mass spectrometry (GC-MS); liquid chromatography – tandem mass spectrometry (LC-MS-MS) have been used to study tryptophan and the degradation metabolites [6-9]. For rapid and routine analysis of tryptophan and kynurenine, HPLC is the preferred method. However for added sensitivity and specificity coupling chromatography to mass spectrometry delivers superior results, such as the utilization of GC-MS for this study.

2.5.1 HPLC

The High Performance Liquid Chromatography (HPLC) technique employed for this study enabled the separation of the intended compounds ideally on a C18 reversed phase column using an acetate buffer as the mobile phase. The method was adopted from a technique developed by Laich et al [6]. Principally a 12cm length, C18 reversed phase column with an internal diameter of 0.5 µm was used for chromatographic separation. A C18 pre-column, with gold line seal filter represented the guard-column.15mmol/L sodium acetate buffer of pH 4 (adjusted with acetic acid) was used for the mobile phase. 25-50mL of acetonitrile was added to the mobile phase to optimize retention times of the

eluted compounds. The mobile phase was programmed to elute isocratically with a flow rate of 1.1mL/min.

The instrument used was an HP 1050 series HPLC system with HP 1050 series autosampler unit. HP 1100 fluorescence (FLD) and HP 1050 UV detectors were used for detection of analytes. The fluorescence detector was set at an excitation wavelength of 286nm and an emission wavelength of 366nm due to the fact that tryptophan and serotonin have indole rings in their chemical structure and thereby fluoresce at the mentioned wavelength [5,6]. The UV detector was set to analyse kynurenine species at a wavelength of 360nm. Both UV and fluorescence detectors were connected in series allowing simultaneous detection of the analytes in one chromatographic run.

The supernatants of the prepared samples were transferred to autosampler amber vials (1.5ml). The injector was set to deliver 40µl of sample into the system. Chromatographic run time was 12 minutes with the retention times of the analytes appearing between 3 and 6 minutes. After a series of chromatographic analyses the system was rinsed from 10% methanol to pure methanol and back using a gradient elution program. The guard column filter was also replaced. All solvents and mobile phases were de-gassed and filtered before pumping into the system. The system was purged at the stage of mobile phase or solvent exchange. General maintenance and system check ensured optimal performance of the system and prevented air getting into the system or blockage of system flow.

2.5.2 GC-MS

Gas Chromatography with mass spectrometry (GC-MS) is a very sensitive analytical instrument that not only allows the chromatographic separation of analytes on a GC column but also separation by mass (mass to charge ratio) using a mass spectrometric detector.

For the purpose of this study, the gas chromatographer was a Hewlett Packard HP GC 6890 series instrument. The GC instrument was coupled to a MS 5973 series mass spectrometer. Two MS ionization sources were employed: electron impact ionization (EI)

and negative chemical ionization (nCI). It is necessary to give details on the functioning and reasons for the use of the two MS sources. A concise explanation of this together with table of comparison (Table 2.2) of the two sources will follow later. The same can be said for the testing and use of two different GC columns for separation of analytes (Table 2.3). For this purpose DB-5MS and ZB 1701 GC columns were used. The ideality of these comparisons was to maximize sensitivity and selectivity of the measuring instrument for optimal and reliable quantification of the relevant analytes from the appropriate matrices. It was also to ascertain which column provided better performance and separation of analyte peaks.

The GC-MS instrument consists of a number of components with subcomponents. Optimal maintenance and performance checks for reliability of these components are essential to ensure valid data to be obtained from an analysis. This works hand in hand with the method validation procedure (see chapter 3) done after method development for this study.

The GC used consisted of the following major components and subcomponents:

- Autosampler unit
- GC injector unit
- GC glass needle and syringe.
- Injection port with rubber septum and temperature control
- Front/back inlet with glass inlet sleeve; gold seal, rubber seal, split/splitless
- Switching valves
- Purge valve/unit
- Column nut with ferrules
- Column
- GC oven
- Interface column nut with ferrule
- GC-MS interface with Auxiliary temperature control

The MS consisted of the following components and subcomponents:

- MS source with interface socket; filaments; lenses; source chamber.
- Quadripole
- Analyzer and Analyzer chamber
- Vacuum chamber
- MS detector

The instrumentation was controlled by the sophisticated software HP ChemStation ® and this allowed monitoring of system integrity such as pressures and temperatures. Integration software also enabled the decoding and encoding of data from the detector as well as peak integration information such as peak area, height, width and retention times for quantification of data.

Figure 2.1 represents a basic illustration for the GC-MS instrument setup and layout. The ideality of the instrument is the GC-MS interface which represents the link between the chromatography (column) and the mass spectrometer (detector) and which allows coupling of the both. This is important because of the pressure differences between the GC and MS. The high vacuum pumps are thus the most critical and sophisticated ingenuity of the system.

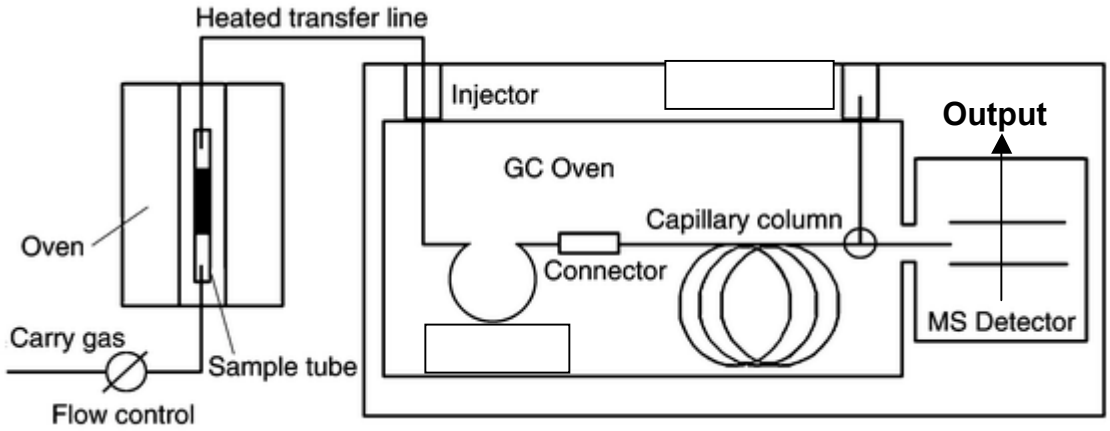

Figure 2.1 Basic line diagram indicating the basic instrument layout for a GC-MS system. Adapted from [12]

It is important that each of the components is in functioning and optimal condition. Regular maintenance and cleaning/replacing of certain components are necessary to ensure that system performance is up to standard and that reproducible data can be obtained.

For the purpose of this study: the specific ion source was cleaned after a determined number of analyses and the relevant filaments were also replaced. The same procedure was followed for the inlet glass sleeves as these are also know to produce peak tailing and decreased sensitivity when dirty [15,16]. The injector needle and syringe were cleaned before each set of analyses. Bad injection techniques and injection-port performances are also known to cause poor results [17]. Thus the inlet rubber septum and inlet gold seal liner were replaced regularly. It is further important to take note of the required temperatures of the injection port, oven, GC-MS interface, ion source and quad according to the type of analyte that is to be determined. This was done by testing and optimization of each condition to obtain acceptable results.

Table 2.2 shows the differences and similarities of the two ion sources that were used for this study. Electron impact ionization was used to identify and obtain structural information about each of the derivatized analytes. Negative chemical ionization on the other hand was used for quantification due to its better sensitivity.

Table 2.2 Comparison of the two MS ionization sources employed [8,13,14]

Parameter	Electron Impact	Negative Chemical Ionization
Sensitivity	Low compared to NCI	Excellent compared to other sources
Selectivity	Unique mass spectra for individual compounds.	Less selective as compared to EI.
Type of ionization	Hard: collision of volatized sample molecules with energetic electrons produces many fragmentary ions.	Soft: reagent gas removes excess energy from electrons which produce fewer, but more stable and abundant ions.
MS structural information	Fragmentation of the molecular ion allows structural identification through interpretation	Adds complementary structural information to EI
Pros/Cons	Absent or low relative abundance of molecular ion. Cost effective allowing usage in many laboratories.	Presence of molecular ion and with improved sensitivity for detection and trace analysis. Availability limited due to high cost of instrumentation.

Table 2.3 Comparison of the two GC columns tested for use

Parameter	DB-5MS	ZB 1701
Type	Capillary Column	Capillary Column
Model Number	Agilent 122-5531	ZB 7GH-G006-11
Nominal Length	30.0 m	30.0 m
Diameter	250.0 µm	250.0 µm
Film thickness	0.10 µm	0.25 µm
Maximum Temperature	350^0C	300^0C
Mode	Constant Flow	Constant Flow
Column Packing	5-MS specifications	1701 specifications

The two GC columns were tested for separation and peak resolution of the individual analytes. Some of the problems encountered with the DB-5MS were peak tailing and poor separation of some of the analytes. Column specifications are given in table 2.3 and results for the two columns are given in the next chapter.

2.6 Method development

A number of analytical techniques have been used for the quantification of tryptophan, as well as the metabolites of the specific pathways as discussed in Chapter 1. Such analytical techniques comprise of chromatographic techniques relating to high performance liquid chromatography (HPLC) and gas chromatography (GC) [5,6,8]. It was decided during the preliminary method consideration of this study to test analyte detection by first employing HPLC.

2.6.1 Sample preparation for HPLC analysis

To 200µl of plasma 20µl each of working standard were added thereby giving rise to spiked plasma standards. The plasma standards were vortexed thoroughly to ensure essential mixing of the spiked standards with the relevant matrix. This was followed by addition of 70µl of the deproteinating reagent trichloroacetic acid (TCA). The standards were further vortexed thus ensuring proper precipitation of proteins. The prepared samples were thereafter centrifuged at 13000rpm for 5 minutes and the supernatants were transferred to amber vials ready for injection into the HPLC system.

Once the method was in order, calibration curves for tryptophan and serotonin were drawn up to test the linearity of the method and to perform quantification at a later stage. It can be seen from figures 2.4 to 2.6 that excellent response could be obtained for peak area, as determined by the HPLC detector, versus the concentrations of the spiked standards thus giving calibrations of r^2 well above 0.999. These curves were however based on standards spiked in water thereby bypassing the analytical interference effects of matrix (whole blood, plasma and serum) samples as in the case of actual patient samples. In order to simulate the matrix as with actual patient samples, standards were spiked in plasma or whole blood generated from pools (random plasma and whole blood collected and pooled together).

Figure 2.2 shows the typical calibration curve obtained for plasma spiked standards. The r^2 value deviates further away from 1 as a result of matrix interference effects in the plasma sample. The mentioned data were however obtained from single prepared standards and generalization from a single set of data would be scientifically and statistically incorrect. This is the concept from which method validation parameters were taken into account during development of a method in order to ensure repeatability and reliability of the performing method. The explanation and data for the method validation will be included in the following chapter. It is however necessary at this point to portray the importance of method validation before heading directly into the mass of data obtained. Substantiation of which can be noted from the unreliability of the single set of data for figures 2.2 to 2.4. These are results which are unreliable as no repeats are performed and no statistical data can be obtained from the data set.

Table 2.4 Results for tryptophan standard calibration curve (spiked water standards)

Concentration in µM	Peak Area
0z	0
15	789.5765
30	1568.841
45	2299.554
60	3090.149
75	3839.532

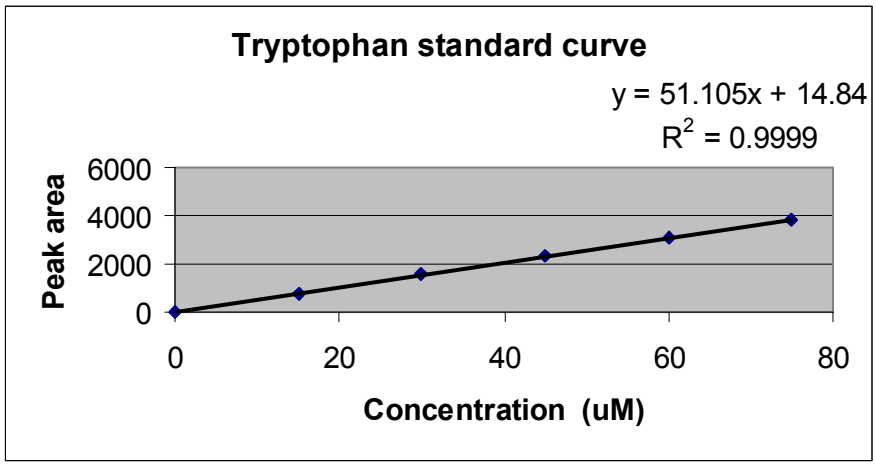

Figure 2.2 Typical standard calibration curve obtained illustrating peak area versus concentration for the analysis of tryptophan via HPLC

Table2.5 Results for serotonin standard calibration curve (spiked water standards)

Concentration in µM	Area
0	0
15	301.5482
30	605.3088
45	932.3464
60	1250.64
75	1559.755

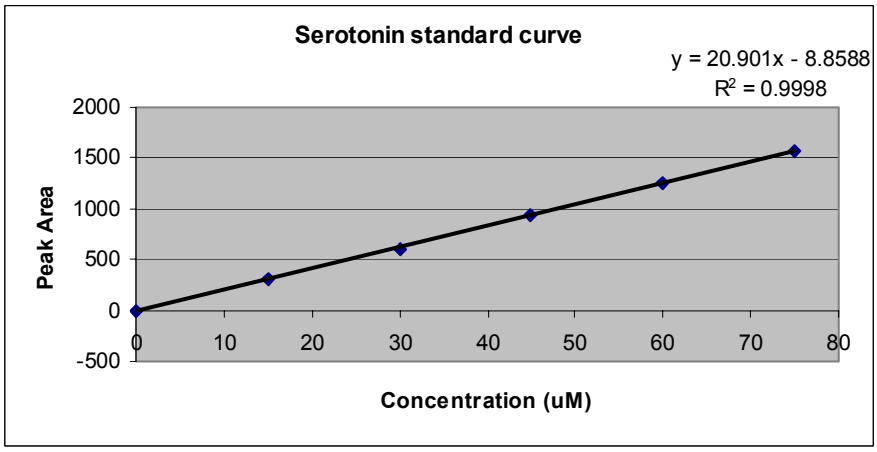

Figure 2.3 Typical standard calibration curve obtained illustrating peak area versus concentration for the analysis of serotonin via HPLC

Table 2.6 Results for tryptophan standard calibration curve (spiked plasma standards)

Concentration in µM	Area
0	9.2225
15	655.2806
30	1440.894
45	2300.003
60	3346.319
75	4255.621

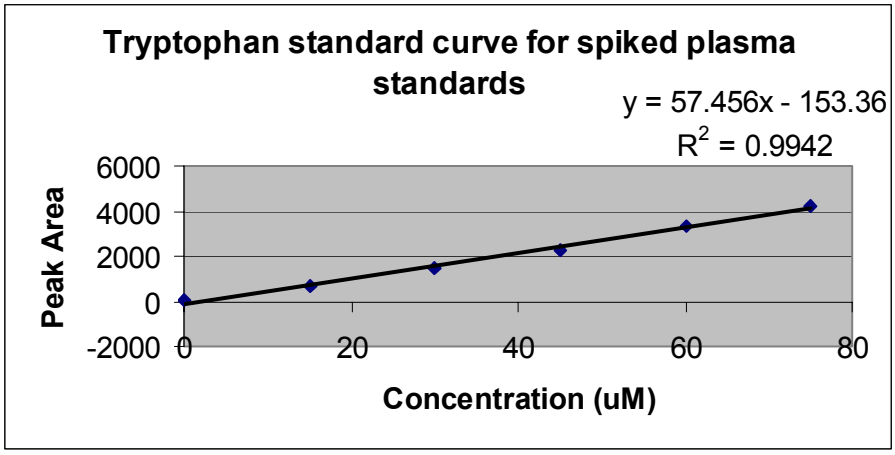

Figure 2.4 Typical standard calibration curve illustrating peak area versus concentration for tryptophan spiked plasma standards

Due to the instability (light and temperature sensitivity) of tryptophan and serotonin a compensatory precaution was needed to consider degradation and loss of sample analyte during a chromatographic analysis. The use of stable labeled isotopes, matched for tryptophan and serotonin, resolved this problem to an extent and therefore GC-MS was chosen as the major technique for analyte quantification. The use of GC-MS also added improved sensitivity and analyte selectivity for the simultaneous separation of tryptophan, kynurenine, quinolinic acid and serotonin during a single chromatographic run.

2.6.2 GC-MS method development

An important consideration prior to the analysis of compounds via GC-MS is to take note of the chemical and structural information of the specific compound. Judging from the fact that detection of compounds is based on the mass to charge ratio (m/z), it is important to determine the molar mass of the compound. When a derivatizing agent is

used, the agent binds to the functional groups of the compound which results in a derivative compound with an added molar mass. As will be explained later, the interpretation of mass spectra is essential for the analysis of compounds using MS. The selection of an appropriate derivatizing agent/s is also of importance and this was shown to be of significance for this study.

2.6.2.1 Derivatization

A question to pose: why is it necessary to derivatize a compound and not just interpret mass spectra from the original molar mass of the compound? A simple but relevant answer is that it would depend on the compound to be analyzed. A second explanation would be to have an understanding of GC coupled to MS and on the mechanism of functioning of GC columns.

During derivatization the interested compound is chemically modified and the result is the production of a new compound which has properties suitable to be analyzed using GC [10]. Certain compounds lack the volatility and stability to be analyzed directly and will therefore require derivatization. The chromatography and behavior of the compound is thus improved upon. In such circumstances the detectability of otherwise undetectable compounds is thereby enhanced [10,11].

The polarity (water solubility) of the compound also plays a role as many compounds have polar functional groups (OH, NH and SH groups). These groups pose a threat to GC analysis because the polar nature of the compound causes it to adsorb onto the walls of the GC column and solid support. By eliminating the presence of these polar functional groups, the correctly chosen derivatizing agent can reduce adsorption and reaction of the compound with the GC column (stationary phase).

2.6.2.2 Derivatizing agents

There are numerous derivatizing agents available and these are classified according to the mechanism of derivatization and the specificity of the agent to derivatize certain functional groups. It can be noted from the information provided above that the choice of

derivatization agent is of utmost importance with regards to the structural properties of the compound. This will determine whether all the functional groups of the compound are completely derivatized thus enabling proper detection and separation.

There are three major categories of derivatizing agents, namely silylation, alkylation and acylation. Silylation gives rise to silyl derivatives by replacing hydrogen atoms with a trimethylsilyl group. These reagents react with water and alcohols first and are specific for the following functional groups: alcohol, phenol, carboxyl, amine, amide and hydroxyl [10]. The most popular silylating reagent is MTBSTFA (N-methyl-N-t-butyldimethylsilyltrifluoroacetamide) [8,10].

With the case of alkylating reagents the polarity of amino, hydroxyl and thiol groups are reduced and replaced with halogenated groups. These reagents target highly polar compounds like amino acids and carbohydrates. For the purpose of this study the fluorinated anhydride pentafluoropropionic anhydride (PFPA) was used. This reacts with alcohols, amines and phenols to give stable volatile compounds. Pentafluoropropanol (PFPOH) is the agent used in conjunction with PFPA and is ideal for derivatization of the functional groups of biological organic compounds [10,11]. The next section will cover the procedure for the preparation of derivatized samples for the GC-MS analysis.

2.6.2.3 Sample preparation for GC-MS analysis

The same procedure was used for the preparation of spiked standards as compared to HPLC sample preparation. Minor differences are attributed to either the use of plasma spikes for the preparation of tryptophan, kynurenine and quinolinic acid standards or whole blood for serotonin. The prepared spike standards were however polar and therefore needed to be extracted, dried and derivatized for GC-MS analysis.

After precipitating of the protein (deproteination) from the prepared spiked standards or samples, the supernatants were collected into separate test tubes. Figure 2.5 illustrates the step by step procedure to be followed for the preparation of derivatized samples suitable for GC-MS analysis.

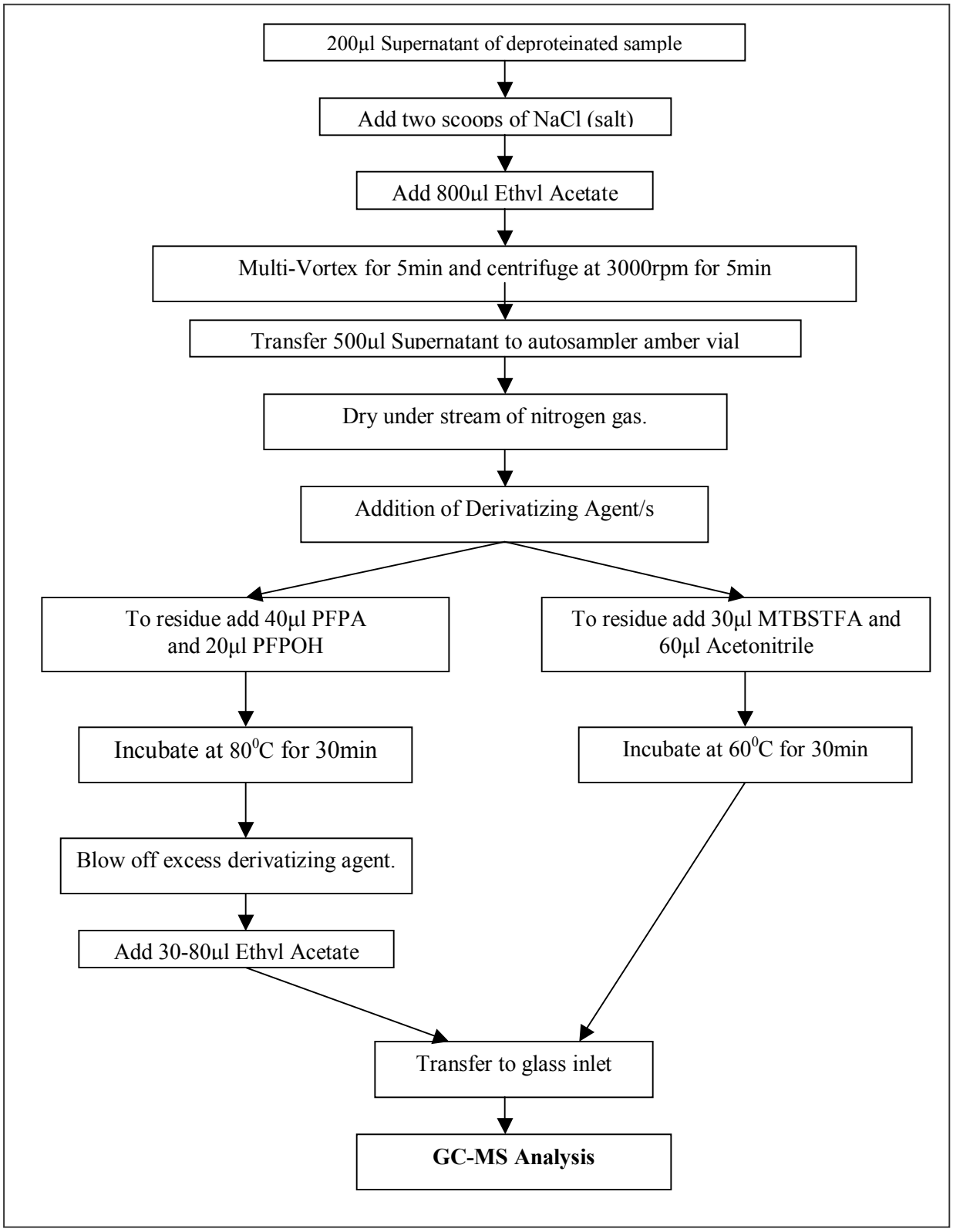
200µl Supernatant of deproteinated sample
Add two scoops of NaCl (salt)
Add 800ul Ethyl Acetate
Multi-Vortex for 5min and centrifuge at 3000rpm for 5min
Transfer 500µl Supernatant to autosampler amber vial
Dry under stream of nitrogen gas.
Addition of Derivatizing Agent/s
To residue add 40µl PFPA and 20µl PFPOH
To residue add 30µl MTBSTFA and 60µl Acetonitrile
Incubate at 80°C for 30min
Incubate at 60°C for 30min
Blow off excess derivatizing agent.
Add 30-80ul Ethyl Acetate
Transfer to glass inlet
GC-MS Analysis

Figure 2.5 Schematic diagram representing steps followed for GC-MS sample preparation

Development of method procedure was in accordance with trial and error measures taken until a suitable method was established. Steps taken were at first to derivatize the actual analyte from its manufactured consistency (powder form). Once peaks for the relevant analytes could be determined an extraction procedure was undertaken to extract water stock standards into a non-polar organic solvent such as ethyl acetate. The method was refined and adjusted using water standard spikes before applying the method to the actual matrix (plasma; whole blood).

Figures 2.6 to 2.9 represent the typical chromatograms, with the respective retention times, obtained for the prepared samples for the analytes and internal standards. These chromatograms illustrate the chromatographic peaks when the most abundant ions were extracted as used for SIM quantification and elution on the DB-5MS column.

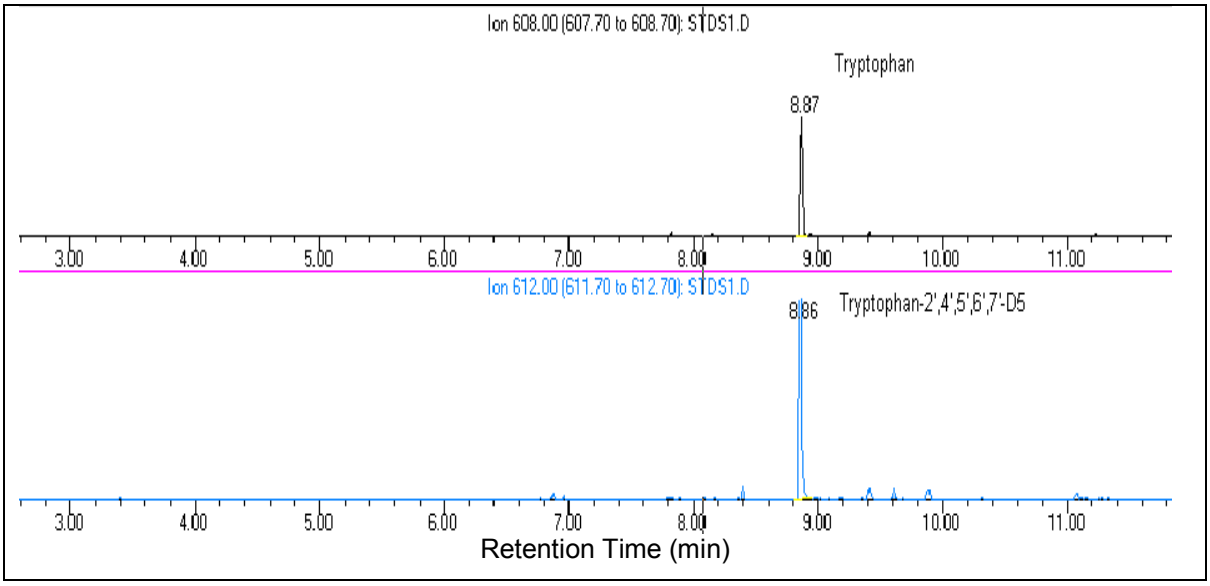

Figure 2.6 Typical chromatogram obtained for tryptophan and internal standard (nCI)

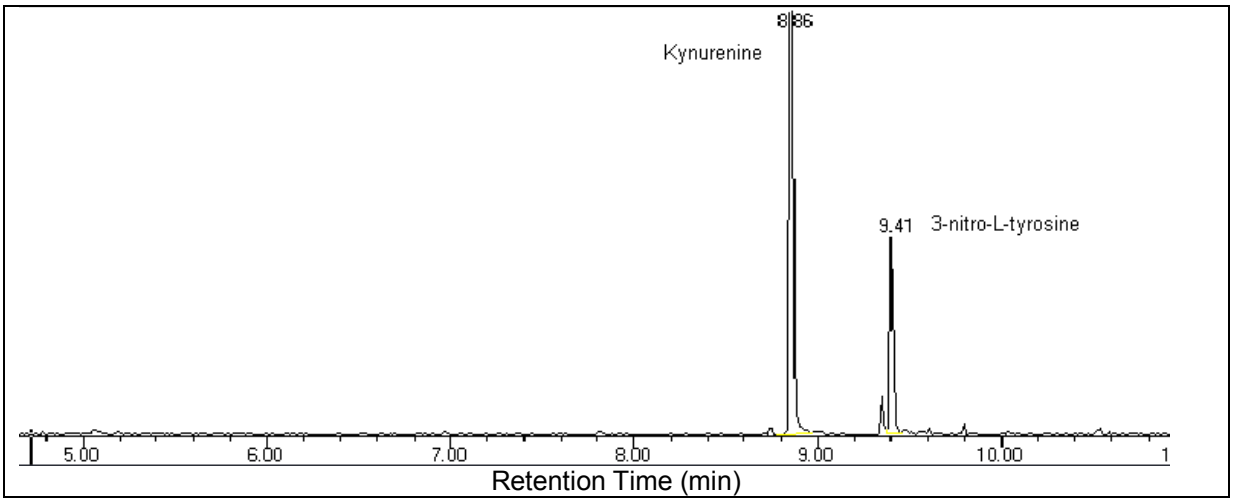

Figure 2.7 Typical chromatogram obtained for kynurenine and internal standard (nCI)

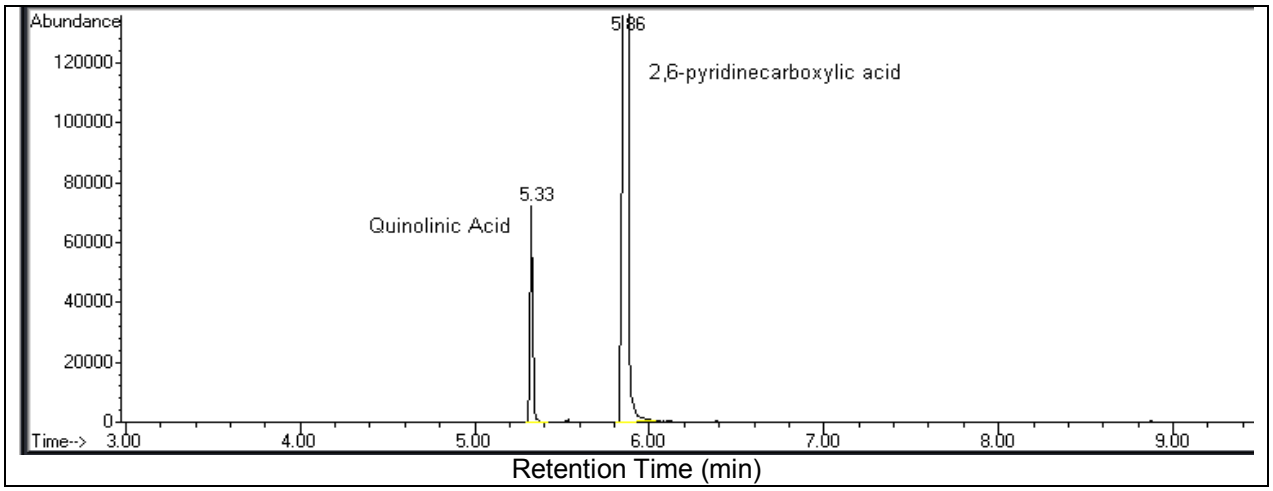

Figure 2.8 Typical chromatogram obtained for quinolinic acid and internal standard (nCI)

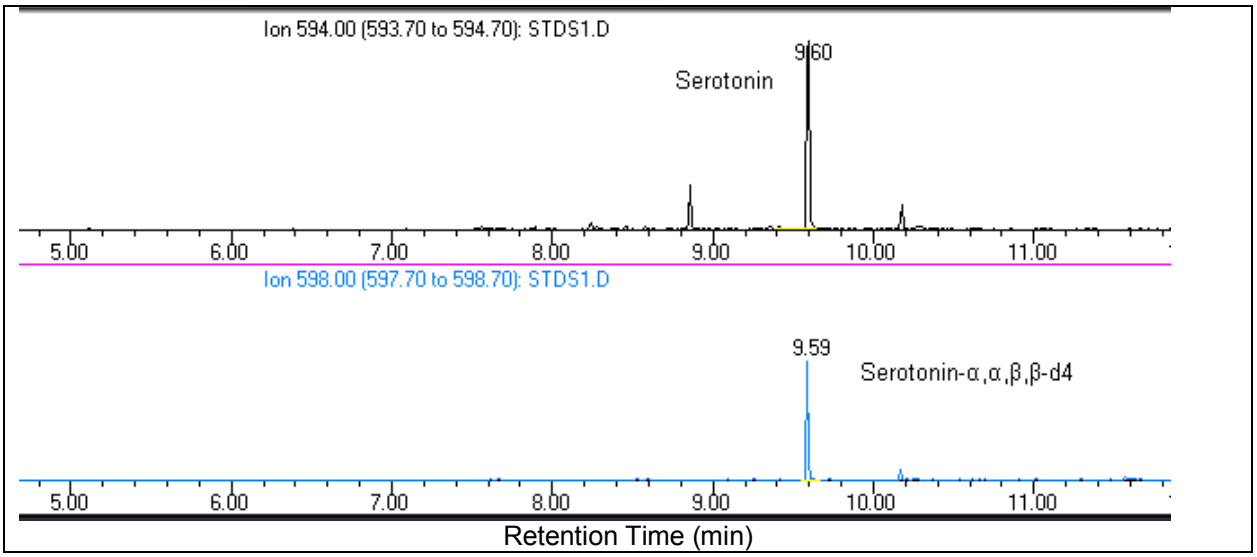

Figure 2.9 Typical chromatogram obtained for serotonin and internal standard (nCI)

The sample preparation procedure plays a vital role to complement the analysis of an analyte. Despite the sensitivity and selectivity of the analytical instrument, inappropriate sample preparation could result in the actual analyte not being detected if at all. Each step for the sample preparation procedure is important with regards to the final determination of the analyte. Extraction ensures anhydrous transfer of analyte. Freeze-drying plays a similar role. Addition of salt (salting out) facilitates the transfer of analyte from one phase to the other via solvent-solvent extraction. Thorough vortexing and centrifuging ensures adequate mixing and transference of analyte. It is important to dry the extraction solvent under nitrogen gas to enable reactivity of the derivatizing agent and prevent volume dilution.

2.6.2.4 GC-MS Instrumentation setup for analysis

Once the method for the sample preparation steps is in order, the derivatized sample can be analyzed using GC-MS. It is however imperative to ensure that the functionality and setup of the instrumentation is appropriate for the analysis. Proper instrument setup will enable detection of the analyte/s with acceptable selectivity and sensitivity. One of the aims for establishing an ideal method design is to maximize the sensitivity and optimize the selectivity of the analysis. Modification of certain instrument parameters can help to

achieve this. Two sets of instrument parameters can be modified and they are GC dependent and MS dependent. GC parameters will opt for good peak resolution with apt separation of peaks in minimum retention time. MS parameters are scanning of the mass spectrum and monitoring of selected ions. There is also proper MS tuning and choice of tuning parameters such as source temperature and as well as choice and reasons for use of a specific ionization source. The parameters which were modified, adjusted and optimized for this analysis study will be addressed in the following sections.

Instrument parameters which can be optimized are: temperature programming; injection volume, inlet mode/type (split or splitless); inlet temperature; column flow rate. There are also minor components such as ferrules and port liner seals which contribute to the functionality of the specific parameter.

Temperature Programming:

It was, for the purpose of this study, noticed that the retention times of the individual analyte peaks increased upon decreasing the oven temperature and the opposite was noted when the initial oven temperature was increased. Appropriate ramping of the oven temperature at specific rates enabled separation and elution of the analyte peaks. During preliminary instrument setup, a basic temperature program was established: initial oven temperature of 50^0C, ramped at 10^0C to 300^0C. Thereafter the program was modified to a suitable program (See table 2.7)

Table 2.7 Temperature program used for optimum separation of analytes

Initial Temperature	**Ramps**		**Final Time**
	Rate	**Final Temperature**	
80^0C for 2 min	20^0C	210^0C	1.00 min
	30^0C	300^0C	2.00 min

Table 2.8 Table indicating injection parameters used

Parameter	**Value/Indication**
Sample Washes	0
Sample Pumps	2
Injection Volume	1.0 microliter
Syringe Size	10.0 microliters
Nanoliter Adapter	Off
Post Injection Methanol Washes	2
Post Injection Dichloromethane Washes	2
Viscosity Delay	0 seconds
Plunger Speed	Fast
Pre Injection Dwell	0 minutes
Post Injection Dwell	0 minutes

Table 2.9 Table indicating front inlet parameters used

Parameter	**Value/indication**
Mode	Splitless
Initial Temperature	280^0C
Pressure	177.2 kPa
Purge Flow	50.0 mL/min
Purge Time	0.60 min
Total Flow	54.7 mL/min
Gas Saver	On/Off
Gas Type	Helium

The thermal auxiliary MSD transfer line heater was set to a temperature of 280^0C at constant timing with no ramping used. The MS quad and MS source were set to temperatures of 150^0C (of maximum 200^0C) and 240^0C (of maximum 300^0C) according to the tune program used for negative CI as well as EI.

The next chapter will give the details, explanation and results for the validation of the methods use for the quantification of the analytes of the tryptophan and kynurenine pathways.

2.7 References

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Chapter 3
Method Validation

This chapter deals with the validation of the developed methods for the quantification of tryptophan and metabolites by means of GC-MS. A brief overview of the topic will be given, followed by the presentation of the steps and results obtained for the validation procedure. A question to pose is what the purpose of method validation is and why it is necessary for a newly developed method?

Answers to the above questions will be handled along the following lines. No analytical method is perfect, i.e., free from any errors. In this regard three types of errors are known to occur during analytical analyses. These are gross, systematic and random errors. The purpose of validation is to identify the sources and quantify the potential errors in the presenting method [1]. The reliability of the analytical data is also of judgment whereby unreliable results can be contested in court or lead to the wrong treatment of the patient in terms of forensic and clinical toxicology respectively [2]. Likewise this applies to other fields of study as well. Thus it is important to assure reliability of the method by providing validation results to confirm that a method is relatively free from the potential errors and meets the relevant criteria for optimum performance. Statistical analysis of the validation data is of importance in order to demonstrate and measure the degree of imprecision and inaccuracy of the method [1].

Gross errors may include sample contamination, instrument failure or incorrect sample preparation. These errors are easily identifiable and indicate that the experiment should be stopped and the results disregarded. Systematic errors affect the accuracy of the method and include consistent errors such as those of the analyst, instrument calibration and reagent introduction, thereby causing results to all be erroneous in the same sense [3]. Bias and accuracy are determined to relate the systematic error. These are defined in terms of calculated formulas and will be defined later. Uncontrollable variables in the measurement procedure give rise to random errors. This pertains to categories of distribution of data over a sample mean as falling on either side of a Gaussian distribution

curve and in terms of standard deviations and so forth [2,3]. No method can be completely free from random errors. However, these errors can be identified with the determination and aid of statistical analysis of the data. Also before validation steps are followed it is important to check the measuring instruments, such as pipettes and scales, to ensure proper performance and analyses. The following paragraphs and details illustrate checking and calibration of the volumetric pipettes.

It is imperative that the measuring tools and equipment used for experimental preparation and method validation be of highest quality to ensure reliable and standardized performance throughout their use. Therefore regular calibration and quality check of the equipment play a vital role in the data to be obtained. Pipettes, mass scales, measuring cylinders, aliquot vials and glassware equipment were used for preparation of samples. Pipettes and scales were regularly calibrated to ensure accuracy and uniformity of measurements. Variations in pipette action can occur as well thus decreasing uniformity in the volume of substance delivered per an action. Intra and extra-user variability needs to be taken into account, as well as within and between (day to day) variations in pipette action.

The within and between pipette action can be tested by a linearity experiment whereby a response to volume is directly proportional to one another. In this case the volume delivered per a pipette action can be correlated with the response as measured on a mass scale given that 1 litre volume is equivalent to 1 kilogram in mass. Pipette precision or repeatability can be determined by repeating pipette action for a single volume and thereby working out the co-efficient of variation (CV) on the measurements. The CV can give an indication about the degree of variation between the repeat measurements whereby a low CV value will be most ideal.

The experiment was designed as follows. The pipette was set to deliver a volume of 20µl. The volumes 20, 60,100, 140, 180 µl were used as the independent variable for linear calibration. The response as measured on the mass scale for each volume was the dependant variable. To obtain volumes of 20 to 180µl, the numbers of 20µl pipette

actions were 1, 3, 5, 7, and 9 respectively. Quality controls of 40 and 160µl were repeated and the measured response was back extrapolated from the calibration curve to determine the difference between the expected (theoretical expected) and actual (accuracy as calculated from curve), which is expressed as bias. Repeat measurements were performed on consecutive days to establish within and between day variations in pipette action.

The results for pipette precision are presented in the following tables and figures.

Table 3a Data Results for n = 5 calibration sets

Volume (µl)	Mass (g)	Mass (g)	Mass (g)	Mass (g)	Mass (g)		Average
0	0	0	0	0	0		**0**
20	0.0206	0.0202	0.0204	0.0207	0.0206		**0.0205**
60	0.0623	0.0616	0.0614	0.0623	0.062		**0.06192**
100	0.1044	0.1035	0.1029	0.1039	0.10154		**0.103248**
140	0.1457	0.1456	0.144	0.1449	0.1454		**0.14512**
180	0.1869	0.1872	0.1858	0.1855	0.1883		**0.18674**

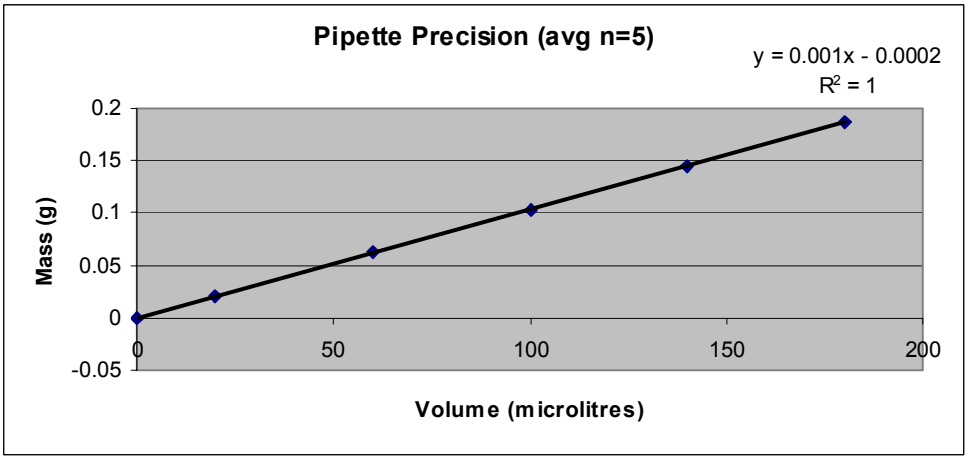

Figure 3a Linear calibration curve illustrating mass versus volume for average of n = 5 data sets

Table 3b Data Results for n=12 data sets. CVs < 1 obtained for each standard volume

Volume	1	2	3	4	5	6	7	8
0	0	0	0	0	0	0	0	0
20	0.0208	0.0208	0.0208	0.0207	0.0206	0.0208	0.0207	0.0207
60	0.063	0.063	0.0627	0.0625	0.0627	0.0622	0.063	0.0632
100	0.1053	0.1046	0.1035	0.1038	0.1043	0.105	0.1051	0.105
140	0.1475	0.1467	0.1467	0.1454	0.1471	0.1473	0.1475	0.147
180	0.189	0.1879	0.1891	0.1882	0.1895	0.1888	0.1897	0.1895

Volume	9	10	11	12	Average	Standard deviation	CV
0	0	0	0	0	**0**	**0**	**0**
20	0.0208	0.0208	0.021	0.0208	**0.020775**	**9.65E-05**	**0.464649**
60	0.0624	0.0626	0.0634	0.063	**0.062808**	**0.00035**	**0.557595**
100	0.1052	0.1047	0.1056	0.1052	**0.104775**	**0.00063**	**0.601054**
140	0.1475	0.1472	0.1474	0.1477	**0.147083**	**0.000618**	**0.419945**
180	0.1893	0.1891	0.1902	0.1898	**0.189175**	**0.000654**	**0.345624**

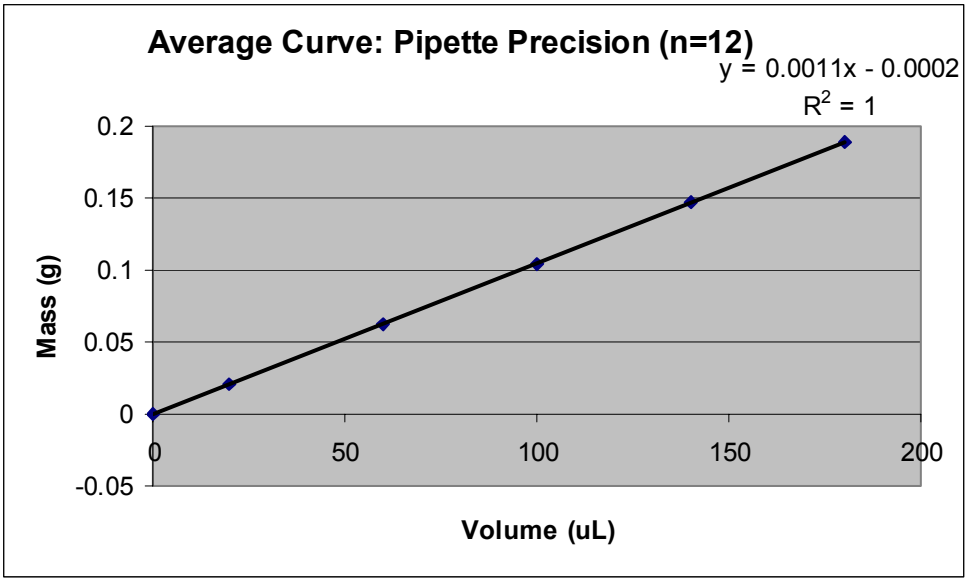

Figure 3b Average linear calibration curve for mass versus volume (n=12). Plotted data obtained from Table 3b

Table 3c DAY 1 - Data Results for repeat quality controls: volumes 40 and 160 µl. Data for actual determined from Figure 3b

Volume	1	2	3	4	5	6	7	8
40 (Expected)	0.0418	0.0423	0.0422	0.0423	0.0425	0.0424	0.0423	0.0423
Actual (Calculated)	38.18182	38.63636	38.54545	38.63636	38.81818	38.72727	38.63636	38.63636
Bias	4.545455	3.409091	3.636364	3.409091	2.954545	3.181818	3.409091	3.409091

Volume	9	10	11	12	Average	SD	CV
40 (Expected)	0.0424	0.0427	0.0426	0.0423	**0.042342**	**0.000223**	**0.527701**
Actual (Calculated)	38.72727	39	38.90909	38.63636	**38.67424**	**0.203125**	**0.52522**
Bias	3.181818	2.5	2.727273	3.409091	**3.314394**		

Volume	1	2	3	4	5	6	7	8
160 (Expected)	0.1693	0.1691	0.1694	0.1693	0.1692	0.1699	0.1698	0.1699
Calc	154.0909	153.9091	154.1818	154.0909	154	154.6364	154.5455	154.6364
Bias	3.693182	3.806818	3.636364	3.693182	3.75	3.352273	3.409091	3.352273

Volume	9	10	11	12	Average	SD	CV
160 (Expected)	0.1698	0.1699	0.17	0.1696	**0.1696**	**0.000322**	**0.189815**
Calc	154.5455	154.6364	154.7273	154.3636	**154.3636**	**0.29266**	**0.189591**
Bias	3.409091	3.352273	3.295455	3.522727	**3.522727**		

Table 3d DAY 2 - Data Results for repeat quality controls: volumes 40 and 160 µl

Volume	1	2	3	4	5	Average	SD	CV
0	0	0	0	0	0	**0**	**0**	
20	0.0205	0.0206	0.0207	0.0208	0.0208	**0.02068**	**0.00013**	**0.630484**
60	0.0622	0.0619	0.0623	0.0627	0.0622	**0.06226**	**0.000288**	**0.462732**
100	0.1037	0.1037	0.1038	0.1035	0.1039	**0.10372**	**0.000148**	**0.143004**
140	0.1454	0.145	0.1458	0.1454	0.1453	**0.14538**	**0.000286**	**0.196971**
180	0.1872	0.187	0.1873	0.1874	0.1873	**0.18724**	**0.000152**	**0.080996**

Volume	1	2	3	4	5	6	7	8
40 (Expected)	0.0418	0.0417	0.0418	0.0417	0.0417	0.0415	0.0418	0.0416
Actual (Calculated)	41.9	41.8	41.9	41.8	41.8	41.6	41.9	41.7
Bias	-4.75	-4.5	-4.75	-4.5	-4.5	-4	-4.75	-4.25

Volume	9	10	11	12	Average	SD	CV
40 (Expected)	0.0415	0.0417	0.0418	0.0416	**0.041683**	**0.000111**	**0.267407**
Actual (Calculated)	41.6	41.8	41.9	41.7	**41.78**	**0.113529**	**0.271731**
Bias	-4	-4.5	-4.75	-4.25	**-4.45**		

Volume	1	2	3	4	5	6	7	8
160 (Expected)	0.1665	0.1664	0.166	0.1664	0.1667	0.1665	0.1668	0.1662
Calc	166.6	166.5	166.1	166.5	166.8	166.6	166.9	166.3
Bias	-4.125	-4.0625	-3.8125	-4.0625	-4.25	-4.125	-4.3125	-3.9375

Volume	9	10	11	12	Average	SD	CV
160 (Expected)	0.1657	0.1667	0.1667	0.1667	**0.166442**	**0.000332**	**0.199198**
Calc	165.8	166.8	166.8	166.8	**166.49**	**0.341402**	**0.205059**
Bias	-3.625	-4.25	-4.25	-4.25	**-4.05625**		

Table 3e DAY 3 - Data Results for repeat quality controls: volumes 40 and 160 µl

Volume	1	2	3	Average	SD	CV
0	0	0	0	**0**	**0**	
20	0.0204	0.0206	0.0205	**0.0205**	**0.0001**	**0.487805**
60	0.0613	0.0615	0.0616	**0.061467**	**0.000153**	**0.248513**
100	0.1026	0.1028	0.1024	**0.1026**	**0.0002**	**0.194932**
140	0.1446	0.1442	0.1437	**0.144167**	**0.000451**	**0.31278**
180	0.1857	0.1859	0.1841	**0.185233**	**0.000987**	**0.532613**

Volume	1	2	3	4	5	6	7	8
40 (Expected)	0.0411	0.0412	0.0411	0.0415	0.0417	0.0417	0.0413	0.0413
Actual (Calculated)	41.2	41.3	41.2	41.6	41.8	41.8	41.4	41.4
Bias	-3	-3.25	-3	-4	-4.5	-4.5	-3.5	-3.5

Volume	10	11	12	Average	SD	CV
40 (Expected)	0.041	0.0412	0.0414	**0.041308**	**0.000227**	**0.550663**
Actual (Calculated)	41.1	41.3	41.5	**41.40833**	**0.22747**	**0.549333**
Bias	-2.75	-3.25	-3.75	**-3.52083**	**0.568674**	

Volume	1	2	3	4	5	6	7	8
160 (Expected)	0.1641	0.1644	0.1639	0.166	0.1658	0.1654	0.1651	0.1658
Calc	164.2	164.5	164	166.1	165.9	165.5	165.2	165.9
Bias	-2.625	-2.8125	-2.5	-3.8125	-3.6875	-3.4375	-3.25	-3.6875

Volume	9	10	11	12	Average	SD	CV
160 (Expected)	0.1655	0.1654	0.1661	0.1652	**0.165225**	**0.000731**	**0.442597**
Calc	165.6	165.5	166.2	165.3	**165.325**	**0.731282**	**0.44233**
Bias	-3.5	-3.4375	-3.875	-3.3125	**-3.32812**	**0.457051**	

Table 3f CVs for within and between day

QC	Within Day CV				Between Day CV
	DAY1	**DAY2**	**DAY3**		
40	0.52522	0.271731	0.549333		**0.448761**
60	0.189591	0.205059	0.44233		**0.278993**

Relatively low CVs were obtained for the within and between day pipette action. This indicates that there was a relatively low degree of variation and bias between the pipette actions. These data also coincide with the calibration of the pipettes used as well as the indication for optimal performance of the measuring instrument.

3.1 Method Validation Parameters

There are certain parameters that are necessary and should be included in the method validation section. These include selectivity, calibration model, stability, accuracy, limit of quantification and detection and recovery [1,3]. In this study these parameters were evaluated quantitatively with statistical inference of the data. Furthermore optimization experiments were performed to determine the best suitable method procedure to be followed in order to obtain the best possible results for the validation and quantification.

A definition of each of the parameters is given below, followed by the procedure undertaken and results obtained. Validation results are presented for each analyte namely tryptophan, kynurenine, quinolinic acid and serotonin. Determination of results from each analyte was in accordance to the developed GC-MS method using the appropriate sample matrix and concentration range.

3.1.1 Selectivity

A definition of selectivity: "… is the ability of the bioanalytical method to measure unequivocally and to differentiate the analyte(s) in the presence of components, which may be expected to be present" [4]. Such components might be metabolites, impurities, matrix components, etc. Matrix components play a major role in validation and are important as analyses are performed by spiking standards in the relevant matrix. It is thus imperative to analyse and distinguish the analytes from the matrix components (proteins, fats, cells etc.).

A way to determine selectivity is to demonstrate the lack of response in blank matrix [3,4,5]. With regards to this method mass spectrometry was used as the detector. It is therefore straightforward to distinguish the analyte in terms of its "unique" mass spectrum obtained on the electron impact (EI) source. Each analyte is differentiated with respect to its chromatographic peak (retention time) and MS helps to distinguish between overlapping peaks.

Each analyte (substance of interest being analysed) showed a unique mass spectrum on the electron impact (EI) ionization source (see figures 3.1 to 3.4). In order to establish selectivity, each analyte was analyzed individually in scan mode to obtain a complete mass spectrum fragmentation pattern of the analyte. Blank matrix samples were analyzed and selectivity was illustrated by a lack of response (absence of specific ions for each analyte) at the relevant chromatographic retention times. Hence, selectivity is also demonstrated when analytes are analyzed simultaneously in one chromatographic analysis. Analytes are distinguished from each other according to their mass spectrum and retention time. Selectivity is important when closely eluting peaks (overlapping) occur and it is also important to demonstrate that no coinciding ions appear at the same retention times. Thus by selecting the most appropriate and abundant ions during selective ion monitoring (SIM), the minor coinciding ions can be eliminated and selectivity thereupon improved. Structural information of the derivatized analyte is obtained from the electron impact spectra. The fragmentation pattern obtained from using the EI source is exceptional in a sense that the actual structure of the derivatized analyte can be easily derived. In Table 3.1 the selected ions chosen for quantification as well as the respective internal standard ions are given. Figures 3.1, 3.2, 3.3 and 3.4 show the electron impact (EI) mass spectrum for tryptophan, kynurenine, quinolinic acid and serotonin respectively.

Table 3.1 Selected ions chosen for quantification with internal standard ions in brackets

Analyte	Electron Impact	Negative Chemical Ionization
Tryptophan	276 (280)	608 (612)
Kynurenine	266 (152)	454 (504)
Serotonin	451 (454)	594 (598)
Quinolinic Acid	282 (282)	431 (431)

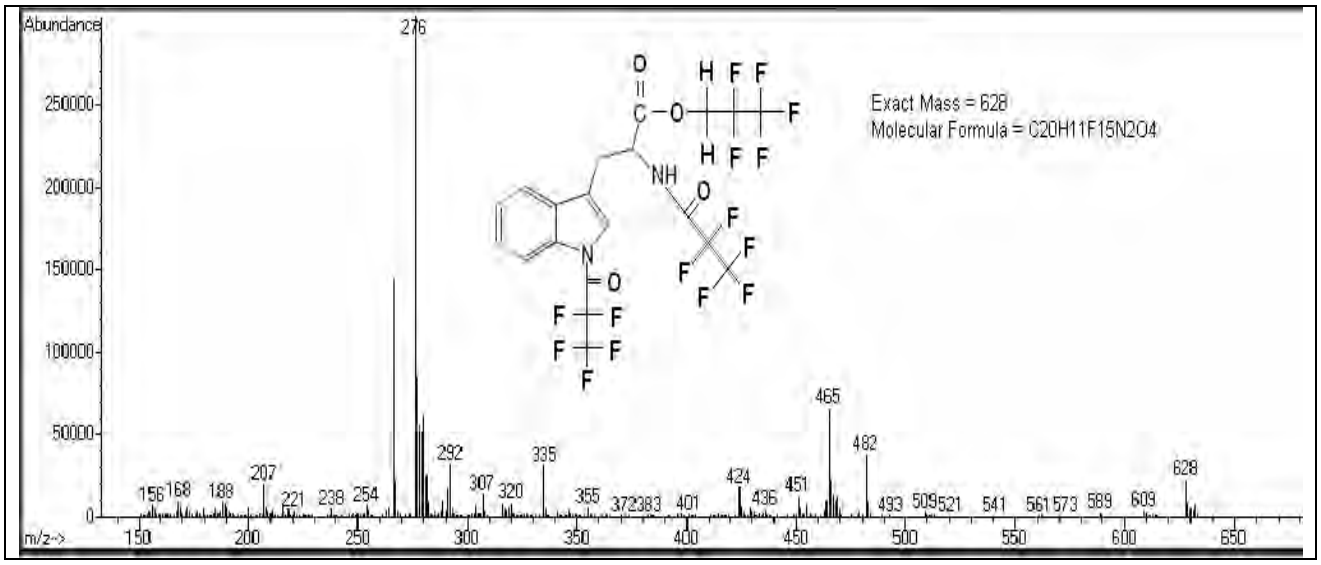

Figure 3.1 Electron Impact (EI) mass spectrum for tryptophan

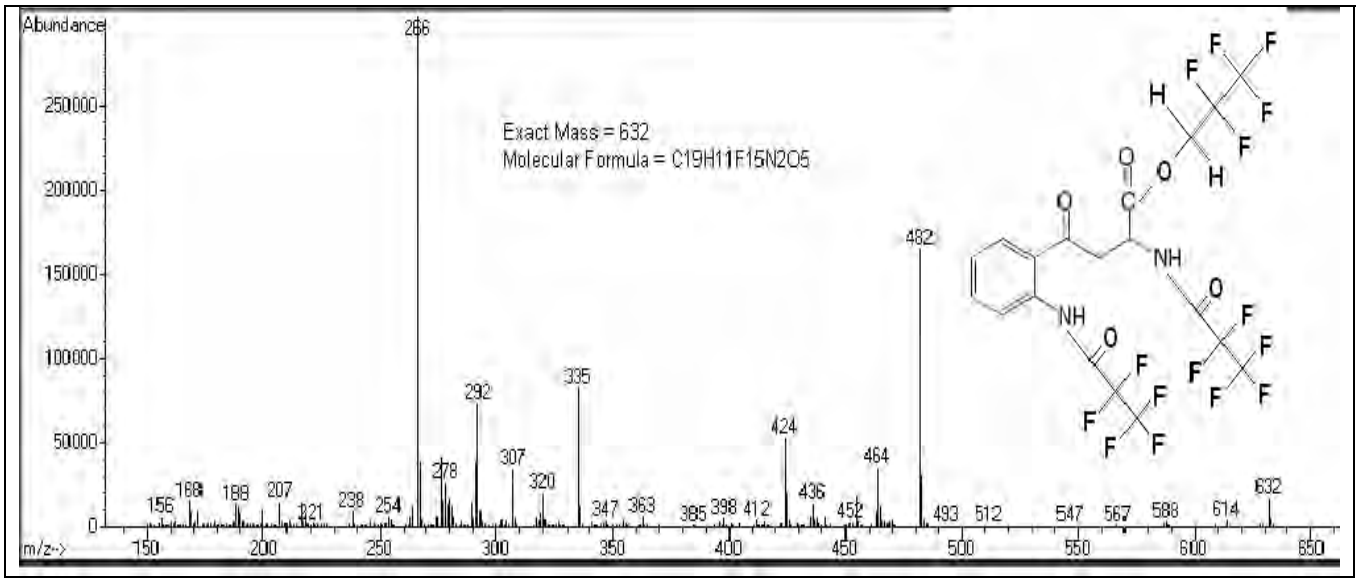

Figure 3.2 Electron Impact (EI) mass spectrum for kynurenine

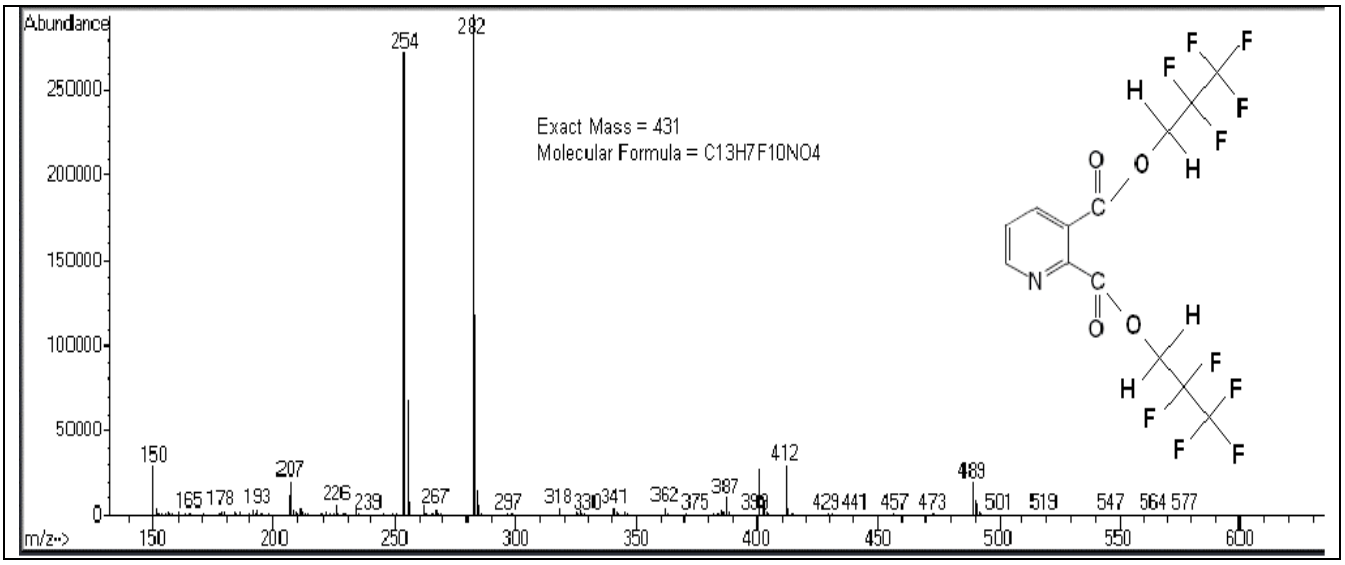

Figure 3.3 Electron Impact (EI) mass spectrum for quinolinic acid

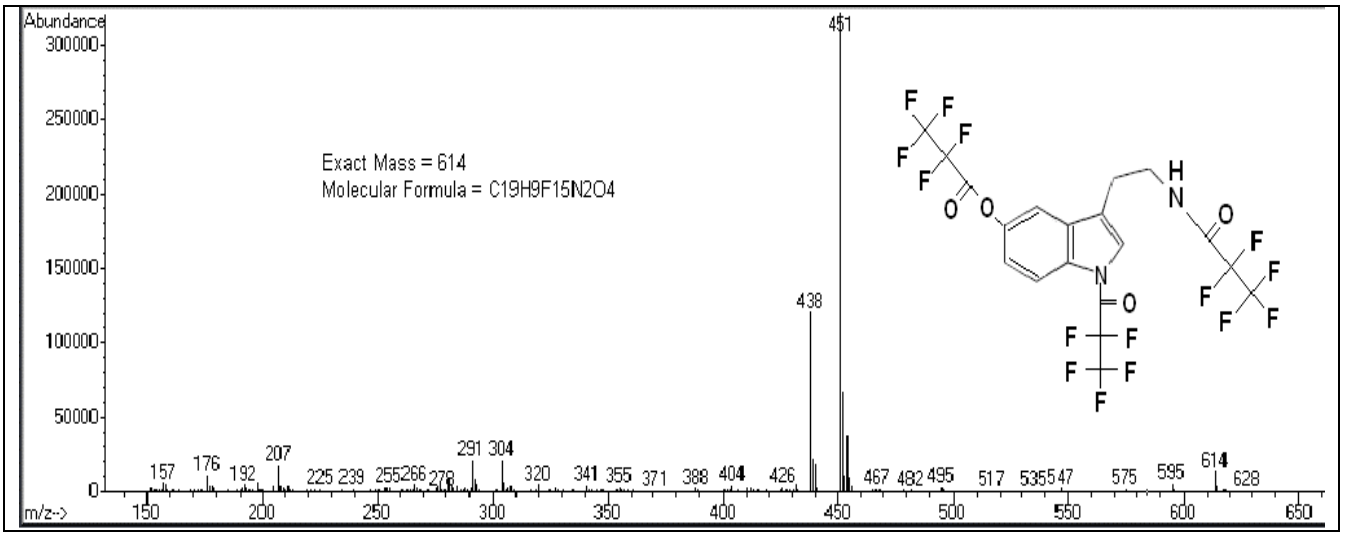

Figure 3.4 Electron Impact (EI) mass spectrum for serotonin

3.1.2 Calibration (Linearity)

Reliable quantification necessitates the application of appropriate calibration models thereby requiring the validated investigation of the relationship between the concentration of the analyte and the response to be obtained from the detector [2]. For this study working concentration standards for each analyte were prepared by spiking the appropriate matrix with the relevant standards. This gave rise to standard solutions

(calibration samples) of equally spaced increments in concentration. The samples were analyzed via the GC-MS or HPLC method and calibration curves (standard curves) were drawn up showing plots of response versus the corresponding concentrations of the analytes.

Owing to the use of internal standards the relative response (ratio of analyte response divided by internal standard response: peak area/height ratios) versus the expected concentration was plotted graphically. The concentration (volume amount) of internal standard must remain constant for each standard spike. By definition: internal standards are compounds added, in equal concentrations, to all standards and test samples. The internal standard is beneficial in the following instances: volume losses, variations in extraction efficiencies, derivatization conditions and pre-treatment of samples [6]. The internal standard should have similar (as closely related) properties to the complementary analyte of interest. Hence, the chemical structures should be similar to match chromatographic behavior and mass spectra for both analyte and internal standard. Likewise the physical properties of the two should be comparable such that specific conditions affect both to the same degree. Due to the light sensitivity and environmental factors influencing serotonin and tryptophan (indole species), the use of isotope labeled internal standards showed excellent compensatory measures. These labeled standards differ from the original analytes by a few mass units via the addition of deuterium subunits. Thus the chemical and physical properties are almost identical. For other analytes, surrogate analogous internal standards were used. These have closely related chemical and physical properties to the original analytes and are sufficient to enable compensatory measures.

Calibration samples should be prepared in blank matrix and the respective concentrations should span over the whole calibration range [2]. There is however an uncertainty on the number of concentration levels and repeats that should be used for drawing up calibration curves and there is a disagreement according to the varying recommendations in the literature [2,4,7-9]. Through personal gain of knowledge from colleagues and over the duration of the study, it was decided to employ five concentration levels as well as a

blank. Thus the calibration curves consisted of 6 calibration points covering a wide range which was expected to fall within and outside of the biological range for each analyte.

Table 3.2 Calibration data for Quinolinic Acid using MTBSTFA derivatization

Concentration (µM)	1	2	3	4	5	Average
0	0.032922	0.001308	0.043806	0.065639	0.048537	0.038442
0.1	0.035577	0.001032	0.042377	0.071553	0.049239	0.039956
2.6	0.127031	0.002876	0.085871	0.146823	0.110634	0.094647
5.1	0.160941	0.004751	0.15527	0.218113	0.185565	0.144928
7.6	0.205467	0.006292	0.239649	0.331317	0.27544	0.211633
10.1	0.275303	0.007562	0.292633	0.420389	0.329641	0.265105

As was discussed in the methods chapter of this write up, one of the derivatizing agents tested during this study was MTBSTFA. Judging from the chemical structures of the analytes analysed, quinolinic acid has the simplest structure with a single type of functional group (carboxylic). The preliminary method design for GC-MS (Electron Impact) detection of analytes was thus performed on this analyte. Once the method was in order a calibration model was performed to establish linearity as well as compensatory measures of its internal standard 2,6 pyridine dicarboxylic acid. Linearity was first verified by spiking concentration standards in water before using the actual matrix (plasma). Results are presented in table 3.2 and figure 3.5. Examination of the calibration curve shows that good linearity was achieved. All calibration points fall within the straight line and the R^2 correlation co-efficient is above 0.99.

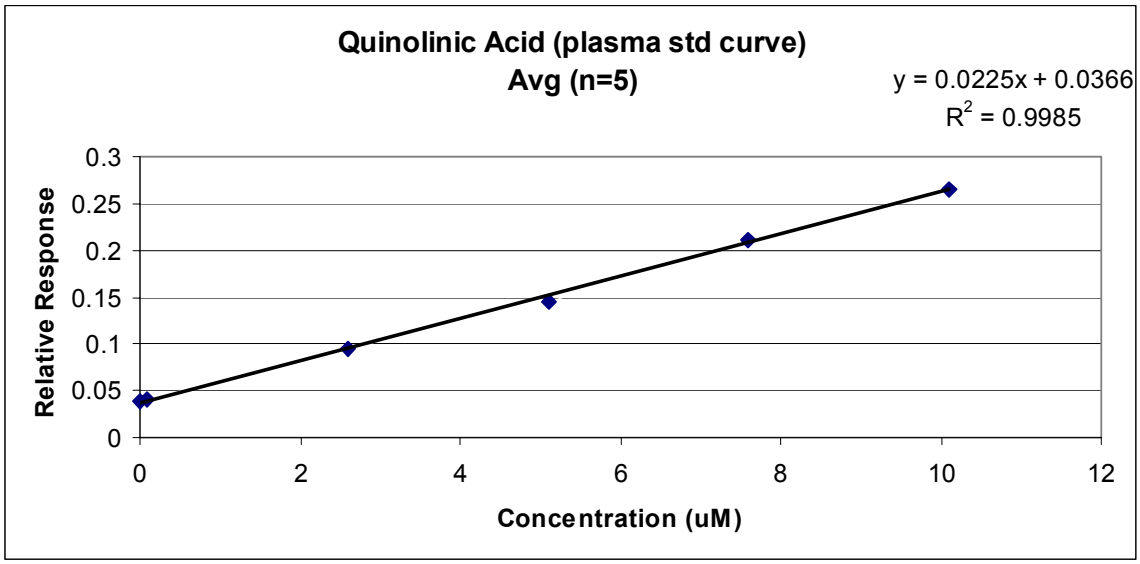

Figure 3.5 Average calibration curve illustrating relative response versus concentration for the analysis of quinolinic acid using MTBSTFA derivatization and EI detection. Data obtained from Table 3.2

In contrast to quinolinic acid, tryptophan, kynurenine and serotonin have more than one type of functional group in their chemical structure. As motivated previously it is necessary to derivatize all of the functional groups. PFPA and PFPOH were chosen because when combined both of the agents are able to derivatize the respective functional groups of all the analytes of concern for this study (see figures 3.1 to 3.4 for derivatized chemical structures). Calibration curves were drawn up for water spikes and matrix spikes using both EI and NCI detection. The following tables and figures illustrate the calibration data and calibration curves for the four analytes.

Table 3.3 Calibration data for analysis of tryptophan using EI detection

Concentration (µM)	Ion 276	Ion 280		Relative Response
0	0	17562		0
5	4664	22940		0.203313
15	12754	19916		0.64039
25	16739	16613		1.007584
35	18078	13152		1.374544
45	52659	27324		1.927207

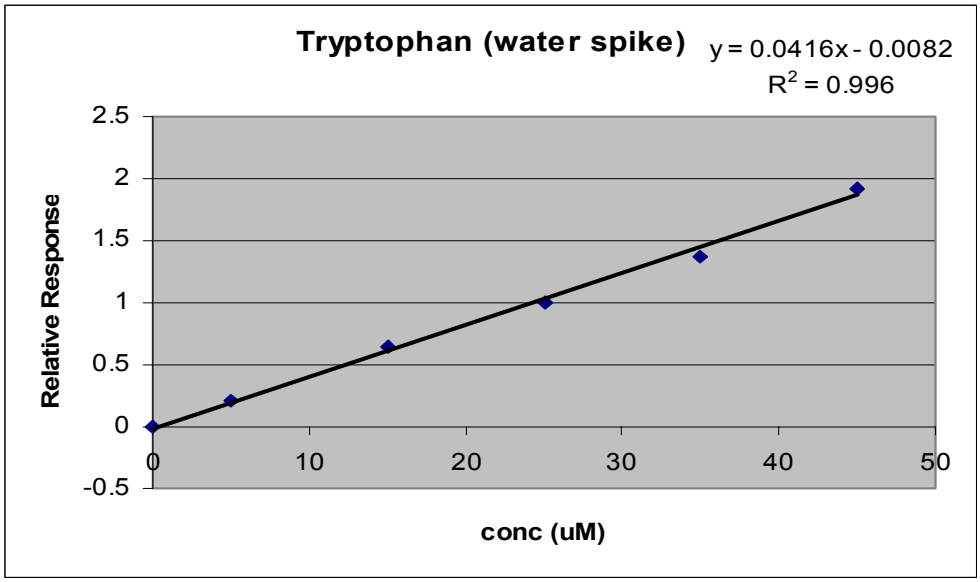

Figure 3.6 Standard calibration curve illustrating relative response versus concentration for tryptophan water spikes using EI detection. Data obtained from Table 3.3

Table 3.4 Calibration data for analysis of tryptophan using nCI detection

Concentration (µM)	608	612		Relative Response
0	0	567198		0
5	79692	861295		0.092526
15	169316	694436		0.243818
25	261762	593141		0.441315
35	248138	430254		0.576724
45	400803	507947		0.789065

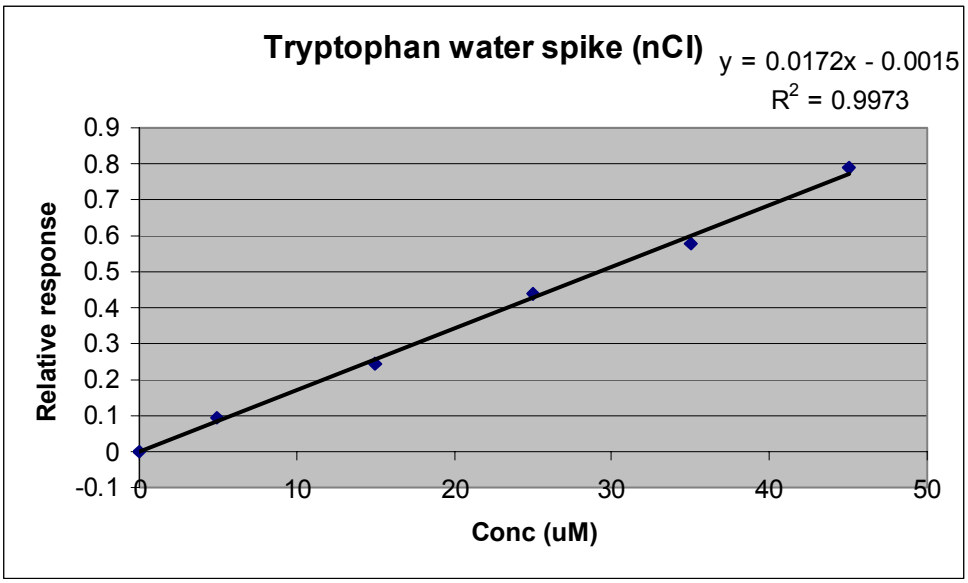

Figure 3.7 Standard calibration curve illustrating relative response versus concentration for tryptophan water spikes using nCI detection. Data obtained from Table 3.4

Table 3.5 Calibration data for analysis of tryptophan in plasma using nCI detection

Concentration (µM)	608	612	Relative Response
0	1197960	1598108	0.749611
5	2053980	1786230	1.149897
15	2439601	1412703	1.726903
25	5774410	2609203	2.213093
35	3444780	1278043	2.695355
45	6229539	1855997	3.356438

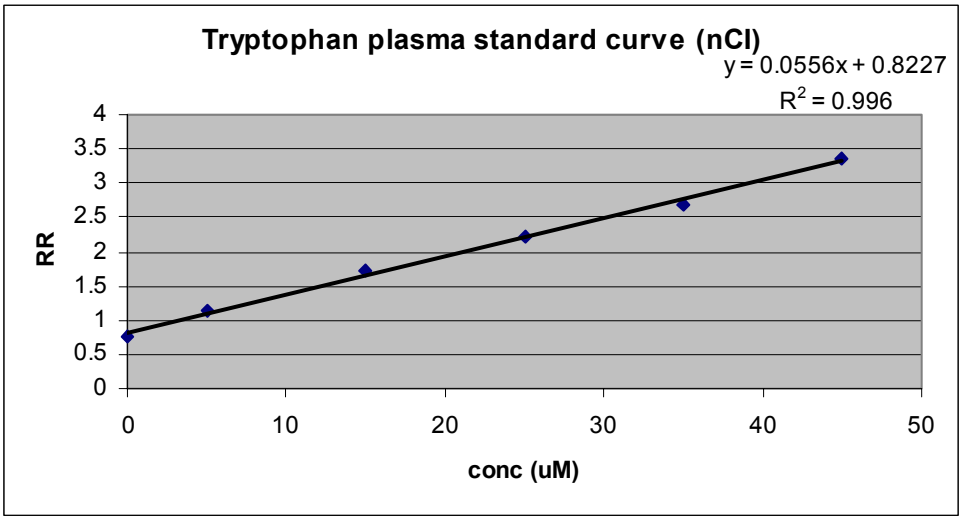

Figure 3.8 Standard calibration curve illustrating relative response versus concentration for tryptophan plasma spikes using nCI detection. Data obtained from Table 3.5

Table 3.6 Average calibration data for analysis of tryptophan in plasma using nCI detection

Concentration (µM)	1	2	3	4	5	Average
0	0.74878	0.79071177	0.77015004	0.76988	0.749611	0.765827
5	1.051795	1.13490072	1.08936131	1.09201	1.149897	1.103593
15	1.530192	1.50509413	1.4253026	1.48686	1.726903	1.53487
25	1.962846	2.07389153	1.9499496	1.99556	2.213093	2.039068
35	2.57042	2.50812961	2.70711919	2.59522	2.695355	2.615249
45	3.081536	3.48642401	3.06790979	3.21195	3.356438	3.240851

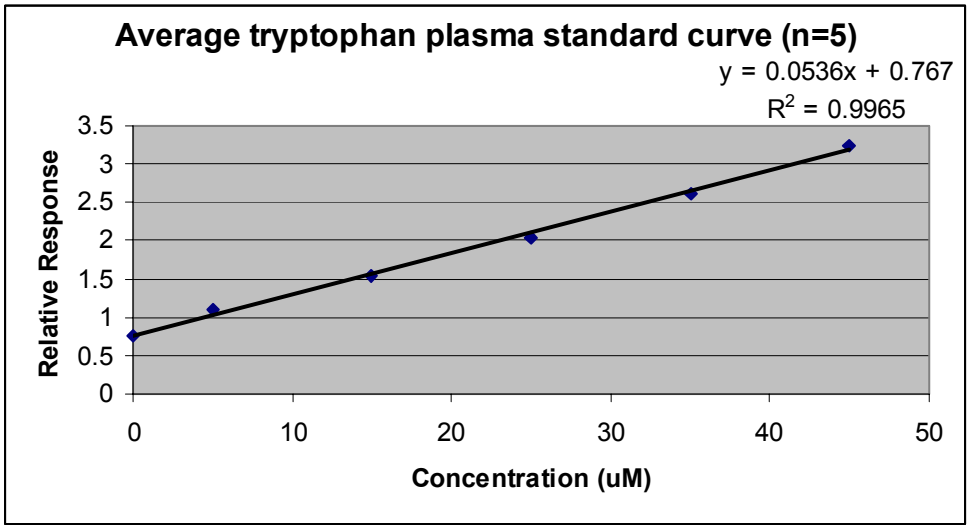

Figure 3.9 Average standard calibration curve illustrating relative response versus concentration for tryptophan plasma spikes using nCI detection. Data obtained from Table 3.6

Evaluation of the correlation coefficients of the standard curves shows that good linearity is achieved (closeness to +1 for a positive gradient), however this acceptance of linearity is insufficient due to a lack of statistical analysis [2,5]. Thus the statistical significance of the correlation coefficient (R^2) is determined by using a paired t-test (data from Table 3.7) and hypothesis (H_0: $\mu = 1$; H_1: $\mu < 1$). The p-value obtained is 0.0767909 (for $p<0.01$ considered as statistically significant) and indicates that there is no significant difference between the obtained correlation coefficient values and 1. Accordingly the standard errors of the slope (m) and intercept (c) can be given together with their respective calculated confidence intervals. Similarly, the same statistical analysis of linearity data was performed on the all other linearity data obtained.

Table 3.7 Linearity data showing individual equations and R^2 for the average plasma curve (see figure 3.9)

Number	Linear equation (y=mx + c)	Correlation Coefficient
1	Y = 0.0512x + 0.7574	R^2 = 0.998
2	Y = 0.056x + 0.7493	R^2 = 0.9768
3	Y = 0.052x + 0.7526	R^2 = 0.9887
4	Y = 0.0531x + 0.7531	R^2 = 0.9949
5	Y = 0.0556x + 0.8227	R^2 = 0.996

The confidence intervals (CI) for the gradient (m) and the intercept (c) are determined by the equations $CI(m)=m \pm t.s_m$ and $CI(c)= c \pm t.s_c$ respectively. Thus a 95% confidence interval for μ is given by (mean $\pm t_{n-1,\ 0.025}\ s/\sqrt{n}$). The 95% confidence intervals for the gradient and intercept for the data set from table 4.7 are 0.05358 ± 0.00265743 and 0.76702 ± 0.038807 respectively. The relative standard deviations (RSD), also know as the coefficient of variance (CV), for m and c are 0.03995074% and 4.075387% respectively.

Table 3.8 Average calibration data for analysis of serotonin in whole blood using nCI detection

	1	2	3	4	5	6	Average
0	0	0	0	0	0	0	0
0.42	0.150468	0.113538	0.223683	0.219511	0.172713	0.274585	0.192416
0.84	0.355454	0.236908	0.397232	0.227773	0.272753	0.270547	0.293445
1.68	0.685691	0.443107	0.808469	0.43937	0.555045	0.547961	0.57994
3.36	1.338018	0.927987	1.546837	0.944399	1.142872	1.164544	1.177443
6.72	2.945401	1.897808	3.363594	1.76858	2.302936	2.385159	2.443913

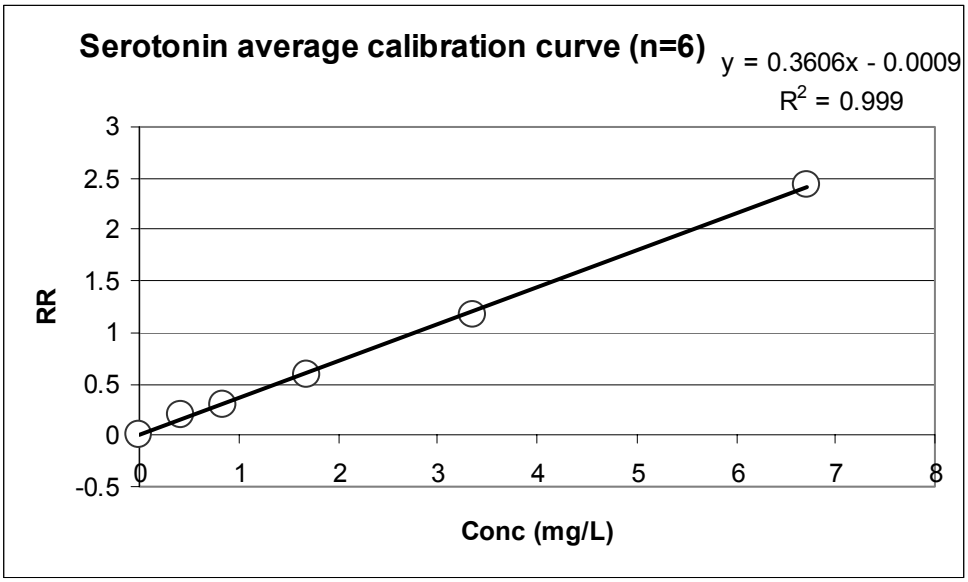

Figure 3.10 Average standard calibration curve illustrating relative response versus concentration for serotonin whole blood spikes using nCI detection. Data obtained from Table 3.8

Table 3.9 Linearity data showing individual equations and R^2 for the average whole blood curve (see figure 3.10)

Number	Linear equation (y=mx + c)	Correlation Coefficient
1	y = 0.0704x - 0.0345	R^2 = 0.9979
2	y = 0.0454x - 0.0043	R^2 = 0.9994
3	y = 0.08x - 0.0202	R^2 = 0.9982
4	y = 0.0418x + 0.0375	R^2 = 0.9953
5	y = 0.0551x - 0.0005	R^2 = 0.9996
6	y = 0.0563x + 0.0167	R^2 = 0.9953

The p-value calculated for the correlation coefficient (R^2) from the data set (table 3.9) is 0.028289 ($p<0.01$). Therefore there is no significant difference between the obtained correlation coefficients and 1. The 95% ($\alpha = 0.025$) confidence intervals for the gradient (m) and the intercept (c) are 0.058167 ± 0.015355 and 0.01895 ± 0.015901, respectively.

Table 3.10 Average calibration data for analysis of tryptophan in plasma using nCI detection (simultaneous analysis with kynurenine and quinolinic acid)

Concentration (mg/L)	1	2	3	4	5	6	Average
0	0.29528	0.251554	0.238716	0.284366	0.280546	0.395598	0.29101
0.42	0.435055	0.394671	0.269897	0.348486	0.34602	0.452663	0.374465
0.84	0.462022	0.425911	0.3423	0.504637	0.442676	0.662912	0.47341
1.68	0.602209	0.545506	0.512452	0.56039	0.493497	0.894942	0.601499
3.36	1.136723	1.004089	0.812436	0.949386	0.810947	1.315662	1.004874
6.72	1.876016	1.723622	1.424774	1.513772	1.035437	2.056783	1.605067

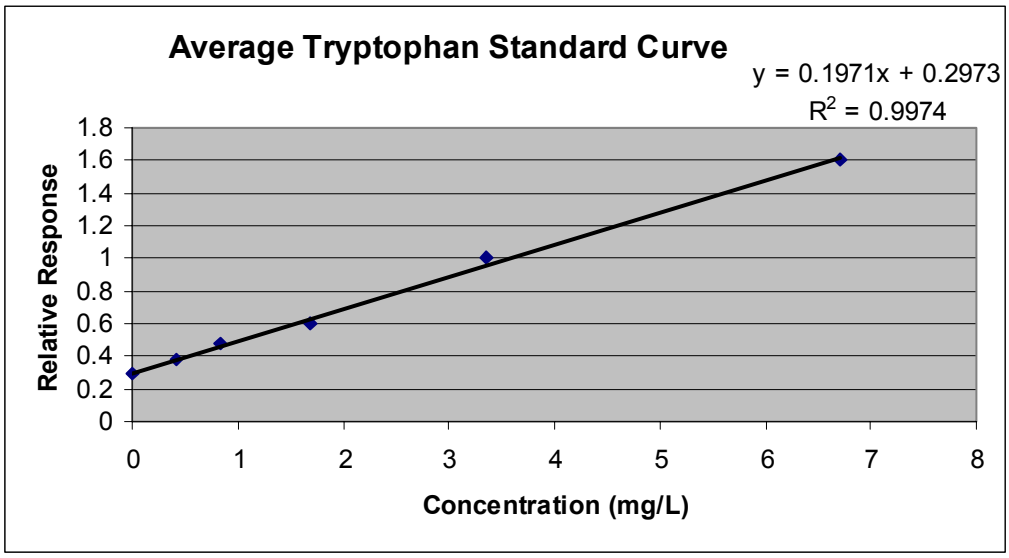

Figure 3.11 Average standard calibration curve illustrating relative response versus concentration for tryptophan plasma spikes using nCI detection. Data obtained from Table 3.10

Table 3.11 Linearity data showing individual equations and R^2 for the average tryptophan plasma curve (see figure 3.11)

Number	Linear equation (y=mx + c)	Correlation Coefficient
1	Y = 0.2375x + 0.2858	0.9930
2	Y = 0.2184x + 0.2503	0.9945
3	Y = 0.1803x + 0.2087	0.9986
4	Y = 0.1832x + 0.2959	0.9933
5	Y = 0.1138x + 0.3212	0.9901
6	Y = 0.2493x + 0.4221	0.9926

The p-value calculated for the correlation coefficient (R^2) from the data set (table 3.11) is 0.027 ($p<0.01$). Therefore there is no significant difference between the obtained correlation coefficients and 1. The 95% ($\alpha = 0.025$) confidence intervals for the gradient (m) and the intercept (c) are 0.197083 ± 0.0519 and 0.297333 ± 0.076181 respectively.

Table 3.12 Average calibration data for analysis of kynurenine in plasma using nCI detection (simultaneous analysis with tryptophan and quinolinic acid)

Concentration (mg/L)	1	2	3	4	5	6	Average
0	0	0	0	0	0	0	0
0.42	0.08315	0.07313	0.08401	0.08401	0.13422	0.05865	0.0862
0.84	0.15797	0.16133	0.17603	0.17603	0.31043	0.10857	0.18173
1.68	0.3855	0.24411	0.25347	0.25347	0.46890	0.28120	0.31444
3.36	0.49581	0.62565	0.71733	0.71733	0.85892	0.59621	0.66854
6.72	1.64445	1.16567	1.20594	1.20594	1.21336	1.03974	1.24585

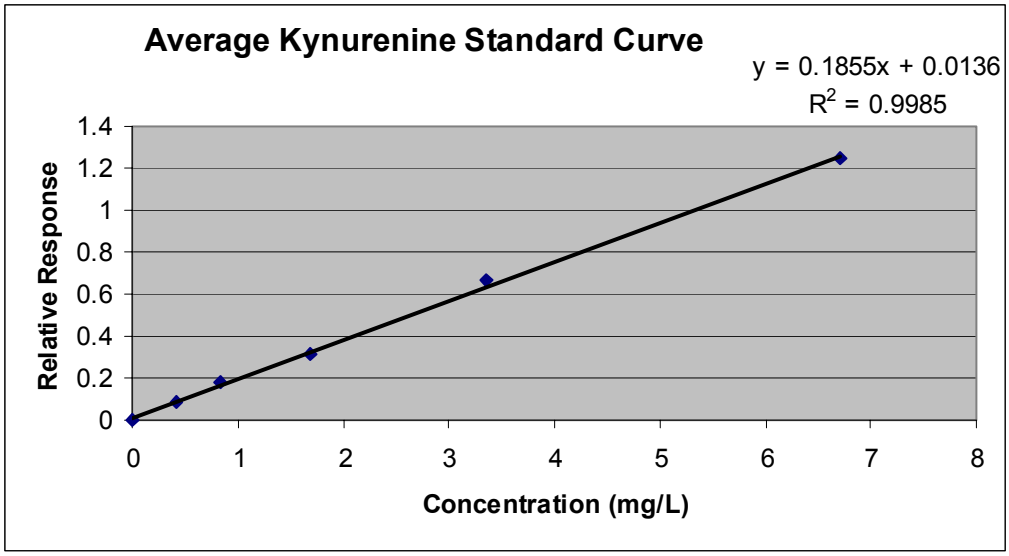

Figure 3.12 Average standard calibration curve illustrating relative response versus concentration for kynurenine plasma spikes using nCI detection. Data obtained from Table 3.12

Table 3.13 Linearity data showing individual equations and R^2 for the average kynurenine plasma curve (see figure 3.12)

Number	Linear equation (y=mx + c)	Correlation Coefficient
1	y = 0.2360x - 0.0509	0.9979
2	y = 0.1751x - 0.0017	0.9879
3	y = 0.1829x + 0.0092	0.9870
4	y = 0.1829x + 0.0092	0.9579
5	y = 0.1776x + 0.1122	0.9907
6	y = 0.1585x + 0.0035	0.9932

The p-value calculated for the correlation coefficient (R^2) from the data set (table 3.13) is 0.057671 ($p<0.01$). Therefore there is no significant difference between the obtained correlation coefficients and 1. The 95% ($\alpha = 0.025$) confidence intervals for the gradient (m) and the intercept (c) are 0.1855 ± 0.027624 and 0.031117 ± 0.045883 respectively.

Table 3.14 Average calibration data for analysis of quinolinic acid in plasma using nCI detection (simultaneous analysis with tryptophan and kynurenine)

Concentration (mg/L)	1	2	3	4	5	6	Average
0	0	0	0	0	0	0	0
0.42	0.021538	0.050854	0.011906	0.013154	0.02308	0.043064	0.027266
0.84	0.069417	0.060686	0.049535	0.030797	0.045815	0.091998	0.058041
1.68	0.131201	0.190685	0.116821	0.067323	0.090904	0.188767	0.13095
3.36	0.263685	0.291998	0.243747	0.194314	0.178747	0.343154	0.252607
6.72	0.607904	0.754223	0.523003	0.300823	0.351823	0.726769	0.544091

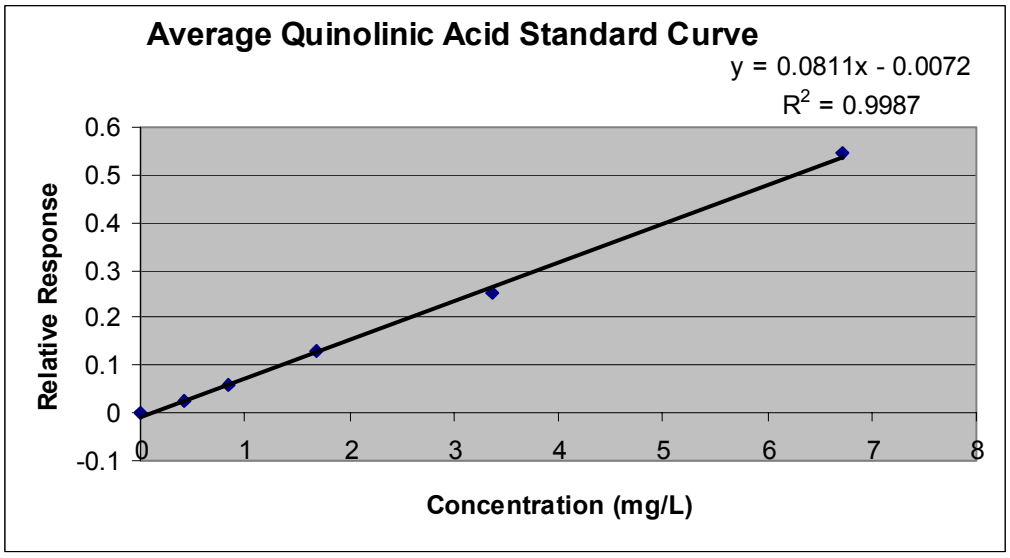

Figure 3.13 Average standard calibration curve illustrating relative response versus concentration for kynurenine plasma spikes using nCI detection. Data obtained from table 3.15

Table 3.15 Linearity data showing individual equations and R^2 for the average quinolinic acid plasma curve (see figure 3.13)

Number	Linear equation (y=mx + c)	Correlation Coefficient
1	y = 0.0905x - 0.014	0.9955
2	y = 0.1102x - 0.0144	0.9844
3	y = 0.0792x - 0.0143	0.9982
4	y = 0.0471x - 0.0012	0.9752
5	y = 0.0523x + 0.0016	0.9999
6	y = 0.1074x - 0.0007	0.9989

The p-value calculated for the correlation coefficient (R^2) from the data set (table 3.15) is 0.108098 ($p<0.01$). Therefore there is no significant difference between the obtained correlation coefficients and 1. The 95% ($\alpha = 0.025$) confidence intervals for the gradient (m) and the intercept (c) are 0.081117 ± 0.028226 and 0.0077 ± 0.007519 respectively.

3.1.3 Precision

In logical terms: no method used for the quantification of a substance will be free from possessing any form of imprecision. This can be imprecision of the analytical instrument or of the analyst or both. By definition precision is "… the closeness of agreement (degree of scatter) between a series of analyses obtained from multiple sampling of the same homogenous sample under the prescribed conditions…" [2]. Precision is expressed in terms of repeatability which is the precision over a short time period, such as the precision for one day's analysis. The within-run or within-day precision gives an indication of the repeatability of the method. In addition to this the between-run or between-day precision will attempt to define the degree of imprecision of the same analysis performed over consecutive days.

Determination of precision was evaluated by performing analyses of both high and low quality controls (QCs) for each of the analytes of concern. Within-day precision was assessed by analyzing six sets of high and low controls for each analyte. Between-day precision was assessed by measuring the six sets of high and low controls on each day over five consecutive days. Statistical inference of the within and between-day data enabled the analysis of precision for the method. This was done by calculating the

relative standard deviations (RSD) or coefficient of variance (CV) of the data sets for each day. Acceptance criteria was taken as precision to be within 10% RSD for high QCs and within 20% for the low QC samples [2,9].

Table 3.16 Precision data for tryptophan high quality controls

Control High	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	Between Day
Tryptophan	4.120842	3.172833	4.286354	6.247261	5.457503	
4.44 mg/L	3.242277	3.514159	4.51681	6.249447	5.413443	
	3.201713	3.132106	4.263685	6.242535	5.411522	
	4.153166	3.602298	4.53143	6.229809	5.443607	
	3.228551	3.16964	4.253223	6.29	5.468358	
	4.073584	3.596397	4.524839	6.287166	5.441961	
Mean	3.670022	3.364572	4.396057	6.257703	5.439399	
SD	0.489225	0.228664	0.141032	0.024886	0.022985	
CV	13.3303	6.796217	3.208156	0.397683	0.422562	**4.830982772**

Table 3.17 Precision data for tryptophan low quality controls

Control Low	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	Between Day
Tryptophan	0.5498	0.246041	0.443823	0.870589	0.626319	
0.56 mg/L	0.520803	0.276729	0.57917	0.860394	0.625602	
	0.512389	0.246709	0.419029	0.866529	0.633518	
	0.528497	0.275327	0.59421	0.871797	0.633283	
	0.507087	0.244751	0.448601	0.873185	0.625843	
	0.542635	0.280669	0.593586	0.875235	0.627835	
Mean	0.526868	0.261704	0.51307	0.869621	0.628733	
SD	0.016821	0.017485	0.083942	0.005377	0.003698	
CV	3.19273	6.681128	16.36066	0.618361	0.588227	**5.488220769**

Table 3.18 Precision data for kynurenine high quality controls

Control High	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	Between Day
Kynurenine	5.033304	1.710663	3.825343	3.860866	2.811444	
4.44 mg/L	3.761913	2.41739	3.950659	4.061665	3.04077	
	3.7737	2.680035	3.677589	4.053999	2.898485	
	4.751512	2.746392	3.888451	3.474964	3.074096	
	3.903939	3.07709	3.342171	3.770315	2.948857	
	4.564889	3.38825	3.717463	3.987258	3.110305	
Mean	4.29821	2.66997	3.733613	3.868178	2.980659	
SD	0.554101	0.578218	0.217313	0.223783	0.114499	
CV	12.89143	21.65636	5.820448	5.785236	3.841384	**9.998972686**

Table 3.19 Precision data for kynurenine low quality controls

Control Low	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	Between Day
Kynurenine	0.377248	0.304866	0.444901	0.646735	0.583686	
0.56 mg/L	0.246082	0.40256	0.37976	0.728836	0.515169	
	0.403697	0.379167	0.425154	0.714078	0.505706	
	0.219218	0.433457	0.386925	0.715056	0.535606	
	0.517777	0.3897	0.429083	0.689485	0.513526	
	0.392219	0.530614	0.387614	0.711008	0.520762	
Mean	0.359373	0.406727	0.408906	0.700866	0.529076	
SD	0.110393	0.074169	0.027396	0.029397	0.028549	
CV	30.71821	18.2355	6.699887	4.194318	5.396087	**13.04880137**

Table 3.20 Precision data for quinolinic acid high quality controls

Control High	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	Between Day
Quinolinic Acid	3.965953	2.994336	4.574527	4.377895	5.130084	
4.44 mg/L	4.159431	4.362561	4.366481	4.511341	5.10699	
	4.119757	4.66463	4.906111	4.820866	5.049293	
	3.965223	4.487651	4.750238	4.669636	5.148853	
	4.165454	4.926126	5.825478	5.641646	5.076344	
	3.823731	4.682872	5.1949	4.289256	5.085156	
Mean	4.033258	4.353029	4.936289	4.71844	5.099453	
SD	0.137068	0.692617	0.519349	0.491577	0.036578	
CV	3.398438	15.91115	10.52104	10.41822	0.717297	**8.193228634**

Table 3.21 Precision data for quinolinic acid low quality controls

Control Low	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	Between Day
Quinolinic Acid	0.611378	0.286474	0.55463	0.905426	0.568225	
0.56 mg/L	0.601223	0.442481	0.566038	0.890129	0.561949	
	0.753949	0.441871	0.561665	0.907738	0.541848	
	0.605292	0.482706	0.564274	0.871969	0.561746	
	0.709241	0.437687	0.342992	0.922716	0.54219	
	0.631259	0.4556	0.466513	1.003938	0.543569	
Mean	0.652057	0.42447	0.509352	0.916986	0.553254	
SD	0.064047	0.069578	0.09003	0.045957	0.011985	
CV	9.822366	16.39169	17.67543	5.011792	2.16619	**10.21349386**

Table 3.22 Precision data for serotonin high quality controls

Control High	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	Between Day
Serotonin	28.12176	28.37864	26.69902	25.88606	27.08835	
27.778 µM	26.81976	28.02785	26.93587	28.02943	27.24946	
	28.08902	27.84378	26.92616	25.95294	27.06407	
	26.66757	28.1313	26.90831	27.90677	27.34453	
	27.95192	27.6166	26.94032	26.13526	27.15697	
	26.73461	28.15933	26.92754	28.1323	27.23664	
Mean	27.39744	28.02625	26.88954	27.00713	27.19	
SD	0.723344	0.266094	0.093979	1.117922	0.106648	
CV	2.64019	0.949447	0.349502	4.139359	0.392234	**1.694146298**

Table 3.23 Precision data for serotonin low quality controls

Control Low	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	Between Day
Serotonin	3.318856	2.872109	2.601043	3.371157	2.950445	
3.087 µM	3.963286	4.133401	2.214141	3.284822	2.95031	
	3.343834	2.984444	2.535397	3.303395	2.973742	
	4.03524	5.167791	2.101831	3.239374	2.943007	
	3.338295	3.084178	2.423918	3.389779	2.812765	
	4.014157	3.726486	2.105359	3.298091	2.957214	
Mean	3.668945	3.661402	2.330281	3.314436	2.931247	
SD	0.368122	0.884383	0.219281	0.056204	0.058972	
CV	10.03346	24.15423	9.410056	1.695719	2.011835	**9.461059408**

Analysis of the precision quality control data shows that the acceptance criteria were met for the within-day RSD for each analyte per a day (with the exception of a few outliers). The between-day precision RSD for each analyte fell within the limits of the acceptance criteria for both high and low controls.

A further way to demonstrate the precision of a method is to plot Levey-Jennings charts of consecutive controls. The charts illustrate the distribution of the controls over the mean. The limits for acceptance are that the controls should be "scattered" over both sides of the mean and should lie within ± 2SD of the mean [10]. Charts for 12 consecutive high and low controls for each analyte were plotted. The 95% confidence intervals for each plot are also indicated.

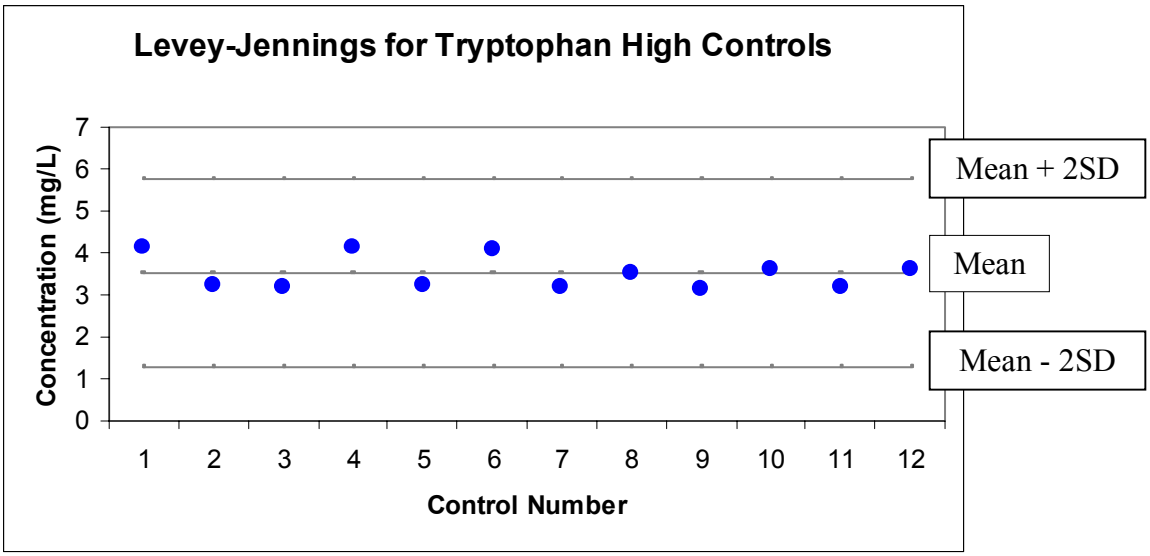

Figure 3.14 Levey-Jennings chart for tryptophan high controls. The 95% confidence interval = 3.5172 ± 0.7163 mg/L ($p<0.025$)

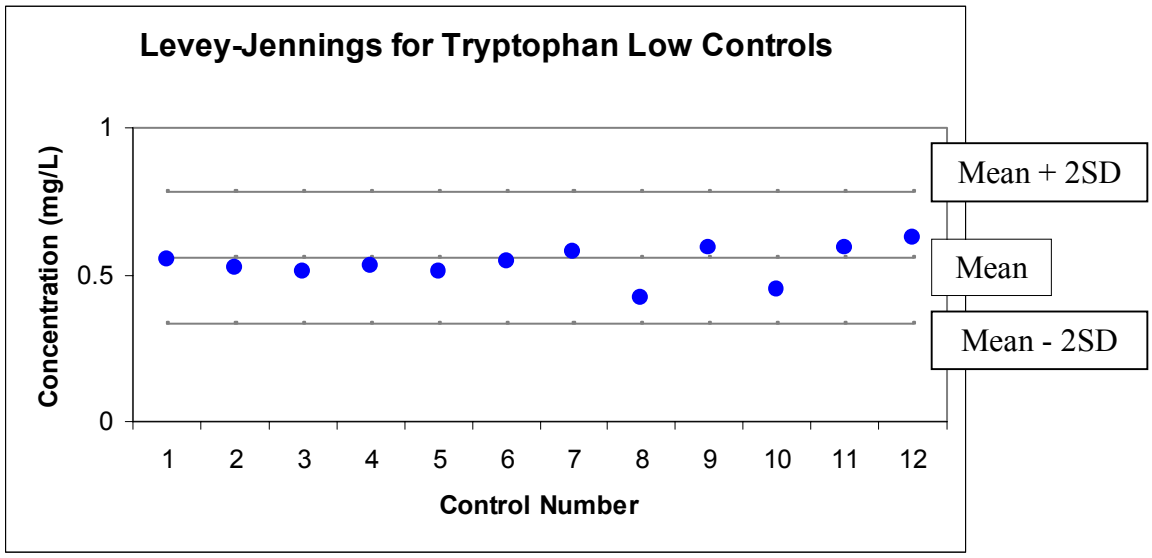

Figure 3.15 Levey-Jennings chart for tryptophan low controls. The 95% confidence interval = 0.55553 ± 0.071467 mg/L ($p<0.025$)

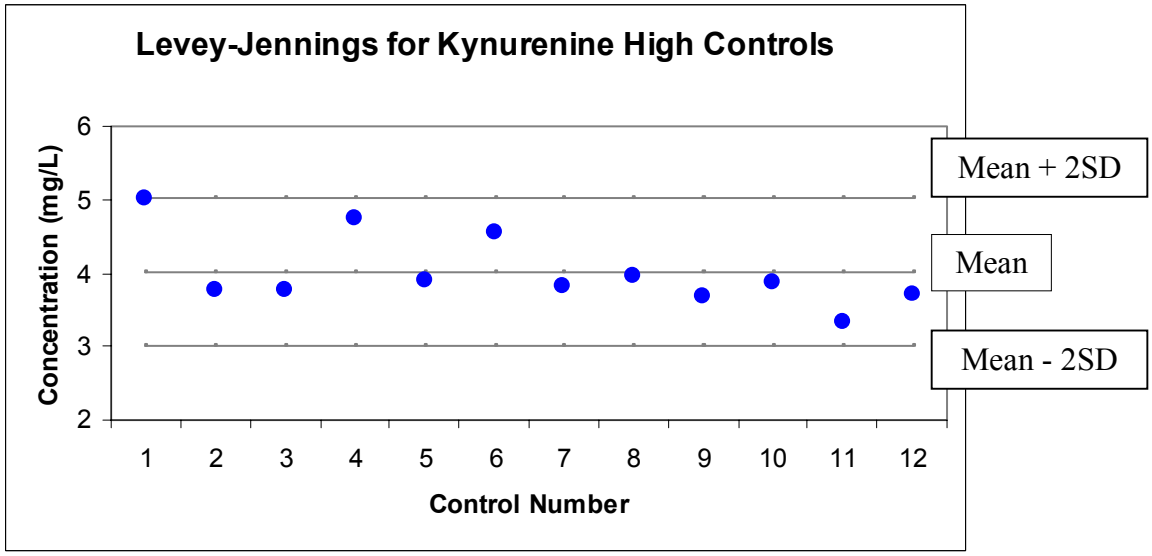

Figure 3.16 Levey-Jennings chart for kynurenine high controls. The 95% confidence interval = 4.01591 ± 0.31639 mg/L ($p<0.025$)

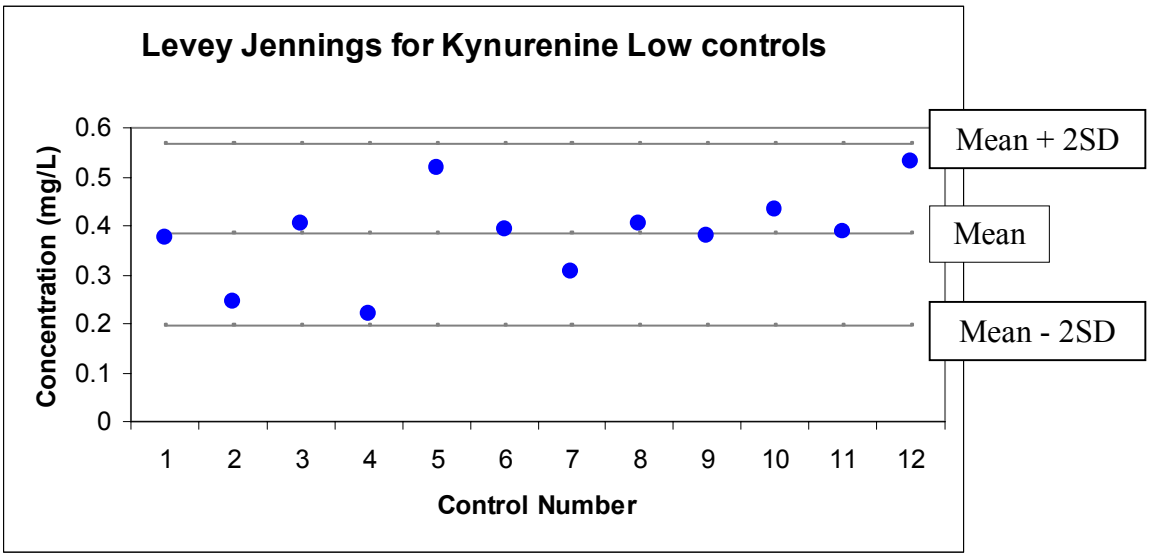

Figure 3.17 Levey-Jennings chart for kynurenine low controls. The 95% confidence interval = 0.38305 ± 0.059097 mg/L ($p<0.025$)

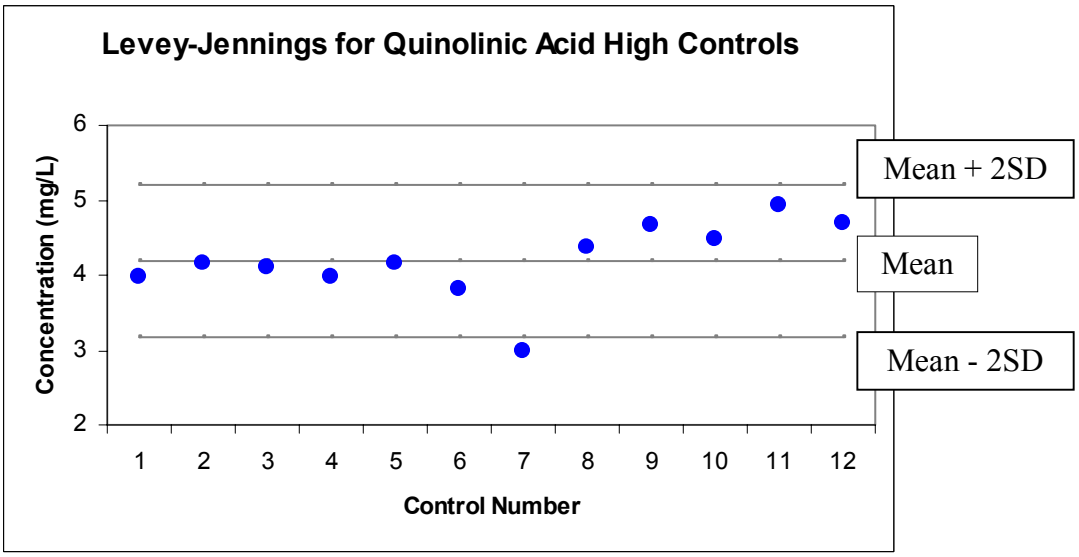

Figure 3.18 Levey-Jennings chart for quinolinic acid high controls. The 95% confidence interval = 4.19314 ± 0.32052 mg/L ($p<0.025$)

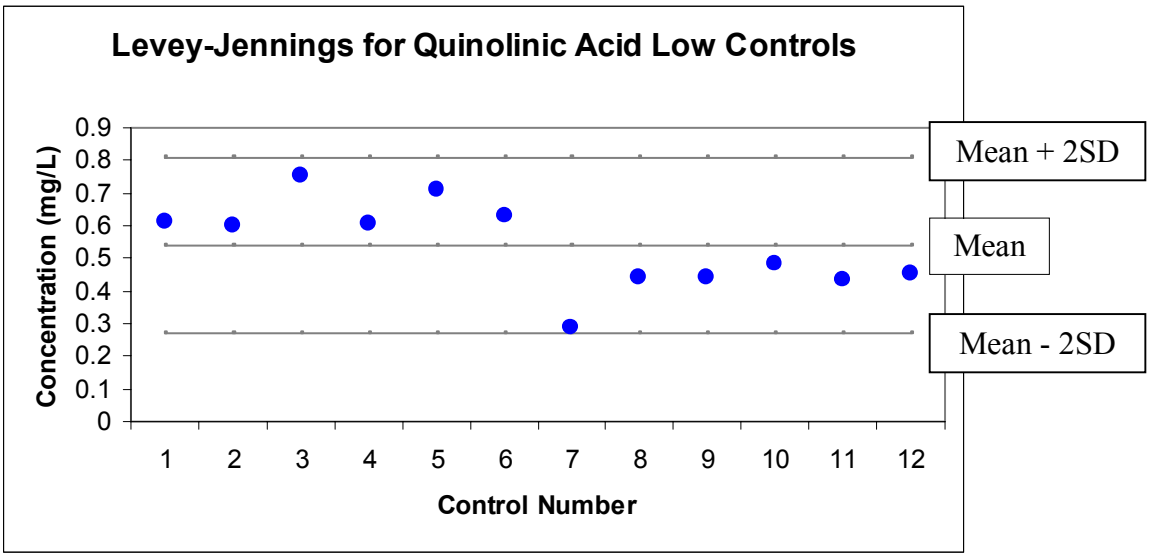

Figure 3.19 Levey-Jennings chart for quinolinic acid low controls. The 95% confidence interval = 0.53826 ± 0.08569 mg/L ($p<0.025$)

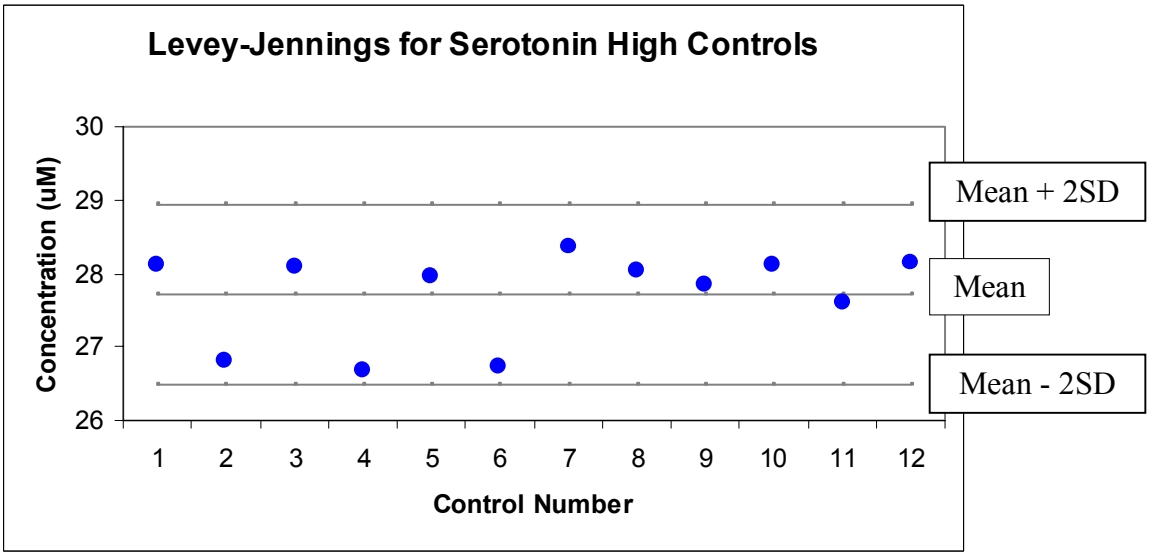

Figure 3.20 Levey-Jennings chart for serotonin high controls. The 95% confidence interval = 27.7118 ± 0.39056 µM ($p<0.025$)

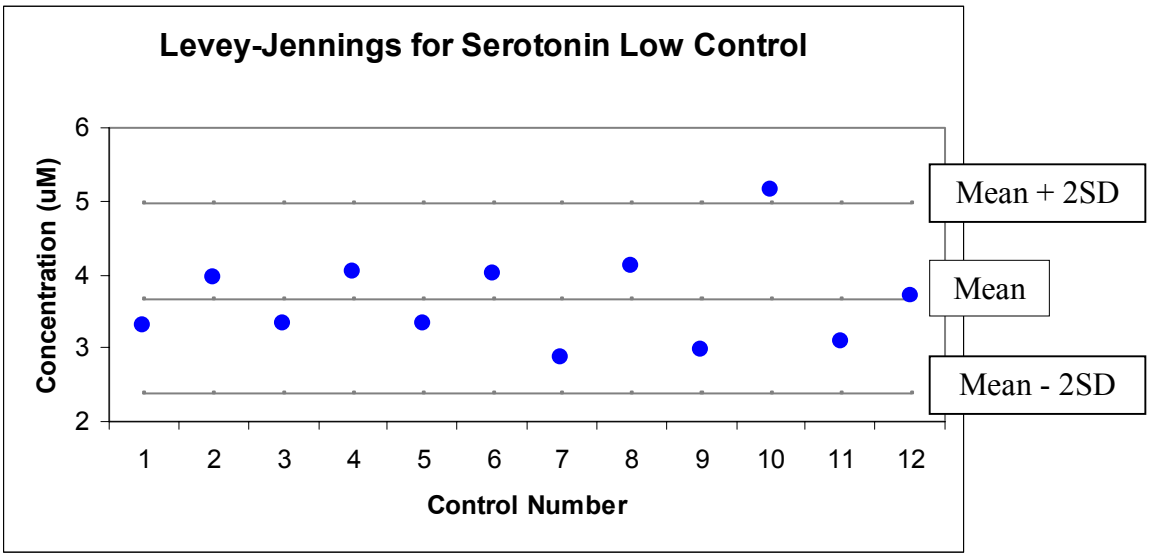

Figure 3.21 Levey-Jennings chart for serotonin low controls. The 95% confidence interval = 3.66517 ± 0.41034 µM ($p<0.025$)

Criteria for the Levey-Jennings plots were met for distribution over the mean and limits within ± 2SD of the mean, with the exception of single outliers in figures 3.18 and 3.21. These are however single outliers and are thus negligible.

3.1.4 Accuracy and bias

Bias is termed as "the difference between the expectation of the test results and an accepted reference value" [11]. The percentage deviation from the accepted reference value gives an indication of bias [2]. Accuracy is in the sense of its meaning: how accurate are the measurements with relation to the true value or mean.

The following formulas were used to calculate bias and accuracy:

$$\textbf{Bias} = (\mu-\chi)/\mu \times 100\%$$

$$\textbf{Accuracy} = (\chi/\ \mu) \times 100\%$$

The true mean (or population mean) is represented by µ. χ is the mean or average of measurements made on a sample taken to be representative of the total population. The acceptance limits for bias were ±15% for both high and low concentration levels for all analytes [2]. The closeness of the percentage value to 100% illustrates accuracy. The

significance of the calculated bias and accuracy was tested by performing t-tests against the accepted true value (μ) for each analyte.

The following tables represent the bias and accuracy results for repeat high and low controls for each analyte. T-test results are presented for each table ($p<0.01$ considered as statistically significant).

Table 3.24 Bias and accuracy data for tryptophan high controls (4.44 mg/L)
P= 0.37474

Tryptophan High Control		Bias	Accuracy
μ	χ		
4.44	4.12084214	7.18824	92.81176
4.44	3.242276883	26.97575	73.02425
4.44	3.201713435	27.88934	72.11066
4.44	4.153166256	6.460219	93.53978
4.44	3.228551078	27.28489	72.71511
4.44	4.073583737	8.252619	91.74738
4.44	3.172832575	28.53981	71.46019
4.44	3.514158672	20.85228	79.14772
4.44	3.132106215	29.45707	70.54293
4.44	3.602297588	18.86717	81.13283
4.44	3.169640473	28.6117	71.3883
4.44	3.59639733	19.00006	80.99994
4.44	4.286354493	3.460484	96.53952
4.44	4.516810483	-1.72997	101.73
4.44	4.26368545	3.971048	96.02895
4.44	4.531430099	-2.05924	102.0592
4.44	4.253222765	4.206694	95.79331
4.44	4.524839351	-1.9108	101.9108
4.44	6.247261002	-40.7041	140.7041
4.44	6.249446698	-40.7533	140.7533
4.44	6.242535025	-40.5976	140.5976
4.44	6.229808679	-40.311	140.311
4.44	6.289999859	-41.6667	141.6667
4.44	6.287165984	-41.6028	141.6028
4.44	5.457503241	-22.9167	122.9167
4.44	5.413442809	-21.9244	121.9244
4.44	5.411522194	-21.8811	121.8811
4.44	5.443606729	-22.6038	122.6038
4.44	5.468358328	-23.1612	123.1612
4.44	5.441961086	-22.5667	122.5667
Mean	**4.625550689**	**-4.17907**	**104.1791**
SD	**1.127333762**	**25.3904**	**25.3904**

Table 3.25 Bias and accuracy data for tryptophan low controls (0.56 mg/L)

P= 0.01410

Tryptophan Low Control		Bias	Accuracy
μ	χ		
0.56	0.54979956	1.821507	98.17849
0.56	0.520802622	6.999532	93.00047
0.56	0.512389321	8.501907	91.49809
0.56	0.528496907	5.625552	94.37445
0.56	0.507086925	9.448763	90.55124
0.56	0.542634733	3.10094	96.89906
0.56	0.579170224	-3.42325	103.4233
0.56	0.419028911	25.17341	74.82659
0.56	0.594209903	-6.10891	106.1089
0.56	0.448600517	19.89276	80.10724
0.56	0.593586353	-5.99756	105.9976
0.56	0.870588509	-55.4622	155.4622
0.56	0.860394005	-53.6418	153.6418
0.56	0.866529196	-54.7374	154.7374
0.56	0.871796731	-55.678	155.678
0.56	0.873184717	-55.9258	155.9258
0.56	0.875234869	-56.2919	156.2919
0.56	0.62631927	-11.8427	111.8427
0.56	0.625602412	-11.7147	111.7147
0.56	0.633518094	-13.1282	113.1282
0.56	0.633283457	-13.0863	113.0863
0.56	0.625842861	-11.7577	111.7577
0.56	0.627834836	-12.1134	112.1134
Mean	**0.642866736**	**-14.7976**	**114.7976**
SD	**0.149051846**	**26.6164**	**26.6164**

Table 3.26 Bias and accuracy data for kynurenine high controls (4.44 mg/L)

P= 0.00000268 (p<0.01)

Kynurenine High Control		Bias	Accuracy
μ	χ		
4.44	5.033304315	-13.3627	113.3627
4.44	3.761913089	15.27223	84.72777
4.44	3.773700373	15.00675	84.99325
4.44	4.751512401	-7.01605	107.016
4.44	3.903939233	12.07344	87.92656
4.44	4.56488906	-2.81282	102.8128
4.44	3.825343388	13.84362	86.15638
4.44	3.950658932	11.0212	88.9788
4.44	3.677589199	17.17141	82.82859
4.44	3.888450764	12.42228	87.57772
4.44	3.342170701	24.72589	75.27411
4.44	3.717463118	16.27335	83.72665
4.44	3.860865531	13.04357	86.95643
4.44	4.061665128	8.521056	91.47894
4.44	4.053999031	8.693716	91.30628
4.44	3.47496395	21.73505	78.26495
4.44	3.770314783	15.083	84.917
4.44	3.987257551	10.1969	89.8031
4.44	2.811443637	36.6792	63.3208
4.44	3.040770074	31.51419	68.48581
4.44	2.89848459	34.71882	65.28118
4.44	3.07409581	30.76361	69.23639
4.44	2.948857211	33.5843	66.4157
4.44	3.110304752	29.94809	70.05191
Mean	**3.720164859**	**16.2125**	**83.7875**
SD	**0.571259673**	**12.86621**	**12.86621**

Table 3.27 Bias and accuracy data for kynurenine low controls (0.56 mg/L)

P= 0.004056239 (p<0.01)

Kynurenine Low Control		Bias	Accuracy
μ	χ		
0.56	0.37724787	32.63431	67.36569
0.56	0.246081794	56.05682	43.94318
0.56	0.403697288	27.9112	72.0888
0.56	0.219217792	60.85397	39.14603
0.56	0.517776859	7.539847	92.46015
0.56	0.392218965	29.9609	70.0391
0.56	0.30486603	45.55964	54.44036
0.56	0.402560419	28.11421	71.88579
0.56	0.379166892	32.29163	67.70837
0.56	0.433457359	22.5969	77.4031
0.56	0.38970001	30.41071	69.58929
0.56	0.530614228	5.247459	94.75254
0.56	0.444900925	20.55341	79.44659
0.56	0.379759809	32.18575	67.81425
0.56	0.425153702	24.0797	75.9203
0.56	0.386925033	30.90624	69.09376
0.56	0.429083472	23.37795	76.62205
0.56	0.387613833	30.78324	69.21676
0.56	0.646734905	-15.4884	115.4884
0.56	0.72883573	-30.1492	130.1492
0.56	0.714077938	-27.5139	127.5139
0.56	0.715056385	-27.6886	127.6886
0.56	0.689485223	-23.1224	123.1224
0.56	0.711008268	-26.9658	126.9658
0.56	0.583685741	-4.2296	104.2296
0.56	0.515169359	8.005472	91.99453
0.56	0.505705832	9.695387	90.30461
0.56	0.535606121	4.35605	95.64395
0.56	0.513526092	8.298912	91.70109
0.56	0.520762417	7.006711	92.99329
Mean	**0.480989876**	**14.10895**	**85.89105**
SD	**0.138656615**	**24.76011**	**24.76011**

Table 3.28 Bias and accuracy data for quinolinic acid high controls (4.44 mg/L)

P= 0.08357

Quinolinic Acid High Control		Bias	Accuracy
μ	χ		
4.44	3.965953037	10.67673	89.32327
4.44	4.159430627	6.31913	93.68087
4.44	4.119757116	7.212678	92.78732
4.44	3.965222547	10.69319	89.30681
4.44	4.165453962	6.183469	93.81653
4.44	3.823730732	13.87994	86.12006
4.44	2.994336285	32.55999	67.44001
4.44	4.362560914	1.744124	98.25588
4.44	4.664630117	-5.05924	105.0592
4.44	4.487650681	-1.07321	101.0732
4.44	4.926126144	-10.9488	110.9488
4.44	4.682871712	-5.47008	105.4701
4.44	4.574526623	-3.02988	103.0299
4.44	4.366480672	1.655841	98.34416
4.44	4.906110853	-10.498	110.498
4.44	4.75023849	-6.98735	106.9874
4.44	5.825478391	-31.2045	131.2045
4.44	5.194899583	-17.0022	117.0022
4.44	4.377894969	1.398762	98.60124
4.44	4.511341162	-1.60678	101.6068
4.44	4.820866293	-8.57807	108.5781
4.44	4.66963605	-5.17198	105.172
4.44	5.641646219	-27.0641	127.0641
4.44	4.289256298	3.395128	96.60487
4.44	5.130083817	-15.5424	115.5424
4.44	5.106989798	-15.0223	115.0223
4.44	5.049293234	-13.7228	113.7228
4.44	5.148852824	-15.9652	115.9652
4.44	5.076344114	-14.3321	114.3321
4.44	5.085155575	-14.5305	114.5305
Mean	**4.628093961**	**-4.23635**	**104.2364**
SD	**0.574905612**	**12.94832**	**12.94832**

Table 3.29 Bias and accuracy data for quinolinic acid low controls (0.56 mg/L)

P= 0.13374

Quinolinic Acid Low Control		Bias	Accuracy
μ	χ		
0.56	0.611377908	-9.17463	109.1746
0.56	0.601223027	-7.36125	107.3613
0.56	0.753948759	-34.6337	134.6337
0.56	0.605292035	-8.08786	108.0879
0.56	0.709241433	-26.6503	126.6503
0.56	0.631259486	-12.7249	112.7249
0.56	0.286473714	48.84398	51.15602
0.56	0.442480565	20.98561	79.01439
0.56	0.441870985	21.09447	78.90553
0.56	0.482705937	13.80251	86.19749
0.56	0.437687466	21.84152	78.15848
0.56	0.455599568	18.64293	81.35707
0.56	0.554630361	0.958864	99.04114
0.56	0.566037966	-1.07821	101.0782
0.56	0.561664517	-0.29724	100.2972
0.56	0.56427386	-0.76319	100.7632
0.56	0.342992437	38.75135	61.24865
0.56	0.466512758	16.69415	83.30585
0.56	0.905425553	-61.6831	161.6831
0.56	0.890129005	-58.9516	158.9516
0.56	0.907737518	-62.096	162.096
0.56	0.87196949	-55.7088	155.7088
0.56	0.922715503	-64.7706	164.7706
0.56	1.003937905	-79.2746	179.2746
0.56	0.568224761	-1.46871	101.4687
0.56	0.56194855	-0.34796	100.348
0.56	0.541847642	3.241492	96.75851
0.56	0.561745975	-0.31178	100.3118
0.56	0.542190409	3.180284	96.81972
0.56	0.543568682	2.934164	97.06584
Mean	**0.611223792**	**-9.14711**	**109.1471**
SD	**0.181863968**	**32.47571**	**32.47571**

Table 3.30 Bias and accuracy data for serotonin high controls (27.778 μM)

P= 0.044519

Serotonin High Control		Bias	Accuracy
μ	χ		
27.778	28.12176	-1.23751	101.2375
27.778	26.81976	3.449651	96.55035
27.778	28.08902	-1.11966	101.1197
27.778	26.66757	3.997525	96.00248
27.778	27.95192	-0.6261	100.6261
27.778	26.73461	3.756172	96.24383
27.778	28.37864	-2.16229	102.1623
27.778	28.02785	-0.89944	100.8994
27.778	27.84378	-0.2368	100.2368
27.778	28.1313	-1.27185	101.2719
27.778	27.6166	0.581019	99.41898
27.778	28.15933	-1.37279	101.3728
27.778	26.69902	3.884303	96.1157
27.778	28.02943	-0.90514	100.9051
27.778	25.95294	6.570178	93.42982
27.778	27.90677	-0.46358	100.4636
27.778	26.13526	5.913818	94.08618
27.778	28.1323	-1.27547	101.2755
27.778	27.08835	2.482722	97.51728
27.778	27.24946	1.902735	98.09727
27.778	27.06407	2.570115	97.42989
27.778	27.34453	1.560496	98.4395
27.778	27.15697	2.235677	97.76432
27.778	27.23664	1.948898	98.0511
27.778	27.84866	-0.25437	100.2544
27.778	27.59018	0.676147	99.32385
27.778	27.45728	1.154576	98.84542
27.778	27.58413	0.697944	99.30206
27.778	27.71878	0.213181	99.78682
27.778	27.42054	1.286859	98.71314
Mean	**27.35227**	**1.53262**	**98.46738**
SD	**0.648557**	**2.334787**	**2.334787**

Table 3.31 Bias and accuracy data for serotonin low controls (3.087 µM)

P= 0.04923

Serotonin Low Control		Bias	Accuracy
µ	χ		
3.087	3.318856	-7.51071	107.5107
3.087	3.963286	-28.3863	128.3863
3.087	3.343834	-8.31986	108.3199
3.087	4.03524	-30.7172	130.7172
3.087	3.338295	-8.14042	108.1404
3.087	4.014157	-30.0342	130.0342
3.087	2.872109	6.961146	93.03885
3.087	4.133401	-33.897	133.897
3.087	2.984444	3.322183	96.67782
3.087	5.167791	-67.405	167.405
3.087	3.084178	0.091403	99.9086
3.087	3.726486	-20.7155	120.7155
3.087	3.371157	-9.20495	109.2049
3.087	3.284822	-6.40823	106.4082
3.087	3.303395	-7.00987	107.0099
3.087	3.239374	-4.93598	104.936
3.087	3.389779	-9.80819	109.8082
3.087	3.298091	-6.83807	106.8381
3.087	2.950445	4.423546	95.57645
3.087	2.95031	4.427937	95.57206
3.087	2.973742	3.66888	96.33112
3.087	2.943007	4.664489	95.33551
3.087	2.812765	8.883554	91.11645
3.087	2.957214	4.204268	95.79573
3.087	3.615628	-17.1243	117.1243
3.087	3.730792	-20.855	120.855
3.087	3.620729	-17.2896	117.2896
3.087	3.747017	-21.3805	121.3805
3.087	3.64717	-18.1461	118.1461
3.087	3.719278	-20.482	120.482
Mean	**3.264402**	**-5.74675**	**105.7468**
SD	**0.627013**	**20.31139**	**20.31139**

3.1.5 Stability

It would be senseless not to determine the stability of an analyte that is intended to be analyzed over a given time frame. This time frame contributes to the essentials of the stability of the analyte. By definition stability is the "chemical stability of an analyte in a given matrix under specific conditions for given time intervals" [4]. Thus over the period

which the analyte is intended to be analyzed, it should remain relatively stable. It is therefore important to investigate whether this is the case and if the analyte is stable given certain conditions and time periods. Stability experiments were performed for certain stages of the analysis. The freeze/thaw, in-process and processed sample stability were tested.

3.1.5.1 Freeze/thaw stability

The storage conditions for the analytes are important to ensure their stability. For purpose of this study samples (plasma, whole blood, stock solutions, controls and standards) were all stored at -70^{0}C. Prior to preparation before analysis the relevant samples were thawed (defrosted) to room temperature (equilibrated). The freeze/thaw experiment was conducted as follows: 4 high and 4 low controls for each analyte were analysed at 3 cycles according to validation criteria. For the first cycle the controls were thawed, prepared and analyzed. The same controls were frozen and served as the controls to be thawed for the second cycle. Similarly the same procedures (and controls) were followed for the third cycle.

Table 3.32 Freeze/thaw data for tryptophan high and low controls. # **(p<0.025)** and *P is the p-value obtained testing the significant difference between 1st and 3rd cycles (p<0.01)

	Tryptophan High		*P=0.43058		Tryptophan Low		*P=0.02443
	Cycle 1	Cycle 2	Cycle 3		Cycle 1	Cycle 2	Cycle 3
1	1.988651311	2.9363187	2.4251855	1	0.66569588	0.41524824	0.894805372
2	2.031615617	2.49201612	1.61957296	2	0.41322645	0.49459776	0.91928806
3	1.789381308	2.51284108	1.58901044	3	0.49256147	0.52062266	0.717110735
4	2.203729555	2.83579304	1.50117917	4	0.45516818	0.70328645	0.677843467
#Mean	2.00 ± 0.27	2.69 ± 0.36	1.78 ± 0.69		0.51 ± 0.18	0.53 ± 0.19	0.80 ± 0.19
SD	0.170244494	0.2254177	0.43056682		0.1108639	0.12177864	0.122460992
CV	8.498014127	8.36664577	24.13847		21.8811919	22.8289816	15.26446548

Table 3.33 Freeze/thaw data for kynurenine high and low controls. # **(p<0.025)** and *P is the p-value obtained testing the significant difference between 1st and 3rd cycles (p<0.01)

	Kynurenine High		*P=0.2814		Kynurenine Low		*P=0.4714
	Cycle 1	Cycle 2	Cycle 3		Cycle 1	Cycle 2	Cycle 3
1	2.082459046	1.1211152	1.08559393	1	0.55043006	0.46739719	0.410070491
2	1.524587881	1.25074632	1.71669443	2	0.3232197	0.35947468	0.413899249
3	1.651250506	1.21217946	1.23906721	3	0.34869273	0.38591787	0.491478305
4	1.418430298	1.24334087	1.30635782	4	0.39289188	0.36835413	0.515624287
#Mean	1.67 ± 0.46	1.21 ± 0.09	1.34 ± 0.43		0.40 ± 0.16	0.40 ± 0.08	0.46 ± 0.09
SD	0.291492327	0.05954643	0.26950735		0.10189759	0.04931383	0.053799796
CV	17.46318489	4.93405555	20.1586979		25.2341315	12.4754821	11.75263162

Table 3.34 Freeze/thaw data for quinolinic acid high and low controls. # **(p<0.025)** and *P is the p-value obtained testing the significant difference between 1st and 3rd cycles (p<0.01)

	Quinolinic Acid High		*P=0.1185		Quinolinic Acid Low		*P=0.9107
	Cycle 1	Cycle 2	Cycle 3		Cycle 1	Cycle 2	Cycle 3
1	0.423191717	0.29965038	0.3469575	1	0.09382984	0.1084684	0.092854545
2	0.394323811	0.28307731	0.22018996	2	0.09383862	0.07969843	0.094249658
3	0.344987642	0.33649621	0.36531242	3	0.09890954	0.09394393	0.092978503
4	0.401917171	0.28169897	0.22753569	4	0.09114559	0.0870743	0.099034036
#Mean	0.39 ± 0.05	0.30 ± 0.04	0.29 ± 0.12		0.09 ± 0.01	0.09 ± 0.02	0.09 ± 0.005
SD	0.033083893	0.02551593	0.07679268		0.00324364	0.01225054	0.00290579
CV	8.459080222	8.49877331	26.4803372		3.43493469	13.2730601	3.065852917

Table 3.35 Freeze/thaw data for serotonin high and low controls. # **(p<0.025)** and *P is the p-value obtained testing the significant difference between 1st and 3rd cycles (p<0.01)

	Serotonin High		*P=0.0592		Serotonin Low		*P=0.10290
	Cycle 1	Cycle 2	Cycle 3		Cycle 1	Cycle 2	Cycle 3
1	2.752229081	3.22660671	3.21821873	1	0.74101408	0.92183578	0.725726598
2	2.277087453	2.00522512	3.70736106	2	0.69045215	0.82595007	0.782851174
3	2.155751232	2.80479939	2.6577109	3	0.69372778	0.84524882	0.869035485
4	2.81441561	2.85057899	3.26277412	4	0.80368833	0.86238611	1.006528716
#Mean	2.45 ± 0.53	2.72 ± 0.82	3.21 ± 0.68		0.73 ± 0.08	0.86 ± 0.07	0.85 ± 0.19
SD	0.332000922	0.51373868	0.43020721		0.05295049	0.04142021	0.122138668
CV	13.28072299	18.874943	13.3957664		7.23149391	4.79480968	14.43658914

According to validation criteria, analysis of a minimum of three cycles is sufficient to determine acceptable results [2]. Tables 3.32 to 3.35 indicate no significant differences between the first and last cycles thereby substantiating stability.

3.1.5.2 In-process stability and processed sample stability

Samples should remain relatively stable over the conditions and time period during their preparation. Environment conditions such as temperature and light conditions all contribute towards the stability of the analyte. The same applies for the length of time for the preparation of samples. External factors are specific for each laboratory; however certain conditions can be kept relatively constant. Use of air-conditioning helped to keep the room temperature within a fare temperature range (25^{o}C). The influence of light affecting the degradation of the indole species was limited by working in a dark room during sample preparation. Samples were shielded from light (outside the dark room) by covering in aluminum foil. The prepared samples were transferred to light protective amber Agilent autosampler glass vials. The time period over which the samples were prepared was kept to minimal and specific preparation procedures such as centrifugal and incubation times were kept constant.

Once the samples are prepared it is important to ensure stability prior to and during analysis (processed sample stability). In this case the autosampler conditions for the maximum expected chromatographic run times were tested. This was done by performing replication experiments for high and low controls of all the analytes. Controls were repeatedly injected over a period of one chromatographic analysis while remaining on the autosampler. The ratios between the relative response of stability samples (results of QC samples after exposure to autosampler conditions) and comparison samples (before exposure to autosampler conditions) were determined. The stability is expressed as a percentage of the ratios with 100% being the ideal stability.

Table 3.36 Processed sample stability for tryptophan high controls

A - Comparison	A1	A2	A3	A4	A5	A6
	3.339165	3.173288	3.136107	3.029044	3.051926	2.980635
B - Stability	B1	B2	B3	B4	B5	B6
	3.078327	2.962772	3.279786	3.009235	2.848765	3.017376
B/A %	**92.18851**	**93.36598**	**104.5815**	**99.34604**	**93.34318**	**101.2327**

Table 3.37 Processed sample stability for tryptophan low controls

A - Comparison	A1	A2	A3	A4	A5	A6
	2.793837	2.888995	2.827322	2.84854	2.811602	2.743273
B - Stability	B1	B2	B3	B4	B5	B6
	2.866313	2.813411	2.769981	2.819212	2.833209	2.781314
B/A %	**102.5941**	**97.38375**	**97.97188**	**98.97042**	**100.7685**	**101.3867**

Table 3.38 Processed sample stability for kynurenine high controls

A - Comparison	A1	A2	A3	A4	A5	A6
	0.478842	0.497814	0.479111	0.495982	0.498947	0.4833
B - Stability	B1	B2	B3	B4	B5	B6
	0.560088	0.537397	0.499678	0.605971	0.616195	0.517611
B/A %	**116.967**	**107.9514**	**104.2927**	**122.1761**	**123.4993**	**107.0994**

Table 3.39 Processed sample stability for kynurenine low controls

A - Comparison	A1	A2	A3	A4	A5	A6
	0.767294	0.779422	0.750531	0.835907	0.896221	0.86668
B - Stability	B1	B2	B3	B4	B5	B6
	0.405496	0.546563	0.633858	0.662399	0.715075	0.735375
B/A %	**52.84756**	**70.12413**	**84.45453**	**79.24313**	**79.78776**	**84.84961**

Table 3.40 Processed sample stability for quinolinic acid high controls

A - Comparison	A1	A2	A3	A4	A5	A6
	0.03908	0.036808	0.040273	0.037414	0.037975	0.0406
B - Stability	B1	B2	B3	B4	B5	B6
	0.037569	0.038393	0.039547	0.038581	0.038446	0.039201
B/A %	**96.13484**	**104.3045**	**98.19752**	**103.1175**	**101.2423**	**96.55377**

Table 3.41 Processed sample stability for quinolinic acid low controls

A - Comparison	A1	A2	A3	A4	A5	A6
	0.015419	0.014059	0.013843	0.013561	0.012026	0.016285
B - Stability	B1	B2	B3	B4	B5	B6
	0.017747	0.014365	0.01676	0.019539	0.01136	0.014963
B/A %	**115.0991**	**102.1783**	**121.0733**	**144.0852**	**94.45641**	**91.88345**

Table 3.42 Processed sample stability for serotonin high controls

A - Comparison	A1	A2	A3	A4	A5	A6
	3.181249	2.948672	3.015001	3.144494	2.984547	3.072051
B - Stability	B1	B2	B3	B4	B5	B6
	3.009923	3.006333	3.061049	2.988794	3.165488	2.98151
B/A %	**94.61452**	**101.9555**	**101.5273**	**95.04849**	**106.0626**	**97.05275**

Table 3.43 Processed sample stability for serotonin low controls

A - Comparison	A1	A2	A3	A4	A5	A6
	3.19555	3.059341	3.081873	3.100651	3.143808	3.143885
B - Stability	B1	B2	B3	B4	B5	B6
	3.239654	3.119898	3.110372	3.163797	3.07106	3.192656
B/A %	**101.3802**	**101.9794**	**100.9247**	**102.0366**	**97.686**	**101.5513**

3.1.6 Recovery

During the method validation recovery of the analytes from the relevant matrix was determined. This was used to estimate the proportional systematic error of the method. The recovery experiment was designed as follows: six high and low controls of each analyte were used to obtain an average recovery. Pairs of controls (sample with addition)

and blanks (sample with dilution) were prepared by spiking the equal volumes control and water to the matrix respectively. Controls and blanks were analysed according to the method and the concentrations were calculated from standard curves. Recovery was calculated as the difference between the sample with addition and sample with dilution divided by the amount of control spiked.

Recovery is expressed as a percentage with an ideal recovery being 100%. The systematic proportional error is calculated as the difference between 100 and the determined percentage recovery. The following tables indicate the calculated recovery and errors for low and high controls of each analyte.

Table 3.44 Average (n=6 repeats) recovery (%) for high and low controls of each analyte

Average Recovery	High Control	Low Control
Tryptophan	98.93721461	88.15990341
Kynurenine	87.12111852	72.62990888
Quinolinic Acid	98.04120065	75.79816173
Serotonin	98.46231815	75.48692739

Table 3.45 Systematic proportional errors (%) for high and low controls of each analyte

Systematic Proportional Error	High Control	Low Control
Tryptophan	1.062785393	11.84009659
Kynurenine	12.87888148	27.37009112
Quinolinic Acid	1.958799349	24.20183827
Serotonin	1.537681848	24.51307261

3.1.7 Limit of detection and limit of quantification

The limit of detection (LOD) indicates the lowest amount of an analyte that gives a signal which can be detected by the detector and still be distinguished from the background noise. For chromatographic analyses acceptable limit of detection is a chromatographic peak with a signal to noise ratio of 3 and above. The limit of quantification (LOQ) is given by a chromatographic peak with a signal to noise ratio of 10 and above [2,3]. Data

for results below the limit of quantification should not be quantified as these may produce inaccurate an invalid results.

The limits of detection and quantification were tested by making serial dilutions of the lowest standard for each analyte and performing repeats of the analyses. The signal to noise ratio was estimated from the abundance of the peak height and the baseline as analysed on the chromatograms. The concentrations of the lowest dilutions for the LOD and LOQ were calculated by multiplying the lowest standard with the respective dilution factor.

Table 3.46 Limit of quantification and detection for the analytes

Analyte	LOQ	LOD
Tryptophan	0.343 µM	0.0428 µM
Kynurenine	0.336 µM	0.084 µM
Quinolinic Acid	0.105 µM	0.0262 µM
Serotonin	1.5435 µM	0.77175 µM

This chapter completes the discussions on the methods and the validation of the methods used for the determinations of tryptophan and its metabolites, as well as that for the cytokines. Before presentation of the results of these analyses on the blood of the patients and the controls, the chapter on the quality of life of the patients studied is presented. There is no logical reason for this – merely that presentation of this chapter at a later stage will break the line of thought between results and discussion of the main experimental work. The possibility of any correlation between the quality of life and the tryptophan metabolism will be presented with the rest of the correlations tested.

3.2 References

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Chapter 4

Quality of Life and Well Being in Chronic Renal Failure

This assessment is an addendum to the study intended only to provide some added value to the illness presentation of the CRF patients at our research site and to test whether any correlation can be seen between the mental health on the one hand and tryptophan and metabolites on the other. It therefore fulfills no criteria or outcomes for the completion of this study.

4.1 Importance of tryptophan and metabolic pathways

Chapter 1 of this dissertation has reviewed tryptophan metabolism in terms of the kynurenine pathway and the production of neurotoxic kynurenine metabolites such as quinolinic acid. Tryptophan also serves as the substrate for the synthesis of serotonin as well as for protein synthesis [1]. Thus tryptophan depletion or alterations in tryptophan metabolism may have a negative influence on an individual's physical and mental well being. A shift of tryptophan metabolism towards the kynurenine pathway is dependant on the enzyme activity. One such enzyme, IDO, is induced under immune activation and pro-inflammatory cytokine activity [2,3]..

Should tryptophan levels be depleted, or should a shift in favour of one, and at the cost of another tryptophan metabolic pathway occur, it could have physical as well as psychological consequences. Therefore, excessive activity of any factor that influences the enzymatic activity involved in tryptophan metabolism can have a widespread influence. Psychopathology as a result of tryptophan depletion, has for instance been described in diseases accompanied by chronic immune activation and inflammation [1,3]. Inflammation and immune activation in HD and PD patients have been discussed in chapter 1.

It can thus be seen that tryptophan depletion as well as alterations in tryptophan metabolism can have an influence on the quality of life and well being in susceptible and compromised individuals. It therefore justifies this study to also evaluate quality of life

and well being in the dialysis patients who, as will be shown in the later chapters, are shown to be tryptophan depleted and have alterations in tryptophan metabolism.

4.2 Study subjects and Methods

4.2.1 Participants

The same patients, who were involved in the main study and from whom blood collections were obtained for the tryptophan and metabolite analysis, were earmarked for this subdivision. Patients were recruited from the Pretoria Academic Hospital, Department of Nephrology, haemodialysis and peritoneal dialysis ward. Ethical clearance for the addition to the study was obtained from the Faculty of Health Sciences Research and Ethics Committee, University of Pretoria, No. S168/2006. Informed consent forms were signed by all patients. Patients were informed about the questionnaires that were to be conducted and about their participation in this study. Questionnaires on the medical short form-36 were completed upon the patients' scheduled visits to the hospital. Peritoneal dialysis patients were scheduled to visit the ward once a month. Questionnaires were completed on the day of each of the PD patient's hospital visit.

4.2.2 Data collection

Information about the study protocol was presented to the dialysis staff prior to commencement of the study. The medical outcomes general health and well being self assessment scale (SF-36) was completed by the patient with the assistance of the dialysis nurse whenever necessary.

4.2.2.1 Medical Outcomes Short Form (SF-36)

The Medical Outcomes Short Form is an index used to assess the general health and well being of the patient. The SF-36 instrument provides scores for general health, pain, mental and physical health, role function (emotional), vitality and social function. It is therefore possible to obtain information with regards to the individual's perception on his/her personal health in general [4].

4.2.3 Data Analysis

The SF36 scores for the haemodialysis and peritoneal dialysis patients were compared to each other using Mann-Whitney ranks (BMDP Statistical Software, Inc.). Statistical inference for the analysis was performed using correction for non-parametric distribution of data with *P* values of < 0.05 considered as statistically significant. In addition to this analysis, the scores of the scales were compared to demographics, such as age, number of years on treatment, and clinical variables to investigate whether trends existed in the scoring patterns.

The SF-36 scores were correlated with the biochemical and immunological results obtained from the main study on tryptophan, kynurenine and immune activation in chronic renal failure patients. This was done by using Pearson and Spearman correlations in order to correlate the SF-36 scores with tryptophan, kynurenine/tryptophan ratio and quinolinic acid for the haemodialysis and peritoneal dialysis groups.

4.3 Results

4.3.1 Demographic and SF-36 scale results

Table 4.1 Gender, age and mean questionnaire results for haemodialysis patients

No.	Gender	Age	Ethnicity	Years on treatment	SF36
HD1	M	48	W	3	122
HD2	M	45	B	7	113
HD3	M	36	W	2	123
HD5	F	54	B	22	102
HD7	M	57	B	8	101
HD8	M	38	B	3	107
HD9	M	32	B	1	125
HD10	M	27	B	5	95
HD15	M	48	B	6	114
HD16	F	24	B	8	120
HD18	F	39	B	2	95
HD21	F	39	B	2	114
HD25	M	25	B	3	95
HD28	M	42	B	2	100
HD32	M	44	B	13	100
Mean		**39.9**		**5.8**	**108.4**
SD		**9.98**		**5.5**	**10.92**

Table 4.2 Gender, age and mean questionnaire results for peritoneal dialysis patients

No.	Gender	Age	Ethnicity	No. Years on treat	SF36
PD1	F	42	B	8	106
PD3	M	57	W	3	106
PD4	F	23	B	3	99
PD6	M	24	B	6	110
PD10	F	39	B	5	109
PD13	M	19	B	2	102
PD14	M	34	B	8	106
PD15	F	30	B	4	114
PD16	M	25	B	8	99
PD17	F	50	B	3	105
PD18	F	51	W	2	116
PD19	M	34	W	2	104
PD21	M	31	W	2	102
PD22	F	47	B	6	102
PD23	M	48	B	3	104
Mean		**36.9**		**4.3**	**105.6**
SD		**11.8**		**2.3**	**4.94**

Demographic and scale score results for haemodialysis and peritoneal dialysis groups are presented in tables 4.1 and 4.2 respectively. For the haemodialysis participants 73% were male and 87% of the group was from the South African Black population. The mean haemodialysis patient age was 40 years (range, 24-57). 53% of the peritoneal dialysis patients were male and 73% of the group was from the South African Black population. The mean age was 37 (range, 19-57). The number of years on treatment for the haemodialysis patients ranged from just over a year to 22 years with a mean of 5.8 years SD 5.544 on treatment. Patients from the peritoneal dialysis group underwent dialysis treatment with the number of years on treatment ranging from 2 to 8 years with a mean of 4.3 SD 2.32 years.

4.3.2 Results for statistical analysis

Table 4.3 gives the age, number of years on treatment and the gender distribution for the haemodialysis and peritoneal dialysis patients. No statistical difference was indicated for age (p=0.4695) and average years on treatment (p=0.3444) between the haemodialysis and peritoneal dialysis groups.

Table 4.3 Age, gender and treatment details of the two patient groups

Patient Group	Mean age in years	SD age	Age Distribution	Average Years on Treatment (SD)	Years on Treatment (range)	Gender Distribution			
						Male		Female	
						n	%	n	%
HD (n=15)	39.9	9.98	24 - 57	5.8 (5.54)	1 - 22	11	73.3	4	26.7
PD (n=15)	36.9	11.8	19 - 57	4.3 (2.32)	2 - 8	8	53.3	7	46.7

Table 4.4 shows the medical short form-36 data for the haemodialysis and peritoneal dialysis patients. The mean scores of the two patient groups were statistically compared with each other.

Table 4.4 SF36 data for haemodialysis and peritoneal dialysis patient groups

SF-36 Scoring	n	Mean	SD	Range	Median	SEM
*# HD	15	109.36	10.66	95 – 125	110	2.85
*# PD	15	105.60	4.934	99 – 116	105	1.28
Test statistics for * Separate T: $P = 0.2443$ and # Mann-Whitney: $P = 0.4976$						

Comparison of SF36 data for HD and PD groups gave insignificant *P* values of 0.2442 and 0.4976 for separate and Mann-Whitney test statistics respectively.

Figure 4.1 indicates a bar graph plot for the break down of the SF-36 scores into eight different categories. The data is obtained from Table 4.8 which shows the mean scores and *P* values obtained for comparison between the two patient groups.

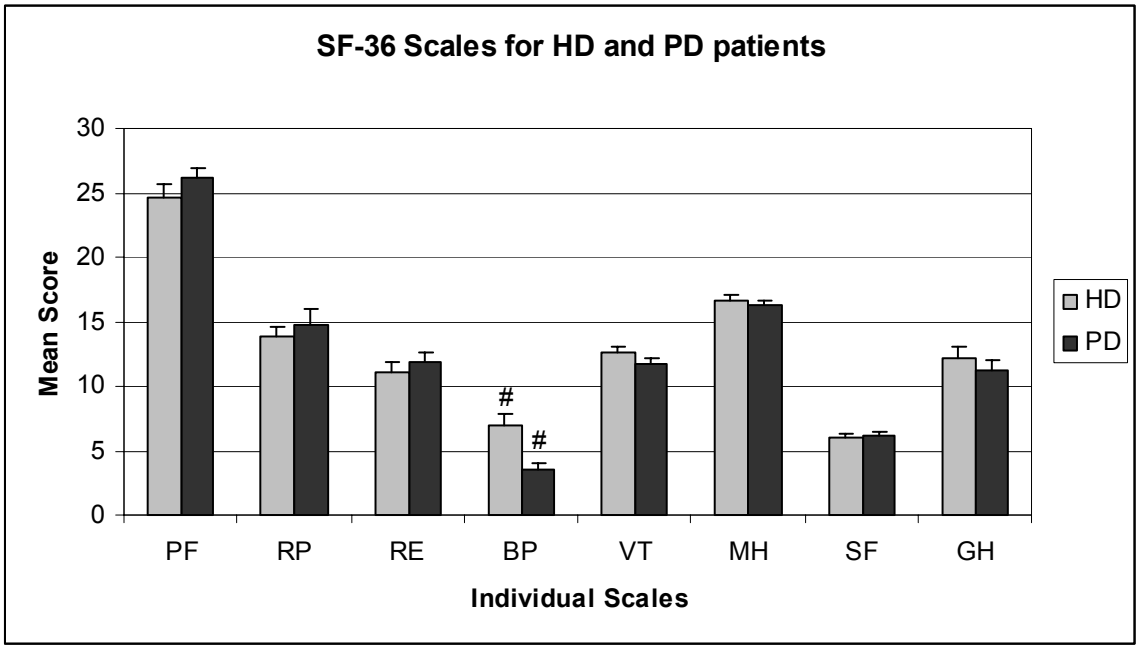

Figure 4.1 SF-36 Measurement Model for HD and PD patients. Error bars indicate standard errors. (# $p<0.05$). Physical Functioning (PF); Role-Physical (RP); Role-Emotional (RE); Bodily Pain (BP); Vitality (VT); Mental Health (MH); Social Functioning (SF); General Health (GH)

Table 4.5 SF-36 scale category breakdown for HD and PD patients

Scales		**HD** (n=15)	**PD** (n=15)	***P*-value**
Functioning (PF)Physical	Mean Score [a]	24.57(3.936)	26.13(3.044)	0.240515
	Range	16 – 30	20 – 30	
	median	26	27	
Role-Physical (RP)	Mean Score [a]	13.79(3.309)	14.8(4.799)	0.516192
	Range	7 – 20	5 – 20	
	median	14	16	
Role-Emotional (RE)	Mean Score [a]	11.14(2.507)	11.87(2.90)	0.479749
	Range	7 – 15	5 – 15	
	median	11	12	
* Bodily Pain (BP)	Mean Score [a]	7(3.397)	3.47(1.959)	* 0.00180
	Range	2 – 11	2 – 9	
	median	7.5	3	
Vitality (VT)	Mean Score [a]	12.57(2.138)	11.73(1.387)	0.218087
	Range	9 – 16	10 – 14	
	median	12.5	12	
Mental Health (MH)	Mean Score [a]	16.57(1.869)	16.27(1.163)	0.599639
	Range	13 – 19	14 – 18	
	median	16.5	17	
Social Functioning (SF)	Mean Score [a]	6(1.109)	6.2(0.775)	0.575971
	Range	4 – 8	5 – 8	
	median	6	6	
General Health (GH)	Mean Score [a]	12.14(3.613)	11.27(2.915)	0.477034
	Range	5 – 19	4 – 15	
	median	12	12	
[a] Reported as mean (standard deviation). * $P < 0.05$				

The statistical analysis results for correlations between the test scores and patient demographics (age and number of years on treatment) gave correlation coefficients of $r < 0.5$ with no statistically significant results ($p>0.05$). The mean total SF-36 scores of the two patient groups were correlated with the urea, albumin and haemoglobin using Spearman correlation coefficients. The correlation coefficients (r value) for the HD group were r= -0.02 (p=0.93), r= -0.44 (p=0.103), r= -0.39 (p=0.15) respectively for urea, albumin and haemoglobin. Correlations for the PD group were r = -0.35 (p=0.197),

r= -0.14 (p=0.615), r=0.45 (p=0.092) respectively for urea, albumin and haemoglobin. There were no statistically significant correlations in these data.

The scores of the scales were subsequently compared to the biochemical values from the tryptophan study. Pearson and Spearman correlation coefficients gave the degree of correlation with the respective r values. Spearman correlations indicated correction for non-parametric distribution of data.

Table 4.6 SF-36 Pearson correlations for the haemodialysis group. Gray shading indicates statistically significant correlation

		Tryptophan	**Quinolinic acid**	**Kyn/Trp**
SF-36	Correlation Coefficient	**0.6088**	- 0.3959	- 0.2933
	P-value	**0.016**	0.1441	0.2887

Table 4.7 SF-36 Spearman correlations for the haemodialysis group. Gray shading indicates statistically significant correlation

		Tryptophan	**Quinolinic acid**	**Kyn/Trp**
SF-36	Correlation Coefficient	**0.5404**	-0.4255	-0.35728
	P-value	**0.0375**	0.1138	0.1911

Table 4.8 SF-36 Pearson correlations for the peritoneal dialysis group. Gray shading indicates statistically significant correlation

		Tryptophan	**Quinolinic acid**	**Kyn/Trp**
SF-36	Correlation Coefficient	-0.16084	-0.10121	**0.61535**
	P-value	0.5669	0.7197	**0.0146**

Table 4.9 SF-36 Spearman correlations for the peritoneal dialysis group. Gray shading indicates statistically significant correlation

		Tryptophan	Quinolinic acid	Kyn/Trp
SF-36	Correlation Coefficient	-0.07748	-0.06487	**0.50993**
	P-value	0.7837	0.8183	**0.0521**

4.4 Discussion

Mental health problems such as depression are often overlooked and therefore untreated in dialysis patients, and this may result in diminished quality of life for the patient [5,6,7]. The objectives for this study were to assess the general mental health and well being in the haemodialysis and peritoneal dialysis groups and to see if any relationship is indicated with the psychological assessments and tryptophan and its metabolites.

General health and well being for the haemodialysis and peritoneal dialysis groups were tested using the Medical Outcomes Short Form 36. 15 haemodialysis and 15 peritoneal dialysis patients underwent SF36 testing. The mean SF-36 scores obtained were 108.4 SD 10.9 and 105.6 SD 4.9 for the haemodialysis and peritoneal dialysis groups respectively. There was no statistically significant difference (p=0.4976) in the SF-36 scores between the haemodialysis and peritoneal dialysis groups. The SF-36 scale is divided into 8 categories and these were individually compared between the haemodialysis and peritoneal dialysis groups. The only statistically significant difference between the two groups was bodily pain (p=0.0018) which was higher in the haemodialysis group.

The SF-36 scores were subsequently compared to the biochemical values. The comparisons were performed in order to determine if there was any link between tryptophan; kynurenine/tryptophan ratio and quinolinic acid with the SF-36 scores. These were indicated by Pearson and Spearman correlation coefficients whereby r-values of 0.8 to 1 gave a ‘strong’ correlation. Correlations of r-values around 0.5 were considered as possible correlations and all statistically significant correlations were indicated by *P*-

values less than 0.05. Non significant but possible correlations of r-values around 0.05 were considered.

The SF-36 scores of the haemodialysis and peritoneal dialysis patients were correlated with the biochemical values. Pearson correlation for the haemodialysis group gave a statistically significant correlation for SF-36 with tryptophan (r=0.6088; p=0.016). The Spearman correlation for the haemodialysis group gave a statistically significant correlation for SF-36 with tryptophan (r=0,5404; p=0.0375). For the peritoneal dialysis group Pearson correlations gave a statistically significant correlation for SF-36 with Kyn/Trp ratio (r=0.6153; p=0.0146). Similarly a Spearman correlation was indicated for SF-36 with Kyn/Trp ratio (r=0.50993; p=0.05).

This study has shown that there is no significant difference in the quality of life, as measured by SF-36 testing, between HD and PD patients with the exception of bodily pain. Bodily pain has been known to have an impact on the quality of life in dialysis patients [8]. This may give indication that HD patients may have a lower quality of life due to the influence of bodily pain as a result of the treatment modality. However further studies focusing particularly on bodily pain between HD and PD patients are required to substantiate this.

The correlations of the SF-36 scoring with the tryptophan and biochemical metabolites have indicated significant correlations for the SF-36 with tryptophan and the Kyn/Trp ratio. This gives reason to believe that tryptophan and the metabolic pathways may have an influence on the quality of life in chronic renal failure patients on dialysis treatment. A limitation to this study is that patients were confined to one study unit, and a broader study population from different dialysis regions is required for future studies in this field.

The next chapter represents the results for the tryptophan and metabolite analysis in the patient and control groups. Results for the statistical analysis and correlation of data are also given.

4.5 References

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Chapter 5

Results for tryptophan and metabolite analysis

This chapter deals with the presentation and statistical analysis of the results obtained from the analysis of tryptophan and kynurenine metabolites for the three study groups. The analyses were divided into the 3 groups, namely the volunteer control (VC) group, the haemodialysis (HD) patient group and the peritoneal dialysis (PD) patient group. The VC group consisted of 12 age, gender and race matched controls. Controls were recruited as volunteers from the general public. All participants gave written informed consent and the clearance number for the project was S168/2006.15 HD and 15 PD patients were recruited from the Pretoria Academic Hospital; Department of Nephrology.

5.1 Statistical Analysis of Results

Results of the volunteer control group, haemodialysis patient group and peritoneal dialysis patient group were compared with each other. Data from the 3 groups were compared by using Kruskal-Wallis one way analysis of variance testing and multiple comparison testing. The 2 patient groups were compared with each other using Mann-Whitney tests. Furthermore specific correlations of results within each group were determined using Pearson and Spearman Correlation Coefficients. Statistical inference for the analysis was performed using correction for non-parametric distribution of data with P values of < 0.05 considered as statistically significant.

5.2 Study groups and information

In table 5.1 the age and gender details for the control group are given. The age, gender and dialysis history for the haemodialysis and peritoneal dialysis groups are given in tables 5.2 and 5.3 respectively. Tables 5.4 and 5.5 represent the blood test results for urea, albumin and haemoglobin, as captured from the patients' hospital files. Figure 5.1 represents a bar graph illustration for the results from tables 5.4 and 5.5.

Table 5.1 Age and gender of the volunteer control group

Volunteer Control	Age (years)	Gender
V1	45	f
V2	34	m
V3	39	m
V4	23	m
V5	34	m
V6	48	m
V7	49	m
V8	20	m
V9	50	f
V10	27	f
V11	35	f
V12	27	m
Mean	**35.9**	
SD	**10.4**	

Table 5.2 Age, gender and dialysis history of the HD patient group

HD patients	Age	Gender	Start of treatment	Years on treatment	Duration of treatment
HD1	47	m	19/10/2005	>2years	3hrs x 3/week
HD2	44	m	28/03/2001	>6years	4.5hrs x 3/week
HD3	35	m	28/06/2006	>1year	3hrs x 3/week
HD5	53	f	1/11/1986	>21years	4hrs x 3/week
HD7	56	m	8/5/2001	>7years	4hrs x 3/week
HD8	37	m	10/5/2005	>2years	4hrs x 3/week
HD9	31	m	14/03/2006	>1year	4hrs x 3/week
HD10	26	m	7/7/2003	>4years	4hrs x 3/week
HD15	47	m	21/05/2002	>5years	4hrs x 3/week
HD16	23	f	4/4/2000	>7years	4hrs x 3/week
HD18	38	f	23/12/2005	>1year	4hrs x 3/week
HD21	38	f	11/3/2006	>1year	4hrs x 3/week
HD25	24	m	15/07/2005	>2years	4hrs x 3/week
HD28	41	m	19/05/2006	>1year	3hrs x 3/week
HD32	43	m	18/10/1995	>12years	4hrs x 3/week
Mean	**38.7**				
SD	**9.98**				

Table 5.3 Age, gender and dialysis history of the PD patient group

PD patients	Age	Gender	Start of treatment	Years on treatment	Duration of treatment/frequency
PD1	41	f	28/06/2001	>7years	3 x2.5%;1 x1.5%/24hrs
PD3	56	m	13/05/2005	>2years	3 x2.5%;1 x1.5%/24hrs
PD4	22	f	2/12/2005	>2years	1 x4.25%;3 x2.5%/24hrs
PD6	23	m	30/08/2002	>5years	1 x4.25%;2 x2.5%/24hrs
PD10	38	f	12/06/2003	>4years	2 x2.5%;2 x1.5%/24hrs
PD13	18	m	21/07/2006	>1year	3 x1.5%;3 x2.5%/24hrs
PD14	33	m	22/06/2001	>7years	1 x1.5%;2 x2.5%/24hrs
PD15	29	f	1/10/2004	>3years	2 x1.5%;1 x4.25%/24hrs
PD16	24	m	30/11/2001	>7years	3 x2.5%/24hrs
PD17	49	f	22/07/2005	>2years	2 x2.5%;1 x1.5%/24hrs
PD18	50	f	30/06/2006	>1year	1 x2.5%;3 x1.5%/24hrs
PD19	33	m	25/06/2006	>1year	3 x1.5%/24hrs
PD21	31	m	4/08/2006	>1year	4 x1.5%/24hrs
PD22	46	f	12/11/2002	>5years	3 x2.5%;1 x1.5%/24hrs
PD23	47	m	20/09/2005	>2years	3 x2.5%;1 x1.5%/24hrs
Mean	**36**				
SD	**11.77**				

Table 5.4 Blood test results from haemodialysis patients' files

HD patients	Urea (mmol/L)	Albumin (g/L)	Hb (g/dL)
HD1	25.9	29	7.4
HD2	34	31	9.4
HD3	34.2	29	7.1
HD5	26.8	36	10.2
HD7	27.4	31	10.3
HD8	16.5	32	14
HD9	22.8	35	7.2
HD10	26.7	36	10.1
HD15	32.9	36	9.8
HD16	27	30	6.5
HD18	20.9	32	8.6
HD21	17.4	34	7.8
HD25	33.9	35	7
HD28	44.7	38	7.7
HD32	25	32	11.4
Mean	**27.74**	**33.07**	**8.97**
SD	**7.32**	**2.84**	**2.05**

Table 5.5 Blood test results from peritoneal dialysis patients' files

PD patients	Urea (mmol/L)	Albumin (g/L)	Hb (g/dL)
PD1	16.5	33	12.3
PD3	29.5	29	13.1
PD4	38.4	36	11.6
PD6	28.1	28	11
PD10	12.3	26	11.9
PD13	10.1	32	10.6
PD14	25.5	34	12.3
PD15	25.8	34	12.8
PD16	49.9	20	8.1
PD17	12.9	34	10.4
PD18	21.1	30	8.7
PD19	17.5	20	8.7
PD21	37.7	39	11.8
PD22	21.2	32	8.4
PD23	31.8	32	8.2
Mean	**25.22**	**30.6**	**10.66**
SD	**11.1**	**5.36**	**1.8**

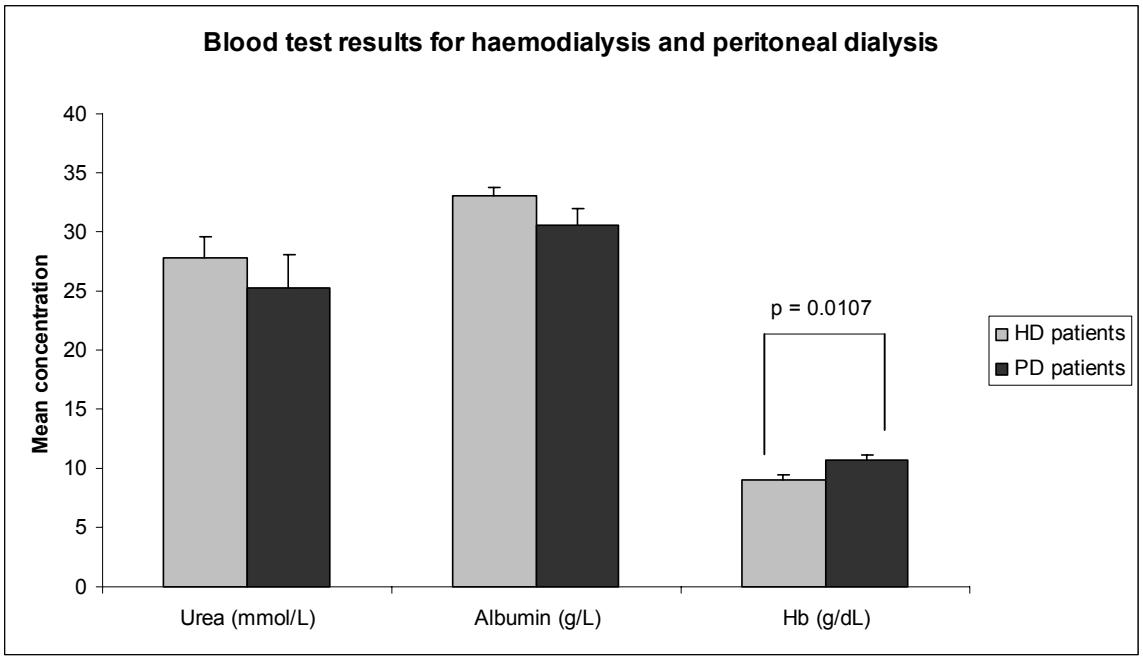

Figure 5.1 Bar graph illustration of urea, albumin and haemoglobin blood test results for the haemodialysis and peritoneal dialysis patient groups. Error bars indicate standard errors

5.3 Results for tryptophan and metabolite analysis

The results obtained for the GC-MS analysis of tryptophan, kynurenine and quinolinic acid. Results are given as the mean of duplicate measurements. The concentrations of each analyte were obtained by extrapolation of data from standard curves drawn up for each day and assay analysis. External quality controls (low and high concentrations) were analyzed between batches (analytical runs) to ascertain acceptance/rejection of the analyses. The concentration results for each analyte per group are presented in the following tables.

Table 5.6 Tryptophan, kynurenine, quinolinic acid and kyn/trp ratio of the control group

Volunteer Control	Tryptophan (µM)	Kynurenine (µM)	Quinolinic Acid (µM)	Kyn/Trp
V1	29.9264	2.86198	0.28661	0.095634
V2	28.1649	2.26947	0.65601	0.080578
V3	32.1472	1.34126	0.2306	0.0417225
V4	24.2556	2.65734	0.219	0.1095557
V5	34.3072	2.01099	0.4612	0.0586171
V6	24.1529	2.52979	0.4752	0.1047406
V7	21.2275	2.26881	0.2793	0.1068807
V8	32.6076	2.63951	0.4145	0.0809477
V9	29.7721	2.0156	0.1926	0.067701
V10	27.0816	1.36511	0.2056	0.0504073
V11	23.6043	0.96458	0.2427	0.0408646
V12	33.1113	2.57572	0.1663	0.0777898
Mean	**28.4**	**2.1**	**0.3**	**0.08**
SD	**4.3**	**0.6**	**0.15**	**0.02**

Table 5.7 Tryptophan, kynurenine, quinolinic acid and kyn/trp ratio of the HD group

HD patients	Tryptophan (µM)	Kynurenine (µM)	Quinolinic Acid (µM)	Kyn/Trp
HD1	2.5489	5.552	4.1738	2.1781945
HD2	12.0266	5.3592	1.8739	0.4456122
HD3	18.0922	6.6203	3.7453	0.3659201
HD5	1.7584	9.123	3.399	5.1882393
HD7	7.1338	5.1671	6.569	0.7243124
HD8	1.9731	5.2837	6.9498	2.6778673
HD9	12.88	4.6351	5.8791	0.359868
HD10	2.1979	1.8447	7.2812	0.8393012
HD15	2.9897	4.7761	5.8639	1.5975181
HD16	5.7769	4.5102	3.8153	0.7807301
HD18	3.0368	6.365	6.9888	2.0959563
HD21	3.5292	3.2458	2.8221	0.9196985
HD25	2.5817	2.7046	7.5882	1.0476043
HD28	2.0233	2.6638	5.7701	1.3165621
HD32	1.6034	2.9175	1.4253	1.8195709
Mean	**5.3**	**4.7**	**4.9**	**1.5**
SD	**5.04**	**1.9**	**2.03**	**1.2**

Table 5.8 Tryptophan, kynurenine, quinolinic acid and kyn/trp ratio of the PD group

PD patients	Tryptophan (µM)	Kynurenine (µM)	Quinolinic Acid (µM)	Kyn/Trp
PD1	13.0312	3.3948	1.2386	0.2605132
PD3	5.6319	2.9919	2.7848	0.5312417
PD4	4.1121	2.0185	2.7294	0.4908684
PD6	4.9969	3.6041	4.001	0.7212672
PD10	4.95255	2.77917	1.8012	0.5611594
PD13	7.9861	4.16072	8.4665	0.5209952
PD14	5.4641	2.199	4.0573	0.4024451
PD15	3.7598	4.1854	3.8801	1.1131975
PD16	3.3444	1.8237	1.3254	0.5452996
PD17	7.9523	3.1033	1.37768	0.3902393
PD18	5.26885	3.4369	1.081	0.6523055
PD19	13.572	3.037	0.38522	0.2237695
PD21	9.783	2.2789	1.6353	0.2329449
PD22	6.39989	2.1696	4.6909	0.3390058
PD23	4.6874	2.2114	2.2372	0.4717754
Mean	**6.7**	**2.9**	**2.8**	**0.5**
SD	**3.2**	**0.8**	**2.03**	**0.2**

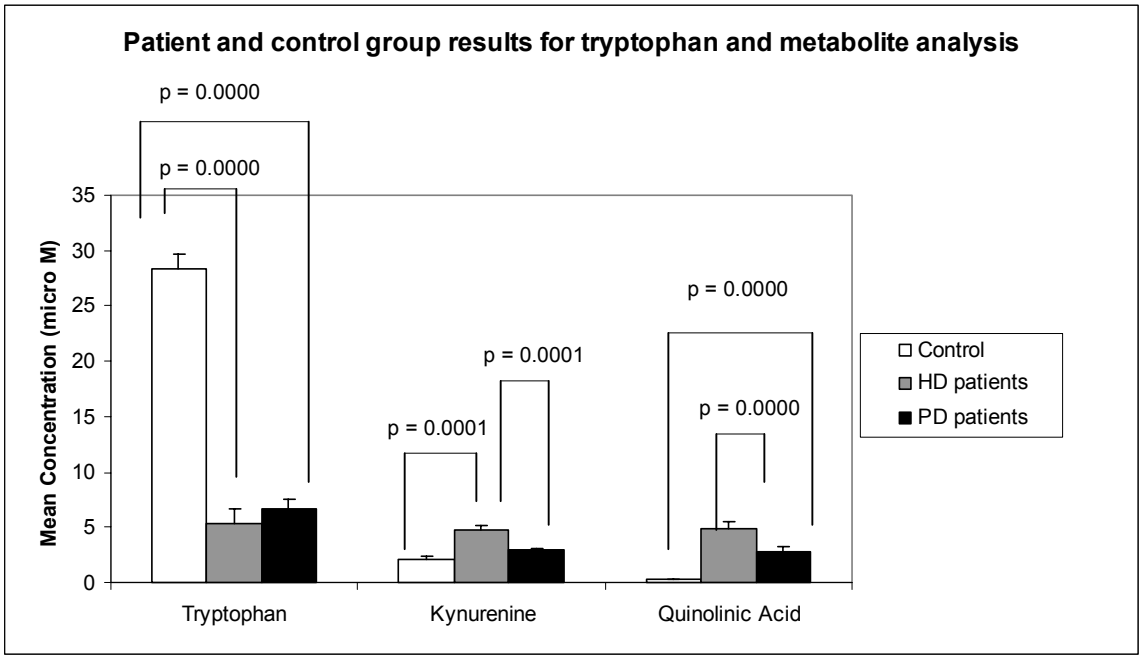

Figure 5.2 Bar graph illustrating tryptophan and metabolites for the 3 study groups. Error bars indicate standard errors

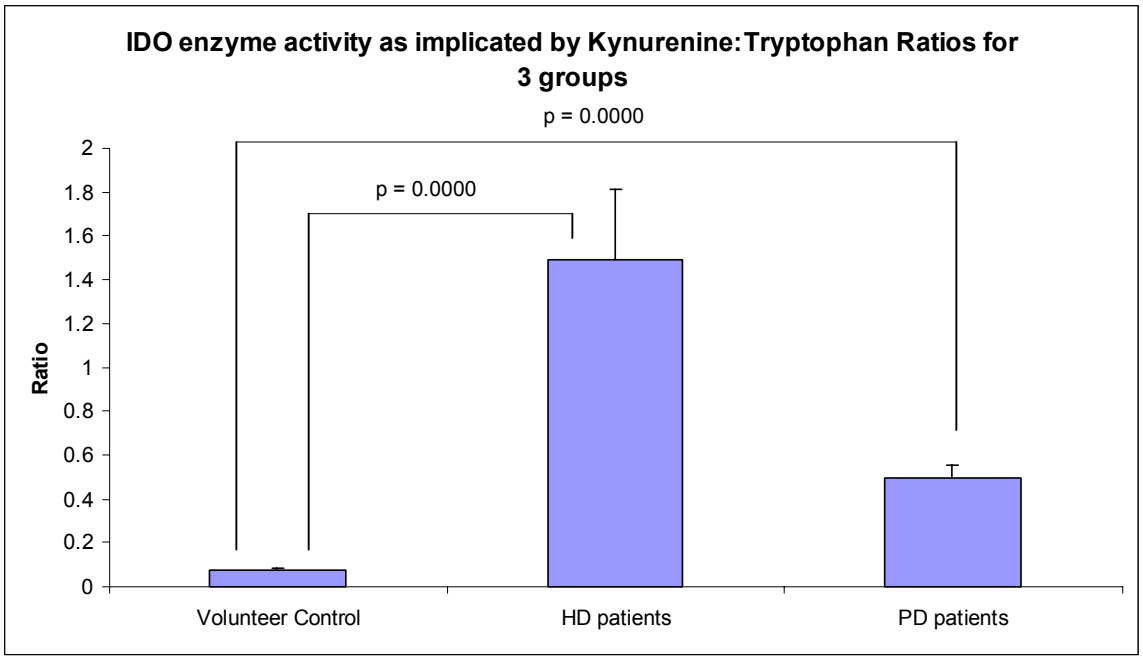

Figure 5.3 Bar graph illustrating kynurenine : tryptophan ratio as an implication of IDO enzyme activity. Error bars indicate standard errors

Figure 5.2 shows the distribution of the tryptophan and metabolite concentrations over the 3 study groups. From the graph it can be seen that there is tryptophan depletion in the HD and PD patient groups as compared to the control group ($p<0.05$). Conversely there is a simultaneous increase in the kynurenine and quinolinic acid concentrations in the two patient groups as compared to the control group ($p<0.05$). An increased kynurenine to tryptophan ratio in the patient groups as compared to the volunteer group ($p<0.05$), is shown in figure 5.3, and this gives an implication for increased IDO enzyme activity.

Table 5.9 C-reactive protein (CRP) values for the haemodialysis and peritoneal dialysis groups. Normal reference range is 0 to 10 mg/L

HD patients	CRP (mg/L)
HD1	3.8
HD2	5
HD3	15.3
HD5	24.7
HD7	22.3
HD8	0.9
HD9	13.9
HD10	1.1
HD15	4.2
HD16	7.6
HD18	2
HD21	0.9
HD25	26.1
HD28	11.5
HD32	12.2
Mean	**10.1**
SD	**8.82**

PD patients	CRP (mg/L)
PD1	26.7
PD3	64.5
PD4	25.8
PD6	19.7
PD10	5
PD13	1.1
PD14	65
PD15	42.3
PD16	1.2
PD17	5
PD18	10.3
PD19	1.8
PD21	7.9
PD22	5
PD23	6
Mean	**19.15**
SD	**21.92**

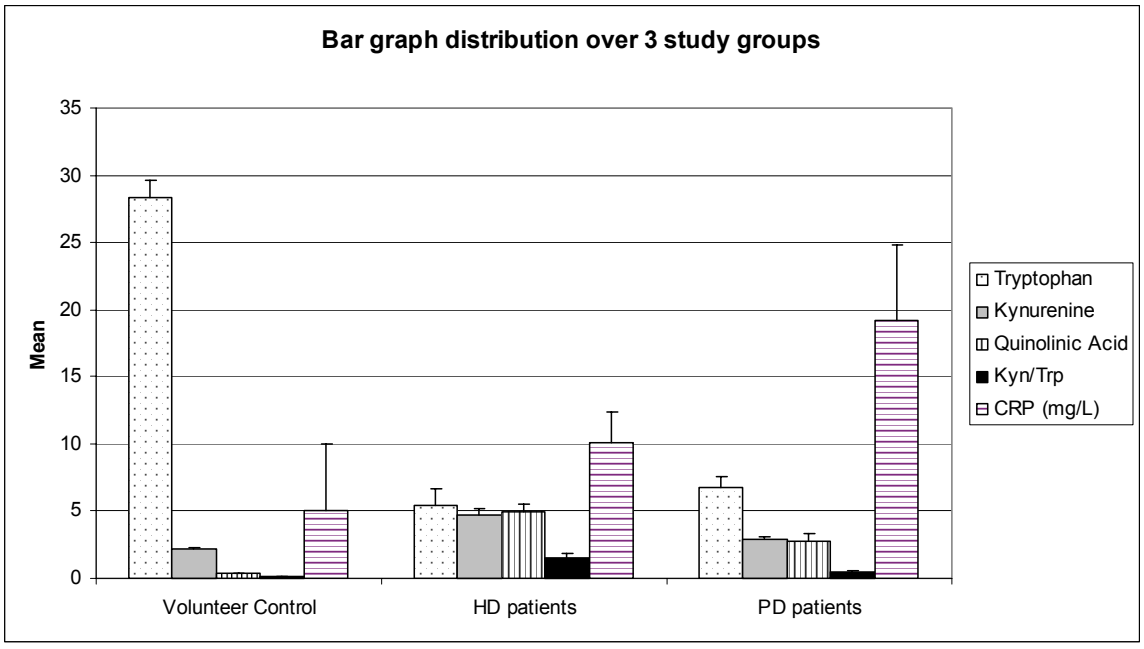

Figure 5.4 Bar graph illustrating CRP, tryptophan and metabolite results for the volunteer control HD and PD groups. CRP for volunteer group derived from normal reference range (0-10mg/L)

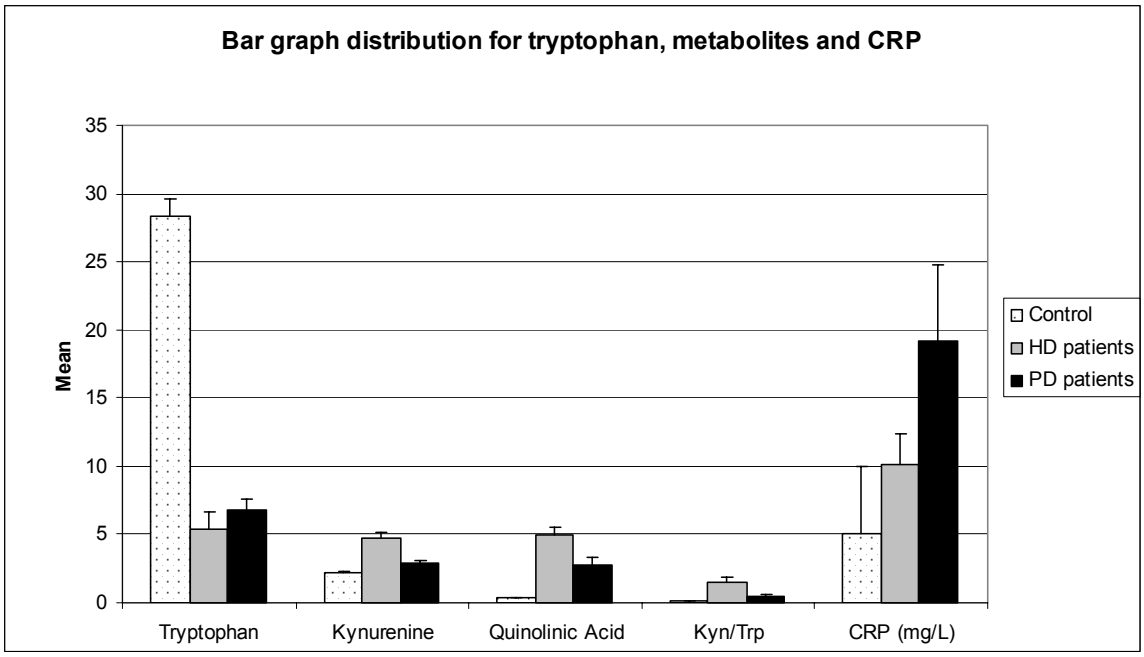

Figure 5.5 Bar graph showing tryptophan, metabolites and CRP according to category

5.4 Kruskal-Wallis test statistic results

Tryptophan, kynurenine, quinolinic acid, kyn:trp ratio were compared between the three groups. The test procedure for multiple comparisons was designed as follows:

The null hypothesis (H_0: there is no difference between the groups) was rejected if the ZSTAT value was larger than the critical Z value. With 3 groups the critical Z value was 2.39 for an overall significance level of 0.05. A Z value of 2.13 for an overall significance of level of 0.10 was also noted. Calculated *P*-values, to indicate whether comparisons were statistically significant, were obtained using Chi-Square distributions with 2 degrees of freedom. Comparisons were determined between VC-HD, VC-PD and HD-PD.

Table 5.10 Kruskal-Wallis test statistic results for 3 way group comparisons.
(# Statistically Significant Difference for overall alpha of 0.05, Z Value of 2.39)

Variable	ZSTAT Value			*P*-value
	VC-HD	VC-PD	HD-PD	
Tryptophan	5.14 #	3.70 #	1.53	0.0000
Kynurenine	4.27 #	1.92	2.49 #	0.0001
Quinolinic Acid	5.25 #	3.39 #	1.98	0.0000
Kyn:Trp	5.49 #	3.35 #	2.28	0.0000

Table 5.11 Kruskal-Wallis test statistic comparison of patient to control age demographic variable

Variable	ZSTAT Value			*P*-value
	VC-HD	VC-PD	HD-PD	
Age (Years)	0.61	0.09	0.74	0.7301

Table 5.11 indicates that there was no significant difference between the ages of the three groups. Hence the control group was appropriately matched to the patient groups.

5.5 Mann-Whitney Test Statistic Results

Data from the hospital files of the haemodialysis and peritoneal dialysis patient groups were compared with each other using Mann-Whitney test statistic (2 group comparison). This was performed using normal two-tail approximations. *P*-values of less than 0.05 were considered as statistically significant.

Table 5.12 Mann-Whitney Test Statistic HD vs. PD comparison. ([#] Statistically Significant Difference)

HD vs. PD	*P*-Value
Urea	0.3837
Albumin	0.2339
HB	0.0107 [#]
Years on treatment	0.8320

5.6 Within group tryptophan and analytes correlations

Correlations were determined from the data for each of the study groups. Tryptophan was correlated to the data of each group using Pearson and Spearman correlation coefficients. Significant correlations were indicated by a *P*-value of less than 0.05. Correlation coefficients range from 0 to 1 with values of 0.8 to 1 considered as 'strong' correlations. Correlations coefficients ranging around 0.5 indicate evidence for possible correlations of data which are however 'weak' correlations.

5.6.1 Correlations for volunteer control group

Table 5.13 Pearson Correlations for VC group

		Kyn	QA	Age
Tryptophan	Correlation Coefficient	0.112	0.062	-0.305
	P-value	0.729	0.848	0.336

Table 5.14 Spearman Correlations for VC group

		Kyn	QA	Age
Tryptophan	Correlation Coefficient	0.154	-0.056	-0.358
	P-value	0.633	0.863	0.253

5.6.2 Correlations for haemodialysis patient group

Table 5.15 Pearson Correlations for HD group

		Kyn	QA	Urea	Alb	Hb	Age	Treat
Tryptophan	Correlation Coefficient	0.268	-0.23	0.183	-0.422	-0.36	-0.11	-0.242
	P-value	0.335	0.41	0.512	0.117	0.189	0.71	0.384

Table 5.16 Spearman Correlations for HD group (Grey shading indicates possible correlations)

		Kyn	QA	Urea	Alb	Hb	Age	Treat
Tryptophan	Correlation Coefficient	0.235	-0.03	0.243	**-0.456**	**-0.52**	-0.20	-0.338
	P-value	0.397	0.92	0.383	**0.087**	**0.044**	0.47	0.218

5.6.3 Correlations for peritoneal dialysis patient group

Table 5.17 Pearson Correlations for PD group

		Kyn	QA	Urea	Alb	Hb	Age	Treat
Tryptophan	Correlation Coefficient	0.199	-0.24	-0.44	-0.03	0.01	0.08	-0.14
	P-value	0.48	0.39	0.10	0.92	0.97	0.78	0.62

Table 5.18 Spearman Correlations for PD group (Grey shading indicates possible correlation)

		Kyn	QA	Urea	Alb	Hb	Age	Treat
Tryptophan	Correlation Coefficient	0.26	-0.18	**-0.53**	0.097	0.093	0.21	-0.34
	P-value	0.35	0.52	**0.044**	0.73	0.742	0.44	0.215

C-reactive protein (CRP) levels for the HD and PD groups were correlated with tryptophan, kynurenine, quinolinic acid, kyn/trp ratio, urea, albumin and haemoglobin. The following tables give the correlation coefficients for the correlations.

Table 5.19 Pearson correlation coefficients for the haemodialysis group

		Trp	Kyn	QA	Kyn/Trp	Urea	Albumin	Hb
CRP	Correlation Coefficient	0.15863	0.2287	0.04803	0.16432	0.36261	0.1409	-0.17454
	P-value	0.5723	0.4123	0.865	0.5584	0.1841	0.6165	0.5338

Table 5.20 Spearman correlation coefficients for the haemodialysis group. Grey shading indicates possible correlation

		Trp	Kyn	QA	Kyn/Trp	Urea	Albumin	Hb
CRP	Correlation Coefficient	0.13047	0.09115	-0.05898	-0.24486	**0.55585**	0.03069	-0.23235
	P-value	0.643	0.7466	0.8346	0.3791	**0.0314**	0.9135	0.4047

Table 5.21 Pearson correlation coefficients for the peritoneal dialysis group. Grey shading indicates a 70% strong correlation

		Trp	Kyn	QA	Kyn/Trp	Urea	Albumin	Hb
CRP	Correlation Coefficient	-0.21537	0.06267	0.12762	0.26058	0.12661	0.27575	**0.70773**
	P-value	0.4408	0.8244	0.6504	0.3482	0.653	0.3198	**0.0032**

Table 5.22 Spearman correlation coefficients for the peritoneal dialysis group. Grey shading indicates a 73% strong correlation

		Trp	Kyn	QA	Kyn/Trp	Urea	Albumin	Hb
CRP	Correlation Coefficient	-0.15412	0.08961	0.1828	0.17921	0.29032	0.43542	**0.73788**
	P-value	0.5834	0.7508	0.5143	0.5228	0.2939	0.1048	**0.0017**

This concludes the chapter for the results of the tryptophan and metabolite analysis. The next chapter deals with the discussion of the method validation and the results of the study.

Chapter 6

Discussion

Tryptophan-related abnormalities are probably much more common than generally assumed. This may perhaps be more applicable in Africa and other Third World Countries than anywhere else as malnutrition, as well as infections and inflammatory disorders may lead to tryptophan depletion and accumulation of toxic metabolites of tryptophan degradation. In spite of this, services for the determination of the said substances in body fluids are not readily available. The primary aim of this study therefore was to develop and validate methods for such determinations. A secondary aim was to examine the levels of these substances in the blood of chronic renal failure patients on dialysis and to compare the levels of tryptophan and that of two metabolites of the kynurenine pathway, a major route of tryptophan metabolism, in the blood of haemodialysis and peritoneal dialysis patients. In addition, certain preliminary investigations were performed in order to obtain guidelines for further investigations in this field. These preliminary studies include: a) a look at possible immune activation to confirm the assumption that chronic renal failure patients usually exist in a pro-inflammatory state – a condition that could, in addition to the poor protein intake, have deleterious effects on the status of tryptophan and its metabolites, b) investigating the quality of life of these patients as the proposed disturbances of tryptophan metabolism are bound to have an effect on quality of life, and c) seeing whether any link is indicated between disturbances in tryptophan metabolism on the one hand, and the degree of uraemia as reflected in urea concentration, the nutritional status as reflected by albumin, and the haemoglobin levels – a parameter known to be influenced by abnormal tryptophan metabolism, on the other hand.

The first objective was therefore to establish the facilities to measure the said metabolites. The measurement of tryptophan and its metabolites requires sensitive and reliable methods which are not readily available. Many pitfalls beyond the chemical analyses are present in the execution of these determinations. For instance, indole species such as tryptophan and serotonin are light and temperature sensitive and this adds to the difficulty

of such measurements. Furthermore the chromatographic and chemical properties of tryptophan and its metabolites differ and this contributes to difficulty in measuring these analytes simultaneously. In addition, stability is a factor to consider and the use of labelled stable isotopes for tryptophan and serotonin had to be employed during quantification as these served as compensatory internal standards. Hence changes in tryptophan and serotonin as a result of environmental and sample preparation were to appropriately reflect and be compensated for by the levels of the labelled internal standards.

Having fulfilled the first aim of this study, i.e., to develop a reliable and repeatable method to determine the blood concentrations of the metabolites of interest, it was necessary to validate the method. Given that no method is 100% accurate, validation was imperative to determine performance and reliability of the method and to identify the proportional errors. Once the method was validated, the metabolites from the patient and control samples were quantified. This was followed by capturing of the data and statistical analysis. The additional assessments, mentioned earlier, were performed around the time of blood sampling.

The first part of this discussion therefore concentrates on the development and evaluation of the methods for the analysis of tryptophan and its metabolites.

6.1 Method development, design and validation

As previously mentioned, a major part of this study was the development of the analytical methods for the quantification of tryptophan and metabolites. This was done by trial and error testing of different methods until a suitable method was obtained. The final method settled on was gas chromatography coupled to mass spectrometry. Gas chromatography when coupled to mass spectrometry is a very sensitive and selective instrumentation that allows identification, separation and simultaneous measurement of specific analytes. The major outcome for this study was the development of a technique for the determination of

tryptophan, kynurenine, serotonin and quinolinic acid from blood samples by GC-MS determination.

Firstly, it was important to obtain unique mass spectrums for each of the analytes to establish the individual chromatographic peaks used for measurements. Electron impact ionization enabled the determination of unique mass spectra from which selected ions were chosen for each specific analyte. The ion with the highest mass to charge ratio on a given mass spectrum is the considered to be the most abundant ion and is used for the quantification of data. With the use of the electron impact ionization source the most abundant selected ions were 276, 266, 451 and 282 for tryptophan, kynurenine, serotonin and quinolinic acid respectively. The same procedure of selecting the most appropriate and abundant ions was carried out for the internal standards and when using the negative chemical ionisation source. A problem posed prior to this was to first select the most appropriate derivatizing agent/s that could bind to the functional groups of all the analytes. After testing the effects of numerous derivatizing agents on all of the analytes, PFPA and PFPOH were chosen because these agents bound to all of the functional groups of all of the analytes. This enabled simultaneous measurements of the analytes during a single chromatographic analysis.

Chromatographic difficulties encountered were peak tailing, poor peak separation and low resolution. These were partially corrected by testing of the DB-5-MS and ZB1701 GC columns and thereafter modifying instrument parameters to obtain ideal chromatographic conditions. Another problem was low sensitivity; therefore the negative chemical ionization source was used for the measurements. However, after performing multiple analyses on the GC-MS, it was noted that the sensitivity dropped and poor chromatography with high background noise resulted. This problem was resolved by cleaning of the ionization source as well as system maintenance and checkups on a regular basis. This once again stresses the importance of proper maintenance when reproducible results are of utmost importance.

In summary the optimal method for the quantification of tryptophan, kynurenine, serotonin and quinolinic acid was GC-MS using negative chemical ionisation detection and derivatization with PFPA and PFPOH. Separation of analytes was achieved a 30m DB-5-MS capillary GC column with a diameter of 250.0 µm and a film thickness 0.10 µm. The GC oven was temperature programmed to start at an initial temperature of 80^0C for 2 minutes, ramped at a rate of 20^0C to 210^0C and held at 210^0C for 1 minute. The final ramp was at a rate of 30^0C to 300^0C and held at 300^0C for 2 minutes. The chromatographic run time for a single analysis was achieved in 12.5 minutes. 1 µl of sample was injected into the system using splitless mode and helium was used as the carrier gas with a flow rate of 2.5 ml/min. The MS quad and MS source were set to temperatures of 150^0C (of maximum 200^0C) and 240^0C (of maximum 300^0C) respectively.

Development of the method was then followed by extensive validation procedures including tests for selectivity (specificity), sensitivity, linearity, bias and accuracy, precision – within and between days, stability, process sample stability and recovery.
Validation data fell within the acceptance criteria in terms of validation for the chromatographic method, and the different factors validated are discussed separately below.

Selectivity is the ability of the method to measure clearly and to differentiate the analyte/s of interest from the presence of matrix components which are expected to be present in the actual samples [1]. For the GC-MS analysis selectivity was achieved with the electron impact mass spectral source used by obtaining unique mass spectra for the individual analytes tryptophan, kynurenine, quinolinic acid and serotonin. Quantification of analyte concentrations was achieved by constructing standard calibration curves. The calibration curves were also used during validation to determine the linearity of the method with regards to relative response versus concentration. Calibration data obtained gave calibration curves with correlation coefficients of $r^2 > 0.99$ for all the analytes. This indicated a good response against concentration on a linear plot. The 95% confidence intervals and relative standard deviations (RSD) were indicated for the gradient and

intercept from the average calibration curves. The RSD for the gradient and intercept for all the calibration data were below 15%. The calibration model was further validated by determining bias and accuracy data for controls by using different calibration curves.

Bias is expressed as the percentage deviation of the calculated results from that of the accepted reference values or the expected results. Accuracy defines how accurate the measurements are with relation to the true value or mean [2]. The validation criteria for accuracy and bias were met and this indicated the appropriateness of calculating concentrations from the standard calibration curves. The average bias and accuracy data for repeat measurements of low and high controls fell within ± 15% for bias and around 100% for accuracy for all of the analytes with the negligible exception of kynurenine with a bias of 16.2 % and accuracy of 83.8% for the high controls. The significance of the bias was also tested by performing t-tests against the actual or expected values. *P*-values of less than 0.01 indicated that there was a significant difference between the calculated mean and the actual values. Ideally it would be accepted if there was relatively no difference between the calculated and actual results. There were no differences between calculated and actual results for tryptophan high ($p=0.3747$) and low ($p=0.0141$) controls. There were differences for kynurenine high and low controls ($p<0.01$). No differences were indicated for quinolinic acid high ($p=0.0836$) and low ($p=0.1337$) controls. Serotonin high ($p=0.045$) and low ($p=0.049$) controls showed similar results.

Precision measurements were performed on high and low quality controls for each analyte. Precision is the closeness of agreement between a series of analyses obtained from repeat measurements of the quality controls [3]. Repeat measurements were performed per day and the same daily repeats were performed over five consecutive days to give the between day precision. Precision is indicated by the co-efficient (CV) of variance whereby low CVs indicate good precision of the measurements. The within and between day precision for all the analytes had coefficient of variances below 10% for high controls and below 15% for low controls. The between day precision for tryptophan, kynurenine, quinolinic acid and serotonin high controls were 4.8%, 9.99%, 8.19% and 1.69% respectively. The low controls for tryptophan, kynurenine, quinolinic acid and

serotonin had between day precisions with CVs of 5.49%, 13.05%, 10.21% and 9.46% respectively.

Precision for low and high controls were also tested by drawing Levey-Jennings plots for measurements of 12 consecutive controls for each analyte. Both low and high controls fell within ± 2 SD of the mean for all the analytes. Furthermore the points on the plots were acceptably scattered over the mean with no more than 6 consecutive points on one half of the mean.

Stability is the chemical stability of the analyte in a given matrix over a defined time period [1]. This tests how stable an analyte is over a time period with the influence of environment and external factors such as storage conditions and after being processed. Stability for each of the analytes at both high and low levels were determined. The freeze/thaw experiments tested stability of the analyte over 3 cycles of repeated freezing and defrosting. A student t-test performed between the first and third cycles indicated whether there was a significant difference between samples analyzed before and after exposure to freeze/thaw cycles. A *P* value of less than 0.01 was considered as statistically significant. There was no significant difference between the first and third freeze thaw cycles for tryptophan high (p=0.43058) and low (p=0.02443) controls. Kynurenine high (p=0.2814) and low (p=0.4714) controls showed no difference between freeze thaw cycles. Similarly there was no difference for quinolinic acid high (p=0.1185) and low (p=0.9107) and serotonin high (p=0.0592) and low (p=0.1029) controls. Therefore the analytes were relatively stable through three freeze thaw cycles for both high and low controls. Processed sample stability showed acceptable percentage ratios for high and low controls exposed to autosampler conditions during each chromatographic analysis.

The recovery experiment illustrates the amount of analyte that is measured or recovered from the actual matrix of the sample. This experiment is also used to determine the proportional errors of the method. The proportional error is the difference of the recovery from 100% and indicates the error of the method from determining the total analyte response after sample workup [3]. Recovery samples for tryptophan high and low

controls were 98.9 % and 88.2% respectively. Kynurenine high and low controls gave recoveries of 87.1% and 76.2% respectively. Quinolinic acid high and low controls had 98% and 75.8% recoveries respectively. 98.4% and 75.5% were the percentage recoveries for serotonin high and low controls respectively. The proportional errors were 1.06%, 12.88%, 1.96% and 1.54% for tryptophan, kynurenine, quinolinic and serotonin high controls respectively. The low controls had proportional errors of 11.84%, 27.37%, 24.20%, and 24.51% respectively for tryptophan, kynurenine, quinolinic acid and serotonin.

The limit of quantification indicates the lowest possible concentration of a given analyte that can be quantified from the sample matrix [3]. Likewise the limit of detection indicates the lowest concentration of a given analyte that can be detected and distinguished from the background noise [3]. Signal to noise ratios of 10 and 3 were considered for the limits of quantification and detection respectively. The limits of quantification for tryptophan, kynurenine, quinolinic acid and serotonin were 0.343, 0.336, 0.105 and 1.543 μM respectively. The limits of detection for tryptophan, kynurenine, quinolinic acid and serotonin were 0.0428, 0.084, 0.026 and 0.771 μM respectively.

In summary it can thus be said that the validation data for tryptophan, kynurenine, serotonin and quinolinic acid were within the acceptance criteria for a newly developed method. In summary the selectivity was warranted by the use of the electron impact ionization source which enabled unique mass spectra with selected ions for each of the analytes. The negative chemical ionisation source used allowed samples to be determined with relatively good sensitivity for all the analytes with the minor exception of serotonin. All the analytes showed good linearity in terms of relative response versus concentration and the r^2 correlation coefficients were > 0.99. Also the RSD for the gradient and intercept for all the calibration data were below 15%. Bias and accuracy were acceptable with bias values of ±15% for both high and low controls and accuracy >90%. The within and between day precision for all the analytes were within acceptable ranges with coefficient of variances below 10% for high controls and below 15% for low controls. All

the analytes were relatively stable over three freeze/thaw cycles. Likewise the analytes were stable under processed conditions. Limits of detection and quantification were considered at single to noise ratios of 3 and 10 respectively. The minimum recoveries were >85% for high controls and >75% for low controls.

After method validation, the actual analyses of patient and control blood samples were performed. Parallel to patient samples high and low quality control samples were also analysed. This was to ensure system performance remained up to standard and that the analysis results were valid. All the metabolites could be detected in the patient samples except that of serotonin. The results, i.e., the levels found in the blood of patients and control subjects will be discussed in the next section.

6.2 Tryptophan and metabolite analysis in chronic renal failure

This part of the study investigated aspects of tryptophan metabolism in end-stage chronic renal failure patients on dialysis. Two groups of end-stage chronic renal failure patients were investigated, a group on haemodialysis treatment and a group on peritoneal dialysis, as well as a control group. The haemodialysis group consisted of 15 end-stage chronic renal failure patients with a mean dialysis period of 278.4 SD 266.1 weeks at three three-hour dialysis treatments per week. They included 11 males and 4 females and the mean age of the group was 38.9 SD 9.98 years. The peritoneal dialysis group consisted of 15 patients who were on treatment for a mean dialysis period of 208 SD 111.3 weeks. This group consisted of 11 males and 4 females with a mean group age of 36 SD 11.77 years. The age, gender and race-matched control group consisted of 12 healthy volunteers with a mean age of 35.9 SD 10.42 years. The control group consisted of 8 males and 4 females. There was no statistically significant difference ($p=0.7301$) in age between the three groups. The mean urea values were 27.47 SD 7.3 mmol/L for the haemodialysis patients and 25.2 SD 11.1 mmol/L for the peritoneal dialysis patients. Mean albumin values were 33.1 SD 2.8 g/L for the haemodialysis patients and 30.6 SD 5.4 g/L for the peritoneal dialysis patients. The mean haemoglobin levels were 8.97 SD 2.05 g/dL for the haemodialysis group and 10.7 SD 1.8 g/dL for the peritoneal dialysis group. The only

statistically significant difference between the two patient groups was indicated for haemoglobin (p=0.0107). The two dialysis groups were therefore largely comparable.

The first, and primary, objective was to investigate the levels of tryptophan and its metabolites in the blood of chronic renal failure patients and of the controls. As discussed, in the introduction to this dissertation, tryptophan is essential for the synthesis of tissue proteins [4,5], and depletion of tryptophan may therefore have serious implications. Although this utilisation of tryptophan is said to account for the fate of a major part of the ingested tryptophan, some of the tryptophan is either utilised or degraded along other pathways. One percent is probably used for the synthesis of serotonin, but a much larger component of the ingested tryptophan is metabolised along the kynurenine pathway [4,6]. The two metabolites measured in this study i.e. kynurenine and quinolinic acid, are from this pathway.

The first substance investigated and compared in this study was tryptophan. In this, the present study, the tryptophan levels were 5.34 SD 5.04 µM for haemodialysis patients, 6.73 SD 3.18 µM for the peritoneal dialysis patients and 28.4 SD 4.31 µM for the control group. Our control group values were similar to two studies published elsewhere. In a study on chronic renal failure patients, plasma tryptophan levels in a control group of 16 healthy subjects were determined by Pawlak *et al* (2001) using reversed phase high performance liquid chromatography and fluorescence detection [7]. The control tryptophan levels were 31.2 SD 7.4 µM which correspond to the control group of our study. Our control values for tryptophan further correspond to that of a study by Myśliwiec *et al* (2002) who, through HPLC found values of 25.15 µM [8]. The tryptophan levels found in our study for HD and PD patients were subsequently compared to that of our control group. Tryptophan levels were significantly lower in our patient groups compared to the control group ($p<0.05$). Statistically significant differences were shown between the levels of HD versus control ($p<0.05$) and PD versus control ($p<0.05$). There were previous indications that renal failure patients, especially those on renal replacement treatment are tryptophan depleted [9,10]. However, only two previous studies exist for tryptophan levels in HD patients – although both these studies

indicated a tryptophan depletion in HD patients, their values were slightly higher. The values and references are shown in Table 6.1. Table 6.1 contains all available data for tryptophan done on humans in chronic renal failure, but do not include experimental work on animals. It has to be stressed that these studies were done in First World countries and that the majority of the patients in our study were from the previously disadvantaged population. Malnutrition could therefore have been a contributing factor. There was no significant difference between the tryptophan levels of the PD and the HD patients of our study. No existing publications could be found for PD patients dialysed within the hospital environment. The only work done on peritoneal dialysis patients is one publication on continuous ambulatory peritoneal dialysis by Cernacek *et al* (1980) [9], who also showed decreased tryptophan levels, but this is not strictly relevant as the patients in our study were on hospital peritoneal dialysis and not continuous ambulatory dialysis. As no previous publications are available on PD patients it is obvious that PD and HD patients were never before compared with regard to their tryptophan levels.

Table 6.1 Metabolite levels of present study and literature for haemodialysis and controls

Author	Tryptophan		Kynurenine		Quinolinic Acid	
	HD	Control	HD	Control	HD	Control
Present Study	5.3 (5.04)	28.4 (4.3)	4.7 (1.9)	2.1 (0.6)	4.9 (2.03)	0.3 (0.15)
Pawlak *et al (2002)* [7]	17.3 (9.7)	31.2 (7.4)	2.7 (1.4)	1.6 (0.9)	7.9 (2.8)	0.26 (0.1)
Myśliwiec *et al* (2002) [8]	15.2	25.15	-	-	6.75	0.42
Niwa *et al* (1991) [11]	-	-	-	-	9.2 (5.3)	0.6 (0.3)
Holmes 1988 [12]	-	-	3.99 (1.68)	2.26 (0.48)	-	-

The second substance assessed was serotonin. In view of the low tryptophan levels found in the patients [13], it was feasible to expect that serotonin, which is synthesised from tryptophan, could have been affected by altered substrate availability. The reason for this is not only that substrate availability would have curtailed serotonin synthesis, but also that tryptophan depletion, as in the case of reduced dietary intake, may alter tryptophan-5-hydroxylase activity, leading to a decreased serotonin synthesis [14]. In this study

serotonin levels could not be determined with any degree of certainty as the levels registered were below the quantification limit for the method. It therefore cannot conclusively be stated that decreased serotonin existed for this haemodialysis and peritoneal dialysis study groups. The sensitivity of the method would thus have to be increased in order to assess the levels in end stage chronic renal failure patients. However, some studies are available which indicate that low serotonin concentrations do indeed occur in chronic renal failure patients undergoing dialysis [15,16]. The methodology, including a study previously done at this renal unit [16], required serotonin to be determined from whole blood. Differences in sample workup of this study as compared to the other studies could have contributed to whole blood serotonin not being detected in present this study. In study samples of the patients and controls differed from the serotonin spiked whole blood standards used. It once again shows both the pre-analytical and analytical stages are of great importance for serotonin sample determinations.

In addition, to tryptophan, two metabolites of the kynurenine pathway, i.e., kynurenine and quinolinic acid were also determined in this study. Kynurenine levels were 4.7 SD 1.9 µM for the haemodialysis patients, 2.9 SD 2.0 µM for the peritoneal dialysis patients and 2.1 SD 0.6 µM for the control group. Kynurenine levels in a control group of another study, as determined by HPLC and ultraviolet detection, were 1.6 SD 0.9 µM [7] and therefore largely corresponds to our values. For this, the present study, the kynurenine levels were much higher in the haemodialysis group, i.e., statistically significant differences for kynurenine were found for the haemodialysis versus control ($p<0.05$) and haemodialysis versus peritoneal dialysis ($p<0.05$) groups. The kynurenine levels in the peritoneal dialysis patients were higher compared to the control group; however this did not reach statistical significance. It can thus be seen from the present study that kynurenine levels are increased in the chronic renal failure patients at the renal unit studied. Our study correlates with evidence from literature indicating that kynurenine levels are indeed elevated in individuals and animals with chronic renal failure [12,17,18,19]. The kynurenine values of the haemodialysis group for our study fell within the same range than that of the only two other studies done for kynurenine in patients on

renal replacement therapy, i.e., Pawlak *et al* (2001) and Holmes (1988) who showed that kynurenine levels of haemodialysis patients were significantly elevated [7,12]. Actual values are shown in Table 6.1. To our knowledge there was no study measuring and comparing kynurenine levels in both HD and PD patients.

The next substance to be determined was quinolinic acid. Quinolinic acid levels were 4.9 SD 2.0 µM for the haemodialysis, 2.8 SD 2.0 µM for the peritoneal dialysis group and 0.3 SD 0.1 µM for the control group. In our present study quinolinic acid levels were significantly elevated in both the patient groups, with statistically significant differences for the haemodialysis versus control ($p<0.05$) and peritoneal dialysis versus control ($p<0.05$) groups. The difference between the two patient groups was negligible. As in the case of other metabolites of the tryptophan-kynurenine pathway the values to be found in literature seem to depend on the analytical methods used and as these substances have only been measured in a limited number of studies absolute consensus remains to be reached. However, the ranges do not seem to differ too much and values similar to those obtained in the present study have previously been reported by Pawlak *et al* (2002), Myśliwiec *et al* (2002) and Niwa *et al* (1991) [7,11,12]. In the study done by Pawlak *et al* (2001) quinolinic acid levels, as determined by HPLC and fluorescence detection, were 0.26 SD 0.10 µM for the control group which are similarly to our study [7]. In another study by Niwa *et al* (1991) quinolinic acid levels, measured by GC-MS and electron impact ionization detection, were 0.6 SD 0.3 µM in a normal control group of 10 subjects [11]. In the study by Myśliwiec *et al* (2002) quinolinic acid levels were 6.75 µM for the normal control group [8]. All three studies showed increased levels of quinolinic acid in HD patients. Actual values can be seen in Table 6.1. No publications could be found for quinolinic acid in patients on PD. In conjunction with the quinolinic acid results of this study and evidence from literature it is clear that quinolinic acid is elevated in chronic renal failure [7,10,11,20]. To our knowledge there was no study looking at quinolinic acid in PD patients and therefore no comparison could be made between quinolinic acid levels in HD and PD.

As reported levels of both tryptophan and its metabolites vary according to the analytical techniques employed it would perhaps be a better indication to calculate the percentage decrease in tryptophan and the percentage increase in metabolite concentrations found by the studies published. When all studies ever performed in this field were scrutinized the values given below were found.

1. Ratios of tryptophan levels in HD patients versus control values for same study in order to demonstrate the degree of tryptophan depletion found by the available studies:

HD: control for **Tryptophan** 55.55 % (1.8 fold decrease) Pawlak *et al* (2002) [7]
HD: control for **Tryptophan** 60.44 % (1.7 fold decrease) Myśliwiec *et al* (2002) [8]
HD: control for **Tryptophan** 58.0 % (1.75 fold decrease) mean from previous publications
HD: control for **Tryptophan** 18.66 % (5.4 fold decrease) present study
HD: control for **Tryptophan** 44.88 % (2.97 fold decrease) mean calculated all including present study

2. Ratios of kynurenine values in HD patients versus control values of same study in order to show the increase in kynurenine levels

HD: control for **Kynurenine** 168.75 % (1.7 fold increase) Pawlak *et al* (2002) [7]
HD: control for **Kynurenine** 176.55 % (1.8 fold increase) Holmes 1988 [12]
HD: control for **Kynurenine** 172.66 % (1.75 fold increase) mean from the previous publications
HD: control for **Kynurenine** 223.81 % (2.2 fold increase) present study
HD: control for **Kynurenine** 189.71 % (1.9 fold increase) mean calculated all including present study

3. Ratios of quinolinic acid levels in HD patients versus control values for same study in order to demonstrate the degree of quinolinic acid increase

HD: control for **QA** 3038.46 % (30.4 fold increase) Pawlak *et al* (2002) [7]
HD: control for **QA** 1533.33 % (15.3 fold increase) Niwa *et al* (1991) [11]
HD: control for **QA** 3068.18 % (30.7 fold increase) Myśliwiec *et al* (2002) [8]
HD: control for **QA** 2546.66 % (25.5 fold increase) mean from the previous publications
HD: control for **QA** 1633.33 % (16.3 fold increase) present study
HD: control for **QA** 2318.33 % (23.2 fold increase) mean calculated all including present study

If all published data, as well as our own, are considered, plasma tryptophan would appear to be about 45% lower in the blood of renal failure patients on renal replacement therapies than in normal individuals. As can be seen in the values the tryptophan levels in our patients were markedly lower than that reported for the two continental groups of renal patients on HD. It is highly unlikely that this could be a technical error as the control values were in agreement. The possible reasons are to be addressed at a later stage. If all published data, as well as our own, are considered, plasma kynurenine would appear to be about 1.9 times higher in the blood of renal failure patients on renal replacement therapies than in the normal population. The increase in kynurenine was found to be slightly higher in our group. If all published data, as well as our own, are considered, plasma quinolinic acid would appear to be about 23 times that of the values in the blood of renal failure patients on renal replacement therapies than in the normal population. The increase in our group corresponded to the study of Niwa *et al* (1991) [11].

As tryptophan is essential for both physical and psychological health (in its own right as well as the fact that it is a precursor of serotonin), a decrease in tryptophan levels may have far reaching consequences. In general, low tryptophan levels, or tryptophan depletion may occur as a result of low intake, abnormal metabolism, or loss from the circulation [4,21,22,23]. In general, the major cause for depletion is assumed to be malnutrition resulting in a reduced tryptophan intake, but it is slowly becoming evident that other factors may play a role in the biological availability of tryptophan including alterations of tryptophan binding to plasma albumin and, perhaps, altered tryptophan metabolism as in the case of certain inflammatory conditions [4,5,13].

Renal failure patients are known to have alterations to their protein intake, both as a result of a decrease in appetite and as a result of certain dietary restrictions imposed by their therapy [24]. Albumin, although not a good indicator of protein intake in renal failure patients, remains a fair indicator of nutritional status. In this study albumin was found to be below normal (reference range = 34-48g/L) in 53.3 % and 67.7% of the haemodialysis and peritoneal dialysis groups, respectively. It is thus highly feasible that a deficit in their

general nutritional status may be the main cause of tryptophan depletion in some, if not most, of these patients. There was no significant difference between the albumin levels of the haemodialysis and peritoneal dialysis groups (p=0.2339), neither, as previously mentioned, could any significant difference be seen between the tryptophan levels of the haemodialysis and peritoneal groups investigated. Although intake may be the major cause, it is generally known that stimulation of the enzyme IDO by immune activation can accelerate tryptophan metabolism along the kynurenine pathway and in this way contribute to a decline in the levels of both serotonin and tryptophan, as well as to an increase in the plasma levels of tryptophan metabolites [25]. Whether this applies to chronic renal failure patients on dialysis treatment is speculative. The possible contribution to the tryptophan depletion and the accumulation of metabolites of the kynurenine pathway is rather difficult to establish without the measurement of enzyme activity in the tissues where the metabolic processes occur. Put as a secondary hypothesis of this study, an attempt was made to see if there were indirect indications that the tryptophan depletion, as well as the increase in metabolites of the kynurenine pathway, may partially be the result of this type of alteration in the metabolic pathway of tryptophan in chronic renal failure patients on haemodialysis and peritoneal dialysis treatment.

The possibility that alterations in tryptophan metabolism may be a contributing factor as a result of increased activity of the enzyme IDO which converts tryptophan to kynurenine can generally be examined a) by looking at the kynurenine to tryptophan (kyn/trp) ratio and b) by looking at the levels of pro-inflammatory cytokines and C-reactive protein (CRP) levels as an indication of inflammation.

The kyn/trp ratio gives an indication of the rate of production/turnover of the first major metabolite kynurenine and the substrate tryptophan, in other words the rate of enzyme activity. The reason why the kyn/trp ratio is thus considered is because it provides an estimate of IDO activity and this is independent of baseline tryptophan concentrations [26,27]. Furthermore the kyn/trp ratio has also been shown to be higher in patients with diseases involving activated immune response [27,28]. Needless to say that this does not really apply to patients where the functionally anephric state contributes to kynurenine

accumulation. However, the ratios were still determined in order to see if any information, beyond IDO activity, could be gleaned from it. In this study the kyn/trp ratio was significantly higher in the two patient groups compared to the control group ($p<0.05$). The kyn/trp ratios were, 1.5 SD 1.2 for the haemodialysis group, 0.5 SD 0.2 for the peritoneal dialysis group and 0.08 SD 0.02 for the control group. Although the ratios were significantly higher for the patient groups than for the control and the HD patients had higher values than the PD patients (which could indicate either stronger stimulation of IDO in the HD than the PD group, or better dialysance through the peritoneal membrane than the artificial kidney membrane) the difference between HD and PD was not statistically significant. With a 3 times higher ratio in the HD than PD group it is highly likely that the non-significance was the result of the large standard deviation found for the HD group and that a larger group of subjects would indeed show differences. We subsequently compared the ratios found in our study to ratios calculated from the results shown by Pawlak, *et al* [7] - the only other study who measured both substances. Their study was however limited to HD patients. Although our control values were just slightly higher than that of Pawlak *et al* (0.08 *vs.* 0.05), the ratio for their HD patients was much lower (0.16 *vs.* 1.5). Whether this points towards a difference in clearance by the dialysis membrane or a higher stimulation of IDO needs to be investigated further. As HD procedures are usually relatively comparable through the world the possibility of a higher IDO stimulation would, on first glance, appear to be the more feasible possibility.

In the second place the possibility that immune activity known to stimulate a shift in the tryptophan metabolic pathways is present was examined by looking at CRP levels in the patient groups. The reason for this is that an increase in the pro-inflammatory activity causes an induction of the enzyme IDO [29] that is in turn responsible for the conversion of tryptophan to kynurenine. Interferon-γ (INF-γ) is involved in the induction of IDO. In addition to this IL-1β, TNF-α and lipopolysaccharide enhance INF-γ-induced IDO activity [30]. The physiological importance for this process in the body is to assist the immune response by the removal of tryptophan from the micro-environment in order to restrict intracellular pathogen growth [29,30]. Tryptophan depletion and accumulation of the metabolites can, in such conditions then be attributed to an increased IDO-induced

breakdown of tryptophan along the kynurenine pathway as a result of immune activation. It is fairly well accepted that patients in chronic renal failure have increased pro-inflammatory activity [31]. In fact, pro-inflammatory cytokines have on occasion been described as uraemic toxins and said to contribute to several of the problems of the uraemic patients, including disturbances in lipid metabolism, increased oxidative stress, cardiac hypertrophy, and a pro-atherogenic state [32,33,34]. It is well established that chronic inflammation is further exacerbated in haemodialysis and peritoneal patients [35]. This is indicated by, amongst others, raised levels of pro-inflammatory cytokines [35,36]. The potential causes of inflammation in haemodialysis and peritoneal dialysis patients are related to uraemia and/or the dialysis procedure itself. Uraemia *per se* relates to a decreased renal clearance of cytokines, the accumulation of advanced glycosylation end-products, continuous infections (*H. pylori*, dental and gingival infections, Hepatitis B and C), as well as genetic factors [35,37]. The haemodialysis procedure itself can be a major cause of inflammation. This can be attributed to the possible contamination of blood from endotoxins in the dialysis fluid [35]. Other haemodialysis causes of inflammation are catheter, graft and fistula infections, as well as bio-incompatibility of the dialyser membranes [35,38,39]. In contrast to haemodialysis, peritoneal dialysis is said to have a lower degree of inflammatory activity. There are, however, some major inflammation-causing aspects related to the peritoneal dialysis procedure. These relate to peritonitis, bio-incompatibility of the dialysate, aspects of peritoneal access and exposure to plasticisers [35,40]. There is thus substantial evidence from literature indicating that inflammation and immune activation is present in chronic renal failure patients on dialysis treatment. In conjunction with tryptophan metabolism it seems likely that the presence of such immune activity in renal dialysis patients may induce the enzyme IDO thereby activating the kynurenine pathway. This may ultimately contribute to tryptophan depletion (due to metabolism) and accumulation of kynurenine end metabolites such as quinolinic acid as well as an increased kynurenine to tryptophan ratio.

In this study CRP levels were used as an indication of inflammation and immune activation in the patient groups. The CRP levels were 10.1 SD 8.8 mg/L for the haemodialysis group and 19.1 SD 21.9 mg/L for the peritoneal dialysis groups. Despite

the large difference in mean values there was no statistically significant difference in CRP levels between the two patient groups (p=0.3089). This could most probably be ascribed to the large standard deviations – especially in the case of the peritoneal dialysis group. The normal reference range for the CRP laboratory test is 0 to 10 mg/L. Seven HD patients and seven PD patients had elevated CRP levels above 10 mg/L. This gave an indication for 46.7% of each of the patient groups as having above normal inflammatory activity.

The question now is whether there are indications that immune activation may be a factor in the activation of IDO and therefore in the tryptophan depletion of our renal groups. It is accepted that the kyn/trp ratio is not really an indicator of IDO activity in patients in renal failure. It is however certain that these patients are immune stimulated and this opens up the possibility that IDO activation may be a factor. A further indirect indication may be seen in the fact that, despite the lower tryptophan ratios found in our study relative to the European studies, the kynurenine levels were higher than theirs. This could perhaps be interpreted as, not only a poorer nutritional state, but also a higher immune activation, or rather a greater shift to a pro-inflammatory state than in the First world population. This possibility is tentatively supported by the fact that populations in Africa are known to have higher rates of infection than European populations. The prevalence of tuberculosis (TB) and HIV infection is, for instance, highest in Africa compared to Europe and some other parts of the world [41,42,43]. In addition, the average individual from Africa is known to be exposed to numerous infectious diseases including bacterial, viral and parasitic infections [44]. TB, malaria and helminth are the most common of these infections which are widely prevalent in most parts of Africa. Therefore it is likely that the general population could have increased immune activity because of the wide occurrence of infections in Africa [44]. Thus for our study there could be a higher incidence of infection and inflammation in the patients compared to the other European studies investigating tryptophan and kynurenine metabolism in renal dialysis patients. The higher presence of immune activation would induce IDO activity to a greater degree thereby increasing the shift of tryptophan metabolism towards kynurenine and this may

partly explain the higher kynurenine levels in our study compared to the European studies.

As mentioned in the first paragraphs of the discussion, a number of relationships were tested between tryptophan and the metabolites on the one hand, and physiological parameters suspected to be influenced by their abnormal levels, on the other. This was done to test for indications for further studies at completion of the work for this dissertation. Probably as a result of the wide standard deviations, which is not abnormal in these patients, but also as a result of the relatively small groups investigated very few indications that were both statistically significant and which could be of clinical value were found between the various biochemical/physiological parameters. What was, however, of interest was the relationship found between the well-being of the patients, as reflected by the SF-36 and the tryptophan levels. The SF-36 is generally used to assess quality of life [45]. It is a 36-item based questionnaire. The scale assesses two main components dealing with general health and well-being, namely physical health and mental health components. These components are subdivided into eight categories based on the individual's perception of his/her own health with regards to physical bodily pain and emotional functioning [46]. The instrument provides scores for mental health (which refers to the evaluation of vitality, social functioning, emotional roles and emotional aspects), physical health (which encompasses the individual's evaluation of physical functioning, physical roles, bodily pain and general health). Further discussion of the SF-36 and the results found were presented in Chapter 4 of this dissertation. When the scores of the two treatment groups were compared for each of the eight aspects measured by the SF-36, namely, physical functioning (PF), role-physical (RF), bodily pain (BP), general health (GH), vitality (VT), social functioning (SF), role-emotional (RE) and mental health (MH) the only significant difference between the HD and PD patients was found for bodily pain where the haemodialysis patients scored significantly higher ($p=0.00180$). This correlates with previous published material that showed bodily pain to be one of the factors to significantly impact on the quality of life of dialysis patients [47]. In short, the results of our study indicated, but for pain, quality of life to be similar in the HD and PD patient groups investigated. The results of the two patient groups were therefore pooled

when tested for a correlation between tryptophan levels and quality of life. A significant correlation was found between the levels of tryptophan and quality of life as indicated by the SF-36 score ($p=0.6088$; $r=0.016$). This result is not unexpected when the impact of tryptophan-depletion on both physical and psychological health is considered.

6.3 Summary of the work done on chronic renal failure patients

The study investigated the levels of tryptophan and two metabolites of the kynurenine pathway in two groups of end stage renal failure patients on renal replacement therapies. This study confirmed the findings of the small number of previous studies that chronic renal failure patients on renal replacement therapies are tryptophan-depleted with a concomitant accumulation of metabolites of the kynurenine pathway. As the published levels of these substances seem to differ depending on the analytical techniques used, an attempt was made to view the degree of tryptophan depletion and metabolite accumulation reported by other studies, as well as in the present study, in relation to the values found for the control group for each study. This showed that tryptophan levels were 5.4 times less in the HD patients of our study in comparison to being 1.8 times less in HD patients of the other studies. Kynurenine levels were 2.2 times more in the HD patients of our study whereas the other studies showed 1.8 times more in kynurenine. For our study quinolinic acid levels were 16.3 times more in the HD patients and the other studies showed an increase in quinolinic acid of 25.5 times for the HD patients. As previously discussed, poorer nutrition and higher infection rate could theoretically have contributed to this. Our study appears to be the first to compare the status quo, with regard to tryptophan and its metabolites, between PD and HD patients. The patient groups were similar with regard to urea and albumin levels, i.e., degree of uraemia and nutritional status. The results showed slightly lower levels of tryptophan in HD than PD patients, but the difference was not of statistical significance. Kynurenine levels were, however, significantly higher in HD than in PD patients. Quinolinic acid levels were significantly higher in the HD and PD patient groups. No significant correlations were found between either the level of tryptophan depletion or the level of metabolite accumulation and that of physical symptoms theoretically associated with disturbances in the tryptophan pathway. To show such correlations may very well require the research

subject numbers only to be found in multi-centre studies. When the SF-36 was used as an index of the patient's perception of their health with regard to physical and emotional functioning, a positive correlation was found between their senses of quality of life and their tryptophan levels.

In conclusion it can be said that the objectives for the study have been met, that is:

- A suitable and reliable method, for the quantification of tryptophan, kynurenine and quinolinic acid in blood, has been developed and validated.
- The tryptophan and metabolite levels have been quantified in the blood of haemodialysis and peritoneal dialysis patients. These levels were also determined in a set of age and gender matched controls.
- The study showed that there is indeed significant tryptophan depletion in haemodialysis and peritoneal dialysis patients, with a concomitant significant accumulation of the metabolites of the kynurenine pathway.
- The study for the first time compared HD to PD patients' blood with regard to tryptophan depletion and accumulation of the metabolites. There was no significant difference between the HD and PD patients in the degree of tryptophan depletion, but differences were seen in the levels of accumulated metabolites of the kynurenine pathway.
- A correlation was shown between a decline in the quality of life of the patients and the degree of tryptophan depletion.

6.4 Limitations and future perspectives

Possibly the most important limitation of this study is the fact that it was confined to one hospital and that not all patients on the HD and PD programs of the hospital were prepared to volunteer for the study. This put a limitation on the number of research subjects. Nevertheless, it is unlikely that larger numbers of patients would have had a significant impact on the core outcome of the study. Questions that arise from this study is whether the peritoneal membrane clears the kynurenine pathway metabolites better than the membranes of the artificial kidney, whether stimulated IDO activity is higher in HD than PD patients, and whether a higher pro-inflammatory activity in patients of the

developing countries contribute to more significant abnormalities in tryptophan/kynurenine metabolism in chronic renal failure patients? Future studies should also aim at measuring all of the tryptophan metabolites of the kynurenine pathway, as well as serotonin levels in dialysis patients, as this may help to identify the complete tryptophan metabolic profile. In addition, should the opportunity arise where liver biopsies can be obtained, the enzymes which induce the tryptophan and kynurenine pathways should be assessed. Measurements of the relevant pro-inflammatory cytokines can further help to identify the degree of immune activation. In addition, it would be of scientific, and possibly clinical, value if these investigations could be performed in patients in the pre-dialysis stages of chronic renal failure. Such a study should preferably be of a longitudinal nature where patients are followed through as their creatinine clearance values decline.

6.5 References

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