Biochemistry Markers of Neuroendocrine Tumours

Mfon Ewang-Emukowhate

Royal Free Hospital

University College London

This thesis is submitted for the degree of MD(Res)

Declaration

I, Mfon Ewang-Emukowhate, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Mfon Ewang-Emukowhate

21st October 2020

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Abstract

Neuroendocrine tumours (NET) are a diverse group of neoplasms originating from cells within the diffuse endocrine system. Urine 5-hydroxyindole acetic acid (5-HIAA), a metabolite of serotonin is commonly used in the diagnosis and monitoring of patients with NET in particular small intestinal neuroendocrine tumour with carcinoid syndrome. The collection of urine 5-HIAA over a 24 hour period, and exposure to the acid preservative in the sample container are limitations in the use of urine 5-HIAA. In this thesis,
I have developed a liquid chromatography tandem mass spectrometry assay for plasma and serum 5-HIAA with acceptable analytical performance. I have also demonstrated that it compares well with the currently used urine 5-HIAA assay.

I compared plasma and serum 5-HIAA with serotonin, chromogranin A (CgA) and N-terminal prohormone of brain natriuretic peptide (NT-proBNP). A significant correlation was observed.

Somatostatin analogues (SSA) are often used as first line treatment in patients with metastatic NET. Diarrhoea and steatorrhoea are adverse effects reported with SSA use, which can lead to malabsorption of fat-soluble vitamins (FSV) and trace elements (TE). I have therefore investigated the prevalence of deficiencies in fat-soluble vitamins and trace elements. Deficiencies especially in vitamin K1 and zinc were observed.

I have shown in this thesis that the measurement of 5-HIAA in plasma or serum is a suitable alternative to urine, and it addresses the inconvenience associated with the timing and collection of the urine. In patients with NET on SSA, monitoring and supplementation of FSV and TE should be considered.

Impact Statement

5-hydroxyindole acetic acid is a biomarker used in the diagnosis and monitoring of patients with neuroendocrine tumours and carcinoid syndrome, in particular those with small intestinal neuroendocrine tumours. It helps identify those at high risk of developing carcinoid heart disease.

In this study, we developed a plasma and serum 5-hydroxyindole acetic acid assay with similar diagnostic performance, and comparable to the currently used urine assay. These blood based assays will be more convenient for patients with neuroendocrine tumours as samples can be collected immediately and at the same time during the same phlebotomy episode with other blood tests required for their clinic review. This is in contrast to a 24-hour urine collection which is time consuming and tedious, with the added risk of having acid being used as a preservative in the specimen container for the urine collection. An important benefit of the blood based assays is that more reliable results will be obtained as errors due to incomplete collection which occurs with urine will be avoided. Dietary restriction of serotonin rich foods prior to sampling of 5-hydroxyindole acetic acid in plasma or serum has been shown to be effective if done either overnight or for 24 hours compared to 72 hours required with urine. This reduced time for dietary restriction will improve adherence and as a consequence, also lead to more reliable results.

Both plasma and serum 5-hydroxyindole acetic acid assays having the same diagnostic performance at the chosen cut-off in our study will be beneficial. These two sample types are commonly used for blood test analysis and being

able to use either of them is an added convenience both for the patients with neuroendocrine tumours and the clinicians.

This study also highlights the importance of monitoring and giving supplements of fat-soluble vitamins and trace elements to patients with neuroendocrine tumours on somatostatin analogues. In addition it stresses the importance of considering other causes of diarrhoea in these patients.

Publications and Presentations

The following publication and presentations are as a result of the work carried out in this thesis and collaboration with another project.

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List of Abbreviations

$^{13}C_6$-5-HIAA	Carbon-13 isotopically-labelled analogue of 5-HIAA
5-FU	5-fluorouracil
5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine/serotonin
5-HTP	5-hydroxytryptophan
AAAD	Aromatic L-amino acid decarboxylase
ALD	Aldehyde dehydrogenase
APCI	Atmospheric pressure chemical ionisation
AUC	Area under the curve
BSA	Bovine serum albumin
CAP	Capecitabine
CAPTEM	Capecitabine and temozolamide
CgA	Chromogranin A
CHD	Carcinoid heart disease
CI	Confidence interval
CLARINET	Controlled Study of Lanreotide Antiproliferative Response in Neuroendocrine Tumours
CRM	Certified reference material
CRP	C-reactive protein
CS	Carcinoid syndrome
CSF	Cerebrospinal fluid
CT	Computed tomography
CTCs	Circulating tumour cells
CV	Coefficient of variation

DNA	Deoxyribonucleic acid
DTIC	Dacarbazine
ECD	Electrochemical detector
EDTA	Ethylenediaminetetraacetic acid
eGFR	estimated glomerular filtration rate
ELECT	Evaluation of Lanreotide depot/autogel Efficacy and safety as a Carcinoid-syndrome Treatment
ELISA	Enzyme-linked immunosorbent assays
EORTC	European Organisation for Research and Treatment of Cancer
EpCAM	Epithelial cell adhesion molecule
EQA	External quality assessment
ESI	Electrospray ionisation
FDA	Food and drug Administration
FN	False negative
FP	False positive
FSV	Fat-soluble vitamins
G1	Grade 1
G2	Grade 2
G3	Grade 3
GEP-NET	Gastroenteropancreatic neuroendocrine tumours
GFR	Glomerular filtration rate
GI	Gastrointestinal
HPLC	High performance liquid chromatography
HR	Hazard ratio
HRQoL	Health related quality of life
ICP-MS	Inductively coupled plasma mass spectrometry

IFN-α	Interferon alpha
IQCs	Internal quality controls
IQR	Interquartile range
LC	Liquid chromatography
LCMS	Liquid chromatography–mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometer
LLOQ	Lower limit of quantitation
m/z	Mass to charge ratio
MALDI	Matrix-assisted laser desorption ionisation
MDRD	Modification of Diet in Renal Disease
MN	Micronutrients
MRM	Multiple reaction monitoring
MS/MS	Tandem mass spectrometer
mTOR	Mammalian target of rapamycin
NAD	Nicotinamide adenine dinucleotide
NET	Neuroendocrine tumours
NETTER-1	Neuroendocrine Tumors Therapy
NT-proBNP	N-terminal prohormone of brain natriuretic peptide
Octreotide LAR	Long acting octreotide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
PEI	Pancreatic exocrine insufficiency
PERT	Pancreatic enzyme replacement therapy

PFS	Progression free survival
PIS	Participant information sheet
PIVKA-II	Protein Induced by Vitamin K Absence or Antagonism-II
PP	Pooled plasma
PPI	Proton pump inhibitors
PPP	Platelet poor plasma
PROMID	Prospective, randomized study on the effect of octreotide LAR in the control of tumor growth in patients with metastatic neuroendocrine midgut tumors
PRP	Platelet rich plasma
PRRT	Peptide Receptor Radionuclide Therapy
QC	Quality control
QLQ	Quality of-life questionnaire
QoL	Quality of life
R0	Complete resection
RADIANT	RAD001 in advanced neuroendocrine tumours
RFA	Radiofrequency ablation
RIA	Radioimmunoassays
ROC	Receiver Operating Characteristic
RRT	Renal replacement therapy
S/N	Signal/noise
SC	Subcutaneous
SD	Standard deviation
SE	Standard error
SEER	Surveillance, Epidemiology and End Results
SIM	Selected ion monitoring

SI-NET	Small intestinal NET
SPE	Solid phase extraction
SPE	Solid phase extraction
SRM	Selected reaction monitoring
SSA	Somatostatin analogues
SSTR	Somatostatin receptors
STZ	Streptozocin
TACE	Trans-arterial chemoembolization
TAE	Trans-arterial embolization
TE	Trace elements
TELESTAR	Telotristat Etiprate for Somatostatin Analogue Not Adequately Controlled Carcinoid Syndrome
TEM	Temozolamide
TN	True negative
TP	True positive
TPH	Tryptophan hydroxylase
TRACE	Time-Resolved Amplified Cryptate Emission
ULN	Upper limit of normal
ULOQ	Upper limit of quantitation
US	United States
w/v	Weight by volume
WDHA	Watery diarrhoea, hypokalemia, and achlorhydria
WS	Working solutions

Chapter 1 Introduction to Neuroendocrine tumours

1.1 History of serotonin and 5-hydroxyindoleacetic acid

5-hydroxyindole acetic acid (5-HIAA) is a metabolite of serotonin. The journey to the discovery of serotonin started as far back as the 19th century, when Ludwig and his colleague Schmidt in 1868 observed increased vascular resistance in the muscle of a dog perfused with defibrinated blood (1). In 1912, O'Connor following his investigation deduced that the vasoconstrictor substance which exerts its effect in serum and not plasma is likely released during the clotting process by platelets (2). A number of groups have been credited for the discovery of serotonin. Irvine Page an American physiologist and his colleagues Maurice Rapport an organic chemist and Arda Green a biochemist, isolated and identified this vasoconstrictor substance as 5-hydroxytryptamine (5-HT) also known as serotonin in 1948 (3, 4). An Italian pharmacologist and physiologist, Vittorio Erspamer in the 1930's discovered a smooth muscle contracting substance found in the enterochromaffin cells of the gastrointestinal tract (GI tract) which he named 'enteramine'. He later identified it in 1952 to be the same substance as serotonin (5). The following year 1953, Dr Betty Twarog with Iirvine Page identified serotonin in brain extracts (6). Much of what we know about the biochemistry of serotonin is credited to Sidney Udenfriend a biochemist who was involved in delineating the metabolic pathway for serotonin including the origin from the precursor amino acid tryptophan through to formation of 5-HIAA (7). He and his team were the first to show 5-HIAA was a normal component of human urine and they went on to describe a method for its measurement (8).

Interestingly, not long after the discovery of serotonin, it was implicated as the major substance secreted by carcinoid tumours. A lot of knowledge acquired over the years about the pathophysiology of serotonin can be traced back to studies carried out on patients with neuroendocrine tumours (9, 10).

1.2 Overview of neuroendocrine tumours

In 1907 the German pathologist Siegfried Oberndorfer first described neuroendocrine tumours (NET) arising from the gastrointestinal tract as a distinct entity with a more benign course. He coined the term karzinoide or “cancer-like” to differentiate these tumours from adenocarcinomas (11). They have more recently been reclassified under the general term of neuroendocrine neoplasms (12). This diverse group of neoplasms originate from cells within the diffuse endocrine system. They have the ability to secrete increased amounts of biologically active products which may be associated with specific hypersecretory syndromes that determine their clinical presentation. These biologically active NET are classified as functioning tumours. Non-functioning NET on the other hand do not secrete excess bioactive substances but may present incidentally or with symptoms that are due to the compression or invasion of surrounding organs or tissues (13). NET are rare, but over the last thirty years there has been an increase reporting in both their incidence and prevalence. A US based population study gives the annual incidence of NET to be approximately 7 per 100,000 persons and the prevalence 0.048% in 2012. This increase may reflect not only a genuine increase but also a rise due to better diagnosis as well as incidental finding in individuals undergoing more investigations e.g. endoscopies and computed tomography (CT) scans. The SEER data also showed an improvement in the

survival of patients with NET with a median overall survival of 9.3 years for all NET. The availability of more effective therapies in the management of patients with NET is responsible for this (14, 15).

1.2.1 Small intestinal neuroendocrine tumours

Small intestinal NET (SI-NET) previously known as midgut NET originate from serotonin secreting enterochromaffin cells. These tumours are often slow growing with a low proliferation rate, and are often diagnosed at an advanced stage when metastasis has occurred (16). They can cause functional symptoms due to carcinoid syndrome (CS) which presents clinically with diarrhoea, flushing, wheezing and dyspnoea as a result of the secretion of serotonin and other vasoactive substances. CS commonly occurs in SI-NET when there is metastasis of the tumour to the liver but it can also be seen in bronchial and more rarely pancreatic and ovarian NET. In 20-30% of patients with SI-NETs and liver metastasis, CS is present. A recent large population based study carried out by Halperin and his group revealed that CS is present in 19% of patients with NET and of these, 32% had SI-NET and 8% bronchial NET (17, 18).

CS is also associated with the development of carcinoid heart disease (CHD). The first case of CHD was described in 1954. 20% of patients with CS have CHD and it has been shown to occur more frequently in patients with SI-NET (19, 20).The pathogenesis of CHD although not completely understood is linked, to the secretion of serotonin by the metastatic tumour leading to formation of fibrous plaques and thickening of the right sided heart valves with

consequent regurgitation or stenosis of the affected valves (19, 21, 22). In patients with CHD, urine 5-HIAA levels are significantly raised compared to those without CHD (median urine 5-HIAA; 576 μmol/24hr versus 233 μmol/24/hr) (23). Plasma 5-HIAA has been shown to correlate with the development of CHD (22), whilst urine 5-HIAA levels can predict the development or progression of CHD (17).

1.3 Biochemical markers of neuroendocrine tumours

Biochemical markers are substances that are usually present or synthesised in the blood, urine, or other body fluids which are produced or secreted in increased amounts in disease states. They can be used as diagnostic markers, in monitoring disease progression and response to treatment, and to determine prognosis (13, 24). Biochemical markers have played an important role in the diagnosis and management of NET over the years (Table 1). They are either general markers such as plasma Chromogranin A (CgA), one of the most extensively investigated and clinically applied biochemical markers for NET, or they are specific markers for example 5-HIAA, vasoactive intestinal peptide, insulin and gastrin which in pathological amounts present with distinct clinical symptoms.

NET markers	Year first isolated	NET type	Associated clinical syndrome
Specific markers			
Serotonin	1948 by Page IH *et al.*	SI-NET	Carcinoid
Urine 5-HIAA	1955 Udenfriend *et al.*	SI-NET	Carcinoid
Gastrin	1905 by Edkins JS	Gastrinoma	Zollinger-Ellison
Insulin	1922 by Banting F *et al.*	Insulinoma	Whipple's triad
Glucagon	1923 by Kimball CP *et al.*	Glucagonoma	None
Vasoactive Intestinal Peptide	1970 by Said S *et al.*	VIPoma	WDHA
Somatostatin	1973 by Brazeau P *et al.*	Somatostatinoma	None
Neurokinin A	1983 by Kimura S *et al.*	SI-NET	None
Non-specific markers			
Chromogranin A	1967 by Blaschko H *et al.*	Most NET	None
Pancreastatin	1986 by Tatemoto K *et al.*	Most NET	None
Neurone-specific enolase	1974 by Marangos PJ *et al.*	Poorly differentiated NET	None
Pancreatic polypeptide	1971 by Kimmel JR *et al.*	GEP-NET	None
NT-proBNP	1987 by Sudoh T *et al.*	SI-NET (CHD)	None
Adrenaline	1899 Abel J & Takamine J	Pheochromocytoma and Paraganglioma	None
Noradrenaline	1946 by Euler V		None
Metanephrine	1960 by LaBrosse EH & Mann JD		None
Normetanephrine			None

NET - neuroendocrine tumour, GEP - gastroenteropancreatic, SI-NET -small intestinal neuroendocrine tumour, WDHA - watery diarrhoea, hypokalemia, and achlorhydria , NT-pro BNP - N terminal pro B type natriuretic peptide, CHD – Carcinoid heart disease (25-36)

Table 1. Biochemical markers of neuroendocrine tumours

1.3.1 Chromogranin A

CgA is an amino acid glycoprotein and was the first to be identified in the granin family of secretory proteins predominantly found in the dense-core granules of neuroendocrine cells (37). It is involved in the regulation of secretory granules, controlling the release of hormones and other biologically active peptides (37-39). The expression of CgA in tissues is a marker of neuroendocrine differentiation. In circulation, increased CgA levels occur in both hormone-secreting and non-hormone-secreting NET hence making it a widely accepted general marker of NET (37, 40, 41). Its level in circulation is determined by the tumour burden and the secretory activity of the neuroendocrine tumour (13, 41, 42). Patients with metastatic SI-NET have been shown to have one of the highest circulating levels of CgA (41, 43).

The diagnostic sensitivity and specificity of CgA as a marker of NET has been reported by various studies to be between 30-100% and 42-100% (37, 39, 41, 42, 44-48). Variation in CgA sensitivity is due to various factors, such as the type of CgA assay used and the cut-off level, the tumour type and activity, as well as the extent of disease. Localised non-functional NET tend to have poor sensitivity compared to functioning and advanced NET especially if liver metastases is present (39, 42).

CgA has been shown to be a prognostic marker in NET. Janson *et al* in a multivariate analysis established that high CgA levels were an independent predictor of poor prognosis in patients with SI-NET (43). In a prospective study of patients with non-functional pancreatic NET with liver metastases, baseline

CgA values above 2.5 times the upper reference limit was shown to be a poor prognostic factor for overall survival (44). In addition to baseline CgA levels, patients with an early reduction of CgA greater than 30% following treatment showed significantly increased overall survival (49). CgA levels were also shown to correlate with survival time (P=0.02, HR=2.4) (40).

There are different assays available for the measurement of CgA. Commonly, enzyme-linked immunosorbent assays (ELISA), radioimmunoassays (RIA), and Kryptor assay (ThermoFisher Brahms, Hennigsdorf, Germany) which employs a Time-Resolved Amplified Cryptate Emission (TRACE) technology which is based on a non-radiative energy transfer between a donor (europium cryptate) and an acceptor [XL665] (50). These assays differ in the forms of CgA they measure due to differences in the antibodies used and the epitopes they bind to. This can limit the clinical utility of these assays as a raised CgA concentration may be missed if the molecular form secreted by the patient is not detected by the particular assay used. It also makes it difficult to compare results between the different assays. Therefore, in the diagnosis and long term monitoring of patients with NET, the same assay should be used (39, 45, 51).

CgA has reduced specificity because elevated levels have been observed in other conditions. Inflammatory bowel disease, chronic atrophic gastritis, helicobacter pylori infection, chronic hepatitis, liver cirrhosis and pancreatitis are all gastrointestinal (GI) causes of elevated CgA. Cardiovascular diseases such as acute coronary syndrome, chronic heart failure and hypertension lead to increased CgA concentrations. Renal failure and the use of proton pump

inhibitors (PPI) are other common causes of elevated CgA concentration (39, 45).

1.4 Novel markers of neuroendocrine tumours

With advances in the field of NET, new biomarkers with better sensitivity and specificity are emerging.

Circulating tumour cells (CTCs) are released into the circulation by a primary or metastatic tumour. Their size and the expression of epithelial cell adhesion molecule (EpCAM) differentiate them from peripheral blood cells (52, 53). Detection of CTCs is done using the CellSearch system approved by the US Food and Drug Administration. Khan *et* al. showed that the presence of ≥ 1 CTCs was associated with a poorer prognosis with regards to progression-free survival and overall survival in patients with NET (54). However, CTCs do not exhibit similar sensitivity for the different types of NET and is neither specific for any NET group (55, 56).

The NETest; a multianalyte reverse transcription PCR (qRT-PCR) test with algorithm analysis is potentially an exciting new assay which involves the simultaneous measurement of 51 neuroendocrine specific marker genes (57, 58). It has a high sensitivity and specificity (>93%) (57, 59) in detecting gastroenteropancreatic (GEP) NET. The NETest has been shown to be a good predictor of response to treatment. In a study assessing the efficacy of Peptide Receptor Radionuclide Therapy (PRRT), the NETest showed a better correlation with treatment response compared to CgA (89% vs 24%) (60). In

patients on SSA, it has also been shown to be a good predictor of response to therapy (79%) (58). The NETest has been observed to give an indication of the extent of surgical resection, with lower scores associated with an R0 (complete) resection (61). It may have a role in the identification of recurrent and progressive disease (58, 61).

1.5 Serotonin

Serotonin (5-HT) is a biogenic amine synthesised from the essential amino acid tryptophan. The majority of the body's 5-HT is produced in the enterochromaffin cells of the GI tract. A small proportion is synthesised in the serotonergic neurons of the central nervous system. Blood 5-HT is almost completely found in platelets where it is stored in dense granules. A small amount is present in plasma. Platelets do not synthesize 5-HT, their 5-HT content is predominantly from the enterochromaffin cells of the GI tract following its release into the circulation. The metabolism of 5-HT is via oxidative deamination by monoamine oxidase to 5-hydroxyindoleacetaldehyde which in turn is oxidised to 5-HIAA the major metabolite or reduced to 5-hydroxytryptophol.

5-HT is an important signalling molecule involved in various physiological processes. It regulates gut motility, and in the central nervous system is involved in temperature control, mood and sleep. It also plays a role in platelet aggregation, vascular tone and metabolic processes such as regulation of bone turnover, lipid metabolism and glucose homeostasis (62, 63).

1.5.1 Serotonin analysis

Various methods have been employed in the analysis of 5-HT. Earlier methods such as paper chromatography and spectrofluorometry were superseded by more sensitive and specific ones including radioimmunoassays and enzyme-linked immunosorbent assays. The use of these assays was limited by cross reactivity and interference from endogenous substances in the samples. High performance liquid chromatography (HPLC) and Liquid chromatography tandem mass spectrometry (LC–MS/MS) methods are now commonly used as they provide better specificity and sensitivity, and they also allow the simultaneous measurement of 5-HT metabolites and other related compounds (63, 64). 5-HT has been measured in whole blood, platelet rich plasma (PRP), platelet poor plasma (PPP), serum and urine but they are challenges surrounding its analysis. Pre-analytically, there are precautions around sample collection and preparation because 5-HT can easily be released from platelets leading to a falsely elevated result. Also, 5-HT is readily oxidised and degraded enzymatically and can lead to erroneously low results. The addition of antioxidants to specimen tubes and the immediate freezing of samples after collection are measures used to ensure the stability of 5-HT. The saturation of platelets at high 5-HT concentration and issues surrounding the reference range for measurement of 5-HT in PPP due to the huge variation in results reported by different studies limits the analysis of 5-HT in platelets (65, 66).

1.6 5-hydroxyindoleacetic acid

5-HIAA is made up of an indole ring with two functional groups, a phenol and a carboxymethyl group, as shown below in Figure 1. The production of 5-HIAA via oxidative deamination is the major metabolic fate of serotonin. It is

synthesised mainly in the kidney and liver, and excreted in the urine because it is water soluble (8, 67). Sjoerdsma, Udenfriend and their colleagues outlined the metabolic pathway for serotonin from tryptophan to 5-HIAA as summarised in Figure 2 (7, 10). In various neurological conditions, 5-HIAA is used as a surrogate for serotonin measurement (68, 69).

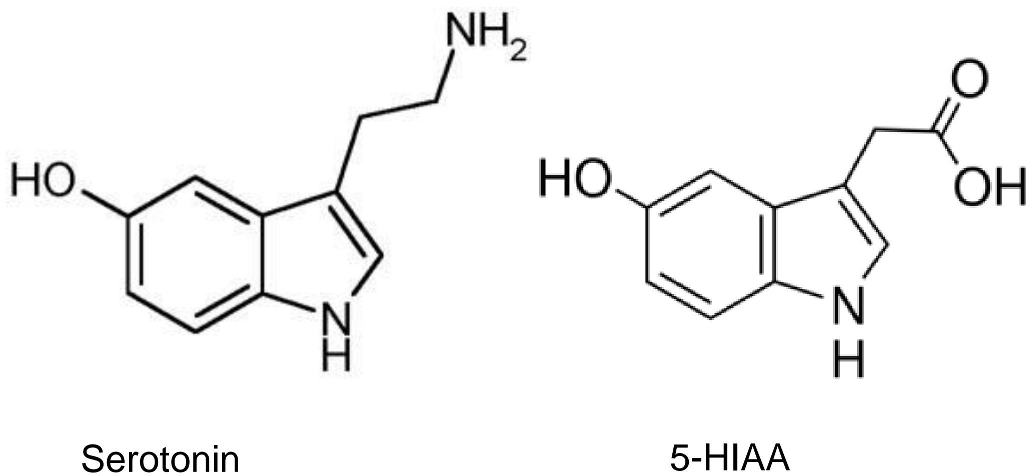


Figure 1. The chemical structures of serotonin and 5-HIAA.

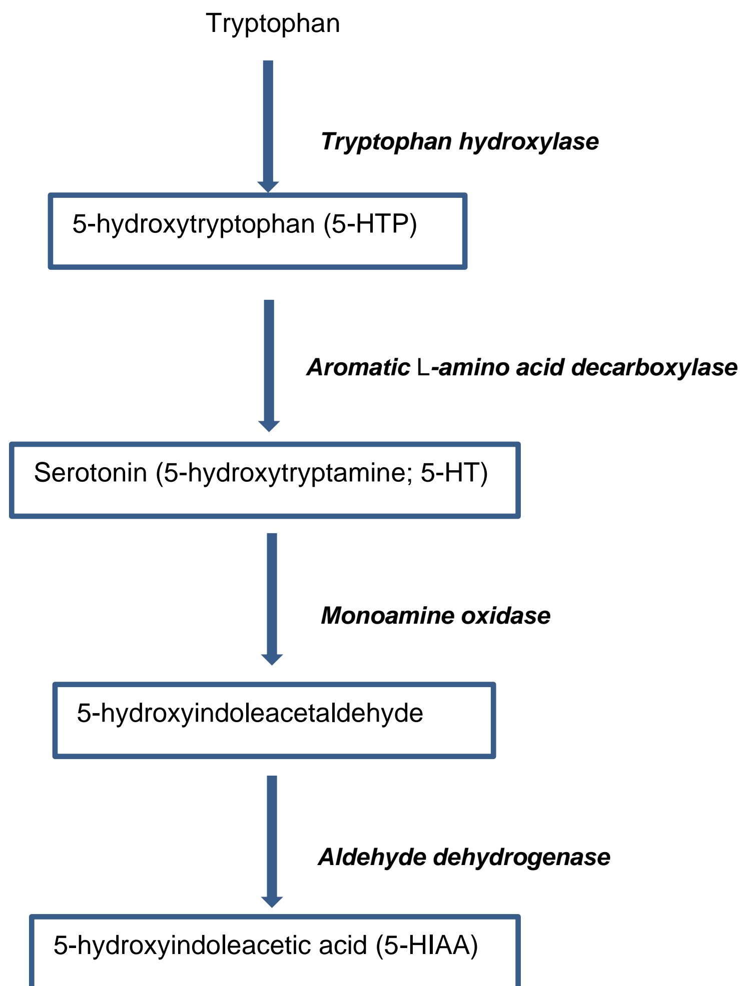


Figure 2. Pathway for 5-HIAA synthesis

Serotonin and 5-HIAA were the initial biochemistry markers used in NET originally identified as carcinoid tumours. This was due to earlier observations which include the discovery of high levels of serotonin in the blood and tissue of patients with metastatic carcinoid tumours, the demonstration of increased urinary 5-HIAA in these group of patients, and the description and association of carcinoid syndrome with 5-HT (10, 70-72). Over the years further work and advances in the NET field corroborated these findings that serotonin is the main substance secreted by carcinoid tumours.

1.6.1 5-HIAA analysis

Historically urine 5-HIAA has been the preferred biochemical marker for carcinoid tumours. Sidney Udenfriend and his group described a colorimetric method for its measurement (8). A problem with the colorimetric method was its poor specificity which required several modifications. Fluorimetric assays were also used in urine 5-HIAA analysis; however, although it is a sensitive method, its use was limited by interference from other urine constituents. Other methods such as immunoassays, thin layer chromatography, gas chromatography, HPLC and tandem mass spectrometry have been described. HPLC assays are now commonly used, with the advantages of improved sensitivity and specificity, and the ability to measure other compounds such as metanephrines simultaneously (67, 73-77).

A 24-hour urine collection is required for the analysis of 5-HIAA as random urine produces varying concentrations. The specimen is collected in an acidified container because 5-HIAA like other 5-hydroxyindoles, easily undergoes oxidation at alkaline pH. Addition of a weak acid such as acetic acid was recommended but it was found to interfere with a colorimetric method. Commonly, hydrochloric acid is used to lower the pH to 3 (67, 73).

Urine 5-HIAA excretion is increased by tryptophan or serotonin rich foods (Table 2). A study on the influence of diet on urine 5-HIAA excretion suggests 5-H1AA levels return to baseline by the second day after a serotonin rich diet is stopped (78). Thus prior to urine 5-HIAA collection, tryptophan and serotonin containing foods should be stopped for about 48 hours. Certain medications

can affect urine 5-HIAA concentration (Table 2). Chemotherapy drugs such as cisplatin cause an increase in urine 5-HIAA excretion presumably due to the release of a large amount of 5-HT by the cancer cells. Other medications such as monoamine oxidase inhibitors exert their effect by acting on the metabolic pathway for serotonin which can alter 5-HIAA excretion.

Interfering factors	Urine 5-HIAA concentration	Mechanism of effect
Foods Bananas, plantain, plums, pineapples, kiwi fruit, figs, dates, cantaloupe melon, honeydew melon, grapefruit, walnuts, pecan, macadamia, and brazil nuts, aubergine, olives, broccoli, spinach, cauliflower	Increased	Rich in serotonin or tryptophan
Medications Glyceryl guaiacolate present in cough remedies, naproxen, paracetamol	Increased	Analytical interference
Cisplatin, fluorouracil, melphalan	Increased	Increased 5-HIAA excretion
Imipramine	Decreased	Blocks serotonin re-uptake
Isoniazid and methyldopa	Decreased	Inhibits 5-HT synthesis
Isocarboxazid and moclobemide	Decreased	Inhibits conversion of 5-HT to 5-HIAA
Levodopa and ethanol	Decreased	Diverts tryptophan and 5-HT to alternative pathways
Chlorpromazine	Decreased	Analytical interference

Table 2. Factors known to interfere with urine 5-HIAA analysis summarised from published literature (67, 73, 79-81))

Interference with analytical methods for urine 5-HIAA is another way in which medications were found to either falsely increase or decrease 5-HIAA

concentration. This is not an issue with the modern 5-HIAA assays which predominantly employ the HPLC method (67, 73, 80).

1.6.2 Diagnostic performance and utility of urine 5-HIAA

Several studies have shown the sensitivity of urine 5-HIAA to be between 35 and 73%, and the specificity 89 to 100% depending on the cut-off used (46, 82, 83). The experience of a single centre has shown that in SI-NET, the sensitivities of both urine 5-HIAA and CgA was similar (69 vs 68%) and in patients with liver metastases, the sensitivity of urine 5-HIAA was greater (86 vs 77%) (42). In carcinoid syndrome, both markers demonstrated good sensitivities (46). A urine 5-HIAA level greater than 300 μmol/24 hours is associated with an increased risk of developing carcinoid heart disease (84).

The prognostic role of urine 5-HIAA was demonstrated in a study of patients with SI-NET (85). However other studies have shown that in multivariate analysis, urine 5-HIAA had no prognostic benefit (86).

1.6.3 Plasma and serum 5-HIAA

Measurement of 5-HIAA in plasma or serum addresses the inconvenience and issues surrounding 24 hour urine collection including the stress associated with the timing and collection of the urine as well as the exposure to a hazardous substance used as a preservative in the sample container. Several methods have been described in the analysis of plasma or serum

5-HIAA, they include HPLC, gas chromatography mass spectometry and LC–MS/MS (87-90).

The limited studies comparing urine and plasma 5-HIAA have shown good agreement and statistically significant correlation between both sample types (90, 91). The diagnostic performance of these tests depend on the chosen cut-off. Urine 5-HIAA at a cut-off of 40 to 56 µmol/24hr showed sensitivities between 74 and 85% compared to 79.6 and 89% in plasma 5-HIAA at a cut-off of 118nmol/L. Specificities were between 90-97% in urine and 74-100% in plasma. Concordance was also shown in their discriminating capacity. The Receiver Operating Characteristic (ROC) curve obtained by Adaway *et al* in their comparison of urine and plasma 5-HIAA showed the area under the curve (AUC) to be 0.920 for urine 5-HIAA and 0.917 for plasma 5-HIAA. AUC for the ROC curve in the study by Carling *et al* was 0.895 and 0.902 for urine and plasma 5-HIAA respectively (83, 89, 91).

Adaway *et* al also compared urine and serum 5-HIAA in 68 patients attending a NET clinic and reported a good agreement between urine and serum 5-HIAA in more than 90% of these patients. Another study comparing urine and serum 5-HIAA showed the sensitivity of urine 5-HIAA at a cut-off of 40 µmol/24hr was 67% with a specificity of 81% compared to serum 5-HIAA with a sensitivity of 57% and specificity of 95% at a cut-off of 123 nmol/L. ROC curve analysis showed similarities in their ability to detect NET, with AUC for urine 5-HIAA 0.83 and 0.81 for serum 5-HIAA (89, 91) .

Comparison of serum and plasma 5-HIAA by Adaway *et al* revealed a higher 5-HIAA concentration in the serum which they explained may be due to its release from cells during clotting (91).

A study looking at the association between biomarkers and CHD showed that the N-terminal prohormone of brain natriuretic peptide (NT-proBNP) and plasma 5-HIAA had similar discriminatory abilities in the diagnosis of CHD. ROC curve analysis showed the AUC for NT-proBNP was 0.82 and plasma 5-HIAA was 0.85 (22).

1.7 Brief Overview of Treatment of neuroendocrine tumours

Surgery is usually the initial treatment for NET diagnosed at an early stage of the disease and it can be curative. However, most patients at the time of diagnosis with SI-NET will present with metastases commonly to the liver, mesentery and peritoneum. In distant metastatic disease, palliative surgery can still be offered (16, 92).

Somatostatin analogues (SSA) are often used as first line treatment in patients with metastatic NET. They have both anti-secretory effects and anti-neoplastic effects. The anti-secretory effects thus reduce the release of peptide causing syndromes e.g. carcinoid syndrome by reduction of serotonin, see below. SSA also have antineoplastic properties reducing tumour growth via different mechanisms including inducing apoptosis and also having anti-angiogenic effects. Long acting octreotide (octreotide LAR) an intramuscular injection and

lanreotide (lanreotide autogel) a deep subcutaneous injection both administered every 28 days, are now the commonly used SSA (93-96).

The effect of SSA on reducing excess hormone secretion has long been established. Several studies have demonstrated the efficacy of SSA in CS. There are five known somatostatin receptors (SSTR 1-5). SSTR-2 and SSTR-5 have a high expression in GEP-NET and octreotide binds with high affinity to SSTR-2 (94). Rubin *et al* in their study comparing the efficacy of different doses of octreotide LAR and subcutaneous octreotide in controlling CS showed comparable effects with the different formulations of octreotide in decreasing the episodes of flushing and diarrhoea due to CS (97). It was observed that 91% of patients with flushing and 62% with diarrhoea achieved a greater than 50% reduction in their symptoms with prolonged release lanreotide four to six months after treatment commenced (98). Another study showed that after 6 months of treatment with prolonged release lanreotide, the mean daily episodes of flushing and diarrhoea had decreased by 43% ($p \leq 0.001$) and 24% ($p \leq 0.001$) respectively (99). Pancreatic NET such as glucagonomas and VIPomas are other functioning NET that are responsive to SSA (94).

The anti-proliferative effect of SSA to control tumour growth was first demonstrated in the PROMID trial where 85 patients with SI-NET were randomly assigned to receive octreotide LAR 30mg or placebo. The medium time to progression was shown to be significant between the two groups, 14.3 months in the octreotide LAR 30mg arm vs 6 months in the placebo group

(HR=0.34; 95% CI, 0.20 to 0.59; P=0.000072) (100). This was also confirmed in the CLARINET study, where 204 patients with NET were randomly assigned to an extended release formulation of lanreotide or placebo. At 24 months, the median progression-free survival was not attained in the lanreotide arm compared to 18 months in the placebo arm, $p < 0.001$. The estimated rates of progression-free survival was 65.1% (95% CI, 54.0 to 74.1) in the lanreotide arm versus 33.0% (95% CI, 23.0 to 43.3) in the placebo arm (101).Studies have shown that octreotide use in SI-NET led to stabilisation of tumour growth in approximately 50% of patients and regression of tumour in about 10% of patients. The majority of patients with CS experienced relief of their symptoms with SSA use (95). In the ELECT phase 3 trial, patients with NET and liver metastases who had stable CS and were either SSA naïve or were responsive to conventional dose of octreotide LAR or SC (subcutaneous) octreotide were randomised to receive either lanreotide 120mg or placebo. The result showed a significant reduction in the mean percentage of days of SC octreotide use as rescue therapy in patients who received lanreotide compared to those on placebo [-14.8 (-26.8 to -2.8); p=0.017)]. Significant improvement was observed in the symptoms of diarrhoea and flushing in the group on lanreotide compared to the group on placebo. This was more evident in the patients who were SSA naïve (102).

Treatment with SSA can cause adverse effects as a result of alteration in hormonal signalling occurring predominantly in the gastrointestinal tract (GI tract). Altered secretion of digestive and exocrine pancreatic enzymes, and interference with cholecystokinin signalling will result in decreased motility in

flow that can lead to formation of sludge in the gall bladder or cholecystolithiasis which are recognised side effects of SSA therapy (103). Deficiency in the exocrine pancreatic enzymes amylase, lipase and protein will lead to malabsorption of carbohydrate, fat and protein, which can present with abdominal symptoms such as nausea, bloating, abdominal discomfort or pain, flatulence and diarrhoea. Significant pancreatic exocrine insufficiency causes steatorrhoea (104). It is important to recognise that loose stools or diarrhoea can occur in patients with NET due to the underlying condition, for example as a result of excess hormone secretion as seen in CS. It is equally important to appreciate the impact of ongoing loose stools or diarrhoea and steatorrhoea that may occur as a result of SSA treatment. A consequence of this will be malabsorption of fat-soluble vitamins and trace elements. The importance of recognising this is discussed in detail in chapter 6.

Telotristat ethyl is an oral systemic inhibitor of tryptophan hydroxylase, the rate limiting step in the conversion of tryptophan to serotonin (Figure 2). The central nervous system effect of telotristat ethyl is limited because its high molecular weight prevents it from crossing the blood-brain barrier. In the USA and Europe, it has been approved in combination with a SSA for the treatment of refractory diarrhoea in patients with CS (21, 95). A multicentre open-label trial was carried out to assess its efficacy and safety over a 12-week period. At the end of the treatment, there was a 74.2% reduction in mean urine 5-HIAA levels from baseline. There was also a statistically significant reduction in the frequency of bowel movements; 43.5% ($p < 0.001$) and stool form; 19.5% ($p < 0.001$). It was generally well tolerated (105). In a post hoc analysis in the phase

III TELESTAR trial, the participants who had established SI-NET and CS, with an average of at least four bowel movements a day, on a stable dose of a SSA were randomly assigned to receive either 250mg dose of telotristat ethyl, 500mg dose of telotristat ethyl or placebo. 78% and 87% of patients on 250mg and 500mg dose of telotristat ethyl three times daily achieved a ≥ 30% reduction in urine 5-HIAA compared with 10% in the placebo group (106).

Peptide receptor radionuclide therapy (PRRT) is recommended when other medical treatments have failed. Treatment is delivered using radiolabelled molecules that bind to specific peptide receptors expressed by the tumour. Integrating SSA in the radiolabelled molecule such as Lutetium (^{177}Lu) oxodotreotide ensures that the tumours that express somatostatin receptors are targeted. In the NETTER-1 trial, patients with advanced and progressive SI-NET were randomly assigned to receive either four cycles of lutetium-177 (^{177}Lu)–Dotatate therapy followed by octreotide LAR 30mg every 4 weeks or high dose octreotide LAR 60mg every 4 weeks. The median progression free survival (PFS) was not reached in the ^{177}Lu–Dotatate group but it was 8.4 months in the group on high dose octreotide (95% CI, 5.8 to 9.1). This means that there was a 79% reduced risk of disease progression or death in the ^{177}Lu–Dotatate group compared to the group on high dose octreotide (107). Health related quality of life (HRQoL) analysis in the NETTER-1 trial showed that PRRT maintains and improves the quality of life in patents with SI-NET. A significant improvement was shown in various domains of the clinical symptoms assessed as part of the HRQoL in the ^{177}Lu–Dotatate group compared to the group on high dose octreotide (strosberg et al, NETTER 1

QOL). (92, 95). A similar finding had been observed by Khan et al in a study of patients with GEP and bronchial NET who were treated with ^{177}Lu–octreotate. Using the European Organisation for Research and Treatment of Cancer (EORTC) quality of-life questionnaire (QLQ)–core module (C30) to assess quality of life (QoL), a clinical significant improvement was observed in eight of the fifteen scales of the EORTC QLQ-C30 following treatment with ^{177}Lu–octreotate. In 19% of patients with diarrhoea, at least a 50% reduction was seen in their urine 5-HIAA levels after ^{177}Lu–octreotate therapy (108).

Other treatment options for NET include; Interferon alpha (IFN-α) which is used as second-line therapy in functionally active NET. IFN-α has anti-proliferative properties and is recommended as an add-on therapy to SSA. In a study assessing the role of IFN-α in patients with NET and liver metastases, 42% of patients on IFN-α had more than a 50% reduction in urine 5HIAA. 15% of these patients also had more than 50% reduction in their tumour size (109). However, the toxicity of IFN-α limits its use.

Novel targeted drugs everolimus and sunitinib are approved for pancreatic NET (110, 111). Although not recommended as a first-line treatment option due to their potential toxicity, it can be used in situations where SSA is not an option or slowly progressive NET growth prior to chemotherapy being considered. In SI-NET, it can be used as second or third-line therapy after the failure of SSA (92, 112). The anti-tumour activity of everolimus an inhibitor of mammalian target of rapamycin (mTOR) has been shown in both pancreatic and non-pancreatic NET. The RADIANT-2 study assessed the effect of the

combination of everolimus and octreotide LAR 30mg every 28 days compared to placebo and octreotide LAR 30mg every 28 days in patients with advanced NET and carcinoid syndrome. The median PFS in the everolimus plus octreotide LAR group was 16.4 months, and 11.3 months in the group given placebo and octreotide LAR. Everolimus plus octreotide LAR led to a 23% reduction in the relative risk of progression [HR 0.77 (95% CI, 0.59- 1.00); p=0.026]. There was no significant difference in overall survival with the addition of everolimus to octreotide LAR (113). In the RADIANT-3 study, patients with advanced pancreatic NET were randomly assigned to receive everolimus and octreotide LAR 30mg every 28 days or placebo and octreotide LAR 30mg every 28 days. The median PFS was 11 months with everolimus compared to 4.6 months with placebo, reflecting a 65% reduction in the estimated risk of progression [HR 0.35 (95% CI, 0.27- 0.45); p<0.001] (110). The estimated PFS at 18 months was 34% (95% CI, 26- 43) with everolimus and 9% (95% CI, 4 to 16) with placebo. Median overall survival was 44 months in the everolimus group compared to 37.7 months in the placebo group, this difference was not statistically significant (114). Patients with advanced and progressive non-functional NET of the lung and GI tract were randomly assigned in the RADIANT-4 study to receive everolimus or placebo in a ratio of 2:1. The median PFS was 11 months in the everolimus arm compared to 3.9 months in the placebo arm, reflecting a 52% reduction in the estimated risk of disease progression or death [HR 0.48 (95% CI, 0.35 to 0.67); p<0.00001]. The estimated PFS at 12 months was 44% with everolimus and 28% with placebo (115). In patients with non-functional GI tumours, a median PFS of 13.1 months was observed in the everolimus group compared to 5.4 months

in the placebo group. In the sub-group analysis of those with SI-NET, a median PFS of 17.28 months was observed with everolimus compared to 10.87 months with placebo. The increase of 6.41months with everolimus was associated with a 29% reduction in the risk of disease progression or death [HR 0.71 (95% CI, 0.40 – 1.26)] (116). Analysis performed in patients with non-functional bronchial NET also showed that everolimus increased the median PFS by 5.6 months over placebo and was associated with a 50% decrease in the risk of disease progression or death [HR 0.50 (95% CI, 0.28 – 0.88)] (117).

Sunitinib a tyrosine kinase inhibitor has shown anti-tumour activity in patients with pancreatic NET. In a phase 3 trial, patients with well-differentiated, advanced or metastatic (or both) pancreatic NET were randomly assigned to receive sunitinib or placebo in a 1:1 ratio. The study was discontinued early due to the high number of deaths in the placebo arm and serious adverse events. The median PFS observed was 11.4 months in the sunitinib group compared to 5.5 months in the placebo group. This was associated with a 58% reduced risk of disease progression or death [HR 0.42 (95% CI, 0.26 – 0.66)] (111).

In advanced pancreatic NET, progressive NET with failure of other treatment options and high grade NET, chemotherapy is recommended (118, 119). The two main classes of chemotherapeutic agents used in the treatment of NET are; alkylating agents such as streptozocin (STZ), dacarbazine (DTIC) and temozolamide (TEM), and antimetabolites such as 5-fluorouracil (5-FU) and capecitabine (CAP) (120). Commonly STZ, 5-FU, doxorubicin and lately CAP

and TEM have been used (118, 119). Pancreatic NET compared to other types of NET are more sensitive to chemotherapy (120). The effect of combination therapy with CAP and TEM (CAPTEM) was assessed in 30 patients with metastatic pancreatic NET. The median PFS was 18 months (95% CI, 9- 31) with an overall radiographic response rate of 70% (95% CI, 54- 86) (121). The same chemotherapy regime in a study of 29 patients with NET showed an estimated median PFS of 12 months (95% CI, 4- 20). There was no difference in PFS in patients with pancreatic NET (12 months) compared to those with other types of NET (13 months). 17% of patients experienced a partial response and in patients with pancreatic NET, it was 20% (122). The role of chemotherapy in the management of G1 and G2 GEP NET has not been clearly established but they are an important treatment modality in G3 GEP NET (118, 119).

Immunotherapy is emerging as a potential option in the management of NET. Expression of the immune check point proteins, programmed cell death 1 (PD-1) and programmed cell death ligand 1 (PD-L1) are associated with high grade or poorly differentiated NET or neuroendocrine carcinoma (123, 124). 100% of different types of grade 3 NET were found to express PD-L1 compared to 0% of grade 1 NET (125). A phase 1b trial to assess the effect and safety of pembrolizumab a monoclonal antibody against PD-1in patients with NET showed an objective response rate of 6.3% (95% CI, 0.2- 30.2) in those with pancreatic NET and 12% (95% CI, 2.5- 31.2) in those with other NET. The median PFS rate at 6 months was 44% and 40% in the group with NET and pancreatic NET respectively, at 12 months it was 27% for both

groups (125). Clinical trials are ongoing as there is currently limited knowledge about the efficacy of immunotherapy in NET (124).

With the exception of appendiceal, gastric and rectal NET, approximately 65 to 95% of patients with GEP NET present with liver metastases at diagnosis (126). Targeted therapies for liver metastases include embolization, ablation techniques. Embolization used for the treatment of non-resectable liver metastases involves the infusion of substances to occlude the hepatic artery which supplies the tumour thus leading to ischaemia and necrosis. In trans-arterial embolization (TAE), an embolic agent such as polyvinyl alcohol is used. In trans-arterial chemoembolization (TACE), chemotherapeutic agents are introduced prior to the embolic substance (126-128). TAE is a better treatment for liver metastases from small intestinal NET. Symptomatic response is achieved in 53-100% at 10 to 55 months and morphological response in 35-74% of patients at 6 to 63 months with these embolization techniques (126).

Ablative techniques utilise the cytotoxic effects of very high or low temperatures to induce a coagulation necrosis. They include radiofrequency ablation (RFA), microwave ablation and cryoablation (127, 129). There is limited experience with microwave ablation and cryoablation is not commonly used due to its increased rate of complication. A mean 5 year overall survival of 53% was shown with RFA. It is recommended for limited unresectable liver metastases (126).

Selective internal radiation therapy (SIRT) with yttrium-90 (^{90}Y) microspheres is another targeted therapy for liver metastases. Intra-arteral injection of ^{90}Y

will cause necrosis of the tumour by releasing a localised dose of ionising radiation. In patients with hepatocellular carcinoma and metastasis from colorectal cancer, SIRT has been shown to delay disease progression (130). A meta-analysis of the efficacy of SIRT in NET patients with liver metastases included 12 studies with 414 participants. The objective response rate defined as both complete and partial response was shown to vary from 12 to 80%. The disease control rate defined as complete and partial response, and stable disease was also shown to vary from 62 to 100% (131).

1.8 Conclusion

There are many different options for treating NET. The choice of treatment will depend on several factors including the primary site of the tumour, the stage and grade at diagnosis and if it is functional or non-functional (119). Biomarkers are commonly used in the diagnosis of NET and in the monitoring of their response to treatment. Urine 5-HIAA is useful in the diagnosis and monitoring of patients, in particular those with SI-NET and carcinoid syndrome. It has also been associated with the identification and progression of CHD. Serum and plasma 5-HIAA have been shown to have similar diagnostic performance and discriminatory capacity as the urine 5-HIAA assay. Analysis of 5-HIAA in plasma or serum will offer a more practical and convenient alternative to analysis in urine.

1.9 Rationale of thesis

The primary aim of this thesis is to develop and validate a plasma and serum 5-HIAA assay and assess its efficacy and correlate with urine 5-HIAA.

To assess the clinical utility of plasma and serum 5-HIAA; comparison will be made with other biochemistry markers including CgA, and NT-proBNP a marker of CHD.

Adverse effects of SSA used in the management of NET include diarrhoea, and steatorrhoea secondary to fat malabsorption which in turn affects the absorption of fat soluble vitamins (94, 104). A secondary aim of this study is to assess for malabsorption in patients on long term SSA, correlating deficiencies in fat soluble vitamins (A,D,E,K) and trace elements such as zinc, selenium and copper.

In Chapter 2, I develop and analytically validate a plasma and serum 5-HIAA assay.

In Chapter 3, I carry out clinical validation of the plasma and serum 5-HIAA assay, comparing its performance with the current urine 5-HIAA assay.

In Chapter 4, I compare and assess the relationship between plasma and serum 5-HIAA with serotonin measured in whole blood.

In Chapter 5, I correlate plasma and serum 5-HIAA with CgA and NT-proBNP, the other biochemical markers used in NET.

In Chapter 6, I investigate the prevalence of fat-soluble vitamins and trace elements in patients with NET on SSA.

Chapter 2 Development and validation of an LC-MS/MS method for the measurement of plasma and serum 5-HIAA

2.1 Introduction

Method development and full method validation is crucial to the introduction of a new assay. The choice of the method selected will depend on factors such as the chemical properties of the analyte(s) of interest, the turnaround time, cost and the analytical instrument available. Validation of the method will reveal if the assay based on its performance, is suitable for the intended use. Suitability will depend on the method producing accurate, reliable and reproducible results. Method validation should include tests for ascertaining precision, matrix effect, recovery, selectivity, detection limits, analyte carryover, and stability.

2.1.1 Liquid Chromatography (132, 133)

Chromatography is a technique that involves the separation of components of a sample mixture by their differential distribution between stationary and mobile phases. The mobile phase carries the sample mixture through a column that contains a stationary phase. This can lead to no migration, where the solutes in the sample mixture with higher affinity for the stationary phase are attracted to it and remain there. The other two options will be migration with the mobile phase, where the solutes with higher affinity for the mobile phase remain in the mobile phase or differential migration which involves distribution between the two phases. When the mobile phase used is liquid, the technique is referred to as liquid chromatography (LC). The stationary phase can either be packed into a tube or coated onto its inner surface. When small diameter

particles are used in the stationary phase in LC so that the resolution is increased, this technique is known as HPLC. It usually employs relatively high pressures to pump the liquid through the LC columns. Resolution is a measure of successful chromatographic separation.

The physical or chemical properties of solutes, such as their polarity, their net charge and size are utilised in chromatographic separation. In adsorption chromatography, separation is achieved by the difference between the adsorption and desorption of the solutes at the surface of the solid stationary phase. In normal-phase HPLC, the solutes to be separated are adsorbed to the stationary phase which is polar, and elution will occur from the least polar to the most polar compound. The mobile phase employed is increasingly hydrophilic if gradient chromatography is used. In reverse-phase HPLC which is more commonly used, the opposite occurs. The solid stationary phase is hydrophobic (typically modified silica) and the mobile phase is increasingly hydrophobic, thereby readily removing non-polar molecules from the stationary phase. The mobile phase is usually made up of one or more solvents with additives such as acids, buffers and ion-pairing reagents.

To achieve optimal chromatography and analyte separation in LC, certain parameters can be varied. They include the mobile phase composition, stationary phase chemistry (column), column temperature, pH, ionic strength and gradient elution. The mobile phase can either be in isocratic mode where the composition remains constant throughout the chromatographic run or in a gradient mode. In gradient elution the mobile phase composition is altered

during the run either in a stepwise or a continuous manner. Therefore as the mobile phase composition changes, it can become a better solvent for the solute of interest which is removed from the stationary to the mobile phase. Increasing the length of a column, increases the efficiency of separation as it allows more opportunity for the solute of interest to interact with the stationary phase. Increasing the column temperature can improve resolution and reduce the time for analysis. Fluctuation in the column temperature can affect the retention time, which is the time from the introduction of a sample to the detection of a given compound. The pH of the mobile phase is important in the retention of a charged analyte of interest and it also has an impact on what state the analyte will be in.

In LC, as the eluent exits from the column, it passes through a detector whose function is to identify and measure the separated analytes. There are different detection methods available that may be coupled to the LC, such as spectrophotometers, fluorometers and electrochemical detectors.

2.1.2 Mass spectrometry

A mass spectrometer generates charged ions and separates them according to their mass to charge ratio (m/z). If it is interfaced to a liquid or gas chromatograph, it provides chemical information in real time about the target analyte(s) eluting from a chromatography column (132). Mass spectrometry is an analytical technique increasingly used to measure a wide variety of clinically important analytes (132). Joseph John Thomson described the fundamental

principles of mass spectrometry in 1897 (134). A mass spectrometer is made up of an ion source, mass analyser and a detector.

The initial step in mass spectrometry involves ionisation of the target molecule. There are different methods that can be used in generating ions, the most common being electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) in LC-MS/MS (Liquid chromatography tandem mass spectrometer), Inductively coupled plasma (ICP) and matrix-assisted laser desorption ionisation (MALDI) are other forms of ionisation. Ionisation can be in a positive (usually generating $[M+H]^+$ ions) or negative (usually generating $[M-H]^-$ ions) mode. The development of ESI by John Fenn who won the Nobel Prize in chemistry in 2002 paved the way for analysis of large biological molecules (134, 135). ESI is one of the most common ionisation techniques used in clinical laboratories. It involves the introduction of a sample, for example an eluent from an HPLC column passing through a metal capillary to which a charge is applied. Outside the capillary, a heated gas commonly nitrogen is applied which causes the eluent to be nebulised at the tip of the capillary to produce charged droplets. The ions generated enter the mass analyser. Quadrupole, time of flight, quadrupole ion trap and linear ion trap are the different types of mass analysers which are available. (132, 135, 136).

Tandem mass spectrometry is a technique where two mass analysers are coupled together. Two mass spectrometers arranged in sequence with a collision cell between them is an example of a tandem mass spectrometer (MS/MS) because two or more mass spectrometers are connected in series

(

Figure 3). These individual mass analysers are also referred to as quadrupoles because each of them is made up of four parallel rods. The first quadrupole selects the precursor ion which is typically in M+1 in positive ionisation mode. This ion is selected to enter into the collision cell where fragmentation into smaller ions occurs after collision with an inert gas such as argon. These smaller ions are called product ions. Fragmentation in the collision cell gives rise to multiple product ions. The second quadrupole selects the product ion that will be detected based on its m/z and this is referred to as the transition.

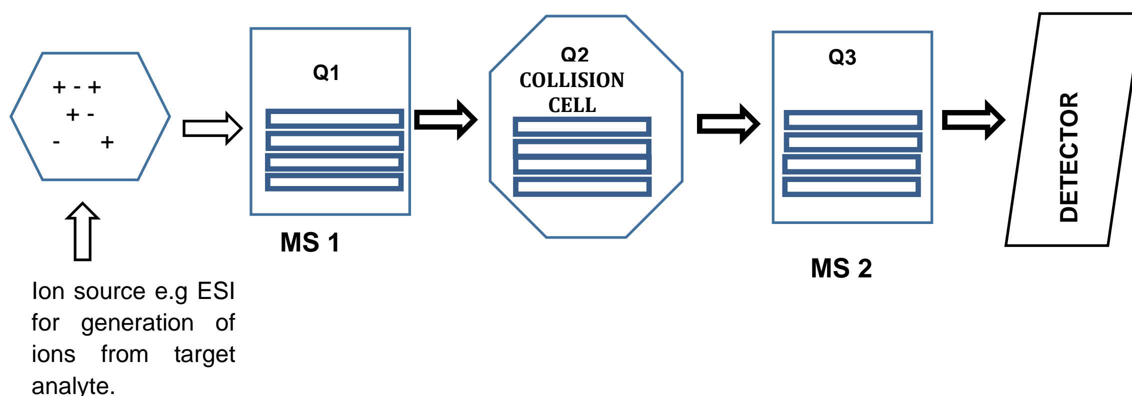


Figure 3. Schematic representation of the components of a tandem mass spectrometer. Q1= First quadrupole, Q2 = Second quadrupole, Q3= Third quadrupole, MS –mass spectrometer

The mass spectrometer can operate in either the scanning or selected ion monitoring (SIM) mode. In the scanning mode, the quadrupoles voltages are continuously varied to scan an entire range of m/z values. In the SIM mode, the mass spectrometer is programmed to monitor only the selected ions of interest. Monitoring of a single transition is termed selected reaction monitoring

(SRM) (132, 135, 136). Multiple precursor/product ion pairs can be generated when analysis involves for instance the analyte of interest and its internal standard or multiple analytes and their internal standards. Monitoring multiple transitions in sequence is known as multiple reaction monitoring (MRM), which is commonly employed in LC-MS/MS (135, 136). The detector converts the signal generated from the mass spectrometer into data that is interpretable by the analyst.

Mass spectrometry has a range of clinical applications. LC-MS/MS is currently used in therapeutic drug monitoring, toxicology and for various other tests such as testosterone, 17-hydroxyprogesterone, plasma metanephrines and 25-hydroxyvitamin D3 and D2. The advantages of using LC-MS/MS for clinical analysis includes its high analytical specificity and sensitivity, the higher throughput that can be obtained compared to other analytical methods, and the capacity to analyse multiple analytes in a single run which can lead to savings in terms of cost, time and sample required. LC-MS/MS analysis can involve manual steps such as the use of pipettes and sample preparation which can introduce errors if not performed accurately. Also, it requires skilled personnel trained in the use of this analytical technique. These are some of the limitations to the use of LC-MS/MS. The initial cost for materials and reagents required for setting up a method can be high, however, in the long-term it can be cost saving (134, 135, 137).

2.1.3 Sample Preparation

Sample preparation is important in HPLC as it helps isolate the target analyte of interest by eliminating interfering substances. It improves the sensitivity and accuracy of the analysis and will help maintain the analytical column for longer (138). Matrix effect is the overall impact of all the components in a sample (including salts, lipids, proteins, organic small molecules etc.) apart from the analyte of interest. The matrix effect depends on the analyte, the LC-MS/MS method and the ion source. The ESI is said to be more prone to matrix effects because of the acquisition of charge in the solution phase and changing to the gas phase in the ESI source. In LC-MS/MS analysis, matrix components can co-elute with the target analyte, interfering with the ionisation of the analyte in the mass spectrometer. This can lead to ion suppression or enhancement (137, 139). The most important matrix components that can alter ionization efficiency of the analyte are salt and lipids, specifically phospholipids. Exogenous compounds such as polymers from plastic collection tubes and gels can also be responsible

Internal standards are added in a constant amount to a sample mixture at the beginning of the sample preparation to account for any variation during the sample preparation. They correct for losses and inefficiencies in sample extraction, ion generation and mass selection. Internal standards are chosen to have physical and chemical properties as close as possible to the target analyte but distinct mass spectrums from the analyte on the chromatogram. Ideally for mass spectrometric assays, the internal standard is a stable isotope-

labelled form of the target analyte labelled with deuterium or carbon-13 (135, 140).

Different sample preparation methods can be employed prior to LC-MS/MS analysis, and they can include protein precipitation, solvent extraction, solid-phase extraction, ultrafiltration and microdialysis (140).

2.2 5-HIAA Assay development

2.2.1 Background

The selection of an appropriate method depends on the chemical properties of the analyte, the matrix being used and the number of analytes. It can also be influenced by the cost of setting up the method, and the analytical performance.

5-HIAA has a molecular weight of 191.18 g/mol with the molecular formula $C_{10}H_9NO_3$. It is a solid white powder and a weak acid, $pK_a = 4.22$ (141, 142). Its chemical structure has been described in a previous chapter (Chapter 1, figure 1).

5-HIAA analysis is commonly performed in urine. However analysis in plasma and serum using LC-MS/MS has previously been described (88, 89). An isocratic HPLC method with fluorimetric detection for plasma 5-HIAA analysis was previously described by Degg and colleagues and is still currently in use for example at Leeds Teaching Hospitals NHS Trust (87). Miller *et al* described an LC-MS/MS method, an improvement on the HPLC method by Degg *et al*

with regards to the sample preparation which was easier, with improved analytical features including a shorter time for analysis. This method employed protein precipitation for sample preparation. Quantification of plasma 5-HIAA by LC-MS/MS was achieved by performing HPLC using an anion-exchange plus reversed-phase chromatography column, and mass spectrometry with ESI in positive ion mode. 5-HIAA and the deuterated internal standard were detected in MRM (88).

2.2.1.1 Aim

The primary aim of this study was to develop and validate a method for plasma and serum 5-HIAA analysis and assess their use and agreement with the current urine 5-HIAA assay.

2.2.2 Method Development

It was decided that in order to save cost and time, off-line sample preparation should be kept as simple as possible. The availability of a two-dimensional-LC-MS/MS system allowed for sample clean-up to be carried out on-board. The system has a third pump (Pump C) which allows loading of the sample extract onto a trap column with a specific chemistry which retains the analyte. It then undergoes a wash period (using mobile phase C) to flush unwanted material from the plasma matrix straight to waste. The valve then switches the flow path so that the binary pump can be used to elute the analyte off the trap column and onto the analytical column for separation and MS/MS detection. This on-line solid phase extraction (SPE) minimises interferences and matrix

effects and also has the advantage of keeping the MS source and lens system clean from contamination.

2.2.2.1 LC Configuration

The LC configuration (Figure 4) offers the capacity to load/flush (Figure 5) and elute off the analyte (Figure 6) from the trap in either direction (forwards or backwards), allowing automated diversion to waste for while the trap is flushed and at the beginning of the chromatographic separation.

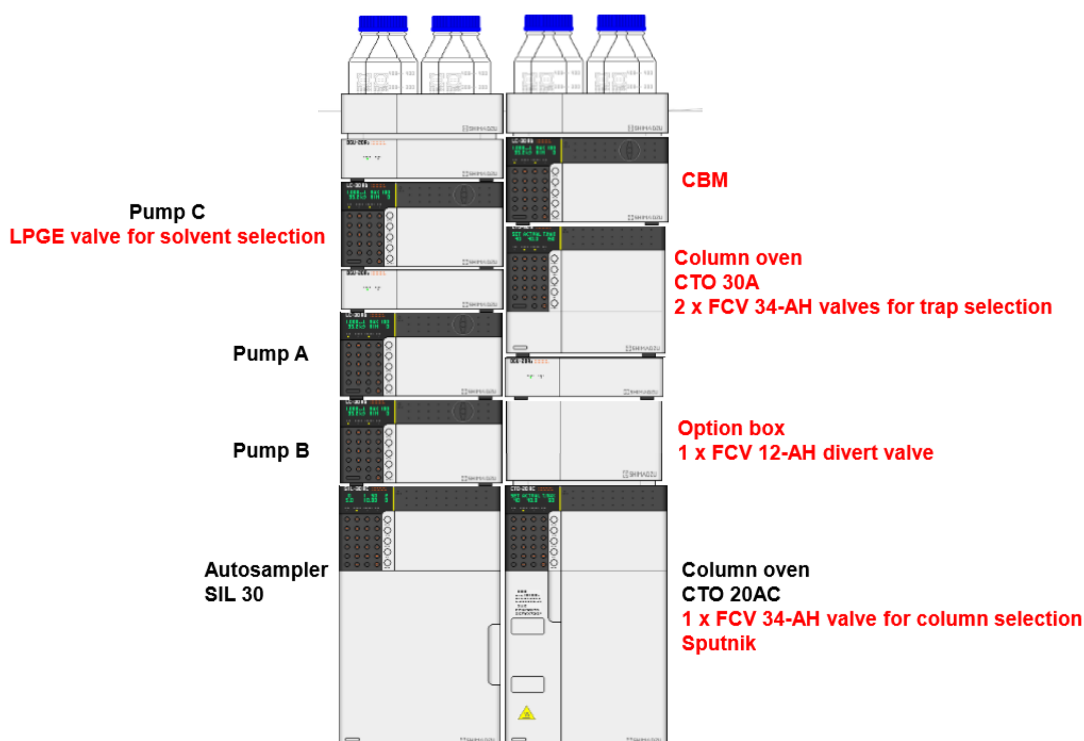


Figure 4. LCMS 8050 configuration. CBM is a system controller that acts as an interface for connecting the instrument to LC workstations or network computers. LPGE – low pressure gradient (143)

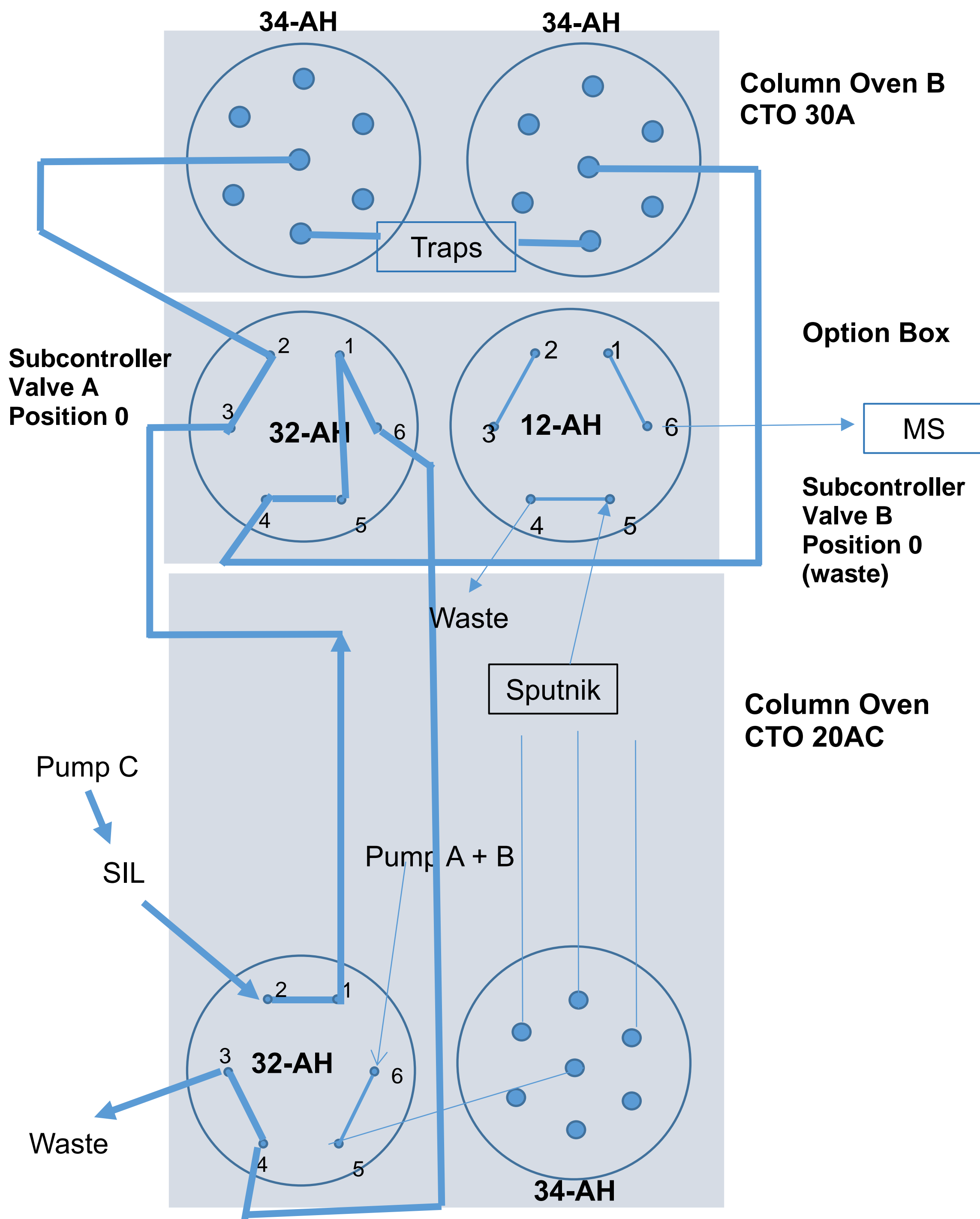


Figure 5. The schematic representation of loading of the sample extract onto the trap column where the analyte of interest is retained. SIL - sample injector. The arrows depict the route the sample extract travels (143)

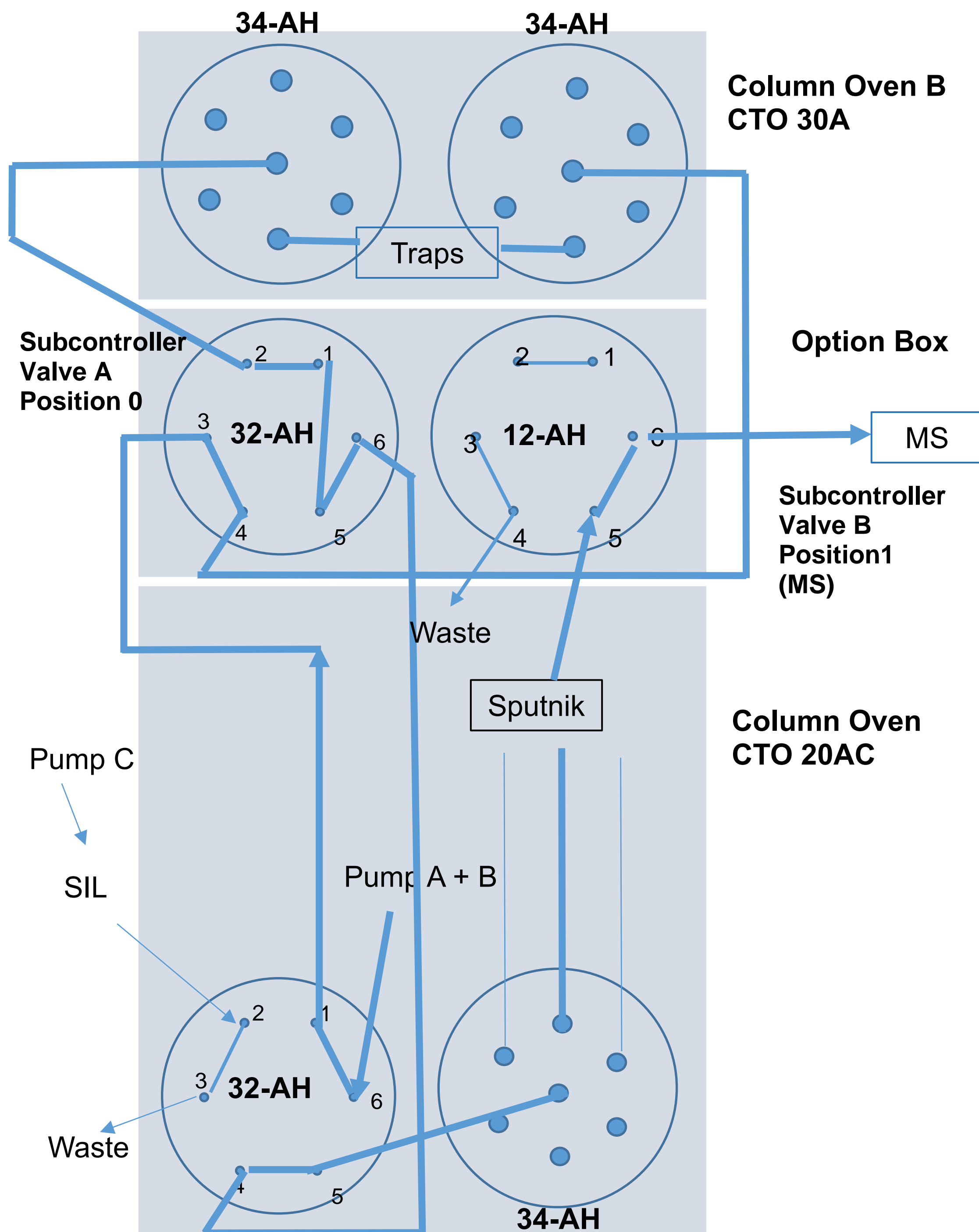


Figure 6. The schematic representation of elution of the analyte of interest from the trap column into the mass spectrometer (MS) using mobile phases A and B (pump A and B). The arrows depict the route the mobile phases travel into the traps and elute the analyte of interest to the MS (143)

2.2.2.2 Trap Column Selection

Anion Exchange

Since 5-HIAA is an acid, a weak anion-exchange polymeric sorbent was the first chemistry trialled for the method (Strata-X-AW). This functionality allows for retention of acidic compounds with pK_a less than 5 using three mechanisms of retention: mainly weak anion exchange via an amine group, but also pi to pi (π-π) bonding and hydrophobic interaction. The analyte is loaded on at neutral pH so that it is in the charged state (anionic) and is retained on the sorbent (attracted to the positively charged amine group). After the trap is washed, with the loading solution (water/methanol, neutral pH), the analyte is then eluted off with acidic mobile phase. The low pH results in re-protonation so that the analyte is no longer charged, and so loses its affinity to the sorbent. Unfortunately there were significant interfering peaks at the quantifier transition (192.10 $\rightarrow$146.20) observed both before and after the analyte peak which could not be resolved chromatographically. This was noted to be present only in the plasma samples and so was likely to be matrix in origin. Alterations in the composition of the loading/wash mobile phase were trialled in an attempt to wash away the contaminants. The analytical column, elution gradient, mobile phase pH, buffer composition and solvent types were all explored without success. It was speculated that the low pH required to elute off the analyte might also be producing or washing off a substance from the matrix which produced a signal at the quantifier transition.

Reverse Phase

Next a reverse phase functionalized polymeric sorbent that claimed to give retention of neutral, acidic or basic compounds was explored. The trap (Strata-X, On-line Extraction Cartridge) contained sorbent which uses three mechanisms of retention: π-π bonding, dipole-dipole interaction (including hydrogen bonding) and hydrophobic interaction. Acidic compounds must be loaded on in their uncharged (neutral) species to optimise affinity with the functional groups. The sample preparation would therefore include acidification to at least pH 2 to ensure that all the analyte was in its desirable form.

Choice of Analytical LC Column

The choice of column was based on the chemical composition of the analyte, as well as a literature search to reveal which stationary phases may be suitable. Column chemistries used previously include mixed mode – reversed-phase plus anion exchange, HILIC, C18 reverse phase (144). Since the analyte would be eluted off the trap as an uncharged species, it was decided that a reverse phase column would be chosen. Several C18 and biphenyl columns were trialled and found to produce similar chromatography. A biphenyl column (2.7µm 2.1mm i.d) from Thames Restek, UK was chosen due to the availability of a short cartridge column (0.5 cm). This allowed a high elution flow rate to be used within an acceptable back pressure range.

2.2.2.3 Mobile Phase

Isocratic separation was chosen since there is only one analyte of interest and so peak spreading of late-eluting compounds is not a consideration. Gradient elution was attempted however it was found to show decreased analyte selectivity. The analyte peak was found to be poorly resolved from an interfering peak when the percentage of organic solvent was increased. The analyte was found to be difficult to elute off and so an increased flow rate was used (0.9 mL/min) in order to decrease the peak width and tailing. The optimal eluting mobile phase composition was found to be 57% B.

2.2.2.4 Injection Volume

Sample injection volume was chosen optimised to achieve acceptable sensitivity while avoiding overloading the column and minimising the amount of matrix introduced into the system. Desirable signal strength was measured by determining the signal/noise (S/N) ratio of the analyte peak. The analyte transition was found to have a relatively high background and while increasing the sample volume resulted in an increase in the absolute analyte signal strength, it also resulted in significant increases in background noise. The minimum S/N ratio at the lower limit of quantitation (LLOQ) is 10:1. However to ensure that even on a day when the assay is not performing at its best, acceptable sensitivity can still be achieved, best practice recommendation is to ensure that the S/N is greater than 20:1 for any signal used for quantitative analyses. It was found that 3uL injection of the sample extract achieved this.

2.2.2.5 Mass Spectrometer Parameter Optimisation

The Shimadzu Lab Solutions software includes a compound optimization program. This is an automated program which optimises the mass spectrometer's parameters to achieve the best analyte response, via multiple injections of a solution of the analyte. These parameters include determining parent ion m/z, product ions m/z, collision energy, quadropole voltages. The program applies incremental changes to a given parameter with every injection to produce an optimisation curve. A methanolic solution of 5-HIAA at a concentration of 10ug/mL was used, a strong signal was observed without saturating the detector. The optimization programme was initially run using 50:50 methanol:water with 0.1% formic acid as the additive at a flow rate of 0.5 mL/min. Optimisation of the internal standard was carried out using the same concentration (10mg/mL) of the isotope. Two SRM transitions were identified for the analyte – a quantifier transition (the transition which produced the most abundant product ion; *m/z* 192.1>146.2) and a qualifier transition (the second most abundant product ion; *m/z* 192.1>118.25) which was used to confirm the identity of the analyte. This will help enhance the selectivity of the measurement. The chromatographic peak for the quantifier and qualifier transitions can be used to calculate the ion ratios for the analyte. Optimisation of the internal standard parameters only produced one viable product ion – the other two m/z identified did not produce visible peaks. Therefore only one SRM was used in the analysis of the internal standard; *m/z* 198.15>152.25.

<u>Capillary Voltage</u>: This is the voltage required to help form the solvent spray from the tip of the electrospray probe. After the chromatography conditions

were determined, the capillary voltage was optimised for the mobile phase composition during analyte elution. The system sensitivity was also optimized in the presence of matrix, by carrying out on-column injections of an extracted sample, and the voltage modulated until the highest S/N ratio was obtained. For both the analyte and internal standard the optimal voltage was 0.4V.

Dwell Time: This is the amount of time spent by the MS measuring a particular product ion. The number of data points collected across a peak is dependent on the dwell time per ion transition. Best practice recommends the collection of 15 – 20 data points across the chromatographic peak. The appropriate dwell time can be calculated by:

Dwell time (miliseconds) = Cycle time/20 x no. of transition

Since the peak for the analyte is relatively broad, the dwell time chosen (100miliseconds) results in approximately one hundred points. It is generally accepted that 100 points fully defines the peak. There are 3 transitions.

Data Processing: The Insight program software employs in-built algorithms for peak integration which determines the area, height and retention time of the peaks. This is then used for subsequent data analysis (construction of standard curves, calculation of unknown concentrations etc.). Due to the noisy baseline observed in this method, samples of lower concentration had to be integrated manually to ensure accuracy. A tangent skim (straight line) is used for integration of the analyte and internal standard peak in all batches.

2.2.3 Reagents and materials

The following reagents and materials were used in the development and validation of the plasma and serum 5-HIAA method. All the reagents were of appropriate analytical grade.

1. Stock powder (500 mg) of 5-HIAA (Sigma-Aldrich, UK)
2. $^{13}C_6$-5-HIAA as Internal standard (Sigma-Aldrich, UK)
3. Normal range (10x5ml) and pathological range (10x5ml) endocrine plasma control, lyophilised; (Chromsystems, instruments & chemicals GmbH, Germany)
4. Phosphate buffered saline tablets (PBS) and bovine serum albumin (BSA) obtained from (Sigma-Aldrich, UK)
5. LCMS Grade Methanol (HoneyWell LC-MS Chromasolv 99.9% Methanol)
6. Acetronitrile (Greyhound Biosolve ULC/MS-CC/SFC)
7. Formic acid (Merck Suprapur Formic acid)
8. Isopropanol (Fisher chemical optimal LC/MS 2-Propanol)
9. Hydrochloric acid (VWR chemicals)

2.2.3.1 Equipment and Consumables

1. Sarstedt micro tubes 1.5ml
2. Sartorius pipettes and tips
3. Pasteur pipettes
4. Universal tubes
5. Graduated glass cylinders
6. Glass bottles

7. Volumetric flasks of different volumes used in the preparation of samples, internal standard, calibrators, quality control materials and the different mobile phase used
8. Vortex mixer by VELP Scientifica
9. Plate shaker by VWR used for mixing the prepared samples
10. MSE micro centaur plus centrifuge used in the centrifugation of samples
11. 96-well plate from Waters (Herts, UK) where the samples were placed for analysis
12. Thermo Scientific heat sealer used to secure the adhesive plate seals on the 96-well plate prior to analysis

2.2.3.2 Instumentation

Shimadzu Nexera X2 LC-30AD high performance liquid chromatography (HPLC) system and a tandem mass spectrometer 8050 (Shimadzu, Japan).

2.2.4 Sample Preparation

A literature search was performed to ascertain sample preparation methods that had been used for the analysis of 5-HIAA in plasma or serum. Protein precipitation and solid phase extraction using weak anion exchange are methods that have been previously described (89, 91).

Protein precipitation was the method chosen for sample preparation, using acetonitrile with 1% formic acid to lower the pH of the mixture. Protein precipitation was chosen because it is a simple sample preparation process

and is appropriate since the LC method includes a trap column for on-board clean-up. Protein precipitation will also release any analyte that is bound to plasma proteins ensuring that the total 5-HIAA concentration is analysed. There is limited understanding of the role of protein binding of 5-HIAA in plasma although it was investigated by Tohmola *et al* in serum. They found that excess 5-HIAA in circulation remains mostly in the free or unbound state. 84% of 5-HIAA is in the unbound state in patients with NET. If the 5-HIAA concentration is low, it is predominantly bound to albumin (83%) (145).

Internal standard was added to the sample, and afterwards incubation time was added to account for any protein binding of the analyte. To precipitate plasma proteins, 150 µl acetonitrile was used. After addition of acetonitrile, the mixture was mixed on a plate shaker, centrifuged for 15 minutes at 14243g and the supernatant removed for injection in the LC-MS/MS. The supernatant was transferred into allocated positions in a 96-well plate according to the plate diagram in Figure 7. Plasma and serum samples, QCs and calibrators were prepared in the same way.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Double Blank	Cal 7	Cal 9_2	PS 23A	Cal 9_4	PS 26A	CS L2_6	CS L1_7	Cal 1_8	IQC3_9	PS31A	Cal 4
B	Blank	Cal 8	IQC3_2	PS 24	IQC3_4	Cal 1_5	CS L1_6	CS L2_7	PS 29	Cal 9_9	PS32	Cal 5
C	Cal 1	Cal 9	CS L2_2	PS 24A	CS L2_4	CS L1_5	Cal 1_6	IQC3_7	PS 29	Cal 9_10	PS32A	Cal 6
D	Cal 2	Cal 1_1	CS L1_2	Cal 1_3	CS L1_4	CS L2_5	PS 27	Cal 9_7	PS 30	IQC3_10	PS33	Cal 7
E	Cal 3	CS L1_1	Cal 1_2	CS L1_3	Cal 1_4	IQC3_5	PS 27A	Cal 9_8	PS 30A	CS L2_10	PS33A	Cal 8
F	Cal 4	CS L2_1	PS 22	CS L2_3	PS 25	Cal 9_5	PS 28	IQC3_8	Cal 1_9	CS L1_10	Cal 1	Cal 9
G	Cal 5	IQC3_1	PS 22A	IQC3_3	PS 25A	Cal 9_6	PS 28A	CS L2_8	CS L1_9	Cal 1_10	Cal 2	Blank
H	Cal 6	Cal 9_1	PS 23	Cal 9_3	PS 26	IQC3_6	Cal 1_7	CS L1_8	CS L2_9	PS31	Cal 3	Double Blank

Figure 7. An example of the layout of a 96-well plate. Cal – Calibrator, CS – Control solution, L1 - level 1, L2 - level 2, IQC – In-house quality control, PS – Patient sample

2.2.5 Calibration standards and quality control material

Calibration describes the relationship between the signal from the instrument (mass spectrometer) and the concentration of the analyte (132). There are no known commercial calibrators for the measurement of 5-HIAA so they were prepared in-house. Calibrators of known 5-HIAA concentrations were used to prepare a linear calibration curve. This was done through the generation of a plot of analyte to internal standard peak area ratios (y-axis) versus the known calibrator concentrations of the analyte (x-axis). This was used to determine unknown concentrations of 5-HIAA in samples and QCs, using their analyte/internal standard ratio to back-calculate against the calibration equation. The accurate quantitation of analyte relies on high-quality standard materials so that gravimetric quantitation of standard material is not compromised during production of calibrators. Certified reference material (CRM) standard material (100µg/mL in methanol) with a certificate of analysis describing the characterisation of the material and its purity was used for the production of standards and IQCs.

Ideally the reference material would have been used to produce spiking solutions to prepare plasma calibrators. However since 5-HIAA is an endogenous compound that is present in health, it was not possible to use purchased plasma as the calibrator matrix, and plasma stripped of 5-HIAA is not available. A ‘proxy’ matrix was therefore employed of phosphate buffered saline with bovine serum albumin. The assay will be validated to demonstrate a lack of differential matrix effects when compared to native patient samples.

The choice of the range and number of standards for the 5-HIAA calibration curve was influenced by the concentration of 5-HIAA that can be expected in patients with NET. It is important that optimal calibration is obtained at concentrations that are important for clinical decisions. The lower and upper limits of the measuring interval were included in the construction of the calibration curve. Nine calibrators including a blank were prepared using the PBS/BSA solution at the following concentrations; 0, 25, 50, 100, 250, 500, 1000, 5000 and 10000 nmol/L.1mg of BSA was dissolved in 1L of PBS (1 pouch dissolved in 1L of deionised water = 0.01M) to give 0.1% weight by volume (w/v) PBS/BSA solution.

A stock solution of 100 mg/L equivalent to 523.067 µmol/L of 5-HIAA was prepared. Two working solutions (WS) were prepared from the stock solution. The first working solution (WS1) contained 50,000 nmol/L of 5-HIAA and the second working solution (WS2) contained 5000 nmol/L. The working solutions were used in preparing the calibrators as shown in Table 3.

Quality control (QC) materials are used in assessing the precision of a method and whether a batch is performing as expected (132). Three concentrations of QC materials were used. Two were commercial lyophilised endocrine controls. These lyophilised controls were based on human plasma spiked with 5-HIAA at two different levels. Level 1(normal) and level 2 (pathological). The mean concentration of 5-HIAA in the spiked normal QC was 41.8 nmol/L and 209 nmol/L in the pathological range. These QCs were reconstituted with deionised water as per the manufacturer's instructions.

An In-house QC (IQC, level 3) with a 5-HIAA concentration of 2000 nmol/L was prepared by spiking pooled plasma. The background concentration of 5-HIAA measured in the pooled plasma was 195 nmol/L. A stock solution of 1mmol/L of 5-HIAA (1.91 mg of 5-HIAA was dissolved in 10 ml of 0.1 M HCL) was used. 20 µl of the stock was added to 10 ml of pooled plasma.

Calibrator (nmol/L)	Dilution factor	Preparation
10000	1 in 52.3	191 µl of 5-HIAA **Stock solution** in 10 mL of PBS/BSA
5000	1 in 104.6	96 µl of 5-HIAA **Stock solution** in 10 mL of PBS/BSA
2000	1 in 25	400 µl of **WS 1** in 10 mL of PBS/BSA
1000	1 in 50	200 µl of **WS 1** in 10 mL of PBS/BSA
500	1 in 100	100 µl of **WS 1** in 10 mL of PBS/BSA
250	1 in 20	500 µl of **WS 2** in 10 mL of PBS/BSA
100	1 in 50	200 µl of **WS 2** in 10 mL of PBS/BSA
50	1 in 100	100 µl of **WS 2** in 10 mL of PBS/BSA
25	1 in 200	50 µl of **WS 2** in 10 mL of PBS/BSA
0	0	10 mL of PBS/BSA

Table 3. The preparation of different concentrations of calibrators including the blank (10 mL of PBS/BSA)

2.2.6 Internal standard

A carbon-13 isotopically-labelled analogue of 5-HIAA was used as the internal standard. To obtain a 60 µg/L concentration of internal standard, 60 µL of a 10 mg/L stock solution of the internal standard was made up to 10 ml with methanol in a 10 ml volumetric flask.

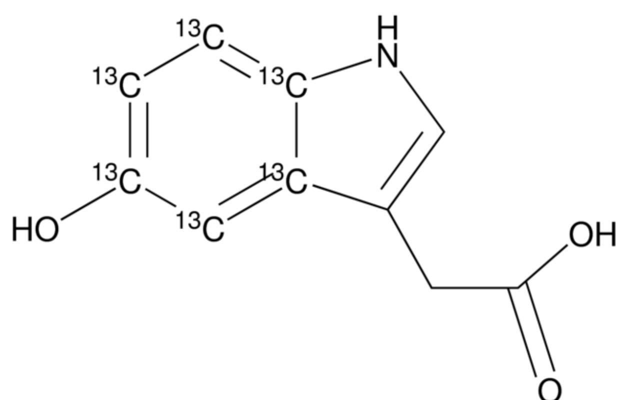


Figure 8. Structure of the carbon-13 isotope for 5-HIAA (146)

The internal standard used was seen to compensate well for ion suppression/enhancement for all analytes.

2.2.7 Liquid chromatography

A Shimadzu Nexera X2 LC-30AD HPLC system coupled to a tandem mass spectrometer 8050 (Shimadzu, Japan) was used for liquid chromatography.

The choice of column and mobile phase (elution solution) used is important as they can have an impact on the shape of the peak produced, adequate retention of the target analyte and the length of time for analysis (140). After sample preparation and centrifugation, the 96-well plate was placed in the auto sampler. Prior to introducing the sample, the HPLC system was purged to flush through all the lines to ensure that the mobile phase for the 5-HIAA analysis replaced any previous ones used and also it helped dislodge air bubbles trapped in the system.

3 µl of the supernatant was injected onto a Strata-X (Phenomenex) on-line extraction column for on-board sample clean up, followed by analytical separation on a Raptor Biphenyl LC column 2.7µm x 2.1mm (Thames Restek, UK).

Mobile phase A was aqueous and it contained 0.1% formic acid in water. It was prepared by adding 1ml of formic acid to deionised water in a 1L volumetric flask and making it up to 1L with deionised water. Mobile phase B was organic and it contained 0.1% of formic acid in methanol. It was prepared by adding 1ml of formic acid to methanol in a 1L volumetric flask and making it up to 1L with methanol. Mobile phase C which was used for loading and washing the trap column contained 20% methanol and 0.1% formic acid in water. It was prepared by adding 1 ml of formic acid to 200 ml of methanol and making it up to 1L with water. A reversed phase separation employing isocratic elution with optimal mobile phase composition of 57% B and 43% A was used. The total flow rate was maintained at 0.9 ml/min, pump pressure for mobile phase A and B was 77 bar, and 92 bar for mobile phase C, the oven temperature was 40 ^{o}C.

2.2.8 Tandem mass spectrometry and optimization of 5-HIAA method

A tandem mass spectrometer 8050 (Shimadzu, Japan) coupled to a Shimadzu HPLC system was used for 5-HIAA analysis. Ionisation was carried out using positive mode ESI and MRM was the method used in selecting the precursor/product ion. The MRM transitions as previously mentioned was *m/z* 192.1>146.2 (quantifier) and 192.1>118.25 (qualifier) for 5-HIAA, and *m/z* 198.15>152.25 for the internal standard ($^{13}C_6$-5-HIAA).

The Lab solution software (Shimadzu, Japan) was used in the method development and data analysis.

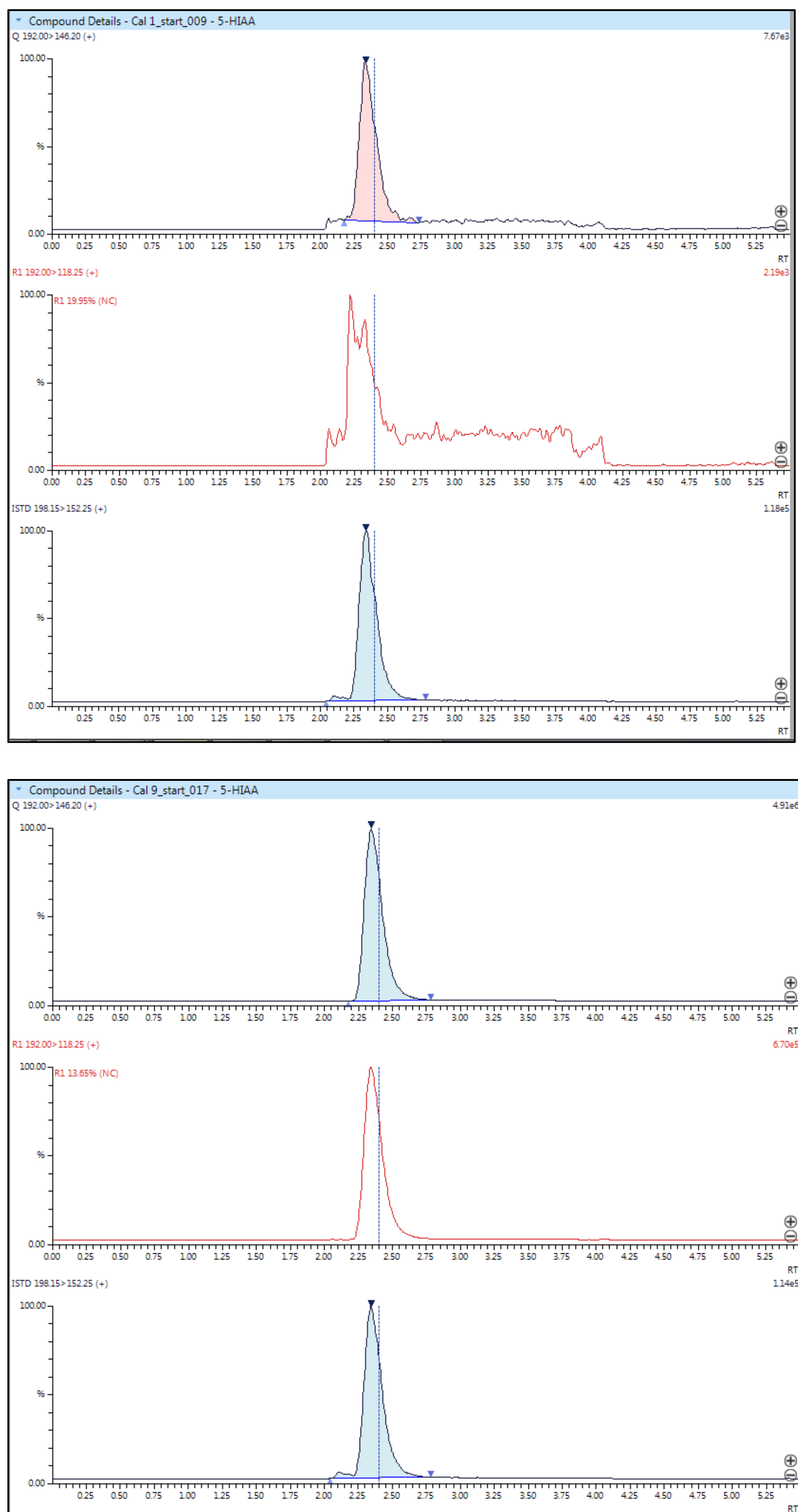


Figure 9. Chromatograms for calibrators 1 (above) and 9 (below) showing the quantifier (m/z 192.0>146.2) and qualifier (m/z 192.0>118.25) peaks for 5-HIAA (top two traces) and the peak for the internal standard (bottom trace)

2.3 Method validation (140, 147)

Method validation was carried out in plasma samples and performed according to the United States Food and drug Administration (FDA) guidance for bioanalytical method validation (147). Statistical analysis was performed using Microsoft Excel, Analyse-It (Analyse-It Software Ltd, Leeds, UK) and SPSS version 25. Test for normality was performed using the Shapiro-Wilk test and a Normal Q-Q plot. A normal distribution was confirmed if the significance value in the Shapiro-Wilk test was greater than 0.05, and the data points were lying on the straight diagonal line of the Normal Q-Q plot. A p value of < 0.05 was considered to be statistically significant.

2.3.1 Selectivity and Interference

Blank plasma or serum which did not contain 5-HIAA was not available at the time of this project. It was therefore not possible to test for endogenous interference in plasma or serum samples.

There are no known isobaric compounds with 5-HIAA. However, we need to assess if similar compounds produce fragments at the transitions for 5-HIAA (*m/z* 192.1>146.2 [quantifier] and 192.1>118.25 [qualifier]), and the internal standard (*m/z* 198.15>152.25). If necessary, any interfering compounds should be resolved chromatographically. The possibility of interference with 5-HIAA precursors was assessed by injecting 1µmol/L concentrations of serotonin and L-tryptophan prepared in methanol. No peaks were observed in the chromatogram (Figure 10) but the trace shows signal fluctuation generated

as a result of the manual injection of the L-tryptophan and methanol mixture into the HPLC pump.

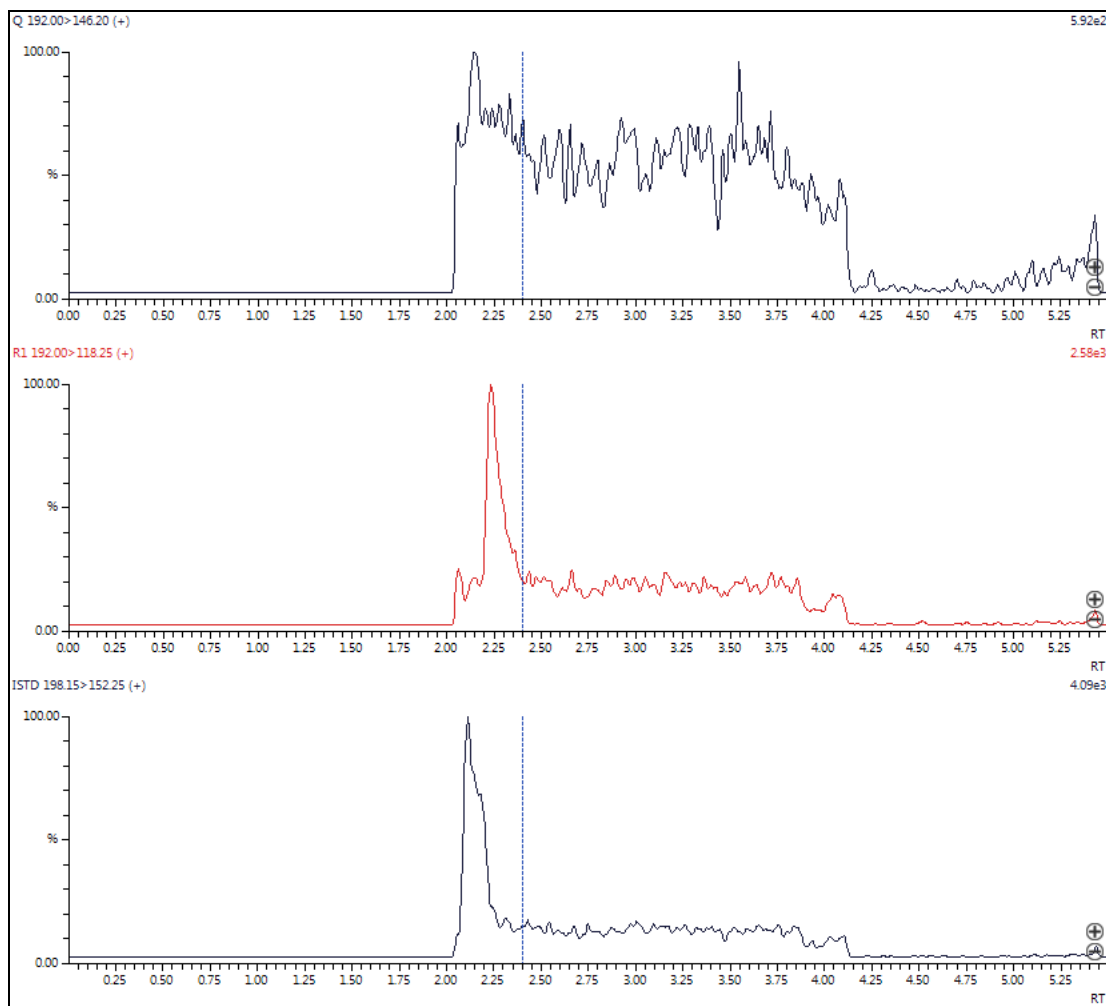


Figure 10. Chromatogram for tryptophan

2.3.1.1 Matrix Effects

Solutions containing the analyte and internal standard at the concentration of the lowest standard were prepared in (i) pooled plasma, and (ii) mobile phase A. The prepared samples were then extracted using the established protein precipitation method and analysed. The peak area of each analyte/internal standard was compared in the presence and absence of matrix to calculate the matrix effect.

Ion suppression is a matrix effect in LC-MS that result in diminishing of analytical signal, independent of the sensitivity or selectivity of the mass analyser. Ion suppression was examined as described by Bonfiglio *et al* (148). Post-column infusion of a methanolic solution containing 5-HIAA (20 ug/L) was performed at a constant flow rate, while the extracted blanks were injected. No ion suppression or enhancement was seen at the retention times of the different analytes.

2.3.2 Calibration (standard) curve

The calibration curve was generated using a blank and nine calibrator standards with the following concentration; 50, 25, 50, 100, 250, 500, 1000, 5000 and 10000 nmol/L. It has previously been shown that the detector response obtained for calibrators prepared in spiked plasma, water and PBS/BSA was similar (88). For consistency, PBS/BSA was chosen for preparation of the calibrators. The calibration was a linear plot (equation y = mx +c) as shown in Figure 11.

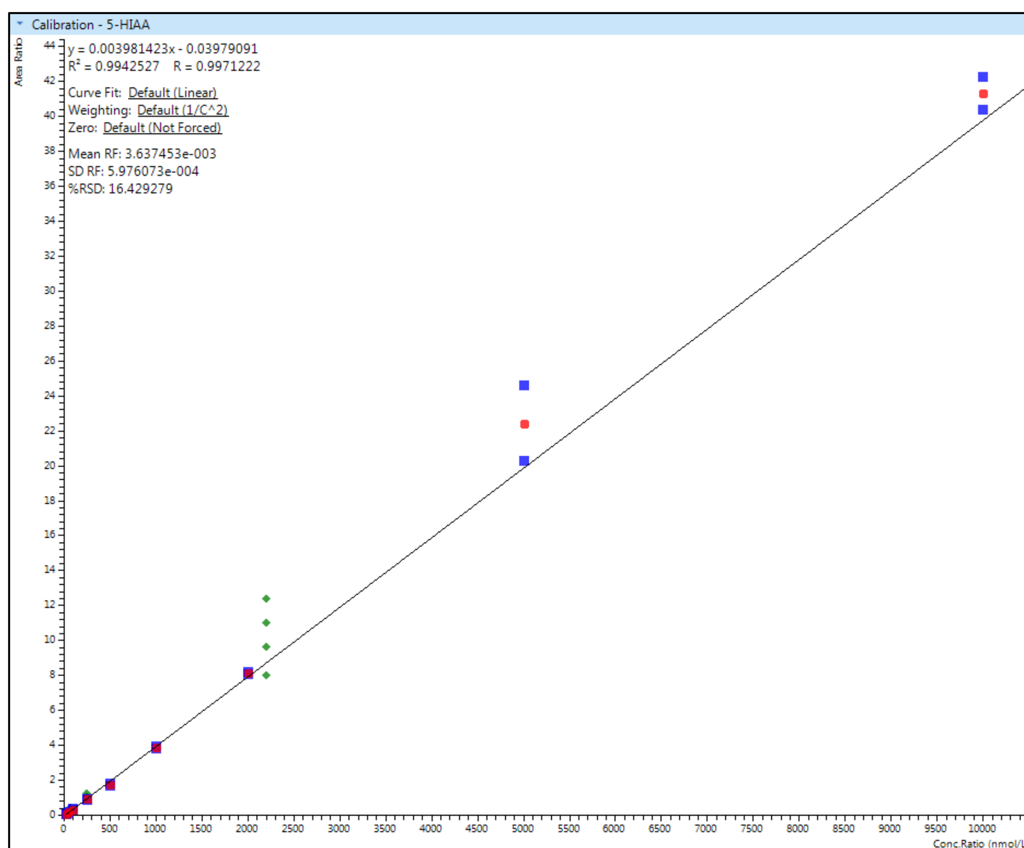


Figure 11. Calibration curve for plasma 5-HIAA. The blue coloured data points represent the two sets of standards analysed during a run and the red data point is the calculated mean of the two standards

2.3.2.1 Lower limit of quantitation (LLOQ)

The lowest calibration standard on the calibration curve is accepted as the LLOQ if the analyte response at that concentration is at least five times when compared to the blank response. Another criteria is that the peak should be identifiable, discrete and reproducible with a precision of 20% and an accuracy of 80-120% (147). 25 nmol/L is the lowest 5-HIAA standard. To access the precision of this calibration standard, it was analysed ten times within the same analytical run. The mean, standard deviation (SD) and coefficient of variation (CV) were calculated (Table 4).

1	2	3	4	5	6	7	8	9	10	**Mean**	**SD**	**%CV**
25	24	24	24	29	24	24	25	23	26	**25**	**2**	**7**

Table 4. The values obtained for ten analyses of the 25 nmol/L calibrator and the calculated mean, SD and CV

The precision obtained was 7%, therefore the 25nmol/l calibrator standard satisfies the FDA guidance criteria for precision.

To assess the concentration-response relationship of the calibration curve, one of the conditions to satisfy is that the deviation of the LLOQ from its nominal value should not exceed 20%. Using the mean value obtained for the lowest standard (25 nmol/L) in Table 4, the mean percentage deviation from its nominal value was 0%.

To assess the concentration-response relationship at concentrations lower than the lowest standard (25 nmol/L), the lowest standard was diluted serially to obtain concentrations of 15, 12.5 and 10 nmol/ (Table 5). The results of the % deviation of the mean values from the target values are recorded in Table 6. Results obtained were greater than 20 %. According to the FDA guidance, they would not be suitable to use for the calibration curve. Therefore, the 25 nmol/L calibrator fulfils the criteria of the LLOQ.

Calibrator (nmol/L)	**Dilution factor**	**Preparation**
15	3 in 5	150 µL of 25 nmol/L Cal + 100 µL of PBS/BSA
12.5	1 in 2	150 µL of 25 nmol/L Cal + 150 µL of PBS/BSA
10	2 in 5	100 µL of 25 nmol/L Cal + 150 µL of PBS/BSA

Table 5. Preparation of standards lower than 25 nmol/L

Calibrator (nmol/L)	15	12.5	10
1	20	21	17
2	19	19	16
Mean	19.5	20	16.5
% deviation of mean value from target	**30**	**60**	**65**

Table 6. Results obtained from analysing calibrators lower than 25 nmol/L

The concentration-response relationship was also determined in the other standards used in developing the calibration curve apart from the 25 nmol/L standard. They were analysed in duplicates and their mean values were used to calculate the percentage deviation from the target value (Table 7). The results obtained showed that six out of seven standards had less than 15% deviation from the target value. This meets the condition for developing a calibration curve as set out by the FDA guidance.

Target 5-HIAA (nmol/L)	10000	5000	1000	500	250	100	50
Measured 5-HIAA (nmol/L)	10248	4595	1047	553	261	113	65.5
% deviation from target calibrator	**2.48**	**-8.1**	**4.7**	**10.6**	**4.4**	**13**	**31**

Table 7. Results showing the percentage deviation of the measured standards from their target values

2.3.2.2 Linearity

The interval between the LLOQ and the upper limit of quantitation (ULOQ) must be demonstrably linear – there must be a defined relationship between the analyte concentration and the signal intensity.

The calibrators used in assessing the linearity of the 5-HIAA assay were made by spiking pooled plasma samples. The top calibrator standard (10,000

nmol/L) was made from a stock solution of 1 mmol/L 5-HIAA. Subsequent non-serial dilution of the top calibrator standard produced different concentrations of calibrators (Table 8).

The calibration curve should be linear regarding regression statistics. Linearity of the assay was confirmed by linear regression analysis with a correlation coefficient; r^2 greater than 0.99 (Figure 12). This assay fulfils all the criteria required for generating a calibration curve.

Calibrator (nmol/L)	Dilution factor	Preparation
10000	1 in 100	100 µl of 5-HIAA stock in 10 mL of pooled plasma (PP)
5000	1 in 2	200 µl of spiked PP + 200 µl of PBS/BSA
1000	1 in 10	40 µl of spiked PP + 360 µl of PBS/BSA
500	1 in 20	40 µl of spiked PP + 760 µl of PBS/BSA
250	1 in 40	125 µl of spiked PP make up to 5000 µl of PBS/BSA
100	1 in 100	50 µl of spiked PP make up to 5000 µl of PBS/BSA
50	1 in 200	50 µl of spiked PP make up to 10000 µl of PBS/BSA

Table 8. Preparation of calibrator standards to assess linearity. PP-pooled plasma

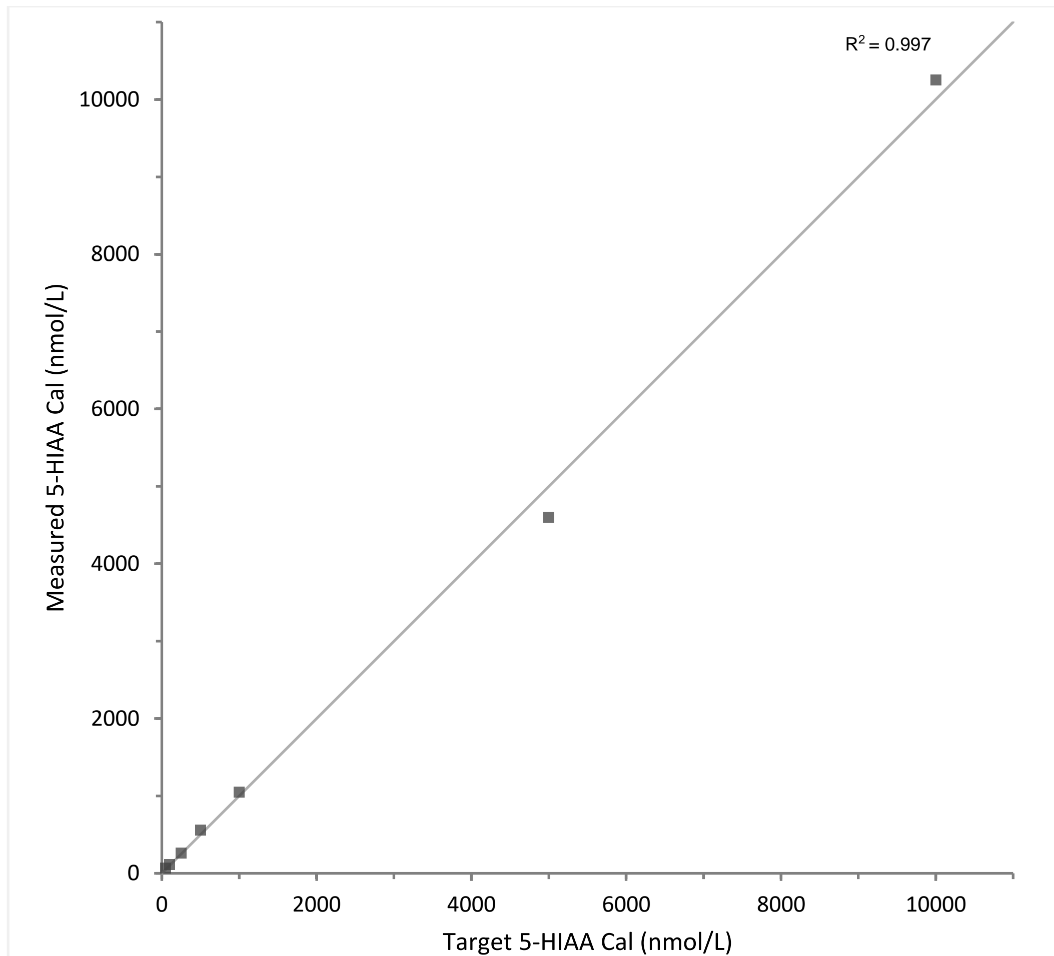


Figure 12. Linear regression graph used to determine the linearity of the plasma 5-HIAA assay

2.3.3 Accuracy

The accuracy of a method describes how well the results obtained from that method agrees with an accepted reference method. Accuracy can be determined in different ways, such as comparison of results with a reference method, analysis of certified reference material, comparison of calibrators, determining the percentage of recovery from a spiked blank or spiked sample. The accuracy of a method is deemed to be acceptable if the mean value obtained is within 15% of the reference value (140, 147).

The preference was to determine the accuracy of this method using a reference method. There is no known reference method for plasma or serum 5-HIAA. External quality assessment (EQA) samples are often used but there is no EQA scheme available.

2.3.4 Recovery

Recovery is a measure of the extraction efficiency of an analytical method within permitted limits of variability. As a measure of accuracy, it can be assessed by spiking samples and determining the percentage of recovery (140, 147). The three different concentrations of 5-HIAA used for spiking were 50, 200 and 1000 nmol/L. A Cerilliant solution of 5-HIAA (1mg/mL in methanol) was used to prepare a 100 mg/L stock solution of 5-HIAA. 500 µL of the Cerilliant solution was pipetted into a 5ml volumetric flask and the volume made up to 5mls with methanol. The 100 mg/L stock solution contained 523.067 µmol/L of 5-HIAA (MW =191.18). Two working solutions of 50000 nmol/L and 5000 nmol/L were prepared from the stock solution.

1. 50,000 nmol/L working solution (**WS1**) → Pipette 478 µL of stock solution into a 5 ml volumetric flask and make up the volume to 5mls with methanol
2. 5000 nmol/L working solution (**WS2**) → Pipette 96 µL of stock solution into a 10 ml volumetric flask and make up the volume to 10 mls with methanol

The working solutions were used to spike pooled plasma and steroid stripped serum.

Solution (nmol/L)	Dilution factor	Preparation
50	1 in 100	50 µL of **WS2** in 5 ml of matrix material
200	1 in 25	80 µL of **WS2** in 2 ml of matrix material
1000	1 in 50	40 µL of **WS1** in 2 ml of matrix material

Table 9. Preparation of the different concentrations of the spiked solution

On analysis of the spiked steroid stripped serum, the results obtained for the different concentrations (50, 200, 1000 nmol/L) were less than that of the unspiked sample. It is unclear why this was the case. It may be due to deterioration of the 5-HIAA added to the sample possibly because of changes to the matrix during the removal of steroids from it. Unfortunately, results were not obtained for the pooled plasma spiked with 200 nmol/L.

Recovery of 5-HIAA from the spiked pooled plasma is shown below in Table 10. It was calculated using this formula;

Concentration of 5-HIAA in [(spiked sample – neat sample)/concentration spiked] x 100

Pooled plasma	Neat	50 nmol/L	1000 nmol/L
	169	215	933
	182	224	975
	169	216	983
	177	216	955
	170	213	1337
Mean total	173	217	1037
Recovery		0.88	0.86
% Recovery		88	86

Table 10. Results obtained from recovery experiment

88 and 86% recovery were obtained in the samples spiked with 50 and 1000 nmol/L of 5-HIAA respectively.

2.3.5 Precision

The precision of a method is the closeness of the results obtained when the procedure is applied repeatedly to multiple extracts of the same sample. There are two types of precision; within run also referred to as intra-batch and between run also known as inter-batch (140, 147).

Precision was carried out using the normal, pathological and in-house QC, and the lowest and highest standards. Within run precision involved analysis of the selected standards and QCs ten times during a batch analysis (Table 12). This was repeated on three consecutive days to assess the between run precision. Within and between run precision is acceptable if the CV is 15% or less. The between run CVs were all acceptable (Table 11).

Day 1	Day 2	Day 3	**Mean**	**SD**	**%CV**
25	25	26	25	0.6	2.3
117	103	106	109	7.4	6.8
259	265	262	262	3.0	1.1
2229	2471	2433	2378	130.1	5.5
10486	10653	10706	10615	114.8	1.1

Table 11. Results obtained for inter-batch precision

Day 1	1	2	3	4	5	6	7	8	9	10	**Mean**	**SD**	**CV**
Cal 1	28	24	28	27	23	23	25	23	25	26	25	2	8
CS QC 1	108	105	102		110	101	103	107	107	108	106	3	3
CS QC 2	265	265	264	267	252	267	248	258	248	251	259	8	3
IQC 3	2475	2023	2308	2372	2179	2268	2236	2369	2274	1782	2229	199	9
Cal 9	10267	10626	10683	10153	10726	10228	10823	10407	10624	10327	10486	237	2

Day 2	1	2	3	4	5	6	7	8	9	10	**Mean**	**SD**	**CV**
Cal 1	25	24	24	24	29	24	24	25	23	26	25	2	7
CS QC 1	105	99	101	104	100	104	100	109	106	106	103	3	3
CS QC 2	254	273	254	290	260	274	264	275	249	261	265	13	5
IQC 3	2493	2499	2511	2517	2291	2512	2454	2592	2488	2349	2471	88	4
Cal 9	9731	10493	11137	11622	10566	10717	10495	10035	10823	10909	10653	534	5

Day 3	1	2	3	4	5	6	7	8	9	10	**Mean**	**SD**	**CV**
Cal 1	27	25		25	24	27	22	22	25	23	24	2	8
CS QC 1	102	103	101	107	105	109	110	105	106	108	106	3	3
CS QC 2	250	251	267	261	255	264	268	262	278	261	262	8	3
IQC 3	2438	1941	2548	2252	2433	2650	2489	2371	2566	2637	2433	211	9
Cal 9	10447	10521	11868	10270	9129	10655	10765	11107	11482	10816	10706	739	7

Table 12. Results obtained for intra-batch precision. Cal 1 – low standard, CSQC1- normal QC, CSQC2 – pathological QC, IQC 3 – in house QC, Cal 9 – highest standard

2.3.6 Carryover

It is important to assess for carryover and its impact on results. Carryover was assessed by the sequential analysis of the lowest (25 nmol/L) and highest (10,000 nmol/l) calibrator standard five times each. This was followed by the alternate analysis of the lowest and highest calibrator standard five times each. The order of analysis can be described as; LLLLLHHHHHLHLHLHLHLH where L represents the lowest standard and H the highest standard. The independent sample t-test was used to analyse the results. This showed that that the lowest calibrator standard (25 nmol/L) when analysed alternately with the highest standard had a higher mean concentration (33.6 ± 1.34 nmol/L) which was statistically significant when compared to the analysis done sequentially (23 ± 2.74 nmol/L), p= 0.0001. According to the 2018 FDA guidance for bioanalytical method validation (149), carryover should not exceed 20% of the LLOQ. The mean concentration of the lowest standard, also the LLOQ in the alternate analysis (LHLHLHLHLH) was 33.6 nmol/L and the carryover was 34%. Significant carryover has been demonstrated in this 5-HIAA assay. To address this, methanol washes are injected between samples analysed consecutively if the preceeding sample is known to have a high 5-HIAA concentration. In these cases, subsequent samples may have to be repeated to ensure that the possibility of carry-over has been eliminated. However, it may not be time and cost effective to re-analyse all samples that fall into this category if the effect of carry-over does not have a clinical impact.

2.3.7 Stability

The stability of an analyte can be assessed at different stages after the sample has been collected. Storage at different temperatures, freeze-thaw cycles and post-extraction stability should be evaluated.

Five patient samples with low to high 5-HIAA concentrations were used to assess stability. Due to time constraints, these samples were only subjected to two freeze-thaw cycles. After the first freeze-thaw cycle and analysis of the samples, they were re-frozen at -20°C for another 72 hours before undergoing a second freeze-thaw cycle. Results were analysed in duplicates and the percentage change of the mean difference was determined. Four out of the five patient samples analysed showed acceptable stability of ± 15% (Table 13), based on the 2018 FDA guidance for bioanalytical method validation (149). The second patient had a result outside the acceptable limit (Table 13). Although it does not rule out a problem with stability, there is a possibility that something inherent in the patient's sample may be responsible for this, thus suggesting they may be a cohort of patients with a similar pattern of results. Further investigations will be required to confirm this.

	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2	Day 1	Day 2
	P1		*P2		P3		P4		P5	
1	78	69	110	140	249	230	513	502	870	952
2	64	73	118	129	242	238	495	532	867	970
Mean	71	71	114	134.5	245.5	234	504	517	868.5	961
Mean difference	0		20.5		-11.5		13		92.5	
% change	0		18.0		-4.7		2.6		10.7	

Table 13. Results obtained for assessment of stability. P – patient, *P2 – patient 2

2.4 Patient Sample Comparison

A plasma 5-HIAA method was developed and validated by Miller *et al* at Manchester University NHS Foundation Trust. Their serum assay has been in routine use since 2012 (91).

A patient sample comparison was carried out between our plasma 5-HIAA assay and that of Manchester University NHS Foundation Trust.

Samples were obtained from 80 patients with NET. The mean age of the patients was 64 years (± 11) and most of the patients were male (62%).

Patient characteristics (n=80)	
Mean age (years)	64 +/-11
Gender	
Male	50 (62%)
Female	30 (38%)
Our median plasma 5-HIAA concentration (nmol/L)	384 (145,1000.5)
MUT median plasma 5-HIAA concentration (nmol/L)	369 (128, 990.2)

Table 14. Baseline characteristics of patients. Categorical data is reported in percentage and continuous data as mean ± standard deviation for normally distributed data or median and interquartile range (IQR) if the data does not follow a normal distribution. MUT = Wythenshawe lab at Manchester University NHS Foundation Trust

Our plasma samples were also analysed at the Wythenshawe lab at Manchester University NHS Foundation Trust and their method was compared with our method. Plasma 5-HIAA did not follow a normal distribution, the median concentration of our plasma 5-HIAA was 384 (145, 1000.5) nmol/L and that of the Wythenshawe lab was 369 (128, 990.2) nmol/L (Table 14).

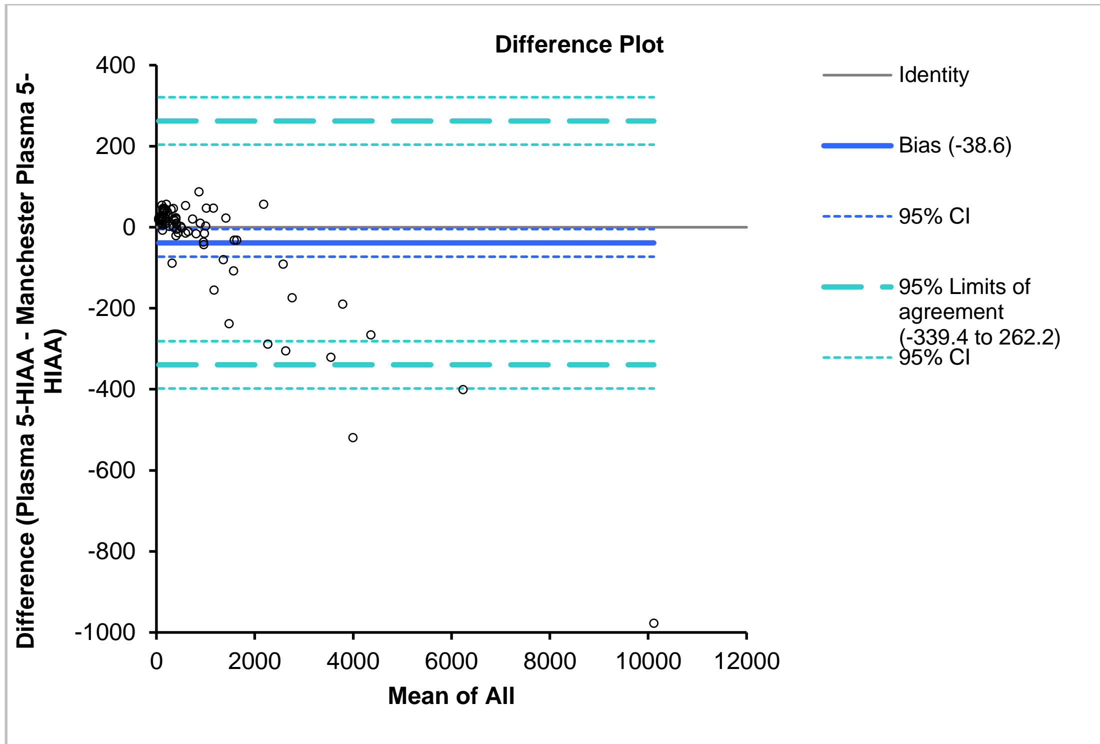


Figure 13. Bland-Altman plot showing the mean difference between our plasma 5-HIAA method and the Wythenshawe lab method

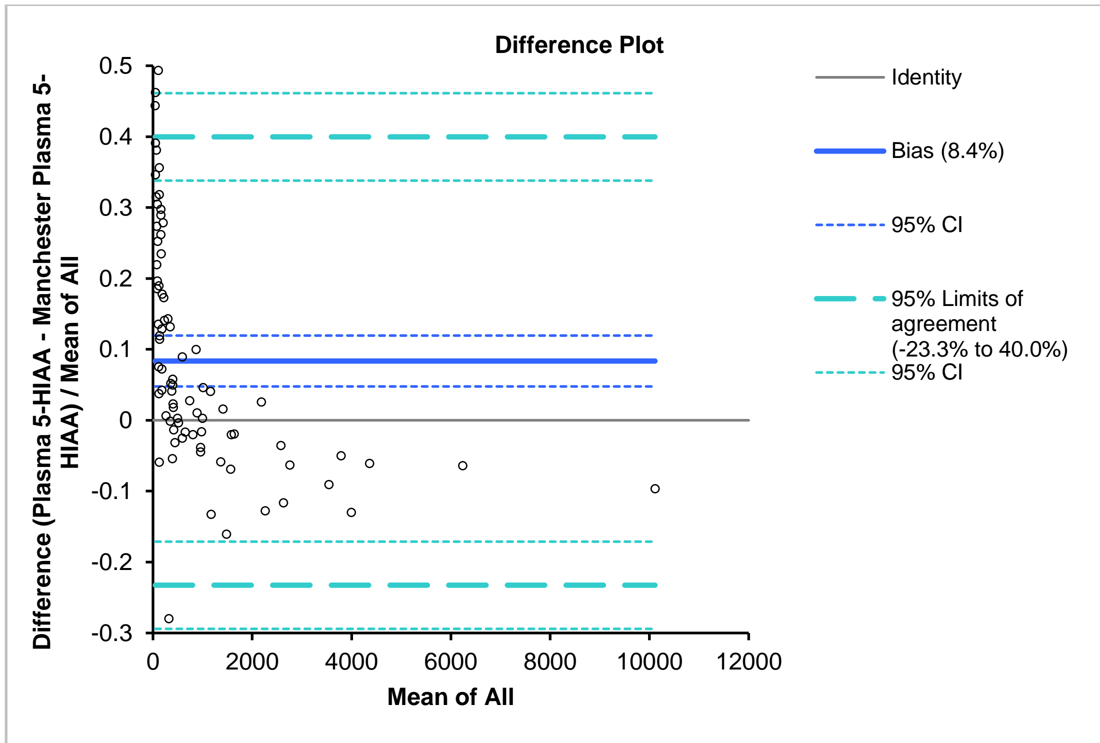


Figure 14. Bland-Altman plot showing the percentage difference between our plasma 5-HIAA method and the Wythenshawe lab method

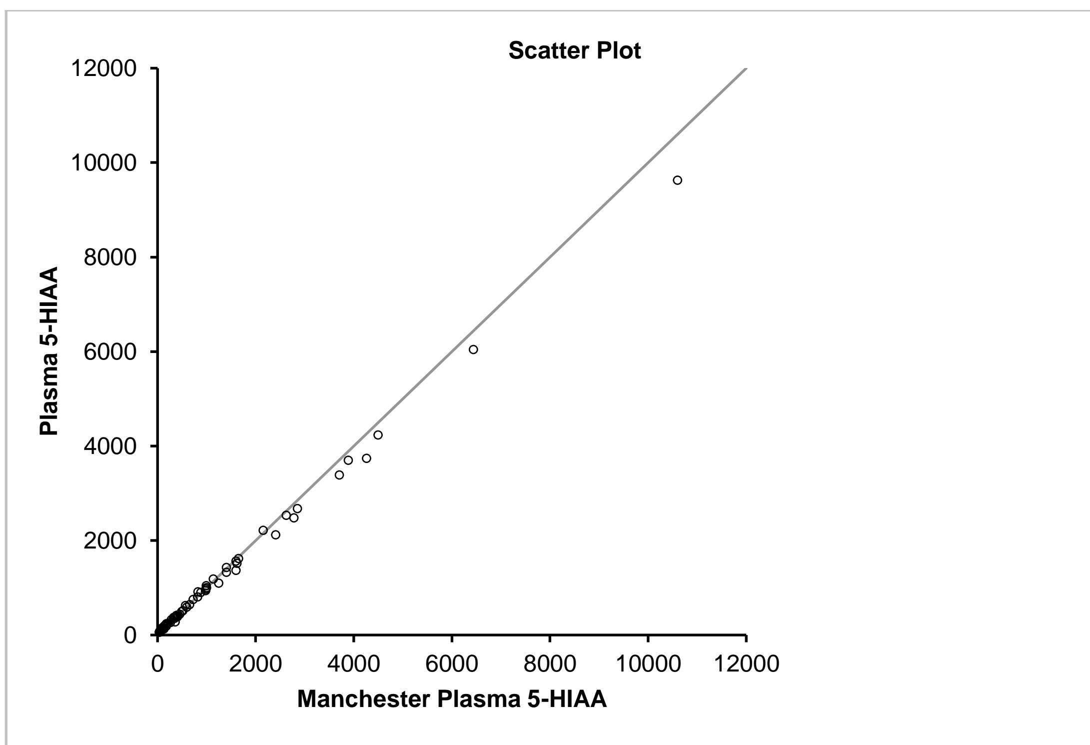


Figure 15. Scatter plot showing the relationship between our plasma 5-HIAA method and that of the Wythenshawe lab

The Bland-Altman analysis comparing the two plasma 5-HIAA methods showed our method has a mean negative bias of 38.6 nmol/L (8.4%) compared to the Wythenshawe method. The percentage difference plot in Figure 14 shows the bias observed is not a concentration dependent bias. The scatter plot (Figure 15) shows there is a good agreement between the two methods.

2.5 Conclusion

The FDA guidance was used to validate this LC-MS/MS plasma 5HIAA method. This assay was shown to be linear across the concentration range 25 to 10,000 nmol/L (R^2=0.997). The lowest calibrator standard was established as the LLOQ. Acceptable inter and intra-assay precision was demonstrated by this plasma 5-HIAA method. Adequate and consistent recovery was achieved

at 50 and 1000 nmol/L. Significant carryover was observed and injection of methanol washes will minimise the impact of this. A limited study of the stability of plasma 5-HIAA showed that following 2 freeze-thaw cycles at -20°C, 5-HIAA was stable. A previous study of 5-HIAA in serum showed stability after five freeze-thaw cycles (89).

The limitations observed were mainly due to time constraints in order to complete my MD thesis (including impact of COVID-19). I was unable to repeat some experiments, and carried out limited analysis for interference and analyte stability. The effect of haemolysis and lipaemia were also not investigated. This was further compounded by technical issues experienced intermittently with the LC-MS/MS analyser and batch failures which led to repeat analysis of some samples.

In order to introduce this assay into routine service, further investigations would be required to ensure that the analysis is robust and confirmatory experiments as well as assessment of stability and effect of haemolysis, icterus and lipaemia are planned.

Other aspects of 5-HIAA stability such as post-extraction stability will also be assessed. This would enable a batch to be prepared in advance of analysis. Establishing post-extraction stability is especially useful where analysis on the LC-MS/MS is delayed or interrupted due to instrument failure. In the absence of this, the batch would have to be re-extracted.

In summary, this new LC-MS/MS method for the determination of plasma-5HIAA has acceptable performance as shown by the method validation.

Chapter 3 Clinical validation of plasma and serum 5-HIAA

3.1 Introduction

Addressing a clinical need is one of the reasons behind the development of an assay in a clinical laboratory. Validation of an assay is essential as this is the evidence required to show that the method employed is suitable for its intended use. Analytical validation describes the performance characteristics of a new assay and at the same time, it can highlight any area that may require improvement so that the performance of the assay is optimised. Clinical validation is usually the next step after analytical validation. It evaluates the clinical utility of the results obtained from a new assay. Therefore, whilst the emphasis on analytical validation is on the analyte, clinical validation focuses on the clinical condition the analyte is associated with (150, 151).

3.2 Aim

The primary aim of this study was to develop an assay for plasma and serum 5-HIAA, assess their efficacy as biochemical markers of neuroendocrine tumour and correlate them with urine 5-HIAA.

The analytical validation of the plasma and serum 5-HIAA assay has been described in chapter 2. This chapter will focus on the clinical validation of this assay.

3.3 Methods

3.3.1 Study design

This was a prospective single centre non-randomised study. Patients with NET and healthy volunteers were required for this study. Eligible participants were expected to attend the clinic once during the study, with no follow up visits. Enrolment into the study lasted for approximately twelve months.

The sample size used in this study was computed by a medical statistician. Agreement of urine with plasma and serum 5-HIAA assays was measured by Cohen's Kappa coefficient (152), where $\kappa = 0$ when assays agree purely by chance and $\kappa = 1$ denotes perfect agreement between the assays. Assuming that 20% of participants were 5-HIAA secretors, we required 80 participants in order to have 90% power to detect a κ of at least 0.90 (strong agreement) compared to κ no greater than 0.60 (weak agreement). This assumes a one-sided hypothesis test and an acceptable type I error (false positive) rate of 2.5%.

To clinically validate the plasma and serum 5-HIAA assay, we recruited 30 healthy volunteers under A'hern's single arm design (153). The diagnostic performances of the plasma and serum 5-HIAA assays were assessed using a ROC curve, and different sensitivities and specificities were determined. This was used to decide a suitable cut-off. This cut-off was used to assess for agreement between the urine and the plasma and serum 5-HIAA assays. If an agreement was observed in 23 or more healthy volunteers, then we could reject the null hypothesis that the probability of agreement is lower than 60%, assuming an acceptable type I error of 5% and at least 99% power to detect

an agreement probability of at least 90% (the probability of agreement here is not the same as Cohen's Kappa).

3.3.2 Ethics and consent

Ethical approval for this study was obtained from South West – Frenchay Research Ethics Committee (17/SW/0131).

Written informed consent was obtained from each participant including the healthy volunteers in accordance with local and national regulatory requirements, and the Declaration of Helsinki. For patients with NET this took place during the clinic visit to the Royal Free Hospital. Each participant had the study details explained including the anticipated benefits and potential risk(s). A participant information sheet (PIS) was given to them and they were informed they could withdraw from the study at any time without obligation.

3.3.3 Patient recruitment

80 participants with NET were recruited from the Neuroendocrine Tumour clinic and the multidisciplinary team meeting at the Royal Free Hospital. They were identified from the NET patient database and the electronic patient record used in the NET clinic by the clinical team. A PIS containing details about the study was given to them and they were given adequate time, at least 7 days to decide if they wished to take part in the study. Informed consent was taken during the clinic visit.

Pregnant women were excluded from the studies because serotonin is known to be elevated in pregnancy (154, 155).

3.3.3.1 Eligibility criteria

Inclusion Criteria

1. 18 years and above
2. Patients with a confirmed diagnosis of neuroendocrine tumour

Exclusion Criteria

1. Pregnant women
2. Inability to give informed consent

3.3.4 Recruitment of healthy volunteers

30 healthy volunteers participated in this study. Some of them were recruited from the Neuroendocrine Tumour Unit and the Clinical Biochemistry department at the Royal Free Hospital. Others were recruited through advertisement from University College London. The study was discussed with them in detail, a PIS was given and informed consent was obtained.

3.3.5 Sample collection and analysis

Patient and healthy volunteer samples were drawn into appropriate evacuated specimen tubes (BD Diagnostics Pre-analytical Systems, Plymouth, PL6 7BP, UK). Serum gel tubes were used for collecting blood tests for renal function and serum 5-HIAA. Potassium EDTA tubes were used for plasma 5-HIAA and serotonin. Prior to the 24 hour urine collection, every participant was given an information sheet about how to collect the sample and the dietary restrictions (to avoid serotonin rich foods) to adhere to 48 hours before and during the 24 hour urine collection. All blood samples were

collected on the day the 24 hour urine collection was completed and returned. Participants were not required to fast for this study.

Glomerular filtration rate (GFR) was estimated using the Modification of Diet in Renal Disease (MDRD) formula, 4-variable version;
GFR (ml/min/1.73 m^2) = 175 x serum creatinine x 0.011312)$^{-1.154}$ x age $^{-0.203}$ (0.742 if female) x (1.212 if Afro-Caribbean).
Creatinine was analysed using the Jaffe kinetic colorimetric assay on an automated Roche analyser.

3.3.5.1 Urine 5-HIAA analysis

Urine 5-HIAA analysis was performed using a Chromsystems kit (Chromsystems, Germany) on a Shimadzu prominence HPLC analyser, an Antec electrochemical detector (ECD) and a liquid chromatography system. Sample preparation involved the addition of an internal standard prior to protein precipitation. After vortex-mixing and centrifugation, an aliquot of the supernatant was diluted with an equal volume of water in a glass HPLC vial and mixed, before being placed onto the HPLC autosampler for analysis. Reverse phase liquid chromatography with isocratic elution was used to separate 5-HIAA and the internal standard from other electrochemically active compounds. The eluted compounds were detected and quantitated by the ECD.

3.3.5.2 Plasma and serum 5-HIAA analysis

Plasma and serum 5-HIAA analysis were performed using an LC-MS/MS method with electrospray ionisation established using a Shimadzu Nexera X2

LC-30AD high performance liquid chromatography (HPLC) system and a tandem mass spectrometer 8050 (Shimadzu, Japan) as described in chapter 2. Sample preparation involved extraction of 50uL of plasma or serum using protein precipitation with acetonitrile and loading this on to a Strata-X (Phenomenex) on-line extraction column for on-board sample clean up. This was followed by analytical separation on a Raptor Biphenyl LC column 2.7µm X 2.1mm (Thames Restek, UK). Mobile phase A contained 0.1% formic acid in water, and mobile phase B contained 0.1% formic acid in methanol. Mobile phase C (for loading and washing of the trap column) contained 20% of methanol and 0.1% formic acid in water. Ionisation was carried out in a positive mode. The ion transitions were *m/z* 192.1>146.2 (quantifier) and 192.1>118.25 (qualifier) for plasma 5-HIAA and *m/z* 198.15>152.25 for the internal standard ($^{13}C_6$-5-HIAA). Detection of 5-HIAA and the internal standard was in a multiple reaction monitoring (MRM) mode.

3.3.6 Statistical analysis

Statistical analysis was performed using Microsoft Excel, Analyse-It (Analyse-It Software Ltd, Leeds, UK) and SPSS version 25. Test for normality was performed using the Shapiro-Wilk test and a Normal Q-Q plot. A normal distribution was confirmed if the significance value in the Shapiro-Wilk test was greater than 0.05, and the data points laid on the straight diagonal line of the Normal Q-Q plot. A p value of < 0.05 was considered to be statistically significant.

3.4 Results

3.4.1 Baseline characteristics

Most participants in this study, 62.5% (50/80) of patients and 57% (17/30) of healthy volunteers were male (Table 15). The healthy volunteers were younger with a mean age of 34 ±14 years compared to 64 ±11 years in the patients with NET. SI-NET was the predominant type of neuroendocrine tumour. Seven (8.75%) patients were not on SSA. Four of them had their primary tumours (SI-NET) resected with no evidence of recurrence. One patient was due to start on SSA (lanreotide autogel) and the last two patients stopped their SSA due to intolerance. One patient stopped their SSA four years before the study and the other, a month before the study. One patient did not have their estimated glomerular filtration rate (eGFR) result recorded because their creatinine result was not available.

A study by Hannedouche *et al* on the potential use of plasma 5-HIAA as an endogenous marker of renal plasma flow revealed high concentrations of plasma 5-HIAA at low renal plasma flow rates, indicating reduced excretion by the kidneys (156). This was confirmed in the study by Adaway *et al* who showed a negative inverse relationship between eGFR and serum 5-HIAA concentration. As the eGFR declined, the median concentration of 5-HIAA increased with markedly raised levels at an eGFR of less than 20 ml/min/1.73 m^2 (91). This has implications for the interpretation of 5-HIAA results in the urine and blood. In view of this, all the results of any study participant with an eGFR of less than 20 ml/min/1.73 m^2 were excluded from statistical analysis required to clinically validate the plasma and serum 5-HIAA assays. Two

patients with NET were affected. One had an eGFR of 16 ml/min/1.73 m^2 and was on renal replacement therapy (RRT), the other patient with an eGFR of 15 ml/min/1.73 m^2 was due to commence on RRT.

Participants	**Patients**	**Healthy volunteers**
Number	80	30
Mean age (Years)	64 ± 11	34 ±14
Gender		
Male	50 (62.5%)	17 (57%)
Female	30 (37.5%)	13 (43%)
NET type		
SI-NET	76 (95%)	
Pancreatic NET	2 (2.5%)	
Bronchial NET	1 (1.25%)	
Hindgut NET	1 (1.25%)	
SSA therapy		
Lanreotide Autogel	38 (47.5%)	
Octreotide LAR	33 (41.25%)	
Lanreotide Autogel + SC Octreotide	2 (2.5%)	
None	7 (8.75%)	30 (100%)
eGFR (90 ml/min/1.73m2)		
≥ 90	22 (27.8%)	19 (63%)
60-89	44 (55.7%)	11 (37%)
45-59	7 (8.9%)	
30-44	3 (3.8%)	
15-29	3 (3.8%)	

Table 15. Baseline characteristics of patients and healthy volunteers. Categorical data is reported in percentages and continuous data as mean ± standard deviation

3.4.2 Diagnostic performance of plasma and serum 5-HIAA

A receiver operating characteristic (ROC) curve was generated for the plasma and serum 5-HIAA assays (Figure 16 andFigure 17). Flushing in the presence of liver metastases or carcinoid heart disease was used to define true 5-HIAA

secretors (carcinoid syndrome). The area under the curve (AUC) for plasma 5-HAA was 0.899 (95% confidence interval (CI) 0.829 to 0.970) and 0.902 (95% confidence interval (CI) 0.833 to 0.972) for serum 5-HIAA.

The ROC curve analysis for the urine 5-HIAA assay (Figure 18) currently in use had an AUC of 0.919 (95% CI 0.857 to 0.982). At the present cut-off of 42 µmol/24 hour, the sensitivity of this assay was 89.5% with a specificity of 81% (Table 18). From our dataset, this cut-off may be sub-optimal because at a slightly lower cut-off of 40 µmo/24 hour, the sensitivity improves to 91% with a specificity of 81%.

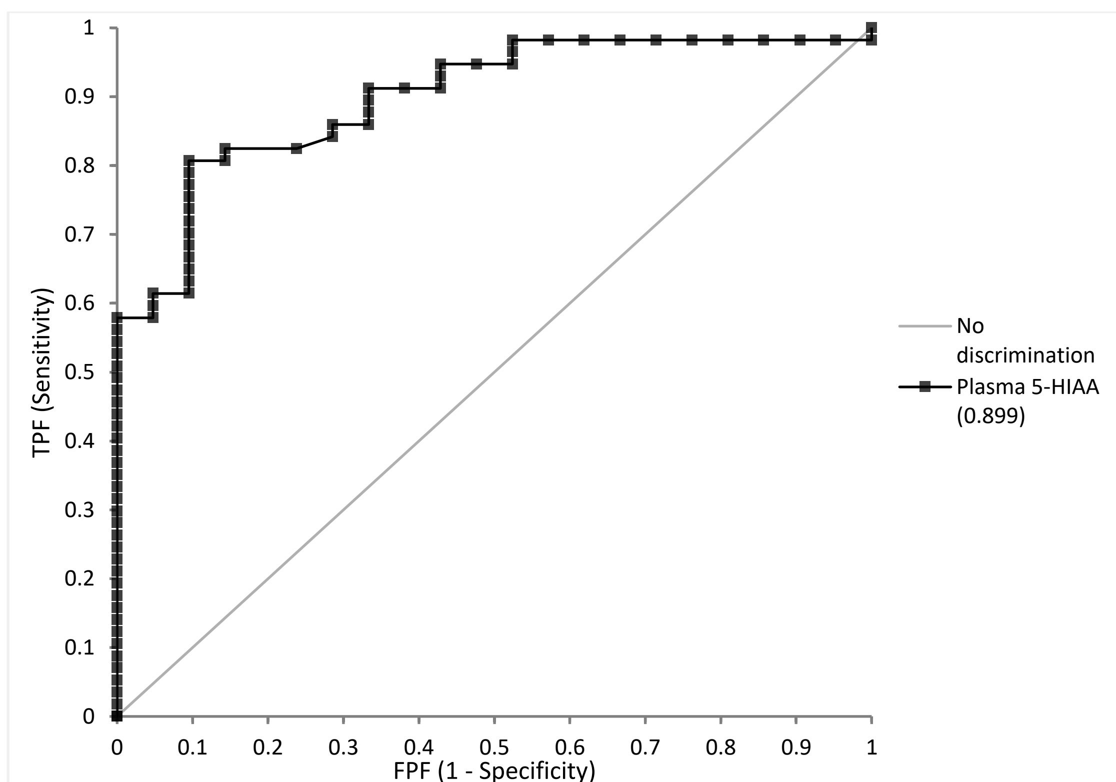


Figure 16. ROC curve analysis for plasma 5-HIAA

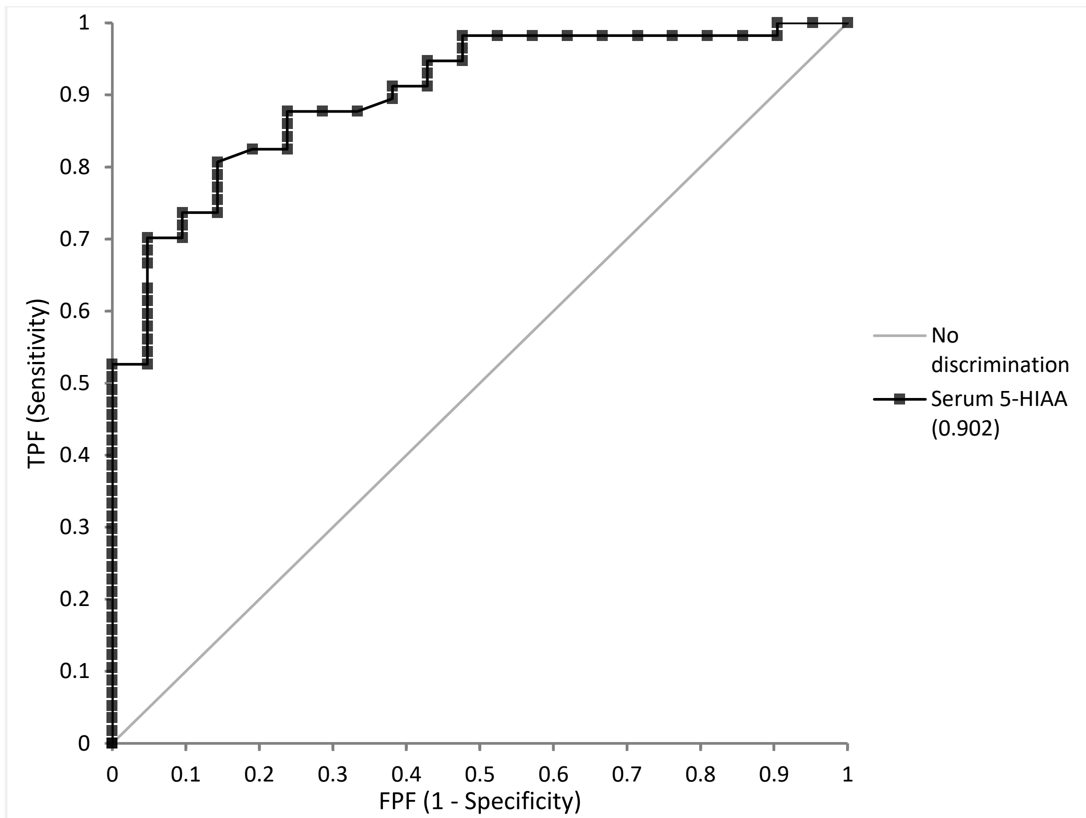


Figure 17. ROC curve analysis for serum 5-HIAA

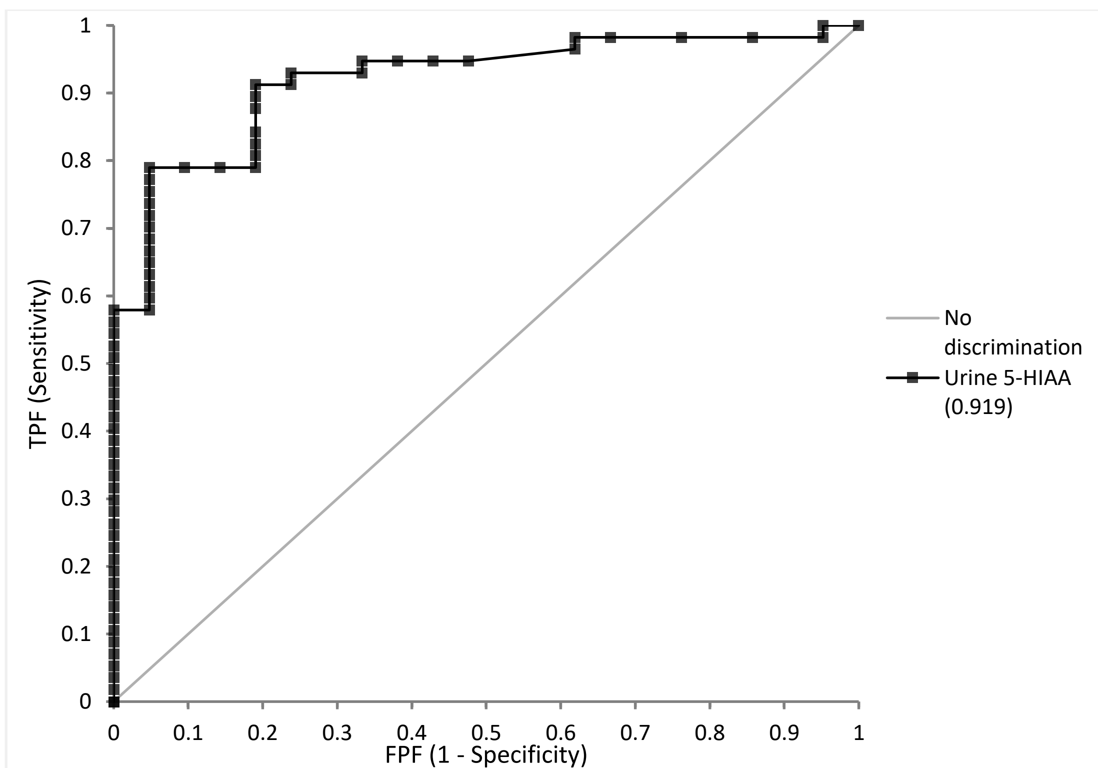


Figure 18. ROC curve analysis for urine 5-HIAA

Plasma 5-HIAA	TP	FP	TN	FN	TP proportion (Sensitivity)	TN proportion (Specificity)
56.5	56	21	0	1	0.982	0.000
57.0	56	20	1	1	0.982	0.048
59.5	56	19	2	1	0.982	0.095
60.5	56	18	3	1	0.982	0.143
72.0	56	17	4	1	0.982	0.190
82.5	56	16	5	1	0.982	0.238
84.5	56	15	6	1	0.982	0.286
85.5	56	14	7	1	0.982	0.333
88.0	56	13	8	1	0.982	0.381
94.5	56	12	9	1	0.982	0.429
99.5	56	11	10	1	0.982	0.476
106.0	55	11	10	2	0.965	0.476
112.5	54	11	10	3	0.947	0.476
122.0	54	10	11	3	0.947	0.524
122.5	54	9	12	3	0.947	0.571
124.5	53	9	12	4	0.930	0.571
134.5	52	9	12	5	0.912	0.571
135.0	52	8	13	5	0.912	0.619
143.0	52	7	14	5	0.912	0.667
144.5	51	7	14	6	0.895	0.667
147.5	50	7	14	7	0.877	0.667
152.5	49	7	14	8	0.860	0.667
179.5	49	6	15	8	0.860	0.714
180.5	48	6	15	9	0.842	0.714
183.0	47	5	16	10	0.825	0.762
188.5	47	3	18	10	0.825	0.857
192.0	46	3	18	11	0.807	0.857
204.5	46	2	19	11	0.807	0.905
231.0	45	2	19	12	0.789	0.905
237.0	44	2	19	13	0.772	0.905
248.0	43	2	19	14	0.754	0.905
262.5	42	2	19	15	0.737	0.905
274.0	41	2	19	16	0.719	0.905
323.5	40	2	19	17	0.702	0.905
349.5	39	2	19	18	0.684	0.905
366.0	38	2	19	19	0.667	0.905
370.0	37	2	19	20	0.649	0.905
383.5	36	2	19	21	0.632	0.905
384.0	35	2	19	22	0.614	0.905

Table 16. Sensitivity and specificity for different cut-off values for plasma 5-HIAA. TP – True positive, FP – False positive, TN- True negative, FN – False negative

Serum 5-HIAA	TP	FP	TN	FN	TP proportion (Sensitivity)	TN proportion (Specificity)
50	57	20	1	0	1.000	0.048
52	57	19	2	0	1.000	0.095
53	56	19	2	1	0.982	0.095
63	56	18	3	1	0.982	0.143
64	56	17	4	1	0.982	0.190
72	56	16	5	1	0.982	0.238
78	56	15	6	1	0.982	0.286
79	56	14	7	1	0.982	0.333
88	56	13	8	1	0.982	0.381
95	56	12	9	1	0.982	0.429
98	56	11	10	1	0.982	0.476
103	56	10	11	1	0.982	0.524
109	55	10	11	2	0.965	0.524
117	54	10	11	3	0.947	0.524
123	54	9	12	3	0.947	0.571
133	53	9	12	4	0.930	0.571
134	52	9	12	5	0.912	0.571
135	52	8	13	5	0.912	0.619
143	51	8	13	6	0.895	0.619
153	50	7	14	7	0.877	0.667
167	50	6	15	7	0.877	0.714
173	50	5	16	7	0.877	0.762
177	49	5	16	8	0.860	0.762
180	48	5	16	9	0.842	0.762
182	47	5	16	10	0.825	0.762
200	47	4	17	10	0.825	0.810
203	46	3	18	11	0.807	0.857
223	45	3	18	12	0.789	0.857
224	44	3	18	13	0.772	0.857
228	43	3	18	14	0.754	0.857
231	42	3	18	15	0.737	0.857
254	42	2	19	15	0.737	0.905
291	41	2	19	16	0.719	0.905
313	40	2	19	17	0.702	0.905
334	40	1	20	17	0.702	0.952
341	39	1	20	18	0.684	0.952
344	38	1	20	19	0.667	0.952
349	36	1	20	21	0.632	0.952
355	35	1	20	22	0.614	0.952
397	34	1	20	23	0.596	0.952
404	33	1	20	24	0.579	0.952
426	32	1	20	25	0.561	0.952
433	31	1	20	26	0.544	0.952
447	30	1	20	27	0.526	0.952
481	30	0	21	27	0.526	1.000

Table 17. Sensitivity and specificity for different cut-off values for serum 5-HIAA. TP – True positive, FP – False positive, TN- True negative, FN – False negative

Urine 5-HIAA	TP	FP	TN	FN	TP proportion (Sensitivity)	TN proportion (Specificity)
7	57	20	1	0	1.000	0.048
9	56	20	1	1	0.982	0.048
13	56	18	3	1	0.982	0.143
18	56	16	5	1	0.982	0.238
19	56	14	7	1	0.982	0.333
21	56	13	8	1	0.982	0.381
26	55	13	8	2	0.965	0.381
27	54	10	11	3	0.947	0.524
28	54	9	12	3	0.947	0.571
32	54	8	13	3	0.947	0.619
33	54	7	14	3	0.947	0.667
37	53	7	14	4	0.930	0.667
38	53	5	16	4	0.930	0.762
39	52	5	16	5	0.912	0.762
40	52	4	17	5	0.912	0.810
42	51	4	17	6	0.895	0.810
43	50	4	17	7	0.877	0.810
44	48	4	17	9	0.842	0.810
45	47	4	17	10	0.825	0.810
46	46	4	17	11	0.807	0.810
47	45	4	17	12	0.789	0.810

Table 18. Sensitivity and specificity for different cut-off values for urine 5-HIAA. TP – True positive, FP – False positive, TN- True negative, FN – False negative

The sensitivities and specificities of the plasma and serum 5-HIAA assays were determined at different cut-offs (Table 16Table 17). At a cut-off of 135 nmol/L, both the plasma and serum 5-HIAA assays had the same performance with both having a sensitivity of 91.2% with a specificity of 61.9%.

3.4.3 Comparison of plasma and urine 5-HIAA

Cohen's kappa (κ) was used to assess the agreement between the plasma and urine 5-HIAA assays in patients with NET. When compared to the null value of kappa = 0 (kappa = 0 is equivalent to random allocation, that is, the plasma and urine 5-HIAA assays agree purely by chance) there was a moderate agreement between the plasma and urine 5-HIAA assays which was statistically significant, $\kappa = 0.675$ (95% CI 0.49 to 0.86), $p < 0.001$. When

compared to the null value of kappa = 0.6, the moderate agreement observed was not statistically significant, κ = 0.675 (95% CI 0.49 to 0.86), p = 0.296. This indicates that the agreement observed is unlikely to be by chance.

To validate the plasma 5-HIAA assay, we assessed the agreement between plasma and urine 5-HIAA in healthy volunteers using A'hern's single arm design. Performing an exact binomial test, 29 out of 30 successes were recorded. Testing against the null hypothesis that the probability of agreement is greater than or equal to 0.6, the probability of agreement obtained was 0.967 (95% CI 0.828 to 0.999), p = <0.001. This indicates a statistically significant agreement.

3.4.4 Comparison of serum and urine 5-HIAA

Similar to the plasma 5-HIAA assay, Cohen's kappa (κ) was used to assess the agreement between the serum and urine 5-HIAA assays in patients with NET. The null value of kappa = 0 (kappa = 0 is equivalent to random allocation, that is, the serum and urine 5-HIAA assays agree purely by chance). The same result as the plasma 5-HIAA was obtained, a moderate agreement was shown between the serum and urine 5-HIAA assays which was statistically significant, κ = 0.675 (95% CI 0.49 to 0.86), p < 0.001. When compared to the null value of kappa = 0.6, the moderate agreement observed was not statistically significant, κ = 0.675 (95% CI 0.49 to 0.86), p = 0.296. Again, this indicates that the agreement observed is unlikely to be by chance.

A similar result was also observed when we used the A'hern's single arm design to assess the agreement between serum and urine 5-HIAA in healthy volunteers. The exact binomial test revealed 29 out of 30 successes. Testing

against the null hypothesis that the probability of agreement is greater than or equal to 0.6, the probability of agreement obtained was 0.967 (95% CI 0.828 to 0.999), p = <0.001. This indicates a statistically significant agreement.

3.4.5 Plasma and serum 5-HIAA

A strong positive correlation which was statistically significant was observed between the plasma and serum 5-HIAA assay. The scatter plot (Figure 19) shows the relationship between them. Spearman's correlation coefficient was 0.99 (p = < 0.001).

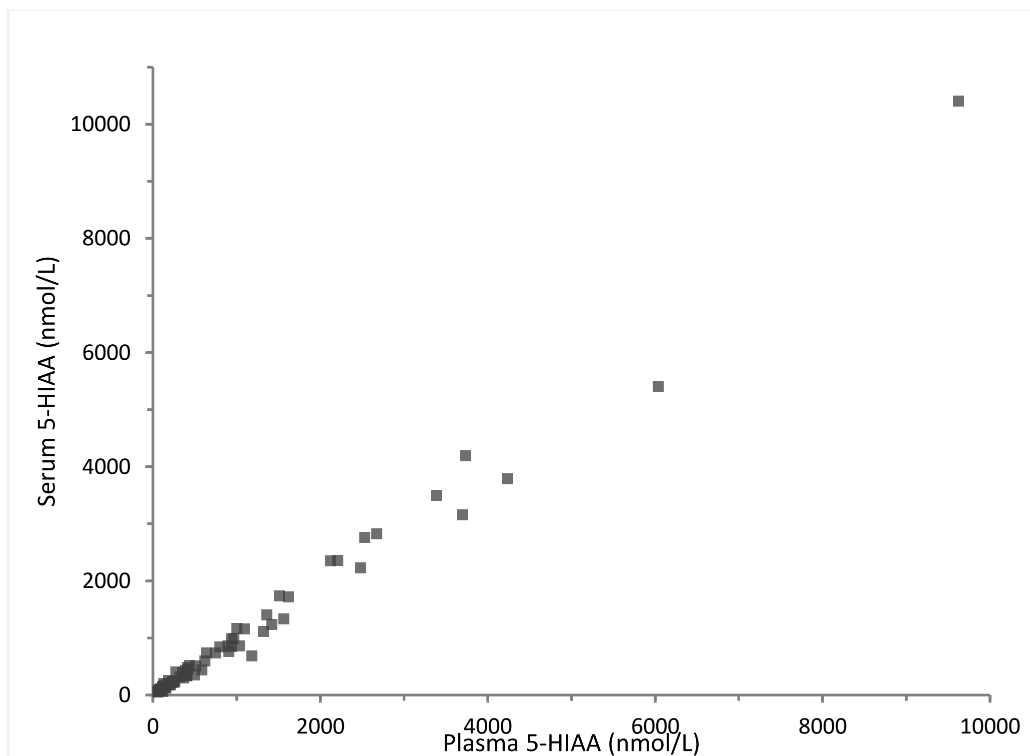


Figure 19. Scatter plot showing the relationship between plasma and serum 5-HIAA

Bland-Altman analysis was used to compare both assays and a good agreement was seen with a mean negative bias of 17.12 nmol/L (2.94%). This means that on average plasma 5-HIAA measures about 17 nmol/L which is 3% higher than the serum 5-HIAA in this dataset. The bias observed is not

concentration dependent; this is best viewed in Figure 21 which shows the percentage difference between the two assays. With increasing concentration, the percentage difference does not increase but the data points are generally spread around the mean.

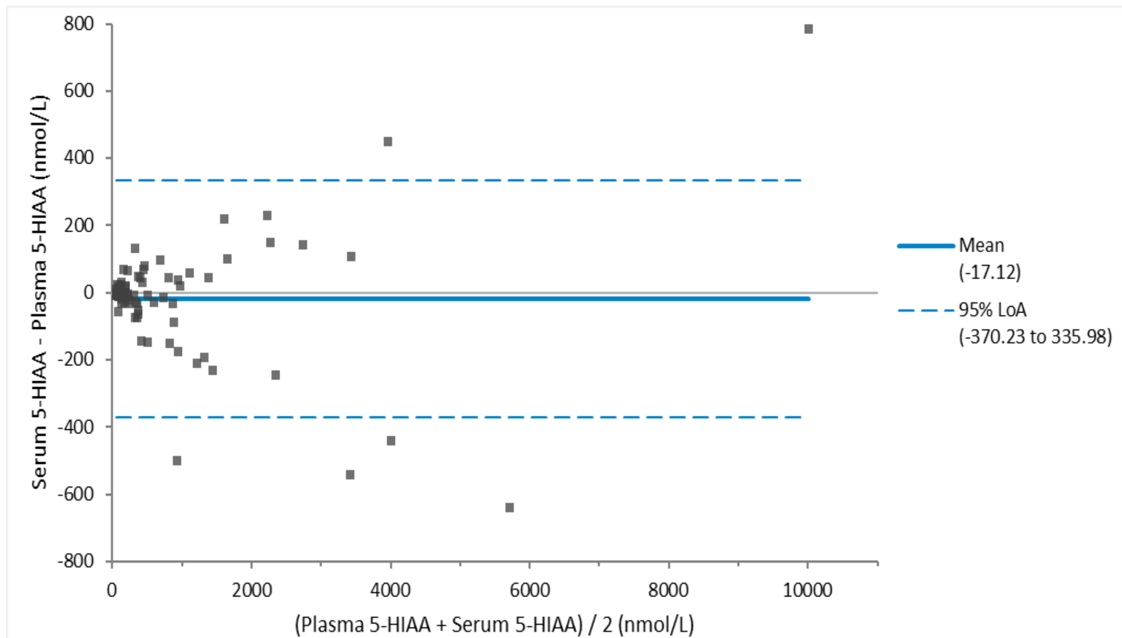


Figure 20. Bland-Altman analysis showing the mean difference between the plasma and serum 5-HIAA assay

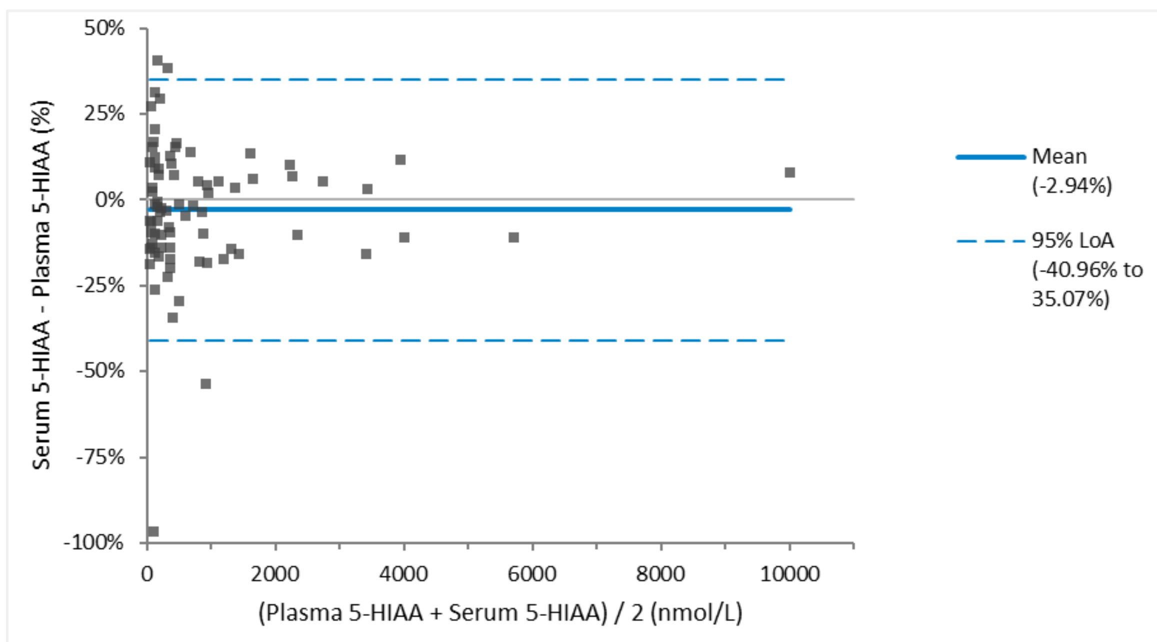


Figure 21. Bland-Altman analysis showing the percentage difference between the plasma and serum 5-HIAA assay

To determine if the difference observed between the plasma and serum 5-HIAA was a true difference, the non-parametric Wilcoxon signed-rank test was performed as both assays do not follow a normal distribution. This showed there was no statistically significant difference between the plasma and serum 5-HIAA assays ($p= 0.714$).

3.5 Discussion

The liver is a common site for metastases from SI-NET (157). CS which occurs in approximately 18% of patients with SI-NET usually occurs when there is metastasis of the tumour to the liver (158). Serotonin is the main hormone implicated in CS. It is normally metabolised in the liver to 5-HIAA but in the presence of liver metastases or ovarian metastases, excess serotonin produced will enter into the systemic circulation leading to CS (157, 159, 160). Flushing occurs in up to 94% of patients with CS and it is usually induced by stress, certain foods such as cheese and meat, and alcohol (157, 160).

The true positive state used in generating the ROC curve was defined as SI-NET with liver metastases and the presence of flushing. Diarrhoea which is another common symptom of CS was not used as a criterion for defining the true positive state because of the complexities that may be involved in the definition of diarrhoea especially in patients with SI-NET who may have other causes for diarrhoea related to the treatment of their NET.

In determining a cut-off for the plasma and serum 5-HIAA assays, a higher sensitivity is obtained with a lower cut-off, whilst at a higher cut-off, the specificity of the assay is better. The cut-off value of 135 nmol/L was chosen because of its high sensitivity (91%) and reasonable specificity (62%), giving a good balance of sensitivity and specificity. A high sensitivity will lead to a high detection rate of patients with CS who have elevated 5-HIAA. Carling et al. obtained a sensitivity of 89% and a specificity of 97% at a cut-off of 118 nmol/L for a plasma 5-HIAA method using HPLC (83). Adaway *et al* adopted this cut-off and reported a sensitivity of 79.6% (91). An LC–MS/MS method for serum 5-HIAA at a cut-off of 123 nmol/L had a sensitivity of 57% and a specificity of 95% (89).

Cohen's kappa used in assessing the agreement between urine 5-HIAA and 5-HIAA in plasma and serum in patients with NET, revealed a strong evidence that the moderate agreement between the assays was better than 0 but little evidence to suggest it was significantly better than 0.6. Comparing 5-HIAA in blood and urine samples for healthy volunteers, there was also strong evidence to reject the null hypothesis that the probability of agreement is less than or equal to 60%. Therefore a good agreement was shown between urine and plasma and serum 5-HIAA.

29 out of the 30 healthy volunteers showed agreement between their urine 5-HIAA and plasma and serum 5-HIAA results. This means that these results were within their reference limits. The only healthy volunteer with a discrepant result had a urine 5-HIAA concentration of 15 µmol/24 hour, a plasma 5-HIAA

of 296 nmol/L and a serum 5-HIAA of 354 nmol/L. All participants in this study were informed they were not to stop dietary restrictions until their blood test was performed on the day they returned their 24 hour urine 5-HIAA collection. As previously listed (Chapter 1, table 2), commonly consumed foods that are rich in serotonin include walnut, banana, pineapple, tomato and plum (78, 79, 87). A possible explanation for the discordant result observed in the healthy participant could be that a serotonin rich food or a pharmacological preparation with a high level of serotonin was consumed prior to the blood test.

Tohmola *et al* observed that serum 5-HIAA concentration was not affected by fasting or having a meal before the blood sample collection. However, the food used in this study was a typical Finnish breakfast which is not rich in serotonin (89). In another study, plasma 5-HIAA levels were monitored in a participant for 8 hours after the consumption of two bananas which are rich in serotonin. Their plasma 5-HIAA concentration was found to return to baseline after 6 hours (87). In a later study by Tohmola *et al,* investigating the duration of 5-HIAA elevation in serum following a diet rich in serotonin, participants were fed a serotonin rich test meal. They observed the highest level of serum 5-HIAA within 2 hours after the meal. Calculation of the half-life of 5-HIAA in circulation was 1.3 hours. Decreased levels of 5-HIAA in the serum was observed within 4 hours after the meal and basal levels were reached from 6 hours after the meal. In participants who consumed test meals with a higher concentration of serotonin, basal levels were reached by the next morning (145). These studies make a case for dietary restriction of serotonin containing meals to be limited to an overnight fast or 24 hours (83, 145).

Plasma and serum 5-HIAA were analysed using the same method. There was a good correlation between both of them as shown by the scatter plot (Figure 19) and Spearman's rank correlation. The concentration of 5-HIAA was on average 3% higher in plasma compared to serum but this difference was not statistically significant, suggesting that measurement of 5-HIAA using plasma or serum is comparable.

Existing literature shows that 5HIAA concentration in plasma or serum is affected by renal function (91, 156) as discussed earlier in this chapter (3.4.1). Further work needs to be carried out to investigate the relationship between eGFR and plasma or serum 5-HIAA. Of particular importance is the level of eGFR at which the rise in plasma or serum 5-HIAA is of clinical relevance.
Our study shows that the serum and plasma 5-HIAA assays have the same performance at the chosen cut-off of 135 nmol/L. Their diagnostic performances were comparable to the urine assay which performed slightly better at the current cut-off of 42 µmol/24 hours (AUC for plasma 0.899, serum 0.902, urine 0.919). Sensitivities of the plasma and serum assays were also marginally better than that of the urine assay which had a better specificity. However, the trade-off is for increased sensitivity to detect more patients with CS.

The process of collecting a 24-hour urine can be tedious and an inconvenience. The addition of acid to the sample container for preservation of 5-HIAA poses a risk. Having a blood based assay for 5-HIAA has the advantage of an immediate collection that can be performed at the same time,

using the same sample tube as other blood tests that may be required. Another benefit as discussed previously is that serotonin rich diets can be restricted for a shorter time in preparation for 5-HIAA sample collection in plasma or serum. Urine 5-HIAA collection requires a total of 72 hours dietary restriction which includes the day of collection. The pre-analytical issues surrounding the 24-hour urine collection can lead to erroneous results either due to incomplete collection or lack of adherence to the dietary restrictions. Blood sampling for 5-HIAA will require venepuncture with the risk of pain at the site which should be short lived or bruising. However due to the routine use of venepuncture in patients with NET for monitoring of other blood based tests, and the convenience compared to the 24-hour urine collection, a blood based assay for 5-HIAA is ideal.

3.6 Conclusion

The plasma and serum 5-HIAA assays developed show good diagnostic accuracy. At a cut-off of 135 nmol/L, both assays show a sensitivity of 91% and a specificity of 62%. We have demonstrated that they compare well with the urine 5-HIAA assay currently in use. The plasma and serum 5-HIAA assays were shown to be comparable, hence either sample type may be used.

The plasma and serum 5-HIAA assays at the cut-off of 135 nmol/L are suitable to use as an alternative to the urine 5-HIAA assay.

Chapter 4 Comparison of plasma and serum 5-HIAA with serotonin (5-hydroxytryptamine)

4.1 Introduction

CS is commonly associated with SI-NET. A hallmark of SI-NET is the occurrence of fibrosis either around or distant from the primary tumour, and there is strong evidence that 5-HT is involved. It is implicated in the pathogenesis of CHD. 5-HT receptors particularly the subtype 2b which is prevalent on the cardiac valves are activated by 5-HT causing fibrotic proliferation, stimulation of inflammatory cytokines and up-regulation of transforming growth factor-β1. This leads to formation of plaques in the cardiac valves predominantly affecting the right side of the heart. The potential involvement of 5-HT in mesenteric fibrosis was demonstrated in a study which showed a correlation between elevated platelet 5-HT and mesenteric fibrosis in patients with SI-NET. Another study showed a significant association between elevated urine 5-HIAA levels and mesenteric fibrosis (161-163).

5-HT quantitation would be ideal in patients with NET especially if CS is present. Analysis of 5-HT has been performed in different biological fluids such as cerebrospinal fluid (CSF), urine and blood. CSF analysis is relevant in the assessment of neuropsychiatric disorders. 5-HT is present in very small concentrations in urine, and may contribute to the challenge of analysis in this matrix (164). Determination of 5-HT concentration in plasma, serum or whole blood as previously discussed (Chapter 1.5.1) is limited by issues surrounding the collection and preparation of samples (165). Variable results have been obtained with 5-HT analysis in platelets. Whole blood analysis is preferred as

it is more sensitive and specific, but Carling *et al* did report saturability of 5-HT in platelets (83, 166). A study that analysed 5-HT in serum found that the sample was more stable and its preparation was less extensive compared to plasma (167).

Few studies have assessed the relationship between 5-HT and 5-HIAA. Yubero-Lahoz *et al* compared these analytes in plasma and PPP of patients with hypertension, end stage renal disease and a control group. As expected, 5-HT and 5-HIAA were lower in the control group and higher in the patients with end stage renal disease. A significant positive correlation was observed between 5-HT and 5-HIAA in the control group (166). Another study observed better performance of the plasma 5-HIAA assay compared to whole blood serotonin (83). Lindstrom *et al* compared serum 5-HT and serum 5-HIAA and on average, 5-HT concentrations were five times higher. ROC curve analysis showed both serum 5-HT and 5-HIAA demonstrated similar diagnostic performance (AUC 0.91 and 0.94) at discriminating between the healthy group and patients with active NET (65).

4.2 Methods

4.2.1 Participants

70 patients with NET and 30 healthy volunteers took part in this study. The patients with NET were recruited from the Neuroendocrine Tumour clinic and the multidisciplinary team meeting at the Royal Free Hospital. The healthy volunteers were recruited from University College London, the

Neuroendocrine Tumour unit and the Clinical Biochemistry department at the Royal Free hospital. Informed consent was obtained from all participants.

Ethical approval for this study was obtained from South West – Frenchay Research Ethics Committee (17/SW/0131).

4.2.2 Sample collection and analysis

Patient and healthy volunteer samples were drawn into appropriate evacuated specimen tubes (BD Diagnostics-Preanalytical Systems, Plymouth, PL6 7BP, UK). A Serum gel tube was used for collecting blood for serum 5-HIAA. Potassium EDTA tubes were used for both plasma 5-HIAA and 5-HT collection. 5-HT samples were collected and stored immediately in the -70°C freezer until analysis. Serotonin result was not available for one of the healthy volunteers.

Plasma and serum 5-HIAA analysis was performed as described in chapter 2.

4.2.2.1 Serotonin analysis

5-HT was analysed in whole blood using a HPLC method at the Neurometabolic Laboratory at the National Hospital for Neurology and Neurosurgery. Ascorbic acid which is an antioxidant was added to every sample before it was thawed, to limit the degradation of 5-HT. To 1 ml of the sample, 2.5 mls of ice cold deionised water was added and the sample was allowed to stand on ice. The deionised cold water was to cause haemolysis of the cell so that 5-HT is released. 1 ml of zinc sulphate was used for protein

precipitation and the mixture was allowed to stand on ice for 10 minutes. 0.5 ml of 1M sodium hydroxide was added to the mixture and allowed to stand on ice for 10 minutes. It was then centrifuged at 14000 rpm for 2 minutes and the supernatant was transferred into vials for injection into the HPLC for analysis with fluorescence detection.

4.2.3 Statistical analysis

Statistical analysis was performed using Microsoft Excel and SPSS version 25. Test for normality was performed using the Shapiro-Wilk test and a Normal Q-Q plot. A normal distribution was confirmed if the significance value in the Shapiro-Wilk test was greater than 0.05, and the data points laid on the straight diagonal line of the Normal Q-Q plot. Logarithmic transformation was performed so that the positively skewed 5-HT, plasma and serum 5-HIAA data becomes more symmetrical. A p value of < 0.05 was considered to be statistically significant.

4.3 Results

4.3.1 Baseline characteristics

Most of the study participants were male, 47 (67%) patients with NET and 17 (57%) healthy volunteers. The patients were older with a mean age of 64 (± 11) years compared to 34 (± 14). SI-NET was the predominant type of neuroendocrine tumour in 66 (94.3%) of the patients. CS was present in 70% (49) of patients, and apart from one patient with bronchial NET, the rest of them had SI-NET. 5-HT, plasma and serum 5-HIAA levels did not follow a normal distribution, the median values and interquartile ranges in patients with

NET and healthy volunteers are shown below in Table 19. Two patients were excluded from statistical analysis due to the reason previously mentioned in chapter 3 (eGFR of less than 20 ml/min/1.73 m^2 therefore not included in the validation of the plasma and serum 5-HIAA assays). One healthy volunteer did not have a serotonin result.

Participants	**Patients**	**Healthy volunteers**
Number	70	30
Mean age (Years)	64 (± 11)	34 (±14)
Gender		
Male	47 (67%)	17 (57%)
Female	23 (33%)	13 (43%)
NET type		
SI-NET	66 (94.3%)	
Pancreatic NET	2 (2.9%)	
Bronchial NET	1 (1.4%)	
Hindgut NET	1 (1.4%)	
Presence of CS	49 (70%)	0 (0%)
Median serotonin concentration (nmol/L)	5205 (2410, 7308)	851 (710, 1040)
Median plasma 5-HIAA concentration (nmol/L)	377 (141, 939)	56 (49, 65)
Median serum 5-HIAA concentration (nmol/L)	337 (134, 852)	63 (56,70)

Table 19. Baseline characteristics of participants. Categorical data is reported in percentages and continuous data as mean ± standard deviation for normally distributed data or median and interquartile range (IQR) if data does not follow a normal distribution.

4.3.2 Comparison of plasma 5-HIAA and 5-HT

To assess the relationship between plasma 5-HIAA and 5-HT in patients with NET, a Spearman's rank correlation was performed as the plasma 5-HIAA did not follow a normal distribution. A strong correlation which was statistically significant was demonstrated between plasma 5-HIAA and 5-HT, Spearman's correlation coefficient was 0.75 ($p = < 0.0001$). In healthy volunteers, there

was no correlation between plasma 5-HIAA and 5-HT. Spearman's correlation coefficient was 0.26 (p = 0.17). A scatter plot performed (Figure 22) showed a positive relationship between the log transformed plasma 5-HIAA and log transformed 5-HT concentrations in patients with NET. The scatter plot in Figure 23 does not show a clear relationship between log transformed plasma 5-HIAA and log transformed
5-HT in healthy volunteers.

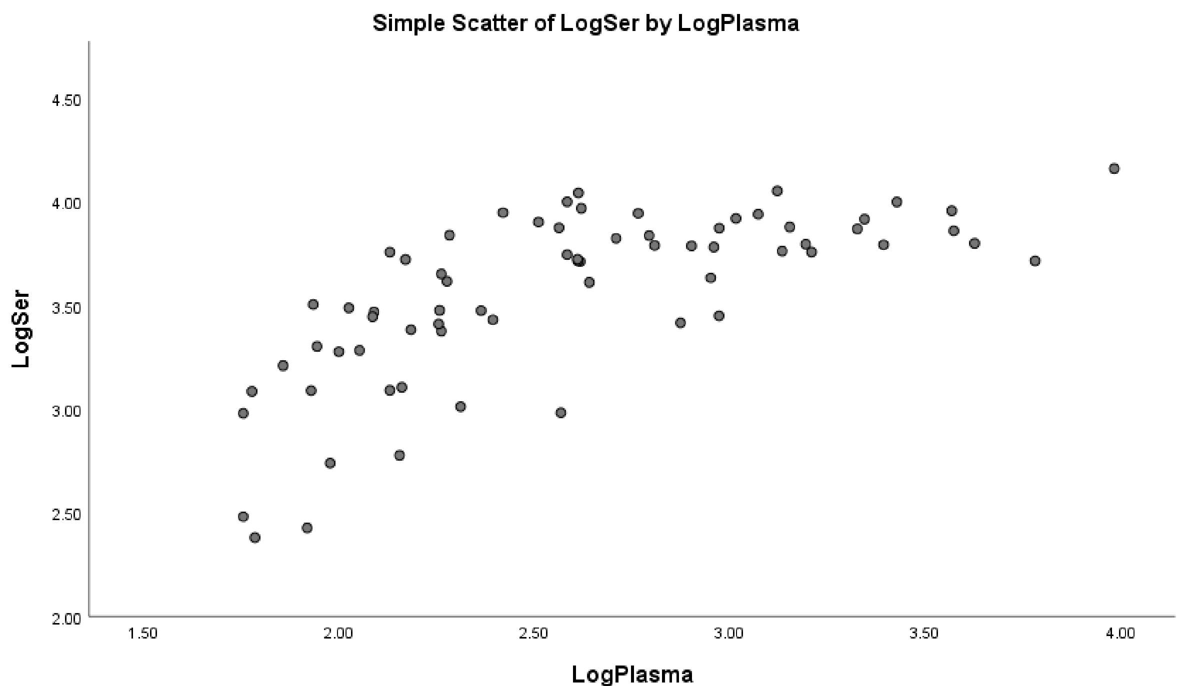


Figure 22. Scatter plot showing the relationship between plasma 5-HIAA (Logplasma) and 5-HT (LogSer) in patients with NET

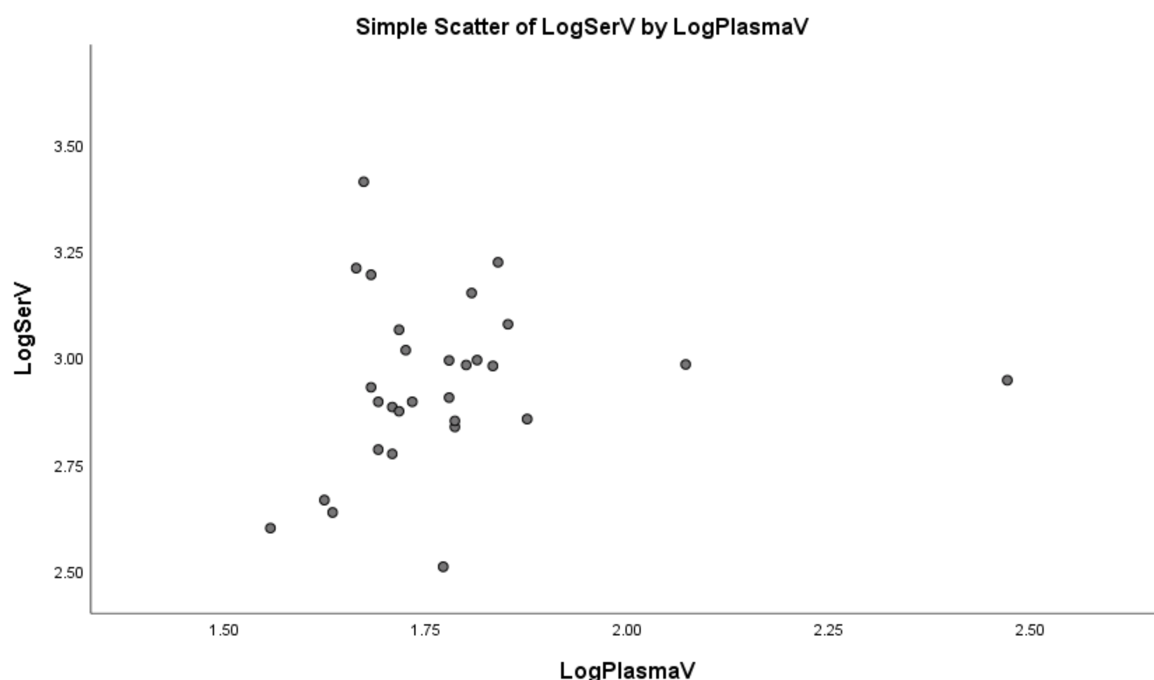


Figure 23. Scatter plot showing the relationship between plasma 5-HIAA(LogPlasmaV) and 5-HT (LogSerV) in healthy volunteers

4.3.3 Comparison of serum 5-HIAA and 5-HT

A Spearman's rank correlation was used to assess the relationship between serum 5-HIAA and 5-HT in patients with NET as serum 5-HIAA did not follow a normal distribution. A strong correlation which was statistically significant was observed, Spearman's correlation coefficient was 0.735 ($p = < 0.0001$). In the healthy volunteers, similar to what was observed with plasma 5-HIAA, there was no correlation between serum 5-HIAA and 5-HT. Spearman's correlation coefficient was 0.331 ($p = 0.079$). The scatter plots in Figure 24 and Figure 25 show the relationship between the log transformed data for serum 5-HIAA and 5-HT in the patients and healthy volunteers. A positive relationship is seen in the patients with NET and no relationship is observed in the healthy volunteers.

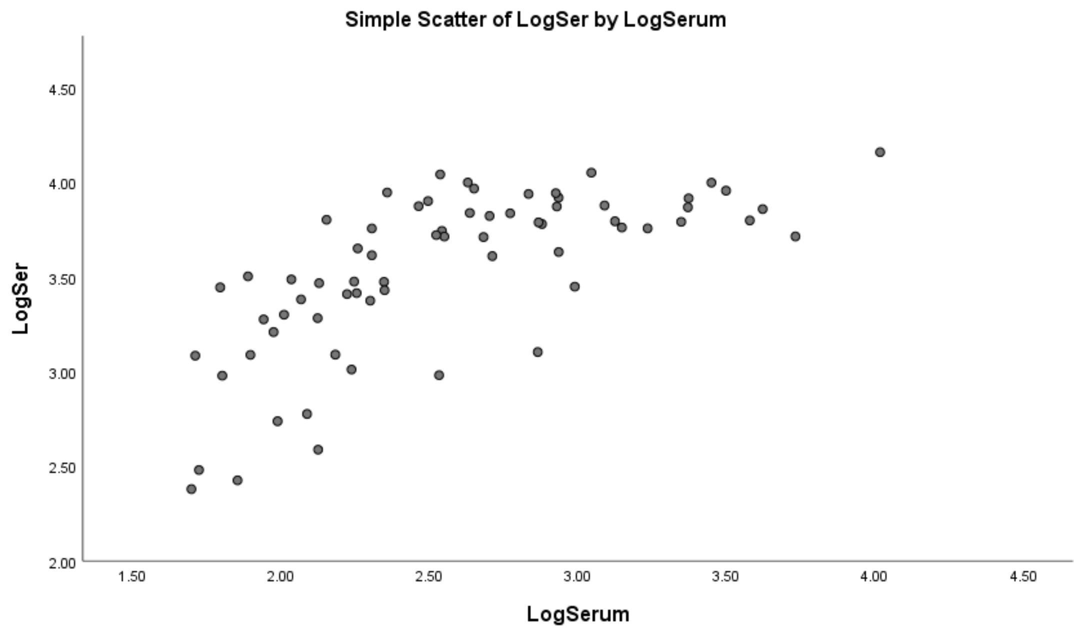


Figure 24. Scatter plot showing the relationship between serum 5-HIAA (LogSerum) and 5-HT (LogSer) in patients with NET

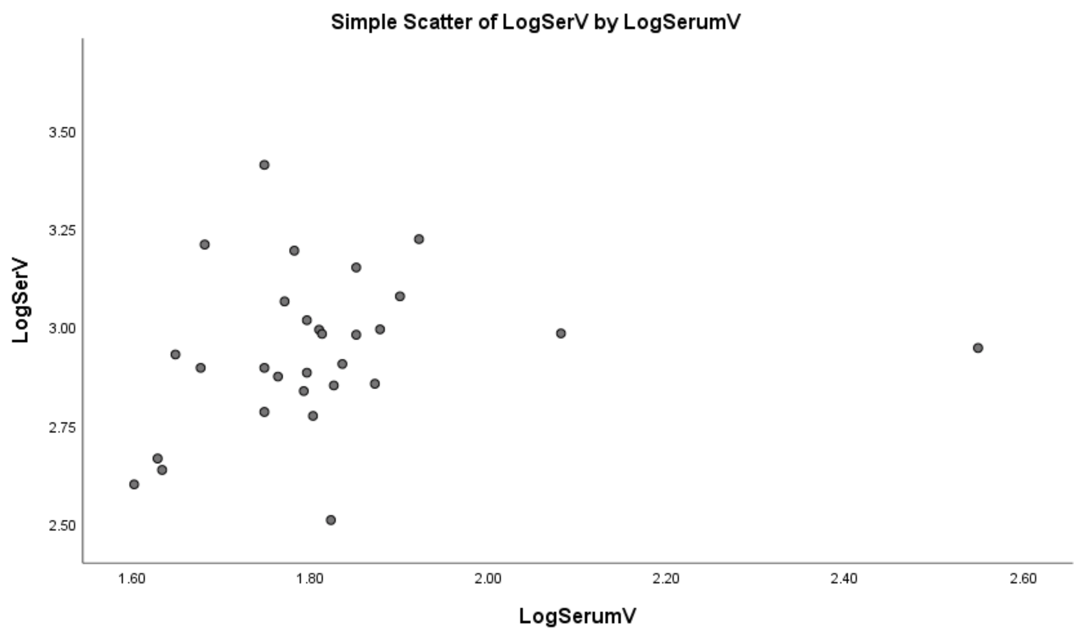


Figure 25. Scatter plot showing the relationship between serum 5-HIAA (LogSerumV) and 5-HT (LogSerV) in healthy volunteers

4.3.4 Comparison of 5-HT between patients with NET and healthy volunteeers.

Patients with NET had higher concentrations of 5-HT compared to the healthy volunteers. The median concentration in patients with NET was 5205 nmol/L and 851 nmol/L in healthy volunteers. The box whiskers plot in Figure 26 shows the distribution of 5-HT concentrations in the patients with NET (Serotonin) and healthy volunteers (Serotonin HV). As indicated by the length of the whiskers, patients with NET had a wider range of 5-HT concentrations. The whisker in the patient group also showed that most of the patients had 5-HT concentrations that were elevated which confirm the data was positively skewed. It is difficult to interpret the data for the healthy volunteers because it was plotted on the same scale as that of the patients with NET. Four of the healthy volunteer values (denoted by 1,2,15 and 17 on the box and whisker plot) were identified as outliers.

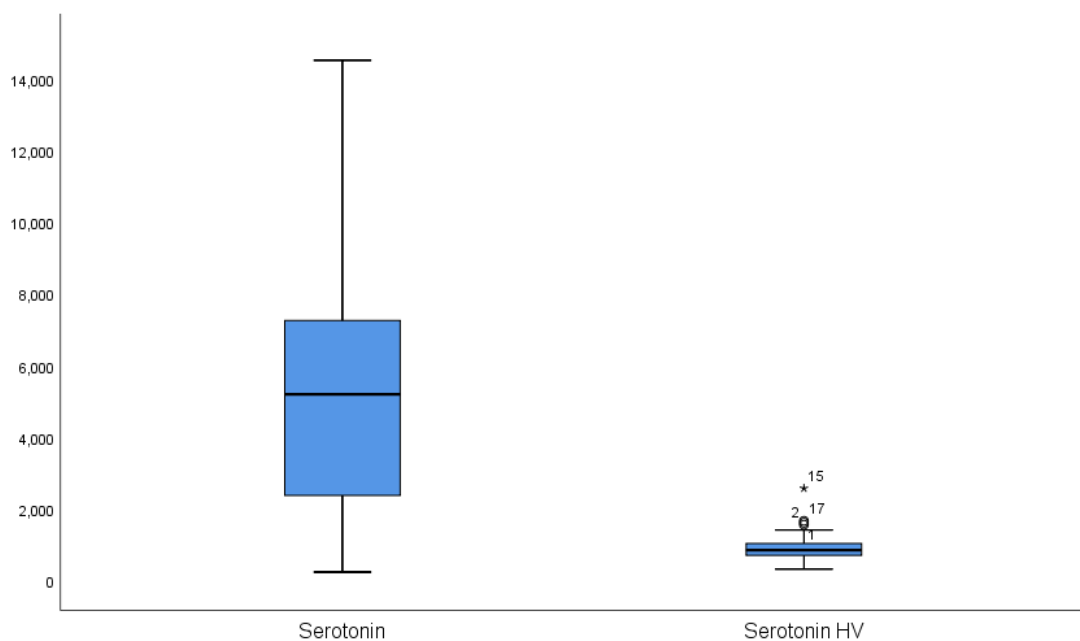


Figure 26. Box whisker plots showing the distribution of 5-HT concentrations in patients with NET and healthy volunteers. Serotonin = patients with NET, Serotonin HV = healthy volunteers

4.4 Discussion

5-HT is a metabolite of tryptophan an essential amino acid. Less than 2% of tryptophan ingested is converted to 5-HT and about 95% is metabolised by the kyruneric pathway to give the end product nicotinamide adenine dinucleotide (NAD), the metabolically active form of niacin (Figure 27). Two thirds of the required amount of NAD is obtained from tryptophan (132). The metabolism of 5-HT from tryptophan involves the hydroxylation of tryptophan to 5-hydroxytryptophan (5-HTP), the rate limiting step, which is catalysed by the enzyme tryptophan hydroxylase (TPH). Two isoforms, TPH1 and TPH2 are known. TPH1 is expressed in enterochromaffin cells and TPH2 in neurones. The next step involves the decarboxylation of 5-HTP catalysed by aromatic L-amino acid decarboxylase (AAAD) into 5-HT (168-170).

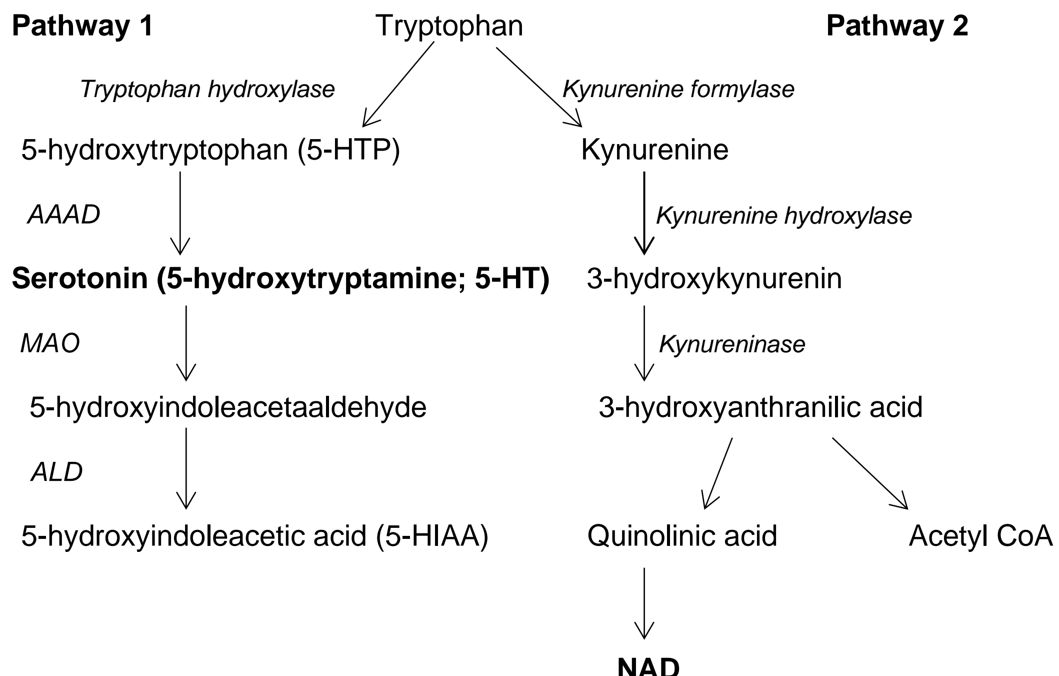


Figure 27. The metabolic fate of tryptophan showing synthesis of serotonin and NAD (nicotinamide adenine dinucleotide). AAAD - aromatic L-amino acid decarboxylase, MAO - monoamine oxidase, ALD - aldehyde dehydrogenase

CS is associated with increased 5-HT production because up to 60% of tryptophan is metabolised to 5-HT (Figure 27; pathway 1) compared to approximately 1% in healthy individuals. This commonly occurs in patients with SI-NET in the presence of hepatic or ovarian metastases because the 5-HT produced enters the systemic circulation directly (160, 168). 58 (83%) of the patients with NET in this study had liver metastases on their most recent imaging. Of these, 46 (66%) of them had carcinoid syndrome based on the presence of flushing or CHD. However, it is estimated that 25% of patients with elevated 5-HT are asymptomatic (171). CS is said to occur in about 18% of patients with SI-NET (158) but our patient group was a biased one, 66 (94%) of them had SI-NET, and 55 (83%) of them had liver metastases.

The increased production of 5-HT and its metabolite 5-HIAA in CS can lead to decreased availability of tryptophan for the kynurenin pathway (Figure 27; pathway 2). In cases of severe CS, this can lead to deficient NAD (niacin) which will manifest clinically as diarrhoea, dermatitis and dementia (168). It is suggested that niacin deficiency may not be given much attention in patients with CS because one of the symptoms (diarrhoea) is also a feature of CS and there is no reliable marker for niacin measurement. Another reason suggested is that in sub-clinical niacin deficiency, patients may be asymptomatic (172). It is most likely that controlling the symptoms of carcinoid syndrome with SSA or improved and effective therapies such as a combination of SSA and Telotristat ethyl in refractory diarrhoea or PRRT reduces the severity of diversion of tryptophan to 5-HT production. This in turn will lead to less niacin deficiency. We did not assess our patients for niacin deficiency. In the absence of reliable

accredited assays for niacin determination, improvement in clinical symptoms following the replacement of niacin (vitamin B3) will confirm a suspected diagnosis.

Our results demonstrated that in patients with NET, there was a good correlation between 5-HT and 5-HIAA in serum or plasma. Lindstrom *et al* showed comparable diagnostic performance between their serum 5-HT and serum 5-HIAA assay with AUC 0.91 and 0.94 respectively. Their mean serum 5-HT concentration was five times that of serum 5-HIAA (65). The median 5-HT concentration in our patients with NET was about fourteen times that of the plasma 5-HIAA and fifteen times that of serum 5-HIAA. In the healthy volunteers the median 5-HT concentration was fifteen and fourteen times higher in comparison to plasma and serum 5-HIAA levels.

We did not show a correlation between 5-HT and plasma or serum 5-HIAA in healthy volunteers. Whole blood 5-HT was analysed in this study and it is a preferred matrix because it has less issues associated with sample handling (165, 166). However, 5-HT levels obtained can be influenced by factors affecting its synthesis such as the concentration of its precursors, enzyme activities involved in its synthesis and catabolism, and sample preparation and handling. The conditions for sample collection, preparation and analysis were standardised in both our patient and healthy volunteer groups. The better relationship and correlation observed between 5-HT and 5-HIAA in serum or plasma in patients with NET compared to healthy volunteers is likely due to the increased production of 5-HT and 5-HIAA, making it easier to assess the

relationship. In the healthy volunteers only about 1% of tryptophan is metabolised by the 5-HT pathway (Figure 27, pathway 1). The low concentrations of 5-HT and in turn 5-HIAA may have influenced the relationship observed between both analytes. Pre-analytical errors may be accentuated in the healthy volunteers because of the lower concentration of 5-HT. The number of healthy volunteers (N=29) was fewer compared to the number of patients with NET (N=70), which may also contribute to the ability to determine a relationship between 5-HT and 5-HIAA in plasma or serum.

4.5 Conclusion

5-HT concentrations are elevated in patients with NET. Our study shows that there is a good correlation between 5-HT and 5-HIAA in plasma or serum of patients with NET. However, 5-HT is a labile analyte and this limits its use in the diagnosis and monitoring of CS. Careful sample handling and preparation, as well as the use of analytical methods with high sensitivity and specificity are required to ensure accurate results are obtained for 5-HT. At present, the use of plasma or serum 5-HIAA which is less error prone is appropriate.

Chapter 5 Comparison of plasma and serum 5-HIAA with other biochemical markers of NET including chromogranin A and NT –ProBNP

5.1 Introduction

The diagnosis of NET usually involves the measurement of circulating biochemical markers in conjunction with radiological investigations and a histological diagnosis. The biochemical markers do not only play a role in the diagnosis of NET but are also useful in monitoring the response to treatment and assessing for disease progression (173).

CgA is an established general biochemical marker for NET. It is present in majority of NET, and of particular importance is its role in the monitoring of non-functioning NET where it may be the only biochemical marker that can be used (37, 40). The highest CgA levels detected, have been in patients with metastatic NET (82, 174), in particular those with SI-NET and liver metastases (37, 42, 43). A statistically significant difference was shown between the mean CgA levels of patients with metastatic NET (3444 ± 16256 µg/L) and those without metastases (174 ± 233 µg/L); $p < 0.001$ (175).

Several studies have compared the diagnostic utility of CgA and 5-HIAA. CgA was shown to correlate better with physical functioning ($p=0.01$) and quality of life ($p=0.03$) in a study monitoring patients during treatment with the long-acting octreotide, Sandostatin LAR (40). Recurrence of NET was detected earlier with increased plasma CgA concentrations at a median time of 8 months following radical resection of NET. The median time indicating

recurrence improved to 5 months when CgA was combined with urine 5-HIAA (176). A significant correlation was observed between the median values of urine 5-HIAA and CgA in patients with SI-NET and liver metastases (42). Post hoc analysis performed on urine 5-HIAA and plasma CgA data from the CLARINET study revealed that biochemical response was achieved in 69% of patients in the lanreotide 120mg SC treatment arm compared to 22% in the placebo arm (p= < 0.0001). For plasma CgA, it was 28% compared to 3% (p= < 0.0001) in the lanreotide versus placebo treatment arm. Biochemical response was defined as 50% or more decrease in urine 5-HIAA and plasma CgA levels from baseline. Baseline values had to be greater than the upper limit of normal (ULN). In the same study, at the end of 96 weeks, patients with urine 5-HIAA and CgA concentrations greater than the ULN at baseline, treated with lanreotide achieved significant reductions in urine 5-HIAA (p= < 0.0001) and CgA (p=0.0043) levels compared to the group receiving placebo. Progression free survival was significantly prolonged amongst responders compared to non-responders, for 5-HIAA (median PFS not reached vs. 16.2 months, p < .0001; HR for progression or death = 0.21; 95% CI, 0.09–0.48) and CgA (median PFS not reached vs. 16.2 months, p = .0070; HR for progression or death = 0.30; 95% CI, 0.12–0.76) (177).

NT-proBNP is derived from the prohormone B-type natriuretic peptide (proBNP) released by the atria and ventricles. The main stimulus for its synthesis is myocardial stretch (178). It is an important marker for CHD with diagnostic and prognostic implications (22, 161). In patients with SI-NET and CS, median NT-proBNP concentration has been demonstrated to be

significantly raised in those with CHD (median 1149 pg/ml, IQR 404 to 1601) compared to those without CHD (median 101 pg/ml, IQR 50 to 169); p= < 0.001 (179).

The diagnostic utility of NT-proBNP has been compared with 5-HIAA in CHD. A urine 5HIAA level of 300 μmol/24 hour gives almost a threefold risk of developing CHD (84). In a study to determine the factors associated with echocardiographic progression of CHD, plasma 5-HIAA concentration at baseline was shown to be independently associated with progression of CHD. More patients with progression of CHD compared to those without, had an increase of more than 50% from baseline in their plasma 5-HIAA (62% vs 31%; p=0.04) and NT-proBNP (46% vs 18%; p=0.035) concentrations. (180). Both biochemical markers demonstrated significant correlations with the severity of CHD but the correlation was stronger with NT-proBNP (22).

5.2 Methods

5.2.1 Participants

80 patients with NET participated in this study. They were recruited from the Neuroendocrine Tumour clinic and the multidisciplinary team meeting at the Royal Free Hospital. Informed consent was obtained from all participants.

Ethical approval for this study was obtained from South West – Frenchay Research Ethics Committee (17/SW/0131).

5.2.2 Sample collection and analysis

Samples from the participants were drawn into appropriate evacuated specimen tubes (BD Diagnostics-Preanalytical Systems, Plymouth, PL6 7BP, UK). A serum gel tube was used for serum 5-HIAA and NT-pro BNP collection. CgA was drawn into a trasylol tube and placed on ice immediately after collection. Plasma 5-HIAA was collected in a potassium EDTA tube. Analysis of plasma and serum 5-HIAA was performed as described in chapter 2.

5.2.2.1 Analysis of NT-pro BNP

NT-pro BNP analysis was performed using an electrochemiluminescence immunoassay on an automated Roche Elecsys® cobas e 801 analyser (Roche Diagnostics, Mannheim).

5.2.2.2 Analysis of CgA

CgA analysis was performed using a NEOLISA™ test kit which employs an ELISA (Euro Diagnostica, Sweden) performed on an automated DS2 analyser. This assay uses two monoclonal antibodies raised against CgA. The capture antibody binds an epitope that begins at the amino acid residue 236 and ends at the amino acid residue 251, whilst the detector antibody binds an epitope from the amino acid residue 264 to 279 (181).

5.2.3 Statistical analysis

Statistical analysis was performed using Microsoft Excel, Analyse-It (Analyse-It Software Ltd, Leeds, UK) and SPSS version 25. Test for normality was

performed using the Shapiro-Wilk test and a Normal Q-Q plot. A normal distribution was confirmed if the significance value in the Shapiro-Wilk test was greater than 0.05, and the data points laid on the straight diagonal line of the Normal Q-Q plot. Logarithmic transformation was performed so that the positively skewed CgA, NT-proBNP, plasma and serum 5-HIAA data becomes more symmetrical. A p value of < 0.05 was considered to be statistically significant.

5.3 Results

5.3.1 Baseline characteristics

80 participants took part in this study, and most of them were male (62.5%). SI-NET was the predominant type of neuroendocrine tumour (95%). 85% of patients had liver metastases. 58 patients (72.5%) had carcinoid syndrome and of these, 57 (98%) of them had SI-NET, and the last patient had bronchial NET. 10 (12.5%) patients with CS had CHD. 50% (5) of them had mild CHD and the remaining 50% had severe CHD as documented in their records. 43.75% (35) of patients were on a PPI. The values for the median and interquartile ranges of CgA, NT-proBNP, plasma and serum 5-HIAA are shown in Table 20. Two patients were excluded from statistical analysis due to the reason previously mentioned in chapter 3 (eGFR of less than 20 ml/min/1.73 m^2). Four patients did not have results for CgA and NT-proBNP.

Characteristics of participants (n=80)	
Mean age (Years)	64 ± 11
Gender	
Male	50 (62.5%)
Female	30 (37.5%)
NET type	
SI-NET	76 (95%)
Pancreatic NET	2 (2.5%)
Bronchial NET	1 (1.25%)
Hindgut NET	1 (1.25%)
Biochemical markers	
Median plasma CgA concentration (U/L)	96 (43, 321)
Median NT-proBNP concentration (ng/L)	106 (50, 234)
Median plasma 5-HIAA concentration (nmol/L)	384 (147, 1010)
Median serum 5-HIAA concentration (nmol/L)	349 (153, 979)
Liver metastases	
Yes	68 (85%)
No	12 (15%)
Proportion of patient with CS	58 (72.5%)
Proportion of patient with CHD	10 (12.5%)
Proportion of patients on PPI	35 (43.75%)

Table 20. Baseline characteristics of participants. Categorical data is reported in percentages and continuous data as mean ± standard deviation for normally distributed data or median and interquartile range (IQR) if data does not follow a normal distribution

5.3.2 Comparison of plasma 5-HIAA and CgA

Spearman's rank correlation coefficient was used to assess the relationship between plasma 5-HIAA and CgA, as neither analyte followed a normal

distribution. A strong positive correlation which was statistically significant was observed between plasma 5-HIAA and CgA, Spearman's correlation coefficient; $r_s = 0.792$ ($p = < 0.0001$). A scatter plot of the log transformed data in Figure 28 showed a positive relationship between both analytes.

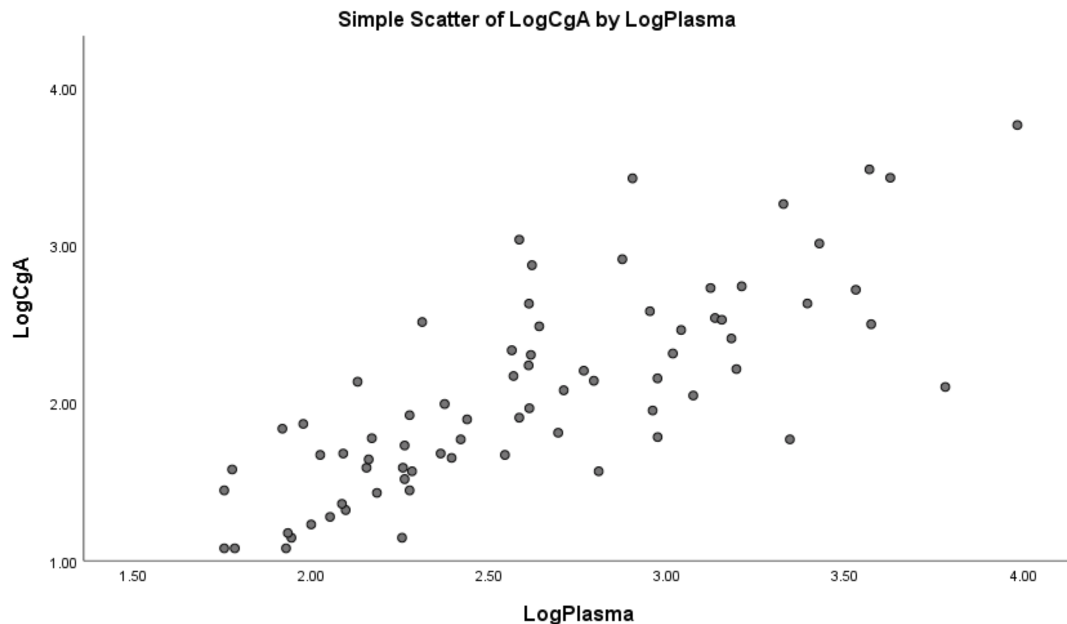


Figure 28. Scatter plot showing the relationship between plasma 5-HIAA (LogPlasma) and CgA (LogCgA) in patients with NET

5.3.3 Comparison of serum 5-HIAA with CgA

The relationship between serum 5-HIAA and CgA was assessed using Spearman's rank correlation which showed a statistically significant strong positive correlation. Spearman's correlation coefficient; $r_s = 0.794$ ($p = < 0.0001$). The scatter plot in Figure 29 demonstrates the positive association between serum 5-HIAA and CgA.

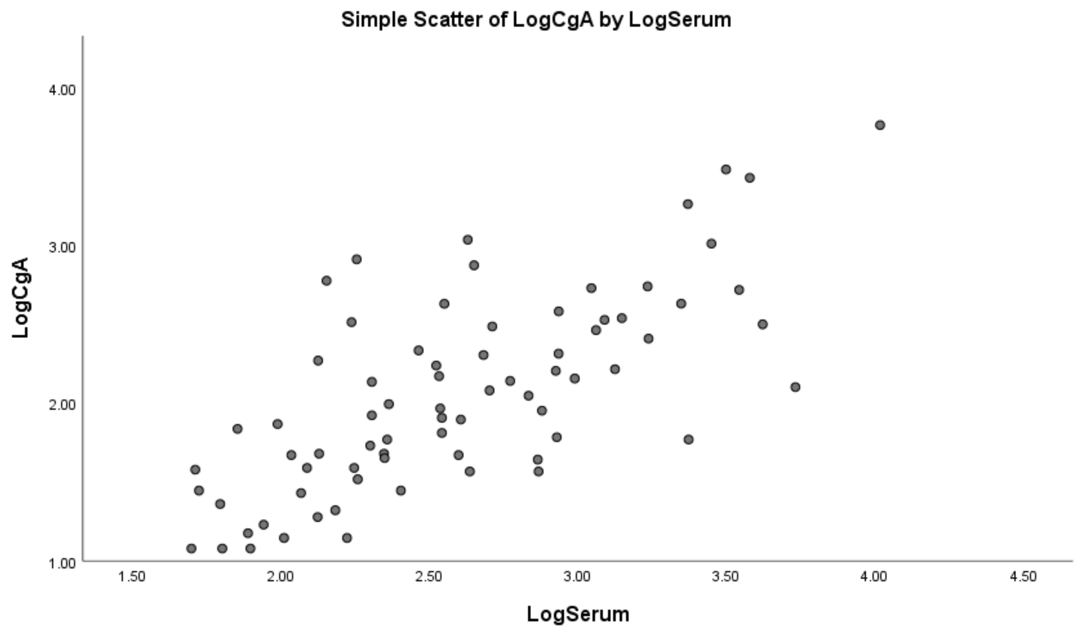


Figure 29. Scatter plot showing the relationship between serum 5-HIAA (LogSerum) and CgA (LogCgA) in patients with NET

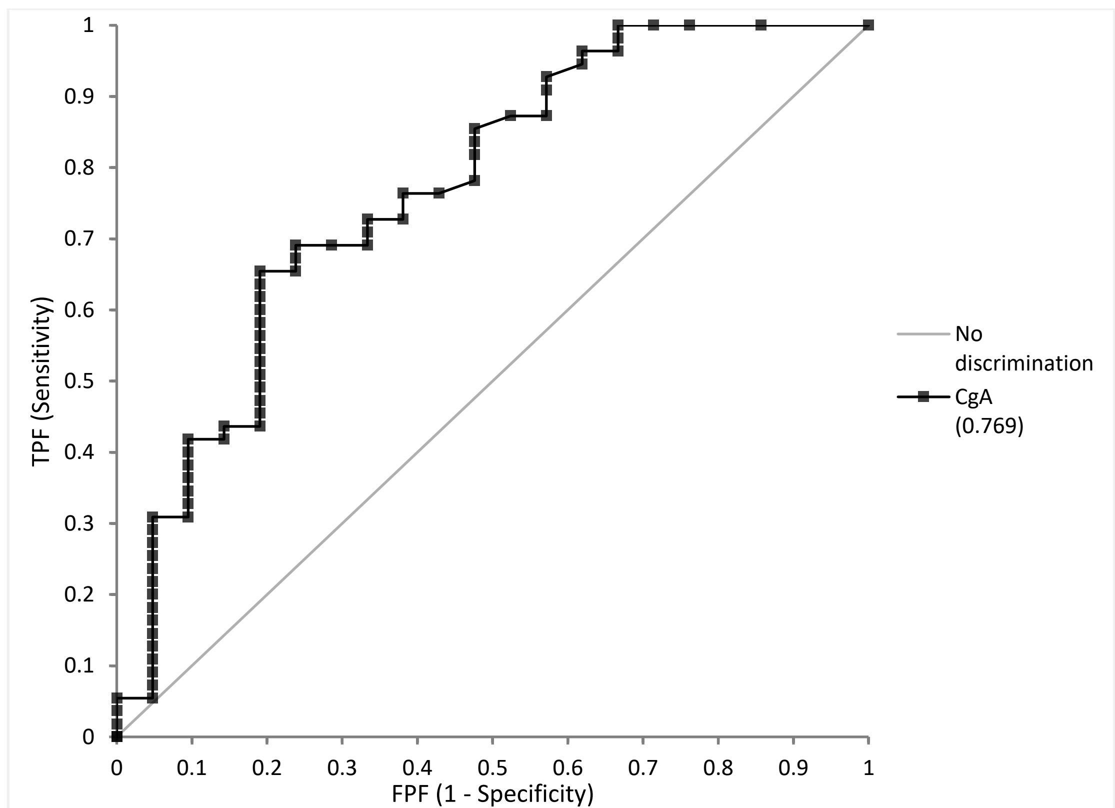


Figure 30. ROC curve analysis for CgA

CgA	TP	FP	TN	FN	TP proportion (Sensitivity)	TN proportion (Specificity)
12	55	18	3	0	1.000	0.143
14	55	16	5	0	1.000	0.238
15	55	15	6	0	1.000	0.286
17	55	14	7	0	1.000	0.333
19	54	14	7	1	0.982	0.333
21	53	14	7	2	0.964	0.333
23	53	13	8	2	0.964	0.381
27	52	13	8	3	0.945	0.381
28	51	12	9	4	0.927	0.429
33	50	12	9	5	0.909	0.429
37	48	12	9	7	0.873	0.429
38	48	11	10	7	0.873	0.476
39	47	10	11	8	0.855	0.524
44	46	10	11	9	0.836	0.524
45	45	10	11	10	0.818	0.524
47	43	10	11	12	0.782	0.524
48	42	9	12	13	0.764	0.571
54	42	8	13	13	0.764	0.619
59	40	8	13	15	0.727	0.619
60	40	7	14	15	0.727	0.667
61	39	7	14	16	0.709	0.667
65	38	7	14	17	0.691	0.667
69	38	6	15	17	0.691	0.714
74	38	5	16	17	0.691	0.762
79	37	5	16	18	0.673	0.762
81	36	5	16	19	0.655	0.762
84	36	4	17	19	0.655	0.810
90	35	4	17	20	0.636	0.810
93	34	4	17	21	0.618	0.810
99	33	4	17	22	0.600	0.810
112	32	4	17	23	0.582	0.810
121	31	4	17	24	0.564	0.810
127	30	4	17	25	0.545	0.810
137	29	4	17	26	0.527	0.810
139	28	4	17	27	0.509	0.810
144	27	4	17	28	0.491	0.810
149	26	4	17	29	0.473	0.810
161	25	4	17	30	0.455	0.810
165	24	4	17	31	0.436	0.810
174	24	3	18	31	0.436	0.857
187	23	3	18	32	0.418	0.857
203	23	2	19	32	0.418	0.905
207	22	2	19	33	0.400	0.905

Table 21. Sensitivity and specificity data for for the different cut-off values for CgA. TP – True positive, FP – False positive, TN- True negative, FN – False negative

5.3.4 Diagnostic performance of the CgA assay

A ROC curve (Figure 30) was generated to assess the diagnostic performance of the CgA assay which is currently in use. The AUC was 0.769 (95% CI 0.643 to 0.896). At our current cut-off of 27 U/L, the CgA assay has a sensitivity of 94.5% and a specificity of 38.1% as shown in Table 21. A sensitivity of 91% and specificity of 62% was obtained for both plasma and serum 5-HIAA respectively at the chosen cut-off of 135 nmol/L.

5.3.5 Comparison of plasma 5-HIAA and NT-proBNP

Correlation between plasma 5-HIAA and NT-proBNP was assessed using Spearman's rank correlation. NT-proBNP did not follow a normal distribution. A weak statistically significant correlation was observed between both analytes, Spearman's correlation coefficient; $r_s = 0.38$ ($p = < 0.001$). A scatter plot of the log transformed data for plasma 5-HIAA and NT-proBNP was used to demonstrate the relationship between them. The scatter plot in Figure 31 shows the relationship between log transformed NT-proBNP and plasma 5-HIAA.

5.3.6 Comparison of serum 5-HIAA and NT-proBNP

Spearman's rank correlation was used to assess the relationship between serum 5-HIAA and NT-proBNP. Similar to the findings observed in the correlation of NT-proBNP with plasma 5-HIAA, a weak statistically significant relationship was seen; Spearman's correlation coefficient; $r_s = 0.421$ ($p = < 0.001$). The relationship between the log transformed data for serum 5-HIAA and NT-proBNP is demonstrated in the scatter plot in Figure 32.

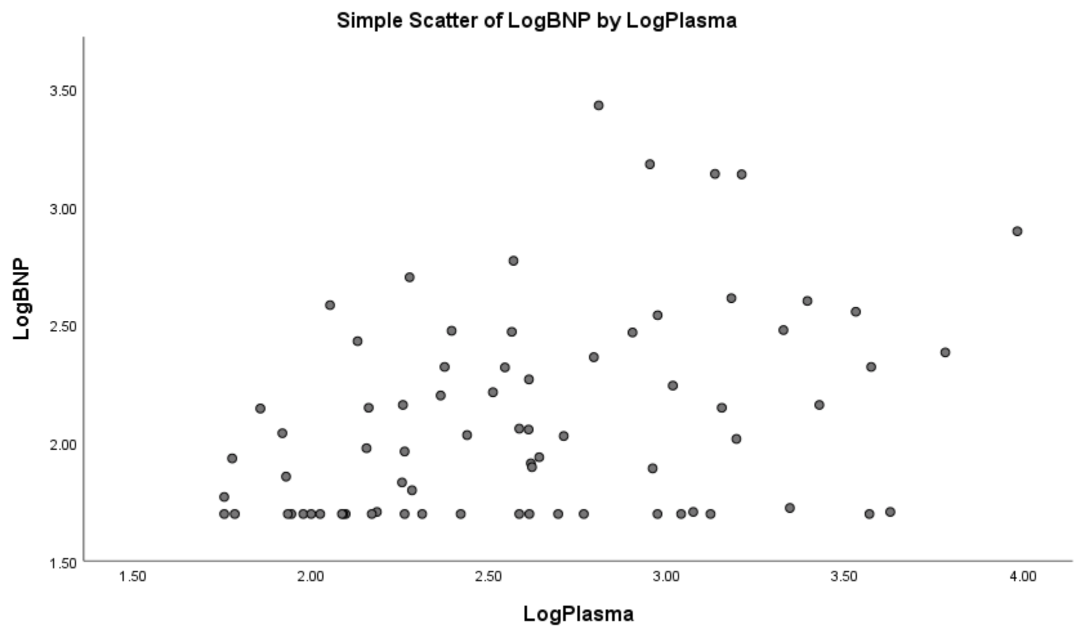


Figure 31. Scatter plot showing the relationship between plasma 5-HIAA (LogPlasma) and NT-proBNP (LogBNP) in patients with NET

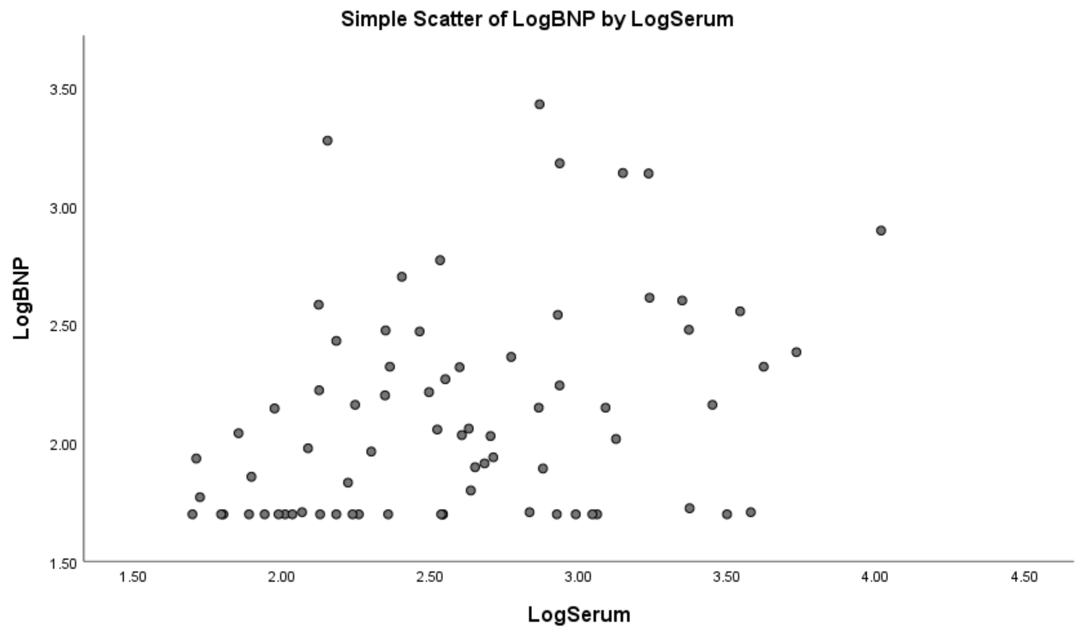


Figure 32. Scatter plot showing the relationship between serum 5-HIAA (LogSerum) and NT-proBNP (LogBNP) in patients with NET

5.4 Discussion

Our CgA assay has a high sensitivity (94.5%) but a low specificity of 38.1% at the current cut-off of 27 U/L. Increasing the cut-off will improve the specificity of the assay. At a slightly higher cut-off of 39 U/L as seen in Table 21, a better compromise will be achieved between sensitivity (86%) and specificity (52%) of the assay. In a study assessing the value of CgA in the diagnosis and monitoring of different types of NET, at a cut-off of 34 U/L, the specificity was 83% with sensitivity varying between 37 to 80% depending on the stage of the tumour (82). Other studies have shown that the diagnostic performance of CgA is determined by the cut-off selected (38, 42, 175). The sensitivity of the CgA assay also depends on the type of neuroendocrine tumour and the stage of the disease. A higher sensitivity has been observed in SI-NET, other functioning NET, and in metastatic disease (42, 82, 175).

Several CgA assays are available which measure different forms of the CgA molecule. Our Neolisa chromogranin assay (Figure 33) which is made up of two monoclonal antibodies targets the epitopes beginning at the amino acid position 236 to 251 (capture antibody) and the amino acid position between 264 to 279 (detector antibody). These CgA assays differ in the method employed which is determined by the type of antibody used and the epitope of the CgA molecule they recognise. Consequently, a lack of standardisation of the CgA assay limits the comparison of results obtained from different methods (51).

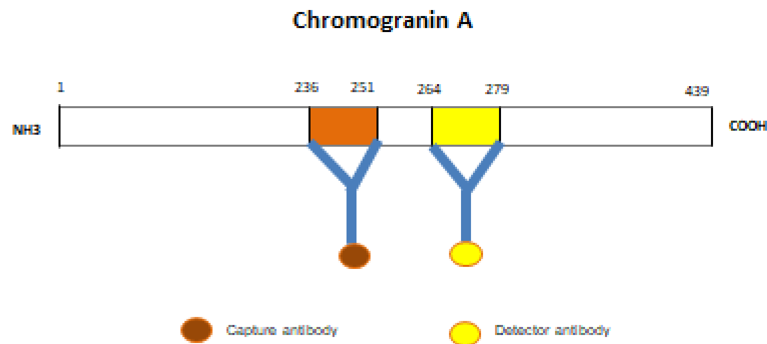


Figure 33. A schematic representation of chromogranin A and the epitopes the capture and detector monoclonal antibodies bind

CgA showed a strong correlation with both plasma and serum 5-HIAA assays. Nolting et al in their study, observed a significant correlation between the median values of urine 5-HIAA and CgA in patients with SI-NET (42). In a sub-group of these patients, with liver metastases, a significant correlation was observed (Spearman correlation coefficient $r_s = 0.696$, $p = < 0.0001$), which was not the case in the group without liver metastases (Spearman correlation coefficient $r_s = 0.036$, $p = 0.915$). Our patient population predominantly had SI-NET (95%) and 86% (65/76) of them had liver metastases. We observed a significant correlation between plasma 5-HIAA and CgA in patients with liver metastases (Spearman correlation coefficient $r_s = 0.751$, $p = < 0.0001$) and in those without liver metastases (Spearman correlation coefficient $r_s = 0.651$, $p = 0.03$).

Several studies have compared the efficacy of CgA and 5-HIAA as biochemical markers of NET (40, 82, 174, 177, 182). Post hoc data on urine 5-HIAA and plasma CgA from the CLARINET study demonstrated significant reductions in these biochemical markers from baseline in patients receiving treatment with subcutaneous lanreotide autogel 120 mg injection. Patients with non-functioning enteropancreatic NET without disease progression, who had baseline levels of urine 5-HIAA or CgA above the ULN, achieved significant reduction on lanreotide treatment compared to placebo at the different time points up to 96 weeks. The median change (IQR) in urine 5-HIAA at 96 weeks in the treatment arm [-39 µmol/24hr (-102.5 to 22.4)] compared to the placebo arm [111 µmol/24hr (-11.2 to 170.8)] was significant ($p = < 0.0001$). For CgA, the median change (IQR) at 96 weeks in the treatment arm [-70 µg/L (-223.9 to -17.2)] compared to the placebo arm [71.4 µg/L (-82.3 to 389.3)] was significant ($p = 0.0043$) (177).

A main limitation of CgA is that it is non-specific, as it is increased in other conditions discussed in chapter 1 (1.3.1). The chronic use of PPI is known to increase CgA concentration. PPI inhibits gastric acid secretion. The absence of gastric acid activates gastrin production. Gastrin stimulates the enterochromaffin like cells in the stomach to secrete CgA (173). Therefore, CgA results cannot be reliably interpreted in patients on PPI. In our study, thirty five (43.75%) patients were on PPI (Table 20). Excluding these patients and eight others we did not have information about their use of PPI from the analysis, a significant correlation was still observed between CgA and 5-HIAA in plasma (Spearman correlation coefficient $r_s = 0.531$, $p = 0.001$) or serum

(Spearman correlation coefficient $r_s = 0.523$, $p = 0.001$) in the patients who were not on PPI.

The median (IQR) concentration of NT-proBNP in our patients was 106 ng/L (50, 234). However, when comparing the patients with CHD and those without CHD, there was a significant difference between their median concentrations. The median (IQR) concentration was 251 ng/L (161, 382) in patients with CHD and in those without CHD, it was 90 ng/L (50, 183); $p = 0.021$. A similar finding was observed by Bhattacharyya et al in a group of patients with SI-NET and CS who were screened for CHD. 39 (19.5%) of the 200 patients had CHD and their median (IQR) NT-proBNP concentration was 1149 pg/ml (404, 1601) compared to 101 pg/ml (50, 169); $p = < 0.001$ (179).

A urine 5-HIAA concentration of 300 μmol/24 hours or more has been demonstrated to be a predictor for the development or progression of CHD (84). As previously mentioned, plasma 5-HIAA concentrations at baseline was associated with progression of CHD (180). We observed a weak but significant correlation between plasma or serum 5-HIAA and NT-proBNP. The scatter plots in Figure 31 and Figure 32 comparing the relationship between the log transformed data for plasma and serum 5-HIAA with NT-proBNP shows that at a log NT-proBNP (LogBNP) value of 1.7, multiple data points are observed across the entire concentration range of the log transformed concentrations of plasma and serum 5-HIAA. This is because 50 ng/L is the lowest limit of quantitation (LLOQ) of our NT-proBNP assay and when it is log transformed gives 1.7. The LLOQ of an assay is the lowest concentration of an analyte that

can be reliably measured with a precision of 20% and an accuracy of 80 – 120 % (140, 149). Therefore, NT-proBNP concentrations less than 50 ng/L are assigned a value of 50 ng/L. This was the concentration in 22 (29%) of the 76 NT-proBNP results available. We can speculate that this could have contributed to the weak association (Spearman's correlation, $r_s = 0.38$; $p = < 0.001$) observed between NT-proBNP and 5-HIAA in plasma or serum.

In a study investigating which biochemical markers correlate better with the presence and severity of CHD, a poor correlation was observed between CgA and the severity of CHD. It was suggested that the low specificity of CgA may be responsible. Plasma 5-HIAA and NT-proBNP were shown to be equally useful in the diagnosis of CHD but NT-proBNP correlated better with the severity of CHD (22). NT-proBNP is a known marker of heart disease. There would be an indirect mechanism of association of 5-HIAA and serotonin with CHD as chronic elevated serotonin is associated with functional tumour and CS and subsequently the potential development of CHD. Unfortunately due to the small number of patients in our study with CHD we were unable to assess the relationship between NT-proBNP and plasma or serum 5-HIAA with disease severity. In patients with CHD, we did not observe a correlation between NT-proBNP and plasma or serum 5-HIAA (Spearman's correlation for both, $r_s = 0.285$; $p = 0.425$), urine 5-HIAA (Spearman's correlation, $r_s = - 0.018$; $p = 0.96$) or CgA (Spearman's correlation, $r_s = 0.35$; $p = 0.356$).

Our study population was predominantly patients with SI-NET and liver metastases. This can be considered a strength of this study, as CgA and NT-

proBNP levels have been shown to be higher in these patient group. However, a weakness of this study is the small number of patients with CHD, which makes it difficult to draw a conclusion about the correlation observed between NT-proBNP and the other analytes.

5.5 Conclusion

Our study shows a significant correlation between plasma 5-HIAA and CgA currently the most widely accepted biochemical marker of neuroendocrine tumour. As previously demonstrated in the literature, we have confirmed that the median values of NT-proBNP are higher in patients with CHD.

Chapter 6 Somatostatin analogues and micronutrient deficiencies

6.1 Introduction

SSA often used as first line treatment in patients with metastatic NET, control symptoms due to excess hormone secretion and have an anti-proliferative effect, slowing down tumour growth (93, 96, 183, 184). The SSA octreotide LAR and lanreotide prolonged release have been shown to reduce the frequency of diarrhoea and flushing associated with CS (97, 99). The anti-proliferative effect of SSA was demonstrated in the PROMID and CLARINET studies (100, 101). The PROMID study showed that treatment of SSA-naïve patients with midgut NET using octreotide LAR 30mg compared with placebo increased the time to tumour progression especially in those who had their primary tumour resected and those with a low hepatic tumour burden. Median time to tumour progression was 14.3 (octreotide LAR) and 6 months (placebo), hazard ratio =0.34; 95% CI, 0.20 to 0.59; p = 0.000072 (100). In the CLARINET study, progression-free survival was significantly extended in patients with metastatic enteropancreatic NET on lanreotide autogel 120 mg compared to placebo (median progression-free survival, not reached vs 18 months, p <0.001). At 24 months, the estimated rate of progression-free survival was 65.1% (95% CI, 54.0 to 74.1) in patients on lanreotide autogel 120mg and 33% (95% CI, 23.0-43.3) in those on placebo (101). Patients are often on SSA for many years.

Vitamins and trace elements referred to as micronutrients (MN) are essential components in the diet. They are involved in various physiological processes

in the body. The biological effects of deficiencies of these micronutrients are usually reversible with supplementation (132, 185, 186). Vitamins are classified into two groups based on their solubility in aqueous or lipid media. Water-soluble vitamins are rapidly absorbed directly by the gut. The fat-soluble vitamins (FSV) are initially dissolved in emulsion particles whose lipid content is predominantly triglycerides, in the upper gastrointestinal (GI) tract. These particles are too large for the absorptive surface of the small intestine. Lipases which are produced in the pancreas catalyse the hydrolysis of these particles to release free fatty acid and monoglycerides, which combine with bile salts to form smaller particles called mixed micelles. The FSV dissolve in the interior of these mixed micelles which facilitate their absorption in the proximal jejunum (185, 187, 188).

Trace elements (TE) such as copper, zinc and selenium obtained from the diet are absorbed via the intestinal mucosa mostly in the small bowel either passively or through active transport mechanisms (185, 188).

Drugs through their effect on the GI tract can impair the absorption of FSV and TE, leading to deficient states (185, 186).

GI side effects are common in patients treated with SSA (94, 95, 189). In the CLARINET study, diarrhoea was the most commonly reported adverse effect in patients who received Lanreotide. It also occurred more frequently in the group of patients that received Octreotide LAR in the PROMID study (100, 101). Steatorrhoea is another recognised adverse effect of SSA therapy (103, 104, 190). SSA have been shown to decrease the secretion of pancreatic enzymes (190). Deficiency of the exocrine pancreatic enzyme

lipase will affect the digestion of fat, which in turn will affect the absorption of FSV. Often patients require pancreatic enzyme replacement therapy (PERT) as treatment for steatorrhoea. Deficient levels of TE such as zinc and copper are associated with diarrhoea which is a common adverse effect of SSA use (188, 191).

Malabsorption and deficiencies of MN can also occur as a result of surgical intervention, such as bowel resection or Whipple's procedure in patients with GEP NET leading to a reduced absorptive surface or bacterial overgrowth (192). MN deficiency can be an adverse effect of chemotherapy treatment in patients with NET. Cisplatin causes increased renal excretion of the MN magnesium and L-carnitine. Paclitaxel increases the breakdown of 25-hydroxycholecalciferol (produced in the liver and the form of vitamin D measured routinely) and 1,25-dihydroxycholecalciferol (the active form of vitamin D produced in the kidneys) to their inactive metabolites (193). Malnutrition due to decreased appetite and food intolerance is another reason for MN deficiencies in patients with NET. It affects approximately 25% of inpatients with GEP NET (194).

Fiebrich *et al.* investigated the prevalence of deficiencies in FSV in 19 patients with acromegaly and 35 patients with carcinoid tumours on SSA. They observed that more than 75% of these patients had a deficiency in at least one FSV (189).

MN deficiencies have been linked to cancer risk (195). Optimal nutrition can have an impact on the response to treatment in patients with cancer. The immunomodulatory and anti-oxidant function of micronutrients has been implicated (193). Vitamin D deficiency has been associated with a worse outcome in patients with cancer. In a study looking at the association between MN and breast cancer risk, it was observed that at higher vitamin D levels of 61.5 nmol/L, the risk of breast cancer decreased by 67% (OR:0.33; 95% CI 0:11-0.97; $p < 0.05$) when adjusted for dietary patterns and confounders that are risk factors for breast cancer (196). A meta-analysis showed a 5% reduction in the risk of lung cancer for every 10 nmol/l increase in vitamin D (RR 0.96, 95% CI 0.94-0.99) (197). Vitamin E and selenium are MN with antioxidant and immunomodulatory properties. They also have apoptosis-inducing effects and are involved in the regulation of cell proliferation and differentiation. Low selenium levels have been linked with increased risk of breast and GI cancers (193). Vitamin A also functions as an antioxidant and its deficiency enhances the effect of DNA damaging agents (198). A lot of DNA binding proteins contain zinc fingers. Deficiency in zinc will have an impact on DNA repair enzymes that contain zinc. Mutation in the tumour protein 53 (TP53) gene commonly occurs in cancer. For the TP53 gene to maintain its conformation and function in the cellular response to DNA damage, binding to zinc is essential (195, 199). Copper-zinc superoxide dismutase a tumour suppressor protein containing copper and zinc has been shown to be a promising candidate in the treatment of breast cancer (200).

6.2 Aims

The aim of this study was to assess the prevalence of FSV and TE deficiencies in patients with NET on SSA.

6.3 Methods

6.3.1 Patient selection

82 patients were recruited to participate in this study. Patients younger than 18 years, pregnant, or unable to give informed consent were not included. Nine patients were excluded because they were not on SSA, a total of 73 patients were eligible. Information was obtained from the patients and their medical records about the duration of SSA use, pancreatic enzyme replacement (PERT), vitamin supplementation and their bowel habit including the presence of steatorrhoea.

This study was approved by the South West – Frenchay Research Ethics Committee (17/SW/0131). Informed consent was obtained from all participants.

6.3.2 Laboratory analysis of fat-soluble vitamins and trace elements

6.3.2.1 Analysis of fat-soluble vitamins (A,D,E,K)

Vitamins A and E concentrations were measured simultaneously in serum using the Chromsystems one step HPLC assay kit (Chromsystems, Germany) at the Neurometabolic Laboratory at the National Hospital for Neurology and Neurosurgery. An ultraviolet (UV/vis) detector which absorbs light at a specific wavelength band was used to quantify the peaks of the analytes. A pre-mixed

reaction tube containing a pre-formulated mixture of all the required reagents was used. The mixture in the reaction tube made the matrix of the serum sample more organic and released the bound vitamin A from retinol-binding protein and vitamin E from very low-density lipoproteins. The cell debris and protein were spun into a pellet and the sample containing vitamin A, vitamin E and the internal standard were injected into the HPLC column for analysis. Vitamin A eluted from the column first at about 2.3 minutes with a λ_{max} of 325nm. Vitamin E eluted at around 8 minutes with a λ_{max} of 295nm.

Vitamin D analysis was performed using a Roche Elecsys® assay (Roche Diagnostics, Mannheim) which employs an electrochemiluminescence method on an automated analyser.

Vitamin K1 concentration was measured using LC-MS/MS at the Nutristasis unit, Viapath at St Thomas' hospital. The sample preparation involved protein crash, extraction and solid phase extraction (SPE) clean-up. Quantitation was done using LC-MS/MS (Agilent, technologies, UK). 30 µl of extracted sample was injected. The chromatographic mobile phases consisted of 0.1% acetic acid (v/v) in water (eluent A) and 0.1% acetic acid (v/v) in methanol (eluent B). A fast gradient elution was performed from 80% B to 100% B in 0.1 minute with an isocratic hold at 100%. Baseline separation of K1 was achieved on the Eclipse Plus C18, 50 mm x 2.1 i.d, 3.5 um with the total run time of 5.5.mins. The column was then equilibrated to baseline conditions and the flow rate was maintained at 0.5ml/min, with the temperature at 50°C throughout the chromatographic run. The mass spectrometer operated in positive ion MRM mode.

6.3.2.2 Analysis of trace elements

The trace elements; copper, zinc and selenium were analysed simultaneously using an Agilent 7700 inductively-coupled plasma mass spectrometry (ICP-MS) analyser (Agilent technologies, Japan). Samples were introduced into the centre of an argon plasma source (a highly charged state formed by argon gas). This very high atomization temperature environment is where the constituent chemical species in the sample were rapidly desolated, atomised and then ionised. The ions produced then passed into the mass spectrometer where a combination of radiofrequency and electric fields were used to allow only ions of a particular mass to charge ratio to go through to the detector. The ICP-MS has a collision gas chamber where helium gas can be introduced and the ion beams from the argon plasma can be directed through. This is useful in reducing interferences from polyatomic ions. Copper (isotope 63Cu) and selenium (isotope 78Se) were measured using the helium gas collision cell with rhodium (isotope 103Rh) and germanium (isotope 72Ge) as the internal standards respectively. Zinc (isotope 66Zn) was measured without using the collision gas with germanium (isotope 72Ge) as the internal standard. The total time for analysis of each sample was approximately 2 minutes.

6.3.3 Statistical analysis

Micronutrient deficiencies were depicted graphically using bar charts. Duration of SSA use was categorised into four groups; ≤ 1 year, 2-5 years, 6-10 years and > 10 years. Correlation between micronutrient deficiencies and the duration of SSA use was analysed using Pearson's correlation for normally distributed variables and Spearman's correlation for variables not normally

distributed. A p value < 0.05 was considered statistically significant. Test for normality was performed using the Shapiro-Wilk test and a Normal Q-Q plot. A normal distribution was confirmed if the p value in the Shapiro-Wilk test was greater than 0.05, and the data points laid on the straight diagonal line of the Normal Q-Q plot. Statistical analysis was performed using Microsoft Excel and SPSS version 25.

6.4 Results

73 patients with a confirmed diagnosis of neuroendocrine tumour on SSA participated in this study. Their baseline characteristics are shown in Table 22. Most of the patients 71/73 (97%) had SI-NET. The mean duration of SSA use was 6.4 years +/- 3.8 years. More patients were treated with lanreotide autogel (56%), with majority of them on the higher dose, 120mg. Those on octreotide were commonly receiving the octreotide LAR 30mg dose. Almost half of the patients were on PERT (47%).

Patient characteristics (n=73)	
Mean age (years)	64 +/-11
Gender	
Male	45 (62%)
Female	28 (38%)
NET type	
SI NET	71 (97%)
Pancreatic NET	1 (1%)
Hindgut NET	1 (1%)
SSA type and dose	
Octreotide LAR/SC	32/73 (44%)
Octreotide ≥ 600 mcg daily	2/73 (3%)
Octreotide LAR 30mg	27/73 (37%)
Octreotide LAR 40mg	1/73 (1%)
Octreotide LAR 50mg	2/73 (3%)
Lanreotide autogel	41/73 (56%)
Lanreotide 90mg	3/73 (4%)
Lanreotide 120mg	38/73 (52%)
Duration of SSA use (years)	
Mean duration of SSA use	6.4+/-3.8
≤1	10 (14%)
2 - 5	25 (34%)
6 -10	27 (37%)
>10	11 (15%)
PERT use	
Yes	34 (47%)
No	36 (49%)
Unknown	3 (4%)
GI Surgery	
Yes	55 (75%)
No	18 (25%)

Table 22. Baseline characteristics of the patients. Categorical data is reported in percentages and continuous data as mean ± standard deviation. GI – gastrointestinal surgery

6.4.1 Deficiencies in micronutrients

74% (54/73) of patients were deficient in at least one micronutrient and 36% (26/73) in multiple micronutrients. A deficiency in at least one FSV was seen in 47% of patients and in at least one TE in 58% of patients. Two patients were deficient in all FSV and TE. 38 (52%) patients were taking multivitamins (13%),

vitamin D3 (79%) or a combination of both supplements (8%). 37 of the 38 patients had their vitamin D results available.

6.4.1.1 Fat-soluble vitamin deficiencies

Vitamin K1 was the most frequent vitamin deficiency, observed in 48% (31/65) of patients (Figure 34). Undercarboxylated prothrombin, Protein Induced by Vitamin K Absence or Antagonism-II (PIVKA-II) an abnormal species of factor II was measured alongside vitamin K1. PIVKA-II provides additional information about the nature and extent of vitamin K1 deficiency. It is raised in vitamin K1 deficiency and in patients taking vitamin K antagonists such as warfarin. In patients with vitamin K1 deficiency, 24 (77%) of them had a low vitamin K1 and a raised PIVKA-II as expected. One of these patients was on warfarin therapy and had a markedly elevated PIVKA-II level. The other patients (7/31) had a low vitamin K1 with PIVKA-II levels within normal limits, which reflects adequate vitamin K1 status with respect to coagulation function but low tissue stores.

12(17%) patients had Vitamin D deficiency (< 25 nmol/L) and 44 (64%) of them had insufficient levels (25-75 nmol/L) as depicted in Figure 34. Only one patient with vitamin D deficiency was on vitamin D supplement. 26 (59%) patients with insufficient levels and 10 (77%) of the 13 patients who were vitamin D replete were either on vitamin D or multivitamins or a combination of both supplements. Only 3 (4.5%) and 5 (8%) patients had deficient levels of vitamin E and A.

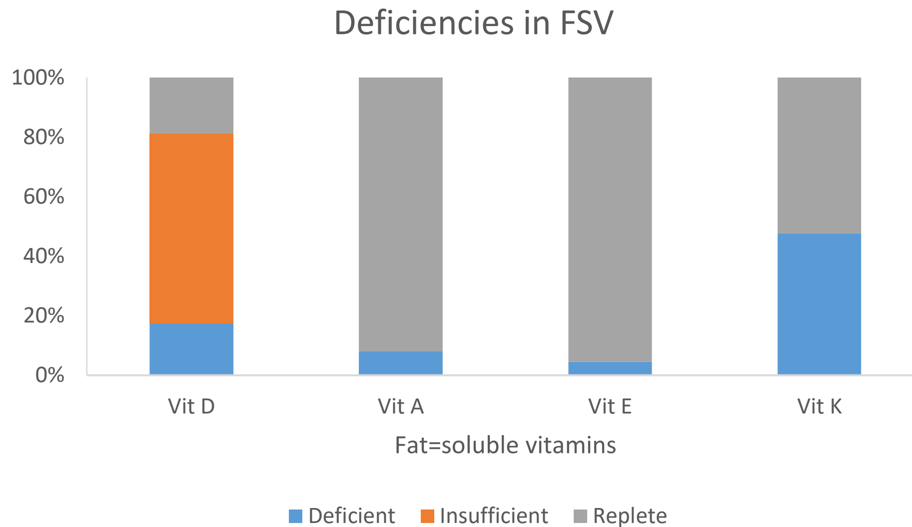


Figure 34. Proportion of patients on SSA with FSV insufficiencies and deficiencies

6.4.1.2 Trace element deficiencies

Zinc was the most common trace element deficiency in 54% (38/71) of the patients. 16% (11/68) had selenium deficiency and 7% (5/71) had copper deficiency (Figure 35).

The concentration of TE can be altered in inflammatory states. Therefore, C-reactive protein (CRP) measurement is recommended in the interpretation of TE (201-203). CRP results were available for 69 (95%) patients. 29% (20) of them had CRP levels above the reference range (5mg/L). 4 (20%) patients with elevated CRP had no TE deficiency.

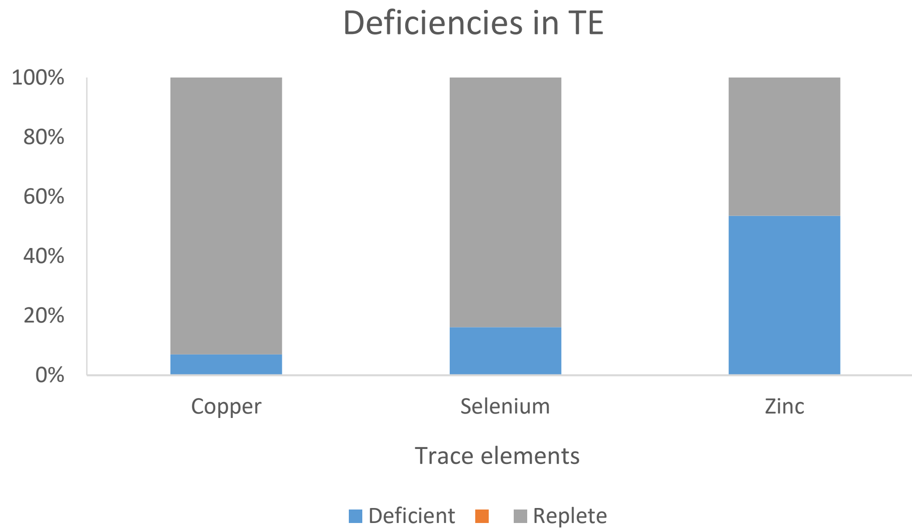
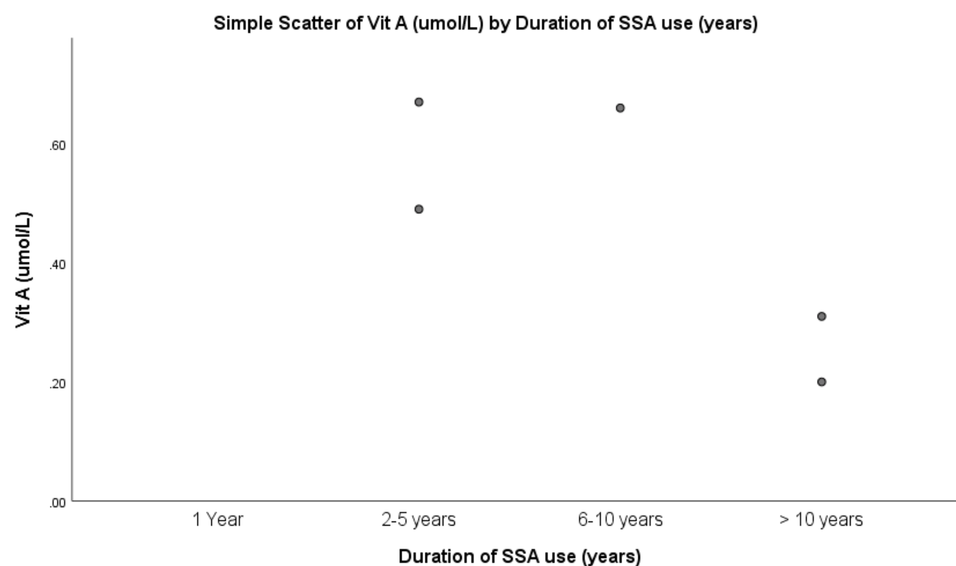


Figure 35. Proportion of patients on SSA with TE deficiencies

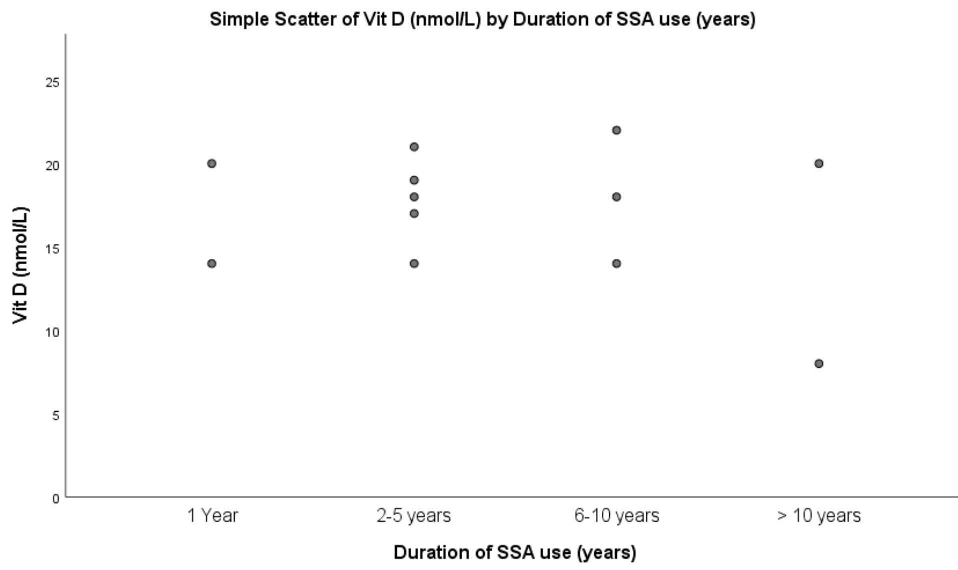
6.4.2 Effect of SSA use

To assess the relationship between micronutrient deficiency and the duration of SSA use, Pearson's correlation was used for the normally distributed FSV A and D and the TE. Spearman's correlation was used for the FSV E and K.

A)

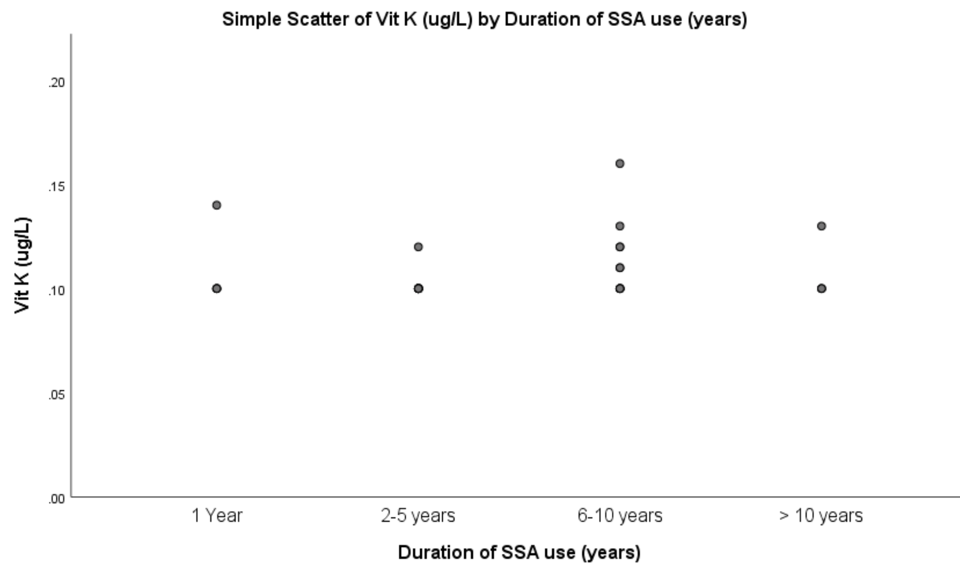


B)

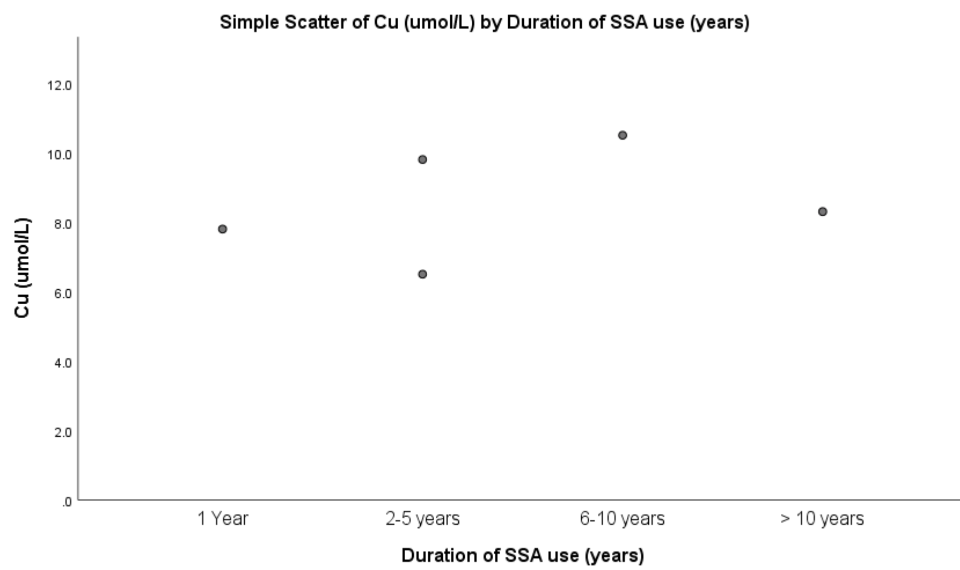


C)

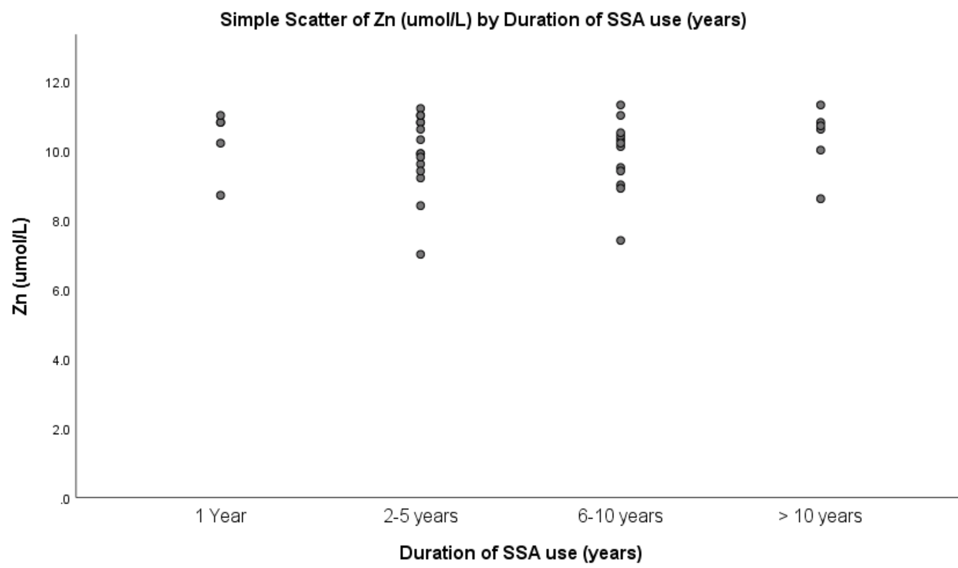
D)



E)



F)



G)

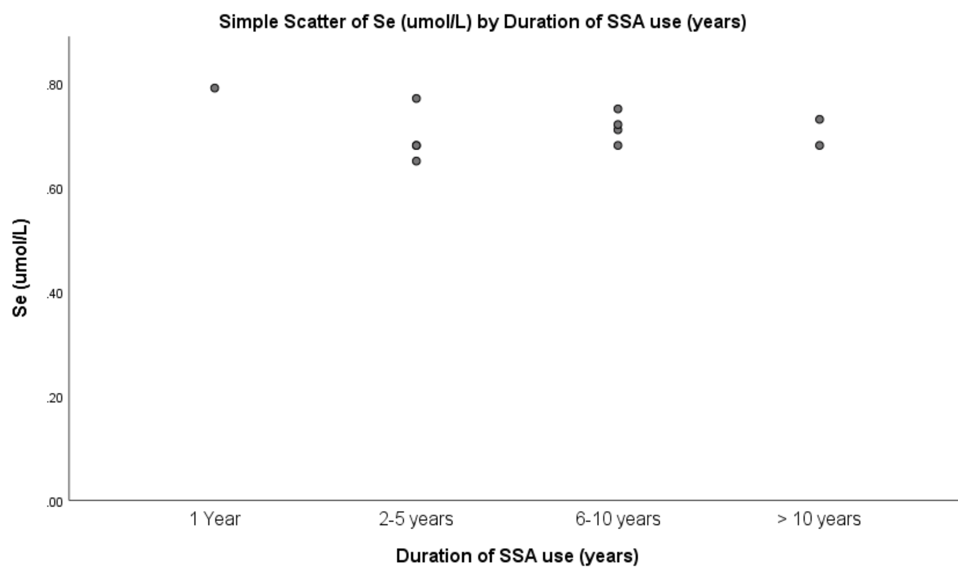


Figure 36. Scatter plots A to G showing the distribution of the deficient micronutrient levels across the duration of SSA use

	N	Mean(+/- SE) of MN	Correlation between SSA use and MN	p-value*
Fat-soluble vitamins				
Vitamin A	5	0.47 (+/-0.21)	-0.8	p=0.12
Vitamin D	12	17.08 (+/-3.97)	-0.2	p=0.50
Vitamin E	3	9.3 (+/-1.90)	0.9	p=0.33
Vitamin K	31	0.11 (+/-0.015)	0.24	p=0.19
Trace elements				
Copper	5	8.6 (+/-1.60)	0.3	p=0.63
Zinc	38	10.0 (+/-1.04)	0.008	p=0.96
Selenium	11	0.71 (+/-0.04)	-0.25	p=0.45

Table 23. Results of the Pearson's and Spearman's correlation between micronutrient (MN) deficiencies and duration of SSA use. * All tests use Pearsons's correlation except those for the fat-soluble vitamins E and K (Spearman's correlation)

The correlation analysis in Table 23 shows a weak relationship between the micronutrients and the duration of SSA use. The correlation coefficient of vitamins A and E appear to suggest a strong relationship with the duration of SSA use. However, few patients (5 and 3) had deficient levels of these vitamins. The p values for the correlation between the micronutrients and duration of SSA use are greater than 0.05, suggesting that if any relationship was observed, it is not statistically significant. Therefore, these results show there is no statistically significant correlation between the micronutrients and the duration of SSA use.

6.4.2.1 SSA and PERT

47% (34/73) of patients were on PERT (Figure 37). 74% (25/34) of them had at least one micronutrient deficiency, while 35% (12/34) of them had multiple deficiencies.

23 (32%) patients reported ongoing loose stools, most of them (65%) were on PERT (Figure 38).

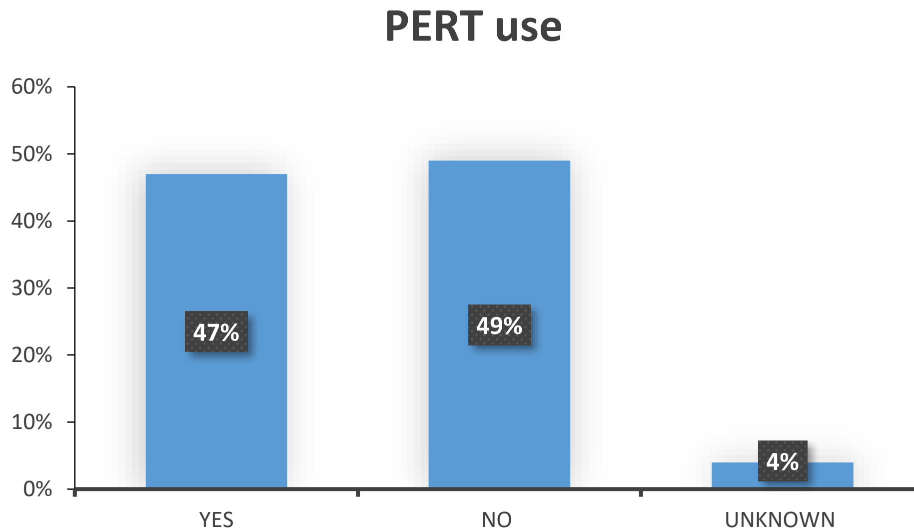


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Figure 37. Proportion of patients on PERT

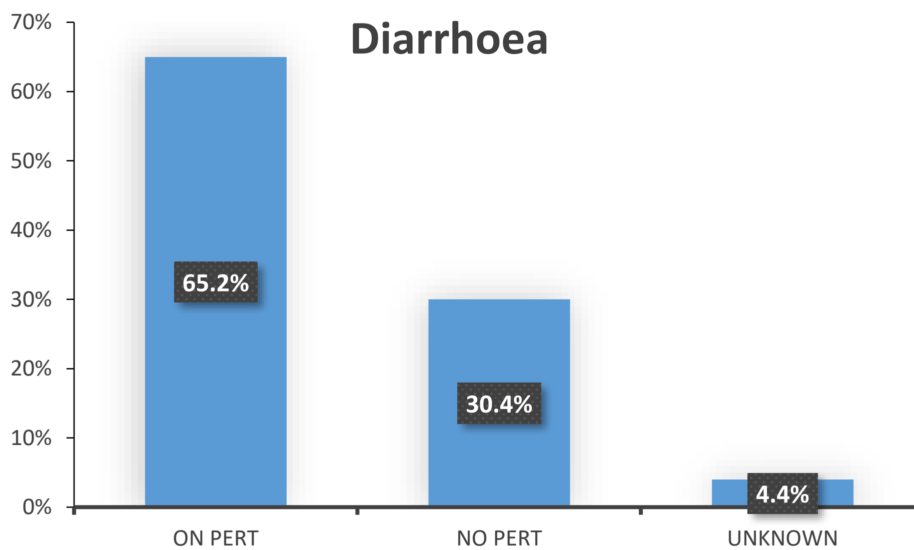

Figure 38. Proportion of patients with diarrhoea categorised by PERT use

6.5 Discussion

MN deficiency is caused by decreased availability, reduced intake or malabsorption (204). There is increasing evidence that MN deficiency is associated with the risk of cancer. They are essential either as substrates or co-factors in regulatory pathways for DNA synthesis and repair. Examples have been demonstrated in in vitro human studies. Treatment with vitamin E stimulated DNA synthesis in human peripheral blood lymphocytes and reduced chromosomal damage in leukocytes (205). Repair of DNA strand breaks was faster when lymphocytes treated with hydrogen peroxide where supplemented with β-carotene compared to when there were not supplemented with β-carotene (206). Therefore, single or multiple deficiencies in these micronutrients will often lead to DNA damage and cancer as a consequence (195, 198).

Vitamin D deficiency is defined as a 25-hydroxyvitamin D [25(OH)D] level of less than 25 nmol/L. Insufficient levels according to the endocrine society is between 25 and 75 nmol/L (207). However, levels greater than 50 nmol/L is said to be sufficient for most people (208, 209). In this study, twelve patients had deficient vitamin D levels. 10 (83%) of them were not on vitamin D or multivitamin supplement. One patient was unsure, and the last patient was on vitamin D supplement which was started shortly before he participated in the study. According to the endocrine society guidelines, only 13 (19%) patients were vitamin D replete. Of these, 10 (77%) of them were on vitamin D or multivitamin supplement at the time of the study, 2 were not on supplements and the last patient was unsure. These patients are usually prescribed a

course of high dose vitamin D supplementation if found to have insufficient or deficient vitamin D levels. This may have been the case with the 2 patients who were not on supplements but we did not enquire about recent supplement use. We did not carry out a quality of life assessment in these patients to assess the impact of vitamin D supplementation with regards to commonly experienced symptoms such as muscle aches and weakness, bone pain and generalised body pain. Patients with cancer have reported improvement in their quality of life following vitamin D supplementation (210).

The pancreatic enzyme lipase and bile acids are crucial for the digestion and absorption of fat. Treatment with SSA suppresses the secretion of pancreatic enzymes and inhibits bile flow (190), subsequently leading to malabsorption of fat and FSV. Deficiencies in the TE zinc, copper and selenium have been associated with malabsorption (188, 191, 211). This study shows that deficiencies in FSV and TE especially vitamin K1 (48%) and zinc (54%) are not uncommon in patients with NET on SSA. Fiebrich *et al* also observed a higher frequency of vitamin K1 deficiency (69% and 53%) in both carcinoid and acromegaly patients on SSA (189). Vitamin K1 (phylloquinone) is the main dietary form of vitamin K and its measurement in serum is used to determine vitamin K status. PIVKA-II is a marker that gives an indication of the cellular utilisation of vitamin K1. Elevated levels of PIVKA-II reflect low hepatic vitamin K stores sufficient to impair effective γ-carboxylation of factor II, which can lead to coagulation abnormalities (212).

Zinc deficiency which has been linked to chronic diarrhoea and malabsorption states (188) was the most common TE deficiency identified in this study. A likely contributory factor may be that its daily requirement is higher than that of the other two TE (213). The concentration of these TE can be altered in conditions such as infection and chronic diseases where an inflammatory response is induced. This inflammatory response also known as an acute phase response is associated with an elevation in CRP concentration. Zinc and selenium are negative acute phase reactants because their concentrations decrease in inflammatory states. Copper is a positive acute phase reactant, as its concentration increases. A study assessing the impact of the degree of inflammatory response on the concentration of micronutrients showed that at a slight CRP elevation of less than 10mg/L, selenium results can be reliably interpreted. At CRP concentrations of less than 20mg/L, reliable interpretation of zinc results can be made (201, 203).

12(60%) of the 20 patients with elevated CRP had levels ≤10mg/L. 4 (33%) of them had no TE deficiency, 4 patients had deficient zinc and selenium levels and the other patients had isolated zinc deficiency. 6 (30%) patients had a CRP concentration greater than 10 but below 20mg/L. 3 of them had isolated zinc deficiency, 1 had both zinc and selenium deficiency, 1 patient had no TE deficiency and the last patient had no TE result. 2(10%) patients had CRP levels above 20mg/L. One of them had zinc deficiency and the other was not deficient in any TE. Interestingly the 2 patients with global micronutrients deficiency had CRP concentrations within the normal limits. Based on the

study by Duncan *et al* (203), one patient with deficient zinc (2.6%) and one patient with deficient selenium (9%) levels may not have true deficiencies.

The use of PERT prescribed for steatorrhoea, a well-recognised adverse effect of SSA was common in this study. Lamarca *et al* reported pancreatic exocrine insufficiency (PEI) which presents as steatorrhoea when pancreatic enzymes are significantly reduced, in 24% of patients with well-differentiated NET on SSA (104). We found that 47% (34/73) of our patients were on PERT. A possible explanation for the increased frequency of PERT use in this study could be because most patients (93%) were on the maximum dose of SSA (120mg lanreotide autogel or ≥ 30mg octreotide LAR), with some of the SSA dose at intervals of less than 4 weeks.

23 (32%) patients reported diarrhoea. Majority of them were on PERT (65%), which may suggest insufficient treatment or the presence of another cause for the persistent diarrhoea. In this study where the patients had predominantly SI-NET (97%) and 75% of them had surgery, there may be other causes for diarrhoea not related to SSA use. The diarrhoea could be due to increased hormone production in CS, or as a result of small bowel resection especially when the primary tumour is around the terminal ileum, reducing the surface of absorption. Bile acid malabsorption and small intestinal bacterial overgrowth which can occur following small bowel resection can also cause diarrhoea (214). 8 (11%) patients had either a diagnosis of bile salt malabsorption (4 patients) or were on treatment for bile salt malabsorption with cholestyramine

(3 patients) or colesevelam (1 patient). Seven of these patients were on PERT simultaneously.

In this study, I did not observe a correlation between the duration of SSA use and deficiency in either FSV or TE. In a multivariate regression analysis, Fiebrich *et al* also found that there was no association between the duration of SSA use and deficiency in FSV (189). I had relatively small numbers of participants in the study to draw a conclusion about the correlation between micronutrient deficiencies and the duration of SSA use. Lamarca *et al* showed that PEI occurred quite early after starting on SSA. Faecal elastase-1 used to diagnose PEI in their study showed statistically significant changes in levels between patients who did and those that did not develop PEI at 6 months (104). 40% (4/10) of our patients who were on SSA for a year or less were on PERT.

The limitations from our study include the following. Most patients in this study had SI-NET (97%), therefore our observations may not necessarily apply to all patients with NET. The majority of the patients (75%) in our study had undergone surgery which makes the cause of diarrhoea likely multifactorial. Some patients had missing results especially for vitamin A, E and K, where 10, 7 and 8 patients had no results available mostly due to the special collection requirement (protection from light) not being adhered to. This may have contributed to the lower number of deficiencies recorded. We did not collect information about common signs and symptoms of micronutrient deficiencies such as easy bruising in vitamin K deficiency; fatigue, muscle aches and bone

discomfort or pain in vitamin D deficiency. A quality of life assessment would have been useful in assessing the impact of these micronutrient deficiencies in our patients.

6.6 Conclusion

The rate of persistent diarrhoea in patients on PERT is high. These patients should be optimised on PERT and actively investigated for alternative causes of diarrhoea including bile acid malabsorption and small intestinal bacterial overgrowth.

Deficiencies in FSV and TE especially vitamin K1 and zinc is common in patients with NET on SSA. 52% of patients were on vitamin supplementation, predominantly vitamin D3 (79%). This is likely to account for the fewer cases of vitamin D deficiency observed in this study. We would recommend that monitoring and supplementation of FSV and TE is considered in patients with NET on SSA.

Summary

NET are a diverse group of neoplasms that arise from cells within the diffuse endocrine system. Small intestinal NET (SI-NET) are often slow growing with a low proliferation rate. They tend to be diagnosed at an advanced stage when metastasis has occurred and cause functional symptoms due to CS. 5-HIAA a metabolite of serotonin is an important biochemical marker used in the diagnosis and monitoring of patients with SI-NET especially in the presence of CS. Its measurement is commonly performed on a 24-hour urine collection. Serum and plasma assays are now being used in the analysis of 5-HIAA. SSA are often used as first line treatment in patients with metastatic NET and their adverse effects include diarrhoea and steatorrhoea which can impair the absorption of FSV and TE.

The primary aim of this thesis was to develop and validate an assay for plasma and serum 5-HIAA. In chapter 2, I developed the assay for 5-HIAA in plasma and serum. This was validated using FDA guidance, and it was shown to have an acceptable performance.

In chapter 3, 5-HIAA analysis was compared in plasma and serum and there was a good agreement between both sample types. Plasma and serum 5-HIAA also performed similarly at the chosen diagnostic cut-off of 135 nmol/L. A clinical validation was carried out using 80 patients with NET and 30 healthy volunteers. I was able to demonstrate that plasma and serum 5-HIAA at the chosen cut-off had comparable performance with the urine assay currently in use.

I assessed the relationship between the plasma and serum 5-HIAA assays with other biochemical markers of NET. In chapter 4, plasma and serum 5-HIAA were compared to whole blood serotonin. In patients with NET a statistically significant correlation was demonstrated between the 5-HIAA assays and whole blood serotonin; Spearman’s correlation coefficient was 0.75 ($p = < 0.0001$) for comparison with plasma and 0.735 ($p = < 0.0001$) with serum 5-HIAA. In healthy volunteers, a correlation was not shown between these assays; Spearman’s correlation coefficient was 0.26 ($p = 0.17$) for comparison with plasma and 0.331 ($p = 0.079$) with serum 5-HIAA.

In chapter 5, I compared plasma and serum 5-HIAA with CgA and NT-proBNP. A statistically significant correlation was observed between the plasma and serum 5-HIAA assays and CgA; Spearman’s correlation coefficient was 0.792 ($p = < 0.0001$) for comparison with plasma and 0.794 ($p = < 0.0001$) with serum. Chromogranin A levels tend to reflect tumour burden. Comparison of the 5-HIAA assays with NT-proBNP demonstrated a statistically weak association. Spearman’s correlation coefficient; 0.38 ($p = < 0.001$) when compared with plasma and 0.421 ($p = < 0.001$) with serum. One would not necessarily expect a strong correlation because NT-proBNP is a marker in this instance for carcinoid heart disease and not all patients with elevated 5-HIAA develop CHD.

A secondary aim of this thesis was to assess for malabsorption in patients on long term SSA, correlating deficiencies in FSV (A,D,E,K) and the TE zinc, selenium and copper. 73 patients with NET on SSA took part in this study. 74%

of them were deficient in at least one micronutrient and 36% in multiple micronutrients. FSV deficiency was observed in 47% and TE in 58% of patients. No relationship was observed between the micronutrient deficiencies and the duration of SSA use.

Future work is required to investigate the relationship between 5-HIAA in serum and plasma with eGFR, to determine the level of eGFR that 5-HIAA measurement in blood becomes unreliable. It will also be useful to know the levels of 5-HIAA in plasma and serum which predicts an increased risk of developing carcinoid heart disease especially as we are likely to move to blood based 5-HIAA measurement. The small number of patients with MN deficiencies in this study limited the ability to detect a correlation between the duration of SSA use and MN deficiencies. Further work is required to evaluate this. A more representative population of GEP-NET patients should be studied. In view of other causes of diarrhoea apart from SSA use identified in our study cohort, the assessment of micronutrient deficiencies should also include water-soluble vitamins and other TE such as vitamins B12, folic acid and iron which can be deficient in these patients.

In conclusion, an assay for the analysis of plasma and serum 5-HIAA has been developed, with acceptable analytical performance. This blood based 5-HIAA assays offer a convenient and practical alternative to the urine 5-HIAA assay.

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