Quantification and analysis of biogenic amines using a liquid chromatographic tandem mass spectrometric method

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DECLARATION OF ORIGINALITY

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Topic of work: Quantification and analysis of biogenic amines using a liquid chromatographic tandem mass spectrometric method

Declaration

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Ethics

Ethical approval, reference number 103/2014, was obtained from the University of Pretoria's Research for the use of drawing of blood from healthy volunteers. Approval was obtained from the Masters Committee, Faculty of Health Sciences, University of Pretoria to conduct this project. See Addendum 1 and 2

Abstract

Keywords: Biogenic amines, Derivatisation, Chromatography, Mass spectrometry, Validation, Method development

Introduction: Biogenic amines are a group of endogenous compounds which play an important role in maintaining homeostasis. These compounds are produced from amino acid precursors and exert strong physiological effects despite being at low concentrations (low pg/ml). There are a wide variety of affects and effects controlled by biogenic amines. Different conditions can be brought on by changes in the concentrations of these biogenic amines that can alter the levels or be brought about by incorrect levels. Thus biogenic amine concentrations may prove to be valuable biomarkers – if the extremely low levels can be accurately and rapidly quantitated. The aim of this study was to develop and validate a method which is able to quantitate multiple biogenic amines in a single run from low sample volumes.

Methods and Materials: Quantification of the blood levels of biogenic amines was performed following extraction using derivatisation and an appropriate liquid chromatography tandem mass spectrometric method. The derivatisation was performed at mild conditions for an hour and extracted using ethyl acetate. The LC-MS/MS system used was an Agilent 1100 series HPLC and an ABSciex 4000 QTrap. The system was operated in positive ESI mode and appropriate MRM precursor-product pairs were selected. In order to validate the method a series of 8 dilutions from 0.05 – 2.5 ng/ml were prepared in solvent and plasma. These were then processed according to the developed method and analysed using Analyst software.

Results and discussion: The validation of the biogenic amines was performed according to the ICH guidelines. The calibration curves were fitted using linear regression (1/x weighting) over the linear range of the calibration curve. Metanephrine and melatonin were validated in plasma between 0.1 – 2.5 ng/ml. Serotonin, histamine and normetanephrine were validated in solvent between 0.25 – 2.5 ng/ml. Adrenaline, dopamine, noradrenaline and 5-hydroxy-3-indoleacetic acid were validated in solvent between 5 – 50 ng/ml. Metanephrine exhibited the highest sensitivity and showed linearity between 0.05 – 2.5 ng/ml in solvent. The catecholamines and 5-HIAA were the least sensitive of the analytes and had estimated LLOQs between 0.5 – 2.5 ng/ml. Stability tests show the derivatised analytes to be stable at -20°C for 5 days and recovery testing shows recoveries of over 70% for metanephrine and melatonin.

Conclusion: Sensitivity still remains an issue when working with biogenic amines. The extremely low physiological concentrations are further exacerbated by the low sample volumes used in this study. Despite the challenges the compounds were all validated according to ICH guidelines and an LC-MS/MS method was developed that was able to analyse 9 biogenic amines of different classes in a single run using only 250 µl of human plasma, after multistep sample preparation whilst achieving high sensitivity.

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Abbreviation list

%CV	coefficient of variance
5HT	5-hydroxytryptophan
5-MT	5-methoxytryptamine
AADC	aromatic acid decarboxylase
APPI	atmospheric pressure photoionization
AUC	area under the curve
BA	biogenic amine
CE	capillary electrophoresis
CNS	central nervous system
CSF	cerebrospinal fluid
DBSS	dry blood spot sampling
DNSCl	dansyl chloride
DOPA	levo dihydroxyphenylalanine
ECD	electrochemical detection
ELISA	enzyme-linked immunosorbent assay
ER	enhanced resolution
ESI	electrospray ionisation
GABA	gamma-amino butyric acid
GC	gas chromatography
GC-MS	gas chromatography mass spectrometry
HPLC	high pressure liquid chromatography
LC-MS/MS	liquid chromatography tandem mass spectrometry
LLE	liquid-liquid extraction
LLOD	lower limit of detection
LLOQ	lower limit of quantitation

LOD	level of detection
m/z	mass-to-charge ratio
MRM	multiple reaction monitoring
MTMAC	methyl trimethylamino caproate,
NHS	N-hydroxysuccinimide ester
OPA	*o*-phthalaldehyde
PNS	peripheral nervous system
QQQ	triple quadrupole
QTOF	quadrupole-time of flight
QTrap	quadrupole Trap
RP	reverse phase
RPC	reversed phase chromatography
RP-IP	reverse phase - ion pair
TH	tryptophan hydroxylase
TLC	thin layer chromatography
TMAC-NHS	trimethyl-aminocaproic acid N-hydroxysuccinimide ester
TOF	time of flight
UPLC	ultra pressure liquid chromatography
UV	ultraviolet visible

Chapter 1 Introduction and literature review

1.1 Introduction, definition and classification of biogenic amines

Biogenic amines (BA) can be broadly defined as a group of biologically active nitrogen containing compounds produced by living systems (Segen, 2005). The name 'biogenic amines' covers numerous sub-groups including catecholamines, polyamines, imidazoles, indoleamines, betaines and ethanolamine derivatives (to name a few.) These substances are produced via numerous pathways, commonly using amino acid precursors (Jia *et al*, 2011). They serve a variety of functions in the living organism - they are involved in signalling between cells but can also alter the physiology of the organism as a whole. A careful homeostasis exists between these compounds and the human physiologic system with alterations in these levels able to cause significant shifts in normal human body functions (De Jong *et al*, 2011).

Biogenic amines are low molecular weight, hydrophilic, organic, basic compounds which contain one or more amine (nitrogen) functional groups. They exist as primary, secondary or tertiary amines, and can have aliphatic, aromatic or heterocyclic structures (Önal, 2007, Zhang *et al*, 2008, De La Torre and Conte-JúNior, 2013). Biogenic amines can be classified into various sub-groups according to their structures or the amine content.

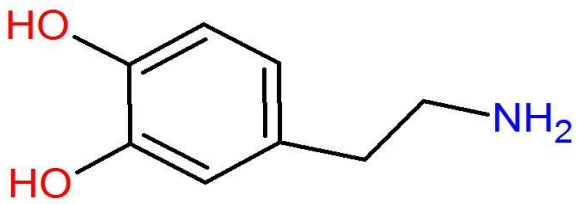
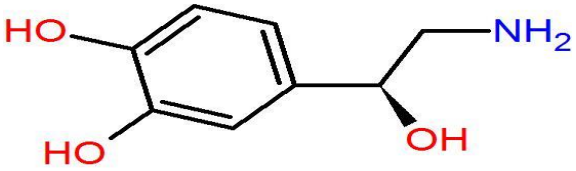
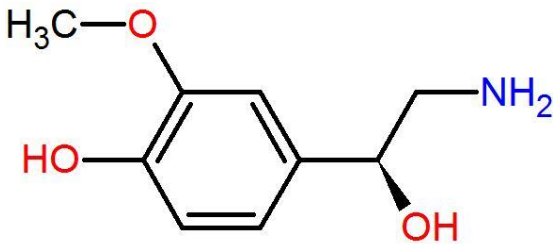
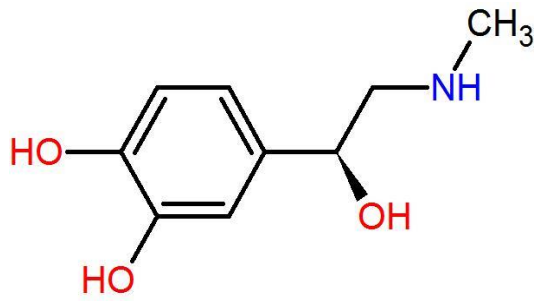
The catecholamine group, is characterised by a phenyl ring with two adjacent phenolic hydroxyls and an aliphatic chain with an amine and hydroxyl (except dopamine) groups (Bourcier *et al*, 2006). They possess a single amine group which is a primary amine group for dopamine and norepinephrine and a secondary amine group for epinephrine. The most important catecholamine BA include dopamine, noradrenaline (norephinephrine) and adrenaline (epinephrine) (Bertil and Goldstein, 1988). The structure of these BA are illustrated in Table 1.

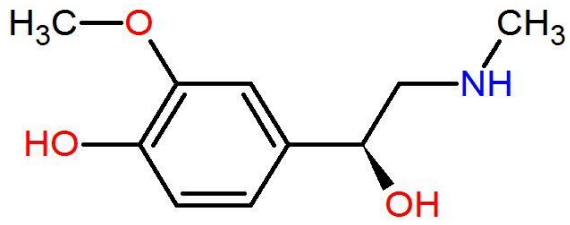
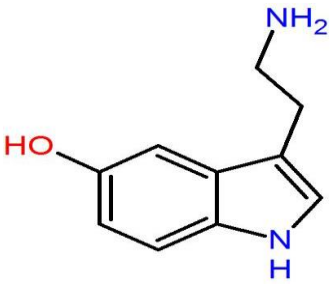
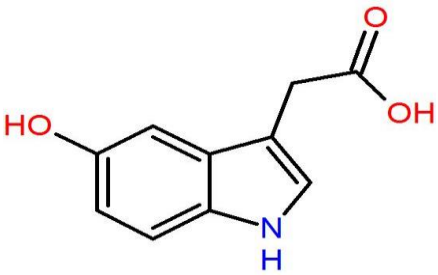
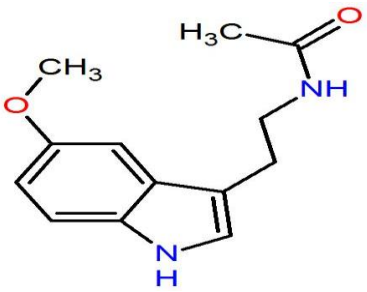
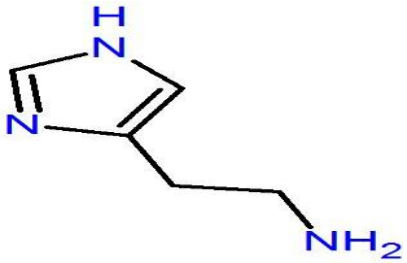
The imidazole group, is characterised by an imidazole ring and an aliphatic amine group. Histamine has three amine groups, namely a tertiary amine and one secondary amine within the imidazole ring as well as a primary amine on the aliphatic tail. The most important imidazole based BA is histamine (Oguri and Yoneya, 2002). This BA is illustrated in Table 1.

The indoleamine group is characterised by an indole group (a fused benzene and pyrrole ring combination) with an aliphatic amine and a single phenolic hydroxyl group (Bourcier *et al*, 2006). The indoleamines have two amines, one secondary amine within the pyrrole ring and one amine, which can be primary or secondary, on the aliphatic tail. The more important indoleamine BAs of indoleamine synthesis include serotonin and melatonin (Cryan and Leonard, 2000). These BA are illustrated in Table 1.

The aliphatic BA include the polyamines such as spermine, spermidine and putrescine (Teti *et al*, 2002). Biogenic amines possess similar structures often containing common chemical moieties between sub-groups which have the same amino acid precursors and have only one or two chemical differences resulting in similar structures. The structures, along with the molar mass as well as derivatisation sites, of various BAs are displayed in Table 1

Table 1: Biogenic amines used in this study. This table shows the structures, the molar mass and number of derivatisation sites of the biogenic amines assayed in this study.

Structure	Biogenic amines
	**Dopamine – 4-(2-aminoethyl)benzene-1,2-diol** Molar Mass: 153.18 g/mol Available sites for derivatisation: 3
	**Noradrenaline – 4-[(1R)-2-amino-1-hydroxyethyl]benzene-1,2-diol** Molar Mass: 169.18 g/mol Available sites for derivatisation: 3
	**Normetanephrine – 4-(2-amino-1-hydroxy-ethyl)-2-methoxy-phenol** Molar Mass: 183.20 g/mol Available sites for derivatisation: 2
	**Adrenaline – (*R*)-4-(1-Hydroxy-2-(methylamino)ethyl)benzene-1,2-diol** Molar Mass: 183.20 g/mol Available sites for derivatisation: 3

	**Metanephrine – 4-(1-hydroxy-2-methylamino-ethyl)-2-methoxy-phenol** Molar Mass: 197.23 g/mol Available sites for derivatisation: 2
	**Serotonin – 3-(2-aminoethyl)-1H-indol-5-ol** Molar Mass: 176.22 g/mol Available sites for derivatisation: 2
	**5-Hydroxyiondole acetic acid – 2-(5-hydroxy-1H-indol-3-yl) acetic acid** Molar Mass: 191.18 g/mol Available sites for derivatisation: 1
	**Melatonin – N-[2-(5-methoxy-1H-indol-3-yl)ethyl)** Molar Mass: 232.28 g/mol Available sites for derivatisation: 2
	**Histamine – 2-(1H-amidazol-4-yl) ethanamine** Molar Mass: 111.15 g/mol Available sites for derivatisation: 2

1.2 Synthesis and metabolism of biogenic amines

The majority of BAs are produced via decarboxylation reactions of amino acid precursors except for the polyamines, which are produced via an initial acetylation reaction of an amino acid precursor (Teti *et al*, 2002). The following section will overview the synthesis of the most important groups of biogenic amines with a detailed pathway overview illustrated in Figure 2.

Catecholamines are produced from the amino acid tyrosine. Tyrosine is converted to levo dihydroxyphenylalanine, DOPA, by tyrosine hydroxylase, which adds a single phenolic hydroxyl group. DOPA is then converted to dopamine by the enzyme aromatic acid decarboxylase (AADC) that removes the aliphatic carboxyl group. Dopamine is converted to noradrenaline via the enzyme dopamine beta-monooxygenase (DMO) which adds a hydroxyl group to the carbon position 2. Noradrenaline is then converted to adrenaline via phenylethanolamine N-methyltransferase, which adds a methyl group to the aliphatic primary amine (De Jong *et al*, 2011). The catecholamines are largely metabolised by two enzymes; monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT) with aldehyde dehydrogenase playing minor roles in metabolism. Dopamine metabolism has a few major products, namely- 3-methoxytyramine, DOPAC and homovanillic acid. Adrenaline is largely metabolised to metanephrine, vanillylmandelic acid and 3-methoxy-4-hydroxyphenylglycol. Noradrenaline is metabolised in a similar fashion to adrenaline with normetanephrine, vanillylmandelic acid and 3-methooxy-4-hydroxyphenylglycol as major metabolic products (Bertil and Goldstein, 1988, De Jong *et al*, 2011, Kurian *et al*, 2011).

The indoleamines are produced from the amino acid tryptophan. Tryptophan is converted to 5-hydroxytryptophan, 5HT, by tryptophan hydroxylase, TH, which adds a single phenolic hydroxyl group. 5HT is converted to serotonin by AADC which removes the carboxyl group. Serotonin is metabolised by MAO into 5-hydroxyindoleacetic acid (De Jong *et al*, 2011, Kurian *et al*, 2011). Pineal melatonin is synthesised from serotonin by two enzymes; N-acetyltransferase to form N-acetyl-serotonin. Hydroxyindole-O-methyltransferase then converts N-acetyl-serotonin to melatonin (De Jong *et al*, 2011). Outside of the pineal gland other enzymes may be involved in the synthesis of melatonin and its regulation may be different to that described for the pineal gland (Hardeland, 2008).

The imidazoles are produced from the amino acid histidine. The production of histamine is a single step process in which histidine is converted to histamine by the removal of the carboxyl group, via AADC (Oguri and Yoneya, 2002). Histamine is largely metabolised via two pathways using the enzymes histamine N-methyltransferase and diamine oxidase.

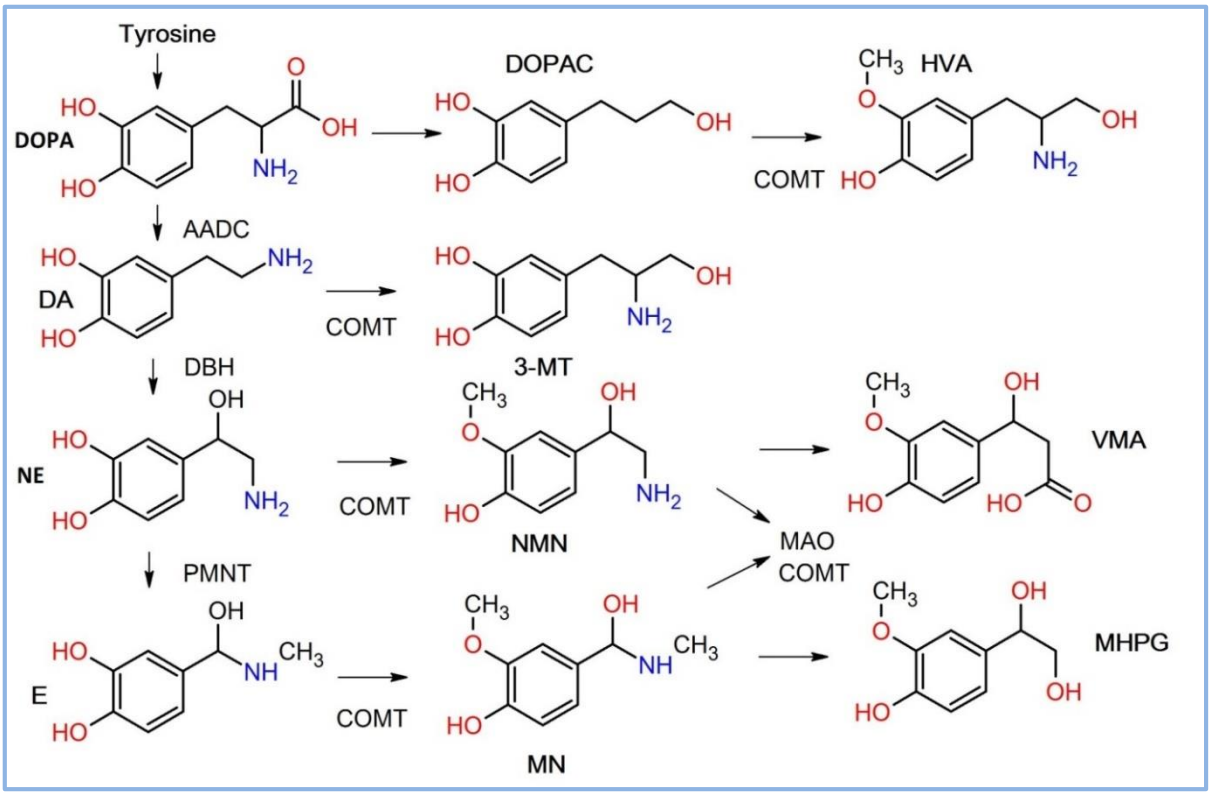


Figure 1: Figure showing the metabolic pathways of the tyrosine derived catecholamines. (Adapted with Permission)

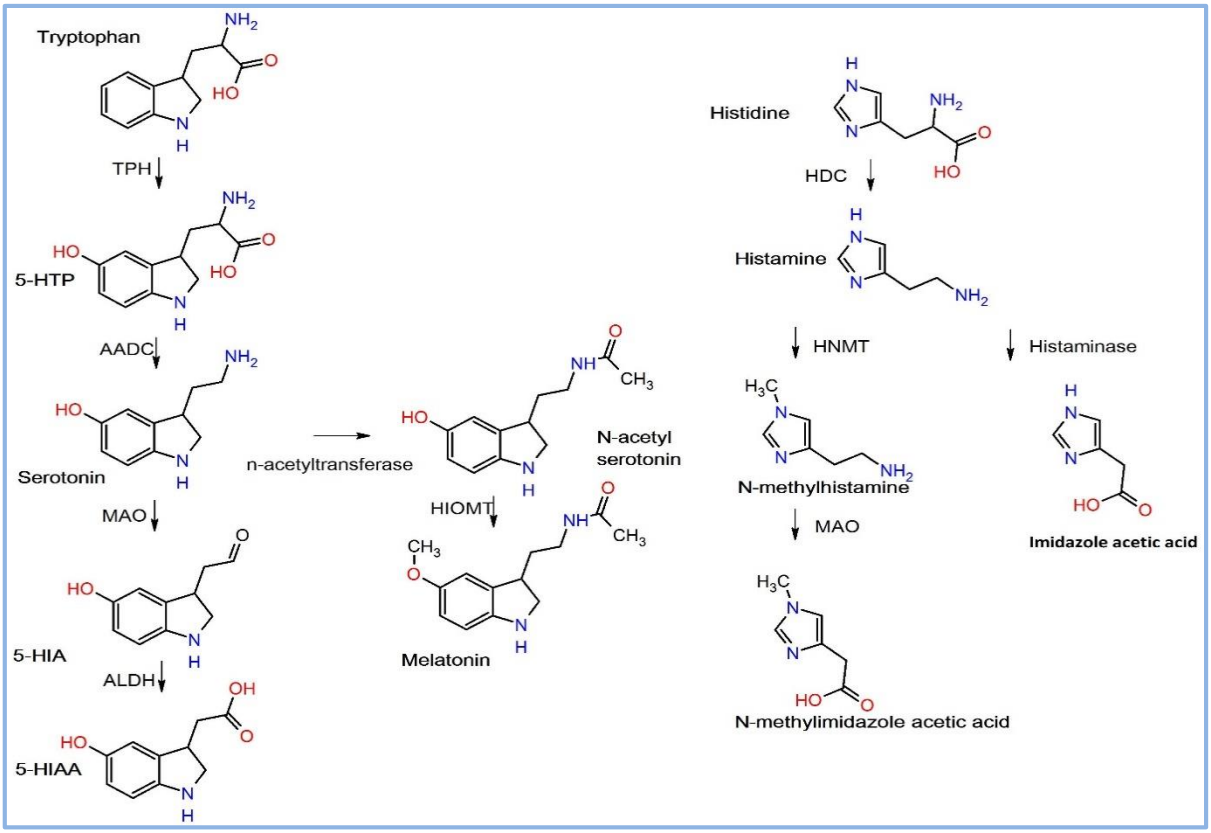


Figure 2: Figure showing the metabolic pathways of the indole and indolylamines. (Adapted with permission)

1.3 Functions of biogenic amines *in vivo*

Biogenic amines have a variety of functions within the human body by interactions with specific receptors. These substances play roles in intercellular communication or affect the physiology of the entire body by acting as hormones (De Jong *et al*, 2011). Receptors with which BA interact are ubiquitous within the body, from serotonin receptors in the central nervous system (CNS) and gastrointestinal tract (Gershon, 2004, Nelson and Gehlert, 2006), histamine receptors in the CNS, GIT and smooth muscle cells (Van Der Goot and Timmerman, 2000, Haas *et al*, 2008), dopamine receptors in the CNS and various receptors that bind adrenaline in heart, lung and muscle tissue (Bertil and Goldstein, 1988, Nelson and Gehlert, 2006). The binding of BA to the various receptors not only maintain the daily functioning of the human body but are of major importance during moments of extreme stress. Excessive release and binding of BA may result in tissue damage and disease states and can elicit detrimental effects within the human body (Bertil and Goldstein, 1988, De Jong *et al*, 2011).

Catecholamines allow for the healthy functioning of the CNS and act as effectors in the sympathetic branch of the autonomic nervous system. The sympathetic nervous system is also referred to as the "fight or flight" system and allows the body to respond in times of stress. Adrenaline and noradrenaline are released by the sympathetic nervous system to act as end stage effectors on alpha and beta adrenergic receptors (Robinson and Hudson, 2000, Davis *et al*, 2008). The effects of such BA release cater toward energy generation and higher physical output by the body. There are two major alpha adrenoreceptor subtypes – 1 and 2. Alpha 1 receptors are located in the CNS and PNS, smooth muscle cells, liver cells as well as in cardiac cells (Robinson and Hudson, 2000, Davis *et al*, 2008). In the CNS the binding of catecholamines to the receptors results in an excitatory effect. The binding to smooth muscle receptors causes contraction of the cells and binding to cardiac cells results in positive ionotropy, the force with which the heart pumps. The effect on liver cell receptors is to increase glycogen phosphorylation (Robinson and Hudson, 2000, Van Der Goot and Timmerman, 2000). The alpha-2 receptors are found in the CNS and PNS. The major result of alpha-2 receptor activation is hypotension, pupil dilation, sedation and anaesthesia. It also causes smooth muscle contraction (Robinson and Hudson, 2000, Davis *et al*, 2008). The beta receptors are also divided into 2 major subtypes – 1 and 2. Beta-1 receptors are found in cardiac and adipose tissue and activation of the receptors results in positive ionotropy and chronotropy, (the force of the heart beat and the rate with which the heart pumps respectively). Beta-2 receptors are located on airway, vascular and uterine smooth muscle and activation of these receptors results in relaxation of the smooth muscle resulting in vasodilation and bronchodilation. There are other receptors activated by dopamine in the peripheral system in the blood vessels, kidneys and heart (Missale *et al*, 1998). The adrenergic receptors in the CNS are activated by noradrenaline and dopamine. The dopaminergic system has numerous roles and these include in the voluntary locomotion, cognitive processes, control of prolactin secretion, and motivated behaviours such as

emotion and reward mechanism (Samuels and Szabadi, 2008). The noradrenergic system has a variety of roles in maintaining homeostasis, some of these include arousal, cognitive processes, wakefulness, anxiety and act on the sympathetic nervous system (Cools *et al*, 2011).

Serotonergic nerves are widespread in the CNS and maintain homeostasis within the brain. The role that serotonin plays is less defined than that of dopamine due to less research directed at the study of serotonin and its various receptors (Cools *et al*, 2011). There are numerous serotonergic receptors, with at least fourteen different receptors characterised (Fuller, 1996, Nichols and Nichols, 2008). The innervation patterns of serotonergic nerves in the CNS implicate serotonin in numerous functions such as impulsivity, depression, motor control, autonomic control of respiration, temperature control, behaviour, arousal, pain and hormone control (Fuller, 1996, Cools *et al*, 2011, Kurian *et al*, 2011). Serotonin receptors in the periphery are widespread in the human body. There are receptors located in the cardiovascular tissue, liver, gastrointestinal tract, blood platelets as well as cerebral blood vessels (Lesurtel *et al*, 2006, Brenner *et al*, 2007, Nichols and Nichols, 2008). These receptors exhibit functions from the proliferation of arterial fibroblasts, arterial vasoconstriction, relaxation of the colon, regulation of intestinal movement, vasoconstriction and allow for the development of the heart and enteric nervous system (Fiorica-Howells *et al*, 2000, Nebigil *et al*, 2000).

Melatonin has been discovered to be highly ubiquitous and with its inter-species variability is no longer seen as a hormone or trace compound. Melatonin has numerous functions in the organism. There is melatonin found outside of the pineal gland, however the function of this melatonin is poorly understood (Hardeland, 2008). Melatonin has two receptors characterised so far- MT_1 and MT_2 (Barrenetxe *et al*, 2004). The primary physiological function of pineal melatonin is to respond to light-dark cycles and is secreted in circadian rhythms in response to the dark phase of the day (Barrenetxe *et al*, 2004, Srinivasan *et al*, 2005, Hardeland, 2008, Hardeland *et al*, 2012). The outputs of this information results in the sleep/wake cycle, feeding, temperature regulation and the control of endocrine rhythms. Melatonin also has a neuro-protective effect acting as an antioxidant and may aid in protecting the brain from neurodegenerative diseases such as Parkinson's disease and Huntington's chorea as well as neurological conditions like strokes and epilepsy (Barrenetxe *et al*, 2004, Hardeland, 2008). This function of melatonin is often seen as a non-classical action of melatonin. Melatonin has been found to occur in the retina, GIT and skin, however the functions within these organs are poorly understood (Hardeland, 2008). Melatonin may be synthesised or absorbed by these organs and then secreted and act to assist in formation of peripheral circadian rhythms. It is important to note however, that in some organs there is little secretion of melatonin, supporting the postulation that it must have other functions. Some functions of peripheral melatonin are believed to play roles in lipogenesis, glucose homeostasis and modulation of the neuropeptides- leptine and ghrelin (Barrenetxe *et al*, 2004, Hardeland *et al*, 2012).

Histamine is a chemical mediator in the periphery and is involved in widespread innervation of the CNS (Van Der Goot and Timmerman, 2000, Oguri and Yoneya, 2002, Lieberman, 2011). Histamine binds to four metabotropic receptors, $H_{1\text{-}4,}$ and is believed to have an ionotropic receptor in the CNS but is postulated to be a gamma-amino butyric acid (GABA) receptor with a histamine sensitive site (Haas *et al*, 2008). Histamine receptors are located in numerous parts of the CNS, and serve a range of functions linked to thermo-regulation, immune regulation, feeding, memory and learning to name a few (Haas *et al*, 2008). Histamine acts on parasympathetic nerve fibres of the peripheral nervous system (PNS) and is believed to modulate neuronal activity, control sympatho-adrenal outflow and also to act as a pruritogen, causing an itch response (Oguri and Yoneya, 2002). Non-neuronal functions of histamine elicit numerous effects throughout the human body, including in the bone marrow, leukocytes, cardiovascular system, skin and respiratory tract (Batmanghelidj, 1990, Van Der Goot and Timmerman, 2000, Haas *et al*, 2008, Lieberman, 2011). The H_1 receptors are located in blood vessels and the sensory nerves in the GIT, cardiac tissue and endothelium. Activation of these receptors results in increased vascular permeability, sensory stimulation, eosinophil chemotaxis and smooth muscle contraction in the GIT and airways (Lieberman, 2011). The H_2 receptor is located in epithelial cells, cardiac cells, mucosal cells of the stomach, uterus and smooth muscle cells. Activation of the H_2 receptor results in stimulation of mucous glands in the airways, increasing vascular permeability, chronotropic and ionotropic effects on the heart and gastric acid secretion. H_3 receptors are located in the airways, GIT and sympathetic adrenergic nerves. Activation of these receptors causes suppression of noradrenaline and nasal mucosal gland secretion. H_4 receptors are located on a variety of white blood cells, bone marrow, spleen and liver. The activation of these receptors results in chemotaxis of white blood cells and up-regulation of adhesion molecule secretion (Haas *et al*, 2008).

1.4 Pharmacological use of biogenic amines

Biogenic amines have numerous applications in treating disease states. An example of this is the use of adrenaline for its agonistic action on β-receptors to relieve symptoms of anaphylactic shock. Adrenaline may also be of use during emergency cardiac cases due to its positive ionotropic and chronotropic effects. Adrenergic BA can be used to treat low blood pressure. Catechol BA may also be used in clinical shock. Adrenaline is a common additive in local anaesthetics to cause vasoconstriction to increase the effective duration of local anaesthetics (Bertil and Goldstein, 1988). Biogenic amines can be used as medication for the treatment of diseases that are caused by a deficiency of those BA. An example is levodopa, a dopamine precursor, can be used as a treatment for Parkinson's disease, which is characterised by a lack of dopamine in specific areas of the CNS (Cools *et al*, 2007, Kurian *et al*, 2011). Depression brought about by serotonergic or adrenergic deficiency can be treated using serotonin or noradrenaline as targets of treatment. Melatonin can also be ingested from exogenous sources, serving to reset and form circadian rhythms and thereby aid people who have sleep disorders (Nakamura *et al*, 1997).

1.5 Diseases states relating to biogenic amines

The biological effects of biogenic amines are widespread and have large impacts on the physiology of the human body, it thus follows that any alteration of the closely controlled levels within the CNS or periphery can cause a disease state. A single type or class of biogenic amine may have effects ranging from psychiatric to neurological or cardiac pathologies. Research indicates a large overlap between activity of different types of biogenic amines and related physiology, often obscuring the true underlying cause of a disease. This implies that a disease may be associated with altered levels or receptor activity of more than one biogenic amine. Diseased states can be brought about by altering the metabolism, synthesis and/or action of one or more of the biogenic amines. Conversely the disease state may bring about a change in the levels of the various biogenic amines. In such a case the altered levels are rather a result of the disease itself which alters the normal synthesis, metabolism and/or action of these compounds. Temporary disease states may be brought about by introduction of the biogenic amine into the body either intentionally or unintentionally through treatment of a patient or ingestion of contaminated consumables.

Levels of the catecholamines may be altered in psychiatric, neurologic and cardiac pathologies. Depression is commonly associated with decreased levels of noradrenaline within the brain, and dopamine has also been implicated in the pathology of depression (Bertil and Goldstein, 1988, Srinivasan *et al*, 2005, Willner *et al*, 2013).

Dopamine is an important role player in addiction and risk-reward systems, with excess levels of dopamine thought to be closely linked to activities such as gambling (Missale *et al*, 1998, Grant *et al*, 2006). Schizophrenia has demonstrated links to dopamine levels, while increased plasma dopamine levels are seen in acutely psychotic patients. Changes in dopamine metabolites have also been associated with changes in schizophrenic symptoms (Bertil and Goldstein, 1988, Davis, 1989, Cai *et al*, 2011a). Dopamine hyperactivity results in anxiety and in addition dopaminergic pathways have been implicated in the behavioural and psychological signs and symptoms of dementia (Vermeiren *et al*, 2013). Dopamine is perhaps most commonly associated with its link to Parkinson's disease, where decreased levels of this amine in specific areas of the CNS are linked to Parkinson's disease symptoms (Davis, 1989, Medina *et al*, 2003).

Post-mortem samples from patients suffering from Alzheimer's disease have been shown to have decreased levels of noradrenaline and serotonin, with possible reduction in dopamine (Davis, 1989, Medina *et al*, 2003). The plasma levels of the catecholamines may be decreased resulting in sympatho-adrenomedullary deficiency disorders such as Addison's disease or glucocorticoid deficiency (Goldstein *et al*, 2003). Alternatively elevated plasma levels of catecholamines can arise from pheochromocytomas which secrete catecholamines in an unregulated fashion. This can result in hypoglycaemia, hypertension, cardiac palpitations and tachycardia (Golestein, 1983, Bertil and Goldstein, 1988, De Jong *et al*, 2011).

The numerous effects serotonin has are often poorly understood and research may be deemed conflicting. Serotonin is correlated to clinical manifestations of schizophrenia, (with decreased levels mediating negative symptoms in schizophrenia) (Medina *et al*, 2003, Cai *et al*, 2011a). Serotonin syndrome, which can arise from excess serotonin in the brain, results in dystonia and is potentially fatal (Kurian *et al*, 2011). Serotonin can be produced by carcinoids resulting in an excess of serotonin and subsequent symptoms such as flushing, diarrhoea and heart disease (De Jong *et al*, 2011). Serotonin is also the main biogenic amine linked to depression, marked by decreased levels of serotonin in the CNS (Medina *et al*, 2003, Jonnakuty and Gragnoli, 2008, Cools *et al*, 2011). Serotonin may also be decreased in patients suffering from addictions and impulsivity problems, however the relationship is not clear. Serotonin is also suggested to be implicated in eating disorders and may result in obesity. Abnormal serotonin secretion can result in behaviour disturbances, anxiety and migraines (Jonnakuty and Gragnoli, 2008).

Abnormal levels of plasma melatonin are related to fatal familial insomnia, thus highlighting the importance of the regulation of melatonin. Melatonin levels may be decreased in some forms of epilepsy and studies show that melatonin may display anticonvulsant action (Claustrat *et al*, 2005). Decreased melatonin is observed in depression and Alzheimer's disease but is increased during manic episodes in bipolar patients and may be increased in Parkinson's disease (Srinivasan *et al*, 2005). Plasma melatonin may be altered in hypothalamic tumours, with conflicting results implicating melatonin in schizophrenia and anorexia nervosa (Claustrat *et al*, 2005). Melatonin levels have also been found to be increased in cases of trauma and strokes, perhaps acting as a free radical scavenger (Srinivasan *et al*, 2005).

Histamine dysfunction has not been specifically or selectively related to a disease state. Histamine is a major neurotransmitter regulating the wake cycle and can play a role in sleep disorders (Haas *et al*, 2008). Histamine's effects on the reward systems of the brain can result in compulsive eating, hypertension and cardiovascular dysfunction associated with metabolic syndromes. Histamine release can result in potentially fatal anaphylaxis and levels of histamine are commonly altered in diseases relating to inflammation like atherosclerosis, encephalopathy and neurogenic inflammations. It is also increased in selective areas of the CNS where decreased H_2 receptors are implicated in Huntington's disease. Histamine or histamine receptors in the CNS can also be implicated in schizophrenia, depression, dementia, epilepsy as well as addiction and compulsions (Haas *et al*, 2008). Histamine ingestion can cause "scromboid poisoning" when ingested from contaminated food sources, especially fish (Self *et al*, 2011, Fda, 2014a, Fda, 2014b).

The importance of biogenic amines is easily appreciated when looking at the number of disease states and the known physiologic effects that are related to biogenic amines. These factors become important in a clinical setting when accurately quantitated levels of biogenic amines can be used for the diagnosis of a disease. The current knowledge of biogenic amine

functions and their importance in maintaining homeostasis is increasing with new research findings adding information continually. The ability of biogenic amines, especially catecholamines, to exert sub-second responses in an individual is also an area of interest as the release of large amounts of adrenaline happens almost instantaneously in moments of stress.

1.6 Other factors relating to biogenic amines

The levels of biogenic amines can be altered in response to disease or can result in disease pathology itself. Other factors exist, however, which may alter the levels of these compounds. The multi-factorial nature of biogenic amines is remarkable and their effects are numerous, which can complicate the use of these compounds in clinical settings.

One of the most important factors influencing biogenic amines is circadian rhythms. Melatonin is implicated in control of these rhythms as the synchronous secretion of melatonin results in the sleep-wake cycle. Thus when testing melatonin levels at night, plasma levels are naturally higher than in the mid-afternoon. Other biogenic amines such as serotonin and the catecholamines also exhibit variations which can be related to rest and activity, feeding patterns and other habitual activities.

A review by Davis in 1989 showed that although some inconsistencies exist there is evidence to show that increasing age has a tendency to increase the concentration of the biogenic amines (Davis, 1989, De Jong *et al*, 2011). In the same paper it was found that males tend to have higher urinary excretion of BA metabolites than in females, but when adjusted for total urinary output these differences seem to be minimal. This difference was postulated to be due to weight and total daily excretion differences between the genders. CSF levels of biogenic amines were found to be higher in females but this may be due to an average height difference. It was also noted that in females, a serotonin metabolite was affected by body size but not in males. A trend of increasing urinary norepinephrine excretion with increasing weight has been reported (Davis, 1989). The author stated that there may be different concentration gradients of the analytes and sampling sites must be standardised for meaningful results. Excluding these factors, many of the BA levels demonstrate differences between individuals.

Another important factor that affects the BA levels found in human fluids is genetics. There are various genetic related diseases which exhibit altered BA levels, further indicating that there are numerous biological control mechanisms directly influenced by genotype. The genes encoding for numerous enzymes involved in the metabolism or synthesis of biogenic amines can be different between individuals and may result in large differences in biogenic amines levels.

The levels of biogenic amines may also be influenced by environmental factors. Diets that are rich in amino acids that are precursors may force feedback mechanisms to increase levels of the related biogenic amine. In the review article by Davis, (1989) differences in

biogenic amines levels were found after consumption of foods high in caffeine and protein. It was also noted that smoking, alcohol, exercise, stress, ambient temperature and even the method of sampling can all alter the measured levels of biogenic amines (De Jong *et al*, 2011).

Although the results reviewed in that paper were not collected using advanced methods in use today, differences could still be detected between individuals and even in the same individual as the biogenic amines responded to influencing factors. This highlights the multitude of factors which may need to be taken into account when analysing biogenic amines from samples acquired from humans.

1.7 Clinical uses of biogenic amines

Analysis of biogenic amines have a wide range of uses in a clinical setting from food safety testing to the use of biomarkers in various disease states. Biogenic amines are quantitated from different complex matrixes such as food, urine, plasma and cerebrospinal fluid (Bourcier *et al*, 2006, Chia and Huang, 2006, Latorre-Moratalla *et al*, 2009, Vermeiren *et al*, 2013).

Microbes are able to convert amino acids to biogenic amines via decarboxylation reactions, which in turn is able to provide a biomarker for the quality of food (Self *et al*, 2011, Önal *et al*, 2013). Biogenic amines are seen as indicators of putrefaction in foods (Hernández-Cassou and Saurina, 2011). At low concentrations biogenic amines have physiological functions but when ingested at high quantities can cause acute illness in an individual. Histamine is a primary chemical indicator of decomposition and has an established limit set by the Food and Drug Administration, and is the only biogenic amine to have such a limit (Slavíková *et al*, 2007, Self *et al*, 2011). Due to the adverse effects of ingesting biogenic amines numerous studies have been undertaken to quantitate those levels to ensure the safe consumption of food products. A review article by Onal, et al, (2011), reviewed numerous articles in which biogenic amines were quantitated in various food stuffs such as wine, cheese, fish, sausage, beer, honey, milk and canned tuna, highlighting the applicability of using biogenic amines in the quality control testing of consumables (Önal *et al*, 2013).

Biogenic amines may also be used in the diagnosis of neuroendocrine tumours. Pheochromocytomas and carcinoids elevate the levels of biogenic amines, and this can be used to aid in the diagnosis of the disease. The tests for pheochromocytomas are based on the levels of catecholamines and their metabolites in plasma and urine (De Jong *et al*, 2011). When testing for carcinoids, urinary 5-hydroxyindoleacetic acid, a primary serotonin metabolite, is used for the diagnosis of carcinoid tumours.

Catecholamines are used when evaluating new drug treatments for diseases such as diabetes, heart disease, pain, anxiety and neurological disorders (Parker *et al*, 1995, Slavíková *et al*, 2007, Flaa *et al*, 2008, Mravec *et al*, 2008, Cotella *et al*, 2009). The

catecholamines are also used in monitoring of the hemodynamic function of patients in intensive care (Ji *et al*, 2010).

Biogenic amine levels when altered, either resulting in a disease or as the result of a disease, may also have the potential to be used as biomarkers to aid in the diagnosis of the disease. The large redundancy in the functions, effects and effectors of biogenic amines means that a diagnosis cannot be conclusively made from the quantitation of these compounds alone but can be used by clinicians to aid in the diagnosis of diseases. Biogenic amines could also be used in monitoring the progress or prognosis of a disease, as different levels can be indicative of the current state of disease. The clinical importance of these compounds can be far reaching, however the challenging analytical aspects need to be addressed and overcome prior to the exploration of these compounds as biomarkers of disease.

1.8 Clinical and analytical challenges to accurate quantitation of biogenic amines

The quantitation of biogenic amines can be highly problematic owing to a number of factors. They are small, polar compounds which can make the separation of these compounds challenging when using reverse phase chromatography. The selection of the chromatographic column is important as some specialised reverse phase columns can separate selected classes of these compounds but can add costs to the method. Another factor that can affect the separation of these compounds is the highly similar structures between compounds in the same sub-group and between the sub-groups. This means that isocratic chromatography may not provide efficient separation, but is required for certain quantitative methods and is problematic when quantitating a number of different biogenic amines simultaneously.

The low physiologic concentration of biogenic amines, sub-ng/ml range, means that a highly sensitive method is required to accurately quantitate the compounds (Ji *et al*, 2010). This problem is compounded further when working with low sample volumes which means the absolute quantities of BA in the total sample are low. The method may also require time consuming and expensive sample preparation before analysis in order to achieve the high level of sensitivity required to quantitate the low concentrations in physiological systems.

The matrix in which biogenic amines are commonly tested is often complex (Latorre-Moratalla *et al*, 2009). Aqueous matrixes such as plasma, urine and CSF are the most common samples tested and usually contain interfering compounds such as protein and lipids (Latorre-Moratalla *et al*, 2009). This can make the extraction and sample preparation of these compounds time consuming. It also means that these compounds require efficient separation and retention away from interfering compounds which can cause further problems with detection.

Biogenic amines are also susceptible to interference from many drugs and their metabolites (Ji *et al*, 2010). Thus they need to be separated from each other and any interfering compounds. The small molecule nature of the biogenic amines means that with mass spectrometric detection they lie within the range of abundant low molecular weight contaminants which can in turn decrease the sensitivity of the mass spectrometric detection. The catecholamine group of adrenaline and noradrenaline are readily oxidised, thus further decreasing the amount of available catecholamines for quantitation (Pyo *et al*, 2006). They also do not show strong absorbance when using ultraviolet visible (UV) detection and require derivatisation when using UV and fluorescent detection. They are non-volatile and so will require derivatisation when using gas chromatography (GC) as a means of separation.

When using biogenic amines for the diagnosis of disease in a clinical setting clinicians will need to take into account the multi-factorial nature of biogenic amines. There are numerous parameters that can affect the levels of biogenic amines and when diagnosing a disease, these factors must be considered so that incorrect conclusions are not drawn. The levels may be elevated as part of their natural cycle or decreased due to the diet of an individual rather than in response to a disease. When using biogenic amines as a target for treatments of chronic diseases (either by introduction of the biogenic amine or by altering the metabolism or synthesis) the underlying pharmacogenetics would play a role in patient responses to treatment. A patient may have defective genes and a resultant alteration in physiological function. This in turn may cause treatment failure. Biogenic amines may also spike in patients during the blood sampling process which can cause high levels of catecholamines to be measured due to a rapid stress response by the body rather than a disease.

Determining whether a certain BA can be used as a biomarker of a disease requires both an analytical method that can accurately determine the levels in the chosen sample as well as the consideration of the many physiological parameters that can alter the measured BA levels. Both clinicians and scientists need to be aware of the many challenges that biogenic amines can pose to the diagnosis or treatment of diseases and to the development of analytical methods used to quantitate these compounds.

1.9 Sampling Methods and Considerations

Common matrices sampled for BA analysis include urine, CSF and venous blood samples. These often involve large volume samples and in the case of venous blood sampling may induce a rapid spike in catecholamine levels. The aim of this study was to increase the sensitivity of the assay to be able to use to use small sample volumes and investigate dry blood spot sampling as a sampling technique if the necessary sensitivity was achieved.

Dry blood spot sampling (DBSS), is a sampling technique in which a simple, minimally invasive finger or ear prick test is done and a small volume of capillary blood is spotted onto

a special filter paper based collection card. The collection card has a variety of properties allowing for the extended stability of the compounds. It provides an easy way to store the samples and air drying the card results in the inactivation of some major pathogens such as HIV. The collection method of the sample is also simple, allowing it to be performed at home by the patient. The ease of collection means this method and can be used when multiple samples need to be taken over a certain time frame such as in therapeutic drug monitoring or the monitoring of concentrations after dose changes, with the added benefit of minimal risk to the patient (Keevil, 2011). This saves both time and cost to the patient and the hospital.

Despite the advantages of using DBSS the method has not gained widespread acceptance in clinical laboratories, except in new born screening laboratories, where larger blood volumes cannot be collected from the new-born infants (Keevil, 2011). Hospitals and laboratories often avoid use of DBSS due to drawbacks related to the type of filter paper used and the requirement of very sensitive analytical methods. This is mainly due to low volumes of sample collected and because serum is generally used for many automated test procedures (Abdulrazzaq and Ibrahim, 2001). Keevil proposed that the use of DBSS may reduce sample integrity, however a study by Burns *et al* claims that DBSS has the ability to maintain sample integrity when working with large molecules (Keevil, 2011, Burns *et al*, 2013). Other limitations include the inability to run multiple assays from a sample while this can be performed when using venous blood due to the higher volumes collected and the common use of automated methods that have been developed using venous plasma or serum. The revalidation of these methods will be time consuming, expensive and may require overhauling of instruments and established routine practices (Keevil, 2011). These changes are often deemed to be unnecessary and to create extra cost implications for validation prerequisites.

There are many factors to consider when using DBSS. These include factors which can change the composition of the blood during the sampling process. Firstly, trauma during sample collection can result in the release of interstitial and intracellular fluids and other chemicals (Keevil, 2011). Applied pressure and squeezing to extract larger amounts of the sample can also influence the levels of the analyte in question. Due to the potential differences in the concentrations, comparative testing and validation needs to be carried out to ensure the method is accurate. Haematocrit may affect the recovery, this is because the viscosity of the blood can affect blood distribution on the collection card and thus may alter detected levels (Abdulrazzaq and Ibrahim, 2001, Holub *et al*, 2006, Keevil, 2011). Another factor that can alter levels is the effect of lysed and intact red blood cells. In some cases lysed red blood cells lead to higher levels of the analyte being measured. Capillary blood also differs to venous blood and is reported to be more similar to arterial blood. In addition differences in levels of certain constituents in capillary blood have been found (Keevil, 2011). Prior to analysis portions of the collected sample need to be punched from the collection card, in the form of small discs, and the analytes eluted from these discs.

Once the analyte is eluted it can be analysed by a variety of methods. One major drawback to using dry blood samples is the small sample size collected and the subsequent selection of only a portion of the collected sample. Due to this limited sample size the analytes of interest are present in extremely small absolute quantities. DBSS thus requires both sensitive equipment as well as a validated method, which may initially increase the costs of using DBSS – both monetarily and in terms of time consumed.

There is a recognised standard procedure for DBSS: (CLSI):LA4-A4 (Keevil, 2011). This outlines the correct procedure to minimise differences due to sampling procedures and external influences on the levels of biogenic amines in the sample are reduced. These include eliminating factors such as squeezing and pressure. The general process is that a drop should be applied to the printed side of the filter paper card and enough blood allowed to fill and saturate the pre-printed circle without the filter paper coming directly into contact with the puncture site. The circle should be filled with a single application of blood. The blood should be uniformly distributed throughout the pre-printed circle. The cards are air dried at ambient temperature for 3 hours and must be kept out of direct sunlight (Keevil, 2011).

There are numerous advantages to both traditional sampling techniques as well as DBSS. This study aimed to assess both sample collection methods to determine which would be the superior method. A major drawback to DBSS is the extremely low sample volumes collected – approximately 30 µl with approximately 10 µl of plasma being analysed. Another drawback is often the sample collection does not meet the sample collection criteria which renders numerous samples unusable. Traditional venous sampling collects larger volumes of blood when compared to DBSS. The aims of this study are to develop a method which using less than 250 µl of plasma for the analysis of multiple biogenic amines from a single sample.

1.10 Current analytical methods used for detecting biogenic amines

Biogenic amines have been quantitated for many years and the modern analytical methods used today have improved markedly. Methods that are currently in use often have the same pitfalls: time-consuming pre-treatment, long run times, use of complicated derivatisation procedures, large sample quantities required, low sensitivity and specificity as well as issues with robustness (Davis, 1989, Bourcier *et al*, 2006, De Jong *et al*, 2011, Wang *et al*, 2011, Romero-González *et al*, 2012, You *et al*, 2012). There have been numerous review articles written highlighting differences between the sample preparation and analysis methods applied to determine the concentrations of biogenic amines in food and human fluids. The most common method of determining biogenic amines is through high pressure liquid chromatography (HPLC) coupled to UV, fluorescent and electrochemical detection (ECD) (Önal, 2007, De Jong *et al*, 2011, Önal *et al*, 2013). The literature indicates a shifting trend in this pattern as mass spectrometric methods become increasingly more accessible and popular.

1.10.1 UV, fluorometric and electrochemical detection

UV detection is based on a principle of passing a beam of light at a specific wavelength through a liquid. The detector constantly measures the intensity of a selected wavelength light that reaches the detector while the analyte absorbs the light in a concentration dependant manner, reducing the detector measured light. The use of UV detection is declining as a form of detection for biogenic amines due to poor sensitivity and lack of selectivity.

A recent paper used capillary electrophoresis (CE) and UV absorbance for the determination of aliphatic amines (Felhofer *et al*, 2013). This method used a derivatisation procedure using an N-hydroxysuccinimide (NHS) ester that was produced by the authors. The authors wanted to quantitate 5 primary aliphatic amines. They achieved a level of detection (LOD) between 16 and 140 µg/ ml. Another report analysed levels of biogenic amines and amino acids in wine and beer samples (Gómez-Alonso *et al*, 2007). In this paper they used diethylethoxymethylenemalonate to form aminoenone derivatives and used reverse phase (RP) HPLC to separate the biogenic amines. The authors achieved LOD below 0.06 mg/L for all the biogenic amines, which was acceptable for the application, but is insufficient for biological samples.

The major drawbacks to using UV and spectrophotometry as detection is non-selectivity and poor sensitivity for detection and quantitation of biogenic amines in biological samples. UV detection is however a straightforward and simple method that can be applied to a wide range of samples.

Fluorescence detection is based on the principle of exciting a fluorophore at a specific wavelength and detecting the light that is emitted at a different wavelength. Fluorescent detection is a highly popular choice used for quantitating biogenic amines. Biogenic amines require derivatisation with a fluorophore in order to confer fluorescent properties. There are a wide variety of fluorescent derivatising agents that can be used with biogenic amines.

A paper published in 2013 analysed 3 biogenic amines along with various amino acids (Vermeiren *et al*, 2013). An N-hydroxysuccinimide ester was used for the derivatisation process. The authors were able to obtain detection limits of 50 pmol/L, which is approximately 8.5 ng/L depending on the biogenic amine, demonstrating high sensitivity. Another report using an N-hydroxysuccinimide ester, with a different fluorophore was able to achieve a reported LLOD of 0.4 nMol, or roughly 44 ng/ml (Guihen *et al*, 2011). Determination of histamine levels in human patients with psoriatic plaques and a LOD of 0.4 nM, which is roughly 44 ng/L for histamine. The detection of seven biogenic amines in fish flesh was achieved by using CE and fluorescent detection (Zhang *et al*, 2008). In this paper, the authors synthesised their own derivatising agent- 3-(4-fluorobenzoyl)-2-quinolinecarboxaldehyde. The authors were able to achieve linear ranges between 0.005 and 10 µM. A method for the detection of histamine and histidine was developed using an

N-hydroxysuccinimide ester (Yoshida *et al*, 2004). A LOD of 2.3 and 3.8 fmol per 5 µL injection volume for histamine and histidine respectively was achieved. To simultaneously determine the levels of four polyamines in beer and wine the author also used an N-hydroxysuccinimide ester (Latorre-Moratalla *et al*, 2009). The method developed was able to achieve linearity between 0.2 and 50 µM. Another paper, regarding the health and safety risks after consuming contaminated foods, quantitated 12 biogenic amines (Latorre-Moratalla *et al*, 2009). In this paper the authors used *o*-pthaldehyde (OPA) as a derivatising agent. The authors were able to achieve LOD of 0.05 mg/L for the majority of the biogenic amines with the least sensitive LOD at 0.3 mg/ L.

Three of the authors mentioned above used ultra-performance (pressure) liquid chromatography (UPLC) (Latorre-Moratalla *et al*, 2009, Guihen *et al*, 2011, Vermeiren *et al*, 2013). One paper was based on the use of capillary electrophoresis (Zhang *et al*, 2008). Two papers were based on the use of HPLC (Lozanov *et al*, 2003, Yoshida *et al*, 2004). Five of the papers were based on RPC separation using C18 bonded phase (Lozanov *et al*, 2003, Zhang *et al*, 2008, Latorre-Moratalla *et al*, 2009, Guihen *et al*, 2011, An *et al*, 2015). The authors of one report used capillary electrophoresis, CE, for separation (Yoshida *et al*, 2004). All of the papers used derivatisation commonly using a N-hydroxysuccinimide ester (Lozanov *et al*, 2003, Yoshida *et al*, 2004, Guihen *et al*, 2011, Vermeiren *et al*, 2013). One paper used a compound synthesised by the authors, which consisted of a N-hydroxysuccinimide ester coupled to a fluorescent pyrene reagent, while one used OPA as a derivatising agent (Yoshida *et al*, 2004, Latorre-Moratalla *et al*, 2009). The two review articles also found most methods were based on RPC after derivatisation (Önal, 2007, Önal *et al*, 2013). The sensitivity of the methods varied greatly from 8.5 ng/L to 0.3 mg/L.

Fluorescent detection is sensitive, however it requires time consuming derivatisation with expensive fluorophores. The reagents used in derivatisation also have shortcomings such as short detection wavelengths, poor stability and sample matrix interferences in biological samples as well as compatibility issues with the analytes (Zhang *et al*, 2008, You *et al*, 2012). This method is also less selective than mass spectrometric methods and can result in poor reproducibility especially when analytes co-elute or elute during periods of contamination. Derivatisation can cause contamination, especially when working with non-specific, highly reactive reagents. These will react with the numerous contaminants which may cause co-elution. Thus the method must also be highly specific to avoid contamination and increase reproducibility.

Electrochemical detection (ECD) works by using the innate oxidative susceptibility of biogenic amines. This process takes place by measuring the electrical current generated by the oxidation reaction. The use of ECD is still highly popular as it is an extremely sensitive method. There are numerous papers using ECD to determine biogenic amines. Two recent papers determined biogenic amines in foods (Fiddes *et al*, 2014, An *et al*, 2015). Using CE the authors separated the biogenic amines found in oysters (An *et al*, 2015). The authors did

not use a derivatisation procedure in this study and were able to achieve a LOD of 0.01 mg/L for two of five BA compounds. The rest of the compounds had LODs of 0.1 mg/L. One paper determined biogenic amines in human brain tissue (Van Dam *et al*, 2014). In order to quantitate 8 biogenic amines and their metabolites the authors of this study used Reverse phase ion pair (RP-IP) HPLC. There was no derivatisation procedure used and the authors were able to achieve LODs in the femtogram/mL range, and detect DOPAC at 40 femtogram/mL. An interesting approach to using ECD detection was coupled to the use of radio frequency identification tags (Fiddes *et al*, 2014). The authors coated conventional RFID tags with a composite that was chemically sensitive to biogenic amines. The coating would swell and increase the resistance of the film and thus be able to detect biogenic amines. Although the method was not very sensitive and could only detect volatile biogenic amines it can still be considered a highly innovative way to quantitate biogenic amines.

Electrochemical detectors are limited in their applications. This is because the compound being analysed must be electrochemically reactive to undergo the oxidation or reduction reaction necessary to generate the signal. Traditionally these detectors required isocratic elution methods, which limited the separating power of these methods, reducing the number of biogenic amines classes that could be analysed in a single run. This methodology can also not differentiate between families of biogenic amines. Thus method development can be time consuming to ensure the accurate quantitation as well as to ensure there is no interference during the detection window due to the lack of specificity of ECD detection. Certain ECD detectors, such as the UltiMate 3000 ECD-3000RS ECD, are gradient compatible which will increase the separating power, which may lead to increase use of ECD detection of biogenic amines (Bailey and Acworth, 2016, Zhang *et al*, 2016).

1.10.2 Enzyme-linked immunosorbent assay (ELISA)

An uncommon method used for the quantitation of biogenic amines is ELISA. ELISA uses primary antibodies that bind to and capture the biogenic amine, while a second labelled antibody is then added to quantitate what has been captured by the primary antibody. The second antibody carries an enzyme which acts to form a quantifiable change when the substrate for the enzyme is added.

A paper describing the detection of biogenic amines in tricyclic antidepressant patients was done using ELISAs as the method of quantitation (Walker *et al*, 2013). The authors obtained commercial ELISA kits and did not mention the LLODs. On searching for similar ELISA kits to those that the authors used, it was noted that the sensitivity of commercially available ELISA kits are in the low ng/mL (Immusmol, Rockymountaindiagnostics).

ELISA often suffers from cross reactivity and with the high LODs will require large samples of human plasma. These kits are also highly expensive and can increase costs dramatically. These kits also are limited in the number of analytes that can be analysed, limiting the use to one analyte per kit.

1.10.3 Mass spectrometry

Mass spectrometry is fast becoming the leading quantitative method for the assessment of numerous compounds. It can be coupled to UPLC, HPLC and GC for the initial separation of compounds to be analysed.

Gas-chromatography tandem mass spectrometry, GC-MS/MS, has high sensitivity combined with a strong separation power and good identification capability. GC-MS however has a major drawback in that only volatile compounds can be analysed. Another limitation of GC-MS for BA is non-specific adsorption resulting in significant peak-tailing and carry-over effects (Oguri and Yoneya, 2002). Furthermore, to overcome these problems requires extensive sample preparation to produce stable, volatile derivatised analytes which are fit for the separation and ionization techniques. The application of GC-MS for the analysis of BA has limitations and is a very specialised field (De Jong *et al*, 2011).

The use of LC-MS/MS to quantitate biogenic amines has had varying success; some methods have low pg/ml LODs whereas others are at µg/mL LODs but analyse only selected classes of the BA. The majority of reports have used derivatisation procedures which have a large positive impact on the chromatographic separation and show increased sensitivity for the BAs. The LODs of methods using derivatisation often have lower LODs, however some methods achieved LODs in the ng/ml range without using derivatisation prior to analysis, although these results are not often achieved.

In order to analyse and to quantitate catecholamine neurotransmitters in rat brain microdialysates, the authors reported the use of derivatisation and HPLC (Nirogi *et al*, 2013). By using dansyl chloride (DNSCl) as a derivatisation agent and a RP column, these authors were able to achieve limits of quantitation of 0.068 pmol/mL for dopamine and 0.059 pmol/mL for noradrenaline. To determine monoamines in human plasma, polyamines in red blood cells as well as biogenic amines in fermented food samples the authors used DNSCl and RP-U/HPLC. They were able to achieve LODs in the low pmol/mL range (Cai *et al*, 2009, Ducros *et al*, 2009, Jia *et al*, 2011). Catecholamine levels could be determined at the ng/mL range in urine by the use of RP-HPLC and derivatisation using chloroformate based derivatising agents (Pyo *et al*, 2006). Chloroformate based derivatising agents were utilised and achieved detection in the fmol range when using RP-HPLC separation (You *et al*, 2012).

To determine neurotransmitters in biological fluids RP-HPLC with no derivatisation procedure were able to achieve LODs of 1 nM (Bourcier *et al*, 2006). No derivatisation procedure were used for several quantitative methods with RP-U/HPLC separation (Hasegawa *et al*, 2006, Sanchez-Lopez *et al*, 2009, Self *et al*, 2011, Wang *et al*, 2011, Zhu *et al*, 2011, Romero-González *et al*, 2012, Sagratini *et al*, 2012). The majority of these papers achieved unremarkable sensitivity in the mg/kg or µg/kg range (Song *et al*, 2004, Sanchez-Lopez *et al*, 2009, Sagratini *et al*, 2012, You *et al*, 2012). However three papers reported

high sensitivity, one in the pg/mg range analysing brain tissue, another in the range of 0.019 ng/ml and the last in the ng/mL range (Self *et al*, 2011, Wang *et al*, 2011, Zhu *et al*, 2011). Derivatisation using 7-fluoro-4-nitrobenzoxadiazole, 4-bromobenzenesulfonyl chloride, 2,6-dimethyl-4-quinolinecarboxylic acid N-hydroxysuccinimide ester, pentafluorobenzaldehyde and D4-acetaldehyde have also been used (Song *et al*, 2004, Chia and Huang, 2006, Huang *et al*, 2009, Croyal *et al*, 2010, Ji *et al*, 2010). Derivatisation was used for analysis by capillary electrophoresis, another GC while the remainder used HPLC or UPLC and RPC. Three reports were able to achieve ng/mL LODs (Song *et al*, 2004, Chia and Huang, 2006, Ji *et al*, 2010). One paper achieved an LOD in the low pM range while the last paper was able to detect levels at 0.02 µMol/L (Huang *et al*, 2009, Croyal *et al*, 2010).

The drawbacks to using mass spectrometry as a form of detection vary between the different types of ionisation sources and the detectors. Some general drawbacks for MS detection include a lack of sensitivity for poorly ionising analytes, severe matrix effects that effect the sensitivity and soft-ionisation techniques prone to ion suppression.

1.11 Derivatisation- Process, advantages and drawbacks

Derivatisation is a chemical reaction in which an analyte of interest is altered by targeting a common chemical moiety in order to meet specific requirements of the analytical method being used. This alteration will add to the existing structure and convey the required properties to the analyte such as greater volatility, ionisability or lipophilicity. This is common practice in GC-MS analysis in order to create stable volatile analytes which can then be separated and analysed, but it is not routinely used in LC-MS due to the solubility of analytes in mobile phases and the wider compatibility of LC separation (Xu *et al*, 2010). Derivatisation is often used in instances when the analyte ionises poorly or is difficult to fragment such as in steroid analysis. This is still widely used to enhance the detectability of biogenic amines (Qi *et al*, 2014). It may also be performed to adjust the retention times on RP columns, or change the shape and symmetry of the peaks, increase the resolution or allow for chiral separation without the aid of a chiral column (Santa, 2011). There are numerous advantages to derivatisation and these include increase sensitivity, improved selectivity and separation, decreased endogenous interference and improved structural elucidation (Qi *et al*, 2014). These advantages are, in part, due to increasing the m/z ratio to above those of abundant, low molecular weight impurities. Derivatisation is used to change the analyte structure to enhance the nebulisation and ionisation by increasing the hydrophobicity (Song *et al*, 2004, Santa, 2011, Qi *et al*, 2014). Derivatisation may stabilise reactive compounds or improve the extraction efficiency and selectivity, if derivatisation is performed prior these steps (Qi *et al*, 2014). Figure 3 depicts a general derivatisation reaction.

Biogenic amines have a common amine group, whether it is aliphatic, aromatic or a primary or secondary amine. This amine group can be targeted by a derivatising reagent in order to

alter the analytes prior to analysis. The amine group will react by nucleophilic substitution and can react with many different derivatising agents in order to alter the analytes.

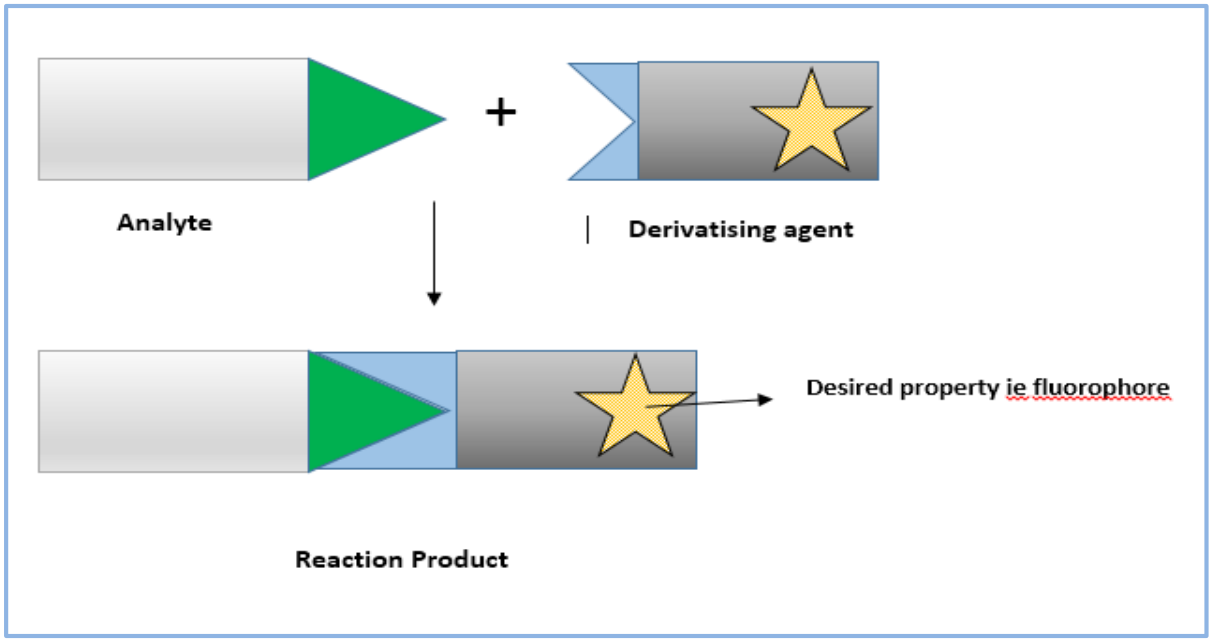


Figure 3: A schematic showing a general derivatisation procedure. The desired property can have different properties such as fluorometric, permanent charge or increased lipophilicity.

Popular choices when derivatising biogenic amines are N-hydroxysuccinimide esters, dansyl chloride and chloroformate based compounds. These compounds react with the amine group to yield amine derivatives.

Ethyl chloroformate is a highly reactive reagent and will react with primary or secondary amines but will also react with phenolic hydroxyl groups. The derivative which is formed is slightly more lipophilic than the precursor and also ionises readily. It is fairly reactive at room temperature and is an inexpensive reagent, however it is not highly selective and can react with a wide range of functional groups depending on the conditions used during the derivatisation reaction. It is also a highly exothermic reaction and can cause eruption of the solution upon addition of large volumes of derivatisation reagent. Figure 4 shows the reaction for an amine group derivatised with ethyl chloroformate.

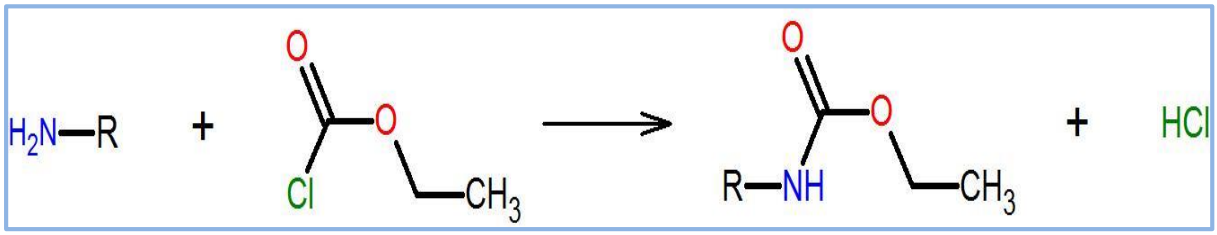


Figure 4: A schematic showing the derivatisation reaction of ethyl chloroformate with a primary amine group

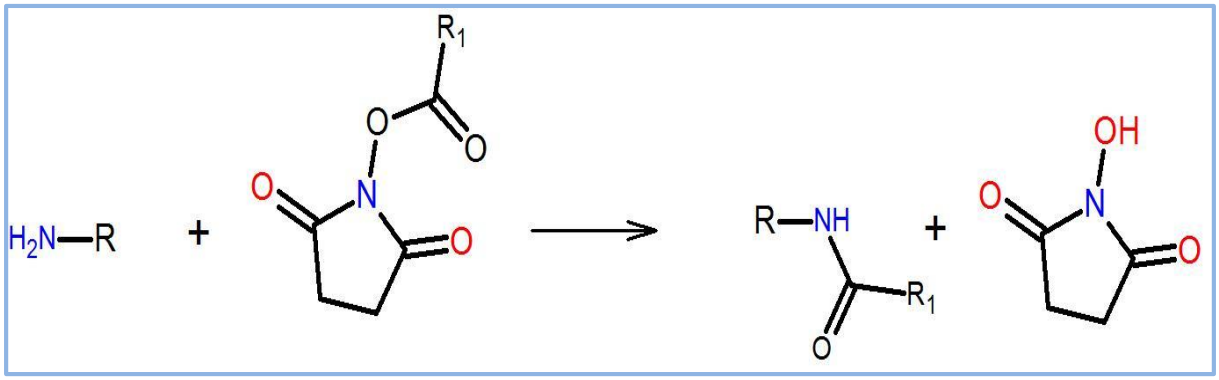


Figure 5: A general reaction schematic showing the derivatisation reaction of a primary amine using an N-hydroxysuccinimide ester. The R1 group can vary depending on the techniques used for analysis

N-hydroxysuccinimide esters are reactive esters that easily react with primary and secondary amines in a trans-esterification reaction reputed to be selective for amine moieties but can also react with other nucleophiles such as phenolic hydroxyl groups. When the NHS-ester reacts with a nucleophile the NHS group acts as a leaving group and forms an acylated amine product. The general reaction scheme is shown above in Figure 5. The R_1 group, as indicated, bonds to the nucleophile, conveying the required properties to the analyte. Using NHS as a leaving group allows for large versatility as the R_1 group can be altered according to the requirements or demands of the researcher. This R_1 group can have longer or shorter aliphatic chains to alter lipophilicity, have permanently charged moieties to increase sensitivity during MS detection or introduce volatile or fluorescent properties to the analyte. The NHS ester used for the derivatisation can be synthesised relatively easily under moderate reaction conditions while utilising a condensing reagent to allow for large customisability of the structure of the derivative by the researcher. NHS esters react readily under neutral pH and moderate temperatures. The main drawback when using NHS-esters are the fact that they require aprotic reaction conditions, as they can react with hydroxyl groups, and derivatives formed can be highly unstable under aqueous conditions, which may affect the sample preparation.

Dansyl chloride is a sulphonyl chloride that reacts with primary and secondary amines but can also react with phenolic hydroxyl groups, as depicted in Figure 6. Dansyl chloride reacts readily with amines at neutral or slight alkaline pH and moderate reaction conditions. A dansyl chloride group increases the lipophilicity of the analyte through the addition of the naphthyl group increasing the retention on RP columns while adding fluorescent properties for increased sensitivity with fluorescent detection. It also has a tertiary amine group that increases the ionisability for increased sensitivity with MS detection in positive ionisation mode. The drawbacks of DNSCl is that is requires aprotic reaction conditions as it will react with any available hydroxyl groups which decrease the availability of DNSCl for the analyte. This can affect the choice of sample preparation conditions, while derivatives show decreased stability under aqueous conditions. The derivatives are usually light sensitive and will degrade when exposed to UV light. When DNSCl is added to analytes with multiple

reactive groups the addition of more than one dansyl group may increase the lipophilicity by a large degree which may significantly increase the chromatographic run times when using RPC. The fragmentation pattern of dansyl derivatives are highly predictable due to the common fragmentation site, which can be advantageous when using tandem mass spectrometry. An interesting pattern that occurs when fragmenting dansyl derivatives in LC-MS/MS systems is that amine derivatives often form a common 170 m/z product ion and when fragmenting oxygen containing derivatives a common 171 m/z product ion is formed. This pattern seems to exist in many reports of dansylated derivatives analysed by MS. The amine derivatives appear to favour the formation of a radical product but the reasons why this occurs have not been determined but may be due to relative proton affinities of amine and hydroxyl groups.

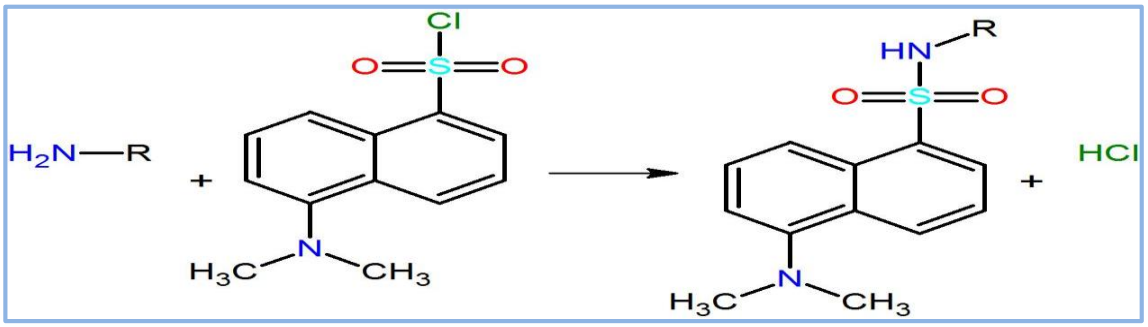


Figure 6: Reaction schematic showing the general derivatisation reaction of a primary amine with of dansyl chloride.

Another derivatisation procedure involves a complex-formation reaction using 2-aminoethyl diphenylborinate. In this reaction the 2-aminoethyl diphenylborinate will dissociate and a diphenyl borate anion will form a complex with the two adjacent catechol hydroxyl groups. This method is used to increase the lipophilicity of catechol compounds so that it can be separated on a C18 column. The complex will break down within the ionisation source of the mass spectrometer and allow the parent catechol class biogenic amine to be analysed by mass spectrometric detection (Griffin *et al*, 2011). This complexing reaction may be used prior to derivatisation with N-hydroxysuccinimide esters or dansyl chloride in order to eliminate the lack of specificity during derivatisation, and may allow for more specificity and selective amine only derivatisation. Although no publications could be found which used a combination of both of these derivatisation procedures it may still be a viable means to ensure that there is specificity for the amine moiety when derivatising the catecholamines. This method will not work with the indolamines and imidazoles which lack the adjacent phenolic hydroxyl groups.

A common challenge when derivatising biogenic amines is that the derivatising agents lack specificity for the amine group. This is because the majority of derivatising agents react by nucleophilic substitution and will react with phenolic hydroxyl groups almost as readily as with the primary amine group. This becomes problematic when derivatising the

catecholamines which possess two phenolic hydroxyl groups and one aliphatic amine group, thus yielding a total of three possible derivatives. This can result in different derivatisation patterns as the same moieties may not be derivatised in each instance. An example is where one amine and only one hydroxyl will be derivatised in one instance, however in another both hydroxyls may be derivatised. The different derivatives will have different lipophilicity and result in chromatographic separation and mass differences of these analytes. This means that not only the number of times the analyte has been derivatised must be taken into account but also the potential reactive sites which were derivatised are to be considered. This problem can be overcome by adding a large excess of the derivatising agents to force the reaction to completion of all possible reaction sites, and can increase the reproducibility of the derivatisation reaction. Another solution is by selection of a more specific derivatising agent, however due to the similar process and reactivity of the amines and phenolic hydroxyls a chance for cross reactivity between these groups will always exists. Other challenges include the formation of by-products, either as a result of the desired reaction or through unwanted side reactions. This may interfere with the analysis and consume the derivatising agent. Some derivatising reactions may require harsh conditions such as high pH, temperatures or pressures. Other issues with derivatisation are that some reactions require long reaction times and derivatisation products formed may not be stable, thus leading to degradation and loss of analytes (Xu *et al*, 2010).

Specific derivatisation reagents such as benzylamine and 1,2-diphenylethylenediamine have been used in studies (Fujino *et al*, 2003, Yoshitake *et al*, 2004). These reagents allow for specific single product formations of serotonin and catecholamine derivatives respectively and have been used successfully in reaching extremely high sensitivity limits – in the attomolar range. A major issue with these reagents is that it limits the number of BA classes that can be analysed in a single run. Benzylamine is specific for serotonin and similar metabolites but is unreactive toward catecholamines. The reduction in the number of analytes reduces the applicability when a broader explorative approach is required in the search for biomarkers but can be an excellent choice when those biomarkers are known and can be selectively derivatised or are limited to a single class of BA compound. Another approach is to combine numerous derivatising reagents. This is done in order to increase the number of analytes that can be analysed with more specific derivatising reagents – however care must be taken to ensure that the reaction conditions are amenable for all reagents, that the reagents are highly specific and that the reagents do not react with each other.

When choosing a derivatising reagent it is preferable that the reaction conditions are mild and proceed rapidly. It is also advantageous that the reaction is facile to ensure the highest possible sensitivity and that derivatives are stable enough to allow for further sample workup and analysis. The reaction process must also be predictable and reproducible with few side reactions to ensure the robustness of the assay. The derivatising agent in the case of clinical settings must preferably be commercially available and the application of the

procedure must be simple and rapid to allow for greater throughput. It is also preferential that the procedure allows for possible future automation.

When derivatisation is applied to analytes which will be analysed using LC-MS/MS the derivatising agent must readily ionise under soft ionisation conditions or add a permanently charged moiety. It must allow for separation of the analytes without sacrificing resolution or cause co-elution of analytes and the by-products of the reaction must not become a source of interference or lead to matrix effects. These requirements are to ensure the sensitivity and integrity of the assay. Derivatisation used in LC-MS/MS analysis allows for the analysis of compounds that may otherwise be unattainable or may increase the sensitivity of previously developed methods.

1.12 Principles of Liquid chromatography tandem mass spectrometry (LC-MS/MS)

Liquid chromatography is a technique in which the analytes of interest are separated from interfering compounds by a differential degree of association with a stationary phase while dissolved in a mobile liquid phase. It can be used prior to analyses to purify and concentrate the sample during sample pre-treatment or used for sample analysis where a variety of detectors can be used, with a tandem mass spectrometer the detector for LC-MS/MS analysis.

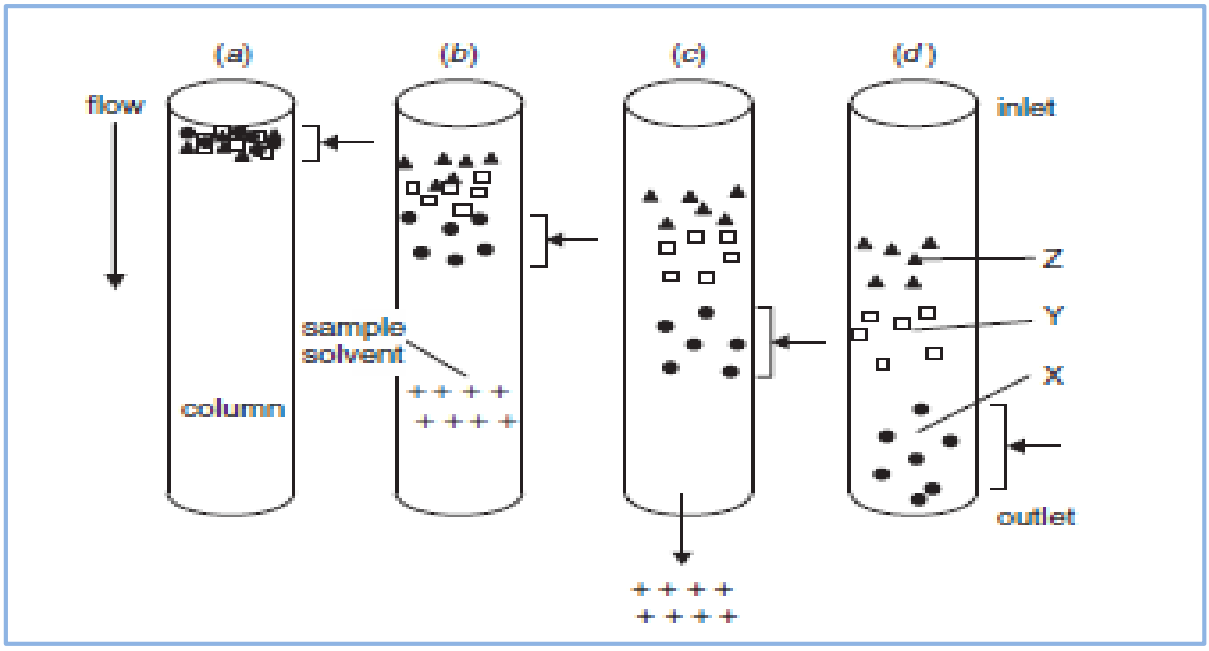


Figure 7: Diagram of the overall chromatographic process. (a-d) Shows sequential separation within the column at different times. (X-Z) represents different analytes of interest. (Snyder et al, 2010) Reproduced with permission.

The principle behind chromatography is based upon an analytes interaction between two phases- a stationary phase and a mobile phase. The stationary phase is used to retard the movement of the analyte using “like-like” interactions. The mobile phase also uses like-like interactions to influence the movement of the analyte through the stationary phase and movement through the stationary phase is retarded when the affinity of the analyte for the mobile phase is lower. The subtle differences in the interactions of the analytes between the two phases means that each analyte will travel through the stationary phase at different rates and will be separated from each other and arrive at the post column detector at different times from interfering compounds under optimal separating conditions. Figure 7 shows an overview of the chromatographic process.

HPLC is a technique in which a sample, along with a mobile phase, is pumped at high pressure through a column which is packed with a small particle size stationary phase. HPLC is often used to separate the analytes before it reaches a detector. It is a simple process which is primarily affected by the following three factors: the column efficiency, selectivity and the linear flow rate of the mobile phase. There are other factors such as additives to the solvents and temperature which may also affect the chromatographic process by changing selectivity. Figure 8 shows a schematic of a basic HPLC system which generally consists of an auto-sampler, pumps, a column and detector. The auto-sampler introduces the sample into a stream of solvent without changing the pressure or flow of the mobile phase. The system of pumps will carry the sample in the mobile phase through the column where separation takes place and then to the detector. The detector can be one of many types such as fluorescent, ECD or MS.

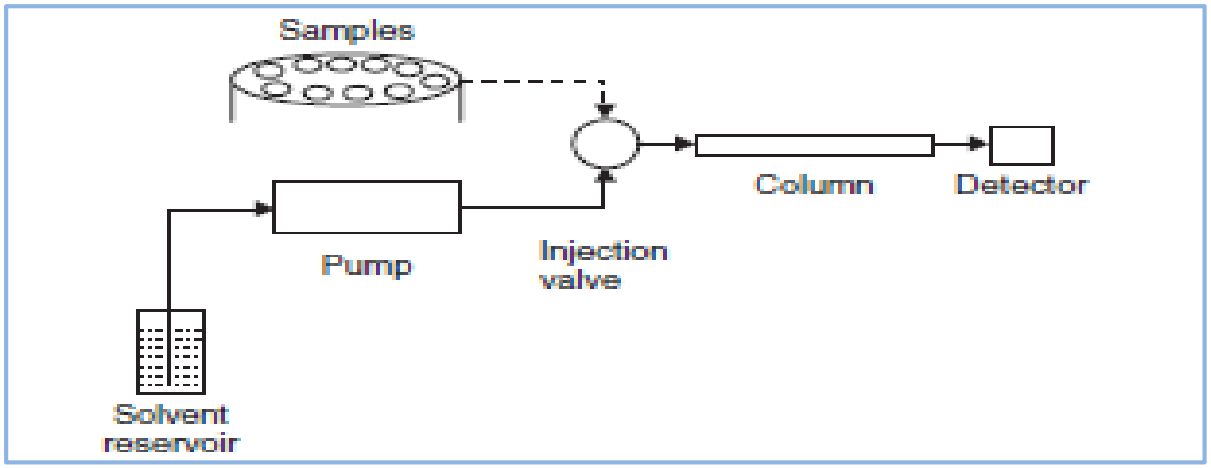


Figure 8: Diagram showing the typical setup of a basic HPLC system. (Snyder et al., 2010) Reproduced with permission.

A column is a cylindrical tube filled with small spherical particles. The column can be various lengths with 2 cm being the shortest to 30 cm columns as some of the longer columns. The diameter of the column also differs and can vary from sizes around 1 mm to larger than 4.6 mm. The column is filled with stationary phase particles ranging in sizes from 1.5 to 10 µm. The particles are often porous and are coated with a bonded phase which determines the

mode of separation. There are numerous column types and chemistries summarised in Table 2.The size of the particles in the columns determines the back pressure of the system and as the particle size decreases there is an increase in the pressure of the system. Particles 3 micron and larger generally used for HPLC. Smaller particle sizes are used for ultra-pressure liquid chromatography (UPLC). There are numerous advantages to using smaller particles and increasing pressure. This increases the speed, sensitivity and resolution of analytical separations. It also decreases solvent use and run times allowing for decreases in the cost of the methods used and increases the number of samples that can be run in a certain time frame. This increases the overall efficiency of analytical laboratories.

The mobile phase can be altered in order to retard or promote analyte movement through the stationary phase. This can be done by altering the solvents used that make up the mobile phase to be more or less eluotropic. This is to ensure that the compounds are retained for long enough that they are separated from interfering compounds and other analytes but still within a reasonable run time.

Table 2: Table summarising the different chromatographic modes available to researchers

Chromatographic modes

Reverse phase chromatography(RPC)	This stationary phase is non-polar and coated in aliphatic chains. Common lengths of these chains include C8 and C18. This is commonly used to separate lipophilic compounds.
Hydrophilic interaction chromatography (HILIC)	This stationary phase is polar using silica or amide, hydroxyl or cyano-bonding. Used to separate polar compounds in non-polar mobile phase .
Ion-exchange chromatography	Stationary phase contains discrete charges on the surface to capture oppositely charged analytes. A change in the pH or ionic strength of the mobile phase then releases these captured analytes.
Ion-pair chromatography	Uses reverse phase stationary phase and ionic lipids to create a dynamic ion-exchange layer.
Size-exclusion chromatography	Separation based on the sieving effect of the analytes with larger molecules eluting first.
Other	These columns can often combine two chemistries ie non-polar, to certain specific chemical moieties such as phenyl, amide, cyano groups to achieve separation that would otherwise be unobtainable.

There are numerous detectors than can be coupled to HPLC. These include UV, fluorescent, refractive index, evaporative light scattering detection, electrochemical detection and mass spectrometry. These detectors each have their benefits and drawbacks and selection of the correct detector is vital.

Mass spectrometry (MS) is based on the premise of a stream of analytes being introduced into an ionisation-source via any one of a variety of different separation techniques. These analytes then become charged and are sent through a mass analyser to a detector. There are a variety of ion sources and mass spectrometers available on the market, each with different capabilities and applications. Triple quadrupole systems, which have superior quantitation capabilities, are composed of three segments as shown in Figure 9. The first segment, or Q1, is a mass analyser. This enables the system to select a range of charged ions based on their mass-charge ratio, and will allow the passage of only the selected ions into the second segment. The second segment, Q2, is a collision cell where a voltage can be applied to a gas introduced into the flight path of the ions. This subsequently causes the selected ions from Q1 to fragment through collisionally induced fragmentation. The last segment, Q3, is a mass analyser which enables only selected ionised fragments from Q2 to travel to the detector. This enables a researcher to determine structures and precursor-product fragmentation patterns and aids in positive identification of known samples (as each analyte has specific fragmentation patterns). This system can allow for one, two or three segments to be simultaneously operational, thus enabling many different assay types to be performed at the same time. The advantages of triple quadrupole systems are a high mass range, high scan speeds and excellent quantitative analysis but are restricted to nominal mass resolution.

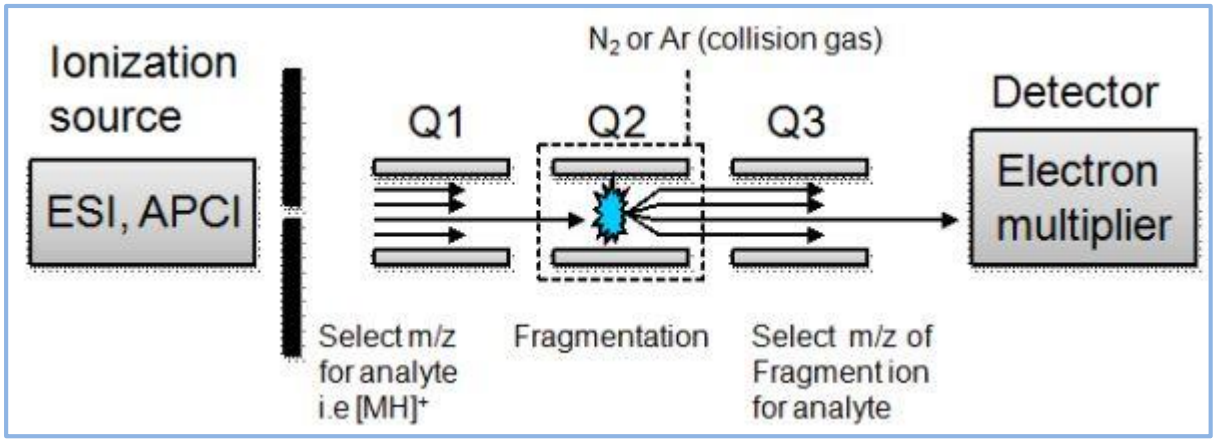


Figure 9: Diagram showing a schematic of a triple quadrupole mass spectrometer. (Paxton, 2012) © 2012 Ni J, Rowe J. Published in [Topics on Drug Metabolism, 2012, InTech under CC BY 3.0 license. Available from: http://dx.doi.org/10.5772/28525]

The ionisation-source is responsible for the ionisation of the analytes and as such can have a major impact on the overall sensitivity of the method. The ability of the ion-source to selectively ionise the analyte while being robust to matrix effects are important consideration when choosing the source. The source must also be compatible with upstream techniques such as HPLC. There are a variety of ion-sources and these differ in the way in which they ionise compounds and thus have varying efficiency in ionising the same compounds. Electrospray ionisation (ESI), is one of the most common sources used due to its sensitivity, wide range of applicability and reliability (Ho *et al*, 2003). Atmospheric pressure photoionisation (APPI), is less commonly used than ESI, but is still highly sensitive and is excellent at ionising low weight, non-polar compounds. Figure 10 shows the different characteristic of ionisation sources and their ability to ionise analytes depending on the molecular mass and polarity.

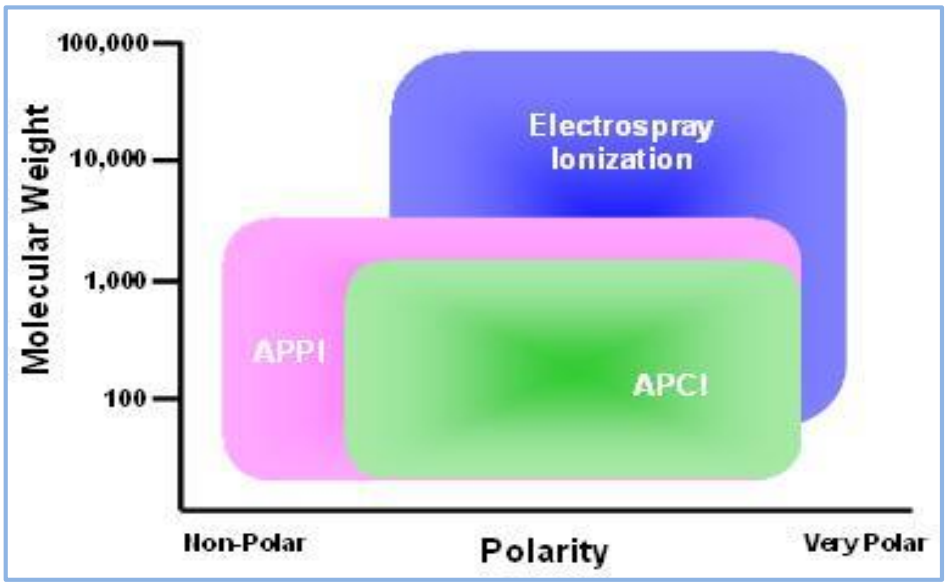


Figure 10: Optimal ionisation source characteristics relative to polarity and molecular weight of the analyte. APCI- Atmospheric pressure chemical ionisation. APPI- Atmospheric pressure photoionisation (Safranmorpho). Reproduced with permission.

In Figure 10 it can be seen that an APPI source is able to ionise non-polar analytes which are unable to be ionised by ESI, however ESI preferentially ionises polar, high weight compounds. The overall process of ESI ionisation is shown in Figure 11. ESI uses electrical energy in order to ionise the analyte. A charge is placed onto the surface of a liquid droplet and as the droplet decreases in size, the droplet becomes unstable and ions are ejected into the gaseous phase (Ho *et al*, 2003). The affinity for this charge differs between molecules and thus the ionisation efficiencies between various compounds differs.

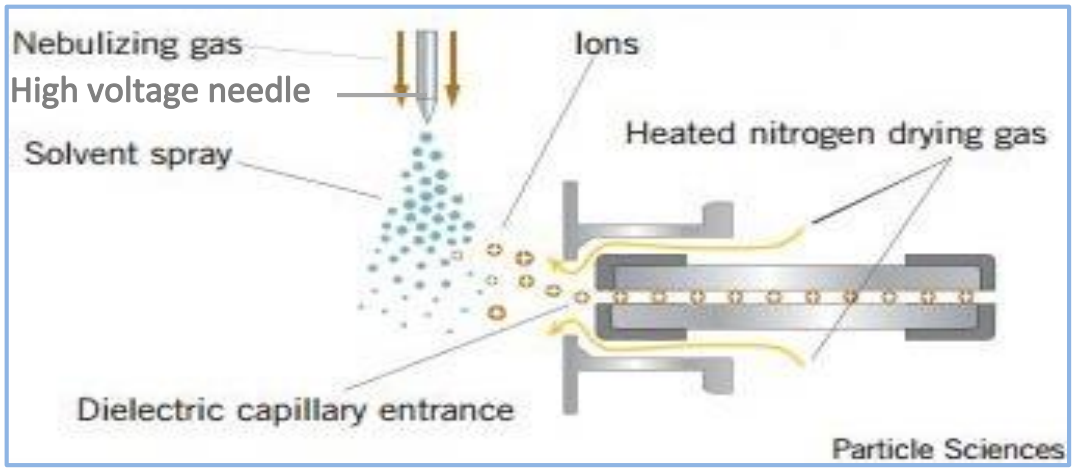


Figure 11: Diagram of the basic functioning of an electrospray ionisation source. (Particlesciences, 2009) All rights to this media is owned by, including the copyright license, Particle Sciences, Inc. This image is reproduced with permission from PSI Inc.

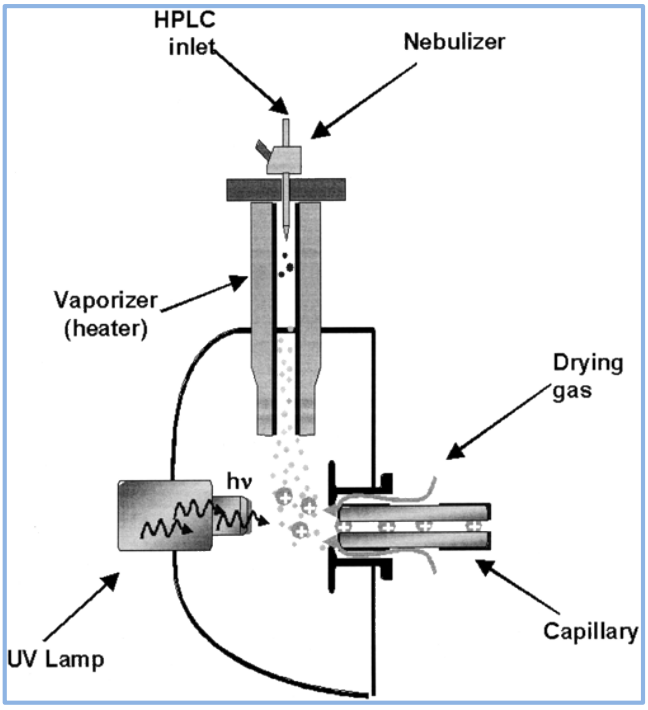


Figure 12: The basic functioning of an atmospheric pressure photoionisation source. Reprinted (adapted) with permission from (Hanold et al, 2004). Copyright (2016) American Chemical Society.

The overall process of APPI ionisation is shown in Figure 12. Photoionisation is based on the interaction of a photon beam with nebulised liquid containing a compound known as a dopant that can absorb the light energy. The dopant molecule absorbs the photon and enters an excited state. An electron can be released while in this excited state forming a charged cation. A dopant is required to improve the ionisation efficiency, and acts to transfer a proton to the analytes (Marchi *et al*, 2009). Theoretically ESI is more prone to matrix effects due to the competition for the charge by all molecules in the source whereas APPI functions differently and is less prone to matrix effects (Safranmorpho). APPI dopants with different proton affinity relative to the analytes proton affinity can be used to manipulate the selectivity to ionise the analyte. Manipulation of various source conditions allows for increased sensitivity due to higher ionisation efficiencies.

There are many other types of mass analysers such as ion traps, ion cyclotron resonance and time-of-flight. They all detect compounds based on their mass/charge ratios but have different applications due to different capabilities such as higher mass resolution, faster analysis times and wider mass ranges which allow for specific or niche applications. These mass analysers can be coupled to quadrupole based systems to combine the strengths of the two mass analysers, such as quadrupole-Time of Flight (QTOF) mass spectrometers.

Time-of-flight (TOF) mass spectrometers operate by pushing ions into a defined length drift path that has a strong voltage gradient. The ions will become resolved in time before reaching the detector based on the actual mass to charge ratio. Ions start at the same point and time and will arrive at the detector at different times, with heavier ions arriving later. This difference in time to reach the detector allows for the masses of the ions to be determined. The TOF chamber can be coupled down line from a quadrupole, thereby coupling the fragmenting and quantitative ability of the triple quadrupole with the high mass accuracy and qualitative ability of the TOF. In this study a Waters Synapt G2 high definition mass spectrometer was compared to an Agilent 4000 QTrap to determine the optimal system to obtain the highest possible sensitivity. Figure 13 is a schematic of the Waters Synapt G2 QTOF. These systems have higher mass accuracy and are able to provide superior qualitative power when compared to a triple quadrupole, and allow for easier positive identification of analytes.

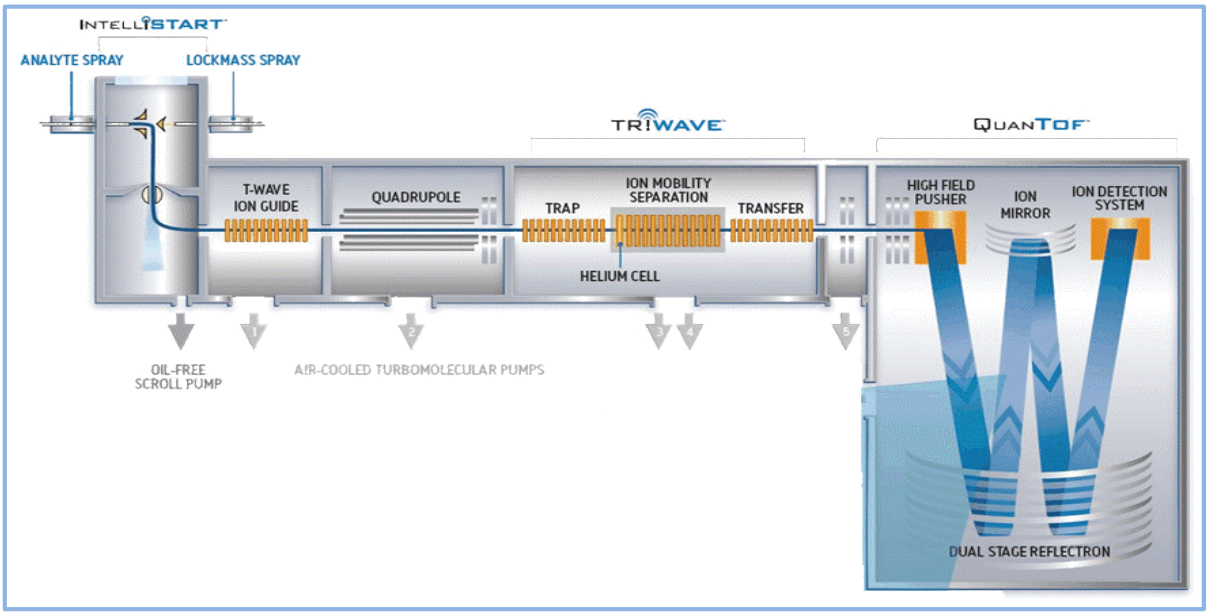


Figure 13: A schematic of the Waters Synapt G2 HDMS, which is a QTOF mass spectrometer(Waters, 2015). Reproduced with permission.

Mass accuracy is an important aspect in the field of mass spectrometry. It is the ability of the mass spectrometer to correctly determine the mono-isotopic mass of the charged ion. The higher the mass accuracy capability the better the system is able to determine the exact mono-isotopic mass of the ion. The mono-isotopic mass is the exact mass of the most abundant lowest mass isotopes of a specific molecular ion. A low mass accuracy system may, for example, yield a mass of 190.2. Looking at significant figures, this ion may have a mono-isotopic mass anywhere between 190.15 and 190.24, which is a large enough discrepancy to identify the molecule with a different empirical formula. At this low mass accuracy 190.2 may have tens of possible elemental compositions, meaning many more possible compounds for a researcher to consider, especially when analysing samples with unknown analytes. As the mass accuracy increases so does the number of significant figures reportable for the molecular ion. A higher mass accuracy system may yield a mass of 190.1637. This mass number will yield for example perhaps five elemental composition options decreasing the list of possible compounds that this molecule of interest may be. Using high mass accuracy researchers are better able to identify unknown analytes as well as to positively identify known analytes from mixtures of unknown samples. This becomes extremely important when working with complex samples in which there may be many contaminants. It also has greater structural characterisation capabilities and allows for superior analysis of metabolic changes to the analyte.

1.13 Advantages of HPLC-MS/MS

LC-MS is becoming an indispensable tool for the quantitation of low molecular weight compounds and is replacing methods using other forms of detection. It is however a specialised field with a high degree of expertise required to develop the requisite methods (De Jong *et al*, 2011). The application of LC-MS/MS has numerous advantages over other forms of detection methods and this is why LC-MS/MS formed the basis of the quantitation methods used in this study.

The advantages of LC when compared to other forms of separation is that it has greater analyte compatibility and does not routinely require derivatisation like GC. LC can thus be applied to different types of analytes more readily than GC. It also has numerous subtypes of stationary phases and modifications of methods like HILIC, RP and ion-pairing and can easily be applied to analytes with wide ranging polarity and chemical properties. The high separating power of H/UPLC also reduces interference and minimises matrix effects that allows for higher sensitivity. The high separating power is able to resolve numerous analytes simultaneously. The use of UPLC instead of HPLC has further advantages of shorter run times, increased resolution and sensitivity and the ability to decrease the cost of analysis in both time and money. U/HPLC can also be automated which is highly advantageous in a clinical laboratory setting.

Mass spectrometric detection has advantages over detection methods such as UV, fluorescence and ECD. It is able to reach or surpass the sensitivity of ECD and fluorescent detection but it has higher specificity due to the detection of the analytes based on mass of the individual analytes. Mass spectrometry can quantitate numerous compounds simultaneously because of the simultaneous mass detection and can also detect and identify unknown compounds from a mixture, which other forms of detection are unable to do. The ability of a mass spectrometer to fragment and detect the mass of the precursor and product allows for the elucidation of the structure of the analyte. Perhaps the greatest advantage of mass spectrometry is the ability to use stable isotope labelled internal standards. Stable isotopes are the identical compounds, however some atoms are replaced by stable isotopes with a higher mass and this increased mass is utilised to differentiate between the standard and analyte using mass spectrometric detection. These identical stable isotope labelled compounds will act in the exact same way as the analyte during analysis. This process allows mass spectrometry to more accurately correct for losses during sample pre-treatment, analyte separation and detection (De Jong *et al*, 2011) . A drawback of stable isotopes is the expense to create these compounds is higher than traditional stable isotopes.

The short run times and superior sensitivity and specificity of LC-MS/MS provide numerous advantages over other forms of separation and detection methods. The use of UPLC further shortens the run times, have greater separating power and superior resolution than HPLC. A QTOF mass spectrometer combines the higher mass accuracy, thus allowing for easier

positive identification and specificity combined with the ability to fragment as well as the quantitative capabilities of a triple quadrupole. This provides a platform able to reach the sensitivity required in this study. Using an LC-MS/MS method is a suitable platform to develop an analytical method in which analytes from low volume blood spot samples are collected and analysed. It serves to combine the separating power of LC with the high sensitivity and specificity of MS. The technique is capable of detecting analytes at concentrations below the picogram levels that is the expected concentration range for the total concentration of specific biogenic amines collected on a dry blood spot card or from low sample volumes.

1.14 Aims

The aim of this study was to develop and validate a quantitative method to analyse adrenaline, noradrenaline, dopamine, metanephrine, normetanephrine, serotonin, melatonin, histamine and 5-hydroxyindole-3-acetic acid in a single run using LC-MS/MS from limited sample quantities such as dry bio-matrix samples or small volumes of human plasma.

Objectives

- To optimise a derivatisation procedure and possible synthesis of a new derivatising agent.
- To determine the optimal precursor-product pair and mass spectrometer conditions for development of an MRM based quantitative method to analyse selected biogenic amine analytes in a single run.
- To optimise the method for native and derivatised biogenic amines focusing on a variety of chromatography columns for optimal separation of the biogenic amine analytes.
- To optimise sample preparation and derivatisation of both native and derivatised biogenic amines for the LC-MS/MS methods to be able to use dry bio-matrix samples of both spiked and collected plasma.
- To develop and validate, according to ICH guidelines, a quantitative LC-MS/MS method that is able to analyse a variety of native or derivatised biogenic amines in a single run.

Chapter 2 Materials and methods

2.1 Materials

Serotonin, dopamine, histamine, adrenaline, noradrenaline, melatonin as well as the metabolites metaneprine, normetanephrine and 5-hydroxyindole acetic acid were purchased from Sigma Aldrich (St Louis, USA). After initial tests mexamine proved to be an unsuitable surrogate standard. The stable isotope labelled surrogate standard D3-metanephrine was purchased from Cambridge Laboratories (Cambridge, USA).

The compounds for the synthesis of the in-house derivatisation agent are: methyl iodide, carnitine, dicyclocarboiimide, N-hydroxysuccinimide, N-aminocaproic acid and phosphorus tribromide. These compounds were all purchased from Sigma Aldrich (St Louis, USA).

MS grade acetonitrile and methanol was purchased from Romil (Cambridge, USA). Double deionised water (>18 MΩ) as produced in-house at the Department of Pharmacology using an Elga Genetics water purification system. LC-MS grade formic acid was purchased from Sigma Aldrich (St Louis, USA).

A Phenomenex Kinetex Biphenyl type column was purchased from Phenomenex (Jhb, RSA).

2.2 Standard solutions

The biogenic amines used are: adrenaline, noradrenaline, dopamine, serotonin, melatonin, histamine, normetanephrine, metanephrine, 5-hydroxy-3-indole acetic acid and D3-metanephrine as the surrogate standard.

The standard solutions were prepared by accurately dissolving the individual analytes as stock solutions in 0.1 M hydrochloric acid at a concentration of 1,0 mg/ml. A mixture of the nine biogenic amines was made by mixing equal volumes of the biogenic amines stock solutions and an equal volume of 0.1 M HCl, thus forming a mixed stock solution of all the biogenic amines. Dilutions were prepared from the individual or mixed stock solutions to form the working solutions. To prepare calibration curves the stock solution was diluted using 0.1 M HCl. To 910 µl of water, 10 µl of the biogenic amines were mixed, to form a concentration of 10 µg/ml. This was then diluted by adding 990 µl of deionised water to 10 µl of the analyte mixture to form a 100 ng/ml solution. This was then used to make the final dilutions, using deionised water, used to spike. The concentrations were 0.5, 1, 2.5, 5, 7.5, 10, 15 and 25 ng/ml, this was performed in triplicate. In order to spike the solvent or plasma 25 µl of the analyte mixture was added to 220 µl of either solvent or plasma, to this 5 µl of the surrogate standard was added. The dilutions were performed to bracket final concentrations which are to be expected in normal and pathological physiological systems.

The range of these analytes from the low pg/ml (roughly 10 pg/ml at the lowest concentration) to the high ng/ml range.

2.3 LC-MS/MS

The system that was used for quantitative analysis and method validation is located at the Department of Pharmacology, University of Pretoria. The system is comprised of an Agilent 1100 series HPLC and an ABSciex 4000 QTrap, triple quadrupole mass spectrometer system. The system used for initial analysis was a Waters Acquity UPLC coupled to a Synpat G2 HDMS QTOF. This system was not used for the quantitative analysis due to the lower sensitivity relative to the triple quadrupole system.

The Agilent 1100 HPLC system was used to achieve separation of the analytes. The system comprises of a binary pump, sample manager, solvent manager and a column oven.

The initial starting column to analyse underivatised biogenic amines was a Waters bridged ethyl hybrid (BEH) C18 column, (100 x 2.1 mm, 1.7 µm). Other columns used for underivatised biogenic amines were a Waters penta-fluorophenyl column (150 x 2.1 mm, 1.7 µm) and a Phenomenex Luna HILIC (150 x 4 mm, 3 µm) and a Waters UPC^2 BEH 2-ethylpyridine HILIC column (100 x 2 mm, 1.7 µm). The BEH C18 column was used as the initial column tested for the separation of the derivatised biogenic amines.

The auto-sampler tray was initially set to a temperature of 4°C. The aqueous mobile phase A consisted of the following: UPLC grade water with 0.1% formic acid. The organic mobile phase B was acetonitrile with 0.1% formic acid. A gradient elution program was used starting with a high aqueous content. The parameters, such as time points of mobile phase composition changes as well as changing the composition of the mobile phase, of the gradient program were altered and optimised to achieve the best separation and peak shape for all the analytes.

Concentrations of the additives and organic modifiers, such as formic acid and ammonium acetate, were also tested to ensure that the best peak shape (peak symmetry at 10 % peak height) was achieved and to ensure favourable ionization conditions to achieve higher sensitivity. The following chromatographic parameters were optimised: peak shape, analyte resolution and retention times.

The Waters Synapt G2 QTOF system was used to identify the compounds and consists of a sample infusion manager, source manager and a time of flight mass spectrometer. The mass spectrometer was set to scan from 100 – 1000 m/z units. The source conditions were optimised by infusing working solutions into the mass spectrometer. The following values were optimised: capillary voltage, cone voltage, desolvation temperature, source temperature and collision energy. Matrix effects were assessed by infusing a sample of mixed standards while running a blank plasma sample through the UPLC system.

The use of an Agilent 1100 series LC and an ABSciex 4000 QTrap was used for analysis of the derivatised biogenic amines including the in-house synthesised derivatising agent. The derivatised biogenic amines were analysed by diluting with 50:50:0.1 ratio of water: methanol: formic acid. These were infused using a Harvard syringe and pump at a flow rate of 10 µl/min. The masses of the analytes of interest were determined using Q1+ and enhanced resolution (ER) scans. Expected isotopic distribution was utilised, along with literature and fragmentation patterns, to confirm the identity of each of the analytes. The declustering potential, the precursor-product pairs and the collision energy were optimised by using the parameter-ramp functions. These optimised MS settings were then used for LC-MS/MS analysis.

A number of different HPLC columns for chromatographic separation of dansyl chloride and trimethyl-aminocaproic acid N-hydroxysuccinimide ester (TMAC-NHS) derivatised biogenic amines were used including: Alltima Grace C18 column (20 x 2 mm, 3 µm), a Phenomenex Gemini C18 (100 x 2 mm, 3 µm), a Phenomenex Synergi MAX-RP (150 x 4.6 mm, 5 µm), a Phenomenex Kinetex C18 (100 x 2.1 mm, 2.6 µm) and a Phenomenex Kinetex Biphenyl (100 x 2.1, 2.6 µm). A Phenomenex Luna HILIC column (150 x 2 mm, 3 µm) was used for the TMAC-NHS derivatised biogenic amines after the use of the RP columns.

The gradient profiles, solvent effects and column temperature, were systematically changed to assess the effects on the analyte separation, retention times as well as peak symmetry and area. The initial solvent tested was acetonitrile as is it a strong solvent which could elute the highly lipophilic analytes within a sufficiently short run time and assays show slightly higher sensitivity when using acetonitrile. This was then changed to methanol to determine if greater resolution between co-eluting compounds could be obtained. Due to the drastically increase run time a combination of these was used. This was done by starting with an initial 50:50 ratio of acetonitrile to methanol and was adjusted appropriately while using the same gradient profile in order to assess the best solvent composition. The gradient profile was adjusted from a basic profile, from high aqueous to organic content, in order to observe both elution order as well as identify co-eluting compounds. This profile was then altered, the same sample run and the changes – positive or negative were noted. The times of composition changes as well as the composition of the mobile phase were altered in order to yield the best resolution and peak shape. Column temperature was changed to determine if higher or lower temperatures had a large impact on the resolution or peak shape. The temperature was not noted to have a large effect on the chromatography but was maintained at 40°C to decrease the back pressure of the system. Peak symmetry was determined by noting the width from the middle of the peak to both the front and reverse sides of the peak at 10% of the peak height. Any peak that has a ratio higher than 2 in asymmetrical and deemed not suitable for the assay.

The final chromatographic method selected for the dansylated BA is described below for the separation using the Phenomenex Kinetex Biphenyl (100 x 2.1, 2.6 µm) column: This column

and method was selected as peak symmetry ratios were acceptable for all peaks, the observed peak height of the same sample was increased when compared to other columns. The resolution between all the analytes was superior when compared to other methods and columns with more time elapsing between eluting analytes.

A gradient elution programme was used as described below. The flow rate was kept at 300 µl/min with a column temperature of 40°C. A 10 µl injection volume was used. Mobile phase A consisted of 0.1% formic acid in double deionised water and mobile phase B consisted of an 0.1% formic acid in a 80:20 ratio of acetonitrile:methanol.

Table 3: Final time program for the pumps of the optimised chromatographic method used during analysis.

Total time (Min)	A%	B%
0.00	85.0	15.0
0.85	85.0	15.0
1.20	55.0	45.0
3.00	27.0	73.0
5.20	20.0	80.0
7.40	10.0	90.0
9.00	10.0	90.0
9.50	85.0	15.0
13.00	85.0	15.0

During the initial testing phase, the following mass spectrometer conditions were found to be optimal for the detection of biogenic amines. The resolution of Q1 and Q3 were set to low. A “scheduled” MRM was setup to allow for more accurate quantitation by controlling the number of data points collected per time. A 60 second detection window was used with a target scan time of 1 second.

Table 4: Final optimised source conditions for dansylated biogenic amine analysis.

Optimised conditions for underivatised biogenic amines	
Ionspray voltage	5000 V
Source temperature	450°C
Ion source gas 1	40
Ion source gas 2	30
Curtain gas	23
Collision Gas	High

Table 5: Final MRM pairs used for the analysis of dansylated biogenic amines

MRM Pair	Q1 mass	Q3 mass	DP (volts)	CE (Volts)
Adrenaline	884.1	397.4	72.0	72.0
Adrenaline1	884.1	537.6	72.0	72.0
Noradrenaline	870.1	636.9	65.0	65.0
Noradrenaline1	870.1	851.7	65.0	65.0
Dopamine	854.1	619.8	65.0	65.0
Dopamine1	854.1	384.4	65.0	65.0
Metanephrine	665.0	646.6	40.0	40.0
Metanephrine1	665.0	412.0	40.0	40.0
d3-metanephrine	668.0	649.6	40.0	40.0
d3-metanephrine1	668.0	415.0	40.0	40.0
Normetanephrine	650.7	554.7	50.0	50.0
Serotonin	643.7	146.2	57.0	57.0
Serotonin1	643.7	235.4	57.0	57.0
Histamine	578.6	234.5	60.0	60.0
Histamine1	578.6	315.0	60.0	60.0
Melatonin	233.3	174.1	26.0	26.0
5-hydroxy-3-indole acetic acid	425.5	381.5	50.0	50.0
5-hydroxy-3-indole acetic acid1	425.5	382.5	50.0	50.0

2.4 Synthesis of the TMAC-NHS ester

To synthesize the n-aminocaproic acid NHS ester 5 grams, 0.03 M, of n-aminocaproic acid was dissolved in a minimum of anhydrous dimethyl formamide with 4 grams, 2 M excess of potassium hydroxide and was heated until it was refluxing under a condenser. Methyl iodide at a 3 M excess was slowly added through a dropping funnel while the solution was stirred and further refluxed under a condenser overnight. The next morning a further 1.2 M excess of methyl iodide was added and the solution allowed to react for a further 24 hours under reflux. TLC and MS was used to monitor the progress of the amine methylation and esterification reaction as described below.

A volume of 100 mL of 1 M NaOH in water was added and the solution was then refluxed overnight to saponify the methyl ester than formed from the carboxylic acid. Again TLC and MS was used to determine the reaction progress.

The solution was then dried down to a sticky powder using a Buchi rotovaporator and water bath. The dried powder was solubilised in a minimum of anhydrous acetonitrile and a 1.1 M

excess of dicyclohexylcarbodiimide and a 1.1 M excess of N-hydroxysuccinimide was added. The solution was refluxed overnight. The final solution was analysed using TLC and MS. After confirmation that the TMAC-NHS ester had formed to apparent completion, the solution was filtered using a Whatman no. 1 filter paper and then dried down to a precipitate using a Buchi rotovaporator to yield 5.151 g of a yellow paste. This was then dissolved in acetonitrile, as required, and then used to derivatise the biogenic amines.

To determine the reaction progress, samples of the reaction solution were monitored at different times by thin layer chromatography (TLC) and the mass spectrometer was used to confirm the identity of the different spots detected. The mobile phase for the TLC was water:methanol:acetonitrile in a 20:40:40 ratio. The plate was then stained with iodine, Dragendorffs and hydroxylamine-ferric chloride reagents to detect the amine group. The hydroxylamine was made up as two different spray solutions. Spray 1 was formed from 2 different solutions. Solution A was made up with 2 g of hydroxylamine dissolved in a minimum of deionised water and then made up to 20 mL of ethanol. Solution B comprised of 0.5 g of potassium hydroxide in a minimum of deionised water and dissolved in 5 mL ethanol. These two solutions where combined in a 1:2 ratio of A:B and this formed one spray. Spray two was made by dissolving 1 g of ferric chloride in 2 mL of concentrated HCl and made up to 20 mL using diethyl ether.

2.5 Derivatisation of Biogenic amines

2.5.1 Ethyl chloroformate derivatisation

To derivatise the biogenic amines with ethyl chloroformate an aliquot of 20 µl of the stock mixture was diluted to a final concentration of 1 ug/ml of which 100 µl/ml was derivatsed. A volume of 300 µl of an ethanol:pyridine in a 4:1 ratio was added to the biogenic amine mixture, in order to neutralise the acidic conditions. To this solution, 100 µl of pure ethyl chloroformate were slowly added in volumes that avoided eruption of the solution. This solution was the vortex mixed for 5 minutes. To extract the biogenic amines 500 µl of water was added as well as 1 mL of diethyl ether was added and the resulting solution was vortex mixed for 15 minutes and centrifuged at 2000 *xg* for 10 minutes. The organic phase was then transferred to another tube, dried down in a Labconco “Centrivap” at 45˚C until a dry residue was formed.

2.5.2 TMAC-NHS derivatisation

The derivatisation of the BA used 100 µL of the 1 mg/mL individual biogenic amines. The reaction was optimised using a variety of buffers, pH ranges, temperatures and reaction times. The final reaction was performed by adding 20 µL of sodium carbonate and 200 µL of a 0.1 M sodium borate buffer at pH 8.3, to the biogenic amines aliquots. To this solution 200 uL of the TMAC-NHS ester at a concentration of 3 mg/mL was added. The reaction was mixed and left to react for 10 minutes at room temperature. The reaction was quenched with ammonia. The final solution was then dried down in a Labconco “Centrivap" at 50°C.

2.5.3 Dansyl chloride derivatisation

Derivatisation of biogenic amines was performed using dansyl chloride. The biogenic amines were derivatised individually. The reaction was performed by reacting 100 µL of biogenic amines with 200 µL of a 0.1 M sodium borate buffer. To this varying amounts of dansyl chloride were added at 20 mg/mL in acetone. The reaction conditions were optimised with respect to temperature, pH, time and molar excess of dansyl chloride in order to yield the highest ratio of product to underivatised starting BA The conditions tested are shown below in Table 6.

Table 6: Various conditions tested to obtain the highest yield of dansylated biogenic amines.

Parameters			
Time (minutes)	**pH**	**Temperature (°C)**	**Molar Excess (x-fold)**
15	8	Room temperature	1
30	9	40	5
45	10	60	10
60			20

The biogenic amines were then derivatised as a mixture and this was done by using 100 µl of mixed stock of the biogenic amines. This solution was derivatised as described above using the optimised conditions for highest fully derivatised product formation.

The samples were protected from sunlight throughout the experiment. The dried biogenic amine derivative residues were then extracted using 500 µl of ethyl acetate and the organic layer was collected. This extraction step was performed twice. The combined organic extracts were then dried down in a Labconco “Centrivap” apparatus at 50°C till a dry residue was obtained.

The resulting residue was reconstituted into 50 µl of acetonitrile and analysed by LC-MS/MS. Initial tests were done by infusion of the samples into the ABSciex 4000 QTrap source using a Harvard syringe pump. Using the optimised mass spectrometer method the samples were analysed and quantitated using the Analyst software. In order to assess the varying methods a simple method was used to separate the biogenic amines. These analytes were then quantitated. The AUC was then determined by using analyst software, ensuring the integration of each peak was accurate and to the baseline. The AUC represents the size of a peak, thus representing the overall amount of the derivatised analyte. The bigger the AUC the higher the yield of that analyte which indicates the optimal conditions to use for derivatisation.

The final conditions for the derivatisation were as follows: The biogenic amines were kept at a temperature of 23°C and were reacted for 60 minutes at pH 8. A 20 M excess of DNSCl was added to force the reaction to completion.

2.6 Human plasma samples

Human plasma was prepared from blood collected from healthy volunteers, using venipunture into heparin collection tubes. The blood was centrifuged at room temperature at 2000 *xg* for 15 minutes. In order to develop the analytical method, assess the sensitivity, limits of detection, recoveries and to determine their ability to avoid matrix effects, numerous extraction and derivatising methods were assessed. These included acetonitrile, methanol and perchloric acid protein precipitation steps as well as various pre- and post-derivatisation liquid-liquid extraction (LLE) procedures. This was done by using the same working stock solutions to form multiple samples at the same concentration. These samples were the extracted using a variety of solvents and acids, derivatised and analysed to assess the pre-derivatisation extraction procedure. Based on those relative signal intensities the same method was applied to different samples with various solvents to determine the optimal post-derivatisation extraction procedure. The combination which gave the highest signal intensity was then used as the extraction method. Other factors considered were issues relating to solubility of the buffer or analytes as well as the ease of use in a high throughput scenario, the equipment available at the time of the experiments as well as compatibility with downstream MS applications.

To develop and validate the method 220 µl plasma was either left unspiked, or was spiked with a constant volume, using a series of increasing concentrations of the analyte mixture to form an 8-point calibration curve. To each sample 5 µl of the stable isotope surrogate standard was added at the same time as the spiking procedure, which resulted in a final surrogate standard concentration of 1 ng/ml. The samples were protein precipitated using an equal volume of acetonitrile. This was then centrifuged at 5000 *xg* for 10 minutes and the supernatant was collected. To the supernatant 200 µl of buffer and 100 µl of 20 mg/ml DNSCl in acetone was added. The samples were then allowed to react at 23˚C for 60 minutes with vortex mixing at 15 minute intervals. Ethyl acetate was used for the extraction, where 500 µl was added, vortex mixed, centrifuged and collected, this step was repeated twice. The combined ethyl acetate extracts were then dried down at 50°C in a Labconco “Centrivap”. The dried residue was then reconstituted in 50 µl of acetonitrile, 10 µl was injected and analysed using the optimised LC-MS/MS method.

2.7 Method Validation

In order to validate the method a number of assays were performed. To assess inter and intra-day precision, accuracy and linearity, calibration curves were made up on three separate days, derivatised and analysed. This was done in triplicate each day on 3 different days resulting in 9 runs total per concentration. Each concentration point was derivatised once per day on three different days resulting in 3 derivatisation repeats. Recoveries were determined by spiking the biogenic amines into both solvent and plasma and the differences determined to yield the percent recovery. Matrix effects were assessed to determine any major sources of interference. This was performed by derivatising biogenic amines in

solvent as well as from unspiked plasma. The solvent based biogenic amine derivatives were then infused at a constant flow rate of 20 µl/min post column via a "T" connector and the derivatised blank plasma sample was injected and analysed using the optimised analytical chromatography method. Stability assays were performed by derivatising a sample which was then analysed immediately. This sample was then aliquoted into two samples. One sample was frozen at -20°C and the other kept in the dark at room temperature and analysed again after 5 days.

The calibration curves were made up by diluting a mixture containing the biogenic amines mixtures to make a set of working solution. The working solutions were then spiked into either plasma or solvent to make the final concentrations to be tested. The final concentrations were chosen to extend to the low pg/ml range as the biogenic amines are often found at these low concentrations in biological samples. The concentrations for all the tested biogenic amines were as follows: 10, 50, 100, 250, 500, 750, 1000 and 2500 pg/ml and the surrogate standard, D3-metanephrine, was added at a constant concentration of 1500 pg/ml. Solvent and plasma samples were also derivatised without spiking to determine background levels. After derivatisation these samples were dried down and reconstituted prior to analyses. The series of plasma samples were run first followed by solvent calibration curves. The samples were run in ascending order of concentration, with each sample run 3 times followed by the next sample. The column was cleaned between plasma and solvent calibration curve by running a 30 minute wash step using a high percentage organic solvent. A blank was also run every 8 samples in order to avoid accumulation and carry over. Carry over was assessed by injecting a blank directly after the highest concentration calibration sample and determining the peak areas of the different analytes.

The samples were then processed and analysed according the optimised method. These were then analysed using Analyst software 1.5.2 (ABSciex, Concord, Canada). The peaks were integrated and then plotted as Analyte/IS response versus analyte concentration. Linear regression was used to determine the formula of the linear response curve and the correlation coefficient and coefficient of determination. Analyst software was also used to determine the accuracy and the coefficient of variation (precision) at each concentration point. The signal to noise ratio, LOD and LLOQ were also determined using the Analyst software.

The method was validated according to International Conference on Harmonization (ICH) guidelines (Ich, 2005). The ICH has a variety of criteria which must be met:

2.7.1 Specificity

Specificity refers to the ability of the assay or analytical procedure to test/detect/quantitate the analyte of interest unambiguously. It also encompasses factors such as whether compounds of a similar structure will interfere and cause false signals (thus affecting the end result).

Identification

Suitable identification tests are able to differentiate between analytes of similar structures which are expected to be present in the same sample. The test may be confirmed by obtaining a positive result, from a sample in which the analyte is present, compared to a negative test in which the analyte is not present within an equivalent test sample. When using LC-MS/MS the retention time, unique mass and predictable fragmentation pattern can all add to the positive identification of the analyte.

Assay and Impurity test

Chromatographic assays require chromatograms that demonstrate single symmetrical peaks at specific retention times that match the standard to prove specificity. Individual components of the chromatogram are labelled accordingly. Critical separations must be investigated at appropriate concentration levels and can be demonstrated by resolution of the two analytes that elute closest to each other at various concentrations. The assay should also be able to differentiate between the analyte and impurities.

2.7.2 Linearity

There must be a linear relationship across the concentration range of the assay. The linearity should be evaluated based on a signal as a function of the analyte concentration. This can be done by a visual inspection or by using the area-under-the-curve (AUC). The linear relationship should be tested using regression analysis. In order to establish a linear relationship a minimum of 5 different concentrations is recommended.

2.7.3 Range

The range is normally established from linearity studies but must include samples at the extremes of the range to be tested. It is derived by confirming that the assay offers acceptable linearity, accuracy and precision when the assay is applied to a sample in which the analyte is present at concentrations within or at the extremes of the range. An intra-assay variability of 15% is acceptable with a 20% variability allowed at the LLOQ.

2.7.4 Accuracy

The confidence of agreement between signals obtained from a sample and a reference value. The accuracy is established by a minimum of 9 separate determinations at a minimum of 3 different concentration levels that fall within the linearity range. Accuracy is reported as percentage of a known true value. Calibration curve accuracy is confirmed by the use of three different levels of quality control standards run during the set of calibrant standards.

2.7.5 Precision

The confidence of agreement between multiple signals obtained from multiple sampling of the same sample under the same conditions

Repeatability

Assessed by a minimum of 9 determinations over a minimum of 3 different concentration levels that fall within or at the extremes of the range

Intermediate Precision

The effects of random events on the precision should be evaluated. These include analysis on different days and by different analysts etc.

Reproducibility

Determined by inter-laboratory trials.

2.7.6 Detection limit

This can be determined based on the assay. Common practice for LC-MS/MS is based on signal-to-noise ratio and can be applied to procedures which have baseline signals. Measured signals of samples with the analyte present are compared to a blank sample run under identical conditions. A signal-to-noise ratio of 3 is considered as the Limit of Detection (LOD).

2.7.7 Quantitation limit

This is determined based on the type of assay. Common practice for LC-MS/MS is based on signal-to-noise ratio. A ratio of 10 is considered as the Lower Limit of Quantitation (LLOQ). Other methods use a statistical assay where the variance must be less than 15%.

2.7.8 Robustness

This should be evaluated during development. It should indicate the reliability of the assay with regards to deliberate variations in method parameters. Some parameters that can be examined are:

- Stability of analyte and analytical solutions
- Extraction time
- pH variations of mobile phase
- Mobile phase composition
- Different columns
- Column temperature and flow rate
- Matrix effects

2.7.9 System Suitability Testing

This test is based on the concept that the equipment, analytical operations, samples and electronics are all part of an integral system and should be evaluated as such. The test parameters that should be established for a particular procedure must ensure the procurement of high quality data. In short the validation process must prove that a particular method of analysis is fit for its intended purpose.

Chapter 3 Results and discussion of method development

3.1 Method development of underivatised biogenic amines

A method for underivatised biogenic amines was first developed. This was to provide a comparison of underivatised to derivatised biogenic amines to determine if derivatisation will increase the sensitivity of the assay.

Mass spectrometric conditions were optimised to ensure the highest possible sensitivity. MRM precursor – product pairs were also optimised. The source was operated in positive electrospray ionisation mode for underivatised biogenic amines. The optimised mass spectrometer conditions are displayed below in Table 7 and the optimised MRM product-precursor pairs in Table 8.

Table 7: Final conditions for the analysis of underivatised biogenic amines.

Optimised source conditions for underivatised biogenic amines

Condition	Value
Capillary voltage	5000 V
Source temperature	450°C
Ion source gas 1	20
Ion source gas 2	15

Optimised MRM pairs of underivatised biogenic amines

Biogenic amines	Precursor	Product	Collision energy (V)	Declustering potential (V)
Adrenaline	184.2	166.3	13.0	30.0
Noradrenaline	170.2	152.3	11.0	25.0
Dopamine	154.4	137.3	15.0	30.0
Metanephrine	198.2	180.1	18.0	30.0
Normetanephrine	184.4	166.3	10.0	24.0
Serotonin	177.2	160.1	15.0	33.0
Histamine	112.2	94.9	25.0	25.0
5-HIAA	192.4	146.2	21.0	34.0
Melatonin	233.4	216.2	22.0	26.0

These optimised mass spectrometric conditions were then used to develop the chromatographic method. Multiple column types were tested however due the highly polar nature of underivatised biogenic amines, minimal retention was observed for the BA compounds on available columns. The retention on all the C18 columns was poor, and the available HILIC column gave broad, tailing peaks which are often observed with larger particle size columns and HILIC separation. The separation of native biogenic amines still remains problematic as numerous columns do not possess the required interaction to retain these compounds using reverse phase columns and normal phase chromatography often suffers from a lack of sensitivity due to high aqueous and non-volatile salts required for successful chromatography. Columns that require the use of ion-pairing agents can either increase costs or are not amenable with specific detectors. The final column selected was the Kinetex Biphenyl (2.1 x 100 mm, 2.6 µm) as it provided retention of some of the aromatic biogenic amines, such as melatonin and mexamine. Figure 14 illustrates a chromatogram obtained of underivatised biogenic amines. The chromatogram shows co-elution of at least five compounds with minimal retention of the analytes, especially those of the catecholamine classes. The reason for assaying the underivatised BAs is to provide a comparison of the sensitivity before and after derivatisation. The mixed sample that was analysed in Figure 14 was diluted approximately 1/50, derivatised and analysed using the optimised LC-MS/MS method developed for dansylated biogenic amines. In Figure 15, a chromatogram obtained for the dansyl derivatives of the same BAs using the optimised HPLC method using the Kinetex Biphenyl column. It was observed that there was a significant increase, between 50 and 100-fold, in the sensitivity of these compounds post-derivatisation. The separation of the derivatised test BAs were also significantly improved compared to native underivatised biogenic amines. This improvement in separation is to be expected on a reverse phase type column as the derivatisation process increased the lipophilicity of the analytes to a large extent through the addition of up to three naphthyl groups per compound. The use of derivatisation increased the sensitivity of the reverse phase chromatographic method without the use of ion pairing agents and with solvents that were amenable to downstream MS applications, which could not be achieved with underivatised BAs.

The chromatograms clearly show that derivatisation improved both the separation of these BA compounds and the overall sensitivity – while still using RP chromatography and with the addition of an easily ionisable moiety. For comparative purposes the methods were kept almost identical using the same injection volumes and flow rates to provide a more direct comparison of parameters. The increased sensitivity will allow for the detection at the extreme low concentrations found in biological samples like human plasma on the biphenyl type column. A study performed on dansyl chloride derivatisation of propofol found a 200-fold increase of signal intensity when compared to underivatised samples (Beaudry et al, 2005). Derivatisation remains a viable solution to increase the sensitivity of a method especially when dealing with analytes which ionise poorly. The increase in signal intensity is

dependent on a variety of factors which include the ionisability of the derivative relative to the ionisability of the original analyte, the reaction efficiency and the chromatographic conditions required to separate the newly formed derivatives. Derivatisation remains viable even when using liquid chromatographic techniques and has been demonstrated to increase the signal intensity of biogenic amines which makes it an invaluable technique to reach low pg/ml concentration ranges.

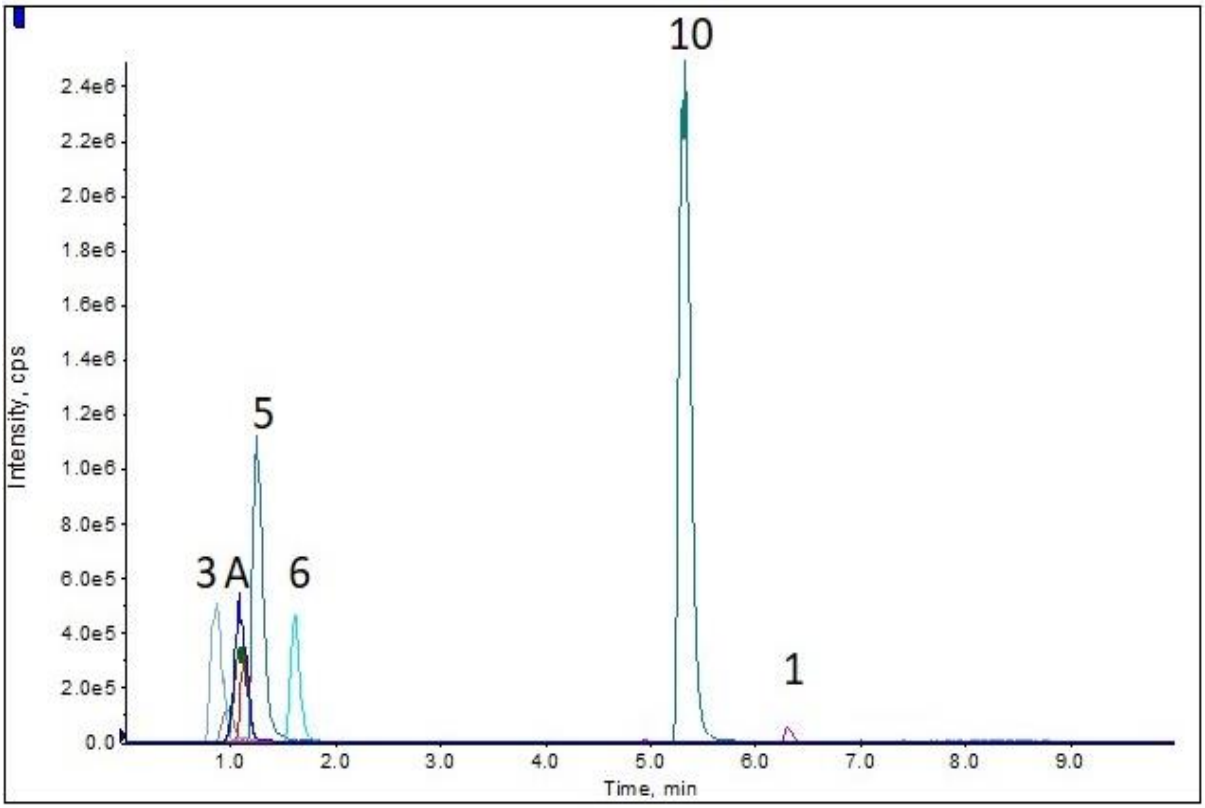


Figure 14: Chromatogram obtained of underivatised biogenic amines using a biphenyl column. The peak are indicated as follows: 1- Melatonin, 3 – Histamine, 5 – Metanephrine, 6 – Serotonin, 10 – Mexamine, A- Adrenaline, Noradrenaline, Dopamine, Normetanephrine

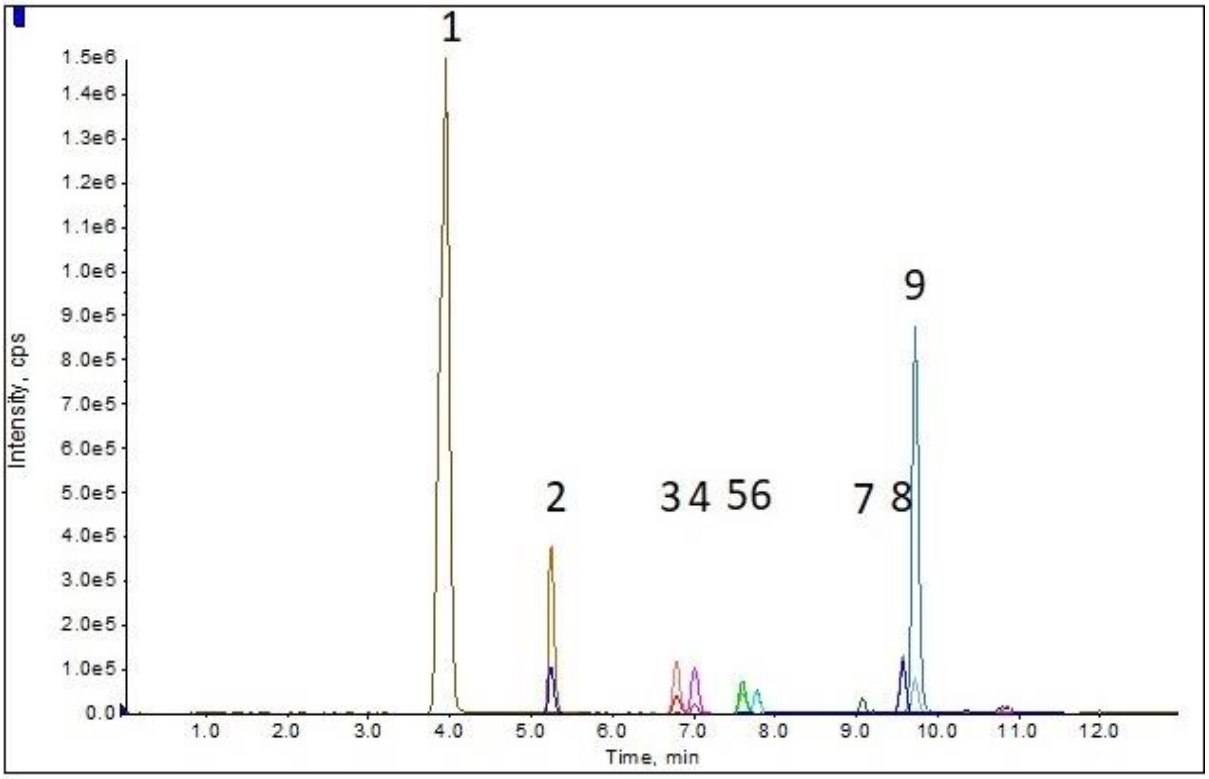


Figure 15: Chromatogram obtained of ten dansylated biogenic amines using a biphenyl column detected by MS/MS with two fragments detected per derivatised analyte and one fragment for melatonin. The analytes are indicated as follows: 1 – Melatonin, 2 – 5HIAA, 3 – Histamine, 4 – Normetanephrine, 5 – Serotonin, 6 – Metanephrine, 7 – Noradrenaline, 8 – Adrenaline, 9 – Dopamine.

3.2 Derivatisation using ethyl chloroformate

Ethyl chloroformate derivatisation was a simple procedure carried under mild conditions. The reaction was performed at room temperature for 10 minutes with a short extraction procedure. This implies that the reaction is easily performed, rapid, has a simple extraction procedure and uses commercially available reagents. A problem with the reaction is that it is highly exothermic and addition of excess molar ratios of ethyl chloroformate must be done drop wise to avoid superheating of the solution even when diluted. Another limitation with the reaction was the use of volatile chemicals and the need to carry out the reaction under a fume hood, which may reduce the applicability in certain testing facilities.

Table 8: Expected masses after ethyl chloroformate derivatisation.

Biogenic amine	Underivatised mass		Derivatised mass		
	M	M+ H^+	1x [M+ H^+]	2x [M+ H^+]	3x [M+ H^+]
Adrenaline	183.2	184.2	256	328	400
Noradrenaline	169.1	170.1	242	314	386
Serotonin	176.2	175.2	249	321	
Histamine	111.1	112.1	184	256	
5-MT	190.2	191.2	263		

Ethyl chloroformate is a non-specific derivatising reagent and reacted with primary and secondary amines as well as phenolic hydroxyl groups. The lack of specificity resulted in numerous derivatives which was more pronounced when derivatising with smaller molar excess ratios. Using UPLC and high mass accuracy detection and using the predicted masses of the derivatives it was possible to identify 5 different individual analytes from the derivatisation of adrenaline. The lack of specificity shown by ethyl chloroformate was especially prominent when working with the catecholamines. Each of the catecholamines treated with ethyl chloroformate had 3 possible derivatisation sites giving rise to the 5 different peaks that were shown to be various ethyl chloroformate derivatives of the parent compound.

In the chromatogram in Figure 16, which represents a derivatised adrenaline standard, the multiple derivatives are easily seen. The high mass accuracy of the system also enabled adducts formation of the derivatives to be identified. A mono-ethyl adrenaline derivative eluted early in the run at a time of 1.14 minutes and had a mass of 238.1063. This mass was identified as $[M+H-H_2O]^+$. The second peak was identified as an adrenaline derivative eluted at a time of 2.05 minutes, at a mass of 310.1326 and was determined to be the $[M+H-H_2O]^+$ of a di-ethylformate adrenaline derivative. The third and fourth peaks determined to be adrenaline derivatives eluted at 2.66 and 3.07 minutes respectively at a mass of 350.1183 and 350.1265 respectively. These derivatives were determined to be the $[M+H+Na]^+$ and identified as di-ethylformate adrenaline derivatives, and are identical in terms of mass. It is worth noting that these two peaks and the previous peak are di-ethylformate adrenaline derivatives but elute at different retention times. The possible reasons for such an observation are that peaks three and four may be positional isomers separated on the chromatographic column. The fact that these analytes have been derivatised at different sites means that although the molecular mass may be the same, the analytes are different with respect to polarity resulting in the observed retention time differences. The final peak identified as an adrenaline derivative eluted at 5.30 minutes at a mass of 422.1385. This derivative was determined to be $[M+H+Na]^+$ and determined to be a tri-ethylformate adrenaline derivative. The formation of this derivative was favoured providing there was a large excess of ethyl chloroformate to react with all available reactive sites on the adrenaline. The mass of adrenaline found in this study has been noted before, and through

the use of the QTOF system an accurate mass was obtained in this study (Pyo *et al*, 2006). Figure 16 also demonstrates how the lipophilicity increases as adrenaline becomes multiply derivatised. The separation was performed on a BEH C18 column using an increasing organic solvent gradient, and thus more lipophilic compounds elute later as they are more retained. The separation of the biogenic amines was unobtainable on the C18 column in their native format, and thus demonstrates the ability of derivatisation to increase retention and separation.

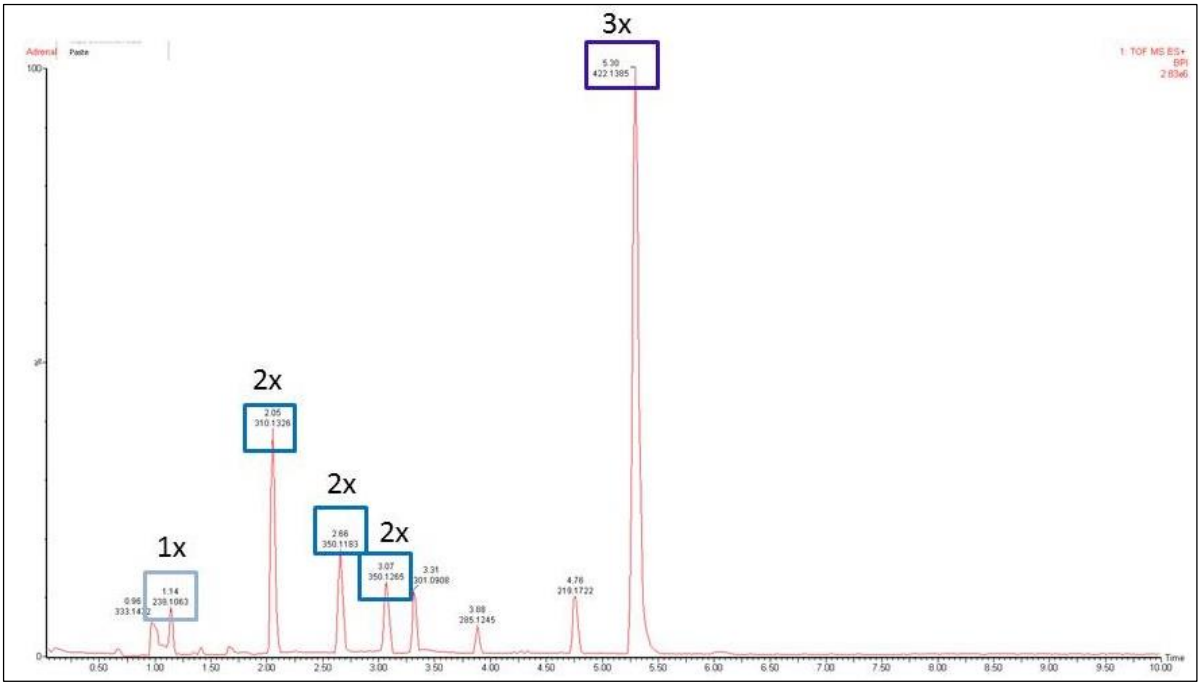


Figure 16: Chromatogram obtained for adrenaline derivatised with a four times molar excess of ethyl chloroformate. The peaks indicated with blue squares are adrenaline derivatives and the number represents the number of ethyl chloroformate molecules that reacted with the parent compound.

In the chromatogram in Figure 17, which represents a mixture of derivatised biogenic amines, it was shown that the majority of the derivatives exhibited an increase in lipophilicity and adequate retention could be achieved on a C18 column. It was noted that histamine eluted early in the run demonstrating a high polarity, despite being derivatised. This lack of retention results in elution close to the void volume along with a high abundance of interfering compounds. This implies that histamine will be more susceptible to matrix effects than those eluting later. These matrix effects may result in non-predictable ionisation of the analytes and impact on the level at which histamine may be accurately determined using this assay. The histamine product was determined to be a mono-ethylformate derivative without adduct formation, the fact that histamine did not form a di-ethylformate derivative may be due to the low molar excess of ethyl chloroformate added, or merely a low abundance of the di-ethylformate derivative that was not detected. A study performed on histamine analysis using GC-MS postulated a di-ethylformate derivative of histamine (Pittertschatscher *et al*, 2002). Serotonin and the internal standard, 5-methoxytryptamine, were determined to be di-ethylformate derivatives and easily formed

sodium adducts. Figure 17 demonstrates the separating power of small particle size and narrow bore columns afforded by the use of UPLC techniques. Despite the fact numerous adducts were formed from each analyte, a basic chromatographic method was able to completely resolve the majority of the peaks. The peaks were sharp and symmetrical for most of the analytes analysed all without time consuming optimisation and while using a standard C18 UPLC column.

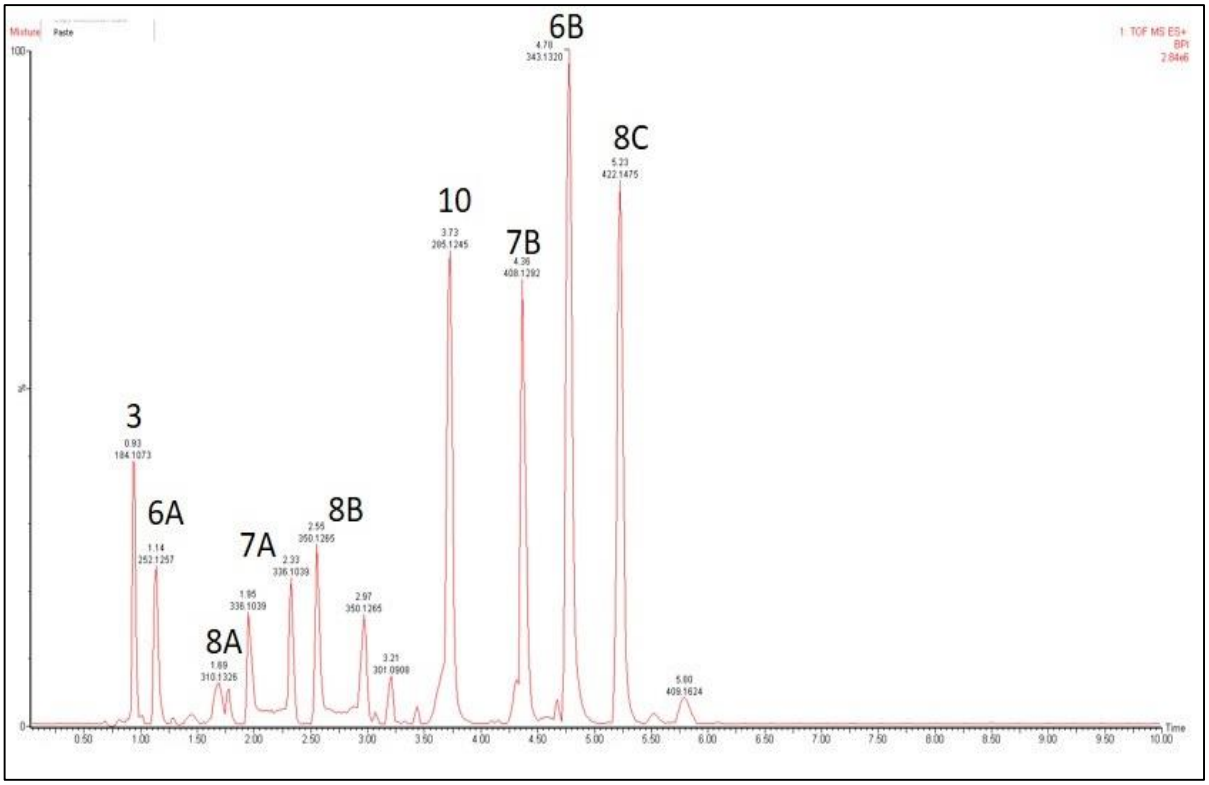


Figure 17: Chromatogram obtained after ethyl chloroformate derivatisation of a mixture of biogenic amines was performed. The peaks are indicated as follows: 3 – Histamine, 6A, B – serotonin (mono and diethyl), 7A, B – Noradrenaline (mono and diethyl), 8A, B, C – Adrenaline (mono, di and triethyl), 10 – Mexamine.

The efficiency of the extraction procedure was also tested and this was done by drying down the residual aqueous phase after removing the organic phase and then reconstituting and analysing the residue obtained. In the chromatograms in Figure 18, it was found that the extraction procedure for histamine was not efficient and a large portion of the histamine was not extracted. The most likely scenario for the poor extraction is that the histamine derivative is polar and is not efficiently extracted into diethyl ether. Thus in order to provide better extraction of histamine a more polar solvent would be required but it would need to be lipophilic enough to extract the remaining analytes and not be miscible with water.

The results show that when five BA analytes were derivatised and run on a standard C18 column baseline separation of all the peaks was achieved and reasonable sensitivity for the majority of the analytes was observed. This assay however was performed when the numbers of analytes tested were limited. The number of analytes being tested increased to

a total of 10 analytes and including various metabolites the ability of this assay to separate and quantitate these compounds would become untenable. The use of an accurate mass MS system allowed the use of elemental composition to identify the various derivatives and could account for adduct formations. This demonstrates the power of a high accurate mass system has over triple quadrupole systems. “Eruption” of the solution became problematic due to the highly exothermic nature of the reaction. Even when adding ethyl chloroformate in a drop wise manner or using diluted ethylchloroformate, sample spattering and loss still occurred. The study was focused around using limited sample volumes and sample loss at any stage of the developed method would negatively affect the sensitivity, reducing the chances of quantitating at pg/ml ranges. Due to the lack of specific derivatisation products being formed when using ethyl chloroformate, the eruption of the solution when adding the ethylchloroformate, as well as the quality of the reagent, the use of other derivatising reagents were explored in order to obtain greater specificity and sensitivity were explored.

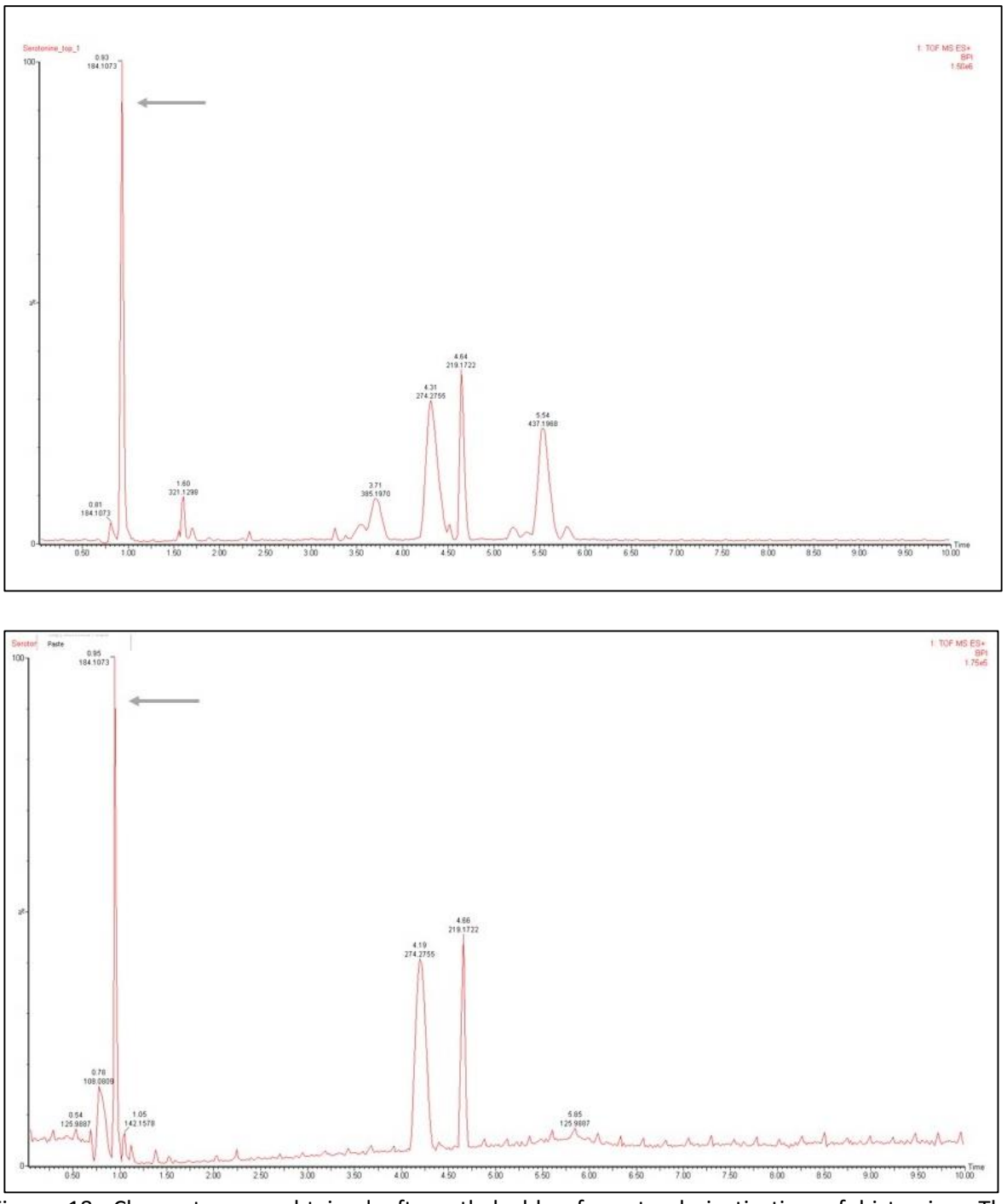


Figure 18: Chromatogram obtained after ethyl chloroformate derivatisation of histamine. The presence of histamine derivatives was detected in both the aqueous (top) and organic (bottom) layers, and is indicated by a grey arrow. The presence of other peaks were not identified as histamine derivatives and are unknown contaminants.

3.3 Synthesis of NHS-ester

The synthesis of the N-hydroxysuccinimide ester of trimethylaminocaproic acid used for the derivatisation of the biogenic amines was performed in the Department of Pharmacology. The initial step was to synthesise a quaternary amine and the second step was to couple the quaternary amine to N-hydroxysuccinimide to form the active derivatising reagent.

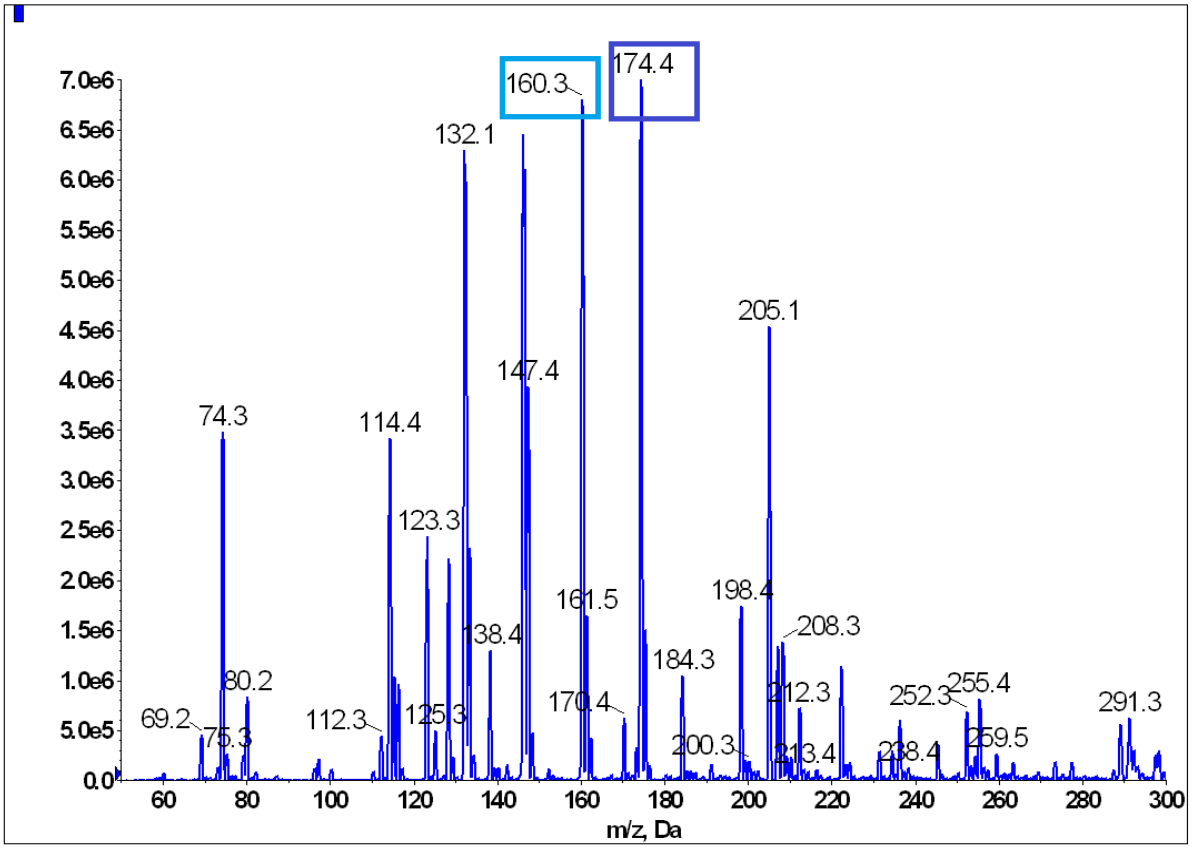


Figure 19: Mass spectrum of trimethylaminocaproic acid infusion. The spectrum was obtained from an infusion of the trimethylaminocaproic acid mixture. The light blue box indicates the unreacted parent compound and the dark blue indicates the trimethylaminocaproic acid compound. The other peaks are unknown, reaction by-products.

The formation of the quaternary amine was performed by reacting ε-aminocaproic acid under a 3 M excess of methyl iodide. The resulting solution was then tested using TLC and mass spectrometry in order to determine the contents of the mixture. Figure 19, shows a spectrum obtained when performing an infusion of the reaction mixture. TLC was also performed to confirm the presence of trimethylaminocaproic acid (not shown). TLC analysis indicated the presence of trimethylaminocaproic acid in the reaction mixture, the presence of ε-aminocaproic acid was confirmed using Dragendorff's reagent which would stain the amine group of the starting material only, indicating that the reaction had not yet completed. The presence of the trimethylaminocaproic acid was confirmed using iodine staining as the methylated caproic acid would not be stained by Dragendorff's reagent. The results showed that there was formation of the trimethylaminocaproic acid, confirmed by the presence of 174.4 m/z, but there was still a large excess of unreacted ε-aminocaproic

acid, confirmed by the presence of 160.3 m/z. The mixture was then reacted with more methyl iodide in order to react the remaining ε-aminocaproic acid. Figure 20, shows that the reaction reacted the remaining ε-aminocaproic acid but also indicated that the methyl iodide had reacted with the carboxylic group to form a methyl ester, methyl trimethylamino caproate, MTMAC, confirmed by the presence of 188.1 m/z. This data was confirmed by using normal phase TLC and a hydroxylamine-ferric chloride stain (not shown). The hydroxylamine-ferric chloride stain selectively stains esters a red/pink colour, which was observed when the TLC plate was stained. The methyl iodide will favour acting upon the more nucleophilic amine group first, which then becomes unreactive when it forms a quaternary amine. Excess methyl iodide will then act upon the carbonyl group of the carboxylic acid, when all amine groups have been converted to quaternary amines, which then forms a methyl ester. The presence of unreacted ε-aminocaproic acid was not seen in the spectrum obtained, and an approximate 85% yield of the trimethylamino caproic acid was obtained. In Figure 21, which is a spectrum obtained after the sample was reacted with sodium hydroxide to remove the methyl ester and acidified to remove the sodium adduct the end result was trimethyl aminocaproic acid, which was confirmed by the observation of an expected mass-charge ratio of 174.3 and the notable absence of 188.1 m/z. The reason behind demethylating the carbonyl group was to form a free acid product that would be able to couple to NHS in the presence of a condensing reagent. If the carboxyl group is not free then the compound cannot be transesterified with the NHS preventing the formation of the active derivatising reagent.

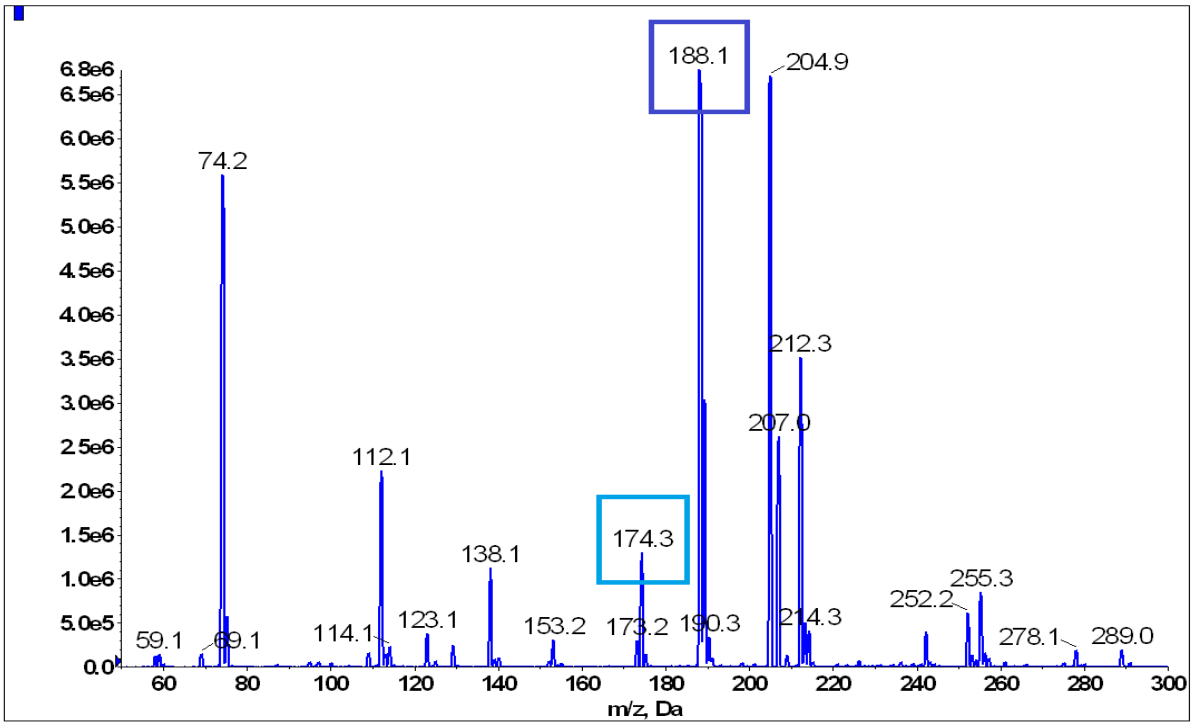


Figure 20: Mass spectrum of methyl-trimethylamino caproate. The spectrum was obtained when performing an infusion of the methyl-trimethylamino caproate mixture. The dark blue box indicates the methyl-trimethylamino caproate compound and the light blue box indicates the trimethylamino caproic acid.

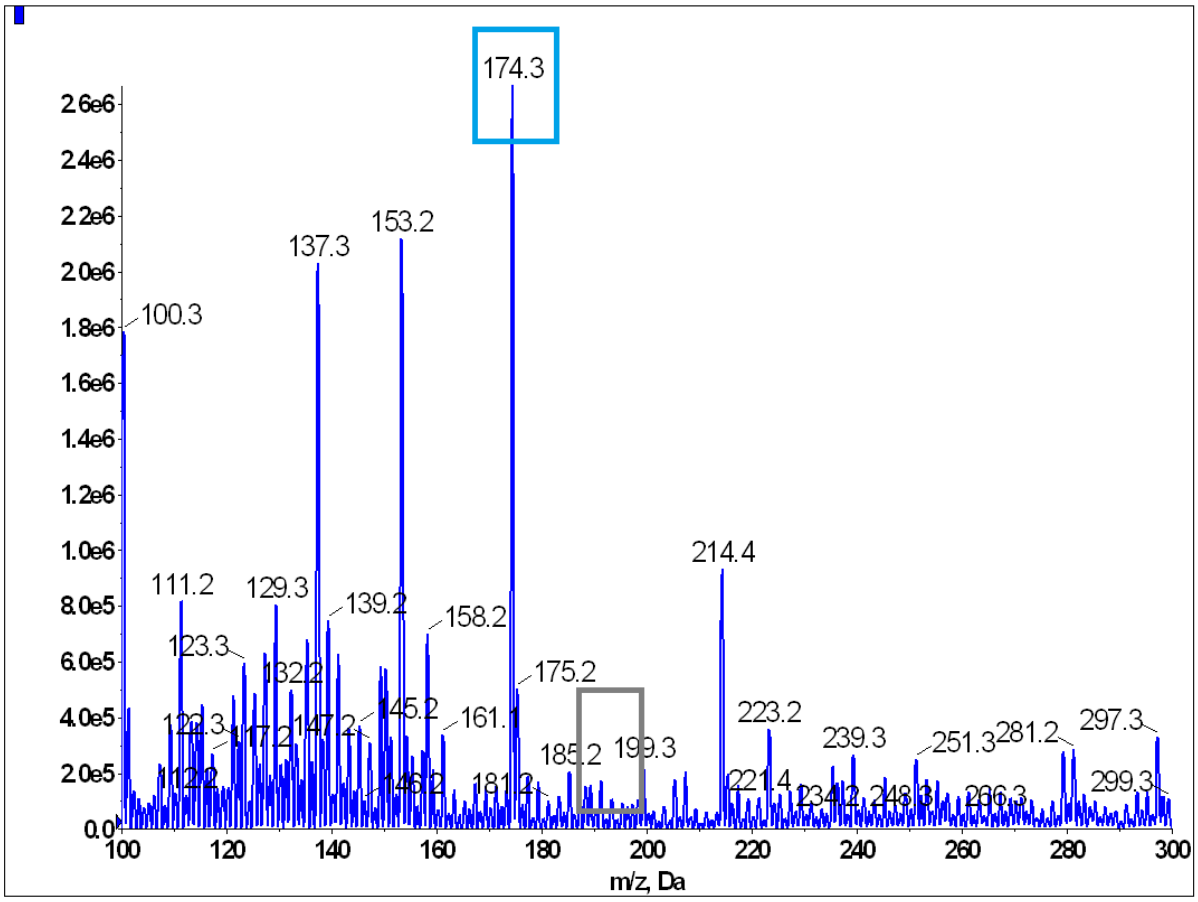


Figure 21: Mass spectrum post-hydrolysis of MTMAC. The spectrum was obtained from an infusion of MTMAC after hydrolysis using sodium hydroxide. The light blue box indicates the trimethylamino caproate compound and the grey box indicates a lack of the methyl ester.

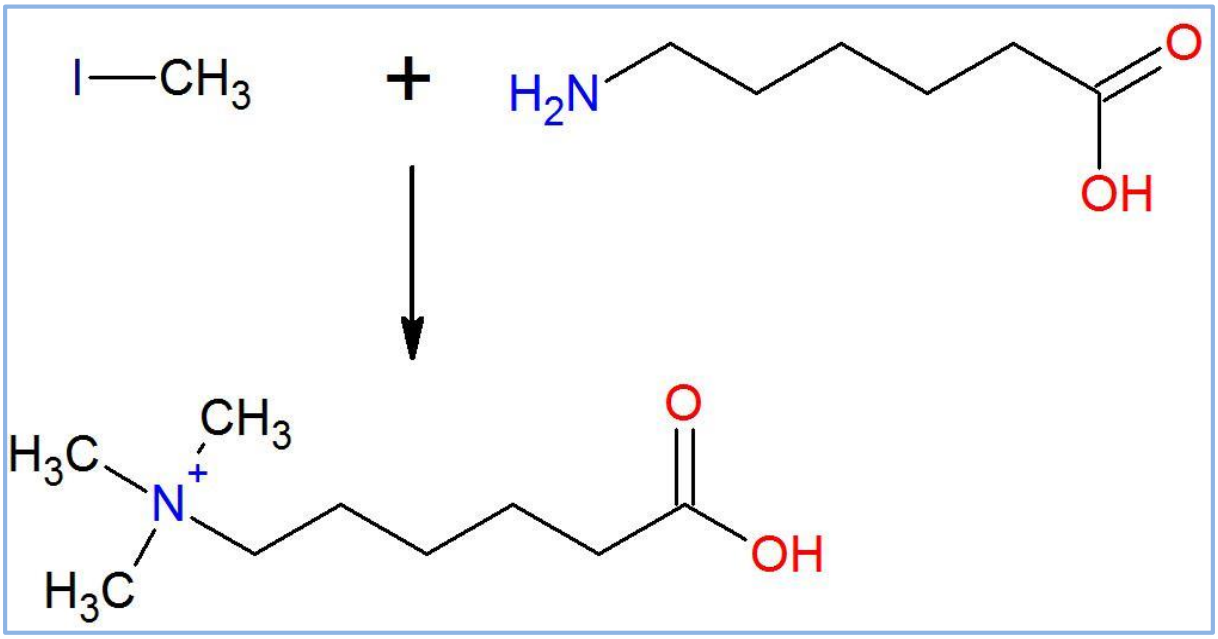


Figure 22: Reaction scehmatic of methyl iodide with ε-aminocaproic acid. Methylation of the primary amine occurs forming a quaternary amine.

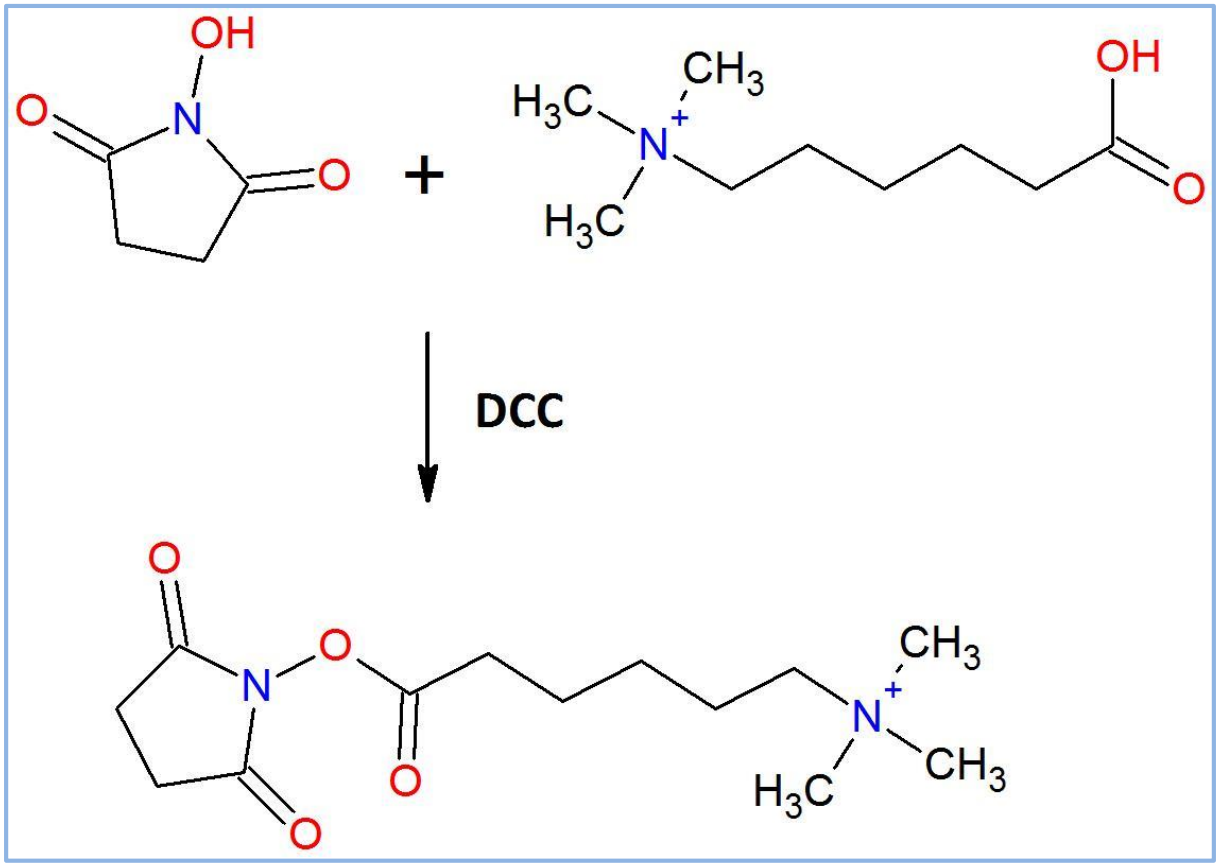


Figure 23: Synthesis of the final active NHS-ester. The figure above is a schematic of the second step of the reaction. This step involved the reaction of trimethylaminocaproic acid with N-hydroxysuccinimide in the presence of DCC to form the active derivatising agent.

The second step of the formation of the NHS-ester was to react the synthesised trimethylaminocaproic acid with N-hydroxysuccinimide with a condensing agent dicyclohexylcarbodiimide. The presence of the synthesised TMAC-NHS ester was tested using TLC and mass spectrometry. The spectrum represented in Figure 24, shows the presence of a mass of 271, which is the mass of the TMAC-NHS ester. The TLC (not shown) also indicated the presence of a TMAC-NHS ester which was located at a different retention factor on the silica plate when compared to the trimethylaminocaproic acid, DCC and NHS alone. The observed results from TLC plates (not shown) results were confirmed using mass spectrometry. The formation of this ester provided an active TMAC-NHS ester that would react with nucleophiles. The resulting solution was the dried down and prepared as needed.

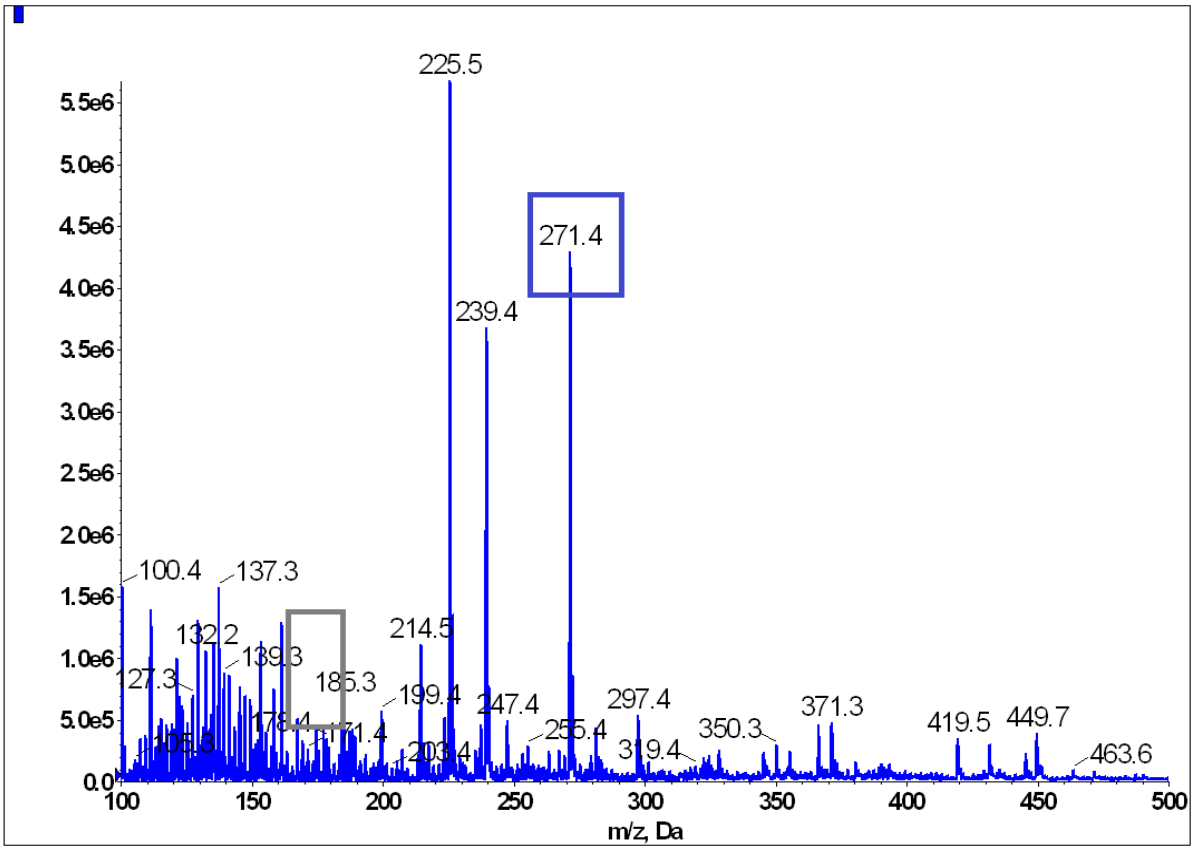


Figure 24: Mass spectrum of the final trimethylaminocaproic N-hydroxysuccinimide derivatising reagent. The spectrum was obtained from an infusion of the solution from the final reaction mixture. The dark blue box indicates the final active derivatising agent, and a lack of the unreacted trimethyl aminocaproic acid ester is indicated in grey. The other peaks are unknown contaminants.

This section emphasises the power that a simple, cheap technique such as TLC can play. TLC was effectively used in this study to provide separation of different reaction products and also selectively stain various reaction products. The use of selective stains that were able to stain free amines and esters were used to determine the status of the reaction, determine reaction products as well as used in conjunction with spectra obtained from the mass spectrometer to monitor the progress of the reaction. The use of these two techniques used for qualitative data provided a simpler way than is traditionally used for reaction monitoring, that although is not as advanced as other techniques, was suitable for use in this study.

3.4 Derivatisation using the NHS-ester

Derivatisation using the synthesised trimethylaminocaproic acid-NHS ester was carried out under mild reaction conditions. The solution was buffered to a pH of 8.3 using a sodium borate buffer and reacted at 40˚C for 10 minutes. This pH was selected to ensure the reaction proceeded rapidly and that the reactive NHS-ester did not rapidly degrade in the presence of water. The sample extraction was done by drying down the reaction solution and then reconstituting in 100 µl of 50:50 methanol:water which was then analysed by LC-

MS/MS. The reaction procedure was simple, using common reagents and could be easily carried out routinely without the use of specialised equipment. In the case of routine laboratory assays there are commercially available NHS based derivatising reagents which would eliminate the need to synthesis the derivatising reagent in-house. Table 9 below indicates the expected masses after derivatisation with the synthesised TM-NHS ester.

Table 9: Expected mass/z values expected to be obtained for TMAC-NHS derivatisation of the BAs

Biogenic amine	Underivatised mass		Derivatised mass		
	M	M+ H^+	1x	2x	3x
Adrenaline	183.2	184.2	339	247	217
Noradrenaline	169.1	170.1	325	240	212
Dopamine	153.1	154.1	309	231	207
Serotonin	176.2	177.2	332	244	
Histamine	111.1	112.1	267	211	
5-MT	190.2	191.2	346	251	

The TMAC-NHS ester proved to be a non-specific reagent as it reacted with the primary and secondary amines as well as the phenolic hydroxyls of the BA. This trend is also noted in other reports during the analysis of amines in which multiple derivatives were noted to form where the derivatisation of phenolic hydroxyl groups also occurred (Yang *et al*, 2006, Kalkhof and Sinz, 2008, Huang *et al*, 2009). This lack of specificity is illustrated in Figure 25. Figure 25 depicts a mass spectrum obtained when derivatising dopamine with the synthesised TMAC-NHS ester. In the spectrum obtained there are four masses that were identified as TMAC derivatives. The first mass is 271.3 m/z, which as noted earlier is the synthesised TMAC-NHS ester. This indicated that there was excess derivatising reagent within the solution for reaction with the dopamine. The second mass of importance noted was 154.4 m/z, which is free ionised dopamine. This means that although there was an excess of the TMAC-NHS ester to react with the dopamine the reaction was not efficient. Possible explanations are the reaction required a higher temperature to force the reaction to completion, the optimal pH of the solution was not maintained or was not optimal or the solution was not allowed to react for long enough. The results indicate that at a temperature of 40°C the reaction was not facile and did not go to completion. This is a major obstacle as any unreacted biogenic amine will not elute at the monitored time and therefore not be detected lowering the sensitivity of the final method and increasing the required sample volumes for quantitative analysis.

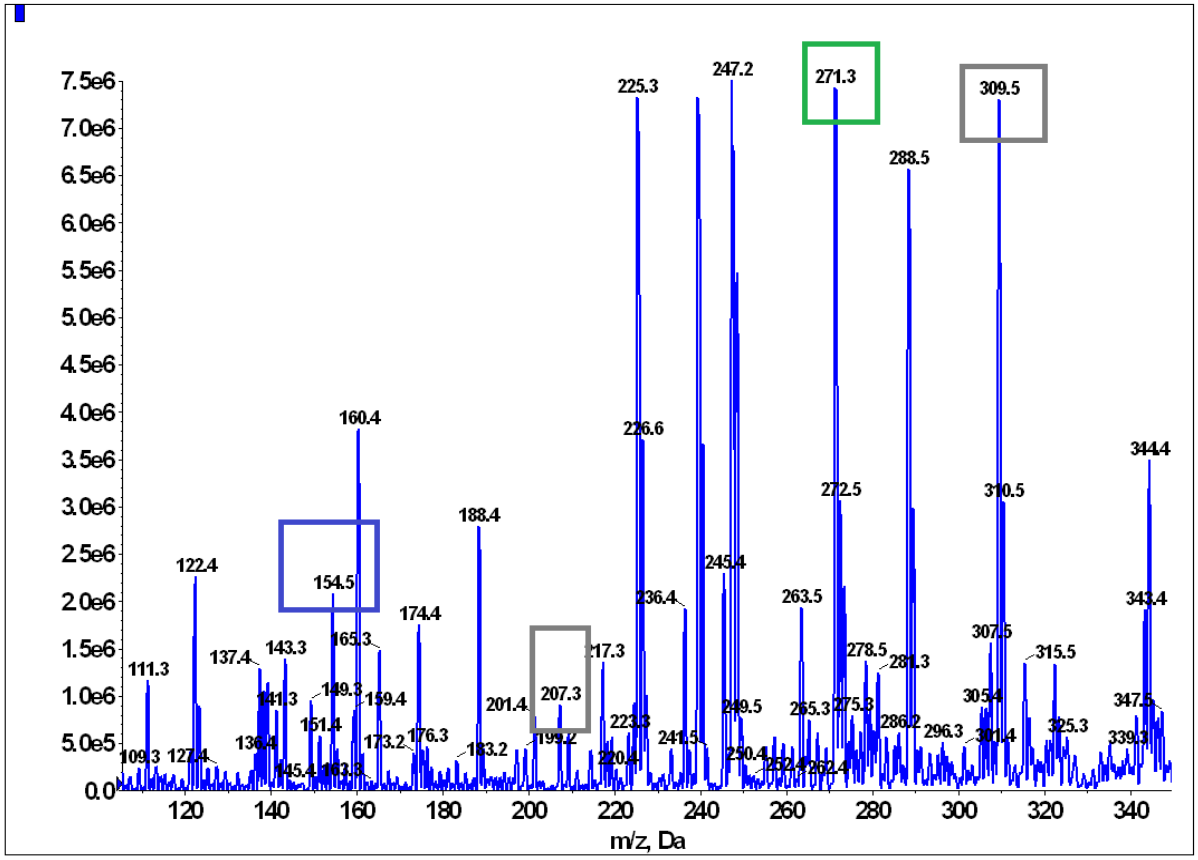


Figure 25: Spectrum of dopamine derivatised with TMCA-NHS ester. The spectrum was obtained from an infusion of dopamine post-derivatisation with the TMCA-NHS-ester. The blue box indicates underivatised dopamine, the grey boxes indicate NHS-ester derivatives and the green box indicates excess TMCA- NHS-ester used for the derivatisation.

The masses of importance noted were 207.2 and 309.5. These masses correspond to dopamine derivatives of the tri- and mono-TMAC-NHS esters respectively. As the number of sites which undergo derivatisation increase the m/z ratio gets smaller, this is due to the permanent positive charge that was introduced by the TMCA derivative. Therefore as the charges increase, the m/z ratio decreases, despite the increasing mass. This effect may be advantageous with overall method sensitivity. Due to the permanent charge which is present, the inlet capillary voltage could be lowered, thus decreasing the ionisation of background and interfering compounds. The multiple charges would decrease the m/z ratio, but during fragmentation may yield products which have a higher m/z ratio, as is often seen in peptide analysis, which will increase the specificity of the assay. These two factors can decrease the noise levels, meaning a larger signal-noise ratio is possible, increasing sensitivity.

This result indicated that the reaction was non-specific and reacted with either the phenolic hydroxyl or amine groups. Although the dopamine was derivatised and formed both mono- and tri-NHS esters, in this instance the presence of a doubly derivatised dopamine was not found. This was also confirmed by chromatographic data (not shown), which depicted the presence of only single and triply derivatised dopamine. This is an unusual observation as

the presence of a triply derivatised catecholamine normally implied the presence of a double derivatised dopamine. The presence of a di-NHS ester was almost certainly formed, but was not detected. This lack of di- and tri-NHS esters can be attributed to the sub-optimal reaction conditions which did not force the reaction to completion. These conditions could be further manipulated in order to increase the reaction efficiency and force the reaction to completion, in-turn yielding a single derivative for these analytes.

The ability of these compounds to separate on a C18 column was tested. This was done by performing multiple reaction monitoring (MRM) at extremely low collision energies, during preliminary testing, in order to prevent fragmentation of the analytes. Figure 26 depicts the chromatogram obtained when attempting to separate the serotonin-TMAC derivatives on a C18 column. The results indicate that although the compound derivatised it remained highly polar and was not effectively retained on the C18 column. The mono-TMAC ester of serotonin eluted on the void volume with underivatised serotonin. This was observed for a majority of the derivatised BA analytes, while those that were able to be retained showed minimal retention on various C18 columns. The low retention of the derivatives are further confirmed by the observation that these compounds were better retained on a HILIC column, as shown in Figure 27. The results show that underivatised serotonin is more polar than the derivatised serotonin, but only marginally. The poor retention on the C18 column may be due to the relatively short aliphatic chains imparting minimal added lipophilicity but adding a permanent charge onto the polar biogenic amines, offsetting the lipophilicity imparted by the aliphatic chain. Broader, tailing peaks observed in the chromatograms are probably due to the larger particle size of the column, 5 µm, as well as secondary reactions due to the positive moiety and adsorption effects often seen with HILIC columns.

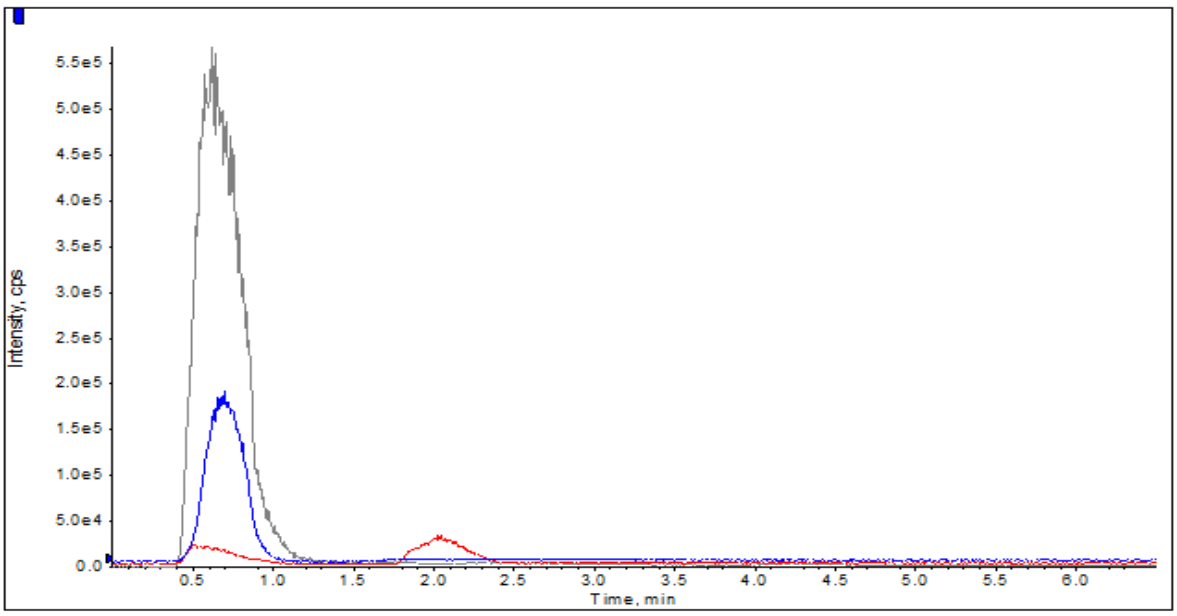


Figure 26: Chromatogram of serotonin derivatised with the TMAC-NHS ester. This chromatogram was obtained using a C18 column. The light blue peak is a mono-NHS derivative while blue is underivatised serotonin. The chromatogram shows the analytes are still polar and are unretained on a C18 column.

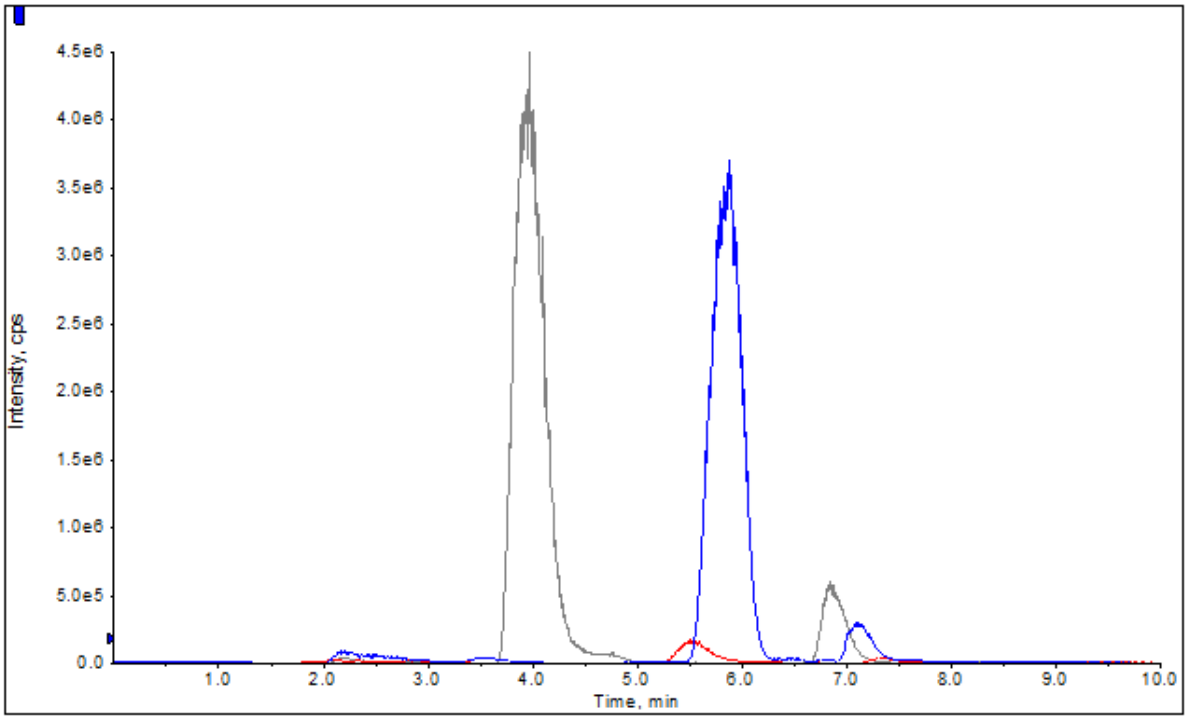


Figure 27: Chromatogram of derivatised serotonin using HILIC separation. This chromatogram was obtained using a HILIC column. The light blue peak is a mono-TMCA derivative while dark blue is underivatised serotonin. The chromatogram shows the analytes are polar and are retained on a HILIC column.

The use of an NHS-ester for derivatisation was an attempt to gain more specificity than ethyl chloroformate. The results indicate that the TMAC-NHS ester was still non-specific and was also prone to multiple derivative formation, especially noted with the catecholamines and equal molar ratios of the TMAC-NHS ester. The results indicated that the reaction was not facile upon the addition of a large molar excess of TMAC-NHS ester, and resulted in a high abundance of underivatised biogenic amine and multiple TMAC-NHS derivatives. The observed results may be attributed to the low reaction temperature, inefficient buffering capacity or the use of an aqueous buffer competing for the TMAC-NHS ester. The reaction conditions were optimum at a neutral pH, and at an alkaline pH the TMAC-NHS ester was not stable and was rapidly hydrolysed. The derivatised biogenic amines were also not stable at an alkaline pH and were hydrolysed when the pH was altered during the derivatisation process, highlighting the need of a suitable buffer with adequate buffering capacity. The derivatised biogenic amines also exhibited poor retention on various C18 columns and a mixture of derivatised biogenic amines was injected onto various C18 columns types to assess the retention of the derivatised compounds. The results indicated that even tri-TMAC derivatives were still highly polar and were unretained on a C18 column. These conclusions regarding the polarity were confirmed when the TMAC-NHS derivatives were analysed using a HILIC column. The derivatised biogenic amines were retained on the HILIC column, indicating that the analytes were polar. The permanent cationic moiety on the TMAC-derivatives decreased the m/z ratio of the analytes into the high abundant low molecular

weight impurities and during Q1 scans were often masked among the abundant impurities. One reason to use derivatisation was to make use of more sensitive RP chromatography conditions due to the higher organic content of the mobile phase, however the fact these analytes were not sufficiently retained on a C18 did not meet the objectives of using derivatisation. This may not be the case when using MRM-based quantitative method, and may increase the sensitivity of the method, but this was not tested during the course of this study. The reaction was not specific nor facile and the required time consuming synthesis of the reactive ester meant that the TMAC-NHS ester did not undergo further testing, in favour for a commercially available and more reactive derivatising agent. The study demonstrates the versatility that an N-hydroxysuccinimide ester can offer, provided the synthesis can be easily performed. The simple reaction of N-hydroxysuccinimide to a carboxyl group can easily be performed, and can provide a unique derivatisation reagent, such as the one synthesised in this study, which can be modified to a specific study. The variety of compounds which could be coupled to N-hydroxysuccinimide are numerous and can impart a variety of unique properties in order to fully utilise the techniques and instrumentation available to researchers.

3.5 Derivatisation using dansyl chloride

Derivatisation with dansyl chloride was a simple procedure and was reacted under relatively mild reaction conditions. The optimum conditions for the reaction were found to be at a slight alkaline pH of 8. The solution was left to react for 60 minutes at 23°C in order to ensure the completion of the reaction. The reaction could easily be carried out routinely and for a large number of samples with commonly used reagents. Dansyl chloride is also commercially available reducing the need for the synthesis of the active derivatisation reagent.

Table 10: Expected masses after derivatisation with dansyl chloride

Biogenic amine	Underivatised mass		Derivatised mass		
	M	M+ H^+	1x	2x	3x
Adrenaline	183.2	184.2	417.7	651.0	884.1
Noradrenaline	169.1	170.1	403.7	637.0	870.1
Dopamine	153.1	154.1	387.7	620.9	854.1
Metanephrine	197.2	198.2	431.2	664.1	
Normetanephrine	183.2	184.2	417.3	650.1	
Serotonin	176.2	177.2	410.7	644.0	
Histamine	111.1	112.1	345.2	578.8	
Melatonin	232.2	233.2	466.2		
5-hydroxyindole acetic acid	191.1	192.1	425.2		

Table 10 describes the expected masses that will be obtained after derivatisation with dansyl chloride and includes all derivatives. Results indicated that dansyl chloride was a non-specific derivatising reagent. As with the already tested derivatisation reagents, dansyl chloride also gave multiple derivatives, and this was more notable for the catecholamine class of BA and when using a low molar excess of DNSCl. This effect is observed for the same reasons as the TMAC-NHS ester and ethyl chloroformate; multiple reaction sites on the BA and the non-specific reaction mechanism of dansyl chloride. Due to the simplicity of the reaction and easy availability of dansyl chloride, the reaction conditions were optimised to yield the highest possible amount of derivatised biogenic amines, which were reacted at all available sites, to increase the sensitivity of the assay. The following reaction conditions were optimised: pH, temperature, reaction time and molar excess of dansyl chloride. Three different pH levels were tested; 8, 9.2 and 10. Higher pH levels were not tested due to the increased hydrolysis of dansyl chloride, which would decrease the availability of the dansyl chloride for derivatisation. At the three different pH levels, three different temperatures were assessed; room temperature, 40°C and 60°C. The combination of these two parameters which yielded the highest amount of derivatised BA were used to assess four different time points; 15, 30, 45 and 60 minutes. The samples were then analysed using the optimised method and the area under the peaks (AUC) of the derivatised biogenic amines were obtained and compared to determine the optimum reaction conditions. The results of the optimisation of the reaction are shown below in Figures 29, 30 and 31.

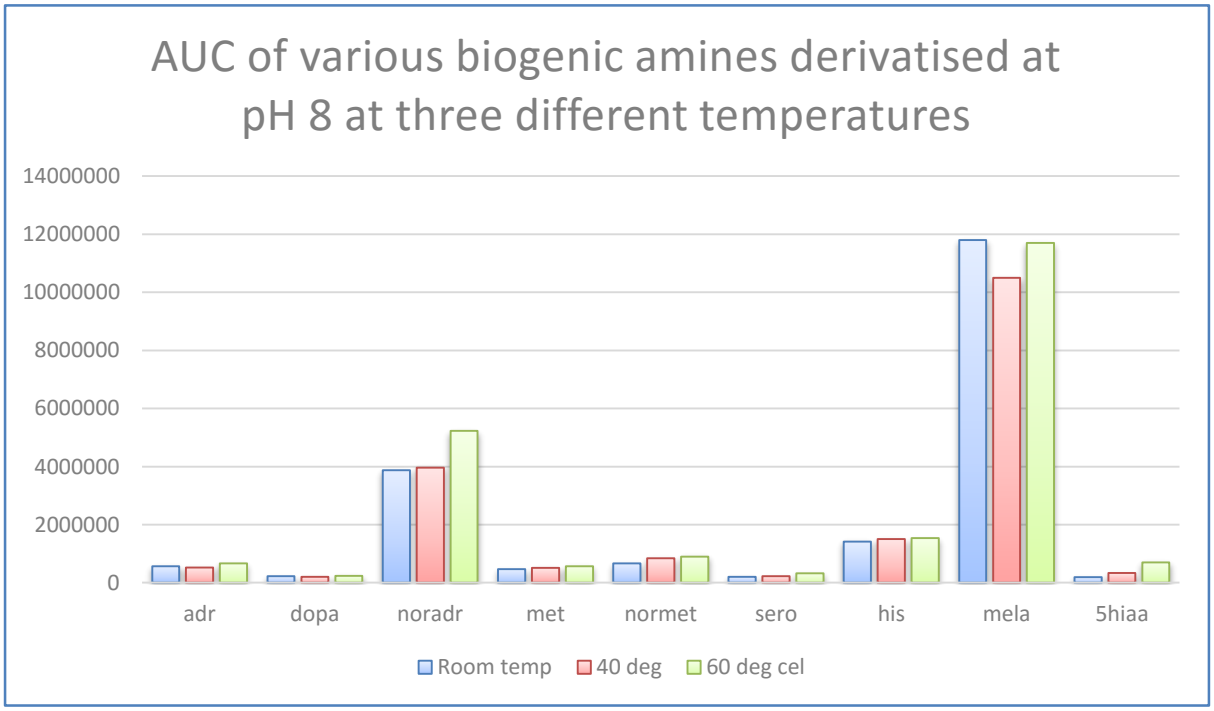


Figure 28: Peak areas of dansyl derivatives derivatised with dansyl chloride reacted at pH 8. Adr – adrenaline; dopa – dopamine; noradr – noradrenaline; met – metanephrine; normet – normetanephrine; sero – serotonin; his – histamine; mela – melatonin; 5HIAA – 5-hydroxy-3-indole acetic acid

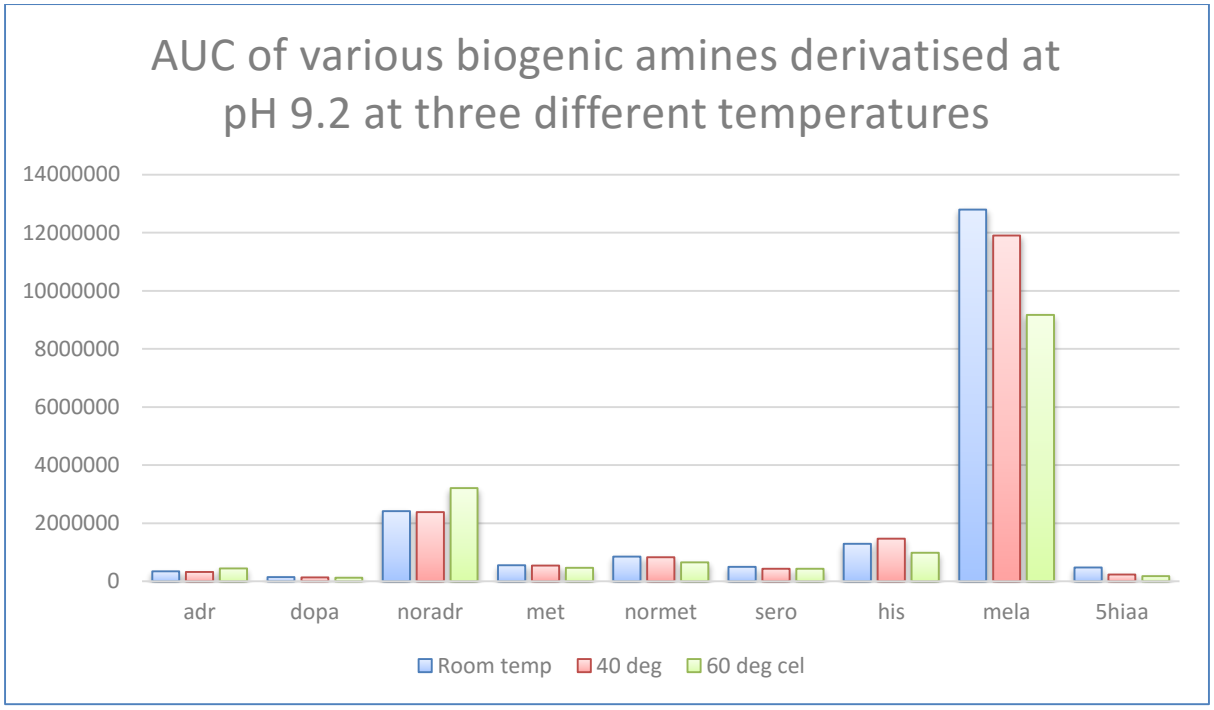


Figure 29: Peak areas of dansyl derivatives derivatised with dansyl chloride reacted at pH 9.2

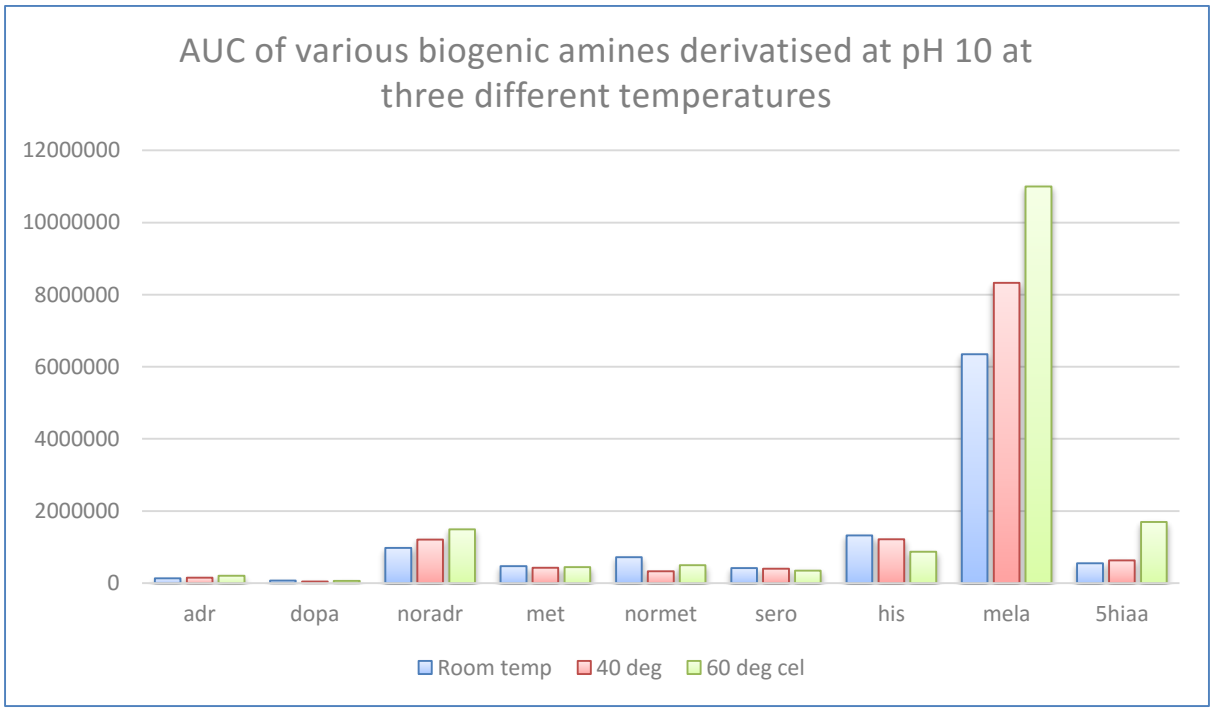


Figure 30: Peak areas of dansyl derivatives derivatised with dansyl chloride reacted at pH 10.

The results show that certain biogenic amines did favour the formation of derivatives under different conditions. The results indicate that at a pH of 10 there was a decrease in the amount of derivatised biogenic amines being formed in the reaction. The reason for this is the high pH causes DNSCl to rapidly hydrolyse in the presence of water, reducing the amount of available DNSCl to react with the biogenic amines. At a pH of 9 the hydrolysis of DNSCl also occurred but at a slower rate, and the results indicated that there were more

derivatised biogenic amines formed from the reaction. At pH 8 the reaction formed increased amounts of derivatised biogenic amines. This is indicated by an increase in the AUC of the majority of the biogenic amines assessed. The reaction proceeds rapidly at a neutral pH and the effect of hydrolysis is minimised at this pH. This effect has been well documented since the year 1969 and has been reported in papers (Gros and Labouesse, 1969). This was also noted upon visual inspection of the samples in which the derivatisation solution turned from orange (dansyl chloride forms an orange solution) to an opaque solution in a shorter time frame at higher pH.

The results indicate that at pH 8 the optimal temperature will vary for each biogenic amine with minimal differences between the three temperatures assessed. Reactions carried out at room temperature allow for easier large scale application of the method in routine analyses. As the reaction temperature increases so does the rate of hydrolyses of DNSCl, which will reduce sensitivity if it exceeds the derivatisation process. The increased presence of dansylated biogenic amines is due to the increased availability of reactive dansyl chloride which has not been hydrolysed. The conditions for the remainder of the study used were pH of 8 and reacting at room temperature. The derivatisation conditions used in other studies vary widely from short reaction times at high pH and temperature (Cai *et al*, 2009, Nirogi *et al*, 2013) to high pH and long reaction times with milder temperatures (Jia *et al*, 2011) to high temperature and lower pH (Kang *et al*, 2006). The difference in observed optimal reaction conditions highlights the need to optimise the derivatisation reaction conditions to ensure a complete derivatisation to increase the sensitivity of the assay. A reason that may explain the difference reaction conditions is the amount of DNSCl added to the analyte. A large excess of DNSCl may force the reaction the completion, which is also seen when heating the reaction solution.

The derivatisation pattern of multiple dansyl derivatives observed in this study has been noted in previous reports on biogenic amines as well as other polyamines and reinforces the non-specific reaction mechanism of dansyl chloride (Cai *et al*, 2009, Ducros *et al*, 2009, Cai *et al*, 2011b, Jia *et al*, 2011, Nirogi *et al*, 2013). At low molar ratios of dansyl chloride mono- and di-dansyl derivatives of the catecholamines has been observed, as well as mono-dansyl derivatives of histamine, serotonin and the metanephrines. Histamine, serotonin, metanephrine and normetanephrine when reacted to completion formed di-dansyl derivatives. Melatonin was the only biogenic amine that was reported to not react with dansyl chloride despite the fact it has two available amine groups. This is due to alteration of the primary amine on the aliphatic chain and the phenolic hydroxyl groups, which have reduced reactivity when compared to serotonin. The amine in the pyrrole ring appears to be stable and does not react under the mild conditions used in this study, however this reaction appears to occur in the case of histamine. To the extent that the literature was reviewed, melatonin is analysed in native format and is not derivatised except a few reports using hydrogen peroxide (Iinuma *et al*, 1999). The reaction conditions did effect the observed amount of melatonin present in solution, indicating that melatonin may be

degraded by a high pH, and at pH 8 the differences in melatonin were negligible. The presence of underivatised (except for melatonin) and partially derivatised biogenic amines was not observed when reacted using the optimised reaction parameters.

Table 11: Summary of the different derivatising agents tested

Derivatising agent	Mass Addition	Charged	Lipophilicity	Multiple derivatives formed
Ethyl Chloroformate	73	No	Increased	Yes
N-Hydroxysuccinimide	156	Yes	Slight increase	Yes
Dansyl Chloride	233	No	Greatly increased	Yes

A total of four different derivatisation reagents were assessed in this study. Table 11 above summarises the various derivatising reagents tested. Ethyl chloroformate was a simple reaction carried out at room temperature, however eruption resulted in sample loss especially when adding large volumes. It did increase the lipophilicity of the analytes and they were retained on a C18 column and despite the formation of multiple derivatives, separation was achieved for the analytes tested with good peak shape. The second reagent tested used 1-fluoro-2, 4-dinitrobenzene, however the final derivatives were not detected in this study. The third reagent tested was an N-hydroxysuccinimide ester. This is the most versatile of all the tested reagents as N-hydroxysuccinimide can be coupled to numerous compounds and be tailored to suit the needs of a specific assay. In this study an aliphatic quaternary amine was synthesised to provide an increase in signal intensity and increased retention on a C18 column. Although it provided an increase in signal intensity the positive charge meant the derivatised analytes were still highly polar and the analytes were minimally retained on a C18 column. This would necessitate the use of a less sensitive HILIC based method. The synthesis was a simple, multistep reaction that may assist with more specific assays rather than large scale analysis or routine laboratory. The last derivatisation reagent tested was dansyl chloride. It has been shown in literature and in this study that it increases the sensitivity of biogenic amine detection as well increases their retention on C18 columns. It was shown that despite the formation of multiple derivatives under sub-optimal reaction conditions, the reaction could be optimised to ensure the reaction was facile and formed a single derivative. The reaction also proceeded to completion under mild reaction conditions without using harsh buffers, catalysts or other compounds that would influence downstream analysis. The fully derivatised analytes were also highly lipophilic and a simple liquid-liquid extraction could be performed from an aqueous derivatisation solution. Due to the commercial availability of this reagent as well as the mild reaction conditions it was selected as the derivatisation reagent of choice.

3.6 Optimisation of mass spectrometric and chromatographic conditions for dansylated biogenic amines

Prior to the optimisation of the derivatisation conditions a mixture of biogenic amines was reacted at pH 8 at 50°C for 30 minutes. The resulting solution was then dried down and reconstituted in an acetonitrile: water mixture. The reconstituted derivatives were initially infused into an ABSciex 4000 QTrap using a Harvard syringe and pump at a constant flow rate of 20 µl/min. During the course of this study two different sources were investigated to determine which provided the greater sensitivity. The use of the APPI source necessitated the introduction of a dopant into the as mobile phase post column to allow for ionisation and detection of the dansyl derivatives during the tuning step. Initial Q1 scans were performed to determine the derivatisation pattern of the biogenic amines, and through the use of the ramp function optimal declustering potentials for each dansyl derivative was determined. Following Q1 scans, enhanced resolution scans were performed to aid in the confirmation of derivatives using observed versus expected mass and isotopic distribution of the dansyl derivatives. The identified dansyl derivatives were then fragmented, using MS^2, to determine the optimal product-precursor pairs and collision energies.

Table 12: Optimised conditions for a quantitative MRM method after biogenic amines were derivatised using dansyl chloride.

MRM Pairs of dansylated biogenic amines

Biogenic amines	Precursor	Product			
		Low energy	Collision energy	High energy	Collision Energy
Adrenaline	884.1	397.4/537.6	45	170.1/171.1	72
Noradrenaline	870.1	636.9/851.7	40	170.1/171.1	70
Dopamine	854.1	384.4/619.8	40	170.1/171.1	70
Metanephrine	665.0	646.6/412.0	30	170.1/171.1	55
D3-Metanephrine	668.1	651.6/415.0	30	170.1/171.1	55
Normetanephrine	650.7	554.7	32	170.1/399.3	45
Serotonin	643.7	146.2/235.4	52	170.1/235.4	55
Histamine	578.4	234.5/315.0	30	170.1/315.0	50
5-hydroxyindoleacetic acid	425.4	381.5/382.5	25	170.1/171.1	45
Melatonin	233.3	174.1	22	216.3/174.4	22

Table 12 shows the optimal collision energies and product-precursor pairs at both low and high collision energies. It was observed that at low collision energy the optimal MRM pairs for the dansyl derivatives was different when compared to high collision energies. It was noted that at high collision energies the emergence of similar fragmented products was observed - 170.1/171.1 m/z for all the BA derivatisation products. The reason this occurs is due to the release of the common dansyl moiety. The fragmentation occurs on the dansyl group between the sulphur and naphthyl groups, resulting in an observed protonated fragment product of 171.1 m/z. Increasing the collision energy causes more fragmentation at this site, forming these common product fragments, which are then detected. The formation of a fragment product of 170.1 m/z is believed to be an ionised radical due to the same fragmentation site as 171.1 m/z fragment products. The reason as to why a dansyl derivatives forms both a protonated and an ionised radical can only be alluded to. It is postulated that the cyclic structure of the naphthyl group can more readily donate or accept electrons allowing for the formation of protonated or ionised radicals. The formation of this ionised radical seem to be tentatively linked to compounds which have more hydroxyl-dansyl derivatisation moieties, with amine-dansyl moieties favouring the formation of the protonated product, this however, is not decisively proven. The formation of the common pairs was seen for both the APPI and ESI ionisation source and has been observed or monitored in quantitative methods in other reports (Cai *et al*, 2009, Nirogi *et al*, 2013).

When using the APPI ionisation source in this study it was observed that the dopant (toluene and acetone) composition and flow rate played a significant role in the ionisation efficiency and pattern of dansylated biogenic amines. The overall sensitivity increased as dopant flow rate increased. The results showed increased ionisation of dansyl derivatives up to a maximum dopant flow rate tested of 300 µl/min. This flow rate was the same as the flow rate of the chromatographic method, which conflicts with the recommended dopant flow rate guidelines of 10% of total chromatographic flow rate. The high dopant flow rates caused safety concerns due to the evaporation of the toluene. Thus the flow rate of the dopant was decreased to 200 µl/min which allowed for safe laboratory conditions as well as reasonable sensitivity of the assay. Dansyl derivatives started forming ionised radicals at higher dopant flow rates. An explanation for this phenomenon is the relative proton affinity of toluene is higher than that dansyl derivatives. At high dopant flow rates the formation of ionised radicals was increased, due to the process of ejecting an electron from the dansyl derivatives rather than the toluene, causing the ionised radical to form. This effect was only be seen at the higher dopant flow rates due to the increased amount of toluene in the mixture, which causes more formation of the ionised radical than at lower flow rates. This effect could be prevented by changing to an acetone: toluene dopant composition. The acetone has a lower proton affinity than toluene and allows effective protonation of dansyl derivatives. Another possible explanation for this phenomenon is that the dansyl moiety is strongly UV absorbent and absorbs the UV light from the APPI lamp, acting as a ‘dopant’. The dansyl derivative would then eject an electron and form a positively charged radical. It

is postulated that this phenomenon occurring with toluene is due to the relative proton affinities and the formation of odd-electron cations of aromatic compounds that has been noted in a review article by Marchi et al, (2008). This effect of charged radical formation is observed when using APPI to analyse estradiols and perfluorinated compounds, which have aromatic moieties (Song *et al*, 2007, Li *et al*, 2008). This effect however has not been noted for dansylated biogenic amine detection in previous literature, due to the prevalence of ESI source selection rather than APPI. The use of the acetone: toluene dopant was used for the remainder of the study when analysing samples using the APPI ionisation source.

The formation of radicals could be manipulated by altering dopant flow rate and composition where the use of an ESI source formed only protonated dansyl derivatives. The fragmentation of these compounds could form either protonated or ionised radical fragment products. It was found that the sensitivity of the method could be increased significantly by turning both the Q1 and Q3 selectors from 'unit' to 'low' settings. This means that the system will include compounds in a wider window to the mass stipulated in the settings. The 'unit' selection allows compounds in a 0.1 Dalton window. Allowing a wider window allows more ions to reach the detector and increases the signal intensity. The use of a wider resolution on the Q1 selector also increases the noise as it can allow more interfering compounds to be detected, and may subsequently decrease the S/N ratio, decreasing overall sensitivity.

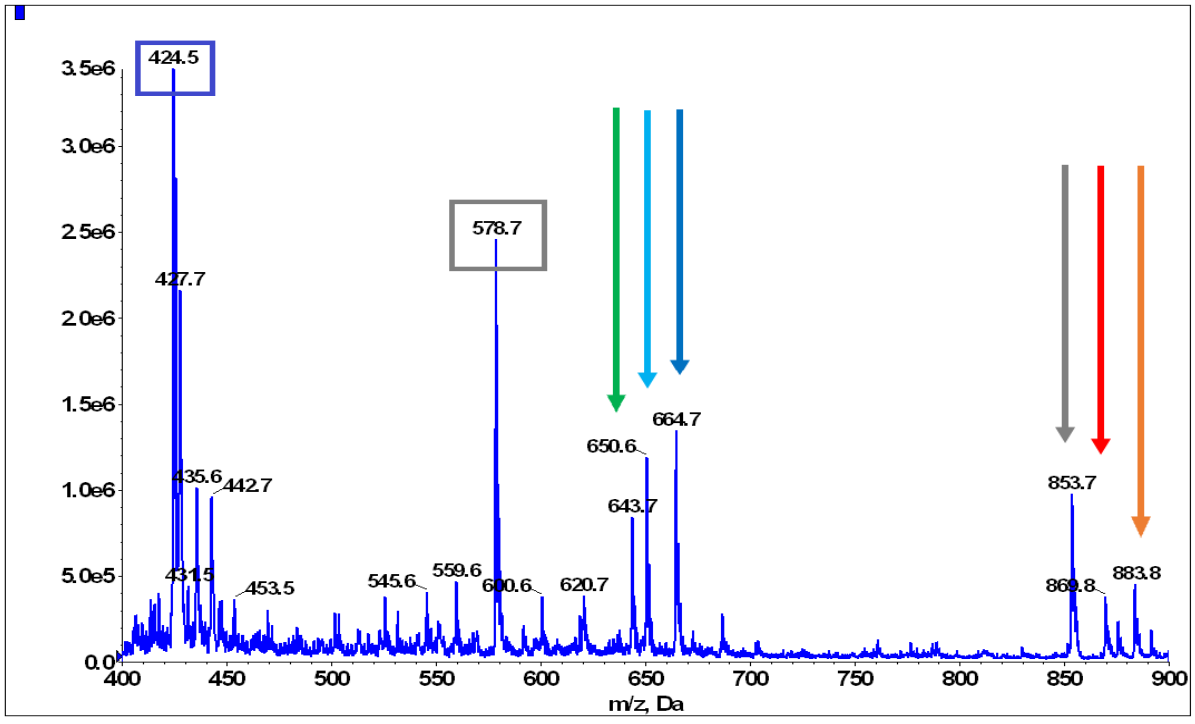


Figure 31: Mass spectrum of BA derivatised with the optimised derivatisation conditions using DNSCl. This spectrum was obtained by an infusion of the derivatisation solution and an ESI source. The analytes are indicated as follows: Blue box- 5-methoxytryptamine and 5-hydroxyindoleacetic acid, Grey box- Histamine. Arrows (Left to right): Green- serotonin, Light blue- Normetanephrine, Blue- Metanephrine, Grey- Dopamine, Red- Noradrenaline, and Orange- Adrenaline

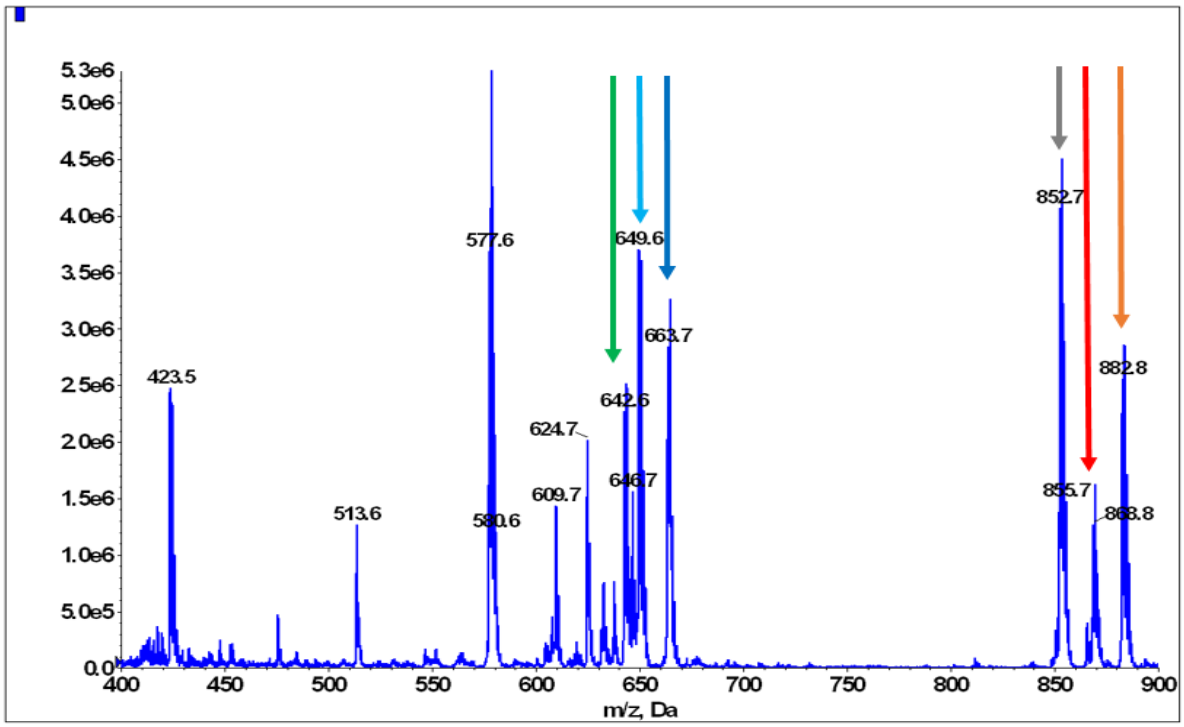


Figure 32: Mass spectrum obtained from an infusion of dansylated BA obtained using an APPI source and a toluene only dopant at high flow rate. The presence of ionised radicals indicated with arrows: Green - serotonin, Light blue – normetanephrine, Blue – metanephrine, Grey – dopamine, Red – noradrenaline and Orange - adrenaline

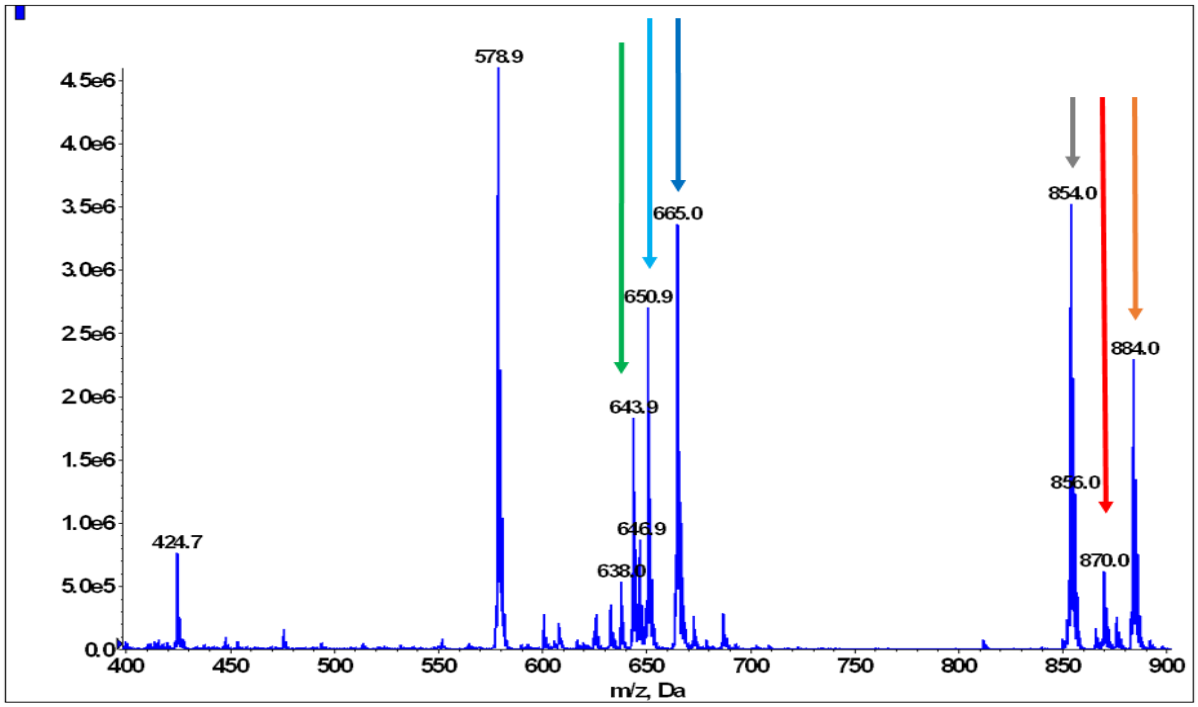


Figure 33: Mass spectrum obtained from an infusion of dansyl derivatives. Obtained using an APPI source and a toluene:acetone dopant. The arrows indicate the absence of ionised radicals: Light blue - serotonin, grey – normetanephrine, green – metanephrine, light blue – dopamine, grey – noradrenaline and green adrenaline

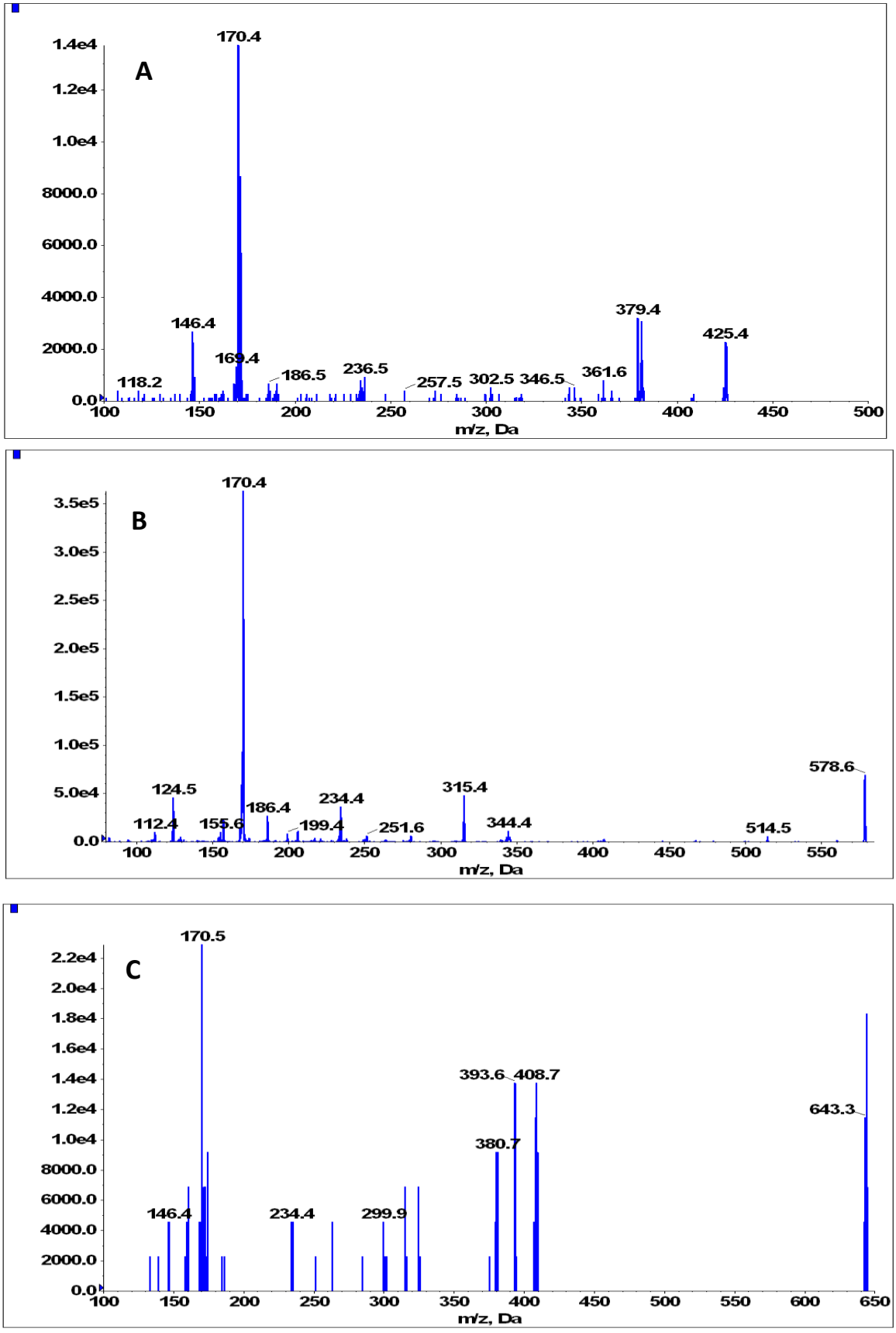


Figure 34A: Mass spectra of precursor-product pairs of dansylated derivatives obtained from an infusion of the derivatisation solution, using an ESI source and MS^2 scans. A – 5-hydroxy-3-indole acetic acid. B – Histamine. C – Serotonin.

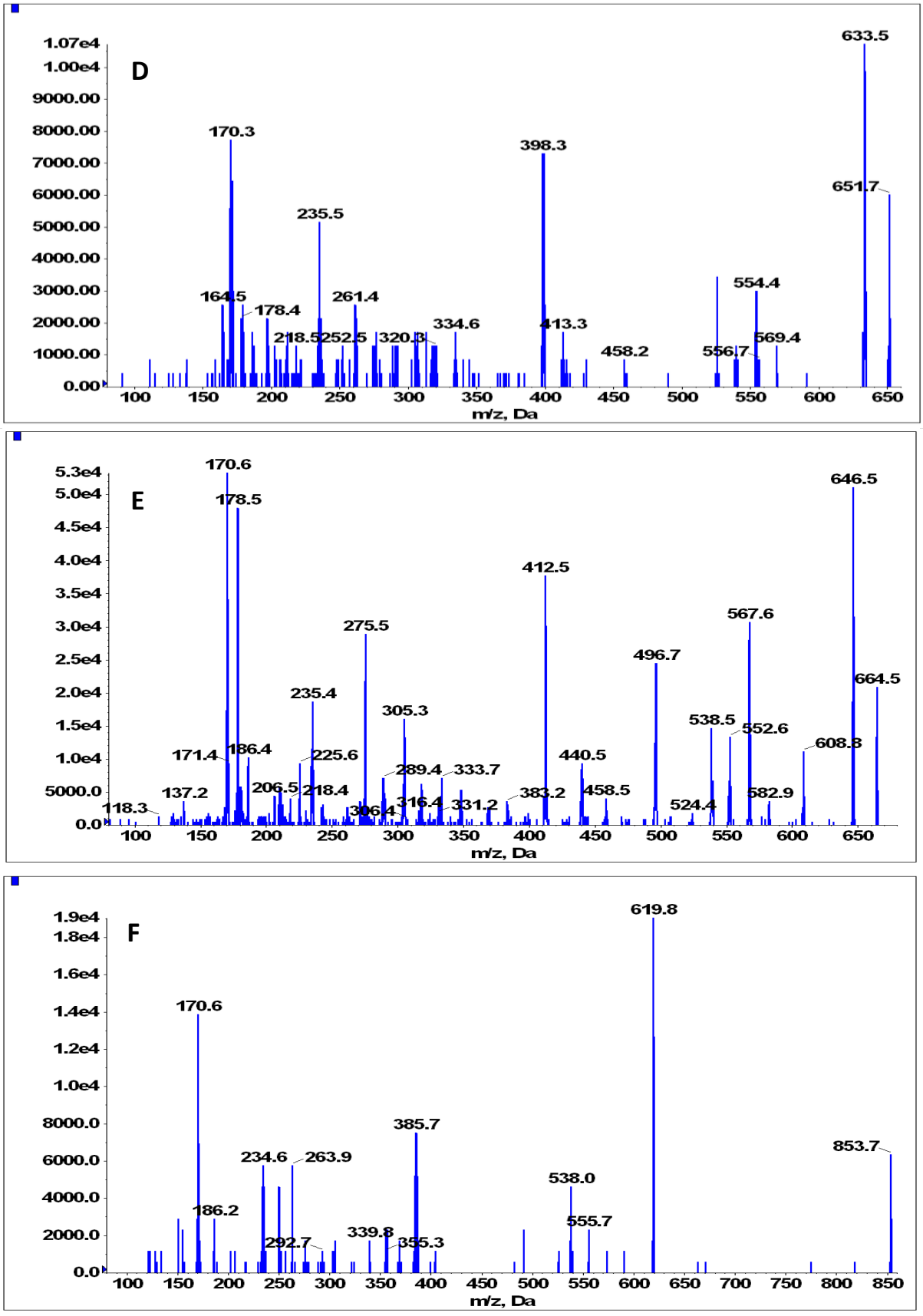


Figure 34B: Mass spectra of precursor-product pairs of dansylated derivatives obtained from an infusion of the derivatisation solution, using an ESI source and MS^2 scans. D – Normetanephrine. E – Metanephrine. F – Dopamine

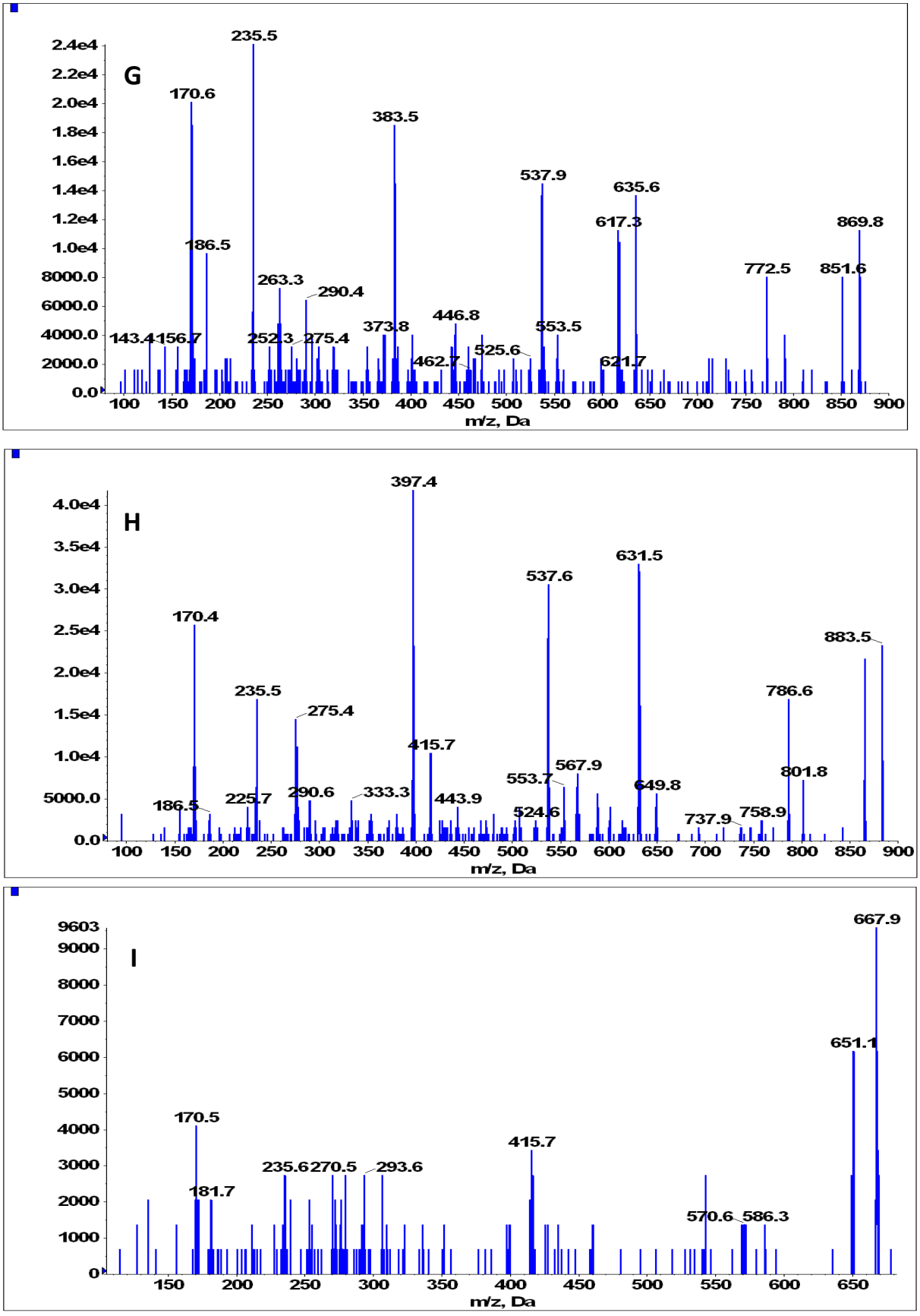


Figure 34C: Mass spectra of precursor-product pairs of dansylated derivatives obtained from an infusion of the derivatisation solution, using an ESI source and MS^2 scans. G – Noradrenaline. H – Adrenaline. I – D3-Metanephrine

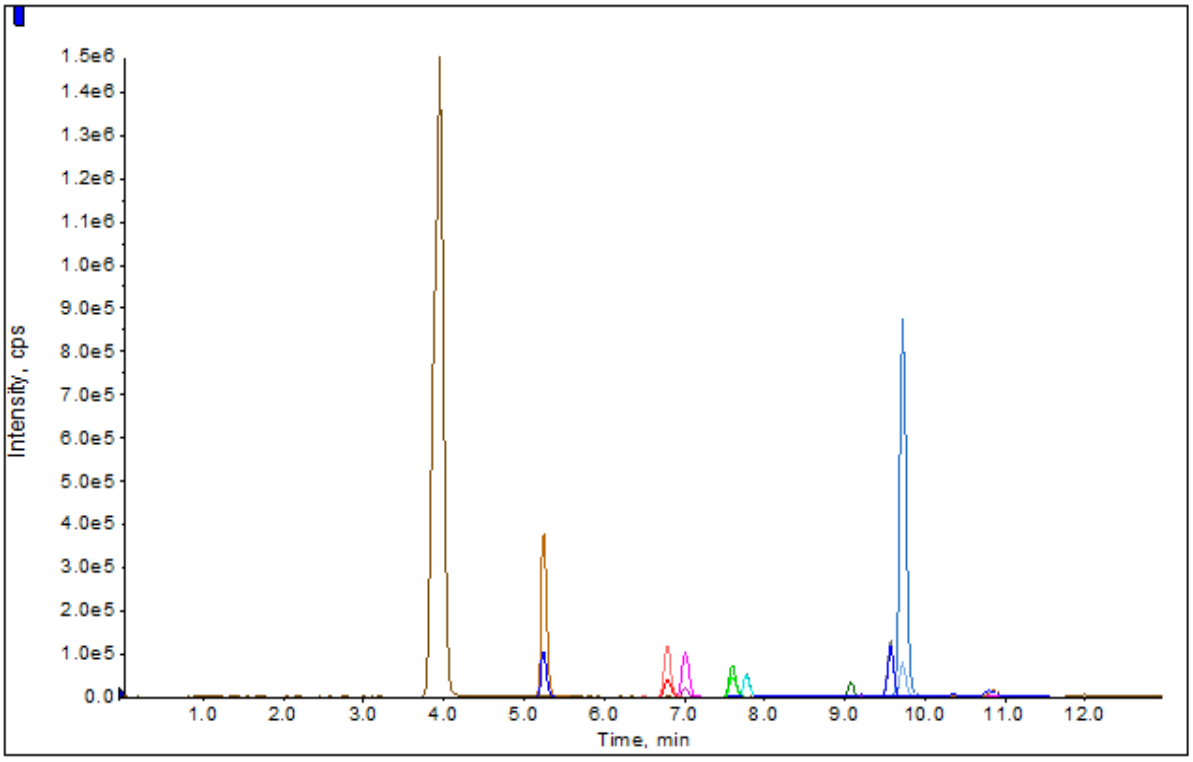


Figure 35: Chromatogram obtained of dansyl derivatives. Derivatised under the optimised conditions and using the optimised LC-MS/MS method. The mass selectors were set to “unit” resolution on Q1 and Q3. The intensity on the y-axis is lower than in Figure 36.

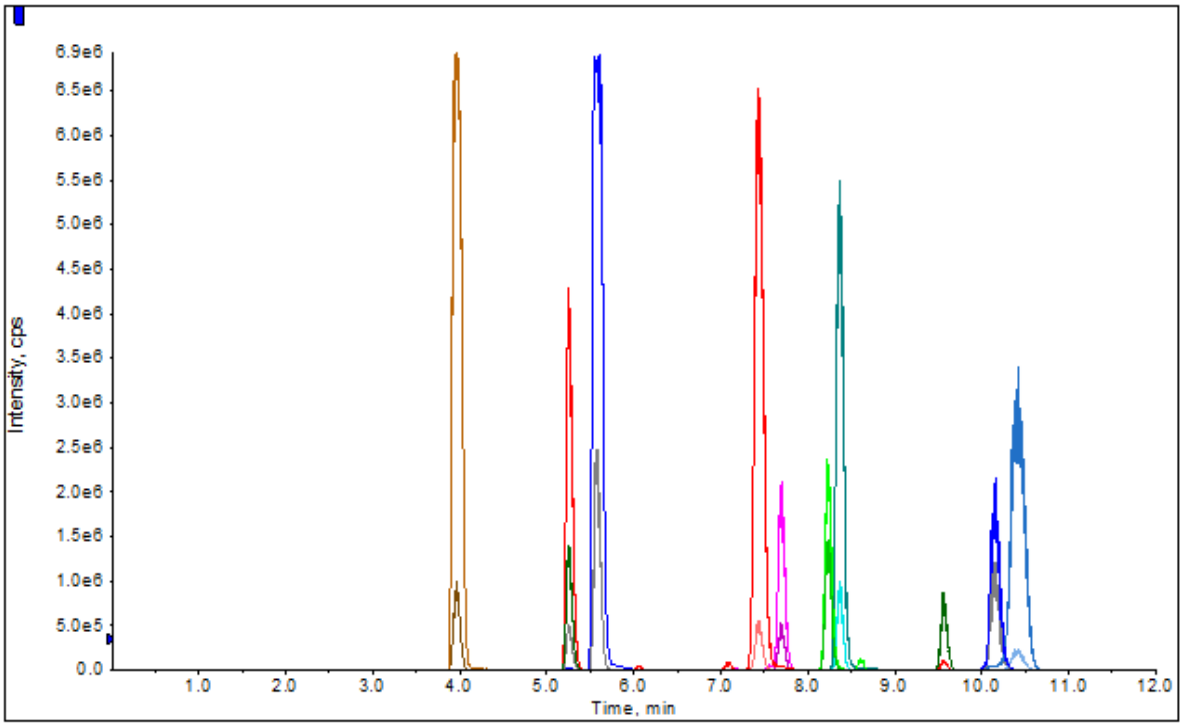


Figure 36: Chromatogram obtained of dansyl derivatives, run from the same sample as in Figure 35 and analysed directly after the previous run ended. Derivatised under the optimised conditions and using the optimised LC-MS/MS method. The mass selectors were set to “low” resolution on Q1 and Q3. The intensity on the y-axis is higher than in Figure 35.

It was observed at an early stage of method development that the fragmentation of dansyl derivatives was problematic when ionising with an ESI source. A simple Q1 total ion chromatogram was performed and the catecholamine m/z were extracted and exhibited high sensitivity. When the same sample was analysed using an optimised MRM method, significant reductions in the sensitivity was observed. This significant sensitivity loss was observed during fragmentation of all the dansyl derivatives. Alternate methods were assessed utilising different MRM pairs at different collision energies but this phenomenon was still observed. The reason for this is still unknown as the sample matrix was relatively pure and there is no source incompatibility that could explain this effect.

The source was changed to an APPI source. It was observed that when fragmenting using an APPI source, using similar conditions and same chromatographic method an increase in the sensitivity was noted. The APPI source performed significantly better when analysing the same samples under similar conditions during this phase of testing. During the next comparison study between the two sources it was observed that the ESI source performed better than the APPI source, and the loss of signal during fragmenting no longer occurred. More testing was performed comparing the two sources and it was noted that the ESI had superior performance in terms of sensitivity and was therefore used for the remainder of the study.

Initial chromatography on the triple quadrupole system was performed on an Alltima Grace C18 column (20 x 2 mm, 3 µm) to assess the retention of the compounds using a C18 column. It was observed that the dansyl derivatives were retained on this short column and eluted under a high percent organic mobile phase. The catecholamines exhibited the highest retention due to the presence of three dansyl groups and were expected to be more lipophilic than the other dansyl derivatives. There was major co-elution as well as broad peak shapes when using this column. The short length of the column did not allow for sufficient interaction of the analytes with the stationary phase for proper separation, and the broad peak shapes were observed due to the large particle size and the fully porous nature of the particles.

The next column assessed was a Phenomenex Kinetex C18 (100 x 2.1 mm, 2.6 µm) and the chromatogram obtained is shown in Figure 37. This was due to the small bore which would sharpen the peak shape, improve sensitivity and superior overall chromatographic performance compared to other columns. The use of this column achieved separation of some of the dansyl derivatives with sharp, narrow peaks. The Kinetex column provided excellent overall peak shape with narrow and symmetrical peaks. This is due to the small particle size as well as the core shell nature of these particles, which increases the performance of the column when compared to fully porous, larger particle size HPLC columns. There was complete resolution for four of the ten total peaks however co-elution was observed at peaks four, five and seven. The dansyl derivatives observed co-eluting were histamine with normetanephrine (peak 4), serotonin with metanephrine (peak 5) and

adrenaline co-eluting with dopamine (peak 7). A possible reason for this co-elution could be due to the lack of secondary retention effects which would provide more selective retention of the dansylated biogenic amines. The structures are highly conserved between the various biogenic amine classes, and this effect is noted post-derivatisation. The structural differences may be masked, depending on the structural folding of the large derivatives, which lead to the co-elution. This co-elution was highly problematic due to the MRM pairs of these analytes, 578.4 > 170.1/315.0 m/z – 650.7 > 170.1/633.7 m/z for histamine and normetanephrine respectively, 643.7 > 170.1/409.6 m/z – 650.7 > 170.1/171.1 m/z for serotonin and metanephrine respectively and 883.7 >170.1/171.1 m/z – 853.7 >170.1/171.1 m/z for adrenaline and dopamine respectively. MRM pairs which have similar product fragments and elute at similar retention times, with short MRM scan cycles, result in cross talk. This is due to the detectors inability to clear previous fragments prior to the introduction of similar fragment products and incorrectly determines the origin of the fragment product. This results in inaccurate quantitation of these analytes and results in poor reproducibility. The assessment of cross talk was performed. The co-eluting dansyl derivatives were derivatised as individual standards and were injected with the developed method, which included MRM pairs from both co-eluting analytes. These samples were diluted with solvent for sample A and with the co-eluting dansyl derivative for sample B. The presence of any ghost peaks was assessed using samples A in comparison to sample B. The ratio of the diluted: non-diluted dansyl derivative was also used to determine if there was a predictable decrease in the samples diluted with solvent and the co-eluting analyte. Cross talk was assumed if the presence of the co-eluting dansyl derivative was observed in sample A or less than a half-fold decrease in either sample A or B when measuring the diluted samples.

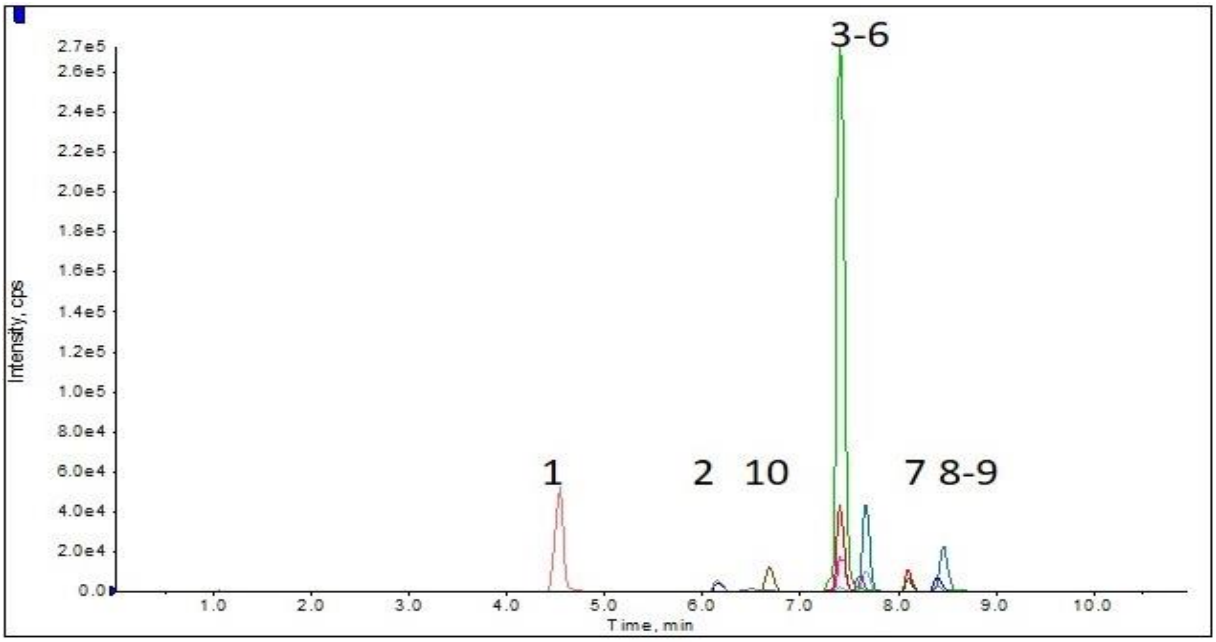


Figure 37: Chromatogram obtained after analysis of dansyl derivatives using a Kinetex C18 column. The dansyl derivatives are as describes according to the retention times (left to right): 1 – Melatonin, 2 – 5HIAA, 3 – Histamine, 4 – Normetanephrine, 5 – Serotonin, 6 – Metanephrine, 7 – Noradrenaline, 8 – Adrenaline, 9 – Dopamine, 10 - Mexamine

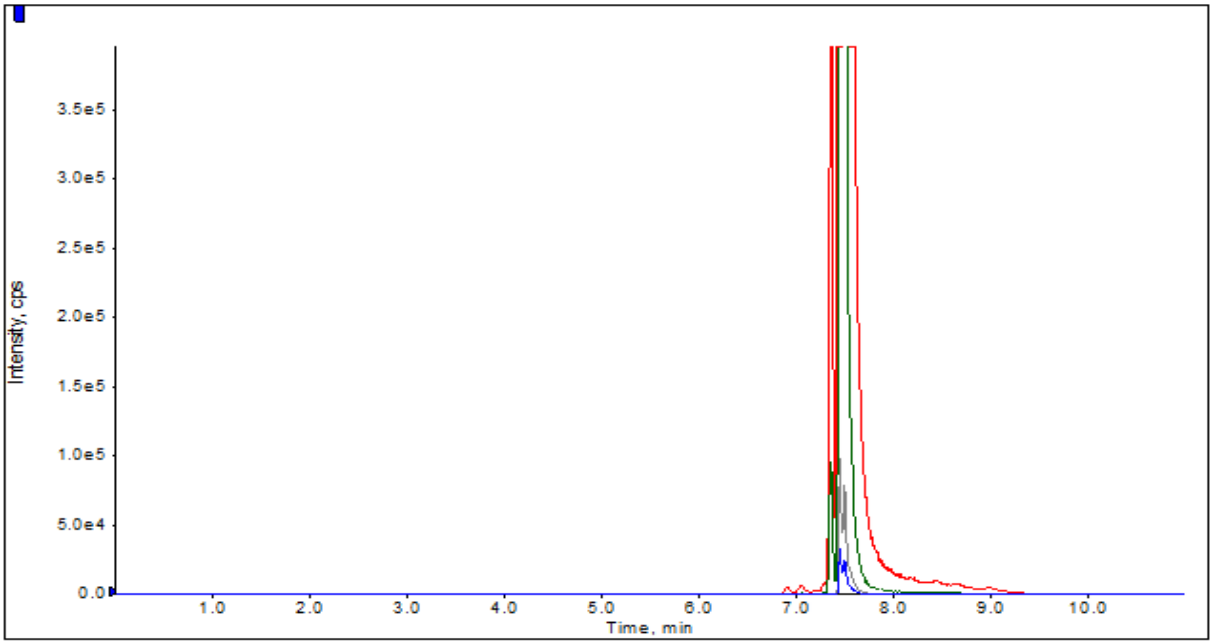


Figure 38: Chromatogram obtained from sample A as described above on a Kinetex C18 column. The presence of cross-talk between histamine and normetanephrine is confirmed due to the system reporting the presence of both dansyl derivatives in a histamine only sample. The blue/grey peak is normetanephrine and the red/brown peak is histamine.

Cross talk was confirmed between histamine and normetanephrine and was absent in the remaining co-eluting dansyl derivatives. The reason this occurs may be due to the complete co-elution of these dansyl derivatives whereas the other co-eluting peaks showed partial co-elution, which decreases the time for the detector to clear of previous fragments. Cross talk can be solved by the selection of different MRM pairs or by further chromatographic resolution of the analytes. Further chromatographic optimisation was assessed using the Kinetex C18 column but complete resolution of these compounds was not achieved. The use of different column chemistries was explored as a means to increase the resolution between co-eluting dansyl derivatives and prevent cross talk. The method developed for the Phenomenex Kinetex C18 was applied to various columns to assess their ability to resolve and separate the co-eluting analytes. These columns assessed were a Phenomenex Synergi Max-RP (150 x 4.6 mm, 5 µm), a Phenomenex Gemini C18 (100 x 2.1 mm, 3 µm), a Supelco Discovery HS F5 (100 x 2.1 mm, 3 µm) and a Phenomenex Kinetex Biphenyl (100 x 2.1 mm, *2.6 µm).*

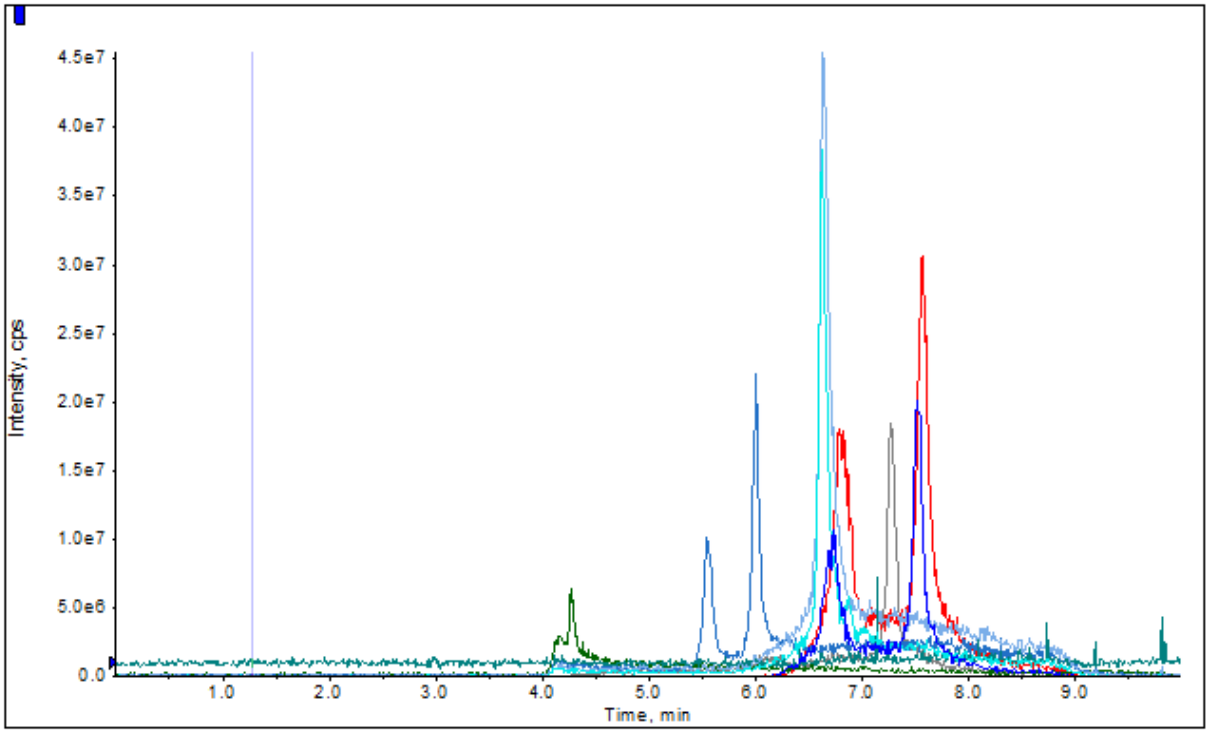


Figure 39: Chromatogram obtained from the analysis of dansyl derivatives on a Phenomenex Synergi Max-RP column. Co-elution for majority of the compounds with broader peaks were obtained than when using the Kinetex C18 column.

Figure 39 is a chromatogram when analysing dansyl derivatives using the Phenomenex Synergi Max-RP column. There is significant co-elution of the majority of the analytes as well as poor peak shape. This column is a C12 column with a large particle size of 5µm, the largest of the columns tested. The column is a wide bore column, which necessitates the use of a higher chromatographic flow rate, decreasing sensitivity and increases solvent use. The wide bore as well as the large, fully porous, particles, reduces the performance of the

column. The reduced performance of the column and the increased flow rate limits the sensitivity and results that can be achieved when using this column compared to the Kinetex column. The tailing is noticeable with the use of this column, suggesting that the end capping was not be sufficient for dansylated biogenic amines. The chromatography was not optimised due to the better peak shape, separation as well as lower solvent usage and improved sensitivity obtained when using the Kinetex column.

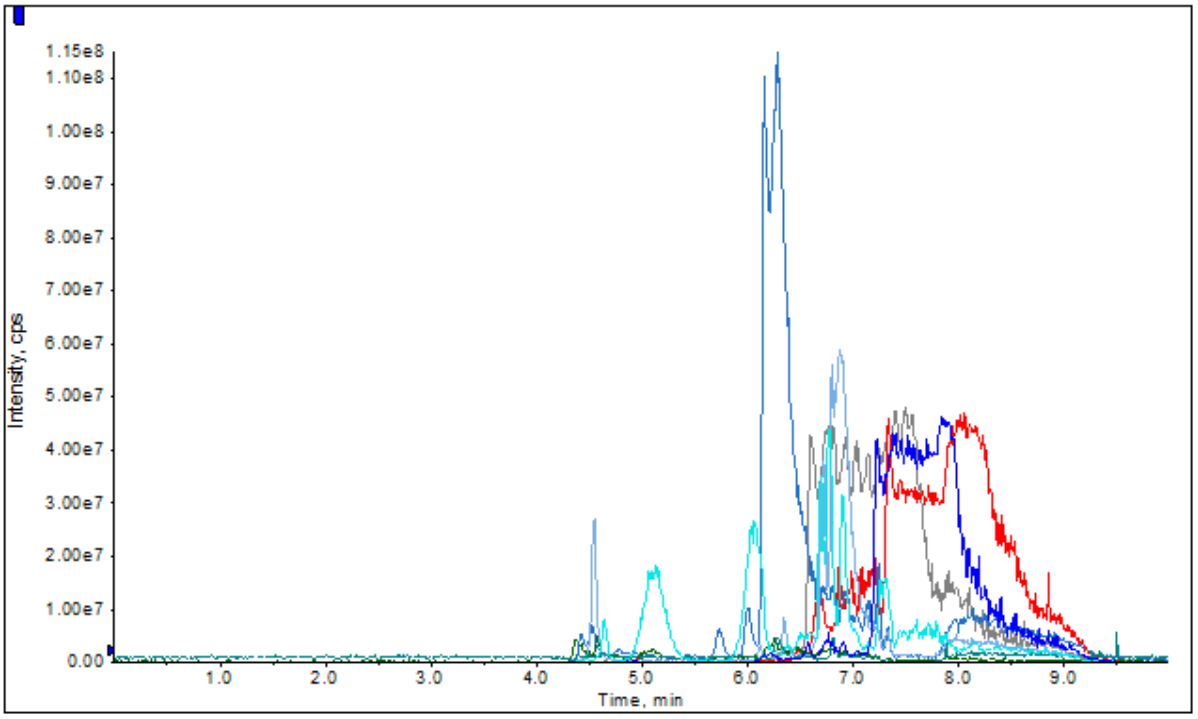


Figure 40: Chromatogram obtained from the analysis of dansyl derivatives using a Gemini column. There is poor peak shape and co-elution of the majority of dansyl derivatives when compared to the Kinetex C18 column.

Figure 40 is a chromatogram obtained from the assessment of the Gemini column. The peak shapes were broad and co-elution for numerous dansyl derivatives was observed. There was overloading of the Gemini column, despite the fact the same sample was run on a number of other columns, despite the possibility of increased loading capacity on this column. The Gemini suffered from the same issues having larger fully, porous particles. The Gemini is a C18 column, with no secondary retention effects, which may result in the co-elution of many compounds. No further optimization was performed on the Gemini C18 column due to the co-elution and better peak shape obtained when using the Kinetex C18 column. The next column assessed was the Discovery HS F5. Figure 41 is a chromatogram obtained from the analysis. This column provided more selective retention than the Synergi and Gemini columns. The peaks were broad and tailing and the symmetry was extremely poor for the catecholamines and histamine. Despite the phenyl group, F5 columns do not resemble phenyl phases in retention, due to the fluorine groups. The column does however offer pi-pi interactions, similar to that offered by the biphenyl column, which does offer more selective

retention to the C18 and C12 columns tested previously. This can be seen in that the chromatography looks most similar to the biphenyl column. The retention of the derivatised analytes are greater using this column compared to the other columns except that of the biphenyl column which provided the greatest retention of the tested columns. This column has large, porous particles which reduce performance is seen in the other columns. The tailing may be due to secondary retention effects caused by the fluorine groups or by poor end capping. There was also co-elution of the majority of the dansyl derivatives due to the broad peaks. Due to the better performance and resolution of the Kinetex C18 under similar conditions further optimization was not performed.

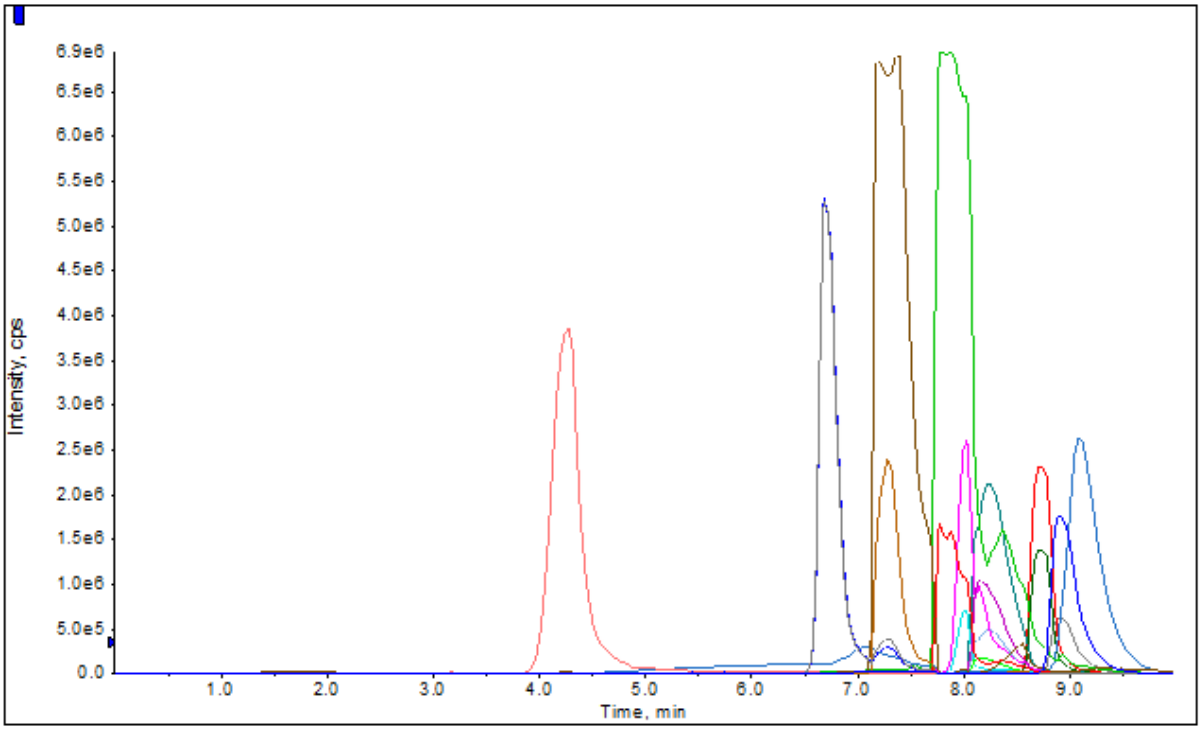


Figure 41: Chromatogram obtained from the analysis of dansyl derivatives using a Discovery HS F5 column. The peaks are broad and tailing with major co-elution of many of the dansyl derivatives when compared to the Kinetex C18 column.

The final column tested was a Phenomenex Kinetex Biphenyl column (100 x 4.6 mm, 2.6 µm) initially on loan from Separations, Johannesburg, RSA. This column proved to be superior when compared to the other columns tested. Under similar conditions to the Phenomenex Kinetex C18 column there was complete resolution of all peaks. The peaks were sharp, narrow and symmetrical. Due to the superior performance, seen in Figure 35, of this column a smaller bore column was purchased and used for the remainder of the study. This column, like the Kinetex C18, uses small 2.6 µm core shell particles, which allows for higher performance to be achieved whilst keeping the back pressure low so that it can be used on HPLC systems. It has a unique retention mechanism, with two electron rich phenyl rings, to interact and retain the naphthyl groups of the dansyl moiety. The narrow bore decreases the required flow rate and if linear flow rate in maintained provides the same retention as

wide bore columns. The use of small bore columns allows for less solvent to be used, saving money, but also increases the resolution observed. A disadvantage to small bore columns is they possess lower loading capacity, but due to the low concentration ranges used in this study is not of concern, and will benefit greatly by an increase is sensitivity and resolution.

Decreased resolution of the dansyl derivatives was observed when using an acetonitrile only mobile phase B. This is due to the dipole moment of acetonitrile, which can disrupt pi-pi interactions, the primary retention mechanism of the biphenyl column. When mobile phase B was changed to include a small percentage of methanol, the resolution of the peaks was improved. A methanol only mobile phase had minimal eluting power and even the use of a high percent methanol gradient was unable to elute the analytes in a reasonable time and was most notable for the tri-dansylated analytes. Acetonitrile, not only being a stronger solvent, through the disruption of the pi-pi interactions allowed for a sufficiently short run time with reasonable resolution when using 80:20 acetonitrile: methanol as mobile phase B. This demonstrates the strong retention of the dansyl groups by the biphenyl column, and that changing the solvent composition of the mobile phase can aid in resolution when unique and selective mechanisms are responsible for retention on the stationary phase.

This section highlights the importance of correct column selection during the development of a chromatographic method. Through the use of a short, small bore column the retention of dansylated biogenic amines in short run times could be assessed, to determine if RP chromatography would provide acceptable resolution of closely eluting compounds. The use of a small bore, small core shell particle, as found in the Kinetex range of columns, allows for reduced flow rate and increased resolution while maintaining a back pressure that can be maintained on HPLC systems. The increased sensitivity seen when using these columns was due to the improved peak shape and resolution but also a lower flow rate, which reduces the dilution effect of the sample and allows for faster evaporation of the mobile phase plume in the ionisation source, which increases the ionisation efficiency and increased entry of ionised particles to enter the mass spectrometer. Several columns were assessed to determine if the chromatographic parameters could be improved relative to that obtained when using the Kinetex biphenyl column. The larger, fully porous particles gave decreased chromatographic performance, with broader peaks and poor resolution. Due to a lack of selective retention mechanisms the standard C18 column could not provide complete resolution of many peaks and resulted in co-elution. The F5 column did show some selective retention of the analytes and more separation between the classes of biogenic amines but due to the broader peaks co-elution was commonly observed. Although not done in this study, a comparison between different core shell phenyl based chemistry columns could provide interesting results to determine the performance of these very different chemistries to aid in the separation of dansylated biogenic amines.

3.7 Application of the developed method to human plasma

The optimised method was then applied to human plasma samples in order to determine the sensitivity, selectivity and recoveries. To assess these parameters 250 µl of "stripped" human plasma, either spiked or unspiked with the BA mixture, was protein precipitated with acetonitrile and then derivatised. The derivatised sample was then vortex mixed with ethyl acetate to extract the dansyl derivatives. The ethyl acetate solution was dried down and reconstituted in 50 µl of acetonitrile.

The method used for the initial human plasma assessment use higher collision energies due to the increased sensitivity of the dansyl fragment when compared to lower collision energies. This method used the 170.4/171.1 m/z MRM fragment products, which arise from the fragmentation of the common dansyl moiety introduced by derivatisation. Although these fragments provided increased sensitivity the method was not selective due to this fragmentation being common to all derivatised compounds including non-biogenic amine type compounds. Major interference was observed when monitoring the MRM pairs with the 170.4/171.1 m/z fragment, which decreased the applicability of this method in human plasma where many unknown compounds also become derivatised during the sample preparation. Figure 42 shows a chromatogram which was obtained when using the high collision energy precursor-product pairs in a derivatised human plasma sample. The results show there was major interference near the elution times of histamine, noradrenaline, 5-HIAA, and serotonin. Human plasma is a highly complex matrix with the presence of numerous proteins and small molecules which have active phenolic hydroxyl and amine reactive sites which can react with dansyl chloride. Due the non-specific nature of dansyl chloride these interfering compounds will become derivatised and can cause major contamination especially when using the added dansyl moiety as a fragment ion. The mass spectrometer method was changed to use lower collision energy, to increase the selectivity of the method but retain the sensitivity by decreasing background noise levels. The chromatograms in Figure 43 are all samples analysed from plasma and the decrease in contamination can be observed when using the more selective MRM pairs. The results show that the interferences were significantly reduced due to the increased selectivity of the fragmentation of the analytes. The low collision energy also prevented cross talk between histamine and normetanephrine. Cross talk was also a problem with the introduction of a dansylated deuterated metanephrine surrogate standard that co-eluted with the unlabelled metanephrine. The selection of the high collision energy method with the common product fragmentation pairs lead to cross talk of metanephrine with the stable isotope standard, decreasing the reproducibility of the method.

The method was able to detect the alternative standard 5-methoxytryptamine, also known as mexamine, in a blank plasma sample. The presence of this analyte in a blank sample would cause interference when using mexamine as an internal standard. Surrogate standards must be added to each sample at a constant concentration and must not be

present in the sample that is being analysed. An alternative stable isotope labelled metanephrine, D3-metanephrine, surrogate standard was selected due to this interference. The use of a stable isotope has numerous advantages when compared to non-labelled surrogate standards and can more correctly account for changes in sample volume, extraction efficiency derivatisation effects, which will decrease the variability of the method. Figure 42 shows a chromatogram of derivatised plasma with the presence of mexamine indicated with a black arrow. Mexamine was removed from the study as an internal standard at this point.

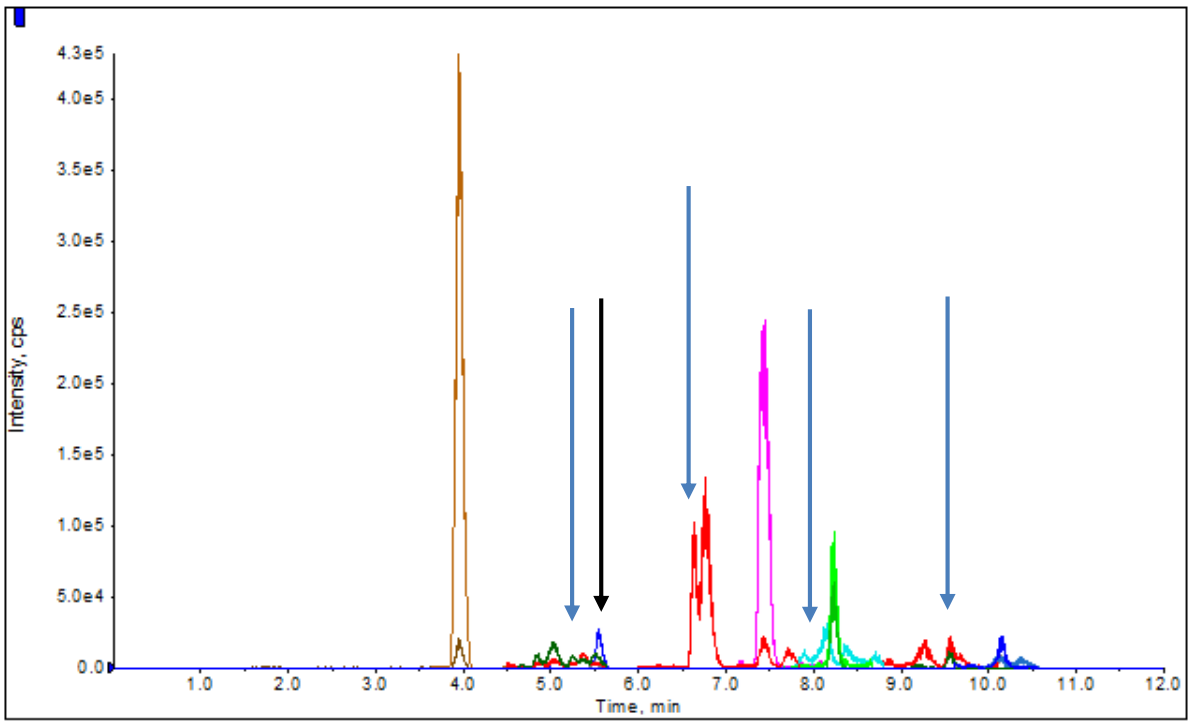


Figure 42: Chromatogram of a derivatised plasma sample analysed using a Kinetex Biphenyl column and an MRM method using high collision energy. The black arrow indicates the presence of mexamine, which was initially selected as the internal standard. The interference can be observed with the more non-selective MRM pairs and is indicated by blue arrows.

In order to assess the recoveries of the biogenic amines, a mixed stock solution of the BAs was spiked into both solvent and stripped plasma at a concentration of 50 ng/ml. These samples were extracted using a variety of different methods, derivatised and processed in the same manner post-derivatisation. The samples were then analysed using the optimised LC-MS/MS method. The results were compared to determine the recoveries of the dansyl derivatives and the method that showed the highest recoveries and lowest background was selected. Various extraction techniques were assessed – including three different protein precipitation techniques using acids and/or organic solvents, liquid-liquid extraction methods and various post-derivatisation extraction methods. It was found that acetonitrile in equal volume to the plasma was the best method for extraction of pre-derivatised biogenic amines compared to a methanol or perchloric acid crash. It sufficiently lowered

the background noise and removed interfering compounds such as proteins. Methanol produced slightly higher recoveries compared to acetonitrile however the increased volumes required to precipitate the protein using acetonitrile meant the volume exceeded the capacity of the micro-reaction tubes. Perchloric acid protein precipitation methods performed the worst of the methods tested, extracting almost no pre-derivatised biogenic amines, despite being used in various previous HPLC based studies. The acetonitrile extracted a relatively high percentage, ±80%, of biogenic amines compared to the other methods assessed and the volumes were still suitable to the equipment available. Due to the limitations of equipment and the reasonable extraction provided by acetonitrile it was used for the remainder of the study. The results can be seen below in Figure 43. Ethyl acetate provided the best performance to extract post-derivatised biogenic amines. The other tested solvents were tertiary butyl methyl ether (MTBE), diethyl ether as well as drying down immediately post derivatisation followed by reconstitution in acetonitrile. The organic solvents all performed similarly when extracting post-derivatised biogenic amines. The method performing worst was drying down the derivatisation reaction solution. It was time consuming and took over 2 hours to dry down to powder, and also did not completely redissolve into acetonitrile during the reconstitution step. Ethyl acetate dried down rapidly reducing the time spent on sample preparation.

Both pre- and post-derivatisation steps were also tested if multiple extraction steps would increase recovery. It was found that performing the ethyl acetate extraction step twice significantly increased the recovery of the post-derivatisation steps when compared to a single extraction step. The dual extraction step also performed better when compared to a single extraction using double the volume of ethyl acetate. Acetonitrile extraction was assessed in the same way, using a single, double extraction and a single larger volume extraction methods. It was found that dual extraction procedures did not increase the overall recovery, similarly larger volumes of acetonitrile did not increase recoveries. This may due to the highly polar nature of the biogenic amines, which required a fairly polar extraction solvent in order to be extracted. This is further supported by the fact that methanol extracted slightly higher percentage of the catecholamine class compared to acetonitrile due to the more polar nature of methanol. It can also be observed in the fact that acetonitrile had better extracted the slightly more lipophilic melatonin and histamine analytes when compared to the highly polar catecholamines.

Sample preparation is such an important step when developing a method but is sometimes overlooked. There are many different sample extraction techniques available, from simple protein precipitation steps, to SPE and molecular imprinted beads. Protein precipitation is the most simple of these methods however it can suffer from a lack of selectivity with the compounds extracted, which can complicate downstream analysis depending on the detector type. It can also dilute the sample, which is problematic with low concentration analytes, and may need to be dried down in order to remain within the linear range of an instrument. Selective extraction mechanisms, like SPE and imprinted beads, are more

expensive than simple solvent protein precipitation. The advantage they offer is through selective extraction of analytes via affinity based interactions and can allow for lower extraction volumes, increasing sensitivity. It is important to explore all options based on the affordability and effectiveness of the sample extraction techniques as well as the challenges for quantitating the analytes. Numerous sample extraction techniques were used in this study and simple protein precipitation was selected, due to its overall effectiveness and affordability. The acetonitrile based method was also highly agreeable to the derivatisation procedure and downstream analysis as it did not use any harsh ion pairing agents or buffers.

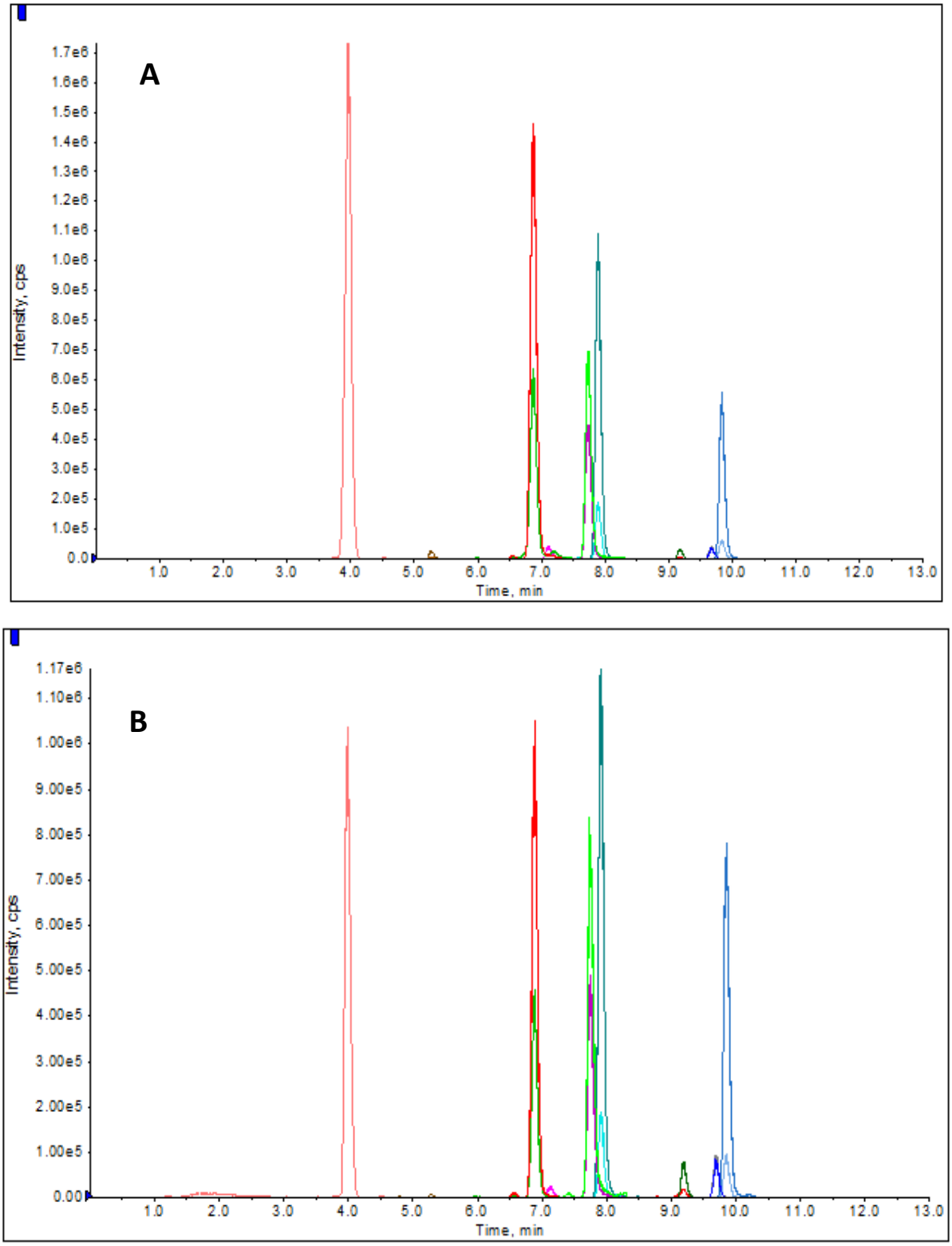


Figure 42A: Chromatograms obtained after analysis using the optimised chromatographic and mass spectrometric methods. These chromatograms were obtained from human plasma samples after various pre- and post-derivatisation extraction methods. A-C: Protein precipitation extraction methods using acetonitrile, methanol and perchloric acid respectively. D-Liquid-liquid extraction using methyl tertiary butyl ether

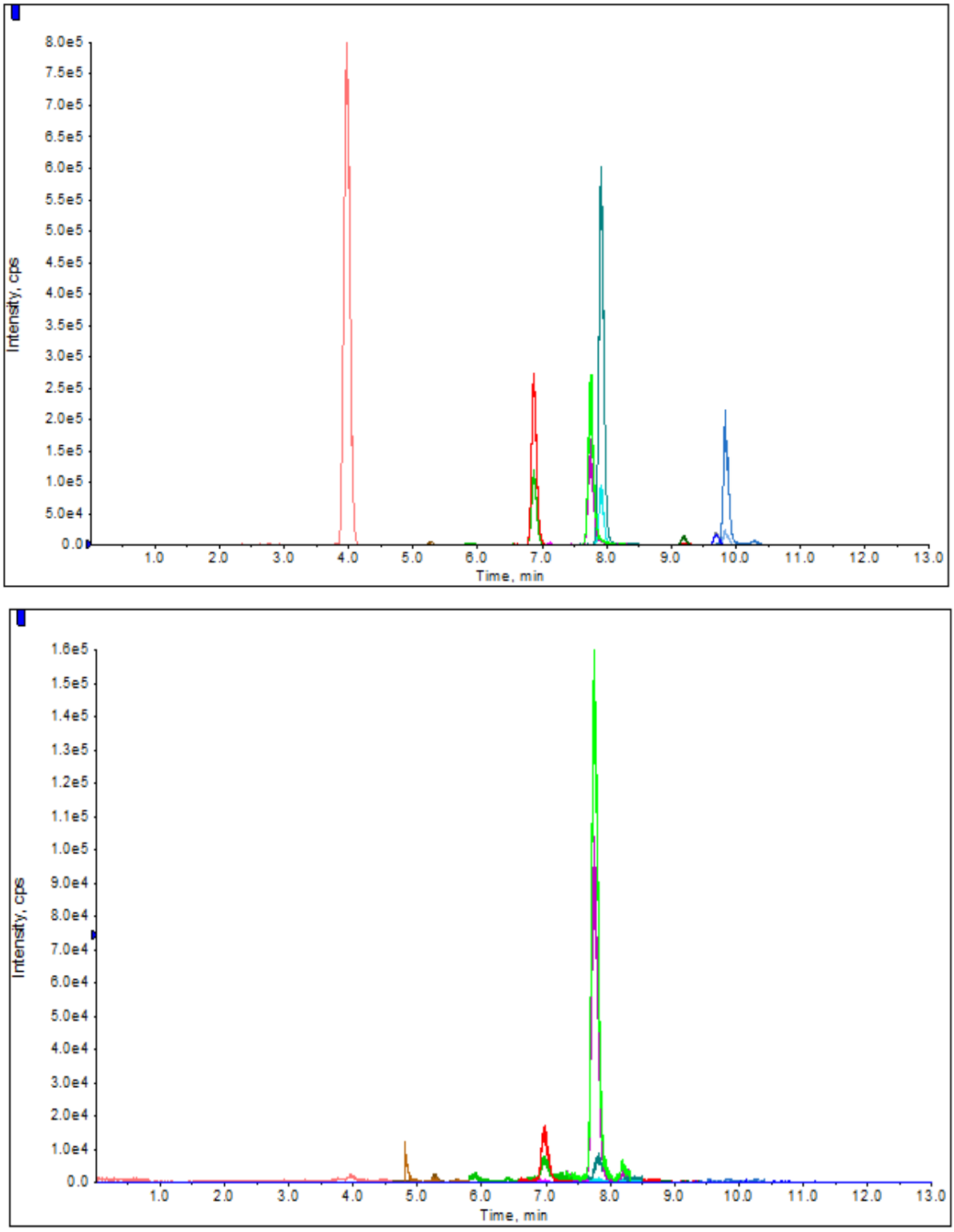


Figure 43B: Chromatograms obtained after analysis using the optimised chromatographic and mass spectrometric methods. These chromatograms were obtained from human plasma samples after various pre- and post-derivatisation extraction methods. A-C: Protein precipitation extraction methods using acetonitrile, methanol and perchloric acid respectively. D-Liquid-liquid extraction using methyl tertiary butyl ether

Chapter 4: Results and discussion of method validation

Method validation was attempted and assessed according to criteria set by the ICH. Matrix effects of the plasma samples were assessed first, due to the complex matrix. Human plasma consists of numerous proteins, lipoproteins and many small molecules including steroidal and biogenic amine like compounds. The next step was to test linearity, LOD, LLOQ, precision and accuracy. These factors were assessed using calibration curves. An 8-point calibration was made between 0.05 – 2.5 ng/ml. These values reflect the typical physiological levels of the biogenic amines and provided insight as to whether the method has the required sensitivity. The next steps was to assess the stability of the dansyl derivatives, which was performed by derivatising a single sample, analysing it immediately and again after 5 days stored on the bench at ambient temperature and frozen at -20°C.

Figure 45 is a chromatogram obtained from the matrix effects assessment. The results show that at 4 minutes there is a steady decline in the signal intensity, indicating ion suppression due to interferences being eluted from the blank plasma extract. This proved to be problematic as this coincides with the retention time of melatonin. The interference eluting between 5 and 5.5 minutes was observed to not negatively influence 5-HIAA detection as the ionisation of these MRM-pairs was constant. The signal intensity is irregular between 7 and 9 minutes. The only compound which was negatively influenced was normetanephrine, which elutes at a time of increasing signal intensity, or ionisation enhancement. The intensity increases gradually and may be due to the increased presence of organic solvent in the mobile phase at this time point which allows more efficient ionisation, rather than due to any matrix effects. The catecholamines eluted between 9 and 10 minutes in which the signal intensity was stable and appeared to not be significantly influenced by matrix effects. The ion suppression at the same time of melatonin could not be resolved chromatographically even with varying starting mobile phase conditions. It appears the retention of melatonin is similar to the contaminant that causes the observed matrix effect at that retention time. Relatively stable ionisation was seen from minutes 6 – 11 with the most matrix effects occurring between 3.5 – 5.5 minutes. This is likely due to contaminants that arose due to the derivatisation process, which would normally elute in the void volume on a reverse phase column. These compounds are slightly non-polar due to the dansyl group and elute later in this window causing the matrix effects observed. Matrix effects can be assessed by injecting a solvent sample by a subsequent plasma sample at the same concentration, and monitoring the subsequent change in sensitivity. This could not be performed in this instance due to the presence of biogenic amines in the plasma used in this study.

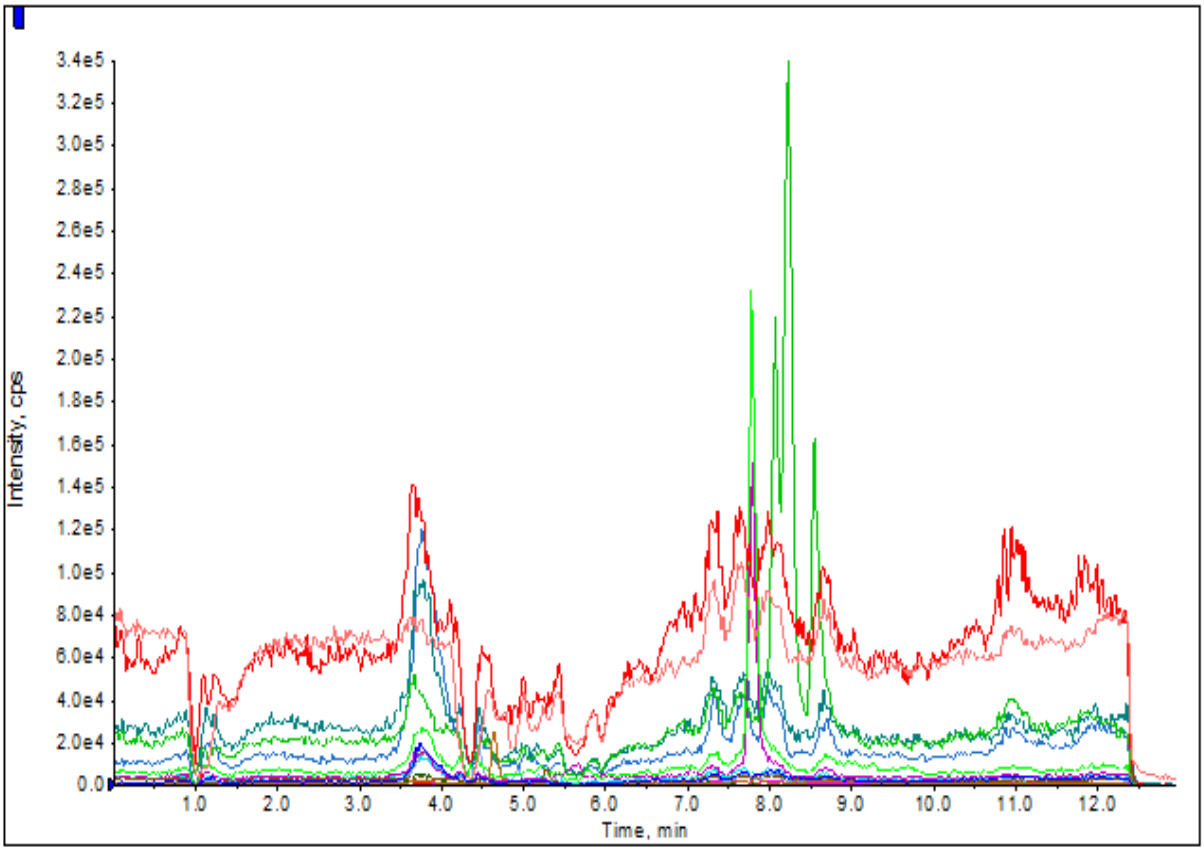


Figure 44: Chromatogram obtained of the assessment of matrix effects. This was assessed by a constant post column infusion of dansylated derivatives, while monitoring an injection of a processed plasma sample while using the optimised chromatographic and mass spectrometric conditions.

The calibration curve standards were made up using both solvent and plasma samples and analysed with the optimised LC-MS/MS method. This allowed for the assessment of a variety of validation parameters simultaneously. The parameters required for method validation are extensive and these include: Linearity, linear dynamic range, inter and intraday precision and accuracy, analyte stability and recoveries. The less stringent requirement of a 20% variability at the LLOQ at the lowest concentrations of the calibration curve, allows for the method to attain analytical limits.

The catecholamines exhibited poor sensitivity in both solvent and plasma. The estimated LODs for these compounds was between 0.5 – 1.5 ng/ml. These values are above the concentrations (0.03 – 0.5 ng/ml) that are expected in physiological systems. Figure 48 depicts the low S/N ratio of noradrenaline, 9.8 at a concentration of 2.5 ng/ml, which was the least sensitive of the catecholamines. Adrenaline and dopamine had slightly higher sensitivity when compared to noradrenaline. Figure 45 and 47 are chromatograms obtained from spiked plasma samples. Dopamine had a S/N ratio of 14 at a concentration of 0.5 ng/ml, which was the estimated LLOQ and adrenaline had a S/N ratio of 28 at 2.5 ng/ml and

an estimated LLOQ 0.75 ng/ml. Figure 46 depicts the calibration curve obtained for dopamine in plasma. Dopamine exhibited a strong linear trend between 0.25 – 2.5 ng/ml in both solvent and plasma. The r-value for inter-day plasma samples was 0.9866, accuracy and precision values exceeded the acceptable limits in solvent but were acceptable in plasma. Visual inspection of the peaks, S/N ratio and integration of numerous samples necessitated the exclusion of the samples and dopamine did not meet required standards for validation. The catecholamines were validated at higher concentrations, between 5 – 50 ng/ml. Despite the fact these values are above the physiological concentration, this was performed to prove that the derivatisation and extraction method is suitable for the quantitation of biogenic amines. Adrenaline, dopamine and noradrenaline could be validated at these concentration ranges. The validation values obtained for the catecholamines are displayed below in Table 13. The validation parameters were all within acceptable limits of precision and accuracy across all three analytes. Dopamine, adrenaline and noradrenaline was within the allowed 20% at the lowest point of 5 ng/ml. The least accurate was adrenaline with an accuracy of 120% at the lowest point in the calibration curve. The rest of the points on the calibration curve were all within the allowed 15% for accuracy and the precision was lower than 11% at all points for adrenaline and noradrenaline and lower than 15% for all points except the lowest concentration for dopamine. The method also showed acceptable linearity exceeding an r-value of 0.9844 for all assessments.

The poor sensitivity exhibited by the catecholamines adversely affected the validation process for these analytes at these low concentrations. Further optimisation is required to validate the quantitation of the catecholamines at the philological concentrations expected in healthy individuals, which is in the range of 0.01 to 1 ng/ml (Grouzmann and Lamine, 2013). The numerous challenges to overcome in reaching these sensitivities mean that very few reports have been published which are able to reach these limits, even when high sample amounts have been used. A report performed on catecholamines using a 96-well plate based assay analysing 250 µl of plasma was able to achieve these sensitivity limits without using derivatisation. In this study the authors used a 6.7 µl injection and a Waters TQD mass spectrometer (Dunand *et al*, 2013).

A possible explanation for this lack of sensitivity is the dansylated analytes may form multiple charge states, due to the presence of three dansyl moieties. The variable formation of these charge states will reduce the sensitivity and require the optimisation and inclusion of these analytes as MRM pairs, taking care to not select similar precursor-product fragment pairs due the same retention times which will result in ghost peaks. Due to the common cleavage sight of the dansyl moiety, the fragments will likely result in the same product being monitored for, which will increase variability of the method. The selection of other products may result in unique MRM pairs, but will result in low sensitivity which means that these MRM pairs may not be detected at all, invalidating their inclusion in the study. The fact that multiple peaks may need to be integrated will further increase the variability of the

method. Despite the challenges these compounds were able to be validated despite using a multistep sample preparation.

Table 13: Interday validation parameters of dopamine in both plasma and solvent. Despite acceptable values obtained for dopamine, visual inspection excluded numerous samples.

Run	Coefficient of determination	%CV	Accuracy
Dopamine			
Interday Plasma	0.9733	7-15%	94-109%
Interday Solvent	0.7485	9-40%	79-129%
Intraday Solvent (5-50)	0.9763	0.2-16%	81-113%
Interday Solvent (5-50)	0.9690	5-17%	96-119%
Adrenaline			
Intraday Solvent (5-50)	0.9805	0.5-7%	84-118%
Interday Solvent (5-50)	0.9793	3-10%	94-120%
Noradrenaline			
Intraday Solvent (5-50)	0.9857	0.4-10%	86-119%
Interday Solvent (5-50)	0.9880	3-11%	87-111%

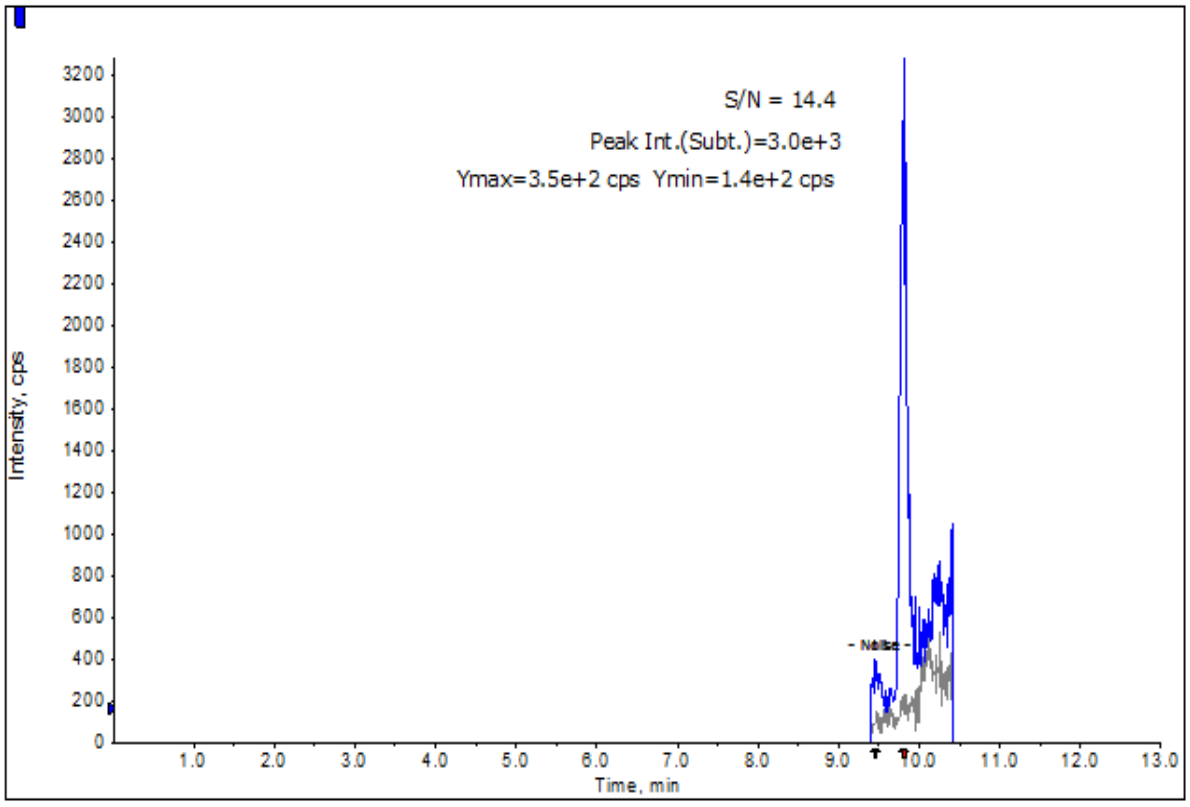


Figure 45: Chromatogram obtained of dansylated dopamine using scheduled MRM at a concentration of 0.5 ng/ml from a plasma sample. The blue and grey traces are the main quantitative dopamine MRM pair. The blue peak was obtained from a spiked sample and the grey peak was obtained from a blank sample, which was processed in the same manner but was not spiked with biogenic amines.

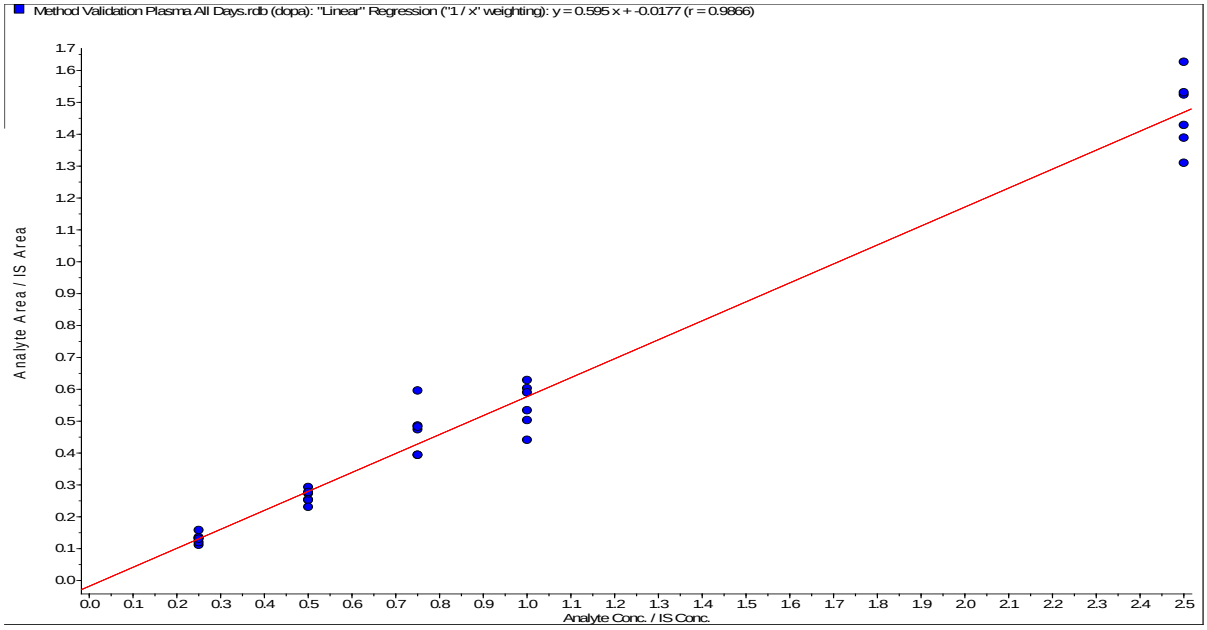


Figure 46: Inter-day calibration curve obtained of dopamine in plasma. The results appear to indicate that the method was linear between 0.25 – 2.5 ng/ml.

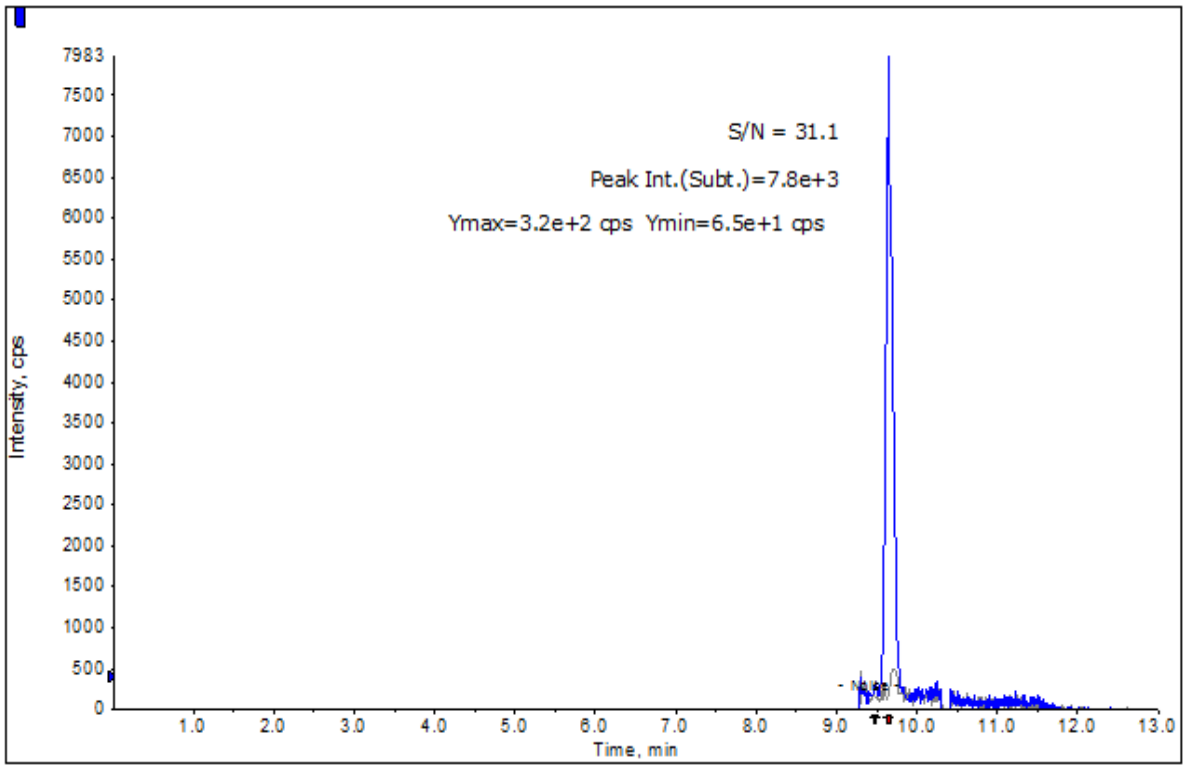


Figure 47: Chromatogram obtained of adrenaline using scheduled MRM in plasma at a concentration of 2.5 ng/ml. The estimated LLOQ for adrenaline is 0.75 ng/ml based on this sample. The blue trace is from the spiked sample while the grey trace is from a surrogate standard blank plasma sample.

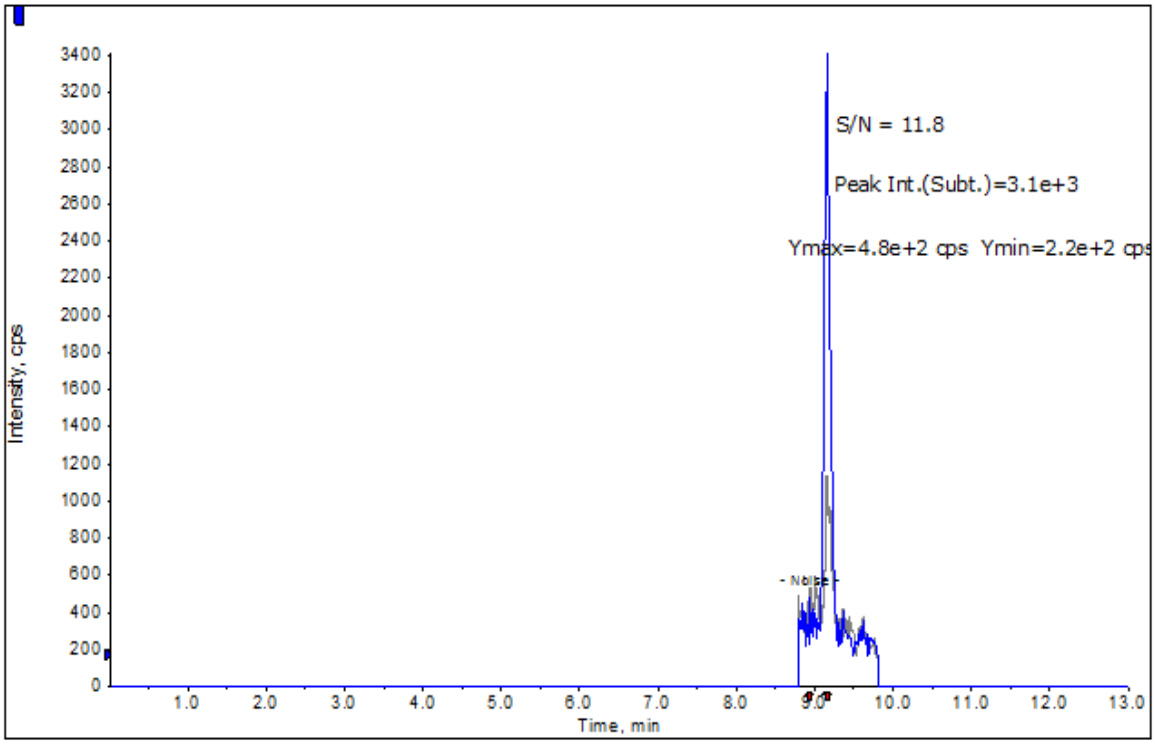


Figure 48: Chromatogram obtained of noradrenaline using scheduled MRM in plasma at a concentration of 2.5 ng/ml. The blue trace was obtained from a spiked sample and the grey trace was obtained from a surrogate standard blank plasma which was derivatised and processed in the same manner.

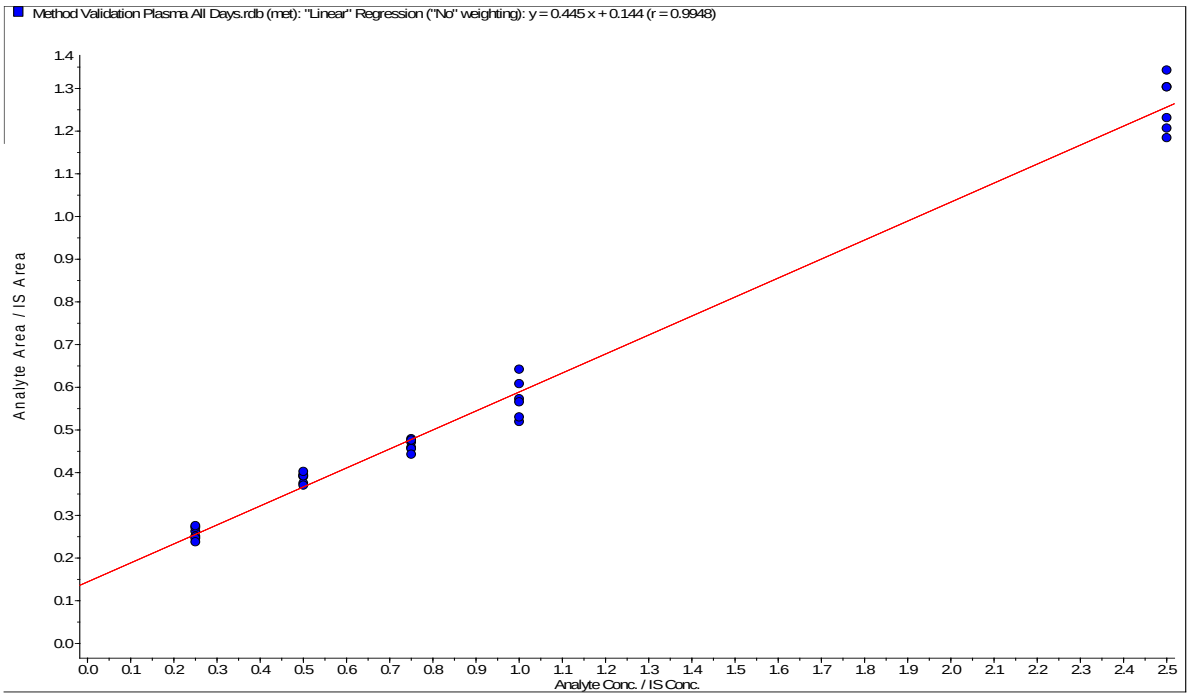


Figure 49: Inter-day calibration curve obtained of metanephrine in plasma. Metanephrine was validated in plasma and solvent and was linear between 0.1 – 2.5 ng/ml.

Figure 49 depicts the calibration curve obtained of metanephrine in plasma. The results show that acceptable accuracy and precision was obtained for metanephrine in both solvent and plasma, and the method was linear between 0.1 – 2.5 ng/ml in plasma. Figure 50 depicts the high sensitivity obtained for metanephrine in plasma, S/N ratio of 10 at 0.25 ng/ml which was determined to be the LLOQ for metanephrine. Table 14 below summarises the validation values obtained for both inter- and intra-day calibration curves. The results show that the precision was acceptable, with the largest variance of 19 % at the lowest concentration of 0.05 ng/ml in solvent. In the plasma the precision was lower than 15% across all points. The accuracy was between 83 % - 108 % in the solvent, and was acceptable for all concentration points in solvent. The accuracy in plasma is between 95 % - 117 %, with an accuracy of 117 % at the lowest concentration which is still within the acceptable limits. The calibration curve shows strong correlation between concentration and signal response with both r and r^2 values > 0.99.

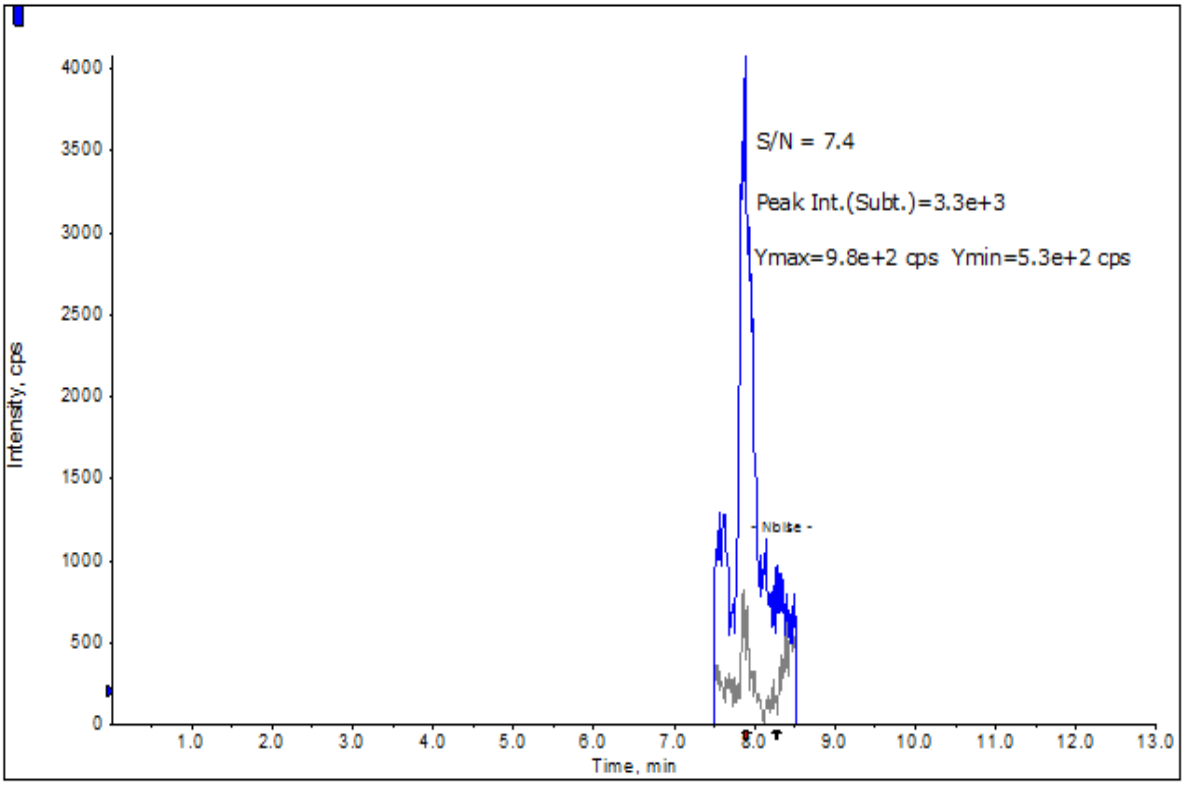


Figure 50: Chromatogram obtained using scheduled MRM of metanephrine in plasma at a concentration of 0.25 ng/ml. The blue trace was obtained from spiked plasma and the grey trace was obtained from surrogate standard blank plasma which was processed in the same manner, but was not spiked with biogenic amines.

Table 14: Inter- and Intra-day validation values of metanephrine obtained in both solvent and plasma

Metanephrine			
Run	**Coefficient of determination**	**% CV**	**Accuracy**
Intraday Plasma	0.9872	1-7%	81-123%
Intraday Solvent	0.9817	1-20%	92-110%
Interday Plasma	0.9930	4-13%	95-117%
Interday Solvent	0.9904	2-10%	83-108%
Normetanephrine			
Interday Plasma	0.9481	9-17%	78-124%
Interday Solvent	0.9716	8-19%	96-105%

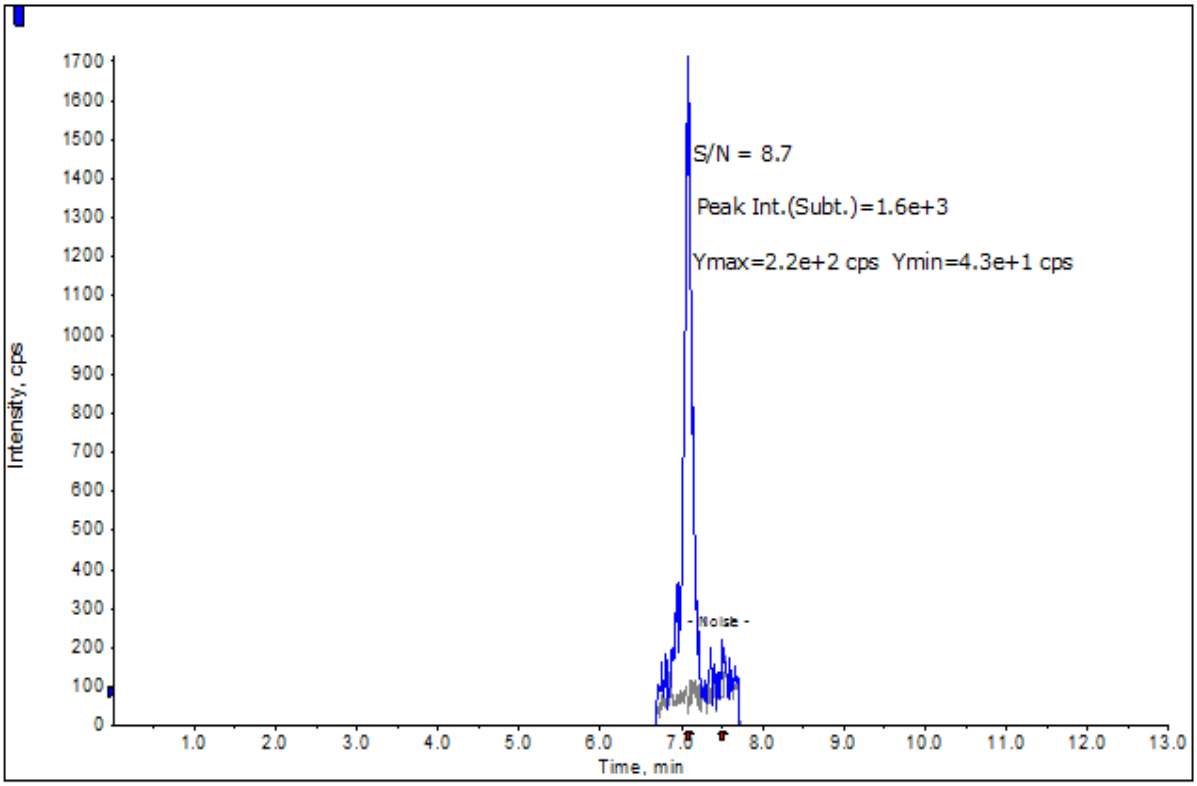


Figure 51: Chromatogram of normetanephrine using scheduled MRM in plasma at a concentration of 2.5 ng/ml. The blue trace was obtained from the spiked plasma samples and the grey trace was the sample obtained from surrogate standard blank plasma.

Table 14 summarises the inter-day validation values obtained for normetanephrine in both plasma and solvent. The values show that reasonable linearity was obtained between 0.25 – 2.5 ng/ml in both solvent and plasma based calibration curves, r-value <0.97 and 0.98 respectively. The accuracy and precision values obtained were however not acceptable for plasma samples. The accuracy was between 96% and 105% in solvent but in plasma exceeded the allowable 15% deviation for the mid-range values. The fact that normetanephrine was validated in solvent samples but did not meet the requirements for plasma samples may be due to matrix effects seen in plasma samples that were noted previously. In Figure 51 the S/N ratio of normetanephrine was only 8.7 at a concentration of 2.5 ng/ml. The matrix effects will need to be addressed using an improved sample preparation method or by using another chromatographic column to enable the validation of normetanephrine in plasma.

The reference range of metanephrine and normetanephrine are 0.09 and 0.2 ng/ml in plasma respectively (Osinga *et al*, 2016). Metanephrine exhibited high sensitivity with an LOD of 50 pg/ml in solvent. Normetanephrine did not exhibit this high sensitivity despite being structurally similar. The LOD is similar to what has been reported when using ECD, which is a highly sensitive technique, despite the fact the sample volume was four times less than used in this report (Nieć and Kunicki, 2015). The sensitivity obtained for both compounds was found to be higher despite using four times less sample volume, however this study was performed on urine (Woo *et al*, 2016). A study performed on saliva was able to achieve excellent LODs of 10 pg/ml, however the volume of saliva was not indicated, with another study being able to achieve 0.2 ng/ml using only 200 µl of plasma with a 35 µl injection volume (Petteys *et al*, 2012, Osinga *et al*, 2016). The results obtained for metanephrine are comparable to what has been previously reported, and in some instances shows higher sensitivity when taking into account the low sample volume and injection volumes used in this study. The high sensitivity demonstrates that derivatisation coupled with an appropriate LC-MS/MS is able to achieve extremely high sensitivity, comparable to that seen by powerful EC detectors.

Table 15: Inter- and Intra-day validation values of serotonin and histamine obtained in solvent and plasma.

Histamine			
Run	**Coefficient of determination**	**%CV**	**Accuracy**
Interday Plasma	-	1-94%	-
Interday Solvent	0.9675	9-18%	89-108%
Serotonin			
Interday Plasma	-	0-72%	-
Interday Solvent	0.9663	8-14%	92-112%

Table 15 depicts the inter- and intra-day validation values obtained for histamine and serotonin for plasma and solvent calibration curves. The physiological levels of histamine (0.8 ng/ml) and serotonin (0.5 – 44 ng/ml) are higher than those of other biogenic amines (Lee *et al*, 2000). A possible explanation for the wide variance of serotonin can be due to the sample processing, as majority of the serotonin is bound to red blood cells and is released during blood clotting. The method of collection and sample processing can significantly alter the observed levels and care must be taken to ensure that the collection meets standard operating procedures.

The plasma samples analysed showed detector saturation of the system and the validation of these compounds could not be performed in plasma despite using stripped plasma. Figure 53 depicts the high background obtained of serotonin and histamine in an internal standard plasma blank sample. Blank plasma completely stripped of serotonin and histamine or artificial plasma will be required in order to validate the method in plasma, as even the use of stripped plasma did not remove the presence of histamine or serotonin when it was used in this study. Serotonin was the analyte that was the most affected by this large background signal. This was observed in the calibration curves for both analytes, which had low or flat gradients – indicating that a proper signal – analyte concentration response was not obtained. Another way to validate these compounds in plasma would be to 'de-tune' the mass spectrometric values in order to reduce the signal reaching the detector. This may then allow for the validation of these analytes in plasma, however this would then limit the applicability in a real world sample as these samples would not be spiked and the loss in sensitivity may not be sufficient to detect these analytes. The values obtained for the calibration curves in solvent were acceptable for serotonin and histamine between 0.25 – 2.5 ng/ml. Figure 52 depicts serotonin with a S/N ratio of 9.9 at a concentration of 0.25 ng/ml in solvent. The precision and accuracy was less than 15 % for all concentrations tested which are within acceptable limits. The precision values for histamine in solvent are under 15 % except at the lower range of the calibration curve, but still lie within acceptable limits and the accuracy is within the acceptable 15% across all concentration points. These are acceptable limits and these analytes can be analysed in solvent with an LLOD that is below that of the reference values. A study performed on 100 µl sample and a 20 µl injection volume was able to achieve a LLOQ of 17 ng/ml and the authors claimed this to be 15-fold higher than the levels used in clinical diagnosis suggesting that the reference values of histamine vary widely and change depending on the technique employed (Laurichesse *et al*, 2016). A study performed on serotonin using LC-ECD was able to detect serotonin and 5- to 10 pg/ml, which is highly sensitive. This report however may be outside of the reference values for serotonin, although this could be due to the sample workup and preparation (Tekes, 2008).

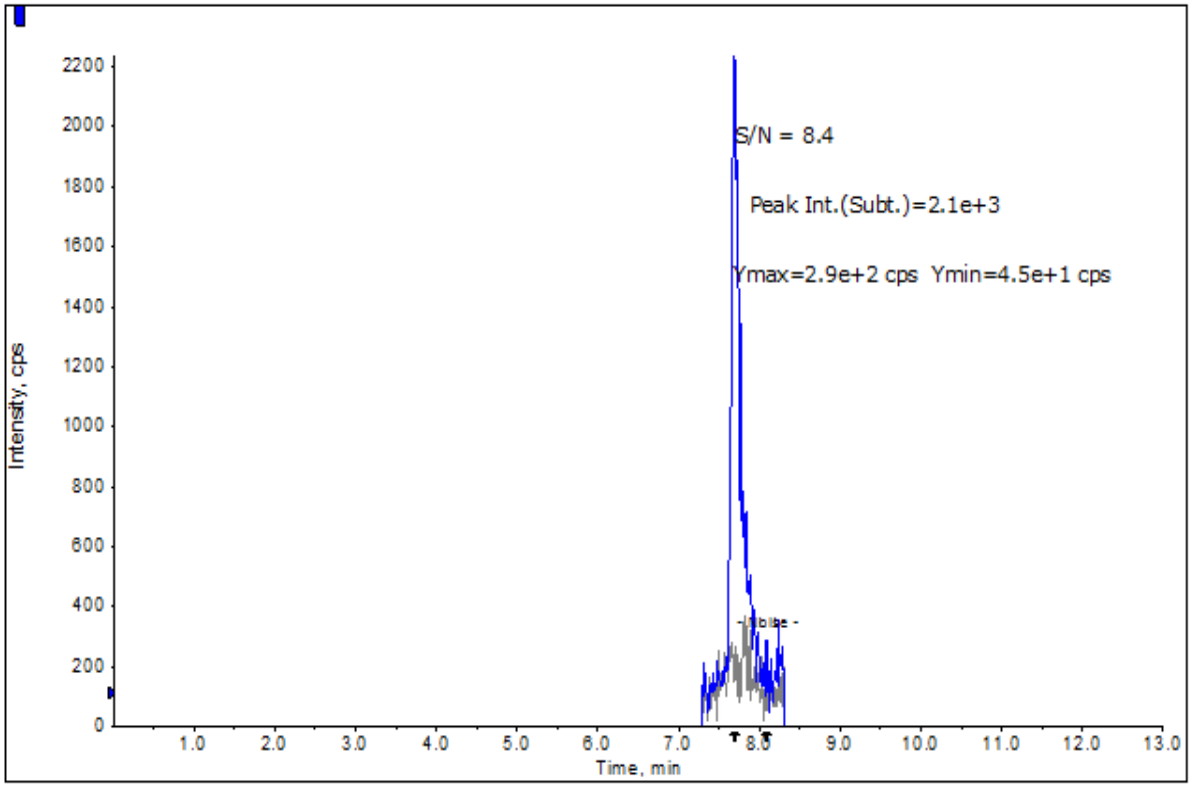


Figure 52: Chromatogram of serotonin obtained using scheduled MRM in solvent at a concentration of 0.25 ng/ml. The blue peak was obtained from spiked solvent and the grey trace was obtained from an unspiked solvent sample.

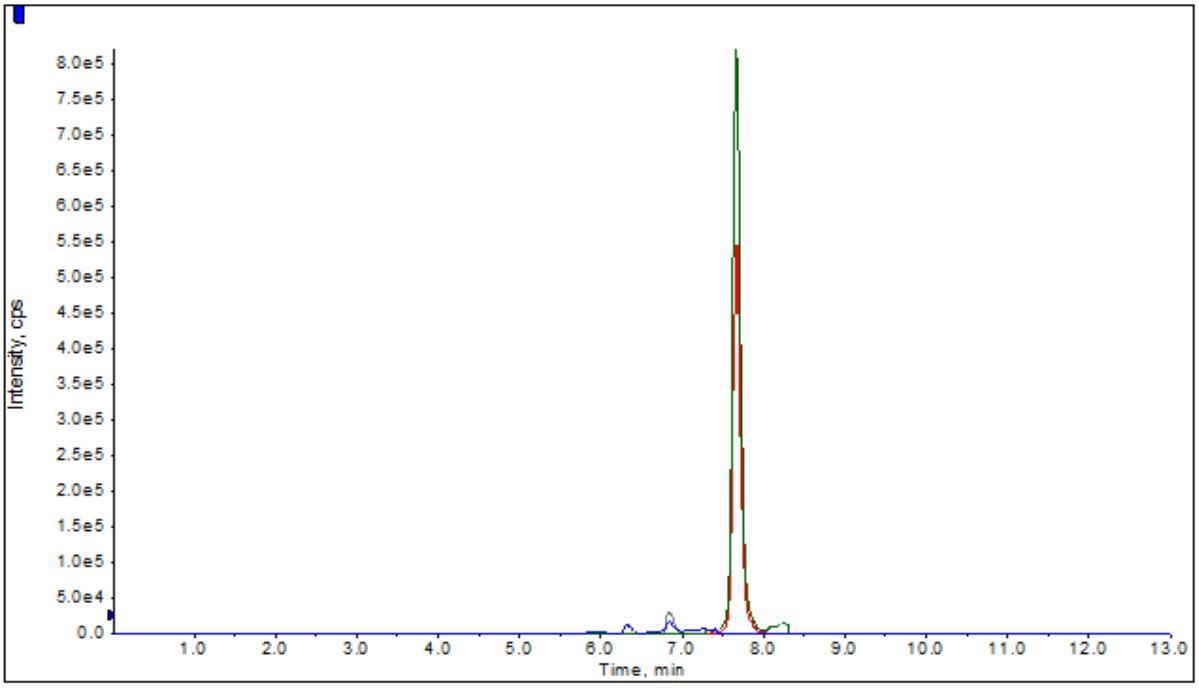


Figure 53: XIC chromatogram of histamine and serotonin obtained from a surrogate standard blank. The chromatogram demonstrates the high background signal from serotonin and histamine. The red trace is serotonin and the blue trace histamine.

Table 16: Validation parameters of melatonin and 5-HIAA in solvent and plasma

Melatonin			
Run	**Coefficient of determination**	**%CV**	**Accuracy**
Intraday Plasma	0.9872	1-10%	86-110%
Intraday Solvent	0.9661	1-20%	87-146%
Interday Plasma	0.9876	6-11%	87-103%
Interday Solvent	0.9761	12-20%	86-113%
5-Hydroxy-3-indole acetic acid			
Intraday Solvent (5-50)	0.9882	0.6-15%	87-119%
Interday Solvent (5-50)	0.9918	1-4%	91-112%

Melatonin showed acceptable intra and interday accuracy as well as precision in solvent and plasma samples. The assay was linear between 0.25 – 2.5 ng/ml. Figure 54 depicts a chromatogram obtained, which shows a high S/N ratio of 41.8 at a concentration of 0.5 ng/ml. The method was validated for melatonin between 0.25 – 2.5 ng/ml. Despite the high sensitivity exhibited by melatonin, it is still marginally above the expected physiological range between 10 – 100 pg/ml (Benloucif *et al*, 2008). Despite the fact that melatonin was underivatised it was one of the most sensitive analytes assessed. Melatonin did have a relatively high background noise level, due to the low m/z ratio of both the product and precursor MRM pairs but it proved highly ionisable. The exact reason melatonin remains unreacted despite the presence of two apparent reactive amine groups is unknown. Melatonin has not been shown to be reactive to derivatisation reactions, except in the instance of hydrogen peroxide, but may be due to the manner in which the structure folds or it is a more stable molecule than serotonin or histamine, which are structurally similar.

5-hydroxy-3-indoleacetic acid was not validated in either solvent or plasma due to the low sensitivity afforded by the chosen MRM pairs, this was further investigate and it was determined that the use of a different MRM pair was required. The method was then changed to use the dansyl fragment ion. The final MRM pair selected for this analyte was 425.4>170.4 m/z, which afforded higher sensitivity. The validation was performed in solvent alongside the catecholamines at a range of 5 – 50 ng/ml. This was not performed in plasma but as stated earlier in Figure 42 the contamination and noise may negatively influence the validation of 5HIAA. The S/N ratio of 5-HIAA at 5 ng/ml was 24, indicating an estimated LLOQ of 2.5 ng/ml in solvent, which is slightly higher than the lower end of the physiological range - 1.8 – 6.1 ng/ml (Fukui *et al*, 2012)

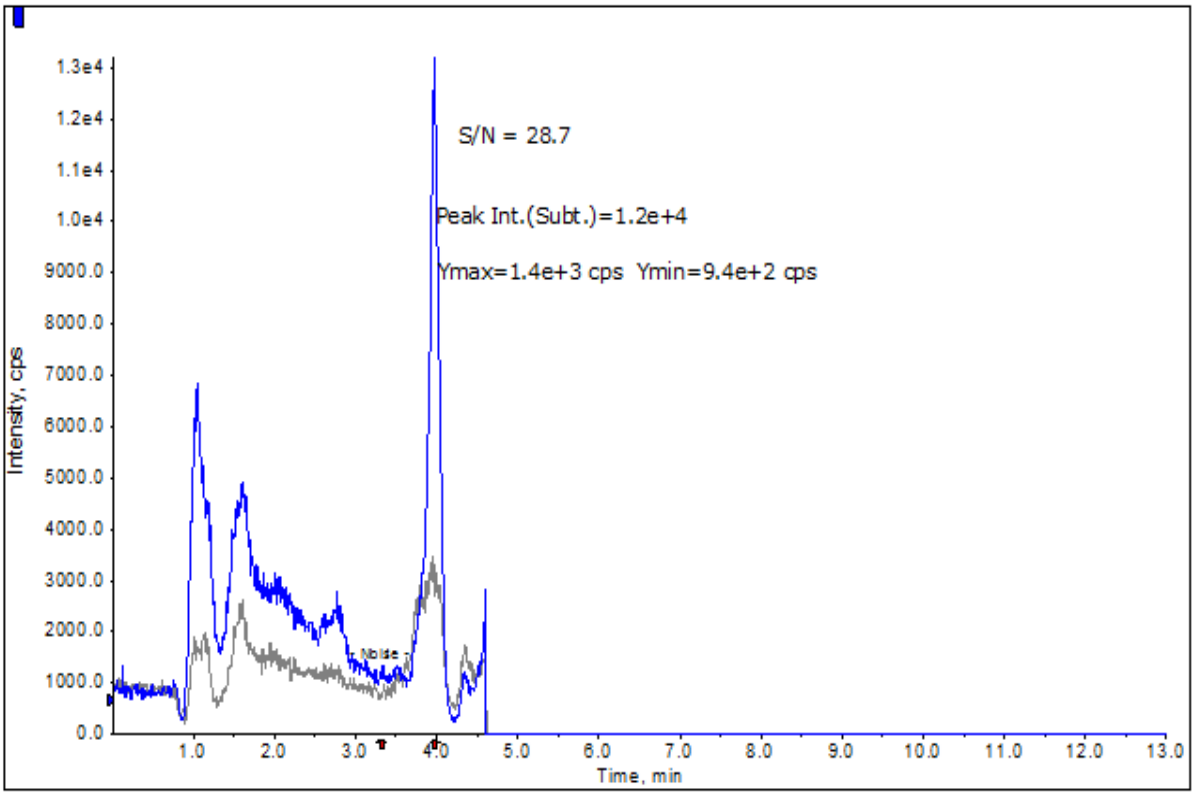


Figure 54: Chromatogram of melatonin obtained using scheduled MRM in plasma at a concentration of 0.5 ng/ml. The blue trace was obtained from the spiked plasma sample and the grey trace peak from blank plasma.

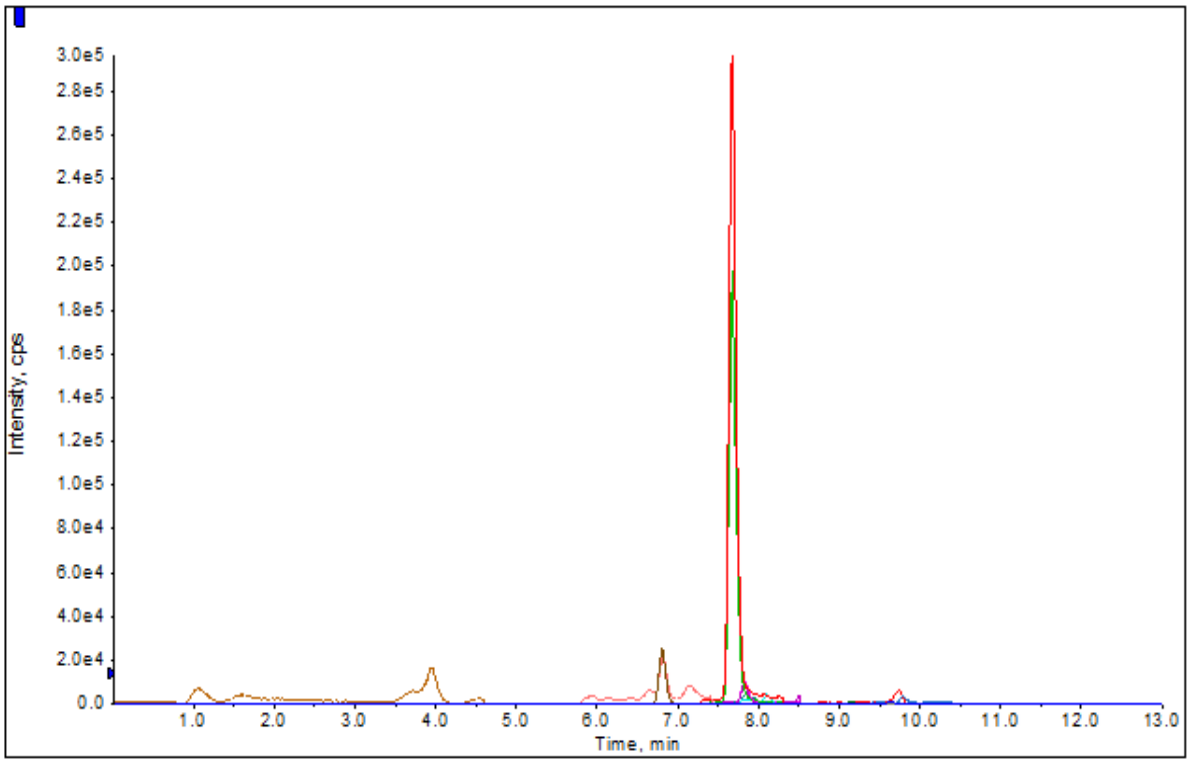


Figure 55: Chromatogram obtained of dansylated biogenic amines at a concentration of 0.25 ng/ml in human plasma. This sample was obtained using the final, optimised method.

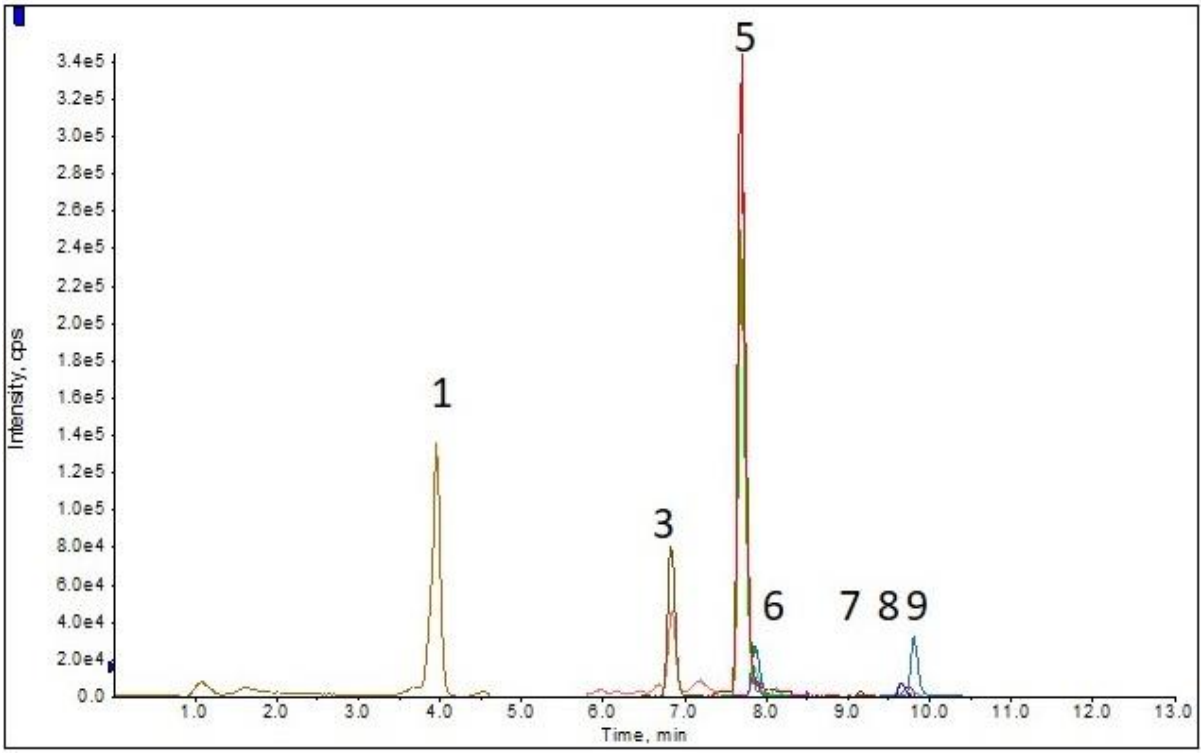


Figure 56: Chromatogram obtained of dansylated biogenic amines at a concentration of 2.5 ng/ml in human plasma. This sample was obtained using the final, optimised method and shows the low sensitivity observed for the catecholamine class compared to the other classes of BAs. The analytes are indicated as follows: 1 – Melatonin, 3 – Histamine, 4 – Normetanephrine, 5 – Serotonin, 6 – Metanephrine, 7 – Noradrenaline, 8 – Adrenaline, 9 – Dopamine.

Figures 55 and 56 depict chromatograms obtained from spiked plasma at concentrations of 0.25 and 2.5 ng/ml respectively. These chromatograms display the significant reduction in contamination when compared to the chromatogram using the common dansyl fragment MRM pairs in Figure 42. The plasma samples maintained the sharp, symmetrical peaks and resolution when compared to the solvent samples. It also displays the relative low sensitivity of the catecholamine class. The high background signal, which lead to analysis complications, from histamine and serotonin can also be observed.

Stability testing was also performed. There was no significant loss of analyte during either the intraday or interday assays performed. The samples that were kept at room temperature (21°C) for five days showed significant analyte loss for dansylated derivatives, when compared to the sample kept at -20°C. The sample kept at -20°C did not show significant loss when compared the initial analysis. It is advisable that the samples are stored at or below -20°C if storage is necessary and must be analysed within five days. The samples once derivatised and prepared were stable for at least 15 hours and so analysis should be performed within a 15 hour window after derivatisation or must be frozen for analysis later.

Table 18 shows the results obtained from recovery assessment of the plasma validated analytes – metanephrine and melatonin. The results show that the recoveries were acceptable for metanephrine, however the recovery of metanephrine at a concentration of 2.5 ng/ml is lower when compared to 0.25 and 0.75 ng/ml, which had 30 and 20% higher recovery respectively. The trend observed is that there is a decrease in the recovery of metanephrine at higher concentrations, however the physiological range is lower than 1 ng/ml which has higher recoveries. Melatonin recoveries are above 70%, and are within acceptable limits, however the method will benefit from higher recoveries especially due to the low concentrations of the analytes being tested. The acetonitrile protein precipitation is inefficient at extracting this biogenic amine at higher concentrations, which significantly reduces the sensitivity of the assay. This may be due to the highly polar biogenic amines not being extracted into the organic solvent, which is further corroborated with data showing that methanol had slightly higher extraction yields. The use of an SPE based extraction technique may allow for increased extraction yields whilst limiting the volume required for extraction the biogenic amines.

Table 17: Results from the stability testing dansyl derivatives. A sample was analysed once and stored at room temperature and at -20°C and reanalysed after 5 days using the optimised chromatographic and mass spectrometric. Results are displayed as a percentage of day 1 AUC.

Analyte	Room temperature	-20°C
Adrenaline	75%	90%
Noradrenaline	77%	88%
Dopamine	72%	95%
Metanephrine	90%	91%
Normetanephrine	87%	95%
Serotonin	88%	87%
Histamine	73%	92%
Melatonin	99%	99%
5-hydroindole-3-acetic acid	89%	98%

Table 18: Results from the recovery assessment of metanephrine and melatonin.

Concentration (ng/ml)	Metanephrine (%)	Melatonin (%)
0.25	107.2	73.6
0.75	82.0	68.9
2.5	73.2	70.3

Table 19 summarises the validation parameters for the biogenic amines. Three factors not discussed in the above table are the linear range, specificity and robustness of the assay. A small linear range was used in this study due to the small range that biogenic amines are physiologically active in. They are potent compounds and have an effect at extremely low concentrations. The use of a wider linear range would not be applicable in this study. The method was highly specific, which was ensured through the use of an MRM technique. The nature of an MRM technique is to ensure that by using a unique precursor and product pairs that numerous contaminating and interfering compounds will not reach the detector. This reduces the noise seen as the mass transitions are often highly specific to the analyte. Derivatisation also increases the mass of these analytes to a higher mass range, further eliminating possible contaminants. Stability and recovery were both assessed and found to be acceptable in both cases. The method shows a high level of robustness. The entire process from sample collection to analyse is a multistep process but the method was still able to achieve sufficient linearity and all analytes were validated.

Table 19: Summary of the validation parameters based on ICH guidelines

Biogenic amine	Linearity (ng/ml)	Co-efficient of determination r^2	Accuracy	Precision	LLOD (ng/ml)	LLOQ (ng/ml)
Adrenaline	5 -50	0.98	94 – 120%	3 – 10%	0.260	0.900
Noradrenaline	5-50	0.98	87 – 111%	3 -11%	0.750	2.500
Dopamine	5-50	0.97	96 – 119%	5 - 17%	0.150	0.500
Metanephrine	0.1 - 2.5	0.99	95 – 117%	4 – 13%	0.080	0.250
Normetanephrine	0.25 – 2.5	0.97	96 – 105%	8 – 19%	0.750	2.500
Histamine	0.25 – 2.5	0.97	89 – 108%	9 -18%	0.080	0.250
Serotonin	0.25 – 2.5	0.97	92 – 112%	8 - 14%	0.080	0.250
5HIAA	5 - 50	0.99	91 – 112%	1 – 4%	0.750	2.500
Melatonin	0.25 – 2.5	0.99	87 – 103%	6 - 11%	0.003	0.010

Dopamine exhibited linearity and appeared to have acceptable validation parameters between 0.25 – 2.5 ng/ml, this range is above the expected biological plasma concentration of 0.03 ng/ml. Dopamine quantitation could not be validated as it failed to meet standards of visual inspection. The lack of sensitivity of the catecholamine group may be due to the selection of the selective MRM pairs as concentrations as low as 0.068 pmol/ml have been reported to be quantitated when using dansyl chloride and mass spectrometry (Nirogi *et al*, 2013). Low limits of quantitation are seen in other studies with dansylated monoamines and are considerably lower when using MRM based mass spectrometric techniques when compared to DAD or QTOF/MS which often necessitate the use of high sample volume of 500 – 1000 µl of plasma or urine (Kang *et al*, 2006, Cai *et al*, 2009, Jia *et al*, 2011).

Metanephrine is validated in both plasma and solvent and normetanephrine was only validated in solvent due to matrix effects experienced in plasma samples. The analysis of the metanephrines in plasma is not routinely used, however more prominence is being placed on analysis to aid in the diagnosis of pheochromocytomas. Reports are emerging indicating that plasma metanephrines do possess similar sensitivities and specificities compared to urine tests and can be used in diagnosis (Grouzmann *et al*, 2010).

Histamine testing is often routinely performed for food safety testing but rarely for medical purposes, although reports are emerging on the role of histamine in neurological disorders as well as chronic allergy and pain assessments (Jutel *et al*, 2005, Marchand *et al*, 2005, Croyal *et al*, 2010, Jia *et al*, 2011). The most common consequence of excessive histamine plasma levels is anaphylactic shock, which does not require analytical confirmation testing. Currently the value of determining histamine in plasma appears to be of little value. In contrast serotonin is routinely tested, often alongside dopamine and norepinephrine, for medical uses especially in neurological instances due to the presence of serotonergic neurons in the nervous system (Cai *et al*, 2009, Cai *et al*, 2011a, Kurian *et al*, 2011). Although both of these analytes were validated to low concentrations in solvent, the presence of excessive amounts in plasma meant the system was saturated and could not be validated. Thus in order to validate these compounds in plasma artificial or blank plasma will need to be used as the reputed BA stripping techniques have been shown to be ineffective at removal of the polar BAs.

Melatonin, like serotonin, is tested for medical purposes due to the effects of melatonin on the wake-sleep cycle and its role in neurological disorders (Srinivasan *et al*, 2005, Hardeland, 2008). Melatonin was validated in this study in both solvent and plasma. Excellent sensitivity was obtained for melatonin despite not reacting with dansyl chloride. A study performed on brain tissue of sea lampreys validated melatonin to an LOD of 0.03 ng/ml which is slightly more sensitive that the optimised method developed in this study, however this was performed on brain tissue and may have more sample available, and with proper concentrating techniques will be able to yield better limits of detection (Wang *et al*, 2011). A study performed on saliva used 250 µl of sample and was able to obtain an LOD of 0.95

pg/ml (Jensen *et al*, 2011). This study used a 25 µl injection, 2.5 times higher than used in this study and an Agilent 6460 triple quadrupole.

The results show that despite the large increase in sensitivity afforded by using derivatisation, the catecholamines and melatonin did not reach the required sensitivity to be able to analyse physiological concentrations in plasma. The low ranges of these compounds are between 0 – 30 pg/ml, which is an extremely challenging range and can be seen in the limited reports that can reach these concentrations. Despite this in many cases excellent sensitivity was achieved and in some analytes could be quantitated to the pg/ml range. The concentrations which need to be achieved are not unrealistic however, and in many instances a slightly more sensitive instrument would have resulted in the sufficient sensitivity to analyse numerous of these analytes at the required reference ranges. This sensitivity was achieved without using large sample volumes as in published papers – in excess of 500 µl, often using 1 mL of human plasma. This study also used a smaller injection volume than is used in numerous reports – only 10 µl compared to 20 and 35 µl injection volumes. The aim of this study was to use low sample volumes in order to assess the viability of being able to analyse biogenic amines. The use of 250 µl is lower than is seen in most other reports and respectable sensitivity was still attained. The sensitivity of this assay may possibly be applied to serotonin and histamine in dry bio-matrix cards due to their higher reference ranges as well as the high sensitivity shown by these analytes – which could not be validated due to saturation of the detector. To the extent literature was reviewed, no report has currently been found which has the sensitivity or has achieved biogenic amine analysis from dry bio-matrix cards, which shows the difficulty in analysing these compounds. The instrumentation available used in this study may not be able to reach the limits when using limited sample sizes from low plasma volumes. Despite this the analytes were all validated after multistep sample preparation involving the BA extraction and derivatisation and demonstrate sensitivity that is close to that required to assay plasma samples within reported reference ranges.

Chapter 5: Conclusion and considerations

5.1 Conclusion

The technical difficulties of reliably detecting and quantitating various classes of biogenic amines in a single injection at the extremely low concentrations in plasma proved to be highly challenging. Despite this fact, in this study metanephrine and melatonin were validated from small plasma volumes while adrenaline, noradrenaline, dopamine, normetanephrine, 5HIAA, histamine and serotonin could only be validated in solvent. The method was able to detect these compounds using a low sample volume of 250 µl and was able to detect them at the pg/ml concentration range.

The varying physiochemical properties of the different classes of biogenic amines can complicate analysis which is compounded by the low physiological concentrations. The lack of specificity of derivatising reagents for biogenic amines, due to multiple available reaction sites, can be problematic. However using optimised reaction conditions the sensitivity was improved in this study. This study demonstrated how formation of multiple derivatives per analyte could be overcome by adding sufficient excess of the derivatising reagent and optimising reaction parameters such as the temperature and reaction time. Ensuring the reaction was facile was a limiting factor when using derivatisation, however this study demonstrated excellent derivatisation reproducibility. Three different derivatisation reagents were assessed as part of this study – ethyl chloroformate, N-hydroxysuccinimide and dansyl chloride. The N-hydroxysuccinimide derivatisation reagent was synthesised in-house at the Department of Pharmacology. Of the tested derivatising reagents, dansyl chloride was found to be the most suitable reagent for the selected biogenic amines used in this study, as it increased the sensitivity of the tested biogenic amines when using an optimised MRM-based mass spectrometric technique. The changes of the various derivatisation reagents on sensitivity was determined by developing methods for native and derivatised biogenic amines which was then used to analyse samples. These samples were initially analysed in native format, then the sample was derivatised using the optimised reaction conditions and compared to the sensitivity obtained from the native samples. This study demonstrates, although derivatisation is not routine for liquid chromatography, it can be used successfully to increase the detection sensitivity of poorly ionising compounds. It was found that the catecholamines were the least sensitive of all the analytes tested, with estimated LLOQs between 1 – 2.5 ng/ml. Metanephrine had the highest sensitivity and had an estimated LLOQ of 0.05 ng/ml. This high sensitivity is within the physiological concentration expected for this compound. Melatonin also displayed high sensitivity despite the fact in remained in an underivatised native format.

The use of a Kinetex Biphenyl column has not been reported in previous publications assaying dansylated biogenic amines and the selection of this column proved to be an excellent choice for the analysis of these analytes. The unique pi-pi interactions between the column stationary phase and dansyl moieties demonstrated superior retention and resolution when compared to a number of tested C18 columns. This biphenyl column allowed for the separation of the majority of the analytes with sharp, symmetrical peaks. This superior peak shape afforded by the biphenyl column allowed for accurate quantitation of the analytes and thus reduced variability during analysis.

In order to develop the method and achieve the highest sensitivity possible two ionisation sources were tested. Initial results showed the APPI source was superior and gave better fragmentation patterns and sensitivity when compared to an ESI source. However further investigation of two sources lead to the conclusion that the ESI source was superior and provided better sensitivity under the optimal conditions. When using the APPI source the ionisation products of the dansylated biogenic amines were influenced by altering the dopant composition or relative flow rate verses the mobile phase, and could yield varying ratios of protonated or radical cations. The use of an APPI source did not have the required sensitivity and demonstrated limits of detection between 1 – 25 ng/ml. The ESI source demonstrated higher sensitivity when compared to the APPI source, however sensitivity remained and issue for numerous analytes analysed. Due to the nature of the ionisation mechanism, ESI produced protonated dansyl derivatives in contrast to the cation radicals seen when using the APPI source, which has not been noted previously for dansylated biogenic amines. There was no adduct formation for either the APPI source or ESI source when analysing dansylated biogenic amines. Sodium adducts were seen when using the Waters Synapt G2 mass spectrometer, which was more prone to adduct formation when compared to the ABSciex 4000 QTrap.

The fragmentation of the dansylated biogenic amine analytes produced different fragments at low and high energies, to yield either increased specificity or sensitivity respectively. The analysis of plasma samples proved problematic due to the large background of interfering compounds in the extracts, exacerbated by the non-specific reaction mechanism of dansyl moiety which produced a common 170.1/171.1 product pair that could not be used for quantitation due to the increase in interfering peaks. The less sensitive but more specific MRM pairs were used, which decreased the sensitivity slightly, as the co-eluting compound or interference could not always be resolved chromatographically. This highlighted the importance of an appropriate sample clean-up/extraction step, which can decrease the interference and allow the use of the slightly more sensitive but common MRM pairs. The study implies that a more selective derivatising reagent may be better suited when sample clean-up is not as selective, as a non-specific reagent such as dansyl chloride will react with a variety of amine containing contaminants.

Adrenaline, noradrenaline and dopamine were all validated in solvent between 5 – 50 ng/ml. Metanephrine was validated in plasma and was found to be linear between 0.1 – 2.5 ng/ml. Normetanephrine, histamine and serotonin were validated, and were linear between 0.25 – 2.5 ng/ml in solvent. The use of artificial plasma to allow for the validation of these analytes, as stripped plasma still contained both serotonin and histamine, as matrix effects were not observed and good peak shape was obtained for these analytes. These analytes are also present at much higher physiological levels between, 1 – 40 ng/ml, compared to the catecholamines. Melatonin was validated in solvent and plasma and was linear between 0.25 – 2.5 ng/ml. The detection of 5-HIAA was not detected even at higher concentrations which was due to non-optimal MRM pair selection, this was then corrected using more sensitive fragment pairs and could be validated between 5 – 50 ng/ml. The validation of the catecholamines and 5-HIAA could be shown at higher concentrations where linearity, accuracy, precision could be proved and that the method was reliably able to extract, derivatise and quantitate these compounds but lacked the required sensitivity to assess physiological plasma concentrations. This shows that although the method lacks sufficient sensitivity the method is suitable for the detection of catecholamines and 5-HIAA but will require an increase of sensitivity to quantitate from small sample volumes like 250 µl of human plasma. The use of a more sensitive instrument would allow for the validation of these analytes at a much lower concentration.

The derivatised analytes demonstrated stability at -20°C for at least 5 days. The samples could be stored at -20°C prior to analysis but are only stable for approximately 15 hours post-derivatisation if left at room temperature. The presence of a large percentage organic solvent in the reconstitution solution required the use of tight fitting vial caps to ensure the sample did not evaporate prior to analysis.

The study found that numerous biogenic amines from different classes of amine could be detected and quantified to as low as 0.1 ng/ml using only 250 µl of human plasma. Sample extraction was able to recover approximately 70% of the biogenic amines in plasma which are within acceptable limits for metanephrine and melatonin. A factor to consider when developing a method is the time it takes for sample processing. The method developed takes approximately two and a half hours from extracting the biogenic amines from plasma to the start of analysis on the LC-MS/MS, and due to the simple extraction and derivatisation procedure can easily be automated or multiplexed.

This highlights the significant sensitivity increase that is obtainable when using an appropriate derivatising reagent and optimised LC-MS method. The study highlights that derivatisation is a viable technique and provides a possible platform for the analyses of biogenic amines from dry bio-matrix samples and proved that it is possible to analyse numerous compounds which are present at extremely low concentrations from small sample volumes for quantitative analysis. This study demonstrates that LC-MS/MS is a powerful technique and the superior ability of MRMs to decrease noise to allow for

quantitation in the pg/ml range. The significant improvement in analyte separation with narrow symmetrical peaks combined with the reduction in noise levels afforded by the MRM technique increased the sensitivity and increased the signal response through reliable derivatisation. This can be observed in the sensitivity differences between the QToF and QTrap systems as the limits of detection on the QToF system was almost 100-fold lower when compared to the QTrap system.

The method developed in this study was able to analyse nine different biogenic amines from different classes in a single 13 minute LC-MS/MS run. The method also used a limited sample volume of 250 µl of human plasma for analysis. All nine biogenic amines were validated according to ICH guidelines at extremely low concentrations. The entire method was optimised including the derivatisation procedure, chromatography and mass spectrometric conditions. These include the testing of almost seven different analytical columns, identification and selection of MRM pairs at both low and high collision energies as well as chromatographic mobile phase choices, compositions and gradient timings to be able to separate and identify these nine analytes, which can't be separated on systems using EC or coulometric detection due to the requirement of isocratic conditions. The derivatisation procedure was optimised, and a new derivatisation reagent was synthesised, to increase sensitivity but compromised the chromatographic separation that could be obtained from the method. Numerous sample preparation methods were examined to extract both pre- and post-derivatised biogenic amines – testing numerous buffers, reaction times and extracting solvents. Overall the study was a success and a method has been developed that can be built upon that will be able to detect and analyse different biogenic amines classes from small sample volumes.

5.2 Considerations

There are a few considerations to take into account, if this project was to be continued. These may help in the successful validation of all the analytes that were used in the study. These are related to the sample preparation, chromatography as well as mass spectrometry.

The first and arguably most important part of this study was the sample preparation. The successful analyte extraction from the matrix, facile derivatisation and subsequent extraction are perhaps the single most important factor to reach the extreme sensitivity limits required for dry blood spot quantitation. The extraction of biogenic amines from dry bio-matrix cards may prove problematic and so time should be spent improving the recoveries from the paper cards where it appears some BAs are selectively extracted. The use of a weak cation exchange solid phase extraction cartridges should be explored, as simple protein precipitation could not sufficiently reduced interferences which were then derivatised with the dansyl chloride reagent. Another possible route of extracting the biogenic amines from plasma is to use a dopamine imprinted molecular bead which can

selectively extract biogenic amines in a similar fashion to SPE. The recovery during the sample preparation is a hugely important step. The analyte extraction should reduce matrix effects and contamination while providing acceptable recoveries. If the sample preparation exhibited better performance this would have allowed for the use of more sensitive but common derivative MRM pairs - improving the overall validation parameters. The extraction procedure used in this study was neither selective nor highly efficient. The complex nature of human plasma meant that even using the highly selective nature of MRM based techniques, interferences were found at the same mass-charge ratio at the same retention times as several of the BAs. The ability of the sample preparation to selectively retain the biogenic amines and reduce the contamination found will prove key to being able to achieve the sensitivities required.

The selection of a proper derivatisation reagent will also be of utter importance. Dansyl chloride proved to be highly sensitive and increased the sensitivity of the method. The problem with dansyl chloride lies in the non-selective nature of the reaction mechanism. If more selective derivatisation reagents could be used such as benzylamine (selective for catechol groups), they will react more specifically with the biogenic amines. This will aid in reducing the interferences and improve the methods sensitivity. The combination of numerous specific reagents and perhaps a non-specific reagent such an dansyl chloride, could after sufficient sample extraction, prove to be a viable method as this could allow for a broad range of biogenic amines to be analysed as well as increase the sensitivity of the method. This will increase the sample treatment time as well as increase costs which are factors which will need to be considered. Another possibility is to use a pre-formed derivatisation reagent with a permanent positive charge but still shows chromatographic retention. The precursor-product pairs may show an increase in the mass-charge ratio, which along with decreasing the S/N ratio may increase the sensitivity of the method.

These are some suggestions which can be implemented in order to validate more compounds in plasma and at lower concentrations. Despite these changes which could be implemented at a later stage to improve on the sensitivity and outcomes of the project the aims and objectives of this study were met. The ability of the assay to analyse multiple biogenic amines of different classes using low sample volumes and still achieve a high level of sensitivity demonstrates the impressive power of derivatisation and MRM based techniques.

Chapter 6: References

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Chapter 7 Addendum

ADDENDUM 1: Ethical Approval

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.
- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences Research Ethics Committee

25/07/2014

Approval Certificate
New Application

Ethics Reference No 103/2014

Title Quantification and analysis of biogenic amines using dry bio-matrix sampling and a UPLC-MS/MS method.

Dear Mr João Carlos Cardeano

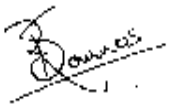
The **New Application** as supported by documents specified in your cover letter for your research received on the 28/02/2014, was Provisionally approved by the Faculty of Health Sciences Research Ethics Committee on the 26/03/2014.

Please note the following about your ethics approval:
- Ethics Approval is valid for 2 years.
- Please remember to use your protocol number (**103/2014**) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

Ethics approval is subject to the following:
- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely



Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

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ADDENDUM 2: Informed consent form

PATIENT / PARTICIPANT'S INFORMATION LEAFLET & INFORMED CONSENT FORM FOR A NON-INTERVENTION STUDY

TITLE OF STUDY:

Quantification and analysis of biogenic amines using a liquid chromatographic tandem mass spectrometric method.

Principal Investigator: John Cardeano
Organization: Pharmacology Department, University of Pretoria
Daytime Number: 076 721 7193
Afterhours: 076 721 7193

Date and time of first informed consent discussion:

dd	**mm**	**year**	**Time**

This Informed Consent Form has three parts:

- **Information Sheet (to share information about the research with you)**
- **Consent form (for signatures if you agree to take part)**

It will take 10 minutes to complete the questionnaire

Dear Mr/Mrs_____ Date of consent procedure_____/_____/_____

PART 1: Information leaflet

1) INTRODUCTION

You are invited to volunteer to take part in a research study where biogenic amines, like adrenaline and serotonin, as well as their metabolites are to be measured in blood samples. This information leaflet is to help you to decide if you would like to participate. Before you agree to take part in this study you should fully understand what is involved. If you have any questions, which are not fully explained in this leaflet, do not hesitate to ask the investigator. You should not agree to take part unless you are completely happy about all the procedures and data collection involved. You are welcome to discuss you possible participation with the researchers or other confidents. In the best interests of your health, it is strongly recommended that you discuss with or inform your personal doctor of your possible participation in this study, wherever possible.

2) THE NATURE AND PURPOSE OF THIS STUDY

You are invited to take part in a research study. The aim of this laboratory based research study will assess whether a method developed in the Department of Pharmacology is able to accurately determine the level of these compounds in blood. The purpose of this study is to determine the level of biogenic amines in the blood using a liquid chromatography tandem mass spectrometry method using dry blood spots as a method of collecting the samples rather than blood samples obtained via the typical veni-puncture.

3) EXPLANATION OF PROCEDURES TO BE FOLLOWED

This study involves answering some questions with regard to your health as well as a finger prick test and veni-puncture.

The blood will be collected via a small finger or ear prick with a total of about 3-6 drops of blood (around 60-120 ul) will be collected onto a special blood spot collection card. The finger prick test is similar to tests that are performed when testing for glucose or cholesterol. A total of 5 mL will also be drawn using a typical veni-puncture method. This is performed as per a standard blood test.

A questionnaire will be required to be filled out and will include your gender, age, weight, perceived stress levels, any medical conditions as well as any medications you are currently taking.

Samples will be analysed using a newly developed LC-MS/MS method. There will be no intervention in this study, meaning that you will not be required to take any pharmaceutical drug or any other substance nor be required to stop any medication that you may be taking.

The results collected will be compared to that of previously determined results and calibration curves that have been used to develop the method to determine whether the method is sensitive enough and has the ability to quantitate biogenic amines in each sample.

4) RISK AND DISCOMFORT INVOLVED

The only risk and discomfort involved will be a finger prick for collection of blood spots. If the area is properly cleaned with a sterilising swab there is very little chance of any infection and minimal chance of any permanent damage. The small needle lance is a single use device that is the same as used for collecting blood to test for glucose levels in diabetic patients.

5) POSSIBLE BENEFITS OF THIS STUDY

There are no benefits for you for participating in this study.

6) WITHDRAWAL

You may at any time withdraw from this study without giving reasons and without any prejudice to your privacy.

7) HAS THE STUDY RECEIVED ETHICAL APPROVAL?

This Protocol was submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria, telephone numbers 012 3541677 / 012 3541330 and written approval has

been granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: October 2008), which deals with the recommendations guiding doctors in biomedical research involving human/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

8) INFORMATION and CONTACT INFORMATION
If I have any questions concerning this study, you should contact:
Prof Duncan Cromarty cell: 0733064220 or duncan.cromarty@up.ac.za
Mr João Cardeano cell: 076 721 7193 or j.cardeano@gmail.com

9) CONFIDENTIALITY
All samples collected will be collected with only a group identity and a code number to link the questionnaire form that is linked to the sample. Any data obtained in this study will be regarded as confidential. Results will be published in peer reviewed journals or presented at a conference in such a fashion that participants remain unidentifiable.

PART 2: Certificate of Consent

10) CONSENT TO PARTICIPATE IN THIS STUDY.
I have read or had read to me in a language that I understand the above information before signing this consent form. The content and meaning of this information have been explained to me. I have been given opportunity to ask questions and am satisfied that they have been answered satisfactorily.
I hereby volunteer to take part in this study.
I have received a signed copy of this informed consent agreement.

Name of Participant_____

Signature of Participant _____

Date _____

Name of Researcher/person taking the consent_____

Signature of Researcher /person taking the consent_____

Date _____

Witness Name_____

Witness Signature_____

Date _____