The detection and quantification of biomolecules in biological sample matrices

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PREFACE

“The fear of **יהוה** is the beginning of knowledge; Fools despise wisdom and discipline.”

Proverbs 1:7 (The Scriptures Bible 2009)

“The surest path to knowing God is through the study of science and for that reason God started the Bible with a description of the creation.”

Moses Maimonides, Sephardic Jewish philosopher (1135-1204).

(http://www.biblemysteries.com/library/biblescience.htm)

“What we know is a drop, what we do not know is a vast ocean. The admirable arrangement and harmony of the universe could only have come from the plan of an omniscient and omnipotent being.”

“God created everything by number, weight and measure.”

“In the absence of any other proof, the thumb alone would convince me of God’s existence.”

Sir Isaac Newton, Theologian, physicist, astronomer and mathematician (1643-1727)

(https://hiddeninjesus.wordpress.com/2018/02/25/god-and-science-until-20th-century/)

“The more I study nature, the more I stand amazed at the work of the Creator. Science brings men nearer to God.

A little science estranges men from God but much science leads them back to Him.”

Louis Pasteur, French chemist and microbiologist (1822-1895)

(https://hiddeninjesus.wordpress.com/2018/02/25/god-and-science-until-20th-century/)

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ABSTRACT

In the bodies of living humans and animals, endogenous biomolecules, and in particular the chemical messengers, play an essential role in the maintenance and functionality of physiological processes (Daintith, 2008; Gault & McClenaghan, 2013). When these chemical messengers are out of balance, however, it can cause a variety of disorders throughout the whole body. The analytical measurement of these biomolecules can play an essential role in our understanding of both the normal physiological and the abnormal pathophysiological processes. This understanding can help with better treatment of these disorders and development of newer and better drugs. In the research environment, costs related to analysis of biomolecules of either chemical messengers or biomarkers for certain disorders is a problem. The current commercial available radioimmunoassay (RIA) (Wassell *et al.*, 1999), enzyme immunoassay (EIA) (Kemppainen *et al.*, 2018) and enzyme-linked immunosorbent assay (ELISA) (Kim *et al.*, 2008) kits are expensive per sample and because of cross contamination, some are unreliable. Chromatography is an analytical technique for the separation of mixtures of compounds and molecules in solutions by a variety of different chemical processes (Licker, 2003), and a variety of sample preparation techniques. The aim of this study was to develop and validate new methods for the analysis of endogenous biomolecules that would be sensitive, specific, reliable and affordable. We developed and validated three new methods, which were published in an international accredited journal. The first method was for the analysis of the monoamines and their metabolites in rat brain tissue. The second was for norepinephrine and its metabolites for the measurement of enzyme COMT activity in rat liver homogenate in the presence of the known COMT inhibitor, entacapone. Lastly, for the analysis of cortisol, corticosterone and melatonin in plasma samples from laboratory animals, the Sprague-Dawley rat and the white rhinoceros, a wild animal. These methods not only adhered to the aims and objectives of the study project but also to the research problem of the need to develop and validate new analytical methods that would be sensitive, specific, reliable and more affordable than commercially available analytical kits. We conclude that these newly developed and validated analytical methods can be applied in practice with great success and with cost saving advantages. We anticipate that these methods will be a great addition especially to our research environment, where these kind of methods are constantly used in a variety of animal behavioural studies in stress or stress-related disorders. These methods can also be used for better drug development and discovery.

Key terms: Endogenous biomolecules, chemical messengers, biological matrices, method development and validation, HPLC, diode array detection, electrochemical detection

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A		
AADC		Aromatic L-amino acid decarboxylase
ACN		Acetonitrile
ADR		Aldehyde reductase
ALDH		Aldehyde dehydrogenase
B		
BDNF	-	Brain derived neurotrophic factor
BLOD		Below limit of detection
C		
CNS	-	Central nervous system
COMT	-	Catechol-*O*-methyltransferase
CSF		Cerebrospinal fluid
D		
DA		Dopamine
DAD		Diode array detection
DBH		Dopamine ß-hydroxylase
DDC		DOPA decarboxylase
DHBA		3,4-dihydroxy-benzylamine
DHPG		3,4-Dihydroxyphenylglycol
DMSO		Dimethyl sulfoxide
Dopac		3,4-dihydroxyphenylacetic acid
E		
ECD		Electrochemical detection
EDTA	-	Ethylenediaminetetraacetic acid
EIA		Enzyme immunoassay
ELISA	-	Enzyme-linked immunosorbent assay
EPI		Epinephrine/adrenaline

LIST OF ABBREVIATIONS

F

FDA	-	Food and Drug Administration
FLD		Fluorescent detection

G

GABA		ɣ-aminobutyric acid
GC		Gas chromatography
GSH		Glutathione reduced
GSSG		Glutathione oxidised

H

$HClO_4$		Perchloric acid
5-HIAA		5-hydroxyindole-3-acetic acid
5-HMT		5-hydroxy-Nω-methyltryptamine oxalate
HPLC	-	High performance liquid chromatography
5-HT		Serotonin
5-HTPD		5-Hydroxytryptophan decarboxylase
HVA		Homovanillic acid
Hz		Hertz

I

K

L

L-DOPA		L-3,4-Dihydroxyphenylalanine
LOD		Limit of detection
LOQ		Limit of quantification

M

LIST OF ABBREVIATIONS

M	Molarity
MAO	Monoamine oxidase
MeOH	Methanol
mg	milligram
MHPG	3-Methoxy-4-hydroxyphenylglycol
Min	Minutes
ml	Millilitre
mM	milli Molar
MN	Metanephrine
MS	Mass-spectrometry
3-MT	3-Methoxytyramine
mV	milli-Volts

N

n	Number
nA	nano ampere
NE	Norepinephrine/noradrenaline
ng	Nanogram
NMN	Normetanephrine

O

OPA	o-Phthalaldehyde

P

PAH	Phenylalanine hydroxylase
PBS	Phosphate buffer saline
pg	Picogram
PNMT	Phenylethanolamine-N-methyltransferase

Q

QC	Quality control

R

rcf	-	Relative centrifugal force

LIST OF ABBREVIATIONS

RIA	Radioimmunoassay
RSD	Relative standard deviation
RP	Reverse phase

S

SAMe	*S*-(5′-sdenosyl)-*L*-methionine
SANS	South African National Standard
SD	Standard deviation
SI units	International system of units
S/N	Signal-to-noise
SOP	Standard operating procedure
SS	Stock solution

T

TH	Tyrosine hydroxylase
TPH	Tryptophan hydroxylase

U

µl	Micro litre
µm	Micro meter
UV	Ultraviolet

V

V	Volt
VAT	Value-added tax
VMA	Vanillylmandelic acid

W

DECLARATION BY STUDENT

Declaration by student

I, Francois Petrus Viljoen, hereby declare that all the literature research, experimental work and data capturing and interpretation of this study were conducted by myself. I further declare that the initial version of this dissertation was also written by myself, and that improvements and corrections were then made as per advice from study guidance. My promoter (Prof. Jan L du Preez) and both he, the co-promoter (Prof. Johanna C Wessels) and assistant promoter (Prof. Marique E Aucamp) assisted me with the proof reading of the articles and dissertation in preparation for the final version.

22 November 2018

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As promoters, Prof. Jan L du Preez, Prof. Johanna C Wessels and Prof. Marique E Aucamp confirm that the declarations stated above, by Mr. Francois P Viljoen, are true and correct.

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CHAPTER 1 INTRODUCTION

1.1 Dissertation layout

This dissertation is written and submitted in the standard *"article"*-format for dissertation submission, as approved by the North-West University. The format outline serves to assist the reader in finding key elements of the study inside the dissertation and is as follows:

Chapter 1: **Introduction**.

Chapter 2: **Aims, objectives and methodology**

Chapter 3: **Manuscript** A, published in *Pharmazie* **73** (2018), an accredited international journal.

Chapter 4: **Manuscript B** submitted to an accredited international journal.

Chapter 5: **Manuscript C** submitted to an accredited international journal.

Chapter 6: **Discussion, conclusion, limitations and future directions**

References: The referencing was done with EndNote X8 software and is cited according to the Harvard style (preferred by the North-West University) and can be found at the end of this dissertation.

Addendum A: Concept Manuscript D.

Addendum B: Conformation of all ethical approval.

Addendum C: Conformation of manuscript acceptance.

Addendum D: Conformation of language proofreading, editing and checked for plagiarism.

The reference list of each manuscript is presented at the end of each manuscript (i.e. Chapter 3-5) and is in accordance with the specific reference style required by the scientific journal to which the manuscripts was submitted to. All of the referencing throughout this dissertation was done with EndNote X8 software. The rest of the dissertation references are cited according to the Harvard style (preferred by the North-West University) and can be found at the end of this dissertation. This dissertation is written in United Kingdom (UK) English.

CHAPTER 1: INTRODUCTION

1.2 Introduction

Chromatography has become a very powerful analytical technique used in a wide range of scientific research areas (Dhanarasu, 2012). Since its discovery in 1906 by Mikhail Tswett, scientists developed and validated a significant number of methods for the detection, separation and quantification of a whole variety of different compounds and molecules in simple and complex matrices (Christian, 2004; Gault & McClenaghan, 2013). These compounds or molecules can range from drugs, pro-drugs, biologically active compounds or molecules, toxins, trace elements, phytochemicals, biomarkers and biological molecules (biomolecules). This study will focus on biological molecules or biomolecules as they are also known.

Biomolecules are organic molecules primarily involved in the maintenance and functionality of physiological processes of living mammals (Daintith, 2008; Gault & McClenaghan, 2013). These molecules are mainly formed out of the following elements carbon (C), hydrogen (H), oxygen (O), nitrogen (N), phosphorus (P) and sulphur (S) with carbon as the main element in all of them (Pratt & Cornely, 2014). Biomolecules are naturally occurring in all living mammals and are also called endogenous molecules because they originate from within the mammal (Gault & McClenaghan, 2013; Dictionary, 2016). Therefore, these molecules form part of the molecular, cellular or biochemical processes in animal and human cells, tissues, fluids, glands and organs. They originate from within the organs, glands, tissues or cells through metabolic processes and are controlled by various physiological processes. They are also organic molecules which can either be macromolecules (for example, nucleic acids, proteins, enzymes, peptides, lipids and complex carbohydrates) or small molecules (for example, nucleotides, amino acids, fatty acids, monosaccharaides, steroids, neurotransmitters, metabolites and hormones) (Raven & Johnson, 2002; Gault & McClenaghan, 2013; Pratt & Cornely, 2014). Small molecules or micro molecules have a molecular weight less than 1000 Daltons (Miller & Tanner, 2013). The small molecules group consists of molecules called monomers which form the building blocks of the macromolecules (polymers) (Garrett & Grisham, 2005). Most of these biomolecules are also biological markers of physiological and pathophysiological processes in humans and animals, the difference is that all biomarkers are biomolecules, but not all biomolecules are biomarkers. The definition of a biomarker is that it is a molecule, gene, or characteristic (cellular, biochemical or molecular alteration) whereby a particular physiological process, pathological disease process or pharmacological intervention can be measured and identified (Mayeux, 2004; Dictionary, 2016). A bioactive molecule or biologically active molecule is a substance or molecule that has an effect on a molecular level on the body (Dictionary, 2016).

The precise analysis and measurement of biomolecules can have a great impact on biological, pharmaceutical and medical sciences. Moreover, the analysis could teach us how certain

physiological processes and pathophysiological processes work as well as certain disease states in humans and animals. Therefore, the analysis of these chemical messengers can be used as a diagnostic tool in the diagnosis of diseases and disorders, to aid in the exact identification of drug mechanisms of action, as well as the development of new drugs or the improvement of existing drugs. There are already a few drugs that have been produced over the past years to mimic or control the release of these chemical messengers in humans and animals. Examples of these chemical messengers and diseases are: insulin in diabetes, gabapentin in epilepsy and adrenaline in anaphylactic shock (Moffat *et al.*, 2011).

1.3 Chemical messengers as biomolecules

Chemical messengers (also known as signalling molecules) and their metabolites are an important part of mammalian biomolecules and are critical for three communication systems, namely: the nervous, endocrine and immune system (Lieberman *et al.*, 2007; Wilkinson & Brown, 2015). These systems interact with each other through specific types of chemical messengers (Wilkinson & Brown, 2015), which communicate with target cells via the following signalling action ways neurocrine, endocrine, paracrine, neuroendocrine, autocrine and intracrine (Wilkinson & Brown, 2015). The process to classify chemical messengers is a constantly changing task because of the continuous discovery of newer messengers and new functions for known messengers (Wilkinson & Brown, 2015). Currently chemical messengers are classified as demonstrated in Figure 1-1. The figure was compiled by making use of a variety of literature resources.

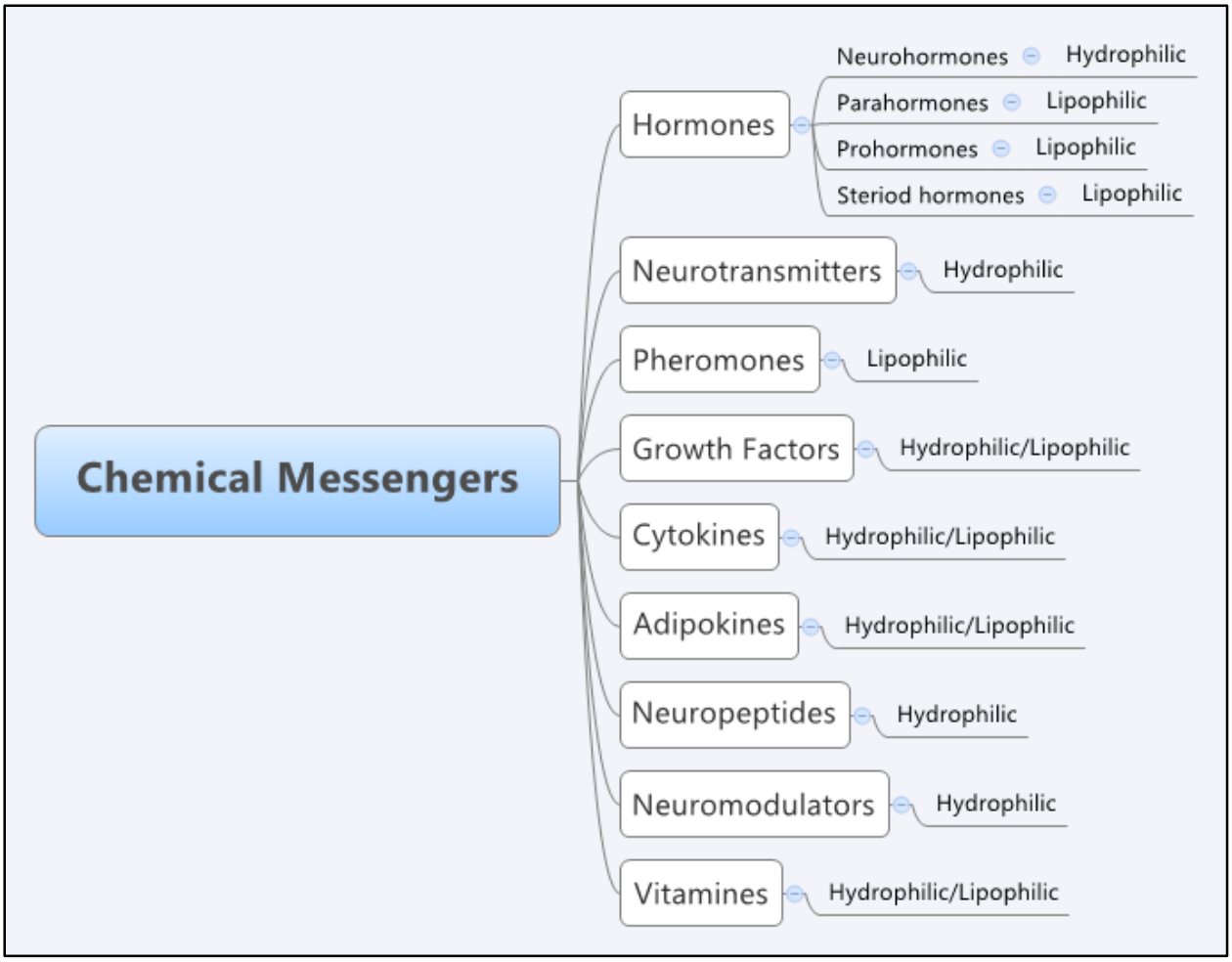

Figure 1-1: The classification of chemical messengers. Modified from literature (Sembulingam & Sembulingam, 2012; Wilkinson & Brown, 2015; Van Putte *et al*., 2016).

These chemical messenger molecules can also be divided into two groups according to their molecular structure namely the ones that are hydrophilic (water soluble/polar molecules) and lipophilic (lipid soluble/non-polar molecules) (Raven & Johnson, 2002; Koolman *et al.*, 2005; Campbell *et al.*, 2009). The hydrophilic chemical messengers are mainly derived from amino acids and the lipophilic ones are steroids and thyroid hormones (Raven & Johnson, 2002; Koolman *et al.*, 2005; Campbell *et al.*, 2009). There are three groups of chemical messengers derived from amino acids and they include amines, proteins and peptides (Campbell *et al.*, 2009). These chemical messengers are formed by biosynthesis in mammals (Koolman *et al.*, 2005). The neurotransmitters, the catecholamines (dopamine, norepinephrine and epinephrine) are biosynthesised from the amino acid tyrosine and all the steroid hormones from cholesterol (Koolman *et al.*, 2005).

1.4 Chromatographic method development for quantitative and qualitative analysis of biomolecules

The on-going development of analytical methods to analyse endogenous biomolecules in biological samples is imperative because it assists in better understanding physiological processes in mammalian bodies and it especially aids in the differentiation between normal and abnormal concentration levels. The constant discovery of newer biomolecules and additional functions of certain biomolecules necessitates the development of more sensitive and more specific methods. The search for alternative ways to treat diseases and the development of novel drugs also plays a significant role.

Analytical methods can be divided into the following three types: (a) qualitative methods, which are performed to confirm that the specific analyte is present in the biological sample, (b) semi-quantitative methods, which are only performed to give an approximate concentration of the specific analyte, and (c) quantitative methods which are used to give a well-defined accurate concentration value of the specific analyte in the biological sample (Saunders & Parkes, 1999). Each one of these has an important role to fulfil, however the focus of this study will be on quantitative analysis.

There are three main factors to consider when developing a method for quantitative analysis of biomolecules. The first one is to find an original biological sample matrix that is free of the specific biomolecule of interest or to find a suitable surrogate replacement sample matrix (Van Merbel 2008). The reason for this is to prepare a reference sample and standard samples to measure the test sample against to get an accurate reading of the concentration level of the biomolecule of interest. The second factor is the concentration of these biomolecules in the biological sample matrix, which mostly falls in the pg/ml (picograms per millilitre) to ng/ml (nanograms per millilitre) concentration ranges. The reason for this is to know what analytical instrument and sample preparation technique will be suitable. The third factor is to effectively extract the specific biomolecule of interest from the biological sample matrix to analyse it quantitatively.

The following steps (Figure 1-2) can help in guiding the analyst in the analytical process to develop an analytical method for these biomolecules:

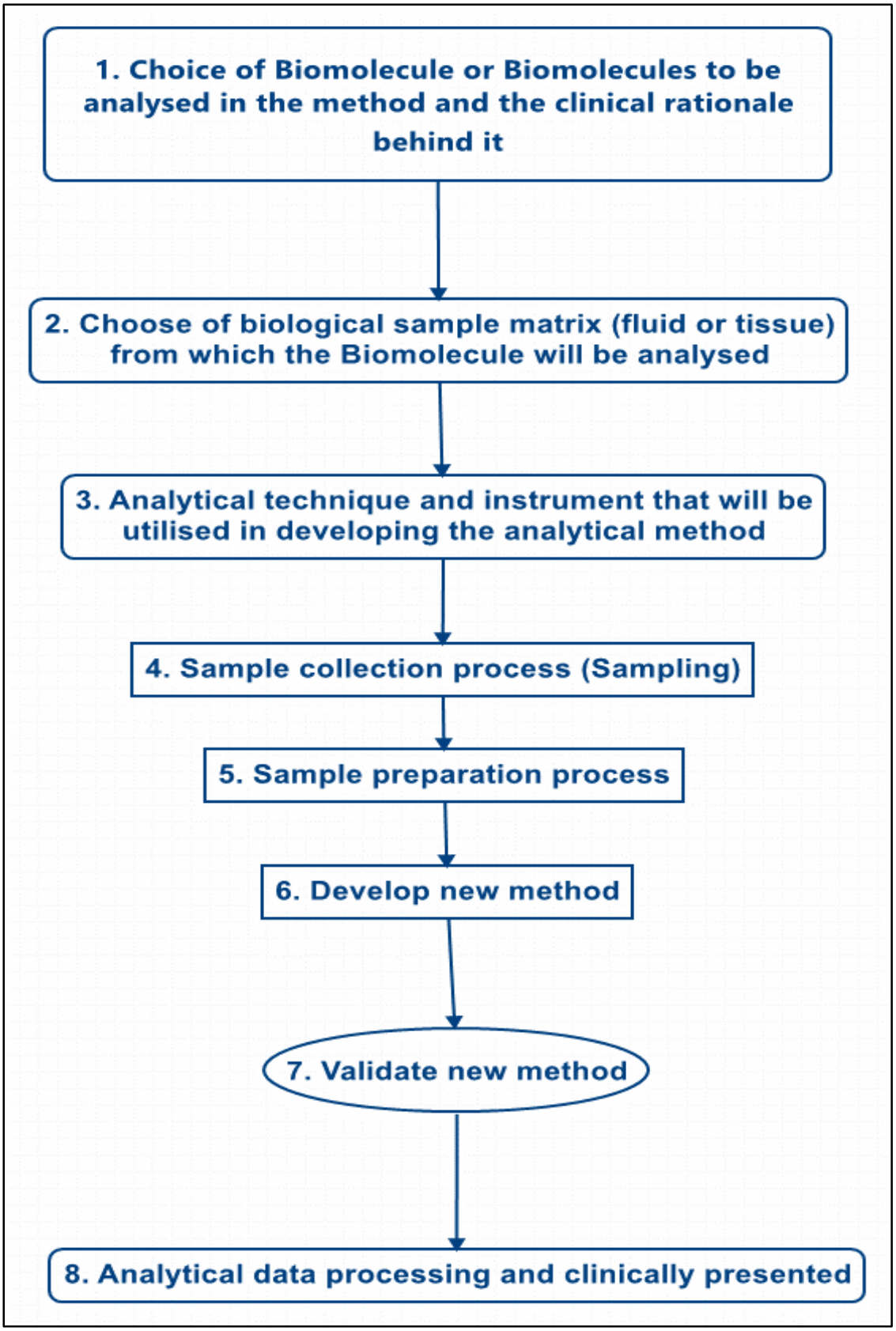

Figure 1-2: Guiding steps for the analyst in the analytical process to develop an analytical method for biomolecules.

1.4.1 Biomolecule characterisation

The first step in method development is to study the biomolecules (chemical messengers) to be analysed as well as the rationale behind it. In sample preparation this information helps to decide which extraction method to use and in chromatography it helps with the selection of the column and the mobile phase. The study must also provide an answer on which biological sample matrix will be best suited for the biomolecule of interest.

1.4.2 Biological sample matrix

The next phase in the process is studying the biological sample matrix from which the biomolecule will be analysed. Whole body analysis in biomedical research is not always possible, so a biological fluid or tissue sample is collected and used (Saude & Sykes, 2007). The sample matrices can range from body fluids (whole blood, plasma, serum, cerebrospinal fluid (CSF), saliva, urine) and tissue. These sample matrices consist of many different components that range from cells, electrolytes, various ions, respiratory gases, proteins, nutrients, hormones, enzymes, antibodies, mucus, waste products (urea and uric acid), creatinine and water (Marieb, 2006). Each of these biological matrices presents its own challenges when it is the sample of choice because of their complex composition of different components and unique characteristics (Su *et al.*, 2013). The reason for this is the molecular composition differs between all the different matrices and this can have an effect on the analytical process, and the stability or conformation of the biomolecule in the sample (Bielohuby *et al.*, 2012). The composition of the components of these biological sample matrices change during age, diseases and disorders, therefore the accurate analytical measurement of these biomolecules is important to understand and manage these changes. In the selection between plasma and serum as a biological sample matrix of choice, both are widely used but there is an ongoing debate regarding which sample matrix is best suited for a specific analyte (Liu *et al.*, 2010; Yu *et al.*, 2011; Bielohuby *et al.*, 2012; Oddoze *et al.*, 2012). When developing a method, it is advisable to test both plasma and serum to find which is best. CSF is a very invasive sample to collect for both humans and animals (Anoop *et al.*, 2010), however, it can be a very important biological sample matrix especially in humans to measure certain chemical messengers (for example neurotransmitter metabolites) for what is going on in the brain due to its close connection to the brain (Hyland, 2006; Anoop *et al.*, 2010; Chatzittofis *et al.*, 2013). Lately, saliva has become a biological sample matrix of interest because it is less invasive, can be collected very easily, has a less complex composition and a variety of chemical messengers and their metabolites can be measured (Chiappin *et al.*, 2007; Gröschl, 2008; Nunes *et al.*, 2015). Urine is another non-invasive sample to collect and is also one of the major routes whereby hormones and their metabolites are excreted from the bloodstream via the kidneys. Through the analysis of urine, important information can be obtained concerning the functioning of other

organs and therefore urine analysis can be used as a diagnostic tool for a variety of systemic and metabolic diseases (Kwasnik *et al.*, 2016).

1.4.3 The analytical technique and instrument

Chromatography is an analytical technique for the separation of mixtures of compounds and molecules in solutions by a variety of different chromatographic techniques (Licker, 2003). As mentioned, the process to develop chromatography methods to detect and quantify biomolecules starts with a study of the biomolecule.

(a) High-performance liquid chromatography (HPLC) coupled to a variety of detectors such as ultraviolet (UV), fluorescence (FLD), electrochemical (ECD) and mass-spectrometry (MS);

(b) Gas chromatography (GC) coupled to mass spectrometry (GC-MS).

HPLC is a technique where a liquid phase, which is called the mobile phase, is forced through a packed column (the stationary phase) with a material in it that can separate and retards molecules and then send it through a detector to detect and quantify it (Hansen *et al.*, 2011). GC is a technique where a molecule is taken through a special GC column in a gas phase that can separate the molecules and then be detected and quantified by the MS with which it is coupled (Hansen *et al.*, 2011).

The chemical structure of the biomolecule is very important in the decision of which detection technique will be suitable for the method development. For example is the biomolecule UV absorbent or electro-active? When this detection (UV, FLD or ECD) methods fall short, derivatisation of the molecule can be performed, such as in the case of ɣ-aminobutyric acid (GABA) which is derivatised with o-phthalaldehyde (OPA) followed by detection through either of the mentioned detection methods (Blau & Halket, 1993; Bartolomeo & Maisano, 2006). When these methods still fall short detection methods such as LCMS or GCMS can be used.

1.4.4 The sample collection and preparation processes

The process of preparing a biological sample for analysis can be challenging due to a variety of factors, such as the sample matrix and the wide range of possible analytes in them (Watson, 1994). The sample preparation process can be divided into the following four steps (Watson, 1994): the collection of the biological sample, the isolation of the biomolecule(s) from the biological sample matrix, the purification of the sample and the final preparation step before the sample is analysed (Watson, 1994). The last and very important step is the sample storage, either before analysis or after.

1.4.4.1 The sample collection process, isolation of the biomolecule(s) and purification of the sample

Sample collection or sampling is the first step and all further steps depend on the accuracy of the sampling procedure. It is important to put procedures in place to ensure sample integrity throughout the whole process and the consistency of the sampling technique is just as important (Watson, 1994). The sampling technique is highly dependent on the physical nature of the sample, liquid or solid. The biological sample amount required depends on the concentration level of the analyte(s) in the sample, the nature of the matrix and the chosen analytical method. The origin of the biological sample matrix is also important. For example, knowing if it is from human, animal or cell culture origin, could help to determine the concentration range in which the analyte(s) can be detected. Part of sample collection is the handling of samples for both human and animal biological samples and here a variety of aspects need to be considered. These aspects include the following: biosafety (does the biological samples contain infectious or contagious disease), is light and temperature protection of the analytes needed, the long-term storage time of the samples and ethical research practices. In table 1-1, the different biological sample types are tabled, the reason for its analysis in terms of what type chemical messengers can be measured in it, the pre-treatment of the sample in the pre-analytical phase, the preparation treatment and most important the storage.

Table 1-1: Examples of different biological sample types, reason for analysis, pre-treatment, preparation treatment and storage.

Biological Sample type	Reason for analysis (Chemical messenger)	Pre-treatment to stop any metabolic processes	Preparation treatment (Adding a chemical or reagent to keep the analyte of choice from degrading)	Storage
Whole blood, Plasma or serum	Hormones, neuropeptides	Liquid nitrogen	EDTA or Heparin blood test tubes	-80ºC
Tissue: Brain, endocrine glands	Hormones, neurotransmitters, neuropeptides, neuromodulators	Liquid nitrogen or pre-cooled isopentane	An acid (perchloric acid or hydrochloric acid) or a buffer (saline or phosphate)	-80ºC
Urine	Hormones	Liquid nitrogen		-80ºC
Saliva	Hormones	Liquid nitrogen		-80ºC
CSF	Neurotransmitters	Liquid nitrogen		-80ºC

Whole blood samples that can be separated into either plasma or serum samples can have both the advantages and limitations of time dependency as analytical samples for biomolecules (Melmed, 2016). From blood samples rapid changes in the bodily systems caused by a variety of stimulus, for example stress, diseases, disorders and also drugs, can be detected (Melmed, 2016). Sample pre-treatment will also play a role in the analysis of chemical messengers. For example, the effects of anticoagulants in blood samples can interfere in the analysis and knowledge of these interferences is very important (Bielohuby *et al.*, 2012). Sample pre-treatment can also involve adding a reagent to the sample to stop all biological processes (inherent enzymatic activity) to ensure that the biomolecule does not undergo any further metabolism or degradation, for example adding acetonitrile or perchloric acid to the sample to precipitate and denature proteins (this can also form part of the sample preparation step) (De la Luz-Hdez, 2012). Accurate and reliable results are obtained if, immediately after blood collection, samples are put on ice followed by immediate centrifuging to separate the blood cell components from the plasma or serum. The plasma or serum is then transferred to another tube and snap frozen in liquid nitrogen. Subsequently the samples are stored at -80°C if not immediately analysed. Some sample collection methods can also have a negative effect on the analytical result for example trauma during blood collection can have an influence on the catecholamine levels if the collection process takes too long (Burtis & Bruns, 2014).

Biological tissue samples must be snap frozen immediately after collection by submerging the specimen (within its sample collection container) into liquid nitrogen or pre-cooled isopentane to stop any metabolic processes, such as enzyme activity which can have a negative influence on the biomolecule's concentration in the sample (De la Luz-Hdez, 2012; Jones, 2014). Subsequently it can be stored at -80°C until analysis. When tissue samples are collected (for example rat brain) clean instruments must be used to prevent any contamination and any blood on the tissue must be quickly cleaned in distilled water. This will prevent any cross contamination from catecholamines in the blood to the brain tissue sample.

Urine can contain important information and be a good source of biomolecules (for example hormones) connecting it to the whole body and its systems (Kwasnik *et al.*, 2016). Urine does not only contain some important biomolecules but also their metabolites which can also have biological activity (Sluss & Hayes, 2016). The task to measure these biomolecules in the urine can be complicated due to significant changes in the body throughout the day, connected to a variety of factors (Kwasnik *et al.*, 2016), such as circadian rhythms, the circulatory levels of the various hormones, exercise, diet and the time of sample collection (Kwasnik *et al.*, 2016). When urine samples are used it is preferred to collect it over a 24-hour period (Burtis & Bruns, 2014), but this is not always possible when the sample is from small animal origin. Urine samples should

also be immediately snap frozen and stored at -20°C until day of analysis as long as the pH remains between 3 and 7, but for long term storage -80°C is recommended (Miki & Sudo, 1998).

In humans saliva is a non-invasive sample and easily collected. Saliva can also reflect the health status of the body with most of the biomolecules also detected in the blood and urine, but at much lower concentrations (Kwasnik *et al.*, 2016). Sample pre-treatment prior to storage is important because protease activity is still present at -20°C storage, therefore storage at -80°C is recommended (Kwasnik *et al.*, 2016).

CSF has a very invasive sample collection process (also known as lumbar puncture or spinal tap), but due to its close proximity to the brain and spinal cord it is the prevalent sample matrix for the monitoring of central nervous system (CNS) disorders and diseases (Kwasnik *et al.*, 2016). After CSF sample collection the sample must be snap frozen in liquid nitrogen to ensure sample integrity and stop any possible sample or analyte degradation. Storage at -80°C is also recommended.

It is important to minimize freeze-thaw cycles of all these biological sample matrices before analysis to preserve sample integrity. The choice of the sample collection container is very important for the storage and preservation of the sample and its analyte(s). The container must be able to handle the snap frozen process in the liquid nitrogen. Another important factor is the timing or time when the sample is collected. The timing of collecting the sample is important, for example when a certain drug's or invasive action's (stressed induced) effect is being tested in a test subject (animals or later in humans). Biomolecule levels may have a minute-to-minute, hour-to-hour or metabolic variation throughout a 24 hour cycle (Holland *et al.*, 2003). There is, for example, a difference in hormones and their various metabolite levels detected in the first morning samples in compared to subsequent collections later in the day (Holland *et al.*, 2003). The important question to answer is how long after the drug has been given or the invasive action was performed must the sample be taken to best analyse the desired effect on the biomolecule. This timing factor can be solved with a good literature study or an appropriate pilot study.

Sample preparation is a vital part of analysis and choosing the correct one is imperative. It forms part of the total analytical process, which has to ensure that the results obtained will give a true reflection of the analyte(s) status in the biological sample. Biological samples (like blood, plasma, serum, urine, tissue, etc.) are complex matrices, which contain many compounds that can interfere in the analysis process (Blahová & Brandsteterova, 2004). It is essential to isolate the desired biomolecule(s) from other endogenous compounds and molecules, such as proteins, enzymes, lipids and complex carbohydrates, which can interfere with the analysis. There is a variety of techniques used either solely or in combination with another technique to help isolate

the biomolecule(s). These techniques include protein precipitation, derivatisation, dilution, filtration, extraction (liquid-liquid extraction or solid phase extraction or solid-liquid extraction) and/or centrifugation to isolate the desired biomolecule(s) to be analysed (Blahová & Brandsteterova, 2004). These vital techniques also help to purify the sample of any possible interference before analysing it.

1.4.4.2 The final preparation step before the analysis of the sample

The final preparation for analysis of the sample involves the correct sample volume needed for the instrument to perform the analysis. In HPLC the sample volume to be injected by the autosampler can vary from 1 µl up to 100 µl, but in HPLCMS and GCMS the injection volume is from 1 µl up to 20 µl. The stability time frame of this final sample and the analytes in it need to be determined. This stability time frame is important for the analyst, as they need to know, how long after the sample was prepared can it still be injected and produce valid results.

1.4.4.3 Sample storage and transportation

Sample storage is imperative because biological samples are more liable to decomposition and degradation of the biomolecules. The following factors must be taken into consideration: light sensitivity, temperature sensitivity and the degradation of the analytes of interest. The time samples can be stored or preserved is also important and here ethical aspects play a roll, especially in the case of human samples. The temperature for long-term storage of biospecimens is at least below -80°C, but to eliminate temperature fluctuations when opening and closing the freezer's door -150°C storage is suggested (Shabihkhani *et al.*, 2014). Transporting the sample in the correct way is also essential. All biological samples must be transported in cooled icebox with dry ice or in liquid nitrogen, in the correct collection container.

1.4.4.4 Processing and presentation of analytical data

The way analytical data is processed and presented is important. There are generally two types of data - the first is the data produced by the method development and validation stages and secondly, the data produced by the experimental or diagnostic testing stages. The following factors play an important role when it comes to presenting the final data: the origin of the biological sample (human, animal or cell culture), the original biological sample (liquid or tissue); is it an acute or chronic study/sample, is it a time dependent study/sample, are the biomolecule and its metabolite(s) measured. In research, will the data be presented in groups or individual results? Analytical data can be presented in various forms depending on the type of data collected, e.g. graphical, chart or table form. In chromatographic analysis there is also no zero value results, those results are either indicated as below LOD (limit of detection) or below LOQ (limit of

quantification). The correct reporting unit of the data or results is vital in the case of endogenous molecules, therefore the use of international reporting units (SI units; International System of Units) is of importance (Taylor & Thompson, 2008) to ensure the results are internationally presentable and applicable in a generic form for the whole research community to understand and make a contribution to research (Miller *et al.*, 2014). The majority of international journals also prefer the use of these universal reporting units. When setting up the study design, a literature study must be done to decide in what units the results will be presented, for example, in mass per litre or moles per litre.

1.5 Chromatographic method validation for the detection and quantification of biomolecules: chemical messengers

The purpose of analytical method validation is to demonstrate through a series of analytical parameters that a newly developed analytical method will be analytically reliable to detect and quantify an analyte or series of analytes in a specific sample matrix (Garofolo, 2004). Method validation in chromatography forms an essential part of the method development process. According to Van de Merbel (2008), there are no official validation guidelines for these endogenous biomolecules. A few researchers have already proposed guidelines to validate methods for endogenous biomolecules (Lee *et al.*, 2006; Bansal & DeStefano, 2007; Kelley & DeSilva, 2007). In general, the validation parameters used are selectivity, specificity, precision, accuracy, linearity, range, robustness and stability (Huber, 2007; van de Merbel, 2008; Swartz & Krull, 2012).

The first challenge is to find a biological sample matrix that is free of the authentic endogenous biomolecule or to develop/use a reliable surrogate matrix to perform the method development and validation. There are a variety of procedures available to deplete the original biological sample matrix of the authentic biomolecule and use this prepared matrix to perform the method validation (van de Merbel, 2008). These procedures include removal by enzymatic action, by keeping the biological sample at room temperature or 37°C for a week so that oxidation takes place or by stripping the biological sample of the endogenous biomolecules with activated carbon (van de Merbel, 2008). There are several disadvantages of these procedures. The first is that it requires a lot of original biological sample matrix, which is not always possible or cost effective and they are not universally applicable to all endogenous biomolecules (van de Merbel, 2008). There are also some risks in these procedures, for example the oxidised product might interfere with the analyte of interest's detection or reductive back-conversion can take place (van de Merbel, 2008). The disadvantage by keeping the biological sample at room temperature or 37°C for a week, especially plasma, serum or urine, is that the biological sample could change physiologically and be unable to be classified as an authentic biological sample matrix. The disadvantage in using

activated charcoal is in the instance of not all the charcoal being removed causing false decreased levels of the interested analyte in the test samples (Thakare *et al.*, 2016). Activated charcoal also cannot efficiently remove all endogenous biomolecules for biological samples (Thakare *et al.*, 2016). The disadvantage in using the enzymatic action technique is that it is expensive in our research environment and increases the cost of research projects. Solid phase extraction cartridges can also be used to deplete the original biological sample matrix of the authentic biomolecule, but the disadvantage is that this can also increase the costs of the project. In literature, there are procedures available to prepare analytic acceptable surrogate matrices (simulated biological fluids) on which to perform the validation for a specific biological matrix (Marques *et al.*, 2011; Thakare *et al.*, 2016). The second challenge is to prepare the analyte free authentic matrix or the surrogate matrix with a specific authentic endogenous analyte or surrogate endogenous analyte. Commercially available analytical or reference standards can be used as the authentic endogenous analyte to prepare samples to develop and validate a new chromatographic method. The preparation with a surrogate analyte can be done by commercially available stable-isotope-labelled forms of a specific analyte (van de Merbel, 2008). Therefore, the following three approaches from literature were proposed to follow when developing and validating a new chromatographic method for endogenous biomolecules using an authentic analyte in an authentic matrix; or an authentic analyte in a surrogate matrix; or a surrogate analyte in an authentic matrix (van de Merbel, 2008; Jian *et al.*, 2013).

When developing new analytical methods, a full validation has to be done (US FDA, 2001). There is a variety of analytical validation guidelines available to follow in literature from governmental and other regulatory authorities. For example: Guidance for Industry Bioanalytical Method Validation (US FDA, 2001; US FDA, 2013; US FDA, 2015; US FDA, 2018); Guidelines for the validation of analytical methods for active constituent agricultural and veterinary chemical product (Australian Pesticides and Veterinary Medicines Authority, 2004); Guide to Quality in Analytical Chemistry An Aid to Accreditation (Barwick, 2016); Guideline on bioanalytical method validation (European Medicines Agency, 2011); the Validation of analytical procedures: text and methodology Q2(R1) (ICH Harmonised Tripartite Guideline, 2005). When a laboratory or analyst uses an already fully validated method, only a partial validation needs to be done. A good literature study on method validation before starting to validate the new method can help the analyst in this process. This full validation process can be divided into three phases. The first phase consists of the following parameters, analytic specificity, selectivity, sensitivity, linearity, precision, accuracy and range (Sluss & Hayes, 2016); the second phase includes stability and robustness (Sluss & Hayes, 2016); the third and final phase consists of the interpretation parameters, namely reportable ranges, reference intervals and diagnostic power (Sluss & Hayes, 2016).

CHAPTER 2 AIMS, OBJECTIVES AND METHODOLOGY

2.1 Research question/problem

Currently, commercially available analytical kits for the analysis of various biomolecules lack reliability (cross contamination) and affordability (as discussed in 2.3). This research project will focus on developing and validating new analytical methods that will be sensitive, specific, reliable and more affordable than commercially available analytical kits.

2.2 Aims and Objectives

The aim of this study was to develop and validate at least three analytical methods to detect and quantify a variety of biomolecules in different biological sample matrices (for example blood, plasma, serum, tissue, CSF and urine) and publish the newly developed and validated methods in the appropriate internationally accredited journals.

2.3 Background

During the last decade, bioanalytical method development and validation had become a fundamental part of drug discovery and development because of the need for newer and better techniques and to adhere to regulatory authorities (Shabir, 2006; Huber, 2007). The discovery and analysis of biomolecules and biomarkers have also become important, especially in clinical diagnosis. Biomedical and pharmaceutical sciences research is strongly dependent on the availability of specific and sensitive analytical methods for a variety of biomolecules (e.g. neurotransmitters, neuromodulators, peptides, proteins, hormones, etc.), also known as chemical messengers. The development of newer, faster and more specific and sensitive analytical methods remains challenging for the bio-analyst due to the wide concentration ranges in which these molecules can be present in the different biological sample matrices, in addition to the need for higher throughput.

The biomolecules discussed in the previous chapter are vital in the mammalian body, and may be of great value if methods can be developed to quantify them in different biological sample matrices. The precise control and the intensity of these molecules' actions at specific molecular targets are required in the intercellular communication in the central nervous system (Masson *et al.*, 1999), and therefore they are important in the field of biological sciences research. This study will specifically focus on the development and validation of analytical methods to analyse these biomolecules in different biological sample matrices.

CHAPTER 2 AIMS, OBJECTIVES AND METHODOLOGY

There are numerous commercially available analytical kits to use for the analyses of biomolecules such as radioimmunoassay (RIA) (Wassell *et al.*, 1999), enzyme immunoassay (EIA) (Kemppainen *et al.*, 2018) and enzyme-linked immunosorbent assay (ELISA) (Kim *et al.*, 2008). After several years of experience, we found these commercially available kits are expensive to use in our research conditions. There are also a few disadvantages with these analytical kits, for example most of the kits are limited to analysing one compound or molecule and do not have the ability to analyse a set of compounds or molecules per sample. These kits are mostly sold only to analyse an average of 100 samples per kit. These 100 samples also include standards and every sample must be analysed in duplicate; in other words, only 50 samples can be analysed by one kit. This means the more samples there are, the more kits need to be bought, and that increases the costs of the research project. The kits also have a very short shelf life, limiting the number of kits that can be stored. Moreover, the analyst using these commercial kits follows a blind recipe and does not know the specific ingredients of the different reagents or buffers within them. When using these kits, not all the reagents or buffers are included and the analyst must prepare some buffers for the analytical procedure. In general, these immunoassay methods are a more rapid technique but less sensitive and specific, while HPLC, GC-MS and LC-MS/MS, which take longer to develop and are more expensive but more sensitive, can analyse more than one compound or molecule per sample and the results obtained are more reliable (Faupel-Badger *et al.*, 2010; Grebe & Singh, 2011; Leung & Fong, 2014; Cross & Hornshaw, 2016).There are also commercially available kits for high performance liquid chromatography (HPLC) (Mathar *et al.*, 2010) and liquid chromatography-mass spectrometry (LCMS/MS) (Grüning & Bonningtonl, 2013), but again, the analyst follows a blind recipe as with the immunoassay kits. Table 2-1 shows a summary of current costs per sample when a commercially available immunoassay or HPLC kits is used, with excluded costs as a note at the bottom of the table.

Table 2-1: Example of current costs per sample for a commercially available Immunoassay kit and HPLC kit.

Immunoassay kits for plasma	**Cost per sample (excluding the standard samples)**
Abnova Epinephrine/Norepinephrine ELISA kit, KA 1877, 96 assays, ± R 9000.00	R 9000-00 ÷ 96/2 = R 187-50
Normetanephrine Elisa kit, KA1892, 96 assays, ± R 9000.00	
GenWay Biotech, Inc. Cortisol ELISA Kit, GWB-5E73AA, 96 assays, ± R9000.00	
Abnova Corticosterone ELISA kit, KA0468, 96 assays, ± R9000.00	
Antibodies-online Melatonin ELISA kit, ABIN2091896, 48 assays, ± R 15000.00	R 15000-00 ÷ 48/2 = R 625-00
HPLC kit for plasma	
ChromSystems HPLC Catecholamine (Norepinephrine, epinephrine and dopamine) in plasma Kit, 200 assays, ± R 40000.00	R 40000-00 ÷ 200 = R 200-00

Note: For ELISA, EIA and RIA kits all samples (standards & test) must be done in duplicate. All the prices exclude import costs, local distributor costs and VAT (Value Added Tax), instrumentation, maintenance of that instrumentation, the costs of reagents and buffers not included in the kit and which the analyst must prepare, and finally the salary of the analyst.

We postulate, that the development of analytical techniques on these (HPLC & LCMS) instruments, not using commercially available analytical kits, could produce a product that is cheaper in the long term and more specific to the exact research needs of the researcher and the research project (Turpeinen *et al.*, 2003; Cross & Hornshaw, 2016). The one biggest advantage of using these analytical techniques is that the number of samples to be analysed is much more than the commercially available analytical kits and that can bring down the costs long term and make it more affordable. These analytical techniques can also save time because most of them today are equipped with temperature-controlled autosamplers, which can continuously analyse big batches of prepared samples. In most biomedical research today, in either animals or humans, large volumes of samples usually need be tested to make the research data statistically significant. A global need in new drug discovery and development requires more research and that makes these instruments and techniques a valid choice.

The bio-analysis of monoamines (dopamine, norepinephrine and epinephrine) and their metabolites by these commercially available kits in various biological matrices has a variety of short falls. These short falls include that most kits can only analyse one or two but not all at once, for example the epinephrine/norepinephrine ELISA kit (plasma and urine) from Abnova (catalogue

number KA1877) and the serotonin ELISA kit (plasma, serum, urine and platelets) from Enzo (catalogue number ADI-900-175). However, there are no kits available to analyse monoamines and their metabolites in rat brain samples. In steroid hormone immunoassays significant cross-reactivity generally occurs with structurally similar endogenous compounds such as 21-deoxycortisol that can accumulate to very high concentrations in certain disease conditions (Krasowski *et al.*, 2014). Cross-reactivity can also occur with similarly structured drugs for example prednisolone in cortisol immunoassays and methyltestosterone in testosterone immunoassays (Krasowski *et al.*, 2014). In the case of measuring corticosterone, the commonly employed RIA techniques use antiserum which significantly cross reacts with precursors and metabolites of corticosterone, and with other endogenous steroids and their metabolites (Wong *et al.*, 1994). Consequently, levels of corticosterone measured by RIA are probably an overestimate of the true levels (Wong *et al.*, 1994). McCullough and co-workers found, for example, that in their evaluation of certain radioimmunoassay and enzyme immunoassay methods to measure oxytocin levels that they lack reliability when used on unextracted samples of human fluids. They also found that these methods tag molecules in addition to oxytocin, yielding measured levels that are wildly discrepant with earlier findings that were obtained using well validated methods (McCullough *et al.*, 2013). Compounds or molecules producing cross-reactivity in steroid hormone and other immunoassays generally have a high degree of structural similarity to the target compound or molecule (Tate & Ward, 2004; Krasowski *et al.*, 2014). The question now is how many more of the above analytical cases are there? The availability of specific, sensitive and accurate analytical methods is of great importance to ensure reliable end results for these biomolecules. Therefore, because of their better analytical performance the desire is rather to apply chromatographic techniques rather than traditional ligand-binding assays (van de Merbel, 2008).

Quantitative analysis of biomolecules in biological sample matrices is more complicated, both analytically and from a validation point of view. It is often difficult, if not impossible, to obtain biomolecule-free samples of the authentic biological matrix or samples with accurately-known biomolecule concentrations (van de Merbel, 2008). According to van de Merbel the validation of analytical methods for biomolecules has been made difficult by the absence of official guidelines (van de Merbel, 2008). Most researchers apply “the method-validation principles for the analysis of drugs issued by the US Food and Drug Administration” (van de Merbel, 2008), to their methods in measuring specific biomolecules, for them to ensure their results have an acceptable and comparable level of quality (van de Merbel, 2008). The problem here is that these principles of the FDA were meant for the analysis of drugs in biological fluids and not for biomolecules and therefore direct application in most cases is not possible (van de Merbel, 2008).

2.4 Research Design and Methodology

In analytical method development the research design and methodology play the main important roles. The research design helps the researcher to set up a plan from the research question or problem statement and rationale of the research project to the end where the question is answered or the research problem is solved. The research methodology will describe all the methodology procedures to be followed to answer the question or to solve the research problem.

The following diagram describes the experimental design that will be followed in the analytical methodology for developing and validating new analytical methods for the analysis of the biomolecules in this study.

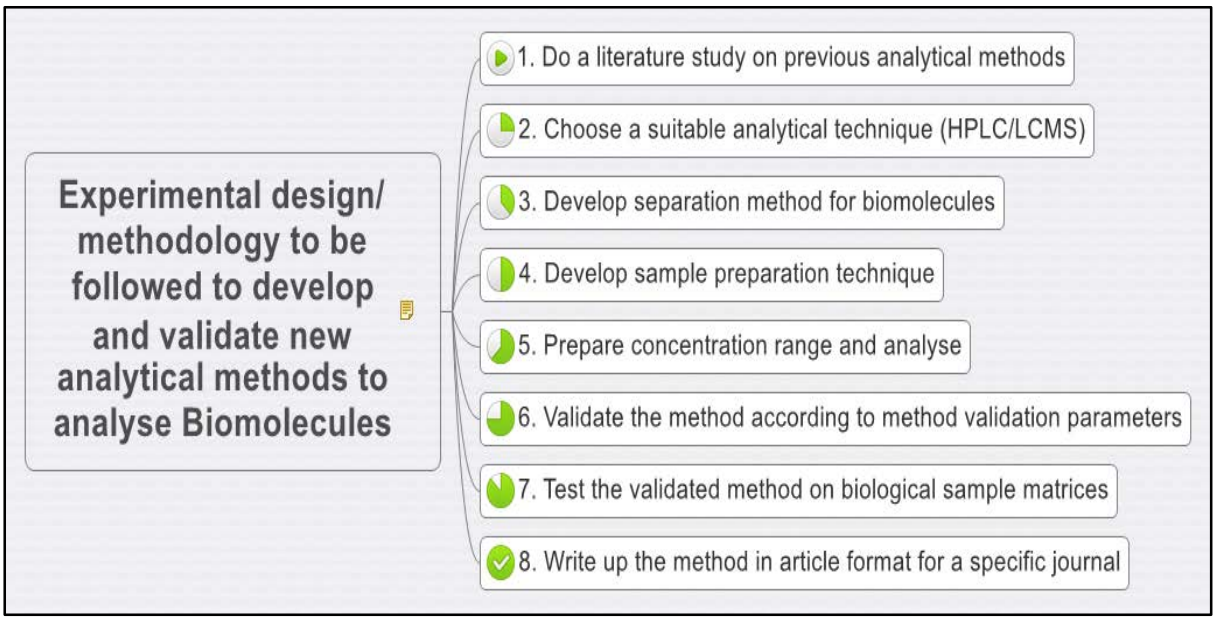

Figure 2-1: Experimental design/methodology that will be followed during the analytical method development and validation process.

2.4.1 Literature study on previous analytical methods

A literature study on previous analytical methods is an essential step in the whole process. Here the researcher can get a better understanding of methods that have already been developed and where there is a need to develop new methods or to better the older methods. This can make the method development part easier, by using an already developed method, but there are a few questions or factors the researcher need to consider first. The following table (2-2) will discuss these questions and factors.

Table 2-2: Questions or factors to consider before using an already developed analytical method from literature.

Question or Factor	Possible Solution
How old is the method/ Is there a method?	Can a better new one be developed?
How old is the instrument on which the method was developed (old UV detector, less sensitive)?	Are there newer instruments (newer UV detector, more sensitive)?
The detection method is less sensitive and selective (UV detector) for the biomolecule.	The detection method is more sensitive and selective (electrochemical detector) for the biomolecule.
Older type of analytical column, separation not good.	Newer type of analytical column, better separation.
Older sample preparation techniques.	Newer sample preparation techniques.
Method developed on older HPLC instrumentation.	Newer and more advance HPLC instrumentation.
Chemicals used toxic?	Can other less toxic chemicals be used?
Sample preparation very complicated, extensive and expensive.	Can a less complicated and expensive sample preparation developed?
The method was developed for pharmaceutical drug testing (for example the drug Hydrocortisone, Cortef®).	Can this method be used to detect and analyse natural occurring Cortisol in animals and humans?
The method was developed for a different biological sample (for example plasma).	Can this method be used also for biological tissue sample or must a new sample preparation method be developed?
Was the method validated?	The method needs to be validated.
Is the literature method valid for the new research project, answer the research question or problem?	Is there a need to modify or develop a new method to fit the research project, answer the research question or problem?
Was the method comprehensive or was some important details of the method missing?	The method needs to be re-developed to make it comprehensive.
Some reagents, chemical or column not commercially available.	The method needs to be re-developed with replacement reagent or chemical or column.

2.4.2 Instrumentation

The following analytical instruments were utilised in this study in order to develop and validate specific analytical methods: High performance liquid chromatography coupled with diode array detection (HPLC-DAD) and HPLC coupled with electrochemical detection (amperometric and coulometric) (HPLC-ECD). HPLC is a powerful and widely used technique for the separation of both large and small biomolecules (Inc., 2010). Its primary abilities over other analytical techniques are high efficiency and that it can separate and distinguish between compounds that are chemically very similar (Inc., 2010). The two detectors have both unique and different

detection capabilities to detect and quantify the biomolecules. The analytical detectors connected to an HPLC system rely on the physico-chemical properties of the analyte molecules to detect them. The diode array detectors are based on the absorbance of ultraviolet (or visible) light by the analyte molecule, which must contain a chromophore; the mobile phase and solvents used must be transparent (Swartz, 2010; Crawford Scientific, 2014). Electrochemical detection is based on the oxidation-reduction property of the analyte molecule and the mobile phase used must be conductive (Swartz, 2010; Crawford Scientific, 2014). Developing analytical methods on these instruments can be costly, but when the method is fully developed and validated, the analyses of biological samples can be cost effective when compared to commercially available analytical kits.

2.4.3 HPLC columns used

A variety of columns were tested, including the Luna C18 (2) 250 x 4.6 mm 5 µm, Synergi Max-RP 4 µm C12 250 x 4.6 mm, Synergi Hydro-RP 4 µm C18 250 x 4.6 mm (Phenomenex) and Venusil XBP C18 4.6 x 250 mm 5 µm (Bonna-Agela Technologies). The following two columns proved to be the best the Venusil ASB C18 4.6 x 250 mm 5 µm and C8 4.6 x 250 mm 5 µm (Bonna-Agela Technologies). The main features of these columns are that they are 100% aqueous compatible and have the capability of handling low pH, even stable at pH 0.8 (Bonna-Agela Technologies).

2.4.4 Quality assurance and control in research laboratories

In the last two decades quality assurance and control in research and development laboratories have become very important (Robins *et al.*, 2006). The researchers must demonstrate that they are conducting their research to the highest of standards (Robins *et al.*, 2006). In the field of research, standard operating procedures and analytical methods need to be written comprehensively to ensure quality and control of research at a specific research institution. The quality and control does not only apply to the experimental, analytical and data processing stages, but to the original research design of the research project. The original research design must be rigorous and well planned to ensure the results are as robust and unambiguous as possible, and to enable the success and reproducibility of studies. Here, good method development and validation plays a crucial role in the whole research process.

2.4.5 Analytical method validation

In analytical method validation for endogenous biomolecules/chemical messengers there are no official documents stating official guidelines or validation parameters (van de Merbel, 2008). In practice, most researchers follow the FDA guidelines on “Bioanalytical method validation:

Guidance for Industry" for these kind of molecules (van de Merbel, 2008). Criteria for test method clinical requirements are usually related to the biological distribution of values observed within a healthy population or to consensus opinions based upon perceived requirements for clinical diagnosis (Lumsden, 2000). This criteria also applies to pre-clinical research in our view. Thus the developed and validated method must be practically applicable in pre-clinical research. In Chapter 1, the different method validation parameters, as described in literature, and how they were to be applied in practice were discussed. In this chapter, the importance of every one of these parameters and their purpose in the method validation process will be discussed.

2.4.5.1 Selectivity and specificity

The selectivity of a method relies on the physico-chemical properties of the analyte molecule to be measured and the correct selective analytical detector to detect it at the desired concentration levels (Crawford Scientific, 2014). The specificity of a method relies on the ability of that method to detect specifically and accurately the molecule of interest on its own or in the presence of other compounds that may also be present in the sample (Swartz & Krull, 2012; US FDA, 2018).

Specificity and **Selectivity** are parameters in validation, which show that the method can detect and accurately measure a specific analyte (biomolecule) in a specific matrix sample in the presence of other analytes (biomolecules) and other possible interferences (US FDA, 2001; Sluss & Hayes, 2016). In chromatography, these parameters show that the chromatographic method can separate the specific analytes (biomolecules) of interest from other analytes (biomolecules) and other possible interferences. **Cross-reactivity** is a major problem when analytes have similar physico-chemical properties, such as for example with the analysis of steroid hormones especially in immunoassay techniques (Krasowski *et al.*, 2014). These parameters become much better when the analysis method moves from immunoassay techniques to HPLC and then to LCMS or GCMS. This improvement is because of the chromatographic separation technique and variety of different detectors in HPLC or GC (for example, ultra-violet, fluorescence, electrochemical and mass spectrometry) therefore the cross-reactivity problem can be eliminated. **Interferences** in chromatography can come from a number of sources and these have to be kept to a minimum to give a high specificity and selectivity; sources such as background noise, baseline noise, different reagents and buffers used in the sample preparation process and different chemicals used in the mobile phase.

2.4.5.2 Sensitivity

The sensitivity of a method relies on the detectable signal response of the detector for a specific molecule. The sensitivity will differ from molecule to molecule. The sensitivity also relies on the

signal-to-noise (S/N) ratio, where the signal response of a specific molecule must be greater than the background noise or the baseline (Swartz & Krull, 2012; Crawford Scientific, 2014). The standard acceptable criteria for the signal-to-noise ratio is between 3:1 and 2:1, in other words the response signal of the molecule must be two to three times greater than the background noise or the baseline (Swartz & Krull, 2012; Crawford Scientific, 2014). The S/N ratio is also used to determine the detection limit of the method. Table 2-3 shows the selectivity and sensitivity of the two different analytical detectors that can be used with an HPLC, and used in this study project.

Table 2-3: Selectivity and sensitivity of the two different analytical detectors used in this study project *(Swartz, 2010; Skoog et al., 2013; Crawford Scientific, 2014).*

Detector	Selectivity	Sensitivity: minimum mass detectable	Relative mass detection range
UV/Visible/DAD	Medium	0.5 – 1.0 ng/ml	µg - ng
Electrochemical	High	50 – 500 pg/ml	ng – pg

Analytic sensitivity consists of two parameters limit of detection (LOD) and limit of quantification (LOQ). LOD is where the lowest detectable concentration of a specific analyte can reliably be detected and distinguished from the background noise and baseline (Garofolo, 2004; Bansal & DeStefano, 2007; Melmed, 2016; Sluss & Hayes, 2016). The analytical method must also be able to reliably differentiate this concentration from the background noise (Garofolo, 2004). The LOD concentration can be determined in three ways. The first is the basic visual evaluation, the second is based on the signal-to-noise ratio which must be between 3 or 2:1 to be accepted, and third is based on the standard deviation (SD) of the response and the slope of the calibration curve (Shrivastava & Gupta, 2011).

The LOQ parameter can be measured with acceptable accuracy and precision and be divided into the lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ) (Garofolo, 2004; Bansal & DeStefano, 2007; Melmed, 2016; Sluss & Hayes, 2016). The response of the analyte peak should be discrete, identifiable and reproducible with an accuracy of 80 to 120% and precision % RSD of 15 to 20% (US FDA, 2001). The dilution of samples should not affect the accuracy and precision. If applicable, dilution integrity can be demonstrated by spiking the matrix with an analyte concentration above the ULOQ and diluting the sample with blank matrix solution. Accuracy and precision should fall within the set criteria with at least five determinations per dilution factor. The dilution integrity should cover also the dilution applied to the test samples (European Medicines Agency, 2011). These two parameters are essential for the quantifiable detection range of the method. In some cases the LLOQ and the LOD will be the same concentration value.

The matrix effect is a validation parameter that most validation processes do not include, but this is an important parameter especially in LCMS (Rogatsky & Stein, 2005). The matrix effect can be described as the effect that the sample matrix has on the sensitivity of the detection signal of a specific analytical detector by either suppressing or enhancing it. In most cases suppression is the result causing a loss in sensitivity for the specific analyte to be analysed. In LCMS sensitivity can be greatly reduced because of the matrix effect which causes suppression of the ionisation efficiency (Rogatsky & Stein, 2005).

2.4.5.3 Accuracy, precision, and recovery

The purpose of accuracy, precision and recovery in method validation is to ensure that the results obtained from test samples will be a true reflection of the molecule (s) of interest concentration analysed in a specific biological sample at a specific time (Swartz & Krull, 2012; US FDA, 2018). These parameters also test the method's reliability, in that the results obtained will be accurate and repeatable (Swartz & Krull, 2012; US FDA, 2018). These three parameters are crucial especially when liquid-liquid extraction, liquid-solid extraction and solid phase extraction are used as sample preparation techniques in biological sample analysis.

Precision or **reproducibility** measures the replication of repeated analysis of the same sample concentration to see how reproducible and repeatable the analytical method is (US FDA, 2001). This parameter consists of two parts, intra-day variance and inter-day variance. Intra-day variance is calculated by measuring a minimum of five determinations per concentration with a minimum of three concentrations (low, medium and high) in the analytical range if expected concentrations are to be analysed on the same day or within 24 hours (Australian Pesticides and Veterinary Medicines Authority, 2004; US FDA, 2013). Inter-day variance is calculated in the same way as intra-day variance, but the five determinations are done on five different days. The % RSD (percentage relative standard deviation) determined at each concentration level should be less than 15% except for concentrations at the LOQ, where it should be less than 20% (US FDA, 2001).

Accuracy consists of a variety of parameters, starting with the **linearity curve**. Linearity can be defined as the ability of an analytical method to obtain a measured result directly proportional to that specific analyte in the tested sample (Bansal & DeStefano, 2007). A minimum of six non-zero concentrations should be used to construct the linearity curve, starting at the LLOQ and ending at the ULOQ. The linear regression value for the curve must not be less than $r^2 = 0.95$ for biologics or biomolecules (Shabir, 2006). The next part in the accuracy parameter describes the closeness of the mean of the analysed results obtained by the analytical method to the true value (concentration) measured over a period of time of the analyte of interest (US FDA, 2001). A

minimum of five determinations of three or four concentrations in the expected analytical range is recommended (US FDA, 2001; Australian Pesticides and Veterinary Medicines Authority, 2004; European Medicines Agency, 2011; US FDA, 2013). The mean value should be within 15% of the actual value except at LLOQ, where it should not deviate by more than 20%. The deviation of the mean from the true value serves as the measure of accuracy (US FDA, 2001). The measurement of accuracy is expressed in the deviation of the mean from the true value. **Recovery** is the next important parameter and here the percentage recovery of the analyte of interest is measured. This parameter measures the extraction efficiency of the whole analytical process from the different sample preparation steps up to the final analysis step and the detector response of the method (US FDA, 2001; Bansal & DeStefano, 2007). A minimum of three concentrations (low, medium and high) must be used. The results must not only be as close as they can to 100% but, must also be consistent and reproducible (US FDA, 2001). The parameter **carryover** is important in analytical methods that involve auto-samplers or automated sample-handling devices (Sluss & Hayes, 2016). The percentage of the specific analyte carryover between samples that are directly analysed after each other must be evaluated otherwise it could result in false higher concentrations for the ensuing sample.

2.4.5.4 Calibration/Linearity

In bioanalytical measurement, method development and validation the calibration/linearity parameter is an important tool (Barwick, 2003; Barwick, 2016). In general, calibration is a methodology followed to establish a calibration curve that represents the relationship between the measurement response output of an analytical instrument and the acceptable values of calibration standards which contain a certain concentration of a specific analyte (Cuadros-Rodríguez *et al.*, 2001; Barwick, 2003; Dong, 2006; Barwick, 2016). Linearity, conversely, is the ability of an analytical method to elicit a test result that is directly or indirectly, by a well-defined mathematical conversion, proportional to the analyte concentration within the given range (Swartz & Krull, 2012). In practice, both rely on a well-defined linear regression line. Linearity only needs to be evaluated when an analytical method initially is developed, validated and put into service, but calibration verification involves all processes especially the assaying calibration standards representing the lower limit, midpoint, and upper limit of the reporting range in the same manner as test samples.

Calibration in general can be divided into two parts qualitative or quantitative calibration (Cuadros-Rodríguez *et al.*, 2001). In quantitative calibration the main purpose is to show that both the instrument and the analytical process are valid for use (Cuadros-Rodríguez *et al.*, 2001). There are basically two ways of setting up a calibration currently in practice and they are one-point or multi-point calibration (Khamis *et al.*, 2018). Both have advantages and disadvantages; for

example, one point calibration can only be used when the analytical data values are in a narrow range and when the sample size is small, but it is the least desirable of the two (Harvey, 2000). Multi-point calibration takes longer to prepare but it is more accurate and reliable. Thus for a multiple-point calibration at least three standards, although more are preferable, should be used (Harvey, 2000).

In method, validation calibration is one of the required criteria with at least a minimum of six non-zero standards and one zero standard or blank (US FDA, 2013), in other words, multi-point calibration. In practice, there are several ways to prepare standards for multi-point calibration; the use of serial or parallel standard dilutions or a combination of the two are used. The debate on which one is better is still ongoing in literature (Khamis *et al.*, 2018). The two dilution techniques have both advantages and disadvantages. The serial dilution method is a progressive dilution of an analyte concentration in a series of volumetric flasks or Eppendorf tubes in predetermined ratios, for example 2-fold or 10-fold dilution steps (see figure 2-2). The parallel dilution method consists of a stock solution from which new solutions are made by transferring different amounts of the stock solution to different volumetric flasks or Eppendorf tubes (figure 2-3).

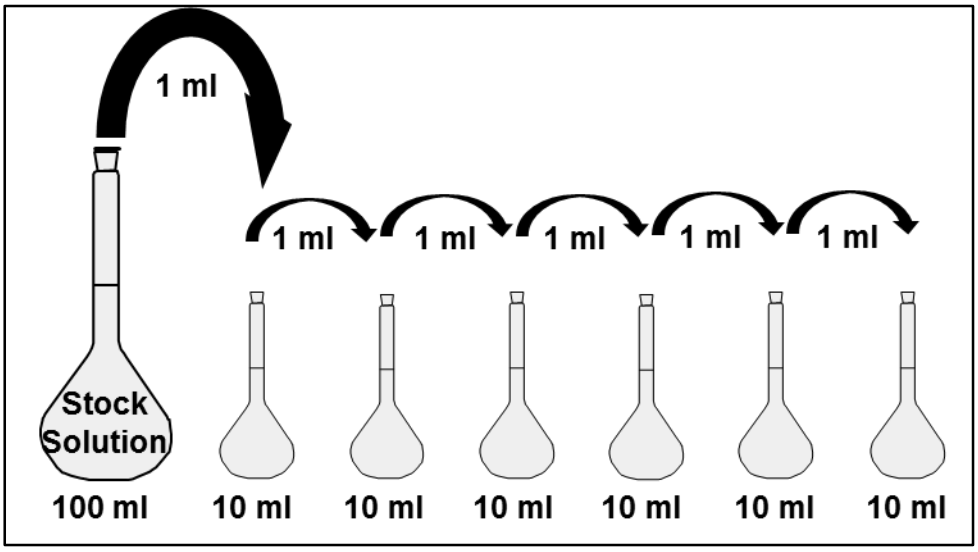

Figure 2-2: Serial range standard dilutions method.

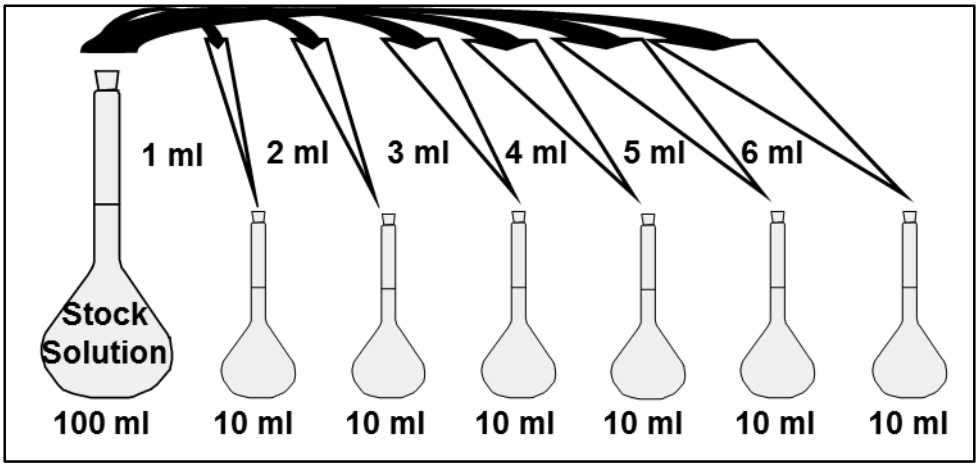

Figure 2-3: Parallel range standards solutions method.

In this study project, the parallel standard dilutions method will primarily be used. The following table shows an example of a range of standards dilutions where the parallel method was followed.

Table 2-4: Example of standard dilutions for standard concentration range

Prepare a stock solution (SS) by weighing of 1 mg of the analyte and dissolve it in the appropriate solvent volume, 10 ml, to prepare a 100 µg/ml concentration stock solution.						
Standard number	**Concentration (ng/ml)**	**Dilution volume**	**+**	**Dilution solvent volume**	**=**	**Total volume**
A	5000	100µl (SS)	+	1900 µl	=	2 ml
9	200	80µl (A)	+	1920 µl	=	2 ml
8	150	60µl (A)	+	1940 µl	=	2 ml
7	100	40µl (A)	+	1960 µl	=	2 ml
6	75	30µl (A)	+	1970 µl	=	2 ml
5	50	20µl (A)	+	1980 µl	=	2 ml
4	25	10µl (A)	+	1990 µl	=	2 ml
3	10	4µl (A)	+	1996 µl	=	2 ml
2	5 (LOQ)	1000µl (3)	+	1000 µl	=	2 ml
1	2,5 (LOD)	500µl (3)	+	1500 µl	=	2 ml

Note: These dilutions can be done with an air-displacement pipette.

2.4.5.5 Stability

The purpose of stability testing is to ensure that the biomolecules of interest and the internal standard will be chemically stable through the whole analytical process from sample collection until final analysis (Garofolo, 2004). This also includes the stability of the biological sample. In biological samples, there are common factors that affect the stability of the sample and the

analytes, and they include temperature, light, pH, oxidation, enzymatic degradation and time until analysis (Jones *et al.*, 2007; Bielohuby *et al.*, 2012; van de Merbel *et al.*, 2014). These factors can cause a loss of the biological molecule of interest over time. The background and baseline interferences can also increase causing detection problems with low concentrations in the sample.

Stability is a time related parameter and measures over time the stability of the analyte(s) within the standards and sample matrices, the reagents, the buffers and the assay itself. The following are crucial: the bench-top stability, freeze and thaw stability of the sample matrices, the short and long term stability of the standards and sample matrices, and the stability of the analyte(s) in the finally prepared sample (US FDA, 2001; Garofolo, 2004). The accuracy and precision parameters of gathered data can also assist the analyst to measure the stability.

2.4.5.6 Robustness/Ruggedness

Robustness/Ruggedness measures the method's stability and capacity to remain unaffected by small, but deliberate variations in the analytical method's parameters (Tiwari & Tiwari, 2010). This will then provide an indication of the reliability with which the analyte(s) can be determined during normal analytical usage. The analytical method's parameters that can be varied are the flow rate of the mobile phase through the LC system, the pH of the mobile phase, the percentage of the inorganic phase in the mobile phase and the column's temperature (Tiwari & Tiwari, 2010). Different sample preparation techniques can also prove the methods robustness. Robustness is not commonly a part of full method validation but it does play an important role during the method development stage (Tiwari & Tiwari, 2010).

2.4.5.7 Interpretation

The **interpretation** parameter is part of the 3rd phase where the method will be applied into practice. Here the first parameter is a **calibration** or **standard curve** (the linearity curve parameter forms the frontrunner to this parameter), which will be used to give a mathematical quantifiable and reliable concentration value to the specific analyte(s) detected and measured in the test sample. The calibration curve must consist of at least six to eight non-zero concentrations and include a blank sample and a zero sample (Garofolo, 2004). The blank sample is a matrix sample prepared without the internal standard and a zero sample is prepared with the internal standard (Garofolo, 2004). The range of concentrations chosen for this curve must span over the expected concentration range for the specific endogenous biomolecule in the specific biological sample matrix. It is also good laboratory practice to run a calibration curve for every batch of unknown samples to be done. The addition of an internal standard to the whole method

development and validation process can make a positive contribution, as well as the interpretation of the unknown or test samples. An internal standard must be a compound or molecule that is structurally related to the endogenous biomolecule that the method will be developed for, and will not interfere with the biomolecule in any way during the method analysis. The internal standard must also adhere to the validation processes. The internal standard is basically a constant concentration standard added to all the samples from the calibration standards to the unknown samples. Quantification of the unknown samples is then based not only on the standards, but on the response ratio of the analyte of interest to the internal standard of the unknown sample compared to the same ratio within the calibration sample (Standardization, 1994). The internal standard method is especially proposed to be used in samples where the concentration range of a specific analyte is low and sensitivity is a problem, and in samples with a complex sample preparation procedure (Standardization, 1994).

The **reportable** or **quantifiable range** of a method mainly spans from the LOQ (or LOD) to the highest standard prepared of the calibration curve (Sluss & Hayes, 2016). This range must cover the whole range of concentration results that can be measured in a certain biological matrix sample for a specific analyte. In the case of a concentration result above the highest calibration level, in for example immunoassays, the test samples have to be diluted (Sluss & Hayes, 2016), but this is not always necessary in chromatography when analysing biomolecules because of their low concentration ranges and the fact that the detectors used in chromatography can handle those signal responses.

Reference intervals or normal ranges describe the expected concentration interval or range that a specific analyte (especially a biomolecule) will be analytically measured within when samples from healthy humans or animals were tested (Sluss & Hayes, 2016). These intervals or ranges will be specific for that analyte in conjunction with the following factors; age, gender, biological sample type, analytical instrument and the analytical method technique followed. These intervals or ranges are essential because they help the research laboratory to differentiate between normal or control or baseline samples and abnormal or test samples (Jones & Barker, 2008; Boyd, 2010). In medical laboratories, these reference intervals or normal ranges are used to differentiate between healthy samples (patients) and unhealthy samples (patients) to diagnose patients with certain diseases.

Diagnostic power can typically be described as the last step in the process of method validation and therefore must show that this method will be clinical useful (Sluss & Hayes, 2016). This means that this method can be successfully applied in a research laboratory to do research or a medical laboratory to diagnose patients.

2.4.5.8 Analytical bias

Analytical bias (also known as trueness) can be seen as a 4th phase in method validation although it includes a combination of accuracy and precision. According to the ISO 5725-1 document of 1994, bias can be defined as “the difference between the expected test results and the accepted reference value” (International Organization for Standardization, 1994) and therefore measures the systematic variation of the method over time (White & Farrance, 2004). The bias (trueness) parameter can be measured as a percentage deviation of the mean value of a large series of measurements from the accepted reference value (Tiwari & Tiwari, 2010). This large series of measurements are not done during the initial full validation but through the analysis of a great number of quality control (QC) samples during routine analysis work (Tiwari & Tiwari, 2010). The determination of bias can also be achieved by performing the method in separate laboratories or with separate analysts and this is known in inter-laboratory quality control.

2.5 The importance of the use of an internal standard

In the process of bioanalytical method development and validation as well as when a new method is applied to test samples of biological origin, an internal standard can play an important role. There are many advantages in the use of an internal standard. The internal standard is a substance that has been added at a fixed concentration to all standards and samples during the sample preparation process (Harvey, 2000). In the past, the internal standard method was used to correct for variability in the injection volume, but today's autosamplers generally have a volumetric imprecision of <0.5%, so such corrections are not needed today (Dolan, 2012). The internal standard plays an important role in the calibration and standardisation of the method (Harvey, 2000). The internal standard can compensate for both systematic and random errors in the analysis process (JoVE Science Education Database, 2018). In the case of random errors, such as instrumental fluctuations during the analysis, these fluctuations will be expected to be the same for both the internal standard and the analyte, thus the ratio of signal responses does not change. In the case of systematic errors, such as matrix effects of the solvent, the ratio again will be unaffected, as long as the effect is equal for both the internal standard and analyte (JoVE Science Education Database, 2018). An internal standard will benefit a method mostly when there are multiple sample preparation steps in order to compensate for any losses during the sample preparation process (Dolan, 2012; Crawford Scientific, 2014). It also serves as an internal marker to indicate that the sample preparation process was successful especially when the process has many steps, as in liquid-liquid or solid phase extraction. The internal standard substance must not be found naturally in the sample especially biological samples and it must be stable (Dolan, 2012). It also must be compatible with the detector’s response and structurally similar to analyte but this is not always possible (Dolan, 2012). The internal standard also serves as a marker of

the relative retention time of analyte peaks of a chromatogram in case of a retention time shift due to changes in temperature for example.

2.6 Practical application/utilisation of the new validated method

After the method is developed and validated, it must be applied to test samples to show that the method will be applicable in practice. This can also be achieved by routinely using the method in research studies in place of the commercially available ELISA, EIA and RIA kits. Studies can also be done by comparing the developed method with the commercially available kits to show that the new method is practically viable.

2.7 The analytical process

In the research fields of biological, medical and veterinary sciences the analytical laboratory plays an essential role in the measurement and analysis of important compounds (for example endogenous biomolecules, biomarkers, drugs etc.). The analytical process in these research fields and the analytical laboratory can be divided into three important phases. The pre-analytical phase, analytical or intra-analytical phase and the post-analytical phase (Lippi *et al.*, 2018). Before the analytical process can commence, the first objective is choosing a research project and writing a research proposal containing, in short, a problem statement, hypothesis, study aims and the research methodology that will be followed in the study. The research methodology will consist of experiments that will be done to obtain data for the study. These experiments can consist of analytical methods done on biological samples to obtain the data needed. The following table will describe the main important steps in the three different phases.

Table 2-5: Three phases of the analytical process

Pre-analytical Phase	Analytical/Intra-Analytical Phase	Post-Analytical Phase
Setting up a sample collection protocol for the study	Setup analytical instrument	Check calibration results
Biological sample collection (Blood, tissue, saliva, urine, CSF).	Calibrate analytical instrument	Check results for re-analysis of test samples
Labelling the samples	Sample preparation before analysis; standards and test samples	Processing of results; converting results to data
If needed blood must be centrifuged immediately to separate the plasma or serum and pipette it to other containers for storage.	Analyse the standards and test samples	Data processing
Correct storage of samples until analysis can commence		Data interpretation
Sample preparation process		
Storage of excess sample		

2.8 Methods Developed and Validated

The following three chapters consist of the first three methods developed and validated for this PhD project. The methods were published in international accredited journals. Future methods for the detection and quantification of other important biomolecules and biochemical messengers will still be developed and validated as ongoing projects to adhere to the need for these kind of analytical methods for our research projects.

Chapter 3: Manuscript A:

HPLC electrochemical detection and quantification of monoamines and their metabolites in rat brain tissue samples

Published in *DIE PHARMAZIE* ***73*** *(2018).*

Chapter 4: Manuscript B:

Development and validation of a HPLC electrochemical detection method to measure COMT activity as a tool in drug development

Submitted to *DIE PHARMAZIE*, and accepted to be published in 2019.

Chapter 5: Manuscript C:

An HPLC-DAD validated method for the detection and quantification of cortisol, corticosterone and melatonin in plasma samples of two different animal species

Submitted to *DIE PHARMAZIE*, and accepted to be published in 2019.

CHAPTER 3 MANUSCRIPT A

3.1 Published article

Article published in ***Pharmazie 73 (2018) - An International Journal of Pharmaceutical Sciences*** titled:

HPLC electrochemical detection and quantification of monoamines and their metabolites in rat brain tissue samples

AUTHOR CONTRIBUTIONS

Francois P Viljoen developed and validated the method. FP Viljoen did all the analytical work and the data processing for the manuscript. FP Viljoen wrote the first draft of the manuscript. FP Viljoen finalized the manuscript for publication and was the corresponding author in the submission of the final manuscript to *Die Pharmazie.*

Jan L du Preez was the promoter. JL du Preez advised on the study design and proofread the final manuscript.

Johanna C Wessels was the co-promoter. JC Wessels assisted in proofreading the manuscript for publication.

Marique E Aucamp was the assistant promoter. ME Aucamp assisted in proofreading the manuscript for publication.

IMPORTANT INFORMATION

- The manuscript was inserted into this document in the word document form it was submitted to the journal. In addendum B the first page as published in the journal was attached.
- Instructions to the author can be viewed online at *http://pharmazie.govi.de/instructions/*
- Conformation of acceptance of this manuscript by *Die Pharmazie* is presented in addendum B.

CHAPTER 3 MANUSCRIPT A

Centre of Excellence for Pharmaceutical Sciences, Faculty of Health Sciences, North-West University, Potchefstroom, South Africa[1], School of Pharmacy, University of Western Cape, Bellville, South Africa[2]

Method development and validation for HPLC electrochemical detection and quantification of monoamines and their metabolites in rat brain tissue samples

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The monitoring of monoamines and their metabolites in CNS samples can be very valuable in pharmaceutical and biomedical research. A specific high performance liquid chromatography, coupled to a coulometric electrochemical detection method, for the assay of monoamines (Dopamine, Norepinephrine, Epinephrine and Serotonin) and their metabolites in rat brain tissue samples was developed. The chromatographic separation was achieved on a C8 reversed phase column with a mobile phase consisting of 0.1 M sodium formate buffer, 5 mM sodium 1-heptanesulfonate, 0.17 mM ethylenediaminetetraacetic acid disodium salt and 5% v/v acetonitrile (pH ± 4.0). The detection was achieved through electrochemical detection, with a coulometric cell potential setting of +650 mV. The flowrate was at 1 ml/min and the total run time was 50 minutes. The method was validated according to validation guidelines. The method was found to be linear (R^2 > 0.99) over the analytical range (5 to 200 ng/ml) for all monoamines and their metabolites. All the other validation parameters were acceptable and within range. The method was applied to three rat brain areas (pre-frontal cortex, hippocampus and striatum), where the monoamines (except for epinephrine) and their metabolites were easily detected.

Keywords:

Monoamines, Metabolites, CNS, Rat brain tissue, HPLC, Electrochemical detection

1. Introduction

In the central nervous system (CNS), monoamines are an important group of biogenic amines, which include dopamine, noradrenaline, epinephrine, serotonin and their metabolites (Raven & Johnson, 2002; Koolman *et al.*, 2005; Noback *et al.*, 2005; Burtis *et al.*, 2012). Their main function is neurotransmission by means of neuronal or hormonal signals in a variety of physiological

processes (Burtis *et al.*, 2012). The imbalances and dysfunction of monoamines are associated with a variety of CNS disorders (Booij *et al.*, 2003; Kurian *et al.*, 2011; Ng *et al.*, 2015). These monoamines are mainly synthesised from two amino acids, tyrosine and tryptophan (Koolman *et al.*, 2005; Burtis *et al.*, 2012). The metabolic pathways of the monoamines are shown in a simplified diagram in Figure 1.

The objective of this study was to develop a high performance liquid chromatography (HPLC) method to detect and quantify the monoamines in CNS samples, more specific rat brain tissue samples. The method was therefore developed and validated for the detection and quantification of dopamine and its metabolites (3,4-dihydroxyphenylacetic acid, 3-methoxytyramine and homovanillic acid), norepinephrine (noradrenaline) and its metabolite (3-methoxy-4-hydroxyphenylglycol), epinephrine (adrenaline) and serotonin and its metabolite (5-hydroxyindoleacetic acid).

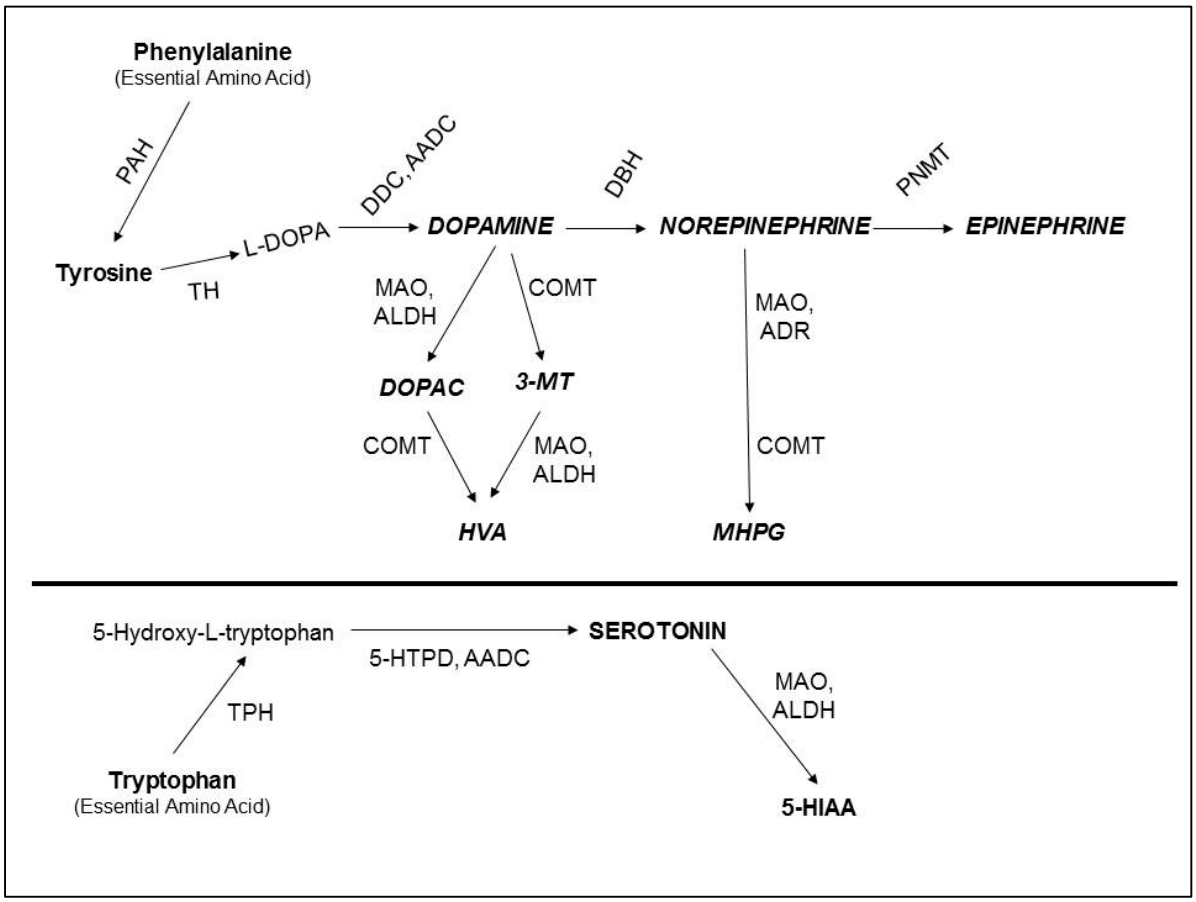

Figure 1: Monoamine metabolic pathways. PAH: Phenylalanine hydroxylase; TH: Tyrosine hydroxylase; L-DOPA: L-3,4-Dihydroxyphenylalanine; DDC: DOPA decarboxylase (Aromatic L-amino acid decarboxylase/AADC); MAO: Monoamine oxidase; ALDH: Aldehyde dehydrogenase; DOPAC: 3,4-dihydroxyphenylacetic acid; COMT: Catechol-O-methyltransferase; 3-MT: 3-Methoxytyramine; HVA: Homovanillic acid; DBH: Dopamine ß-hydroxylase; ADR: Aldehyde

reductase; MHPG: 3-Methoxy-4-hydroxyphenylglycol; PNMT: Phenylethanolamine N-methyltransferase; TPH: Tryptophan hydroxylase; 5-HTPD: 5-Hydroxytryptophan decarboxylase (Aromatic L-amino acid decarboxylase/AADC; 5-HIAA: 5-Hydroxyindoleacetic acid (Modified from literature (Allen *et al.*, 2009; Burtis *et al.*, 2012).

2. Investigations and results

2.1. Methods (standards, mobile phase and sample preparation)

2.1.1. Solution A

Solution A (consisting of 0.1 M perchloric acid; 0.5 mM sodium metabisulphite and 0.3 mM Ethylenediaminetetraacetic acid disodium salt) was used as the preparation solvent for all samples. The purpose of this solution was to keep the monoamines protected from auto-oxidation and for the precipitation of proteins in the biological samples, thus keeping the samples stable for longer (Willemsen *et al.*, 2007; Burtis *et al.*, 2012).

2.1.2. Preparation of standard solutions

Standard stock solution was prepared with a concentration of 100 µg/ml for each of the following analytes; norepinephrine (NE), 3-methoxy-4-hydroxyphenylglycol (MHPG), epinephrine (EPI), dopamine DA), 3,4-dihydroxyphenylacetic acid (Dopac), 3-methoxytyramine (3-MT), homovanillic acid (HVA), serotonin (5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA), using solution A as the solvent. From this stock solution, a range (5 ng/ml to 200 ng/ml) of concentrations were prepared to setup a standard calibration curve.

2.1.3. Preparation of the internal standard solution

Standard stock solution of the internal standard, 5-hydroxy-Nω-methyltryptamine oxalate (5-HMT), with a concentration of 100 µg/ml using solution A as the solvent was prepared. The working internal standard solution, with a final concentration of 1500ng/ml, with an appropriate dilution from the internal standard stock solution using solution A as solvent was prepared.

2.1.4. Mobile phase preparation

A mobile phase consisting of 0.1 M sodium formate buffer, 5 mM sodium 1-heptanesulfonate, 0.17 mM ethylenediaminetetra- acetic acid disodium salt and v/v 5% acetonitrile was prepared. The pH of the mobile phase was set at ± pH 4.00 with ortho-phosphoric acid. The mobile phase was filtered through a 0.22 µm nylon filter before use (Agela Technologies).

2.1.5. HPLC Instrument and electrochemical detector settings

Table 1: Instrumentation settings	
HPLC instrument settings:	
Flow rate	1.0 ml/min
Injection volume	20 µl
Run time	50 minutes
Electrochemical detector settings	
Cell potential settings	Test electrode 1 (E1):-150 mV (to eliminate background noise)
	Test electrode 2 (E2): +650 mV (to analyse the monoamines)
	Guard Cell (E^{GC}): +350 mV
	Detection range: 500 nA
	Filter: 0.5 seconds
	Offset: 0%
	Signal output: 0.1 V
Data collection rate	20 Hz

2.1.6. Sample preparation of brain tissue samples (Harvey et al., 2006)

Brain tissue samples of different brain areas (hippocampus, pre-frontal cortex and striatum), collected from laboratory animals, male Sprague–Dawley rats, *via* dissection, were transferred to 1.5 ml Eppendorf tubes, immediately snap frozen with liquid nitrogen and stored at -80º C until the day of analysis. On the day of analysis, the brain tissue sample was weighed and 1 ml of solution A was added to the tube. The tissue in the tube was then ruptured by sonication (2 x 12 seconds, at an amplitude of 14 µ (Keller *et al.*, 1976). The tube was placed in ice for 20 minutes to complete perchlorate precipitation of proteins and extraction of monoamines from the brain tissue. The sample was then centrifuged at 4ºC for 25 minutes at 20 817 rcf. The supernatant fluid (tissue extract) was removed and pipetted into 2 ml amber Eppendorf tube. The pH of the sample was adjusted to 5.0 with the addition of 1 drop of 10 M potassium acetate. An aliquot of 200 µl of the tissue extract, or standard, was pipetted into 1.5 ml Eppendorf tube and 20 µl of the internal standard, 5-HMT, was added to the sample. The rest of the extracted brain tissue sample was stored at -80°C. The final sample was vortexed and centrifuged for 5 minutes at 20 817 rcf and transferred to HPLC vial insert.

2.2. Method validation parameters

The method was validated according to the guidelines of the FDA concerning linearity, sensitivity (quantification and detection limits), precision (reproducibility), accuracy (repeatability), recovery and stability (US FDA, 2013). Selectivity was performed by injecting samples of solution A without any standards or brain tissue. Surrogate matrices (for example artificial cerebrospinal fluid) were used instead of the authentic sample matrix as it is free of monoamines (van de Merbel, 2008).

2.2.1. Linearity/Calibration curve

Linearity was done by preparing eight standard concentrations as described in section 2.1.2. Six replicates of each standard were injected to establish linear regression for each analyte. The linear regression value (coefficient of determination, r^2) for the calibration curve must not be less than 0.95 for endogenous biomolecules (Shabir, 2006). For calibration, the internal standard method was used with 5-HMT as the chosen internal standard.

2.2.2. Quantification and detection limits (Sensitivity)

The lower limit of quantification (LLOQ) and the lower limit of detection (LLOD) can be defined as the minimum concentration where the signal-to-noise ratio is at least 10:1 and 3:1 greater than the average background noise of an unspiked blank (only containing the internal standard), at the retention time of each analyte, respectively (Shrivastava & Gupta, 2011).

2.2.3. Precision and Accuracy

Four concentrations (5, 10, 75 and 200 ng/ml) were chosen and six determinants for each were done for precision and accuracy. Precision results were expressed in %RSD (relative standard deviation from the mean) and the acceptability criterion for each concentration level was not to exceed 15% (US FDA, 2013). The accuracy results for each concentration level were determined by comparing the closeness of the mean test concentration result to that of the true concentration value. The accuracy results were expressed as % recovery. The acceptability criterion for accuracy for each concentration level was to fall between 90 to 110% (US FDA, 2013; Shabir, 2006).

2.2.4. Recovery

The percentage recovery of the extraction procedure was determined by preparing four spiked concentrations (5, 10, 75 and 200 ng/ml) in solution A. The peak areas were compared to those of prepared standards. This was done in triplicate for each concentration and the mean %

recovery was calculated. The % recovery for each concentration must be above 90% and consistently reproducible to adhere to the acceptance criterion.

2.2.5. Stability

Stability was determined by injecting the eight standard solutions and two CNS samples directly after sample preparation. The initial injection set was assayed immediately and served as reference values. The samples were reinjected after 24 h and 48 h, while keeping the samples at room temperature in the HPLC auto-sampler tray. Percentage stability for each analyte will be given as the mean % stability of the samples analysed and ± SD at the given hour.

2.3. Method validation results

2.3.1. Linearity/Calibration curve

The calibration curve constructed was evaluated by means of its linear regression value. Linearity was excellent over the respective calibration ranges (Table 2), with corresponding coefficient of determination (R^2) consistently greater than 0.99 for all the analytes.

Table 2: Linear regression line equation and coefficient of determination		
Monoamines	y = mx + c	Coefficient of Determination (R^2)
Norepinephrine	y = 0.0042x + 0.0208	R^2 = 0.9988
MHPG	y = 0.0069x + 0.0345	R^2 = 0.9989
Epinephrine	y = 0.0029x + 0.0167	R^2 = 0.9989
Dopamine	y = 0.0052x + 0.0288	R^2 = 0.9991
Dopac	y = 0.0040x + 0.0234	R^2 = 0.9989
3-MT	y = 0.0045x + 0.0250	R^2 = 0.9986
HVA	y = 0.0047x + 0.0346	R^2 = 0.9988
Serotonin	y = 0.0073x + 0.0309	R^2 = 0.9993
5-HIAA	y = 0.0064x + 0.0274	R^2 = 0.9989

2.3.2. Quantification and detection limits (Sensitivity)

The lower limit of detection (LLOD) and the lower limit of quantification (LLOQ) were determined to be 2.5 ng/ml and 5.0 ng/ml for all the analytes respectively.

2.3.3. Precision and Accuracy

The precision and accuracy results of the four concentrations tested are provided in Table 3. Both the precision and accuracy results were within the acceptable criteria ranges set by the method

validation parameters in Point 2.2. Throughout these four concentrations, the %RSD for both the intra-day and inter-day precision was below 4%. The accuracy of all concentration levels for all of the analytes tested was between 90.11% and 109.16%.

Table 3.Accuracy and precision results in solution A						
Analyte Concentration (ng/ml)	**Intra-day (n=6)**			**Inter-day (n=6)**		
	Measured concentration (ng/ml) (mean±SD)	**Precision (%RSD)**	**Accuracy (%)**	**Measured concentration (ng/ml) (mean±SD)**	**Precision (%RSD)**	**Accuracy (%)**
Norepinephrine						
5 (LLOQ)	5.06 ± 0.16	3.25	101.17	4.83 ± 0.10	1.98	96.57
10	10.54 ± 0.11	1.07	105.42	9.86 ± 0.15	1.48	98.63
75	74.86 ± 0.74	0.98	99.81	76.53 ± 0.54	0.71	102.04
200	199.09 ± 2.04	1.03	99.55	202.80 ± 0.45	0.22	101.40
MHPG						
5 (LLOQ)	4.92 ± 0.15	3.05	98.31	4.86 ± 0.09	1.92	97.28
10	10.44 ± 0.22	2.13	104.36	9.65 ± 0.09	0.93	96.49
75	75.30 ± 0.45	0.60	100.40	75.80 ± 0.60	0.80	101.07
200	201.36 ± 1.64	0.81	100.68	199.70 ± 0.91	0.45	99.85
Epinephrine						
5 (LLOQ)	5.38 ± 0.16	3.04	107.62	4.50 ± 0.19	4.15	89.99
10	10.89 ± 0.28	2.60	108.86	9.05 ± 0.10	1.12	90.46
75	76.27 ± 0.40	0.52	101.70	73.77 ± 0.88	1.20	98.36
200	198.36 ± 1.76	0.89	99.18	203.45 ± 0.58	0.29	101.73
Dopamine						
5 (LLOQ)	5.05 ± 0.17	3.27	101.07	4.61 ± 0.09	2.04	92.19
10	10.82 ± 0.31	2.88	108.18	9.36 ± 0.15	1.60	93.58
75	75.80 ± 1.43	1.89	101.06	71.96 ± 0.51	0.71	95.59
200	198.50 ± 1.89	0.95	99.25	201.15 ± 1.59	0.79	100.58
Dopac						
5 (LLOQ)	4.85 ± 0.17	3.56	97.07	4.66 ± 0.11	2.44	93.14
10	10.93 ± 0.37	3.40	109.26	9.36 ± 0.18	1.92	93.55
75	75.53 ± 0.75	0.99	100.71	74.57 ± 0.89	1.19	99.43
200	199.91 ± 1.58	0.79	99.96	201.41 ± 0.77	0.38	100.70
3-MT						
5 (LLOQ)	5.46 ± 0.08	1.44	109.16	4.51 ± 0.18	4.04	90.11
10	9.80 ± 0.23	2.39	97.96	10.29 ± 0.19	1.85	102.90
75	79.62 ± 0.81	1.01	106.17	73.49 ± 1.44	1.96	97.99

200	198.29 ± 3.20	1.61	99.14	201.35 ± 1.98	0.98	100.68
HVA						
5 (LLOQ)	5.05 ± 0.17	3.30	100.99	4.64 ± 0.09	1.98	92.81
10	10.81 ± 0.36	3.33	108.08	9.51 ± 0.27	2.82	95.13
75	78.03 ± 1.43	1.84	104.03	71.87 ± 1.61	2.24	95.82
200	200.37 ± 2.05	1.02	100.19	196.14 ± 1.15	0.59	98.07
Serotonin						
5 (LLOQ)	5.32 ± 0.21	3.86	106.40	4.65 ± 0.22	4.82	93.00
10	10.83 ± 0.20	1.81	108.32	9.48 ± 0.21	2.21	94.76
75	76.68 ± 0.81	1.05	102.24	72.01 ± 0.59	0.82	98.74
200	201.10 ± 2.97	1.47	100.55	196.20 ± 0.97	0.49	98.10
5-HIAA						
5 (LLOQ)	5.43 ± 0.13	2.35	108.56	4.96 ± 0.11	2.30	99.13
10	10.21 ± 0.34	3.37	102.11	9.40 ± 0.13	1.40	93.99
75	76.43 ± 0.83	1.08	101.91	71.80 ± 0.81	1.12	95.73
200	203.01 ± 2.61	1.28	101.51	189.99 ± 2.24	1.18	95.00

2.3.4. Recovery

The mean absolute recovery for each analyte, measured in triplicate for all four concentrations tested, were constantly above 90%, with the mean recovery indicated in Table 4.

Table 4: Percentage Recovery results		
Monoamines	Concentration (ng/ml)	% Recovery (mean ± SD)
Norepinephrine	5.0 (LLOQ)	96.86 ± 2.59
	10.0	98.14 ± 2.36
	75.0	100.69 ± 0.10
	200.0	97.49 ± 3.01
MHPG	5.0 (LLOQ)	96.32 ± 2.50
	10.0	98.44 ± 0.23
	75.0	96.48 ± 6.51
	200.0	97.35 ± 6.12
Epinephrine	5.0 (LLOQ)	99.14 ± 1.72
	10.0	98.54 ± 2.40
	75.0	98.24 ± 1.29
	200.0	99.65 ± 1.16

Dopamine	5.0 (LLOQ)	96.04 ± 0.73
	10.0	97.22 ± 0.73
	75.0	101.13 ± 0.30
	200.0	97.52 ± 1.26
Dopac	5.0 (LLOQ)	98.16 ± 2.72
	10.0	101.77 ± 1.88
	75.0	101.03 ± 1.35
	200.0	99.33 ± 0.80
3-MT	5.0 (LLOQ)	98.54 ± 0.72
	10.0	97.86 ± 1.85
	75.0	100.61 ± 1.38
	200.0	99.77 ± 0.22
HVA	5.0 (LLOQ)	98.76 ± 2.32
	10.0	98.28 ± 3.20
	75.0	100.86 ± 1.62
	200.0	98.79 ± 0.78
Serotonin	5.0 (LLOQ)	97.52 ± 1.33
	10.0	99.87 ± 1.06
	75.0	97.85 ± 1.20
	200.0	97.44 ± 3.26
5-HIAA	5.0 (LLOQ)	98.55 ± 0.87
	10.0	95.87 ± 1.53
	75.0	94.50 ± 0.08
	200.0	96.08 ± 5.57

2.3.5. Stability

In Table 5, the mean percentage stability results for the all the analytes tested in the eight standards and the two brain tissue samples are demonstrated. All the monoamines and their metabolites were stable when tested at 24 h and 48 h stored at room temperature in the auto-sampler tray for both the standards and the two rat brain tissue samples. Epinephrine however was below the limit of detection in the brain tissue samples and therefore not detected.

Table 5: Stability results for the eight standards		
% Stability (mean ± SD) of monoamines analytes at room temperature after 24 h and 48 h.		
Time:	24 Hours*	48 Hours*
Norepinephrine	99.89 ± 1.46	99.85 ± 1.86
MHPG	99.81 ± 0.57	99.73 ± 1.35
Epinephrine	99.09 ± 1.66	98.68 ± 1.90
Dopamine	99.03 ± 1.89	98.85 ± 2.18
Dopac	99.32 ± 1.16	99.18 ± 1.65
3-MT	99.34 ± 1.28	99.23 ± 2.46
HVA	98.12 ± 2.27	97.71 ± 2.70
Serotonin	99.26 ± 1.76	99.12 ± 1.79
5-HIAA	99.05 ± 1.05	98.72 ± 0.84
Stability results for the two brain tissue samples		
Time:	24 Hours*	48 Hours*
Norepinephrine	99.78 ± 0.08	97.23 ± 0.07
MHPG	98.01 ± 0.27	93.46 ± 3.57
Epinephrine	Not detected in brain tissue samples	
Dopamine	99.59 ± 0.26	98.98 ± 0.45
Dopac	99.20 ± 0.16	98.62 ± 0.36
3-MT	99.89 ± 1.02	95.86 ± 1.28
HVA	99.11 ± 0.77	96.23 ± 2.39
Serotonin	99.11 ± 0.34	97.48 ± 1.14
5-HIAA	99.75 ± 1.81	95.21 ± 0.29

*Compared to 100% at t = 0, mean ±SD.

2.4. Chromatographic results

Table 6 presents the relative retention times of all the monoamines, their metabolites and the internal standard for this method.

Table 6: The relative retention times of the monoamines and their metabolites and the internal standard are presented	
Analytes	Relative retention times (minutes)
1. Norepinephrine	± 6.378
2. MHPG	± 7.414
3. Epinephrine	± 8.148
4. Dopac	± 10.833
5. Dopamine	± 13.091
6. 5-HIAA	± 21.535
7. HVA	± 25.632
8. 3-MT	± 30.653
9. Serotonin	± 35.929
10. Internal standard (5-HMT)	± 46.413

The following three chromatographic figures are representative of a blank sample containing only the internal standard, 5-HMT (Figure 2), a 5ng/ml standard (Figure 3) and a 200ng/ml standard (Figure 4).

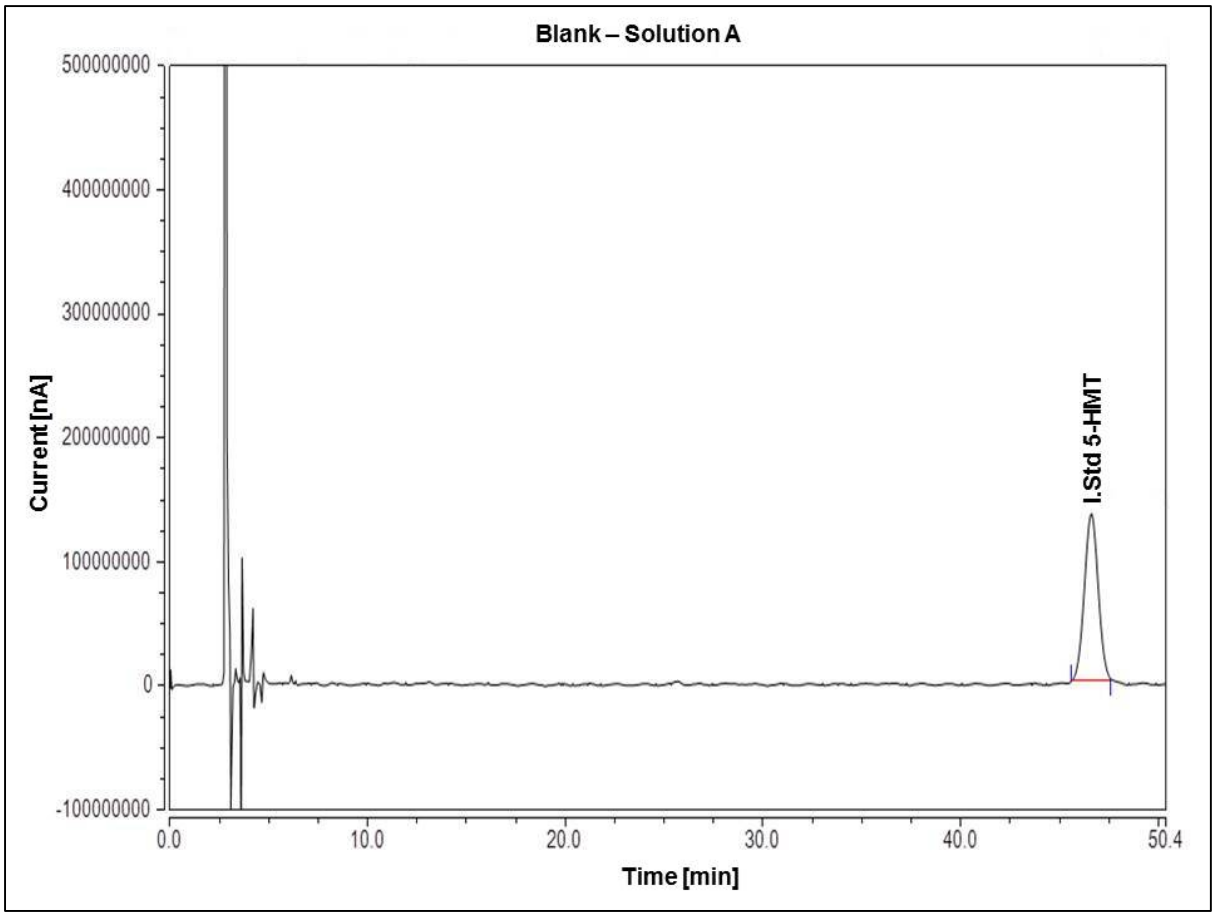

Figure 2: Blank sample with internal standard (5-HMT).

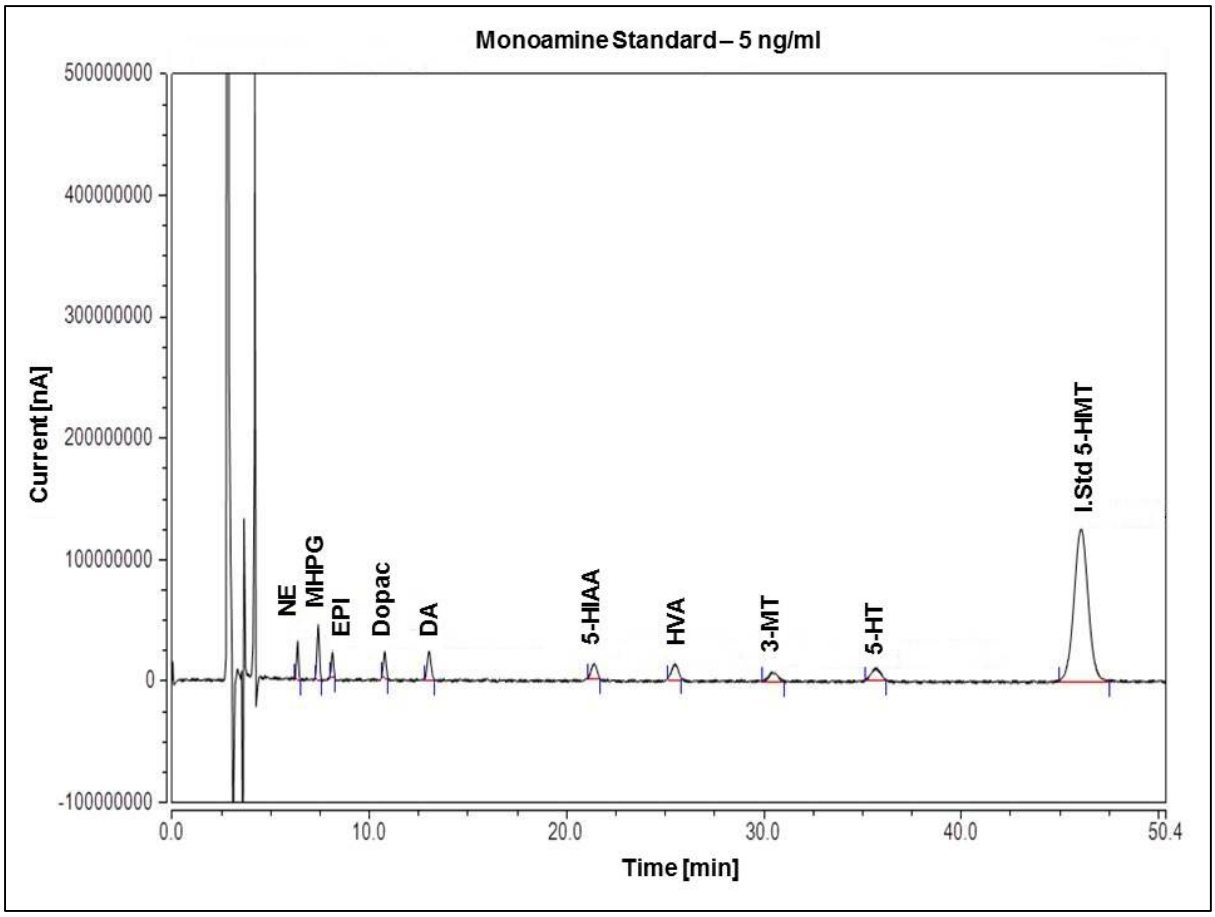

Figure 3: Monoamine standard – 5 ng/ml.

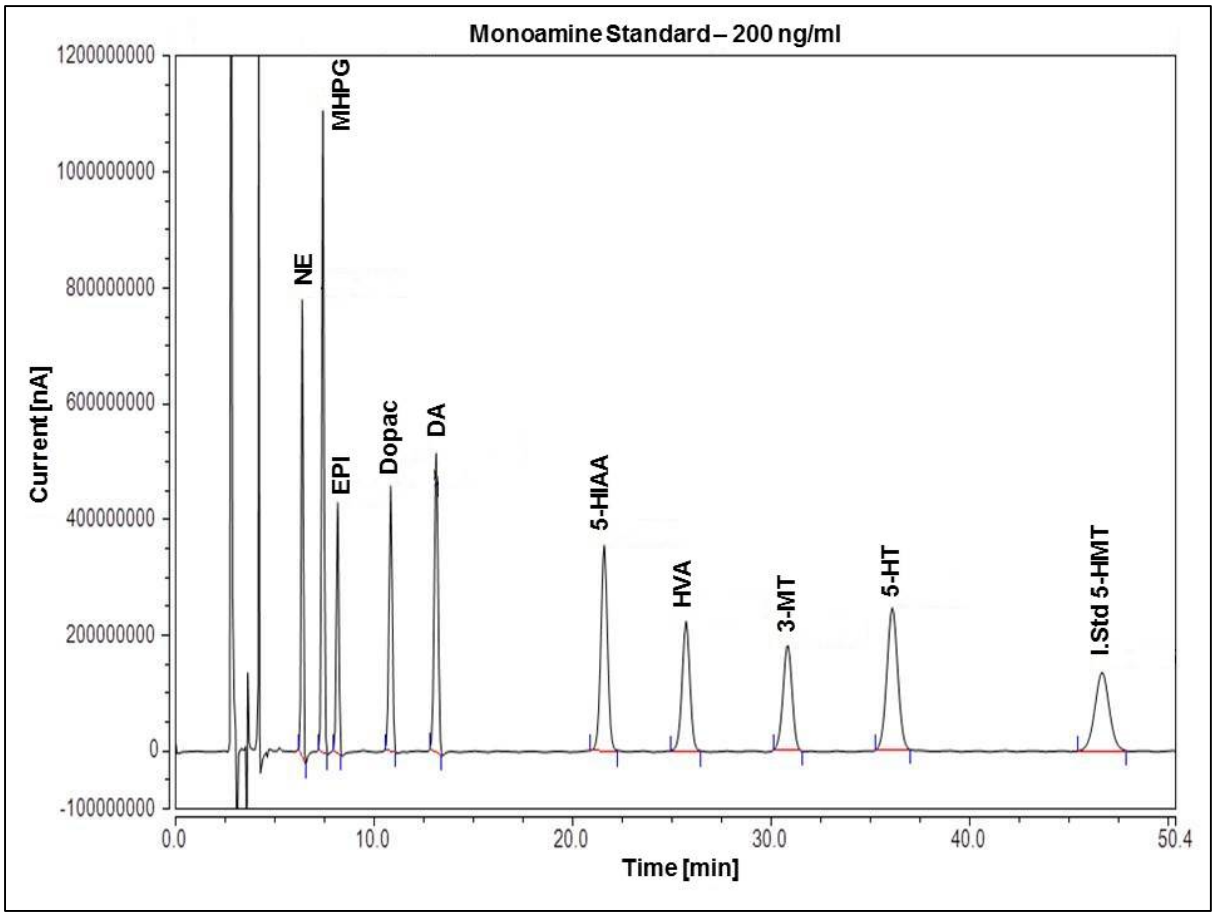

Figure 4: Monoamine standard – 200 ng/ml.

2.5. Application

The validated method was applied to three different rat brain tissue areas: hippocampus, pre-frontal cortex and striatum. The samples from the different rat brain tissue areas were removed and prepared according to section 2.1.6, and analysed. The internal standard method was used to calculate the concentration of each analyte in the brain tissue samples (Scientific, 2014).

The following three chromatographic figures represent the different rat brain tissue areas that were analysed. The first represents a 69 mg hippocampus (Figure 5), the second an 85 mg pre-frontal cortex (Figure 6) and the third a 30 mg striatum (Figure 7).

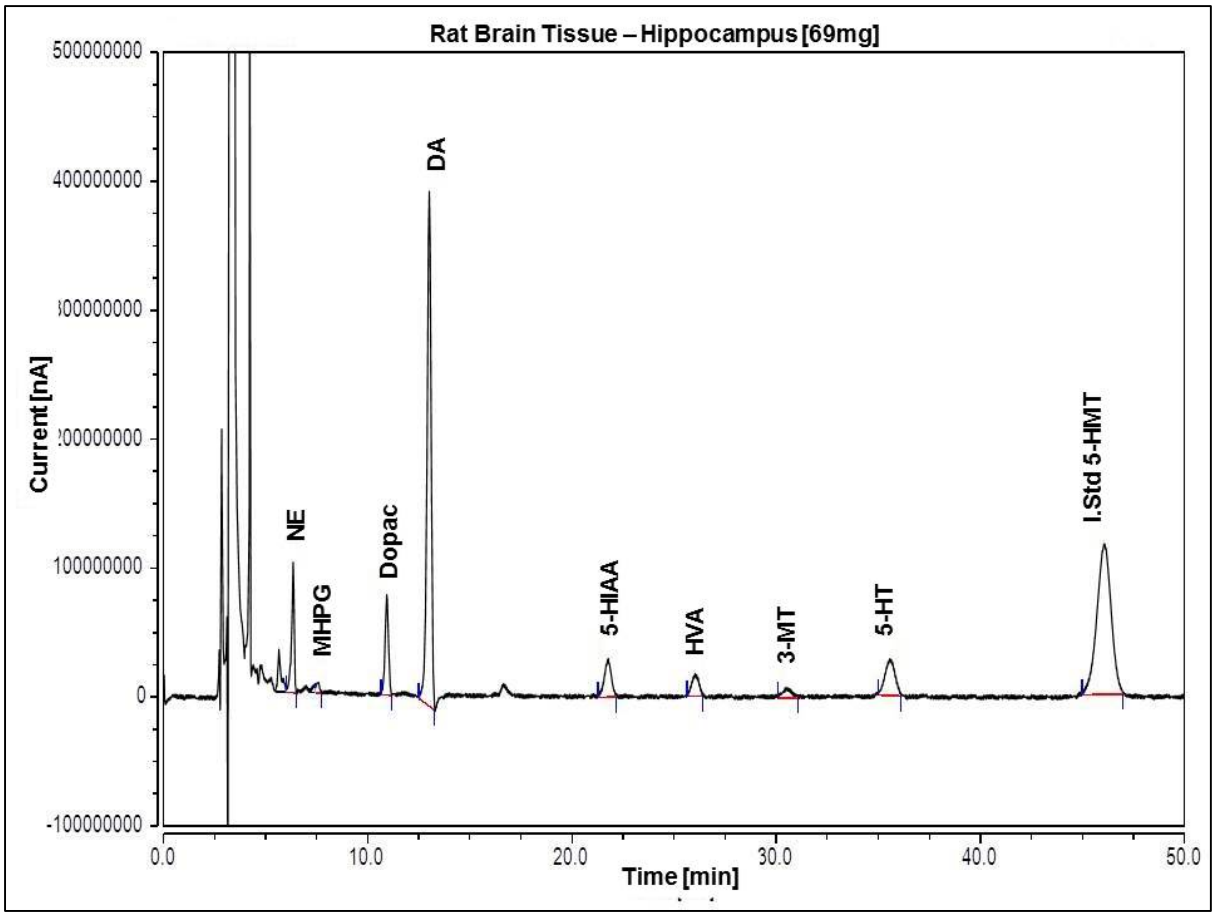

Figure 5: Rat brain tissue – hippocampus [69 mg].

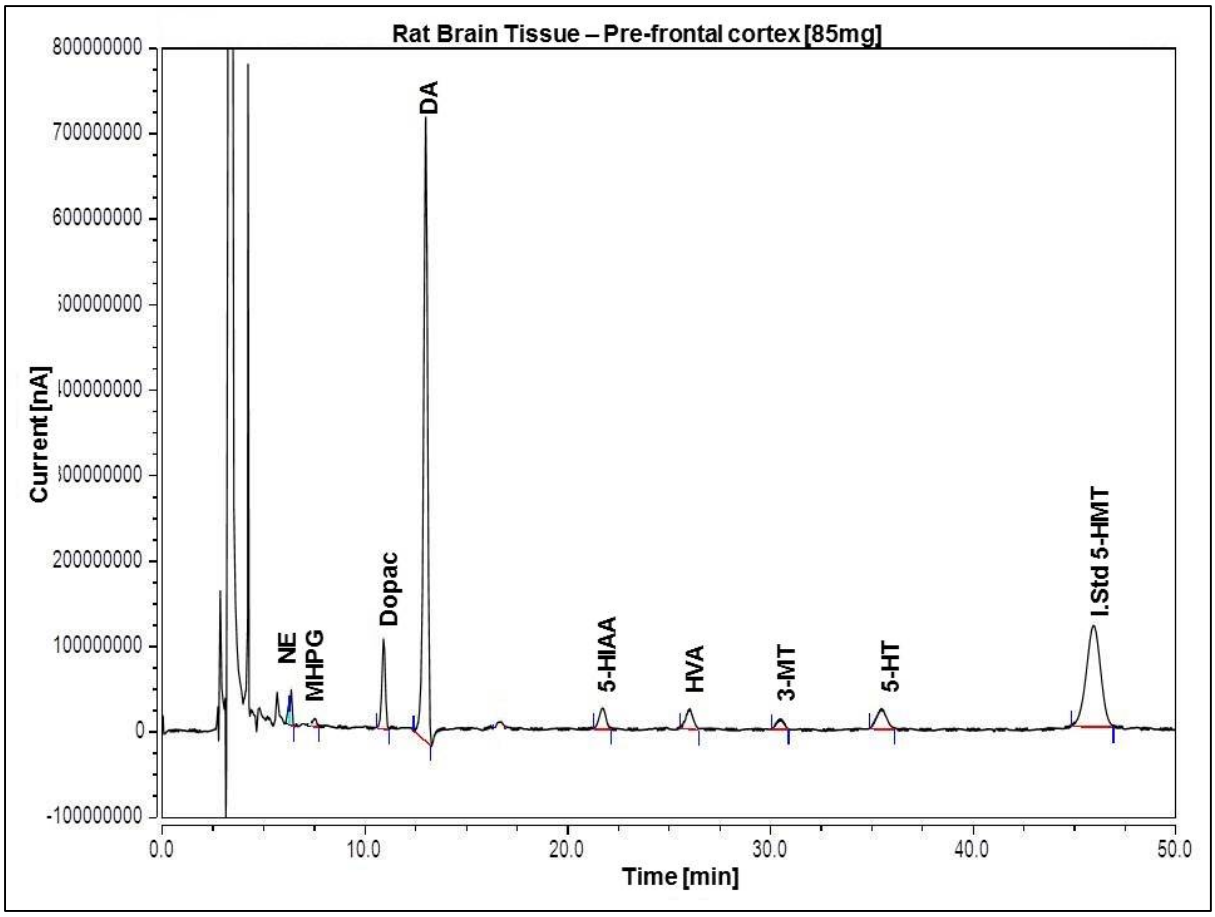

Figure 6: Rat brain tissue – pre-frontal cortex [85 mg].

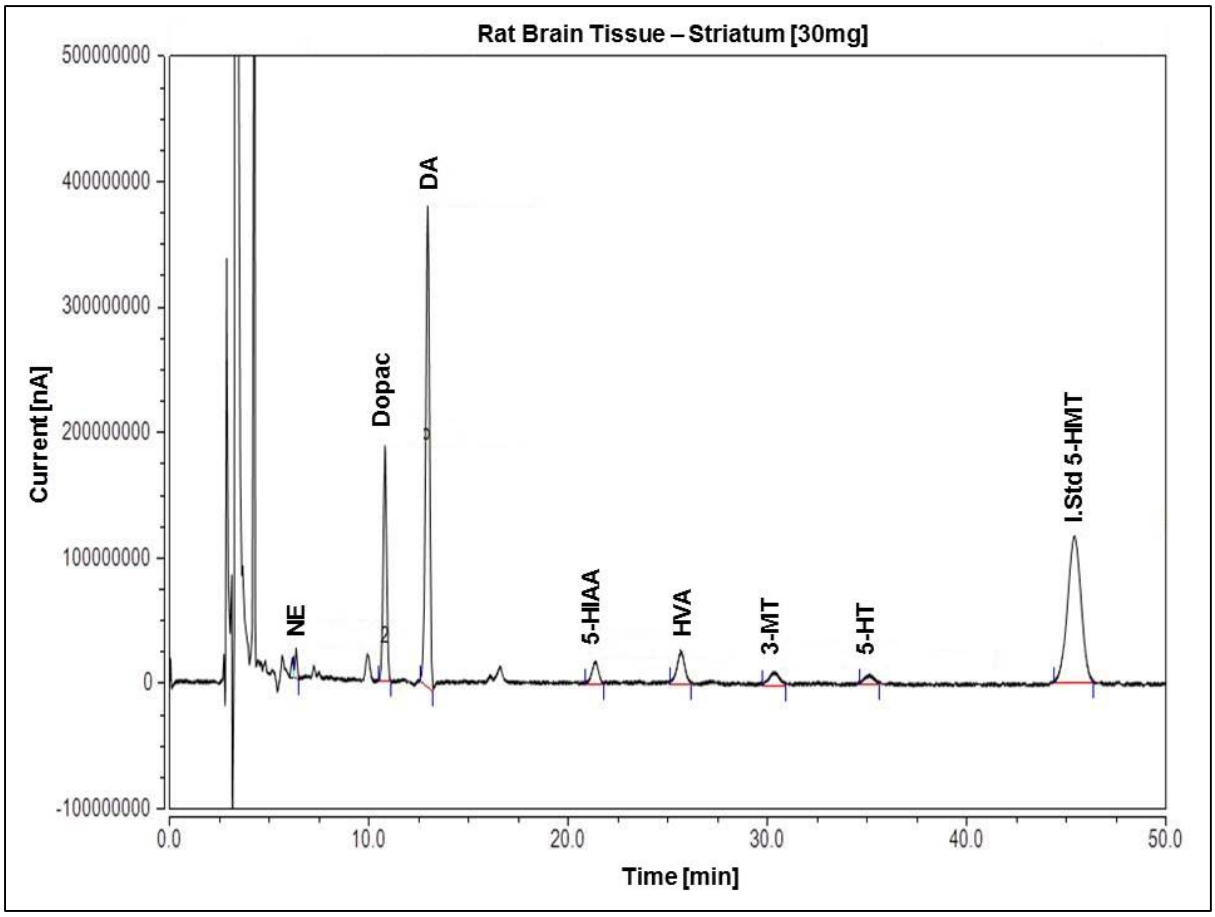

Figure 7: Rat brain tissue – Striatum [30 mg].

In Table 7, the concentrations of the monoamine analytes analysed in the three different brain tissue areas (Figures 5 to 7) are shown. The results were expressed in ng/g wet weight tissue. Epinephrine levels were below the limit of detection of the method in all three brain tissue samples and MHPG in the striatum.

Table 7: Rat brain tissue results in ng/g wet weight tissue			
Rat brain tissue area	Hippocampus [69 mg]	Pre-frontal cortex [85 mg]	Striatum [30 mg]
Analyte			
Norepinephrine	337.86	33.00	30.84
MHPG	13.96	29.41	BLOD
Epinephrine	BLOD	BLOD	BLOD
Dopamine	2196.43	3243.68	4712.20
Dopac	390.51	463.18	2675.72
3-MT	20.85	28.29	24.88
HVA	64.51	123.77	508.56
Serotonin (5-HT)	253.57	163.92	27.23
5-HIAA	170.28	96.55	179.43

(BLOD - below limit of detection).

3. Discussion

A specific HPLC, coupled to a coulometric electrochemical detection method, was developed for the assay of monoamines and their metabolites in rat brain tissue samples. This method was validated for linearity, sensitivity, precision, accuracy, recovery and stability. The linearity for all respective analytes' calibration ranges was excellent and the coefficient of determination (r^2) was consistently above 0.95. The LLOQ and LLOD were respectively 5.0 ng/ml and 2.5 ng/ml for the method. The % recovery was acceptable for all the analytes at all the concentrations measured, and the % stability was also acceptable for both 24 and 48 hours testing for the standards and the rat brain tissue samples. The method was found to be selective and specific for norepinephrine, dopamine, serotonin and their metabolites in rat brain tissue samples. In the rat brain samples, epinephrine was below the limit of detection. This method can also be used to determine the analytes in cerebrospinal fluid (CSF) samples (Marais *et al.*, 2006) of laboratory animals, however method development and validation must be done in future studies with CSF samples.

We propose that this method can be applied to drug and behaviour studies in laboratory animals (for example rats and mice) to determine the effect on the two monoamine pathways in rodent brain tissue samples. The quantitative analysis of monoamines in CNS samples can provide important information in a variety of studies focusing on CNS related disorders.

4. Experimental

4.1. Chemicals, reagents, materials and instrumentation

4.1.1. Chemicals and reagents

L-Noradrenaline hydrochloride, 3-methoxy-4-hydroxyphenylglycol hemipiperazinium salt, epinephrine bitartrate salt, 3-hydroxythyramine hydrochloride, 3,4-dihydroxyphenylacetic acid, homovanillic acid, serotonincreatinine sulphate, 5-hydroxyindole-3-acetic acid and the 5-hydroxy-Nω-methyltryptamine oxalate (internal standard) were obtained from Sigma-Aldrich Pty (Ltd) (Johannesburg, South Africa). Chemicals used for the mobile phase were HPLC grade deionised water and HPLC grade acetonitrile (ACN); sodium formate; 1-heptanesulphonic acid sodium salt; ethylenediaminetetra-acetic acid disodium salt; orthophosphoric acid (H_3PO_4, 85%). Chemicals used for the sample preparation were ethylenediaminetetra-acetic acid disodium salt; sodium metabisulphite; perchloric acid (60%); potassium acetate. All the chemicals were obtained from Merck (Pty) Ltd (Johannesburg, South Africa).

4.1.2. Materials

The analytical HPLC column used was a Venusil ASB C8 (purchased from Bonna-Agela Technologies, USA), 4.6 x 250 mm, a particle size of 5 µm, pore size of 150 Å and a surface area of 200 m^2/g.

4.1.3. Instrumentation

An Agilent 1200 series HPLC (Agilent Technologies Inc., Santa Clara, CA USA), equipped with an isocratic pump and autosampler, coupled to an ESA Coulochem III Electrochemical detector with a coulometric flow cell (Model 5011A High Analytical Cell and Guard cell 5020) and Chromeleon® Chromatography Management System version 6.8 (obtained from Thermo Fisher Scientific, Waltham, MA USA).

Conflict of interest

The authors have no conflict of interest to declare.

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CHAPTER 4 MANUSCRIPT B

4.1 Submitted and accepted article

Article submitted to, and accepted for publication in ***Pharmazie - An International Journal of Pharmaceutical Sciences*** titled:

Development and validation of a HPLC electrochemical detection method to measure COMT activity as a tool in drug development

AUTHOR CONTRIBUTIONS

Francois P Viljoen developed and validated the method. FP Viljoen did all the analytical work and the data processing for the manuscript. FP Viljoen wrote the first draft of the manuscript. FP Viljoen finalized the manuscript for publication and was the corresponding author in the submission of the final manuscript to *Die Pharmazie.*

Jan L du Preez was the promoter. JL du Preez advised on the study design and proofread the final manuscript.

Anél Petzer was a co-author: A Petzer prepared the rat liver homogenate test samples, also proofread the final manuscript.

Johanna C Wessels was the co-promoter. JC Wessels assisted in proofreading the manuscript for publication.

Jacques P Petzer was a co-author. JP Petzer was the prime investigator for obtaining ethical approval for collecting the rat liver tissue samples for another study and the tissue left over was used in this study to adhere to the three R's (replacement, Reduction and refinement) in animal research according to the **South African National Standard (**SANS) document for **the care and use of animals for scientific purposes**.

Marique E Aucamp was the assistant promoter. ME Aucamp assisted in proofreading the manuscript for publication.

IMPORTANT INFORMATION

- The manuscript was inserted into this document in the word document form it was submitted to the journal. In addendum B the first page as published in the journal was attached.

CHAPTER 4 MANUSCRIPT B

- Instructions to the author can be viewed online at *http://pharmazie.govi.de/instructions/*
- Conformation of acceptance of this manuscript by *Die Pharmazie* is presented in addendum B.

Centre of Excellence for Pharmaceutical Sciences, Faculty of Health Sciences, North-West University, Potchefstroom, South Africa[1], School of Pharmacy, University of Western Cape, Bellville, South Africa[2]

Development and validation of a HPLC electrochemical detection method to measure COMT activity as a tool in drug development

F.P. Viljoen [1], J.L. du Preez [1], A. Petzer [1], J.C. Wessels [1], J. Petzer [1] and M. E. Aucamp [2]

The determination of catechol-O-methyltransferase (COMT) activity is considered valuable for various pharmaceutical and biomedical research projects. A specific high performance liquid chromatography-coulometric electrochemical detection method, for the assay of COMT activity was developed by measuring the formation of normetanephrine from norepinephrine. The chromatographic separation was achieved on a C_{18} reversed phase column with a mobile phase consisting of 10 mM sodium dihydrogen phosphate buffer, 4 mM sodium 1-octanesulfonate, 0.17 mM ethylenediaminetetra-acetic acid disodium salt, 6% methanol and 4% acetonitrile (pH ± 4.0). The detection of normetanephrine was achieved through electrochemical detection, with a coulometric cell potential setting of +450 mV. The flow rate was at 1 ml/min and the total run time was 45 minutes. The method was validated according to validation guidelines (Shabir, 2006; European Medicines Agency, 2011; US FDA, 2018). The method was found to be linear (R^2 > 0.99) over the analytical range (100 to 2500 ng/ml) for all the analytes. All the other validation parameters (sensitivity, precision, accuracy, recovery and stability) were acceptable and within range. The method was applied for the determination of COMT activity in rat liver homogenate test samples. The known selective COMT inhibitor, entacapone was used as test inhibitor. The results confirmed the ability of entacapone to inhibit COMT activity by decreasing the production of all the metabolites of norepinephrine.

Keywords:
COMT; norepinephrine; normetanephrine; epinephrine; metanephrine; HPLC; electrochemical detection; entacapone.

1. Introduction

The development of new drugs relies on good analytical methods to facilitate the development process. Throughout the drug discovery and development processes, reliable analytical assay methods play a crucial role, whether used to determine in vitro inhibition potencies during library

screening and optimization of potency or to determine if drug candidates possess in vivo potency at a specific protein target. For the development of novel inhibitors of the enzyme, catechol-O-methyltransferase (COMT), specific and sensitive analytical methods are needed to facilitate the above mentioned goals of drug discovery and development. For the assay of COMT activity, a variety of analytical methods has been developed over the years and range from enzyme-linked immunosorbent assays (ELISA), spectrophotometric and spectrofluorometric assays, and high performance liquid chromatographic (HPLC) methods. There are a variety of ELISA methods which are commercially available as testing kits, but these are expensive and only a certain number of samples can be analysed per ELISA kit. The spectrophotometric methods generally have low sensitivity, are expensive and need large sample size. Although an HPLC method might be relatively expensive, these methods are highly sensitive, specific for a particular analyte and yield reliable and accurate measurements. For HPLC, the three detection methods most often used are ultraviolet (UV), fluorescent (FLD) and electrochemical detection (ECD) (Kaplan *et al.*, 1992; Reenilä *et al.*, 1995; Tsunoda *et al.*, 2001). The COMT enzyme is a key enzyme in the metabolism of the neurotransmitters norepinephrine and epinephrine, (Figure 1) which are important chemical messengers (Sembulingam & Sembulingam, 2012; Wilkinson & Brown, 2015). A better understanding of these metabolic pathways and how drugs influence them can assist in the discovery and development of improved or more potent drugs for a variety of illnesses.

The aim of this study was to develop and validate a method to measure COMT activity in our laboratory which will be selective, specific, reliable and affordable under our conditions.

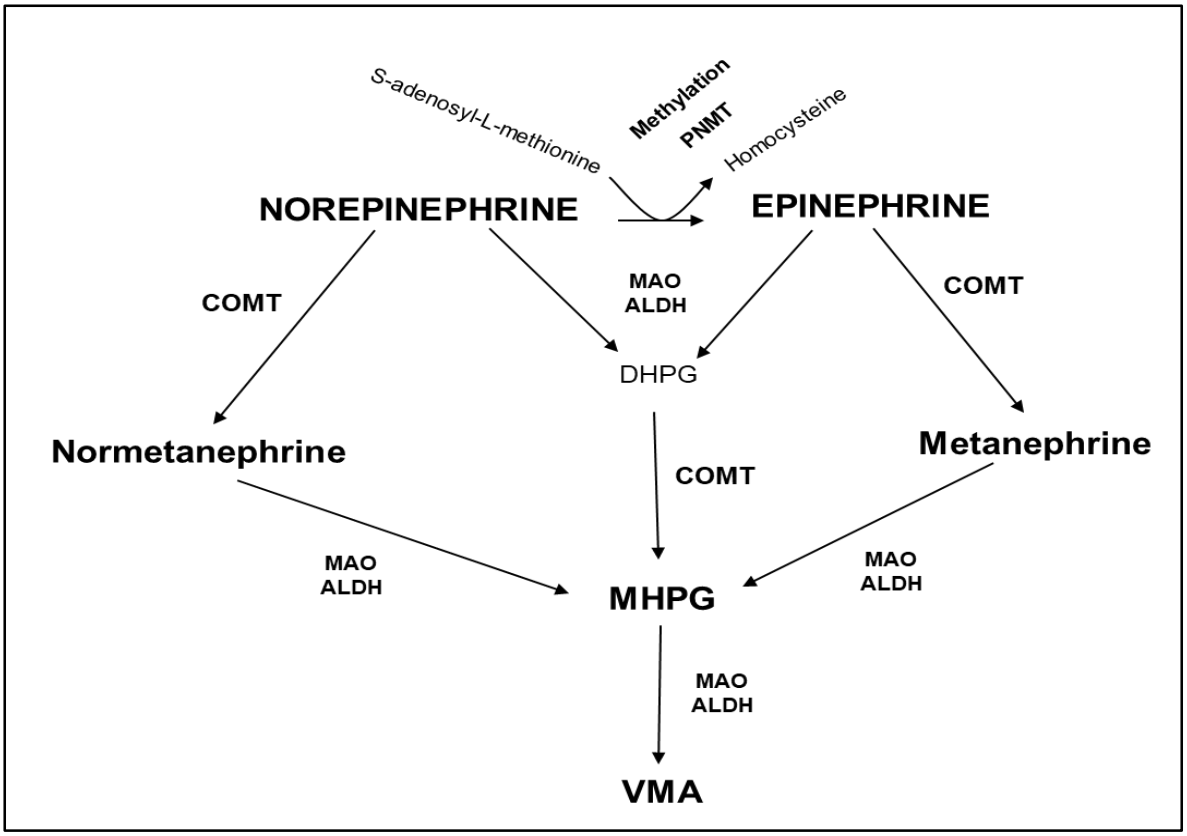

Figure 1: Simplified diagram illustrating the metabolic pathways of norepinephrine and epinephrine. PNMT: Phenylethanolamine-N-methyltransferase; COMT: Catechol-O-methyltransferase; MAO: Monoamine oxidase; ALDH: Aldehyde dehydrogenase; DHPG: 3,4-Dihydroxyphenylglycol; MHPG: 3-Methoxy-4-hydroxyphenylglycol; VMA: Vanillylmandelic acid (Zianni *et al.*, 2004).

2. Investigations and results

2.1. Methods (standards, mobile phase and sample preparation)

2.1.1. Preparation of standard solutions

A standard stock solution with a concentration of 100 µg/ml for each of the analytes, norepinephrine (NE), normetanephrine (NMN), 3-methoxy-4-hydroxyphenylglycol (MHPG), vanillylmandelic acid (VMA), epinephrine (EPI) and metanephrine (MN) was prepared using a sodium phosphate buffer (di-sodium hydrogen orthophosphate; 25 mM; pH 7.8) as the solvent.

From this stock solution, 8 solutions ranging from 100 ng/ml to 2500 ng/ml were prepared (Table 1). These solutions were used to establish the linear regression of the method.

Table 1: Concentrations of the different analytes (ng/ml well as in µM)						
Concentration in ng/ml	Concentration in µM					
	VMA	MHPG	NE	NMN	EPI	MN
100	0,50	0,27	0,59	0,55	0,55	0,51
150	0,76	0,41	0,89	0,82	0,82	0,76
200	1,01	0,54	1,18	1,09	1,09	1,01
300	1,51	0,81	1,77	1,64	1,64	1,52
500	2,52	1,35	2,96	2,73	2,73	2,54
750	3,78	2,03	4,43	4,09	4,09	3,80
1000	5,05	2,70	5,91	5,46	5,46	5,07
2500	12,62	6,76	14,78	13,65	13,65	12,68

2.1.2. Preparation of the internal standard solution

A stock solution of the internal standard, 3,4-dihydroxy-benzylamine (DHBA) was prepared at a concentration of 100 µg/ml using the sodium phosphate buffer as the solvent as described for the standards above. The working internal standard solution with a final concentration of 2000 ng/ml was prepared in 1 M $HClO_4$ with an appropriate dilution from the internal standard stock solution. This solution was used to terminate the enzyme reactions as the final steps in the sample preparation.

2.1.3. Mobile phase preparation

A mobile phase consisting of 10 mM sodium dihydrogen phosphate buffer, 4 mM sodium 1-octanesulfonate, 0.17 mM ethylenediaminetetra-acetic acid disodium salt, 6% (v/v) methanol and 4% (v/v) acetonitrile was prepared. The pH of the mobile phase was set at ± pH 4.00 with ortho-phosphoric acid (85%). The mobile phase was filtered through a 0.22 µm nylon filter before use (Agela Technologies).

2.1.4. HPLC Instrument and electrochemical detector settings

Table 2: Instrumentation settings	
HPLC instrument settings:	
Flow rate	1.0 ml/min
Injection volume	25 µl
Run time	45 minutes
Column temperature	23 °C (column compartment)
Electrochemical detector settings:	
Cell potential settings	Test electrode 1 (E1): -150 mV (to eliminate background noise)
	Test electrode 2 (E2): +450 mV (to analyse the analytes)
	Guard Cell (E^{GC}): +350 mV
	Detection range: 500 nA
	Filter: 0.5 seconds
	Offset: 0%
	Signal output: 0.1 V
Data collection rate	20 Hz

2.1.5. Sample preparations for standards and test samples modified from literature (Aoyama et al., 2005)

The standard samples were prepared in 1.5 ml microcentrifuge tubes. For the standards, DMSO replaced the test inhibitor drug while distilled water replaced the COMT enzyme solution.

Table 3: Sample preparation steps for standards		
Steps	**Reagents**	**Volume (Total = 137.5 µl)**
1.	2 mM $MgCl_2$	10 µl
2.	Standard (100 – 2500 ng/ml)	35 µl
3.	200 µM *S*-(5′-sdenosyl)-*L*-methionine (SAMe)	25 µl
4.	DMSO	5 µl
5.	Sample was vortexed for 5 seconds and allowed to incubate at 37 °C for 10 minutes	
6.	Distilled water instead of COMT	50 µl
7.	Samples were vortexed for 5 seconds and allow to incubate at 37 °C for 60 minutes	
8.	1 M $HClO_4$ (to terminate reaction, containing the internal standard)	12.5 µl

Table 4: Sample preparation steps for test liver tissue samples		
Steps	**Reagents**	**Volume (Total = 137.5 µl)**
1.	2 mM $MgCl_2$	10 µl
2.	250 µM substrate (norepinephrine)	35 µl
3.	200 µM SAMe	25 µl
4.	Test inhibitor (entacapone) dissolved in DMSO	5 µl
5.	Sample was vortexed for 5 seconds and allow to incubate at 37 °C for 10 minutes	
6.	COMT solution	50 µl
7.	Sample was vortexed for 5 seconds and allow to incubate at 37 °C for 60 minutes	
8.	1 M $HClO_4$ (To terminate reaction, containing the internal standard)	12.5 µl

After the stop solution (1 M $HClO_4$) was added, the samples were centrifuged at 4°C for 10 minutes at 20 817 rcf. The supernatants were subsequently transferred to HPLC vial inserts. The Chromeleon® Chromatography Management System software was then programmed to inject 25 µl of the sample into the HPLC system.

2.2. Method validation parameters

Electrochemical detection techniques coupled to an HPLC instrument offers high sensitivity and selective detection for catecholamines and their metabolites (Peaston & Weinkove, 2004; Dong, 2006; Swartz, 2010).

The method was validated according to the guidelines of the FDA for linearity, sensitivity (quantification and detection limits), precision (reproducibility), accuracy (repeatability), recovery and stability (US FDA, 2013).

2.2.1. Linearity (Calibration curve)

Linearity was evaluated by preparing eight standard concentrations as described in section 2.1.1. Six replicates of each standard were injected to establish linear regression for each analyte. The linear regression value (coefficient of determination, R^2) for the calibration curve should not be less than 0.95 for endogenous biomolecules (Shabir, 2006). For calibration, the internal standard method was used with DHBA as the selected internal standard.

2.2.2. Quantification and detection limits (sensitivity)

The lower limit of quantification (LLOQ) and the lower limit of detection (LLOD) can be defined as the minimum concentration where the signal-to-noise ratio is at least 10:1 and 3:1 greater than

the average background noise of an unspiked blank (only containing the internal standard), at the retention time of each analyte, respectively (Shrivastava & Gupta, 2011).

2.2.3. Precision and Accuracy

Four concentrations ranging from low to high (100, 200, 500 and 2500 ng/ml) were selected and six samples of each concentration was prepared and injected to determine precision and accuracy. Precision was expressed in %RSD (relative standard deviation from the mean) and the acceptability criterion for each concentration level was not to exceed 15% (US FDA, 2013). The accuracy for each concentration level was determined by comparing the mean test concentration to that of the true concentration value. The accuracy was expressed as percentage recovery. The acceptability criterion for accuracy for each concentration level was 90 to 110% (Shabir, 2006; US FDA, 2013).

2.2.4. Recovery

The percentage recovery of the extraction procedure was determined by preparing four spiked concentrations (100, 200, 500 and 2500 ng/ml) in solution A. The peak areas were compared to those of the prepared standards. This was done in triplicate for each concentration and the mean percentage recovery was calculated. The percentage recovery for each concentration must be above 90% and consistently reproducible to adhere to the acceptance criterion.

2.2.5. Stability

Stability was determined by injecting the eight standard solutions and two test samples directly after sample preparation. The initial injection set was assayed immediately and served as reference values. The samples were reinjected after 24 h and 48 h, while keeping the samples at room temperature in the HPLC auto-sampler tray. Percentage stability for each analyte is given as the mean percentage stability (± SD) of the samples analysed at the given time point.

2.3. Method validation results

2.3.1. Linearity/Calibration curve

The calibration curves constructed for all analytes were evaluated by means of linear regression analysis. Six replicates of each standard were injected and the mean used to establish linear regression for each analyte. Linearity was excellent over the calibration ranges (100 to 2500 ng/ml), with corresponding coefficient of determination (R^2) consistently greater than 0.99 for all analytes (Table 5).

Table 5: Linear regression line equation and coefficient of determination		
Analytes	**y = mx + c**	**Coefficient of Determination (R^2)**
Norepinephrine (NE)	y = 0,0012x + 0,0251	R^2 = 0,9997
Normetanephrine (NMN)	y = 0,0013x + 0,0100	R^2 = 0,9999
3-Methoxy-4-hydroxyphenylglycol (MHPG)	y = 0,0015x + 0,1119	R^2 = 0,9926
Vanillylmandelic acid (VMA)	y = 0,0009x - 0,0266	R^2 = 0,9987
Epinephrine (EPI)	y = 0,0014x + 0,0547	R^2 = 0,9988
Metanephrine (MN)	y = 0,0012x + 0,0100	R^2 = 0,9999

2.3.2. Quantification and detection limits (Sensitivity)

The limit of detection (LOD) and the limit of quantification (LOQ) were determined to be 10 ng/ml and 100 ng/ml respectively for all the analytes that were analysed and evaluated in this study.

2.3.3. Precision and Accuracy

The results for the determination of precision and accuracy of all four selected concentrations are provided in Table 6. Both the precision and accuracy were within the acceptable criteria ranges set by the method validation parameters in section 2.2.3. For all analytes, at these four concentrations the %RSD for both the intra-day and inter-day precision was smaller than 9%, which is well below the 15% criteria. The accuracy of all concentration levels for all of the analytes tested, ranged from 90.47 to 108.45%, which is within the set criteria of 90 to 110%.

Table 6. Accuracy and precision results						
Analyte concentration (ng/ml)	**Intra-day (n=6)**			**Inter-day (n=6)**		
	Measured concentration (ng/ml) (mean ± SD)	**Precision (%RSD)**	**Accuracy (%)**	**Measured concentration (ng/ml) (mean ± SD)**	**Precision (%RSD)**	**Accuracy (%)**
Norepinephrine (NE)						
100 (LLOQ)	101.36 ± 1.60	1.58	101.36	90.47 ± 5.95	6.57	90.47
200	209.95 ± 1.49	0.71	104.97	182.91 ± 11.50	6.29	91.46
500	508.62 ± 2.11	0.42	101.72	487.60 ± 12.12	2.43	97.46
2500	2557.27 ± 19.77	0.77	102.29	2471.01 ± 56.82	2.30	98.84
Normetanephrine (NMN)						
100 (LLOQ)	105.70 ± 2.94	2.78	105.70	94.61 ± 4.29	4.53	94.61
200	208.81 ± 2.70	1.29	104.40	191.57 ± 7.12	3.71	95.78

500	501.37 ± 2.39	0.48	100.27	498.64 ± 6.29	1.26	99.73
2500	2519.36 ± 16.37	0.83	100.76	2481.08 ± 67.05	2.70	99.24
Vanillylmandelic acid (VMA)						
100 (LLOQ)	108.03 ± 4.06	3.76	108.03	91.74 ± 7.38	8.04	91.74
200	204.29 ± 8.74	4.28	102.15	188.9 ± 15.68	8.30	94.49
500	503.63 ± 14.81	2.94	100.73	484.08 ± 33.99	7.02	96.82
2500	2711.35 ± 13.76	0.51	108.45	2260.41 ± 41.12	1.82	90.42
3-Methoxy-4-hydroxyphenylglycol (MHPG)						
100 (LLOQ)	103.54 ± 3.60	3.48	103.54	96.58 ± 2.29	2.37	96.58
200	207.25 ± 7.25	3.50	103.62	193.01 ± 5.66	2.93	96.50
500	511.40 ± 4.96	0.97	102.28	498.60 ± 12.12	2.43	99.72
2500	2501.59 ± 45,71	1.83	100.06	2498.41 ± 63.59	2.55	99.94
Epinephrine (EPI)						
100 (LLOQ)	106.88± 1.85	1.73	106.88	90.72 ± 6.77	7.46	90.72
200	200..41 ± 3.72	1.86	100.20	181.32 ± 15.85	15.85	90.66
500	500.95 ± 2.86	0.57	100.19	467.45 ± 17.79	3.81	93.49
2500	2502.44 ± 16.16	0.65	100.10	2469.27 ± 49.21	1.99	98.77
Metanephrine (MN)						
100 (LLOQ)	106.05 ± 3.40	3.20	106.05	93.12 ± 1.67	1.79	93.12
200	208.75 ± 9.06	4.34	104.37	191.62 ± 5.04	2.63	95.81
500	503.31 ± 8.72	1.73	100.66	496.71 ± 10.14	2.04	99.34
2500	2527.62 ± 23.64	0.94	101.10	2472.69 ± 56.08	2.27	98.91

2.3.4. Recovery

The mean absolute recovery for each analyte, measured in triplicate for all four concentrations tested, were constantly above 90%, with the mean recovery indicated in Table 7.

Table 7: Percentage Recovery results		
Analytes	***Concentration (ng/ml)***	***% Recovery (mean ± SD)***
Norepinephrine (NE)	100 (LLOQ)	98.60 ± 4.40
	200	98.80 ± 2.54
	500	97.90 ± 2.84
	2500	98.29 ± 2.10
Normetanephrine (NMN)	100 (LLOQ)	95.02 ± 9.14
	200	97.20 ± 1.01
	500	100.91 ± 1.53
	2500	99.61 ± 2.38
Vanillylmandelic acid (VMA)	100 (LLOQ)	101.88 ± 3.34
	200	96.43 ± 5.97
	500	100.81 ± 3.55
	2500	99.34 ± 3.84
3-Methoxy-4-hydroxyphenylglycol (MHPG)	100 (LLOQ)	99.22 ± 1.84
	200	97.24 ± 1.50
	500	106.68 ± 4.37
	2500	97.82 ± 3.90
Epinephrine (EPI)	100 (LLOQ)	98.32 ± 6.21
	200	98.81 ± 5.50
	500	97.44 ± 1.74
	2500	99.73 ± 2.41
Metanephrine (MN)	100 (LLOQ)	94.15 ± 1.87
	200	95.69 ± 1.17
	500	102.48 ± 1.97
	2500	99.65 ± 2.34

2.3.5. Stability

In Table 8, the results of the stability study are provided as the mean percentage values. For all the analytes, the stability of eight standard solutions as well as the stability of the analytes in three test rat liver homogenate samples are demonstrated. All the analytes in the standard solutions as well as in the test tissue samples were stable when tested at 24 h and 48 h after being stored at room temperature in the auto-sampler tray. In contrast poor stability was recorded for the analytes after 48 h in the presence of rat liver homogenate. The 48 hour poor stability of the analytes in the rat liver homogenate samples can be due to the storage at room temperature. Room

temperature of ± 23°C is high enough to cause natural degradation of the analytes in the samples (Shabihkhani *et al.*, 2014). This can be resolved by using a temperature controlled auto-sampler which can help to keep the samples stable for longer, but good laboratory practice will be to analyse the samples rather within 24 hours.

Table 8: Stability results for the eight standards		
% Stability (mean ± SD) of monoamines analytes at room temperature after 24 h and 48 h.		
Time:	***24 Hours****	***48 Hours****
Norepinephrine (NE)	98.54 ± 2.52	101.85 ± 4.07
Normetanephrine (NMN)	101.51 ± 0.95	104.37 ± 3.02
Vanillylmandelic acid (VMA)	95.40 ± 5.96	103.74 ± 9.82
3-Methoxy-4-hydroxyphenylglycol (MHPG)	102.52 ± 1.70	108.61 ± 3.09
Epinephrine (EPI)	98.45 ± 2.73	101.38 ± 4.15
Metanephrine (MN)	99.81 ± 1.86	104.50 ± 4.60
Stability results for the three test rat liver homogenate samples		
Time:	***24 Hours****	***48 Hours****
Norepinephrine (NE)	99.27 ± 3.71	83.84 ± 4.89
Normetanephrine (NMN)	99.37 ± 2.47	85.26 ± 13.14
Vanillylmandelic acid (VMA)	100.43 ± 4.40	81.88 ± 4.21
3-Methoxy-4-hydroxyphenylglycol (MHPG)	94.73 ± 6.06	70.74 ± 9.92
Epinephrine (EPI)	90.06 ± 5.95	58.88 ± 13.32
Metanephrine (MN)	Stability could not be calculated because MN was below LOD in most of the rat liver samples tested.	

*Compared to 100% at t = 0, mean ±SD.

2.4. Chromatographic results

Table 9 present the relative retention times of norepinephrine, epinephrine, their metabolites and the internal standard for this method.

Table 9: The relative retention times of the monoamines and their metabolites and the internal standard	
Analytes	**Relative retention times (minutes)**
1. Vanillylmandelic acid (VMA)	± 3.488
2. 3-Methoxy-4-hydroxyphenylglycol (MHPG)	± 5.0424
3. Norepinephrine (NE)	± 13.015
4. Epinephrine (EPI)	± 17.044
5. Internal Standard (DHBA)	± 22.009
6. Normetanephrine (NMN)	± 24.347
7. Metanephrine (MN)	± 34.152

The following three chromatograms are representative of the following samples: a blank sample containing only the internal standard, DHBA (Figure 2); a standard solution containing 100 ng/ml of each analyte (Figure 3); a standard solution containing 2500 ng/ml of each analyte Figure 4).

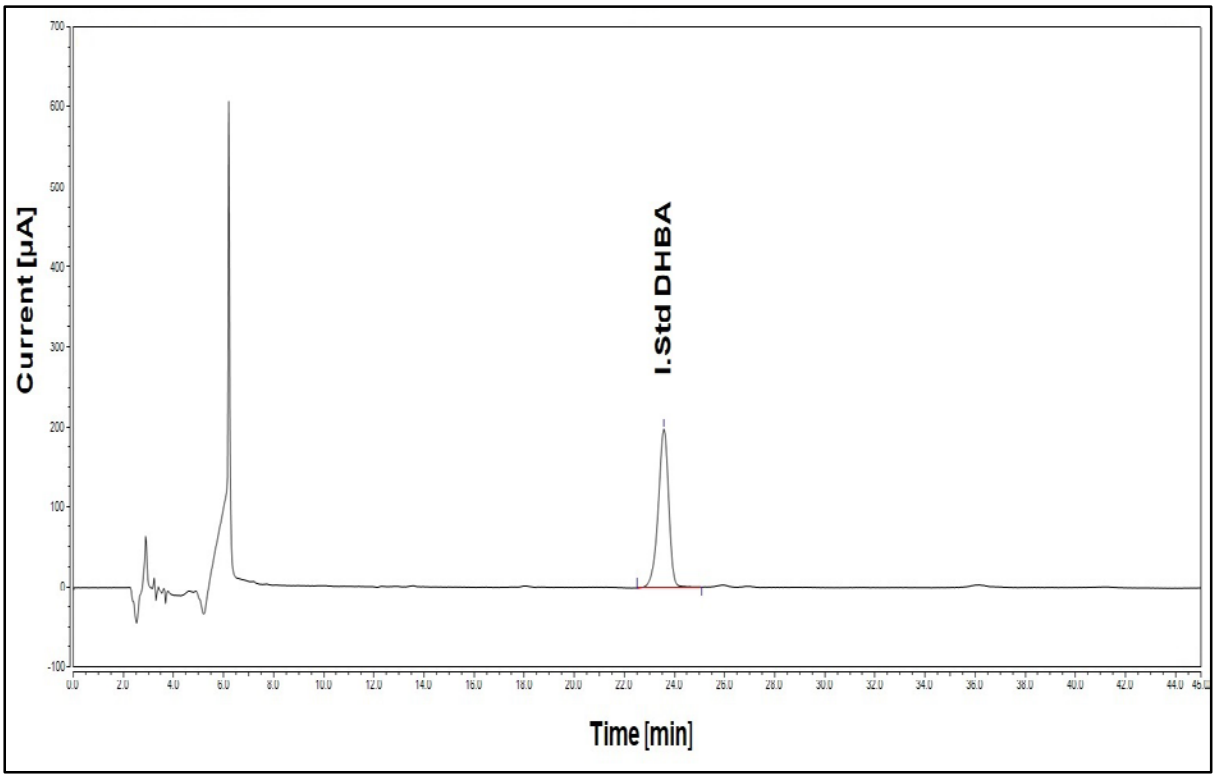

Figure 2: Blank sample with internal standard (DHBA).

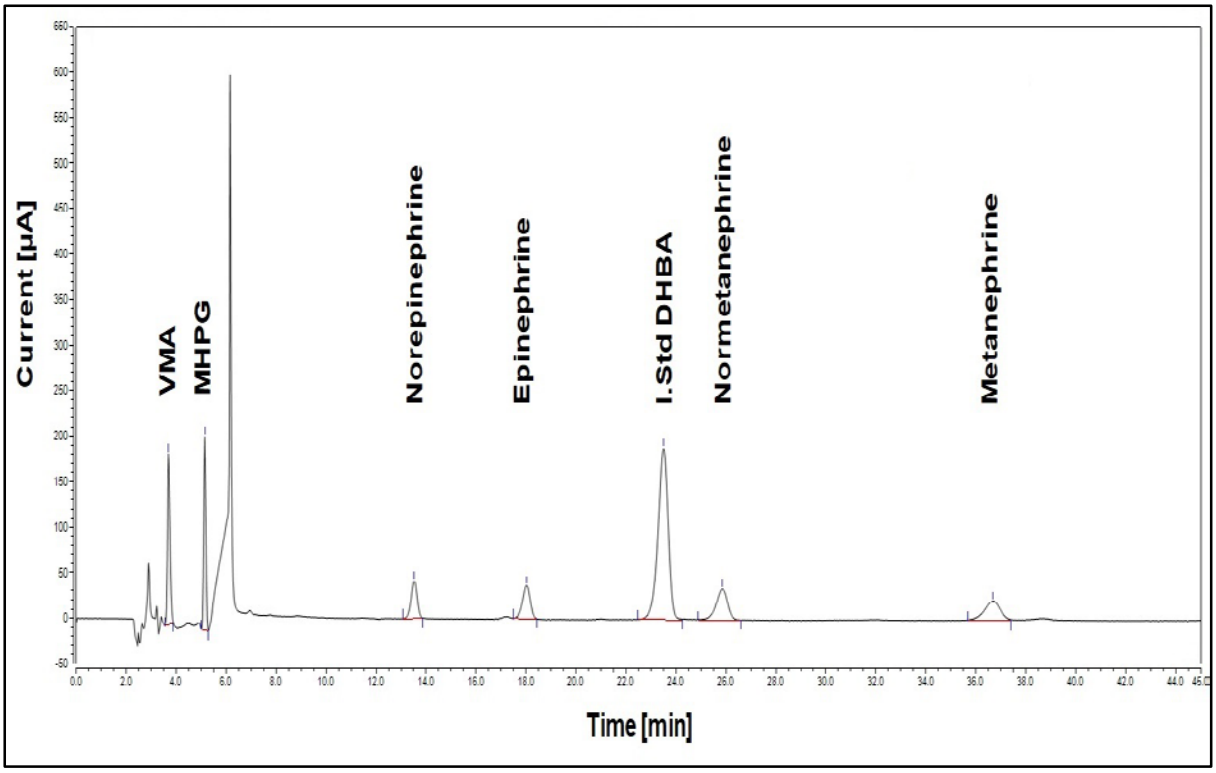

Figure 3: Standard solution containing 100 ng/ml of each analyte.

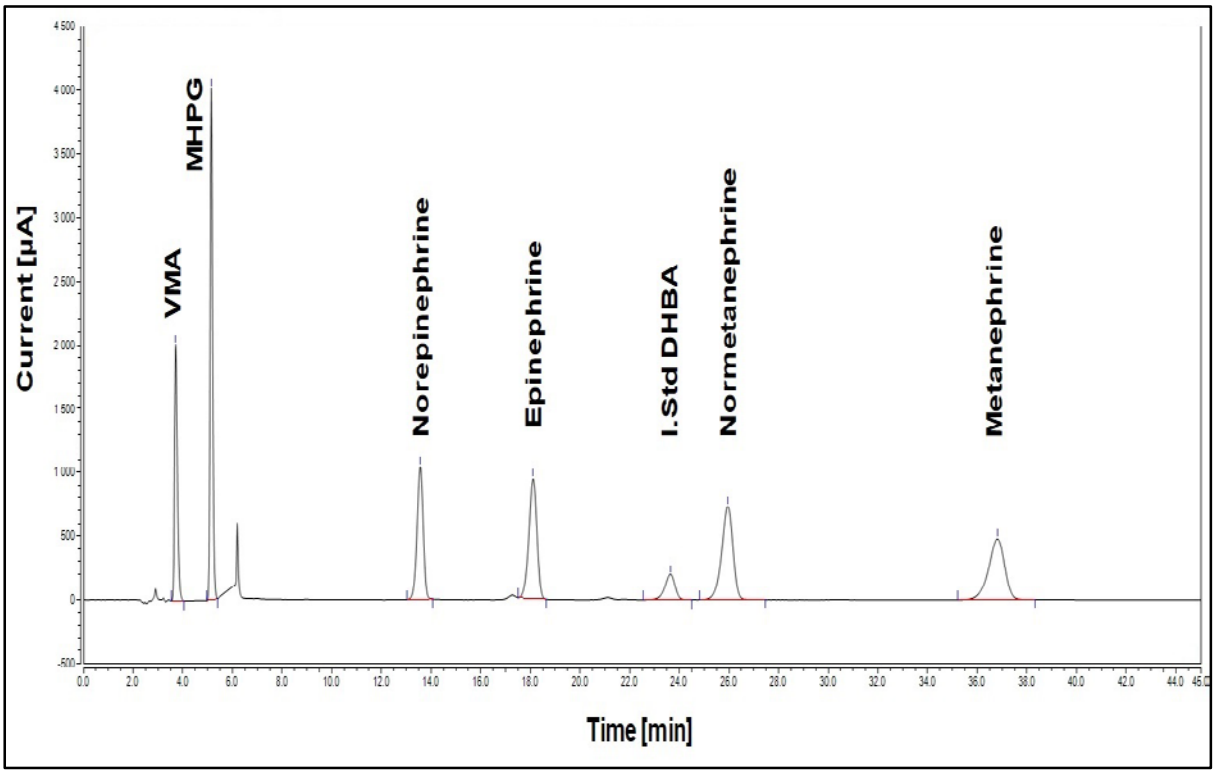

Figure 4: Standard solution containing 2500 ng/ml of each analyte.

2.5. Application of the method to prepared rat liver tissue test samples

The following three chromatograms are representative of the prepared rat liver homogenate test samples that were treated with norepinephrine and incubated for 20 min (see section 2.1.5). The samples were also treated with a range of concentrations (from 0 µM to 100 µM) of the selective COMT inhibitor, entacapone (Katzung, 2018).

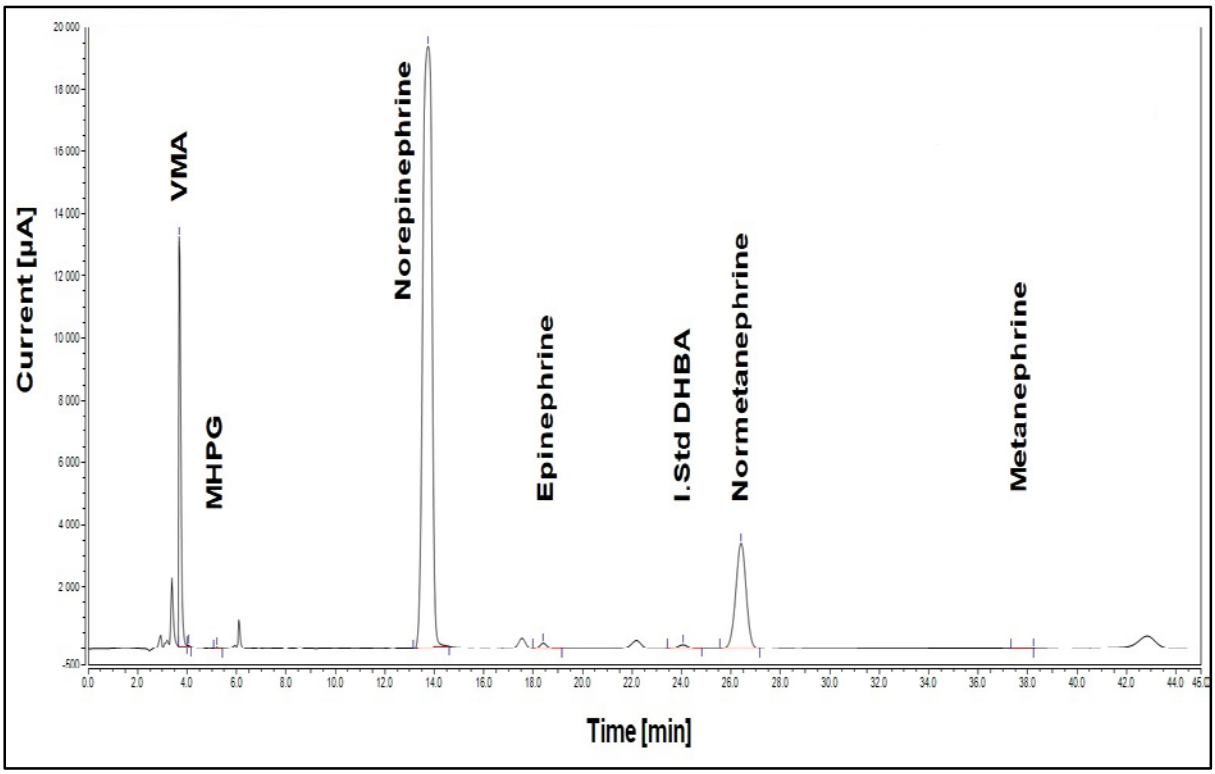

Figure 5: Rat liver homogenate sample treated with 0 µM entacapone.

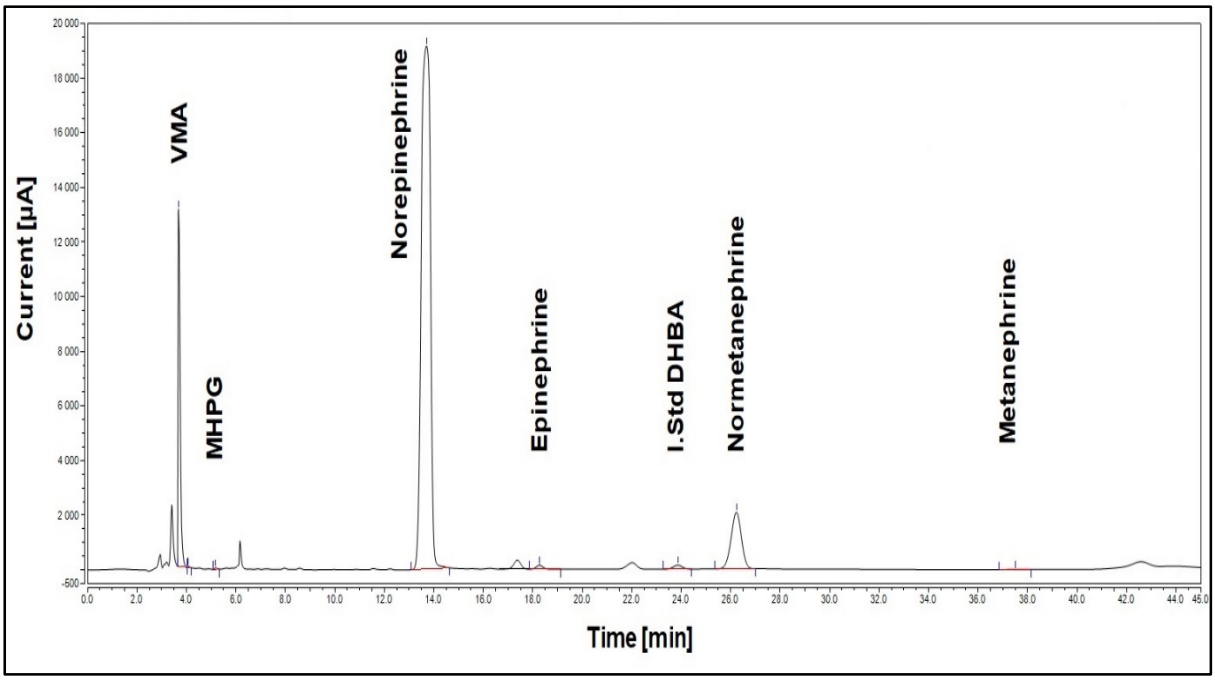

Figure 6: Rat liver homogenate sample treated with 0.3 µM entacapone.

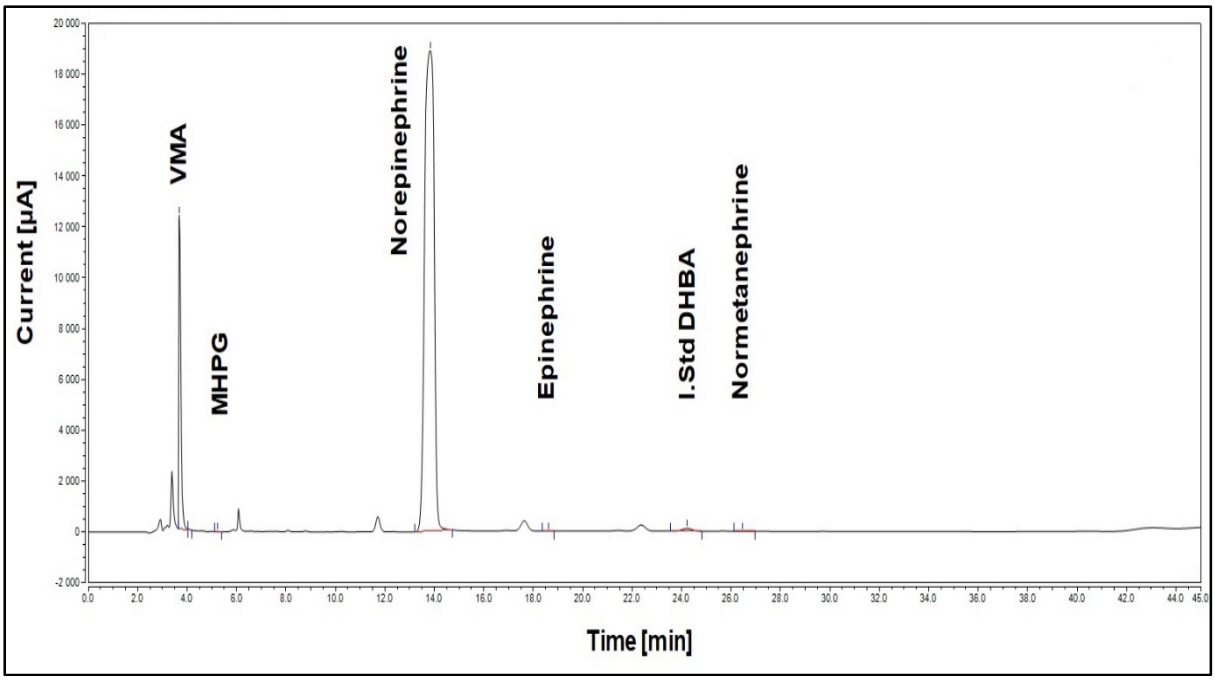

Figure 7: Rat liver homogenate sample treated with 100 µM entacapone (metanephrine in this sample was below LOD).

In chromatographic separation the ratio (fluctuations) of the retention times of the analytes and internal standard must correspond to that of standard solution within a tolerance of ±2.5% for LC (European Commission, 2002).

Table 10 shows the results of the COMT assay that was performed to illustrate the inhibition of COMT by entacapone. Entacapone successfully inhibited the COMT activity by decreasing not only the production of normetanephrine, but also indirectly decreasing the production of MHPG and VMA. The reduction of metabolite concentration correlated with the increased concentration of entacapone.

Table 10: COMT assay results done on rat liver homogenate with Entacapone used as the inhibition drug						
Entacapone concentration added (µM)	**Norepinephrine**	**NMN**	**MHPG**	**VMA**	**Epinephrine**	**MN**
	Concentration measured (µM)					
0	758,60	126,70	0,28	125,44	4,26	0,05
0.01	595,65	109,29	0,22	103,33	3,57	0,16
0.1	582,46	84,15	0,15	100,80	3,33	0,38
0.3	715,66	46,52	0,11	122,82	3,45	0,12
1.0	563,87	1,66	0,02	88,93	0,52	BLOD
3.0	644,78	0,32	0,03	104,53	0,20	BLOD
10.0	653,03	0,06	0,02	102,91	0,06	BLOD
100.0	657,90	0,05	0,02	99,79	0,03	BLOD

BLOD (Below limit of detection)

3. Discussion

In this study an HPLC method to measure COMT activity was successfully developed and validated. A specific HPLC method, coupled to a coulometric electrochemical detection method, was developed for the measurement of COMT activity in rat liver homogenate. This method was validated for linearity, sensitivity, precision, accuracy, recovery and stability. The linearity over the relevant calibration ranges for all analytes was excellent and the coefficient of determination (R^2) was consistently above 0.95. The LLOQ and LLOD were respectively 100 ng/ml and 10 ng/ml for all analytes. The percentage recovery was acceptable for all the analytes at all the concentrations measured. The percentage stability was also acceptable for both the standards and test samples at 24 hours, but not for the test samples at 48 hours. The method was found to be very sensitive and specific for all the analytes tested, although metanephrine was below LOD in some of the rat liver homogenate test samples.

The newly developed and validated method was applied to measure COMT activity in rat liver homogenate and to illustrate with the inhibition of COMT by the known selective COMT inhibitor entacapone, at a concentration range of 0 µM to 100 µM. Entacapone inhibited the activity of COMT in a concentration-dependent manner and decreased the production of normetanephrine, MHPG and VMA. A decrease in epinephrine and metanephrine was also observed in the study. In future studies other known COMT inhibitors may also be used to further validate and test this new method.

We propose that this new method can be applied to future studies where COMT activity can be measured with norepinephrine as a substrate.

4. Experimental

4.1. Chemicals, reagents, materials and instrumentation

4.1.1. Chemicals and reagents

L-Noradrenaline hydrochloride, DL-normetanephrine hydrochloride, 3-methoxy-4-hydroxyphenylglycol hemipiperazinium salt, vanillylmandelic acid, epinephrine bitartrate salt, D,L-metanephrine hydrochloride and the 3,4-dihydroxy-benzylamine (internal standard) were obtained from Sigma-Aldrich Pty (Ltd) (Johannesburg, South Africa). Chemicals used for the mobile phase were HPLC grade deionised water and HPLC grade methanol (MeOH) and acetonitrile (ACN); sodium dihydrogen phosphate buffer; 1-octanesulphonic acid sodium salt; ethylenediaminetetra-acetic acid disodium salt; orthophosphoric acid (H_3PO_4, 85%). Chemicals used for the sample preparation were magnesium chloride, *S*-(5′-sdenosyl)-*L*-methionine (SAMe) chloride dihydrochloride, dimethyl sulfoxide (DMSO); perchloric acid (70%). All the chemicals were obtained from Merck (Pty) Ltd (Johannesburg, South Africa) and Sigma-Aldrich Pty (Ltd) (Johannesburg, South Africa).

4.1.2. Materials

The analytical HPLC column used was a Venusil ASB C_{18} (purchased from Bonna-Agela Technologies, USA), 4.6 x 250 mm, a particle size of 5 µm, pore size of 300 Å and a surface area of 200 m^2/g.

The developed and validated method was applied to prepared Sprague-Dawley rat liver homogenate samples.

4.1.3. Instrumentation

The chromatographic system consisted of an Ultimate 3000 UHPLC system, equipped with an ISO-3100SD isocratic pump and WPS-3000TSL analytical autosampler, coupled to an ECD-3000RS rapid separation electrochemical detector with 2-Channel Coulometric Cell 6011RS and Chromeleon® chromatography management system version 7.2 (all obtained from Thermo Fisher Scientific, Waltham, MA USA).

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Conflicts of interest:

The authors declare no conflict of interest.

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CHAPTER 5 MANUSCRIPT C

5.1 Submitted article

Article submitted to, and accepted for publication in ***Pharmazie - An International Journal of Pharmaceutical Sciences*** titled:

An HPLC-DAD validated method for the detection and quantification of cortisol, corticosterone and melatonin in plasma samples of two different animal species

AUTHOR CONTRIBUTIONS

Francois P Viljoen developed and validated the method. FP Viljoen did all the analytical work and the data processing for the manuscript. FP Viljoen wrote the first draft of the manuscript. FP Viljoen finalized the manuscript for publication and was the corresponding author in the submission of the final manuscript to *Die Pharmazie.*

Jan L du Preez was the promoter. JL du Preez advised on the study design and proofread the final manuscript.

Johanna C Wessels was the co-promoter. JC Wessels assisted in proofreading the manuscript for publication.

Marique E Aucamp was the assistant promoter. ME Aucamp assisted in proofreading the manuscript for publication.

Leith C R Meyer was a co-author. LCR Meyer was the prime investigator for obtaining the blood plasma samples from the White Rhinoceros (Ceratotherium simum). Ethical approval was obtained from both the University of Pretoria and North-West University for this study.

Friederike Pohlin was a co-author. F Pohlin was responsible for collecting the blood plasma samples from the White Rhinoceros (Ceratotherium simum).

IMPORTANT INFORMATION

- The manuscript was inserted into this document in the word document form it was submitted to the journal. In addendum B the first page as published in the journal was attached.
- Instructions to the author can be viewed online at *http://pharmazie.govi.de/instructions/*

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- Conformation of acceptance of this manuscript by *Die Pharmazie* is presented in addendum B.

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An HPLC-DAD validated method for the detection and quantification of cortisol, corticosterone and melatonin in plasma samples of two different animal species

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The monitoring of endogenous hormone plasma levels could be very valuable in biomedical, veterinary and pharmaceutical research. A specific high performance liquid chromatography method with diode array detection, for the assay of cortisol, corticosterone and melatonin in animal plasma was developed and validated. The chromatographic separation was achieved on a C_8 reversed phase column with a mobile phase consisting of HPLC-grade water and 35% v/v acetonitrile (pH ± 3.36). The detection was achieved through diode array detection, with two set wavelengths; 245 and 275 nm. The flow rate was at 1 ml/min and the total run time was 50 minutes. The method was validated according to validation guidelines (Shabir, 2006; US FDA, 2013). The method was found to be linear ($R^2 > 0.99$) over the analytical range (10 to 500 ng/ml) for all three analytes. All the other validation parameters were acceptable and within range. The method was applied to plasma samples from Sprague-Dawley rats and white rhinoceros.

Keywords:

Cortisol, Corticosterone, Melatonin, Plasma, HPLC-DAD, HPLC-ECD, Liquid-liquid extraction

1. Introduction

The measurement of endogenous hormone plasma levels forms a very important part of laboratory and wild animal research. These hormones help to understand and interpret a variety of animal disorders and diseases which are often similar, or related to those seen in other animals,

including humans. It also helps in the process of developing newer drugs for certain pathological conditions to either help cure them or treat the symptoms. Hormones are mediator biomolecules secreted by a variety of endocrine glands that are located all over the mammalian body (Tortora & Derrickson, 2014). The hormone is secreted from its endocrine gland and enters the bloodstream via the interstitial fluid (Tortora & Derrickson, 2014). The circulating blood system then delivers hormones to their related target cells throughout the body (Tortora & Derrickson, 2014). The hormone system's time of onset of action takes from seconds to hours or days and its duration of effect is generally longer than its release (Tortora & Derrickson, 2014). One of the major areas of animal research is stress and stress related disorders. A multitude of hormones (e.g., catecholamines, hormones secreted from the adrenal gland, etc.) are involved in the stress response (Möstl & Palme, 2002). Stressful situations trigger the adrenals to respond, which results in an increase in glucocorticoid, mineralocorticoid and/or catecholamine secretion (Möstl & Palme, 2002; Koren *et al.*, 2012). These increases form part of the front-line endocrine mechanisms to defend an organism against stressful conditions (Möstl & Palme, 2002). In addition, hormones which increase during stress periods are also part of the hormonal cascade causing parturition in some species (Möstl & Palme, 2002). During short-term stress, glucocorticoids improve fitness by energy mobilisation and may change behaviour (Möstl & Palme, 2002). However, severe chronic stress (prolonged periods of high cortisol concentrations) may decrease individual fitness by causing immunosuppression and atrophy of tissues (Möstl & Palme, 2002). Gong and associates (2015) show that serum cortisol and corticosterone in mice are closely correlated with the dynamics that occur during different physiological or pathological (stressful) conditions (Gong *et al.*, 2015). They hypothesised that corticosterone is a more adaptation-related biomarker than cortisol during chronic stress, and found that cortisol was a quicker responder than corticosterone during severe acute stress (Gong *et al.*, 2015).

Cortisol and corticosterone are part of the steroid hormone group called corticosteroids (Marieb, 2006). These hormones are produced and secreted by the adrenal cortex gland and are both classified as glucocorticoids (Marieb, 2006; Sembulingam & Sembulingam, 2012). The glucocorticoids are all 21 carbon steroid hormones (Sembulingam & Sembulingam, 2012). Cortisol is the glucocorticoid predominantly secreted in larger animals, including humans, whereas corticosterone is the predominant one in rodents (Granner, 1985). Two animal species plasma (Sprague-Dawley rat and white rhinoceros) were used to develop and validate the method because of this phenomenon. Figure 1 shows the biosynthesis of cortisol and corticosterone that takes place in the adrenal cortex.

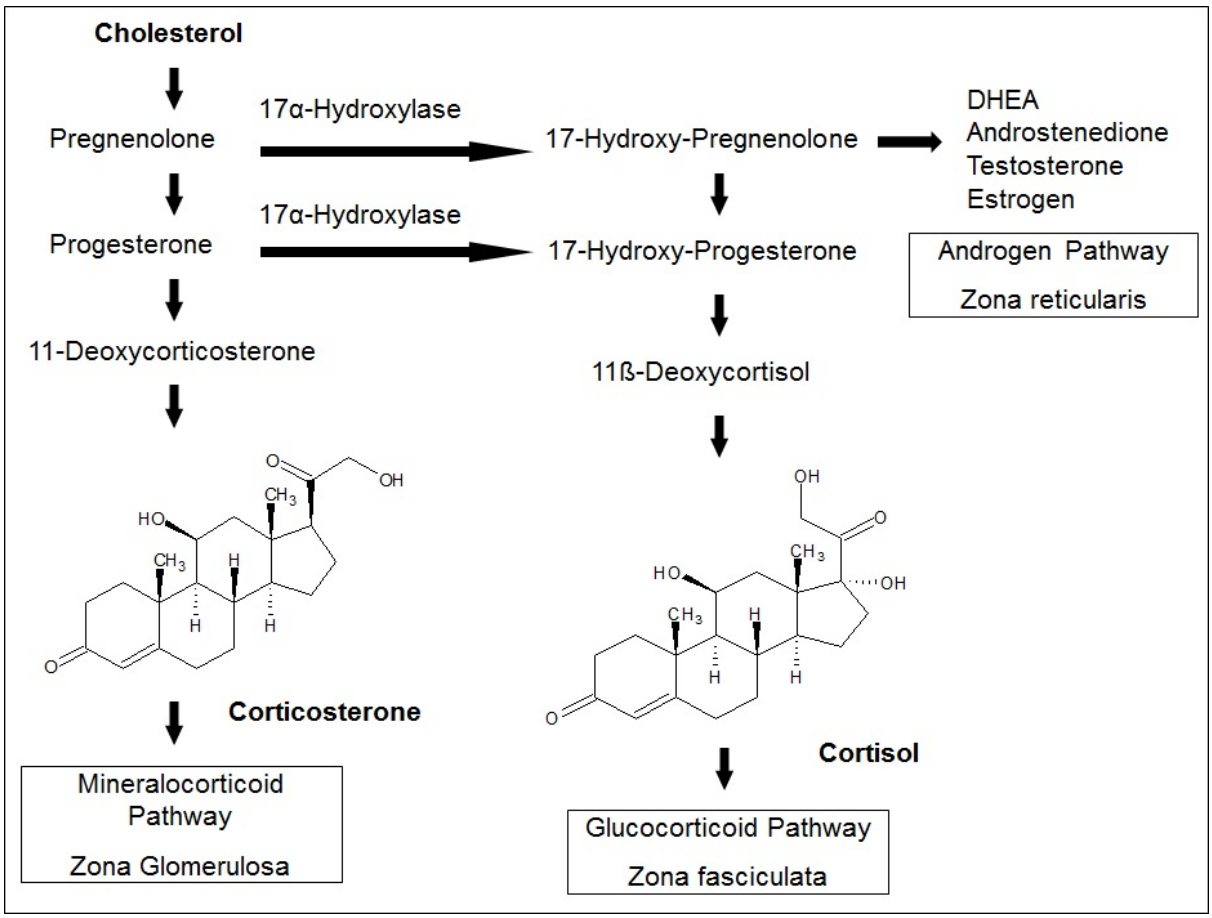

Figure 1: Adrenocortical hormone biosynthesis, modified from the literature (Sembulingam & Sembulingam, 2012).

Melatonin is an indolamine secreted by the pineal gland and it plays an important role in the regulation of the circadian sleep–wake rhythm (Sastre Toraño *et al.*, 2000; Çetin *et al.*, 2018). Figure 2 shows that melatonin is synthesised via serotonin from the amino acid tryptophan (Claustrat *et al.*, 2005; Tortora & Derrickson, 2014). Research in rats has linked low plasma levels of melatonin to acute stress (physical activity) (Paredes *et al.*, 2005). In another study in rats increased melatonin plasma levels were linked to chronic stress (Dagnino-Subiabre *et al.*, 2006).

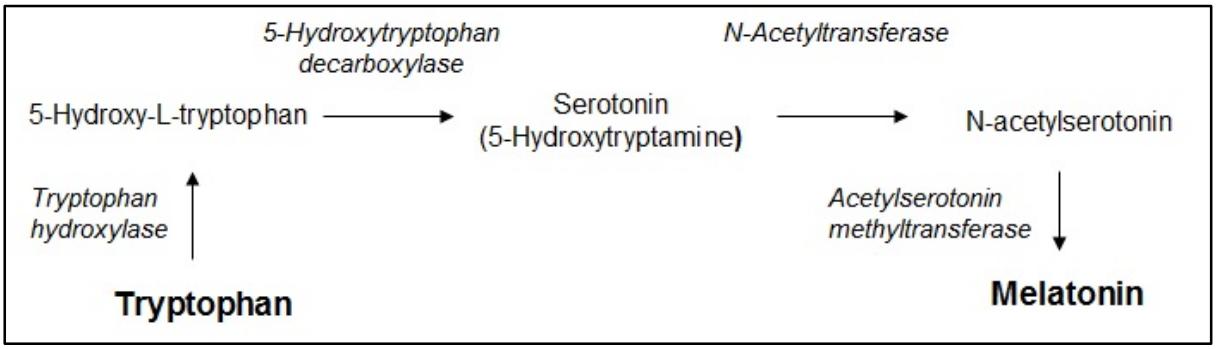

Figure 2: Melatonin synthesis from tryptophan, modified from the literature (Burtis *et al.*, 2012; Zagajewski *et al.*, 2012).

The HPLC-DAD method detected melatonin concentrations in units (ng/ml) greater than those reported (pg/ml) by Paredes *et al* (2005) and Haritou *et al* (2008). The melatonin molecule is electro-active and can also be easily detected with electrochemical detection (Vieira *et al.*, 1992). This detection technique was thus used to verify the data obtained with DAD detection.

2. Investigations and results

2.1. Methods (standards, buffer, mobile phase and sample preparation)

2.1.1. Preparation of standard solutions

Standard stock solution was prepared with a concentration of 100 µg/ml for each of the following analytes; cortisol, corticosterone and melatonin using HPLC-grade water (with 2% methanol) as the solvent. From this stock solution, a range (10 ng/ml to 500 ng/ml) of concentrations were prepared to setup a standard calibration curve, only with HPLC-grade water.

2.1.2. Preparation of the internal standard solution

Standard stock solution of the internal standard, dexamethasone, with a concentration of 100 µg/ml using HPLC-grade water as the solvent was prepared. The working internal standard solution, with a final concentration of 2.5 µg/ml, with an appropriate dilution from the internal standard stock solution using HPLC-grade water as solvent was prepared.

2.1.3. Phosphate buffer saline solution (PBS)

To prepare PBS add 8 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g disodium phosphate and 0.24 g of monopotassium phosphate to 800 ml of HPLC-grade water and mix. After all the buffer salts were dissolved top up the solution to 1 litre. Adjust the pH of the solution to pH 7.4 with hydrochloric acid (Top Tip Bio, 2018).

2.1.4. Mobile phase preparation

Mobile phase for the HPLC coupled to the diode array detector (DAD): A mobile phase consisting of HPLC-grade water and v/v 35% acetonitrile was prepared. The pH of the mobile phase was set at ± pH 3.36 with glacial acetic acid or formic acid. The mobile phase was filtered through a 0.45 µm nylon filter before use (Agela Technologies).

2.1.5. HPLC-DAD instrument settings

Table 1: Instrumentation settings	
HPLC instrument settings:	
Flow rate	1.0 ml/min
Injection volume	100 µl
Run time	55 minutes
Diode array detector settings:	
Wavelength 1	245 nm
Wavelength 2	275 nm
Data collection rate	5 Hz

Cortisol and corticosterone were monitored at 245 nm and melatonin at 275 nm (Rizzo *et al.*, 2002; Izquierdo-Hornillos *et al.*, 2005; Viljoen *et al.*, 2012).

2.1.6. Sample preparation of the plasma samples

Add 4.5 ml of the PBS solution to a 10 x 100 mm screw-capped glass tube followed by 1ml of the standard solution or test plasma and also 250µl of the internal standard dexamethasone (2.5 µg/ml). The analytes were extracted with 5ml of ethyl acetate by mixing the samples for 30 minutes with a rotating mixing wheel. Hereafter, the samples were centrifuged at 4500 rcf for 15 minutes. After centrifugation the organic upper layer was transferred to conical tubes and evaporated to dryness under a stream of nitrogen at a temperature of 40°C. The residue was reconstituted with 125µl of mobile phase where after the final sample was centrifuged at 1620 rcf for 5 minutes. The 125µl final sample was transferred into inserts in vials and placed in the autosampler of the HPLC system for analysis.

2.2. Method validation parameters

The method was validated according to the guidelines of the FDA concerning linearity, sensitivity (quantification and detection limits), precision (reproducibility), accuracy, recovery and stability (US FDA, 2013).

2.2.1. Linearity/Calibration curve

Linearity was done by preparing seven standard concentrations as described in section 2.1.2 and analysed in triplicate. The linear regression value (coefficient of determination, R^2) for the calibration curve must not be less than 0.95 for endogenous biomolecules (Shabir, 2006). For

calibration, the internal standard method was used with dexamethasone as the chosen internal standard.

2.2.2. Quantification and detection limits (Sensitivity)

The limit of quantification (LOQ) and the limit of detection (LOD) can be defined as the minimum concentration where the signal-to-noise ratio is at least 10:1 and 3:1 greater than the average background noise of an unspiked blank (only containing the internal standard), at the retention time of each analyte, respectively (Shrivastava & Gupta, 2011).

2.2.3. Precision and Accuracy

Four concentrations (5, 10, 75 and 200 ng/ml) were chosen and five determinants for each were done for precision and accuracy. Precision results were expressed as %RSD (relative standard deviation from the mean) and the acceptability criterion for each concentration level was not to exceed 15% (US FDA, 2013). The accuracy results for each concentration level were determined by comparing the closeness of the mean test concentration result to that of the true concentration value. The accuracy results were expressed as % recovery. The acceptability criterion for accuracy for each concentration level was to fall between 90 to 110% (Shabir, 2006; US FDA, 2013).

2.2.4. Recovery

The percentage recovery of the liquid-liquid extraction procedure was determined by preparing four standard concentrations (10, 75, 250 and 500 ng/ml). These four concentrations were analysed without going through the liquid-liquid extraction procedure and their peak areas determined. Here after the same four concentrations were put through the liquid-liquid extraction process and analysed. The percentage recovery of the liquid-liquid extraction procedure was then calculated with the following equation.

$$\text{Percentage Recovery} = \frac{\text{Peak Area of extracted sample}}{\text{Peak Area of unextracted sample}} \text{ X } 100$$

The % recovery for each concentration must be above 90% and consistently reproducible to adhere to the acceptance criterion.

2.2.5. Stability

Stability was determined by injecting the three standard solutions (low, medium and high) and two plasma samples directly after sample preparation. The initial injection set was assayed

immediately and served as reference values. The samples were re-injected after 24 h and 48 h, while keeping the samples at room temperature in the HPLC auto-sampler tray. Percentage stability for each analyte will be given as the mean % stability of the samples analysed and ± SD at the given hour.

2.3. Method validation results

The method was fully validated on a Venusil ASB C_8, 4.6 x 250 mm, 5 µm analytical column.

2.3.1. Linearity/Calibration curve

The calibration curve constructed was evaluated by means of its linear regression value. Linearity was excellent over the respective calibration ranges (Table 2), with corresponding coefficient of determination (R^2) consistently greater than 0.99 for all the analytes.

Table 2: Linear regression line equation and coefficient of determination with the diode array detector		
C_8 analytical column	**y = mx + c**	**Coefficient of determination (R^2)**
Cortisol	y = 0.0025x + 0.0054	R^2 = 0.9993
Corticosterone	y = 0.0027x + 0.0078	R^2 = 0.9993
Melatonin	y = 0.0018x – 0.0099	R^2 = 0.9991
Linear regression line equation and coefficient of determination with the electrochemical detector		
C_8 analytical column	**y = mx + c**	**Coefficient of determination (R^2)**
Melatonin	y = 1.2003x + 5.8288	R^2 = 0.9999

2.3.2. Quantification and detection limits (Sensitivity)

The lower limit of detection (LOD) and the lower limit of quantification (LOQ) were determined to be 5 ng/ml and 10 ng/ml for all the analytes respectively.

2.3.3. Precision and Accuracy

The precision and accuracy results of the four concentrations tested are provided in Table 3. Both the precision and accuracy results were within the acceptable criteria ranges set by the method validation parameters in Point 2.2.3. Throughout these four concentrations, the %RSD for both the intra-batch and inter-batch precision was below 4%. The accuracy of all concentration levels for all of the analytes tested was between 92.64% and 106.78%.

Table 3.Accuracy and precision results						
Analyte Concentration (ng/ml)	**Intra-batch (n=5)**			**Inter-batch (n=5)**		
	Measured concentration (ng/ml) (mean±SD)	**Precision (%RSD)**	**Accuracy (%)**	**Measured concentration (ng/ml) (mean±SD)**	**Precision (%RSD)**	**Accuracy (%)**
Cortisol						
10 (LOQ)	10.47 ± 1.24	11.82	104.71	10.03 ± 1.17	11.68	100.32
75	76.86 ± 4.71	6.13	102.47	73.19 ± 4.15	5.67	97.59
500	505.72 ± 4.88	0.96	101.14	494.34 ± 17.90	3.62	98.87
Corticosterone						
10 (LOQ)	10.46 ± 1.21	11.61	104.62	9.47 ± 1.21	12.79	94.73
75	74.02 ± 1.82	2.46	98.69	72.33 ± 6.19	8.55	96.44
500	500.79 ± 18.06	3.61	100.16	499.21 ± 14.47	2.90	99.84
Melatonin						
10 (LOQ)	10.17 ± 0.47	4.63	101.74	9.83 ± 1.30	13.27	98.29
75	79.86 ± 5.88	7.36	106.48	69.48 ± 3.83	5.51	92.64
500	529.51 ± 36.81	6.95	105.90	533.88 ± 44.26	8.29	106.78

Note: Intra-batch is where all the standard concentrations were prepared from one standard stock solution and analysed. Inter-batch is where all the standard concentrations were prepared from 5 separate prepared standard stock solutions.

2.3.4. Recovery

The percentage recovery for each analyte, measured at the four concentrations tested, were constantly above 90%.

2.3.5. Stability

In Table 4, the mean percentage stability results for the all the analytes tested in the three standards and the plasma samples are demonstrated. All the analytes were stable when tested at 24 h and 48 h stored at room temperature in the autosampler tray for the standard samples analysed. The plasma samples analysed for stability had mixed results but in the case of these types of biological samples it is better to analyse them within 24 hours after the samples are prepared to ensure sample integrity.

Table 4: Stability results for the three standards of the HPLC-DAD method		
% Stability (mean±SD) of the analytes at room temperature after 24 h and 48 h.		
Time:	**24 Hours***	**48 Hours***
Melatonin (275 nm)	99.14 ± 5.02	87.60 ± 4.70
Cortisol	96.25 ± 5.05	94.12 ± 5.00
Corticosterone	93.97 ± 4.37	92.76 ± 8.12
Stability results of the analytes in plasma samples		
Time:	**24 Hours***	**48 Hours***
Melatonin (275 nm)	79.36 %	83.59 %
Cortisol	98.51 %	79.85 %
Corticosterone	85.04 %	89.15 %

*Compared to 100% at t = 0, mean ±SD.

2.4. Chromatographic results

Table 5 present the relative retention times of all the analytes and the internal standard for this method.

Table 5: The relative retention times of the analytes and the internal standard are presented	
Analytes	**Relative retention times (minutes)**
1. Melatonin (HPLC-DAD method)	10.0 ± 0.5
2. Cortisol	16.75 ± 0.5
3. Internal standard (dexamethasone)	31.01 ± 0.5
4. Corticosterone	37.45 ± 0.5

The following two chromatographic figures are representative of a 75 ng/ml standard (Figure 3), and a 500ng/ml standard (Figure 4), both done with the HPLC-DAD method.

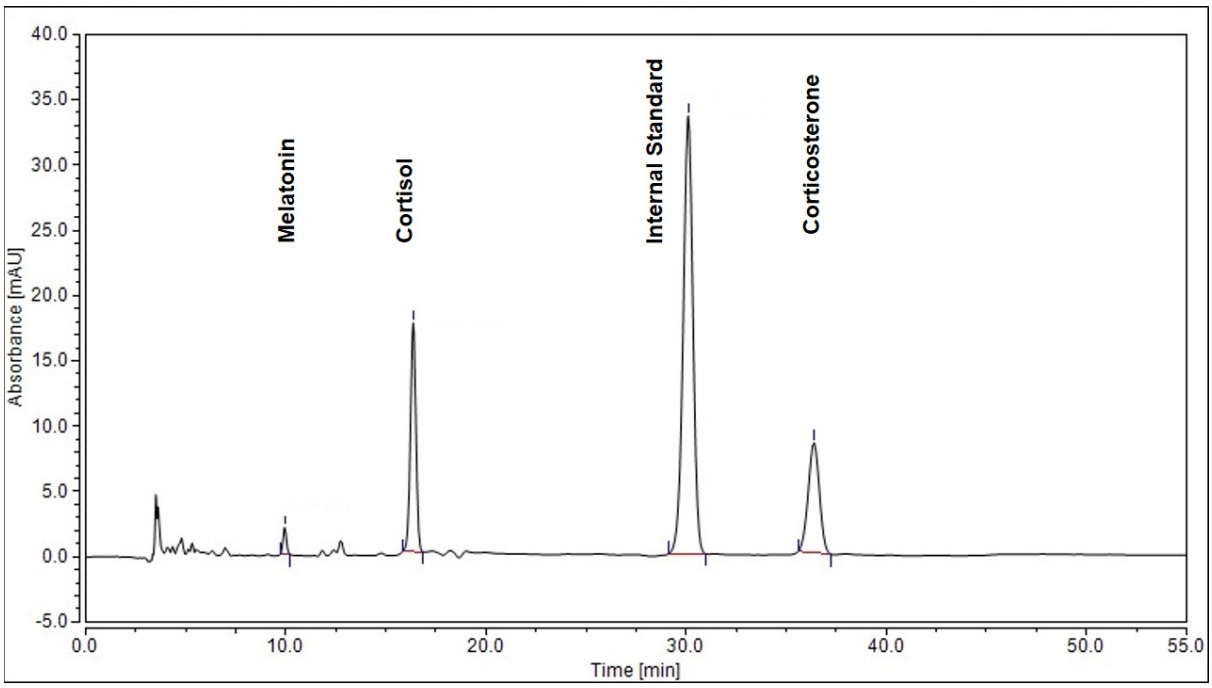

Figure 3: 75 ng/ml standard monitored at 245 nm with the HPLC-DAD method.

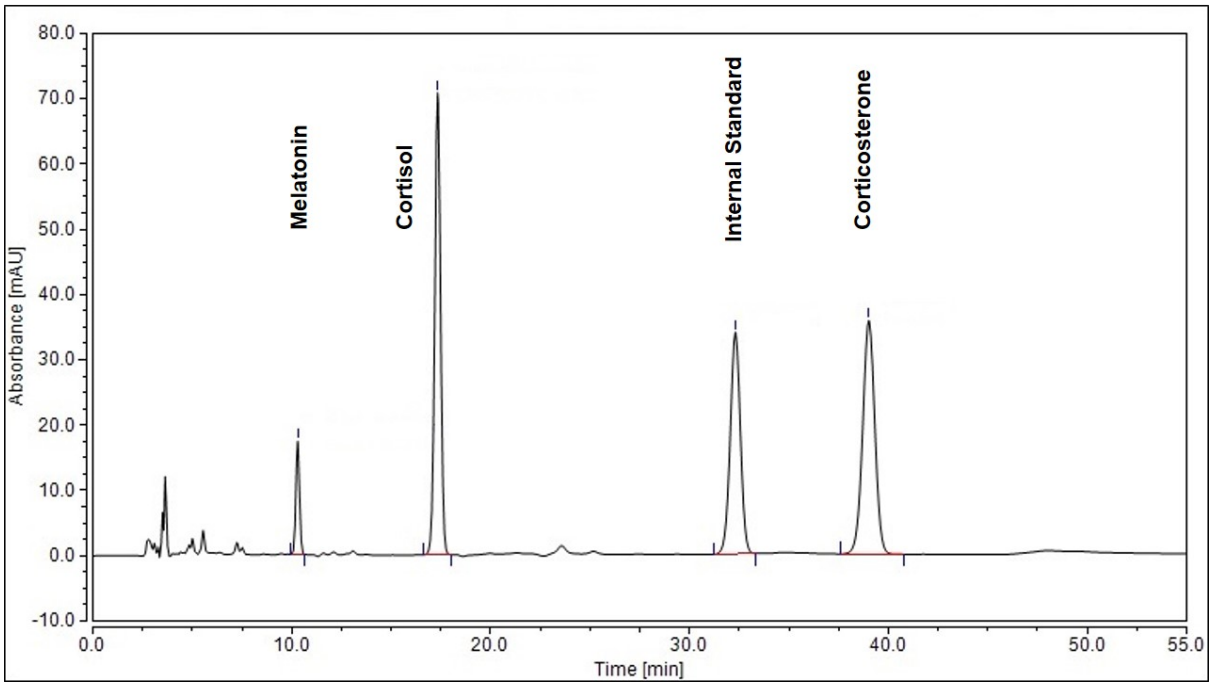

Figure 4: 500 ng/ml standard monitored at 245 nm with the HPLC-DAD method.

2.5. Application

The validated method was applied to plasma samples from white rhinoceros and Sprague-Dawley rats that was collected in lithium-heparin (green top) and K_3EDTA (purple top) blood collection test tubes respectively. The internal standard method was used to calculate the concentration of each analyte in the samples (Scientific, 2014).

The following three chromatographic figures represents the two animal species plasma liquid-liquid extraction results.

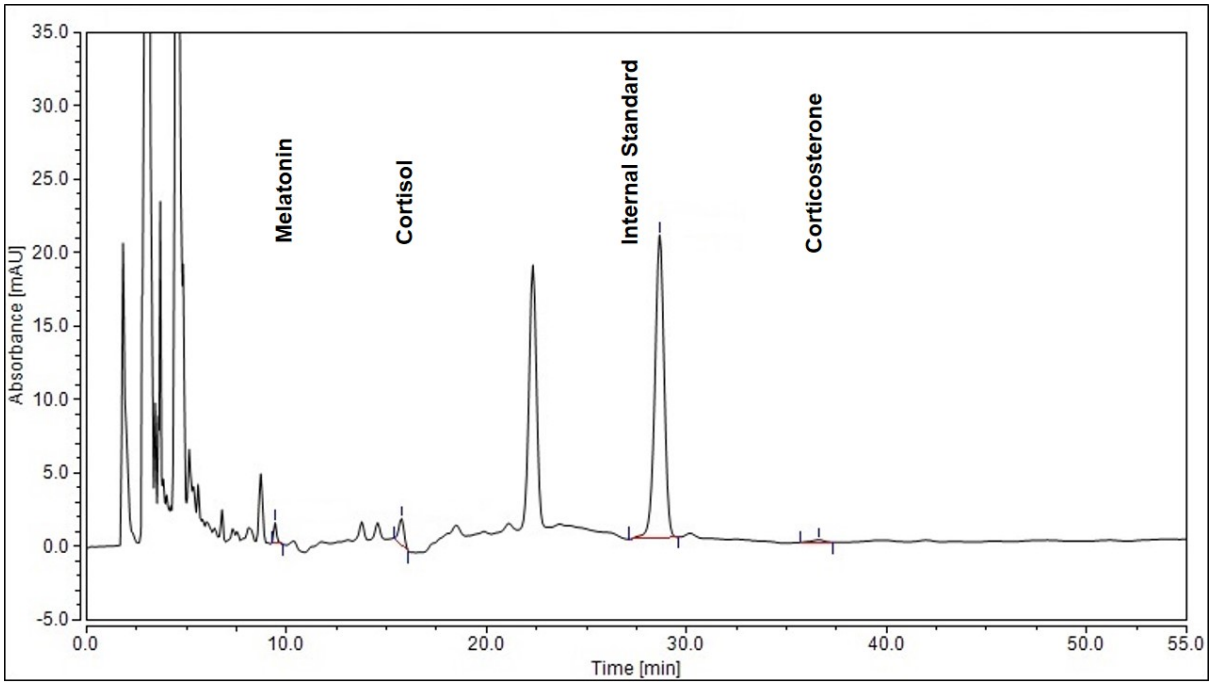

Figure 5: Rhinoceros plasma sampled at capture and monitored at 245 nm with the HPLC-DAD method.

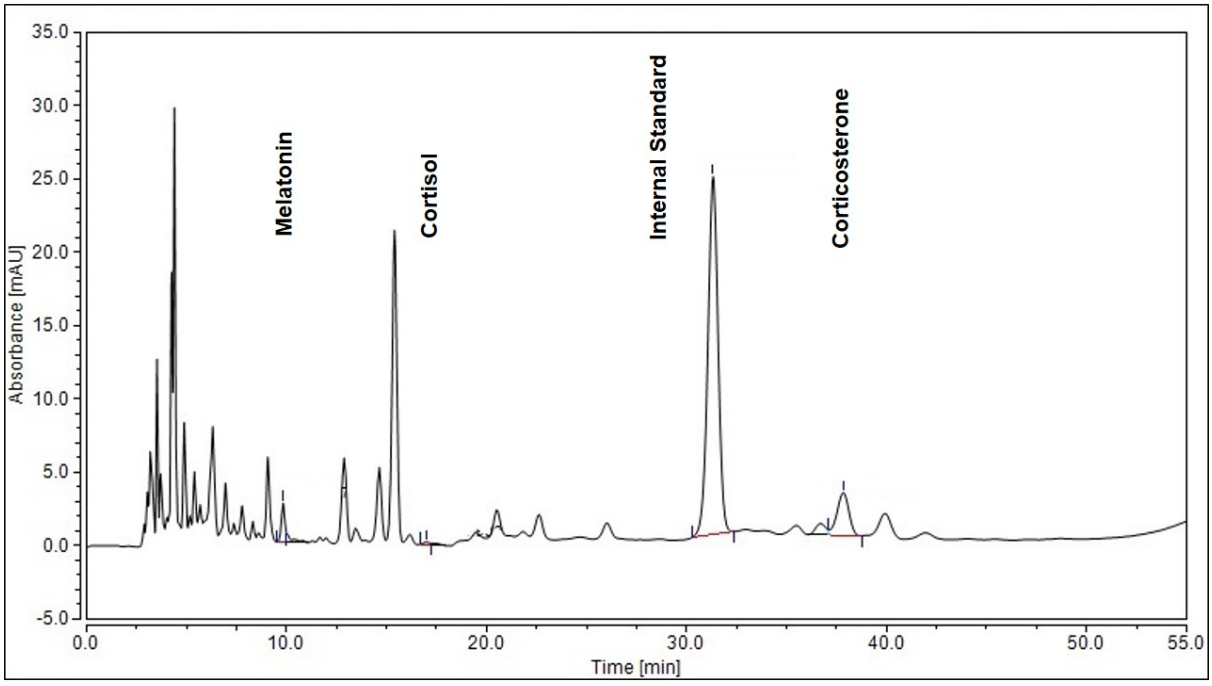

Figure 6: Rat plasma sample monitored at 245nm with the HPLC-DAD method.

In Table 6, the concentrations of cortisol, corticosterone and melatonin that were detected and quantified in the plasma samples. The results were expressed in ng/ml. Cortisol however was below the LOD in some of the rat samples. The cortisol and corticosterone results were comparable with that found in the literature (Van Heerden *et al.*, 1985; Koren *et al.*, 2012; Viljoen

et al., 2012). However, the melatonin concentrations measured in both the species were different to those reported in literature, both the HPLC-DAD and HPLC-ECD method measured the melatonin concentrations in units (ng/ml) greater than those reported (pg/ml) by Paredes *et al.* (2005) and Haritou *et al.* (2008) (Paredes *et al.*, 2005; Haritou *et al.*, 2008). However, the melatonin concentrations measured by the two methods, compared well with each other, especially in rhinoceros number 3 after 6 hours of transport, and the mixture of rhinoceros plasma samples (Table 6). Furthermore a pharmacokinetic study in human volunteers that were given an oral dosage of a 20 mg capsule of melatonin also measures melatonin concentrations in the ng/ml (mean C_{max} of ± 70 ng/ml) analytical unit (Jenjirattithigarn *et al.*, 2014).

Table 6: Rhinoceros and rat plasma extraction results obtained (ng/ml)						
	White Rhinoceros samples				2 Male Rats	
Analyte	**Animal 1 captured**	**Animal 1 at released**	**Animal 2 captured**	**Animal 2 after 6 hours of transport**	**Animal 1 sample A**	**Animal 2 sample B**
Cortisol	8,95	20,74	8,93	7,36	BLOD	4,08
Corticosterone	3,50	3,46	2,05	0,69	41.99	31,32
Melatonin with HPLC-DAD method	27,42	22,17	23,25	240,58	105,59	234,22
	Animal 3 captured	**Animal 3 after 6 hours of transport**		**Mixture of rhinoceros plasma samples**		**Mixture of rat plasma samples**
Cortisol	11,51	7,41		17,22		0,91
Corticosterone	2,21	1,37		1,82		30,82
Melatonin with HPLC-DAD method	19.02	42.64		30,33		96,05
Melatonin with HPLC-ECD method	13.89	16.31		19,49		123,18

(BLOD - below limit of detection).

3. Discussion

A specific HPLC, coupled to a diode array detector combined with liquid-liquid extraction method, was developed and validated for the quantitative analysis of cortisol, corticosterone and melatonin in rat and white rhinoceros plasma samples. This method was validated for linearity, sensitivity, precision, accuracy, recovery and stability. The linearity for all respective analytes calibration ranges was excellent and the coefficient of regression (R^2) was consistently above 0.95. The LOQ and LOD were respectively 10.0 ng/ml and 5 ng/ml for the method. The percentage recovery was

acceptable for all the analytes at all the concentrations measured, and the percentage stability was also acceptable for both 24 and 48 hours testing for the standards and the plasma samples, however, plasma samples ideally should be analysed within 24 hours after sample preparation. The method was found to be selective and specific for cortisol, corticosterone and melatonin, although cortisol was below the limit of detection in some of the rat samples. The higher than expected melatonin levels measured on the HPLC-DAD method were correlated with a HPLC-ECD method. The melatonin levels measured by both methods compared well with each other. That melatonin concentrations can be measured, using these methods, at a higher analytical unit than reported in the literature is novel and requires further investigation once ethical approval is given to get more plasma samples for this analysis.

We propose that this method can be applied to drug and behaviour studies in laboratory animals (for example rats and mice) as well as wild animals to determine the stress response to management procedures, and other related disorders in these animals.

4. Experimental

4.1. Chemicals, reagents, materials and instrumentation

4.1.1. Chemicals and reagents

Melatonin, cortisol (hydrocortisone), corticosterone and dexamethasone (internal standard) were obtained from Sigma-Aldrich Pty (Ltd) (Johannesburg, South Africa). Chemicals used for the mobile phase were HPLC grade deionised water, HPLC grade methanol (MeOH), glacial acetic acid and formic acid. Chemicals used for the sample preparation were sodium chloride, potassium chloride, disodium phosphate, monopotassium phosphate, hydrochloric acid and ethyl acetate. All the chemicals were obtained from Merck (Pty) Ltd (Johannesburg, South Africa).

4.1.2. Materials

The analytical HPLC columns used was a Venusil ASB C_8, 4.6 x 250 mm, a particle size of 5 µm, pore size of 150 Å and a surface area of 200 m^2/g (purchased from Bonna-Agela Technologies, USA).

The developed and validated method was applied to prepared white rhinoceros (Ceratotherium simum) plasma collected in Lithium-heparin blood collection tubes and Sprague-Dawley rat plasma collected in EDTA blood collection tubes. The blood samples were immediately centrifuged after collection to separate the plasma from the blood components. The plasma was

transferred to Eppendorf tubes and immediately snap frozen in liquid nitrogen, transferred in dry ice and stored in a -80°C freezer until day of analysis.

4.1.3. Instrumentation

The chromatographic system for the HPLC-DAD method, consisted of an Ultimate 3000 UHPLC system, equipped with an pump and WPS-3000TSL analytical autosampler, coupled to an diode array detector and Chromeleon® chromatography management system version 7.2 (all obtained from Thermo Fisher Scientific, Waltham, MA USA).

Conflict of interest

The authors have no conflict of interest to declare.

Ethics approval

Ethical approval for this project to use animal (rat and white rhinoceros) plasma was obtained from the animal care, health and safety in research ethics committee (AnimCare) of the Faculty of Health Sciences, from the North-West University (NWU-00252-17-A5), as well as from the Animal Ethics Committee from the University of Pretoria (V067-17).

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CHAPTER 6 DISCUSSION, CONCLUSION, LIMITATIONS AND FUTURE DIRECTIONS

6.1 Discussion

Bioanalytical techniques refer to a variety of methods, assays, and procedures that enable researchers to detect and analyse endogenous biomolecules found in living organisms and the chemical reactions underlying life processes. To perform a comprehensive bioanalysis of a biomolecule in a biological process or system, the researcher typically needs to design a strategy to detect the specific biomolecule, isolate it in its pure form from among thousands of molecules that can be found in an extract from a biological sample, characterise it, and analyse its function. An analytical technique, the biochemical test that detects and measures a molecule whether quantitative or semi-quantitative, is important to determine the presence and quantity of a biomolecule at each step of a particular study. Detection methods may range from the simple type of spectrophotometric measurements to ELISA or RIA methods and chromatographic methods. Chromatography techniques these days are sensitive and effective in separating and measuring minute components of a mixture, and are widely used for quantitative and qualitative analysis in biomedical, industrial processes, and other fields. The utilisation of HPLC with diode array detection and electrochemical detection provided a platform to develop these methods. Understanding the biomolecules' molecular structure helped with the choice of correct detection method. The challenge however was also to choose and develop the appropriate sample preparation technique for the specific biological sample matrix. The essential part sample preparation plays is to provide a final sample matrix compatible with the HPLC system and detector without much interference. Sample preparation plays also an essential role in ensuring good and reliable validation results. The practical application of the new method not only proves that the method is working, it also supports the method validation of the method.

In this study, three new methods were successfully developed and validated for the quantitative analysis of a variety of endogenous biomolecules (chemical messengers). The first one was the method for the analysis of the monoamines and their metabolites in rat brain tissue. The second was a method for norepinephrine and its metabolites for the measurement of enzyme COMT activity in rat liver homogenate in the presence of the known COMT inhibitor, entacapone. Lastly, the method for the analysis of cortisol, corticosterone and melatonin in plasma samples from laboratory animals, the Sprague-Dawley rat, and the white rhinoceros, a wild animal. These new methods were published in an international accredited journal, Die Pharmazie (http://pharmazie.govi.de/). These methods not only adhered to the aims and objectives of the study project but also to the research problem of the need to develop and validate new analytical

methods that will be sensitive, specific, reliable and more affordable than commercially available analytical kits.

6.1.1 Affordability

Were these new methods more affordable than the commercially available variety of analysis kits? The answer is "Yes". In following three tables (6-1 to 6-3) a budget for each of the three new methods was done to show its affordability.

Table 6-1: The cost per single run analysis per rat brain area sample done on HPLC-ECD for the monoamines (Chapter 3: Manuscript A).

Components	**Price per item**	**Price per sample**	**Possible number of samples**
Analyte Compounds for Standard Samples (100mg)	R 10000.00	R 5.00	± 1500
HPLC Column	R 4 000.00	R 5.00	± 1500 samples injected per column per year
HPLC Mobile Phase Buffer (1 litre per month or project prepared)		R 10.00	
Sample Preparation:		R 50.00	Per sample
Solvents, buffers, consumables, Eppendorf tubes and internal standard			
Instrument yearly service with parts	R 25 000.00	R 20.00	± 1500 samples injected per year
Coulometric cell for HPLC	R 25 000.00	R 5.00	± 5000 samples injected per cell
Total Price per Sample		**R 100.00**	
Abnova ELISA kit		**R 187.50**	
ChromSystems HPLC Catecholamine kit		**R 200.00**	

Note: The price per sample excludes VAT (Value Added Tax) and the salary of the analyst.

The cost per sample for the rat brain monoamines analysis was budgeted at ± R 100.00 as shown in Table 6-1. This method can detect and quantify the three monoamines, dopamine, norepinephrine, epinephrine, serotonin and their metabolites in rat brain tissue samples, which amounts to nine biomolecules and involves the whole monoamines metabolic pathways. The commercially available kits can only detect two to three biomolecules, for a higher price of ± R187.50 for the Abnova Epinephrine/Norepinephrine (no metabolites) ELISA kit and ± R200.00

for the ChromSystems HPLC Catecholamine (Norepinephrine, epinephrine and dopamine, but no metabolites) in Plasma kit, per sample respectively (from Table 2-1).

Table 6-2: The cost per single run analysis per sample prepared for the analysis of the COMT enzyme activity done on HPLC-ECD (Chapter 4: Manuscript B).

Components	**Price per item**	**Price per sample**	**Possible number of samples**
Analyte Compounds for Standard Samples (100mg)	R 10000.00	R 5.00	± 1500
HPLC Column	R 4 000.00	R 5.00	± 1500 samples injected per column per year
HPLC Mobile Phase Buffer (1 litre per month or project prepared)		R 10.00	
Sample Preparation:		R 100.00	Per sample
Solvents, buffers, reagents, consumables, Eppendorf tubes and internal standard			
Instrument yearly service with parts	R 25 000.00	R 20.00	± 1500 samples injected per year
Coulometric cell for HPLC	R 25 000.00	R 5.00	± 5000 samples injected per cell
Total Price per Sample		**R 150.00**	
Abnova ELISA kit		**R 187.50**	

Note: The price per sample exclude VAT (Value Added Tax) and the salary of the analyst.

The cost per sample prepared for the analysis of the COMT enzyme activity done on HPLC-ECD was budgeted at ± R 150.00 as shown in Table 6-2. This method can detect and quantify the norepinephrine and epinephrine and their metabolites (normetanephrine, MHPG, VMA and metanephrine) in rat liver homogenate samples, which amounts to six biomolecules, compared to the commercially available kits for a higher price of ± R187.50 per sample for the Abnova Epinephrine/Norepinephrine (no metabolites) ELISA kit and the Abnova Normetanephrine Elisa kit, KA1892, respectively (from Table 2-1). Here again the new method can detect the whole norepinephrine/epinephrine metabolic pathway of biomolecules from the same sample, the kit is limited to only one or two biomolecules.

Table 6-3: The cost per single run analysis per plasma sample done on HPLC-DAD and HPLC ECD for cortisol, corticosterone and melatonin (Chapter 5: Manuscript C).

Components	**Price per item**	**Price per sample**	**Possible number of samples**
Analyte Compounds for Standard Samples and internal standard (100mg)	R 5000.00	R 3.50	± 1500
HPLC Column	R 4 000.00	R 5.00	± 1500 samples injected per column per year
HPLC Mobile Phase Buffer (1 litre per month or project prepared)		R 10.00	
Sample Preparation:		R 75.00	Per sample
Solvents, buffers, consumables, Eppendorf tubes.			
Instrument yearly service with parts	R 25 000.00	R 20.00	± 1500 samples injected per year
D2-Lamp DAD 2-Channel Coulometric Cell 6011RS	R12000.00 R 25 000.00	R 8.00 R 5.00	± 1500 samples injected per lamp ± 5000 samples injected per cell
Total Price per Sample		**R 126.50**	
cortisol or corticosterone ELISA kit		**R 187.50**	
Melatonin ELISA kit		**R 625.00**	

Note: The price per sample exclude VAT (Value Added Tax) and the salary of the analyst.

The cost per plasma sample done on HPLC-DAD for cortisol, corticosterone and melatonin was budgeted at ± R 126.50, as shown in Table 6-3. This method can detect and quantify these three biomolecules in plasma samples, which amounts to three biomolecules that are all influenced by physiological stressors. This is in comparison to commercially available kits, which can only detect one of these biomolecules per kit and with a price of ± R187.50 per sample for the cortisol or corticosterone and ± R625.00 per sample for melatonin (from Table 2-1).

6.1.2 Method validation

What parameters are important for the method validation of chromatographic methods for the analysis of biomolecules (chemical messengers)?

We propose the following parameters must be investigated for a full method validation: specificity and selectivity, sensitivity, linearity, range, precision, accuracy, stability and an appropriate practical application. The parameters to be done for a partial method validation are precision, accuracy and the linearity. Partial method validation is applicable when a new analyst is going to perform analysis with the method, change from one HPLC system to another, change in sample matrix between species (animal to animal to humans) and changes in sample volume (US FDA, 2013). When major changes to the method are made, for example, the biological sample matrix changes, changes in the sample preparation process, changes to the mobile phase composition and changes between different column packing materials, a full method validation must be performed again. In Table 6-4, the proposed method validation guideline parameters for HPLC methods for the quantification of endogenous biomolecules from biological sample matrixes is tabled with the number of samples to be tested and limits for each parameter.

Table 6-4: Proposed method validation guideline parameters for HPLC quantification of endogenous biomolecules (chemical messengers) in biological sample matrixes

Parameters to be evaluated		**Number of Samples Tested and limits for each parameter**
Specificity/Selectivity	Interferences, matrix effect	6 Blank and zero sample runs to show no interferences.
Analytical sensitivity	Limit of detection (LOD)	Determine by injecting decreasing injection volumes of the LOQ. The response signal of the analyte molecule must be two to three times greater than the background noise or the baseline (Swartz & Krull, 2012; Crawford Scientific, 2014).
	Limit of quantification (LOQ)	Lowest concentration of the calibration curve range.
Linearity (Calibration curve) and range	With an appropriate internal standard	6-8 Concentrations (3 determinations) The linear regression value for the curve must not be less than $r^2 = 0.95$ for biologics or biomolecules (Shabir, 2006).
Precision (Reproducibility and repeatability) and Accuracy	Intra-sample, intra-batch or Intra-day variance	3-4 Concentrations: low, medium and high (5 determinations) The % RSD (percentage relative standard deviation) determined at each concentration level should be less than 15% except for concentrations at the LOQ,
	Inter-sample, intra-batch or Inter-day variance	

		where it should be less than 20% (US FDA, 2013).
	Recovery	3-4 Concentrations: low, medium and high (3 determinations) The results must not only be as close as they can to 100% but, must also be consistent and reproducible (US FDA, 2013).
Stability	Standards and Test samples	3-4 Concentrations: low, medium and high. 1-2 Test samples. Injections at 0 hours, 24 hours and 48 hours at room or autosampler temperature. The results must be above 90%, but in the case of these types of biological samples it is better to analyse them within 24 hours after the samples are prepared to ensure sample integrity.
Practical application	Appropriate test samples	From 3 to 5 test samples or a whole study.

When the method development and the first validation is fully completed, it is essential to write up the method with full details in a standard operating procedure (SOP) format, highlighting important points to guide any analyst who will be using the method. When an analyst uses the method, any modifications or improvements must be noted and the method and SOP updated. One of the advantages of developing and validating own methods, is the analyst or researcher can trust the method if it is well maintained and each time the method is employed a partial validation can be done to make sure the results obtained will be trustworthy.

6.2 Conclusion

The quantitative analysis of these endogenous biomolecules (chemical messengers) is essential for a variety of health science research areas in both humans and animals. It can be an asset for the better understanding of health disorders and diseases in both humans and animals. We conclude that these newly developed and validated analytical methods can be applied in practice with great success and with cost saving advantages. We anticipate that these methods will be a great addition, especially to our own research environment, where these kind of methods are constantly used in a variety of animal behavioural studies in stress or stress-related disorders. These methods can also be utilised to test already registered drugs on the markets and for better drug development and discovery.

6.3 Limitations

A limitation to this study was that these methods could not be compared to the commercially available kits in a study where test animal samples were tested simultaneously on both, and the results compared. This limitation was due to the costs of these commercially available kits. This comparative investigation can be performed as part of future studies.

6.4 Future directions

Although this study is concluded, the aim of this researcher is to develop and validate more analytical methods to adhere to the need in our own research environment, and globally, for these kinds of methods to analyse endogenous biomolecules (chemical messengers). There are many chemical messengers in need of being quantitatively analysed, and this will be a great asset to biomedical and pharmaceutical research. The following are essential and will be looked at first: peptides such as oxytocin, arginine-vasopressin and brain-derived-neurotropic factor (BDNF), which is under the spotlight in neuroscience research. They are all currently measured by commercially available ELISA or RIA kits separately, but not combined into one kit. Other chemical messengers of interest are acetylcholine, the tryptophan and kynurenine pathway and glutathione oxidised (GSSG) and reduced (GSH). These types of methods form an essential part our research environment to contribute to pharmaceutical and biomedical research globally.

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ADDENDUM A CONCEPT MANUSCRIPT D

This is a concept manuscript that will be finished next year and published in an international accredited journal. The method still requires an appropriate internal standard. The method validation is not fully completed. The linearity parameter and the intra-batch precision and accuracy was the only validation parameters that's completed. The method will also be applied to test plasma samples of variety of animal species.

Centre of Excellence for Pharmaceutical Sciences, Faculty of Health Sciences, North-West University, Potchefstroom, South Africa[1], School of Pharmacy, University of Western Cape, Bellville, South Africa[2], Centre for Veterinary Wildlife Studies and Department of Paraclinical Sciences, University of Pretoria, Pretoria, South Africa[3]

An HPLC-ECD validated method for the detection and quantification of melatonin in plasma samples of animal species

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The monitoring of endogenous hormone plasma levels could be very valuable in biomedical, veterinary and pharmaceutical research. A specific high performance liquid chromatography method coulometric electrochemical detection, for the assay of melatonin in animal plasma was developed and validated. The chromatographic separation was achieved on a C_8 reversed phase column with a mobile phase consisting of 50 mM di-sodium hydrogen phosphate, 0.05 mM ethylenediaminetetraacetic acid disodium salt and 35% v/v acetonitrile was prepared. The pH of the mobile phase was set at ± pH 4.12 with ortho-phosphoric acid. The detection was achieved through coulometric electrochemical detection with the test electrode set at +600 mV. The flowrate was at 1 ml/min and the total run time was 15 minutes. The method was validated according to validation guidelines (Shabir, 2006; US FDA, 2013). The method was found to be linear (R^2 > 0.99) over the analytical range (10 to 500 ng/ml).

Keywords:

Melatonin, Plasma, HPLC-DAD, HPLC-ECD, Liquid-liquid extraction

ADDENDUM A CONCEPT MANUSCRIPT D

1. Introduction

Melatonin is an indolamine secreted by the pineal gland and it plays an important role in the regulation of the circadian sleep–wake rhythm (Sastre Toraño *et al.*, 2000; Çetin *et al.*, 2018). Figure 2 shows that melatonin is synthesized from the amino acid tryptophan through the monoamine serotonin (Claustrat *et al.*, 2005; Tortora & Derrickson, 2014). Research in rats has linked low plasma levels of melatonin to acute stress (physical activity) (Paredes *et al.*, 2005). In another study in rats increased melatonin plasma levels was linked to chronic stress (Dagnino-Subiabre *et al.*, 2006).

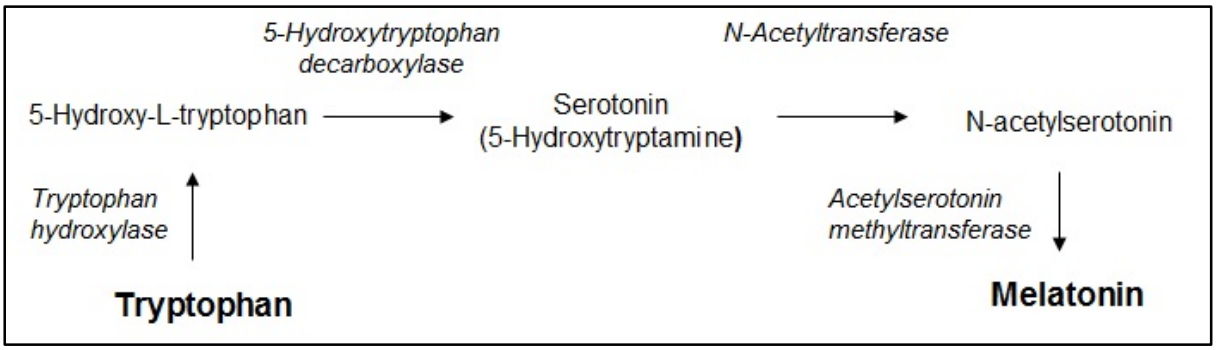

Figure 2: Melatonin synthesis from tryptophan, modified from the literature (Burtis *et al.*, 2012; Zagajewski *et al.*, 2012).

2. Investigations and results

2.1. Methods (standards, buffer, mobile phase and sample preparation)

2.1.1. Preparation of standard solutions

Standard stock solution was prepared with a concentration of 100 µg/ml for melatonin using HPLC-grade water (with 2% methanol) as the solvent. From this stock solution, a range (10 ng/ml to 500 ng/ml) of concentrations were prepared to setup a standard calibration curve, only with HPLC-grade water.

2.1.2. Preparation of the internal standard solution

The method still requires an appropriate internal standard

2.1.3. Phosphate buffer saline solution (PBS)

To prepare PBS add 8g of sodium chloride, 0.2 g of potassium chloride, 1.44 g disodium phosphate and 0.24 g of monopotassium phosphate to 800 ml of HPLC-grade water and mix.

After all the buffer salts were dissolved top up the solution to 1 litre. Adjust the pH of the solution to pH 7.4 with hydrochloric acid (Top Tip Bio, 2018).

2.1.4. Mobile phase preparation

Mobile phase for the HPLC coupled to the electrochemical detector (ECD): A mobile phase consisting of 50 mM di-sodium hydrogen phosphate, 0.05 mM ethylenediaminetetraacetic acid disodium salt and 35% v/v acetonitrile was prepared. The pH of the mobile phase was set at ± pH 4.12 with ortho-phosphoric acid. The mobile phase was filtered through a 0.22 µm nylon filter before use (Agela Technologies).

2.1.5. HPLC Instrument, diode array detector and electrochemical detector settings

Table 1: Instrumentation settings	
HPLC instrument settings:	**HPLC-ECD method**
Flow rate	1.0 ml/min
Injection volume	10 µl
Run time	15 minutes
Electrochemical detector settings	
Coulometric cell potentials	
Test electrode 1 (E1)	-250 mV
Test electrode 2 (E2)	+600 mV
Data collection rate	20 Hz

2.1.6. Sample preparation of the plasma samples

Add 4.5 ml of the PBS solution to a 10 x 100 mm screw-capped glass tube followed by 1ml of the standard solution or test plasma and also 250µl of the internal standard Dexamethasone (2.5 µg/ml). The analytes were extracted with 5ml of ethyl acetate by mixing the samples for 30 minutes with a rotating mixing wheel. Hereafter, the samples were centrifuged at 4500 rcf for 15 minutes. After centrifugation the organic upper layer was transferred to conical tubes and evaporated to dryness under a stream of nitrogen at a temperature of 40°C. The residue was reconstituted with 125µl of mobile phase where after the final sample was centrifuged at 1620 rcf for 5 minutes. The 125µl final sample was transferred into inserts in vials and placed in the autosampler of the HPLC system for analysis.

2.2. Method validation parameters

The method was validated according to the guidelines of the FDA concerning linearity, sensitivity (quantification and detection limits), precision (reproducibility), accuracy, recovery and stability (US FDA, 2013).

2.2.1. Linearity/Calibration curve

Linearity was done by preparing seven standard concentrations as described in section 2.1.2. Three replicates of each standard were injected to establish linear regression for each analyte. The linear regression value (coefficient of determination, R^2) for the calibration curve must not be less than 0.95 for endogenous biomolecules (Shabir, 2006). For calibration, the internal standard method was used with Dexamethasone as the chosen internal standard.

2.2.2. Quantification and detection limits (Sensitivity)

The limit of quantification (LOQ) and the limit of detection (LOD) can be defined as the minimum concentration where the signal-to-noise ratio is at least 10:1 and 3:1 greater than the average background noise of an unspiked blank (only containing the internal standard), at the retention time of each analyte, respectively (Shrivastava & Gupta, 2011).

2.2.3. Precision and Accuracy

Four concentrations (5, 10, 75 and 200 ng/ml) were chosen and six determinants for each were done for precision and accuracy. Precision results were expressed in %RSD (relative standard deviation from the mean) and the acceptability criterion for each concentration level was not to exceed 15% (US FDA, 2013). The accuracy results for each concentration level were determined by comparing the closeness of the mean test concentration result to that of the true concentration value. The accuracy results were expressed as % recovery. The acceptability criterion for accuracy for each concentration level was to fall between 90 to 110% (Shabir, 2006; US FDA, 2013).

2.2.4. Recovery

The percentage recovery of the liquid-liquid extraction procedure was determined by preparing four standard concentrations (10, 75, 250 and 500 ng/ml). These four concentrations were analysed without going through the liquid-liquid extraction procedure and their peak areas determined. Here after the same four concentrations were put through the liquid-liquid extraction process and analysed. The percentage recovery of the liquid-liquid extraction procedure was then calculated with the following equation.

$$\text{Percentage Recovery} = \frac{\text{Peak Area of extracted sample}}{\text{Peak Area of unextracted sample}} \text{ X } 100$$

The % recovery for each concentration must be above 90% and consistently reproducible to adhere to the acceptance criterion.

2.2.5. Stability

Stability will be determined by injecting the three standard solutions (low, medium and high) and two plasma samples directly after sample preparation. The initial injection set was assayed immediately and served as reference values. The samples were re-injected after 24 h and 48 h, while keeping the samples at room temperature in the HPLC auto-sampler tray. Percentage stability for each analyte will be given as the mean % stability of the samples analysed and ± SD at the given hour.

2.3. Method validation results

The method was fully validated on a Venusil ASB C_8, 4.6 x 250 mm, 5µm.

2.3.1. Linearity/Calibration curve

The calibration curve constructed was evaluated by means of its linear regression value. Linearity was excellent over the respective calibration range (Table 2), with corresponding coefficient of determination (R^2) consistently greater than 0.99 for melatonin.

Table 2: Linear regression line equation and coefficient of determination with the electrochemical detector

C_8 analytical column	**y = mx + c**	**Coefficient of Determination (R^2)**
Melatonin	y = 1.1419x + 9.9035	R^2 = 0.9993

2.3.2. Quantification and detection limits (Sensitivity)

The limit of quantification (LOQ) was10 ng/ml.

2.3.3. Precision and Accuracy

The precision and accuracy results of the four concentrations tested are provided in Table 3. Both the precision and accuracy results were within the acceptable criteria ranges set by the method validation parameters in Point 2.2.3. Throughout these four concentrations, the %RSD for the

intra-batch precision was below 7%. The accuracy of all concentration levels for melatonin was between 99.30% and 106.06%.

Table 3.Accuracy and precision results of the HPLC-ECD method			
Analyte Concentration (ng/ml)	**Intra-batch (n=5)**		
	Measured concentration (ng/ml) (mean±SD)	**Precision (%RSD)**	**Accuracy (%)**
Melatonin			
10 (LOQ)	10.09 ± 0.68	6.75	100.89
50	50.15 ± 2.70	5.38	100.30
100	100.06 ± 6.21	6.21	106.06
500	496.51 ± 6.49	1.31	99.30

Note: Intra-batch is where all the standard concentrations were prepared from one standard stock solution and analysed.

2.3.4. Recovery

This parameter must still be validated.

2.3.5. Stability

This parameter must still be validated.

2.4. Chromatographic results

Table 5 present the relative retention times of all the analytes and the internal standard for this method.

Table 5: The relative retention times of the melatonin and the internal standard are presented	
Analytes	**Relative retention times (minutes)**
1. Melatonin (HPLC-ECD method)	6.59 ± 0.5

The following chromatographic figure are representative of a 10ng/ml standard (Figure 3) done with the HPLC-ECD method.

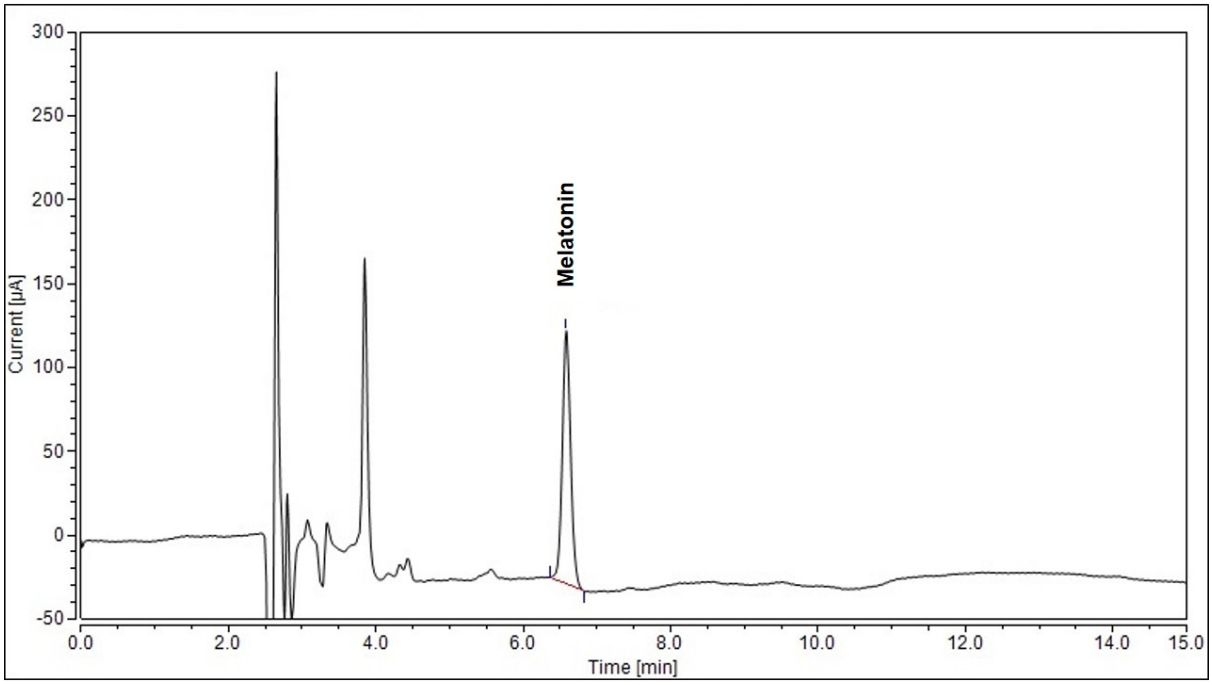

Figure 3: 10ng/ml Standard monitored at +600 mV with the HPLC-ECD method.

2.5. Application

The method must still be applied to a range of animal plasma samples.

The following chromatographic figure represents the plasma liquid-liquid extraction results.

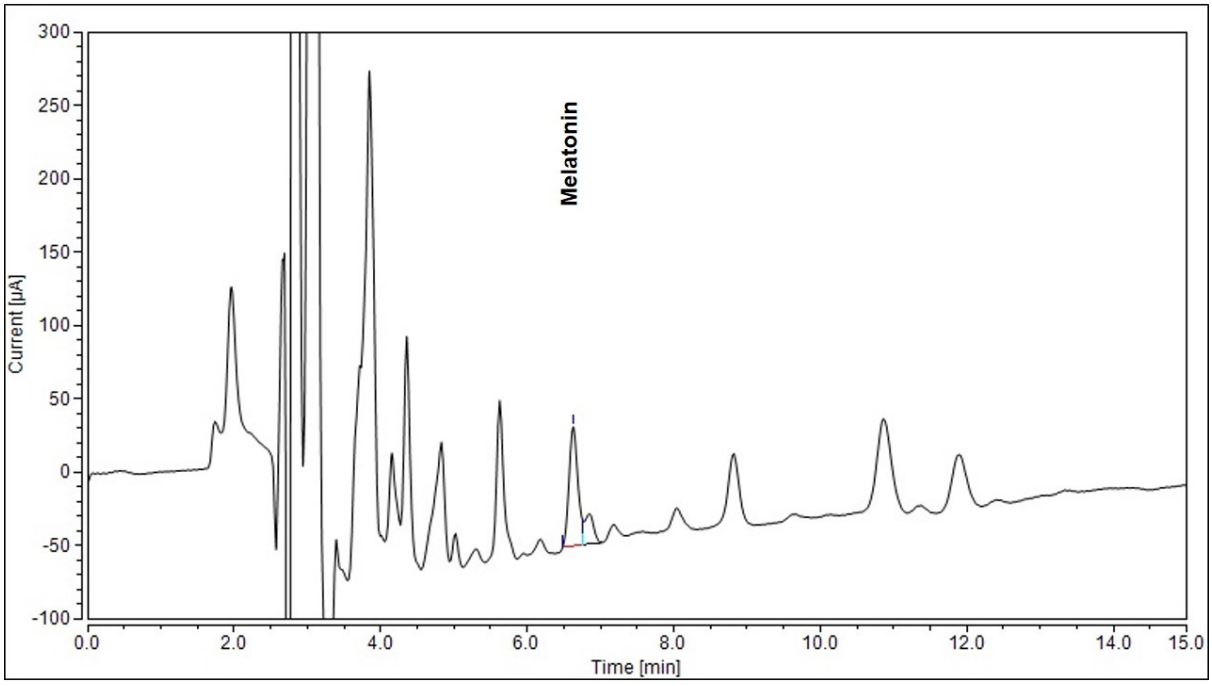

Figure 4: Rhinoceros plasma sampled at 6 hours during transport and monitored at +600 mV with the HPLC-ECD method.

3. Discussion

A specific HPLC, coupled to a coulometric electrochemical detector combined with liquid-liquid extraction method, was developed quantitative analysis of melatonin in plasma samples. The linearity for melatonin calibration range was excellent and the coefficient of regression (R^2) was above 0.95. The LOQ was 10.0 ng/ml for the method.

The finished developed and validated method will be compared to literature and discussed, before submitting it for publication.

4. Experimental

4.1. Chemicals, reagents, materials and instrumentation

4.1.1. Chemicals and reagents

Melatonin, Cortisol (hydrocortisone), Corticosterone and Dexamethasone (internal standard) were obtained from Sigma-Aldrich Pty (Ltd) (Johannesburg, South Africa). Chemicals used for the mobile phase were HPLC grade deionised water, HPLC grade methanol (MeOH), HPLC grade acetonitrile (ACN) and ortho-phosphoric acid. Chemicals used for the sample preparation were sodium chloride, potassium chloride, disodium phosphate, monopotassium phosphate, hydrochloric acid, ethyl acetate, di-sodium hydrogen phosphate and ethylenediaminetetraacetic acid disodium salt. All the chemicals were obtained from Merck (Pty) Ltd (Johannesburg, South Africa).

4.1.2. Materials

The analytical HPLC columns used was a Venusil ASB C_8, 4.6 x 250 mm, a particle size of 5 µm, pore size of 150 Å and a surface area of 200 m^2/g (purchased from Bonna-Agela Technologies, USA).

The developed and validated method was applied to prepared white rhinoceros (Ceratotherium simum) plasma collected in Lithium-heparin blood collection tubes and Sprague-Dawley rat plasma collected in EDTA blood collection tubes. The blood samples were immediately centrifuged after collection to separate the plasma from the blood components. The plasma was transferred to Eppendorf tubes and immediately snap frozen in liquid nitrogen, transferred in dry ice and stored in a -80°C freezer until day of analysis.

4.1.3. Instrumentation

The chromatographic system for the HPLC-ECD method, consisted of an Ultimate 3000 UHPLC system, equipped with an ISO-3100SD isocratic pump and WPS-3000TSL analytical autosampler, coupled to an ECD-3000RS rapid separation electrochemical detector with 2-Channel Coulometric Cell 6011RS and Chromeleon® chromatography management system version 7.2 (all obtained from Thermo Fisher Scientific, Waltham, MA USA).

Conflict of interest

The authors have no conflict of interest to declare.

Ethics approval

Ethical approval for this project to use animal (rat and white rhinoceros) plasma was obtained from the animal care, health and safety in research ethics committee (AnimCare) of the Faculty of Health Sciences, from the North-West University (NWU-00252-17-A5), as well as from the Animal Ethics Committee from the University of Pretoria (V067-17).

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ADDENDUM B CONFORMATION OF ALL ETHICAL APPROVAL

Ethics approval letter from the animal care, health and safety in research ethics committee (AnimCare) of the Faculty of Health Sciences, of the North-West University.

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Faculty of Health Sciences
Ethics Office for Research, Training and Support
Animal Care, Health and Safety in Research
Ethics Committee (AnimCare)

Tel: 018 299 2234
Fax: 018 299 2225
Email: Tiaan.Brink@nwu.ac.za

17 February 2017

Dear Prof JL Du Preez

APPROVAL OF YOUR APPLICATION BY THE ANIMAL CARE, HEALTH AND SAFETY IN RESEARCH ETHICS COMMITTEE (ANIMCARE) OF THE FACULTY OF HEALTH SCIENCES

Ethics Number: NWU-00252-17-A5

Kindly use the ethics reference number provided above in all correspondence or documents submitted to the AnimCare secretariat.

Study Title:	The detection and quantification of biomolecules in biological sample matrices
Study leader/Supervisor:	Prof. Jan L Du Preez
Student:	Mr. Francois P Viljoen
Application type:	New Application - Category 0

Project Category *(impact on animal wellbeing)*	**Not applicable**	**0**	**1**	**2**	**3**	**4**	**5**
		X					

The abovementioned application has been through the expedited review process and discussed by the AnimCare, Animal Research Ethics Committee Potchefstroom Campus, North-West University, Potchefstroom.

The commencement date for this study is **17 February 2017**. Continuation of the study is dependent on receipt of the annual (or as otherwise stipulated) monitoring report and the concomitant issuing of a letter of continuation up to a maximum period of three years when extension will be facilitated during the monitoring process.

After ethical review

The AnimCare, Faculty of Health Sciences requires immediate reporting of any aspects that warrants a change of ethical approval. Any amendments, extensions or other modifications to the proposal or other associated documentation must be submitted to the AnimCare, Faculty of Health Sciences prior to implementing these changes. Any adverse/unexpected/unforeseen events or incidents must be reported on either an adverse event report form or incident report form sent to Ethics-AnimCareIncident-SAE@nwu.ac.za

A monitoring report should be submitted within one year of approval of this study (or as otherwise stipulated) and before the year has expired, to ensure timely renewal of the study. A final report must be provided at completion of the study or the AnimCare committee, Faculty of Health Sciences must be notified if the study

is temporarily suspended or terminated. The monitoring report template is obtainable from the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-Monitoring@nwu.ac.za.

The AnimCare, Faculty of Health Sciences has the authority and responsibility to initially approve and subsequently monitor animal activities to confirm on-going compliance with and adherence to the approved protocol in terms of section 5.2.7 of the SANS 10386:2008.The AnimCare, Faculty of Health Sciences reserves the right to visit sites where approved protocols will be conducted and any animal housing facility under the authority of NWU as often as it deem necessary either announced or unannounced.

Please note that for any permits/permission must still be obtained from relevant authorities and provided to the AnimCare, Faculty of Health Sciences. Ethics approval is required BEFORE approval can be obtained from these authorities.

The AnimCare Committee, Faculty of Health Sciences complies with the South African National Health Act 61 (2003), the Regulations on Research with Human Participants (2014), the Ethics in Health Research: Principles, Structures and Processes (2015), the SANS 10386:2008 document, the Belmont Report and the Declaration of Helsinki (2013).

We wish you the best as you conduct your research. If you have any questions or need further assistance, please contact the Faculty of Health Sciences Ethics Office for Research, Training and Support at Ethics-AnimCare@nwu.ac.za.

Yours sincerely

Prof Christiaan B Brink
Chair: AnimCare

Prof Minrie Greeff
Head: Ethics Office

ADDENDUM B CONFORMATION OF ALL ETHICAL APPROVAL

Ethics approval letter from the Animal Ethics Committee of the University of Pretoria.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Animal Ethics Committee

PROJECT TITLE	**Pharmacological management of stress and its pathophysiological consequences during the transport of the free-ranging white rhinos *(Ceratotherium simum)***
PROJECT NUMBER	**V067-17**
RESEARCHER/PRINCIPAL INVESTIGATOR	**F Pohlin**

STUDENT NUMBER (where applicable)	**U_17310441**
DISSERTATION/THESIS SUBMITTED FOR	**PhD**

ANIMAL SPECIES	**White rhinos *(Ceratotherium simum)***	**White rhinos (Ceratotherium simum)**
NUMBER OF SAMPLES	**3-6 (Pilot study)**	**50 Experiment**
Approval period to use animals for research/testing purposes		**June 2017- June 2018**
SUPERVISOR	**Prof. L Meyer**	

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s - please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

APPROVED	Date 26 June 2017
CHAIRMAN: UP Animal Ethics Committee	Signature

S4285-15

ADDENDUM C CONFORMATION OF MANUSCRIPT ACCEPTANCE.

Conformation of all the three articles submitted to the international accredited journal, *Die Pharmazie* (http://pharmazie.govi.de/).

Die Pharmazie

An International Journal of Pharmaceutical Sciences

Online submission / Tracking area

Check manuscript status

Ref. Nr.	Manuscript title	PDF as submitted	Submission date	Current status
P 8225	An HPLC-DAD validated method for the detection and quantification of cortisol, corticosterone and melatonin in plasma samples of two different animal species	manuscript13238.pdf	23.11.2018 09:39:10	Finally accepted

Show details Show correspondence

Ref. Nr.	Manuscript title	PDF as submitted	Submission date	Current status
P 8218	Development and validation of a HPLC electrochemical detection method to measure COMT activity as a tool in drug development	manuscript13207.pdf	14.11.2018 11:13:18	Finally accepted

Show details Show correspondence

Ref. Nr.	Manuscript title	PDF as submitted	Submission date	Current status
P 8099.3	Method development and validation for HPLC electrochemical detection and quantification of monoamines and their metabolites in rat brain tissue samples	manuscript12726.pdf	04.07.2018 17:34:15	Scheduled for publication Issue: October 2018

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ORIGINAL ARTICLES

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HPLC electrochemical detection and quantification of monoamines and their metabolites in rat brain tissue samples

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The monitoring of monoamines and their metabolites in CNS samples can be very valuable in pharmaceutical and biomedical research. A specific high performance liquid chromatography, coupled to a coulometric electrochemical detection method, for the assay of monoamines (dopamine, norepinephrine, epinephrine and serotonin) and their metabolites in rat brain tissue samples was developed. The chromatographic separation was achieved on a C8 reversed phase column with a mobile phase consisting of 0.1 M sodium formate buffer, 5 mM sodium 1-heptanesulfonate, 0.17 mM ethylenediaminetetraacetic acid disodium salt and 5% v/v acetonitrile (pH ±4.0). The detection was achieved through electrochemical detection, with a coulometric cell potential setting of +650 mV. The flow-rate was at 1 ml/min and the total run time was 50 min. The method was validated according to validation guidelines. The method was found to be linear ($R^2 > 0.99$) over the analytical range (5 to 200 ng/ml) for all monoamines and their metabolites. All the other validation parameters were acceptable and within range. The method was applied to three rat brain areas (pre-frontal cortex, hippocampus and striatum), where the monoamines (except for epinephrine) and their metabolites were easily detected.

1. Introduction

In the central nervous system (CNS), monoamines are an important group of biogenic amines, which include dopamine, noradrenaline, epinephrine, serotonin and their metabolites (Raven and Johnson 2002; Koolman et al. 2005; Noback et al. 2005; Burtis et al. 2012). Their main function is neurotransmission by means of neuronal or hormonal signals in a variety of physiological processes (Burtis et al. 2012). The imbalances and dysfunction of monoamines are associated with a variety of CNS disorders (Booij et al. 2003; Kurian et al. 2011; Ng et al. 2015). These monoamines are mainly synthesised from two amino acids, tyrosine and tryptophan (Koolman et al. 2005; Burtis et al. 2012). The metabolic pathways of the monoamines are shown in a simplified diagram in Fig. 1.

The objective of this study was to develop a high performance liquid chromatography (HPLC) method to detect and quantify the monoamines in CNS samples, more specific rat brain tissue samples. The method was therefore developed and validated for the detection and quantification of dopamine and its metabolites (3,4-dihydroxyphenylacetic acid, 3-methoxytyramine and homovanillic acid), norepinephrine (noradrenaline) and its metabolite (3-methoxy-4-hydroxyphenylglycol), epinephrine (adrenaline) and serotonin and its metabolite (5-hydroxyindoleacetic acid).

2. Investigations and results

2.1. Method validation parameters

The method was validated according to the guidelines of the FDA concerning linearity, sensitivity (quantification and detection limits), precision (reproducibility), accuracy (repeatability), recovery and stability (US FDA 2013). Selectivity was performed by injecting samples of solution A without any standards or brain tissue. Surrogate matrices (for example artificial cerebrospinal fluid) were used instead of the authentic sample matrix as it is free of monoamines (van de Merbel 2008).

2.1.1. Linearity/calibration curve

Linearity was tested by preparing eight standard concentrations as described in section 4.2.2. Six replicates of each standard were injected to establish linear regression for each analyte. The linear regression value (coefficient of determination, r^2) for the calibration curve must not be less than 0.95 for endogenous biomolecules (Shabir 2005). For calibration, the internal standard method was used with 5-HMT as the chosen internal standard.

2.1.2. Quantification and detection limits (sensitivity)

The lower limit of quantification (LLOQ) and the lower limit of detection (LLOD) can be defined as the minimum concentration where the signal-to-noise ratio is at least 10:1 and 3:1 greater than the average background noise of an unspiked blank (only containing the internal standard), at the retention time of each analyte, respectively (Shrivastava and Gupta 2011).

2.1.3. Precision and accuracy

Four concentrations (5, 10, 75 and 200 ng/ml) were chosen and six determinants for each were done for precision and accuracy. Precision results were expressed in %RSD (relative standard deviation from the mean) and the acceptability criterion for each concentration level was not to exceed 15% (US FDA 2013). The accuracy results for each concentration level were determined by comparing the closeness of the mean test concentration result to that of the true concentration value. The accuracy results were expressed as % recovery. The acceptability criterion for accuracy for each concentration level was to fall between 90 to 110% (US FDA 2013; Shabir 2005).

2.1.4. Recovery

The percentage recovery of the extraction procedure was determined by preparing four spiked concentrations (5, 10, 75 and 200

ADDENDUM D CONFORMATION OF LANGUAGE PROOFREADING, EDITING AND CHECKED FOR PLAGIARISM

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