QUANTIFICATION OF 3-METHOXY-4-HYDROXYPHENYLGLYCOL IN HUMAN SALIVA BY AN OPTIMISED HPLC METHOD WITH ELECTROCHEMICAL DETECTION

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“The fear of the Lord is the start of knowledge: but the foolish have no use for wisdom and teaching.”
Proverbs 1:7 (Bible in basic English)

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

“The more I study nature, the more I stand amazed at the work of the Creator.”
Louis Pasteur.

Statement Regarding Independent Work

I, **Francois Petrus Viljoen**, identity number: [redacted] and CUT student number: **9300147**, hereby declare that all work done in this research project, submitted to the Central University of Technology, Free State, for the degree MAGISTER TECHNOLOGIAE: BIOMEDICAL TECHNOLOGY, is my own independent work. It complies with the Code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the University and has not been submitted before to any other institution by myself or any other person in fulfilment (or partial fulfilment) of the requirements for the attainment of any qualification.

_____ _____

Signature of student **Date**

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Above all I would like to thank the Lord Almighty for giving me the opportunity, ability, strength, wisdom and courage to accomplish this milestone in my life.

I want to dedicate this work to my Father and Mother who already passed away, for the great parents they were and for the good life they had given me when they were still alive.

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Abstract

One of the greatest challenges in the field of biomedical technology, is to find new techniques to detect, diagnose and interpret complex human disease conditions. The need to analyze or measure endogenous biological markers related to different diseases has stimulated the continuous search for more effective and sensitive analytical methods which will eventually also contribute to better therapy.

The development of reliable bio-analytical methods is of utmost importance for the total care of a patient and if such a method can be developed to aid in the diagnosis of especially psychiatric conditions (e.g. stress-related disorders, depression etc.) the benefits will be incalculable.

In the central nervous system catecholamines are naturally occurring chemicals called neurotransmitters and in the blood they are hormones secreted by the adrenal medullae. They are very important for proper brain and body function because they relay signals between nerve cells. Norepinephrine is one of the main catecholamines and 3-methoxy-4-hydroxyphenylglycol (MHPG) is one of its metabolites. Several studies from literature have confirmed that analysis of MHPG in saliva may be a biological marker for detecting changes in the central and peripheral catecholamine metabolism while also reflecting some aspects of mental functioning in humans.

The current study was undertaken as part of a target population comparative study which commenced in February 2008 and which main aim was to investigate biological markers associated with higher sympathetic nervous system activity in urbanised teachers with a specific coping style, viz. the SABPA project (Sympathetic activity and Ambulatory Blood Pressure in Africans).

The design and methodology was to optimise an existing high performance liquid chromatography electrochemical detection method for the quantification of catecholamines and their metabolites in rat brain tissue for the quantification of MHPG in human saliva. The new optimised method was validated and applied to human saliva samples.

The major changes that were made for the new optimised method were the following: the organic part of the mobile phase was changed from 10% to 4% acetonitrile. The pH of the mobile phase was changed from pH 3.2 to pH 4.1. A longer column was used and the flowrate was changed from 1.0 ml/min to 0.85 ml/min. The injection volume was changed from 100 µl to 5 µl. The sample preparation for the saliva samples was also different from the rat brain tissue sample preparation.

The key findings were that the new method was sensitive and specific enough for the quantification of MHPG in human saliva samples. The validation parameters showed that the method was sensitive, specific and reliable. The method could detect MHPG levels in human saliva from as low as 3.85 ng/ml and with a lower limit of detection of 1 ng/ml. The regression value for the linearity curve was r^2 = 0.998. The accuracy and precision results gave percentage relative standard deviation values less than 15%. The % recovery results were all above 80 %, which is good for the analysis of endogenous compounds in biological samples.

The recommendations include that in order to increase the sensitivity and selectivity of the method even more, the amperometric electrochemical detector can be replaced with a coulometric electrochemical detector or a coularray electrochemical detector. The rest of the unknown peaks in the saliva samples should be identified, because it may broaden the range of endogenous compounds that may be analysed through this method.

Opsomming

Een van die grootste uitdagings in biomediese tegnologie is om nuwe tegnieke te vind vir die deteksie, diagnose en die interpretasie van komplekse menslike siektetoestande. Die behoefte vir die analise van endogene biologiese merkers van verskillende siektetoestande stimuleer die voortdurende soeke na meer effektiewe en sensitiewe analitiese metodes wat uiteindelik tot beter behandeling sal bydra.

Die ontwikkeling van betroubare bio-analitiese metodes is van uiterste belang vir die totale sorg van 'n pasiënt en as so 'n metode ontwikkel ter stawing van die diagnose van veral psigiatriese toestande (bv. stress-verwante siektetoestande, depressie ens.) kan word, sal die voordele onberekenbaar wees.

In die sentrale senustelsel kom katesjolamiene as natuurlike chemiese middels genaamd neurotransmitters voor en in die bloed funksioneer hulle as hulle hormone wat deur die adrenale medulla vrygestel word. Hulle is belangrik vir die goeie funksionering van die brein en liggaam want hulle herlei seine tussen senuselle. Norepinefrien is een van die primêre katesjolamiene en 3-metoksi-4-hydroksifenielglikol (MHPG) is een van die metaboliete daarvan. Verskeie studies het die belang van MHPG in spesiaal as 'n biologiese merker vir veranderinge in die sentrale en perifere katesjolamienmetabolisme ondersteun, terwyl dit van sommige aspekte van verstandelike funksionering in die mens kan weerspieël.

Hierdie studie, wat in Februarie 2008 begin het, is onderneem as deel van 'n vergelykende studie, met 'n teikenpopulasie waarvan die hoofdoel was om die biologiese merkers van hoër aktiwiteit van die simpatiese senustelsel in verstedelikte onderwysers met 'n spesifieke oorlewingstyl te ondersoek (nl. die SABPA-projek).

Die hoofdoelwit was die optimalisering van ‘n bestaande hoëverrigting vloeistof-chromatografiese elektrochemiese deteksiemetode om MHPG in menslike speeksel monsters te kwantifiseer.

Die ontwerp en metodiek was die optimalisering van ‘n bestaande hoë verrigting vloeistofchromatografiese elektrochemiese deteksiemetode vir die kwantifisering van katesjolamiene en hul metaboliete in rotbreinweefsel vir die kwantifisering van MHPG in menslike speeksel. Dit het ook die validering van die nuwe geoptimaliseerde metode en die toepassing van die metode op menslike speekselmonsters behels.

Die belangrikste veranderinge wat vir die nuwe geoptimaliseerde metode gemaak is, was die volgende: die organiese gedeelte van die mobiele fase is van 10% na 4% asetonitriel verander. Die pH van die mobiele fase is vanaf pH 3.2 na pH 4.1 aangepas. ‘n Langer kolom is gebruik en die vloeisnelheid is van 1.0 ml/min na 0.85 ml/min verander. Die inspuitingsvolume is vanaf 100 µl na 5 µl verander. Die voorbereiding van die speekselmonsters was ook anders as dié van die rotbreinweefsel.

Die sleutelbevindinge was dat die nuwe metode sensitief en spesifiek genoeg vir die kwantifisering van MHPG in menslike speekselmonsters is. Die validasieparameters het getoon dat die metode sensitief, spesifiek en betroubaar is. Die metode kon MHPG-vlakke in menslike speeksel tot so laag as 3.85 ng/ml met ‘n ondergrens van 1 ng/ml bepaal. Die regressiewaarde van die lineêre kurwe was r^2 = 0.998. Die akkuraatheids- en presisieresultate het ‘n relatiewe persentasie standaard afwyking van minder as 15% gegee. Die persentasie herwinning was meer as 80%, wat goed is vir die analise van endogene komponente in biologiese monsters.

Die aanbevelings is onder meer dat die metode se sensitiwiteit en selektiwiteit verhoog kan word deur die amperometriese elektrochemiese detektor met ‘n coulometriese elektrochemiese detektor of ‘n CoulArray® elektrochemiese detektor te vervang. Die ander onbekende pieke in die speekselmonsters kan geïndentifiseer word, want dit kan die reeks endogene komponente vergroot wat deur die metode ontleed kan word.

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List of Abbreviations

aCSF	Artificial cerebrospinal fluid
AD	Aldehyde dehydrogenase
ADH	Alcohol dehydrogenase
AECD	Amperometric electrochemical detection
AR	Aldehyde reductase
ATL	Analytical Technology Laboratory
$D_{\beta}H$	Dopamine-β-hydroxylase
DHBA	3,4-Dihydroxy-benzylamine
CNS	Central nervous system
COMT	Catechol-O-methyltransferase
CSF	Cerebrospinal fluid
DA	Dopamine
DOPAC	3,4-dihydroxyphenylacetic acid
E	Cell potential
ECD	Electrochemical detection
EGTA	Ethylene glycol-bis(β-aminoethylether)-N,N',N'-tetraacetic acid
Epi	Epinephrine (adrenaline)
FDA	Food and Drug Administration
GCMS	Gas chromatography mass spectrometry
gs (g)	Gravitational acceleration

HCl	Hydrochloric acid
$HClO_4$	Perchloric acid
HPLC	High performance liquid chromatography
H_3PO_4	Orthophosphoric acid
I.Std	Internal standard
LAMB	Laboratory for Applied Molecular Biology
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
HVA	Homovanillic acid
M	Molar
MAO	Monoamine oxidase
ml	Milliliter
ml/min	Millilitre per minute
MN	Metanephrine
MHPG	3-Methoxy-4-hydroxyphenylglycol
mV	Millivolt
n	Quantity
Na_2EDTA	Ethylenediaminetetra-acetic acid disodium salt
NE	Norepinephrine (noradrenaline)
ng/g	Nanograms per gram
ng/ml	Nanograms per millilitre
NMN	Normetanephrine
pA	Picoampere
PNMT	Phenylethanolamine N-methyltransferase

r^2	Regression
rpm	Revolutions per minute
% RSD	Percentage relative standard deviation
SABPA	**S**ympathetic activity and **A**mbulatory **B**lood **P**ressure in **A**fricans
µl	Micro litre
VMA	Vanillylmandelic acid

Chapter 1
Introduction

One of the greatest challenges in the field of biomedical technology is to find new techniques to detect, diagnose and interpret complex human disease conditions. The need to analyse or measure endogenous biological markers related to different diseases has stimulated the continuous search for more effective and sensitive analytical methods which will eventually also contribute to better therapy.

The development of reliable bio-analytical methods is of utmost importance for the total care of a patient and if such a method can be developed to aid in the diagnosis of especially psychiatric conditions (e.g. stress-related disorders, depression, etc.) the benefits will be incalculable.

The current study was undertaken as part of a target population comparative study that commenced in February 2008 and whose main aim was to investigate biological markers associated with higher sympathetic nervous system activity in urbanised teachers with a specific coping style, *viz.* the SABPA (Sympathetic activity and Ambulatory Blood Pressure in Africans) project (see appendix B: Information and consent form and Ethics approval certificate of the SABPA project).

The search for a non-invasive method to detect biological markers associated with higher sympathetic nervous system activity in humans is a major problem, especially when it comes to stress and stress-related disorders.

Everly and Lating (2002) stated: "If we cannot reliably and validly measure the human stress response, what degree of credibility do we place upon investigations into its phenomenology?"

In order to get an indication of the levels of biological markers associated with stress-related disorders, viz. the catecholamines, a few methods are described in the literature to quantify these substances and their metabolites (Okumura *et al.*, 1997; Yang *et al.*, 1997; Yamada *et al.*, 2000). Most of these methods however involve invasive sampling procedures (plasma, CSF) or urine samples. While some of the catecholamines and their metabolites can be detected in saliva (Yamada *et al.*, 2000; Kennedy *et al.*, 2001), we aimed to develop a novel bio-analytical method to quantify the levels of the primary catecholamine metabolite in saliva, *viz.* 3-methoxy-4-hydroxyphenylglycol (MHPG).

AIM

The aim of the current study was therefore to develop or optimise an existing method to quantify 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in human saliva.

While we have a validated and reliable method in place in our laboratory for the determination of catecholamines and metabolites in brain and cerebrospinal fluid (Coetzee *et al.*, 2006; Harvey *et al.*, 2006; Marais *et al.*, 2006), we hypothesised that this method can be modified and optimised to be sensitive and specific to determine 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in human saliva samples.

OBJECTIVES

The objectives of the current study include the following:

1. To optimise an existing HPLC electrochemical detection method for the quantification of catecholamines and their metabolites in rat brain tissue for the quantification of 3-methoxy-4-hydroxyphenylglycol (MHPG) in human saliva.
2. To validate the new optimised method according to point 2.9 in chapter 2.
3. To apply this validated method to human saliva samples.

RELEVANCE

This new method can be utilised as a screening procedure to monitor the body's reaction in response to sympathetic activity and as such it may be of value to determine the body's response to stress and aid as a diagnostic tool for stress-related disorders. Pharmacologically, it may be of value in the search for novel and more effective treatment regimens for stress and stress related disorders such as anxiety, post-traumatic-stress-disorder and depression.

RESEARCH LAYOUT

The study was divided into three phases:

- Optimising the existing HPLC electrochemical detection method.
- Validation of the new method in saliva.
- Application of the validated method to human saliva samples obtained in the SABPA project.

In order to achieve the aims of the study, an HPLC coupled to an electrochemical detector was used throughout the study. Human saliva samples collected from participants in the SABPA project were finally used to evaluate the applicability of the new method. Not all the collected samples were analysed in the current study, but only five samples representative of the group.

A thorough discussion of the available and relevant literature regarding analysis of catecholamines, especially the metabolite MHPG in biological samples with the focus on saliva, as well as current methods available to quantify MHPG in this matrix is given in the following chapter. In chapter 3 the research methodology followed in this study is described in detail, while discussion of the results and conclusions from the study is given in chapters 4 and 5. This dissertation is concluded with two appendices in which appendix A consists of a summary on troubleshooting using HPLC with

electrochemical detection and appendix B includes information on the SABPA project and the Ethics approval certificate.

Chapter 2 Literature Review

In medical science there is a considerable need for new and better analytical methods to detect and quantify endogenous biomarkers in biological samples in order to diagnose and treat human disease states. There are a few important key aspects when a new method is developed or a current method is optimised. Knowledge of the endogenous biomarker, the biological sample matrix, the analytical instrument, previous developed methods, sample collection, sample preparation and previous results are essential. The validation of the new analytical method is also very important. The focus of the current study was on the development and optimisation of a method for the determination of MHPG (an important catecholamine metabolite) in saliva.

2.1 3-Methoxy-4-hydroxyphenylglycol (MHPG)

In the central nervous system (CNS) catecholamines are naturally occurring chemicals called neurotransmitters and in the blood they are hormones secreted by the adrenal medullae. They are very important for proper brain and body function because they relay signals between nerve cells. These neurochemicals play an important role in a person's mental, physical and emotional status. They also affect functions like sleep, behaviour, mood, digestion, weight, focus and learning ability. When there are imbalances of these neurochemicals it can usually be traced to stress or a poor diet. Genetics and environmental toxins can also cause imbalances. When a patient has an imbalance it will usually involve more than one neurotransmitter (Marcantel, 2008).

The 3 main catecholamines with their metabolites are dopamine (DA, metabolites: DOPAC, HVA), norepinephrine (NE) and epinephrine (Epi). 3-

Methoxy-4-hydroxyphenylglycol (MHPG, MOPEG) is a major metabolite of the degradation of NE in the CNS. The aldehyde intermediate DHPG (3,4-dihydroxyphenylglycol), produced by the action of the enzyme monoamine oxidase (MAO) on norepinephrine is reduced to a glycol with the aid of the enzyme, catechol-O-methyltransferase (COMT).

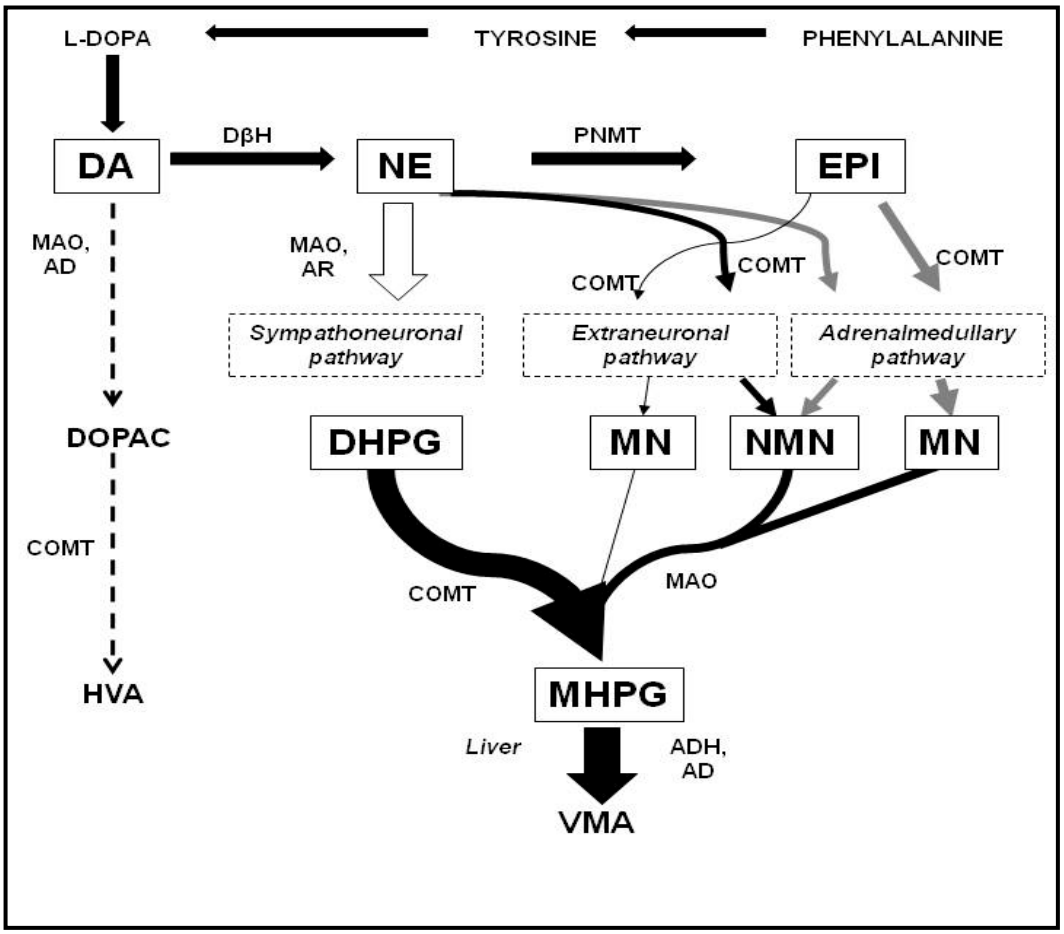

Figure 1 : Synthesis of MHPG (Degradation of NE). Catecholamines: DA (dopamine), NE (norepinephrine), EPI (epinephrine), Metabolites: Dopac (3,4-dihydroxyphenylacetic acid), HVA (homovanillic acid) , DHPG (3,4-dihydroxyphenylglycol), MN (metanephrine), NMN (normetanephrine), MHPG (3 -methoxy-4-hydroxyphenylglycol), VMA (vanillylmandelic acid), Enzymes and co-factors: DβH (dopamine-β-hydroxylase), PNMT (Phenylethanolamine N -methyltransferase), MAO (monoamine oxidase), COMT (catechol-O-methyltransferase), AD (aldehyde dehydrogenase), AR (aldehyde reductase), ADH (alcohol dehydrogenase) (Eisenhofer *et al* ., 2004).

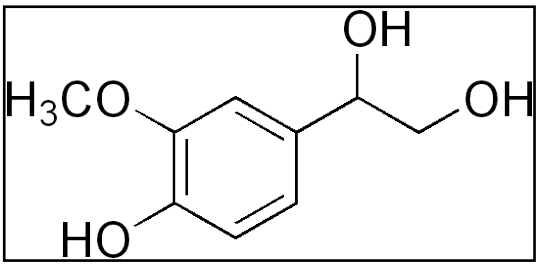

Figure 2: MHPG molecule.

Catecholamines and their metabolites are classified as either basic or acidic, but MHPG is a neutral molecule (molar mass of 184.19). It is readily released into the bloodstream and cerebrospinal fluid (CSF) and therefore a blood or a CSF sample may be an indication of recent sympathetic nervous system activity (Cooper *et al.,* 2003*;* Li *et al.*, 2006).

In human blood, catecholamines and their metabolites are water-soluble hormones. They are dissolved in the plasma and transported from their sites of synthesis to target tissues throughout the body. Here they diffuse out of the blood capillaries, into the interstitial fluid, and ultimately into the target cells (Guyton *et al.*, 2005).

Hormones that are commonly measured in plasma are for example steroids, non-steroids, peptides and protein hormones but they can also be detected in saliva. The existence of a connection between the circulating hormone levels and the hormone levels in saliva must first be defined before any significance can be attached to the measured hormone level in the saliva. The information of the origin of the salivary hormone allows for a definition of its role and connection with pathological and physiological states (Chiappin *et al.*, 2007).

The correlation of MHPG levels in the different bodily fluids, the blood (plasma) and saliva is very important and may give valuable information about the sympathetic activity in the body as well as possible pathological states. Mitoma *(*2008) and coworkers reported on a variety of studies that connected increased MHPG plasma levels with psychological stress, depressed patients and the state of anxiety of a patient. They also found in

their own study that subjects with a greater psychological job stress had higher plasma MHPG levels than subjects who suffered less from psychological job stress.

All three major catecholamines can be detected in saliva with levels varying from 250 up to 800 pg/ml, but their origin is still largely unknown. Part of salivary catecholamines seems to originate from plasma *via* diffusion and part of it is derived from sympathetic nervous terminals *via* direct release. Due to uncertainty of their origin, concentrations of the major catecholamines in saliva are usually poorly correlated with catecholamine levels in plasma (Chiappin *et al.*, 2007).

Previous studies did, however, find good correlations between plasma and saliva catecholamine metabolite levels, especially MHPG (Yang *et al.*, 1997; Yamauchi *et al*., 2001). Yamauchi and co-workers reported on a number of studies in which the MHPG level in saliva correlated significantly with its level in plasma, while other studies confirmed that catecholamine metabolites of NE in saliva mainly result from diffusion from the bloodstream into the saliva. Strong correlations have also been reported for levels of the NE metabolite MHPG between plasma and saliva, with the total levels not higher in the saliva than in the plasma. MHPG levels in saliva may therefore be used as a useful and non-invasive biomarker to detect changes in the central and peripheral catecholamine metabolism (Yamada *et al*., 2000; Kennedy *et al.*, 2001; Yamauchi *et al.*, 2001).

Yamada and co-workers reported in 2000: "The concentration of saliva MHPG seems to be regulated by a balance between peripheral sympathetic (noradrenergic which supplies MHPG to saliva) and parasympathetic (cholinergic which regulates the volume of saliva) nerve activities. Thus, the greater changes in saliva MHPG level response to anxiety or stress could be detected in comparison with those in plasma MHPG level, because anxiety or stress should reduce the volume of saliva, resulting in a robust increase in the concentration of saliva MHPG". Therefore the MHPG levels detected in saliva appeared to be non-specific and representative of the anxiety state in humans (Yamada *et al.*, 2000).

MHPG concentrations in both plasma and CSF are frequently used to assess noradrenergic metabolism in clinical studies (Reuster *et al*., 2002). Recent studies have shown that MHPG can be reproducibly assayed in saliva. While the brain contributes about 30% of plasma MHPG and furthermore 50% of plasma MHPG is converted to VMA, saliva MHPG ultimately reflects both plasma MHPG levels and local MHPG synthesis (Reuster *et al*., 2002). Significant correlations were found between plasma and saliva MHPG concentrations, further confirming results of previous studies that salivary MHPG can reflect plasma MHPG levels (Reuster *et al*., 2002). These results provide grounds to assume that salivary MHPG can indeed also reflect CSF MHPG levels and enhance the utility of salivary MHPG measurements as a non-invasive means for investigating the noradrenergic system in a clinical setting (Reuster *et al*., 2002).

Several studies in which the potential clinical benefits of measuring MHPG levels in plasma, saliva and CSF were investigated as indicators of brain noradrenergic activity related to drug therapy and neuropsychiatric disorders have been reported (Reuster *et al*., 2002). Li *et al*. (2006) reported e.g. that while the level of MHPG in plasma reflects noradrenergic neuronal tone in humans the MHPG level in saliva correlates significantly with levels thereof in plasma and CSF and it may therefore be a useful marker for detecting changes in the central and peripheral catecholamine metabolism while also reflecting some aspects of mental functioning in humans.

Hormone quantities required to control most metabolic and endocrine functions in the body are incredibly small and in the blood their concentrations range from as little as 1 picogram (which is 1×10^{-12}) up to at most a few micrograms (1×10^{-6}) per millilitre of blood. The secretion rates of the various hormones are extremely small, and are usually measured in micrograms or milligrams per day (Guyton *et al*., 2005).

2.2 The sample matrix - Saliva

Analytical studies done on saliva are not novel, but the physiological importance of saliva as a sample matrix has only been recognized lately. The pace of salivary research has accelerated in the past 50 years because of new techniques that illuminate the biochemical and physical-chemical properties of saliva (Schipper *et al.*, 2007). The finding that saliva was filled with hundreds of components that may serve to detect even more systemic diseases or evidence of exposure to various harmful substances, as well as providing biomarkers of health and disease status, caused the interest in saliva to increase even more. Nowadays, due to novel approaches that include metabolomics, genomics, proteonomics and bioinformatics the research field of saliva is rapidly advancing. But, due to its complex biochemical and physical-chemical properties and its inherent variability and instability, the usage of saliva as a research material may pose a few problems (Schipper *et al.*, 2007).

The assay of human saliva as a diagnostic tool has implications for both basic and clinical investigations and has provided an extra tool for identification of disease processes and disorders (Chiappin *et al.*, 2007).

The blood flow to salivary glands is increased by parasympathetic and peptidergic stimulation and therefore enhances saliva formation. The levels in saliva of compounds such as catecholamines and their metabolites derived from the blood can therefore provide a useful index of blood levels of the same compounds (Kennedy *et al.*, 2001).

There are quite a few advantages of using saliva rather than blood or urine as a diagnostic tool. This includes a simpler, non-invasive and less traumatic collection method than that of blood or urine, especially for infants, children and the elderly. There are also many circumstances in which the sampling of blood and urine is not possible (Chiappin *et al.*, 2007).

The oral fluid in humans is called saliva and it is an exocrine secretion with a pH range from 6.2 to 7.4, with the higher pH caused by an increased rate of secretion. Saliva consists mainly of water, a variety of electrolytes, mucus, proteins and enzymes (Höld *et al.*, 1995; de Almeida *et al.*, 2008). Human saliva production per day is from 1 to 1.5 litres. Its primary purpose is to aid in digestion of food, the protection and lubrication of the oral tissues, the dilution of certain substances (for example sugars) and the mechanical cleansing of the mouth (Höld *et al.*, 1995; de Almeida *et al.*, 2008). It possesses several functions involving homeostasis and maintaining oral health through an active protective role (Chiappin *et al.*, 2007). This oral fluid originates from mainly three pairs of major salivary glands (parotid, sublingual and submandibular) and also from a large number of minor salivary glands (Chiappin *et al.*, 2007).

The compositional analysis of saliva can be an important tool for physiology, pathology and for the diagnosis of oral and systemic diseases. There are a variety of inorganic, organic, non-protein, protein or polypeptide compounds in saliva, as well as hormones and lipid molecules (Chiappin *et al.*, 2007). The interrelationship between salivary hormones and the circulating ones, especially catecholamines (Chiappin *et al.*, 2007) emphasises the fact that saliva could therefore be an important diagnostic tool for research in a variety of medical research fields.

The composition and output of saliva depend on the autonomic nervous system's activity. The sympathetic system controls the serous part of the glands whereas both the parasympathetic and sympathetic systems control the mucous part (Chiappin *et al.*, 2007). The quantity, viscosity and ionic composition of saliva can be modified by pharmacological and neural stimuli (α – β adrenergic and cholinergic) and the protein concentrations can vary as well (Chiappin *et al.*, 2007).

Upon parasympathetic stimulation, a high flow of saliva that contains low levels of organic and inorganic compounds results. Sympathetic stimulation produces saliva which is low in volume and has high concentrations of

protein and potassium. Food presence may affect the salivary composition in the mouth. Following food intake, selective protein release is stimulated thereby increasing the total amount of proteins and the enzyme α-amylase in saliva (Chiappin *et al.*, 2007).

There are primarily three processes involved in the clearance of compounds from plasma into saliva. The first is ultra filtration through the gap junctions in between cells of the secretory units (the intercellular nexus). The only molecules that will filter through are molecules with a molecular weight smaller than 1900 g/mol. The molecules involved here are water, ions, and hormones (such as catecholamines and steroids). The concentration of these molecules in the saliva is 300 to 3000 times lower than in plasma (Chiappin *et al.*, 2007). The second is transudation of the compounds in the plasma into the oral cavity and thirdly is through cellular membranes by means of selective transport (Chiappin *et al.*, 2007).

2.3 Analytical methods for catecholamine detection

There are several analytical methods described in literature to detect catecholamines and their metabolites in human saliva, *viz.* radio-immunoassay procedures, gas chromatography-mass spectrometry (GCMS) (Yamada *et al.*, 2000), high performance liquid chromatography (HPLC) coupled to a fluorescent or ultraviolet detector (Okumura *et al.*, 1997), and HPLC coupled to a electrochemical detector (ECD) (Yang *et al.*, 1997).

HPLC coupled to an electrochemical detector is the preferred analysis method, because it is more sensitive than ultra-violet or fluorescent detectors, and also much cheaper than GCMS and radio-immunoassay procedures. It is also a direct analysis method and no derivatisation procedures are needed.

The method which will be optimised in this particular study for analysis of salivary catecholamines, and specially MHPG, is an HPLC-ECD method for quantifying catecholamines and their metabolite levels in brain tissue, CSF

and plasma and has been used in our department for more than 20 years (Coetzee *et al.*, 2006, Harvey *et al.*, 2006, Marais *et al.*, 2006).

2.4 High performance liquid chromatography coupled to electrochemical detection

High performance liquid chromatography (HPLC)

The method or technique rapidly becoming the choice for analysis and the separation of different matrixes in many research areas today is high performance liquid chromatography (HPLC) (McMaster, 2007).

HPLC coupled to a variety of different detectors is a major analytical tool in biomedical and pharmacological research today. The fast and effective ways for the development of analytical HPLC methods is undertaken more efficiently with a thorough understanding of HPLC principles, theory and instrumentation (Kazakevich *et al*., 2007). HPLC is a technique that allows the analyst to separate complex mixtures of analytes on a chromatographic column connected *via* a pump and an autosampler to a specific detector in order to determine the number, identity and quantity of those analytes. (Huber, 2009).

Basic theory of high performance liquid chromatography

HPLC is the chromatographic technique widely used in analytical research. The type of chromatography which is mostly used is reversed-phase chromatography, which is the reverse of the normal-phase mode (Hanai, 1991). HPLC utilises a liquid mobile phase for the separation of components (analytes) of a solution (sample). The sample with analytes are prepared, and then forced to flow through a chromatographic column (stationary phase) under a high pressure. In reversed-phase HPLC the retention of a component on the column is mainly the result of hydrophobic interactions between the component and the hydrophobic stationary phase surface of the column. In the case of catecholamines and their metabolites, which

comprise of acidic, basic and neutral compounds, reversed-phase ion-pair HPLC is used. Here the formation of neutral ion pairs, with the aid of ion-pairing agents (e.g. sodium 1-heptanesulfonate), which is added to the mobile phase, helps to separate the different compounds on a reversed-phase column (Lim, 1991; Lough and Wainer, 1996).

The reverse-phase columns are usually packed with silica-based solid particles in which octadecylsilyl (C18) or octylsilyl (C8) groups had been chemically bonded to the silica surface. In the column, the mixture is resolved into its separate components following migration of the analytes through the column. The interaction of the solutes (analytes) with mobile and stationary phases can be manipulated through different choices of both solvents and stationary phases. As a result, HPLC acquires a high degree of versatility not found in other chromatographic systems and it has the ability to easily separate a wide variety of chemical and biological samples. Each analyte is shown as a single peak on the final chromatogram as long as there is good separation between all the analytes in the sample (Lim, 1991; Lough and Wainer, 1996).

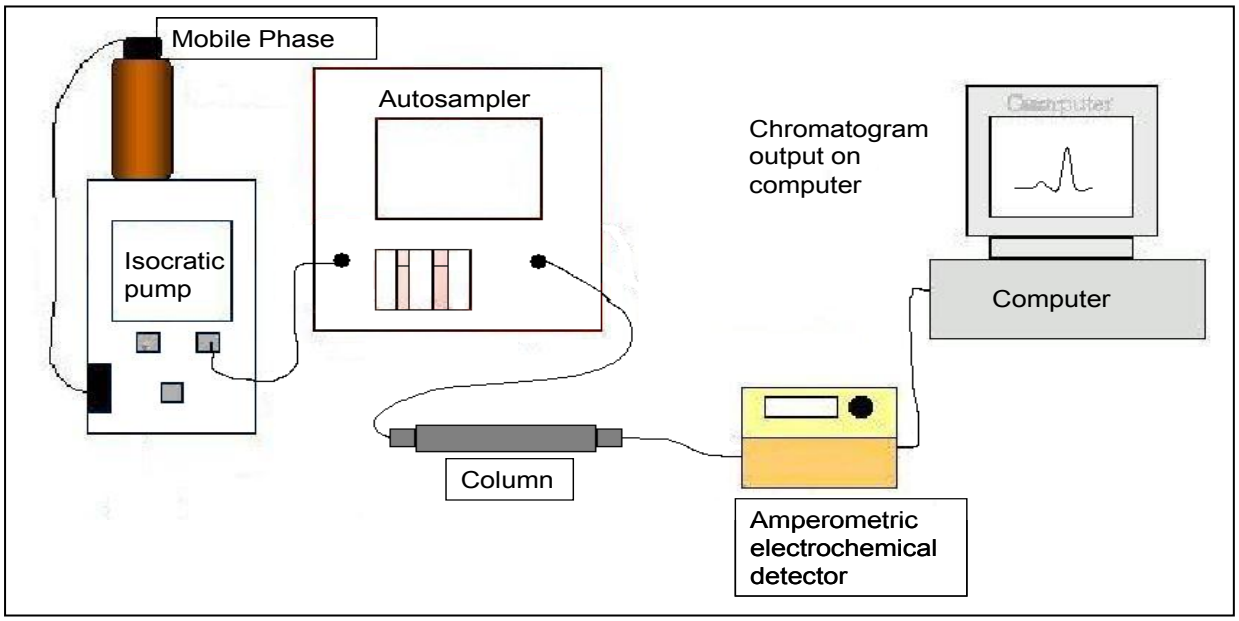

Figure 3: Simple diagram of an HPLC system with amperometric electrochemical detector (AECD).

Currently, most HPLC instruments are connected to a computer with a software program that converts the analog signal from the detector to a digital signal in order to give an electronic interpretation of the data found. The output is in the form of a chromatogram (see figure 3). In HPLC the solvents, mobile phase, stationary phase, pH and temperature play a very important role in the outcome of how the different analytes will separate into individual peaks on the final chromatogram picture.

Electrochemical detection

In electrochemical detection the oxidation or reduction of an analyte in the redox process is the resulting current monitored in ampere in a flow-through cell where the applied potential (volts) across a working electrode surface is held constant (GBC Scientific Equipment Pty, 1995; Lough and Wainer, 1996; ESA, Inc., 2004). Thus, this technique can be utilised for analytes that are readily oxidizable or reducible or ionic e.g. proteolytic organic compounds such as amines (catecholamines), carboxylic acids, inorganic ions, alcohols, phenols and thiols (Kazakevich *et al*., 2007). There are two kinds of electrochemical detection methods available, *viz.* amperometric and coulometric. This study will utilise amperometric electrochemical detection.

Basic theory of amperometric electrochemical detection (AECD)

Amperometric electrochemical detection is based on the measurement of the current resulting from an oxidation or reduction reaction of the analyte at a suitable electrode. When an amperometric electrochemical detector is used, the potential (in volts) is kept constant and the current produced from the electrochemical reaction is then measured in ampere (micro- or nano- or pico-ampere) - the term used for this process is potentiostatic amperometry.

The level of the current (and the area or height of the analyte's peak on the chromatogram) is directly proportional to the concentration of the analyte. The eluent (or mobile phase) that flows through the electrochemical cell

should contain electrolytes (analytes) of a sample and be electrically conductive. Most of the analytes require pH adjustments to the mobile phase to be successfully detected (GBC Scientific Equipment Pty, 1995; Lough and Wainer, 1996).

The areas of application of electrochemical detection are restricted. Sensitivities for compounds such as phenols, catecholamines, nitrosamines, and organic acids are in the pico-mole (nano-gram) range. The specificity and sensitivity make it a very useful tool for monitoring these compounds in complex matrices such as body fluids and natural products. The purity of the eluent is very important, because the presence of oxygen, metal contamination and halides may cause significant background current and interferences (GBC Scientific Equipment Pty, 1995; Lough and Wainer, 1996).

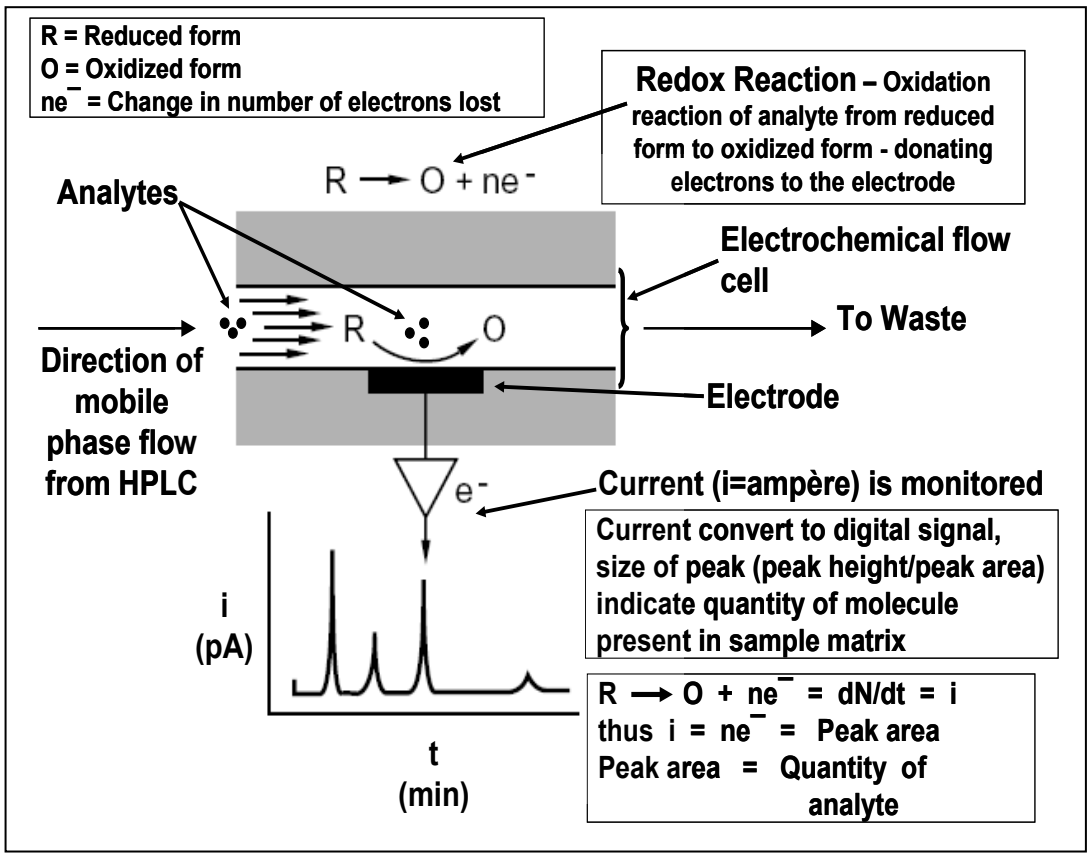

Figure 4: Principle of electrochemical detection in an electrochemical flow cell. The redox reaction takes place inside the electrochemical flow cell of the electrochemical detector (Bioanalytical Systems, Inc. 1994).

Oxidation reaction at the electrode of an amperometric electrochemical detector

The reaction of a specific analyte that takes place at the electrode is called the oxidation reaction. This is where the analyte change from its reduced form to its oxidized form and donates electrons to the electrode. This reaction is caused by the applied potential of the electrode. The next 2 figures show the oxidation reaction for norepinephrine and its metabolite MHPG.

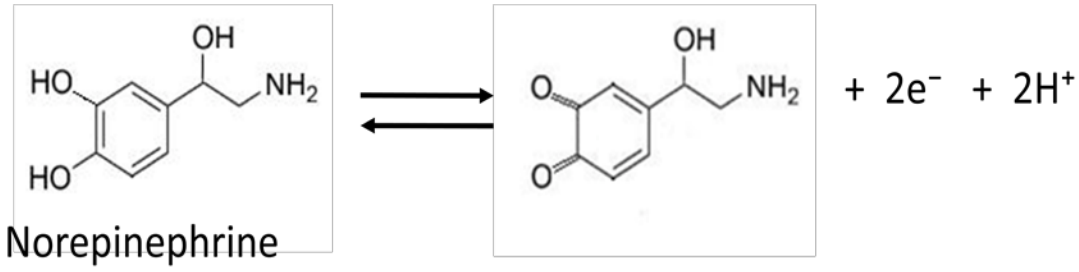

Figure 5: Oxidation reaction of norepinephrine (Lough and Wainer, 1996; Flanagan *et al.*, 2005).

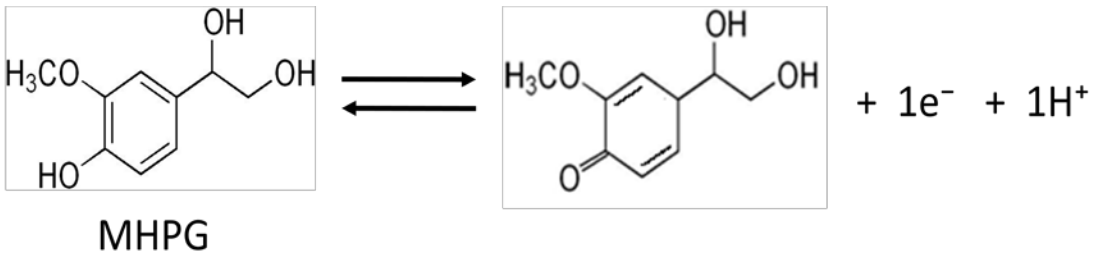

Figure 6: Oxidation reaction of MHPG (as adapted from examples in literature, Lough and Wainer, 1996; Flanagan *et al.*, 2005 and Robinson *et al.*, 2008).

2.5 Method development or the optimisation of an HPLC method

Substances that are biomedically important in human samples have a variety of structures with different substituents. They have aromatic rings, aliphatic rings, long alkyl chains and some are acidic or basic compounds. These differences make it very difficult to develop or optimise an HPLC method of biomedically important compounds. Although many related reports have been published, there is still no simple solution for separating such complicated mixtures in HPLC (Lim, 1991).

When developing a new method or optimising an existing HPLC method, knowledge about the analyte, the sample matrix, the instrumentation and the existing methods in literature are very important.

Important information of the analyte is the size of the molecule, the structure, purity, solubility, stability, presence in the sample, synthesis, metabolism and protein binding (Kelly *et al*., 2007) (discussed in 2.1). Important information of the sample matrix is the biological origin, pH, composition and stability (discussed in 2.2). It is also very important to know the working principle of the instrumentation (discussed in 2.4).

During HPLC analysis the factors in table 1 are important for good analysis, as they may all have an effect on the elution of the analytes through the HPLC column and also on the baseline detected by the detector (in this case electrochemical detection). These factors all together play an important role to produce a valid chromatogram for a specific analyte or group of analytes. The influence of the different contributing factors on the resulting chromatogram is shown in the next table.

Table 1: Influences of different contributing factors on the resulting chromatogram

Factor	**Change**	**Effect on peak elution through the HPLC column**
Room temperature	Increase	Faster peak elution
	Decrease	Slower peak elution
Mobile phase pH	More acidic	Faster peak elution for basic analytes
	More alkaline	Slower peak elution for acidic analytes
% Organic content	More	Faster peak elution
	Less	Slower peak elution
HPLC column length	Longer	Slower peak elution
	Shorter	Faster peak elution
HPLC column diameter:	The smaller the diameter	Produces better sensitivity and resolution
Mobile phase flowrate	Higher	Faster peak elution
	Lower	Slower peak elution
Factor	**Change**	**Effects on the baseline**
Room temperature	Increase	Upwards drift of baseline
	Decrease	Downwards drift of baseline
Mobile phase flowrate	Too high	Analytes pass electrode too quickly and cause poor sensitivity
	Too Low	Analytes pass electrode too slowly and cause an overload on electrode
Sample injection volume	Too much	Will flood the flowcell, peaks to big
	Too little	Peaks too small or not detected

2.6 Sample collection and preparation

Human saliva samples can usually easily be collected in special salivettes. It is however very important that the patient receives detailed information of the collection protocol, such as the exact timing of sampling. Patients should not brush their teeth and also avoid any food and fluid intake for at least 30 minutes before sample collection. They must also rinse their mouths with distilled water just before the sample is collected (Chiappin *et al.*, 2007).

There are various ways of collecting saliva, from sucking it up with a pipette to chewing a cotton swab. There are several oral fluid collectors and commercial devices available for collecting saliva samples, the one to be used in this study, are polyester salivettes. This device is most commonly used and consists of a sterile cotton dental roll inside a sterile polyester tube called a salivette. This product is commercially available from Sarsted (Newton, NC) (Chiappin *et al.*, 2007).

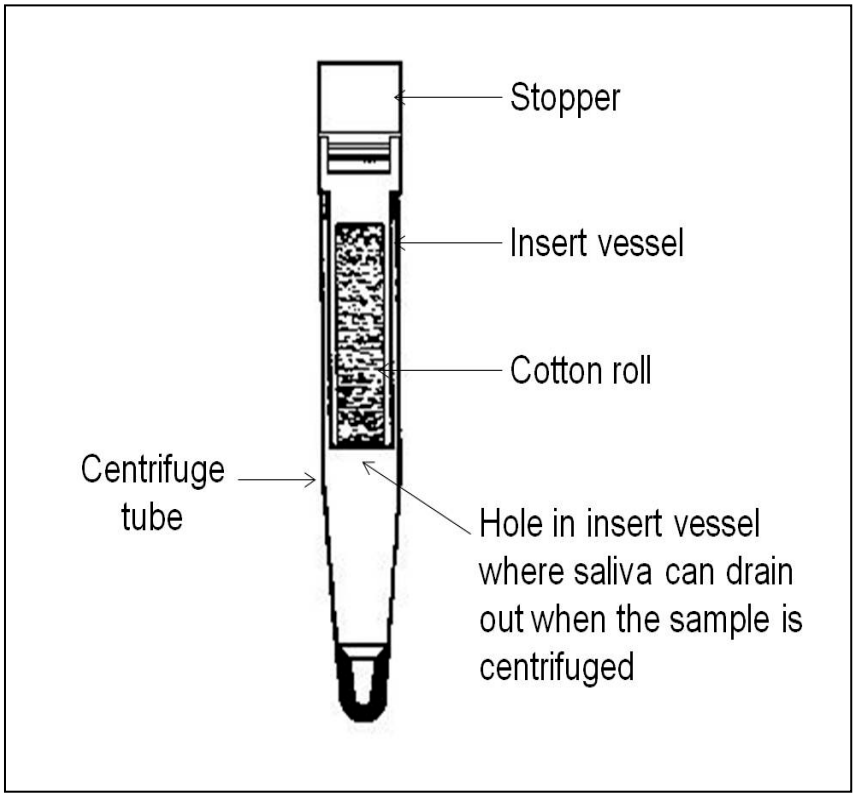

Figure 7: Polyester salivette with cotton roll.

The cotton roll is placed inside the patient's mouth with a sterile forceps. The patient then gently chews it for 2 minutes, where after it is removed again with a sterile forceps and placed into the polyester tube.

After sample collection the saliva specimen should be snap frozen with liquid nitrogen, kept on ice and immediately stored at -80°C to maintain the sample integrity until the day of sample analysis (Chiappin *et al.*, 2007).

2.7 Internal standard

The internal standard is a test substance which is added in a known concentration to both the calibration standards and test samples. The internal standard is used to facilitate in the calculation of the unknown concentration of the analyte in the test samples during the qualitative identification and/or quantitative determination of the sample components. The test substance used as the internal standard must be in structure similar to the test compounds and it must be a stable substance (Garofolo, 2004). The internal standard also serves as a marker or insurance in the way that when there is something wrong with the system or analysis procedure both the internal standard and analytes peak would not show on the chromatogram. But when there is nothing wrong with the system or the analysis procedure and only the internal standard's peak show on the chromatogram and not the analyte of interest's peak that means that the sample does not contain any of the analytes of interest or the concentration of the analyte is below the detection limit.

2.8 Previous results from literature

Table 2: Previous results

Author	**MHPG level in saliva**	**Comment**
Yang *et al.*, 1997	7.03 ± 3.26 ng/ml (n = 10)	HPLC-ECD
Yamada *et al.*, 2000	Men and women (10.7 ± 3.1 ng/ml, n = 59 and 10.3 ± 3.6 ng/ml, n = 137) in normal controls. Patients with anxiety disorders, male patients 20.6 ± 8.0 ng/ml (n = 20) and women patients 16.1 ± 6.8 ng/ml (n = 22).	GCMS
Yamauchi *et al.*, 2001	Panic disorder patients, 19.3 ± 8.4 ng/ml (n = 8) Generalized anxiety disorder patients, 14.4 ± 5.5 ng/ml (n = 10) Adjustment disorder patients, 18.4 ± 9.3 ng/ml (7)	GCMS
Reuster *et al.*, 2002	12.85 ± 2.02 ng/ml (n = 21), patients from a psychiatry department.	HPLC-ECD
Hamer *et al.*, 2006	Resting MHPG saliva levels of 7.4 ± 4.6 ng/ml (n = 32) for patients with low depressive symptoms and 9.6 ± 4.7 ng/ml (n = 23) for patients with high depressive symptoms.	GCMS
Li *et al.*, 2006	Controls of men and women respectively (11.0 ± 5.5 ng/ml, n = 162 and 10.3 ± 5.5 ng/ml, n = 108).	GCMS

2.9 Analytical method validation

The objective of validation of an analytical method is to demonstrate that it is suitable for its intended purpose. In order to demonstrate that the new optimised method is accurate and specific for the quantification of 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in human saliva, the procedures followed for the validation will be described in the following section.

Chromatographic techniques play a predominant role in the quantitative determination of non-endogenous (drugs and their metabolites) and endogenous (naturally occurring) compounds in biological samples. However, the quantitative determination of endogenous compounds is more complicated, analytically and from a validation point of view (Van de Merbel, 2008).

In the literature there is no clear cut methodology for the validation of the quantitative determination of endogenous compounds in biological samples, whereas for non-endogenous compounds there are a few. The methodology for validation of an analytical method that was followed as guidance was according to the following documents: The Guidance for Industry, Bioanalytical Method Validation of the Food and Drug Administration (FDA) of the U.S. Department of Health and Human Services (FDA, 2001) and the "Bioanalytical method development and validation" by Singh and co-workers (Singh *et al*., 2008), as well as chapter 8 on Bioanalytical Method Validation in the book "Analytical Method Validation and Instrument Performance Verification" (Garofolo, 2004).

2.9.1 Method validation parameters

There are a few fundamental parameters for analytical method validation which include the following: selectivity, sensitivity, accuracy, precision, reproducibility and stability. The validation process also involves documenting the performance characteristics of the method to show that it is suitable and reliable for the intended analytical application, and this is

done through the use of specific laboratory investigations. The acceptability of the analytical data is ensured by the fact that it corresponds directly with the criteria used to validate the method (FDA, 2001).

The parameters used to validate the new optimised method, are the following.

2.9.1.1 Specificity / Selectivity

When a method is specific it means that the method produces a response for a single analyte only, but when a method is selective it means that the method provides responses for a number of analytes that may or may not be distinguished from one another. Selectivity differentiates the analyte of interest from all the other components in a mixture. The other components do not need to be differentiated from each other. When a method is selective it refers to the ability of that analytical method to differentiate and quantify the analyte or analytes of interest in the presences of other components in a sample for example metabolites, impurities, and degradants or matrix components.

The analyses of blank samples of the appropriate biological matrix, in this case saliva, are important for selectivity. The blank samples should be tested for any interference. The lower limit of quantification (LLOQ) must be ensured for selectivity.

In the case of quantitative determination of endogenous compounds it is in some cases difficult and sometimes impossible to obtain the authentic biological matrix (in this case saliva) that is free of the endogenous compound (van de Merbel, 2008). To solve this problem when no compound free samples of the authentic matrix are available, blank samples and calibration standards can be prepared with artificial or surrogate matrix. There are a variety of surrogate matrices available, for example distilled water, a buffer such as artificial cerebrospinal fluid with specific pH (± pH = 7.4) or any other solution close to the authentic biological matrix (van de Merbel, 2008).

2.9.1.2 Accuracy

Accuracy describes the closeness of mean test results obtained by the analytical method to the true value (concentration) of the analyte of interest. Accuracy is determined by replicate analysis of samples containing a known amount of the analyte. Accuracy is subdivided into the following 2 categories, *viz.* intra-day accuracy and inter-day accuracy. Intra-day accuracy is calculated by measuring a minimum of 5 determinations per concentration and a minimum of 3 concentrations in the range of expected concentrations is to be used on the same day. Inter-day accuracy is calculated in the same way as intra-day accuracy, but the 5 determinations are done on 5 different days. The % RSD (percentage relative standard deviation) of the mean value result should be less than 15% of the actual value except for values at LLOQ, where it should be less than 20% (FDA, 2001). The measurement of accuracy is expressed in the deviation of the mean from the true value.

2.9.1.3 Precision (reproducibility)

Precision describes the closeness of individual measurements of an analyte of interest when the analytical procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision is calculated by measuring a minimum of 5 determinations per concentration and a minimum of 3 concentrations in the range of expected concentrations to be used. The % RSD determined at each concentration level should be less than 15% except for concentrations at the LLOQ, where it should be less than 20% (FDA, 2001).

Precision can be subdivided into following categories: within-run, intra-batch precision or repeatability. These categories assess precision during a single analytical run, and between-run, interbatch precision or repeatability. Precision can also be measured over time, and may involve different analysts, equipment, reagents, and laboratories. Precision measures the repeatability of the whole analytical procedure, including sample preparation under various conditions.

2.9.1.4 Lower limit of detection (LLOD)

The lower limit of detection is the lowest concentration of the analyte of interest that can be detected in a sample but not necessarily be quantified under the stated analytical conditions. It is also defined as the lowest concentration that can be distinguished from the baseline and background noise with a certain degree of confidence.

2.9.1.5 Lower limit of quantification (LLOQ)

The lower limit of quantification is the lowest concentration of the analyte of interest which can be quantitatively determined with suitable accuracy and precision. The LLOQ value is determined by the presence of a baseline or background signal (accuracy) and the precision of the analytical method (reproducibility). The LLOQ should be the lowest concentration value on the calibration curve.

2.9.1.6 Calibration curve / Linearity

The relationship between an instrument's response and the known concentrations of the analyte of interest is expressed in the calibration (standard) curve. Each analyte in a sample should have its own calibration curve. A minimum of 6 concentrations (standards) should be used to generate a calibration curve covering the expected analytical concentration range, including the LLOQ. The standards for the calibration curve should be prepared in a matrix close to the biological matrix of the endogenous compound.

The range of standards that is used to construct the calibration curve is a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations of the standards should be chosen on the basis of the analytical concentration range expected in the particular study. The calibration curve data should consist of a blank sample (matrix sample analysed without an internal standard) and a zero sample (matrix sample analysed with an internal standard).

It is important that the calibration (standard) curve should have a linear regression value not less than $r^2 = 0.95$, as this curve will be used to calculate the values of MHPG in the test samples in the study.

2.9.1.7 Range

The range of a specific analyte for analysis and the range used for the calibration curve is usually the same. This range is determined by the concentrations found of the endogenous compound in a particular biological matrix. The lower level (also the LLOQ) must be a concentration 10 times less than the expected lowest endogenous concentration. The upper level must be a concentration 10 times higher than the expected highest endogenous concentration. This is not always possible and therefore the range must be chosen in a way that most of the unknown values will fall within it. The values of the chosen range are expressed in the same units as the test results (e.g. nanogram per millilitre, percentage, parts per million) obtained by the analytical method.

2.9.1.8 Robustness / Ruggedness

Robustness measures the analyte of interest's capacity to remain unaffected by small, but deliberate variations in the analytical method's parameters. This will then provide an indication of the reliability with which the analyte can be determined during normal analytical usage. The analytical method's parameters that can be varied are the flow rate of the mobile phase, the pH of the mobile phase, the percentage of the inorganic phase in the mobile phase and the column's temperature.

2.9.1.9 % Recovery (percentage recovery)

The % recovery of a specific analyte does not need to be 100%. The % recovery of the analyte must rather be consistent, precise and also reproducible. Experiments to determine % recovery should be done on at least 3 concentrations (low, medium and high) of the standard range and

also on test samples. The % recovery for endogenous compounds can be obtained by means of different methods.

2.9.1.10 Stability

The stability of the analyte of interest either as standard or as test samples is very important. Stability is a function of the sample collection, storage conditions, and chemical properties of the analyte, the matrix, and the storage container system. The stability of the analyte should be evaluated during sample collection, after short-term storage, long-term storage, after being frozen and following thaw (unfreezing) cycles and also during the analytical process. The stability of the standard's stock solution must also be tested. The conditions used in stability experiments should reflect situations to be encountered during actual sample handling, preparation and analysis.

Chapter 3 Research Design & Methodology

As previously stated in chapter 1, was the current study undertaken as part of a target population comparative study investigating biological markers e.g. MHPG, associated with higher sympathetic nervous system activity.

The aim of the current study was to develop or optimise a method to quantify 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in human saliva.

HYPOTHESIS

We anticipate that the new method will be sensitive and specific enough to determine 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in human saliva samples.

3.1 Research design

The research design was structured in order to achieve the following objectives.

1. To evaluate the existing method for catecholamine determinations in brain tissue in terms of its ability to quantify MHPG in samples.
2. To optimise the existing method to quantify catecholamines and their metabolites in another sample matrix, *viz.* human saliva.
3. To validate the new method.
4. To apply the new method to human saliva samples.

3.2 Materials and Instrumentation

Table 3: Materials and consumables

Materials & consumables	**Brand Name**
Polyester salivettes	Sarsted
200 µl Yellow pipette tips	Merck
1000 µl Blue pipette tips	Merck
2 ml amber polypropylene tubes	Merck (Eppendorf)
1.5 ml polypropylene tubes	Merck (Eppendorf)
300 µl Glass inserts	Separations
1.5 ml Amber vails	Separations
Blue nitrile examination gloves	Merck

Table 4: Equipment and instrumentation

Equipment & instrumentation	**Brand Name**	**Location**
Air-Displacement Pipettes	Eppendorf Research	ATL
Volumetric glassware	Blue Brand	ATL
Centrifuges	Eppendorf & Sigma	ATL & LAMB
Ultra Low Temperature Freezer	New Brunswick Scientific	Physiology
-86°C Cryo Freezer	Forma	LAMB
Vortex	Gemini	ATL
HPLC	Agilent 1200 Series: isocratic pump & autosampler	ATL
Amperometric Electrochemical Detectors	GBC LC1260 electrochemical detector & ESA Coulochem III electrochemical detector	ATL
Column	Luna C18-2 column, 250 x 4.6 mm, 5 µm	Separations
Data Acquisition & Analysis Software on Computer connected to HPLC-AECD	Agilent Chemstation Rev.A.06.02 Data Acquisition & Analysis Software and Chromeleon® Chromatography Management System version 6.8	ATL

All the materials and equipment to be used are available in the Analytical Technology Laboratory (ATL) and Laboratory for Applied Molecular Biology (LAMB) of the School of Pharmacy at the North-West University, Potchefstroom Campus.

3.3 Methodology

3.3.1 The existing HPLC electrochemical detection method

The analysis technique which was optimised in this particular study is an HPLC coupled to an amperometric electrochemical detector method used in our department for more than 20 years for quantifying catecholamines and their metabolite levels in brain tissue, CSF and plasma (Coetzee *et al*., 2006; Harvey *et al.*, 2006; Marais *et al.*, 2006) (see table 5).

3.3.1.1 Chromatographic conditions

Table 5: Chromatographic conditions of the existing method

Analytical Instrument	Agilent 1100 series HPLC, equipped with an isocratic pump, autosampler, GBC LC 1260 electrochemical detector (with amperometric wall jet flow cell, flow cell volume is ± 200 µl, glassy carbon target electrode and Ag/AgCl reference electrode) and Chemstation Rev. A.06.02 data acquisition and analysis software.
Column	Luna C18-2 column, 150 x 4.6 mm, 5 µm, 100 Å pores, 17.8% carbon load, endcapped, Phenomenex, Torrance, CA (Column L1, USP 24, 2000, p 1925).
Guard column	SecurityGuard™, HPLC Guard Cartridge System, with SecurityGuard Cartridges, C18, 4.0 x 3.0 mm, Phenomenex, Torrance, CA.

Mobile phase	0.1 M sodium formate buffer, 5 mM sodium 1-heptanesulfonate, 0.5 mM Ethylenediaminetetra- acetic acid disodium salt, 6% v/v methanol and 4% v/v acetonitrile. The pH of the mobile phase was set at ± pH 3.2 with ± 6 ml of orthophosphoric acid (H_3PO_4) (85%).
Flow rate	1.0 ml/min.
Injection volume	100 µl
Amperometric electrochemical detector settings	Settings for the GBC instrument: Cell potential (E): + 600 mV, Range: 5 nA, Polarity: Positive, Filter: 64 Point, Filter (backside): 0.5 Hz, Reaction: Oxidation, Signal output: 1.0 V
Analog to digital converter settings	Signal 1, Description: AECD, Source: Signal Unit: pA (i), Units/Volt: 1000.00, Peak width (Data Rate): 0.133 Min (2.00 Hz), Stop Time: No Limit, Data Storage: All

3.3.1.2 Reagents

Solution A (for protein precipitation)

Contents:

- 0.5 mM sodium metabisulphite (E Merck, Midrand).
- 0.3 mM ethylenediaminetetra-acetic acid disodium salt (Na_2EDTA) (E Merck, Midrand).
- 0.1 M perchloric acid ($HClO_4$) 60% strong solution (E Merck, Midrand).

Preparation:

1. Weigh 0.047525 g sodium metabisulphite and 0.055836 g Na_2EDTA and dissolve it in 400 ml distilled water.
2. Add 5.435 ml perchloric acid to above solution and make up to 500 ml with distilled water.

Note: All standards and internal standard were prepared within this solution (Solution A).

3.3.1.3 Sample preparation of standards and test samples

1. Following dissection of a brain from a laboratory animal (for example a rat), the brain samples were placed in a 1.5 ml polypropylene tube and immediately snap frozen with liquid nitrogen and stored in a -80°C freezer until day of analysis.
2. On the day of analysis the brain samples were weighed and thawed.
3. 1ml of solution A was added to the tube. The brain tissue was then ruptured by sonication.
4. The sample was allowed to stand on ice for 20 minutes to complete perchlorate precipitation of protein and the extraction of the catecholamines.
5. The sample was then centrifuged at 15300 rpm for 20 minutes at 4°C.
6. The supernatant fluid was removed and pipetted into a 2 ml amber polypropylene tube.
7. The pH of the sample was adjusted to 5 with the addition of 1drop/ml of 10 M potassium acetate.
8. An aliquot of 200 µl of the sample (or standard) was pipetted into a 1.5 ml polypropylene tube. The rest of the sample was stored at -80°C.
9. 20 µl of the Internal Standard (isoprenaline with concentration of 1500 ng/ml, made up in solution A) was added.
10. The final sample was vortexed and then centrifuged at 7 000 rpm for 5 minutes.
11. 100 µl of the final sample was injected onto an HPLC column.
12. The results were expressed in ng/g (nanograms per gram) wet tissue.

3.3.2 Optimisation of the existing HPLC electrochemical detection method

The following factors were taken into account during the process of optimising the method (Kromidas, S., 2006).

1. The composition of the mobile phase.
2. The pH of the mobile phase.
3. The analytical column.
4. The flow rate of the mobile phase through the HPLC system.
5. The optimal injection volume of the standards and samples.
6. The sample preparation.
7. The calibration range for the standards.
8. The lower limit of detection (LLOD).

3.3.3 Method validation parameters

The following validation parameters were followed to validate the new method.

3.3.3.1 Specificity / Selectivity

Determinations of specificity and selectivity were done on solution A and artificial cerebrospinal fluid (aCSF, ± pH 7.4) because no compound (in this case MHPG) free samples of the authentic matrix (saliva) are available.

Plasma and saliva were also used to verify the results from the two surrogate matrixes, *viz.* solution A and artificial CSF.

3.3.3.2 Accuracy

Intra-day and inter-day accuracy were done on the following three standard concentrations: 2.5; 7.5; and 10 ng/ml (as described in 2.9.1.2).

3.3.3.3 Precision (reproducibility)

Precision was done on the following three standard concentrations: 2.5; 7.5; and 10 ng/ml (as described in point 2.9.1.3).

3.3.3.4 Lower limit of detection (LLOD)

The lower limit of detection for this analytical method is the smallest analyte peak detected by the amperometric electrochemical detector and can be distinguished from the baseline and background noise with a certain degree of confidence.

3.3.3.5 Lower limit of quantification (LLOQ)

The lower limit of quantification for this analytical method is the lowest value on the calibration curve.

3.3.3.6 Calibration curve / Linearity

The calibration curve range that was chosen for this analytical method was according to previous literature published and also according to previous results found for MHPG in saliva samples (see references in table 2).

3.3.3.7 Range

The range of MHPG concentrations that was chosen for this analytical method is the same as for the calibration curve mentioned in 3.3.3.6 above.

3.3.3.8 Robustness / Ruggedness

The parameters that were changed to test for robustness were the flow rate of the mobile phase, the pH of the mobile phase, the percentage of the inorganic phase in the mobile phase, the column's temperature or the room temperature.

3.3.3.9 % Recovery (percentage recovery)

% Recovery was determined for the analyte (MHPG) out of the salivette as well as for the complete method. For % recovery from the salivette, only the standards were used.

Table 6: % Recovery from the salivette

Samples analysed for % Recovery	
A standard sample	Sample A
A standard sample that was put through a salivette	Sample B

The following equation was used to calculate the % recovery.

$$\% \text{ Recovery} = \frac{\text{Sample B}}{\text{Sample A}} \times 100$$

The answer has to be close to 100% but not less than 80%.

To determine the % recovery for the analyte (MHPG) for the completed new method, standards and test saliva samples were used.

Table 7: % Recovery for MHPG for the complete new method

Samples analysed for % Recovery			
Blank aCSF sample	+		
Blank aCSF sample	+	Spiked with known concentration MHPG	Sample A
Standard or test sample	+		Sample B
Standard or test sample	+	Spiked with known concentration MHPG	Sample C

The blank sample should be in a matrix close to the test sample for example saline or artificial CSF.

The % recovery for the known spiked concentration was determined by means of the following equation:

$$\% \text{ Recovery} = \frac{(\text{Sample C} - \text{Sample B})}{\text{Sample A}} \times 100$$

The answer has to be close to 100 % but not less than 80 %.

3.3.3.10 Stability

The stability of MHPG was tested for under the following conditions: the stock solution for the standards was tested for long term stability at -80°C. The standards used for the calibration curve and linearity were tested for long term stability at -20°C, freeze and thaw (unfreezing) stability and also stability in the autosampler at room temperature (18-24°C) over 24 hours.

3.3.4 Application of new optimised method to human saliva samples and data analysis

This new optimised HPLC electrochemical detection method was applied to human saliva samples from the SABPA project to quantify MHPG concentration levels.

The human saliva samples were prepared according to the new method's sample preparation, where-after the prepared samples were analyzed on the HPLC amperometric electrochemical detector system.

The MHPG peak area data of each human saliva sample with its internal standards peak area data were used to calculate the MHPG concentration of each human saliva sample.

The results were calculated according to the calibration curve's straight line equation and expressed in ng/ml (nanograms per millilitre).

While this study was focussed on method development and validation, no statistical analysis of human sample data was preformed.

Chapter 4
Results & Discussions

4.1 The New Optimised HPLC Amperometric Electrochemical Detection Method

The factors mentioned in 3.3.2 were taken into account and changed in the process to optimise the existing method (see 3.3.1) in order to analyse MHPG successfully.

4.1.1 Chromatographic conditions

Table 8: Chromatographic conditions of the new method

Analytical instrument: **Instrument 1 used**	Agilent 1200 series HPLC, equipped with an isocratic pump, autosampler, coupled to a GBC LC 1260 Electrochemical detector (with amperometric wall jet flow cell, flow cell volume is ± 200 µl, glassy carbon target electrode and Ag/AgCl reference electrode) and Chemstation Rev. A.06.02 data acquisition and analysis software.
Analytical instrument: **Instrument 2 used**	Agilent 1200 series HPLC, equipped with an isocratic pump, autosampler, coupled to an ESA Coulochem III Electrochemical detector (with thin layer amperometric flow cell, flow cell volume is ± 10.5 µl, glassy carbon/ceramic target electrode) and

	Chromeleon® Chromatography Management System version 6.8.
Column	Luna C18-2 column, 250 x 4.6 mm, 5 µm, 100 Å pores, 17.8% carbon load, endcapped, Phenomenex, Torrance, CA (Column L1, USP 24, 2000, p 1925).
Guard column	SecurityGuard™, HPLC Guard Cartridge System, with SecurityGuard Cartridges, C18, 4.0 x 3.0 mm, Phenomenex, Torrance, CA.
Mobile phase	0.1 M Sodium formate buffer (6,801 g/l), 5 mM Sodium 1-heptanesulfonate (1,01125 g/l), 0.17 mM Ethylenediaminetetra-acetic acid disodium salt (20 mg/l), 4% v/v acetonitrile. The pH of the mobile phase was set at ± pH 4.1 with orthophosphoric acid (H_3PO_4) (85%).
Flow rate	0.85 ml/min
Injection volume for instrument 1	10 µl (flow cell volume = ± 200 µl)
Injection volume for instrument 2	5 µl (flow cell volume = 10.5 µl)
Amperometric electrochemical detector settings for instrument 1	Settings for the GBC instrument: Cell potential (E): + 750 mV, Range: 200 pA, Polarity: Positive, Filter: 64 Point, Filter (backside): 0.5 Hz, Reaction: Oxidation, Signal output: 1.0 V.

Amperometric electrochemical detector settings for instrument 2	Settings for the ESA Coulochem III instrument: Cell potential (E): + 750 mV, Range: 200 pA, Filter: 0.5 sec, Offset: 0%, Signal output: 1.0 V.
Analog to digital converter settings for the GBC instrument	Signal 1, Description: AECD, Source: Signal, Unit: pA (i), Units/Volt: 1000.00, Peak width (Data Rate): 0.133 Min (2.00 Hz), Stop Time: No Limit, Data Storage: All.
Relative retention times	3-Methoxy-4-hydroxyphenylglycol (MHPG) ± 9 minutes. 3,4-Dihydroxy-benzylamine (DHBA) - Internal Standard ± 24 minutes.

4.1.2 Reagents

Solution A

Contents:

- 0.5 mM sodium metabisulphite (E Merck, Midrand).
- 0.3 mM ethylenediaminetetra-acetic acid disodium salt (Na_2EDTA) (E Merck, Midrand).
- 0.1 M per chloric acid ($HClO_4$) 60% st rong so lution (E Me rck, Midrand).

Preparation:

1. Weigh 0.047525 g sodium metabisulphite and 0.055836 g Na_2EDTA and dissolve it in 400 ml distilled water.
2. Add 5. 435 ml per chloric acid t o ab ove s olution a nd m ake up t o 500 ml with distilled water.
3. Note: All standards were prepared in this solution (solution A).

Solution C (Inhibitor solution)

This solution was used by Okumura and co-workers to stabilize catecholamines in saliva samples (Okumura *et al.*, 1997).

Contents:

- 0.3 mM ethylene glycol-bis(β-aminoethylether)-N,N',N'-tetraacetic acid (EGTA) (Sigma- Aldrich, Aston Manor).
- 0.5 mM L-glutathione reduced form (Sigma- Aldrich, Aston Manor).
- 0.1 M hydrochloric acid (HCl) 32% strong solution (E Merck, Midrand).

Preparation:

Weigh 0.011412 g EGTA and 0.0153665 g *L*-glutathione and dissolve it in 80 ml distilled water. Add 984 µl of HCl and make up with distilled water to 100 ml. The EGTA and *L*-glutathione was used to stabilize MHPG in saliva samples.

4.1.3 Preparation of standard solutions

3-Methoxy-4-hydroxyphenylglycol hemipiperazinium salt = 445.45 MW (Sigma- Aldrich, Aston Manor).

3-Methoxy-4-hydroxyphenylglycol (MHPG) = 184.19 MW (40.50%)

Weigh 1.23 mg and dissolve it in 10 ml of solution A, 40.50% of the 1.23 mg will be 0.5 mg that will represent 3-methoxy-4-hydroxyphenylglycol (Gupta *et al.*, 1987).

This solution will be the stock solution (SS) for all the standards and will have a concentration of 50 µg/ml.

All of the working standards will be prepared from this solution, see table below.

Table 9: Preparation of standard solutions

Working Standards	Concentration (ng/ml)	Dilution	+	Solution A	=	Total Volume
1	**1**	20 µl (B)	**+**	1980 µl	**=**	2 ml
2	**2.5**	50 µl (B)	**+**	1950 µl	**=**	2 ml
3	**5**	100 µl (B)	**+**	1900 µl	**=**	2 ml
4	**7.5**	150 µl (B)	**+**	1850 µl	**=**	2 ml
5	**10**	200 µl (B)	**+**	1800 µl	**=**	2 ml
6	**15**	300 µl (B)	**+**	1700 µl	**=**	2 ml
7	**20**	400 µl (B)	**+**	1600 µl	**=**	2 ml
B	**100 ng/ml**	200 µl (A)	**+**	9800 µl	**=**	10 ml
A	**5 µg/ml**	200 µl (SS)	**+**	1800 µl	**=**	2 ml

4.1.4 Preparation of internal standard (I. Std)

3,4-Dihydroxy-benzylamine (DHBA) = 220. 1 M W (Sigma- Aldrich, Ast on Manor)

Weigh off 1 mg and dissolve it in 10 ml of solution A, this solution will be the stock solution for the internal standard. Take 50 µl of the stock solution and make up to 10 ml with a 0.25 M perchloric acid solution, this solution will be the w orking i nternal st andard so lution. The f inal concentration of t he working internal standard solution will be 500 ng/ml.

4.1.5 Sample preparation of standards and test samples

1. The collected saliva samples were immediately snap frozen with liquid nitrogen and stored in a -80°C freezer until day of analysis.
2. On the day of analysis the salivettes with saliva samples will be removed from the -80°C freezer to thaw (to unfreeze) on ice.
3. Add 15 µl of solution C to the bottom part of the salivette.
4. The samples were centrifuged at 5500 rpm (5445 gs) for 35 minutes at 4°C to ensure that all the saliva is drained out of the cotton roll.
5. All the saliva was transferred to 2ml amber polypropylene tube.
6. 100 µl of a standard or a saliva Sample was pipetted into a 1.5 ml polypropylene tube. The rest of the saliva sample was stored at -80°C.
7. Add 15 µl of the internal standard (DHBA with concentration of 500 ng/ml), made up in 0.25 M perchloric acid solution. The reason for the 0.25 M perchloric acid is to precipitate any proteins in the sample.
8. Mix the sample with a vortex and centrifuge at 15300 rpm (21460 gs) for 15 minutes.
9. The whole sample was pipetted into a 300 µl glass insert, which fits into an amber glass vial which is placed into the sample tray of the Agilent 1200 series autosampler.
10. The instrument's software was programmed to inject 5 µl of the sample onto the column.
11. The software will collect the data.
12. The peak area data of each sample were typed in to a Microsoft Excel spreadsheet to be processed.
13. The results were expressed in ng/ml (nanograms per millilitre).

4.2 Chromatographic results

The following typical chromatographic results are examples of a blank solution A sample (figure 8), a blank artificial cerebrospinal fluid (aCSF) sample (figure 9), a blank aCSF sample spiked with a known concentration of MHPG (figure 10), 2 standard MHPG samples (figures 11 & 12), a pooled saliva sample (figure 13), a pooled plasma sample (figure 14), a saliva sample (figure 15) and a spiked saliva sample (figure 16).

The standard samples runtime on the HPLC was only 30 minutes long, but the saliva samples was 50 minutes long because of unknown peaks that eluted later than 30 minutes.

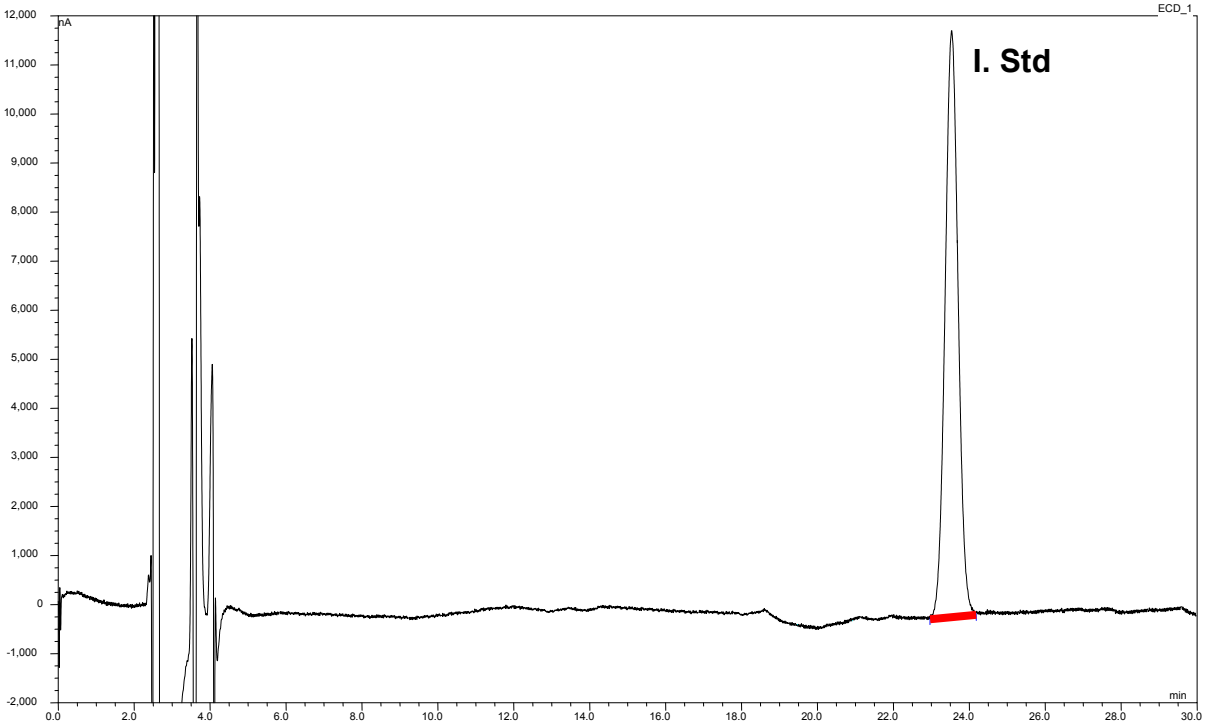

Figure 8: Chromatogram of a blank solution A sample with internal standard.

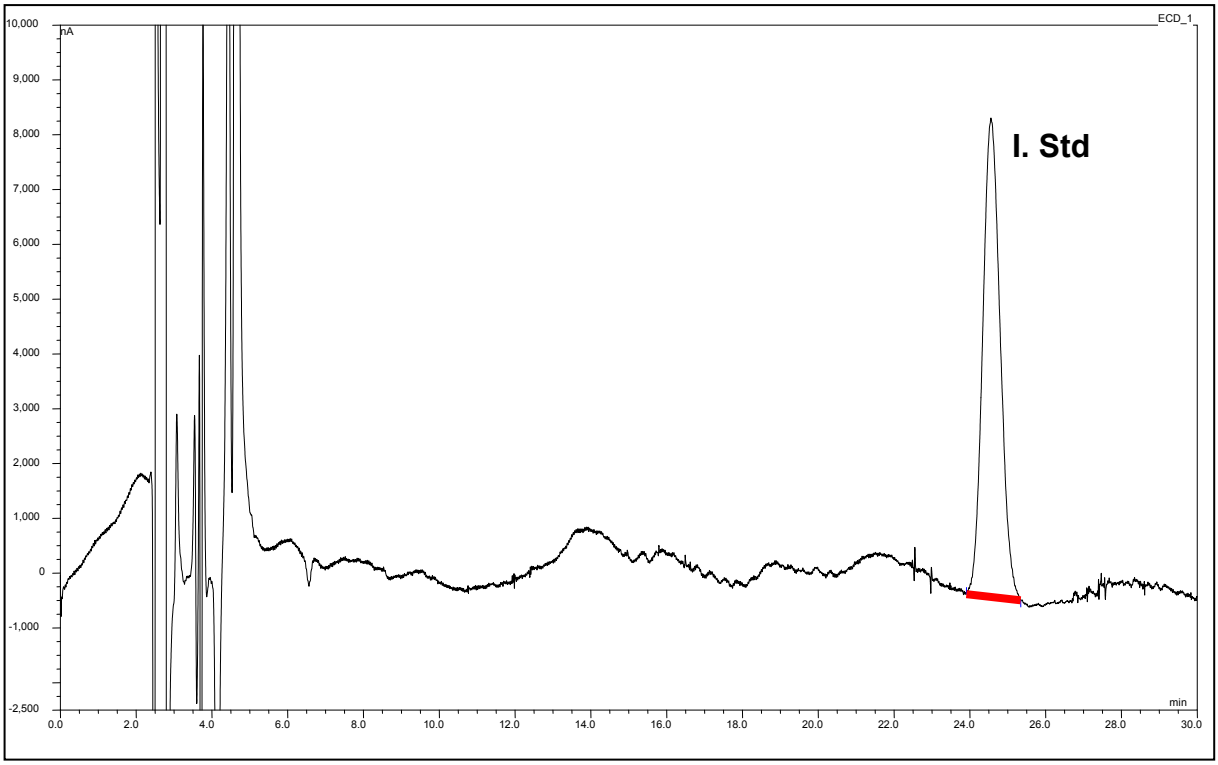

Figure 9: Chromatogram of a blank aCSF sample with internal standard.

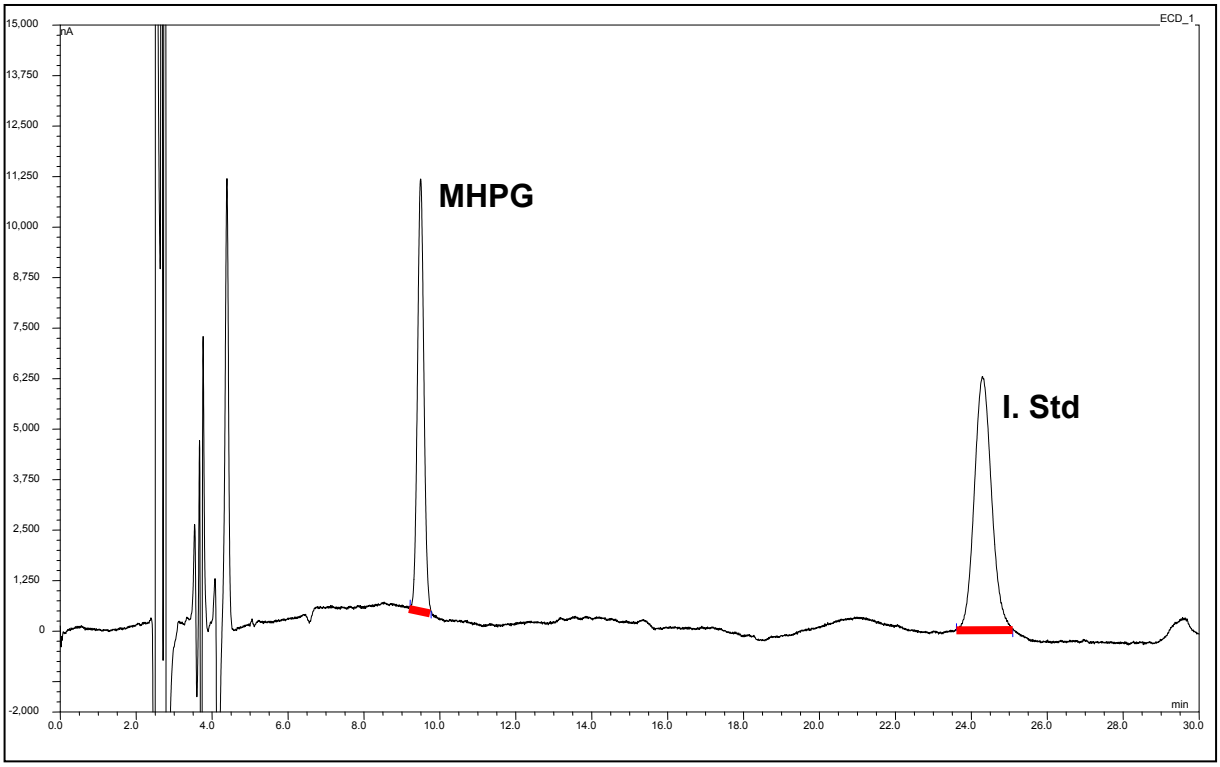

Figure 10: Chromatogram of a blank aCSF sample spiked with 5 µl of a 500 ng/ml MHPG with final MHPG concentration of 25 ng/ml.

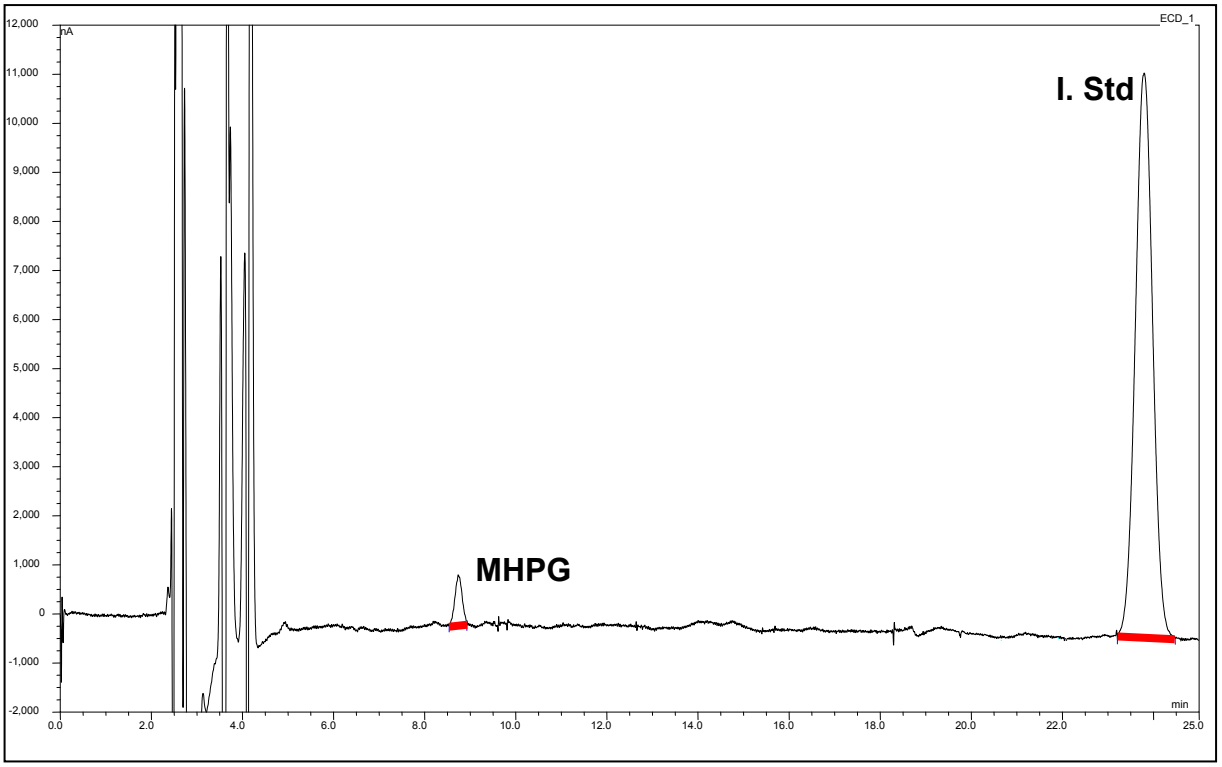

Figure 11 : C hromatogram of a 2. 5 ng/ml M HPG s tandard sample (in solution A).

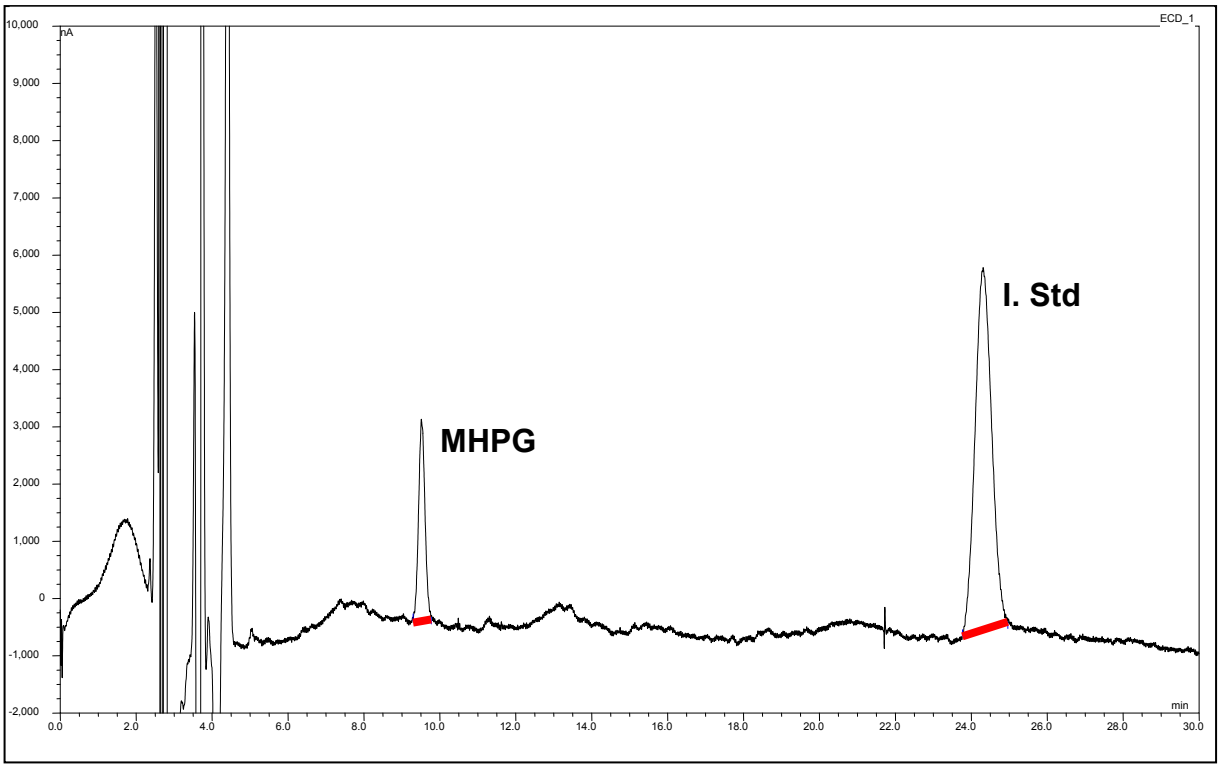

Figure 12: Chromatogram of a 10 ng/ml MHPG standard sample (in aCSF).

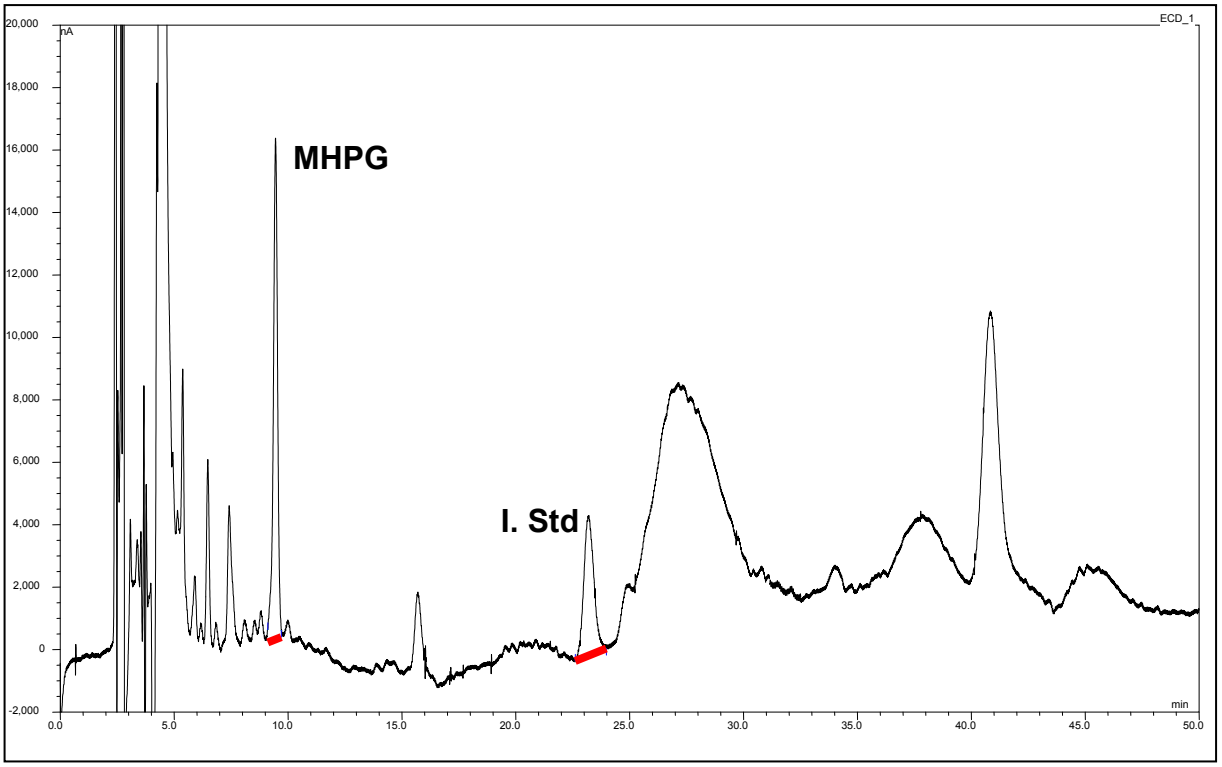

Figure 13: Chromatogram of a pooled saliva sample.

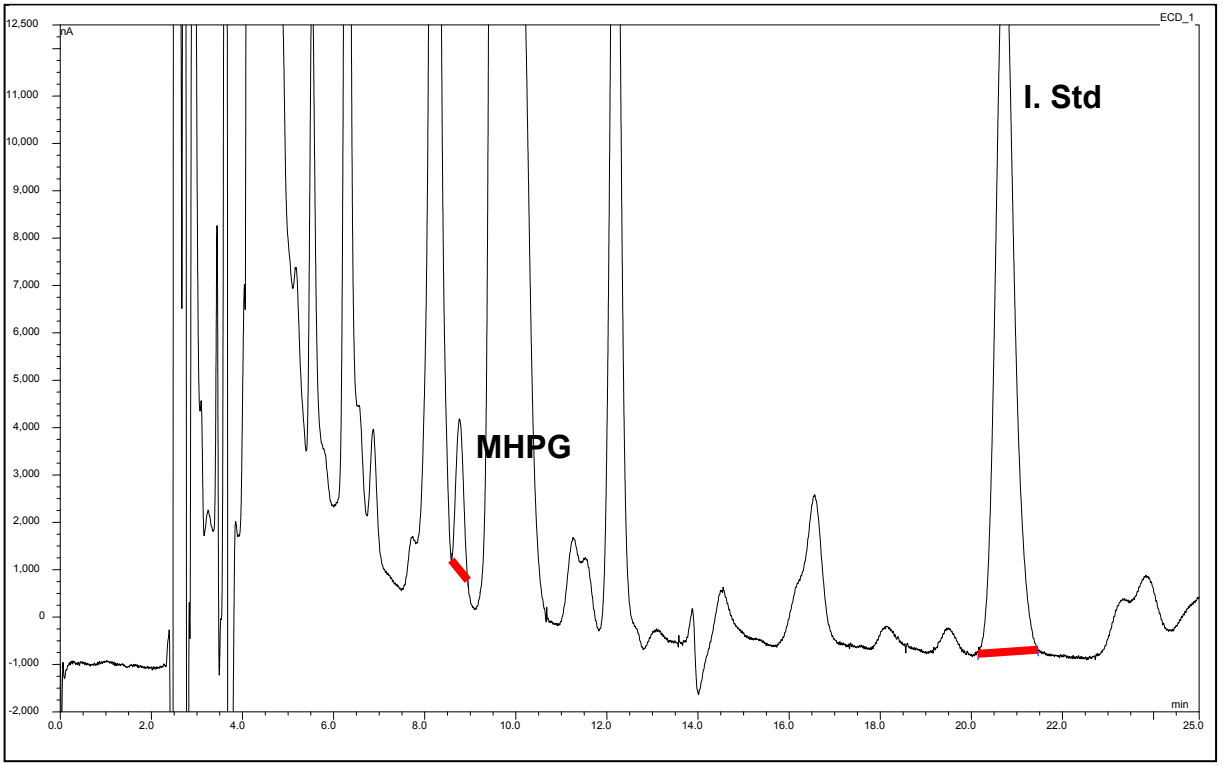

Figure 14: Chromatogram of a pooled plasma sample.

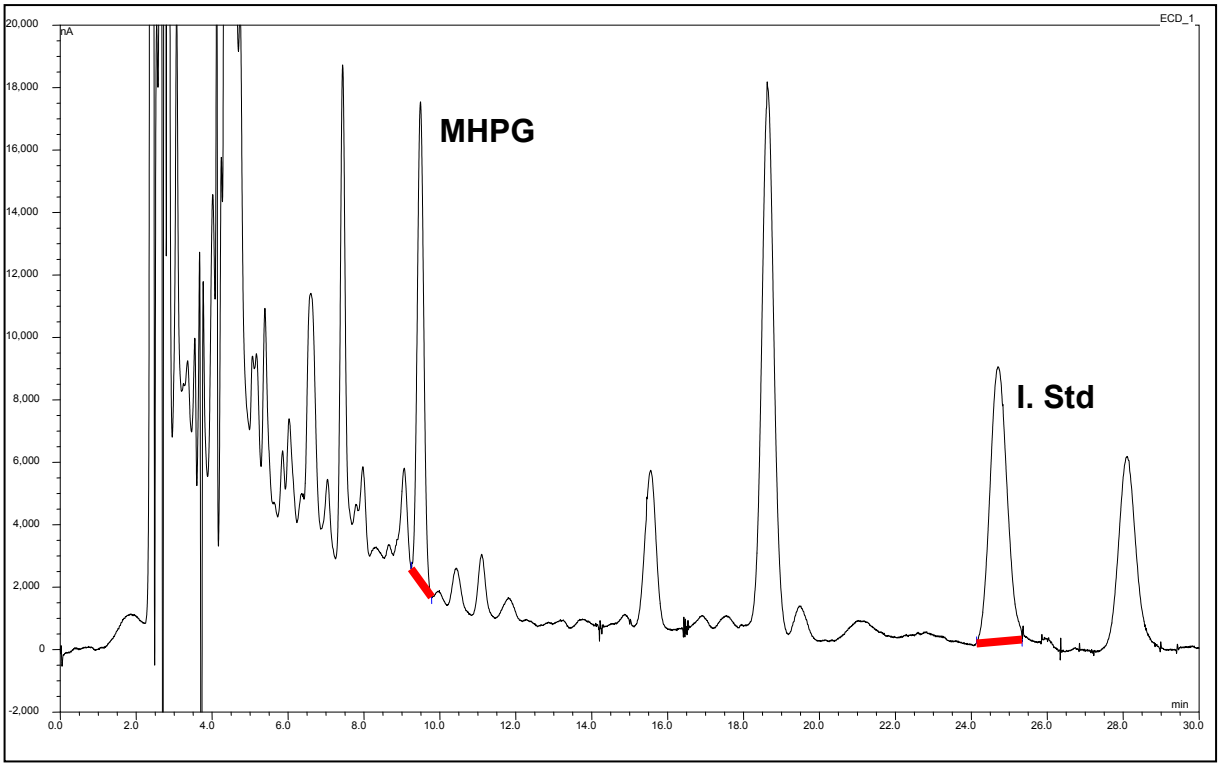

Figure 15: Chromatogram of a saliva sample with a MHPG concentration of 25.88 ng/ml.

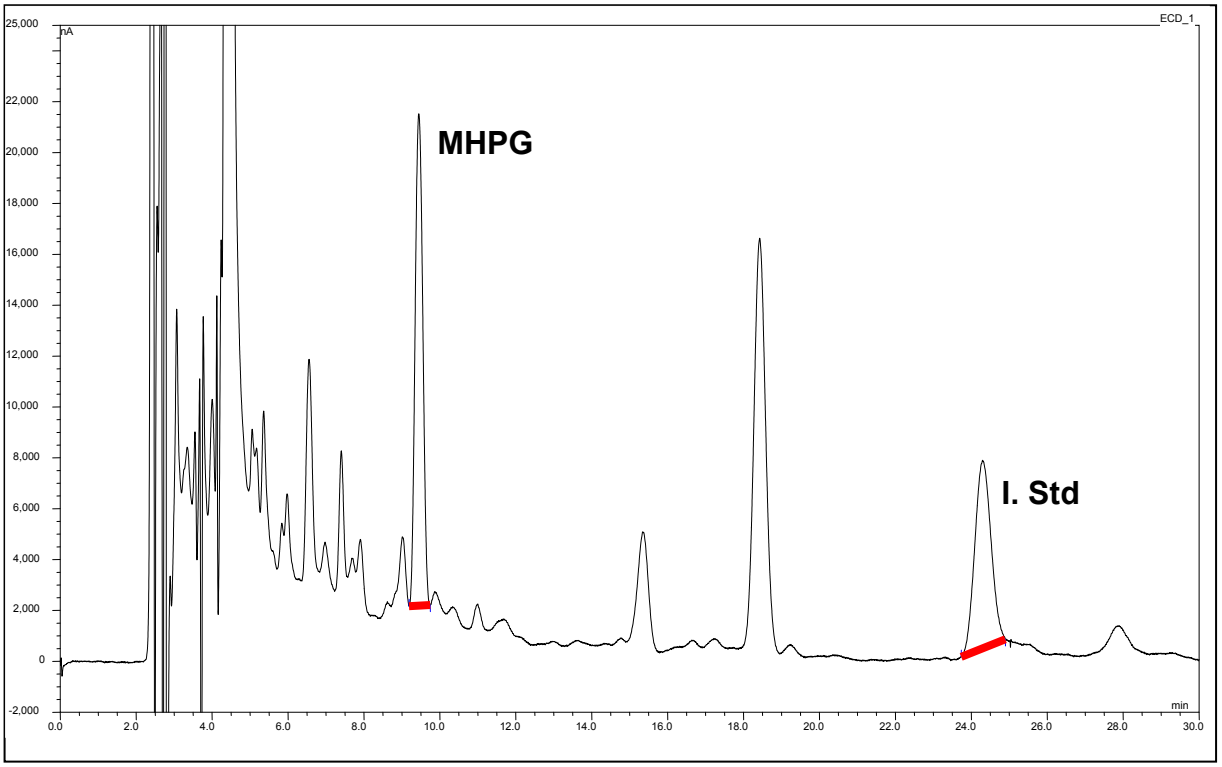

Figure 16: Chromatogram of the same saliva sample as figure 14, spiked with 5 µl of a 500 ng/ml MHPG, with a final MHPG concentration of a 46.51 ng/ml.

4.3 Validation results

4.3.1 Specificity / Selectivity

This new optimised method could detect MHPG in all the matrices (solution A, a rtificial ce rebrospinal f luid, plasma an d saliva samples) without any interference (see figures 8 to 16).

4.3.2 Accuracy and precision

Table 10: Accuracy and precision

Accuracy & precision	Validation Samples	Concentration added (ng/ml)	Mean concentration found (ng/ml)	RSD (%)	Accuracy (%)	*n*
Intra-day	2.5	2.5	3.11	12.67	124.40	5
	7.5	7.5	8.10	3.07	108.00	5
	10.0	10.0	10.19	4.93	101.90	5
Inter-day	2.5	2.5	2.77	6.77	110.80	5
	7.5	7.5	7.63	10.23	101.73	5
	10.0	10.0	10.58	4.86	105.80	5

4.3.3 Lower limit of detection (LLOD)

The lower limit of detection was found to be 1.0 ng/ml.

4.3.4 Lower limit of quantification (LLOQ)

The lower limit of quantification was found to be 2.5 ng/ml, which was also the lowest concentration on the calibration curve.

4.3.5 Calibration curve / Linearity

The calibration curve used in this validation process comprised of the following concentrations: 2.5, 5.0, 7.5, 10.0, 15.0, and 20.0 ng/ml. Each standard was analysed in triplicate.

The calibration graph was plotted using the peak area of the MHPG standards divided by the peak area of that specific standard's internal standard.

Table 11: Data of the calibration curve

Calibration curve data										
MHPG Standards	**Standards**			**Internal standard**			**Standard / Internal standard**			
Conc.	**Peak area**						**1**	**2**	**3**	**Avg.**
ng/ml	**1**	**2**	**3**	**1**	**2**	**3**				
2.5	1185	1128	1374	9302	10368	10794	0.127	0.109	0.127	0.121
5	1656	1680	2053	9611	10896	10649	0.172	0.154	0.193	0.173
7.5	2128	2269	2748	8854	10267	9842	0.240	0.221	0.279	0.247
10	3086	3300	3407	9646	12217	10308	0.320	0.270	0.331	0.307
15	3858	4212	4268	8838	10402	10339	0.437	0.405	0.413	0.418
20	5384	5587	5343	8997	11226	11036	0.598	0.498	0.484	0.527

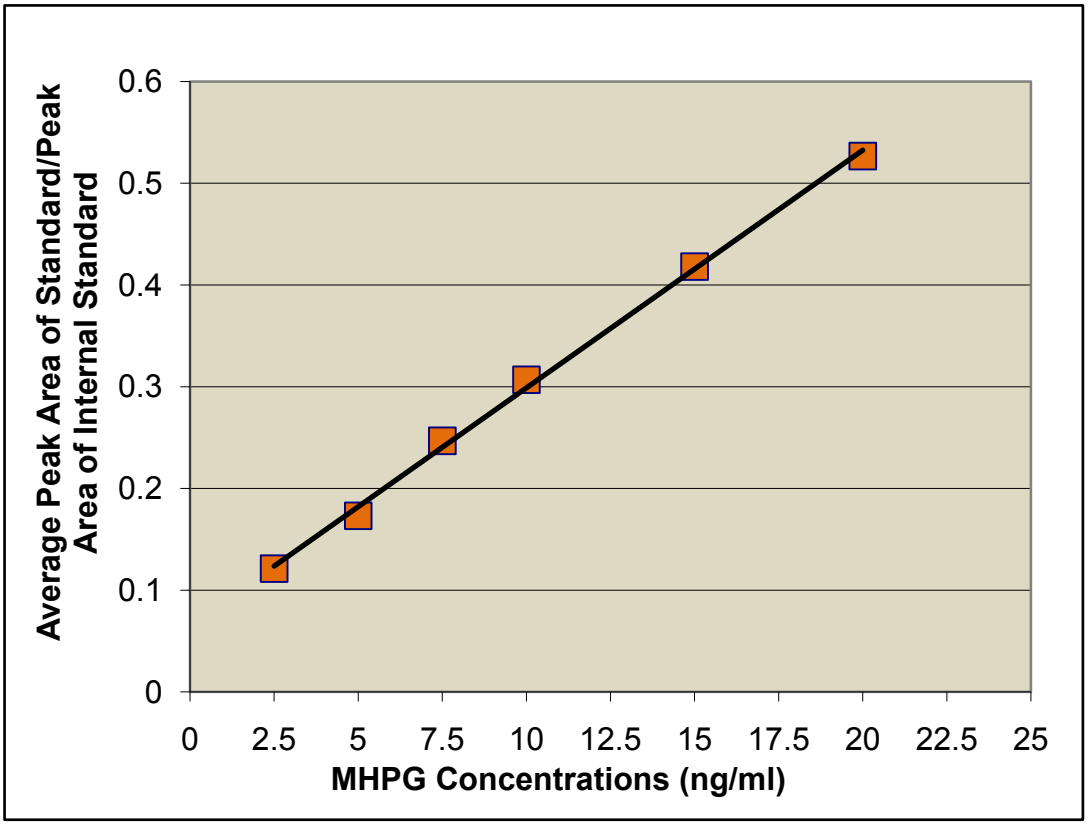

Figure 17: Calibration curve (Linearity curve).

The linearity value of the calibration curve was good (r^2 = 0.998) and was better than the criteria set for it.

The equation for the straight line was y = 0.0234x + 0.0651.

4.3.6 Range

The range chosen and used for this study was from 2.5 to 20.0 ng/ml a range that correlates with the values usually found in saliva.

4.3.7 Robustness / Ruggedness

The new method was found to be robust. The parameters that were changed to test for robustness did not have any notable effects on the

chromatogram or the MHPG peak. There was only a slight change in the retention time of the MHPG. The size and the shape of the peak stayed the same each time.

4.3.8 % Recovery

Table 12: Results of % recovery of MHPG out of the salivette:

MHPG standard sample	% Recovery
1 ng/ml (LLOD)	98.96
2.5 ng/ml (LLOQ)	99.09
5.0 ng/ml	94.46
7.5 ng/ml	88.44
10.0 ng/ml	85.83
15.0 ng/ml	91.22

Table 13: % Recovery results for MHPG following the whole methodological procedure:

Samples that was spiked with a known concentration of MHPG (5µl of 500ng/ml) (n = 2)	% Recovery
2.5 ng/ml (LLOQ)	104.99
7.5 ng/ml	92.65
10.0 ng/ml	109.18
15.0 ng/ml	83.36
Saliva sample 1	91.41
Saliva sample 2	81.57
Saliva sample 3	88.89
Saliva sample 4	91.95
Saliva sample 5	91.25

4.3.9 Stability

The MHPG stock solution as well as standard samples prepared from the stock solution tested for long term stability at -80°C was found to be 100% stable for at least a month.

The standard samples that were used for the calibration curve and linearity that was tested for long term stability at -20°C was found to be 100% stable for at least a month.

The standard samples were found to be 100 % stable after the freeze and thaw (unfreezing) processes.

The standard samples were found to 100 % stable when left for a 24 hour period in the autosampler, at room temperature (18-24°C).

4.4 Application to human saliva samples

The MHPG concentrations obtained in the human saliva samples were between the ranges of 3 ng/ml to 20 ng/ml.

Table 14: Results from human saliva samples

Quantification of MHPG Peaks in human Saliva Samples				
Human saliva samples	MHPG peak area	I. Std peak area	MHPG/I. Std peak area	Calculated concentration in ng/ml (y=0.0234x+0.0561)
Patient 1	43451.37	296839.85	0.146379826	3.86
Patient 2	42533.71	230609.46	0.184440452	5.48
Patient 3	67655.41	280270.18	0.241393532	7.92
Patient 4	91905.81	274794.15	0.334453276	11.89
Patient 5	124453.61	247785.93	0.502262602	19.07

The following chromatograms of the human saliva samples analysed are typical examples of what was found throughout the study.

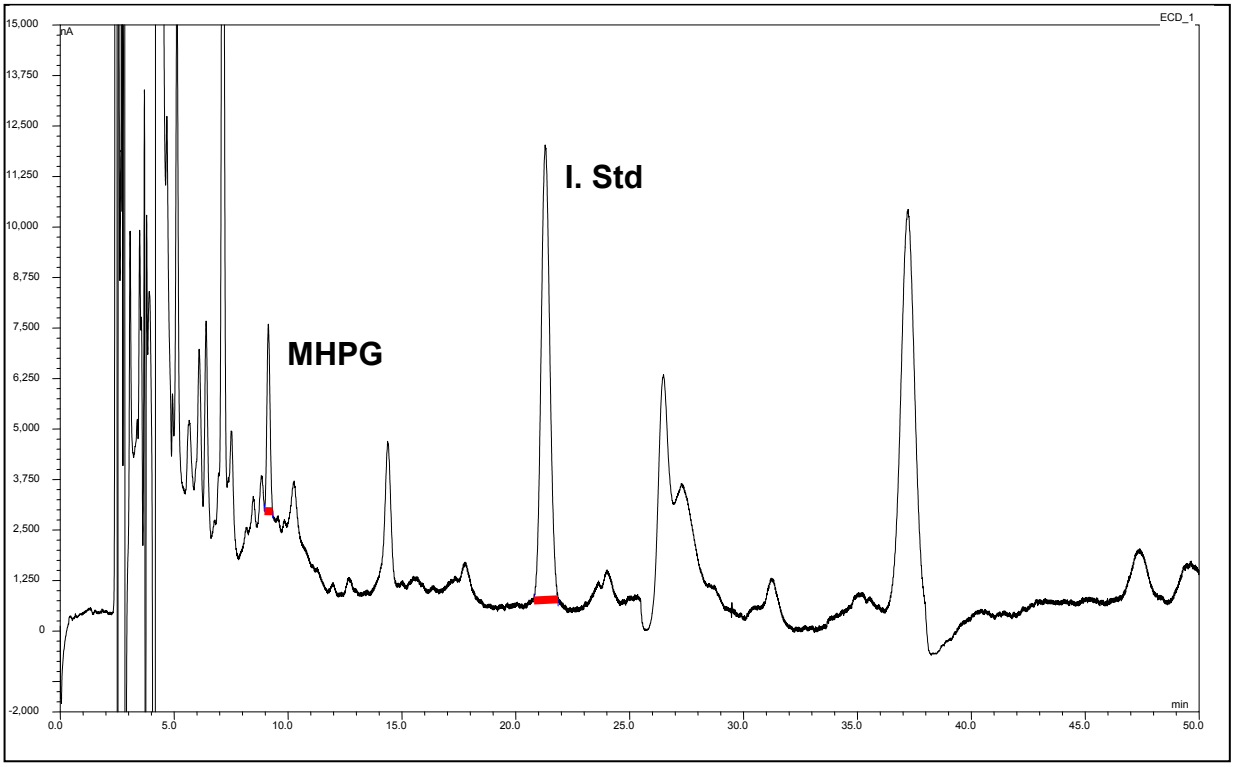

Figure 18 : Chromatogram of a human saliva sample with a MHPG concentration of 3.85 ng/ml (Patient 1).

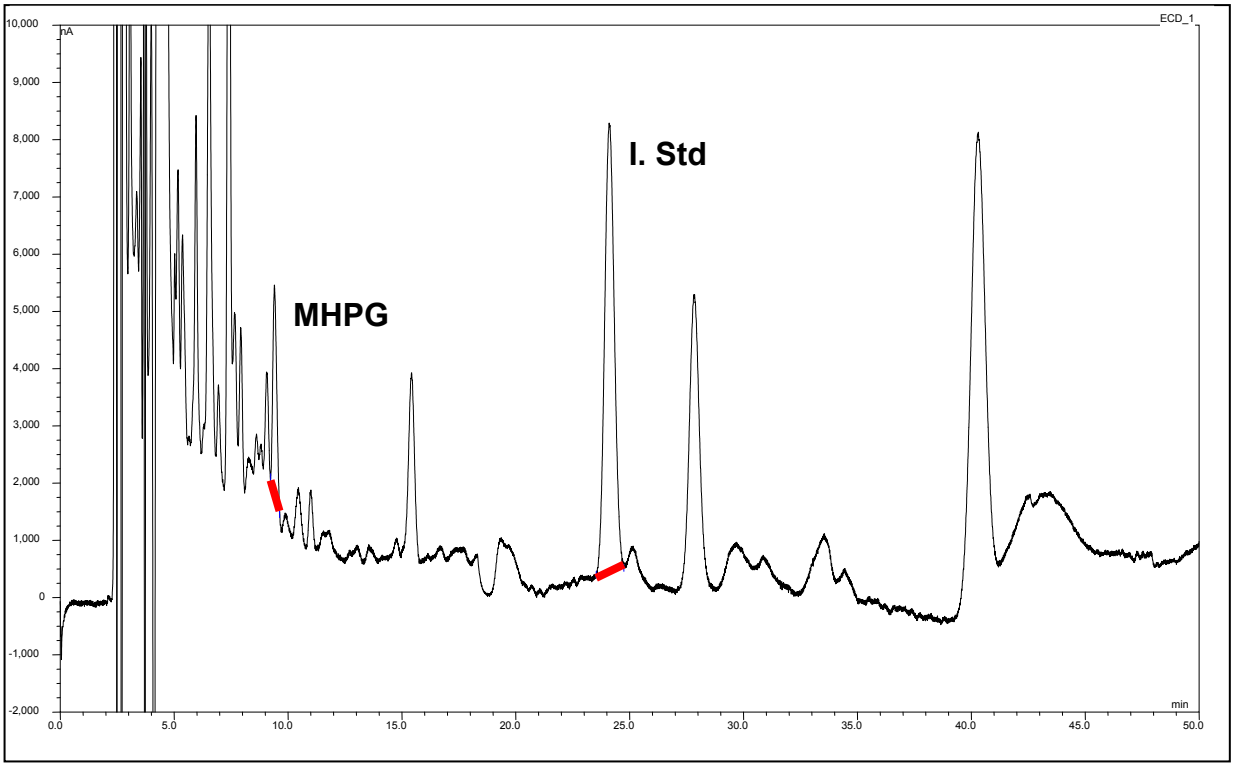

Figure 19: Chromatogram of a human saliva sample (MHPG = 5.48 ng/ml, Patient 2).

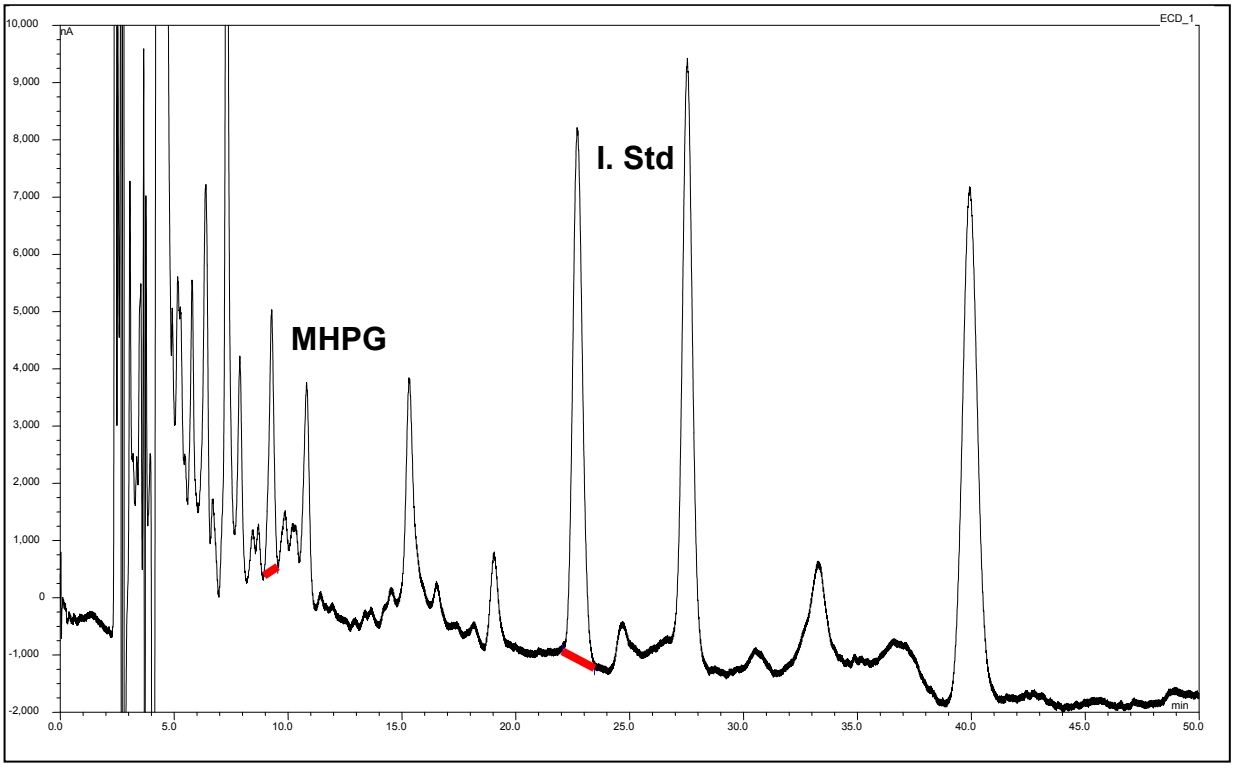

Figure 20: Chromatogram of a human saliva sample (MHPG = 7.91 ng/ml, Patient 3).

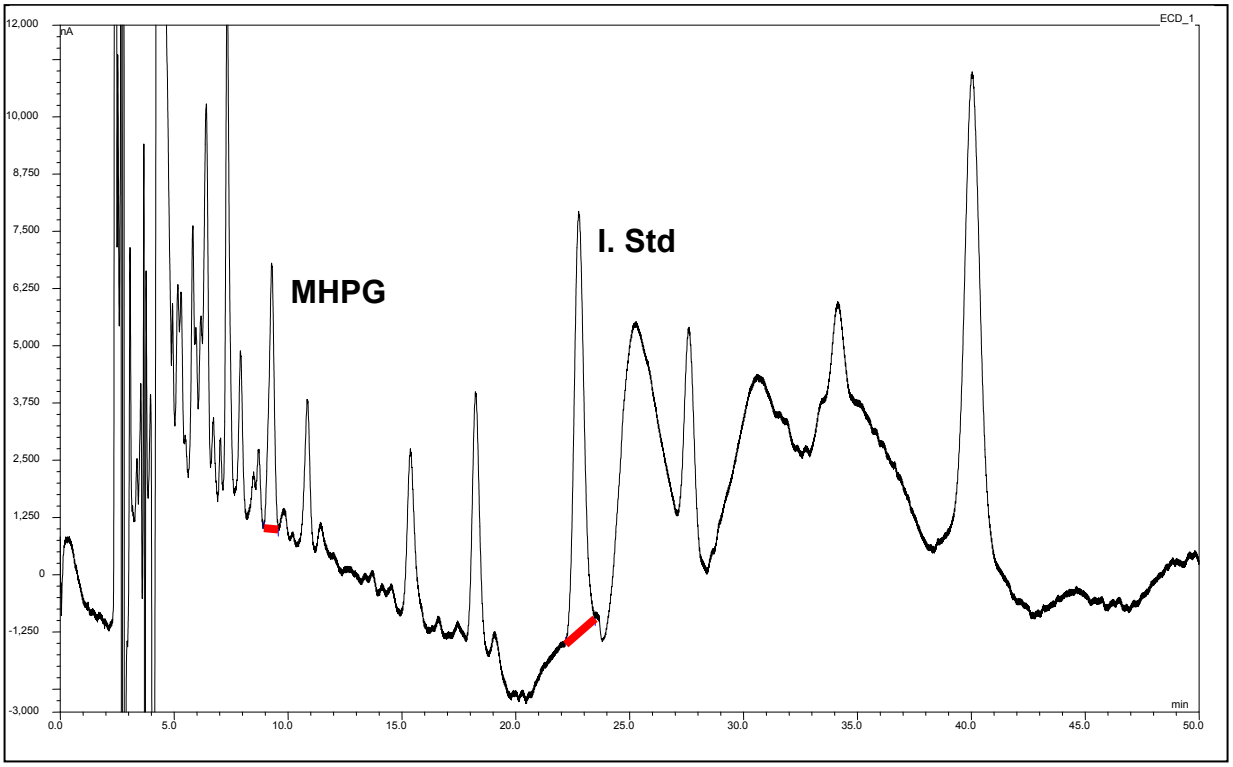

Figure 21: Chromatogram of a human saliva sample (MHPG = 11.89 ng/ml, Patient 4).

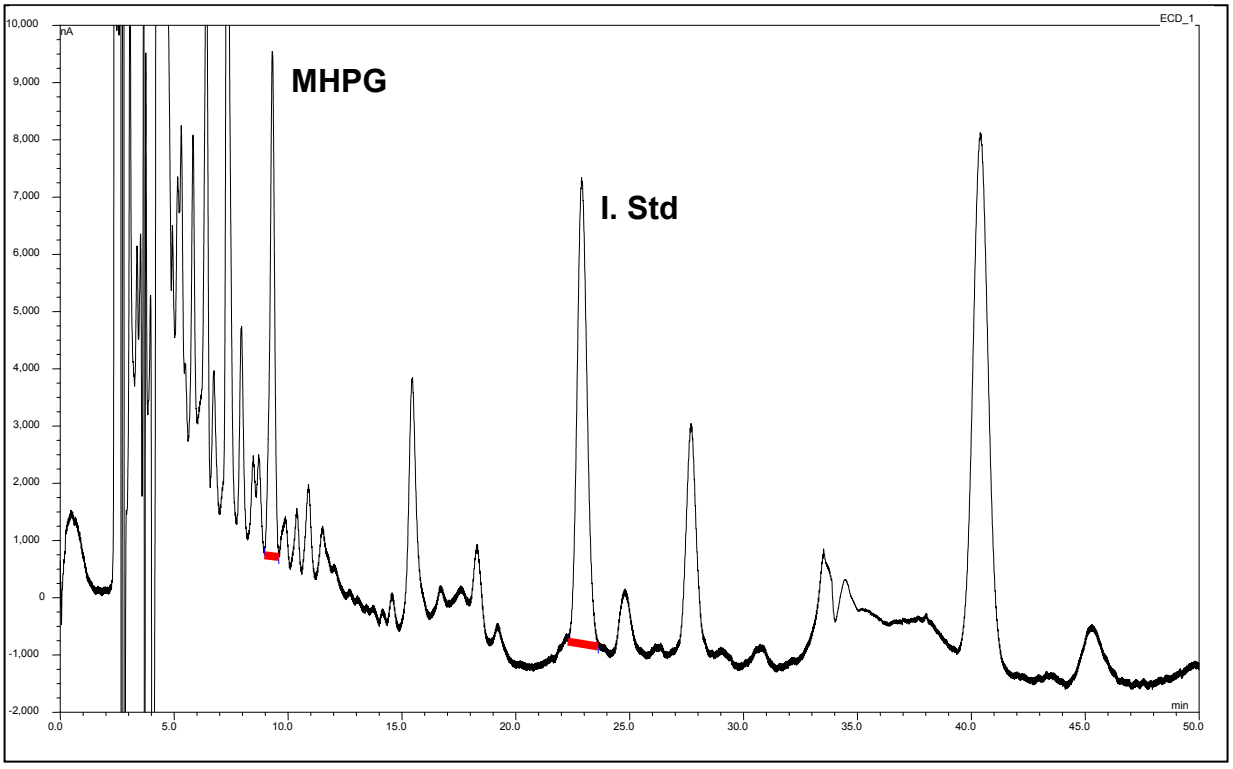

Figure 22: Chromatogram of a human saliva sample (MHPG = 19.06 ng/ml, Patient 5).

4.5 Discussion

4.5.1 The new optimised HPLC amperometric electrochemical detection method

4.5.1.1 The composition of the mobile phase

The composition of the mobile phase stayed more or less the same as the one used in the existing method, except that the organic part was changed from 10% methanol/acetonitrile mixture to only 4% acetonitrile. The pH was made a little less acidic and was changed from pH 3.2 to pH 4.1. Both these changes were necessary because the MHPG peak elutes very fast due to its molecular characteristics.

4.5.1.2 The column

The column's length used in the optimised method was changed form 150 mm to 250 mm. This change was also necessary due to the fast elution of the MHPG's peak.

4.5.1.3 The flow rate

The flow rate was changed from 1.0 ml/min to 0.85 ml/min, the slower flow rate also decreased the speed of elution of MHPG's peak.

4.5.1.4 The optimal injection volume

The injection volume was changed from 100 µl to 10 µl for the GBC instrument and to 5 µl for the Coulochem III instrument. This change from 10 µl to 5 µl was because this instrument's flow cell is much smaller than that of the GBC electrochemical detector.

4.5.1.5 The cell potential setting of the electrochemical detector

The cell potential (in millivolt) setting of the electrochemical detector was changed from +600mV to +750mV. This setting was used because it was

found to be the most sensitive setting for the MHPG molecule, being more electrically conductive at +750mV than at +600mV. The MHPG peak on the chromatogram was also greater in size at this new setting than at the previous one.

4.5.1.6 The sample preparation

The sample preparation differed a considerably from the original method because saliva is a fluid and brain tissue is solid. Solution C was added to the saliva to stabilize the sample and to neutralize any enzymes in the saliva sample. The internal standard solution was added to the saliva sample not only to add the internal standard but also to precipitate any proteins in the sample. This was done to ensure that the sample is clean from any proteins that can harm the HPLC column by clogging it.

4.5.1.7 The calibration range for the standards

The calibration range for MHPG in saliva was chosen according to results found in literature (see table 3).

4.5.1.8 The lower limit of detection (LLOD) and lower limit of quantification (LLOQ)

The lower limit of detection for the optimised method was found to be 1 ng/ml and the lower limit of quantification was 2.5 ng/ml.

4.5.1.9 The validation of the method

The optimised method was found to be sensitive and selective for MHPG in all the matrixes it was analysed (see figures 8 to 16 and 18 to 22). The accuracy and precision gave percentage relative standard deviation (% RSD) values, all less than 15%, that were within the criteria set for it (see points 2.8.1.2 and 2. 8.1.3). The calibration curves (linearity) gave a regression value (r^2 = 0.998) that was very good. The method was found to be robust. The percentage recovery results were also good, all above 80%, which is good for the analysis of endogenous compounds in biological

samples. The stability of MHPG has met all the requirements that were set for it (see point 4.3.9).

4.5.1.10 Application of the method to human saliva samples

The MHPG concentrations obtained with the optimised method applied to human saliva samples, correlated well with the range of MHPG values found in the literature (see table 3) with values between 7 ng/ml to 20 ng/ml. While the five human samples taken from the SABPA project were representative of both baseline and following a stress procedure, the MHPG values ranged also from low to high. This is not an unexpected finding for a study like this (SABPA – Appendix B).

Chapter 5 Conclusions & Recommendations

5.1 Conclusions

The aim of the current study was to develop or optimise an existing HPLC-ECD method to quantify 3-methoxy-4-hydroxyphenylglycol (MHPG) levels in human saliva. This aim was achieved by the study, as we were able to:

- Optimise an existing HPLC-ECD method for the quantification of catecholamines and their metabolites in rat brain tissue to be used for the quantification of 3-methoxy-4-hydroxyphenylglycol (MHPG) in human saliva.
- Validate the optimised method in our laboratory and showed it to be selective and sensitive for MHPG in saliva giving reproducible and repeatable results.
- Apply this optimised method to samples of human saliva and found that the MHPG levels quantified by this method correlated extremely well with the values reported in literature.

Finally the new optimised HPLC amperometric electrochemical detection method may be used in future studies to quantify MHPG in human saliva samples.

5.2 Recommendations

There are a few recommendations to be made for future studies.

- A filter in the salivette, between the insert vessel and the centrifuge tube may help to filter out any impurities and proteins, thereby obtaining a cleaner sample. The samples can be put through a solid

phase extraction procedure but this will make the method more expensive.

- Identification of the rest of the unknown peaks in the saliva samples may broaden the range of endogenous compounds that may be analysed through this method.
- In order to increase the sensitivity and selectivity of the method even more, the amperometric electrochemical detector can be replaced with a coulometric electrochemical detector or a coularray electrochemical detector.
- It may be of value to investigate ways in which a shorter runtime can be achieved (e.g. other mobile phase or column).

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Appendix A

High performance liquid chromatography electrochemical detection troubleshooting

The following table gives a summary of all the most general troubleshooting an an alyst w ill co me acr oss when w orking w ith an HPLC co upled t o an electrochemical det ector (ESA, Inc., 2004; GBC Sci entific Equipment PTY, 1995).

Problem	Possible Cause	Solution
Baseline drift	Electrochemical detector not equilibrated	Wait until detector is equilibrated; stable baseline.
	HPLC column not properly equilibrated	Properly equilibrate HPLC column
	Variation on in mobile phase flow	Check HPLC pump
	Reference electrode worn	Replace reference electrode
	Fluctuation in temperature	Make sure room temperature stays stable
Noisy baseline and Very fast noise spikes	Flowcell has air bubble	Remove bubble through flushing; make sure mobile phase is thoroughly degassed.
	Poor working electrode	Replace or repolish the working electrode's surface
	Reference electrode worn	Replace reference electrode

Problem	Possible Cause	Solution
Output signal constantly pegged at the maximum level	Poor working electrode	Replace or repolish the working electrode's surface
	Reference electrode worn	Replace reference electrode
High Background current	HPLC water quality is poor	Use freshly prepared HPLC water
	Reagents quality is poor	Use highest purity reagents
	Electro-active material slowly eluting from column	Replace or clean column
Gradual loss/decrease in signal response	Fouling working electrode	Repolish or the working electrode's surface
	Reference electrode worn	Replace reference electrode
	Sample unstable	Use a fresh sample
	Change in mobile phase pH	Check mobile phase pH and adjust or freshly prepare new mobile phase
	Change in mobile phase composition	Check mobile phase and prepare fresh mobile phase
	Accidental change of a parameter on the recorder or detector	Check parameter settings and correct setting
	Compounds of interest starting to decompose	Prepare fresh standards

Problem	Possible Cause	Solution
Changes in retention times of compounds of interest	HPLC column not properly equilibrated	Properly equilibrate HPLC column
	Fluctuation in temperature	Make sure room temperature stays stable
	Change in mobile phase pH	Check mobile phase pH and adjust or freshly prepare new mobile phase
	Change in mobile phase composition	Check mobile phase and prepare fresh mobile phase
	Flowrate setting not correct	Set flowrate correct
Back pressure increases	Clogged injector or column or guard column	Clean injector, clean or replace column, replace guard column

Appendix B

NORTH-WEST UNIVERSITY
POTCHEFSTROOM CAMPUS
SCHOOL FOR PHYSIOLOGY, NUTRITION AND CONSUMER SCIENCES

The SABPA Project (Sympathetic activity and Ambulatory Blood Pressure in Africans)

PARTICIPANT INFORMATION AND CONSENT FORM

PART 1

PRINCIPAL RESEARCHER: Dr Leoné Malan, Subject Group Physiology

PROJECT LEADER: Dr Leoné Malan, Subject Group Physiology

Associate Researcher(s): The postdoctoral fellow involved in this trial is Dr. P Szabolcs. Other persons assisting in the study are Dr. Hugo W. Huisman, Prof. Johannes M. van Rooyen, Prof. Nico T. Malan, Mrs. Carla M.T. Fourie, Mrs. Tina Scholtz (Cardiovascular research group, Physiology), Prof. Salomé Kruger & Dr. Ramoteme Mamabolo, (Physical activity), Proff. Hans de Ridder (Anthropometry), Marié Wissing (Psychology), Linda Brand & Brian Harvey (Pharmacology), Kobus Mentz (Education), Francois van der Westhuizen (Biochemistry), Hester Klopper (Nursing), Nancy Frasure-Smith & Francois L espérance (Psychology, Canada), Alaa Alkerwi (Epidemiology, Luxembourg), Yackoob Seedat (ECG, Kwazulu Natal), Paul Rheeder (Sonar, Pretoria Univeristy), Drs. Johan Potgieter & Michael Temane & Mr Thumi Khumalo (Psychology), Mrs Gedina de Wet (Nursing).

This Participant Information and Consent Form is **8** pages long. Please make sure you have all the pages.

Your Consent

You are invited to take part voluntarily in this research project.
This participant information document contains detailed information about the research project which has been explained to you verbally. Its purpose is to explain to you as openly and clearly as possible all the procedures involved in this project before you decide whether or not to take part.
Please read this *Participant Information Form* carefully. Feel free to ask questions about any information in the document. You may also wish to discuss the project with a relative or friend or your local health worker. Feel free to do this.
Once you understand what the project is about and if you agree to take part in it, you will be asked to sign the Consent Form. By signing the Consent Form, you indicate that you understand the information and that you give your consent to participate in the research project.
You will be given a copy of the Participant Information and Consent Form to keep as a record.

What is the study about?

The aim of this project is to have an impact on the eventual prevention and treatment of lifestyle diseases in Africans from South Africa. New knowledge regarding the relationship between higher nervous system activity implicating cardiovascular, metabolic and psychological well-being will improve understanding and change strategies at the roots of treatment and prevention of lifestyle diseases.
Our research has shown that lifestyle diseases in urbanised Africans present higher obesity levels, high blood pressure or hypertension prevalence rates and the experiencing of more stress. This pattern is enhanced during psychosocial stress/urbanisation in participants with a specific coping style. Hence the planned SABPA project, which is the first study in South Africa where coping and direct markers of nervous system activity in Africans will be measured.

Purpose of study

The purpose of this study is to investigate biological markers associated with higher sympathetic nervous system activity in urbanised teachers with a specific coping style.
To investigate the relationship between blood pressure, inflammation, obesity, stress and coping in more detail we are going to perform this study in 400 men and women from the North-West province, aged 25-60 years. A comprehensive assessment of the cardiovascular and nervous systems by means of non-invasive painless techniques will be performed and a blood sample will be taken by an experienced research doctor and nurse to determine your blood sugar, cardiovascular, inflammation and stress hormone levels amongst other health markers.

Procedures

All measurements are performed in the Metabolic Unit (lipid clinic) of the University. A researcher has explained the entire procedure in detail and while you are reading this information document you have time to ask questions and to have clarified matters. If you are fine with the explained procedure you are requested to sign a *consent form (at the end of this document). Remember all personal data will be handled with care and remain confidential.
**By consenting to participate in this study, you consent to the storage and later analysis and testing of your stored blood samples for the purposes noted above. Your blood will also be tested for preliminary results on HIV status, since your HIV status may directly influence the main purposes of this study. If you would like to know what your HIV-status is, we will provide it. If tested positive we will refer you to your doctor and he/she will perform the necessary tests which will allow you to apply for chronic medication benefit. Also, the blood cells from your donated blood sample will be used to investigate the molecular genetics of higher nervous system activity and type 2 diabetes in order to enable pre-symptomatic diagnosis of hypertension and diabetes in the long term.*

Why was I chosen?

Teachers are exposed to changing curricula and disciplinary problems whilst living in an urbanised environment adding to higher stress experiencing and nervous system activity.

How was I chosen?

Inclusion criteria:
Phase I: 200 black Africans aged 25-60 years (male=100, female = 100)
Phase II: 200 white Africans (n = male, 100 = female) aged 25-60 years.

Exclusion criteria: *pregnancy, lactation, any acute/chronic medication (e.g. high blood pressure, TB/tuberculosis, high sugar/diabetes, arthritis, anti-clotting/stroke factors, epilepsy/mental diseases or being treated for it as well being addicted to the medicine). You can not be included if you have been vaccinated in the previous 3 months and if you are a regular blood donor.*

What will be expected of me?

You, as participant will be screened once by a registered nurse to be eligible complying to the inclusion criteria. The following procedures will be followed:

- Recruitment, screening and informed sessions with all participants will be done two months prior to the study (October - November 2007, Phase I, and November, 2008, Phase II) and informed consent forms will be signed.
- After selection of all participants, the details of the project will be discussed with you in English or your home language, i.e. what the exact objectives of the study are, what procedures will be taken and what will be expected from each of you (e.g. overnight stay, resting blood pressure procedures and fasting urine and blood samples are required, importance of complying with the correct sampling methods, incentives). You will be given the opportunity to ask questions.
- Data collection for each participant will involve two days (15min in the morning and 2½ hours in the evening) on Day I; and 2 hours on Day II):

DAY I

- On day I at 07:00, the blood pressure apparatus, which will measure your blood pressure and heart function as well as a physical activity meter will be applied to your arm and waist at your school and you can then resume your normal daily activities. In the afternoon you must complete the Neethling Brain Instrument questionnaire which measures thought processes of the brain.
- At the end of Day I (± 16:30) you will be transported from your schools to overnight in the Metabolic Unit Research Facility of the North-West University. This unit is a research unit for human studies and equipped with 10 well furnished bedrooms, a kitchen, two bathrooms and a television room. Each of you will be subjected to the following procedures:
  - At the end of Day I between ± 17:15 and 18:00 you will be welcomed and each of you will receive your own private bedroom.
  - The procedures, which will be done, will be explained again and each of you will then complete a general socio-demographic health questionnaire. Afterwards you will receive dinner.
  - After dinner, psychological questionnaires will be completed under supervision of registered education specialists and psychologists. Completion of questionnaires will take approximately 40 min, including a break of 20 minutes with coffee/tea and biscuits. This willl be you last meal for Day I as you must be fasting on Day II for obtaining good results.
  - Thereafter, you can relax and watch television or socialise with your co-participants. It will be wise to go to bed not later than 22:00 as the blood pressure apparatus will take measurements every hour during the night and it can be tiring.

DAY II

- At 06:45 on Day II the AMBP will be removed and an urine sample collected. Once this has been done you will be directed to the anthropometric station where your weight, height and body circumferences will be measured.
- The next station involves the blood pressure measurement station. Whilst in a sitting position your blood pressure will be taken in duplicate with the sphygmomanometer (the same as used at clinics) with a resting period of 5 minutes in between. Our registered research doctor/nurse will take a fasting saliva sample as well as a blood sample of 45ml from a vein in your dominant arm. The infusion set will be left in your arm to lessen the effect of inserting a needle again for blood sampling after exposure to the two stressors. A small amount of diluted heparin will be left in the infusion set in your arm to prevent clotting.

Next the cardiovascular measurements will follow consisting of three separate procedures:

- The 1st measurement involves an ECG apparatus, which measures heart function, with 12 leads, which will be placed into position on your rib cage/front part of the body.
- The 2nd measurements are non-invasive and will be done by means of the Finometer device which also involves the assessment of heart functioning such as pulse (beats per minute), stroke volume (blood volume ejected by the heart per beat), cardiac output (blood volume ejected by the heart per minute), total peripheral resistance (resistance against the blood flow created by small arteries), central resistance (resistance against which the heart has to work while ejecting the blood into the aorta) as well as the elasticity of your large arteries (compliance). For this procedure a blood pressure cuff will be placed around your left arm and middle finger which will be inflated and stepwise deflated. You will not have more discomfort than during a common blood pressure measurement. This will take about 5 minutes.
- The stressor application procedure follows: You will now be exposed to a stressor for 1 minute whilst your blood pressure and ECG will still be taken. After exposure a saliva and blood sample (45ml) will be taken. After 10 minutes another saliva sample will be taken. Then the stressor application procedure will be repeated with the second stressor.
- At another station your 3rd measurement includes the assessment of pulse wave velocity, i.e. how fast your blood travels through your arteries. This measurement gives us an indication about how stiff your vessel walls are. The stiffer your vessel wall is the faster the blood travels from one point of your body to another. These painless measurements will require two technicians using blunt probes (tonometer) putting light pressure on the neck and on the foot to measure the velocity of the pulse waves. This takes only a few minutes. An ultrasound device will be taken of your arteries in the neck with a blunt probe to indicate the intrinsic thickness of your arteries which contributres to high blood pressure.

The two stressors you will be exposed to for one minute include:

1. The *Colour-Word-Conflict Chart (applied for 1 minute)* is written in various colours. You must say or select the ink colour rather than the name of the colour spelled out by the word. A sliding scale with monetary incentives (maximum of R55.00) will be given if you can complete reading the chart.

2. The Cold Pressor Test (Foot) (applied for 1 minute): Immersion of your foot up to the wrist in ice water (4 degrees Celcius). As the cold can make you hold your breath you must quietly count to yourself during cold exposure to breath more rythmic.

- You have reached the end of the sampling phase.

- **Thank you for your participation! You now will have the opportunity to shower and a take away breakfast will be given**.
- Immediate feedback on your HIV/AIDS status, obesity, blood pressure and blood glucose/sugar values will be given. *HIV/AIDS post-test counselling will be arranged if you are tested positive.*
- You are now transported back to your school and after one week you will receive your Neethling Brain Instrument and 24-hour blood pressure reports.

Possible Risks

The measurements performed in our study will include only non-invasive techniques that are not expected to reveal any risks but might cause little discomfort. The taking of blood samples is an invasive procedure with a minimal risk of bleeding. Thus the procedure may cause only a few seconds of light discomfort. All tests will be performed by experienced research nurses of our department. There may be additional unforeseen or unknown risks.

Precautions to protect the participant

The Metabolic Unit facility of the NWU is fully equipped, and in case of an emergency which could not be handled by the registered nurse, the supervising medical doctor Emile Kotzé will be contacted. Dr. Kotzé was notified before the study commenced that this study will be taking place, and that there is a slight possibility that he may be contacted. Supporting medical treatment care facilities will be at hand anytime if needed.

Other Treatments Whilst on Study

It is important to tell the research staff about any treatments or medications you may be taking, including non-prescription medications, vitamins or herbal remedies during your participation in the study.

Incentives

1. All teachers will receive feedback on their health profile and if necessary references will be given to physicians/clinics/hospitals.
2. Blood pressure and ECG monitoring report (normally costing R 637.60). Your benefit of participation is a comprehensive assessment of the cardiovascular and metabolic condition including investigation of blood pressure, inflammatory status and psychological well-being. These examinations will help us to assess the degree of vascular impairment of the arteries and to predict your risk of possible cardiovascular events such as heart attacks and stroke. The results may assist your doctor in decision making for further treatment or for instituting preventive measures. Our study will also contribute to the identification of possible factors leading to high blood pressure. As 24 hour ambulatory blood pressure monitoring is required for the diagnosis of hypertension, medical aids insist on this method of diagnosis to qualify for chronic medication. Additional testing could also reveal illnesses of a chronic nature and would serve as a motivation to qualify for chronic medication, such as metabolic syndrome, anti-inflammatory and cholesterol-lowering drugs.
3. Monetary incentive on completion of the colour word conflict chart (± R55.00).
4. Dinner and breakfast (± R24.00).
5. Neethling Brain Instrument profiles done by registered user of the Whole Brain (normally costing ± R350.00).
6. Coping skills workshop will be arranged on request.

Privacy, Confidentiality and Disclosure of Information

By consenting to participate in this study, you consent to the storage and later analysis and testing of your stored blood samples for purposes noted above. Your blood samples will be discarded immediately after analysis. All information provided by you and the results of tests will be treated in the strictest confidence, and will only be used for the purpose of this research project. It will only be disclosed with your permission, except as required by law. The results of your medical tests will be labelled only with a code number, and will be stored separately from any identifying information. When the results are analysed we will be looking for differences between groups of people, not at the results of individuals. No information that could identify any person taking part in the study will be revealed when the results are reported.

Participation is Voluntary

Participation in any research project is voluntary. If you do not wish to take part you are not obliged to. If you decide to take part and later change your mind, you are free to withdraw from the project at any stage.
Your decision whether to take part or not to take part, or to take part and then withdraw, will not affect your routine treatment, your relationship with those treating you or your relationship with the North-West University.
Before you make your decision, a member of the research team will be available so that you can ask any questions you have about the research project. You can ask for any information you want. Sign the Consent Form only after you have had a chance to ask your questions and have received satisfactory answers.
If you decide to withdraw from this project, please notify a member of the research team before you withdraw.

Ethical Guidelines

This project will be carried out according to Ethical Guidelines of the Helsinki declaration from 2000, with additional notes in 2002. This statement has been developed to protect the interests of people who agree to participate in human research studies.
The ethical aspects of this research project have been approved by the Human Research Ethics Committee of ***North-West University Potchefstroom.***

Further Information or Any Problems

If you require further information or if you have any problems concerning this project, you can contact the principal researcher or *the other* researchers responsible for this project.

Dr Leoné Malan (018-299 2438) Signature:

Sr. Chrissie Lessing (018-299 2480) Project Leader: Dr Leoné Malan

PART 2

To the subject signing the consent as in part 3 of this document

You are invited to participate in a research project as described in paragraph 2 of Part 1 of this document. It is important that you read/listen to and understand the following general principles, which apply to all participants in our research project:

1. **Participation in this project is voluntary.**
2. **It is possible that you personally will not derive any benefit from participation in this project, although the knowledge obtained from the results may be beneficial to other people.**
3. **You will be free to withdraw from the project at any stage without having to explain the reasons for your withdrawal. However, we would like to request that you would rather not withdraw without a thorough consideration of your decision, since it may have an effect on the statistical reliability of the results of the project.**
4. **The nature of the project, possible risk factors, factors which may cause discomfort, the expected benefits to the subjects and the known and the most probable permanent consequences which may follow from your participation in this project, are discussed in Part 1 of this document.**
5. **We encourage you to ask questions at any stage about the project and procedures to the project leader or the personnel, who will readily give more information. They will discuss all procedures with you.**
6. **We require that you indemnify the University from any liability due to detrimental effects of treatment by University staff or students or other subjects to yourself or anybody else. We also require indemnity from liability of the University regarding any treatment to yourself or another person due to participation in this project, as explained in Part 1. Lastly it is required to abandon any claim against the University regarding treatment of yourself or another person due to participation in this project as described in Part 1.**
7. **If you are married, it is required that your spouse abandon any claims that he/she could have against the University regarding treatment or death of yourself due to the project explained in Part 1.**
8. **Participation in this project is voluntary.**

Consent

Title of the project:
"THE SABPA STUDY (**S**YMPATHETIC A CTIVITY AND **A**MBULATORY **B**LOOD **P**RESSURE IN **A**FRICANS)".

I, the undersigned ……
(full names)
read/listened to the information on the project in PART 1 and PART 2 of this document and I declare that I understand the information. I had the opportunity to discuss aspects of the project with the project leader and I declare that I participate in the project as a volunteer. I hereby give my consent to be a subject in this project.

(Signature of the subject)

Signed at ..... on ……2008

Witnesses

1. .....

2. .....

Signed at ..... on ……2008

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Ethics Committee
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Dr L Malan

Dear Dr Malan

6 February 2008

ETHICS APPROVAL OF PROJECT

The North-West University Ethics Committee (NWU-EC) hereby approves your project as indicated below. This implies that the NWU-EC grants its permission that, provided the special conditions specified below are met and pending any other authorisation that may be necessary, the project may be initiated, using the ethics number below.

Project title:.SABPA (Sympathetic activity and Ambulatory Blood Pressure in Africans)

***Ethics* number**: N W U - 0 0 0 3 6 - 0 7 - S 6

Approval date: 12 November 2007 **Expiry date**: 11 November 2012

Special conditions of the approval (if any): None

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, please note the following:

- *The project leader (principle investigator) must report in the prescribed format to the NWU-EC:*
  - *annually (or as otherwise requested) on the progress of the project,*
  - *without any delay in case of any adverse event (or any matter that interrupts sound ethical principles) during the course of the project.*
- *The approval applies strictly to the protocol as stipulated in the application form. Would any changes to the protocol be deemed necessary during the course of the project, the project leader must apply for approval of these changes at the NWU-EC. Would there be deviated from the project protocol without the necessary approval of such changes, the ethics approval is immediately and automatically forfeited.*
- *The date of approval indicates the first date that the project may be started. Would the project have to continue after the expiry date, a new application must be made to the NWU-EC and new approval received before or on the expiry date.*
- *In the interest of ethical responsibility the NWU-EC retains the right to:*
  - *request access to any information or data at any time during the course or after completion of the project;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the project are revealed or suspected,*
    - *it becomes apparent that any relevant information was withheld from the NWU-EC or that information has been false or misrepresented,*
    - *the required annual report and reporting of adverse events was not done timely and accurately,*
    - *new institutional rules, national legislation or international conventions deem it necessary.*

The Ethics Committee would like to remain at your service as scientist and researcher, and wishes you well with your project. Please do not hesitate to contact the Ethics Committee for any further enquiries or requests for assistance.

Yours sincerely

Prof M M J Lowes
(chair NWU Ethics Committee)