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# Quantitative analysis of catecholamines and their metabolites in human urine by gas chromatography - mass spectrometry as a screening method for sympatho - adrenal tumors.

by

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Submitted in fulfillment of the requirements for the degree, MSc Chemical Pathology in the Faculty of Health Sciences, University of Pretoria

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Dedicated to Jean,

*"…may your hands always be busy*
*may your feet always be swift*
*may you have a strong foundation when the winds of change shift*
*may your heart always be joyful*
*may your song always be sung*
*may you stay forever young…"*

*Bob Dylan*

# Table of Contents

## Chapter 3



## *Gas Chromatography-Mass Spectrometry*

**Summary**

The endogenous catecholamines and their metabolites play an integral role in establishing the presence or absence of a suspected sympatho-adrenal tumor. Highly elevated metabolites excreted in the urine are indicative of a tumor. For this reason numerous analytical methods has been developed to accurately quantify the levels of these compounds. However, current methods usually make use of conventional HPLC methods. Although effective, these methods require tedious sample preparation and are usually plagued by interferences. It was the aim of this work to develop a gas chromatographic – mass spectrometric (GC-MS) method that allow for the simultaneous analysis of the endogenous catecholamines, their basic and acidic metabolites using a single extraction procedure (which is easy to use and not tedious) with minimal derivatization steps. Furthermore, to develop GC-MS methods which do not require tedious sample preparation and yet be sensitive and accurate and allow for rapid analysis in the clinical pathology laboratory. Four different gas chromatographic - mass spectrometric methods were developed for the analysis of the catecholamines and their metabolites and are discussed in detail.

**Opsomming**

Benewens hul funksie as hormone in die endokriene stelsel van die mens, is die katecholamiene ook waardevolle tumor merkers. Wanneer uitermatige hoë vlakke van die metaboliete aangetref word

is die teenwoordigheid van ‘n tumor gewoonlik vanselfsprekend. Verskeie chromatografiese metodes is oor die jare ontwikkel wat die katecholamiene asook hul metaboliete kan kwantifiseer. Die metodes is doeltreffend maar die monster voorbereiding is tydsaam en weens die gebruik van vloeistof chromatografiese detektors is steuring van ander produkte ‘n algemene verskynsel. Die doel van hierdie werk was om ‘n gas chromatografiese metode te ontwikkel wat gelyktydig katecholamiene asook hul basiese en suur metaboliete kan analiseer met behulp van massa spektrometrie deur gebruik te maak van minimale monster voorbereiding. Benewens laasgenoemde was die doel ook om metodes te ontwikkel wat vinnig en eenvoudig, tog sensitief en doeltreffend is, en wat met gemak gebruik kan word in die kliniese laboratorium. Vier verskillende gas chromatografiese metodes wat gebruik maak van massa spektrometrie is ontwikkel en word beskryf in hierdie werk.

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# Chapter 1

## *Introduction*

The endogenous catecholamines and their metabolites are renowned biological markers for the assessment of tumors in mankind[1]. It is well known that hyper excretions of these compounds in urine are indicative of tumors arising in the sympatho-adrenal system. Currently almost every clinical pathology laboratory has an analytical screening method available for detecting catecholamine secreting tumors.

The last two decades saw a plethora of analytical methods developed to screen for sympatho-adrenal tumors. It is generally accepted that chromatographic techniques, either liquid or gas chromatographic, surpassed early spectrophometric methods which were flawed by interferences. Many of these chromatographic techniques were "fine tuned" over the last two decades to minimize interferences and to allow for adequate detection of the low levels of catecholamines and metabolites found in human urine. Attempts were made, and are still currently underway, to minimize tedious sample preparation to allow for fast and efficient analysis in the laboratory.

As time passed specific catecholamine and metabolites excretion patterns were identified[1] for the specific tumors arising in the sympatho-adrenal system allowing for fast and specific screening techniques. In fact, we have by far a better understanding of the tumors as well as which catecholamines and metabolites to screen for than twenty years ago.

This work describes and investigates the analytical techniques employed in screening for catecholamine secreting tumors. It focuses on gas chromatographic techniques coupled to mass spectrometry for the analysis of catecholamines and their metabolites in human urine. It was the aim of this work to develop a gas chromatographic – mass spectrometric (GC-MS) method that allow for the simultaneous analysis of the endogenous catecholamines, their basic and acidic metabolites using a single extraction procedure (which is easy to use and not tedious) with minimal derivatization steps. Furthermore, to develop methods which do not require tedious sample preparation and yet be sensitive and accurate and allow for rapid analysis in the clinical pathology laboratory.

# Chapter 2

## *The sympatho-adrenal system and its tumors*

2.1 Introduction

- 2.1.1 The adrenal gland
- 2.1.2 The peripheral nervous system
- 2.1.3 Catecholamines and the nervous system.

2.2 Biochemistry of the catecholamines

- 2.2.1 Synthesis and storage
- 2.2.2 Metabolism and excretion
- 2.2.3 Physiological properties

2.3 The pheochromocytoma

- 2.3.1 Introduction
- 2.3.2 Prevalence
- 2.3.3 Histopathology
- 2.3.4 Disorders associated with pheochromocytomas
- 2.3.5 Catecholamine secretion in pheochromocytomas

2.4 The neuroblastoma

- 2.4.1 Introduction
- 2.4.2 Prevalence
- 2.4.3 Catecholamine secretion in neuroblastomas

2.5 Conclusion on sympatho-adrenal tumors

2.6 Current analytical techniques and their shortcomings

## 2.1 Introduction

The sympatho-adrenal system includes the medulla of the adrenal gland as well as the ganglia of the sympathetic nervous system[1]. It is embryologically derived from the neural crest tissue and therefore the designation: Tumors of the neural crest, when generally speaking of catecholamine secreting tumors.

### 2.1.1 The adrenal gland

The adrenal gland is composed of two endocrine glands, the inner medulla as well as the outer cortex. Whereas the medulla is responsible for the synthesis and secretion of epinephrine and norepinephrine, the cortex is arranged into three layers responsible for the synthesis and secretion of the steroid hormones (figure 2.1) [2]. The three layers of the cortex include: the zona reticularis and zona fasiculata that secrete the glucocorticoids (corticosterone and cortisol) as well as the androgens (dehydro-epiandrosterone and androstenedione) and the zona glomerulosa, which is responsible for the secretion of aldosterone.

It might seem ludicrous why the adrenal gland is responsible for secreting two different types of hormones; therefore one might ask whether there is any physiological connection between the medulla and the cortex. In fact there is adequate scientific proof to support

the hypothesis that the adrenal cortex plays a role in the formation of catecholamines in the medulla.

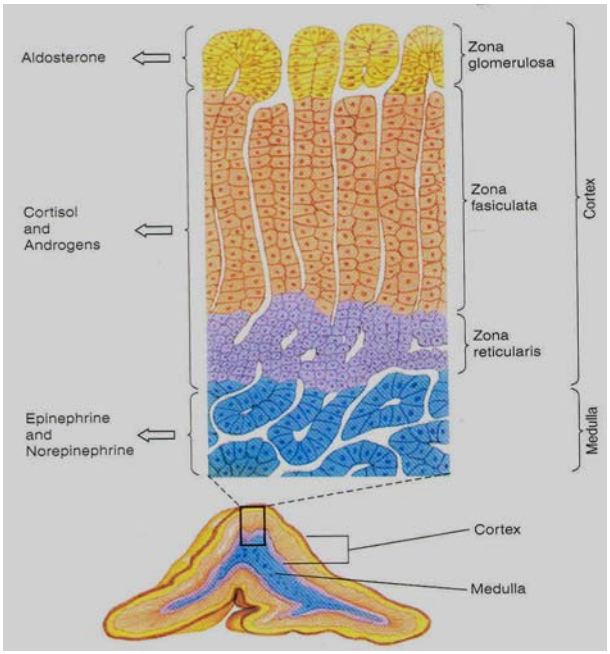



Figure 2.1: Section through the adrenal gland showing the medulla as well as the three layers of the cortex and the hormones secreted [2].

R.E Coupland [3] was the first to suggest that high concentrations of glucocorticoids are responsible for the synthesis of epinephrine due to an increase in phenylethanolamine-N-methyl transferase (PNMT), the enzyme responsible for the conversion of norepinephrine to epinephrine.

This hypothesis was drawn from the fact that the medulla has a dual blood supply, however, both these blood supplies first cross the cortex before reaching the medulla. The first route is via the

medullary arteries, which lead directly through the cortex into the medulla. The second and more gradual supply is via small vessels that permeate through the zona reticularis. These vessels subsequently connect to venous sinuses, which flows into the medulla. It was shown that the chromaffin cells of the adrenal gland, which contain epinephrine, receive most of their blood supply via the cortico-medullary sinuses and therefore contain high concentrations of corticosteroids[3]. The corticosteroids are thought to increase the PNMT levels thus resulting in the conversion of norepinephrine to epinephrine.

Later work also supported this view; when rats were injected with hydrocortisone, epinephrine emerged in extra-adrenal chromaffin tissue[4] and the PNMT activity in extra-adrenal chromaffin tissue also increases when hydrocortisone is administered[5]. Furthermore, high concentrations of corticosterone stimulated the storage of epinephrine in extra-adrenal chromaffin tissue, which mainly store norepinephrine (noradrenaline) rather than epinephrine[6] (adrenaline). Therefore, there is adequate evidence to suggest that the adrenal medulla and cortex are dependent on each other and not merely two separate glands apart from each other despite their difference in hormone secretion.

### 2.1.2 The peripheral nervous system

All vertebrates maintain their physiological homeostasis by two control systems: the endocrine - and the nervous -systems. The

nervous system is anatomically divided into two divisions: the central nervous system, which includes the brain and spinal cord, as well as the peripheral nervous system. The peripheral nervous system includes all the nerves responsible for transmitting signals between the central nervous system and the body's glands, muscles and sense organs collectively known as effector organs.

The peripheral nervous system includes 43 pairs of nerves. These include 12 pairs of cranial nerves and 31 pairs of spinal nerves. A nerve consists of nerve fibers, which includes the axons of efferent - or afferent -neurons or a combination of the two. Therefore the peripheral nervous system is further divided into two divisions: the efferent division, which relays signals from the central nervous system towards an effector organ, and the afferent division which transmits signals from receptors towards the central nervous system.

The efferent division of the peripheral nervous system is further divided into two divisions as shown in figure 2.2. These include: the somatic nervous system which innervates skeleton muscle, and the autonomic nervous system which innervates smooth or cardiac muscle, glands and gastrointestinal neurons. The main differences between the somatic and autonomic nervous system is outlined in figure 2.3.

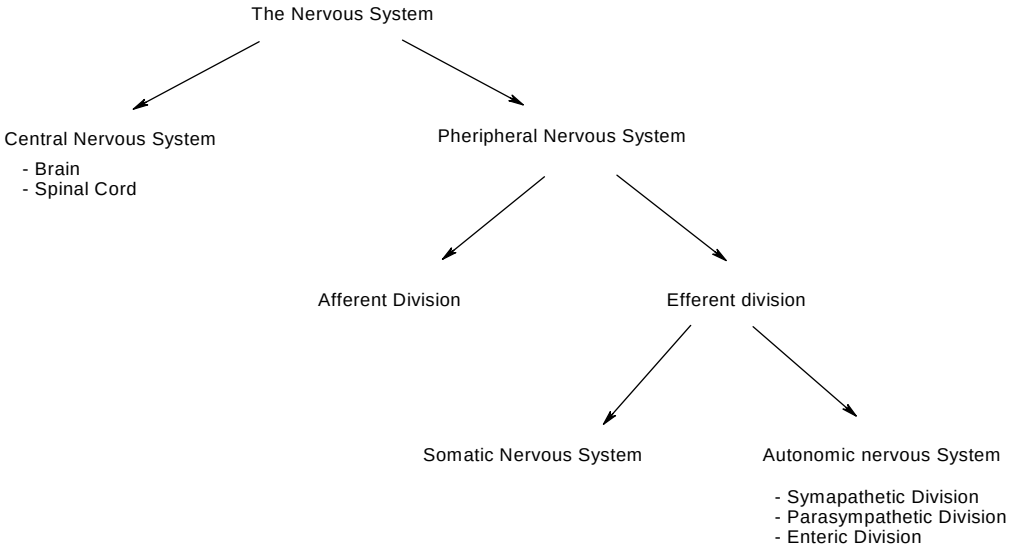



Figure 2.2: Organization of the nervous system. Functional differences of afferent and efferent neurons divide the peripheral nervous system in two divisions. Anatomical and physiological differences divide the efferent division further in to the somatic and autonomic nervous systems, which differences are illustrated in figure 2.3.

Physiological and anatomical differences divide the autonomic nervous system further into three divisions: sympathetic, parasympathetic and enteric. The sympathetic and parasympathetic nerve fibers originate and leave the central nervous system at different levels. Sympathetic nerve fibers originate in the gray matter of the spinal cord and leave the central nervous system from the thoracic and lumbar regions of the spinal cord. Parasympathetic nerves originate and leave the central nervous system in the brain and in the sacral region of the spinal cord.

Integration of the adrenal medulla and sympathetic nervous system is shown figure 2.4. The adrenal medulla has been called a special

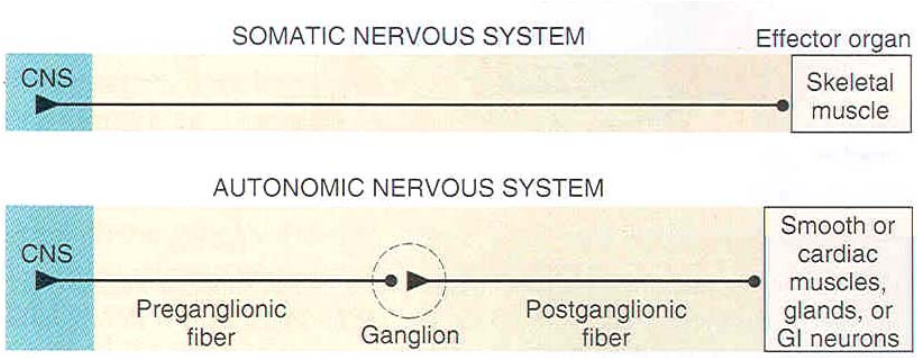



Figure 2.3: The main differences of the somatic and autonomic nervous system[2]. The somatic nervous system is composed of a single neuron, which innervates skeleton muscle (the effector organ). It can only lead to excitation of its effector organ. The autonomic nervous system is composed of a link (via a ganglion) between the central nervous system and the effector organs. It can lead to either excitatory or inhibitory actions of its effector organs.

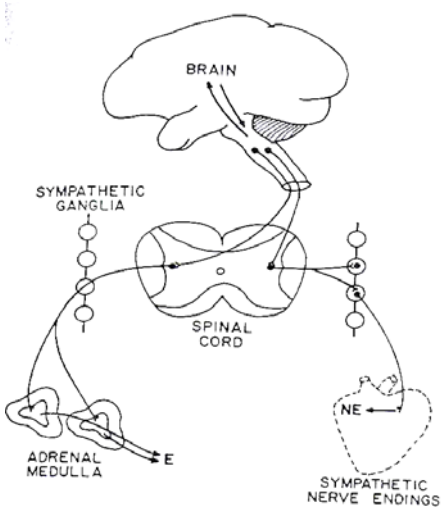



Figure 2.4: Integration of the adrenal medulla and the sympathetic nervous system[1]. A sympathetic stimulus results in release of catecholamines from nerve endings and the adrenal medulla. This however is not the only way of catecholamine release as later discussed. (Figure adapted from reference 1).

"ganglion" since it can be classified as a group of postganglionic neurons in the sympathetic nervous system that never developed axons and secondly, because secretion of epinephrine and

norepinephrine is under control of sympathetic preganganglionic nerve fibers.

### 2.1.3 Catecholamines and the nervous system.

Besides their well known physiological functions as hormones, the catecholamines also play a vital role as neurotransmitters in the nervous system. Norepinephrine is the major neurotransmitter between the postganglionic nerve fiber and effector cell in the sympathetic system whereas acetylcholine is the major neurotransmitter in the parasympathetic system[2]. Acetylcholine also acts as the major neurotransmitter between the pre- and post-ganglionic fibers in both the sympathetic and parasympathetic systems. Dopamine acts as a neurotransmitter in the central nervous system.

## 2.2 Biochemistry of the catecholamines

### 2.2.1 Synthesis and storage

Chemically the catecholamines can be defined as 1-R-3,4-dihydroxybenzenes where R signifies the position of an amino group[7]. It is perhaps appropriate to give credit to Vulpian[8] who in 1856 first "identified" the presence of epinephrine in the adrenal glands when he observed a "substance" which yielded a green color when exposed to iron (III) chloride. Not surprisingly he thought it to be phenol but failed to extract it. Much later, in 1897, the so-called "*blood pressure raising constituent of the suprarenal capsules*" was identified as a catecholamine[9]. Shortly thereafter, Aldrich and

Takamine simultaneously and independently identified this "substance" as 3,4-dihydroxyphenyl-2-methylaminoethanol[10,11]. Aldrich named it epinephrine and Takamine adrenaline and ever since this potent hormone has been stuck with two names.

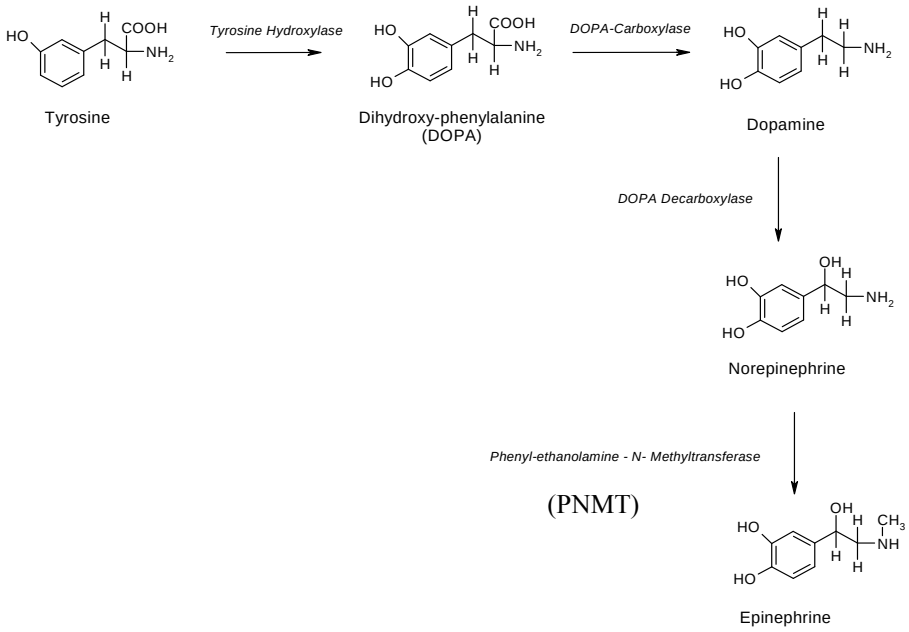



Figure 2.5: Biosynthesis of the catecholamines as originally described by Blaschko. Even though he had no proof to support his hypothesis that the pathway went from tyrosine via DOPA to a primary amine that would be methylated later work using radioactive tracers confirmed his hypothesis.

Biosynthesis of the catecholamines occurs mainly in the adrenal medulla, the sympathetic neurons and the brain. The precursor of all catecholamines is L-tyrosine. The biosynthesis of catecholamines, as shown in figure 2.5, were first postulated by Blaschko [12] and although he had no proof to support his hypothesis, later work with radioactive tracers showed that his hypothesis was indeed correct[13].

Subsequent to their synthesis the catecholamines are stored in either the chromaffin granules of the adrenal gland or in membrane bound vesicles of post ganglion neurons[14]. Here they are protected from enzymatic degradation by adhering to proteins called chromogranins prior to their exocytotic release by sympathetic stimulation. Note that this is not the only way of catecholamine release, a dynamic equilibrium exists between release and re-uptake of catecholamines by the vesicular monoamine transporters as will be discussed later. Non-exocytotic release of norepinephrine has been postulated[7,15]. The latter is as a result of tyramine, a so-called sympathomimetic amine, which apparently has the ability to displace norepinephrine from its storage vesicles[15].

The main storage site for epinephrine is the chromaffin tissue of the adrenal medulla where it makes up 85 % of the stored catecholamines. As mentioned previously extra-adrenal chromaffin tissue usually contains only norepinephrine. The main site of norepinephrine storage is the post-ganglionic sympathetic nerve endings while dopamine is found throughout the central nervous system. Although known to be present in the sympatho-adrenal system, it should be noted that this is not the only site of dopamine synthesis. In fact, the gastrointestinal tract, pancreas and spleen accounts for a large part of dopamine production[16,17].

### 2.2.2 Metabolism and excretion patterns in urine

Metabolism of norepinephrine and epinephrine.

Early studies on catecholamine metabolism were mainly done by istotopic tracer studies. Much of these pathways are still accepted as being correct and even published in textbooks and review articles, even though research over the last two decades showed that catecholamine metabolism is more complex as previously thought[18, 19]. A recent article summarized these *facts and fallacies* regarding catecholamine metabolism[17].

The most common misunderstanding is the deamination of norepinephrine and epinephrine by monoamine oxidase (MOA) to yield vanillylmandelic acid (VMA). The pathway is generally described as deamination of norepinephrine and epinephrine to form 3,4-dihydroxymandelic acid (DOMA) followed by o-methylation to form VMA as shown in figure 2.6. This is generally considered as the main route of VMA production. In fact, VMA is mainly produced by oxidation of 3-methoxy-4-hydroxyphenylglycol (MHPG) [2, 20] as shown in figure 2.7.

There is however reason for this misunderstanding. Early work on catecholamine synthesis and metabolism is accredited to Blaschko[12] who later showed the involvement of amine oxidases on a range of amines that led to the formation of acidic end products[21]. A few

years later, in 1957, Armstrong *et al* found a patient with a norepinephrine tumor who excreted large amounts of VMA in the urine[22]. Therefore, VMA was shown to be the end product of norepinephrine. Furthermore, Homovanilic acid (HVA) was shown to be the end product of dopamine[23]. With these discoveries it is easy to understand why the notion still persist that metabolism occur mainly by two enzymatic pathways: catechol o-methyltransferase and monoamine oxidase as shown by figure 2.6.

In addition, it is now also clear that the liver plays an important role in VMA production[24]. The liver contributes to more than 94% of the total amount of VMA formed. The majority of the VMA produced in human liver originates by metabolism of MHPG and DHPG, only a small percentage accounts for metabolism by norepinephrine and epinephrine[25], indicating that VMA is produced mainly by oxidation of MHPG and not by oxidation of DOMA, nor – and – metanephrine as shown in numerous textbooks.

The second common misunderstanding is that catecholamines are metabolised at sites different from their synthesis and secretion. This can be attributed to the so-called *vesicular leaking* of catecholamines from their storage granules into the cytoplasm. However, the majority of cateholamine metabolism occurs in the cells where they are synthesized [17].

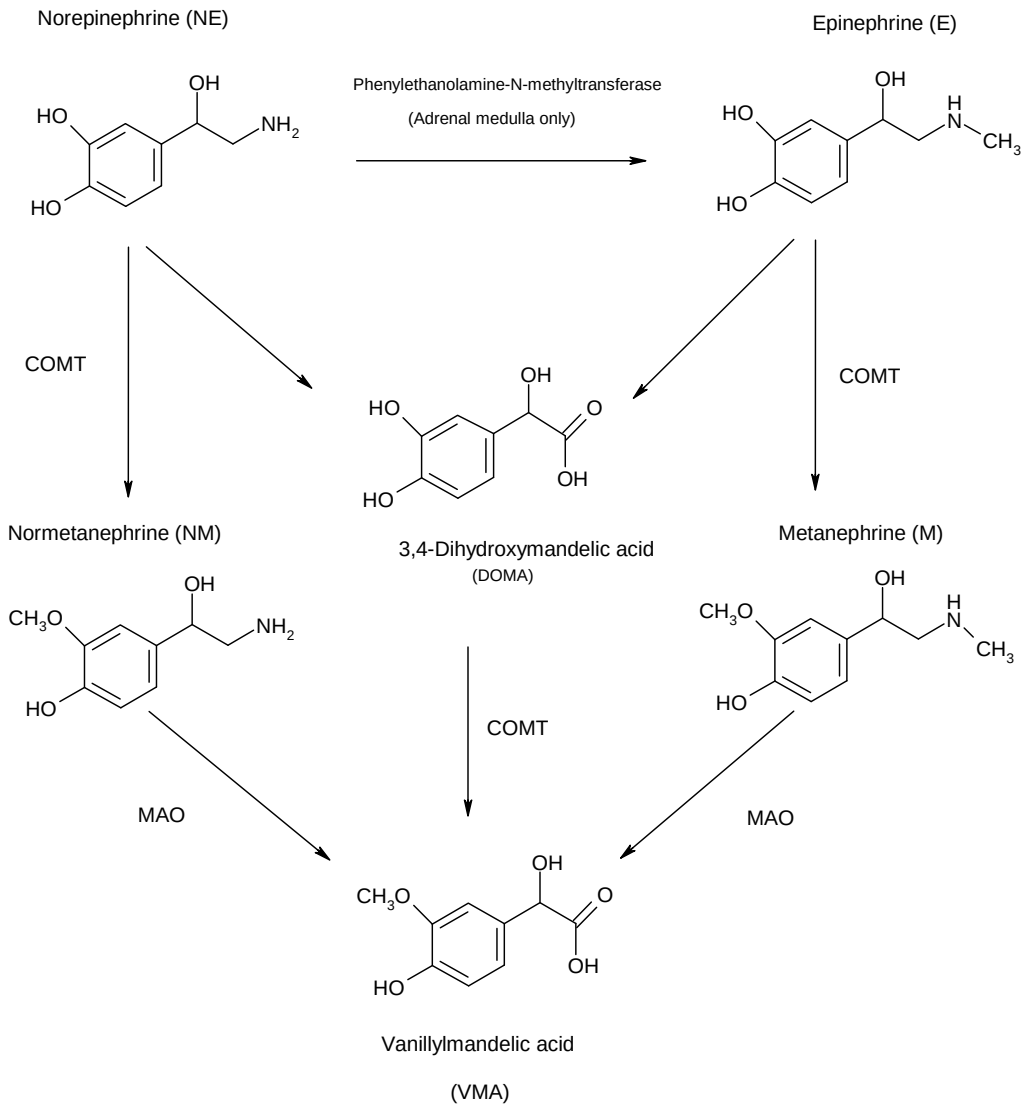



Figure 2.6: Incorrect presentation of norepi – and – epinephrine metabolism as shown by textbooks[17]. The major route of VMA production is not via DOMA but via MHPG and DHPG as shown in figure 2.7.

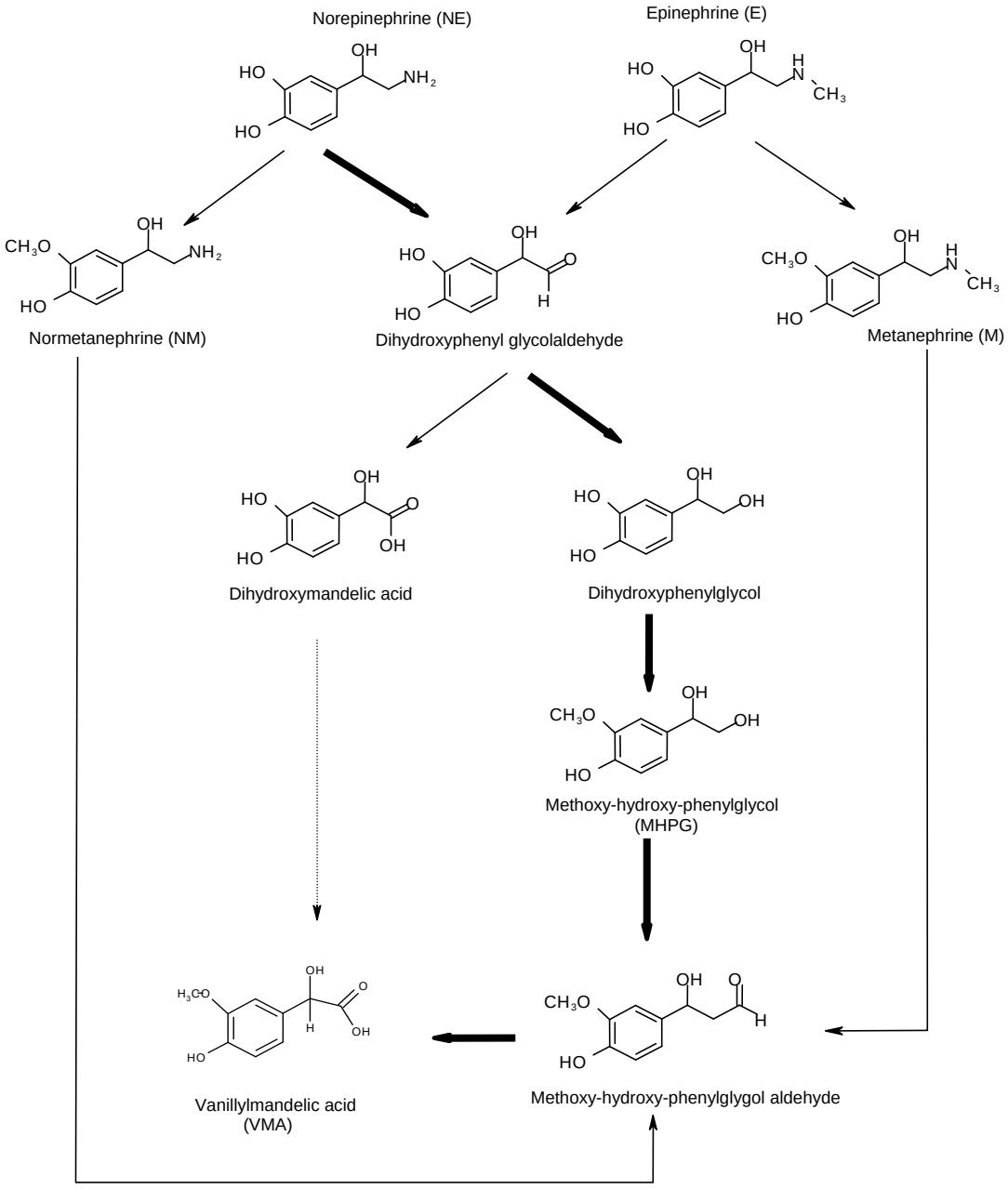



Figure 2.7: Correct metabolism of norepinephrine and epinephrine as determined during the last two decades. The "bold" arrow indicates the major route of VMA production. Only a small percentage of VMA is formed by DOMA[17].

This is due to the vesicular monoamine transporter, which transport the *leaking* catecholamines back to the vesicles where they were originally synthesized. For example, in the heart the monoamine transporter transports approximately 90 % of the *leaking catecholamines* back into the storage vesicles[26]. This is also true for norepinephrine secreted from sympathetic nerves where approximately 70 % is transported back to the site of synthesis[27].

The third false impression is that the vesicular stores of catecholamines remain inactive until a stimulus causes secretion. As described above, and as with most biological systems, there is in fact a fine equilibrium between catecholamines in the extra-cellular space and those in vesicular stores, which is regulated finely by the vesicular monoamine transporters.

Dopamine metabolism.

Metabolism of dopamine occurs by two enzymatic pathways, monoamine oxidase (MAO) and catechol -o- methyl transferse (COMT) to ultimately form homovanillic acid (HVA) as well as 3,4-dihydroxyphenyl acetic acid (DOPAC). Homovanillic is the major end product of dopamine. The majority HVA is formed outside the liver by the two enzymes MAO and COMT. Metabolism outside neural tissue by COMT results in the formation of 3-

methoxytyramine. The metabolism of dopamine is shown in figure 2.8.

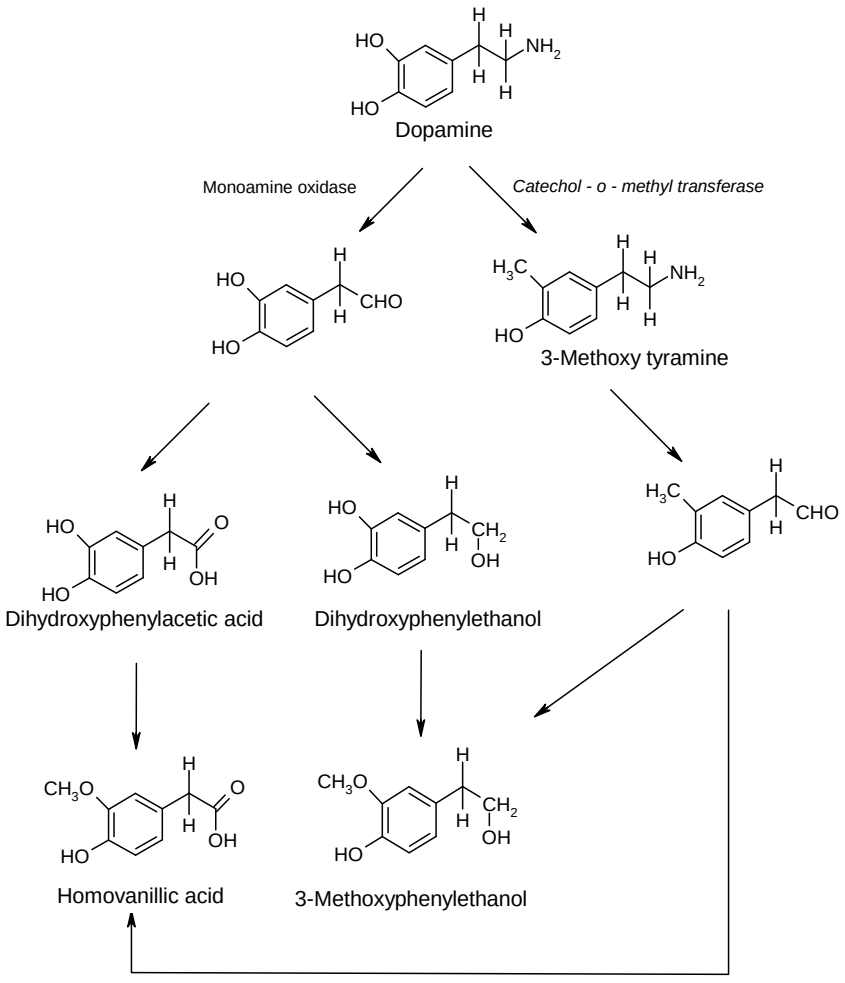



Figure 2.8: Metabolism of dopamine[7]. The major acidic end product is HVA. The formation of 3-methoxytyramine occurs outside neural tissue via COMT.

Excretion patterns of catecholamines in urine.

The catecholamines and their metabolites are excreted in urine either in the free (unconjugated) amine form or as sulfur - and

glucuronide - conjugates. Dopamine is the major unconjugated catecholamine excreted in urine while norepinephrine, epinephrine and the metanephrines are excreted in their conjugated form. The acidic metabolites, HVA and VMA, are not conjugated in urine [28].

In urine the amount of metabolites excreted predominates over the catecholamines with HVA and VMA the most abundant metabolites followed by the metanephrines. Epinephrine is the least abundant catecholamine found in urine.

### 2.2.3 Physiological properties

Catecholamines exert their physiological response by binding to adrenergic and dopaminergic receptors[28]. The adrenergic receptors include α- and β-subtypes which are responsible for binding epinephrine and norepinephrine. The α-receptors are capable of binding both epinephrine and norepinephrine while the β-receptors interact mainly with epinephrine[28].

Apart from their function as sympathetic neurotransmitters the catecholamines play a central role in maintaining optimal physiological conditions. Both epinephrine and norepinephrine have cardiovascular and metabolic effects. While epinephrines' are mainly metabolic, norepinephrine has mainly cardiovascular effects[29].

Norepinephrine functions as a hypertensive agent. It is responsible for vasoconstriction and causes an increase in peripheral

resistance[29]. However, norepinephrine's physiological effect on the heart is much smaller than epinephrine's. While epinephrine causes an increase in pulse rate as well as cardiac output norepinephrine doesn't, in fact the cardiac output may even fall due to the rise in peripheral resistance.

Epinephrine and norepinephrine both share metabolic functions but epinephrine is more efficient in causing the response. Generally speaking epinephrine causes a rise in overall metabolism. It aids in the conversion of glycogen to glucose, helps in the breakdown of fat to free fatty acids and glycerol and promotes glycogenolysis in skeletal muscle [2]. The glucose, free fatty acids and glycerol all serve as substrates for the increase in metabolism.

Perhaps the most renowned physiological effect of epinephrine is the so-called "*fight or flight*" response caused during a rapid onset of stress or anger. In such a condition epinephrine causes the vessels in the heart and skeleton muscle to dilate while the vessels in the skin contract resulting in a fall of peripheral resistance, and an increase in heart rate as well as systolic blood pressure[25]. Why some people "*fight*" while others "*flight*" remain a mystery, the physiological effect however is mediated by epinephrine.

Dopamine's main physiological function is that of a neurotransmitter in the central nervous system. Together with serotonin it also functions as a hormone of euphoria, for example:

during smoking nicotine binds to the acetylcholine receptors thereby blocking their function as "control gates" for the flow of dopamine; the result: a continues flow of dopamine through the channel and thus the feeling of euphoria associated with smoking[30]. Dopamine also functions as a hypo-physiotropic hormone since its secretion from the hypothalamus inhibits the secretion of prolactin from the anterior pituitary [2].

The major metabolites of the above mentioned catecholamines have no significant physiological properties. They do however hold clinical importance as markers for tumors arising in the sympatho-adrenal system as described below.

**2.3 The Pheochromocytoma**

2.3.1 Introduction

"*In 1954, the physicians at a British teaching hospital asked the clinical chemistry laboratory to set up a test for detecting phaeochromocytomas. The head of the department undertook the task himself. After calibrating the method with standard solutions of noradrenaline, the clinical chemist decided to establish a reference range for normal individuals. He obtained specimens of urine from fit members of his staff, and assayed them. They all had only small effects on the test object. Finally, he decided to assay his own urine. To his surprise, the test object produced an enormously exaggerated response. Since he felt well, he evidently did not think highly of the specificity of the method and did not introduce it into his*

*laboratory's repertoire. About 2 years later, the laboratory bought its first fluorimeter and by this time there was a fluorimetric method available for measuring catecholamines in urine. The clinical chemist set up this method and again assayed urine specimens from members of his staff and from himself. As before, he found that his own urine contained vastly more catecholamines than that of any member of his staff. Although he still felt well, he decided to consult a physician colleague, but only a few days later, and before he had arranged for a formal consultation, he was sitting at home quietly reading his news paper in the evening when he had a cerebral infarction. He died shortly afterwards, and at the subsequent post mortem examination he was found to have a phaeochromocytoma"*[29]

The above situation shows the insidious onset of pheochromocytomas which may stay dormant before they suddenly develop to their full activity. It also illustrates the complexity of identifying a sympatho-adrenal tumor. In this case the patient "*found himself to be fit*" and although it is not clear whether he was suffering from hypertension it shows that the symptoms of sympatho-adrenal tumors are sometimes so small that patients simply don't seek medical advice.

### 2.3.1 Prevalence

Pheochromocytomas are rare tumors. Their incidence was once reported as 0.8 per 100 000 persons per year [31]. However, one should not be misguided by these statistics since another study,

conducted over a 50-year period, showed that out of 54 autopsy cases only 13 (24%) patients were diagnosed correctly in life[32]. Reasons for these "clinically unsuspected" pheochromocytomas include:

1. The ability of the tumor to stay dormant before suddenly developing into full activity as shown in the above case. They are predominant during the fourth and fifth decades of life.
2. The pheochromocytoma is a secondary cause of hypertension making it difficult to diagnose since clinical features include: sweating, palpitations and headaches, the same as for primary hypertension.
3. Laboratory tests are not specific for pheochromocytoma. For example, reports are now available indicating pheochromocytomas with normal urinary catecholamine excretion patterns. In these cases the measurement of the metanephrines have shown to increase the sensitivity.[33,34]
4. Although rare, the so-called normotensive pheochromocytomas do exist. Several attempts have been made to explain these normotensive pheochromocytomas. These include:

- Pheochromocytomas that secrete large amounts of epinephrine,
- Tissue receptors that show tolerance towards catecholamines
- Pheochromocytomas with predominant secretion of dihydroxyphenylalanine as well as dopamine[35].

The above mentioned clearly indicates that diagnoses of pheochromocytoma is not merely a routine exercise of screening every patient with hypertension, careful and specific steps are needed to make a proper diagnosis. The reader is referred to a recent article that explains this scientific approach and difficulties in diagnosing a pheochromocytoma[36].

### 2.3.2 Histopathology

The pheochromocytomas first description dates back to 1886 and is accredited to Frankel[37]. The term "pheochromocytoma" was invented by Pick in 1912[38] and originates from the pheochromocyte (a cell that develops a dark color when exposed to chromium salts) while cytoma denotes cancer.

The tumor arises from the chromaffin cells of the sympatho-adrenal system. It is found predominantly in the adrenal medulla (85-90%) while extra-adrenal tumors are rare (10-15%) [29]. The main extra-adrenal sites include the paraganglia cells of the sympathetic nervous system or the organ of Zuckerkandl [31]. For reasons not known one study showed that right-sided pheochromocytomas occur twice as much as left sided ones[39].

On a morphological level the adrenal tumors are much larger, average 100 mg in weight and 10 cm in diameter, as opposed to extra-adrenal pheochromocytomas that are less than 5 cm in diameter [29]. On a microscopic level the tumor consists of chromaffin

cells and it contains large amounts of chromaffin granules, which are known to store catecholamines. It is generally accepted that the adrenal pheochromocytomas are mainly benign, less than 10 % are malignant while the extra-adrenal, hereditary and those found in children have a higher incidence of malignancy [29].

### 2.3.3 Disorders associated with pheochromocytomas

Neoplasm disorders associated with pheochromocytomas are not uncommon. Pheochromocytoma has been associated with multiple endocrine neoplasia (MEN) type 2A and 2B, von Hippel-Lindau disease (VHL) and neurofibromatosis[40]. Both MEN-2 and VHL are autosomal dominant inherited diseases associated with pheochromocytoma. MEN-2A is associated with carcinoma of the thyroid, pheochromocytoma and hyperparathyroidism and was first described by Sipple[41] (Sipple's syndrome). MEN-2B is associated with carcinoma of the thyroid, pheochromocytoma and mucosal neuromas while VHL includes renal carcinoma, pancreatic cysts and pheochromocytomas.

It should be noted that the pheochromocytomas associated with MEN-2 and VHL differ in their biochemical, morphological and clinical presentation[42]. On a biochemical level, patients with MEN-2 pheochromocytomas show higher m-RNA expression of both phenylethanolamine *N*-transferase and tyrosine hydroxylase, which subsequently lead to higher plasma epinephrine and norepinephrine levels as opposed to VHL tumors. Not surprisingly, MEN-2

pheochromocytomas show distinctively higher plasma metanephrine levels when compared to VHL pheochromocytomas, which only show elevated normetanephrine. Morphologically, secretory granules in MEN-2 pheochromocytomas are more dense than those found in VHL pheochromocytomas. Furthermore, MEN-2 pheochromocytomas contain both norepinephrine and epinephrine secretory vesicles whereas VHL pheochromocytomas contain mostly norepinephrine granules. With these biochemical and morphological differences taken into account the clinical picture associated with MEN-2 and VHL pheochromocytomas become clearer: Patients with MEN-2 pheochromocytomas show periodic spells of hypertension, attributed to the higher tyrosine hydroxylase activity and phenylethanolamine *N*-transferase, as opposed to sustained hypertension in VHL pheochromocytomas. To date, the reason for these tumor differences in MEN-2 and VHL are unknown but a possible, and logic, explanation is specific mutation-dependant differences in the gene expression relating to catecholamine synthesis.

### 2.3.4 Catecholamine secretion in pheochromocytomas

In 1926 Dr Mayo draw attention to "*toxins*" that are "*affecting the sympathetic"* when evaluating the first disectable pheochromocytoma at the Mayo clinic[25]. Shortly thereafter it was established that the tumor secreted substances that are similar to epinephrine and in 1949 it was shown that apart from epinephrine the pheochromocytoma also secretes norepinephrine.

The last decade saw numerous scientific papers being published with reference to which catecholamines one should measure to accurately determine the presence of a pheochromocytoma. However, before explaining which test is superior, it is perhaps appropriate to distinguish between free, fractionated and total catecholamine metabolites prior to explaining the findings of these scientific articles.

Much of this "free" versus "total" confusion arises from earlier spectrophometric methods which couldn't distinguish between the catecholamine metabolites and therefore quantified the metabolites together and reported the value as a single entity, hence the terminology "total metanephrines". This term was replaced by "fractionated metanephrines" a designation for meta – and -normetanephrine since chromatographic methods are able to distinguish the two compounds from each other [43]. The fractionated metanephrines can be further divided into two groups, free and conjugated, since conjugation of metanephrine and normetanephrine occur via a sulfutransferase isoenzyme (SULT1A3) in the gastrointestinal tract[43]. Acid hydrolysis is sufficient for deconjugation of the metanephrines. Therefore, in modern scientific articles (employing chromatographic techniques), fractionated metanephrines denotes to the sum of the free and conjugated metanephrines, following an acid hydrolysis step. A recent article explained this difference with regard to the diagnoses of

pheochromocytomas [43]. The figure below illustrates the difference between free, total and fractionated catecholamine metabolites.

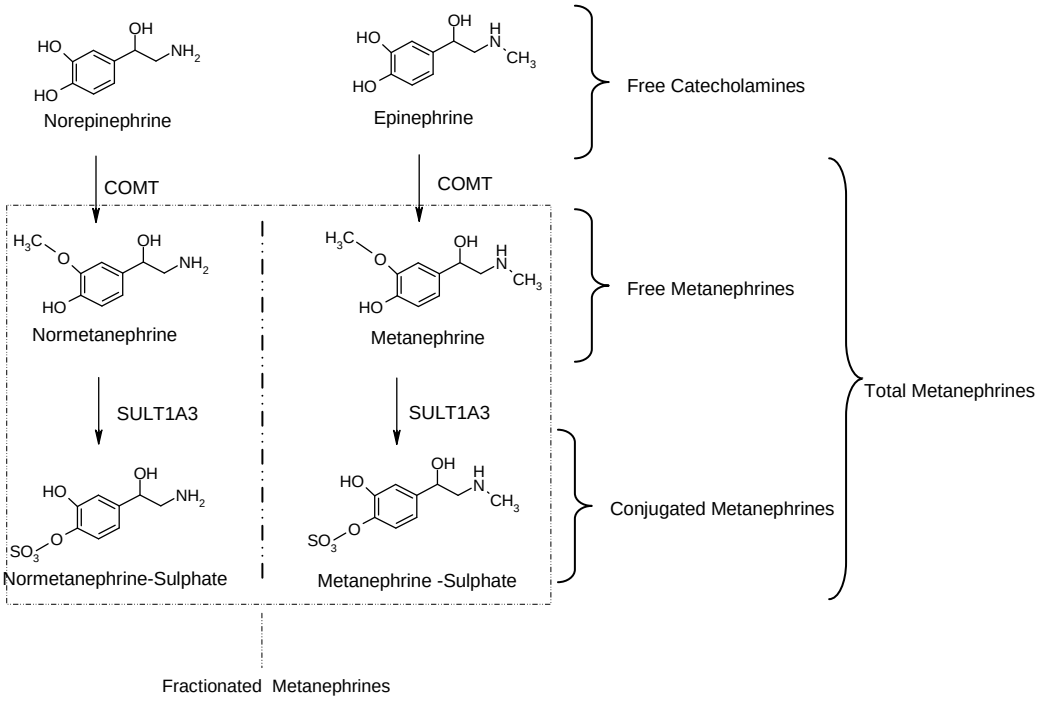



Figure 2.9: The difference between free, total and fractionated metanephrines[43]. The term total metanephrines was replaced by fractionated metanephrines, denoting to the sum of the free and conjugated metanephrines following acid hydrolysis.

Accurate and rapid detection of pheochromocytomas are now required from analytical laboratories and therefore numerous articles have focused on the value of analytes providing the best sensitivity and specificity in screening for these tumors. In screening for pheochromocytomas the sensitivity of the assay is of more importance than selectivity since a false-positive result can be ruled out during the imaging technique, after a positive screening result.

However, an assay with low sensitivity will result in a high rate of false-negative results, which can be fatal towards human life.*

A recent article[44] evaluated the plasma and urinary excretion values of 859 patients of whom 214 had confirmed pheochromocytomas. The diagnostic sensitivity of the plasma metanephrines assay was the highest (99%) followed by urinary fractionated metanephrines (97%). Urinary VMA had the lowest sensitivity (64%), i.e. it is a poor screening test. The highest specificity was obtained by the analysis of urinary VMA (95%) followed by total urinary metanephrines (93%) and plasma catecholamines (81%) while urinary fractionated metanephrines had the lowest specificity (69%). These results agreed with other findings, for example: In a report of 19 patients with pheochromocytoma the sensitivity of fractionated urinary metanephrines was 100 %[45]. However, two patients showed normal urinary VMA excretion values and three patients had no increase in norepinephrine and epinephrine. Furthermore, five patients showed only a marginal increase in the urinary norepinephrine values and three patients only a marginal increase in urinary epinephrine values.

In another study[46], with 6 confirmed pheochromocytoma cases, the urinary VMA values were only elevated in two cases and in one case only marginally increased. Fractionated urinary metanephrines

* Specificity and sensitivity are two terms that differ in the analytical and diagnostic settings. For this reason these differences are clearly described in appendix 1 of this work.

were significantly increased in three cases; one case had no data for the metanephrines. This significant increase of fractionated metanephrines agreed with a previous study which showed a 100 % sensitivity value when analyzing fractionated urinary meta – and – normetanephrine[47]. The authors also warned against the use of VMA as the only screening test for pheochromocytomas due to the low sensitivity.

The above findings yield two important, yet explainable, questions. Firstly, why is VMA not a sensitive marker? Secondly, why is plasma free and urinary fractionated metanephrines not implemented in every screening laboratory?

The answer of the first question lies in the metabolism of nor – and –epinephrine. The majority of VMA produced during metabolism originate from metabolism of circulating MHPG and DHPG in the liver and not in the adrenal glands making it a relatively insensitive marker towards a pheochromocytoma. However in patients with pheochromocytomas more than 94 % of the increased plasma metanephrines come from metabolism of catecholamines within the tumor itself since the tumor cells contain the enzyme catechol-o-methyltransferase[48]. Thus the production of the metanephrines in the pheochromocytoma is described as an independent ongoing process in the tumor itself[48]. This also explains why in the examples above the metanephrines were shown to be considerably elevated.

The reason why plasma metanephrines are superior to urinary fractionated metanephrines in diagnosis of pheochromocytomas, is due to the fact that a large amount of sulfate-conjugated normetanephrine is produced in other parts of the body than the adrenal medulla or pheochromocytoma cells[48] i.e. elevated non - conjugated plasma metanephrines can only be due to a tumor.

Why plasma and / or fractionated urinary metanephrines are not the first choice of screening method is difficult to answer. Perhaps the most appropriate answer is due to the skill required to quantify these metabolites via high performance liquid or gas chromatographic methods as opposed to conventional spectrophometric methods used to quantify VMA. Furthermore, the concentration of these metabolites in urine and in plasma is in the parts per million and parts per billion-region respectively making sample handling and sample work-up a challenge for most laboratories. Finally, early analytical techniques described were aimed at only quantifying the catecholamines; a notion that still persists in numerous laboratories.

## 2.4 The Neuroblastoma

### 2.4.1 Introduction.

Perhaps the most common association with the neuroblastoma is the fact that it is the most common malignant tumor found in children. The original discovery of the neuroblastoma in an infant dates back to 1901 when a post mortem on a four-week-old infant uncovered a tumor of the right suprarenal gland together with an enlarged liver [49]. Unlike the pheochromocyoma the neuroblastoma is known to be malignant. It's a fast growing tumor, spreading rapidly through the body making early diagnosis vital. It has been reported that if the tumor is detected in the advance stages, the five-year survival rate falls to less than 50 % even with the help of extensive therapy[50]. Furthermore, prognosis is low if the tumor is detected after the age of two and has been estimated to be as low as 20 %[51]. The reason for the poor prognosis is not merely coupled to age, but rather to the stage of the tumor; in children over the age of two the tumor has already spread to the distant lymph nodes, bone marrow and soft tissue as shown in table 2.1.

### 2.4.2 Prevalence

Data of the prevalence vary from 10 per 1 million births[52] to 1 in 30 000 – 40 000[53]. One scientific article states that 1 out of 7600

children in Austria are affected with a neuroblastoma[54]. For reasons unknown the prevalence is higher in boys than in girls[29].

### 2.4.3 Histopathology

The neuroblastoma is a tumor derived from the sympathetic ganglia, which arise from the sympathagonia cells[29]. The latter spreads from the neural crest tissue during the embryonic stages of life and therefore a neuroblastoma can be found wherever there is sympathetic nervous tissue. The tumor is found predominantly in the abdomen regions of the infant. The main site of the tumor is the adrenal glands, contributing to 39 % of all neuroblastomas found in children. Other sites include the chest (14.6 %), neck (3.2 %) and head (0.2%)[55]. An international staging system, as originally described by Evans *et al*[56], classifies the tumor into five stages as shown in table 2.1.

### 2.4.4 Catecholamine secretion properties of neuroblastomas

The catecholamine secretion patterns of neuroblastomas differ considerably from that of pheochromocyomas. In fact after its original discovery in 1901[49] it was only in 1957 when it was shown that a neuroblastoma secreted large amounts of adrenaline[57]. Shortly thereafter, a neuoblastoma was found to sectrete large amounts of norepinephrine[58]. Currently the neuroblastoma is labeled as a "dopamine secreting" tumor with elevated levels of the acidic

Table 2.1: Staging system of neuroblastoma as originally described by Evans[55].

| Stage: | Description of tumor |
|---|---|
| 1. | Tumor confined to the original organ of origin |
| 2. | Tumor extended beyond the organ of origin, tumor does not cross the midline |
| 3. | Tumor extended across the midline; lymph nodes may be affected. |
| 4. | Metastasis of tumor to distant lymph nodes, skeleton, bone marrow and soft tissue. |
| 4S. (Special) | Patients under the age of 1 with a localized tumor as in stages 1 and 2, however metastasis is restricted to the liver, skin and bone marrow. |

catecholamine end products, HVA as well as VMA, used in the diagnoses[50-53].

Screening projects initiated in Japan[59] as well as in Canada[60] used the analysis of both VMA and HVA in order to establish the presence of a neuroblastoma. The study in Quebec Canada reported a specificity of 99% using thin layer chromatography as an initial screening method followed by gas chromatography-mass spectrometry (GC-MS) for borderline and positive cases. Interestingly, the Quebec study reported 15 cases diagnosed clinically under the age of 3 weeks, merely due to increased awareness of the neuroblastoma. Furthermore, the value of plasma

L-DOPA, the precursor of the catecholamines, in screening for neuroblastomas has been documented [61,62]. In both studies the plasma DOPA levels were increased in patients with neuroblastomas.

## 2.5 Conclusion on sympatho-adrenal tumors

Sympatho-adrenal tumors are life threatening if not detected early. Although rare, pheochromocytomas have the ability to stay "silent" before suddenly developing into their full malignant capacity. Fortunately, there is adequate scientific evidence published during the last decade with reference to which metabolites one should measure to diagnose a pheochromocytoma accurately.

Unlike pheochromcytoma the majority of neuroblastomas found in children are malignant. Early detection of this tumor in infants is vital since prognosis decrease as the child grows older. Screening programs for neuroblastomas have been implemented and these have shown a decrease in fatality rates of children born with neuroblastomas.

Various analytical methods to screen for these tumors have been documented. These techniques, their application in a clinical laboratory, as well as their shortcomings are discussed in the following section.

## 2.6 Current analytical techniques and their shortcomings

### 2.6.1 Early methods

The chemical quantification of epinephrine date back as far as 1935 when J.C Whitehorn used a colometric assay for the determination of epinephrine in blood[63]. Proteins were first precipitated and the epinephrine was measured by the blue color produced on heating the sample with arsenomolybdic acid. Later Lund used an oxidation technique to form adrenolutine, which was measured by fluorescence detection[64]. In 1952 Weil-Malherbe and Bone developed an ethylenediamine condensation technique, which measures epinephrine, norepinephrine and dopamine utilizing fluorescence detection[65]. The above-mentioned methods formed the basis for the detection of catecholamines. Other early methods included radiochemical techniques such as oxidation techniques[66], radio-enzymatic techniques[67] as well as radio immunoassays[68]. Although sensitive, these methods have been replaced by more conventional chromatographic techniques since the earlier methods required labeling techniques to quantify the catecholamines. Furthermore, antibodies intended for the catecholamines also interfere with other compounds of similar structure in the immunoassays.

### 2.6.2 High performance liquid chromatography

Currently the method of choice in numerous clinical laboratories is high performance liquid chromatography (HPLC) coupled with an electrochemical detector (ED). This method has found many applications in the analyses of catecholamines due to the high sensitivity and selectivity obtained during analyses. Much of the selectivity lies in the electrochemical properties of the catecholamines, which are utilized to their full extent to quantify the catecholamines[68]. For the catecholamines these electrochemical reactions occur quickly resulting in high signals during analyses. Few other substances interfere electrochemically with the catecholamines, thus a high signal to noise ratio is obtained.

#### HPLC-electrochemical detection methods

Hawley *et al* set the hallmark of electrochemical detection after they studied the electrochemical properties of catecholamines under different pH conditions[69]. Over the years numerous articles were published utilizing HPLC techniques with electrochemical detection.

Riggin and Kissinger used acid washed alumina combined with ion-exchange chromatography with HPLC-ED to detect E, NE and D in human urine[70]. However, the recovery of this method was low (51%) mainly due to the extraction with alumina, which is known to

yield low recoveries. Odink *et al* were able to detect E, NE, D and salsolinol, a metabolite of D, using a single clean-up procedure by cation-exchange chromatography[71] while Binder and Sivorinovsky used anion-exchange chromatography as an extraction technique to quantify VMA and HVA in urine[72]. Simultaneous measurement of the free cateholamines and the metanephrines were carried out by Violin[73] using electrochemical detection.

It is clear from the above-mentioned that only selected metabolites are measured in each of the analyses. This is mainly due to the chemical properties (acid and basic) of the catecholamines and their metabolites.

**HPLC- Fluorescence detection methods**

HPLC techniques with fluorescence detection (HPLC-FLD) also found widespread use[74,75,76] since the catechol group has fluorescence activity with an excitation wavelength of 285 nm and an emission wavelength of 320-325 nm. Although fluorescence detection is sufficient for quantitative analyses, the detection limit of HPLC-ED out performs HPLC-FLD. One advantage of fluorescence detection above electrochemical detection is simplified method development. HPLC-ED requires volt-amperograms to establish the optimal voltage for more sensitivity a task that is tedious. This is not required during fluorescence detection.

It is clear that current HPLC techniques have the following limitations:

1. They require extensive sample clean-up prior to analysis which makes the procedure laborious and expensive to incorporate in the clinical pathology laboratory.
2. Beside tedious sample preparation techniques chromatographic interferences still persist due to detector limitations.
3. Only selected metabolites can be analyzed due to mobile phase and detector limitations.

For these reasons it was decided to employ GC-MS methods to circumvent these shortcomings and thus it was the aim of this work to develop a method that allow for the simultaneous analysis of the endogenous catecholamines, their basic and acidic metabolites using a single extraction procedure (which is easy to use and not tedious) with minimal derivatization steps. Furthermore, to develop GC-MS methods which do not require tedious sample preparation and yet be sensitive and accurate and allow for rapid analysis in the clinical pathology laboratory.

The fundamentals of GC-MS are described in the following chapter. Derivatization techniques applied in the analysis of the catecholamines and their metabolites are also discussed.

# Chapter 3

*Gas Chromatography-Mass Spectrometry*

3.1 Gas Chromatography

3.2 Design and operation of the GC system

3.3 Parameters affecting gas chromatography
- 3.3.1 Column Length
- 3.3.2 Internal diameter
- 3.3.3 Film thickness
- 3.3.4 Stationary phase
- 3.3.5 Carrier gas and flow rate

3.4 Gas chromatography inlet techniques used in this work

- 3.4.1 Split injection
- 3.4.2 Split less injection

3.5 Column selection and applications for the analysis of catecholamines

3.6 Mass Spectrometry

3.7 Design and operation of the mass spectrometer

3.8 Ionization techniques

- 3.8.1 Electron Impact ionization
- 3.8.2 Negative Chemical Ionization

3.9 The MS quadrupole

3.10 Coupling gas chromatography to mass spectrometry: A powerful technique

3.11 Derivatization techniques used in gas chromatography- mass spectrometry

3.12 Derivatization techniques applied for the analysis of catecholamines and their metabolites

3.13 Conclusion

## 3.1 Gas chromatography

In 1901 the Russian botanist, Mikhail Semyonovivich Tsvet, first described the technique of chromatography at the XI Congress of Naturalists and Doctors in St. Petersburg[77]. His first written account of this separation technique was in 1903 in the Proceedings of the Warsaw Society of Naturalists[78]. However, it was not until 1906 that he first used the term chromatography in his paper entitled: "Physical chemical studies on chlorophyll adsorption"[79]:

> *"Like the light rays of the spectrum, the different components of a pigment mixture in the calcium carbonate column will be separated regularly from each other, and can be determined qualitatively and also quantitatively. I call such a preparation a chromatogram and the corresponding method the chromatographic method…"*[79]

In his second paper of 1906 he uses both the terms *chromatograms* as well as *chromatographic analysis*, yet he doesn't describe the meaning of these terms [80]. It is accepted that he developed the term from Greek nomenclature, chroma (colour) and graphein (to write), an appropriate name if one visualizes what Tsvet saw during the separation of chlorophyll and carotenoids from plant mixtures. However, in Russian the name "Tsvet" literally means "colour" and therefore chromatography can be translated to: *Tsvet's writing.* Whether Tsvet had the intention to name the technique after himself remains a mystery. Today, the term chromatography is defined as "*a separation method that exploits the differences in partitioning*

*behavior between a mobile phase and a stationary phase to separate the components in a mixture* [77]"

The science of chromatography has expanded into a multi-million dollar industry ranging from routine analysis towards sophisticated analysis in the forensic, clinical and pharmaceutical – industries. This chapter describes basic chromatographic concepts as well the application of gas chromatography mass spectrometry towards the analysis of catecholamines in the clinical laboratory.

Basic chromatographic concepts such as: the distribution coefficient, chromatographic separation, capacity factor and the concept of theoretical plates are not within the scope of this work and are explained in detail in any textbook relating to chromatography [81].

## 3.2 Design and operation of the GC system

Gas chromatography, the most widely used analytical technique in analytical chemistry, has become a pivotal tool for any analytical chemistry laboratory. Its popularity is mainly due to its high sensitivity, high resolution obtained and relative ease of operation. In gas chromatography the mobile phase is an inert gas, usually helium or nitrogen, which is responsible for the transfer of volatile compounds through the column. The compounds of interest interact whith the stationary liquid phase where they separate from each

other due to different absorptive properties for the stationary phase as well as different vapour pressures.

A basic gas chromatographic (GC) system is shown in figure 3.3.

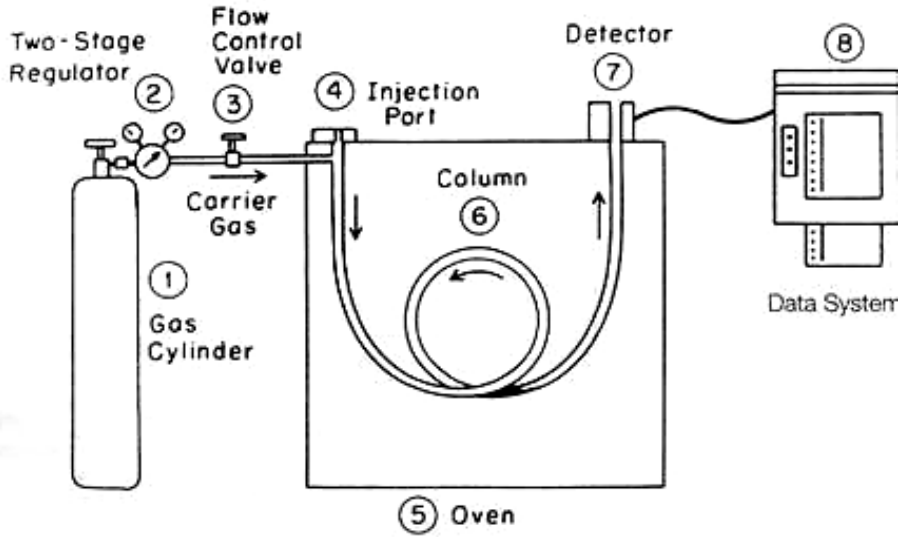



Figure 3.1: The basic design of a GC system, regulated gas supply by a gas cylinder is used as the mobile phase while separation occur in the column which is situated in the oven[81].

## 3.3. Parameters affecting gas chromatography

### 3.3.1 Column length

Column length in gas chromatography plays an important role in the efficiency as well as speed of chromatography. Since the number of plates, *N,* is related to the column length, *L,* increasing the length will increase the number of theoretical plates and therefore the efficiency of the column. However, doubling the column length only increases the resolution by the square root of the length change, i.e. doubling the column length increase resolution by the square root of two[81]. Long column length does help in improving resolution; however, the drawback is of course long analysis time. Table 3.1 summarise the effects of column length on resolution and analysis time[81].

Table 3.1: The effect column length has on resolution and the speed of analysis

| Column Length | Resolution | Speed of Analysis |
|---|---|---|
| Long (60 – 100 m) | High | Slow |
| Medium (25 – 30 m) | Medium | Medium |
| Short (5 – 10 m) | Moderate | Fast |

### 3.3.2 Internal diameter

As previously described capillary columns can be viewed as "open tubes" with a stationary "lining" on the inside. Thus, decreasing the internal diameter (i.d.) of this tube will increase efficiency as well as retention since the compounds in the gas phase has less open space to travel, i.e. more interaction between the stationary liquid phase occur. The speed of chromatographic analysis also increases. One disadvantage of columns with small internal diameter is over loading due to limited capacity for the compounds to be separated. The standard i.d. of capillary columns are 250µm, narrow bore columns generally have i.d.'s of 100µm while macrobore i.d.'s are in the order of 530µm. Table 3.2 summarise the effects of column i.d on GC analysis.

Table 3.2: The effect of internal diameter on the resolution, speed and capacity of GC analysis.

| Column Diameter | Resolution | Speed | Capacity |
|---|---|---|---|
| 100 µm | Excellent | Excellent | Fair |
| 250 – 320 µm | Good | Good | Good |
| 530 µm | Fair | Good | Excellent |

### 3.3.3 Film thickness

Film thickness can be defined as the amount of liquid stationary phase of the column. A thick film will allow for a large capacity and increases the retention time of the compound. Thick films are generally used for the separation of natural gas components such as methane, ethane and propane. One disadvantage of thick films is decrease in efficiency. Furthermore, due to the fact that the compounds now spend more time in the stationary phase high temperatures are required to elute the compounds and this causes column bleed.

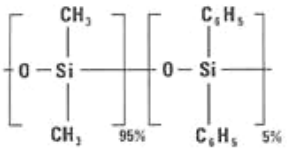
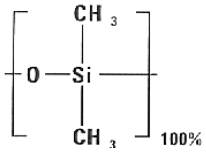
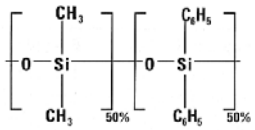
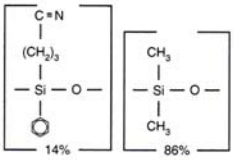
The effect of a thin film thickness, short column and small diameter GC column was applied in section 4.3.4 of this work.

### 3.3.4 Type of stationary phase

Before any analysis an important fact to consider is which stationary phase should be used. The primary requirement of the stationary phase is to insure adequate interaction between the stationary phase and the compounds to be separated. More specifically the stationary phase should have a high selectivity, $\alpha$ - factor, towards the compounds of interest. A general rule is usually that “like dissolves like” i.e. for non-polar compounds a non-polar stationary phase will be used. The polarity of the stationary phase also influences the order of elution; in fact the order of elution can be reversed when

using a stationary phase with different polarity. The main stationary phases used in GC analysis are shown in table 3.3 and the column selection applied for the work conducted is described in more detail in section 3.6

Table 3.3: The main stationary phases used in GC analysis and some of their applications.

| Stationary Phase: | Polarity | General Applications |
|---|---|---|
| DB – 5<br>(5%-phenyl-95%-dimethylpolysiloxane)<br>  | Non Polar | Most widely used column<br>Applications include: drugs of abuse, general hydrocarbons |
| DB – 1<br>(100% dimethylpolysiloxane)<br>  | NonPolar | Hydrophobic compounds such as steroids, hydrocarbons, petroleum products |
| DB 17<br>(50%-phenyl-50%-methylpolysiloxane)<br>  | Mid Polar | Suitable for compounds with benzene rings such as pesticides, phenols |
| DB 1701<br>(14%-cyanopropylphenyl-86%-dimethyl-polysiloxane)<br>  | Mid Polar to Polar | Alcohols, acrylates, Organochloride pesticides |

### 3.3.5 Carrier gas and flow rate

The effect of carrier gas and flow rate in GC analysis can be explained by using a van Deemter curve. The curve illustrates the effect of average linear velocity (*u*) on column efficiency (*HETP*). The Van Deemter curves for the three most commonly used carrier gases: helium (He), hydrogen ($H_2$) and nitrogen ($N_2$) is shown in figure 3.5. For each gas an optimum efficiency ($u_{opt}$) exist at a specific linear velocity. This $u_{opt}$ value is where the efficiency is highest (smallest HETP value) and is on the lowest point of the parabolic curve.

The curve illustrates that when using $N_2$ as carrier gas the efficiency will be highest, however, note that a small change in velocity causes a large change in efficiency which and that the $u_{opt}$ value is the lowest for $N_2$ causing analysis time to be lengthy. Consequently $N_2$ is not the preferred gas in GC analysis. The $u_{opt}$ for He is slightly better than that of $N_2$, however, increasing the average linear velocity doesn't cause a dramatic loss in efficiency. From the curve it is evident that $H_2$ would be the best carrier gas for GC analysis. It is not used often due to safety aspects (explosive nature) in the laboratory, which make He the most commonly used gas. Helium was used as carrier gas in this work.

In general, GC analysis is performed at average linear velocities slightly above the $u_{opt}$ value, the so-called optimal practical gas

velocity (OPGV). This OPGV is approximately 1.5 - 2 times the $u_{opt}$ and allows for a decrease in analysis time with only a small loss in efficiency. For this reason the average linear velocity used in this work was approximately 40 cm/sec.

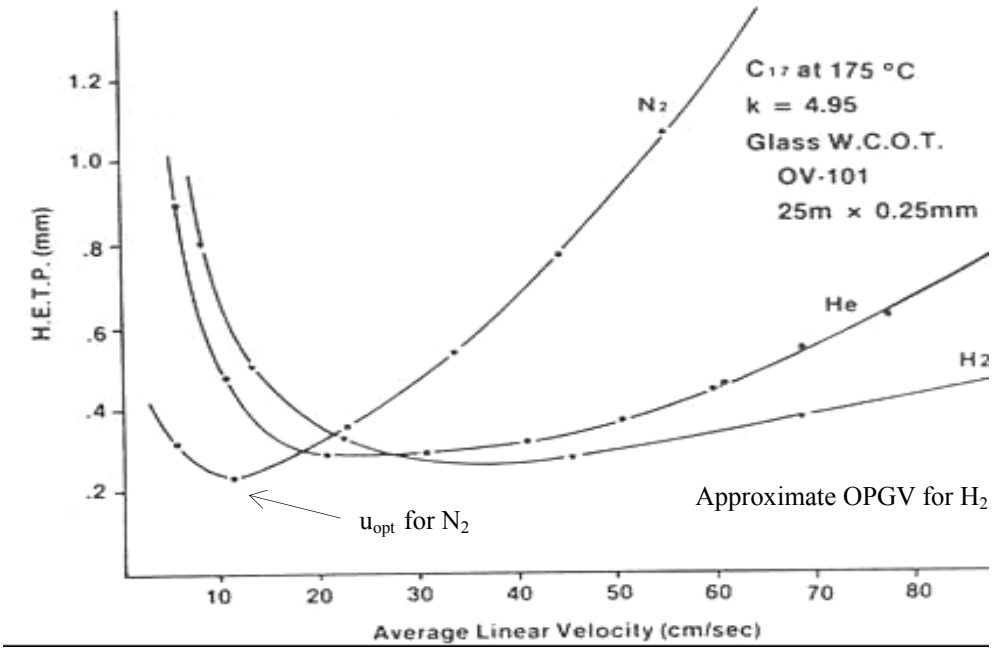



Figure 3.2: A typical van Deemter curve illustrating the effect average linear velocity on the columns efficiency for the three most common gases used in GC analysis[81].

## 3.4 Gas chromatography inlet techniques used in this work

There are two main injection types in GC analysis: split and split-less injection. Their advantages and disadvantages will be described in the following section as well as how to choose the correct injection mode.

### 3.5.1 Split injection

In split injection mode most of the sample injected into the injection port is swept away by the split (purge) valve so that only a fraction of the sample introduced into the system actually reaches the column. Split injection is the injection type used mostly in GC analysis mainly due to the ease of operation. The operator merely has to control the split ratio electronically as opposed to split-less injection where factors such as purge flow rate and septum purge time play an important factor.

The main advantage of split injection is the high resolution achieved due to the small sample amount introduced and the high flow rate before the split. Split injection also has the advantage that the sample concentration can be high; the split injection prevents over load of the column. The major disadvantage of split injection is that it is not particularly feasible for trace analysis since the majority of sample is lost during the split analysis. Figure 3.3 depicts a representation of an inlet system operating in the “split” mode.

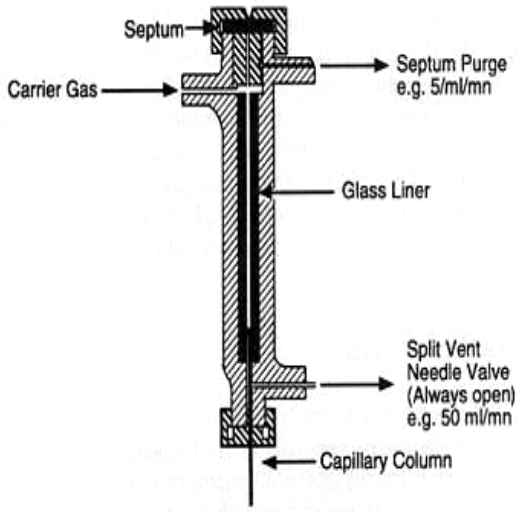



Figure 3.3: Schematic representation of an inlet in the "split" mode [81].

Split injection was used in the analysis of the acidic catecholamine metabolites (VMA and HVA) since the concentration levels of these metabolites were high for GC analysis (mg/L)

### 3.5.1 Split-less injection

In split-less injection the split valve is closed during the injection procedure allowing the entire sample to reach to column. The split valve is only opened after a few seconds. Not surprisingly the main advantage of split less injection is that more sample enters the column making it suitable for trace analysis. One disadvantage is that over loading of the column and the time of opening of the purge valve needs to be investigated; opening it too early will cause sample to be swept away before even entering the column, leaving it closed too long will cause over loading.

Ideally split-less injection requires lower initial oven temperatures to ensure that the entire sample injected is focussed on the first section of the column before starting the temperature program. Split-less injection can therefore result in longer analysis times. Figure 3.4 depicts a representation of an injector in the "split-less" mode.

Split-less injection was used in this work for the analysis of the endogenous catecholamines (E, NE and D) and their basic metabolites (M and NM).

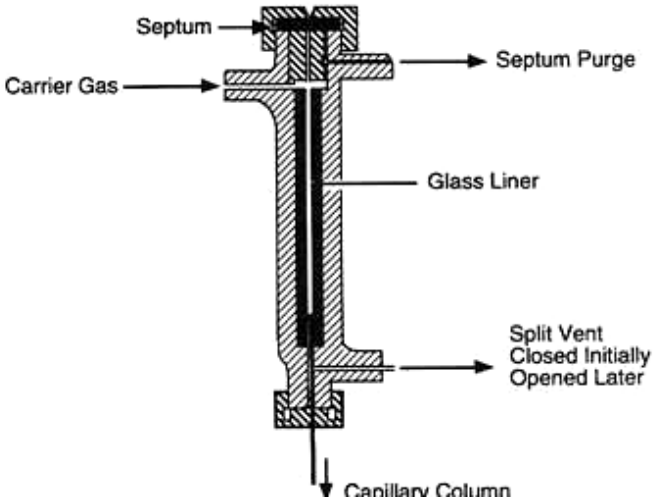



Figure 3.4 Schematic representation of an inlet in the "split-less" injection [81].

## 3.5 Column selection for the analysis of catecholamines

As described in section 3.4, the general rule for selecting a stationary phase is that like dissolves like. Therefore, GC columns

used in the analysis of catecholamines and their metabolites generally include DB-5 and DB-17 capillary columns. The DB-5 stationary phase column is also employed for general use in a clinical laboratory and was therefore employed for catecholamine analysis i.e. other compounds can be analysed without changing the column specifically for catecholamine analysis. The added ring structure in the DB 17 stationary phase makes it suitable for catecholamine analysis. For convenience a DB-5 column should be suitable for catecholamine analysis, however, if the analysis of catecholamines has a dedicated GC instrument (such as in a laboratory screening solely for adrenal tumours) the use of a DB-17 column is advised.

Gas chromatography holds the position as the most widely used analytical technique in the world. An achievement that is likely to remain due to the reliability and numerous applications it has found over the years. In conjunction with highly selective detectors (such as the mass spectrometer described in the following section) it is the analytical method of choice for numerous laboratories. With the ever-demanding competition between manufactures of GC systems it has now become even more affordable and economically feasible to have a GC system for routine analysis in the laboratory.

## 3.6 Mass Spectrometry

> "*At first there were very few who believed in the existence of these bodies smaller than atoms. I was even told long afterwards by a distinguished physicist who had been present at my 1897 lecture at the Royal Institution that he thought I had been 'pulling their legs*."[82] J.J. Thomson (1936).

The origin of mass spectrometry is accredited to Sir Joseph John Thomson who was awarded the Nobel Prize in 1906 for the discovery of the electron[83].

He was also responsible for the construction of the first mass spectrometer, which was initially called a parabolic spectrograph. Figure 3.5 illustrates Thomson performing *m*/*z* experiments on a cathode ray tube[82].

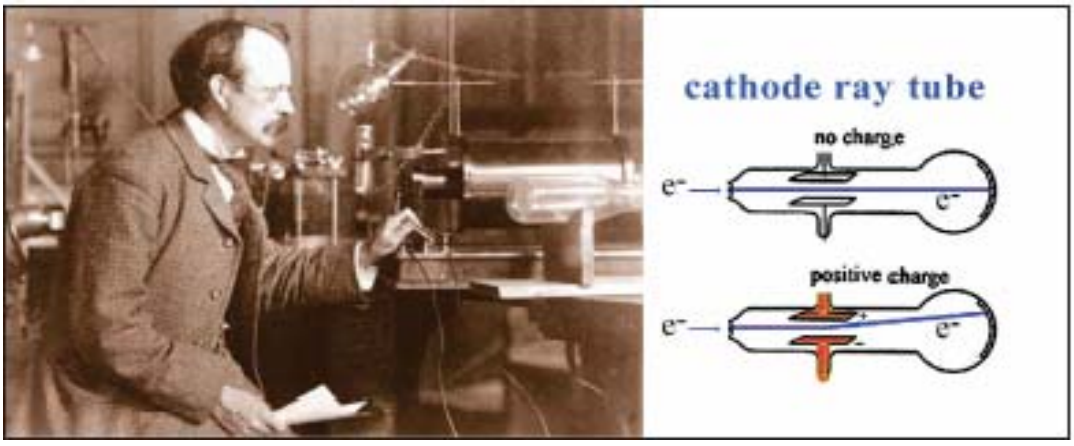



Figure 3.5: Sir Joseph John Thomson performing the "first" mass spectrometry experiments. An electron showed a deflection once an electrical field was applied.[82]

Mass Spectrometry involves the use of a mass spectrometer to measure the mass to charge *(m/z)* ratio of ions. The amount of charged ions created and analyzed is a reflection of the amount of compound of interest and is depicted as a mass spectrum. Since most compounds analyzed has only one charge, the generated *m/z* signal is equivalent to the mass itself.

### 3.7 Design and operation of the mass spectrometer

The basic mass spectrometer of modern days have three basic sections: 1) the ionization compartment, which contains the ion source and focusing lenses, 2) the analyzer, which in this case is a quadruple, and 3) the detector, which is responsible for developing the signal[84].

In mass spectrometry only charged ions are analyzed, therefore a means must exist where the molecules of the sample to be analyzed are converted into charged molecules. This is the function of the ion source in the mass spectrometer, here the molecules of the sample is bombarded with electrons by heated filaments in the ion source. These high-energy electrons literally knock off electrons from the molecules leaving positively charged ions. Only a fraction of the molecules develop a charge, which subsequently needs to be "pulled" towards the quadruples; the function of the positively charged repeller in the ion source. Subsequently the ions travel through a small hole, known as the slit or skimmer, and through an electrically charged focusing lens towards the quadruple.

The quadruple has been described as the *heart of the mass spectrometer* [84]. It consists of four quartz rods spaced opposite each other in the mass spectrometer. Both direct current (DC) and oscillating radio frequency (RF) signals are applied across the rods in order to generate an electric field which is used to select specific ions. A basic quadruple mass spectrometer is shown in figure 3.6.

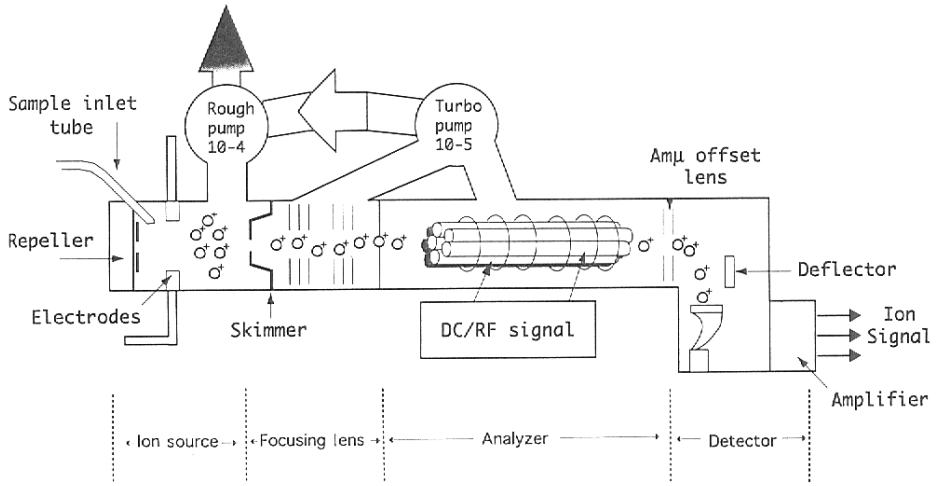



Figure 3.6: The basic components of a quadruple mass spectrometer[84]

## 3.8 Ionization techniques

### 3.8.1 Electron impact ionization

In electron impact ionization (EI) the volatile molecules are bombarded with a high electron field, generally 70 (eV). This high electric field not only develops positively charged ions but also has enough energy to fragment the molecule. These fragments formed are highly characteristic of the ionized molecule and are used to interpret the specific compound of interest. EI is also the most

common ionization technique used in MS analysis and was used in this work.

### 3.8.2 Negative Chemical Ionization.

In negative chemical ionization (NCI) a gas (usually methane or buthane) is delivered into the source and used to attract the ionized electron i.e. the molecules loses an electron by interacting with the positively charged gas leaving them with a negative charge. The NCI technique is generally known as a "soft" ionization technique since the ionization energy is lower than that of EI. One advantage of NCI is the generation of a strong molecular ion that can be used to identify the molecular weight of a specific compound. Secondly, NCI generally has increased sensitivity over EI analysis. Of course not all compounds will yield a strong molecular ion since some compounds simply don't produce stable molecular ions under NCI conditions.

## 3.9 The MS Quadrupole

The ionized molecules subsequently enter the mass spectrometer's quadrupole. In the first step these ionized molecules are literally pushed towards the quadruple, by a repeller plate situated at the back of the ionization source. Subsequently, the ions are focused by

the electrical focusing lenses and are now ready to enter the quadruple.

A MS quadrupole functions by delivering both direct current (DC) and radio frequency (RF) across the rods of the quadruple with adjacent rods having opposite charge.[85] The mechanism of action can be described as follow:

> " *The ion stream entering the quadruple is forced into a corkscrew, three- dimensional sine wave by the quadruple electromagnetic field of the analyzer. The combined DC/RF field applied to the rods is swept together higher (or lower) field strength by the DC/RF generator, upsetting this standing wave for all but a single fragment mass at a given frequency.*"[84]

Once the ions have passed through the MS quadrupole their journey ends by entering the ion detector. An atomic mass unit offset (Amu) is applied behind the MS causing the ions to deflect from their linear path towards the detector. The ions now hit the conductive surface of the detector. Subsequently, a cascade of ions is generated inside the detector that basically enhances the original single-fragment signal. A signal is generated and delivered to the data system, which nowadays is a powerful computer.

## 3.10 Coupling gas chromatography to mass spectrometry: a powerful analytical technique

With the sections on gas chromatography and mass spectrometry completed it is clear that both techniques are powerful tools in the laboratory. It comes as no surprise that a combination of these two instruments (GC-MS) would yield a powerful system; capable of numerous applications in analytical chemistry.

The first successful assembly of a GC-MS was by Roland Gohlke and Fred McLafferty at the Dow Chemical Research Laboratory in 1958.[86] Ever since this breakthrough, GC-MS has become more than a research tool, it has developed into a commercialized field of its own having applications in the clinical, pharmaceutical, forensic and industrial sectors.

In GC-MS analysis the mass spectrometer can be viewed as a highly selective detector with gas chromatography merely a means of separating compounds before entering the MS. Of course poor chromatography will void the use of a powerful MS and vice versa, excellent mass spectra without proper chromatography are futile. It is therefore important not to focus solely on the GC or MS part but rather to find a mean that will result in a highly sensitive means of analysis.

As is with any analytical tool, GC-MS does have its shortcomings. One of these is that derivatization is required for most compounds before analysis. A topic discussed in more detail in the following section. Furthermore, only volatile compounds can be analyzed since a gas is used as mobile phase in GC analysis. Due to the fact that the compound must be in the gaseous phase, GC analysis is not suitable for thermally labile compounds. Mass spectrometers also have their pitfalls: quadruple mass spectrometres have mass limitations (typically up to 800 amu) which make analysis of large molecules (such as peptides) virtually impossible. Of course other mass analyzers such as Time of Flight (TOF) have virtually no mass limits. Furthermore, highly effective vacuum pumps are required for mass spectrometry, which can increase cost of analysis. Finally, interpretation of mass spectra can be described as a skill required over years, which make GC-MS analysis impractical if a skilled person is not available in the laboratory.

### 3.11 Derivatization techniques used in gas chromatography mass spectrometry.

Derivatization is the process where a compound is chemically modified in order to make it suitable for GC analysis. The main reason for derivatization is to create volatile compounds before GC analysis. The second main reason is "*blocking*" polar groups (OH, NH, and SH) on the compound, which tend to adsorb on the active surfaces of the chromatographic system. Derivatization can also aid in enhancing detection of compounds, for example, in NCI detection

halogenated compounds such as fluoride or chloride increase electron affinity. Derivatization also aids in making the compound more stable during the GC analysis. The main derivatization techniques in GC-MS analysis will be described in the following section.

Derivatization by silylation

Silylation is the most common method of derivatization and produces silyl derivatives which are known for their stability. In silylation, the active hydrogen groups are replaced by trimethylsilyl (TMS) groups[87]. The reaction occur by nucleophilic attack and the mechanism is shown below in figure 3.7.

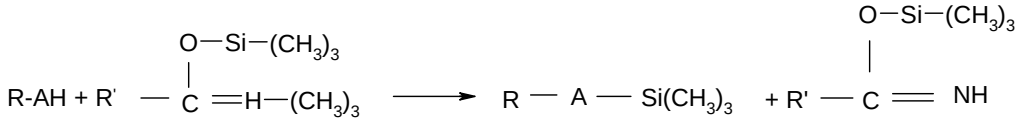



Figure 3.7: Reaction mechanism of silylation where A indicates either –O, -S or -NH groups. The reaction is driven by a good leaving group, the better the leaving group the better the derivatization[87].

One disadvantage of this derivatization procedure is that silylation reagents will react with water and alcohols first, i.e. it is imperative that the sample must be as dry as possible (preferably freeze-dried) before derivatization occur.

Derivatization by acylation.

In acylation the polarity of hydroxyl, amino and thiol groups are reduced by converting the active hydrogen groups into esters. The derivatization technique is usually employed in GC systems with electron capture detectors (ECD). The halogenated compounds substituted onto the compound increases electron affinity for the ECD. One disadvantage is that the acylation reaction is sensitive to moisture, and that the derivatization reaction produces acid by-products, which need to be removed before GC analysis to prevent column damage. A schematic representation of derivatization by acylation with pentafluoropropionic anhydride (PFPA) used in this work is shown below.

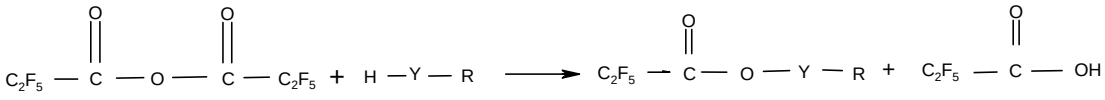


Figure 3.8: Reaction mechanism of acylation by pentafluoropropionic anhydride as used in this work. Y represents – O, -S or –NH groups.

Derivatization by alkylation

Alkylation is generally employed to modify compounds with acidic groups such as carboxylic acid groups and phenols. The active hydrogen is replaced with an alkyl group by nucleophilic displacement. One major advantage of alkyl esters is their stability; the derivatized samples can be stored for longer periods before analysis. The alkylation reaction can also be used in conjunction with acylation or silylation. Furthermore, some alkylation reactions can be done in aqueous solution, such as the use of methyl chloroformate discussed in this work. The major disadvantage is that the reaction mechanism is limited to acidic hydroxyls and amines.

## 3.12 Derivatization techniques applied to the analysis of catecholamines and their metabolites

There are numerous techniques available for the derivatization of the catecholamines and their metabolites. Due to their amphoteric nature, each (or a combination) of the above derivatization procedures methods can be applied. The advantages and disadvantages of each will be discussed in chapter 4 and 5.

Derivatization of catecholamines by silylation

There are not many silylation applications recorded in literature for catecholamines and their metabolites. This may be due to the widespread use of acylation derivatization techniques for these compounds. Some applications of silylation include those of Muskiet *et al* who utilized bis(trimethylsilyl)trifluoroacetamide to derivatize the catecholamines in urine [88]. Hattox and Murphy intensively studied the trimethylsilyl derivatives of the catecholamines and related compounds [89]. The use of *Tert*-butyldimethylchlorosliane/imidazole was also utilized for determination of the acidic metabolites VMA, HVA, MOPEG, 3,4-dihydroxyphenylacetic acid in urine [90] . A recent article used N-methyl-N-(trimethylsilyl)trifluoroacetamine (MSTFA) and N-methyl-bis-heptafluorobutryamide (MBHFBA) to derivatize metanephrine and normetanephine for analysis in urine samples[91]. The duel derivatization increased sensitivity due to the higher molecular weight of the parent fragments. GC-MS analyses were also compared with a standard HPLC assay and it was found that drug interferences in the HPLC method were absent during GC-MS analyses.

Derivatization of catecholamines by acylation.

Acylation reactions have found widespread use in catecholamine analysis. Its popularity is mainly due to the stability and excellent mass spectra that are produced by acylation. In particular, the use of pentafluoro-propionic acid anhydride (PFPA) and pentafluoro propanol (PFP-OH) was found to be the derivatization reagents of choice. The PFP derivatives and mass spectrometric fragmentation patterns for meta – and –normetanephrine were studied by Martin *et al*.[92] Modifications of the derivatization by PFPA were also introduced. This led to enhanced fragmentation patterns of the endogenous catecholamines by incorporating benzylic-o-methylation prior to PFPA derivatization[93]. Other examples of successful PFP derivatization include the work performed by Canfell *et al*, who used a solid phase extraction technique followed by PFPA derivatization for the analysis of metanephrine and normetanephrine in urine[94] and that of Ehrhart and Schwartz who utilized PFPA derivatives for the analysis plasma catecholamines[95] The use PFPA combined with PFP-OH derivatization has also been successfully employed for the derivatization of the acidic metabolites, vanilmandelic and homovannilic acid [96].

Derivatization of catecholamines by alkylation

Derivatization by alkylation has the advantage that derivatization can occur within the aqueous phase, which can aid in the extraction of catecholamines. For example, De Jong and Cramers utilized methyl chloroformate to successfully extract catecholamines from urine samples[97]. Another example is the use of ethyl chloroformate for the derivatization of phenolic amines metanephrines, and indole amines prior to GC analysis[98].

## 3.13 Conclusion

Analysis by GC-MS allow for sensitive quantification of selected compounds due to the high resolving power of each individual technique. The combination of GC-MS analysis allow for relative simple analysis of the catecholamines and their metabolites. These methods, applied to the clinical laboratory setting, will be described in the following chapter.

# Chapter 4

*The application of gas chromatography-mass spectrometry for the analysis of catecholamines and their metabolites.*

4.1 Introduction

4.2 Quantification by the method of standard addition.

4.3 Analytical procedures

4.3.1 A method for the simultaneous analysis of the catecholamines, their basic and acidic metabolites using methylchloroformate followed by silylation.

4.3.2 Analysis of the catecholamines, epinephrine (E), norepinephrine (NE) and dopamine (D) as well as their basic metabolites (M+NM) using solid phase extraction followed by methylation and acylation derivatization techniques.

4.3.3 Extractive acylation of basic catecholamine metabolites, metanephrine (M) and normetanephrine (NM), using pentafluoropropionic anhydride (PFPA).

4.3.4 Analysis of acidic catecholamine metabolites, Vanillmandelic (VMA) – and – Homovanillic Acid (HVA) using pentafluoropropionic acid (PFPA) and pentafluoropropanol (PFP-OH) for the screening of Neuroblastoma.

## 4.1 Introduction

This chapter describes selective GC-MS methods that were developed for accurate quantification of the catecholamines and their metabolites in urine. It was the aim to develop extraction – and – derivatization procedures that are fast, yet sensitive to be incorporated in any clinical laboratory. Each method has its own advantages and disadvantages. Some have more selective extraction procedures with less interference (i.e. good selectivity) and stronger mass spectrometry signals while others are less time consuming but designed for specific metabolites only.

Quantification of all compounds was done by the method of standard addition with the use of deuterium labeled internal standards as discussed in the following section.

## 4.2 Quantification by the method of standard addition.

One of the most important factors that need to be taken before quantification is matrix effects. Urine, generally considered to be a complex matrix, contains a vast amount of salts and numerous other metabolic compounds that can interfere in the analysis. Calibration standards should therefore simulate the matrix as far as possible. A selective extraction procedure should be used, followed by the chosen derivatization procedure. The extraction procedure will not only extract the compounds of interest but also other compounds

with the similar chemical properties. However, selective derivatization and the subsequent chromatographic separation by gas chromatography followed by mass spectrometric detection is usually sufficient to resolve the compounds of interest from the background - a definite advantage of GC-MS analysis.

Stable isotope labeled compounds as internal standards are well known to improve accuracy and precision of assays, since these standards endures the same chemical changes as the compounds of interest during the extraction and derivatization procedures. The "labels" can either be deuterium ($D_x$) or carbon-13 ($C^{13}$). Therefore the use of an isotopically labeled internal standard in an assay is an excellent means of compensating for sample loss and matrix effects during the work-up procedure.

Figure 4.1 illustrates quantification by the method of standard addition with the use of a deuterium labeled internal standard. Peak area was used as a measure of response.

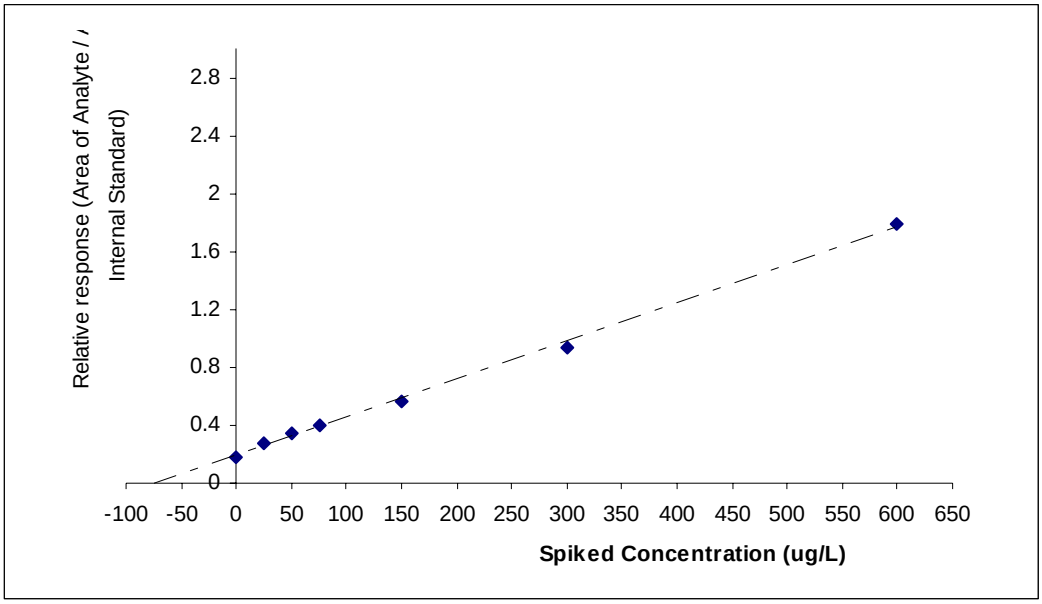



Figure 4.1: Schematic representation of the method of standard addition. The calibration curve crosses the y-intercept at 0.2, indicating the presence of endogenous compound in the matrix.

A pooled urine sample is used to simulate the matrix in the calibration standards. Subsequently, increasing amounts of target analyte is added to aliquots of the sample while the amount of internal standard (deuterium labeled in these assays) is kept constant. As shown in figure 4.1, a calibration curve of these spiked samples in the urine pool indicated an increasing linear response. The blank sample (only spiked with the internal standard) will also show a response; if of course there is endogenous analyte present. The concentration of this endogenous analyte can be obtained by the method of "standard addition". However, employing the spiked samples as calibration standards requires addition of the target analyte to the endogenous sample. In this way the real amount (concentration) can be used as a calibration concentration.

## 4.3 Analytical procedures used

### 4.3.1 A method for the simultaneous analysis of the catecholamines their basic and acidic metabolites

Introduction

As with any chromatographic analysis one always strives to achieve the maximum amount of results from a multi analyte approach. This is not always possible due to extraction as well as derivatization limitations. However, it would be rewarding to have a method available which allow for the simultaneous analysis of the endogenous catecholamines, their basic and acidic metabolites using a single extraction procedure (which is easy to use and not tedious) with minimal derivatization steps. At first glance this might seem a daunting task; the chemical properties of the different catecholamine metabolites differ considerably, making extraction as well as derivatization difficult. Yet such a method is proposed in this section by liquid-liquid extraction and derivatization by means of alkylation followed by silylation.

The procedure involves the use of derivatization of the catecholamines and their metabolites in urine as described by De Jong and Cramers[99] followed by silylation. The method is easy to use and allows for simultaneous analysis of the catecholamines, their basic as well as acidic metabolites in human urine.

## Experimental

### Chemicals and reagents

Epinephrine (E), norepinephrine bitartate salt (NE), dopamine (D), metanephrine – HCl (M), normetanephrine – HCl (NM), vanilmandelic acid (VMA) and homovanillic acid (HVA) were all purchased from Sigma Chemical Co. The derivatization reagents: methyl chloroformate and N-methyl-N-[*tert*-butyldimethylsilyl]-trifluoroacetamine (MTBSTFA) were purchased from the Merck Chemical Co (Germany) and Fluka (Buchs, Switzerland) respectively. Sodium Carbonate ($Na_2CO3$), di-Potasium hydrogen phosphate ($K_2HPO_4$) and ethyl acetate were obtained from Saarchem-Merck.

### Solutions

A stock solution (40 mg/L) were prepared for each compound in HCl solution (0.01 M) and stored at -20$^0$C before use. Working solutions were prepared by serial dilutions of the stock solution and stored at -20$^0$C before use. The deuterium internal standard solutions were prepared in methanol and stored at -80$^0$C.

Calibration Curves

A urine pool from a healthy volunteer was used to construct the calibration curves for E, NE, D, M, NM, VMA and HVA. To an aliquot (800µl) of urine, 100 µl of the working calibration standard as well as 100 ul of the internal standard solution were added. Six-point calibration curves were constructed by using the relative response (peak area) of the analyte versus the deuterium labeled internal standard.

Deconjucation

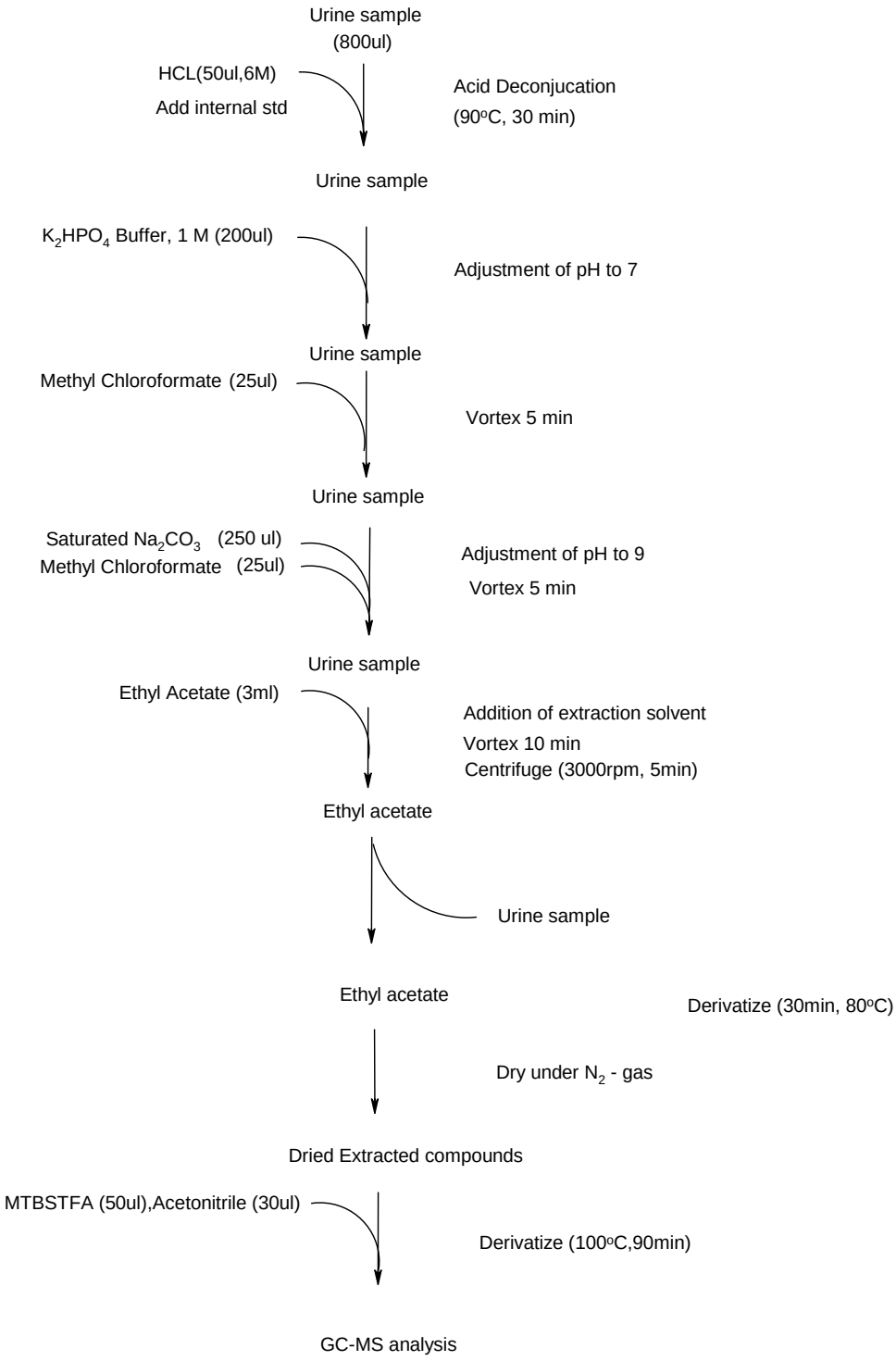
Acid deconjucation was performed by adding HCl (50 µl, 6M) to the sample and by heating at 90°C for 25 min. The sample was allowed reach room temperature before the extraction procedure commenced.

Extraction procedure

The extraction procedure involves the use of liquid-liquid extraction for the simultaneous extraction of the catecholamines and their metabolites by selected derivatization within the aqueous phase.

A flow diagram of this extraction procedure is shown on the following page.

Figure 4.2: Flow diagram of the extraction procedure for simultaneous analysis of the catecholamines their basic and acidic metabolites.



First the pH is adjusted to pH 7 with the addition of a $K_2HPO_4$ buffer (1 M, 200 µl). This pH adjustment is necessary to aid in the first reaction which involves the derivatization of the catechol groups via the addition of 25 µl methyl chloroformate. The samples are shaken on a multi vortex for 5 min at room temperature. Subsequently a pH shift to pH 9 is introduced to enhance the reaction of the methyl chloroformate with the secondary amine groups. This is accomplished by the addition of a saturated solution $Na_2CO_3$ (250µl) followed by a second derivatization with methyl chloroformate (25 µl).

It is perhaps appropriate to mention that the first reaction under mild pH conditions (pH7) is first used to derivatize the catechol groups and benzylic hydroxyl groups of E, NE, M and NM. Direct derivatization at pH 9 will cause the catechol groups to degrade (a fact which was also observed in the extractive acylation experiments).

With the catechol and amine groups derivatized, the compounds of interest are now extracted with ethyl acetate (3ml) by vigorous shaking for 10 min at room temperature. The organic phase is removed, dried under a stream of dry nitrogen gas and subjected to the silylation reaction.

Silylation using MTBSTFA were carried out by the addition of MTBSTFA (50 µl) and Acetonitrile (30µl) followed by subsequent

incubation (100$^{O}$C, 90min). The silylation reaction allows for substitution of the O-carbamate by a tBDMS group while the amine group is not affected by the silylation. This provide: *"a powerful tool to increase selectivity"* as noted by the original authors. A reaction scheme of this alkylation and silylation procedure is shown in figure 4.3.

With the extraction procedure completed and the successful analysis of NE, E, M and NM as described by De Jong and Cramers[99]attempts were made to include dopamine and the acidic metabolites VMA and HVA. The reason was to obtain a single extraction procedure which allows for the simultaneous analysis of all the compounds of interest.

Gas Chromatography-Mass Spectrometry

A Hewlett Packard (HP6890) GC system equipped with a HP 7683 auto injector and HP5973 mass selective detector (Agilent Technologies, Palo Alto, CA, USA) was used for chromatographic separation and recording of mass spectra. A DB17ms fused silica capillary column (5m x 100µm; $d_f$ 0.1 µm) (Varian, Middelburg, The Netherlands) was used. Data collection and integration was performed with the HP Chem Station Software.

A 2µl-volume of the derivatized sample was injected into the GC using the split-less mode. The inlet temperature was 250°C and

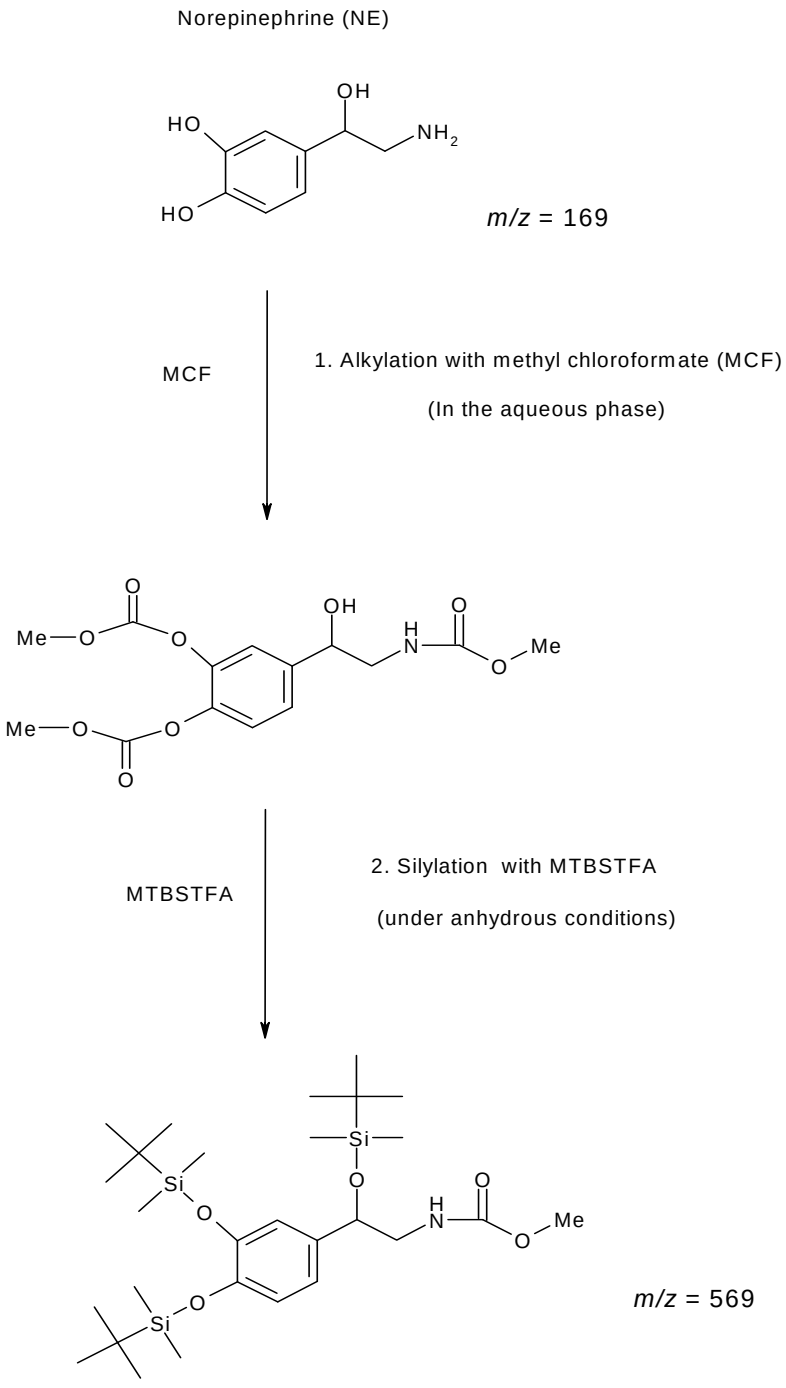



Figure 4.3: A reaction scheme of the alkylation with methyl chloroformate followed by silylation with MTBSTFA.

helium was used as carrier gas at a constant flow of 0.2 ml/min. The average velocity was 40 cm/s.

The GC oven was held at 80°C for 0.5 min and ramped at 30°C/min to 300°C.

Quantification by the MSD was performed in the SIM mode. The MSD transfer line was set at 280°C. The source and quadruple temperatures were 230°C and 150°C respectively. The mass spectra were collected in the electron impact mode at 70eV. The mass-to-charge ratios (*m/z*) of the ions used for quantification in SIM mode were: Epinephrine, 481 *m/z*, 526 *m/z*; Norepinephrine, 481 *m/z*, 512 *m/z*; Dopamine 351 *m/z*, 382 *m/z*; VMA and HVA.

Results

Gas Chromatography

A gas chromatogram obtained for the simultaneous analysis of the compounds of interest is shown in figure 4.4. Sufficient resolution was obtained using a DB-17MS column. Dopamine is the only compound which delivered two chromatographic peaks; a phenomenon which cannot be explained. The increase in signal as concentration increases is illustrated in figure 4.5. It is clear that dopamine delivers two separate peaks.

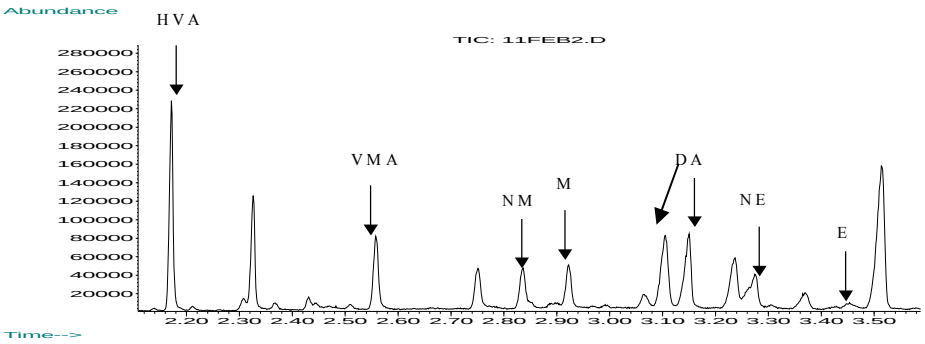



Figure 4.4: A gas chromatogram obtained in the selected ion monitoring mode of the simultaneous analysis of the catecholamines, their basic and acidic metabolites within 4 minutes.

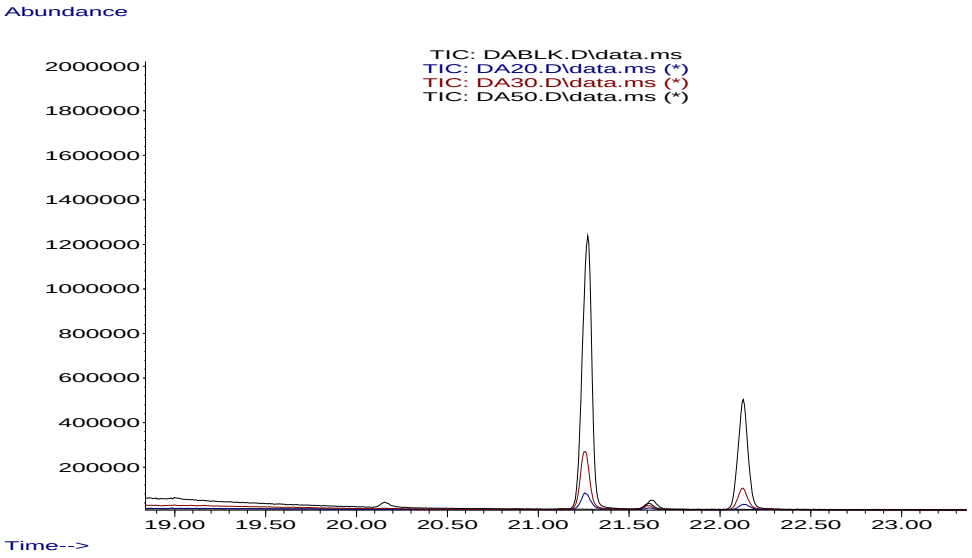



Figure 4.5: A gas chromatogram of dopamine which shows two distinctive peaks as the concentration increase. Four concentration levels were included, a blank, 20 ppm, 30 ppm, and 50 ppm. The samples were analyzed in the scan mode.

Mass Spectrometry

Whether the methyl choroformate derivatizes the carboxyl acid groups of VMA and HVA are not known, what is known is that the carboxyl acid group will be derivatized by the silylation procedure and for this reason VMA and HVA show derivatives containing only tBDMS groups. A reaction scheme of the derivatization procedure is shown on the following page.

The mass spectra obtained for the compounds analyzed are shown in the figures below.

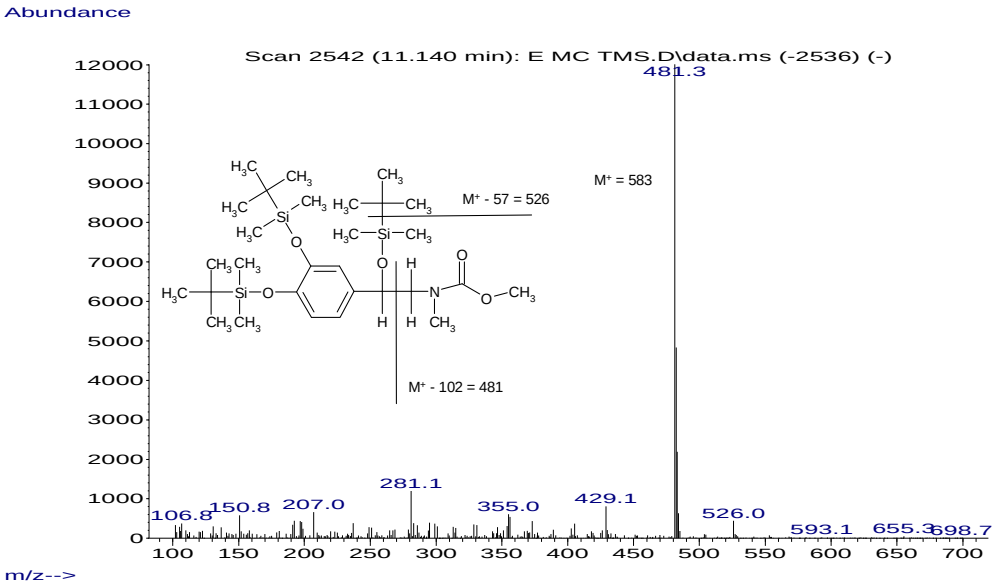



Figure 4.6: Full scan mass spectrum of epinephrine derivatized with methyl chloroformate followed by silylation with MTBSTFA.

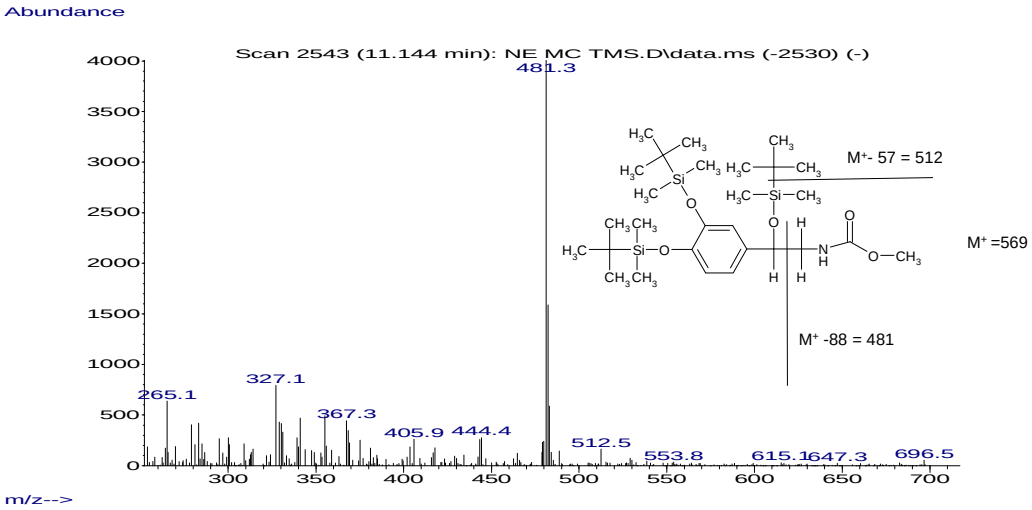



Figure 4.7: Full scan mass spectrum of norepinephrine derivatized with methyl chloroformate followed by silylation with MTBSTFA.

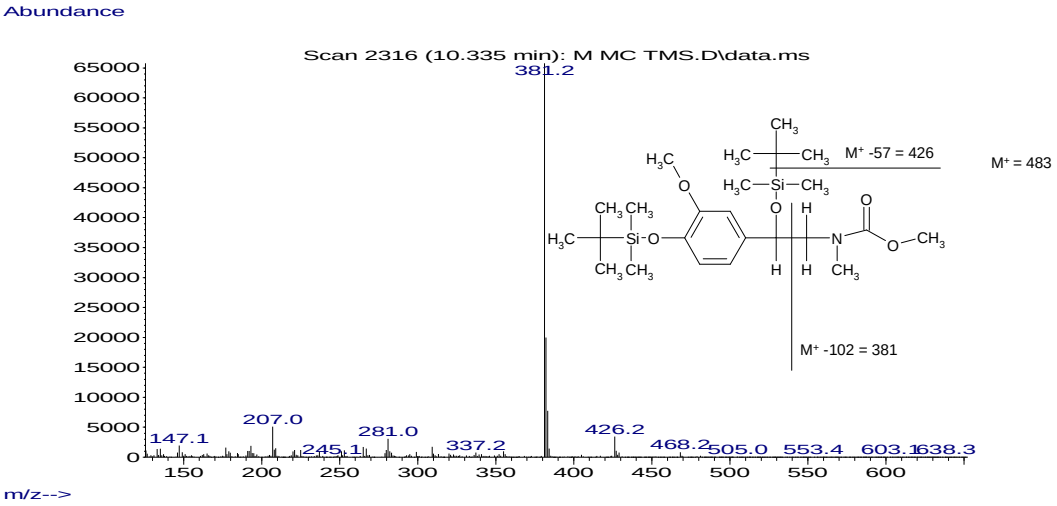



Figure 4.8: Full scan mass spectrum of metanephrine derivatized with methyl chloroformate followed by silylation with MTBSTFA.

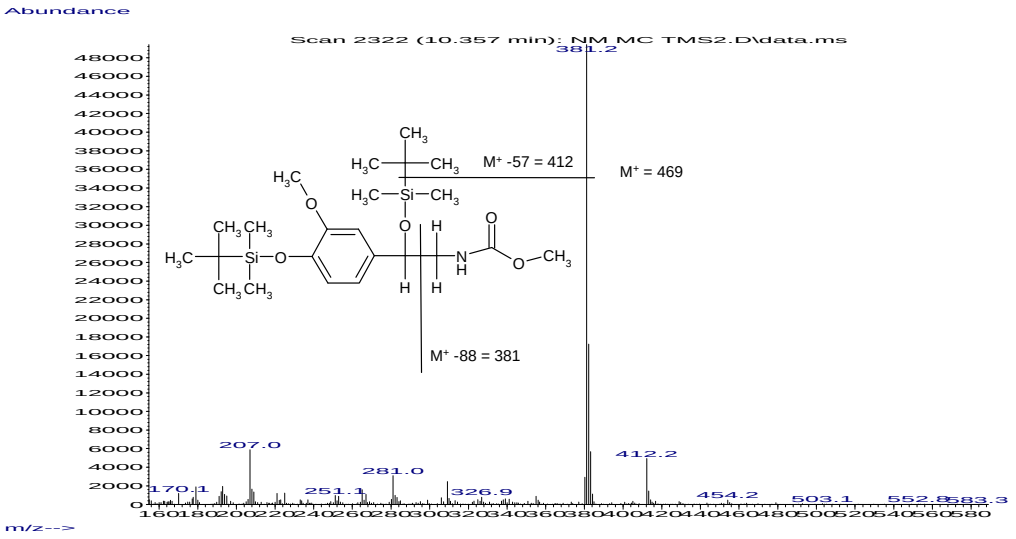



Figure 4.9: Full scan mass spectrum of normetanephrine derivatized with methyl chloroformate followed by silylation with MTBSTFA.

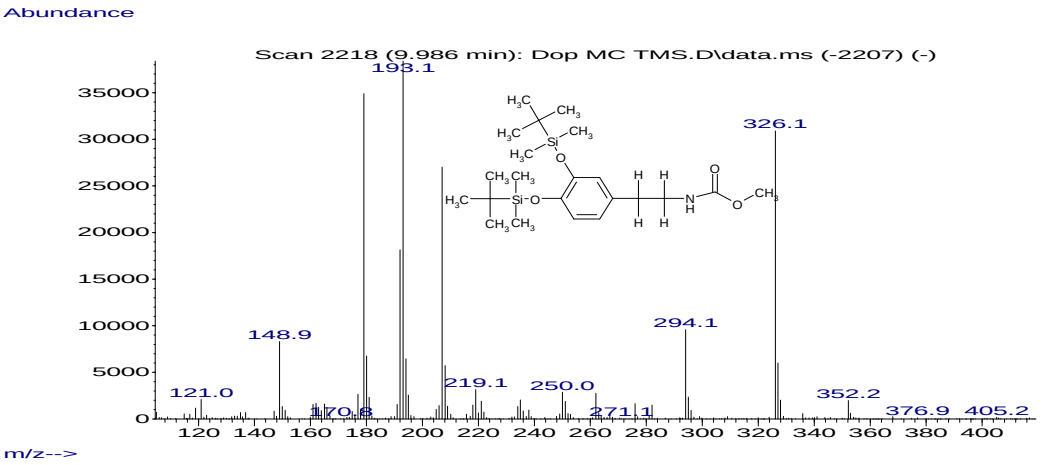



Figure 4.10: Full scan mass spectrum of dopamine derivatized with methyl chloroformate followed by silylation with MTBSTFA.

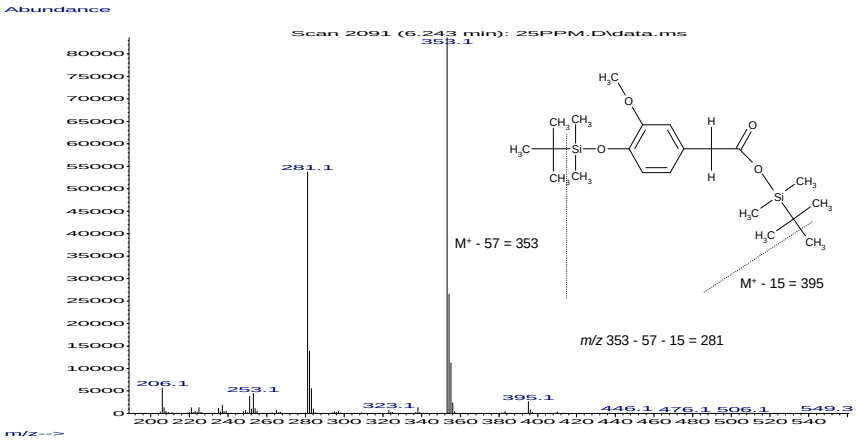



Figure 4.11: Full scan mass spectrum of vanilmandelic acid derivatized with methyl chloroformate followed by silylation with MTBSTFA.

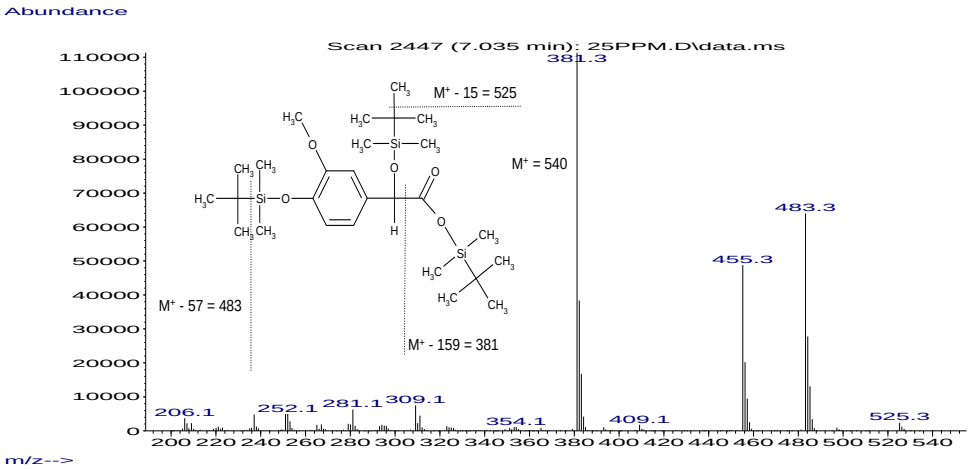



Figure 4.12: Full scan mass spectrum of homovanillic acid derivatized with methyl chloroformate followed by silylation with MTBSTFA.

The simultaneous analysis of all the compounds mentioned was achieved on a DB-17MS column. An attempt to separate the compounds on a conventional DB-5 column was unsuccessful; co – elution and poor chromatography being the main reasons for the use of a DB-17MS column.

Calibration curves constructed in the urine matrix is shown below and it is perhaps appropriate to discuss the capabilities of the method by these calibration curves. For example, the figures show calibration for VMA, HVA, M, NM, E, NE and DA in the urine, yet on a different occasion calibration curve attempts failed for the same metabolites.

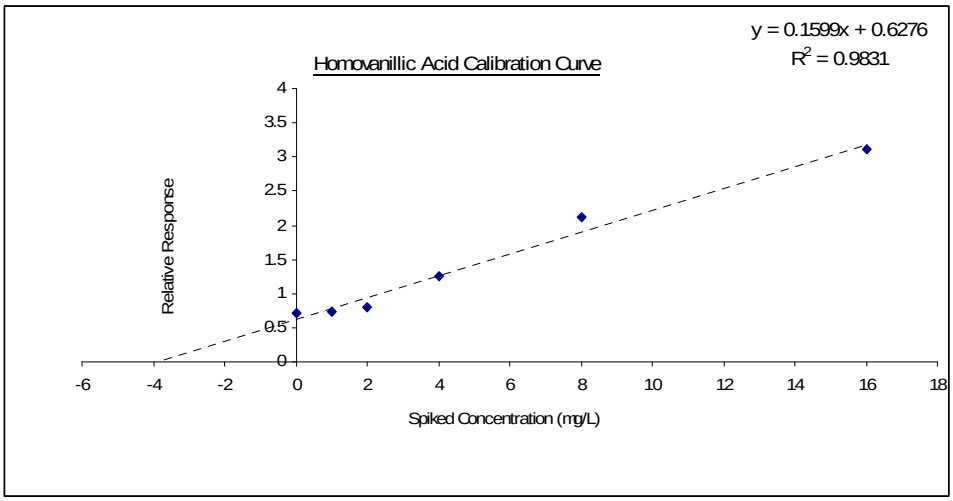



Figure 4.13: Calibration curve for Homovanillic acid in urine

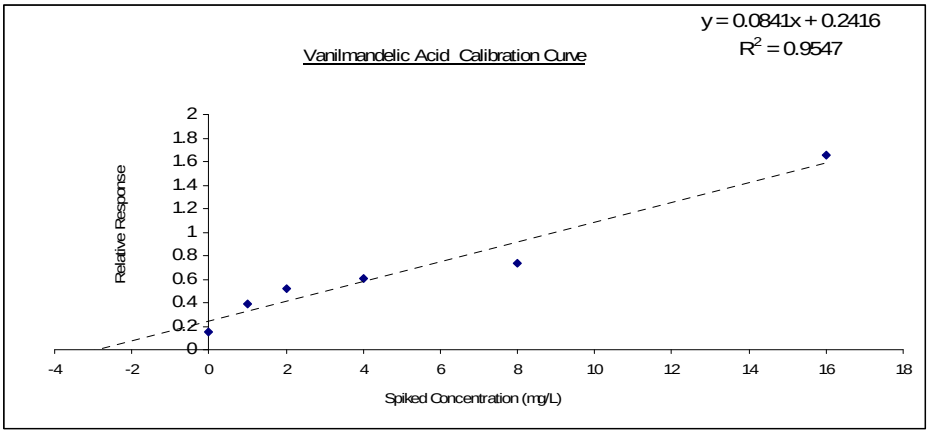



Figure 4.14: Calibration curve for Vanilmandelic acid in urine

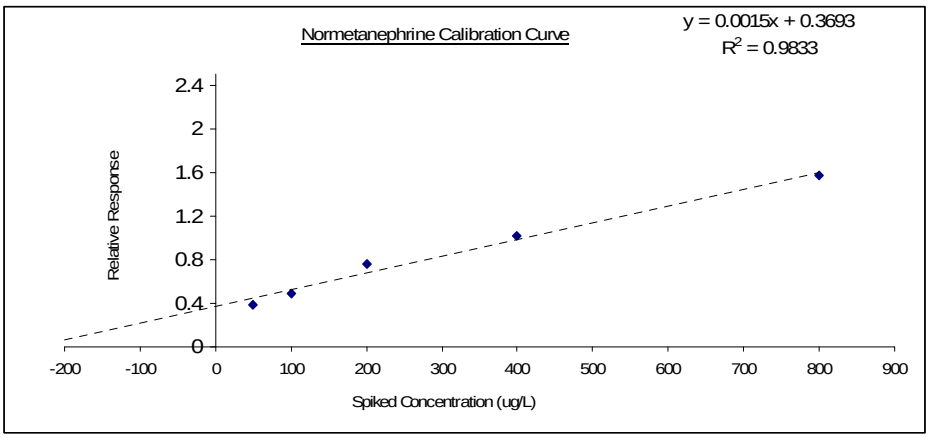



Figure 4.15: Calibration curve for Normetanephrine in urine

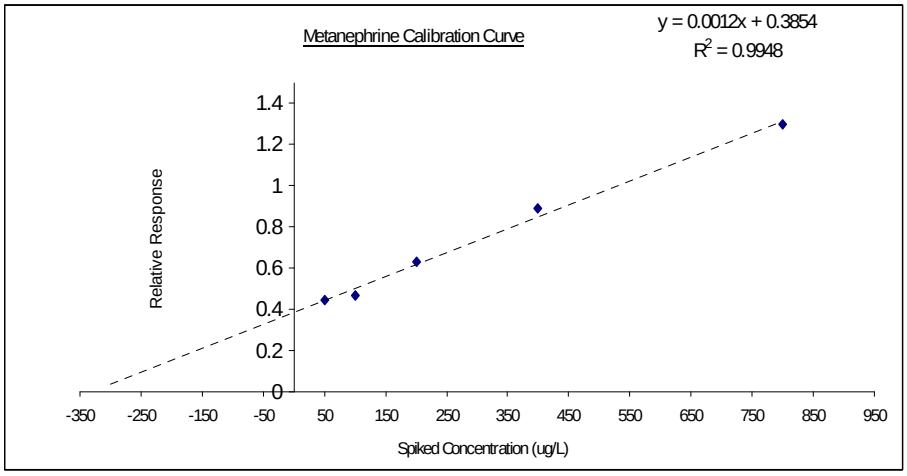



Figure 4.16: Calibration curve for Metanephrine in urine

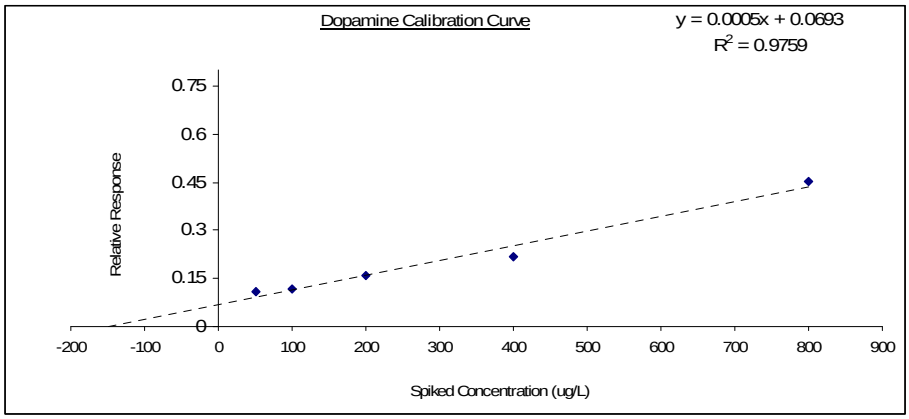



Figure 4.17: Calibration curve for Dopamine in urine

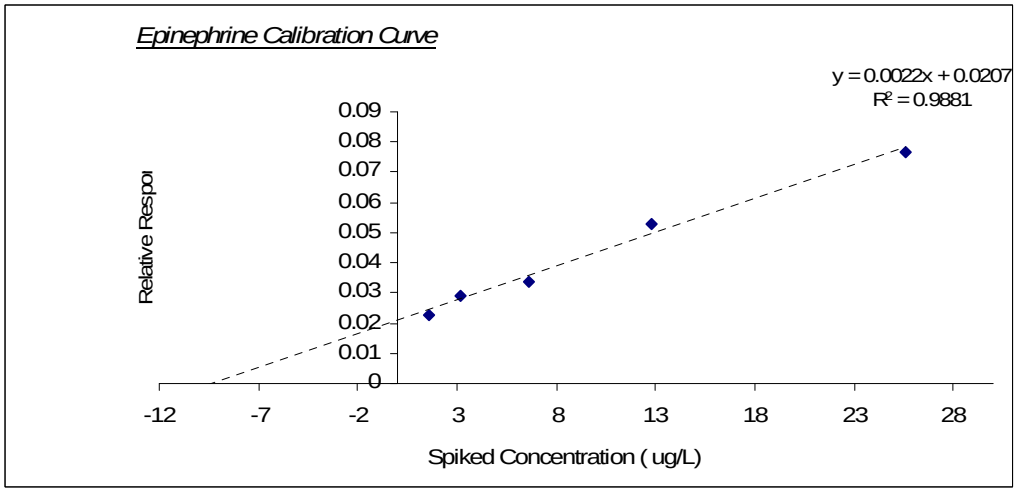



Figure 4.18: Calibration curve for epinephrine in urine

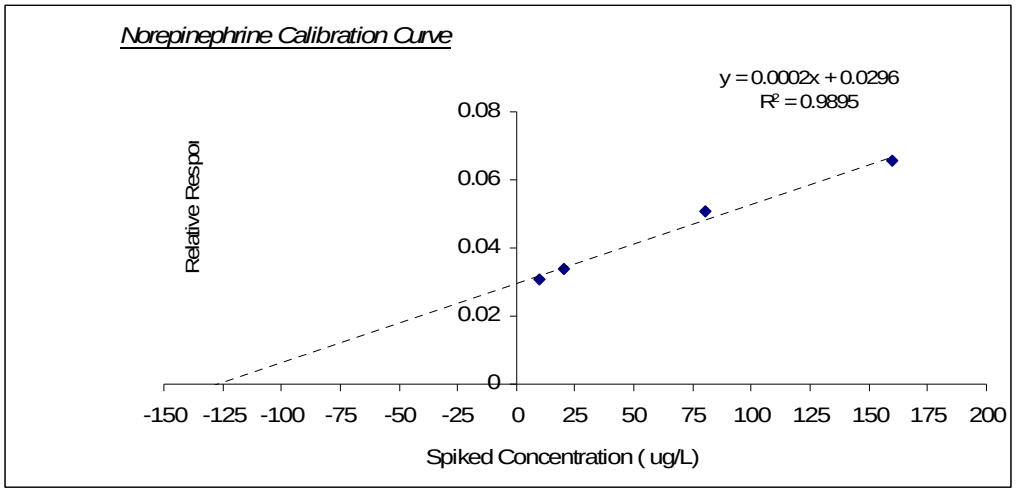



Figure 4.19: Calibration curve for norepinephrine in urine

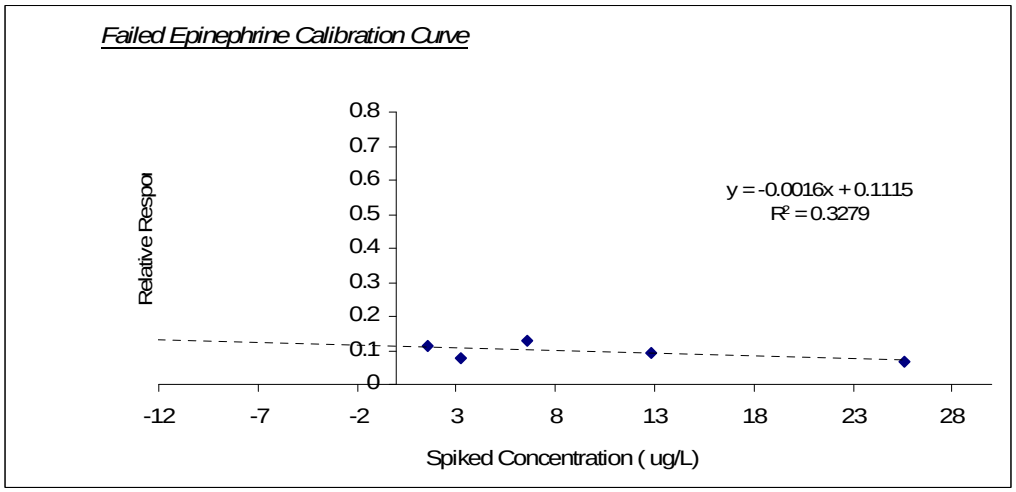



Figure 4.20: Example of a failed calibration curve for epinephrine in urine

This highlights one of the assays draw backs, derivatization with chloroformates are known to deteriorate the stationary phase which in turn will yield high back ground noise as is evident from these calibration curves. A note of caution is also the large range in concentration which was used to allow for all the analytes in one

single analysis. The acidic metabolites (VMA and HVA) are in the mg/L concentration range, while endogenous catecholamines (E, NE and D) and basic metabolites (M and NM) are in the low µg/L concntration range, which can explain the higher background noise obtained. However, the extraction procedure has the advantage of simultaneous analysis of endogenous catecholamines their basic and acidic metabolites.

Conclusion

The method used show potential as a rapid screening technique in the clinical laboratory. However, it is not always possible to obtain calibration for all the compounds in the assay due to limitations of the assay as described. With the careful selection of the correct GC – column, analysis was possible for the catecholamines, their basic and acidic metabolites using a single extraction procedure by derivatization with methyl chloroformate followed by silylation with MTBSTFA.

Due to the limitations of this method it was decided to develop a more specific extraction technique. For this reason a solid phase extraction (SPE) technique was employed since SPE techniques are known to be more specific as opposed to liquid-liquid extractions.

This solid phase extraction procedure is discussed in the following section.

### 4.3.2 Analysis of the catecholamines, epinephrine (E), norepinephrine (NE) and dopamine (D) as well as their basic metabolites (M+NM) using solid phase extraction followed by methylation and acylation derivatization techniques.

Introduction

Solid phase extraction (SPE) has played and will in future play an integral part in sample preparation prior to chromatographic analysis. In fact it is estimated that between 60 – 80 % of the work flow and cost in an analytical laboratory is dedicated to sample preparation.[100] It is therefore no surprise that the first commercialized SPE columns were introduced as early as 1977 by the Waters Chemical Company[100]. Nowadays there are numerous applications, each with its own selective SPE technique, available for sample clean up by SPE prior to chromatographic analysis.

The three main functions of SPE include:

i) Elimination of contaminants in the sample matrix which can interfere chromatographically with the compounds of interest.

ii) Selective extraction of compounds due to their chemical nature by means of selecting a specific stationary phase for the compounds of interest.

With the above mentioned, it is clear why SPE has found numerous applications in the analysis of catecholamines and their metabolites.

This is mainly due to the selectivity obtained by manipulation of the amine functional group with the use of cation exchange SPE. A second reason for the extensive use is employment of HPLC techniques requiring more sample clean-up to detect the compounds of interest.

As mentioned, catecholamines are usually extracted by a cation exchange mechanism a technique which has found widespread use in HPLC with electrochemical[101,102,103] - and - fluorescence[104,105] detection as well as in GC-MS analysis. The principle of the technique is discussed in the following section.

Solid phase extraction by a cation exchange mechanism.

The ion-exchange mechanism is based on the principle that charged analytes are extracted from the aqueous phase by interaction with a charged solid phase sorbent. In this method a cation exchange mechanism was used so that the positively charged amine groups are retained on the negatively charged cation exchanger. For the simultaneous extraction of the catecholamines, and their basic metabolites meta – and - normetanephrine from urine a strong cation exchange column from Varian Associates, Inc was chosen. The reasons for this choice were as follows: i) The presence of a benzene ring incorporated in the resin which has a higher potential for non-polar interactions. Thus the primary interaction is by ion-exchange with an additional non-polar interaction between the benzene rings

of the analytes and that of the resin. ii) The cation exchange group ($R\text{-}SO_3^- H^+$) has a low pKa value i.e. it is always charged in aqueous solution ($R\text{-}SO_3^-$). A schematic representation of the solid phase extraction procedure is shown in figure 4.21.

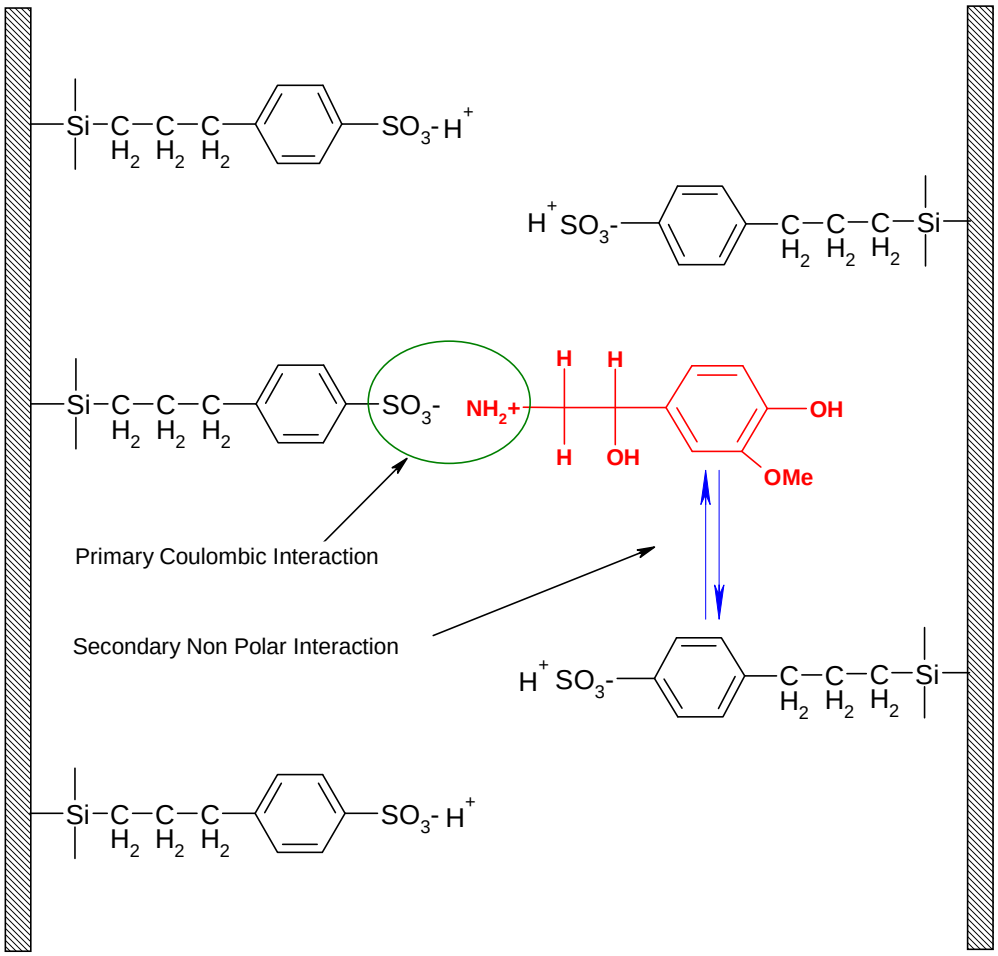



Figure 4.21: Schematic representation of the solid phase extraction technique used for the extraction of the catecholamines and their basic metabolites from urine. Extraction selectivity is obtained by ion-exchange (coulombic) and non-polar interaction.

It is important to realize that SPE by an ion-exchange mechanism is more complex than conventional reversed phase extraction as is

commonly used for hydrophobic compounds where long chain carbon ($C_n$) sorbents such as $C_{18}$ are used. The main reason is due to the fact that ion exchange mechanisms rely on specific coulombic interactions between the analyte and the solid phase sorbent. Therefore, only compounds with an appropriate charge will be retained on the solid phase sorbent while the "*interferences*" simply have no interaction and are eluted. The retained compounds of interest are subsequently eluted from the sorbent by strong acids / bases or high ionic strength buffers. For this reason, ion exchange SPE has the advantage of improved selectivity towards specific compounds but the disadvantage that more time is utilized during method development.

Selectivity of the counter ion is also an important factor to consider before extraction occurs[106]. The counter ion should have a lower selectivity than that of the charged group on the compound to be extracted. Therefore, buffers with appropriate pH levels and selected counter ions are passed through the sorbent before the sample is added. The relative counter-ion selectivity for cation exchange procedures is shown in table 4.1.

The last important factor to consider, especially when extracting from a urine matrix, is the amount of salt content in the matrix. High salt concentrations can hamper the extraction efficiency since the salts present, such as NaCl, can interfere with the ion-exchange mechanism; therefore it is recommended to dilute the urine sample

with an appropriate buffer before loading the sample on the extraction cartridge.

Table 4.1: Counter ion selectivity used for a cation solid phase exchange mechanism[107]. To change to a counter ion which has higher ion selectivity than the one on the sorbent, two to three bed volumes of an appropriate buffer containing the counter ion of choice is passed through the solid phase extraction cartridge

| Counter ion | Relative Ion Selectivity |
|---|---|
| $Li^+$ | |
| $H^+$ | |
| $Na^+$ | |
| $NH_4^+$ | |
| $Mn^{2+}$ | |
| $K^+$ | |

Experimental

Chemicals and reagents

The standards; epinephrine - HCl, norepinephrine - bitartate salt, dopamine, metanephrine – HCl and normetanephrine – HCl, were purchased from Sigma-Aldrich Chemical Co. Deuterium labeled internal standards, $d_3$-α,α,β-Epinephrine and $d_3$-α,α,β-metanephrine-HCl were purchased from Cambridge Isotope

Laboratories Inc, $d_2$-α,α,-Dopamine were from CDN isotopes and $d_3$-α,α,β-Normetanephrine-HCl, were purchased from Medical Isotopes Inc. Derivatization reagent, pentafluoropropionic anhydride (PFPA) was purchased from Fluka (Buchs, Switzerland). Anhydrous methanolic hydrochloric acid (1M) was prepared by the addition of acetyl chloride to methanol, both obtained from Merck. Solid phase extraction cartridges (SCX, LRC, 200mg) were purchased from Varian Associates, Inc. Ammonium phosphate as well as mono sodium phosphate and dibasic sodium phosphate for the preparation of the buffer were from Saarchem Merck.

Solutions

Stock solutions were prepared in Hydrochloric acid (0.01 M) and stored at -20$^{o}$C until used. The deuterium internal standards were prepared in methanol and stored at -20$^{o}$C until used. Serial dilutions of the stock solution were made in Hydrochloric acid (0.01 M) to obtain the working calibration standards as shown in table 4.2.

Calibration curves

A urine pool from a healthy volunteer was used to construct the work standards for E, NE, D, M and NM. To an aliquot of urine (1600µl), of the working standard (200 µl) as well as the internal standard stock solution (200 ul) of were added. Six-point calibration curves were constructed by using the relative response (peak area) of the analyte versus the deuterium labeled internal standards. The

samples subsequently underwent acid deconjugation as described below

Table 4.2: Work standard concentrations for the solid phase extraction procedure

| | Standard 1 | | Standard 2 | | Standard 3 | | Standard 4 | | Standard 5 | | Standard 6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | µg/L | nmol/L | µg/L | nmol/L | µg/L | nmol/L | µg/L | nmol/L | µg/L | nmol/L | µg/L | nmol/L |
| M | 250 | 1250 | 500 | 2500 | 750 | 3750 | 1500 | 7500 | 3000 | 15000 | 6000 | 30000 |
| NM | 250 | 1375 | 500 | 2750 | 750 | 4125 | 1500 | 8250 | 3000 | 16500 | 6000 | 33000 |
| D | 500 | 3250 | 750 | 4875 | 1000 | 6500 | 2000 | 13000 | 4000 | 26000 | 8000 | 52000 |
| E | 5 | 27.5 | 25 | 137.5 | 50 | 275 | 100 | 550 | 250 | 1375 | 500 | 2750 |
| NE | 100 | 590 | 150 | 885 | 200 | 1180 | 400 | 2360 | 800 | 4720 | 1600 | 9440 |

Deconjugation

Acid deconjucation was performed by the addition of HCl (6M, 50µl) and subsequent heating (90°C for 25 min). The sample was allowed to reach room temperature before the extraction procedure commenced.

Solid phase extraction procedure

One milliliter of the acid hydrolyzed sample was diluted six times with an ammonium phosphate buffer (10 mM, pH 6.5). The pH of the sample was adjusted to pH 6.5 ± 0.2 with the addition of sodium hydroxide. The cation-exchange solid phase extraction cartridges were conditioned as follow:

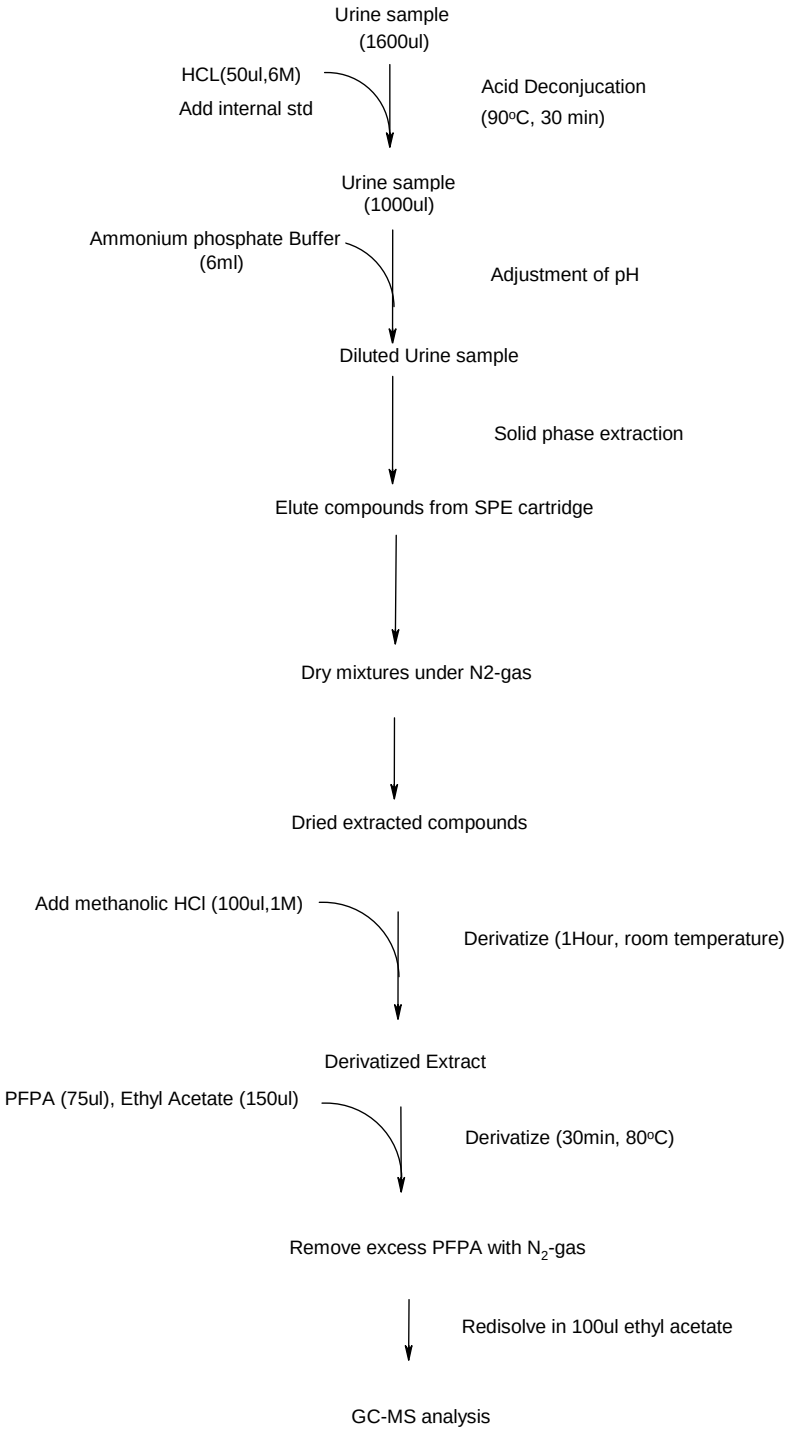
1. Activate with 5 ml HCl (0.1 M) in MeOH
2. Wash with distilled $H_2O$ (10 ml)
3. Condition with Ammonium phosphate buffer (10mM, 10ml).
4. Load sample (7 ml)
5. Wash with distilled $H_2O$ (10 ml)
6. Dry under vacuum for 2 min
7. Elute the compounds of interest with HCl (0.1 M, 1 ml) in MeOH.

A flow diagram of this solid phase extraction procedure is shown on the following page.

Mass Spectrometry

The advantage of derivatization by acylation with PFPA has been described in the previous two sections (stable derivatives, short incubation times and high *m/z* ions in the EI spectra). Yet with the derivatization of catecholamines it was found that pentafluoropropionyl derivatives (PFP) especially that of epinephrine and norepinephrine didn't yield "favorable" mass spectra. By this is meant that the most abundant ions produced results from the derivatization reagents molecular back bone, i.e. the abundance of distinctive ions are low while non characteristic ions dominate which make quantification difficult. The mass spectra of epinephrine and norepinephrine derivatized with PFPA are shown in figures 4.23 and 4.24.

Figure 4.22: Flow diagram of the solid phase extraction procedure used



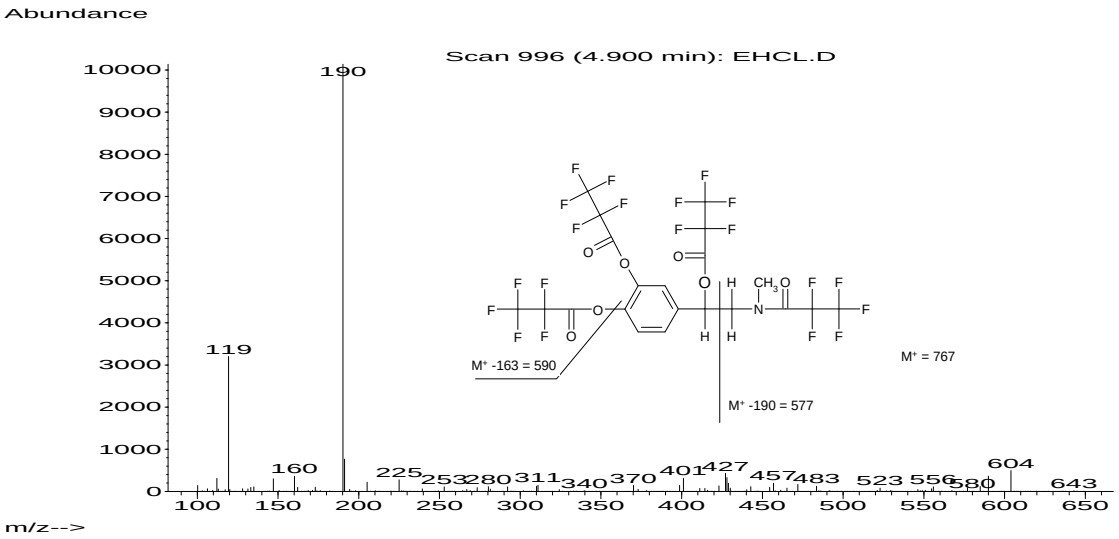



Figure 4.23: Full scan mass spectrum of epinephrine derivatized with PFPA.

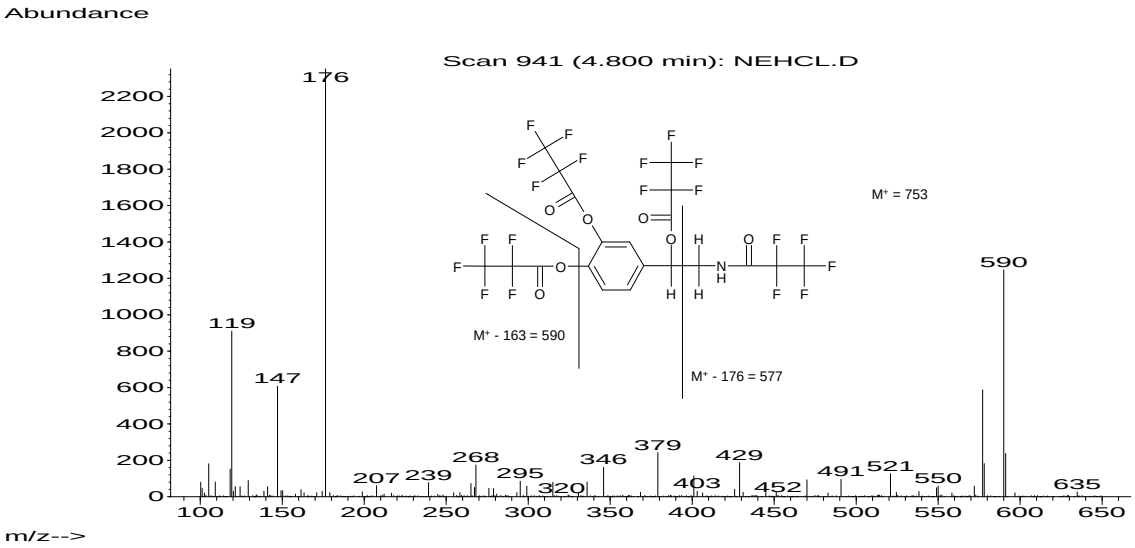



Figure 4.24: Full spectrum of norepinephrine derivatized with PFPA.

The mass spectra as shown above makes quantification difficult since the ions that dominate (*m/z* 176 and *m/z* 190) yields high backgrounds and are not distinctive for each individual analyte. The PFP-derivative of dopamine, figure 4.25, illustrates a more "favorable" spectrum, the reason: the absence of a benzylic

hydroxyl group, hence a more characteristic mass spectrum with a distinctive *m/z* 428 ion dominating as opposed to the *m/z* 176.

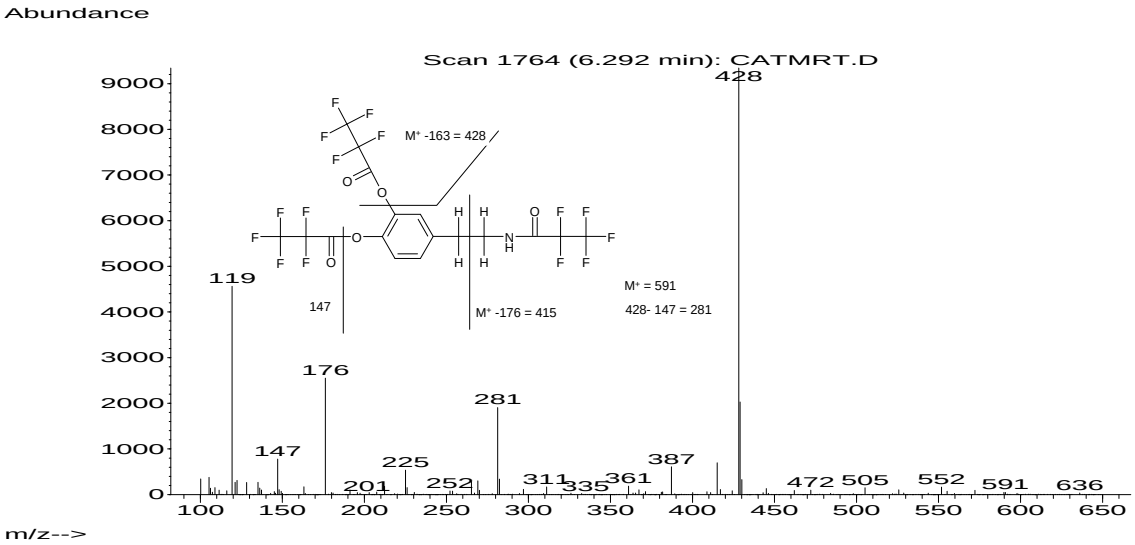



Figure 4.25: The PFP-derivative of dopamine.

To circumvent the problem of the derivatization the following solutions can be presented:

1. The ion energy of the electron impact ionization source can be lowered in an attempt to yield more ions in the higher mass range i.e. having a "softer" ionization. However, 70 (eV) is the ion energy of choice for an EI source and by changing the ion energy would require "tuning" the MS if it is not dedicated for this analysis.
2. A different derivative can be used. This will remove the favorable advantages: high *m/z* ions, low

incubation temperatures and stable derivatives, of PFP-derivatives as previously explained.

3. The derivatization reaction can be manipulated to yield "favorable" mass spectra. This procedure was chosen and is explained below.

The PFP-mass spectrometric behavior of catecholamines has been described by Tas *et al*[108] and Ehrhardt[109]. Derivatization of the benzylic hydroxyl by methylation yielded more favorable mass spectra. The procedure for derivatization was as follows:

To the dried eluate, a solution of methanolic hydrochloride (1M, 100µl) was added[†]. The sample was stored at room temperature for 1 hour. Subsequently, the methanolic hydrochloric acid was removed by a stream of $N_2$ – gas. The derivatized extract was subjected to acylation by PFPA (150µl PFPA, 75 µl ethyl acetate) at 80$^{o}$C for 30 min. The excess PFPA was removed by $N_2$ – gas and dissolved in 100 µl ethyl acetate before injected into the GC-MS.
A reaction scheme of the derivatization procedure is shown in figure 4.26.The mass spectra are shown in figures 4.27 – 4.30 and include the methylated PFP-derivatives of epinephrine, norepinephrine, metanephrine and normetanephrine respectively. Dopamine is the only compound not influenced since it has no benzylic hydroxyl group.

[†] The anhydrous methanolic hydrochloride solution was prepared by adding acetyl chloride drop wise to methanol cooled over ice in advance.

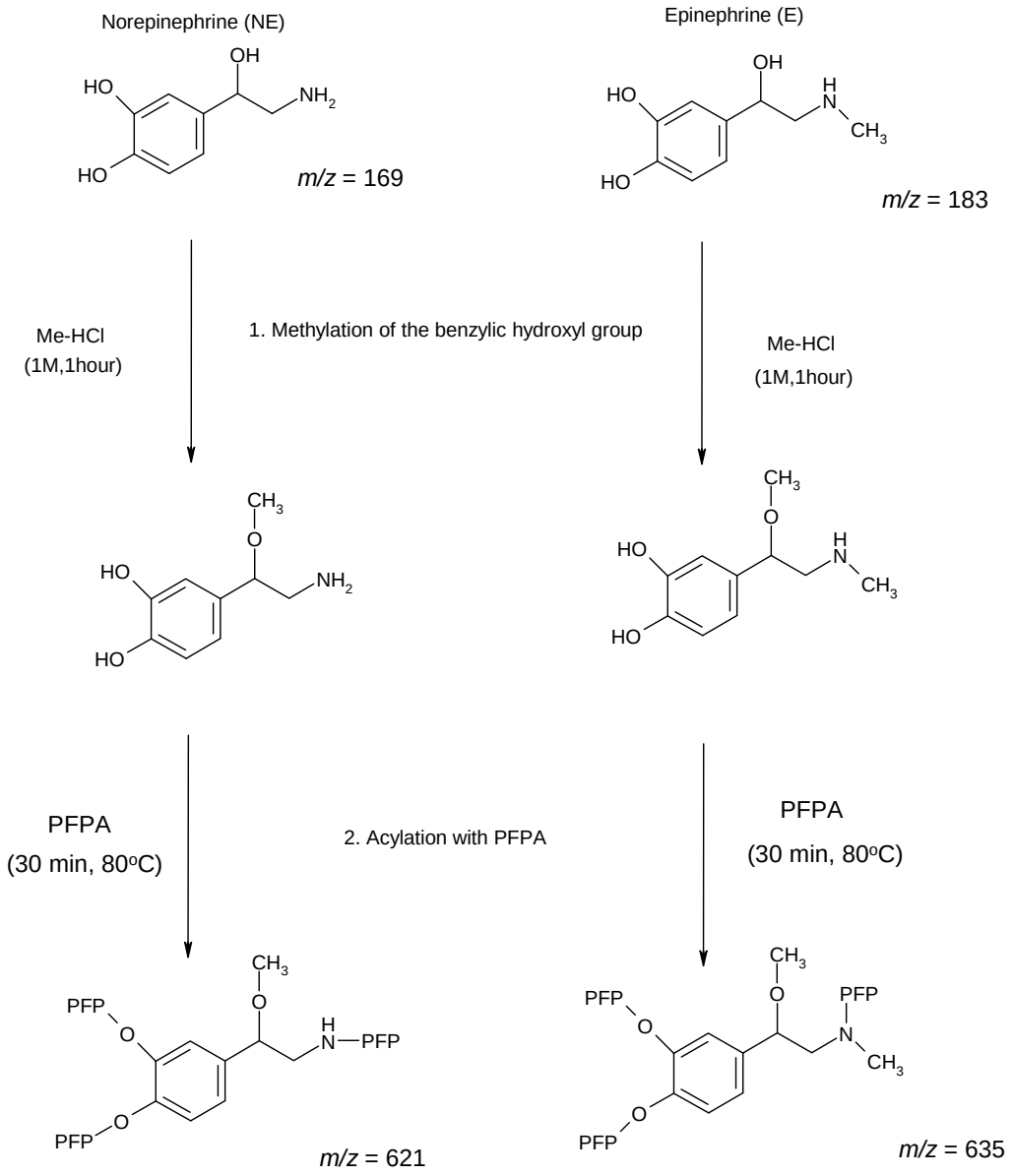



Figure 4.26: Reaction scheme for the reaction of E and NE with methanolic hydrochloric acid (methylation of the benzylic hydroxyl group) followed by acylation with PFPA.

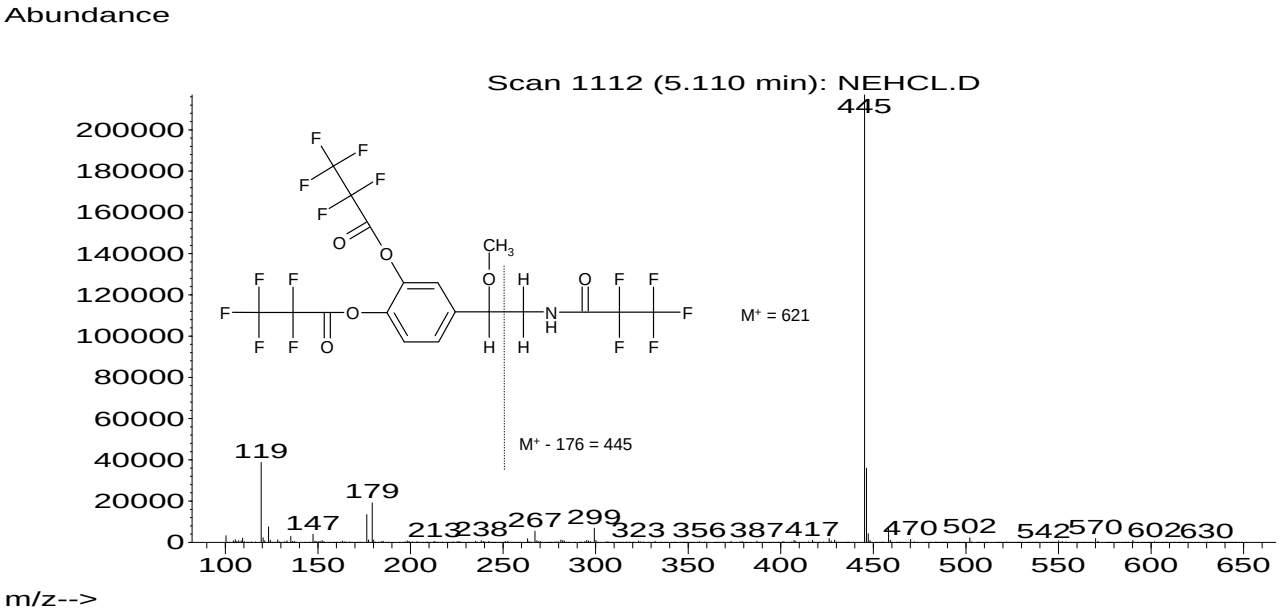



Figure 4.27: Norepinephrine PFP-derivative following methylation of the benzylic hydroxyl group. A more distinctive *m/z* 445 ion is now observed.

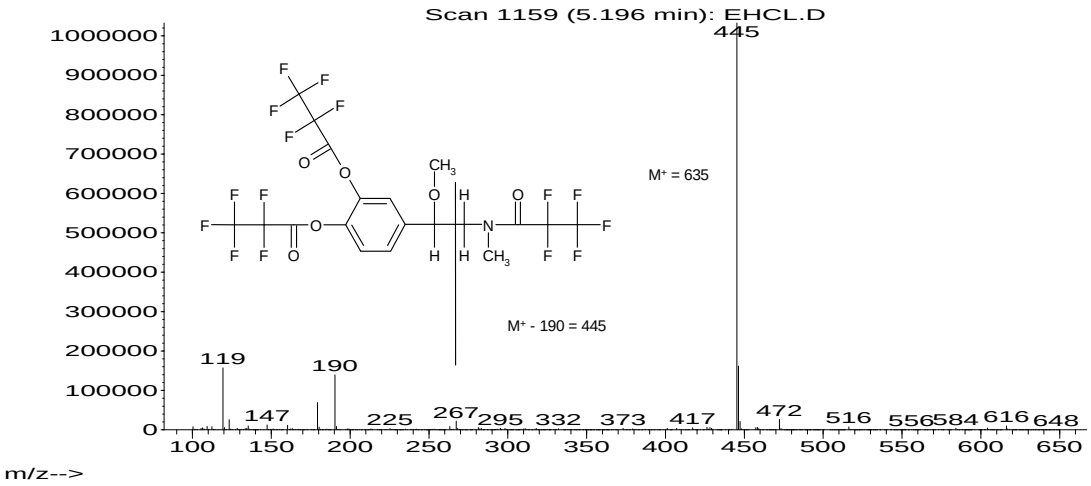



Figure 4.28: Epinephrine PFP-derivative following methylation of the benzylic hydroxyl group. A more distinctive *m/z* 445 ion is now observed.

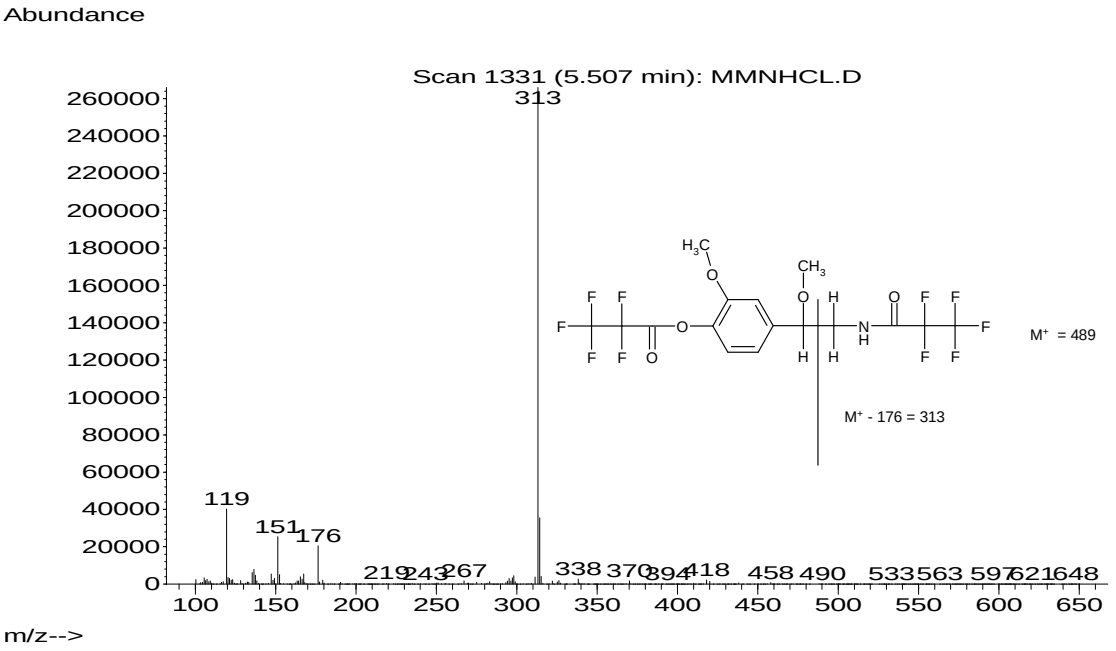



Figure 4.29: Normetanephrine PFP-derivative following methylation of the benzylic hydroxyl group.

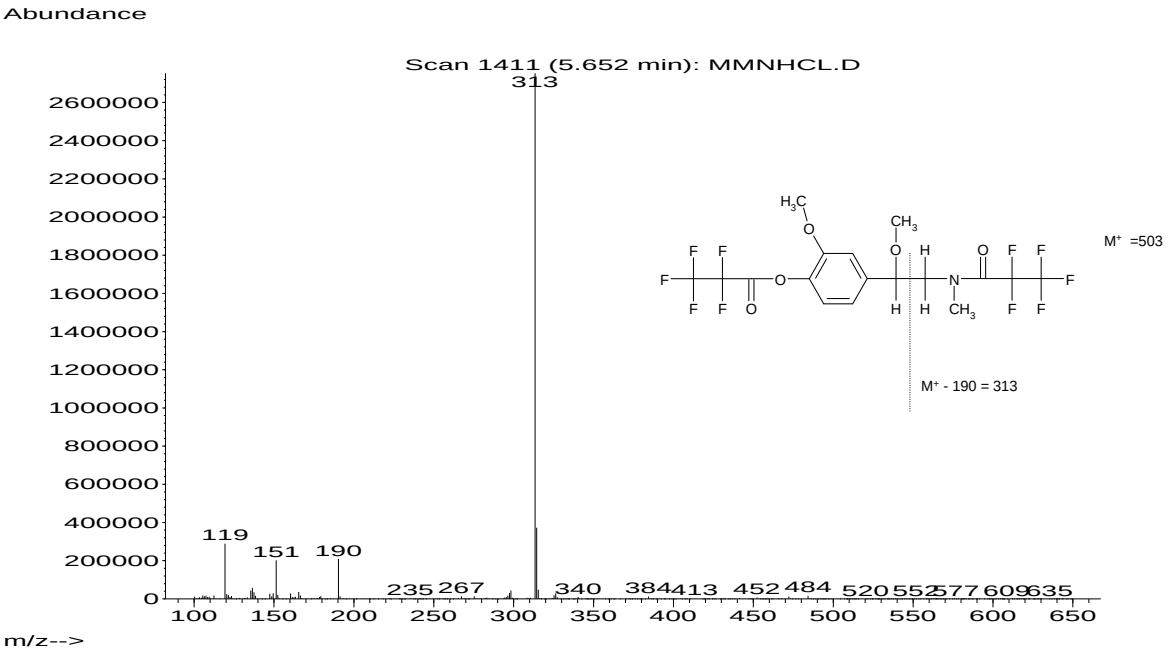



Figure 4.30: Metanephrine PFP-derivative following methylation of the benzylic hydroxyl group.

Results

Gas Chromatography

A gas chromatogram obtained by selected ion monitoring of a urine sample extracted by the solid phase extraction procedure is shown in figure 4.31. The ions used were: *m/z* 313 for M and NM, *m/z* 445 for E and NE and *m/z* 428, 281 for D. The chromatogram indicates the successful extraction of M and NM; however extraction of the endogenous catecholamines was not as successful. At first glance it doesn't seem to be true; there are two distinctive peaks for *m/z* 445, the ions used for the methylated epinenephrine and norepinephrine. However, it should be noted that this error may be catastrophic if one merely assumes that these are in fact the extracted ions for epinephrine and norepinephrine.

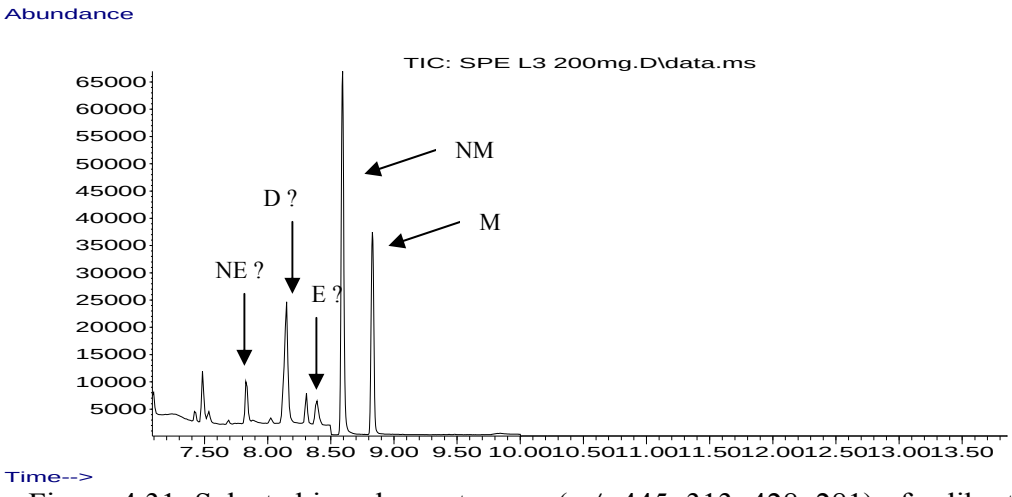



Figure 4.31: Selected ion chromatogram (*m/z* 445, 313, 428, 281) of calibration level 3 extracted by the solid phase extraction procedure. The question marks relates to what is believed to be NE, D and E, see text for further explanations.

What is known[108, 109] is the fact that the methylation of the benzylic hydroxyl group does not yield 100% fully derivatized samples, neither by increasing the concentration of the methanolic hydrochloric acid nor by increasing the incubation time. Subsequently the mass spectra of M and NM without methylation of the benzylic hydroxyl group will also be present in the chromatogram. These mass spectra of both M and NM have ions correlating to *m/z* 445 when derivatized with PFPA in the absence of methylation. To try and elucidate whether these ions are in fact the methylated E and NE derivatives, working solutions were freeze dried and subjected to the derivatization process without any solid phase extraction. The results are shown in figure 4.32 and indicate the presence of four in stead of two *m/z* 445 ions; the extra two ions eventually determined to be those of M and NM.

Since the extraction of the endogenous catecholamines was not possible an attempt was made to optimize the solid phase extraction further. It was decided to change the conditioning step with ammonium phosphate buffer to a sodium phosphate buffer. The reason for this was due to the lower affinity of the $Na^+$ counter ions than that of the $NH_4^+$ ions as shown in table 4.1 i.e. the catecholamine should have a higher affinity towards the $R\text{-}SO_3^-$ group. The results however didn't yield improvement of the extraction. A possible explanation might be due to the high salt content observed prior to derivatization due to the choice of the sodium phosphate buffer.

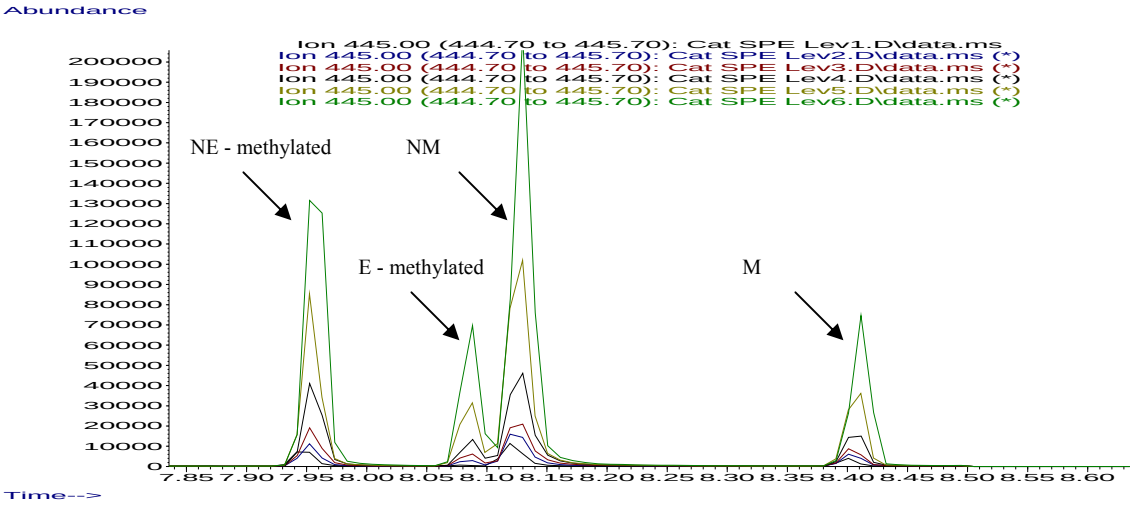



Figure 4.32: Increase in concentration of four *m/z* 445 ions due to the fact that the methylation of the benzylic hydroxyl group does not yield 100% fully derivatized products. The two extra *m/z* 445 ions were determined to be those of M and NM.

The solid phase extraction procedure did however yield promising results for M and NM extraction; figure 4.33 shows a selected ion chromatogram of calibration level 3 (spiked with 150µg/L). High signal to noise as a result of the selective extraction procedure is evident.

The recovery and coefficient of variance for the solid phase extraction procedure for metanephrine and normetanephrine are summarized in table 4.3.

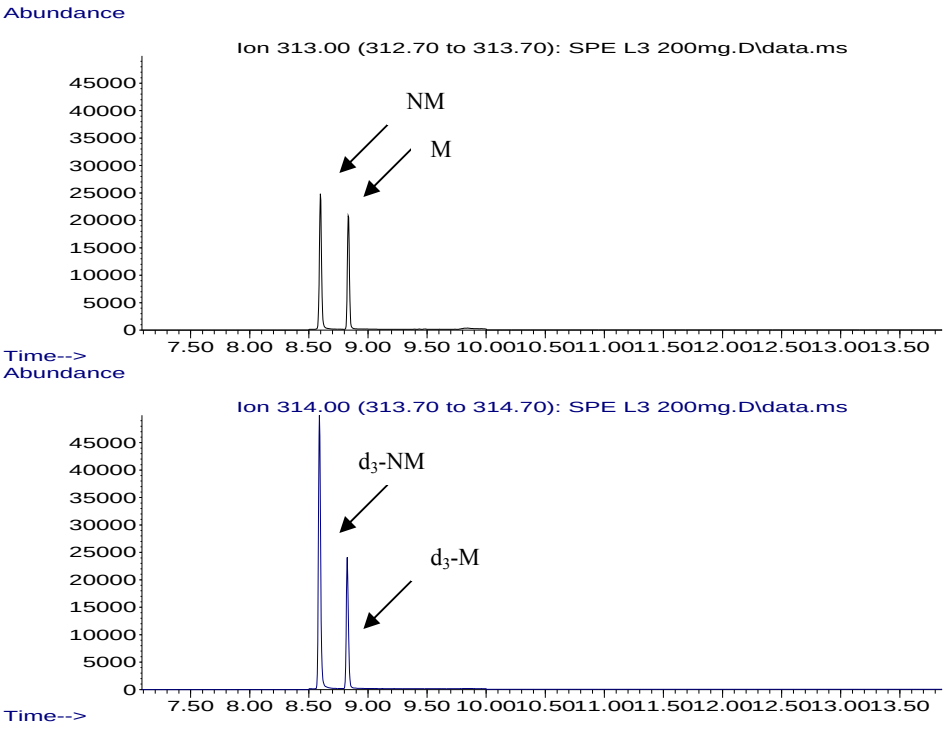



Figure 4.33: Selected ion chromatogram of M and NM (*m/z* ions 313 and 314) at a spiked concentration of 150 µg/L extracted from urine by the solid phase extraction procedure.

Table 4.3: Validation parameters obtained for metanephrine and normetanephrine extracted by the solid phase extraction procedure.

| Metabolite: | $r^2$ | Concentration | CV ,% (n=5) | Mean Recovery,% (n =5) |
|---|---|---|---|---|
| M | 0.99 | 50 µg/L | 2.23 | 140.48 |
| | | 300 µg/L | 2.43 | 105.56 |
| NM | 0.99 | 50 µg /L | 1.86 | 137.49 |
| | | 300 µg/L | 1.12 | 104.65 |

Conclusion

Solid phase extraction has the advantage of superior clean – up as opposed to liquid-liquid extraction methods. Correct utilization may assist in the analysis of compounds in low concentration ranges. However, SPE has its disadvantages:

1. It takes time to develop a method before this method can be employed in the routine laboratory.
2. It is expensive and for this reason the cost of analysis need to be taken into consideration.
3. It requires more skill and training than liquid-liquid extraction

It is not fully understood why the endogenous catecholamines were not extracted successfully by this method, it is known that catecholamines do extract optimally at pH 6.5 on cation exchange cartridges. Yet this was achieved for metanephrine and normetanephrine and it is clear that solid phase extraction has the advantage of superior sample clean up.

Furthermore, to allow for optimal quantification of the catecholamines and their basic metabolites, derivatization procedures had to be adjusted which does make this method labor intensive. One advantage is superior clean-up prior to analysis, this resulted in a high signal to noise for M and NM and low coefficient of variance (<5%) when using this assay.

Due to the tedious sample preparation and derivatization procedures required in this assay it was decided to develop a more selective liquid-liquid extraction procedure that requires minimal sample preparation and uncomplicated derivatization prior to GC-MS analysis. For these reasons an extractive acylation procedure was developed since this procedure fulfill these criteria. This method is described in the following section.

### 4.3.3 Extractive acylation of the basic catecholamine metabolites, metanephrine (M) and normetanephrine (NM), using pentafluoropropionic anhydride (PFPA)

Introduction

As previously described, quantification of M and NM provide a sensitive means for the detection or exclusion of a pheochromocytoma. This assay focuses on a previously described extractive acylation procedure by Beck *et al* [110], which is now applied towards the quantification of M and NM in human urine.

The procedure is based on the Schotten-Baumann reaction[111]whereby acylation of amines and phenolic functional groups will take place under aqueous conditions employing an anhydride as acylation reagent. Neutralization of the excess acid formed during the reaction is required to maintain the basic conditions. Neutralization in this case was effected by the addition of sodium bicarbonate. The derivatives were subsequently extracted from the matrix with an organic solvent. A second derivatization reaction, under anhydrous conditions, is required, however, to derivatize the benzylic hydroxyl groups[110] in the case of M and NM.

The assay utilizes the potential of acylation by PFPA combined with a liquid-liquid extraction procedure, which is less time consuming than conventional solid-phase extraction procedures.

## Experimental

### Chemicals and reagents

Metanephrine-HCl and normetanephrine-HCl were purchased from Sigma-Aldrich Chemical Co. Deuterium labeled internal standards $d_3$-α,α,β-Normetanephrine-HCl and $d_3$-α,α,β-metanephrine-HCl were purchased from Medical Isotopes Inc, and Cambridge Isotope Laboratories Inc, respectively. Derivatization reagent, pentafluoropropionic anhydride (PFPA) was purchased from Fluka (Buchs, Switzerland). Dichloromethane (GC Grade), methanol as well as ethyl acetate were obtained from Fluka (Buchs, Switzerland). Hydrochloric acid was purchased from Merck (Darmstadt, Germany). Sodium hydrogen carbonate ($NaHCO_3$) was obtained from Saarchem-Merck. De-ionized water from a Milli-Q (Millipore, USA) was used for the preparation of standards.

### Solutions

A stock solution (40mg/L) of M and NM were prepared in HCl (0.01M) and stored at 4°C until used. The internal standard stock solution (25mg/L) of $d_3$-M and $d_3$-NM were prepared in methanol and stored at -20°C until used. Serial dilutions of the M and NM stock solution were prepared in HCl (0.01M) to obtain the work standards as shown in table 4.4.

Table 4.4: Concentrations of the work standards. The calibration standards were diluted twenty times in the urine matrix to construct calibration curves ranging from 25 – 600µg/L.

| | Standard 1 | | Standard 2 | | Standard 3 | | Standard 4 | | Standard 5 | | Standard 6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | µg/L | nmol/L | µg/L | nmol/L | µg/L | nmol/L | µg/L | nmol/L | µg/L | nmol/L | µg/L | nmol/L |
| M | 500 | 2500 | 1000 | 5000 | 1500 | 7500 | 3000 | 15000 | 6000 | 30000 | 12000 | 60000 |
| NM | 500 | 2750 | 1000 | 5500 | 1500 | 8250 | 3000 | 16500 | 6000 | 33000 | 12000 | 6600 |

## Calibration curves

To an aliquot of urine (800µl), work standard (50µl) as well as the internal standard solution (50µl) was added. Six-point calibration curves were constructed utilizing the relative response of the signal obtained (peak area of analytes / peak area of internal standard). The concentration range of the calibration curves ranged from 127–3041 nmol/L and 136 – 3275 nmol/L for M and NM respectively. This concentration range included the normal human urinary reference ranges of M (375 -1506 nmol/day) and NM (573 - 1903 nmol/day)[112]. The samples subsequently underwent acid deconjugation as described below.

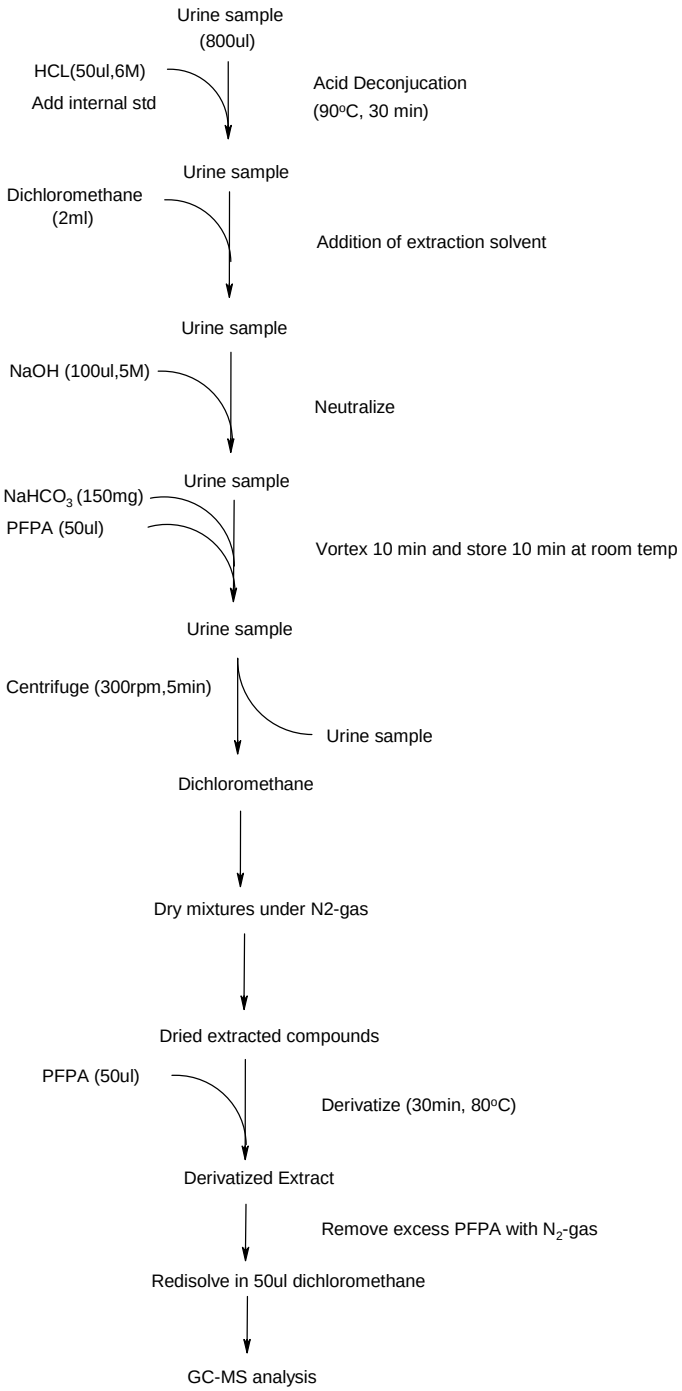
Deconjugation

Acid deconjucation[113] was performed by the addition of HCl (6M, 50µl) to the sample and subsequent heating at (90°C, 25 min). The sample was allowed reach room temperature before the extraction procedure commenced.

Extractive acylation

To the acid hydrolyzed samples, dichloromethane (2ml) were added and the samples were neutralized with NaOH (5M, 100µl). Thereafter, $NaHCO_3$ (150 mg) were added, followed by PFPA (50 µl). The mixtures were capped with a PTFE lined cap and vortexed for 10 min at room temperature. The mixtures were stored an additional 10 min at room temperature to allow the reaction to complete. Mixtures were centrifuged (3000 rpm, 5 min), the organic layer removed with a pasteur pipette and transferred to a clean reaction vial. The sample extract was dried under a stream of dry nitrogen gas. An additional PFPA (50µl) was added and the dried extract was derivatized for 30 min at 80°C. The derivatized extracts were dried under a stream of dry nitrogen gas and reconstituted in dichloromethane (50 µl) before subjected to GC-MS analysis. A flow diagram of this extraction procedure is shown on the following page.

Figure 4.34: Flow diagram of the extractive acylation of M and NM from urine.



Gas Chromatography Mass Spectrometry

A Hewlett Packard (HP6890) GC system equipped with a HP 7683 auto injector and HP5973 mass selective detector (Agilent Technologies, Palo Alto, CA, USA) was used for chromatographic separation and recording of mass spectra. A fused silica capillary column (DB17ms, 5m x 100µm; $d_f$ 0.1 µm) (Varian, Middelburg, The Netherlands) was used to effect separation. Data collection and integration was performed with the HP Chem Station Software.

A 2µl-volume of the derivatized sample was injected into the GC using the split-less mode. The inlet temperature was 250°C and helium was used as carrier gas at a constant flow of 0.4 ml/min. The average velocity was 40 cm/s. The GC oven was held at 80°C for 0.5 min and ramped at 30°C/min to 145°C. The second ramp rate was 15°C/min to 185°C. The final ramp was 30°C/min to an isotherm of 300°C, which was held for 0.5 min. The total separation time was 12 minutes.

Mass spectrometric quantification was performed in the single - ion - monitoring mode (SIM). The transfer line was set at 280°C and the source and quadruple temperatures were 230°C and 150°C respectively. The mass spectra were collected in the electron impact

mode at 70eV. The mass-to-charge ratios (*m/z*) of the ions used for quantification in SIM mode were: M, 445 *m/z*, 458 *m/z* and 635 *m/z*; NM, 445 *m/z*, 458 *m/z*, 621 *m/z* for the endogenous M and NM. The ions used for the deuterium labeled internal standards were $d_3$-M, 446 *m/z*, 460 *m/z*, and 638 *m/z*; $d_3$-NM, 446 *m/z*, 460 *m/z*, 624 *m/z*.

## Results

### Gas Chromatography

A gas chromatogram of (150ug/L), recorded in the SIM mode, is shown in figure 4.35. A chromatographic separation was effected within 4 minutes with sufficient resolution

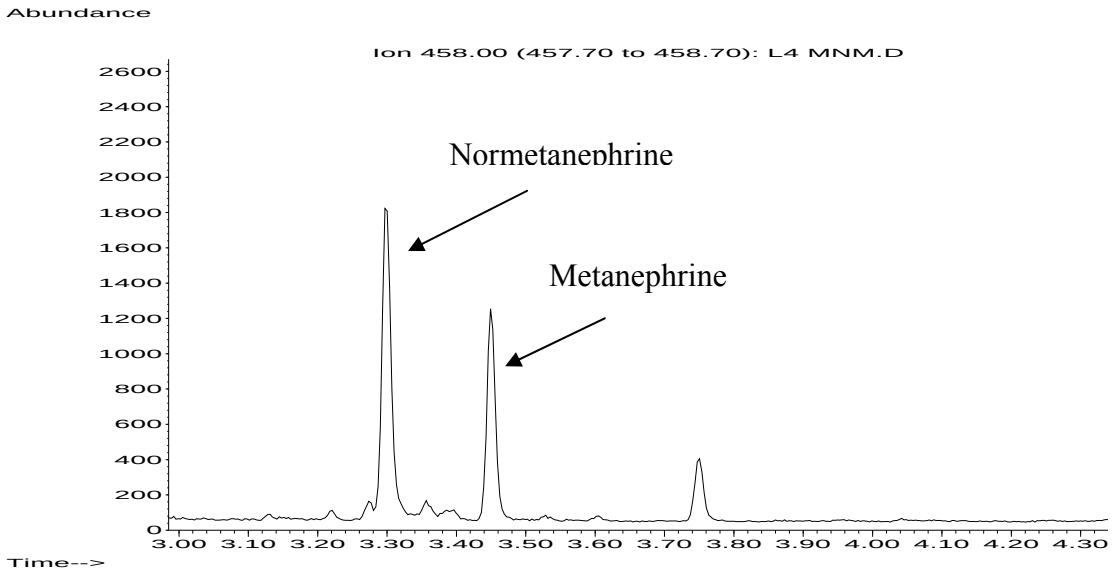



Figure 4.35: A gas chromatogram obtained in the selected ion monitoring mode of a calibration standard (150µg/L) extracted by the acylation procedure

Mass Spectrometry

A full scan mass spectrum of M and NM is shown in figures 4.36 and figure 4.37 respectively. A reaction scheme of the derivatization procedure is shown in figure 4.38.

The molecular ion of NM, 621 *m/z*, could be observed in the electron impact ionization mode, however, since the 458 *m/z* ion dominate in abundance this ion was used for quantification while 621 *m/z* was used for identification. For M, 635 *m/z* was used for identification and 458 *m/z* for quantification.

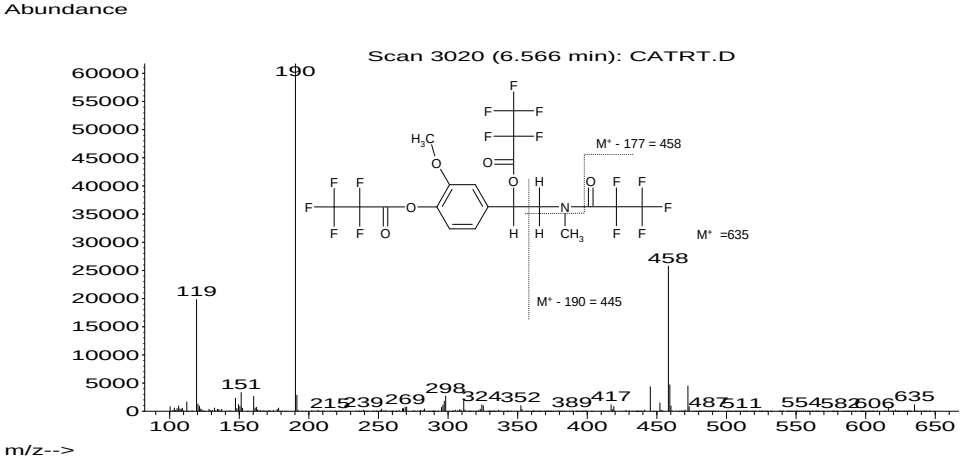



Figure 4.36: Full scan mass spectrum of metanephrine following derivatization by PFPA

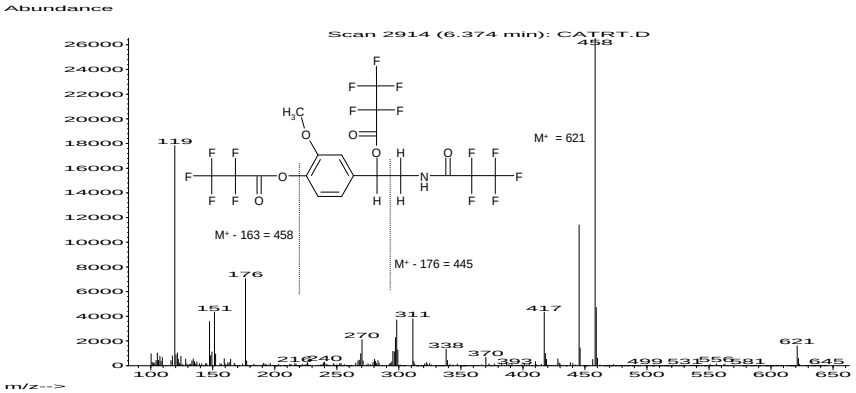



Figure 4.37: Full scan mass spectrum of normetanephrine following derivatization by PFPA.

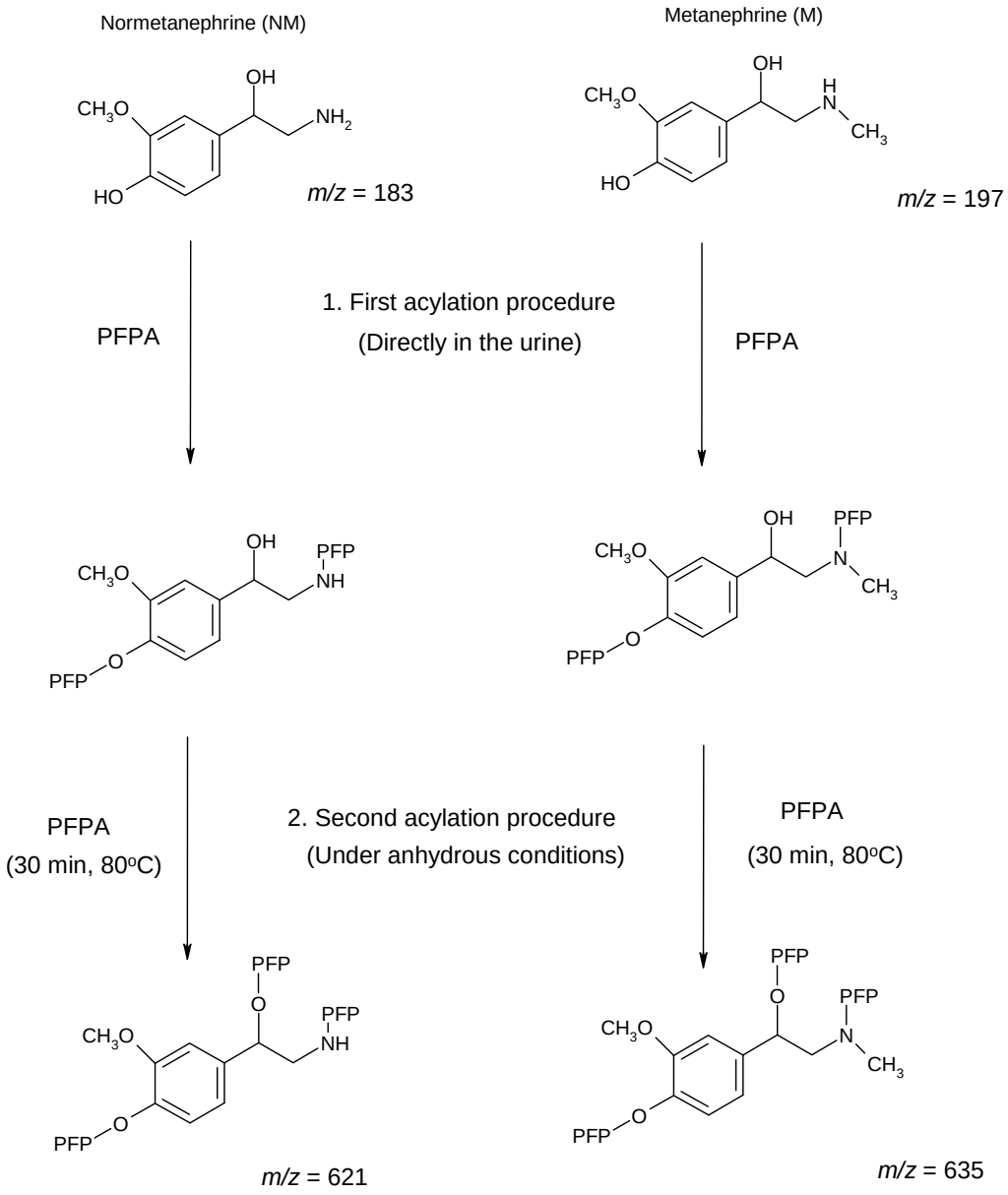



Figure 4.38: Reaction scheme of the acylation procedure with PFPA

## Validation

Validation parameters such as linearity, recovery, coefficient of variation (CV), and stability of controls over a period of time were investigated. Tables 4.5 and 4.6 provide a summary of the data obtained.

The assay was found to be linear from 25 – 600 µg/L, which covers the endogenous levels of M and NM[112], in a 24-hour urine collection. Recovery experiments were determined by spiking M and NM standards at two different concentration levels (50 µg/L, and 300 µg/L) in urine. These controls were stored at -20°C and analyzed against the work standards.

Table 4.5: Within batch validation parameters of M and NM using the extractive acylation procedure.

| Metabolite: | $r^2$ | Concentration | CV ,% (n=5) | Mean Recovery,% (n =5) |
|---|---|---|---|---|
| Metanephrine | 0.99 | 50µg/L | 4.47 | 103.91 |
| | | 300 µg/L | 4.06 | 95.73 |
| Normetanephrine | 0.99 | 50µg/L | 3.05 | 95.09 |
| | | 300 µg/L | 4.35 | 90.63 |

Table 4.6: Between batch validation parameters of M and NM using the extractive acylation procedure.

| Metabolite: | $r^2$ | Concentration | CV ,% (n=7) | Mean Recovery,% (n =7) |
|---|---|---|---|---|
| Metanephrine | 0.99 | 50µg/L | 9.46 | 93.99 |
| | | 300 µg/L | 9.39 | 82.89 |
| Normetanephrine | 0.99 | 50µg/L | 13.53 | 99.77 |
| | | 300 µg/L | 2.83 | 74.87 |

To establish the stability of the acylated derivatives, a spiked sample (M and NM, 75 µg/L) were subjected to the extractive derivatization procedure and the derivatized extract was injected 10 times over a period of 5 hours. The absolute and relative responses are plotted in figure 4.39 and 4.40 respectively. The variance in the absolute signal was due to variation in the instrument parameters (CV = 12.23 % and 10.36 % for NM and M respectively). It can be seen that the isotope labeled standard compensated for this variation in the relative response plot (CV = 5.42% and 2.07% for NM and M respectively). A similar sample, (M and NM, 75 µg/L) stored at room temperature for 24 hours yielded concentration values of 81.89 µg/L and 86.28 µg/L for NM and M respectively.

It was also noted that the assay is sensitive for interferences originating from the GC inlet. This may be due to contamination from other routine analysis. This was particularly the case when silylated derivatives were analyzed prior to the M and NM analysis.

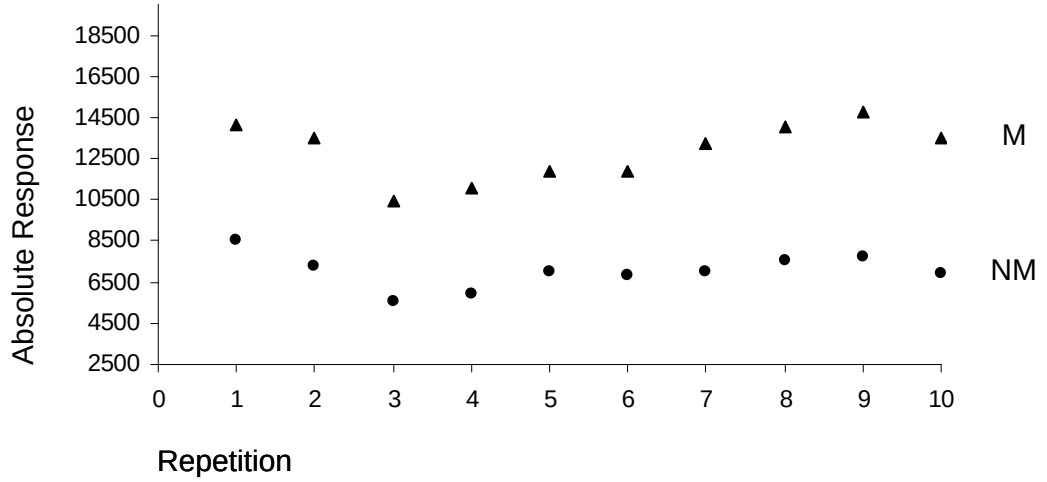



Figure 4.39: Absolute stability of the PFPA derivative of M and NM over a period of 5 hours with 10 injections of the same sample.

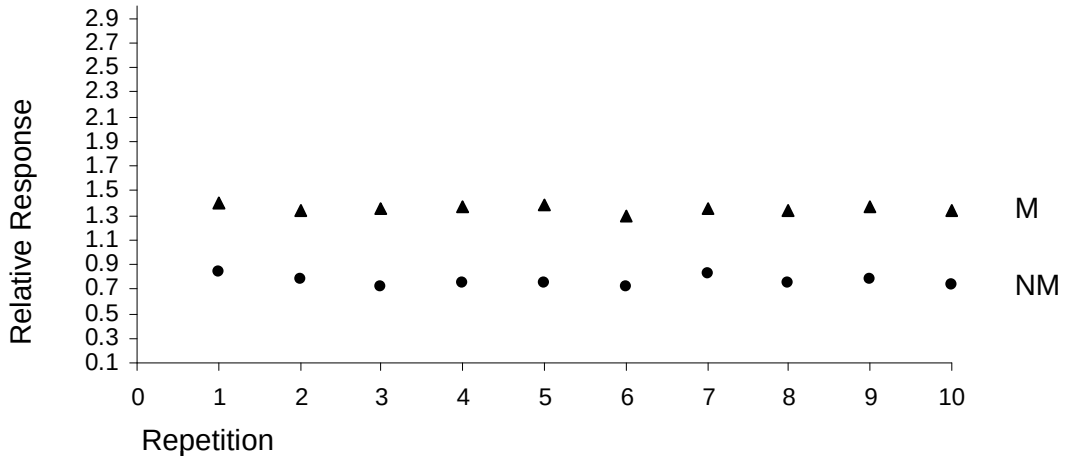



Figure 4.40: Relative stability of the PFPA derivative of M and NM over a period of 5 hours with 10 injections of the same sample.

It is therefore recommended to monitor the status of the inlet prior to analysis.

Care should also be taken not to transfer any bicarbonate particles prior to the second anhydrous derivatization procedure. Figure 4.41 illustrates the effect of a dirty inlet on the analysis, loss of signal and poor chromatography is evident of a dirty inlet.

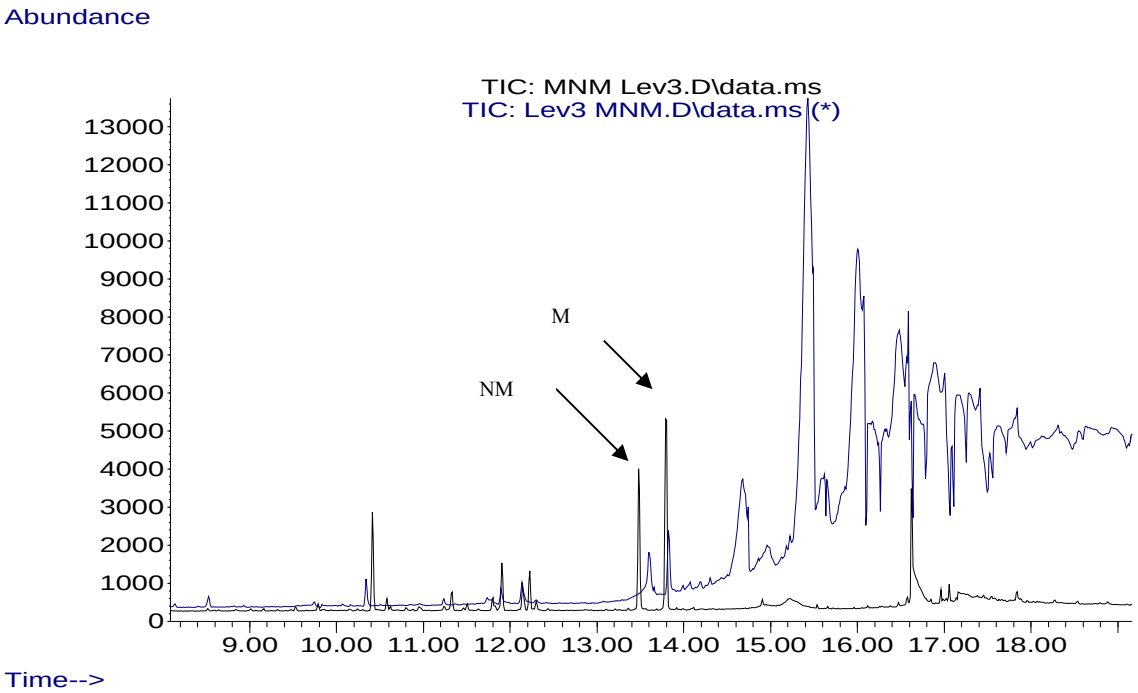



Figure 4.41: The effect of a dirty inlet on the chromatography and signal intensity

The method does however have its shortcoming: Attempts to analyze the endogenous catecholamines, epinephrine, norepinephrine and dopamine in urine by this method was ineffective, not even by incorporating deuterium labeled internal standards. A possible explanation is due to the use of the sodium bicarbonate which is required to drive the reaction mechanism and

removes excess acid formed. With the addition of the bicarbonate the pH increases and the endogenous catecholamines are subsequently degraded. Virtually no signal was observed when attempts were made to extract the endogenous catecholamines.

## Conclusion

A validated extractive acylation procedure for the analysis M and NM in human urine is proposed. The assay utilizes the potential of acylation by pentafluoropropionic anhydride (PFPA) combined with a liquid-liquid extraction procedure followed by GC-MS analysis with deuterium labeled internal standards. The use of GC-MS required for minimal sample preparation and decreased analysis time compared to more conventional HPLC analysis.

Furthermore, the use of PFPA as derivatization reagent also allowed for stable derivatives, permitting analysis of numerous samples in a batch-like manner without degradation over time. The molecular ions of the M and NM derivatives, 635 m/z and 621 m/z, could be observed in the electron impact ionization mode. Although the use of the molecular ion has the advantage that less interference is evident; the signal to noise is lower and therefore the 458 m/z ion was used for quantification while the molecular ions were used for identification. Below a concentration of 253 nmol/L (50µg/L) for M and 273 nmol/L (50µg/L) for NM the molecular ion signal could not be observed.

This method is proposed as the method of choice for quantitative analysis of M and NM in the clinical pathology laboratory. It is the preferred method when screening for pheochromocytoma due to the ease of operation, low coefficient of variation, acceptable recoveries, stable derivatives and cost effectiveness. This method was employed in the screening of a patient who presented with sustained hypertension. A case study of this patient is presented in chapter 6 of this work.

With the above methods completed and validated a method was developed for the simultaneous analysis of VMA and HVA. Even thought it was possible to analyze these two metabolites with the first proposed liquid-liquid extraction method, the method had limitations and high background noise for VMA and HVA. Although VMA is a poor marker for pheochromocytoma it has been mentioned in chapter 2 of this work that VMA and HVA are appropriate markers when screening for Neuroblastoma in children. For this reason a rapid GC-MS method was developed for the analysis of VMA and HVA. This method is described in the following section of this work.

### 4.3.4 Analysis of acidic catecholamine metabolites Vanillmandelic (VMA) – and – Homovanillic Acid (HVA) using pentafluoropropionic acid (PFPA) and pentafluoropropanol (PFP-OH) for the screening of Neuroblastoma

Introduction

In contrast with pheochromocytoma, the neuroblastoma is found predominantly in children and elevated levels of the acidic catecholamine metabolites vanilmandelic acid (VMA) and (HVA) is indicative of a tumor. From an extraction point of view the analyses of these two compounds are relatively easier than that of metanephrines simply because they are excreted in larger quantities than the metanephrines i.e. present at larger concentrations. However, due to their chemical nature the derivatization procedure needed to be adapted and the chromatographic conditions required some alterations to allow for successful analysis of both compounds.

## Experimental

### Chemicals and reagents

Vanilmandelic acid (VMA) and Homovanillic acid (HVA) were were purchased from Sigma-Aldrich Chemical Co. Deuterium labeled internal standards 4-Hydroxy-3-methoxymandelic -2- $d_1$-Acid ($d_1$-VMA) and 4-Hydroxy-3-methoxyphenylacetic -2,2-$d_2$ acid ($d_2$-HVA) were purchased from Cambridge Isotope Laboratories Inc. Derivatization reagent, pentafluoropropionic anhydride (PFPA) and pentafluoropropanol (PFP-OH) was purchased from Fluka (Buchs, Switzerland). GC grade ethyl acetate and hydrochloric acid were form Merck (Darmstadt, Germany). De-ionized water from a Milli-Q (Millipore, USA) was used for the preparation of standards.

### Solutions

A stock solution (100 mg/L) of VMA and HVA were prepared in Hydrochloric acid (0.01M) and stored at 4°C until used. The internal standard stock solution (100 mg/L) of $d_2$-VMA and $d_2$-HVA were prepared in acetonitrile and stored at -20°C until used. Serial dilutions of the VMA and HVA stock solution were prepared in hydrochloric acid (0.01 M) to obtain the work standards as shown in table 4.7.

Table 4.7: Concentrations of the working calibration standards. The calibration standards were diluted ten times in the urine matrix to construct calibration curves ranging from 0.5 – 10.0 mg/L.

| | Standard 1 | | Standard 2 | | Standard 3 | | Standard 4 | | Standard 5 | | Standard 6 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mg/L | μmol/L | mg/L | μmol/L | mg/L | μmol/L | mg/L | μmol/L | mg/L | μmol/L | mg/L | μmol/L |
| VMA | 5 | 25 | 10 | 50 | 15 | 75 | 40 | 202 | 80 | 404 | 100 | 505 |
| HVA | 5 | 27 | 10 | 54 | 15 | 82 | 40 | 220 | 80 | 440 | 100 | 549 |

Calibration curves

A urine pool from a healthy volunteer was used to construct the calibration curves for VMA and HVA by the method of standard addition. To an aliquot of urine (400μl), work standard (50 μl) as well as internal standard (50μl) were added. Six-point calibration curves were constructed by using the relative response (peak area) of the analyte versus the deuterium labeled internal standard. The concentration range of the calibration curves ranged from 0.5 – 10.0 mg/L which covers the amount of endogenous VMA and HVA found in human urine[112].

Extraction procedure

The catecholamine metabolites VMA and HVA were extracted by ethyl acetate under acidic conditions. To an aliquot of urine (400μl), spiked with the internal standard (50μl), NaCl (50 mg) and ethyl

acetate (2 ml) were added. The samples were capped with a PTFE lined cap and vortexed for 5 min at room temperature. Thereafter they were centrifuged (3000 rpm, 5 min) and the organic layer removed with a pasteur pipette and transferred to a clean reaction vial. The extract was dried under a stream of dry nitrogen gas.

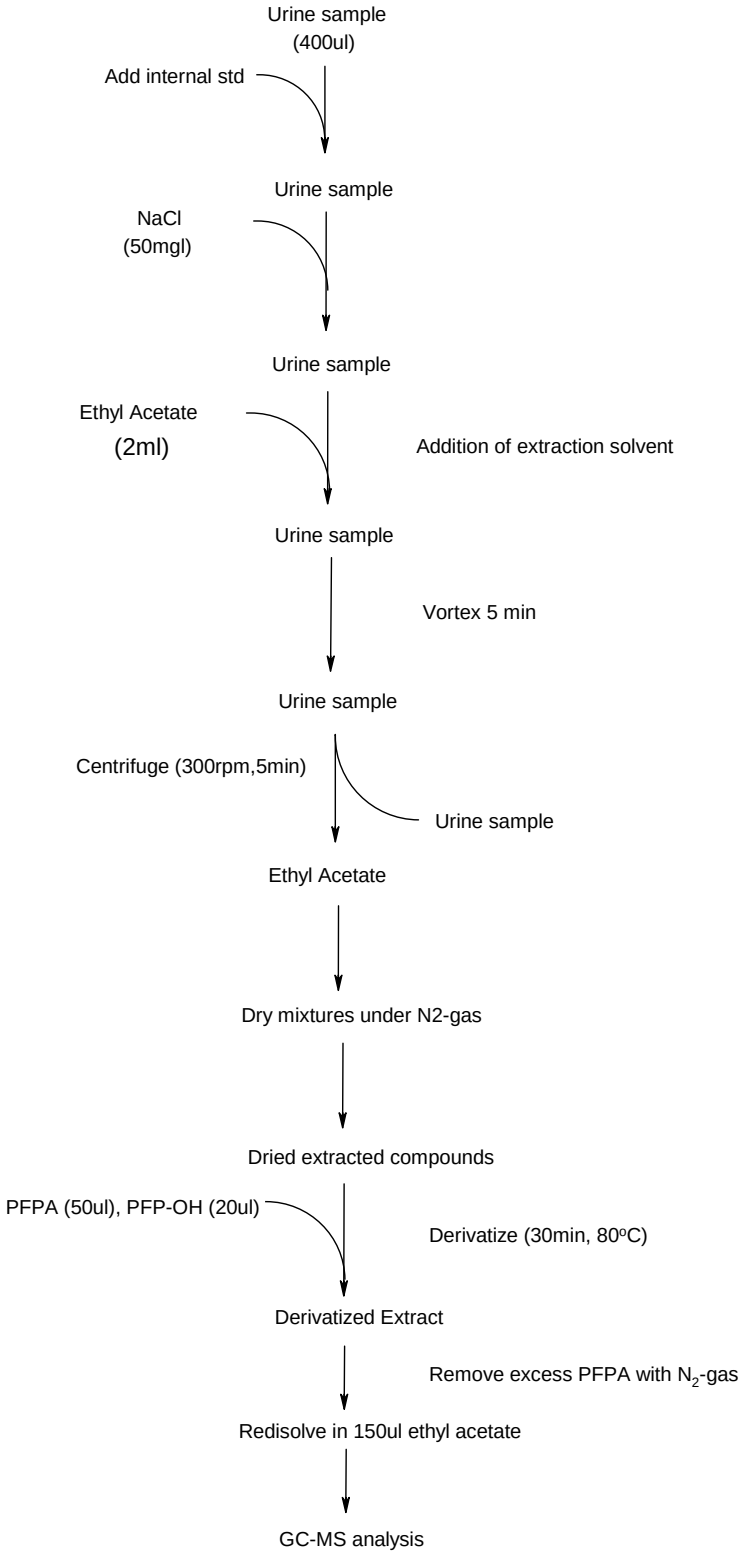
Derivatization

Derivatization of VMA and HVA was performed as described by Watson *et al.*[114] In short; the dried extract was derivatized with 50 µl PFPA and 20 µl PFP-OH at 80$^{o}$C for 30 min. The excess derivatized reagent was removed under a gentle stream of dry $N_2$-gas. The derivatized compounds were reconstituted in 150 µl ethyl acetate before subjected to GC-MS analysis. A flow diagram of this extraction procedure is shown on the following page.

Gas Chromatography-Mass Spectrometry

A Hewlett Packard (HP6890) GC system equipped with a HP 7683 auto injector and HP5973 mass selective detector (Agilent Technologies, Palo Alto, CA, USA) was used for chromatographic separation and recording of mass spectra. A DB5 fused silica capillary column (5m x 100µm; $d_f$ 0.1 µm) (Agilent Technologies) was used. Data collection and integration was performed with the HP Chem Station Software.

Figure 4.42: Flow diagram of the extraction procedure of VMA and HVA from urine



A 2µl volume of the derivatized sample was injected in split mode. A split ratio of 100:1 was used. The inlet temperature was 250°C and helium was used as carrier gas at a constant flow of 0.2 ml/min. The average velocity was 40 cm/s. The GC oven was held at 85°C for 0.25 min and ramped at 40°C/min to 140°C. The second ramp rate was 25°C/min to 185°C. The final ramp was 50°C/min to an isotherm of 300°C, which was held for 0.5 min. The chromatographic analysis was completed in 6.2 minutes.

Mass spectrometric quantification was performed in the single – ion- monitoring mode (SIM). The transfer line was set at 280°C and the source and quadruple temperatures were 230°C and 150°C respectively. The mass spectra were collected in the electron impact mode at 70eV. The mass-to-charge ratios (*m/z*) of the ions used for quantification in SIM mode were: VMA , 445 *m/z*, 622 *m/z* and 417 *m/z*; HVA, 283 *m/z*, 313 *m/z*, 460 *m/z* for the endogenous VMA and HVA. The ions used for the deuterium labeled internal standards were $d_1$-VMA, 446 *m/z*, 623 *m/z*, and 418 *m/z*; $d_2$-HVA, 285 *m/z*, 315 *m/z*, 460 *m/z*.

Results

Gas Chromatography

A gas chromatogram obtained by selected ion monitoring of VMA and HVA (1.5mg/L) is shown in figures 4.43 and 4.44 respectively. Note the high signal to noise ratio obtained. This advantage is due to

the use of the molecular ions for quantification since their abundance in the electron impact mode was sufficient to be used for quantification. Chromatographic analysis of VMA and HVA was completed in 2.2 min due to the short column length, small internal diameter and thin film thickness.

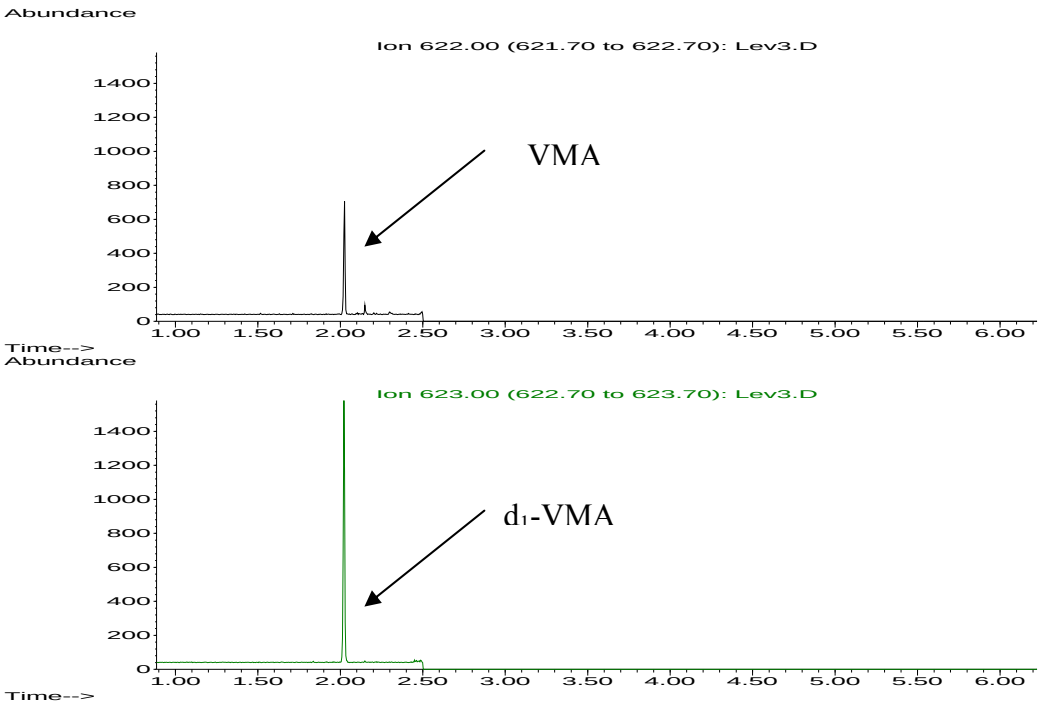



Figure 4.43: Selected ion chromatogram of a urine extract at a concentration of 1.5 mg/L for VMA and $d_1$-VMA.

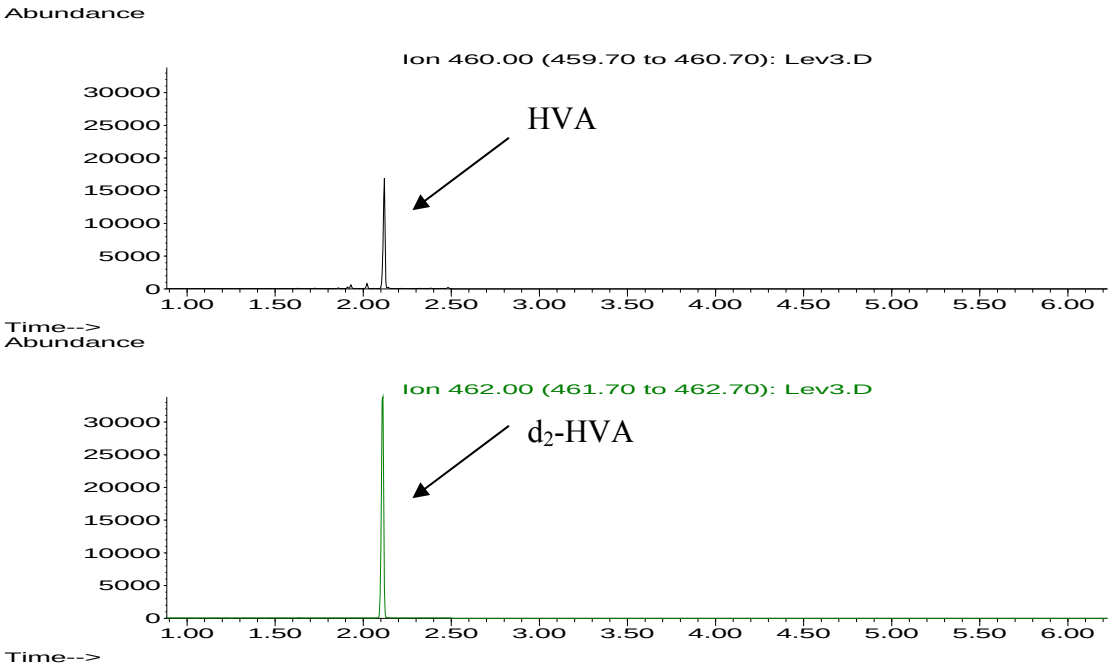



Figure 4.44: Selected ion chromatogram of a urine extract at a concentration of 1.5 mg/L for HVA and $d_2$-HVA

## Mass Spectrometry

Full scan mass spectra of VMA and HVA are shown in figures 4.45 and 4.46 respectively. A reaction scheme of the derivatization procedure is shown in figure 4.47.

The molecular ion as well as three distinctive ions was produced in the electron impact mode. The molecular ion of both compounds had sufficient intensity for quantification.

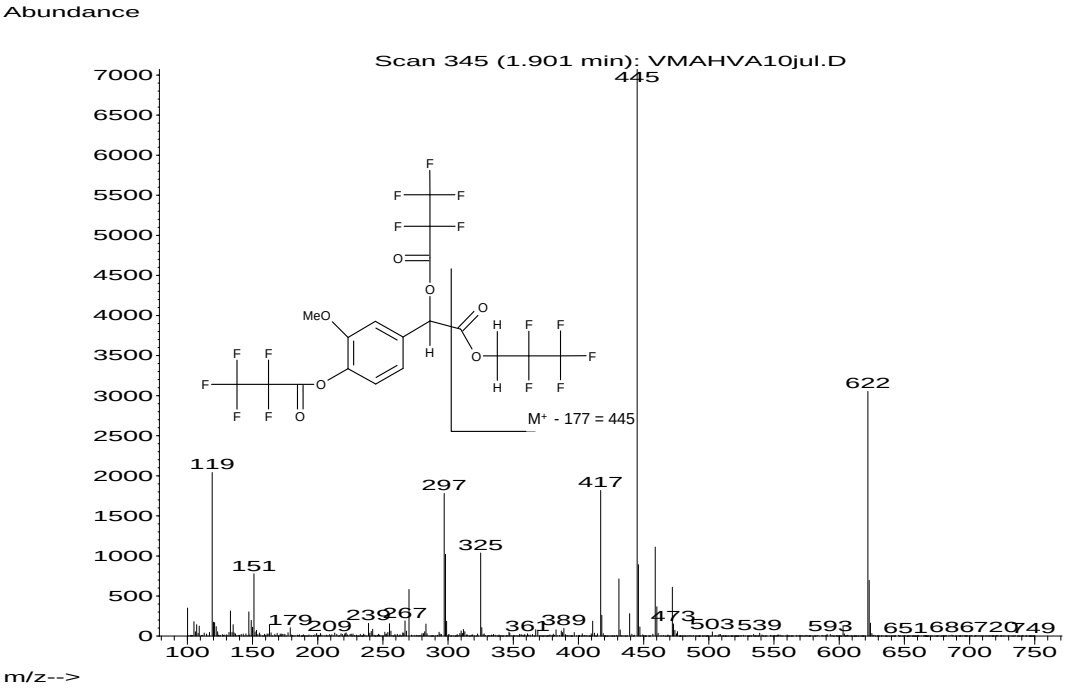



Figure 4.45: Full scan mass spectrum of VMA derivatized with PFPA and $H_2PFP$

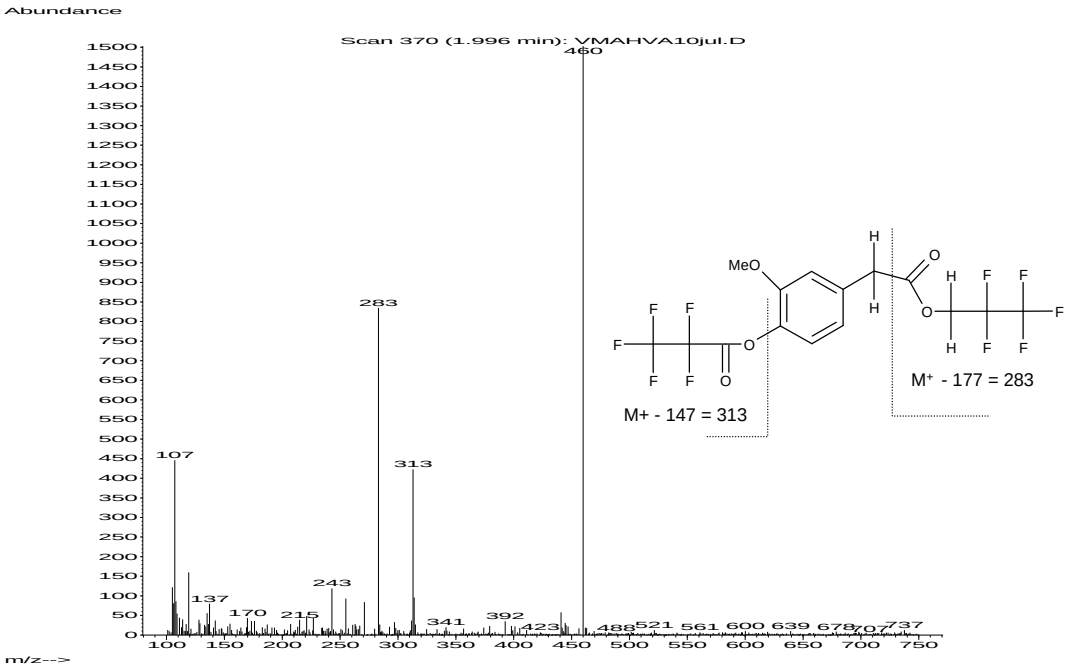



Figure 4.46: Full scan mass spectrum of HVA derivatized with PFPA and $H_2PFP$

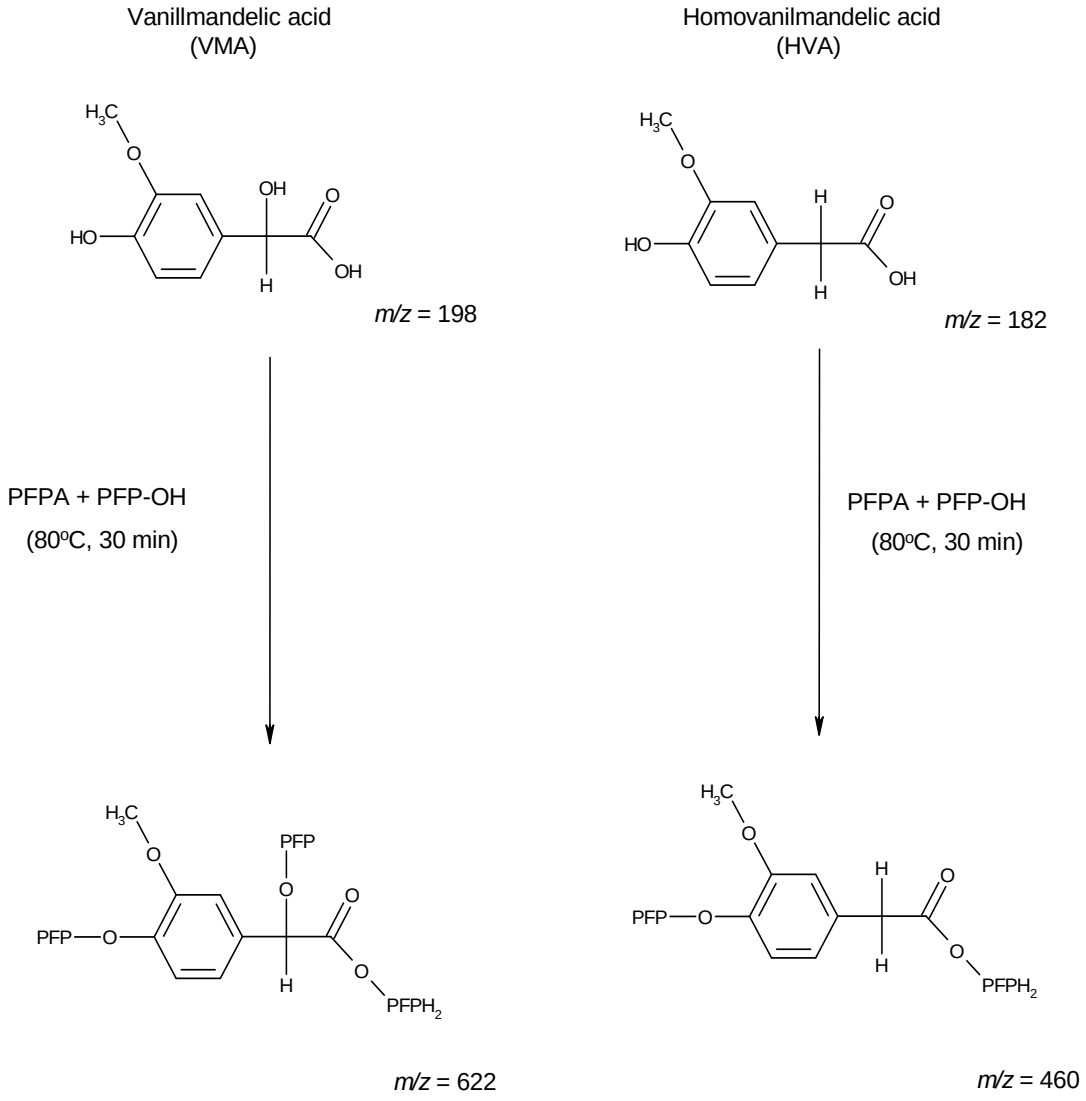



Figure 4.47: Reaction scheme for the reaction of VMA and HVA with PFPA and PFP-OH.

## Validation

Validation parameters such as linearity, recovery and coefficient of variation (CV) were investigated. Table 4.8 provides a summary of the data obtained.

The assay was found to be linear from 0.5 – 10 mg/L, which covers the endogenous levels of VMA (1.4 – 6.5 mg/day) and HVA (1.4 – 8.8 mg/day), in a 24-hour urine collection[88]. Recovery experiments were performed by spiking VMA and HVA standards at two different concentration levels (0.5 mg/L, 10 mg/L) in urine. These controls were stored at -20°C and analyzed against the work standards.

Table 4.8: Validation parameters of VMA and HVA obtained using the extraction procedure described.

| Metabolite: | $r^2$ | Concentration CV ,% | Within Batch (n=5) | Mean Recovery,% (n =5) |
|---|---|---|---|---|
| VMA | 0.99 | 0.5mg/L | 6.61 | 93.82 |
| | | 10 mg/L | 2.43 | 100.65 |
| HVA | 0.99 | 0.5mg /L | 2.94 | 110.00 |
| | | 10 mg/L | 2.20 | 100.29 |

## Conclusion

Quantification of the acidic catecholamine metabolites, HVA and VMA, are known to be sensitive markers for neuroblastoma, one of the most common tumors found in children. Although pheochromocytomas are capable of secreting large amounts of VMA it is well known that VMA is in fact a poor marker for pheochromocytomas. Therefore this method is proposed as a screening technique for neuroblastoma in children rather than for pheochromocytoma. The method has the advantages that it allow for minimal sample preparation followed by derivatization in 30 minutes which makes it a suitable screening technique for laboratories that process large batch analysis. Furthermore, acceptable recoveries and coefficient of variance values were obtained for this method.

# Chapter 5: Case Study - Confirmation of a sympatho-adrenal tumor in a patient suffering from consistent hypertension by GC-MS.

## 5.1 Introduction

A twenty four hour urine sample from a patient (female, age 14) was received from the Pretoria Academic Hospital (PAH) for the analysis of VMA, M and NM and 5-Hydroxyindole acetic acid (5-HIAA). Clinically, the patient suffered from excessive hypertension and complained from abdominal pain. The blood pressure values upon admission to the hospital are shown in figure 5.1.

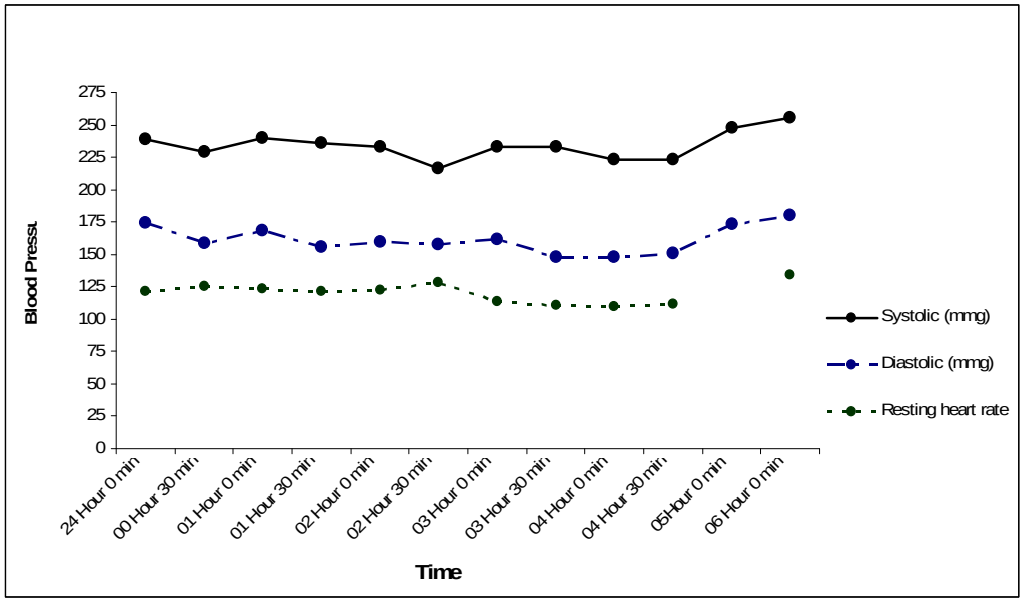



Figure 5.1: Blood pressure values of a patient suspected of having a pheochromocytoma over a period of six hours following admission to the hospital

Subsequently, the sample was subjected to GC-MS analysis using methods 4.3.3 and 4.3.4 as described in this work.

5.2 Explanation of data obtained.

The extractive acylation of M and NM indicated highly elevated levels of NM as illustrated in figure 5.2. Furthermore, elevated levels of VMA were observed using the liquid-liquid extraction method. The patient showed normal urinary 5-HIAA excretion patterns which excluded the possibility of a carcinoid tumor. The sample was also analyzed using conventional HPLC techniques. These results are in comparison with those obtained by GC-MS. Both HPLC and GC-MS analysis showed highly elevated levels of NM and VMA with moderately elevated levels of M. It is well known that pheochromocytomas may secrete large amounts of NE as opposed to E, which are the metabolic precursors of NM and M respectively. This is in agreement of data obtained in this specific case. The calculated concentration levels of VMA, 5-HIAA, M and NM are shown in table 5. 1.

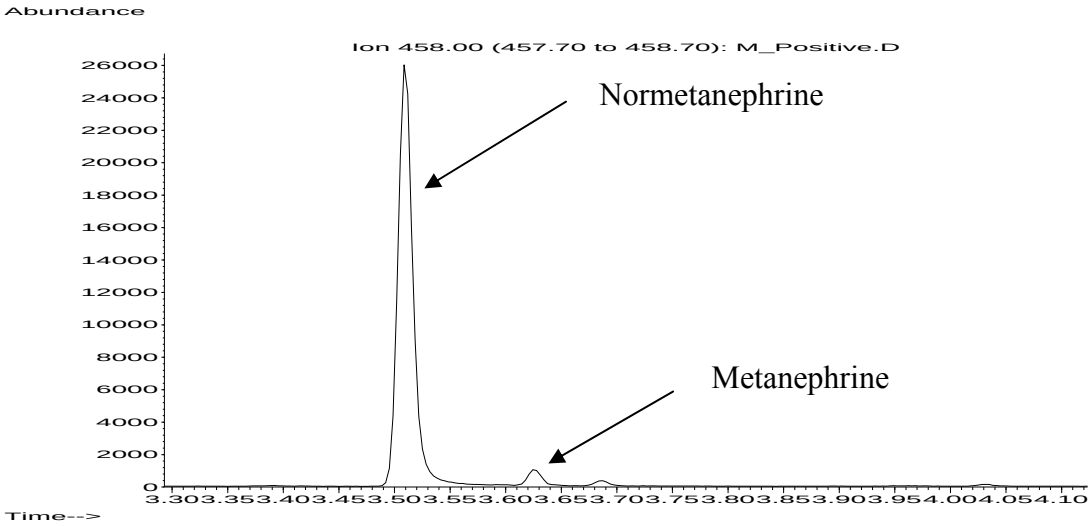



Figure 5.2: A gas chromatogram obtained in the selected ion monitoring mode of a patient's urine diagnosed with a sympatho-adrenal tumor. Note the elevated levels of Normetanephrine in the urine.

Table 5.1: Calculated concentration levels of VMA, 5-HIAA, M and NM.

| Metabolite used | Normal Range (24 Hour urine) | Concentration Reported | Technique |
|---|---|---|---|
| VMA | 7 – 33 µmol/Day | 190 µmol/Day | GC-MS |
| | | 332 µmol/Day | HPLC |
| 5HIAA | 10 – 31 µmol/Day | 13 µmol/Day | GC-MS |
| | | 24 µmol/Day | HPLC |
| M | 375 – 1506 nmol/Day | 1540 nmol/Day | GC-MS |
| | | 1999 nmol/Day | HPLC |
| NM | 573 – 1932 nmol/Day | 82769 nmol/Day | GC-MS |
| | | 123332 nmol/Day | HPLC |

The difference in concentration values obtained by the two techniques can be explained as follow:

1. HPLC techniques are more prone to interferences due to detector limitations as opposed to more sensitive GC-MS detection.
2. In both instances the concentration levels are enormously elevated i.e. accurate quantification becomes difficult since the concentration levels are outside the calibration range.
3. Both techniques however show highly elevated NM levels, moderately elevated M levels and highly elevated VMA levels.

The patient was transferred two days after admission to Montana Private Hospital where magnetic resonance imaging (MRI) was performed on the abdomen and pelvis. The MRI scan revealed that: "*a round well circumscribed mass is visible lying adjacent to the aorta and anterior to the origin of the psoas muscle on the left. This mass measures 4 x 3.9 x 4.5 cm. In this patient with M.E.N syndrome as well as blood pressure instability, this picture is consistent with a left paravertebral phaeochromocytoma. The adrenal glands appear normal*". [115]

The patient was transferred back to PAH and surgery was scheduled for the excision of an extra adrenal pheochromocytoma.

5.3 Histology Report.

The histology report revealed the following results:

The tumor and surrounding lymph nodes was removed. The clinical information revealed that the tumor was intra-abdominal, extra adrenal and removed from the sympathetic chain.

On a macroscopic level the specimen was labeled as a tumor "*and consists of a soft yellow nodule, measuring 55 x 40 x 35 mm and weighing 32g. The outer surface is smooth. On cut section the mass appears to be well encapsulated. The tissue is very soft and haemorrhagic with focal areas of cystic degeneration having an average diameter of 6 mm.*"[116] On a microscopic level the specimen showed "*the presence of a tumor having packeted appearance. Focal haemorrhage and cystic degeneration are identified. Vascular channels are easily identified. The features are those of a paraganglioma*"[118]

5.4 Concluding remarks:

It was later revealed that the patient had loss off sight following surgical removal of the tumor. Loss of sight is not a common finding in patients with sympatho-adrenal tumors, however in this specific case the prolonged and sustained hypertension led to hypertensive retinopathy which subsequently caused the loss of sight in the patient.

Following surgical removal of the pheochromocytoma two more VMA analysis were carried out. These values were both below 25µmol/L and within the normal urinary reference range.

The above case study again shows the importance of rapid detection of sympatho-adrenal tumors. In this instance the patient was "fortunate" since adequate medical and analytical action was taken rapidly to establish the cause of the secondary hypertension. However not all patients are so fortunate.

In one specific case study[117] a patient suffered from consistent hypertension for two years. Following knee surgery the patient suffered a cardiac arrest and died. The cause of death was due to acute heart failure and hypokalaemia secondary to the presence of a supra-renal pheochromocytoma only discovered during the autopsy. The case was settled for the sum of R 1,272,900.00 due to negligence for not correctly monitoring the hypokalemia following the operation and for not referring the patient when anti-hypertensive therapy didn't yield the results as expected. This case study again shows the importance of accurate and rapid screening for sympatho-adrenal tumors.

# Chapter 6

## *Conclusion on methods used for the analysis of catecholamines and their metabolites.*

### 6.1 Introduction

Analysis of the catecholamines and their metabolites is hampered by the low concentration in which they occur in human urine as well as their amphoteric chemical properties. Time and effort is of essence in the development of selective extraction procedures towards these metabolites to screen effectively for sympatho-adrenal tumors. Tedious sample preparation is a time constraint in the clinical laboratory which requires results in the shortest possible time.

Four analytical methods were developed and utilized for the analysis of the endogenous catecholamines, their basic and acidic metabolites by gas chromatography – mass spectrometry. The extraction procedures included liquid – liquid, as well as solid phase extraction procedures while the derivatization techniques covered three main derivatization procedures namely: acylation, alkylation and silylation procedures. Each method has its own advantages and disadvantages which are discussed in this final chapter.

*6.2 Simultaneous analysis of the catecholamines their basic and acidic metabolites by a single extraction procedure.*

As a rapid screening technique for the simultaneous analysis of the endogenous catecholamines their basic as well as acidic metabolites a method is proposed by alkylation followed by silylation.
The method involves derivatization of the amine groups with methyl chloroformate in the urine matrix followed by a basic pH shift to enhance the derivatization procedure and subsequent extraction using ethyl actetate. The subsequent dried extract is derivatized with MTBSTFA and analysis is performed on a DB-17MS column.

The possibility exists that the analysis can be completed in less than five minutes on a GC-MS. Further derivatization with MTBSTFA yielded stable derivatives with distinctive mass spectra for the compounds analyzed.

The method is however proposed as a screening technique for tumors or preliminary analysis due to extraction and chromatographic capabilities as discussed previously.

*6.3 Analysis of catecholamines and their basic metabolites by solid phase extraction*

Solid phase extraction techniques are known for their superior extraction capabilities due to the selectivity obtained by selecting the correct stationary phase prior to extraction.

For this reason an attempt to extract the catecholamines and the metanephrines by a cation –exchange mechanism using a strong cation exchange solid phase extraction cartridge were made. Following extraction, the compounds were derivatized first by methylation of the benzylic hydroxyl group followed by acylation by PFPA. This duel derivatization technique caused a "*shift"* in the mass spectra obtained with increase the sensitivity of the assay. Of course a draw back of this assay is the skill required for solid phase extraction, the adjustment of the aqueous phase to the correct pH prior to extraction and duel derivatization. One main advantage of this assay is the superior clean –up prior to analysis on the GC-MS, allowing for less interference. The method did yield high recoveries for M and NM and low coefficient of variance in the assay.

*6.4 Analysis of Metanephrine and Normetanephrine by extractive acylation*

The analysis of metanephrine and normetanephrine provide a sensitive means for the detection of the pheochromocytoma.

However, analysis is complicated due to the low concentration levels and the amine group which hinders extraction. To circumvent these problems an extractive acylation procedure was utilized to effectively derivatize the amine group in the aqueous matrix followed by a liquid – liquid extraction with dichloromethane. A second derivatization procedure was required to derivatize the benzylic hydroxyl group.

The method is easy to use and allows for rapid analysis of metanephrine and normetanephrine without tedious sample preparation. Of caution is the fact that the method is sensitive towards "*interferences*" from other derivatization reagents used on the same GC-MS system; the result being loss of signal as discussed previously. This method is the method of choice to use when screening for pheochromocytoma due to the low CV%, high recoveries, ease of operation and stability of the derivative.

### *6.5 Rapid analysis of Vanilmandelic – and – Homovanillic Acid by GC-MS*

This method was developed specifically for the analysis of VMA and HVA to screen for the neuroblastoma, since the acidic metabolites are the best marker for this type of sympatho – adrenal tumor.

The method involves a rapid extraction procedure by ethyl acetate followed by derivatization via PFPA and PFP-OH. The extraction method performs optimally under acidic conditions and the metabolites are extracted efficiently with the addition of sodium chloride to effectively "*salt out*" the compounds in the organic phase. The derivatization technique employed, allowed for the detection of HVA and VMA using the molecular ions as well as two qualifier ions with a high *m/z* ratio – the result being less interference since low *m/z* ratios a prone to interference from compounds with low molecular mass.

It should be noted that the extraction by ethyl acetate is a fairly non specific extraction procedure from the urine matrix, causing the inlet liner of the GC to foul quickly. For this reason a high split ratio is recommended with an injection volume of 1 µl. Even though a relatively non specific extraction method was used the assay did yield acceptable recoveries and low coefficient of variance making it a suitable method for the screening of neuroblastomas in the routine clinical laboratory.

GC-MS has been, and in future will remain one of the most utilized analytical techniques in the laboratory whether it is for clinical, forensic, food or industrial purposes. With the advent of more affordable GC-MS systems available it remains to be seen whether this powerful analytical technique will be utilized more for catecholamine research.

It is hoped that the GC-MS methods discussed in this work will aid in the future analysis of the catecholamine and their metabolites in the clinical laboratory.

**Appendix 1: Sensitivity and specificity, the confusion in the medical and analytical professions.**

Sensitivity and specificity are described here since these terms have different meanings in the medical profession and analytical chemistry.

Diagnostic sensitivity can be defined as the percentage of patients who have a disorder and are correctly diagnosed by the assay used[118,119]. On the contrary, analytical sensitivity merely refers to the smallest amount (expressed as a concentration) of a substance that can be accurately measured by the assay; more commonly known by analytical chemists as the limit of detection.

Diagnostic specificity can be defined as the percentage of patients who do not have a disorder and who are correctly diagnosed as negative by the assay used. On the contrary, the term specificity and selectivity is used interchangeably in analytical chemistry. The difference of these two terms is discussed in detail by Riley and Rosanske[120]. In analytical chemistry selectivity is defined as: "*the ability of an analytical method to differentiate various substances in the sample and is applicable to methods which two or more components are separated and quantified in a complex matrix*."[117] In contrast the term specificity strictly uses the term "*to measure unequivocally the analyte of interest in the presence of all other*

*components which might be present.*"[117] For this reason the term selectivity is appropriate in chromatographic techniques since the compounds are physically separated from each other, whereas specificity should be used for analytical techniques in which only a single parameter can be measured; for example the measurement of radioactivity by a radioimmunoassay.

# Appendix 2: List of figures and tables

## Figures:

## Tables:

# List of References

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