NEAR-BODY DRUGS OF ABUSE TESTING IN A POST MORTEM MEDICO-LEGAL POPULATION IN SOUTH AFRICA: A PILOT STUDY

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, in partial fulfilment of the requirements for the degree

of

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DECLARATION

I, **Candice Geraldine Hansmeyer**, declare that this research report is my own work. It is being submitted for the degree of Master of Medicine at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

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On this date

ABSTRACT

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NEAR-BODY DRUGS OF ABUSE TESTING IN A POST MORTEM

MEDICO-LEGAL POPULATION IN SOUTH AFRICA: A PILOT STUDY

Background:

Forensic toxicological analysis in SA is fraught with delay, partly due to lack of equipment as well as lack of experienced staff, but also due to the overwhelming number of backlogged Forensic Chemistry Laboratory (FCL) cases, as well as new cases being submitted to the FCL for generalised untargeted toxicology screening using gold-standard chromatographic techniques. These delays continue to result in significant adverse legal, social and administrative consequences. Reliable rapid near-body screening toxicological tests would reduce the load on the Forensic Chemistry Laboratories and improve efficiency of the South African criminal justice system.

Methods:

This was a prospective, observational and transverse study examining a sample population from the Johannesburg Forensic Pathology Service (FPS) Medico-Legal Mortuary in South Africa. Whole blood samples were taken from femoral vessels of decedents. Blood samples were processed and qualitatively analysed using near-body tests (Randox Drugs of Abuse (DOA) I and Narcotics Detector(ND) immunoassays) and liquid chromatography coupled with mass spectrometry (LC-MS). Amphetamines, tetrahydrocannabinol (THC) and opiates were the drugs of abuse examined in this study.

Results:

In total 102 whole blood samples were collected and processed from 102 decedents between March – May 2014 and subsequently analysed.

Randox and ND performed well when compared to LC-MS. The poorest performance was in the diagnosis of THC, where the specificity of Randox was low (84%) and the sensitivity of ND was very low (28.6%). Although the confidence intervals were wide positive predictive values for THC were also poor using both Randox (32%, 95% CI 0.15-0.55) and ND(40%, 95% CI 0.07-0.83). However, negative predictive values for THC were good with narrow confidence intervals using both Randox (100%, 95% CI 0.94-1) and ND(94%, 95% CI 0.88-0.98) .

Negative predictive values for opiates (ND - 98% and Randox - 94.8%) and amphetamines (ND - 100% and Randox - 100%) using both ND and Randox were also good with narrow confidence intervals. The results for amphetamines should be treated with caution however, as the overall positivity rate was very low.

Positive predictive values for opiates and amphetamines appeared good, but due to the low positive test prevalence in this population, the confidence intervals were wide and these values are possibly not accurate.

There was significant correlation shown between LC-MS, Randox and ND using Cohen's kappa (fair to substantial correlation) and the McNemar two-tailed test ($p<0.05$) for all drugs with the exception of the use of Randox to detect THC.

Conclusion:

Narcotics Detector and Randox DOA I were found to be viable near-body tests that have good negative predictive value and specificity. This makes them promising screening tests that could potentially be added to the armamentarium of the forensic pathologist.

PRESENTATIONS ARISING FROM THIS PROJECT

POSTER PRESENTATION:

- School of Clinical Medicine Research day, Health Sciences, University of the Witwatersrand, 2015.

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LIST OF ABBREVIATIONS

DAD- Photodiode array detection

DOA – Drugs of abuse

DoH – Department of Health

℃ - Degrees centigrade

CEDIA - Cloned Enzyme Donor Immunoassay

EDTA - Ethylenediaminetetraacetic acid

EMIT - Enzyme Multiplied Immunoassay Techniques

FCL – Forensic Chemistry Laboratory

FPIA - Fluorescent Polarisation Immunoassay

FPS – Forensic Pathology Service

GC – Gas chromatography

GC-MS – Gas chromatography – mass spectrometry/spectroscopy

GUS- General unknown screening

HPLC – High performance liquid chromatography

KIMS - Kinetic Interaction of Micro particles in Solution

LC – Liquid chromatography

LC-DAD – Liquid chromatography – photodiode array detection

LC-MS – Liquid chromatography – mass spectrometry/spectroscopy

MDMA - 3,4-methylenedioxy-methamphetamine (“Ecstasy”)

MEIA - Micro Particle Enzyme Immunoassay

mg – Milligrams

μg – Micrograms

μl - Microliters

ml – Millilitres- Litres

MS – Mass spectrometry/spectroscopy

MS-MS – Mass spectrometry-mass spectrometry (tandem mass spectrometry)

ND – Narcotics Detector

PMI – Post mortem interval

PMR – Post mortem redistribution

Randox DOA I – Randox Drugs of Abuse I assay

RPM – Revolutions per minute

SACENDU - South African Community Epidemiology Network on Drug Use

SA – South Africa

SAPS – South African Police Service

STA – systematic toxicological analysis

THC- Δ9 –tetrahydrocannabinol

TOF – Time of flight mass spectrometry

WHO – World Health Organisation

CHAPTER 1 - INTRODUCTION

This chapter forms the introductory chapter. A short introduction outlines the motivation for the study followed by the aims, objectives, that were used in the study. The chapter is concluded by the ethical considerations of the study.

1.1 INTRODUCTION

Forensic toxicological analysis in South Afirca(SA) is fraught with delay, partly due to lack of equipment and experienced staff, but also due to the overwhelming number of backlogged Forensic Chemistry Laboratory (FCL) cases. New cases are continuously being submitted to the FCL for generalised untargeted toxicology screening using gold-standard chromatographic techniques. These delays continue to result in significant adverse legal, social and administrative consequences. Reliable, rapid and near-body screening toxicological tests would reduce the load on the FCL and improve efficiency of the South African criminal justice system.

1.2 AIM

The aim of this study is to determine if Narcotics Detector (ND) point of care testing can be used as a reliable screening tool in a South African post mortem population for the presence of targeted drugs of abuse by measuring its performance against the gold standard liquid chromatography and mass spectrometry (LC-MS) technique. We also aim to compare the performance of the Narcotics Detector kit with another point of care test namely the Randox Drugs of Abuse (DOA) I biochip immunoassay. Lastly, this study will attempt to confirm the reliability of the Randox DOA I biochip immunoassay against gold standard LC-MS in a South African post mortem population.

1.3 OBJECTIVES

- To determine the presence in blood samples of opiates, cannabinoids and methamphetamine in a South African post mortem population, using the Randox DOA I immunoassay.

- To determine the presence in blood samples of opiates, cannabinoids and methamphetamine in a South African post mortem population, using the ND screening test kit.

- To determine the presence and blood concentration of opiates, cannabinoids and methamphetamine in a South African post mortem population, using LC-MS.

- To compare the specificity, sensitivity, negative predictive and positive predictive values of the ND screening results with that of the Randox DOA I immunoassay results.

- To compare the performance of the Narcotics Detector screening results against the LC-MS results.

- To compare the performance of the Randox DOA I immunoassay results against the LC-MS results.
- To compare performance of the Randox DOA I immunoassay results against the ND results.

1.4 LITERATURE REVIEW

1.4.1 DEFINITION OF FORENSIC TOXICOLOGY

The term 'forensic toxicology' is used where the results of toxicological studies may be required for medico-legal purposes or judicial investigations. [1]

All therapeutic agents have the potential to be toxic in various doses. These agents together with the illicit range of substances forms an enormous arsenal of compounds, with which toxicology is concerned. These compounds also include common household, agricultural or horticultural chemicals with which people come into contact with on a daily basis.

There are sub-disciplines of toxicology, most of which encompass the realm of forensic toxicology. Forensic toxicology is the "application of toxicology to cases and issues where those adverse effects have administrative or medico-legal consequences and where the results are likely to be used in court".[1] The discipline of forensic toxicology can be divided into three domains: post mortem or death investigation toxicology; behavioural or human performance toxicology and forensic workplace drug testing or drug urinalysis.[2]

Another discipline within toxicology is that of clinical toxicology which is largely hospital based. This discipline is most noticeably utilised by emergency physicians and critical care specialists. As new agents are added to the pharmacopoeia, treating physicians need to be able to utilise drug results and levels with speed in order to implement the appropriate anti-dotes and treatments. The testing requirements are largely different from the needs of the forensic toxicologists. An initial screen is often sufficient in the emergency room; therefore, qualitative immunoassays provide the necessary information, despite being less specific. In larger centres therapeutic drug monitoring is performed using equipment with high-end analytic capabilities.[2]

There are pertinent differences between forensic and clinical toxicology. The most important difference is in the fact that forensic toxicology requires quantification and confirmation of drug findings. Since there is always the potential that any result may be used as evidence in a court of law, there is greater emphasis on producing results that are highly accurate and specific. To this end there is the implementation of multiple fail proof mechanisms. These include the utilisation of screening tests as well as the use of confirmatory tests on the same sample. Furthermore, these tests should be performed using two different analytical procedures. Should this not be feasible, then confirmation may be established using two different methods of extraction, or by demonstration of the presence of the same drug or metabolite in two different specimens.[2] This results in a more expensive and time consuming process than is utilised for the purposes of clinical toxicological analysis.

No matter what the purpose of the testing, all analytic tests require validation and must adhere to stringent quality control procedures.

1.4.2 THE HISTORY OF TOXICOLOGY

The history of toxicology is colourful and interesting spanning numerous decades beginning with the early cave dwellers. The historical development of toxicology begins with the early humans who had begun to recognise and understand the use of poisonous plants and extracts from animals. These extracts were primarily used for hunting and warfare purposes.[3]

The earliest medical record of poisons (circa 1500 BC) was “The Ebers” papyrus, which contains centuries old information about poisons. The use of poisons in antiquity is further highlighted in Sumerian texts and classical Greek literature and mythology. Hippocrates (400 BC) who is often considered the earliest forefather of medicine, wrote numerous

instructions that might be considered the primitive principles of toxicology. His instructions included attempts in influencing the absorption of toxic materials for therapeutic purposes and in cases of overdose.[4]

The Romans also demonstrate understanding of poisons, using them for political gain. Historical documentation shows that Cleopatra, Queen of Egypt (69 – 30 BC) had experimented with strychnine and other poisons on prisoners and the poor. However, it was her act of suicide using the poison of the Egyptian asp that has gained much recognition and has been romanticised in the works of Shakespeare. A famous physician during the reign of the Roman Empire, Dioscorides attempted to classify poisons. His classification of poisons into plant, animal and mineral compounds, has remained a reference for numerous centuries and is still used today. The execution of Socrates (470 – 399 BC), who had been poisoned by a compound containing *alkaloid coniine,* a powerful neuromuscular agent, can be heralded as possibly the first of many homicides in ancient Greece and Rome.[4]

The knowledge and use of toxins / toxicants used in antiquity continued to be used by the Byzantines in the middle ages. The '*Treatise on Poisons and their Antidotes'* written by Moses Maimonides (1135 – 1204) can be cited as the first first-aid guide to the treatment of accidental or intentional poisonings.[4]

It was the words of Paracelsus (Philippus Aureolus Theophrastus Bonbastus von Hohenheim), that still rings true today "*all substances are poisons; there is none which is not a poison. The right dose determines the poison from a remedy*". He was perhaps the first scientist to apply chemicals and minerals to medicines. Furthermore, his views remain integral to the present understanding of toxicology. He highlighted the importance of the "toxicon" or toxic agent as a chemical entity, as well as emphasising the first description of the dose-response relation of poisons and their effects.[4]

Mattieu Joseph Bonaventura Orfila (1787 – 1853), a Spanish physician attempted systematic correlation between the chemical and biological components of known poisons and also delved into the intricacies of combining chemistry and jurisprudence. Due to his insights he is recognised the forefather of forensic toxicology.[4]

The development of the chemical and industrial revolution of the mid-19th century brought

with it an awareness of the effect of chemicals being released into the environment. This awareness also led to the awareness of occupation related illnesses.[4]

The knowledge of the effect of chemicals on the environment was further addressed and highlighted towards the mid-late 20th century, particularly in the development of environmental toxicology.

To date and currently, toxicology has become concerned with the use of chemical agents for the purposes of biological warfare, and continues to focus on environmental issues.

1.4.3 TYPES OF TOXINS / TOXICANTS

A toxicant can be defined as any chemical which could be harmful or poisonous and is generally considered a man-made or synthetic product. A toxin can be defined as a poisonous substance produced by living organisms.[5] Venoms on the other hand are poisonous compounds secreted by animals such as snakes, spiders and insects, which typically inject their venom into their prey.[6] No matter the source a limitless range of toxins / toxicants are freely available to the public. These include but are not limited to therapeutic drugs, illicit drugs, agricultural compounds and household chemicals.

1.4.4 IMPORTANCE AND IMPACT OF TOXINS / TOXICANTS AND DRUGS OF ABUSE

The aim of forensic or post mortem toxicology is to determine the involvement of a chemical in the death of a person. It needs to be ascertained as to whether the chemical has a direct causal link or has contributed to the death in some way. Examples include being under the influence of drugs or alcohol whilst driving; the death of a person suspected of overdosing on prescription or over-the-counter medication; the individual who has been allegedly poisoned or the young child who has been treated by a traditional healer.

The United Nations Office on Drugs and Crime(2012) estimates that between 150 and 300 million people aged 15-64 use an illicit substance at least once per year. There are anywhere between 15 million and 39 million problem drug users at any one time globally. There are between 99 000 and 253 000 drug related deaths every year. Unfortunately the majority of African countries have poor reporting systems, and these statistics are predominantly from developed nations. However, according to the same UN report, the rate of illicit drug use is significantly increasing in Africa.[7] SA is no exception with the availability of illicit drugs

becoming more of a problem. In addition, other toxins such as street pesticides (which refer to pesticides labelled for agricultural use which are decanted into unlabelled containers and made available for domestic rodent control), have become freely available from road-side vendors and markets. These compounds find their way into homes, where children are frequently exposed to them with deleterious and often fatal consequences. [8]

1.4.4.1 TRADITIONAL AND HERBAL MEDICINES IN SA

In SA a large portion of the population seeks the advice and services of traditional healers or herbalists. It has been estimated that at least 80% of the South African population makes use of traditional remedies at some stage of their lives.[9,10] The reasons being that there is deemed to be the provision of not only physical and medical care but also spiritual care, as well the accessibility and affordability of these services. These healers make use of time honoured plants and herbs, many of which are not known to modern medicine. These remedies are often prescribed in combinations, as oral solutions, inhalants or as enemas. Numerous efforts have been made by government and the World Health Organisation(WHO) to recognise, institutionalise and regulate the practice and use of traditional healers. These efforts include the establishment of the Traditional Health Practitioners Act 22 of 2007 which aims to ensure the quality, safety and efficacy of these so called remedies.[11] Despite these interventions, the compounds, pharmacological effects and doses of these substances are unknown, which often leads to patient morbidity and in some cases mortality.

1.4.4.2 ALCOHOL AND ILLICIT DRUGS IN SA

In addition to traditional remedies well known drugs such as alcohol, amphetamines, opiates and Δ9-tetrahydrocannabinol (THC) also pose a significant problem. In his opening address to parliament in 1994, former South African president Nelson Mandela emphasised that alcohol and drug abuse was a large problem among social pathologies that needed to be addressed.[12] Furthermore, the prevalence of alcohol and drug abuse is regarded as a fuel for crime, poverty, reduced productivity, unemployment, dysfunctional family life, political instability, the escalation of chronic infectious diseases, injury and premature death. [13]

- **THE DEMOGRAPHICS OF ILLICIT DRUG USE IN SA**

There is accurate, comprehensive and up-to-date data on alcohol abuse, however there is paucity in statistics with regard to the prevalence of illicit and prescription drug abuse in SA . The Central Drug Authority commissioned a national household survey on the prevalence of

drug abuse in SA , but for reasons beyond their control, the survey was never conducted. South African Community Epidemiology Network on Drug Use(SACENDU)data in 2015, revealed interesting regional details about the common drugs of abuse.[14,15] Cannabis was the most common drug of abuse with up to 39% of patients in the Kwazulu Natal region indicating that this was their primary drug.[15] Cocaine use was highest in the Eastern Cape with 6% of patients reporting this as their primary drug. [15] Mpumalanga and Limpopo provinces had the highest prevalence of heroin users with 30% indicating this as their primary drug. Although there has been as substantial decrease in the proportion of heroin abuse in KwaZulu-Natal(5%), this province has traditionally shown high rates of heroin use attributed to the use of "sugars" or nyaope (a low-quality heroin and cocaine mixture) by young Indian males in the south of Durban. [14,15] The SACENDU data also reveals that intravenous use of heroin is not uncommon. With regard to the period January to June 2015, 7% (Western Cape), 22% (Gauteng), 1% (KZN) and 5% (Mpumalanga-Limpopo) of patients admitted to treatment centres reported that they injected this drug. [15]

Between 0,1% (KwaZulu-Natal) and 35% (Western Cape) of patients reported methamphetamines as their primary drug of abuse. Methamphetamine also known as “Tik” was also the most commonly reported primary drug of abuse among patients admitted to treatment centres in the Western Cape.[15]

Numerous factors are found to be responsible for the increase of illicit drugs in SA . Peltzer et al, stipulate that availability and easy accessibility of drugs are present due to multiple reasons.[16] These include a tolerant and limited law enforcement regarding drugs within society, growing wealth amongst emergent populations after the implementation of democracy in 1992, porous borders due to poor policing, better infrastructures for transportation as well as the age of first use and diversity of available drugs. Cannabis is one of two drugs produced in SA . Altogether 22% of the world's harvest of cannabis comes from Africa. The largest producer is SA with about 2 500 metric tons of the total, 8 900 metric tons produced, i.e. 28% of the African production and 7% of the world production. Alarmingly, SA is one of the world's largest importers of illicit ephedrine and pseudoephedrine, two of the precursor chemicals used to manufacture methamphetamine.[13]

The abuse of illicit drugs in transport related fatalities was recently further highlighted by a study performed at the Johannesburg FPS Medico-Legal Mortuary(results unpublished). On

01 October 2013, Mr Mbayu, the lead investigator of this study communicated preliminary results. These showed a high prevalence of illicit drug detection in post mortem blood of individuals who died as a result of motor vehicle crashes with the prevalence of different types of illicit drugs detected being the following: 31% cannabis; 37% opiates; 23% methamphetamines and 6% amphetamines. This emphasises the involvement of illicit drugs in activities of daily living, highlighting the necessity for appropriate screening and confirmatory testing not only in forensic toxicological settings but in clinical settings too.

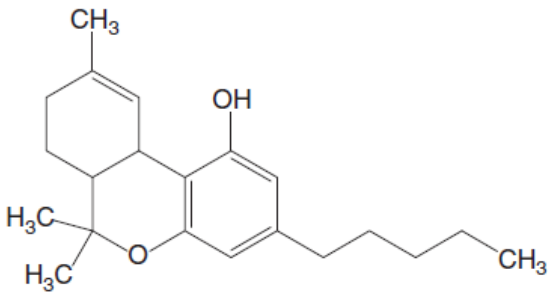
1.4.5 DRUGS OF ABUSE

1.4.5.1 CANNABINOIDS

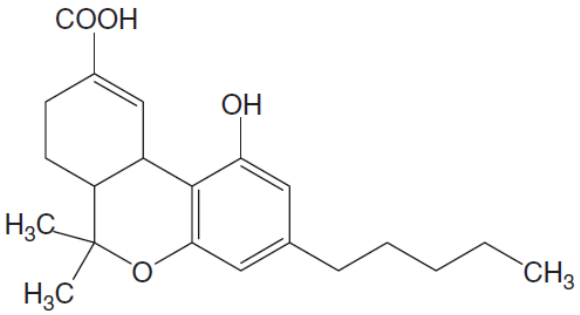
For over 4 000 years the psychoactive products with their resultant euphoric effects obtained from the *Cannabis sativa* (marijuana) plants have been used across the world.[21] Currently in SA, according to the admission data at substance abuse related treatment centres in the period 2008 – 2010, it is stated that between 11,2% (Western Cape) and 50,2% (Mpumalanga/Limpopo) of patients reported cannabis as their primary drug of abuse.[14] In SA, marijuana is commonly known as "dagga".[14]

The generic group of Cannabinoids refers to the more than 100 related compounds found in the extract of the cannabis plant. These compounds are lipid soluble; the most psychoactive compound being Δ9 -tetrahydrocannabinol (THC). The molecular structure of THC is shown in figure 1. The leaves and flowering tops of the plant are smoked and commonly referred to as marijuana, while hashish and hash oil are prepared from a concentrated resin and a lipid-soluble extract. [21]

The most potent form of marijuana, known as *sinsemilla*, is prepared from dried parts of mostly indoor-grown female plants. [21]



THC



THC-COOH

FIGURE 1: MOLECULAR STRUCTURE OF THC AND ITS METABOLITE THC-COOH[21]

- **MODE OF ACTION**

When smoked, THC is rapidly absorbed from the lungs into the bloodstream, from which it is then distributed into the tissues. THC exerts its effect by binding to specific cannabinoid receptors in the brain, largely the CB1 and CB2G coupled-protein receptors.[17] THC has an analgesic effect through G-protein coupled mechanisms that block propagation of neurotransmitters, which register pain in the brain and spinal cord. It is also assumed that there is an interaction with delta and kappa opiate receptors, responsible for the analgesic effect of THC.[18]

- **PHARMACOKINETICS**

THC reaches maximum plasma concentrations within minutes and psychotropic effects are reached within 15–30 minutes and may last for 2–3 hours. The half-life of THC is approximately 7 days.[19] THC is rapidly metabolized by cytochrome P 450 enzymes (mostly CYP3A4, CYP2C9 and CYP2C11) to 11-hydroxy-9-tetrahydrocannabinol (11-OH−THC) which is also an equipotent psychoactive metabolite and to 11-nor-9-carboxy-9-tetrahydrocannabinol (THC−COOH), an inactive metabolite. Smaller quantities of other metabolites have also been isolated. It has been established that 65% of the drug is excreted in the faeces and 20% is eliminated in the urine.[20] THC−COOH is found in the urine in the conjugated form which can be detected up to 4 days after inhalation. [21]

The primary metabolite of significant importance in toxicological analysis in urine is THC-COOH, however the metabolite used for analysis in blood are THC-COOH and 11-OH-THC.[21]

- **CLINICAL EFFECTS**

THC impairs cognition, psychomotor skill and driving performance in a dose related manner. Epidemiological studies performed by Raemakers et al, established that the combined use of THC and alcohol produces severe cognitive and psychomotor impairment, together with decrease in actual driving performance, sharply increasing the risk of an accident.[22] Furthermore, MacInnes et al reported fatal coronary artery thrombosis associated with cannabis smoking.[23]

- **AUTOPSY FINDINGS**

There are almost no specific post mortem signs of cannabinoid use, however there may well be evidence of blunt force injuries as a result of cognitive and psychomotor impairment, which predisposes users to accidents and violent encounters.[24]

1.4.5.2 OPIOIDS AND OPIATES

Opioids are produced from the crude extracts of the latex (a milky fluid) collected from the immature seed capsules of the seedpods of the *Papaver somniferum*. Opiates, on the other hand, are produced from the synthetic acetylation of morphine in clandestine laboratories. Opioids form part of the more than 20 alkaloids that have been isolated from *Papaver Somniferu.* Three of these alkaloids (morphine, codeine and noscapine) are used clinically.[21] Despite the discrepancy in the terminology, both essentially have the same effects on the human body, due to their similar molecular structures. The molecular structure of these agents are shown in figure 2.

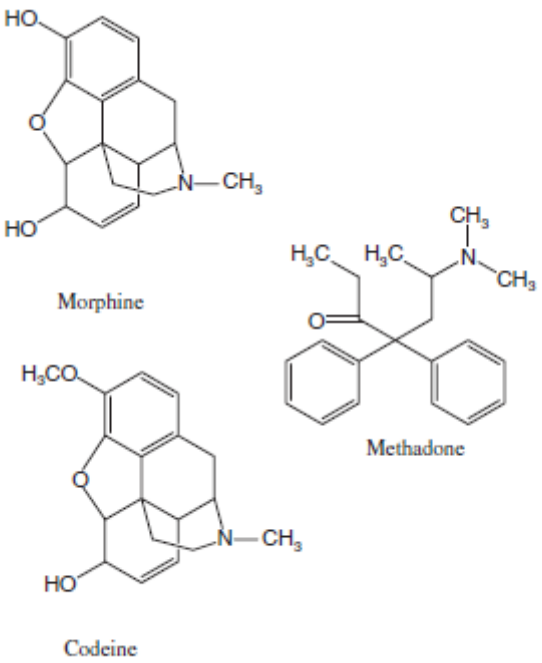


FIGURE 2: MOLECULAR STRUCTURES OF MORPHINE, CODEINE AND METHADONE[21]

- **MODE OF ACTION**

The effects of these molecules are facilitated by the interaction with the family of opiate receptors (mu, delta and kappa) in the central nervous system and the gastro-intestinal tract). Opiate receptor agonists typically produce analgesia, while antagonists block this response. In addition to potent analgesic properties, opiates can also cause sedation, euphoria, and respiratory depression. Long-term use can lead to tolerance and both physical and psychological dependence.[21]

- **PHARMACOKINETICS**

Morphine is the structural building block for many of the semi-synthetic opiates including heroin, oxycodone, oxymorphone, hydrocodone, hydromorphone and levorphanol.

Morphine is available for administration in oral form, although its effect is usually diminished in this form, or administered as an intravenous injection. Morphine is conjugated in the liver by the action of uridine diphosphate glucoronyl transferase and 90% of the drugs is excreted in the urine as morphine-3-glucuronide, which is an active metabolite. A minor metabolite of morphine (less than 5%) is nor-morphine but the majority of morphine is excreted in urine as morphine-3-glucuronide. The rest of the drug is excreted in the bile. [25]

Heroin is metabolized to MAM and then to morphine by hydrolysis

of ester linkages by pseudocholinesterase in the serum. It is also hydrolysed in the liver by the enzymes, human carboxylesterase-1 and carboxylesterase-2.

Heroin has little oral bioavailability because it is subjected to complete first pass metabolism. MAM is detected in the urine for only up to 8 hours after administration. However, 80% of the drug is excreted as mostly morphine 3-glucoronide in the urine within 24 hours.[25]

Codeine is metabolised to morphine in the liver mostly by the actions of CYP2D6.[26] Codeine is principally eliminated unchanged in the urine within 24 hours.[25]

The interpretation of results pertaining to the presence and significance of opiate/opiate use is difficult. A positive result in a screening immunoassay merely indicates the presence of morphine, it does not indicate which opiate or opioid was consumed. Opium, heroin, codeine and morphine share common metabolic pathways, of which all maybe sources of morphine, morphine 3- and 6-glucoronide. The detection of heroin use is therefore based on the presence of morphine and MAM in biological specimens. The added difficulty is that MAM has an extremely short half-life and is labile, undergoing deacetylation in room temperature depending on the pH of the specimen it is in.[27]

- **ADVERSE EFFECTS**

Opiate and opioid related fatalities are largely dependent on three mechanisms namely respiratory depression; cardiovascular collapse and bronchoconstriction.[28] Furthermore, a large percentage of cases are often exacerbated by the concurrent use of other drugs, including alcohol. SACENDU showed that between 7% (Eastern Cape) and 31% (Mpumalanga-Limpopo) of patients who reported for treatment in the period January to June 2015 indicated more than one substance of abuse other than heroin.[15] For the purpose of this study and in the interest of not being repetitive, the term 'opiate' in lieu of both opiates and opioids will be used for the remainder of this report.

- **AUTOPSY FINDINGS**

There may well be pertinent findings in keeping with opiate abuse, depending on the route of administration. External examination may reveal "tram track" marks due to chronic intravenous needle puncture wounds and sclerosis of vessels. There may be numerous 'concealed' needle puncture wounds, between the toes, fingers, groin folds and even the external genitalia. Reflection of the skin on internal examination may reveal the presence of

significant subcutaneous haemorrhage in keeping with evidence of external needle puncture marks.[24] Another sign of opiate use may be the presence of pulmonary oedema, although this can be seen in other non-opiate related cases and is therefore not at all pathognomonic. Venous congestion of the internal organs is often noted, this being equally non-specific. Microscopic examination may reveal the presence of foreign body granulomas within lymph nodes and the lungs, due to the presence of diluents ("cutting agents") in the vasculature. Diluents include fructose, corn starch, talc and even strychnine or quinine. There may also be histopathological evidence of a hepatic triaditis. In addition, microbiological and virological studies may reveal the presence of hepatitis or HIV – the consequence of sharing of needles and syringes. The findings may appear numerous and conclusive, yet they are largely non-specific and may well be found in an array of drugs of abuse.[24,28]

1.4.5.3 AMPHETAMINES

The amphetamine-type stimulants are a collective group of several stimulants and hallucinogens which are chemically related to phenylethylamine.

Amphetamines are sympathomimetic amines and are often optically active. In general, the D-enantiomers stimulate the central nervous system, while L-enantiomers act peripherally.

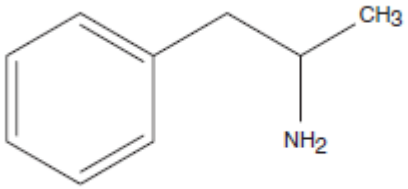
The history of amphetamines spans over a century. It has transformed from a drug that was freely available for a range of disorders into a highly restrictive controlled drug with definite therapeutic applications, and is only available by prescription.[21] It has also become a worldwide recreational drug. Recent statistics revealed evidence of misuse and abuse in SA with particular reference to the Western Cape.[15]

Amphetamines were discovered as early as 1910, but it was not until 1927 that the molecule was first synthesised in an attempt to provide a less costly and more easily synthesised substitute for ephedrine. The synthesised molecules came into popularity due to its ability to reverse drug induced anaesthesia and produce arousal and insomnia. The synthesised molecules where registered by a pharmaceutical company under the trade name of Benzedrine. Benzedrine was used as a treatment for narcolepsy, depression, post-encephalitic Parkinson's and numerous other disorders.[29] Various reviews of the late 30's comment on the cognitive enhancing properties of amphetamines including improvements in intelligence.[30,31] It was widely used to reduce stress, improve concentration and improve intellectual performance. The benefits of amphetamines seemed overwhelming

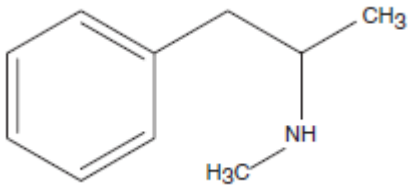
despite the concerns of possible addiction. Due to this, it was estimated that 150 million Benzedrine tablets were supplied to the Allied forces during the course of World War II.[32] It was Bradley in 1937, in a study of 30 subjects, who first reported on the use of amphetamines amongst children with severe behavioural abnormalities.[33] Since then amphetamines have been used in the treatment of attention deficit hyperactivity disorder (ADHD).

Despite its recognised predilection towards addiction, it was only until a classic study performed by Connell in 1966, reporting the deleterious side-effects of amphetamines in causing paranoia, that the wide use of amphetamines was questioned.[34] The use of prescription amphetamines continues today in the field of psychiatry not only in developed countries but also in SA.

Although legislation has attempted to control and limit the sale of amphetamines into the black market, there is still a continual insurgence of new synthetic amphetamine based designer drugs. These include an analogue of amphetamine, 3,4-methylenedioxymethamphetamine (MDMA), colloquially known as "ecstasy". Other drugs include 3,4-methylenedioxyamphetamine (MDA) para-methoxy-amphetamine (PMA) and paramethoxy-methamphetamine (PMMA).[29] The molecular structure of amphetamine and methamphetamine are shown in figure 3.



Amphetamine



Methamphetamine

FIGURE 3: MOLECULAR STRUCTURE OF AMPHETAMINE AND METHAMPHETAMINE[21]

- **MODE OF ACTION**

The action of amphetamines is largely based on the mechanisms that increase synaptic dopamine concentrations, primarily by stimulation of presynaptic release rather than by

blockade of reuptake. Increased levels of dopamine in the brain elicit euphoria, contributing to the addictive properties of amphetamines. Amphetamines demonstrate good bioavailability and the protein binding of amphetamine and methamphetamines is less than 20%.[21]

Recreational drug abusers and independent users are known to administer psycho stimulants at doses which are much higher than those stipulated for therapeutic use. This is in order to achieve the maximal pharmacological effect in the least amount of time.

It is this phenomenon which often leads drug abusers to progress from relatively safe methods of administration to increasingly dangerous routes, ultimately leading to intravenous usage. Another factor recognised in the development of abuse is the desire for instant gratification, which often determines the choice of a particular drug based on its ability to produce its desired effect within minutes.[29]

- **PHARMACOKINETICS**

Amphetamines belong to the class of drugs called the B-phenylethylamines. The molecule is structurally similar to the biologically active molecule of ephedrine. It therefore shows similarities between the chemical structures of the catecholamine neurotransmitters, noradrenaline and dopamine.[21]

Amphetamines and methamphetamines enter into the bloodstream either by being inhaled when smoked, ingested or injected intravenously. Hepatic and renal clearance contribute to the elimination of amphetamine and methamphetamines. They are found to have an elimination half-life of between 6 and 12 hours. Hepatic metabolism is extensive however a large percentage of both drugs is excreted unchanged via the urine. Amphetamine and related compounds are weak bases with a pKa of around 9.9 and a relatively low molecular weight, allowing amphetamine and related compounds to diffuse through cell membranes and lipid layers to tissues and body fluids which have a lower pH than blood. In addition to urine and blood, amphetamine-like compounds can also be detected sweat, saliva, hair and nails.[21]

Amphetamine undergoes aromatic hydroxylation to parahydroxyamphetamine and oxidative deamination to produce benzoic acid.

The major metabolite of methamphetamine is amphetamine.[21] A significant portion of both amphetamine and methamphetamine are excreted in the urine unchanged. Amphetamine

also undergoes aromatic hydroxylation to parahydroxyamphetamine and oxidative deamination to produce benzoic acid.[21]

- **CLINICAL EFFECTS**

These are variable ranging from restlessness and confusion to hallucinations, convulsions, coma and arrhythmias. Recently due to the popularity of large electronic music dance parties ("raves"), an increasing awareness of the deleterious effects of amphetamine-like substances on the hypothalamus has been highlighted by Byard et al, who reported several fatalities from ecstasy abuse where severe hyperthermia (temperatures of 41.5^{o}C–46.1^{o}C) was the cause of three deaths.[35] Furthermore, the chronic use of these substances can lead to the development of myocardial fibrosis as well as hepatic necrosis. Another important factor is the increased incidence of traumatic injuries amongst users, due to the development of potentially violent and aggressive behaviour and loss of inhibition.[28]

- **AUTOPSY FINDINGS**

Findings at autopsy are usually non-specific, revealing only the presence of venous congestion of the internal organs. Histological evaluation may prove fruitful in revealing evidence of myocardial fibrosis and hepatic necrosis. There may also be evidence of traumatic related injuries, including blunt force injuries.[28]

1.4.6 THE PRINCIPLES OF FORENSIC TOXICOLOGICAL ANALYSIS

The detection of drugs of abuse in a post mortem setting has numerous challenges. Interpretation of toxicological results requires knowledge of the stability of the substances investigated in decomposing biological matrices. The following factors are most important when conducting a toxicological investigation in a deceased individual: choice of matrix (tissue or fluid) to sample; post mortem interval and post mortem drug metabolism; storage and transport of specimens; sample analysis; interpretation and reporting of results.

1.4.6.1 CHOICE OF SAMPLE MATRIX

Post mortem toxicology is concerned with collection of the most appropriate sample; as numerous biological sample choices are available. Drummer stipulates that the case being investigated, dictates the sample that should be used.[36]

The use of blood, urine and liver is deemed the most appropriate for the analysis of drugs of abuse.[36] Qualification and quantification of drugs and their metabolites can be performed

and interpreted by analysis of whole blood. A positive result can be interpreted as the recent use of or exposure to a specific compound, bearing in mind that many drugs of abuse are rapidly metabolised and so are quickly eliminated from the circulation.

- **BLOOD**

The use of blood is readily available in an autopsy setting. In order to acquire meaningful result, the site of collection is an important factor to consider in order to limit post mortem contamination due to post mortem redistribution (PMR). In post mortem cases, diffusion of drugs from high concentrations to low concentrations occurs due to the disruption of cellular membranes. This can then potentially affect the determination of the concentration of drugs being investigated in blood. This process is particularly significant for drugs with high lipid solubility or high tissue concentrations relative to blood taken from the heart.[36]

TABLE 1: PHARMACOKINETIC PROPERTIES AND LIKELY POST MORTEM REDISTRIBUTION FOR SELECTED DRUGS OF ABUSE [36]

Drug/Drug Class	Common Dose (mg)	Usual therapeutic blood levels (mg/l)	Main active metabolite or bio-marker in blood	Vd (l/kg)	Extent of redistribution
Amphetamine	10-100	0.2	None	3-5	Low
Methamphetamine	50-2000	0.2	Amphetamine (~10%)	3-4	Low
Heroin	10-100	-	Morphine, 6-AM	See Morphine	Low-moderate
Morphine	10-100	0.5	None	2-4	Low-moderate
Methadone	10-120	1.0	EDDP (urine)	3-5	Moderate
Codeine	8-60	0.2	Morphine (10%)	4	Low-moderate
Cocaine	10-100	0.5	Benzoylecgonine	1-3	Low

			, EME		
THC	5-25	50	11-Carboxy-THC	9-11	Low-moderate

THC- Tetrahydrocannabinol; mg- milligram; l-litre; kg-kilogram; Vd- volume of distribution; 6-AM – 6-monoacetylmorphine; EDDP – 2-ethylidene-1.5-dimethyl-3,3-diphenylpyrrolidine; EME – Ecgonine methyl ester

THC is the drug with the highest lipid solubility and volume of distribution. The volume of distribution is a term used to describe the theoretical volume that would be necessary to contain the total amount of an administered drug at the same concentration that it is observed in plasma. [36] However, in his body of work, Hilberg demonstrated that it did not show consistent increases in blood concentration after death. [37]

Variability in the volume of distribution amongst the opiates has been demonstrated, with methadone demonstrating a high volume of distribution, therefore exhibiting moderate increases in blood concentration after death, as opposed to the more water-soluble morphine which showed little change in blood concentration after death.[36] Milroy et al demonstrated a range of up to a fourfold increase in blood concentration of methadone after death however there appeared to be significant site to site variability.[38]

Drummer demonstrated a low extent of redistribution with regards to methamphetamines.[36] Miyazaki et al had previously demonstrated at least a twofold increase in the heart blood samples, when femoral and heart blood specimens were compared.[39] A study conducted by Gerostamoulos et al demonstrated that few drugs exhibited particularly large changes in drug concentration when femoral blood was collected, but went on to comment on the largest change noted which was the decrease of concentrations of methamphetamines and amphetamine in the femoral blood specimens.[40]

Despite conflicting evidence, peripheral blood is preferred as the most stable and least affected by post mortem re-distribution despite the apparent abundance of thoracic and abdominal blood available for sampling.

- **OTHER TISSUES**

As has been already stated, the choice of matrix to be tested is dependent on the case. In cases of advanced decomposition, muscle tissue, hair, nails and bone can be used as specimens. Body fluids in putrefied bodies, comprises largely of liquefied tissue, so although these samples can be useful in testing for the presence of drugs, they cannot be used for

quantification purposes.

In some centres, liver specimens are submitted for analysis in order to supplement other results. There is limited redistribution due to the close proximity of the liver to the bowel. It is therefore recommended that samples should be taken from within the deeper substance of the right lobe of the liver.[41] When urine is available in a post mortem setting, it can be utilised in supporting blood based analysis, but may be negative if the time of death is soon after the ingestion of the compound as there would not be enough time for metabolism and excretion to have occured.

Bile can be utilised as a matrix, however there are factors which influence results and must be born in mind. A rise in molecular weight of a compound correlates with an increase in concentration within bile relative to blood, together with the influences of liver perfusion and biliary secretion. Despite the interpretative information obtained through bile analysis being limited, a review by Kalter et al revealed that it was possible to differentiate between acute and chronic heroin use based on the concentration of bile morphine. The authors concluded that decedents with lower bile morphine concentrations were less tolerant to the effects of the drug.[42]

Muscle tissue can be used in toxicological analysis; however unequal perfusion of the tissue, together with the presence of post mortem artefact, makes interpretation of results difficult due to variability.

Bone has been used to determine drug exposure in human remains as demonstrated by Drummer et al,[43] as well as Nagata et al,[44] who did animal experiments to detect the presence of methamphetamines within bone, which revealed promising results.

Vitreous humour has attracted much attention due to the presence of numerous factors, which lend themselves to forensic toxicological analysis. These include a relatively large specimen volume and accessibility; it appears to be relatively inert and stable to fluctuation in blood chemistry and it is relatively resistant to bacterial contamination.[45,46] A review on the post mortem applications of vitreous humour compiled by Baniak et al, revealed that apart from its use in various forensic applications including biochemical analysis, it could be used to estimate the post mortem interval utilising potassium.[47] Vitreous humour has also been shown to be of value in the determination of ante mortem ethanol concentrations

from post mortem concentrations.[48] In terms of using vitreous humour in the detection of illicit drugs, there appears to be conflicting evidence. There may to be a correlation in morphine levels between femoral blood and vitreous humour however this was not confirmed in a subsequent study.[49,50] Further positive correlations have however been found in the analysis of cocaine.[51]

1.4.6.2 POST MORTEM INTERVAL AND DRUG METABOLISM

Robertson et al highlighted that the extent of chemical change in the post mortem interval and the presence of post mortem metabolism can affect the interpretation of results. [52]
This is further compounded by the fact that some drugs are unstable.[53]
Post mortem breakdown will occur for all drugs of abuse. Cocaine and heroin rapidly convert into their respective hydrolytic products in life, and also undergo rapid bioconversion after death.[36] It is therefore imperative that careful sample collection, handling and storage must be ensured in order to halt the hydrolysis of these compounds. Important factors such as the post mortem interval can further lead to degradation of samples as for example in cases of decomposition and eventual degradation of tissues. There is evidence demonstrating that anaerobic bacterial action can have an effect on the concentration of drugs, noticeably on nitrobenzodiazepines.[52] There is sparse literature on the time dependence regarding detectability of drugs or poisons in a putrefying body.[54] There are, however, studies that have revealed the presence of drugs in specimens from embalmed corpses and formalin stored tissues.[55]

1.4.6.3 THE PRE-ANALYTICAL PHASE (SAMPLE COLLECTION, STORAGE AND TRANSPORT)

It has been emphasised that appropriate sample collection is essential in order to ensure post mortem drug stability as in the analysis for cocaine and heroin. Not only is site of collection important but so too the appropriate collection vessel and storage container. These factors form part of the pre-analytical phase of post mortem toxicology.[54] In this article Skopp states that the concept of a pre-analytical phase was first described in 1922 by Jansch. He highlighted the importance of the appropriate amount of tissue specimens and the use of appropriate containers for long-distance transportation.[54] The use of inappropriate storage could potentially lead to a loss of compound within the specimen and loss of valuable information. Fuller in 1992 and Grinstead in 1994 confirmed that storage conditions could change metabolite concentrations within a sample.[56,57] They demonstrated

that the intermediate metabolite of heroin, 6-acetyl morphine (6–AM), undergoes deacetylation to morphine at room temperature but remains stable in urine frozen at -20^{o}C for at least 12 months.

Despite technological advances in analytic and measurement procedures, there is increasing evidence, which shows that the reliability of the analytical result depends largely on the pre-analytical phase of post mortem toxicology.[58]

In jurisprudence, the chain of custody for toxicological evidence begins in the pre-analytical sample collection phase, and must continue up to and including the interpretation of the results by the appropriate authority.

1.4.7 ANALYSIS OF SAMPLES

In SA, deceased individuals anecdotally present with little or no background history, leaving the forensic pathologist with a dilemma regarding further toxicological investigations and even interpretation of results. The currently recognised gold standard for the measurement of toxicological compounds is mass spectrometry coupled with liquid or gas chromatography. However, laboratories that can conduct this testing are not easily accessible and are usually overburdened. The development of a screening test, near the body of a deceased individual, together with select samples requiring confirmatory, preferably targeted, quantitative analyses with LC/GC-MS, is a logical development in forensic toxicology. In a clinical setting, systematic toxicological analysis (STA) is largely used. The International Association of Forensic Toxicologists Committee of Systemic Toxicological Analysis defines STA as an adequate analytical application for the detection and identification of numerous possible potentially toxic compounds.[1] It is therefore a stepwise approach to toxicological investigation. A test screening for the presence of a wide range of toxins / toxicants is initially performed. Thereafter, positive identified samples will undergo confirmatory and, if necessary, quantitative testing.

In the post mortem population, a reliable screening test has yet to be validated in SA. The reason for this is largely multifactorial. As post mortem toxicological analysis is fraught with difficulties a more comprehensive and sophisticated STA is often required, which results in financial implications. This means that currently in the FPS and FCL, every sample sent for toxicological analysis undergoes general unknown screening (GUS). GUS is a laboratory based procedure using chromatography coupled with mass spectrometry on every sample to

test for the presence of a very wide range of compounds, which is a lengthy and exceedingly costly procedure.[59] This "blue sky" approach refers to the seemingly limitless assessment of compounds in a screening and even confirmatory test panel. As already alluded to, this process is extremely expensive, time consuming and a burden on resources. However, in the absence of a definitive history, GUS is currently a necessary first step. Sensitive near-body tests could potentially be used to conduct the initial screening process, thus eliminating the excess resource and time wastage of conducting GUS using chromatography/mass spectroscopy methods. There are no available near-body tests specifically designed to be used on a post-mortem population and the most readily available tests that could potentially be used for screening purposes are those used clinically as near-patient tests. The near-patient toxicological screening drug tests currently being used in the clinical setting consist typically of immunoassays.

1.4.7.1 IMMUNOASSAY SCREENING TESTS

When referring to immune mediated assays, both laboratory based and near-patient/near-body tests are available. Enzyme-linked immunosorbent assays (ELISA) are preferred over near-patient chromatographic test kits displaying higher sensitivity and are considered to be less error prone.[60] Formal ELISA testing is only available in the laboratory setting.

However, near-patient tests based on ELISA principles have been developed. These immunoassays are useful laboratory methods in any toxicological setting and can be conducted in a laboratory or on-site. They are fast, sensitive and accurate, allowing for the rapid quantification of drugs.[61] Immunoassays are based on the principle that drug molecules present in the patient sample bind to specific antibodies suspended in regions on the test matrix. This reaction is coupled with an indicator based system producing either chemoluminescence (for example, Randox DOA I immunoassay) or a colour change, or lack thereof, in an indicator strip (for example, ND).

The choice of immunoassays is wide. It is also important to remember that immunoassays can only provide presumptive results. This is due to a variety of issues such as analytical sensitivity and specificity, false positive and negative results, assay cross-reactivity, analytical cut-off concentrations and analytical interference and specimen adulteration.[62]

Although it is not possible within this manuscript to discuss each aspect in detail, there have been significant developments in the use of immunoassays. These developments include

improved labelling, monoclonal antibody techniques, automated systems and improved sensitivities. Examples include the emergence of automatic immunoanalyses; the use of enzyme multiplied immunoassay techniques (EMIT); fluorescent polarisation immunoassay (FPIA); micro particle enzyme immunoassay (MEIA); cloned enzyme donor immunoassay (CEDIA) and lastly kinetic interaction of micro particles in solution (KIMS).

- **RANDOX IMMUNOASSAY SCREENING SYSTEM**

The Randox immunoassay screening test is an automated system which is able to use whole blood or urine as sample matrix.

This system requires the use of an automated instrument. The Evidence Investigator is a compact, laboratory based, semi-automated benchtop platform which utilises the Biochip Array Technology. This analyser accommodates simultaneous detection of multiple drug metabolites from a single sample, with the ability to consolidate a number of immunoassay tests. The system is kit based and provides for all the necessary components such as the chips, chemicals, calibrators and developing agents.

It consists of nine 9 x 9 mm biochips in wells on a cassette. Already present in the biochips are drug molecules labelled with horseradish peroxidase (HRP). The drug molecules present in the patient sample compete for antibody binding with the HRP-labelled drug molecules (see figure 4). When these HRP-labelled molecules bind to the antibodies in the presence of a substrate, a resultant chemoluminescent reaction occurs. The amount of chemoluminescence is inversely proportional to the concentration of drug in the sample that is being tested.[63] If the HRP-labelled molecules cannot bind the antibodies due to the presence of competing drug molecules from the patient sample, chemoluminescence will not occur. The degree of chemoluminescence is detected by a digital camera, and this image is processed by the software within the instrument. The kit provides a range of calibrators with known concentrations of drugs and this is then used to establish a calibration curve. Therefore, information from this image of an unknown specimen is determined from the established calibration curve through the use of proprietary software, a drug concentration is calculated.[63] This analytical process is obtained within an hour dependant on the number of specimens batched and analysed.[64]

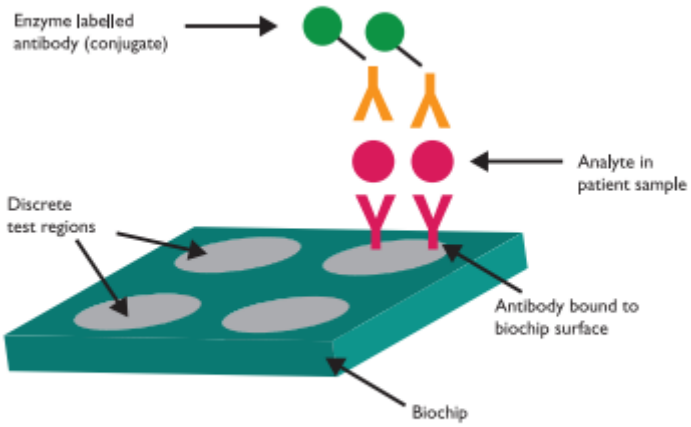


FIGURE 4: RANDOX IMMUNOASSAY BIOCHIP TECHNOLOGY[64]

McLaughlin et al examined the use of the Randox biochip immunoassay technology for use in a European post mortem population.[63] In this study liver, psoas muscle, femoral blood, vitreous humour and urine from 75 post mortem cases was screened for amphetamine, barbiturates, benzodiazepines, benzoylecgonine, buprenorphine, cannabinoids, fentanyl, ketamine, lysergic acid, diethylamide, methadone, methamphetamine, methaqualone, methylenedioxymethylamphetamine (ecstacy), oxycodone, phencyclidine and propoxyphene using the Randox DOA I and II arrays. Positive results were confirmed using GC-MS, LC-DAD and LC-MS/MS. This study showed that there was excellent concordance between the immunoassay and gold-standard methodology, with correlations between 98 and 100 percent with the various drug groups. There were a few false negative results in certain matrices (for example, cannabinoid and benzodiazepines in vitreous humor). The authors attribute this largely to drug redistribution, drug accumulation and selective membrane permeability.[63] They also mention that in cases of low drug concentrations, especially with respect to opiates and methadone; the immunoassay cut-off levels were higher than the actual blood levels. This resulted in false negative results in those cases. The only false positives detected were for amphetamines. These were detected in two heavily decomposed bodies, which comprised 2 percent of the study population. This is consistent with what is expected using other methods to detect amphetamines, as the putrefactive amines that are released during decomposition are similar in structure to the amphetamine group of drugs resulting in false positive tests. The authors conclude that the Randox DOA I

and II assays can be used in a post mortem population to screen blood, amongst other specimens, for the drugs that the assay is designed to detect [64].

Although this process is laboratory based. It can be used off site and facilitated in an area within the mortuary that is conducive to toxicological analysis. This allows for on-site simultaneous screening of various drugs metabolites, with a high level of accuracy. It also accommodates the application of different specimen matrices.[64]

Although the results from the Mclaughlin study are encouraging, these samples were taken from a population in the United Kingdom. South African forensic services are over-burdened and autopsies can be delayed, potentially resulting in a longer post mortem interval. As discussed in 1.4.6.2, drug redistribution and metabolism occurring during this period may impact on the levels of detectable drug in various tissues. Thus, a critical evaluation of the Randox test in the South African setting where anecdotally, longer post mortem intervals are common, is warranted.

Randox and other such systems require some laboratory resources in order to store and prepare samples. The technology requires skilled technicians in order to operate, however as these tests are batched, staff numbers can be limited. Despite its obvious advantages, the technology requires maintenance and the use of assay kits, which are expensive, in addition to the initial financial outlay of the equipment.

1.4.7.2 NON-AUTOMATED NEAR-PATIENT TESTS

Numerous non-automated near patient tests have found their way onto the commercial market. One of the first devices to be scientifically scrutinised for its effectiveness was the KDI Quik Test (Keystone Medical Corporation, Columbia, USA). It was based on a paper chromatography technique and allowed for the screening of urine specimens for the presence of amphetamines, cocaine, phencyclidine and morphine. The sensitivity and specificity of this test was poor.[65] Since then numerous tests have been developed which range in effectiveness and application. Studies have been conducted reviewing and comparing the performance of multiple tests. One such study was conducted by Hino et al, which looked at the performance of three modified immunoassay methods. The study also used blood specimens after precipitation of proteins with acetone, as the study sample. The

results revealed good correlation with results obtained from confirmation by GC-MS.[68] However, despite being convenient, it must be remembered that these tests present with pitfalls which include difficulties in interpretation, the presence of potential cross reactivity and the possibility of false positive and negative results.

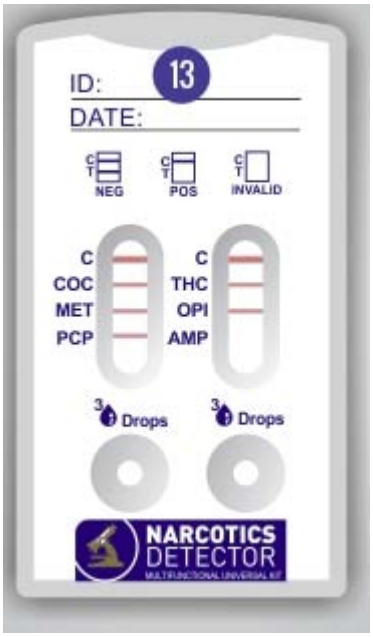
- **DISADVANTAGES OF NON-AUTOMATED NEAR-PATIENT TESTS**

The disadvantages of near patient testing devices include the following: Costly when using individual tests to create a multiple drug screen; limited specificity leading to problematic interpretation; inconsistent quality control of the testing devices; poor record-keeping following testing with near patient testing devices and subjective interpretation in tests using endpoint colouration.[62] Primarily due to their subjective interpretation, these limitations may make these screening test results difficult to defend in court.

- **NARCOTICS DETECTOR IMMUNOASSAY**

Narcotics Detector (ND) is a competitive binding immunoassay based point of care test for drugs of abuse available in SA. It has been marketed as a reliable screening test for use by law enforcement and the general public. Urine and saliva samples are collected from surfaces or other containers using a sponge provided in the kit. The sample from the sponge is diluted with a phosphate-buffered saline (PBS) solution. This diluted specimen is then applied into the wells on the test device. Manufacturers of the kit have indicated that a sample matrix such as blood can be utilised in this test kit however no verified or validated data is available. The kit tests for the presence of MDMA ("Ecstasy"), methamphetamines, cocaine, cannabinoids, opiates and phencyclidine.[66]

The device consists of a membrane, the proximal part of which is impregnated with drug antibodies labelled with a coloured conjugate. Further down the membrane are areas containing drug-protein conjugates. The sample is wicked by the membrane and moves across the strip with the drug antibodies. Should there be drug in the sample, the drug antibodies will be bound proximally, and there will be no colour change in the areas where the drug-protein conjugates for that specific drug are contained (no stripe will be visible). If there is no drug in the sample (a negative test), the drug antibodies will move with the sample across the membrane unbound. When these antibodies cross the areas where the drug-protein conjugate is impregnated, they will bind and a colour change will occur (a stripe will be visible, see figure 5).[66]



ID – Identity number, C – Control, COC- cocaine, MET- methamphetamine, PCP-phencyclidine, THC-tetrahydrocannibinol, OPI-opiates, AMP-amphetamines

FIGURE 5: AN EXAMPLE OF A POSITIVE TEST FOR THE PRESENCE OF AMPHETAMINES IN A SAMPLE USING ND[66]

The use of this device does not require any off site laboratory facilities and does not required trained staff to utilise or interpret it. Result are obtained within 10 minute and can contribute significantly to the performance of the autopsy as it can virtually be used at the gurney side.

There are no studies validating the use of ND with blood as a sample matrix. However, the manufacturers of the test have indicated that provided a correct dilution method is used, blood can be a suitable sample matrix for testing using ND. ND has not of yet been tested in a post mortem population.

1.4.7.3 LABORATORY BASED SCREENING TESTS

Despite the advent of near-body testing, some authors feel that comprehensive chromatographic screenings should also additionally be used specifically in order to detect basic or basic/neutral substances.[59] The use of these tests falls into the realm of laboratory based screening.

The use of GC-MS screening of compounds in blood combined with free automated high performance liquid chromatography (HPLC) has also been found to be effective in screening

for a large number of analytes.[67] Photodiode or multi-wavelength UV detection (DAD) coupled to HPLC has been used to detect specific and neutral compounds and is a useful adjunct to a GC-MS screening method.[68] Recently high resolution mass spectrometry has also been used as a screening tool to detect and identify drugs and drug metabolites.[69] This allows more accurate identification of compounds; an example of this being time-of-flight(TOF) mass spectrometry. It is now also common practice to use established toxin databases in order to assist with the identification of drugs in specimens. Software manipulation and algorithm application are available for use for a variety of mass spectrometry and UV/IR spectrometry based techniques.[68]

New research and reviews continue to be produced, concerning more advanced methods for the detection of substances. These studies are variants of the same theme and all take their origin from the knowledge reaped from the chemists of the past.

1.4.7.4 CONFIRMATORY TESTING

Liquid or gas chromatography coupled with mass spectrometry (LC/GC-MS) are considered gold-standard analytical techniques in order to conduct GUS or as a confirmatory step in STA.

The general principal of chromatography is the separation of a mixture of substances or analyte into its individual constituents. The analyte to be tested is dissolved in a fluid called the “mobile phase”, either gas or liquid. This phase carries the analyte through another material called the “stationary phase”. As the mobile phase moves along the stationary phase, the individual constituents of the analyte move at different speeds, based on their mass and partition coefficients, causing them to separate from each other. These individual constituent molecules are then detected and identified using an ultraviolet absorbance detector or a mass spectrometer.[70] This type of analysis can be qualitative, semi-quantitative or quantitative. Qualitative analysis is the cheapest, but it only can give an indication of whether the compound being tested is present or absent in the sample. Quantitative analysis can give an accurate concentration of the compound in the sample, but this is the most expensive, labour intensive and slowest analysis to conduct. It is conducted using a set of purchased certified reference materials in a range of concentrations. The best compromise between cost and information yield seems to be a semi-quantitative analysis where a more limited set of reference materials is used, and the compound is said to be

present or absent around a set of specific cut-off concentration values.[67]

- **GAS CHROMATOGRAPHY**

In gas chromatography, the mobile phase is a carrier gas. The gas used is usually inert or unreactive such as helium or nitrogen. The stationary phase is a microscopic layer of liquid or solid polymer on an inert support structure. This is usually contained within a piece of glass or metal tubing, referred to as a column. A known volume of gas or liquid analyte is introduced into the column using a syringe. The carrier gas sweeps the analyte through the column. The speed of the molecules within the analyte is slowed by adsorption of these molecules onto the liquid microfilm or solid polymer stationary phase. The rate at which the molecules of the analyte progress along the column depends on the strength of adsorption of these molecules to the stationary phase. After different time periods, known as the "retention times", each type of molecule reaches the end of the column. As the molecule exits the column a detector is used to monitor the elution of the components, allowing a qualitative identification of the substance. The detector (e.g. mass spectrometer, flame ionisation detector or electron capture detector) is used to identify the molecules as they elute from the column and also aid in the unique identification of the compounds. Qualitative, semi-quantitative and quantitative data can be obtained from this analysis. [70]

- **LIQUID CHROMATOGRAPHY**

In liquid chromatography, also known as high performance liquid chromatography (HPLC), the mobile phase is a liquid. Typically, the liquid phase consists of a mixture of polar solvents (e.g. acetonitrile, water, methanol). The stationary phase consists of a non-polar silica and polymer based sorbent. Each sorbent particle is 2-50 μm in size. The sorbent is packed into a column constructed of an inert material. [71]

The analyte is injected into the liquid mobile phase percolating through the column. The molecules of the analyte are adsorbed onto the stationary phase. The retention time of the molecules onto the sorbent depends on factors such as molecular weight, hydrophilicity and partition coefficients. As specific component molecules of the analyte leave the column, they are detected using a detector. Detectors include ultraviolet absorbance based detectors or mass spectrometer. Quantitative, semi-quantitative and qualitative data are obtained using this technique.[71]

Liquid chromatography offers the advantage of analysing a wider range of components than gas chromatography (which is only able to analyse thermally stable and volatile compounds). It is therefore now largely replacing gas chromatography in clinical use. [71]

- **MASS SPECTROMETRY**

Mass spectrometry (MS) allows the identification of each of the molecules emerging from either a gas or liquid chromatograph. If MS follows chromatography, compounds are fed directly into the ion source of the mass spectrometer as they are separated. Typically, during MS analysis, a sample is first ionised by bombarding it with high energy electrons. The ion source differs according to whether gas or liquid chromatography is used. The sample will break up into unique charged fragments. These charged fragments are then accelerated and subjected to an electric and magnetic field. Ionised molecules of the same mass to charge ratio will undergo the same amount of deflection. Results are displayed as a graph of the relative abundance of detected ionised fragmented molecules as a function of the mass to charge ratio (a spectral pattern). The molecules within the sample can then be identified by correlating known spectral fragment patterns to the sample spectral pattern, thus allowing identification of the analyte in question. [71]

- **EVIDENCE FOR THE CLINICAL AND FORENSIC USE OF CHROMATOGRAPHY AND MASS SPECTROMETRY**

A high level of specificity and sensitivity is required from any gold standard test. For this reason, chromatography coupled with mass spectrometry continues to be recommended as confirmatory testing in toxicological analyses.[59] It is still well recognised that GC-MS can be used as a qualitative reference analytical method. However, LC-MS is becoming increasingly important in routine analysis especially for quantitative analyses.[72] LC-MS has been demonstrated to be equivalent to, and in some cases more suitable, for the quantification of thermolabile compounds in plasma ; that occur in low concentrations; have high molecular masses or are polar in nature.[73,74] More recently, Maurer further highlighted the fact that the use of these hyphenated techniques , such as GC-MS and LC-MS provide high sensitivity, specificity and universality, are all criteria paramount to forensic toxicology.[75] A further development is the establishment of low resolution and high resolution mass spectrometry (HRMS). High resolution mass spectrometers can measure mass so accurately that they can detect minute differences in mass between two compounds that, on a regular

low-resolution instrument, would appear to be identical. The use of HRMS is primarily for the detection of or confirmation of molecular formulas of new compounds.[76] The future of forensic toxicological analysis is heading towards the standardised implementation of high resolution MS-MS. This method allows for identification of drugs and metabolites with infinitely small masses and can provide highly accurate, sensitive and specific results. Unfortunately, this analytical method is extremely expensive although for now it is considered the so-called "holy grail" of toxicological analysis.

1.4.8 INTERPRETATION OF RESULTS AND FACTORS AFFECTING INTERPRETATION

1.4.8.1 INTEPRETATION

Once analysis has been performed, the toxicologist or forensic pathologist is faced with the arduous task of date and result interpretation. The interpretation of a result can be supported by a thorough investigation of the scene of death together with a comprehensive background history and details of the possible circumstances surrounding death. Anecdotally, in a South African forensic setting, few scenes are available to toxicologists and forensic pathologists for investigation and/or sample and/or evidence collection and few cases are accompanied by a comprehensive history. Despite the paucity in background information, significant information can be gathered from a thorough autopsy. Results should seldom be used in isolation and interpretation should be a culmination of clinicopathological diagnoses supported by death scene information and toxicological evidence.

1.4.8.2 FACTORS AFFECTING INTEPRETATION OF RESULTS

There are several factors which may influence the interpretation of a result for forensic purposes. These include:

the presence of drug-drug interactions; polymorphisms and pharmacogenomics; the presence of tolerance or loss thereof; and lastly the difference between therapeutic versus toxic levels. [77]

- **DRUG-DRUG INTERACTIONS**

Detoxification of drugs and chemicals takes place in the liver by reactions known as phase I and phase II metabolism. Phase I is mostly oxidation, reduction or hydrolysis of substrates. Phase II concerns the conjugation of substrates with small molecules. Induction or inhibition

of metabolism of one drug by another is often a consequence of phase I enzymes, noticeably hepatic cytochrome P-450 mixed function oxidases. Numerous drugs function as substrates, inducers, and/or inhibitors of this enzyme system. The induction of metabolism of one drug by another results in the production of more enzyme, this may then decrease the concentration and efficacy of the drug in question. Conversely, inhibition of cytochrome P-450 metabolism by a drug can lead to an increase in toxicity of another drug. A good understanding of which drugs serve as substrates, inducers and/or inhibitors is essential to the understanding of drug-drug interactions and the associated results they may produce.

A second form of drug-drug interactions is as a result of free and bound substances. The primary sites of drug storage are plasma proteins and tissues. If drugs bind to plasma proteins and/or tissues, they are no longer available to assert their pharmacologic effect. When another drug with a greater affinity for the binding site is introduced, the bound drug will become free and available to act.

Lastly the third mechanism involving drug-drug interactions is the development of increased toxicity because of an additive or synergistic effect. An example of this is best demonstrated by the pharmacological effects of drugs on the central nervous system. Depression of the central nervous system occurs as a direct or indirect result of numerous drugs including opiates and alcohol. The synergistic effect of substances can result in severe central nervous system depression resulting in cardiorespiratory depression and ultimately death.[77]

- **POLYMORPHISMS AND PHARMACOGENOMICS**

Polymorphism is the difference in phenotype between individuals, and pharmacogenomics is the study of the genetic variations that cause differences in the drug response among individuals.[78] The more familiar terms of “fast” and “slow” metabolisers are often utilised. An example of such a polymorphism is N-acetyltransferase 2, one of the first isozymes characterized, which causes the acetylation of isoniazid.

There are various other agents which show variable metabolism and these include succinylcholine (defects in pseudocholinesterase) and nortriptyline and codeine (CYP2D6) amongst others.[77]

- **TOLERANCE**

Tolerance is an acquired phenomenon as a result of continued exposure to a chemical substance. Mechanisms of tolerance may include an increase in

metabolism of a drug as a result of metabolic enzyme induction and desensitization of the receptor with a decrease in pharmacologic response.[77] The presence or loss of tolerance must be established before interpretation of results particularly in cases of alleged overdose. Habitual drug users who develop tolerance require much higher levels of drug to achieve the same effect than individuals who do not use that particular drug.

The best example of this is the loss of tolerance particularly towards opiates by individuals who abstain. When these individuals resume their addiction at the same level as before, there is often inadvertent toxicity resulting in dire consequences.[77]

- **DIFFERENCE BETWEEN THERAPEUTIC AND TOXIC LEVELS**

Lastly, for accurate interpretation of post mortem toxicology it is important to have quantitative knowledge of the levels of therapeutic drugs in blood. There are numerous references available for referral including those published by The International Association of Forensic Toxicologists (TIAFT). The dilemma, however, is that some of these references are commonly derived from plasma or serum concentrations from *in vivo* and therapeutic circumstances or from pharmacokinetic studies. These referral lists do not consider the phenomenon of post mortem redistribution, together with the possible discrepancy between plasma/serum and full blood concentrations.[54] Furthermore, although references are available to determine whether a drug concentration is therapeutic, toxic or lethal, these references are largely based on case reports provided in the literature and on the experience of the forensic pathologist and toxicologist. An example of this is a reference list compiled by Musshoff et al providing fatal blood and tissue concentrations of more than 200 drugs. [79]

Despite knowledge of therapeutic levels, the interpreter also needs to consider factors such as a single dose versus steady state, timing of sampling in relation to drug intake, the development of tolerance, the usual dose range and the possibility of atypical responses. The usual dose range or level refers to “therapeutic drug levels measured in serum”. It is a therapeutic range which is deemed as being safe and effective in a particular population.[80,81] The single dose versus steady state of therapeutic doses is established in initial pharmacokinetic studies which tend to be lower. Furthermore, the usual dose range is based on initial pharmacokinetic studies which may be based on smaller doses than those which are adapted to in clinical practice. Atypical or paradoxical reactions to drugs may lead to an

intoxicated-like state despite the presence of the usual drug level. This is best exemplified by the combination of benzodiazepines with alcohol.

In conclusion, the interpretation of toxicological data is fraught with difficulties and is dependent on numerous factors. Review of the literature highlights that correct interpretation should follow a multi-disciplinary approach, with collaboration of the death scene investigators, forensic pathologist, relevant family members and toxicologist, in order to obtain the most accurate determination of a result, so as to aid the courts in the fight towards justice.

1.4.9 THE SOUTH AFRICAN DILEMMA AND A POSSIBLE SOLUTION

Advances in toxicological analysis are evident due to the calibre of research that is emerging from international forensic centres. This work is a culmination of available resources, trained laboratory staff, well established protocols and available statistics and data. By and large most international centres implement GUS on all unnatural deaths. There is an increase in the use of STA (screening followed by confirmatory testing) as near-patient testing methods improve. There are centres that have on site laboratory services, providing accurate and rapid results. One such centre is the Office of the Chief Medical Examiner in the state of Maryland, USA, which comprises not only of administrative and mortuary facilities but also makes provision for the numerous ancillary forensic departments of which toxicology is one (discussed in person with Professor Fowler in 2013).

In SA the picture is less than pleasing. Newspaper reports echo the frustration of the law enforcement agents, the forensic pathologists and importantly the public.[82] Forensic Chemistry Laboratories(FCL) in SA have been placed under an enormous amount of media and government pressure. Alarming details have been made available within the public domain. A nationwide backlog of 62 261 forensic toxicological samples has been reported as at November 2014.[83] It has been our experience as part of the Forensic Pathology Services within the Gauteng Southern Cluster, that cases requiring toxicological analysis have taken up to 8 years to conclude. A report by the Auditor General in 2009, commented on the dilapidated state of the FCL infrastructure, which was found to be in contravention of the Occupational Health and Safety Act of 1993. Furthermore, there has been a large amount of criticism towards the administration of this service, with regard to their high staff turnover

and vacancy rates, inadequate information systems and poor management.[83] This, together with the increasing pressure of unnatural deaths due to crime and increasing alcohol and illicit drug use,[84] has resulted in poor performance of these FCL services and significant delays within the Forensic Pathology Service and justice system.

If validated in the South African post mortem population, near-patient/near-body screening tests, such as Randox or ND, would reduce the workload of the National Department of Health FCL, which in turn would improve throughput timeframes through the criminal justice system.

CHAPTER 2 – MATERIALS AND METHODS

2.1 INTRODUCTION

This chapter discusses the methodology of the study. It begins by elaborating on the study design, period, context and sampling strategy. It then discusses the materials and techniques used to acquire, store, process and analyse samples. Finally, it concludes with details about the statistical analysis and ethical principles of the study.

2.2 STUDY DESIGN

The study was designed as a prospective, observational and transverse study. This study design was chosen to answer the research question and satisfy the aims of the project best. The study concept is best explained by defining each of the separate three terms.

The term "prospective" provides the temporal context of the data collection. This means that we enrolled candidates before conducting the study, and collected data as events, which happened during a pre-defined data collection period. [85]

The term "observational" means that the independent variables within the study sample are beyond the control of the researcher. [85]

A transverse study is by definition observational. The term “transverse” means that data collected from the study population is analysed at one specific point in time. Furthermore, the data gathered is representative of the entire population under research. [85]

2.3 STUDY PERIOD

The recruitment and data collection was conducted over a three-month period during 2014.

2.4 STUDY SITE AND CONTEXT OF THE STUDY

The study was conducted at the Johannesburg Forensic Pathology Service (FPS) Medico-Legal Laboratory in Braamfontein, Johannesburg, which is a service provision and training site affiliated to the University of the Witwatersrand. The facility is classified as an M6A facility, which denotes accreditation to conduct more than 2000 autopsies per year in an academic institution. All unnatural deaths in SA require a medico-legal post mortem examination in accordance with the Inquests Act 58 of 1959. This FPS mortuary allows delivery of a comprehensive service including scene investigations, autopsy; collection of-, preliminary processing-, storage- and dispatch of samples for ancillary forensic examinations

(such as forensic toxicology, histology, anthropology and DNA samples); the performance of radiological investigations, teaching, training and research and administrative functions.

2.5 STUDY POPULATION

The study sample was drawn from decedents admitted to the Johannesburg FPS Medico-Legal Laboratory in Johannesburg.

2.6 STUDY SAMPLE AND RECRUITMENT

The study was designed to recruit 100 candidates. As discussed within the ethical considerations further below, additional familial consent was not required for the performance of the tests used in this study, since the information gleaned could be utilised towards determining with greater certainty the cause or circumstances of death in these decedents, as mandated by the Inquests Act 58 of 1959. Once the decedent met the inclusion and exclusion criteria, it was given a specific study number. This number was the only identifier used during the study. No other identifying information was collected. Recruitment was performed on a convenience basis. No randomisation was required for this study. All recruitment and sample collections were performed by the author. Sample analyses were performed by individuals specifically trained for this purpose in collaboration with the author.

The recruitment of candidates occurred during the performance of the medico-legal autopsy examination. Prior to the performance of autopsies, the pathologists are assigned an allocated number of cases. It is the duty of the forensic pathologist to peruse the information pertaining to the deceased. This information must include a SAPS180 “history” form and FPS death scene forms and, where relevant, may include hospital D28 and GW7/24 clinician’s forms. In addition, any other information deemed important by the police may be included. The medico-legal autopsy can then be conducted once the necessary background information has been acquired. Details of the utility and contents of these forms are tabulated in table 2.

The information gathered from these documents allowed the author to identify study candidates. Figure 7 summarises the recruitment process of the study.

TABLE 2: DETAILS OF THE FORMS REQUIRED PRIOR TO PERFORMANCE OF A MEDICO-LEGAL AUTOPSY

Form	Details
SAPS180	- Mandatory form in order to authorise the FPS to conduct the medico-legal post mortem examination. - Completed by the attending police Investigating Officer in whose jurisdiction the death occurs. - Provides information pertaining to the scene of death as well as circumstances and manner of death.
D28 and GW7/24	- Completed by the primary clinician and/or the anaesthesiologist involved in the care of the deceased prior to death. - Provides information regarding treatment, therapeutic interventions and progress of the deceased during their admission to a health care facility. - Can also provide information about the initial circumstances surrounding the hospitalisation of the individual and their demographics.
FPS Scene of Death Form	- Completed by the Forensic officers who attend the scene of death. - Provides limited information pertaining to the circumstance and possible manner of death.

2.6.1 INCLUSION CRITERIA

- Any decedent between the ages of 18 -70 years.
- Any decedent who died of unnatural causes and required a medico-legal post mortem examination (as per the Inquests Act 58 of 1959 and as defined in the Regulations to the National Health Act 61 of 2003).

2.6.2 EXCLUSION CRITERIA

- Any decedent who showed signs of late decomposition, including putrefaction, adipocerous formation or mummification.
- Any decedent who was extensively burnt.

- Any decedent who had been hospitalised immediately prior to death.

2.7 STUDY MATERIALS

The Johannesburg Medico-Legal Laboratory acquired a Randox Evidence Investigator Analyser in 2012 through grant funding. During 2014, the National Department of Health undertook to provide Randox Kits and controls for one year to the Johannesburg FPS Medico-Legal Mortuary in order to facilitate toxicological screening in all unnatural deaths admitted to this facility, using the Randox equipment at this facility. The Randox Kits and controls used in this study were effectively funded through this arrangement. The manufacturer of ND sponsored the kits used in the study.

The performance of the confirmatory stage of the study using LC-MS, was conducted and sponsored by the Horse Racing Authority (HRA). The HRA is an organisation which regulates and maintains good practice in thoroughbred horse racing in Southern Africa. The HRA has an accredited forensic toxicology laboratory service, which can perform a range of methodologies including sensitive immune-diagnostic methods to drug specific mass spectrometric methods. The HRA laboratory is also actively involved in research and development, and welcomed the collaboration with the FPS.

2.8 SPECIMEN ACQUISITION AND PROCESSING

After a decedent was recruited into the study, anonymised demographic details and other required data were recorded as per appendix 1. These records were labelled with a unique number. Blood samples were collected from femoral vessels of decedents during autopsy using aseptic technique and universal precautions. Three millilitres of blood was taken and dispensed into 2 ethylenediaminetetraacetic acid (EDTA) preserved tubes. These samples were labelled with the same unique number correlating to the patients recorded study information and were stored in plastic containers on a test tube rack under controlled conditions at -20°C. Whole blood was used as the matrix to be tested, since this is the most commonly tested matrix in the literature concerning near-body drugs of abuse testing. It is also easily collected, requires minimal processing, and is least likely to produce false negative results. [86]

2.9 TESTING

2.9.1 MATERIALS

Certified reference materials purchased from Cerilliant® (Sigma-Aldrich, Texas, USA) included Amphetamine 1mg/ml, MDMA 1mg/ml, Methamphetamine 1mg/ml, Morphine 1mg/ml, Dihydrocodeiene HCL 1mg/ml, MAM 1mg/ml, Codeine 100 100µg/mL, Hydromorphone 1mg/ml and Cannabinol 1mg/ml. Beta glucuronidase/arylsulphatase were purchased from Roche (Basel, Switzerland). Di-potassium hydrogen orthophosphate, sodium carbonate, sodium dihydrogen orthophosphate, and sodium hydroxide were purchased from Associated Chemical Enterprises (ABC, Johannesburg, SA). Acetonitrile and methanol were purchased from Honeywell Burdick and Jackson (New Jersey, USA). 1-chlorobutane was purchased from Minema (Johannesburg, SA). Ethanol was purchased from Radchem (Johannesburg, SA). Dichloromethane, ethyl acetate, ethyl ether, hexane, and isopropanol were purchased from RCI Labscan (Bangkok, Thailand). Methyl tert-butyl ether was purchased from Honeywell Riedel-de Haën (New Jersey, USA). 1-chlorobutane, acetic acid, formic acid, and sodium acetate were purchased from Sigma-Aldrich(Texas, USA). The LC-MS column used was the X Select CSH (C18 5 µm, 2.1 x 150 mm) column from Waters (Milford, UDA). The centrifuges used included Nuve NF200, Beckman TJ-6, Beckman JS-6, and Beckman Coulter Allegra X-12R. Double deionised water was made from a Siemens LaboStar evoqua water purifier.

2.9.2 NARCOTICS DETECTOR

Narcotics Detector (ND) is a competitive binding chromatographic immunoassay based point of care test for drugs of abuse available in SA.

The ND kit comprises of a buffer tube (phosphate buffered saline), collection tube, collector and the test device. This is a multifunctional kit testing for the presence of cocaine, methamphetamines, phencyclidine, THC and opiates. With regards to the testing of the opiates, ND does not single out specific drugs within the opiate group but rather performs a general screen.

2.9.2.1 SAMPLE PREPARATION

After samples were removed from the freezer and allowed to thaw to room temperature, they were centrifuged using a Nuve NF200 at 5000rpm for 10 minutes.

2.9.2.2 ANALYSIS

Fifty microlitres of sample from the centrifuged specimen was pipetted in a numerically labelled plastic collection tube provided by the manufacturer. One hundred and fifty microlitres of the manufacturers buffer was added resulting in a four-fold dilution. This tube containing the diluted specimen was then agitated for 30 seconds using a vortex. One hundred and fifty microlitres of diluted specimen was then pipetted into the two wells within the test device. Results were observed and recorded after 10 minutes.

Interpretation criteria for this test were as follows (see figure 6):

- A positive result was represented by a coloured line in the control region, but no line in the test line region for a specific drug on the test device. A positive result indicates that the concentration of the drug in the specimen, exceeds the designated cut-off value for that specific drug in this device namely 50 ng/mL for amphetamines; 12 ng/mL for cannabinoids and 40 ng/mL for opiates.

  A coloured line no matter how faint was regarded as positive. [66]
- If a coloured line was not represented in the control region, the test was excluded and repeated. After recording the result on the result sheet (appendix 1), surplus sample was removed using standard precautions.

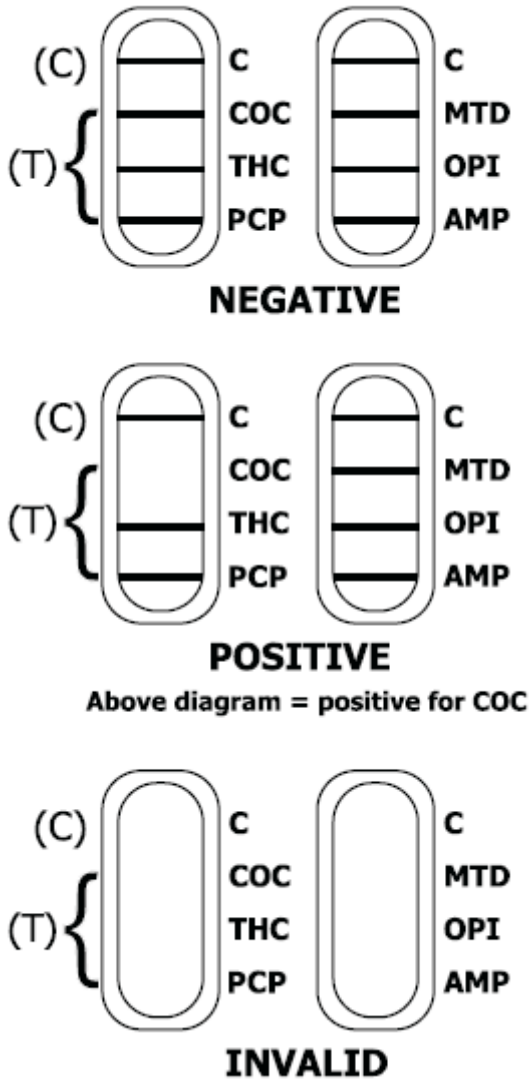


FIGURE 6: INTERPRETATION OF THE RESULTS USING NARCOTICS DETECTOR [66]

2.9.3 RANDOX BIOCHIP IMMUNOASSAY

The Randox immunoassay screening test is an automated system which is able to use whole blood or urine as sample matrix. It is currently most commonly used to conduct point of care toxicological screens in emergency departments.

The Randox DOA I Whole Blood Plus (WBP) kit comprises 10 mL of assay diluents, 10 mL of conjugate, 54 biochips, nine 1mL calibrators, 10 mL of luminal-EV841 and 10mL of peroxide, 10 mL of phosphate buffer (pH 7.2), 20 mM Tris buffered saline (pH 7.4) containing surfactant and preservatives, a single calibrator disc, and barcodes. [64]

The DOA I WBP quality control consists of two components. These are control 1 and control 2. These controls are lyophilised materials. These controls contain the same range and variety of analytes tested for within the kit at two different concentrations levels, which

cover the cut-off ranges. The list of drug assay classes is listed in table 3.

TABLE 3: LIST OF ASSAY CLASSES TESTED FOR BY THE RANDOX DOA I WBP KIT [4]

- Amphetamine (amphetamine/methamphetamine)
- Barbiturates
- Benzodiazepine 1 & 2 (oxazepam and lorazepam)
- Benzoylecgonine (cocaine, benzoylecgonine, cocaethylene)
- Methadone
- Opiates (morphine, MAM, codeine, morphine-3-glucuronide, hydromorphone, hydrocodone, dihydrocodeine)
- Phencyclidine (PCP)
- MDMA
- Cannabinoids (11-nor-delta9 –THC-9-carboxylic acid, 11-hydroxy-delta9-THC, cannabinol, delta9-THC)
- Tricyclic antidepressants (nortriptyline)
- Buprenorphine

2.9.3.1 SAMPLE PREPARATION

Sample preparation was carried out as per the manufacturer's instructions. [64] The DOA I WBP kit and controls were removed from the refrigeration unit and left to reach room temperature. The calibrators and controls were reconstituted with 1 mL of deionised water in each bottle and placed on an ABC rocking roller for 30 minutes. The numerically labelled blood samples were removed from the freezer and allowed to thaw to room temperature. The specimens were then centrifuged using a Nuve NF200 at 13000rpm for 10 minutes. 50µl of specimen from the centrifuged specimen was added to 150µl of sample diluent resulting in a four-fold dilution, according to the manufacturer's instructions. The biochip carriers were removed from their packaging, numerically labelled and then placed on the carrier holder. [64]

2.9.3.2 ANALYSIS

Analysis was carried out as per the manufacturer's instructions.[64] 120µl of assay diluent was pipetted into each biochip well of the biochip carriers. This was followed by 60µl of

reconstituted calibrators, controls and prepared specimen, into their respective wells. 120µl of conjugate (enzyme-labelled antibody) was then pipetted into each well. The biochip carrier holder containing the biochip carriers was then placed into the thermos shaker for incubation for 30 minutes at 25 ℃ at a speed of 330 rpm.

During the incubation, the signal reagent (luminol and peroxide mixed in a 1:1 ratio) was prepared and placed in a light protected container. The signal reagent was then place on a rocking roller to mix for 15 minutes.

After incubation the surplus mixtures in each biochip carrier were discarded and the biochip carriers were washed using the wash buffer solution. The wash cycle consisted of six quick rinses and six 2 minute soaks. After the final soaking and wash the biochip carriers were filled with wash buffer solution to prevent the biochips from drying out.

The biochip carriers were processed individually for imaging. The awaiting carriers were covered by aluminium foil to protect them from light exposure. Prior to imaging the biochip carrier was tapped onto dry paper towel to remove residual wash buffer. Then 250µl of working signal reagent was added to each biochip well and covered to protect from light exposure. This was left for 2 minutes. After this time the biochip carrier was placed into the Randox Evidence Investigator for imaging. The images were automatically captured and processed by the on-board software. Calibration curves were established for each drug or drug group and specimen results were displayed as concentrations and these were recorded on the result sheet (appendix 1). Both quality control results were verified, according to manufacturer specifications and quality assurance data, prior to the acceptance of the results of the specimens. The cut-off levels applied to the analysis were as per Randox recommendations. There are listed in table 4.

TABLE 4: DRUGS OF ABUSE ARRAY I WHOLE BLOOD PLUS ANALYTE CUT-OFF LEVELS [64]

Analyte	Cut-off levels (ng/mL)
Amphetamines	**25**
Barbiturates	50
Benzodiazepine	50
Buprenorphine	1
Tetrahydrocannibinol	**10**
Benzoylecgonine	50
3,4-Methylenedioxymethamphetamine	60
Methadone	25
Methamphetamine	**50**
Opiates	**25**
Phencyclidine	5
Tricylclic Antidepressants	60

2.9.4 LIQUID CHROMATOGRAPHY-MASS SPECTOMETRY

Liquid chromatography coupled with mass spectrometry (LC-MS) is considered a gold-standard analytical technique to detect the presence of any substance. The general principle of chromatography is that a sample mixture is separated by a liquid or gas column into component molecules. As the components of this sample have different masses they appear at different times at the end of the column. Each component is detected by a mass spectrometer as it exits the column and accurate identification of the component can then be made.

2.9.4.1 SAMPLE PREPARATION

The extraction of whole blood samples was adapted from previously published techniques.[87]

The numerically labelled samples were removed from the freezer and allowed to thaw to room temperature. Each sample was then vortex agitated for 30 seconds. One millilitre of blood sample was then pipetted into corresponding, numerically labelled B24 glass tubes. To the blood specimen, 2ml of sodium carbonate buffer (pH 9) was then pipetted. Fifty microlitres of the HRA in-house internal standard solution (these comprising compounds cannot be divulged, due to a confidentiality agreement which were undertaken between HRA and the researcher) was then added to the sample. Finally, 10ml of 1-chlorobutane: isopropanol (90:10) was added. This mixture was agitated for 30 seconds and centrifuged (Beckman Model TJ6) for 15 minutes at 3000rpm. Samples were then frozen using dry ice in an ice bath. After freezing, the organic layer was pipetted into clean glass tubes. The supernatant was then evaporated to dryness, using a concentration evaporator (Turbovap LV) at 40^{o}C using nitrogen as the ambient gas. The samples were subsequently reconstituted by adding 100µL of methanol. [87]

2.9.4.2 QUALITY CONTROL PREPARATION

Included in the 100 samples were 5 control blood samples. These samples were drawn from consenting staff members. These samples did not contain any prescription or illicit drugs. Two external controls (labelled QC1 and QC2) were also added to the test pool. QC1 consisted of a mixture, which was prepared from the certified reference materials listed in table 5 below. The concentration of each compound was 300ng/ml in methanol. QC2 consisted of a HRA in-house external standard solution (comprising of 250 compound routinely screened for in jockeys and horses by the HRA which cannot be divulged, due to a confidentiality agreement which were undertaken between HRA and the author).

TABLE 5: COMPOUNDS PRESENT IN THE EXTERNAL CONTROL: QC1

Manufacturer	Drug name and dose	Lot number	Expiry date
Cerilliant®	Amphetamine 1mg/mL	FE072712-02	08/2017
Cerilliant®	MDMA 1.0mg/mL	FE012111-07	02/2016
Cerilliant®	Methamphetamine 1.0mg/mL	FE082712-03	05/2017
Cerilliant®	Morphine 1.0mg/mL	FE080411-01	08/2016
Cerilliant®	Dihydrocodeine HCl 1.0mg/mL (as free base)	FE070910-01	07/2015 *
Cerilliant®	6-Acetylmorphine 1.0mg/mL	FE060412-13	07/2017
Cerilliant®	Codeine 100µg/mL	FE101310-02	10/2015 *
Cerilliant®	Hydromorphone 1.0mg/mL	FE110711-04	11/2016
Cerilliant®	Cannabinol 1.0mg/mL	Fe092711-04	09/2015 *

**** sample analysis was performed between in 2014***

2.9.4.3 ANALYSIS

Compounds were separated using a high performance liquid chromatography (HPLC) system (Agilent 1260 Infinity) with a 150 x 2 mm (5µm) column (Waters XSelectCSH C18). The mobile phase consisted of a gradient of water to acetonitrile, both containing 5 mM ammonium acetate buffer and 0.1% formic acid. The linear gradient (0.3 mL/min) started at 2 minutes from 2% to 98% acetonitrile at 20 minutes, held at 98% until 26 minutes, followed by a return to the initial conditions at 28 minutes. The column was allowed to recondition until 35 minutes before the next injection was started.

A high resolution mass spectrometer (Thermo Fisher Q Exactive) was used for detection. The detection software (XCalibur) was used to set a scan range of 110-700 *m/z*; resolution of 70 000; automatic gain control target balanced; and a maximum injection time of 500ms. A heated electrospray (HESI-II) ion source was employed using scan to scan positive to negative ion switching. Processing of the data was done using the Thermo Fisher's ToxID automated screening software The ToxID software requires an Excel spread sheet comprising the compound name, elemental composition, polarity, and expected retention time and from this it generates a PDF report. A mass to charge ratio (m/z) window of 5 mu and retention time window of 0,3 min were used to identify compounds.

Compounds were identified as being positive if they meet the following criteria:

- The compound was at a concentration of more than or equal to 20ng/m l(limit of detection applied to compounds on the instrument with the utilised method).
- If associated compound metabolites were present.
- The presence of expected compound ratios e.g. morphine/codeine.

This analytical test method is a semi-quantitative method employed by the HRA for routine screening of over 250 compounds.

The results sheet (appendix 1) was completed with the measured data.

2.10 QUALITY CONTROL

Strict adherence to inclusion and exclusion criteria ensured appropriate selection of candidates. All sample collection, storage and testing was overseen by either the primary investigator of the study or the FPL/FCL analytic staff and supervisors, ensuring that samples were processed in the correct manner. All equipment was checked for functionality and calibrated if necessary prior to use.

2.11 DATA ANALYSIS AND MANAGEMENT

After all the samples were processed, data was transferred from the results sheets to an electronic spreadsheet (Microsoft Excel, 2015, Microsoft Corporation, USA). Results sheets were stored in a secure location by the primary researcher. Both physical and digital copies will be kept for 5 years before destruction. All data was analysed in consultation with a statistician.

2.11.1 STATISTICAL ANALYSIS

Mean and standard deviation were used to describe baseline demographic variables. Sensitivity, specificity, negative predictive value and positive predictive value was calculated for ND and Randox, against the gold standard LC-MS. Cohen's Kappa test as well as the McNemar test was used to compare Randox, ND and LC-MS.

Mean is the mathematical average of the sample in question, and standard deviation is a mathematical description of the positive and negative variation from the mean within the sample. Specificity and sensitivity are probabilities used to measure the ability of a test to truly reflect an accurate result. Negative and positive predictive values are the probabilities that samples with a negative or positive test result truly reflect the absence or presence of the substance in question respectively. [88]

Cohen's Kappa coefficient test measures agreement or inter-rater reliability between two categorical, qualitative variables.[89] The McNemar chi-squared two-tailed comparison is a non-parametric (distribution-free) test, which assesses if a statistically significant change in proportions has occurred on a dichotomous trait, at two time points on the same population. It is applied using a 2×2 contingency table with the dichotomous variable at time 1 and time 2.[90]

Although both Cohen's Kappa and McNemar tests have limitations, both were used to examine correlation in an attempt to reduce error in interpretation. Statistica 12.7 (2015, StatSoft, SA) was used to process the data and apply the statistical tests. A *p* value of less than 0.05 was taken as being significant.

2.12 ANTICIPATED LIMITATIONS OF THE STUDY

2.12.1 SAMPLE SIZE

Due to a paucity of literature in the data examining near-body testing for illicit drug use in a post mortem population, this study is forced to be a pilot study in nature, with the sample size estimated using unpublished drug analysis data obtained from the Johannesburg FPS Mortuary in-house toxicology laboratory.

2.12.2 POST MORTEM REDISTRIBUTION

Drug redistribution occurs between different body compartments in the post mortem period. This means that blood concentrations of drug at the time of sampling may be low due to movement of the drug out of the blood since the time of death.

2.12.3 LIMITED TESTING MATRICES

Due to limited funding, we were only able to perform analyses on blood samples. There is a possibility that this could cause false negative results. This was taken into account during data analysis and result interpretation.

2.12.4 DRUG ASSAY CUT-OFF CONCENTRATIONS

ND has higher cut-off concentrations than the Randox DOA I immunoassay, and this may result in false negative tests when using ND.

2.12.5 CROSS REACTIVITY WITH OTHER DRUGS

There is a possibility that other drugs or compounds present in the sample could cross react with the antibodies used in ND or the Randox DOA I immunoassay. If this occurs, it will result in false positive result which will be shown during the confirmatory chromatography.

2.13 ETHICAL CONSIDERATIONS

The Human Research Ethics Committee and the Post Graduate Committee from the University of the Witwatersrand approved the conduct of this study, with reference number M131191 (appendix 2 and 3). The Inquests Act 58 of 1959 authorises and mandates the FPS to perform ancillary toxicological analyses in relevant unnatural death investigations, towards determining causes or circumstances of death, without the need for additional familial consent. In addition, the chief specialist, Head: Clinical Department of the FPS Southern Cluster and the Chief Executive Officer of Forensic Services of Gauteng approved

the conduct of this study (appendix 4). There was no risk that decedents received less than the established standard of investigation.

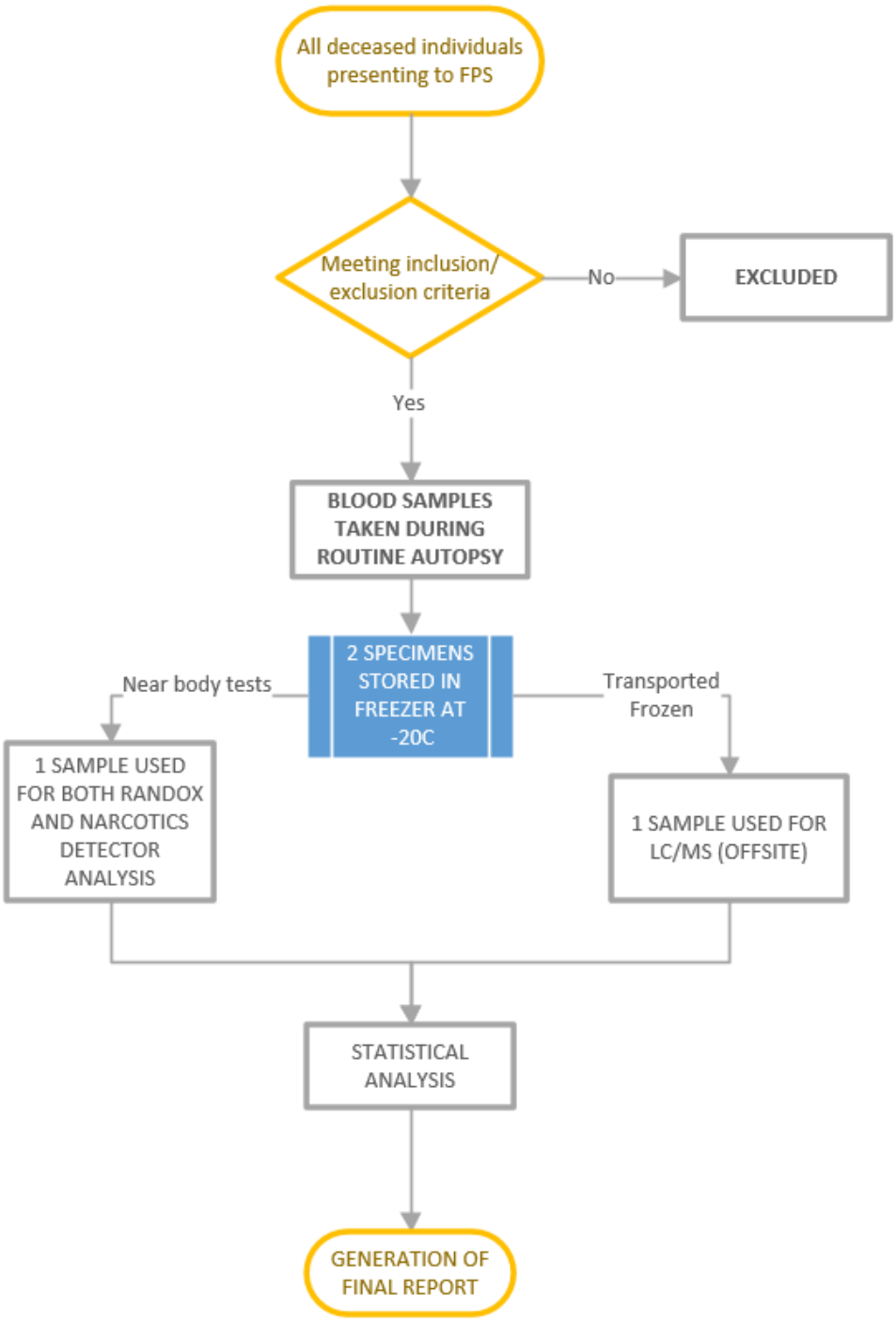


FIGURE 7: FLOW DIAGRAM DESCRIBING THE METHODOLOGY OF THE STUDY

CHAPTER 3 – RESULTS

3.1 INTRODUCTION

This chapter consists of the presentation of results. The chapter begins a description of demographic data across the sample. The results are then presented using the objectives laid out in Chapter 2. The chapter is then concluded with a short summary of important findings.

3.2 DEMOGRAPHIC DATA

The sample size comprised of 102 samples. Demographic analysis revealed that of the 102 samples collected, 77 (76.23%) of the candidates where male, 17 (16.80%) were female and in 7 (6.93%) samples the gender was unknown due to inadequately completed result forms. The mean age of the individuals was 36 years (SD ±12.05 years) with a range of 18 to 70 years. The mean post mortem interval (PMI) was determined from the date of death to the date of autopsy. This time period was merely an approximation as the exact time of death and that of autopsy were not known. This estimation was under taken to provide a rough guide, as prolonged PMI may influence the presence of substrate in the blood.[91] The mean PMI was 2.3 days (SD ±1.54 days).

The causes of death in the study population were: sharp force injury (10, 9.90%); gunshot wound (21, 20.80%); blunt force injury (29, 28.71%); mild burns (1, 0.99%); hanging (9, 8.91%); overdose (7, 6.93%); gassing (1, 0.99%) and natural deaths (8, 7.92%). Of the sample population 15 cases were still under investigation (14.85%). These data are summarised in Table 6.

TABLE 6: DEMOGRAPHIC DATA OF SAMPLE

Characteristic	
Post mortem interval(SD)	2.3(± 1.54) days
Mean Age (SD)	36 (±12.05) years
Gender (#, %)	- Male (77, 76.23%) - Female (17, 16.80%) - Unknown (7, 6.93%)
Cause of death (#, %)	- Sharp force (10, 9.90%) - Gunshot wound (21, 20.80%) - Blunt force (29, 28.71%) - Natural (8, 7.92%) - Mild burns (1, 0.99%) - Hanging (9, 8.91%) - Overdose (7, 6.93%) - Gassing (1, 0.99%) - Under investigation (15, 14.85%)

** SD- Standard Deviation, #-number*

3.3 THE PRESENCE OF OPIATES, CANNABINOIDS AND METHAMPHETAMINE USING THE ND IMMUNOASSAY

The number and percentage of positive tests for THC, opiates and amphetamines using ND, Randox and LC-MS are summarised in Table 7.

TABLE 7: POSITIVE TESTS FOR THC, OPIATES, AMPHETAMINES USING ND, RANDOX AND LC-MS

	ND	**Randox**	**LC-MS**
THC	5 (4.9%)	22 (21.5%)	7 (6.86%)
Opiates	7 (6.8%)	4 (3.90%)	9 (8.82%)
Amphetamines	4 (3.9%)	1 (0.98%)	2 (1.96%)

3.3.1 THC

Of the 102 samples tested ND displayed positivity in 5 (4.9%) samples for THC. Analysis revealed that ND displayed a sensitivity of 28% (95% CI 0.05-0.7), but specificity of 96% (95% CI 0.9-0.99). The negative predictive value for ND in the detection of THC was 94% (95% CI 0.88-0.98). The positive predictive value was 40% (95% CI 0.07-0.83). These results are summarised in table 8.

TABLE 8: ANALYSIS OF DETECTION FOR THC

	Sensitivity (95% CI)	**Specificity (95% CI)**	**Negative predictive value (95% CI)**	**Positive predictive value (95% CI)**
Randox	100% (0.56-1)	84% (0.75-0.9)	100% (0.94-1)	100% (0.4-1).
ND	28% (0.05-0.7)	96% (0.9-0.99)	94% (0.88-0.98)	40% (0.07-0.83).

3.3.2 OPIATES

There were only 7 (6.8%) positive results detected using ND to test for opiates. ND revealed a sensitivity of 87% (95% CI 0.47-1) and a specificity of 100% (95%, CI 0.95-1). The negative predicative value was 98% (95%, CI 0.93-0.99) and the positive predictive value was 100% (95%, CI 0.56-1). These results are summarised in table 9.

TABLE 9: ANALYSIS OF DETECTION FOR OPIATES

	Sensitivity (95% CI)	**Specificity (95% CI)**	**Negative predictive value (95% CI)**	**Positive predictive value (95% CI)**
Randox	45% (0.15-0.77)	100% (0.95-1)	94.8% (0.88-0.98)	100% (0.4-1).
ND	87% (0.47-1)	100% (0.95-1)	98% (0.93-0.99)	100% (0.56-1).

3.3.3 AMPHETAMINES

There were 4 (3.9%) positive tests for the presence of amphetamines. The sensitivity of ND in this drug group was 100% (95%, CI 0.19-1) and the specificity was 97% (95% CI 0.92-0.99). The negative predictive value was 100% (95%, CI 0.95-1) and the positive predictive value was 50% (95%, CI 0.09-0.91). These results are summarised in table 10.

TABLE 10: ANALYSIS OF DETECTION FOR AMPHETAMINES

	Sensitivity (95% CI)	**Specificity (95% CI)**	**Negative predictive value (95% CI)**	**Positive predictive value (95% CI)**
Randox	50% (0.03-0.97)	100% (0.95-1)	99% (0.93-0.99)	100% (0.05-1)
ND	100% (0.19-1)	97% (0.92-0.99)	100% (0.95-1)	50%(0.09-0.91)

3.4 THE PRESENCE OF OPIATES, CANNABINOIDS AND METHAMPHETAMINES USING THE RANDOX DRUGS OF ABUSE ASSAY I IMMUNOASSAY

3.4.1 THC

There were 22 (21.5%) positive samples using the Randox method. The sensitivity and specificity for the detection of THC was 100% (95% CI 0.56-1) and 84% (95% CI 0.75-0.9) respectively. The negative predictive value for Randox was 100% (95% CI 0.94-1) and the positive predictive value was 32% (95% CI 0.15-0.55). These results are summarised in table 8.

3.4.2 OPIATES

Only 4 (3.9%) samples tested positive for opiates using Randox. The sensitivity and specificity for the detection of opiates was 45% (95% CI 0.15-0.77) and 100% (95% CI 0.95-1) respectively. The negative predictive value was 94.8% (95% CI 0.88-0.98) and the positive predictive value was 100% (95% CI 0.4-1). These results are summarised in table 9.

3.4.3 AMPHETAMINES

Only 1 (0.98%) sample tested positive for amphetamines opiates using Randox. The sensitivity and specificity for the detection of opiates using Randox was 50% (95%, CI 0.03-0.97) and 100% (95%, CI 0.95-1) respectively. The negative predictive value was 99%

(95%, CI 0.93-0.99) and the positive predictive value was 100% (95%, CI 0.05-1). These results are summarised in table 10.

3.5 THE PRESENCE AND BLOOD CONCENTRATION OF OPIATES, CANNABINOIDS AND METHAMPHETAMINE USING LC-MS

3.5.1 THC

Of the 102 samples tested, LC-MS displayed positivity in 7 (6.86%) samples for THC.

3.5.2 OPIATES

There were 9 (8.82%) positive results detected using LC-MS.

3.5.3 AMPHETAMINES

There were 2 (1.96%) positive results for the presence of amphetamines.

Sensitivity, specificity, negative and positive predictive values were not calculated for LC-MS as this is considered the gold standard test.

3.6 COMPARISON OF THE SPECIFICITY, SENSITIVITY, NEGATIVE PREDICTIVE AND POSITIVE PREDICTIVE VALUES OF ND SCREENING WITH THAT OF THE RANDOX ASSAY.

3.6.1 THC

The presence of THC tested positive in 22 of samples using Randox, however only 7 were confirmed positive on LC-MS. The sensitivity and specificity for THC using Randox was 100% (95%, CI 0.56-1) and 84% (95%, CI 0.75-0.9) respectively. ND demonstrated poor sensitivity (28%, 95% CI 0.05-07) but adequate specificity (96%, 95% CI 0.9-0.99). Randox testing for THC demonstrated a better negative predictive value (100%, 95% CI 0.94-1) when compared with ND (94%, 95% CI 0.88-0.89). The positive predictive value for Randox was poor, with a value of 32% (95%, CI 0.15-0.55) when compared with a similarly poor value of 40% for ND (95%, CI 0.07-0.83).

3.6.2 OPIATES

Using ND 7 samples tested positive for opiates and 9 tested positive using LC-MS. However, only 4 samples tested positive using Randox. Two samples that were confirmed on LC-MS,

tested negative using Randox and ND. The sensitivity for the detection of opiates using Randox was 45% (95%, CI 0.15-0.77). Narcotics detector displayed better sensitivity 87.5% (95% CI 0.47-1). Randox displayed a specificity of 100% (95%, CI 0.95-1), with ND offering a comparable specificity of 100% (95%, CI 0.95-1). Both testing methods proved to have good comparable negative predictive values. Randox was analysed as having a value of 94.8% (95%, CI 0.88-0.98) and ND as having a value of 98% (95%, CI 0.93-0.99). The positive predictive values of each fared well, as both demonstrated a value of 100%. Randox had a value of 100% (95%, CI 0.4-1) and ND a value of 100% (95%, CI 0.56-1).

3.6.3 AMPHETAMINES

Of the samples studies, three tested positive for amphetamines, and were confirmed positive using LC-MS. One sample tested positive for amphetamines using Randox, however, was negative when later tested on LC-MS. The sensitivity and specificity for the detection of amphetamines using Randox was 50% (95%, CI 0.03-0.97) and 100% (95%, CI 0.95-1) respectively. ND had a higher sensitivity 100% (95%, CI 0.19-1) and a comparable specificity 97% (95%, CI 0.92-0.99). Both tests demonstrated good negative predictive values of 99% (95%, CI 0.93-0.99) and 100% (95%, CI 0.95-1) respectively. Despite the small numbers of positivity, these tests demonstrated acceptable positive predictive values. Randox demonstrated a value of 100% (95%, CI 0.05-1) and ND had a value of 50% (95%, CI 0.09-0.91).

Sensitivity, specificity, negative predictive values and positive predicted values are graphically shown in figures 8,9,10 and 11 respectively.

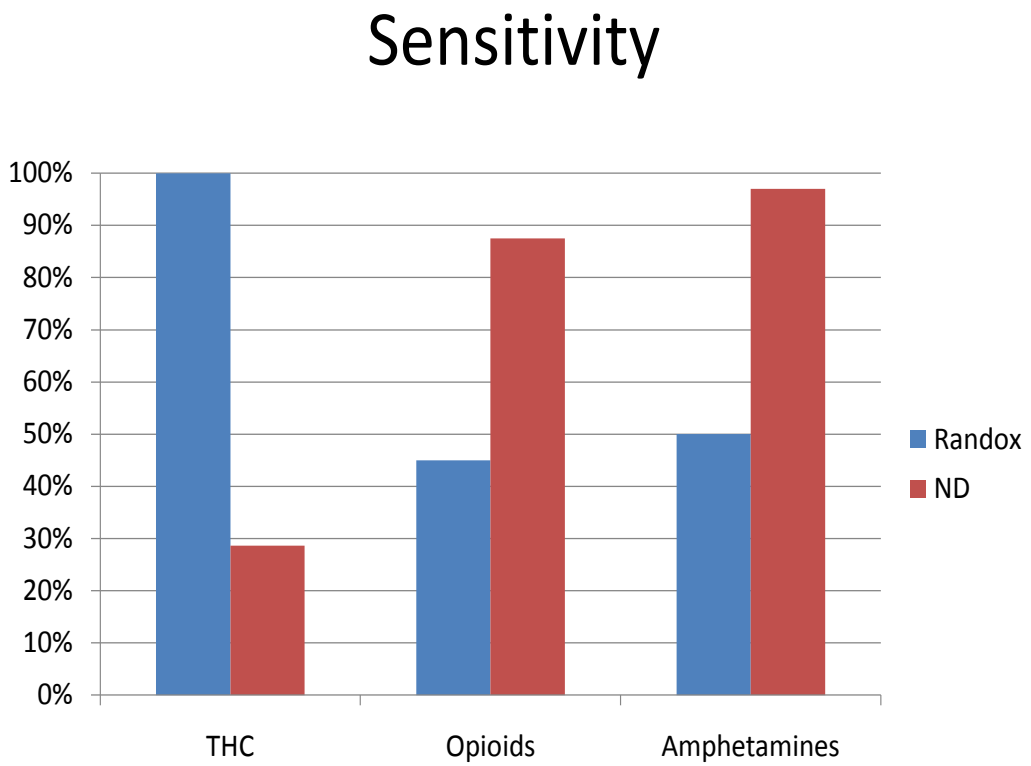


FIGURE 8: REPRESENTATION OF THE SENSITIVITY OF ND AND RANDOX IN THE DETECTION OF THC, OPIATES AND AMPHETAMINES

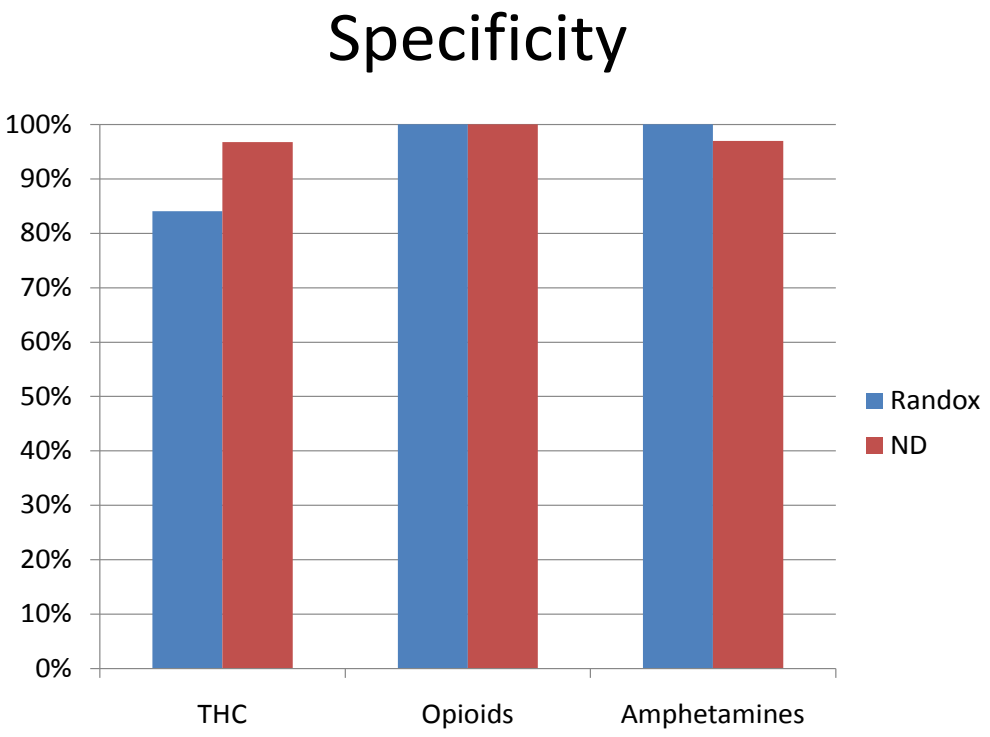


FIGURE 9: REPRESENTATION OF THE SPECIFICITY OF ND AND RANDOX IN THE DETECTION OF THC, OPIATES AND AMPHETAMINES

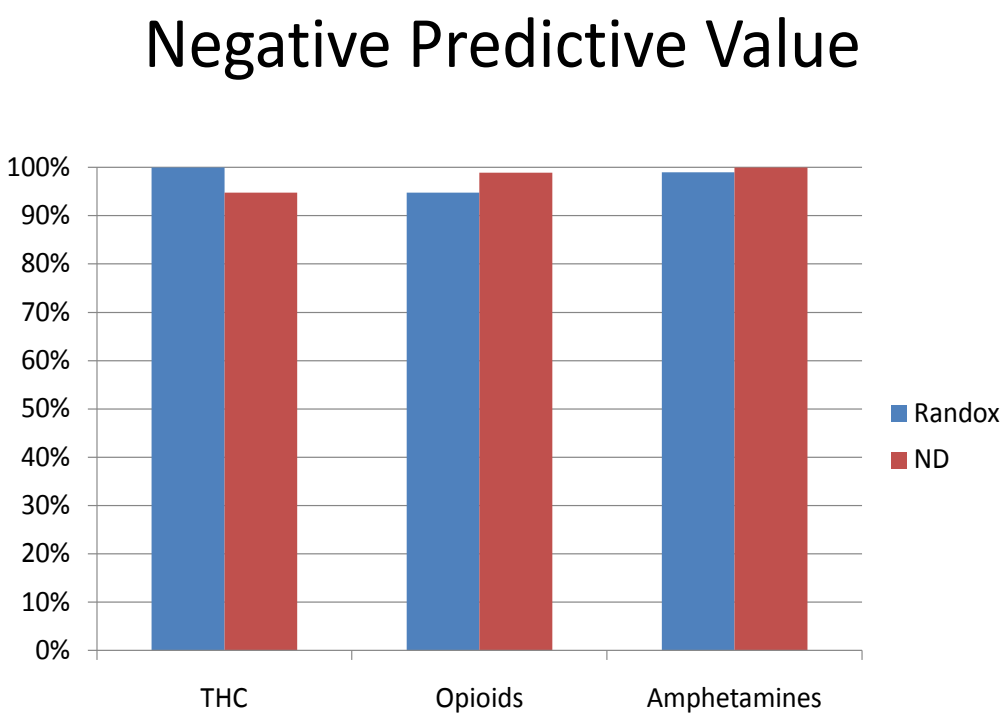


FIGURE 10: REPRESENTATION OF THE NEGATIVE PREDICTIVE VALUES OF ND AND RANDOX IN THE DETECTION OF THC, OPIATES AND AMPHETAMINES.

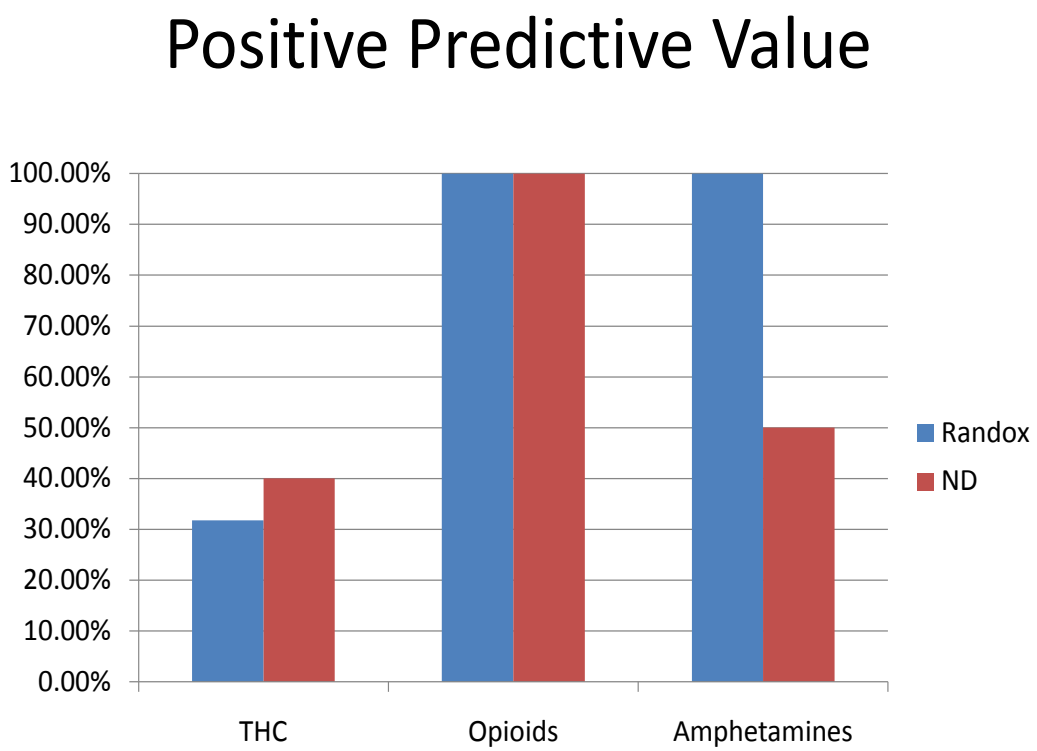


FIGURE 11: REPRESENTATION OF THE POSITIVE PREDICTIVE VALUES OF ND AND RANDOX IN THE DETECTION OF THC, OPIATES AND AMPHETAMINES.

3.7 THE CORRELATION OF THE NARCOTICS DETECTOR SCREENING RESULTS WITH THAT OF CHROMATOGRAPHY / MASS SPECTROMETRY RESULTS.

3.7.1 THC

Cohen's Kappa demonstrated a fair correlation between ND and LC-MS for THC detection with a Kappa value of 0.29 (SE 0.24, 95%, CI 0-0.76). The McNemar two-tailed comparison indicated no difference in the detection of THC by ND or LC-MS (p=0.72).

3.7.2 OPIATES

Cohen's Kappa comparison between ND and LC-MS revealed an almost perfect correlation with a Kappa value of 0.92 (SE 0.07, 95%, CI 0.78-1). The McNemar two-tailed comparison confirmed this (p=1).

3.7.3 AMPHETAMINES

Cohen's Kappa comparison between ND and LC-MS again revealed a substantial correlation with a Kappa value of 0.66 (SE 0.24, 95%, CI 0.19-1). The McNemar two-tailed comparison confirmed this (p=1).

3.8 THE CORRELATION OF RANDOX IMMUNOASSAY RESULTS WITH THAT OF CHROMATOGRAPHY / MASS SPECTROMETRY RESULTS

3.8.1 THC

Cohen's Kappa comparison between Randox and LC-MS for the detection of THC revealed a moderate correlation with a kappa value of 0.422 (SE 0.14, 95%, CI 0.14-0.15). However, the McNemar two-tailed comparison between Randox and LC-MS revealed a significant difference between the two measurements ($p<0.001$).

3.8.2 OPIATES

Cohen's Kappa comparison between Randox and LC-MS revealed a moderate correlation with a kappa value of 0.59 (SE 0.18, 95%, CI 0.25-0.94). The McNemar two-tailed comparison indicated that there was no difference between Randox and LC-MS detection for opiates (p=0.06).

3.8.3 AMPHETAMINES

Cohen's Kappa comparison between Randox and LC-MS revealed a substantial correlation with a kappa value of 0.67 (SE 0.33, 95%, CI 0-1). The McNemar two-tailed comparison indicated that there was no difference between Randox and LC-MS (p=1).

3.9 COMPARE THE GOLD STANDARD CORRELATION OF THE RANDOX IMMUNOASSAY RESULTS WITH THAT THE NARCOTICS DETECTOR

Randox and ND performed well when compared to LC-MS. The poorest performance was in the diagnosis of THC, where the specificity of Randox was low (84%) and the sensitivity of ND was very low (28.6%). Although the confidence intervals were high, positive predictive values for THC were also poor using both Randox and ND. However, negative predictive values were substantial, with narrow confidence intervals.

Negative predictive values for opiates and amphetamines using both ND and Randox were also good with narrow confidence intervals.

Positive predictive values for opiates and amphetamines appeared good, but due to the low positive test prevalence in this population, the confidence intervals were wide and these values are possibly not accurate.

There was significant correlation shown between LC-MS, Randox and ND using Cohen's kappa (fair to substantial correlation) and the McNemar two-tailed test ($p<0.05$) for all drugs with the exception of the use of Randox to detect THC.

CHAPTER 4 – DISCUSSION AND CONCLUSION

4.1 INTRODUCTION

This chapter contains a discussion of the significance of the results, limitations of the study and proposes future directions of research in this field.

4.2 SIGNIFICANCE OF THE RESULTS

The aim of the study was to compare the performance of two available screening tests, namely ND and the Randox Biochip DOA I WBP immunoassay, against a gold standard, currently being LC-MS. Although the Randox DOA I WBP immunoassay has been previously tested in a post mortem population by McLaughlin et al in the UK,[63] we sought to confirm their results in the South African context where PMI's before sample collection, are known to be longer. A total number of 102 blood samples was assessed for the possible detection of tetrahydrocannibanoids, opiates and amphetamines.

In this study population there was a low prevalence of positive tests for drugs of abuse, particularly with regard to opiates and amphetamines. This was most likely due to the convenience serial sampling methodology and sample size. If samples had only been taken from decedents in high risk groups, as individuals in cases of alleged drug overdoses, the yield of positive results could have been higher. With the low yield of positives in mind, the interpretation of the Cohen's kappa correlation and calculated values needs to be carefully considered. Despite these limitations, ND and Randox fared well when compared to LC-MS. Overall, across the three drug groups, the tests displayed good negative predictive values with narrow confidence intervals. The positive predictive value was good; however, the confidence intervals were wide due to a low positive prevalence and a small study sample, which lead to possible inaccuracy of these results. The poor performance demonstrated in the THC group exemplifies the above-mentioned factors leading to poor positive predictive values, and therefore cannot be interpreted meaningfully.

In the setting of this study, a substantial negative predictive value is valuable, in the sense that in near-body testing, a negative result can confidently exclude the unnecessary testing for compounds, which are not present. This benefits the forensic pathologist, laboratory,

family members of the patient and the state administrative and legal mechanisms.

Although not planned in the initial study design, a small subset of results was examined for interesting demographic details that could help plan future study design. Decedents with suicide as the manner of death were chosen for this analysis. Only 5 cases in this group were positive for THC, amphetamines or opioids. One opiate positive case correlated with a history of known heroin addiction and alleged overdose. Three positive results including a combination of THC and methamphetamines had hanging as the cause of death. The alarming reality however is that only the individual with the history of heroin abuse, had a sample submitted by the pathologist for toxicological analysis to the state laboratory. This highlights the possible dilemma of "missed toxicology" which may be contributing factors in a cause of death. It also emphasises the need for research toward accessible screening tests possibly leading to protocol driven routine screens being performed on cases with suspicious case histories or manner of death, without a decision from the pathologist.

4.2.1 THE SOUTH AFRICAN CONTEXT

The FCL's in SA have a large post mortem specimen workload and limited resources. The wait for definitive toxicology results often leads to significant delay in administrative and legal proceedings following an individual's unnatural demise. This then has a knock on effect with regards to the decision to perform toxicological analysis. The forensic pathologist may be hesitant to perform ancillary tests in order to investigate possible contributory factors in a death due to the laboratory backlog and hence inability to provide finalisation to a case. A reliable near-body screening test could obviate the need to send all specimens for toxicology analysis, thus resulting in targeted testing and improved efficiency of the criminal justice system and appreciable cost savings. This study evaluated the performance of two such near-body tests for use in a South African post mortem population and found both ND and Randox to have a good negative predictive value when testing for THC, opiates or amphetamines. This finding supports the use of ND or Randox as a screening test for THC, opiates or amphetamines in a post mortem South African population.

4.3 LIMITATIONS OF THE STUDY

In order to discuss the significance of the results it is important to bear the study limitations in mind, before a meaningful interpretation can be made. Sample size, poor positivity rate, a

limited test panel, the post mortem interval, additional processing for blood samples and test interpretation issues were recognised as potential limitations to this study.

4.3.1 SAMPLE SIZE

Although the study is of relevance in the South African context, the sample size is small owing to the financial costs involved in toxicological analysis. Based on this there was a restriction on the total number of samples that could be processed. However, this study still bears important findings which can be used as motivation for larger, well-funded forensic toxicology projects.

4.3.2 POOR POSITIVITY RATE

The rate of positivity was low largely due to the fact that the samples were not collected from a targeted group. An ideal group would be decedents presenting with a known history of illicit or prescription drug use; or presenting with suggestive circumstances of possible drug use surrounding the time of death. Samples were taken as per the inclusion criteria, without additional extensive historical investigation and on a convenience basis. Additionally, it is unknown as to how many drug related deaths occur in SA and it is likely that only a handful of truly drug related deaths eventually present to the FPS Medico-Legal Laboratories because in the author's experience clinicians either do not recognise certain deaths as drug-related, or they incorrectly ascribe such deaths due to natural causes. A further reason could be that clinicians yield to family pressures to not refer such cases to the FPS. Lastly, the small sample size impacts on the amount of truly positive tests available within the sample.

4.3.3 LIMITED TEST PANEL

Toxicological analysis has significant cost implications due to the expense of equipment, controls and consumables, as well as the necessity for trained staff. It was due to these restraints that only a limited panel of illicit drugs could be analysed. A wide panel of illicit substances and prescription drugs would have been the ideal.

4.3.4 POST MORTEM INTERVAL

As alluded to, any delay in toxicological analysis using biological matrices is sub-optimal, as there is the possibility of sample degradation. Due to circumstances which include limited resources with regards to manpower and facility provisions, a routine medico-legal service

cannot be provided 24 hours a day including weekends and public holidays. This results in the potential delay of a minimum of 48 - 72 hours, before an autopsy can be conducted. Even with adequate storage and refrigeration of deceased individuals, continued post mortem effects such as redistribution and degradation of the potential drug or its metabolites, can result in erroneous interpretation of the test result. There is no available published data on post mortem intervals in SA. Although McLaughlin et al's study did not specifically mention the post mortem interval,[63] it is widely accepted that post mortem intervals to autopsies and specimen collections in developing countries, such as SA, are longer that those in developed countries.

4.3.5 SPECIAL PROCESSING FOR BLOOD SAMPLES

The term "near-body test" infers ease of sample processing and analysis. However, when using blood as a sample for either Randox or ND tests, further preparatory steps such as centrifugation and addition of buffer solutions are required in order to commence the analysis. Therefore, although the location of conducting these tests is near the body of the deceased individual, trained staff with an analytical background are required for this specialised processing and therefore these tests are not as convenient as may be inferred.

4.3.6 TEST INTERPRETATION DIFFICULTIES

The interpretation of some results utilising ND was difficult. Although designed for urine samples, it was deemed that blood could be a suitable alternative after discussions with the manufacturer. The viscosity of blood is higher than that of urine despite the addition of a buffer. In turn this lead to difficulty in the interpretation of a negative or positive result. The test line was in some cases barely visible, leading to analyser subjectivity. However, the manufacturer insert states the following, "A very, very faint line, one that requires you to look twice to see such a line, must be regarded as a positive result". On this basis any suggestion of a test line, was taken as a positive result, no matter how faint.

In addition, the use of blood as a sample matrix may pose difficulties should the sample be too viscous. Incorrect dilution of this sample may prevent analysis. This then in turn may require additional sampling in order to repeat the test at an appropriate viscosity. This may be impossible if the samples have already been taken, are batched and the body has been released from the facility.

4.4 IMPLICATIONS OF THIS STUDY

The results of this study imply that near-body testing could provide additional insight into the death of an individual, both to the forensic pathologist and law enforcer. The knowledge that an individual has tested positive for a particular compound, at the time of autopsy, may lead to greater insight into the pathophysiological and circumstantial aspects of the death. This may then in turn lead to further complementing ancillary procedures and ultimately a more comprehensive, deeper understanding of the demise of an individual.

Near-body testing in general may allow for screening in settings where financial constraints limit comprehensive toxicological analysis, with only positive samples being sent for confirmatory analysis. This will allow resources to be appropriately allocated to cases that require confirmation only. For example, a Randox DOA I array testing kit for 11 drugs allowing for the analysis of 43 specimens is an estimated R10 000. This equates to R232 per specimen. LC-MS/MS is far more expensive, costing between R600 and R3000 per compound, depending on the laboratory used and whether the analysis is qualitative or quanititative. Lastly Narcotics detector is a mere R100 per test, testing for 6 drug groups. Furthermore, a wide-ranging investigation including near-body testing may leave little room for the defence to question the validity and thoroughness of an examination. This may in turn positively impact the justice system.

4.5 FUTURE RESEARCH SUGGESTIONS

A more comprehensive study, utilising a large sample size with a complete compound panel including prescription drugs, could be invaluable. This may confirm the findings of this study, have a better sample size to correctly analyse the positive predictive value, provide evidence for the use of near-body screening tests for other compounds, as well as assist in more appropriate utilisation of financial resources. Further data could be gleaned, resulting in the much needed demographic assessment of drug related deaths in SA.

4.6 CONCLUSION

Randox DOA 1 array and ND have good negative predictive values for THC, opiates and amphetamines in a post mortem population in SA. Although both near-body tests had good positive predictive values, the limitation of a low positive test prevalence makes the interpretation of this result difficult. These tests are therefore suitable as alternative, cheap

screening tests, particularly in the South African climate where resource constraints limit rapid formal laboratory testing. It must be noted that there are limitations to this study, and further studies may need to be performed prior to unequivocal use in daily practice.

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APPENDIX 1: DATA COLLECTION TEMPLATE

Study sample number:	**Sample:** **Site:** **Randox □** **Drug Detective □** **Chromatography □**
Date of Autopsy	
Date of Death	
Age:	**Sex: M/F**
Estimated Post Mortem Interval (PMI):	**_____hours**
History of drug use: Y/N	**Type of drugs used:**
Cause of death:	**Past medical history:**
Randox result: Cannabis: + / - level: Opiates: + / - level: Methamphetamine: + / - level:	**Drug Detective Result:** Cannabis: + / - Opiates: + / - Methamphetamine: + / -_____
Chromatography result: Cannabis: + / - level: Opiates: + / - level: Methamphetamine: + / - level:	

APPENDIX 2: ETHICS APPROVAL

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M131191

NAME: (Principal Investigator)	Dr Candice Geraldine Hansmeyer
DEPARTMENT:	Forensic Medicine and Pathology Johanneburg Forensic Pathology Service
PROJECT TITLE:	Near-Body Drugs of Abuse Testing in a Post-Mortem Medico-Legal Population in South Africa: A Pilot Study
DATE CONSIDERED:	29/11/2013
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Ms Ildi Fenyvesi and Prof Jeanine Vellema
APPROVED BY:	Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 31/01/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature M131191Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX 3: POSTGRADUATE COMMITTEE APPROVAL

UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

Private Bag 3 Wits, 2050
Fax: 027117172119
Tel: 02711 7172076

Reference: Ms Thokozile Nhlapo
E-mail: thokozile.nhlapo@wits.ac.za

09 January 2015
Person No: 9901118E
PAG

Dr CG Hansmeyer
PO Box 85046
Emmaretia
2193
South Africa

Dear Dr Hansmeyer

Master of Medicine: Approval of Title

We have pleasure in advising that your proposal entitled *Near-body drugs of abuse testing in a post-mortem medico-legal population in South Africa: A pilot study* has been approved. Please note that any amendments to this title have to be endorsed by the Faculty's higher degrees committee and formally approved.

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

APPENDIX 4: APPROVAL OF THE HEAD OF DEPARTMENT

University of the Witwatersrand
Faculty of Health Sciences
Division of Forensic Medicine
Forensic Pathology Service: Southern Gauteng

Adjunct Professor Jeanine Vellema
Head Clinical Department (Chief Specialist) and Head of Division
Tel: 011 - 489 1659/00
Cell: 082 777 0737
Fax: 086 656 8413
E-mail: vellema@telkomsa.net

University of the Witwatersrand
Faculty of Health Sciences
HREC

7 November 2013

TO WHOM IT MAY CONCERN

RE: RESEARCH REQUEST BY DR CANDICE HANSMEYER

Permission is hereby granted for Dr. Candice Hansmeyer to conduct her research as proposed, at the Johannesburg Forensic Pathology Service (JHB FPS) Medico-legal Mortuary Facility. She is granted access to the JHB FPS autopsy suite to view autopsies as they are carried out as well as access to the scribe notes/ autopsy report for the purposes of her research as outlined in her proposal.
She also understands that the strictest patient confidentiality must be maintained and that all data must remain anonymous and unlinked.

Yours sincerely,

……

Professor Jeanine Vellema
Adjunct Professor and Head of Division of Forensic Medicine & Pathology
Wits Faculty of Health Sciences
Head Clinical Department (Chief Specialist): Forensic Pathology Service – Southern Gauteng