

**THE SIMULTANEOUS DETECTION OF NARCOTIC ANALGESICS AND NON-  
STEROIDAL ANTI-INFLAMMATORY DRUGS IN HUMAN URINE USING HIGH  
PERFORMANCE LIQUID CHROMATOGRAPHY - TANDEM MASS  
SPECTROMETRY**

BY

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### **Declaration of Independent Work**

I, André Coetzee, do hereby declare that this dissertation is submitted for the degree **Masters of Medical Science**, at the University of the Free State, is my own independent work that has not been submitted before to any university/faculty by me as part of any qualification. I do hereby give author's rights to The University of the Free State.

.....  
Signature of Student

.....  
Date

# Abbreviations

APCI	Atmospheric pressure chemical ionisation
API	Atmospheric pressure ionisation
CE	Collision energy
CNS	Central nervous system
DAD	Photo diode array detector
DP	Declustering potential
ELISA	Enzyme-linked immunosorbent assay
ESI	Electro spray ionisation
EXP	Exit potential
FIA	Flow injection analysis
FP	Focusing potential
GC-MS	Gas chromatography Mass spectrometry
HPLC	High pressure liquid chromatography
ISTD	Internal standard
LC	Liquid chromatography
LC-ES-MS/MS	Liquid chromatography electro spray mass spectrometry
LC-MS/MS	Liquid chromatography mass spectrometry
LOD	Limit of detection
MBTFA	<i>N</i> -Methyl-bis(trifluoroacetamide)
MP	Mobile phase
MRM	Multiple reaction monitoring

MS	Mass spectrometry
MSTFA	<i>N</i> -Methyl- <i>N</i> -(trimethylsilyl)trifluoroacetamide
NSAIDs	Non-steroidal anti-inflammatory drugs
OTCs	Over the counter
SIM	Single ion monitoring
SOP	Standard operation procedure
SPE	Solid phase extraction
TLC	Thin layer chromatography
TS	Thermo spray
WADA	World Anti-Doping Association

# Chapter 1

## Narcotic analgesics

Narcotic analgesics are a natural or synthetic compound that produces morphine-like effects. The term “opiates” is reserved for drugs such as morphine and codeine, obtained from the juice of the opium poppy. All of these drugs act by binding to specific opioid receptors in the central nervous system (CNS) to produce the effect that mimics the action of the endogenous peptide neurotransmitters, for example endorphins and enkephalins. Opioids have a broad range of effects, but their primary use is to relieve intense pain and the anxiety that accompanies it, whether it be from surgery or as result of injury or a disease, such as cancer. However, their widespread availability has led to abuse of those opioids with euphoric properties.

Opioids interact stereospecifically with protein receptors on the membranes of certain cells in the CNS, on nerve terminals in the periphery and on cells of the gastrointestinal tract. The major effects of the opioids are mediated by 4 families of receptors, namely  $\mu$ ,  $\kappa$ ,  $\sigma$  and  $\delta$ , each exhibiting a different specificity for the drugs it binds. All opioid receptors are coupled to inhibitory G proteins, and inhibit adenylyl cyclase. They may also be associated with ion channels to increase  $K^+$  efflux (hyperpolarization) or reduce  $Ca^{2+}$  influx, this impeding neuronal firing and transmitter release. The five general areas where the opioid receptors are present on the CNS are involved in the integrating information about pain. Opioid receptors in the brainstem mediate respiration, coughing, nausea and vomiting, maintenance of blood pressure and control of stomach secretions. The medial thalamus mediates deep pain that is poorly localized and emotionally influenced. Receptors in the spinal cord are involved with receipt and integration of incoming sensory information, leading to the reduction of painful afferent stimuli. Receptors in the hypothalamus have an effect on neuroendocrine secretion.

## **1.1 Mechanism of action**

There are many different mechanisms through which opioids have an effect on the receptors on the cell membranes to modulate the stimuli of pain. The action of the  $\kappa$  receptors of the spinal cord decreases the release of substance P, which modulates pain perception in the spinal cord, raising the pain threshold at the spinal cord and altering the perception of the brain's perception of pain may relieve pain. The awareness of the pain is still present, but the sensation is not unpleasant. Morphine, for example, causes euphoria by stimulating the ventral tegmentum. Some opioids have antitussive properties and relieve diarrhoea and dysentery by decreasing the motility of the smooth muscle and increasing tone, also in the anal sphincter. Such opioids increase the pressure in the biliary tract. This leads to constipation when not managed correctly.

## **1.2 Therapeutic uses**

Opioids are very effective for the treatment of pain. They may also induce sleep, where pain leads to sleep deprivation, and sleep is necessary to aid the sleep-inducing properties of benzodiazepines. Morphine decreases the motility of the smooth muscle and increases tone for the treatment of diarrhoea. Relieving coughing is caused by suppressing the cough reflex. Methadone is used in the controlled withdrawal of addicts from heroin and morphine. It causes a milder withdrawal syndrome, which develops more slowly than that of morphine.

## **1.3 Pharmacokinetics**

The absorption of, for example, morphine from the gastrointestinal tract is slow and erratic and the drug is not usually given orally. Significant first pass metabolism takes place in the liver, therefore intramuscular, subcutaneous or intravenous injection produces the most reliable responses. It enters the body tissues very rapidly, but only a small

percentage crosses the blood-brain barrier. Morphine is the least lipophilic opioid. Opioids are metabolized in the liver to glucuronides and the conjugates are excreted primarily in the urine, with very few in the bile. Other opioids, for example codeine, are well absorbed when given orally and absorbed from the gastrointestinal tract. Narcotic analgesics have mixed duration of actions. Some of them, for example methadone, have a long duration of action as they accumulate in tissues, where they remain bound to the protein from which they are slowly released. By contrast, Fentanyl has a rapid onset and short duration of action.

#### **1.4 Adverse effects**

Adverse effects include nausea and vomiting, anorexia, constipation, dysphoria and allergy-enhanced hypotensive effects. The elevation of intracranial pressure, particularly in head injuries, can be serious. Morphine, for example, enhances cerebral and spinal ischemia. Acute urinary retention may be present. Tremors, muscle twitches and convulsions may be caused by larger doses. Dilation of the pupil causes hyperactive reflexes. Severe hypotension can occur when the drug is administered postoperatively. Depression may be enhanced when used with neuroleptics. The combination of monoamine oxidase inhibitors and opioids is not advisable due to convulsions and hyperthermia. Cross-tolerance with other opioids occurs.

#### **1.5 Toxicity**

Severe respiratory depression, convulsions, hallucinations, confusion, cardiotoxicity, pulmonary edema occur with toxic doses. High doses cause the drug to increase blood pressure and can cause tachycardia and dizziness.

#### **1.6 Tolerance and physical dependence**

Repeated usage produces tolerance to the respiratory depressant, analgesic, euphoric and sedative effect. Physical and psychologic dependence readily occurs.

## 1.7 References

- Mycek M.J, Harvey R.A, Champe P.C., (2000). Chapter 14: Opioid Analgesics and Antagonists. Lippencott's Illustrated Reviews: *Pharmacology*. Second edition. 133-142.

## Chapter 2

# Non-Steroidal Anti-Inflammatory Drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are chemical agents that differ in their antipyretic, analgesic and anti-inflammatory activities. They act primarily by inhibiting the cyclo-oxygenase enzymes. The antipyretic and anti-inflammatory effects are due primarily to the blockage of prostaglandin synthesis at the thermoregulatory centres in the hypothalamus and at the peripheral target sites. By decreasing the prostaglandin synthesis, sensitization of pain of the pain receptors for both mechanical and chemical stimuli is decreased. NSAIDs have three major therapeutic actions, namely they reduce inflammation (anti-inflammation), pain (analgesia) and fever (antipyrexia). Not all of the NSAIDs are equally potent in each of these actions.

### 2.1 Mechanism of actions

The anti-inflammatory action inhibits the cyclo-oxygenase activity, it diminishes the formation of prostaglandins and thus modulates those aspects of inflammation in which prostaglandins act as mediators.

The analgesic action decreases the Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) synthesis at the sensitive nerve ending where bradykinin, histamine and other chemical mediators are released by the inflammation process. The decrease of PGE<sub>2</sub> represses the sensation of pain.

The antipyretic action is where fever occurs when the set point of the anterior hypothalamic thermoregulatory centre is elevated. This can be caused by the synthesis of PGE<sub>2</sub>, stimulated when an endogenous fever-producing agent such as cytokine is released from the white cells that are activated by the infection; hypersensitivity; malignancy, or inflammation. The NSAIDs, especially the salicylates, lower the body

temperature in patients with fever by decreasing the PGE<sub>2</sub> synthesis and release. This action increases heat dissipation as a result of peripheral vasodilatation and sweating.

## 2.2 Other effects

Other effects include respiratory actions through the increase of alveolar ventilation by acting on the respiratory centre in the medulla, resulting in hyperventilation and respiratory alkalosis during toxic levels. This may cause respiratory paralysis and respiratory acidosis caused by continued production of CO<sub>2</sub>.

The gastro-intestinal system inhibits prostacyclin (PGI<sub>2</sub>) and the gastric acid secretion, whereas PGE<sub>2</sub> and PGF<sub>2α</sub> stimulate the synthesis of protective mucus in both the stomach and small intestine. In the presence of aspirin, these prostaglandins are not formed, resulting in the increase of gastric acid secretion and decreased mucus production. This may cause gastric distress, ulceration and haemorrhage.

Thromboxane A<sub>2</sub> (TXA<sub>2</sub>) enhances platelet aggregation, whereas PGI<sub>2</sub> decreases it. Low doses of aspirin irreversibly inhibit thromboxane production in the platelets without markedly affecting TXA<sub>2</sub> production in the endothelial cells of the blood vessel. Because platelets lack nuclei, they cannot synthesize new enzymes, and the lack of thromboxane persists for the lifetime of the platelet. As a result of the decrease in TXA<sub>2</sub>, platelet aggregation is reduced, producing an anticoagulant effect with a prolonged bleeding time.

Cyclo-oxygenase inhibitors prevent the synthesis of PGE<sub>2</sub> and PGE<sub>2</sub>-prostaglandins that are responsible for maintaining renal blood flow, particularly in the presence of circulating vasoconstrictors. This can result in retention of sodium and water and may cause oedema and hyperkalemia in some patients.

Some NSAIDs are beneficial for the treatment of postoperative pain, for example, ophthalmic procedures, uveitis, and indomethacin can delay labour by suppressing uterine contractions, and the treatment of ductus arteriosus.

## **2.3 Therapeutic uses**

NSAIDs are used as antipyretics and analgesics in the treatment of spondylitis, rheumatic fever and rheumatoid arthritis, osteoarthritis and ankylosing spondylitis. Common treatment conditions are headache, arthralgia and myalgia. External applications are used to treat corns, calluses and epidermophytosis. Cardiovascular applications are used to inhibit platelet aggregation. Aspirin is used prophylactically to decrease the incidence of transient ischemic attack, unstable angina and coronary artery thrombosis.

## **2.4 Pharmacokinetics**

The substances are absorbed through intact skin. After oral administration, passive absorption in the stomach and small intestine take place. The dissolution of the tablets is aided by the higher pH of the gut. Some of them may cross the blood-brain barrier and the placenta. Rectal absorption is irregular and not accurate. This is the option for children with nausea and vomiting.

## **2.5 Dosage**

The salicylates exhibit analgesic activity at low doses and at higher doses do these drugs show anti-inflammatory activity. Low dosages of salicylates it are converted by the liver to water soluble conjugates that are rapidly cleared by the kidney, and eliminated with first-order kinetics and a serum half-life of 3.5 hours. At higher anti-inflammatory dosages, the hepatic metabolic pathway becomes saturated and zero-order kinetics is observed, with a drug half-life of 15 hours or more.

## **2.6 Adverse effects**

The most common gastro-intestinal effects are epigastric distress, gastro-intestinal bleeding, nausea and vomiting, diarrhoea, abdominal pain or anorexia. The inhibition of platelet aggregation may lead to prolonged bleeding during injure or surgery. Toxic

dosages may lead to respiratory depression or respiratory and metabolic acidosis. Hypersensitivity reactions are a big problem, especially with salicylates. In very rare instances hepatitis and jaundice may be present; however, very rarely, neutropenia, thrombocytopenia and aplastic anaemia may also be present. Cross reactivity with some NSAIDs and other drugs may occur, for example, indomethacin with furosemide,  $\beta$ -blocking drugs and ACE inhibitors.

## **2.7 Toxicity**

Intoxication may be mild or severe. The mild Intoxication is characterized by nausea, vomiting, hyperventilation, headache, mental confusion, dizziness and tinnitus. Higher dosages may cause restlessness, delirium, hallucinations, convulsions, coma, respiratory and metabolic acidosis and death from respiratory failure.

## 2.8 References

- Mycek M.J, Harvey R.A, Champe P.C., (2000). Chapter 14: Opioid Analgesics and Antagonists. Lippencott's Illistrated Reviews: *Pharmacology*. Second edition. 133-142.

# Chapter 3

## Literature and Method Survey

### 3.1 Literature survey

There is an increasing need for fast and reliable analytical identification and quantification of unknown compounds in numerous types of sample matrixes. The reasons for this are numerous. The use of over-the-counter products (OTCs) has increased over the years. This is evident from the wide range of products available and also the easy availability of these OTCs in pharmacies, health shops and even supermarkets in South Africa and elsewhere. Therefore the procurement of these products is easy and they are used by many consumers for self-treatment of numerous conditions. This has given rise to the problem of irresponsible use of these products by consumers. This may be due to a lack of appropriate medical knowledge or awareness of how to use these products; incorrect and unsafe storage of medication at home, and the low cost of these medicines. Many of these OTCs are sometimes used in combination with various drugs. This situation may lead to adverse reactions or other medical complications, for example, gastric bleeding and gastric ulcers. Abuse of these OTCs is also on the increase. This is due to the uncontrolled selling of OTCs to persons who use the medicines for purposes that they were not originally intended for. This may cause accidental overdose, allergic reactions, cardiovascular risks, deaths and other complications, to name only a few.

Some of the groups of OTCs sold are non-steroidal anti-inflammatory drugs (NSAIDs) and narcotic analgesics. Fosbøl *et al.*, (2008) reported on a study in which they investigated and determined NSAID use patterns in the Danish population. The results showed that NSAIDs were very commonly used by the Danish population. The authors

underlined the need for risk assessment associated with the increased use of NSAIDs because the drugs are easily acquired as OTCs in many countries. The authors also stated that previous studies suggested an increased risk associated with dosage and that the increased use of NSAIDs is unfavourable. Some of the risks included cardiovascular and gastro-intestinal risks, like ulcers. The authors suggested the re-evaluation of the evidence that NSAIDs are not a harmless class of drugs. More than 175 million adults in the United States consume OTCs daily. Of these 14% take OTC medicines several times a week and another 15% take OTCs for pain daily (Ajuoga *et al.*, 2008). NSAIDs make up 5.6% of the South African market breakdown and narcotic analgesics 18.3% (Labat/CMCS, 2000). A study by Truter, (1997) reported that analgesics were the most frequently prescribed group, representing 12.3% of the total amount of medication prescribed, and this amounted to 14.2% of the total budget spent in 1995, according to a medical aid fund in South Africa. More than 56.8% of all the medication was available without prescription from a medical practitioner. This may be due to the fact that pain is the most common pharmacological challenge encountered by the medical practitioner and therefore treatment for pain is frequently prescribed. The intake of this medication is safe, but these agents, when used chronically and in higher than recommended dosages, is unsafe. In 2000 The Commission on Narcotic Drugs acknowledged the need for an effective response to drug problems. The treatment with OTC and prescription medicine abuse was very high in South Africa. Benzodiazepines and analgesics are the most common classes of medicine that are abused in South Africa (Parry *et al.*, 2002). Between 5% and 8% of substance abusers in South Africa need treatment for addictions to OTC or prescription drugs. These medications include codeine- and dextropropoxyphene-containing analgesics, cough mixtures and paracetamol. The treatment is complicated due to the fact that drug screens test positive for opiates, without identifying the substance used, therefore special testing is needed for synthetic and unknown opioids (Weich *et al.*, 2008).

### **3.2 Pain**

Pain is a complex occurrence that is experienced by all individuals. Pain is primarily a protective mechanism meant to bring the conscious awareness of the fact that tissue

damage is occurring or is about to occur. It is accompanied by motivated behavioural responses such as withdrawal or defence as well as emotional reactions such as crying or fear. Pain is divided into two groups – acute pain and chronic pain. Acute pain normally follows stress factors, an acute injury, disease or some types of surgery. Acute pain usually has a sudden onset, a “sharp” sensation at a defined area, and a quick response to treatment. Examples of acute pain includes post operative pain, post burn pain, pain associated with internal diseases. Acute pain left untreated or managed ineffectively may lead to chronic pain. Chronic pain is pain that persists beyond the expected time of healing. Chronic pain may be continuous or recurs at intervals of months or years (Sherwood, 1997).

### **3.3 Pharmacological treatment options for pain**

Pharmacological treatment options for pain management include opioid analgesics, non-opioid analgesics, and combination analgesic products. Opioid analgesics are the primary class of agents used in the management of acute to chronic pain. Analgesics are drugs that relieve moderate to severe pain. The dosage of opioids should be adjusted according to the severity of the pain and the response or tolerance of the patient. Another group of drugs are the non narcotic analgesics – Non steroidal anti-inflammatory drugs (NSAIDs). They are called non steroidal because they do not belong to the steroid group of drugs and do not possess the adverse reactions associated with the steroids, and yet they have anti-inflammatory, analgesic and antipyretic properties. Opioid analgesics are classified as low-efficacy, high-efficacy and also intermediate-efficacy opioids. Low-efficacy opioids, when used alone, may seldom be adequate in the treatment of severe pain. Their analgesic efficacy is usually enhanced when combined with other opioids or NSAIDs. High efficacy opioids may be relied on to relieve severe and chronic pain. Intermediate-efficacy agents fall between the high and low-efficacy groups. They may be useful in many pain situations and should be considered before using stronger agents. The NSAID group contains a large number of drugs divided into different groups, with new drugs continually becoming available (Sherwood, 1997).

### 3.4 Analytical Techniques

During drug development the use of analytical procedures is very important for the determination of drugs from bio-samples obtained from pharmacokinetic, toxicological, clinical, forensic toxicology and doping control. In such samples the analytes are unknown and therefore analytical methods are important to identify and quantify the compound of interest. Efficient analysis and high throughput procedures mean that numerous analytes need to be screened simultaneously using one single procedure (Maurer, 2000).

Thin layer chromatography (TLC) is a simple technique used for the detection and identification of unknown compounds. TLC is not favourable during mass screening procedures (Liu *et al.*, 2001).

Immunoassays (IA) may be used to differentiate between negative and positive samples in drugs of abuse tests in urine. The procedures are not so sensitive for low concentrations. Enzyme-linked immunosorbent assays (ELISA) and enzyme multiplied immunoassay techniques (EMIT) may be used, however the detection limit is not low enough. Not all drugs can be detected by IA, including the opioids. The detection of opioids in whole blood, plasma or serum by non chromatographic methods is rare, because of the need of sensitivity and selectivity which it does not provide. IA is not specific in identification of a compound in a sample, and this is also the case for opioids.

In recent years high pressure liquid chromatography (HPLC) coupled to a diode-array detector (DAD) has been used. The DAD detector is problematic for polar compounds, but these techniques still provide good levels of specificity and sensitivity for screening and confirmations.

HPLC techniques is useful in separating complex sample medium into separate compounds that are easily identified. The performance of the HPLC systems was until recently limited to single wavelength UV, fluorimetric and electrochemical detectors and the diode detector. The possibility of false positives due to insufficient purification and variable specificity and selectivity is problematic in the identification of compounds. However, HPLC techniques are sensitive, selective, reproducible and well understood

with easy operation. Therefore, HPLC is the better choice for the purpose of time saving and better performance (Liu *et al.*, 2001).

The demanding applications of analytical chemistry in toxicology led to the increased need for detection, identifying and quantifying of any xenobiotic, responsible for the intoxicification, at very low concentrations. The requirements of sensitivity and specificity were met in the past by various immunochemical, radio immunological and physiochemical techniques. These techniques are limited to a small number of therapeutic drugs or drugs of abuse. For years GC-MS was the preferred choice and it has performed with good selectivity and sensitivity, but it is not applicable to polar, thermolabile or high mass molecules. The preparation for GC-MS is often time-consuming and most often requires extraction with derivatization procedures.

In GC-MS techniques, sensitivity may be lost due to chemical oxidation or derivatization and are limited to volatile, non-polar and thermally stable compounds. Identification of unknowns is based on comparing their mass spectra with special mass spectral libraries and fragmentation patterns from electron ionization. However, single MS does not always provide sufficient information for confirmation of unknowns. The oxidation and derivatization process may complicate the process of finding a corresponding match in the literature and mass spectrum database (library). When the identification is performed by non-specific extraction from the sample and detected by GC-MS, the results for some polar, especially acidic, compounds are poor where derivatization is performed.

The LC-MS system development started in the early 1970s and developed through thermospray (TS), electrospray (ES), ionspray (IS) to the late 1990s with atmospheric pressure chemical ionization (APCI), where it was first used in toxicology. In clinical toxicology and forensics the rapid identification and quantification of compounds are important. This is due to the abuse of both medications sold over the counter and prescription drugs. This abuse, for example, leads to overdose, severe side effects, toxicity and possible death. Deaths under suspicious conditions may have legal

implications. The method of intoxication, the compound that led to the intoxication and the dosage provide important evidence (Concheiro and de Castro, 2006). The need to solve toxicological and forensic problems more effectively relies on fast and effective methods to identify the compound responsible for the situation. The determination of human pharmaceuticals in the environment, for example, waste water irrigation systems/treatment plants or surface water, is very important. Siemens *et al.*, (2008) suggested that the assessment of the concentrations and retention of pharmaceutically active compounds is crucial for assessing the environmental risk of medication of humans. The proposed assessment included the use of solid phase extraction (SPE) and liquid chromatography tandem mass spectrometry (LC-MS/MS) detection. This was supported by Lacey *et al.*, (2008). Pharmaceuticals are continuously introduced into the environment by industrial and domestic use. This leads to possible environmental contamination and pollution by pharmaceuticals. Their toxicity to the environment is relatively unknown and needs investigation. Therefore an effective analytical procedure has been developed using solid phase extraction and LC-MS/MS detection to assist in this problem.

Marquet and Lachâtre, (1999) reported that the limit of detection (LOD) was lower with LC-ES-MS-MS than the LOD reached by GC-MS, also for intra- and inter-assay precision and accuracy. This establishes the need for LC-MS systems in forensic toxicology to confirm compounds with spectral information that is not suited for GC-MS or HPLC techniques. They also reported on a study where ES and APCI in the positive single ion monitoring (SIM) mode, were compared. The APCI in that study was significantly more sensitive. The advantages are in the simple sample preparation and a short chromatographic run with high sensitivity. The authors reported on other studies performed with LC-MS for the determination of heroin and its metabolites, including the glucuronides. This also applies to morphine and codeine. The identification of unknown compounds is difficult, especially when the sample volume for sample preparation is limited. Alternatively, the HPLC-DAD is used, but is limited to compounds in which UV spectra are influenced by sample pH where little or no UV absorbance has taken place. HPLC techniques are complementary to GC-MS techniques where unknowns are present.

HPLC coupled to a mass spectrometer (LC-MS) is very important in analysing complex sample matrices where compounds may be present in very low concentrations. Very small molecular size compounds and very large molecular size compounds are easily detected by LC-MS. The introduction of atmospheric pressure ionization (API), electrospray ionization (ESI) and other approaches improved the direct detection and identification of compounds with fast method development. These systems have the ability to operate at high pressure with very thin columns with high flow rates. Improved sensitivity and selectivity makes LC-MS the better choice for identification and confirmation of multiple compounds in one single sample and shortens the multi-residue analysis runtime. This improves sensitivity and selectivity (Gentili, 2007). Unfortunately the identification of metabolites, impurities and degrading products is difficult and time consuming. The main objective of metabolomics is the analysis of large numbers of cellular metabolites for comparison with metabolite levels in organisms under given conditions. LC-MS/MS is the most commonly used, due to its sensitive ability in detecting polar compounds and its ability to provide structural information. This information is needed to identify the differences in metabolite content of biological samples. These metabolites belong to diverse chemical classes in diverse pharmaceutical and environmental matrices, therefore the LC-MS is the better choice because of better selectivity, sensitivity, versatility and the ability to identify unknown analytes (Bajad and Shulaev, 2007).

The application of LC-MS is also useful in the characterizing of food proteins and derived peptides. This is evident in the study reported by Léonil *et al.*, (2000) on milk, egg, meat and cereal proteins. This is possible because of the improved sensitivity of the LC-MS systems to large biomolecules with high mass ranges and the capacity to analyse complex mixtures to give structural information by possible chemical induced modifications. It is stated that LC-MS has advantages due to the fact that it can provide structural information in complex medium without the purification of the proteins and peptides as done in this study. This was also confirmed by Marquet *et al.*, (1999) in respect of the use of LC-ES-MS for the detection of high molecular mass compounds for the screening and quantification of peptides and proteins with therapeutic and toxic properties. The data from proteolytic digestion of protein and peptide are used to create

protein and peptide search databases. These databases are routinely used in the fields of biochemistry and molecular biology. There are papers available regarding the use of LC-MS for the detection and quantification of various myotoxins and marine toxins. LC-MS/MS techniques are important in clinical pharmacology and drug monitoring. The sphere of pharmacogenomics is also investigated for possible application in drug monitoring in hospitals. The LC-MS/MS based phenotyping, for example polymorphism, in patients on certain drugs, will become important. In hospitals in which patients with organ failure or chronically ill patients are admitted, the pharmacokinetic properties (absorption, distribution, metabolism, excretion) may differ from normal conditions. The toxic drug accumulation or low blood concentrations of the drug may influence the treatment of the patient. The LC-MS/MS is important in the routine monitoring of all the essential drugs used in hospitals. Endogenous small molecular compounds have been recognized as potentially diagnostically useful analytes. This also applies to small molecule markers and the growing number of methods for the quantification of peptides and therapeutic oligonucleotides (Vogeser and Seger, 2008).

The presence of peptide hormones in sport doping (adrenocorticotropin, human growth factor, chorionic gonadotropin, erythropoietin and their releasing factors) can be confirmed by mass spectrometry, after ionization in an electrospray source. An LC-MS separation can be applied for the detection of IGF-1 as a pure solution with good sensitivity for the detection of doping (Marquet *et al.*, 1999).

The focus in recent studies has been on determining as many classes of compounds in a single sample with a single method, without compromising the quality of the results obtained. The identification and quantification results must be accurate and cost effective. Improved accuracy is achieved by combining high sensitivity with high specificity from gas chromatography, liquid chromatography and mass spectrometry. This is important for identification and quantification of compounds in very low concentrations in samples with good sensitivity and selectivity (Huestis and Smith, (2006) and Manini *et al.*, (2006). These authors stated that LC-MS success is due to the fact that it kept the advantages of liquid chromatography from aqueous matrices with high efficiency and selectivity in separation and the characteristics of mass

spectrometry. The disadvantages of LC-MS are the high cost and shortage of experienced operation of the systems. However, the phase I and II metabolites, as well as analytical artifacts, can be characterized using APCI and ESI ionization. This shows that the application of the LC-MS/MS for this characterizing is very useful (Manini *et al.*, 2006).

The advantages and disadvantages of the LC-MS/MS were also outlined in the article from Vogeser and Seger, (2008). In this article it was stated that extensive training is needed for operation of the LC-MS/MS systems to limit the high maintenance cost and prevent intervention from the service engineer. This can be prevented by regular maintenance visits for re-optimization and tuning of the instruments. This leads to downtime of the instruments which influences the response time. Another negative aspect is the downtime during column or mobile phase switching for different assays in a routine analysis laboratory. The positive advantage is the improvement of the quality of the analytical results obtained through better instrument improvements, for example, the improvement of the stationary phase for improvement of peak formation. This lowers the limit of detection for compounds to much lower levels that can be obtained from GC-MS. Other improvements include the optimization of the ion source, collision cell and mass selectors through selective ion transfer into a very pure vacuum system for increase in signal-to-noise ratios. The result is better regarding sensitivity and selectivity. Another advantage in electrospray techniques is its compatibility with chromatographic gradients and capillary electrophoresis. However, the drawback is the noise in the low masses and low fragmentation. The electrospray is suitable for polar compounds, including metabolites of therapeutic or abused drugs.

It is evident that the strength of the LC-MS/MS technology lies in its specificity and its applicability to high polar compounds, easy sample preparation without derivatization and shorter runtimes for faster sample throughput. The LC-MS/MS is a flexible technique for method development within a short time with a larger number of quantitative or qualitative results in a single analytical run.

### **3.5 The anti-doping tests: an outline of the present situation**

Anti-doping regulation is based on a list maintained by the World Anti-Doping Authority (WADA). The prohibited list includes all the prohibited classes of substances and methods that need to be tested for doping control laboratories in sport. One of the prohibited classes of substances is narcotic analgesics. Not all narcotic analgesics are prohibited. There are many narcotic analgesics commercially available, but not on the prohibited list of substances which are used by athletes. Another group of substances not on the prohibited list is the NSAIDs. Both the narcotic analgesics and the NSAIDs are under investigation with a lot of research ongoing on these classes of substances.

The use of performance-enhancing substances and methods in sport is prohibited. Anti-doping control in sport is based on the list provided by WADA, which is continually updated with new possible doping substances or methods. The narcotic analgesics is on the prohibited list provide by WADA. Opioids are very effective for the treatment of pain. These compounds decrease the motility of smooth muscle and change the perception of pain, by raising the pain threshold. The pain experienced during excessive exercise and during a sport event is reduced by using such medication. Athletes abusing these medications have an advantage over athletes not using such medication because these athletes can exercise and continuing competing with the advantage of the reduced sensation of pain. The pain may be the result of an injury and this may be aggravated by continuing competing, putting his health at risk and prolonging the healing time period for this injury. There are other health risks involved from using this medication. Some of the risks included respiratory depression, convulsions, confusion and increased blood pressure. Severe risks included tolerance, physical and psychological dependence.

The advantage from the use of NSAIDs is the reduction of inflammation in the injured muscles. This inflammation may be from excessive training or muscle injury. The usage of this medication reduces the pain associated with inflammation and this gave the athlete an advantage over non-using athletes during a sporting event or training. Other health risks included gastric-intestinal effects like peptic ulcers, gastro-intestinal bleeding and prolonged bleeding during injury or surgery.

The aim of effective anti-doping control is the development of reliable analytical methods to reduce the number of substances not detected by current testing methods. Testing laboratories is continuously improving current analytical testing methods and procedures to increase the number of detectable analytes and identify new potential doping agents, to reduce the possibility of cheating in sport. These improvements are obtained through evaluation of currently available analytical methods to exploit the advantage of different analytical techniques. The LC-MS/MS and GC-MS play an important role in this evaluation process to determine which technique is most suitable for doping control purposes (Botrè, 2003).

### **3.6 Method Survey for NSAIDs and Narcotic Analgesics**

The detection of opioids in whole blood, plasma or serum by non-chromatographic methods is rare, because of the need of sensitivity and selectivity which it does not provide. Positive samples must be confirmed by a second independent method that is very sensitive and provide reliable results. GC-MS is widely used for confirmation of positive screening results, as it provides high specificity and selectivity. GC-MS is still the choice over HPLC with UV, DAD or FLD detectors. However, recent development of the HPLC into the improved LC-MS/MS systems improved the screening capabilities.

A GC-MS method for the analysis of some common used NSAIDs in urine was published by El Haj *et al.*, (1999). Various GC-MS methods in splitless mode were used to determine the compounds. The sample preparation was performed with methanol and ethyl acetate and TMS derivatization were used. Three extraction methods used a total of 25 ml of urine and two injections were done into the GC-MS. In this study it was evident that some NSAIDs behave differently to the temperature conditions in the GC-MS methods. In GC-MS analysis the urine must first be hydrolyzed due to the presence

of their glucuronide conjugates in the excreted urine. This is a long extraction method and a large volume of urine is needed with different GC-MS detection methods needed to analyze the urine for the detection of NSAIDs in urine. This makes the method not suitable for fast routine screening of NSAIDs in urine.

A study was undertaken by Kim and Yoon, (1996) to investigate the optimal conditions for GC-MS analysis and quantitative screening for trace amounts of acidic NSAIDs using selected-ion monitoring mode (SIM). In this study 18 acidic NSAIDs were investigated in urine. GC-MS analysis with SIM mode was used where one base peak was selected for detection of the NSAIDs in a 20 minute run time. SIM mode was used to limit the interference of the organic acids present in the urine. Complete resolution, efficient and selective detection of each drug was possible in trace amounts in presence of urinary organic acids. However the extraction procedure is very long and complex derivatization solutions were used and the runtime is very long for GC-MS analysis. The detection of NSAIDs requires derivatization prior to chromatographic separation. A second method was developed for the determination of NSAIDs in equine plasma and urine by GC-MS. The screening analysis was performed in SIM mode monitoring 3 characteristic ions for each compound. Sample extraction was performed on 2 ml of urine, but blood may also be used. The sample was acidified and extracted with diethyl ether. The acidic nature of NSAIDs allows the extraction from biological matrix by liquid-liquid procedures under acidic conditions.

During the 24<sup>th</sup> Olympic Games in 1988 in Korea, phenolalkylamines, narcotic analgesics and beta-blockers were determined by GC-MSD (Lho and Hong *et al.*, 1990). Sample preparation was done on 5 ml of urine with 2 separate pH adjustments and 2 separate derivatization steps included during the sample preparation. This method was long and complex. This may be justified by the long list of different groups of compounds that was tested for.

Systematic analysis of stimulants and narcotic analgesics is also done by gas chromatography with nitrogen specific detection and mass spectrometry. The detection of nitrogen containing compounds is specifically performed by the use of gas chromatographs equipped with a nitrogen phosphorus detector (NPD). This type of detection is sensitive for nitrogen containing compounds. Urine was used and a complex derivatization mixture was added for selective derivatization. In this method the compounds were detected in their free form in high concentrations and their metabolites at very low, sometimes undetectable concentrations (Lho and Shin *et al.*, 1990).

More recently a single method was published by Van Thuyne *et al.*, (2008) where 150 compounds on the WADA list were analyzed by GC-MS. The groups of compounds on this list included narcotics, stimulants, anabolic androgenic steroids, some anti-estrogenic and beta-agonist compounds. The aim of this method was to reduce the amount of analytical preparation needed for the urine samples, and saving preparation time and life span of the apparatus. This method led to the reduction of the amount of urine needed for sample preparation without the loss in sensitivity and selectivity. However, the sample was hydrolyzed overnight at 42 °C. This was a very long hydrolysis time with 2 pH adjustments to pH 9.5, and extracted with ethyl ether and pH 14 and extracted with *tert*-butyl methyl ether. The final residue was derivatized with a complex derivatization solution. The injection volume was very small in splitless mode and SIM scan mass spectrometry parameters were used. It provided adequate sensitivity. The derivatization mixture resulted in bad chromatography and decreased sensitivity for narcotic agents like morphine. Different organic solvents were tested. For narcotic agents and anabolic steroids ethyl acetate provided the best results but very high interference of the urea and glycerol in the start of the chromatogram for the volatile stimulants occurred. The combination of dichloromethane and methanol used for conjugated narcotics and stimulants gave bad extraction recoveries for the anabolic

steroids. The best solvent was diethyl ether although the use of this solvent resulted in the loss of benzoylecgonine, the metabolite of cocaine.

In the study of Van Thuyne *et al.*, (2007), the method allows the detection of more than 90 different components, including all narcotics from the World Anti-Doping Agency (WADA) doping list and also numerous stimulants on the list. This preparation method was very long with overnight hydrolysis followed by further sample preparation which included rolling for 1 hour and a derivatization step with *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA). Single step derivatization is necessary to save time and prolong column lifetime. This may not be the case when *N*-Methyl-

bis(trifluoroacetamide) (MBTFA) or MSTFA are used, due to the decomposition of the stationary phase leading to bad chromatography and decreased sensitivity.

In the study of Solans *et al.*, (1995) the simultaneous isolation of stimulants, narcotics,  $\beta$ -agonists,  $\beta$ -blockers and many of their metabolites using solid phase extraction (SPE) were investigated. The sample preparation started with a 2 hour hydrolysis phase followed with SPE. This was followed with a 30 minute waiting period in the desiccator. Two different derivatization solutions were used and the derivatization can result in multiple derivatives. In this study there was a lack of information for the metabolites of the compounds, but it didn't mean that it is not present in the samples or that there was detection problems with the analytical method.

An article by Hirai *et al.*, (1997) compared methods from various authors using HPLC methods for only a single compound or a few compounds. Although methods were published applying gradients elution, the compounds were overlapping. Some methods used different chromatographic conditions in the composition of the mobile phase and detection wavelengths for each compound. These methods lack sensitivity. The authors established a sample preparation method using SPE. The residue was resuspended in mobile phase and 10-30  $\mu$ l was injected onto the HPLC. The separation decreased as

the pH of the mobile phase increased above pH 5. The HPLC was connected to a variable-wavelength detector. In this article 3 different hydrolysis procedures were compared. This included enzymatic hydrolysis, acidic hydrolysis and alkaline hydrolysis. The alkaline conditions were applicable for the common hydrolysis of the conjugates, although the acidic hydrolysis yield the most compounds. This was the same result as described by Solans *et al.*, (1995), stated earlier. It was stated again that the main urinary excretion of the parent compound is through phase II metabolism by conjugation to their glucuronides or sulphates.

The comparison of UV and tandem mass spectrometric detection for the determination of diclofenac were reported by Mayer *et al.*, (2003) after topical application. This was done to determine the better of the two methods on small-volume microdialysis (MD) samples with low analyte concentrations. Both the methods, HPLC-UV and LC-MS/MS, were compared in regard to sensitivity, selectivity, accuracy, precision and their suitability for the analysis of biological samples. A complex mobile phase was used and the run time lasted 23 minutes with 2.5 minutes equilibration time between each run. The LC-MS/MS analysis were performed on a API 3000 triple quadrupole mass spectrometer operated in positive mode and selected reaction monitoring were performed with 5 minutes runtime and 1 minute equilibration time between runs. Sample extraction were performed on 25 µl MD samples and 25 mM formic acid and 20 µl directly injected into the LC-MS. A comparison of the results from the two methods clearly showed that the HPLC-UV provided both false and negative values for diclofenac. The reason for this may be components in the sample matrix disturbed the assay and generate too high concentration levels, although no reasonable explanation for to low values were found by UV detection. The method failed when applied to biological samples from healthy volunteers. The LC-MS method should be preferred due to its excellent and superior selectivity of the selected mass transitions of the parent ions to the product ions and reduced sample preparation and run times, allowing higher sample through-puts.

A study was done by Panusa *et al.*, (2007) for the analysis of anti-inflammatory pharmaceuticals with UV and electrospray-mass spectrometry in counterfeit homeopathic medicinal products. The anti-inflammatory compounds in this study included naproxen, ketoprofen, ibuprofen, piroxicam, diclofenac, nimesulide and paracetamol. These compounds were chosen, because they are the most utilized in allopathy. In this study a HPLC method with UV and ESI-MS detection were developed. UV detection is the first method of detection used. For substances at lower concentrations and for mass confirmation of compounds, the use of ESI-MS is more suitable. The mobile phase used in the chromatographic separation consisted of a binary mixture of solvents with a gradient with a long total runtime. The ions monitored were in selected-ion-monitoring mode (SIM). Both positive and negative ionization mode were used to obtain the best fragments for each compound.

Pirnay *et al.*, (2006) published a paper about the LC-ESI-MS analysis of buprenorphine, norbuprenorphine, nordiazepam and oxazepam in rat plasma. The reason for this was that the GC-MS methods were not sufficient enough for the detection in very low concentrations and for the detection of metabolites. The problem was overcome by LC-MS which provide good separation and high sensitivity and selectivity. In this paper liquid-liquid extraction was performed on plasma. The HPLC conditions include the use of a C18 column with a mobile phase consisted of 2 mM aqueous ammonium formate at pH 3 with formic acid (solvent A) and acetonitrile (solvent B). A gradient in the mobile phase was used. The total run time was 35 minutes. The MS conditions were in electron spray ionization mode (ESI) and single ion storage (SIS) mode and the most abundant ions ( $m/z$ ) were used for identification. Huynh *et al.*, (2005) reported that HPLC and immunoassays did not provide high enough sensitivity required for very low dosages of fentanyl. In this study the sample volume was small and a triple quadrupole mass spectrometer electrospray technique in positive mode was reported with a single step liquid-liquid extraction (LLE) sample preparation. The chromatographic conditions for separation included a gradient.

In the study by Thieme *et al.*, (2003) improved screening capabilities were investigated by implementing LC-MS/MS. A mass spectrometer with atmospheric pressure ionization (API) was applied for analysis. A C<sub>18</sub> column connected to a guard column was applied for chromatographic separation. The mobile phase consisted of ammonium acetate buffer and acetonitrile with a gradient. Default settings of the MS were used in the method. This method was developed for detection of drug of abuse which included narcotic analgesics, antidepressants and benzodiazepines.

A list of 29 multi-class pharmaceuticals, including analgesics and NSAIDs, using solid phase extraction followed by LC-MS/MS analysis was developed (Gros *et al.*, 2006). This method was optimized using ground water. The samples were collected and filtered. Different SPE materials were compared to optimize the extraction method. Samples were spiked prior to extraction with standard solutions of the analytes. Recoveries of the analytes were investigated after pH adjustment, to determine if any pH adjustment is necessary prior to extraction. Therefore, samples which pH was adjusted to pH 2 were compared with samples with no pH adjustment at neutral pH. LC-MS analysis was performed using a triple quadrupole mass spectrometer with a RP-18 end capped column and a C<sub>18</sub> guard column. The analysis was in negative ionization mode with a gradient, with re-equilibration for 15 minutes before another injection. For the analysis in positive ionization mode the composition of the mobile phase was changed and the column was re-equilibrated before. The data was collected in both negative and positive ionization mode in multiple reaction mode (MRM) to increase the sensitivity and selectivity. For both these methods the total runtime was long and the composition of the mobile phase was changed between the 2 ionization modes. Only recoveries of the most abundant analytes were investigated to determine the optimal extraction procedure. The results showed that basic and neutral analytes yield higher recoveries without the acidifying the sample. However, for acidic compounds the results were the same. Using the sample without the acidifying step was determined as the best option. The optimal conditions for the most optimal separation of samples were observed in positive ionization.

There are many commercial methods available for the detection of various compounds, proving that there is no universal or common method available for the detection of such a long list of different groups of compounds in any industry. The confirmation of a positive sample need to be performed, therefore, better sensitivity, selectivity, robustness and linearity is needed. LC-MS/MS analysis can be implemented to provide good, rapid and sensitive analysis with single mobile phase compositions. The advantage is the short runtime and multiple analyses in a single run of various substances and their metabolites. Another advantage is the exclusion of the derivatization step of the analytes prior to instrument analysis and the associated problems of reproducibility.

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## **Chapter 4**

### **Aim of the study**

From the cited literature it is clear that improvement of analytical methods was obtained with the introduction of the GC-MS. This is clear from the vast number of published GC-MS methods. This is a cost effective way of analyzing samples with reliable results. The problem with GC-MS is that some compounds are thermally labile while polar substances like NSAIDs needs derivatization. Recent studies involving LC-MS/MS are on the increase. This technique has the advantage that no derivatization is needed and results are therefore obtained faster.

There are no comprehensive methods for the simultaneous detection of narcotic analgesics and NSAIDs in urine and therefore the aim of this study was to develop a method to identify the presence of narcotic analgesics and NSAIDS in human urine using LC-MS/MS.

In order to reach this goal several specific objectives were set.

The first objective was to develop a specific method using the mass spectrometry properties of the reference substances.

A second objective included the optimizing of an extraction method.

The third objective was to validate the new method

The last objective was to determine the application of this method.

This will be determined by using this method on urine available from excretion studies. A second application will be by using this method for the determination of the presence of narcotic analgesic and NSAIDs in urine from competitors in several sporting events.

# Chapter 5

## Instrumental and chromatographic conditions

### 5.1 Reference substances

A list of the narcotic analgesics and NSAIDs available in South Africa was compiled. Reference substances were obtained from the reference standards of the Department of Pharmacology, UFS. Reference substances not available from the department were obtained from pharmaceutical companies.

### 5.2 Chemicals and reagents

**Table 1:** List of reagents and chemicals used

Reagent	Supplier
Acetic acid	Merck
Ammonium acetate	Merck
Diethyl ether	Burdich & Jackson
di-Potassium hydrogen phosphate ( $K_2HPO_4$ )	Merck
di-Sodium hydrogen phosphate ( $Na_2HPO_4$ )	Merck
Ethyl acetate	Burdich & Jackson
Formic acid	Merck
Methanol	Merck

**Table 1** continued

Reagent	Supplier
Potassium carbonate ( $K_2CO_3$ )	Merck
Potassium di-hydrogen phosphate ( $KH_2PO_4$ )	Merck
Sodium acetate ( $CH_3COONa$ )	Fluka
Sodium hydrogen carbonate ( $NaHCO_3$ )	Merck
Sodium hydroxide ( $NaOH$ )	Merck
Sodium sulfate ( $Na_2SO_4$ )	Merck
$\beta$ -glucuronidase	Roche
$\beta$ -glucuronidase-arylsulphatase	Roche

### 5.3 Buffers and solutions

#### Acetate buffer

Acetic acid (25.2 ml) is diluted with water (500ml) to give Solution 1.

Sodium acetate (129.5 g) is dissolved in distilled water (500 ml) to give Solution 2.

To Solution 1 add Solution 2 until the desired pH of 5.2 is reached. This results in a 2M acetate buffer stock solution. For a 0.25M solution, take 12.5 ml of the solution and make up to a volume of 100 ml using distilled water.

#### Potassium carbonate buffer

$K_2CO_3$  (20 g) and  $NaHCO_3$  (20 g) is dissolved in distilled water (160 ml) (pH = 9.6)

### **Phosphate buffer at pH 7**

Sodium hydrogen phosphate (14.1 g) is dissolved in distilled water and dilute to 1 litre to give Solution 1.

Potassium hydrogen phosphate (13.6 g) is dissolved in distilled water and dilute to 1 litre to give Solution 2.

To 1 litre of (1) add sufficient of (2) to bring the pH to 7. This buffer is stored at 4°C until used.

### **20% sodium hydroxide solution**

Sodium hydroxide (200 g) is dissolved in distilled water (1000 ml).

### **0.01% formic acid solution**

Formic acid (2 ml) is diluted with water to 100 ml to give a 2 % formic acid solution. Five ml from the 2 % formic acid solution is diluted to a volume of 1000 ml using distilled water to give a 0.01 % formic acid solution.

### **Injection solution**

Stock solutions of the reference substances were prepared at a concentration of 1 mg/ml in methanol. These solutions were stored in screw-capped bottles at approximately -20°C in a freezer.

Dilutions of the stock solutions were prepared at a concentration of 5 ug/ml in methanol and 2 % formic acid solution (50/50, v/v).

Apomorphine was used as the internal standard (ISTD). Two mg of apomorphine was dissolved in 100 ml methanol and stored at approximately -20°C in a freezer.

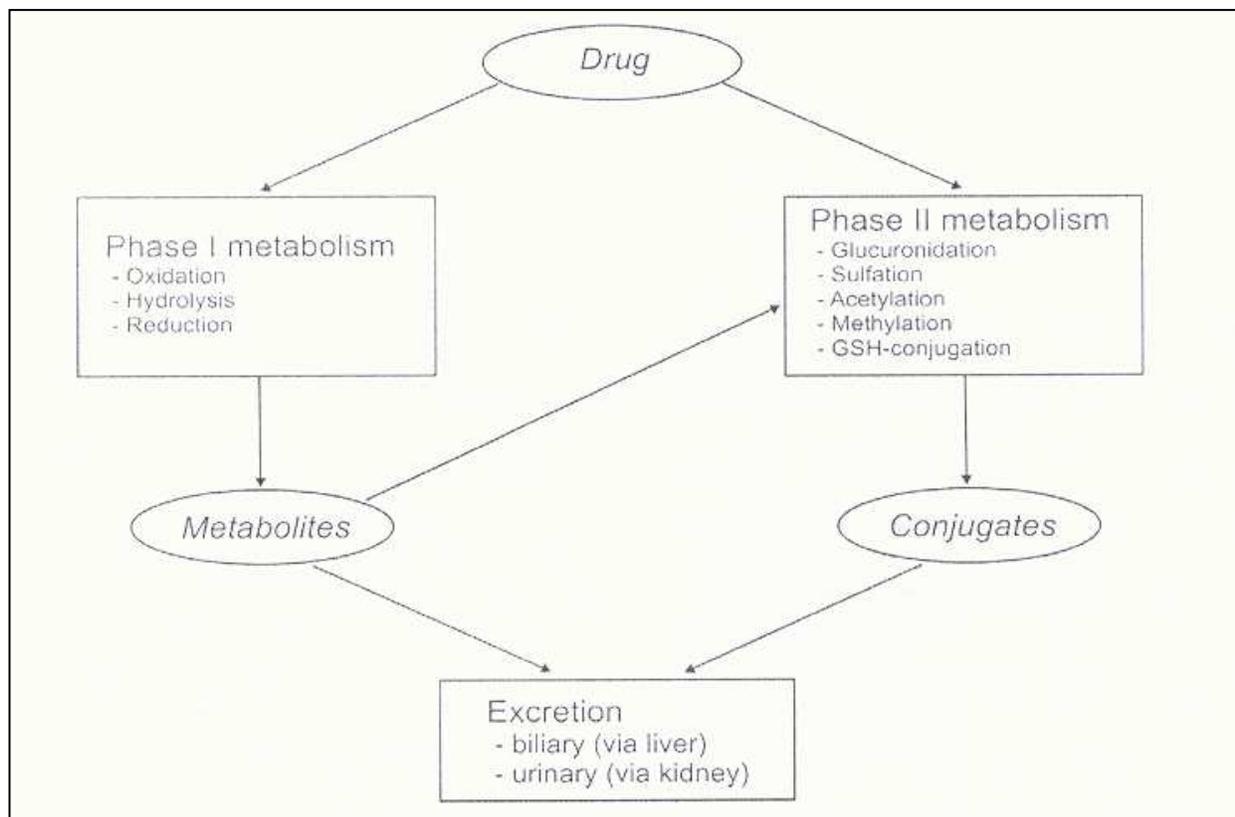
## 5.4 Injection Procedure (infusion)

Each individual dilution solution is directly injected into the ion spray source of the LC-MS/MS system. Ions are selected according to the molecular weight of each compound and their resulting mass values ( $m/z$ ). These ions are protonated at electron impact (EI). The mass spectrometry (MS) parameters for each precursor ion are optimized in the collision cell of the MS system. The protonated molecule  $(M + H)^+$  is chosen as the base peak. This is followed by the optimisation of the resulting product ions (fragment ion) in the collision cell, and detecting the resulting product ions using a multiplier detector. These parameters are further optimised using Analyst® software 1.4. This is called Multiple Reaction Monitoring (MRM). These optimal instrument conditions are used for the identification of the compounds. The parameters included are declustering (DP), focusing (FP), exit potential (EXP) and the collision energy (CE). The largest product ion is chosen, because this provides the highest abundance during detection. If two compounds have the same product ions, the second largest product of the relevant compounds is chosen to distinguish between the two compounds.

## 5.5 Metabolites

In the human body the substance is transformed into one or more metabolites and excreted as a mixture of the parent substance and/or metabolites. The transformation of the parent substance may be through phase I metabolism or phase II metabolism. The metabolite may be active, but in some cases it may also be inactive. Most of the narcotic analgesics and NSAIDs are metabolized in the liver to the glucuronide conjugates and excreted in the urine as a metabolite and unchanged drug. The conjugates are water soluble and are therefore rapidly cleared by the kidney. The unchanged substance or metabolites are excreted from the body and therefore may be detected with the help of analytical methods to identify and quantify the compound in the body fluid (Mycek M.J., Harvey R.A., Champe P.C., 2000). Figure 1 shows a simplified scheme of drug metabolism in the human body with phase I and phase II metabolism leading to excretion through the liver and kidney.

**Figure 1.** Simplified scheme of drug metabolism in the human body (Pérez S., Barceló D., 2007)



## 5.6 Instrumental conditions

The mass spectrometry system consisted of an Applied Biosystem API 2000 MS/MS system equipped with an Agilent 1100 Series HPLC consisting of a binary pump and autosampler. A Dell computer with Windows 2000 equipped with Analyst® software 1.4. was used. The column used was an Agilent Eclipse 1.8  $\mu\text{m}$  XDB-C<sub>18</sub> column (50 mm x 2.1 mm) with a guard column from Phenomenex®.

The compounds are divided into 4 groups (controls) and are shown in Table 2.

**Table 2:** List of the compounds used in the control samples.

Control 1	Control 2	Control 3	Control 4
Dextropropoxyphene	Ethoheptazine	Buprenorphine	Diclofenac
Fenoprofen	Hydromorphone	Codeine	Ibuprofen
Fentanyl	Indomethacin	Dextromoramide	Paracetamol
Ketoprofen	Meloxicam	Hydrocodone	Salicylic acid
Ketorolac tromethamine	Nalbuphine	Mefenamic acid	
Morphine	Naproxen	Methadone	
Oxycodone	Oxymorphone	Nabumetone	
Pentazocine Base	Piroxicam	Tiaprofenic acid	
Pethidine	Sulindac	Tilidine	
Phenylbutazone	Tramadol		
	Tenoxicam		

This is done to ensure good separation between the many compounds on the list during the method development stages. The 4 control samples were prepared at a concentration of 1 µg/ml in methanol. Ten µl of these 4 control solutions were separately evaporated in ampoules and dissolved in 100 µl mobile phase. Ten µl of each control solution was injected into the LC-MS/MS system. The runtime was 10 minutes, during the development stages, to ensure that each compound is eluted from the column for detection. The retention time and mass spectrum properties (m/z values) were used to differentiate between the different compounds. The mobile phase consisted of 0.01% formic acid (A) and acetonitrile (B).

## 5.7 Results

### Instrumental Conditions

**Table 3:** The compounds and their precursor and product ions (fragment ions)

<b>Compound Name</b>	<b>Precursor ion (screening)</b>	<b>Product ions (screening)</b>	<b>Other ions (confirmation)</b>
Apomorphine (ISTD)	268	237	-
Buprenorphine	468	187	179, 397, 415
Codeine	300	128	152, 165, 215
Dextromoramide	393	306	152, 164, 236
Dextropropoxyphene	340	266	115, 128, 143
Ethoheptazine	262	234	129, 188, 216
Fentanyl	337	188	103, 105
Hydrocodone	300	199	115, 171, 141
Hydromorphone	286	185	129, 152, 157
Methadone	310	265	117, 219, 223
Morphine	286	115	128, 152, 165
Nalbuphine	358	340	185, 254, 296
Oxycodone	316	298	241, 256
Oxymorphone	302	284	198, 227, 242
Pentazocine	287	218	159, 172, 174
Pethidine	248	220	105, 115, 175
Tilidine	274	155	130, 156, 213
Tramadol	264	58	57, 91, 247

**Table 3** continued

<b>Compound Name</b>	<b>Precursor ion (screening)</b>	<b>Product ion (screening)</b>	<b>Other ions (confirmation)</b>
Diclofenac	296	252	215, 278, 93
Fenoprofen	243	197	79, 91, 105
Ibuprofen	207	161	91, 105, 119
Indomethacin	358	111	139, 174, 312
Ketoprofen	255	209	105, 177, 194
Ketorolac Tromethamine	256	105	178, 210
Mefenamic acid	242	224	152, 178, 180
Meloxicam	352	115	115, 141, 200
Nabumetone	229	171	102, 128, 156
Naproxen	231	185	115, 155, 170
Paracetamol	152	110	94, 108, 134
Phenylbutazone	309	160	106, 120, 210
Piroxicam	332	121	123, 153, 164
Salicylic acid	139	93	64, 75
Silundac	357	233	234, 247, 340
Tenoxicam	338	164	143, 162, 200
Tiaprofenic acid	261	105	138, 182, 215

**Metabolites**

In this study reference substances available in the department were used to compile this method. The transitions used in this method are shown in Table 3. During the literature investigation it was evident that research was done on some of these

substances and their metabolites on the LC-MS/MS. Reference standards for the metabolites are limited and information for these metabolites are therefore limited to the literature. Table 4 summarise the MS parameters obtained from the literature for the metabolites of some of the listed compounds.

**Table 4:** Data of the metabolites as obtained from the literature

<b>Metabolite Name</b>	<b>Precursor ion</b>	<b>Product ion</b>	<b>Reference</b>
Norbuprenorphine	414	225	Mueller <i>et al</i> , (2005)
6-Oxycodol	318	282	Baldacci <i>et al</i> , (2004)
Acetylcodeine	342	225	Rook <i>et al</i> , (2005)
Nor-6-oxycodol	304	243	Baldacci <i>et al</i> , (2004)
Norcodeine	286	268	Boleda <i>et al</i> , (2007)
Nordextropropoxyphene	326	252	Lakso and Norström (2003)
4' Hydroxydiclofenac	311	267	Stülten <i>et al</i> , (2008)
Norfentanyl	233	177	Koch <i>et al</i> , (2004)
Norhydromorphone	272	185	Zheng <i>et al</i> , 2004)
Hydroxyibuprofen	221	177	Weigel <i>et al</i> , (2004)
Carboxyibuprofen	264	145	Weigel <i>et al</i> , (2004)
Dimethylhydroxy Ketoprofen	284	284	Alkatheeri <i>et al</i> , (1999)
Methyl Ketoprofen	268	268	Alkatheeri <i>et al</i> , (1999)
Dimethyl-3-hydroxybenzol Ketoprofen	298	298	Alkatheeri <i>et al</i> , (1999)
Hydroxymeloxicam	366	302	Ho <i>et al</i> , (2006)

**Table 4** continued

EDDP (Methadone metabolite) <sup>(1)</sup>	278	234	Etter <i>et al</i> , (2005)
EMDP (Methadone metabolite) <sup>(2)</sup>	264	220	Rook <i>et al</i> , (2005)
Normethadone	296	251	Mueller <i>et al</i> , (2005)
6-Mono-acetylmorphine	328	268	Dienes-Nagy <i>et al</i> , (1999)
Dihydromorphine	288	213	Wey and Thorman (2001)
di-hydromorphone	288	185	Zheng <i>et al</i> , (2004)
Ethylmorphine	314	229	Mueller <i>et al</i> , (2005)
Nordihydromorphine	274	231	Wey and Thorman (2001)
Normorphine	272	254	Boleda <i>et al</i> , (2007)
6-HNA (Nabumetone metabolite) <sup>(3)</sup>	203	157	Mueller <i>et al</i> , (2005)
6-MNA (Nabumetone metabolite) <sup>(4)</sup>	217	199	Mueller <i>et al</i> , (2005)
Noroxycodone	302	229	Edwards and Smith (2005)
Oxycodone-N-oxide	332	315	Baldacci <i>et al</i> , (2004)
Noroxymorphone	288	173	Wey and Thorman (2002)
Salicylic acid	137	93	Segarra <i>et al</i> , (2006)
Gentisic acid	153	109	Parker <i>et al</i> , (2004)
Salicyluric acid	193	150	Parker <i>et al</i> , (2004)
M1 (Tramadol metabolite) <sup>(5)</sup>	250	58	Juzwin <i>et al</i> , (2000)
M5 (Tramadol metabolite) <sup>(6)</sup>	236	44	Juzwin <i>et al</i> , (2000)
M6 (Tramadol metabolite) <sup>(7)</sup>	280	58	Juzwin <i>et al</i> , (2000)

(1) 2-Ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine

(2) Ethyl-5-methyl-3,3-diphenyl-1-pyrroline

(3) 6-hydroxy-2-naphthylacetic acid

(4) 6-methoxy-2-naphthylacetic acid

- (5) (dl)-1-(hydroxyphenyl)-2(dimethylaminomethyl)-cyclohexan-1-ol-monohydrochloride hemihydrates
- (6) 3(‘1-hydroxy-2-[(methylamino) methyl] cyclohexyl)phenol hydrochloride
- (7) 1(3-methoxyphenyl)-2-(dimethylaminomethyl)cyclohexan-1,4-diol

## 5.8 Chromatographic conditions

An extended list of compounds was used during this study (Table 3, page 40). A shorter column of 50 mm, in stead of 150 mm, was used during the development phase of the method to determine if this column would be effective in separation and detection for such an extended list of compounds.

This is the summary of the initial method used for the development phase.

<b>Instrument:</b>	Applied Biosystems API 2000 LC-MS/MS	
<b>Computer:</b>	Intel Pentium 4 Dell computer with Microsoft Window’s 2000	
<b>Software:</b>	Analyst 1.4.	
<b>Column:</b>	Agilent Eclipse 1.8 µm XDB-C <sub>18</sub> column (50 mm x 2.1 mm)	
<b>Printer:</b>	Hewlet-Packard Laserjet 2300	
<b>Parameters:</b>	Scan type:	MRM
	Polarity:	Positive
	Ion Source:	Turbo Spray
	Curtain Gas:	25.0 psi
	Ion Spray Voltage:	5500 Volt
	Temperature:	400°C
	Ion source gas 1:	40 Psi

Ion source gas 2: 20 psi

Collision gas: 4.00 psi

**Autosampler properties: Agilent 1100 Autosampler**

Injection volume: 10 µl

Runtime: 10 minutes

**Pump method properties: Agilent 1100 Binary Pump**

**Mobile phase:** **Solvent A:** 0.01% formic acid

**Solvent B:** acetonitrile

Step	Time (min)	Flow rate (µl/min)	A (%)	B (%)
0	0.00	200	40.0	60.0
1	10.00	200	40.0	60.0

The criteria for the positive identification of the compounds were set. The criteria included (a) the retention times of the compounds in the controls, (b) the intensity of the precursor-product ion transition and (c) good peak shape.

After injection of the 4 control samples using the initial method, the following problems were encountered with the separation.

Mobile phase 1 (MP 1): The first MP consisted of 40:60 (v/v) of solvent A (0.01% formic acid) and solvent B (acetonitrile). This composition of the mobile phase was used in the laboratory and was therefore the starting mobile phase.

Outcome: The separation was not very good between all the compounds. The elution of the compounds from the column was too early with retention times of the compounds to close too one another, less than 5 minutes, resulting in overlaying of peaks with poor separation and identification. Some of the compounds were not detected at all.

Mobile phase 2 (MP 2): The composition of the mobile phase was changed to 45:55 (v/v) of solvent A (0.01% formic acid) and solvent B (acetonitrile).

Outcome: The retention times of compounds were, however, still too short and too close to one another and some of them still overlay each other, but with good peak shape.

Mobile Phase 3 (MP 3): The composition of the mobile phase was changed to 65:35 (v/v) of solvent A (0.01% formic acid) and solvent B (acetonitrile).

Outcome: For most of the narcotic analgesics the retention times were still too early. Most of them eluted in less than 2 minutes, but with better peak shape. For the NSAIDs the retention times were later, with all of them eluting in less than 7 minutes with good peak shape.

Mobile Phase 4 (MP 4): The composition was changed to 80:20 (v/v) of solvent A (0.01% formic acid) and solvent B (acetonitrile).

Outcome: For the narcotic analgesics and the NSAIDs the retention times did not shift very significantly from the previous run. The retention times for the NSAIDs were less than 8 minutes.

Mobile Phase 5 (MP 5): The composition of the mobile phase was changed to 30:70 (v/v) of solvent A (0.01% formic acid) and solvent B (acetonitrile). Another mobile phase was investigated. The composition of the mobile phase changed to 20:80 (v/v) of solvent A (0.01% formic acid) and solvent B (acetonitrile). The result for these two compositions of the mobile phase was compared to the previous results.

Outcome: For the narcotic analgesics the retention times did not change significantly from the previous runs. However, for the NSAIDs the separation between these compounds was better. All of the compounds eluted under less than 8 minutes after injection.

It was evident that the change in the composition of the mobile phase did not have any significant effect on the retention times of the narcotic analgesics in the control samples. The retention times were too close to one another, in less than 2 minutes. The retention

times of the NSAIDs in the controls were much later with better separation and peak shape.

During the development phase of the method it was decided to change the 0.01% formic acid solution to 4mM ammonium acetate buffer as solvent A and acetonitrile as solvent B as described in the literature.

Outcome: The same compositions of the mobile phases were tested as described for the 0.01 % formic acid solution in MP 1 to MP 5. The retention times for the narcotic analgesics shifted later, in less than 3 minutes, with good peak shapes. However, for the NSAIDs the retention times shifted earlier, in less than 5 minutes, and peak shapes of some of the compounds was not very good. The peak shapes widened and were not very sharp (tailing). It was decided to revert back to the original mobile phase of 0.01% formic acid (solvent A) and acetonitrile (solvent B).

Mobile phase gradients were described in the literature and therefore some of these gradients were investigated during the development phase of this method.

Mobile Phase 6 (MP 6): The first gradient was tested. The gradient started at 40% solvent A (0.01% formic acid), linearly increased to 55% solvent A in 3.5 minutes. Thereafter the gradient linearly increased to 70% solvent A in 2 minutes where it was kept for 2.5 minutes at 70% solvent A, where after it linearly decreased to 40% solvent A in 2 minutes. Solvent B was acetonitrile.

Outcome: The retention times for the narcotic analgesic compounds were less than 2 minutes with retention times for the NSAIDs much later. All the compounds were detected under less than 8 minutes after injection. The peak shape was good for most of the listed compounds.

Mobile phase 7 (MP 7): The second gradient was tested to determine if the retention times for the narcotic analgesics would shift away from one another. The gradient started at 15% solvent A (0.01% formic acid) for 3 minutes, linearly increased to 30% solvent A in 7 minute, increased linearly to 90% solvent A in 20 minutes, and re-

equilibrated with 15% solvent A for 10 minutes, as described in the literature. Solvent B was acetonitrile.

Outcome: The retention times for the narcotic analgesics and NSAIDs did not change significantly from the previous injection.

Mobile phase 8 (MP 8): The composition in the mobile phase was changed significantly. The gradient decreased linearly from 70 to 30% solvent A (0.01% formic acid) in 7 minutes; linearly increased to 70% in 8 minutes, and held for 20 minutes at 70% for re-equilibration. Solvent B was acetonitrile.

Outcome: The significant change in the composition of the mobile phase provided good results. The retention times for the narcotic analgesics shifted away from one another and the retention times for the listed compounds were in less than 8 minutes. A good peak shape was maintained for all of the listed compounds.

Mobile phase 9 (MP 9): Another change in the mobile phase was investigated by applying a more significant change in the gradient to determine if the retention times of the narcotic analgesics would shift away from one another. The gradient started at 90% solvent A (0.01% formic acid) for two minutes. The gradient linearly decreased to 10% solvent A in 6 minutes. The gradient linearly changed back to the original composition of 90% solvent A in 1 minute. Solvent B was acetonitrile.

Outcome: This significant change in mobile phase provided good results. The retention times for most of the narcotic analgesics shifted away from one another and all of the retention times for the listed compounds were in less than 8 minutes. Good separation was obtained between the compounds with good peak shapes. This gradient in the mobile phase was better for the separation and identification of the listed compounds.

The re-equilibration time between injections was investigated to shorten the total runtime. Re-equilibration times from 1 minute to 12 minutes were investigated. A series of the 4 control samples were injected repeatedly. Each series of the control samples was separated with different re-equilibration times starting from 1 minute to 12 minutes. The retention times for each of the listed compounds were compared to one another

between the series of injections. The re-equilibration time where the retention times did not differ significantly between the injections were chosen as the better re-equilibration time.

Outcome: The best re-equilibration time were determined to be 11 minutes. The 4 control samples were injected repeatedly to ensure that the method was effective in separation and detection of the listed compounds.

The results were satisfactory during the development phase for the shorter column by shortening the total runtime for such an extended list of compounds. The column used through the development phase was the Agilent Eclipse 1.8  $\mu\text{m}$  XDB-C<sub>18</sub> column (50 mm x 2.1 mm) with a guard column from Phenomenex<sup>®</sup>.

The final chromatographic conditions for this study are as follows:

**Autosampler properties: Agilent 1100 Autosampler**

Injection volume: 10  $\mu\text{l}$

Runtime: 20 minutes

**Pump method properties: Agilent 1100 Binary Pump**

**Mobile phase:**                      **Solvent A:** 0.01% formic acid

**Solvent B:** acetonitrile

Step	Time (min)	Flow rate ( $\mu\text{l}/\text{min}$ )	A (%)	B (%)
0	0.00	200	90.0	10.0
1	2.00	200	90.0	10.0
2	8.00	200	10.0	90.0
3	9.00	200	90.0	10.0
4	20.0	200	90.0	10.0

## 5.9 Sample preparation

Sample preparation methods in the literature were investigated to obtain a possible method for the extraction of the list of narcotic analgesics and NSAIDs. The listed compounds contained basic, acidic and neutral properties. The pH of the extraction procedure was therefore important. The detection of metabolites was also investigated. It was stated in the literature that the metabolites are excreted in the urine in glucuronide form. It is therefore important to investigate some enzymatic hydrolysis steps before detection on the LC-MS/MS. In the literature extraction procedures were described for basic, acidic and neutral compounds. These methods were investigated to determine the optimal extraction conditions for the listed compounds. Specific changes were made especially for this study. A urine control sample at concentration of 1  $\mu\text{g}/\text{ml}$  for each compound was used during the extraction procedures.

The criteria for the positive identification of the compounds were set. The criteria included (a) the retention times of the compounds in the controls, (b) the abundance (area) of the precursor-product ion transition and (c) good peak shape.

The extraction methods were evaluated by comparing abundance for each compound in every extraction method after injection into the LC-MS/MS.

Extraction procedure 1 (EP 1): Two ml of urine control sample was used, the urine pH was adjusted to approximately 14 and extraction was performed with diethyl ether. The

organic phase was transferred to an ampoule, evaporated under a stream of nitrogen gas, dissolved in mobile phase and injected into the LC-MS/MS.

Outcome: The extraction procedure did not provide high enough abundance for many of the listed compounds. The sample pH was adjusted to approximately 14. This suggested that the urine pH may be too high for efficient extraction of some compounds in the urine.

Extraction procedure 2 (EP 2): This extraction procedure included both basic and acidic urine sample conditions. One part consisted of 2 ml of urine for extraction of the basic compounds and 2 ml urine for the acidic compounds. The basic extraction procedure included the adjusting of the urine pH to approximately 9.6. The acidic extraction procedure included the adjusting the urine pH to approximately 5.2. Both these parts were extracted with a solution of diethyl ether and ethyl acetate (3:2, v/v). The organic phase of the basic and acidic extraction was evaporated separately and injected into the LC-MS/MS. Another extraction was performed in which the organic phase of the basic extraction was transferred to an ampoule and evaporated to dryness under a stream of nitrogen. The organic phase of the acidic extraction was transferred to the same ampoule containing the basic extraction and evaporated to dryness under a stream of nitrogen gas. This residue was dissolved in mobile phase and injected into the LC-MS/MS.

Outcome: When comparing the basic and acidic extraction procedures, the abundance of the listed compounds was found to be different. Some of the narcotic analgesics provided higher abundance in the basic extraction than in the acidic extraction, and vice versa.

As described earlier, the metabolites of these compounds are excreted as a mixture of the parent compound and/or metabolites. The metabolites are transformed through phase I and phase II metabolism in the liver to glucuronide conjugates. For this reason some enzymatic hydrolysis steps were investigated to remove the glucuronide conjugates, as described in the literature.

Extraction procedure 3 (EP 3): This extraction procedure included the enzymatic hydrolysis of the urine control sample for 2 hours at 50°C with  $\beta$ -Glucuronidase-arylsulphatase at a urine pH of 5.2. The pH of the urine was thereafter changed to approximately 9.6 and extracted with a solution of diethyl ether and ethyl acetate (3:2, v/v). The organic phase was transferred to an ampoule and evaporated under a stream of nitrogen gas. The residue was dissolved in mobile phase and injected into the LC-MS/MS.

Outcome: Good abundance was obtained for most of the narcotic analgesics, however, for the NSAIDs some of the listed compounds did not provide good abundance, although it was better than at pH 14, as described earlier.

Extraction procedure 4 (EP 4): An extraction procedure including solid phase extraction of the urine followed by enzymatic hydrolysis was investigated. The solid phase clean-up was performed with Sep-Pack C<sub>18</sub> columns. The residue was dissolved in phosphate buffer solution at pH 7 and  $\beta$ -Glucuronidase enzymatic hydrolysis was performed for 2 hours at 50°C. The extraction was performed with diethyl ether followed by freezing of the water layer in a freezer bath. The organic layer was transferred to an ampoule and evaporated to dryness under a stream of nitrogen gas. The residue was dissolved in mobile phase and injected into the LC-MS/MS.

Outcome: Better results were obtained for the listed compounds after hydrolysis at pH 7 and extraction with diethyl ether. The extraction procedure used solid phase extraction in the preparation phase of the urine sample. This part of the procedure was not investigated further due to the longer extraction time and the cost implications of the cartridges needed for the solid phase extraction.

## 5.10 Result evaluation

The evaluation of the chromatographic conditions showed that MP 9 using a gradient provided the best results during the chromatographic development. The gradient started at 90% solvent A (0.01% formic acid) for two minutes. The gradient linearly decreased to 10% solvent A in 6 minutes. The gradient linearly changed back to the original composition of 90% solvent A in 1 minute. Solvent B was acetonitrile. The retention times for the listed compounds were in less than 8 minutes. Good separation was obtained between the compounds with good peak shapes. The best re-equilibration time were determined to be 11 minutes. The 4 control samples were injected repeatedly to ensure that the method was effective in separation and detection of the listed compounds.

The results were satisfactory during the development phase for the shorter column by shortening the total runtime for such an extended list of compounds (Table 3, page 39). The column used through the development phase was the Agilent Eclipse 1.8  $\mu\text{m}$  XDB- $\text{C}_{18}$  column (50 mm x 2.1 mm) with a guard column from Phenomenex<sup>®</sup>.

The evaluation of the extraction methods showed that EP 3 was the best option for extraction of the narcotic analgesics and NSAIDs from the urine. However, some of these compounds were more effectively extracted by the method in which the urine pH was adjusted to approximately pH 7 (EP 4). Therefore, extraction procedure 3 was selected with a small change. The hydrolysis step was performed as described previously, where after 1 ml of phosphate buffer was added to the sample to adjust the sample pH to approximately 7, as described in EP 4. This neutral pH was selected because it provided the best results for most of the NSAIDs on the list.

The final extraction procedure is as follows:

- 2 ml urine
- 1 ml acetate buffer pH = 5.2
- 25  $\mu\text{l}$   $\beta$ -glucuronidase/arylsulfatase enzyme

- Hydrolyse at 50 °C for 2 hours
- Add 1 ml phosphate buffer pH = 7
- Add 5 ml diethyl ether
- Shake horizontally for 5 minutes on a mechanical shaker
- Centrifuge for 5 minutes
- Transfer organic phase to an ampoule containing 50 µl internal standard (Apomorphine)
- Evaporate to dryness
- Dissolve in 100 µl mobile phase.
- Inject 10 µl into LC-MS/MS

The mobile phase for solution of the residue consisted of 90:10 (v/v) 0.01% formic acid and acetonitrile.

Numerous control samples were extracted and analysed repeatedly to ensure that the extraction procedure provided reliable results according to that described in the criteria (a), (b) and (c) for positive identification.

## 5.11 References

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# Chapter 6

## Validation

Validation is important in any method to ensure that the results obtained using such a method are reliable. There are two kinds of methods used for determination of compounds, namely qualitative and quantitative methods. Qualitative methods are used for identification of compounds in any sample without determining any concentration of the compounds present as performed in quantitative methods.

There are different parameters to validate in each method. In this study the developed method is an identification method with no concentration determination for the list of specified compounds and therefore it is a qualitative method. For validation of this analytical procedures specificity, limit of detection, precision (repeatability) and, if possible, stability of compounds under storage conditions must be taken into consideration.

The criteria for the positive identification of the compounds were set. The criteria were: (a) the retention time of each compound in the control sample, (b) the abundance of the precursor-product ion transition and (c) good peak shape.

The following section is to provide a short description of each parameter on what it means and how it was executed in this study.

### 6.1 Specificity

Specificity is defined as the ability of a method to differentiate the analyte(s) in the presence of other constituents in the sample. This refers to the ability of the method to produce a response for a single analyte of interest. This is to proof that there are no interfering signals between the analyte(s), the internal standard or other closely related constituents present in the sample.

In this study 10 different sources of blank urine samples were used with different characteristics. These characteristics included SGs, pH and gender. These samples were selected randomly from our routine samples. The compounds used in this study were added at a specific concentration (Table 1) in the selected urine samples for validation. These urine control samples were extracted and analysed together with the corresponding blank urine samples using the method described previously. The identification of the compounds must meet the criteria for positive identification under different sample characteristics to prove the specificity of the method. In Figure 1 the chromatogram of the blank sample is shown with its corresponding chromatogram of the control sample and Table 1 summarises the retention times obtained for each of the listed compounds.

## **6.2 Repeatability**

Repeatability is defined as the “closeness” of identification of the compounds from a series of aliquots from the same homogeneous sample. The same extraction and analytical procedure should be followed with each series so as to compare results.

In this study sufficient volume of urine for the control samples were prepared to analyse 5 aliquots from the same control sample at each concentration. That is a low concentration at 100 ng/ml, a medium concentration at 400 ng/ml and a high concentration at 800 ng/ml. The 5 samples in each concentration group were compared with one another and had to meet the stipulated criteria for positive identification stipulated earlier. The coefficient of variation (CV%) was calculated using the average of the area of the precursor-product ion transitions of the 5 samples and the standard deviation over the range of these 5 samples. Table 2 summarises the calculated CV values for each listed compound at low, medium and high concentration.

### **6.3 Limit of detection**

Limit of detection (LOD) is defined as the lowest concentration of the compound present in the sample which can be detected (identified) from the background of the sample without interference from other compounds or constituents in the extracted sample. The signal to noise ratio (S/N) is calculated and the LOD concentration determined where the S/N is higher than 3. LOD in this study was determined by starting at a high concentration and diluting the urine 1:1 sequentially with blank urine. The sequent dilution was begun at a high concentration of 800 ng/ml and the last dilution concentration was a low 1.563 ng/ml. These samples were extracted and analysed by the described method. The signal-to-noise was calculated using the Analyst® software. The calculated LOD values are summarised in Table 3. The results in the dilution series were checked closely to ensure that the identification of the compounds met the stipulated criteria for positive identification, especially at the low concentrations in the dilution series.

### **6.4 Stability of compounds under storage conditions**

Stability is defined as the chemical stability of a compound in a given matrix under specific conditions for given time intervals. These testing procedures are included in the validation process to determine stability of the compounds prior to extraction and analysis at various stages of storage. This is important for reliable identification. If published literature is not available for the compounds used, it is advisable to determine stability for the compounds under your specific conditions.

In this study two stability parameters were investigated, namely freeze-thaw stability and long term stability.

Freeze and thaw compound stability was determined during three freeze and thaw cycles. The cycle started by using blank urine and adding the specific compounds at a concentration of 100 ng/ml each. One aliquot of this sample was analysed immediately. The rest of the sample was stored in glass vials at approximately -20 °C in a freezer for

24 hours. The sample was then thawed unassisted on the work bench at room temperature. When the sample had completely thawed the sample was transferred back to the same freezer and kept frozen for another 24 hours. The same procedure was repeated twice and the sample was extracted and analysed in the third cycle. This is done to include conditions in which samples may be frozen and then thawed to be re-analysed. The area ratio for each compound was calculated by dividing the area of the precursor-product ion pair for each compound by the area of the precursor-product ion pair of the internal standard. This was performed for the immediate analysis of the control sample and after the freeze-thaw cycle was completed. The freeze-thaw area ratio after 3 cycles was compared with the area ratio of the sample analysed immediately.

Long-term stability was determined by starting with one urine control sample at a concentration of 100 ng/ml for each compound and extracting and analysing one aliquot from this control sample immediately. Sufficient volume of control sample was prepared to allow several aliquots to be analysed from the same control sample. The rest of the control sample is stored at approximately -20°C in a freezer. A second aliquot from the same stored sample is analysed after one month of storage at -20°C under the same conditions as the first aliquot. This was repeated after two months and three months of storage using the same control sample. The samples were thaw unassisted at room temperature on a bench top before being analyzed with the described method. The area ratios of the first, second and third month were compared with the area ratios of the immediate analysis for each compound. These area ratios were calculated for each compound and compared with the immediate area ratios, to identify any significant change in the area ratios from the immediate to the third month of storage. The results are summarised in Table 5. The criteria for positive identification stated earlier were applying for all the samples during the 3 months of storage. During the 3 months of storage the retention time of the compounds, the intensity of the precursor-product ion transition and peak shape should not have changed significantly.

## 6.5 Results

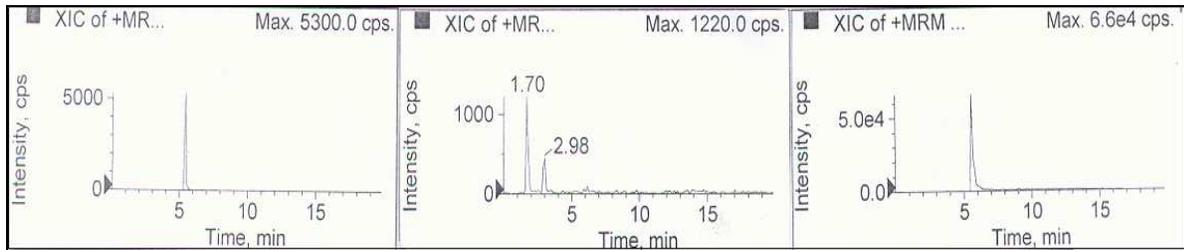
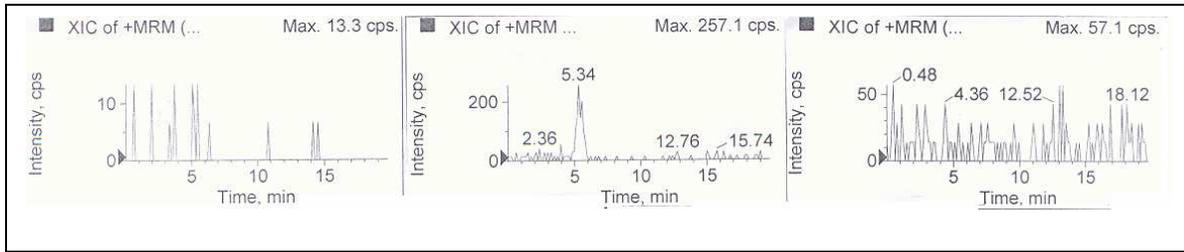
### Specificity

**Figure 1:** Chromatograms of the blank sample (top) and of the corresponding control sample (bottom).

Buprenorphine

Codeine

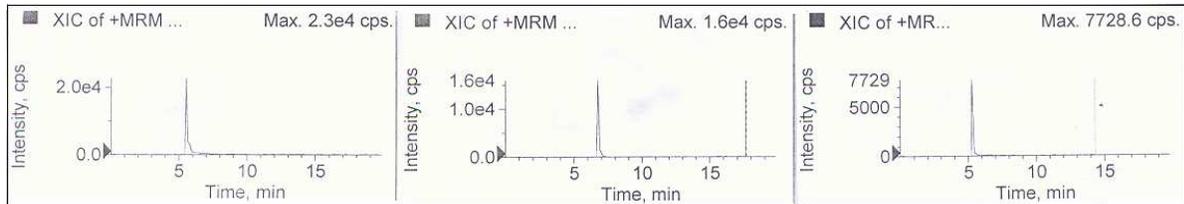
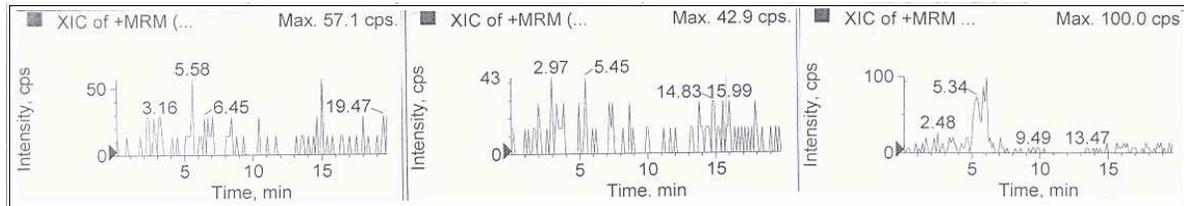
Dextromoramide



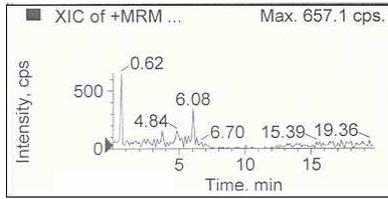
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Diclofenac

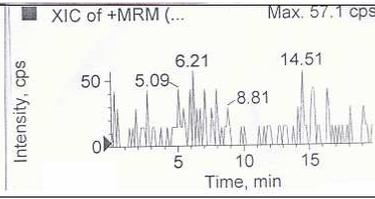
Ethoheptazine



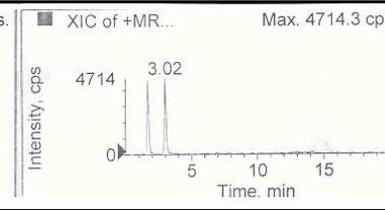
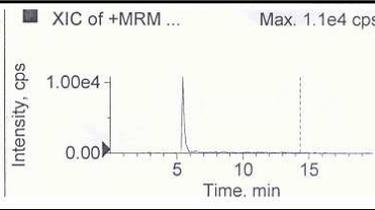
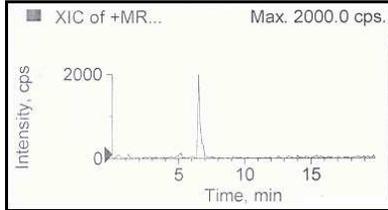
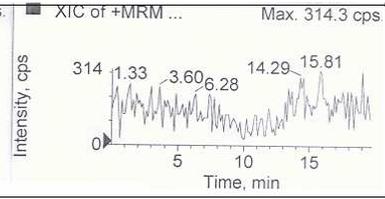
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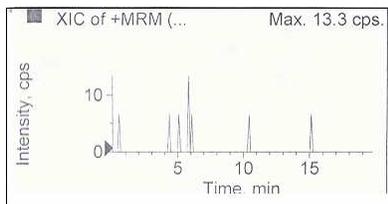
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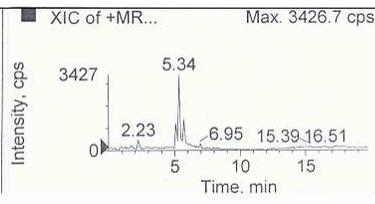
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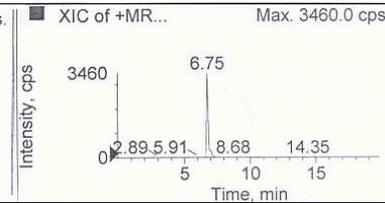
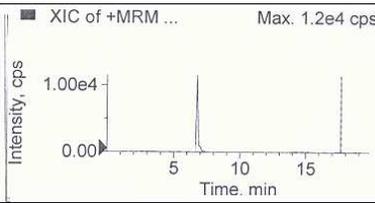
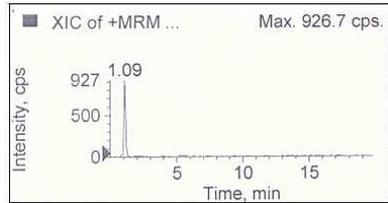
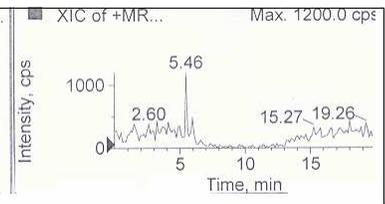
### Hydromorphone



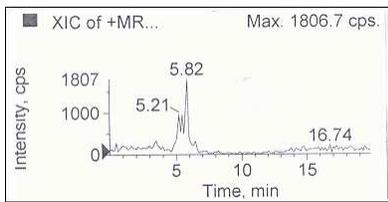
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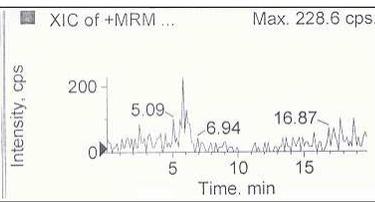
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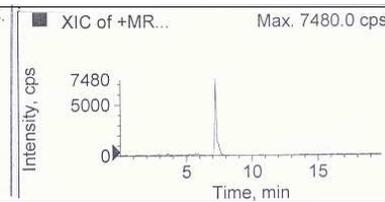
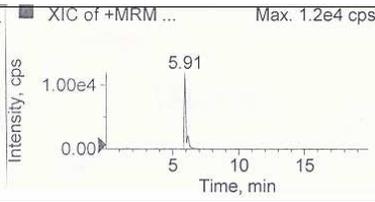
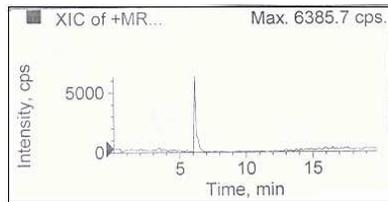
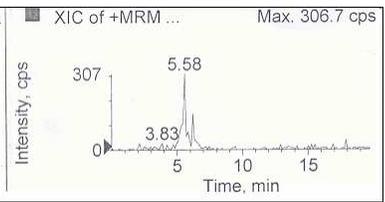
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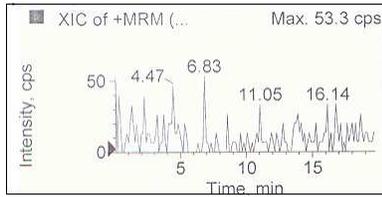
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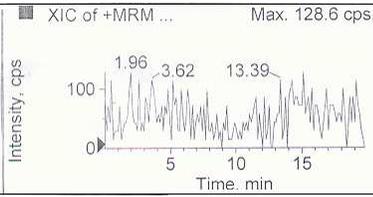
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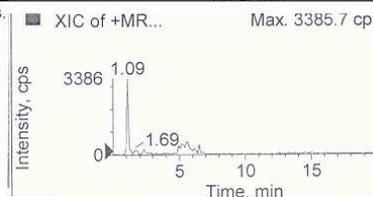
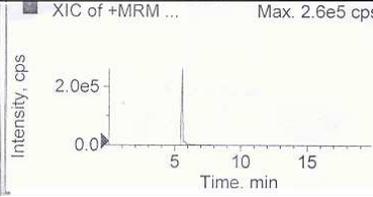
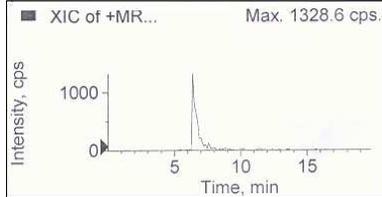
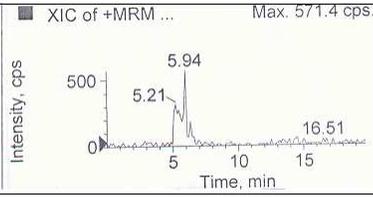
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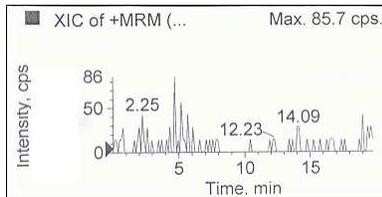
### Methadone



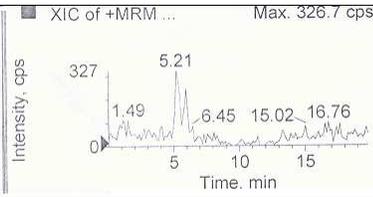
### Morphine



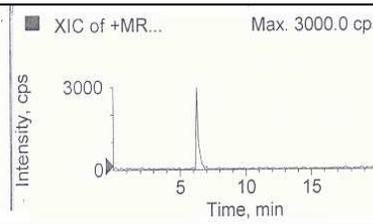
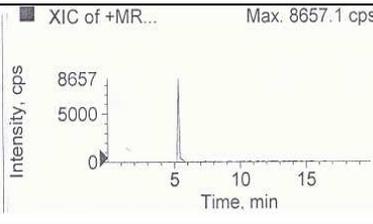
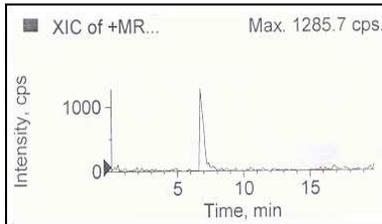
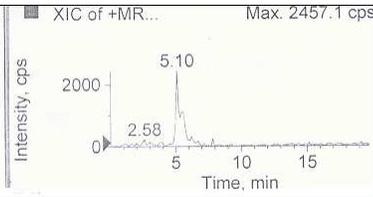
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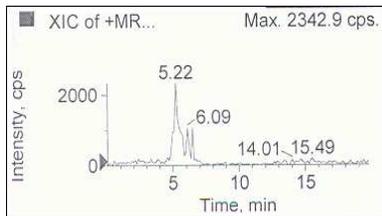
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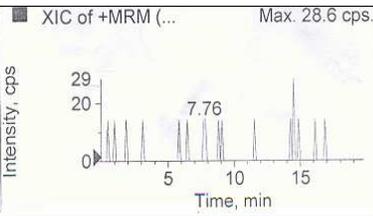
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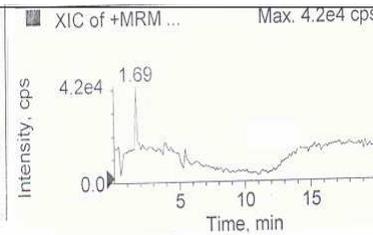
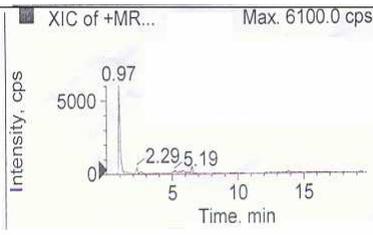
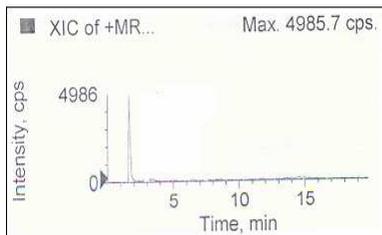
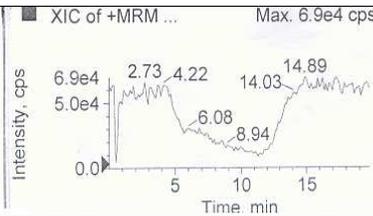
### Oxycodone



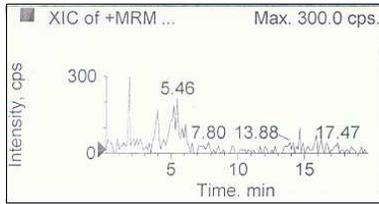
### Oxymorphone



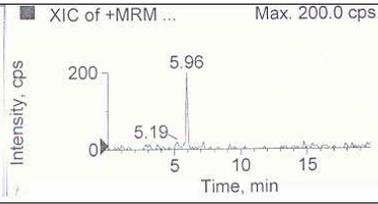
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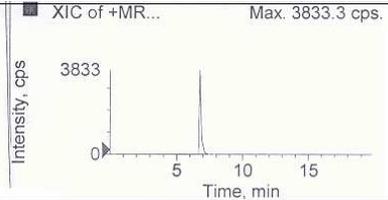
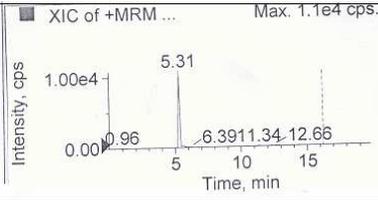
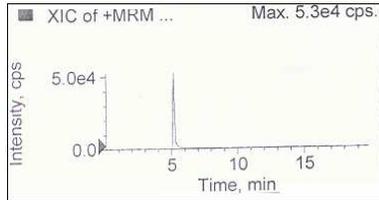
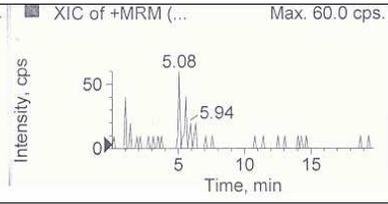
### Pentazocine Base



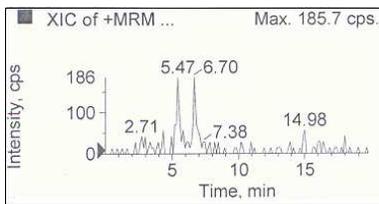
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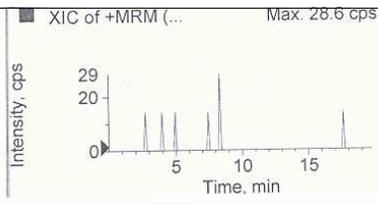
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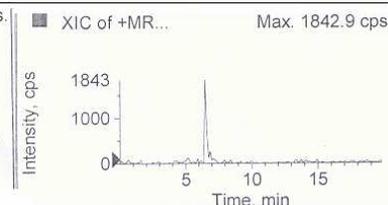
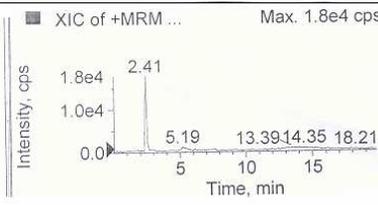
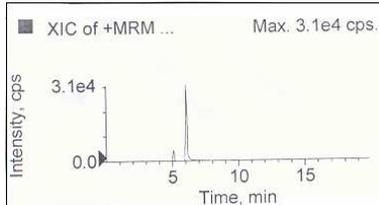
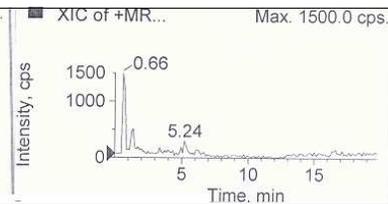
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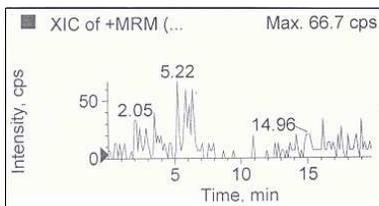
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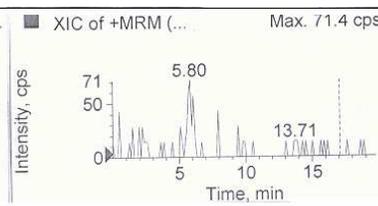
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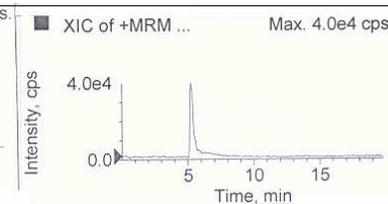
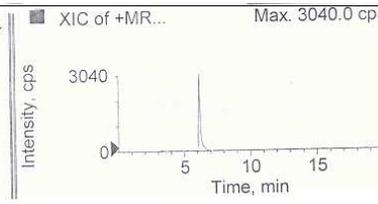
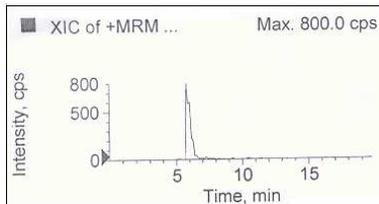
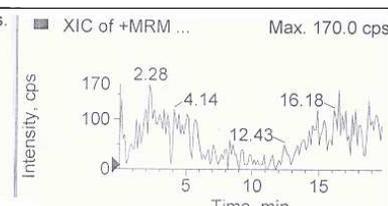
### Tenoxicam



### Tiaprofenic acid

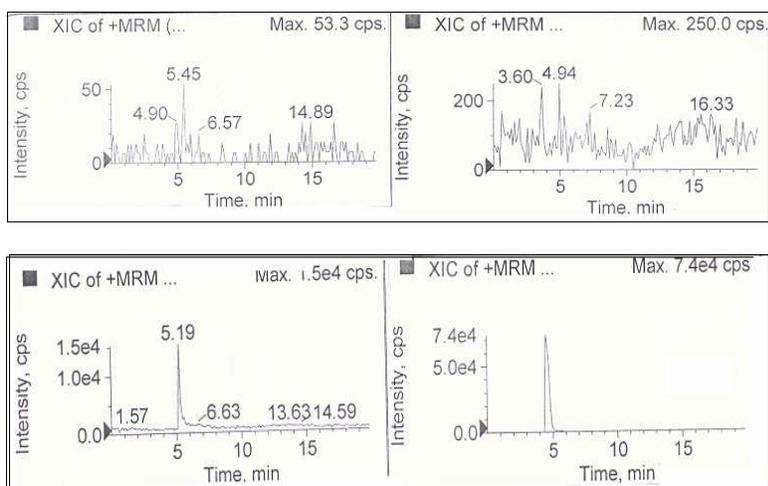


### Tilidine



Tramadol

Apomorphine (ISTD)



**Table 1:** Concentration of the compounds in the control and their retention times used for determination of specificity (n=10)

Compound name	Concentration in urine (µg/ml)	Average Retention time (minutes)
Apomorphine (ISTD)	20.0	4.45
Buprenorphine	100.0	5.43
Codeine	40.0	1.73
Dextromoramide	20.0	5.62
Dextropropoxyphene	20.0	5.58
Diclofenac	60.0	6.79
Ethoheptazine	20.0	5.32
Fenoprofen	180.0	5.54
Fentanyl	20.0	5.45
Hydrocodone	70.0	1.65
Hydromorphone	80.0	1.08

**Table 1** continued

Ibuprofen	360.0	6.90
Indometacin	80.0	6.80
Ketoprofen	60.0	6.18
Ketorolac Tromethamine	60.0	5.97
Mefenamic acid	40.0	7.25
Meloxicam	30.0	6.39
Methadone	20.0	5.56
Morphine	40.0	1.07
Nabumetone	40.0	6.78
Nalbuphine	40.0	5.73
Naproxen	180.0	6.35
Oxycodone	60.0	2.39
Oxymorphone	60.0	0.98
Paracetamol	60.0	1.69
Pentazocine Base	50.0	5.32
Pethidine	20.0	5.28
Phenylbutazone	60.0	6.99
Piroxicam	20.0	6.04
Salicylic acid	60.0	2.38
Sulindac	45.0	5.90
Tenoxicam	45.0	5.62
Tiaprofenic acid	30.0	6.20
Tilidine	20.0	5.36
Tramadol	40.0	5.21

## Repeatability

**Table 2:** Calculated coefficient of variation (CV%) for the listed compounds at low, medium and high concentration (n=5)

Name	CV% (100 ng/ml)	CV% (400 ng/ml)	CV% (800 ng/ml)
Apomorphine-ISTD	2	2	4
Buprenorphine	3	7	8
Codeine	7	3	4
Dextromoramide	1	4	5
Dextropropoxyphene	9	1	9
Diclofenac	4	2	13
Ethoheptazine	3	1	8
Fenoprofen	7	4	4
Fentanyl	4	2	5
Hydrocodone	6	6	7
Hydromorphone	7	14	2
Ibuprofen	6	5	11
Indomethacin	7	5	5
Ketoprofen	6	6	8
Ketorolac	7	5	2
Mefenamic acid	5	3	3
Meloxicam	9	7	4
Methadone	1	2	2

**Table 2** continued

Morphine	10	7	6
Nabumetone	9	2	13
Nalbuphine	5	9	8
Naproxen	10	5	4
Oxycodone	2	5	6
Oxymorphone	9	1	12
Paracetamol	4	1	3
Pentazocine Base	5	1	7
Pethidine	5	6	9
Phenylbutazone	15	10	4
Piroxicam	1	2	2
Salicylic acid	9	3	9
Sulindac	6	7	7
Tenoxicam	7	8	3
Tiaprofenic acid	8	2	3
Tilidine	1	5	2
Tramadol	2	5	6

## Limit of detection

**Table 3:** LOD values calculated for the listed compounds

Name of compound	LOD (ng/ml)
Buprenorphine	0.6
Codeine	0.7
Dextromoramide	0.8
Dextropropoxyphene	0.5
Diclofenac	0.5
Ethoheptazine	0.6
Fenoprofen	0.5
Fentanyl	0.9
Hydrocodone	2.7
Hydromorphone	1.7
Ibuprofen	0.5
Indomethacin	0.6
Ketoprofen	0.7
Ketorolac	1.7
Mefenamic acid	0.9
Meloxicam	0.9
Methadone	0.4
Morphine	0.9
Nabumetone	0.8
Nalbuphine	0.5

**Table 3** continued

Naproxen	3.0
Oxycodone	1.9
Oxymorphone	0.8
Paracetamol	18
Pentazocine Base	0.5
Pethidine	1.3
Phenylbutazone	0.5
Piroxicam	34
Salicylic acid	0.8
Sulindac	0.8
Tenoxicam	2.0
Tiaprofenic acid	2.0
Tilidine	0.5
Tramadol	0.6

## Stability of compounds under storage conditions

### Freeze and thaw stability

**Table 4:** Results obtained for freeze and thaw compound stability after 3 cycles

Compound name	Area ratio (Immediately) (A)	Area ratio (freeze-thaw cycle) (B)	Ratio B / A
Apomorphine (ISTD)	1.000	1.000	1.00
Buprenorphine	0.012	0.011	0.92
Codeine	0.010	0.008	0.80
Dextromoramide	0.115	0.112	0.97
Dextropropoxyphene	0.063	0.062	0.98
Diclofenac	0.048	0.047	0.98
Ethoheptazine	0.017	0.016	0.94
Fenoprofen	0.033	0.031	0.94
Fentanyl	0.086	0.082	0.95
Hydrocodone	0.010	0.008	0.80
Hydromorphone	0.002	0.002	1.00
Ibuprofen	0.010	0.009	0.90
Indomethacin	0.012	0.010	0.83
Ketoprofen	0.045	0.045	1.00
Ketorolac	0.062	0.060	0.97
Mefenamic acid	0.075	0.073	0.98
Meloxicam	0.050	0.048	0.96
Methadone	0.310	0.303	0.98

**Table 4** continued

Morphine	<b>0.001</b>	<b>0.001</b>	<b>1.00</b>
Nabumetone	0.011	0.010	0.91
Nalbuphine	0.042	0.037	0.88
Naproxen	0.044	0.041	0.93
Oxycodone	0.029	0.027	0.93
Oxymorphone	0.021	0.020	0.95
Paracetamol	2.355	2.260	0.96
Pentazocine Base	0.027	0.024	0.89
Pethidine	0.352	0.329	0.93
Phenylbutazone	0.007	0.006	0.86
Piroxicam	0.041	0.040	0.98
Salicylic acid	0.004	0.003	0.75
Sulindac	0.030	0.029	0.97
Tenoxicam	0.109	0.105	0.96
Tiaprofenic acid	0.048	0.040	0.83
Tilidine	0.076	0.074	0.97
Tramadol	0.056	0.053	0.95

## Long term stability

**Table 5:** Results for long-term stability of compounds over 3 months

Compound name	Area ratio (0 Month) (A)	Area Ratio (1 Month)	Area ratio (2 Months)	Area ratio (3 Months) (B)	B/A
Apomorphine	1.000	1.000	1.000	1.000	1.00
Buprenorphine	0.041	0.042	0.032	0.030	0.73
Codeine	0.025	0.026	0.019	0.019	0.76
Dextromoramide	0.542	0.557	0.543	0.526	0.97
Dextropropoxyphene	0.670	0.601	0.514	0.518	0.77
Diclofenac	0.031	0.030	0.029	0.022	0.71
Ethoheptazine	0.056	0.055	0.052	0.049	0.88
Fenoprofen	0.082	0.083	0.066	0.061	0.74
Fentanyl	0.071	0.069	0.063	0.059	0.83
Hydrocodone	0.041	0.036	0.030	0.027	0.66
Hydromorphone	0.019	0.016	0.014	0.013	0.68
Ibuprofen	0.036	0.036	0.031	0.027	0.75
Indomethacin	0.025	0.024	0.021	0.020	0.80
Ketoprofen	0.101	0.097	0.089	0.09	0.89
Ketorolac	0.073	0.073	0.059	0.058	0.79
Mefenamic acid	0.188	0.193	0.180	0.170	0.90
Meloxicam	0.051	0.049	0.046	0.044	0.86
Methadone	1.004	0.992	0.841	0.761	0.76
Morphine	0.001	0.001	0.001	0.001	1.00

**Table 5** continued

Nabumetone	0.091	0.088	0.069	0.067	0.74
Nalbuphine	0.305	0.308	0.288	0.277	0.91
Naproxen	0.021	0.018	0.015	0.015	0.71
Oxycodone	0.016	0.016	0.014	0.011	0.69
Oxymorphone	0.058	0.058	0.054	0.052	0.90
Paracetamol	0.511	0.489	0.431	0.463	0.91
Pentazocine Base	0.081	0.071	0.065	0.064	0.79
Pethidine	0.081	0.069	0.067	0.064	0.79
Phenylbutazone	0.061	0.061	0.057	0.054	0.88
Piroxicam	0.074	0.079	0.056	0.058	0.78
Salicylic acid	0.032	0.032	0.030	0.030	0.94
Sulindac	0.036	0.035	0.029	0.030	0.83
Tenoxicam	0.028	0.026	0.023	0.021	0.75
Tiaprofenic acid	0.027	0.021	0.016	0.021	0.78
Tilidine	0.349	0.345	0.333	0.336	0.96
Tramadol	0.250	0.261	0.250	0.243	0.97

# Chapter 7

## Application of the method

### 7.1 Excretion studies

Urine from an excretion study was available where indomethacin and diclofenac were administered to healthy volunteers. Diclofenac (50 mg) and indomethacin (50 mg) were administered separately during 2 excretion studies and urine was collected at different time intervals as shown in Table 1 below.

**Table 1:** Time intervals for sample collection

Day	Time after administration (hours)	Urine Sample
1	0	U0
	2	U1
	4	U2
	6	U3
	8	U4
	12	U5
2	24	U6
	36	U7
3	48	U8

The urine was extracted and analyzed with the new developed method (page 49 and 54) in 24 hours after the last urine sample was collected. The purpose was to determine if the method was able to identify the known compounds in the urine samples according

to the stipulated criteria for positive identification as described during method validation. The area ratio for diclofenac was plotted against the time interval during the sample collection period. The excretion profile for diclofenac was determined from the results of the excretion study. The area ratio for the metabolite of diclofenac, 4'-hydroxydiclofenac, was plotted against the same time period as used for diclofenac, to determine the excretion profile of the metabolite, 4'-hydroxydiclofenac.

This was also performed for indomethacin. The area ratio for indomethacin was plotted against the time interval during the sample collection period. The excretion profile for indomethacin was determined from the results of the excretion study.

## **7.2 Urine from sports events**

Urine samples from competitors were tested for the listed compounds. These samples were collected from competitors for testing of prohibited substance abuse in sport as determined by WADA. Permission was obtained from the national testing authority, South African Institute for Drug Free Sport, to use some of the urine samples for research purposes during this study. The competitor information for the samples is confidential and not available to the testing facility, therefore no written consent is needed.

The criteria for selection of the urine samples for research purposes included samples collected during sport events where narcotic analgesics and NSAIDs may be used for the treatment of pain associated with excessive muscle use and inflammation. The criteria for selection of the sport events are (a) endurance events, (b) contact sport and (c) strength sport. The samples were randomly selected from these sport events and analyzed without knowing if medication was used during a sport event by the competitor.

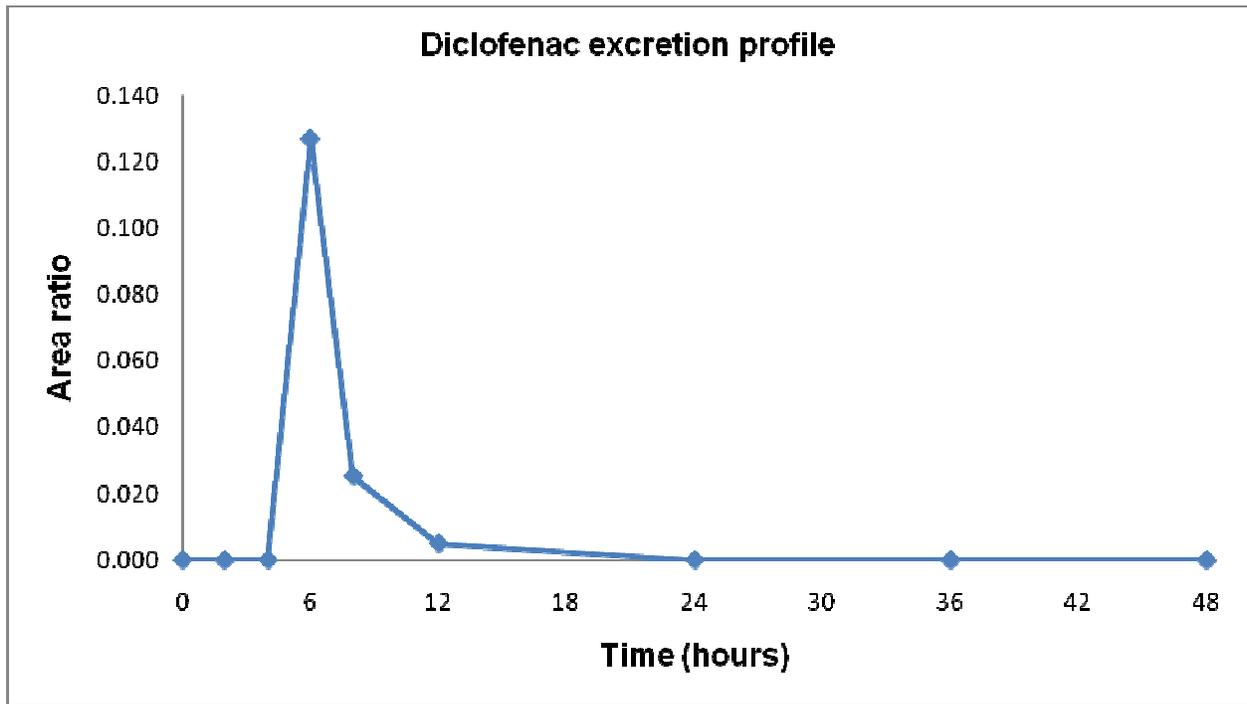
## 7.3 Results

### Urine from excretion studies

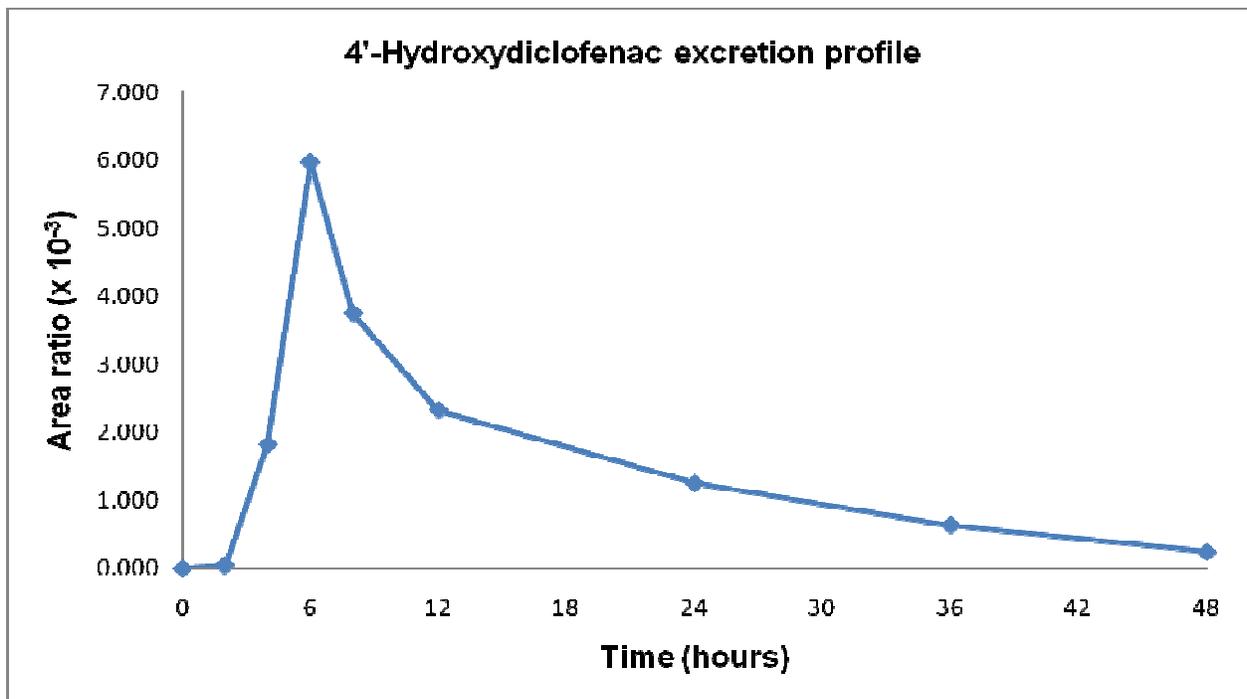
**Table 2:** Summary of the data determined for diclofenac and 4'-hydroxydiclofenac during the excretion study.

<b>Time after administration (hours)</b>	<b>Diclofenac Area ratio</b>	<b>4'-hydroxydiclofenac Area ratio (x 10<sup>-3</sup>)</b>
0	0.000	0.000
2	0.000	0.040
4	0.000	1.830
6	0.127	5.970
8	0.025	3.750
12	0.005	2.320
24	0.000	1.250
36	0.000	0.637
48	0.000	0.245

**Graph 1:** The excretion profile of diclofenac.



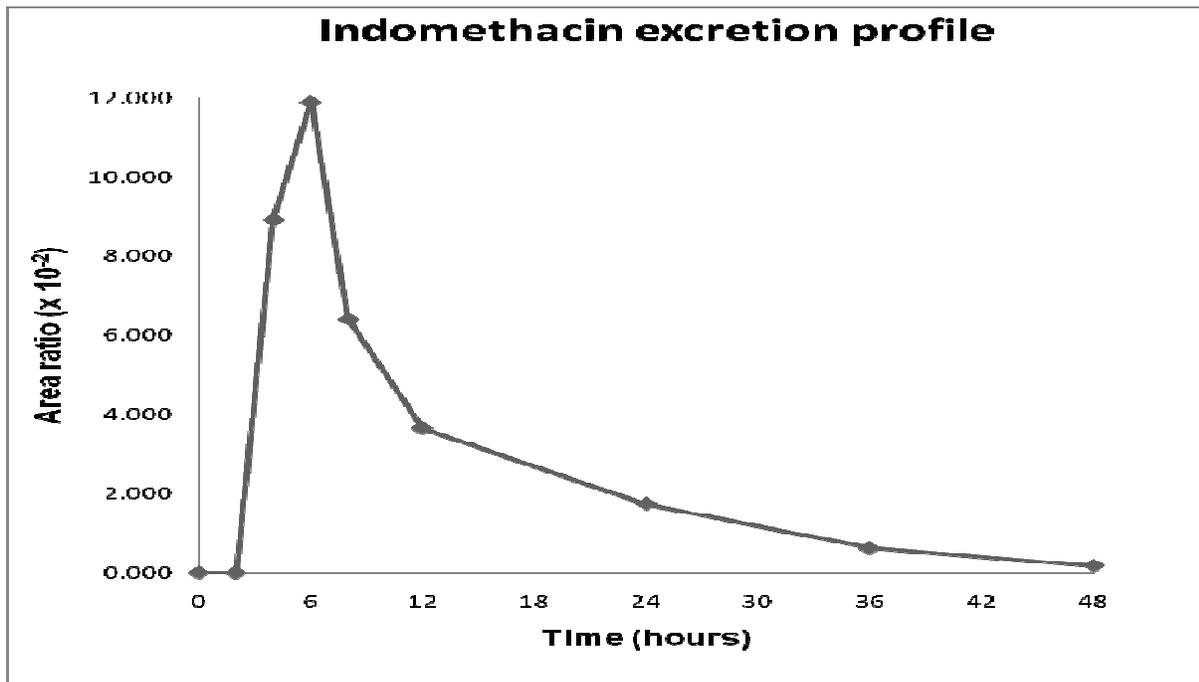
**Graph 2:** The excretion profile of 4'-Hydroxydiclofenac.



**Table 3:** Summary of the data determined for indomethacin during the excretion study.

Time after administration (hours)	Indomethacin Area ratio ( $\times 10^{-2}$ )
0	0.000
2	0.000
4	8.930
6	11.900
8	6.420
12	3.660
24	1.740
36	0.627
48	0.181

**Graph 3:** The excretion profile of indomethacin.



## Urine from sports events

A total of 137 samples were randomly tested for the listed compounds (Table 3, page 40). Table 4 represents the sport events chosen according to the criteria for selection of the sport events and the number of positive samples obtained during the testing of a series of urine samples.

**Table 4:** Summary of sports events and number of samples tested for each event.

Sport event	Number of samples	Number of positive samples	% positive samples
Athletics	27	8	30
Triathlon	8	6	75
Cycling	10	10	100
Rugby	27	20	74
Soccer	17	10	59
Boxing	20	2	10
Wrestling	5	1	20
Powerlifting	15	10	67
Weightlifting	8	8	100

Table 5 represents the compounds identified during the random testing of the series of urine samples. This table lists the compounds identified, the number of samples that were tested positive for that specific compound and the number of samples where a metabolite was detected.

**Table 5:** Compounds identified in samples

<b>Compound name</b>	<b>Number positive samples</b>	<b>Number metabolites identified</b>	<b>% positive samples</b>
Codeine	11	11	22
Diclofenac	12	12	24
Ibuprofen	19	10	37
Paracetamol	23	0	45
Salicylic acid	9	0	18

# Chapter 8

## Discussion

The aim of this study was to develop a method to identify the presence of narcotic analgesics and NSAIDs in urine using LC-MS/MS. To reach this aim several objectives were set.

One of the objectives of this study was to develop one specific method for the identification of an extended list of narcotic analgesics and NSAIDs as described in Chapter 5 page 49 and 54. An extended list of compounds was used and their mass spectrometric properties were determined successfully as summarised in Chapter 5, Table 3, page 40. Not many of the product ions overlapped with one another and enough ions were optimized for identification and confirmation of the listed compounds. Some metabolites were also successfully included in the identification method given in Chapter 5, Table 4, page 42. An Agilent Eclipse 1.8  $\mu\text{m}$  XDB-C<sub>18</sub> column (50 mm x 2.1 mm) with a guard column from Phenomenex® was used during the development phase of this study instead of a 150 mm long column. The purpose was to determine whether this shorter column would be successful in detection and separation of such an extended list of compounds. A mobile phase gradient with 0.01% formic acid (solvent A) and acetonitrile (solvent B) was applied with this shorter column during this study. The specificity for all of the listed compounds was satisfactory, as can be seen in the validation results in Chapter 6, Figure 1, page 62. This shorter column was successfully applied during this study.

The final chromatographic conditions for this study are as follows:

**Autosampler properties:      Agilent 1100 Autosampler**

Injection volume:              10  $\mu\text{l}$

Runtime:                          20 minutes

**Pump method properties: Agilent 1100 Binary Pump**

**Mobile phase: Solvent A:** 0.01% formic acid

**Solvent B:** acetonitrile

Step	Time (min)	Flow rate (µl/min)	A (%)	B (%)
0	0.00	200	90.0	10.0
1	2.00	200	90.0	10.0
2	8.00	200	10.0	90.0
3	9.00	200	90.0	10.0
4	20.0	200	90.0	10.0

A second objective included the optimizing of an extraction method for the detection of the listed compounds. This included the exclusion of the derivatization steps during sample preparation. This exclusion of the derivatization steps was favourable because LC-MS/MS was used during the identification, and no derivatization was therefore needed. A liquid-liquid extraction procedure with diethyl ether was used after enzymatic hydrolysis for 2 hours at 50°C. A hydrolysis step was necessary as some of the compounds and their metabolites are extracted in the urine in the conjugated form, as either glucuronide or sulphates. This objective was achieved because these modifications shortened the sample preparation time needed for rapid analysis of the extended list of compounds.

The final extraction procedure is as follows:

- 2 ml urine
- 1 ml acetate buffer pH = 5.2
- 25 µl β-glucuronidase/arylsulfatase enzyme
- Hydrolyse at 50 °C for 2 hours

- Add 1 ml phosphate buffer pH = 7
- Add 5 ml diethyl ether
- Shake horizontally for 5 minutes on a mechanical shaker
- Centrifuge for 5 minutes
- Transfer organic phase to an ampoule containing 50 µl internal standard (Apomorphine)
- Evaporate to dryness
- Dissolve in 100 µl mobile phase.
- Inject 10 µl into LC-MS/MS

The third objective included the validation of the new method. Every listed compound was identified in every sample according to the criteria for positive identification. The criteria included (a) the retention times of the compounds in the controls, (b) the intensity of the precursor-product ion transition and (c) good peak shape. The validation parameters included in this study were specificity, repeatability, LOD and stability.

Specificity is the ability of the method to detect the compound of interest in urine without the interference of the background or co-extracted compounds. Specificity was determined by extracting 10 control samples together with the corresponding blank sample to identify the listed compounds in the control samples. Good specificity was obtained for each compound using the new method. All of the listed compounds were detected in less than 8 minutes after injection, as can be seen in Chapter 6, Figure 1 (page 62) and Table 1 (page 66). There were no interfering peaks for any of the compounds. If retention times were nearly the same, transitions made the identification of these compounds possible.

For repeatability 5 aliquots from the same control sample at three concentrations (low, medium and high) were analysed. The coefficient of variation (CV%) was calculated for each compound (Chapter 6, Table 2, page 68). For all the listed compounds the CV% was under 15% and therefore the repeatability of the method is good at low, medium and high concentrations.

Limit of detection (LOD) is defined as the lowest concentration of the compound present in the sample which can be detected (identified) from the background of the sample without interference from other compounds or constituents in the extracted sample. The signal to noise ratio (S/N) is calculated and the LOD concentration determined where the S/N is higher than 3. LOD in this study was determined by starting at a high concentration and diluting the urine 1:1 sequentially with blank urine. Relatively low LOD values were calculated for most of the listed compounds (Chapter 6, Table 3, page 70). The dosages of these compounds are relatively high and therefore the excretion in the urine of these compounds and their metabolites is relatively high. The LOD values for paracetamol are relatively high in this method. For paracetamol the background is very high, as can be seen in Chapter 6, Figure 1, page 64, and that contributes to the higher LOD in this method. The LOD value for piroxicam is also higher in this study. A possible reason for this may be that the extraction procedure is not that effective to extract this compound from the urine. The pH adjustment during the extraction procedure may not be effective for extraction of this compound from the urine. However, these LOD values are low enough for the purpose of identification during this study as the compounds are excreted in the urine in relatively high concentrations.

Good freeze and thaw stability were obtained for all of the compounds. An aliquot of the control sample was analysed immediately and the rest of the control sample was frozen at approximately  $-20^{\circ}\text{C}$  for 24 hours. The control sample was defrosted unassisted and placed back into storage. This was repeated three times and the control sample was analysed after the third cycle. The area ratio of the third cycle was compared to the area ratio of the first analysis. The area ratio calculated after 3 cycles showed that the listed compounds (Chapter 5, Table 3, page 40) are stable under freeze and thaw conditions and the method is able to identify the listed compounds after 3 cycles of freeze and thaw. The only area ratio out of the range of 20% was for salicylic acid at 0.75 (Chapter 6, Table 4, page 72). The stability of the compounds in urine was further established during long-term storage. An aliquot of the control sample was analysed immediately and the rest of the control samples was frozen at approximately  $-20^{\circ}\text{C}$  in a freezer. Aliquots of the control sample was analysed after 1, 2 and 3 months of storage. The area ratio's were calculated and compared to the first aliquot analysed (Chapter 6,

Table 5, page 74). Good long-term stability was obtained after 3 months of storage at -20°C for some of the listed compounds. The area ratio between the immediately analysis and the third month of storage were calculated. Eighteen of the listed compounds had a calculated area ratio below 0.8. From these compounds 15 had a ratio between 0.7 and 0.8 and 3 samples between 0.6 and 0.7. After 3 months of storage at -20°C this method can still be used for identification of this extended list of compounds, because only a few compounds calculated area ratios were under 0.7. The results show that the samples can be stored for several months at -20°C without a huge decrease in concentration.

The last objective of this study was to assess the effectiveness of this method during the testing of a series of urine samples. Urine from excretion studies after administration of diclofenac and indomethacin were available. The excretion profile for diclofenac showed that the peak concentration was at 6 hours (U3) after administration of a 50 mg Voltaren dosage (Chapter 7, Graph 1, page 79). The excretion of diclofenac decreased dramatically after 6 hours of administration, with the result that no diclofenac was detected 24 hours (U6) after administration. For 4'-hydroxydiclofenac, the metabolite of diclofenac, the concentration was the highest at 6 hours (U3) after administration of diclofenac, as can be seen in Graph 2, page 79. For the metabolite the excretion decreased slowly from 6 hours and could be still be detected after 48 hours.

For indomethacin the excretion profile showed that the peak concentration of indomethacin was detected 6 hours (U3) after administration (Chapter 7, Graph 3, page 80). The excretion of indomethacin decreased slowly and could still be detected after 48 hours (U8) of administration of a 50 mg dosage of indomethacin.

These results showed that the new method is effective for detection of diclofenac, its metabolite, and indomethacin in urine samples and could also be applied for pharmacokinetic studies.

A further application of the method was done by analysing real samples obtained from competitors in sporting events.

From the 137 urine samples tested, 51 of the samples contained some of the listed compounds (Table 3, page 40). That is 37% of the total samples tested. From these 51 positive samples, 31 samples tested positive for 1 substance used, 14 samples tested positive for 2 substances used and 6 samples contained 3 or more substances used by the competitor. The results are summarised in Chapter 7, Table 4, page 81, where it can be seen that weightlifting and cycling was the sports disciplines that had the most positive samples, followed by triathlon and rugby. Wrestling and boxing had the fewest positive samples. Some of these samples contained more than one compound. Paracetamol was identified in most of the samples, as can be seen in Chapter 7, Table 5, page 82. That is 45% of the total positive samples, followed by ibuprofen which was present in 37% of the total positive samples.

Some samples tested positive for codeine and morphine. Codeine is the pro-drug for morphine, therefore the positive samples for morphine may derive from codeine use, because all the samples that contained codeine, were also positive for morphine. None of the samples contained morphine alone. In all the samples which were positive for diclofenac, the metabolite 4'-hydroxy-diclofenac was also present. Regarding the ibuprofen positive samples, some samples were also positive for the metabolites, hydroxyibuprofen and carboxyibuprofen.

These results showed that the newly developed method is effective in detection of narcotic analgesics and NSAID and can be applied to test urine samples obtained from competitors in sport. This method also showed that some the metabolites mentioned in Chapter 5, Table 4, page41, could be detected with this newly developed method.

The objectives for this study were all successfully achieved.

# Summary

The use of over-the-counter products (OTCs) has increased over the years. This is evident from the wide range of products available and also the easy availability of these OTCs in pharmacies, health shops and even supermarkets in South Africa and elsewhere. Therefore the procurement of these products is easy and they are used by many consumers for self-treatment of numerous conditions. This has given rise to the problem of irresponsible use of these products by consumers. Pain is the most common pharmacological challenge encountered by the medical practitioner and therefore treatment for pain is frequently prescribed. Narcotic analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) are drugs used to relieve pain.

The use and misuse of drugs and medications by competitors in sport has been recognized as an important problem. Ethical aspects of competing under non-equal opportunities are of concern. Elite athletes who turn to doping take the greatest risk to satisfy their burning desire for gold regardless of the health risks involved. Deaths under the influence of drugs and combinations thereof are not uncommon in sport. Athletes might use substances to eliminate any obstacle they might encounter during their training. One of the obstacles they might encounter is pain felt from injury or excessive training. The misuse of drugs for sport enhancement is a health risk because of the kinds of drugs used and the large doses given. The narcotic analgesics and the NSAIDs are some of the drugs abused by competitors to overcome the effects of pain.

The aim of this study was to develop a screening method for the simultaneous detection of an extended list of narcotic analgesics and NSAIDs in human urine using high performance liquid chromatography – tandem mass spectrometry.

An extended list of some of the narcotic analgesics and NSAIDs available in South Africa was compiled. Literature research was done on these reference substances and their metabolites. It was found that information from the literature was limited. The mass spectrometry properties of each reference standard were determined using a LC-MS/MS system in positive electron ionization mode. A new method was successfully optimised using these mass spectrometric properties. Information regarding extraction procedures for these compounds was collected. The extraction procedure included a hydrolysis step with  $\beta$ -glucuronidase/arylsulfatase followed by liquid-liquid extraction with diethyl ether at pH 7. No derivatization steps were necessary during sample preparation, which shortens the preparation time. This extraction procedure was successfully combined with the LC-MS/MS method for the screening of the list of narcotic analgesics and NSAIDs.

The final chromatographic conditions for this study are as follows:

**Autosampler properties: Agilent 1100 Autosampler**

Injection volume: 10  $\mu$ l  
 Runtime: 20 minutes

**Pump method properties: Agilent 1100 Binary Pump**

**Mobile phase:**  
**Solvent A:** 0.01% formic acid  
**Solvent B:** acetonitrile

Step	Time (min)	Flow rate ( $\mu$ l/min)	A (%)	B (%)
0	0.00	200	90.0	10.0
1	2.00	200	90.0	10.0
2	8.00	200	10.0	90.0

3	9.00	200	90.0	10.0
4	20.0	200	90.0	10.0

The final extraction procedure is as follows:

- 2 ml urine
- 1 ml acetate buffer pH = 5.2
- 25 µl β-glucuronidase/arylsulfatase enzyme
- Hydrolyse at 50 °C for 2 hours
- Add 1 ml phosphate buffer pH = 7
- Add 5 ml diethyl ether
- Shake horizontally for 5 minutes on a mechanical shaker
- Centrifuge for 5 minutes
- Transfer organic phase to an ampoule containing 50 µl internal standard (Apomorphine)
- Evaporate to dryness
- Dissolve in 100 µl mobile phase.

Good results were obtained during the validation. Good specificity was obtained for each compound without the interference of the background or co-extracted compounds. Good repeatability was obtained for the listed compounds. The calculated CV% for all the compounds was under 15% for low, medium and high concentrations. The calculated LOD values was relatively low for most of the compounds, with exception of paracetamol, which has a high back-ground and piroxicam which could not be extracted effectively from the urine. The freeze and thaw stability of the listed compounds was satisfactory with only salicylic acid which was outside the range of 20% after 3 cycles of freeze and thaw. For long term stability the results were different. The results for the analysis of the control sample after 3 months of storage at -20°C were below 80% for 18 compounds when compared to the sample analysed immediately before storage. From these, 15 compounds were between 70 and 80% and 3 compounds were between 60 and 70%.

The method was applied to obtain the excretion profiles for diclofenac, its metabolite, 4'-hydroxydiclofenac, and indomethacin. For diclofenac the peak excretion was determined at 6 hours after administration with a sharp decrease of excretion until 24 hours after administration. For its metabolite, 4'-hydroxydiclofenac, the peak excretion was also determined at 6 hours after administration, but with a slower decrease from 6 hours and can still be detected after 48 hours. For indomethacin the peak excretion was determined at 6 hours after administration with a slow decrease from 6 hours and can still be detected after 48 hours.

A further application of this screening method was done by analyzing a series of 137 real urine samples collected from competitors from different sporting events. The results showed that there is a large number of these substances used by competitors in sport. The number of positive samples was 37% of the total number of samples tested. Rugby with 39% of the positive samples was identified as the sporting event with the highest usage of these substances, with paracetamol identified as the substance that is the most frequently used.

It can be concluded that this method is effective in detection of the listed narcotic analgesics and NSAIDs in urine from human.

**Keywords:** pain, narcotic analgesics, non-steroidal anti-inflammatory drugs, analytical method, metabolites, screening, LC-MS/MS, prohibited list, validation, excretion profile

# Opsomming

Daar is 'n toename in die gebruik van oor-die-toonbank geneesmiddels. Dit word waargeneem in die groot verskeidenheid van geneesmiddels beskikbaar in apteke, gesondheidswinkels en kettingwinkels in Suid Afrika en elders. Die geneesmiddels is maklik sonder voorskrif verkrybaar en daarom is die gebruik van die geneesmiddels vir verskeie mediese toestande baie maklik. Pyn is een van die algemeenste uitdagings waarmee die algemene praktisyn te doen kry en daarom word baie van die geneesmiddels vir die behandeling van pyn voorgeskryf. Narkotiese analgetika en nie-steroïed anti-inflammatoriese middels word vir die behandeling van pyn gebruik.

Die misbruik van hierdie geneesmiddels deur atlete is as 'n groot probleem geïdentifiseer. Die etiese aspekte om deel te neem onder nie-gelyke vermoëns is 'n probleem. Die atlete misbruik verskeie geneesmiddels om hul doel te bereik en neem nie hulle gesondheid in ag nie. Ernstige nagevolge a.g.v die misbruik van die geneesmiddels in sport is nie ongewoon nie. Atlete gebruik die geneesmiddels om enige struikelblokke te oorkom wat hulle mag teëkom gedurende oefensessies of gedurende 'n sportbyeenkoms. Pyn is geïdentifiseer as een van hierdie struikelblokke. Die pyn mag die gevolg wees van oormatige oefening of 'n besering. Narkotiese analgetika en nie-steroïed anti-inflammatoriese geneesmiddels word deur die atlete vir die behandeling van pyn gebruik.

Die doel van hierdie studie is die ontwikkeling van 'n metode vir die gelyktydige identifisering van 'n uitgebreide lys van narkotiese analgetika en nie-steroïed anti-inflammatoriese middels in uriene deur middel van hoë-druk-vloeistof- chromatografie en tandem massaspektometrie.

'n Uitgebreide lys van beskikbare narkotiese analgetika en nie-steroïed anti-inflammatoriese middels in Suid Afrika is saamgestel en literatuurstudies is gedoen oor die middels en hulle metaboliete. Die beskikbare inligting is egter beperk. Massaspektrometrie is op elk van die geneesmiddels uitgevoer deur middel van positiewe elektronionisasie gedurende vloeistofchromatografie en hierdie massa spektrometriese inligting is gebruik om 'n nuwe metode te optimiseer. Inligting i.v.m ekstraksiemetodes is verkry uit die literatuurstudies. Die ekstraksiemethode sluit 'n hidrolisestap met  $\beta$ -glukuronidase/arielsulfatase ensiem in, wat gevolg word deur 'n vloeistof-vloeistof ekstraksie met diëtleter by pH 7. Geen derivatisering is nodig nie, wat die voorbereidingstyd verkort. Die ekstraksie- en chromatografiemethode is suksesvol gekombineer vir die identifisering van narkotiese analgetika en nie-steroïed anti-inflammatoriese middels in uriene.

Die finale chromatografiese toestande vir die studie sluit in:

**Autosampler toestande:      Agilent 1100 Autosampler**

Inspuitvolume:                      10  $\mu$ l  
 looptyd:                                20 minute

**Pomp metode : Agilent 1100 Binary Pump**

**Mobiele fase:    Oplossing A:**            0.01% mieresuur

**Oplossing B:**            acetonitriël

Step	Tyd (min)	Vloeitempo ( $\mu$ l/min)	A (%)	B (%)
0	0.00	200	90.0	10.0
1	2.00	200	90.0	10.0
2	8.00	200	10.0	90.0
3	9.00	200	90.0	10.0
4	20.0	200	90.0	10.0

Die finale ekstraksiemetode is soos volg:

- 2 ml urien
- 1 ml asetaat buffer pH = 5.2
- Voeg 25  $\mu$ l  $\beta$ -glucuronidase/arylsulfatase ensiem by
- Hidroliseer by 50 °C vir 2 uur
- Voeg 1 ml fosfaatbuffer (pH = 7) by
- Voeg 5 ml diethyleter by
- Skud horisontaal vir 5 minute op a meganiese skudmasjien
- Sentrifugeer vir 5 minute
- Dra organisefase oor na 'n ampule wat 50  $\mu$ l interne standaard (Apomorphine) bevat
- Damp droog
- Los op in 100  $\mu$ l mobiele fase
- Spuit 10  $\mu$ l in op LC-MS/MS

Betroubare resultate is gedurende validasie van die metode verkry. Betroubare spesifisiteit vir die geneesmiddels is verkry met geen inmenging vanaf endogene verbindings nie. Betroubare herhaalbaarheid is verkry gedurende validasie van die metode. Die koëffisiënt van variasie vir al die geneesmiddels is bereken en was minder as 15% vir lae, medium en hoë konsentrasies van die geneesmiddels. Die berekende konsentrasie vir die laagste konsentrasie benodig vir identifisering is baie laag vir al die geneesmiddels, met uitsondering van parasetamol wat 'n hoë agtergrond het en piroksikaam wat nie voldoende ekstraheer vanuit die uriene nie. Die vries-ontvries stabiliteit van die lys geneesmiddels was voldoende, met uitsondering van salisielsuur wat buite die perk van 20% was na 3 siklusse van vries-ontvries. Die langtermynstabiliteit van die kontrolemonster na 3 maande van berging by -20°C was vir 18 geneesmiddels onder 80% as dit vergelyk word met die kontrolemonster voor dit geberg is. Van hierdie 18 geneesmiddels was 15 tussen 70 en 80% en 3 geneesmiddels tussen 60 en 70%.

Hierdie metode is gebruik om die uitskeidingsprofiel van diklofenak, en sy metaboliet, 4'-hidroksidiklofenak, asook indometasien te verkry. Die hoogste uitskeiding van diklofenak is 6 uur na toediening verkry, met 'n skerp afname tot by 24 uur na toediening. Vir die 4'-hidroksidiklofenakmetaboliet is die hoogste uitskeiding 6 uur na toediening verkry, met 'n geleidelike afname na 6 uur. Die metaboliet kan na 48 uur nog aangetoon word. Vir indometasien is die hoogste uitskeiding 6 uur na toediening verkry en kon dit 48 uur na toediening nog steeds ge-identifiseer word.

'n Verdere toepassing van die metode was die toets van 137 sportmonsters versamel vanaf atlete wat deelgeneem het aan verskeie sportbyeenkomste. Die resultate dui aan dat 'n groot aantal van die geneesmiddels gebruik word deur atlete. Van die sportmonsters wat getoets is, was 37% positief vir die gebruik van die geneesmiddels. Rugby met 39% van die positiewe resultate, is ge-identifiseer as die sportsoort waar die meeste geneesmiddels gebruik word. Parasetamol is geïdentifiseer as die geneesmiddel wat die meeste gebruik word.

Die ontwikkelende metode is 'n omvattende en veelsydige metode wat effektief gebruik kan word vir die identifisering van narkotiese analgetika en nie-steroïed anti-inflammatoriese middels in uriene.

**Sleutelwoorde:** pyn, narkotiese analgetika, nie-steroïed anti-inflammatoriese middels, analitiesemetode, metaboliete, siftingsmetode, LC-MS/MS, verbode lys, validasie, uitskeidingsprofiel