METHOD DEVELOPMENT AND APPLICATION OF NOVEL ANALYTICAL TECHNIQUES FOR DETERMINING ILLICIT AND THERAPEUTIC DRUG USE

Thesis Submitted in partial fulfilment for the degree of Doctor of Philosophy

By

Syeda Amena Batool Shah

Faculty of Science, Engineering and Computing

School of Pharmacy and Chemistry

Kingston University London, UK

July 2014

Dedicated to my parents Syed Zulfiqar Ali Shah and Sameena Yasmeen

ABSTRACT

The research described in this thesis is based on novel analytical approaches to develop new methods for determining psychoactive and therapeutic drugs in biological matrices. All the analytical methods developed were according to Food and Drug Administration (FDA) guidelines.

The first project involved development and validation of an analytical method for quantification of psychoactive drug mephedrone and its two metabolites 4-methylephedrine and 4-methylnorephedrine in human hair using liquid chromatography tandem mass spectrometry (LC–MS/MS). Recent abuse of designer drugs such as mephedrone has presented a requirement for sensitive, reliable and reproducible analytical methods for the detection of these controlled drugs in different matrices. Based on the similar structure of mephedrone to other methcathinones and amphetamines, the study also proposed a similar metabolic pathway for mephedrone. The method developed can be of great value for the future detection of mephedrone and its two metabolites in human hair.

Having completed the above, another analytical method was developed capable of detecting 0.6 ng/mg abacavir and tenofovir in human hair using LC-MS/MS. The method was successfully validated for the intraday precision, interday precision, and limit of detection, accuracy and extraction recovery. This is the first full report of a method for the simultaneous determination of these two key antiretroviral drugs in hair. The newly developed method is useful for future routine analysis of tenofovir and abacavir in human hair and could be used in therapeutic drug monitoring and adherence to medicines studies, which would be helpful in decision making regarding treatment change in combination anti-retroviral therapies.

The last project focused on analysing dietary substances such as green and white tea, fruit juices along with catechins present in tea and corticosteroids in order to investigate potential inhibitory effects on the glucuronidation of β_2- agonists clenbuterol and formoterol. β_2- agonists are frequently prescribed for the treatment of asthma in athletes. Due to performance-enhancing effects, these β_2- agonists have been subjected to restrictions in sport. A glucuronidation method using human liver microsomes (HLM) and uridine 5'-diphospho-glucuronosyltransferase (UGT2B17) has been used. The study shows that common dietary products and the catechins present in tea inhibit the glucuronidation activity of formoterol which effects/alters the actual drug concentration when testing in human body/urine and may help in masking formoterol misuse therefore having implications on current doping control in sport.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

DECLARATION

This is to declare that this doctoral thesis and the work presented in it is based upon original research conducted by the author in the School of Pharmacy and Chemistry at Kingston University London between July 2010 and July 2013. The work done has not been submitted anywhere for any award other than part of it for the publication in scientific literature. Other contributions made to the thesis have been acknowledged as text or by references.

LIST OF PUBLICATIONS

Publications arising from Chapter 3:

1. **Shah SA**, Mullin R, Jones G, Shah I, Barker J, Petroczi A, et al. Simultaneous analysis of antiretroviral drugs abacavir and tenofovir in human hair by liquid chromatography tandem mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis 2013;74:308-313

2. Petróczi A, Nepusz T, Cross P, Taft H, **Shah S**, Deshmukh N, et al. New non-randomised model to assess the prevalence of discriminating behaviour: a pilot study on mephedrone. Substance Abuse Treatment, Prevention, and Policy 2011;6(1):20

Publication arising from Chapter 4:

3. **Shah SA**, Deshmukh NI, Barker J, Petróczi A, Cross P, Archer R, et al. Quantitative analysis of mephedrone using liquid chromatography tandem mass spectroscopy: Application to human hair. Journal of Pharmaceutical and Biomedical Analysis 2012;61:64-69

LIST OF ABBREVIATIONS

β-ketoamphetamine	Beta-ketoamphetamine
APCI	Atomic pressure chemical ionisation
API	Atmospheric pressure ionisation
APPI	Atomic pressure photonisation
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
cART	Combination antiretroviral therapy
CID	Collision induced dissociation
CNS	Central Nervous System
DC	Direct-current
DNA	Deoxyribonucleic acid
EIA	Exercised-induced asthma
ESI	Electrospray ionisation
FDA	Food and drug administration
GC	Gas chromatography
GC-MS	Gas chromatography mass spectrometry
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HLM	Human liver microsomes
HPLC	High performance liquid chromatogrpahy
HPLC-UV	High-performance liquid chromatography with UV Detector
I_0	Incident light
IS	Internal standard
LC	Liquid chromatography

LC-MS	Liquid chromatography - mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLE	Liquid-liquid extraction
LLOD	Lower limit of detection
LLOQ	Lower limit of quantification
mg	Milligram
mL	Millilitre
mM	Millimolar
MS	Mass spectrometry
m/z	Mass to charge ratio
µL	Microlitre
µm	Micrometer
µM	Micromolar
µL/min	Microlitre per minute
mg/kg	Milligram per kilogram
mg/mL	Milligram per millilitre
min	Minute
ng/mg	Nanogram per milligram
NNRTI	Non-nucleoside reverse transcriptase inhibitor
NRTI	Nucleoside reverse transcriptase inhibitor
NtRTI	Nucleotide reverse transciptase inhibitor
PDA	Photodiode array
PI	Protease inhibitors
pg/mg	Picogram per milligram
psi	Pressure per square inch

QC	Quality control
RF	Radiofrequency
r^2	Regression coefficient
RSD	Relative standard deviation
RT	Retention time
SIM	Selected ion monitoring
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
S/N	Signal to noise ratio
TB	Tuberculosis
TDM	Therapeutic drug monitoring
TIC	Total ion current
TUE	Therapeutic use exemption
UDPGlcA	UDP-glucuronic acid
UGT	Uridine 5'-diphospho-glucuronosyltransferase/ UDP-glucuronosyltransferases
UV	Ultra violet
WADA	World anti-doping agency

ACKNOWLEDGEMENTS

First of all my utmost thanks to my supervisors Dr. James Barker, Professor Declan Naughton and Professor Andrea Petroczi for their consistent guidance and support throughout my research and also helping in providing samples for the studies.

Thanks to Dr Julian Swindon for his advice and technical assistance in the laboratory. Finally, I would also like to acknowledge my research colleagues and family for their continued support and words of encouragement throughout my research.

CHAPTER 1

INTRODUCTION

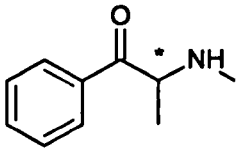
1.1 Psychoactive drugs

Psychoactive drugs are chemical substances that primarily act upon the Central Nervous System (CNS) by altering the brain function leading to temporary changes to perception, behaviour, and consciousness. Recently, new classes of designer drugs synthesised to mimic the effects of psychoactive drugs have become very popular among drug users (1). These drugs can be referred to as "bath salts", "plant feed" or 'legal high', a term given to chemicals, which possess psychoactive activity in humans (2). In the last few years, buying legal highs from the Internet has become common practice for drug users. This is, in part, due to their structural similarity to controlled psychoactive substances such as phenethylamines and cathinones (3-5).

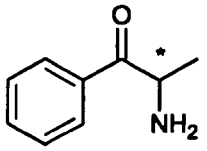
Among these designer drugs are the synthetic cathinones (1). Cathinone is a naturally occurring beta-ketone amphetamine analogue that can be extracted from the leaves of an African and Middle Eastern plant *Cathaedulis* or 'Khat' (3,6). The alkaloid content of the fresh leaves is responsible for the psychoactive stimulating effects. Alterations to the core chemical structure and functional group substitutions of the parent cathinone compound has led to a large number of these synthetic cathinone derivatives. Among these cathinone psychostimulants, mephedrone has gained most popularity and is very commonly abused (1).

1.1.1 Mephedrone

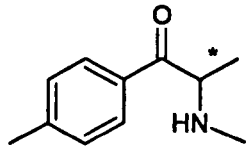
Mephedrone, sometimes referred to as 'meow', 'mcat' and 'bubbles' is a β-ketoamphetamine and has a structure similar to methcathinone, which in turn is the N-methyl analogue of the natural product cathinone (4).



Methcathinone



Cathinone



Mephedrone

In April 2010, it was specifically named and hence controlled under the Misuse of Drugs Act 1971 as a Class B substance in the UK (7-9). It has also been banned in the majority of other European countries (3).

The popularity of use of these psychoactive drugs has created a demand for sensitive, robust and reliable analytical methods for their identification and quantification in different matrices. Gas chromatography mass spectrometry (GC-MS) has routinely been used for the detection of mephedrone in matrices such as blood and urine (5,8,10).

1.1.2 Source

The major source for these designer drugs is the Internet and local dealers. But after classification of the drug in the UK, there is a significant decrease in the number of online selling websites of mephedrone (11). Although, some studies suggest that after the legal classification of the drug, there is no significant effect on control and availability of mephedrone as users have started buying the drug from street dealers at double the price instead of buying it from the Internet. One study suggested that after the restrictions by law within the UK, the demand of buying from local dealers

has increased (11-13). These synthetic cathinones are available in pill, capsule as well as in powder form. The change in legal status has also caused in an increase in the price of these drugs including mephedrone (2).

1.1.3 Prevalence and administration routes

The main route of administration for these legal highs is nasal ingestion. For mephedrone one of the ways is called "bombing" where it is wrapped in a cigarette paper and swallowed. Other ways include rectal, gingival, inhalation, intramuscular and intravenous injections. The use of mephedrone is more common in males than females and is reported to be one of the most commonly abused psychotropic drugs (2,7). A British crime survey showed that the prevalence of mephedrone is more common in young people, aged 16-24 at 4.4%, which is similar to the prevalence of cocaine and has been chosen as their favourite drug (11).

The effects of mephedrone are similar to those of cocaine, amphetamine and MDMA. Users of this drug report the peak effect is usually after about 20 – 30 minutes and is followed by a strong urge for another dose (14).

1.1.4 Mechanism of action

Compared to its parent compound cathinone, mephedrone is a weak psychomotor stimulant. Studies in rats showed that brain concentrations of dopamine after giving a single dose of 3 mg/kg of mephedrone peaked in 20 minutes and returned to initial conditions in 100-120 minutes. This rate when compared to MDMA and amphetamine is much faster. When compared to MDMA mephedrone was less potent at increasing serotonin concentrations in brain but caused a greater increased in dopamine concentrations (2).

1.1.5 Pharmacology and toxicity

Only a few studies have been carried out on laboratory animals, which show that mephedrone affects the extracellular dopamine and serotonin in the nucleus accumbens and enhances the two, which leads to locomotor activation (13).

In vitro studies on rat brain show that mephedrone is a non-selective substrate for plasma membrane monoamine transporters. Mephedrone also induces transport-mediated monoamine release through the reversal of normal transporter flux. In rats, doses that stimulate locomotor activation also increase extracellular dopamine levels in nucleus accumbens. Locomotor stimulant properties are dependent on increased dopamine transmission (13). Mephedrone stimulates the release and uptake of monoamine neurotransmitter (15). A recent study has suggested that mephedrone displays lower selectivity to release dopamine and norepinephrine as compared to methcathinone (16).

Due to the structural similarity between mephedrone and methyl cathinone, it is very likely that mephedrone will have the same effects and could lead to neurotoxicity. A recent detailed study in the structural similarities between mephedrone and other psychoactive drugs showed that street mephedrone is a racemic mixture, which gives rise to a possibility of its toxicity towards dopamine and serotonin neurons. This could be the reason behind the number of death cases caused by this drug (17).

1.1.6 Metabolism of mephedrone

There is limited information available on the metabolism of mephedrone in humans. As the structure of mephedrone is substantially similar to other psychoactive drugs such as methcathinone and methamphetamine, one can tentatively propose a metabolism of mephedrone on a similar basis. There are a number of different

pathways by which this drug could be metabolised. The two metabolites analysed in this study are 4-methylephedrine and 4-methylnorephedrine (10,18-21). More detail about the nature, prevalence, pharmacology and toxicity of mephedrone has been discussed in Chapter 3.

Due to recent abuse of these designer drugs, there is a requirement for sensitive, reliable and reproducible methods for the detection of these controlled drugs in different matrices. Therefore this thesis describes the development of a quantitative method for the detection of mephedrone and its two metabolites in hair using LC-MS/MS.

1.1.7 Aims of the study

Owing to the frequent use and popularity of psychoactive drugs such as mephedrone, a requirement exists for sensitive and reliable analytical methods for the detection of these compounds in biological samples other than blood and urine. The validity of blood and urine analysis is dependent on the elimination half-life of the compound in question. A drug with a short elimination half-life is not likely to be detected for more than a few days in either blood or urine. Therefore, urine and blood testing will only provide a reliable answer for short-term recent exposure (22). Hair analysis has the potential to reliably determine psychotropic drugs covering prolonged periods depending on the length of hair analysed. Hence, this study presents the development of a novel sensitive analytical method for quantification of mephedrone and its metabolites 4-methylephedrine and 4-methylnorephedrine in human hair using LC-MS/MS.

1.2 Antiretroviral drugs

The very first antiretrovirals used against human immunodeficiency virus (HIV) infection are nucleoside reverse transcriptase inhibitors (NRTIs). They prevent the synthesis of viral deoxyribonucleic acid (DNA) by the reverse transcriptase enzyme (23,24). The treatment usually comprises of two or three combination NRTIs for an active antiretroviral therapy (23).

The major therapeutic families that are used for antiretroviral therapy comprise of a combination of nucleotide reverse transciptase inhibitors (NtRTIs), NRTIs, non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs).

These are used as combination antiretroviral therapy (cART)/highly active antiretroviral therapy (HAART) for treating HIV. These act to inhibit multiple viral targets and are the most recently approved integrase inhibitors used for antiretroviral treatment for patients with viral resistance (25-27). The development of resistance to these drugs when used in combination therapy is complex, with each class being postulated to develop resistance at varying levels of adherence to therapy (25).

Abacavir and tenofovir are prodrugs that belong to these families. Tenofovir is a nucleotide reverse transcriptase inhibitor and abacavir is a nucleoside reverse transcriptase inhibitor and thus are key components of cART regimes. One of these two drugs or both are co-administered with other NRTI's and PI's in the present patients' samples used in Chapter 4 (23,28). The effect of these drugs depends on the degree of metabolic phosphorylation (29).

1.2.1 Therapeutic Drug Monitoring (TDM)

Compliance to medication is very important for improved clinical outcomes for a range of conditions including tuberculosis (TB) infections and treating HIV patients

(23,25,29-33). Therapeutic drug monitoring (TDM) has been used to aid cART/HAART in order to check adherence, monitor viral resistance and to individualise dose regimens (26,34). The major reasons for the failure of these therapeutic drugs are the non-compliance to the treatment and resistance mutations to these drugs. For anti-HIV treatments, a major reason for lower efficacy of these drugs is non-compliance to the treatment associated with the development of drug resistance (25). In this thesis, a quantitative method was developed for the above HIV drugs in hair using LC-MS/MS in order to monitor patient's compliance to medical treatment and also to inform clinical practises where they can arrange programmes that can help such patients to regularly follow their treatment.

Different analytical methods have been developed using a range of instrumentation for the analysis of antiretroviral drugs. Amongst these, LC-MS/MS has gained an increased popularity due to its high sensitivity, specificity and shorter analysis times (34). LC-MS/MS is a very useful instrument and plays a major role in HIV TDM in different matrices such as hair, plasma and urine (31,35). Due to the advantages associated with this technique, LC-MS/MS is widely used for TDM of antifungals, antidepressants, antibacterial and antivirals (31).

1.2.2 Mechanism of action

After metabolic phosphorylation to their monophosphate, diphosphate and triphosphate forms of NRTIs, they interact with the substrate-binding site of the enzyme. The triphosphate will act as a competitive inhibitor where it will inhibit incorporation of the substrate into the growing DNA chain and will lead to the termination of the chain (36).

NtRTIs differentiate from the NRTIs in their phosphorylation steps. Compared to NRTIs these only need two phosphorylation steps in order to convert to their active form. Phosphorylation of tenofovir to its diphosphate acts as an obligate chain terminator in the reverse transcriptase reaction (36).

1.2.3 Aims of the study

Past studies have shown quantification of antiretroviral drugs in matrices such as plasma, which provides short-term information on the exposure to these medications. Hair analysis provides information over a longer period of time and can help in better predictive treatment outcomes of HIV therapy (37).

The aim of this study is to develop and validate an LC-MS/MS method for the quantification of abacavir and tenofovir in human hair. This is a part of a larger observational study and could help investigate the factors that may lead to adherence/non-adherence to medical therapy in HIV patients (38).

1.3 β_2 – adrenoceptors and β_2 – agonists

In humans, the β – adrenoceptor is located on the arm of chromosome 5 and is a member of 7 – transmembrane family of receptors. It is composed of 413 amino acid residues. These adrenoceptors are subdivided into three categories i.e. β_1, β_2 and β_3, where the second category is for airway smooth muscles. β – adrenoceptors are widely found in airway smooth muscle cells, lung epithelial and endothelial cells as well as type II and mast cells (39).

Biologically active β_2-agonists must consist of a substituted six-membered aromatic ring, a hydroxyl group bonded to a β – carbon in the R configuration, a positively charged nitrogen in the ethylamine side chain and a bulky substituent on the aliphatic

nitrogen in order to have specificity for the β – adrenergic receptors. The physiological activity of β_2 – agonists depends on its binding to the receptor at three points i.e. β – hydroxyl group, aliphatic nitrogen and the aromatic ring as well as its absorption, metabolism, distribution and elimination (40).

1.3.1 β_2 – agonists in sports

Beta-2-agonists play a vital role in the management of asthma for the athletes in order for them to perform their respective sports. These β_2 – agonists, if misused, can have doping effects that can both improve performance and have anabolic effects on the muscles. Therefore, the World Anti-Doping Agency (WADA) has put restrictions on the use of these β_2 – agonists. Long-acting beta-2-agonists such as clenbuterol and formoterol have been forbidden or restricted for use in sports (41,42). Some of these β_2 – agonists can still be obtained for medical treatment by applying for Therapeutic Use Exemption (TUE) process, where athletes can obtain approval to use a prescribed prohibited substance for the treatment of any medical condition (43).

1.3.2 Clenbuterol

Clenbuterol is a potent β_2 – agonist and its main use is as tocolytic and bronchodilator agent. Clenbuterol belongs to a family of compounds that were firstly used for their anti-asthamatic properties (39,44). However, WADA prohibits its use in sports due to its anabolic properties. Its administration could lead to other physiological effects such as increase in muscular mass and decrease in the body fat. The therapeutic use of clenbuterol is prohibited in humans but is illegally used as a growth-promoting agent in farm animals owing to its anabolic effects and may pose a potential risk for consumer health (44,45). Therefore use of β_2 – agonists are banned

in the EU (45,46). Consumption of contaminated meat can lead to the detection of clenbuterol in human urine samples, which can have very serious consequences for athletes (44). It can also enhance aerobic capacity and stimulate the CNS as well as blood pressure and oxygen transportation (39,44).

1.3.3 Mechanism of action

Clenbuterol is a sympathomimetic agent with specific affinity for the β_2 – adrenoceptors with a prolonged bronchodilator activity. Pharmacokinetic studies in humans showed a plasma half-life of about 35 hours (47). Other than bronchodilator activity, two major contributions towards performance enhancement of clenbuterol are rate of muscle growth and lipolysis. Though there are contradictions and specie dependent responses between different studies carried out regarding its mode of action, the above two advantages are what interests athletes the most (48). Studies on rats have shown that clenbuterol causes increase in volume of skeletal, cardiac muscle, rather than cell profileration (48). Having a balance of protein production with protein degradation enhances the efficacy of muscle growth. Some studies have proved that there is an increase in concentration of specific inhibitors of proteolytic proteinases (48).

1.3.4 Formoterol

Formoterol is a long acting, potent selective β_2 – agonist prescribed for inhalation only. Compared to short acting β_2 – agonists, this drug has a rapid onset of action with a long duration of 12 hours. β_2 – agonists generally possess potential performance enhancing activity (49,50). Owing to this, it is very beneficial for

asthmatic athletes performing in endurance sports (41). For this reason formoterol has been placed on the list of prohibited substances by WADA (51,52).

Structurally, formoterol ((RR)-(9/)-N-[2-hydroxy-5-[1-hydroxy-2-[[2-(4-methoxypheny)-1-methylethyl]amino]ethyl]phenyl] formamide) is a phenyl ethylamine derivative with one phenolic hydroxyl and one secondary amino group (Table 2.1). It is marketed as racemate of RR + SS enantiomers, where RR is the active form of the drug that has anti-bronchoconstrictor activity (42).

1.3.5 Mechanism of action

Inhalation of formoterol acts in the lung as a bronchodilator. The pharmacologic effects includes stimulation of intracellular adenyl cyclase, enzyme that catalyzes the conversion of adenosine triphosphate (ATP) to cyclic-3', 5'-adenosine monophosphate (cyclic AMP) (53).

Increased cyclic AMP levels leads to relaxation of bronchial smooth muscle and inhibition of release of mediators of immediate hypersensitivity from cells, especially from mast cells. An *in vitro* study showed that formoterol is an inhibitor for the release of mast cell mediators, such as histamine and leukotrienes, from the human lung. Formoterol also inhibits histamine-induced plasma albumin extravasation in anesthetized guinea pigs and inhibits allergen-induced eosinophil influx in dogs with airway hyper-responsiveness (53).

1.3.6 Aims of the study

Recent studies revealed that green tea could suppress testosterone metabolism, *in vitro*, by inhibiting a key enzyme UGT2B17, which may result in toxicity or false negative doping tests. The final section of this thesis aims to investigate whether

substances containing phenolic compounds such as green and white tea, fruit juices and also the pharmaceutical products that are given along with these β_2 – agonists for the treatment of asthma have an effect on the glucuronidation of clenbuterol and formoterol and its implications for doping control (54).

Tea contains a number of polyphenol compounds including catechins. White tea has shown remarkable activities in a range of enzyme assays pertaining to oxidative damage and inflammation (54). Fruit juices are rich in different phenolic compounds such as flavanols, flavan-3-ols, anthocyanins, hydroxycinnamates and some catechins (55).

Corticosteroids are sometimes given with β_2 – agonists as combination therapy for the treatment of asthma (56). In recent years, the use of inhaled corticosteroids along with β_2- agonists as combination therapy has become more common in athletes (57). Therefore, along with dietary components, we will investigate the effects of some selected corticosteroids on the rate of glucuronidation.

Currently, there is no published literature for the inhibition of glucuronidation activity of clenbuterol and formoterol using any dietary substance. Given the inhibitory effects of green tea and catechins present in green tea on testosterone, it is conceivable that green and white tea along with other dietary products (fruit juices), and also catechins contained in tea and pharmaceutical products (corticosteroids) could have an effect on the metabolism of clenbuterol and formoterol.

1.4 Hair analysis

Using hair analysis for the detection of drugs has been in practice for more than two decades and has gained attention in several fields such as toxicology, forensic science, clinical chemistry as well as doping control (58). It is popular due to its

convenience of sample collection and storage, as it does not decompose like other matrices such as fluids and tissues (58).

1.4.1 Hair physiology

Hair is considered as an annex of skin and originates from the hair follicle where matrix cells form the germination centre (58). Hair consists of a hair shaft made up of tightly compact cells; these are produced from follicles that are small sac-like organs (59). These hair follicles are found 3-4 mm below the skin surface, where blood capillaries surround the follicles. The hair follicles are associated with three major glands i.e. apocrine, sebaceous (these two gland keeps the hair moist within the skin surface) and sweat (keeps the hair moist above the skin surface) glands (58). The inner core of the follicle is called the bulb where there is very fast cell division (every 23-72 hours) (59). The major components of hair are keratins, which is a fibrous protein, melanin and lipids. These matrix cells produce several layers of hair shaft i.e. cuticle, cortex and medulla (58).

The volume of the cell in the bulb increases with time and become elongated and starts to move up into the keratin consisting area. At this stage, the cells start to produce melanin (pigmentation) and keratinize i.e. start enriching in sulphur-rich proteins (keratins). The long fibres produced by keratin are bound together and interlinked with other proteins forming disulphide bonds (-S-S) (59).

The hair shaft consists of three different types of keratin rich cells as shown in Figure 1.1. The outer most layer is known as the cuticle and is responsible for protecting the inner layers from external damages such as heat, light etc. The second layer is called the cortex and is responsible for forming the hair shaft; this layer also contains the

pigment granules that contain melanin. The inner layer is the medulla consisting of medullar cells (59).

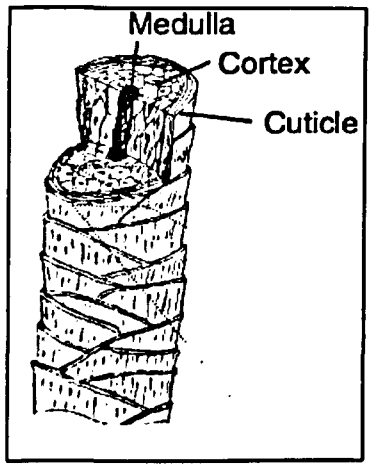


Figure 1.1: Structure of human hair shaft (60)

1.4.2 Hair growth

Hair grows in three stages; the active growing stage known as the anagen, during which the hair follicle is developed to produce hair. The second stage is the transition stage, also known as the catagen, where the follicle bulb activity is halted and lastly the resting stage called the telogen, after which another cycle starts (Figure 1.2). The anagen stage for humans are 4-8 years, the catagen is several weeks and the telogen 4-6 months. The scalp hair growth rate is 0.6-1.4 cm per month. Hair from different parts of the body has different growth rates and also depends on race, sex, age and health (58,61).

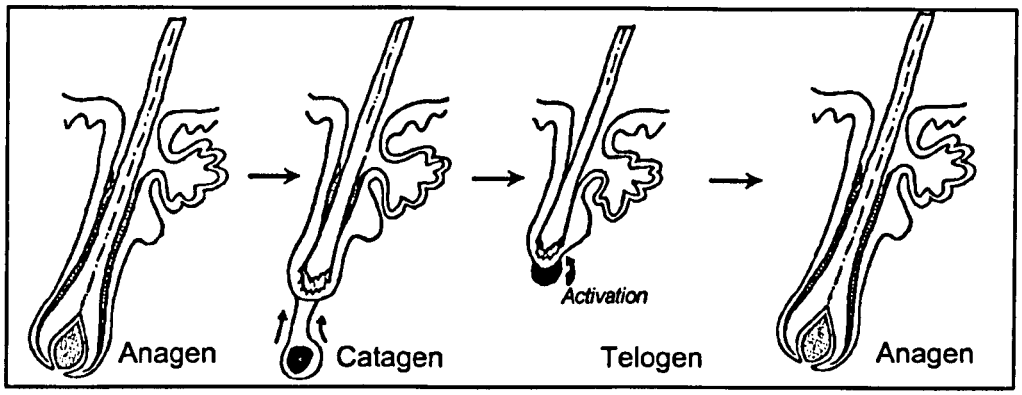


Figure 1.2: Different stages of hair growth (61)

1.4.3 Drug incorporation

Drugs can get incorporated into the hair through various mechanisms depending on their location and source. The three major factors affecting the incorporation of the drugs into hair are its melanin content, drug lipophilicity and basicity, hair structure as well as its colour. Few studies have shown that the drug concentration in pigmented hair is much higher as compared to non-pigmented blonde or grey hair after the same dosage had been given (58).

There are three major pathways that a drug can enter the hair as shown in Figure 1.3:

1. It can get incorporated actively or passively through the blood stream. As the hair follicle has a good blood supply due to rapid cell division, it diffuses across the cell membrane and enters the hair matrix (61).
2. There is a possibility of diffusion from sweat or other sebum secretions into the growing or mature hair fibre, as the drugs and their metabolites can be excreted in sweat (61).
3. Contamination from the external environment is also a very important drug incorporation path (58). It can get incorporated externally from vapours or powders into the mature hair fibre (61).

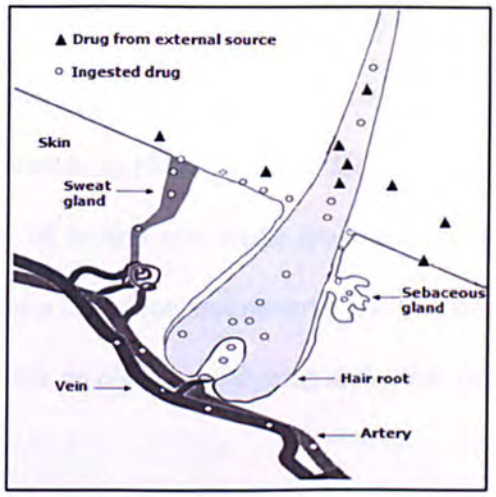


Figure 1.3: The three pathways for incorporation of drugs into hair (61)

Among these three routes, the first route is of greater importance, as it can give information about the time of intake of the drug or the dose taken (61).

Drugs bind to melanin, which is the major component of hair. There are several possible mechanisms that a drug can get bound to the melanin. Drug incorporation usually happens during the synthesis of melanin, where it gets bound to the melanin polymer. The molecules can also possibly migrate to the granules rather than binding only to the surface of melanin (61). Apart from melanin, there are other components that can be responsible for the incorporation of drugs within the hair matrix, such as keratin and other proteins (61). Thus, hair is gaining potential interest for the detection of parent drugs in doping control (62).

Incorporation of drug also depends on what racial/ethnic group the individual belongs to. Research shows that a group of individuals exposed to the same amount of drug show a diverse amount of incorporation of these drugs (61).

The drugs can also get deposited from deep skin compartments into hair growing cells as well as during the formation of the hair shaft into the keratogenous area (58). The polarity of the drug and its metabolites also play a major role in incorporation. As metabolites are more polar, they get incorporated to a lesser extent than the drug

itself. Another major aspect that influences the incorporation of the drugs is its acidity and basicity. The matrix of hair is more acidic when compared to blood pH, which is 7.4. Therefore the resulting pH gradient prefers convenient transfer of bases as compared to the transfer of neutral and acidic molecules (58). The drugs can be stably retained in the hair for a long time, but nevertheless can be affected if there are any cosmetic treatments, such as bleaching, dyeing and other treatments are carried out (58).

1.4.4 Hair digestion methods

The recovery of the incorporated drugs in the hair is a very crucial procedure for hair analysis. In order to isolate these drugs from the hair matrix, it is necessary to either digest the hair or use other extraction procedures. After a decontamination step, the next step in hair analysis is the extraction of the drugs from the hair matrix. Different extraction procedures have different recovery and suitability for the drugs as some compounds can deteriorate under certain conditions. The typical extraction procedures used for hair analysis include acidic extraction, alkaline digestion, enzymatic digestion and solvent extraction.

Alkali Digestion – this applies to alkaline stable compounds such as amphetamines, morphine and cannabinoids. The general procedure involves incubation of finely cut/ground hair in 0.1~2.5 M sodium hydroxide (NaOH). The temperature is normally set to 37 ^{0}C and pH and duration of the incubation often need to be optimised. After adjusting the pH with acid, the aqueous solution is extracted with solid phase extraction (SPE) or liquid-liquid extraction (LLE) (59,63). Another similar protocol involves placing 2 mg of hair in a 10 mL glass tube and adding 0.5 mL of 1 M NaOH along with 20 μL internal standard. The contents of the tube are

heated at 95 ^{0}C for 10 minutes in order to completely digest the hair. After the contents are cooled down to room temperature, liquid-liquid extraction with 1-chlorobutane is carried out. The organic layer is transferred to a separate glass tube and is mixed with 3 drops of 2-propanol: concentrated hydrochloric acid (HCl) (10:1, v/v), followed by evaporation and reconstitution of the sample (64).

Acidic extraction – this procedure involves taking 2 mg of hair and adding 1 mL of methanol:5M hydrochloric acid (20:1, v/v) along with 20 μL of internal standard. The contents of the tube are sonicated for 30 minutes and incubated overnight at room temperature. The tubes are again sonicated for 30 minutes the following day and supernatant is transferred to a separate glass tube and evaporated using nitrogen gas at 45 ^{0}C. The residue is then reconstituted with 100 μL water:acetonitrile (9:1, v/v) (64). Acidified methanol under ultra-sonication had been proved to give good extraction recovery in hair for some drugs (59). Other protocols involve the extraction of the drugs using 0.1~0.6 M HCl or 0.05 M sulphuric acid (H_2SO_4) at room temperature or 37 ^{0}C overnight. After acid treatment, the solution is neutralized, centrifuged and extracted with SPE (63).

Solvent extraction – solvent extraction can also be used for some drugs such as corticosteroids and antiretroviral drugs (65,66). A sample of 50 mg hair is incubated with 1 mL methanol for 2 hours in an ultrasonic bath at +60 ^{0}C along with the internal standard (65). Methanolic incubation under sonication is a commonly used extraction process. In some protocols the hair specimen are incubated for around 16~18 hours or are left overnight at room temperature (67). The process then involves centrifugation and transferring of the supernatant into a separate glass tube

followed by evaporation using nitrogen gas or compressed air. The sample is then reconstitution with methanol or acetonitrile.

Enzymatic Digestion – there are several enzymes that could be used to achieve digestion of the hair. These include proteinase K, protease E and β-glucoronidase/arylsulfatase etc. They cause complete dissolution of the hair matrix and hence can give very good recovery without any degradation of the drugs in question (59,63). The procedure generally involves hydrolysis of 10-50 mg hair with enzyme for 2 hours at 37~40 ^{0}C. The mixture is then centrifuged and the supernatant is extracted using SPE or LLE (63).

1.4.5 General procedure for sample collection

Before sample collection ethical approval should be sought from the responsible authority. The preferred collection area is the posterior vertex of the scalp and should be cut as close to the skin as possible. While collecting the sample information such as length and weight of the hair, assigned sample id etc. should be taken down if possible. The samples can be stored under dry conditions at room temperature. Decontamination of the collected samples prior to analysis is an integral step in order to eliminate contamination. Prior to analysis hair should be finely ground or cut into very small segments in order to expose the inner surface of the hair matrix (58,67).

1.4.6 Advantages of hair analysis

Urinalysis is commonly used to determine the presence of controlled drugs and their metabolites. The disadvantage with urine and blood analysis is that the matrix effects of these matrices are much greater than hair (62). Compared to urine and blood, hair samples can provide information on the long-term history (from weeks to months) of the individual's intake of drugs depending on the pharmacokinetic properties of the drug in question (68,69). Hair grows approximately half an inch per month which is equivalent to approximately 1.2 cm a month, so one and a half inch of the hair sample could give the history of individuals drug use of the previous 90 days (58,61,70). Also, blood and urine samples for drugs such as mephedrone require storage at -20 ^{0}C in the presence of preservatives until analysis (70). Compared to blood or urine samples, collection, handling and storage of hair samples is much easy (61).

Hair analysis is a very useful technique in investigating the history of drug use rather than recent use. The time window is much wider (depending on the length of hair) i.e. weeks to months as compared to blood and urinalysis, which only gives a time window of hours to days (58). One useful technique being widely used is sectional hair analysis, which can give a detailed history of drug use as hair keeps the information trapped in for a long time. This could also help in Therapeutic Drug Monitoring (TDM) by evaluating the compliance of the patients with their therapy regime (59,71). Hair analysis is best suited to identify individuals with complete absence rather than ascertaining the exact level of compliance. In addition, sample collection is non-invasive, is easy to perform and prevent adulteration (59).

1.4.7 Drawbacks of hair analysis

Where there are many advantages of hair analysis, there are few limitations associated to this technique. Generally, hair analysis is not useful in the case of single or very recent exposure of drug i.e. if the drug has been taken in the last two weeks. This is due to the fact that it takes time for drug to reach and get incorporated in hair, which is why the drug will not be detected if the hair testing is carried out straight after the dose taken. Also, in the case of single dose of a drug there is a possibility that drug might not reach the hair or the concentration in hair might be very low for it to get detected (72).

In some cases accidental or environmental exposure may be a reason for the presence of a particular drug in hair samples (72). The hair analysis results cannot distinguish substances that have been deposited in hair (such as from hair care products, dust) from the substances that might have been distributed into hair after the intake of that particular drug (73). This inability to distinguish between exogenous contaminants and endogenous can increase likelihood of false positive test results (74). Another drawback is the use of hair dyes or other cosmetic procedures that may involve the use of chemicals that can affect the incorporation of the drug or may affect the already incorporated drugs (61).

In addition, hair analysis may require a high sensitivity that only selective instrumentation can provide in order to achieve good analytical results. If the sensitivity is poor the analytical method will not be able to detect the drug in question. Compared to urine and blood, achieving a calibration in hair is more difficult as hair is a solid heterogeneous matrix and requires spiking with the drug. Spiked hair is not the same as using real samples where the drugs are incorporated during hair growth. An alternative to this is the use of a standard material such as

melanin. Also, contamination of hair is very easy due to its high surface to volume ratio (67).

1.5 Chromatography Techniques

Gas chromatography (GC) is a very common technique for the analysis of compounds as it provides faster results but many compounds cannot be analysed using GC because of their volatility or thermal instability, where they decompose under the conditions used. The advantage of liquid chromatography (LC) over GC is that there are no limitations as far as the volatility and thermal stability of the compound is concerned, therefore it is more suited for a wide variety of compounds that are less stable such as proteins, nucleic acids, amino acids, dyes, polar lipids, plant and animal metabolites etc. (75).

1.5.1 High-Performance Liquid Chromatography (HPLC)

HPLC is widely used for separation of many compounds (organic, inorganic, biological compounds, polymers, chiral compounds) as well as analysing impurities, volatile, non-volatile compounds. It is a very useful technique for qualitative and quantitative methods and could also separate very closely related compounds (76).

There are many applications of HPLC such as; purification, analysing environmental samples, quality control, measuring concentrations of certain compounds in physiological samples and many others (76).

Chromatographic separation depends on the interactions between sample molecules and the stationary phase. The main factors affecting the HPLC results is the combination of the mobile phase, the size of the column and its packing, the flow-rate of the mobile phase, column temperature etc. (75).

The sample is separated into its individual components by distribution between a fixed stationary phase and a mobile phase. Some compounds in the sample mixture have a greater affinity for the stationary phase than the mobile phase and are retained in the column longer. The compounds that are not retained as strongly in the column are carried by the mobile phase down the column. The longer the column the more opportunities there are for analyte to interact with the stationary phase and the greater the separation (77).

Researchers aim to use smaller columns, higher flow rates and higher working temperatures in order to increase the diffusion process of the analytes, but a problem with pressure can occur. Concerning all these purposes an advanced technology has been developed - Ultra High Performance Liquid Chromatography (UPLC). Both HPLC and UPLC involve liquid chromatography used to separate the components from mixtures. Two main properties that make UPLC more practical and more popular are having high-quality small porous packing material and capability of high pressures. In UPLC, particle sizes less than 2 μm can be used allowing better separation when compared to 5 μm particle size used in HPLC. The fundamental principle of UPLC is governed by the Van Deemter equation (discussed in section 1.5.9.9). The equation confirms the relationship between linear velocity (flow-rate) and plate height (column efficiency) (78).

Disadvantages associated with this technique include sample preparation, which is sometimes required. Compounds may require longer analysis time in order to separate, however good resolution is sometimes difficult to attain in complex mixtures, and there may be problems with identification of some compounds in the absence of relevant standards (76).

1.5.2 Preparing the sample

Sample preparation involves various steps such as dissolving, diluting, different extraction methods etc. The sample is injected as in a liquid form only and solid samples are dissolved in solvents that will not interfere with the analysis and can be used with the mobile phase as well as the stationary phase (76).

1.5.3 LC equipment

Modern LC equipment consists of integrated units that are capable of efficient separation, good precision, are convenient to use and involve less labour. To produce high quality separation of complex mixtures a combination of high efficiency columns along with good quality equipment is very necessary (75).

1.5.4 Mobile phase reservoir

Modern LC equipment contains mobile phase reservoirs. The tubing inlet carrying the solvent to the pump has a filter that prevents any particles going into the pump. Degassing of the mobile phase helps prevent the formation of any bubbles that can travel to the pump and detector hence leading to better chromatographic baseline. The solvents used for mobile phase are usually HPLC grade in order to avoid as many impurities as possible (75).

1.5.5 Column

The column is the stationary phase of a chromatographic system and plays a major role in separation of the compounds. Many different columns are commercially available in different lengths, bore sizes and packing materials. In order to achieve the best separation, the use of the correct combination of column length, packing

material along with the appropriate mobile phase is very important. These columns are available in various dimensions, and include preparative, normal-bore, micro- and mini-bore as well as capillary columns. Different packing materials and flow rates can be used to achieve good separation. The most commonly used column material is octadecyl-silica (ODS-silica), which contains C18 coating. Other columns containing C1, C2, C4, C6, C8 and C22 packing material are also available. There are several other packings available such as polymer-based etc.

Maintainance of the column can help prolong its life e.g. by flushing a column with mobile phase following sample runs, also keeping it capped so it doesn't dry out when not in use. In addition, filtering of samples is essential and a guard column can be used to avoid column blocking (79).

1.5.6 LC Pump

The mobile phase is consistently pumped at a fixed flow rate through the system and mixed (if required) by the pump (77). Several types of pumps are available depending on suitability of the analysis e.g. constant flow-rate pumps and constant pressure pumps (75). The most common pump used is the constant flow pump, which usually consists of a pair of reciprocating pistons (76). High-pressure pumps are necessary as LC columns are packed with small particles, which give a high resistance to column flow. The main requirements for a pump are that it must have chemical resistance to the mobile phase, have a pressure output of up to 5000 psi and must be capable of producing reproducible flow-rate (75). Isocratic or gradient elution can be used. Isocratic elution involves use of one solvent system whereas gradient elution is for more complex samples and involves changing the strength of mobile phase composition throughout the analysis (76).

1.5.7 Autosampler

In modern LC equipment an autosampler is used, as it is capable of automatic sampling and injection. It is very useful where large numbers of samples are analysed on a routine basis. It also excludes the need of an operator; hence overnight runs can be set on the instrument and is usually coupled to automatic data handling systems. The sample tray can accommodate a large number of sample vials and the temperature can also be controlled (75). The injection volume used could range from 1-100 μL depending on the sensitivity and the detector used (76).

1.5.8 Data Acquisition

Different softwares are used from different manufacturers; data acquisition and analysis is usually carried out on a computer carrying the software connected to the detector. These are used for routine purposes and offer method optimisation parameters for qualitative and quantitative analyses. It plots the chromatogram upon receiving an electronic signal.

Figure 1.4 below illustrates a schematic of a typical HPLC system.

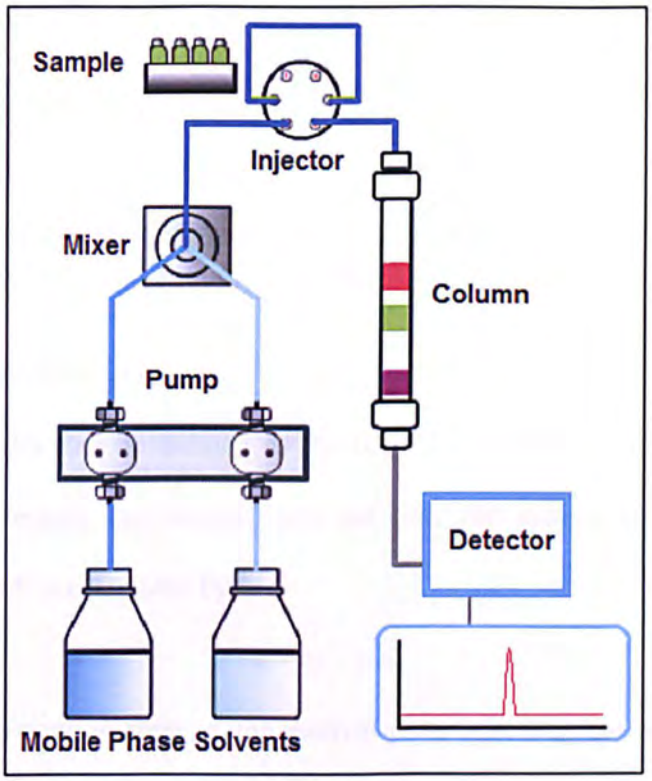


Figure 1.4: Schematic of typical HPLC system (77)

1.5.9 Chromatographic principles

In order to utilize practical application of HPLC effectively, it is necessary to have an understanding of some basic concepts and parameters of chromatography theory, which are described below:

1.5.9.1 Retention parameters

The time between the injection of the sample to the peak resulting from a component of the sample is called the retention time (t_R). It is the total time that the solute spends in the stationary phase and mobile phase. The void time (t_0) is defined as time spent by any component in the mobile phase (80). The time spent by the solute in stationary phase can be defined as

$$t'_R = t_R - t_0$$

1.5.9.2 Void volume (v_0)

Even if the analyte does not interact with the stationary phase it still has to travel through this volume before it enters the detector. The volume of the liquid phase in the column is called void volume (v_0) (80).

1.5.9.3 Capacity factor (k)

The capacity factor can be defined as the time the analyte resides in the moving mobile phase (through the column) and the time the analyte is retained on the stationary phase. It is calculated by

$$k = t_R - t_0/t_0$$

where t_R is the retention time of the analyte peak and t_0 is the retention time of unretained peak

Capacity factor can be used to evaluate column efficiency. The longer the column retains the analyte, the greater is the capacity factor (79,80).

1.5.9.4 Selectivity factor (α)

The selectivity can be described as the ability of the chromatographic system to discriminate between different analytes. To achieve optimum selectivity for two analytes, the value must be more than 1 (80).

$$\alpha = k_2/k_1 = t_{R2} - t_0/t_{R1} - t_0$$

where k_1 and k_2 are the respective retention factors.

Selectivity is dependent on the nature of the analytes and their interaction with the stationary phase. Also the column packing material and mobile phase composition can influence the selectivity factor (80).

1.5.9.5 Resolution (Rs)

Resolution is the degree of separation of two components on the chromatograph. The width of the chromatographic peak reflects the system band broadening and thus efficiency. Resolution describes both efficiency and selectivity as it is described as the ratio of the distance between two peaks on the average width of these peaks at baseline. It can be measured by using the following equation (79,80).

$$Rs = (t_{R2} - t_{R1})/0.5\ (w_{b1} + w_{b2})$$

Where t_R is the retention time of the analyte peak, and wb is the peak width at base.

1.5.9.6 Column efficiency (N)

The efficiency is the measure of band broadening and the number of theoretical plates (N) in the column. It can be calculated using the following equation.

$$N = 16(t_R/w_b)^2 = 5.54\ (t_R/w_{1/2})^2$$

Where N is the number of theoretical plates, t_R is the retention time, w_b is the peak width and $w_{1/2}$ is the peak width at half height. Figure 1.5 shows a schemetic of the efficiency measurements.

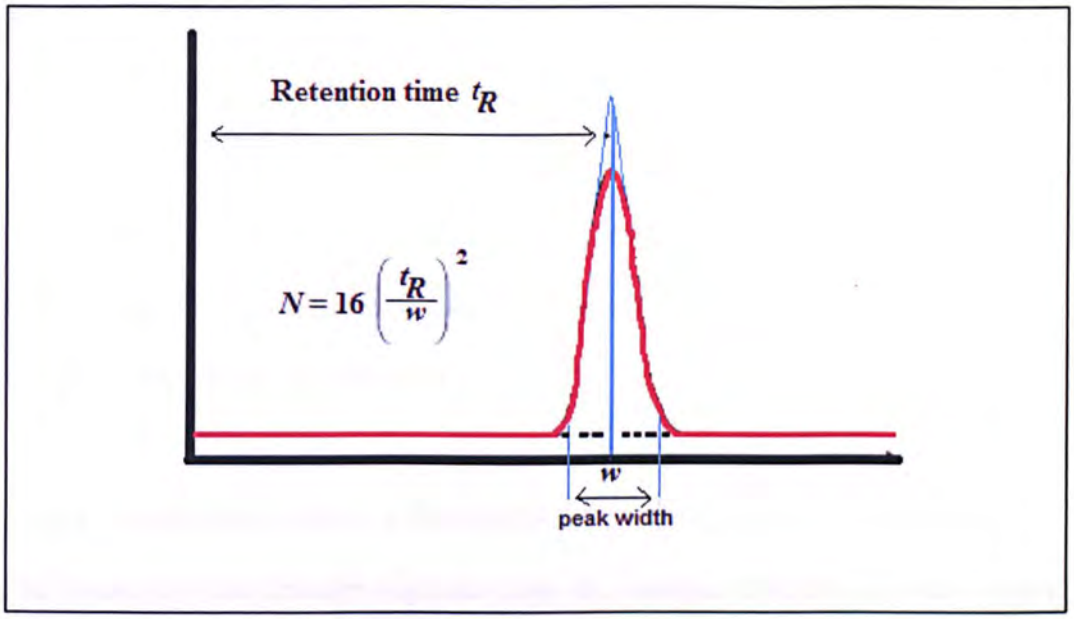


Figure 1.5: Schematic of the efficiency measurements (80)

The efficiency of the column is mainly dependent on the kinetic factors of the chromatographic system such as molecular diffusion, properties of the column packing bed, flow rate etc. the smaller the particles and more uniform its packing in the column, the higher the efficiency. Optimum flow rate is required in order to achieve best efficiency. Very fast flow rate will allow less time for analyte molecule to diffuse and can lead to band broadening. On the other hand the faster the flow rate, the further analyte molecules are from the thermodynamic equilibrium with the stationary phase (79,80).

1.5.9.7 Peak Symmetry: Asymmetry Factor (A_s)

Under ideal conditions, chromatographic peaks should be symmetrical and should have Gaussian peak shape but in reality most peaks exhibit either fronting or tailing. The asymmetry factor (A_s) is used to quantify peak symmetry, calculated at peak width of 10% of peak height (81).

$$A_s = B/A$$

A value of 1 indicates a perfect symmetry, anything <1.0 is fronting and >1.0 is tailing. Peak tailing is a result of adsorption or extra column band broadening, whereas peak fronting is typically caused by column over loading or chemical reaction of the analyte during chromatography. For example many basic compounds exhibit some peak tailing due to their polar interaction with residual acidic silanol groups in silica-based columns (81).

1.5.9.8 Height Equivalent to a Theoretical Plate (HETP) or Plate Height (H)

The theoretical plate concept originates from the industrial distillation process where a distillation column consisting of individual plates were used. A longer column would have more plates and would give better separation. The height equivalent to a theoretical plate (*HETP*) or plate height (*H*) is a measure of a column's efficiency and is expressed as:

$$HETP = L/N$$

The HETP is equal to the length of column (*L*) divided by the number of plates (*N*). Columns packed with smaller particles are usually more efficient and have a higher plate number (81).

1.5.9.9 Band Broadening

The Van Deemter Equation

Band broadening leads to poor chromatographic performance and reduces the efficiency of the separation being carried out. Van Deemter equation describes this phenomenon and the factors that contribute to band broadening. The equation combines the individual sources of band broadening and represents them as the

dependence of height equivalent to a theoretical plate (HETP) on the mobile phase linear velocity as shown in Figure 1.6 (82).

$$HETP = A + B/u + Cu$$

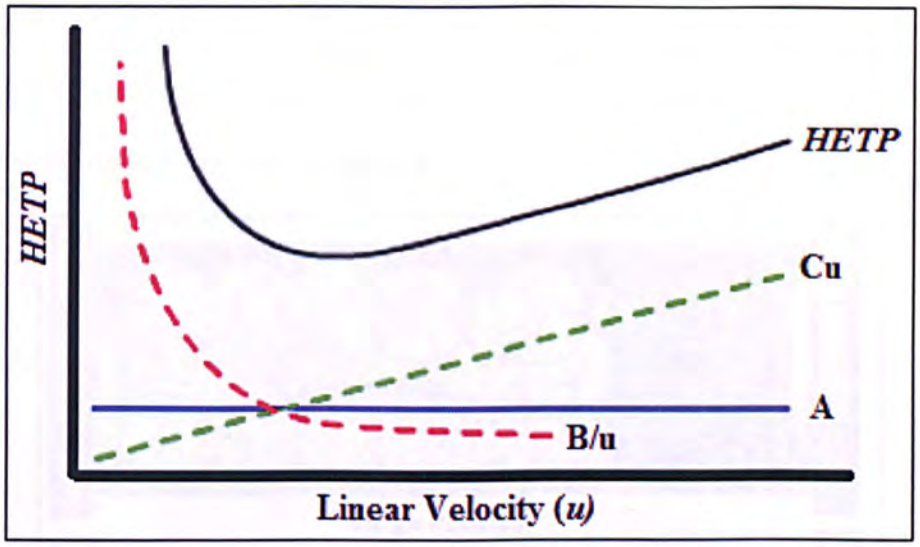


Figure 1.6: Typical Van Deemter curve showing contribution from three dispersion mechanisms(82)

Where,

A = Eddy Diffusion

B = Contribution from longitudinal or axial diffusion

C = Mass transfer

a) Eddy Diffusion (A)

Eddy diffusion reflects mainly the quality of column packing. It relates to variations in the flow rate of mobile phase or the flow path of the analyte within the column. An analyte molecule within a band of analytes can take one of many paths through the column. This is due to the fact that column packing has small variations in the particle size and is inhomogeneous. This leads to band broadening of the analytes as they travel through the column (82).

In addition, when liquids flow under pressure through the column, they tend to adopt laminar flow profile. Linear velocity is low near the inner walls of the tubing when compared to the centre of the column, which also leads to band broadening. Eddy diffusion can be minimised by using well-packed column (82). Figure 1.7 gives example of how large and small particle in the column packing can affect the path taken by the analyte through the column.

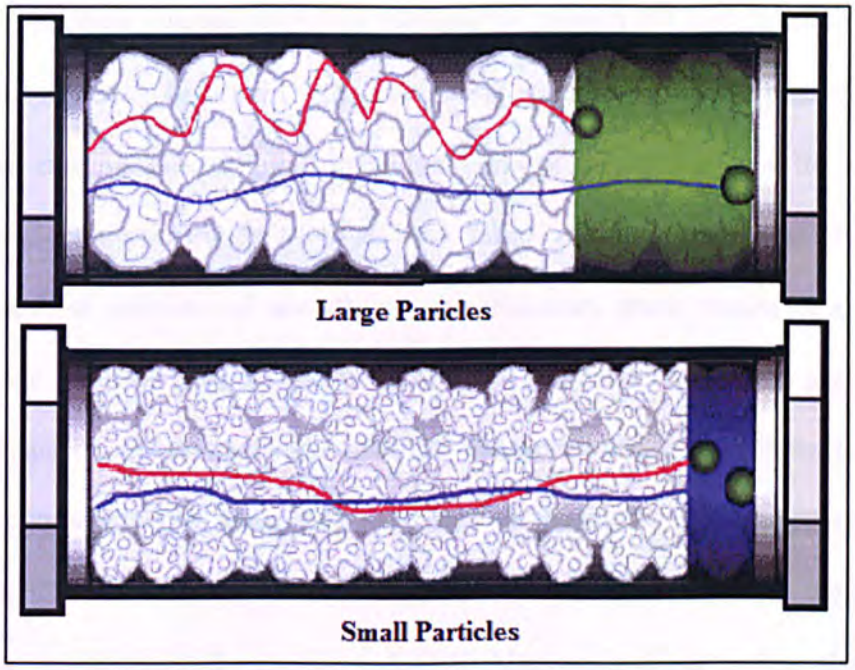


Figure 1.7: Illustration of Eddy diffusion (82)

b) Longitudinal Diffusion (*B/u*)

Longitudinal diffusion occurs due to unnecessary internal volumes within a HPLC system. The greatest scope for broadening is along the axis flow. Some of the factors that contribute to this are, if the tubing length is too long or the internal diameter of the tubing is very wide. Also, if the tubing is joined by unions and use of wrong column nuts and ferrules can also affect the chromatography. Longitudinal diffusion has a much larger effect due to low flow rate of mobile phase. Using higher mobile

phase flow rates and using correct nuts and short tubing can minimise band broadening (82).

c) Mass Transfer (*C*)

The stationary phase material is porous and the mobile phase within the pores is stagnant. The fact that the packing material is porous it allows a very large surface area for separation. During migration through the column the analyte molecules are constantly diffusing between the mobile phase and the stationary phase. Analyte molecules entering the pore, and those that penetrate more deeply in the pore and those that do not enter the pore will all have variation in the flow. These differences in the depth of diffusion of analyte into the stationary phase results in a variable delay in the diffusion back into the mobile phase. The deeper an analyte has diffused in, the longer it will take to diffuse back into the mobile phase and slower the rate of transfer compared with those that are close to the surface of stationary phase. Reducing the size of the packing material packing size in order to make pores shallower can minimise the band broadening. Also, using lower mobile phase flow rates and heating the column can help minimising mass transfer as at higher temperatures diffusion process is speeded up and the variation in elution time from the particle pore is reduced (82). Figure 1.8 shows how different velocities of mobile phase can affect the band broadening.

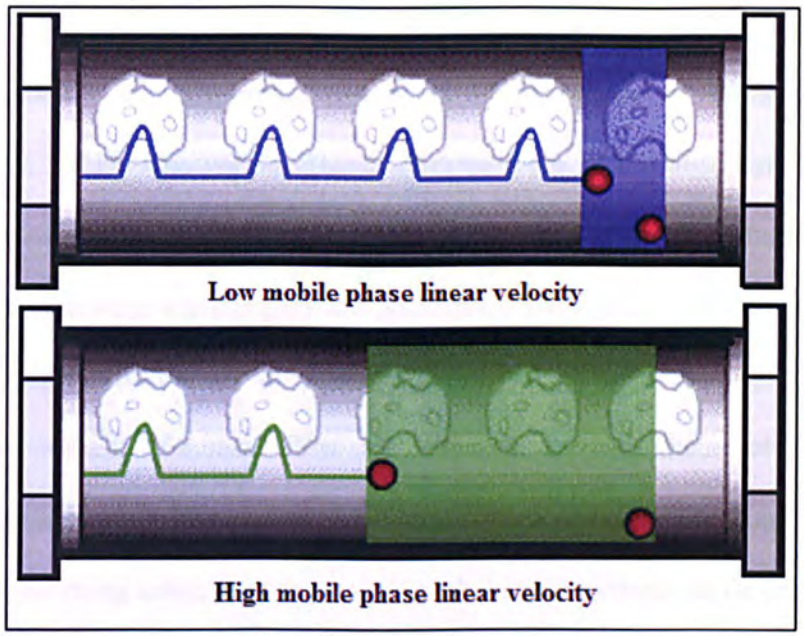


Figure 1.8: Illustration of mass transfer (using high and low mobile phase velocity)

(82)

One needs to use optimum chromatographic conditions to carry out a separation on HPLC. System backpressure needs to be carefully monitored when using small diameter particles in conjunction with higher mobile phase flow rates.

1.6 Detection Techniques

This is an integral part of LC equipment as it is responsible for monitoring and analysing the column effluent. An ideal detector should have various qualities such as high sensitivity, reproducible results, should offer good linearity, reliable, convenient, be able to provide qualitative as well as quantitative results on detected peaks. Several detectors are used including UV-Vis, fluorescence, electrochemical, mass spectrometer etc. (75).

1.6.1 UV Detector

UV-Vis examines the electronic transitions of molecules as a result of light absorption in the UV-Vis region. There are three types of UV absorbance detectors i.e. fixed-wavelength (does not allow change of wavelength), variable wavelength (selection of operating wavelength) and photodiode array (PDA) (single wavelength, range of wavelengths or entire spectrum).

The detector consists of a small flow cell, where the radiation beam of the UV/Vis spectrometer is located. The mobile phase passes through this cell and upon detection of UV-Vis absorbing solute generates a signal that is proportional to its concentration (76). These light absorbing functional groups are called chromophores (76). An electromagnetic field can interact with the electrons, which causes their excitation and can lead them to move to a higher energy level, it can also excite the molecular bonds that causes the vibration or rotation of their functional group. The intensity of the signal decreases as the analyte travels through the flow cell upon absorption (83). Absorbance is the logarithm of the ratio of the intensity of the incident light (I_0) and the light transmitted (I) (83). The Beer-Lambert law describes the absorption as a function of concentration:

$A = \log (I_0)/I) = \varepsilon cl$

A = absorbance, ε = molar extinction coefficient, c=concentration of the analyte, l = path length of the cell (76)

1.6.2 Mass Spectrometer (MS)

There are a number of detectors available that can be connected to HPLC, but among these MS has gained popularity due to its versatility. The mass spectrometer even though more expensive has many advantages associated to it compared to other detectors. The LC on its own is a technique that is used for the separation of the

compounds in a mixture. It can also be used to identify these compounds, however the identification can only be determined depending on the retention time of a compound. Identification of the compound can be made by running pure standards of the compound in question and using exactly the same conditions. Sometimes, for more complex mixtures, identification based on retention time is not sufficient as more than one analyte in a mixture could have the same retention time. MS is very useful in identifying the compound as it gives structural information as well as the molecular weight of the compound (84). A mass spectrometer analyses by measuring the gas phase molecules according to their mass-to-charge ratio (m/z). The charge is produced by addition or loss of a proton, cations, anions or electrons (85). It is more sensitive and is able to provide a full scan spectrum as compared to UV and other detectors (84).

The main advantage of having MS connected to HPLC is that it separates as well as identifies the compound in the same step. There are many advantages associated with this technique such as:

- Selective detection of a variety of compounds and identification of metabolites
- Separation and identification of a variety of compounds (non-volatile, organic and inorganic compounds, thermally unstable compounds).
- Molecular weight determination, qualitative and quantitative analysis
- Analysis and confirmation of drugs, toxin and other exogenous and endogenous compounds present in biological samples.

A few disadvantages associated with this technique are that co eluting isomers are difficult to discriminate. In addition, the sample must be soluble in the electrospray solvent (76,86).

There are different types of mass analysers available such as the quadrupole mass analyser, the quadrupole ion-trap mass analyser, and the time-of-flight mass analyser. The quadrupole is ideal as it is able to do fast scanning and uses low voltages. The quadrupole mass analyser has four rods and opposite pairs are connected electrically and a voltage consisting of radiofrequency (RF) and direct-current (DC) components is applied. Ions of a particular m/z follow a stable trajectory through the rods and reach the detector when the voltage reaches a specific value; hence a mass spectrum is produced (84).

1.6.3 Tandem Mass Spectrometry (MS-MS)

In tandem or hyphenated MS-MS, the ion of interest is isolated at one stage of mass spectrometry and the second stage helps in finding out the relationship of the ion to the one that had generated this particular ion.

A triple quadrupole is the most commonly used MS-MS device and consists of three sets of quadrupoles (84). A quadrupole consists of four parallel rods with fixed DC and alternative RF potential applied to it (85). Ions are produced in the source and are passed along the parallel array, under the influence of the fields (complex trajectories). Some of these trajectories are unstable and due to infinite displacement from the centre, ions are lost, for example, when colliding with an electrode. Only ions of a specific mass-to-charge ratio (m/z) with stable trajectories reach the detector as shown in Figure 1.9 (87). Alternating RF potential focuses ions of different mass to charge ratio (m/z) on to the detector hence producing a mass spectrum (85).

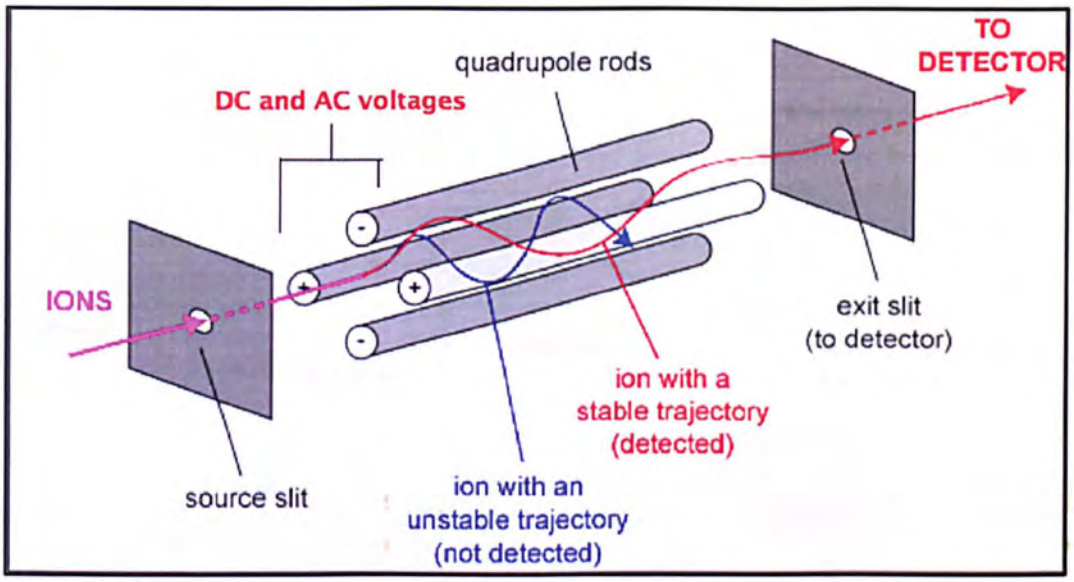


Figure 1.9: Quadrupole filter (88)

At present, the triple quadrupole is the most commonly used tandem mass spectrometer consisting of a linear assembly of three quadrupoles. Figure 1.10 illustrates the process of triple quadrupole. The first and third quadrupole serve as mass analysers operated with the combination of RF and DC potentials required for mass selection. The second quadrupole is the collision cell and has a fixed RF voltage only (89). The first quadrupole separates the original precursor ion; the second set is used as a collision device, where fragmentation of the ions is carried out and the third quadrupole separates the fragments produced in the collision cell (84,90).

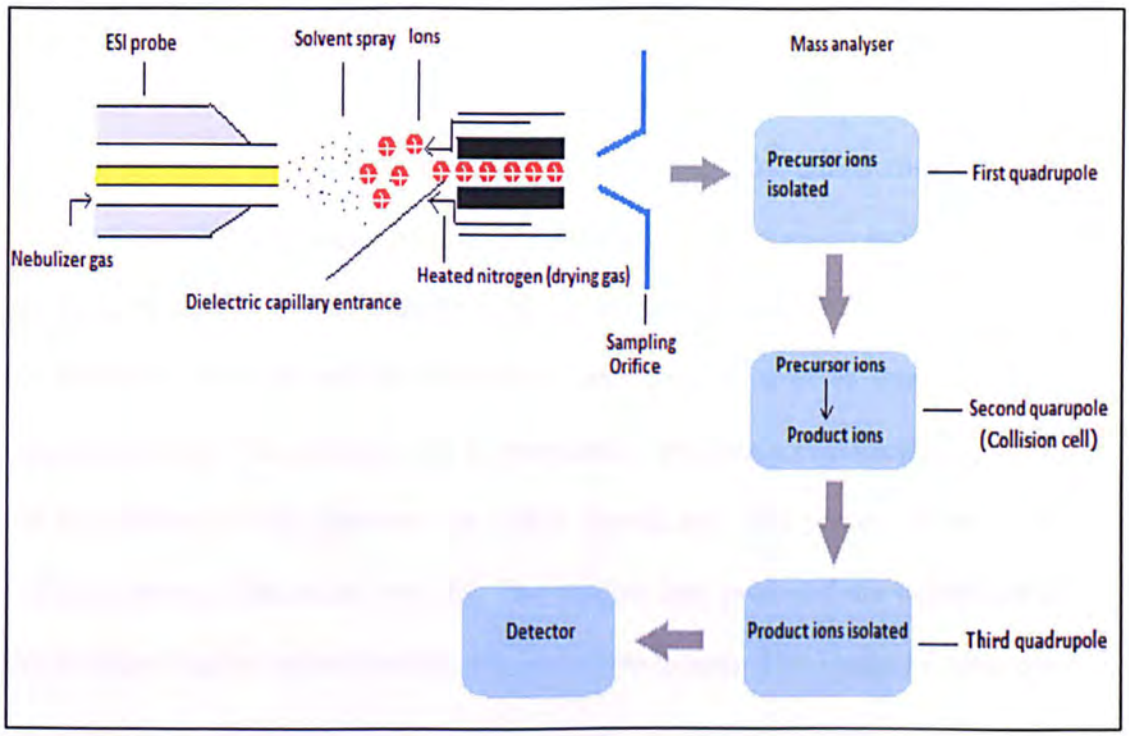


Figure 1.10: Schematic of triple quadrupole mass spectrometry – adapted from Hoffmann (89)

There are four main scan modes available using tandem mass spectrometers, product ion scan, precursor ion scan, neutral loss scan and selected reaction monitoring (SRM) (91). The product ion scan is the most common, where ions of a given m/z value are selected by the first quadrupole. The selected ions are then passed on to the collision cell where they get activated by collision and produce fragmentation. The third quadrupole then analyses these product ions and is set to scan over a suitable mass range (89).

In the precursor ion scan, a product ion is chosen and precursor ions (parent ions) are determined. The second spectrometer is focussed on a selected ion whereas the first spectrometer scans the masses. Hence, precursor ions that produce ions with the selected mass through reactions or fragmentations are detected (91).

The neutral loss scan selects a neutral fragment and detects all the fragmentation leading to the loss of that neutral. This scan requires both mass spectrometers to scan together, with a constant mass offset between the two (91).

SRM is the most common method that is mainly used for quantitative purposes (Figure 1.11). This scan mode consists of selecting a fragmentation reaction and both the first and second analysers are focused on selected masses. The ions selected by the first mass analyser are only detected if they produce a given fragment, by a selected reaction. The collision cell is optimised to produce a characteristic product ion by collision of the precursor ion with a neutral gas. This process is known as Collision Induced Dissociation (CID). The product ions produced are transferred to the third quadrupole, where specific m/z is allowed to pass. This method is analogue to Selected Ion Monitoring (SIM), where a single quadrupole is operated (89,92). It identifies a unique fragment ion and the plot usually contains a single peak. It is an ideal scan mode for sensitive and selective quantification of the compounds (93).

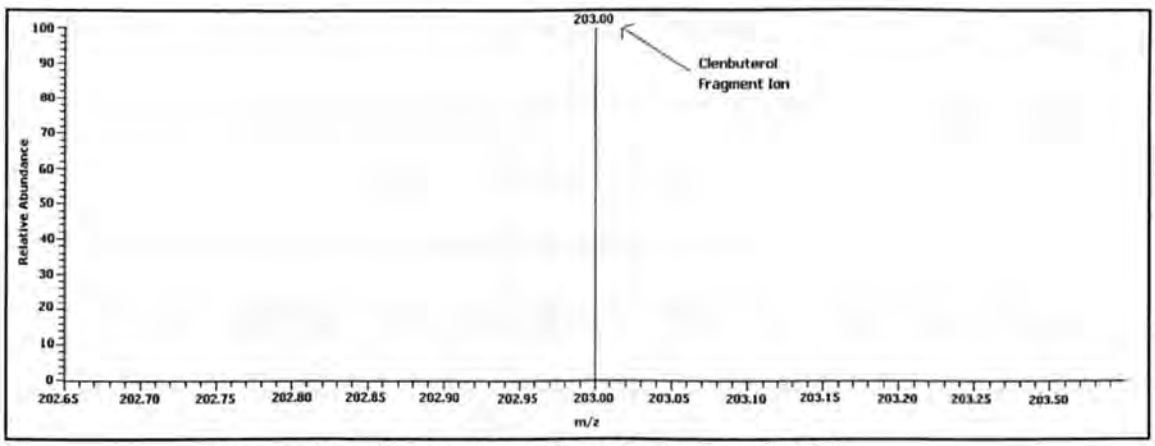


Figure 1.11: Selected Reaction Monitoring (SRM)

1.6.4 Ionisation

Atmospheric Pressure Ionisation (API) offers soft ionisation with positive or negative ion generation and detection. It can operate using Electrospray Ionisation

(ESI), Atomic Pressure Chemical Ionisation (APCI) and Atomic Pressure Photoionisation (APPI) (85).

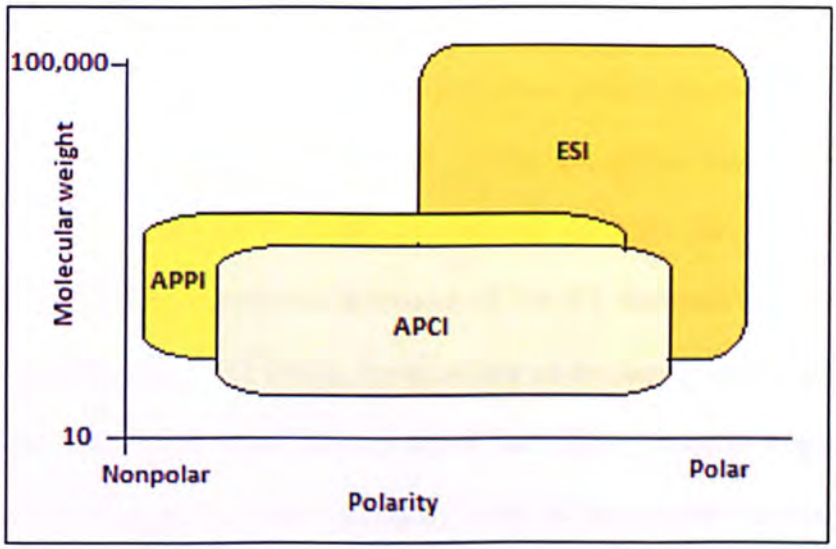


Figure 1.12: Various LC-MS Operating Techniques– adapted from Agilent (94)

Figure 1.12 shows the three techniques and their suitability for the compounds. APCI is applicable for poorly ionisable moderately non-polar to polar molecules that uses gas phase ion molecule reactions at atmospheric pressure; the principal involves sample vaporisation at temperatures up to 600 °C. Ion molecule reactions take place with the reagent ions in the plasma (produced by solvent vapor, by applying high electrical potential). A flow rate of 0.1 mL/min to 2 mL/min could be used (91).

APPI uses photons in order to ionize gas-phase molecules. Unlike APCI where corona discharge needle is used to emit electrons, APPI source uses discharge lamp emitting photons. The heated nebulizer vaporizes the solvent and the remaining analyte interacts with the emitted photons from a discharge lamp where it leads to series of gas-phase reactions causing the ionization of the analyte. APPI is able to ionize mainly non-polar compounds and also compounds that cannot be ionized by APCI and ESI (91).

ESI has gained popularity over the years due to its easy coupling to HPLC and its high sensitivity. This is called soft ionisation technique as very little residual energy is retained by the analyte upon ionisation (91). It is a very useful technique for thermo labile molecules as the ionisation takes place without heating (95). In ESI, a strong electric field is applied to the liquid passing through the capillary tube with a slow flow rate (1-10 µl/min) under atmospheric pressure. The electric field is obtained by applying a potential difference of 3-6 kV between the capillary and counter electrode. There is a charge accumulation on the liquid surface at the end of the capillary; and these liquid droplets break into highly charged droplets. These droplets move towards the source-sampling cone on the counter electrode and the solvent is evaporated from the droplet when passed through a curtain of heated capillary and heated inert gas. After solvent evaporation, the droplet becomes smaller and the charge (91) density on the droplet surface increases to the so-called "Rayleigh instability limit" that causes the droplets to rip apart into even smaller droplets until the gas phase ions are left. The ions formed by this technique are either protonated molecules (positive ion mode) $[M+H]^+$or adducts $[M+NH_4]^+$, $[M+Na]^+$ or deprotonated molecules $[M-H]^-$ (negative ion mode) (95). Figure 1.13 illustrates the process of ESI.

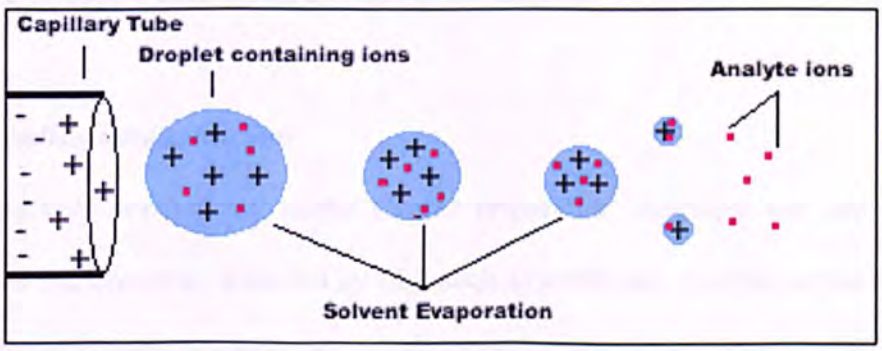


Figure 1.13: Schematic of Electrospray Ionisation (ESI) – adapted from Agilent (94)

1.7 Sample Preparation

Sample preparation is an integral step for analysis of compound using LC-MS/MS and provides the analyte in the solution. It helps in reducing matrix effect as it eliminates the unwanted elements present in the matrix. Matrix effects could affect the analysis in a negative way such as accuracy, precision, and ion suppression that could also lead to loss in signal (96). The sample preparation depends on the type of analytical techniques employed. The major possible sample preparation steps are:

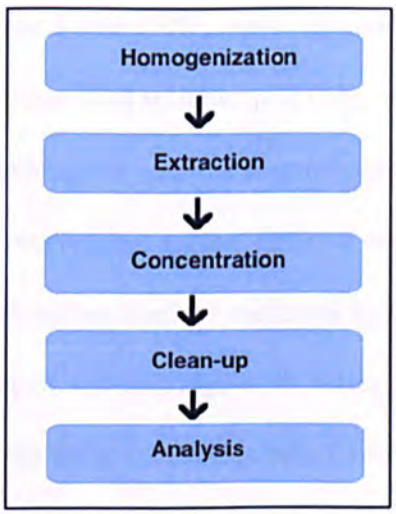


Figure 1.14: Basic steps in sample preparation (97)

Sample preparation could involve various steps such as dilution, protein precipitation, liquid-liquid extraction (LLE), solid-phase extraction (SPE) (96). Basic steps involved in sample preparation are shown in Figure 1.14.

1.7.1 Solid-phase extraction

SPE is a very popular and useful sample preparation technique and can resolve problems that cannot be achieved by LLE such as inefficient recovery of the analytes and disposal of large quantities of organic solvents (98). In SPE, the cartridge has the stationary phase embedded inside. The cartridge consists of a chromatographic stationary phase that has solid particulates to separate the analytes from its matrix

(99). The choice of the stationary phase depends on the analyte in question as well as the matrix that it is present in (100). There are different SPE procedures used depending on the retention mechanism of the analyte in question and their extraction from the matrix its present in. It is time consuming because often method development is required in order to achieve ideal extraction of the analyte (93).

SPE cartridges are available with various packings mostly based on bonded silica material with specific functional groups such as hydrocarbon chains of different lengths mainly used in reversed phase SPE, amino groups for anion exchange and for cation exchange carboxyl groups and sulfonic acid (93).

There are various SPE mechanisms such as normal-phase, reversed-phase and ion exchange SPE. The steps involved in a typical SPE are conditioning of the stationary phase such as C_{18}-silica with solvent such as methanol in order to activate the bonded phase followed by washing of the cartridge with water. The crude sample is then placed in the cartridge and the polar substances pass through easily whereas the other components stick to the embedding. The column is then rinsed again to remove any polar substances (impurities) left behind. Elution of the analyte is finally achieved by solid-liquid extraction by washing the cartridge with desired solvent that is able to elute the analyte that is retained on the stationary phase (99). SPE helps in removal of unwanted matrix effects (99).

In normal phase SPE there is usually a polar analyte, a relatively non-polar matrix and a polar stationary phase. The retention of the analyte in question is due to the interactions between polar functional groups present in analyte and polar groups on the sorbent surface. The elution of the analyte is achieved by using a solvent that is more polar than the sample's matrix (98).

Ion exchange SPE is used for ionisable compounds where pH is very important to consider; as the ionisation state of the compound has a very serious impact on its retention and elution characteristics (93). The elution is carried out by a solution, which has a specific pH in order to neutralise either the functional group of the analyte or the stationary phase. Upon neutralisation of these, the binding electrostatic forces are disturbed and the analyte is then eluted (98).

Reversed-phase SPE involves a polar sample matrix and hydrophobic silica based stationary phase. The hydrophilic silanol groups are modified with hydrophobic alkyl or aryl functional groups. The retention of the sample is due to attraction forces between the carbon-hydrogen bonds in the analyte and the functional groups on the silica surface. These hydrophobic attractions between the two are called dispersion or van der Waals forces. A non-polar solvent is used to disrupt the packing in order to elute the analyte (98).

1.7.2 Liquid-liquid extraction

This technique is simple and is based on two liquid phases that are immiscible. One phase is aqueous, whereas second phase is an organic solvent such as hexane, pentane, chloroform and many others. The bottom layer is the denser phase (100) and solvents that are less dense than water will form the top layer. For extraction to occur the analyte has to have favourable solubility in the organic phase. Some solvents are somewhat soluble in each other and become saturated when mixed with each other (101).

The procedure involves mixing the solution containing aqueous and organic layers, which helps in creating a large interfacial area between the two layers, which enables the mass transfer of analyte into the organic layer. The two phases can then be

centrifuged and the organic layer can be separated (93). The choice of solvent depends on the solubility of the analyte. For compounds that prefer to stay in aqueous layer; more polar organic solvent can be used.

1.7.3 Protein precipitation

This technique is mainly used for removing unwanted proteins from biological samples. The procedure involves adding a solvent such as acetonitrile or methanol or strong acid or base to the biological samples leading to precipitation of these proteins and other biological materials. The sample is then centrifuged and a pallet of protein is formed which can be separated from the supernatant. The supernatant is then dried down and reconstituted with desired solvent or can be injected directly. The major drawbacks of this technique are the lack of specificity and selectivity and hence significant matrix effects and ion suppression can occur (102).

1.8 Validation method for LC-MS/MS analysis

Method validation is very necessary for analytical studies as it proves whether the analytical method developed and employed for a specific analysis is acceptable for use. One can follow Food and Drug Administration (FDA) guidelines to validate all the parameters in the method (103). The results for method validation confirm the quality and reliability of the method produced. Once the method and all the parameters of the instrument are optimised, the next step is to validate this method for its reliability, accuracy, precision, robustness, linearity, lower limit of quantification, lower limit of detection and its reproducibility. Method re-validation has to be carried out if there is a change in instrument or any change in conditions or if there is a change in sample matrix e.g. if the column temperature used was 40 °C,

if for some reason it is changed to higher or lower temperature, the method has to be revalidated (104).

1.8.1 Reference standards

To develop a method for any compound on the chromatographic instruments, it is very important to have an authenticated reference standard. It is a highly purified compound that is well characterised and is used to spike samples in order to obtain calibration. The reference standard used should either be same as the analyte, where this is not possible then it should be as a freebase or acid or salt or ester (103,105).

1.8.2 Internal standard (IS)

Use of internal standard is very essential in quantitative studies. It compensates for common losses during analysis such as sample manipulation, variability caused by autosampler, degradation etc. When the analyte and internal standard go through same losses and same matrix effects, these two cancel when the ratio of IS to analyte is calculated. The internal standard needs to have similar extraction charactertistics, stability and detector response to the analyte, which is why the internal standard should be physically and chemically similar to the analyte. Usually, an isotopically labelled IS is used, other IS can be chemical isomers and analogues of the analyte in question. The quantitation is based on the response ratio of the analyte to the response ratio of internal standard. It is suitable for methods where the analyte has to go through certain extraction steps. The concentration of the IS should be kept close to the concentration of the analyte (105).

1.8.3 Blanks

Matrices with no presence of analyte are referred to as blank samples. Blank samples are crucially important for the validation of an analytical method as these give the information on any interferences that might be encountered while analysing the actual test samples (104).

1.8.4 Calibration curve

A calibration or standard curve consists of at least six standard points including lower limit of quantification (LLOQ) and should be obtained for each analyte in the sample. It represents the relationship between the instrument and known concentrations of the analyte. It should be prepared in same manner as the actual samples to be analysed i.e. by spiking the matrix with a known concentration of the drug and extracting it in exactly the same way. The standard points of the concentrations should be within the range expected for the study itself. It should consist of a blank sample with no internal standard, another blank with internal standard and at least six calibrants including LLOQ covering the range of calibration curve (103). This could be achieved by diluting the stock solution over a range of concentrations. The response for LLOQ should not deviate by more than 20% from the actual concentration where as for other standards, it should not deviate by more than 15%. At least four out of six standards should meet the above criteria (103).

1.8.5 Linearity and range

Linearity should be evaluated across the range of the method and is defined as method's ability to produce a response that is directly proportional to the concentration of the analyte in the matrix and is determined graphically as well as

mathematically. A regression line can be used to obtain mathematical data on the degree of linearity. The ideal correlation coefficient of the linear regression should be around 1 and the intercept should be 0. Where the intercept is significantly different to 0, it should be shown that it has no effect on the accuracy of the method. The range is the interval between the higher concentration and the lower concentration of analyte in the sample with a suitable level of precision, accuracy and linearity (106).

1.8.6 Selectivity

The ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample is referred to as selectivity. Selectivity can be determined by analysing six blank samples at different concentration levels as well as LLOQ for any interference such as the matrix effect. If there are more than one analyte to be analysed then each component should be tested for the presence of any interferences (103).

1.8.7 Specificity

Specificity is defined as the ability of an analytical method to distinguish the analyte within the sample that contains other components as well.

1.8.8 Sensitivity

Sensitivity is defined as the ability of an analytical method to discriminate between small changes and differences caused in the concentration of the analyte or its mass (104). It is the change in signal per unit change in the concentration of analyte (93).

1.8.9 Stability

Checking the stability of an analyte is very useful as many chemical compounds can decompose under certain conditions. These conditions could be during the sample preparation, storage conditions (e.g. temperature), matrix or the manufacturing material used for the vials and sometimes UV light. Stability helps in determining the time allowed between the sample collections till it has been analysed. The experiment should consist of all the steps involved in the actual study that is from collecting the samples to handling, to short or long term storage. The procedure should include a freshly prepared stock solution and a set of samples should be prepared from that in an appropriate solvent at known concentrations. The stock solution of the analyte as well as the internal standard should be investigated at room temperature for six hours and then the response of these should be compared to the response of a freshly prepared stock solution. The stability is then determined by calculating the % RSD (relative standard deviation) of the replicates that should not exceed more than 20%. In addition to this, stability can also be determined by freeze and thaw cycles. Three freeze and thaw cycles can be used and could be for short or long-term stability. Three replicates of low and high concentration each can be stored at required storage temperature and thawed after 24 hours at room temperature and kept at this temperature based on how long it will be out in the actual study at this temperature. If the sample is out for 4-24 hours it is considered as short-term stability whereas for long term stability testing should be when the sample was first collected and when the last analysis is carried out. The concentrations should be compared to the concentrations of the standards from first day of the stability testing. In addition, the stability of the analyte in question and the internal standard should also be

investigated over the anticipated run time for the batch size in validation samples by determining concentrations on the basis of original calibration standards (106).

1.8.10 Accuracy

Accuracy is defined as closeness of the experimental value to the 'true value'. The true value could be the accepted reference value or could be a conventional true value. There are several ways that accuracy of a method can be tested. One alternative is to compare the results obtained from the current method with results obtained from an established reference method (106). Samples with a known amount of analyte should be analysed and the response should be compared to the response produced by a standard and blank solution. The accuracy is then calculated as the percentage of analyte recovered. Accuracy in this thesis was determined by analysing quality control (QC) samples in replicates (six per concentration level) at three concentration levels, equally distributed over the linear range. For accuracy, the determined values are compared with the known concentration values (103). According to FDA, the mean value for the calibrants should be within 15% of the actual value and should be 20% for LLOQ. The deviation of the mean from the true value is the measure of accuracy (103).

1.8.11 Precision

Precision is one of the important parameters of judging an analytical method and is measured by monitoring the repeatability and reproducibility of an analytical method (104). Precision is defined as closeness of the data values at difference concentrations under the same conditions (105). Repeatability of the samples help in measuring the variance in the method; smaller variance means better precision.

Precision is usually referred to as standard deviation or RSD. RSD is the standard deviation as a fraction of the mean (S/x) and, when multiplied by 100, it gives the % relative standard deviation, which is a more reliable expression for precision. Repeatability and reproducibility of the results are dependent on the range of analyte concentrations to establish the estimate of a true value. Accuracy and precision are the two main parameters that determine the error (104).

1.8.12 Recovery

Recovery is measured by spiking the matrix with a known concentration of the analyte followed by extraction of the analyte and injecting it into the instrument. The response produced can be compared to the response produced by injecting a reference material dissolved in pure solvent (104). A comparison of a signal produced by extracted to non-extracted pure analyte should be at three concentration levels i.e. low, medium and high and should be able to represent 100% recovery.

1.8.13 Lower limit of detection

LLOD is the lowest possible concentration of the analyte that can be detected, but not necessarily quantified, by the analytical method used. The peak or the response for the LLOD should be at least two or three times higher than the baseline noise level (104). It is generally referred to as signal to noise ratio and in this case it should be 3:1 (93). The LLOD for a reference material dissolved in solvent and injected in the instrument could be different to the spiked sample with same concentration. This is because the baseline noise for extracted analyte from matrix could vary to the baseline noise of the same concentration of the reference material (104,105). As

LLOD is dependent on the signal to noise ratio, it can be improved by reducing the baseline noise and hence enhancing the analyte signal (93).

Noise is the width of the baseline and there are different parameters that contribute towards this such as detector electronics causing background fluctuations, pump oscillations, dirty column and the baseline signal in the absence of the analyte. Noise can be reduced by using high sensitivity detectors that have low noise and drift characteristics and pumps with low pulsation (93).

1.8.14 Lower limit of quantification

LLOQ is defined as the lowest concentration of an analyte that an analytical procedure is capable of quantifying within the limits of accuracy and precision. This can be achieved by analysing known concentrations of the analyte and determining the minimum level of concentrations that can be quantified accurately (106). The signal to noise ratio for LLOQ should be 10:1 (93).

1.8.15 Robustness

It is defined as when a method remains unaffected by causing deliberate variations in method parameters, hence proving the analytical method's reliability. One can examine the effect of deliberate changes in these parameters on an analytical method and its results (106). It helps in identifying the effective variables in methods as one can closely control them and in case of improvements to be made in the method these specific parameters can be changed accordingly (104).

1.9 Overall aims of this research project

- To develop an analytical method for the detection of social drug mephedrone and its two metabolites 4-methylephedrine and 4-methylnorephedrine using LC-MS/MS
- To apply this novel analytical method to human hair samples that were collected as part of an independent study
- To develop a method for simultaneous detection of two antiretroviral drugs abacavir and tenofovir using LC-MS/MS
- To apply the novel developed analytical method to human hair samples donated by HIV positive patients
- To develop a HPLC-UV method for the detection of β_2-agonist in enzymatic assay
- To employ this method to investigate the inhibitory effects of dietary products containing phenolic compounds (various teas and fruit juices) on the glucuronidation activity of clenbuterol and formoterol
- To also apply this method to investigate potential inhibitory effects of catechins present in tea and corticosteroids on the glucuronidation activity of the two β_2-agonist clenbuterol and formoterol

1.10 Preface

The work presented in this Ph.D thesis involves development and application of novel analytical methods to detect social and antiviral drugs as well as β_2-agonists using LC-MS/MS and HPLC-UV, respectively. The following work is reported over five Chapters.

- Chapter one provides a general introduction and aims for the three projects reported in this thesis
- Chapter two provides detailed materials and methods employed in the three reported projects
- Chapter three reports the development and validation of an analytical method for the detection of mephedrone and its two metabolires 4-methylephedrine and 4-methylnorephedrine using LC-MS/MS. The Chapter also contains application of the developed method to human hair
- Chapter four details the development of novel analytical method for simultaneous quantification of abacavir and tenofovir using LC-MS/MS and its application to human hair
- Chapter five represents method development of β_2-agonist using HPLC-UV. The Chapter also reports effects of dietary products (tea and fruit juices) and catechins present in tea as well as corticosteroids on the glucuronidation activity of clenbuterol and formoterol
- The last part of this thesis provides the concluding remarks and information on potential future work arising from this work

CHAPTER 2

MATERIALS AND METHODS

This Chapter contains a detailed overview of all the chemicals and consumables used along with the methodologies and instrumentations for all the three studies.

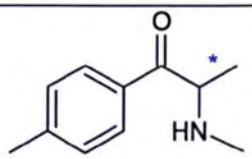
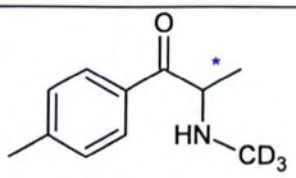
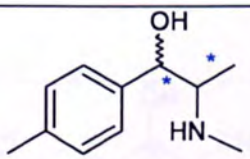
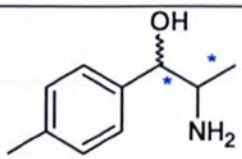
2.1 Chemicals and reagents

2.1.1 Reference materials

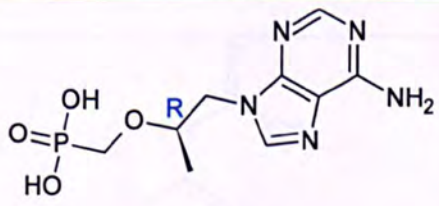
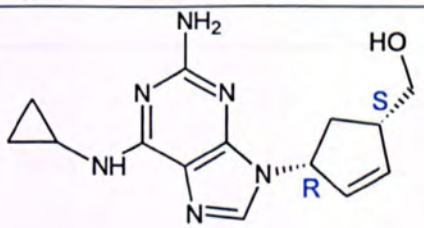
Mephedrone hydrochloride (1.0 mg/mL) (as free base) in methanol, mephedrone d3 hydrochloride (0.1 mg/mL) (as free base) in methanol, tenofovir (15 mg), abacavir sulphate (5 mg), abacavir – d4 (1.0 mg) and clenbuterol hydrochloride (30 mg) were purchased from LGC standards (Teddington, UK). Paracetamol, 4-methylephedrine hydrochloride and 4-methylnorephedrine hydrochloride were synthesised at Kingston University (London, UK). Formoterol fumarate dehydrate (10 mg) was purchased from Sigma Aldrich (Dorset, UK). Figure 2.1 shows the structures and molecular formulae for all the reference materials used along with their molecular weights.

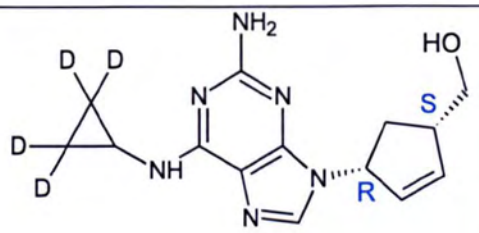
Table 2.1: Structures of all the drugs used in the three studies and their chirality

Drugs used in Chapter 3

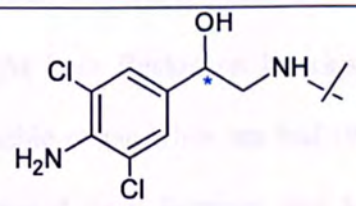
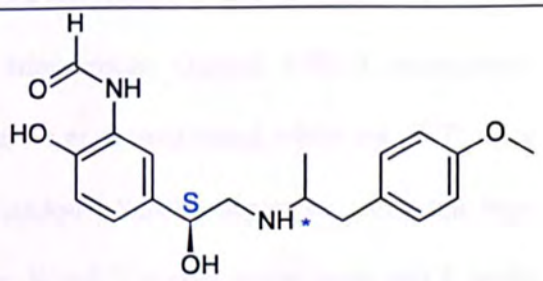
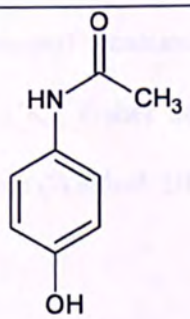
 Mephedrone Molecular formula: $C_{11}H_{15}NO$ Molecular weight: 177.70 g/mol	 Mephedrone – D3 (IS) Molecular formula: $C_{11}H_{12}D_3NO$ Molecular weight: 180.72 g/mol
 4-methylephedrine Molecular formula: $C_{11}H_{17}NO$ Molecular weight: 179 g/mol	 4-methylnorephedrine Molecular formula: $C_{10}H_{15}NO$ Molecular weight: 165.72 g/mol

Drugs used in Chapter 4

 Tenofovir Molecular formula: $C_9H_{14}N_5O_4P$ Molecular weight: 287.213 g/mol	 Abacavir Molecular formula: $C_{14}H_{18}N_6O$ Molecular weight: 286.332 g/mol

 Abacavir – D4 (IS) Molecular formula: $C_{14}H_{18}D_4N_6O$ Molecular weight: 290.36 g/mol	

Drugs used in Chapter 5

 Clenbuterol Molecular formula: $C_{12}H_{18}Cl_2N_2O$ Molecular weight: 277.65 g/mol	 Formoterol Molecular formula: $C_{19}H_{24}N_2O_4$ Molecular weight: 344.404 g/mol
 Paracetamol (used as IS) Molecular formula: $C_8H_9NO_2$ Molecular weight: 151.163 g/mol	

2.1.2 Solvents and other general chemicals

HPLC grade methanol (MeOH), acetonitrile (ACN), water, and hexane, reagent grade ethyl acetate, ethanol, dichloromethane, chloroform and formic acid were all purchased from Fisher Scientific UK Ltd (Loughborough, UK). Tris HCl buffer and Cleland's reagent were obtained from VWR (Lutterworth, UK). Proteinase K, sodium hydroxide, hydrochloric acid, sodium hydrogen phosphate heptahydrate and sodium phosphate monobasic dehydrate were purchased from Sigma Aldrich. Pooled liver microsomes, UGT2B17, solution A (containing uridine -5'- diphosphoglucronic acid) and solution B (containing $MgCl_2$, Tris HCl (pH) 7.5 and alamathecin) were all bought from Beckinson Dickinson (BD Biosciences, Oxford, UK). Commercially available yunan white tea leaf (WTL) and silver dragon beard white tea (WT) were purchased from Fortnum and Mason (London). Sencha Japanese green tea bags (GT), ocean spray classic cranberry juice, Welch's purple grape juice and Copella Apple (cloudy) juice were all purchased from local supermarket (London).

2.1.3 Essential Consumables

All the essential consumables listed in Table 2.2 were bought from Agilent (Stockport, UK), Fisher Scientific (Loughborough, UK), Capital Analytical (Leeds, UK), Millipore (Watford, UK) and Biochemic Diagnostics, Inc (NY, USA).

Table 2.2: List of all the consumable used in the three studies

Consumables	Description and Brand
SB-C18 column Zorbax eclipse XDB-C18 column Supelco C18	2.1 µm × 150 mm × 1.8 µm, Agilent 2.1 µm x 150 mm x 1.8 µm, Agilent 5 µm x 250 mm x 4.6 µm, Supelco
C18 SPE catridges GV-65C SPE cartridges	Varian, Agilent Biochemical Diagnostics
Syringe driven micro-filter	0.2 µm, PTFE membrane syringe filter, Millipore 0.45 µm, PTFE membrane syringe filter, Millipore
Silanised amber glassware	4 mL with PTFE lined screw caps, Sigma Aldrich
Silanised glass inserts	200 µL and 300 µL, Capital Analytical
Amber vials Clear glass vials	1.5 mL with screw caps, Fisher Scientific 1.5 mL with screw caps, Fisher Scientific
Glass centrifuge tubes	10 mL with PTFE lined screw caps, Fisher Scientific

2.2 Instruments

The instruments used for the studies were bought from Thermo Scientific (Loughborough, Leicestershire, UK), Fritsch (Germany), PerkinElmer (Cambridge, UK), SPEware Corporation (California, USA), Ideal Scientific (Ancaster, Toronto), Jones Chromatography Ltd (Wales, UK) and Fisher Scientific (Loughborough, UK).

Table 2.3: List of all the instruments used in all the three studies

Instruments used	Model and Brand
LC-MS/MS	Accela UPLC pump, Thermoscientific Triple Quadrupole TSQTM mass spectrometer, Thermo Eclectro Corp, Thermoscientific
HPLC	Pump (series 200), PerkinElmer Autosampler (series 225), PerkinElmer Diode array UV detector (series 200 EP), PerkinElmer
Centrifuge	Heraeus Labofuge 400R, Thermoscientific
Ball mill	Mini-mill pulverisette, Fritsch
SPE instrument	Cerex System 48 Pressure Processor, SPEware Corporation
Vortex mixer	ZX Wizard with infrared sensor, Fischer Scientific
pH meter	Seven Easy S20, Mettler Toledo, Fischer Scientific
Sample concentrator	Dri-block DB-3D , Technne, Fischer Scientific
Column oven for HPLC	Column oven, Jones Chromatography Ltd
Orbital Shaker	IKA – KS 130 Basic, Ideal Scientific

2.3 Overview of the Methodologies used

2.3.1 Chapter 3 – Methodology

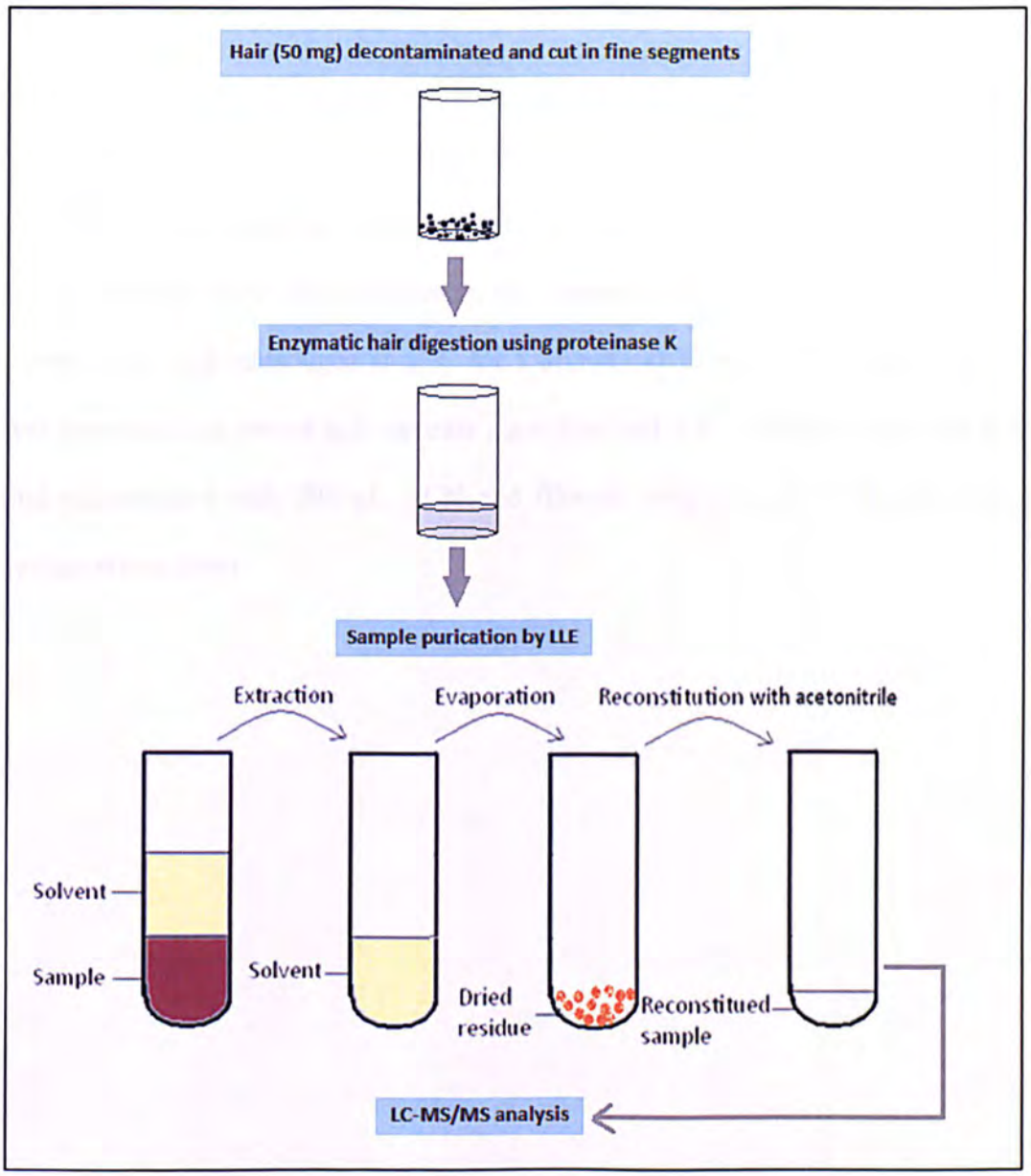


Figure 2.1: Sample preparation for extracting mephedrone and its metabolites from human hair

Figure 2.2 shows that hair samples were decontaminated by rinsing with 2 mL dichloromethane for 2 min (twice) at room temperature followed by drying of the samples. The hair samples were then cut into very fine segments (*ca.*1 mm length).

Enzymatic digestion consisted of placing the finely cut hair segments (50 mg) in a glass vial containing Cleland's reagent (100 mg), enzyme proteinase K (15 mg) and internal standard (100 μL). The mixture was finally incubated with Tris buffer (1 mL) for 2 hours at 37.5 ^{0}C with constant stirring using a water bath.

The third step of sample preparation involved transferring of the digested hair mixture to glass centrifuge tubes in order to carry out LLE. For LLE different organic solvents were used (Chapter 3), the contents of the tube were mixed using a vortex mixer and centrifuged at 5 ^{0}C for 5 minutes at 1750 x g. The organic layer was separated and placed in a separate glass tube and was dried using nitrogen gas and reconstituted with 200 μL ACN and filtered using 0.2 μm, PTFE membrane syringe driven filters.

2.3.2 Chapter 4 – Methodology

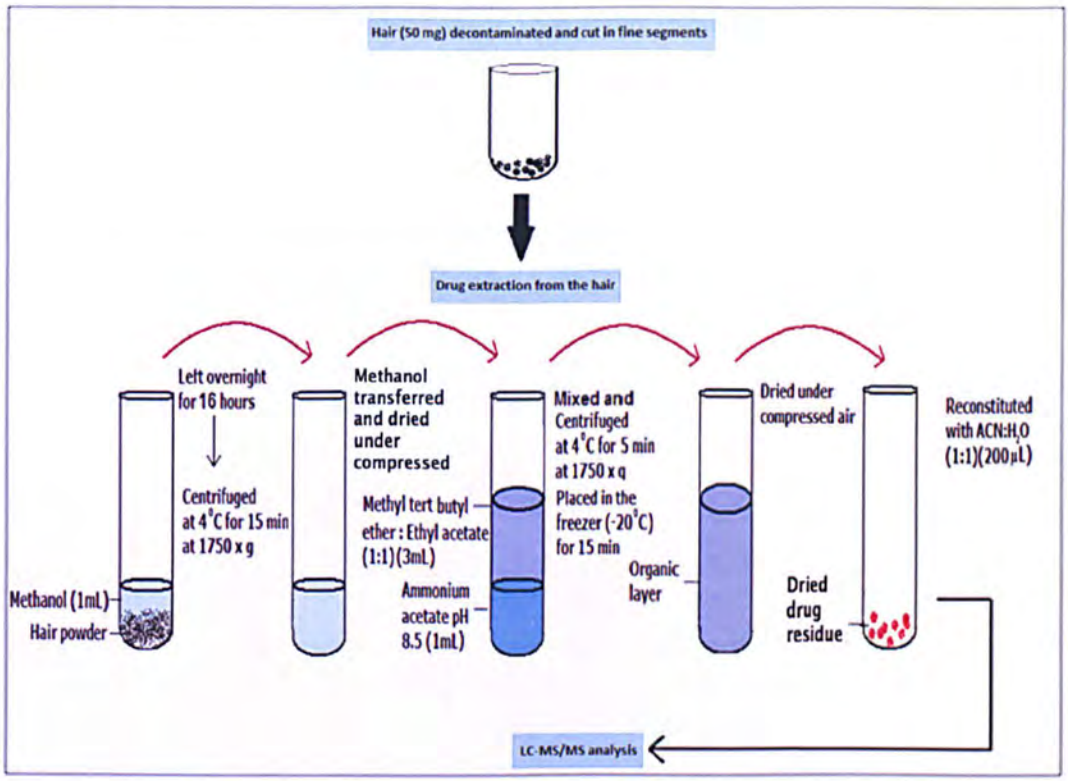


Figure 2.2: Sample preparation process for extracting tenofovir and abacavir from patient's hair

Decontamination of hair involved rinsing hair samples with 2 mL of dichloromethane twice for 2 minutes at room temperature. After drying, the hair samples were pulverised into a fine powder using a mini-mill for 5 minutes.

For the extraction of drug, hair (50 mg) was placed in a glass tube with methanol (1 mL) and 100 µL internal standard. After mixing the contents, the mixture was agitated in an orbital shaker for 16 hours overnight at room temperature. The mixture was then centrifuged at 4 ^{0}C for 15 minutes at 1750 x g and was evaporated using compressed air. After drying, ammonium acetate buffer pH 8.5 (1 mL) and 3 mL mixture of methyl tert butyl ether: ethyl acetate (1:1) was added to the tubes. The content of the tubes was vortex-mixed for 2 minutes and centrifuged again at 4 ^{0}C for

5 minutes at 1750 x g. In order to separate the organic layer, tubes containing the mixture were place in the freezer (-20 ^{0}C) for about 15-20 minutes. After the aqueous layer was frozen the organic layer was transferred in a separate glass tube and was dried using compressed air. The samples were then reconstituted with 200 μL acetonitrile: water and filtered using 0.2 μm, PTFE membrane syringe filter (1:1) (37).

2.3.3 Chapter 5 – Methodology

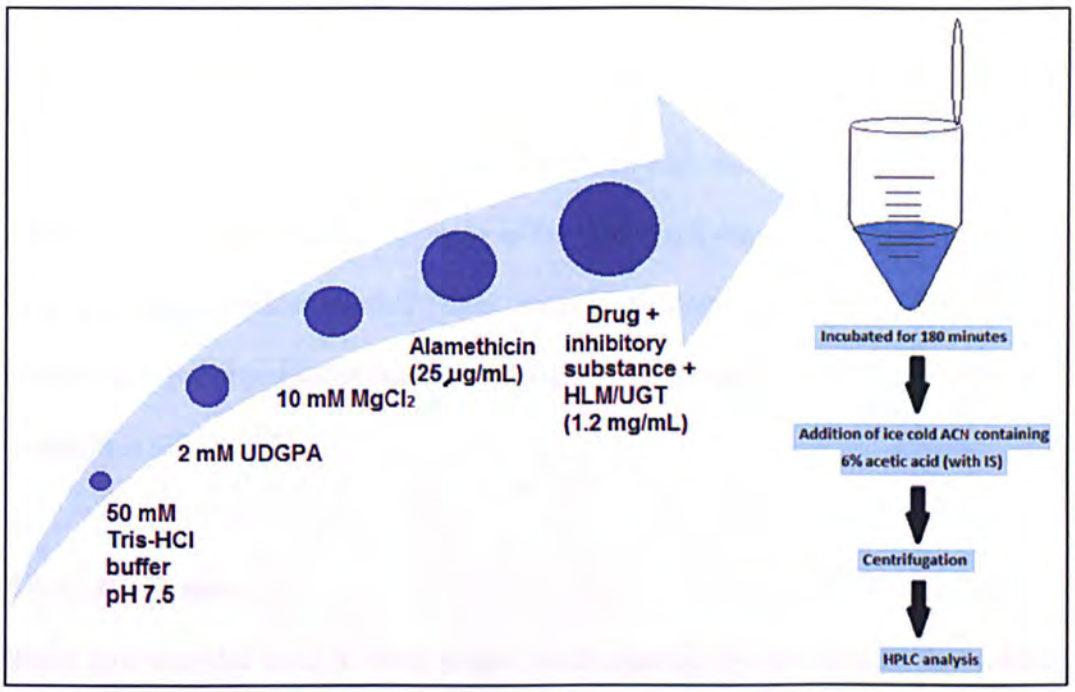


Figure 2.3: Enzymatic assay preparation

50 mM Tris-HCl buffer (pH 7.5), 2 mM UDPGA, 8 mM $MgCl_2$, 25 µg/mL alamethicin, β_2-agonist, and HLM or UGT protein were mixed and made up to 0.2 mL of final incubation volume with water. The mixture was initially incubated for 5 minutes at 37 ^{0}C and the reaction was started by addition of HLM or UGT (1.2 mg/mL) protein. The reaction was terminated at 90 and 180 minutes by transferring the aliquot of incubation mixture to a separated vessel and adding ice cold ACN containing 0.6% acetic acid and IS (final concentration 100 µg/mL). The aliquots in the vessel were vortexed mixed and the protein was precipitated by centrifugation at 4 ^{0}C for 5 minutes at 3939 x g. The supernatant was analysed by HPLC-UV. In order to determine any potential inhibition of the glucuronidation process, the water amount in the reaction mixture was replaced by adding the potential inhibitor.

2.4 Hair Specimen

The hair samples provided for the two studies were collected from the volunteers as part of independent studies. The author did not participate in collecting the hair samples for any of the two studies. The amount of hair collected ranged between 30 – 100 mg in weight and were cut close to the skin. Each sample collected was stored in a separately labelled sealed paper envelopes. Both studies were carried out according to the approved protocols of Kingston University Faculty Research Ethics Committee (38,72,107).

2.4.1 Blank hair

Blank hair samples used in both studies were donated by two healthy individuals with no history of any social drug intake. The hair samples were free from any treatments such as dyeing, perming and bleaching.

2.4.2 Mephedrone hair samples

Hair samples for this study were obtained from 154 healthy volunteers (95 male, 59 females) after two higher education institutions provided the ethical approval. The age range of the volunteers was from 18 to 56 years old (72,107).

2.4.3 HIV patient's hair samples

Ethical consent was granted by the NHS (UK) National Research Ethics Service (Ref. No. 09/H0712/86) for collecting the hair samples from HIV patients. Hair samples were donated by 32 HIV positive patients undergoing combination therapies at the time (38).

2.5 Preparation of potential inhibitory products

2.5.1 Tea extracts

Tea samples used were all commercially available brands and consisted of green tea leaf, white tea leaf and white tea stem, where tea (0.8 g) was ground using pestle and mortar and the powder was dissolved in 40 mL of boiling water. After 5 minutes, extracts were filtered using a 0.45 μm PTFE membrane syringe filter. Tea extracts were added to the reaction mixture at 10% v/v of the final volume.

2.5.2 Fruit juices

Fruit juices used in the study (Chapter 5) were purple grape juice, cloudy apple and cranberry juice. All the juices were filtered using a 0.45 μm PTFE membrane syringe filter and added to the reaction mixture at 10% v/v of the final volume.

2.5.3 Catechins

Catechin gallate (CG), epicatechin (EC) and epigallocatechin gallate (EGCG) were prepared by dissolving each sample in water. These catechins were added to the reaction mixture by replacing the amount of water at the initial concentration of 1 mM.

2.5.4 Corticosteroids

Each corticosteroid was prepared by dissolving it in water and added to the reaction mixture by replacing the amount of water, at the initial concentration of 2 mM.

2.6 Instrumentation

For Chapter 3 and 4, analyses were performed on a Thermoscientific liquid chromatography – tandem mass spectrometry (LC-MS/MS) system consisting of an Accela UPLC system coupled to a Triple Quadrupole TSQTM mass spectrometer. The UPLC system consisted of a quaternary pump, column heater, thermostated autosampler and an automatic degasser. The MS had an electrospray ionisation source (ESI), which was operated in positive ion mode for both studies. The data acquisition software used for the analysis was Xcaliber version 2.0. The Figure 2.4 below shows the LC-MS/MS system used for the analysis.

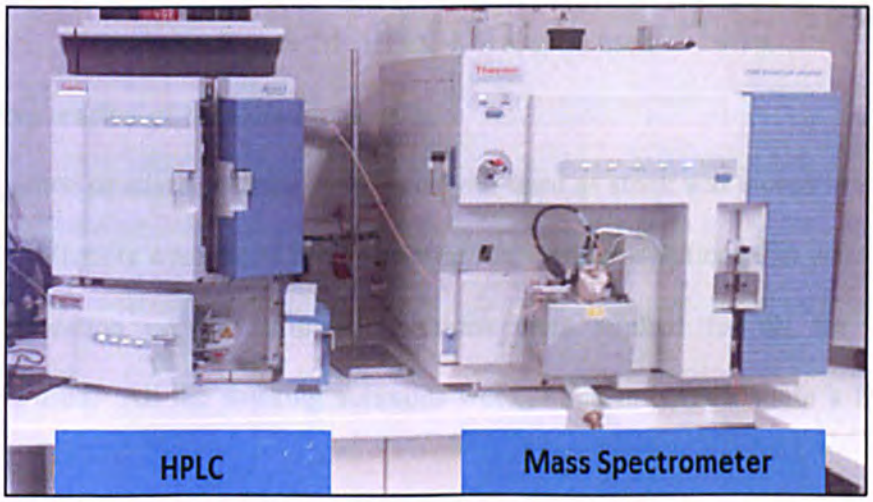


Figure 2.4: LC-MS/MS system used

For Chapter 5 a PerkinElmer HPLC system consisting of a pump, an auto sampler and a quaternary pump; coupled to a diode array UV/Vis detector were used. The data acquisition software was Totalchrom. Figure 2.5 shows the HPLC-UV system used in this project.

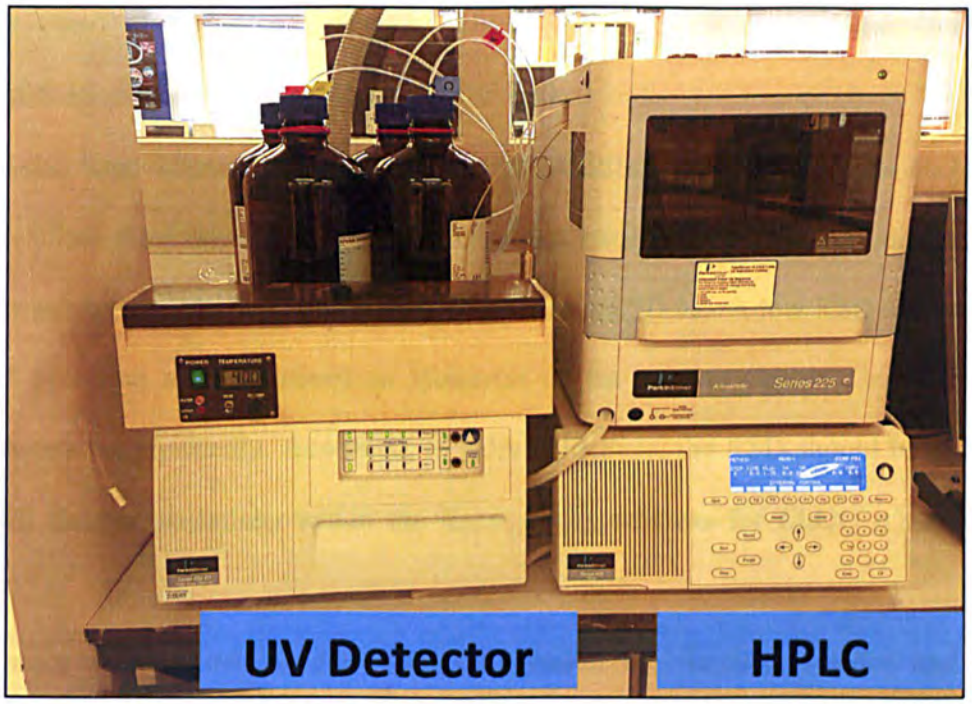


Figure 2.5: HPLC-UV system used

2.7 Preparation of the standards

The reference standard solution for the analytes used as stock was stored in a freezer (- 20 ^{0}C). Aliquots were taken for making further working solutions that were used to make calibration curves. Similar conditions were applied for all the internal standards used. All the working solutions were stored in glass vials in a fridge (4 ^{0}C).

2.8 Method Validation

Method validation was performed for each study according to FDA guidelines in order to provide assurance for the reliability and reproducibility of the method (103). The parameters that were validated for each study are, as follows:

The sensitivity of the method was defined by its LLOD and LLOQ. LLOD is the lowest concentration level that can be detected for an analyte with the S/N (signal to

noise ratio) of > 3 (103), whereas, LLOQ is the lowest concentration for the quantification of an analyte with the S/N of > 10.

Precision was validated at four different concentrations with six replicates each; on consecutive three days. The four concentrations used were within the linear range and was expressed by a relative standard deviation (RSD %). A low RSD indicates a good precision and is defined as closeness of the analyte signals for the same concentration repeatedly. According to FDA guidelines, the RSD should be ± 15% for all the concentrations within the linear range, whereas for LLOQ it's ± 20% (103).

Accuracy was defined as the closeness between the theoretical value and the calculated value under the same conditions. Theoretical value here refers to the calculated area of the analyte when dissolved in a solvent, whereas calculated value refers to the area calculated for the same concentration of the analyte after going through the sample preparations step i.e. in the actual sample matrix. The RSD percentage should be ± 15% for all the concentrations within the linear range, whereas for LLOQ its ± 20%.

The method should be able to give a linear calibration curve in a given concentration range. The ratio was calculated by dividing the response of the analyte by the response of the internal standard. The regression coefficient, r, describes the linearity of the equation. The closer it is to 1, the better the linearity.

Specificity was identified as absence of any matrix interfering peaks at the same retention time as the analyte. It was investigated by treating a blank sample in exactly the same manner as the actual samples in the absence of any analyte or internal standard. Selectivity was determined in order to investigate if there are any interfering peaks at the same retention time as the analytes (103). The robustness was

defined as the reproducibility of the method without getting affected by any minor variations that could be instrumental as well as external.

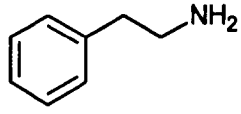
Extraction recovery was determined by spiking a blank with the known amount of analyte and internal standard followed by extraction process and then comparing the area to the area of the un-extracted sample containing the same concentration in the solvent (103).

CHAPTER 3

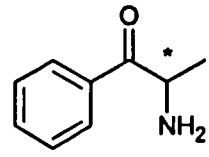
ANALYSIS OF MEPHEDRONE AND ITS METABOLITES IN HUMAN HAIR USING LC-MS/MS

3.1 Introduction

'Legal high' refers to chemicals that possess psychoactive activity in humans, and are not subject to prohibition by law (7). Compared to other traditional psychoactive drugs such as cocaine, phencyclidine, opiates, there is not much information available in the literature about these emerging class of psychotropic drugs (15). Due to the structural similarity of these legal highs to controlled psychoactive substances such as phenethylamines and cathinones, it has become common practice for the drug users to buy these legal highs from the Internet (3-5).



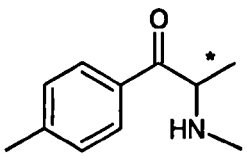
Phenethylamine



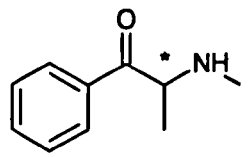
Cathinone

Mephedrone also known as 'meow', 'mcat' and 'bubbles' was initially available to users and was the most widely experienced 'legal high' (7). It is a β-ketoamphetamine and has close structural similarity to methcathinone, which in turn is the N-methyl analogue of the natural product cathinone (4). Methcathinone, which

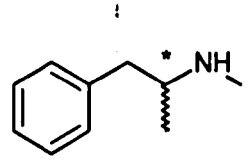
is a Class B drug is also a cathinone derivative and is analogous to methamphetamine. Mephedrone is a synthetic ring-substituted cathinone, very similar to the phenethylamine family, differing only by a keto functional group at the beta carbon. Mephedrone first synthesised in 1933, is a stimulant extracted from *Catha edulis* and the leaves of this plant are chewed in Somali, Yemeni and Ethiopian countries (17,108). The alkaloid content of the fresh leaves is responsible for the psychoactive stimulating effects (3).



Mephedrone



Methcathinone



Methamphetamine

Mephedrone has been readily available on the European recreational drug market for the past 5 years. The drug was initially drawn to attention in December 2008 after the death of 18-year-old woman whose post mortem report-identified mephedrone (14). Initially, it was legally available to users, but in April 2010 it was classed as a controlled drug under the Misuse of Drugs Act 1971 as a Class B, Schedule 1 substance (7,8,109). Other brand names under which mephedrone is sold in the UK are 'plant feeder' or 'plant food', whereas in the USA it is mostly named as 'bath salt'. It is available in powder form as well as in a form of tablet or capsule (11). The use of mephedrone is more common in males than females and it is reported to be one of the most commonly abused psychotropic drugs (7).

Drug designers produce these derivatives by modifying the functional groups of the actual drugs or make changes to the substitutions and change the moieties of the substances repeatedly (15). Synthesis of cathinone derivatives is carried out by modifying cathinone, which is structurally very similar to amphetamine (14).

3.1.1 Source

Cathinone derivatives are widely available from the common sources such as Internet websites and are sold as bath salts and plant food with an indication "not suitable for human consumption" to avoid any legal action against them (14). The most common way to obtain mephedrone is from Internet and high street drug dealers. An Internet survey shows that mephedrone is more common in UK compared to other European countries. Studies show that it is the sixth most frequently used drug (11-13).

3.1.2 Mode of administration and dose

The most common way the users take mephedrone is by intranasal insufflation or ingestion. The doses vary according to the route of administration and this, in turn, affects the impact of experience. Nasal insufflation doses range from 25 mg to 75 mg, and the effect is very fast, with peak efficacy in less than half an hour and fading rapidly thereafter (15). Ingestion doses range from 150 mg to 250 mg, the effect onset is between 45 minutes and 2 hours, and the effects last for 2 to 4 hours. According to the users, among all these routes of administration, intranasal dosing has more impact with a strong desire for the next dose. Mephedrone has been the cause of several deaths in Europe and USA (15).

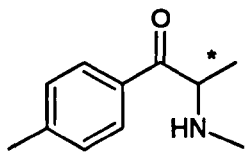
The most common effects of mephedrone abuse are head rush, lack of concentration and this can affect visual focus and memory loss. It could also cause nasal irritation, can affect body temperature and can cause chest pain, nausea, vomiting and nose bleed. In addition, some users have also reported tremors, elevated heart rate, unusual behaviour, agitation, insomnia and hallucinations, sweating and could even lead to death (5,11,13).

3.1.3 Metabolism

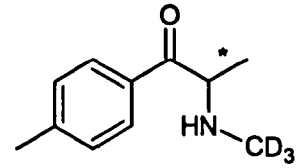
In the scientific literature, there is limited information available on the metabolism of mephedrone in humans. Pharmacokinetics studies in rats and humans suggest that metabolism of mephedrone is very similar to ring substituted amphetamines (13). As the structure of mephedrone is substantially similar to other psychoactive drugs such as methcathinone and methamphetamine, one can tentatively propose a metabolism of mephedrone on a similar basis. There are a number of different pathways by which this drug could be metabolised.

In order to determine the presence of metabolites of the controlled drugs in humans, urinalysis is the traditional technique used however it is often difficult to detect the parent drug. In addition, the matrix effects for urine and blood are much greater than hair (68).

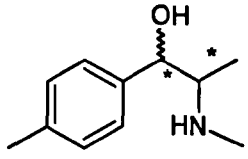
The two metabolites analysed in this study are 4-methylephedrine and 4-methylnorephedrine (8,19,21,62). The structures of mephedrone and its two metabolites are depicted in Figure 3.1.



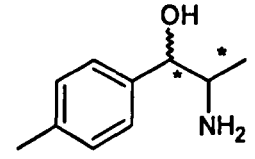
Mephedrone



Mephedrone – D3



4-methylephedrine



4-methylnorephedrine

Figure 3.1: Structures of mephedrone, 4-methylephedrine, 4-methylnorephedrine and mephedrone – d3 (internal standard) with their chiral centre

There has been very little research carried out on the metabolism of mephedrone. Due to the structural similarities of mephedrone to methcathinones and methamphetamines the metabolism of mephedrone has been proposed to follow a similar pathway. The two metabolites analysed in this study could be postulated via two different metabolic pathways. Both metabolites are produced by phase I metabolic pathway (19,110). The pathway involves the reduction of the methylene group and the second metabolite is formed by N-demethylation, thus forming a primary amine as shown in Figure 3.2 (10,19,62).

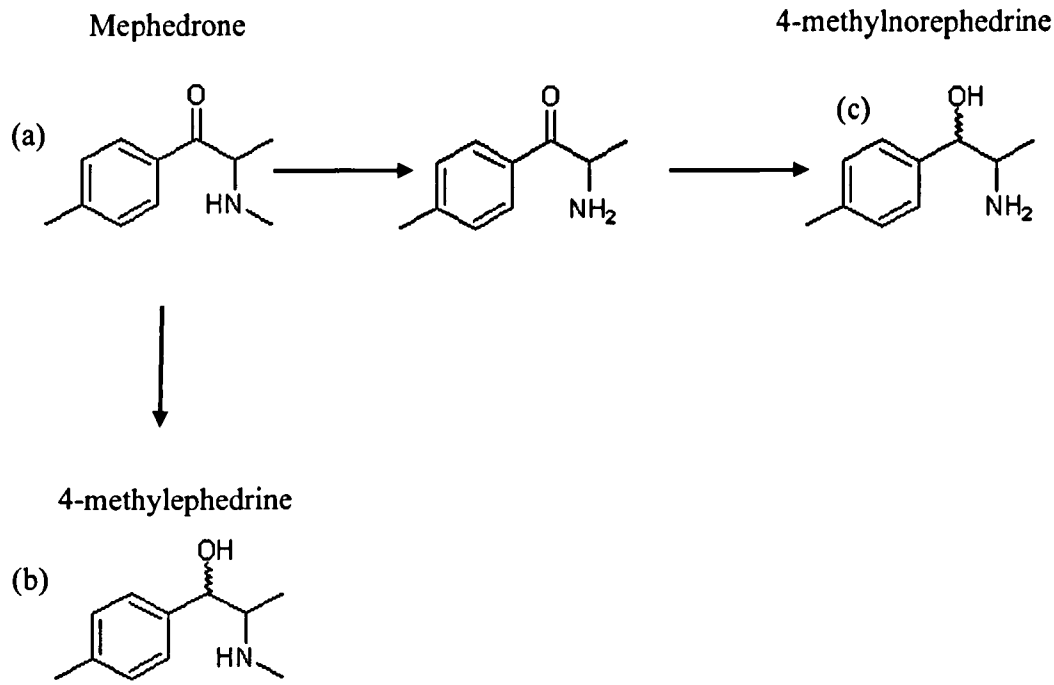


Figure 3.2: Proposed metabolism pathway for (a) mephedrone (b) 4-methylephedrine (c) 4-methylnorephedrine (19,21,62)

However there is a possibility of other pathways for metabolism of mephedrone. Some other metabolites that are deduced in another study are nor mephedrone, nor-dihydromephedrone, hydroxytolyl mephedrone and nor-hydroxytolyl mephedrone (10).

The frequent use of these psychoactive drugs has created a demand for sensitive, robust and reliable analytical methods for their identification and quantification in different matrices. GC-MS has been the common analytical technique that has routinely been used for the detection of mephedrone in matrices such as blood and urine (5,6,61,68,69). In the recent years, studies have shown the use of liquid chromatography mass spectroscopy (LC-MS) for analysing blood samples for the detection of mephedrone (6,111). Compared to the common techniques such as urine and blood analysis, hair analysis is a good method of providing information on long-term history of the individual's intake of controlled drugs depending on the pharmacokinetic properties of the drug in question (68,69). In addition, blood and urine samples require storage at -20 ^{0}C in the presence of preservatives until analysis, for mephedrone (5,68).

The aim of the study was to develop and validate a sensitive and reproducible analytical method for the quantitative analysis of mephedrone and its two metabolites 4-methylephedrine and 4 - normethylephedrine in human hair using liquid chromatography – mass spectrometry.

3.2 Materials and methods

3.2.1 Hair specimen

Following ethical approval from two higher education institutions, hair samples were obtained from 154 healthy volunteers (95 male, 59 females), as part of an independent study (72). The age of the volunteers ranged between 18 to 56 years old. Blank hair samples were donated by two healthy individuals with no history of any intake of social drugs (72).

3.2.2 Chemicals and consumables

HPLC grade methanol (MeOH), acetonitrile (ACN), water, and hexane, along with reagent grade ethyl acetate, ethanol, dichloromethane, chloroform and formic acid were all purchased from Fisher Scientific UK Ltd (Loughborough, UK). Tris HCl buffer and Cleland's reagent were obtained from VWR (Lutterworth, UK). Proteinase K, sodium hydroxide, hydrochloric acid, sodium hydrogen phosphate heptahydrate and sodium phosphate monobasic dehydrate were purchased from Sigma Aldrich (Dorset, UK). The metabolites 4-methylephedrine hydrochloride and 4-methylnorephedrine hydrochloride were synthesised by borohydride reduction of mephedrone and 4-methylcathinone respectively at Kingston University (London, UK). Reference standards Mephedrone hydrochloride and mephedrone – d3 hydrochloride (internal standard) were purchased from LGC standards (Teddington, UK).

3.2.3 Instrumentation

Analyses were performed on a Thermoscientific LC-MS/MS system consisting of an Accela UPLC system (Thermo Scientific, UK Ltd, Loughborough, Leicestershire, UK) coupled to a Triple Quadrupole TSQTM mass spectrometer (Thermo Electron Corp, UK). The data acquisition software used was Xcaliber version 2.0. An Agilent SB-C18 UPLC column (2.1 μm × 150 mm × 1.8 μm) was purchased from Agilent Technologies UK Ltd. (Wokingham, UK). Vortex mixer from Fischer Scientific (Loughborough, UK) was used to shake the mixture. A Thermo Scientific Centrifuge, Heraeus Labofuge 400R was used for separating the mixtures. Sample concentrator, Dri-block DB-3D purchased from Fischer Scientific was used for the evaporation of solvents using nitrogen gas.

3.2.4 LC conditions

ACN and water were used as mobile phase solvents. A solvent gradient was used to achieve the separation of compounds (Table 3.1). The column oven was used to maintain the column temperature at 50^0C and the auto sampler tray was set at 10 ^{0}C. The flow rate of the mobile phase was 200 µL/min and the injection volume 3.0 µL. An Agilent SB-C18 column (2.1 µm × 150 mm × 1.8 µm) with 0.2 µm inline filter was used to carry out analysis, in order to filter the particles coming from the mobile phase and the sample matrix. A 50:50 mixture of ACN and water was used as needle and syringe wash. Following LC conditions were used for the separation of the drugs (Table 3.1).

Table 3.1: Chromatography conditions used for the separation of the compounds

LC run time (min)	Acetonitrile (%)	Water (%)
0	65	35
3	100	0
4	100	0
5	65	35
10	65	35

3.2.5 MS conditions

The mass spectrometer was operated in positive ionisation mode using ESI source. The capillary temperature was maintained at 300 ^{0}C and a voltage of 4000 V was employed. The collision pressure was kept at 1.5 mTorr. Nitrogen was used as the source gas and also for the collision cell. This was operated in SRM mode to detect precursor and product ions. The protonated molecules $[M+H]^+$ of the analytes were used as precursor ions in order to achieve collision induced dissociation (CID). The

quantification was carried out by using Quan browser using Xcalibur software, which automatically integrates the peak area of the SRM transitions. The analytes' retention time (RT), ion transitions and optimum collision energy required by the precursor ions to produce the product ions are given in Table 3.2. The fragmentation pathways of all the analytes are shown in Figure 3.4.

Table 3.2: Ion transitions, RT and collision energies used for the analysis of the analytes and the internal standard

Compound	(RT) min	Precursor ions (m/z)	Product ions (m/z)	Collision energy (eV)
Mephedrone	2.07	178.1	160.1	10
			145.1	18
4-methylephedrine	1.93	180.2	147.2	19
			131.3	18
			115.2	39
4-methylnorephedrine	1.85	166.2	131.2	14
			115.2	33
			90.79	24
Mephedrone – d3	1.98	181.2	163.2	17
			148.2	20

3.2.6 Preparation of standards

The reference standard solution for mephedrone (1 mg/mL) in acetonitrile (ACN) and the internal standard mephedrone – d3 were used as stock and were stored in a freezer (- 20 ^{0}C). For 4-methylephedrine and 4-normethylephedrine, stock solutions were prepared by weighing out the powder and dissolving it in MeOH to the concentration of 1 mg/mL which were then stored in amber vials in a freezer (-20

^{0}C). Metabolites gave better sensitivity when dissolved in MeOH when compared to dissolving it in ACN. Working standards of mephedrone, mephedrone – d3 and the metabolites were prepared from the stock solutions at the concentration of 1 µg/mL for the optimisation of the LC-MS/MS parameters. All the working solutions were stored in glass vials in a fridge (4 ^{0}C). The calibrants for standard curve as neat and in hair were prepared from stock solutions. The QC solutions were also prepared from stock solution.

3.2.7 Hair digestion and extraction

a) Decontamination

Decontamination of the hair samples was carried out by rinsing with 2mL dichloromethane for 2 minutes (twice) at room temperature. This step ensured the removal of any contaminants from the surface of hair, which may interfere with the analysis(72). Generally, the less polar the solvent, the less that solvent swells the hair therefore is more difficult for the solvent to wash away the incorporated drug. After decontamination, the hair samples were completely air dried before cutting into very fine segments (*ca.*1 mm length) using scissors.

b) Hair Digestion Methods

i) Alkali digestion

Initially, hair digestion was carried out by incubating the hair samples (50 mg) with 1 M sodium hydroxide solution (1 mL) at 95 ^{0}C for 10 minutes. After the samples were cooled down to room temperature, the mixture was than neutralised by adding 1 M hydrochloric acid (ca. 0.9 mL) followed by addition of 0.2 M phosphate buffer (2 mL), pH 7.0.

ii) Enzymatic digestion

Alkali digestion led to the complete degradation of the analytes, mainly mephedrone. It could be due to the high temperature at which the alkali digestion is carried out. Other possible reason is pH of the solution when carrying out the digestion, as the solution is only neutralised after the hair is completely digested at 95 ^{0}C. Hence, enzymatic method was chosen alternatively to digest hair completely, which assures the extraction of majority of the analytes.

Enzymatic digestion was carried out by placing the fine hair segments (50 mg) in a glass vial containing Cleland's reagent (100 mg) and enzyme Proteinase K (15 mg). The internal standard mephedrone – d3 (100 µL) at 40 pg/mg was added to the mixture. The mixture was finally incubated with Tris buffer (1 mL) for 2 hours at 37.5 ^{0}C with constant stirring using a water bath.

c) Extraction

The digested hair mixture was then transferred to glass centrifuge tubes. For mephedrone, hexane (3 mL) was added to the digested hair in order to carry out liquid-liquid extraction. The contents were mixed for 2 minutes using a vortex mixer and centrifuged at 5 ^{0}C for 5 minutes at 1750 x g. The supernatant organic layer was separated and transferred into a glass tube. For 4-methylephedrine and 4-normethylephedrine, a mixture of chloroform, ethanol and ethyl acetate (3 mL in total) in the ratio of 3:1:1 (v/v/v) was used to carry out LLE. All the above steps used for mephedrone were repeated. The bottom organic layer were separated and pooled together in the same glass tube as used for mephedrone. The organic layer was then dried using nitrogen gas and reconstituted with 200 µL acetonitrile by using a syringe driven, 0.2 µm PTFE membrane filter.

3.2.8 Method validation

Method validation was carried out in order to establish the sensitivity, specificity, selectivity, linearity, LLOD, LLOQ, inter and intraday precision, accuracy and percentage recoveries. FDA guidelines were followed for the validation of measuring the analytes in hair matrix (103).

A blank hair sample was used as a negative control for the validation of mephedrone, 4-methylephedrine and 4-methylnorephedrine. This was confirmed by running the blank hair sample on LC-MS/MS.

Any matrix effects were investigated by digesting and extracting blank hair samples in the same manner as actual hair samples in the absence of any analyte or internal standard. Selectivity was determined in order to investigate if there are any interfering peaks at the same retention time as the analytes.

In order to construct a calibration curve, blank hair was spiked with known concentrations of the analytes along with internal standard and put through the same hair digestion and extraction process as used for the actual hair samples. The QC's and LLOQ were prepared in the same manner as calibrants at four different concentrations within the linear range of the calibration curve. The linearity of the curve was obtained by using linear regression analysis and by calculating the ratio of known concentration of mephedrone and its two metabolites to the internal standard. The ratio was calculated by dividing the peak area of the analyte by the peak area of the internal standard. A calibration curve was constructed in the range of 5 pg/mg to 100 pg/mg for mephedrone and 10 pg/mg to 150 pg/mg for both 4-methylephedrine and 4-methylnorephedrine. The internal standard mephedrone – d3 (100 µL) was added to all the standards with a final concentration of 40 pg/mg. The quantified precision for the calibrants was within ±15% of the RSD, whereas for the LLOQ

precision and accuracy was ±20%. LLOQ was determined as the lowest quantified concentration with an S/N ratio of at least 10, whereas LLOD was the lowest detectable concentration with an S/N of 3.

Interday precision was calculated by measuring six replicates at four different known concentrations on three consecutive days, whereas intraday precision was calculated by calculating six replicates of each of the four known concentrations within one day.

Accuracy was calculated by comparing the mean values calculated for the six replicates of each four known concentrations with the actual value calculated for that known concentration.

The extraction recovery was validated for mephedrone and its two metabolites at three different concentrations using six replicates by comparing the extracted samples with non- extracted samples.

3.3 Results and discussion

3.3.1 Validation results

Blank (control) hair that was analysed after decontamination with DCM and extraction using the chromatographic conditions stated in section 3.2.4 gave no chromatographic interferences due to the endogenous substances present in the hair. The precision, accuracy and recoveries lie within the limits set by the FDA guidelines (103). The detection and quantification of all the analytes and internal standard was carried out according to their SRM transitions. The internal standard was used to compensate for any loss the analytes may go through while carrying out LLE or any unknown matrix effects or any variations in the instrumental response. In order to get the optimum conditions and highest sensitivity possible, different LC

and MS conditions were tried such as choice of mobile phase. Acetonitrile gave better sensitivity as compared to methanol. Addition of formic acid (0.1%) to water resulted in a poor baseline (Figure 3.3).

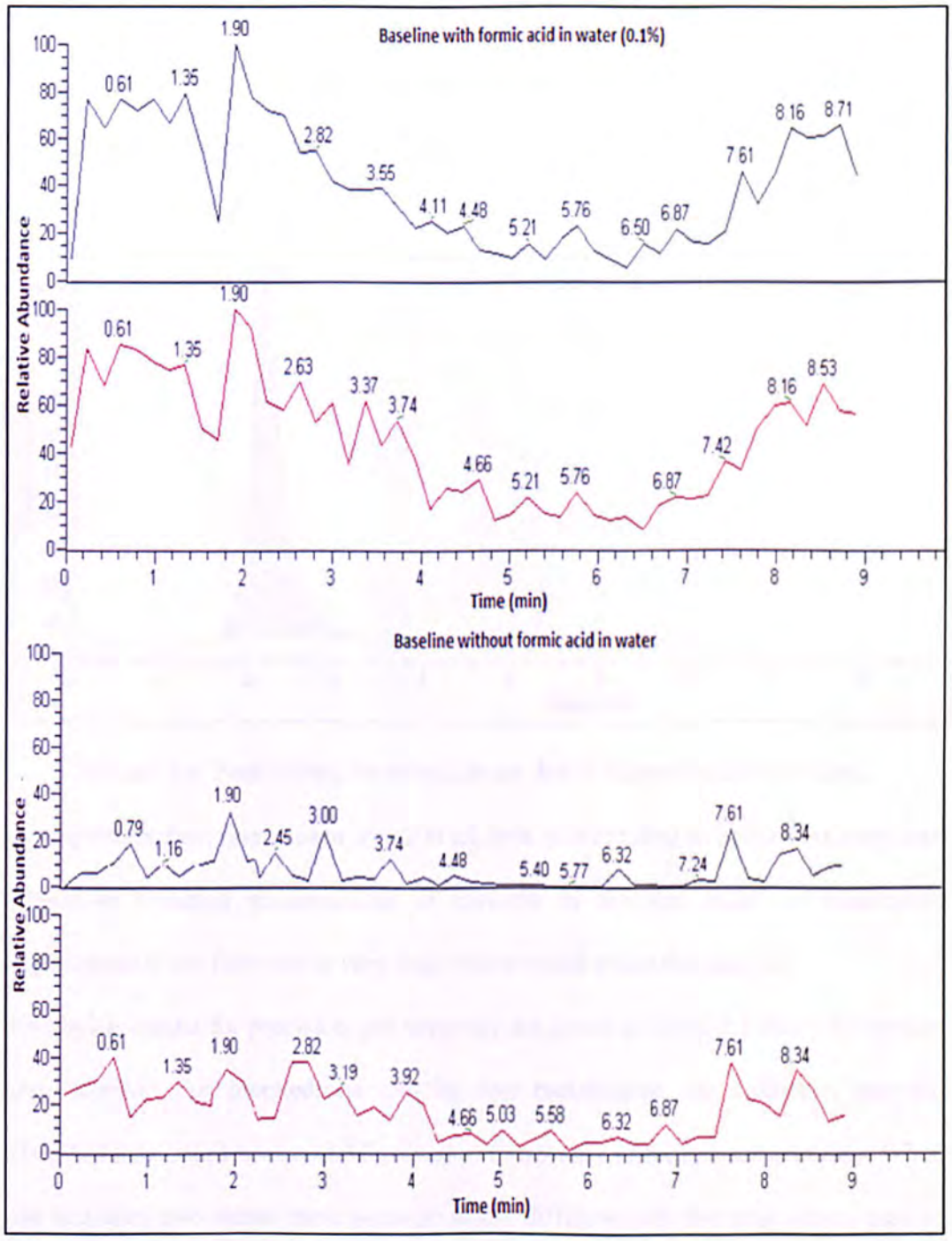


Figure 3.3: LC–MS/MS chromatogram showing baseline with formic acid (0.1%) in water and without formic acid in water

As compared to its metabolites, mephedrone gave better sensitivity with the conditions used for the analysis. The metabolites when dissolved in methanol gave better sensitivity compared to when dissolved in acetonitrile, whereas mephedrone gave a similar sensitivity when dissolved in both solvents. The optimum injection volume was 3.0 µL. Increasing the injection volume caused peak tailing and little broadening of the peaks as shown in Figure 3.4 below. A lower injection volume lead to poor sensitivity.

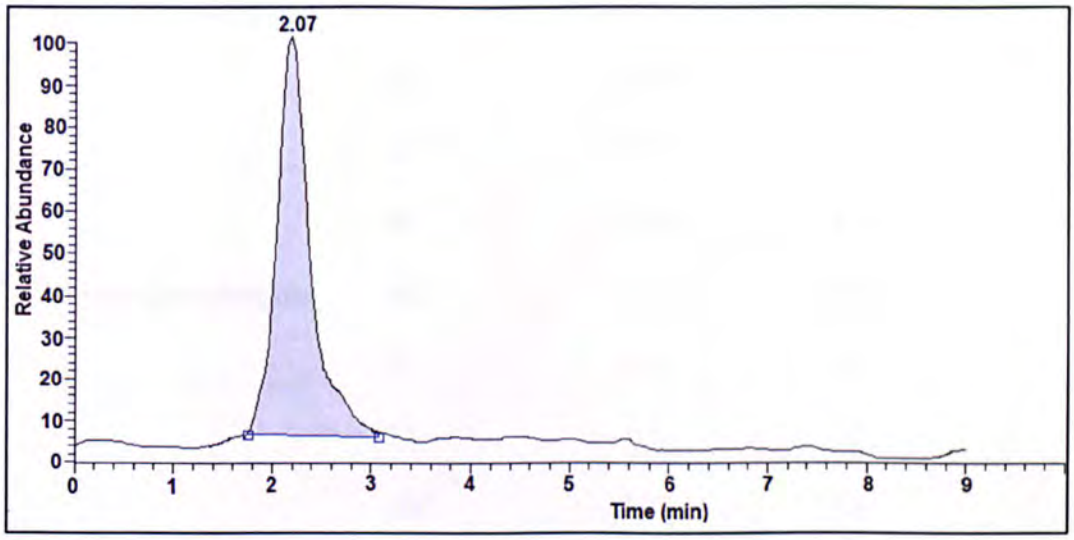


Figure 3.4: Peak tailing for mephedrone due to higher injection volume

The optimum flow rate chosen was 200 µL/min as it resulted in better sensitivity and helped in avoiding accumulation of solvents in the ion source as sometimes experienced if the flow rate is very high which could affect the analysis.

Validation results for precision and accuracy are given in Table 3.3 for both intraday and interday. For mephedrone and its two metabolites, the calibrants and the extracted hair samples were stable for up to two weeks in the autosampler tray. When injected after two weeks there were no major differences in the peak shape, area or ratio of the analyte to internal standard.

Table 3.3: Precision and accuracy for intraday and interday of the three drugs at four different concentrations

Compound	Concentration (pg/mg)	Precision RSD (%)		Accuracy %
		Intraday N=6+6+6 +6	Interday N=18+18+18+18	
Mephedrone	5	0.62	0.8	99.15
	10	0.4	0.7	106.34
	20	2.11	1.7	103.16
	40	2.56	4.5	107.53
4-methylephedrine	10	0.6	0.9	114.59
	20	0.75	0.7	110.76
	50	0.8	4	107.02
	100	4.92	3.4	98.52
4-methylnorephedrine	10	0.54	1.1	107.98
	20	0.2	0.7	106.51
	50	2.91	2	97.6
	100	6.97	7.3	100.67

Table 3.4: Extraction recoveries of the three drugs at three different concentrations

Compound	Concentrat -ion (pg/mg)	Recovery (%) N= 6+6+6	Regression Coefficient	Regression equation
Mephedrone	5	90.36 ± 4.76	0.999	y = 0.112x
	20	110.37 ± 6.33		+0.429
	40	108.10 ± 5.03		
4-methylephedrine	10	106.16 ± 5.80	0.990	y = 0.012x +
	20	98.37 ± 8.60		0.033
	100	101.87 ± 5.90		
4-methylnorephedrine	10	100.23 ± 3.21	0.990	y = 0.008x +
	20	96.78 ± 10.12		1.389
	100	101.32 ± 6.0		

Figure 3.5 shows the calibration curves for mephedrone, 4-methylephedrine and 4-methylnorephedrine in hair. The linearity for all three analytes was within the quantification range.

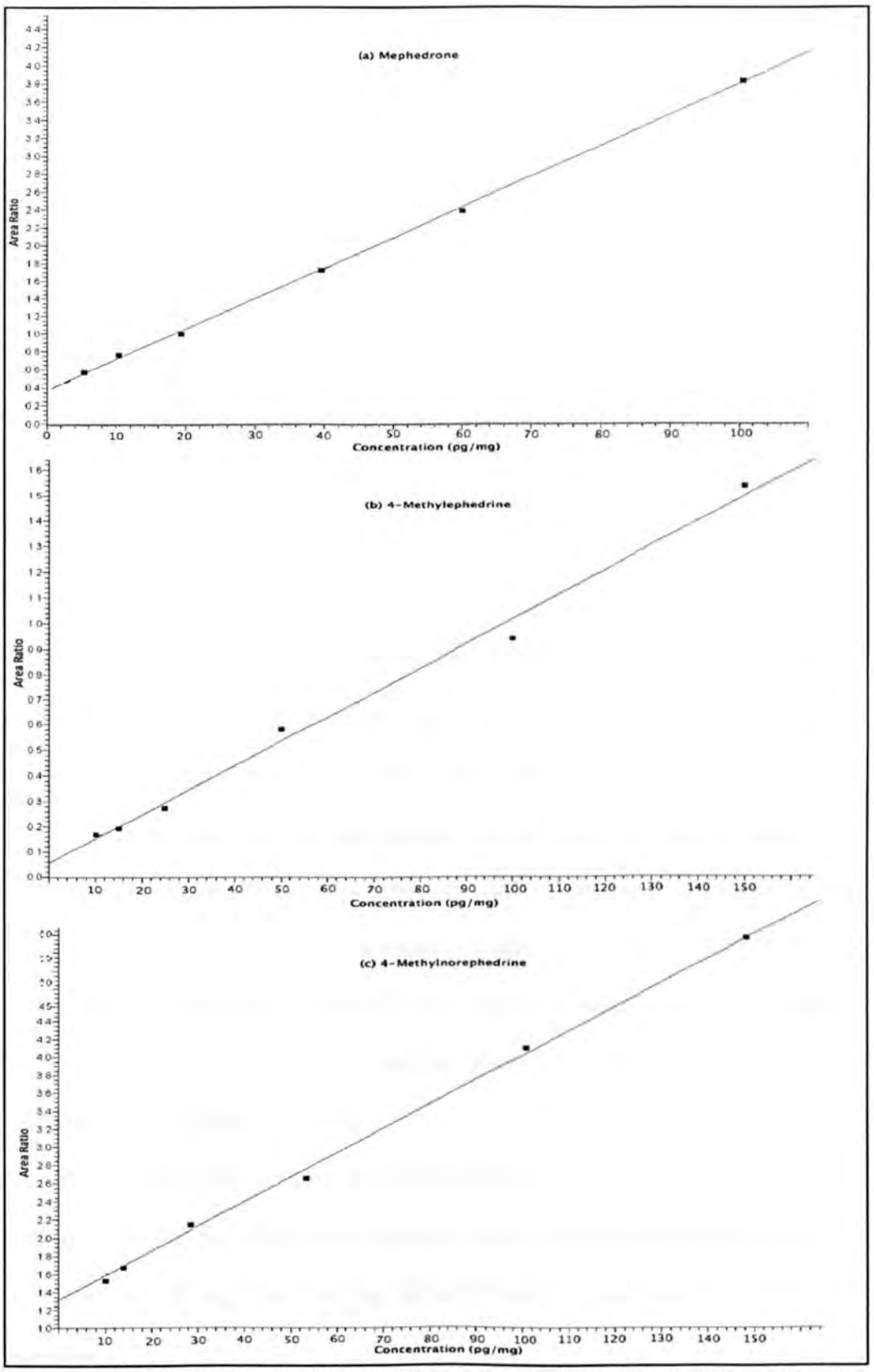


Figure 3.5: Calbration curves for (a) mephedrone, (b) 4-methylephedrine and (c) 4-methylnorephedrine

LLOD and LLOQ values for mephedrone were 2.5 pg/mg and 5 pg/mg respectively. For 4-methylephedrine and 4-methylnorephedrine, the LLOD was 5 pg/mg and the LLOQ 10 pg/mg. The signal to noise ratio for the LLOD and LLOQ are given in Table 3.5.

Table 3.5: Signal to noise ratios (S/N) for the LLOD and LLOQ

Compound	S/N for LLOD N=6	S/N for LLOQ N=6
Mephedrone	7	11
4-methylephedrine	5	10
4-methylnorephedrine	4	10

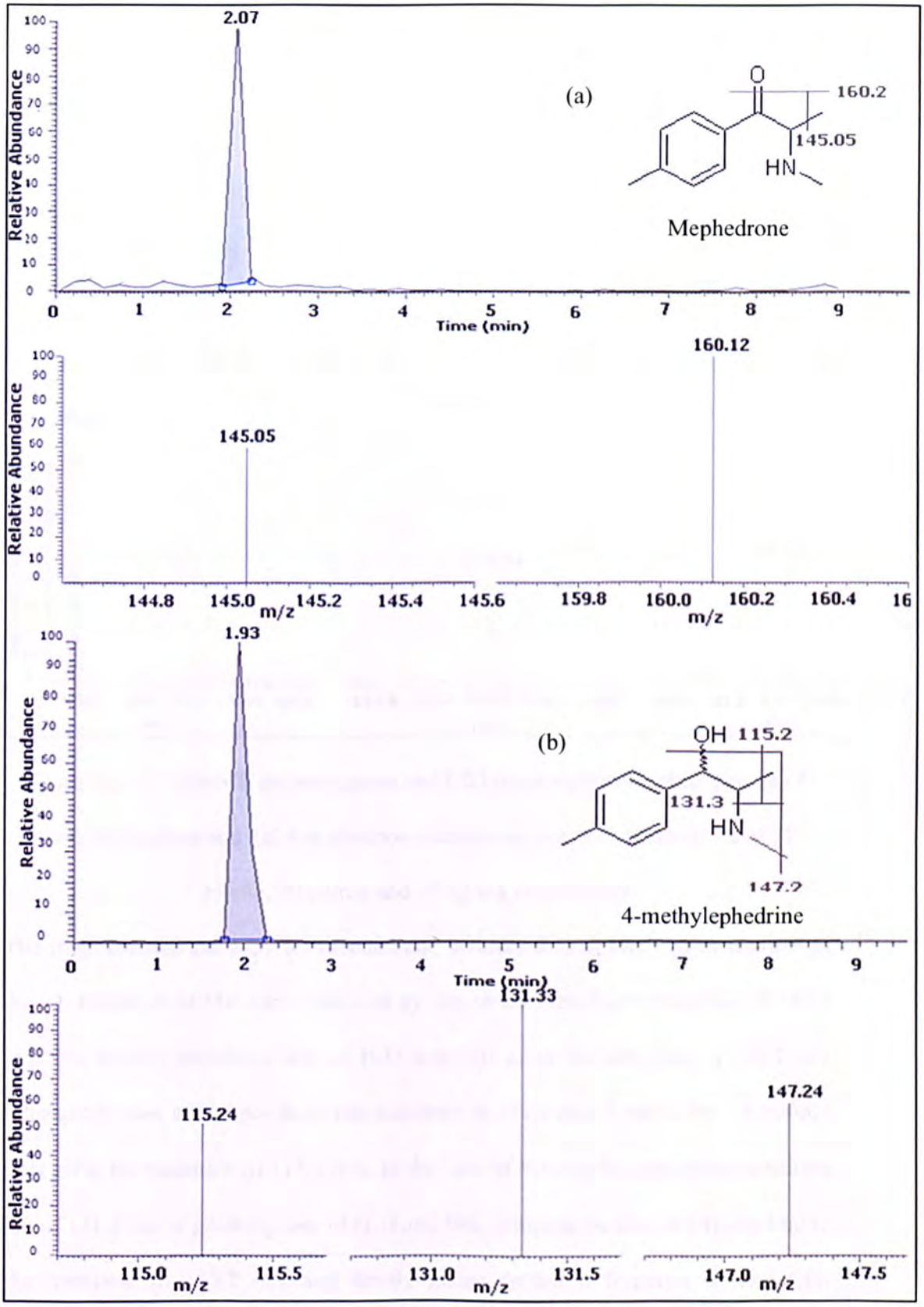
Mephedrone was extracted using different solvents such as pentane, chloroform, and mixture of chloroform, ethanol and ethyl acetate with different ratios including (3:1:1, 2:1:1), but hexane gave the best results. For the metabolites, 4-methylephedrine and 4-methylnorephedrine, hexane was not a good choice of a solvent as the extraction recoveries were very poor. Pentane gave very similar results with poor extraction recoveries. The mixture of chloroform, ethanol and ethyl acetate (3:1:1) gave the best results. Hexane is not as polar as the mixture of solvents used and as metabolites are more polar than the parent drug these tend to prefer to be extracted with more polar solvents.

The LLE with the solvents used for the mephedrone and the metabolites enhanced the purification of the solution and got rid of any unwanted components in the hair matrix. Vortex mixing while carrying out LLE helped in maximum extraction of the analytes.

For extraction of mephedrone the top organic layer was decanted whereas for the extraction of the two metabolites the bottom organic layer was decanted. When separating the organic layer in extraction process it was ensured that there were no drops of aqueous layer transferred along with the organic layer. Blank ACN was injected after every two sample runs to avoid any contamination or carry over effects of the column.

The analytical method gave reproducible and highly sensitive results for mephedrone and 4-methylephedrine and 4-methylnorephedrine.

Figure 3.6 below shows LC–MS/MS chromatogram and CID spectra of mephedrone and its two metabolites 4-methylephedrine and 4-methylnorephedrine in hair at concentrations of 10 pg/mg, 20 pg/mg and 20 pg/mg respectively.


2.07
Relative Abundance
Time (min)
(a)
O
160.2
145.05
HN
Mephedrone
160.12
145.05
m/z
1.93
(b)
OH 115.2
131.3
HN
147.2
4-methylephedrine
131.33
115.24
147.24

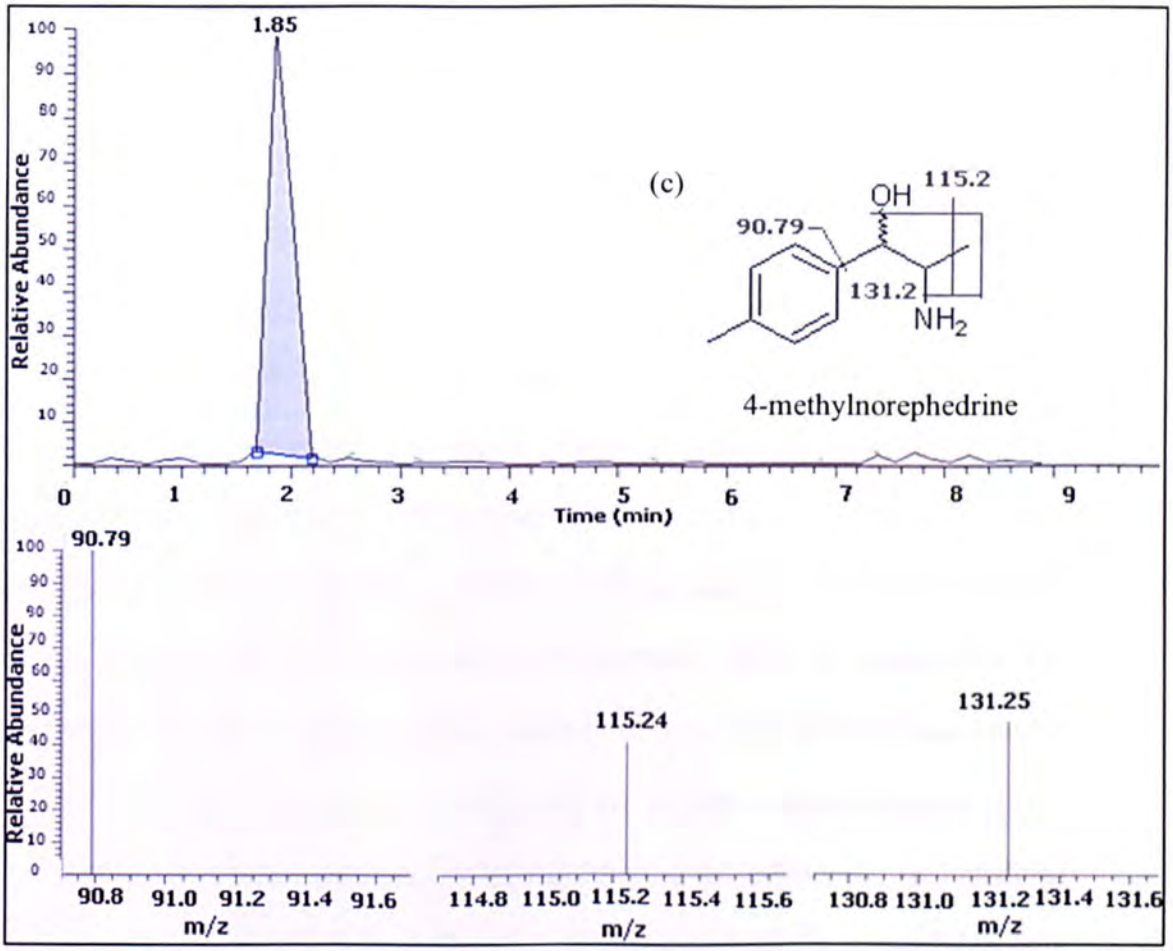


Figure 3.6: LC–MS/MS chromatogram and CID spectra of (**a**) mephedrone, (**b**) 4-methylephedrine and (**c**) 4-methylnorephedrine in hair at concentrations of 10 pg/mg, 20 pg/mg and 20 pg/mg respectively

The fragmentation pathway for mephedrone includes loss of H_2O which resulted in the ion transition of 160.1 m/z followed by loss of CH_3 leading to transition of 145.1 m/z. For 4-methylephdrine, loss of H_2O and CH_3 gives the transition of 147.2 m/z followed by loss of NH produces ion transition of 131.3 m/z. Finally, loss of another CH_3 gives the transition of 115.2 m/z. In the case of 4-methylnorephedrine transition ion of 131.2 m/z is given by loss of H_2O and NH_2 followed by loss of CH_3 leading to the transition of 115.2 m/z and finally losing further a fragment of two CH_2 producing an ion transition of 90.79 m/z.

3.3.2 Application to human hair

The method was applied to human hair samples that were collected from healthy volunteers. Out of the 154 samples analysed, mephedrone was detected in only five samples. However, the metabolites were not detected in any of the samples analysed. In this study, the metabolites could not be detected in the hair samples. In general, high polarity metabolites, which are destined to be excreted in urine, are difficult to detect in hair. In contrast, the parent drugs are lipophilic and can become incorporated into the hair (8). Due to that reason, sometimes it is difficult to detect metabolites in hair, though they could travel and get incorporated in hair. Another possible reason could be the sensitivity of the method to detect the metabolites. The concentration of the metabolites in hair could be very low and therefore could not be detected. However, owing to the method used for data and sample collection (72), it is impossible to link admission of mephedrone use to individuals, or individual samples. Out of five samples, mephedrone could be successfully quantified in only one sample. It was found to be present at a concentration of 21.11 pg/mg. For the rest of the four positive samples, mephedrone was present below the level of LLOQ, but above the level of LLOD. Figure 3.7 shows the chromatogram and mass spectra of the quantified positive sample.

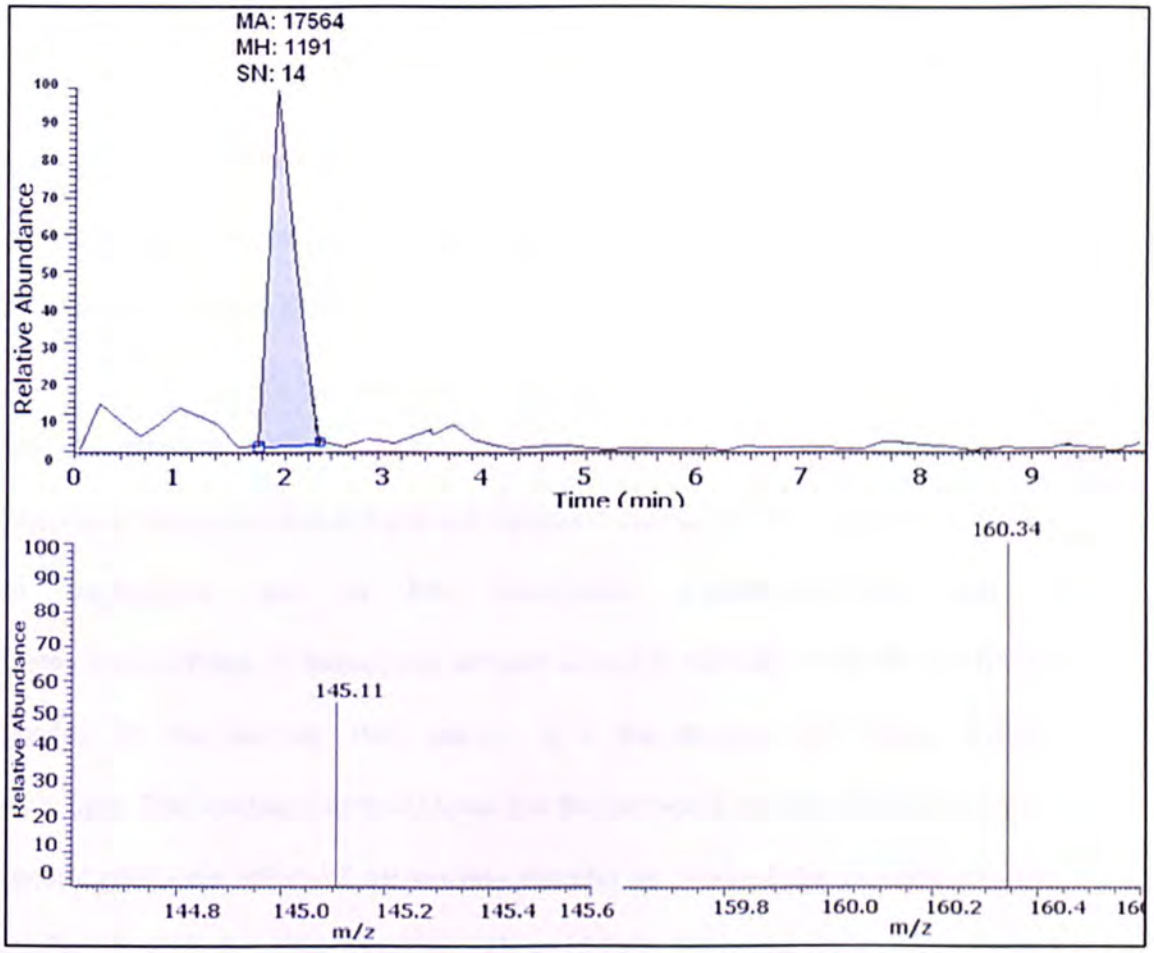


Figure 3.7: LC–MS/MS chromatogram and CID spectra of the positive samples at concentrations of 21.11 pg/mg

The results confirm that the LC-MS/MS method developed for mephedrone and its two metabolites is selective, reproducible and reliable. The hair analysis results showed that only 50 mg hair is required for the detection of mephedrone and its metabolites. The novelty of this method is that this method provides simultaneous sensitive detection of mephedrone, 4-methylephedrine and 4-methylnorephedrine using LC-MS/MS with very less hair sample requirement. Compared to previously published methods on GC-MS this method doesn't require any sample derivatisation, hence it is more economical, rapid and less time consuming. Hair analysis is capable of providing a long-term history of drug use and any kind of hair treatment such as

bleaching cannot affect the incorporation of the drug into hair. Thus, it can be used to compliment urinalysis for identifying routine drug users (70).

The study is also affiliated with the investigation to estimate doping prevalence by asking respondents to complete questionnaires. Hair analysis was employed to validate these surveys (72).

3.4 Conclusion

The study describes a novel, rapid and replicable method for the quantitative analysis of mephedrone and its two metabolites, 4-methylephedrine and 4-methylnorephedrine, in human hair samples using LC-MS/MS. Only 50 mg hair was needed for the analysis. Hair analysis is a non-invasive and human friendly technique. The validated method shows that the method is reproducible and does not require additional effort of derivatising samples as required for GC-MS/MS. The method developed is quite sensitive with an LLOQ of 5 pg/mg for mephedrone and 10 pg/mg for its two metabolites. The absence of any interference on the chromatograms shows that the method is highly selective with no matrix effects. The method was successfully applied to the human hair and is suitable for the future detection and quantification of mephedrone and its metabolites for the given sensitivity in hair and could complement urine and blood analysis.

CHAPTER 4

SIMULTANEOUS QUANTIFICATION OF ANTIRETROVIRAL DRUGS ABACAVIR AND TENOFOVIR IN HUMAN HAIR BY LC-MS/MS

4.1 Introduction

4.1.1 Antiretroviral therapy

At present, there are three major therapeutic families that are being used in "highly active antiretroviral therapy" (HAART). Some are used in combination as antiretroviral therapy (cART) or HAART for treating HIV. These comprise of a combination of nucleoside reverse transcriptase inhibitors (NRTIs), non – nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs) and most recently approved integrase inhibitors for the antiretroviral treatment for patients with viral resistance. All these inhibitors act against multiple viral targets (25-27,29). Nucleoside reverse transcriptase inhibitors (NRTIs) are the first antiretrovirals used against HIV infection. Their role is to prevent the synthesis of viral DNA by the reverse transcriptase enzyme (23,24).

Nucleoside/nucleotide reverse transcriptase inhibitors prevent replication by inhibiting the synthesis of viral DNA by the reverse transcriptase. These compounds

have to be phosphorylated at the intracellular level by endogenous kinases in order to obtain the active triphosphate moieties. These active anabolites then competitively inhibit the incorporation of endogenous nucleoside triphosphate by the viral reverse transcriptase enzyme, therefore blocking the synthesis of new viral DNA strand(23) Abacavir and tenofovir are prodrugs that belong to the NRTI's family and need metabolic activation. Tenofovir is a nucleotide reverse transcriptase inhibitor and abacavir is a nucleoside reverse transcriptase inhibitor and thus are key components of cART regimens. The effect of these drugs depends on the degree of metabolic phosphorylation that they undergo and thus they need metabolic activation (32,33,112).

4.1.2 Treatment adherence and compliance

There are many different clinical situations that require quantification of these therapeutic drugs in patients, such as therapeutic drug monitoring (TDM), drug interactions, patients with resistant genes and compliance testing (23,31). One of the major reasons for the failure of these therapeutic drug treatments is the non-compliance to the treatment with the development of drug resistance (25). The development of resistance to these drugs when used in combination therapy is complex, with each class being postulated to develop resistance at varying levels of adherence to therapy (25). The combination therapy helps patients with adherence to medication regimens and to improve the efficacy (113).

A major reason for the failure of anti-HIV treatment is the non-compliance or poor adherence to the medication. Adherence to medication is especially important for improved clinical outcomes for a range of conditions including tuberculosis (TB) infections and treating HIV patients. This has led to intense interest in developing

analytical methods to determine drug levels in patient samples (25,29-33). Quantification of these NRTIs is very useful where a patient's compliance to the treatment needs to be monitored. The treatment usually comprises of a combination of three or four different drugs for an active antiretroviral therapy (30).

4.1.3 Quantification of antiretroviral drugs using hair analysis

The quantification of these antiretroviral drugs is very important for monitoring of HIV therapy. There have been many studies carried out in the past for the measurement of these drugs in human plasma, but the drawback of this technique is that it only provides short – term information on the exposure to these medications. On the other hand, hair analysis provides information over a longer period (from weeks to months), which could help in better predictive treatment outcomes of HIV therapy and give information on efficacy and toxicity of these drugs. Also, compared to blood or urine samples, hair samples are easy to collect and there are fewer hazards associated when storing or handling these samples (37). Hair grows at approximately 1 cm per month and can give the history of an individuals drug use of the previous days e.g. 3 cm hair could give 90 days history of an individual's drug use (58,61,70).

To date, there has not been any published method for simultaneous quantification of abacavir and tenofovir using hair analysis, as hair is more suitable for monitoring individual's repeated drug uptake over time (38). However, hair analysis has been used for the quantitative and qualitative analysis of many different social drugs (70,107). TDM has been used to aid cART/HAART in order to check adherence, monitor viral resistance and to individualise dose regimens (26,34).

Due to its high sensitivity and specificity, LC-MS/MS has gained increased popularity for analysis of many different drugs, including antiretroviral drugs (34). LC-MS/MS is a very useful instrument and plays a major role in therapeutic drug monitoring (TDM) in different matrices such as hair, plasma and urine. Due to the advantages associated with this technique, it is widely used for TDM of antifungals, antidepressants, antibacterials and antivirals (31).

Measurement of these drugs in plasma can be carried out by different analytical techniques (114-116). Although a previous study reported a sensitive LC-MS/MS method for the analysis of the anti-HIV drugs, efavirenz, lopinavir and ritonavir (37) and tenofovir (23,29) in human hair, no hair-based method was available for the simultaneous determination of the two key drugs used in this study, abacavir and tenofovir. Depending on the antiretroviral therapy regimen prescribed to patients, tenofovir and abacavir could be taken together along with other NRTI's and PI's or could be taken separately in combination therapies with other antiretroviral drugs (23,28).

This aim of this study is to develop a hair-based analytical method for two common NRTI's abacavir and tenofovir using LC–MS/MS. As one of the two drugs or in some cases both of the drugs are co-administered with other NRTI's and PI's in the present patients (29). This is a component of a larger observational study, which has the aim of investigating predictors of the adherence and non-adherence to prescribed exercise and medication therapy in HIV seropositive patients (117).

4.2 Materials and methods

4.2.1 Hair samples

Ethical consent for the broader clinical study on adherence was granted by the NHS (UK) National Research Ethics Service (Ref. No. 09/H0712/86) which included hair sampling and analyses (38). Thirty-two HIV positive patients undergoing combination therapies donated the hair samples. All hair samples were stored in paper envelopes at room temperature. A blank hair sample was obtained from one healthy individual and was used to prepare calibrants and quality controls (QCs) in order to validate the study.

4.2.2 Chemicals and consumables

Abacavir sulphate, tenofovir and abacavir – d4 (internal standard) were purchased from LGC standards (Teddington, UK). HPLC grade methanol and reagent grade hexane, ethyl acetate, ethanol, dichloromethane and formic acid were purchased from Fisher Scientific UK Ltd (Loughborough, UK). Methyl *tert*-butyl ether, sodium hydroxide, hydrochloric acid, trifluoroacetic acid, sodium phosphate dibasic heptahydrate, sodium phosphate monobasic dihydrate and ammonium acetate solution were purchased from Sigma Aldrich (Dorset, UK). Acetonitrile and water were of LC-MS grade and were purchased from VWR (Lutterworth, UK).

4.2.3 Instrumentation

A Thermo Scientific LC-MS/MS system consisting of an Accela UPLC system (Thermo Scientific, UK Ltd, Loughborough, Leicestershire, UK) coupled to a Triple Quadrupole TSQ Quantum Access mass spectrometer (Thermo Electron Corp, UK) was used. Varian C18 SPE cartridges and an Agilent zorbax eclipse XDB-C18

column (2.1 μm x 150 mm x 1.8 μm) was purchased from Agilent Technologies UK Ltd (Wokingham, UK). A Cerex System 48 Pressure Processor was used from SPEware Corporation (California). GV-65C solid-phase extraction (SPE) cartridges were purchased from biochemical diagnostics, Inc (NY, USA). A vortex mixer from Fischer Scientific (Loughborough, UK) was used. A Thermo Scientific Centrifugation system, Heraeus Labofuge 400R was used for separating the mixtures. A sample concentrator from Technne, Fischer Scientific (Loughborough, UK) was used for the evaporation of solvents using compressed/nitrogen gas. A Fritsch mini-mill 'pulverisette 23' (Idar-Oberstein ,Germany) was used to pulverise hair.

4.2.4 LC-MS/MS conditions

To achieve the optimum conditions for the analysis of tenofovir and abacavir; ACN and water were used as mobile phase. The column temperature was maintained at 30 ^{0}C and the autosampler tray was set at 10 ^{0}C. The flow rate was kept at 200 μL/min and the injection volume of 3.0 μL was used. ACN was used as needle and syringe wash. The mobile phase composition and gradient conditions are given in Table 4.1.

Table 4.1: Chromatography conditions (gradient elution) used for the separation of the compounds

LC run time (min)	Acetonitrile (%)	Water (%)
0	85	15
3	85	15
4	50	50
7	50	50
8	85	15
12	85	15

The mass spectrometer was set to electrospray ionization in positive ion mode. A voltage of 4000 V was employed to the capillary. The collision pressure was kept at 0 mTorr and the capillary temperature was maintained at 300 ^{0}C. Nitrogen gas was used as the source gas and also for the collision cell. The instrument was operated in SRM.

4.2.5 Hair decontamination

Decontamination is essential for the hair samples in order to remove undesired contaminants from the hair surface that may lead to interferences with the analysis. The process involved rinsing hair samples with 2 mL of DCM twice for 2 minutes at room temperature. The hair samples were then dried and pulverised into a fine powder using a Fritsch mini-mill for 5 minutes.

4.2.6 Extraction method

A number of extraction methods were examined for abacavir and tenofovir in order to get the optimum extraction recovery.

4.2.6.1 Alkali digestion

Hair (50 mg) was placed in a glass vial with 1 M sodium hydroxide (NaOH) (1 mL) at 95 ^{0}C. The mixture was incubated for 10 minutes where all the hair was dissolved. The solution was then transferred to glass centrifuge tubes and was neutralised with 1M hydrochloric acid (HCl) (1 mL) and 0.2 M phosphate buffer (2 mL). The mixture was vortex mixed for 2 minutes.

4.2.6.2 LLE

LLE was applied to the above hair mixture, where different solvents and their mixtures were used such as hexane, and a mixture of chloroform: ethyl acetate: ethanol 3:1:1 (v/v/v). The extraction solvent (3 mL) was added to the mixture and was vortex mixed thoroughly for about 1 minute. The digested hair solutions were centrifuged for 20 minutes at 3939 x g at 4 ^{0}C. The top layer was decanted for LLE using hexane, whereas for the mixture of solvents, the bottom layer was decanted using a pasteur pipette and placed in a separate glass test tube. The extracted samples were then dried using nitrogen gas and reconstituted with 200 µL methanol and water (1:1, v/v).

4.2.6.3 SPE

SPE was also applied instead of LLE with different SPE cartridges such as cation exchange/non-ionic and C18. The method was adapted from previously published

methods. The cartridges were preconditioned with methanol (3 mL) and 0.6 % TFA (v/v in water) (3 mL). Digested hair solution (1 mL) was added to the cartridges and these were rinsed twice with 0.6 % TFA (2 mL) followed by elution of the drugs with methanol (2.5 mL). The solution was then evaporated using nitrogen gas and reconstituted with water (200 µL) (118). A pressure processor was used in order to balance the elution of the analytes from the cartridges.

4.2.6.4 Methanolic extraction

The above three ways of isolation of the drugs from hair matrix did not give good extraction recovery. Methanolic extraction was used alternatively and was adapted from a previous study where hair (50 mg) was placed in a borosilicate glass tube containing methanol (1 mL) and 100 µL abacavir – d4 internal standard at a final concentration of 0.15 ng/mg (37). The contents were vortex-mixed for 1 minute and were agitated in an orbital shaker for 16 hours at room temperature overnight. The mixture was then centrifuged at 4 ^{0}C for 15 minutes at 1750 x g and was then evaporated using compressed air. Ammonium acetate (250 mM), pH 8.5 (1 mL) was added to the tubes followed by addition of a mixture (3 mL) of methyl tert butyl ether: ethyl acetate (1:1). The contents were vortex-mixed for 2 minutes and centrifuged again at 4 ^{0}C for 5 minutes at 1750 x g. The samples were then placed in the freezer (- 20 ^{0}C) for 15-20 minutes, in order to freeze the aqueous layer. The top organic layer was transferred in a separate glass tube and dried using compressed air. The samples were reconstituted with 200 µL acetonitrile: water and filtered with 0.2 µm, PTFE membrane syringe filters (1:1) (37).

4.2.7 Preparation of standard solutions

Stock solutions for abacavir and tenofovir were prepared by weighing out 1 mg of each and dissolving it in MeOH (1 mL) to make up to the concentration of 1 mg/mL. The stock solutions were stored in a freezer (- 20 ^{0}C) in amber glass vials. Working standards of the two drugs were than prepared from stock solutions at the concentration of 1 μg/mL and were stored in amber glass vials in a fridge (4 ^{0}C). The same solution was used to optimise the LC-MS/MS parameters. The six calibrants for the standard curve for the two drugs tenofovir and abacavir as neat were prepared from stock solutions. The internal standard abacavir – d4 was added to all the standards at a final concentration of (0.15 ng/mg hair). The QC's and LOQ were prepared in similar manner using separate stock solutions.

4.2.8 Method validation

The major parameters for the validation of the analytical method involve recovery, specificity, linearity, sensitivity, stability, accuracy and precision. Both drugs were analysed in hair and the method was validated for all the above parameters in accordance with FDA guidelines (103).

The extraction recovery was validated for the two drugs by comparing the peak area ratio of the internal standard versus the peak area ratio of the analyte of the extracted samples of hair that was spiked with known concentration of the analytes with the un-extracted samples at three different concentrations.

Selectivity was established to investigate any ghost peaks appearing at the same retention time as the two analytes. This was achieved by extracting six replicates of blank negative hair samples and analysing these. Matrix effects were examined by spiking the extracted blank hair samples with the know amount of drug. The

resulting peaks were compared to the peaks produced by analysing an un-extracted standard sample containing same concentration of the drug. Here an un-extracted sample is referred to a standard sample (in solvent) freshly prepared from stock solution of the drug without going through any extraction process.

Six point calibration curves were constructed in hair in order to investigate the linearity of the method. This was achieved by spiking the blank hair extract with known concentrations of the analytes in the range of 0.12, 0.25, 0.5, 1.0, 2.0 and 4.0 ng/mg of drug/hair and with internal standard at a final concentration of 0.15 ng/mg hair. QC's were also prepared in exactly the same manner. The drugs were extracted as mentioned in the method. The calibration curve was constructed by calculating analyte to internal standard ratio against the known concentration added to each calibrant. This is achieved by dividing the peak area for the analytes by peak area of the internal standard. The range for QC's and LLOQ were within the calibration range and were validated at three different concentrations. The three sets of calibration curves with their QC's were prepared on three different days. Linear regression was used to evaluate the linearity of the calibration curves for both drugs.

To investigate the interday and intraday precision, the analysis was carried out by carrying out measurements of six replicates at four different known concentrations on three consecutive days. Intraday precision was calculated by analysing six replicates of each of the four known concentrations within one day. The mean values for these six replicates were compared to the known concentrations. The precision was validated by calculating the RSD % at each concentration level.

Accuracy was determined by comparing the mean values calculated for the six replicates of each of the four known concentrations with the actual value calculated

for that known concentration. Again, it was calculated within the day and on three consecutive days.

The samples were kept in the freezer and stability tests of the two drugs were performed by 3 freeze (at -20 ^{0}C) and thaw cycles of three replicates at three different concentrations of QCs (1, 0.5 and 0.25 ng/mg). It was examined to see if there are any variations in the results.

4.3 Results and discussion

4.3.1 Method optimisation

4.3.1.1 Chromatographic conditions optimisation

Initially methanol and water with (0.1%) formic acid were used as mobile phase to examine the analysis of abacavir and tenofovir, which resulted in a high column backpressure. Before adapting the LC method stated in Table 4.1, different gradient and isocratic conditions were examined along with different flow rates. Acetonitrile and water as mobile phase gave the optimum sensitivity. Above 200 µL/min of flow rate resulted in poor peak shapes as shown in Figure 4.1, whereas a flow rate of 200 µL/min gave the best peak shape and sensitivity.

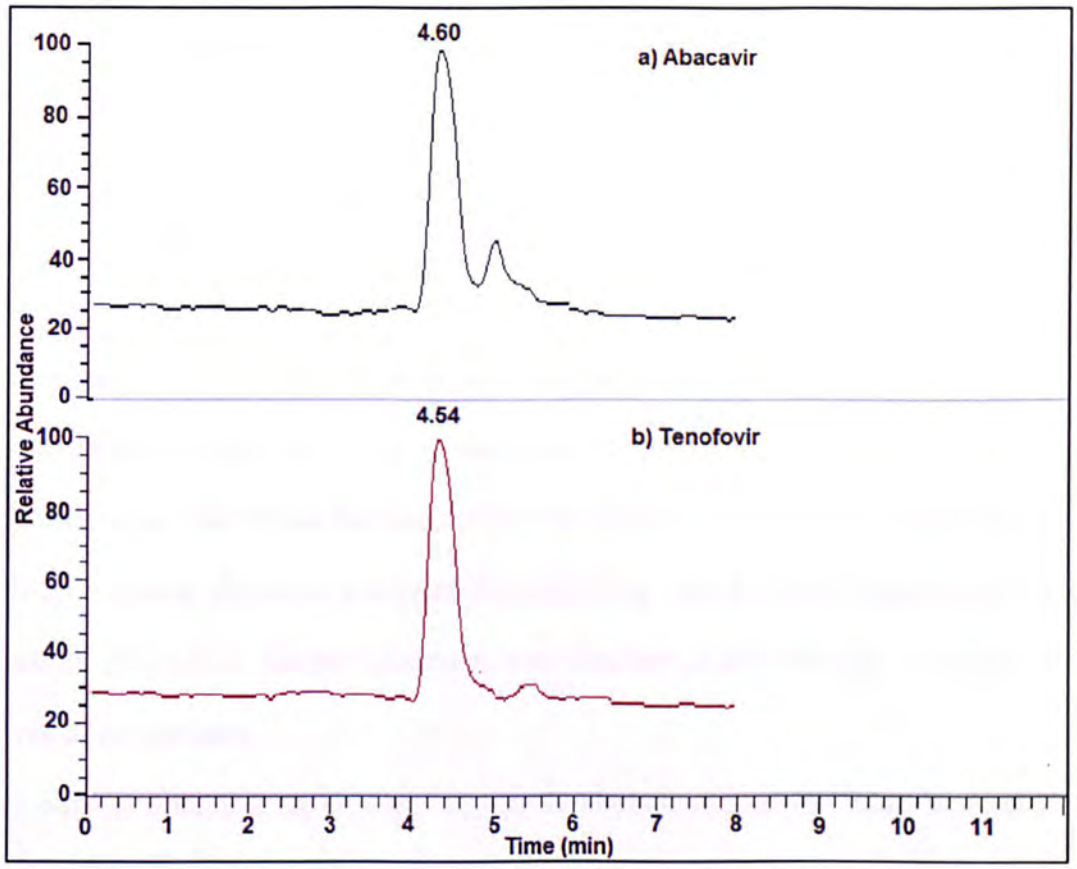


Figure 4.1: Poor peak shape for abacavir and tenofovir due to higher flow rate

This could be due to the mass transfer effect of Van Deemter equation as discussed in Chapter 1. The residence time of an analyte in the stationary phase is variable and can cause band broadening. The analyte molecules enter the pores in stationary phase by diffusion. Some molecules penetrate deeper into the pores than others causing variability in elution time. This can be minimised by reducing the size (diameter) of the packing material and also by lowering linear velocity (flow rate) of the mobile phase (82).

4.3.2 Mass spectrometer method optimisation

The mass spectrometer was operated in positive ion mode using an ESI source. Different parameters were optimised in order to determine the precursor and product ions of abacavir and tenofovir. The key parameters affecting the optimisation were

the spray voltage, capillary temperature; skimmer offset voltage, sheath and auxiliary gas pressure, mobile phase and its flow rate. The optimum spray voltage was kept at 4000 V for both drugs with the skimmer offset of 14 V. Sheath and auxiliary gas pressures were 30 psi and 5 psi, respectively. The optimisation was carried out initially by directly injecting 1 μg/mL solution prepared as standard for each drug separately, in order to determine the precursor ion. A full scan mode was used for the determination of precursor ions in the range of m/z 50 to 350, as both drugs had molecular weights within this range. Once the precursor ion was determined through direct injection, the drugs were then injected along with the mobile phase at a flow rate of 200 μL/min. The precursor ions were determined for both drugs separately as well as in a mixture.

In order to determine the product ions, the SRM mode was chosen on the instrument, where the voltage is applied in the collision cell and the product ions are produced. The product ions reach the third quadrupole where the ions are detected. Different collision energy values were applied and collision pressure was set at 1.5 mTorr. The efficiency of formation of products ions in MS/MS depends on the nature of the collision gas, collision energy and collision gas pressure. By increasing the collision energy and collision pressure, one can achieve greater production and intensity of fragment ions. Increase in gas pressure leads to increase in the number of ions produced under single-collision conditions and increasing the energy of deposition into the parent ion leads to more fragmentation under multiple-collision conditions (119,120).

The most abundant product ions seen were the m/z 191 for abacavir and m/z 176.1 for tenofovir. The internal standard abacavir – d4 was optimised in a similar manner

as the two drugs using the same optimum conditions and the most abundant product ion was m/z 197.1.

Initially, to optimise the mass spectrometer method, product ions were determined and the above conditions were used, but due to interferences at the similar retention time as the drugs, only the precursor ions were used for the analysis of the two drugs. At higher collision energies and collision pressures, there were interference peaks at around same retention time as the analytes as shown in Figure 4.2, which is why the method was run in SRM mode, but only the precursor ions were used.

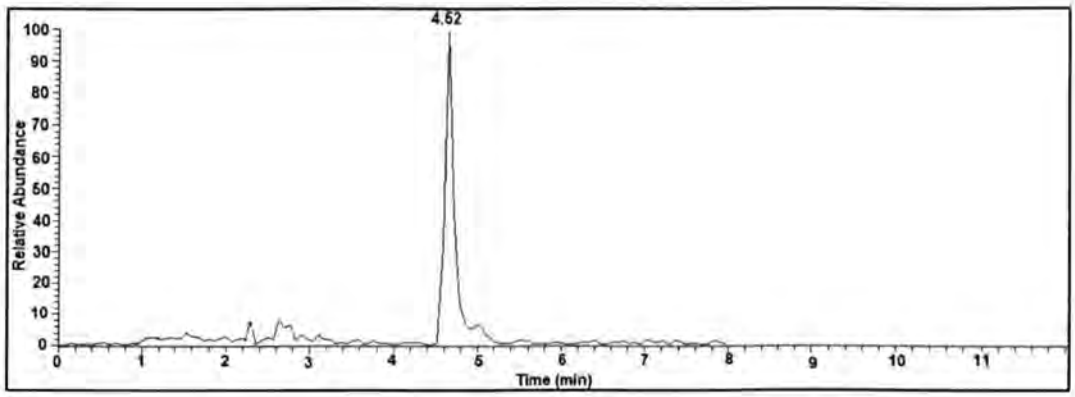


Figure 4.2: Interfering peak at the same retention time as the analyte

Lower collision energies were used so there is no fragmentation of the precursor ions. Also, it was not possible to chromatographically separate abacavir from the internal standard abacavir – d4, which led to the use of SRM instead of SIM, while the collision energies were kept at minimum. SRM enabled the filtering of the precursor ions for the drugs and the internal standard (Figure 4.1). Water and ACN were used as mobile phase. The column temperature and tray temperature were set at 30 ^{0}C and 10 ^{0}C, respectively. The mobile phase flow rate was kept at 200 µL/min and the injection volume of 3.0 µL was used. The collision pressure was kept at 0 mTorr where as the capillary temperature was maintained at 300 ^{0}C. Figure 4.3 shows the retention time for abacavir and tenofovir along with their precursor ions.

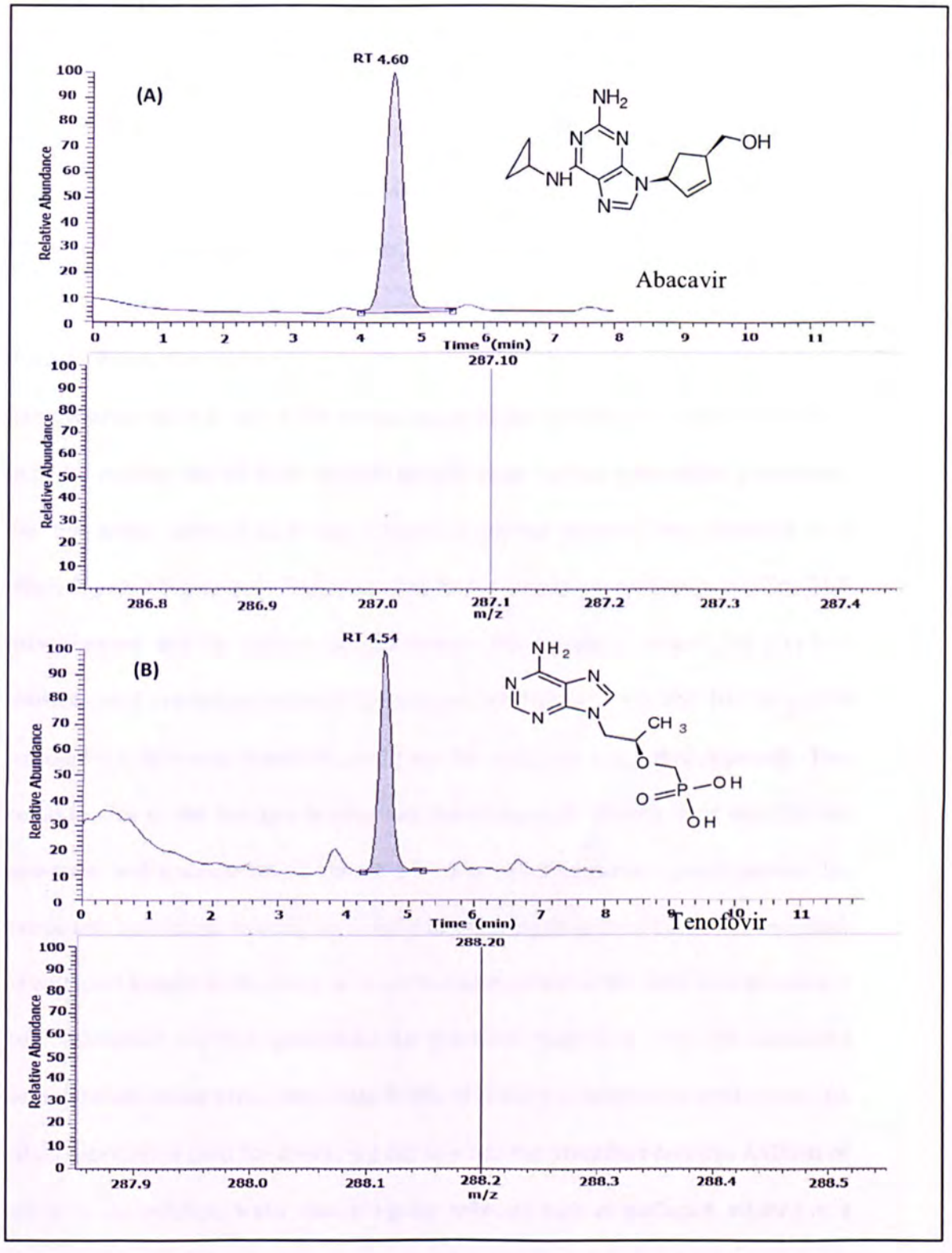


Figure 4.3: LC-MS/MS chromatogram of hair samples spiked with 1.0 ng/mg of (A) abacavir and (B) tenofovir along with their precursor ions and structures of the parent drugs

4.3.3 Method validation results

The method was fully validated for several parameters including extraction recovery, accuracy, sensitivity, linearity and intraday and interday precision was performed according to FDA guidelines and was found to be within the set limits (103). All the parameters validated are detailed below.

4.3.3.1 Extraction recovery

Sample preparation is one of the crucial stages of any bioanalytical procedure, which includes making use of most reliable sample clean up and preparation procedures. For this study, initially LLE was adapted to extract abacavir and tenofovir from alkali digested human hair. Different extraction solvents were investigated. The LLE using hexane and the mixture of chloroform: ethyl acetate: ethanol 3:1:1 (v/v/v) showed good extraction recovery for abacavir of 103 % $\pm$ 4.6 and 101 % $\pm$ 1.69 respectively, however, tenofovir could not be extracted using this approach. This could be due to the fact that hexane and the mixture of solvents used for LLE are non-polar and because of the nature of tenofovir, it requires a polar solvent for extraction. Extraction recovery is calculated by comparing the response of the mean of extracted sample to the mean of un-extracted standard of the same concentration x 100. According to FDA guidelines the precision limit is $\pm$ 15% for calculated concentration values hence more than 100% of extraction recovery in some cases. As alkali digestion is used for dissolving the hair and the procedure requires addition of buffer to the solution, water miscible polar solvents such as methanol, ethanol or a mixture of chloroform: ethyl acetate: ethanol 3:1:3 (v/v/v) were added however the two layers were miscible with each other therefore, it was not possible to carry out LLE using these solvents.

Instead SPE was applied using Cerex System 48 Pressure Processor for balanced elution of the analytes through the cartridge. SPE cartridges cation exchange/ non-ionic and C18 were used to determine a good extraction recovery. The cartridges were initially preconditioned with 3 mL of methanol and 0.6% of TFA (v/v in water) (3 mL). The digested hair sample (1 mL) was placed in the cartridge and was allowed to run through the cartridge using pressure processor. These were then rinsed with 0.6% TFA (v/v in water) (2 mL) and the elution of the drugs was carried out with 2.5 mL of methanol. The extracted sample was then dried using nitrogen gas and reconstituted with 200 µL of water.

There was very poor extraction recovery for tenofovir, of about 9% $\pm$ 26.25 for cation exchange/non-ionic cartridge and 7% $\pm$ 36.87 for C18 cartridge. The drug was either not retained by the cartridge or eluted into the waste in the wash up step or not eluted using the elution solvent. One way to investigate this was by injecting the waste part of the sample after the clean-up procedure, but as the digestion method involved NaOH, it was not possible as injecting any Na^+ into the instrument could lead to the formation of adduct ions that could interfere with the analysis of the drugs (121). The extraction of abacavir using cation exchange/non-ionic and C18 cartridges gave recovery values of 84% $\pm$ 5.78 and 96% $\pm$ 16.80 respectively.

One way to improve the recovery for abacavir was to carry out the above LLE using mixture of chloroform: ethyl acetate: ethanol 3:1:1 (v/v/v) and then applying the SPE method using C18 cartridges to the same samples and pool together the organic layers. The pooled organic layers were dried down and reconstituted with methanol (200 µL). This approach was used due to the fact that different organic solvents were used when carrying out LLE and SPE for extracting the two drugs. By carrying out LLE and then applying SPE to the same digested sample using different solvents can

help achieve maximum recovery possible. But LLE and SPE both extraction methods gave poor extraction recovery for tenofovir so pooling the organic layers made no improvements in recovering the drugs.

Finally, methanolic extraction was applied and afforded the best extraction recovery results in comparison to the other extraction methods tested. This kind of technique can extract entrapped drugs from hair without dissolving the hair (122). This approach proved to be a reliable, less laborious and more economical alternative as compared to LLE and SPE.

The extraction recoveries were determined at three different concentrations with six replicates each. The mean extraction recovery data studied over the concentration range are given in Table 4.2.

Table 4.2: The extraction recoveries for the two drugs at three different concentrations

Compound	Concentration (ng/mg)	Absolute Recovery (%) N= 6+6+6
Abacavir	0.12	96.15 ± 11.67
	0.50	108.16 ± 3.60
	1.00	111.0 ± 4.02
Tenofovir	0.12	95.23 ± 13.24
	0.50	117.27 ± 10.82
	1.00	99.09 ± 0.00

4.3.3.2 Specificity

The method was shown to be specific for tenofovir and abacavir. Specificity is the ability of an analytical method to distinguish the analyte within the sample that

contains other components as well. Six blank hair samples from one healthy individual were subjected to the entire extraction procedure and analysed. Figure 4.4 shows the absence of any peaks arising due other components in the matrix.

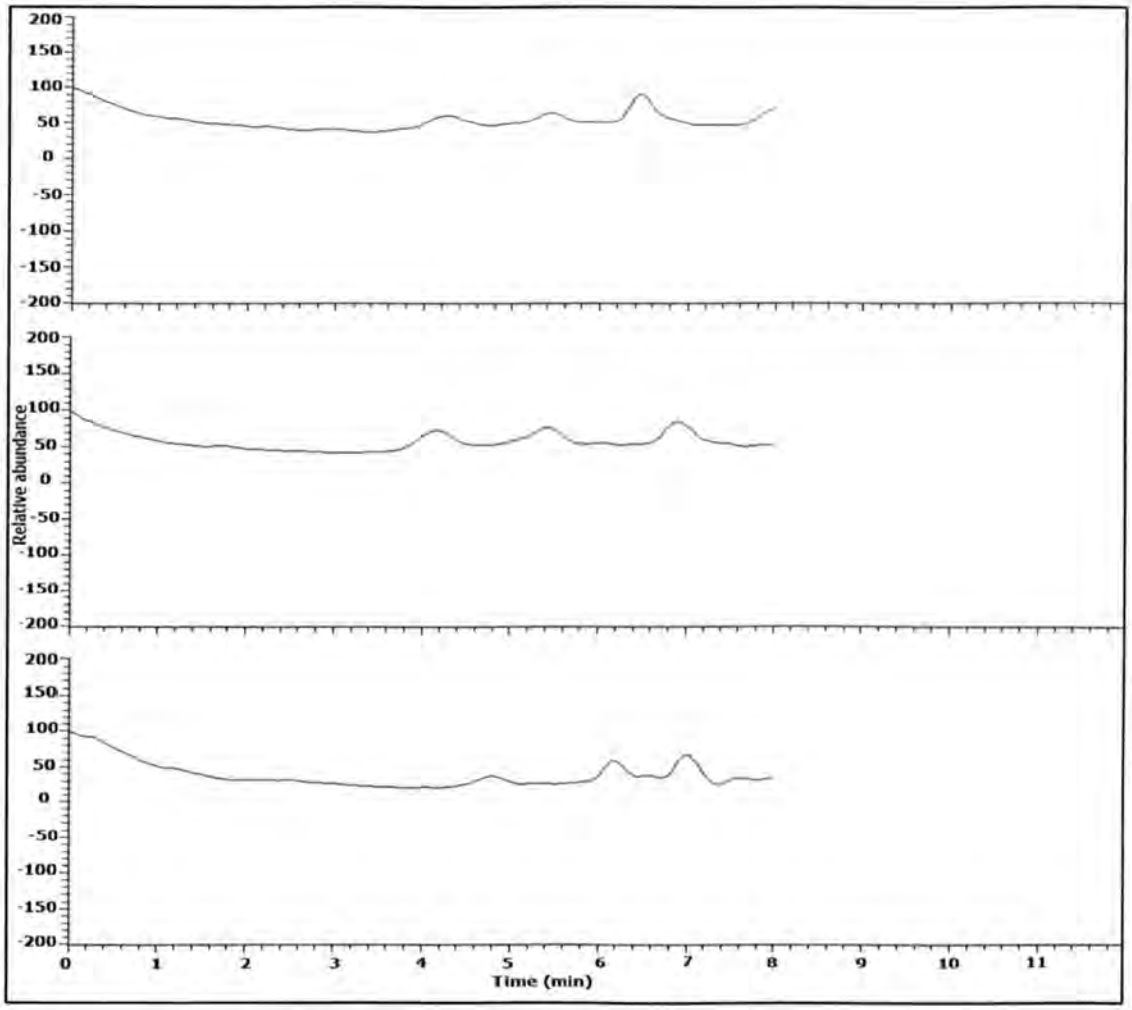


Figure 4.4: Chromatograms of three blank hair samples showing specificity

Analysis of these blank samples confirmed that there were no endogenous interfering peaks for abacavir, tenofovir and abacavir – d4 at the retention times 4.60, 4.54 and 4.58 min, respectively, therefore confirming specificity of the method.

4.3.3.3 Linearity

The calibration curves were shown to be linear over the concentration range of 0.12 – 4 ng/mg hair for both drugs (Figure 4.3). The regression coefficient (r^2) for abacavir and tenofovir were both 0.999 and the equations were (y 0.290504+3.74077*x), (y= 0.0224784+0.597258*x), respectively, showing the linearity of the method. The calibration solutions contained the extracted hair samples with internal standard abacavir – d4 (0.15 ng/mg hair). A blank solution containing the mobile phase was run after every two injections in order to avoid any carry over effects or instrumental contamination. The standard curves for abacavir and tenofovir in hair are shown in figure 4.5.

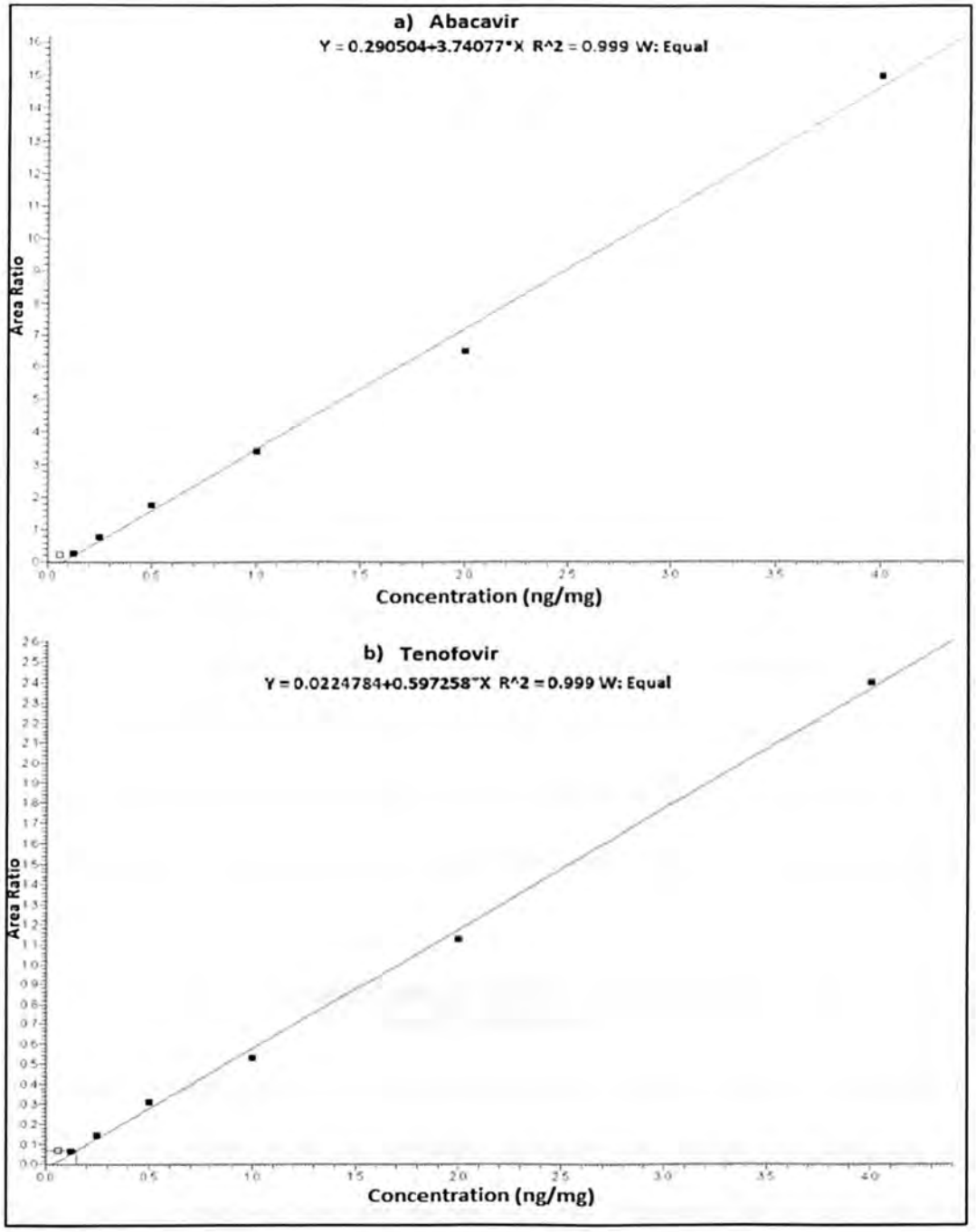


Figure 4.5: Standard curve for (a) abacavir and (b) tenofovir

4.3.3.4 Sensitivity

The respective values for LLOD and LLOQ were 0.06 and 0.12 ng/mg (drug/hair) for both drugs (Table 4.3). The LLOQ was considered as the lowest concentration that produced a peak with the signal to noise ratio of at least 10 against the

background noise. The LLOD was defined as lowest concentration that had the signal to noise ratio against the background of at least 3.

Table 4.3: LLOD, LLOQ for the two drugs

Compound	LLOD (ng/mg)	LLOQ (ng/mg)
Abacavir	0.06	0.12
Tenofovir	0.06	0.12

4.3.3.5 Accuracy and precision

Accuracy was validated for the method at four different concentration levels of abacavir and tenofovir (0.12, 0.25, 0.50 and 1.00 ng/mg) using blank hair. The accuracy was calculated by dividing the calculated mean amount of the drug by the actual amount x 100. All the calculated values were within 15 % precision limit (Table 4.4).

$$\text{Accuracy} = \frac{\text{Calculated mean concentration of six replicates}}{\text{Actual concentration}} \times 100$$

It is possible to get accuracy values of more than 100%. This is because the mean of calculated concentrations of six replicates is taken into account and according to FDA guidelines the precision limit for this is $\pm$ 15%. Therefore out of six replicates there is a possibility of some calculated concentration values to be under or over ($\pm$ 15) actual concentration value.

The precision was determined for both drugs abacavir and tenofovir at four different concentrations for intraday and interday variations. The RSD% for intraday and interday was calculated by taking in account the average for the replicates of each concentration. The intraday precision was calculated by processing six separate

replicates of each concentration within the day. For interday studies six replicates of each concentration were determined on three consecutive days. For both intraday and interday studies the RSD% calculated were within 15% precision. RSD% was calculated by dividing the standard deviation by the mean of the replicates x 100. The accuracy and precision for intraday and interday variations for each concentration are given in Table 4.4.

Table 4.4: Intraday/interday precision and accuracies for the drugs at four different concentrations

Compound	Concentration (ng/mg)	Precision RSD (%)		Interday Accuracy (%)
		Intraday N=6+6+6 +6	Interday N=18+18+18+18	
Abacavir	0.12	0.03	0.02	95.6
	0.25	0.28	0.08	95.0
	0.50	0.02	0.10	105.9
	1.00	0.05	0.09	112.5
Tenofovir	0.12	0.07	0.04	94.8
	0.25	0.01	0.02	105.0
	0.50	0.01	0.04	107.3
	1.00	0.02	0.10	110.7

4.3.3.6 Stability

To determine the stability of abacavir and tenofovir, three freeze/thaw cycles were evaluated. The samples were stored at - 20 ^{0}C and thawed to room temperature. Three different concentrations with six replicates each were analysed on three different days. The samples were compared to the analysed spiked samples freshly prepared on the day for the same concentrations. Table 4.5 shows the stability data for abacavir and tenofovir at three different concentrations on three different occasions. Longer storage periods are possible but only three cycles were analysed for this study. The data in Table 4.5 is the RSD % of the QCs and shows that the three freeze/thaw cycles had no significant impact on the analysis. The results were within the acceptance criteria of $\pm$ 15% of their respective nominal values. Hence, the samples could be freeze/thaw several times and not have any effect on the analysis of the drugs.

Table 4.5: Stability results of tenofovir and abacavir

Freeze /thaw cycles	1 ng/mg (QCH)		0.5 ng/mg (QCM)		0.25 ng/mg (QCL)	
	CV %					
	A	*T*	*A*	*T*	*A*	*T*
1	5.28	1.87	5.28	2.89	3.54	4.22
2	4.56	5.58	4.56	5.52	3.17	5.94
3	1.8	3.24	1.8	3.21	6	5.15

A = Abacavir

T = Tenofovir

4.3.4 Application to patient's hair

The hair samples that were quantitatively analysed for abacavir and tenofovir using the above validated analytical method were obtained from 32 HIV positive patients. Few of these patients provided multiple hair samples at different times. Based on prescription data, 25 samples were expected to be positive for tenofovir, 5 for abacavir and 2 for both drugs owing to change in treatment regime over the sampling period.

Out of 32 samples, 22 samples were positive and were above the level of quantification, these were 15/27 (55.5%) for tenofovir, 7/7 (100%) for abacavir. However, 3 samples for tenofovir were above the limit of detection, but below the limit of quantification. To summarise the above results, based on prescribed data, the drugs were detectable for tenofovir and abacavir in 66.6% and 100% of expected samples, which may reflect the adherence to medication. The levels of each drug (where quantified) found in hair are represented in Figure 4.7 as a dot plot. The chromatograms of one of the patients with a positive hair sample and their concentrations are shown in Figure 4.6. The background noise is a little higher at around 0-1 minute in case of abacavir. The sample was found to be positive for both drugs. For some patients more then one hair samples were obtained at different point in time. Some samples that were collected at earlier date were negative for the drugs and same patients samples taken at a later date were positive. The reason for negative results could be that the drug had not reached the hair at that point in time.

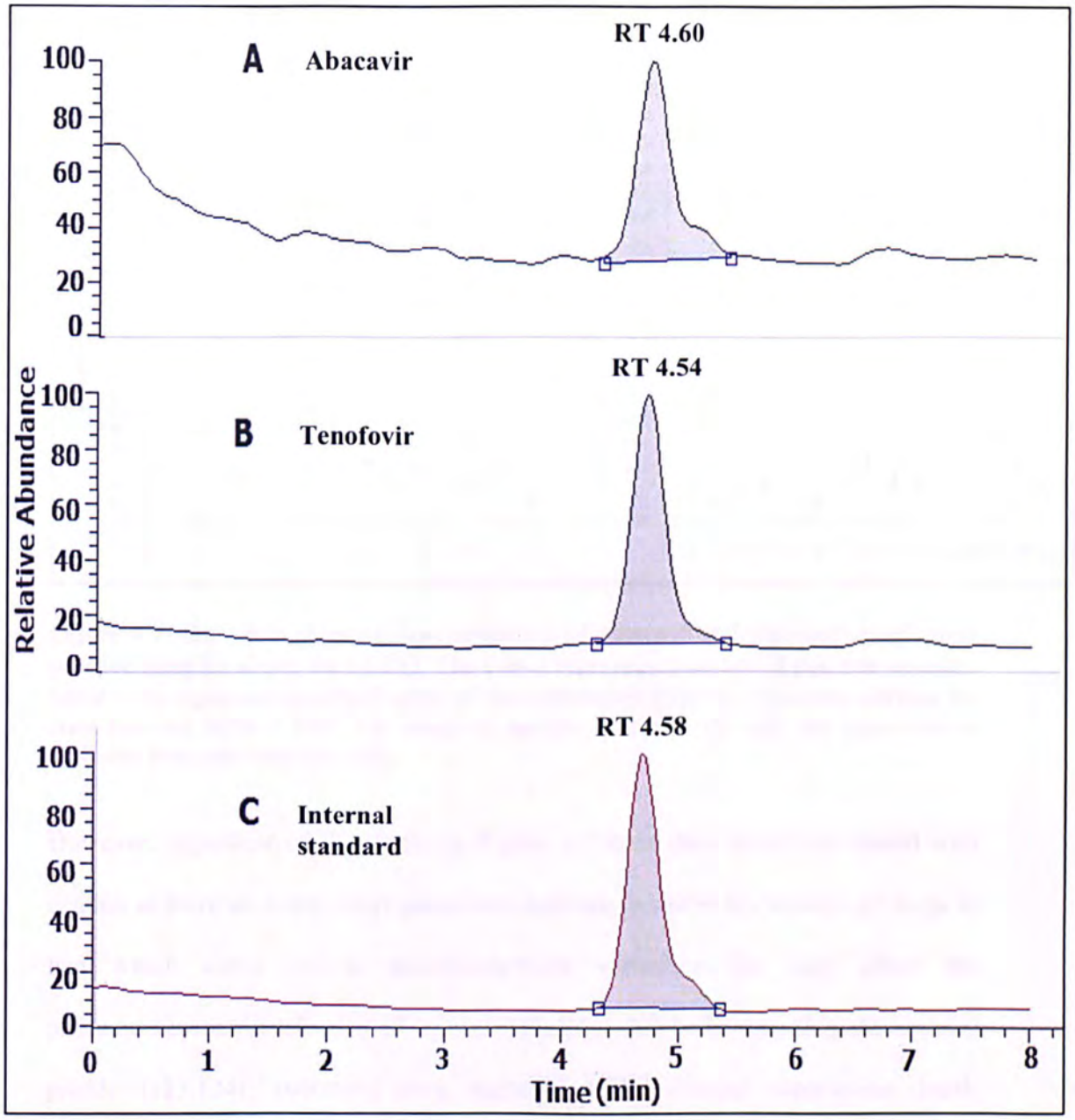


Figure 4.6: LC-MS/MS chromatogram of positive hair sample for both drugs (A) abacavir and (B) tenofovir and internal standard (C) abacavir – d4

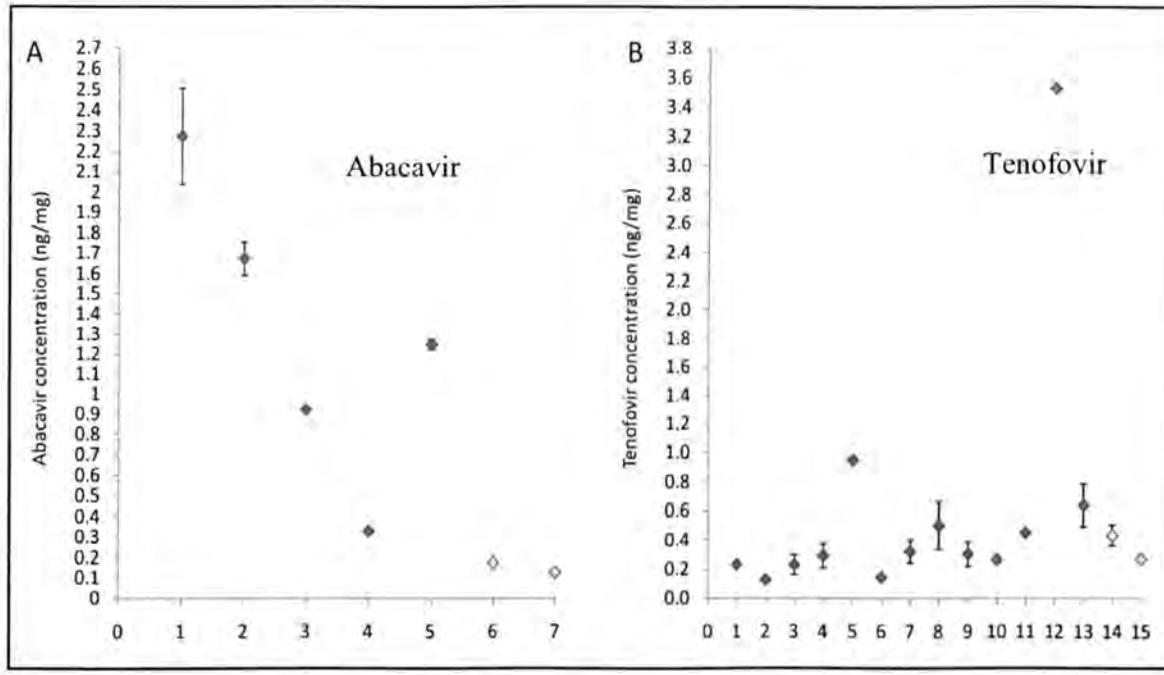


Figure 4.7: Dot plots showing concentrations of abacavir and tenofovir confirming positive samples above the LLOQ. The x-axis represents number of positive samples. Error bars represent standard error of measurements (SEM). * Samples without the error bars are SEM < 0.05. For tenofovir samples 1, 2 and 12, only one measurement available, hence no error bars (38).

However, regardless of the results in Figure 4.7 these data should be treated with caution as there are many other parameters that may result in the absence of drugs in hair which could include pharmacogenetic variations that may affect the pharmacokinetics/distribution of a drug throughout the body including the toxicity profile (123,124); switching drug regimens under clinical supervision, harsh chemical hair treatments (such as dyeing or bleaching the hair), potential interactions of dietary components (54) and drug – drug interactions (60,118,125).

This is the first study to report a method for the simultaneous quantification of tenofovir and abacavir in hair. Numerous analytical methods have been developed in the past for the quantification of these two drugs in different matrices such as plasma (30). Hair analysis is a non-invasive technique with very less influence from matrix effects as compared to urine and plasma (68,70,107,123,126). It is a very useful

technique for providing long-term history of the anti-HIV drugs' intake. Quantification of levels of anti-HIV drugs in hair may enhance our knowledge of adherence to the treatments (25). This method may be a useful tool in order to regulate the drug adherence of the patients and also to ascertain cases of drug avoidance. To elucidate the information such as pharmacokinetic data; more work is required. The validation data show that the method is rapid, highly sensitive, specific and selective.

Due to the intra and inter - individual variations and a possible relationship between the drug concentrations and its efficacy or toxicity, it is difficult to establish a detailed TDM. Therefore, a complete adherence study would necessitate hair sectioning in a longitudinal study, which is beyond the scope of this study (38).

4.4 Conclusion

To the best of author's knowledge this is the first report on the simultaneous quantification of tenofovir and abacavir in human hair. The newly developed successfully validated analytical method is highly sensitive and reproducible and is suitable for quantifying abacavir and tenofovir in human hair. The method only required 50 mg of hair for analysis and was successfully applied to the HIV positive patient's hair and may be useful where drug compliance of the patients is monitored. The method successfully identified the drugs in the hair sample.

In conclusion, a novel, fast and sensitive method has been developed with no matrix effects and has been proven to be accurate and precise. The high precision, accuracy and robustness make it suitable for future application of this method in clinical trials and TDM. The method could also be used to compliment urinanalysis for the quantification of these anti-HIV drugs.

CHAPTER 5

EFFECTS OF DIETARY SUBSTANCES ON THE GLUCURONIDATION OF β_2-AGONISTS AND ITS IMPLICATIONS FOR DOPING CONTROL

5.1 Introduction

The medical commission of the IOC first introduced the list of prohibited substances in sports in 1967, which has now been taken over and annually revised by WADA (127). These substances are those that have potential performance enhancing effects and/or are against the spirit of sport (127). For those athletes that have medical conditions and require treatment can obtain Therapeutic Use Exemption (TUE) for using these pharmacological products (127,128). In some cases, athletes tend to misuse TUE by feigning medical conditions (127).

β_2-agonists are widely used for the treatment of respiratory diseases such as asthma (129). The prevalence of asthma is higher in athletes as compared to general population (130). In UK, for athletes to obtain TUE for asthma, they need to prove that they have a clinical need to use the asthma inhaler containing prohibited β_2-agonists. In addition, there is a requirement for lung function test results by athletes that could be obtained from specific specialist asthma screening centres (43).

Physical exercise may sometimes lead to exercise-induced asthma (EIA) and in order to treat these asthmatic diseases athletes take β_2-agonists. Use of β_2-agonists is prohibited in athletes however EIA athletes use this prior to exercise via TUE. Studies show that in recent years the percentage of athletes taking these β_2-agonists in Olympic games has increased (130). However, this is an arguable point whether it is a real condition or if it's a misuse of these drugs for performance enhancing purposes (130).

Since drug metabolism is an enzymatic process, there are sometimes substances that can lead to its inhibition and alter the drug glucuronidation within individuals. This in return can lead to various situations such as an increase in plasma concentration of the drug, decrease in the metabolite concentration and can also cause increased toxicity (131). Several studies have shown that anti-inflammatory drugs, diclofenac and ibuprofen, have shown to competitively inhibit the action of UGT2B15 and UGT2B17 leading to a reduction in the rate of testosterone glucuronidation (132,133).

In addition, studies have also shown that dietary components such as green and white tea can inhibit the glucuronidation activity of UGTs and can have an effect on the levels of drugs within individuals, which can lead to false doping results in sports (133). Effect of dietary products on glucuronidation enzyme activity could in return affect the rate of drug excretion and its bioavailability. In the light of previous studies carried out on the inhibitory effects of dietary products on testosterone glucuronidation, the aim of this study was to investigate the inhibitory effects of dietary products containing phenolic compounds such as tea and fruit juices. Along with dietary products, catechins present in tea and pharmaceutical products that are taken along with β_2-agonists (corticosteroids) were investigated for their potential

effects on the glucuronidation activity of the β_2-agonists (134). In this study, formoterol and clenbuterol were the two β_2-agonists in question, where the potential inhibitory effects of these dietary and pharmaceutical products on the glucuronidation activity of the two drugs were investigated.

5.1.1 Drug metabolism

There are different organs such as liver, gut, lungs skin and kidneys that take part in drug metabolism, but the major site for the drug metabolism is the liver. The metabolism is the stage where endogenous compounds as well as drugs and xenobiotics get converted into more hydrophilic compounds and get eliminated from the body and their biological activity is terminated. Drug metabolism is carried out by drug metabolising enzymes responsible for changing the chemical structure of lipophilic compounds into more hydrophilic compounds, in order to eliminate and detoxify these exogenous compounds from the body. These metabolic reactions are divided into two phases i.e. Phase I and Phase II reactions. The role of Phase I metabolism is to prepare chemicals for Phase II metabolism, whereas Phase II metabolism is an actual detoxification step with excretion through urine and bile (131,135,136). Phase I and Phase II enzymes are present in most of the tissues and organs of the body and are there to protect the body from potential harm from these xenobiotics (135). These Phase I and Phase II enzymes are usually found in the endoplasmic reticulum (ER) and cytosol (131,137).

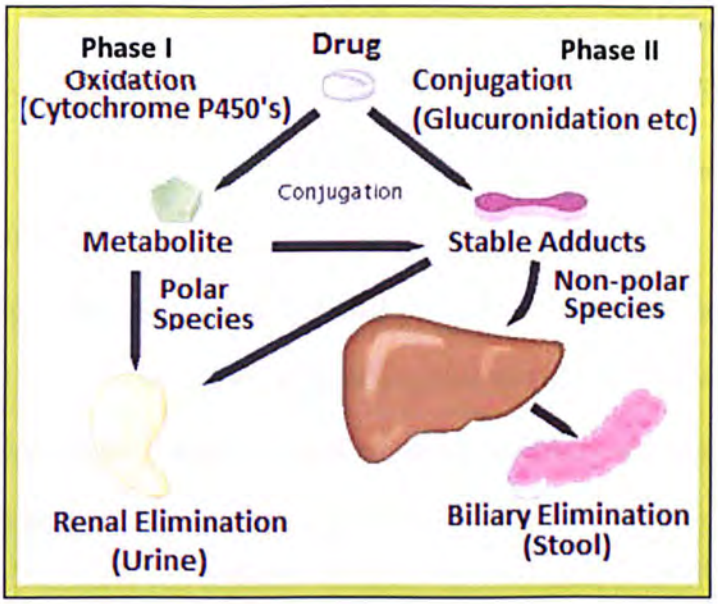


Figure 5.1: Drug metabolism (138)

5.1.1.1 Phase I metabolism

Major Phase I reactions are functionalization reactions such as oxidation, reduction and hydrolysis where a functional group is added to the parent drug molecule, converting it into a more polar metabolite. Phase I reactions usually lead to loss of pharmacological activity of the drug or it might lead to a more active metabolite than the drug itself. Major Phase I enzymes are cytochrome P450 (CYP) isoenzymes and flavin-containing monooxygenase (FMO) (136).

Oxidation reactions

Cytochrome P450 that is the microsomal mixed-function oxidase performs many different functionalisation reactions. These reactions require the presence of molecular oxygen, NADPH and complete mixed-function oxidase system that includes cytochrome P450, NADPH-cytochrome P450 reductase and lipid. All reactions involve initial insertion of a single oxygen atom into the drug molecule. A subsequent rearrangement or decomposition of the product may occur, leading to the

final products. Reactions performed by the microsomal mixed-function oxidase system involve aromatic and/or aliphatic hydroxylation, epoxidation, N-dealkylation, S-dealkylation, oxidative deamination, O-oxidation, S-oxidation as well as alcohol oxidation (139).

Other oxidation reactions can be carried out by a number of enzymes in the body that are not related to cytochrome P450. These enzymes include alcohol dehydrogenase, aldehyde dehydrogenase, xanthine oxidase, amine oxidases, aromatases as well as alkylhydrazine oxidase (139).

Reduction reactions

Reductive reactions can be catalysed by hepatic microsomal enzymes and usually require NADPH and are generally inhibited by oxygen. Compounds that undergo reduction process by hepatic microsomes are azo- and nitro- compounds, epoxides, heterocyclic ring, halogenated hydrocarbons as well as some steroids (139).

Hydrolosis

This involves the cleavage of a bond in a molecule by the addition of water. Esters, amides, hydrazides and carbamates can undergo hydrolysis by the help of various enzymes (139).

5.1.1.2 Phase II metabolism

Phase II enzymes are responsible for conjugating xenobiotic itself or its metabolite, hence increasing its hydrophilicity (140,141). In this, a covalent bond is formed between a functional group of parent compound or phase I metabolite and an

endogenous substrate such as glucuronic acid, sulphate, acetate or an amino acid (136).

Glucuronidation

It is the major phase II metabolism reaction. In this process, substrate is conjugated to glucuronic acid (142). Glucuronidation is the most widespread conjugation reaction due to the relative abundance of the co-factor for the reaction, UDP-glucuronic acid and the nature of the enzyme, UDP-glucuronosyltransferase(139). This glucuronide formation leads to inactivation of the drug by terminating its pharmacological activity (143). Glucuronidation of the drugs helps inactivating the drug and is responsible for the excretion of most pharmaceutical products (144). Conjugation can occur at hydroxyl, carboxylic acid, amino and at carbon centers giving β-glucuronide metabolites that are more polar (143). These glucuronides can also be synthesised as these are needed in many research areas such as analytical studies and can be used as reference materials. Another way of achieving glucuronides synthesis is with the help of enzymes. Different kinds of enzyme preparations can be used such as rat liver, human liver microsomes (HLM), recombinant human Uridine 5'-diphospho-glucuronosyltransferase (UGTs) for carrying out the glucuronide synthesis (143).

For *in vitro* metabolic studies; microsomes are mostly derived from the liver; the tissue is homogenized followed by fractionation by differential centrifugation. The process involves removal of other denser substances such as mitochondria and nuclei at a lower centrifugation force 10000 x g, where the enzymes are left in the supernatant and are obtained at higher centrifugation force (142,145).

Uridine 5'-diphospho-glucuronosyltransferase (UGTs):

UDP-glucuronosyltransferases (UGTs) are a superfamily of membrane-bound enzymes found mainly in the liver that catalyse glucuronidation reactions. These UGTs inactivate and convert dietary components, drugs, endogenous as well as exogenous substances into hydrophilic glucuronides and help in elimination of these substrates via urine or faeces (137,146). The human UGTs are classified into two major families according to their amino acid sequence and gene structure; these are UGT1 and UGT2. UGT1A is located on chromosome 2 whereas UGT2 is found on chromosome 4. These UGTs use UDP-glucuronic acid (UDPGlcA) as the co-substrate and are found in the endoplasmic reticulum where 95% of polypeptide chain including the catalytic part is located on the luminal side (137,142,144,146,147). The active site of the UGTs resides in the lumen of ER and causes *in vitro* 'latency' of enzymatic activity i.e. ER membrane forms a diffusional barrier for substrates, cofactors and products which is why this barrier has to be somehow disturbed/removed when carrying out microsomal incubation and hence maximising the glucuronidation activity (142,143,148). UGT's play a major role in conjugation of metabolites from oxidation reaction, but in case of a suitable electrophilic group, UGTs can also conjugate drugs directly without any former oxidation steps (148).

The availability of these recombinant enzymes has enabled wide research in metabolism studies. In addition, recombinant human UGTs have been expressed in baculovirus-infected insect and mammalian cells and are used to study tissue specific glucuronidation as well as to evaluate structure-function relationships of UGTs (149).

Other conjugation reactions other than glucuronidation are sulfation, methylation, actylation, amino acid conjugation, glutathione conjugation as well as fatty acid conjugation. All these reactions involve a diverse group of enzymes usually involving a co-factor or substrate derivative that lead to water-soluble products which can be excreted in bile or urine.

5.1.2 Clenbuterol

Clenbuterol is one of the most potent β2-agonists mainly used for the treatment of respiratory diseases and has been misused in sports as an anabolic agent for performance enhancing purposes (129,150). Therefore, it is placed in list of prohibited substances published by WADA. Clenbuterol is classified as a Schedule 2 (part 2), Class C drug (151). A large amount of clenbuterol intake results in enhanced protein synthesis and loss of body fat. Therefore, it is also misused in farm animals like cattle in order to increase the meat mass and enhance lipolysis. Misuse of clenbuterol has been very common in athletes and most recently in horse racing. Its more common in body-builders, weight lifters and athletes, where there is more requirement for muscle strength and increased lean body mass (152). Clenbuterol is approved in USA for veterinary purposes in nonfood animals (153). Part of the dosage is excreted as the parent compound that in return is dependent on the species, the route of administration, and the dosage (147).

5.1.3 Formoterol

Formoterol is a highly potent β_2- adrenoceptor agonist. β_2- agonists are used clinically for the treatment of asthma and chronic pulmonary disease. These drugs along with corticosteroids have been used as combination inhalers (154,155). The

major metabolism of this drug in human is mainly glucuronidation and is stereoselective (154,156).

Due to their stimulating performance-enhancing effects, beta2-agonists have been subjected to restrictions in sports (49,51). Besides its therapeutic activity, formoterol has potential performance enhancing activity hence could be beneficial to athletes (49). Studies have shown that it improves the muscle growth in animals (50). Formoterol has been placed on the list of prohibited substance by WADA since 1992 (51,52). The therapeutic drug dose allowed for inhaled formoterol is maximum dose of 54 μg over 24 hours (150). According to WADA, the presence of formoterol in urine beyond 40 ng/mL is presumed not to be an intended therapeutic use of the substance(150). Administration of formotoral by oral or parenteral route at very large inhaled doses is forbidden due to its anabolic activity (51,150).

Formoterol is primarily metabolised to its glucuronides (144). UDP-glucuronosyltransfertases (UGTs) are the biological catalysts of glucuronidation reaction. The microsomal fractions prepared from liver, kidney and intestine show higher activity for these UGTs (157). Previous studies have shown that UGT2B17 is the most active isozyme for the glucuronidation of (R, R)-Formoterol followed by 1A9, 2B7 and 1A1 in decreasing order of activity (144).

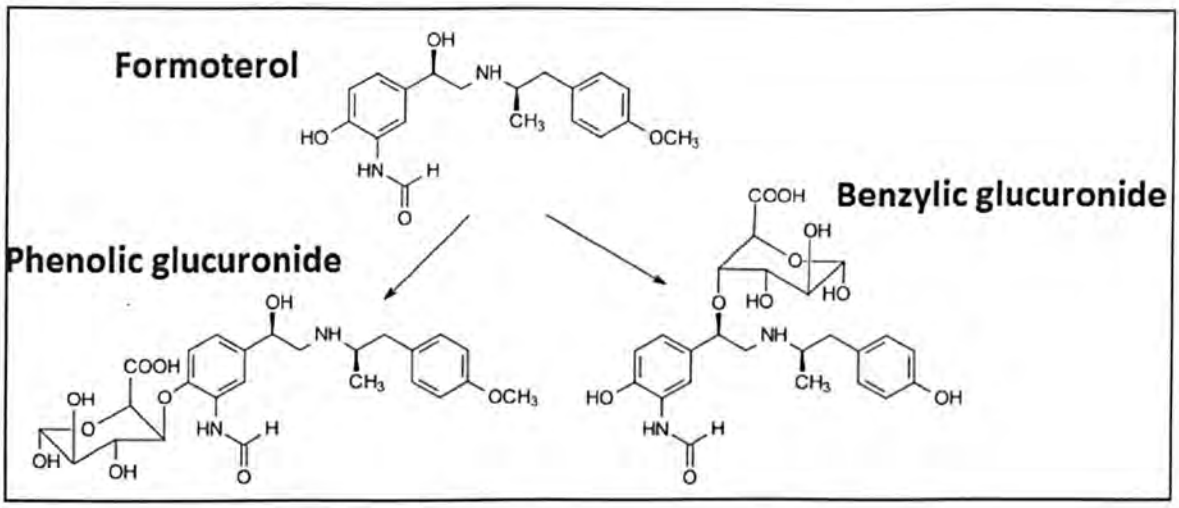


Figure 5.2: (R,R) - Formoterol Glucuronides (144)

Study on various fruit juices and drinks have shown that purple grape juice contained the highest number of phenolic and other antioxidant compounds (55). These phenolic compounds have proven to be very useful for decreased risk of several diseases as these compounds have potent antioxidants activities. Other juices rich in these phenolic compounds include apple and cranberry juice. The choice of fruit juices used in the current project was based on their phenolic content described in previous study (55).

Corticosteroids are the most effective drugs for long-term control of asthma where they improve the lung function (158). Sometimes these are given along with bronchodilators as combination therapy for improved results (56). Over the last few years, there has been a significant increase in the number of athletes using inhaled corticosteroids along with β_2- agonists, as combination therapy. Corticosteroids help in regulating the function of β_2 – receptors; this combination therapy results in enhanced anti-inflammatory activity (57).

Green tea and catechins present in green tea have shown inhibitory effects on testosterone glucuronidation, therefore it is conceivable that tea, fruit juices and corticosteroids could also affect the drug metabolism of clenbuterol and formoterol.

Dietary products and selected corticosteroids will be added separately to the incubation mixture, in order to investigate their inhibitory effects. The inhibition of formoterol glucuronidation by any of these dietary substances could affect the actual drug concentration when the urine is tested and thus may help in masking formoterol misuse by athletes (133,134). The dietary products included green and white tea leaf, white tea stem, selected catechins present in these tea samples, fruit juices (purple grape, cranberry, apple). In this study pooled HLM and UGT2B17 are the main enzymes used for the glucuronidation process.

5.2 Materials and Methods

5.2.1 Chemicals and consumables

Reference standards formoterol fumarate dihydrate (10 mg), cortisone, hydrocortisone, dexamethasone, epigallocatechin gallate (EGCG), catechin gallate (CG), epicatechin (EC), saccharic acid 1,4-lactone, magnesium chloride, sodium phosphate dibasic heptahydrate, sodium phosphate monobasic dihydrate and acetic acid were purchased from Sigma–Aldrich (Poole, Dorset). Clenbuterol hydrochloride (30 mg) was purchased from LGC standards (Teddington, UK). Pooled human liver microsomes (HLMs), and UGT2B17 enzymes (as supersomes), were purchased from Becton Dickinson (BD Biosciences, Oxford). Solution A containing urdine -5'-diphosphoglucronic acid and solution B containing Tris HCl, magnesium chloride and alamethicin were purchased as a UGT reaction mix from BD Biosciences. HPLC grade methanol (MeOH), water and acetonitrile (ACN) were purchased from Fisher Scientific (Loughborough). The tea samples used were a commercially available silver dragon beard white tea (WT), yunan white tea leaf (WTL), sencha Japanese green tea bags (GT). WT and WTL were purchased from Fortnum and Mason

(London) and the GT was purchased from a local supermarket (London). Paracetamol was synthesised at Kingston University (London, UK). Ocean spray classic cranberry, Welch's purple grape and Copella Apple (cloudy) was all purchased from local supermarket (Sainsbury, London).

5.2.2 Enzymatic assay for glucuronidation of clenbuterol

This glucuronidation method was adapted from a previously published study that was carried out on clenbuterol. The enzymatic assay contained 5 mM saccharic acid 1,4 lactone and $MgCl_2$, 50 mM phosphate buffer (pH 7.4), 5 mM UDPGA, 5 mM $MgCl_2$, Human Liver Microsomes/ UGT enzymes (0.625 mg) and varying concentrations of clenbuterol as dimethylsulfoxide solution (2% of the final reaction volume). The sample volume was 250 µL. Control samples were prepared in the same manner as enzymatic assay and the same concentration of clenbuterol was added but without adding any enzyme. All samples were prepared in duplicates. The reaction mixture was then incubated at 37 ^{0}C for 4 hours. After the first 2 hours an aliquot of 100 µL was withdrawn and placed in a separate microtube. The reaction was terminated by adding icecold ACN containing 6% acetic acid. The sample was then left on ice for 5 minutes followed by centrifugation (at 4 ^{0}C for 5 minutes at 3939 x g) and analysed using HPLC-UV. The same procedure was followed for the last 2 hours of the reaction (142,147).

5.2.3 Enzymatic assay for glucuronidation of the drugs

The glucuronidation of formoterol and clenbuterol was adapted from a previously published literature using HLMs and UGT2B17 supersomes expressed in baculovirus infected insect cells. The enzymatic assay contained 50 mM Tris-HCl buffer (pH

7.5), 2 mM UDPGA, 8 mM $MgCl_2$, 25 μg/mL alamethicin and formoterol at various concentrations. The final incubation volume was made up to 200 μL with HPLC grade water. After incubating the mixture for 5 minutes at 37 ^{0}C the reaction was initiated by adding the HLM or UGT (1.2 mg/mL) protein. Aliquots of the incubated reaction mixture were transferred after 90 and 180 minutes to a separate vessel and the reaction was terminated by adding ice cold ACN containing 6% acetic acid and 100 μg/mL final concentration of IS to each. The mixture was then vortex mixed and protein was precipitated by centrifugation (at 4 ^{0}C for 5 minutes at 3939 x g) and an aliquot of the supernatant was analysed by HPLC-UV. The control samples were prepared in the same manner with same concentration for the drug as used in the actual reaction mixture but without adding any enzymatic protein (UGTs/HLMs). All the samples including controls were prepared in duplicates (144). Alamethicin and other membrane-disrupting agents such as detergents help increase the glucuronidation activity due to the greater access to the enzyme active site and UDPGA.

5.2.4 Preparation of tea extracts, catechin, corticosteroid solutions and fruit juices

The dietary products, catechins and coricosteroids were added to the above enzymatic assays as potential inhibitory agents for the glucuronidation of formoterol. Tea extracts and catechins were prepared by adapting a previously used method where tea samples were finely grounded manually (pestle and mortar) and 0.8 g of each sample was dissolved in 40 mL of boiling water. The solution was then left for 5 minutes. Extracts were then filtered using a 0.45 μm PTFE membrane syringe filter (133).

Catechin gallate (CG), epicatechin (EC) and epigallocatechin gallate (EGCG) were prepared by dissolving each catechin in water, and adding it to the above-mentioned enzymatic assay (section 5.2.3) at the initial concentration of 1 mM. The corticosteroids were prepared similarly by dissolving each i.e. cortisone, hydrocortisone and dexamethasone in water to aid dissolution and adding it to the reaction mixture at 2 mM concentration. Fruit juices were filtered using 0.45 μm PTFE membrane syringe filter.

The above tea extracts, catechins, fruit juices and corticosteroids were added to the incubation mixture by replacing the amount of water used in the assay. Catechins and corticosteroids were added to the reaction vessel at 1 mM and 2 mM concentration, respectively. Tea extracts and fruit juices were added to the reaction mixture at 10% v/v of the final 0.2 mL reaction volume. Inititial starting concentration of formoterol used was 1 mM. For the termination of the reaction, ice cold ACN (6% acetic acid) containing IS at the final concentration of 100 ug/mL was used. The termination of the reaction was conducted by taking out aliquot (70 uL) of the reaction mixture and adding ice cold ACN (280 uL) for tea extract, corticosteroids and fruit juices where as for catechins the volume of 700 uL was used.

5.2.5 Instrumentation

PerkinElmer HPLC system consisting of a pump (series 200) and autosampler (series 225); coupled to a diode array UV detector (series 200 EP) was used for the analysis. A Supelco C18 column (5 μm x 250 mm x 4.6 mm) was used for the separation of compounds.

HPLC was used to determine the remaining concentrations of the drug left after stopping the glucuronidation reaction in order to work out how much drug has been

glucuronidated as well as determining the inhibitory activity of the tea extracts, catechins, corticosteroids and fruit juices.

5.2.5.1 LC conditions

The method was optimised by trying different LC conditions such as mobile phase composition, column temperature and injection volume in order to obtain best separation for the drug and internal standard. The optimum conditions and mobile phase composition used for the separation are as follows:

Table 5.1: Chromatography conditions (gradient elution) used for the separation of formoterol and internal standard (paracetamol)

LC run time (min)	MeOH (%)	Water with 0.1% Formic acid (%)
0.5	11.3	88.7
3.0	11.3	88.7
1.0	50.0	50.0
1.0	35.0	65.0
1.0	25.0	75.0
2.0	11.3	88.7
1.0	11.3	88.7

The column temperature was maintained at 40 ^{0}C and the flow rate was set at 1.35 mL/min. The injection volume was 10 µL and MeOH:H_2O (50:50) was used as syringe wash whereas the autosampler was maintained at room temperature. Detection of formoterol and paracetamol was at 244 nm using a Perkin Elmer diode array detector.

5.2.6 Preparation of standard solutions

A stock solution for formoterol was prepared by weighing out 5 ± 0.003 mg powder and dissolving it in MeOH:H_2O (50:50) at a concentration of 5 mg/mL where as for clenbuterol, 30 ± 0.003 mg was weighed and dissolved in H_2O at a concentration of 30 mg/mL. A stock solution for the paracetamol internal standard was prepared by dissolving 1 ± 0.003 mg in 1 mL MeOH. All the prepared stock solutions were stored in the freezer (- 20 ^{0}C) in glass vials. A calibration was prepared using the above solution. QC samples were prepared similarly, but from separate stock solutions. These calibration curve and QC samples prepared were used for spiking negative control samples for method development and validation.

5.2.7 Method validation

Although a HPLC method was developed for clenbuterol it was not fully validated according to FDA guidelines. This was due to the poor glucuronidation results for clenbuterol and therefore the inhibitory effects of the dietary products could not be investigated.

The performance of the analytical method for formoterol and paracetamol as internal standards was validated following FDA guidelines for the stated parameters: linearity, LLOD, LLOQ, accuracy, selectivity, interday precision and intraday precision as well as recovery. The analysis for the drug and IS was carried out in enzymatic assay. Drug free assay was used as blank and was analysed for any interfering or ghost peaks. The calibrants were prepared by spiking the assay with a known amount of formoterol and IS. QC samples were prepared similarly at three known concentrations distributed over the linear range. All the calibration curves prepared for validation were obtained by plotting the analyte to IS ratio against the

known concentrations of analyte in each sample; this was obtained by dividing the peak area of analyte by the peak area of IS. Linear regression analysis was performed for evaluating the linearity of the method.

Accuracy of the method was determined by analysing six replicates of the QCs at three different concentrations and comparing the mean calculated values with the actual concentration values accordingly. Selectivity was investigated by preparing the assay in replicates without any spiking of the drug or internal standard and confirming the absence of any interference peaks at the same retention time as of the drug and IS. Matrix effect was determined by spiking the incubated enzymatic assay with the drug and IS and comparing it to the drug and IS as standard solution at the same concentration.

A six point calibration curve was constructed by spiking the enzymatic assay with known concentrations of the drug and IS. The linearity of the calibration curve was determined by using a linear regression method. Measuring six replicates for the QCs at each concentration level on the same day assessed intraday precision and the interday precision was determined by analysing six replicates for each concentration on three consecutive days. The precision was measured in terms of RSD % and the limit was set at 15% for all the concentrations other than LLOQ where the precision limit was 20%. The LLOQ was the lowest quantifiable amount of analyte with a S/N $\geq$ 10 and LLOD was the lowest detectable concentration level with a S/N $\geq$ 3.

Recovery was determined by spiking the six replicates of enzymatic assay with the drug and IS without addition of any protein at three different known concentration levels and following the whole procedure of incubation. The mean was than compared to the standard solutions prepared in MeOH at the same concentrations.

5.3 Results and Discussion

5.3.1 Formoterol method development and validation results

Different gradient and isocratic conditions were examined using different flow rates in order to achieve optimum LC conditions for the analysis of formoterol. The HPLC column was equilibrated for an hour each time prior to analysis. The method was fully validated for accuracy, inter and intraday precision, linearity, recovery, sensitivity and selectivity. All the parameters were within the limit set according to FDA guidelines.

The HPLC-UV method for the analysis of formoterol and IS gave no interfering peaks. Figure 5.3 shows blank 100% (MeOH) solution ran using the LC conditions described in section 5.2.5.1.

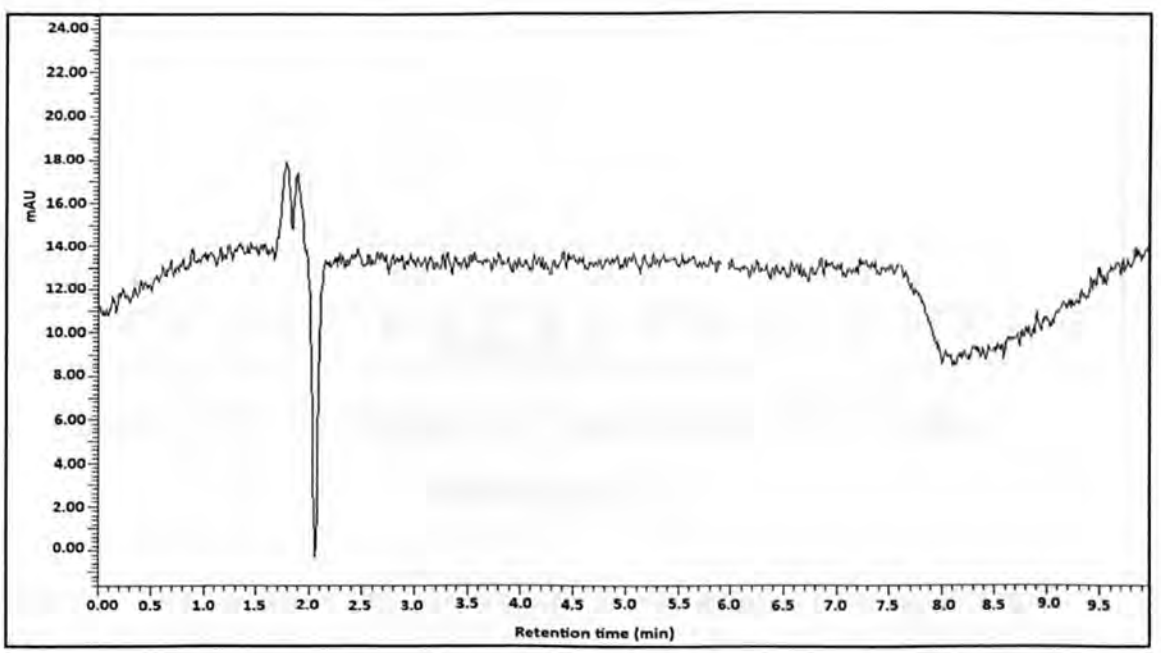


Figure 5.3: HPLC-UV chromatogram of a blank (100% Methanol) showing baseline

Figure 5.3 shows small splitted ghost peak at around 1.7 minutes, which is due to the absorbance of methanol by UV detector. The lower the absorbance of an organic solvent used for mobile phases, the lower the noise in UV detection (159).

Selectivity was achieved by investigating the assay in replicates in the absence of drug and IS and the absence of any interfering peaks at the same retention time as the drug confirmed that the method was selective for formoterol. In addition, any matrix effect was determined by analysing the enzymatic incubated assay spiked without any drug and IS and comparing the chromatogram to the one for the drug and IS as standard solution at the same concentration. The Figure 5.4 shows that there is a peak at around 2 minutes, but is not interfering with the drug or IS peak.

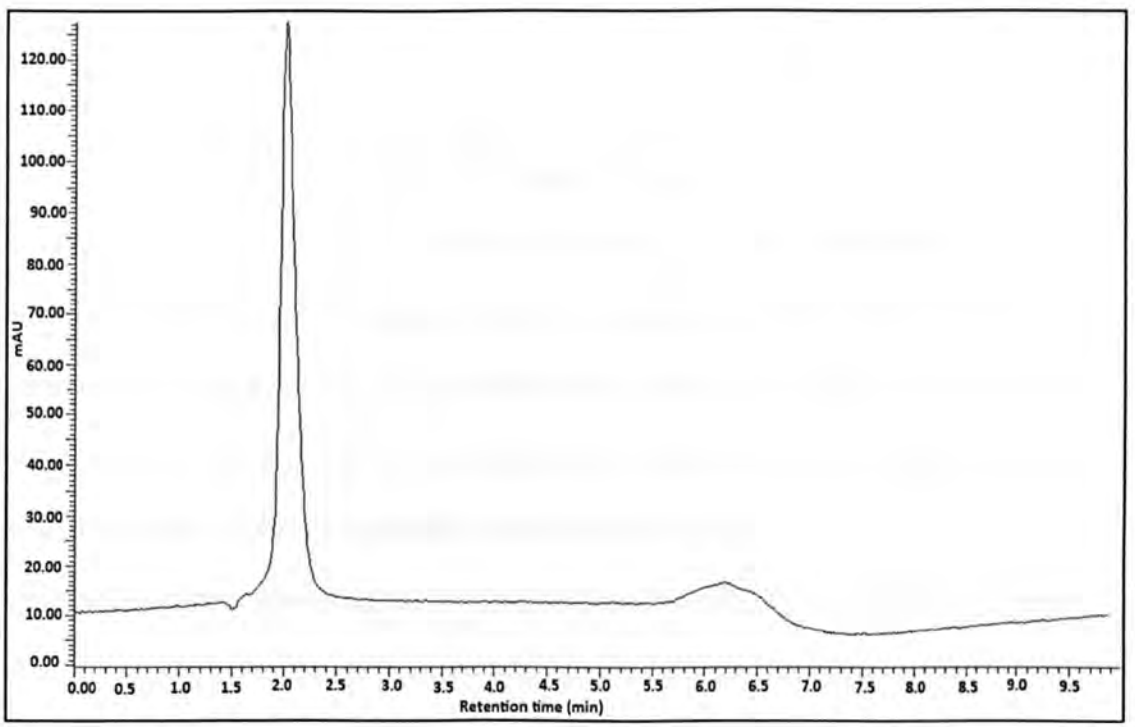


Figure 5.4: HPLC-UV chromatogram of enzymatic assay without any drug or internal standard

Figure 5.5 shows peaks for formoterol and IS at concentrations of 500 µg/mL and 25 µg/mL respectively. The small peaks at 1.5 – 3.2 minutes correspond to matrix effects.

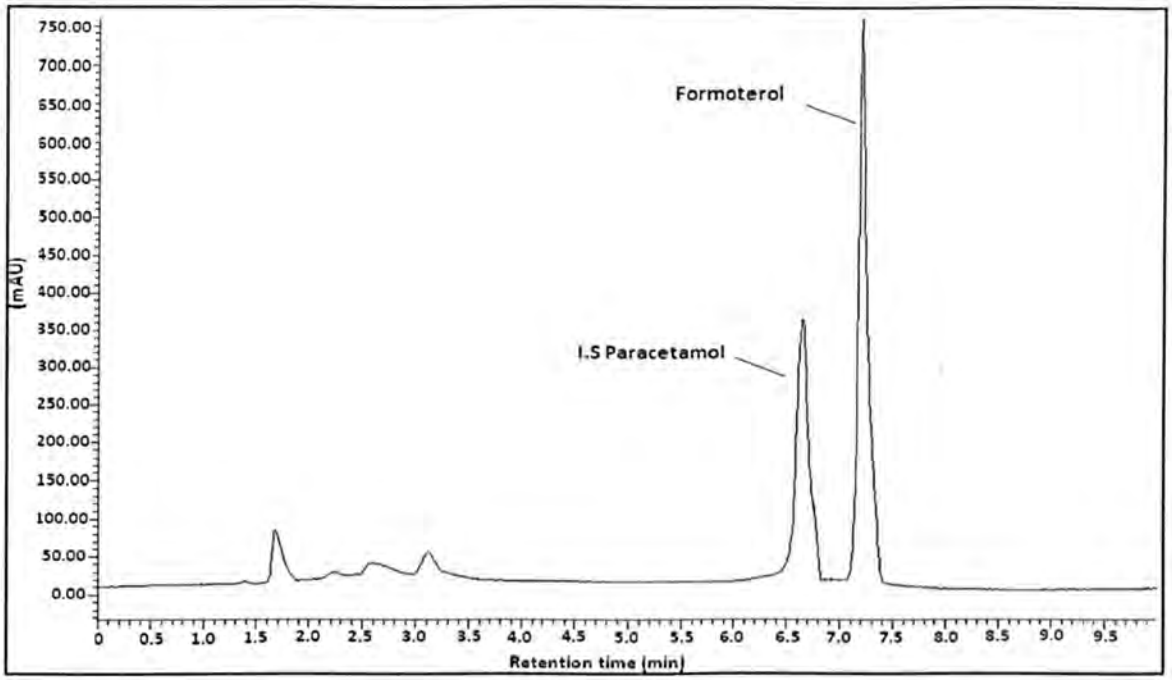


Figure 5.5: HPLC-UV chromatogram of formoterol and IS in enzymatic assay

Although there was no extraction of the drug involved in this study, the internal standard was added in case of any instrumental variations. The internal standard is added along with cold ACN when stopping the reaction instead of adding it along with formoterol to avoid any possible glucuronidtion of IS.

Calibration curves consisting of six points along with QC's and LLOQ at three different days were achieved by preparing it in the assay in the absence of enzyme in order to determine the linearity of the method. Enzyme was not added to the assay as addition of any enzyme may lead to glucuronidation of the drug, which can affect the actual concentration hence leading to poor calibration curve. The assay was spiked with known concentrations of the drug in the range of 20, 30, 100, 125, 250 and 500 μg/mL and with internal standard at a final concentration of 100 μg/mL. LLOQ and QC's were similarly prepared and were within the calibration range at three different concentrations. The calibration curves were constructed by dividing the peak area of the drug by the peak area of the IS and the ratio obtained was plotted against the

known concentration of each calibrant. The regression coefficient (r^2) stated in Figure 5.6 was $\geq$ 0.999 for each calibration curve and showed that the analytical method was linear for formoterol. A blank solution containing the mobile phase was run after every two injections in order to avoid any carry over effects or instrumental contamination.

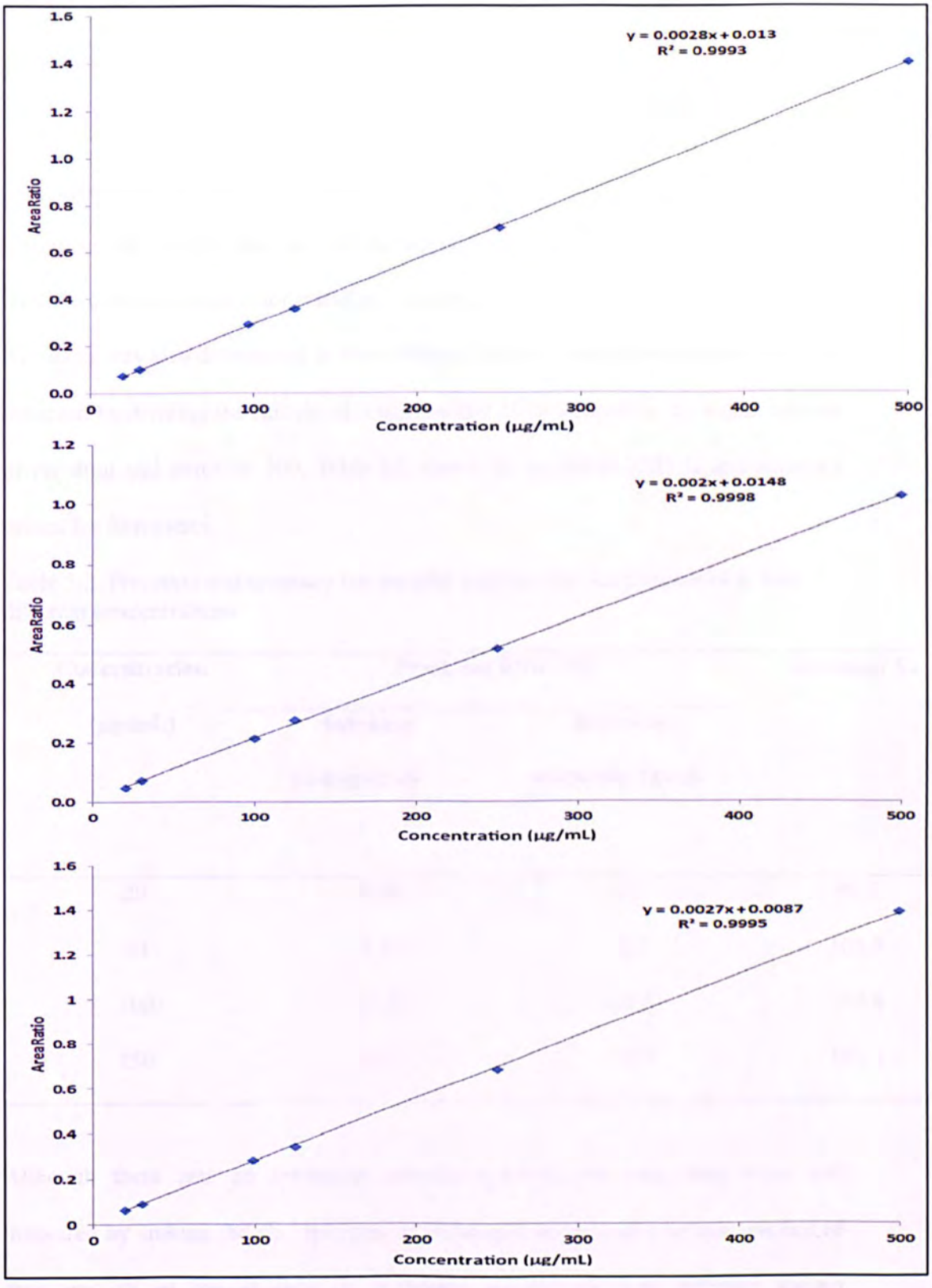


Figure 5.6: Typical calibration curves of formoterol in assay without any presence of enzymes

The interday and intraday precision and accuracy results were also within the FDA limit i.e. 15% precision. The precision was determined at four different known concentrations. The RSD% was calculated by taking the average for the replicates of each concentration and by processing six separate replicates of each concentration within the day for intraday and six replicates of each concentration was determined on three consecutive days for interday, accordingly.

Accuracy was also determined at four different known concentration levels and was achieved by dividing the calculated mean amount of formoterol by the actual amount of the drug and times by 100. Table 5.2 shows the precision RSD % and accuracy values for formoterol.

Table 5.2: Precision and accuracy for intraday and interday for formoterol at four different concentrations

Concentration (µg/mL)	Precision RSD (%)		Accuracy %
	Intraday N=6+6+6 +6	Interday N=18+18+18+18	
20	0.64	1.1	99.7
50	2.49	2.7	103.5
100	13.54	11.5	100.0
250	13.57	13.3	103.3

Although there was no extraction process involved, the recoveries were still measured by spiking the six replicates of enzymatic assay with a known amount of drug and IS in the absence of enzymatic protein at three different known concentration levels followed by the incubation process. After the analysis, the signal

produced was compared to the signal produced by the standard solutions prepared in MeOH at the same concentration.

Table 5.3: Recovery of formoterol at three different concentrations following the incubation process without addition of any enzyme, LLOD and LLOQ values for formoterol

Concentration (µg/mL)	Recovery (%) N= 6+6+6
50	106.02
100	95.01
250	103.61

LLOD and LLOQ values for formoterol are given in Table 5.4 were and the signal to noise ratio was 5 and 10, respectively.

Table 5.4: LLOD and LLOQ values for formoterol

	Concentration (µg/mL)	S/N
LLOD	10	5
LLOQ	20	10

5.3.2 Clenbuterol glucuronidation results

Previous studies have shown that clenbuterol glucuronide conjugates may be present as minor metabolites. There have been reports on the O- and N- glucuronides of clenbuterol in dog and calf urine (160,161). In this study, clenbuterol glucuronidation was carried out by following two different literature methods, using human UGT 2B17 and HLMs (142). The choice of UGTs used was dependent on their availability

and previously published literature (142,144,147). UGT2B17 has shown good enzymatic activities towards testosterone and previous studies have shown good glucuronidation activities in liver microsomes of other animals, hence UGT2B17 and HLMs were used for glucuronidation. (133,142). However, there was extremely low glucuronidation activity found in case of clenbuterol and was not as good as could have been expected. Based on data obtained by the experiments for the given UGTs and HLM, it was difficult to predict the amount of glucuronidation achieved for clenbuterol in this study due to the very low percentage. The best glucuronidation activity was achieved with HLMs that was approx. 5%, which was considered very low in the case of investigating inhibitory effects, as it would be very difficult to distinguish between the actual glucuronidation and if there is any inhibition. In order to investigate any inhibitory effect, first there needed to be enough glucuronidation activity. Some studies have shown that the glucuronidation activity can be affected by different factors such as alamethicin, UDPGA, buffer, concentration of the detergent as well as pH (143,162). Glucuronidation of the drugs is also specie dependant and there are major differences in their glucuronidation properties between different species (142). As the aim of the investigation is based on examining the inhibitory effects in terms of doping controls in athletes, only HLMs were used for glucuronidation rather than using liver microsomes from other animals.

In light of these poor results for glucuronidation of clenbuterol, it was decided not to further investigate the inhibitory activities of different dietary products; beverages and corticosteroid on the glucuronidation activity. Preliminary experiments were performed to examine the glucuronidation activity of clenbuterol but due to very poor results the HPLC method was not further validated.

5.3.3 Formoterol glucuronidation results

Inhibitory effects on the glucuronidation of formoterol were investigated, as it is another commonly used β_2- agonists for the treatment of asthma. It was subjected to the previously published glucuronidation method. Based on the previous data UGT2B17 and HLMs were chosen and formoterol showed good affinity for both enzymes (133,144).

Following the method described in section 5.2.3, formoterol was successfully glucuronidated with HLMs and UGT2B17 about 33% and 25%, respectively. Compared to UGT2B17, pooled HLMs showed better glucuronidaton activities, this could be due to the fact that pooled HLMs contain a wide variety of metabolic enzymes. The glucuronidation was monitored by preparing a control at the same known concentration level as the sample in the absence of any enzymatic protein. The amount of protein was replaced by water and the peak area ratio of the control was compared to the sample containing enzymatic protein, after the incubation. The difference between the area ratio of the drug and IS corresponds to the glucuronidation activity. As there was good glucuronidation activity achieved for formoterol using UGT2B17 and pooled HLMs, the inhibitory effects of products in question were investigated using same incubation method.

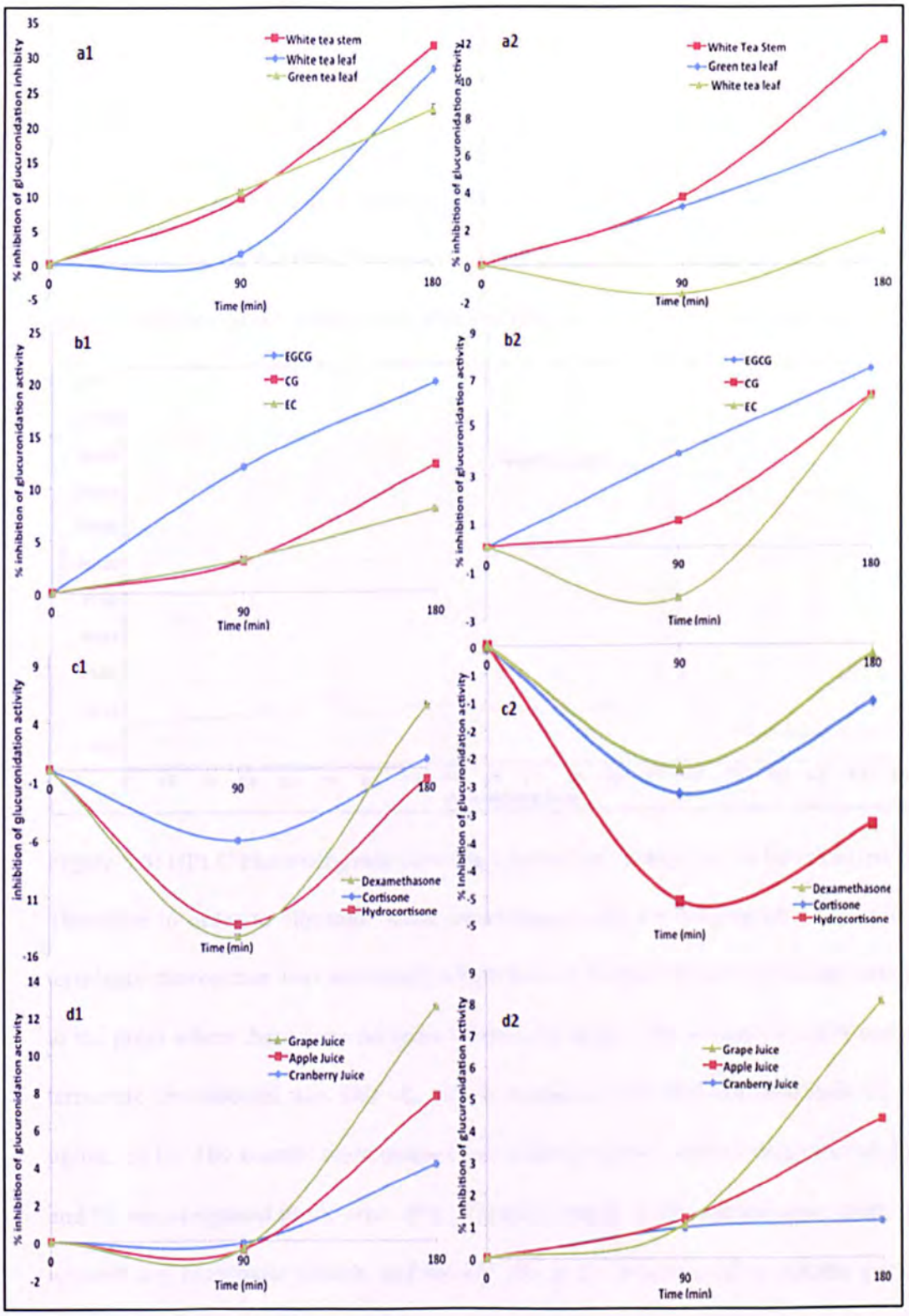


Figure 5.7: *In vitro* inhibition of glucuronidation activity over time with different tea samples, catechins present in tea, corticosteroids and fruit juice samples - a1, b1, c1, d1 for HLMs and a2, b2, c2, d2 for UGT2B17. The results represent an average of duplicate assay samples at each of the two time points. The control here is represented as 0 for both 90 minutes and 180 minutes.

5.3.3.1 Inhibition using tea samples

White tea leaf, white tea stem and green tea leaf were used to investigate the potential inhibitory effects on the glucuronidation of formoterol. Initially tea samples were dissolved in water as described in section 5.2.4 and the solution containing tea extracts was ran on the HPLC method used for formoterol. The tea extracts gave very small interfering peaks at the same retention time as the drug as shown in Figure 5.8.

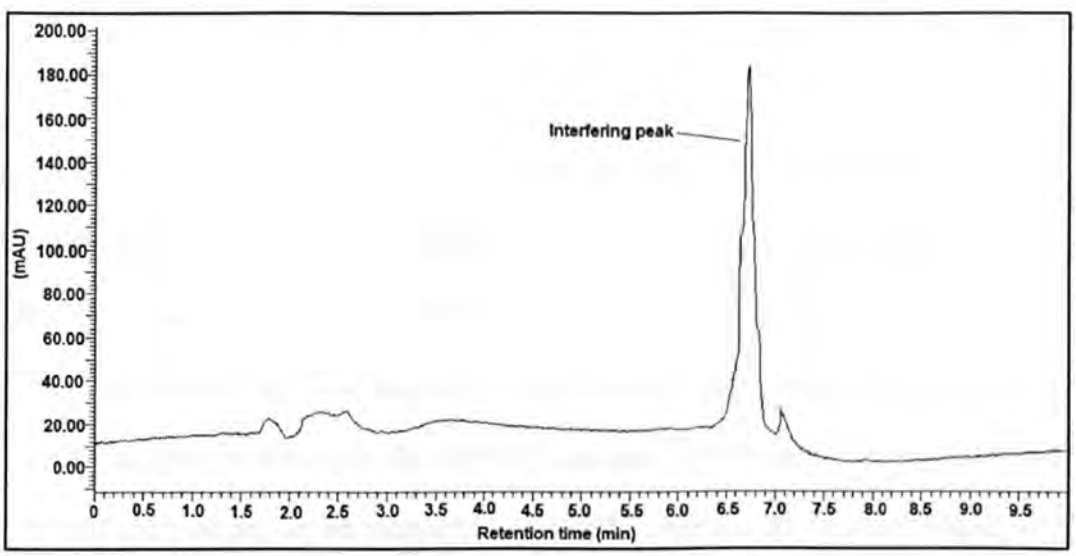


Figure 5.8: HPLC chromatogram showing interferring peaks caused by tea extracts

Therefore in order to eliminate these interfering peaks the volume of ACN used to terminate the reaction was increased, which lead to further dilution of the tea extracts to the point where there were no more interfering peaks. The volume of ACN used to terminate the reaction was 280 uL, which contained the final concentration of 100 ug/mL of IS. The control were prepared in similar manner and its area ratio of drug and IS was compared to the area ratio of actual sample. Two controls were used; one without any enzymatic protein and second one in the presence of enzymatic protein and these were compared to the sample containing enzymatic protein as well as the inhibitory product in question.

The results shown in Figure 5.7 (a1 and a2) indicate that over 180 minutes white tea stem was the most potent inhibitor and inhibited the glucuronidation of formoterol up to 31% for HLMs and around 12% for UGT2B17. White tea leaf only inhibited about 28% for HLMs and less than 2% for UGT2B17, whereas green tea leaf showed better result in case of UGT2B17, where the inhibition was around 7% and for HLMs it was around 22%.

5.3.3.2 Inhibition using catechins

CG, EC and EGCG samples were added to the reaction mixture at 1 mM concentration. In the case of catechins, the interfering peaks were larger which is why the final solution was diluted further and then examined. Preliminary experiments were conducted in order to optimise the right volume for the stopping solution in order to eliminate the interfering peaks. For catechins, the reaction was terminated by taking out an aliquot (70 uL) of the reaction mixture and adding 700 uL of ice cold ACN. This ensured that there were no interfering peaks.

For catechins, after 180 minutes of glucuronidation, EGCG gave the best results for both HLMs and UGT2B17, where it inhibited 20% and about 8% of formoterol glucuronidation, respectively as shown in Figure 5.7 (b1 and b2). CG showed about 12% inhibition for HLMs, whereas for UGT2B17 CG and EC showed same results i.e. below 7%. For HLMs, only about 8% inhibition was seen for EC.

5.3.3.3 Inhibition using corticosteroids

Corticosteroids are given to athletes with respiratory problems such as asthma in conjunction with β_2-agonists. It is useful to know whether these have any effect on the metabolic activity of formoterol. Dexamethasone, cortisone and hydrocortisone

were prepared similarly to catechins at the initial concentration of 1 mM. There were no interfering peaks observed for any of the corticosteroids when examined using the same HPLC conditions as used for formoterol. For both HLMs and UGT2B17, all the three corticosteroids gave negative results except for dexamethasone and cortisone in the case of HLMs, where only about 5% and below 1% inhibition was observed as shown in Figure 5.7 (c1 and c2). Otherwise, all the results were negative with no inhibition at all and instead showed an increase in glucuronidation activity. This study was carried out in light of the previous two studies where commonly used dietary products such as green tea and red wine lead to a reduction in the glucuronidation activity of testosterone (133,134). The results correspond to a reduction of formoterol glucuronidation in the presence of the inhibitory product, at 1 mM initial formoterol concentration. All the samples including controls were examined as duplicates and the results displayed in Figure 5.7. All the dietary products and catechins showed reduction in glucuronidation activity apart from corticosteroids, which showed an increase in glucuronidation activity.

5.3.3.4 Inhibition using fruit juices

Fruit juices were added into the reaction mixture in a similar way as for the tea extracts. There were no interfering peaks found at the same retention time as the drug. Figure 5.7 (d1 and d2) shows the inhibition of glucudonidation activity using fruit juices. Purple grape juice showed about 12% and 8% inhibition of formoterol glucuronidation for HLMs and UGT2B17, accordingly, followed by apple juice where inhibition was about 8% and 4%. Cranberry juice showed about 4% and below 2% inhibition of glucuronidation activity for HLMs and UGT2B17, respectively. A previous study showed that these juices contain different phenolic levels, where

purple grape juice showed the highest levels of phenolic compounds followed by apple and cranberry juices. The results obtained from this study showed the purple grape juice was the most potent inhibitor of enzyme assisted glucuronidation activity of formoterol (55).

Inhibitory effects of tea extracts, catechins and fruit juices can lead to alteration of formoterol and its metabolite concentration within an athlete's body, hence masking the actual drug concentration. This can lead to false doping results and can hide the β2- agonist misuse.

5.4 Conclusion

Formoterol is primarily eliminated through direct glucuronidation. Tea and fruit juices are rich in phenolic compounds; this is likely to be the reason for reduction in enzyme assisted glucuronidation activity of formoterol. Inhibitory effects of these dietary products along with catechins and corticosteroids on the glucuronidation activity of clenbuterol could not be investigated due to poor glucuronidation results of the drug using HLMs and UGT2B17.

In conclusion, it has been found that daily dietary products such as tea and fruit juices that contain phenolic compounds can inhibit formoterol glucuronidation over time, hence altering the levels of the drug within the body and excretion of its metabolites and hence can lead to false doping results. The inhibitory effects of each tea extract, catechins and fruit juices on HLMs and UGT2B17 resulted in an increased concentrations of remaining formoterol as compared to controls, hence the decrease in their glucuronidation activity. On the other hand, corticosteroids used in the study enhanced the glucuronidation activity of formoterol.

Further research is required to establish which of the flavonoids and phenolic compounds in fruit juices are not substrates of these enzymes and lead to inhibition

of glucuronidation. For clenbuterol, further optimisation of the glucuronidation reaction is required to achieve better results. Also, developing a method for the detection of formoterol glucuronides can help in determining exactly how much glucuronide is produced over a certain time period.

CONCLUDING REMARKS AND FUTURE WORK

To conclude, new highly sensitive, specific, reproducible and reliable analytical methods have been developed under challenging conditions in order to detect psychoactive and therapeutic drugs in complex matrices. The novel analytical methods were validated according to FDA guidelines. Novel assays were developed for quantifying mephedrone and its two metabolites 4-methylephedrine and 4-methylnorephedrine in human hair using LC-MS/MS. The method was capable of detecting mephedrone and its two metabolites by using only 50 mg of hair. In addition, a possible metabolic pathway for mephedrone has also been proposed. The method developed was successfully employed to analyse human hair samples for detection of mephedrone and its metabolites. Amongst the 154 hair samples analysed, five were confirmed to be positive for mephedrone. The method is suitable for the future detection and quantification of mephedrone and its metabolites, thus supporting various studies on psychoactive drug use. This research project also reports novel analytical method for the simultaneous quantification of two anti-retroviral drugs (tenofovir and abacavir) in human hair using LC-MS/MS. Again, only 50 mg hair was required for the analysis. This specific project did not require a hair digestion process, but instead used methanolic extraction which gave good drug recoveries for both antiretroviral drugs. The easy sample preparation and fast separation makes this assay highly suitable for TDM. The method was employed to 32 HIV patients' hair samples. Quantification of these antiretroviral drugs can be

useful for monitoring patient's compliance to the treatment and other clinical trials. Both assays can complement urinalysis to determine individual's drug use.

This research project also explored the influence of dietary substances, catechins and corticosteroids on the concentration level of β_2-agonists, *in vitro*, using HPLC-UV. It was observed that green and white tea had an effect on the glucuronidation of formoterol using HLM and UGT2B17. Fruit juices and catechins also showed some inhibition in the glucuronidation activity, whereas corticosteroids accelerated the glucuronidation process of formoterol. The inhibitory effects of these products could not be tested for clenbuterol due to unsuccessful glucuronidation. The method was useful for future doping control in sport as altering the glucuronidation activity of formoterol can mask the actual concentration in human body and urine.

Future work

Future work could involve method development for simultaneous detection of mephedrone and its metabolites in different matrices such as blood and urine using LC-MS/MS. In addition, further research needs to be carried out on the metabolic pattern of mephedrone, *in vitro*. The method could also be extended for detection of other psychoactive drugs of the same category.

It would be advantageous to develop analytical methods for the simultaneous detection of abacavir and tenofovir in different matrices with enhanced sensitivity, which could be very useful in monitoring drug compliance. Also, close monitoring of the relationship between drug intake and levels in hair by sectioning the hair over the course of certain time periods could be carried out to facilitate a complete longitudinal adherence study.

Future work could also involve improved optimisation of the glucuronidation process for clenbuterol in order to investigate the potential inhibitory effects of tea, fruit juices, catechins and corticosteroids on the glucuronidation activity. Also, additional dietary products that are rich in phenolic compounds could be analysed to investigate any potential inhibitory effects. Other UGTs could also be investigated to achieve better glucuronidation activity for formoterol and clenbuterol, hence investigating any potential inhibition of the glucuronidation activity of these β_2-agonists using potential inhibitory products. Developing a method for detecting the glucuronides of both drugs could help in determining how much glucuronide of the respective drug is produced in a particular time period and could help in glucuronidation process optimisation.

REFERENCES

(1) Gregg RA, Rawls SM. Behavioral pharmacology of designer cathinones: A review of the preclinical literature. Life Sciences 2013;97(1):27-30.

(2) Prosser JM, Nelson LS. The toxicology of bath salts: a review of synthetic cathinones. Journal of Medical Toxicology 2012;8(1):33-42.

(3) Sørensen LK. Determination of cathinones and related ephedrines in forensic whole-blood samples by liquid-chromatography–electrospray tandem mass spectrometry. Journal of Chromatography B 2011;879(11–12):727-736.

(4) Santali EY, Cadogan A, Daeid NN, Savage KA, Sutcliffe OB. Synthesis, full chemical characterisation and development of validated methods for the quantification of (±)-4′-methylmethcathinone (mephedrone): A new "legal high". Journal of Pharmaceutical and Biomedical Analysis 2011;56(2):246-255.

(5) Lusthof KJ, Oosting R, Maes A, Verschraagen M, Dijkhuizen A, Sprong AGA. A case of extreme agitation and death after the use of mephedrone in The Netherlands. Forensic Science International 2011;206(1–3):e93-e95.

(6) Wood DM, Davies S, Puchnarewicz M, Button J, Archer R, Ovaska H, et al. Recreational use of mephedrone (4-methylmethcathinone, 4-MMC) with associated sympathomimetic toxicity. Journal of Pharmaceutical and Biomedical Analysis 2010;6(3):327-330.

(7) Vardakou I, Pistos C, Spiliopoulou C. Drugs for youth via Internet and the example of mephedrone. Toxicology Letters 2011;201(3):191-195.

(8) Torrance H, Cooper G. The detection of mephedrone (4-methylmethcathinone) in 4 fatalities in Scotland. Forensic Science International 2010;202(1-3):e62-3.

(9) Dargan PI, Albert S, Wood DM. Mephedrone use and associated adverse effects in school and college/university students before the UK legislation change. QJM: International Journal of Medicine 2010;103(11):875-879.

(10) Meyer MR, Wilhelm J, Peters FT, Maurer HH. Beta-keto amphetamines: studies on the metabolism of the designer drug mephedrone and toxicological detection of mephedrone, butylone, and methylone in urine using gas chromatography–mass spectrometry. Analytical and Bioanalytical Chemistry 2010;397(3):1225-1233.

(11) Wood DM, Dargan PI. Mephedrone (4-methylmethcathinone): What is new in our understanding of its use and toxicity. Progress in Neuro-Psychopharmacology and Biological Psychiatry 2012;39(2):227-233.

(12) Winstock A, Mitcheson L, Marsden J. Mephedrone: still available and twice the price. The Lancet 2010;376(9752):1537.

(13) Lisek R, Xu W, Yuvasheva E, Chiu Y, Reitz AB, Liu-Chen L, et al. Mephedrone ('bath salt') elicits conditioned place preference and dopamine-sensitive motor activation. Drug and Alcohol Dependence 2012;126(1):257-262.

(14) Morris K. UK places generic ban on mephedrone drug family. The Lancet 2010;375(9723):1333-1334.

(15) Johnson LA, Johnson RL, Portier R. Current "Legal Highs". The Journal of Emergency Medicine 2013;44(6):1108-1115.

(16) Bonano J, Glennon R, De Felice L, Banks M, Negus S. Abuse-related and abuse-limiting effects of methcathinone and the synthetic "bath salts" cathinone analogs methylenedioxypyrovalerone (MDPV), methylone and mephedrone on intracranial self-stimulation in rats. Psychopharmacology 2014;231(1):199-207.

(17) Gibbons S, Zloh M. An analysis of the 'legal high' mephedrone. Bioorganic & Medicinal Chemistry Letters 2010;20(14):4135-4139.

(18) Kamata H, Shima N, Zaitsu K, Kamata T, Miki A, Nishikawa M, et al. Metabolism of the recently encountered designer drug, methylone, in humans and rats. Xenobiotica 2006;36(8):709-723.

(19) Pawlik E, Plässer G, Mahler H, Daldrup T. Studies on the phase I metabolism of the new designer drug 3-fluoromethcathinone using rabbit liver slices. International Journal of Legal Medicine 2012;126(2):231-240.

(20) Caldwell J, Dring L, Williams R. Metabolism of [^{14}C] methamphetamine in man, the guinea pig and the rat. Biochemical Journal 1972;129(1):11-22.

(21) Williams DA, Foye WO, Lemke TL. Foye's principles of medicinal chemistry. 5th ed. USA: Lippincott Williams & Wilkins; 2002.

(22) Klein J, Karaskov T, Koren G. Clinical applications of hair testing for drugs of abuse — the Canadian experience. Forensic Science International 2000 1/10;107(1–3):281-288.

(23) Le Saux T, Chhun S, Rey E, Launay O, Weiss L, Viard J, et al. Quantification of seven nucleoside/nucleotide reverse transcriptase inhibitors in human plasma by high-performance liquid chromatography with tandem mass-spectrometry. Journal of Chromatography B 2008;865(1):81-90.

(24) King T, Bushman L, Kiser J, Anderson PL, Ray M, Delahunty T, et al. Liquid chromatography–tandem mass spectrometric determination of tenofovir-diphosphate in human peripheral blood mononuclear cells. Journal of Chromatography B 2006;843(2):147-156.

(25) Bangsberg DR, Moss AR, Deeks SG. Paradoxes of adherence and drug resistance to HIV antiretroviral therapy. Journal of Antimicrobial Chemotherapy 2004;53(5):696-699.

(26) McColl DJ, Chen X. Strand transfer inhibitors of HIV-1 integrase: bringing IN a new era of antiretroviral therapy. Antiviral Research 2010;85(1):101-118.

(27) Cocohoba J, Dong BJ. Raltegravir: the first HIV integrase inhibitor. Clinical Therapeutics 2008;30(10):1747-1765.

(28) Gilliam BL, Sajadi MM, Amoroso A, Davis CE, Cleghorn FR, Redfield RR. Tenofovir and abacavir combination therapy: lessons learned from an urban clinic population. AIDS Patient Care and STDS 2007;21(4):240-6.

(29) D'Avolio A, Simiele M, Siccardi M, Baietto L, Sciandra M, Oddone V, et al. A HPLC–MS method for the simultaneous quantification of fourteen antiretroviral agents in peripheral blood mononuclear cell of HIV infected patients optimized using medium corpuscular volume evaluation. Journal of Pharmaceutical and Biomedical Analysis 2011;54(4):779-788.

(30) Gehrig A, Mikus G, Haefeli WE, Burhenne J. Electrospray tandem mass spectroscopic characterisation of 18 antiretroviral drugs and simultaneous quantification of 12 antiretrovirals in plasma. Rapid Communications in Mass Spectrometry 2007;21(16):2704-2716.

(31) Müller DM, Rentsch KM. Therapeutic drug monitoring by LC–MS–MS with special focus on anti-infective drugs. Analytical and Bioanalytical Chemistry 2010;398(6):2573-2594.

(32) Checa A, Oliver R, Hernández-Cassou S, Saurina J. Determination of HIV drugs in biological matrices: a review. Analytica Chimica Acta 2009;647(1):1-13.

(33) Rezk NL, Crutchley RD, Kashuba AD. Simultaneous quantification of emtricitabine and tenofovir in human plasma using high-performance liquid chromatography after solid phase extraction. Journal of Chromatography B 2005;822(1):201-208.

(34) Pruvost A, Théodoro F, Agrofoglio L, Negredo E, Bénech H. Specificity enhancement with LC-positive ESI-MS/MS for the measurement of nucleotides: application to the quantitative determination of carbovir triphosphate, lamivudine triphosphate and tenofovir diphosphate in human peripheral blood mononuclear cells. Journal of Mass Spectrometry 2008;43(2):224-233.

(35) Taylor PJ, Tai C, Franklin ME, Pillans PI. The current role of liquid chromatography-tandem mass spectrometry in therapeutic drug monitoring of immunosuppressant and antiretroviral drugs. Clinical Biochemistry 2011;44(1):14-20.

(36) De Clercq E. Anti-HIV drugs: 25 compounds approved within 25 years after the discovery of HIV. International Journal of Antimicrobial Agents 2009;33(4):307-320.

(37) Huang Y, Gandhi M, Greenblatt RM, Gee W, Lin ET, Messenkoff N. Sensitive analysis of anti-HIV drugs, efavirenz, lopinavir and ritonavir, in human hair by liquid chromatography coupled with tandem mass spectrometry. Rapid Communications in Mass Spectrometry 2008;22(21):3401-3409.

(38) Shah SA, Mullin R, Jones G, Shah I, Barker J, Petroczi A, et al. Simultaneous analysis of antiretroviral drugs abacavir and tenofovir in human hair by liquid chromatography tandem mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis 2013;74:308-313.

(39) Palmieri TL. Use of beta-agonists in inhalation injury. Journal of Burn Care and Research 2009;30(1):156-159.

(40) Smith DJ. The pharmacokinetics, metabolism, and tissue residues of beta-adrenergic agonists in livestock. Journal of Animal Science 1998;76(1):173-194.

(41) Carlsen K, Hem E, Stensrud T, Held T, Herland K, Mowinckel P. Can asthma treatment in sports be doping? The effect of the rapid onset, long-acting inhaled β_2-agonist formoterol upon endurance performance in healthy well-trained athletes. Respiratory Medicine 2001;95(7):571-576.

(42) Akapo SO, Asif M. Validation of a RP-HPLC method for the assay of formoterol and its related substances in formoterol fumarate dihydrate drug substance. Journal of Pharmaceutical and Biomedical Analysis 2003;33(5):935-945.

(43) UKAD. Asthma Advice. Available at: http://www.ukad.org.uk/support-personnel/asthma/. Accessed May,21, 2014.

(44) Nicoli R, Petrou M, Badoud F, Dvorak J, Saugy M, Baume N. Quantification of clenbuterol at trace level in human urine by ultra-high pressure liquid chromatography–tandem mass spectrometry. Journal of Chromatography A 2013;1292:142-150.

(45) Kuiper HA, Noordam MY, van Dooren-Flipsen MM, Schilt R, Roos AH. Illegal use of beta-adrenergic agonists: European Community. Journal of Animal Science 1998;76(1):195-207.

(46) Blanca J, Muñoz P, Morgado M, Méndez N, Aranda A, Reuvers T, et al. Determination of clenbuterol, ractopamine and zilpaterol in liver and urine by liquid chromatography tandem mass spectrometry. Analytica Chimica Acta 2005;529(1):199-205.

(47) Anderson G, Wilkins E. A trial of clenbuterol in bronchial asthma. Thorax 1977;32(6):717-719.

(48) Prather ID, Brown DE, North P, Wilson JR. Clenbuterol: a substitute for anabolic steroids? Medicine and Science in Sports and Exercise 1995;27(8):1118-1121.

(49) Carlsen K-, Hem E, Stenstrud T, Held T, Herland K, Mowinckel P. Can asthma treatment in sports be doping? The effect of the rapid onset, long-acting inhaled β_2-agonist formoterol upon endurance performance in healthy well-trained athletes. Respiratory Medicine 2001;95(7):571-576.

(50) Gunnar AK, Faweett PJ, inventors. AnonymousMethods of accelerating muscle growth and improving feed efficiency in animals by using optically pure eutomers of adregenic beta-2 receptor agonists, and food supplements containing the same. United States patent 6,110,974. 2000 29 August.

(51) Deventer K, Pozo OJ, Delbeke FT, Van Eenoo P. Quantitative detection of inhaled formoterol in human urine and relevance to doping control analysis. Drug Testing and Analysis 2012;4(6):449-454.

(52) Mazzarino M, de la Torre X, Fiacco I, Pompei C, Calabrese F, Botrè F. A simplified procedure for the analysis of formoterol in human urine by liquid chromatography–electrospray tandem mass spectrometry: Application to the characterization of the metabolic profile and stability of formoterol in urine. Journal of Chromatography B 2013;931:75-83.

(53) Arizona Physicians IPA. Foradil Aerolizer (formoterol fumarate inhalation powder) 12mcg. Available at: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Enforce

mentActivitiesbyFDA/WarningLettersandNoticeofViolationLetterstoPharmaceuticalCompanies/ucm054662.pdf. Accessed March,17, 2014.

(54) Jenkinson C, Petroczi A, Barker J, Naughton DP. Dietary green and white teas suppress UDP-glucuronosyltransferase UGT2B17 mediated testosterone glucuronidation. Steroids 2012;77(6):691-695.

(55) Mullen W, Marks SC, Crozier A. Evaluation of phenolic compounds in commercial fruit juices and fruit drinks. Journal of Agricultural and Food Chemistry 2007;55(8):3148-3157.

(56) Suissa S, Ernst P. Inhaled corticosteroids: impact on asthma morbidity and mortality. Journal of Allergy and Clinical Immunology 2001;107(6):937-944.

(57) Johnson M. Interactions between corticosteroids and β2-agonists in asthma and chronic obstructive pulmonary disease. Proceedings of the American Thoracic Society 2004;1(3):200-206.

(58) Balíková M. Hair analysis for drug abuse. Plausibility of interpretation. Biomedical Papers-Palacky University in Olomouc 2005;149(2):199-207.

(59) Boumba VA, Ziavrou KS, Vougiouklakis T. Hair as a biological indicator of drug use, drug abuse or chronic exposure to environmental toxicants. International Journal of Toxicology 2006;25(3):143-163.

(60) Pragst F, Balikova MA. State of the art in hair analysis for detection of drug and alcohol abuse. Clinica Chimica Acta 2006;370(1):17-49.

(61) Kronstrand R, Scott K. Drug Incorporation into Hair. In: P.Kintz, editor. Analytical and practical aspects of drug testing in hair Boca Raton: CRC Press; 2007. p. 1-23.

(62) Kamata H, Shima N, Zaitsu K, Kamata T, Miki A, Nishikawa M, et al. Metabolism of the recently encountered designer drug, methylone, in humans and rats. Xenobiotica 2006;36(8):709-723.

(63) Nakahara Y. Hair analysis for abused and therapeutic drugs. Journal of Chromatography B 1999 10/15;733(1–2):161-180.

(64) Miyaguchi H, Kakuta M, Iwata YT, Matsuda H, Tazawa H, Kimura H, et al. Development of a micropulverized extraction method for rapid toxicological analysis of methamphetamine in hair. Journal of Chromatography A 2007 9/7;1163(1–2):43-48.

(65) Gaillard Y, Vayssette F, Pépin G. Compared interest between hair analysis and urinalysis in doping controls: Results for amphetamines, corticosteroids and anabolic steroids in racing cyclists. Forensic Science International 2000 1/10;107(1–3):361-379.

(66) Heine Rt, Beijnen J, Huitema A. Bioanalytical issues in patient-friendly sampling methods for therapeutic drug monitoring: focus on antiretroviral drugs. Bioanalysis 2009;1(7):1329-1338.

(67) United Nations. Guidelines for testing drugs under international control in hair, sweat and saliva. 2001; Available at: https://www.unodc.org/pdf/publications/st-nar-30.pdf. Accessed March,14, 2014.

(68) Deshmukh N, Hussain I, Barker J, Petroczi A, Naughton DP. Analysis of anabolic steroids in human hair using LC–MS/MS. Steroids 2010;75(10):710-714.

(69) Kintz P, Cirimele V, Dumestre-Toulet V, Ludes B. Doping control for nandrolone using hair analysis. Journal of Pharmaceutical and Biomedical Analysis 2001;24(5):1125-1130.

(70) DuPont RL, Baumgartner WA. Drug testing by urine and hair analysis: complementary features and scientific issues. Forensic Science International 1995;70(1):63-76.

(71) Tsatsakis AM, Tzatzarakis M. Sectional hair testing. Judicial and clinical applications. Pure and Applied Chemistry 2000;72(6):1057-1066.

(72) Petróczi A, Nepusz T, Cross P, Taft H, Shah S, Deshmukh N, et al. New non-randomised model to assess the prevalence of discriminating behaviour: a pilot study on mephedrone. Substance Abuse Treatment, Prevention, and Policy 2011;6(1):20.

(73) Health Care Providers. Analysis of hair samples: How do hair sampling results relate to environmental exposure? 2003; Available at: http://www.atsdr.cdc.gov/HAC/hair_analysis/03-0330HairSampleTesting-Scientific.pdf. Accessed Febuary/26, 2015.

(74) Frisch M, Schwartz BS. The pitfalls of hair analysis for toxicants in clinical practice: three case reports. Environmental Health Perspectives 2002 Apr;110(4):433-436.

(75) Snyder LR, Kirkland JJ, Dolan JW. Introduction to modern liquid chromatography. 2nd ed. USA: John Wiley & Sons; 1979.

(76) Brown P, DeAntonis K. Separation methods. In: Settle F, editor. Handbook of instrumental techniques for analytical chemistry New Jersey: Prentice-Hall, Inc.; 1997. p. 147-160.

(77) Crawford Scientific. The Theory of HPLC - Introduction. Available at: http://forensicscienceeducation.org/wp-content/uploads/2013/04/Theory_Of_HPLC_Introduction.pdf. Accessed November,25, 2014.

(78) Gumustas M, Kurbanoglu SU, Bengi, Ozkan SA. UPLC versus HPLC on Drug Analysis : Advantageous, Applications and their Validation parameters. Chromatographia 2013;76(21-22):1365-1427.

(79) Kupiec T. International Journal of Pharmaceutical Compounding 2004;8(3):223-227.

(80) Kazakevich Y, Lobrutto R editors. HPLC for Pharmaceutical Scientists. New Jersey: John Wiley & Sons; 2007.

(81) Dong MW. Modern HPLC for Practicing Scientists. New Jersey: John Wiley & Sons; 2006.

(82) Crawford Scientific. Theory of HPLC - Band Broadening. Available at: http://www.chromacademy.com/lms/sco3/Theory_Of_HPLC_Band_Broadening.pdf. Accessed November,25, 2014.

(83) Rimmer CA. Lloyd R. Snyder, Joseph J. Kirkland, John W. Dolan: Introduction to modern liquid chromatography. Analytical and Bioanalytical Chemistry 2011;399(5):1809-1810.

(84) Ardrey RE. Liquid chromatography-mass spectrometry: an introduction. England: John Wiley & Sons; 2003.

(85) Westman-Brinkmalm A, Brinkmalm G. A mass spectrometer's building blocks. In: Ekman R, Silberring J, Westman-Brinkmalm A, Kraj A, editors. Mass Spectrometery Instrumentation, Interpretation, and Applications New Jersey: John Wiley & Sons, Inc.; 2009. p. 15-87.

(86) Dass C. High performance liquid chromatography electrospray ionization mass spectrometry. In: Settle F, editor. Handbook of instrumental techniques for analytical chemistry New Jersey: Prentice-Hall, Inc.; 1997. p. 647-663.

(87) March R, Todd J. A historical review of early development of the quadrupole ion trap. In: Winefordner J, editor. Quadrupole Ion Trap Mass Spectrometry. 2nd ed. New Jersey: John WIley & Sons, Inc.; 2005. p. 1-25.

(88) Gates P. Quadruple & Triple Quadrupole (QQQ) Mass Analysis. May, 2009; Available at: http://www.chm.bris.ac.uk/ms/theory/quad-massspec.html. Accessed March,19, 2014.

(89) Hoffmann E. Special feature tutorial: Tandem mass spectrometry: a primer. Journal of Mass Spectrometry 1996;31:129-137.

(90) McMaster M. LC/MS: a practical user's guide. New Jersey: John Wiley & Sons; 2005.

(91) Hoffmann E, Stroobant V. Mass Spectrometry: Principles and Applications 2007. 3rd ed. England: John Wiley & Sons Ltd; 2007.

(92) Schreiber A. Advantages of using triple quadrupole over single quardupole mass spectrometry to quantify and identify the presence of pesticides in water and soil samples. Available at: http://www.absciex.com/Documents/Downloads/Literature/mass-spectrometry-TripleQuad-Pesticides-Testing-0701310.pdf. Accessed May/20, 2014.

(93) Deirdre Fox. Development and application of novel approaches to quantitate therapeutic drugs in human bloodNational Institute for Cellular Biotechnology Dublin City University; 2012.

(94) Agilent Technologies. Basic of LC-MS. Available at: http://ccc.chem.pitt.edu/wipf/Agilent%20LC-MS%20primer.pdf. Accessed February 23, 2014.

(95) Tiina Kauppila. Atmospheric Pressure Photoionization-Mass Spectrometry. Helsinki: Faculty of Pharmacy University of Helsinki Finland; 2004.

(96) Danaceau J. Successful Sample Preparation Strategies for LC/MS/MS Analysis of Drugs in Complex Biological Matrices for Forensic Toxicology Applications. Available at: http://www.waters.com/webassets/cms/events/docs/FINALSamplePrepStrategiesClinical_Forensic11_5_13.pdf. Accessed February,22, 2014.

(97) Mitra S. In: Winefordner J, editor. Sample Preparation Techniques in Analytical Chemistry New Jersey: John Wiley & Sons; 2003. p. 3.

(98) SUPELCO Bulletin. Guide to Solid Phase Extraction. Available at: www.sigmaaldrich.com/Graphics/Supelco/objects/4600/4538.pdf. Accessed February 23, 2014.

(99) Harris DC. Quantitative chemical analysis. 7th ed. New York: WH Freeman and Co.; 2007.

(100) Harvey D. Modern analytical chemistry. USA: McGraw-Hill Companies, Inc.; 2000.

(101) Wells M. Principles of Extraction and the Extraction of Semivolatile Organics from Liquids. In: Winefordner J, editor. Sample Preparation Techniques in Analytical Chemistry New Jersey: John Wiley & Sons, Inc.; 2003. p. 57.

(102) Bakhtiar R, Majumdar TK, Tse FL. The Role of Liquid Chromatography–Mass Spectrometry in Pharmacokinetics and Drug Metabolism. HPLC for Pharmaceutical Scientists 2007:605-640.

(103) FDA. Guidance for Industry Bioanalytical Method Validation. Available at: http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf. Accessed February 26, 2014.

(104) Kapil Kalra. Method Development and Validation of Analytical Procedures. Available at: http://www.intechopen.com/download/get/type/pdfs/id/23463. Accessed February 26, 2014.

(105) Center for Drug Evaluation and Research. Reviewer's Guide
 Validation of Chromatographic Methods. Available at: http://www.fda.gov/downloads/Drugs/Guidances/UCM134409.pdf. Accessed Februaury 26, 2014.

(106) Ludwig Huber. Validation of Analytical Methods
Agilent Technologies. Available at: http://www.chem.agilent.com/Library/primers/Public/5990-5140EN.pdf. Accessed February 26, 2014.

(107) Shah SA, Deshmukh NI, Barker J, Petróczi A, Cross P, Archer R, et al. Quantitative analysis of mephedrone using liquid chromatography tandem mass spectroscopy: Application to human hair. Journal of Pharmaceutical and Biomedical Analysis 2012;61:64-69.

(108) Martínez-Clemente J, López-Arnau R, Carbó M, Pubill D, Camarasa J, Escubedo E. Mephedrone pharmacokinetics after intravenous and oral administration in rats: relation to pharmacodynamics. Psychopharmacology 2013;229:295-306.

(109) Brandt SD, Sumnall HR, Measham F, Cole J. Analyses of second-generation 'legal highs' in the UK: Initial findings. Drug Testing and Analysis 2010;2(8):377-382.

(110) R Meyer M, H Maurer H. Metabolism of designer drugs of abuse: an updated review. Current Drug Metabolism 2010;11(5):468-482.

(111) Wood DM, Davies S, Greene SL, Button J, Holt DW, Ramsey J, et al. Case series of individuals with analytically confirmed acute mephedrone toxicity. Clinical Toxicology 2010;48(9):924-927.

(112) Fung HB, Stone EA, Piacenti FJ. Tenofovir disoproxil fumarate: a nucleotide reverse transcriptase inhibitor for the treatment of HIV infection. Clinical Therapeutics 2002;24(10):1515-1548.

(113) Nannini L, Cates CJ, Lasserson TJ, Poole P. Combined corticosteroid and long-acting beta-agonist in one inhaler versus placebo for chronic obstructive pulmonary disease. Cochrane Database Syst Rev 2010;4:1-53.

(114) Takahashi M, Kudaka Y, Okumura N, Hirano A, Banno K, Kaneda T. Determination of plasma tenofovir concentrations using a conventional LC-MS method. Biological and Pharmaceutical Bulletin 2007;30(9):1784-1786.

(115) Delahunty T, Bushman L, Robbins B, Fletcher CV. The simultaneous assay of tenofovir and emtricitabine in plasma using LC/MS/MS and isotopically labeled internal standards. Journal of Chromatography B 2009;877(20):1907-1914.

(116) Gomes NA, Vaidya VV, Pudage A, Joshi SS, Parekh SA. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for simultaneous determination of tenofovir and emtricitabine in human plasma and its application to a bioequivalence study. Journal of Pharmaceutical and Biomedical Analysis 2008;48(3):918-926.

(117) Jones G, Hawkins K, Mullin R, Nepusz T, Naughton D, Sheeran P, et al. Understanding how adherence goals promote adherence behaviours: a repeated measure observational study with HIV seropositive patients. BMC Public Health 2012;12(1):587.

(118) Barkil ME, Gagnieu M, Guitton J. Relevance of a combined UV and single mass spectrometry detection for the determination of tenofovir in human plasma by HPLC in therapeutic drug monitoring. Journal of Chromatography B 2007;854(1):192-197.

(119) Charles JM, * Marbury DG. Collision Energy, Collision Gas, and Collision Gas Pressure Effects on the Formation of 2,3,7,8-Tetrachlorodibenzo-p-dioxin and 2,3,7,8-Tetrabromodibenzo-p-dioxin Product Ions. Analytical Chemistry 1991;63:713-721.

(120) MacLean B, Tomazela DM, Abbatiello SE, Zhang S, Whiteaker JR, Paulovich AG, et al. Effect of collision energy optimization on the measurement of peptides by selected reaction monitoring (SRM) mass spectrometry. Analytical Chemistry 2010;82(24):10116-10124.

(121) DeHaven CD, Evans AM, Dai H, Lawton KA. Organization of GC/MS and LC/MS metabolomics data into chemical libraries. Journal of Cheminformatics 2010;2(1):9.

(122) Tutudaki M, Tsakalof AK, Tsatsakis AM. Hair analysis used to assess chronic exposure to the organophosphate diazinon: a model study with rabbits. Human & Experimental Toxicology 2003;22(3):159-164.

(123) Pavlos R, Phillips EJ. Individualization of antiretroviral therapy. Pharmacogenomics and Personalized Medicine 2011;2012(5):1-17.

(124) Lubomirov R, Colombo S, di Iulio J, Ledergerber B, Martinez R, Cavassini M, et al. Association of pharmacogenetic markers with premature discontinuation of first-line anti-HIV therapy: an observational cohort study. Journal of Infectious Diseases 2011;203(2):246-257.

(125) Gram LF. The dose–concentration–effect relationships—the basis for TDM. A critical appraisal. International Congress Series 2001;1220:117-123.

(126) Deshmukh NI, Barker J, Petroczi A, Naughton DP. Detection of testosterone and epitestosterone in human hair using liquid chromatography–tandem mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis 2012;67:154-158.

(127) Overbye M, Wagner U. Between medical treatment and performance enhancement: An investigation of how elite athletes experience Therapeutic Use Exemptions. International Journal of Drug Policy 2013;24(6):579-588.

(128) WADA. Therapeutic Use Exemptions. December,2011; Available at: http://www.wada-ama.org/en/Science-Medicine/TUE/. Accessed March, 25, 2014.

(129) Domínguez-Romero JC, García-Reyes JF, Martínez-Romero R, Martínez-Lara E, Del Moral-Leal ML, Molina-Díaz A. Detection of main urinary metabolites of β2-agonists clenbuterol, salbutamol and terbutaline by liquid chromatography high resolution mass spectrometry. Journal of Chromatography B 2013;923–924:128-135.

(130) Kindermann W. Do Inhaled B2-Agonists have an Ergogenic Potential in Non-Asthmatic Competitive Athletes? Sports Medicine 2007;37(2):95-102.

(131) Virginia A. Introduction to drug metabolism. Available at: http://www.healthsci.jmu.edu/common/Knitter/powerpoint/Drug%20Metabolism.ppt. Accessed March, 26, 2014.

(132) Sten T, Finel M, Ask B, Rane A, Ekström L. Non-steroidal anti-inflammatory drugs interact with testosterone glucuronidation. Steroids 2009;74(12):971-977.

(133) Jenkinson C, Petroczi A, Barker J, Naughton DP. Dietary green and white teas suppress UDP-glucuronosyltransferase UGT2B17 mediated testosterone glucuronidation. Steroids 2012;77(6):691-695.

(134) Jenkinson C, Petroczi A, Naughton DP. Red wine and component flavonoids inhibit UGT2B17 *in vitro*. Nutrition Journal 2012;11(67).

(135) Xu C, Li CY, Kong AT. Induction of phase I, II and III drug metabolism/transport by xenobiotics. Archives of Pharmacal Research 2005;28(3):249-268.

(136) University of California, San Diego. Drug metabolism. Available at: http://classes.biology.ucsd.edu/bimm118.WI12/PPT%20Lecture%20Notes/Lecture%203.ppt . Accessed March, 26, 2014.

(137) Strassburg CP, Kneip S, Topp J, Obermayer-Straub P, Barut A, Tukey RH, et al. Polymorphic gene regulation and interindividual variation of UDP-glucuronosyltransferase activity in human small intestine. Journal of Biological Chemistry 2000;275(46):36164-36171.

(138) Peter L. Anderson. The ABCs of Pharmacokinetics. Available at: http://www.thebody.com/content/art875.html. Accessed March/26, 2014.

(139) Gibson GG, Skett P editors. Introduction to Drug Metabolism. 3rd ed. Cheltenham, United Kingdom: Nelson Thornes Publisher; 2001.

(140) Iyer KR, Sinz MW. Characterization of Phase I and Phase II hepatic drug metabolism activities in a panel of human liver preparations. Chemico-Biological Interactions 1999;118(2):151-169.

(141) Olinga P, Elferink MGL, Draaisma AL, Merema MT, Castell JV, Pérez G, et al. Coordinated induction of drug transporters and phase I and II metabolism in human liver slices. European Journal of Pharmaceutical Sciences 2008;33(4–5):380-389.

(142) Alonen A. Glucuronide Isomers: Synthesis, Structural Characterization, and UDP-glucuronosyltransferase Screening. 2009.

(143) Fisher MB, Campanale K, Ackermann BL, VandenBranden M, Wrighton SA. In vitro glucuronidation using human liver microsomes and the pore-forming peptide alamethicin. Drug Metabolism and Disposition 2000;28(5):560-566.

(144) Dehal SS, Ho T, Patten CJ, Stresser DM, Abolin C. Glucuronidation of (R,R)-Formoterol and (S,S)-Formoterol is Catalyzed by cDNA-expressed Human Uridine 5´-Diphospho-Glucuronosyltransferases (UGTs) and Human Liver Microsomes. International Society for the Study of Xenobiotics Meeting 2006:1-9.

(145) Venkatakrishnan K, von Moltke LL, Obach R, Greenblatt DJ. Drug metabolism and drug interactions: application and clinical value of in vitro models. Current Drug Metabolism 2003;4(5):423-459.

(146) Strassburg CP, Nguyen N, Manns MP, Tukey RH. Polymorphic expression of the UDP-glucuronosyltransferase UGT1A gene locus in human gastric epithelium. Molecular Pharmacology 1998;54(4):647-654.

(147) Alonen A, Gartman M, Aitio O, Finel M, Yli-Kauhaluoma J, Kostiainen R. Synthesis, structure characterization, and enzyme screening of clenbuterol glucuronides. European Journal of Pharmaceutical Sciences 2009;37(5):581-587.

(148) Kilford PJ, Stringer R, Sohal B, Houston JB, Galetin A. Prediction of drug clearance by glucuronidation from in vitro data: use of combined cytochrome P450 and UDP-glucuronosyltransferase cofactors in alamethicin-activated human liver microsomes. Drug Metabolism and Disposition 2009;37(1):82-89.

(149) Radominska-Pandya A, Bratton S, Little JM. A historical overview of the heterologous expression of mammalian UDP-glucuronosyltransferase isoforms over the past twenty years. Current Drug Metabolism 2005;6(2):141-160.

(150) WADA. THE 2014 PROHIBITED LIST INTERNATIONAL STANDARD. 2014; Available at: http://www.wada-ama.org/Documents/World_Anti-Doping_Program/WADP-Prohibited-list/2014/WADA-prohibited-list-2014-EN.pdf. Accessed March, 25, 2014.

(151) Controlled Drug List 2013-06-12. Available at: http://www.ch.cam.ac.uk/sites/ch/files/controlled-drugs-list-june-2013.pdf. Accessed March, 27, 2014.

(152) Machnik M, Geyer H, Horning S, Breidbach A, Delahaut P, Schänzer W. Long-term detection of clenbuterol in human scalp hair by gas chromatography–high-resolution mass spectrometry. Journal of Chromatography B 1999;723(1–2):147-155.

(153) Spiller HA, James KJ, Scholzen S, Borys DJ. A descriptive study of adverse events from clenbuterol misuse and abuse for weight loss and bodybuilding. Substance Abuse 2013;34(3):306-312.

(154) Campestrini J, Lecaillon JB, Godbillon J. Automated and sensitive method for the determination of formoterol in human plasma by high-performance liquid chromatography and electrochemical detection. Journal of Chromatography B 1997;704(1–2):221-229.

(155) Assi KH, Tarsin W, Chrystyn H. High performance liquid chromatography assay method for simultaneous quantitation of formoterol and budesonide in Symbicort Turbuhaler. Journal of Pharmaceutical and Biomedical Analysis 2006;41(1):325-328.

(156) Zhang M, Fawcett JP, Kennedy JM, Shaw JP. Stereoselective glucuronidation of formoterol by human liver microsomes. Journal of Clinical Pharmacology 2000;49(2):152-157.

(157) Ritter JK. Roles of glucuronidation and UDP-glucuronosyltransferases in xenobiotic bioactivation reactions. Chemico-Biological Interactions 2000;129(1-2):171-193.

(158) International Olympic Committee. IOC Consensus statement on asthma in elite athletes

 . Available at: http://www.olympic.org/Documents/Reports/EN/en_report_1301.pdf. Accessed May, 21, 2014.

(159) Shimadzu. Differences between using acetonitrile and methanol for reverse phase chromatography. Available at: http://www.shimadzu.com/an/hplc/support/lib/lctalk/35/35lab.html. Accessed February/13, 2015.

(160) Schmid J, Prox A, Zimmer A, Keck J, Kaschke S. Biotransformation of clenbuterol. Fresenius' Zeitschrift für analytische Chemie 1990;337(1).

(161) Shelver WL, Smith DJ. Evaluation of commercial immunoassays for cross-reactivity to clenbuterol stereoisomers and bovine metabolites. Food Additives & Contaminants 2000;17(10):837-845.

(162) Boase S, Miners JO. In vitro–in vivo correlations for drugs eliminated by glucuronidation: investigations with the model substrate zidovudine. British Journal of Clinical Pharmacology 2002;54(5):493-503.

APPENDIX

Petróczi et al. Substance Abuse Treatment, Prevention, and Policy 2011, 6:20
http://www.substanceabusepolicy.com/content/6/1/20

SUBSTANCE ABUSE TREATMENT, PREVENTION, AND POLICY

METHODOLOGY Open Access

New non-randomised model to assess the prevalence of discriminating behaviour: a pilot study on mephedrone

Andrea Petróczi[1,2*], Tamás Nepusz[3], Paul Cross[4], Helen Taft[4], Syeda Shah[5], Nawed Deshmukh[5], Jay Schaffer[6], Maryann Shane[6], Christiana Adesanwo[1], James Barker[5] and Declan P Naughton[1]

Abstract

Background: An advantage of randomised response and non-randomised models investigating sensitive issues arises from the characteristic that individual answers about discriminating behaviour cannot be linked to the individuals. This study proposed a new fuzzy response model coined *'Single Sample Count'* (SSC) to estimate prevalence of discriminating or embarrassing behaviour in epidemiologic studies.

Methods: The SSC was tested and compared to the established Forced Response (FR) model estimating Mephedrone use. Estimations from both SSC and FR were then corroborated with qualitative hair screening data. Volunteers (n = 318, mean age = 22.69 ± 5.87, 59.1% male) in a rural area in north Wales and a metropolitan area in England completed a questionnaire containing the SSC and FR in alternating order, and four questions canvassing opinions and beliefs regarding Mephedrone. Hair samples were screened for Mephedrone using a qualitative Liquid Chromatography-Mass Spectrometry method.

Results: The SSC algorithm improves upon the existing item count techniques by utilizing known population distributions and embeds the sensitive question among four unrelated innocuous questions with binomial distribution. Respondents are only asked to indicate *how many* without revealing *which* ones are true. The two probability models yielded similar estimates with the FR being between 2.6% - 15.0%; whereas the new SSC ranged between 0% - 10%. The six positive hair samples indicated that the prevalence rate in the sample was at least 4%. The close proximity of these estimates provides evidence to support the validity of the new SSC model. Using simulations, the recommended sample sizes as the function of the statistical power and expected prevalence rate were calculated.

Conclusion: The main advantages of the SSC over other indirect methods are: simple administration, completion and calculation, maximum use of the data and good face validity for all respondents. Owing to the key feature that respondents are not required to answer the sensitive question directly, coupled with the absence of forced response or obvious self-protective response strategy, the SSC has the potential to cut across self-protective barriers more effectively than other estimation models. This elegantly simple, quick and effective method can be successfully employed in public health research investigating compromising behaviours.

Keywords: random response technique, non-random model, Mephedrone, survey, illicit substances, epidemiology

* Correspondence: A.Petroczi@kingston.ac.uk
[1]School of Life Sciences, Kingston University, UK
Full list of author information is available at the end of the article

Background

Outcome based evaluation of interventions, which play a central role in public health prevention, need to show the effect the policy or intervention makes at the public level. Whilst a plethora of literature focuses on evaluating various social marketing campaigns that tackle public health and safety issues such as drug use, health compromising lifestyle choices, unprotected or risky sexual behaviour, or unsafe driving practices, tend to rely on self reports, regardless of whether or not they were conducted in laboratory or field settings [1-3]. The issues that may hinder an evaluation of any health promotion [4] are further complicated by the influence of social desirability that may cast doubt over the validity of self-reported information when to the study topic relates to socially sensitive behaviour [5]. In addition to public health concerns where obtaining accurate information on drug use is vital in establishing the need for and to evaluate preventive measures or intervention strategies, policy makers in public service utilities and law enforcement agencies also require the most accurate estimates of the problematic behavioural choices as possible in order to make informed choices.

The need to obtain the maximum intelligence on health related behaviours stems from the necessity to develop and deploy optimal intervention measures to counteract consistent failures to attain acceptable levels of behaviour across a wide range of health practices. These range from adherence to medication, resistance to addiction, avoidance of exploration of social drug use through to uptake of illegal and health damaging performance enhancement agents. The immense health, financial and social consequences of enhancing these health related behaviours has led to decades of investigation into improved approaches to obtain accurate data on sensitive personal behaviours.

Investigating the epidemiology of socially sensitive or transgressive behaviours such as illicit drug use, unhealthy weight management practices, risky behaviour, cheating, doping or non-adherence to prescribed medication or treatment, is hindered by respondents evasively answering questions about sensitive behaviours [6]. A recent research programme provides further evidence for self-protective strategic responding, even under anonymous answer conditions [7-9]. Consequently, much effort has been made to develop reliable methods to collect valid epidemiological data in these sensitive behavioural domains.

Approaches range from techniques such as the Bogus Pipeline [10] to providing incentives for honest answers such as the Bayesian Truth Serum (BTS) [11]. Whilst the Bogus Pipeline has been used for decades and accumulated reasonable evidence that the BPL shifts self-reports toward veracity [12], the BTS approach is relatively new and in need for further refinements [13,14]. Based on empirical evidence, Barrage and Lee [13] also suggest that to be effective, respondents may need to have a positive experience with and trust in the BTS method, which can lead to respondents learning how to maximise their incentives and therefore their answers might be biased towards maximum income at the expense of telling the truth. Although these methods possess the potential to overcome to an extent, self-protective response bias by either evoking fear of exposure of lying or providing financial gain for truthfulness, their feasibility in self-administered epidemiological scale studies appears to be compromised. An alternative approach has made notable progress in collecting data on sensitive behaviours through the development of indirect methods using randomisation or deliberate uncertainty to provide respondent protection over and above ensuring anonymity [6].

The concept behind randomised response models (collectively termed RRT) rests on introducing a randomising element to the survey question by using some device (e.g. by rolling a dice, flipping a coin or picking a card) which determines how the respondent should answer [15]. Since the researcher has no control over this randomising device, answers cannot be directly traced back to any particular individual, which in turn heightens the respondents' sense of increased protection. A common characteristic of RRTs is that to obtain useful data on the sensitive question, the technique requires respondents to answer directly, in some form, the sensitive question. By contrast, non-random models (NRM) do not require a direct answer as they rely on implicit uncertainty rendering impossible the link between an individual and the sensitive behaviour. Whilst NRMs build on combining the sensitive question with unrelated innocuous questions, some RRTs also incorporate innocuous questions where the population prevalence may or may not be known. When population prevalence needs to be established, it requires an independent sample randomly selected from the same population.

Randomised response models

The RRT aims to elicit sensitive, embarrassing or compromising information that may portray respondents unfavourably. The common characteristic of the RRT is that sensitive behaviour estimation can only be made at the aggregated population level. The method is based on the principle introduced by Warner [16] using a spinner as a randomising device to gauge the proportion of the sample with a compromising behaviour. The method assumed that any person in a sample is either characterised by the behaviour (group A) or is not (group B). The respondents, hidden from the interviewer, were asked to use the spinner which either landed on group

A or on group B and answer with a simple 'yes' or 'no' depending on whether the spinner pointed to the group he/she belonged to. Whilst the outcome of the spinner exercise for each individual was not known to the interviewer, hence protecting the individual, the chance that a spinner points to group A or B was known (p and $1-p$). Thus compared to the observed pattern of 'yes' and 'no' answers Warner was able to determine the proportion of respondents in the sample admitting the sensitive behaviour.

Subsequent adaptations of the RRT have covered a wide range of sensitive issues along with numerous attempts to refine the approach [15]. Among the wide array of models, the Forced Alternative/Response model, used only when the sensitive question is presented [16], has been found to be one of the most efficient variants of Warner's original conception [17]. Recently the RRT method has been expanded to multi-item scales and tested with male date rape attitude [18] and alcohol abuse [19]. The extension of the RRT to multi-item scales allows its application to psychological measures such as attitudes toward sensitive issues. This approach can be expanded to areas where honest responding might be compromised by self-protective lying, for example illegal substance dependence, domestic violence, disordered eating or cheating and doping use in sport.

Non-randomised methods

Research has shown that whilst respondents understand the reason behind the use of the RRT approach in surveys, they generally find it obtrusive and favour simpler approaches [20]. Contrary to the RRT, non-random models present a more straightforward approach that provides protection by asking the number or combination of behaviours respondents are engaged in rather than asking about each behaviour in turn.

The non-randomised model (NRM) has received increased attention lately. A recent review [21] showed that NRMs appear to successfully address many of the limitations typically associated with RRTs such as the need for a randomisation device which often requires interviewers; forcing participants to say 'yes' to an embarrassing question when their honest answer would be the opposite or requiring a direct answer to the same question. Contrary to the RRT, in the NRM every participant is required to answer the research question in an evasive way. The fact that a response is required to the research question can help participants to feel that they have made a contribution by volunteering to take part in the research whereas with many RRT variations, a significant proportion of respondents are simply instructed to ignore the research question and just say 'yes' or 'no'. Owing to this characteristic, NRMs can also be more efficient with comparable or even increased privacy protection levels.

Alternative approaches have been progressively developed which preclude the need for the randomising device. These include an item count method [22], later termed the 'item count technique' [19] and later the 'unmatched count technique' [23]. In a similar concept to the unrelated question (UQ) method [16,24], item counts (IC) utilise a simple response task whilst embedding the sensitive question in a list of innocuous questions. In place of the randomising device the experimental group receives all questions with instruction and are asked to indicate only the number of affirmative answers. As a control sample is required to establish the population prevalence of the innocuous questions, respondents are randomly assigned to one of two groups (experimental and control), where the control sample receives the identical list of questions minus the sensitive question. The mean number of 'yes' responses are compared between the two groups. Assuming that the innocuous behaviour is equally manifest in both groups, the difference between the observed proportion of 'yes' answers must be due to the presence of the sensitive question in one of the groups and not the other.

Using prior knowledge of the population prevalence for an innocuous question, has led to the development of a number of competing techniques over the past five years. In these models, the innocuous question is outside the researcher's control, independent of the research question but the population prevalence is already established such as birth month or season, geographical location for the person or a family member. The Triangular Model (TM) and the Crosswise Model (CWM) use a combination of a sensitive and an innocuous question with known population prevalence [25]. The question and answer options are then placed in a 2 × 2 contingency table where two 'quadrants' relate to the innocuous questions are with known population prevalence (e.g. 3/12 and 9/12 if someone's birth month is used as the innocuous question). The other two quadrants represent the binomial response options to the sensitive question. In the TM respondents are asked to indicate whether they belong to the No-No quadrant or any of the other three quadrants (Yes-No, Yes-Yes or No-Yes). The CWM asks people to indicate whether they belong to any of the mixed categories (Yes-No and No-Yes) which only reveals that *one of the two* statements is true but *which* one remains hidden. Similarly, the Hidden Sensitivity (HS) model for two sensitive questions with binary outcomes using one quadrant such as season for birthday or geographic location (e.g. South/West/North/East, East/West side of a river or any criteria that creates meaningful and useable groups)

[26]. In this technique two response pathways are provided. Respondents are required to either answer truthfully or are forced to an option for the non-sensitive question (e.g. about birth date or place of living) based on their answers to the two sensitive behaviours. The drawback of this technique is that only those who belong to the category of not having a sensitive behaviour (0,0) are asked to answer the innocuous question honestly, whereas others (0,1; 1,0; 1,1) are forced to select an answer for the innocuous question based on their sensitive behaviour. Therefore, people admitting to a sensitive behaviour (or both) are protected by the true answers of those who do not have a sensitive behaviour to declare. The advantage of the HS model over the Triangular or Crosswise models is that HS allows two sensitive questions to be simultaneously investigated [27].

Other models such as the Unmatched Count Technique (UCT) [28] or the Cross-Based Method (CBM) and the Double Cross-based Method (DCBM) [29] work with unknown population prevalence. The common characteristic of these models is that an independent sample randomly drawn from the same population is required to establish the prevalence rate for the innocuous questions in order to estimate the prevalence rate for the sensitive question. The UCT [28] contains two parallel questionnaires with several innocuous questions but only one version of the questionnaire features the sensitive questions. The total number of endorsed answers is calculated for each version independently, and then compared. The difference between the two sample means indicates the proportion of the respondents who endorsed the sensitive question.

Currently, studies comparing the performance of the item count method to other NRM or RRT models, or direct self-reports, are inconclusive. Coutts and Jann [28] found that the UCT outperformed the RRT counterparts in assessing many sensitive behavioural domains. By contrast, Tsuchiya et al. [30], using a web-based survey, compared the item counts to direct self reports and concluded the item count technique yielded lower numbers of endorsed behaviour. However, Tsuchiya's [30] list of behaviours contained items to which over-reporting can reasonably be expected (e.g. donating blood), which might have skewed upwards the total numbers of reported behaviours in direct self-reports. Where differences were found between self-reports and item counts (using CBM and shoplifting) the differences were explained by the sample demographic. The largest difference was found among the middle-aged, domiciled in urban areas and highly-educated (e.g. in or completed tertiary education) female respondents [30].

Practical issues

Constraints of each approach were associated with whether or not the population prevalence used for the non-sensitive questions was known. When this information is not available, the research requires an independent sample of significant size to establish this, parallel to collecting a sample to answer the research question about some sensitive issue. Furthermore, the chosen probability that requires respondents to answer truthfully determines the proportion of the sample that is directly useable to answer the research question. Finally, the actual prevalence rate of the target behaviour also has an effect on the minimum required sample size.

Investigating the efficiency of the RRT, Lensvelt-Mulders et al. [17] compared five RRT methods and found the Forced Response method and a special from of the Unrelated Question design the most efficient requiring about 2.2 times the sample size required of a direct self-report method. Sample sizes for the Crosswise model were estimated for a number of combinations of power and population prevalence [31] where estimates for minimum required sample sizes ranged between 2.5 and 19.3 times the sample size required for direct questioning surveys. Based on these simulations, the Crosswise model's efficiency compared favourably to Warner's [32] model.

An alternative way to think about efficiency is to consider the proportion of the population sample solely used to provide an estimate of the population prevalence for the non-sensitive questions. This 'waste', which accompanies most models, is the acceptable efficiency cost of providing the added anonymity. The proportion of the sample inefficiency ranges between 25% and 75%, depending on the research design. Consequently, in order to achieve a sample size with sufficient statistical power for meaningful analysis there is a requirement for more extensive data collection than in a typical survey.

Aims

The recent change in legal status (in the UK) of the drug Mephedrone provided an opportunity to explore a novel approach to data collection on a sensitive issue. Mephedrone is a central nervous system stimulant that produces effects similar to amphetamines. It produces a euphoric effect, and has been reported to increase empathy, stimulation and mental clarity, but can lead to adverse effects such as nasal irritation, tachycardia and restlessness [33]. Although limiting in scope (i.e. we asked about the use of one specific drug), Mephedrone was a topical choice at the time of the study's conception as it had been reclassified as a Schedule 1 Class B drug on April 16th 2010 [34], making it unlawful to possess, produce, and/or distribute without licence and carrying a five year prison sentence for possession and up to 14 years for producing, selling or distributing. The ban generated considerable debate, with some expressing discontent about the hastened reaction and the

generic ban [35] along with a concern that the ban may not stop Mephedrone use, but could make the demand and supply clandestine, leading to unintended consequences from the addition of toxic excipients (through "cutting" or chemical by-products) and thus present an even greater danger to health [36]. In spite of the new legislation, internet retailers appear to have continued to sell products under different brand names that contain, albeit unlabelled, Mephedrone-like substances [37]. This case is a good illustration of the situation when the change in regulation could (and should) have been supported with at least an estimation of what proportion of the population uses Mephedrone and is at risk.

Recent inter-disciplinary approaches to estimating doping prevalence in sporting sub-populations has led to advances in estimation through improved efficiencies [38]. The current study aimed to develop and test a new research tool for use at the epidemiological scale. To achieve this aim, a fuzzy response model, Single Sample Count (SSC), was proposed.

Methods

The study utilised a mixed design questionnaire method with chemical analysis of hair samples collected from the questionnaire respondents. This approach has been successfully employed in research investigating social cognitive factors in prohibited performance enhancing and illicit drug use [7,8].

To establish validity and reliability, the SSC was compared to an established RRT model, the Forced Response (FR), estimating Mephedrone use in a three-month period preceding the data collection. Estimations from both SSC and FR were then corroborated with qualitative hair analysis. Ethical approval was obtained from the two HEIs' Research Ethics Committees. Data were collected in two sites: a rural area in north Wales (51.3% of the surveys; 92.8% of the hair samples) and a metropolitan area in England (48.7%) from 318 volunteers (mean age 22.69 ± 5.87, 59.1% male). Of the 153 hair samples, 95 (61.7%) were donated by males. The majority of the data (91.5% of the questionnaires and 92.2% of the hair samples) were collected in May-June 2010, capturing the period in which Mephedrone has become a controlled substance in the UK. The remaining samples were collected up to February 2011.

Measures

Along with the newly developed SSC, the questionnaire consisted of an established RRT, the Forced Response model [16], incorporated into the questionnaire in alternating order to mitigate any potential learning or priming effect, and always separated by four single questions evaluating the respondents' understandings and social projection of Mephedrone among a student population.

To establish prevalence of recent use, the sensitive question asked respondents to indicate whether they have used Mephedrone in the last three months. One-hundred and fifty-three (48.43%) of the questionnaire respondents were asked to provide a hair sample for Mephedrone analysis to determine the drug's use over approximately three months prior to the study survey.

Forced Response model

The FR method has been shown to be one of the most efficient designs [15,17] and was consequently considered suitable as a validation tool for the new method. This variation of the FR [39] requires a pair of ordinary D6 dice. Respondents were instructed to shake the die in an opaque container in order to hide the score from all other observers and then to answer the following question 'Have you used Mephedrone in the previous three months? (Yes/No)' according to the outcome. If the combined score from the two dice is

- 2 - 4 = ignore the question and tick the 'Yes'
- 11, 12 = ignore the question and tick 'No'
- 5-10 = answer the question truthfully by ticking either 'Yes' or 'No'

As for scores 5-10 there are more variations (27/36) for suitable dice outcome than for scores 2-4 (6/36) or 11 and 12 (3/36), theoretically 75% of the respondents were instructed, by chance, to answer the target research question honestly.

Additional questions

In addition, three questions were included to gauge directly reported opinion, belief about health hazards and social projection. The questions were:

- In your opinion, should Mephedrone be a controlled substance? (Yes/No)
- What percentage of students in the UK do you think use Mephedrone (0% = nobody, 100% = everybody)? (Yes/No)
- On a scale of 1 (not harmful at all) to 10 (very harmful), how harmful do you think Mephedrone is for your health?

These questions were also used to establish that the two samples collected at different locations differed significantly.

Analyses

Statistical analyses

Prevalence rates for the last three months were estimated using model specific formulae (detailed below). Testing sample means against the pre-set value was performed using single sample t-tests. The 95%CI for the binomial distribution was calculated using the Wilson

interval. Simulations for establishing the required sample size for the SSC model were performed using varying levels of prevalence rates. Statistical analyses were performed using PASW 18.0, R and Minitab.

Hair analysis

Hair samples were screened for the presence of Mephedrone using a qualitative method developed in-house, Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). Sample preparation included enzymatic digestion to preserve the drug and liquid-liquid extraction as detailed below. All solvents/chemicals apart from Mephedrone and Mephedrone -d3 were of analytical or general purpose reagent grade and purchased from Sigma-Aldrich UK Ltd (Gillingham, Dorset, UK). Mephedrone and Mephedrone-d3 were purchased from LGC Ltd, (Teddington, Middlesex, UK). Mephedrone-d3, the triply deuterated form is used as a standard reference for mass spectrometric measurements.

Hair digestion Hair (50 mg) was cut into fine segments and Cleland's Reagent (100 mg) was added followed by the addition of the enzyme Proteinase K (15 mg). Internal standard Mephedrone-d3 (100 μL) with 5 ng total concentration was added to the mixture and finally incubated with Tris buffer (1 mL) for 2 hours at 37.5°C with constant stirring.

Liquid Liquid Extraction (LLE) The digested hair solution was then placed in a centrifuge tube for Liquid-liquid extraction with hexane (3 mL). The contents of the tube were mixed using a vortex mixer and centrifuged for 5 min at 1750 × g. The top layer was decanted using Pasteur pipettes and placed in a glass test tube. The extracted samples were dried completely with nitrogen gas and reconstituted with 100 μL acetonitrile.

Qualitative analysis Qualitative analysis was carried out using a Thermoscientific liquid chromatography - tandem mass spectrometry (LC-MS/MS) system (Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK). Three microlitres of reconstituted sample solution were injected into an Agilent SB-C18 column (Agilent Technologies UK Ltd, Wokingham, Berkshire, UK), (maintained at 45°C) for analysis. Acetonitrile (with 0.1% v/v formic acid) and water were used as mobile phase solvents. Total flow rate through the column was 200 μL/min. The LC mobile phase gradient composition is detailed in Table 1.

The mass spectrometer was operated in selective reaction monitoring (SRM) mode to confirm the presence of Mephedrone. One precursor > two product ion transitions for Mephedrone (m/z = 178.1 > 160.1, 145.1) and Mephedrone-d3 (m/z = 181.2 > 163.2, 148.2) were monitored for qualitative analysis. The retention times for Mephedrone and Mephedrone-d3 were found to be 1.68 and 1.92 minutes, respectively. The calibration curve of Mephedrone was found to be linear in the range 1 ng/mL to 80 ng/mL (Lower limit of detection 0.5 ng/mL). Qualitative analysis of 154 hair samples was carried out using this calibration curve. Blank (control) hair without any Mephedrone was analysed to detect any artefact peaks that might elute at the same retention time or have similar isobaric transitions and thus lead to false results. However, no such interferences were observed. Thus, retention time and the most abundant SRM transitions were used to qualitatively determine the presence of Mephedrone.

Sampling

Respondents completed either version of the question in randomly allocated order, separated by three questions soliciting responses to social projection and opinions to the target drug. Participants were recruited at universities and social spaces such as clubs and sport grounds outside the higher education institutions in the UK.

Respondents were approached by a data collector (two in total, one in each study region). The participation was voluntary. Participants who provided hair samples received a small monetary compensation (value of £5) for any inconvenience incurred in completing the survey. A hair sample was requested from each respondent upon completion of the questionnaire survey. Approximately half of the respondents provided usable hair samples. The exclusion criteria included treated (e.g. dyed or permed) or too short hair (less than 3 cm). Over 80% of the hair samples were dark in colour. The different sample sizes are owing to 70 volunteers receiving a 4-question (four innocuous questions only) version for the Single Sample Count (data not shown).

Table 1 LC-MS Methods for Mephedrone-d3

LC run time (min)	Acetonitrile in presence of (0.1% Formic acid)	Water (%)
0	60	40
3	100	0
4	100	0
5	60	40
10	60	40

Retention time (min)	Lower Limit of Detection (ng)	Flow rate (μL/min)	Injection volume (μL)	Column Temperature (°C)
1.92	0.5	200	3.0	45

Results

Results from the survey

Using the full dataset (n = 318), no gender*region interaction effect was observed in social projection ($F(1,310) = 1.547$, $p = 0.211$; partial $eta^2 = 0.004$) or in perceived harm ($F(1,308) = 1.242$, $p = 0.266$; partial $eta^2 = 0.005$). Participants in the metropolitan area gave significantly higher estimates for others using Mephedrone ($F(1,310) = 16.90$, $p < 0.001$) but no difference was evidenced by gender ($F(1,310) = 0.506$, $p = 0.478$; Cohen's $d = 0.100$). The main effect for gender and region in perceived harm was significant ($F(1,308) = 5.237$, $p = 0.023$; $F(1,308) = 5.000$, $p = 0.026$, respectively). The slight discrepancies in sample sizes are due to missing values. Means and standard deviations by area and gender are shown in Table 2. The opinion regarding the legal status of Mephedrone overwhelmingly favoured control (81.7%), independent of area (Fisher's Exact Test = 2.104, $p = 0.370$) but not of gender (Fisher's Exact Test = 7.731, $p = 0.011$), with the preference for non-control of Mephedrone being higher amongst males (21.8%), compared to 11.6% amongst females.

Higher estimation of prevalence by participants in the metropolitan area is likely to be due to them holding different descriptive norms arising from the person's social context. Declared drug use among the active population (16-59) in England and Wales is consistently around twice as high in males than females and higher prevalence rates have been documented for urban compared to rural areas in last year's usage; with a similar but slightly more ambiguous trend for the 16-24 age group [40-42]. Biased social projection is one of the most intriguing areas in social cognition research. On the one hand, it suggests that the repeatedly observed association between self-reported behaviour or personality characteristics is explained by an egocentric bias (i.e. finding comfort in false consensus) [43], which is in keeping with the Bayesian approach [11]. On the other hand, particularly regarding the chosen sensitive and/or transgressive behaviours, it is suggested that the distorted perception of what eventually leads to a behavioural choice is congruent with this perception [44,45]. Conversely, recent research provides evidence showing that the prediction of population prevalence relates to the behaviour or characteristics the respondents wish to project about themselves, but not the actual behaviour [46,47].

Table 2 Social projection (0: nobody - 100%: everybody) and perceived harm (1: not harmful at all - 10: very harmful)

		Area		
		Rural	Metropolitan	ALL
Social projection	Male	28.00 ± 23.690	35.51 ± 23.231	31.45 ± 23.717
	Female	26.56 ± 20.780	40.68 ± 22.898	33.79 ± 22.926
	ALL	27.45 ± 22.572	37.74 ± 23.155	
Health risk	Male	5.87 ± 2.415	6.71 ± 1.912	6.26 ± 2.139
	Female	6.73 ± 1.968	7.01 ± 2.303	6.87 ± 2.139
	ALL	6.20 ± 2.286	6.84 ± 2.083	

Age was significantly negatively related, with the prevalence estimate (Spearman's $r = -.150$, $p = 0.01$) suggesting that younger people consider Mephedrone to be more prevalent. This is in line with the notion that Mephedrone is a drug for the young [33]. The correlation between age and the belief that Mephedrone was harmful was positive and significant (Spearman's $r = .190$, $p = 0.001$). As regional differences were not significant, the data from the two collection sites was combined and treated as one unified sample for future analyses.

Estimation using the Forced Response model

Subsequent to completing the questionnaire, the prevalence rate for Mephedrone use, using the formula suggested by Tourengeau & Yan [6] was calculated as follows:

$$\widehat{p} = \frac{\lambda - \pi_1}{\pi_2}$$

where:

π_1 = probability that the respondent is forced to say 'yes'

π_2 = probability that the respondent is forced to answer a sensitive question honestly

λ = observed percent that responded 'yes'

From the dice instructions, we see that $\pi_1 = 1$ out of 6 and $\pi_2 = 3$ out of 4. There were 74 'yes' responses out of 318 total, thus

$$\widehat{p} = \frac{74/318 - 1/6}{3/4} = 0.0881$$

The estimated prevalence rate for Mephedrone is 8.81%. The variance and standard error of this estimator are calculated as:

$$Var(\widehat{p}) = \frac{\widehat{\lambda}(1-\widehat{\lambda})}{n(\pi_2)^2}$$

$$Var(\widehat{p}) = \frac{74/318(1-74/318)}{318(3/4)^2} = 0.000998$$

$$SE = \sqrt{0.000998} = 0.034159$$

A 95% CI for the prevalence rate of Mephedrone would be the estimated prevalence rate ± the product of the $z_{\alpha/2}$ value and the standard error: 1.96 × 0.034159 = 0.061925, yielding the 95% CI of 0.026175 and 0.150025. Thus, the prevalence rate as determined by the Forced Response model with a standard error of 0.034159 and a 95% confidence interval of (0.02611, 0.14999) is estimated to be between 2.6% and 15.0%.

Hair analysis

Among the available 154 hair samples, the presence of Mephedrone was found in six samples giving a 3.9% positive rate. As the quantity of substance potentially used and time of exposure is not known, it is plausible that the actual positive rate is higher than 3.9%. It is likely that the hair analysis would only capture 3 months preceding drug use and could not detect a single exposure, nor any use that might have taken place in the immediate two weeks preceding the sample collection during which the hair is still in the scalp. Thus this period is considered as a 'blind period' for hair analysis.

Combining these positive samples with known use from the questionnaire where respondents accidentally give away this information by either answering each question on the Single Sample Count/Unmatched list five or answered each question on the same individually, the prevalence rate rises to 5.7% (9/157). Two of the nine known positive cases overlap between analytical and questionnaire results.

The simplified SSC algorithm

The fuzzy response SSC model is a new method and uses known population prevalence to estimate the proportion of affirmative answers to the sensitive question. As such, it is a simplified and more economical version of the Unmatched List Count using only one (experimental) sample. In order to avoid the need for a control sample (which inevitably leads to 50% loss of the sample), we embedded the target sensitive question into a set of four questions with 50-50 probability and benchmarked the sum of the number of observed 'yes' responses against the expected sum of the number of 'yes' responses for the four questions.

The benchmark questions were:

- My birthday is in the first 6 months (January - June) of the year.
- My house number is an even number.
- The last digit of my phone number is even
- My mother's birthday falls between July and December

The probability of a 'yes' answer to each of the four questions is therefore 50%, the expected average (sum of the number of 'yes' responses divided by the total number of responses) is two. Any upward deviation from this benchmark figure is the estimated proportion of 'yes' answers to the target question.

The target research question was:

- I have taken Mephedrone at least once in the previous three months

Respondents were instructed to indicate only the total number of their affirmative answers to the five questions without revealing which ones.

Based on the nature of the four non-sensitive questions, it was assumed that the population distribution for each question follows a binomial distribution, thus the distribution of the total number of 'yes' responses for non-sensitive questions is B(4*k, 0.5) where k is the sample size. In other word, the probability of an honest 'yes' response to each of the four non-sensitive questions is 50%. Assuming that there are equal numbers of 'yes' and 'no' responses to each of these four non-sensitive questions, it is possible to calculate the expected value of responses for the baseline non-sensitive questions:

$$E[response] = 4 \times [0.5(1)] = 2$$

Thus, if the probability distributions are exactly the same for all non-sensitive questions individually (assumed to be 0.5 in this case), the mean response for the four non-sensitive questions is expected to equal two, thus obtaining a mean response value greater than two is the indication of the estimated prevalence rate for the sensitive question. The prevalence rate estimation is calculated as:

$$d = (\lambda/n) - 2$$

where d is the estimated population distribution of the 'yes' answers to the sensitive question, λ is the observed number of 'yes' answers; and n is the sample size. The observed probability distribution of the number of 'yes' answers is shown in Table 3.

The three-month prevalence rate and 95%CI for Mephedrone use, using the SSC method, was calculated as follows:

The observed number of 'yes' answers is derived from the sum of two random variables with distribution of B (4*237, 0.5) and B(237, d), where d is the population distribution of the sensitive key question and 237 was the number of respondents in the sample. The observed number of 'yes' answers in the sample was 469.

Whilst the distribution of the sum of these two random variables is unknown, we can make use of the normal approximation for a binomial distribution. A rule of thumb is that the normal approximation is applicable if

Table 3 Observed probability distribution of X = the number of 'yes' answers

X	Observed P(X)
0	0.063
1	0.270
2	0.376
3	0.215
4	0.068
5	0.008

$np > 5$ and $n^*(1-p) > 5$, $d > 0.021$ and $d < 0.979$, where n and p are the distribution of the two binomial parameters. The normal approximation is derived as mean = np and variance = $n^*p^*(1-p)$. Thus B(4*237, 0.5) is approximately the same as N(2*237, 237) and B(237, d) is N(237*d, 237*d*(1-d)). Since the maximum likelihood approximation of the mean of the normal distribution is the sample mean, 237*(d+2) = 469, hence d = -0.021097. Note that the estimated d is negative, since the observed number of 'yes' responses (469) is less than the expected number of 'yes' responses for the non-sensitive questions (474). This does *not* mean that the prevalence rate for Mephedrone is negative, only that the random fluctuations in the sample were too large and mask the expected upward bias in the number of observed 'yes' responses. We can nevertheless calculate the 95%CI for d, which is 469 ± $Z(0.95)^*\sqrt{(237^*(1+d^*(1-d)))}$, where $Z(0.95)$ = 1.959964. Thus 95%CI is d ± 0.12731334 = -0.021097 ± 0.12731334 = 0, 0.099634. Therefore the estimated prevalence rate for Mephedrone use is between 0 and 10.0%.

T-test statistics indicated that the mean score (1.9789, 95%CI 1.85, 2.11) obtained on the SSC did not differ significantly from 2, thus there was no evidence that the prevalence rate for Mephedrone use in the population would differ significantly from zero (t(236) = -0.3113, p = 0.7558, Cohen's d = 0.041). This non-significant test result can be explained by the relatively small sample size. Notably, the sample prevalence was estimated to be between 0 and 10%.

The above calculation holds if the probability distribution of answers to each baseline question is equal (e.g. 50/50 in all 4 cases), thus we can assume that the sum of the binomial distributions is also binomial. However, the sum of the binomials is not necessarily binomial if the probabilities vary among the questions. Therefore, in such cases the normal approximation is calculated individually for each question before the probabilities from the baseline questions are added together, as we know that the sum of the normal distributions also follows normal distribution.

SSC algorithm taking the divergence from the 50/50 distribution into consideration

In order to test whether the estimation from the simplified SSC algorithm differs significantly from the estimation that takes the observed likely distribution for the 4 innocuous questions into consideration, we calculated d in a two-step process.

Firstly, we assumed that the probabilities of the innocuous binomial variables are not the same, so we estimated the probability distribution for each baseline question independently. In order to calculate the probabilities of the 4 innocuous binomial questions, we used the following datasets. For distribution of house and phone numbers, we used 7,500,000 UK residential data (usable dataset for house numbers: n = 6,859,957 and for phone numbers: n = 6,895,960) purchased from a commercial provider, whereas for birthdays, we used anonym datasets from two UK universities (n = 495,870 and n = 11,157). For the subsequent analysis, we used the large UK university dataset (n = 495,870) for birthdays. Details are presented in Table 4.

House numbers (including apartment/flat number in the absence of house number) were split as 3,405,322 even (p = 0.4964057) and 3,454,635 (p = 0.5035943) odd numbers. 0.5 (t = -18.828, df = 6859956, p-value < 2.2e-16, 95% CI: 0.4960316, 0.4967799). Among the listed phone numbers, the last digit of the phone number was an even number in 3,429,497 cases (p = 0.4973197) with 3,466,463 last digits being an odd number (p = 0.5026803). The probability of a birthday falling on the first half of the year was p = 0.5004075 (247,447 cases) vs. 248,423 (p = 0.499016) birthdays registered for the second half of the year. Single sample t-test statistic testing H_0: p = 0.5 for the 4 innocuous questions are as follows.

1. *My birthday is in the first 6 months* (January - June) of the year (t = -1.386, df = 495869, p = 0.1657; with estimated probability of 0.4990159 (95% CI = 0.4976242, 0.5004075)
2. *My house number is an even number* (t = -18.6633, df = 6952970, p < 0.001; with estimated probability of 0.49646115 (95% CI = 0.4960895, 0.4968328)
3. *The last digit of my phone number is even* (t = -14.077, df = 6895959, p < 0.001); with estimated probability of 0.4973197 (95% CI = 0.496946, 0.4976929)
4. *My mother's birthday falls between July and December* (t = 1.386, df = 495869, p = 0.165); with estimated probability of 0.5009841 (95% CI: 0.4995925, 0.5023758)

Table 4 Birthday distributions

	Frequency count	Probability	Frequency count	Probability
Birthday on/in[a]				
odd/even days	245,269	0.509872	235,771	0.490128
first half (up to and including the 15th)/second half of the month	239,157	0.497167	241,883	0.502833
first half/second half of the year	232,666	0.483673	248,374	0.516327
odd/even numbered months	242,683	0.504497	238,357	0.495503
Birthday on/in[b]				
odd/even days	253,438	0.511098	242,432	0.488902
first half (up to and including the 15th)/second half of the month	247,927	0.499984	247,943	0.500016
first half/second half of the year	247,447	0.499016	248,423	0.500984
Odd/even numbered months	251,226	0.506637	244,644	0.493363
Birthday on/in[c]				
odd/even days	5,739	0.514386	5,418	0.4856144
first half (up to and including the 15th)/second half of the month	5,562	0.498521	5,595	0.501479
first half/second half of the year	5,606	0.502465	5,551	0.497535
Odd/even numbered months	5,731	0.513669	5,426	0.486331

[a]US life insurance application data (n = 481,040)
[b]UK university registration data (n = 495,870)
[c]UK university registration data (n = 11,157)

Therefore, we used these empirically derived probabilities to approximate normal distribution.

The number of 'yes' answers for the

1^{st} question is binomial, B(k, 0.4990159) → N (k*0.4990159, k*0.4990159*0.5009841)

2^{nd} question is binomial, B(k, 0.4964611) → N (k*0.4964611, k*0.4964611*0.5035389)

3^{rd} question is binomial, B(k, 0.4973197) → N (k*0.4973197, k*0.4973197*0.5026803)

4^{th} question is binomial, B(k, 0.5009841) → N (k*0.5009841, k*0.4990159*0.5009841)

Sensitive question is binomial, B(k, d) → N(k*d, k*d*(1-d))

Therefore, by adding these approximations together, the distribution of the 'yes' answers are

$$N\left(k^{*}\left(1.9937808+d\right),\ k^{*}\left(0.999978355+d^{*}\left(1-d\right)\right)\right)$$

The Mephedrone dataset contained 469 'yes' answers from 237 respondents, therefore k = 237, and 237*(1.9937808+d) = 469, thus d = -0.0148779. The 95%CIs for the number of 'yes' answers with the above estimated mean and variance are439.0453 and 498.9547, thus d is between -0.1412 and 0.1115. Consequently, d (the estimated prevalence of Mephedrone use) is, indeed, between 0% and 11%, which is in keeping with the estimation we received using the simple algorithm with assumed p = 0.5 for 'yes' answers in all baseline non-sensitive questions. Therefore, applying the principles of Occam's razor, the simple algorithm should prevail.

Triangulating the SSC with the FR and hair analysis

The single most useful aspect of the hair analysis was to provide evidence that the sample prevalence of Mephedrone use was higher than zero. Figure 1 shows the combination of information available from the sample on Mephedrone use including an objective chemical analysis based on the presence of the drug in hair, accidental exposure via direct self-reports and two estimates representing two different indirect models. Combining these prevalence rates and estimates, we can conclude that the prevalence of Mephedrone use in the sample ranges between 5.7% and 15.0%. The two models yielded similar estimates with the FR up to 15% and the new SSC up to 10%. The close proximity of these estimates provides evidence that supports the validity of the new SSC model.

Implementation

Practical issues relating to the indirect estimation methods are i) the chance of exposure, ii) minimum and optimal sample sizes required to achieve a desirable power, iii) efficiency and iv) potential to eliminate or detect noncompliance. This section discusses these in the context of implementing the SSC approach.

Potential exposure

One notable drawback of the Single Sample Count model (as well as for the Unmatched List) is the scenario in which a respondent happens to have 'yes'

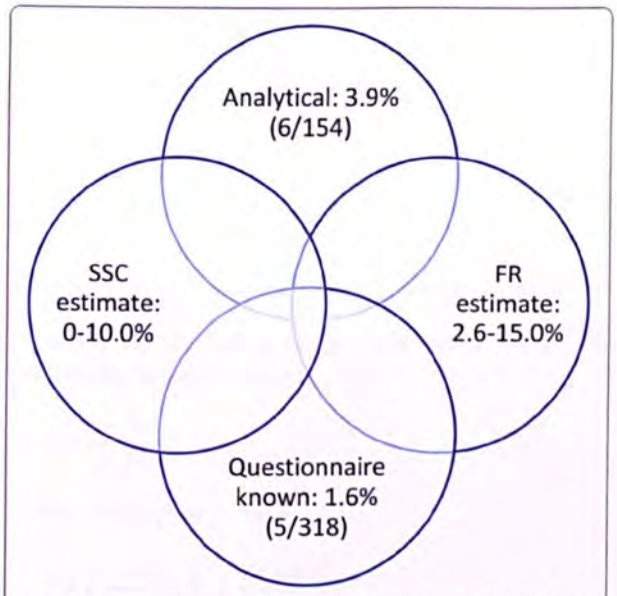


Figure 1 Comparison of the two estimation methods (FR and SSC) hair sample results and limited information on Mephedrone use directly available from self-reports.

answers to all innocuous questions and a 'yes' answers to the sensitive question. In this case, the respondent, if he/she answers truthfully, would reveal the information about the compromising behaviour. Note that the level of exposure in this situation becomes equivalent of the risk of exposure in an anonymous direct self-report.

This potential exposure situation can be mitigated by either increasing the number of innocuous questions (thus reducing the probability that such a scenario occurs (Table 5), or by offering an option of a new set of questions. Naturally, this latter option requires a bank of innocuous questions and only works in face-to-face interview settings or computer-assisted self-administration. Selecting the number of questions should take into consideration not only the probability but also the cognitive demand on respondents.

Table 5 The percentage of respondents potentially required to answer in a revealing way as the function of model design and prevalence rate of the sensitive question

Design	Innocuous	Sensitive[a]						
		5%	10%	15%	20%	30%	40%	50%
1 + 1	50.00	2.50	5.00	7.50	10.00	15.00	20.00	25.00
2 + 1	25.00	1.25	2.50	3.75	5.00	7.50	10.00	12.50
3 + 1	12.50	0.62	1.25	1.87	2.50	3.75	5.00	6.25
4 + 1	6.25	0.31	0.62	0.94	1.25	1.87	2.50	3.12
5 + 1	3.12	0.16	0.31	0.48	0.62	0.94	1.25	1.56
6 + 1	1.56	0.08	0.16	0.23	0.31	0.47	0.62	0.78
7 + 1	0.78	0.04	0.08	0.12	0.16	0.23	0.31	0.39
8 + 1	0.39	0.02	0.04	0.06	0.08	0.12	0.16	0.19

[a] for illustration we assume that the compromising behaviour is proportionally distributed

In cases where d is large, the potential exposure might be significantly high enough to consider alternative approaches. One example would be where answer options either combine 0 and 5 or allow respondent with answer '5' to select any answer options (0-4). Comparing the distribution of a hypothetical honest answer scenario with d = 0.2 prevalence rate for the sensitive question to the two proposed solutions using Kolmogorov-Smirnov's maximum divergence of the cumulative distribution function, no statistically meaningful preference was found between the two options (KS = 0.0125 for '0& 5'; and KS = 0.0125 for 'any option'). Using Root Mean Square (RMS) indicated a slight preference towards the 'any other' option (RMS = 0.0027 vs. RMS = 0.0035 for the '0 & 5'). Probabilities for the three scenarios are presented in Table 6. Simulations with 1 million data responses also showed very similar distributions (Figure 2). For simplicity, we assumed a 0.5 probability for each innocuous question. Given these results and taking practical issues into consideration, the combined 0 and 5 answer option is suggested for its relative simplicity. As one might expect, this solution to the '5-yes' problem affects the complexity of the computation to derive the estimated probability for the target sensitive question.

Required minimum sample size

Owing to the relatively small sample sizes, estimates using either the Forced Responses or the Single Sample Count method yielded negative values, making the lower bound of the 95%CI set to zero. The sample size required for the SSC model is chiefly determined by the sample size required to obtain a mean value for the four non-sensitive baseline questions to be as close as possible to two. Figure 3 shows that the bin width did not change significantly unless a significant increase in n is in place. Table 7 gives the exact values for the lower and upper 95%CI for selected sample sizes. From the practical point of view, the gain from increasing the sample by 500 is negligible compared to the potential cost of generating 500 samples. For comparison, 95%CIs are also calculated for 5 and 6 baseline questions, where the same logic applies as in the 4+1 model.

Power analysis

The first four (non-sensitive) questions will be distributed B(n = 4, p = 0.5) while the 5th question (sensitive question) will follow the Bernoulli distribution with a success probability of p, where d is the unknown prevalence rate. Let X = number of 'yes' answers out of the 5 questions, using the 4+1 SSC design.

Table 6 Probability of answer distributions if i) questions are honestly answered, ii) 0 and 5 answers are combined and iii) respondents are instructed to select any response option; *d* = probability of doping

	Questions are honestly answered	0 and 5 answers are combined	Any other response options are selected
0	1/16 - d/16	1/16	1/16 - d/20
1	1/4 - 3d/16	1/4 - 3d/16	1/4 - 7d/40
2	3/8 - d/8	3/8 - d/8	3/8 - 9d/80
3	1/4 + d/8	1/4 + d/8	1/4 - 11d/80
4	1/16 + 3d/16	1/16 + 3/16d	1/16 + d/5
5	d/16		

Testing H_0: $\mu = 2.0$ vs. H_1: $\mu > 2.0$ with $\alpha = 0.05$, the test statistic is calculated as:

$$t = \frac{\bar{x} - 2.0}{s/\sqrt{n}}$$

Solve this equation for n:

$$n = \left(\frac{t * s}{\bar{x} - 2.0}\right)^2 = \left(\frac{t * s}{\Delta}\right)^2$$

With $\alpha = 0.05$, the critical value t would be equal to 1.645. Substituting $t = 1.645$, n becomes a function of the effect size, Δ and standard deviation of X, s. We can calculate s from simulations using different prevalence levels. We will use $d = 0.05, 0.10, 0.15, \ldots 0.50$. This in turn will allow us to calculate the standard deviation of X, s.

From Table 8 below, it can be shown that when p = 0.15, s can estimated though 10,000 simulations to be 1.063. From this, recommended sample sizes can be developed. For example, to detect a significant effect size, $\Delta = 0.04$ with $\alpha = 0.05$ and $d = 0.10$, Table 8 shows that the simulated standard deviation would be s = 1.043 and the recommended sample size would be n = 1,839.

Mapping the information from Table 8 to Figure 3 and Table 7, it is easy to see the minimum required sample size is a direct function of the achieved bin width of the baseline questions. In practical terms, the required sample size ensures that the sample mean for the SSC is above the 95%CI for the baseline questions. The same logic applies for the models where the sensitive question is embedded in five or even the six

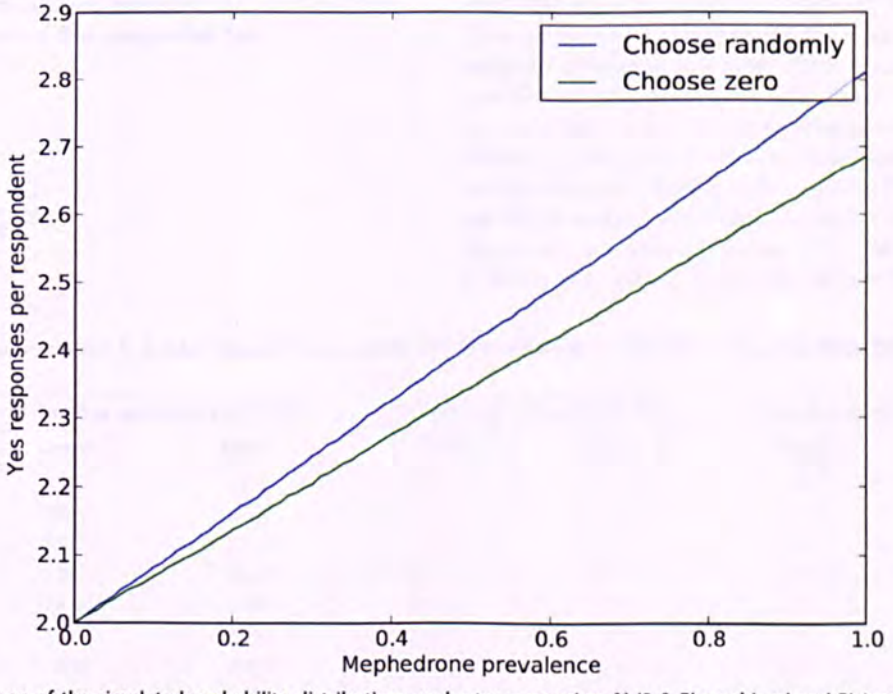


Figure 2 Comparison of the simulated probability distributions under two scenarios: A) '0 & 5' combined and B) 'any other option' to avoid exposure (n = 1,000,000).

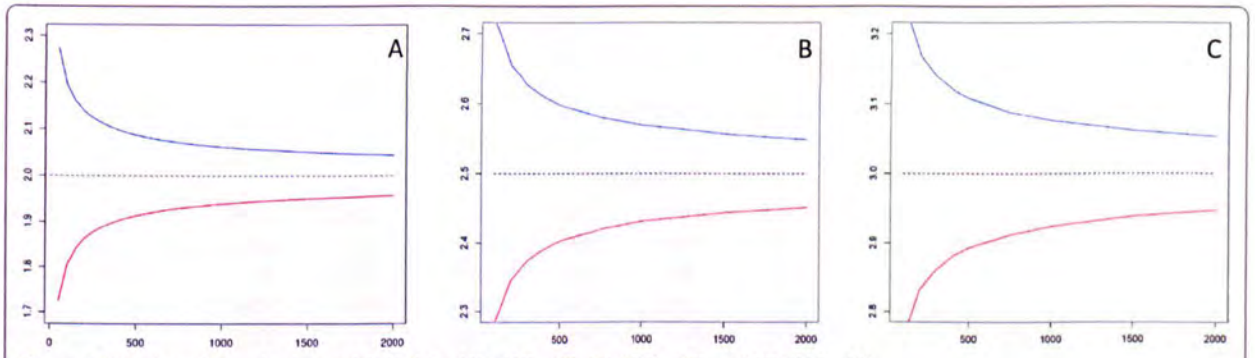


Figure 3 Function of sample size and 95%CI for (A) B(4*k, 05), (B) B(5*k, 05) and (C) B(6*k, 05)

innocuous questions. Table 9 displays the sample size values for the 5+1 SSC model.

Comparing the 4+1 model to the 5+1 model, the price that must be paid for the reduced chance of exposure is a slight increase in the required sample size. More importantly, however, this is in addition to the increased cognitive load on respondents which should be taken into consideration when designing the questionnaire.

In comparison, the minimum sample size for the FR is presented in Table 10 for SE {0.01, 0.02, 0.03, 0.04}, where we calculate a minimum sample size to form a confidence interval with varying levels of confidence. The conservative estimate of the minimum necessary sample size was calculated as follows:

π_1 = P(forced to say yes)

π_2 = P(forced to answer honestly)

λ observed percent that responded 'yes'

$$\widehat{p} = \frac{\lambda - \pi_1}{\pi_2}$$

$$Var(\widehat{p}) = \frac{\widehat{\lambda}(1-\widehat{\lambda})}{n(\pi_2)^2}$$

Solve for n:

$$n = \frac{\lambda(1-\lambda)}{Var(\widehat{p})(\pi_2)^2}$$

Maximize this equation by using $\lambda = 0.5$ and $\pi_2 = 3/4$. Setting $\lambda = 0.5$ creates the maximum variance possible thus result in a conservative estimate establishing the necessary sample size assuming the worst case scenario.

Using a 95% confidence interval, we get:

$$\widehat{p} \pm 1.96SE(\widehat{p})$$

Now use a standard error = {0.05, 0.04, 0.03, 0.02, 0.01}. Values are presented in Table 10.

For example, to obtain a 95% CI with width of 9.8 percentage points, n = 178 is required. To obtain a 95% CI with width of 1.96 percentage points, the required sample size rises to n = 4,445. This is comparable to the sample size required for the SSC to obtain a sufficiently narrow 95% CI for the innocuous questions (Table 7), which in turn, is very reassuring for the SSC as the FR model has been shown to be one of the most efficient model in terms of sample size with some 2.2 times of the direct question equivalent [17]. For example, Table 7 shows that with n = 400, the SE is 0.050 (n = 336 for

Table 7 95%CI intervals for 4, 5 and 6 baseline question models when n = 100, 200, 300, 400, 500, 750, 1500 and 2000

Sample size	4 baseline questions B(4*k, 05)		5 baseline questions B(5*k, 05)		6 baseline questions B(6*k, 05)	
	Lower	Upper	Lower	Upper	Lower	Upper
100	1.800	2.200	2.280	2.720	2.760	3.240
200	1.860	2.140	2.345	2.655	2.830	3.170
300	1.887	2.113	2.373	2.627	2.860	3.140
400	1.903	2.098	2.390	2.610	2.880	3.120
500	1.912	2.088	2.402	2.598	2.892	3.108
750	1.928	2.072	2.420	2.580	2.912	3.088
1000	1.938	2.062	2.431	2.569	2.924	3.076
1500	1.949	2.051	2.443	2.557	2.938	3.062
2000	1.956	2.044	2.451	2.549	2.947	3.054

Table 8 Minimum sample sizes as the function of difference (denoted by Δ) for the 4 baseline question SSC model

	Prevalence Rate									
	0.05	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.45	0.5
	Standard Deviation (s)									
	1.022	1.043	1.063	1.080	1.092	1.104	1.112	1.118	1.118	1.121
Effect Size (Δ)	Minimum Sample Size (n)									
0.01	28247	29421	30566	31563	32251	32975	33479	33799	33823	33993
0.02	7062	7355	7641	7891	8063	8244	8370	8450	8456	8498
0.03	3139	3269	3396	3507	3583	3664	3720	3755	3758	3777
0.04	1765	1839	1910	1973	2016	2061	2092	2112	2114	2125
0.05	1130	1177	1223	1263	1290	1319	1339	1352	1353	1360
0.1	282	294	306	316	323	330	335	338	338	340
0.15	126	131	136	140	143	147	149	150	150	151
0.2	71	74	76	79	81	82	84	84	85	85
0.25	45	47	49	51	52	53	54	54	54	54
0.3	31	33	34	35	36	37	37	38	38	38
0.35	23	24	25	26	26	27	27	28	28	28
0.4	18	18	19	20	20	21	21	21	21	21
0.45	14	15	15	16	16	16	17	17	17	17
0.5	11	12	12	13	13	13	13	14	14	14

this scenario in Table 10). Similarly, SSC n = 500, 1000 and 2000 give SE = 0.041, 0.032 and 0.023, respectively. These sample sizes map well onto those presented in Table 10 as n = 525, 934 and 2101, respectively. This congruence only holds for the 4+1 SSC design. As the number of the innocuous questions increases, so does the minimum required sample size. For example, reading from Table 7 (and in comparison to Table 10), we see that the sample required for SE ~ 0.03 is around 1,000 for the FR model and for the 4+1 SSC model, but reaching 1,500 for the 5+1 SSC with a further increase for the 6+1 SSC models. Thus the increase in sample size is the consequence of the increased security provided to respondents. Similarly, reducing the proportion in the FR model where honest answer is required results in increased security as well as in increased sample size. Notably, however, the large sample approximations of the proposed SSC method, along with other randomised response and non-random models, will provide reasonably close coverage for larger sample sizes, but may

Table 9 Minimum sample sizes as the function of difference (denoted by Δ) for the 5 baseline question SSC model

	Prevalence Rate									
	0.05	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.45	0.5
	Standard Deviation (s)									
	1.138	1.157	1.172	1.190	1.202	1.203	1.214	1.219	1.224	1.227
Effect Size (Δ)	Minimum Sample Size(n)									
0.01	35063	36243	37157	38294	39129	39155	39914	40224	40548	40713
0.02	8766	9061	9289	9574	9782	9789	9979	10056	10137	10178
0.03	3896	4027	4129	4255	4348	4351	4435	4469	4505	4524
0.04	2191	2265	2322	2393	2446	2447	2495	2514	2534	2545
0.05	1403	1450	1486	1532	1565	1566	1597	1609	1622	1629
0.1	351	362	372	383	391	392	399	402	405	407
0.15	156	161	165	170	174	174	177	179	180	181
0.2	88	91	93	96	98	98	100	101	101	102
0.25	56	58	59	61	63	63	64	64	65	65
0.3	39	40	41	43	43	44	44	45	45	45
0.35	29	30	30	31	32	32	33	33	33	33
0.4	22	23	23	24	24	24	25	25	25	25
0.45	17	18	18	19	19	19	20	20	20	20
0.5	14	14	15	15	16	16	16	16	16	16

Table 10 Minimum required sample size as a function of standard error (SE) with 95% confidence interval.

Standard error (SE)	Percentage points (1.96SE)	Minimum n
(0.05)	±0.0980	178
(0.04)	±0.0784	278
(0.03)	±0.0588	494
(0.02)	±0.0392	1112
(0.01)	±0.0196	4445

deviate from 95%CI for smaller sample sizes because of the discrete nature of the events.

Efficiency

Unlike other RRT/NR models, the Singe Sample Count model uses every single response in the sample to estimate the prevalence rate for the sensitive question. As the population distribution is known *a priori*, there is no need to generate an independent sample from the same population to establish population prevalence. Thus the SSC model comes with no waste of any proportion of the sample. This aspect is unique among the RRT/NRM models.

Non-compliance

The key driver for improving the random response and non-random models has been the hope that such techniques will be able to eliminate socially desirable responses. Social desirability (SD) is a known confounding factor in self reported research design, stemming from either the research tool or the person but equally resulting in dishonest responses [48,49].

Contrary to this desire, overwhelming evidence demonstrates that RRT/NRM are not cheating free [50,51]. Böckenholt et al. [51] used two separate methods, namely the Forced response and Kuk's [52] rather complicated card colour naming technique. Both demonstrated that accounting for non-compliance bias doubled the estimated prevalence. This finding is in-line with a medication non-adherence study that showed that almost half of the respondents did not follow the questionnaire instructions thus considerably distorting the prevalence rate without correcting for cheating [53]. This study used a variation of the forced response model linked to a rather low percentage when respondents have to answer honestly. The instructions were that if the respondent's father's birthday occurred in January or February then a truthful answer was requested, with a forced 'yes' for all other months. Therefore only 16.7% (2/12) of the respondents were asked to answer the sensitive question.

Self-protective no saying (SPN) is a known pattern in which respondents say 'no' without considering the instructions or truth. Considerable effort has been made to estimate the effect of dishonesty or correct for such effects [54-60]. Triggers for noncompliance could be the forced 'yes' answers in situations when respondents do not identify themselves with the discriminating behaviour; or complicated instructions which respondents are unable or unwilling to follow [51,61].

At this stage, we do not have data to ascertain what proportion of the responses on the SSC might have been affected by dishonest answering. Nonetheless, SSC does not offer an obvious self-protective response option as respondents who wish to deceive in their answers may simply chose entering zero, or any other number that is less than their true response would be. The somewhat higher estimate received using the SSC compared to the FR suggests that the SSC might be less affected by self-protective responding. Qualitative feedback received during data collection supports this assumption. Upon prompting for feedback in one group, respondents felt that they are more protected under the SSC model because as they phrased it: they *"didn't really have to answer the sensitive question"*. This is, by design, was not the case in the FR model where depending on the outcome of the dice roll, 75% of the respondents were asked to answer the sensitive question.

Potential innocuous questions

The SSC method builds on the innocuous question where the population distribution is assumed to be approximately close to 50/50. Such questions could be related to the last digits of a phone number, possibly house numbers or postcodes (even though these may vary from country to country), as well as birthdays. Selection of the most appropriate question must be informed by the research design, taking the target sample characteristics into consideration. Below, we present statistics derived from worldwide empirical data (n = 1,379), a publicly available dataset on birthdays (n = 481,040) and birthday data extracted from a UK university database (n = 495,870) to assist this process.

Empirical data were collected via Amazon Mechanical Turk in May-June 2011, with the Human Intelligence Tasks (HITs) made accessible worldwide to those with at least a 80% HITs acceptance rate [62]. The majority of the information was provided by people in India (59.2%), followed by the USA (28.4%), Canada (1.5%), Pakistan (1.1%) and the UK (1.0%). The remaining 51 countries contributed to a total of 8.8%. House numbers were odd numbers in 50.8%, whereas the last digits of the phone numbers were odd numbers in 48.6% of all records. Our results showed that more people prefer odd numbers for a lucky number (65.6%). The day of the birthday being odd occurred in 51.10% of the sample.

The publicly available birthday dataset was collected by Roy Murphy based on insurance policy applications to a Life Insurance Company between 1981 and 1994 http://www.panix.com/~murphy/bday.html and over 500 thousand birthdays captured in the internal information management system of two UK universities. The overall distribution of the birthdays in all three available datasets is remarkably similar to another database containing over 135 million records http://anybirthday.com/.

Using Roy Murphy's insurance application data, the results suggest that the 'first half vs. second half of the month' appears to give the closest split to 50/50, followed by the 'odd/even numbered month'. The analysis of two UK university population datasets of 495,870 and 11,157 birthdays provided further evidence that in the large dataset 'first half vs. second half of the month' lead to a closest split to 50/50, with the next closest distribution to 50/50 was the 'first vs. second half of the year' with the smaller dataset (n = 11,157) showing the opposite positions for the top two places. Frequency counts and probability distributions for birthdays falling on odd vs. even days and months; first vs. second half or the month and years, independently, are reported in Table 4.

Discussion

The overarching advantage of both randomised response and non-random models is that they provide greater respondent anonymity protection as question responses cannot be traced to the individual. This anonymity also removes any ethical or legal obligation from the interviewer to act upon sensitive information disclosed to them as part of the research process.

Further advantages of the SSC method are:

- The model is simple to administer, offering a self-administration option without any sense of deception.
- The SSC model reduces the complexity in instructions and places low cognitive demands upon respondents.
- Unlike the FR model, SSC asks each respondent to answer, in a fuzzy way, the (sensitive) research question and hence improves the face validity of the research tool.
- Unlike other RRT/NR models, the SSC avoids a forced 'yes' response, which can be off-putting for people whose honest answer would normally be 'no' to the sensitive question. Also, respondents are not required to answer the sensitive question directly.
- In the SSC model, no obvious self protective strategy is present (e.g. self-protective 'no' saying), thus this approach can overcome the 'self protective no' bias.

The challenges with the SSC model arise from finding a suitable set of baseline questions where the population prevalence and distribution is known to be 50-50% and adequately addressing the chance of potential exposure. There is a small but existing chance that someone encounters a situation in which the answer would be revealing. This is not only a problem for the newly proposed Single Sample Count but also affects the classic Unmatched Count technique. The potential of exposure can be mitigated by either increasing the number of baseline questions to reduce the likelihood of having affirmative answers to all baseline questions or by offering different sets of baseline questions. This latter approach requires computerised administration or personal interviews.

This study would have further benefited from an increased sample size as confidence intervals were limited to the upper bounds. Further studies are required to improve the evidence base for testing the methodological validity and reliability. The sample size was confined by a number of specific criteria. The focus of the study was restricted to the use of a single substance (Mephedrone), which on the one hand held the advantage of requiring a single screening in hair, but on the other placed limits on the study for two reasons. Firstly, the population prevalence rate of illicit substance use was low because the study restricted itself to a specific substance. As the method for detecting Mephedrone in human hair is newly developed, it is not yet known what consumed quantity of the drug signals a positive analytical result, or how natural hair colour, sex or ethnicity, for instance, might affect the deposition of the drug in human hair. However, hair analysis may provide the ultimate gold standard for validating the SSC approach for substance use. If such is the case, then careful consideration must be accorded to the research design to ensure effective synergy between social, analytical and statistical approaches. It is important that the sensitive question considers the limitations imposed by the hair analysis. For example, some drugs deposit into hair with more ease than others and stay longer. Accidental or environmental exposure may be a contributing factor in explaining the presence of a given drug in hair samples. Hair analysis is normally not suitable to detect single or very recent (i.e. last two weeks) exposure - but if research remit requires knowledge of these aspects then urinalysis may be a viable alternative. The timeframe afforded by the selected biochemical analysis must be carefully matched in the question. Exploring these issues is beyond the scope of this paper.

Secondly, whilst the hair analysis component was useful to prove that the sample prevalence is larger than zero, its labour and costs implications placed limits on the sample size. We have compensated for this

limitation with simulations to calculate the required sample sizes. The SSC model should also be tested investigating other discriminating behaviours with differing expected prevalence rates. If dishonest response patterns are known, prevalence estimation could incorporate a statistical correction component to account for this bias. Further refinement of the SSC model could include two variations where the sensitive question is positively (e.g. 'I have used drugs' or 'I do take my medications') or negatively framed (e.g. 'I have never used drugs' or 'I do not take my medications') to test whether giving confirmation, albeit indirect, of the desired or undesired behaviour has an effect on the results.

The research design could also benefit in some cases from the inclusion of a priming task to investigate whether or not the indirect approach and the additional protection afforded by the fuzzy response mode itself generates the maximum achievable admission of the discriminating behaviour. Alternatively, lie detector Implicit Associations Tests (e.g. [63,64]) could be combined with the SSC models for contrasting and comparing prevalence rates obtained via different methods from the same sample.

Conclusion

The major advantage of the Single Sample Count method over other models such as the Forced Response model is rooted in its simplicity, equal face validity for each respondent, simple calculations and maximum use of the data. This elegantly simple, quick and cost effective method can be successfully employed in public health research aiming to establish the epidemiology of potentially compromising behaviours. Notwithstanding, this approach, akin to other randomised and non-random models, is suitable to establish group level prevalence.

Acknowledgements

The authors thank Iltaf Shah for his advice and assistance in conducting the hair analysis.

Author details

[1]School of Life Sciences, Kingston University, UK. [2]Department of Psychology, University of Sheffield, UK. [3]Department of Biological Physics, Eötvös Loránd University, Hungary. [4]School of Pharmacy and Chemistry, Kingston University, UK. [5]School of the Environment, Natural Resources and Geography, Bangor University, UK. [6]Applied Statistics and Research Methods, University of Northern Colorado, USA.

Authors' contributions

AP and DPN initiated the project. AP devised the study, contributed to analyzing the data and prepared the first draft of the manuscript. PC, HT and CA collected the data and contributed to the final draft of the manuscript. TN, MS and JS analysed the survey data and provided the additional statistical information. SH, ND, DPN and JB developed the method for and conducted the hair analysis; and prepared the relevant section of the manuscript. All authors have contributed to drafting the paper; read and approved the final version of the manuscript.

Conflicting interests

The authors declare that they have no competing interests.

Received: 8 May 2011 Accepted: 3 August 2011
Published: 3 August 2011

References

1. Pan W, Bai H: **A Multivariate Approach to a Meta-Analytic Review of the Effectiveness of the D.A.R.E. Program.** *Int J Environ Res Public Health* 2009, 6(1):267-277.
2. Snyder LB: **Health Communication Campaigns and their Impact on Behavior.** *J Nutr Educ Behav* 2007, 39(25):S32-S39.
3. Keller PA, Lehman DR: **Designing effective health communication: a meta-analysis.** *J Public Policy Mark* 2008, 27(2):1-26.
4. Merzel C, D'Afflitti J: **Reconsidering community-based health promotion: promise, performance, and potential.** *Am J Public Health* 2003, 93:557-574.
5. Robling M, Ingledew DK, Greene G, Sayers A, Shaw C, Sander L, Russell IT, Williams JG, Hood K: **Applying an extended theoretical framework for data collection mode to health services research.** *BMC Health Serv Res* 2010, 10:180.
6. Tourangeau R, Yan T: **Sensitive questions in surveys.** *Psychol Bull* 2007, 133:859-883.
7. Petróczi A, Aidman EV, Hussain I, Deshmukh N, Nepusz T, Uvacsek M, Tóth M, Barker J, Naughton DP: **Virtue or pretense? Looking behind self-declared innocence in doping.** *PLoS One* 2010, 5(5):e10457.
8. Petróczi A, Uvacsek M, Deshmukh N, Shah I, Nepusz T, Aidman EV, Tóth M, Barker J, Naughton DP: **Incongruence in doping related attitudes, beliefs and opinions in the context of discordant behavioural data: In which measure do we trust?** *PLoS One* 2011, 6(4):e18804.
9. Petróczi A, Nepusz T: **Methodological considerations regarding response bias effect in substance use research: is correlation between the measured variables sufficient?** *Subs Abuse Treat Prev Policy* 2011, 6:1.
10. Jones E, Sigall H: **The Bogus Pipeline: A new paradigm for measuring affect and attitude.** *Psychol Bull* 1971, 76(5):349-364.
11. Prelec D: **A Bayesian Truth Serum for subjective data.** *Science* 2004, 306:462-466.
12. Roese NJ, Jamieson DW: **Twenty years of bogus pipeline research: a critical review and meta-analysis.** *Psychol Bull* 1993, 114:363-375.
13. Barrage L, Lee MS: **A penny for your thoughts: inducing truth telling in stated preference elicitation.** *Economics Letters* 2010, 106:140-142.
14. Offerman T, Sonnemans J, van de Kuilen , Wakker PP: **Truth Serum for non-Bayesians: correcting proper scoring rules for risk attitudes.** *Rev Econ Studies* 2009, 76(4):1461-1489.
15. Lensvelt-Mulders GJLM, Hox JJ, van der Heijden PGM: **Meta-analysis of randomized response research. Thirty-five years of validation.** *Sociol Methods Res* 2005, 33:319-347.
16. Warner SL: **Randomized response: A survey technique for eliminating evasive answer bias.** *J Am Stat Assoc* 1965, 60:63-69.
17. Lensvelt-Mulders GJLM, Hox JJ, van der Heijden PGM, Maas CJM: **How to improve efficiency of randomised response designs.** *Qual Quant* 2005, 39:253-265.
18. Himmelfarb S: **The multi-item randomized response technique.** *Sociol Methods Res* 2008, 36:495-514.
19. Fox J-P, Wyrick C: **A mixed effects randomized item response model.** *J Educ Behav Stat* 2008, 33:389-414.
20. Droitcour J, Caspar RA, Hubbard ML, Parsely TL, Vicer W, Ezzati TM: **The item count technique as a method of indirect questioning: a review of its development and a case study application.** In *Measurement errors in surveys.* Edited by: Biemer P, Groves RM, Lyberg LE, Mathiowetz NA, Sudman S. New York: Wiley; 1991:185-210.
21. Ming T-T, Tian G-L, Tang M-L: **Sample surveys with sensitive questions: a nonrandomized response approach.** *Am Stat* 2009, 63(1):9-16.
22. Miller JD: **A new survey technique for studying deviant behavior.** *PhD thesis* The George Washington University; 1984.
23. Dalton DR, Wimbush JC, Daily CM: **Using the Unmatched List Technique (UCT) to estimate base rates for sensitive behavior.** *Pers Psychol* 1994, 47:817-828.

24. Horvitz DG, Shah BV, Simmons WR: **The unrelated question randomized response model.** *Social Stat Sect Proc Am Stat Assoc* 1967, 65-72.
25. Yu JW, Tian G-L, Tang M-L: **Two new models for survey sampling with sensitive characteristic: design and analysis.** *Metrika* 2008, **67**:251-263.
26. Tian G-L, Yu J-W, Tang M-L, Geng Z: **A new non-randomized model for analyzing sensitive questions with binary outcomes.** *Stat Med* 2007, **26**:4238-4252.
27. Tian G-L, ChuenYuen K, Tang M-L, Tan MT: **Bayesian non-randomized response models for surveys with sensitive questions.** *Stat and Interface* 2009, **2**:13-25.
28. Coutts E, Jann B: **Sensitive questions in online surveys: experimental results for the Randomized Response Technique (RRT) and the Unmatched Count Technique (UCT).** *Sociol Method Res* 2011, **40**:169-193.
29. Tsuchiya T: **Domain estimators for the item count technique.** *Surv Methodol* 2005, **31**:41-51.
30. Tsuchiya T, Hirai Y, Ono S: **A study of the properties of the item count technique.** *Public Opin Quart* 2007, **71**:253-272.
31. Tian G-L, Tang M-L, Liu Z, Ming Tan M, Tang N-S: **Sample size determination for the non-randomised triangular model for sensitive questions in a survey.** *Stat Methods Med Res* 2009.
32. Warner SL: **The linear randomized response model.** *J Am Stat Assoc* 1971, **66**:884-888.
33. Vardakou I, Pistos C, Spiliopoulou Ch: **Drugs for youth via Internet and the example of Mephedrone.** *Toxicol Lett* 2011, **201(3)**:191-195.
34. Home Office UK: **A change to the Misuse of Drugs Act 1971: Control of mephedrone and other cathinone derivatives.** *Circular (010/2010) Misuse of Drugs Act 1971 (amendment) order 2010 (S.I. 2010/1207): Misuse of Drugs (designation) (amendment) (England, Wales and Scotland) order 2010 (s.i. 2010/1143); Misuse of Drugs (amendment) (England, Wales and Scotland) regulations 2010 (S.I. 2010/1144)* .
35. Morris K: **UK places generic ban on Mephedrone drug family.** *Lancet* 2010, **375**:1333-1334.
36. Winstock A, Mitcheson L, Marsden J: **Mephedrone: still available and twice the price.** *Lancet* 2010, **376**:1537.
37. Brandt SD, Sumnall HR, Measham F, Cole J: **Analyses of second-generation 'legal highs' in the UK: Initial findings.** *Drug Test Anal* 2010, **2**:337-382.
38. Petroczi A, Naughton D: **Impact of multidisciplinary research on advancing anti-doping efforts.** *Int J Sport Policy* 2011, **3**:231-254.
39. Boruch R: **Assuring confidentiality of responses in educational research: A note on strategies.** *Am Sociologist* 1971, **6**:308-311.
40. **Drug Misuse Declared: Findings from the 2007/08 British Crime Survey. England and Wales.** Home Office Statistical Bulletin; 2008, ISSN 1358-510X.
41. **Drug Misuse Declared: Findings from the 2008/09 British Crime Survey. England and Wales.** Home Office Statistical Bulletin; 2009, ISSN 1358-510X.
42. **Drug Misuse Declared: Findings from the 2009/10 British Crime Survey. England and Wales.** Home Office Statistical Bulletin; 2010, ISSN 1358-510X.
43. Ross L, Greene D, House P: **The false consensus effect: An egocentric bias in social perception and attribution processes.** *J Experiment Soc Psychol* 1977, **13**:279-301.
44. Borsari B, Carey KB: **Descriptive and injunctive norms in college drinking: a meta-analytic integration.** *J Stud Alcohol* 2003, **64(3)**:331-341.
45. LaBrie JW, Grossbard JR, Hummer JF: **Normative misperceptions and marijuana use among male and female college athletes.** *J Appl Sport Psychol* 2009, **21(Supp 1)**:S77-S85.
46. Petróczi A, Aidman EV, Hussain I, Deshmukh N, Nepusz T, Uvacsek M, Tóth M, Barker J, Naughton DP: **Virtue or pretense? Looking behind self-declared innocence in doping.** *PLoS One* 2010, **5(5)**:e10457.
47. Petróczi A, Uvacsek M, Nepusz T, Deshmukh N, Shah I, Aidman EV, Barker J, Tóth M, Naughton DP: **Incongruence in doping related attitudes, beliefs and opinions in the context of discordant behavioural data: in which measure do we trust?** *PLoS One* 2011, **6(4)**:e18804.
48. McCrae RR, Costa PT: **Social desirability scales: More substance than style.** *J Consult Clin Psych* 1983, **51**:882-888.
49. Uziel L: **Rethinking social desirability scales: From impression management to interpersonally oriented self-control.** *Perspect Psychol Sci* 2010, **5(3)**:243-262.
50. Lensvelt-Mulders GJLM, Boeije HR: **Evaluating compliance with computer assisted randomized response technique: a qualitative study into the origins of lying and cheating.** *Comput Human Behav* 2007, **23**:591-608.
51. Böckenholt U, Barlas S, van der Heijden PGM: **Do randomized-response design eliminate response biases? An empirical study of non-compliance behavior.** *J Appl Econom* 2009, **24**:377-392.
52. Kuk AYC: **Asking sensitive questions indirectly.** *Biometrika* 1990, **77**:346-438.
53. Ostapczuk M, Musch J, Moshagen M: **Improving self-report measures of medication non-adherence using a cheating detection extension of the randomized-response-technique.** *Stat Methods Med Res* 2010.
54. Clark SJ, Desharnais RA: **Honest answers to embarrassing questions: detecting cheating in the randomized response model.** *Psychol Methods* 1998, **3**:160-168.
55. Böckenholt U, van der Heijden PGM: **Item randomized-response models for measuring noncompliance: risk-return perceptions, social influences and self-protective responses.** *Psychometrika* 2007, **72**:245-262.
56. van den Hout A, Böckenholt U, van der Heijden PGM: **Estimating the prevalence of sensitive behavior and cheating with dual design for direct questioning and randomized response.** *Appl Stat* 2010, **59**:723-736.
57. Cruyff MJLF, van den Hout A, van der Heijden PGM, Böckenholt U: **Log-linear randomized-response models taking self-protective response behavior into account.** *Sociol Method Res* 2007, **36**:266-282.
58. Cruyff MJLF, Böckenholt U, van den Hout A, van der Heijden PGM: **Accounting for self-protective responses in randomized response data from a social security survey using the zero-inflated Poisson model.** *Ann Appl Stat* 2008, **2**:136-331.
59. Frank LE, van den Hout A, van der Heijden PGM: **Repeated cross-sectional randomized response data: Taking design change and self-protective responses into account.** *Methodology* 2009, **5**:145-152.
60. van den Hout A, Klugkist I: **Accounting for non-compliance in the analysis of randomized response data.** *Aust N Z J Stat* 2009, **51**:353-372.
61. Boeije H, Lensvelt-Mulders G: **Honest by chance: a qualitative interview study to clarify respondents' (non-)compliance with computer assisted randomized response.** *Bull Methodol Sociolog* 2002, **75**:24-39.
62. Buhrmester M, Kwang T, Gosling SD: **Amazon's mechanical Turk. A new source of inexpensive, yet high-quality, data?** *Persp Psychol Sci* 2011, **6(1)**:3-5.
63. Sartori G, Agosta S, Zogmaister C, Ferrara SD, Castiello U: **How to accurately detect autobiographical events.** *Psychol Sci* 2008, **19**:772-780.
64. Gregg AP: **When vying reveals lying: the Timed Antagonistic Response Alethiometer.** *Appl Cognitive Psych* 2007, **21**:621-647.

doi:10.1186/1747-597X-6-20
Cite this article as: Petróczi *et al.*: New non-randomised model to assess the prevalence of discriminating behaviour: a pilot study on mephedrone. *Substance Abuse Treatment, Prevention, and Policy* 2011 **6**:20.