Forensic and Clinical Toxicology Studies Focusing on Drug Analysis in Hair and Other Biological Matrices

Thesis Submitted in Accordance with the Requirements of the University of London, Queen Mary for the Degree of Doctor of Philosophy

By

Jaber Al Jaber

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Clinical Pharmacology, WHRI

Dedication

To my father who passed away 32 years ago

To my mother

To my disabled brother

To my wife and my children

To all the kind people I have met in my life

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Abstract

Clinical and forensic toxicology analysts rely heavily in their daily tests on the analysis of the conventional samples (blood and urine). However, these specimens are limited in the time scale they reflect with regard to drug intake history and also in terms of drug stability within the matrices. Alternative matrices such as hair, oral fluids and dried blood spots (DBS) provide new horizons and new opportunities. Drugs incorporated within hair are very stable. Hair also provides a very long detection window, for at least one year, if not a lot longer. Oral fluids on the other hand are non-intrusive, easy to collect and much cleaner sample matrix than blood or urine. DBS also offer great drug stability, are easy to collect, faster to analyse and suitable for automated analysis. However, a number of studies are needed to assess the limits of these alternative samples in terms of the correlation of their results with the results of conventional samples and with regard to drug stability. Such studies will enable a more reliable and confident interpretation of results obtained from these matrices especially for medico-legal purposes.

The main aims of this research were: to develop and validate analytical methods for detection and quantitation of drugs of use and abuse in hair, oral fluids, blood and DBS samples, to investigate the correlation between dose and drug concentration in hair, blood and oral fluids after controlled chronic drug administration, to investigate the stability of anti-psychotic drugs in DBS (from patients) stored under different conditions and the effect of addition of preservative, and to investigate the alcohol intake prevalence among Kuwaiti drug addicts and correlate these results with self-reported intake.

As the majority of drugs were basic, an extraction method based on methanolic incubation was developed for detection of basic/weak basic drugs in hair. It was compared to alkaline digestion (with NaOH) followed by liquid-liquid extraction (LLE). Detection was achieved by LC-MS/MS (Sciex2000) after separation on a C18 column. When applying both methods on positive authentic hair samples the results showed that the methanolic method was capable of extracting most basic drugs in hair but only partially, while the alkaline digestion method was found to degrade

some unstable drugs like sulpiride, but was capable of fully extracting the alkaline stable drugs such as quetiapine.

After development and validation of the LLE-LC-MS(Exactive) method for the analysis of anti-psychotics in blood, oral fluids and hair, an investigation was carried out on the correlation pattern between trough concentrations in those three matrices. The most significant correlation coefficients (r) found were those between blood and hair concentrations, procyclidine r=0.83 (18 subjects p=<0.001), risperidone r=0.96 (14 subjects p=<0.001), haloperidol r=0.90 (10 subjects p=<0.001), OH-risperidone r=0.24 (13 subjects p=>0.44), quetiapine r=0.28 (14 subjects p=>0.33) and chlorprothixene r=0.32 (13 subjects p=>0.32). Among the interesting results was the strong correlation found between drugs half-lives and the mean ratio of hair concentration/dose (r=0.96, p=<0.003).

The stability of anti-pyschotics in DBS from patients' samples was assessed by storing them at four different temperatures (25, 4, -20 and -80°C) with and without prior impregnation of the DBS cards with sodium fluoride. After development and validation of the LLE-LC-MS method, samples were analysed at days 0, 45, 90 and 180. Results showed good stability of all the compounds (procyclidine, quetiapine, risperidone, OH-risperidone, chlorprothixene and haloperidol) in all the different storage conditions and no significant increase or decrease in drug concentrations with sodium fluoride impregnation.

Finally, after trials with five different HPLC columns, two SPE cartridges, two LLE extraction procedures and two mass spectrometer instruments, a method was developed and validated for the detection and quantitation of alcohol's minor and specific metabolite in hair, ethyl glucuronide (EtG). The method has a limit of detection (LOD) of 3pg/mg and lower limit of quantitation (LLOQ) of 9pg/mg. This method was applied to 59 hair samples from patients at a general addiction centre and alcohol prevalence was investigated and its correlation with self-reported use was investigated.

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11-OH-THC	11-hydroxy-Δ9-tetrahydrocannabinol
5-HTOL	5-hydroxytryptophol
6-MAM	6-monoacetylmorphine
95% CI	95% confidence interval
γGT	γ-glutamyl-transferase
ACN	acetonitrile
AEME	anhydroecgonine methyl ester
ALT	alanine aminotransferase
amu	atomic mass unit
AST	aspartate aminotransferase
AUC	area under the curve
BE	benzoylecgonine
BEH	ethylene bridged hybrid particle
BDB	3,4-methylendioxyphenyl-2-butanamine
BTCA	benzothiazolecarboxylic acid
CDT	carbohydrate-deficient transferring
CE	cocaethylene
CV	coefficient of variation
DBS	dried blood spot
ELISA	enzyme-linked immunosorbent assay
EME	ecgonine methylester
ESI	electrospray ionisation
EtG	ethyl glucuronide
EtS	ethyl sulfate
FAEE	fatty acid ethyl esters
FDA	food and drug administration
GC	gas chromatography
GC-MS-EI	gas chromatography mass spectrometry electron impact ionization
GC-MS-NCI	gas chromatography mass spectrometry negative chemical ionization
GHB	gammahydroxybutrate

GMF	glass microfiber
HFBA	heptafluorobutyric anhydride
HPLC	high performance liquid chromatography
HS–SPME	headspace/solid phase microextraction
IS	internal standard
K	keratin
LC	liquid chromatography
LLE	liquid-liquid extraction
LOD	limit of detection
LogP	partition coefficient
LLOQ	lower limit of quantitation
MAF	methanol/acetonitrile/formic acid
MA	methamphetamine
MBDB	N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine
MCV	mean corpuscular volume
MDA	3,4-methylendioxyamfetamine
MDEA	3,4methylendioxyethylamfetamine
MDMA	3,4methylendioxymethamfetamine
ME	matrix effect
MeOH	methanol
MRM	multiple reaction monitoring
MS	mass spectrometry
MTBE	methyl-tert-butyl-ether
MW	molecular weight
NC	norcocaine
NCE	norcocaethylene
pHILIC	polymeric column for hydrophilic interaction liquid chromatography
psi	pound per square inch
PTCA	pyrroletricarboxylic acid
PTFE	polytetrafluroethylene
PVDF	polyvinylidenefluoride
QC	quality control

RBC	red blood cells
rpm	round per minute
RSD	relative standard deviation
SD	standard deviation
SDS	sodium dodecylsulfate
SoHT	hair testing society
SPDE	solid phase dynamic extraction
SPE	solid phase extraction
SRM	selected reaction monitoring
TDM	therapeutic drug monitoring
TFA	trifluoroacetic acid
THC	tetrahydrocannabinol
THC-COOH	11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid
TIC	total ion count
Tris	trisaminomethane
TTCA	Thiazoletricarboxylic
UGT	uridine diphosphate glucuronyl transferase
ZIC-HILIC	zwitterionic hydrophilic interaction liquid chromatography

1. Chapter 1 - Introduction

1.1. Introduction to hair

Hair is a unique continuously regenerating mini-organ covering the human and animal body. It serves many physiological and psychosocial functions. The average human displays an estimated number of 5 million hair follicles, of which 80,000 to 150,000 are located on the scalp (Krause, Foitzik 2006). These hairs are divided into three basic types: terminal, vellus and intermediate hair. Each one of these hair types has its own characteristics of length, colour, texture, shape and diameter. The terminal hair is usually coloured, long hair and has a wide cross sectional area which is mostly found in the scalp, beard & moustache, eyelashes, eyebrows, pubic hair and axilla. The vellus hair is the very small colourless hair that covers almost the whole human body, clearly seen on the bald scalp or forehead. The intermediate hair is that found on the hands and legs of adults that has an intermediate size and length and intermediate cross sectional area. However, the main focus of this research is mostly on the terminal scalp hair.

1.1.1. Hair structure

Hairs are mini shafts that are cylindrical in shape which grow from small pouches known as hair follicles. The morphology of the hair differs between individuals and within individuals. For instance, African hair structure is clearly different from Caucasian hair, both microscopically and in its natural appearance. It differs as well within the body regions of the same individual. It can exist as single strand or double or even group of strands emerging from the skin (Montagna, Ellis 1959). Basically this tiny structure consists of three main layers of cells as can be seen in Figure 1.

The outermost layer, the cuticle, serves as a protector to the inner layers and cells of the hair shaft. It consists of elongated single overlapping layers of melanin free cells pointing outward and upward like palm trunk shingles. These cells have a

diameter of about 45µm and thickness of about 0.7µm (Harkey 1993). Although the cuticle layer protects and preserves the components of the hair, it can be vulnerable against environmental, chemical or physical insults.

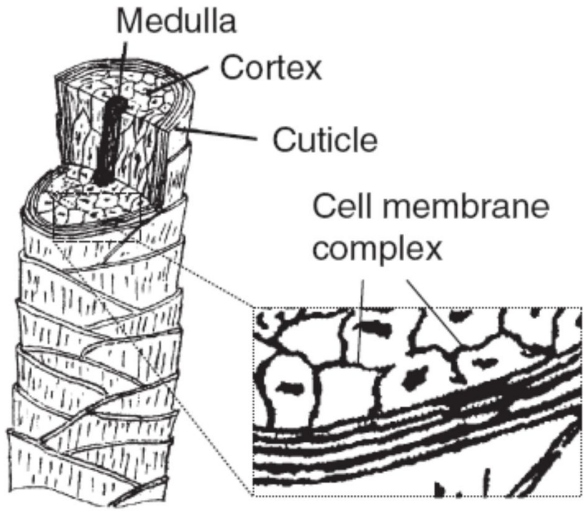


Figure 1: Cross section of the human hair shaft (Pragst, Balikova 2006)

The middle and the bulk layer is the cortex where the melanin and the keratinized cells are located. The melanin granules are the source of colour and its intensity in the hair. It is divided into two main types: eumelanin and pheomelanin, which will be discussed further under separate headings. Within the cortex cells there are irregular-shaped airspaces of varying sizes called cortical fusi filled with fluid and replaced by air as the hair grows and dries out (Harkey 1993).

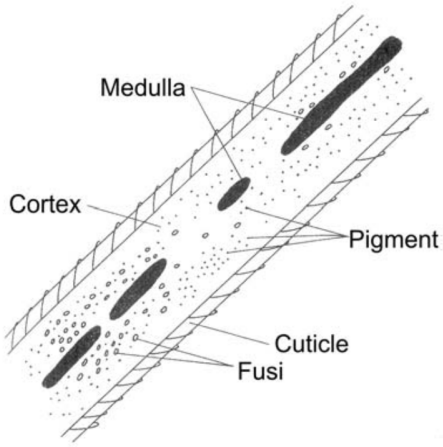


Figure 2: Longitudinal-section of hair shaft (Bisbing 2002)

As shown in Figure 2, the innermost layer of the hair shaft is the medulla. It is not always continuous along the shaft; it is sometimes discontinuous or absent. The medulla layer holds many keratinized cells. Intra and intercellular airspaces can exist as well in this layer (Montagna, Ellis 1959). It is also possible to find granules of melanin present in the medulla.

1.1.2. Hair composition

The main component of hair is the keratin proteins which produce the structure of the hair. These proteins are rich in sulphur to form the disulphide bonds between the amino acids which are required in shaping the hair. Proteins account for 65-95% of the total hair composition while the remaining constituents include water (15-35%), lipids (1-9%), melanin/pigment (0.1-5%), trace elements and minerals (0.25-0.95%) and small amounts of polysaccharides (Robbins 2002). These percentages vary between individuals.

1.1.3. Hair follicle

Hair follicles are rooted 3-4mm underneath the skin's surface within the dermis layer. This small sac is surrounded by apocrine and eccrine sweat and sebaceous glands that lubricate the hair shaft through secretary ducts in an upward direction just before the skin opening. These sweat and lipid secretions can contain body excreted toxins. The eccrine glands can be located on the surface of the whole body but higher in density on soles of feet, forehead and palms, whereas apocrine glands are mainly found in axillaries, eyelids, perineal region and in the external auditory canal. The hair follicle can be divided into three parts: innermost, middle and uppermost part. The innermost part is the place of hair cell synthesis, proliferation and differentiation, which is the lower segment in and around the bulb. The middle segment or the second part is the keratinization area where keratin gene expression, pigment incorporation, hair hardening, disulfide bonding, and dehydration are carried out. The last part is the zone of hair maturation, the uppermost segment of the hair follicle, where degradation of the inner root sheath and the dehydrated cornified hair cells shape up the shaft as seen in Figure 3 (Krause, Foitzik 2006, Harkey 1993, Pragst, Balikova 2006).

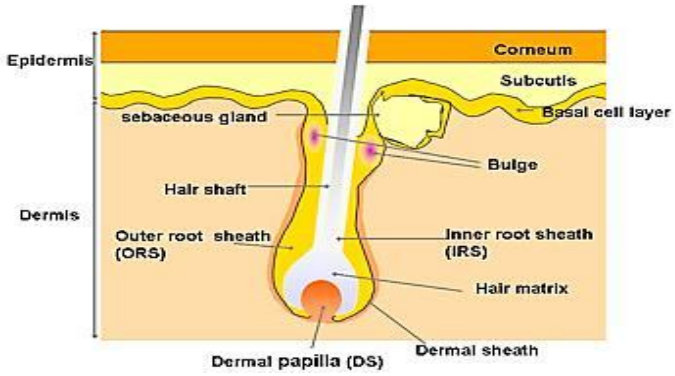


Figure 3: Hair follicle (Phoenixbio 2008)

There are three types of follicles according to Harkey (Harkey 1993) that generate different hair on the human skin. The first type are the nonsexual hair follicles, which produce the intermediate hair. These follicles are not affected by hormones and are

the same before and after puberty. The second type are the ambosexual hair follicles which are sensitive to hormones, such as pubic hair, axilla and temple hair of the scalp. Consequently these are altered after puberty. The third are the male hair follicles which play a role in the characteristic features of men which include hair in beard, moustache, abdomen, chest, nose, ears and some part of the scalp.

1.1.4. Hair growth cycle

The hair follicle cycle goes through three different phases.

1.1.4.1. Anagen

The anagen phase is the growth phase of the cycle when the follicle becomes metabolically active and the matrix cells are in production and the hair shaft is getting elongated. The keratinization and hair cell production are actively processing. Under normal circumstances the duration of this phase is from 2-4 years and usually depends on the body region with the growing rate varying as well. Drugs, metals and toxins which are transported by blood capillaries can be incorporated into hair during this phase.

1.1.4.2. Catagen

The catagen phase is the regression and dynamic transition phase between anagen and telogen (resting phase). Generally, this phase lasts only 4 to 6 weeks. The root of the hair becomes keratinized and starts to split from the hair follicle's bulb and starts forming what is known as the club hair. Apoptosis of epithelial cells in the bulb and outer root sheath will take place and a white node will form as shown in Figure 4. The follicle will start shrinking and getting shorter.

1.1.4.3. Telogen

The telogen phase is the resting phase which is typified as the club. In this phase the hair will became even shorter and easily plucked as it is completely separated from the bulb. This phase will last normally for 2 to 3 months, but it differs between body regions and can be longer with old individuals or certain conditions.

The anagen and telogen phases account for more than 95% of the hair growth process in the scalp at any one time. In the catagen phase the new growing hair may force the old one out and let it fall out naturally (Krause, Foitzik 2006, Harkey 1993, Nakahara 1999). The three phases is shown in Figure 4.

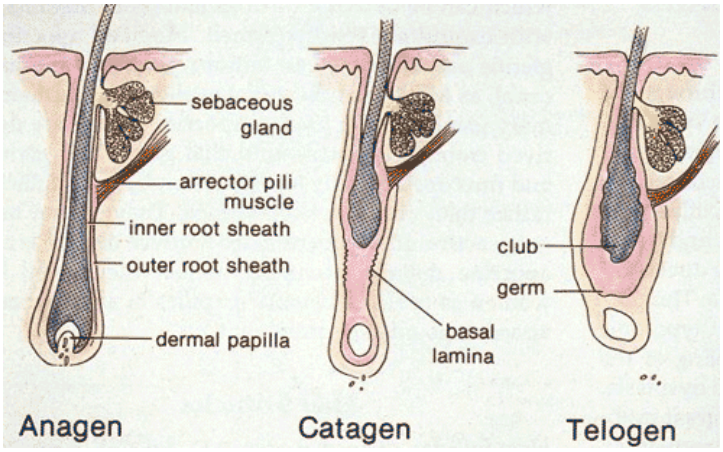


Figure 4: Phases of hair cycle (Furdon, Clark 2003)

1.1.5. Hair pigmentation

The hair pigment melanin (synthetic pathway shown in Figure 5) is formed in the cytoplasmic melanosomes of the melanocytes, melanin cells in the hair bulb. These organelles biosynthesize the melanin and condense it in a process called follicular melanogenesis. They then transfer it into the keratinocytes of the cortex and the medulla of the hair shaft. A number of enzymes, proteins, transporters and receptors are involved in this process which takes place during the anagen phase. One of the known enzymes is tyrosinase. It has been found that pigment dilution results

primarily from a reduction in tyrosinase activity within hair bulbar melanocytes (Cheze, Villain & Pepin 2004). Melanin is mostly found in the cortex with some in the medulla and seldom in the cuticle (Slominski et al. 2005).

Table 1: Average melanin content in hair (Uhl, Scheufler 2005)

Hair colour	Samples (n)	Total melanin (µg/mg hair)	Median (µg/mg hair)
Light blond	20	0.8 – 2.4	2
Medium blond	15	1.2 – 5.0	3
Reddish	5	3.8 – 5.7	4
Brown	15	3.3 – 11.5	5.7
Black	10	8.0 – 20.0	12

There are two types of melanin in human hair; eumelanin and pheomelanin. One study suggests that two other types exist, oxyeumelanin and oxypheomelanin, which both represent the oxidative products of the earlier types (Prota 2000). According to that study eumelanin is predominant in black hair and it gets lightened as the percentage of oxyeumelanin increase. So, blond hair has a large quantity of oxyeumelanin, while the pheomelanin and oxypheomelanin content in hair reflects the red/carroty colour and its degree (Kintz 2007a). The hair colour classification is shown in Tables 1 and 2.

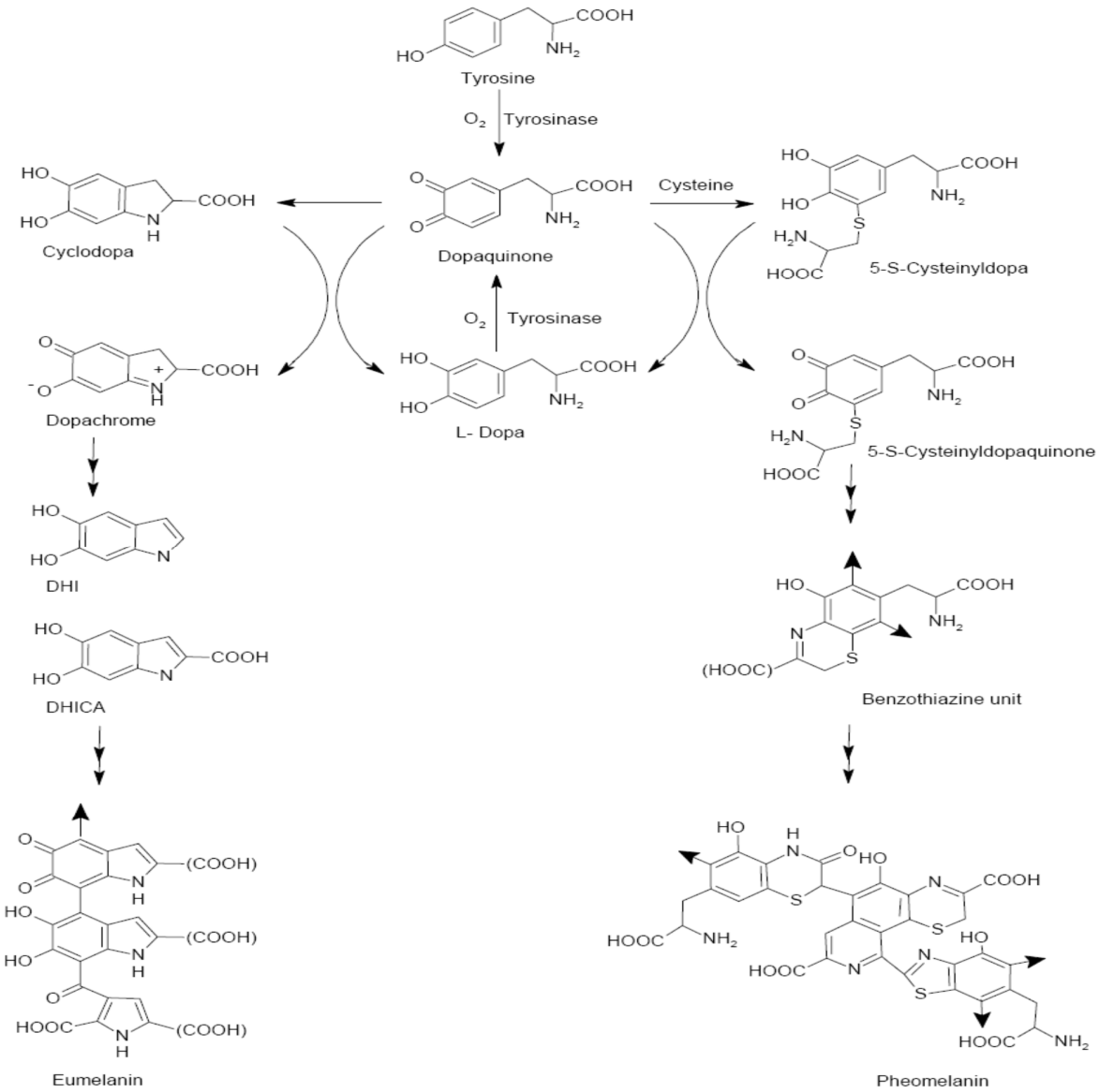


Figure 5: General outline of melanin formation (Dernroth 2009)

Table 2: Classification of human hair pigmentation according to melanin type (Kintz 2007a)

Pigmentation	Colour	PTCA*	BTCA*	TTCA*
Eumelanin	Black to dark brown	100-300	-	-
Oxyeumelanin	Brown to blond	50-80	-	≥200
Pheomelanin	Fiery/carroty red	-	1000-2500	-
Oxypheomelanin	Other red hues	-	-	100-300

*Pyrroletricarboxylic acid (PTCA), Benzothiazolecarboxylic acid (BTCA), Thiazoletricarboxylic (TTCA). Yields (ng/mg)

1.1.6. Hair ageing

As humans get older, their hair physical appearance changes. The dominantly clear characteristics of hair ageing are dryness of hair, thinning and loss of colour. Surprisingly, there are only few studies that have addressed hair ageing. From the hair growth perspective, Courtois et al observed the effect of ageing on 10 men during a period of 8-14 years and they found a noticeable decrease in the duration of hair growth and in the diameter of hair shafts with prolongation in the time between the loss of a hair in the telogen phase and the appearance of a substitute hair in the anagen phase (Courtois et al. 1995). All these signs contribute to overall progressive impoverishment in hair growth.

1.1.6.1. Pigmentary unit ageing

Follicular melanogenesis is thought to be as important regulator of hair growth. It was found that white beard hair grows at up to three times faster than the adjacent pigmented hair (Van Neste, Tobin 2004). The intact follicular pigmentary unit goes through cyclic reconstruction and maintenance throughout the first 10 hair cycles, which roughly equates to 40 years of age, with subsequently a genetically regulated exhaustion of the pigmentary potential of each individual hair follicular unit leading to the formation of white and grey hair (Van Neste, Tobin 2004). Melanocytic stem cells of the secondary hair germ involved in apoptosis might be an important factor for hair greying as well (Krause, Foitzik 2006).

1.1.7. Rate of hair growth

As this research mainly focuses on the scalp hair, the growth of the scalp hair in general is mosaic or asynchronous (Chamberlain, Dawber 2003). This means each hair is in a different phase of growth to the neighbouring hairs with approximately 85% to 90% of all scalp hairs being in the anagen phase (Krause, Foitzik 2006). Therefore for assessing the rate of hair growth a number of studies have been conducted. These studies concluded that scalp hair in general grows in the range of

0.6-1.4cm per month in normal healthy individuals from different ethnicities (Pecoraro, Astore 1990, Pragst et al. 1998). However, Potsch found that in alopecic individuals it may get to as low as 0.02 cm/month and at the same time it can get to 2.3cm/month in healthy individuals with the majority (82%) of the examined population falling in the range of 1-1.4 cm/month (Potsch 1996). In addition to that scalp hair growth was also found to slightly differ in its anatomical site as it been suggested that the posterior vertex region is the most constant in the scalp while the margins are the least (Chamberlain, Dawber 2003). The other hair sites in the body such as axillary and pubic hair have more telogen and catagen phases (~50%) which make their growth rate slower and fluctuating or heterogenic (Pragst, Balikova 2006).

1.2. Hair analysis

1.2.1. Chronology

In the fifties, hair analysis was used for the analysis of drugs when Goldblum et al first analysed barbiturates in guinea pig hair (Goldblum, Goldbaum & Piper 1954). This was followed in the sixties and seventies by metals and trace element analysis (Smith, Forshufvud & Wassen 1962, Cumbie, Jenkins 1974, Hambidge et al. 1976). Hair analysis was also proposed at that time as a means of assessing nutritional status but Sorenson et al soon disputed that by claiming difficulties in agreeing normal levels due to the variations among laboratories and possibilities of external contamination from cosmetic products, air or water (Sorenson et al. 1973). In the same decade Forrest et al investigated the passage of some drugs through the hair of mammals (Forrest et al. 1972). Detection of drugs of abuse in hair was initiated in late seventies by Baumgartner et al who investigated opiate addiction (Baumgartner et al. 1979). A number of studies followed during the eighties on detection of other drugs of abuse. In 1989, Sato et al suggested the possible use of hair analysis for therapeutic drug monitoring (Sato et al. 1989). From the nineties onward the

attention to hair analysis has increased considerably owing to the advances in detection sensitivity. Immunoassays, GC-MS and tandem mass spectrometry instruments (GC-MS/MS and LC-MS/MS) enabled the detection of even a single dose or single hair strand (Kintz 2007a).

1.2.2. Introduction

Hair analysis is becoming increasingly popular in forensic laboratories especially those dealing with drug facilitated crime cases. Hair differs from other traditional biological samples used for human toxicological analysis such as urine, blood, liver, or oral fluids because of its significantly longer detection window (months to years) allowing retrospective investigation and measurement of chemical/drug consumption. Hair analysis is now accepted in many of the developed countries for substance consumption related issues in a wide range of sectors; medico-legal sector, workplace, forensics, treatment monitoring, schools, research, insurance and driving licence.

Due to the fact that hair testing can provide evidence of drug facilitated crimes the analytical laboratories are assumed to be highly responsible in providing the most accurate results to the justice system. This accuracy requires implementation of a series of procedures that have been adequately validated and reviewed. Furthermore, these procedures need to be undertaken in a highly organized and precise manner to avoid any possible error. Extraction of drugs from hair is considered the most important step in the whole process. The drugs are enclosed tightly in the hair shaft and to a certain extent could be bound to proteins, melanin or even lipids of the cell membrane complex. The hair matrix type, structure of the drug, method and duration of extraction, and solvent used are all important factors affecting the final extraction yield (Musshoff, Madea 2007b). In this research the extraction strategies for the main illicit drug groups have been reviewed. In addition, the steps preceding extraction, sampling, storage, decontamination and digestion are also covered.

1.2.3. Mechanism of drug incorporation

The precise mechanism of drug incorporation into the hair shaft is still unclear. However, most scientists if not all of them in this field, agree on the possible routes by which drugs can be entrapped in hair (Kintz 2007a). The multi compartment model which has been suggested more than 15 years ago stated that ingested or injected drug can be incorporated into hair mainly through the blood stream feeding to newly growing hair cells in the dermal papilla part of the hair's root. The other routes are sweat and sebum secretions that lubricate the hair shaft and also diffusion from the surrounding tissue is possible (Henderson 1993). To elucidate the theory, Pragst et al mentioned that during the anagen phase of the hair growth a drug dose can be incorporated, within the hair follicle, to a drug-containing zone of approximately 2-5mm width from the base of the hair follicle, which appears at the skin surface 1-5 days after drug exposure and moves regularly away following the hair shaft's growth rate (Pragst et al. 1998). This process may be followed by sweat and sebum baths which possibly carry drugs that can be incorporated as well. In addition to these routes an external source of drug is probable through environmental contamination with drug smoke, powder or even solution. Nevertheless, these routes differ significantly in their relative contribution or importance among drugs and individuals. Some drugs distribute more in lipid compartments and are excreted more in sebum, while others favour aqueous compartments and so are excreted more in sweat. However, interpretively the most important one is the bloodstream route because it can help in predicting the time of drug intake when analyzing hair samples in segments. These segments can serve as pages of the hair's chronological diary note telling retrospectively about an individual's drug intake history.

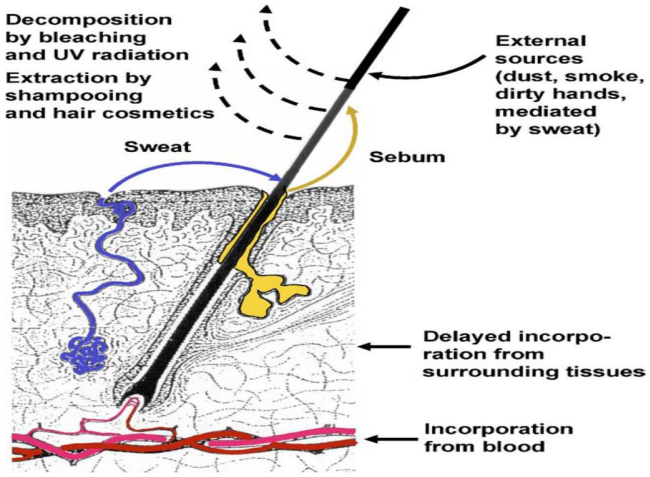


Figure 6: Drug incorporation mechanism (Pragst, Balikova 2006)

1.2.3.1. Blood stream incorporation

The assumed model for drug incorporation from blood is that drug enters the growing matrix hair cells by passive diffusion from the capillaries surrounding and feeding these cells. The area of the contact is estimated to be between 1.2 to 1.5 mm in length covering the main zones of the hair follicle root (zone of cell proliferation and differentiation, zone of keratinization and pigmentation, and zone of hardening and disulfide bonding) (Pragst, Balikova 2006). The matrix hair cells are exposed to the drug during the time when the drug is present in the blood, which is represented by drug area under the curve (AUC). So, theoretically the higher the drug AUC the longer exposure time and consequently the higher incorporation rate but practically this is not always the case, because lots of other elements are involved in the incorporation rate which will be discussed later. It is worth mentioning as well that blood AUC for any given drug has possibly poor inter-individual correlation with the dose according to Pragst (Pragst, Balikova 2006), so it will not be a surprise if the concentration of a given drug in the hair for the same given dose differs between individuals. Some studies showed that no correlation between blood concentration and hair concentration for drugs such as tricyclic antidepressants (Pragst et al. 1997) and methadone (Paterson et al. 2003). Kinz et al also could not find a clear correlation between the dose of heroin and the concentration of total

opiates in hair after controlled administration (Kintz et al. 1998). In contrast, Musshoff et al repeated the experiment by injecting the subjects with pharmaceutical heroin and found a correlation between the dose and the total opiate concentration in the hair (r=0.66) and as the analyte's plasma half-life increased the correlation was increased (Musshoff et al. 2005). Another controlled study supported these findings with codeine and cocaine (Scheidweiler et al. 2005). Another study also showed some correlation between plasma and hair concentrations for clozapine (r=0.542) (Cirimele et al. 2000).

1.2.4. Bias in hair analysis

The main sources of bias in hair analysis are cosmetic treatment and hair colour, which both can be related to race in many cases. Cosmetic treatment of hair, such as bleaching, dyeing, permanent waving and chemical straightening, can cause structural damage which will make it more penetrable. Consequently, incorporated drugs can be removed by for example normal hygiene practices or oppositely drug can penetrate and bind to hair from external sources like smokes for instance leading to false negative or positive results. Study showed that after such hair treatments, drug concentrations can decrease by 50-80% of their original concentration (Kintz 2004). This issue is of concern particularly for interpretation of recreational drug analysis. Cocaine and codeine were found to be 3 fold higher in concentration in brown hair strand compared to bleached ones of the same female subject (Joseph, Su & Cone 1996). On the other hand many studies showed that melanin content in hair play major role in drug incorporation particularly basic drugs. Therefore, there may be a bias in drug analysis with hair from different ethnicities. African American hair samples were found to contain significantly higher cocaine samples than hair from black/brown Caucasian hair (Kidwell, Lee & DeLauder 2000). Animal studies as well showed that pigmented hair incorporate more basic drugs than non-pigmented hair (Pötsch, Skopp & Moeller 1997).

1.2.5. Sampling

Most hair analysts agreed that all sampling should be done preferably by laboratory staff with a clearly written sampling protocol provided to the collectors. Along with sampling, a good documentation of all the relevant case history information (e.g. date and time of drug intake) and all the hair characteristics should be noted such as hair colour, type, ethnicity of the person and the length of hair sample after cutting. After sampling the proximal side of the hair tuft should be clearly identified and labelled. Ensuring alignment of hair at the root is very important. Date and site of collection on the head must be recorded as well as any cosmetic treatment history.

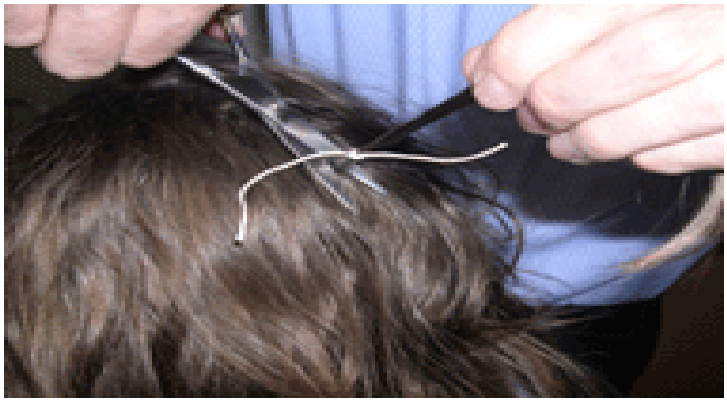


Figure 7: Sampling from the posterior region of the scalp (ChemTox 2009)

A recommendation of the hair testing society (SoHT 2004) states that a tuft with a thickness of a pencil or several small tufts with a thickness of straw should be taken from the occipital region (least variation in growth rate) of the scalp hair as close as possible from the skin. A fine-tipped pair of surgical scissors would be a good tool. Preferably two separate samples tied with string should be taken to allow analysis and conformation or reanalysis. Alternative hair, such as pubic and axillary hair, can be sampled, if head hair is not available, but they differ in the growth rate and are less prone to cosmetic exposure. Therefore, estimating the period of drug ingestion would be very difficult. When taking a postmortem sample it should be done before the autopsy to avoid any source of contamination like body fluids. Wet hair samples from autopsies stored in a fridge were found to have remarkably decreased concentrations of heroin and 6-monoacetylmorphine (6-MAM) due to hydrolysis after

sampling (Musshoff, Madea 2007a). The sampling and analysis process should not be carried out where the drugs being tested for are handled to protect samples from external contamination. A hair sampling kit is available commercially. In cases of criminal assaults it is necessary to wait for a few weeks (4 to 5) after the offence before sampling to ensure the drug has been incorporated in the hair (Kintz 2007a).

1.2.6. Storage

The first issue with storage is adequate labelling which is highly important to avoid a mix up with other samples. Most, if not all, scientists working in this field are recommending the following storing protocol (Pragst, Balikova 2006, Musshoff, Madea 2007a). Hair samples must be stored dry and light must be avoided. A study done by Skopp et al on the stability of cannabinoids in hair samples clearly showed significant reduction and degradation of their initial concentrations after 10 weeks of exposure to sunlight (Skopp, Potsch & Mauden 2000). A dry sample should be wrapped in aluminium foil, then put into a labelled paper envelope and stored at room temperature. By these measures most drugs are very stable and will be detected even years after sampling (Pragst, Balikova 2006). Plastic containers and bags should be avoided as they may extract lipophilic drugs or the softeners or the stabilizers may interact with hair samples if not protected with foil (Peters 2007).

1.2.7. Decontamination

This step of the hair analysis process is necessary to ensure that all the drugs detected in hair are those which have been ingested and not incorporated from environmental sources such as smoke, laboratory contamination or any other form of drug contact. Decontamination or washing will also help in removing hair cosmetic products, dust, sweat and sebum, therefore improving the analytical sensitivity by working with a cleaner sample. Washes should be kept for analysis, especially the first wash, to investigate if there was any external drug incorporation. One of the ways to assure consumption is the measurement of metabolite to parent drug concentration ratios, a metabolite that cannot be formed by hydrolysis.

In terms of solvent choice there is no general agreement on which solvent should be used but the SoHT guidelines recommend an initial organic solvent wash to remove oils, followed by aqueous washes (SoHT 2004). The most popular and widely used organic solvent is dichloromethane. Because it is a non-protic solvent it will not swell the hair and thus extract the drugs. Acetone is another non-protic solvent but is being used to a lesser degree or mostly as a last step drying off solvent. Protic solvents like methanol and phosphate buffers can swell the hair and encourage the extraction of the drugs (Kintz 2007a). One study found that three phosphate buffer washes removed up to 20% of the total drug (codeine and cocaine) present in hair (Ropero-Miller et al. 2000). Wilkins et al (Wilkins et al. 1995) found that a methylene chloride wash procedure reduced the measured THC concentration by up to 50%. Several publications reported the use of shampooing in postmortem samples as a first step in washing. This is obviously due to the fact that autopsy samples are usually less hygienic. Detergents like 0.1% sodium dodecylsulfate (SDS) have been reported to remove external methamfetamine (MA) contamination under ultrasonication, when control hair was soaked in an aqueous solution containing 10mg/ml of MA hydrochloride for 24 hours, but when the concentration doubled to 20mg/ml a few ng/mg was found in hair (Nakahara 1999). However, it seems difficult to recommend a particular washing procedure based on the conclusion of a study comparing different washing practices for morphine (Marsh et al. 1992). Paulsen et al concluded that the use of improper solvents can significantly affect the analytical results of hair analysis (Paulsen et al. 2001). Another study showed that the hair type can influence the decontamination process. It concluded that thick black hair is more resistant to decontamination than lighter hair and if it is dyed it will be more susceptible to external drug incorporation than lighter hair (Blank, Kidwell 1995) this could be due to the damages to hair structures with basic dyes. It worth mentioning as well that a formula proposed by Tsanaclis (Tsanaclis, Wicks 2008) suggested the use of analyte concentration ratio found in the washes (W) with that found in hair (H) and dividing it into three categories. If W/H is less than 0.1 or null, it may indicate genuine drug use, while if the W/H ratio is between 0.1 and 0.5 this indicates possible use or combined with external contamination, but a W/H ratio more than 0.5 is likely to indicate external contamination.

To sum up no single procedure could be recommended (Kintz 2007a) but to help in excluding possible external contamination from the environment four criteria should be considered: use of non-intensive multiple washing steps for not more than 15 minutes each with no temperature higher than 37°C, analysis of the washes after washing, identification of unambiguously endogenous metabolites with their ratios to the parent drug and finally use of cut-off levels.

Segmentation of hair usually follows the decontamination process and tentatively every centimetre of hair represents a month based on an average growth rate of 1cm/month.

1.2.8. Homogenization

The hair homogenization process seems to be controversial in the literature as some reports prefer cutting the hair into small snippets and others prefer grinding. Almost two thirds of the hair analysis published studies have used the cutting into snippets method and mostly with scissors, while roughly the remaining third used the pulverizing method by ball mills (Goulle et al. 1995), mortar and pestle (Hadidi et al. 2003) or beadbeaters (Joseph et al. 1997). However, the use of grinding is noticeably increasing in recent publications. Some other studies used a direct application of hair solubilising enzymes or digestive agents such as concentrated NaOH, pronase and proteinase K to hydrolyze hair proteins (Musshoff, Madea 2007b, Offidani, Strano Rossi & Chiarotti 1993). Incongruously, Pragst mentioned that grinding generally results in loss of sample material and does not improve the extraction process (Pragst, Balikova 2006), in contrast to a study done by Eser et al, which showed that pulverizing hair has a lower risk of missing drugs than using hair snippets (Eser et al. 1997). Also, in another hair test done on healthy volunteers, a higher amount of bromozepam in powdered hair than scissors cut hair into 1-2mm snippets was found (Cheze, Villain & Pepin 2004). Moreover, analysts working in minerals detection in hair reported the appropriateness of using powdered hair samples (Okamoto et al. 1985, Kamogawa et al. 2001). Scientists researching in the organic pollutants field also stated that the more powdered is the hair sample, the more efficient will be the subsequent extraction (Schramm 2008). In another option,

1M NaOH digestion for 1 h at 80°C offers high yields from alkaline-stable drugs such as cannabinoids, amfetamines, nicotine, some antidepressants and some neuroleptics, in addition to several other drugs, but generally cannot be used for the analysis of cocaine, heroin, 6-monoacetylmorphine (6MAM) and other ester compounds in hair (Nakahara 1999). For example cocaine will convert to benzoylecgonine under these alkaline conditions (Musshoff, Madea 2007b). The digestive enzymes work by breaking down the hydrogen and disulfide bonds within the hair and cause degradation and destruction of its fibre structure. These enzymes are proteinase K, protease E and VIII. The advantage of these enzymatic methods is to solubilise the hair sample without degradation of the unstable compounds like cocaine and heroin/6MAM (Nakahara 1999). The disadvantage of this method is that it is quite expensive. Normally the process consists of mixing the enzyme with 10-30mg aliquot of hair and incubating it at a relatively high temperature (40-60°C) for ~2 hours, then centrifugation and the supernatant is extracted by either solid phase extraction (SPE) or liquid-liquid extraction (LLE) (Nakahara 1999).

The sample size to be used for these homogenizing methods and the rest of the extraction process varies noticeably among laboratories, normally depending on the drug to be analyzed and the test protocol. For instance, if GHB or benzodiazepines are tested, the sample size should be at least 5 or 20mg per segment, respectively (Kintz 2007a).

1.2.9. Extraction

After decontamination and homogenization, comes the challenging step which is the release of the drug substance from the hair matrix. Drugs are incorporated and entrapped most likely within the hair's keratin, melanin, lipids and fibres and can form a stable complex that may be preserved for years. Therefore, a reliable extraction method should be implemented in order to release these compounds from hair. Ideally, the extraction conditions have to make possible the extraction of 100% of the compounds present without significant degradation (Kintz 2007a). Many extraction techniques have been published. The most appropriate and

recommended ones for each major forensically important drug groups are discussed in the following paragraphs.

1.2.9.1. Opiates

Musshoff & Madea (Musshoff, Madea 2007b) have reviewed and outlined the recent extraction procedures for opiates in the literature and they found three dominating methods; heat assisted hydrolysis by HCl acid overnight introduced by Kintz and Mangin (Kintz, Mangin 1995) followed by liquid-liquid extraction (LLE), enzymatic hydrolysis introduced by Moeller et al (Moeller, Fey & Wennig 1993) with β-glucuronidase/arylsulfatase followed by SPE and methanolic ultrasonication (Kauert, Rohrich 1996). The first two are enhanced with derivatization and both have the drawback that heroin and 6-acetylmorphine can be hydrolyzed to morphine. The third is the only method that has been suggested to identify heroin itself in hair samples and it could be improved if followed by clean-up procedures with LLE or SPE to reduce the noise (Musshoff et al. 2009). Ultrasonic methanolic incubation at 45°C for several hours was found to be good because methanol swelled the hair samples and facilitated the release of drugs as Eser et al reported (Eser et al. 1997). Balikova et al (Balikova, Habrdova 2003) also evaluated the opiate extraction methods and concluded that 1M NaOH or 0.1M HCl digestions produced the highest recoveries of morphine and codeine but decomposed 70–86% of the acetylated opiates, while the use of the neutral Soerensen buffer (pH7.4) incubation has lower recovery but did not change the ratios of labile metabolites and/or parent compounds in the original sample, with minor (7-18%) decomposition of acetylated opiates. Kronstrand et al (Kronstrand et al. 2004) proposed a simpler extraction method for opiates, cocaine and amfetamines by incubating the hair samples in the mobile phase (acetonitrile:methanol:20mM formate buffer, pH3 (10:10:80)) overnight at 37°C and it had a good recoveries (~84%) for morphine, codeine, 6-acetylmorphine and ethylmorphine.

1.2.9.2. Cocaine

There are many successful extraction protocols for the analysis of cocaine and its family, benzoylecgonine BE, ecgonine methylester EME, norcocaine NC, cocaethylene CE and norcocaethylene NCE in hair samples, along with the pyrolysis product of cocaine the anhydroecgonine methyl ester AEME which differentiates crack from cocaine (Musshoff, Madea 2007b, Kintz, Mangin 1995). Similar to opiates most of the methods are based on treatment with acid, enzymes, methanol or buffers, but not alkaline treatment as it hydrolyses cocaine to BE and EME. Most acid extractions (e.g. 0.01, 0.1M HCl, 0.05M sulfuric acid) are adequate but all can cause partial hydrolysis of cocaine (Musshoff, Madea 2007b). The method of enzymatic treatment showed good performance and high recoveries in general and the only two hold-ups are the cost and some large interfering peaks noticed throughout the whole run when tried with proteinase and pronase (Kintz 2007a, Musshoff, Madea 2007b). Extraction by phosphate buffer 0.1N at pH5 showed good recoveries (all >81%) for cocaine, BE and opiates (Romolo et al. 2003). Methanol has similar extractability to the buffers, which is relatively lower than the previous methods, and can extract all types of drugs. However the addition of trifluoroacetic acid TFA significantly improved the recoveries (Musshoff, Madea 2007b). Two separate studies tested the extractability of methanol/TFA (9:1) in comparison with methanol, 0.1M phosphate buffer and 0.1M HCl and both confirmed that methanol/TFA has the highest recoveries for cocaine and BE and high recoveries also for opiates (Romolo et al. 2003, Jurado et al. 2004). In terms of stability Romolo et al found almost no degradation of cocaine and 6-MAM with methanol/TFA (1.4%), while the other incubation media results were methanol or buffer (pH5-6)=1.3-4.2%, 0.01M HCl=3%, 0.1M HCl=12% and buffers pH>7=50%< (Romolo et al. 2003). For clean-up, SPE is considered to be the gold standard, and many such procedures are useful for the simultaneous determination of cocaine and its metabolites as well as opiates (Musshoff, Madea 2007b).

1.2.9.3. Amphetamines

The method previously mentioned (Kronstrand et al. 2004) for simultaneous detection of opiates, cocaine and amfetamines through incubation with the mobile phase has been shown to detect amfetamine (A), methamfetamine (MA), 3,4methylendioxymethamfetamine (MDMA) and 3,4-methylendioxyamfetamine (MDA) with high extraction recoveries (91-100%). However, the majority of published methods use alkaline (1-10M NaOH), acidic (0.25-5M HCl), or enzymatic digestion with some using methanol. Methanol/TFA seems to work well with amfetamines as it has high recoveries, very low detection and quantitation limits (LOD/LOQ) and the precision of intra- and inter-day assay as the relative standard deviation (RSD) were in the range 1.5–6.8% and 2.7–4.7%, respectively, for A, MA, MDA and MDMA (Nakamura et al. 2006). Kintz & Cirimele compared four different procedures; methanol sonication, acid (0.1M HCl), alkaline (1M NaOH) and β-glucuronidase/arylsulfatase hydrolysis (Kintz, Cirimele 1997). They found highest recoveries with the alkaline hydrolysis. A method proposed by Cirimele et al for detection of A, MA, MDMA, MDA, and MDEA together with N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine (MBDB) and its metabolite 3,4-methylendioxyphenyl-2-butanamine (BDB) in hair samples was found to have low LODs (0.02–0.05 ng/mg) (Musshoff, Madea 2007b). It consists of digestion with 1M NaOH followed by extraction with ethyl acetate and evaporation to dryness, in the presence of 2-propanol-HCl (99:1, v/v) and the target compounds were derivatized with HFBA (Musshoff, Madea 2007b).

The HS–SPME technique for determining semi-volatile amfetamines in hair samples has been shown to reduce time and effort and was introduced by Koide and co-workers (Koide et al. 1998). A modern SPDE technique with solventless extraction in a so-called inside-needle capillary absorption trap (a hollow needle with an internal coating of polydimethylsiloxane/charcoal) was found to have a higher extraction rate and faster operation, especially in combination with GC–MS/MS (Musshoff, Madea 2007b). Finally the vast majority of amfetamine extraction methods included clean up and derivatization steps.

1.2.9.4. Cannabinoids

Cannabinoids testing in hair is considered a challenge because of the low incorporation rate of especially the metabolites (Musshoff, Madea 2007b). It has been found that 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) has an incorporation rate that is 3600-fold lower than cocaine (Nakahara, Takahashi & Kikura 1995). In addition cannabinoids are present in smoke and sweat and therefore there is a high possibility of contamination. The selection of an appropriate extraction and decontamination method is difficult (Uhl 2006). To indicate definite endogenous cannabinoids use a detection of one or both of the metabolites, 11-hydroxy-THC and THC-COOH, is essential to prevent false positives (Uhl 2006). The majority of the published extraction methods depend on dissolving the hair matrix in alkaline solutions (0.1-2M NaOH or 10M KOH) with some preferring enzymatic digestion and very few using methanolic ultrasonication (Kintz 2007a). The general step of the alkaline digestion for cannabinoids is incubating the samples with the alkali for about half an hour at >70°C, then extraction with n-hexane after adjusting the pH to 5±0.5 and some prefer the addition of SPE to clean up. Baptista et al compared the use of GC-MS-EI and GC-MS-NCI for detection of THC-COOH and found the latter to be more convenient with a minimum chance of false negatives (Baptista et al. 2002). They extracted the samples after β-glucuronidase/arylsulfatase hydrolysis for 2h at 40°C followed by a liquid-liquid extraction procedure (with chloroform/isopropyl alcohol, after alkalinization, and n-hexane/ethyl acetate, after acidification). A different method proposed by Uhl & Sachs consists of mixing methanol and 10M KOH (1:1) and adding it to the hair sample with ultrasonication and vortexing for 30 minutes at 70°C then adjusting the pH to 4-5 and cleaning up with SPE (Uhl, Sachs 2004). As a final point in this context Wilkins et al validated the alkaline digestion method (18h in 1N NaOH at 37°C) followed by LLE and detected the 11-OH-THC, THC-COOH and THC with intra-assay and inter-assay imprecision (2.1 to 11.2% and 4.4 to 13.0%) and limit of detection 10pg/mg for THC and THC-COOH and 100pg/mg for 11-OH-THC (Wilkins et al. 1995). However, the regulation proposed by the US federal regulations for the

detection of THC-COOH in hair requires a confirmatory detection level of 0.05pg/mg (Moore et al. 2006), which is fairly difficult.

1.2.9.5. Benzodiazepines

Hypnotic drugs such as benzodiazepines are at the top of the list for drug facilitated crimes. Their extraction from hair samples is mainly through incubation with phosphate buffers, methanol or digestive enzymes (Musshoff, Madea 2007b). Most reviews (Musshoff, Madea 2007b, Kintz 2007b, Villain, Kintz 2006) focus on the method proposed by Villain et al which is incubation in phosphate buffer (pH8.4) overnight followed by LLE with methylene chloride/diethylether (9:1) (Villain et al. 2005). This method was able to screen 14 benzodiazepines plus zolpidem and zaleplon with LOQ ranges 0.5-5pg/mg and extraction recovery ranges from 32 to 76% and imprecision <20%. With a different incubation medium, Laloup et al validated a new method capable of identifying 26 benzodiazepines plus zopiclone and zolpidem simultaneously (Laloup et al. 2005). The method works simply by incubating the pulverized samples with methanol at 45°C for 2 hours with orbital shaking followed by LLE with 1-chlorobutane/ammonium chloride buffer (4:1). The LOQ was between 0.5 and 10 pg/mg, precision (CV <20% in most cases) and extraction recoveries from 53.0% to 103.8%.

1.2.9.6. Alcohol

From the ethanol metabolites two markers have been suggested for the identification of alcohol consumption, fatty acid ethyl esters (FAEEs) and ethyl glucuronide (EtG). FAEEs are lipid soluble and incorporated into the hair from the sebum, so segmentation will be of no use because their incorporation does not associate with the growth rate of the hair and they are also found in the hair of strict teetotallers (Auwarter et al. 2001). Therefore generally they can be classified as co-indicators of general drinking behaviour if detected in abnormal levels. As a consequence EtG attracted attention because it is water soluble and incorporated from blood. Although only 0.02–0.06% of ingested alcohol is eliminated as EtG it

can still indicate alcohol abuse (Pragst, Yegles 2006). The general extraction method for EtG in hair is simply incubation in water preferably with ultrasonication. Jurado et al showed that this method was superior to methanol, methanol/H_2O or aqueous trifluoroacetic acid (Jurado et al. 2004).

1.2.9.7. Screening

There are numerous accounts of screening strategies published for analyzing different forensic drug groups, most of which are designed for screening by immunochemical, GC-MS and LC-MS/MS assays. As in this review, the interest is mainly in the extraction part of the process. Four of the recent, with the widest detection window protocols, are discussed below plus one extraction method for immunoscreening. Starting with the most recent one, Miller et al validated a quantitative method for the detection of 17 drugs (amfetamines, diazepam and its metabolites, cocaine and its metabolites and opiates) by LC-MS/MS with total extraction recoveries >76%, LOD were between 0.02 and 0.09ng/mg and imprecisions were generally <20% (Miller, Wylie & Oliver 2008). Their extraction method was based on incubation of hair samples in phosphate buffer (pH5) for 18 h at 45°C and then clean up with SPE. Phosphate buffer (pH5) was chosen because of the high stability of cocaine and 6-monoacetylmorphine in this medium. The second method was proposed by Hegstad et al for the detection of 22 drugs and metabolites (nicotine, cotinine, morphine, 6-MAM, codeine, A, MA, MDMA, cocaine, BE, zopiclone, zolpidem, carisoprodol, meprobamate, buprenorphine, methadone and 6 benzodiazepines) with validation results of <15% RSD for within- and between-assay, imprecision range from 16-24% and recoveries between 25-100% (Hegstad et al. 2008). The extraction was through incubating the samples with acetonitrile/25mM formic acid (5:95 v/v) in a water bath at 37°C for 18 h. Similarly, four years earlier Kronstrand et al introduced incubation in the mobile phase (acetonitrile:methanol:20mM formate buffer, pH3 (10:10:80)) at 37°C for 18 h but their method detected only 13 drugs from the 22 detected by Hegstad et al's method plus ethylmorphine (Kronstrand et al. 2004). Cordero & Paterson also proposed a simultaneous quantification method for opiates, amfetamines, cocainics, diazepam

and nordiazepam (14 drugs) with RSD results <25% (inter/intra-day), LOQ 0.1-0.2ng/mg sample (Cordero, Paterson 2007). They used 0.1M HCl incubation overnight at 50°C followed by SPE clean-up, but they adjusted the pH to 7±0.4 with phosphate buffer (0.1M, pH7) and potassium hydroxide (1M). Finally Tsanaclis & Wicks showed a two steps extraction method for ELISA screening and confirmation with GC-MS or GC-MS/MS (Tsanaclis, Wicks 2007). The method was mainly incubating the samples in methanol with sonication for ≥6 h. The methanol was then decanted into a clean test tube and then the hair sample was digested using sodium hydroxide and extracted with a mixture of chloroform:iso-propanol (9:1). The two solvent extracts were combined and after solvent evaporation the residues reconstituted in phosphate buffer pH7.2. The reconstituted sample extract was screened using an ELISA test. Positive samples were cleaned up with SPE and derivatized prior to GC-MS confirmation analysis. This method can be used for the detection of 28 drugs and metabolites (opiates, cocainics, amfetamines, cannabinoids, benzodiazepines and methadones).

1.2.10. Society of hair testing main guidelines

Please see appendix 8.3.

1.3. Aims and objectives

The application of alternative matrices in analytical toxicology is a novel field of study. The main aim of this research is to investigate the value of these matrices, hair, oral fluid and dried blood spots, with the following objectives, as described below:

- To develop a method to enable the detection and quantification of basic and weakly basic drugs in hair simultaneously based on a methanolic solution extraction technique and to compare its efficiency with an alkaline digestion technique followed by liquid-liquid extraction (LLE) through applying them on real hair samples.

- To investigate incorporation rate of some psychoactive drugs into hair and to look into the correlation pattern, if it exists between the daily dose, plateau in blood, plateau in oral fluids and plateau in hair. Also, to see if drug half-life has an effect on the incorporation.

- To develop and validate a method to enable the use of the dried blood spots (DBS) technique for the analysis and quantification of some of the common psychoactive drugs used in psychiatric clinics in Kuwait. Also, to investigate the stability of these drugs DBS stored for 6 months in freezers (-20 and -80°C), refrigerator (4°C) and at room temperature (25°C). Another objective was to see the effect of impregnating the cards with sodium fluoride on the stability of the drugs.

- To develop and validate a sensitive and specific method for ethyl glucuronide EtG detection and quantitation in hair based on comparing different extraction methods (LLE and SPE) and also different separation columns (hydrophilic interaction liquid chromatography HILIC and reversed phase column C18).

- To determine the alcohol prevalence or consumption behaviour among Kuwaiti addicts and to draw a picture of addiction trends starting with this alcohol focused study. Also, to analyse the data statistically to look for any patterns or correlations between alcohol consumption, age and other drugs of abuse.

- To compare the results with self-reports received from addicts and see if they are in agreement, in other words, to investigate if addicts tend to reveal their alcohol consumption or they try not to.

2. Chapter 2 - Analysis of basic drugs in human hair

2.1. Introduction

Drug analysis in hair has grabbed the attention of toxicology analysts and researchers in recent years. This is mainly because it has provided some ability of proving drug ingestion when conventional samples could not. Hair differs from other traditional biological samples used for human toxicological analysis such as urine, blood, liver or oral fluids with its significantly longer detection window (months) allowing retrospective investigation and measurement of drug consumption. Hair analysis is becoming accepted in many developed countries for substance consumption related issues in a wide range of sectors; the medico-legal sector, workplace testing, treatment monitoring, schools, forensics, research, insurance companies, environmental biomonitoring and driving licensing (Pragst, Balikova 2006, Kintz 2007a, Kempson, Lombi 2011, Smolders et al. 2009).

Extraction of drugs from hair is considered one of the most important steps in hair analysis. Apart from external drug deposition on hair, drugs are mainly enclosed tightly in the hair shaft and to a certain extent maybe bound to proteins, melanin or lipids of the cell membrane complex. Therefore, hair matrix type, structure of the drug, method and duration of extraction, and solvent used are all important factors affecting the final extraction yield (Musshoff, Madea 2007b).

There are numerous reports of screening strategies published for analysing different forensic basic drugs groups. Hypnotic drugs such as benzodiazepines are at the top of the list of drug facilitated crimes. These are weak basic drugs and have been reported to be extracted from hair by different methods, with phosphate buffers, methanol or digestive enzymes (Musshoff, Madea 2007a, Villain et al. 2005, Laloup et al. 2005). Other basic drug groups like anti-psychotics, antidepressants and amfetamines have frequently been reported to be extracted with alkaline digestion

(NaOH) (Couper, McIntyre & Drummer 1995b, Josefsson et al. 2003, Villamor et al. 2005, Gottardo et al. 2007). However, recently there has been some focus on using methanolic solutions as an extraction medium consisting of varying ratios of methanol, acetonitrile and formate buffer for extraction of several groups of basic drugs simultaneously (Hegstad et al. 2008, Broecker, Herre & Pragst 2011, Nielsen et al. 2010). However, the extent of their efficiency has not been examined most of the time.

2.1.1. Liquid chromatography-mass spectrometry

In the last few decades there has been a huge increase in the use of liquid chromatography coupled with mass spectrometric detection that is primarily due to the development of suitable interfaces that allowed the liquid mobile phase to enter the mass spectrometer with the help of vacuum negative pressure. This successful combination of liquid chromatography-mass spectrometry (LC-MS) became almost essential in many analytical fields. This instrument was recognized initially as a complementary analytical tool to gas chromatography-mass spectrometry (GC-MS) for the analysis of non-volatile compounds and high molecular weight compounds. However, it been estimated now that more than seventy percent of toxicology analysis can be done by this machine (Van Bocxlaer et al. 2000). In addition LC-MS has several advantages such as fast running time, normally no derivatization required, and optimization of the ionisations and the MS parameters and tuning are performed semi-automatically.

A triple quadrupole mass spectrometer (MS-MS) comprises of two mass analysers (Q1 and Q3) separated by a collision cell (Q2). Q1 filters parent or precursor ions (ions of interest), while Q3 filters (second mass analyser) product or daughter ions which are produced after fragmentation of the parent ions in the collision cell (Q2). Ions finally pass into a mass detector and specialist software is used to process the data qualitatively and quantitatively. Illustrating figure is shown in appendix 8.4.

The most common interface used in LC-MS(MS) to carry the analytes from the liquid phase to the gas phase is electrospray ionisation (ESI). In electrospray process ions

are generated from droplets after spraying of the mobile phase that passes through high electric field needle. With the assistant of warm gas (nitrogen) flow droplets vaporize and leaves highly charged analyte molecules. The charging of the analytes is achieved with nebulizing needle at ground potential surrounded with electrodes with a high voltage. The potential difference between the nebuliser and the electrode produces a strong electric field that charges the surface of the mobile phase droplets (Ho et al. 2003). Illustrating figure is shown in appendix 8.5.

ESI known as a soft ionisation method, because the amount of energy transferred to the analyte during the ionisation process is small. Thus pseudo-molecular ions $[M + H]^+$ or $[M + H]^-$ will normally be formed and that will lead to a small degree of molecular fragmentation or even no fragmentation. So the amount of structural information it can reveal will be very scant. On the other hand LC-MS, especially with tandem MS/MS, is highly sensitive and very useful quantitatively, as most of the total ion count (TIC) relates to one precursor ion in the MS/MS mode. However, it is possible as well with this technique to find cluster of charged ions formed between the analyte and the solvent (e.g. acetonitrite ACN $[M + 42]^+$ and methanol $[M + 32]^+$) or with the adducts (sodium $[M + 23]^+$, potassium $[M + 39]^+$ or ammonium $[M + 18]^+$). As a result it is advisable to use the protonated or deprotonated pseudomolecular ions as precursor ions instead of using cluster ions or adducts as they can cause poor reproducibility of the assay.

Application of LC-ESI-MS/MS is wide and varied. It is been used in chemical and drug detection in toxicological, pharmacokinetic, environmental, proteomics and therapeutic drug monitoring studies. Most of the analysis of the drugs of abuse is carried out currently by LC-MS as well as this research. LC-MS drugs of abuse screening methods have been developed and published already (Kintz 2007a, Kronstrand et al. 2004, Hegstad et al. 2008).

2.2. Hair analysis

2.2.1. Aim of the study

In this study the aim was to develop a method to enable the detection and quantification of basic and weakly basic drugs in hair simultaneously based on the methanolic solution extraction technique and to compare its efficiency with the alkaline digestion technique which was followed by liquid-liquid extraction (LLE).

2.2.2. Materials and reagents

2.2.2.1. Chemicals

All chemicals were of HPLC or analytical grade. Methanol, acetonitrile, acetone, propanol, methyl-tert-butyl-ether (MTBE), citalopram, clobazam, clonazepam, cocaine, codeine, desmethyldiazepam, 3,4-methylenedioxyamfetamine (MDA), 3,4-methylenedioxy-N-ethylamfetamine (MDEA), 3,4-Methylenedioxymethamfetamine (MDMA), midazolam, sulpiride, zaleplon and zolpidem were purchased from Sigma-Aldrich Co, Dorset UK. 7-aminoclonazepam, amfetamine, amfetamine-D11, benzoylecgonine-D8, benzoylecognine, codeine-D3, diazepam, diazepam-D5, ecognine methyl ester, medazepam, oxazepam and temazepam were purchased from LGC standards, Middlesex UK. Other prescription drug standards were supplied from their manufacturer. Deionised water was obtained on site (ELGA Limited). Dichloromethane, 7.5M ammonium acetate solution and all consumables (tubes, etc) were from VWR International Ltd, Lutterworth UK. The rest of the chemicals used in the solutions below were from Sigma-Aldrich Co, Dorset UK.

2.2.2.2. Solutions

Standard solutions

1000µg/mL or 100µg/mL working standards were made with MS grade methanol of each analyte and internal standard. All were stored at -20^{0}C and diluted to the required concentration prior to the experiment.

0.1M phosphate solution

One litre of solution was prepared by dissolving 1.7g of anhydrous disodium orthophosphate and 12.14g of sodium dihydrogen orthophosphate monohydrate in 800mL of de-ionized water. The pH was adjusted to 6 with 1M potassium hydroxide and the solution made up to volume with de-ionized water.

0.1% formic acid (v/v)

100µL of concentrated formic acid (98/100) was mixed with 99.9mL of de-ionized water, mixed well and stored at room temperature.

Methanolic extraction solution

60mL of methanol, 10mL of acetonitrile and 30mL of 0.1% formic acid were measured into a measuring cylinder, mixed well and stored at room temperature.

1M NaOH solution

40g of NaOH (MW 40.0) pellets were measured into a 1000mL volumetric flask and made up to the 1000mL mark with de-ionized water. This was mixed thoroughly and stored at room temperature.

2M ammonium acetate

266.6mL of 7.5M ammonium acetate solution were measured into a 1000mL volumetric flask and made up to the 1000mL mark with de-ionized water. This was mixed thoroughly and stored at room temperature.

2.2.3. Hair preparation

The decontamination process was by using three washing steps with two solvents and one aqueous solution: 0.01M phosphate solution (pH6), dichloromethane and propanol/acetone (1:1), used in the respective order. The uncut hair was put in a 6ml glass tube, then 2mL of the first solution added and vortex mixed for 1 minute. After that the solvent was decanted and then the next solvent was added. After the last washing step (2mL propanol/acetone) the hair was left to dry at room temperature or the process was speeded up with the speed-vac. This decontamination method was found to be quick and very efficient when used for cleaning some encountered post-mortem hair samples.

After the hair had dried segments of 0-0.6cm each were cut into small pieces (1–3mm) with scissors and ideally 25mg were weighed directly, larger segments were considered in some cases (e.g. 0-1.5cm). If this was not possible any amount between 10 and 80mg was considered and placed into a 2mL glass tube with a screw cap.

2.2.4. Methanolic extraction

Five hundred µL of the methanolic extraction solution (described earlier) was added to the hair in each tube and left in a sonicator for 16 hours at 40°C. In the case of calibrators, the required concentrations were made based on adding 25µL of drugs standard (e.g. 1µg/mL in methanol) to the calibrators' tubes containing 25mg blank hair (from lab personnel not on any drug for ~1year) each and these 25µL were deducted from the extraction solution (475µL). Also 25µL of internal standards (bromperidol and the deuterated standards amfetamine-D11, benzoylecgonine-D8, codeine-D3 and diazepam-D5) were added as well at a concentration similar to the middle calibrator. After centrifugation for 10 minutes the extracted solutions were transferred to auto-sampler vials and evaporated to dryness followed by reconstitution with 125µL of 25% methanol and filtered with the syringeless mini-uniprep filters (Whatman GE). Finally 20µL of this filtrate was injected into the LC-MS/MS system.

2.2.5. Alkaline digestion

Twenty five mg of washed blank/patient hair was incubated with 0.5mL of 1M NaOH for 3 hours in a water bath (50°C) to allow for digestion of the hair. Samples were then extracted after digestion with 2mL MTBE by 30 minutes rotary mixing and 10 minutes centrifugation. Solvent layers were transferred into clean tubes containing 150µL 1% formic acid, then mixed on a rotary mixer for 20 minutes and removed after 10 minutes of centrifugation by aspiration. From the remaining aqueous layer 20µL was injected into the LC-MS/MS system.

2.2.6. Instrumentation and ion identification

The LC-MS/MS analysis was carried out with an HPLC system consisting of a Perkin Elmer PE200 series autosampler, pump and column oven. The MS/MS part was a SCIEX API 2000 Triple Quadrupole MS/MS instrument (Applied Biosystems) equipped with an electrospray interface (Turbo Ion Spray) set on the positive mode. Ion spray voltage was set to 5500V. Nitrogen was used as the nebulizer gas-GS1 (30psi), auxiliary gas-GS2 (20psi heated to 300°C), curtain gas (20psi) and as the CAD gas (set on 3).

Chromatography was achieved using a C18 column (Altech Alltima C18, 15cm x 2.1mm, 5µm) maintained at 50°C. The mobile phase consisted of (A) 950mL methanol, 50mL de-ionised water, 1mL 2M ammonium acetate and 1mL formic acid (98/100) and (B) 998mL de-ionised water, 1mL 2M ammonium acetate and 1mL formic acid (98/100). The running of the system was isocratic and the percentage of A and B of the mobile phase was kept at 50% of (A) and was pumped at 0.25mL/min. The output from the column was split 10:1 before entering the mass spectrometer. The volume of injection was 20µL.

Analytes were infused at 0.25mL/minute to the LC-MS/MS system at a concentration of 1µg/mL in 50% methanol/2mM ammonium acetate solution. The precursor and product ion identification and parameter tuning were completed by

automatic and manual tuning. Optimum tuning parameters, precursor and product quantitation ions are shown in Table 3.

2.2.6.1. Ion Identification results

Table 3: Optimized MRM and MS/MS parameters for analytes and internal standards

Analyte	Q1 (m/z)	Q3 (m/z)	Internal Standard	Retention Time (min)	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
7-Aminoclonazepam	285.92	120.94	Diazepam_D5	2.9	51	370	10.5	41	2.0
Alprazolam	308.98	280.84	Diazepam_D5	11.2	71	240	10.5	35	4.0
Amfetamine	135.99	91.15	Amfetamine_D11	2.7	11	340	5.5	21	8.9
Amfetamine_D11	147.29	98.20	-	2.6	36	360	4.0	25	12.0
Amisulpiride	370.05	242.07	Diazepam_D5	2.2	51	370	10.5	37	10.0
Amlodipine	408.90	237.90	Bromperidol	8.5	35	330	3.6	35	10.0
Aripiprazole	448.23	176.20	Bromperidol	13.6	76	370	6.5	43	2.0
Benzoylecgonine	289.92	168.04	Benzoylecgonine_D8	3.4	26	350	10.0	27	6.5
Benzoylecgonine_D8	298.15	171.10	-	3.3	20	200	10.0	30	6.6
Bromperidol	420.17	123.20	-	6.3	66	370	8.5	59	0.0
Carbamazepine	237.02	194.06	Diazepam_D5	7.3	56	290	10.5	29	4.0
Chlordiazepoxide	299.98	227.03	Diazepam_D5	6.8	56	170	10.5	35	4.0
Citalopram	325.05	109.14	Diazepam_D5	4.3	61	170	10.5	37	2.0
Clobazam	300.97	259.18	Diazepam_D5	9.1	56	370	10.5	31	4.0
Clonazepam	315.91	269.98	Diazepam_D5	7.5	66	250	10.5	37	4.0
Cocaine	303.92	182.04	Benzoylecgonine_D8	3.2	26	350	10.0	27	6.8
Codeine	300.09	165.00	Codeine_D3	2.2	26	350	10.5	63	12.0
Codeine_D3	303.00	165.00	-	2.2	31	370	10.5	63	16.0
Dehydroaripiprazole	446.02	284.90	Bromperidol	11.9	76	350	10.0	33	8.0
Desmethyldiazepam	270.97	140.02	Diazepam_D5	14.1	66	350	8.5	41	6.0
Diazepam	284.98	193.05	Diazepam_D5	18.1	46	350	8.5	45	8.0
Diazepam_D5	290.02	198.04	-	18.1	46	330	8.5	43	4.0
Ecgonine M.Ester	200.07	182.10	Benzoylecgonine_D8	2.0	51	340	5.5	25	28.0
Lorazepam	320.86	274.89	Diazepam_D5	13.3	46	360	10.5	33	6.0
Lormetazepam	334.89	288.87	Diazepam_D5	13.6	46	280	10.5	31	6.0
MDA	180.10	105.10	Amfetamine_D11	2.6	26	340	4.5	31	14.0
MDEA	208.11	163.10	Amfetamine_D11	2.7	36	350	6.0	19	24.0
MDMA	194.22	163.10	Amfetamine_D11	2.5	36	360	6.0	17	8.0
Medazepam	270.99	91.00	Diazepam_D5	5.5	56	350	10.5	49	2.0
Midazolam	325.95	290.89	Diazepam_D5	5.6	81	270	10.5	39	6.0
Mirtazapine	265.92	195.11	Bromperidol	3.4	36	350	8.5	35	8.0
Nitrazepam	282.01	235.89	Diazepam_D5	7.4	66	370	10.5	33	4.0
OH-risperidone	427.11	207.09	Bromperidol	3.6	46	360	7.5	39	8.0
Oxazepam	286.96	240.89	Diazepam_D5	10.2	96	350	8.5	35	8.0

Analyte	Q1 (m/z)	Q3 (m/z)	Internal Standard	Retention Time (min)	DP (V)	FP (V)	EP (V)	CE (V)	CXP (V)
Phenazepam	350.79	206.04	Diazepam_D5	15.3	61	350	8.0	49	24.0
Quetiapine	384.22	253.24	Bromperidol	7.2	76	350	10.5	31	12.8
Risperidone	411.18	191.05	Bromperidol	4.5	51	120	10.5	39	11.3
Sulpiride	342.02	213.90	Bromperidol	2.1	41	370	10.0	51	24.0
Temazepam	300.99	255.03	Diazepam_D5	12.4	41	270	8.5	33	10.0
Zaleplon	305.92	235.96	Diazepam_D5	5.5	56	350	8.5	39	8.1
Zolpidem	308.06	234.93	Diazepam_D5	3.2	66	270	10.5	49	4.0

Q1=precursor ion, Q3=product ion Q1=precursor ion, Q3=product ion, DP = declustering potential, FP = focusing potential, EP = entrance potential, CE = collision energy, CXP = collision cell exit potential

2.2.7. Methods and results with partial validation for selected analytes

2.2.7.1. Comparison of Mini-Uniprep filter media

Three filter materials were compared by using spiked blank hair samples at concentrations of 0.5ng/mg hair. 25mg of blank hair were weighed out in glass tubes. Then the whole extraction/incubation method was applied to all the samples. The three materials were 0.45µm pore size glass microfiber (GMF), 0.2µm pore size polytetrafluroethylene (PTFE) and 0.2µm pore size polyvinylidenefluoride (PVDF) used in manufacturing Whatman Mini-UniPrep Syringeless Filters. The filters efficiency was calculated by comparing the percentage of extraction recovery through their peak area ratio of product ion/external standard for each drug after filtration.

Table 4 shows clearly that the PTFE filter medium has the greatest recovery (mean=100%) of all the analytes while the GMF and PVDF filters (mean=89 and 72%, respectively) reduced significantly the response of amisulpride and citalopram. Also the responses of medazepam, midazolam and zolpidem were considerably reduced by the PVDF filter.

Table 4: Mini-UniPrep filter media: comparison of results for % recovery of 0.5ng/mg spiked analytes

Analyte	GMF	PTFE	PVDF
7-Aminoclonazepam	114	94	93
Alprazolam	100	98	101
Amisulpiride	7	92	21
Carbamazepine	108	98	94
Chlordiazepoxide	116	99	84
Citalopram	7	113	<1
Clobazam	106	100	94
Clonazepam	103	104	93
Desmethyldiazepam	99	100	101
Diazepam	103	99	98
Lorazepam	104	97	99
Lormetazepam	109	96	94
Medazepam	86	100	2
Midazolam	59	104	25
Nitrazepam	109	97	93
Oxazepam	105	102	92
Phenazepam	105	103	92
Temazepam	100	101	99
Zaleplon	106	96	98
Zolpidem	100	96	18
Mean	**92.3**	**99.5**	**78.5**

GMF= Glass Microfiber, PTFE= Polytetrafluroethylene, PVDF= Polyvinylidenefluoride

2.2.7.2. Efficiency of the selected filter medium (PTFE)

To further check the efficiency of PTFE media filters two unextracted standards were prepared at the same concentration in the mobile phase and submitted to the LC-MS/MS with another two unextracted standards but filtered with PTFE Mini-UniPrep filters. Filter efficiency was calculated as the percentage of total extraction recovery by dividing the filtered over the unfiltered standards (n=3). Also blank samples (mobile phase) filtered and unfiltered were run in conjunction to see if any interfering peaks eluted from the filter.

2.2.7.2.1. PTFE filter results

Interestingly, as seen in Table 5 most of the drugs showed good recoveries when repeating the experiment with PTFE filtered standards in addition to blank standards and comparing them against the unfiltered standards except for medazepam and citalopram. Their recoveries were reduced by almost 50%. Also, no eluting peaks were found in any of the drugs' detection windows.

Table 5: Mini-UniPrep filters with PTFE media comparison results for 0.5 ng/mg benzodiazepine standards (n=2)

Analyte	Blank	Blank-Filtered	Filtered Standard
7-Aminoclonazepam	0	0	122.1
Alprazolam	0	0	116.2
Amisulpiride	0	0	97.1
Carbamazepine	0	0	127.7
Chlordiazepoxide	0	0	112.1
Citalopram	0	0	54.4
Clobazam	0	0	115.3
Clonazepam	0	0	132.3
Desmethyldiazepam	0	0	97.3
Diazepam	0	0	89.5
Lorazepam	0	0	125.4
Lormetazepam	0	0	94.7
Medazepam	0	0	30.4
Midazolam	0	0	84.2
Nitrazepam	0	0	128.2
Oxazepam	0	0	108.9
Phenazepam	0	0	85.9
Temazepam	0	0	115.6
Zaleplon	0	0	129
Zolpidem	0	0	107.3
Mean	0	0	101.6

PTFE= Polytetrafluroethylene

2.2.7.3. Linearity

The linearity study reflects the relationship between change of spiked analyte concentration and detector response to that change (Hartmann et al. 1998). It was attained by spiking hair samples with 50, 75, 100, 500, 1000 and 2000pg/mg of each

analyte. The linear correlation coefficients (r^2) values were better than 0.99 for all analytes.

2.2.7.4. LOD and LOQ

Limit of detection and limit of quantification were assessed by spiking blank hair with the analytes in decreasing concentrations. The LOD and LOQ were calculated at a signal to noise ratio of 3 and 10, respectively. LOD values for all analytes were 0.5–23pg/mg and LOQ values were 1.7–76.7pg/mg (Table 7).

2.2.7.5. Selectivity

Selectivity in drug analysis is the ability of an analytical method to differentiate and quantify the analyte of interest in the presence of other components in the sample (Peters, Drummer & Musshoff 2007). In order to achieve that, a low standard (0.1ng/mg) of each drug from the group was injected individually into the LC-MS/MS system and all the other drug detection windows and retention times were observed to see if there were any interfering peaks or false positive results that might appear in the same retention time and same window as the injected analyte. No interference was found in the same detection window at the retention times for any of the analytes. Also drug-free hair samples showed no false positive results.

2.2.7.6. Matrix effect

The matrix effect (ME) is basically the ion suppression or enhancement of the analyte peak caused by co-eluting compounds from the biological matrix (urine, blood, hair, etc). The aim of this experiment was to acquire more accurate data on the extent of matrix influence on the results when matrices come from different types of hair. As proposed by Matuszewski et al the ME was assessed by comparing the mean peak area of product ion of extracted drug-free hair samples from 5 different sources (black, baby-brown, black dyed, white and blonde) spiked with 0.2ng/mg low standard solution following filtration (i.e. after extraction/incubation) with the

mean peak area of product ion of neat unextracted standards prepared in the mobile phase (Matuszewski, Constanzer & Chavez-Eng 2003). The percentage of the ME was calculated by dividing the mean of the extracted samples by the mean of unextracted ones according to Equation 1.

Equation 1

$$\mathbf{ME}\ (\%) = \frac{\textbf{spiked after incubation}}{\textbf{unextracted standard}}\ \mathbf{X\,100}$$

This percentage represents the absolute ME (both ion suppression and ion enhancement).

The ME results shown in Table 6 indicate that there was no significant ion suppression or enhancement affecting the analytes, except with amisulpiride and the 7-amino metabolite of clonazepam. Some of the analytes such as diazepam and clobazepam showed slight ion enhancement, but the rest of the drugs were within 91.4 - 110.2% ME. Also the %CV was in the range of 1.1 – 12.1% showing a good agreement level between the different hair samples.

Table 6: Matrix effect (ME) results for 0.2ng/mg hair samples spiked with the analytes (n=5)

Analyte	Black	Brown	Dyed	White	Blond	%ME	%CV
7-Aminoclonazepam	91	92	65	62	75	77	18
Alprazolam	103	104	93	92	91	97	7
Amisulpiride	46	52	58	28	62	49	27
Carbamazepine	120	106	96	98	98	103	10
Chlordiazepoxide	109	104	93	95	94	99	7
Citalopram	97	120	99	99	109	105	9
Clobazam	121	121	108	112	110	114	5
Clonazepam	112	99	95	93	95	99	8
Desmethyldiazepam	118	111	107	100	106	109	6
Diazepam	117	120	113	113	116	116	3
Lorazepam	118	112	100	102	102	98	10
Lormetazepam	113	114	106	110	108	107	7
Medazepam	98	116	96	102	94	110	3
Midazolam	100	109	95	93	96	101	8
Nitrazepam	112	105	95	96	100	99	6
Oxazepam	113	111	97	97	100	101	7
Phenazepam	117	114	92	97	98	104	1
Temazepam	119	115	109	109	109	104	7
Zaleplon	99	99	87	86	87	112	4
Zolpidem	95	98	91	92	94	91	8
Mean	**106**	**106**	**95**	**94**	**97**	**100**	**8**

2.2.7.7. Absolute recovery with methanolic incubation

Recoveries were estimated by determining peak areas of analytes obtained when the drugs spiked with blank hair (in extraction solution) at three concentrations 0.5, 1, and 2ng/mg (n=3) were compared with unextracted/unfiltered standards. Therefore, this recovery takes into account all the extraction steps including filtration, incubation and matrix effect (absolute). The recovery was calculated as a percentage by dividing the average peak area ratio of the extracted standards over the average peak area ratio of the unextracted standards and multiplied by 100.

The results in Table 7 show that all the analytes had good recoveries (> 58%) at all three concentrations with %CV below 13.4%, except for three analytes which gave low recoveries (12.9 - 29.8%) and high %CV (< 34.7%).

Table 7: Linearity, LOD, LOQ and Absolute Recovery (RE%) results for 0.5, 1 and 2ng/mg of analytes (n=3)

				Absolute Recovery (RE%)						
Analyte	**Linearity (r^2)**	**LOD pg/mg**	**LOQ pg/mg**	**0.5 ng/mg**	**%CV**	**1 ng/mg**	**%CV**	**2 ng/mg**	**%CV**	**%CV for 3 Conc.**
7-Aminoclonazepam	0.991	2.5	8.3	67.4	7.3	62.6	5.6	64.2	1.9	3.8%
Alprazolam	0.997	1.5	5.0	98.3	0.8	93.7	1.3	93.1	0.7	3.0%
Amisulpiride	0.997	1.0	3.3	28.4	3.4	29.0	6.0	29.8	2.6	2.4%
Carbamazepine	0.996	1.3	4.3	98.0	2.7	93.0	1.6	94.0	1.8	2.8%
Chlordiazepoxide	0.999	2.6	8.7	78.9	4.1	75.6	6.8	72.5	4.0	4.2%
Citalopram	0.991	4.8	16.0	12.9	11.3	16.5	34.7	13.9	2.5	12.9%
Clobazam	0.999	1.2	4.0	103.8	1.6	98.7	2.5	96.8	2.1	3.6%
Clonazepam	0.999	2.2	7.3	94.6	5.0	89.3	5.5	90.7	0.9	3.0%
Desmethyldiazepam	0.999	12.0	40.0	98.4	0.2	92.8	4.8	91.3	3.9	4.0%
Diazepam	0.999	2.6	8.7	87.6	3.8	87.6	9.9	82.4	3.6	3.5%
Lorazepam	0.996	2.7	9.0	79.9	4.2	72.4	6.3	75.3	6.0	5.0%
Lormetazepam	0.995	3.4	11.3	101.0	7.4	94.8	4.8	94.3	4.7	3.9%
Medazepam	0.996	7.0	23.3	104.4	3.9	103.1	6.3	94.8	2.0	5.2%
Midazolam	0.998	1.0	3.3	60.4	13.4	57.8	6.6	60.1	6.4	2.4%
Nitrazepam	0.998	6.5	21.7	102.0	2.0	98.1	3.2	100.3	4.3	2.0%
Oxazepam	0.996	23.0	76.7	118.8	5.0	100.1	2.3	100.1	4.2	10.2%
Phenazepam	0.998	6.5	21.7	90.2	5.9	79.0	6.2	81.1	6.6	7.1%
Temazepam	0.999	2.6	8.7	95.8	0.4	93.1	2.1	91.8	2.8	2.2%
Zaleplon	0.998	1.3	4.3	80.5	1.4	77.7	2.8	78.7	1.8	1.8%
Zolpidem	0.999	0.5	1.7	73.5	4.0	71.2	2.2	68.3	3.1	3.7%

2.2.7.8. Stability during methanolic extraction

All analytes were prepared at 0.5µg/mL (~=ng/mg) of the extraction solution (mentioned in solutions section) and incubated/sonicated with slightly harsher conditions at 50^0C for 18h without the blank hair. Then the mean peak area ratio of

product ion/internal standard was compared with the mean peak area ratio of product ion/internal standard of unextracted/unincubated standards prepared in 25% MeOH at the same concentration (n=3).

2.2.7.9. Stability/recovery with alkaline digestion

Extraction of hair with various solvents following alkaline digestion has been widely mentioned in many publications (Villamor et al. 2005, Pelander et al. 2008, Villain 2004, Stanaszek, Piekoszewski 2004). In order to investigate whether the proposed method has any advantages over the alkaline digestion method, the first step was to check how stable the analytes would be during the incubation/extraction with NaOH/MTBE. Twenty five mg of blank/washed hair was incubated for 3 hours at 50°C with the analytes at a concentration of 1ng/mg and mixed with 0.5mL 1M NaOH. Extraction was with 2mL of MTBE on an orbital shaker for 30 minutes followed with back extraction with 150µL of 1% formic acid. 20µL were injected into the LC-MS/MS system.

The stability results shown in Table 8 indicate that almost all the analytes are stable under the extraction/incubation conditions of the methanolic method. The least stable analyte was oxazepam with 56.1% recovery. The average recovery was 89.6% with a coefficient of variation of 4.2%. The stability of the analytes under alkaline conditions was much lower with an average of 51.7%.

Table 8: Stability of the analytes after sonication/incubation at 50^{0}C/18h (n=3) and after extraction and digestion with 1M NaOH at 50^{0}C/3h (n=2)

Analyte	% Stability with methanolic extraction	% CV	% Stability with NaOH digestion	%CV
7-Aminoclonazepam	98.3	3	19.7	36
Alprazolam	97.5	2	67.2	8
Amisulpiride	93.6	4	25.0	41
Carbamazepine	101.8	3	66.3	2
Chlordiazepoxide	70.3	9	83.6	19
Citalopram	101.3	7	28.3	37
Clobazam	104.5	4	61.3	7
Clonazepam	92.3	0	50.4	7
Desmethyldiazepam	76.3	4	76.4	28
Diazepam	98.5	1	51.1	16
Lorazepam	64.2	4	52.6	19
Lormetazepam	98.2	1	60.7	13
Medazepam	100.6	6	30.5	5
Midazolam	100.5	4	32.6	12
Nitrazepam	73.3	6	49.2	14
Oxazepam	56.1	8	54.4	30
Phenazepam	86.1	5	75.6	41
Temazepam	81.1	1	60.4	20
Zaleplon	97.7	7	36.7	12
Zolpidem	98.9	4	52.0	15
Mean	89.6	4.2	51.7	19.04

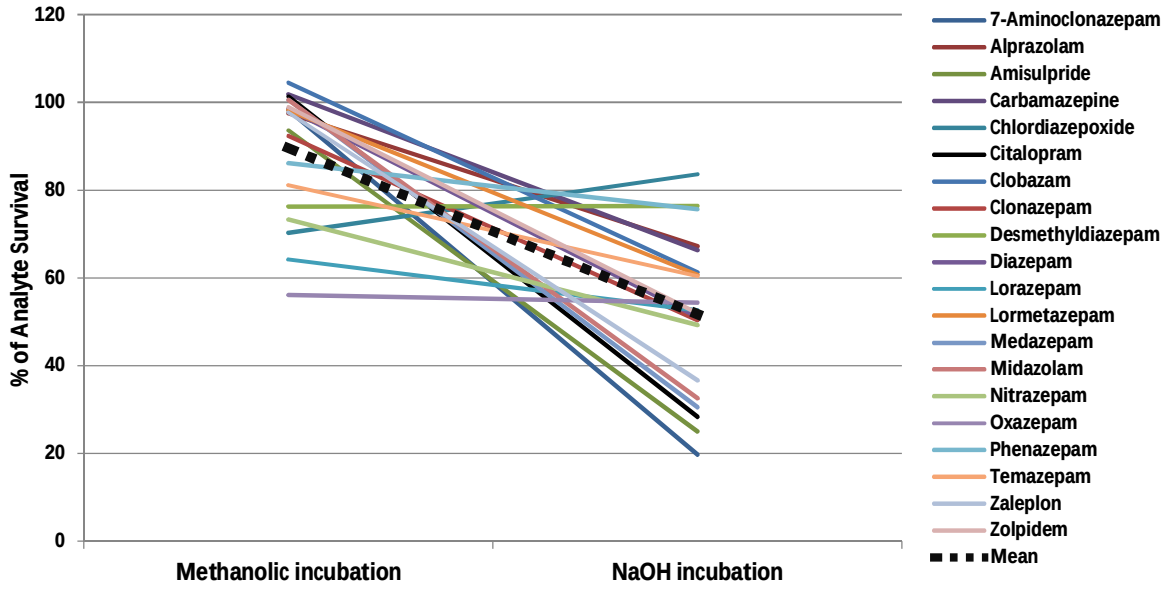


Figure 8: Stability of analytes during methanolic and alkaline conditions

2.2.8. Real samples (methanolic versus alkaline extraction)

The methanolic method was applied to 10 cases, one of them postmortem. Hair samples were taken from the posterior vertex region of the scalp. Nine of these cases were patients taking doses of different classes of drugs. Seven of the nine were addicts under rehabilitation.

About a 0.6 cm segment from the root end (proximal) of each sample was decontaminated and extracted by the methanolic method. However, there was an additional step added to the analysis of those cases. After full incubation and extraction steps, hair sample remains in the tubes were not discarded. Instead they were re-extracted with the alkaline digestion method described earlier. The measurement was based on one-point calibration (cut-off).

Case 1 NR

Age: 32yrs **Hair Colour:** Black **Sample Collection Date:** 26 May 09

Drug name	Strength	Daily dose	Duration of use	Addiction history
Quetiapine	400mg	1600mg	07-26May09	Benzodiazepines
Valproic acid	500mg	2000mg	07-26May09	
Atrovastatin	20mg	20mg	07-26May09	
Aspirin	100mg	100mg	07-26May09	

Case 2 MD

Age: 28yrs **Hair Colour:** Black **Sample Collection Date:** 26 May 09

Drug name	Strength	Daily dose	Duration of use	Addiction history
Fenetylline *	50mg	~300mg	1year	Amfetamines

*Fenetylline is metabolized in the body to amfetamine and theophylline.

Case 3 AZ

Age: 34yrs **Hair Colour:** Dark Brown **Sample Collection Date:** 26 May 09

Drug name	Strength	Daily dose	Duration of use	Addiction history
Quetiapine	400mg	800mg	11-12,17-26May09	Benzodiazepines
Valproic acid	500mg	1000mg	17-26May09	Cannabis
Chlordiazepoxide	10mg	30mg	11-15May09	Alcohol (1/2 bottle/day of whisky)
Sulpiride	300mg	600mg	12-17May09	

Case 4 ED

Age: 45yrs **Hair Colour:** Black **Sample Collection Date:** 26 May 09

Drug name	Strength	Daily dose	Duration of use	Addiction history
Risperidone	2mg	4mg	07-26May09	Poly-Addict
Procyclidine	5mg	10mg	07-26May09	

Case 5 MN

Age: 38yrs **Hair Colour:** Black **Sample Collection Date:** 26 May 09

Drug name	Strength	Daily dose	Duration of use	Addiction history
Risperidone	2mg	2mg	03-26May09	Poly-Addict
Mirtazapine	30mg	30mg	03-20May09	

Case 6 HR

Age: 37yrs **Hair Colour:** Black **Sample Collection Date:** 26 May 09

Drug name	Strength	Daily dose	Duration of use	Addiction history
Quetiapine	200mg	400mg	20-26May09	Benzodiazepines
Mirtazapine	30mg	30mg	20-26May09	

Case 7 AR

Age: 35yrs **Hair Colour:** Black **Sample Collection Date:** 26 May 09

Addiction Drug	Strength	Daily dose	Duration of use	Addiction history
Heroin	1-2g	1-2g	April07-April08	Poly-Addict
Cannabis cigarette	-	1-10	April07-April08	
Cocaine	1g	1g	April07-April08	
Flunitrazepam	2mg	4mg	April07-April08	
Alprazolam	0.5mg	1mg	April07-April08	
Diazepam	10mg	20mg	April07-April08	
Amfetamines	-	Rarely	April07-April08	

Case 8 MQ

Age: 44yrs **Hair Colour:** Black **Sample Collection Date:** 26 May 09

Drug name	Strength	Daily dose	Duration of use	Addiction history
Mirtazapine	30mg	30mg	14-26May09	Benzodiazepines
Aripiprazole	15mg	15mg	14-26May09	

Case 9 DH

Age: 66yrs **Hair Colour:** Grey **Sample Collection Date:** July 09

Drug name	Strength	Daily dose	Duration of use	Other Drug
Amlodipine	5-10mg	10mg	>5years	-

Case 10 SK

Age: 35yrs **Hair Colour:** Brown **Sample Collection Date:** May 08

Addiction Drug	Strength	Daily dose	Duration of use	Addiction history
Cannabis	-	-	-	Cannabis
Cocaine	-	-	-	Cocaine
Amfetamines	-	-	-	Amfetamines

Table 9: Real samples (cases): comparison of results

Case	Hair Colour	Hair Segments	MeOH/ACN/FA Incubation	NaOH Digestion
1 NR	Black	20.3mg (0.6cm)	0.134ng/mg Codeine 2.53ng/mg Quetiapine	0.763ng/mg Codeine 31.7ng/mg Quetiapine
2 MD	Black	17.5mg (0.6cm)	42.3ng/mg Amfetamine	0.173ng/mg Amfetamine
3 AZ	Dark Brown	22.6mg (0.6cm)	19.1ng/mg Amfetamine 0.88ng/mg Sulpiride 3.41ng/mg Quetiapine	0.123ng/mg Amfetamine Negative Sulpiride 11.46ng/mg Quetiapine
4 ED	Black	17.4mg (0.6cm)	13.5ng/mg Amfetamine 0.019ng/mg Mirtazapine 0.039ng/mg OH-risperidone 0.059ng/mg Risperidone	0.036ng/mg Amfetamine 0.010ng/mg Mirtazapine 0.035ng/mg OH-risperidone 0.18ng/mg Risperidone
5 MN	Black	24.5mg (0.6cm)	16ng/mg Mirtazapine 0.138ng/mg OH-risperidone 1.37ng/mg Risperidone	40ng/mg Mirtazapine 0.134ng/mg OH-risperidone 4.1ng/mg Risperidone
6 HR	Black	19.9mg (1.5cm)	14.8ng/mg Mirtazapine 1.26**p**g/mg Risperidone 6.58ng/mg Quetiapine	43.2ng/mg Mirtazapine 0.022ng/mg Risperidone 33ng/mg Quetiapine
7 AR	Black	33.6mg (0.6cm)	0.08ng/mg Mirtazapine 0.016ng/mg Amfetamine	0.3ng/mg Mirtazapine Negative Amfetamine
8 MQ	Black	33.4mg (0.6cm)	3.7ng/mg Amfetamine 0.067ng/mg Codeine 18.9ng/mg Mirtazapine Negative Aripiprazole Negative Dehydroaripiprazole	0.015ng/mg Amfetamine 1.225ng/mg Codeine 41.4ng/mg Mirtazapine Positive Aripiprazole Positive Dehydroaripiprazole
9 DH	Grey	80mg (~1.5cm)	0.015ng/mg Amlodipine	Negative Amlodipine
10 SK*	Dark Brown	27mg (1cm) 1st	4.52ng/mg Amfetamine 6.46ng/mg MDMA 0.258ng/mg MDA 0.143ng/mg MDEA 2.68ng/mg Cocaine 28.42ng/mg Benzoylecognine 0.644ng/mg Ecognine methyl ester	
		35mg (2cm) 2nd	4.75ng/mg Amfetamine 6.72ng/mg MDMA 0.272ng/mg MDA 0.147ng/mg MDEA 1.434ng/mg Cocaine 27.83ng/mg Benzoylecognine 0.165ng/mg Ecognine methyl ester	

* Results of this subject are from combined method (methanolic and alkaline extraction)

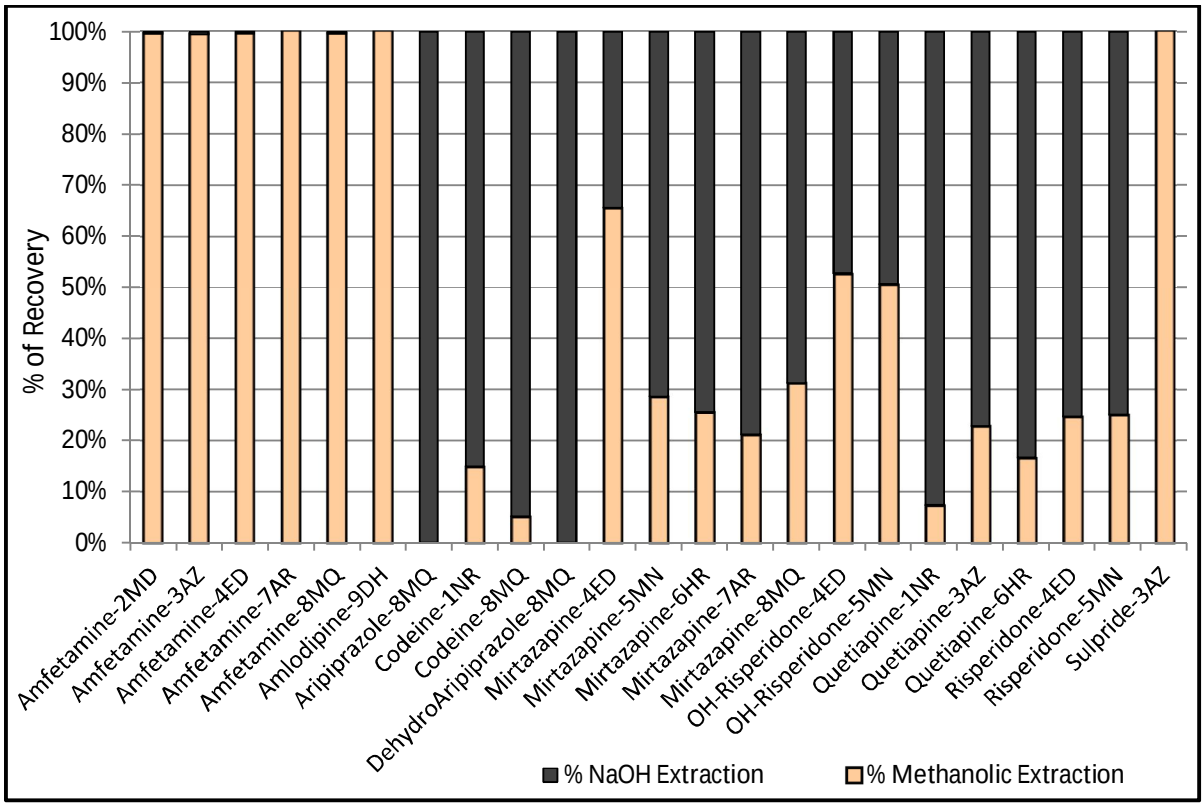


Figure 9: Real samples: comparison of results for methanolic and alkaline extraction (Drug-Case No.)

2.3. Discussion and conclusion

The development of the proposed basic drugs extraction method was based on previous studies showing that methanolic and aqueous solutions are capable of extracting drugs from hair samples (Kintz 2007a). However, a comparison between this extraction solution and the digestion with NaOH was not probably suggested in this specified way, particularly with general basic drugs screening. Therefore, the mixture of methanol, 0.01M formic acid and acetonitrile (methanolic solution) was proposed to extract basic drugs. Methanol and 0.01M formic acid are protic solvents and capable of swelling the hair and attracting/ionizing basic drugs entrapped inside the hair shaft, at least theoretically. Acetonitrile was added in a small amount (10%) as it was recommended by some articles (Kronstrand et al. 2004, Hegstad et al. 2008).

The first early recovery experiments with the methanolic method produced extracts that were turbid in colour which brought about the necessity of adding a filtration step to the method and checking the effect of these filters on the drugs' recovery. Mini-UniPrep filters were chosen as they are syringeless, simple and fast. As these filters come in different materials, a comparison study was carried out and showed that PTFE based filters gave the highest recoveries for the analytes.

It was seen from the stability results that digestion with NaOH reduces the stability of most of the analytes whereas methanolic incubation has no such effect on stability as the great majority of drugs showed >90% stability with or without blank hair.

Some method validation steps have been carried out on the methanolic method, such as recovery, LOD/LOQ, matrix effect and selectivity. Absolute recovery experiments showed that all the analytes have a recovery ranging between 59.4 and 106.3% with a CV from 0.2 to 13.4% after excluding the results of four drugs. Amisulpiride and citalopram gave low recoveries (14.4 - 29.1%, CV 2.5-34.7%). During the matrix effect study five different sources of hair have been incorporated (different colours and ethnicities). Results showed that all the drugs have ME ranging between 91.4 and 115.6% (CV 1.1 – 12.1%) except 7-aminoclonazepam and amisulpiride (ME 77 and 49.1%, CV 18.4 and 26.8%, respectively). The selectivity study also showed no major problem affecting the method selectivity as no analyte eluted in the same retention time and window of the other analytes and there were no false positives from blank hair.

Last but not least, analysis of authentic hair samples from a group of subjects taking basic or weak basic drugs revealed the usefulness of the proposed method at least for the detection of basic drugs in hair. To further examine the extent of extraction the same samples were digested with 1M NaOH after the methanolic extraction to compare the extractability of both methods on the administered drugs by using the exact same samples. Results revealed that the methanolic method was able to extract most of the investigated drugs from hair samples, but the downside about it was the fact that it only extracted part of the incorporated drug in hair. In addition,

this part or this percentage of the incorporated drug varied between the investigated compounds. Results showed that 100 to 50.7% of found amfetamine, amlodipine, sulpiride and 9-OH-risperidone were extracted by the methanolic method, whereas, the percentage of extraction for the rest of the investigated drugs was from 0 to 31.3% as shown in Figure 9, with the exception of mirtazapine which gave a high percentage of extraction (65%) in one subject out of five. These results suggest that the incorporated drugs in hair need either complete digestion of the hair samples or maybe powdering the samples to ensure complete release of the entrapped compounds. Although some drugs have been extracted or revealed only by the methanolic method without digestion, like sulpiride and amlodipine, it does not mean that the percentage of recovery was really 100%. It is likely that these drugs were not stable under NaOH digestion which may led to their degradation and disappearance. High pH possibly has caused hydrolysis to both amlodipine's ester moiety and sulpiride's amide type link.

This way of comparison between alkaline and methanolic methods is different from the direct comparison with two matching hair samples. It was design specifically this way to investigate the amount of residual drug left behind from the methanolic extraction, especially because not all drugs are stable under alkaline digestion and would not be detected if only alkaline digestion was used.

With regard to the analysis of hair washes, it was not been done due to a number of reasons. The washes are three per sample and that will add up a large amount of samples to be analysed and instrument time was extremely limited. Also the research was focusing mainly on drug incorporated from blood so external contamination or incorporation from sebum or sweat were beyond the scope of the research. Lastly, extraction of drugs through washes is very unlikely because each washing step takes less than 1 min.

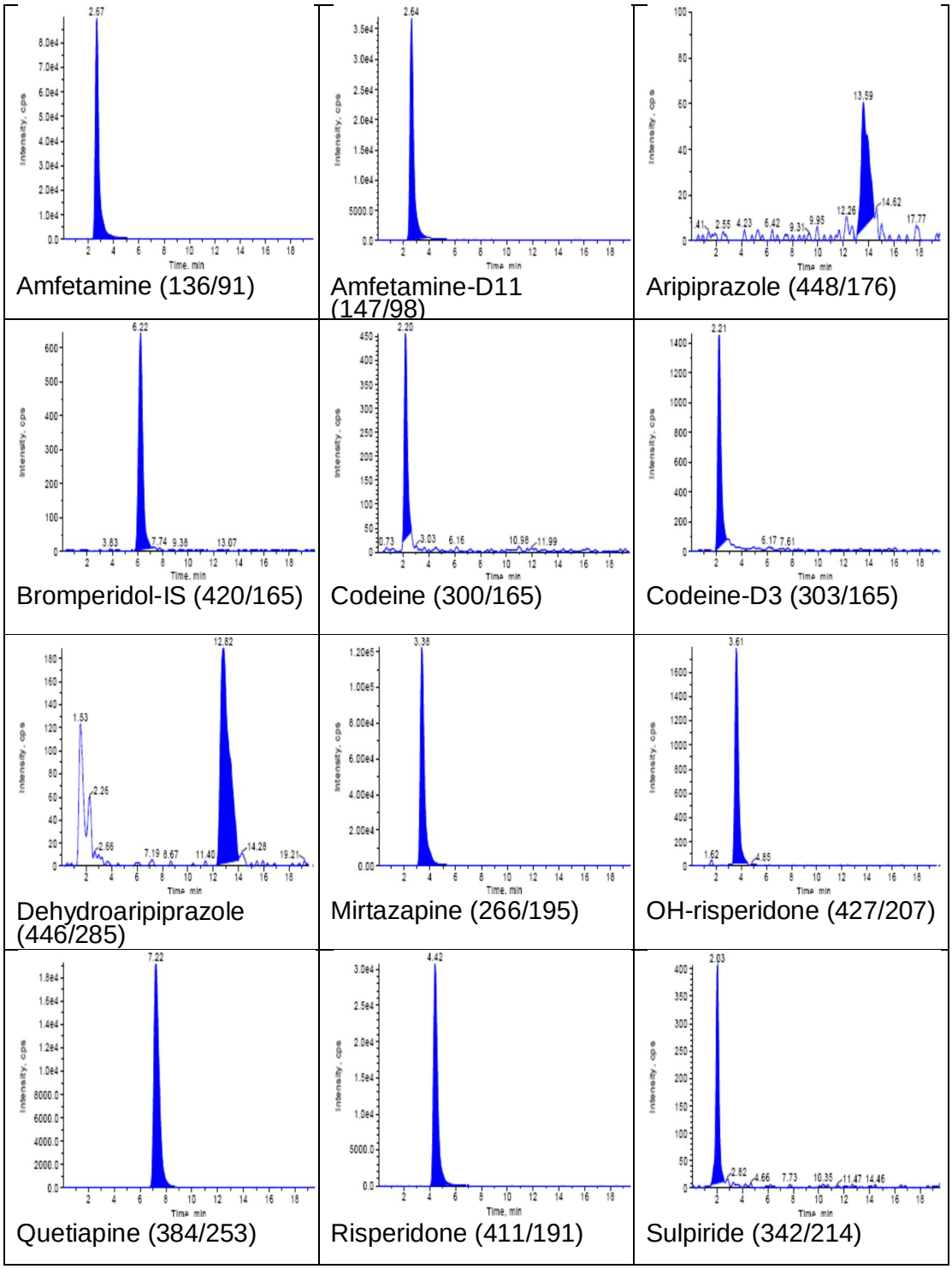


Figure 10: Some of the real sample chromatograms, between brackets are precursor ion/product ion

In conclusion, the methanolic method is capable of extracting most basic drugs in hair samples but only part of the total incorporated drug. Therefore, these results

suggest that a combination of both methods (methanolic and alkaline extractions) in hair sample processing for the detection of basic and weak basic drugs seems to provide better extraction yield than either method alone. However, not all basic drugs are stable to alkaline digestion. A further study with more real samples was needed and preferably in comparison with other hair extraction techniques, such as powdering the hair and other digestion methods.

Limitations

Ideally, the method should be applied to more samples especially from subjects taking the same drugs as the drugs used in the method development. Unfortunately, only those patients who were studied agreed to participate. Also, the access time on the LC-MS/MS was very limited and optimization of every variable was not feasible. Moreover, the method is intended to analyse basic drugs in general and basic drugs are very common so it is almost impossible to include all of them. Alkaline digestion has limited application to only analytes stable under basic conditions, while methanolic extraction showed partial extraction ability even after 18 hours of incubation. Also due to limited access time to LC-MS/MS analysis of hair washes could not be included in the study. Finally, in this study MS/MS data were based on only one product ion transition, which should be acceptable for clinical purposes, compared to that of forensic purposes, where a minimum of two is required. In the clinic, the drug therapy will be known and hence, to monitor patient compliance, it may be argued that one major ion transition will suffice, simplifying data analysis, especially for those not so skilled in mass spectrometry. By contrast, in forensic toxicology, whether a particular drug has been administered or not, is often unknown, and once a drug (or its metabolites) is detected in a biological matrix, confirmatory analysis is required so that the results can withstand the scrutiny of a legal challenge, necessitating the use of several ion transitions (or full scan).

3. Chapter 3 - Dose/concentration relationships in hair, blood and oral fluids from patients under controlled psychoactive drug administration

3.1. Introduction

As mentioned in the hair analysis review given in chapter 1, this bioanalytical tool is getting more interest and attention by the toxicology(ists) community particularly as it has shown proven histories of single and chronic drug consumption (Kintz 2007a). However, this bio-monitoring tool lacks some features such as the ability of its results to reflect or estimate the relative amount of drug consumption or a clear association with blood drug concentration. This in fact is due to a number of reasons, one of them is the lack of extensive research in this area, principally controlled administration studies. Other reasons/factors are the effects of hair treatments, hair colour, growth rate, external contamination and the heterogeneity of samples.

However, in the case of the other biological matrices like, for example oral fluids, drug level correlation with blood does exist at least for some investigated drugs such as voriconazole (correlation, r=0.95), chloramphenicol (r=0.8), ampicillin (r=0.78), lacosamide (r=0.8), alcohol (r=0.88), salicylic acid (r=0.94), theophylline (r=0.86) (Michael et al. 2010, Koup et al. 1979, Akimoto et al. 1985, Greenaway et al. 2010, Degutis et al. 2004, Levy et al. 1980, Tewthanom et al. 2005). On the other hand, in case of urine it is a completely different story as the drug concentration there is significantly affected by a number of variables; pH of the urine, creatinine clearance, frequency of bladder voiding, water consumption, diabetes, etc (Jones, Karlsson 2005). Therefore, drug concentration in urine would possibly have low correlation with blood concentration and mostly used as an indication of recent drug use.

From the limitations mentioned earlier not all drugs are suitable for detection by hair testing although the majority is. The more basic and lipophilic the drug the higher the concentration will be found in hair. Also the darker the hair the more of the basic drug will be incorporated (Pragst, Balikova 2006). All these characteristics have been taken into consideration in this study as most of the patients have very dark hair and the consumed drugs are basic drugs. In addition to that this study has the advantage of having closely monitored administration of the drugs unlike other published studies which relied on possibly inaccurate self-reported administration.

The choice of specimen type for the present study (whole blood, oral fluids and hair) was based on the following facts:

Whole blood rather than plasma or serum because in a fairly recent bioanalytical meeting scientists highlighted that it could be more accurate to measure drug concentrations in all blood components (whole blood) as most drugs do not solely bind to plasma proteins. In addition, the advances in analytical techniques and the instrumentation ruled out the need to separate the plasma to get cleaner samples or reduce the noise level. Moreover, when measuring plasma it is important to relate it back to blood drug concentration as well as all the pharmacokinetic parameters, whereas in whole blood this is not the case (Beaumont 2010).

Oral fluids and hair are non-invasive promising alternative matrices. Oral fluids have several advantages over the conventional samples (blood and urine), such as the ease of collection and multiple sampling especially with the new collection kits/devices available, providing less risk of infection compared to blood venipuncture, less prone to tampering, much cleaner sample than blood and urine and so may require a shorter extraction time.

Results of some of the previous hair to dose or hair to blood correlation investigation studies in controlled administration settings showed that generally the correlations found were weaker in case of dose to hair and slightly better with hair to blood/plasma concentration. In France, Kintz et al for example found dose/hair correlation coefficients for heroin, 6-MAM and morphine to be r=0.12, 0.25 and 0.64, respectively, in 20 patients receiving controlled heroin doses in a heroin

maintenance programme (Kintz et al. 1998), whereas the total opiate correlation was r=0.346. Kintz's study was repeated in 2005 by a German group on a larger number of subjects (n=46) in a very similar controlled program and they found much better correlation (r=0.66) between total opiates in hair and the given dose. Plasma/hair concentration correlation coefficients for each analyte were r=0.42, 0.58 and 0.69 for heroin, 6-MAM and morphine, respectively (Musshoff et al. 2005). Two other groups have investigated the correlation for methadone between hair concentration and the given dose and they found no significant relationship exists (r=0.1 and r=0.34) (Paterson et al. 2003, Girod, Staub 2001), respectively. Although Girod et al found the correlation very weak between dose and hair level they remarkably found it strong between blood and hair (r=0.8). Moreover, the dose of the anti-psychotic drug clozapine was found to correlate with hair drug concentration at r=0.54 (Cirimele et al. 2000). Three years earlier Pragst and his group suggested that there was no way to estimate the dose or steady state concentration from hair for amitriptyline, clomipramine, doxepine, imipramine and maprotiline (Pragst et al. 1997). In contrast, the antiepileptic phenytoin was found to have a good dose to hair concentration relationship (r=0.88) and dose to plasma (r=0.78) as well, while valproic acid and carbamazepine showed a lower relationship coefficient (r=0.72 and r=0.46, respectively) and this was even lower with plasma (r=0.13 and r=0.14, respectively) (Mieczkowski et al. 2001).

These conflicting results bring up many questions such as were the subjects taking any illicit heroin while in the heroin maintenance program? Was the hair extraction method efficient enough with real samples? Was the number of patients participating enough to draw conclusion from their results? Was the length of hair appropriate or representative of the drug administration period? Answering these questions maybe quite difficult but these things need to be taken into consideration particularly when interpreting results and drawing conclusions, especially if needed or requested by court or any judicial authorities. There are as well inter-individual variations in metabolism which might have contributed also to those inconsistent results.

Procyclidine (Kemadrin – brand name) is a synthetic antagonist for muscarinic and nicotinic cholinergic receptors. It is been used as an anti-dyskinetic, anti-epileptic and anti-Parkinson agent. Currently procyclidine is used widely with psychiatric patients especially those on anti-schizophrenic medications as it has a neuroprotective characteristic that controls the extrapyramidal side effects caused by those medications. It has a half-life of 12.6 hours and a normal dosage regimen of 2.5 or 5mg two to four times a day. Published therapeutic plasma levels ranged from 0.116 to 0.650µg/mL and toxicity level >4µg/mL. Procyclidine is a phenylpropylamine with a tertiary amine group responsible for its basic characteristics (Mehta, Royal Pharmaceutical Society of Great Britain 2000, Ettinger et al. 2003, Hadidi 2004, Kim et al. 2002, Whiteman et al. 1985, Brocks 1999).

Due to its basic nature, extensive prescribing, reported abuse potential and lack of appearance in analytical publications, procyclidine was chosen for this study (El-Haj, Al-Amri & Ali 2011).

Quetiapine (Seroquel) is a clozapine-like dibenzothiazepine compound, an atypical anti-psychotic used for treatment of schizophrenia, schizoaffective disorders, acute mania and depression. Pharmacokinetic data show that it has a half-life ranging between 5 and 7 hours and steady state level is achieved after 48 hours. Normal quetiapine doses range from 100 and up to 800mg per day, while therapeutic serum levels are reported between 70 and 170ng/mL with a toxicity level above 1.8µg/mL. The abuse of this drug has been found to be related to its anxiolytic and sedative properties. (Gerlach et al. 2007, Khazaal et al. 2007, Schulz, Schmoldt 2003, PIERRE et al. 2004, DeVane, Nemeroff 2001a, Winter et al. 2008).

Risperidone (Risperdal), is an atypical anti-psychotic drug belonging to the chemical class of benzisoxazole derivatives. It is commonly used in the treatment of psychoses connected with schizophrenia, Alzheimer's disease, bipolar disorders and irritability in people with autism. It has both selective serotonin 5-HT2 and a dopamine D2 receptor antagonism activity. Wide inter-individual pharmacokinetic variability for risperidone is quite normal. Its average half-life is 20±3 hours. Normal doses range from 2 and up to 8mg per day, while therapeutic serum levels are reported between 10 and 30ng/mL with a toxicity level above 90ng/mL. Paliperidone

(9-OH-risperidone) therapeutic serum level is from 15 and 60ng/mL and its toxicity level is above 80ng/mL. Some papers suggest that most patients taking oral risperidone have 5–10 times more paliperidone in their blood than the parent drug (Schulz, Schmoldt 2003, Xiang et al. 2010, Leon, Wynn & Sandson 2010, Huang et al. 1993, Scordo et al. 1999, Heykants et al. 1994).

Chlorprothixene is a thioxanthene compound from the earlier generation of anti-psychotics still commonly used in some countries for the treatment of schizophrenia, senile dementia and bipolar disorders. It has good sedative properties as well. A recent study showed that it was associated with higher mortality rates in long term treatment behind haloperidol compared to other anti-psychotic medications. The half-life of chlorprothixene is between 8 and 12 hours. The clinical oral dosage range is 50 to 400mg/day. The therapeutic serum level is reported between 30 and 300ng/mL and the toxicity level is above 400ng/mL, while the lethal concentration is reported at 800ng/mL. (Schulz, Schmoldt 2003, Bagli et al. 1999, Raaflaub 1975, Lehman, Steinwachs 1998, Gjerden, Slørdal & Bramness 2010, Di Giovanni, Di Matteo & Esposito 2008).

Haloperidol is a butyrophenone compound and a member of the typical anti-psychotic drugs. It is a potent antidopaminergic and narcoleptic drug very active against severe psychosis symptoms such as delusions, hallucinations and agitation. It possesses a good sedative effect as well on patients and was found to have the highest association with mortality risk among a list of anti-psychotics investigated in Norway. The official recommended dosage is from 1 up to 30mg/day in cases of severe schizophrenia. It has a half-life of 10-30 hours and a therapeutic serum level reported between 4 and 25ng/mL, a toxicity level above 50ng/mL and a lethal level of 500ng/mL (Schulz, Schmoldt 2003, Gjerden, Slørdal & Bramness 2010, Di Giovanni, Di Matteo & Esposito 2008, Hugenholtz et al. 2006, Froemming et al. 1989, Desai et al. 2003).

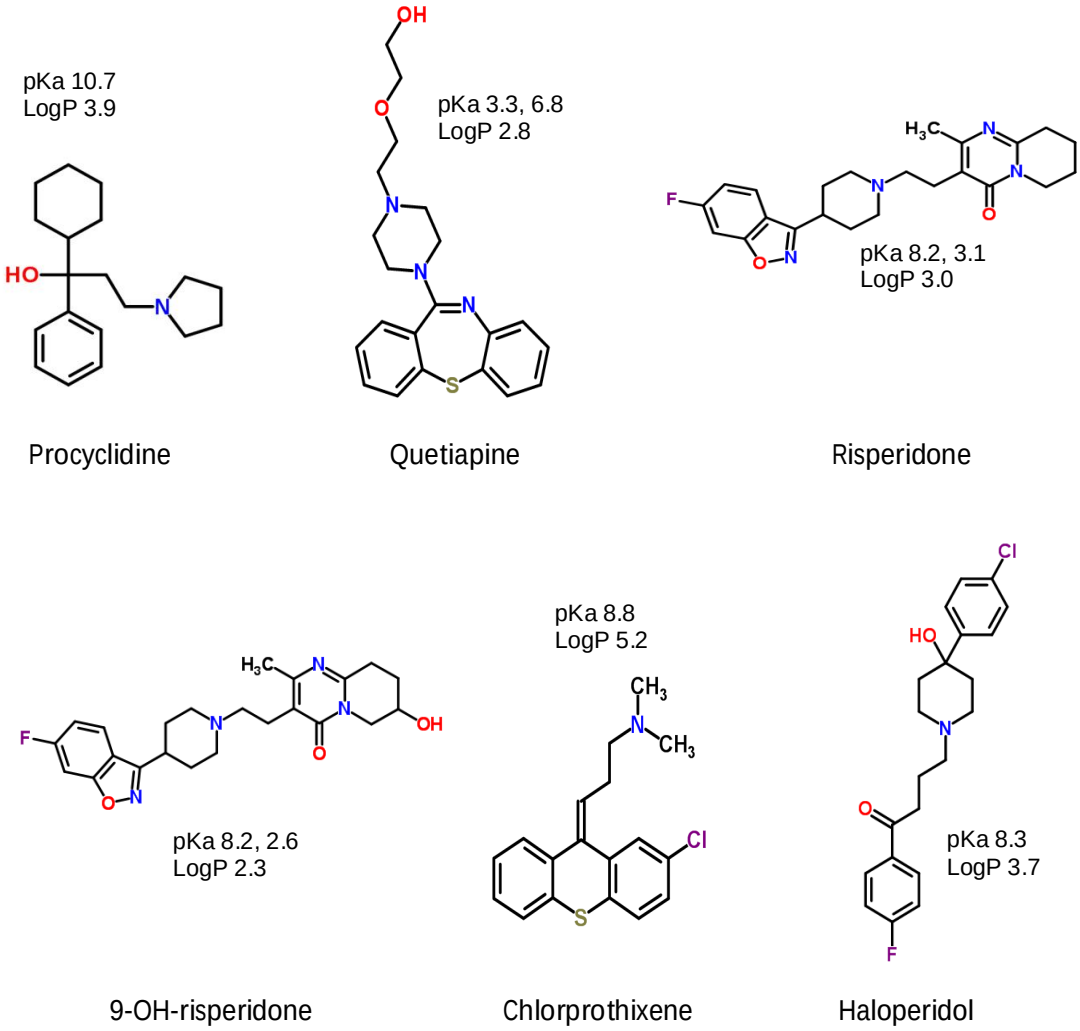


Figure 11: Chemical structures of the analytes (ChemSpider 2011, Baselt 2004)

3.1.1. Patients' compliance and hair analysis

Many factors can play roles in patients' non-adherence to their medication such as alcohol or drug abuse, unawareness of possible health consequences, fear of medication side effects, etc. However, the results of non-compliance can be serious

in some occasions particularly cases like psychiatry where patients have potential of violent behaviour. Recent study showed also that anti-psychotic therapy compliance lower the risk of death by 25% in addition to the management of schizophrenic symptoms (Cullen 2012). Therefore observing compliance with the use of hair analysis can have a great potential if studies carried out and showed significant correlations between doses, blood and hair concentrations of the given medications.

3.1.2. Hydrophilic interaction liquid chromatography

Hydrophilic interaction liquid chromatography (HILIC) is an alternative high-performance liquid chromatography (HPLC) mode for separating polar compounds. It is based on a mixed mode retention mechanisms and it has been reported that HILIC is a modified normal phase liquid chromatography NP-LC, but the separation mechanism used in HILIC is more complicated than that in NP-LC. Like NP-LC, HILIC employs conventional polar stationary phases such as silica, amino or cyano, however the mobile phase used is like those used in the reversed phase RP-LC mode, aqueous-organic (Buszewski, Noga 2012). The retention, unlike traditional RP materials, is based on the formation of a stagnant aqueous layer on the surface of the stationary phase. Analytes of a hydrophilic nature will then partition between the eluent and the aqueous phase, with subsequent phase interactions would be dipole–dipole, ion exchange and/or hydrogen bonding (Dejaegher, Mangelings & Vander Heyden 2008). This separation technique will be employed in this study as its usage with the study's analytes has not been found in literature.

3.2. Aim of the study

The main aim is to investigate drug incorporation rate into hair and to look into the correlation pattern, if it exists between the daily dose, plateau in blood, plateau in oral fluids and plateau in hair. Clinical/forensic bioanalysts/toxicologists would interpret their results more efficiently and more confidently if more data of this kind were available.

3.3. Research subjects and drug administration

The population of the study were middle-eastern male patients (17 for procyclidine, 17 for quetiapine, 14 for risperidone, 14 for chlorprothixene and 10 for haloperidol) aged between 18 and 68 years from the psychology hospital and the addiction treatment centre in Kuwait. Almost all were receiving long term management of their psychological conditions with oral doses of medication. A minimum of 2 weeks treatment with a constant dose was a pre-inclusion condition for hair samples. However, almost all of the patients received the same dose for months. Therefore, the concentration of the drug in their hair should have reached a constant or steady-state level in the near root hair segment. Ethical approval was granted by the research ethics committee at the ministry of health in Kuwait and all the consent forms were signed by the patients both in Arabic and English, copies are shown in Appendix 8.1 and 8.2. Doses of the drugs were different from one patient to another as shown and detailed in Tables 24, 27, 30, 35 and 38. Almost all the patients gave all three samples; hair, blood and oral fluids, except some special cases. Data treatment was carried out anonymously and patients were given total freedom of choice to participate or not and many declined participation.

3.4. Materials and reagents

All chemicals were of HPLC or analytical grade. Procyclidine, risperidone, 9-OH-risperidone, chlorprothixene and haloperidol were purchased from Sigma-Aldrich Co. UK. Quetiapine was purchased from Kinesis UK. Deuterated internal standards haloperidol-D4 and nortriptyline-D3, were purchased from LGC standards, Middlesex UK. Deionised water was prepared on site (ELGA Limited). All consumables (tubes, etc) are from VWR UK. The rest of chemicals used in the solutions below are from Sigma-Aldrich Co. UK.

Other solutions used in this chapter have been mentioned in section 2.2.2.2.

Standard solutions

1000µg/mL or 100µg/mL working standards were prepared with MS grade methanol for procyclidine, quetiapine, risperidone, 9-OH-risperidone, chlorprothixene, haloperidol, nortriptyline-D3 and haloperidol-D4, respectively. All were stored at -20ºC.

Quality control samples (QCs)

QCs were prepared in two concentrations, medium 0.5µg/mL and low 0.05µg/mL. The medium QC samples were prepared by dissolving 50µL of 50µg/mL stock standard of the analyte in 4.95mL pooled drug free blood/oral fluids. The low QCs were prepared by diluting 0.5mL of the medium QC mixture into 4.5mL pooled drug free blood/oral fluids. All the mixtures were thoroughly mixed and divided into 150µL aliquots stored in 0.5mL Eppendorf tubes and kept at -80°C until analysis.

1M phosphoric acid

6.5mL of concentrated 14.7M phosphoric acid (85/100) was mixed with 93.5mL of deionized water, mixed well and stored at room temperature.

Hair extraction solution (MAF)

80mL of 1% formic acid methanol, 10mL of acetonitrile and 10mL of deionized water were measured in a measuring cylinder, mixed well and stored at room temperature.

1M Sodium sulfide solution

24g of Na_2S*9H_2O (MW 240g/mol) pellets were measured into a 100ml volumetric flask and made up to the 100ml mark with deionized water. This was mixed thoroughly and stored at room temperature.

Proteinase K extraction solution (Lucas et al. 2000)

The working solution was based on mixing 25µL of 20mg/mL proteinase K solution with 0.475mL of 5mg/mL dithiothreitol prepared in 10mM Tris buffer pH9 (readymade solution) to make a final concentration of 1mg/ml.

Mobile phase

(A) 999mL de-ionised water and 1mL formic acid (98/100).

(B) 999mL acetonitrile and 1mL formic acid (98/100).

3.5. Samples and data collected

Blank (drug free) hair, blood and oral fluids samples were obtained from laboratory personnel and friends not taking any medication that interfered with the study. Patients' hair samples were taken from the occipital area of the scalp where hair is strongly and constantly growing even in bald subjects. Colours of the hair were black, dark brown or grey and none of the patients had any kind of hair treatment. Each sample was cut as close as possible from the scalp with a targeted amount of around 100mg hair of at least 0.5cm in length. This study focused mainly on the first hair segment (proximal end 0-0.6cm), and should represent roughly drug consumption in the last 2 weeks. All blood and oral fluids samples collected from patients were taken just before their next dose (trough level), in most cases at 6-7am in the morning. Oral fluids samples were taken without stimulation simultaneously with blood samples. The amounts of blood and oral fluids samples were a minimum of 2mL collected in sodium fluoride/potassium oxalate vacutainer and frozen at -20°C for few days then in -80°C until analysis. All the samples were collected in the hospitals by the researcher himself with some help from the nursing staff. Other information such as age, dose and duration of therapy are listed in Tables 24, 27, 30, 35 and 38.

3.6. LC-MS method

Mass spectrometry was chosen for the detection and quantitation in this study as it can provide a very sensitive and very low detection limit (ng/mL and pg/mg)

compared to other analyte detection instruments. The LC-MS analysis was carried out with an HPLC system consisting of a Thermo Scientific Accela 600 pump, autosampler and column compartment. The MS part was an Orbitrab Exactive Mass Spectrometer (ThermoElectron UK) equipped with an electrospray interface set in the positive mode. The ion spray needle was set to 4500 voltage. The heated capillary temperature was set on 320°C, sheath gas flow of 50 arbitrary units and auxiliary gas flow of 17 arbitrary units. The Exactive mass spectrometer is a high resolution instrument with mass accuracy less than 5ppm and capable of running negative and positive scans simultaneously (Moulard et al. 2011).

Chromatography was achieved using a ZIC-HILIC column (150 x 4.6mm 5µm particle size, Hichrom UK) preceded by a guard ZIC-HILIC column (20 x 4.6mm 5µm). The mobile phase consisted of: (A) 999mL de-ionised water and 1mL formic acid (98/100) and (B) 999mL acetonitrile and 1mL formic acid (98/100). The running of the system was isocratic and the percentage of A and B of the mobile phase was 40% of (A) and was pumped at 0.5mL/min. The column oven was off and auto-sampler tray set on 4°C. The volume of injection was 10µL and on no waste mode. Total run time was 7 min.

The high resolution Exactive MS instrument was running on full scan, range from 75 to 1200 amu. Extracted ion chromatograms were based on less than 5 ppm mass deviation window. Chromatographic qualitative and quantitative data acquisition and processing was carried out using Xcalibur 2.2 software (Thermo Fisher Scientific, UK).

3.6.1. Identification of ions results

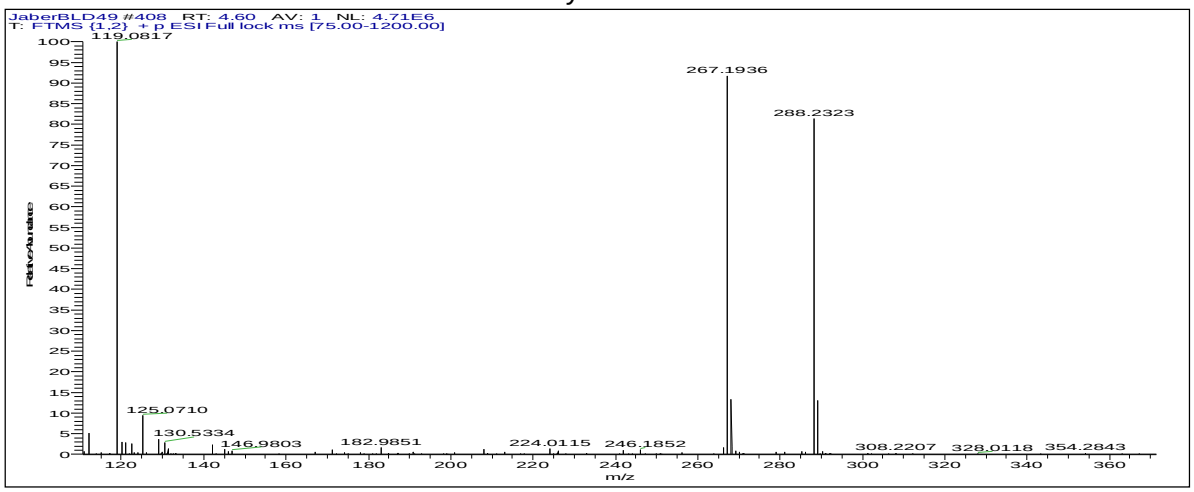
Table 10 shows the analytes' molecular formula, accurate masses, retention times and mass accuracies. Mass accuracy with Exactive MS was generally below 1ppm, except on some occasions below 2ppm, which means that the potential for interference from other components was very little. Figure 12 shows the mass

spectra of each analyte ion after extraction from full scan (75-1200 amu) at the retention time. Retention chromatograms are shown in patients' results, section 0.

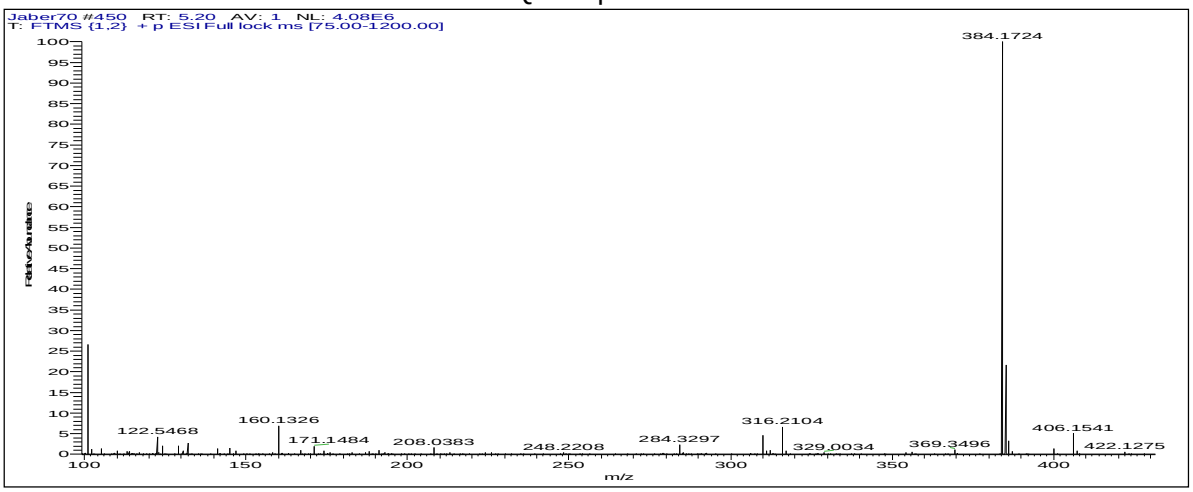
Table 10: Extracted ions of target analytes, internal standards and their relevant data.

Analyte	Formula (+H)	Accurate mass (M+H)	Retention time	Mass accuracy
Procyclidine	$C_{19}H_{30}NO$	288.2323	4.5	0.109 ppm
Quetiapine	$C_{21}H_{26}O_2N_3S$	384.1724	5.2	-1.624 ppm
Risperidone	$C_{23}H_{28}FN_4O_2$	411.2193	5.7	0.219 ppm
9-OH-risperidone	$C_{23}H_{28}FN_4O3$	427.2141	5.0	0.105 ppm
Chlorprothixene	$C_{18}H_{19}ClNS$	316.0922	4.4	0.065 ppm
Haloperidol	$C_{21}H_{24}ClFNO_2$	376.1476	4.4	0.229 ppm
Haloperidol-D4	$C_{21}H_{20}\mathbf{D}_4ClFNO_2$	380.1727	4.4	0.182 ppm
Nortriptyline-D3	$C_{19}H_{19}\mathbf{D_3}N$	267.1936	4.5	0.093 ppm

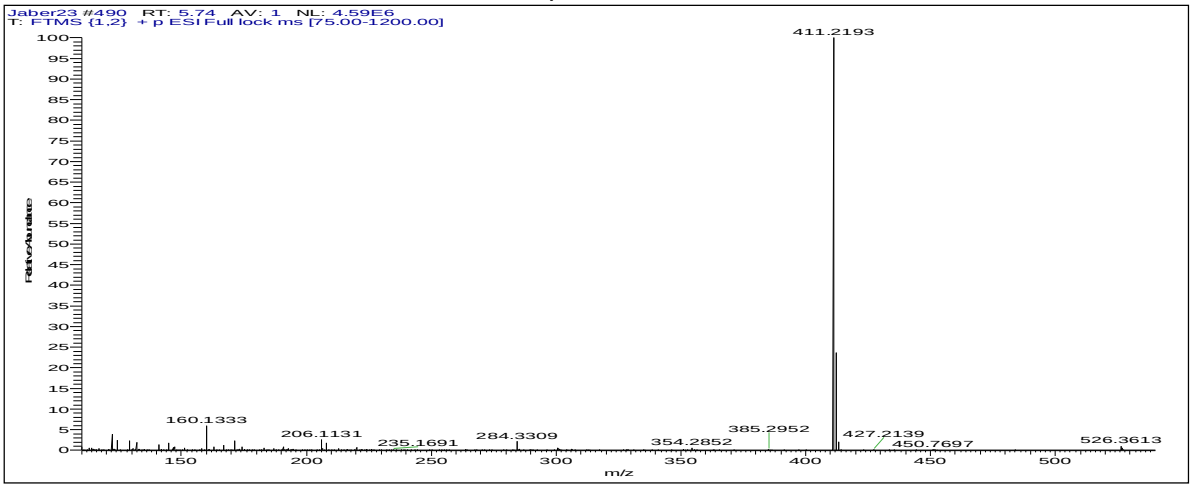
Procyclidine



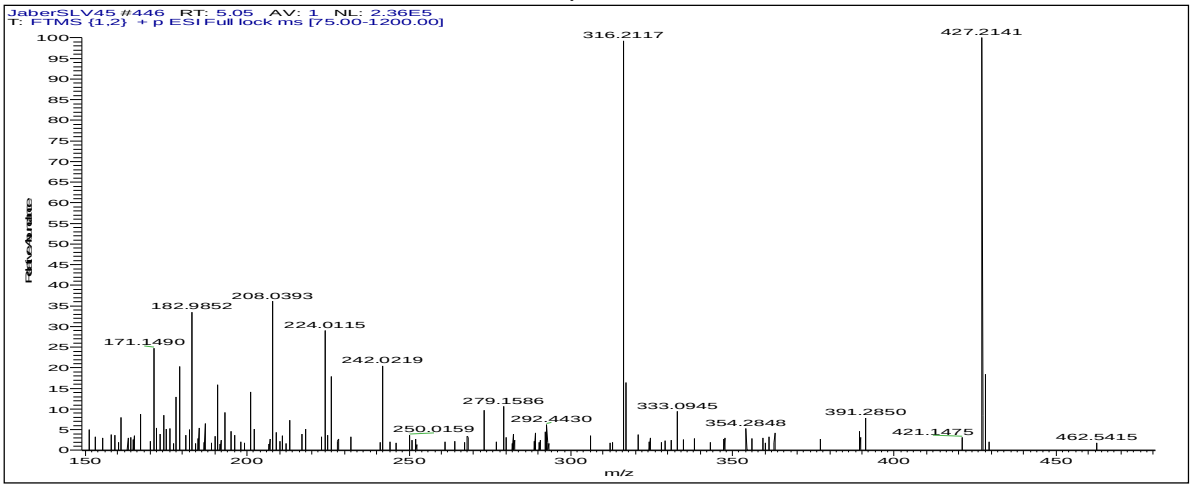
Quetiapine



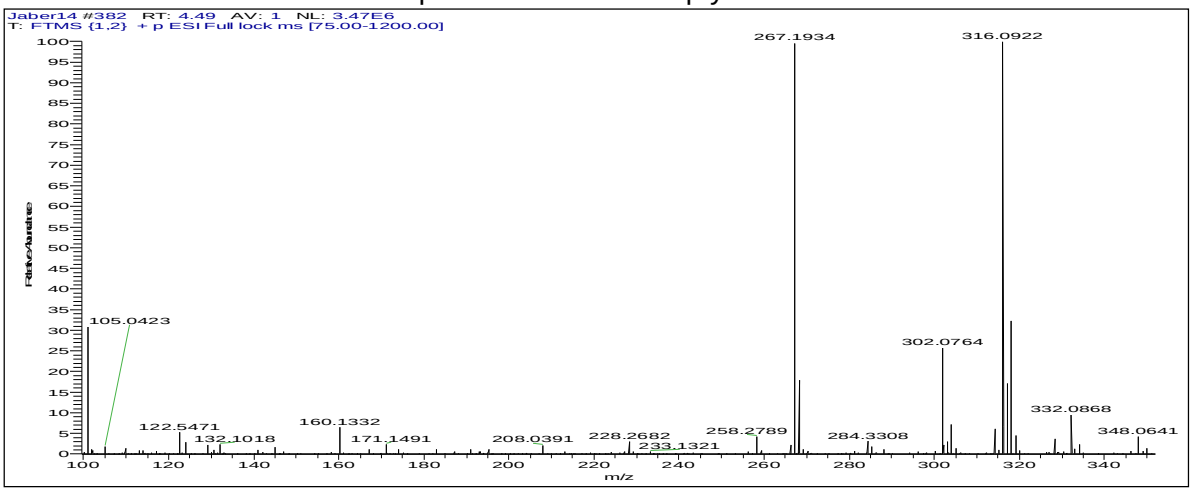
Risperidone



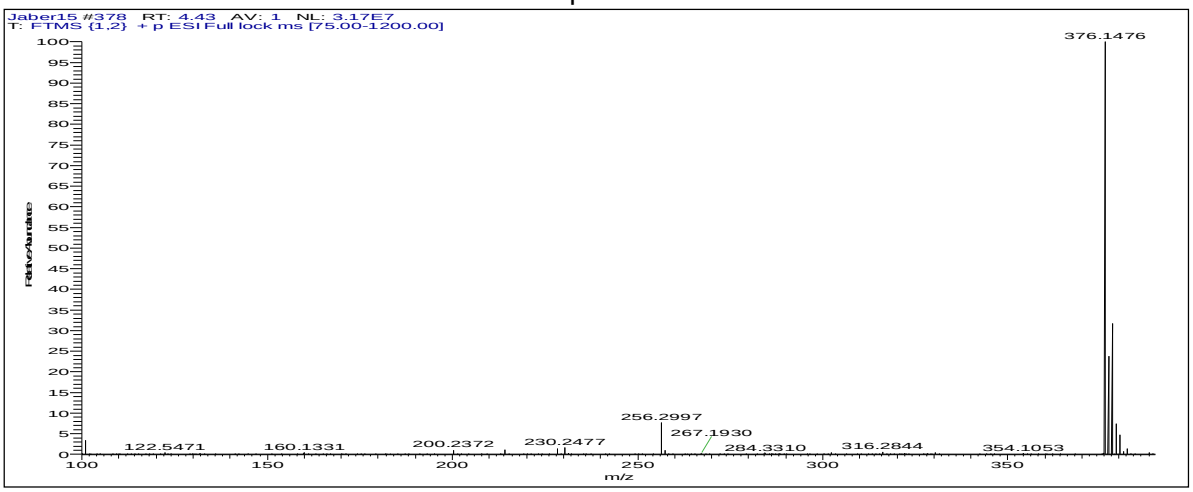
OH-risperidone



Chlorprothixene and Nortriptyline-D3



Haloperidol



Haloperidol-D4

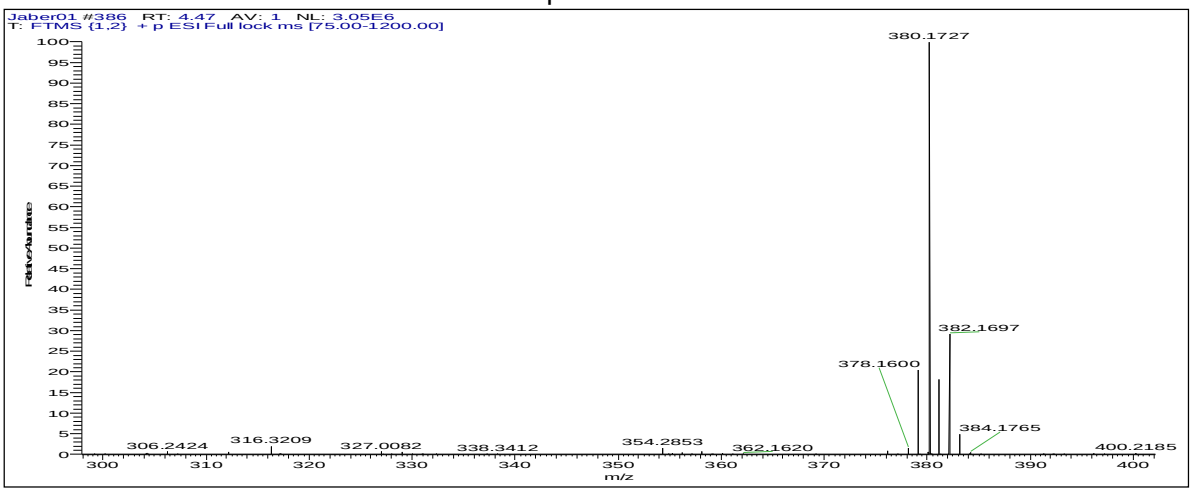


Figure 12: Extracted mass spectra of each analyte and internal standard ion

3.7. Blood and oral fluids analysis

3.7.1. Method development

Methods 1, 2, 3 (0.01, 1M NaOH, Tris Buffer pH9)

Three types of extraction solutions were tested on spiked blood and oral fluids samples in three triplicates. The general extraction procedure was based on placing 100µL of blood/oral fluids samples spiked with 1µg/mL of the analyte (10µL of blood/oral fluids sample was replaced with 10µL of 10µg/mL standard in deionized water and mixed properly) in 4mL polypropylene tubes. Two hundred fifty µL of extraction solution (0.01, 1M NaOH or Tris buffer pH 9) plus 2mL MTBE were added to the samples, mixed on a rotary mixer for 30 min and then centrifuged for 10 min. The solvent layer was transferred to tubes containing 200µL 0.1M phosphoric acid and shaken again for 15 min and centrifuged. Finally, the solvent layer was removed or aspirated, 150µL of 0.01, 1M NaOH or Tris buffer was added plus 0.3mL MTBE, vortex mixed for 2 min and centrifuged. Solvent layers were transferred to 0.3mL auto-sampler vials, evaporated to dryness under nitrogen and reconstituted in 120µL of acetonitrile and then 10µL was injected into the LC-MS system. At the same time, two replicates of unextracted 100µL of calibrators containing 1µg/mL of analytes in methanol were placed in two vials, evaporated to dryness and reconstituted in 120µL of acetonitrile and injected alongside the samples.

Method 4 (Protein crash)

Three replicates of 100µL of blood/oral fluids samples spiked with 1µg/mL of the analyte (10µL of blood/oral fluids sample was replaced with 10µL of 10µg/mL standard in deionized water and mixed properly) placed in 2mL Eppendorf tubes. Acetonitrile (350µL) was added and vortex mixed for 20 min and centrifuged for 30 min. Solvent layers were transferred to vials, evaporated and reconstituted in 120µL acetonitrile. 10µL was injected into the LC-MS system. At the same time, two replicates of unextracted 100µL of calibrators containing 1µg/mL of analytes in methanol were placed in two vials, evaporated to dryness and reconstituted in 120µL of acetonitrile and injected alongside the samples.

3.7.1.1. Results

Extraction with 1M NaOH (Method 2) showed the highest extraction recovery with mean percentages ranging between 58 and 110 for all the analytes. Next was the protein crash method with a percentage of recovery ranging from 46 to 96, while the other two methods (1 and 3) failed to produce any acceptable recoveries as shown in Table 11.

Table 11: Percent of recoveries with four different extraction conditions for 1µg/mL of analytes spiked in blood and oral fluids (n=3)

	Method 1		Method 2		Method 3		Method 4	
Analyte	**0.01M NaOH**	**%CV**	**1M NaOH**	**%CV**	**Tris buffer**	**%CV**	**Protein crash**	**%CV**
Procyclidine	**0.4**	6	**110**	6	**1.4**	46	**49**	8
Quetiapine	**0.1**	132	**90**	6	**0.2**	88	**86**	2
Risperidone	**0.1**	87	**103**	5	**0.2**	68	**77**	11
9-OH-risperidone	**0.1**	140	**85**	2	**0.2**	95	**96**	11
Chlorprothixene	**4.8**	19	**58**	11	**10.6**	51	**46**	20
Haloperidol	**2.4**	27	**93**	6	**8.2**	27	**61**	7
Mean	**1.3**	69	**90**	6	**3.4**	63	**69**	10

3.7.2. Method validation

3.7.2.1. Linearity

Linearity was defined in section 2.2.7.3. It was attained by spiking blank blood and oral fluids samples with a range from 0, 10, 100, 1000 and 10,000ng/mL of each analyte. One µg/mL deuterated internal standard was used. The resulting ratio of peak areas of standards/internal standard was plotted versus the spiked analyte concentrations and linear correlation coefficient (r^2) was calculated. The correlation coefficient (r^2) values indicate the degree of linearity (r^2 should be >0.99).

3.7.2.2. Recovery

Recoveries were estimated by measuring peak areas obtained when the drugs were spiked into blank blood/oral fluids at three concentrations 0.1, 0.5, and 2µg/mL (n=3)

against unextracted/unfiltered standards. This recovery takes into account all the extraction steps including cleaning and matrix effect (absolute). The recovery was calculated as a percentage by dividing the average peak area of the extracted standards over the average peak area of the duplicate unextracted standards and multiplied by 100.

3.7.2.3. Intra-day precision

Precision is the closeness of agreement between a series of tests results obtained under a set of prescribed conditions (Peters, Drummer & Musshoff 2007). The intra-day precision was calculated by spiking blank blood/oral fluids samples with three levels of concentration (low, medium and high, 0.1, 0.5 and 2µg/mL) in five replicates. After extraction, precision was measured by calculating the coefficient of the variation expressed as a percentage (CV %).

3.7.2.4. Inter-day precision

The inter-day precision experiment was a replicate of the intra-day experiment over 5 days and the coefficient of the variation percentage (CV %) was calculated between concentrations of each analyte over these 5 days.

3.7.2.5. LOD and LLOQ

Limit of detection (LOD) and lower limit of quantification (LLOQ) were assessed by spiking blank blood/oral fluids with the analytes in decreasing concentrations. LOD is defined as the lowest detectable concentration of the analyte that differentiates it from the background noise, whereas LLOQ is lowest quantitative concentration that fulfils the required accuracy and precision. The spiked concentrations were 0, 1, 2.5, 5, 10 and 25ng/mL of each analyte and 1µg/mL deuterated internal standard. Ten microliters of the final extracts were injected into the LC-MS system. LOD and LLOQ signal-to-noise cut-offs ratios were 3 and 10, respectively.

LODs were calculated using Equations 2 and 3 (Alfazil, Anderson 2008).

Equation 2

$$Y_{LOD} = Y_B + 3S_B$$

Equation 3

$$LOD = (Y_{LOD} - Y_B)/m$$

where Y_B is the intercept, S_B is the standard error of the regression line and m is the gradient.

LOQs were calculated using Equations 4 and 5.

Equation 4

$$Y_{LOQ} = Y_B + 10S_B$$

Equation 5

$$LOQ = (Y_{LOQ} - Y_B)/m$$

3.7.2.6. Method validation results

Linearity results showed a good regression with r^2>0.99 for all analytes over the calibration line ranging from LOQ to 10,000ng/mL in spiked blood and oral fluids samples as listed in Table 12. LOD and LLOQ were 0.3-0.9 and 1-3.1ng/mL, respectively, for all analytes as summarized in Table 12.

Table 12: Linear correlation coefficients, LODs and LLOQs of analytes in blood and oral fluids

Analyte	Blood			Oral fluids		
	Linear correlation coefficient (r^2)	LOD ng/mL	LLOQ ng/mL	Linear correlation coefficient (r^2)	LOD ng/mL	LLOQ ng/mL
Procyclidine	0.999	0.8	2.7	0.999	0.5	1.6
Quetiapine	0.999	0.3	1.1	0.999	0.4	1.2
Risperidone	0.998	0.3	1.0	0.998	0.3	1.0
9-OH-risperidone	0.998	0.8	2.7	0.998	0.8	2.7
Chlorprothixene	0.999	0.6	2.0	0.999	0.3	1.0
Haloperidol	0.999	0.7	2.3	0.999	0.3	1.0

When the absolute recovery experiment was carried out taking into account all the extraction steps and the matrix effect it gave a lower recovery with spiked blood than with oral fluids. Blood recovery results were between 48-75% with CVs from 3-19% for the means of all the three concentrations (0.1, 0.5 and 2µg/mL), while oral fluids recoveries ranged from 57 to 93% with CVs from 6-26% for the means of the three levels. The results are summarized in Tables 13 and 14.

Table 13: Absolute recovery (RE%) results for 0.1, 0.5 and 2µg/mL of analytes spiked in blood (n=4)

Analyte	100ng/mL (RE%)	%CV	500ng/mL (RE%)	%CV	200ng/mL (RE%)	%CV	Mean RE (CV)
Procyclidine	46	4	48	4	49	4	48 (3)
Quetiapine	65	5	72	3	87	4	75 (15)
Risperidone	60	6	80	1	75	5	72 (15)
9-OH-risperidone	48	9	68	2	65	4	60 (18)
Chlorprothixene	44	4	53	4	64	5	54 (19)
Haloperidol	62	1	75	1	70	4	69 (10)

Table 14: Absolute recovery (RE%) results for 0.1, 0.5 and 2µg/mL of analytes spiked in oral fluids (n=4)

Analyte	100ng/mL (RE%)	%CV	500ng/mL (RE%)	%CV	200ng/mL (RE%)	%CV	Mean RE (CV)
Procyclidine	72	4	90	8	79	2	80 (11)
Quetiapine	51	3	85	3	84	3	73 (26)
Risperidone	87	4	97	2	95	3	93 (6)
9-OH-risperidone	81	3	97	4	87	2	88 (9)
Chlorprothixene	44	2	64	3	62	1	57 (19)
Haloperidol	97	1	97	1	86	1	93 (7)

Over three concentrations, 100, 500 and 2000ng/mL, intra- and inter-day precision results for blood QC samples showed acceptable CV percentages of ≤17% for all analytes except only the set of 500ng/mL for inter-day results where the precision was between 9-38% as shown in Table 15. Similarly, with oral fluids QC intra- and inter-day precision results showed good precision (≤12%) with all samples apart from only three occurrences as listed in Table 16.

Table 15: Inter-day and intra-day precision results in blood

Analyte	Inter-day mean (CV%) n=4			Intra-day mean (CV%) n=4		
	100ng/mL	500ng/mL	2000ng/mL	100ng/mL	500ng/mL	2000ng/mL
Procyclidine	92 (17)	502 (9)	1995 (6)	87 (7)	515 (4)	2030 (9)
Quetiapine	82 (10)	374 (38)	1992 (7)	89 (7)	430 (3)	1942 (6)
Risperidone	90 (10)	444 (26)	1926 (12)	95 (15)	502 (8)	2152 (7)
9-OH-risperidone	93 (15)	411 (29)	1987 (7)	100 (8)	498 (4)	2053 (8)
Chlorprothixene	93 (13)	469 (32)	2029 (7)	92 (11)	526 (7)	2052 (5)
Haloperidol	99 (8)	451 (27)	1945 (8)	95 (7)	508 (9)	1941 (8)

Table 16: Inter-day and intra-day precision results in oral fluids

Analyte	Inter-day mean (CV%) n=4			Intra-day mean (CV%) n=4		
	100ng/mL	500ng/mL	2000ng/mL	100ng/mL	500ng/mL	2000ng/mL
Procyclidine	94 (12)	459 (10)	1870 (6)	90 (5)	466 (10)	1813 (2)
Quetiapine	95 (28)	501 (8)	2205 (5)	104 (6)	525 (5)	2135 (6)
Risperidone	97 (10)	489 (9)	2034 (6)	98 (6)	453 (5)	2115 (3)
9-OH-risperidone	101 (7)	469 (5)	1932 (4)	99 (4)	470 (4)	1916 (5)
Chlorprothixene	96 (30)	394 (33)	2014 (6)	111 (3)	500 (6)	2081 (4)
Haloperidol	100 (10)	493 (12)	2177 (5)	98 (7)	522 (5)	2249 (2)

3.8. Hair analysis

3.8.1. Sample preparation

3.8.1.1. Decontamination

The decontamination process was achieved by using three washing steps with three solvents and one aqueous solution: 0.01M phosphate solution (pH6), dichloromethane and propanol/acetone (1:1). The uncut hair was put in a 6ml glass tube, then 2mL of the first solvent added, followed by 1 min vortexing. After that the solvent was decanted into a separate tube where all the following washing waste solvents were collected for the same sample. The decanting process was done with the use of a plastic pipette or in case of short hair where the hair was scattered in the tube, a piece of fabric or textile was used to hold the hair inside the tube and filter the washing solution out. After the last washing step (2mL propanol/acetone) the hair was left to dry at room temperature. This decontamination method was found to be the quickest and most efficient by the researcher, as there is no

agreement or consensus on specific decontamination method among hair analysts or in literature (Kintz 2007a).

3.8.1.2. Segmentation

After the hair dried segments of 0-0.5cm each were cut in small pieces (1–2mm) with scissors onto a piece of paper and weighed directly. Mostly to ease the calculation 25 or 30mg of hair was taken but, if not, any available weight was considered, and then segment snippets were placed into a 2ml Chromacol glass vial with a screw cap.

3.8.2. Optimization of extraction

Four extraction methods have been tested by applying them on authentic patient samples reduced into 1-2mm snippets and mixed thoroughly after washing. These patients' hair samples were chosen based on their availability in large amount, being darker in colour and being from patients taking high doses of the analytes. The recovery of the following four extraction methods was measured by the use of five 50µL unextracted calibrators 0.01, 0.02, 0.05, 0.1, 0.5, 1, 2µg/mL (~ng/mg) with 50µL of 1µg/mL IS (nortriptyline-D3) prepared at the same time as the samples but kept in the fridge during sample incubation and then evaporated with the samples and reconstituted in 160µL of acetonitrile and run simultaneously with the samples.

Extraction method 1 (1M NaOH)

Twenty five mg of washed blank(drug free)/patient hair was incubated in triplicate with 0.5mL of 1M NaOH for 6 hours in a water bath (45°C) to allow for homogenization of the hair. Homogenates were then extracted with 2mL MTBE by 30min rotary mixing and 10 min centrifugation (3.5krpm). Solvent layers were transferred into new tubes containing 200µL 0.1M phosphoric acid then mixed on a rotary mixer for 15min and the solvent layer was removed afterward. 100µL of 1M NaOH and 300µL of MTBE were added and vortex mixed for 1 min and centrifuged.

The solvent layer evaporated and the residue reconstituted in 100µL of acetonitrile. 10µL was injected to the LC-MS system.

Extraction method 2 (1M Na_2S)

Similar to method 1, 25mg of washed blank/patient hair was incubated in triplicate with 0.5mL of 1M Na_2S for 6 hours in a water bath (45°C) to allow for homogenization of the hair. Homogenates were then extracted with 2mL MTBE by 30min rotary mixing and 10 min centrifugation. Solvent layers were transferred into new tubes containing 200µL 0.1M phosphoric acid, then mixed on rotary mixer for 15min and the solvent layer was removed afterwards. 100µL of 1M NaOH and 300µL of MTBE were added and vortex mixed for 1 min and centrifuged. The solvent layer evaporated and the residue reconstituted in 100µL of acetonitrile. 10µL was injected to the LC-MS system.

Extraction method 3 (MAF)

After weighing 25mg of washed blank/patient hair, samples were incubated in three replicates in 1mL of extraction solution (0.1% formic acid methanol:acetonitrile:deionized water/80:10:10) for 6 hours at 45°C. After centrifugation at 3.5krpm for 10 min the extraction solutions were dried out and reconstituted in 300µL 0.1M NaOH and 300µL MTBE were added and vortex mixed for 1 min and centrifuged. The solvent layer was evaporated and the residue reconstituted in 100µL of acetonitrile. 10µL was injected to the LC-MS system.

Extraction method 4 (Proteinase K)

Twenty five milligrams of washed blank/patient hair was incubated in triplicate with 0.5mL of Proteinase K extraction solution (described in section 3.4) for 6 hours in a water bath (45°C) to allow for homogenization of the hair. Homogenates were then extracted with 2mL MTBE by 30min rotary mixing and 10 min centrifugation (3.5krpm). Solvent layers were transferred into new tubes containing 200µL 0.1M phosphoric acid, then mixed on a rotary mixer for 15min and the solvent layer was removed afterwards. 100µL of 1M NaOH and 300µL of MTBE were added and vortex mixed for 1 min and centrifuged. The solvent layer evaporated and the

residue reconstituted in 100µL of acetonitrile. 10µL was injected into the LC-MS system.

3.8.2.1. Extraction optimization results

Comparison between the four methods revealed that method 3 (MAF) gave the highest recoveries from the authentic hair samples for procyclidine, chlorprothixene and haloperidol, 982, 368 and 61pg/mg, respectively. Method 1 (1M NaOH) gave the highest recoveries for quetiapine and risperidone, 3125 and 1192pg/mg, respectively. Method 4 showed superiority only with 9-OH-risperidone (22pg/mg), while method 2 (1M Na_2S) showed no lead in recovery of any of the analytes as shown in Table 17 and Figure 13. Accordingly, a combination of method 1 and 3 was considered for the analysis of the analytes as described below.

Developed extraction method (Combined methods 1 and 3)

Incubation with 1mL MAF solution (16hr/40°C)

↓

Centrifugation 7min

supernatant transferred to new tubes (1st extraction tubes) ↓ dried under nitrogen and reconstituted in 0.4mL deionized water

to remaining residue 0.4mL 1M NaOH added incubated (3hr/50°C)→Centrifuge ↓ liquid layer transferred to 1st extraction tubes

2mL MTBE added

windmill mixed 20min→centrifuged

solvent transferred to tubes containing 0.2mL 0.1M phosphoric acid

windmill mixed 15min→centrifuged→ solvent removed→ 0.1mL 1M NaOH and 0.3mL MTBE added vortex mixed 1min→centrifuged

solvent transferred to 0.3mL vials → dried →

reconstituted in 100µL acetonitrile

10µL injected into LC-MS

Table 17: Extraction optimization results from authentic/patient hair samples in pg/mg of analytes (n=3)

	Method 1		Method 2		Method 3		Method 4	
Analyte	**1M NaOH**	**% CV**	**1M Na_2S**	**% CV**	**MAF**	**% CV**	**Proteinase K**	**% CV**
Procyclidine	457	16	132	40	**982**	4	635	15
Quetiapine	**3125**	6	1461	17	655	3	871	13
Risperidone	**1192**	5	206	18	235	3	592	9
9-OH-risperidone	0	0	0	0	12	11	**22**	4
Chlorprothixene	355	13	117	32	**368**	10	36	21
Haloperidol	40	15	26	5	**61**	13	9	9

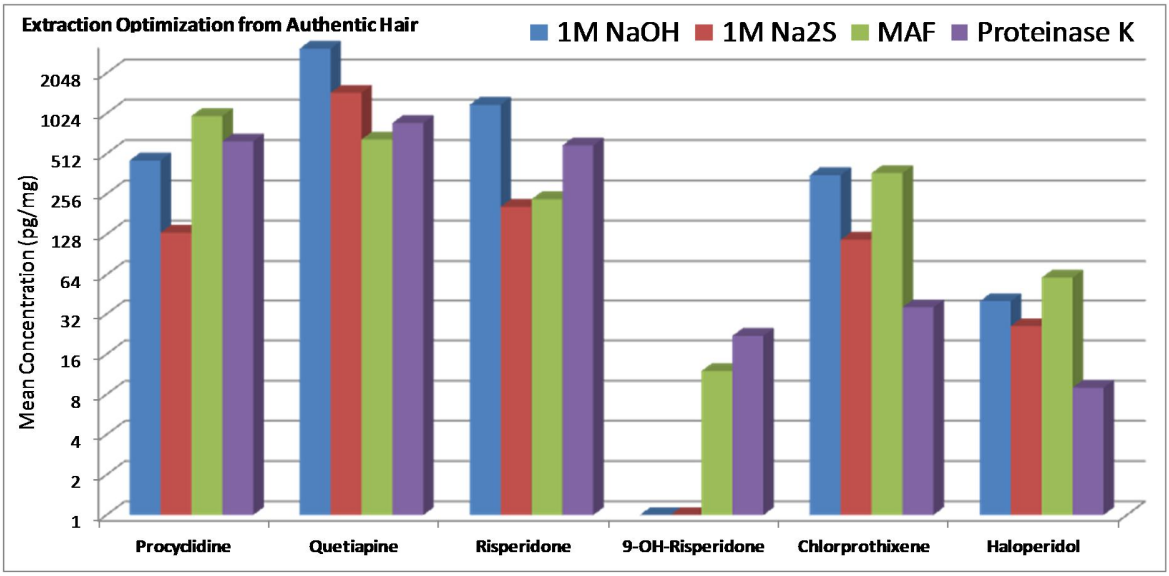


Figure 13: Extraction optimization results from authentic/patient hair samples

3.8.3. Method validation

Essential method validation experiments were carried out for the combined method and achieved by following the industrial guidelines for bioanalysis method validation

stated by the FDA and other sources (Peters, Drummer & Musshoff 2007, FDA 2001, Shah et al. 2000).

3.8.3.1. Linearity

Linearity was described in section 2.2.7.3. It was assessed by spiking blank hair samples with 0, 10, 50, 100, 500, 1000, 10000, 40000pg/mg of each analyte and 1ng/mg deuterated internal standard. The resulting ratio of peak areas of standards/internal standard was plotted versus the spiked analyte concentrations and linear correlation coefficient (r^2) calculated. The correlation coefficient (r^2) value indicates the degree of linearity (r^2 should be >0.99).

3.8.3.2. Stability

Two experiments testing the stability of the analytes were been done, stability with method 1 (NaOH) and stability during the whole extraction/incubation process with the selected optimized extraction method (combined 1 and 3). Both were assessed by preparing matrix-free QC samples at a concentration of 0.5ng/mg in triplicate. Deuterated external standards were added at the end of the extraction procedure and samples were run together with unextracted standards spiked at the same concentrations.

3.8.3.3. Recovery

Recoveries were estimated by measuring peak areas obtained when the drugs were spiked into blank hair (in extraction solution) at three concentrations 0.1, 0.5, and 2ng/mg (n=3) against unextracted/unincubated standards. This recovery takes into account all the extraction steps including clean up, incubation and matrix effect (absolute). The recovery was calculated as a percentage by dividing the average peak area ratio of the extracted standards by the average peak area ratio of the duplicate unextracted standards and multiplied by 100.

3.8.3.4. Matrix effect

The matrix effect is principally the ion suppression or enhancement of analyte ionisation caused by co-eluting compounds from the biological matrix (urine, blood, hair, etc). The aim of this experiment was to acquire more accurate data on the extent of matrix influence on the results when matrices come from different individuals or different types of hair. As proposed by Matuszewski et al the matrix effect was assessed by comparing the mean peak area ratio of product ion/internal standard of extracted blank hair samples from three different sources (dark brown, baby-brown and white) spiked with 0.1ng/mg low standard solution following filtration (i.e. after extraction) with the mean peak area ratio of product ion/internal standard of unextracted standards prepared in mobile phase at equivalent concentrations (Matuszewski et al., 2003). The samples were decontaminated, segmented and extracted according to the procedures described in sections 3.8.1.1 and 3.8.1.2. The percentage of the matrix effect was calculated by dividing the mean of the extracted samples by the mean of unextracted ones according to Equation 1 (page 59).

This percentage represents the absolute ME taking into account ion suppression and ion enhancement. It shows the difference in response between a standard present in the sample of hair extract and the same standard present in a neat solution without the presence of the matrix. A 100% value indicates that the standard's MS response from the neat solution was the same as the standard response from the hair extracts and no matrix effect was observed. Accordingly <100% value indicates ion suppression while >100% value indicates ion enhancement. Because hair was from different types of hair, they may contain different amounst of endogenous compounds that maybe undetected and co-elute with the analytes of interest. Therefore the ionisation of the analytes of interest could be affected, resulting in a decreased or increased MS response.

3.8.3.5. Intra-day precision

Precision was described in section 3.7.2.3. However, the intra-day precision for hair analysis was made by spiking the blank hair samples with three levels of concentration (low, medium and high) in five replicates. After extraction, precision was measured by calculating the coefficient of variation expressed as a percentage (CV%).

3.8.3.6. Inter-day precision

The inter-day precision experiment was a replicate of intra-day experiment over 5 days and the coefficient of variation percentage (CV%) was calculated between concentrations of each analyte over these 5 days.

3.8.3.7. LOD and LLOQ

Limit of detection (LOD) and lower limit of quantification (LLOQ) were assessed by spiking blank hair with the analytes in decreasing concentrations. LOD is defined as the lowest detectable concentration of the analyte that differentiates it from the background noise, whereas LLOQ is lowest quantitative concentration that fulfills the required accuracy and precision. The spiked concentrations were 1, 2.5, 5, 10, 25, 50 and 100pg/mg of each analyte and 1ng/mg deuterated internal standard. All were mixed with 25mg of washed blank hair, incubated and extracted according to the selected method. Ten µL were injected into the LC-MS system. Signal-to-noise ratio was considered to calculate LOD and LLOQ, and 3 and 10, respectively, were used as cut-offs. LODs and LLOQs were calculated using

Equation 2-5 (page 91).

3.8.3.8. Method validation results

Linearity results showed a good regression with r^2>0.99 for all analytes over the calibration line ranging from LLOQ to 40,000pg/mg in spiked hair samples as listed

in Table 18. LOD and LLOQ were 1.4-5.8 and 4.7-19.5pg/mg, respectively, for all analytes as summarized in Table 18. Lower calibrants graphs are listed in appendix 8.6.

Table 18: Linear correlation coefficients, LODs and LLOQs of analytes in hair

Analyte	Linear correlation coefficient (r^2)	LOD pg/mg	LLOQ pg/mg
Procyclidine	0.998	2.0	6.6
Quetiapine	0.994	1.4	4.7
Risperidone	0.999	2.5	8.6
9-OH-risperidone	0.996	1.9	6.4
Chlorprothixene	0.997	5.8	19.5
Haloperidol	0.998	4.4	14.7

Three QC concentrations, 100, 500 and 2000pg/mg were analysed in five replicates for intra- and inter-day precision from spiked hair and revealed acceptable CV percentages of <20% for all analytes excluding three samples (≤26%) as shown in Table 19.

Table 19: Inter-day and intra-day precision results in hair

Analyte	Interday mean (CV%) n=5			Intraday mean (CV%) n=5		
	100pg/mg	500pg/mg	2000pg/mg	100pg/mg	500pg/mg	2000pg/mg
Procyclidine	105 (24)	516 (9)	1808 (7)	99 (1)	563 (3)	1752 (2)
Quetiapine	76 (10)	465 (10)	2280 (3)	99 (2)	510 (4)	2013 (2)
Risperidone	64 (26)	506 (7)	1966 (2)	121 (21)	444 (5)	1855 (2)
9-OH-risperidone	88 (14)	552 (1)	1889 (5)	110 (2)	572 (1)	2152 (6)
Chlorprothixene	102 (8)	617 (19)	2049 (7)	95 (3)	556 (4)	2258 (2)
Haloperidol	102 (11)	480 (3)	2041 (3)	102 (7)	467 (6)	2186 (2)

Results of stability during incubation/extraction with method 1 (NaOH) showed that all analytes were stable under the alkaline conditions with stability of >52% except OH-risperidone (~30% stability) as illustrated in Table 20. However, stability of the selected combined method gave >78% recovery for all analytes as shown in Table 21.

Table 20: Analytes stability during 1M NaOH incubation (n=3)

Analyte	0.5ng/mg (%)	%CV
Procyclidine	0.30 (60)	20
Quetiapine	0.47 (94)	10
Risperidone	0.49 (98)	11
9-OH-risperidone	0.16 (32)	21
Chlorprothixene	0.26 (52)	17
Haloperidol	0.44 (88)	8

Table 21: Analytes stability during optimized analysis method (n=3)

Analyte	0.5ng/mg (%)	%CV
Procyclidine	0.39 (78)	5.5
Quetiapine	0.49 (98)	5.9
Risperidone	0.53 (106)	3.5
9-OH-risperidone	0.52 (104)	3.4
Chlorprothixene	0.39 (78)	9.0
Haloperidol	0.39 (78)	4.9

The mean absolute recovery results of the combined method after spiking hair samples with three different concentrations in triplicate were between 71 and 101% and 6-13 CV%, details shown in Table 22.

Table 22: Combined method absolute recovery (RE%) results for 0.1, 0.5 and 2ng/mg of analytes spiked in hair (n=3)

Analyte	0.1ng/mg (RE%)	%CV	0.5ng/mg (RE%)	%CV	2ng/mg (RE%)	%CV	Mean RE (CV)
Procyclidine	67	5	77	6	69	4	71 (7)
Quetiapine	83	2	98	6	80	16	87 (11)
Risperidone	104	3	106	4	94	12	101 (6)
9-OH-risperidone	92	6	104	3	92	6	96 (7)
Chlorprothixene	62	10	77	9	80	11	73 (13)
Haloperidol	74	21	78	5	63	9	72 (11)

Some ion suppression was shown during the matrix effect study with procyclidine results only, 85% (7%CV). However, other analytes showed no effect of matrix with 98 to 104% recovery and 1-4%CV as illustrated in Table 23.

Table 23: Matrix effect (ME) results for 0.1ng/mg spiked hair samples (n=3)

Analyte	% of ME	% CV
Procyclidine	85	7
Quetiapine	98	3
Risperidone	104	3
9-OH-Risperidone	104	4
Chlorprothixene	98	1
Haloperidol	99	2

3.9. Patients' results

3.9.1. Procyclidine

Blood results from the 17 patients receiving procyclidine were all positive with concentrations ranging from 30 to 357ng/mL. However, the corresponding oral fluids samples were short of two samples not given by those patients. Oral fluids samples were generally within relatively similar pre-dose concentration range as blood, from 7 to 395ng/mL. As for hair, all patients were positive except one and drug levels were between 118 and 5624pg/mg as shown in Table 24. Some chromatograms of patients' results and calibrators are shown in Figure 14.

Table 24: Procyclidine patients' hair, blood and oral fluids trough levels

				Proximal (0-0.6 cm)		Pre-dose levels	
Subject	Age (years)	Daily dose	Duration of therapy	Hair colour	Hair conc. pg/mg	Blood conc. ng/mL	Oral fluids conc. ng/mL
1	46	15mg (5X3)	>Month	Black	**1101**	**67.7**	**9.6**
2	53	15mg (5X3)	>Month	Grey	**2959**	**245.6**	**32.9**
3	32	15mg (5X3)	>Month	Black	**1295**	**164.6**	**34.3**
4	48	15mg (5X3)	>Month	Grey	**3521**	**119.8**	**52.9**
5	68	10mg (5X2)	>Month	Grey	**1062**	**48.9**	**144.8**
6	58	5mg (5X1)	>Month	Grey	**1450**	**61.5**	**8.2**
7	38	10mg (5X2)	>Month	Black	**5624**	**356.7**	**125.3**
8	52	10mg (5X2)	>Month	Black	**4790**	**246.9**	**NA**
9	47	5mg (5X1)	>Month	Dark Grey	**939**	**30.2**	**6.7**
10	41	5mg (5X1)	>Month	Black	**118**	**45**	**12.5**
11	48	10mg (5X2)	>Month	Black	**1962**	**73.4**	**13.6**
12	28	15mg (5X3)	>Month	Black	**2024**	**187.2**	**19**
13	46	5mg (2.5X2)	29 days	black	**395**	**63.2**	**NA**
14	44	15mg (5X3)	>Month	Black	**271**	**138.8**	**57.8**
15	33	15mg (5X3)	>Month	Black	**2722**	**165**	**395.2**
16	56	10mg (5X2)	>Month	Grey	**2441**	**38.9**	**34.1**
17	50	10mg (5X2)	>Month	Dark brown	**4446**	**274.6**	**9.2**

NA: Sample not available. NF: Drug not found.

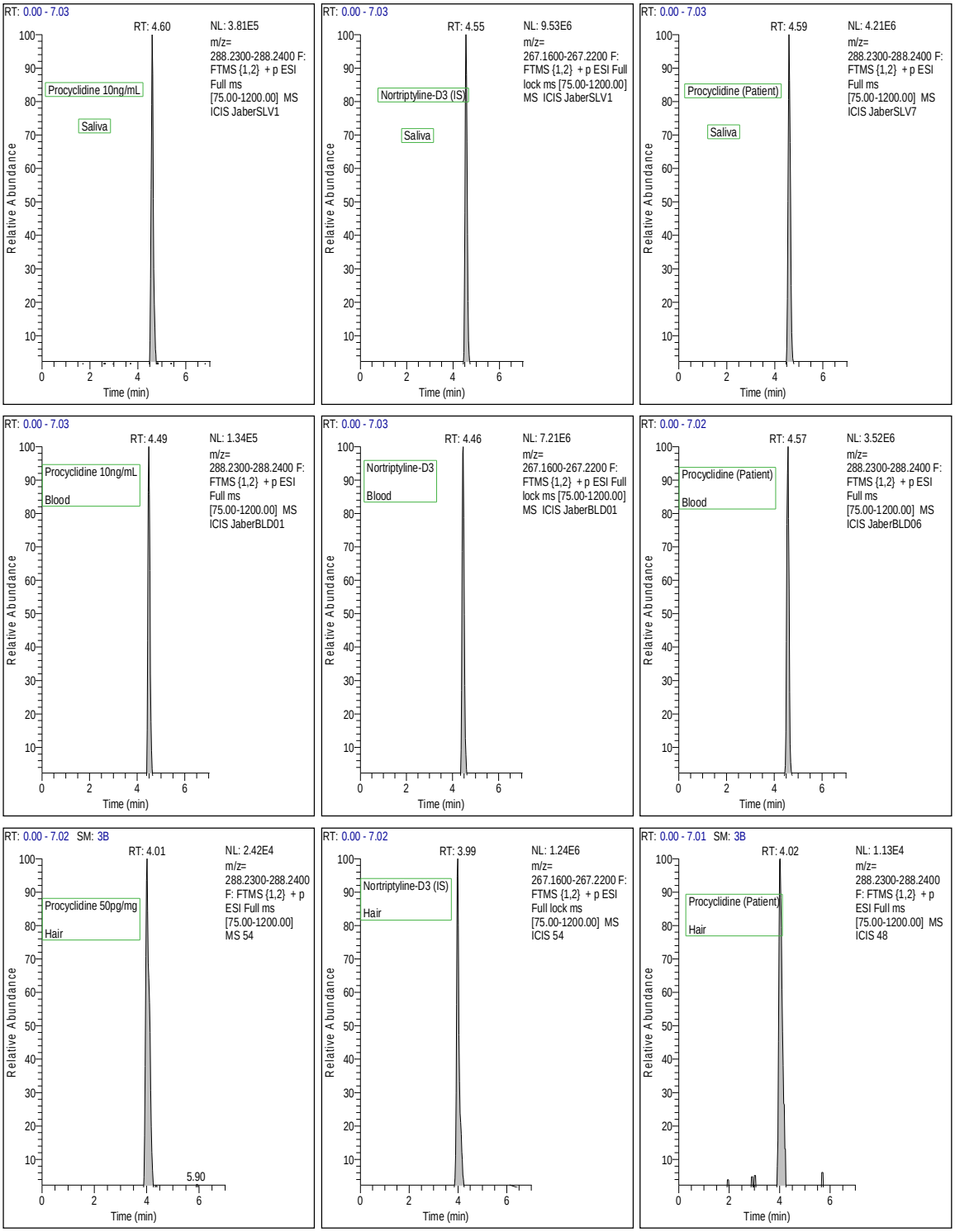


Figure 14: Chromatograms of extracted procyclidine and IS from low calibrators and patient's oral fluids, blood and hair samples

The Pearson correlation coefficient (r) test used for calculating the correlations between results showed that procyclidine's dose and blood level has r=0.38 with p=0.125 , dose/oral fluids level has r=0.28 with p=0.307 and dose/hair level has

r=0.22 with p=0.391 . However, hair and blood levels revealed significant correlation with r=0.83 and p=<0.001, while, hair/oral fluids levels and blood/oral fluids levels did not show good correlation with r=0.21 (p=0.446) and r=0.19 (p=0.491), respectively. All the details are shown in Figure 15, Tables 25 and 26.

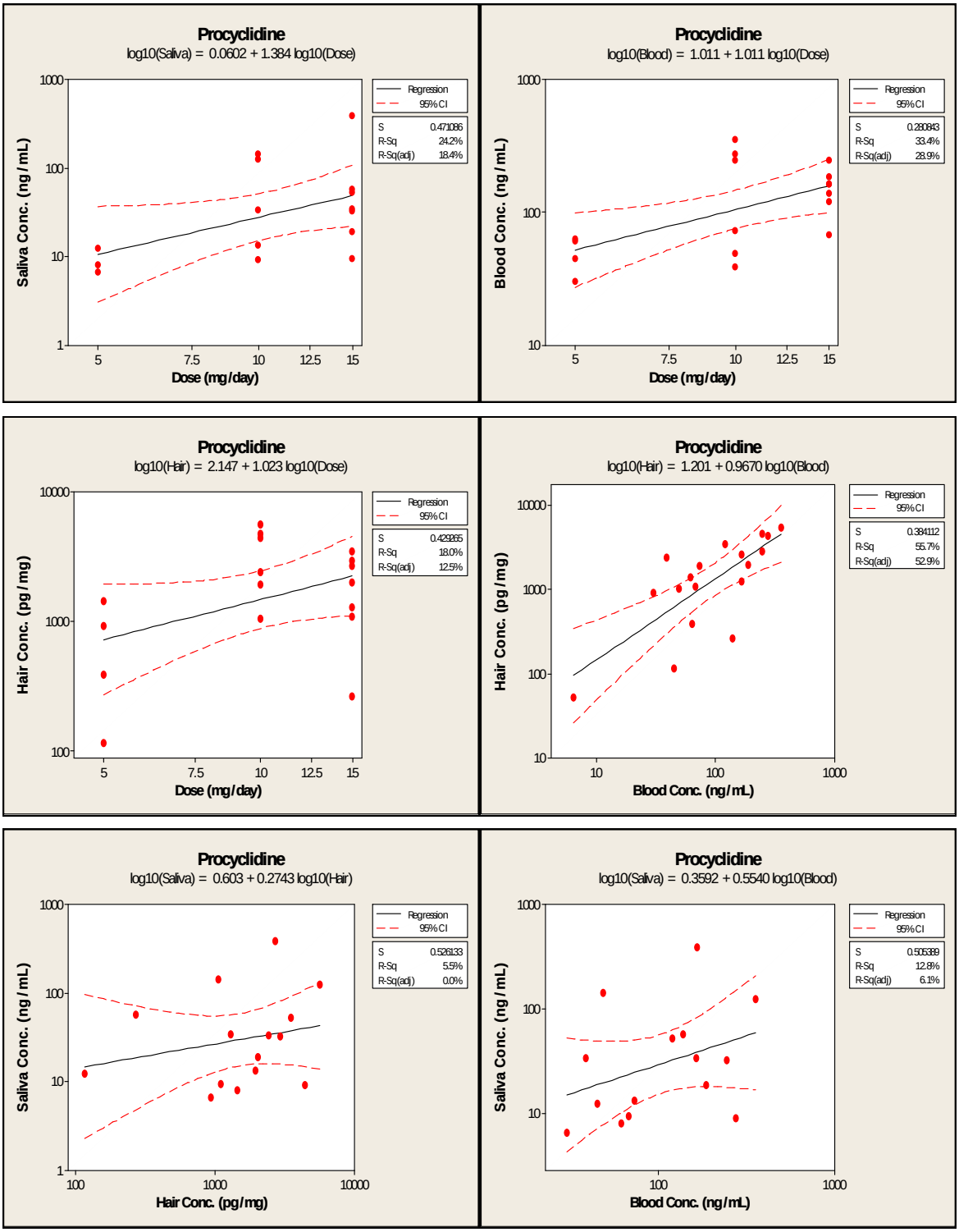


Figure 15: Relationship plots between procyclidine concentrations in hair, blood, oral fluids and daily dose

Table 25: Procyclidine

Procyclidine	Pearson correlation coefficient (r)	P-value (r)	n	Regression equation	Slope (Log)	Regr.
				Log(y) = Log(c) + mLog(x)	CI 95% (m)	P value
Hair vs Dose	0.215	0.391	17	log10(Hair)= 2.15 + 1.023 log10(Dose)	(-0.172 - 2.218)	0.090
Blood vs Dose	0.375	0.125	17	log10(Blood)= 1.01 + 1.011 log10(Dose)	(0.229 - 1.793)	0.015
Oral fluids vs Dose	0.283	0.307	15	log10(Oral fluids)= 0.06 + 1.384 log10(Dose)	(-0.072 - 2.840)	0.062
Hair vs Blood	0.825	0.000	17	log10(Hair)= 1.20 + 0.9670 log10(Blood)	(0.512 - 1.422)	0.000
Oral fluids vs Hair	0.213	0.446	15	log10(Oral fluids)= 0.60 + 0.2743 log10(Hair)	(-0.402 - 0.951)	0.400
Oral fluids vs Blood	0.193	0.491	15	log10(Oral fluids)= 0.36 + 0.5540 log10(Blood)	(-0.306 - 1.414)	0.190

Table 26: Correlation coefficient (r) and P-value for log transformed procyclidine results

(r) / P-value	Log(Dose)	Log(Hair)	Log(Blood)	Log(Oral fluids)
Log(Dose)		0.424	0.578	0.492
		0.090	0.015	0.062
Log(Hair)	0.424		0.746	0.234
	0.090		0.000	0.400
Log(Blood)	0.578	0.746		0.358
	0.015	0.000		0.190
Log(Oral fluids)	0.492	0.234	0.358	
	0.062	0.400	0.190	

3.9.2. Quetiapine

The results from blood samples of the 17 patients taking quetiapine were all positive with concentrations ranging from 1.2 to 955ng/mL, except two unavailable samples and one undetected. Likewise, oral fluids samples were all positives except one negative and two unavailable. Concentrations varied between 5 and 212ng/mL and were markedly lower than in blood. With regard to hair results, they were all positive and ranged from 2.4 to 22ng/mg as shown in Table 27. Selected samples calibrators and patients' results chromatograms are shown in Figure 16.

Table 27: Quetiapine patients' hair, blood and oral fluids trough levels

				Proximal (0-0.6 cm)		Pre-dose levels	
Subject	Age (yrs)	Daily dose	Duration of therapy	Hair colour	Hair conc. pg/mg	Blood conc. ng/mL	Oral fluids conc. ng/mL
1	34	600mg (300X2)	19 days	Black	**5435**	**72**	**41**
2	54	500mg (250X2)	>Month	Dark-grey	**11362**	**15.4**	**8.7**
3	48	300mg (100X3)	>Month	Black	**15399**	**270**	**56.1**
4	37	200mg (200X1)	>Month	Black	**2371**	**23.8**	**22.3**
5	34	200mg (200X1)	4 weeks	Black	**14422**	**NF**	**5**
6	38	400mg (200X2)	>Month	Black	**21998**	**337.4**	**76.7**
7	35	200mg (200X1)	19 days	Black	**2853**	**NA**	**NA**
8	35	200mg (200X1)	>3 weeks	Dark brown	**3183**	**7**	**5.2**
9	34	400mg (400X1)	17 days	Black	**6283**	**17.2**	**9.3**
10	25	200mg (200X1)	>3 weeks	Black	**2437**	**1.2**	**NF**
11	28	200mg(200X1)	>Month	Black	**7750**	**33.8**	**9.4**
12	32	200mg(200X1)	17 days	Black	**3009**	**18.2**	**14.8**
13	35	100mg (100X1)	2 weeks	Black	**5545**	**2.2**	**5.2**
14	38	200mg (200X1)	>3 weeks	Black	**6852**	**NA**	**NA**
15	35	200mg (200X1)	19 days	Black	**7207**	**13.8**	**211.7**
16	34	200mg (200X1)	>2 weeks	Black	**13730**	**17.8**	**18.9**
17	52	200mg (200X1)	>3 weeks	Dark brown	**7169**	**954.7**	**9.1**

NA: Sample not available. NF: Drug not found.

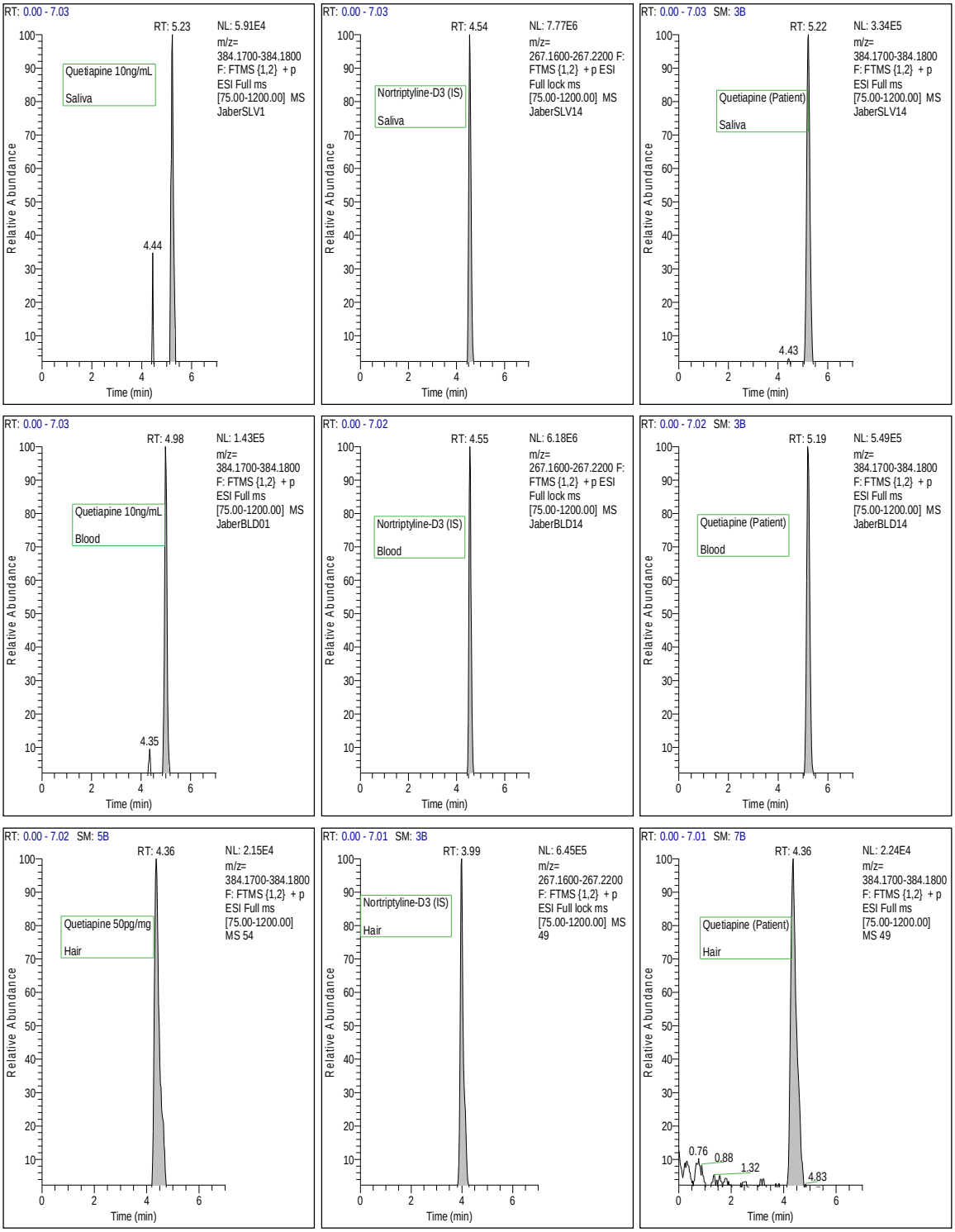


Figure 16: Chromatograms of extracted quetiapine and IS from low calibrators and patient's oral fluids, blood and hair samples

The Pearson correlation coefficient (r) calculated for the correlations between results showed that quetiapine had no correlation at all between its dose and blood level (r=-0.02 with p=0.949), its dose and oral fluids level (r=0.03 with p=0.933) nor its

dose and hair level (r=0.29 with p=0.264). Correlations between hair and blood levels also revealed no significant correlation with r=0.28 and p=0.326. Also hair/oral fluids levels and blood/oral fluids levels did not show a correlation with r=0.16 (p=0.576) and r=-0.05 (p=0.864), respectively. Only the log results revealed a correlation between hair and blood levels, r=0.56 and p=0.036. All the details are shown in Figure 17, Tables 28 and 29.

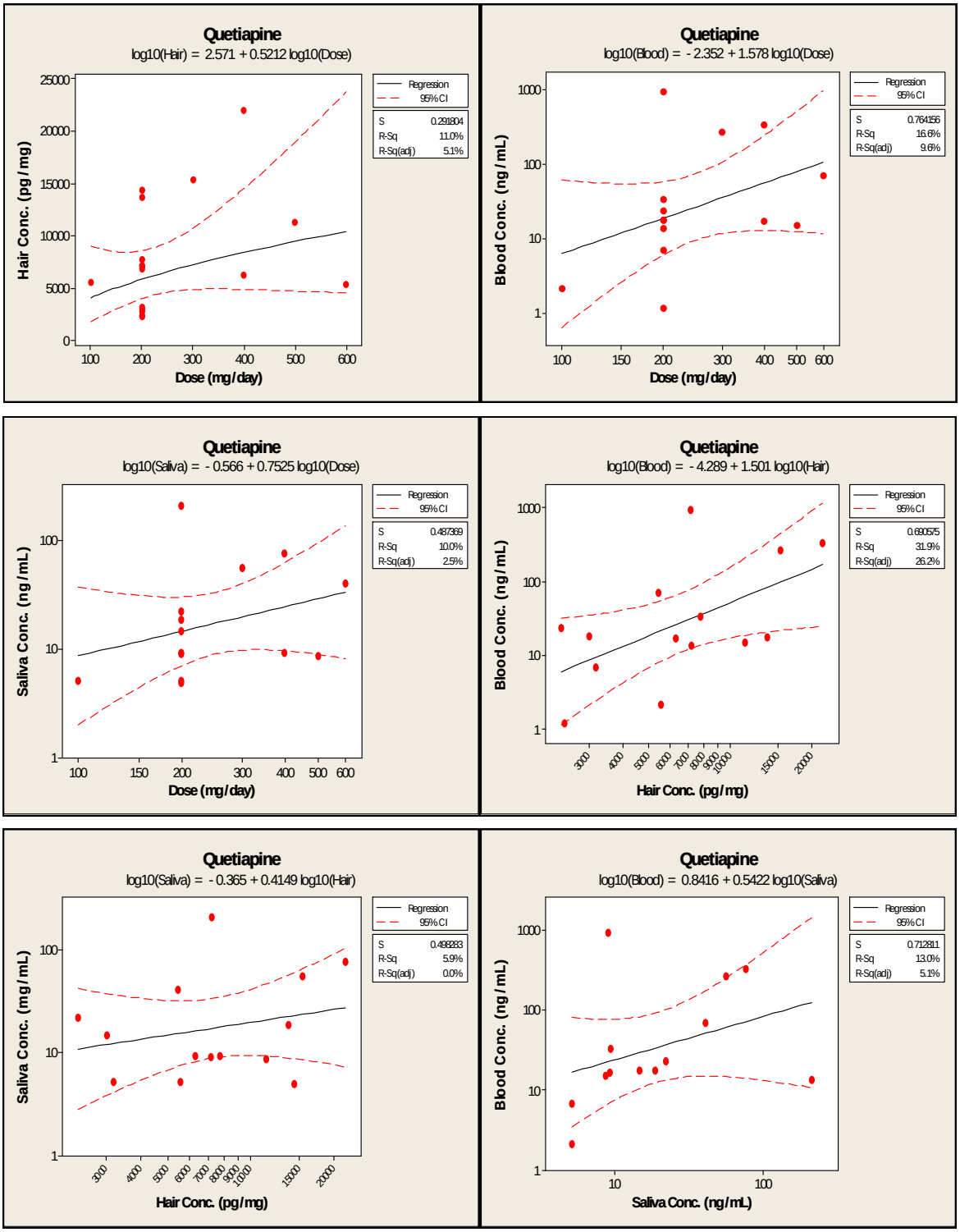


Figure 17: Relationship plots between quetiapine concentrations in hair, blood, oral fluids and daily dose

Table 28: Quetiapine

Quetiapine	Pearson correlation coefficient (r)	P-value (r)	n	Regression equation	Slope (Log)	Regr.
				Log(y) = Log(c) + mLog(x)	CI 95% (m)	P value
Hair vs Dose	0.287	0.264	17	log10(Hair)= 2.57 + 0.521 log10(Dose)	(-0.290 – 1.332)	0.193
Blood vs Dose	-0.019	0.949	14	log10(Blood)= -2.35 + 1.578 log10(Dose)	(-0.632 – 3.788)	0.149
Oral fluids vs Dose	0.025	0.933	14	log10(Oral fluids)= -0.57 + 0.753 log10(Dose)	(-0.656 – 2.162)	0.271
Blood vs Hair	0.284	0.326	14	log10(Blood)= -4.29 + 1.501 log10(Hair)	(0.132 – 2.870)	0.036
Oral fluids vs Hair	0.164	0.576	14	log10(Oral fluids)= -0.36 + 0.415 log10(Hair)	(-0.617 – 1.447)	0.402
Blood vs Oral fluids	-0.053	0.864	14	log10(Blood)= 0.84 + 0.542 log10(Oral fluids)	(-0.370 – 1.454)	0.225

Table 29: Correlation coefficient (r) and P-value for log transformed quetiapine results

(r) / P-value	Log(Dose)	Log(Hair)	Log(Blood)	Log(Oral fluids)
Log(Dose)		0.332	0.407	0.316
		0.193	0.149	0.271
Log(Hair)	0.332		0.564	0.243
	0.193		0.036	0.402
Log(Blood)	0.407	0.564		0.361
	0.149	0.036		0.225
Log(Oral fluids)	0.316	0.243	0.361	
	0.271	0.402	0.225	

3.9.3. Risperidone and OH-risperidone

Blood results of the fourteen patients on risperidone were positive in all the samples for both the drug and its metabolite with concentrations ranging from of 1-68ng/mL and 4-44ng/mL, respectively. In the same way, results from oral fluids samples were all positive for both analytes and concentrations were found to be between 1 and 31ng/mL for risperidone and 7.5 to 110ng/mL for OH-risperidone. With regard to hair results, they were all positive for risperidone ranging from 63 to 5318pg/mg whereas for OH-risperidone all samples were positive except one and concentrations ranged from 8 to 79pg/mg as shown in Table 30. Selective sample calibrators and patients' results chromatograms are shown in Figures 18 and 19.

Table 30: Risperidone and OH-risperidone patients' results

					Risperidone			9-OH-risperidone		
					Proximal 0-0.6cm	Pre-dose levels		Proximal 0-0.6cm	Pre-dose levels	
Subject	Age (yrs)	Daily dose	Duration of therapy	Hair colour	Hair conc. pg/mg	Blood conc. ng/mL	Oral fluids conc. ng/mL	Hair conc. pg/mg	Blood conc. ng/mL	Oral fluids conc. ng/mL
1	48	7mg (3.5X2)	>Month	Black	**1462**	**10.1**	**31.1**	**16.5**	**44.4**	**110**
2	32	6mg (2X3)	>Month	Black	**955**	**9.6**	**8.9**	**71**	**21.8**	**61.7**
3	48	6mg (2X3)	>Month	Grey	**1195**	**1.7**	**10**	**54.7**	**17**	**60.8**
4	68	4mg (2X2)	>Month	Grey	**105**	**1.6**	**9.2**	**49.8**	**40.8**	**36.1**
5	18	3mg(1.5X2)	>Month	Black	**85**	**0.3**	**1**	**13.1**	**4.7**	**12**
6	38	4mg (2X2)	24 days	Grey	**110**	**0.3**	**1.4**	**50.3**	**3.9**	**31.4**
7	24	6mg (2X3)	22 days	Black	**198**	**1.4**	**2.9**	**NF**	**8.7**	**14.3**
8	48	4mg (2X2)	>Month	Black	**1469**	**5**	**10.6**	**79.4**	**26.3**	**49.1**
9	28	6mg (2X3)	>Month	Black	**849**	**5.8**	**10.7**	**10**	**15.8**	**21.9**
10	46	4mg (2X2)	25 days	Black	**99**	**5.2**	**NA**	**24**	**25.2**	**NA**
11	44	3mg(1.5X2)	>Month	Black	**651**	**1.4**	**5.4**	**57.7**	**17.7**	**43.3**
12	51	4mg (2X2)	>Month	Black	**63**	**1**	**8.8**	**0.7**	**10.8**	**90.1**
13	30	6mg (2X3)	>Month	Dark brown	**468**	**1.6**	**6**	**7.9**	**7.6**	**41.3**
14	50	8mg (4X2)	>Month	Dark brown	**5318**	**68.4**	**23.4**	**23.5**	**15.9**	**7.5**

NA: Sample not available. NF: Drug not found.

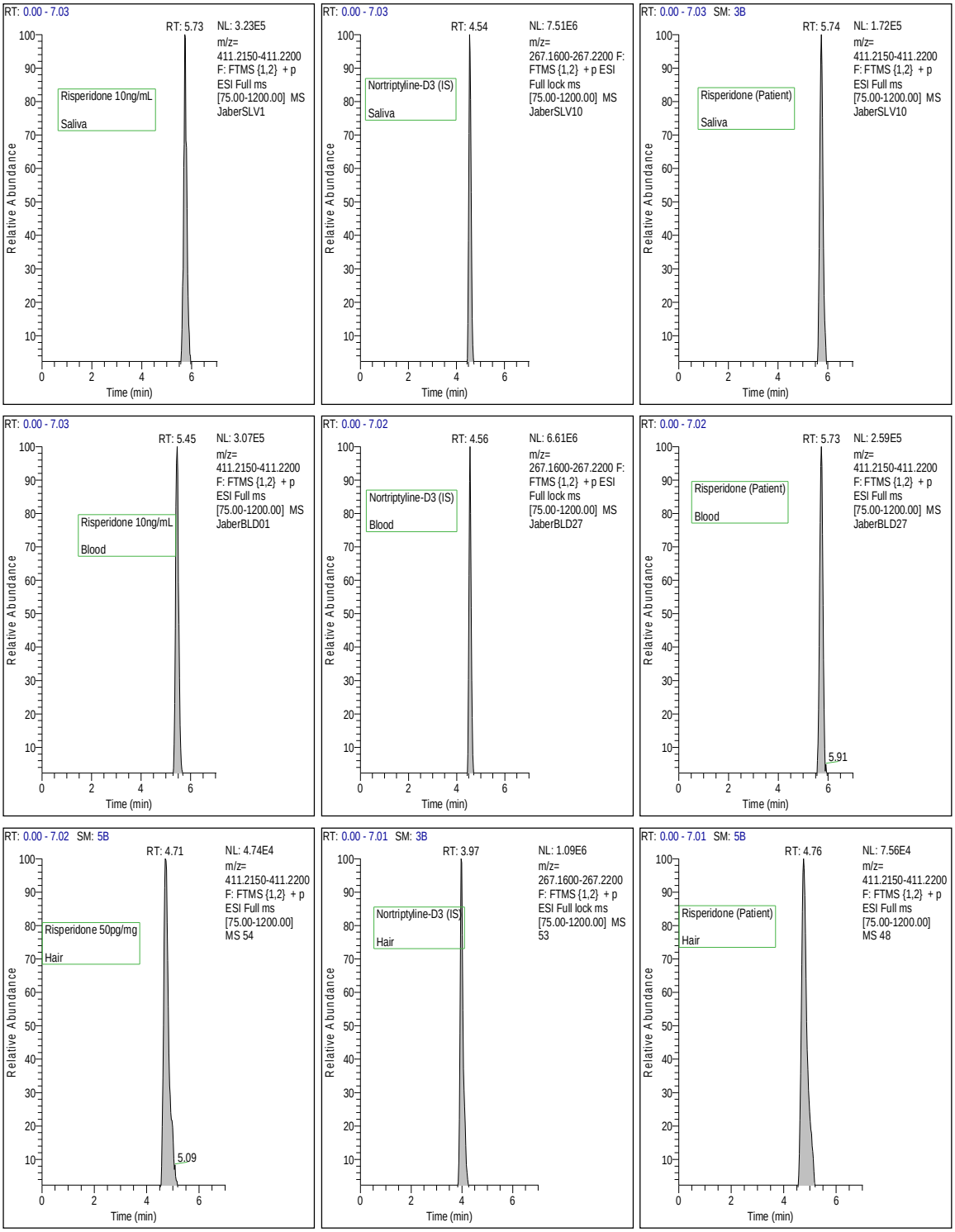


Figure 18: Chromatograms of extracted risperidone and IS from low calibrators and patient's oral fluids, blood and hair samples

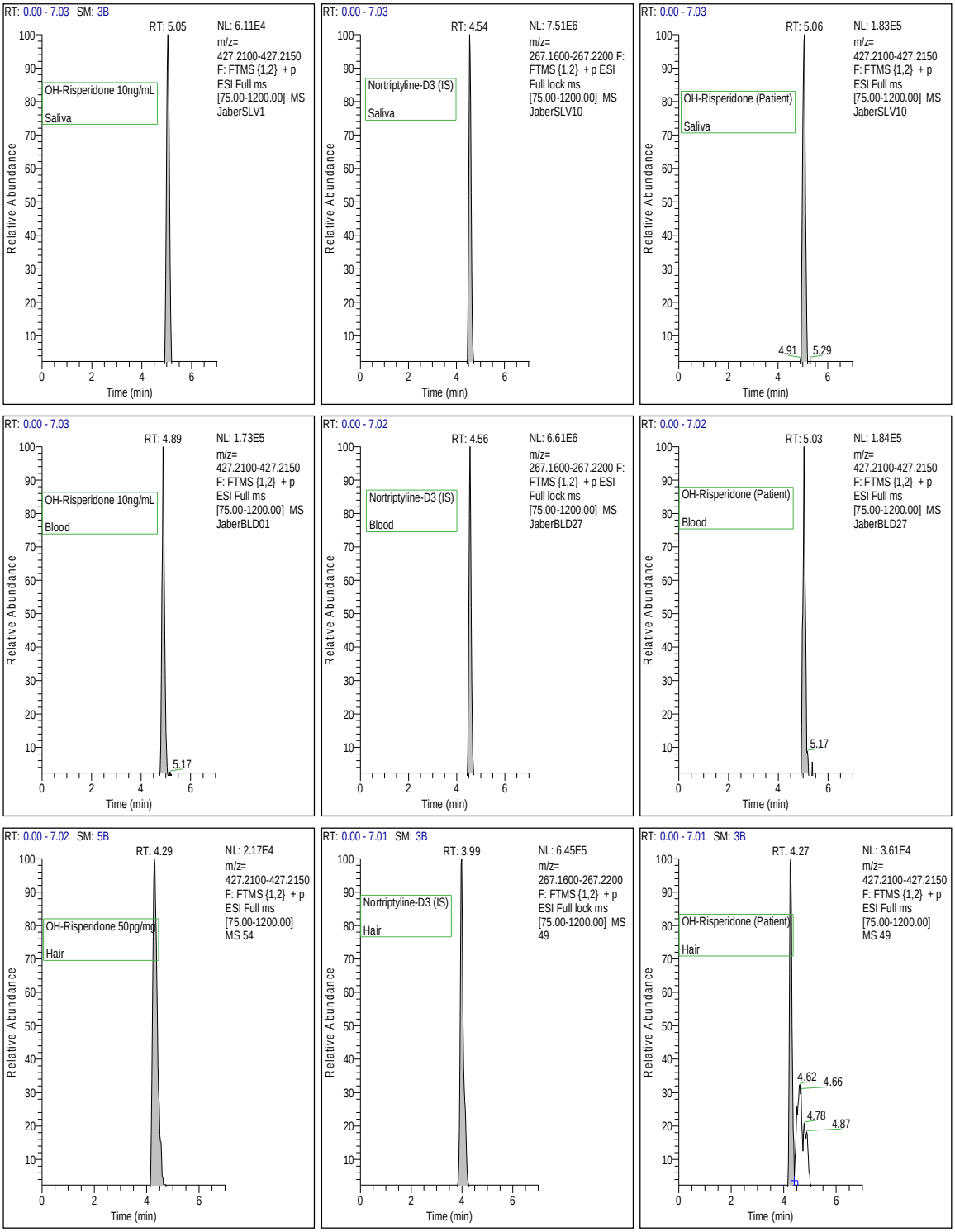


Figure 19: Chromatograms of extracted OH-risperidone and IS from low calibrators and patient's oral fluids, blood and hair samples

Risperidone results showed good correlations between the dose and all the sample matrices. Correlation coefficients for dose/blood concentration, dose/oral fluids concentration and dose/hair concentration were r=0.62 with p=0.009, r=0.67 with p=0.012 and r=0.67 with p=0.017, respectively. The strongest correlation was between blood and hair concentrations, r=0.96 with p=0.000, followed by oral fluids concentration/hair concentration with r=0.66 and p=0.013, while the weakest but still significant was between blood and oral fluids concentrations, r=0.59 and p=0.035.

Unlike its parent drug OH-risperidone did not show any correlation between the dose and any matrix level as correlation coefficients were r=0.15 with p=0.610 , r=0.06 with p=0.838 and r=-0.20 with p=0.515 for dose/blood concentration, dose/oral fluids concentration, dose/hair concentration, respectively. Also no significant correlations were found between blood concentration/hair concentration, oral fluids concentration/hair concentration and blood concentration/oral fluids concentration as coefficients results were r=0.24 with p=0.437, r=-0.03 with p=0.937 and r=0.51 with p=0.075, respectively. All the details are shown in Figures 20 and 21, Tables 31-34.

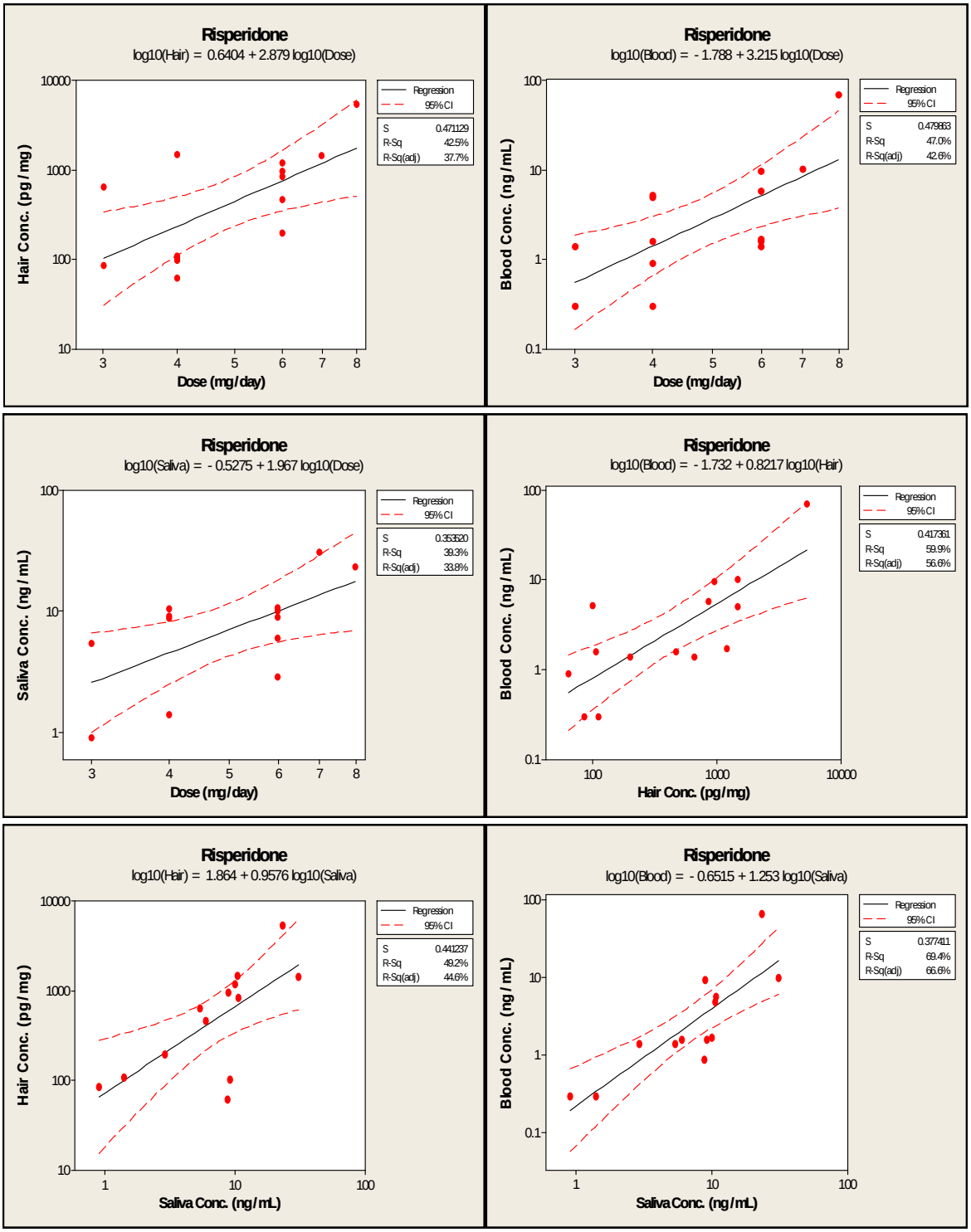


Figure 20: Relationship plots between risperidone concentrations in hair, blood, oral fluids and daily dose

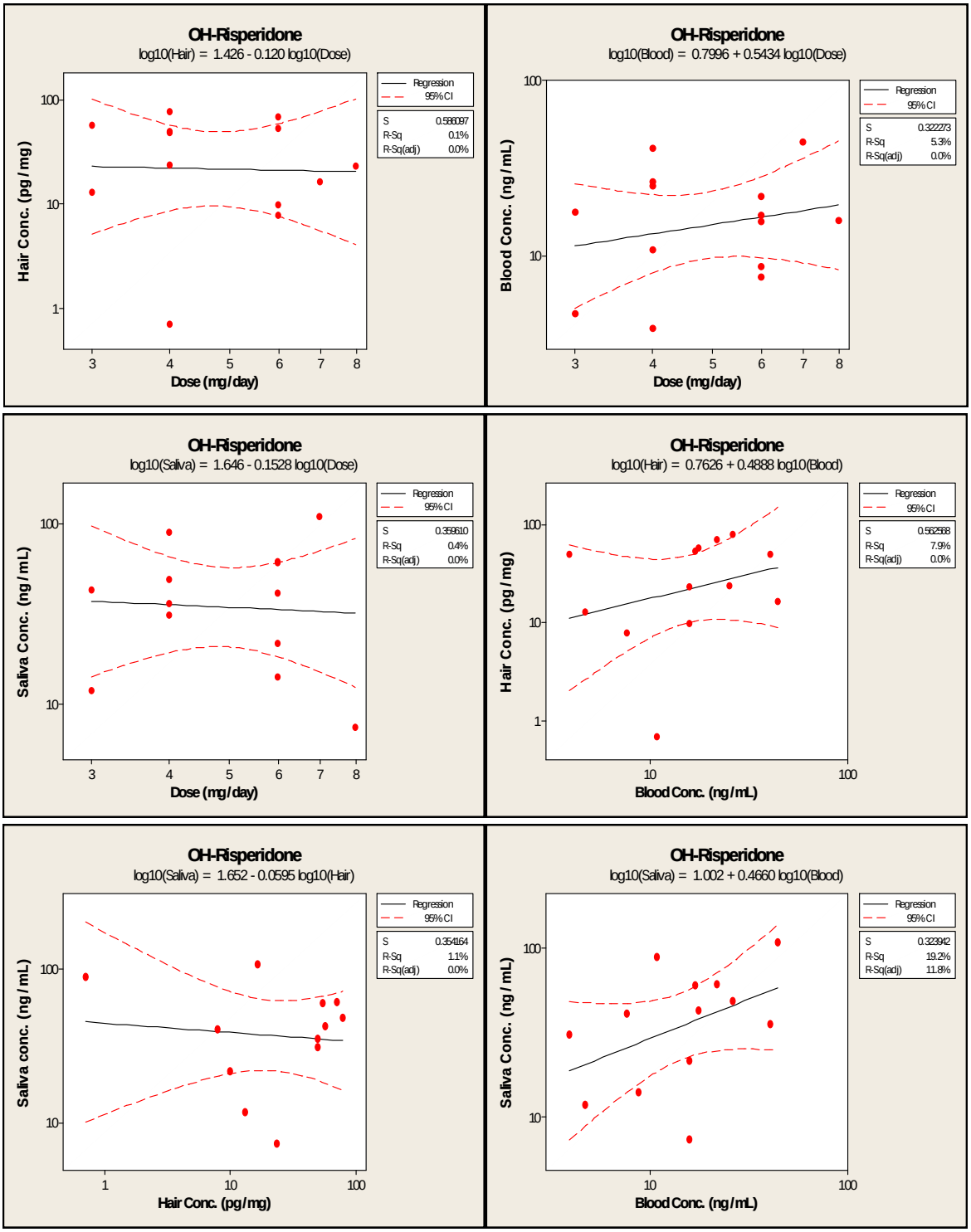


Figure 21: Relationship plots between OH-risperidone concentrations in hair, blood, oral fluids and daily dose

Table 31: Risperidone

Quetiapine	Pearson correlation coefficient (r)	P-value (r)	n	Regression equation	Slope (Log)	Regr.
				Log(y) = Log(c) + mLog(x)	CI 95% (m)	P value
Hair vs Dose	0.668	0.009	14	log10(Hair)= 0.64 + 2.879 log10(Dose)	(0.790 - 4.968)	0.012
Blood vs Dose	0.624	0.017	14	log10(Blood)= -1.79 + 3.215 log10(Dose)	(1.088 - 5.343)	0.007
Oral fluids vs Dose	0.673	0.012	13	log10(Oral fluids)= -0.53 + 1.967 log10(Dose)	(0.360 - 3.574)	0.022
Blood vs Hair	0.955	0.000	14	log10(Blood)= -1.73 + 0.8217 log10(Hair)	(0.403 - 1.241)	0.001
Oral fluids vs Hair	0.664	0.013	13	log10(Hair)= 1.86 + 0.9576 log10(Oral fluids)	(0.318 - 1.597)	0.008
Blood vs Oral fluids	0.586	0.035	13	log10(Blood)= -0.65 + 1.253 log10(Oral fluids)	(0.707 - 1.799)	0.000

Table 32: OH-risperidone

Quetiapine	Pearson correlation coefficient (r)	P-value (r)	n	Regression equation	Slope (Log)	Regr.
				Log(y) = Log(c) + mLog(x)	CI 95% (m)	P value
Hair vs Dose	-0.199	0.515	13	log10(Hair)= 1.43 - 0.120 log10(Dose)	(-2.794 - 2.554)	0.924
Blood vs Dose	0.149	0.610	14	log10(Blood)= 0.80 + 0.5434 log10(Dose)	(-0.886 -1.972)	0.427
Oral fluids vs Dose	0.063	0.838	13	log10(Oral fluids)= 1.65 - 0.1528 log10(Dose)	(-1.788 - 1.482)	0.842
Hair vs Blood	0.236	0.437	13	log10(Hair)= 0.76 + 0.4888 log10(Blood)	(-0.604 - 1.582)	0.351
Oral fluids vs Hair	-0.026	0.937	13	log10(Oral fluids)= 1.65 - 0.0595 log10(Hair)	(-0.457 - 0.338)	0.751
Oral fluids vs Blood	0.510	0.075	13	log10(Oral fluids)= 1.00 + 0.4660 log10(Blood)	(-0.163 - 1.095)	0.135

Table 33: Correlation coefficient (r) and P-value for log transformed risperidone results

(r) P-Value	Log(Dose)	Log(Hair)	Log(Blood)	Log(Oral fluids)
Log(Dose)		0.652	0.686	0.627
		0.012	0.007	0.022
Log(Hair)	0.652		0.774	0.702
	0.012		0.001	0.008
Log(Blood)	0.686	0.774		0.833
	0.007	0.001		0.000
Log(Oral fluids)	0.627	0.702	0.833	
	0.022	0.008	0.000	

Table 34: Correlation coefficient (r) and P-value for log transformed OH-risperidone results

(r) P-Value	Log(Dose)	Log(Hair)	Log(Blood)	Log(Oral fluids)
Log(Dose)		-0.029	0.231	-0.061
		0.924	0.427	0.842
Log(Hair)	-0.029		0.282	-0.103
	0.924		0.351	0.751
Log(Blood)	0.231	0.282		0.438
	0.427	0.351		0.135
Log(Oral fluids)	-0.061	-0.103	0.438	
	0.842	0.751	0.135	

3.9.4. Chlorprothixene

Results from blood samples of the 14 patients on chlorprothixene were all positive except one negative and their trough concentrations were between 13 and 41ng/mL. Four oral fluids samples were negative and one was unavailable. The rest of the oral fluids samples gave results ranging between 16 and 22ng/mL. Only one hair sample was unavailable and the remaining 13 were positive and their concentrations range was from 0.3 to 25.3ng/mg as shown in Table 35. Selected sample calibrators and patients' results chromatograms are shown in Figure 22.

Table 35: Chlorprothixene patient results

				Proximal (0-0.6 cm)		Pre-dose levels	
Subject	Age (yrs)	Daily dose	Duration of therapy	Hair colour	Hair conc. pg/mg	Blood conc. ng/mL	Oral fluids conc. ng/mL
1	46	50mg (50X1)	>Month	Black	**13159**	**17.3**	**16.4**
2	53	100mg (100X1)	>Month	Grey	**12196**	**32.4**	**18.2**
3	48	100mg (50X2)	>Month	Grey	**11680**	**16.1**	**16.4**
4	58	100mg (100X1)	>Month	Grey	**19457**	**25.5**	**17.6**
5	47	50mg (50X1)	>Month	Dark grey	**25257**	**23.9**	**NF**
6	41	50mg (50X1)	>Month	Black	**5114**	**NF**	**NF**
7	34	50mg (50X1)	19 days	Black	**6731**	**17.3**	**16.7**
8	24	50mg (50X1)	3 days	NA	**NA**	**40.6**	**18.8**
9	46	50mg (50X1)	12 days	Black	**981**	**29.4**	**NA**
10	32	65mg (65X1)	>Month	Black	**7432**	**24.2**	**17.9**
11	54	150mg (75X2)	>Month	Dark-grey	**13095**	**35.5**	**21.8**
12	30	50mg (50X1)	>Month	Dark-brown	**6789**	**17.0**	**17.0**
13	56	50mg (50X1)	>Month	Grey	**3561**	**15.8**	**NF**
14	32	50mg (50X1)	17 days	Black	**290**	**13.3**	**NF**

NA: Sample not available. NF: Drug not found.

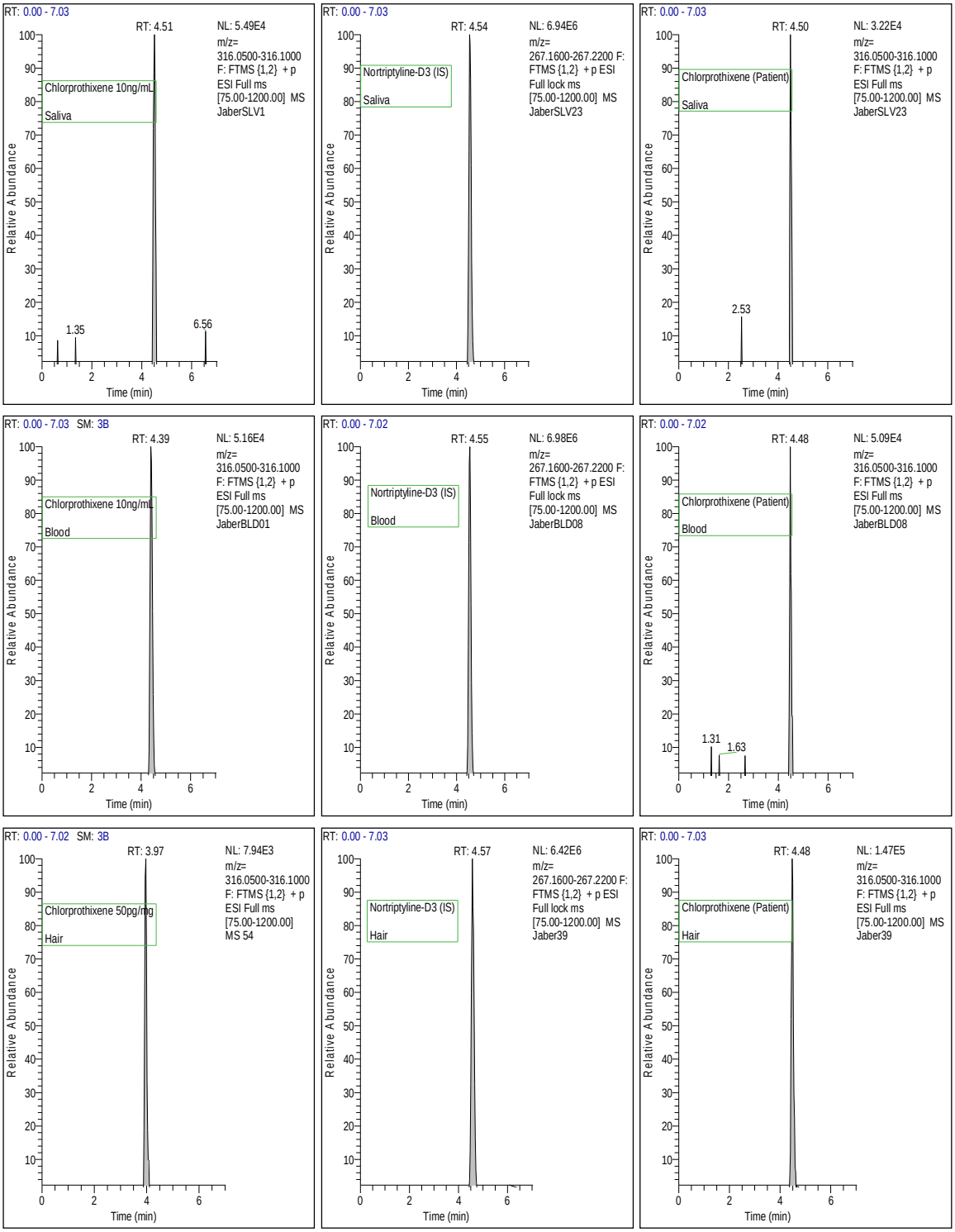


Figure 22: Chromatograms of extracted chlorprothixene and IS from low calibrator's and patient's oral fluids, blood and hair samples

No significant correlations have been found between chlorprothixene dose and blood or hair trough levels, r=0.37 with p=0.211 and r=0.40 with p=0.176, respectively. Conversely, dose/oral fluids concentration showed some significant correlation, r=0.68 and p=0.045. Also blood concentration/oral fluids concentration were correlated with r=0.79 and p=0.011. However, hair concentration/blood concentration and hair concentration/oral fluids concentration revealed insignificant relations, r=0.32 with p=0.319 and r=0.21 with p=0.625, respectively. All the details are shown in Figure 23, Tables 36 and 37.

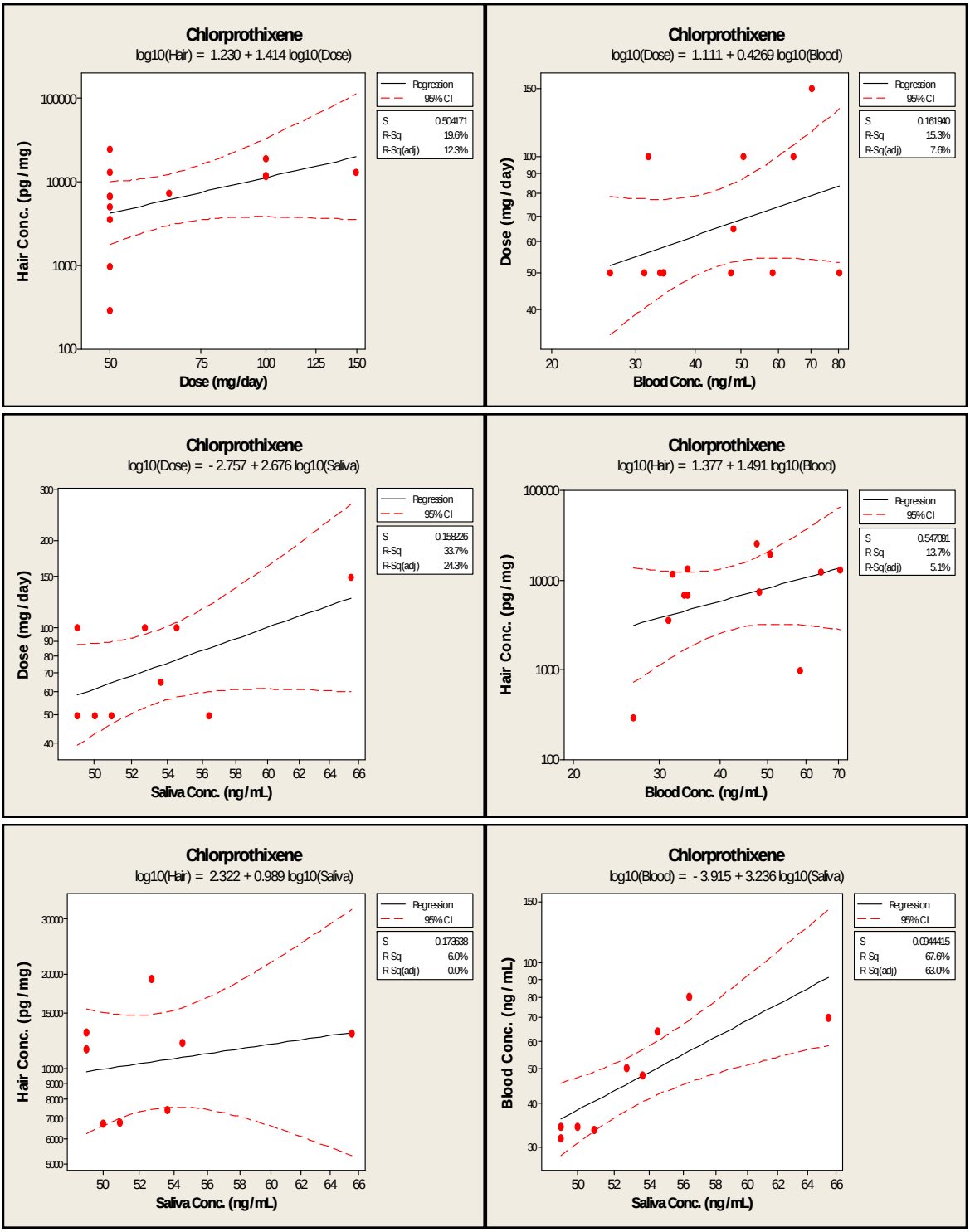


Figure 23: Relationship plots between chlorprothixene concentrations in hair, blood, oral fluids and daily dose

Table 36: Chlorprothixene

Quetiapine	Pearson correlation coefficient (r)	P-value (r)	n	Regression equation	Slope (Log)	Regr.
				Log(y) = Log(c) + mLog(x)	CI 95% (m)	P value
Hair vs Dose	0.372	0.211	13	log10(Hair)= 1.23 + 1.414 log10(Dose)	(-0.468 - 3.296)	0.130
Dose vs Blood	0.400	0.176	13	log10(Dose)= 1.11 + 0.4269 log10(Blood)	(-0.232 - 1.086)	0.186
Dose vs Oral fluids	0.678	0.045	9	log10(Dose)= - 2.76 + 2.676 log10(Oral fluids)	(-0.594 - 5.946)	0.101
Hair vs Blood	0.315	0.319	13	log10(Hair)= 1.38 + 1.491 log10(Blood)	(-1.087 - 4.069)	0.236
Oral fluids vs Hair	0.206	0.625	9	log10(Hair)= 2.32 + 0.989 log10(Oral fluids)	(-2.698-4.676)	0.559
Blood vs Oral fluids	0.792	0.011	9	log10(Blood)= - 3.92 + 3.236 log10(Oral fluids)	(1.284 - 5.188)	0.007

Table 37: Correlation coefficient (r) and P-value for log transformed chlorprothixene results

(r) / P-Value	Log(Dose)	Log(Hair)	Log(Blood)	Log(Oral fluids)
Log(Dose)		0.443	0.392	0.581
		0.130	0.186	0.101
Log(Hair)	0.443		0.370	0.245
	0.130		0.236	0.559
Log(Blood)	0.392	0.370		0.822
	0.186	0.236		0.007
Log(Oral fluids)	0.581	0.245	0.822	
	0.101	0.559	0.007	

3.9.5. Haloperidol

All the blood samples analysed from patients on haloperidol were positive with pre-dose levels ranging from 7 to 27ng/mL. Oral fluids samples as well were all positive except one undetected and one unavailable. Positive oral fluids results were in the range of 6-38ng/mL. Interestingly, hair samples, which were all positive, revealed relatively high levels of haloperidol ranging from 15.3 to 80.3ng/mg as shown in Table 38. Selected samples calibrators and patients' results chromatograms are shown in Figure 24.

Table 38: Haloperidol Patients

				Proximal (0-0.6 cm)		Pre-dose levels	
Subject	Age (yrs)	Daily dose	Duration of therapy	Hair colour	Hair conc. pg/mg	Blood conc. ng/mL	Oral fluids conc. ng/mL
1	46	20mg (10X2)	>Month	Black	**39645**	**11.8**	**7.3**
2	53	30mg (10X3)	>Month	Grey	**52299**	**23.4**	**14**
3	32	30mg (10X3)	>Month	Black	**38446**	**11.2**	**15**
4	48	20mg (10X2)	>Month	Grey	**43451**	**9.5**	**12.2**
5	38	30mg (10X3)	>Month	Black	**80314**	**27.7**	**37.9**
6	52	10mg (5X2)	>Month	Black	**34250**	**14.6**	**NA**
7	47	10mg (10X1)	>Month	Dark grey	**15284**	**6.6**	**NF**
8	41	10mg (10X1)	>Month	Black	**15990**	**7**	**6.2**
9	56	30mg (10X3)	>Month	Grey	**29310**	**11.3**	**18.4**
10	50	15mg (5X3)	>Month	Dark brown	**36363**	**13.5**	**8.1**

NA: Sample not available. NF: Drug not found.

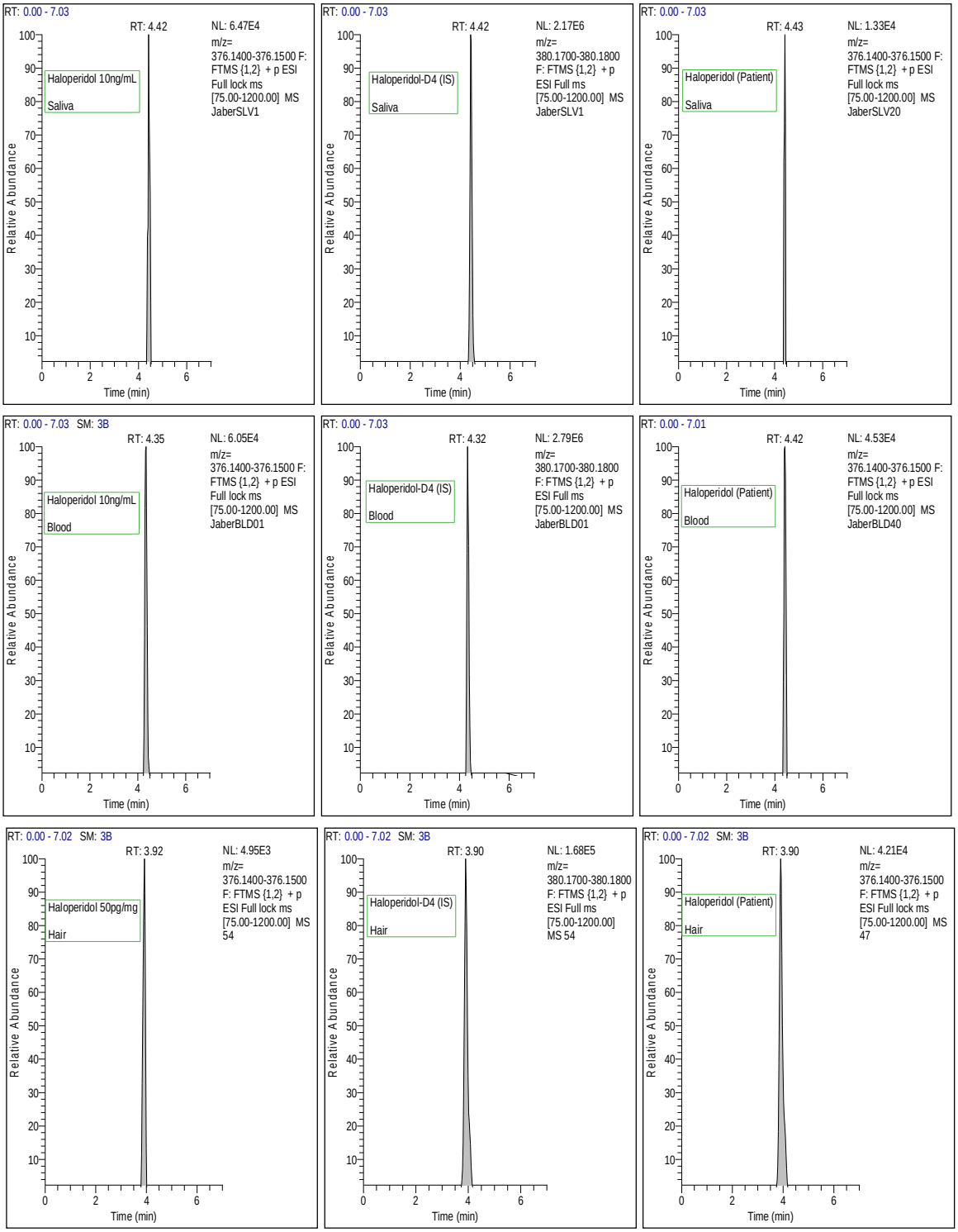


Figure 24: Chromatograms of extracted haloperidol and IS from low calibrators and patient's oral fluids, blood and hair samples

Haloperidol results showed weak correlations existed with dose/blood concentration and dose/oral fluids concentration with coefficients of r=0.58 with p=0.080 and r=0.66 with p=0.073, respectively. With regard to dose/hair concentration the correlation was similar (r=0.65) but with a better p-value (p=0.042). In the other hand, blood concentration/hair concentration showed very significant correlation, r=0.90 with p=0.000. The other two sets also showed significant correlations, oral fluids concentration/hair concentration r=0.83 with p=0.012 and oral fluids concentration/blood concentration r=0.76 with p=0.03. All the details are shown in Figure 25, Tables 39 and 40.

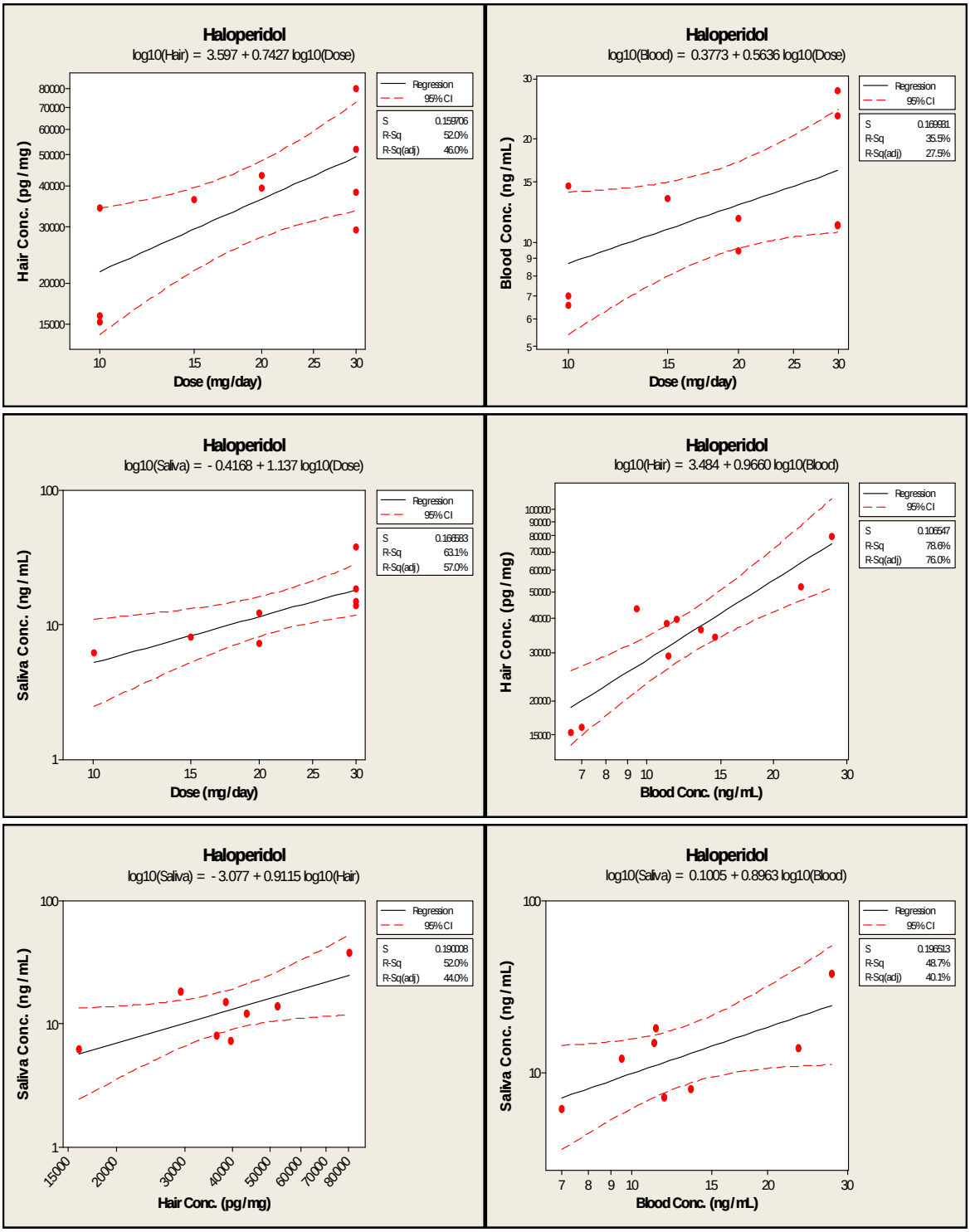


Figure 25: Relationship plots between haloperidol concentrations in hair, blood, oral fluids and daily dose

Table 39: Haloperidol

Quetiapine	Pearson correlation coefficient (r)	P-value (r)	n	Regression equation	Slope (Log)	Regr.
				Log(y) = Log(c) + mLog(x)	CI 95% (m)	P value
Hair vs Dose	0.649	0.042	10	log10(Hair)= 3.60 + 0.7427 log10(Dose)	(0.172 - 1.313)	0.019
Blood vs Dose	0.578	0.080	10	log10(Blood)= 0.38 + 0.5636 log10(Dose)	(-0.043 - 1.170)	0.069
Oral fluids vs Dose	0.663	0.073	8	log10(Oral fluids)= - 0.42 + 1.137 log10(Dose)	(0.298 - 1.976)	0.018
Hair vs Blood	0.901	0.000	10	log10(Hair)= 3.48 + 0.9660 log10(Blood)	(0.563 - 1.369)	0.001
Oral fluids vs Hair	0.825	0.012	8	log10(Oral fluids)= - 3.08 + 0.9115 log10(Hair)	(0.066 - 1.757)	0.043
Blood vs Oral fluids	0.757	0.030	8	log10(Oral fluids)= 0.10 + 0.8963 log10(Blood)	(0.008 - 1.785)	0.054

Table 40: Correlation coefficient (r) and P-value for log transformed haloperidol results

(r) / P-Value	Log(Dose)	Log(Hair)	Log(Blood)	Log(Oral fluids)
Log(Dose)		0.721	0.596	0.795
		0.019	0.069	0.018
Log(Hair)	0.721		0.887	0.721
	0.019		0.001	0.043
Log(Blood)	0.596	0.887		0.698
	0.069	0.001		0.054
Log(Oral fluids)	0.795	0.721	0.698	
	0.018	0.043	0.054	

3.10. Discussion

3.10.1. Aim and the subjects

In this chapter the main aim was to investigate and evaluate the relationship between the given dose and the concentration detected in hair, blood and oral fluids of subjects under controlled drug administration (in-patients). Five drugs and one active metabolite have been studied (procyclidine, quetiapine, chlorprothixene, haloperidol, risperidone and OH-risperidone). These drugs were chosen because of their wide prescription to psychiatric patients, high potential of short and long term side effects and toxicity and also for their resemblance to some degree with the majority of drugs of abuse, which target mostly brain cells.

Addressing and evaluating the research question should provide a better confidence or understanding when interpreting forensic and clinical toxicological case results. This is particularly true for hair and oral fluids results, as hair is the most suitable sample with a wide window for retrospective investigation of drug intake, while collection of oral fluids is non-invasive, easy to provide, easy to collect and easier to extract/process. However, few studies in the literature have dealt with these issues and most of them were based on animal models or based on self-reported drug history. Therefore, their results may not be categorical or conclusive. In contrast, in this study the main criteria were to have human subjects and under controlled drug administration. Nevertheless, the only issue was the population size for those who agreed to participate. The number of patients for procyclidine was 17, 17 for quetiapine, 14 for risperidone, 14 for chlorprothixene and 10 for haloperidol. These patients were chosen primarily for their long and constant treatment regimen, which should have provided a steady-state level of drug in their biological system. Blood and oral fluids samples have been taken at the trough levels (pre-dose), while hair ones were the first segment (<1cm proximal end of hair strands).

Patients' drug history, age, hair colour and other relevant information have been taken. However, their possible influence on the results was not investigated as it clearly require a larger sample size to observe their real effect.

3.10.2. LC-MS

Table 10 shows the accurate masses of the targeted analytes and internal standards extracted from the full scan (75-1200amu) run on the LC-MS system. The mass accuracy for all the analytes was less than 2ppm due to the high resolution (+50,000) of the Exactive (Orbitrap) system have provided a great selectivity, sensitivity and confidence in the results. On the other hand, the chromatography separation which was achieved by the HILIC condition is considered relatively unique as no paper was found in literature reporting analysis of these drugs with these conditions. The HILIC method showed good separation of the peaks with a short run time (7 min). In fact one of the advantages of HILIC is the low back pressure which allows for increasing the flow rate (0.5mL/min) making the run relatively fast without compromising the results as the resolution is very high. The isocratic mobile phase with high acetonitrile content has also contributed to the quick separation without the need of re-equilibrating the column as in a gradient run.

3.10.3. Blood and oral fluids analysis

Blood and oral fluids samples were treated in the same way throughout the method development and validation as these matrices are composed mainly of water. Method development results showed that low strength NaOH solution (0.01M) could not provide enough alkalinisation to extract analytes from the aqueous phase into the solvent phase. Therefore, a very low recovery was seen, as shown in Table 11. Similarly, Tris buffer pH 9 did not produce any recovery except only 11% and 8% for chlorprothixene and haloperidol, respectively. However, 1M NaOH solution has provided the required pH to give the highest extraction yield for all analytes. The analytes have pKa ranging from 8.2-10.7 except Quetiapine (6.8) and 1M NaOH solution has pH of 14 so enough alkalinisation to ensure all analytes are non-ionization in order to be extracted by the MTBE solvent.

On the other hand, the fourth method of extraction, the protein crash, did show good recovery for four analytes (>60%) but procyclidine and chlorprothixene were not as good (<50%). One explanation could be a possible effect from ion suppression by

co-eluting endogenous compounds or metabolites as the crashing solvent dissolves many impurities alongside the targeted analytes. Unluckily, there was no possibility to investigate that effect further and the 1M NaOH showed enough recovery to be chosen as the successful extraction method.

With regard to method validation for the successful method, linearity for blood and oral fluids calibrators was very good and showed r^2=>0.998 for all analytes. LOD and LOQ were low enough for this type of study (below the drugs' therapeutic concentrations range). They were found to be between 0.3 - 0.9ng/mL and 1 - 2.7ng/mL, respectively for all analytes as shown in Table 12. They were calculated with the Equations 2-5 (page 91). Absolute recovery results for analytes in spiked oral fluids samples showed an acceptable level of >60% for all the analytes except in two cases with the low calibrator (0.1ng/mL) where quetiapine and chlorprothixene showed 51 and 44% recovery. In blood the absolute recoveries for all the analytes were generally lower, especially for procyclidine (80% in oral fluids and 48% in blood). Precision results for intra-day as shown in Table 15 were good with a CV below 15% for all analytes. However, inter-day precision was not as good. On some occasions (8 out of 72 results' means) at the 0.1 and 0.5ng/mL concentrations the CV reached up to 38%.

3.10.4. Hair analysis

Conducting a hair analysis always starts with a decontamination step to reduce the interference from external contaminants, especially if the analysed drug presents in sebum or sweat. A very quick and effective decontamination step was applied to all samples as described in section 3.8.1.1 and discussed as well earlier in chapter 2. Following decontamination and reduction into 1-2mm snippets (25mg), authentic hair samples taken from patients on high chronic doses of procyclidine, quetiapine, risperidone, chlorprothixene and haloperidol were used for method optimization. Four different extraction methods were tested in triplicate on these authentic samples as described in the methods section. Method 3 (MAF) gave highest recoveries for procyclidine, chlorprothixene and haloperidol and second highest for OH-risperidone while method 1 (1M NaOH) gave highest recoveries for quetiapine

and risperidone. Therefore, these two methods combined got almost all the highest recoveries for the compounds of interest and the other two tested methods showed lower recoveries. Similar findings for similar drugs have been reported in the literature for the alkaline method (0.1M NaOH) (Shen et al. 2002a). MAF solution seems to be able to swell the hair and attract the basic drugs with its slightly acidic composition while NaOH dissolves the hair and frees the entrapped compounds. Summary of the findings can be seen in Figure 13. Combining methods 1 and 3 was chosen to ensure most if not all the amount of drugs present in hair were extracted.

As the combined method (NaOH/MAF) was chosen validation was carried out and showed acceptable results. All analytes' calibration lines were linear (r^2>0.99). LOD and LOQ were 1.4-5.8 and 4.7-19.5pg/mg which were low enough for the purpose of this study. Intra- and inter-day precision from spiked hair revealed acceptable CV percentages of <20% for all analytes excluding 3 samples (≤26%). As for accuracy it can be seen clearly from Table19 that QC concentrations are within 80-120% except two results at low QC. Absolute recoveries were between 71 and 101% (CV<15%) which considered within acceptable ranges. Also no matrix effect was shown (98-104%) and could only be seen slightly with procyclidine (85%).

Analytes showed 78-106% (CV<10%) stability under the combined method extraction conditions. However, stability of the analytes under the alkaline conditions only was not great but that does not matter so much as these conditions are applied after the samples have been extracted initially with MAF incubation.

3.10.5. Patients' results

Patients' whole blood trough levels (pre-dose) of procyclidine results ranged from 30 to 357ng/mL (dose 5-15mg/day). No whole blood range could be found in the literature for procyclidine. The reported therapeutic range in the literature, 80 to 650ng/mL, is based on plasma levels (Hadidi 2004, Schulz et al. 2012). However, procyclidine is roughly 100% protein bound and plasma normally constitutes approximately 50% of the whole blood (Wishart et al. 2008). Therefore, the present study's whole blood results should represent a 50% of the actual plasma

concentrations and consequently correspond very well with the literature plasma procyclidine range. Based on that almost all the patients are within the therapeutic drug level. With regard to oral fluids results, procyclidine pre-dose levels ranged from 7 to 395ng/mL and no procyclidine oral fluids data could be found in the literature. Analysis of procyclidine in patients' hair revealed a concentration range from 0.12 to 5.62ng/mg and also no published procyclidine levels in hair were found.

Blood trough levels of quetiapine (dose 100-600mg/day) for the studied subjects were between 1.2 and 337ng/mL except one patient with a high result (955ng/mL). However, nine of the patients had quetiapine concentrations <25ng/mL. Considering the published therapeutic serum level which is between 70 and 500ng/mL, these nine patients are possibly below the therapeutic target level even though quetiapine is mainly in the serum with reported protein binding of approximately 83% (Gerlach et al. 2007, Schulz et al. 2012), (DeVane, Nemeroff 2001b). As to subjects' oral fluids quetiapine, the range was between 5 and 212ng/mL and no oral fluids data were found in the literature with regard to this drug. Hair results showed that quetiapine presence in relatively high amount in patients' hair with a concentration range from 2.4 to 22ng/mL. Comparable results were reported in a previous study (Al Jaber, Holt & Johnston 2012).

Risperidone (dose 3-8mg/day) and its active metabolite OH-risperidone blood results were found to be in the range of 1 to 10ng/mL, except for one subject (68ng/mL) who was on the highest daily dose (8mg), and 4 to 44ng/mL, respectively. No whole blood results were found in literature except one study measuring the drug in plasma and in red blood cells (RBC) separately (Aymard et al. 2002). The ranges they found for risperidone were 1-23 and 1-8ng/mL in plasma and RBC respectively, whereas for OH-risperidone they were 8-70 and 2.6-22.5ng/mL for plasma and RBC, respectively. The reference therapeutic plasma levels are 2-20ng/mL and 10-100ng/mL for risperidone and OH-risperidone, respectively (Schulz et al. 2012). Results from oral fluids samples for both analytes were found to be between 1 and 31ng/mL for risperidone and 7.5 to 110ng/mL for OH-risperidone. Comparable results were found in published studies (Mandrioli et al. 2011, Saracino et al. 2010). With regard to hair results, they were ranging from

63 to 5318pg/mg and from 8 to 79pg/mg for both risperidone and its metabolite, respectively. Very similar ranges were reported in published studies (Al Jaber, Holt & Johnston 2012, Schneider et al. 2009). These results indicate that OH-risperidone has a much lower incorporation rate into hair than its parent drug and also the second alkaline extraction might have some effect on its results. A previous study showed that OH-risperidone gave similar recoveries from positive hair samples when extracted firstly with MAF (~50%) and secondly with NaOH digestion (~50%) (Al Jaber, Holt & Johnston 2012). It is also known that polar compounds show lower incorporation, compared to their less polar homologues (Nakahara, Kikura 1996). The focus in this research was mainly on parent drugs as they incorporate in hair more than their water-soluble metabolites.

Trough concentrations (dose 50-150mg/day) for chlorprothixene in blood samples were between 13 and 41ng/mL. Only a serum and plasma therapeutic reference range was reported in the literature and was between 20 to 300ng/mL (Hiemke et al. 2011). As chlorprothixene is known to bind to plasma protein to the extent of 90 to 95% it would be normal to see lower concentrations with whole blood analysis (Ebadi 2007). Results from oral fluids samples ranged from 16 to 22ng/mL and no oral fluids range or data could be found in the literature. Hair results were between 0.3 and 25ng/mg for the subjects and a slightly higher result (30ng/mg) has been reported in one publication (Shen et al. 2002b).

Haloperidol trough blood concentrations (dose 10-30mg/day) were between 7 and 27ng/mL. The clinically recommended therapeutic window is between 4 and 26ng/mL in plasma or serum (Oliveira et al. 1996). Only one study was found reporting whole blood haloperidol levels and was 2.7ng/mL for a patient on a 3mg/day dose (Seno et al. 1993). However, the present study's subjects had an average of a 20mg/day dose. It is worth mentioning that haloperidol is around 90% bound to protein and therapeutic drug monitoring (TDM) is highly recommended for patients on it (Wishart et al. 2008, Hiemke et al. 2011). Subjects' oral fluids haloperidol levels were between 6 and 38ng/mL. Similar oral fluids levels were found in the literature (Jain et al. 2011). Haloperidol seems to have the highest accumulation in hair among the studied drugs as patients' results were between 15

and 80ng/mg. A published case has a concentration within the present study range (Shen et al. 2002b). Some published results were as high as 242ng/mg (Couper, McIntyre & Drummer 1995a).

3.10.6. Correlation between blood, oral fluids, hair and dose

As mentioned earlier the relationships between the given dose and the steady-state concentrations in hair, blood and oral fluids of subjects under controlled drug administration were investigated. Blood and oral fluids were pre-dose samples whereas hair samples were the proximal end (near the scalp) 0-0.6cm segments which should represent roughly drug consumption within the last two weeks. Starting with procyclidine, the only clear significant relationship found with this drug was between trough blood and hair concentrations (Pearson r=0.825, p=<0.001). Correlation between blood level and daily dose was weak (r=0.375, p=0.125), however, the logarithmically transformed results showed significant correlation with r=0.578, p=0.015 and slope of 1.011 (95% CI, 0.229-1.793). All the other procyclidine correlations were very weak and insignificant, between blood and oral fluids trough levels r=0.193 (p=0.491), oral fluids and dose r=0.283 (p=0.307), oral fluids and hair r=0.213 (p=0.446), and hair and dose r=0.215 (p=0.391). The clearly significant correlation between blood and hair levels indicates that procyclidine incorporation into hair is mainly through blood and the other ways of incorporation like sebum and sweat must be minimal. Inter-individual pharmacokinetic and pharmacodynamic variations are possibly the cause of the insignificant correlations found in the other specimens.

Quetiapine on the other hand showed no correlations among all the types of matrices and daily dose with normal data. Correlations coefficients were r=-0.019 (p=0.949) for dose and blood level, r=0.025 (p=0.933) for dose and oral fluids levels, r=0.287 (p=0.264) for dose and hair level, r=0.284 (p=0.326) for hair and blood levels, r=0.164 (p=0.576) for hair and oral fluids levels and r=-0.053 (p=0.864) for blood and oral fluids levels. However, when logarithmically transformed a significant

correlation appeared between blood and hair concentrations r=0.564 (p=0.036) and slope of 1.501 (95% CI, 0.132-2.870). These results also could be due to inter-individual variations.

Contrary to quetiapine, risperidone results have shown remarkable and significant correlations among concentrations in all types of matrices tested and daily dose, both in normal data and in the logarithmically transformed data. Correlation coefficients for dose/blood level, dose/oral fluids level and dose/hair level were r=0.62 with p=0.009, r=0.67 with p=0.012 and r=0.67 with p=0.017, respectively. The strongest correlation was between blood and hair levels, r=0.96 (p=<0.001), followed by oral fluids and hair levels with r=0.66 (p=0.013), whereas the weakest, but still significant, was between blood and oral fluids concentrations, r=0.59 (p=0.035).

Unlike its parent drug, OH-risperidone did not show any correlation between the dose and any matrix level as correlation coefficients were r=0.15 (p=0.610), r=0.06 (p=0.838) and r=-0.20 (p=0.515) for dose/blood level, dose/oral fluids level, dose/hair level, respectively. Also no significant correlations were found between blood with hair levels and oral fluids with hair levels as coefficients results were r=0.24 (p=0.437) and r=-0.03 (p=0.937), respectively. Only weak correlation was observed between blood and oral fluids levels, r=0.51 (p=0.075).

Chlorprothixene correlation results were significant only between dose and oral fluids level (r=0.678, p=0.045) and between blood and oral fluids levels (r=0.792, p=0.011). Correlation coefficients between chlorprothixene dose and blood or hair trough levels were r=0.37 (p=0.211) and r=0.40 (p=0.176), respectively. Chlorprothixene hair and blood levels had an insignificant correlation (r=0.32, p=0.319). Similarly, hair and oral fluids drug levels showed a correlation coefficient of r=0.21 with p=0.625. One of the possible reasons would be the limited number of participants which were not enough to soften the pharmacokinetic/dynamic variations between subjects.

Haloperidol correlation results were similar to those of risperidone. Significant correlations existed between blood and oral fluids trough drug levels (r=0.76,

p=0.03), oral fluids with hair levels (r=0.83, p=0.012) and daily dose with hair drug level (r=0.65, p=0.042). An even stronger correlation was found between blood and hair concentrations, r=0.90 with p=<0.001. However, between the daily dose and blood and oral fluids drug levels the correlations were slightly weaker, r=0.58 with p=0.080 and r=0.66 with p=0.073, respectively. Correlation results significantly increased when they were logarithmically transformed e.g. dose and oral fluids gave r=0.795 with p=0.018. Matsuno et al conducted a similar study and found significant correlations as well between both haloperidol hair concentration and daily dose (r=0.682, p=<0.001) and with plasma concentration (r=0.558, p=<0.001) (Matsuno, Uematsu & Nakashima 1990). However, they did not find a significant correlation between plasma drug level and daily dose.

Regardless of pharmacokinetic and hair growth inter-individual variations and the risperidone metabolite, in this study four out of the five investigated drugs showed significant correlation between hair and blood concentrations. Therefore this study supports that the bloodstream must be playing a major role in hair drug incorporation. Similarly with oral fluids four out of the six compounds (including OH-risperidone) showed correlation between blood and oral fluids. Therefore, compiling data from such studies could be used to establish therapeutic limits for the alternative matrices, as alternative samples have many advantages.

The lack of a correlation between the dosage of the drugs given to the patients and the blood concentrations should be taken into great consideration as it showed clearly that the assigned therapeutic levels should remain tentative due to the great inter-individual variations observed with these drugs.

Table 41: Summary of all Pearson correlation coefficients and their P-values

	r Pearson correlation coefficient (P-value)					
Drug	**Blood/Dose**	**Oral fluids/Dose**	**Hair/Dose**	**Blood/Oral fluids**	**Oral fluids/Hair**	**Hair/Blood**
Procyclidine	**0.38** (0.13)	**0.28** (0.31)	**0.22** (0.39)	**0.19** (0.49)	**0.21** (0.45)	**0.83 (0.001)**
Quetiapine	**-0.02** (0.95)	**0.03** (0.93)	**0.29** (0.26)	**-0.05** (0.86)	**0.16** (0.58)	**0.28** (0.33)
Risperidone	**0.62 (0.02)**	**0.67 (0.01)**	**0.67 (0.01)**	**0.59 (0.04)**	**0.66 (0.01)**	**0.96 (0.001)**
OH-risperidone	**0.15** (0.61)	**0.06** (0.84)	**-0.20** (0.52)	**0.51** (0.08)	**-0.03** (0.94)	**0.24**(0.44)
Chlorprothixene	**0.40** (0.18)	**0.68 (<0.05)**	**0.37** (0.21)	**0.79 (0.01)**	**0.21** (0.63)	**0.32** (0.32)
Haloperidol	**0.58** (0.08)	**0.66 (0.07)**	**0.65 (0.04)**	**0.76 (0.03)**	**0.83 (0.01)**	**0.90 (0.001)**

Green: significant correlations. Yellow: weak correlations or significant when Log transformed.

3.10.7. Effect of t ½ on drug incorporation into hair

It is known that a number of factors contribute to drug incorporation into hair such as basicity and lipophilicity of the drug and the melanin content of the hair (Pragst, Balikova 2006). In the present study another factor has been looked at which is the drug half-life in blood and its effect on drug incorporation into hair. Kintz et al mentioned that when drug plasma half-life increases the correlation between hair concentration and dose increases (Kintz et al. 1998). However, they did not look at the hair incorporation effect. When the ratios of hair concentration over daily dose of the investigated drugs were plotted against their blood half-lives it showed a very remarkable and significant correlation (r^2=0.96, p≤0.003) as seen in Figure 26. Procyclidine, quetiapine, risperidone, chlorprothixene and haloperidol half-lives were 12, 6, 8.5, 10 and 36 hours, respectively (Whiteman et al. 1985, DeVane, Nemeroff 2001a, Raaflaub 1975, de Leon et al. 2004, Feng et al. 2008).

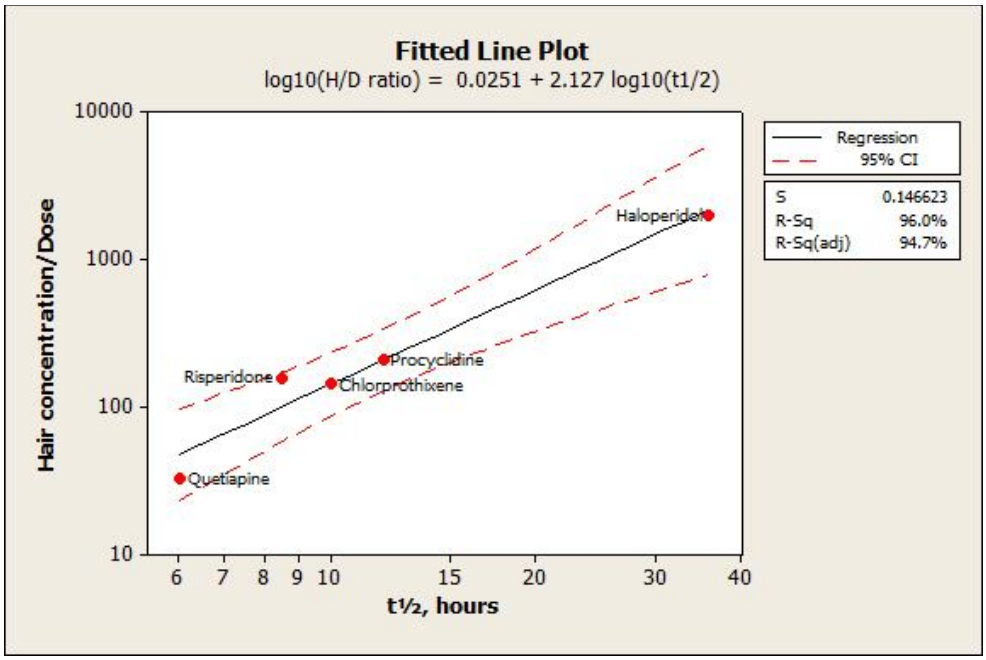


Figure 26: The correlation between concentration in hair over dose ratio and the elimination half-life of the drugs (r^2 = 0.96, p ≤ 0.003)

3.11. Conclusion

After development and validation of the LLE-LC-MS method for the analysis of anti-psychotics in blood, oral fluids and hair, an investigation was carried out on the correlation pattern between trough levels in those three matrices. The most significant correlation coefficients (r) found were those between blood and hair concentrations, procyclidine r=0.83 (18 subjects p=<0.001), risperidone r=0.96 (14 subjects p=<0.001), haloperidol r=0.90 (10 subjects p=<0.001), OH-risperidone r=0.24 (13 subjects p=>0.44), quetiapine r=0.28 (14 subjects p=>0.33) and chlorprothixene r=0.32 (13 subjects p=>0.32). Among the interesting results is the strong correlation found between drug half-lives and the mean ratio of hair concentration/dose (r= 0.96, p≤0.003).

4. Chapter 4 - Stability of some psychoactive drugs in dried blood spots from real patients' samples

4.1. Introduction

Dried blood spot (DBS) sample collection is a well-established practice for the identification of metabolic errors or congenital disorders of metabolism for neonates since almost half a century. This technique was published in the sixties by Dr Robert Guthrie, and the filter paper cards used for blood collection are named after him (Guthrie, Susi 1963). Dr Guthrie used the cards for the collection of newborn blood to detect phenylalanine. Subsequently, a number of screening tests for different metabolic disorders in the newborn have emerged using the DBS technique (Mei et al. 2001). In recent years the applications of DBS have been expanded further to include drugs and their metabolites in pharmacokinetic studies and therapeutic drug monitoring (AbuRuz, Millership & McElnay 2006, Edelbroek, Heijden & Stolk 2009). Furthermore giant pharmaceutical companies are adapting this technique for clinical drug development particularly in their toxicokinetics and pharmacokinetics studies (Spooner, Lad & Barfield 2009), as DBS sampling has many advantages over the traditional way of venipuncturing or cannula sampling. DBS require only a drop of blood from a finger, toe or heel pricking whereas in conventional sampling normally ≥ 0.5mL is needed. DBS decreases the risk of infections with blood-borne viruses. Moreover, transportation of DBS samples is predominantly easier than liquid biological samples (e.g. plasma, blood, etc) since the analytes are generally much more stable and do not require dry ice or refrigeration; they can even be posted by mail (La Marca et al. 2009). Sample stability is of paramount importance in order to ensure accurate results. Degradation of analytes in the sample is usually caused by three possible reactions: hydrolysis, oxidation and reduction (Drummer 2004). The commonest among them is the hydrolysis of amide and ester groups within drugs' structures (Waterman, Adami 2005). For instance, it has been found that

hydrolytically labile drugs were much more stable in DBS compared to liquid samples after 1 year in different storage temperatures (Alfazil, Anderson 2008). In addition, the non-invasive DBS technique made it easier for volunteers to participate in clinical studies, particularly when paediatric patients are needed. Another great advantage of DBS analysis is the great potential for automation of sample processing. A number of companies have already launched machines that automatically punch the cards, do the extraction/cleaning and introduction/injection into the LC-MS/MS system as shown in Figure 27 and 28 below.

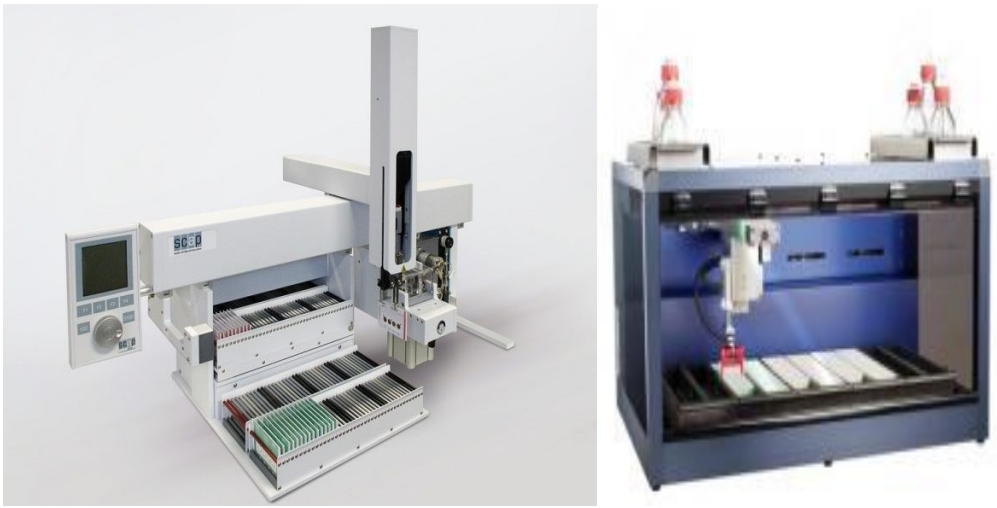


Figure 27: Dried blood spot analysis automation (Karolia 2011, Camag AG 2011)

Figure 28: Specially designed cards for microvolume DBS analysis (ichrom.com 2011, Whatman.com 2009)

4.1.1. Filter papers/cards

Currently, there are a number of different types of DBS cards available on the market. For drugs and small molecules analysis Guthrie cards (Schleicher & Schuell 903®) can be used, they are made of 100% pure cotton linters with no wet-strength additives (Li, Tse 2010). They have been used widely for newborn screening and other uses. The other types such as FTA and FTA Elute are pre-treated cards with chemicals to denature proteins, lyse cells and protect samples from bacterial growth (http://www.whatman.com).

4.1.2. Haematocrit

Haematocrit is the proportion volume of red blood cells out of the total volume of whole blood (all components). The percentage of haematocrit in blood varies with age, sex and general health (Jopling et al. 2009). The ranges are between 0.37 and 0.51 among adults, 0.35 and 0.42 among children and 0.28 to 0.67 in neonates (Kayiran et al. 2003, Wilhelm et al. 2009). The importance of haematocrit effect lies on its effect on the pattern of distribution of the blood drop and its diffusion in the filter paper. Haematocrit has great impact on the viscosity of the blood. Thus, if the blood has a high haematocrit percentage it would result in a smaller and more concentrated spot on the filter paper and vice versa in the case of a low haematocrit sample. However, a number of studies have been conducted on evaluating the haematocrit effect on DBS samples and the conclusion is that it seems that the effect maybe case dependent as it not predominantly significant with most of the tested analytes (Li, Tse 2010).

4.1.3. Spot volume

Due to the advances in the sensitivity of mass spectrometry detectors, especially the tandem ones, it became no longer necessary to have a large volume of samples (e.g. 1mL) for extraction of analytes. Some new DBS methods use only 10µL blood spot (micro-whole blood analysis) (AbuRuz, Millership & McElnay 2006, Déglon et al. 2010, Déglon et al. 2009).

Unlike the recent trend in the published DBS studies, where they punch the filter cards with a 3mm punch in the centre of the spot after drying out, in this study the whole spot was taken and precise and consistent amount of blood (50µL) has been used/spotted for each sample. The reason behind the adoption of this method is to prevent the possible inaccuracy in the results when an uneven distribution of blood on the paper occurs. Therefore, this way will reduce the effect of haematocrit difference between samples.

4.2. Dried blood spot analysis

4.2.1. Aim of the study

The first aim of this study was to develop and validate a method to enable the use of the DBS technique for the analysis and quantification of some of the common psychoactive drugs used in psychiatric clinics in Kuwait. Secondly, to investigate the stability of these drugs in 50µL of whole blood spotted, dried and stored for 6 months in freezers (-20 and -80ºC), refrigerator (4ºC) and at room temperature (25ºC). Another objective was to see the effect of impregnating the cards with sodium fluoride prior to spotting on the stability of the drugs.

4.2.2. Study analytes

The analytes of interest in this study are the same as those covered in the previous chapter. The structures and brief information about each analyte are found in chapter 3. These analytes were procyclidine, quetiapine, risperidone, 9-OH-risperidone, chlorprothixene and haloperidol.

4.2.3. Source of samples

Blank blood (drug free) was collected from laboratory personnel. The source of blood positive samples used in this study was a selection of patients' samples with the highest drug concentrations found in the previous study (chapter 3) for which consent and ethical approval had been already granted. The reason behind that selection is to empower the study's results with authentic patients' samples and avoid building conclusions based on spiked samples even though they are generally and widely accepted. More than one patient sample has been selected for each drug with the highest concentrations as illustrated in results' tables (section 4.5.5). Forty spots from each patient sample were spotted on the cards to cover the different storage time/temperature regimens.

4.2.4. Materials and reagents

All chemicals were of HPLC or analytical grade. All consumables (tubes, etc) are from VWR UK. Whatman 903 neonatal Guthrie cards were purchased from Whatman USA. The rest of chemicals used in the solutions below are from Sigma-Aldrich Co. UK. For drug and solvent standards see section 2.2.2 and 3.4.

Other solutions used in this chapter have been mentioned in section 2.2.2.2.

Standard solutions

1000µg/mL or 100µg/mL working standards of procyclidine, quetiapine, risperidone, 9-OH-risperidone, chlorprothixene, haloperidol, nortriptyline-D3 and haloperidol-D4 were made with MS grade methanol. All were stored at -20ºC.

Quality control samples (QCs)

QCs were prepared in two concentrations, lower 0.5µg/mL and higher 2µg/mL. The higher QC samples were prepared by dissolving 10µL of 1000µg/mL stock standard of the analyte in 4.990mL pooled drug free blood (containing fluoride/oxalate as preservative and anticoagulant). Whereas the lower QCs were prepared by diluting

1mL of the higher QC mixture with 3mL pooled drug free blood. All the mixtures were thoroughly mixed and divided into 150µL aliquots, stored in 0.5mL Eppendorf tubes and kept at -80°C until analysis. Prior to experiments QC aliquots were thawed at room temperature and 50µL was spotted on the filter paper 3-18 hours before extraction and analysis.

DBS extraction solution MAF

80mL of 0.1% formic acid methanol, 10mL of acetonitrile and 10mL of deionized water were measured in measuring cylinder, mixed well and stored at room temperature.

NaF solution 0.5% (w/v)

100mg of sodium fluoride salt was dissolved in 20mL of deionized water, mixed properly and stored in the fridge. (20µL was used for preserving a filter paper card spot).

Calibration standards

Calibration samples were prepared by dissolving 50µL of 1000µg/mL stock standard of the analyte in 4.95mL pooled drug free blood (containing fluoride/oxalate as preservative and anticoagulant) to produce 10µg/mL. A series of dilutions followed with blank blood to produce a range of 2.5 to 2000ng/mL. Fifty milliliters of the spiked blood were spotted on the cards.

4.2.5. Blood spotting

50µL of blank, spiked (calibrators/QCs) or patient blood samples were spotted on the Whatman 903 neonatal cards using a volumetric pipette after 30 minutes of proper mixing to ensure homogeneity and equal distribution of analytes in the samples. The cards were allowed to dry at room temperature for >3 hours. Each card then was packed in a sealable plastic bag containing a desiccant pad and packed again in a paper envelope and stored until the stability analysis due date.

The storage temperatures were varied for stability experiments (25, 4, -20 and -80˚C).

Figure 29: 50µl aliquots of spiked blood spotted on Guthrie cards, dried, and punched out

4.2.6. Method development

4.2.6.1. Extraction optimization

Four liquid-liquid extraction solutions were used and their extraction efficiencies for the analytes were evaluated. 2mL blank blood was spiked with 1µg/mL of analytes and mixed thoroughly, then spotted on the cards (50µL for each spot) and allowed to dry at ambient temperature on a drying rack. After drying, dried blood spots were then punched out with 1.3cm diameter punch. Each disc was placed in an 8mL tube, 60µL of internal standard mixture (1µg/mL of haloperidol-D4 and nortriptyline-D3) was added and the following methods were applied in triplicate.

DBS extraction method 1 and 2

One and a half mL of either MAF (method 1) solution (0.1% formic acid methanol/acetonitrile/deionized water - 8:1:1) or MTBE (method 2) was added to the tubes containing 60µL of internal standard with the discs and vortex mixed for 15 minutes and then sonicated for 1 hour at 40˚C. The discs were then removed and

the remaining solution was evaporated under nitrogen. The extracts were then reconstituted in 0.3mL of 0.1M NaOH solution, followed with 0.3mL MTBE and vortex mixed for 2 minutes, centrifuged and then the solvent layer were transferred into 0.3mL auto-sampler vials. The solvent then was evaporated under nitrogen and finally reconstituted in 120µL of acetonitrile and 10µL was injected into the LC-MS system.

DBS extraction method 3 and 4

One and half mL of 0.1M or 0.05M phosphate buffer (extraction method 3 or 4, respectively) was added to the tube containing 60µL internal standard with the disc and vortex mixed for 15 minutes and then sonicated for 1 hour at 40˚C. The disc was then removed and to the remaining solution 0.5mL of 1M NaOH was added, vortex mixed for 30 seconds, followed with 2mL MTBE and mixing on a rotary mixer for 30 minutes, centrifuged thereafter and the solvent layer was transferred into new tubes and evaporated/reduced under nitrogen to ~0.25mL and transferred finally to 0.3 mL auto-sampler vials. The solvent then was evaporated completely under nitrogen and reconstituted in 120µL of acetonitrile and 10µL was injected into the LC-MS system.

4.2.6.2. Extraction optimization results

Following the application of the four methods, results showed that method 1 (MAF) produced the highest recoveries for most of the analytes with recoveries ranging from 79 to 106% except chlorprothixene (54%). Method 2 (MTBE) also showed good recoveries for most the analytes ranging from 61 to 122% except for risperidone and its metabolite, 47 and 26% respectively. The other methods, 3 and 4, gave generally poor recoveries with mean recoveries for all analytes of 37 and 41% respectively. Details are shown in Table 42.

Table 42: Recovery of (1µg/mL) drugs from DBS using four different solutions (n=3)

Analytes	Mean recovery % (CV %)			
	Method 1 MAF	**Method 2 MTBE**	**Method 3 Phos 0.1M**	**Method 4 Phos 0.05M**
Procyclidine	**106 (7)**	**122 (12)**	59 (5)	63 (3)
Quetiapine	**83 (20)**	61 (9)	31 (5)	33 (8)
Risperidone	**79 (16)**	47 (5)	47 (6)	54 (2)
9-OH-risperidone	**79 (4)**	26 (10)	32 (15)	40 (15)
Chlorprothixene	54 (12)	**76 (14)**	8 (6)	9 (7)
Haloperidol	**84 (14)**	**92 (5)**	43 (7)	47 (7)
Mean	**81**	**71**	37	41

4.2.6.3. Successful method

Method 1 (MAF) showed the best general recoveries compared to the other methods and also better reproducibility. MAF solution also showed the ability to partially extract/release haemoglobin from the spots unlike MTBE. In addition methods 1 and 2 were simpler and faster than methods 3 and 4. Therefore, method 1 was chosen for the rest of this project.

4.2.7. LC-MS method

The LC-MS method is described in section 3.6.

4.2.7.1. Ion identification results

Table 43 shows the molecular formula of the analytes, accurate masses, retention times and mass accuracies. Mass accuracy with Exactive MS was <1ppm which means that the potential for interference from unknown components was very little. Figure 30 shows extracted ion chromatograms for spiked analytes at 10ng/mL, while, Figure 31 shows the mass spectra of each analyte ion after extraction from full scan (75-1200 amu) at retention time. Retention chromatograms are shown in stability results, section 4.4.

Table 43: Target analytes plus internal standards and their relevant data

Analyte	Formula (+H)	Accurate mass (M+H)	Retention time	Mass accuracy
Procyclidine	$C_{19}H_{30}NO$	288.2322	4.5	0.066 ppm
Quetiapine	$C_{21}H_{26}O_2N_3S$	384.1739	5.2	-0.115 ppm
Risperidone	$C_{23}H_{28}FN_4O_2$	411.2190	5.7	-0.148 ppm
9-OH-risperidone	$C_{23}H_{28}FN_4O_3$	427.2141	5.0	0.155 ppm
Chlorprothixene	$C_{18}H_{19}ClNS$	316.0921	4.4	0.017 ppm
Haloperidol	$C_{21}H_{24}ClFNO_2$	376.1476	4.4	0.448 ppm
Haloperidol-D4	$C_{21}H_{20}\mathbf{D_4}ClFNO_2$	380.1722	4.4	-0.732 ppm
Nortriptyline-D3	$C_{19}H_{19}\mathbf{D_3}N$	267.1934	4.5	-0.324 ppm

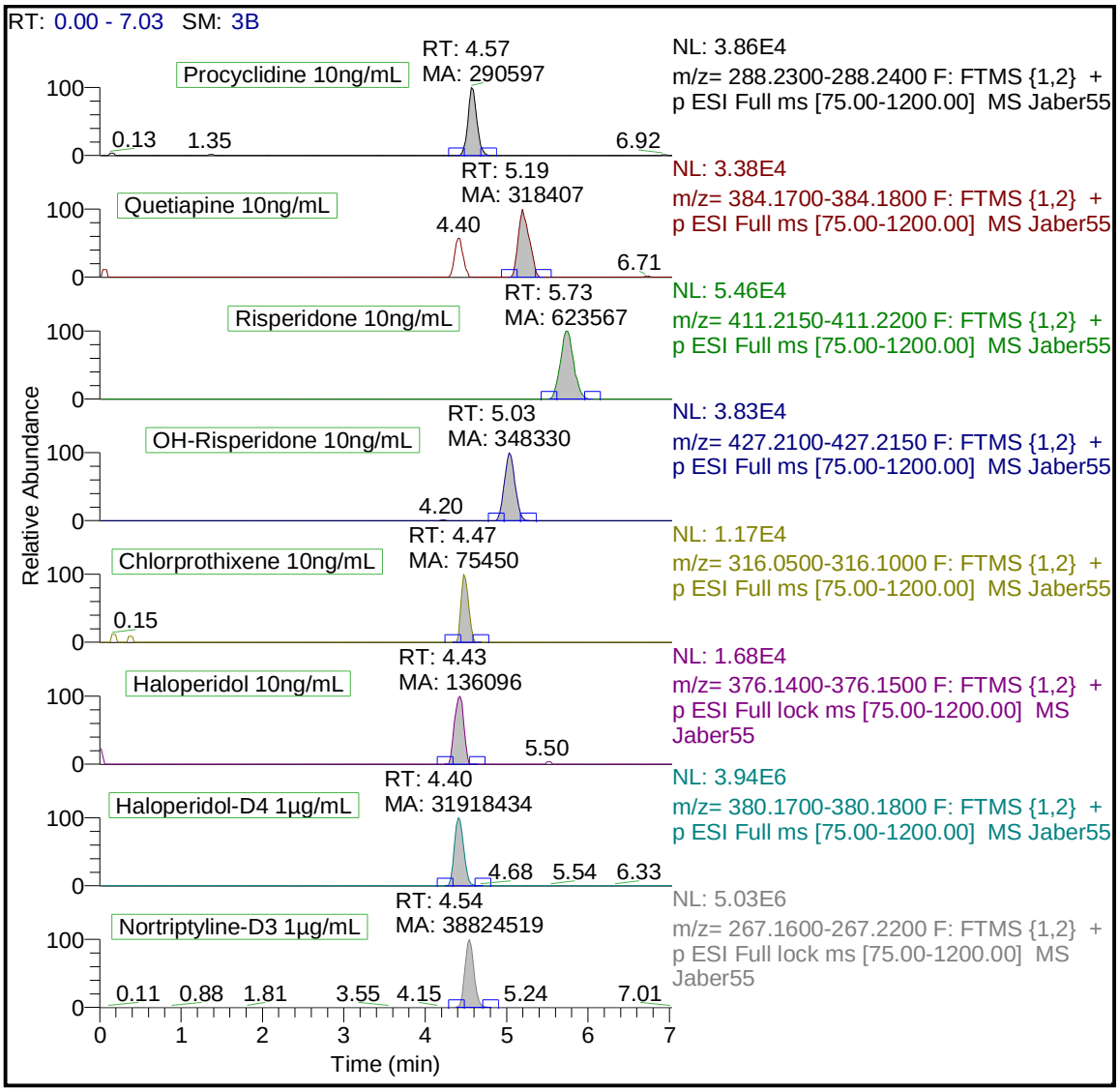


Figure 30 : Set of extracted ion chromatograms, sample spiked with 10ng/mL analytes and 1µg/mL ISs

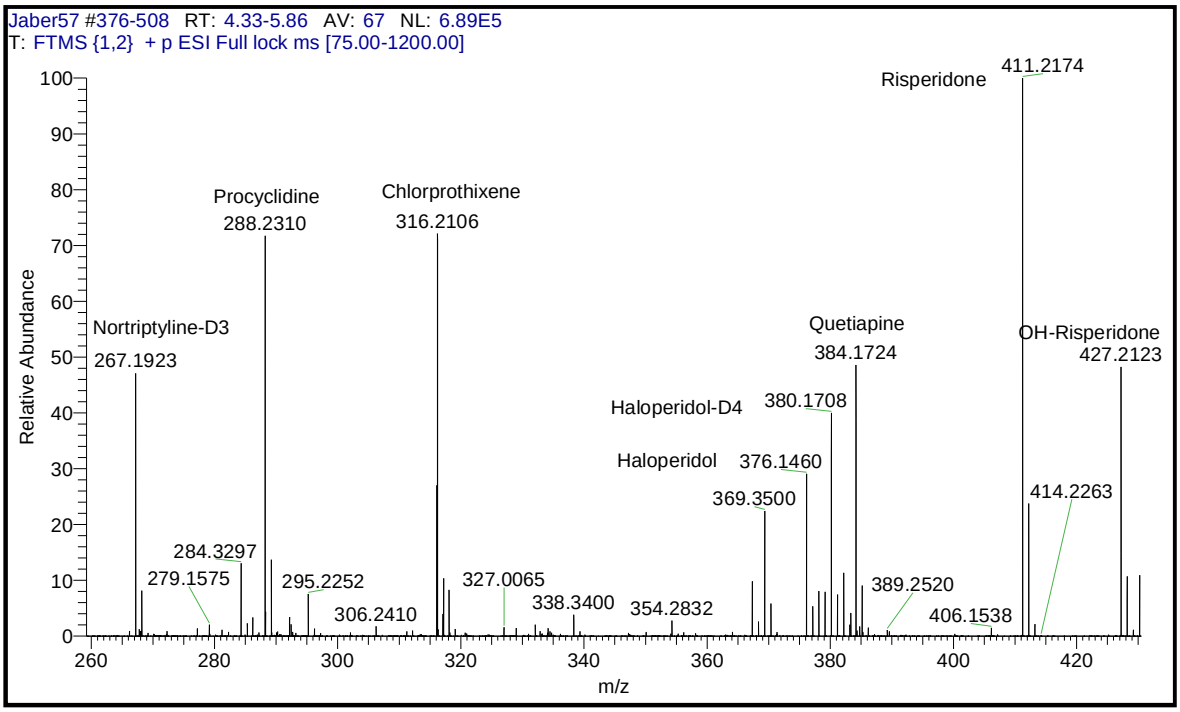


Figure 31: Mass spectra for analytes (1µg/mL) at their mass and retention range

4.2.8. Method validation

4.2.8.1. Linearity

Linearity was attained from spotting 50µL of blank blood samples spiked with concentrations ranging from 2.5, 5, 10, 25, 100, 500, 1000 and 2000ng/mL of each analyte. Deuterated internal standards were 1µg/mL. The resulting ratio of peak areas of analytes/internal standard plotted versus the spiked analyte concentrations were plotted and linear correlation coefficients (r^2) calculated. The correlation coefficient (r^2) value indicate the degree of linearity (r^2 should be >0.99).

4.2.8.2. Recovery

Recoveries were estimated by measuring peak areas obtained when the drugs were spiked into blank blood at three concentrations 100, 500, and 2000µg/mL (n=3) before spotting them on cards against unextracted neat standards dissolved in

acetonitrile. This recovery takes into account all the extraction steps including matrix effect (absolute recovery). The recovery was calculated as a percentage by dividing the average peak area of the extracted standards by the average peak area of the duplicate unextracted standards and multiplied by 100.

4.2.8.3. Intra-day precision

The intra-day precision was made by spiking blank blood samples with two concentrations (medium and high) in five replicates, then spotting them on cards. After extraction and analysis, precision was measured by calculating the coefficient of variation expressed as a percentage (CV %).

4.2.8.4. Inter-day precision

Inter-day precision experiment was done through replicate analysis of intra-day experiments over 5 days and the coefficient of variation percentage (CV %) was calculated between concentrations of each analyte over these five days.

4.2.8.5. LOD and LLOQ

Limit of detection (LOD) and lower limit of quantification (LLOQ) was assessed by spiking blank blood with the analytes in decreasing concentrations before spotting them on cards. LOD is defined as the lowest detectable concentration of the analyte that differentiates it from the background noise by three fold, whereas LLOQ is lowest quantitative concentration that fulfils the required accuracy and precision (10 fold higher than the noise). The spiked concentrations were 1, 2.5, 5, 10 and 25ng/mL of each analyte and 1000ng/mL was the concentration of deuterated internal standard. Ten µL of final extract was injected into the LC-MS system. Signal-to-noise ratio is the method that has been used to calculate LOD and LLOQ, and 3 and 10 respectively were the cut-offs. LODs and LLOQs were calculated using

Equation 2 to Equation 5 (page 91).

4.2.8.6. Method validation results

The relationship between analyte concentrations and peak area ratios was linear over the range of 2.5 to 2000ng/mL and the linear correlation coefficients (r^2) were better than 0.99 for all analytes as shown in Table 44 and Figure 32. LOD and LLOQ were calculated using Equations 2-5 and also from the chromatograms. LOD values for all analytes were 0.1-1.5ng/mL and LLOQ values were 0.4-4.5ng/mL. The details presented in Tables 44 and 45.

Table 44: Linear correlation coefficients, LODs and LLOQs for analytes in DBS calculated using Equations 2-5

Analyte	Linear correlation coefficient (r^2)	LOD ng/mL	LLOQ ng/mL
Procyclidine	0.9938	0.1	0.4
Quetiapine	0.9979	0.7	2.4
Risperidone	0.9977	1.0	3.4
9-OH-risperidone	0.9967	1.1	3.7
Chlorprothixene	0.9924	0.6	1.9
Haloperidol	0.9992	0.2	0.7

Table 45: Linear correlation coefficients, LODs and LLOQs for analytes in DBS calculated from the chromatograms

Analyte	Linear correlation coefficient (r^2)	LOD ng/mL	LLOQ ng/mL
Procyclidine	0.9938	1.5	4.5
Quetiapine	0.9979	0.5	1.5
Risperidone	0.9977	0.2	0.6
9-OH-risperidone	0.9967	0.3	1.0
Chlorprothixene	0.9924	0.5	1.5
Haloperidol	0.9992	0.7	2.0

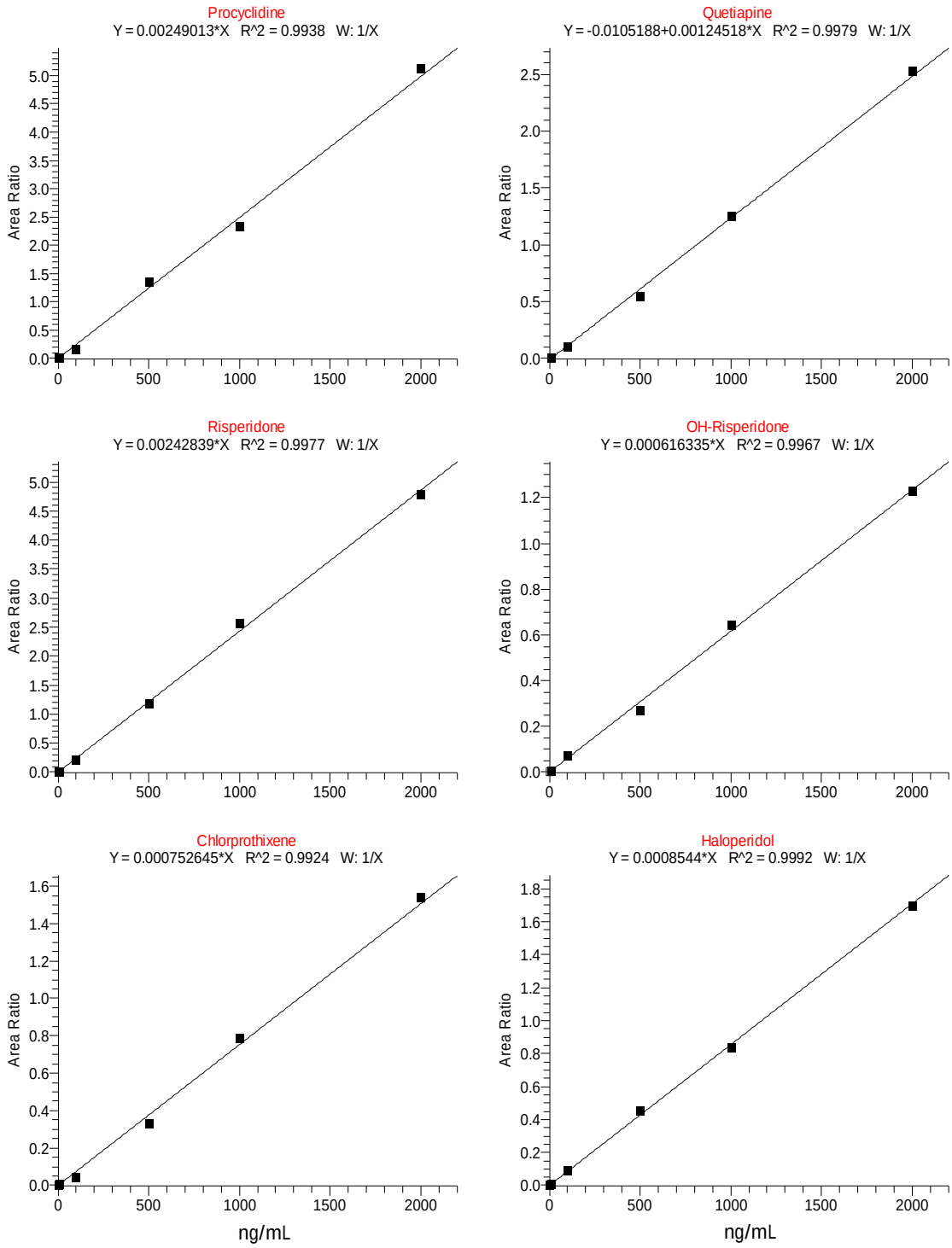


Figure 32: DBS calibration lines for all analytes

As shown in Table 46 the method revealed good precision and the coefficient of variation (CV%) for intra- and inter-day precision was in the range 2-15 and 2-12% respectively, which are considered to be acceptable.

Table 46: Inter-day and intra-day precision results in DBS

Analyte	Inter-day mean (CV%) n=5		Intra-day mean (CV%) n=5	
	0.5µg/mL	2µg/mL	0.5µg/mL	2µg/mL
Procyclidine	0.506 (3)	2.142 (2)	0.536 (2)	2.174 (5)
Quetiapine	0.456 (5)	1.901 (2)	0.448 (6)	2.106 (9)
Risperidone	0.463 (8)	2.084 (3)	0.492 (3)	2.107 (7)
9-OH-Risperidone	0.491 (10)	1.925 (3)	0.457 (15)	2.132 (8)
Chlorprothixene	0.476 (12)	2.077 (5)	0.473 (13)	2.152 (3)
Haloperidol	0.442 (7)	1.924 (5)	0.525 (5)	2.139 (7)

The overall means of absolute recoveries for all analytes at the three concentrations were in the range of 64 to 94% as shown and detailed in Table 47.

Table 47: Absolute recovery (RE%) results for 0.1, 0.5 and 2µg/mL of analytes spiked in DBS (n=3)

Analyte	0.1µg/mL (RE%)	%CV	0.5µg/mL (RE%)	%CV	2µg/mL (RE%)	%CV	Mean (%CV) for 3 Conc.
Procyclidine	63	3	65	8	63	3	64 (2)
Quetiapine	46	4	77	4	70	2	64 (25)
Risperidone	59	5	91	2	85	2	78 (22)
9-OH-risperidone	62	2	86	3	75	4	74 (16)
Chlorprothixene	69	4	66	10	67	6	67 (2)
Haloperidol	102	3	98	1	83	2	94 (11)

4.3. Impregnation/pre-treatment with sodium fluoride

In order to look at the efficacy of pre-treating Guthrie cards with preservative such as sodium fluoride, half of the stability study spots/circles were pre-treated with 20µL of 5mg/mL NaF solution. This amount was enough to spread all over the spot (13mm). The pre-treated cards/spots were allowed to dry completely before applying/spotting patients' blood samples. Data analysis was performed using Microsoft Excel 2010. A t-test was done to test whether the analyte concentrations from patients' blood spotted on NaF treated cards and the corresponding untreated cards were significantly different. The threshold chosen for statistical significance was $p=<0.05$.

4.4. Stability results

4.4.1. Procyclidine

Table 48: Stability of procyclidine in DBS from patient 1

Time (day)	Storage condition							
	Room temperature (25 °C) (ng/mL)		Refrigerator (4 °C) (ng/mL)		Freezer (-20 °C) (ng/mL)		Freezer (-80 °C) (ng/mL)	
Pre-Treated	-	NaF	-	NaF	-	NaF	-	NaF
0	260.7	268.7	260.7	268.7	260.7	268.7	260.7	268.7
45	180.1	177.5	206.1	240.4	272.8	284.2	257.1	272.5
90	185.3	223.3	229.5	241.5	225.4	210.2	197.5	201.4
180	198.6	216.2	223.4	209.0	222.1	218.0	231.7	227.5

Table 49: Stability of procyclidine in DBS from patient 2

Time (day)	Storage condition							
	Room temperature (25 °C) (ng/mL)		Refrigerator (4 °C) (ng/mL)		Freezer (-20 °C) (ng/mL)		Freezer (-80 °C) (ng/mL)	
Pre-Treated	-	NaF	-	NaF	-	NaF	-	NaF
0	97.8	119.1	97.8	119.1	97.8	119.1	NA	NA
45	117.3	96.5	105.5	98.7	112.9	105.4	NA	NA
90	119.3	102.3	120.2	116.1	112.8	112.1	NA	NA
180	125.4	124.5	139.1	120.4	139.5	139.3	NA	NA

NA, not available (amount of the patient sample was not enough to cover all conditions)

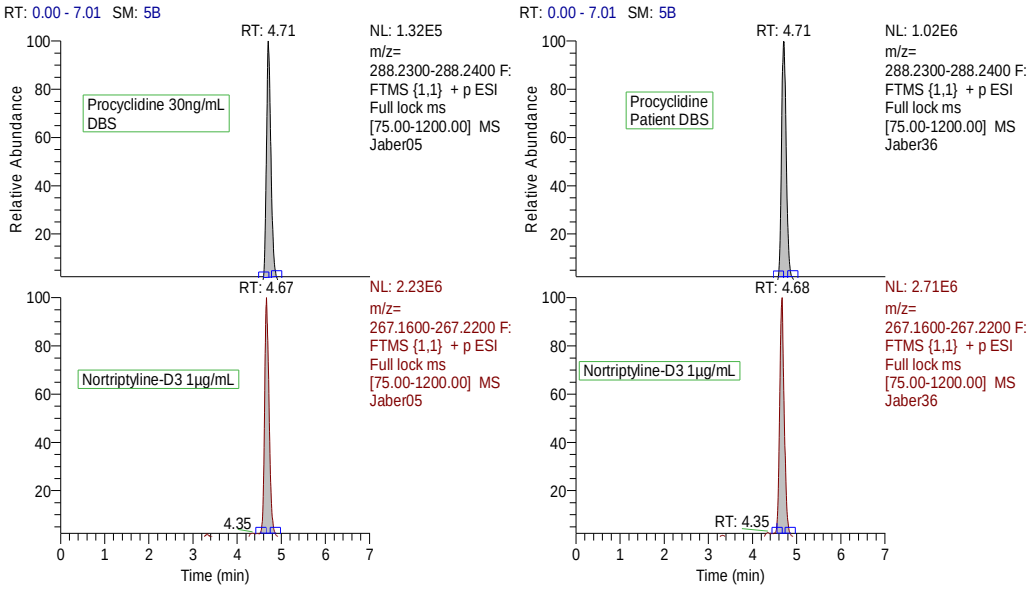


Figure 33: Procyclidine and IS chromatograms from low calibrator and patient 2 DBS samples

4.4.2. Quetiapine

Table 50: Stability of quetiapine in DBS from patient 1

Time (day)	Storage condition							
	Room temperature (25 °C) (ng/mL)		Refrigerator (4 °C) (ng/mL)		Freezer (-20 °C) (ng/mL)		Freezer (-80 °C) (ng/mL)	
Pre-Treated	-	NaF	-	NaF	-	NaF	-	NaF
0	324.5	331.8	324.5	331.8	324.5	331.8	324.5	331.8
45	221.6	194.7	299.6	276.1	272.3	268.4	273.1	276.0
90	230.3	187.5	178.7	208.4	194.4	239.7	249.4	218.5
180	215.1	227.1	242.1	204.9	264.9	212.8	248.8	245.6

Table 51: Stability of quetiapine in DBS from patient 2

Time (day)	Storage condition							
	Room temperature (25 °C) (ng/mL)		Refrigerator (4 °C) (ng/mL)		Freezer (-20 °C) (ng/mL)		Freezer (-80 °C) (ng/mL)	
Pre-Treated	-	NaF	-	NaF	-	NaF	-	NaF
0	164.0	166.4	164.0	166.4	164.0	166.4	164.0	166.4
45	133.6	138.4	152.3	154.1	153.6	140.2	157.5	150.9
90	150.6	151.3	123.2	152.4	126.7	159.5	141.5	144.2
180	148.1	149.5	168.5	156.2	135.7	144.9	142.4	146.5

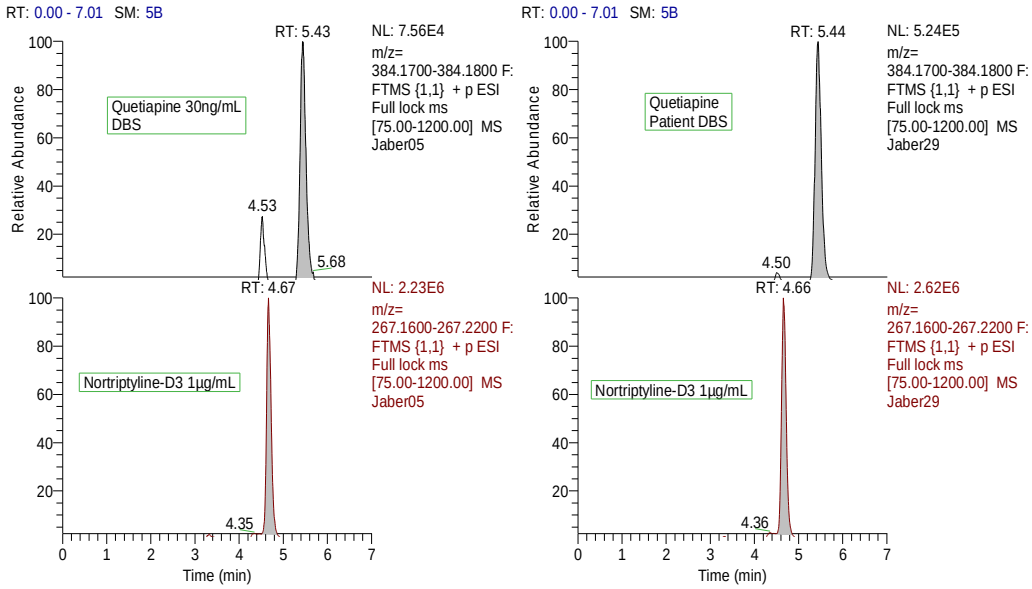


Figure 34: Quetiapine and IS chromatograms from low calibrator and patient 2 DBS samples

4.4.3. Risperidone

Table 52: Stability of risperidone in DBS from patient 1

Time (day)	Storage condition							
	Room temperature (25 °C) (ng/mL)		Refrigerator (4 °C) (ng/mL)		Freezer (-20 °C) (ng/mL)		Freezer (-80 °C) (ng/mL)	
Pre-Treated	-	NaF	-	NaF	-	NaF	-	NaF
0	3.9	3.7	3.9	3.7	3.9	3.7	3.9	3.7
45	4.3	4.5	5.4	5.5	5.2	4.2	3.7	4.5
90	3.5	3.6	3.2	3.6	3.7	3.5	3.5	3.3
180	4.0	4.2	4.7	4.5	3.4	4.4	3.9	4.0

Table 53: Stability of risperidone in DBS from patient 2

Time (day)	Storage condition							
	Room temperature (25 °C) (ng/mL)		Refrigerator (4 °C) (ng/mL)		Freezer (-20 °C) (ng/mL)		Freezer (-80 °C) (ng/mL)	
Pre-Treated	-	NaF	-	NaF	-	NaF	-	NaF
0	48.8	45.5	48.8	45.5	48.8	45.5	NA	NA
45	50.6	51.1	42.6	46.4	43.2	45.3	NA	NA
90	42.7	44.6	42.3	46.0	40.4	42.1	NA	NA
180	45.3	39.7	43.4	40.6	40.9	43.3	NA	NA

NA, not available (amount of the patient sample was not enough to cover all conditions)

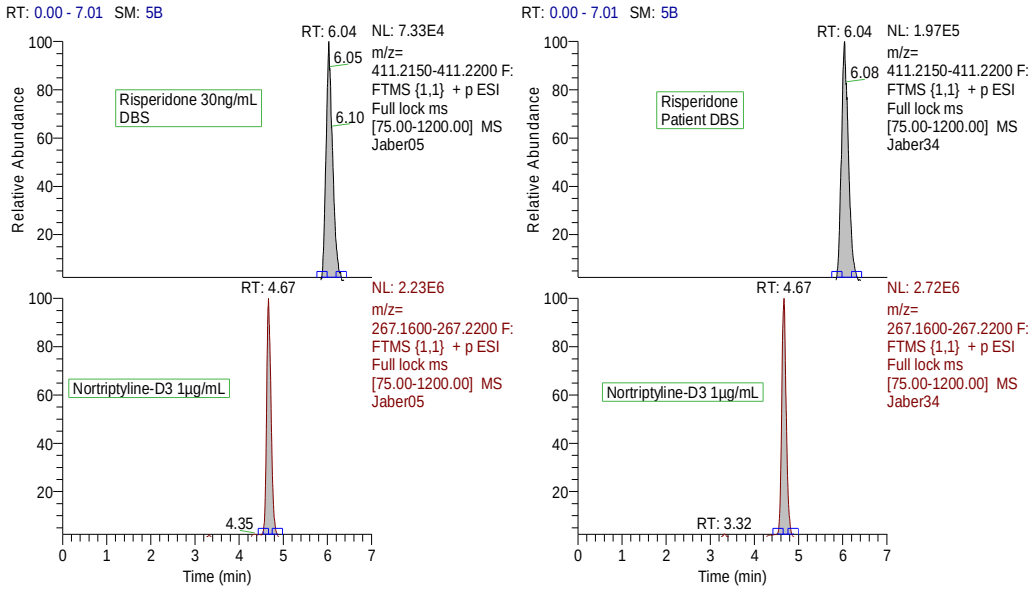


Figure 35: Risperidone and IS chromatograms from low calibrator and patient 2 DBS samples

4.4.4. OH-risperidone

Table 54: Stability of OH-risperidone in DBS from patient 1

Time (day)	Storage condition							
	Room temperature (25 °C) (ng/mL)		Refrigerator (4 °C) (ng/mL)		Freezer (-20 °C) (ng/mL)		Freezer (-80 °C) (ng/mL)	
Pre-Treated	-	NaF	-	NaF	-	NaF	-	NaF
0	17.6	16.0	17.6	16.0	17.6	16.0	17.6	16.0
45	17.8	16.1	14.7	14.5	18.1	18.4	18.6	16.9
90	21.1	18.1	19.0	19.9	17.0	19.9	NF	20.3
180	24.7	21.7	27.9	22.8	23.3	20.5	22.9	22.2

*NF (not found).

Table 55: Stability of OH-risperidone in DBS from patient 2

Time (day)	Storage condition							
	Room temperature (25 °C) (ng/mL)		Refrigerator (4 °C) (ng/mL)		Freezer (-20 °C) (ng/mL)		Freezer (-80 °C) (ng/mL)	
Pre-Treated	-	NaF	-	NaF	-	NaF	-	NaF
0	40.3	42.2	40.3	42.2	40.3	42.2	40.3	42.2
45	39.2	33.4	42.4	42.8	38.6	38.9	43.4	41.1
90	35.0	35.7	24.3	34.1	28.6	34.6	32.7	27.6
180	39.2	38.1	41.4	40.4	32.9	36.3	37.1	37.3

Table 56: Stability of OH-risperidone in DBS from patient 3

Time (day)	Storage condition							
	Room temperature (25 °C) (ng/mL)		Refrigerator (4 °C) (ng/mL)		Freezer (-20 °C) (ng/mL)		Freezer (-80 °C) (ng/mL)	
Pre-Treated	-	NaF	-	NaF	-	NaF	-	NaF
0	23.0	20.1	23.0	20.1	23.0	20.1	NA	NA
45	17.3	19.0	16.9	16.6	15.2	14.2	NA	NA
90	13.5	15.5	18.2	13.4	12.8	12.2	NA	NA
180	16.1	13.4	14.3	13.5	14.3	14.9	NA	NA

NA, not available (amount of the patient sample was not enough to cover all conditions)

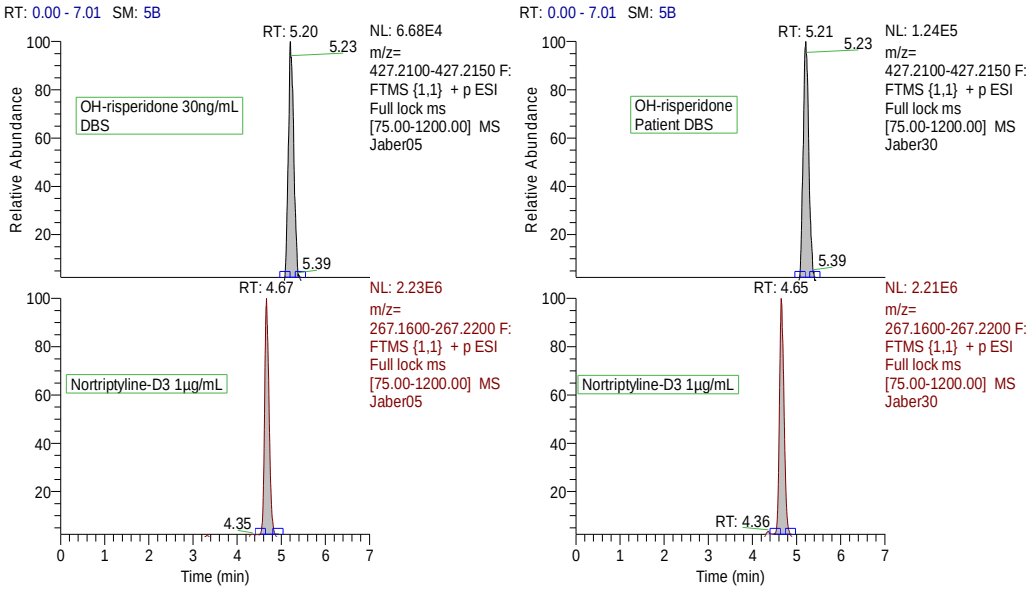


Figure 36: OH-risperidone and IS chromatograms from low calibrator and patient 2 DBS samples

4.4.5. Chlorprothixene

Table 57: Stability of chlorprothixene in real patient DBS sample

Time (day)	Storage condition							
	Room temperature (25 °C) (ng/mL)		Refrigerator (4 °C) (ng/mL)		Freezer (-20 °C) (ng/mL)		Freezer (-80 °C) (ng/mL)	
Pre-Treated	-	NaF	-	NaF	-	NaF	-	NaF
0	8.1	8.9	8.1	8.9	8.1	8.9	8.1	8.9
45	3.7	7.4	7.4	5.7	6.9	8.6	7.1	7.7
90	8.3	9.5	8.4	9.2	8.1	7.5	NF	7.2
180	11.7	11.2	14.6	13.0	12.9	14.7	12.0	12.4

*NF (not found).

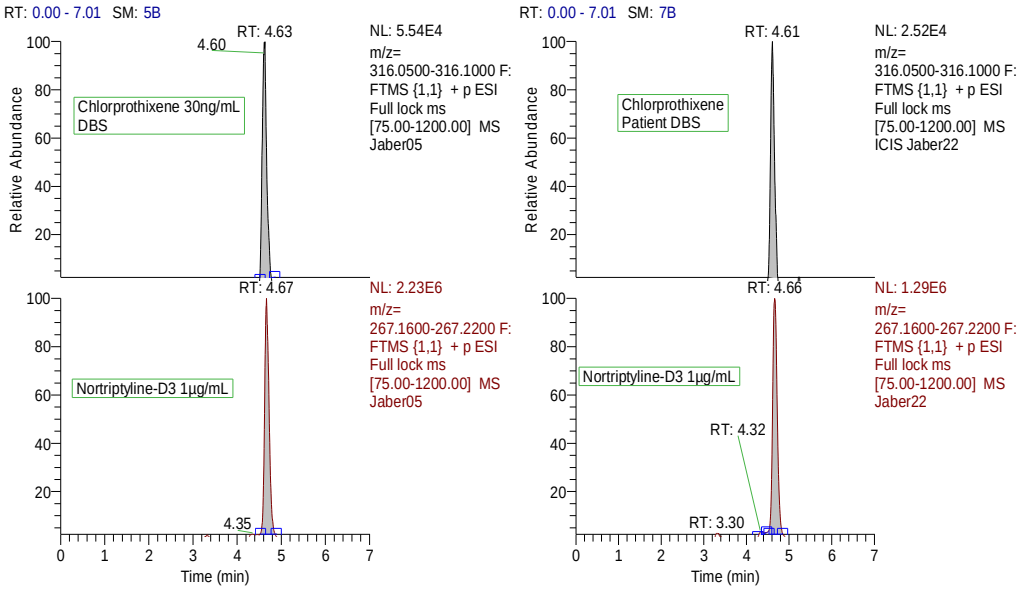


Figure 37: Chlorprothixene and IS chromatograms from low calibrator and patient DBS samples

4.4.6. Haloperidol

Table 58: Stability of haloperidol in DBS from patient 1

Time (day)	Storage condition							
	Room temperature (25 °C) (ng/mL)		Refrigerator (4 °C) (ng/mL)		Freezer (-20 °C) (ng/mL)		Freezer (-80 °C) (ng/mL)	
Pre-Treated	-	NaF	-	NaF	-	NaF	-	NaF
0	30.7	31.4	30.7	31.4	30.7	31.4	30.7	31.4
45	27.3	26.0	32.6	27.8	29.5	30.4	33.9	31.0
90	28.6	29.5	27.1	27.7	29.9	29.0	29.3	30.7
180	28.5	27.7	26.4	23.3	31.2	25.7	27.8	28.7

Table 59: Stability of haloperidol in DBS from patient 2

Time (day)	Storage condition							
	Room temperature (25 °C) (ng/mL)		Refrigerator (4 °C) (ng/mL)		Freezer (-20 °C) (ng/mL)		Freezer (-80 °C) (ng/mL)	
Pre-Treated	-	NaF	-	NaF	-	NaF	-	NaF
0	8.9	7.3	8.9	7.3	8.9	7.3	NA	NA
45	4.9	6.5	5.2	5.6	3.7	4.4	NA	NA
90	8.3	8.6	7.8	9.3	8.2	8.4	NA	NA
180	8.0	7.9	8.5	8.0	8.0	8.2	NA	NA

NA, not available (amount of the patient sample was not enough to cover all conditions)

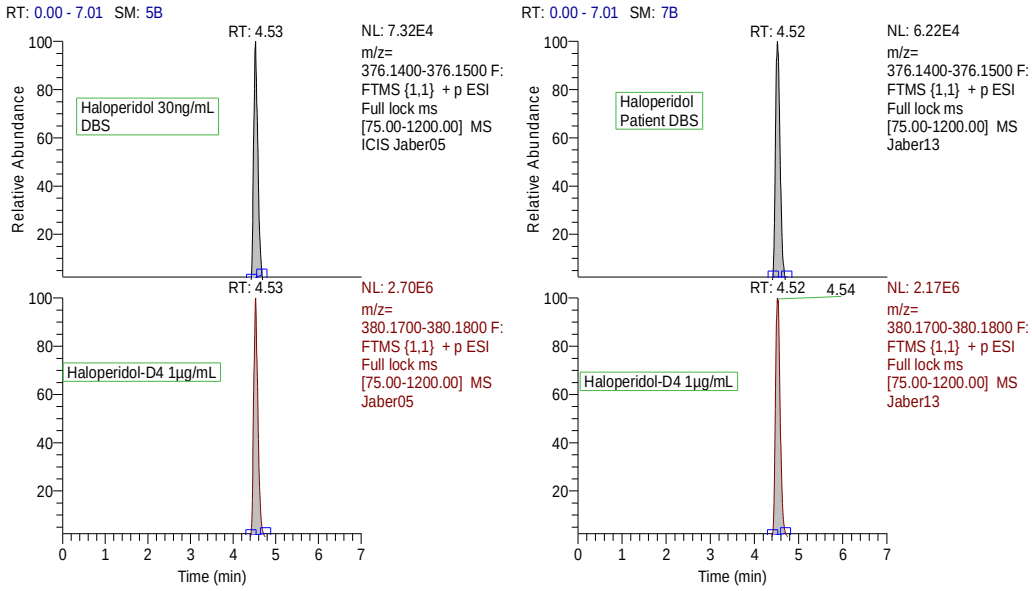


Figure 38: Haloperidol and IS chromatograms from low calibrator and patient 1 DBS samples

4.4.7. Stability results summary

Analytes were found to be stable in dried blood spots stored at room temperature (25±2°C) over 180 days and the loss observed was minimal. Testing on day 45

showed analyte stability to be between 72 and 108% compared to initial concentrations, while testing on days 90 and 180 showed stabilities ranges from 80 to 104% and 79 to 134%, respectively. Details are shown in Table 60.

Table 60: Stability of analytes in DBS stored at 25ºC

Analyte	Percentage of stability for all patients (CV%)			
	Day 0	Day 45	Day 90	Day 180
	DBS	DBS	DBS	DBS
Procyclidine	100	72 (11)	80 (10)	87 (17)
Quetiapine	100	78 (10)	85 (14)	79 (17)
Risperidone	100	108 (4)	93 (6)	99 (12)
9-OH-risperidone	100	91 (12)	96 (19)	81 (19)
Chlorprothixene	100	64 (40)	104 (2)	134 (11)
Haloperidol	100	79 (21)	100 (12)	95 (9)

Analysis of patients' dried blood spots after 6 months of storage in the refrigerator (4±1°C) revealed similar stability results to those of ambient temperature storage. Analysis of days 45, 90 and 180 showed stability ranging from 90-96%, 77-104% and 82-142%, respectively, as shown in Table 61.

Table 61: Stability of analytes in DBS stored at 4ºC

Analyte	Percentage of stability for all patients (CV%)			
	Day 0	Day 45	Day 90	Day 180
	DBS	DBS	DBS	DBS
Procyclidine	100	90 (14)	100 (16)	88 (13)
Quetiapine	100	90 (5)	77 (19)	91 (16)
Risperidone	100	96 (11)	92 (10)	106 (17)
9-OH-risperidone	100	90 (14)	84 (21)	82 (25)
Chlorprothixene	100	91	104	142
Haloperidol	100	91 (16)	98 (20)	92 (16)

No change in results was observed when analysing patients DBS stored in -20°C freezer from the previous ones. The percent of stability for the analytes at day 45 was between 86 and 104% while for day 90 and day 180 it was 82-100% and 81-97%, respectively. Chlorprothixene gave an outlier result for day 180 which was 161% and other details are shown in Table 62.

Table 62: Stability of analytes in DBS stored at -20ºC

Analyte	Percentage of stability for all patients (CV%)			
	Day 0	Day 45	Day 90	Day 180
	DBS	DBS	DBS	DBS
Procyclidine	100	104 (11)	94 (17)	95 (21)
Quetiapine	100	86 (6)	82 (15)	84 (4)
Risperidone	100	101 (12)	92 (6)	97 (16)
9-OH-risperidone	100	91 (15)	83 (15)	81 (7)
Chlorprothixene	100	90 (1)	92 (8)	161 (13)
Haloperidol	100	97 (1)	100 (10)	97 (13)

Also similar stability results were seen for analytes from patients' DBS stored at -80°C for 180 days. Testing on day 45 indicated analyte stability to be between 87 and 108% compared to starting concentrations whereas testing on days 90 and 180 showed stabilities ranges of 76 to 104% and 82 to 144%, respectively. Details are shown in Table 63.

Table 63: Stability of analytes in DBS stored at -80ºC

Analyte	Percentage of stability for all patients (CV%)			
	Day 0	Day 45	Day 90	Day 180
	DBS	DBS	DBS	DBS
Procyclidine	100	100 (2)	76 (1)	87 (3)
Quetiapine	100	89 (7)	83 (7)	82 (9)
Risperidone	100	108 (17)	89 (0)	104 (6)
9-OH-risperidone	100	104 (4)	104 (31)	104 (22)
Chlorprothixene	100	87 (1)	81	144 (4)
Haloperidol	100	106 (8)	98 (2)	92 (1)

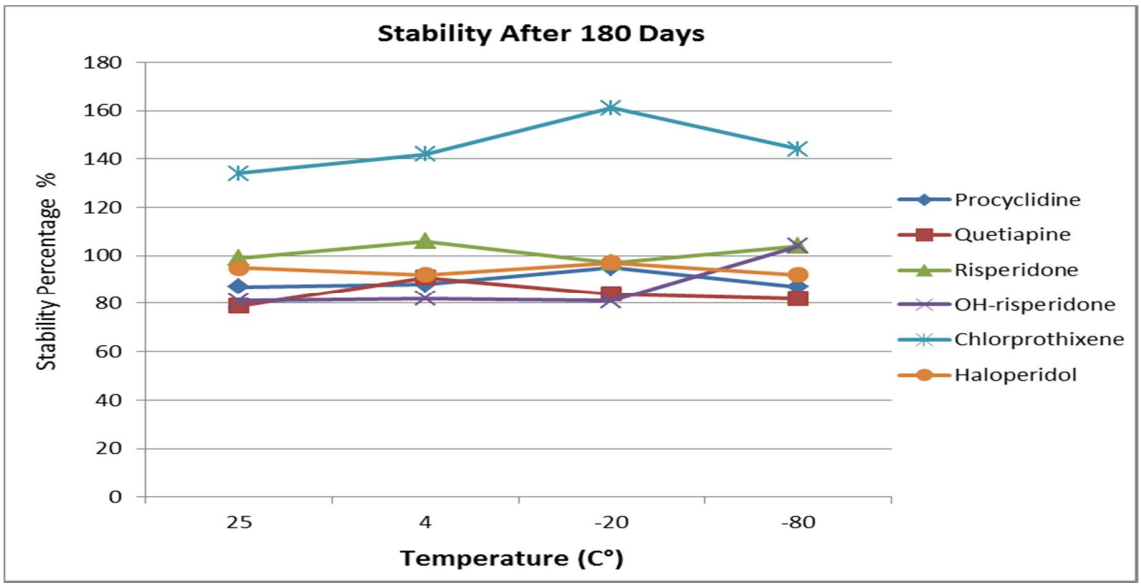


Figure 39: Analyte stability after 180 days over different storage temperatures

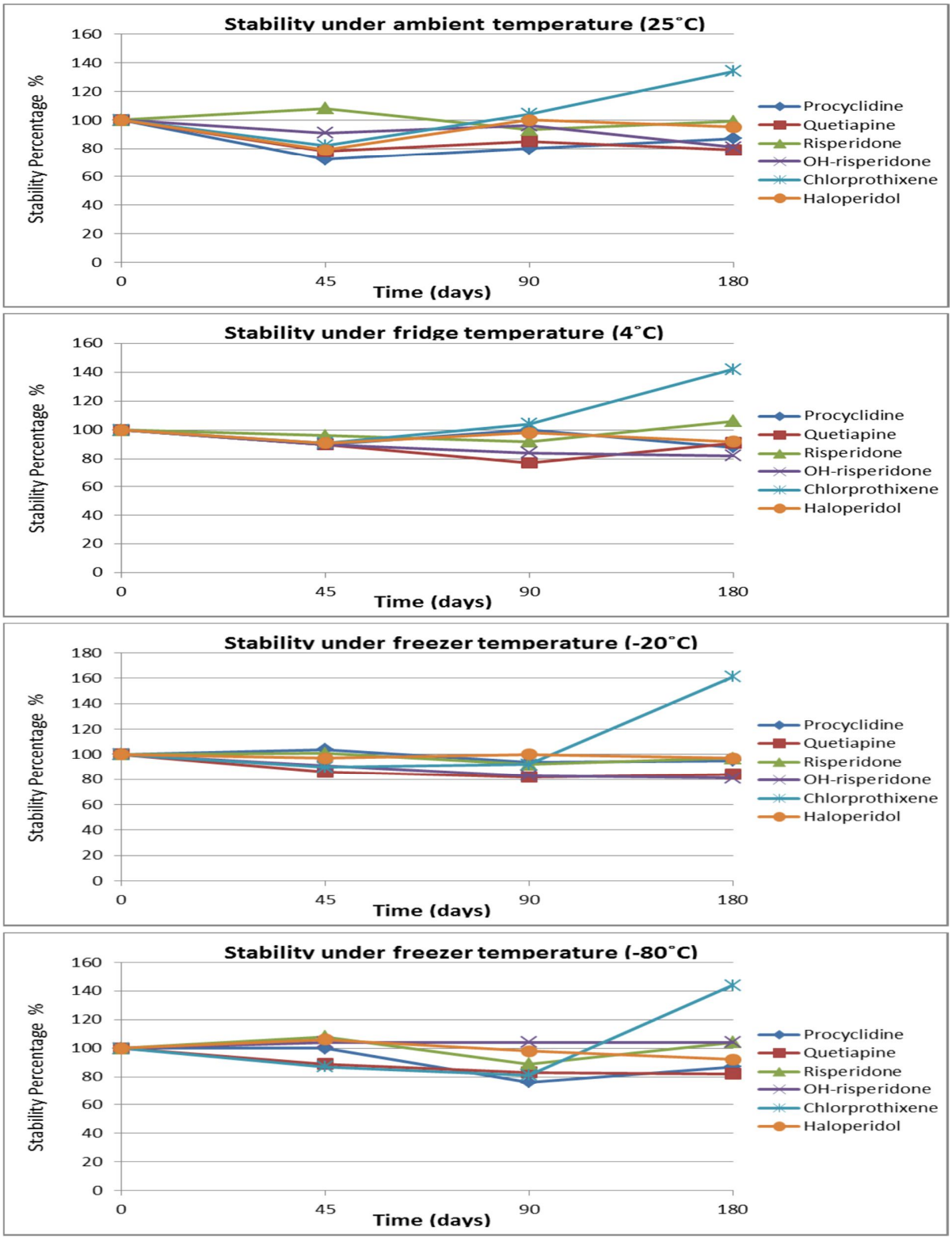


Figure 40: Stability of the analytes overtime under four different temperatures (25, 4, -20 and -80°C)

Table 64: Paired t-test analysis results for the difference between analyte stability in NaF treated and untreated cards

Analyte	NaF treated versus untreated			
	P-values from paired 2 tailed t-test (all patients)			
	25ºC	4ºC	-20ºC	-80ºC
Procyclidine	0.463	0.560	0.702	0.253
Quetiapine	0.473	0.970	0.750	0.557
Risperidone	0.398	0.843	0.656	0.633
9-OH-risperidone	0.093	0.785	0.492	0.158
Chlorprothixene	0.235	0.590	0.195	**0.035**
Haloperidol	0.927	0.304	0.410	0.981

4.5. Discussion

4.5.1. Introduction, aim and study design

As covered in the introduction, storing DBS on paper has been used since at least the 1960s for the screening of metabolic disorders (Guthrie, Susi 1963). However, the usage of this technique has been expanded to cover other purposes such as therapeutic monitoring and clinical drug development (Spooner, Lad et al. 2009). DBS have many advantages over traditional blood samples as described in the introduction. Drug stability is one of the most important ones. Drugs can degrade easily in liquid samples especially if not kept frozen. One of the most common degradation processes is hydrolysis which uses water to split an amide or ester bond within drugs structures (Waterman, Adami 2005).

In recent years, the prescribing of anti-psychotics has increased and their link to sudden cardiac death could explain their high prevalence in forensic cases (Ray et al. 2009). Stability of anti-psychotics in blood samples stored in different conditions have been studied and published reports disclosed that quetiapine, risperidone, OH-risperidone, chlorprothixene and haloperidol showed significant losses after 140

days under at least one of the storage conditions (Saar et al. 2012). No DBS study could be found in the literature investigating these drugs. In the present study the stability of procyclidine, quetiapine, risperidone, OH-risperidone, chlorprothixene and haloperidol in DBS stored at different temperatures at a number of time intervals over a 6 months period was tested. The present study is distinct from other studies as well in the use of real patients' blood samples. It is worth mentioning that stability studies in the literature are generally based only on spiked plasma (Saar et al. 2012).

4.5.2. Extraction optimization

The development of a suitable method for the extraction of the investigated analytes from DBS was started with the use 50µL spiked blood. This amount of blood was used as it filled the marked circles in the cards and was very similar to the actual volume of a blood drop (Morita, Folkers 1993). Four liquid-liquid extraction solutions were tested and they were mainly based on initial incubation with four different solutions (MAF, MTBE, 0.1M or 0.05M phosphate buffer). MAF, which was successful in the previous chapter, and MTBE have shown superiority over the other two solutions for the extraction of the targeted analytes from DBS cards. However, MTBE showed lower recoveries with quetiapine, risperidone and its metabolite. Phosphate buffer 0.1M and 0.05M results were very similar and showed generally low recoveries between 8 and 63% for all analytes. Therefore the MAF solution was chosen for carrying out the rest of the study. It worth mentioning that all the solutions provided a clean extract as the DBS technique has the rarely mentioned advantage of removing many of the unwanted macromolecules which stick to the cards, so they are not extracted alongside the analytes.

4.5.3. LC-MS

Table 43 shows the accurate masses of the targeted analytes and internal standards extracted from the full scan (75-1200amu) run on the LC-MS system. The mass accuracy for all the analytes was less than 1ppm which due to the high

resolution (+50,000) of the Exactive (Orbitrap) system provided great sensitivity and confidence in the results. Detection and quantitation with 1ppm mass window using high resolution mass spectrometry are adequate to generate a high selectivity equal or sometimes superior to that produced by MRM or SRM acquisition at unit mass resolution (Kaufmann et al. 2011). On the other hand, the chromatography separation which was achieved by HILIC conditions is considered rare as no paper has been found in the literature reporting analysis of these drugs under these conditions. The HILIC method showed good separation of the peaks with short run time (7 minutes), in fact one of the advantages of HILIC is the low back pressure which allows for increasing the flow rate (0.5mL/min) making the run relatively fast without compromising the results as the resolution is very high. The isocratic mobile phase with high acetonitrile content has also contributed to the quick separation without the need of re-equilibrating the column as in a gradient run.

4.5.4. Validation

Generally method validation for this optimized method was very successful. Linearity for DBS calibrators was very good and showed r^2=>0.99 for all analytes over the range of 2.5 to 2000ng/mL. LOD values for all analytes were 0.1-1.5ng/mL and LLOQ values were 0.4-4.5ng/mL. Similar range was reported for risperidone and its metabolite (Jantos, Skopp 2011). Overall absolute recovery results for analytes in spiked DBS have been found to be >64% for all the analytes. Only at low calibrators (0.1ng/mL), quetiapine showed reduced recovery (47%). Intra- and inter-day precision from spiked DBS revealed acceptable CV percentages of <15% for all analytes. With regard to accuracy it can be seen clearly from Table 46 that QC concentrations are within 90-110%.

4.5.5. Stability results

4.5.5.1. Procyclidine

From two different patients who were on procyclidine, whole blood with concentrations of 265 and 110ng/mL were spotted on Whatman neonatal cards and stored in conditions described earlier and analysed over a specified time scale in order to assess their stability. Combined results from the two procyclidine patients' DBS showed that mean losses of drug after 180 days under all the storage conditions (25, 4, -20 and -80ºC) were ≤13%. This result also could be due to analytical errors rather than a real loss or degradation of the drug, as at day 45 in the room temperature group, procyclidine concentration had dropped to 72% compared to day 0, while at day 180 it was 87%. Similar patterns have been noticed in literature (Saar et al. 2012, Heller et al. 2004). Other explanation would be the low concentrations of the patients' samples, as small changes in concentration can lead to large differences in the calculated stability results. Nevertheless, these results indicate that there is no need for refrigerating or freezing DBS samples for the analysis of procyclidine for at least 6 months. Unfortunately, no procyclidine stability studies in biological samples were found in literature to be compared with the present findings. However, dehydration of the samples on the cards should generally improve the stability by minimizing the hydrolysis of the compounds.

4.5.5.2. Quetiapine

As for quetiapine, two patients were involved with initial blood concentrations of 328 and 165ng/mL. After the spotting of 50µL patients' blood aliquots onto DBS cards and storing them under the assigned conditions, combined results of both patients showed a mean quetiapine concentration loss of 21% for those stored at room temperature after 180 days. However, the 4ºC batch showed only 9% loss, while -20 and -80ºC had a 16 and 18% reduction, respectively. Analytical and data processing variations could possibly have caused slight deviation in these findings, as normally the lowest storage temperature should have the lowest loss. According to these

findings refrigeration only is enough to retain 90% quetiapine in DBS for at least 6 months if not more. It has been reported that after only 14 days 50% of quetiapine was lost in plasma samples kept at room temperature (Peters 2007). No DBS published study could be found for quetiapine.

4.5.5.3. Risperidone

The initial blood levels for the two risperidone patients' samples were 4 and 47ng/mL. Following 180 days of storage in different temperatures DBS were analysed and risperidone maintained a stability of ≥89% throughout all the tests days and all the storage conditions. At day 180 test stability was 99% for the room temperature batch and 106% for the refrigerated batch. Likewise the freezing at -20 and -80ºC batches showed 97 and 104% stabilities, respectively. Accordingly, these results indicate that there is no need for refrigerating or freezing DBS samples for the analysis of risperidone within at least 6 months. One study has reported risperidone loss of 15-35% in whole blood spiked samples from the starting concentration after 20 weeks of storage under all the range of conditions, 20 to -60ºC (Saar et al. 2012). The only DBS study found in literature for risperidone was focused on the reliability of DBS results and they found the ratio between blood/DBS results to be very close to 1 which means almost equal (Jantos, Skopp 2011).

4.5.5.4. OH-risperidone

Results for OH-risperidone, also known as paliperidone, were from three patients and the starting concentrations were 17, 41 and 21ng/mL. Analysis of DBS after the 6 months study period revealed that the mean losses of OH-risperidone were ≤19% for those stored at ambient (25±2ºC) temperature, refrigerator (4±1ºC) and freezer (-20±1ºC), while the -80ºC freezer batch maintained a constant level of 104% stability throughout the 6 months evaluation. However, day 90 analysis for the ambient temperature batch showed a mean stability of 96%. Such discrepancy has been reported in the literature and one of the suggested explanations would be the method not maintaining a high enough level of accuracy. By and large -80ºC

freezers provide the highest stability but are costly and thus uncommon. A published study has reported that between 15-30% losses have been seen with OH-risperidone stored at 20ºC for 10 weeks whereas at ≤4ºC the loss was ≤15% (Saar et al. 2012). One DBS study for OH-risperidone investigating the reliability of DBS results found the concentration ratio between blood/DBS to be 1.04 which means almost equal (Jantos, Skopp 2011).

4.5.5.5. Chlorprothixene

Samples included in the chlorprothixene DBS stability study were from one patient with a starting concentration of 9ng/mL. Subsequent to 180 days of storage in different temperatures DBS were analysed. On all the tested dates (day 45, 90 and 180) and under all the storage conditions chlorprothixene showed a stability of not less than 81%. However, due to the low concentration of the chlorprothixene originally in the patient sample small variations in the quantitation can lead to outliers in the stability results. This was seen clearly with the day 180 test which showed stabilities between 134 and 161% for all the storage temperatures. This phenomena was seen in some publications (Saar et al. 2012, Heller et al. 2004). In view of this, these results can indicate that there is no need for refrigerating or freezing DBS samples for the analysis of chlorprothixene within at least 6 months. A published study pointed out that chlorprothixene in whole blood lost 55% after 140 weeks of storage at 20ºC and 50 and 40% after storage in 4ºC and -20ºC, respectively (Saar et al. 2012). No DBS published study could be found for chlorprothixene.

4.5.5.6. Haloperidol

With regard to haloperidol, two patients were involved with initial concentrations of 31 and 8ng/mL. After spotting the patients' blood samples onto the DBS cards and storing them at the different temperatures, the combined results of both patients showed that mean haloperidol stabilities were more than 91% for all the storage conditions and all the tests days except day 45 ambient temperature results (79%),

which could be considered as an outlier. According to these results haloperidol is stable within DBS for as long as 6 months without refrigerating or freezing. In literature haloperidol concentrations in blood samples stored at room temperature (20±2ºC) for 140 days were found to drop by 25% of their original level, while at the lower temperatures (4, -20 and -60ºC) the loss was generally below 16% (Saar et al. 2012). No haloperidol DBS study was found in the literature.

4.5.6. Sodium fluoride impregnation

Sodium fluoride is widely used for preserving drugs in samples in both clinical and forensic toxicology. It helps in the inhibition of bacterial growth and subsequently reduction in the rate of sample decomposition (Rees et al. 2011). Half of the stability study DBS cards were pre-treated with 5mg/mL NaF solution to see whether it has any stability enhancement effect on the drugs' concentrations. A paired t-test was used to determine whether a difference between mean values determined from DBS on treated and untreated cards exists or not. Results showed that no significant difference was found between the two groups at all the storage conditions and with all the investigated drugs as seen on Table 64. Therefore, addition of a preservative showed no effect in terms of stability as the drugs were already stable even at room temperature. This could also mean that a much longer storage period might be needed in order to see the long term effect. The limited amount of samples, time and access to the mass spectrometry hindered the long term investigation.

4.6. Conclusion

A validated method has been developed which can be used for the extraction and quantitative analysis of drugs in small volumes of blood spotted and dried on filter papers or Guthrie cards. The DBS technique provides a suitable procedure for the storage and analysis of samples in therapeutic drug monitoring and forensic toxicology because they are easy and safer to handle, transport and store in the laboratory, even in the absence of refrigeration. The stability of antipyschotics in

DBS from real patients' samples was assessed by storing them at four different temperatures (25, 4, -20 and -80°C) with and without prior impregnation of the DBS cards with sodium fluoride. After the development and validation of a LLE-LC-MS method, samples were analysed at days 0, 45, 90 and 180. Results showed a significant stability of all the compounds (procyclidine, quetiapine, risperidone, OH-risperidone, chlorprothixene and haloperidol) in all the different storage conditions and no significant increase or decrease in drug concentrations with sodium fluoride impregnation. Possibly a much longer period than 6 months is needed to see if NaF has an effect on drug stability.

5. Chapter 5 - Prevalence of alcohol use among Kuwaiti patients undergoing drug rehab revealed by hair analysis

5.1. Introduction

Alcohol misuse is a worldwide issue affecting most countries including those where alcohol is considered as a controlled and illegal substance such as Kuwait and Saudi Arabia for instance. Alcohol can be smuggled or produced at home by fermenting sugars in the fruits in these countries. Therefore, they are not in a complete safe haven from alcohol problems. Globally, alcohol related mortality is about 2.5 million each year (WHO 2011). With regards to the Western world, it is estimated that in Western Europe, where extensive alcohol studies have been carried out, 20% of the population are considered alcohol abusers (Rehm et al. 2003). Heavy drinking can lead to several physical and mental health problems in addition to the social problems it can create (Derauf, Katz & Easa 2003), namely, road accidents, violence, abuse of family members, child neglect, absenteeism in workplaces, etc. In fact, the second largest risk factor for disease burden in Europe is alcohol (WHO 2011). In a Scandinavian study they found that the majority of sexually assaulted females had been drinking alcohol (Jones et al. 2008). Therefore, it is not surprising that alcohol has strongly drawn the attention of the scientific community to tackle or minimize its burden.

Table 65: Prevalence of common drugs of abuse in the United States (Abudu 2008)

Drug of misuse	Prevalence (%)
Alcohol	75 - 80
Cannabis	20 – 26
Cocaine	5 – 13
Benzodiazepines	1 - 5
Barbiturates	0.5 - 5
Opiates	0.1 - 2
Phencyclidine	0.1 - 2
Amphetamines	0.1 - 1
Other stimulants	0.8 - 2
Other sedatives	0.6 - 2

In the last two decades alcohol biomarker research was and still is very active. The research is aiming to find reliable markers in human biological matrices. The first generation or traditional markers are mainly detected in urine and blood, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl-transferase (γGT), mean corpuscular volume (MCV) and carbohydrate-deficient transferrin (CDT) (Bearer, Bailey & Hoek 2010). These generally serve as qualitative indicators and require several weeks or more to be elevated. Moreover, their values can be raised by several other non-alcoholic disorders which render their sensitivity and specificity to be quite low or limited which sometimes leads to false positive results. On the other hand, the new biomarkers such as the 5-hydroxytryptophol (5-HTOL) and the metabolites of ethanol; ethyl glucuronide (EtG), ethyl sulfate (EtS) and fatty acid ethyl esters (FAEEs) are generally more specific and more sensitive (Litten, Bradley & Moss 2010). The main focus in the latest publications is on EtG specifically as it seems more robust, sensitive and reflective of actual alcohol consumption. It allows a flexible window of detection from hours after consumption (14 to 24 hours in blood) to several days (up to 102 hours in urine) (Kissack, Bishop & Roper 2008). EtS has similar advantages and window of detection as EtG, however the latter was found recently to have roughly twice the level of EtS in urine and serum (Lostia, Vicente & Cowan 2013). Also EtS detection in hair could not be retrieved from literature.

The detection of ethanol itself in urine has only a short window of 12 to 14 hours. Also ethanol can be formed in urine through fermentation especially in diabetic patients, whereas EtG is only produced after alcohol passage through liver where it is exposed to hepatic enzymes.

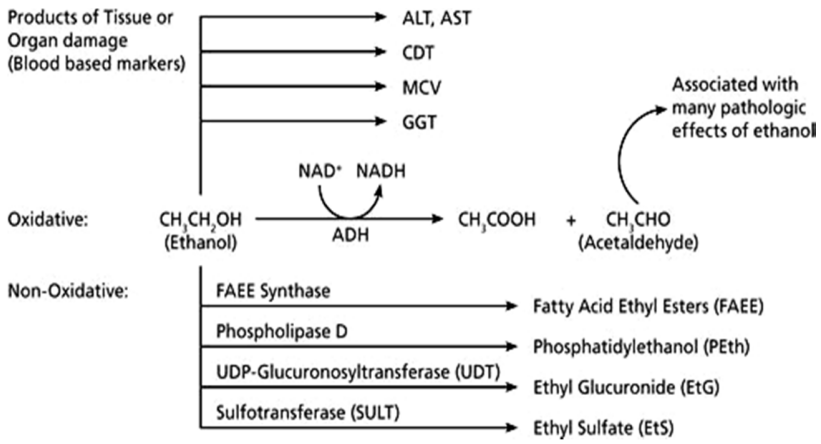


Figure 41: Alcohol metabolic pathways (oxidative, non-oxidative) and blood markers (Abudu 2008)

In fact, the EtG window of detection has been expanded into several months after the introduction of its detection in hair (Skopp et al. 2000, Alt et al. 2000). To be more impartial, hair alcohol analysis studies were not only focused on EtG but also on FAEEs. However, FAEEs are lipid soluble and incorporate into hair from the sebum, so segmentation, for instance, will be of no use because their incorporation is not associated with the growth rate of the hair and consequently may not help in estimating the time or amount of consumption and they also can be found in the hair of strict teetotallers (Auwarter et al. 2001). Thus, generally they can be classified as co-indicators of general drinking behaviour if detected in abnormal levels. In view of that, EtG attracted attention because it is water soluble and incorporated from blood, but although less than 0.1% of ingested alcohol is eliminated as EtG, it can indicate alcohol abuse (Pragst, Yegles 2006). According to a recent paper EtG in hair is not significantly influenced by gender, body mass index, age, or hair colour (Kharbouche et al. 2011, Kintz 2010). One of the other reasons is that EtG has been found to reflect to some extent the actual daily consumption of alcohol (Kharbouche et al. 2010, Appenzeller et al. 2007). As a matter of fact, the society of hair testing

(SoHT) has set a cut-off value for the detection of EtG in hair (Kintz 2010). The proposed cut-off is that detection of >30pg/mg is reflective of chronic heavy/excessive drinking (>60g ethanol/day) and <30pg/mg reflective of social drinking (<60g ethanol/day). Another group has added another threshold of 7pg/mg to differentiate between teetotalers and social drinkers (Liniger et al. 2010). More recently, Kharbouche et al conducted a prospective self-monitoring study on 125 subjects in order to investigate more accurately the thresholds limits and the reliability of EtG as a diagnostic test (Kharbouche et al. 2011). They found that EtG diagnostic performance was significantly better ($P<0.05$) than any of the traditional biomarkers alone and had a stronger or similar diagnostic performance in detecting at-risk (>20/30g/day) or heavy drinkers (>60g/day), respectively, than the best combination of traditional biomarkers (CDT and γGT). They also suggested a 25pg/mg detection threshold for heavy drinkers and 9pg/mg for at-risk drinkers. On the other hand, a Swedish group concluded their controlled alcohol administration study with the fact that EtG detection in hair cannot completely exclude minor daily alcohol ingestion and lacks sensitivity for the purpose of monitoring complete abstinence (Kronstrand, Brinkhagen & Nyström 2011a). In this context of mentioning weak points of EtG power or reliability, it is worth noting that EtG is formed by a conjugation reaction of alcohol with glucuronic acid in the presence of the enzyme uridine diphosphate glucuronyl transferase (UGT) (Wurst, Skipper & Weinmann 2003). However, there are 16 functional human genes of UGT, raising the possibility that the formation rate of EtG may vary by genotype (Litten, Bradley & Moss 2010). The last, but maybe not least, weak point is cosmetic hair treatment which may cause some reduction of the EtG level in hair (Pascal 2011). Nonetheless, an interesting study showed that EtG is not significantly washed out in the distal segments longer than 3cm (Agius, Ferreira & Yegles 2011).

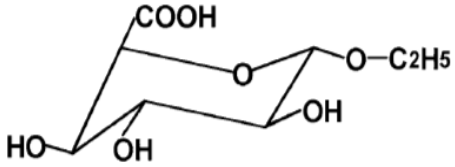


Figure 42: Structure of ethyl glucuronide (Janda, Alt 2001)

Solid phase extraction (SPE)

As EtG is a minor metabolite of ethanol (<0.1%), its concentration in body fluids is mostly measured in ng/mL or ppb and in hair as pg/mg which is a very minute amount. Therefore, EtG extraction maybe better achieved with SPE as it provides a cleaner sample upon concentration than liquid-liquid extraction (LLE). Hence, it should apparently increase the sensitivity.

The SPE technique first appeared in the seventies of last century as an alternative to the traditional liquid-liquid extraction, which is labour and solvent intensive plus not very environmentally friendly (Thurman, Snavely 2000). Thurman et al define SPE as “a method of sample preparation that concentrates and purifies analytes from solution by sorption onto a disposable solid-phase cartridge, followed by elution of the analyte with a solvent appropriate for instrumental analysis” (Thurman, Mills 1998). Therefore, SPE essentially is based on binary on and off separation strategy (Svec 2006).

In the present study an EtG specific SPE column (Clean-Screen® EtG) has been tested. This column is made of proprietary carbon blend sorbent of a porous immobilised graphitic carbon in which the graphite is immobilised on a silica structure with a high attraction for organic polar compounds from both polar and non-polar matrices (Lamoureux et al. 2009). It has microporous surface areas holding polar groups primarily appropriate for the extraction of phenolic-polar compounds such as EtG from the biological matrices such as hair and urine. Precisely, the retention of the EtG on the sorbent is achieved by the attraction that takes place between the functional groups and the EtG through the carbon–hydrogen bonds when the EtG is carried by a polar solution (Lamoureux et al. 2009). Also, Waters Oasis Max (mixed-mode anion exchange) SPE cartridges have been used and compared to the Clean-Screen® EtG cartridges. Oasis Max is a mixed mode polymeric sorbent that combines reversed phase and ion exchange interaction (Fontanals, Marcé & Borrull 2007).

5.2. Ethyl glucuronide (EtG) analysis

5.2.1. Aim of the study

There are several objectives behind this study. Firstly, to determine the alcohol prevalence or consumption behaviour among Kuwaiti addicts and to draw a picture of addiction trends starting with this alcohol focused study. Also to possibly analyse the data statistically to look for any patterns or correlations between alcohol consumption, age and other drugs of abuse. Availability of these kinds of data or studies will facilitate the work of other departments in the country dealing with drugs and addiction. However, as yet no similar study has been conducted covering these issues in Kuwait.

Secondly, to develop and validate a sensitive and specific method for EtG detection and quantitation in hair from samples based on comparing different extraction methods (LLE and SPE) and also different separation columns (hydrophilic interaction liquid chromatography HILIC and reversed phase column C18).

Thirdly, to compare the results with self-reports received from addicts and see if they are in agreement, in other words, to investigate if addicts tend to reveal their alcohol consumption or they try not to.

5.2.2. Sample and data collection

Hair samples were collected from middle-eastern male drug addicts aged between 24 and 54 years attending for treatment or rehabilitation in the addiction treatment centre. Collection permission was granted from the permanent committee of health research ethics at the ministry of health in Kuwait to which the centre belongs. Participants were given an explanation of the study and total freedom of choice to participate or not. Out of almost 120 attending the centre who were interviewed, 59 agreed to participate and signed the consent forms. Information on present and past drug history, age, sex, hair colour and sample collection date were recorded during interviews and can be found in the appendix. Data treatment was carried out

anonymously. All samples were tied with string and kept inside folded tin foil and placed in paper envelope. Blank samples were collected from alcohol abstinent volunteers.

Subjects' hair samples were taken from the occipital area of the scalp where hair is strongly and constantly growing even in bald subjects. Colours of the hair were black, dark brown or grey and none of the subjects had any kind of hair treatment, except one who had a henna (herbal) dye. The minimum amount of collection was about 50mg hair and at least ~1cm in length. However, in 3 cases lesser amounts have been considered.

5.2.3. Materials and reagents

All chemicals were of HPLC or analytical grade. UK. Ethyl β-D-glucuronide and deuterated internal standard ethyl β-D-glucuronide-D5 were purchased from Kinesis UK. Clean Screen EtG extraction columns were purchased from United Chemical Technologies through Presearch, UK. Oasis MAX cartridges were purchased from Waters Limited (Hertfordshire, UK). The rest of chemicals and consumables are mentioned in sections 2.2.2 and 3.4.

Other solutions used in this chapter were mentioned in sections 2.2.2.2 and 3.4.

Standard solutions

100µg/mL stock standards were made with MS grade methanol for ethyl β-D-glucuronide and ethyl β-D-glucuronide-D5 and stored at -20^{0}C.

Mobile phases

0.1% formic acid in water

1mL of concentrated formic acid (98/100) was mixed with 999mL of deionized water, mixed well and sonicated.

0.1% formic acid in acetonitrile

1mL of concentrated formic acid (98/100) was mixed with 999mL of acetonitrile, mixed well and sonicated.

20mM ammonium acetate

1mL of concentrated 2M ammonium acetate stock solution was mixed with 999mL of deionized water, mixed well and sonicated.

20mM Ammonium carbonate

This solution was provided by a collaborative laboratory.

5.2.4. LC-MS(MS) method development

5.2.4.1. Exactive Orbitrap LC-MS method

The extracts were analysed initially by using Exactive Mass Spectrometer (Thermo Electron UK) operated in negative ion ESI mode interface with a needle voltage of 4.5kV, a heated capillary temperature of 320°C, sheath gas flow of 50 arbitrary units and auxiliary gas flow of 17 arbitrary units. Scanning was from 75-1200 amu. Chromatography was carried out using a HPLC system consisting of a Thermo Scientific Accela 600 pump, autosampler and column compartment fitted with either ZIC-pHILIC column (150 x 4.6mm 5µm particle size, Hichrom UK) plus guard ZIC-pHILIC column (20 x 2.1mm 5µm) or ZIC-HILIC column (150 x 4.6mm 5µm particle size, Hichrom UK) plus guard ZIC-HILIC column (20 x 4.6mm 5µm). These two were tested in two different isocratic mobile phases with a flow rate of 0.5 mL/min. The mobile phase for pHILIC was of (A) 20mM ammonium carbonate in water and (B) acetonitrile, while HILIC was (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. A summary of the LC-MS(MS) methods is shown in Table 68. Data analysis was carried out using Thermo Xcalibur 2.2 SP1 software (Thermo Electron, UK).

Table 66: Extracted ions of EtG and internal standard and their mass spectrometry data

Analyte	Formula (-H)	Accurate Mass (M-H)	Retention Time	Mass Accuracy
Ethyl glucuronide	$C_8H_{13}O_7$	221.0668	2.8	5.749 ppm
Ethyl glucuronide-D5	$C_8H_8D_5O_7$	226.0983	2.8	5.825 ppm

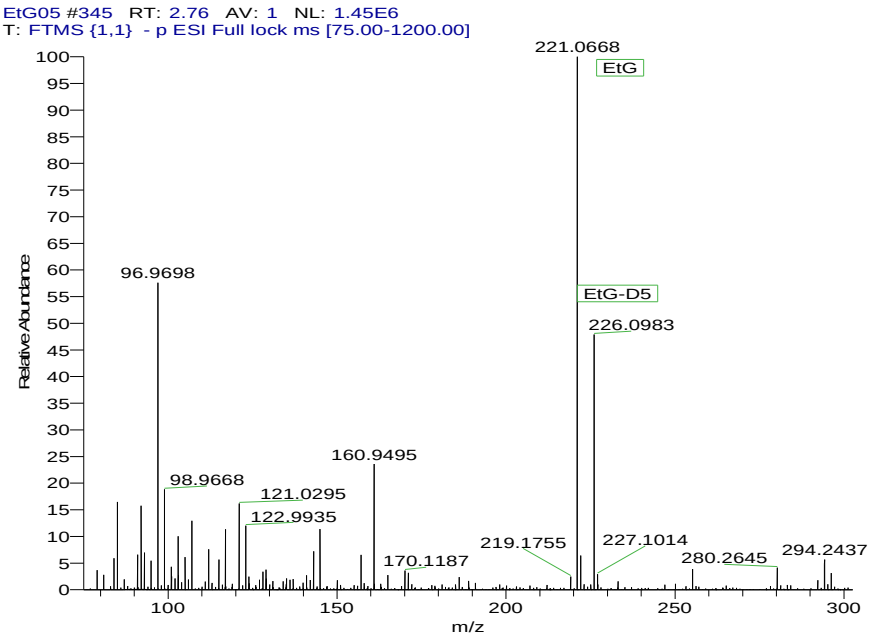


Figure 43: Mass spectrum for EtG and EtG-D5 on Exactive Orbitrap

5.2.4.2. TSQ Vantage triple quad LC-MS/MS method

The other instrument which was tried for this study was of triple quadrupole mass spectrometer TSQ Vantage (Thermo Fisher Scientific, USA) equipped with an electrospray interface (Turbo Ion Spray) set on the negative mode. The ion spray voltage was set to 3500 V. The sheath/auxiliary gas was nitrogen. The sheath gas pressure was 35 arbitrary units and auxiliary gas pressure was 10 arbitrary units. The ion transfer capillary temperature was 270°C and the vaporizer temperature 150°C. The collision gas was argon with a pressure of 1.5 mTorr.

Table 67: Ethyl glucuronide and ethyl glucuronide-D5 SRM scans conditions

Analyte	Parent	Product	Width	Time	CE	Q1 PW	Q3 PW	S-Lens
EtG	221.114	75.042	1.200	0.100	16	0.70	0.70	87
		85.060	1.200	0.100	19	0.70	0.70	87
EtG-D5	226.133	75.052	1.200	0.100	18	0.70	0.70	84
		85.101	1.200	0.100	20	0.70	0.70	84

* PW (peak width), CE (collision energy), Q (quadrupole), S-Lens (stacked-ring ion guide lens)

The TSQ Vantage analyser was coupled with an Accela 1250 U-HPLC system. Extract separation was compared between three different LC columns. The first tested column was Ascentis Express (10cm x 4.6mm x 2.7µm, from Sigma-Aldrich Co. UK) with a mobile phase started with 95% (A) 0.1% formic acid in water (B, 0.1% formic acid in acetonitrile) to 5% (A) in 3 min linear gradient, held for 2 min before returning to original conditions of 95% in 0.1 min followed by 2.9 min equilibration time to make a total run time of 8 min pumped at 0.5mL/min. The second column was a Kinetex HILIC (50mm x 2.1mm x 1.7µm, from Phenomenex Inc. CA, USA) tested with mobile phase (A) 20mM ammonium acetate and (B) acetonitrile. The column was tried on several isocratic runs ranging from 1% to 60% (A) pumped at 0.5mL/min. The last column was an Acquity BEH C18 (50mm x 2.1mm x 1.7µm, Waters Ltd, Hertfordshire UK) with gradient run mobile phase (A) 0.1% formic acid in water and (B) 0.1% formic acid in acetonitrile. The gradient run was started with 99% of (A) held for 1 min, followed by linear gradient to 20% (A) in 2 min and held again for 1 min before returning back to the initial conditions at 99% (A) and kept for 2.5 min for equilibration. The total run time was 6.5 min with a flow rate of 0.25mL/min. The injection was set on no waste mode with an injection volume of 20µL. A list of all mobile phases and LC columns are summarized in Table 68.

The five methods were compared through the injection of 10, 100 and 1000ng/mL EtG standard dissolved in acetonitrile in the case of HILIC/pHILIC columns and in water in the case of C18 columns. The system producing the highest peak area response for EtG was recorded as being 100 % and the peak area responses for the other systems were calculated as a percentage of the optimum system response.

5.2.4.3. Results

As shown in Table 68 the combination of an Acquity BEH C18 column with TSQ Vantage LC-MS/MS provided the highest peak area responses for EtG detection compared to the other combinations. This column and instrument combination was therefore selected for the further EtG method development.

Table 68: Instrument methods evaluated by relative % peak area response for optimal determination of EtG from neat standards

Instrument	Exactive Orbitrap		Triple Quad TSQ Vantage		
Column	ZIC-pHILIC	ZIC-HILIC	Acquity BEH C18	Kinetex HILIC	Ascentis Express C18
Mobile phase	(60%) 20mM ACB (40%) ACN	(60%) 0.1%FA (40%) 0.1%FA in ACN	(99-20%) 0.1%FA (1-80%) 0.1%FA in ACN (gradient)	(40%) 20mM ACT (60%) ACN	(95-5%) 0.1%FA (5-95%) 0.1%FA in ACN (gradient)
Injection	25µL Full loop	25µL Full loop	20µL No waste	20µL No waste	10µL No waste
Relative % Peak Area Response					
Retention	2.8	2.8	1	0.3*	3
10ng/mL	64.6	33.3	100.0	20.4	4.9
100ng/mL	37.7	-	100.0	77.0	-
1000ng/mL	45.8	25.3	100.0	94.0	-

ACB: ammonium carbonate, ACT: ammonium acetate, ACN: acetonitrile, FA: formic acid
* No retention was achieved between 1-95% ACN

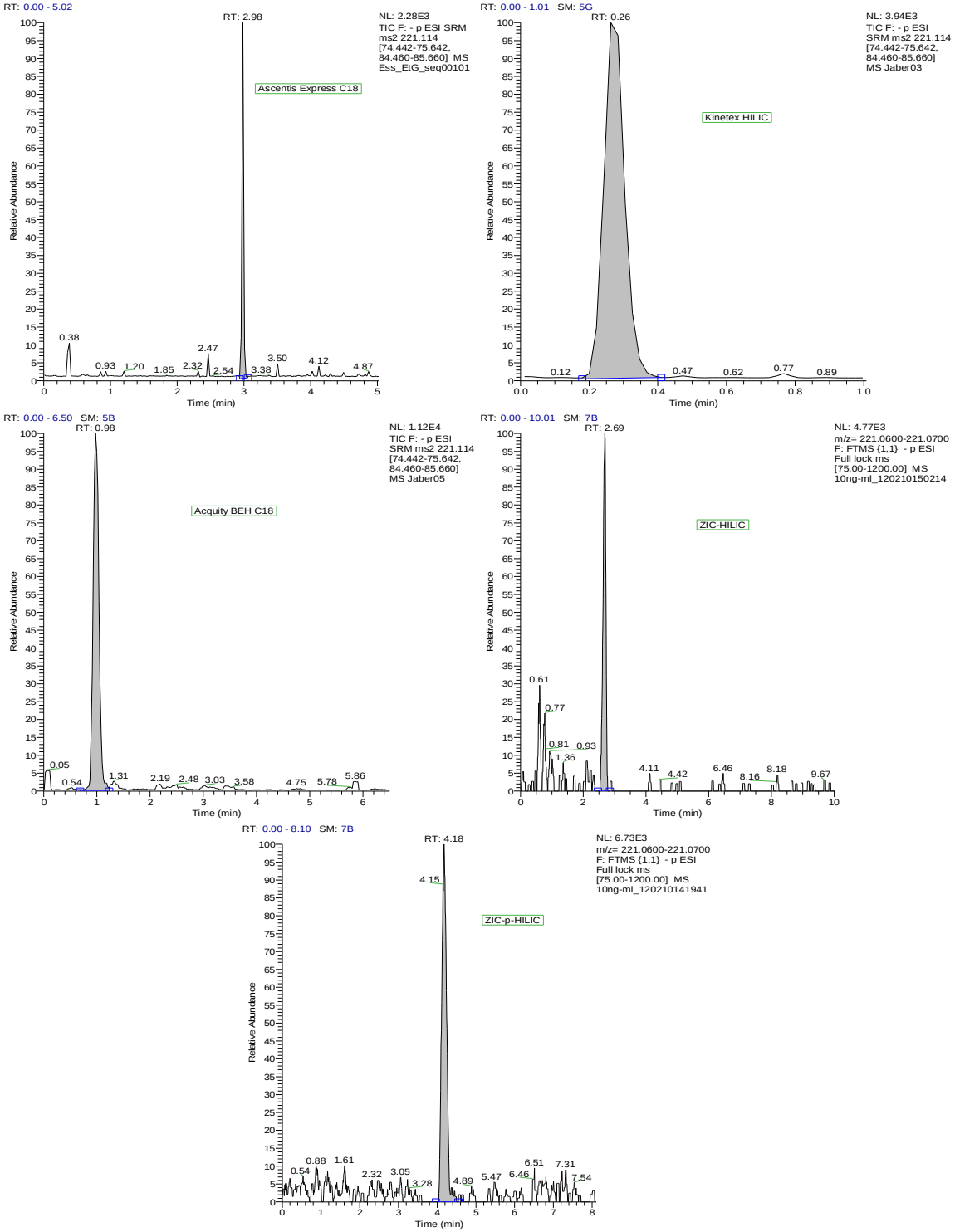


Figure 44: Chromatograms of 10ng/mL EtG standards analysed by the 5 different systems

5.2.5. Sample preparation

5.2.5.1. Decontamination

Morini et al found that washing uncut EtG hair samples with dichloromethane followed by methanol (10 minutes sonication and 30 seconds vortex mixing, respectively) resulted in negligible amount of EtG loss (<2.5%) and significantly reduced matrix interference by removing external contaminants (Morini et al. 2006). However, the 2011 SoHT proficiency testing scheme for EtG did not recommend sonication during washing steps. Besides that, there is no agreement or consensus on a specific decontamination method among hair analysts. Accordingly, the decontamination process was achieved by using three washing steps with three solvents and one aqueous solution: dichloromethane, 0.01M phosphate buffer and propanol/acetone (1:1). The uncut hair was put in a 6ml glass tube and then 2mL of the first solvent was added, followed by 30 seconds vortexing. After that the solvent was decanted. The decanting process can be done with the use of plastic pipette or in the case of short hair where the hair are scattered in the tube a piece of disposable fabric or textile has been used to hold the hair inside the tube and filter the washing solution out. After the last washing step (2mL propanol/acetone) the hair was left to dry at room temperature in a fume hood or speeded up with the speed-vac in low temperature mode. This decontamination method was found to be the quickest and most efficient by the researcher.

5.2.5.2. Segmentation

Segmentation was performed only if hair was longer than 3 centimetres, as the recommendation by the SoHT consensus was that the cut-off concentration should be used when analysing a 3 cm segment (Pascal 2011). After the hair became dry, segments were cut in small pieces (1–3mm) with scissors and weighed directly. To ease the calculation later, 60mg hair was usually taken, if not, any available weight was considered, and then each segment snippets were placed into a 2ml chromacol glass vial with a screw cap.

5.2.6. Optimization of extraction

The main extraction method was based on incubation in water followed by SPE suggested by United Chemical Technology, Inc. (UCT) and recommended by SoHT EtG proficiency scheme and number of papers (Kronstrand, Brinkhagen & Nyström 2011a, Kharbouche et al. 2009, Yegles et al. 2004). The other extraction methods were Oasis Max SPE, LLE and a filtration method. Extraction methods have been tested by applying them on authentic samples from known heavy drinkers. These samples were reduced into 1-3mm snippets and mixed thoroughly after washing. The recoveries of the following three extraction methods were calculated by the use of five 60µL unextracted calibrators 0, 1, 10, 100, 1000ng/mL (~pg/mg) with 50µL of 0.2µg/mL IS (ethyl β-D-glucuronide-D5) prepared at the same time as the samples but kept in the fridge during sample incubation and then evaporated with the samples and reconstituted in 60µL of mobile phase and run simultaneously with the samples.

SPE method1 (CleanScreenEtG)

As mentioned before, after reducing hair into 1-3mm lengths, 60mg of blank/authentic hair was incubated in triplicate with 1.2 mL of deionised water for 18 hours in a water bath (35°C + darkness) including 1 hour of sonication and after the addition of the internal standard. Afterward, samples were centrifuged at 3000rpm for 5 min and the supernatant was loaded into SPE CleanScreenEtG cartridges after conditioning. Conditioning was achieved with 3mL of methanol containing 1% formic acid followed by 3mL of 1% formic acid in deionised water (aspiration < 3 inches Hg) according to protocol provided by the manufacturer. Samples were loaded at 1-2mL/minute and washed with 3mL of deionized water and columns were left to dry for 10 minutes with ≥10 inches Hg aspiration. Finally, analytes were eluted with two 2mL loads of 1% formic acid in methanol, evaporated to dryness under nitrogen (40ºC) and residues were reconstituted in 60µL of the mobile phase.

SPE method 2 (Oasis Max)

Similar to the previous method, 60mg of blank or patient hair (1-3mm) was incubated in triplicate with 1.2mL of deionized water for 18 hours in a water bath (35°C + darkness) including 1 hour of sonication and after the addition of the internal standard. Afterward, samples were centrifuged at 3000rpm for 5 minutes and supernatant was loaded into SPE Oasis Max cartridges after conditioning. Conditioning was achieved with 2mL of methanol followed by 2mL of deionized water. Samples were loaded at 1-2mL/min and washed with 1mL of deionized water and 2mL of cyclohexane. Then columns were left to dry for 10 min with ≥10 inches Hg aspiration. Finally, analytes were eluted with 2mL of 2% formic acid in methanol, evaporated to dryness and residues were reconstituted in 60µL of the mobile phase.

Method 3 (A:W:M incubation)

In this method 60mg of blank or patient hair (1-3mm) was incubated in 1mL of a mixture of acetonitrile: deionized water: methanol (A:W:M, 5:4:1). The hair was incubated overnight in a water bath (35°C + darkness) including 1 hour of sonication and after the addition of the internal standard. After centrifugation the supernatant was dried under nitrogen to ~200µL and then filtered with Mini-UniPrep Syringeless filters. The filtrate was evaporated and reconstituted in 60µL of the mobile phase.

Method 4 (A:M incubation)

60mg of blank or patient hair (1-3mm) was incubated in 1mL of a mixture of acetonitrile: methanol (A:M, 1:1). It was incubated overnight in a water bath (35°C + darkness) including 1 hour of sonication and after the addition of internal standard. After centrifugation the supernatant was dried under nitrogen to ~100µL and 100µL of deionized water was added (to ensure complete EtG transfer) and then filtered with Mini-UniPrep Syringeless filters. The filtrate was evaporated and reconstituted in 60µL of the mobile phase.

Optimization of extraction started with an efficiency comparison between the two SPE methods mentioned earlier. The two methods were applied to two sets of EtG standards, 100 and 1000ng/mL each in triplicate, and their responses were

compared against unextracted standards. Following this stage the highest efficiency SPE method was then compared to methods 3 and 4 by applying them to real samples as described earlier (section 5.2.6).

5.2.6.1. Results

Even though a number of improvements and changes were tried in order to get better results with the use of Clean-Screen®ETG cartridges only 5% recovery was achieved in both tested concentrations (100 and 1000ng/mL). This contrasted with the Oasis Max columns which maintained a good extraction efficiency at both low and high concentrations as shown in Table 69.

Table 69: Extraction efficiency results using two different SPE cartridges (% of recovery, n=3)

Recovery / Analyte	Oasis Max	% CV	EtG CleanScreen	% CV
Ethyl glucuronide 1000ng/mL	103%	7	5%	22
Ethyl glucuronide 100ng/mL	86%	5	5%	23

As method 1 was excluded, results between the other 3 methods when applied to real positive hair samples showed that method 2 (Oasis Max SPE) revealed the highest recovery (26pg/mg) while method 3 (A:W:M) showed 20% lower recovery (22pg/mg) and method 4 showed very low recovery, ~3pg/mg as shown in Table 70 and Figure 45.

Table 70: Extraction optimization results with authentic hair sample extracted in different methods (% of recovery, n=2)

	Method 2		Method 3		Method 4	
Recovery / Analyte	SPE Oaisis Max	% CV	A:W:M	% CV	A:M	% CV
Ethyl glucuronide (pg/mg)	26	31	22	9	3	42

A:W:M, acetonitrile:deionized water:methanol. A:M, acetonitrile:methanol

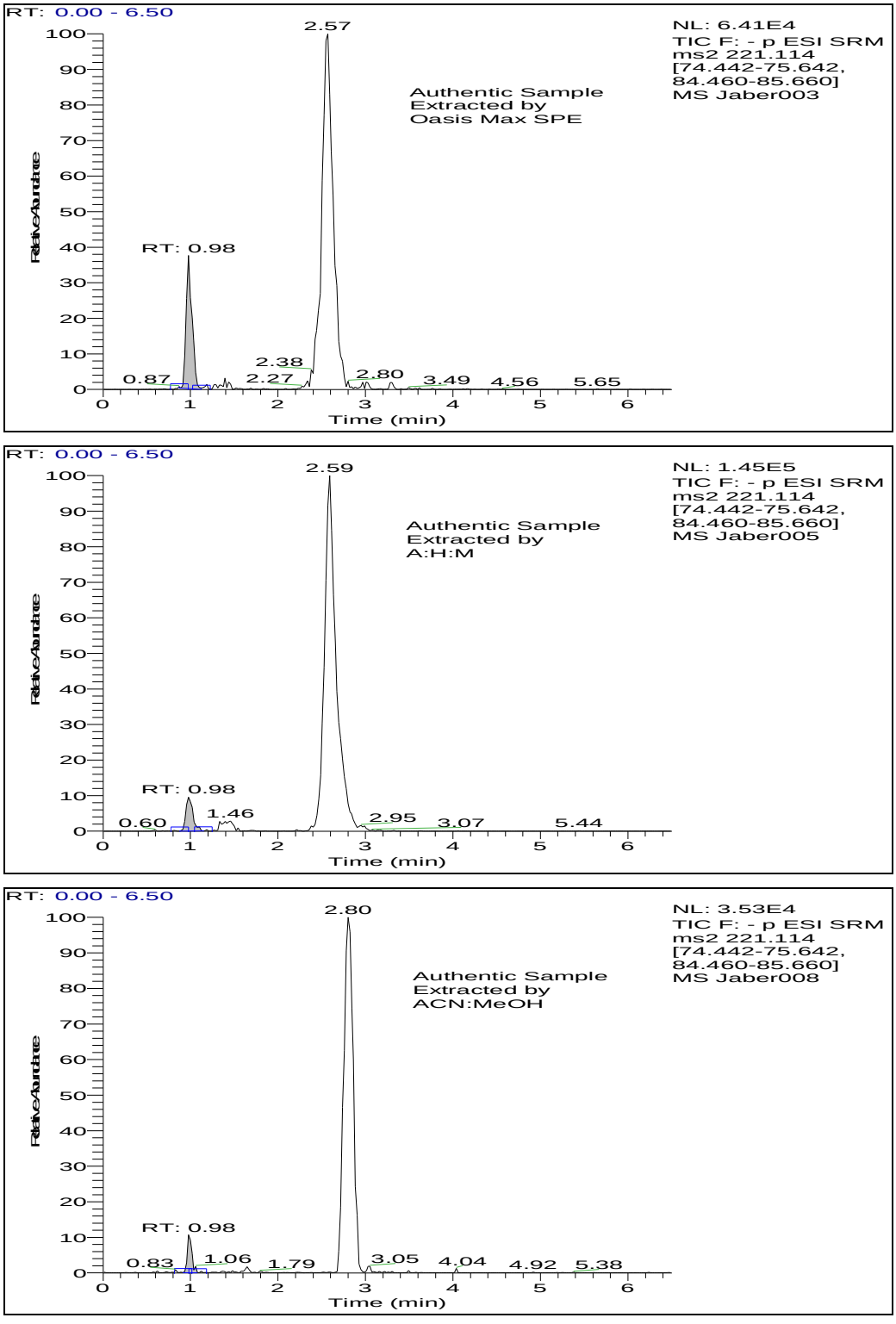


Figure 45: Chromatograms of extraction optimization results of methods 2, 3 and 4 using authentic hair samples

5.2.7. Method validation

Method validation experiments were achieved by following the industrial guidelines for bioanalysis method validation stated by the FDA and other sources (Peters, Drummer & Musshoff 2007, FDA 2001, Shah et al. 2000).

5.2.7.1. Linearity

Linearity was assessed by spiking blank hair samples with 10, 30, 100, 300 and 1000pg/mg of ethyl glucuronide and 0.25ng/mg EtG-D5. The resulting ratio of peak areas of standards/internal standard was plotted versus the spiked analyte concentrations and the linear correlation coefficient (r^2) calculated. The correlation coefficient (r^2) value indicates the degree of linearity (r^2 should be >0.99).

5.2.7.2. LOD and LLOQ

Limit of detection (LOD) and lower limit of quantification (LLOQ) were assessed by spiking blank hair with the EtG in decreasing concentrations. LOD was defined as the lowest detectable concentration of the analyte that differentiated it from the background noise, whereas LLOQ is lowest quantitative concentration that fulfils the required accuracy and precision. The spiked concentrations were 1, 3, 10, 30, 100, 300 and 1000pg/mg of the analyte and 250pg/mg IS (EtG-D5). Signal-to-noise ratio was the method that was used to calculate LOD and LLOQ, and 3 and 10, respectively, were the cut-offs. LODs and LLOQs were calculated using

Equation 2-5 (page 91).

5.2.7.3. Stability (no matrix)

Stability during the whole extraction/incubation process of the analyte in the selected optimized extraction method was assessed by spiking the analyte in 3 different concentrations (100, 300 and 1000pg/mg). Deuterated external standards

(0.25ng/mg) were added at the end of the extraction procedure and samples were run together with unextracted standards made at the same concentrations.

5.2.7.4. Recovery

Recoveries were estimated by measuring peak areas obtained when the EtG spiked into blank hair (in extraction solution) at three concentrations 100, 300, and 1000pg/mg (n=3) against unextracted/unincubated standards. Therefore this recovery takes into account all the extraction steps including incubation and matrix effect (absolute). The recovery was calculated as a percentage by dividing the average peak areas of the extracted standards over the average peak areas of the unextracted standards and multiplied by 100.

5.2.7.5. Intra-day precision

The intra-day precision was achieved by spiking the blank hair samples with three concentrations of analytes (low, medium and high) in five replicates. After extraction and analysis precision was measured by calculating the coefficient of the variation expressed as a percentage (CV %).

5.2.7.6. Inter-day precision

The inter-day precision experiment was a replicate of the intra-day experiment over 5 days and the coefficient of the variation percentage (CV %) was calculated between concentrations of each analyte over these 5 days.

5.2.7.7. Method validation results

Table 71: Linear correlation coefficients, LODs and LLOQs of EtG in hair

Analyte	Linear correlation coefficient (r^2)	LOD pg/mg	LLOQ pg/mg
Ethyl glucuronide	1.0	3.0	9.0

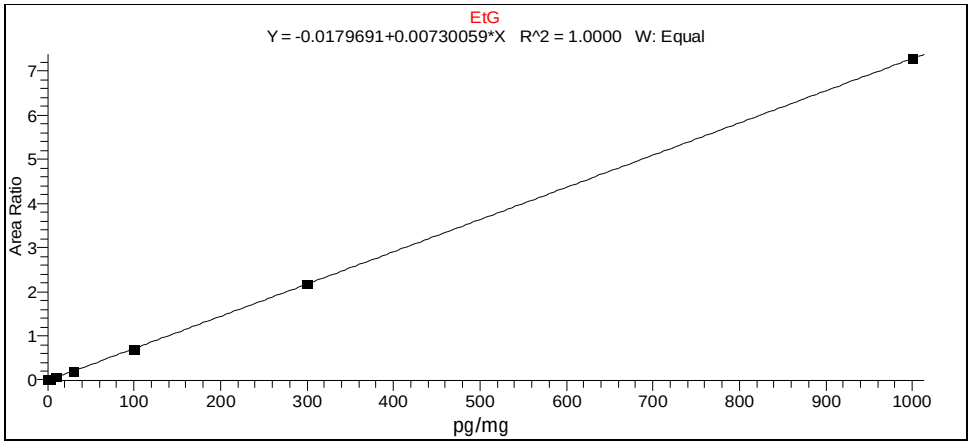


Figure 46: Calibration line for EtG in hair

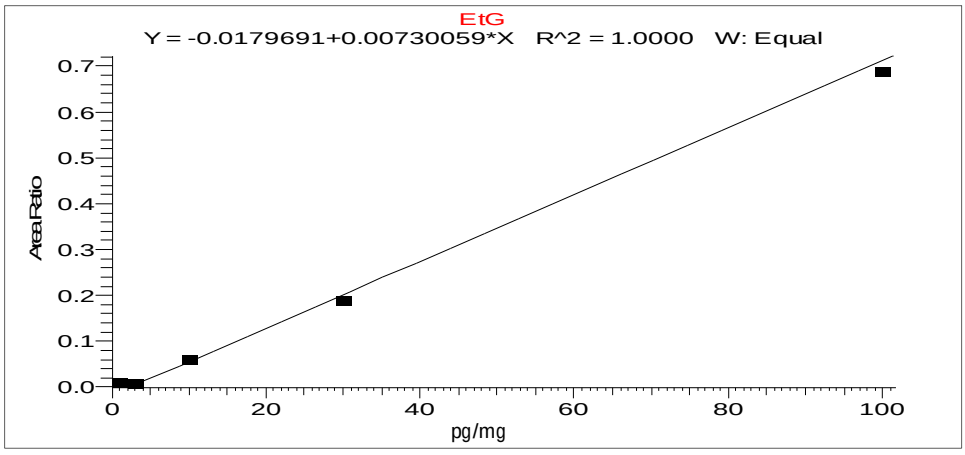


Figure 47a: Magnified lower calibration line for EtG in hair

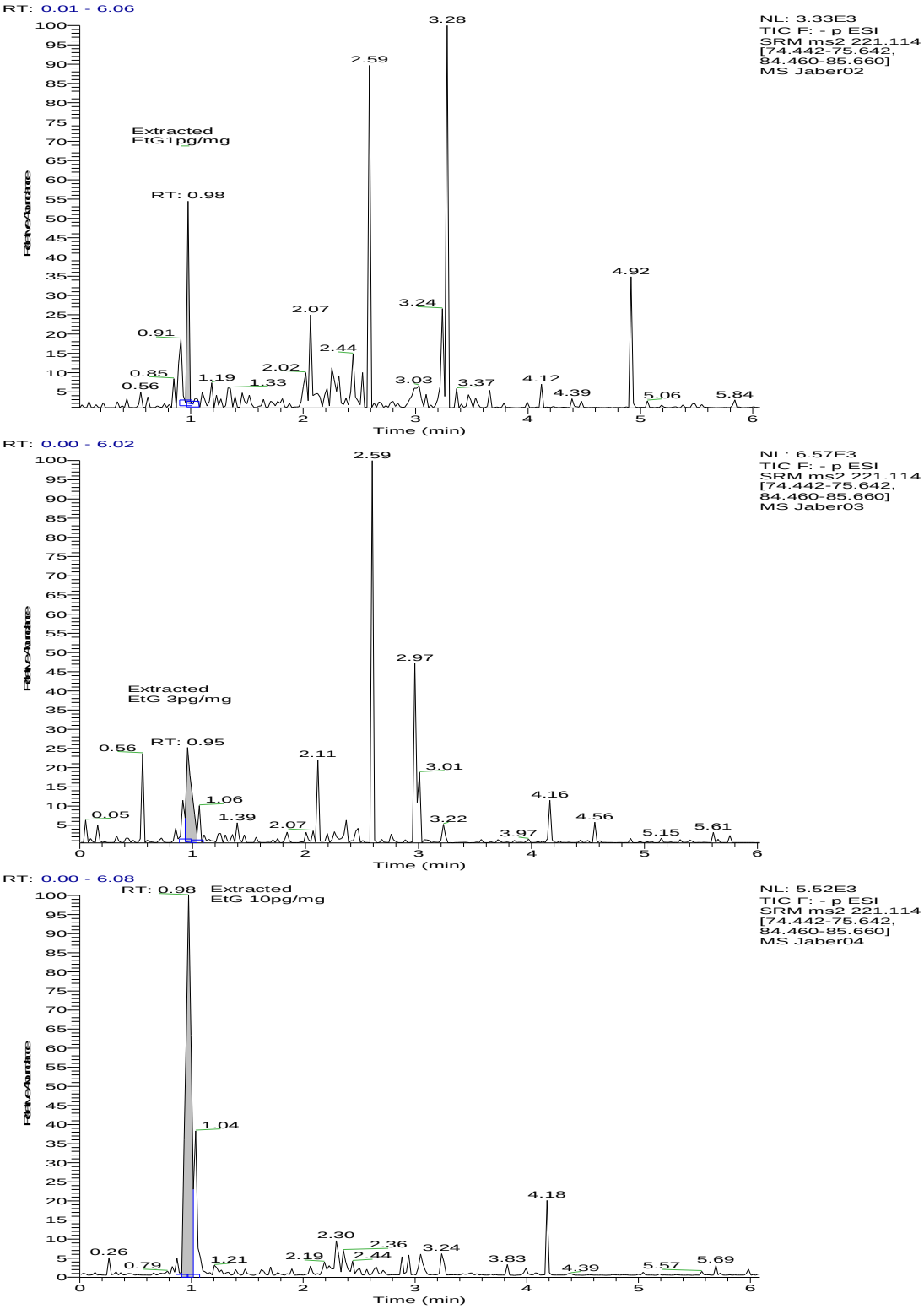


Figure 48: Chromatograms of extracted 1, 3 and 10pg/mg EtG

Table 72: EtG percentage of stability during optimized analysis method

Analyte	100 pg/mg	300 pg/mg	1000 pg/mg	Mean(CV) for 3 Conc.
Ethyl glucuronide	94%	86%	91%	90% (4)

Table 73: Absolute recovery (RE%) results for 100, 300 and 1000pg/mg of EtG spiked in hair (n=3)

Analyte	100 pg/mg	%CV	300 pg/mg	%CV	1000 pg/mg	%CV	Mean(CV) for 3 Conc.
Ethyl glucuronide	70%	9	60%	12	62%	18	64% (8)

Table 74: Inter-day and intra-day precision results in hair

Analyte	Inter-day mean (CV%) n=5			Intra-day mean (CV%) n=5		
	100 pg/mg	300 pg/mg	1000 pg/mg	100 pg/mg	300 pg/mg	1000 pg/mg
Ethyl glucuronide	93 (6)	291 (6)	942 (5)	78 (4)	278 (3)	1051 (3)

5.3. Subjects' EtG results

5.3.1. Comparisons of self-reports and hair analysis

The analysis of patients' hair samples revealed that 37 out of 59 were EtG positive with concentrations ranging from 9 to 339pg/mg, 8 were below the LLOQ and 14 were negative. From these 37 positives, only 16 have clearly self-reported alcohol use or abuse, and 21 had denied alcohol use in their report. However, from the 14 negative subjects 4 were teetotallers as they reported, 7 were unspecified and 3

were from the self-reported use group. Lastly, from the 8 samples with EtG below the LLOQ 5 were from denied use group and 3 from unspecified alcohol use group.

Table 75: Subjects' hair EtG results and alcohol use self-report

Subject	Age (years)	Hair colour	Hair segment	EtG pg/mg	Self-reported alcohol use
1	32	Black	0-3 cm	NF	Yes, poly-addict
2	38	Grey	0-3 cm	NF	Yes, poly-addict
3	32	Black	0-3 cm	NF	Yes, poly-addict
4	24	Black	0-3 cm	<LLOQ	Yes, poly-addict
5	25	Black	0-3 cm	NF	Yes, poly-addict
6	34	Black	0-3 cm	13.9	Yes
7	33	Black	0-3 cm	49.7	Yes
8	28	Dark brown	0-3 cm	19.9	Yes
9	42	Brown	0-3 cm	NF	No
10	40	Black	0-3 cm	9.6	Yes
11	46	Black	0-3 cm	NF	Yes, poly-addict
12	35	Black	0-3 cm	75.4	Yes
13	24	Black	0-3 cm	NF	Yes
14	49	Grey	0-3 cm	NF	Yes, poly-addict
15	34	Dark grey	0-3 cm	18.3	No
16	32	Black	0-3 cm	<LLOQ	No
17	33	Dark grey	0-3 cm	13.0	No
18	36	Black	0-3 cm	53.7	No
19	27	Black	0-3 cm	13.8	No
20	30	Black	0-3 cm	9.3	No
21	41	Grey/ henna	0-3 cm	NF	Yes, rarely
22	54	Grey	0-3 cm	339.3	Yes
23	32	Black	0-3 cm	9.1	Yes
24	38	Dark brown	0-3 cm	12.6	Yes, poly-addict
25	29	Black	0-3 cm	14.4	Yes, rarely
26	35	Black	0-3 cm	NF	No
27	26	Dark brown	0-3 cm	16.0	Yes
28	53	Grey	0-3 cm	31.0	Yes, poly-addict
29	30	Black	0-3 cm	17.1	No

Subject	Age (years)	Hair colour	Hair segment	EtG pg/mg	Self-reported alcohol use
30	32	Brown	0-3 cm	NF	No
31	45	Dark grey	0-3 cm	23.6	Yes
32	25	Black	0-3 cm	38	Yes
33	35	Black	0-3 cm	14.1	Yes, poly-addict
34	25	Black	0-3 cm	57.6	Yes
35	37	Black	0-3 cm	NF	Yes
36	30	Black	0-3 cm	18.9	Yes, poly-addict
37	33	Dark grey	0-3 cm	<LLOQ	Yes, poly-addict
38	29	Black	0-3 cm	14.4	Yes, poly-addict
39	36	Black	0-3 cm	28.5	Yes
40	26	Black	0-3 cm	23.4	Yes
41	38	Black	0-3 cm	32.8	Yes, poly-addict
42	28	Black	0-3 cm	<LLOQ	No
43	35	Black	0-3 cm	NF	No
44	38	Black	0-3 cm	23.2	Yes, poly-addict
45	45	Black	0-3 cm	81.5	Yes, poly-addict
46	32	Black	0-3 cm	24.6	No
47	34	Dark brown	0-3 cm	19.2	Yes
48	44	Black	0-3 cm	24.1	No
49	34	Black	0-3 cm	NF	Yes, poly-addict
50	35	Black	0-3 cm	16.0	No
51	35	Dark brown	0-3 cm	26.4	No
52	34	Black	0-3 cm	17.0	No
53	25	Black	0-3 cm	15.6	No
54	32	Black	0-3 cm	24.2	Yes
55	35	Black	0-3 cm	10	No
56	38	Black	0-3 cm	<LLOQ	No
57	35	Black	0-3 cm	<LLOQ	No
58	34	Black	0-3 cm	<LLOQ	Yes, poly-addict
59	52	Dark brown	0-3 cm	<LLOQ	No

***** Poly-addict taking multi-substances and did not specify if he drinks or not. (NF) not found.

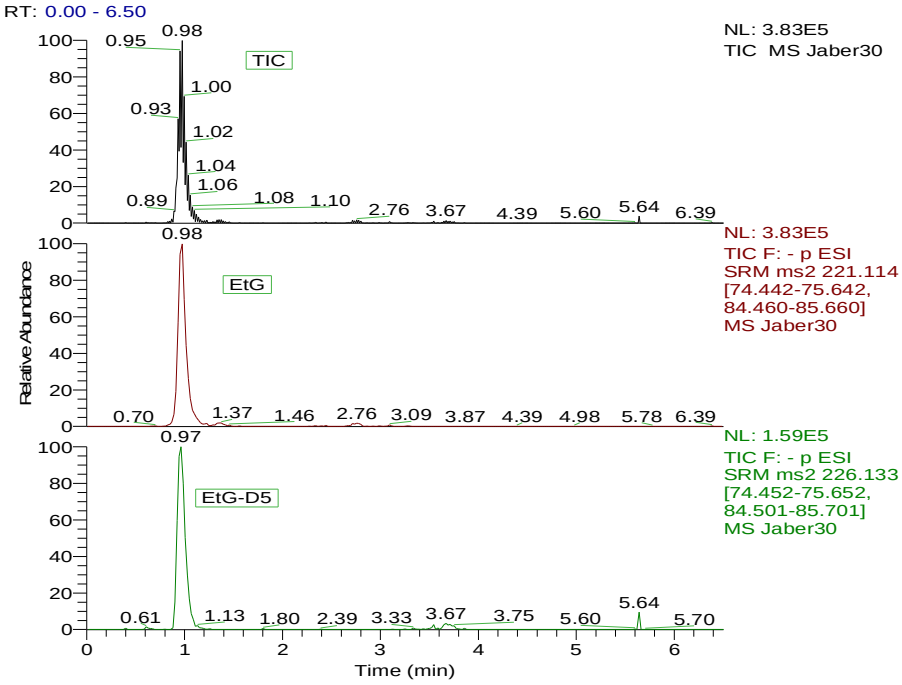


Figure 49: Chromatogram of subject 22 sample

5.4. Statistical analysis

The data collected from this study have been presented in a number of statistical graphs and tables as shown next. Some of the information given in these tables is explained below according to literature (Carletta 1996, Ledgerwood et al. 2008):

- Hair EtG specificity is the percent of total negative self-reports out of negative results from hair analysis.
- Hair EtG sensitivity is the percent of total positive self-reports out of positive results from hair testing.
- Self-report specificity is the percent of total negative hair reports out of negative results from self-reports.
- Self-report sensitivity is the percent of total positive hair reports out of positive results from self-reports.

- Kappa coefficient is a statistical measure of inter-rater agreement or inter-annotator agreement.

$$\text{Kappa} = \frac{Pr(a)-Pr(e)}{1-Pr(e)}$$

where Pr(a) is the relative observed agreement among raters, and Pr(e) is the hypothetical probability of chance agreement.

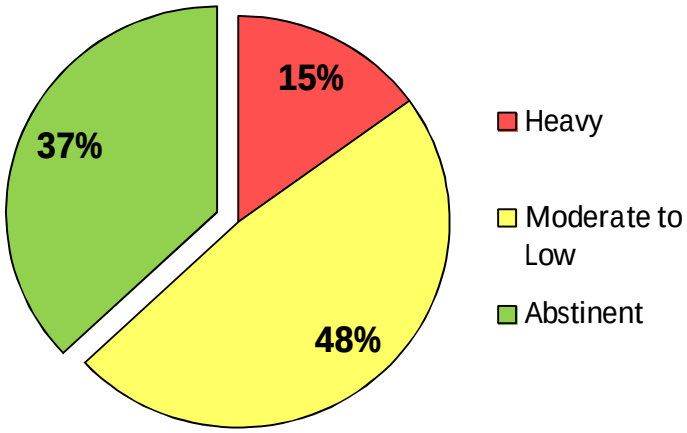


Figure 50: Addicts' alcohol use estimated from hair EtG results

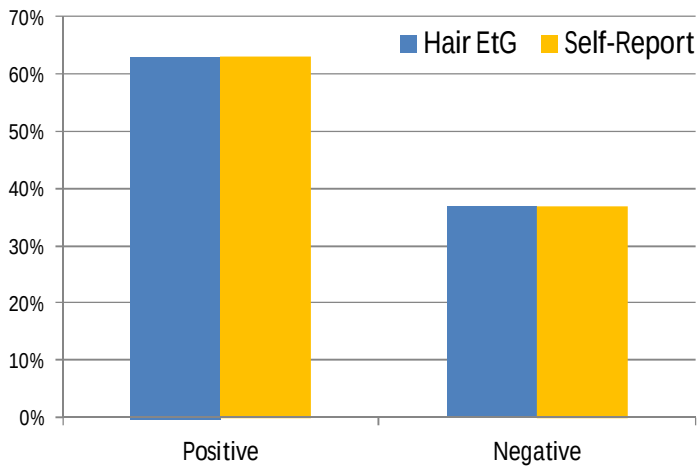


Figure 51: Percent of positive and negative cases found with hair EtG analysis and self-report

Table 76: Correlation of results between hair EtG and self-report

	Positive self-report	Negative self-report	Total
Positive EtG	24	13	37
Negative EtG	13	9	22
Total	37	22	59

Table 77: Measurement of degree of agreement using Kappa

Kappa	Standard error	95% confidence interval	
		Lower limit	Upper limit
0.058*	0.131	0	0.314

* Poor agreement (Kappa range from 0-1)

Table 78: Percentages of specificity and sensitivity for both hair EtG results and self-report

Hair EtG specificity	-SR/-EtG	(9/22)	41%
Hair EtG sensitivity	+SR/+EtG	(24/37)	65%
Self-report specificity	-ET/-SR	(9/22)	41%
Self-report sensitivity	+ET/+SR	(24/37)	65%

-SR= number of negative self-reports, +EtG= number of positive EtG results

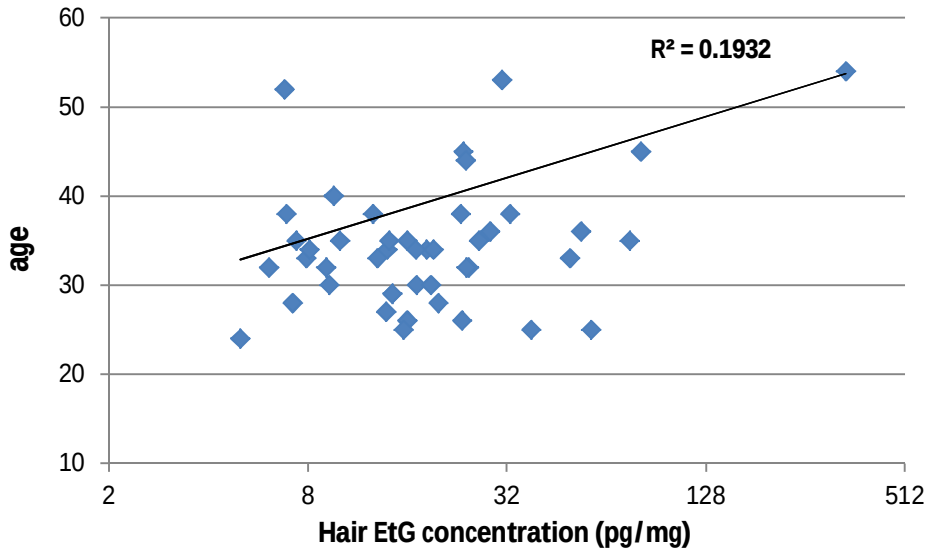


Figure 52: Relationship between addicts' age and EtG concentration in hair

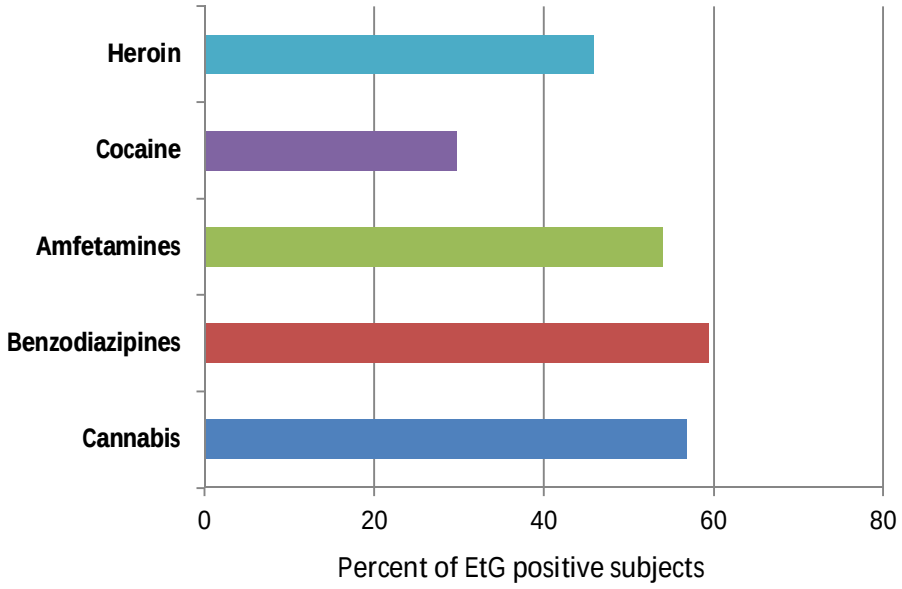


Figure 53: Percent of self-reported concomitant use of illegal drugs by EtG positive subjects

5.5. Discussion

Alcohol is a non-controlled substance in the vast majority of the world's countries. However, its health, social, economic and crime related problems have prompted the need for forensic and clinical screening strategies to be available whenever requested. Research into alcohol detection has led to the focus on EtG noticeably in recent years. Even though, EtG is a minor metabolite of alcohol it can reflect to some extent the actual alcohol intake as it is a direct non-volatile and water-soluble metabolite of the rapidly eliminated ethanol (Kharbouche et al. 2010, Appenzeller et al. 2007). Also the research on the detection of EtG in hair has led to a consensus on suggested cut-offs or thresholds that allow for better interpretation of the results. SoHT cut-offs are shown in Table 79.

Table 79: SOHT consensus on EtG level and its interpretation in hair

Hair sample length	EtG concentration in hair	Interpretation
0-3 cm	<7 pg/mg	Abstinent
0-3 cm	7-30 pg/mg	Social drinker
0-3 cm	>30 pg/mg	Excessive drinker

Based on this information this study was designed and undertaken to fulfil a number of objectives. Each research objective and its finding are discussed in the following paragraphs.

Method development for EtG analysis

Firstly, in this study two different mass spectrometer instruments were tried with different columns. The Exactive Orbitrap system allowed precise ion extraction of EtG and the deuterated internal standard (EtG-D5) with mass accuracy within 6ppm. The instrument showed its best sensitivity when the injector was set on full loop with an injection volume of 25µL. The two columns which were tested on Exactive were Zic-HILIC and Zic-pHILIC. The latter provided almost double the sensitivity with similar retention time (3 min) as seen from the results in Table 68. Both columns are zwitterionic HILIC and were chosen based on the fact that HILIC chromatography is capable of separating polar compounds such as EtG. The retention mechanism is based on hydrophilic partitioning of analytes between an organic solvent-rich mobile phase and an aqueous layer formed on the stationary phase (Jandera 2011). The better performance seen with pHILIC could be due to the pH conditions of the mobile phase (pH 9). The other instrument, TSQ Vantage, has provided almost double the sensitivity of pHILIC-Exactive system without the need of injecting on full loop mode when the correct column was used. Initial trials with Ascentis Express C18 column showed weak response for 10ng/mL EtG. Also preliminary trials with Kinetex HILIC were not successful as no retention could be achieved even though the mobile phase was tried on from 40 to 99% acetonitrile. Thus, Acquity BEH C18 column coupled to TSQ Vantage gave the highest possible sensitivity.

Secondly, with regard to extraction optimization, two SPE methods and two non-SPE methods were compared. Disappointingly, several trials with EtG CleanScreen cartridges ended up with very low recoveries (5%) despite changing and increasing elutants as mentioned in the literature (Lamoureux et al. 2009, Kronstrand, Brinkhagen & Nyström 2011b). In contrast, Oasis Max cartridges showed an average recovery of 94%. Several published methods have opted for this type of cartridge as well (Martins Ferreira, Binz & Yegles 2011, Tarcomnicu et al. 2010, Kintz et al. 2008). However, a real sample comparison was carried out between

Oasis max SPE and two non-SPE methods (A:W:M and A:M) as mentioned in the experimental section. Oasis Max recovery from the real sample not only was 15% higher than the A:W:M incubation/filtration method but also gave cleaner chromatograms with less background noise. A similar finding was reported in a published paper (Tarcomnicu et al. 2010).

Method validation

As the SPE/BEH-C18/TSQ-Vantage method was chosen, essential validation was carried out with as much adherence to FDA and other published bioanalytical guidelines as feasible (Peters, Drummer & Musshoff 2007, FDA 2001). Linearity was excellent with r^2=1 and LOD and LLOQ were calculated to be 3 and 9pg/mg, respectively. The LLOQ was not low enough according to SOHT guidelines. However due to the very limited instrument time allowed the method could not be refined further. Results below the LLOQ were only considered with age/alcohol correlation. Process efficiency or stability during extraction showed good results with an average of 90% (CV=4%) EtG recovery. Inter- and intra-day precision were less than 10% CV for all the three level concentrations. Average absolute recovery, which took into account the matrix effect and stability during incubation, was 64% (CV=8%). It could have been improved if there had been more instrument time.

Alcohol prevalence among addicts

Out of around 120 addicts residing temporarily for rehabilitation in the drug addiction centre in Kuwait 59 agreed to participate in this study. Their EtG results ranged from 9 to 339pg/mg (after excluding <LLOQ results) which were within the range of those reported in the literature, 2-13200pg/mg (Janda, Alt 2001, Morini et al. 2006, Kintz et al. 2008). These results are presumed to represent the last 2-3 months as the analysed hair segments were either 0-2 or 0-3cm from the scalp. The study findings showed also that 63% (15% heavy and 48% moderate to low) of the included addicts had used alcohol in the last 2-3 months. Regardless of the degree of alcohol use, alcohol is an illegal substance in Kuwait and these results can be a further proof of the smuggling or clandestine local production of this substance.

Unfortunately, there was no published data that can be compared with our findings except two studies. One looked at the toxicological screening of blood and urine between 1992-1997 in Kuwait where they found only 10% of the addicts were alcohol positive (Radovanovic et al. 2001). The other one was an aviation related study which was a questionnaire type study. They reported that alcohol use among flying crews was 37% and among clerks working in a ministry it was 4% (Omar et al. 2005). In the first study the analysis was carried out during the 1990s and they tested ethanol (not the EtG), which has a short half-life, so their data refer only to the very recent use of alcohol, the previous 1-2 days. The second study was based on self-report only. Therefore, there is no proven data of how prevalent alcohol abuse is in Kuwait. The present study should present some reliable evidence of alcohol behaviour among the studied population.

Assessment of self-report validity

Alcohol use is not only illegal in Kuwait but also culturally and religiously unacceptable behaviour in that particular region of the world, even though in real life there is a considerable percentage of the population that uses alcohol discreetly. Therefore, studying or investigating alcohol prevalence through self-report would have some degree of unreliability. In the present study comparison between self-report and EtG results gave a low agreement index result (Kappa=0.058), even though positive EtG and positive self-report results have a 65% match. Both hair EtG sensitivity and self-report sensitivity have a score of 65%. On the other hand, negative EtG and negative self-report results have a 41% match, while hair EtG specificity and self-report specificity both scored 41%. Previous studies have as well indicated that under-reporting is common even for pure research (non-medicolegal) purposes (Lees et al. 2012, Johnson, Fendrich 2005).

Correlation with age and other drugs of abuse

Analysing hair EtG results in relation to age has shown a weak positive correlation (r=0.2) between alcohol consumption and age of the addicts as seen in Figure 51. Similar findings were reported in a Spanish study, where correlation between alcohol positive cases and age was r=0.14 (Gómez-Talegón et al. 2012). Higushi et al studied 3 different groups, Japanese, Japanese-Americans and Caucasians

(Higuchi et al. 2006). Their findings were only similar to the present study findings with the Japanese group, where alcohol consumption was more in older than younger drinkers. However, on the contrary the percentage of heavier drinkers was higher among younger people in the case of Japanese-Americans and Caucasians. A possible explanation to the slight increase of alcohol level with age among the Kuwaiti addicts could be due to the very high prices of the smuggled alcohol, as older men normally have more financial capability than younger men.

With respect to the consumption of other substances by the studied subjects, it worth mentioning that benzodiazepines, cannabis and amfetamines were found to be the most used substances with alcohol (59, 57 and 54%, respectively) according to the self-reports and hair EtG results, while heroin and cocaine were the least, 46 and 30%, respectively, as seen in Figure 52. A recent Swedish study examining a toxicological data base for drinking drivers had very similar findings (Jones, Holmgren 2012). Benzodiazepines were the major substances co-ingested with alcohol, followed by cannabis, amfetamine, cocaine and heroin, respectively. These findings might be similar to the prevalence of these illicit drugs globally regardless of the concomitant alcohol use.

5.6. Limitations

The main limitation of the study was the LLOQ which should be lower than 9pg/mg and the percentage of absolute recovery was not too high, but the use of a deuterated internal standard can compensate for the losses. All those issues were due to the very limited instrument time available. Also the number of recruited subjects was limited and could not be increased because many addicts have very short hair/hairless or refused to participate.

5.7. Conclusion

Alcohol abuse is a worldwide problem that needs to be assessed and studied carefully within each country. The detection of a minor and specific metabolite of

alcohol, ethyl glucuronide in hair provides paramount information with regard to alcohol consumption. A SPE-LC-MS/MS method was developed and validated for the detection and quantitation of EtG in hair. The method was used to investigate alcohol prevalence among addicts attending a general addiction centre in Kuwait. The main findings are that 63% of addicts were EtG positive, self-reports and hair EtG results have a weak agreement and a weak correlation was found between age and alcohol consumption.

6. Chapter 6 - Conclusion and future work

6.1. Conclusion

A number of investigations were carried out during this project on the potential use of alternative clinical and toxicological samples, namely, hair, oral fluids and dried blood spots. Special consideration has been given to hair as three chapters were mostly focusing on it.

The main aim of the second chapter was to develop and validate an analytical screening method for detection and quantitation of basic and weak basic drugs in hair samples. The method was based on the extraction of drugs from hair through incubation in methanolic solution containing mainly methanol with acetonitrile and formic acid in deionized water. Due to the very limited access to instrumentation during the research the aim of method validation was not fully achieved, and not for all the investigated basic drugs. One of the main investigations in this chapter was to compare the methanolic method with an alkaline digestion method (1M NaOH) that was followed by liquid-liquid extraction with MTBE. A filtration step with filtration vials was added during method development and their materials were compared. After filtration, extracts were injected directly onto a C18 column coupled to a Sciex ABI 2000 MS/MS. The mobile phase was based on 50% methanol with 0.1% formic acid and 2mM ammonium acetate (isocratic). Both methods were compared by applying them to real samples from ante-mortem and post-mortem cases. Development and validation results showed that calibration was linear with r^2 of 0.991 - 0.999 for 20 tested analytes. The matrix effect was assessed to be between 91.4% - 110.2% for 18 analytes. PTFE filter material showed better recoveries over the GMF and PVDF based filters. Stability of analytes during extraction in general was better with methanolic incubation than alkaline digestion. With regard to real sample recovery, 6 out of 10 analytes were recovered better with alkaline digestion. However, the methanolic method was capable of extracting most of the targeted drugs (8 out of 10) in the real hair samples but only part of the total incorporated drug, as for

instance codeine and mirtazapine had around 10% and 30% of the recovery compared to the alkaline digestion. Therefore, these results suggest that a combination of both methods (methanolic and alkaline extractions) in hair sample processing for general detection of basic and weak basic drugs may produce better results. However, not all basic drugs are stable under alkaline digestion, as sulpiride and amlodipine, for example, were completely undetected under these conditions.

In the third chapter, five drugs and one active metabolite have been chosen (procyclidine, quetiapine, chlorprothixene, haloperidol, risperidone and OH-risperidone) for investigation into the relationship between the given dose and the concentration detected in hair, blood and oral fluids of subjects under controlled drug administration. These drugs were chosen because of their wide prescription to psychiatric patients, high potential of short and long term side effects and toxicity and also for sharing some similarity with most of drugs of abuse, which target generally the brain cells. Nearly all the subjects have been taking these drugs for at least two weeks and should have established a steady-state level of the drugs. In order to carry out the investigations an analytical method for all the three types of samples had to be developed and validated. As for blood and oral fluids samples, four extraction methods have been tested, Tris buffer pH9, protein crash, 0.01M and 1M NaOH. NaOH (1M) and protein crash showed the highest recoveries. Extraction with 1M NaOH and MTBE solvent was chosen with total analyte recoveries during method development of 58-110%. This method was validated with acceptable results. With regards to the hair method also four extraction methods were tested, digestion with NaOH, Na_2S and proteinase K and incubation with MAF. NaOH digestion and MAF incubation gave the highest recoveries for the analytes. However, due to the previous chapter findings and recommendations, the two methods were combined to ensure the highest possible drug extraction yield from hair samples. The combined method was validated subsequently. After analysing patients samples correlations between drug level in each biological matrix and with dose were investigated. The most significant correlation coefficients (r) found were those between blood and hair concentrations, procyclidine r=0.83 (18 subjects p=<0.001), risperidone r=0.96 (14 subjects p=<0.001), haloperidol r=0.90 (10 subjects p=<0.001), OH-risperidone r=0.24 (13 subjects p=>0.44), quetiapine r=0.28

(14 subjects p=>0.33) and chlorprothixene r=0.32 (13 subjects p=>0.32). Risperidone and haloperidol showed remarkable correlation results as nearly all the correlation coefficients between dose/hair, blood and saliva, hair/blood and oral fluids, and blood/oral fluids drug levels were significant with r ≥0.58. Among the interesting results is the strong correlation found between drugs half-lives and the mean ratio of hair concentration/dose (r= 0.96, p≤0.003).

Remaining patients' blood samples from chapter 3 studies with positive results for the six compounds investigated in the chapter have been used for chapter 4 DBS stability evaluation studies. A validated method has been developed which can be used for the extraction and quantitative analysis of drugs in small volumes of blood spotted and dried on filter papers or Guthrie cards. The DBS technique provides a suitable procedure for the storage and analysis of samples in therapeutic drug monitoring and forensic toxicology because they are easy and safer to handle, transport and store in the laboratory, even in the absence of refrigeration. The stability of antipyschotics in DBS from real patients' samples was assessed by storing them at four different temperatures (25, 4, -20 and -80°C) with and without prior impregnation of the DBS cards with sodium fluoride. After the development and validation of a LLE-LC-MS method, which was based on 1hr incubation in MAF solution, samples were analysed at days 0, 45, 90 and 180. Results showed a significant stability of all the compounds (procyclidine, quetiapine, risperidone, OH-risperidone, chlorprothixene and haloperidol) in all the different storage conditions and no significant increase or decrease in drug concentrations with sodium fluoride impregnation. Possibly a much longer period than 6 months is needed to see if NaF has an effect on drug stability.

Finally, in chapter 5, the analysis of minor and specific metabolite of alcohol in hair EtG was explored. Fifty nine hair samples were collected from patients attending the addiction treatment centre in Kuwait for evaluating alcohol prevalence among Kuwaiti drug addicts and to correlate the results with their self-reported intake. After trials with five different HPLC columns, two SPE cartridges, two LLE extraction procedures and two mass spectrometer instruments a SPE-LC-MS/MS method was developed and validated for the detection and quantitation of EtG in hair. The

method has a limit of detection (LOD) of 3pg/mg and limit of quantitation (LLOQ) of 9pg/mg. Following the application of the developed method with the subjects samples, the main findings were that 63% of the addicts were EtG positive, self-reports and hair EtG results have a weak agreement (Kappa=0.058) and a weak correlation was found between age and alcohol consumption.

6.2. Future Work

A simpler and fast general screening method for basic drugs in hair would be of great benefit to the forensic toxicology laboratories. Although the methods developed in chapters 2 and 3 are of extensive capability of extracting basic drugs, they are relatively lengthy requiring two working days to complete analysis of a set of hair samples including calibrators and QCs. Therefore there is plenty of room for improvement especially if access to instrumentation is not restricted.

Studies in chapter 3 aimed to look mainly into the relationships of the steady-state drug levels in the three different matrices with the given dose and with each other. As some ideas have been gathered and some significant correlations were shown the next or future studies should have a much larger number of participants. Also analysis of different hair segments should be considered in order to see if the steady-state level in hair is constant during long term therapy. Other future work should include a single dose study for a large group of subjects as well to see the drug disposition pattern in hair. Also research on the development of a faster and simpler hair analysis method for basic, acidic and neutral drugs would be very beneficial. A large scale study looking into and assessing hair growth variation among different health, age and ethnic backgrounds would be helpful, as the 1cm per month average hair growth used quotation has not been investigated thoroughly especially with respect to all the factors affecting the growth. There is also a need for more alternative matrices (e.g. oral fluids and hair) data from ante-mortem and post-mortem cases in order to establish reference drug ranges.

With regards to DBS, a further evaluation of the stability of the compounds in DBS samples over longer periods (one or two years) in similar conditions to those in

chapter 4 with possible comparison to conventional blood samples would be of interest. Also investigating the usefulness of the addition of preservatives to DBS cards on long term storage and the optimum concentration needed would add a valuable insight.

Finally, further research on the development of a more sensitive method for quantitation of EtG in hair with lower LOD and LLOQ would be of interest. Although solid phase extraction has shown superiority over liquid-liquid extraction for the analysis of EtG as shown in chapter 5, LLE seems to have potential for improvement. A further investigation with different solutions could be of interest especially if that would reduce the cost of EtG analysis. Another interesting research project would be comparing EtG levels in hair with other biological matrices. Investigation into other alcohol markers would be a possibility as well. Lastly, the developed EtG analysis in hair method could be used for the investigation of alcohol prevalence in other groups in society.

7. References

Abudu, N. 2008, "Ethyl Glucuronide: A Sensitive Marker for Alcohol Consumption.", *Archive Issue,* [Online], vol. 19, no. 3. Available from: http://www.wardelab.com/19-3.html#1.

AbuRuz, S., Millership, J. & McElnay, J. 2006, "Dried blood spot liquid chromatography assay for therapeutic drug monitoring of metformin", *Journal of Chromatography B,* vol. 832, no. 2, pp. 202-207.

Agius, R., Ferreira, L.M. & Yegles, M. 2011, "Can ethyl glucuronide in hair be determined only in 3cm hair strands?", *Forensic science international,* .

Akimoto, Y., Nishimura, H., Komiya, M., Shibata, T., Kaneko, K., Fujii, A. & Tamura, T. 1985, "Concentrations of ampicillin and cefadroxil in human serum and mixed saliva following a single oral administration of talampicillin and cefadroxil, and relationships between serum and mixed saliva concentrations", *General Pharmacology: The Vascular System,* vol. 16, no. 3, pp. 273-275.

Al Jaber, J., Holt, D. & Johnston, A. 2012, "Method Development for the Detection of Basic/Weak Basic Drugs in Hair by LCMSMS: Comparison between Methanolic and Alkaline Extraction on Real Samples", *Pharmacology & Pharmacy,* vol. 3, no. 3, pp. 271-282.

Alfazil, A.A. & Anderson, R.A. 2008, "Stability of benzodiazepines and cocaine in blood spots stored on filter paper", *Journal of analytical toxicology,* vol. 32, no. 7, pp. 511-515.

Alt, A., Janda, I., SEIDL, S. & WURST, F. 2000, "Determination of ethyl glucuronide in hair samples", *Alcohol and Alcoholism,* vol. 35, no. 3, pp. 313.

Appenzeller, B.M.R., Agirman, R., Neuberg, P., Yegles, M. & Wennig, R. 2007, "Segmental determination of ethyl glucuronide in hair: a pilot study", *Forensic science international,* vol. 173, no. 2-3, pp. 87-92.

Auwarter, V., Sporkert, F., Hartwig, S., Pragst, F., Vater, H. & Diefenbacher, A. 2001, "Fatty acid ethyl esters in hair as markers of alcohol consumption. Segmental hair analysis of alcoholics, social drinkers, and teetotalers", *Clinical Chemistry,* vol. 47, no. 12, pp. 2114-23.

Aymard, N., Viala, A., Clement, M.N., Jacquot, M., Vacheron, M.N., Gauillard, J. & Caroli, F. 2002, "Long-term pharmacoclinical follow-up in schizophrenic patients treated with risperidone: plasma and red blood cell concentrations of risperidone and its 9-hydroxymetabolite and their relationship to whole blood serotonin and tryptophan, plasma homovanillic acid, 5-hydroxyindoleacetic acid, dihydroxyphenylethyleneglycol

and clinical evaluations", *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 26, no. 5, pp. 975-988.

Bagli, M., Süverkrüp, R., Quadflieg, R., Höflich, G., Kasper, S., Möller, H.J., Langer, M., Barlage, U. & Rao, M.L. 1999, "Pharmacokinetic-pharmacodynamic modeling of tolerance to the prolactin-secreting effect of chlorprothixene after different modes of drug administration", *Journal of Pharmacology and Experimental Therapeutics*, vol. 291, no. 2, pp. 547.

Balikova, M.A. & Habrdova, V. 2003, "Hair analysis for opiates: evaluation of washing and incubation procedures", *J Chromatogr B Analyt Technol Biomed Life Sci*, vol. 789, no. 1, pp. 93-100.

Baptista, M.J., Monsanto, P.V., Pinho Marques, E.G., Bermejo, A., Avila, S., Castanheira, A.M., Margalho, C., Barroso, M. & Vieira, D.N. 2002, "Hair analysis for delta(9)-THC, delta(9)-THC-COOH, CBN and CBD, by GC/MS-EI. Comparison with GC/MS-NCI for delta(9)-THC-COOH", *Forensic Science International*, vol. 128, no. 1-2, pp. 66-78.

Baselt, R.C. 2004, *Disposition of toxic drugs and chemicals in man 7th ed.* Biomedical Publications.

Baumgartner, A., Jones, P., Baumgartner, W. & Black, C. 1979, "Radioimmunoassay of hair for determining opiate-abuse histories", *Journal of Nuclear Medicine*, vol. 20, no. 7, pp. 748.

Bearer, C.F., Bailey, S.M. & Hoek, J.B. 2010, "Advancing alcohol biomarkers research", *Alcoholism: Clinical and Experimental Research*, vol. 34, no. 6, pp. 941-945.

Beaumont, K. 2010, "Who Decided We Should Measure Plasma?", *Connecting Strategies on Dried Blood Spots*European Bioanalysis Forum, , 17-18 June 2010.

Bisbing, R.,Richard E. 2002, *Forensic science handbook*, 2nd Ed. edn, Prentice Hall.

Blank, D.L. & Kidwell, D.A. 1995, "Decontamination procedures for drugs of abuse in hair: are they sufficient?", *Forensic Science International*, vol. 70, no. 1-3, pp. 13-38.

Brocks, D.R. 1999, "Anticholinergic drugs used in Parkinson's disease: An overlooked class of drugs from a pharmacokinetic perspective", *J Pharm Pharm Sci*, vol. 2, no. 2, pp. 39-46.

Broecker, S., Herre, S. & Pragst, F. 2011, "General unknown screening in hair by liquid chromatography–hybrid quadrupole time-of-flight mass spectrometry (LC–QTOF-MS)", *Forensic science international*, .

Buszewski, B. & Noga, S. 2012, "Hydrophilic interaction liquid chromatography (HILIC)—a powerful separation technique", *Analytical and bioanalytical chemistry*, vol. 402, no. 1, pp. 231-247.

Camag AG 2011, Aug. 11, 2011-last update, Workshop “Automatization of DBS Analysis” in Basel [Homepage of G.I.T. Laboratory], [Online]. Available: http://www.laboratory-journal.com/news/company-news/workshop-automatization-dbs-analysis-basel [2011, 9/5].

Carletta, J. 1996, "Squibs and Discussions Assessing Agreement on Classification Tasks: The Kappa Statistic", *Computational linguistics*, vol. 22, no. 2, pp. 249-254.

Chamberlain, A.J. & Dawber, R.P. 2003, "Methods of evaluating hair growth", *Australasian Journal of Dermatology*, vol. 44, no. 1, pp. 10-8.

ChemSpider 2011, "ChemSpider: An Online Chemical Information Resource", *Journal of chemical education*, .

ChemTox, L. 2009, *Hair Sampling*, , Strasbourg.

Cheze, M., Villain, M. & Pepin, G. 2004, "Determination of bromazepam, clonazepam and metabolites after a single intake in urine and hair by LC-MS/MS. Application to forensic cases of drug facilitated crimes", *Forensic Science International*, vol. 145, no. 2-3, pp. 123-30.

Cirimele, V., Kintz, P., Gosselin, O. & Ludes, B. 2000, "Clozapine dose-concentration relationships in plasma, hair and sweat specimens of schizophrenic patients", *Forensic science international*, vol. 107, no. 1-3, pp. 289-300.

Cordero, R. & Paterson, S. 2007, "Simultaneous quantification of opiates, amphetamines, cocaine and metabolites and diazepam and metabolite in a single hair sample using GC–MS", *Journal of Chromatography B*, vol. 850, no. 1-2, pp. 423-431.

Couper, F.J., McIntyre, I.M. & Drummer, O.H. 1995a, "Detection of antidepressant and antipsychotic drugs in postmortem human scalp hair.", *Journal of forensic sciences*, .

Couper, F.J., McIntyre, I.M. & Drummer, O.H. 1995b, "Extraction of psychotropic drugs from human scalp hair.", *Journal of forensic sciences*, .

Courtois, M., Loussouarn, G., Hourseau, C. & Grollier, J.F. 1995, "Ageing and hair cycles", *British Journal of Dermatology*, vol. 132, no. 1, pp. 86-93.

Cullen, B. 2012, 11/01/2012-last update, *Use of antipsychotic drugs improves life expectancy for individuals with schizophrenia* [Homepage of Johns Hopkins], [Online]. Available: http://www.hopkinsmedicine.org/news/media/releases/study_use_of_antipsychotic_drugs_improves_life_expectancy_for_individuals_with_schizophrenia [2012, .

Cumbie, P. & Jenkins, J. 1974, "Mercury accumulation in native mammals of the southeast", *Conference: 28. annual conference of the Southeastern Association of Game and Fish Commissioners, White Sulfur Springs, WV, USA, Nov 1974*, pp. 639.

de Leon, J., Diaz, F.J., Wedlund, P., Josiassen, R.C., Cooper, T.B. & Simpson, G.M. 2004, "Haloperidol half-life after chronic dosing", *Journal of clinical psychopharmacology*, vol. 24, no. 6, pp. 656-660.

Déglon, J., Lauer, E., Thomas, A., Mangin, P. & Staub, C. 2010, "Use of the dried blood spot sampling process coupled with fast gas chromatography and negative-ion chemical ionization tandem mass spectrometry: application to fluoxetine, norfluoxetine, reboxetine, and paroxetine analysis", *Analytical and Bioanalytical Chemistry*, vol. 396, no. 7, pp. 2523-2532.

Déglon, J., Thomas, A., Cataldo, A., Mangin, P. & Staub, C. 2009, "On-line desorption of dried blood spot: A novel approach for the direct LC/MS analysis of [mu]-whole blood samples", *Journal of pharmaceutical and biomedical analysis*, vol. 49, no. 4, pp. 1034-1039.

Degutis, L.C., Rabinovici, R., Sabbaj, A., Mascia, R. & D'Onofrio, G. 2004, "The saliva strip test is an accurate method to determine blood alcohol concentration in trauma patients", *Academic Emergency Medicine*, vol. 11, no. 8, pp. 885-887.

Dejaegher, B., Mangelings, D. & Vander Heyden, Y. 2008, "Method development for HILIC assays", *Journal of separation science*, vol. 31, no. 9, pp. 1438-1448.

Derauf, C., Katz, A.R. & Easa, D. 2003, "Agreement between maternal self-reported ethanol intake and tobacco use during pregnancy and meconium assays for fatty acid ethyl esters and cotinine", *American Journal of Epidemiology*, vol. 158, no. 7, pp. 705.

Dernroth, D. 2009, Pheomelanin markers in melanoma, Linköping University.

Desai, M., Tanus-Santos, J., Li, L., Gorski, J., Arefayene, M., Liu, Y., Desta, Z. & Flockhart, D. 2003, "Pharmacokinetics and QT interval pharmacodynamics of oral haloperidol in poor and extensive metabolizers of CYP2D6", *The pharmacogenomics journal*, vol. 3, no. 2, pp. 105-113.

DeVane, C.L. & Nemeroff, C.B. 2001a, "Clinical pharmacokinetics of quetiapine: an atypical antipsychotic", *Clinical pharmacokinetics*, vol. 40, no. 7, pp. 509-522.

DeVane, C.L. & Nemeroff, C.B. 2001b, "Clinical pharmacokinetics of quetiapine: an atypical antipsychotic", *Clinical pharmacokinetics*, vol. 40, no. 7, pp. 509-522.

Di Giovanni, G., Di Matteo, V. & Esposito, E. 2008, *Serotonin-dopamine interaction: experimental evidence and therapeutic relevance*, Elsevier Science.

Drummer, O.H. 2004, "Postmortem toxicology of drugs of abuse", *Forensic science international*, vol. 142, no. 2-3, pp. 101-113.

Ebadi, M. 2007, *Desk reference of clinical pharmacology*, CRC.

Edelbroek, P.M., Heijden, J. & Stolk, L.M.L. 2009, "Dried blood spot methods in therapeutic drug monitoring: methods, assays, and pitfalls", *Therapeutic drug monitoring,* vol. 31, no. 3, pp. 327.

El-Haj, B., Al-Amri, A. & Ali, H.S. 2011, "Target Urinary Analytes for the Gas Chromatographic Mass Spectrometric Detection of Procyclidine and Benzhexol in Drug Abuse Cases", *Journal of analytical toxicology,* vol. 35, no. 2, pp. 92-98.

Eser, H.P., Pötsch, L., Skopp, G. & Moeller, M.R. 1997, "Influence of sample preparation on analytical results: drug analysis [GC/MS] on hair snippets versus hair powder using various extraction methods", *Forensic Science International,* vol. 84, no. 1-3, pp. 271-279.

Ettinger, U., Kumari, V., Zachariah, E., Galea, A., Crawford, T.J., Corr, P.J., Taylor, D., Das, M. & Sharma, T. 2003, "Effects of procyclidine on eye movements in schizophrenia", *Neuropsychopharmacology,* vol. 28, no. 12, pp. 2199-2208.

FDA, U. 2001, "Guidance for industry: bioanalytical method validation", *Rockville, MD: CDER, .*

Feng, Y., Pollock, B.G., Coley, K., Marder, S., Miller, D., Kirshner, M., Aravagiri, M., Schneider, L. & Bies, R.R. 2008, "Population pharmacokinetic analysis for risperidone using highly sparse sampling measurements from the CATIE study", *British journal of clinical pharmacology,* vol. 66, no. 5, pp. 629-639.

Fontanals, N., Marcé, R. & Borrull, F. 2007, "New materials in sorptive extraction techniques for polar compounds", *Journal of Chromatography A,* vol. 1152, no. 1, pp. 14-31.

Forrest, I., Otis, L., Serra, M. & Skinner, G. 1972, "Passage of 3H-chlorpromazine and 3H--tetrahydrocannabinol into the hair (fur) of various mammals", Proc West Pharmacol Soc, , pp. 83.

Froemming, J.S., Lam, Y.W., Jann, M.W. & Davis, C.M. 1989, "Pharmacokinetics of haloperidol", *Clinical pharmacokinetics,* vol. 17, no. 6, pp. 396-423.

Furdon, S.A. & Clark, D.A. 2003, "Scalp hair characteristics in the newborn infant", *Adv Neonatal Care,* vol. 3, no. 6, pp. 286-96.

Garg, U. & Dasouki, M. 2006, "Expanded newborn screening of inherited metabolic disorders by tandem mass spectrometry: Clinical and laboratory aspects", *Clinical biochemistry,* vol. 39, no. 4, pp. 315-332.

Gerlach, M., Hunnerkopf, R., Rothenhofer, S., Libal, G., Burger, R., Clement, H., Fegert, J., Wewetzer, C. & Mehler-Wex, C. 2007, "Therapeutic drug monitoring of quetiapine in adolescents with psychotic disorders", *Pharmacopsychiatry,* vol. 40, no. 2, pp. 72-76.

Girod, C. & Staub, C. 2001, "Methadone and EDDP in hair from human subjects following a maintenance program: results of a pilot study", *Forensic science international*, vol. 117, no. 3, pp. 175-184.

Gjerden, P., Slørdal, L. & Bramness, J.G. 2010, "Prescription persistence and safety of antipsychotic medication: a national registry-based 3-year follow-up", *European journal of clinical pharmacology*, , pp. 1-7.

Goldblum, R., Goldbaum, L. & Piper, W. 1954, "Barbiturate Concentrations in the Skin and Hair of Guinea Pigs1", *Journal of Investigative Dermatology*, vol. 22, no. 2, pp. 121-128.

Gómez-Talegón, T., Fierro, I., González-Luque, J.C., Colás, M., López-Rivadulla, M. & Javier Álvarez, F. 2012, "Prevalence of psychoactive substances, alcohol, illicit drugs, and medicines, in Spanish drivers: A roadside study", *Forensic science international*, .

Gottardo, R., Bortolotti, F., De Paoli, G., Pascali, J.P., Miksík, I. & Tagliaro, F. 2007, "Hair analysis for illicit drugs by using capillary zone electrophoresis-electrospray ionization-ion trap mass spectrometry", *Journal of Chromatography A*, vol. 1159, no. 1-2, pp. 185-189.

Goulle, J.P., Noyon, J., Layet, A., Rapoport, N.F., Vaschalde, Y., Pignier, Y., Bouige, D. & Jouen, F. 1995, "Phenobarbital in hair and drug monitoring", *Forensic Science International*, vol. 70, no. 1-3, pp. 191-202.

Greenaway, C., Ratnaraj, N., Sander, J.W. & Patsalos, P.N. 2010, "Saliva and serum lacosamide concentrations in patients with epilepsy", *Epilepsia*, .

Guthrie, R. & Susi, A. 1963, "A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants", *Pediatrics*, vol. 32, no. 3, pp. 338.

Hadidi, K.A. 2004, "Development of a screening method for the most commonly abused anticholinergic drugs in Jordan; trihexyphenidyl, procyclidine and biperiden", *Legal medicine*, vol. 6, no. 4, pp. 233-241.

Hadidi, K.A., Almasad, J.K., Al-Nsour, T. & Abu-Ragheib, S. 2003, "Determination of tramadol in hair using solid phase extraction and GC-MS", *Forensic Science International*, vol. 135, no. 2, pp. 129-36.

Hambidge, K., Walravens, P., Brown, R., Webster, J., White, S., Anthony, M. & Roth, M. 1976, "Zinc nutrition of preschool children in the Denver Head Start program", *American Journal of Clinical Nutrition*, vol. 29, no. 7, pp. 734.

Harkey, M.R. 1993, "Anatomy and physiology of hair", *Forensic Science International*, vol. 63, no. 1-3, pp. 9-18.

Hartmann, C., Smeyers-Verbeke, J., Massart, D. & McDowall, R. 1998, "Validation of bioanalytical chromatographic methods", *Journal of pharmaceutical and biomedical analysis,* vol. 17, no. 2, pp. 193-218.

Hegstad, S., Khiabani, H.Z., Kristoffersen, L., Kunøe, N., Lobmaier, P.P. & Christophersen, A.S. 2008, "Drug Screening of Hair by Liquid Chromatography-Tandem Mass Spectrometry", *Journal of Analytical Toxicology,* vol. 32, no. 5, pp. 364-372.

Heller, S., Hiemke, C., Stroba, G., Rieger-Gies, A., Daum-Kreysch, E., Sachse, J. & Härtter, S. 2004, "Assessment of storage and transport stability of new antidepressant and antipsychotic drugs for a nationwide TDM service", *Therapeutic drug monitoring,* vol. 26, no. 4, pp. 459-461.

Henderson, G.L. 1993, "Mechanisms of drug incorporation into hair", *Forensic Science International,* vol. 63, no. 1-3, pp. 19-29.

Heykants, J., Huang, M.L., Mannens, G., Meuldermans, W., Snoeck, E., Van Beijsterveldt, L., Van Peer, A. & Woestenborghs, R. 1994, "The pharmacokinetics of risperidone in humans: a summary", *The Journal of clinical psychiatry,* vol. 55, no. MAI, pp. 13-17.

Hiemke, C., Baumann, P., Bergemann, N., Conca, A., Dietmaier, O., Egberts, K., Fric, M., Gerlach, M., Greiner, C. & Gründer, G. 2011, "AGNP consensus guidelines for therapeutic drug monitoring in psychiatry: update 2011", *Pharmacopsychiatry,* vol. 44, no. 6, pp. 195Y235.

Higuchi, S., Parrish, K.M., Dufour, M.C., Towle, L.H. & Harford, T.C. 2006, "Relationship Between Age and Drinking Patterns and Drinking Problems Among Japanese, Japanese-Americans, and Caucasians", *Alcoholism: Clinical and Experimental Research,* vol. 18, no. 2, pp. 305-310.

Ho, C., Lam, C., Chan, M., Cheung, R., Law, L., Lit, L., Ng, K., Suen, M. & Tai, H. 2003, "Electrospray ionisation mass spectrometry: principles and clinical applications", *The Clinical Biochemist Reviews,* vol. 24, no. 1, pp. 3.

Huang, M.L., Van Peer, A., Woestenborghs, R., De Coster, R., Heykants, J., Jansen, A.A.I., Zylicz, Z., Visscher, H.W. & Jonkman, J.H.G. 1993, "Pharmacokinetics of the novel antipsychotic agent risperidone and the prolactin response in healthy subjects", *Clinical Pharmacology & Therapeutics,* vol. 54, no. 3, pp. 257-268.

Hugenholtz, G.W.K., Heerdink, E.R., Stolker, J.J., Meijer, W.E.E., Egberts, A.C.G. & Nolen, W.A. 2006, "Haloperidol dose when used as active comparator in randomized controlled trials with atypical antipsychotics in schizophrenia: comparison with officially recommended doses", *Journal of Clinical Psychiatry,* vol. 67, no. 6, pp. 897-903.

ichrom.com 2011, , Dry Blood Spot Card Autosampler [Homepage of ©2011 iChrom Solutions], [Online]. Available: http://ichrom.com/dry-blood-spot-card-autosampler/ [2011, 9/5].

Jain, T., Bhandari, A., Ram, V., Sharma, S., Parakh, M. & Parakh, M.C. 2011, "Correlation of haloperidol levels between saliva and plasma of acutely ill schizophrenic patients", *Clinical biochemistry,* vol. 44, no. 8, pp. 675-680.

Janda, I. & Alt, A. 2001, "Improvement of ethyl glucuronide determination in human urine and serum samples by solid-phase extraction", *Journal of Chromatography B: Biomedical Sciences and Applications,* vol. 758, no. 2, pp. 229-234.

Jandera, P. 2011, "Stationary and mobile phases in hydrophilic interaction chromatography: a review", *Analytica Chimica Acta,* vol. 692, no. 1, pp. 1-25.

Jantos, R. & Skopp, G. 2011, "Comparison of drug analysis in whole blood and dried blood spots", *Toxichem Krimtech,* vol. 78, pp. 268-275.

Johnson, T. & Fendrich, M. 2005, "Modeling sources of self-report bias in a survey of drug use epidemiology", *Annals of Epidemiology,* vol. 15, no. 5, pp. 381-389.

Jones, A.W. & Holmgren, A. 2012, "What non-alcohol drugs are used by drinking drivers in Sweden? Toxicological results from ten years of forensic blood samples", *Journal of Safety Research,* .

Jones, A.W., Kugelberg, F.C., Holmgren, A. & Ahlner, J. 2008, "Occurrence of ethanol and other drugs in blood and urine specimens from female victims of alleged sexual assault", *Forensic science international,* vol. 181, no. 1-3, pp. 40-46.

Jones, A. & Karlsson, L. 2005, "Relation between bloodand urine-amphetamine concentrations in impaired drivers as influenced by urinary pH and creatinine", *Human & Experimental Toxicology,* vol. 24, no. 12, pp. 615.

Jopling, J., Henry, E., Wiedmeier, S.E. & Christensen, R.D. 2009, "Reference ranges for hematocrit and blood hemoglobin concentration during the neonatal period: data from a multihospital health care system", *Pediatrics,* vol. 123, no. 2, pp. e333.

Josefsson, M., Kronstrand, R., Andersson, J. & Roman, M. 2003, "Evaluation of electrospray ionisation liquid chromatography-tandem mass spectrometry for rational determination of a number of neuroleptics and their major metabolites in human body fluids and tissues", *Journal of Chromatography B,* vol. 789, no. 1, pp. 151-167.

Joseph, R.E., Su, T.P. & Cone, E.J. 1996, "In vitro binding studies of drugs to hair: influence of melanin and lipids on cocaine binding to Caucasoid and Africoid hair", *Journal of analytical toxicology,* vol. 20, no. 6, pp. 338-344.

Joseph, R.E., Tsai, W.J., Tsao, L.I., Su, T.P. & Cone, E.J. 1997, "In vitro characterization of cocaine binding sites in human hair", *Journal of Pharmacology and Experimental Therapeutics,* vol. 282, no. 3, pp. 1228-1241.

Jurado, C., Soriano, T., Gimenez, M.P. & Menendez, M. 2004, "Diagnosis of chronic alcohol consumption. Hair analysis for ethyl-glucuronide", *Forensic Science International*, vol. 145, no. 2-3, pp. 161-6.

Kamogawa, M.Y., Nogueira, A.R.A., Costa, L.M., Garcia, E.E. & Nóbrega, J.A. 2001, "A new strategy for preparation of hair slurries using cryogenic grinding and water-soluble tertiary-amines medium", *Spectrochimica Acta Part B: Atomic Spectroscopy*, vol. 56, no. 10, pp. 1973-1980.

Karolia, S. 2011, March 2011-last update, *Dried Blood Spot Analysis Automation* [Homepage of SCAP], [Online]. Available: http://www.flickr.com/photos/55796561@N07/5508560139/in/photostream/ [2011, 9/5].

Kauert, G. & Rohrich, J. 1996, "Concentrations of delta 9-tetrahydrocannabinol, cocaine and 6-monoacetylmorphine in hair of drug abusers", *International Journal of Legal Medicine*, vol. 108, no. 6, pp. 294-9.

Kaufmann, A., Butcher, P., Maden, K., Walker, S. & Widmer, M. 2011, "Quantitative and confirmative performance of liquid chromatography coupled to high-resolution mass spectrometry compared to tandem mass spectrometry", *Rapid Communications in Mass Spectrometry*, vol. 25, no. 7, pp. 979-992.

Kayiran, S., Özbek, N., Turan, M. & Gürakan, B. 2003, "Significant differences between capillary and venous complete blood counts in the neonatal period", *Clinical & Laboratory Haematology*, vol. 25, no. 1, pp. 9-16.

Kempson, I.M. & Lombi, E. 2011, "Hair analysis as a biomonitor for toxicology, disease and health status", *Chem.Soc.Rev.*, vol. 40, no. 7, pp. 3915-3940.

Kharbouche, H., Faouzi, M., Sanchez, N., Daeppen, J.B., Augsburger, M., Mangin, P., Staub, C. & Sporkert, F. 2011, "Diagnostic performance of ethyl glucuronide in hair for the investigation of alcohol drinking behavior: a comparison with traditional biomarkers", *International journal of legal medicine*, , pp. 1-8.

Kharbouche, H., Steiner, N., Morelato, M., Staub, C., Boutrel, B., Mangin, P., Sporkert, F. & Augsburger, M. 2010, "Influence of ethanol dose and pigmentation on the incorporation of ethyl glucuronide into rat hair", *Alcohol*, vol. 44, no. 6, pp. 507-514.

Kharbouche, H., Sporkert, F., Troxler, S., Augsburger, M., Mangin, P. & Staub, C. 2009, "Development and validation of a gas chromatography–negative chemical ionization tandem mass spectrometry method for the determination of ethyl glucuronide in hair and its application to forensic toxicology", *Journal of Chromatography B*, vol. 877, no. 23, pp. 2337-2343.

Khazaal, Y., Tapparel, S., Chatton, A., Rothen, S., Preisig, M. & Zullino, D. 2007, "Quetiapine dosage in bipolar disorder episodes and mixed states", *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, vol. 31, no. 3, pp. 727-730.

Kidwell, D.A., Lee, E.H. & DeLauder, S.F. 2000, "Evidence for bias in hair testing and procedures to correct bias", *Forensic science international,* vol. 107, no. 1, pp. 39-61.

Kim, Y.B., Cheon, K.C., Hur, G.H., Phi, T.S., Choi, S.J., Hong, D. & Kang, J.K. 2002, "Effects of combinational prophylactics composed of physostigmine and procyclidine on soman-induced lethality, seizures and brain injuries", *Environmental toxicology and pharmacology,* vol. 11, no. 1, pp. 15-21.

Kintz, P. 2010, "Consensus of the Society of Hair Testing on hair testing for chronic excessive alcohol consumption 2009", *Forensic science international,* vol. 196, no. 1, pp. 2-2.

Kintz, P. 2004, "Value of hair analysis in postmortem toxicology", *Forensic science international,* vol. 142, no. 2-3, pp. 127-134.

Kintz, P., Villain, M., Vallet, E., Etter, M., Salquebre, G. & Cirimele, V. 2008, "Ethyl glucuronide: unusual distribution between head hair and pubic hair", *Forensic science international,* vol. 176, no. 1, pp. 87-90.

Kintz, P. 2007a, *Analytical And Practical Aspects of Drug Testing in Hair,* CRC Press.

Kintz, P. 2007b, "Bioanalytical procedures for detection of chemical agents in hair in the case of drug-facilitated crimes", *Anal Bioanal Chem,* vol. 388, no. 7, pp. 1467-74.

Kintz, P., Bundeli, P., Brenneisen, R. & Ludes, B. 1998, "Dose-concentration relationships in hair from subjects in a controlled heroin-maintenance program", *Journal of Analytical Toxicology,* vol. 22, no. 3, pp. 231-236.

Kintz, P. & Cirimele, V. 1997, "Interlaboratory comparison of quantitative determination of amphetamine and related compounds in hair samples", *Forensic Science International,* vol. 84, no. 1-3, pp. 151-156.

Kintz, P. & Mangin, P. 1995, "Simultaneous determination of opiates, cocaine and major metabolites of cocaine in human hair by gas chromotography/mass spectrometry (GC/MS)", *Forensic Science International,* vol. 73, no. 2, pp. 93-100.

Kintz, P., Villain, M., Vallet, E., Etter, M., Salquebre, G. & Cirimele, V. 2008, "Ethyl glucuronide: unusual distribution between head hair and pubic hair", *Forensic Science International,* vol. 176, no. 1, pp. 87-90.

Kissack, J.C., Bishop, J. & Roper, A.L. 2008, "Ethylglucuronide as a biomarker for ethanol detection", *Pharmacotherapy,* vol. 28, no. 6, pp. 769-781.

Koide, I., Noguchi, O., Okada, K., Yokoyama, A., Oda, H., Yamamoto, S. & Kataoka, H. 1998, "Determination of amphetamine and methamphetamine in human hair by headspace solid-phase microextraction and gas chromatography with nitrogen–phosphorus detection", *Journal of Chromatography B: Biomedical Sciences and Applications,* vol. 707, no. 1-2, pp. 99-104.

Koup, J.R., Lau, A.H., Brodsky, B. & Slaughter, R.L. 1979, "Relationship between serum and saliva chloramphenicol concentrations", *Antimicrobial Agents and Chemotherapy*, vol. 15, no. 5, pp. 658.

Krause, K. & Foitzik, K. 2006, "Biology of the hair follicle: the basics", *Seminars in Cutaneous Medicine and Surgery*, vol. 25, no. 1, pp. 2-10.

Kronstrand, R., Brinkhagen, L. & Nyström, F.H. 2011a, "Ethyl glucuronide in human hair after daily consumption of 16 or 32 g of ethanol for 3 months", *Forensic science international*, .

Kronstrand, R., Brinkhagen, L. & Nyström, F.H. 2011b, "Ethyl glucuronide in human hair after daily consumption of 16 or 32 g of ethanol for 3 months", *Forensic science international*, .

Kronstrand, R., Nyström, I., Strandberg, J. & Druid, H. 2004, "Screening for drugs of abuse in hair with ion spray LC-MS-MS", *Forensic Science International*, vol. 145, no. 2-3, pp. 183-190.

La Marca, G., Malvagia, S., Filippi, L., Luceri, F., Moneti, G. & Guerrini, R. 2009, "A new rapid micromethod for the assay of phenobarbital from dried blood spots by LC-tandem mass spectrometry", *Epilepsia*, vol. 50, no. 12, pp. 2658-2662.

Laloup, M., Ramirez Fernandez Mdel, M., De Boeck, G., Wood, M., Maes, V. & Samyn, N. 2005, "Validation of a liquid chromatography-tandem mass spectrometry method for the simultaneous determination of 26 benzodiazepines and metabolites, zolpidem and zopiclone, in blood, urine, and hair", *Journal of Analytical Toxicology*, vol. 29, no. 7, pp. 616-26.

Lamoureux, F., Gaulier, J., Sauvage, F.L., Mercerolle, M., Vallejo, C. & Lachâtre, G. 2009, "Determination of ethyl-glucuronide in hair for heavy drinking detection using liquid chromatography-tandem mass spectrometry following solid-phase extraction", *Analytical and Bioanalytical Chemistry*, vol. 394, no. 7, pp. 1895-1901.

Ledgerwood, D.M., Goldberger, B.A., Risk, N.K., Lewis, C.E. & Price, R.K. 2008, "Comparison between self-report and hair analysis of illicit drug use in a community sample of middle-age men", *Addictive Behaviors*, vol. 33, no. 9, pp. 1131.

Lees, R., Kingston, R., Williams, T.M., Henderson, G., Lingford-Hughes, A. & Hickman, M. 2012, "Comparison of ethyl glucuronide in hair with self-reported alcohol consumption", *Alcohol and Alcoholism*, vol. 47, no. 3, pp. 267-272.

Lehman, A.F. & Steinwachs, D.M. 1998, "Translating research into practice: the Schizophrenia Patient Outcomes Research Team (PORT) treatment recommendations", *Schizophrenia bulletin*, vol. 24, no. 1, pp. 1.

Leon, J., Wynn, G. & Sandson, N.B. 2010, "The pharmacokinetics of paliperidone versus risperidone", *Psychosomatics*, vol. 51, no. 1, pp. 80.

Levy, G., Procknal, J.A., Olufs, R. & Pachman, L.M. 1980, "Relationship between saliva salicylate concentration and free or total salicylate concentration in serum of children with juvenile rheumatoid arthritis", *Clinical Pharmacology and Therapeutics,* vol. 27, no. 5, pp. 619-627.

Li, W. & Tse, F.L.S. 2010, "Dried blood spot sampling in combination with LC-MS/MS for quantitative analysis of small molecules", *Biomedical Chromatography,* vol. 24, no. 1, pp. 49-65.

Liniger, B., Nguyen, A., Friedrich-Koch, A. & Yegles, M. 2010, "Abstinence monitoring of suspected drinking drivers: ethyl glucuronide in hair versus CDT", *Traffic Injury Prevention,* vol. 11, no. 2, pp. 123-126.

Litten, R.Z., Bradley, A.M. & Moss, H.B. 2010, "Alcohol biomarkers in applied settings: recent advances and future research opportunities", *Alcoholism: Clinical and Experimental Research,* vol. 34, no. 6, pp. 955-967.

Lostia, A.M., Vicente, J.L. & Cowan, D.A. 2013, "Measurement of Ethyl Glucuronide, Ethyl Sulphate and Their Ratio in the Urine and Serum of Healthy Volunteers after Two Doses of Alcohol", *Alcohol and Alcoholism,* vol. 48, no. 1, pp. 74-82.

Lucas, A., Bermejo, A., Tabernero, M., Fernandez, P. & Strano-Rossi, S. 2000, "Use of solid-phase microextraction (SPME) for the determination of methadone and EDDP in human hair by GC-MS", *Forensic Science International,* vol. 107, no. 1-3, pp. 225-232.

Mandrioli, R., Mercolini, L., Lateana, D., Boncompagni, G. & Raggi, M.A. 2011, "Analysis of risperidone and 9-hydroxyrisperidone in human plasma, urine and saliva by MEPS-LC-UV", *Journal of Chromatography B,* vol. 879, no. 2, pp. 167-173.

Marsh, A., Carruthers, M.E., Desouza, N. & Evans, M.B. 1992, "An investigation of the effect of washing upon the morphine content of hair measured by a radioimmunoassay technique", *Journal of Pharmaceutical and Biomedical Analysis,* vol. 10, no. 1, pp. 89-93.

Martins Ferreira, L., Binz, T. & Yegles, M. 2011, "The influence of ethanol containing cosmetics on ethyl glucuronide concentration in hair", *Forensic science international,* .

Matsuno, H., Uematsu, T. & Nakashima, M. 1990, "The measurement of haloperidol and reduced haloperidol in hair as an index of dosage history", *British Journal of Clinical Pharmacology,* vol. 29, no. 2, pp. 187-94.

Matuszewski, B.K., Constanzer, M.L. & Chavez-Eng, C.M. 2003, "Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS", *Analytical Chemistry,* vol. 75, no. 13, pp. 3019-30.

Mehta, D. & Royal Pharmaceutical Society of Great Britain 2000, *British national formulary,* British Medical Association.

Mei, J.V., Alexander, J.R., Adam, B.W. & Hannon, W.H. 2001, "Use of filter paper for the collection and analysis of human whole blood specimens", *The Journal of nutrition*, vol. 131, no. 5, pp. 1631S.

Michael, C., Bierbach, U., Frenzel, K., Lange, T., Basara, N., Niederwieser, D., Mauz-Körholz, C. & Preiss, R. 2010, "Determination of saliva trough levels for monitoring voriconazole therapy in immunocompromised children and adults", *Therapeutic Drug Monitoring*, vol. 32, no. 2, pp. 194.

Mieczkowski, T., Tsatsakis, A.M., Kruger, M. & Psillakis, T. 2001, "The concentration of three anti-seizure medications in hair: the effects of hair color, controlling for dose and age", *BMC clinical pharmacology*, vol. 1, pp. 2.

Miller, E.I., Wylie, F.M. & Oliver, J.S. 2008, "Simultaneous Detection and Quantification of Amphetamines, Diazepam and its Metabolites, Cocaine and its Metabolites, and Opiates in Hair by LC-ESI-MS-MS Using a Single Extraction Method", *Journal of Analytical Toxicology*, vol. 32, no. 7, pp. 457-469.

Moeller, M.R., Fey, P. & Wennig, R. 1993, "Simultaneous determination of drugs of abuse (opiates, cocaine and amphetamine) in human hair by GC/MS and its application to a methadone treatment program", *Forensic Science International*, vol. 63, no. 1-3, pp. 185-206.

Montagna, W. & Ellis, R. 1959, "The biology of hair growth", *The American Journal of the Medical Sciences*, vol. 237, no. 5, pp. 670.

Moore, C., Rana, S., Coulter, C., Feyerherm, F. & Prest, H. 2006, "Application of two-dimensional gas chromatography with electron capture chemical ionization mass spectrometry to the detection of 11-nor-Delta9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH) in hair", *Journal of Analytical Toxicology*, vol. 30, no. 3, pp. 171-7.

Morini, L., Politi, L., Groppi, A., Stramesi, C. & Polettini, A. 2006, "Determination of ethyl glucuronide in hair samples by liquid chromatography/electrospray tandem mass spectrometry", *Journal of mass spectrometry*, vol. 41, no. 1, pp. 34-42.

Morita, M. & Folkers, K. 1993, "A New Method to Determine the Level of Coenzyme Q< sub> 10</sub> in One Drop of Human Blood for Biomedical Research", *Biochemical and biophysical research communications*, vol. 191, no. 3, pp. 950-954.

Moulard, Y., Bailly-Chouriberry, L., Boyer, S., Garcia, P., Popot, M.A. & Bonnaire, Y. 2011, "Use of benchtop exactive high resolution and high mass accuracy orbitrap mass spectrometer for screening in horse doping control", *Analytica Chimica Acta*, .

Musshoff, F., Lachenmeier, K., Wollersen, H., Lichtermann, D. & Madea, B. 2005, "Opiate concentrations in hair from subjects in a controlled heroin-maintenance program and from opiate-associated fatalities", *Journal of analytical toxicology*, vol. 29, no. 5, pp. 345-352.

Musshoff, F., Lachenmeier, K., Lichtermann, D. & Madea, B. 2009, "Cocaine and opiate concentrations in hair from subjects in a heroin maintenance program in comparison to a methadone substituted group", *International Journal of Legal Medicine,* vol. 123, no. 5, pp. 363-9.

Musshoff, F. & Madea, B. 2007a, "Analytical pitfalls in hair testing", *Anal Bioanal Chem,* vol. 388, no. 7, pp. 1475-94.

Musshoff, F. & Madea, B. 2007b, "New trends in hair analysis and scientific demands on validation and technical notes", *Forensic Science International,* vol. 165, no. 2-3, pp. 204-15.

Nakahara, Y. 1999, "Hair analysis for abused and therapeutic drugs", *Journal of Chromatography B: Biomedical Sciences and Applications,* vol. 733, no. 1-2, pp. 161-180.

Nakahara, Y. & Kikura, R. 1996, "Hair analysis for drugs of abuse XIII. Effect of structural factors on incorporation of drugs into hair: the incorporation rates of amphetamine analogs", *Archives of Toxicology,* vol. 70, no. 12, pp. 841-849.

Nakahara, Y., Takahashi, K. & Kikura, R. 1995, "Hair analysis for drugs of abuse. X. Effect of physicochemical properties of drugs on the incorporation rates into hair", *Biological and Pharmaceutical Bulletin,* vol. 18, no. 9, pp. 1223-7.

Nakamura, S., Tomita, M., Wada, M., Chung, H., Kuroda, N. & Nakashima, K. 2006, "Quantification of MDMA and MDA in abusers' hair samples by semi-micro column HPLC with fluorescence detection", *Biomedical Chromatography,* vol. 20, no. 6-7, pp. 622.

Nielsen, M.K.K., Johansen, S.S., Dalsgaard, P.W. & Linnet, K. 2010, "Simultaneous screening and quantification of 52 common pharmaceuticals and drugs of abuse in hair using UPLC-TOF-MS", *Forensic science international,* vol. 196, no. 1-3, pp. 85-92.

Offidani, C., Strano Rossi, S. & Chiarotti, M. 1993, "Improved enzymatic hydrolysis of hair", *Forensic Science International,* vol. 63, no. 1-3, pp. 171-174.

Okamoto, K., Morita, M., Quan, H., Uehiro, T. & Fuwa, K. 1985, "Preparation and certification of human hair powder reference material", *Clinical Chemistry,* vol. 31, no. 10, pp. 1592-1597.

Oliveira, I.R., Sena, E.P., Pereira, E., Miranda, A., Oliveira, N.F., Ribeiro, M., Castro-e-Silva, E., Dardennes, R., Samuel-Lajeunesse, B. & Marcilio, C. 1996, "Haloperidol blood levels and clinical outcome: a meta-analysis of studies relevant to testing the therapeutic window hypothesis", *Journal of clinical pharmacy and therapeutics,* vol. 21, no. 4, pp. 229-236.

Omar, M.M., Al-Mulla, K.F., Al-Seleem, T.A., Murad, B. & Radovanovic, Z. 2005, "Middle east aircrew use of alcohol, tobacco, coffee, and medicaments", *Aviation, Space, and Environmental Medicine*, vol. 76, no. 4, pp. 395-398.

Pascal, K. 2011, "Consensus of the Society of Hair Testing on hair testing for chronic excessive alcohol consumption 2011", *Forensic science international*, , no. 0.

Paterson, S., Cordero, R., McPhillips, M. & Carman, S. 2003, "Interindividual dose/concentration relationship for methadone in hair", *Journal of Analytical Toxicology*, vol. 27.

Paulsen, R.B., Wilkins, D.G., Slawson, M.H., Shaw, K. & Rollins, D.E. 2001, "Effect of four laboratory decontamination procedures on the quantitative determination of cocaine and metabolites in hair by HPLC-MS", *Journal of Analytical Toxicology*, vol. 25, no. 7, pp. 490-6.

Pecoraro, V. & Astore, I. 1990, "Measurements of hair growth under physiological conditions", *Hair and Hair Diseases*, , pp. 237-254.

Pelander, A., Ristimaa, J., Rasanen, I., Vuori, E. & Ojanperä, I. 2008, "Screening for basic drugs in hair of drug addicts by liquid chromatography/time-of-flight mass spectrometry", *Therapeutic drug monitoring*, vol. 30, no. 6, pp. 717.

Peters, F.T., Drummer, O.H. & Musshoff, F. 2007, "Validation of new methods", *Forensic science international*, vol. 165, no. 2-3, pp. 216-224.

Peters, F. 2007, "Stability of analytes in biosamples—an important issue in clinical and forensic toxicology?", *Analytical and Bioanalytical Chemistry*, vol. 388, no. 7, pp. 1505-1519.

Phoenixbio 2008, , *Hair Regeneration Project* [Homepage of PhoenixBio], [Online].

Pierre, J.M., Shnayder, I., Wirshing, D.A. & Wirshing, W.C. 2004, "Intranasal quetiapine abuse", *American Journal of Psychiatry*, vol. 161, no. 9, pp. 1718.

Pötsch, L., Skopp, G. & Moeller, M. 1997, "Influence of pigmentation on the codeine content of hair fibers in guinea pigs", *Journal of forensic sciences*, vol. 42, pp. 1095-1098.

Potsch, L. 1996, "A discourse on human hair fibers and reflections on the conservation of drug molecules", *International Journal of Legal Medicine*, vol. 108, no. 6, pp. 285-93.

Pragst, F. & Balikova, M.A. 2006, "State of the art in hair analysis for detection of drug and alcohol abuse", *Clinica Chimica Acta*, vol. 370, no. 1-2, pp. 17-49.

Pragst, F., Rothe, M., Hunger, J. & Thor, S. 1997, "Structural and concentration effects on the deposition of tricyclic antidepressants in human hair", *Forensic Science International*, vol. 84, no. 1-3, pp. 225-236.

Pragst, F., Rothe, M., Spiegel, K. & Sporkert, F. 1998, "Illegal and therapeutic drug concentrations in hair segments-- A timetable of drug exposure?", *Forensic Science Review,* vol. 10, no. 2, pp. 81-111.

Pragst, F. & Yegles, M. 2006, "14 Alcohol Markers in Hair", *Analytical And Practical Aspects of Drug Testing in Hair,* , pp. 287.

Prota, G. 2000, "Melanins, melanogenesis and melanocytes: looking at their functional significance from the chemist's viewpoint", *Pigment Cell Research,* vol. 13, no. 4, pp. 283-93.

Raaflaub, J. 1975, "On the pharmacokinetics of chlorprothixene in man", *Cellular and Molecular Life Sciences,* vol. 31, no. 5, pp. 557-558.

Radovanovic, Z., Pilcher, C.W.T., Al-Nakib, T. & Shihab-Eldeen, A. 2001, "On substance abuse in Kuwait (1992–1997): Evidence from toxicological screening of patients", *Journal of substance abuse,* vol. 12, no. 4, pp. 363-371.

Ray, W.A., Chung, C.P., Murray, K.T., Hall, K. & Stein, C.M. 2009, "Atypical antipsychotic drugs and the risk of sudden cardiac death", *New England Journal of Medicine,* vol. 360, no. 3, pp. 225-235.

Rees, K.A., Jones, N.S., McLaughlin, P.A. & Osselton, M.D. 2011, "The effect of sodium fluoride preservative and storage temperature on the stability of 6-acetylmorphine in horse blood, sheep vitreous and deer muscle", *Forensic science international,* .

Rehm, J., Gmel, G., Sempos, C.T. & Trevisan, M. 2003, "Alcohol-related morbidity and mortality", *Alcohol Research and Health,* vol. 27, no. 1, pp. 39-51.

Robbins, C.R. 2002, *Chemical and Physical Behavior of Human Hair,* Springer.

Romolo, F.S., Rotolo, M.C., Palmi, I., Pacifici, R. & Lopez, A. 2003, "Optimized conditions for simultaneous determination of opiates, cocaine and benzoylecgonine in hair samples by GC–MS", *Forensic Science International,* vol. 138, no. 1-3, pp. 17-26.

Ropero-Miller, J.D., Goldberger, B.A., Cone, E.J. & Joseph Jr, R.E. 2000, "The disposition of cocaine and opiate analytes in hair and fingernails of humans following cocaine and codeine administration", *Journal of Analytical Toxicology,* vol. 24, no. 7, pp. 496-508.

Saar, E., Gerostamoulos, D., Drummer, O.H. & Beyer, J. 2012, "Assessment of the stability of 30 antipsychotic drugs in stored blood specimens", *Forensic science international,* vol. 215, no. 1, pp. 152-158.

Saracino, M.A., de Palma, A., Boncompagni, G. & Raggi, M.A. 2010, "Analysis of risperidone and its metabolite in plasma and saliva by LC with coulometric detection and a novel MEPS procedure", *Talanta,* vol. 81, no. 4, pp. 1547-1553.

Sato, R., Uematsu, T., Yamaguchi, S. & Nakashima, M. 1989, "Human scalp hair as evidence of individual dosage history of haloperidol: Prospective study", *Therapeutic Drug Monitoring*, vol. 11, no. 6, pp. 686.

Scheidweiler, K.B., Cone, E.J., Moolchan, E.T. & Huestis, M.A. 2005, "Dose-related distribution of codeine, cocaine, and metabolites into human hair following controlled oral codeine and subcutaneous cocaine administration", *Journal of Pharmacology and Experimental Therapeutics*, vol. 313, no. 2, pp. 909-915.

Schneider, S., Sibille, E., Yegles, M., Neels, H., Wennig, R. & Mühe, A. 2009, "Time resolved analysis of risperidone and 9-hydroxy-risperidone in hair using LC/MS-MS", *Journal of Chromatography B*, vol. 877, no. 24, pp. 2589-2592.

Schramm, K.W. 2008, "Hair-biomonitoring of organic pollutants", *Chemosphere*, vol. 72, no. 8, pp. 1103-11.

Schulz, M., Iwersen-Bergmann, S., Andresen, H. & Schmoldt, A. 2012, "Therapeutic and toxic blood concentrations of nearly 1,000 drugs and other xenobiotics", *Critical Care*, vol. 16, no. 4, pp. R136.

Schulz, M. & Schmoldt, A. 2003, "Therapeutic and toxic blood concentrations of more than 800 drugs and other xenobiotics", *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, vol. 58, no. 7, pp. 447-474.

Scordo, M.G., Spina, E., Facciolà, G., Avenoso, A., Johansson, I. & Dahl, M.L. 1999, "Cytochrome P450 2D6 genotype and steady state plasma levels of risperidone and 9-hydroxyrisperidone", *Psychopharmacology*, vol. 147, no. 3, pp. 300-305.

Seno, H., Kumazawa, T., Ishii, A., Sato, K., Suzuki, O., 妹尾洋, 熊澤武志, 石井晃, 佐藤啓造 & 鈴木修 1993, "Determination of some butyrophenones in body fluids by gas chromatography with surface ionization detection", .

Shah, V., Midha, K., Findlay, J., Hill, H., Hulse, J., McGilveray, I., McKay, G., Miller, K., Patnaik, R. & Powell, M. 2000, "Bioanalytical method validation—a revisit with a decade of progress", *Pharmaceutical Research*, vol. 17, no. 12, pp. 1551-1557.

Shen, M., Xiang, P., Wu, H., Shen, B. & Huang, Z. 2002a, "Detection of antidepressant and antipsychotic drugs in human hair", *Forensic science international*, vol. 126, no. 2, pp. 153-161.

Shen, M., Xiang, P., Wu, H., Shen, B. & Huang, Z. 2002b, "Detection of antidepressant and antipsychotic drugs in human hair", *Forensic science international*, vol. 126, no. 2, pp. 153-161.

Skopp, G., Schmitt, G., Pötsch, L., Drönner, P., Aderjan, R. & Mattern, R. 2000, "Ethyl glucuronide in human hair", *Alcohol and Alcoholism*, vol. 35, no. 3, pp. 283.

Skopp, G., Potsch, L. & Mauden, M. 2000, "Stability of cannabinoids in hair samples exposed to sunlight", *Clinical Chemistry*, vol. 46, no. 11, pp. 1846-8.

Slominski, A., Wortsman, J., Plonka, P.M., Schallreuter, K.U., Paus, R. & Tobin, D.J. 2005, "Hair follicle pigmentation", *Journal of Investigative Dermatology*, vol. 124, no. 1, pp. 13-21.

Smith, H., Forshufvud, S. & Wassen, A. 1962, "Distribution of arsenic in Napoleon's hair", *Nature*, vol. 194, no. 4830, pp. 725–726.

Smolders, R., Schramm, K.W., Nickmilder, M. & Schoeters, G. 2009, "Applicability of non-invasively collected matrices for human biomonitoring", *Environmental Health*, vol. 8, no. 1, pp. 8.

SoHT 2004, "Recommendations for hair testing in forensic cases", *Forensic Science International*, vol. 145, no. 2-3, pp. 83-4.

Sorenson, J., Melby, E., Nord, P. & Petering, H. 1973, "Interferences in the determination of metallic elements in human hair. An evaluation of zinc, copper, lead, and cadmium, using atomic absorption spectrophotometry", *Archives of Environmental Health*, vol. 27, no. 1, pp. 36.

Spooner, N., Lad, R. & Barfield, M. 2009, "Dried blood spots as a sample collection technique for the determination of pharmacokinetics in clinical studies: considerations for the validation of a quantitative bioanalytical method", *Analytical Chemistry*, vol. 81, no. 4, pp. 1557-1563.

Stanaszek, R. & Piekoszewski, W. 2004, "Simultaneous determination of eight underivatized amphetamines in hair by high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry (HPLC-APCI-MS)", *Journal of analytical toxicology*, vol. 28, no. 2, pp. 77-85.

Svec, F. 2006, "Less common applications of monoliths: Preconcentration and solid-phase extraction", *Journal of Chromatography B*, vol. 841, no. 1-2, pp. 52-64.

Tarcomnicu, I., Van Nuijs, A.L.N., Aerts, K., De Doncker, M., Covaci, A. & Neels, H. 2010, "Ethyl glucuronide determination in meconium and hair by hydrophilic interaction liquid chromatography-tandem mass spectrometry", *Forensic science international*, vol. 196, no. 1-3, pp. 121-127.

Tewthanom, K., Thongnopnua, P., Pitragool, W., Lukanajuntachod, P., Koikul, J., Jainark, P., Hongdul, P. & Chesho, Y. 2005, "Relationship Between Serum And Saliva Theophylline Level In Thai Patients With Asthma Or Copd: A Preliminary Study: 177", *Therapeutic Drug Monitoring*, vol. 27, no. 2, pp. 256.

Thurman, E.M. & Mills, M.S. 1998, *Solid-phase extraction: principles and practice*, Wiley New York.

Thurman, E. & Snavely, K. 2000, "Advances in solid-phase extraction disks for environmental chemistry", *TrAC Trends in Analytical Chemistry*, vol. 19, no. 1, pp. 18-26.

Tsanaclis, L. & Wicks, J.F. 2008, "Differentiation between drug use and environmental contamination when testing for drugs in hair", *Forensic Science International*, vol. 176, no. 1, pp. 19-22.

Tsanaclis, L. & Wicks, J.F.C. 2007, "Patterns in drug use in the United Kingdom as revealed through analysis of hair in a large population sample", *Forensic Science International*, vol. 170, no. 2-3, pp. 121-128.

Uhl, M. 2006, "5 Determination of Cannabinoids in Human Hair", *Analytical And Practical Aspects of Drug Testing in Hair*, , pp. 127.

Uhl, M. & Sachs, H. 2004, "Cannabinoids in hair: strategy to prove marijuana/hashish consumption", *Forensic Science International*, vol. 145, no. 2-3, pp. 143-7.

Uhl, M. & Scheufler, F. 2005, "Effects of hair color on the drug incorporation into human hair", , pp. 279–284.

Van Bocxlaer, J., Clauwaert, K., Lambert, W., Deforce, D., Van den Eeckhout, E. & De Leenheer, A. 2000, "Liquid chromatography—mass spectrometry in forensic toxicology", *Mass Spectrometry Reviews*, vol. 19, no. 4, pp. 165-214.

Van Neste, D. & Tobin, D.J. 2004, "Hair cycle and hair pigmentation: dynamic interactions and changes associated with aging", *Micron*, vol. 35, no. 3, pp. 193-200.

Villain, M. 2004, "Determination of bromazepam, clonazepam and metabolites after a single intake in urine and hair by LC-MS/MS:: Application to forensic cases of drug facilitated crimes", *Forensic science international*, vol. 145, no. 2-3, pp. 123-130.

Villain, M., Concheiro, M., V, C. & Kintz, P. 2005, "Screening method for benzodiazepines and hypnotics in hair at pg/mg level by liquid chromatography-mass spectrometry/mass spectrometry", *Journal of Chromatography B*, vol. 825, no. 1, pp. 72-78.

Villain, M. & Kintz, P. 2006, "Applications of hair in Drug-Facilitated crime evidence", *Analytical And Practical Aspects of Drug Testing in Hair*, , pp. 255.

Villamor, J., Bermejo, A., Fernandez, P. & Tabernero, M. 2005, "TECHNICAL NOTE: A New GC-MS Method for the Determination of Five Amphetamines in Human Hair", *Journal of analytical toxicology*, vol. 29, no. 2, pp. 135-139.

Waterman, K.C. & Adami, R.C. 2005, "Accelerated aging: Prediction of chemical stability of pharmaceuticals", *International journal of pharmaceutics*, vol. 293, no. 1-2, pp. 101-125.

Whatman.com 2009, [Homepage of © Whatman Ltd 2007-2009], [Online]. Available: http://www.whatman.com/DMPK.aspx [2011, 9/5].

Whiteman, P., Fowle, A.S.E., Hamilton, M., Peck, A., Bye, A., Dean, K. & Webster, A. 1985, "Pharmacokinetics and pharmacodynamics of procyclidine in man", *European journal of clinical pharmacology*, vol. 28, no. 1, pp. 73-78.

WHO 2011, 2011-last update, *Global status report on alcohol and health. World Health Organization, Geneva*. [Homepage of WHO], [Online] [2011, 10].

Wilhelm, A., den Burger, J., Vos, R., Chahbouni, A. & Sinjewel, A. 2009, "Analysis of cyclosporin A in dried blood spots using liquid chromatography tandem mass spectrometry", *Journal of Chromatography B*, vol. 877, no. 14-15, pp. 1595-1598.

Wilkins, D., Haughey, H., Cone, E., Huestis, M., Foltz, R. & Rollins, D. 1995, "Quantitative analysis of THC, 11-OH-THC, and THCCOOH in human hair by negative ion chemical ionization mass spectrometry", *Journal of Analytical Toxicology*, vol. 19, no. 6, pp. 483-91.

Winter, H.R., Earley, W.R., Hamer-Maansson, J.E., Davis, P.C. & Smith, M.A. 2008, "Steady-state pharmacokinetic, safety, and tolerability profiles of quetiapine, norquetiapine, and other quetiapine metabolites in pediatric and adult patients with psychotic disorders", *Journal of child and adolescent psychopharmacology*, vol. 18, no. 1, pp. 81-98.

Wishart, D.S., Knox, C., Guo, A.C., Cheng, D., Shrivastava, S., Tzur, D., Gautam, B. & Hassanali, M. 2008, "DrugBank: a knowledgebase for drugs, drug actions and drug targets", *Nucleic acids research*, vol. 36, no. suppl 1, pp. D901-D906.

Wurst, F.M., Skipper, G.E. & Weinmann, W. 2003, "Ethyl glucuronide—the direct ethanol metabolite on the threshold from science to routine use", *Addiction*, vol. 98, pp. 51-61.

Xiang, Q., Zhao, X., Zhou, Y., Duan, J.L. & Cui, Y.M. 2010, "Effect of CYP2D6, CYP3A5, and MDR1 genetic polymorphisms on the pharmacokinetics of risperidone and its active moiety", *The Journal of Clinical Pharmacology*, vol. 50, no. 6, pp. 659.

Yegles, M., Labarthe, A., Auwärter, V., Hartwig, S., Vater, H., Wennig, R. & Pragst, F. 2004, "Comparison of ethyl glucuronide and fatty acid ethyl ester concentrations in hair of alcoholics, social drinkers and teetotallers", *Forensic science international*, vol. 145, no. 2-3, pp. 167-173.

8. Appendix

8.1. Consent forms

Informed Consent

اقرار مستنير

عنوان البحث/ دراسة مستويات بعض ادوية الطب النفسي والادمان في عينات الشعر واللعاب مقارنة مع عينات الدم

اسم الباحث/ جبر الجبر

عزيزي/عزيزتي .....

لك كامل الحرية في الموافقة أو عدم الموافقة على المشاركة في البحث.

في حالة الموافقة يتعهد الباحثون بالموافقة على سرية المعلومات وعدم تداولها خارج إطار البحث.

وفي حالة الموافقة سيقوم الباحث عن طريق الطبيب المعالج بأخذ بعض البيانات من ملفك الطبي وأخذ عيينة دم مقدارها 3مل وعيينة لعاب مقدارها 1مل وعيينة شعر من المنطقة الخلفية للرأس وهذه العيينات سيتم تحليلها بمختبرات السموم في كلية الطب بجامعة كوين ماري بلندن. ولن يتم اجراء أي فحوصات وراثية أو أي تحاليل أخرى عليها إلا بعد الرجوع لك.

في حالة الموافقة يمكنك الإنسحاب من الدراسة بأي وقت.

وفي حالة عدم الموافقة لن يؤثر هذا على حقك في تلقي الرعاية المقررة لك.

يرجى التوقيع بالمكان المناسب:

☐ أوافق الإسم التوقيع

☐ لا أوافق الإسم التوقيع

Case #

Age: **Hair Colour:** **Sample Collection Date:**

Drug name	Strength	Dosage	Duration of use	Addiction history

KUWAIT UNIVERSTIY
COMMITTEE FOR THE PROTECTION OF
HUMAN SUBJECTS IN RESEARCH
HEALTH SCINCES CENTRE

CONSENT FORM

FACULTY OF …Medicine……
DEPARTMENT …Pharmacology..

Brief description about the project: (**Kindly simplify the language as it is for non medical individuals**)

The study is a comparison study of drug level between the alternative biological matrices (hair & saliva) and the traditional specimen (e.g. blood). Common advantages of the alternative samples include longer drug detection window (up to several months) as well as being noninvasive, but further assessment and investigation is required. In this research we will focus on the analysis of addictive drugs and antipsychotics taken by patients in the psychiatric hospital and the addiction treatment centre. Hair, saliva and blood samples will be taken from these patients to be analyzed.

I _____ agree giving a hair, saliva (1ml.) and blood sample (3 ml.) from myself for the performance of the below mentioned research, Name of patient _____

Research study entitled:
_Investigation of the level of antipsychotics and addictive drugs in hair and saliva in comparison to their blood level._____to be performed by or under the direction of Mr. ___Jaber Al Jaber_____and such assistants as may be chosen by him/her. (The nurse in duty will be asked to help in collecting the blood samples; no other medical staff is required)

1. The nature and purpose of the research have been fully explained to me. No guarantees or assurance have been given by anyone as to results that may be obtained.

2. I have had the opportunity to ask questions and they have been answered to my satisfaction.

3. I understand that I have the right to withdraw from the project at any time without affecting my case

_____ (or _____)
Patient Signature **Guardian Signature (in case of children)**

I have explained to the patient **(guardian or representative)** the nature of the above research. I believe the patient or representative understands.
___ Jaber Al Jaber _____

8.2. Ethics approval

STATE OF KUWAIT
MINISTRY OF HEALTH
Asst. Undersecretary for Public Health Affairs

دولـــة الكويـــت
وزارة الصحـــــة
الوكيل المساعد لشئون الصحة العامة

Reference : وع/ع/٦٨٤

Date : ١٠/٥/٢٠١٠

المرجع :

التاريخ :

السيد / مدير منطقة الصباح الطبية التخصصية المحترم

تحية طيبة وبعد،

الموضوع : تسهيل مهمة الباحث / جبر أحمد العبدالله الناصر الجبر
لإجراء دراسة بمستشفى الطب النفسي
تحت إشراف د. عادل أحمد الزايد
استشاري الطب النفسي
بحث رقم (427)

بالإشارة لبروتوكول البحث المقدم من الباحث / جبر أحمد العبدالله الناصر الجبر (معيد عضو بعثة بكلية الطب / جامعة الكويت) والمبعوث لدراسة الدكتوراه clinical pharmacology بجامعة كوين ماري بلندن / المملكة المتحدة تحت عنوان:

"Drug Analysis of Antipsychotics and addictives in Alternative Biological Samples "

وقد قام الباحث بتعديل البروتوكول والإقرار المستنير وفقا" لتوصيات اللجنة وأعاد تقديمه بتاريخ 2010/5/5 .

يرجى التكرم بالاحاطة بأن اللجنة الدائمة لتنسيق البحوث الطبية والصحية قد أوصت باجتماعها السابع (2010/7) المنعقد بتاريخ الأحد 2010/5/9 بالموافقة على إجراء البحث مع مراعاة تعهد الباحث بالمحافظة على حقوق المرضى بالخصوصية وسرية المعلومات وعدم تداولها خارج إطار البحث والحصول على الموافقة المستنيرة الحرة المسبقة من المشاركين بالبحث (Informed Consent) والتنسيق مع إدارة خدمات المختبرات الطبية بشأن إرسال العينات للخارج ويتم البحث تحت إشراف د. عادل الزايد استشاري الطب النفسي بمستشفى الطب النفسي على عينة قوامها حوالي 30 مريض ويتم البحث بجمع المعلومات المطلوبـــــة

P.O.Box : 33262 Rawda 73453 , Kuwait
Tel.: 24863581 - 24863722 - Fax: 24862535

ص. ب : ٣٣٢٦٢ الرمز البريدي ٧٣٤٥٣ الروضة ، الكويت
تلفون : ٢٤٨٦٣٥٨١ - ٢٤٨٦٣٧٢٢ - فاكس: ٢٤٨٦٢٥٣٥

STATE OF KUWAIT
MINISTRY OF HEALTH
Asst. Undersecretary for Public Health Affairs

دولـــة الكويـــت
وزارة الصحـــــة
الوكيل المساعد لشئون الصحة العامة

Reference : ..... المرجع : .....

Date : ..... التاريخ : .....

للدراسة عن طريق الطبيب المعالج وبعد الحصول على الموافقات اللازمة من المرضى وأخذ عينة دم مقدارها 3ml وعينة لعاب مقدارها 1-2ml لتقدير تركيز العقاقير بها ويتم إرسال العينات لمختبرات الجامعة بلندن لإجراء الفحوصات اللازمة عليها .

ولا يتضمن بروتوكول البحث إعطاء أي أدوية أو إجراء أي فحوصات أو تداخلات تشخيصية أو علاجية للمرضى .

لذلك يرجى التكرم بالموافقة على تسهيل مهمة الباحثين لإجراء البحث تحت إشراف د. عادل أحمد الزايد استشاري الطب النفسي بمستشفى الطب النفسي وفقا" للضوابط والإجراءات الموضوعة لذلك والإمكانيات المتاحة مع مراعاة التنسيق مع إدارة خدمات المختبرات الطبية بشأن إرسال العينات إلى خارج البلاد وعدم تحمل الوزارة لأي تكاليف إضافية .

وتفضلوا بقبول فائق الاحترام ،،،

رئيس اللجنة الدائمة لتنسيق
البحوث الطبية والصحية

د. يوسف عبد الرحمن النصف
وكيل وزارة الصحة المساعد
لشئون الصحة العامة

نسخة : للسيد / وكيل الوزارة المحترم
نسخة :للسيد مدير إدارة المختبرات الطبية المحترم
نسخة : للباحث المحترم

د. عبد اللطيف خالد السهلي
مدير منطقة الصباح الطبية التخصصية

الدكتور / عادل الزايد
رئيس قسم الطب النفسي

P.O.Box : 33262 Rawda 73453 - Kuwait
Tel.: 24863581 - 24863722 Fax: 24862535

ص. ب : ٣٣٢٦٢ الرمز البريدي ٧٣٤٥٣ الروضة ، الكويت
تلفون : ٢٤٨٦٣٥٨١ - ٢٤٨٦٣٧٢٢ - فاكس: ٢٤٨٦٢٥٣٥

State of Kuwait

Ministry of Health

Asst. Undersecretary for Public Health Affairs

Ref.: MPH/682

Date: 11/05/2010

Director, Al-Sabah Specialized Medical Zone

Dear Sir,

Subject: Facilitation of the mission of Jaber Ahmed Al-Abdullah Al-Nasser Al-Jaber to conduct a study at Psychiatry Hospital under supervision of Dr. Adel Ahmed Al-Zaid, Psychiatry Consultant

Research No. (427)

With reference to the research protocol submitted by the researcher Jaber Ahmed Al-Abdullah Al-Nasser Al-Jaber (lecturer and scholarship member in Faculty of Medicine, Kuwait University) who is delegated in scholarship for Ph.D. in Clinical Pharmacology at Queen Mary University in London, United Kingdom entitled:

"Drug Analysis of Antipsychotics and addictives in Alternative Biological Samples"

The research amended the protocol and informed acknowledgement in accordance with the committee recommendations and resubmitted the same on 05/05/2010.

Please be advised that Permanent Committee on Coordination of Medical & Health Research recommended in its 7th meeting (7/2010) held on Sunday, 09/05/2010 the approval to conduct the research subject to the researcher's undertaking to maintain the patients' rights to privacy, confidentiality of information, not to circulate the same beyond the scope of research framework, obtain prior informed consent from participants in the research, and coordinate with Medical Labs Services Department in connection with dispatching samples abroad. The research will be

conducted under supervision of Dr. Adel Al-Zaid, Psychiatry Consultant, Psychiatry Hospital, on sample of 30 patients. The research will be conducted by gathering the information required for the study through the attending doctor after obtaining necessary consents from patients and taking blood sample of 3 ml and saliva sample of 1 – 2 ml to assess drugs concentration therein. Samples will be sent to the University Labs in London to conduct necessary investigations thereon.

The research protocol doesn't include administering any medicines or conducting any diagnostic or treatment investigations or interventions for patients.

Therefore, you are kindly requested to approve facilitating the researcher's mission to conduct the research under supervision of Dr. Adel Al-Zaid, Psychiatry Consultant, Psychiatry Hospital, in accordance with rules and procedures established for this purpose and available facilities subject to coordination with Medical Labs Services Department in connection with dispatching samples abroad and the Ministry will not bear any additional costs.

Best regards,

Chairman, Permanent Committee on
Coordination of Medical & Health Research
Signed/ stamped

Cc: Undersecretary
Cc: Director, Medical Labs Services Department
Cc: the researcher

Ministry of justice
Administrative Affairs Dpt
Translation Section
(2)
The papers Translated by Burhan the officially accredited office at the Ministry of Information are certified without any liability concerning the accuracy of the translation or the document.
Head of Section:
Date 25-8-2011

BURHAN TRANSLATION

ابو الوفا ابراهيم محمد
مترجم أول

8.3. Copy of SoHT recommendations

Recommendations for Hair Testing in Forensic Cases

Society of Hair Testing

Introduction
On Tuesday October 7th 2003, representatives from 15 countries gathered in Heraklion, Crete, to discuss some issues involved in the analysis of hair for drugs of abuse. All representatives were either currently engaged in the analysis of hair samples in their own laboratories, or were conducting hair analysis through a third party laboratory facility.

The countries represented were as follows (in alphabetical order):
Canada, Chile, France, Germany, Greece, Italy, Luxembourg, Norway, Poland, Portugal, Spain, Sweden, Switzerland, United Kingdom, and the USA.

While the Consensus was discussed, there was no final result. The Board of the Society met, and agreed upon the wording of the Consensus in Sevilla, Spain, on January 24th 2004. They included the sections upon which the Assembly had agreed.

Areas for discussion

1. **Sampling, Shipping, Storage**
2. **Decontamination**
3. **Hair disintegration and extraction**
4. **Screening test**
5. **Criteria for mass spectrometric analysis**
6. **Specific Drug Classes**
   - **6.1. Opiates**
   - **6.2. Cocaine**
   - **6.3. Amphetamines**
   - **6.4. Cannabinoids**
7. **Internal Quality Control**
8. **External Quality Control**

1. ***Sampling, Shipping, Storage***
These areas were addressed in a previous Consensus from the Society of Hair Testing
Forensic Science International 84 (1997) 3-6

- The sample should be cut from the posterior vertex region of the head, as close as possible to the scalp, since this is the region of least variation in growth rate. If not, the source of the sampling should be described. In general, head hair is estimated to grow at approximately 1.0 cm per month..
- The sampling does not need to be performed by a physician
- The colour, length, body site and any obvious cosmetic treatment of the hair should be recorded
- Root (proximal) and tip (distal) sections of the hair should be clearly defined
- If segmental analysis is required, a lock of hair must be fixed before cutting
- Head hair is the preferred specimen. Alternative hair (e.g. pubic, axillary) can be collected if head hair is unavailable
- The sample and any aliquots or extracts must be handled and stored in a manner so as to minimize degradation, loss of analyte, or contamination from other sources. Dry hair should be stored in the dark at room temperature.
- There must be adequate sample to allow initial testing, followed by confirmatory or re-testing of the sample if necessary. A lock of hair, with the thickness of a pencil, or several locks with the thickness of a straw is recommended.
- In post-mortem cases, hair should be collected at the beginning of an autopsy

2. *Decontamination*

When hair analysis is being used to identify drug use, the major limitation is external contamination, which if not removed, can confuse exposure with actual drug use. The issue of external contamination must be addressed through multiple methodologies and cannot be solved through the simple application of any single approach.

- Areas of possible contamination must be considered before and during the analysis. These may include, but are not limited to, external drug exposure and laboratory contamination
- A simple use of cut-off levels is insufficient because external contamination can be at any level.
- In general, a decontamination strategy must include an initial organic solvent, to remove oils, followed by aqueous washes.
- The washings should be stored for later analysis, if necessary
- In autopsy or exhumation cases, additional pre-treatment of the hair in the laboratory may be necessary, depending on the condition of the sample

3. *Hair disintegration and extraction*

- Different analytical procedures can produce different quantitative results. Each laboratory has the choice of disintegrating the hair matrix before extraction, or of extracting the drug directly from the solid hair after suitable preparation
- Degradation compounds may be produced during the assay. In order to assess the degree of conversion, the laboratory must include adequate controls

4. *Screening Test*

- If a screening test is used, an appropriate method validation including calibrators and controls in a hair matrix must be performed
- Analytes of interest must be identified to minimize false negatives

5. *Criteria for mass spectrometric analysis*

- The method must be validated according to good laboratory practice.
- The possible influence of the internal standard at low concentrations must be assessed and documented
- For information on valid criteria for mass spectrometric analysis, refer to recommended rules from scientific organizations or national guidelines

6. *Specific Drug Classes*

Drugs and metabolites in hair should be analyzed using valid methods. Detection levels will be different between drug classes. Some examples for specific drugs are presented below.

6.1 Opiates

- *Immunochemical test*:
  - A morphine or 6-acetylmorphine level of 0.2 ng/mg must produce a positive result
- *Chromatographic test*:
  - Recommended Limit of Quantification (LOQ): ≤ 0.2 ng/mg for each compound
  - Heroin consumption must be differentiated from codeine or morphine use by the presence of 6-acetylmorphine

6.2 Cocaine

- *Immunochemical test*:
  - A cocaine level of 0.5 ng/mg must produce a positive result
- *Chromatographic test*:
  - Recommended Limit of Quantification (LOQ): ≤0.5 ng/mg for cocaine, ≤0.05 ng/mg for other compounds
  - The chromatographic analysis should include cocaine, and at least one of the following: benzoylecgonine, cocaethylene, norcocaine or ecgonine methyl ester

6.3 Amphetamines

- *Immunochemical test*:
  - A concentration of 0.2 ng/mg of each substance must separately produce a positive result: MDMA, methamphetamine, amphetamine, MDEA or MDA
- *Chromatographic test*:
  - Recommended Limit of Quantification (LOQ): ≤0.2 ng/mg for each compound

Note: Laboratories should be aware of the possible ingestion of legal drugs producing positive results for methamphetamine and amphetamine

6.4 Cannabinoids

- *Immunochemical test*:
  - A THC concentration of 0.1 ng/mg must produce a positive result
- *Chromatographic test*:
  - Recommended Limit of Quantification (LOQ):
  - THC:≤ 0.1 ng/mg
  - THC-COOH:≤ 0.2 pg/mg
  - Confirmation of THC-COOH is required to definitively prove the use of cannabinoids

7. Internal Quality Control

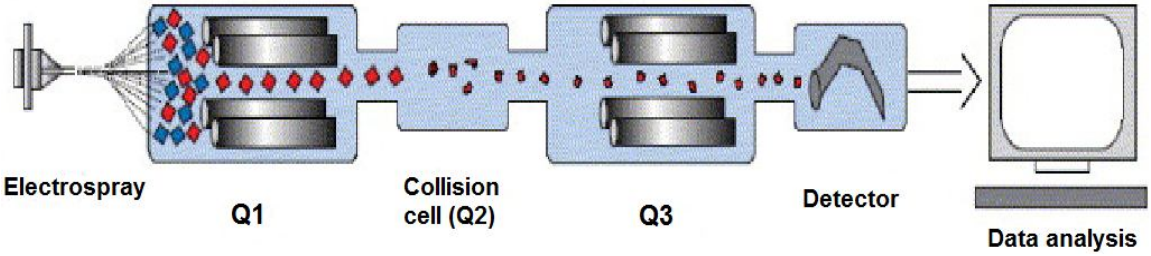
Internal Quality Control for hair is more difficult than for other homogenous body fluids, since spiked control samples cannot substitute for the actual hair of a drug user. However, spiked controls may be substituted for hair from drug users if properly prepared.

- One technique is to expose drug-free hair to aqueous solutions of drugs at high concentrations, for several days and then thoroughly wash the hair before drying and analysis. When suitably homogenized, these spiked samples can be used for precision studies, routine QCs, and as internal degradation controls. Various hair types should be employed.
- Although controls may be homogenized pools, the substitution of properly prepared spiked controls is acceptable
- For endogenous drugs, controls may be prepared using an alternative medium, for example, synthetic melanin

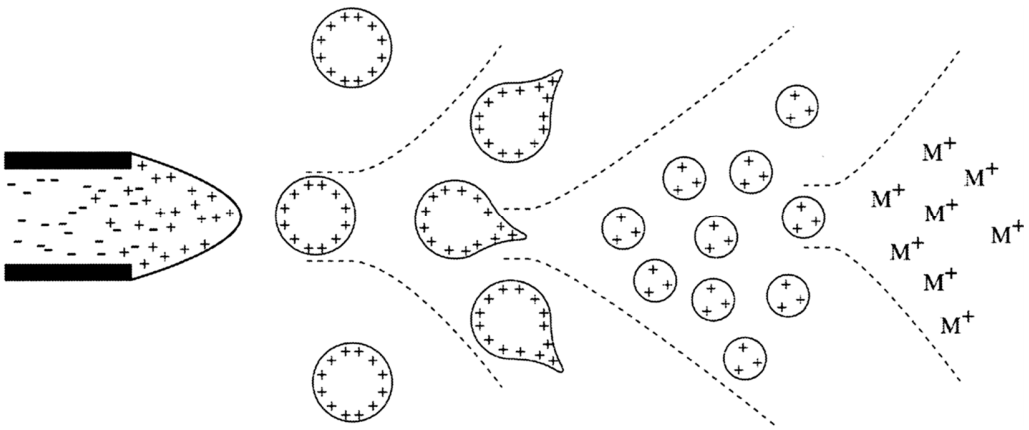
8. External Quality Control

- For external quality control, the laboratory should enrol in a proficiency testing program, where **authentic standard** hair specimens are sent for testing
- The laboratory must analyze proficiency specimens in the same way as routine samples
- If a laboratory does not consistently perform adequately in external proficiency programs, corrective actions must be taken
- In the case of the Society of Hair Testing, reference laboratories will be named, and their results will be used as a reference method and result source
- The results of their analysis are collated and compared to those from selected reference laboratories

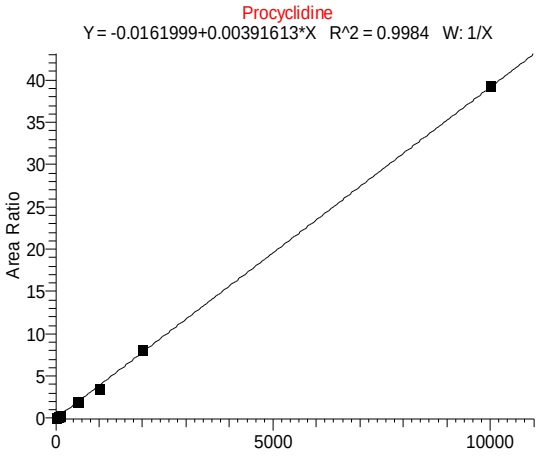
8.4. Illustration of Triple Quadrupole Mass Spectrometry (Garg, Dasouki 2006)

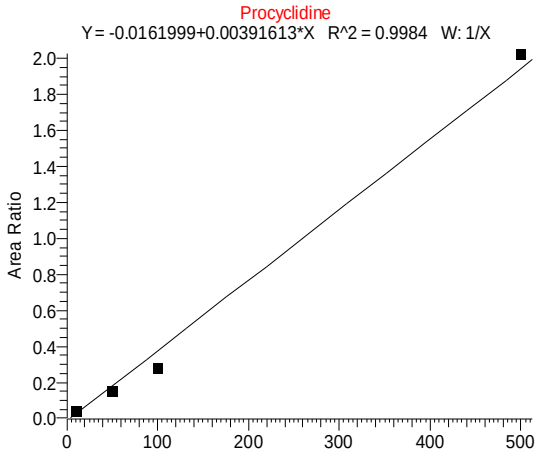


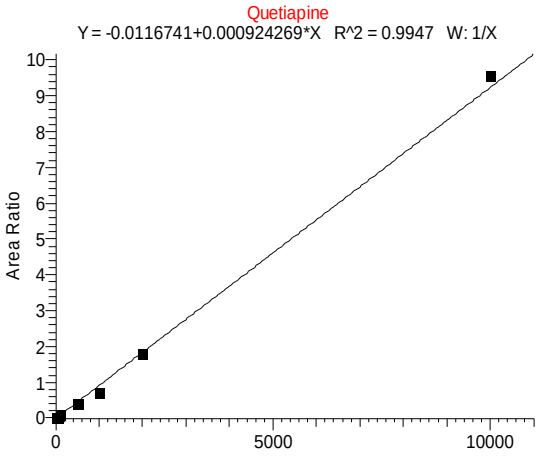
8.5. Mechanism of electrospray ionization (Ho et al. 2003)

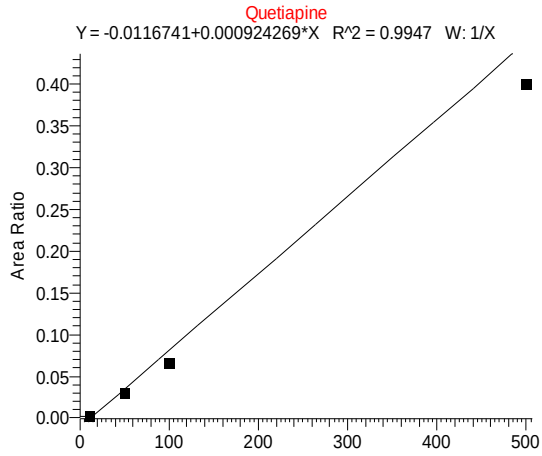


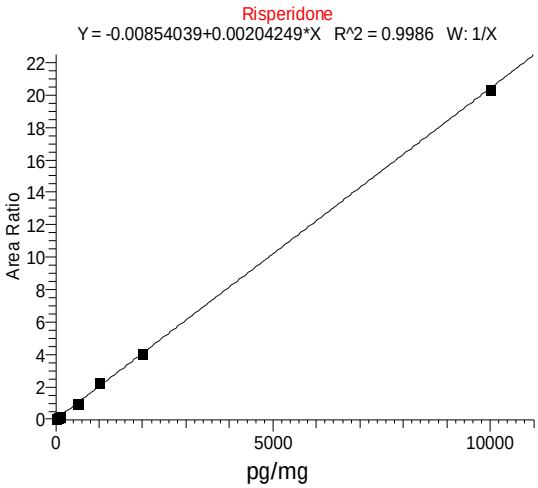
8.6. Hair calibration lines for all analytes

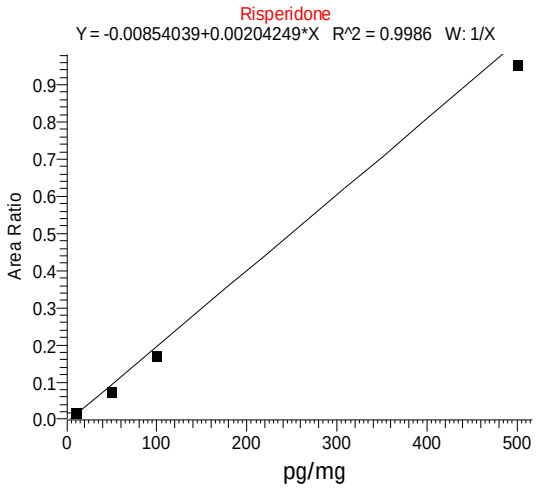


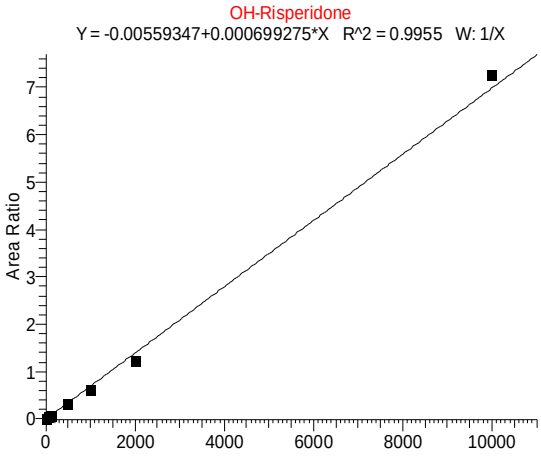








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Y = -0.00559347+0.000699275*X R^2 = 0.9955 W: 1/X
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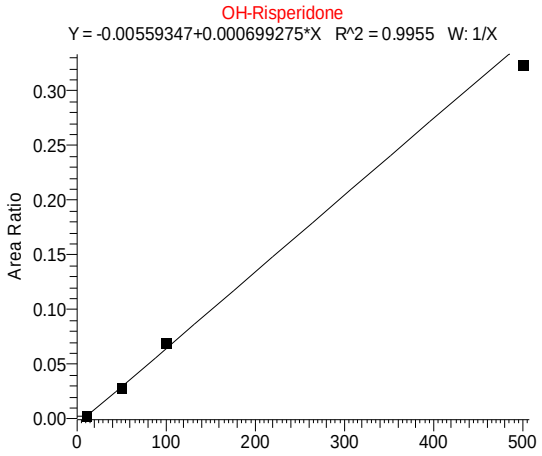
2
3
4
5
6
7
0
2000
4000
6000
8000
10000


OH-Risperidone
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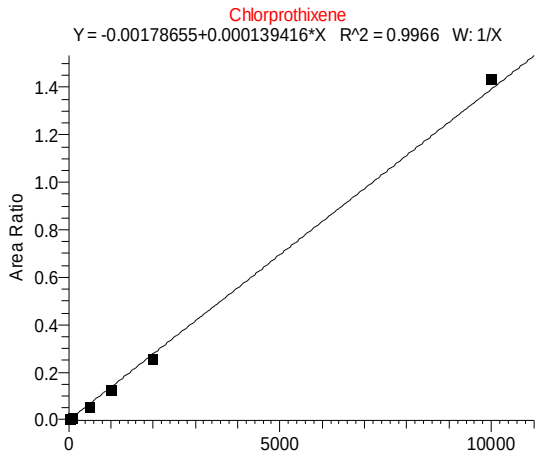
0.10
0.15
0.20
0.25
0.30
0
100
200
300
400
500


Chlorprothixene
Y = -0.00178655+0.000139416*X R^2 = 0.9966 W: 1/X
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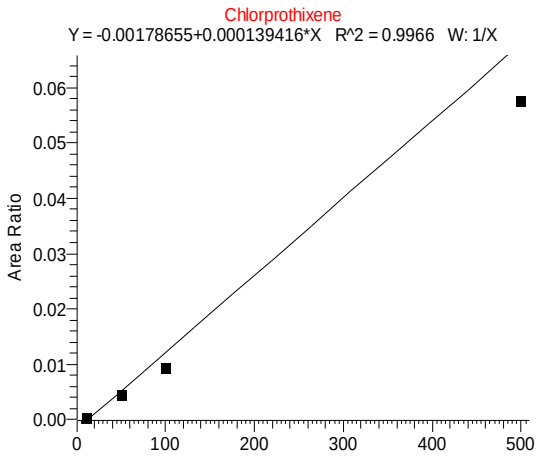
0.6
0.8
1.0
1.2
1.4
0
5000
10000


Chlorprothixene
Y = -0.00178655+0.000139416*X R^2 = 0.9966 W: 1/X
Area Ratio
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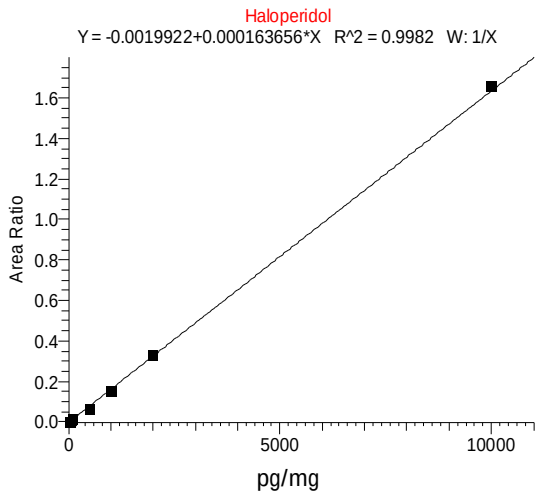
0.05
0.06
0
100
200
300
400
500


Haloperidol
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Area Ratio
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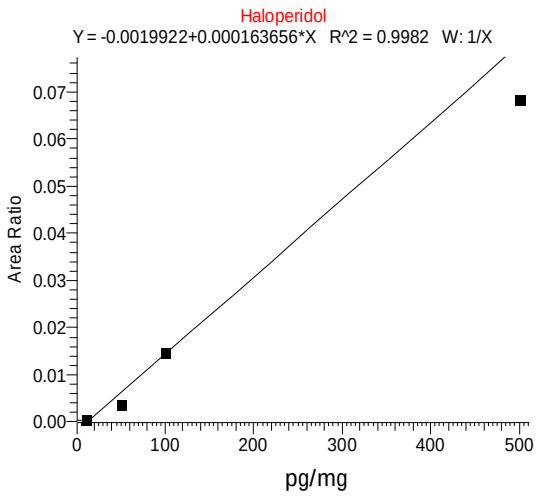
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
5000
10000
pg/mg


Haloperidol
Y = -0.0019922+0.000163656*X R^2 = 0.9982 W: 1/X
Area Ratio
0.00
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0
100
200
300
400
500
pg/mg

8.7. Publication

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Scientific Research

Method Development for the Detection of Basic/Weak Basic Drugs in Hair by LCMSMS: Comparison between Methanolic and Alkaline Extraction on Real Samples

Jaber Al Jaber[1,2], David Holt[3], Atholl Johnston[1,3]

[1]The William Harvey Research Institute, Barts & The London School of Medicine & Dentistry, Queen Mary University of London, London, UK; [2]Department of Pharmacology and Toxicology, Faculty of Medicine, Kuwait University, Jabriya, Kuwait; [3]Analytical Services International Ltd, St George's University of London, London, UK.
Email: j.aljaber@qmul.ac.uk

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ABSTRACT

Detection of drugs in hair has become popular in recent years. The significantly long drug detection window (months) in hair has allowed the retrospective investigation and measurement of past consumption of drug. As the majority of drugs are basic, an extraction method was developed based on a methanolic solution for detection of basic/weak basic drugs in hair. It was compared with alkaline digestion (NaOH) followed by LLE. A filtration step with filtration vials was added and their materials were compared. After filtration, extracts were injected directly onto a C18 column coupled to Sciex ABI 2000 MSMS. The mobile phase was 50% methanol, 0.1% formic acid and 2 mM ammonium acetate (isocratic). Both methods were compared by applying them to real samples. Results showed that calibration was linear with r^2 of 0.991 - 0.999 for 20 tested analytes. The matrix effect was assessed to be between 91.4% - 110.2% for 18 analytes. PTFE filter material showed better recoveries over the GMF and PVDF based filters. Stability of analytes during extraction in general was better with methanolic incubation than alkaline digestion. With regard to real sample recovery, 6 out of 10 analytes recovered better with alkaline digestion. In conclusion, the methanolic method is capable of extracting most basic drugs in hair samples but only part of the total incorporated drug. Therefore, these results suggest that a combination of both methods (methanolic and alkaline extractions) in hair sample processing for general detection of basic and weak basic drugs may produce better results. However, not all basic drugs are stable under alkaline digestion.

Keywords: Hair Analysis; Basic Drugs; LCMSMS; Benzodiazepines; Method Development; LLE

1. Introduction

Drug analysis in hair has grabbed the attention of toxicology analysts and researchers in recent years. This is mainly because it has provided some ability of proving drug ingestion when conventional samples could not. Hair differs from other traditional biological samples used for human toxicological analysis such as urine, blood, liver or saliva with its significantly longer detection window (months) allowing retrospective investigation and measurement of drug consumption. Hair analysis is becoming accepted in many developed countries for substance consumption related issues in a wide range of sectors; the medico-legal sector, workplace testing, treatment monitoring, schools, forensics, research, insurance companies, environmental biomonitoring and driving licensing [1-4].

Extraction of drugs from hair is considered one of the most important steps in hair analysis. Apart from external drug deposition on hair, drugs are mainly enclosed tightly in the hair shaft and to a certain extent maybe bound to proteins, melanin or lipids of the cell membrane complex. Therefore, hair matrix type, structure of the drug, method and duration of extraction, and solvent used are all important factors affecting the final extraction yield [5].

There are numerous reports of screening strategies published for analysing different forensic basic drugs groups. Hypnotic drugs such as benzodiazepines are at the top of the list of drug facilitated crimes. These are weak basic drugs and have been reported to be extracted from hair by different methods, with phosphate buffers, methanol or digestive enzymes [6-8]. Other basic drug groups like antipsychotics, antidepressants and amfetamines have frequently been reported to be extracted with alkaline digestion (NaOH) [9-12]. However, recently

