The Diagnostic Use of Alternate Samples in Forensic Toxicology

Thesis submitted in Accordance with the Requirements of the University of Glasgow for the Degree of Doctor of Philosophy

by

Fiona Mary Wylie
BSc (HONS), A.M.R.S.C.

Department of Forensic Medicine and Science
November 2001

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

Acknowledgements

I would like to thank Dr. John S. Oliver for his support and guidance as supervisor and for giving me encouragement to complete this. Thanks also to the Department of Forensic Medicine and Science, University of Glasgow for financial support.

I would also like to take this opportunity to express my gratitude to the pathologists of the Department of Forensic Medicine and Science, University of Glasgow, Prof. D. Stott, Glasgow Royal Infirmary, Dr. McQueen, Drug Problem Service, Glasgow and medical staff at Lightburn Hospital and Bridgeton Health Centre for the collection of biological specimens and to all of those who kindly donated samples to make this work possible.

Thanks to all members of staff and students in the Department of Forensic Medicine and Science for help, advice, entertaining tea-breaks and nights-out. A special thanks to Alison Seymour who has listened to many a problem and who always makes me look on the bright side when all seems at a loss and to Gail Cooper for her enthusiasm which never fails to encourage me.

Finally, thanks to Kirsty and Colin for managing to live with me for so long, the rest of my friends for keeping me entertained, my brother, Ewan, for not finishing before me and my mum and dad for so many things I would not know where to start - thanks for everything.

List of Figures

List of Tables

SUMMARY

The use of biological specimens other than blood and urine for the detection of drugs, usually referred to as *alternative specimens*, has been the focus of many scientific studies over the past two decades. The type of biological sample analysed affects the type of information obtained and the aims of this project were to investigate hair, saliva and nail as alternative specimens and to evaluate their diagnostic use in Forensic Toxicology.

The potential of hair analysis as a diagnostic tool in Forensic Toxicology was investigated by developing and validating a robust and reliable method for the simultaneous determination of opiates and methadone in hair samples. An assessment was made of the ability of three different pretreatment steps involving enzymatic hydrolysis, acid hydrolysis and solvent washing to extract morphine, codeine, 6-monoacetylmorphine, methadone and EDDP from hair. The methanol procedure was the only one that did not hydrolyse a percentage of 6-monoacetylmorphine to morphine and this was used for subsequent analyses, followed by solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC/MS).

Post-mortem hair samples from suspected heroin overdose cases were analysed using the validated method to determine whether each individual was a heroin user and their history of heroin exposure. An overall indication of drug use was produced for each individual from the results of analyses of hair and post-mortem blood. Known drug use history was established from police reports and the hair analysis results were evaluated against the available information. The ranges of concentrations of analytes found in hair were: morphine 0.10 - 9.79 ng/mg, codeine 0.12 - 3.47 ng/mg, 6-monoacetylmorphine 0.14 - 70.07 ng/mg, methadone 0.15 - 1.15 ng/mg and EDDP 0.27 - 1.55 ng/mg. A total of 31 hair samples was analysed, the majority of which were shown to be from regular heroin users. Criteria were established from the data which allowed users to be categorised into low, medium and high regular users with respect to their heroin habit. Periods of compliance with methadone programmes or drug rehabilitation programmes were also evident. The data supported the conclusion that hair analysis can successfully determine the drug use history of heroin users.

The number of cases involving fluoxetine and paroxetine has increased in the last 5 years and has required the development of improved methods to quantitate these drugs in whole blood. In addition, blood analysis provides the reference data for evaluation of analytical

results from alternative specimens. An SPE method using cyanopropyl columns was developed and evaluated initially for the extraction of fluoxetine and its metabolite, norfluoxetine. This method was compared with existing liquid-liquid extraction methods and validated using both high performance liquid chromatography (HPLC) and GC/MS. The HPLC method was found to work well with clinical blood samples but was subject to problems caused by co-extracted interferences from some post-mortem blood samples. By contrast, the GC/MS method did not exhibit this problem and was selected for further analyses. The method was validated for the simultaneous analysis of fluoxetine, norfluoxetine and paroxetine and was shown to achieve significantly improved limits of detection for each of these analytes of approximately 1 ng/ml. Post-mortem and clinical blood samples from antidepressant users were successfully analysed using this improved method. These results provided the foundation for further research into the diagnostic use of alternative biological specimens and were compared with data from hair, saliva and urine from the same cases.

The method developed for antidepressants in blood was also found to extract these analytes efficiently from saliva. Saliva samples were collected by expectoration and with Omni-Sal® devices. Validation of the Omni-Sal® method included investigating the in-vitro recovery of drugs from the swab. Recoveries were determined to be reproducible but much lower than those obtained from saliva collected by expectoration and this was also true for. clinical saliva samples. Saliva samples were collected serially from volunteers who were given a single dose of 20 mg Prozac® or Seroxat® and concentration-time curves were produced. Peak saliva concentrations were reached at approximately 7 hours following administration of fluoxetine for three individuals and 5 and 7 hours for two individuals who were administered paroxetine. The saliva half-lives were found to be 24-48 hours for fluoxetine and 5 - 8 hours for paroxetine. The saliva concentrations of drugs found in individuals prescribed 20 mg/day varied from 0.022 - 0.223 mg/L for fluoxetine and 0.011 - 0.143 mg/L for norfluoxetine. In one paroxetine sample a concentration of 0.031 mg/L was detected. In some of these cases blood samples were also collected simultaneously and saliva/blood concentrations were determined. Four out of six saliva/blood ratios for fluoxetine were shown to fall within the theoretical range calculated for a saliva pH range of 6.0-7.2 using the Henderson-Hasselbalch equation and the saliva/blood ratio for paroxetine in one case also fell within its theoretical limits. One conclusion from this

study was that saliva can be used as an alternative specimen to blood or urine to indicate recent drug exposure.

The analysis of hair as a means of determining chronic administration of prescription drugs was assessed by developing a procedure for the analysis of fluoxetine, norfluoxetine and paroxetine in hair samples obtained from geriatric and clinical patients. Methanol wash and acidic hydrolysis pre-treatments were assessed for these drugs. The latter produced higher recoveries without deterioration of the analytes and was used for analysis of hair samples from antidepressant users, to give a history of their drug administration, and these results were assessed against information obtained from each patient's medical records. Hair concentrations of each drug were evaluated to find if there was any correlation between individuals receiving the same doses. Concentrations of fluoxetine, norfluoxetine and paroxetine were found to vary from 0.34 - 5.52 ng/mg, 0.15 - 8.09 ng/mg and 0.22 - 6.23 ng/mg respectively and the drug use history as given by the hair samples correlated well with the patients' medical records. There was no dose-concentration correlation between individuals and therefore no information could be derived from hair analysis about the amount of drug consumed. However, within an individual, a constant dose-concentration correlation was apparent and this could be used to test for compliance. The influence of hair colour on the amount of drug incorporated was also investigated in these cases. Darker hair was found to incorporate a greater amount of drug than grey or white hair. The diagnostic use of hair as an indicator of chronic drug use was established using the validated method.

The potential of nail as an alternative to hair for the determination of chronic drug use was studied by validating a simple extraction method and using it to determine levels of antidepressants in nails obtained from clinical patients. Analyte concentrations in nail were evaluated using each patient's medical records and were assessed against the concentrations determined in the hair of each individual, to find if there was any correlation between the concentrations in these different biological samples. Nail concentrations were found to vary from 0.16 - 4.49 ng/mg for fluoxetine, 0.06 - 2.37 ng/mg for norfluoxetine and 0.01 - 0.84 ng/mg for paroxetine and in most cases a good correlation was seen with medical records. Hair and nail concentrations were compared for each individual and these generally showed that, as the hair concentration increased, the nail concentration also increased. The use of nails for the detection of these drugs was demonstrated to be a good alternative to hair as an indicator of chronic use.

Analytical Methods

ECD	Electron Capture Detection
GC	Gas Chromatography
HPLC	High Performance Liquid Chromatography
LLE	Liquid-Liquid Extraction
MS	Mass Spectrometry
NPD	Nitrogen Phosphorus Detection
RIA	Radioimmunoassay
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
UV	Ultra Violet Detection

Drugs and Materials

BSTFA	bis(trimethylsilyl)trifluoroacetamide
CAN	cannabinol
CLO	clomipramine
cod	codeine
d_3	tri-deuterated
d_9	nona-deuterated
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EMDP	2-ethyl-5-methyl-3,3-diphenylpyrrolidine
FLU	fluoxetine
HFBA	heptafluorobutyric anhydride
6-MAM / MAM	6-monoacetylmorphine
MAP	maprotiline
Meth	methadone
mor	morphine
MSTFA	*N*-methyl-*N*-trimethylsilyl-trifluoroacetamide
NFLU	norfluoxetine
PAR	paroxetine
PFPA	pentafluoropropionic anhydride
TMCS	trimethylchlorosilane
TUM	tumoxetine

Other

COD	Cause of Death
rev/min.	revolutions per minute
RSD	Relative Standard Deviation
R^2	Linear Correlation Coefficient

1 Introduction

The use of alternate biological samples for the detection of drugs has been the focus of many scientific investigations in the past two decades. The decision as to which biological specimen is to be analyzed is important because different specimens will render different types of information. Traditional biological samples used in toxicological investigations are blood and urine. These specimens give information about an individual's recent drug use. However, these samples can be awkward to collect and information about the chronic use of drugs cannot be obtained from them. In the case of urine analysis, the detected drug concentrations do not give the same information as blood concentrations. Urine concentrations are accumulated over a period of time and do not give a true reflexion of the amount of drug in the body at the time of collection. They only indicate that the individual has taken a particular drug. On the other hand, blood concentrations can indicate the amount of drug in the system at a specific time. Saliva concentrations have also been shown to be a better indicator of concentrations in the system at a specific time than urine.

Saliva, as a biological sample for the testing of drugs gives an indication of the short term use of drugs in an individual. Its major advantage over blood analysis lies in its non-invasive collection procedure. The collection of blood samples requires a medically qualified person to carry out a standard venepuncture procedure which can often cause discomfort to the person giving the sample and urine collection is intrusive. Saliva testing for drugs has been used in the past for therapeutic drug monitoring.[1] However, in recent years it has become of interest to law enforcement agencies for the roadside testing of potentially intoxicated drug drivers.[2, 3] In the region of Strathclyde, Scotland, the number of blood samples obtained in incidents involving intoxicated drivers which were found positive for drugs increased from 86 cases in 1996 to 139 cases in 1999.[4] In many of these cases the identified drugs were substances controlled under the Misuse of Drugs Act 1971. However, drugs of interest in drivers do not only include controlled drugs, but also prescription drugs and over the counter drugs which have the ability to impair driving performance either alone or in combination with other drugs. It is therefore important to be able to measure them as well as controlled drugs in saliva. The primary disadvantages of using saliva as a test medium are that drug excretion in saliva can vary due to salivary pH changes and contamination of the oral cavity due to drug administration by oral, smoked or intranasal routes can lead to high saliva concentrations of the drug.

Blood, urine and saliva samples can all provide information on the recent use of different drugs. However, hair analysis can give a different perspective on drug consumption in that chronic drug use can be determined. Drugs may be incorporated into hair via several routes including passive diffusion from blood into the hair follicle, by transfer from sweat and sebum and by external contamination. Much of the incorporated drug will remain in the hair during its life. For the average individual, hair grows at approximately one centimetre per month. Sectioning of the hair from the root end towards the tip provides segments which are approximately equivalent to a period of time and this means that it can be estimated when a person was using or not using a particular drug. This has proven useful in monitoring individuals involved in drug maintenance programmes, employee screening, child custody and adoption cases, criminal cases and athletics to either prove or disprove the use of drugs. Hair has the added advantages over conventional fluids that it is relatively stable and can retain drugs over long periods of time. Collection is simple and can be carried out under close supervision and adulteration is not a problem as it can be with urine analysis. However, there are still many controversies surrounding hair analysis and these include drug loss through chemical treatment of hair or environmental factors, the possibility of an ethnic bias or hair colour affecting the amount of drug incorporated and external contamination to hair through smoking or by secondary transfer.

Nail analysis for drugs is also a means of detecting chronic use. It has many of the same merits as hair analysis. Its collection procedure is non-invasive and the stability of the matrix allows drugs to be stored within the structure for long periods of time. However, it cannot give the same information as hair analysis can about when a drug was used. This can be explained by recent studies that have provided evidence into the way in which drugs are incorporated into nails. Incorporation of drugs into nail may be through cells in the nail bed and also in the nail matrix. Therefore, drugs can be detected in nail a few weeks after administration.

The understanding of the structures of each biological matrix and the incorporation of drugs into these samples is crucial in evaluating and interpreting the results from their analysis. In the following chapters these areas amongst others will be addressed for each alternate biological sample.

2 Hair

2.1 INTRODUCTION

Hair testing for drugs has been used in Europe and the United States for various purposes including monitoring individuals who are involved in drug maintenance programmes, employee screening, criminal cases, athletics and child custody cases. In Italy hair analysis has been used to test the hair of drivers who have lost their licences through drug use prior to reissuing their licence.[5, 6, 7]

The hair matrix is a relatively stable matrix which can retain drugs over long periods of time, and can provide a much longer record of drug use in comparison to matrices such as blood, urine and saliva.[8] Collection of hair samples is non-invasive, can be carried out under close supervision and invasion of privacy is not an issue as with urine sampling. Hair samples cannot be adulterated and temporary abstention from drug use only means that the drug will not be detected over that short period, but hair growing at the time of drug use will still indicate drug exposure. A second hair sample can always be collected if required. The hair matrix, unlike other biological matrices is stable even under extreme environmental conditions and it can be easily stored and transported without refrigeration.

For drug analysis in hair, the understanding of the anatomy of human hair appears to be an important factor in evaluating and interpreting hair analysis data. For this reason an overview of hair anatomy, hair growth cycle and drug incorporation into hair is given.

2.2 STRUCTURE OF THE HAIR SHAFT

The hair shaft is composed of three regions, namely the *cuticle*, the *cortex* and the *medulla* (Figure 2.1). Within the cortex and medulla (and very rarely in the cuticle) there exist *pigment granules*. Each of these components will be discussed separately.

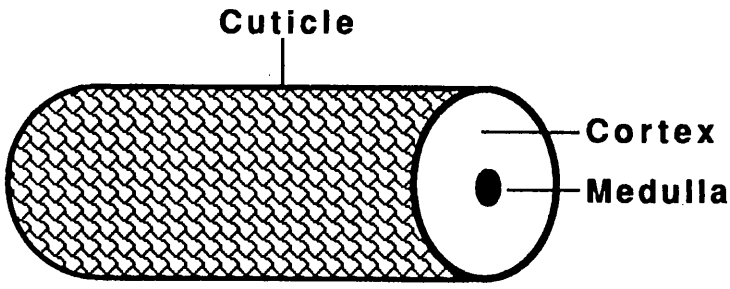


Figure 2.1: Cross-section of a hair shaft showing the three principle regions (Copyright © Elsevier Scientific Publishers, Harkey, M.R. "Anatomy and Physiology of hair." Forensic Science International 1993, 63, p9-18.

2.2.1 CUTICLE

The *cuticle* is made up of six to ten layers of cells that overlap one another from root to tip. The average hair has 4 μm of cuticular covering surrounding the cortex.[9] Its purpose is to shield the inner fibres from damage and to fix the hair shaft in the *hair follicle*, a small sac-shaped secretory gland that contains the hair root.[10]

2.2.2 CORTEX

The *cortex* constitutes the bulk of the hair. Closely packed elongated cells are aligned along the axis of the hair to form long keratin fibres that are contained in the cortex. In the proximal end of the cortex, fluid filled spaces exist between the long keratinized cells. As the hair grows, the fluid dries up and air spaces called *fusi* are formed.[10]

2.2.3 MEDULLA

The *medulla* forms the central core of the hair and it has a tendency to vary along the hair strand. At the follicle end it consists of loosely packed cells that dehydrate leaving vacuoles along the fibre further away from the follicle. In general, the larger the fibre diameter, the more medullar cells are present, such that fine hair may contain no medullar cells whereas thick hair may contain many. In human hair the *medulla* can be continuous, discontinuous or absent and in beard hair a double *medulla* can sometimes be present.[9, 10]

2.2.4 PIGMENT GRANULES

Pigment granules exist in the cortex and the medulla. The main pigment in hair is *melanin* which is produced by cells called melanocytes during the growth or anagen phase of the hair. These melanin granules are fixed between keratinous fibrils in the cortex and are distributed throughout the cortex with a higher density occuring towards the outer edges of the cortex.[9] It is these melanin granules which define hair colour depending on the amounts, size, distribution and types of melanin present. These factors vary for different races for example melanin granules are more abundant in Negroid hair than Caucasian hair.

2.3 THE HAIR FOLLICLE

The hair follicle is a small sac-shaped secretory gland containing the hair root. It extends 3-4 mm below the surface of the skin and it is associated with the sebaceous and apocrine glands and also the sweat glands in axillary and pubic regions.

The follicle is composed of three sections. Hair synthesis takes place in the bulb area, which extends furthest into the skin. Above the bulb is the keratogenous zone where keratinization or hair solidification takes place and in the final region dehydrated, cornified cells make up the hair shaft (Figure 2.2).[10]

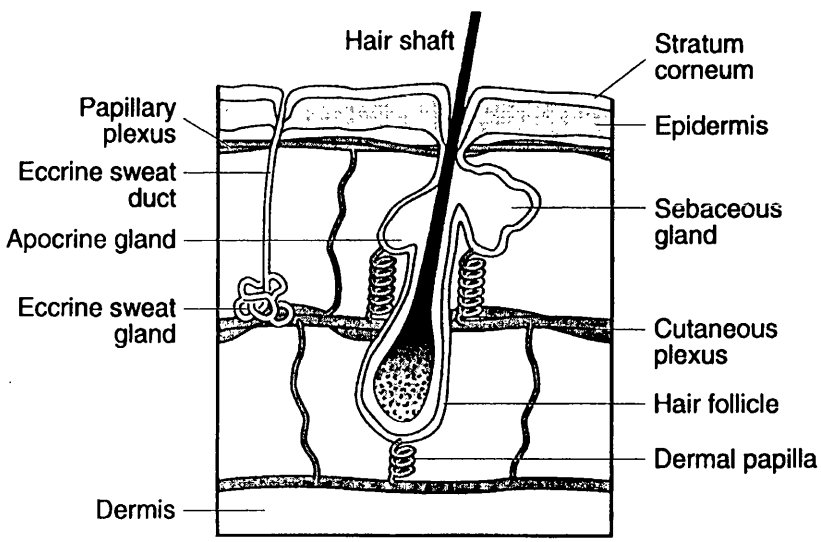


Figure 2.2: The hair follicle and surrounding components (Copyright © Elsevier Scientific Publishers, Harkey, M.R. "Anatomy and Physiology of hair." Forensic Science International 1993, 63, p9-18.

2.4 HAIR GROWTH CYCLE

Hair growth is an intermittent, regenerative process made up of a series of cycles alternating between irregular periods of growth and rest.[11] The growth and rest periods vary between areas of the body and also in any one specific area.[10] There are three main stages involved in the hair growth cycle: anagen, catagen and telogen phases.

2.4.1 ANAGEN PHASE

The anagen phase is the growing stage of the hair which involves mitotic activity of the matrix cells in the hair follicle.[12] Newly produced cells elongate forming fine filaments which are forced up into the follicular canal. At this stage protein synthesis or keratinization occurs and new hair is formed. The growing stage lasts between 2 to 3 years. In the vertex region of the scalp, a maximum of 85 % of hair is in the growing stage at any one time, with the remaining 15 % in the resting stage.[10]

2.4.2 CATAGEN PHASE

The mitotic activity of the matrix cells suddenly ceases and the catagen phase commences. This is a transitional phase between active growth and a resting phase. During this phase, the hair root is partially destroyed: a mass of keratin is formed at the base of the hair shaft and this becomes separated from the bulb of the hair follicle. The hair follicle eventually returns to its normal length.[12]

2.4.3 TELOGEN PHASE

The telogen phase is one where all hair growth activity has ceased and at this stage the hair is completely detached from the bulb with the hair lying in the upper section of the follicular canal. As a result, hair can be easily pulled or plucked at this stage.[10] Individual hairs have their own growth cycle of 2-8 years including the telogen stage of about 3-4 months.[13] For hair analysis, it is preferable to sample hair from a position where the minimum amount of hair is in the telogen or resting phase because telogen hairs will cause a shift in the distribution of drugs found that may be equivalent to several months.

2.5 HAIR GROWTH RATE

Reported hair growth rates for head hair have varied greatly, most likely due to the complex, non-continuous growth mechanism of hair and the fact that growth rate varies between individuals and amongst the hair shafts within an individual. However, it is

generally accepted that head hair grows at a rate of 1 cm/month.[14] Factors affecting hair growth rate primarily are hair type and location and to a certain extent age, race and sex. Growth rate is highest for head hair, followed by axillary and pubic hair and then beard hair. Head hair growth rate decreases with age, is faster in Caucasians than Asians and is faster in females than males.[10]

2.6 DRUG INCORPORATION INTO HAIR

The exact mechanism of the incorporation of drugs into hair has not yet been established. However, the model commonly proposed is one of passive diffusion of drug via blood capillaries into the base of the hair follicle where hair formation takes place. As hair strands are produced they solidify and drug is encapsulated in the hair shaft.[15] Then, as the hair grows, the encapsulated drug moves away from the bulb with the piece of hair to which it has been affixed. Using this model, it is assumed that the amount of drug incorporated into the hair is proportional to the amount in the blood and therefore to that ingested and also, that hair growth is constant so a history of drug use can be established. Methoxyphenamine was administered to individuals and was shown to move along the hair shaft according to hair growth, without diffusion.[16]

However, it has been brought to light through experimental evidence and speculation that the process of drug incorporation may not be as straightforward as first believed and that the amount of drug deposited in hair is due to the contribution of several factors. The simple passive diffusion model assumes that all of the drug enters the hair follicle via blood capillaries. However, it has been speculated that external contamination, from sweat or sebum, or skin surrounding the hair as it is produced could be a source of drug transfer into hair.[15, 17] Other routes of external contamination are by drugs which can be smoked such as nicotine, cannabis, cocaine and heroin. In these cases careful wash procedures must be employed to remove as much external contamination as possible.

2.7 DRUG PROPERTIES AFFECTING INCORPORATION INTO HAIR

Drug incorporation into hair appears to be the result of three main physicochemical properties, namely, lipophilicity, basicity and melanin affinity. Membrane affinity has also been a proposed factor.

It has been suggested that drug incorporation into hair can be put down to a combination of two factors, melanin affinity and lipophilicity. The aspect of membrane affinity can be associated with lipophilicity, and likewise basicity could be strongly linked with melanin affinity since the chemical structure of melanin is composed of many carboxylic acid groups that will attract basic structures.[18] However, this idea has not been entirely proven and for the purpose of this thesis lipophilicity, basicity and melanin affinity will be discussed and also structural factors that seem to have a role in incorporation of drugs into hair.

2.7.1 LIPOPHILICITY

The lipophilicity of a drug will have a large influence on how it enters the hair. In order to pass from the blood capillary to the hair follicle, the drug must be able to cross the cell membrane. Substances that are most likely to be successful at this are chemically nonpolar parent drugs.[17] This may well explain why in many cases parent drugs are detected in higher quantities in hair than their respective hydrophilic metabolites.

The results of a study to test the incorporation of a group of amphetamine analogues into hair indicated that small structural changes increasing the lipophilicity also increased the concentration of drug found in hair.[19]

The lipophilicity of 19 basic drugs of abuse was shown to correlate with their incorporation rate into hair giving a correlation factor of 0.766.[20]

2.7.2 BASICITY

The ionization of a drug at physiological pH affects how much drug incorporates into the hair. In a study involving rats, hair and blood concentration ratios were compared for codeine and phenobarbital. The weak base, codeine, which is cationic at physiological pH incorporated into hair to a greater extent than the weak acid, phenobarbital which has no

cationic properties at physiological pH, concluding that at the same plasma concentration, cations will incorporate into hair with a higher concentration than anions.[17]

Another study investigated the incorporation rates of a range of acidic, basic and neutral drugs into rat hair. Basic drugs had the highest incorporation rates with cocaine featuring highest out of those tested. 11-nortetrahydrocannabinol-9-carboxylic acid had the lowest incorporation rate. It was concluded that the higher incorporation rates of basic drugs over neutral or acidic drugs was highly associated to the membrane permeability of the drug.[20]

2.7.3 MELANIN AFFINITY

In an investigation involving 20 drugs, it was shown that different drugs have different affinities to bind to melanin and the evidence pointed to the fact that melanin in hair could be associated with drug incorporation into hair. Cocaine, benzphetamine and phencyclidine were shown to have the highest affinity for melanin out of those drugs investigated.[20]

The incorporation of seven phenylamines was investigated in white and black rat hair. Six of these were detected at high concentrations in black hair, however, hardly any were detected in white hair indicating that for basic drugs to incorporate into hair, melanin is an essential component.[21]

Melanin affinity was shown to be affected by different groups substituted at the para-position on the benzene ring of methamphetamine. Nitro-, amino-, and methylene dioxy-groups all showed significantly higher affinities compared with the unsubstituted methamphetamine.[18]

In an experiment investigating the different incorporation rates of phenethylamines, it was shown that drugs incorporated into black rat hair in four different manners: rapidly over a long period of time, rapidly over a short period of time, slowly over a long period of time and slowly over a short period of time. However, these same drugs were shown to be scarcely incorporated into white hair. This showed that the presence of melanin was essential for effective incorporation into hair.[21]

2.7.4 STRUCTURAL FACTORS

Small changes in chemical structure can have a large effect on whether a substance is easily incorporated into hair. A group of methamphetamines with different para-

substituents on the benzene ring (nitro-, hydroxy-, bromo-, amino-, methoxy- and methylenedioxy- groups) all possesed different drug incorporation rates which showed the strong effect that functional groups place on drug incorporation.[18] A series of these types of experiments was carried out to evaluate different structural factors influencing the incorporation rate into hair and these again showed that small changes in functional groups alter the incorporation rate and have a considerable influence on whether a drug is easily detected or not.[22]

2.7.5 OTHER FACTORS AFFECTING DRUG INCORPORATION INTO HAIR

There are still many unresolved issues involving drug transfer into hair. Factors such as gender[23], age, differences in hair structure between individuals, seasonal variation, condition of hair, for example, damage by exposure to sunlight[24] have all been questioned as having an effect on incorporation.

2.8 COLLECTION OF HAIR

Several considerations need to be taken into account when collecting hair samples from humans. For instance, is the sample being collected from a live or dead individual? What size of sample is required? Can any type of body hair be used? These will be discussed below.

2.8.1 METHODS

In postmortem cases, it is the pathologist who takes the specimens and it is necessary for the hair sample to be taken prior to the postmortem to prevent unwarranted contamination from blood or other bodily fluids. This hair can be pulled which will include the roots in the sample or cut close to the scalp. Collecting hair from living individuals will involve cutting the hair close to the scalp and this will usually be carried out by a General Practitioner. Whichever method is used, the root end should be labelled clearly (although this is often obvious where the roots are included in the sample), the sample tied with string or rolled in cigarette wrappers to hold it together and placed in a labelled tube.

2.8.2 SAMPLE SIZE

The sample size is important and will depend on the type of investigation and how many analytical investigations are to be carried out. Preferably a bundle of hair at least 0.5

centimetres in diameter is required for each analysis, but this will be dependent on the amount of hair an individual has.

2.8.3 HAIR TYPE

Location of the hair sample is another important factor. For head hair, this should be taken from the posterior vertex where hair growth is at its densest and most consistent in its growth pattern, except in some older males. Other hair types have also been used for analysis of drugs. Pubic hair can be used where there is no other hair available, but this has the disadvantage of providing a sample contaminated by urine. Drug concentrations in various types of hair of an individual vary according to the location of the hair sample.[25] The most likely explanation of this difference is due to the varying growth rates of these different hair types. Beard hair would be the preferable hair type for detection of drugs a few days after use, in addition to urine analysis.[26] On the whole, pubic, axillary and beard hair hold no major advantages over head hair. They are still susceptible to contamination through urine, sweat and the environment and so if obtainable, head hair is the preferred sample for analysis.

2.9 STABILITY OF DRUGS IN HAIR

The stability of drugs in hair has been examined by several authors who have shown that positive opiate levels decreased to produce negative results in hair samples which were exposed to soil and water for a period of six months.[27] Concentrations detected in hair have been shown to decrease when hair was bleached, coloured or treated with perming reagents.[28, 29] Other studies have also shown that when a constant amount of drug is administered, the concentration of drug detected in hair decreases with time. These decreases were explained by some of the drug being removed by washing hair that had been damaged because of environmental factors[16] and hair having been treated with henna products.[30] These findings infer that quantitative results of drugs in hair positioned further away from the root require careful interpretation.

2.10 ETHNIC BIAS

It has already been mentioned in 2.2.4 that the composition of hair from different races varies in the number of melanin granules such that Negroid hair contains more granules than Caucasian hair. Studies have shown that drugs have a tendency to incorporate into

hair which has a higher melanin content, and therefore certain races, African-Americans, Asians, Hispanics could experience a bias in drug testing. Many studies have been set up to resolve this controversial issue. Some authors have concluded from their work that there was no conclusive evidence of hair colour or racial bias and that differences were a result of the varying drug use of different races and cultures.[31] However, several animal investigations have pointed towards the fact that hair colour and therefore melanin content has a large part to play in drug binding. Drugs such as methadone[32], buprenorphine[33], haloperidol[34], codeine[35] and phencyclidine[17] did not incorporate into white hair to the same extent as black hair.

Statistical evaluations have been applied to reported analytical results where hair colour was clearly identified. Although there were apparent differences in the drug levels detected within the various samples, they found that there was no significant difference between the drug levels measured in different hair colours. The authors concluded that hair colour does have a role to play in the incorporation of drugs, however, it only contributes a fraction in the process which is statistically undetectable. Attention should be given, however, to cosmetic treatments and laboratory procedures that may impact on the amount of drug measured in the sample.[36]

2.11 CONTAMINATION

External contamination of hair by drugs through smoking or by secondary transfer from handling drugs and then hair can cause problems for the analyst. This contamination should be eliminated by decontamination wash procedures prior to analysis. Washes should be monitored to ensure that all of the contamination is removed. It has been reported that certain wash procedures may remove drugs from within the hair shaft,[37] whereas others have reported the failure to remove all external contamination from hair. This questioned whether some of the drug had adsorbed into the hair shaft and not solely onto the surface.[38] Several mechanisms have been indicated to be responsible for the binding of drugs to hair. Hair is composed of keratin, melanin and lipids which may all play a part in the binding process. Keratin is composed of several basic and acidic amino acid residues and multiple hydrogen bonds which will have the potential to interact with drugs. The most abundant amino acid in keratin is cystine which containes a disulphide bond which can potentially bind nucleophilic molecules. Also, dipole-dipole interactions

may be possible between peptide bonds in keratin and polarizable molecules. It has already been mentioned that different drugs appear to have different affinities to bind to melanin. The actual structures of pheomelanin and eumelanin have not yet been determined. However, the quantity and type of melanin in hair should dictate the extent of drug binding. Hair consists of 1-9 % lipid content. It has also been suggested that non-specific binding of drugs to lipid components in hair cell membranes can occur. A constituent of lipid membranes are fatty acids containing negatively and positively charged groups which can bind cationic and anionic drugs respectively.[39]

Other authors have also reported the failure to remove all external contamination using different wash procedures.[40, 41, 42, 43] As a consequence, it is not always easy to differentiate environmental contamination from actual drug use. It has been reported that the hair of cigarette smokers and non-smokers could not be differentiated because of contamination due to secondary smoke.[44] However, in another report aimed to distinguish smokers from non-smokers a cut-off level of 2 ng/mg for nicotine was suggested, based on the authors' results.[45]

For certain drugs, the identification of metabolites can be used to distinguish between passive exposure and actual drug use.[46] Metabolites of drugs such as cocaine, however, are also produced by degradation and have been found on the surface of hair of non-users. The idea to use unique metabolites which are only found when the drug has been injested have also been evaluated,[41] however it was suggested that even these metabolites could be found in illicit cocaine samples.[47]

2.12 ANALYSIS

In general, four main steps are used in hair analysis. These will be discussed briefly.

2.12.1 DECONTAMINATION

Before hair samples are extracted, they must be washed to remove extraneous material including oils, grime and externally applied drug. This procedure should help clean the extract whilst preventing false positives from occurring, but should not remove drug which has been incorporated into hair through drug use. This stage is necessary to produce the correct drug historical record of an individual. Wash steps often involve using a detergent and a combination of solvents.

2.12.2 REMOVAL OF DRUG FROM HAIR MATRIX

Drug can be removed from the hair matrix by alkaline hydrolysis, acid hydrolysis, enzymatic hydrolysis or by treatment with solvent, for example methanol. The choice of extraction procedure is influenced by the drug and its chemical stability. Drugs which are chemically unstable are usually extracted using enzyme or solvent methods since alkali hydrolysis is generally too harsh a method and destroys the drug. Also, if the hydrolysis product is a metabolite of the analyte then this can lead to erroneous results. With drugs which are chemically stable, alkaline hydrolysis has often been the preferred method as it efficiently solubilizes the keratin matrix. Enzyme, solvent and acid methods do not completely solubilize the hair and can lead to lower recoveries.[48]

Various types of enzyme digestion have been used. The advantages of this method are that the extraction can be carried out at an optimum pH and the results are often better than those obtained with solvents only. Enzymes act on keratins without altering the chemical structure of the analytes in hair.[49]

2.12.3 EXTRACTION

Clean-up procedures generally involve using either liquid-liquid or solid phase extraction procedures. However, other less conventional methods involving solid-phase microextraction and supercritical fluid extraction are now also being used.

2.12.4 ANALYSIS

Analytes in hair are generally detected using mass spectrometry, following chromatographic separation because it is a sensitive and specific means of detection. Gas chromatography-mass spectrometry has been the most useful tool in the past, but now newer techniques involving tandem mass spectrometry, high resolution mass spectrometry and liquid-chromatography mass spectrometry are becoming more popular because of their increased sensitivity. However, these instruments are not available in most laboratories.

2.13 INTERPRETATION OF AMOUNTS OF DRUG DETECTED IN HAIR

2.13.1 DOSE-CONCENTRATION CORRELATIONS

Many studies have been carried out to find if there is a relationship between the amount of a drug administered to the concentration detected in hair. Some of these studies were conducted in humans and some in animals. Findings have shown that there is a significant

correlation for some drugs such as methadone,[30] nicotine and cotinine,[50] methoxyphenamine,[16] opiates,[24, 51, 52] cocaine,[24, 52] methamphetamine,[52] amphetamine,[52] 6-acetylmorphine,[52] and phencyclidine.[24] On the otherhand, a lack of correlation was observed for drugs morphine,[53] cocaine,[54] dothiepin,[55] amitriptyline,[55] cannabis,[24] nicotine,[56] and benzodiazepines.[57] It has been suggested that lack of correlation is not surprising since several of the studies have employed self-reported usage which could easily be underestimated, the purity of street-drugs is unknown and there is a high degree of inter-individual variation which can affect drug incorporation.[53] Whilst there are many unknown factors remaining about drug incorporation into hair, determining the amount of drug consumed from quantitative measurements in hair will remain inapplicable.

2.13.2 CUT-OFF LEVELS

Cut-off levels are very important when controversial issues such as racial bias or hair colour bias have not been fully resolved. If incorporation rates are different in, for example, two different hair colours, black hair and blond hair, then at low concentrations the drug may be detected in the black hair but not in the blond hair because it is below the cut off point. Cut-off levels must provide an acceptable balance between false positives and false negatives. A false positive result is one in which a drug is found present in a sample when in fact it is not and a false negative result is one in which a certain drug is not found to be present in a particular sample when in fact it is. Both of these are undesirable factors in the analysis of drugs. The finding of false positives is especially of great concern in drug analysis because of the consequences they can have on the individual concerned. After its third meeting in Texas, May, 1999, the Hair Testing Work Group reached a final consensus recommending cut-off levels for different drugs of abuse in hair.[58]

3 Hair Analysis for Opiates and Methadone

3.1 AIM

The aims of the following project were to develop a reliable method for the analysis of opiates and methadone in hair and to investigate postmortem hair samples from suspected heroin overdose cases for the presence of morphine, codeine, 6-monoacetylmorphine (6-MAM), methadone and its metabolite, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP).

Hair samples were to be analyzed in sections to give a history of each individual's drug use and these results compared with the concentrations found in postmortem blood. From these results it could then be ascertained whether the overdose victim was a regular or naïve drug user.

3.2 INTRODUCTION

3.2.1 OPIATES

Opiates are naturally occurring compounds which are derived from opium in the poppy plant, *Papaver somniferum.* These include morphine and codeine which are pharmacologically active alkaloids and these have the primary effect of analgesia. Pure form heroin or diamorphine can be synthesized by acetylation of morphine and it is this highly potent substance which is the major active component in street heroin. Opiates are controlled under the Misuse of Drugs Act 1971 making possession or supplying without a prescription and unauthorised production, import or export illegal.

Heroin abuse is a growing health problem in many countries including Germany,[59] Australia,[60] England and Wales[61] and Scotland. In recent years the increase in availability and the cheaper cost of heroin have been largely responsible for this growth in Scotland. In the region of Strathclyde, the number of drug related deaths where morphine was implicated rose from 48 % of the total drug related deaths in 1990 to 71 % of the total drug related deaths in 1999.[4]

3.2.2 METHADONE

Methadone is a synthetic opioid which has been available clinically in the United States since 1947.[62] It is used in detoxification and maintenance programmes for the treatment of opiate dependence in heroin addiction.[63] Similar to the opiates, it is controlled under the Misuse of Drugs Act 1971 and it is illegal to possess or supply it without a prescription.

Up until 1996, methadone was dispensed by pharmacists, but administration was not under the pharmacists' supervision. This led to some of the prescribed drug being diverted by the patient by various methods towards illicit users. In 1996, it became good practice for prescribed methadone users to be supervised whilst taking their dose to prevent illicit use as far as possible. In the region of Strathclyde, the number of drug related deaths where methadone was implicated rose from 0 % of the total drug related deaths in 1990 to 37 % of the total drug related deaths in 1996. This figure then fell to 19 % by 1999. This decline was largely due to supervised methadone administration. The Drug Problem Service in Glasgow have 99 % supervised methadone administration.[4]

3.3 CHEMISTRY

3.3.1 OPIATES

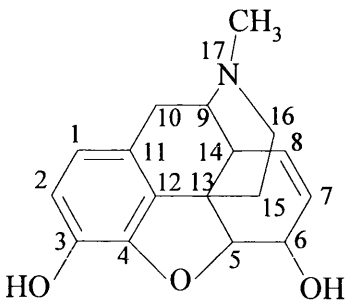


Figure 3.1: Chemical structure of morphine

The structure of morphine is given in Figure 3.1. Many semisynthetic derivatives are made by modifications to its structure. Codeine is methylmorphine where the methyl group is substituted on the phenol group at position 3. Diamorphine is made by acetylation at the 3 and 6 positions[64] and 6-monoacetylmorphine, metabolite of diamorphine is acetylated in position 6.

3.3.2 METHADONE

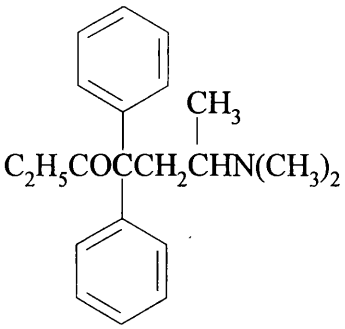


Figure 3.2: Chemical structure of methadone

Methadone is chemically known as 6-dimethylamino-4,4-diphenyl-3-heptanone and is a synthetic opioid, structurally unrelated to morphine. Its structure is shown above in Figure 3.2.

3.4 METABOLISM AND EXCRETION

3.4.1 OPIATES

Administration of heroin is usually through intravenous or intramuscular injection, by smoking or snorting. After administration, heroin is rapidly metabolized to 6-monoacetylmorphine (6-MAM) by deacetylation and 6-MAM is further hydrolyzed to morphine. Morphine metabolizes in the liver to mostly morphine-3-glucuronide, with smaller amounts of morphine-6-glucuronide and normorphine, which also further metabolizes to normorphine-glucuronide. The metabolic route of diamorphine is shown in Figure 3.3.

The primary metabolites of heroin found in urine are morphine and conjugated morphine, but diamorphine and 6-MAM can also be found depending on the dose and time of collection. Codeine can also be found in the urine of a heroin user due to illicit heroin containing acetylcodeine and codeine.

The presence of codeine and morphine in a sample is not conclusive evidence of heroin intake. Both of these opiates are found naturally in poppy seeds and concentrations found in urine can be similar to those found in heroin users. The presence of the metabolite 6-monoacetylmorphine is indicative of heroin exposure.

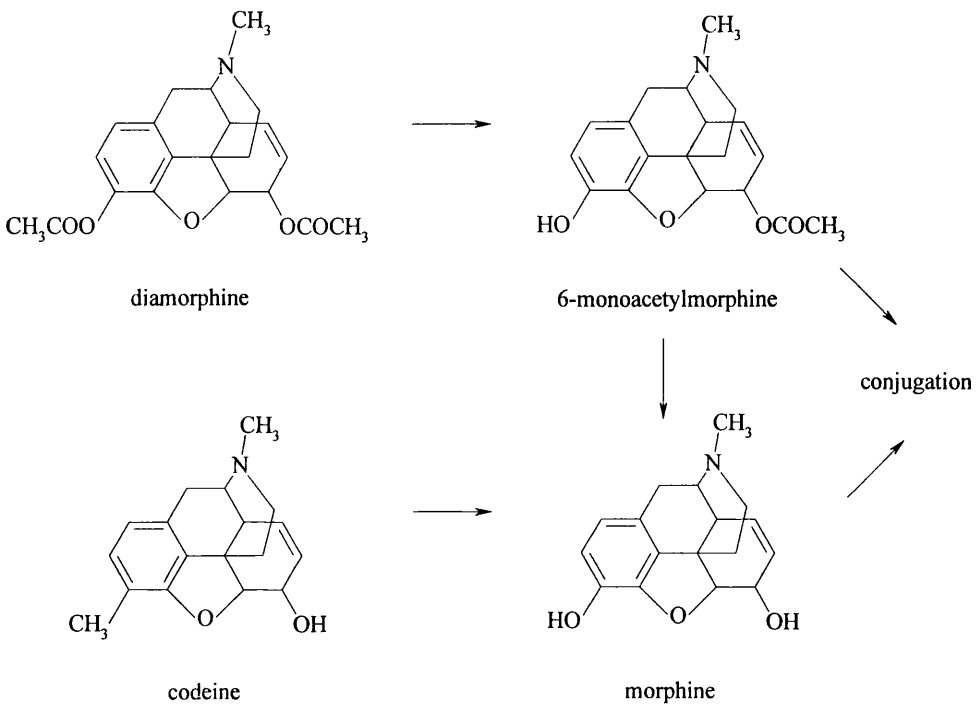


Figure 3.3: Metabolic route of diamorphine

3.4.2 METHADONE

Methadone is generally taken orally due to its complete oral bioavailability. It is metabolized in the liver mainly by demethylation with immediate cyclization to give 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) which is further demethylated producing 2-ethyl-5-methyl-3,3-diphenylpyrrolidine (EMDP). Hydroxylation of the three compounds occurs to a small extent and glucuronide formation follows. A minor proportion of methadone is metabolized to methadol and then normethadol which are the only metabolites with pharmacologic activity (Figure 3.4).

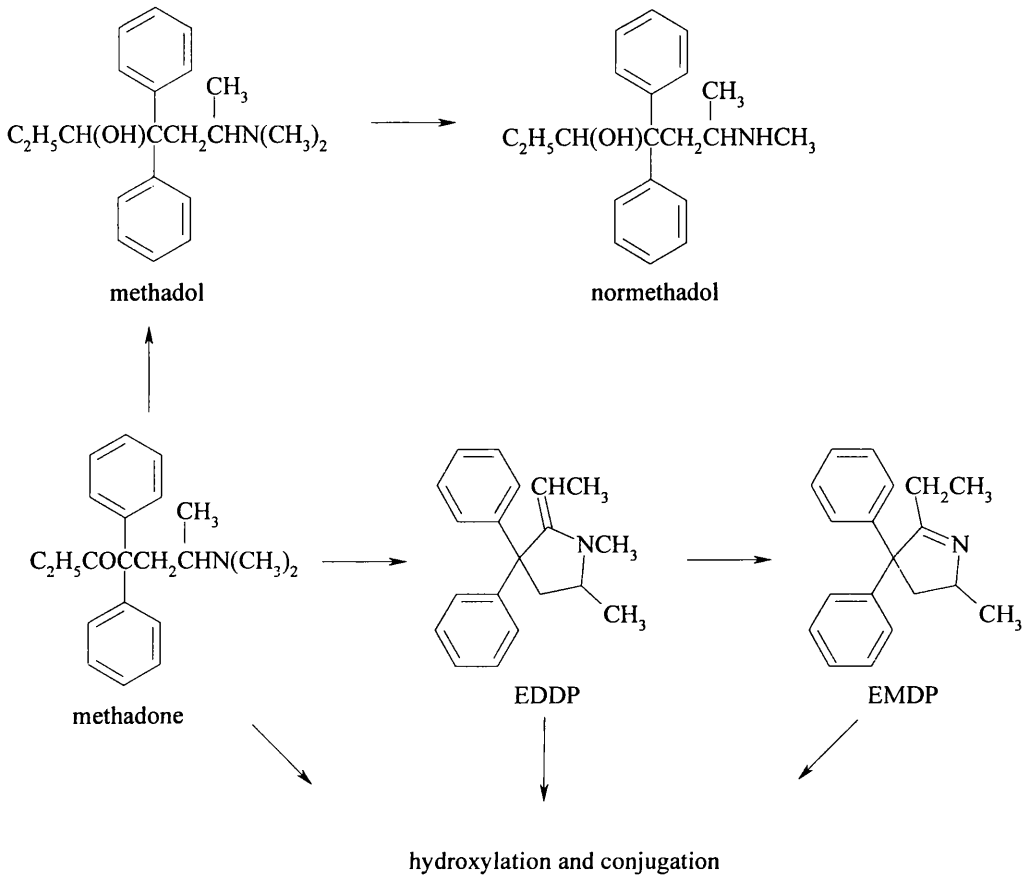


Figure 3.4: Metabolic route of methadone

3.5 PHARMACOKINETICS

3.5.1 OPIATES

Peak diamorphine concentrations are reached rapidly (1 to 5 minutes) after smoking or intravenous use.[65] The half-life of diamorphine is very short as it is rapidly deacetylated to 6-monoacetylmorphine. Pharmacokinetic data for opiates is summarized in Table 3.1.[62]

Table 3.1: Pharmacokinetic data for opiates

	Diamorphine	Morphine	Codeine	6-MAM
Half life	2-6 min	2-3 hours	1.9-3.9 hours	6-25 min
Vd (L/kg)	25	2-5	3.5	-
Protein Binding (%)	40	35	82	-
pKa	7.6	8.1, 9.5	8.2	-

3.5.2 METHADONE

Methadone is generally administered orally and reaches peak plasma levels four hours after ingestion.[64] A peak plasma concentration of 75 ng/ml was reported[66] after a single oral dose of 15 mg and concentrations were shown to decrease slowly with a half-life of 15 hours. Methadone is highly plasma protein bound and distributes rapidly to tissues. Pharmacokinetic parameters for methadone are summarized in Table 3.2.[62]

Table 3.2: Pharmacokinetic data for methadone

	Methadone
Half life	15-55 hours
Vd (L/kg)	4-5
Protein Binding (%)	87
pKa	8.6

3.6 TOXICITY

3.6.1 OPIATES

Sedation is the first sign of opiate intoxication. Excessive doses of opiates can cause stupor and coma and lead to respiratory failure especially in cases where a loss of tolerance or unexpected potency has been experienced or when other depressant drugs are present.[67] Where street heroin is involved there are a number of uncertainties such as the composition and actual purity of the sample and adverse reactions not only due to the active component, but adulterants are possible. For morphine, administration of 30 mg parenterally and 100 mg orally are toxic to a nontolerant individual, and death may occur following a 120 mg dose of morphine.[62] In the Department of Forensic Medicine and Science, University of Glasgow, blood concentrations found in individuals where morphine was implicated in their deaths either alone or in combination with other drugs ranged from 0.02 - 4.7 mg/L.[4]

3.6.2 METHADONE

Overdosage with methadone can produce stupor, respiratory depression, muscle flaccidity, cold and clammy skin, pupillary constriction, hypotension, coma and circulatory collapse.[62] The Department of Forensic Medicine and Science, University of Glasgow has detected methadone concentrations ranging from 0.02 - 11.3 mg/L in postmortem cases where methadone was considered a cause of death either alone or in combination with other drugs.[4]

3.7 DECONTAMINATION OF HAIR

Before hair samples are extracted, they must be washed to remove extraneous material including oils, grime and externally applied drug. This procedure should help clean the extract whilst preventing false positives from occurring. This stage is necessary to produce the correct drug historical record of an individual.

3.7.1 OPIATES

Numerous wash procedures have been carried out on hair samples being tested for opiates. All of these have involved several combinations of solvents such as methanol, ethanol, dichloromethane, acetone, distilled water and sodium dodecyl sulphate solution. However, few of these procedures have been rigorously tested for their efficiency.

3.7.2 METHADONE

Wash procedures have been compared for methadone analysis. These showed that the concentration detected in hair decreased when the hair was washed with a methanol-water method or a dodecylsulphate-water method.[68] These steps appeared to remove drug which was bound to the surface of the hair which had come from sweat contamination or possible drug handling. Like the opiates, various wash steps have been used by different authors. Water and acetone washes were a common combination, but methanol, sodium dodecylsulphate, dichloromethane and hexane have all been used as well.

3.8 SEPARATION OF ANALYTES FROM HAIR MATRIX

Analytes can be separated from the hair matrix in one of four ways: alkaline hydrolysis, acid hydrolysis, enzyme hydrolysis or direct solvent extraction with methanol or ethanol.

3.8.1 OPIATES

The first published method to extract opioids from hair involved refluxing the hair in methanol for 2 hours and subsequent analysis used radioimmunoassay (RIA).[69] Since this initial discovery, other authors have made comparisons of several methods to find the most efficient method of separation. These methods have included treatment with 0.1M hydrochloric acid,[70, 71, 72] 0.1M sodium hydroxide solution,[70] methanol,[42, 70, 72, 73] water,[70] pH 7.4 buffer,[70] 1M sodium hydroxide solution,[74, 75, 76] methanol-5M HCl,[72] methanol-trifluoroacetic acid,[72] enzymes such as 1 mg/ml pronase solution[76] or helicase.[72] When the detection of 6-MAM in hair was discovered complete hydrolysis of the hair using strong

alkaline solutions could not be used as a method due to the hydrolysis of 6-MAM to morphine by this technique. In one study, a mixture of methanol-TFA was shown to be the best solvent for extracting 6-MAM and morphine from hair with minimum hydrolysis and maximium efficiency.[77] Other authors[78] demonstrated that out of ten solvents of varying polarities and hydrophilicities, the highest recoveries for morphine were obtained using methanol with 1 % acetic acid or methanol with 1 % triethylamine. However, a reduction in heroin recoveries occurred and in the majority of cases a reduction in 6-MAM was also reported. These solvent systems undoubtly caused hydrolysis of heroin to 6-MAM and hydrolysis of 6-MAM to morphine and it was concluded that methanol was the most suitable solvent for opiate extraction from hair.

3.8.2 METHADONE

The first method used to remove methadone from hair was acid hydrolysis using hydrochloric acid.[79, 80, 81] Other methods have included mechanical pulverization followed by solid phase extraction,[30] alkaline hydrolysis prior to solid phase microextraction,[82, 83] enzyme hydrolysis, [30, 84, 85] methanol [86] and methanol-trifluoroacetic acid extraction.[87]

3.9 EXTRACTION

3.9.1 OPIATES AND METHADONE

The majority of hair analysis papers have involved using liquid-liquid extraction (LLE) or solid phase extraction (SPE). Several authors have reported the advantages of using SPE over LLE. However, other newer extraction methods such as supercritical fluid extraction for opiates[88] and solid phase microextraction for methadone[82, 83, 85, 89] have been employed.

3.10 ANALYSIS

Analytical methods for hair analysis require sensitive techniques to measure the small quantities of drug incorporated into hair. GC/MS is the most commonly used technique for the detection of drugs in hair although the use of more sensitive methods such as liquid chromatography-mass spectrometry, tandem mass spectrometry, high resolution mass spectrometry and capillary electrophoresis is increasing. However, these instruments are not often available in many laboratories due to their expense.

3.10.1 OPIATES

Opiates have been detected by immunoassay methods such as radioimmunoassay,[69, 70, 71, 74, 76] enzyme immunoassay (EIA)[75, 87] and fluorescence polarization immunoassay (FPIA).[75] High Performance Liquid Chromatography (HPLC) is less commonly used for detection of drugs in hair. HPLC with fluorescence detection,[90] HPLC with an electrochemical coulometric-array detector,[91] tandem mass spectrometry,[92, 93] ion trap GC/MS in chemical ionization mode[94] and capillary electrophoresis[95, 96] have all been used for morphine, but most methods have employed GC/MS analysis.

3.10.2 METHADONE

Immunoassay procedures for methadone have included radioimmunoassay[79, 80, 81] and enzyme-linked immunosorbent assay (ELISA).[87] High performance liquid chromatography with electrochemical coulometric-array detection [91] has been used to detect methadone in hair and liquid chromatography/ion spray-mass spectrometry [84] has been used to separate optical isomers of methadone and its metabolite. In most analyses of methadone in hair, gas chromatography-mass spectrometry has been used.

3.11 DRUG LEVELS IN HAIR

3.11.1 OPIATES

Levels found for morphine, codeine and 6-monoacetylmorphine in head hair from heroin abusers have been reported as 1.0 - 21.8 ng/mg morphine, 0.1 - 4.0 ng/mg codeine and 2.0 - 74.2 ng/mg 6-monoacetylmorphine.[97] Other authors reported morphine levels higher than these along with lower 6-monoacetylmorphine levels. In these cases sodium hydroxide hydrolysis was used in the method and a considerable amount of 6-monoacetylmorphine would have been hydrolyzed to morphine.

3.11.2 METHADONE

RIA methods found levels in the region of 0.2 - 10.63 ng/mg for methadone in head hair.[68] Levels of methadone and EDDP detected in hair using solid phase extraction as the extraction method ranged from 0 - 42.0 ng/mg and 0 - 2.4 ng/mg.[30] In one case where solid phase microextraction (SPME) was used as the extraction method, methadone and EDDP levels as high as 78.1 ng/mg and 7.76 ng/mg were reported.[85] For another SPME method EMDP was also reported positive in two cases at concentrations 0.18 and 0.84 ng/mg.[83]

3.12 EXPERIMENTAL

3.12.1 CHEMICALS

Standard solutions of morphine-d_3, codeine, codeine-d_3, methadone, methadone-d_3 were supplied by Sigma® Chemicals Co. (St. Louis, USA). Morphine, 6-monoacetylmorphine, 6-monoacetylmorphine-d_3, methadone-d_9, EDDP, and EDDP-d_3 were produced by Radian International and obtained from Promochem Ltd. (Hertfordshire, England). Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) with 1 % trimethylchlorosilane was obtained from Pierce (Illinois, USA). HPLC grade acetone, chloroform, dichloromethane, methanol were obtained from Lab-Scan Analytical Sciences (Dublin, Ireland). Analytical grade potassium dihydrogen phosphate, sodium hydroxide and sodium bicarbonate were obtained from BDH Laboratory Supplies (Poole, England). Sodium dodecyl sulphate, β-glucuronidase (Helix pomatia) and pentafluoropropionic anhydride (PFPA) were obtained from Sigma® Chemicals Co. (U.K.) and analytical grade concentrated ammonia was obtained from Merck (Poole, England).

3.12.2 STANDARDS

Stock standard solutions were obtained at concentrations 1 mg/ml or 100 μg/ml in methanol for all standards except 6-monoacetylmorphine and its deuterated standard which were dissolved in acetonitrile. Working solutions were made up at 10 μg/ml and 1 μg/ml by appropriate dilution of the stock standards. All standard solutions were stored at 4 °C.

3.12.3 SOLUTIONS

0.1 M Phosphate buffer, pH 6.0

Potassium dihydrogen phosphate (6.81 g) was dissolved in 450 ml of distilled water and the pH adjusted to 6.0 with 1.0 M potassium hydroxide solution. The solution was made up to the 500 ml mark with distilled water.

3.12.4 GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Two different Gas Chromatography-Mass Spectrometry (GC/MS) instruments were used to complete this work. The first of these was involved solely in the method development stage and the second of these in the method development and validation stages.

The first instrument used was a Hewlett-Packard model 5890 Gas Chromatograph with a VG Analytical VG 70-250S Mass Spectrometer. The GC was fitted with a Zebron-1, 30 m x 0.25 mm x 0.25 μm film thickness and the temperature programme was set from 180 to 300 °C at 10 °C/minute. The injector temperature was 280 °C and the carrier gas was helium. The temperature of the source and interface were 200 °C and 250 °C respectively. The trap current was set at 200 μA. The injection volume was 1 μl.

The second instrument was a ThermoQuest Trace GC with a Finnigan Trace MS. The GC was fitted with a HP-5 column, 30 m x 0.25 mm x 0.25 μm film thickness and the temperature programme was set from 150 to 300 °C at 10 °C/minute. The injector temperature was 250 °C and the carrier gas was helium. The temperature of the source and interface were 200 °C and 250 °C respectively. The emission current was 350 μA. The injection volume was 1 μl.

3.12.5 DERIVATIZATION

Two derivatization methods were used. An unextracted standard containing 100 μl of morphine, morphine-d_3, codeine, codeine-d_3, 6-monoacetylmorphine at a concentration of 10 μg/ml and 40 μl of methadone and EDDP at a concentration of 10 μg/ml was made up in duplicate. Methadone-d_3, 6-MAM-d_3 and EDDP-d_3 were not available at this stage. One standard was derivatized with PFPA and the other with BSTFA as detailed below and these were run on the VG-Analytical MS using full scan analysis.

3.12.5.1 Derivatization with PFPA

The standard was evaporated to dryness under nitrogen at 50 °C. 100 μl ethyl acetate and 100 μl PFPA were added to the residue, vortex mixed and derivatized at 60 °C for 30 minutes. The solvent was evaporated off under nitrogen at 50 °C and the residue was redissolved in 50 μl of ethyl acetate.

3.12.5.2 Derivatization with BSTFA

The standard was evaporated to dryness under nitrogen at 50 °C and 50 μl BSTFA with 1 % TMCS was added, vortex mixed and derivatized at 70 °C for 15 minutes.

3.12.5.3 Results

The following retention times and ions were observed for the derivatized analytes:

Table 3.3: GC/MS data for opiates and methadone

Analyte	Retention time (min:sec)		Main Ions observed (m/z)	
	PFPA	BSTFA	PFPA	BSTFA
EDDP*	6:52	6:52	178, 220, 277	178, 220, 277
Methadone*	7:44	7:44	72, 165, 294	72, 165, 294
Codeine	9:02	10:22	282, 304, 423, 445	178, 196, 234, 371
Codeine-d_3	8:59	10:19	207, 285, 448	181, 199, 237, 374
Morphine	8.37	10:47	414, 577	236, 371, 414, 429
Morphine-d_3	8.34	10:44	417, 580	239, 417, 432
6-MAM	9:37	11:18	204, 361, 414, 473	207, 340, 399

* Analytes which do not derivatize

Symmetrical peaks were obtained for all analytes except for codeine which had been derivatized with PFPA. Frontal tailing was observed for this peak so derivatization by BSTFA was used for all further analyses. Mass spectra for the analytes and internal standards derivatized with BSTFA are shown in Figures 3.5 and 3.6.

3.12.6 GAS CHROMATOGRAPHY/MASS SPECTROMETRY – SELECTED ION MONITORING (SIM)

A selected ion monitoring programme was set up for the analytes. Ions monitored were m/z 277 for EDDP, 371 for codeine, 374 for codeine-d_3, 429 for morphine, 432 for morphine-d_3, 399 for 6-MAM and 402 for 6-MAM-d_3. For methadone m/z 72 was used because of its higher sensitivity compared with other methadone ions. This meant that a separate programme had to be set up since the VG-Analytical Mass Spectrometer which was the only instrument available at the time could not simultaneously carry out single ion monitoring for the range of ions selected. Each sample was injected twice using the same temperature programme for methadone in the first run and the other analytes in the second run. Perfluorokerosene (PFK) was used to calibrate the instrument and lock ions 68.9952 and 268.9824 a.m.u. were used for analysis of methadone and other analytes respectively. Switch and dwell times were set at 60 ms and 40 ms respectively for each monitored ion.

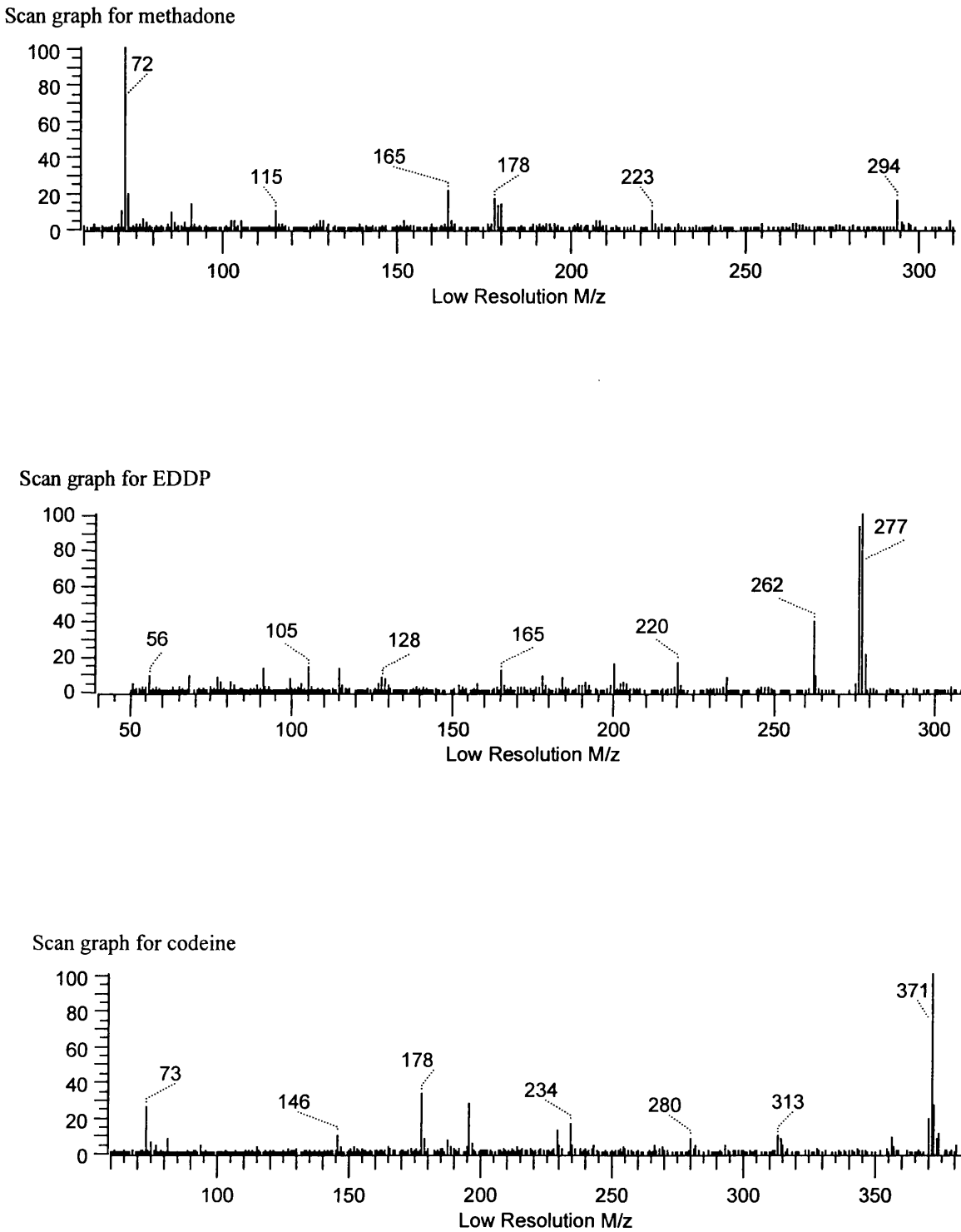
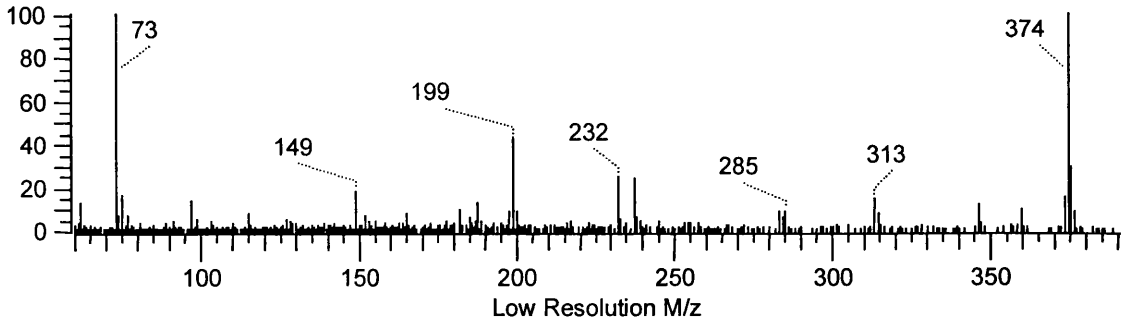
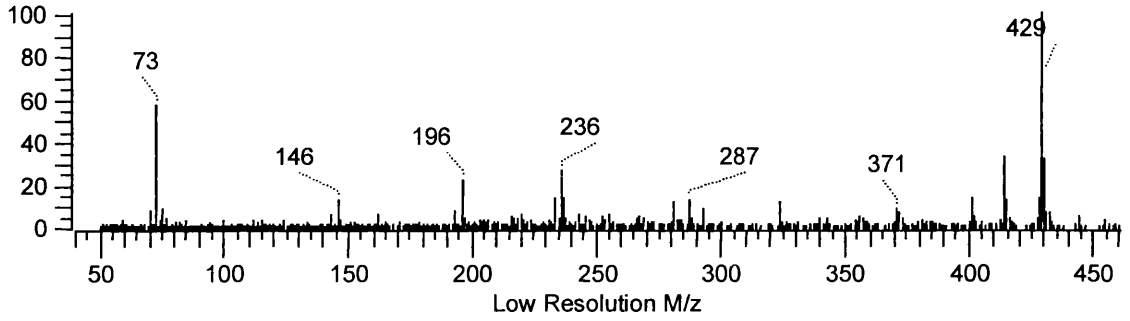


Figure 3.5: Ion graphs for methadone, EDDP and codeine as trimethylsilyl derivative

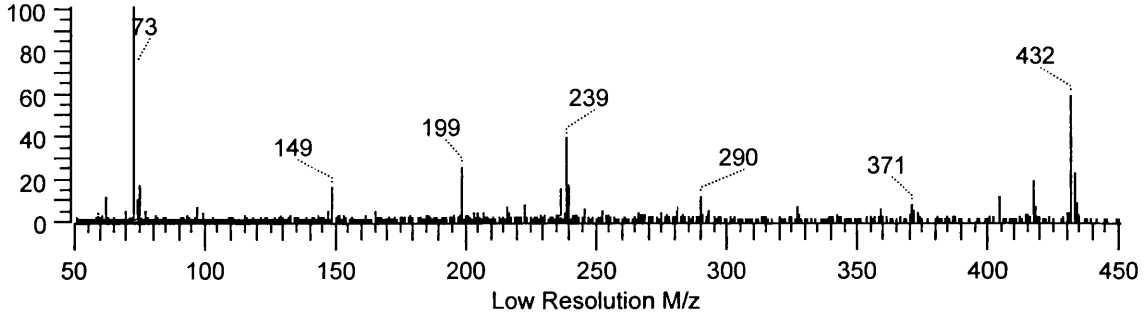
Scan graph for codeine-d_3



Scan graph for morphine



Scan graph for morphine-d_3



Scan graph for 6-monoacetylmorphine

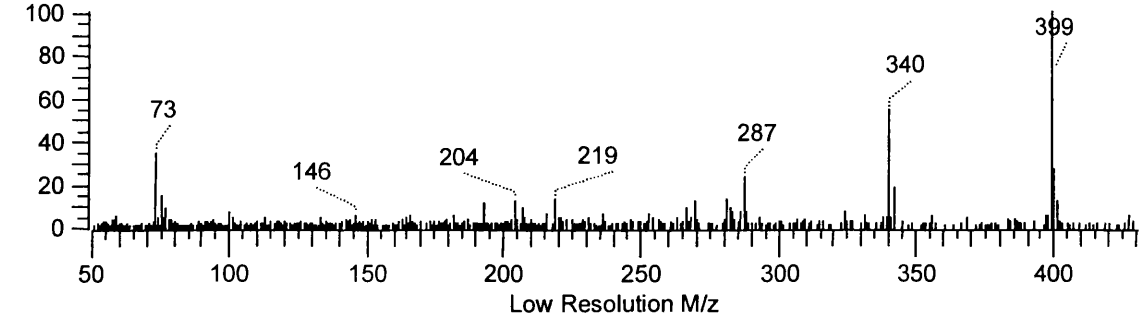


Figure 3.6: Ions graphs of codeine-d_3, morphine, morphine-d_3 and 6-monoacetylmorphine as trimethylsilyl derivatives

3.12.7 DECONTAMINATION OF HAIR

Blank hair was obtained from a volunteer of Malay-Chinese origin. Hair was cut finely into 1-2 mm pieces with scissors and was decontaminated using several sequential wash procedures:

(i) One ten minute wash with sodium dodecyl sulphate solution (0.1%).
(ii) Three ten minute washes with distilled water.
(iii) Three two minute washes with methanol.

Each step was sonicated in 5 ml of solvent and then the solvent was decanted. The hair was allowed to dry in an oven at 40 °C.

3.12.8 SPIKING HAIR

Hair was spiked in one of two ways. Single portions of blank washed hair were weighed out, each at 30 mg, and the appropriate amount of each drug was pipetted into the glass vial containing the weighed hair. The hair was then covered with excess methanol (if required) and the samples were sonicated at room temperature for 15 minutes. The methanol was allowed to evaporate off at room temperature. The alternative method was to spike blank washed hair in bulk to give standards equivalent to 10, 25, 50, 75, 100 ng/30 mg. The method of spiking was the same as described previously. The appropriate amount of sample was weighed out accurately.

3.12.9 METHANOLIC TREATMENT OF HAIR SPIKED WITH OPIATES FOLLOWED BY LIQUID-LIQUID EXTRACTION

3.12.9.1 Methanolic Treatment

A published method[42] was initially used to extract the drugs from hair. Two 50 mg samples of hair spiked with 25 ng and 100 ng of morphine, codeine and 6-MAM were weighed into vials. Methanol (1 ml) was added to each along with 100 µl of morphine-d_3, 100 µl codeine-d_3 and 100 µl 6-MAM-d_3 both at a concentration of 1 µg/ml. The hair was then incubated at 37 °C for 18 hours. After incubation, the methanol was removed using a glass pipette and evaporated at 50 °C under nitrogen.

3.12.9.2 Liquid-liquid extraction

The methanolic extracts were purified further by liquid-liquid extraction. To the residue, 2 ml of distilled water followed by 2 ml of saturated sodium bicarbonate buffer, adjusted to pH 8.4 with 1 M sodium hydroxide solution were added. This was vortex mixed for 15 seconds and 7 ml of toluene-heptane-isoamylalcohol 70:20:10 were added followed by mixing for 15 minutes and centrifuging for 10 minutes at 2000 rev/min. The organic layer was transferred and evaporated at 50 °C under nitrogen. The extracts were derivatized with 40 µl of BSTFA with 1 % TMCS at 70 °C for 15 minutes and analyzed using GC/MS with selected ion monitoring as described in 3.12.6.

3.12.9.3 Results

All three analytes were extracted. A blank extract showed that no extracted hair impurities interfered with the codeine, morphine or 6-MAM peaks. Ratios of the analytes to internal standards were calculated and are displayed in Table 3.4. Methadone was now to be included in the procedure.

Table 3.4: Ratios of opiate analytes/internal standards

Sample	codeine/codeine-d_3 m/z 371/374	morphine/morphine-d_3 m/z 429/432	6-MAM/6-MAM-d_3 m/z 399/402
100 ng/40 µl unextracted standard	0.955	0.987	0.966
100 ng/50 mg extracted standard	0.991	1.127	0.811
25 ng/50 mg extracted standard	0.309	0.208	0.171

3.12.10 METHANOLIC TREATMENT OF HAIR SPIKED WITH OPIATES, METHADONE AND EDDP FOLLOWED BY LIQUID-LIQUID EXTRACTION

3.12.10.1 Experimental

A method to extract morphine, codeine, 6-MAM, methadone and EDDP simultaneously from hair was required. The method used previously (3.12.9) was applied to hair samples which had been spiked with 0, 10, 25, 50, 75, 100 ng/50 mg of each of the analytes. Extractions were also carried out using the same amount of each drug without the presence of hair. Internal standards morphine-d_3, codeine-d_3, 6-MAM-d_3 and methadone-d_3 were added at a concentration of 50 ng/50 mg prior to the methanol incubation step.

3.12.10.2 Results

Extracts without presence of hair showed that all analytes were successfully extracted using this method. However, when the hair spikes were analysed it was shown that EDDP coeluted with peaks resulting from the hair matrix. Quantitation of EDDP therefore was not possible using this extraction method. At the lower concentrations, it also became more difficult to quantitate codeine, morphine and 6-MAM. This corresponds to previous reports using liquid-liquid extraction as a technique to extract drugs from hair where the assay was limited by interference.[98] Solid phase extraction is therefore a better technique to use since cleaner extracts are generally produced. For this reason further analyses used solid phase extraction as the method of extraction.

3.12.11 METHANOLIC TREATMENT OF HAIR SPIKED WITH OPIATES, METHADONE AND EDDP FOLLOWED BY SOLID PHASE EXTRACTION

3.12.11.1 Methanolic Treatment

Hair spiked to give concentrations 50 and 100 ng/50 mg of morphine, codeine, 6-MAM, methadone and EDDP was incubated at 37 °C for 18 hours in 1 ml of methanol. After incubation the methanol was removed and a further 1 ml portion was added to the hair, sonicated briefly and combined with the first fraction. The methanol was evaporated to dryness at 50 °C under nitrogen.

3.12.11.2 Solid Phase Extraction

This solid phase extraction method was currently being used in the laboratory to extract opiates from blood and saliva.

The residues were re-dissolved in 2 ml of distilled water and 2 ml of phosphate buffer, pH 6.0. They were vortex mixed and centrifuged for 10 minutes at 2500 rev/min. World Wide Monitoring Clean Screen® columns (ZSDAU020) were conditioned with 3 ml of methanol, followed by 3 ml of distilled water and then 1 ml phosphate buffer. The samples were applied to the columns at 2 ml/min. which were then washed with 2 ml distilled water, 2 ml 0.1 M hydrochloric acid and 3 ml methanol. The columns were dried under full vacuum for 10 minutes and the analytes were eluted with 4 ml of dichloromethane/

isopropanol/concentrated ammonia (78:20:2). The solvent was evaporated off and the residue derivatized in 40 µl BSTFA with 1% TMCS.

3.12.11.3 Results

The extraction was very clean for morphine, codeine, 6-MAM and EDDP, but comparison of extracts containing no hair with those containing hair showed that peaks from the hair matrix coeluted with methadone and methadone-d_3 ions m/z 72 and 75 respectively.

Several steps were attempted to improve the method for methadone. The first of these was to add another clean-up step to the solid-phase extraction procedure.

3.12.12 METHANOLIC TREATMENT OF HAIR SPIKED WITH OPIATES, METHADONE AND EDDP FOLLOWED BY SOLID PHASE EXTRACTION (METHOD II)

3.12.12.1 Experimental

Blank hair samples and hair samples spiked to give 50 ng/30 mg were extracted using the procedure in 3.12.11 which was modified to include an additional acetone/chloroform step. The acetone/chloroform step involved adding 4 ml of acetone/chloroform (1:1) to the columns before the final elution step. Acetone/chloroform and dichloromethane/ isopropanol/ammonia fractions were collected and analyzed to find if the additional step removed peaks that coeluted with methadone.

3.12.12.2 Results

The acetone/chloroform fraction step cleaned up the the final extract considerably. This was shown by analyzing the acetone/chloroform extract obtained from blank hair which indicated that the peak which had previously coeluted with methadone m/z 72 eluted in this fraction. Analysis of the dichloromethane/isopropanol/ammonia fraction for ion m/z 72 showed that the methadone peak was now separated. However, this was not the case for the methadone-d_3 ion m/z 75. The blank dichloromethane/isopropanol/ammonia fraction still showed coelution with peaks resulting from the hair matrix.

Analysis of the two fractions for opiates and EDDP revealed that these analytes only eluted in the dichloromethane/isopropanol/ammonia fraction.

These results showed that the methadone-d_3 m/z 75 ion was not ideal for quantification using this method and two other options were considered. Alternative ions, for instance m/z 294 and 297 could be used for methadone and methadone-d_3 respectively, however the intensities of these ions are greatly reduced from ions m/z 72 and 75 and therefore using these ions for quantitation would mean the limit of detection would be much higher. Alternatively a different internal standard such as methadone-d_9 could be used to see if ion m/z 78 could be used. It was the second of these options which was considered first. Also, it has been suggested by other authors that methanolic treatment of hair prior to extraction provides less clean extracts than other methods such as acid hydrolysis. A comparison of these two methods using methadone-d_9 as the internal standard was carried out.

3.12.13 ACIDIC HYDROLYSIS VERSUS METHANOLIC TREATMENT

3.12.13.1 Sample Preparation

Blank hair samples and hair samples spiked with 50 ng/30 mg and 75 ng/30 mg of codeine, morphine, 6-MAM, methadone and EDDP were weighed out in duplicate to give 30 mg samples. Internal standards codeine-d_3, morphine-d_3, 6-MAM-d_3, methadone-d_9 and EDDP-d_3 were added at a concentration of 50 ng/30 mg.

3.12.13.2 Methanolic Treatment

One set of samples was treated with 1 ml of methanol and incubated in the oven at 45 °C for 18 hours. The samples were cooled and the methanol was drawn off and the hair rinsed with another 1 ml aliquot of methanol. The second methanol aliquot was combined with the corresponding first aliquot.

3.12.13.3 Acid Hydrolysis

The other set of hair samples was hydrolysed with 0.1M hydrochloric acid for 12 hours at 45 °C. The samples were cooled, neutralized with 0.1M sodium hydroxide solution and 1 ml phosphate buffer (0.1M, pH 6) was added. The sample was shaken, centrifuged at 2500 rev/min. and the solution was removed from the hair. The hair was rinsed with 1 ml phosphate buffer, centrifuged and the phosphate buffer fractions were combined.

3.12.13.4 Extraction

The extraction procedure used was that in 3.12.12 (including the acetone/chloroform step). Ions m/z 72 and 294 were monitored for methadone and m/z 78 and 303 were monitored for methadone-d_9.

3.12.13.5 Results

All analytes were extracted by both methods and no interfering peaks were identified at corresponding retention times to analytes in the blank samples.

Similar ratios of analyte:internal standard were obtained for all the analytes, however it was noticed that the peak area of 6-MAM and 6-MAM-d_3 were much lower for the acid hydrolysis method than the methanol method. This may have been due to hydrolysis of the 6-MAM to morphine during the acidic step, however the morphine peak areas did not clearly reflect this and a recovery study was required to investigate this.

3.12.14 RECOVERY STUDY OF EXTRACTION PROCEDURE

3.12.14.1 Gas Chromatography-Mass Spectrometry

At this stage in the method development a new GC/MS instrument was available in the laboratory which made it possible to analyze the chosen ions for methadone and opiates simultaneously. This instrument was a ThermoQuest Trace GC with a Finnigan Trace MS The GC was fitted with a HP-5 column, 30 m x 0.25 mm x 0.25 μm film thickness and the temperature programme was set from 150 to 300°C at 10 °C/minute. The injector temperature was 250°C.

All further analyses were run on this instrument since this reduced the analysis time.

3.12.14.2 Extraction

A recovery study was carried out to determine the recovery of each drug using the solid phase extraction procedure without the presence of hair. Ten standards containing 50 ng of each analyte were made up. The methanol was evaporated to dryness and five of the vials were redissolved in 2 ml of distilled water and 2 ml of phosphate buffer, pH 6.0. The remaining five were redissolved in 2 ml distilled water and 2 ml of phosphate buffer, pH 7.0. These were then extracted using the solid phase extraction method.

3.12.14.3 Results

The recoveries obtained using this method were high for all of the drugs using both pH's of buffer (Table 3.5). However, EDDP and codeine gave higher recoveries with pH 6.0 and the other analytes gave comparable recoveries at the two pH's.

Table 3.5: Recovery study of SPE method without presence of hair

Sample	EDDP %	Methadone %	codeine %	morphine %	6-MAM %
pH 6.0	96.0	98.3	96.9	95.2	99.5
pH 7.0	87.7	96.6	88.4	99.8	99.4

Phosphate buffer at pH 6.0 was used for further extractions.

3.12.15 RECOVERY STUDY: METHANOLIC TREATMENT VERSUS ACID HYDROLYSIS VERSUS ENZYME HYDROLYSIS

Recoveries using three different pre-extraction treatments were compared. These included the methanolic treatment and hydrochloric acid methods (3.12.13.2 and 3.12.13.3) and also an enzymatic method using β-glucuronidase.

3.12.15.1 β-glucuronidase method

To 30 mg hair samples was added 1 ml phosphate buffer, pH 6.0 followed by 50 µl β-glucuronidase solution. β-glucuronidase solution was made up by adding 1 ml β-glucuronidase (Helix pomatia 132 500 units/ml) to 4 ml phosphate buffer, pH 6.0 and vortexing briefly. The hair samples were incubated at 40 °C for 2 hours and after cooling, the solution was drawn off. The hair was washed with 1 ml phosphate buffer and this solution was combined with the first. The combined phosphate buffer fractions were applied to the solid phase extraction columns.

3.12.15.2 Results

The β-glucuronidase method clearly hydrolyzed 6-MAM to morphine, giving morphine a recovery of 182 % (Table 3.6). The acid hydrolysis method also hydrolyzed some of the 6-MAM to morphine. Methanol treatment extracted the analytes from spiked hair with high recoveries and no or minimal hydrolysis of 6-MAM to morphine.

Table 3.6: Recoveries on spiked hair using methanol treatment, acid and enzyme hydrolysis

Method	EDDP %	Methadone %	codeine %	morphine %	6-MAM %
Methanol	82.4	78.3	87.1	82.3	91.5
Hydrochloric acid (0.1M)	76.0	80.7	87.6	112.2	58.2
β-glucuronidase	76.7	67.2	90.3	182.6	3.8

3.12.16 INVESTIGATION INTO HYDROLYSIS OF 6-MAM TO MORPHINE BY ACID HYDROLYSIS METHOD

3.12.16.1 Experimental

Nine 30 mg portions of blank hair were weighed out into separate vials. Three were spiked with 6-MAM only, three with morphine only and three with both 6-MAM and morphine at a concentration of 50 ng/30 mg. These were all treated using the acid hydrolysis method and internal standards were added after the extraction.

3.12.16.2 Results

Recoveries were calculated for morphine and 6-MAM in each sample and these are displayed below in Table 3.7. They clearly show that under these hydrolysis conditions 6-MAM is hydrolyzed to morphine giving a conversion of almost 45 %. Therefore this method cannot give a true reflection of the concentrations in hair.

Table 3.7: Recovery of morphine and 6-MAM after acidic hydrolysis

	% 6-MAM recovered	% Morphine recovered
6-MAM only	23.3 ± 6.8	43.5 ± 2.8
Morphine only	-	73.3 ± 6.6
6-MAM and morphine	24.8 ± 7.7	111 ± 0.4

3.12.17 METHOD VALIDATION

3.12.17.1 Linearity

Since the enzyme and acidic methods caused a certain degree of hydrolysis of 6-MAM to morphine it was decided to validate the methanolic method. Linearity was determined for the concentration range of 10 to 100 ng/30 mg hair. A larger quantity of hair could have been chosen, but since the hair case samples were to be sectioned the likelihood of obtaining more than 30 mg per centimetre of hair was low. Five samples at each concentration were extracted. Ratios of drug to internal standard are displayed in Table 3.8 along with the coefficient of variation in parenthesis. Values of correlation coefficient, R^2 and intercept and slope values for regression equations are displayed in Table 3.9.

Table 3.8: Intra-day Variation

Concentration (ng/30 mg hair)	meth/meth- d_9 m/z 72/78	EDDP/EDDP- d_3 m/z 277/280	cod/cod-d_3 m/z 371/374	mor/mor-d_3 m/z 429/432	MAM/MAM- m/z 399/402
10	0.217 (8.9)	0.264 (7.8)	0.188 (5.4)	0.123 (9.7)	0.186 (6.7)
25	0.522 (6.4)	0.537 (5.6)	0.415 (2.4)	0.386 (9.7)	0.510 (7.3)
50	1.201 (8.8)	0.963 (8.5)	0.977 (8.4)	0.820 (5.1)	1.012 (6.9)
75	1.793 (8.1)	1.504 (7.6)	1.465 (8.6)	1.244 (6.7)	1.473 (5.7)
100	2.266 (5.2)	2.049 (2.7)	1.932 (5.7)	1.679 (6.9)	1.887 (8.8)

Table 3.9: Linear Regression Values

	Methadone	EDDP	Codeine	Morphine	6-MAM
R^2	0.9963	0.9970	0.9983	1.0000	0.9978
Intercept	- 0.0138	0.0348	-0.0327	-0.0469	0.0297
Slope	0.0233	0.0198	0.0198	0.0172	0.0189

3.12.17.2 Inter-day variation

Inter-day variation was assessed for the same concentration range as the intra-day variation (Table 3.10). These values are based on ratios obtained over five days and these were less than 13 % for all the drugs over the concentration range.

Table 3.10: Inter-day Variation

Concentration (ng/30 mg hair)	meth/meth- d_9 m/z 72/78	EDDP/EDDP- d_3 m/z 277/280	cod/cod-d_3 m/z 371/374	mor/mor-d_3 m/z 429/432	MAM/MAM-d_3 m/z 399/402
10	0.195 (9.8)	0.244 (8.2)	0.186 (12.9)	0.131 (7.7)	0.183 (5.7)
25	0.485 (9.7)	0.543 (2.8)	0.415 (8.4)	0.349 (6.5)	0.520 (2.5)
50	1.148 (8.8)	1.152 (7.9)	1.081 (8.8)	0.788 (3.5)	1.143 (9.3)
75	1.627 (8.9)	1.698 (7.6)	1.553 (5.4)	1.220 (6.7)	1.607 (7.4)
100	2.169 (4.3)	2.243 (6.6)	2.028 (4.9)	1.665 (6.1)	2.201 (8.3)

3.12.17.3 Recovery

The recovery of the method from spiked hair samples has already been mentioned in 3.12.15. However, the recovery from actual hair samples where the drug is bound within the hair will be lower than this. A better indicator of the true recovery can be calculated using fortified hair samples but these were not available. However, even these values are not precise. Recoveries of drugs from hair will vary to a certain extent for each individual due to parameters such as hair diameter, degree of preservation of cuticle and chemical changes to hair as a result of treatment.

3.12.17.4 Limit of Quantitation and Detection

Limits of detection and quantitation were determined for each of the drugs on spiked hair. 30 mg hair samples were spiked with each drug to give concentrations 2.0, 1.0, 0.75, 0.5, 0.4, 0.3, 0.2, and 0.1 ng/30 mg. These were extracted as previously. The limit of detection was defined as the concentration of drug giving a signal to noise ratio of 3 and the limit of quantitation was defined as the concentration of drug giving a signal to noise ratio of 5 (Table 3.11).

Table 3.11: Limit of Quantitation and Detection

	Methadone ng/30 mg	EDDP ng/30 mg	Codeine ng/30 mg	Morphine ng/30 mg	6-MAM ng/30 mg
LOD	0.75	2.0	1.0	1.0	1.0
LOQ	1.0	5.0	2.0	2.0	2.0

3.12.18 CONCLUSIONS

Comparisons of methanolic, β-glucuronidase and hydrochloric acid methods to extract opiates and methadone from hair showed that there was a considerable amount of hydrolysis of 6-MAM to morphine using the β-glucuronidase method and to lesser extent with the hydrochloric acid method. This would be accounted for using the deuterated internal standards, however, in cases where 6-MAM is present in hair at a low concentration then a negative result could be obtained. This would affect the interpretation of the results since a sample found positive for 6-MAM is indicative of heroin use. The methanolic extraction method did not give any significant conversion of 6-MAM to morphine and for this reason was the preferred method. This method successfully extracted morphine, codeine, 6-MAM, methadone and EDDP from spiked hair samples. Calibration curves were linear for all analytes producing correlation coefficients > 0.996. Intra-day and inter-day variation were assessed and coefficients of variation were shown to be < 10 % and < 13 % respectively for all analytes. Limits of quantitation and detection were found to be suitably sensitive. This method was applied to 31 postmortem case samples to assess the quantities of each analyte present in hair to give an indication of the extent of drug use of each individual and a drug use history (Chapter 8.1).

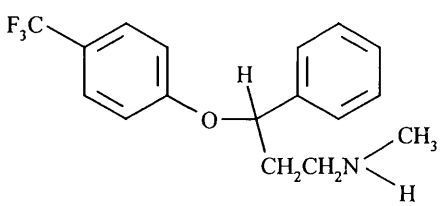
4 Antidepressants in blood

4.1 FLUOXETINE

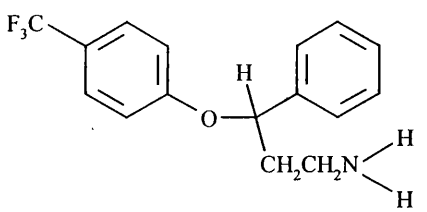
Fluoxetine (Prozac®) is an antidepressant which was first introduced into the United States in 1988 for the treatment of depression. It is a selective serotonin reuptake inhibitor (SSRI) which has been found to be as effective as the traditional antidepressants, but due to its safer pharmacological profile has become one of the most widely prescribed antidepressants. It has not only been used in the treatment of depression, but has also had efficacy in the treatment of obsessive compulsive disorders, bulimia nervosa and obesity.

4.2 CHEMISTRY

Fluoxetine is chemically known as (±)-N-methyl-γ-3-phenyl-3-[(α-α-α-trifluoro-p-tolyl)-oxy] propylamine and has a molecular formula $C_{17}H_{18}F_3NO$ and a molecular weight of 309. Norfluoxetine is produced by demethylation of fluoxetine and has a molecular weight of 295. The structures of fluoxetine and its metabolite are given in Figure 4.1.



Fluoxetine



Norfluoxetine

Figure 4.1: Chemical structures of fluoxetine and norfluoxetine

Fluoxetine and norfluoxetine can be obtained as hydrochloride salts. The hydrochloride salt of fluoxetine is soluble in water at 50 mg/ml and is stable at temperatures up to 50 °C for the duration of 2 years.

4.3 METABOLISM AND EXCRETION

Fluoxetine is rapidly transformed to norfluoxetine which has pharmacological activity equivalent to the parent drug. In a study where radiolabelled fluoxetine was administered to healthy volunteers at a dosage of 60 mg daily for 30 days, 60 % was excreted in the urine over a five week period. Only 2.5 % of this was unmetabolized drug, 10 % norfluoxetine and the remainder other conjugated metabolites (Figure 4.2).[99]

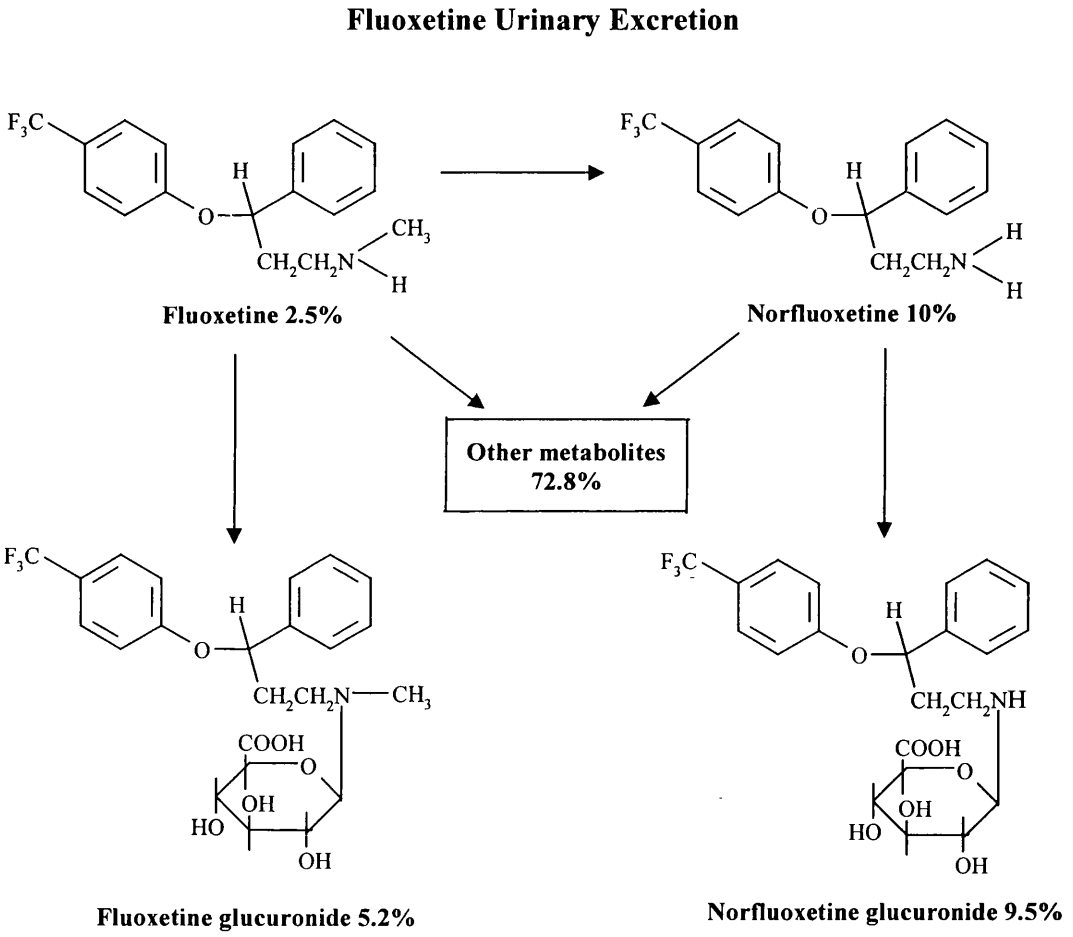


Figure 4.2: Fluoxetine metabolism and excretion

4.4 PHARMACOKINETICS

After oral dosing, fluoxetine is well absorbed and pharmacokinetic studies have shown that peak plasma concentrations are reached within 6 to 8 hours. A single oral dose of 40 mg produced peak plasma levels ranging from 0.015 - 0.055 mg/L within this time.[62] Daily doses of 20 - 60 mg have produced steady state levels ranging from 0.025 - 0.473 mg/L

fluoxetine and 0.018 - 0.466 mg/L norfluoxetine.[100] It has also been shown that at steady state, the plasma concentration of norfluoxetine is greater than that of fluoxetine and that steady state concentrations of fluoxetine or norfluoxetine do not change when the drug is administered over a long period of time.[99] The distribution of fluoxetine to tissues is extensive and the volume of distribution is large after single or multiple dosing. This combined with a low clearance rate leads to the long half-life which the drug possesses. Pharmacokinetic data is summarized in Table 4.1. [62]

Table 4.1: Pharmacokinetic data for fluoxetine and norfluoxetine

	Fluoxetine	**Norfluoxetine**
Half life	1-3 days	7-15 days
Vd (L/kg)	26	-
Protein Binding (%)	94	-
pK_a	9.5	-

4.5 TOXICITY

Side effects of fluoxetine treatment include nausea, anxiety, insomnia, headache and dry mouth. Excessive doses of fluoxetine causes symptoms such as lethargy, tachycardia and hypertension. Few authors have reported fluoxetine deaths where it was the sole drug detected in the blood. Drug concentrations in 6 fluoxetine fatalities (4 of these cases involving another drug) were found to range from 1.3 - 6.8 mg/L blood fluoxetine and 0.9 - 5.0 mg/L blood norfluoxetine.[62]

Although, fluoxetine is considered to be a much safer drug than the tricyclic antidepressants, the long half lives of fluoxetine and norfluoxetine can result in potentially serious drug interactions which are caused by substantial quantities of fluoxetine remaining in the plasma weeks after administration has ceased.

Fluoxetine in combination with monoamine oxidase inhibitors (MAOIs) can cause a serious interaction known as the Serotonin Syndrome. As a result of these interactions with fluoxetine, whenever treatment is changed from using fluoxetine to a MAOI, a period of five weeks between treatments is necessary to allow fluoxetine to be flushed from the system. The Serotonin Syndrome has also been observed with fluoxetine and lithium,[101] fluoxetine and carbamazepine,[102] and the serotonin precursor L-tryptophan.[103] Symptoms

of this interaction include euphoria, drowsiness, sustained rapid eye movement, overreaction of the reflexes, clumsiness, restlessness, feeling drunk and dizzy, muscle contraction and relaxation in the jaw, sweating, intoxication, rigidity, high body temperature, confusion, hypomania, shivering, diarrhea, loss of consciousness and death.[104]

4.6 ANALYSIS

Many analytical methods have been published for the analysis of fluoxetine and its metabolite in plasma or serum. Both gas and liquid chromatographic methods have proved popular choices.

One of the first methods to be published involved a liquid-liquid extraction procedure followed by derivatization with pentafluoropropionic anhydride and analysis by gas chromatography with electron capture detection (GC-ECD).[105] Other authors used heptafluorobutyric anhydride as an alternative derivatizing agent and reported an increase in sensitivity.[106] Other GC/ECD methods used solid phase extraction prior to analysis using either Bond Elut Certify[107] or Bond Elut C8[108] columns. These methods[107, 108] reported higher recoveries than liquid-liquid extraction methods.

Both fluoxetine and norfluoxetine exist as enantiomers. GC-ECD has also been used to analyse these enantiomers following their extraction from plasma and urine of humans, and liver and brain tissue of rats.[109] (S)-(-)-N-trifluoroacetylprolyl chloride was the chiral derivatizing agent employed. Other authors modified this method to allow simultaneous determination of fluvoxamine and the enantiomers of fluoxetine and norfluoxetine by gas chromatography-mass spectrometry.[110] Recoveries of these compounds ranged from 50 to 66 % and the limit of quantitation for the (R) and (S) enantiomers of fluoxetine and norfluoxetine was 1 ng/ml.

Methods for the analysis of fluoxetine and norfluoxetine by gas chromatography with nitrogen-phosphorus detection (GC-NPD) have also been developed.[111, 112] The nitrogen containing structure of fluoxetine is compatible with a high sensitivity by NPD and has proved to be a good method for analysing low concentrations of the drug in plasma. Detection limits of 0.3 ng/ml for fluoxetine and 2 ng/ml for norfluoxetine have been obtained using NPD detection.[112] Other authors used GC-NPD to simultaneously analyse

fluoxetine and desipramine which can be coadministered.[111] Limits of detection were not reported in this paper.

High performance liquid chromatography (HPLC) analysis for the determination of fluoxetine has been the most common method used for the identification and quantitation of the drug and its metabolite. These methods have involved the use of solid phase or liquid-liquid extraction with ultra violet (UV) or fluorescence detection.

One of the first methods developed to determine fluoxetine and norfluoxetine in serum used reversed phase HPLC with UV detection.[100] A multi-step extraction was used to isolate the drug and metabolite from the serum followed by reversed phase chromatography using a phenyl column. Recoveries of 80 % were achieved and the limit of detection was 15 ng/ml. A number of regularly administered drugs given to psychiatric patients were evaluated for their ability to interfere in the assay. Imipramine, desipramine, protriptyline and loxapine all coeluted with one of the analytes.

Another reversed phase HPLC assay using a cyano column and UV detection was established for the purpose of correlating serum fluoxetine and norfluoxetine concentrations to clinical response.[113] From results on thirteen patients receiving doses of 20 - 60 mg/day the fluoxetine and norfluoxetine serum concentrations were 73 - 453 ng/ml and 54 - 362 ng/ml respectively and it was found that antidepressant response and serum concentration were not correlated.

Other HPLC methods have involved pre-derivatization of fluoxetine and norfluoxetine with fluorescent detection providing more sensitive and selective assays.[114] Fluoxetine and its metabolite were extracted from plasma by liquid-liquid extraction and derivatized at room temperature with dansyl chloride. The fluorescent derivatives were chromatographed using a reversed phase C-18 column. Advantages of this method over UV methods are that the derivatizing agent is specific for primary and secondary amines and so no major antidepressants interfere in the assay. Therefore, the problem of co-elution of tricyclic antidepressants and secondary amines is resolved.

Another more recent method involved pre-column derivatization with dansyl chloride.[115] Simultaneous determination of fluoxetine, D-fenfluramine and D-norfenfluramine in

plasma, brain tissue and brain microdialysate was carried out using fluoxetine as the internal standard. The limit of quantitation for fluoxetine in plasma was 3.1 ng/ml. Although fluoxetine was used as an internal standard it was reported that this technique can be used for the analysis of fluoxetine using fenfluramine as the internal standard. Although these procedures involving a pre-derivatization step increase sensitivity and selectivity, the time taken to prepare the sample is extended.

Many of the reported assays require multi-step extraction methods to provide a sufficiently pure sample to be analysed by HPLC. A rapid, sensitive micromethod using a reversed phase liquid chromatographic procedure to separate and quantify fluoxetine and norfluoxetine in plasma after a single extraction step using mefloquine as an internal standard has been developed.[116] The method used a reversed phase C-8 column and detection at 226 nm, and was found to give an improved detection limit (2 ng/ml for both fluoxetine and norfluoxetine) over other UV methods, whilst determining the same specificity. A selection of drugs which are commonly co-administered with fluoxetine were extracted using the method and were shown not to interfere.

An HPLC assay with automated extraction was developed to quantitate fluoxetine and norfluoxetine in serum.[117] This method combined both solid phase and liquid-liquid extractions with a back extraction followed by reversed phase chromatography with a C-8 column and UV detection. Clean chromatograms with few interferences were obtained and the sample preparation time was significantly reduced from that of non-automated methods. However, automated machines are not available in many laboratories.

Few authors reported solid phase extraction with HPLC analysis. A method using solid phase extraction with Bond Elut C18 columns followed by a reversed phase liquid chromatographic procedure with fluorescence detection was developed.[118] A recovery of 85 % was obtained and the method allowed the detection of 20 ng/ml of fluoxetine and norfluoxetine. Protriptyline, maprotiline and its desmethyl metabolite were the only compounds among traditional antidepressants and benzodiazepines which showed fluorescence under the conditions selected for fluoxetine detection.

The most recently reported method on the detection of fluoxetine in plasma has used liquid chromatography with tandem mass spectrometric detection. The analytes were extracted

from plasma by liquid-liquid extraction, separated on a C18 column and ionised by TurboIonSpray ionisation. Recoveries were greater than 97 % and the lower limit of quantification was 0.15 ng/ml.

All of the methods mentioned so far have extracted fluoxetine from serum, plasma or brain tissue. However, in forensic toxicology it is important to have methods capable of measuring substances in whole blood. None of the previously mentioned methods were validated to analyze whole blood samples. Such samples can prove difficult to analyze by HPLC-UV due to the decomposition of the sample and more specific techniques are required.

One of two methods to analyze fluoxetine in whole blood used liquid-liquid extraction followed by derivatization with PFPA and analysis by gas chromatography-mass spectrometry (GC/MS).[119] The limit of detection for this method was 12.5 ng/ml and this was the only reported method to use a deuterated internal standard for identification and quantitation of fluoxetine and its metabolite.

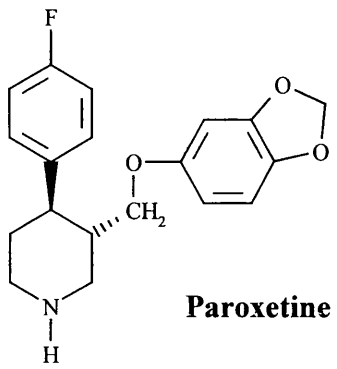
One other method which reported the extraction of fluoxetine from whole blood used HPLC with fluorescence detection following SPE.[120] This method simultaneously extracted citalopram, paroxetine, fluoxetine and metabolites in plasma or whole blood. The limits of detection of these were 8.1 ng/ml, 8.2 ng/ml, and 30 ng/ml respectively.

4.7 PAROXETINE

Paroxetine (Seroxat®) was first introduced into the United Kingdom in 1991 as a treatment for depression.[121] Since then it has also been prescribed to treat obsessive compulsive disorder, panic disorder and several other psychiatric and physical disorders. It has been found to be as effective as the tricyclic antidepressants such as imipramine[122] but with fewer side effects.

4.8 CHEMISTRY

Paroxetine is chemically known as (-)-trans-4-(p-fluorophenyl)-3-[(3,4-(methylenedioxy)-phenoxy)methyl]piperidine and has molecular formula $C_{19}H_{20}O_3NF$ and molecular weight 329.4. It has a phenylpiperidine structure as shown in Figure 4.3. Paroxetine is administered as its hydrochloride salt in doses of 30 mg.



Paroxetine

Figure 4.3: Chemical structure of paroxetine

4.9 METABOLISM AND EXCRETION

Paroxetine undergoes first pass metabolism and is metabolized to several inactive compounds as shown in Figure 4.4. The principal metabolic pathway is thought to be through oxidation to an unstable catechol intermediate which is in turn methylated and conjugated to a glucuronide or sulphate. Less than 2 % of the paroxetine dose remains unchanged in the urine.[123]

4.10 PHARMACOKINETICS

After oral dosing, paroxetine is almost completely absorbed and peak plasma concentrations are reached within 3 to 8 hours after administration. Steady state concentrations are reached within seven to fourteen days after multiple dosing of 20 or 30 mg daily. A single oral dose of 20 mg produced peak plasma levels ranging from 0.008 - 0.033 mg/L within 3 to 8 hours. Daily doses of 30 mg produced an average steady state level of 0.062 mg/L at 5.2 hours.[123] The distribution of paroxetine to tissues is extensive and the volume of distribution is large. Pharmacokinetic data is summarized in Table 4.2.[62]

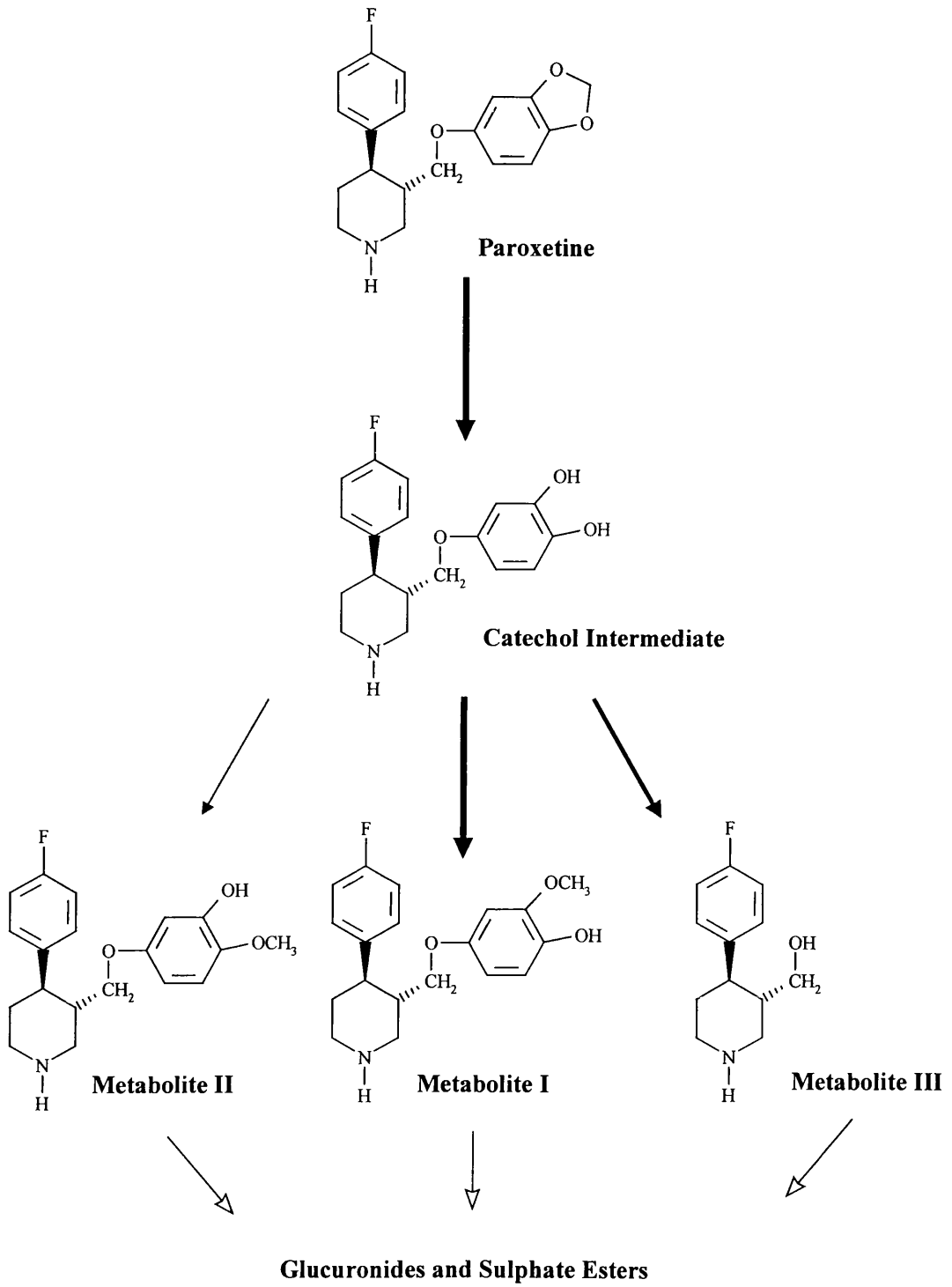


Figure 4.4: Paroxetine metabolism and excretion

Table 4.2: Pharmacokinetic data for paroxetine

	Paroxetine
Half life	1-3 days
Vd (L/kg)	3-28
Protein Binding (%)	95
pK_a	9.9

4.11 TOXICITY

Side effects of paroxetine have included nausea, somnolence, tremor, insomnia, headache and dry mouth. Excessive doses of paroxetine cause symptoms such as lethargy, tachycardia and hypertension. Drug concentrations detected in blood in 4 paroxetine fatalities ranged from 1.4 - 4.0 (mean 2.7) mg/L.[62] In two of these cases imipramine or moclobemide were detected in significant quantities.

Paroxetine, like fluoxetine should not be taken concurrently with monoamine oxidase inhibitors or L-Tryptophan since this can cause the potentially fatal Serotonin Syndrome.

4.12 ANALYSIS

Several methods for the analysis of paroxetine in human plasma or serum have been published. The most common method of analysis has been by HPLC either with ultra-violet detection[124, 125, 126] or fluorescence detection.[120, 127, 128, 129, 130, 131] Fluorescence detection methods all produced limits of detection lower than those of the ultra-violet detection. One of the ultra-violet methods used fluoxetine as the internal standard, however for this method the limit of quantitation was only 5 ng/ml. Another method which used fluoxetine as the internal standard used GC-ECD.[132] The extraction method was a simple liquid-liquid extraction method followed by derivatization with heptafluorobutyric anhydride. The limit of detection for this method was 8.5 ng/ml. A lower limit of detection of 3 ng/ml was obtained using GC-NPD to detect paroxetine in monkey plasma, but this method involved a multi-step extraction.

Most extraction methods for paroxetine used LLE, but SPE and automated column switching methods have also been employed. SPE methods have several advantages over LLE methods. Higher selectivity can be obtained since there is now a wide choice of

sorbents and solvents which can be selected depending on the application. Cleaner extracts can be produced since analytes can be selectively retained on and eluted from the column. Extraction can be less time consuming and since samples can be processed simultaneously, the system can be automated. SPE avoids the problem of emulsions forming which can reduce recoveries. Reproducibility is usually good since specific molecular interactions are involved. Previous SPE methods used either Bond Elut C18 [128, 131] or IST C8 [120] columns for analysis and gave recoveries greater than 90 %.

The only method which has reported the extraction of paroxetine from whole blood used HPLC with fluorescence detection following SPE.[120] The limit of detection for this method was 8 ng/ml. This method has already been mentioned previously since it simultaneously extracted fluoxetine, paroxetine, citalopram and metabolites in plasma or whole blood.

As a result of an increase in the number of whole blood samples containing fluoxetine into our department it was necessary to develop an improved method to extract fluoxetine from whole blood and to apply this method to postmortem blood samples. Solid phase extraction was used to extract the analytes from whole blood since this technique can often provide cleaner extracts than liquid-liquid extraction and produce high recoveries. Previous methods used Bond Elut C8 or Bond Elut C18 columns. Since cyanopropyl columns had previously been successful in separating the analytes by HPLC, it was decided to try solid phase columns of this nature to extract the analytes from whole blood. Initially, HPLC with UV detection was used to validate the method, but in order to obtain lower limits of detection and the required selectivity a GC/MS method was later validated.
Further to this, an increase in the number of paroxetine samples received into the laboratory was recognized and the method originally developed for fluoxetine was later applied to paroxetine. The method was evaluated using postmortem and clinical samples obtained from patients receiving treatment for depression. The developed method can simultaneously analyze fluoxetine, norfluoxetine and paroxetine in whole blood using solid phase extraction followed by gas chromatography-mass spectrometry with selected ion monitoring.

4.13 EXPERIMENTAL

4.13.1 CHEMICALS

Fluoxetine, norfluoxetine and tumoxetine were obtained as hydrochloride salts from Eli Lilly (Windlesham, England). Paroxetine hydrochloride was obtained from SmithKline Beechams (Herts, England) and maprotiline from Promochem (Herts, England). Clomipramine was supplied by Geigy Pharmaceuticals (Macclesfield, England). HPLC grade methanol, acetonitrile, acetone, chloroform and dichloromethane were obtained from Lab-Scan Analytical Sciences (Dublin, Ireland). Analytical grade potassium dihydrogen phosphate, sodium hydroxide, HPLC grade *n*-amyl alcohol, toluene and triethylamine were obtained from BDH laboratory supplies (Poole, England). HPLC grade isopropanol was obtained from Fisher Scientific U.K. Ltd. (Leicestershire, England) and HPLC grade hexane from Rathburn Chemicals Ltd. (Walkerburn, Scotland). Analytical grade concentrated ammonia and glacial acetic acid were obtained from Merck (Poole, England). Heptafluorobutyric anhydride was obtained from Sigma® Chemicals Co. (U.K.).

4.13.2 STANDARDS

Stock standard solutions of fluoxetine, norfluoxetine, tumoxetine, paroxetine, maprotiline and clomipramine were prepared in methanol at a concentration of 1 mg/ml and stored at - 20 °C. Each of these stock solutions was diluted with methanol to give working standard solutions at 1 μg/ml and 10 μg/ml and these were refrigerated at 4 °C.

4.13.3 SOLUTIONS AND BUFFERS

1 M Sodium carbonate buffer, pH 10

10.6 g of sodium carbonate (FW = 105.99) was weighed out into a 100 ml volumetric flask and made up to the mark with distilled water. 8.4 g of sodium hydrogen carbonate was weighed out into a 100 ml volumetric flask and made up to the mark with distilled water. These two solutions were combined, mixed well and stored in a bottle at room temperature.

0.5 M Sodium phosphate buffer, pH 10

7.8 g of sodium phosphate monobasic dihydrate (FW = 119.98) was weighed out into a 100 ml volumetric flask and made up to the mark with distilled water. The pH was adjusted to pH 10 using 10 M sodium hydroxide solution followed by 0.1 M hydrochloric acid while mixing on a magnetic stirrer. The buffer was stored at room temperature.

0.1 M Potassium Phosphate buffer, pH 6.0

6.81g of potassium dihydrogen phosphate (FW = 136.09) was weighed out into a 0.5 l volumetric flask and approximately 450 ml of distilled water were added. The pH was adjusted to 6.0 with 1.0 M potassium hydroxide solution while stirring. The solution was then made up to 0.5 l with distilled water.

4.13.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC conditions were set-up using a cyano column to separate fluoxetine and norfluoxetine.[113] This method avoided using the odourous solvent triethylamine which is often used to reduce peak tailing when separating basic drugs such as fluoxetine. This analytical technique was used to validate current liquid-liquid extraction techniques and a new solid phase extraction method for analysis of fluoxetine and norfluoxetine in whole blood.

Analysis of fluoxetine and norfluoxetine was carried out using a Gilson system consisting of a 307 pump with 5SC pumphead and a 118UV detector. The column selected was a Supelcosil LC-PCN 25 cm x 4.6 mm i.d., 5 μm particle size. The mobile phase was an acetonitrile/methanolic buffer mixture which was prepared by buffering 0.01M potassium dihydrogen phosphate to pH 7 using 1M potassium hydroxide solution and mixing it with HPLC grade methanol in the ratio 5:4. 167 ml of acetonitrile was added to 90 ml of the methanol buffer and this was degassed under helium for 15 minutes and pumped at a flow rate of 2 ml/min. The mobile phase was allowed to equilibrate for 30 - 60 minutes and a detection wavelength of 226 nm was used.

A mixed standard solution containing 100 μl of fluoxetine, norfluoxetine and tumoxetine each at a concentration of 1 μg/ml and three standard solutions containing each drug separately were evaporated to dryness at 40 °C under nitrogen. The residues were redissolved in 200 μl of mobile phase and sonicated at room temperature for 5 minutes. 20 μl of each standard was injected into the HPLC system.

The chromatographic conditions described produced good separation of the analytes and internal standard. The order of elution was norfluoxetine, fluoxetine then tumoxetine and retention times were found to be 4.9, 6.8 and 7.4 minutes respectively.

4.13.5 CURRENT METHODOLOGY

Liquid-liquid extraction has been the preferred extraction method for fluoxetine and norfluoxetine from whole blood. Two methods from the literature were chosen and compared with solid phase extraction.

4.13.6 LIQUID-LIQUID EXTRACTION METHOD 1[106]

4.13.6.1 Sample Preparation

Packed red blood cells from the blood bank which was time expired was used as blank blood. These were suspended in a ratio of 1:1 with isotonic saline solution which was made up by dissolving 9.5 g of sodium chloride in 1 litre of distilled water.

Blood samples were prepared by spiking 1 ml of blank blood with fluoxetine and norfluoxetine to give concentrations 100, 200, 400, 1000, 1400, 2000 ng/ml. A 1 ml blank blood sample containing no drug was also pipetted into a centrifuge tube. Unextracted standards at equivalent concentrations were also prepared. Each concentration was prepared in triplicate.

4.13.6.2 Extraction

Carbonate buffer (2 ml, 1 M, pH 10) was added to each blood sample followed by 6 ml of chlorobutane. This was mixed on a rocking and rolling mixer for 10 minutes and centrifuged at 3000 rev/min. for 10 minutes. The upper chlorobutane layer was transferred to a clean test tube and 2 ml of 0.2 M hydrochloric acid was added. Mixing and centrifuging were carried out as previously and the chlorobutane layer was removed and discarded. The acidic layer was basified using 2 ml of pH 10 carbonate buffer and 5 ml toluene were added. After mixing and centrifuging, the toluene layer was removed and evaporated to dryness at 50 ^{o}C under nitrogen. 200 ng of clomipramine was added to each vial as internal standard, evaporated to dryness and redissolved in 200 µl of mobile phase. 20 µl of each extracted and unextracted standard was injected into the HPLC system.

4.13.6.3 Results

Relative recoveries of fluoxetine and norfluoxetine from whole blood were determined by comparing the peak heights obtained from spiked whole blood extracts with unextracted standards at equivalent concentrations. The mean percentage recoveries were calculated at each concentration and it was found that the recoveries of fluoxetine were higher than

those of its metabolite by approximately 20 % (Table 4.3). Recoveries of the metabolite were less than 50 %.

Table 4.3: Recoveries using liquid-liquid extraction method 1

Concentration in blood (ng/ml)	Mean % Recovery Norfluoxetine	Mean % Recovery Fluoxetine
100	49.4	73.2
200	50.0	68.0
400	43.2	68.3
1000	37.5	60.7
1400	33.0	58.4
2000	38.4	52.0

Since the recoveries were relatively low for this liquid-liquid extraction method, a different method was used and the recoveries compared.

4.13.7 LIQUID-LIQUID EXTRACTION METHOD 2[100]

4.13.7.1 Sample Preparation

Standards were prepared as in 4.13.6.1.

4.13.7.2 Extraction

To the spiked blood sample, 2 ml of sodium phosphate buffer (0.5 mol/L, pH 10) were added and vortex mixed for 5 seconds. 5 ml of hexane:*n*-amyl alcohol 97:3 (*v/v*) were then added, shaken for 20 minutes and centrifuged for 10 minutes at 3000 rev/min. The organic phase was transferred and 1 ml of 0.1M hydrochloric acid added, shaken for 20 minutes and centrifuged for 10 minutes at 3000 rev/min. The organic phase was removed and discarded. To the aqueous phase, 1 ml of sodium hydroxide was added followed by hexane:*n*-amyl alcohol 97:3 (*v/v*). This was shaken for 20 minutes and centrifuged for 10 minutes at 3000 rev/min. The organic layer was transferred and one drop of methanolic hydrochloric acid added. The solvent was evaporated to dryness and reconstituted in 200 µl of mobile phase for HPLC analysis or derivatized for GC/MS analysis.

4.13.7.3 Results

Relative recoveries of fluoxetine and norfluoxetine from whole blood were determined and the mean percentage recoveries are given in Table 4.4. The recoveries using this method

were similar for fluoxetine and norfluoxetine and also much higher than the previous liquid-liquid method.

Table 4.4: Recoveries using liquid-liquid extraction method 2

Concentration (ng/ml)	Mean % Recovery Norfluoxetine	Mean % Recovery Fluoxetine
100	79.2	79.2
200	86.0	92.9
400	81.4	82.1
1000	87.7	88.4
1400	95.9	91.0
2000	89.5	93.6

4.13.8 SOLID PHASE EXTRACTION

The solid phase extraction columns chosen for this analysis consisted of a cyanopropyl bonded phase. This type of phase has the potential to extract a wide variety of drugs under the correct analytical conditions. Therefore, this type of sorbent can be used for solid phase extraction provided the correct conditions are applied to achieve retention or elution of analytes when required. The cyanopropyl bonded phase possesses an intermediate polarity which allows analytes to be separated from an aqueous matrix or a non-polar medium and should also allow elution to be easier than that obtained with non-polar sorbents such as C18. The primary interactions of the sorbent in polar solvent surroundings are due to hydrophobicity and the retention of analytes is largely due to non polar Van der Waals binding forces. Secondary interactions which play a role are electrostatic interactions at residual silanol groups and in aqueous solutions there is the possibility of ionic interactions with protonated bases. Also dipole-dipole interactions may occur at the cyano functional group. Fluoxetine could potentially interact with the phase by all of these mechanisms.

4.13.8.1 Sample Preparation

Four blood samples were prepared by addition of 20 µl of fluoxetine and 20 µl of norfluoxetine, both at a concentration of 10 µg/ml to centrifuge tubes. The samples were evaporated to dryness, reconstituted in 1 ml of blank blood and sonicated for 5 minutes. Phosphate buffer, pH 7.92 (6 ml, 0.1M) was added to two of the tubes and phosphate

buffer, pH 6.5 was added to the other two tubes. These were vortex mixed for 30 seconds and then centrifuged at 3500 rev/min. for 15 minutes.

4.13.8.2 Solid Phase Extraction

Bond Elut cyanopropyl columns (100 mg, 10 ml reservoir) were conditioned with 2 ml of methanol followed by 2 ml of potassium dihydrogen phosphate buffer (0.1 M, pH 7.92 or 6.5 corresponding to pH of phosphate buffer used to dilute blood sample). The diluted blood samples were applied to the columns and the vacuum was applied to give a flowrate of 1.5 ml/min. The column was washed with 1 ml deionized water followed by 0.5 ml acetic acid (0.01M, pH 3.3) at a flowrate of 1.5 ml/min. The sorbent was dried for 5 minutes under a full vacuum followed by addition of 50 µl methanol and a further 1 minute with the vaccuum on full. A further wash was carried out using 4 ml of acetone-chloroform (1:1) at a flowrate of 0.8 ml/min. This fraction was collected, evaporated to dryness at 40 °C. The drugs were eluted with 2 ml of ethyl acetate containing 2 % concentrated ammonia at a flowrate of 0.5 ml/min. A further 1 ml of ethyl acetate containing 2 % concentrated ammonia was added to the columns and these samples were collected separately. Samples were evaporated to dryness at 40 °C. To all fractions, 20 µl of clomipramine at 10 µg/ml was added and this was evaporated to dryness under nitrogen and the residue was reconstituted in 200 µl of mobile phase for HPLC analysis.

4.13.8.3 Results

The acetone-chloroform extracts were analysed and showed that no significant amount of fluoxetine or norfluoxetine was eluted in this step. Peak height ratios were calculated for fluoxetine and norfluoxetine to internal standard and the recoveries calculated for the extraction using phosphate buffer at two different pH's. The extraction using phosphate buffer pH 6.5 gave higher recoveries than at pH 7.92 for the ethyl acetate extracts (Table 4.5). Recoveries were much lower for norfluoxetine than fluoxetine at both pH's which suggested that either the pH of buffer should be altered or an alternative elution solvent should be used. The third millilitre of ethyl acetate with 2 % concentrated ammonia used to elute any remaining drugs was analysed, but no fluoxetine or norfluoxetine was identified.

Table 4.5: Recoveries using phosphate buffer, pH 6.5 and pH 7.9.

pH of phosphate buffer	Mean % Recovery Norfluoxetine	Mean % Recovery Fluoxetine
6.5	25.9	67.1
7.92	5.3	50.4

The experiment was repeated using different pH's of phosphate buffer.

4.13.9 METHOD DEVELOPMENT: ALTERING PH OF PHOSPHATE BUFFER

Phosphate buffer was prepared as described in 4.13.3 and the pH was adjusted to 5.0, 6.0 and 7.0 with potassium hydroxide solution. Blood was spiked and extracted as before and the recoveries were calculated.

Table 4.6: Recoveries using phosphate buffer, pH 5.0, pH 6.0 and pH 7.0.

pH of phosphate buffer	Mean % Recovery Norfluoxetine	Mean % Recovery Fluoxetine
5.0	36.0 (7.1)	63.6 (6.4)
6.0	53.0 (10.9)	89.4 (6.1)
7.0	14.8 (4.6)	37.9 (5.7)

The acetone-chloroform fractions showed no presence of fluoxetine or norfluoxetine. Recoveries were highest using phosphate buffer pH 6.0. These were 53.0 % and 89.4 % for norfluoxetine and fluoxetine respectively. Since the recovery obtained for norfluoxetine was still low, the elution solvent was changed to try and elute a higher percentage of drug.

4.13.10 METHOD DEVELOPMENT: CHANGING THE ELUTION SOLVENT

The elution solvent was changed to dichloromethane:isopropanol 8:2 with 2 % concentrated ammonia. This elution mixture was used as an alternative to ethyl acetate.

The recoveries were 78.6 and 88.2 % for norfluoxetine and fluoxetine respectively. The fluoxetine recovery was similar to that in the previous experiment, however, the norfluoxetine recovery increased by more than 20 %.

4.13.11 RECOVERY OF INTERNAL STANDARD

Three 1 ml samples of blank blood were spiked with 20 µl of clomipramine at 10 µg/ml. This was extracted as in 4.13.8.2 except using the new elution mixture and fluoxetine in

this case was added after the extraction procedure. 20 µl of each extract was injected into the HPLC system and unextracted standards at the same concentration were injected. The recovery of the internal standard was 80 %.

4.13.12 METHOD VALIDATION

4.13.12.1 Linearity

Fluoxetine and norfluoxetine were added to centrifuge tubes to give concentrations of 25, 50, 100, 200, 400, 1000, 2000 ng/ml blood. Internal standard, clomipramine was added to give a concentration of 200 ng/ml blood. Each concentration was extracted in triplicate. Unextracted standards and solid phase extracted standards containing equivalent concentrations of fluoxetine and norfluoxetine were analyzed. Calibration graphs were constructed for the compounds using the values displayed in Table 4.7. These were linear producing regression coefficients of 0.999 and 0.998 and intercepts - 0.0096 and - 0.0024 for fluoxetine and norfluoxetine respectively.

Table 4.7: Intra-day Variation

Concentration (ng/ml blood)	Extracted standards		Unextracted standards	
	PH ratio NFLU/CLO (RSD %)	PH ratio FLU/CLO (RSD %)	PH ratio NFLU/CLO (RSD %)	PH ratio FLU/CLO (RSD %)
25	0.099 (8.4)	0.099 (9.6)	0.094 (13.6)	0.092 (14.7)
50	0.189 (5.6)	0.193 (4.2)	0.203 (7.1)	0.183 (8.7)
100	0.381 (4.0)	0.395 (9.2)	0.370 (7.1)	0.336 (5.0)
200	0.655 (7.1)	0.735 (4.3)	0.752 (3.9)	0.667 (7.7)
400	1.563 (2.1)	1.474 (3.1)	1.526 (6.4)	1.312 (8.3)
1000	3.694 (3.2)	3.595 (9.1)	4.10 (5.2)	3.650 (4.1)
2000	7.332 (3.4)	6.967 (5.7)	7.910 (3.9)	7.067 (4.1)

*PH = Peak Height, RSD = Relative standard deviation as a percent

NFLU = norfluoxetine, FLU = fluoxetine, CLO = clomipramine

4.13.12.2 Recovery

Relative recoveries of fluoxetine and norfluoxetine were obtained by extracting the compounds at three different concentrations (100, 200, 400 ng/ml) and extractions at each concentration were carried out in triplicate. 20 µl clomipramine at 10 µg/ml was added

after the extraction process. Mean recoveries were determined by comparing the ratio of peak heights obtained from spiked whole blood extracts with the ratio of peak heights obtained from unextracted standards at equivalent concentrations and were found to be approximately 95 % and 85 % for fluoxetine and norfluoxetine respectively (Table 4.8).

Table 4.8: Recoveries of fluoxetine and norfluoxetine

Concentration (ng/ml blood)	Mean % Recovery NFLU (RSD %)	Mean % Recovery FLU (RSD %)
100	84.3 (4.0)	95.3 (2.7)
200	85.2 (4.1)	93.2 (4.1)
400	86.3 (4.2)	97.4 (5.4)

4.13.12.3 Limit of Detection

Blank blood was spiked to give concentrations 10, 15, 20 ng/ml of fluoxetine and norfluoxetine. The limit of detection was taken to be the concentration of drug giving a signal to noise ratio of 3 and this was found to be 10 ng/ml for both analytes using this method.

4.13.13 EXTRACTION OF POSTMORTEM BLOOD

4.13.13.1 Experimental

The method was tested using two postmortem blood samples known to contain fluoxetine. These were extracted using the SPE method. Spiked blood standards at 50 ng/ml and 200 ng/ml and a blank blood sample were extracted at the same time.

4.13.13.2 Results

The blank blood extract gave a small peak at 6.6 minutes which in previous blank samples was not present. This peak was also present in the spiked blood extracts. However, it did not interfere with internal standard, norfluoxetine or fluoxetine which eluted at 4.5, 5.3 and 7.3 minutes respectively.

Analysis of the two postmortem blood samples showed peaks which interfered with fluoxetine, norfluoxetine and clomipramine. Since one of these case samples was known to contain only fluoxetine and norfluoxetine, interference resulting from the presence of other drugs was ruled out. These other peaks were caused by the degradation of the blood

samples which is a problem with postmortem blood samples. The method would therefore need to be improved to avoid this problem. Initially, protein precipitation with acetonitrile was investigated to clean up these samples.

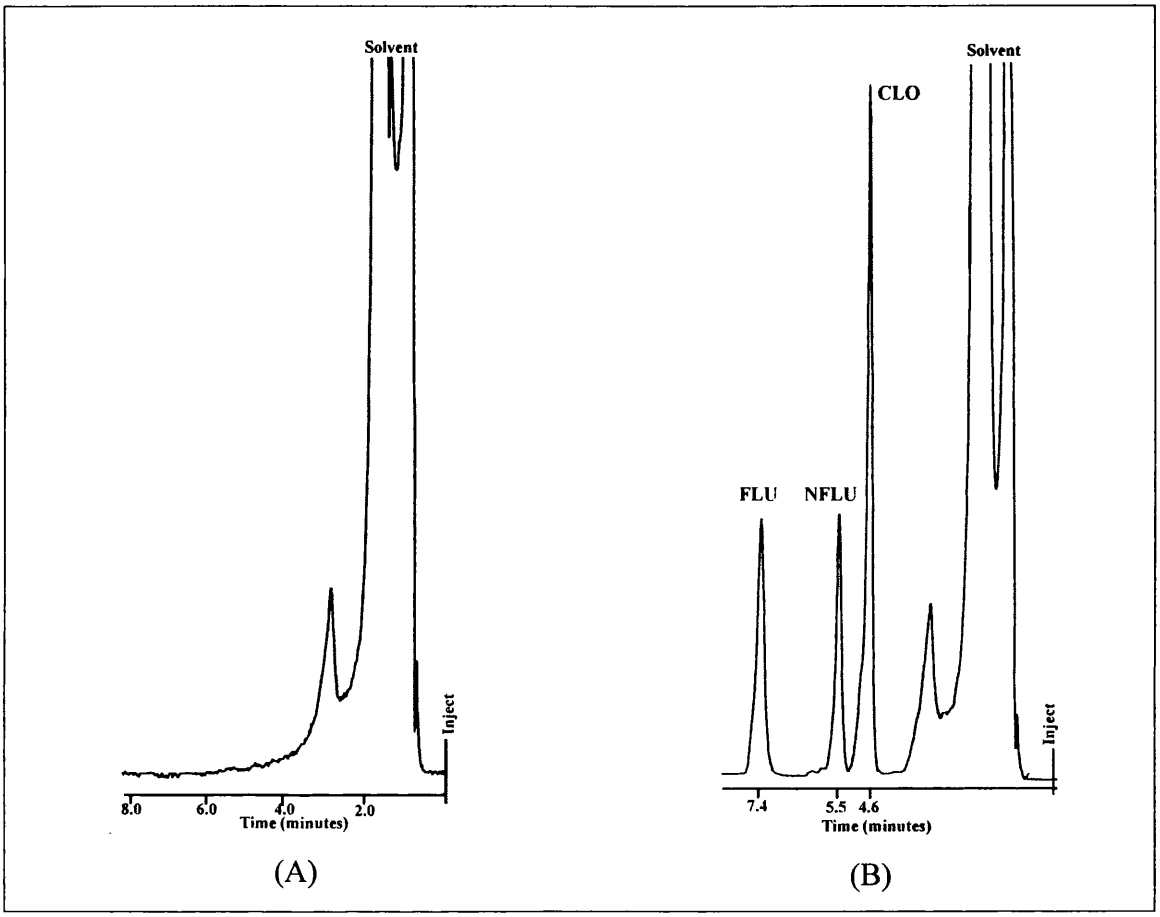


Figure 4.5: High performance liquid chromatograms of a blank blood extract (A) and a blood extract containing 100 ng/ml of fluoxetine (FLU) and norfluoxetine (NFLU) and 200 ng/ml clomipramine (CLO) (B).

4.13.14 PROTEIN PRECIPITATION

4.13.14.1 Experimental

A blank blood sample, a 200 ng/ml spiked blood sample, a blank methanol sample, a 200 ng/ml methanol standard, a postmortem blood sample which was known to be negative for fluoxetine and two case samples which were positive for fluoxetine and norfluoxetine were extracted using freshly made up phosphate buffer and solvents.

Another set of the above samples was extracted similarly, but an extra acetonitrile step was included in the sample preparation to see if this made a difference to the chromatograms. Acetonitrile is sometimes used in methods prior to extraction to precipitate proteins from blood.

1 ml of blood (case sample or spiked sample) was vortex mixed with 1 ml phosphate buffer, pH 6.0 whilst 4 ml of acetonitrile were added gradually. This was centrifuged for 15 minutes at 3500 rev/min, evaporated down to 1 ml and 6 ml phosphate buffer, pH 6.0 was added. SPE was carried out.

4.13.14.2 Results

Methanol extracts where no blood was present and the blood spikes produced no interferences which would affect quantitation of fluoxetine and norfluoxetine with or without the acetonitrile step. The postmortem case sample which was negative for fluoxetine also produced no interfering peaks. Out of the two other postmortem samples which were known to be positive for fluoxetine it was only possible to quantify one. The sample which produced many interfering peaks previously still showed these even when the acetonitrile step was included in the method, although it did reduce some of these interferences. This experiment showed that although it would be possible to quantify some postmortem samples by this method others could present a problem and therefore the method was not satisfactory for postmortem cases.

4.13.15 COMPARISON WITH LIQUID-LIQUID EXTRACTION METHOD 2

4.13.15.1 Experimental

The postmortem case samples were extracted using liquid-liquid extraction method 2 which was previously investigated.

4.13.15.2 Results

The liquid-liquid extraction method gave similar interferences on analysis by HPLC and fluoxetine and norfluoxetine were masked or only partially separated from other peaks. At this stage it was decided that the HPLC method was not selective enough for analysing postmortem samples. Analysis by GC/MS was carried out.

4.13.16 GAS CHROMATOGRAPHY MASS SPECTROMETRY

4.13.16.1 Equipment

A Hewlett-Packard model 5890 Gas Chromatograph with a VG Analytical VG 70-250S Mass Spectrometer was used. The GC was fitted with a HP-5 X-link 5% PH Me silicone column, 30 m x 0.32 mm x 0.25 μm film thickness. The temperature programme was increased from 150 to 300 °C at 10 °C/min and the injector temperature was 225 °C. The temperature of the source and interface were 200 °C and 250 °C respectively. The trap current was 200 μA. The injection volume was 1 μl.

4.13.16.2 Derivatization

To the extracted residue, 500 μl toluene, 100 μl of triethylamine (0.05M) and 10 μl of heptafluorobutyric anhydride (HFBA) were added. The vial was sealed and heated at 50 °C for 15 minutes. After heating, the solvent was evaporated off at 37 °C under nitrogen and 1 ml distilled water and 500 μl toluene were added to the vial and shaken for one minute. The toluene layer was removed and evaporated to dryness and the residue reconstituted in 50 μl toluene.

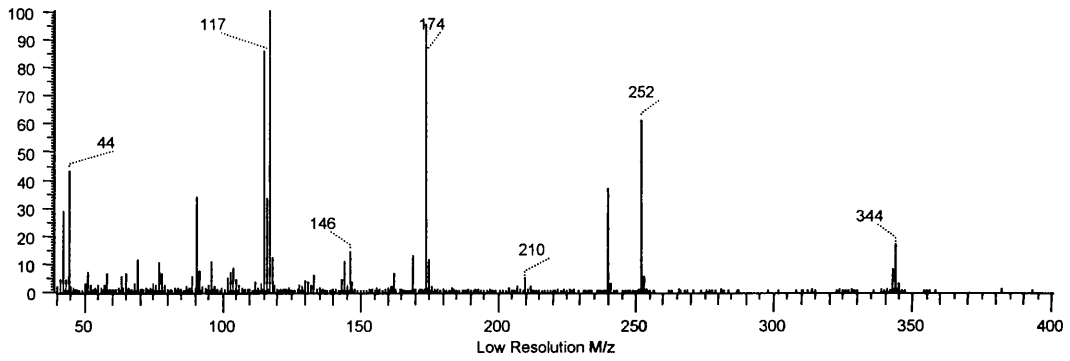
4.13.16.3 Results

The following retention times and ions were observed for the derivatized analytes using full scan analysis:

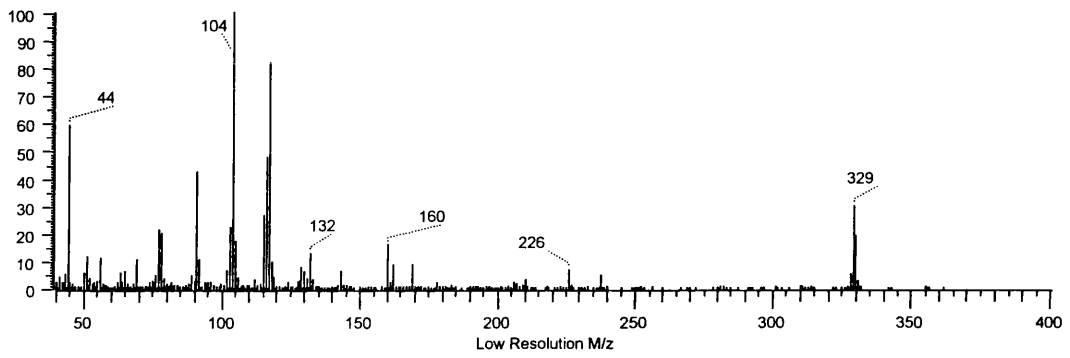
Table 4.9: Retention times and ions observed after GC/MS analysis

Drug	Retention time (min.) 150 - 300 °C	Retention time (min.) 180 - 280 °C	Main ions observed (m/z)
Fluoxetine	6.43	4.50	344
Norfluoxetine	6.08	4.08	330
Tumoxetine	7.24	5.17	344

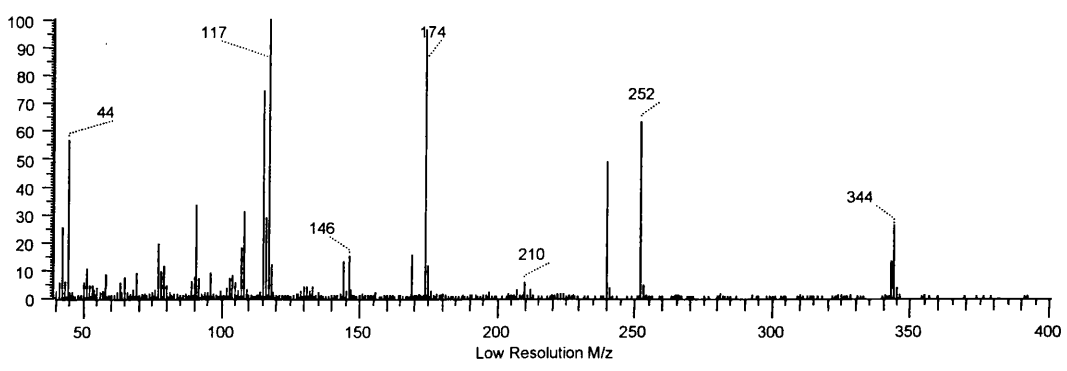
The temperature programme was altered to reduce the analysis time. The initial temperature was 180 °C and this was increased at 10 °C/min to 280 °C. Retention times using this temperature programme are also displayed in Table 4.9. Mass spectra for the derivatized samples are shown in Figure 4.6. From these it was decided to monitor ions m/z 344 for FLU and tumoxetine (TUM) and m/z 330 for NFLU and use these for quantification. Figure 4.7 shows how these drugs fragment to produce these ions.



Fluoxetine-HFB derivative.

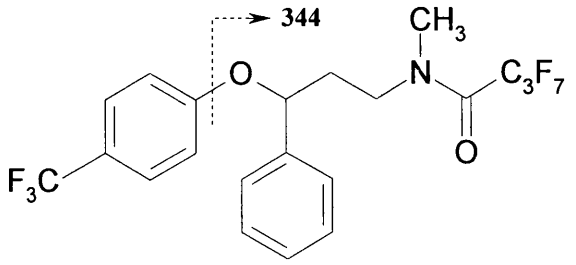


Norfluoxetine-HFB derivative.

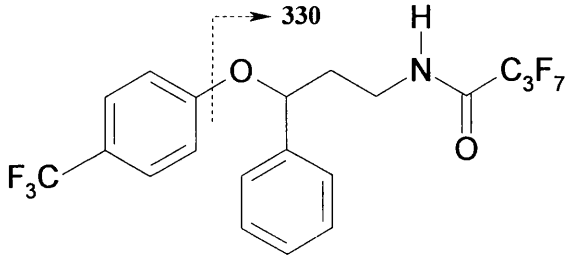


Tumoxetine-HFB derivative.

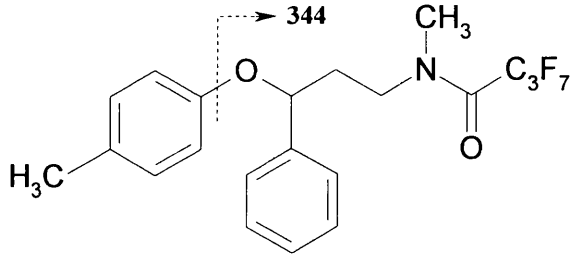
Figure 4.6: Ion graphs of HFB derivatives



Fluoxetine-HFB



Norfluoxetine-HFB



Tumoxetine-HFB

Figure 4.7: Derivatization of fluoxetine, norfluoxetine and tumoxetine with heptafluorobutyric anhydride.

4.13.17 GAS CHROMATOGRAPHY MASS SPECTROMETRY - SELECTED ION MONITORING (SIM)

A selected ion monitoring programme was set up for the analytes. Ions monitored were 344 for fluoxetine and tumoxetine and 330 for norfluoxetine. Perfluorokerosene (PFK) was used to calibrate the instrument and lock ion 318.9792 a.m.u. was used. The initial magnet position was 317 a.m.u. and switch and dwell times were set at 20 ms and 50 ms respectively for each monitored ion. Selected ions were monitored in the electron ionization mode at 70 eV and the trap current was set at 200 μA.

4.13.18 METHOD VALIDATION USING GC/MS

The developed solid phase extraction method including the acetone-chloroform step was fully validated.

4.13.18.1 Linearity

Fluoxetine and norfluoxetine were added to centrifuge tubes to give concentrations of 10, 25, 50, 75, 100, 200, 400 ng/ml blood. Internal standard, tumoxetine was added to give a concentration of 100 ng/ml blood. Each concentration was extracted in triplicate.

Table 4.10: Intra-day Variation

Concentration (ng/ml blood)	Extracted standards		Unextracted standards	
	m/z 330/344 NFLU/TUM (RSD %)	m/z 344/344 FLU/TUM (RSD %)	m/z 330/344 NFLU/TUM (RSD %)	m/z 344/344 FLU/TUM (RSD %)
10	0.056 (15.0)	0.052 (16.0)	0.058 (12.9)	0.053 (16.8)
25	0.178 (5.2)	0.165 (3.6)	0.224 (2.8)	0.202 (3.4)
50	0.387 (5.2)	0.368 (5.8)	0.530 (5.9)	0.476 (2.8)
75	0.560 (6.6)	0.586 (3.5)	0.774 (5.7)	0.694 (3.3)
100	0.893 (7.9)	0.891 (3.8)	1.217 (5.8)	0.988 (4.0)
200	1.785 (3.5)	1.720 (2.9)	2.051 (4.8)	2.179 (4.8)
400	3.225 (3.1)	3.423 (4.6)	4.402 (1.6)	4.437 (5.9)

NFLU = norfluoxetine, FLU = fluoxetine, TUM = tumoxetine

RSD = relative standard deviation as a percent

Coefficients of correlation for fluoxetine and norfluoxetine extracted standards were > 0.999 and > 0.998 and x-axis intercepts were -0.03884 and -0.03824 respectively.

Coefficients of correlation for fluoxetine and norfluoxetine unextracted standards were > 0.999 and > 0.997 and x-axis intercepts were -0.09551 and -0.03038 respectively.

4.13.18.2 Inter-day variation

Inter-day variation was assessed for the same concentration range as the intra-day variation. These ratios are based on ratios obtained over five days and were less than 15 % for both drugs over the concentration range (Table 4.11).

Table 4.11: Inter-day variation

Concentration (ng/ml blood)	Extracted standards		Unextracted standards	
	m/z 330/344 NFLU/TUM (RSD %)	m/z 344/344 FLU/TUM (RSD %)	m/z 330/344 NFLU/TUM (RSD %)	m/z 344/344 FLU/TUM (RSD %)
10	0.055 (4.8)	0.054 (5.2)	0.060 (10.4)	0.055 (9.6)
25	0.192 (14.3)	0.142 (15.0)	0.218 (5.7)	0.211 (7.3)
50	0.344 (12.5)	0.340 (8.2)	0.524 (9.2)	0.456 (5.0)
75	0.522 (13.0)	0.550 (6.4)	0.804 (6.1)	0.725 (6.0)
100	0.888 (4.8)	0.848 (4.1)	1.229 (5.3)	1.034 (3.7)
200	1.614 (6.3)	1.646 (6.8)	2.288 (9.1)	2.006 (5.3)
400	3.442 (5.6)	3.373 (2.2)	4.534 (5.1)	4.516 (4.8)

NFLU = norfluoxetine, FLU = fluoxetine, TUM = tumoxetine

RSD = relative standard deviation as a percent

4.13.18.3 Recovery

The recovery of the method was assessed at three concentrations, 25, 50 and 75 ng/ml. The recoveries were higher than previously seen for the HPLC method and also higher than either of the liquid-liquid methods which were initially investigated.

Table 4.12: Recovery

Concentration (ng/ml blood)	Mean % Recovery Norfluoxetine (RSD %)	Mean % Recovery Fluoxetine (RSD%)
25	98.2 (2.3)	95.2 (7.8)
50	97.0 (8.9)	95.9 (11.6)
75	92.2 (10.8)	97.1 (9.4)

RSD = relative standard deviation as a percent

4.13.18.4 Limit of detection

The limit of detection was determined as the concentration of drug giving a signal to noise ratio of 3. These were found to be 1 ng/ml for fluoxetine and norfluoxetine. This method therefore has the advantage of a lower limit of detection than the HPLC method by a factor of 10.

4.13.18.5 Interference

Analysis using GC/MS eliminated interference from postmortem blood samples. In papers reporting the analysis of tricyclic antidepressants by HPLC there were often reports of other antidepressants or other types of drugs interfering with the analysis.[133] The use of GC/MS produces a higher specificity and therefore will significantly reduce the incidence of interference from other drugs which extract using this method. Selective ion monitoring means that ions can be chosen which are specific for a certain drug. The combination of this and the resultant retention time decrease the likelihood of interference from other drugs. However, if this was a problem the method could easily be carried out using fullscan analysis to allow the possibility of alternative quantitative ions to be chosen.

4.14 PAROXETINE EXPERIMENTAL

4.14.1 DERIVATIZATION

Paroxetine (500 ng) was derivatized using heptafluorobutyric anhydride as described in 4.13.16.2. This derivative was injected into the GC/MS using the same temperature programme used for fluoxetine, 180 - 300 °C at 10 °C/min. The mass spectrum of the derivatized sample is shown in Figure 4.8. From this it was decided to monitor m/z 525 for quantification and use m/z 388 as a qualifier ion. Figure 4.9 shows the derivatized molecule.

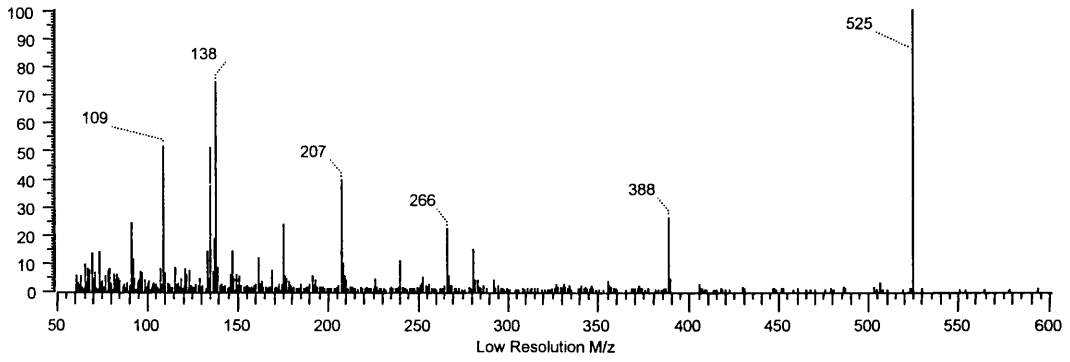


Figure 4.8: Mass spectrum of paroxetine-HFB derivative

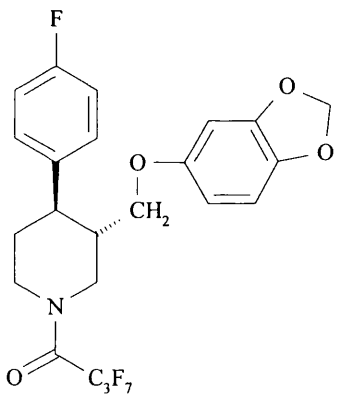


Figure 4.9: Structure of paroxetine-HFB derivative

4.14.2 METHOD VALIDATION FOR PAROXETINE BY GC/MS

The developed method for fluoxetine was carried out on paroxetine and the method was validated for this drug.

4.14.2.1 Linearity

Linearity for paroxetine was evaluated by extracting blood samples spiked to give concentrations 10, 25, 50, 75, 100, 200, 400 ng/ml blood. Internal standard, maprotiline

was added to give a concentration of 50 ng/ml blood. Each concentration was extracted in triplicate.

Table 4.13: Intra-day Variation for Paroxetine

Concentration (ng/ml blood)	Extracted m/z 525/445 PAR/MAP (RSD %)	Unextracted m/z 525/445 PAR/MAP (RSD %)
10	0.038 (12.8)	0.035 (6.9)
25	0.077 (5.1)	0.078 (5.8)
50	0.161 (4.0)	0.160 (4.5)
75	0.215 (2.4)	0.218 (5.8)
100	0.291 (7.5)	0.307 (7.2)
200	0.669 (3.6)	0.649 (3.4)
400	1.420 (6.3)	1.420 (4.8)

PAR = paroxetine, MAP = maprotiline, RSD = relative standard deviation as a percent

Coefficient of correlation for paroxetine extracted standards was > 0.997 and x-axis intercept was -0.02906.

4.14.2.2 Inter-day variation

Inter-day variation was assessed for the same concentration range as the intra-day variation. The coefficient of variation were determined over five days and were less than 15 % across the concentration range (Table 4.14).

Table 4.14: Inter-day variation for Paroxetine

Concentration (ng/ml blood)	m/z 525/445 PAR/MAP (RSD %)
10	0.033 (14.1)
25	0.080 (7.8)
50	0.154 (9.2)
75	0.221 (7.4)
100	0.326 (11.5)
200	0.722 (11.7)
400	1.553 (7.4)

PAR = paroxetine, MAP = maprotiline, RSD = relative standard deviation as a percent

4.14.2.3 Recovery

The recovery of the method was assessed at three concentrations, 25, 50 and 75 ng/ml and the results are displayed in Table 4.15.

Table 4.15: Recovery of Paroxetine

Concentration (ng/ml blood)	Mean % Recovery Paroxetine (RSD %)
25	84.2 (5.4)
50	83.2 (6.2)
75	81.5 (3.7)

RSD = relative standard deviation as a percent

4.14.2.4 Limit of detection

The limit of detection was determined as the concentration of drug giving a signal to noise ratio of 3. This was found to be 1 ng/ml.

4.15 CONCLUSIONS

The developed method successfully extracted fluoxetine, norfluoxetine and paroxetine simultaneously from spiked whole blood samples using cyanopropyl columns. Calibration curves were linear for all the analytes. Intra-day variation gave coefficients of variation < 12 % at higher concentrations and < 17 % at lowest concentration and inter-day variation < 15 % in all cases. The limit of detection was found to be lower than previous methods which simultaneously analyzed these compounds in plasma or whole blood.

The method was applied to postmortem case samples and clinical samples to assess the quantities of these drugs in whole blood samples (see Chapter 8.3).

5 Antidepressants in Saliva

5.1 INTRODUCTION

Saliva testing for drugs was first used about 30 years ago to identify and quantitate lithium and anticonvulsant drugs for the purpose of pharmacokinetic studies and therapeutic drug monitoring. Therapeutic drug monitoring is useful for drugs which have a narrow therapeutic concentration margin. However, to be able to use saliva for such a purpose a correlation between plasma and salivary drug concentrations must exist.

In more recent years there has been growing interest into the measurement of drugs of abuse in saliva[134] and as a result saliva testing has become of interest to law enforcement agencies for road-side testing of potentially intoxicated drivers.[135, 136] Most of the commonly used drugs of abuse, cannabinoids, cocaine, opioids, benzodiazepines, amphetamines, barbiturates have been measured in saliva by various techniques.

A European project called the Roadside Testing Assessment (ROSITA) project was set up in 1999 to investigate oral fluid in road side testing. Eight countries took part in assessing 12 urine tests, 3 saliva tests and 1 sweat test. During this project a survey was conducted into the preferences of police officers involved in collecting samples. The outcome of this was that oral fluid was the preferred sample to be tested at the roadside. Drug classes which were tested were cannabinoids, amphetamines, benzodiazepines, opiates and cocaine. The onsite tests were confirmed by gas chromatography-mass spectrometry, high performance liquid chromatography-diode array detection or gas chromatography-electron capture detection.

However, it is not only illicit drugs which can cause driving impairment. Many widely prescribed drugs have the ability to impair driving performance. Also, combinations of drugs or drugs and alcohol can enhance driving impairment. Therefore, to assess the implications a drug or a combination of drugs has on the driving population and especially those who are impaired, it is important to be able to measure all prescription and over the counter drugs as well as illicit drugs in saliva. Until recently, the importance of saliva analysis of prescription drugs has been in therapeutic drug monitoring, but now saliva testing of these drugs is relevant in a forensic context.

The interest in saliva testing has stemmed from the fact that the collection of saliva is much easier than that of blood. Collection is non-invasive and spares the patient from the discomfort of repeated venipuncture and avoids the privacy issues involved with urine collection. Samples can be stored at room temperature and transportation of samples requires no special needs such as refrigeration. Direct observation of the sample collection can prevent adulteration or possible swapping of samples, a problem sometimes encountered with urine collection. No training requirements are necessary by staff collecting samples and the risk of infection is low. This contrasts with blood collection which requires a medically qualified person to take the sample, and the risk of infection is always present. Saliva has a low protein content compared to blood so binding of drugs will be less and it will have the advantage of giving an improved indication of the degree of intoxication of an individual compared with urine.

However, the disadvantages of using such a matrix are that the mucopolysaccharides and mucoproteins in saliva can cause it to have a viscous, sticky consistency which make handling more difficult. Sufficient volume of sample for analysis can be a problem with the elderly and with patients who have been administered certain drugs or who have an illness that can cause dry mouth. Drugs can cause contamination of the oral cavity if they are administered orally, intranasally or smoked. The method of saliva collection and variability of saliva pH between individuals can both have a large influence on the concentrations of drugs detected in the saliva sample.

5.2 COMPOSITION OF SALIVA

Saliva is a filtrate of blood and a combination of several oral fluids secreted by three principal glands known as the parotid, submandibular and sublingual glands. Parotid gland secretions are watery and contain the enzyme, amylase, which catalyzes starch breakdown. In comparison, the sublingual and submandibular secretions contain mucoid and serous because of the presence of mucous and serous cells in these glands.[137]

Whole saliva is 99 % water, but also contains sodium, potassium, calcium and magnesium cations, chloride, phosphate and bicarbonate anions, urea, proteins and traces of ammonia, uric acid, lipid, cholesterol, fatty acids, amino acids, sialic acid, mucins and enzymes for digestion. The total protein content in saliva is less than 1 % that of blood. Each type of

salivary gland produces saliva with a different composition of these constituents and compositions vary between individuals depending on several factors including age, sex, time of day, diet and changes in flow rate. [138]

Under resting conditions, 65, 23 and 4 % of mixed saliva is produced by the submandibular, parotid and sublingual glands with the further 8 % produced by the minor mucous glands.[139, 140] Under stimulated conditions, saliva flow is increased mainly from the parotid glands and this saliva makes up about 50 % of the mixed saliva which will now be more watery than before stimulation.[139]

5.3 SALIVA FLOW RATE

Humans produce between 0.5 and 1.5 litres of saliva per day.[141] The rate of secretion varies throughout the day, it being lowest during sleep and highest in mid-afternoon.[142] It also varies with season, with flow being higher in winter than in summer.[143] Saliva flow has been shown to change with age, younger people producing greater rates than the elderly. [144]

Saliva flow rate can be increased by ingestion of food and using stimuli which will be mentioned in 5.9. This increase in flow rate causes a change in salivary composition which is independent of the stimulus used. An increase in salivary flow will increase the concentrations of sodium cations, chloride and bicarbonate anions, but decrease potassium cations. In general, an increase in flow rate will cause a decrease in the viscosity.

Some diseases such as congenital xerostomia or Sjogren's syndrome[145] directly affect the salivary glands causing decreased salivation. Changes in saliva flow may also result from illnesses such as chronic rheumatoid arthritis[146], alcoholic cirrhosis[147], cystic fibrosis and conditions such as pregnancy.[148] Certain types of drugs: anticholinergic[149], antidepressant[150] and anticonvulsant[151] have also proven to have a side effect in decreasing saliva secretion.

5.4 SALIVA pH

In humans, saliva pH can range from 6.2 to 7.4.[152] Parotid saliva pH is between 5.45-6.06 and submandibular pH between 6.02-7.14 under resting conditions.[153] After stimulation, the acidity of the saliva can decrease by up to 2 pH units because of the increase of bicarbonate anions. Changes in salivary pH are entirely dependent on changes in flow rate.

A decrease in salivary pH is the result of a decrease in flowrate and an increase in salivary pH is the result of an increase in flowrate.

5.5 SECRETION OF DRUGS INTO SALIVA

Drugs are transferred from the blood into the salivary duct by transportation through the capillary wall, the basement membrane and the membrane of the glandular epithelial cells. However, it is the transportation of drugs across the epithelial membrane which is the rate determining step for the process. The principal method of transportation of drugs across this membrane is believed to be by passive diffusion, a process which depends on the drug existing in a non-ionized state. However, active transport and ultrafiltration may also play a role.

Many drugs are weak electrolytes which are only partially dissociated at physiological pH. The non-ionized form of drug is lipid soluble and will therefore cross the phospholipid bilayer which separates blood and saliva. An equilibrium will form between the blood and saliva for the non-ionized form and equilibria between the ionized and non-ionized forms will exist in both blood and saliva. The extent of ionization in the blood and saliva will depend on the pH on each side of the membrane. Figure 5.1 shows the transfer of an acidic drug across the epithelial membrane.

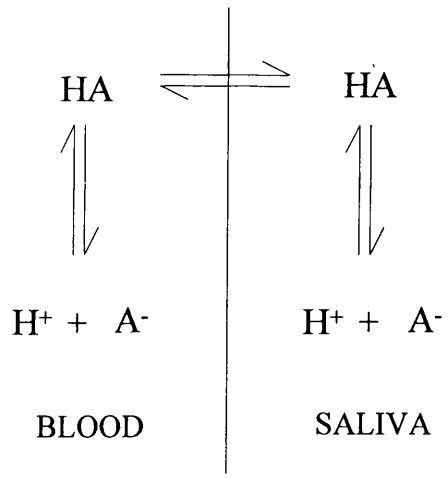


Figure 5.1: Equilibria between ionized and unionized forms of an acidic drug partitioning between blood and saliva by passive diffusion.

The saliva:plasma concentration ratio of acidic and basic drugs can be derived by considering the following nine equations. The derivation is for an acidic drug, but the derivation for basic drugs is obtained in a similar manner.

The ionization of an acidic drug can be given by the following equation:

$$K_a = \frac{[H^+][A^-]}{[HA]}$$

Equation 1

where K_a is the dissociation constant of the drug

$[H^+]$ is the hydrogen ion conentration

$[A^-]$ is the concentration of ionized species

[HA] is the concentration of unionized species

Taking logarithms of both sides of the equation forms the Henderson-Hasselbalch equation:

$$pH = pK_a + \log\frac{[A^-]}{[HA]}$$

Equation 2

where pK_a is the pH at which the drug is 50 % ionized

From equation 2, taking exponentials of both sides yields equation 3:

$$\frac{[A^-]}{[HA]} = 10^{(pH - pK_a)}$$

Equation 3

It is known that the amount of ionized drug and the amount of unionized drug are equivalent to the total concentration of the acidic drug :

$$[A^-] + [HA] = [A]$$

Equation 4

where [A] is the total concentration of the acidic drug (assuming no binding to protein).

Solving simultaneous equations 3 and 4 yields equation 5:

$$[A] = 1 + 10^{(pH - pKa)}$$

Equation 5

The saliva/plasma drug concentration ratio of an acidic drug for paired saliva and plasma samples can therefore be expressed as:

$$S:P = \frac{[A]_s}{[A]_p} = \frac{1 + 10^{(pH_s - pKa)}}{1 + 10^{(pH_p - pKa)}}$$

Equation 6

The saliva/plasma drug concentration ratio of a basic drug for paired saliva and plasma samples can be calculated in a similar manner and can be expressed as:

$$S:P = \frac{[B]_s}{[B]_p} = \frac{1 + 10^{(pKa - pH_s)}}{1 + 10^{(pKa - pH_p)}}$$

Equation 7

Equations 6 and 7 do not take into account that many drugs are highly protein bound in plasma and that some may also bind to protein in saliva. Correcting for protein binding gives equation 8 for acidic drugs and equation 9 for basic drugs.

$$S:P = \frac{[A]_s}{[A]_p} = \frac{1 + 10^{(pH_s - pKa)}}{1 + 10^{(pH_p - pKa)}} \times \frac{f_p}{f_s}$$

Equation 8

$$S:P = \frac{[B]_s}{[B]_p} = \frac{1 + 10^{(pKa - pH_s)}}{1 + 10^{(pKa - pH_p)}} \times \frac{f_p}{f_s}$$

Equation 9

where f_p is the non protein bound fraction of the total drug in plasma

f_s is the non protein bound fraction of the total drug in saliva

Protein binding in saliva is usually negligible and it is assumed that f_s is equivalent to 1.

It has been established from these equations and other calculations[154] that for acidic drugs with pK_a values greater than 8.5 and for basic drugs with pK_a values less than 5.5, the value of unbound saliva/plasma ratio remains the same even with a change in saliva pH. The saliva/plasma ratio for acidic drugs with pK_a values less than 8.5 and basic drugs with pK_a values greater than 5.5 will vary when there is an alteration in saliva pH. This is summarized in Table 5.1.

Table 5.1: Summary of how saliva/plasma ratios are affected by change in saliva pH for acidic and basic drugs

Acidic Drugs	**$pK_a > 8.5$** Change in saliva pH **does not** affect saliva/plasma ratio	**$pK_a < 8.5$** Change in saliva pH **does** affect saliva/plasma ratio
Basic Drugs	**$pK_a < 5.5$** Change in saliva pH **does not** affect saliva/plasma ratio	**$pK_a > 5.5$** Change in saliva pH **does** affect saliva/plasma ratio

5.6 DRUG PROPERTIES AFFECTING SECRETION INTO SALIVA

The saliva/plasma ratio is determined by the mechanism which transfers drugs from the blood into saliva. It has already been mentioned that for most drugs simple diffusion is the mode of transport. In order that the drugs can cross the epithelial membrane, the drugs must be lipid-soluble, non-ionized and not bound to proteins. However, this is a simplified version and other factors must also be taken into consideration. These include molecular weight and stereochemistry, lipid solubility, pKa of ionized compounds and protein binding in plasma and saliva.

5.6.1 MOLECULAR WEIGHT

In theory, smaller molecules diffuse more easily than larger ones. For highly water soluble drugs, a molecular weight of 100 has been shown to be a critical size.[139]

5.6.2 SOLUBILITY

The diffusion of a drug from blood to saliva is dependent on the lipophilicity of the drug. Lipophilic drugs tend to diffuse more easily than lipophobic drugs.

5.6.3 pK_a OF IONIZED COMPOUNDS

Partition theory states that the pKa value of a particular drug and the pH of the medium in which the drug exists dictate the relative amounts of ionized and unionized forms on both sides of the separating membrane. With acidic drugs, the saliva/plasma ratio decreases with decreasing saliva pH and with basic drugs, the saliva/plasma ratio decreases with increasing saliva pH.

In humans, saliva is usually more acidic than blood and physiological pH of blood is 7.4. Therefore:

for acidic drugs, more drug is in the non-ionized form in the acidic saliva matrix which causes diffusion across the membrane into blood. As a result of the higher pH on the blood side of the membrane, more of the drug is present in an ionized form (Figure 5.2). It follows that unbound acidic drugs have a saliva/plasma ratio < 1.

for basic drugs, more drug is in the ionized form in the acidic saliva matrix. This means that non-ionized drug can diffuse from the blood compartment to the saliva compartment (Figure 5.3). It follows that unbound basic drugs have a saliva/plasma ratio > 1.

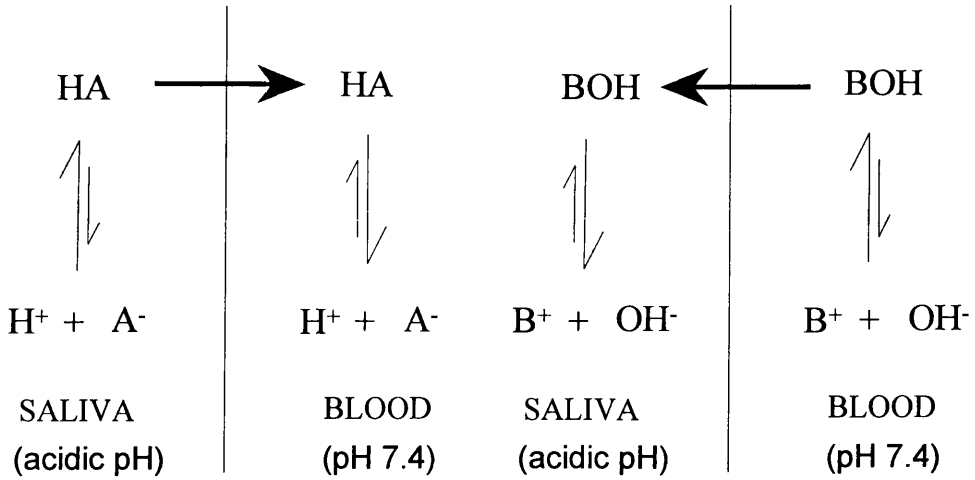


Figure 5.2: Diffusion of non-ionized acidic drug from saliva to blood.

Figure 5.3: Diffusion of non-ionized basic drug from blood to saliva.

5.6.4 PROTEIN BINDING

Drugs can bind to proteins to different extents and are often bound to either albumin or α_1-acid glycoprotein, the concentrations of which can vary between individuals. When more than one protein-binding drug is present in the system these can compete for the protein binding sites. This explains why drugs with the same pKa values can have completely different saliva/plasma ratios. Alterations in the binding of a drug to saliva or blood proteins will affect the concentrations of unbound drug found in saliva and blood.

5.7 CONTAMINATION

Different routes of administration will have an effect on the saliva/plasma ratios observed for the same drug. This was observed for cocaine which was administered intravenously, intranasally and smoked. High saliva/plasma ratios were seen for intranasal administration and for smoking as a result of contamination of the nasal and oral cavities. However, contamination by these methods was shown to clear rapidly and saliva/plasma ratios were comparable with those obtained for intravenous administration two hours after snorting or smoking.[155]

Also, there is the possibility of external contamination from the surrounding environment due to passive inhalation of drugs which can be smoked. However, to date no studies have been carried out to prove or disprove this.

5.8 PHARMACOKINETIC PARAMETERS

Since the mode of transfer of drugs into saliva is mainly by passive diffusion of drugs from the blood into saliva then the amount of a drug present in blood will have an effect on the amount in saliva. For cocaine the shape of saliva drug concentration curves corresponded with the plasma drug concentration curves. Half-life values could be estimated for cocaine in saliva and these were generally found to be lower than the corresponding half-lives in plasma after intravenous injection. After smoking or intranasal administration, the half-lives were comparable.[155]

5.9 COLLECTION OF SALIVA

5.9.1 METHODS

Collection of saliva is non-invasive and it is largely due to this that the analysis of this biological fluid for drugs has become popular. Special collection methods have been designed to improve sampling and make handling easier. To be of general clinical value, whole saliva is required to measure drug levels and it can be collected by a number of methods, either by stimulatory or non-stimulatory techniques. Stimulatory techniques are used to increase the flow of saliva and reduce the collection time.

Non-stimulated methods include allowing saliva to accumulate in the mouth and spitting it into a container or using the draining method which simply involves allowing the saliva to drip from the mouth. Repeated expectoration causes bubbles in the saliva which may affect the pH and collection by this method also has the disadvantage of collecting mucoid proteins and mouth debris. Saliva can also be collected by a suction method which aspirates saliva from the floor of the mouth as it is produced. Other methods involve using absorbent pads to soak up saliva as it is produced.[156] The pH of non-stimulated saliva has been reported to be more variable than that of stimulated saliva.[157]

Stimulated methods have included using citric acid, chewing paraffin wax, parafilm, washed rubber bands, chewing gum or sucking on teflon or a glass marble to stimulate saliva flow. Stimulated methods have been found not only to increase the volume of saliva, but also reduce the variability in pH and therefore less change in S/P ratio is observed. The use of parafilm, however, can cause some absorption to the stimulus and hence loss of drug[158] and chewing gum can contain ingredients which may interfere with the assay.

Saliva samples from six cocaine users collected by stimulated and non-stimulated conditions were analyzed for cocaine and its metabolites. These samples showed that nonstimulated saliva contained more cocaine than stimulated samples most likely due to an increase in saliva pH as a result of the increased saliva flow rate.[159]

Codeine concentrations using different collection techniques were found to be highest for spitting. Saliva collected after chewing on gum gave the next highest concentrations and lower concentrations were detected after acidic stimulation.[160]

A number of collection devices have been designed to facilitate collection using an absorbent pad or swab, such devices include Omni-Sal®, Salivette®, OraSure®, Accu-Sorb™. These collection devices have been found to have an influence on the amount of drug detected compared with saliva collected by spitting. Concentrations of tetrahydrocannabinol in a study in Belgium were higher in the salivette sample in comparison with the spitting saliva sample due to the presence of solid tetrahydrocannabinol in the mouth which was collected on the salivette.[161] On the other hand codeine concentrations detected on the salivette were lower than that obtained by spitting.[160]

The method of saliva collection is therefore very important and must be taken into consideration when organizing saliva tests.

5.9.2 VOLUME

It has already been mentioned that the volume of saliva which an individual can produce will be affected by certain diseases or the administration of certain drugs. Alcohol is another factor that acts to reduce salivary flow.[135]

5.10 PRE-ANALYTICAL AND ANALYTICAL CONSIDERATIONS

5.10.1 FREEZING AND THAWING

A newly taken sample of saliva can be difficult to pipette due to the mucoproteins and mucopolysaccharides. However, after freezing and thawing, the sample is easier to handle due to the denaturation of such constituents.

5.10.2 CENTRIFUGATION

Centrifugation can help to separate protein. Also, studies have shown that the stability of tetrahydrocannabinol in centrifuged blank saliva is excellent but in non-centrifuged saliva stability was poor.

5.10.3 COLLECTION DEVICES

If collection devices are used then it is important to assess the use of these for a specific drug. In a study it was found that after centrifugation of the salivette, less than 1 % of tetrahydracannabinol was recovered from the cotton role. Therefore, an assessment of in vitro recovery of the drug is important.

5.11 INTERPRETATION

5.11.1 DOSE-CONCENTRATION CORRELATIONS

A significant correlation was observed between saliva and plasma concentrations for codeine within an individual, but between individuals there was considerable variation.[160]

Early studies showed that the concentration of drugs in saliva was proportional to the concentration in plasma for some drugs including paracetamol,[162] theophylline,[163] digoxin,[164] amobarbital,[165] phenytoin and phenobarbital,[166] antipyrine, aminopyrine and phenacetin.[167] A correlation was indicated for cocaine and phencyclidine[168] concentrations in blood and saliva. However, poor correlations have been observed for tetrahydrocannabinol because it would appear that THC does not pass from plasma to saliva[169] but is sequestered in the buccal cavity after smoking.

Candidates for salivary therapeutic drug monitoring are phenytoin, carbamazepine, theophylline, digoxin, cyclosporin.[1]

5.12 ANTIDEPRESSANTS IN SALIVA

The first reports into the analysis of tricyclic antidepressants in saliva involved the detection of amitriptyline, desipramine and nortriptyline for the purposes of therapeutic drug monitoring.

Correlations between blood and saliva concentrations of individuals who were administered desipramine were found to be good. However, correlations of saliva/blood ratios between individuals were poor. The authors concluded that if saliva/plasma ratios were determined for each patient then drug concentrations could be monitored using saliva.[170]

Other authors reported poor correlations between total plasma and saliva concentrations of amitriptyline and nortriptyline. However, saliva pH was not taken into account. The authors concluded that the plasma concentration may be predicted from those detected in saliva if saliva pH was taken into account.[171, 172]

To date, there have been no other papers on the detection of antidepressants in saliva, including the newer, safer types which were developed after the tricyclic antidepressants.

5.13 AIMS

The aim of this study was to validate the blood method for antidepressants in saliva and to apply it to samples obtained from single and chronic users of fluoxetine and paroxetine. Saliva collection was to be carried out using Omni-Sal® collection devices and by spitting in order to assess the relative merits of each technique.

5.14 EXPERIMENTAL

5.14.1 SALIVA COLLECTION

Saliva samples were collected using two methods. The first of these methods involved using Omni-Sal® collection devices. These consisted of an absorbent pad which was placed under the tongue until an indicator turned from white to blue, signifying that 1 ml of saliva had been collected. The collection device was then inserted into a tube containing 1 ml of buffer with 0.2 % sodium azide as preservative. The second collection method involved allowing the saliva to collect in the mouth and this was collected by spitting.

Blank saliva samples were collected from volunteers who were known not to have taken fluoxetine, paroxetine or other antidepressant treatment. Both methods were used to collect these samples.

Samples were refrigerated at - 4 °C until analysis was carried out.

5.14.2 SPIKING SALIVA

5.14.2.1 Omni-Sal® method

Blank saliva was squeezed from the absorbent pad which was in buffer using a plunger fitted with a filter so that as much of the liquid was removed from the pad as possible. 100 µl of fluoxetine, norfluoxetine and paroxetine all at a concentration of 1 µg/ml in methanol were added to a clean test tube. The methanol was evaporated off under nitrogen at 40 °C and the blank saliva was poured into the test tube. This gave a spiked saliva sample with a concentration of 100 ng/ml of each drug. The sample was sonicated in an ultrasonic bath for 5 minutes. Saliva was spiked in a similar manner to give concentrations in the range of 5 - 100 ng/ml.

5.14.2.2 Spitting method

1 ml of saliva was pipetted into a test tube and fluoxetine, norfluoxetine and paroxetine were added at concentrations ranging from 5 - 100 ng/ml saliva. The samples were briefly vortex mixed.

Initially, the saliva was used without centrifugation or freezing and thawing and it was found to be difficult to handle. The viscosity and "stringy" nature of some samples made it difficult to pipette and also caused blockage of solid phase extraction cartridges. After collection, the samples were frozen and thawed and then centrifuged to separate the "stringy" substance and oral debris from the rest of the sample. This made it easier for the sample to be taken up by the pipette and also prevented further blockage of solid phase extraction columns.

5.14.3 RECOVERY STUDY OF EXTRACTION PROCEDURE FOR SALIVA

5.14.3.1 Extraction

The solid phase extraction method which was developed in Chapter 4 was used to extract the drugs from saliva. Initially five samples spiked to give a concentration of 100 ng/ml fluoxetine, norfluoxetine and paroxetine were extracted and the recovery of each was calculated using tumoxetine as the internal standard for fluoxetine and norfluoxetine and maprotiline as internal standard for paroxetine.

5.14.3.2 Results

The recoveries for norfluoxetine, fluoxetine and paroxetine were found to be 86.5, 98.4 and 90.0 % respectively. The acetone-chloroform fractions were also analyzed and were shown to only contain a small percentage of the drugs, 1.7 % norfluoxetine, 0.5 % fluoxetine and 0 % paroxetine.

Since the blood extraction method also gave high recoveries for saliva samples this method was fully validated for these drugs in saliva using both the Omni-Sal® devices and the spitting collection methods.

5.14.4 VALIDATION OF OMNI-SAL® METHOD

5.14.4.1 Linearity

Seven point calibration curves were produced with concentrations 5, 10, 15, 25, 50, 75, 100 ng/ml saliva. Internal standards, tumoxetine and maprotiline were added to give concentrations 50 ng/ml and 25 ng/ml saliva. Each concentration was extracted five times. The mean ratios and correlation coefficients were calculated and displayed in Table 5.2.

Linear calibration graphs were obtained and coefficients of correlation for fluoxetine, norfluoxetine and paroxetine extracted standards were 0.998, 0.999 and 0.999 and x-axis intercepts were - 0.22382, - 0.01101 and - 0.0093 respectively.

Table 5.2: Intra-day Variation (Omni-Sal®)

Concentration (ng/ml saliva)	m/z 330/344 NFLU/TUM (RSD %)	m/z 344/344 FLU/TUM (RSD %)	m/z 525/445 PAR/MAP (RSD %)
5	0.085 (8.9)	0.083 (10.9)	0.046 (7.9)
10	0.134 (6.2)	0.164 (2.7)	0.095 (6.8)
15	0.255 (3.9)	0.270 (8.3)	0.126 (5.8)
25	0.428 (5.8)	0.472 (5.1)	0.236 (4.9)
50	0.782 (1.4)	0.980 (5.1)	0.472 (6.6)
75	1.217 (1.7)	1.409 (3.8)	0.752 (6.5)
100	1.680 (1.2)	1.959 (6.5)	0.965 (3.7)

NFLU = norfluoxetine, FLU = fluoxetine, TUM = tumoxetine

RSD = relative standard deviation as a percent

5.14.4.2 Inter-day variation

The between-day variation was monitored by extracting the linearity curve on five different days over a period of two months. The relative standard deviation values were less than 12 % for all cases (Table 5.3).

Table 5.3: Inter-day Variation (Omni-Sal®)

Concentration (ng/ml saliva)	m/z 330/344 NFLU/TUM (RSD %)	m/z 344/344 FLU/TUM (RSD %)	m/z 525/445 PAR/MAP (RSD %)
5	0.079 (11.8)	0.087 (6.7)	0.050 (7.5)
10	0.127 (8.7)	0.172 (5.3)	0.096 (4.2)
15	0.241 (7.8)	0.272 (6.6)	0.136 (9.2)
25	0.409 (5.6)	0.461 (6.9)	0.221 (7.0)
50	0.773 (8.1)	0.928 (7.3)	0.461 (10.8)
75	1.213 (8.8)	1.434 (1.9)	0.696 (9.5)
100	1.613 (6.2)	2.026 (3.0)	0.947 (2.7)

NFLU = norfluoxetine, FLU = fluoxetine, TUM = tumoxetine

RSD = relative standard deviation as a percent

5.14.4.3 Recovery

A more extensive recovery study was carried out to cover the range of the calibration curve. Blank saliva was squeezed from the absorbent pad and spiked to give concentrations 5 - 100 ng/ml. Each concentration was extracted five times and 50 µl of tumoxetine and 25 µl of maprotiline at 1 µg/ml was added after the extraction.

Table 5.4: Recovery of fluoxetine, norfluoxetine and paroxetine from spiked saliva

Concentration (ng/ml saliva)	% Recovery Norfluoxetine (RSD %)	% Recovery Fluoxetine (RSD %)	% Recovery Paroxetine (RSD %)
5	86.0 (8.6)	89.5 (9.3)	91.3 (4.8)
10	89.9 (4.5)	99.9 (10.8)	92.4 (10.3)
15	90.3 (7.2)	90.0 (6.6)	92.6 (6.4)
25	90.5 (5.5)	94.8 (3.3)	90.5 (5.4)
50	96.1 (4.9)	97.7 (2.8)	94.3 (3.4)
75	92.8 (11.8)	94.5 (4.0)	90.9 (6.3)
100	91.9 (3.7)	97.1 (1.7)	92.8 (3.3)

RSD = relative standard deviation as a percent

Recoveries greater than 86 % for norfluoxetine, 89 % for fluoxetine and 90 % for paroxetine were achieved for all points on the linear calibration curve and the relative standard deviations were no higher than 12 %.

5.14.4.4 Recovery of analytes from Omni-Sal® swab

The method of collection of saliva can have an influence on the amount of drug detected in an individual at a specific time. As a result it is necessary to know the limitations of a device and the in vitro recovery of the drug is very important. The following experiment was carried out to test the recovery of the drugs from the device.

Blank saliva was collected from volunteers who were not receiving Prozac® or Seroxat® treatment. The saliva was collected by spitting into a plastic container. These samples were freezed and thawed and 6 ml samples were pipetted into plastic universals and spiked to give concentrations equivalent to 10, 50 and 100 ng/ml. The Omni-Sal® cotton swab was dipped into the saliva and removed when the indicator turned blue. The swab was placed in the buffer solution and the swab squeezed to give the saliva sample in buffer. These were then extracted and derivatized.

The results of this experiment showed that the recovery from the swab was reproducible, but much lower than the recovery obtained from saliva spiked after being squeezed from the pad.

Table 5.5: Recoveries from Omni-Sal® swab

Concentration (ng/ml saliva)	% Recovery Norfluoxetine (RSD %)	% Recovery Fluoxetine (RSD %)	% Recovery Paroxetine (RSD %)
10	25.6 (6.4)	29.7 (7.3)	23.2 (7.6)
50	24.2 (2.4)	29.1 (3.5)	23.3 (4.5)
100	25.1 (7.2)	30.4 (6.5)	26.3 (8.7)

RSD = relative standard deviation as a percent

The mean recovery of norfluoxetine, fluoxetine and paroxetine from the swab was found to be 25.0 %, 29.7 % and 24.3 % respectively. These recoveries were taken into account when case samples were analyzed and adjusted accordingly.

5.14.4.5 Recovery of analytes from Omni-Sal®swab after sonication in methanol

The recovery experiment in 5.14.4.4 was repeated for concentrations 50 and 100 ng/ml except this time the swab was rinsed with methanol and sonicated in an ultrasonic bath for

10 minutes after it had been squeezed to remove the saliva/buffer mixture. This experiment was carried out to see if the recovery from the swab could be improved.

Table 5.6: Recoveries from Omni-Sal® swab after sonication in methanol

Concentration (ng/ml saliva)	% Recovery Norfluoxetine (RSD %)	% Recovery Fluoxetine (RSD %)	% Recovery Paroxetine (RSD %)
50	59.2 (1.3)	51.9 (4.7)	68.5 (4.3)
100	70.5 (5.9)	66.2 (3.3)	49.7 (11.3)

This method was reproducible at both concentrations for each drug. Coefficients of variation were less than 6.0 % for fluoxetine and norfluoxetine and less than 12 % for paroxetine. However, the recoveries at the different concentrations were not reproducible. There were differences of 11 – 19 % in recovery at the two different concentrations. These variations may be a result of the swab being squashed and therefore access of methanol to the bound drug may be hindered more in some cases than in others. A larger volume of methanol and a longer period of sonication could help to release a higher percentage of each drug. However, this would increase the length of time of the step.

5.14.4.6 Limit of Detection and Quantitation

Saliva samples were spiked to give concentrations 1.0, 0.5, 0.3, 0.2 and 0.1 ng/ml saliva. The limit of detection was defined as the concentration of drug giving a signal to noise ratio of 3. This was found to be 0.1 ng/ml for the analytes. The limit of quantitation was defined as the concentration of drug giving a signal to noise ratio of 5 and this was found to be 0.2 ng/ml for all analytes.

5.14.4.7 Blank saliva controls

Blank saliva controls were extracted to make sure that no constituents in the sample coeluted or interfered with the analytes. The extracts were initially analysed by fullscan GC/MS and then by selected ion monitoring.

5.14.5 VALIDATION OF SPITTING METHOD

5.14.5.1 Linearity

1 ml aliquots of saliva were spiked to give concentrations equivalent to 5, 10, 15, 25, 50, 75, 100 ng/ml saliva. Internal standards, 50 ng of tumoxetine and 25 ng of maprotiline were added. Each concentration was extracted five times and calibration graphs of each compound were produced. The mean ratios and correlation coefficients were calculated and displayed in Table 5.7.

Table 5.7: Intra-day Variation (Spitting)

Concentration (ng/ml saliva)	Mean m/z 330/344 ratio NFLU/TUM (RSD %)	Mean m/z 344/344 ratio FLU/TUM (RSD %)	Mean m/z 525/445 ratio PAR/MAP (RSD %)
5	0.096 (14.1)	0.130 (4.8)	0.045 (10.8)
10	0.224 (12.4)	0.246 (6.3)	0.089 (6.3)
15	0.345 (9.2)	0.360 (3.9)	0.133 (0.9)
25	0.591 (10.9)	0.599 (4.4)	0.217 (1.7)
50	1.171 (4.1)	1.23 (4.7)	0.452 (4.1)
75	1.958 (5.7)	1.924 (2.2)	0.696 (2.2)
100	2.633 (3.5)	2.585 (6.4)	0.923 (3.2)

NFLU = norfluoxetine, FLU = fluoxetine, TUM = tumoxetine

RSD = relative standard deviation as a percent

Coefficients of correlation for fluoxetine, norfluoxetine and paroxetine extracted standards were 0.999, 0.998 and 0.999 and x-axis intercepts - 0.0265, - 0.064 and - 0.0068 respectively.

5.14.5.2 Inter-day variation

The linearity curve was extracted five times over a period of two months in order to assess the inter-day variation of the method. The relative standard deviation values were less than 14 % for all cases (Table 5.8).

Table 5.8: Inter-day Variation (Spitting)

Concentration (ng/ml saliva)	Mean m/z 330/344 ratio NFLU/TUM (RSD %)	Mean m/z 344/344 ratio FLU/TUM (RSD %)	Mean m/z 525/445 ratio PAR/MAP (RSD %)
5	0.112 (13.5)	0.114 (13.4)	0.042 (13.0)
10	0.228 (4.0)	0.241 (5.9)	0.095 (5.2)
15	0.350 (3.5)	0.330 (12.2)	0.141 (7.5)
25	0.613 (9.6)	0.585 (6.4)	0.208 (6.8)
50	1.219 (9.3)	1.110 (9.4)	0.497 (9.5)
75	1.963 (5.2)	1.839 (13.6)	0.695 (9.8)
100	2.491 (6.3)	2.389 (9.7)	0.966 (4.5)

NFLU = norfluoxetine, FLU = fluoxetine, TUM = tumoxetine

RSD = relative standard deviation as a percent

5.14.5.3 Recovery

A recovery study was carried out to cover the range of the calibration curve. Blank saliva was spiked to give concentrations 5 - 100 ng/ml. Each concentration was extracted five times and 50 µl of tumoxetine at 1 µg/ml and 25 µl of maprotiline at 1 µg/ml were added after the extraction.

Recoveries greater than 90 % were obtained for all three analytes over the linear concentration range and relative standard deviations were all less than 12 %.

Table 5.9: Recovery of fluoxetine, norfluoxetine and paroxetine from spiked saliva

Concentration (ng/ml saliva)	% Recovery Norfluoxetine (RSD)	% Recovery Fluoxetine (RSD)	% Recovery Paroxetine (RSD)
5	90.5 (9.6)	91.0 (11.6)	95.2 (9.3)
10	90.7 (3.7)	96.6 (3.9)	96.4 (4.4)
15	95.2 (7.4)	91.6 (11.2)	94.2 (6.9)
25	96.6 (9.0)	96.9 (7.2)	96.5 (4.6)
50	97.8 (5.7)	98.4 (4.8)	90.6 (9.4)
75	95.5 (2.5)	97.8 (3.8)	93.4 (8.3)
100	98.1 (4.2)	95.8 (5.7)	96.0 (5.8)

RSD = relative standard deviation

5.14.5.4 Limit of Detection and Quantitation

Limit of detection and quantitation were defined as in 5.13.4.6 and these were found to be the same as the Omni-Sal® method.

5.14.5.5 Blank saliva controls

Blank saliva controls which were extracted and run on fullscan showed that there were no interfering peaks arising from the saliva matrix.

5.15 CONCLUSIONS

The solid phase extraction method developed to extract fluoxetine, norfluoxetine and paroxetine from blood also successfully extracted these analytes from saliva. Saliva samples were collected by two methods, spitting and with Omni-Sal® devices and both methods were fully validated. The largest difference in the two collection methods was in the recovery. In-vitro recovery from the Omni-Sal® swab was found to be reproducible, but much lower than the recovery obtained from the sample collected by spitting. This low recovery is a result of the analytes not being completely removed from the swab. Sonication of the swab in methanol for a period of 10 minutes removed a higher percentage of the drugs from the swab. Also, when the saliva is squeezed from the swab, 100 % of the sample will not be obtained. When using this device to collect samples it is therefore necessary to know its limitations and careful interpretation of data is required when analyzing saliva samples which have been collected by this method. Both collection methods were used to analyze case samples for fluoxetine and paroxetine.

6 Antidepressants in Hair

6.1 INTRODUCTION

One of the major advantages of using hair as a biological matrix for drug testing is that drugs are accumulated and stored in the hair shaft for a long time. This led investigators to believe that it may be possible to use hair analysis for compliance monitoring as an alternative to blood or urine testing. However, one of the main criteria for this is that there is a correlation between the administered dose and the drug concentration in hair.

The reports which have been published providing information on certain drugs and compliance monitoring have proved controversial. Some papers have reported correlations for drugs such as amitriptyline,[173] carbamazepine[174] and haloperidol[175] whilst others have reported no correlation largely due to inter-individual differences in drug incorporation into hair.[176] Consequently, hair analysis cannot be used for compliance monitoring.

However, hair analysis can be used to assess the constant use of a drug by an individual. If an individual is prescribed a constant daily dose of a drug then the measured levels incorporated into that individual's hair should remain relatively constant along the length of the sample.

Depression is one of the most common mental disorders in the world today. Treatment can be carried out with antidepressants, by psychotherapies or a combination of both. As a result, antidepressants have become one of the most commonly prescribed drugs worldwide.[177] Many different groups of people including geriatric patients, prisoners and drug addicts are prescribed antidepressants at sometime or other. In some of these cases supervision of drug taking is possible, for example in geriatric hospitals. However, for the majority of people who are prescribed these drugs supervision is not possible and they are assumed to be taking them as prescribed by their general practitioner. In reality, some people do not like to be reliant on these drugs and stop taking them. Hair testing can provide information on compliance with the use of such drugs.

Also, the incorporation of prescription drugs into hair may be useful in forensic cases, for example, in cases involving decomposed or skeletal remains where identification may be difficult.

A history of drug use in hair can provide information corresponding to a patient's drug history as given in medical records.

6.2 ANALYSIS

Tricyclic antidepressants, amitriptyline, nortriptyline and imipramine were identified in the hair of psychiatric patients who had been on long-term treatment with these antidepressants.[178] Amitriptyline, nortriptyline and clomipramine were detected in postmortem hair samples at concentrations of 0.42 ng/mg, 0.91 ng/mg, and 0.37 - 0.79 ng/mg respectively.[179] A study was conducted on sixty hair samples taken from patients receiving amitriptyline treatment to assess the possibility of using hair concentrations to monitor patients' therapeutic compliance. Hair concentrations of amitriptyline were found to range from 0 - 17.21 ng/mg. Correlation between dose and concentration was not good enough to estimate an individual's therapeutic compliance.[173] These methods all used alkaline hydrolysis to solubilize the hair and liquid-liquid extraction to remove the drugs from the matrix.

In another study, amitriptyline, nortriptyline, doxepin, dothiepin, imipramine, mianserin and trimipramine were detected in postmortem hair samples.[55, 180] Comparisons were made of methods involving solubilization of hair using sodium hydroxide solution, dilute hydrochloric acid, methanol or subtilisin Carlsberg. Stability studies indicated that nortriptyline degraded slightly in strong alkaline conditions, but the other antidepressant drugs showed no significant losses. The acidic, methanolic and enzymatic methods were shown to be less effective than the alkaline method at recovering antidepressants from case hair samples. Recoveries of the acidic, enzymatic and solvent methods were given as a percentage of the alkaline method and these were 49 %, 51 % and 21 % respectively.[101] Concentrations detected in hair were in the range 3.5 - 34 ng/mg for amitriptyline, 3.8 - 9.2 ng/mg for nortriptiline, 7.7 - 87 ng/mg for doxepin, 6.7 - 137 ng/mg for dothiepin, 104 ng/mg for imipramine and 9.2 ng/mg for mianserin.[55]

Tricyclic antidepressants, amitriptiline, clomipramine, doxepine, imipramine and maprotiline were detected in human hair. Concentrations varied between 0.4 and 40 ng/mg for the drugs. In the majority of cases the nor-metabolites were found to accumulate less in

the hair than the parent drug. No correlation between the administered dose and the concentration found in hair was established.[181]

Doxepin and its metabolite, desmethyldoxepin were extracted from hair samples by acidic hydrolysis followed by solid phase extraction. GC/MS was used to detect the analytes and concentrations varied from 0.09 - 0.59 ng/mg for doxepin and 0.04 - 0.40 ng/mg for desmethyldoxepin within a segmented hair sample obtained from a patient receiving a 25 mg daily dose. The metabolite concentrations was always less than that of the parent drug.[182]

Fluoxetine, clomipramine, amitriptyline and moclobemide were included in a screening procedure used to identify and quantify a wide range of drugs in human hair. The method involved acidic hydrolysis of hair followed by solid phase extraction on a C18 phase. Analysis was carried out using GC/MS and recovery of fluoxetine was found to be 86 %. A presented case sample showed the presence of 4.3 ng/mg of fluoxetine.[183]

Fluoxetine has also been detected in the hair of rats. Male rats were injected with 10 mg/kg fluoxetine for 15 consecutive days and hair samples were shaved from the neck area on days five and fifteen. The hair was digested using sodium hydroxide and heated at 56 °C for 2 hours. Liquid-liquid extraction was used to clean up the digested supernatant and samples were derivatized with pentafluoropropionic anhydride. Fluoxetine concentrations were found to be 9.96 ng/mg and 1.12 ng/mg for days five and fifteen respectively.[184]

The active metabolite, norfluoxetine was not sought for in either of these fluoxetine studies.

6.3 AIM

The purpose of this study was to develop a reliable method to extract selective serotonin reuptake inhibitor antidepressants, fluoxetine, its metabolite, norfluoxetine and paroxetine from hair and to apply the method to case samples to find if a correlation between dose and concentration exists between individuals and within an individual. These concentrations were to be compared with those detected in the nails of the individuals supplying the hair samples. Ethical approval was obtained for this study.

6.4 EXPERIMENTAL

6.4.1 CHEMICALS

Fluoxetine, norfluoxetine and internal standard, tumoxetine were obtained as hydrochloride salts from Eli Lilly (Windlesham, England). Paroxetine hydrochloride was obtained from SmithKline Beechams (Herts, England) and maprotiline from Promochem (Herts, England).

6.4.2 STANDARDS

Stock standard solutions of fluoxetine, norfluoxetine, tumoxetine, paroxetine and maprotiline were prepared in methanol at a concentration of 1 mg/ml and stored at - 20 °C. Each of these stock solutions was diluted with methanol to give working standard solutions at 1 μg/ml and 10 μg/ml which were refrigerated at 4 °C.

6.4.3 SOLUTIONS AND BUFFERS

0.1 M Potassium Phosphate buffer, pH 6

6.81 g of potassium dihydrogen phosphate (FW=136.09) was dissolved in 450 ml distilled water. The pH was increased to 6 with 1 M potassium hydroxide solution and this was made up to 500 ml with distilled water.

β-glucuronidase solution

Crude β-glucuronidase solution, type helix pomatia (132 500 units/ml) was diluted with phosphate buffer in the ratio 1:4 and vortex mixed briefly.

6.4.4 GAS CHROMATOGRAPHY-MASS SPECTROMETRY

A ThermoQuest Trace GC with a Finnigan Trace MS was used to analyze samples. The GC was fitted with a HP-5 column, 30 m x 0.25 mm x 0.25 μm film thickness and the temperature programme was set from 150 to 270 °C at 10 °C / min. The injector temperature was 280 °C and the carrier gas was helium. The temperature of the source and interface were 200 °C and 250 °C respectively. The emission current was 350 μA. 1μl was injected.

Ions monitored were m/z 240 and 344* for fluoxetine and tumoxetine, m/z 226 and 330* for norfluoxetine, m/z 388 and 525* for paroxetine and m/z 445* and 446 for maprotiline. Ions marked * were used for quantitation.

6.4.5 DECONTAMINATION OF HAIR

Blank hair was obtained from a volunteer of Malay-Chinese origin. Hair was cut finely into 1-2 mm pieces with scissors and decontaminated using several sequential wash procedures:

(iv) One ten minute wash with sodium dodecyl sulphate solution (0.1 %).

(v) Three ten minute washes with distilled water.

(vi) Three five minute washes with methanol.

Each step was sonicated in 5 ml of solvent and then the solvent was decanted. The washed hair was dried in an oven at 40 °C and used for blank controls and for spiking with the drugs of interest.

6.4.6 SPIKING HAIR

Blank washed hair was spiked in bulk with fluoxetine, norfluoxetine, and paroxetine in methanol to give standards equivalent to 5 ng/30 mg, 10 ng/30 mg, 25 ng/30 mg, 50 ng/30 mg, 75 ng/30 mg, 100 ng/30 mg and 200 ng/30 mg. The samples were ultra-sonicated at room temperature for 15 minutes and the methanol was allowed to evaporate off at room temperature.

6.4.7 EXTRACTION OF ANTIDEPRESSANTS FROM SPIKED HAIR

Twelve 30 mg portions of hair spiked to give concentration 100 ng/ 30 mg were weighed out accurately. Four sets of three samples were treated by methanolic treatment, acid hydrolysis, alkaline hydrolysis or enzyme hydrolysis as described below. All of the samples were then extracted by solid phase extraction.

6.4.7.1 Methanolic treatment

1 ml of methanol was added to 30 mg of spiked hair and this was incubated for 18 hours at 45 °C. The sample was allowed to cool and the methanol was transferred to a vial and 0.5 ml methanol was added to hair, sonicated for 5 minutes and combined with the first fraction.

6.4.7.2 Hydrochloric acid hydrolysis

Hydrochloric acid (1 ml, 0.1 M) was added to 30 mg spiked hair and incubated for 12 hours at 45 °C. The sample was allowed to cool and 1 ml, 0.1 M sodium hydroxide solution was added to neutralize the acid. 2 ml of phosphate buffer was added and the tube shaken briefly. The solution was transferred to a clean test tube by pipette. The hair was washed with 1 ml phosphate buffer and this was combined with the first fraction.

6.4.7.3 Sodium hydroxide hydrolysis

Sodium hydroxide (1 ml, 1 M) was added to 30 mg spiked hair and this was incubated at 80 °C for 30 minutes or until the hair had completely dissolved. This was allowed to cool and 1 ml of hydrochloric acid (1 M) was added to neutralize the alkali. Phosphate buffer (2 ml) was added to the solution.

6.4.7.4 β-glucuronidase hydrolysis

Phosphate buffer (1 ml) was added to 30 mg spiked hair followed by 50 µl β-glucuronidase solution. This was incubated for 2 hours at 45 °C. The sample was allowed to cool and the solution was transferred to a clean test tube. The hair was washed with a further 2 ml phosphate buffer, sonicated for 5 minutes and this was combined with the first fraction.

6.4.7.5 Solid Phase Extraction

Each of the above treated samples was subsequently extracted by solid phase extraction. The procedure used to extract opiates and methadone from hair provided clean extracts using ZSDAU020 Clean Screen columns. This method was tested for the extraction of antidepressants from hair.

World Wide Monitoring Clean Screen® columns (ZSDAU020) were conditioned with 3 ml of methanol, followed by 3 ml of distilled water and then 1 ml phosphate buffer. The samples were applied to the columns at 2 ml/min which were then washed with 2 ml distilled water, 2 ml 0.1 M hydrochloric acid and 3 ml methanol. The columns were dried under full vacuum for 10 minutes and the drugs were eluted with 4 ml of dichloromethane/isopropanol/concentrated ammonia (78:20:2). The solvent was evaporated to dryness and internal standards, tumoxetine and maprotiline were added

(100 ng of each) and the methanol evaporated. The residue was derivatized with HFBA as previously described.

The samples were injected into the GC/MS (1 µl) along with unextracted standards at concentration 100 ng/50 µl.

6.4.7.6 Results

Recoveries using the four methods on spiked hair samples showed that the methanol based method gave the highest recoveries followed by the acid hydrolysis, then β-glucuronidase and then alkaline hydrolysis (Table 6.1).

Table 6.1: Recoveries of extraction methods on spiked hair

Method	% Recovery (RSD)		
	Fluoxetine	**Norfluoxetine**	**Paroxetine**
Methanol extraction	92.2 (7.5)	95.4 (9.4)	86.5 (2.5)
Acid hydrolysis	87.2 (4.1)	85.1 (1.3)	80.8 (6.3)
Alkali hydrolysis	80.1 (4.6)	82.0 (4.3)	73.2 (7.9)
Enzyme hydrolysis	84.8 (7.7)	85.7 (4.5)	85.7 (10.8)

RSD = Relative standard deviation

However, these recoveries are only an indication of the extraction method since the hair was spiked and the drugs were not actually embedded within the hair shaft. For a more realistic indication of the efficiency of the extraction method, the technique had to be tested on a real case sample.

Blank extracts from each method were run on the GC/MS using fullscan. The alkali hydrolysis and enzyme methods provided less clean extracts than the other two methods. In the case of the alkali hydrolysis this is due to the complete dissolution of the hair matrix. The other two methods were comparable. For this reason, it was decided to compare the methanol and hydrochloric acid methods using a real case sample. Lack of sample prevented all four methods being tested. The use of these less extreme methods could also allow the possibility of using this method to screen for other drugs which may be unstable in harsh alkaline conditions.

6.4.8 COMPARISON OF METHANOL EXTRACTION AND ACID HYDROLYSIS

Two postmortem hair samples were taken from a fluoxetine user (Case HF/1) and a paroxetine user (Case HP/1). The hair samples are described below.

Case HF/1: Hair sample was 16.5 cm long. The first 1.5 cm from root end was naturally brown in colour and rest of length was bleached blond.

Case HP/1: Hair sample was 11.5 cm long and naturally dark brown.

6.4.8.1 Case HF/1

The roots were cut off (0.5 cm) and the rest of the hair was segmented into 1 cm segments. For this experiment segments 1 (0.5-1.5 cm) and 2 (1.5-2.5 cm) were selected since these had no bleaching and minimum amount of bleaching on them respectively. These were washed using the decontamination protocol (see 6.4.5) and the methanolic washes were analyzed. Washes were carried out until no drug was detected in the final wash. Segments 1 and 2 were split into two fractions for analysis by methanolic extraction and acidic hydrolysis. The weights of each fraction are shown in Table 6.2.

Table 6.2: Weight of hair samples

Case, Segment	Methanol extraction Weight (mg)	Acid hydrolysis Weight (mg)
HF/1, 0.5-1.5 cm	9.01	9.35
HF/1, 1.5-2.5 cm	9.48	9.07
HP/1, 0.5-2.5 cm	9.72	9.70

6.4.8.2 Case HP/1

The roots were cut off (0.5 cm) and the rest of the hair was segmented into 2 cm segments. These larger segments were chosen because the sample was not nearly as thick as the previous one. Using 1 cm segments may have prevented detection due to lack of sample. Segment 1 (0.5-2.5 cm) was selected for this experiment. This was washed using the decontamination protocol and the methanolic washes were analyzed. Washes were carried out until no drug was detected in the final wash. The segment was split into two fractions

for analysis by methanolic extraction and acidic hydrolysis. The weights of each fraction are shown in Table 6.2.

The samples were extracted as previously along with blank and spiked samples at concentrations 5 ng/30 mg, 10 ng/30 mg, 25 ng/30 mg, 50 ng/30 mg, 75 ng/30 mg, 100 ng/30 mg and 200 ng/30 mg.

6.4.8.3 Results

Fluoxetine and norfluoxetine were detected in the first two methanol wash fractions of case HF/1, but neither was detected in the third wash fraction. No paroxetine was detected in the washes of case HP/1. Calibration graphs were constructed for the three analytes by the two extraction methods and for unextracted standards. The results are displayed in Table 6.3.

Table 6.3: Linearity for spiked hair samples

		Fluoxetine	**Norfluoxetine**	**Paroxetine**
Methanol	$y = mx + c$	$y = 0.020x - 0.033$	$y = 0.017x - 0.066$	$y = 0.005x + 0.029$
	R^2	0.999	0.994	0.995
Acid	$y = mx + c$	$y = 0.019x - 0.038$	$y = 0.016x - 0.001$	$y = 0.006x + 0.010$
	R^2	1.000	0.992	0.998
unextracted	$y = mx + c$	$y = 0.019x - 0.059$	$y = 0.014x - 0.027$	$y = 0.007x + 0.017$
	R^2	0.999	0.997	0.995

Analysis of the two case samples showed that the acid based method provided higher recoveries of fluoxetine, norfluoxetine and paroxetine from hair than the methanolic method. In the fluoxetine case the acidic method improved the recovery by a factor of 4 for norfluoxetine and 2.5 for fluoxetine and in the paroxetine case it improved the recovery by a factor of 2.5. The results of the extractions are given in Table 6.4.

Table 6.4: Concentrations of analytes from hair by methanol and acid methods

		Fluoxetine (ng/mg)	**Norfluoxetine (ng/mg)**	**Paroxetine (ng/mg)**
Methanol	HF/1, 0.5-1.5 cm	2.36	2.57	- ve
	HF/1, 1.5-2.5 cm	2.28	2.17	- ve
	HP/1, 0.5-2.5 cm	- ve	- ve	0.17
Acid	HF/1, 0.5-1.5 cm	5.55	10.92	- ve
	HF/1, 1.5-2.5 cm	6.35	8.41	- ve
	HP/1, 0.5-2.5 cm	- ve	- ve	0.43

For the fluoxetine case sample, concentrations of fluoxetine and norfluoxetine were similar when extracted by the methanol method but norfluoxetine was much higher than fluoxetine when the acid hydrolysis was used. A further experiment was conducted to ensure that none of the fluoxetine has been hydrolyzed to norfluoxetine using this method.

6.4.9 RECOVERY OF FLUOXETINE AND NORFLUOXETINE AFTER ACID HYDROLYSIS

An initial recovery study of the acidic method was carried out. Three vials were spiked with 50 ng of fluoxetine and 50 ng of norfluoxetine, another three vials with 50 ng of fluoxetine only and a further three vials with norfluoxetine only. 1 ml of 0.1 M hydrochloric acid was added to each vial and incubated in the oven for 12 hours at 50 °C. The samples were further treated as previously mentioned in 6.4.7.5.

Table 6.5: Recoveries of fluoxetine and norfluoxetine after acidic hydrolysis

	% Fluoxetine	**% Norfluoxetine**
Fluoxetine and norfluoxetine	94.9	95.7
Fluoxetine only	96.4	-
Norfluoxetine only	-	96.2

Recoveries were high for both analytes and the acid method did not appear to cause any chemical alteration of the drugs. The acid hydrolysis method was chosen for all further extractions.

6.4.10 METHOD VALIDATION

6.4.10.1 Intra-day variation

Each calibration concentration, 5 ng/30 mg, 10 ng/30 mg, 25 ng/30 mg, 50 ng/30 mg, 75 ng/30 mg, 100 ng/30 mg and 200 ng/30 mg was extracted five times to calculate the intra-day variation of the assay. Ratios of drug to internal standard are displayed in Table 6.6 along with the coefficient of variation in parenthesis. Values of correlation coefficient, R^2 and intercept and slope values for regression equations are displayed in Table 6.7.

Table 6.6: Intra-day variation

Concentration (ng/30 mg hair)	Mean FLU/TUM ratio m/z 344/344 (RSD %)	Mean NFLU/TUM ratio m/z 330/344 (RSD %)	Mean PAR/MAP ratio m/z 525/445 (RSD %)
5	0.069 (5.7)	0.090 (10.1)	0.028 (7.9)
10	0.148 (6.9)	0.165 (9.8)	0.054 (8.8)
25	0.410 (6.6)	0.419 (7.7)	0.158 (5.4)
50	0.813 (6.3)	0.724 (4.8)	0.339 (4.3)
75	1.266 (5.8)	1.233 (5.2)	0.488 (6.2)
100	1.787 (4.3)	1.778 (7.9)	0.653 (4.8)
200	3.721 (6.6)	3.595 (3.4)	1.228 (5.3)

FLU = fluoxetine, NFLU = norfluoxetine, PAR = paroxetine, TUM = tumoxetine, MAP = maprotiline
RSD = relative standard deviation as a percent

Table 6.7: Linear Regression Values

	Fluoxetine	Norfluoxetine	Paroxetine
R^2	0.9987	0.9971	0.9981
Intercept	- 0.0726	- 0.0585	0.0099
Slope	0.0188	0.0181	0.0062

6.4.10.2 Inter-day variation

The calibration curve was extracted over five different days and the inter-day variation was assessed and displayed in Table 6.8.

Table 6.8: Inter-day variation

Concentration (ng/30 mg hair)	Mean FLU/TUM ratio m/z 344/344 (RSD)	Mean NFLU/TUM ratio m/z 330/344 (RSD)	Mean PAR/MAP ratio m/z 525/445 (RSD)
5	0.077 (11.0)	0.084 (6.4)	0.032 (9.8)
10	0.172 (9.6)	0.154 (6.4)	0.069 (12.2)
25	0.438 (8.3)	0.377 (9.8)	0.168 (5.7)
50	0.884 (6.1)	0.813 (11.8)	0.336 (8.8)
75	1.355 (4.3)	1.240 (11.5)	0.507 (11.3)
100	1.857 (4.0)	1.772 (8.4)	0.679 (9.2)
200	3.894 (4.7)	3.444 (7.5)	1.269 (8.1)

FLU = fluoxetine, NFLU = norfluoxetine, PAR = paroxetine, TUM = tumoxetine, MAP = maprotiline
RSD = relative standard deviation

6.4.10.3 Limit of Detection (LOD)

Hair was spiked to give concentrations 1, 0.5 and 0.1 ng/30 mg. These were extracted and analyzed by GC/MS to find out the limit of quantitaion and detection. 1.0 ng/30 mg was easily quantified for all drugs. All were detected at concentration 0.5 ng/30 mg, but none at 0.1 ng/30 mg.

6.5 CONCLUSIONS

Extraction of fluoxetine and paroxetine from spiked hair samples was carried out using alkaline hydrolysis, enzyme hydrolysis, acid hydrolysis and methanol extraction. As a result of complete dissolution of hair, it was shown using fullscan analysis that alkaline hydrolysis provided less clean extracts than the other three methods. Acid hydrolysis and methanol methods were compared using a real case sample to indicate which method provided the highest recovery from hair. The acidic method provided fluoxetine, norfluoxetine and paroxetine concentrations 2.5, 4 and 2.5 times respectively higher than those detected using the methanol method. The acidic method was successfully validated for fluoxetine, its metabolite and paroxetine and used to extract these antidepressants from hair case samples.

7 Antidepressants in Nails

7.1 INTRODUCTION

Nails have been used to detect poisons for the past two centuries. Elemental analysis of nails has been investigated for various reasons. Cadmium, copper, lead, zinc, iron and magnesium have been detected in the nails of children for the purpose of environmental exposure,[185, 186] lead in the nails of smelt workers for occupational exposure[187] and mercury in the nails for dental, industrial, domestic and medical exposure.[188] Arsenic has been detected in forensic cases.[189, 190, 191] Nail analysis has also been investigated for therapeutic drug monitoring of the drug haloperidol.[192] Drugs of abuse which have been investigated in nail to date have included amphetamine[193, 194, 195], cocaine[196, 197, 198] cannabinoids[199, 200] and opiates.[201] The methods used for analysis of these drugs will be discussed later.

Nail as a biological specimen for drug analysis has similar advantages to hair analysis. Collection is non-invasive, it can provide a longer record of drug use than conventional fluids and the stability of the matrix means that drugs can be retained over long periods of time. In cases where decomposed remains are involved, conventional fluids may produce negative results due to the instability of the drugs in these fluids or there may be not be any fluids to analyze. Nail analysis can be used as an alternative to hair for the detection of chronic drug use. The samples can be easily stored and transported without special requirements such as refrigeration and one added advantage over hair analysis is that bias resulting from different amounts of hair pigment is not an issue with nails.

7.2 THE NAIL AND SURROUNDING COMPONENTS

The *nail plate* has a complex chemical structure made up of keratin cells which are closely packed together. Along the length of the plate on the outer and inner surfaces are parallel striations, fine ones on the outer surface and thicker ones on the inner surface. The *nail bed* consists of soft tissue which lies beneath the nail plate and its surface mirrors that of the inner surface of the *nail plate*. The proximal part of the nail is set into a depression on the dorsal surface and it is this part of the nail which separates the *dorsal* and *ventral matrices* which make up the *nail fold*. The skin on the dorsal surface of the *nail fold* is called the *nail wall* from which a thin membrane, the *eponychium* (*cuticle*) emerges onto the dorsum

of the nail. The *lunula*, a white convex shaped area extends from the *eponychium* and may or may not be present on all digits. The *hyponychium* lies beneath the distal nail, in between the continuous *nail bed* and epidermis which covers the ventral surface of the digits (Figure 7.1).[202, 203]

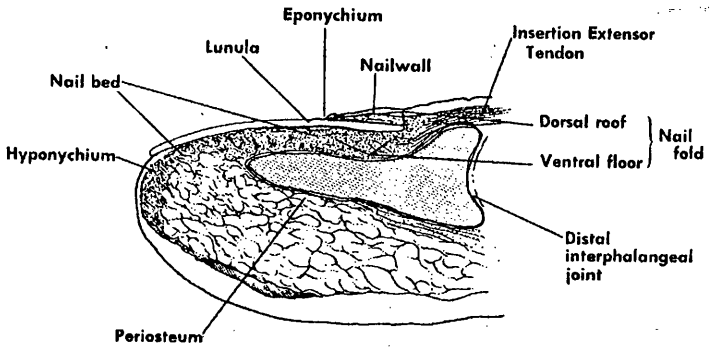


Figure 7.1: Structure of the nail (Copyright © American Society for Surgery of the Hand, 1980)

The mature nail consists of three layers: the dorsal nail, the intermediate nail and ventral nail which are formed in different regions of the nail fold by different processes. [203]

7.3 COMPOSITION OF NAILS

Nails are primarily made up of a substance called keratin, which is a complex mixture of proteins. Other keratin containing substances include hair, claws, feathers, scales and hooves. Substances containing keratin are naturally insoluble, pliable and durable.

Keratin can exist in a soft or hard form. The former is present in stratum corneum, corns, callouses and the eponychium of nails whilst the latter occurs in hair, nails, claws, beaks and quills. These forms have different compositions, the hard keratins containing more sulphur than the soft ones.[204]

7.4 NAIL FORMATION

There have been conflicting views as to whether the nail is produced solely by the nail matrix or if the nail bed contributes to the nail formation. Initial studies into nail production led scientists to believe that the nail matrix was the sole origin of nail production.[205] Further investigations using a silver protein staining method suggested that the nail was made up of three layers produced by a combination of the matrix, the bed and

the proximal nail fold.[206] Other studies later supported this work.[207, 208, 209] A thioflavin T-fluorochroming technique provided evidence that the three nail layers were developed from different sites.[210] However despite these results, conclusions were drawn from radioautographic experiments that the matrix was the only source of nail formation.[211] Glycine-labelled cells were traced from the matrix into the plate, but none were traced from the proximal nail fold or nail bed to the plate. Other authors also used radioautography to label cells.[212] They found that labelled cells were not present in the nail bed after one hour, but were present after one and two week intervals. This was explained to be the result of cell migration from the nail matrix to the nail bed.

A more recent study has shown that only 80 % of nail was produced by the germinal matrix of the lunula and that 20 % was produced by the nail bed.[213] These authors found that nail thickness and mass increased along the length of the nail indicating that the nail bed must contribute to the formation of the nail plate. In a further study, it was concluded that nail is continuously formed along the length of the nail as it grows out. These findings provide a source of entry of drugs into the distal nail plate and provided an explanation for the rapid access of antifungal drugs into the nail plate. [214]

7.5 DRUG INCORPORATION INTO NAILS

The mechanism of drug incorporation into nails is not fully understood, but it is believed that drugs are incorporated into the cells which form nails. In light of recent evidence into the formation of the nail this would mean that drugs are incorporated into cells producing the nail matrix and the nail bed. Rapid access to the distal nail would arise from incorporation of drugs into cells forming the nail bed.[214]

Other possible routes of drug incorporation could be through environmental contamination and biological fluids such as sweat, urine, saliva and sebum.

Drug properties which influence incorporation of drugs into hair such as lipophilicity, basicity and structural factors and the composition of matrix may also play a role in the incorporation of drugs into nails.

7.6 NAIL GROWTH

Complete growth from the production in the nail fold to the free margin has been reported differently by several authors as 130 - 160 days[215], 70 - 138 days[216] and 1.5 inches per year.[217] Growth rate differs depending on a number of factors. It has been reported that the growth rates of the five digits on an individual's hand are different and that the third digit has been found to be consistently greater and the little finger nail consistently lower than that of the others. Age plays a large part in nail growth and has been reported to be highest for those between 3 and 30 years. Above 30 years, rate is inversely proportional to age. Below 3 years and above 80 years growth rate is lowest.[217] In another study using Japanese subjects it was reported that nail growth was most rapid in the first two decades of life and decreased steadily thereafter. Growth was also affected by the season, faster in summer than in winter and has been found to be more rapid in nail biters. [218]

Injury to the nail has been reported to alter the thickness of the nail over 100 days of growth and the speed of nail growth was also altered.[219] Fingernails have been shown to grow faster than toenails by a ratio of 4 to 1.[216]

7.7 COLLECTION OF NAILS

The considerations which are necessary when collecting nail samples are the location from which the nail is taken. Is the sample fingernail or toenail and from which digit was the sample taken? Also, the sample should be taken with clean, non-contaminated scissors or clippers. In reality if several samples are being taken at once from different people it may not be convenient to wash these tools in between samples. To prevent the possibility of contamination between samples a new pair of scissors or clippers should be used.

7.8 ANALYSIS

Nail analysis for drugs involves four steps similar to hair analysis.

7.8.1 DECONTAMINATION

Prior to extraction, nail specimens should undergo a decontamination protocol to ensure that any external drug material along with extraneous material such as dirt are removed. This procedure should remove any drug bound to the outer surface without removing drug from within the nail. Wash steps often use methanol and water and sometimes a weak detergent.

7.8.2 REMOVAL OF DRUG FROM THE NAIL MATRIX

Drugs have been removed from nail by alkaline hydrolysis,[199] methanol reflux,[197] soaking in phosphate buffer,[198] crushing the nail with acid[194] and by a cryogenic method.[200] The alkaline hydrolysis is the only method which completely dissolves the nail but can only be used if the drug is stable in strong alkaline conditions. Methanol and phosphate buffer methods require the nail to be cut into small pieces.

7.8.3 EXTRACTION

Extraction procedures used for nail analysis are usually conventional liquid-liquid or solid phase extraction methods.

7.8.4 ANALYSIS

Analytes in nails are usually detected using gas chromatography-mass spectrometry, but other methods such as high performance liquid chromatography with electrochemical detection[201] have also been used.

7.9 NAIL ANALYSIS FOR DRUGS

Methamphetamine and amphetamine were the first drugs of abuse to be detected in fingernails and toenails. Nails were decontaminated by scraping and sonication in a mixture of methanol and water 1:1 and were then dissolved by alkaline hydrolysis with 2.5 N sodium hydroxide solution at 80 °C. This solution was extracted by liquid-liquid extraction with *n*-pentane and trifluoroacetic derivatives were prepared and detected by gas chromatography-mass spectrometry with chemical ionisation. Recoveries for this method were 91.6 % and 88.9 % for methamphetamine and amphetamine respectively and it was found that the two drugs were more concentrated in toenails than in fingernails. This observation was explained by the differences in growth rates of toenails and fingernails which were quoted as 1.1 mm/month and 3-5 mm/month respectively. The methamphetamine and amphetamine levels detected were 0.32 - 17.7 ng/mg and 0.03 - 0.40 ng/mg respectively in fingernails and 0.06 - 9.93 ng/mg and 0.03 - 1.60 ng/mg respectively in toenails. The methamphetamine levels were similar to those found in hair.[193]

Other authors have also detected methamphetamine and amphetamine in nails. Decontamination was carried out using alternate water and methanol washes. The nails were then crushed with 0.6 N hydrochloric acid in a mortar and pestle, the pH adjusted

with sodium carbonate solution and the drugs extracted using chloroform:isopropanol 3:1. Trifluoroacetic derivatives were prepared and detected using gas chromatography-mass spectrometry with electron ionisation. Methamphetamine was detected in 13 out of 20 nail samples and amphetamine in 3 out of the 13 positive methamphetamine cases. The other major metabolite of methamphetamine, *p*-hydroxymethamphetamine was not detected in any of these cases. The concentrations of methamphetamine and amphetamine detected ranged from 0.4 - 642 ng/mg and 0.3 - 23.2 ng/mg respectively.[194]

Other authors have identified amphetamine, 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA) in a fingernail. The nail was scraped and washed in dichloromethane and incubated in sodium hydroxide at 95 °C for 10 minutes. The homogenate was extracted using liquid-liquid extraction and derivatized using pentafluoropropionic anhydride and pentafluoropropanol. The analytes were detected using gas chromatography-mass spectrometry. Levels of amphetamine, MDA and MDMA in the fingernail were 10.2 , 8.0 and 53.4 ng/mg respectively and these values were found to be similar to those detected in the hair sample of this individual.[195]

Cocaine and benzoylecognine have been detected in nails. Cocaine was extracted from the hair, nails and other tissues of a cocaine overdose victim. Nails were washed for two minutes in methanol. They were cut into small pieces and extracted with methanol in an ultrasonic bath for 5 hours at 55 °C. The extracts were quantified using gas chromatography with nitrogen phosphorus detection and confirmed by gas chromatography-mass spectrometry. The concentration of cocaine detected in the left and right hand fingernail tips were 2300 and 2200 ng/mg respectively.[196]

A reflux method using methanol at 40 °C for 16 hours was used to extract cocaine, benzoylecognine and other cocaine metabolites from toe and fingernail clippings. Prior to this step the nails were cut into small pieces and washed with methanol. Solid phase extraction was used to clean up the extracts following the reflux stage and bis-(trimethylsilyl)trifluoroacetamide (BSTFA) derivatives were prepared. Gas chromatography-mass spectrometry analysis in the SIM mode allowed a limit of detection of 0.10 ng/mg and a limit of quantitation of 0.25 ng/mg for cocaine and benzoylecognine which were the predominant analytes in the positive samples. The range of cocaine and benzoylecognine found in fourteen samples was less than 0.25 to 16.1 ng/mg and 0.27 to

greater than 10.0 ng/mg respectively and the concentration of cocaine analytes was generally greater in fingernails than in toenails. Hydrolysis of cocaine using this method was reported to be less than 5 %. Wash fractions were positive in five out of fourteen positive samples and ranged from 0.10 to 10.0 ng/mg. Blood and urine samples were found to be positive for 27.7 % compared with 82.3 % for nails. This indicated the usefulness of nail analysis for long term exposure to cocaine.[197]

Cocaine, benzoylecognine, norcocaine, cocaethylene, morphine, 6-monoacetylmorphine, codeine and hydrocodone were all detected in postmortem nail clippings. The nails were cut into small pieces, decontaminated with methanol and solubilized in 0.1 M potassium phosphate, pH 5.0. Solid phase extraction was used to clean the extracts, followed by derivatization with *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA). Gas chromatography-mass spectrometry gave a limit of quantification of 0.3 ng on column. Concentrations ranged from 0.20 to 140.17 ng/mg for cocaine and 0.30 to 315.44 ng/mg for benzoylecgonine. Two samples were found to contain norcocaine (6.78 and 0.66 ng/mg) and cocaethylene (2.60 and 0.73 ng/mg). Morphine and 6-monoacetylmorphine were detected in three cases with average concentrations 0.37 ng/mg and 0.89 ng/mg. Codeine was detected in two samples (3.07 and 1.02 ng/mg) and hydrocodone in one sample (0.62 ng/mg). The phosphate buffer, pH 5.0 was reported to give less than 3 % loss of analyte.[198]

Morphine has been detected in fingernails by other authors. The fingernails were washed by a series of steps involving sodium dodecyl sulphate, water and methanol. Alkaline hydrolysis with 1 M sodium hydroxide solution at 60 °C was used and the homogenate was evaporated to dryness, redissolved in water and extracted using Toxi Tubes A. Initial analysis was carried out using radioimmunoassay and confirmation using high performance liquid chromatography with electrochemical detection (HPLC-ECD). Positive radioimmunoassay results were obtained in 25 out of 26 cases and the levels ranged from 0.06 – 4.69 ng/mg. Using HPLC-ECD, 22 of the 26 cases were positive with levels ranging from 0.14 to 6.90 ng/mg.[201]

The same authors also detected cannabis in fingernails of users. The washing protocol was the same as that previously mentioned, a series of steps involving sodium dodecyl sulphate, water and methanol and nails were incubated in 1 M sodium hydroxide at 95 °C.

A simple extraction method was used and analytes were detected by radioimmunoassay and confirmation by gas chromatography-mass spectrometry after derivatization with BSTFA. Cannabinoids were detected in 6 cases by RIA and levels ranged from 0.23 - 2.80 ng/mg. Tetrahydrocannabinol was detected in 11 out of 14 case samples by GC/MS and levels ranged from 0.13 - 6.97 ng/mg. The metabolite, 11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid was not detected under basic pH but under acidic pH was found positive in two out of three cases with concentrations ranging from 9.82 - 29.67 ng/mg.[199]

In a further study of cannabinoids in nails these authors cryogenically ground the nail in liquid nitrogen using a freezer mill fitted with a micro vial grinding set and washed the resulting nail powder from the micro-vial set with various solvents. Methanol was selected as the solvent of choice for extraction. Gas chromatography-mass spectrometry following derivatization with BSTFA was used to analyze the extracts and this method was compared with the previously reported cannabinoid method. The extracts produced by alkaline hydrolysis were found to contain mean concentrations of 1.73 ng/mg tetrahydrocannabinol, 2.48 ng/mg cannabidiol and 0.10 ng/mg 11-hydroxy-tetrahydrocannabinol. The results obtained by crygenic grinding were comparable, but this method also detected the carboxylic acid metabolite. Average concentrations obtained using this method were 0.54 ng/mg tetrahydrocannabinol, 0.7 ng/mg 11-nor-delta-9-tetrahydrocannabinol-9-carboxylic acid, 3.2 ng/mg cannabidiol and 0.15 ng/mg 11-hydroxy-tetrahydrocannabinol.[200]

To date the number of works published on the detection of drugs in nail is far fewer than those for hair. Hair analysis for drugs has not only included drugs of abuse, but many prescription drugs including antidepressants, antipsychotics and hallucinogens have been investigated. On the other hand, nail analysis for drugs has mainly involved the detection of drugs of abuse and research into prescription drugs in nails is sparse.

7.10 AIM

The aim of this study was to use a simple method to extract fluoxetine, norfluoxetine and paroxetine from the nails of users and to compare the concentrations detected in nails with those detected in the hair. Thereby, the possibility of using nail analysis as an alternative to hair analysis to indicate chronic use of these drugs was assessed.

7.11 EXPERIMENTAL

Several methods have been used to separate drugs from the nail matrix. The choice of method depends on the stability of the drug and on the capability of the method to recover as much of the drug as possible from the matrix. Alkaline hydrolysis of the nail matrix has the ability to completely dissolve the nail and assuming that the drugs within the nail are not destroyed then the recovery by this method has the potential to be high. The possibility of using this method[199] to extract antidepressants from nails was investigated.

7.11.1 DECONTAMINATION OF NAIL

Blank fingernails were obtained from volunteers undergoing no treatment with Prozac® or Seroxat® and decontaminated using the same wash protocol as that used for hair samples:

(i) One ten minute wash with sodium dodecyl sulphate solution (0.1 %).

(ii) Three ten minute washes with distilled water.

(iii) Three five minute washes with methanol.

Each step was sonicated in 5 ml of solvent and the solvent was decanted after sonication. The washed nail samples were dried at room temperature and used for blank controls and for spiking with the drugs of interest.

7.11.2 ALKALINE HYDROLYSIS

Drug free nails were dried in an oven at 40 °C and weighed. The nail was hydrolyzed using 1 M sodium hydroxide solution. 1 ml of solution was added for every 10 mg of nail and the sample was incubated at 80 °C for approximately 1 hour or until the nail was completely hydrolyzed.

7.11.3 SPIKING NAILS

When the temperature of the solution had fallen to room temperature, 1 ml portions of this hydrolysate were pipetted into vials and spiked with fluoxetine, norfluoxetine and

paroxetine to give standards with concentrations 5, 10, 15, 25, 50, 75 and 100 ng/10 mg nail. Internal standards tumoxetine and cannabinol were added at concentrations 50 ng/10 mg and 116 ng/10 mg respectively.

7.11.4 EXTRACTION AND DERIVATIZATION

The analytes were extracted from the nail matrix using a simple liquid-liquid method.[199] Several solvent mixtures were tested to find which gave the highest recoveries without the nail matrix interfering with the analytes. This involved mixing 5 ml of solvent with the spiked hydrolysate for 15 minutes, centrifuging the samples for 5 minutes at 2500 rev/min., separating the organic layer and evaporating the solvent to dryness under nitrogen at 40 °C. Derivatization of the nail extracts was carried out as previously described in 4.13.16.2. GC/MS conditions were the same as for the hair analysis of these drugs (6.4.4). Solvents tested included ethyl acetate, dichloromethane, dichloromethane/isopropanol 8:2 and chloroform. Initially, only fluoxetine and norfluoxetine were investigated.

Table 7.1: Recoveries of fluoxetine and norfluoxetine from nail hydrolysate

Solvent	% Recovery Fluoxetine (RSD %)	Norfluoxetine (RSD %)
Ethyl acetate	88.1 (2.3)	84.4 (6.8)
Dichloromethane	65.3 (7.1)	78.9 (3.2)
Chloroform	25.6 (5.5)	35.0 (5.8)
Dichloromethane/ isopropanol 8/2	33.8 (2.1)	49.9 (9.1)

RSD = Relative Standard Deviation as a percent

Ethyl acetate gave the highest recoveries of the solvents tested for both analytes followed by dichloromethane. The extracts obtained with these two solvents were injected into the GC/MS and analysed using fullscan analysis. Although the recoveries were higher for the ethyl acetate extracts, the amount of extracted impurities was also greater than for the dichloromethane extracts. However, since these did not interfere with the derivatized fluoxetine and norfluoxetine peaks it was decided to use ethyl acetate because of the higher recoveries obtained.

7.11.5 ALKALINE TREATMENT OF DRUGS

The hydrolysis step was investigated to find if there was any loss of drug as a result of the harsh alkaline conditions. 100 µl of fluoxetine, norfluoxetine and paroxetine at 1 µg/ml were put into separate vials and 1 M sodium hydroxide solution was added to each. This was carried out in triplicate. The samples were placed in the oven at 80 °C for 30 minutes. Another set of standards at the same concentration was prepared in 1 ml of water. Both sets of standards were extracted using ethyl acetate, derivatized and injected into the GC/MS. It was found that there were no significant differences in recovery between the two sets of standards and therefore alkaline treatment did not appear to destroy these drugs using these conditions. Further validation of recovery was carried out later on (7.11.6.3).

7.11.6 METHOD VALIDATION

7.11.6.1 Intra-day variation

Calibration standards at concentrations 5 ng/10 mg, 10 ng/10 mg, 25 ng/10 mg, 50 ng/10 mg, 75 ng/30 mg, 100 ng/10 mg and 200 ng/10 mg were extracted five times to calculate the intra-day variation of the assay. Drug/internal standard ratios and relative standard deviations were calculated for each concentration and linear calibration curves were produced for each drug (Table 7.2). Values of correlation coefficient, R^2 and intercept and slope values for the regression equations are displayed in Table 7.3. Relative standard deviations were less than 13 % across the calibration curve.

Table 7.2: Intra-day variation

Concentration (ng/10 mg nail)	Mean FLU/TUM ratio m/z 344/344 (RSD)	Mean NFLU/TUM ratio m/z 330/344 (RSD)	Mean PAR/CAN ratio m/z 525/506 (RSD)
5	0.082 (6.6)	0.076 (5.4)	0.219 (9.3)
10	0.154 (7.4)	0.198 (8.9)	0.291 (7.6)
15	0.266 (2.9)	0.281 (6.1)	0.439 (5.8)
25	0.494 (8.1)	0.498 (0.5)	0.673 (8.8)
50	1.067 (8.8)	0.974 (8.5)	1.282 (5.7)
75	1.641 (5.9)	1.450 (2.5)	2.009 (9.9)
100	2.096 (5.3)	1.961 (8.8)	2.749 (12.8)

FLU = fluoxetine, NFLU = norfluoxetine, PAR = paroxetine, TUM = tumoxetine, CAN = cannabinol
RSD = relative standard deviation as a percent

Table 7.3: Linear Regression Values

	Fluoxetine	Norfluoxetine	Paroxetine
R^2	0.9985	0.9997	0.9976
Intercept	- 0.0439	- 0.0082	0.0282
Slope	0.0218	0.0196	0.0267

7.11.6.2 Inter-day variation

The calibration curve was extracted over five different days and the inter-day variation was assessed and displayed in Table 7.4. Relative standard deviations were less than 11 % for the concentrations at the higher end of the calibration curve and less than 23 % for the lowest calibrator.

Table 7.4: Inter-day variation

Concentration (ng/10 mg nail)	Mean FLU/TUM ratio m/z 344/344 (RSD)	Mean NFLU/TUM ratio m/z 330/344 (RSD)	Mean PAR/CAN ratio m/z 525/506 (RSD)
5	0.070 (9.8)	0.085 (11.1)	0.176 (22.6)
10	0.148 (4.4)	0.203 (7.7)	0.261 (10.1)
15	0.253 (4.7)	0.319 (13.1)	0.399 (11.5)
25	0.455 (8.4)	0.543 (7.2)	0.597 (13.3)
50	0.979 (8.8)	1.245 (1.9)	1.216 (11.8)
75	1.488 (10.4)	1.657 (8.1)	1.969 (8.7)
100	1.962 (4.8)	2.160 (5.6)	2.556 (10.6)

FLU = fluoxetine, NFLU = norfluoxetine, PAR = paroxetine, TUM = tumoxetine, CAN = cannabinol

RSD = relative standard deviation as a percent

7.11.6.3 Recovery

Nail hydrolysate was spiked to give the concentrations used to produce the calibration curves. Each of these was extracted five times and internal standards were added following the extraction. Recoveries were calculated for each concentration and these are displayed in Table 7.5 with relative standard deviations in parentheses. Recoveries were greater than 80 % in all cases.

Table 7.5: Recoveries from spiked nail hydrolysate

Concentration (ng/10 mg nail)	% Recovery Fluoxetine (RSD %)	% Recovery Norfluoxetine (RSD %)	% Recovery Paroxetine (RSD %)
5	81.0 (11.4)	81.4 (7.8)	82.9 (10.4)
10	90.2 (0.7)	87.9 (4.9)	89.6 (4.6)
15	93.8 (0.3)	84.2 (8.5)	87.0 (7.0)
25	91.3 (1.9)	79.9 (4.6)	90.7 (8.6)
50	88.1 (6.7)	83.1 (5.3)	88.8 (8.8)
75	85.7 (10.1)	83.6 (3.0)	84.2 (4.4)
100	90.6 (5.8)	84.3 (1.7)	88.2 (8.8)

7.11.6.4 Limit of Detection (LOD) and limit of quantitation (LOQ)

Hydrolyzed nail was spiked to give concentrations 0.5, 0.75, 1.0 and 2.0 ng/10 mg nail and these were extracted to determine the limit of quantitation and limit of detection. The limit of detection was taken to be three times the baseline noise of a blank nail hydrolysate sample and the limit of quantitation five times the baseline noise of a blank nail hydrolysate sample at the relevant retention time of each drug. The limits of quantitation of the drugs was 2.0 ng/10 mg and the limit of detection was 0.75 ng/10 mg for norfluoxetine and paroxetine and 1.0 ng/ 10 mg for fluoxetine.

7.12 CONCLUSIONS

A simple method was used to extract fluoxetine, norfluoxetine and paroxetine from nails. Alkaline hydrolysis was used to completely dissolve the nail and the drugs were extracted with ethyl acetate followed by derivatization with heptafluorobutyric anhydride and subsequent analysis by gas chromatography-mass spectromety. Alkaline hydrolysis was shown to cause no significant destruction of the drugs. Recoveries greater than 80 % were obtained for each drug along the calibration curve and limits of detection were suitably sensitive. The method was used to analyze case samples.

8 Case Samples

8.1 OPIATES AND METHADONE IN HAIR

8.1.1 SAMPLE COLLECTION

Hair samples were collected from cadavers at Glasgow City Mortuary by pathologists from the Department of Forensic Medicine and Science, University of Glasgow. Hairs were plucked prior to postmortem to ensure that the hair had the least amount of exposure to blood which causes external contamination to hair. Prevention of hair contamination by blood, however, is not always possible especially in cases involving head injuries. The hair samples were placed in individual plastic tubes and labelled. Blood samples were collected during the postmortem.

8.1.2 SECTIONING OF HAIR

Hair samples matched to blood samples which were found positive for opiates and/or methadone were analyzed. The analytical results obtained from the blood samples from the selected cases are displayed in Table 8.1. The root end was cut 0.5 cm from the root and each successive sample was cut at 1 or 2 cm intervals, depending on the bundle of hair received. Some samples were analyzed in entirety because of the lack of sample.

8.1.3 DECONTAMINATION OF HAIR

Hair case samples were decontaminated after sectioning as in 3.12.7. The third methanol wash was evaporated to dryness at room temperature under a stream of nitrogen. Internal standards were added (50 ng), evaporated to dryness and derivatized with 40 μl of BSTFA with 1 % TMCS. The washes were analyzed by GC/MS and if they were found to contain morphine, codeine, 6-MAM, methadone or EDDP then the methanol washing procedure was repeated until the washes were free of drug. It has been postulated that there are different regions in the hair which are accessible and inaccessible to the external environment. These same authors have suggested that when hair is exposed to large amounts of external contamination that a small percentage of the drug will be incorporated within the hair shaft, an accessible interior region of the hair whilst the majority will remain on the hair surface. If the hair is analyzed immediately after this exposure then much of the surface drug would be removed and a high external contamination would be detected. This portion of removed drug would have a low binding affinity to the hair. On

the otherhand, if the hair was analyzed several weeks later after numerous normal hygiene washes then most of the loosely bound surface drug would have been removed leaving only the drug within the hair shaft. The decontamination procedure in this case may indicate that there is no external contamination in this case because of the high affinity of drug in hair. This could provide a possible explanation for decreasing amounts of drug being found in successive wash solutions

8.1.4 EXTRACTION OF CASE SAMPLES

The decontaminated hair was allowed to dry at room temperature, weighed and cut into 1-2 mm pieces with scissors. The samples were extracted as in 3.12.12.

8.1.5 RESULTS

31 hair samples were segmented to give a total of 109 sections. Each section was washed and analyzed separately. Of the 31 hair cases, 27 had at least one segment positive for morphine (87.1 %), 23 for codeine (74.2 %), 23 for 6-MAM (74.2 %), 8 for methadone (25.8 %) and 6 for EDDP (19.3 %).

Out of the 109 sections, 85 were found positive for morphine (78.0 %), 72 for codeine (66.0 %), 67 for 6-MAM (61.5 %), 19 for methadone (17.4 %) and 11 for EDDP (10.1 %).

8.1.5.1 Root Analysis

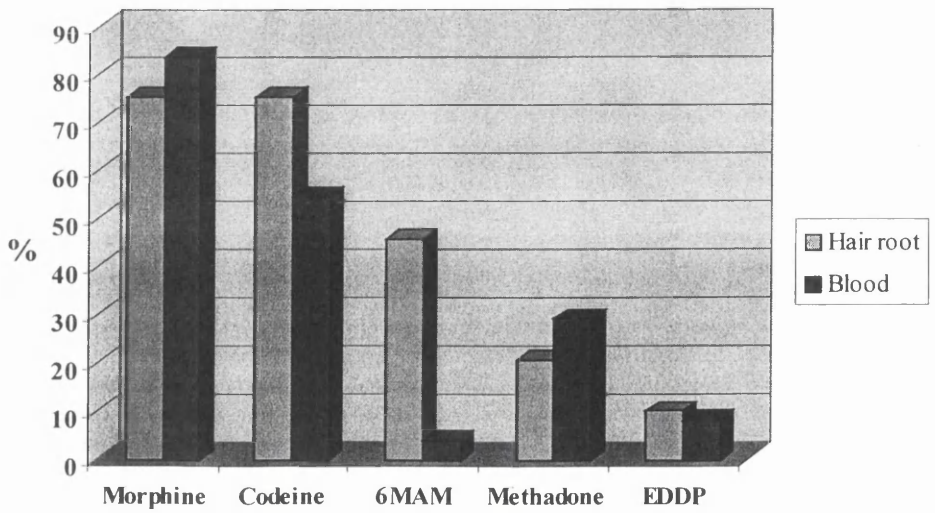
Roots were analyzed for 24 out of the 31 cases. Morphine was found positive in 18 of these cases (75 %), codeine in 18 cases (75 %), 6-MAM in 11 cases (45.8%), methadone in 5 (20.8 %) and EDDP in 4 (16.7 %). For these same 24 cases, 20 blood samples were found positive for morphine (83.3 %), 13 for codeine (54.2 %), one for 6-MAM (4.2 %), 7 for methadone (29.2 %) and 2 for EDDP (8.3 %). These values are displayed in Figure 8.1.

In 14 cases (58.3 %), the hair roots and corresponding blood samples were both found positive for morphine. In 4 cases (16.7 %), the hair root was positive and the blood sample negative and in 6 (25 %) cases, the hair root was negative and the blood sample positive (Figure 8.2). For the latter cases it may be expected that the hair sample should be positive since the blood is positive. It has been shown that codeine can incorporate into the hair bulb within 30 minutes after administration.[23] However, in 4 cases out of the 6 there was a history of sudden collapse immediately after injecting, or there was the possibility of

sudden collapse. In the latter of these cases, the position the deceased was found suggested that sudden collapse was likely. In situations such as these, the drugs would not have had time to incorporate into the hair and if the user had not taken heroin in the time it took for this section of hair to grow then no morphine would be detected. In cases where there was no history of sudden collapse this may be accounted for by the root sample size being too small and in the case of occasional heroin users the low concentrations present in the sample may be below the limit of detection of the method.

Out of 8 cases where methadone was positive in blood and/or root hair samples, the number of methadone positives in hair root and corresponding blood sample was three (37.5 %), positive hair root and negative blood was one (12.5 %) and negative hair root and positive blood was four (50.0 %) (Figure 8.3). Again for some of the root samples, there was insufficient sample available, only 1 - 2 mg.

Concentration ranges detected in root samples were 0.51 - 4.66 ng/mg (mean 1.75 ng/mg) morphine, 0.32 - 3.93 ng/mg (mean 1.27 ng/mg) codeine, 0.35 - 7.80 ng/mg (mean 2.03 ng/mg) 6-MAM, 0.21 - 2.78 ng/mg (mean 1.32 ng/mg) methadone and 0.42 - 1.85 ng/mg (mean 1.07 ng/mg) EDDP. 6-MAM/morphine ratios for the roots varied from 0.09 - 3.01 (mean 0.87). Morphine/codeine ratios in roots varied from 0.69 - 2.80 (mean 1.59). The ratio of methadone/EDDP in root samples was 1.07 - 2.23 (mean 1.43). The results for the root analysis are displayed in Tables 8.2 - 8.3 for opiates and Table 8.4 for methadone.



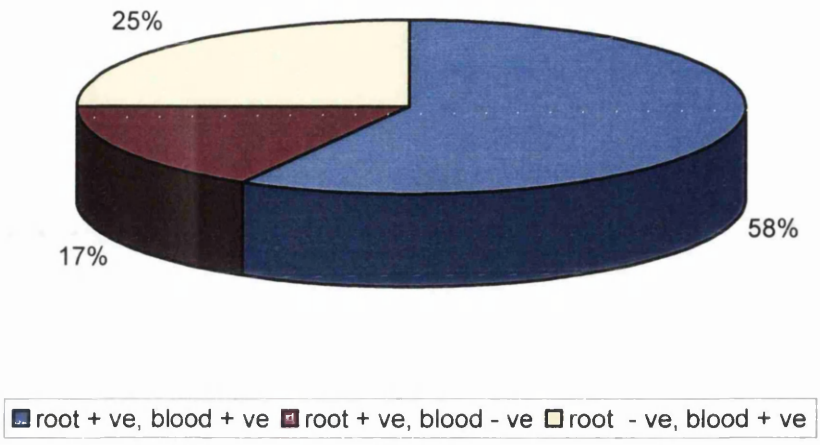


Figure 8.2: Morphine findings in hair root and corresponding blood sample.

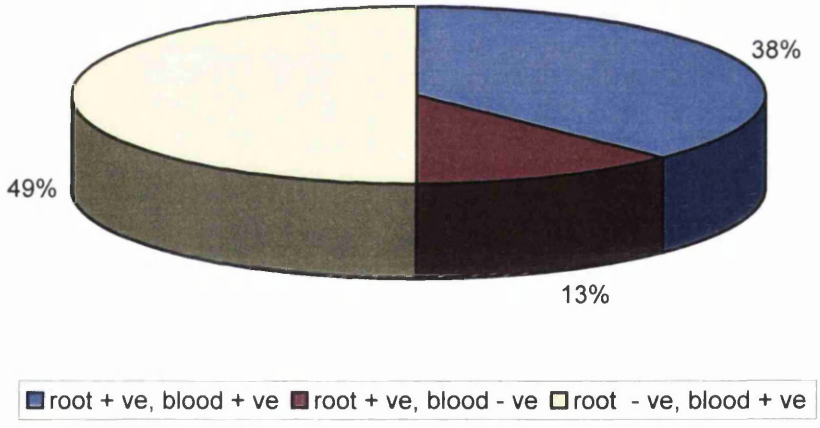


Figure 8.3: Methadone findings in hair root and corresponding blood sample.

8.1.5.2 Hair Segments excluding roots

Concentration ranges detected in segments excluding roots were 0.10 - 9.79 ng/mg morphine, 0.12 - 3.47 ng/mg codeine, 0.14 - 70.07 ng/mg 6-MAM, 0.15 - 1.15 ng/mg methadone and 0.27 - 1.55 ng/mg EDDP. These values correspond with other studies which reported concentrations as high as 21.8 ng/mg morphine, 4.0 ng/mg codeine and 74.2 ng/mg 6-MAM. Methadone and EDDP levels as high as 78.1 ng/mg and 7.76 ng/mg respectively have been reported using solid phase microextraction.[85] However, the levels detected in these case samples were found to be much lower.

In cases where a constant amount of morphine was detected along the hair shaft, it was also noticed that the concentration of 6-MAM increased the further away from the root end. This was particularly noticeable in cases HO/06 (Figure 8.4), HO/07 and HO/08. Also, as the morphine concentration increased towards the tip of the hair, a larger difference in 6-MAM was noted as shown by cases HO/01, HO/09 (Figure 8.5), and HO/26. 6-MAM/morphine ratios varied from 0.08 - 19.33 (mean 3.94), and were generally greater than 1. This range was much broader than the range obtained for the root sections.

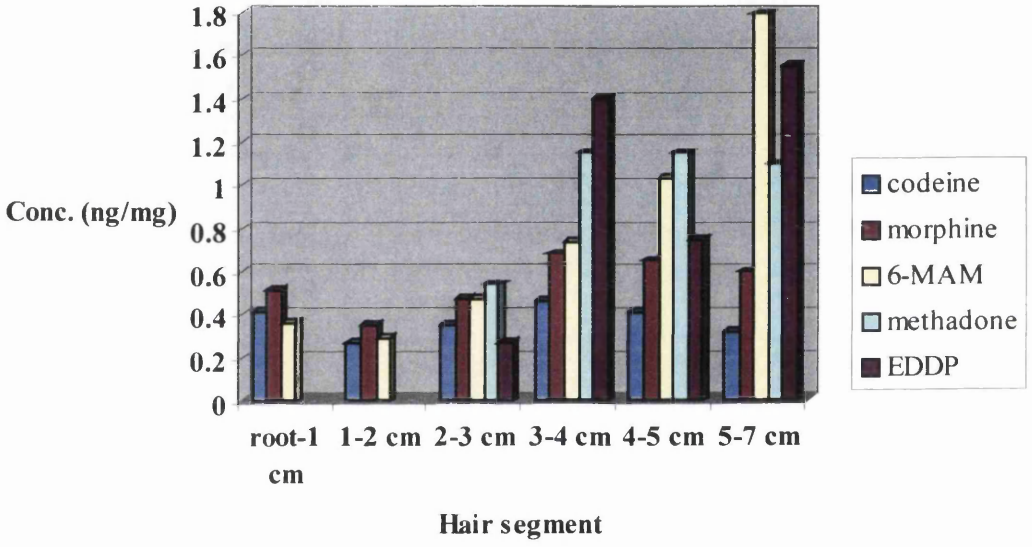


Figure 8.4: Hair case HO/06

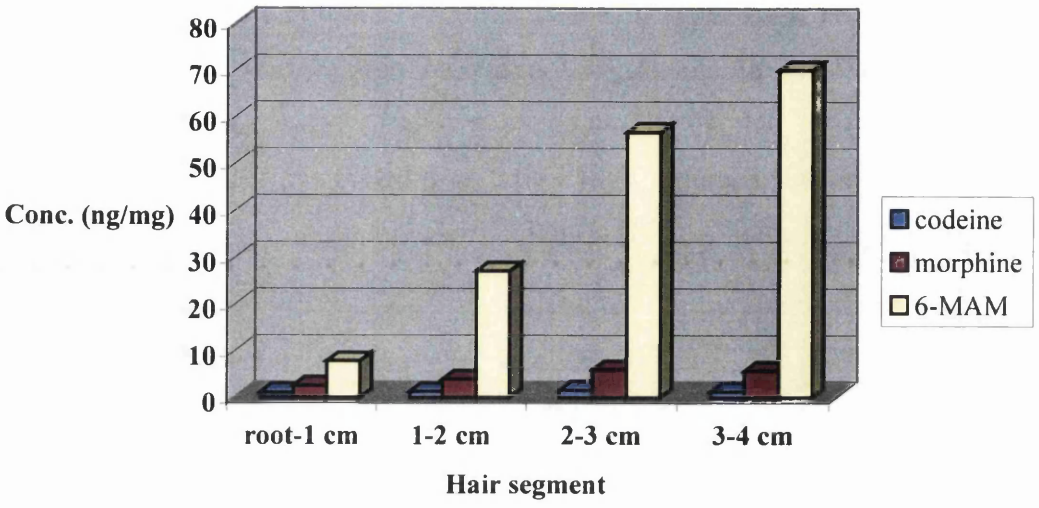


Figure 8.5: Hair case HO/09

In the majority of cases (87 %) where morphine was found positive the concentration of morphine was greater than that of codeine. The morphine/codeine ratio varied from 0.14 - 4.41 (mean 1.96).

The ratio of methadone/EDDP in segment samples varied from 0.71 - 2.36 (mean 1.45). Figure 8.4 also shows a good example of a previous regular methadone user. Concentrations of methadone over three consecutive one centimetre segments were found to be similar and these sections of hair would have grown approximately 3 to 7 months prior to death. EDDP was also detected in these segments, but the concentrations in the separate segments were found to be more variable.

The results for the hair segments excluding roots are displayed in Tables 8.5 - 8.8 for opiates and Table 8.9 for methadone.

8.1.5.3 Interpretation of hair findings

The analysis of hair from heroin addicts can prove long term abuse, give a history of the use of the drug and to a certain extent give an indication of the drug consumption. The presence of 6-MAM in hair is definitive evidence of heroin exposure. In 23 of the 31 cases tested, at least one section of hair was found positive for 6-MAM indicating that 23 of the

individuals had used heroin at some point. Out of the other 8 cases, 3 were negative for opiates and 5 had at least one section positive for codeine and/or morphine. For these 5 cases the ratio of codeine to morphine was studied.

Other authors have defined criteria to distinguish between heroin use and other forms of opiate use in hair. This criteria stated that for cases where no 6-MAM is detected and the morphine concentration is < 1 ng/mg then the morphine/codeine ratio must be > 5 and for cases where no 6-MAM is detected and the morphine concentration is > 1 ng/mg then the morphine/codeine ratio must be > 2.[97] In applying these criteria to the 5 cases, heroin use could not be established solely on the information provided from the hair results since the morphine/codeine ratios did not uphold these rules. However, the drug histories of these five cases showed that two were known heroin users although the extent was not known, one was a casual user, another had a long history of heroin use and only one had no known drug use history. These cases will be discussed further in 8.1.5.4.

In a study investigating dose and hair concentration findings, good proportionality was observed between the quantities of heroin a drug user self-reported to have taken and the amount of 6-MAM detected in the hair. These authors proposed three levels of consumption in terms of the concentration of 6-MAM detected in hair. These were low < 2 ng/mg, medium 2-10 ng/mg and high > 10 ng/mg.[220] Other authors have also reported good correlation between self-report and amount of codeine, morphine and 6-MAM detected in hair.[221] Using this criteria, out of the 23 cases where 6-MAM was detected, 10 (43.5 %) could be classed as low users, 8 (34.8 %) as medium users and 5 (21.7 %) as high users.

A final consensus of the Hair Testing Work Group after its third meeting in Texas, 1999 recommended that cut-off levels for morphine, codeine and 6-MAM in hair should be 0.2 ng/mg and that 6-MAM could not be reported unless another opiate analyte was present above its cut-off level.

In 7 of the hair cases, levels were detected below this cut-off level for at least one segment of hair and in one case 6-MAM was detected without another opiate present.

8.1.5.4 Regular, intermittent or naïve user?

The hair findings, blood findings and information about drug use history obtained from police reports were analyzed for each case to obtain an overall picture of drug use. The interpretation of these results are tabulated in Tables 8.10 - 8.13.

Hair findings provided evidence of whether the user was a regular user. The term "regular heroin user" is used where concentrations of morphine and 6-MAM were detected throughout the length of the sectioned hair. In cases where whole hair was analyzed this term was not used. An indication of the extent of drug use was given by the terms "low", "medium" or "high" as previously discussed in 8.1.5.3 and an estimation of the length of time of use was given by using the approximation that 1 cm of hair is equivalent to 1 months hair growth. For whole hair samples which were classified as "low user" it must also be recognised that this could indicate intermittent use. However, since the hair was not sectioned this could not be proven.

Blood findings provided evidence of recent use of heroin and/or methadone. For blood findings, the term "heroin user" was only used when codeine and morphine concentrations were detected in blood with a morphine/codeine ratio greater or equal to 2.

Drug use history was taken from police reports. For this part it was essential to include any information about methadone or drug rehabilitation programmes to assist with the interpretation.

Out of the 31 cases analyzed, 9 were found to be low users, 8 medium users and 5 high users of heroin. 3 cases were found to be intermittent users, one of which was on a methadone programme, one on drug rehabilitation programmes and the other on unknown tablets to help with withdrawal. Only one of the users was described as a naïve user having no previous history of drug use and the hair was found entirely negative.

Five of the cases were termed inconclusive. Part of the problem for these samples was the small sample size of hair available. For one of these cases (HO/04) low concentrations of codeine and morphine were detected in a hair segment equivalent to three months growth (hair equivalent to months 4-7 prior to death) and morphine was detected in the blood. The deceased was known to have taken heroin prior to death and he was a known heroin

abuser. The amount of hair available for this case was low. The root section was less than 2 mg and the other two sections 6.8 mg and 9.1 mg respectively. These results indicated a possible low user or intermittent user, however the codeine/morphine ratio in hair did not fulfil criteria meeting heroin use. Hair findings could therefore not be used to categorize the extent of heroin use. The drug history indicated that this individual was a heroin user but the extent was not known.

In the second of these cases, (HO/24), the roots were found negative. The rest of hair was found positive for morphine and codeine, however the morphine/codeine ratio again did not meet criteria and the morphine concentration was below the cut-off level recommended by the Hair Testing Work Group. A sample size of 17 mg was tested for the bulk of the hair which is adequate to determine medium or high usage. This indicates that the user was most likely an occasional user in which case concentrations in hair would be low. Blood findings were conclusive of heroin use and the drug use history indicated that the deceased was an occasional user. However, hair findings could not conclusively determine this.

For case HO/28, hair findings could not conclusively indicate heroin use. In the blood, morphine was detected and the drug use history indicated that this individual was intermittently taking heroin with tablets prescribed for withdrawal. It was probable that this was an intermittent user.

For cases HO/29 and HO/30 morphine/codeine ratios detected in hair were again not conclusive of heroin use. Blood findings were both positive for heroin use and in one case drug use history was not known and in the other case a long history of heroin use was known. However, hair findings did not prove heroin use.

For case HO/11, only 6-MAM was detected in the tip section of hair analyzed. The Hair Testing Work Group defined criteria stating that a lone 6-MAM concentration could not be reported. In this case the blood findings were indicative of a heroin user. The drug history confirmed this and the deceased had also been known to have attended several drug rehabilitation programmes. The combined evidence indicated that this hair sample belonged to an intermittent drug user.

Case HO/12 was the only example of an intermittent heroin user where methadone was detected in hair, but opiates were not detected in the hair at the same time. In other words this individual was complying with the methadone programme and from the drug history it was indicated that methadone prescription had ceased two weeks prior to death. Methadone was not detected in the root sample. Blood findings indicated heroin use, but no use of methadone.

8.1.6 CONCLUSIONS

Hair testing for drugs provides a longer time window than urine or blood analysis and can be used to provide information on an individual's drug use which blood and urine analysis fail to achieve. A drug use history can be compiled by sectioning the hair to indicate extent of use, periods of abstinence, periods of treatment with methadone or alternatively unprescribed use of methadone.

For the majority of the hair cases tested, the results were conclusive of regular heroin use and it could be established if these users were low, medium or high users, this being determined from the quantities detected in the hair. For cases which were determined inconclusive, the underlying problem was generally due to a lack of hair samples. In some cases only a few milligrams of hair were available and it was therefore necessary to analyze larger sections equivalent to several months of hair growth. An adequate size of greater than 10 mg per section of hair would be preferable.

The levels of opiates and methadone detected using the developed method were in the range of those seen by other authors.

Table 8.1: Opiate and methadone findings in blood case samples

Case sample	Codeine (mg/L)	Morphine (mg/L)	6-MAM (mg/L)	Methadone (mg/L)	EDDP (mg/L)
HO/01	- ve	- ve	-ve	0.3	0.3
HO/02	- ve	0.5	-ve	- ve	- ve
HO/03	- ve	0.3	-ve	- ve	- ve
HO/04	- ve	0.3	-ve	- ve	- ve
HO/05	0.1	0.2	-ve	- ve	- ve
HO/06	- ve	0.1	-ve	0.03	- ve
HO/07	- ve	0.2	-ve	- ve	- ve
HO/08	0.01	0.1	-ve	- ve	- ve
HO/09	0.02	0.2	-ve	- ve	- ve
HO/10	- ve	0.04	- ve	- ve	- ve
HO/11	0.1	0.3	- ve	- ve	- ve
HO/12	0.04	0.4	- ve	- ve	- ve
HO/13	0.01	0.1	- ve	- ve	- ve
HO/14	0.02	0.1	- ve	- ve	- ve
HO/15	0.1	0.5	- ve	- ve	- ve
HO/16	0.004	0.1	- ve	- ve	- ve
HO/17	-ve	0.3	-ve	0.02	-ve
HO/18	0.02	0.1	0.01	-ve	-ve
HO/19	- ve	- ve	- ve	0.02	- ve
HO/20	0.1	0.5	- ve	- ve	- ve
HO/21	- ve	- ve	- ve	0.14	- ve
HO/22	0.01	0.1	- ve	- ve	- ve
HO/23	0.03	0.29	- ve	0.02	0.001
HO/24	0.06	0.4	0.01	- ve	-ve
HO/25	-ve	0.38	-ve	- ve	-ve
HO/26	0.02	0.18	- ve	0.05	-ve
HO/27	0.02	0.11	-ve	- ve	-ve
HO/28	-ve	0.03	-ve	- ve	-ve
HO/29	0.01	0.02	-ve	- ve	-ve
HO/30	0.08	0.56	-ve	- ve	-ve
HO/31	-ve	-ve	-ve	-ve	-ve

Table 8.2: Blood, hair root and drug history results for opiates

Case sample	Blood	Hair roots					Drug history
	Morphine (mg/L)	Morphine (ng/mg)	6MAM (ng/mg)	Codeine (ng/mg)	6MAM/ Morphine	Morphine/ Codeine	
HO/01	- ve	1.49	0.59	0.89	0.396	1.674	Known illicit drug user
HO/02	0.5	1.28	1.05	0.71	0.820	1.802	Known heroin user for 12 years
HO/03	0.3	NA	NA	NA	-	-	Known heroin user for two years
HO/04	0.3	- ve	- ve	- ve	-	-	Known heroin user
HO/05	0.2	0.34	- ve	0.32	-	1.062	
HO/06	0.1	0.51	0.36	0.41	0.706	1.243	Known heroin user
HO/07	0.2	0.87	0.71	0.52	0.816	1.673	Known heroin user for 20 years
HO/08	0.1	1.03	0.63	0.62	0.611	1.661	Known heroin user
HO/09	0.2	2.59	7.80	1.14	3.011	2.272	Known heroin user
HO/10	0.04	NA	NA	NA	-	-	Known heroin user for 3 years
HO/11	0.3	- ve	- ve	- ve	-	-	Known heroin user for 5 years
HO/12	0.4	- ve	- ve	- ve	-	-	Known heroin user for 5 years
HO/13	0.1	NA	NA	NA	-	-	Known drug user
HO/14	0.1	NA	NA	NA	-	-	Long history of drug abuse
HO/15	0.5	NA	NA	NA	-	-	History of drug abuse 2-3 years
HO/16	0.1	NA	NA	NA	-	-	Known heroin user
HO/17	0.3	- ve	- ve	- ve	-	-	Possible heroin user 1 month prior to death
HO/18	0.1	- ve	- ve	- ve	-	-	Known drug user
HO/19	- ve	2.86	3.18	1.40	1.111	2.042	Known drug user over 18 years
HO/20	0.5	4.66	6.05	3.93	1.298	1.186	Known heroin user over 5 years
HO/21	- ve	1.05	1.21	0.55	1.152	1.909	Known heroin user
HO/22	0.1	- ve	- ve	- ve	-	-	Known heroin user 10 years prior to death
HO/23	0.29	0.55	- ve	0.80	-	0.688	Occassional heroin user
HO/24	0.4	- ve	- ve	- ve	-	-	Occassional user of heroin
HO/25	0.38	3.95	0.37	1.45	0.094	2.724	History of morphine addiction
HO/26	0.18	1.66	- ve	1.18	-	1.407	Registered heroin addict

Table 8.3: Blood, hair root and drug history results for opiates

Case sample	Blood	Hair roots					Drug history
	Morphine (mg/L)	Morphine (ng/mg)	6MAM (ng/mg)	Codeine (ng/mg)	6MAM/ Morphine	Morphine/ Codeine	
HO/27	0.11	0.81	- ve	0.46	-	1.761	Known regular heroin user
HO/28	0.03	0.67	- ve	1.32	-	0.508	Known heroin user prescribed withdrawal tablets
HO/29	0.02	3.36	- ve	2.80	-	1.200	Overdose 3 days prior to death (tablets not known)
HO/30	0.56	3.95	- ve	3.82	-	1.034	Long history of heroin abuse
HO/31	-ve	1.54	0.35	0.55	0.227	2.800	Not known

Table 8.4: Blood, hair root and drug history results for methadone

Case sample	Blood		Hair roots			Methadone history
	Methadone (mg/L)	EDDP (mg/L)	Methadone (ng/mg)	EDDP (ng/mg)	Methadone/ EDDP	
HO/01	0.3	0.3	1.14	0.51	2.235	No methadone history
HO/06	0.03	- ve	- ve	- ve	-	Took wife's methadone prescription day prior to death
HO/17	0.02	- ve	- ve	- ve	-	No methadone history
HO/19	0.02	- ve	1.98	1.85	1.070	Placed on numerous methadone programmes
HO/20	- ve	- ve	2.78	1.50	1.853	Referred to drug rehabilitation unit
HO/21	0.14	- ve	0.47	0.42	1.119	Not on a methadone programme
HO/23	0.02	0.001	0.21	- ve	-	No methadone history
HO/26	0.05	- ve	- ve	- ve	-	No methadone history

Cases which are not mentioned for methadone were negative in blood and hair

Table 8.5: Blood, hair segment and drug history results for opiates

Case sample	Hair segments excluding roots					Blood	Drug history
	Morphine (ng/mg)	6MAM (ng/mg)	Codeine (ng/mg)	6MAM/ Morphine	Morphine/ Codeine	Morphine (mg/L)	
HO/01 1-2 cm	2.34	5.11	0.87	2.183	2.690	- ve	Known illicit drug user
HO/01 2-3 cm	2.09	4.56	0.67	2.181	3.119		
HO/01 3-4 cm	2.81	7.27	0.93	2.587	3.022		
HO/02 1-2 cm	2.86	2.15	1.46	0.751	1.959	0.5	Known heroin user for 12 years
HO/02 2-3 cm	2.16	2.21	1.04	1.023	2.077		
HO/03 whole	0.16	0.16	0.12	1.000	1.333	0.3	Known heroin user for 2 years
HO/04 1-4 cm	- ve	- ve	- ve	-	-	0.3	Known heroin user
HO/04 4-7 cm	0.39	- ve	0.16	-	2.438		
HO/05 1-2 cm	0.37	0.49	0.39	1.324	0.949	0.2	
HO/05 2-3 cm	- ve	- ve	- ve	-	-		
HO/06 1-2 cm	0.35	0.29	0.27	0.829	1.296	0.1	Known heroin user
HO/06 2-3 cm	0.47	0.47	0.35	1.000	1.342		
HO/06 3-4 cm	0.68	0.74	0.46	1.088	1.478		
HO/06 4-5 cm	0.65	1.03	0.41	1.584	1.585		
HO/06 5-7 cm	0.60	1.79	0.32	2.983	1.875		
HO/07 1-2 cm	1.04	1.89	0.63	1.817	1.651	0.2	Known heroin user for 20 years
HO/07 2-3 cm	1.14	3.43	0.73	3.009	1.561		
HO/07 3-4 cm	1.18	5.22	0.74	4.423	1.595		
HO/07 4-5 cm	1.19	6.60	0.76	5.546	1.566		
HO/07 5-6 cm	1.19	7.52	0.72	6.319	1.653		
HO/07 6-7 cm	1.23	9.50	0.78	7.724	1.577		
HO/07 7-9 cm	1.36	12.53	0.84	9.213	1.619		
HO/08 1-2 cm	1.80	2.04	0.85	1.133	2.118	0.1	Known heroin user
HO/08 2-3 cm	1.60	3.09	0.70	1.931	2.286		
HO/08 3-4 cm	0.81	3.44	0.39	4.247	2.077		
HO/08 4-5 cm	0.74	3.94	0.33	5.324	2.242		

Table 8.6: Blood, hair segment and drug history results for opiates

Case sample	Hair segments excluding roots					Blood	Drug history
	Morphine (ng/mg)	6MAM (ng/mg)	Codeine (ng/mg)	6MAM/ Morphine	Morphine/ Codeine	Morphine (mg/L)	
HO/09 1-2 cm	3.94	27.04	1.23	6.863	3.203	0.2	Known heroin user
HO/09 2-3 cm	5.96	56.75	1.60	9.522	3.725		
HO/09 3-4 cm	5.71	70.07	1.30	12.271	4.392		
HO/10 0.5-1.5 cm	- ve	0.91	- ve	-	-	0.04	Known heroin user for 3 years
HO/10 1.5-2.5 cm	0.81	1.24	- ve	1.530	-		
HO/10 2.5-3.5 cm	1.55	2.21	- ve	1.426	-		
HO/10 3.5-5.5 cm	0.47	2.68	- ve	5.700	-		
HO/11 root-0.5 cm	- ve	- ve	- ve	-	-	0.3	Known heroin user for 5 years
HO/11 0.5-1.5 cm	- ve	- ve	- ve	-	-		
HO/11 1.5-2.5 cm	- ve	- ve	- ve	-	-		
HO/12 0.5-1.5 cm	- ve	- ve	- ve	-	-	0.4	Known heroin user for 5 years
HO/12 1.5-2.5 cm	- ve	- ve	- ve	-	-		
HO/13 Whole hair	0.10	0.28	- ve	2.800	-	0.1	Known drug user
HO/14 Whole hair	0.35	0.54	2.16	1.542	0.162	0.1	Long history of drug abuse
HO/15 Whole hair	0.19	0.40	- ve	2.105	-	0.5	History of drug abuse 2-3 years
HO/16 Whole hair	2.95	27.3	1.14	9.250	2.588	0.1	Known heroin user
HO/17 0.5-2.5 cm	- ve	-ve	-ve	-	-	0.3	Possible heroin user 1 month prior to death
HO/17 2.5-4.5 cm	- ve	-ve	-ve	-	-		
HO/17 4.5-6.5 cm	- ve	-ve	-ve	-	-		
HO/17 6.5-8.5 cm	- ve	-ve	-ve	-	-		
HO/17 8.5-10.5 cm	- ve	-ve	-ve	-	-		
HO/17 10.5-13.5 cm	- ve	-ve	-ve	-	-		
HO/18 Whole hair	- ve	-ve	-ve	-	-	0.1	Known drug user
HO/19 0.5-2.0 cm	1.42	2.12	0.75	1.490	1.893	- ve	Known drug user over 18 years
HO/20 0.5-2.0 cm	0.17	1.58	0.24	9.294	0.708	0.5	Known heroin user over 5 years

Table 8.7: Blood, hair segment and drug history results for opiates

Case sample	Hair segments excluding roots					Blood	Drug history
	Morphine (ng/mg)	6MAM (ng/mg)	Codeine (ng/mg)	6MAM/ Morphine	Morphine/ Codeine	Morphine (mg/L)	
HO/21 0.5-1.5 cm	0.22	0.24	0.16	1.091	1.375	- ve	Known heroin user
HO/21 1.5-2.5 cm	0.25	0.22	0.25	0.880	1.000		
HO/21 2.5-3.5 cm	0.21	0.22	0.23	1.048	0.913		
HO/21 3.5-4.5 cm	0.39	0.15	0.59	1.307	0.661		
HO/21 4.5-5.5 cm	0.19	0.42	0.27	2.211	0.704		
HO/21 5.5-6.5 cm	- ve	- ve	- ve	-	-		
HO/22 1-3 cm	0.26	- ve	- ve	-	-	0.1	Known heroin user 10 years prior to death
HO/22 3-5 cm	0.15	0.23	- ve	1.533	-		
HO/22 5-7 cm	0.13	- ve	- ve	-	-		
HO/22 7-9 cm	0.10	- ve	- ve	-	-		
HO/22 9-11cm	0.10	- ve	- ve	-	-		
HO/22 11-13 cm	0.11	- ve	- ve	-	-		
HO/22 13-15 cm	0.10	- ve	- ve	-	-		
HO/22 15-17 cm	0.10	- ve	- ve	-	-		
HO/22 17-19 cm	0.10	- ve	- ve	-	-		
HO/23 1-3 cm	0.35	3.31	0.43	9.457	0.814	0.29	Occassional heroin user
HO/24 0.5-2.0 cm	0.10	- ve	0.22	-	-	0.4	Occassional heroin user
HO/25 1-2 cm	8.15	2.42	3.08	0.297	2.646	0.38	History of morphine addiction
HO/25 2-3 cm	9.79	7.62	3.47	0.778	2.821		
HO/26 1-2 cm	2.31	5.62	0.79	2.433	2.924	0.18	Registered heroin addict
HO/26 2-3 cm	2.43	5.53	0.79	2.276	3.076		
HO/26 3-4 cm	3.39	15.60	1.10	4.602	3.081		
HO/26 4-5 cm	4.18	33.48	1.31	8.009	3.191		
HO/27 1-3 cm	0.40	6.78	0.36	16.950	1.111	0.11	Known regular heroin user
HO/27 3-5 cm	0.59	10.59	0.33	17.949	1.787		
HO/27 5-7 cm	0.70	13.53	- ve	19.329	-		

Table 8.8: Blood, hair segment and drug history results for opiates

Case sample	Hair segments excluding roots					Blood	Drug history
	Morphine (ng/mg)	6MAM (ng/mg)	Codeine (ng/mg)	6MAM/ Morphine	Morphine/ Codeine	Morphine (mg/L)	
HO/28 1-3 cm	- ve	- ve	0.49	-	-	0.03	Known heroin user prescribed withdrawal tablets
HO/28 3-5 cm	- ve	- ve	- ve	-	-		
HO/29 1-3 cm	0.42	- ve	2.23	-	0.188	0.02	Overdose 3 days prior to death (tablets not known)
HO/29 3-5 cm	0.85	- ve	0.43	-	1.977		
HO/29 5-8 cm	0.13	- ve	0.93	-	0.140		
HO/30 1-4 cm	0.57	- ve	0.51	-	1.118	0.56	Long history of heroin abuse
HO/31 1-2 cm	1.33	0.14	0.36	0.105	3.694	- ve	Not known
HO/31 2-3 cm	2.03	0.18	0.46	0.008	4.413		
HO/31 3-4 cm	2.27	0.18	0.52	0.008	4.365		

Table 8.9: Blood, hair segment and drug history results for methadone

Case sample	Hair segments excluding roots			Blood	Drug history
	Methadone (ng/mg)	EDDP (ng/mg)	Methadone/ EDDP	Methadone (mg/L)	
HO/01 1-2 cm	- ve	-ve	-	0.3	No methadone history
HO/01 2-3 cm	- ve	-ve	-		
HO/01 3-4 cm	- ve	-ve	-		
HO/06 1-2 cm	- ve	- ve	-	0.03	Took wife's methadone prescription day prior to death
HO/06 2-3 cm	0.54	0.27	2.000		
HO/06 3-4 cm	1.15	1.40	0.821		
HO/06 4-5 cm	1.15	0.75	1.533		
HO/06 5-7 cm	1.10	1.55	0.710		
HO/17 0.5-2.5 cm	- ve	-ve	-	0.02	No methadone history
HO/17 2.5-4.5 cm	- ve	-ve	-		
HO/17 4.5-6.5 cm	- ve	-ve	-		
HO/17 6.5-8.5 cm	- ve	-ve	-		
HO/17 8.5-10.5 cm	- ve	-ve	-		
HO/17 10.5-13.5 cm	- ve	-ve	-		
HO/19 0.5-2.0 cm	0.67	0.52	1.288	0.02	Placed on numerous methadone programmes
HO/20 0.5-2.0 cm	0.52	0.22	2.364	- ve	Referred to drug rehabilitation unit
HO/21 0.5-1.5 cm	0.15	- ve	-	0.14	Not on a methadone programme
HO/21 1.5-2.5 cm	- ve	- ve	-		
HO/21 2.5-3.5 cm	- ve	- ve	-		
HO/21 3.5-4.5 cm	- ve	- ve	-		
HO/21 4.5-5.5 cm	- ve	- ve	-		
HO/21 5.5-6.5 cm	- ve	- ve	-		
HO/23 1-3 cm	0.34	- ve	-	0.02	No methadone history
HO/26 1-6 cm	- ve	- ve	-	0.05	No methadone history

Table 8.10: Conclusions drawn from hair and blood findings and drug use history

Case	Hair Findings	Blood Findings	Drug use history	Conclusions
HO/01	Regular, medium user of heroin for at least 4 months. Methadone user for 1 month.	Methadone user	Known cannabis and other illicit drug user.	Regular, medium heroin user
HO/02	Regular, medium user of heroin for at least 3 months.	Morphine user	Known heroin user.	Regular, medium heroin user
HO/03	Heroin user. Low concentrations.	Morphine user	Known heroin user. Previously on methadone programme.	Low heroin user
HO/04	Low concentrations of morphine and codeine in months 4-7 prior to death. Insufficient hair root sample. Inconclusive of heroin use	Morphine user	Known heroin user	Findings indicate not a regular user, but may be due to insufficient hair sample. Heroin user, extent inconclusive
HO/05	Low user of heroin for 2 months.	Heroin user	Not known.	Low heroin user
HO/06	Regular, low user of heroin for at least 7 months. Methadone user for months 2-7 prior to death.	Morphine and methadone user	Known heroin user.	Regular low heroin user
HO/07	Regular, high user of heroin for at least 9 months.	Morphine user	Known heroin user for 20 years.	Regular, high heroin user
HO/08	Regular, medium user of heroin for at least 5 months.	Heroin user	Known heroin user.	Regular, medium heroin user
HO/09	Regular, high user of heroin for at least 4 months.	Heroin user	Known heroin user for 5 years. Prescribed methadone one month prior to death.	Regular, high heroin user
HO/10	Regular, medium user of heroin for at least 5 months. Methadone user for months 2-5 prior to death.	Morphine user	Known heroin user for 3 years (smoked). Came off methadone programme 2 months prior to death.	Regular, medium heroin user

Table 8.11: Conclusions drawn from hair and blood findings and drug use history

Case	Hair Findings	Blood Findings	Drug use history	Conclusions
HO/11	6-MAM only detected 1.5-2.5 months prior to death. Insufficient hair root sample.	Heroin user	Known heroin user. Attended several drug rehabilitation programmes.	Intermittent heroin user
HO/12	Methadone user. Not detected in root sample.	Heroin user	Known heroin abuser for 5 years. On methadone programme. Intake reduced from 60 to 8 ml and had stopped taking it 2 weeks prior to death.	Intermittent heroin user
HO/13	Low heroin user	Heroin user	Known drug user	Low heroin user
HO/14	Low heroin user	Heroin user	Long history of drug abuse	Low heroin user
HO/15	Low heroin user	Heroin user	Known drug abuser for 2-3 years	Low heroin user
HO/16	Regular, high heroin user. Methadone user.	Heroin user	Known heroin user	Regular, high heroin user. N.B. Cause of death was hanging.
HO/17	No indication of heroin or methadone use. (Root sample size very small.)	Morphine and methadone user	Possible diamorphine user over 4 weeks prior to death.	Naive user of heroin.
HO/18	No indication of heroin use.	Heroin user	Known drug user. Drug rehabilitation programme. Claimed he had been clean for 4 weeks (one month prior to death).	Intermittent heroin user

Table 8.12: Conclusions drawn from hair and blood findings and drug use history

Case	Hair Findings	Blood Findings	Drug use history	Conclusions
HO/19	Regular, medium user of heroin for at least 2 months. Methadone user for same period.	Methadone user	Long history of drug abuse. Numerous methadone and detoxification programmes.	Regular, medium heroin user
HO/20	Regular medium user for at least two months. Methadone user for same period.	Heroin user	Known heroin user for 5 years	Regular, medium heroin user
HO/21	Low heroin user for at least 6 months. Methadone user for two months prior to death.	Methadone user	Known drug user. Not on methadone programme.	Low heroin user.
HO/22	Low concentrations of morphine detected in hair. 6-MAM detected only in one segment.	Heroin user	Regular heroin user 10 years prior to death. Denied using heroin in last 10 years.	Low heroin user.
HO/23	Medium heroin and methadone user for at least two months	Heroin and methadone user	Occasional heroin user. Not known if regular user of heroin.	Regular, medium heroin user.
HO/24	Low concentrations of morphine detected. Inconclusive of heroin use	Heroin user	Occasional heroin user.	Findings indicate not a regular user, but may be due to insufficient hair sample. Heroin user, extent inconclusive
HO/25	Regular, medium heroin user for at least 3 months.	Morphine user	History of morphine addiction.	Regular, medium heroin user.
HO/26	Regular, high heroin user for at least 6 months.	Heroin and methadone user	Known drug user. Registered heroin addict.	Regular, high heroin user.
HO/27	Regular, high heroin user for at least 7 months.	Heroin user	Known heroin user.	Regular, high heroin user.

Table 8.13: Conclusions drawn from hair and blood findings and drug use history

Case	Hair Findings	Blood Findings	Drug use history	Conclusions
HO/28	Low concentration of morphine detected. Inconclusive of heroin use	Morphine user	Known heroin user in past. Believed to be intermittently taking heroin with tablets prescribed for withdrawal.	Intermittent heroin user
HO/29	Low concentration of morphine detected. Inconclusive of heroin use	Heroin user	Not known	Low heroin user
HO/30	Morphine detected over a 4 month period. Inconclusive of heroin use	Heroin user	Long history of heroin use.	Low heroin user
HO/31	Low heroin user for at least 4 months.	Not analyzed	Not known	Regular, low heroin user

8.2 CASE SUMMARIES

Case HO/01 **Sex:** Male **Age:** 25 years

Drug abuse history: Known to have abused cannabis and other illicit drugs

Medication at time of death: Not known

Circumstances of death: Released from prison 6 weeks prior to death. Last contact with deceased was phone call with sister. Deceased could not be awakened. Ambulance technicians found him dead. CPR. Life pronounced extinct at hospital. No track marks indicating intravenous drug abuse.

Toxicology: desmethyldiazepam 1.7 mg/L, diazepam 0.4 mg/L, EDDP 0.3 mg/L, methadone 0.3 mg/L

COD: Ia Methadone and diazepam intoxication

Case HO/02 **Sex:** Male **Age:** 32 years

Drug abuse history: Abused various drugs for entire adult life. At age 13 he abused solvents then made transition to harder drugs, injecting Temegesic tablets and at age 20 started injecting heroin.

Medication at time of death: Not known

Circumstances of death: Booked into a hostel and was last seen alive by a member of staff. Following day found lying on floor apparently dead, with trousers around his ankles. Signs of drug misuse about the room. Surgeon pronounced life extinct. Hypodermic needle found protruding from groin. No suspicious circumstances.

Toxicology: desmethyldiazepam 0.3 mg/L, morphine 0.5 mg/L

COD: Ia Heroin intoxication

Case HO/03 **Sex:** Male **Age:** 28 years

Drug abuse history: Drug misuse problem became apparent in 1999. Prescribed methadone, 40 mg/day at start of programme. He was not on methadone at time of death.

Medication at time of death: None

Circumstances of death: Deceased found lying on floor of toilets in hostel. CPR. Ambulance crew did not carry out further medical assistance. Casualty surgeon

pronounced life extinct. Diazepam tablets and a paper wrap containing a small quantity of white crystals were found on body. Syringe was also later obtained.

Toxicology: desmethyldiazepam 0.7 mg/L, diazepam 0.6 mg/L, 11-nor-delta-9-THC-COOH 75 ng/ml, codeine 0.1 mg/L, morphine 0.7 mg/L

COD: Ia Acute heroin poisoning

Case HO/04 **Sex:** Male **Age:** 46 years

Drug abuse history: Known heroin abuser and known to drink alcohol frequently.

Medication at time of death: Betablockers and antidepressant, Cipramil

Circumstances of death: Deceased consumed 6 pints of beer and 2 whiskeys in bar with friends. Went to friend's house to snort heroin. Deceased appeared to fall asleep, breathing heavily and looked unwell. CPR. Ambulance staff found him dead. Casualty surgeon pronounced life extinct. A set of miniature scales and assorted literature in relation to HIV and drugs was found.

Toxicology: alcohol 194 mg/100 ml (blood), alcohol 294 mg/100 ml urine, morphine 0.3 mg/L

COD: Ia Heroin and alcohol intoxication

Case HO/05 **Sex:** Male **Age:** 24 years

Drug abuse history: Known intravenous drug user.

Medication: diazepam, dihydrocodeine, thioridazine, prothiaden.

Circumstances of death: Two friends of deceased arrived at house and noticed that deceased was under influence of drugs. They smoked a bag of heroin. Deceased appeared "out of it" and his speech was slurred. Deceased found lying on bed with eyes open, but not responding. CPR. Ambulance technicians could not revive deceased. Two packs of diazepam tablets lying empty on deceased's bed and a spoon lying beside a hypodermic syringe next to the bed. Police surgeon pronounced life extinct.

Toxicology: desmethyldiazepam 0.4 mg/L, diazepam 0.4 mg/L, codeine 0.1 mg/L, dihydrocodeine 0.3 mg/L, morphine 0.2 mg/L

COD: Ia Heroin , dihydrocodeine and diazepam intoxication

Case HO/06 **Sex:** Male **Age:** 33 years

Drug abuse history: Known heroin abuser

Medication at time of death: None

Circumstances of death: Released from prison four days prior to death. Two days after release common law wife gave deceased 10 ml of her methadone prescription. He later gave her 5 blue valium and at this point he appeared "out of it". Deceased and friend injected heroin. Deceased found lying face down, snoring. Following day deceased still lying in face down position, apparently dead. Police surgeon pronounced life extinct. A spoon with a substance and a sharp bin with used needles and syringes were found. A needle and syringe were found beneath deceased where groin area had been.

Toxicology: diazepam 0.4 mg/L, methadone 0.03 mg/L, morphine 0.1 mg/L

COD: Ia Heroin intoxication

Case HO/07 **Sex:** Male **Age:** 39 years

Drug abuse history: Known intravenous heroin abuser for 20 years.

Medication at time of death: Amoxycillin

Circumstances of death: Two weeks previous to his death deceased injected heroin into muscle. He injured leg by falling on stairs. A few days later a hole appeared on knee which increased in size over next few days. Doctor prescribed amoxycillin. Three days later deceased was in a lot of pain but refused to see doctor again. Friend saw deceased injecting heroin into groin. Later no response was obtained from deceased. Friend attempted CPR. Police surgeon pronounced life extinct. Drug paraphernalia found in flat.

Toxicology: alcohol 11 mg/100 ml (blood), diazepam 0.2 mg/L, temazepam 0.1 mg/L, dihydrocodeine 0.5 mg/L, morphine 0.2 mg/L, paracetamol 3.2 mg/L

COD: Ia Multiple organ failure due to

Ib clostridium novyi toxaemia/necrotising faciitis due to

Ic chronic injecting drug abuse

Case HO/08 **Sex:** Male **Age:** 44 years

Drug abuse history: History of heroin abuse. No details of seeking assistance for problem.

Medication at time of death: None

Circumstances of death: Deceased was at the flat where he was residing and injected heroin. Friend noticed the deceased walking with limp. Deceased complained of pain in his left groin. Deceased's breathing was laboured and limping was more noticeable. Friend was aware of deceased being sick. Deceased refused to go to see a doctor and sickness continued. Following day deceased showed friend that his nails were blue in colour and friend advised he go visit doctor. He was later found lying on kitchen floor. CPR. Surgeon pronounced life extinct.

Toxicology: codeine 0.01 mg/L, morphine 0.1 mg/L

COD: 1a Staphylococcus necrotising fasciitis due to

1b Chronic Injecting Drug Abuse

Case HO/09 **Sex:** Male **Age:** 26 years

Drug use history: History of heroin abuse since 1995. Previously prescribed methadone. He had a £70-80 /day habit in January 1999 and by May 2000 £30-50/day habit (24 days prior to death).

Medication: Prescribed 60 ml/day methadone

Circumstances of death: Deceased attended hospital and was diagnosed as having a leg ulcer, given antibiotics and released the following day. He injected heroin in front of friend. Hostel assistants later found deceased lying on bed and he appeared to be dead. Ambulance crew confirmed that deceased was dead. Police surgeon pronounced life extinct. Heroin bag and contents retrieved by police.

Toxicology: desmethyldiazepam 0.2 mg/L, diazepam 0.2 mg/L, codeine 0.02 mg/L, morphine 0.2 mg/L

COD: Ia Multiple organ failure due to

Ib clostridium novyi toxaemia due to

Ic cellulitis/necrotising faciitis of right groin due to

Id chronic injecting drug abuse

Case HO/10 **Sex:** Male **Age:** 25 years

Drug abuse history: Heroin user for 3 years (smoked). Placed on a methadone programme from May 1999 - March 2000 until he was accepted into a rehabilitation centre.

Medication at time of death: Amitriptyline and diazepam

Circumstances of death: Deceased last seen alive by flatmate. Flatmate returned from work to find deceased lying on floor face down. Police surgeon pronounced life extinct. Surgeon pronounced life extinct. Plastic bottle adapted for smoking and two white pills were found in room.

Toxicology: morphine 0.04 mg/L

COD: Ia Heroin intoxication

Case HO/11 **Sex:** Male **Age:** 25 years

Drug abuse history: Abused drugs for 5 years, mainly injecting heroin and taking diazepam tablets. He was not on a methadone programme. And had attended several drugs rehabilitation programmes in the past, all which failed.

Medication at time of death: Diazepam

Circumstances of death: Deceased was in a snooker/pool hall. He asked to use toilets. After twenty minutes he had not returned and worker on the premises became concerned He found door locked and requested assistance. Deceased was found slumped on floor with trousers at ankles and belt tied round left arm. CPR. Police surgeon pronounced life extinct.

Toxicology: diazepam 0.3 mg/L, codeine 0.1 mg/L, morphine 0.3 mg/L

COD: Ia Heroin and diazepam intoxication

Case HO/12 **Sex:** Male **Age:** 32 years

Drug abuse history: Registered heroin addict in 1995, suffered from an addiction for five years. Also known to have abused diazepam in past. He was on a methadone programme. Reduced methadone intake from 60 ml to 8 ml. Two weeks prior to death, deceased had stopped taking methadone altogether.

Medication at time of death: None

Circumstances of death: Deceased had some cans of lager with girlfriend in park then left her to go home. She knocked on door on several occasions and got no reply. She gained access to house via neighbour's veranda and found deceased lying face down in bedroom. Police surgeon pronounced life extinct. Drug paraphernalia were found at scene.

Toxicology: alcohol 141 mg/100 ml (blood), alcohol 228 mg/100 ml (urine), codeine 0.04 mg/L, morphine 0.4 mg/L

COD: Ia Suspected drug related death due to

Ib Heroin and alcohol intoxication

Case HO/13 **Sex:** Male **Age:** 38 years

Drug abuse history: Known drug user

Medication at time of death: None

Circumstances of death: Released from prison 1 day prior to death. Deceased arrived at friend's house with "4 tenner bags of heroin" They both took drugs intravenously and friend left agreeing to meet up later with deceased. They met up and the deceased smoked another bag of heroin. Deceased informed friend he was going to buy more heroin and they met up back at flat and injected a bag each. Deceased fell asleep on couch. Following morning deceased found lying face down on couch and believed he was dead. Life was pronounced extinct by police surgeon. Drug paraphernalia was seized.

Toxicology: codeine 0.01 mg/L, morphine 0.1 mg/L

COD: Ia Suspected drug overdose due to

Ib heroin intoxication

Conditions contributing to death:

II Bronchopneumonia

Case HO/14 **Sex:** Male **Age:** 23 years

Drug abuse history: Long history of drug abuse. Overdosed on drugs on four different occasions.

Medication at time of death: None

Circumstances of death: Deceased released from prison one day prior to death. He returned home with intention of sleeping rough following fall out with mother over drug problem. Mother spoke to deceased who was lying in the rear garden. Later that day, brother awakened and saw deceased lying face down at back of home. Hypodermic needle was found in groin and no vital signs of life. CPR. Life pronounced extinct. Drug paraphernalia and brown powder seized.

Toxicology: desmethyldiazepam 0.4 mg/L, diazepam 0.4 mg/L, temazepam 0.5 mg/L codeine 0.02 mg/L, morphine 0.1 mg/L

COD: Ia Morphine toxicity

Case HO/15 **Sex:** Male **Age:** 24 years

Drug abuse history: History of heroin abuse for 2-3 years (intravenous).

Medication at time of death: None

Circumstances of death: Released from prison 2 weeks prior to death. Deceased's girlfriend left him in house and when she returned an hour later she could not gain access to house. Contacted concierge. Joiner gained access to home and the deceased was found lying face down on living room floor. No signs of life. Surgeon pronounced life extinct. A needle puncture mark was found on left arm and hypodermic syringe and other drug paraphernalia were seized.

Toxicology: desmethyldiazepam 0.3 mg/L, diazepam 0.3 mg/L, codeine 0.1 mg/L, morphine 0.5 mg/L

COD: Ia Suspected drug related death to

Ib Heroin and diazepam intoxication

Case HO/16 **Sex:** male **Age:** 61 years

Drug abuse history: History of heroin abuse and heavy drinker.

Medication at time of death: dihydrocodeine, atenolol, aspirin, glipizide

Circumstances of death: Deceased had not met neighbour as rearranged. Neighbour contacted concierge at deceased's flat. Attended flat but could not enter flat. Contacted police. Police found deceased sitting on floor hall with ligature round neck. Surgeon pronounced life extinct. Drug paraphernalia were found.

Toxicology: desmethyldiazepam 0.1 mg/L, diazepam 0.4 mg/L, codeine 0.004 mg/L, morphine 0.1 mg/L

COD: Ia Hanging

Case HO/17 **Sex:** Female **Age:** 25 years

Drug abuse history: According to partner she was abusing diamorphine over past 4 weeks.

Medication at time of death: venlafaxine

Circumstances of death: Deceased told partner she was going to buy heroin. She returned later and stated that she had bought two bags of heroin. They snorted one of the bags. Friend arrived to prepare the second bag for smoking. They smoked the contents of

this bag and left the house before midnight. Deceased settled to sleep on floor with partner. Partner woke up two hours later and noted that subject did not move. Partner slept on couch and subject had not moved by next morning. Life pronounced extinct. No injection sites detected.

Toxicology: alcohol 18 mg/100 ml (blood), alcohol 67 mg/100 ml (urine), temazepam 0.2 mg/L, methadone 0.02 mg/L, morphine 0.3 mg/L

COD: Ia Morphine toxicity

Case HO/18 **Sex:** Male **Age:** 51 years

Drug abuse history: Past intravenous drug abuser and alcoholic. Previously abused heroin, temazepam, diazepam and cannabis. He was in drug rehabilitation for 4 weeks.

Medication at time of death: None

Circumstances of death: Deceased and friend bought a "bag" of heroin and went to friend's house to inject. They went into the close and injected half of the deal each into their own arms. Deceased suddenly collapsed. CPR. Life was pronounced extinct that day at hospital.

Toxicology: 194 mg/100 ml alcohol (blood), codeine 0.02 mg/L, morphine 0.1 mg/L, 6-MAM 0.01 mg/L

COD: Ia Heroin and alcohol abuse

Case HO/19 **Sex:** male **Age:** 44 years

Drug abuse history: Long history of alcohol and drug abuse since age of 16. He had been on numerous methadone programmes and in and out of drug detoxification programmes.

Medication at time of death: dihydrocodeine, diazepam

Circumstances of death: Friend entered flat and found deceased sitting on couch in living room with hypodermic syringes lying by his side CPR. Surgeon pronounced life extinct.

Toxicology: alcohol 162 mg/100 mL (blood), alcohol 154 mg/100 mL (urine) desmethyldiazepam 1.0 mg/L, diazepam 0.5 mg/L, oxazepam 0.5 mg/L, temazepam 0.2 mg/L, methadone 0.02 mg/L, morphine 0.2 mg/L

COD: Ia Heroin, alcohol and diazepam intoxication.

Case HO/20

Sex: male **Age:** 24 years

Drug abuse history: Habitual drug user who started taking temazepam tablets at 18, then heroin at age 19. Referred to drug rehabilitation unit.

Medication at time of death: None

Circumstances of death: Deceased was observed sitting on landing outside flat. Witness later found him lying on landing and thought he was asleep through drink. When he was still lying there sometime later witness became concerned and called for assistance. Surgeon pronounced life extinct and a fresh puncture wound/needle mark was noticed on the deceased's left arm. Drug paraphernalia were found in flat and outside main door of flat.

Toxicology: desmethyldiazepam 0.8 mg/L, diazepam 0.6 mg/L, oxazepam 0.4 mg/L temazepam 0.2 mg/L, codeine 0.1 mg/L, morphine 0.5 mg/L

COD: Ia Heroin and diazepam intoxication

II Bronchopnemonia

Case HO/21

Sex: male **Age:** 23 years

Drug abuse history: Visited doctor on three occasions about drug abuse. Deceased informed doctor he was using opiates, diazepam, cannabis and ecstacy. Not placed on methadone programme.

Medication at time of death: Ventolin and Becotide

Circumstances of death: Deceased was drinking heavily with two friends at his home address. Two friends arrived at house later on in day and tried to rouse deceased. When he failed to wake they summoned an ambulance. Police surgeon pronounced life extinct.

Toxicology: alcohol 40 mg/100 ml (blood), alcohol 84 mg/100 ml (urine), desmethyldiazepam 0.28 mg/L, diazepam 0.37 mg/L, oxazepam 0.36 mg/L, delta-9-THC 0.8 ng/mL, 11-nor-delta-9-THC-COOH 10.5 ng/ml, methadone 0.14 mg/L

COD: Ia Fatty degeneration of the liver

Case HO/22

Sex: female **Age:** 41

Drug abuse history: Regular heroin user from 17-30 years. Denied abusing drugs since 30 years of age. Chronic alcohol user since 30 years of age.

Medication at time of death: Human Mixtard Insulin, Vitamin B Strong, dihydrocodeine, thiamine, frusemide, spironolactone, lactulose

Circumstances of death: Deceased's aunt was at her house assisting her taking a bath. She noticed a severe swelling on her back around site of a recently removed kidney. She made a doctor's appointment. Four days later the aunt received a call from deceased claiming she was very unwell. She was admitted to hospital with sepsis and swelling to the right, loin area. Condition deteriorated over next few days and she lapsed into multi-organ failure. Doctor pronounced life extinct. Prior to death deceased vehemently denied abusing drugs over past 10 years.

Toxicology: codeine 0.01 mg/L, morphine 0.1 mg/L

COD: Ia Multiple Organ Failure due to

Ib Staph aureus sepsis due to

Ic cirrhosis of the liver and chronic drug abuse

Case HO/23

Sex: male **Age:** 33

Drug abuse history: Known heavy drinker. Not known if he was a regular drug abuser but had smoked heroin on at least one occasion.

Medication at time of death: Not known

Circumstances of death: Deceased was last seen alive by friend and at this time he was heavily under the influence of alcohol. Three days later the deceased's brother became concerned about the him, went to his house and found the deceased in a kneeling position bent forwards with hands underneath his head. Deceased had cuts to his head, blood around his nose. Police surgeon pronounced life extinct. A search of the flat produced drug paraphernalia (a burnt spoon, a shoe lace and silver foil).

Toxicology: alcohol 143 mg/100 ml (blood), alcohol 202 mg/100 ml (urine), EDDP 0.001 mg/L, methadone 0.02 mg/L, codeine 0.03 mg/L, morphine 0.29 mg/L

COD: Ia Heroin intoxication

Case HO/24

Sex: male **Age:** 37

Drug abuse history: Alcohol abuser for 6 years. Casual drug user who occasionally injected heroin.

Medication at time of death: None

Circumstances of death: Deceased consumed two one litre bottles of cider at ex-girlfriend's house. He left the flat with girlfriend and was believed to have purchased a "tenner bag of heroin" They returned to the flat and injected some heroin. Shortly after injecting, the deceased lapsed into unconsciousness with his eyes rolling and stopped breathing. CPR. Deceased was taken to hospital and pronounced dead on arrival. Police found a stained teaspoon, reefer type cigarettes, cling film and three blister packs containing 50, 17 and 106 tablets

Toxicology: alcohol 193 mg/100 ml (blood), alcohol 298 mg/100 ml (urine), 6-MAM 0.01 mg/L, codeine 0.06 mg/L, morphine 0.4 mg/L

COD: Ia Heroin and alcohol intoxication

Case HO/25

Sex: male **Age:** 42

Drug abuse history: History of intravenous morphine addiction

Medication at time of death: None

Circumstances of death: Deceased complained of sickness and diahorrea to friend for which he had been taking anadin and imodium. Friend tried to contact deceased and after several attempts visited with other friends. On receiving no reply they forced the door and found him dead on bed. Police surgeon pronounced life extinct.

Toxicology: alcohol (blood) 308 mg/100 ml, desmethyldiazepam 0.17 mg/L, diazepam 0.09 mg/L, morphine 0.38 mg/L, paracetamol 15.41 mg/L

COD: Ia Heroin and alcohol intoxication

Case HO/26

Sex: male **Age:** 42

Drug abuse history: History of intravenous drug abuse. Registered heroin addict (£100 per day habit). Known alcohol abuser.

Medication at time of death: None

Circumstances of death: Wife of deceased returned from local shop to find deceased lying unconscious on kitchen floor. His trousers were round his ankles and needles were lying at his sides. CPR. Life was pronounced extinct at hospital. Recent injection marks found on groin.

Toxicology: desmethyldiazepam 0.35 mg/L, diazepam 0.25 mg/L, methadone 0.05 mg/L, codeine 0.02 mg/L, morphine 0.18 mg/L

COD: Ia Heroin, diazepam and methadone intoxication

Case HO/27

Sex: male **Age:** 19

Drug abuse history: Known drug user. Admitted to doctor that he was smoking 4 bags of heroin a day. Referred to detoxification clinic.

Medication at time of death: None

Circumstances of death: Deceased and friend came to Glasgow to attend Drug Rehabilitation Unit to get off heroin. The deceased had in his possession 1 gram of heroin and blue valium tablets. Deceased left room where they were staying to buy some beer and sweets. An hour later he returned and appeared to be under the influence of drugs. Two hours later, the friend went to his bed leaving the deceased sitting in the chair. The following morning friend returned to the room after breakfast with guest house manager who was collecting payment for the room. At this time, they observed the deceased sleeping on the chair, snoring. Friend went for a shower and on returning to the room he noticed the deceased had stopped breathing. CPR. Life was pronounced extinct at hospital. Police found a small metal smoking pipe in the deceased's bag.

Toxicology: desmethyldiazepam 1.11 mg/L, diazepam 0.95 mg/L, oxazepam 0.1 , mg/L, codeine 0.02 mg/L, morphine 0.11 mg/L

COD: Ia Heroin and diazepam intoxication

Case HO/28

Sex: male **Age:** 25

Drug abuse history: Known heroin user. Prescribed tablets to help with withdrawal from heroin

Medication at time of death: Not known

Circumstances of death: Deceased was ejected from night club for being too drunk. About two and a half hours later he appeared at dwelling house of two friends. They smoked three cannabis joints supplied by the deceased and the deceased informed them that he had just taken a bag of heroin. They all fell asleep. The following morning the two friends awoke and commented on the deceased's loud snoring and tried to wake him, but could not. They both left the house with the deceased still sleeping. Returning later in the evening the friends found the deceased lying in same position as they had left him and found his body very cold. Paramedics found deceased lying in living room floor apparently dead. Life was pronounced extinct by police surgeon. A syringe cap was found near the deceased's body.

Toxicology: alcohol 23 mg/100 ml (blood), alcohol 137 mg/100 ml (urine), desmethyldiazepam 0.22 mg/L, diazepam 0.18 mg/L, dihydrocodeine 0.04 mg/L, morphine 0.03 mg/L

COD: Ia Multiple drug and alcohol intoxication

Case HO/29

Sex: male **Age:** 31

Drug abuse history: Not known. Three days prior to death he was admitted to hospital after taking an overdose of tablets (type not known).

Medication at time of death: Not known

Circumstances of death: Deceased and brother reported missing by parents. Brother phoned parents telling them that deceased had commited suicide. He returned home next day informing his parents his brother was dead in a dwelling house. The body was found in a bedroom in dwelling house, lying on back on bed. Police surgeon pronounced life extinct. Drug paraphernalia (sin bin containing syringes) were found at scene. Brother admitted buying three valium tablets and a bag of heroin for the deceased so he could take his own life. Householder of dwellinghouse entered bedroom occupied by deceased and watched brother inserting syringe into deceased's arm. Householder and brother checked on deceased regularly and found him asleep, breathing heavily and on one occasion he had been sick.

Toxicology: diazepam 0.07 mg/L, codeine 0.01 mg/L, morphine 0.02 mg/L

COD: Ia Bronchopneumonia

Case HO/30

Sex: male **Age:** 32

Drug abuse history: Long history of heroin abuse and had also been treated for injecting diazepam. Last time he was seen by doctor he was treated for injecting heroin.

Medication at time of death: None

Circumstances of death: Deceased was released from prison four months prior to death and since then he had been residing in a hostel. He appeared normal the day prior to death. On the following day a hostel worker entered the deceased's room and observed the deceased with his head under a dressing table and his trousers round his ankles. A needle was found on the dressing room table. Police surgeon arrived and pronounced life extinct. Police seized a syringe and a wrap which was suspected to have contained controlled drugs.

Toxicology: desmethyldiazepam 0.5 mg/L, codeine 0.08 mg/L, morphine 0.56 mg/L paracetamol 6.11 mg/L

COD: Ia Heroin intoxication

Case HO/31

Sex: male

No other information available

8.3 ANTIDEPRESSANTS IN BLOOD

8.3.1 WHOLE BLOOD

The developed solid phase extraction method in Chapter 4 was used to extract fluoxetine and paroxetine from postmortem peripheral blood samples and also blood samples obtained from live patients which were collected by standard venipuncture procedure.Ethical approval was obtained for this study.

8.3.2 FLUOXETINE CASES

21 postmortem blood samples were analyzed for fluoxetine and its metabolite. The results of these analyses are displayed in Table 8.14. Fluoxetine concentrations were found to vary from 0 - 1.916 mg/L and norfluoxetine concentrations from 0.018 - 1.736 mg/L. Fluoxetine/norfluoxetine ratios varied from 0.181 - 14.406.

Drug concentrations in 6 fatalities have ranged from 1.3 - 6.8 (mean 3.8) mg/L blood for fluoxetine and 0.9 - 5.0 (mean 2.1) mg/L blood for norfluoxetine.[62] However, in four of these cases at least one other drug codeine, meperidine, clozapine or diphenhydramine was detected in the blood.

There were three cases where the levels of fluoxetine and/or norfluoxetine were higher than normal therapeutic values. For case BP/10, an overdose level of fluoxetine and norfluoxetine was detected at 0.631 and 1.736 mg/L respectively. The half-life of fluoxetine is 1 - 3 days. Since a higher level of metabolite was detected than parent drug then this individual must have lived for several hours after taking the dose. On the other hand, case BP/20 showed an overdose level of fluoxetine at 1.916 mg/L and a much lower concentration of norfluoxetine 0.133 mg/L. This person was either a poor metabolizer of fluoxetine or died relatively shortly after taking the dose.

Case BP/13 also gave values higher than maximum therapeutic levels. All other cases gave values within the therapeutic range.

Examples of chromatograms obtained for the fluoxetine blood analyses are shown in Figures 8.6-8.9.

Table 8.14: Fluoxetine Postmortem Blood Cases

Case sample	Fluoxetine (mg/L)	Norfluoxetine (mg/L)	Fluoxetine/ Norfluoxetine
BP/01	0.180	0.210	0.857
BP/02	0.100	0.130	0.769
BP/03	0.070	0.090	0.778
BP/04	0.110	0.296	0.372
BP/05	0.125	0.197	0.635
BP/06	0.064	0.098	0.653
BP/07	0.179	0.369	0.485
BP/08	0.210	0.018	11.667
BP/09	0.017	0.094	0.181
BP/10	0.631	1.736	0.363
BP/11	0.122	0.195	0.626
BP/12	0.103	0.154	0.669
BP/13	0.664	0.752	0.883
BP/14	0.429	0.442	0.971
BP/15	0.073	0.052	1.403
BP/16	- ve	0.072	-
BP/17	0.057	0.047	1.213
BP/18	0.007	0.028	0.250
BP/19	0.114	0.317	0.360
BP/20	1.916	0.133	14.406
BP/21	0.054	0.058	0.931

Twelve clinical blood samples obtained from patients who were known to have been prescribed Prozac® were analyzed. The results obtained using the solid phase extraction procedure are displayed in Table 8.15 along with the ratio of parent drug to metabolite in each case. For these cases, fluoxetine concentrations were found to vary from 0.013 - 0.196 mg/L and norfluoxetine concentrations from 0.011 - 0.199 mg/L. Fluoxetine/norfluoxetine ratios varied from 0.155 - 2.649. All of these patients had been prescribed 20 mg/day. Steady state serum concentrations of fluoxetine and its metabolite have been shown to vary from 0.025 - 0.473 (mean 0.109) mg/L and 0.018 - 0.466 (mean 0.130) mg/L respectively for patients who were receiving 20-60 mg of the drug daily.[100] One of the samples produced negative results for fluoxetine and norfluoxetine. Medical notes indicated that this patient had commenced treatment 24 hours prior to the sample being taken. A single 40 mg oral dose in adults produced peak plasma fluoxetine levels of 0.015 - 0.055 mg/L withing 6-8 hours.[62] Therefore, a 20 mg oral dose would produce significantly lower peak plasma levels and after 24 hours these levels may have decreased to levels below the limit of detection of the method.

Table 8.15: Fluoxetine Clinical Blood Cases

Case sample	Fluoxetine (mg/L)	Norfluoxetine (mg/L)	Fluoxetine/ Norfluoxetine
BV/21	0.042	0.185	0.227
BB/22	0.156	0.068	2.294
BB/23	0.196	0.074	2.649
BL/02	0.114	0.109	1.046
BL/03	0.024	0.155	0.155
BL/04	0.103	0.199	0.518
BL/05	0.079	0.128	0.617
BL/06	- ve	- ve	-
BL/07	0.059	0.119	0.496
BL/12	0.013	0.015	0.867
BL/13	0.062	0.075	0.827
BL/15	0.029	0.011	2.636

8.3.3 PAROXETINE CASES

14 postmortem blood samples which were known to be positive for paroxetine were analyzed. The results obtained using the solid phase extraction procedure are displayed in Table 8.16. Paroxetine concentrations were found to vary from 0.021 - 0.593 mg/L. Peak plasma paroxetine concentrations were found to have a mean value of 0.062 mg/L after treatment with 30 mg/day. It would therefore appear that at least 10 of these cases were overdose cases with values from 0.143 - 0.593 mg/L.

Drug concentrations in 4 fatalities have ranged from 1.4 - 4.0 (mean 2.7) mg/L blood. However, in two of these cases imipramine or moclobemide were detected in significant quantities in the blood.[62]

Table 8.16: Paroxetine Postmortem Blood cases

Case sample Postmortem	Paroxetine (mg/L)	Case sample Postmortem	Paroxetine (mg/L)
BP/22	0.165	BP/29	0.398
BP/23	0.202	BP/30	0.327
BP/24	0.258	BP/31	0.143
BP/25	0.593	BP/32	0.021
BP/26	0.085	BP/33	0.078
BP/27	0.558	BP/34	0.367
BP/28	0.310	BP/35	0.027

1 clinical blood sample obtained from a patient who was known to have been prescribed Seroxat® was analyzed. For this case the concentration of paroxetine detected was 0.048 mg/L. This patient had been prescribed 20 mg/day for a period of one year and this level is within the therapeutic range for this dose. Examples of chromatograms obtained for the paroxetine blood analyses are shown in Figures 8.10-8.12.

8.3.4 CONCLUSIONS

The developed method was successfully evaluated using clinical and postmortem blood samples. Fluoxetine, norfluoxetine and paroxetine levels determined in postmortem samples gave values ranging from therapeutic to overdose levels and in clinical samples only therapeutic levels were detected.

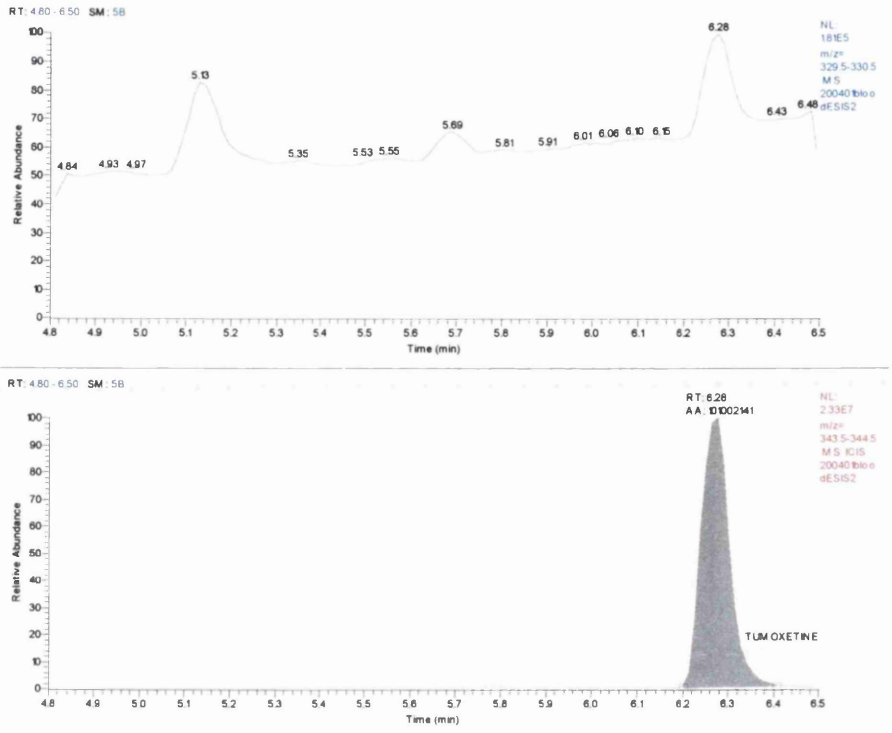


Figure 8.6: Chromatogram of extract of blank blood spiked with 100 ng/ml internal standard

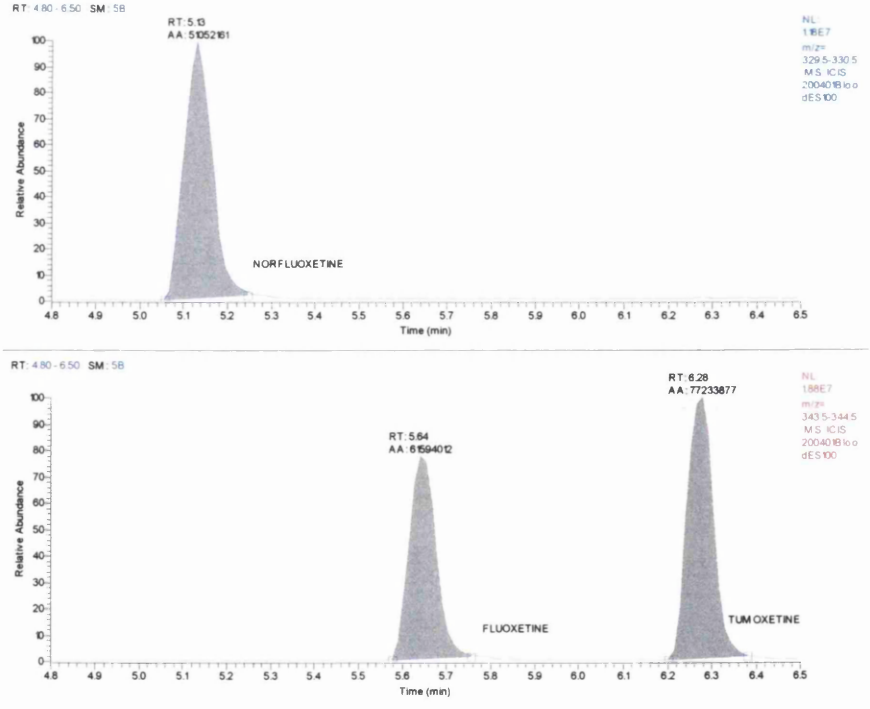


Figure 8.7: Chromatogram of extract of blood spiked to give 100 ng/ml fluoxetine and norfluoxetine

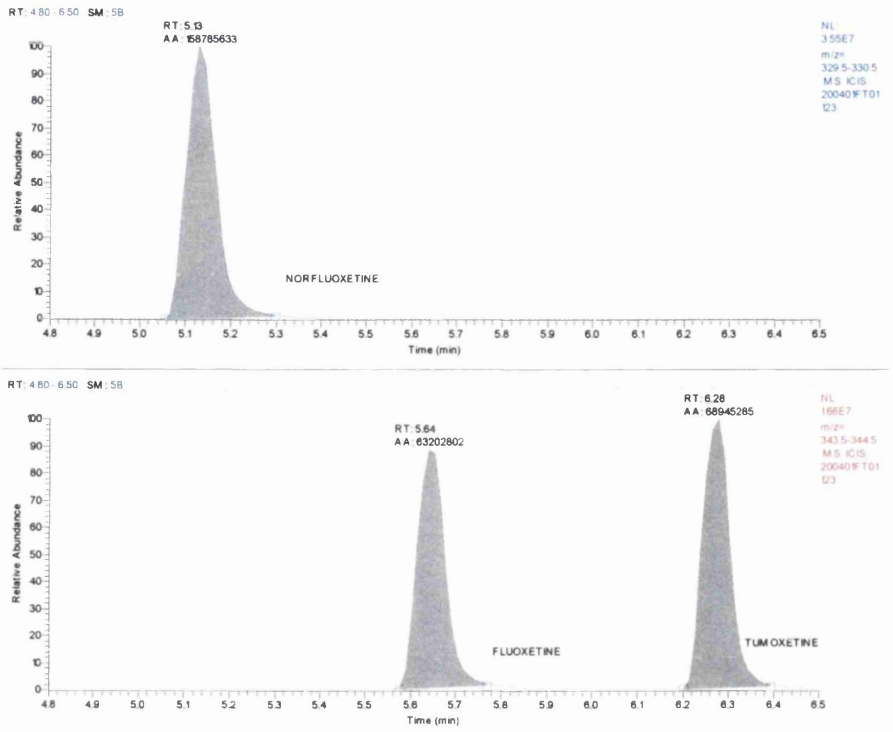


Figure 8.8: Chromatogram of extract of blood from Case BP/19

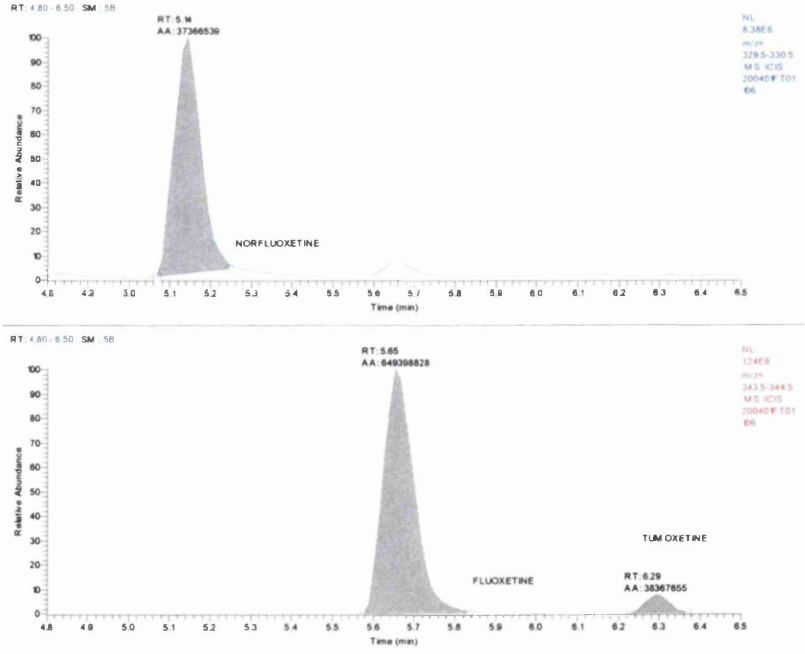


Figure 8.9: Chromatogram of extract of blood from Case BP/20

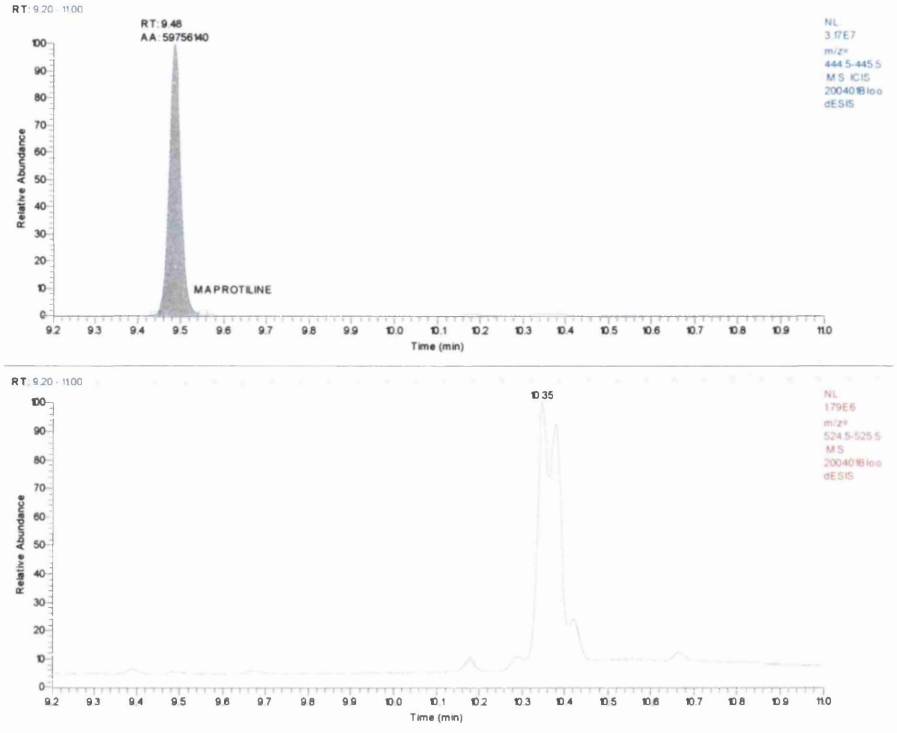


Figure 8.10: Chromatogram of extract of blank blood spiked with 50 ng/ml internal standard

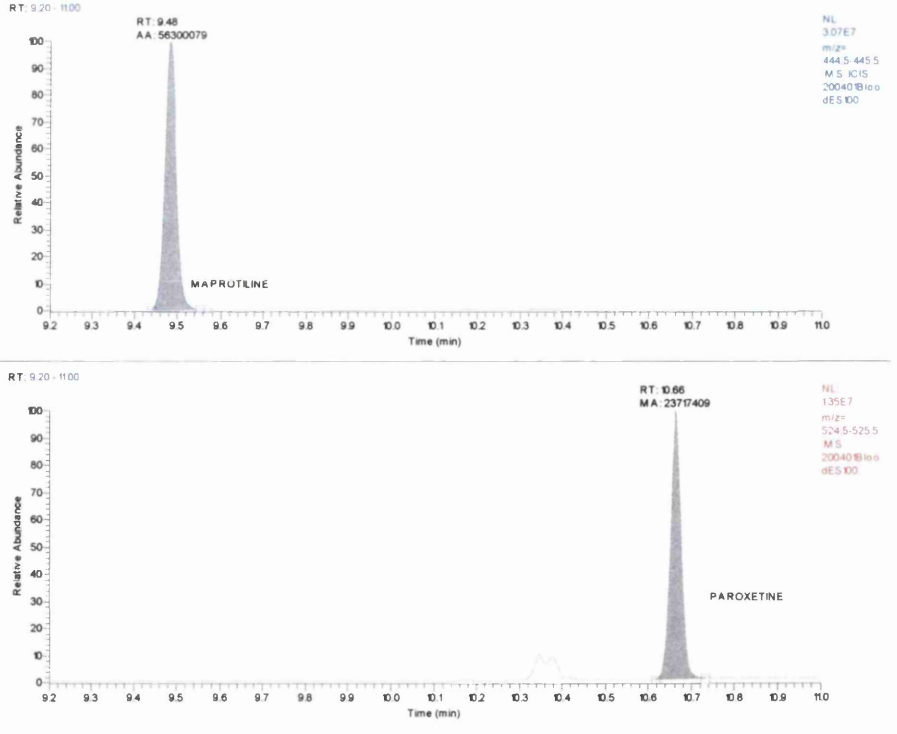


Figure 8.11: Chromatogram of extract of blood spiked to give 100 ng/ml paroxetine

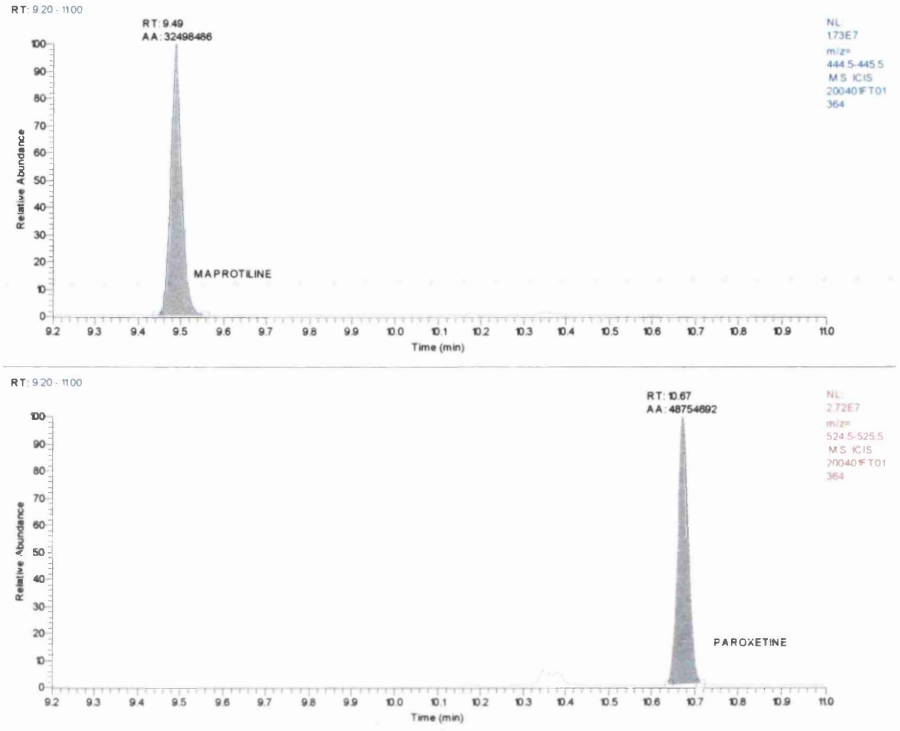


Figure 8.12: Chromatogram of extract of blood from case BP/34

8.4 ANTIDEPRESSANTS IN SALIVA

8.4.1 SAMPLE COLLECTION AND STORAGE

Saliva and blood samples were collected simultaneously where possible from patients attending clinics and geriatric patients who were receiving Prozac® or Seroxat® treatment for depression. Blood was collected by fully qualified medical staff by standard venipuncture procedure and saliva samples were collected using Omni-Sal® devices. Samples were transferred to the laboratory and stored at - 4 °C.

8.4.2 EXTRACTION OF SAMPLES

Blood and saliva samples were extracted using the validated method in Chapter 5 and analyzed by GC/MS.

8.4.3 PATIENT DETAILS

Twelve saliva samples were obtained from nine patients receiving treatment with Prozac®. One of these volunteers (SV/1) provided five samples over different days and the other seven provided one sample each. Only seven blood samples were collected from the patients simultaneously. One patient receiving Seroxat® treatment provided a saliva and blood sample. Information received from the volunteers where possible was age, sex, dosage of drug, length of time drug had been prescribed (Table 8.17), when the last dose was taken and information regarding other medications which may affect analysis.

Table 8.17: Patient details

Patient	Sex	Age	Drug prescribed	Dosage (mg/day)	Duration patient has been prescribed drug
SV/1	F	58	Fluoxetine	20	1 year
SV/2	F	56	Fluoxetine	40	2 years
SB/1	M	58	Fluoxetine	20	4 years
SB/3	F	41	Fluoxetine	20	3 months
SL/2	M	68	Fluoxetine	20	5 weeks
SL/3	M	56	Fluoxetine	20	10 weeks
SL/11	F	68	Fluoxetine	20	16 weeks
SL/12	M	88	Fluoxetine	20	1 week
SB/2	M	71	Paroxetine	20	1 year

8.4.4 RESULTS

8.4.4.1 Fluoxetine Saliva Samples

Fluoxetine and its metabolite were detected in all saliva samples collected by Omni-Sal® devices. The results of these and corresponding blood samples are given in Table 8.18. Fluoxetine concentrations in saliva were found to range from 22.1 - 223.2 ng/ml (mean 62.8 ng/ml) and norfluoxetine concentrations 11.0 - 143.3 ng/ml (mean 69.2 ng/ml). Out of these twelve cases, seven gave higher concentrations for the metabolite and five gave higher concentrations for the parent drug. In one case, there was a fluoxetine concentration of 223.2 ng/ml which was considerably higher than for other cases. It may have been suggested that this was due to oral contamination, however, the individual claimed not to have taken his medication in 24 hours. He had also been prescribed the drug for a longer period of time than the other volunteers and this may account for a certain amount of drug accumulation. It was postulated that this individual was in fact a poor metabolizer of fluoxetine.

Table 8.18: Fluoxetine in blood and saliva

Case Sample	Time lapse	Saliva pH	Blood (ng/ml)		Saliva (ng/ml)		Saliva/Blood	
			FLU	NFLU	FLU	NFLU	FLU	NFLU
SV/1a	5 hr	-	NA	NA	38.9	53.4	-	-
SV/1b	1 hr	-	NA	NA	57.5	83.4	-	-
SV/1c	NK	-	NA	NA	22.1	33.2	-	-
SV/1d	NK	-	NA	NA	35.9	44.5	-	-
SV/1e	NK	-	51.8	101.3	57.0	48.6	1.10	0.48
SV/2	NK	-	NA	NA	67.5	143.3	-	-
SB/1	24 hr	-	155.8	68.2	223.2	73.7	1.43	1.10
SB/3	3 hr	-	73.7	196.4	73.8	117.8	0.38	1.60
SL/2	1 hr	6.2	114	109	40.2	22.8	0.35	0.21
SL/3	1 hr	6.5	24	155	41.7	139.8	1.74	0.90
SL/12	2 hr	-	13.4	14.8	27.6	11.0	2.06	0.74

NK - Not Known, NA - Not Analyzed

Time lapse is the time between the patients last dose and the time the saliva sample was taken.

Saliva/blood ratios were calculated for six case samples and were found to vary from 0.35 - 2.06 for fluoxetine and 0.21 - 1.60 for norfluoxetine. These variations in ratios are a result of the differences in salivary pH of individuals. The pH of blood and saliva in an individual will dictate the amount of ionization a specific drug will have in each of these biological specimens. The theoretical range of saliva/blood ratios for fluoxetine can be calculated using the Henderson-Hasselbalch equation for basic drugs (Chapter 5.5). For fluoxetine the pK_a is 9.5. The non protein bound fraction of total drug in plasma, f_p is 0.06 for fluoxetine since 94 % of drug is plasma bound. Saliva/blood ratios were calculated for the saliva pH varying from 6.0 - 7.2. Using these values the theoretical saliva/blood ratio can vary from 1.495 - 0.095. Therefore a relatively small change in pH can result in a significant change in saliva/blood ratio. It can be noticed from these results that two of the experimental saliva/blood values fell out with this theoretical range.

The drug/metabolite ratios in blood and saliva for the six sets of corresponding samples ranged from 0.15 - 2.28 (mean 0.88) and 0.30 - 3.03 (mean 1.16) respectively. In each case the drug/metabolite ratio for saliva was always higher than that calculated for blood (Table 8.19).

Table 8.19: Fluoxetine/norfluoxetine ratios in blood and saliva

Case Sample	Drug/metabolite ratio	
	Blood	Saliva
SV/1a	-	0.73
SV/1b	-	0.69
SV/1c	-	0.67
SV/1d	-	0.81
SV/1e	0.51	1.17
SV/2	-	0.47
SB/1	2.28	3.03
SB/3	0.38	0.63
SL/2	1.05	1.76
SL/3	0.15	0.30
SL/12	0.90	2.51

8.4.4.2 Paroxetine Saliva Samples

One saliva sample containing paroxetine was collected. The time between administration of the drug and sample collection was approximately 12 hours and the amount detected in saliva was 31.5 ng/ml. A saliva/blood ratio of 0.65 was calculated. This volunteer had been prescribed the medication for 1 year.

Table 8.20: Paroxetine in blood and saliva

Patient/ Sample No.	Time lapse	Saliva pH	Blood (ng/ml)	Saliva (ng/ml)	Saliva/Blood Ratio
P/1	12.5 hr	-	48.3	31.5	0.65

The theoretical range of saliva/blood ratios for paroxetine was calculated using the Henderson-Hasselbalch equation for basic drugs (Chapter 5.5). For paroxetine, the pK_a is 9.9. The non protein bound fraction of total drug in plasma, f_p is 0.05 for paroxetine since 95 % of drug is plasma bound. The saliva/blood ratio was calculated for the saliva pH varying from 6.0 - 7.2. Using these values the theoretical saliva/blood ratio was found to vary from 1.252 - 0.079. The experimental saliva/blood value fell in between these limits.

8.4.5 EFFECT OF COLLECTION METHOD ON SALIVA FLUOXETINE CONCENTRATION

One 20 mg Prozac® tablet was administered to three healthy volunteers who had not previously taken this medication. Saliva samples were collected at intervals of 1, 3, 5, 7, 12, 24, 48, 72 hours by spitting and by collection with Omni-Sal® devices. These samples were analysed for fluoxetine and norfluoxetine. Tables 8.21 and 8.22 give the concentrations detected using the two collection methods.

Table 8.21: Fluoxetine saliva concentrations detected using Omni-Sal® devices

Time after administration (hours)	Volunteer 1 (ng/ml)		Volunteer 2 (ng/ml)		Volunteer 3 (ng/ml)	
	FLU	NFLU	FLU	NFLU	FLU	NFLU
1	0.11	-	0.16	-	0	-
3	0.33	-	1.54	-	0.1	-
5	1.06	0.28	2.79	-	0.69	-
7	2.0	0.28	4.06	0.21	0.84	-
12	1.7	0.47	3.67	0.24	0.57	-
24	1.37	1.26	2.44	0.30	0.48	0.15
48	1.0	2.47	1.55	0.32	0.41	0.38
72	0.28	1.32	1.0	0.35	0.40	0.78

Table 8.22: Fluoxetine saliva concentrations detected in spitting samples

Time after administration (hours)	Volunteer 1 (ng/ml)		Volunteer 2 (ng/ml)		Volunteer 3 (ng/ml)	
	FLU	NFLU	FLU	NFLU	FLU	NFLU
1	0.21	-	0	-	0	-
3	0.89	-	1.37	-	0	-
5	1.8	0.29	2.83	-	0.74	-
7	3.4	0.29	4.58	0.80	2.58	-
12	3.16	0.38	4.38	0.82	1.91	-
24	2.32	0.54	4.0	0.70	0.97	-
48	1.42	1.42	2.34	-	0.40	-
72	0.63	1.26	1.16	-	0.30	0.29

Peak saliva concentrations were reached at approximately 7 hours for the three volunteers. Concentrations obtained at 7 hours after administration varied for the three individuals and these ranged from 0.84 - 4.06 ng/ml using the Omni-Sal® devices. Norfluoxetine concentrations were not detected until at least 5 hours after administration. The fluoxetine graphs of concentration detected in saliva versus time are shown below in Figure 8.13 and 8.14.

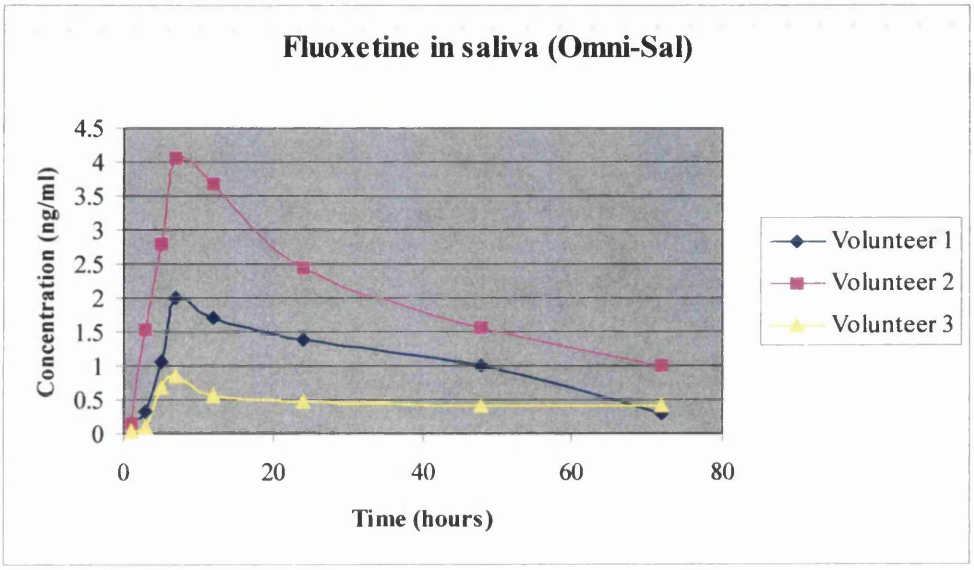


Figure 8.13: Graphs of concentration versus time for fluoxetine in saliva using Omni-Sal® collection method

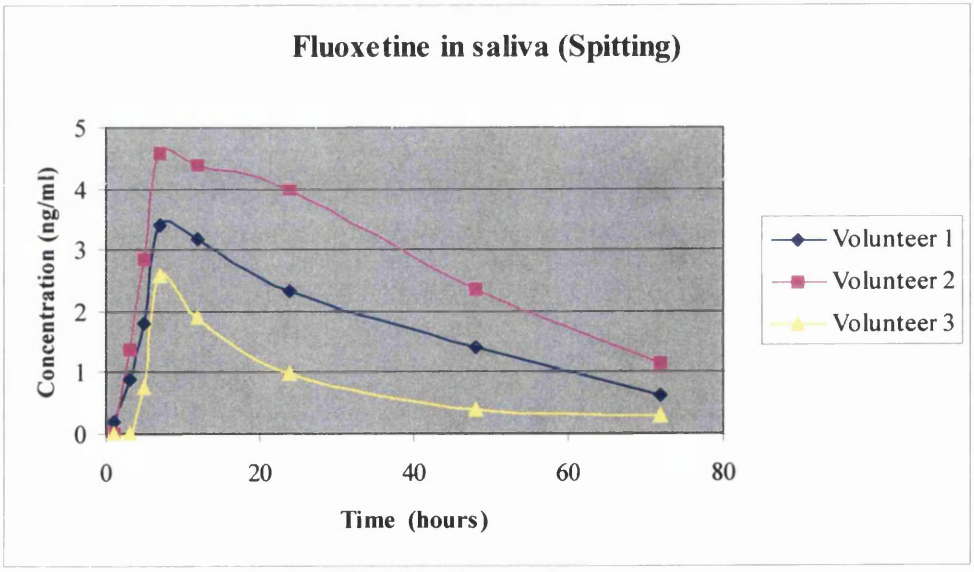


Figure 8.14: Graphs of concentration versus time for fluoxetine in saliva using spitting collection method

Peak saliva concentrations detected in whole saliva collected by spitting were higher than those detected with the Omni-Sal® devices. This can be explained by the lower recovery obtained from the Omni-Sal® cotton swab. The peak concentrations detected for the three volunteers varied from 2.58 - 3.4 ng/ml, with volunteer 2 still giving the highest peak concentration and volunteer 3 giving the lowest (Figure 8.14).

The saliva elimination half-life for fluoxetine was determined to be between 1 and 2 days for the three individuals by both collection methods. This is comparable to plasma the plasma elimination half-life which is between 1 and 3 days. [62]

8.4.6 EFFECT OF COLLECTION METHOD ON SALIVA PAROXETINE CONCENTRATION

One 20 mg Seroxat® tablet was administered to two healthy volunteers who had not previously taken this medication. Saliva samples were collected at intervals of 1, 3, 5, 7, 12, 24, 48, 72 hours by spitting and by collection with Omni-Sal® devices. These samples were analysed for paroxetine. Table 8.23 gives the concentrations detected using the two collection methods.

Table 8.23: Paroxetine saliva concentrations detected in saliva samples

Time after administration (hours)	Paroxetine in saliva (Omni-Sal®)		Paroxetine in saliva (Spitting)	
	Volunteer 1 (ng/ml)	Volunteer 2 (ng/ml)	Volunteer 1 (ng/ml)	Volunteer 2 (ng/ml)
1	2.53	0.65	3.89	3.06
3	0.71	0.16	1.67	0.42
5	0.52	0.65	0.83	4.17
7	0.99	0.52	1.81	4.31
12	0.71	0.26	0.97	1.69
24	0.1	0.11	0.28	0.69
48	0	0	0	0.28
72	0	0	0	0

Graphs of concentration versus time after administration for the two collection methods are shown below (Figures 8.15 and 8.16). These graphs differ from those obtained for the fluoxetine samples in that the highest concentration detected for each volunteer was observed one hour after administration. This concentration rapidly fell within 3 hours after administration and an increase in concentration was seen with peak levels obtained at 5 and 7 hours for the two individuals. This initial high concentration at one hour following administration is a result of oral contamination. The Seroxat® tablets unlike the Prozac® capsules do not have the protective capsule covering which seemed to prevent oral contamination.

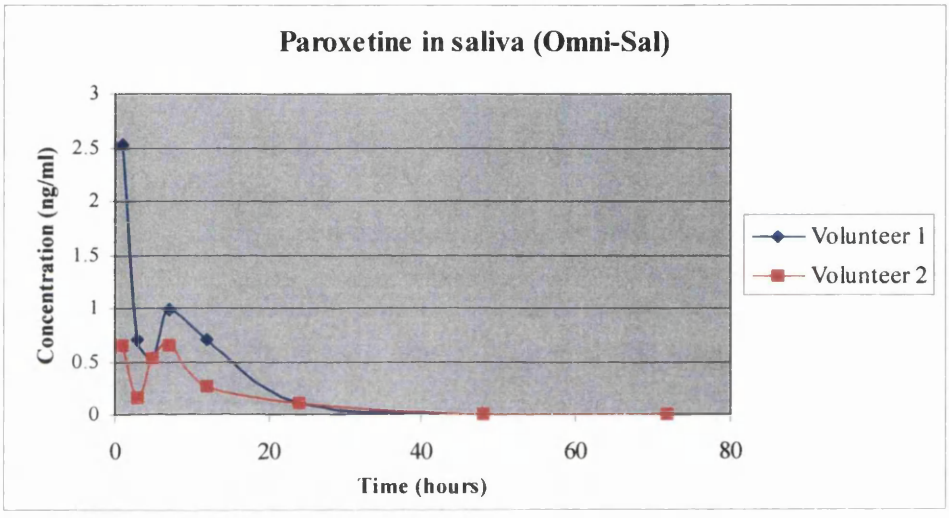


Figure 8.15: Graphs of concentration versus time for paroxetine in saliva using Omni-Sal® collection method

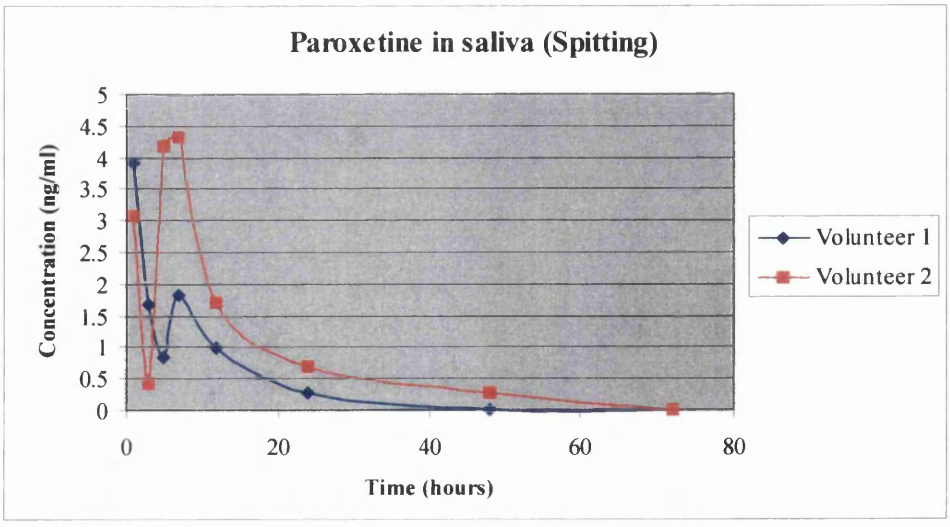


Figure 8.16: Graphs of concentration versus time for paroxetine in saliva using spitting collection method

Similarly to the fluoxetine study, peak saliva paroxetine concentrations in saliva collected by spitting were higher than those detected with the Omni-Sal® devices. The peak concentrations detected for the two volunteers by Omni-Sal® collection were 0.99 and 0.65 ng/ml and by spitting collection were 1.81 and 4.31 ng/ml respectively. Figures 8.17 and 8.18 show this difference in concentration.

The apparent saliva elimination half-life for paroxetine was 5 - 8 hours. Plasma elimination half-lives have been reported as 7 - 37 hours.[62]

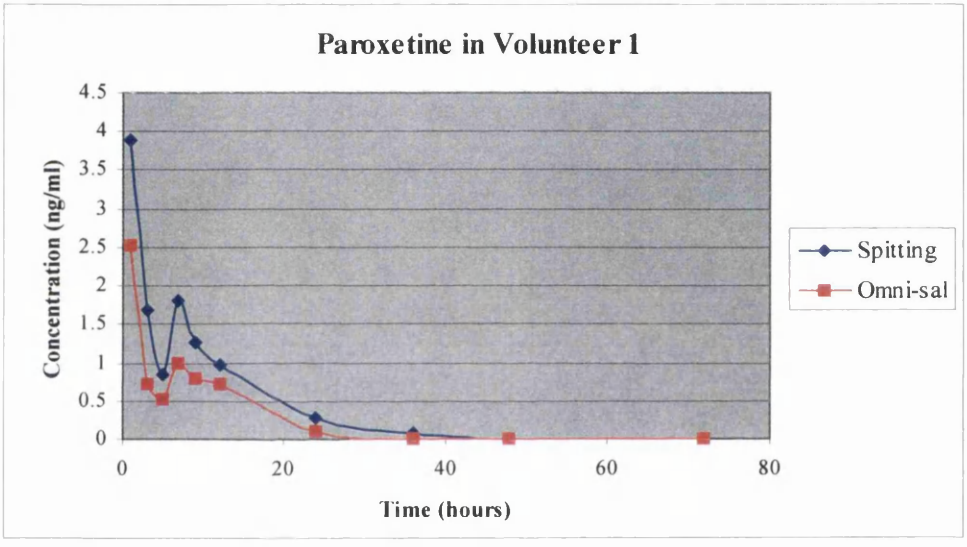


Figure 8.17: Graphs of concentration versus time for paroxetine in volunteer 1

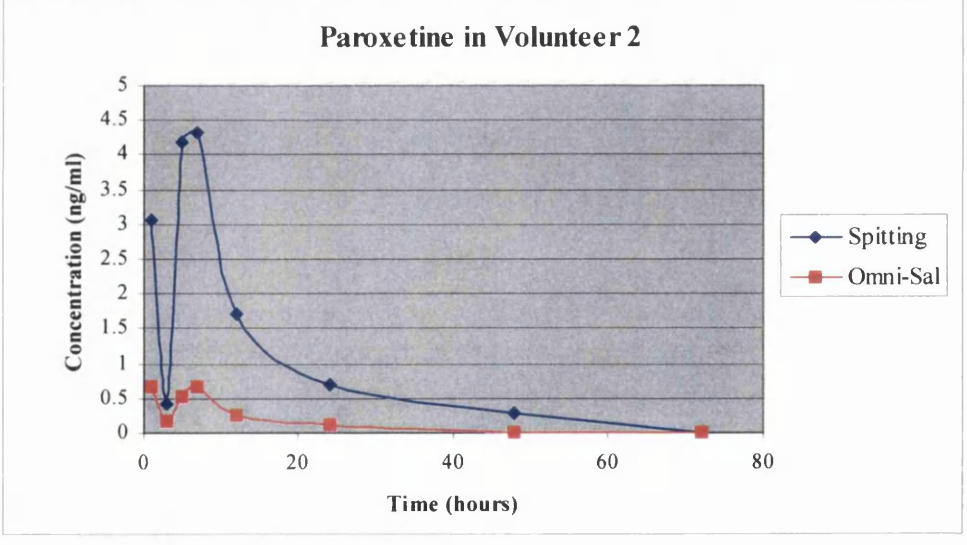


Figure 8.18: Graphs of concentration versus time for paroxetine in volunteer 2

Examples of chromatograms obtained for the fluoxetine and paroxetine saliva analyses are shown in Figures 8.19-8.24.

8.4.7 CONCLUSIONS

Fluoxetine and norfluoxetine or paroxetine were detected in the saliva samples obtained from patients receiving chronic treatment with Prozac® or Seroxat®. Saliva/blood ratios for fluoxetine and norfluoxetine were shown to vary between individuals as a result of differences in salivary pH. Fluoxetine/norfluoxetine ratios in blood and saliva within an individual were always shown to be higher in saliva than in blood.

Saliva samples obtained from volunteers taking a single 20 mg dose of Prozac® or Seroxat® were collected by spitting and by Omni-Sal® devices. Concentrations in saliva were found to vary between the individuals. Peak concentrations were found to be less than 5 ng/ml and were obtained at 7 hours for fluoxetine and 5 - 7 hours for paroxetine. The elimination half-lives were 1 - 2 days for fluoxetine and approximately 5 - 8 hours for paroxetine.

Concentrations were always found to be higher in the samples collected by spitting because of the lower recovery obtained using the Omni-Sal® collection devices. At these low concentrations the collection method is important because it could mean the difference between detecting and not detecting a substance especially in cases where a drug has been administered as a single dose. However, for the majority of people being prescribed these drugs, chronic administration is necessary and in these cases the concentrations are easily detected using the Omni-Sal® collection devices.

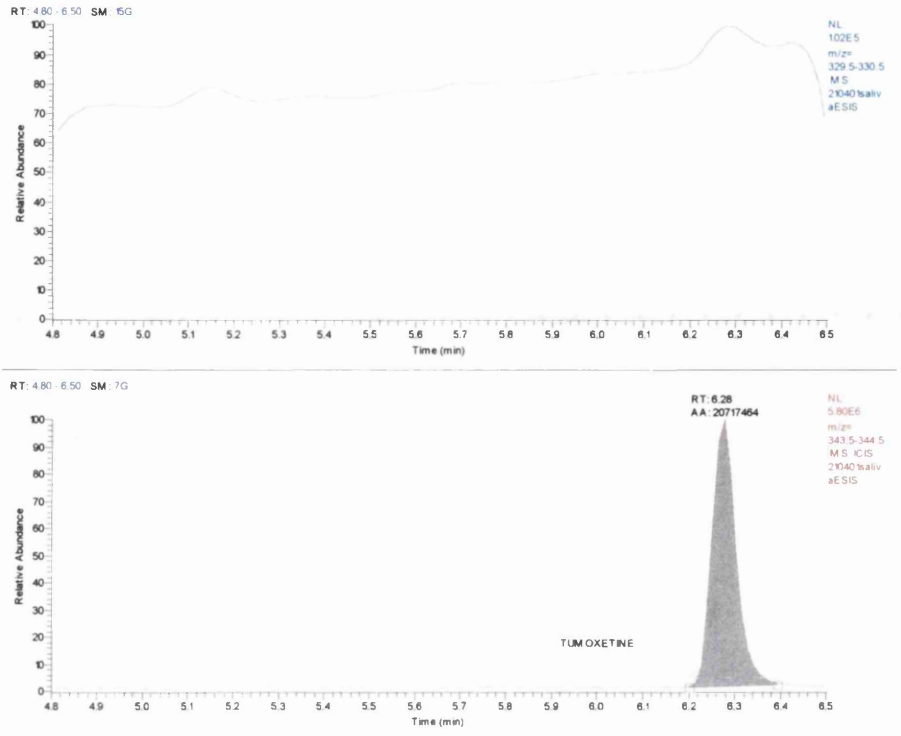


Figure 8.19: Chromatogram of extract of saliva spiked with 50 ng/ml tumoxetine

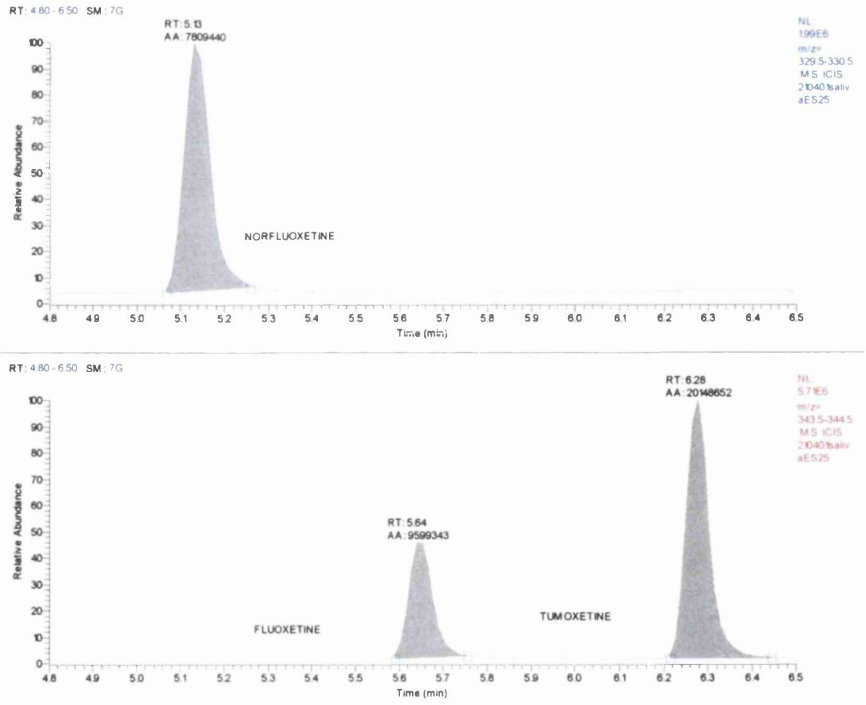


Figure 8.20: Chromatogram of extract of saliva spiked with 25 ng/ml fluoxetine and norfluoxetine

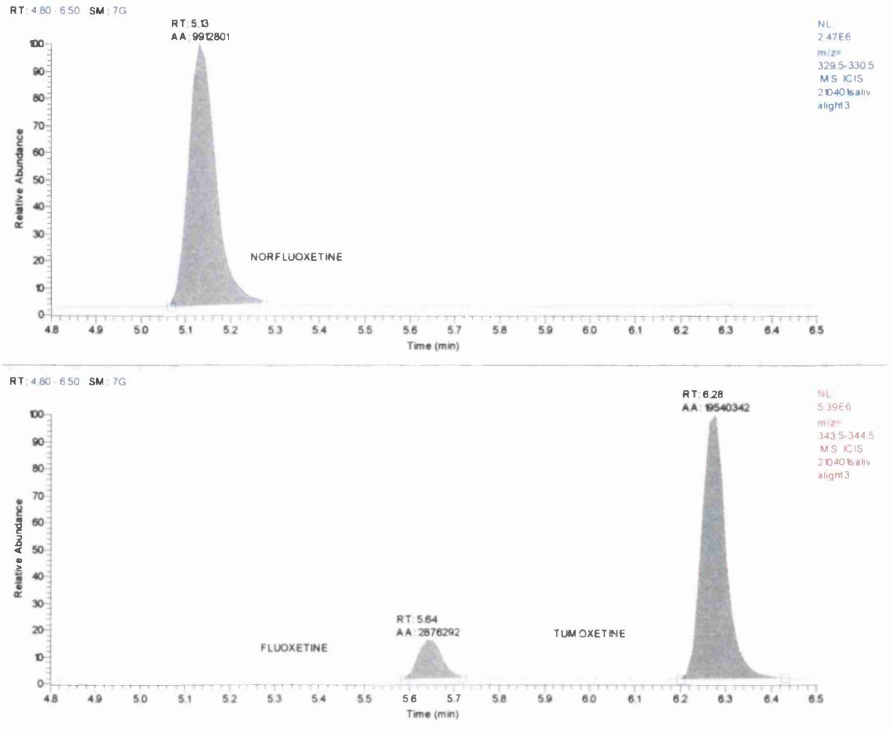


Figure 8.21: Chromatogram of extract of saliva case sample SL/03

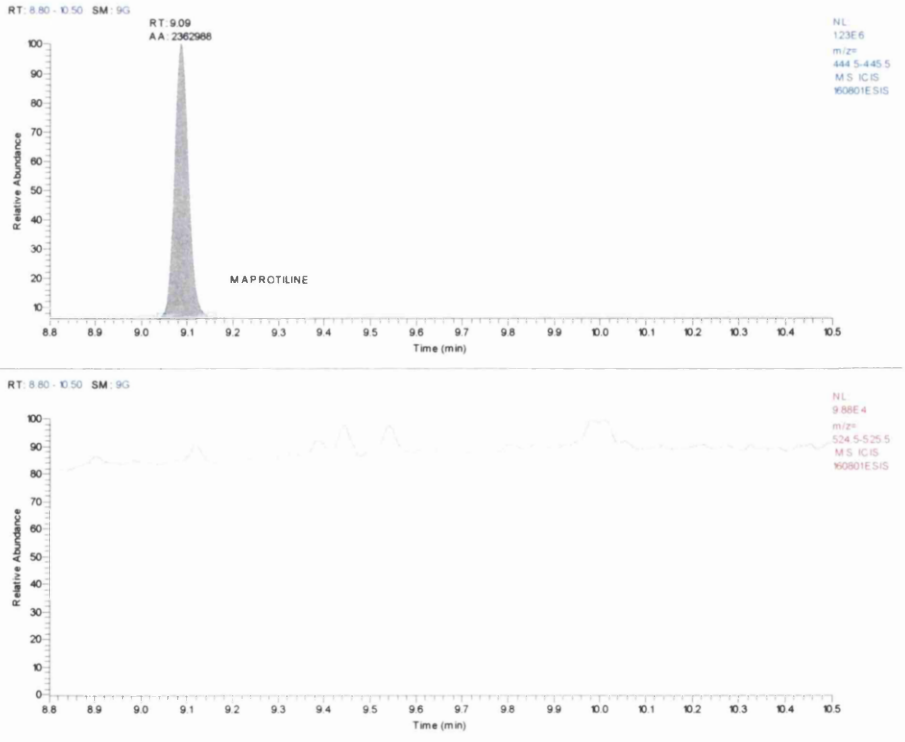


Figure 8.22: Chromatogram of extract of saliva spiked with 25 ng/ml maprotiline

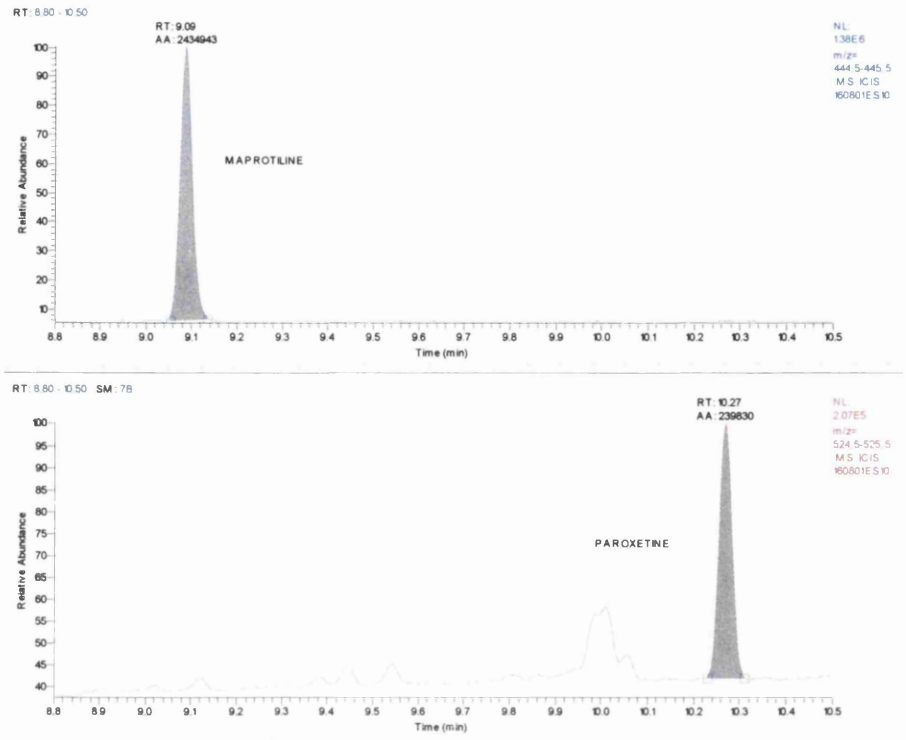


Figure 8.23: Chromatogram of extract of saliva spiked with 10 ng/ml paroxetine

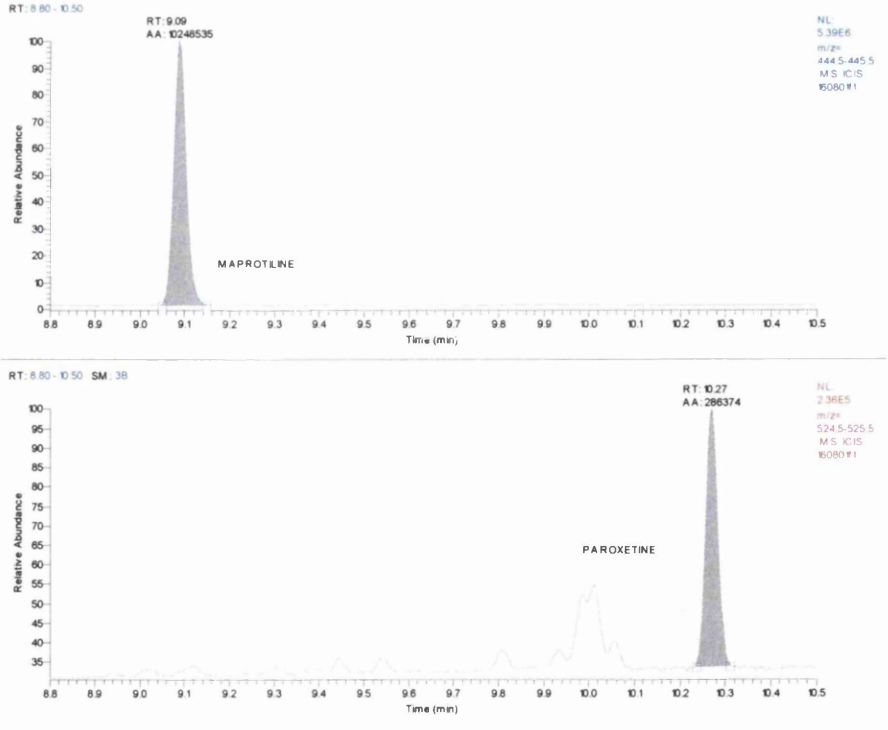


Figure 8.24: Chromatogram of extract of saliva sample obtained from volunteer 1, one hour after 20 mg single dose

8.5 ANTIDEPRESSANTS IN HAIR

8.5.1 POSTMORTEM CASES

Certain segments of postmortem hair samples HF/1 and HP/1 were extracted by methanol extraction and acid hydrolysis by the method described in 6.4.7 and compared in a recovery validation experiment. The other segments of these samples were extracted using acid hydrolysis and the results are tabulated in Tables 8.24 and 8.25.

8.5.2 RESULTS AND DISCUSSION

Fluoxetine and norfluoxetine were detected in all the segments of case HF/1. The concentration of fluoxetine was found to increase from the root to the second segment and thereafter it decreased with length of hair. The concentration of norfluoxetine along the length behaved similarly, increasing from root to first segment and thereafter decreasing with length. Assuming that fluoxetine was used regularly over the period of hair growth, then this decrease in concentration is most likely due to drug leaching from hair as a result of damage from bleaching. Bleaching hair causes the hair to become more porous and therefore the possibility of drugs being removed from the hair by washing is greater. The concentrations of cocaine, benzoylecgonine, methylecgonine, codeine, morphine and 6-MAM in the hair of a drug user were shown to be a third or less in bleached hair than in natural hair.[222]

In a period of five months, the fluoxetine hair concentration was found to decrease by 50 %. A similar decrease in concentration was reported for the drug methoxyamphetamine in natural hair.[16]

The concentration of metabolite, norfluoxetine was higher than fluoxetine for the first four segments. Steady state concentrations of fluoxetine and norfluoxetine in blood will affect the levels which are detected in hair. At steady state, concentrations of norfluoxetine in plasma are generally higher than that of fluoxetine and these do not vary greatly within an individual when the drug is administered for a long time. Factors affecting the incorporation of a drug into hair include the lipophilicity of the drug. It may be expected that chemically nonpolar parent drugs are more likely to be successful at crossing the cell membrane from blood capillary to hair follicle and in this case would imply that fluoxetine would incorporate more easily than norfluoxetine. However, if steady state concentrations in plasma are higher for norfluoxetine this may compensate for the decrease in

incorporation compared to fluoxetine and produce a higher concentration of norfluoxetine in hair. Out of 43 cases determining tricyclic antidepressants, amitriptyline, clomipramine, doxepine and imipramine in hair only three showed a higher concentration of nor-metabolite in hair than the parent drug.[181] The parent drug was most frequently at a higher concentration than the metabolite and this was explained by the polar metabolites having a higher affinity to bind to the hair matrix than the parent drug. For the remaining twelve segments of hair it was found that fluoxetine concentrations were greater than norfluoxetine concentrations. The rate of decrease in fluoxetine concentration with length was also less than the rate of decrease of norfluoxetine with length. The parent drug/metabolite ratios were calculated for the length of the hair and these were found to increase with length. The bleaching of the hair seems to not only have caused a loss of drug, but also appeared to cause a larger loss of the metabolite compared to the parent drug as the section of hair got further away from the root. This is shown in Figure 8.25.

Table 8.24: Findings for case HF/1

Segment	Weight (mg)	Fluoxetine (ng/mg)	Norfluoxetine (ng/mg)	Drug/Metabolite Ratio
Roots	9.16	3.69	10.08	0.37
0.5-1.5	9.35	5.55	10.92	0.51
1.5-2.5	9.07	6.35	8.41	0.76
2.5-3.5	17.0	4.78	4.95	0.96
3.5-4.5	18.81	3.06	3.14	0.97
4.5-5.5	19.78	2.26	2.04	1.11
5.5-6.5	16.78	2.00	1.69	1.18
6.5-7.5	17.25	1.72	1.24	1.39
7.5-8.5	19.47	1.54	1.16	1.33
8.5-9.5	17.86	1.42	0.91	1.56
9.5-10.5	18.19	1.22	0.70	1.75
10.5-11.5	16.97	1.26	0.72	1.75
11.5-12.5	16.27	1.16	0.60	1.92
12.5-13.5	18.96	1.03	0.44	2.33
13.5-14.5	15.45	1.00	0.43	2.33
14.5-15.5	14.46	1.06	0.47	2.27
15.5-16.5	14.62	1.20	0.64	1.89

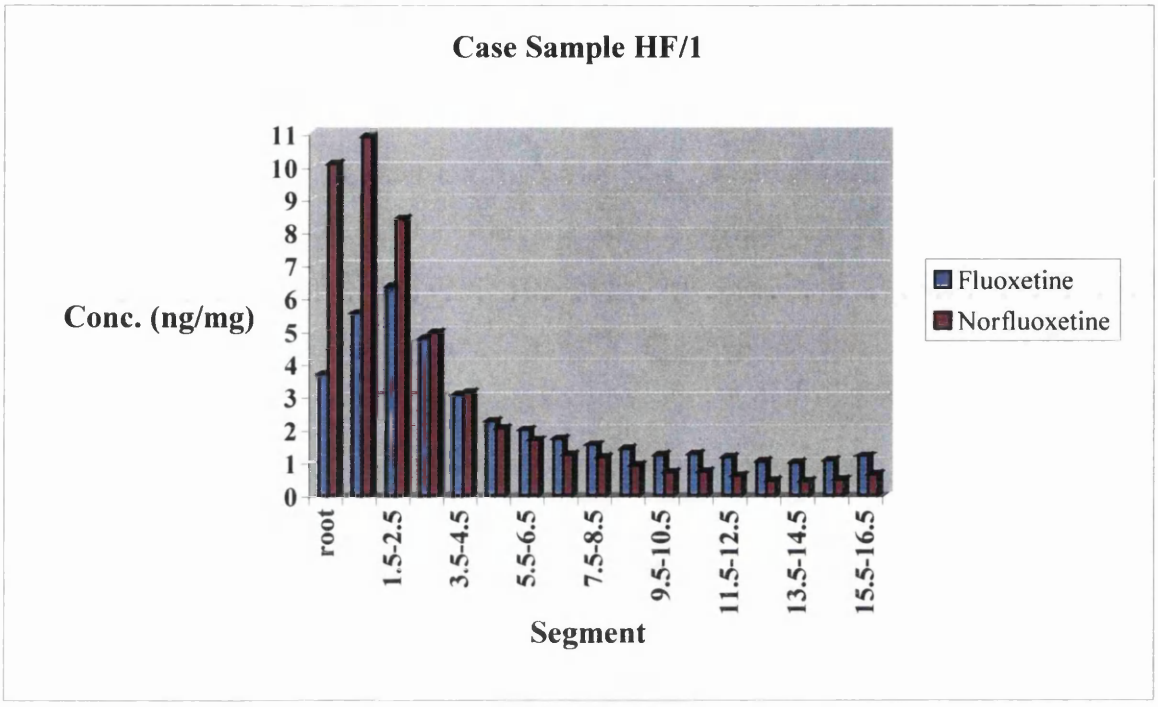


Figure 8.25: Graph showing concentrations of fluoxetine and norfluoxetine along length of hair sample HF/1.

Paroxetine was detected in case HP/1. The concentration was highest in the root section and was found to decrease in the following section and was not detected in the second or following segments. A medical history was not available to check how long the individual had been taking paroxetine, but from the hair results it appears that paroxetine treatment could only have been for about two months (Table 8.25).

Table 8.25: Findings for case HP/1

Segment	Weight (mg)	Paroxetine (ng/mg)
Roots	4.82	4.32
0.5-2.5	9.70	0.43
2.5-11.5	Approx. 10 mg, 2 cm sections	- ve

8.5.3 CLINICAL SAMPLES

Hair samples were obtained from volunteers receiving fluoxetine or paroxetine treatment. The patients were either in a geriatric hospital or attending clinics. Hair was cut from the posterior vertex as close to the scalp as possible. It was then wrapped in cigarette papers, the root end labelled and inserted into a plastic universal. A total of 19 samples were collected and information including the age, sex, hair colour, dosage and length of time the drug had been prescribed was recorded. This information is tabulated in Table 8.26.

8.5.4 RESULTS

19 hair samples were taken from patients receiving fluoxetine or paroxetine treatment. These samples were segmented into 60 sections. Of the 19 hair samples, 14 (73.7 %) were found positive for fluoxetine, 14 (73.7 %) for norfluoxetine, 4 (21.0%) for paroxetine and one (5.3 %) was found entirely negative for these drugs. The results of these are tabulated in Tables 8.27 - 8.29.

Concentration ranges detected in the segments were 0.34 - 5.52 ng/mg for fluoxetine, 0.15 - 8.09 ng/mg for norfluoxetine and 0.22 - 6.23 ng/mg for paroxetine. The fluoxetine/norfluoxetine concentration ratio was determined to vary from 0.30 - 3.85 (mean 1.33). In all cases where fluoxetine was detected, the metabolite was also detected. Out of the 27 sections found positive for fluoxetine and norfluoxetine, 15 (55.6 %) gave a parent drug to metabolite ratio > 1 and 12 (44.4 %) gave values < 1.

Table 8.26: Patient details

Case Number	Sex / age (Years)	Drug	Dosage mg/day	Length of time Prescribed *	Hair Colour
HB/1	M / 58	Fluoxetine	20	4 years	Dark grey
HB/2	M / 71	Paroxetine	20	1 year	White
HB/3	F / 41	Fluoxetine	20	3 months	Dark brown
HL/1	M / 71	Fluoxetine	20	10 weeks, stopped 5 weeks prior	White/grey
HL/2	M / 68	Fluoxetine	20	6 weeks	Grey
HL/3	M / 56	Fluoxetine	20	10 weeks	Black
HL/4	F / 71	Fluoxetine	20	4 weeks	Grey/white
HL/5	M / 74	Fluoxetine	20	6 weeks	Grey
HL/6	F / 89	Fluoxetine	20	2 days	White/few black
HL/7	F / 89	Fluoxetine	20	6 weeks	White
HL/8	M / 80	Fluoxetine	20	5 weeks	Grey
HL/9	M / 60	Fluoxetine	20	12 weeks	Grey
HL/10	F / 68	Fluoxetine	20	16 weeks	Dark grey
HL/11	F / 68	Paroxetine	20	16 weeks	Grey
HL/12	M / 88	Fluoxetine Paroxetine	20 20	1 week 5 days, stopped 6 weeks prior	White/few black
HL/13	F / 83	Fluoxetine	20	4 weeks	Black/few white
HL/14	F / 86	Fluoxetine	20	9 weeks	White
HL/15	M / 72	Fluoxetine	20	3 days	Light grey/white
HD/1	F / 32	Fluoxetine	20	3 months	Brown

* Length of time drug was prescribed previous to hair being sampled unless stated otherwise.

Table 8.27: Fluoxetine, norfluoxetine and paroxetine findings in hair case samples

Case sample	Fluoxetine (ng/mg)	Norfluoxetine (ng/mg)	Fluoxetine/ Norfluoxetine	Paroxetine (ng/mg)
HB/1 Scalp-1 cm	5.52	1.64	3.37	- ve
HB/1 1 - 2 cm	5.7	1.48	3.85	- ve
HB/2 Scalp-1 cm	- ve	- ve	-	1.48
HB/3 Scalp-1 cm	5.38	7.24	0.74	- ve
HB/3 1 - 2 cm	5.53	6.94	0.80	- ve
HB/3 2 - 3 cm	3.18	3.37	0.94	- ve
HL/1 Scalp-1 cm	3.02	7.55	0.40	- ve
HL/1 1 - 2 cm	2.83	5.33	0.53	- ve
HL/2 Scalp-1 cm	1.51	1.42	1.06	- ve
HL/2 1 - 2 cm	0.22	0.15	1.47	- ve
HL/2 2 - 3 cm	- ve	- ve	-	- ve
HL/3 Scalp-1 cm	2.58	8.09	0.32	- ve
HL/3 1 - 2 cm	2.09	6.90	0.30	- ve
HL/3 2 - 3 cm	0.97	2.80	0.35	- ve
HL/4 Scalp-1 cm	0.81	1.35	0.60	- ve
HL/4 1 - 2 cm	- ve	- ve	-	- ve
HL/4 2 - 3 cm	- ve	- ve	-	- ve

Table 8.28: Fluoxetine, norfluoxetine and paroxetine findings in hair case samples

Case sample	Fluoxetine (ng/mg)	Norfluoxetine (ng/mg)	Fluoxetine/ Norfluoxetine	Paroxetine (ng/mg)
HL/5 Scalp-1 cm	4.30	3.56	1.21	- ve
HL/5 1 – 2 cm	1.95	1.14	1.71	- ve
HL/6 Scalp-1 cm	- ve	- ve	-	- ve
HL/6 1 – 2 cm	- ve	- ve	-	- ve
HL/6 2 - 3 cm	- ve	- ve	-	- ve
HL/6 3 - 4 cm	- ve	- ve	-	- ve
HL/6 4 - 5 cm	- ve	- ve	-	- ve
HL/6 5 – 6 cm	- ve	- ve	-	- ve
HL/7 Scalp-1 cm	0.57	0.56	1.05	- ve
HL/7 1 – 2 cm	- ve	- ve	-	- ve
HL/7 2 - 3 cm	- ve	- ve	-	- ve
HL/8 Scalp-1 cm	1.81	0.99	1.83	- ve
HL/8 1 – 2 cm	- ve	- ve	-	- ve
HL/8 2 - 3 cm	- ve	- ve	-	- ve
HL/9 Scalp-1 cm	3.51	1.45	2.42	- ve
HL/9 1 – 2 cm	2.13	0.95	2.25	- ve
HL/9 2 - 3 cm	0.45	0.42	1.07	- ve
HL/10 Scalp-1 cm	1.23	1.03	1.19	- ve
HL/10 1 – 2 cm	0.91	0.30	3.03	- ve
HL/10 2 - 3 cm	0.80	0.45	1.78	- ve

Table 8.29: Fluoxetine, norfluoxetine and paroxetine findings in hair case samples

Case sample	Fluoxetine (ng/mg)	Norfluoxetine (ng/mg)	Fluoxetine/ Norfluoxetine	Paroxetine (ng/mg)
HL/11 Scalp-1 cm	- ve	- ve	-	5.73
HL/11 1 – 2 cm	- ve	- ve	-	6.23
HL/12 Scalp-1 cm	- ve	- ve	-	- ve
HL/12 1 – 2 cm	- ve	- ve	-	0.22
HL/12 2 - 3 cm	- ve	- ve	-	0.79
HL/12 3 - 4 cm	- ve	- ve	-	1.59
HL/13 Scalp-1 cm	0.47	0.51	0.93	- ve
HL/13 1 - 2 cm	- ve	- ve	-	- ve
HL/13 2 - 3 cm	- ve	- ve	-	- ve
HL/13 3 - 4 cm	- ve	- ve	-	- ve
HL/13 4 - 5 cm	- ve	- ve	-	- ve
HL/14 Scalp-1 cm	1.85	1.97	0.94	- ve
HL/14 1 - 2 cm	0.34	0.33	1.03	- ve
HL/14 2 - 3 cm	- ve	- ve	-	- ve
HL/15 Scalp-1 cm	- ve	- ve	-	0.29
HL/15 1 - 2 cm	- ve	- ve	-	0.41
HD/1 Scalp-1 cm	0.48	0.63	0.76	- ve
HD/1 1 - 2 cm	- ve	- ve	-	- ve
HD/1 2 - 3 cm	- ve	- ve	-	- ve
HD/1 3 - 4 cm	- ve	- ve	-	- ve
HD/1 4 - 5 cm	- ve	- ve	-	- ve
HD/1 4 - 5 cm	- ve	- ve	-	- ve

8.5.5 CORRELATION OF HAIR SAMPLES WITH MEDICAL HISTORY

The majority of hair samples provided positive or negative results which correlated with information obtained from patients' medical records. Hair growth was estimated to be 1.0 cm/month. In cases HL/2 and HL/5 where fluoxetine had been prescribed for 6 weeks, it would be expected to find the drug in the first 1.5 cm from the scalp if the growth rate was 1.0 cm/month. Fluoxetine was in fact detected in two sections of hair for these cases. The section closest to the scalp contained a higher concentration of the drug and metabolite than the second centimetre section which would be expected. However, in one other case where fluoxetine had been prescribed for 6 weeks, the drug was only detected in the first one centimetre section. Reasons for this could include the hair sample not having been cut as close as possible to the scalp or simply that hair growth was slower for this individual compared to the average individual. It should also be noted that hair growth is slower in the elderly and this hair sample was obtained from an individual who was 89 years old, 21 and 15 years older than the individuals who supplied hair samples HL/2 and HL/5 respectively.

In two cases, HL/12 and HL/15 where fluoxetine was prescribed 5 and 3 days respectively prior to sampling no fluoxetine or metabolite was detected in the hair. This is most likely because the section of hair where the drug was incorporated was not present. The sample may not have been cut close enough to the scalp or the hair had not had sufficient time to grow. In the first of these cases, however, paroxetine was detected in sections 2, 3 and 4 from the scalp. Information in the supplied medical cards only indicated that paroxetine had been taken for 5 days, 6 weeks prior to sampling and there was no drug information in the medical cards regarding months prior to this. The hair sample, however, indicated that the patient had been taking paroxetine for at least 2 months. In case HL/15 paroxetine was also detected in the two centimetres of hair closest to the scalp. In the centimetre section closest to the scalp the concentration detected was less than that detected in the second centimetre section indicating that the treatment had been ceased for approximately one to two weeks prior to sampling. This information was not available from the available medical cards.

The only other case where medical information did not correlate directly with the results was case HD/1. In many of the other cases, hair was obtained from geriatric patients who

were supervised when given medication to ensure it was taken regularly. This sample, however, was obtained from a heroin drug user and the hair results showed that this individual had not taken the medication as instructed by a general practitioner. This individual had been prescribed Prozac® for 3 months, but fluoxetine and its metabolite were only detected in the centimetre section closest to the scalp.

All other hair samples appeared to give a reasonable correlation with the medical history of each individual.

In cases where fluoxetine had been taken for a period of 8 weeks or longer (HB/1, HB/3, HL/1 and HL/3) it was shown that the concentrations detected in the two centimetre sections were similar indicating that the amount of drug incorporated into hair was consistent within an individual. These samples also showed that the drug/metabolite ratio was constant within an individual. The amount of drug incorporated into hair, however, varied greatly between individuals despite them being administered the same amount of drug. Also, the drug/metabolite ratios widely varied and therefore such an analysis would not be of value for compliance monitoring.

8.5.6 HAIR COLOUR

All of the hair samples were taken from patients receiving the same quantity of drug on a daily basis. However, the quantities of drug detected in the different hair samples were found to vary. This is largely due to inter-individual differences such as hair growth rate, the stage of hair growth and possibly also hair colour or the quantity of pigment in the hair. The majority of samples were obtained from elderly patients and therefore there was less variation in hair colour than within a younger population. However, hair colour and the concentrations detected in the hair were assessed for this group of samples.

One of the problems with assessing hair colour is the subjective nature of such a study. The majority of the samples collected were obtained from geriatric patients many of which contained a mixture of white and/or grey and/or black hairs in different shades and proportions. The assessment of proportions of colours and shade of colour is very subjective and for this reason the concentration of drug detected in hair was compared for

three groups of hair. The first of these groups was hair which was completely white in colour, the second group included hair which contained any grey hairs and the third group hair which was brown or black in colour. None of the samples obtained were blond or red in colour.

As a result of the large variation in drug/metabolite ratios between individuals, the total amount of fluoxetine and norfluoxetine detected in an individual's hair was calculated. Mean values of these total concentrations were also calculated for each group. These results showed that white hair incorporated the least amount of drug. Also, hair which had a proportion of grey hair in it incorporated a larger amount of drug than white hair, but less than that incorporated into brown or black hair (Figure 8.26).

There were insufficient paroxetine case samples to assess hair colour and concentration adequately. However, one sample containing a large proportion of black hair with a small proportion of grey was found to have a much higher concentration of paroxetine than white hair.

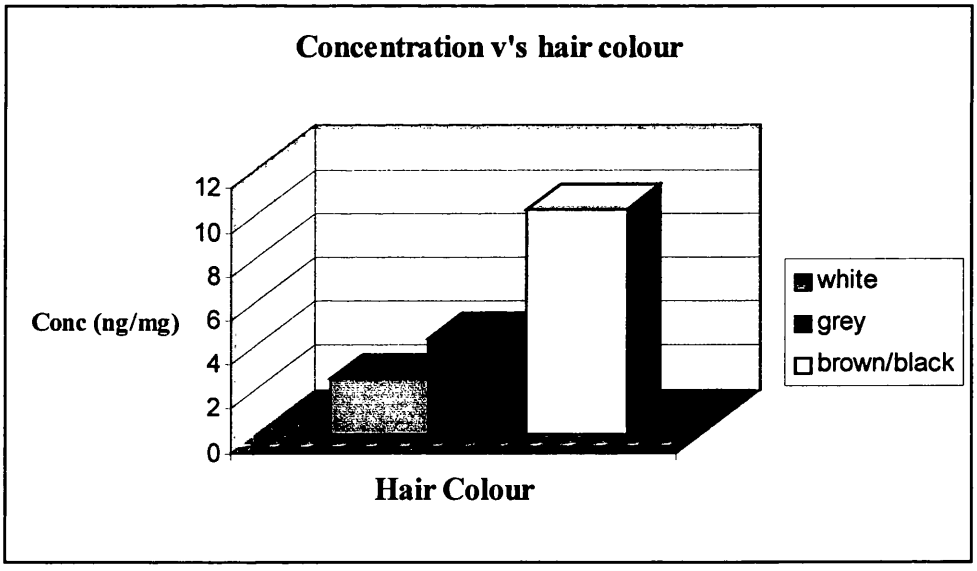


Figure 8.26: Mean concentrations of total fluoxetine and norfluoxetine in different hair colours

Examples of chromatograms obtained for extraction of fluoxetine and paroxetine from hair are shown in Figures 8.27-8.32.

8.5.7 CONCLUSIONS

The developed extraction method easily detected fluoxetine and paroxetine in the hair of users and it was found that the majority of hair cases tested for these drugs gave results which corresponded well with patients' case notes. The concentrations of the drugs detected in the various hair samples showed variation between individuals, not only in the amounts found, but in the case of fluoxetine, also in the ratio of parent drug/metabolite. The ratio of fluoxetine to norfluoxetine was found to vary from 0.30 - 3.85 and a preference for incorporation of one or other into hair was not highlighted. Other factors such as bleaching and washing of hair clearly affected this ratio. Hair colour or amount of pigment in hair appeared to affect the amount of drug incorporated into the hair. The results obtained indicated that dark hair incorporated more drug than grey hair which in turn incorporated more than white hair. This corresponds with other studies investigating the incorporation of drugs into hair.

Concentrations detected within an individual who had taken fluoxetine over two or three months were found to be relatively constant, indicating that incorporation is constant within an individual as is the parent drug to metabolite ratio. Factors which affected this included bleaching of hair.

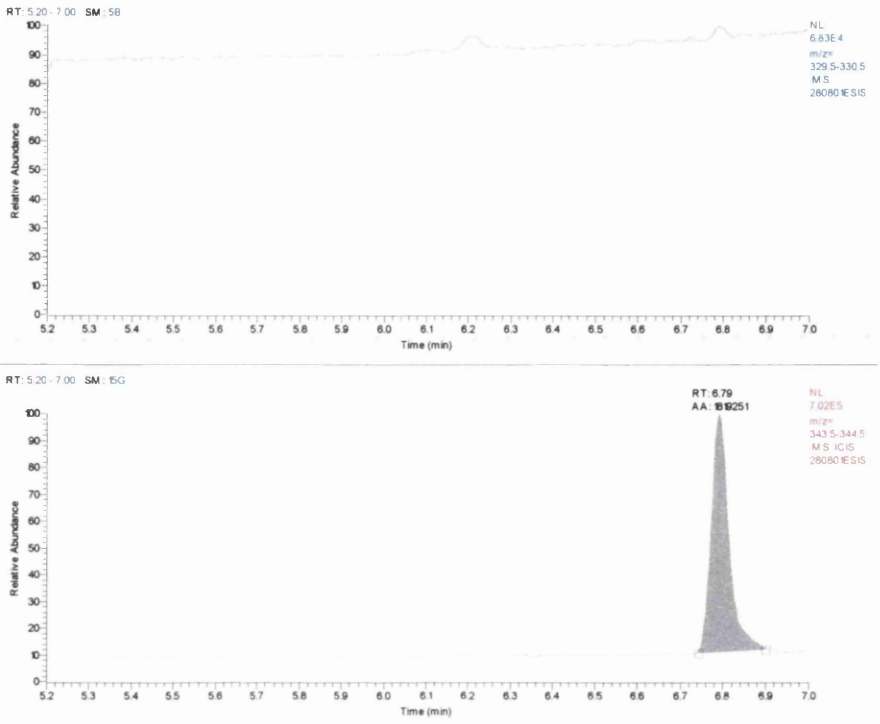


Figure 8.27: Chromatogram of extract of hair spiked with 50 ng/30 mg tumoxetine

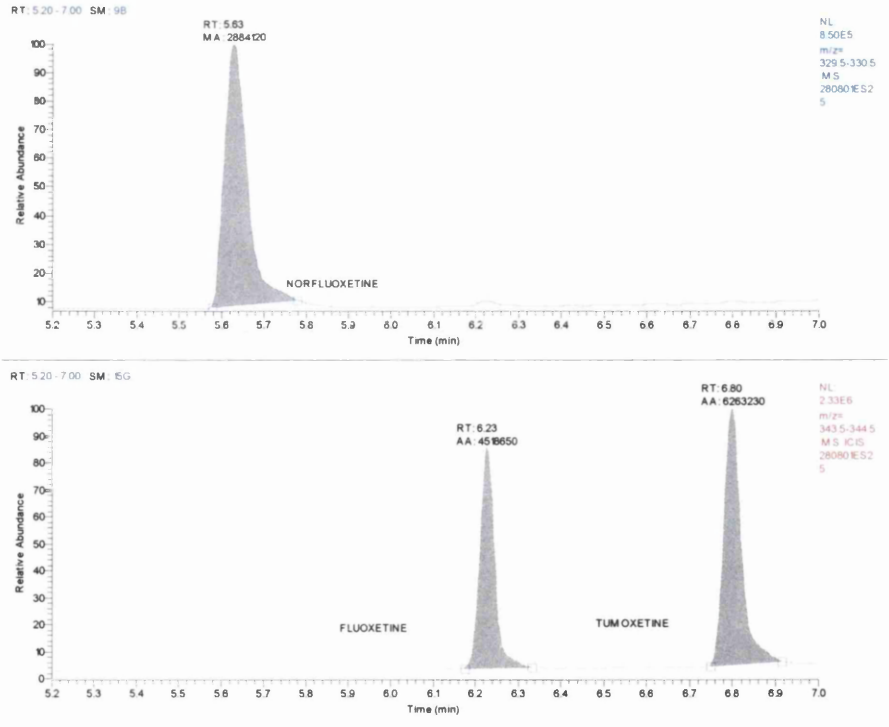


Figure 8.28: Chromatogram of extract of hair spiked with 25 ng/30 mg fluoxetine and norfluoxetine

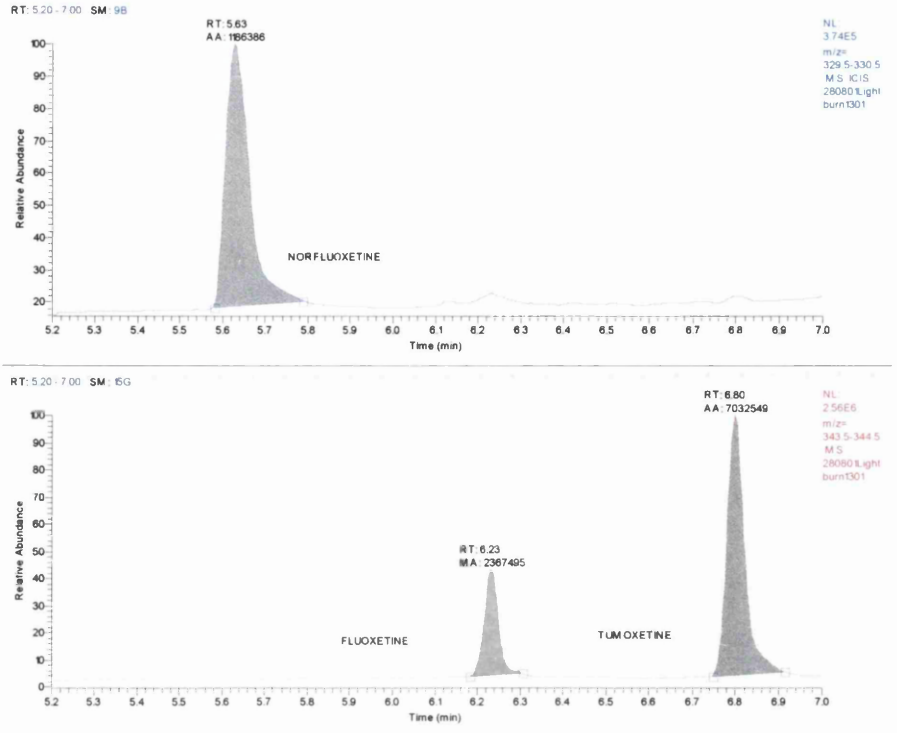


Figure 8.29: Chromatogram of extract of hair case sample HL/13, section 0-1 cm

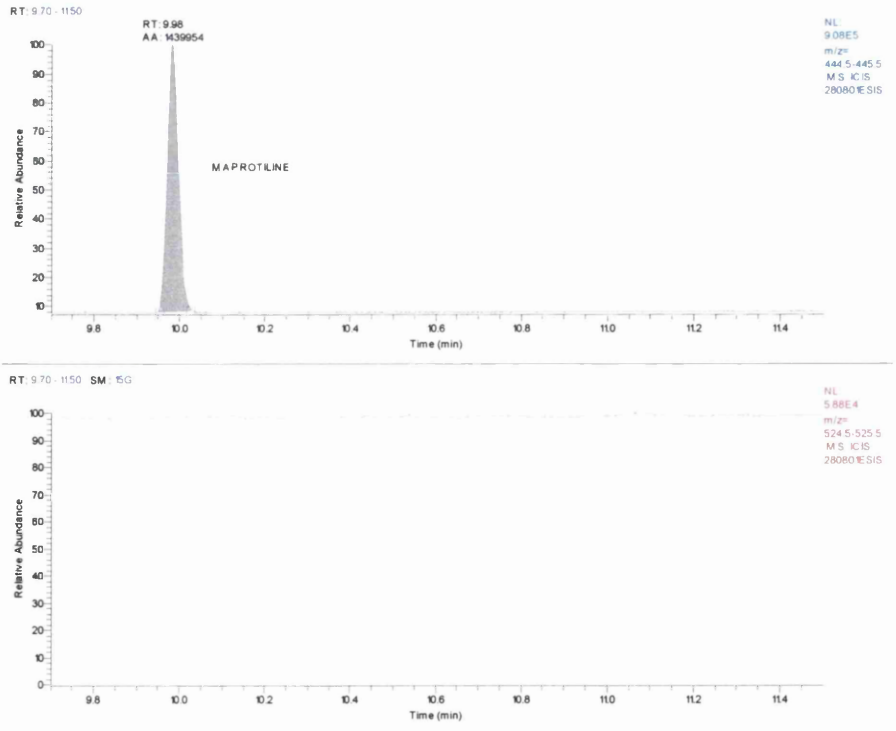


Figure 8.30: Chromatogram of extract of hair spiked with 25 ng/30 mg maprotiline

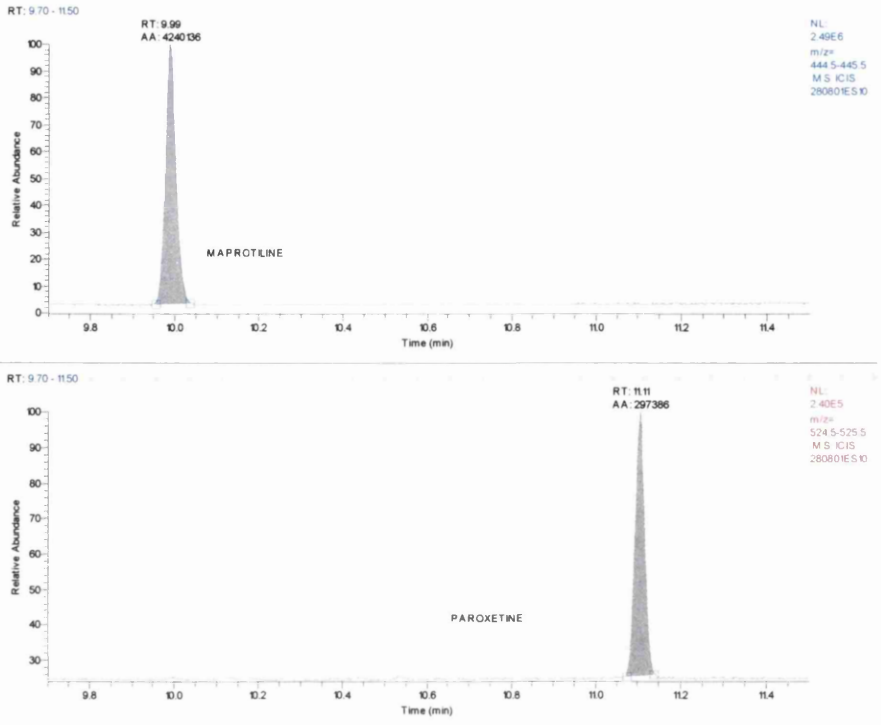


Figure 8.31: Chromatogram of extract of hair spiked with 10 ng/30 mg paroxetine

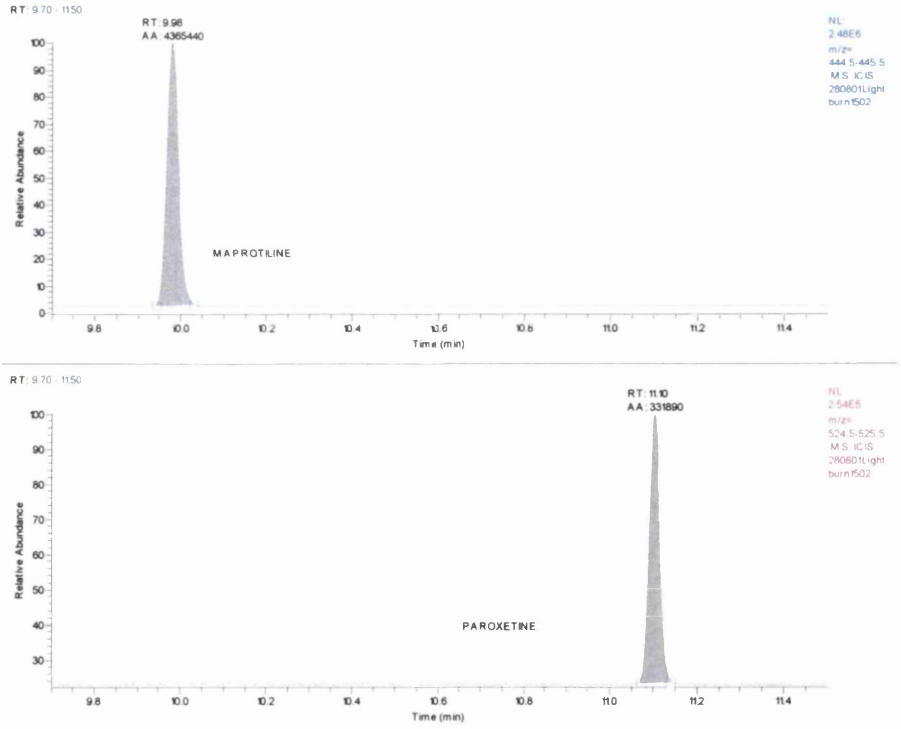


Figure 8.32: Chromatogram of extract of hair case sample HL/15, section 1-2 cm

8.6 ANTIDEPRESSANTS IN NAIL

8.6.1 CLINICAL SAMPLES

Nail samples were obtained from volunteers receiving fluoxetine or paroxetine treatment. Both hair and nails were collected simultaneously from patients. A total of 23 nail samples were collected and information including the age, sex, hair colour, dosage and length of time the drug had been prescribed was recorded. This information has already been tabulated in Table 8.26 of Antidepressants in Hair. However, other nail samples were collected from two other patients who did not provide hair samples and their details are displayed in Table 8.30. The samples were extracted using the method described in Chapter 7.11.

Table 8.30: Patient details

Case Number	Sex / age (Years)	Drug	Dosage mg/day	Length of time Prescribed *
NV/1	F/ 58	Fluoxetine	20	1 year
NV/2	F/ 56	Fluoxetine	20	2 years

8.6.2 RESULTS

23 nail samples were taken from 20 patients receiving fluoxetine or paroxetine treatment. One of these volunteers provided three samples, another volunteer two samples and the rest provided one sample each. Of the 23 nail samples tested, 17 (73.9 %) were found positive for fluoxetine, 17 (73.9 %) for norfluoxetine, 4 (17.4 %) for paroxetine and two (8.7 %) were found entirely negative for these drugs. In one of these cases only 2 mg of nail was available for analysis. The results of these are tabulated in Table 8.31.

Concentration ranges detected in the segments were 0.16 - 4.49 ng/mg for fluoxetine, 0.06 - 2.37 ng/mg for norfluoxetine and 0.01 - 0.84 ng/mg for paroxetine. The fluoxetine/norfluoxetine concentration ratio was determined to vary from 0.39 - 4.19 (mean 1.69). In all cases where fluoxetine was detected, the metabolite was also detected. Out of the 17 nails found positive for fluoxetine and norfluoxetine, 12 (70.6 %) gave a parent drug to metabolite ratio greater than 1 and 5 (29.4 %) gave values less than 1. Three of these 5 gave values greater than 0.90.

Table 8.31: Fluoxetine, norfluoxetine and paroxetine findings in nail case samples

Case sample	Fluoxetine (ng/mg)	Norfluoxetine (ng/mg)	Fluoxetine/ Norfluoxetine	Paroxetine (ng/mg)
NB/1	4.49	1.07	4.19	- ve
NB/2	- ve	- ve	-	0.34
NB/3	1.24	1.34	0.93	- ve
NL/1	1.53	2.37	0.65	- ve
NL/2	0.75	0.29	2.59	- ve
NL/3	0.39	1.01	0.39	- ve
NL/4	0.16	0.17	0.94	- ve
NL/5	1.16	1.05	1.10	- ve
NL/6	- ve	- ve	-	- ve
NL/7	0.39	0.15	2.60	- ve
NL/8	- ve	- ve	-	- ve
NL/10	0.52	0.34	1.53	- ve
NL/11	- ve	- ve	-	0.84
NL/12	- ve	- ve	-	0.14
NL/13	0.19	0.06	3.17	- ve
NL/14	0.66	0.45	1.47	- ve
NL/15	- ve	- ve	-	0.01
ND/1	0.19	0.11	1.73	- ve
NV/1a	0.72	0.51	1.41	- ve
NV/1b	0.34	0.38	0.89	- ve
NV/1c	0.30	0.18	1.67	- ve
NV/2a	2.63	1.71	1.54	- ve
NV/2b	3.27	1.72	1.90	- ve

These results have shown that the parent drug tended to incorporate into nail at a higher concentration than the metabolite.

8.6.3 CORRELATION OF NAIL CONCENTRATIONS WITH CASE HISTORY

Most of the results obtained from the analysis of the nails correlated well with medical records. Out of the 23 nails analyzed, two gave entirely negative results. For one of these cases (NL/8) there was less than 2 mg of nail available for analysis. In the other case (NL/6) the patient had been prescribed fluoxetine for only two days. The section of nail collected would have grown well before the drug was taken and since it was no longer attached to the nail bed then the possibility of drug incorporating through the bed is unlikely.

Case NL/12 was found to be negative for fluoxetine and norfluoxetine, but positive for paroxetine. Fluoxetine had been prescribed for 1 week prior to sampling and similar to the previously mentioned case the drug would not have incorporated into the section of nail collected. On the other hand, paroxetine had been prescribed for 5 days, but this was stopped at a time 6 weeks before sampling. The nail with the incorporated paroxetine therefore had time to grow away from the nail bed and this section was collected in the sample. This sample indicates that one week was not sufficient time for the nail with incorporated drug to grow from the bed. Also, this case shows that paroxetine could be detected in nail after 5 days of use.

Cases NL/4 and NL/13 showed that fluoxetine and norfluoxetine could be detected in nail 4 weeks after administration was started although in these patients the concentrations were very low. Nail growth in the elderly is slower than that in younger adults and it is possible that these concentrations would be higher in younger patients.

Case NL/15 was found positive for paroxetine, but negative for fluoxetine. Fluoxetine had only been administered for three days prior to sampling and so would not have been detected in sampled nail. Paroxetine was not mentioned in the supplied medical records, but for this case paroxetine was also detected in the hair of this individual.

All of the other cases were found positive for the drug which the patient was prescribed as given in their medical records.

8.6.4 COMPARISON BETWEEN HAIR AND NAIL SAMPLES

The range of antidepressant concentrations determined within one hair sample and the mean concentration detected in positive hair sections were calculated for each hair sample. These are shown along with the levels determined in nail samples (Table 8.32).

Table 8.32: Comparison of nail and hair concentrations

Case sample	Nail (ng/mg)			Hair (ng/mg)		
	FLU	NFLU	PAR	FLU	NFLU	PAR
NB/1	4.49	1.07	- ve	5.52-5.70 (5.61)	1.48-1.64 (1.56)	- ve
NB/2	- ve	- ve	0.34	- ve	- ve	1.48
NB/3	1.24	1.34	- ve	3.18-5.38 (4.70)	3.37-7.24 (5.85)	- ve
NL/1	1.53	2.37	- ve	2.83-3.02 (2.93)	5.33-7.55 (6.44)	- ve
NL/2	0.75	0.29	- ve	0.22-1.51 (0.87)	0.15-1.42 (0.79)	- ve
NL/3	0.39	1.01	- ve	0.97-2.58 (1.88)	2.80-8.09 (5.93)	- ve
NL/4	0.16	0.17	- ve	0.81	1.35	- ve
NL/5	1.16	1.05	- ve	1.95-4.30 (3.13)	1.14-3.56 (2.35)	- ve
NL/6	- ve	- ve	- ve	- ve	- ve	- ve
NL/7	0.39	0.15	- ve	0.57	0.56	- ve
NL/10	0.52	0.34	- ve	0.80-1.23 (0.98)	0.30-1.03 (0.59)	- ve
NL/11	- ve	- ve	0.84	- ve	- ve	5.73-6.23 (5.98)
NL/12	- ve	- ve	0.14	- ve	- ve	0.22-1.59 (0.87)
NL/13	0.19	0.06	- ve	0.47	0.51	- ve
NL/14	0.66	0.45	- ve	0.34-1.85 (1.10)	0.33-1.07 (1.15)	- ve
NL/15	- ve	- ve	0.01	- ve	- ve	0.29-0.41 (0.35)
ND/1	0.19	0.11	- ve	0.48	0.63	- ve

FLU = fluoxetine, NFLU = norfluoxetine, PAR = paroxetine

The concentrations of fluoxetine, norfluoxetine and paroxetine in nails and hair were of the same magnitude. Graphs of concentration in nail against mean concentration in hair were plotted for each drug. Correlation coefficients for these plots are displayed on the graphs in Figure 8.33. These were 0.711, 0.731, and 0.960 for fluoxetine, norfluoxetine and paroxetine respectively. All of the plotted graphs gave reasonable correlation. The concentration detected in hair generally increased as the nail concentration increased.

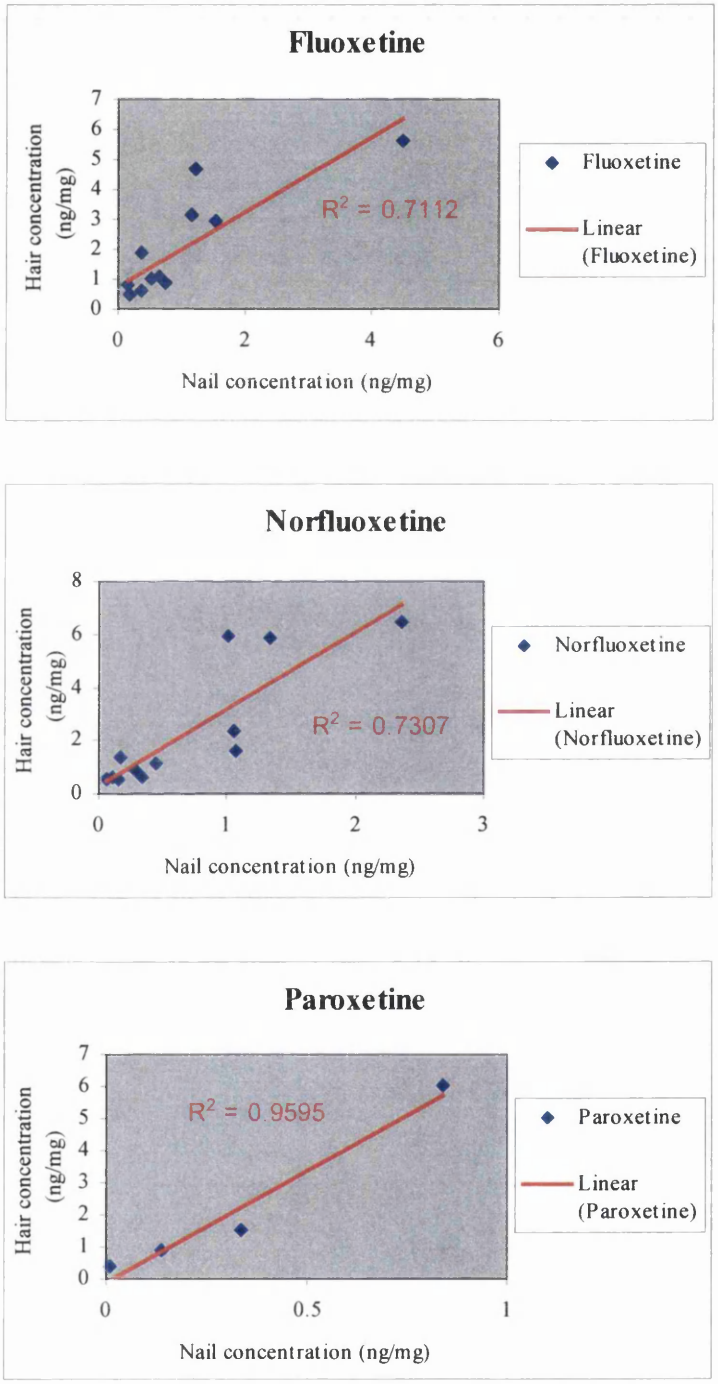


Figure 8.33: Correlations between nail and hair concentrations

Nail/hair concentration ratios were calculated for each drug and are displayed in Table 8.33. The range of these for fluoxetine was 0.20-0.86 (mean 0.49), for norfluoxetine was 0.12-0.69 (mean 0.33) and for paroxetine 0.03-0.23 (mean 0,14). For every sample tested the concentration detected in nails was lower than the mean concentration detected in the hair. Mean values of nail/hair concentrations indicated that fluoxetine levels were 2 times, norfluoxetine concentrations 3 times and paroxetine concentrations 7 times those in nails.

The ratio of parent drug to metabolite was generally found to be higher for nail than hair samples within an individual. This was the outcome in 10 out of 12 cases and is shown more clearly in Figure 8.34.

Examples of chromatograms obtained for the extraction of fluoxetine and paroxetine from nails are shown in Figures 8.35-8.40.

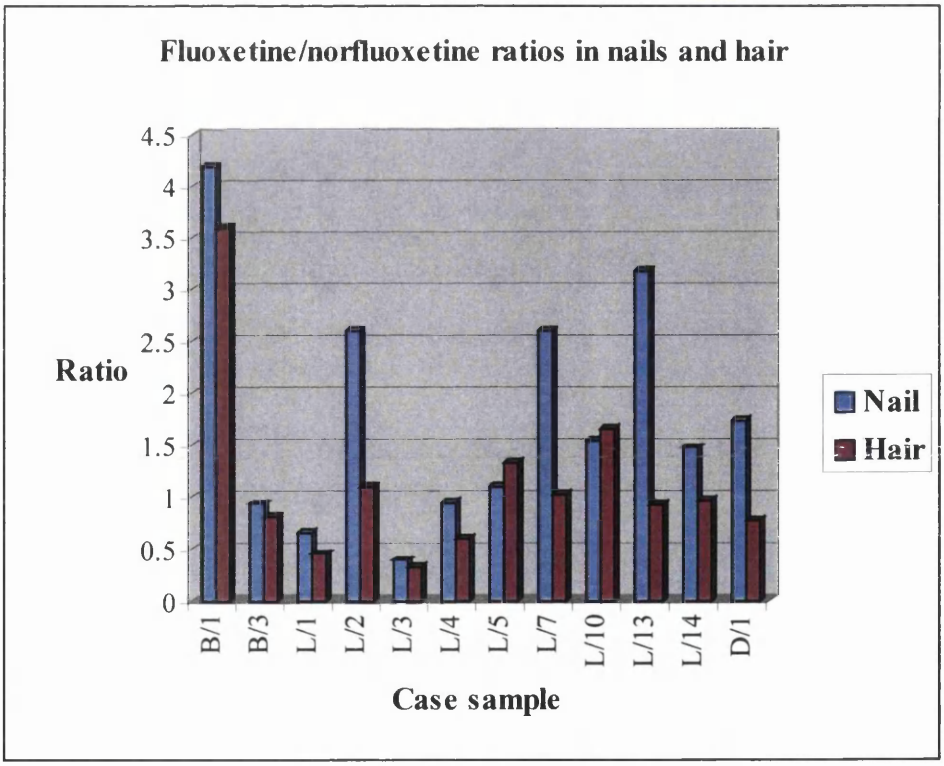


Figure 8.34: Comparison of fluoxetine/norfluoxetine ratio in hair and nail samples

Table 8.33: Nail/hair concentration ratios and drug/metabolite ratios

Case sample	Nail/hair ratio			Nail	Hair
	FLU	NFLU	PAR	FLU/NFLU	FLU/NFLU
NB/1	0.80	0.69	-	4.19	3.60
NB/2	-	-	0.23	-	-
NB/3	0.26	0.23	-	0.93	0.80
NL/1	0.52	0.37	-	0.65	0.45
NL/2	0.86	0.37	-	2.59	1.10
NL/3	0.21	0.17	-	0.39	0.32
NL/4	0.20	0.13	-	0.94	0.60
NL/5	0.37	0.45	-	1.10	1.33
NL/6	-	-	-	-	-
NL/7	0.68	0.27	-	2.60	1.02
NL/10	0.53	0.58	-	1.53	1.66
NL/11	-	-	0.14	-	-
NL/12	-	-	0.16	-	-
NL/13	0.40	0.12	-	3.17	0.92
NL/14	0.60	0.39	-	1.47	0.96
NL/15	-	-	0.03	-	-
ND/1	0.40	0.17	-	1.73	0.76

FLU = fluoxetine, NFLU = norfluoxetine, PAR = paroxetine

8.6.5 CONCLUSIONS

In cases where fluoxetine was detected in nails, similar to hair cases, the metabolite, norfluoxetine was always detected. Correlation between detection of fluoxetine, norfluoxetine and paroxetine in nail with case history was excellent. Cases where negative results were obtained were explained by a lack of sample or a short drug administration time which did not allow an adequate time for the nail to grow beyond the nail bed. The fluoxetine, norfluoxetine and paroxetine hair and nail concentrations were of similar magnitude, but the amounts detected in hair were always greater than those detected in nail. The ratios of nail to hair concentrations showed reasonable correlations for the three drugs with the concentration of drug detected in nail increasing as the concentration detected in hair increased. Therefore, nail analysis appears to be a good alternative to hair analysis to demonstrate chronic use of these drugs.

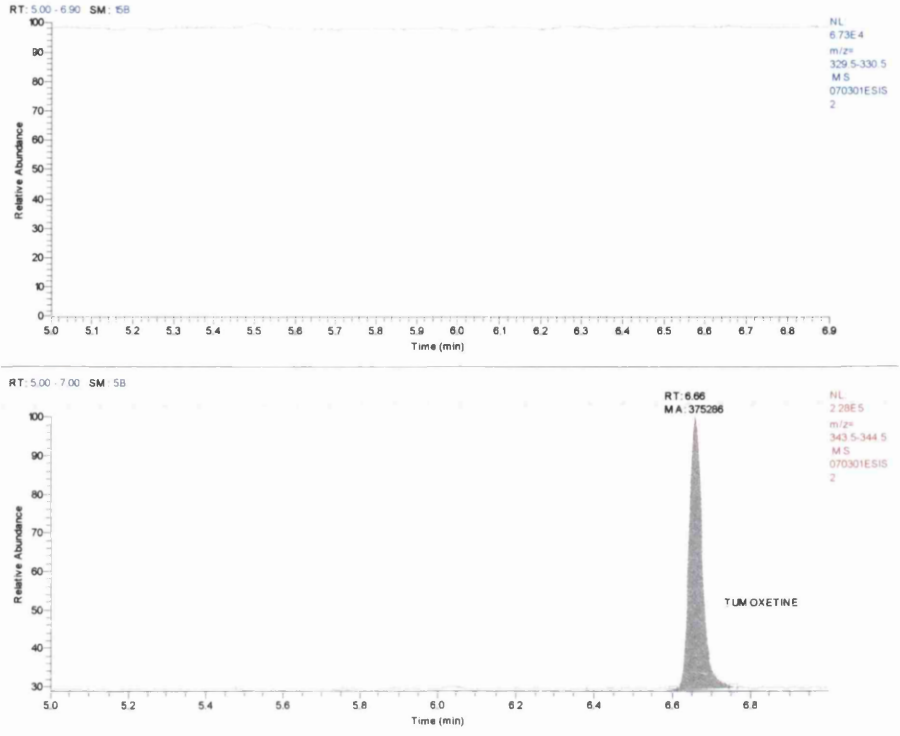


Figure 8.35: Chromatogram of extract of nail hydrolysate spiked with 50 ng/10 mg tumoxetine

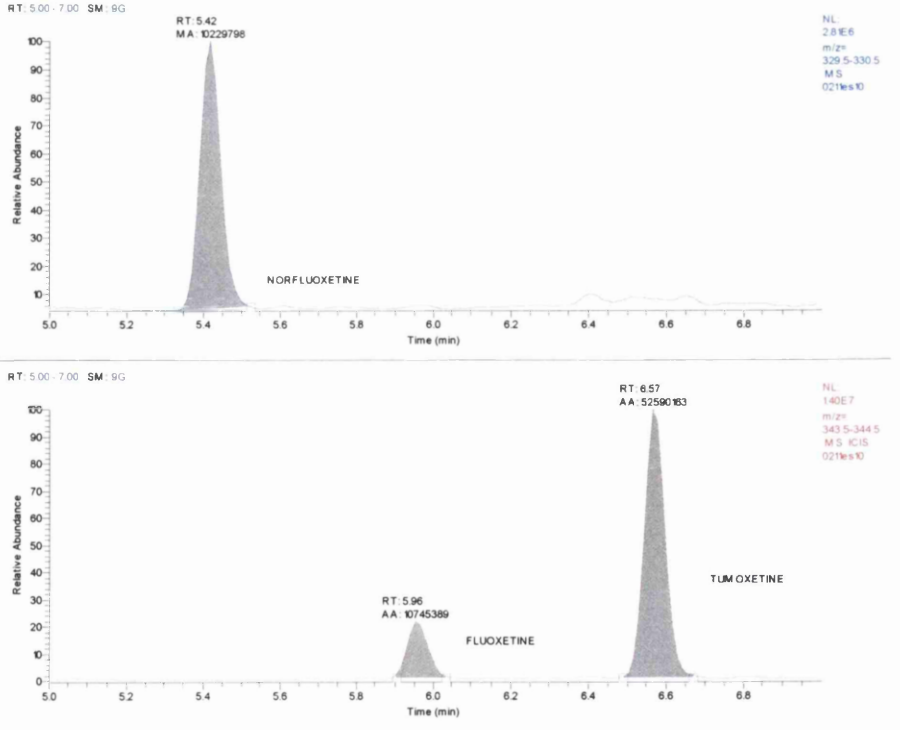


Figure 8.36: Chromatogram of extract of nail hydrolysate spiked with 10 ng/10 mg fluoxetine and norfluoxetine

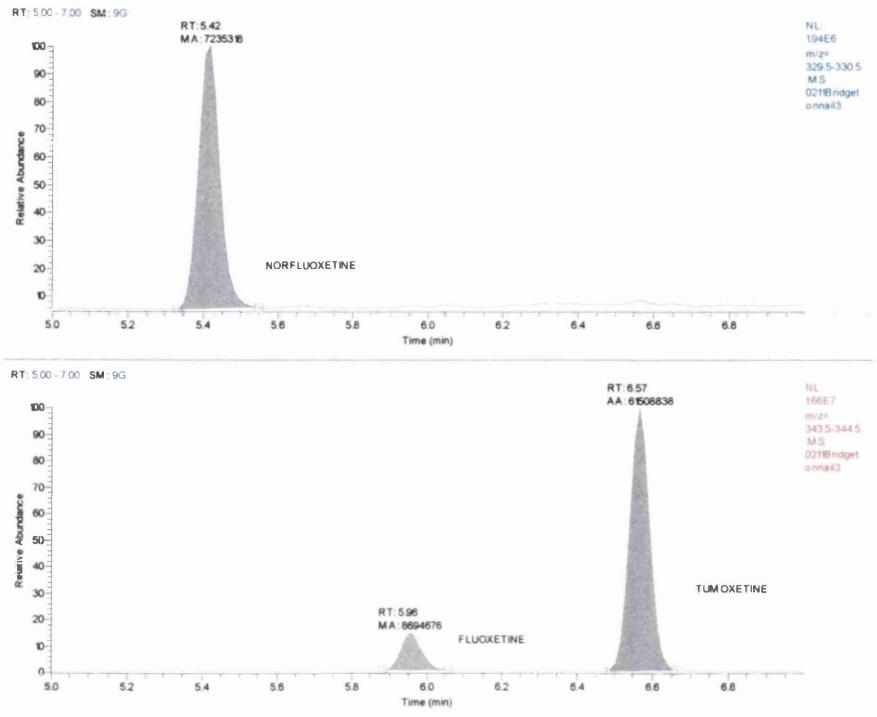


Figure 8.37: Chromatogram of extract of nail case sample NB/03

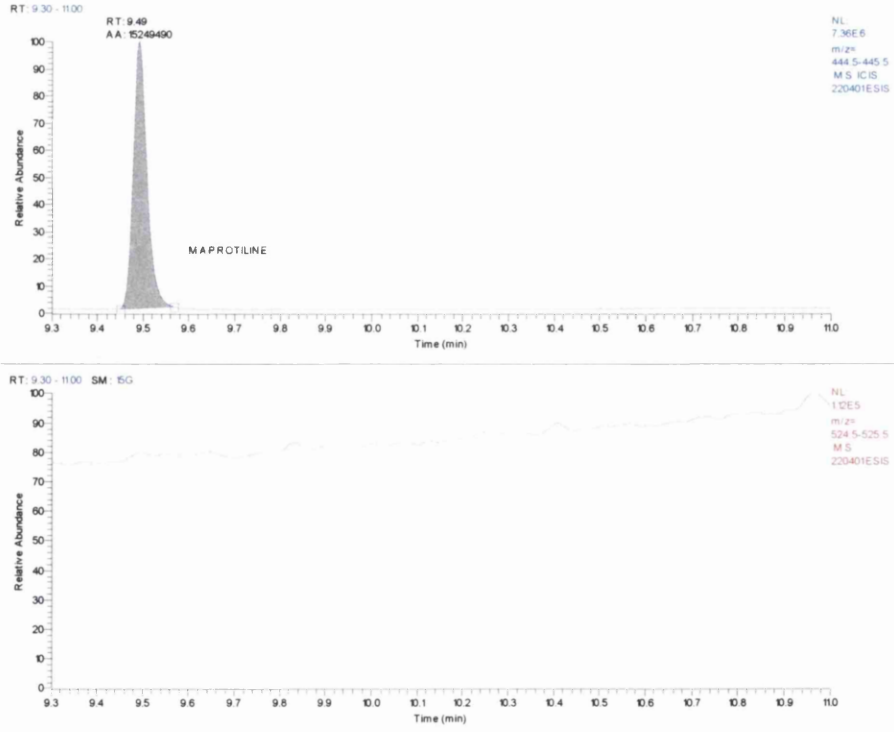


Figure 8.38: Chromatogram of extract of nail hydrolysate spiked with 25 ng/10 mg maprotiline

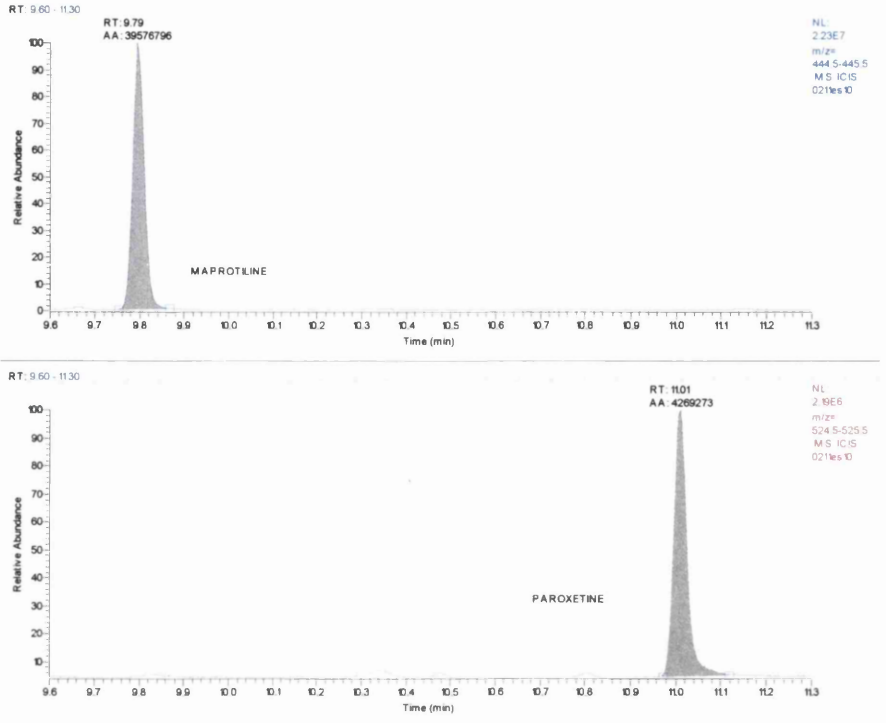


Figure 8.39: Chromatogram of extract of nail hydrolysate spiked with 10 ng/10 mg paroxetine

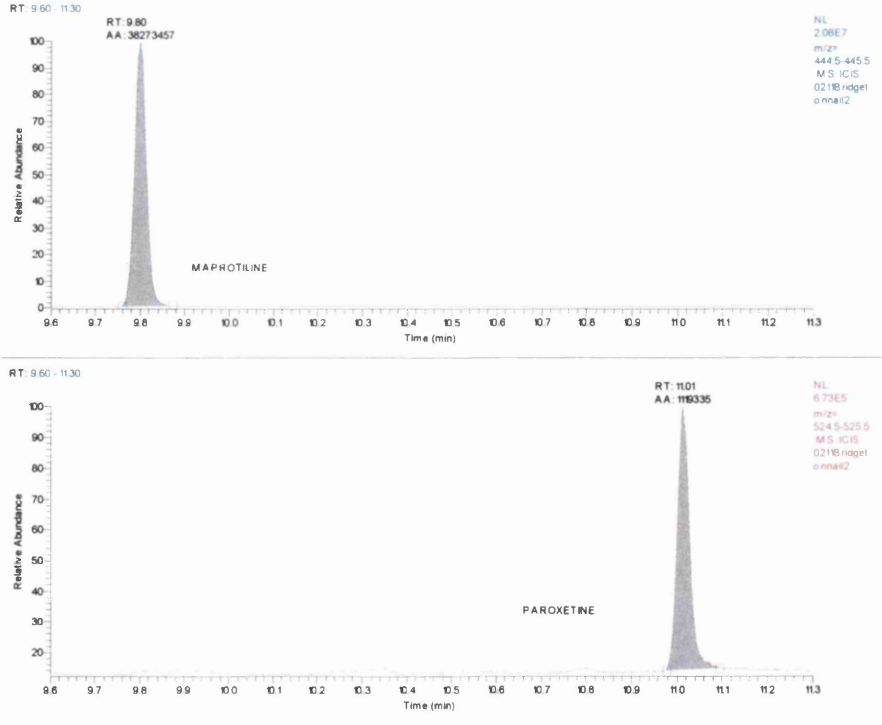


Figure 8.40: Chromatogram of extract of nail case sample NB/03

9 Conclusions

The potential use of hair analysis as a diagnostic tool in Forensic Toxicology was investigated by developing a reliable method for the simultaneous analysis of opiates and methadone in hair. The development of this method included assessing different pretreatment steps to extract the drugs from hair to find which method could be used without chemically altering any of the drugs. Out of three possibilities, methanol treatment of hair was shown to be the only tested pretreatment method which did not hydrolyze a proportion of 6-monoacetylmorphine to morphine. The validated method was used to determine the presence of morphine, codeine, 6-monoacetylmorphine, methadone and its metabolite, EDDP in postmortem hair samples obtained from suspected heroin overdose cases. Following analysis, the hair concentrations were evaluated to investigate whether an individual was a heroin user and to what extent. A drug use history was established for each individual from their hair and postmortem blood results and this was evaluated against any known drug use history obtained from the police report associated with the relevant case. In total, 31 hair case samples were investigated using the validated method which corroborated regular heroin usage in a high proportion of these cases. The extent of use was shown to vary between users and periods of compliance or non-compliance with methadone programmes or drug rehabilitation programmes were also apparent. On the whole, the developed method successfully determined the drug use history of heroin users. In addition, it provides a means of testing for chronic drug use in cases where heroin use is not obvious.

As a result of an increase in the number of postmortem blood cases received into our laboratory which required investigation for the antidepressant drugs fluoxetine and paroxetine it was necessary to develop an improved method to simultaneously analyze for these drugs in whole blood. A solid phase extraction procedure was developed and fully validated using high performance liquid chromatography and gas chromatography-mass spectrometry. This extraction procedure was shown to produce higher recoveries than existing liquid-liquid extraction methods and the use of gas chromatography-mass spectrometry achieved higher sensitivities than previous methods used to simultaneously extract these drugs from whole blood samples. This improved method was used to successfully determine the concentrations of these drugs in postmortem and clinical

samples and provided the basis for further studies into the diagnostic use of alternative biological matrices.

The use of saliva as an alternative sample to blood or urine to indicate recent drug exposure was investigated by validating the developed blood extraction method for the determination of these antidepressants in saliva samples. Saliva samples were collected either by spitting into a universal container and/or using the Omni-Sal® device. The in-vitro recovery of the Omni-Sal® devices was assessed and this was determined to be reproducible. The recoveries, however, were lower than those obtained from saliva collected by the spitting method. Clinical samples were successfully studied using both validated collection methods. The concentrations in saliva were found to vary between 0.022 - 0.223 mg/L for fluoxetine and 0.011 - 0.143 mg/L for norfluoxetine. For one paroxetine saliva sample, the concentration detected was 0.031 mg/L. Despite the lower recoveries obtained using the Omni-Sal® swabs, all samples tested using this collection method were shown to be positive. In some of the cases, blood and saliva samples were collected simultaneously and saliva/blood concentration ratios were determined. These were found to vary widely due to the differences in salivary pH of individuals. Four out of six saliva/blood ratios for fluoxetine were shown to give values within the theoretical range calculated for a saliva pH range of 6.0-7.2. Fluoxetine/norfluoxetine ratios were calculated and were shown to be consistently higher for saliva than for blood. The saliva/blood ratio obtained for paroxetine fell within the calculated theoretical range. These cases demonstrated how inter-individual variability affects the detected saliva concentrations and subsequently the saliva/blood ratios.

In addition, saliva samples were collected using both methods, spitting and with Omni-Sal® devices, from volunteers who were prescribed either a 20 mg dose of fluoxetine or paroxetine. Concentration - time curves were produced for each volunteer and compared. It was apparent that fluoxetine capsules did not seem to produce any noticeable contamination of the oral cavity. However, paroxetine tablets did produce this type of contamination which was shown by a higher concentration of paroxetine being detected at one hour after administration and then a large decrease by 3 hours. Peak saliva concentrations were reached at approximately 7 hours following consumption for fluoxetine and the saliva elimination half-life was determined to be between 1 and 2 days

for three individuals. Paroxetine peak saliva concentrations were reached at approximately 5 and 7 hours for two individuals and the apparent saliva elimination half-life for paroxetine was 5 - 8 hours. Saliva was demonstrated to be a useful alternative specimen to provide the clinician with a non-invasive procedure to obtain information on recent drug exposure.

For many people who are prescribed antidepressants, there is a tendency to either stop taking them before they are advised to by their general practitioner or not to fully comply by taking them only when they believe they are required. Hair testing as a means of providing information on compliance or on the chronic use of such drugs was assessed by modifying the method which was developed to extract opiates from hair. Pretreatment steps to extract the drugs from hair were investigated. The developed method involved using acid hydrolysis as a pretreatment step since this was shown to produce higher recoveries of these drugs from the hair matrix compared with the methanolic treatment. The solid phase extraction method was successfully extended to provide clean extracts. Initially two postmortem cases were analyzed. In one of these cases fluoxetine and norfluoxetine were detected along the hair shaft, but the concentrations were found to decrease along the length of the hair. This was partly due to the hair being constantly bleached which also caused a larger loss of the metabolite compared to the parent drug as the selected section of hair was obtained further away from the root. This hair sample demonstrated how chemical treatment affects the concentrations detected along the hair shaft.

Hair samples from antidepressant users were investigated using the developed method to provide a history of drug use for each individual and these results were evaluated against information obtained from each patient's medical records. The quantities of antidepressants determined in hair samples were evaluated using the developed method to find if there was any correlation between individuals receiving the same dose. Concentrations detected in hair were found to vary between 0.34 - 5.52 ng/mg for fluoxetine, 0.15 - 8.09 ng/mg for norfluoxetine and 0.22 - 6.23 ng/mg for paroxetine. The hair samples were shown to provide a history of antidepressant drug use which corresponded to the patient's history as given in their medical records. Also, the dose-concentration relationship within an individual was found to be relatively constant. However, from this data it was also shown that a dose-concentration relationship between individuals was non-existent. Hair colour

was shown to have some influence on the amount of drug detected in hair. Dark hair was seen to incorporate a higher concentration of drug than white or grey hair. The diagnostic use of hair as an indicator of chronic drug use was successfully established using the validated method. However, no information could be derived from the hair analysis relating to the quantity of the drugs administered.

The potential of using nail analysis as an alternative to hair analysis for the determination of chronic drug use was studied by validating a simple extraction method and using it to determine levels of antidepressants in nails obtained from clinical patients. The detected concentrations were evaluated using each patient's medical records and assessed against the concentrations determined in the hair of each individual to find if there was any correlation between the concentrations in these different biological samples. Nail concentrations were found to be of the same magnitude as hair concentrations and overall the detected concentrations showed a good correlation with medical records. Comparisons between hair and nail samples showed that the concentration in hair generally increased as the nail concentration increased. The use of nails for the detection of these drugs was shown to be a good alternative to hair as an indicator of chronic use.

Overall, this project has demonstrated that the use of alternative biological matrices for the detection of drugs is an important tool for both forensic and clinical practitioners. With further research this importance will increase.

10 Further Work

The developed blood method should be extended to include other selective serotonin reuptake inhibitor drugs such as fluvoxamine, sertraline and citalopram. A literature search indicated that although there have been various combinations of these drugs and selected metabolites with fluoxetine and/or paroxetine there has not been a method which extracts the five main selective serotonin reuptake inhibitors simultaneously. This method should also be applied to these drugs in saliva.

Blood, saliva and urine studies have shown that saliva is a better indicator of blood concentration than urine. In order to use saliva values to predict antidepressant blood concentrations further studies would be useful to thoroughly investigate the variation of pH with saliva concentration.

The hair method for opiates has the potential to include other analytes. The methanolic treatment method allows the possibility to extract a wider range of drugs from hair without causing hydrolysis or destruction of the drug. This method has in fact been used to include other opiates, dihydrocodeine and propoxyphene.

11 References

[1] Drobitch, R.K. and Svensson, C.K. "Therapeutic Drug Monitoring in Saliva." Clinical Pharmacokinetics, 23 (5) (1992) p365-379.

[2] Peel, H.W., Perrigo, B.J. and Mikhael, N.Z. "Detection of saliva of impaired drivers." Journal of Forensic Science, 29 (1984) p185-189.

[3] Skopp, G. and Potsch, L. "Perspiration versus saliva – basic aspects concerning their use in roadside drug testing." Int. J. Legal Med., 112 (1999) p213-221.

[4] Seymour, A. Personal communication.

[5] Ricossa, M.C., Bernini, M. and De Ferrari, F. "Hair analysis for driving licence in cocaine and heroin users. An epidemiological study." Forensic Science International, 107(1-3) (2000) p301-308.

[6] Montagna, M., Stramesi, C., Vignali, C., Groppi, A. and Polettinni, A. "Simultaneous hair testing for opiates cocaine and metabolites by GC/MS: A survey of applicants for driving licenses with a history of drug use." Forensic Science International, 107(1-3) (2000) p157-167.

[7] Tagliaro, F., Valentini, R., Manetto, G., Crivellente, F., Carli, G. and Marigo, M. "Hair analysis by using radioimmunoassay, high-performance liquid chromatography and capillary electrophoresis to investigate chronic exposure to heroin, cocaine and/or ecstasy in applicants for driving licences." Forensic Science International, 107(1-3) (2000) p121-128.

[8] Cone, E.J., Darwin, W.D. and Wang, W-L. "The occurrence of cocaine, heroin and metabolites in hair of drug abusers." Forensic Science International, 63 (1993) p55-68.

[9] Potsch, L. "On physiology and ultrastructure of human hair." Proceedings of the 1995 International Conference and Workshop for Hair Analysis in Forensic Toxicology, (1995) p1-27.

[10] Harkey, M.R. "Anatomy and physiology of hair." Forensic Science International, 63 (1993) p9-18.

[11] Chase, H.B. "Cycles and waves in hair growth." In Lyne, A.G., Short, B.F. (eds.) "Biology of the skin and hair growth." Halstead Press, Sydney, (1965) p461-465.

[12] Bullough, W.S. and Laurence, E.B. "The Mitotic Activity of the Follicle." In Montagna, W. and Ellis, R.A. (eds.) "The biology of hair growth." Academic Press, New York, (1958) p171-175.

[13] Bost, R.O. "Hair analysis-perspectives and limits of a proposed forensic method of proof:a review." Forensic Science International, 63 (1993) p31-42.

[14] Meyers, R.J. and Hamilton, J.B. "Regeneration and rate of growth of hairs in man." Annals of New York Academy of Sciences, 53 (1951) p562-568.

[15] Henderson, G.L. "Mechanisms of drug incorporation into hair." Forensic Science International, 63 (1993) p19-29.

[16] Nakahara, Y., Shimamine, M. and Takahashi, K. "Hair Analysis for Drugs of Abuse. III. Movement and stability of methoxyphenamine (As a model compound of methamphetamine) along hair shaft with hair growth." Journal of Analytical Toxicology, 16 (1992) p253-257.

[17] Rollins, D.E., Wilkins, D.G., Gygi, S.P., Slawson, M.H. and Nagasawa, P.R. "Testing for Drugs of Abuse in Hair." Forensic Science Review, 9 (1997) p23-35.

[18] Nakahara, Y. and Hanajiri, R. "Hair analysis for drugs of abuse XXI. Effect of para-substituents on benzene ring of methamphetamine on drug incorporation into rat hair." Life Sciences, 66(7) (2000) p563-574.

[19] Nakahara, Y. and Kikura, R. "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, 70 (1996) p841-849.

[20] Nakahara, Y., Takahashi, K. and Kikura, R. "Hair analysis for drugs of abuse. X. Effect of physicochemical properties of drugs on the incorporation rates into hair." Biological Pharmaceutical Bulletin, 18(9) (1995) p1223-1227.

[21] Nakahara, Y., Kikura, R. and Takahashi, K. "Hair analysis for drugs of abuse XX. Incorporation and behaviours of seven methamphetamine homologs in the rat hair root." Life Sciences, 63(10) (1998) p883-893.

[22] Nakahara, Y. "The effects of physiochemical factors on the incorporation of drugs into hair and the behavior of drugs in hair root." In Mieczkowski (ed.) "Drug testing technology:assessment of field applications." CRC Press, Florida, (1999) p50-72.

[23] Wilkins, D.G., Haughey H.M., Krueger, G.G. and Rollins, D.E. "Disposition of codeine in female human hair after multiple-dose administration." Journal of Analytical Toxicology, 19 (1995) p492-498.

[24] Baumgartner, W.A., Hill, V.A. and Blahd, W.H. "Hair analysis for drugs of abuse." Journal of Forensic Sciences, 34(6) (1989) p1433-1435.

[25] Mangin, P. and Kintz, P. "Variability of opiates concentrations in human hair according to their anatomical origin: head, axillary and pubic regions." Forensic Science International, 63 (1993) p77 - 83.

[26] Nakahara, Y., Takahashi, K. and Konuma, K. "Hair Analysis for drugs of abuse VI. The excretion of methoxyphenamine and methamphetamine into beards of human subjects." Forensic Science International, 63 (1993) p109-119.

[27] Potsch, L., Skopp, G. and Becker, J. "Ultrastructural alterations and environmental exposure influence the opiate concentrations in hair of drug addicts." Int. J. Legal Med., 107 (1995) p301-305.

[28] Jurado, C., Kintz, P., Menendez, M. and Repetto, M. "Influence of the cosmetic treatment of hair on drug testing." Int. J. Legal Med., 110 (1997) p159-163.

[29] Potsch, L and Skopp, G. "Stability of opiates in hair fibres after exposure to cosmetic treatment." Forensic Science International, 81 (1996) p 95-102.

[30] Moeller, M.R., Fey, P. and Wennig, R. "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, 63 (1993) p185-206.

[31] Kelly, R.C., Mieczkowski, T., Sweeney, S.A. and Bourland, J.A. "Hair analysis for drugs of abuse. Hair colour and race differentials or systematic differences in drug preferences." Forensic Science International, 107 (2000) p63-86.

[32] Green, S.J. and Wilson, J.F. "The effect of hair colour on the incorporation of methadone into the hair in the rat." Journal of Analytical Toxicology 20, (1996) p121-123.

[33] Wilkins, D.G., Valdez, A.S., Nagasawa, P.R., Gygi, S.P., and Rollins, D.E. "Incorporation of drugs for the treatment of substance abuse into pigmented and nonpigmented hair." Journal of Pharmaceutical Sciences, 87(4) (1998) p435 - 440.

[34] Lyden, A., Larsson, B.S. and Lindquist, N.G. "Studies on the melanin affinity of haloperidol." Arch. Int. Pharmacodyn., 295 (1982) p230-243.

[35] Potsch, L., Skopp, G., and Moeller, M.R. "Influence of pigmentation on the codeine content of hair fibres in guinea pigs." Journal of Forensic Sciences, 42(6) (1997) p1095-1098.

[36] Mieczkowski, T. and Newel, R. "Statistical examination of hair colour as a potential biasing factor in hair analysis." Forensic Science International, 107 (2000) p13 - 38.

[37] Wilkins, D.G., Valdez, A.S., Krueger, G.G. and Rollins, D.E. "Quantitative analysis of 1-α-acetyl-*N*-normethadol, and 1-α-acetyl-*N*,*N*-dinormethadol in human hair by positive ion chemical ionisation mass spectrometry." Journal of Analytical Toxicology, 21 (1997) p420-426.

[38] Nakahara, Y., Takahashi, K., Shimamine, M. and Takeda, Y. "Hair analysis for drug abuse:I. Determination of methamphetamine and amphetamine in hair by stable isotope dilution gas chromatography/mass spectrometry method." Journal of Forensic Science, 36 (1991) p70-78.

[39] Cone, E.J. and Joseph, R.E. "The potential for bias in hair testing for drugs of abuse." In Kintz, P. (ed.) "Drug Testing in Hair"The biology of hair growth." CRC Press, Inc., Florida, (1996) p79-82.

[40] Blank, D.L. and Kidwell, D.A. "Decontamination procedures for drugs of abuse in hair: are they sufficient?" Forensic Science International, 70 (1995) 13-38.

[41] Cone, E.J., Yousefnejad, D., Darwin W.D. and Maguire, T. "Testing human hair for drugs of abuse. II. Identification of unique cocaine metabolites in hair of drug abusers and evaluation of decontamination procedures." Journal of Analytical Toxicology, 15 (1991) p250-255.

[42] Goldberger, B.A., Caplan, Y.H., Maguire, T. and Cone, E.J. "Testing human hair for drugs of abuse. III. Identification of heroin and 6-acetylmorphine as indicators of heroin use." Journal of Analytical Toxicology, 15 (1991) p226-231.

[43] Welch, R.J., Sniegoski, L.T., Allgood, C.C. and Habram, M. "Hair analysis for drugs of abuse:evaluation of analytical methods, environmental issues, and development of reference materials." Journal of Analytical Toxicology, 17 (1993) p389-398.

[44] Haley, N.J. and Hoffman D. "Analysis for nicotine and cotinine in hair to determine cigarette smoker status." Clinical Chemistry, 31 (1985) p1598-1600.

[45] Kintz, K., Ludes, B. and Mangin, P. "Evaluation of nicotine and cotinine in human hair." Journal of Forensic Sciences, 37(1) (1992) p72-76.

[46] Kidwell, D.A. "Analysis of phencyclidine and cocaine in human hair by tandem mass spectrometry." Journal of Forensic Sciences, 38(2) (1993) p272-284.

[47] Janzen, K. "Concerning norcocaine, ethylbenzoylecgonine, and the identification of cocaine use in human hair." Journal of Analytical Toxicology, 16 (1992) p402.

[48] Drummer, O. H. and Cooper, F.J. "Extraction of Psychotropic Drugs in Hair." Proceedings of the 1995 International Conference for Hair Analysis in Forensic Toxicology, Abu Dhabi (1995) p326-333.

[49] Offidani, C., Strano Rossi, S. and Chiarotti, M. "Improved enzymatic hydrolysis of hair." Forensic Science International, 63 (1993) p171-174.

[50] Eliopoulos, C., Klein, J. and Koren, G. "Validation of self-reported smoking by analysis of hair for nicotine and cotinine." Therapeutic Drug Monitoring, 18 (1996) p532-536.

[51] Cone, E. "Testing human hair for drugs of abuse I. Individual dose and time profiles of morphine and codeine in plasma, saliva, urine and beard compared to drug-induced effects on pupils and behavior." Journal of Analytical Toxicology, 16 (1992) p253-257.

[52] Nakahara, Y., Kikura, R., Takahashi, K. and Konuma, K. "GC-MS analysis of drugs and metabolites in hair for diagnosis of chronic methamphetamine abuse." Adv. Chem. Diagnos. Metab. Disease, 2 (1994) p187.

[53] Kintz, P., Cirimele, V. and Mangin, P. "Lack of relationship between morphine intake and morphine concentration in hair of carcinoma patients." Annales de Biologie Clinique, 53 (1995) p565-567.

[54] Henderson, G.L., Harkey, M.R., Zhou, C., Jones, R.T. and Jacob, P. III. "Incorporation of isotopically labeled cocaine and metabolites into human hair: I. Dose-response relationships." Journal of Analytical Toxicology, 20 (1996) p1-12.

[55] Couper, F.J., McIntyre, I.M. and Drummer, O.H. "Detection of Antidepressant and Antipsychotic Drugs in Postmortem Human Scalp Hair." Journal of Forensic Sciences, 40 (1995) p87-90.

[56] Klein, J., Chitayat, D. and Koren, G. "Hair analysis as a marker for fetal exposure to maternal smoking." New England Journal of Medicine, 328 (1993) p66-67.

[57] Sramek, J.J., Baumgartner, W.A., Ahrens, T.N., Hill, V.A. and Cutler, N.R. "Detection of benzodiazepines in human hair by radioimmunoassay." Annals of Pharmacotherapy, 26 (1992) p469-472.

[58] Muller R.K. and Thieme D. (Ed.) "Progress in Hair Analysis for Illegal Drugs: Workshop Proceedings of the International Society of Hair Testing." (2000) p68.

[59] Penning, R., Fromm, E., Betz, P., Kauert, G., Drasch, G. and Von Meyer, L. "Drug death autopsies at the Munich Institute of Forensic Medicine (1981-1992)" Forensic Science International, 62 (1993) p135-139.

[60] Zador, D., Sunjic, S. and Darke, S. "Heroin-related deaths in New South Wales, 1992: Toxicological findings and circumstances." MJA, 164 (1996) 204.

[61] Neeleman, J. and Farrell, M. "Fatal methadone and heroin overdoses: time trends in England and Wales." Journal of Epidemiology and Community Health, 51 (1997) 435-437.

[62] Baselt, R.C. and Cravey, R.H. "Disposition of Toxic Drugs and Chemicals in Man." Fifth Edition, Chemical Toxicology Institute, Foster City, California, (2000).

[63] Kintz, P., Eser, H.P., Tracqui, A., Moeller, M., Cirimele, V. and Mangin, P. "Enantioselective separation of methadone and its main metabolite in human hair by liquid chromatography/ion spray-mass spectrometry." Journal of Forensic Sciences, 42(2) (1997) 291-295.

[64] Goodman Gilman, A., Rall, T.W., Nies, A.S. and Taylor, P. "The Pharmacological Basis of Therapeutics." Eighth Edition, Pergamon Press, (1990).

[65] Jenkins, A.J., Keenan, R.M., Henningfield, J.E. and Cone, E.J. "Pharmacokinetics and pharmacodynamics of smoked heroin." Journal of Analytical Toxicology, 18 (1994) p317-330.

[66] Inturrisi, C. and Verebely, K. "Disposition of methadone in man after a single oral dose." Clin. Pharmacol. Ther., 13 (1972) p923-930.

[67] "Drug Abuse Briefing: A Guide to the effects of drugs and to the social and legal facts about their non-medical use in Britain" 5th Edition, Institute for the Study of Drug Independence, (1994).

[68] Marsh, A., Evans, M.B. and Strang, J. "Radioimmunoassay of drugs of abuse in hair. Part 2: The determination of methadone in the hair of known drug users." Journal of Pharmaceutical and Biomedical Analysis, 13 (1995) p829-839.

[69] Baumgartner, A.M., Jones, P.F., Baumgartner, W.A. and Black, C.T. "Radioimmunoassay of hair for determining opiate-abuse histories." J. Nuclear Medicine, 20 (1979) p748.

[70] Valente, D., Cassini, M., Pigliapochi, M. and Vansetti, G. "Hair as the sample in assessing morphine and cocaine addiction." Clin Chem, 27 (1981), p1952.

[71] Marsh A. and Evans M.B. "Challenging declarations of abstinence by the determination of morphine in hair by radioimmunoassay." J. Pharm. Biomed Anal., 11 (1993) p693.

[72] Nakahara, Y., Kikura , R. and Takahashi, K. "Hair analysis for drugs of abuse. VIII. Effective extraction and determination of 6-acetylmorphine and morphine in hair with trifluoroacetic acid-methanol for the confirmation of retrospective heroin use by gas chromatography-mass spectrometry." Journal of Chromatography B, 657 (1994) p93.

[73] Cone, E.J., Darwin, W.D. and Wang, W-L. "The occurrence of cocaine, heroin, and metabolites in the hair of drug abusers." Forensic Science International, 63 (1993) p55.

[74] Sachs, H. and Arnold, W. "Results of comparative determination of morphine in human hair using RIA and GC/MS." Clin. Chem. Clin. Biochem, 27 (1989) p873.

[75] Kintz P. and Mangin P. "Analysis of Opiates in Human Hair with FPIA, EMIT and GC/MS." Adli Tip Derg., 7 (1991) p129.

[76] Offidani, C., Carnevale, A. and Chiarotti, M. "Drugs in hair: a new extraction procedure." Forensic Science International, 41 (1989) p35.

[77] Nakahara, Y. "Hair analysis for abused and therapeutic drugs." Journal of Chromatography B, 733 (1999) p161-180.

[78] Rothe, M. and Pragst, F. "Solvent optimization for the direct extraction of opiates from hair samples." Journal of Analytical Toxicology, 19 (1995) p236-240.

[79] Balabanova, S. and Wolf, H.U. "Determination of methadone in human hair by radioimmunoassay." Z. Rechtsmed., 102 (1989) p1.

[80] Balabanova, S. and Wolf, H.U. "Methadone concentrations in human hair of the head, axillary and pubic hair." Z. Rechtsmed., 102 (1989) p293.

[81] Balabanova, S., Arnold, P.J., Brunner, H., Luckow, V. and Wolf, H.U. "Detection of methadone in human hair by gas chromatography/mass spectrometry." Z. Rechtsmed., 102 (1989) p495.

[82] Spokert, F. and Pragst, F. "Use of headspace solid-phase microextraction (HS-SPME) in hair analysis for organic compounds." Forensic Science International, 107 (1-3) (2000) p129-148.

[83] Spokert, F. and Pragst, F. "Determination of methadone and its metabolites EDDP and EMDP in human hair by headspace solid-phase microextraction and gas chromatography-mass spectrometry." Journal of Chromatography B, 746 (2000) p255-264.

[84] Kintz, P., Eser, H.P., Tracqui, A., Moeller, M., Cirimele, V. and Mangin, P. "Enantioselective separation of methadone and its main metabolite in human hair by liquid chromatography/ion spray-mass spectrometry." Journal of Forensic Sciences, 42(2) (1997) p291-295.

[85] Lucas, A.C.S., Bermejo, A.M., Tabernero, M.J., Fernandez, P. and Strano-Rossi S. "Use of solid phase microextraction (SPME) for the determination of methadone and EDDP in human hair by GC-MS." Forensic Science International, 107 (1-3) (2000) p225-232.

[86] Goldberger, B.A., Darraj, A.G., Caplan, Y.H. and Cone, E.J. "Detection of methadone, methadone metabolites, and other illicit drugs of abuse in hair of methadone-treatment subjects." Journal of Analytical Toxicology, 22 (1998) p526-530.

[87] Segura, J., Stramesi, C., Redon, A., Ventura, M., Sanchez, C.J., Gonzalez G., San, L. and Montagna M. "Immunological screening of drugs of abuse and gas

chromatographic-mass spectrometric confirmation of opiates and cocaine in hair." Journal of Chromatography B, 724(1) (1999) p9-21.

[88] Cirimele, V., Kintz, P., Majdalani, R. and Mangin, P. "Supercritical fluid extraction of drugs in drug addict hair." Journal of Chromatography B, 673 (1995) p173-181.

[89] Strano-Rossi, S. and Chiarotti, M. "Solid-phase microextraction for cannabinoids analysis in hair and its possible application to other drugs." Journal of Analytical Toxicology, 23 (1999) p7-10.

[90] Marigo, M., Tagliaro, F., Poiesi, C., Lafisca, S. and Neri, C. "Determination of morphine in the hair of heroin addicts by high performance liquid chromatography with fluorimetric detection." Journal of Analytical Toxicology, 10 (1986) p158.

[91] Achilli, G., Cellerino, G.P., Mezli d'Eril, G.V. and Tagliaro, F. "Determination of illicit drugs and related substances by high-performance liquid chromatography with an electrochemical coulometric-array detector." Journal of Chromatography, 729 (1-2) (1996) p273-277.

[92] Welch, M.J., Sniegoski, L.T., Allgood, C.C. and Habram, M. "Hair analysis for drugs of abuse:evaluation of analytical methods, environmental issues, and development of reference materials." Journal of Analytical Toxicology, 17 (1993) p389.

[93] Uhl, M. "Tandem mass spectrometry:A helpful tool in hair analysis for the forensic expert." Forensic Science International, 107 (1-3) (2000) p169-179.

[94] Girod, C. and Staub, C. "Analysis of drugs of abuse in hair by automated solid-phase extraction, GC/EI/MS and GC/ion trap/CI/MS." Forensic Science International, 107 (1-3) (2000) p261-271.

[95] Tagliaro, F., Poiesi, C., Aiello, R., Dorizzi, R., Ghielmi, S. and Marigo, M. "Capillary electrophoresis for the investigation of illicit drugs in hair: determination of cocaine and morphine" J of Chrom, 638 (1993) p303.

[96] Tagliaro, F., Valentini, R., Manetto, G., Crivellente, F., Carli, G. and Marigo, M. "Hair analysis by using radioimmunoassay, high performance liquid chromatography and capillary electrophoresis to investigate chronic exposure to heroin, cocaine and/or ecstasy in applicants for driving licences." Forensic Science International, 107 (1-3) (2000) p121-128.

[97] Moeller, M.R., Fey, P., and Sachs, H. "Hair analysis as evidence in forensic cases." Forensic Science International, 63 (1993) p43.

[98] Goldberger, B.A., Darwin, W.D., Grant, T.M., Allen, A.C., Caplan, Y.H., and Cone, E.J. "Measurement of heroin and its metabolites by isotope-dilution electron-impact mass spectrometry." Clinical Chemistry, 39 (1993) p670.

[99] Lemberger, L., Bergstrom, R.F., Wolen, R.L., Farid, N.A., Enas, G.G., and Aronoff, G.R. "Fluoxetine: Clinical Pharmacology and Physiologic Disposition." Journal of Clinical Psychiatry, 46 (3) (1985) p14-19.

[100] Orsulak, P.J., Kenney, J.T., Debus, J.R., Crowley, G. and Wittman, P.D. "Determination of the Antidepressant Fluoxetine and its Metabolite Norfluoxetine in Serum by Reversed-Phase HPLC, with Ultraviolet Detection." Clinical Chemistry, 34(9) (1988) p1875-1878.

[101] Nakra, B.R.S., Szwabo, P. and Grossberg, G.T. "Mania Induced by Fluoxetine." American Journal of Psychiatry, 146(11) (1989) p1515-1516.

[102] Dursun, S.M., Mathew, V.M. and Reveley, M.A. "Toxic Serotonin Syndrome after Fluoxetine plus Carbamazepine." The Lancet, 342 (1993) p442-443.

[103] Feighner, J.P. and Boyer, W.F. "Selective Serotonin Re-uptake Inhibitors" Second Edition, Wiley, Chichester, (1996).

[104] Sternbach, H. "The Serotonin Syndrome." American Journal of Psychiatry, 146(6) (1991) p705-713.

[105] Nash, J.F., Bopp, R.J., Carmichael, R.H., Farid, K.Z. and Lemberger, L. "Determination of Fluoxetine and Norfluoxetine in Plasma by Gas Chromatography with Electron-Capture Detection." Clinical Chemistry, 28(10) (1982) p2100-2102.

[106] Lantz, R.J., Farid, K.Z., Koons, F.J., Tenbarge, J.B. and Bopp, R.J. "Determination of Fluoxetine and Norfluoxetine in Human Plasma by Capillary Gas Chromatography with Electron-Capture Detection." Journal of Chromatography B, 614 (1993) p175-170.

[107] Dixit, V., Nguyen, H. and Dixit, V.M. "Solid-phase extraction of fluoxetine and norfluoxetine from serum with gas chromatography-electron capture detection." Journal of chromatography, 563 (1991) p379-384.

[108] Lopez, C., Lykissa, D. and Kammerer, R.C. "Fluoxetine and norfluoxetine determination in serum by capillary GC-ECD with solid phase extraction." Clinical Chemistry, 35(6) (1989) p1169.

[109] Torok-Both, G.A., Baker, G.B., Coutts, R.T., McKenna, K.F. and Aspeslet, L.J. "Simultaneous determination of fluoxetine and norfluoxetine enantiomers in biological samples by gas chromatography with electron-capture detection." Journal of Chrom. B, 579 (1992) p99-106.

[110] Eap, C.B., Gaillard, N., Powell, K. and Baumann P. "Simultaneous determination of plasma levels of fluvoxamine and of the enantiomers of fluoxetine and norfluoxetine by gas chromatography-mass spectrometry." Journal of Chrom. B, 682 (1996) p265-273.

[111] Goodnough, D.B., Baker, G.B. and Coutts, R.T. "Simultaneous quantification of fluoxetine, norfluoxetine and desipramine using gas chromatography with nitrogen-phosphorus detection." Journal of Pharmacological and Toxicological Methods, 34 (1995) p143-147.

[112] Fontanille, P., Jourdil, N., Villier, C. and Bessard, G. "Direct analysis of fluoxetine and norfluoxetine in plasma by gas chromatography with nitrogen-phosphorus detection." Journal of Chromatography B, 692 (1997) p337-343.

[113] Kelly, M.W., Perry, P.J., Holstad, S.G. and Garvey, M.J. "Serum fluoxetine and norfluoxetine concentrations and antidepressant response." Therapeutic Drug Monitoring, 11 (1989) p165-170.

[114] Suckow, R.F., Zhang, M.F. and Cooper, T.B. "Sensitive and selective liquid-chromatographic assay of fluoxetine and norfluoxetine in plasma with fluorescence detection after precoloumn derivatization." Clinical Chemistry, 38(9) (1992) p1756-1761.

[115] Clausing, P., Rushing, L.G., Newport, G.D. and Bowyer, J.F. "Determination of D-fenfluramine, D-norfenfluramine and fluoxetine in plasma, brain tissue and brain microdialysate using high-performance liquid chromatography after precolumn derivatization with dansyl chloride." Journal of Chrom. B, 692 (1997) p419-426.

[116] Thomare, P., Wang, K., Van Der Meersch-Mougeot, V. and Diquet, B. "Sensitive micromethod for column liquid chromatographic determination of fluoxetine and norfluoxetine in human plasma." Journal of Chrom. B., 583 (1992) p217-221.

[117] Nichols, J.H., Charlson, J.R. and Lawson, G.M. "Automated HPLC Assay of Fluoxetine and Norfluoxetine in Serum." Clinical Chemistry, 40(7) (1994) p1312-1316.

[118] Gupta, R.N. and Steiner, M. "Determination of fluoxetine and norfluoxetine in serum by liquid chromatography with fluorescence detection." Journal of Liquid Chrom., 13(19) (1990) p3785-3797.

[119] Crifasi, J., Le, N.X. and Long, C. "Simultaneous identification and quantification of fluoxetine and its metabolite, norfluoxetine, in biological samples by GC/MS." Journal of Analytical Tox., 21 (1997) p415-419.

[120] Kristoffersen, L., Bugge, A., Lundanes, E., and Slordal, L. "Simultaneous determination of citalopram, fluoxetine, paroxetine and their metabolites in plasma and whole blood by high-performance liquid chromatography with ultra-violet and fluorescence detection." Journal of Chrom. B., 734 (1999) p229-246.

[121] Corby, C.L. and Dunne, G. "Paroxetine: a review." Journal of Serotonin Research, 4 (1997) p47-64.

[122] Cohn, J.B. and Wilcox, C.S. "Paroxetine in Major Depression: A Double-Blind Trial with Imipramine and Placebo." J. of Clin. Psychiatry, 53 (2 suppl) (1992) p52.

[123] Kaye, C.M., Haddock, R.E., Langley, P.F., Mellows, G., Tasker, T.C.G., Zussman, B.D. and Greb, W.H. "A review of the metabolism and pharmacokinetics of paroxetine in man." Acta Psychiatry Scand., 80 (suppl. 350) (1989) p60-75.

[124] Hartter, S., Hermes, B., Szegedi, A. and Hiemke, C. "Automated Determination of Paroxetine and Its Main Metabolite by Column Switching and On-Line High-Performance Liquid Chromatography." Therapeutic Drug Monitoring, 16 (1994) p400-406.

[125] Foglia J.P., Sorisio D., Kirshner M. and Pollock B.G. "Quantitative Determination of Paroxetine in Plasma by High-Performance Liquid Chromatography and Ultraviolet Detection." Journal of Chromatography B, 693 (1997) p147-151.

[126] Knoeller, J, Vogt-Schenkel, R. and Brett, M.A. "A Simple and Robust HPLC Method for the Determination of Paroxetine in Human Plasma." Journal of Pharmaceutical and Biomedical Analysis, 13 (4/5) (1995) p635-638.

[127] Brett M.A., Dierdorf H.D., Zussman B.D. and Coates P.E. "Determination of Paroxetine in Human Plasma, Using High-Performance Liquid Chromatography with Fluorescence Detection." Journal of Chromatography, 419 (1987) p438-444.

[128] Gupta R.N. "Column Liquid Chromatographic Determination of Paroxetine in Human Serum using Solid-Phase Extraction." Journal of Chromatography B, 661 (1994) p362-365.

[129] Ramaiya, A. and Karnes, H.T. "Simultaneous measurement of serotonin and paroxetine in rat brain microdialysate by a single-pump column-switching technique." Journal of chromatography B, 691 (1997) p119-129.

[130] Shin, J-G., Kim, K-A., Yoon, Y-R., Cha, I-J., Kim Y-H. and Shin S-G. "Rapid simple high-performance liquid chromatographic determination of paroxetine in human plasma." Journal of Chromatography B, 713 (1998) p452-456.

[131] Lopez-Callul, C. and Dominguez, N. "Determination of paroxetine in plasma by high-performance liquid chromatography for bioequivalence studies." Journal of Chromatography B, 724 (1999) p393-398.

[132] Lai, C-T., Gordon, E.S., Kennedy, S.H., Bateson, A.N., Coutts, R.T., Baker, G.B. "Determination of paroxetine levels in human plasma using gas chromatography with electron-capture detection." J. of Chromatography B, 749 (2000) p275-279.

[133] Proelss, H.F., Lohmann, H.J. and Miles, D.G. "High-Performance LiquidChromatographic Simultaneous Determination of Commonly Used Tricyclic Antidepressants." Clin. Chem., 24(11) (1978) p 1948-1953.

[134] Malamud, D. and Tabak, L. (Ed.) "Saliva as a Diagnostic Fluid." Annals of the New York Academy of Sciences, 694 (1993) p1-318.

[135] Peel, H.W., Perrigo, B.J. and Mikhael, N.Z. "Detection of saliva of impaired drivers." Journal of Forensic Science, 29 (1984) p185-189.

[136] Skopp, G., Potsch, L. "Perspiration versus saliva – basic aspects concerning their use in roadside drug testing." Int. J. Legal Med., 112 (1999) p213-221.

[137] Jacobson, E.D. "Salivary Secretion." In Johnson L.R. (Ed.) "Gastrointestinal physiology", 2nd edition, CV Mosby, St Louis, (1981) p46-54.

[138] Mandel, I.D. "Relation of Saliva and Plaque to Caries." Journal of Dental Research, 53 (1974) p246-266.

[139] Mucklow, J.C. "The Use of Saliva in Therapeutic Drug Monitoring." Therapeutic Drug Monitoring, 4 (1982) p229-247.

[140] Baselt, R.C. (Ed.) "Advances in Analytical Toxicology." Volume 1, Biomedical Publications, Foster City, California, (1984) p198-254.

[141] Ritschel, W.A. and Tompson, G.A. "Monitoring of drug concentrations in saliva: a non-invasive pharmacokinetic procedure." Meth. Find. Exp. Clin. Pharmacol., 5 (1983) p511-525.

[142] Schneyer, L.H., Pigman, W., Hanahan, L. and Gilmore, R.W. "Rate of flow of human parotid, sublingual and submaxillary secretions during sleep." Journal of Dentistry Research, 35 (1956) p109-114.

[143] Shannon, I.L. "Climatological effects on human parotid gland function." Arch. Oral Biol., 11 (1966) p451-453.

[144] Gutman, D. and Ben-Aryeh, H. "The influence of age on salivary content and rate of flow." International Journal of Oral Surgery, 3 (1974) p324-317.

[145] Blitzer, A. "Inflammatory and obstructive disorders of salivary glands." Journal of Dental Research, 66 (1987) p675-679.

[146] Matthews, R.W., Bhoola, K.D., Rasker, J.J. and Jayson, M.I. "Salivary secretion and connective tissue disease in man." Annals of the Rheumatic Diseases, 44 (1985) p20-26.

[147] Dutta, S.K., Dukehart, M., Narang, A. and Latham, P.S. "Functional and structural changes in parotid glands of alcoholic cirrhotic patients." Gastroenterology, 96 (1989) p510-518.

[148] Hugoson, A. "Salivary secretion in pregnancy." Acta Odontol. Scand., 30 (1972) p49-66.

[149] Rashid, M.U., Bateman, D.N. "Effect of intravenous atropine on gastric emptying, paracetamol absorption, salivary flow and heart rate in young and fit elderly volunteers." British Journal of Clinical Pharmacology, 30 (1990) p25-34.

[150] Gelenberg, A.J., Wojcik, J.D., Falk, W.E., Spring, B. and Brotman, A.W. "Clovoxamine in the treatment of depressed outpatients: a double-blind, parallel-group comparison against amitriptyline and placebo." Comprehensive Psychiatry, 31 (1990) p307-314.

[151] Glass, B.J. "Drug-induced xerostomia as a cause of glossodynia." Ear, Nose and Throat Journal, 68 (1989) p779-781.

[152] Knoebel, L.K. "Secretion and action of digestive juices, absorption". In Selkurt (Ed.) "Physiology" Little Brown, Boston, (1966) p571-602.

[153] Schnidt-Nielsen, B. "The pH in parotid and mandibular saliva." Acta Physiol Scand, 11 (1946) p104-110.

[154] Dvorchik, B.H. and Vesell, E.S. "Pharmacokinetic Interpretation of data gathered during therapeutic drug monitoring." Clinical Chemistry, 22 (1976) p868-878.

[155] Cone, E.J., Oyler, J. and Darwin, W.D. "Cocaine disposition in saliva following intravenous, intranasal, and smoked administration." Journal of Analytical Toxicology, 21 (1997) p465-475.

[156] Navazesh, N. "Methods for Collecting Saliva." Ann. N.Y. Acad. Sci., 694 (1993) p73-77.

[157] Danhof, M. and Breimer, D.D. "Therapeutic Drug Monitoring in Saliva." Clinical Pharmacokinetics, 3 (1978) p39-57.

[158] Chang, K. and Chiou, W.L. "Interactions between drugs and saliva-stimulating parafilm and their implications in measurements of saliva drug levels." Res. Comm. Chem. Path. Pharmacol., 13 (1976) p357-360.

[159] Jufer, R.A., Wstadik, A., Walsh, S.L., Levine, B.S. and Cone, E.J. "Elimination of cocaine and metabolites in plasma, saliva, and urine following repeated oral administration to human volunteers." Journal of Analytical Toxicology, 24(7) (2000) p467-477.

[160] O'Neal, C.L., Crouch, D.J., Rollins, D.E. and Fatah, A.A. "The effects of collection methods on oral fluid codeine concentrations." Journal of Analytical Toxicology, 24(7) (2000) p536-542.

[161] Communication Nele Samyn The International Association of Forensic Toxicologists: Young Scientists Meeting, Gent, Belgium (2001).

[162] Glynn, G.P. and Bastain, W. "Salivary excretion of paracetamol in man." J. Pharm. Pharmacol., 25 (1973) p420.

[163] Koysooko, R., Ellis, E.F. and Levy, G. "Relationship between theophylline concentration in plasma and saliva of man." Clin. Pharmacol. Ther., 15 (1974) p454.

[164] Huffman, D.H. "Relationship between digoxin concentrations in serum and saliva." Clin. Pharmacol. Ther., 17 (1975) p310.

[165] Inaba, T. and Kalow, W. "Salivary excretion of amobarbital in man." Clin. Pharmacol. Ther., 18 (1975) p588.

[166] Cook, C.E., Amerson, E. and Poole, W.K. " Phenytoin and phenobarbital concentrations in saliva and plasma measured by radioimmunoassay." Clin. Pharmacol. Ther., 18 (1975) p742.

[167] Vesell, E.S., Passananti, G.T., Glenwright, P.A. and Dvorchik, B.H. "Studies on the disposition of antipyrine, aminopyrine and phenacetin using plasma, saliva and urine." Clin. Pharmacol. Ther., 18 (1975) p259.

[168] McCarron, M.M., Walberg, C.B., Soares, J.R., Gross, S.J., and Baselt, R.C. "Detection of phencyclidine usage by radioimmunoassay of saliva." Journal of Analytical Toxicology, 8 (1984) p197-201.

[169] Hawks, R.L. "The constituents of cannabis and the disposition and metabolism of cannabinoids." Natl. Inst. Drug Abuse Res. Monogr. Ser., 42 (1982) p125-137.

[170] Cooper, T.B., Bark, N. and Simpson, G.M. "Prediction of steady state plasma and saliva levels of desmethylimipramine using a single dose, single time point procedure." Psychopharmacology, 74 (1981) p115-121.

[171] Baumann, P., Tinguely, D., Koeb, L., Schopf, J. and Le, P.K. "On the relationship between free plasma and saliva amitriptyline and nortriptyline." Int. Pharmacopsychiatry, 17 (1982) p136-146.

[172] Baumann, P., Koeb, L., Tinguely, D. and Rivier, L. "A method for the analysis of free, total plasma and saliva amitriptyline and nortriptyline by dialysis and GC/MS." European Journal of Mass Spectrometry in Biochemical, Medicine and Environmental Research, 2 (1/2) (1982) p19-26.

[173] Tracqui, A., Kreissig, P., Kintz, P., Pouliquen, A. and Mangin, P. "Determination of amitriptyline in the hair of psychiatric patients." Human and Experimental Toxicology, 11 (1992) p363-367.

[174] Kintz, P., Marescaux, C. and Mangin, P. " Testing human hair for carbamazepine in epileptic patients: is hair investigation suitable for drug monitoring." Human Exp. Toxicol., 11 (1992) p363.

[175] Matsuno, H., Uematsu, T., and Nakashima, M. "The measurement of haloperidol and reduced haloperidol in hair as an index of dosage history." Br. J. of Clin. Pharmacol, 29 (1990) p187.

[176] Tracqui, A., Kintz, P., and Mangin, P. "Hair analysis: a worthless tool for therapeutic compliance monitoring." Forensic Science International, 70 (1995) p183.

[177] http://www.depression-net.com/dep.html

[178] Ishiyama, I., Nagai, T. and Toshida, S. "Detection of Basic Drugs (Methamphetamine, antidepressants, and nicotine) from human hair." Journal of Forensic Sciences, 28 (1983) p380-385.

[179] Kintz, P. and Mangin, P. "L'analyse des medicaments et des stupefiants dans les cheveux." J. Med. Strasbourg, 22(9) (1991) p518-522.

[180] Couper, F.J., McIntyre, I. and Drummer, O.H. "Extraction of Psychotropic Drugs from Human Scalp Hair." Journal of Forensic Sciences, 40 (1995) p83-86.

[181] Pragst, F., Rothe, M. Hunger, J. and Thor S. "Structural and concentration effects on the deposition of tricyclic antidepressants in human hair." Forensic Science International, 84 (1997) p225-236.

[182] Negrusz, A., Moore, C.M. and Perry, J.L. "Detection of doxepin and its major metabolite desmethyldoxepin in hair following drug therapy." Journal of Analytical Toxicology, 22 (1998) p531-536.

[183] Gaillard, Y. and Pepin, G. "Screening and identification of drugs in human hair by high-performance liquid chromatography-photodiode-array UV detection and gas chromatography-mass spectrometry after solid-phase extraction." Journal of Chromatography A, 762 (1997) p251-267.

[184] Lefebvre, M., Marchand, M., Horowitz, J.M. and Torres, G. "Detection of fluoxetine in brain, blood, liver and hair of rats using gas chromatography-mass spectrometry." Life Sciences, 64 (9) (1999) p805-811.

[185] Wilhelm, M., Hafner, D., Lombeck, I. and Ohnesorge, F.K. "Monitoring of cadmium, copper, lead and zinc status in young children using toenails: comparison with scalp hair." Sci. Total Environ., 103 (1991) p199-207.

[186] Alexiou, D., Koutselinis, A., Manolidis, C., Boukis, D., Papadatos, J., and Papadatos, C. "The content of trace elements (Cu, Zn, Fe, Mg) in fingernails of children." Dermatologica, 160 (1980) p380-382.

[187] Gerhardsson, L., Englyst, V., Lundstrom, N.G., Nordberg, G., Sandberg, S. and Steinvall F. "Lead in tissues of deceased lead smelt workers." J. Trace Elem. Med. Biol., 9 (1995) p136-143.

[188] Howie, R.A. and Hamilton Smith. "Mercury in Human Tissue." Journal of The Forensic Science Society, 7 (1967) p90-96.

[189] Lander, H., Hodge, P.R. and Crisp, C.S. "Arsenic in the hair and nails. Its significance in acute arsenical poisoning." Journal of Forensic Medicine, 12 (1965) p52-67.

[190] Shapiro, H.A. "Arsenic content of human hair and nails. Its interpretation." Journal of Forensic Medicine, 14 (1967) p65-71.

[191] Pounds, C.A., Pearson, E.F. and Turner, T.D. "Arsenic in fingernails." Journal of Forensic Science, 19 (1979) p165-173.

[192] Uematsu, T., Sato, R., Suzuki, K., Yamaguchi, S. and Nakashima, M. "Human scalp hair as evidence of individual dosage history of haloperidol : method and retrospective study." Eur. J. Clin. Pharmacol., 37 (1989) p239-244.

[193] Suzuki, O., Hattori, H. and Asano, M. "Nails as useful materials for detection of methamphetamine or amphetamine abuse." Forensic Science International, 24 (1984) p9-16.

[194] Suzuki, S-I., Inoue, T., Hori, H. and Inayama, S. "Analyisis of methamphetamine in hair, nail, sweat and saliva by mass fragmentography." Journal of Analytical Toxicology, 13 (1989) p176-178.

[195] Cirimele, V., Kintz, P. and Mangin, P. "Detection of amphetamines in fingernails: an alternative to hair analysis." Arch. Toxicol., 70 (1995) p68-69.

[196] Tiess, D., Wegener, R., Rudolph, I., Steffen, U., Tiefenbach, B., Weirich, V. and Zack, F. "Cocaine and benzoylecgonine concentrations in hair, nails and tissues: A comparative study of ante and post mortem materials in a case of an acute lethal cocaine intoxication." Proceedings of the 32nd T.I.A.F.T. Meeting, Tampa, Florida (1994).

[197] Garside, D., Ropero-Miller, J.D., Goldberger, B.A., Hamilton, W.F. and Maples, W.R. "Identification of cocaine analytes in fingernail and toenail specimens." Journal of Forensic Sciences, 43(5) (1998) p974-979.

[198] Engelhart, D.A., Lavins, E.S. and Sutheimer, C.A. "Detection of drugs of abuse in nails." Journal of Analytical Toxicology, 22 (1998) p314-318.

[199] Lemos N.P., Anderson R.A. and Robertson J.R. "Nail analysis for drugs of abuse: extraction and determination of cannabis in fingernails by RIA and GC/MS." J. Anal. Tox., 23 (1999) p146-52.

[200] Lemos, N.P., Takaichi, K., Anderson, R.A. and Robertson, J.R. "Evaluation of alternative cannabinoid extraction procedures for nail clippings from chronic cannabis smokers." Proceedings of the 53rd American Academy of Forensic Sciences, Seattle, Washington (2001) p318-319.

[201] Lemos, N.P., Anderson, R.A., Valentini, R., Tagliaro, F., Scott, R.T.A. "Analysis of morphine by RIA and HPLC in fingernail clippings obtained from heroin users." Journal of Forensic Sciences, 45 (Suppl 2) (2000) p407-412.

[202] Montagna, W. and Parakkal, P.F. "The Structure and Function of Skin." Third Edition, Academic Press New York and London (1974).

[203] Zook, E.G., Allen, M.D., Van Beek, M.D., Russell, R.C. and Beatty, M.E. "Anatomy and physiology of the perionychium: A review of the literature and anatomic study." The Journal of Hand Surgery, 5(6) (1980) p528-536.

[204] Fraser, R.D.B., MacRae, T.P. and Rogers, G.E. "Keratins: Their Composition, Structure and Biosynthesis." Charles C. Thomas, Illinois, U.S.A. (1972)

[205] Pinkus, F. "Die normale anatomie der Haut." In "Handbuch der Haut und Geschlechtskrankheiten" Jadassohn, J. (Ed.), Springer-Verlag, Berlin and New York (1927) p1-378.

[206] Lewis, B.L "Microscopic studies of foetal and mature nail and surrounding soft tissue." Arch. Dermatol. Syphilol., 70 (1954) p732-744.

[207] Samman, P.D. "The ventral nail." Arch. Dermatol., 84 (1961) p1030-1033.

[208] Lewin, K. "The normal fingernail." British Journal of Dermatology, 77 (1965) p421.

[209] Achten, G. "L'ongle normal et pathologique." Dermatologica, 126 (1963) p229-245.

[210] Jarret, A., and Spearman, R. "Histochemistry of the human nail." Archives of Dermatology, 94 (1966) p652-657.

[211] Zaias, N., and Alvarez, J. "The formation of the primate nail plate: an autoradiographic study in squirrel monkey. J. Invest. Dermatol., 51 (1968) p120-136.

[212] Norton, L.A. "Incorporation of thymidine-methyl-H3 and glycine-2-H3 in the nail matrix and bed of humans. J. Invest. Dermatol., 56 (1971) p61-68.

[213] Johnson, M., Comaish, J.S. and Shuster, S. "Nail is produced by the normal nail bed:a controversy resolved." British Journal of Dermatology, 125 (1991) p127-129.

[214] Johnson, M. and Shuster, S. "Continuous formation of nail along the bed." British Journal of Dermatology, 128 (1993) p277-280.

[215] Pardo-Castello, V. "Diseases of the Nail." Second Edition, Springfield, Illinois (1972), Charles C. Thomas.

[216] Jones, F.W., "The principals of anatomy as seen in the hand." Second Edition, London (1942), Balliere Tindall Cox, p121-134.

[217] Le Gros Clark, W.E. and Dudley Buxton, L.H. "Studies in Nail Growth." British Journal of Dermatology and Syphilis, L (1938) p221-235.

[218] Hamilton, J.B., Terada, H., Mestler, G.E. "Studies of growth throughout the lifespan in Japanese:growth and size of nails and their relationship to age, sex, heredity, and other factors." (1955) p401-415.

[219] Baden, H.P. "Regeneration of the nail." Archives of Dermatology, 91 (1961) p619-620.

[220] Pepin, G. and Gaillard, Y. "Concordance between self-reported drug use and findings in hair about cocaine and heroin." Forensic Science International, 84 (1997) p37-41.

[221] Jurado, C., Gimenez, M.P., Menendez, M. and Repetto, M. "Simultaneous quantification of opiates, cocaine and cannabinoids in hair." Forensic Science International, 70 (1995) p165-174.

[222] Cirimele, V., Kintz, P. and Mangin, P. "Drug concentration in human hair after bleaching." Journal of Analytical Toxicology, 19 (1995) p331-332.

Appendix 1: Publications in Support of this thesis

Wylie, F.M. and Oliver, J.S. "Solid Phase Extraction of Fluoxetine and Norfluoxetine from Whole Blood Using HPLC." Proceedings of The American Academy of Forensic Sciences 51st Meeting, Orlando, Florida, USA (1999).

Wylie, F.M. and Oliver, J.S. "Detection of Fluoxetine and Norfluoxetine in Saliva and Nails." Proceedings of The American Academy of Forensic Sciences 53rd Meeting, Seattle, Washington, USA (2001).

Solid Phase Extraction of Fluoxetine and Norfluoxetine from Whole Blood using HPLC.

Fiona M. Wylie and John S. Oliver

Department of Forensic Medicine and Science, University of Glasgow, G12 8QQ.

The objective of this research was to develop an efficient solid phase extraction procedure to isolate fluoxetine and its active metabolite, norfluoxetine from whole blood.

Fluoxetine (Prozac®) is a selective serotonin reuptake inhibitor which was first marketed in the United States in 1988 for the treatment of depression. Since then it has become the most widely prescribed antidepressant in the U.S. and one of the most commonly prescribed in the U.K. As a result, the analysis of fluoxetine (FLU) and its active metabolite, norfluoxetine (NFLU) have become increasingly important in clinical environments and also in forensic toxicology.

Both liquid-liquid extraction and solid phase extraction (SPE) methods have been published for the isolation of FLU and NFLU from serum and plasma. However, SPE methods are far fewer and the majority of these involve analysis by gas chromatography with electron capture detection (GC-ECD) which requires a derivatization step. Analysis by high performance liquid chromatography with ultra-violet detection (HPLC-UV) avoids derivatization, therefore reducing the time required for sample preparation. An SPE method using cyanopropyl columns was developed for FLU and NFLU and analysis was carried out using HPLC-UV.

Blank blood was spiked with FLU, NFLU and internal standard, clomipramine. The samples were homogenized, diluted with 0.1M phosphate buffer, pH 6.0 and were applied to the preconditioned cyanopropyl columns. These were selectively washed with various solvent and aqueous mixtures. FLU and NFLU were eluted with dichloromethane/isopropanol 8:2 containing 2 % concentrated ammonia. The eluates were evaporated under nitrogen at 40 ^{o}C and reconstituted in 200 µl of mobile phase. Analysis of the samples was carried out using HPLC with UV detection at 226 nm. Separation was achieved on a Supelcosil LC-PCN 25 cm x 4.6 mm i.d., 5 µm particle column using acetonitrile/methanolic buffer as the mobile phase and a flow rate of 2 ml/min. The methanolic buffer was prepared by buffering 0.01M potassium dihydrogen phosphate to

pH 7.0 using 1M potassium hydroxide solution and mixing it with HPLC grade methanol in the ratio 5:4.

This SPE method was compared with a published liquid-liquid extraction (LLE) method. Spiked blood was alkalinized with 0.5 mol/L sodium phosphate buffer, pH 10. FLU and NFLU were extracted into hexane/n-amyl alcohol (97:3) followed by back extraction into 0.1M hydrochloric acid. Sodium hydroxide (1M) was added to basify the aqueous layer followed by hexane/n-amyl alcohol (97:3). The organic phase was separated, evaporated to dryness and reconstituted in 200 μl of mobile phase. These extracts were analysed using the same chromatographic conditions.

Linear quantitative response curves were produced for FLU and NFLU over the concentration range 0 to 2000 ng/ml for both SPE and LLE methods. Relative recoveries of FLU and NFLU from whole blood were determined by comparing the peak heights obtained from spiked whole blood extracts with unextracted standards. Recoveries > 85% were achieved using the SPE method which were higher than those obtained by the LLE method: 79% and 80% for FLU and NFLU respectively. The limit of detection for the procedure was 15ng/ml.

This SPE method using cyanopropyl columns provided a simple, rapid, clean and selective procedure for the determination of FLU and its active metabolite, NFLU from whole blood achieving consistent analytical recoveries throughout the assay range. The technique can be applied to detect the drug at therapeutic and toxic levels.

Keywords: Fluoxetine, Norfluoxetine, Solid Phase Extraction

Detection of Fluoxetine and Norfluoxetine in Saliva and Nails

Fiona M. Wylie and John S. Oliver

Department of Forensic Medicine and Science, University of Glasgow, G12 8QQ, Scotland, U.K.

Fluoxetine and norfluoxetine were identified in the saliva and nails of patients receiving treatment for depression. Saliva analysis, like blood and urine analysis can be used to indicate recent drug use but has the advantage that sample collection is non-invasive. Nails can be used as an alternative matrix to hair in cases where no hair is available and can give an indication of long-term drug usage.

Saliva samples were collected with Omni-Sal® devices and the analytes were extracted using solid phase extraction with tumoxetine as the internal standard. Samples were derivatized with heptafluorobutyric anhydride and detected by gas chromatography/mass spectrometry using selective ion monitoring. Ions monitored were m/z 344 for fluoxetine and internal standard and m/z 330 for norfluoxetine. Recoveries greater than 86% were achieved for both analytes over a linear range of 0-100 ng/ml saliva. The limit of detection was 1 ng/ml. Six case samples were analysed and concentrations in the range of 6.6-20.1 ng/ml (mean 13.9 ng/ml) were obtained for fluoxetine and 8.2-35.4 ng/ml (mean 16.8 ng/ml) for norfluoxetine. Five out of six of these cases gave higher concentrations for the metabolite.

Nail clippings were decontaminated, dried and hydrolyzed with 1M sodium hydroxide solution. Tumoxetine was added to the cooled hydrolysates and the analytes were extracted using ethyl acetate. Derivatization and detection were carried out as previously. Recoveries from nail were greater than 80% for fluoxetine and norfluoxetine over the linear range of 0-100 ng/10 mg. The limit of detection was 1 ng/10 mg. Five case samples from two patients gave nail concentrations ranging from 0.30-3.27 ng/mg (mean 1.45 ng/mg) for fluoxetine and 0.18-1.72 ng/mg (mean 0.9 ng/mg) for norfluoxetine. In four out of five of these cases the fluoxetine concentration was higher than that of the metabolite.

Keywords: Fluoxetine, Saliva, Nails