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Analysis of Bile and Nail as Alternative Biological Specimens in Forensic Toxicology

A thesis submitted for the Degree of

Doctor of Philosophy

by

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"Στον καθένα μας δίνεται μια ζωή. Το να ζήσουμε με τιμιότητα και ν'αφήσουμε τα σημάδια μας όταν πεθάνουμε είναι το μόνο ουσιαστικό που μπορούμε να κάνουμε. Τίποτα δεν υπάρχει όταν περάσει η ζωή. Ο πλούτος είναι σπουδαίος μόνο για κείνους που έχουν στενό μυαλό. Το σπουδαιότερο πράγμα είναι να κάνουμε το καλύτερο που μπορούμε μ'αυτά που έχουμε."

Άγνωστος

To my beloved parents, Chryssanthee and Petros, and the rest of my family who have offered much support and understanding throughout all my University days in America and Britain and to Manoli and my close friends who have shared the best and the worst with me -

This is for you with all my love.

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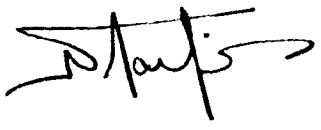

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Abbreviations

mg/l	milligram per litre	No.	number
mM	millimolar	ml	millilitre
ml/min	millilitre per minute	M	molar
µg/l	microgram per litre	°C	degrees centigrade
°C/min	degrees centigrade per minute	r^2	correlation factor
mg	milligram	m	metre
rpm	revolutions per minute	mm	millimetre
ng/ml	nanogram per millilitre	µm	micrometre
eV	electrovolts	µl	microlitre
ng/µl	nanogram per microlitre		
ng/mg	nanogram per milligram		
mg/l	milligram per litre		
µg/ml	microgram per millilitre		
mg/Kg	milligram per kilogram		

Summary

This thesis examines the use of bile and nail as alternative biological specimens in forensic toxicology. In chapter 1, a brief overview of the mechanisms of substance passage into and out of membranes and fluids and matrices was presented followed by a review of the use of alternative biological specimens for analytical purposes.

The experimental part of this thesis begins in chapter 2 where a simple and rapid method for the detection and quantification of dextropropoxyphene in bile and its major metabolite, norpropoxyphene, is presented. The use of bile as an analytical specimen in forensic laboratories is well established but, as yet, little work has been done on the extraction of bile samples using current solid-phase techniques. This study was conducted to develop a gas chromatographic method based on solid-phase extraction for the simultaneous analysis of dextropropoxyphene and its major metabolite, norpropoxyphene, in bile. Bile samples were diluted with phosphate buffer (pH 6.0) and triphenylamine was added as the internal standard. Bile was then extracted using the 200 mg Clean Screen® Extraction Columns. The resulting extract was then analysed by GC-FID using a CHROMPAK CP-SIL 5 CB capillary column. The method was validated and proved to be rapid, specific and reproducible and made dextropropoxyphene analysis in bile readily available as a valuable analytical tool.

Following this, nail is examined as an alternative biological specimen in cases of medico-legal interest. Firstly, two major cannabinoids, Δ^9-tetrahydrocannabinol and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid were identified in the nail (chapter 3) by means of RIA and GC-MS. Cannabinoids were found to be present in all six cases that were analysed for cannabinoids by RIA and their concentration ranged from 0.23 ng/mg to 2.80 ng/mg (average: 1.03 ng/mg). Using GC-MS, Δ^9-tetrahydrocannabinol

was detected in 11 out of the 14 nail clipping hydrolysates after basic extraction with concentrations ranging from 0.13 ng/mg to 6.97 ng/mg (average: 1.44 ng/mg). 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid was not detected in any of these nail clipping hydrolysates but was detected in two of the three fingernail clipping hydrolysates extracted under acidic pH with concentrations ranging from 9.82 ng/mg to 29.7 ng/mg (average: 19.8 ng/mg)

In chapter 4, the detection and quantification of diazepam, the most commonly encountered benzodiazepine, were described. This study offered a protocol involving the analysis of human nails for the presence of diazepam by ECD-GC. Using ECD-GC, diazepam was detected in hydrolysates of all 6 sets of nail clippings in concentrations ranging from 4.37 to 87.8 ng/mg (average: 25.7 ng/mg).

In chapter 5, an analytical protocol for the detection and quantification of morphine in nail clippings of known heroin abusers was presented based on initial screening by RIA and confirmation by HPLC. Morphine was determined in hydrolysates of decontaminated nail clippings by RIA (mean: 1.67 ng/mg) and confirmed by HPLC (mean: 2.11 ng/mg).

Finally, a protocol for the detection and quantification of methadone in nail clipping of patients attending a methadone maintenance program based on screening by EIA and confirmation by GC-MS was presented in chapter 6. Methadone was determined in hydrolysates of decontaminated nail clippings by EIA (mean: 32.84 ng/mg) and confirmed by GC-MS (mean: 26.92 ng/mg).

The conclusions of the thesis were presented in chapter 7 together with suggestions for future research on the topics addressed.

1. ALTERNATIVE FLUIDS & MATRICES

1.1. Introduction

At present, drug-testing laboratories around the world rely heavily on the analysis of blood or urine for the detection and quantification of drugs and/or their metabolites. It may, however, be of interest or may be needed, in certain areas of drug monitoring to determine these analytes in biological fluids and matrices other than blood or urine.

Traditionally, systematic forensic toxicology used a wide selection of body tissues and fluids in order to optimise the detection of drugs with the limited analytical techniques available. However, this approach fell out of use due to the advent of more sensitive methods and techniques that were suitable for the analysis of low concentrations of drugs in blood. Also, there has been an increased need for faster (and more cost efficient) turn-around times in obtaining results, and, in most forensic laboratories dealing with the cause of death, interpretation of concentrations of drugs and other substances in these tissues was problematic. The time-consuming and laborious analyses of these tissues did not always contribute significantly to an interpretation based on blood concentrations alone.

In contrast to this past experience where it was not always possible or desirable to sample nonconventional fluids and matrices, drug monitoring in these specimens has been recently reconsidered and in many instances successfully performed. Often this work has grown from different needs, for example, for drug abuse monitoring rather than forensic autopsy cases (Pichini et al., 1996). Drug analyses in specimens such as

amniotic fluid, breast milk and foetal blood may provide evidence of foetal drug exposure (Pacifici et al., 1995). To account for drug elimination by routes other than the renal one, biliary and faecal drug concentrations are monitored (Agarwal et al., 1996). Hair and nails may provide an insight into an individual's past licit or illicit drug use history (Suzuki et al., 1989), while in other cases specimens such as bronchial secretions, nails and tears may confirm drug presence at the site of action (Wong et al., 1975; Willemsen, et al., 1992).

Drug monitoring in nonconventional biological specimens, although still in its early stages, offers significant advantages, for example, extended drug retention and non-invasive sampling. Furthermore, it may reveal important information to the analyst which otherwise could have passed unnoticed (Pichini et al., 1996). Finally, drug determination in fluids and matrices other than blood or urine may be useful in bioavailability studies, drug organ targeting, doping control and localised effect monitoring. For all these reasons, drug monitoring in nonconventional biological specimens is becoming a common research topic around the world and should also be included in the standard operating procedures of laboratories.

The purpose of the present thesis was to establish the usefulness of two alternative biological specimens, bile and nail, in various routine drug tests in forensic toxicology.

1.2. Mechanisms of transport of drugs into fluids or matrices

1.2.1. Membranes

Up to the first part of this century it was believed that the skin provided an effective barrier preventing toxic substances from entering the body (Amdur et al., 1991). We now know that, given enough time, almost every substance can enter the body via one or more routes. Deleterious and other substances pass through several natural barriers during entry into the body which may vary from the relatively thick skin to the relatively thin lung membranes (Gossel et al., 1994). All membranes are basically similar and are described by the unifying cell membrane concept first postulated by Davson and Danielli in 1935. Among the relatively few modifications of the first model, the most significant one is the now well-established fluidity of membranes. All membranes appear to be comprised of two layers of lipids with proteins variously located throughout the lipid bilayers. This amphipathic nature of membranes produces a barrier for ionised, highly polar compounds - but not an absolute barrier. Membranes are much less permeable to compounds in the ionised state than those in the nonionised form (Mullins, 1981; Hodgson et al., 1987).

1.2.2. Ionisation

The relative amounts of the ionised and nonionised forms of a substance depend upon the pK_a of that substance and the pH of the surrounding medium. When the pH of the medium equals the pK_a of the substance, half of the substance is in the ionised form and half is in the nonionised form (Schanker, 1971). The Henderson-Hasselbach equation describes the degree of ionisation for weak acids or weak bases:

$$\log \frac{\text{nonionised form}}{\text{ionised form}} = pK_a - pH \qquad \text{(for weak acids)}$$

$$\log \frac{\text{ionised form}}{\text{nonionised form}} = pK_a - pH \qquad \text{(for weak bases)}$$

A weak acid is half ionised and half nonionised when its pK_a equals the pH of the surrounding environment. When the pH of the surrounding environment is lower than the acid's pK_a (i.e., more acidic environment), most of the acid is in the nonionised or lipid soluble form. Thus, a weak acid readily diffuses though membranes in acidic environments.

Similarly, a weak base is half ionised and half non-ionised when its pK_a equals the pH of the surrounding environment. When the pH of the surrounding environment is greater than the base's pK_a (i.e., more basic environment), most of the base is in the nonionised or lipid soluble form. Thus, a weak base readily diffuses though membranes in basic environments.

1.2.3. Partition Coefficient

The lipid solubility of a substance is measured by the logarithm of its partition coefficient, P, which is a measure of the partition of this substance between aqueous and lipid phases:

$$P = \frac{\text{concentration in lipid phase}}{\text{concentration in aqueous phase}}$$

Thus, a high partition coefficient is indicative of greater lipophilicity. The organic solvent used for the measurement of partition coefficient is usually octanol but other systems have been evaluated (Hansch et al., 1979; Goodman Gilman et al., 1990). Obviously, each lipid solvent used may give a different value and there is often little consistency between penetration and partition coefficients derived from different organic solvents.

For substances which are relatively hydrophilic, the partitioning coefficient, P, is usually calculated after preparation of the sample in octanol-saturated buffer according to the equation:

$$P = \frac{A_B - A_A}{A_A} \times \frac{V_{aq}}{V_{org}}$$

where, A_B is the is the amount of the substance in the octanol-saturated aqueous sample before partitioning between the two phases, A_A is the amount of the substance in the octanol-saturated aqueous sample after partitioning between the two phases, V_{aq} is the volume of the aqueous phase and V_{org} is the volume of the octanol-saturated buffer used (Quye, 1989).

The logarithm of P applies to nonionised substances. This is due to the fact that the partition coefficient of an ionised substance will almost certainly be lower than expected since more of the substance will be in the aqueous phase. To correct for this discrepancy, a correction factor must be included in the calculations and the partition coefficient then becomes the dissociation coefficient, log D:

$$\log D = \log P - \log (1 + 10^{pH-pKa}) \qquad \text{(for weak acids)}$$

$$\log D = \log P - \log (1 + 10^{pKa-pH}) \qquad \text{(for weak bases)}$$

In order to avoid misinterpretation of data, the state (ionised versus nonionised) of the substance at the time of lipophilicity measurements should always be noted.

1.2.4. Passive Transport as a Mechanism of Transport

Passive transport appears to be the predominant mode of transport across membranes for most toxic substances. The rate of movement for a compound with appropriate water/lipid partition coefficient is largely determined by simple diffusion. This may vary considerably among compounds and is not always predictable. Compounds in the ionised form do not move readily through membranes in this fashion (Mehlman, 1976; Benz et al., 1980).

1.2.5. Filtration as a Mechanism of Transport

Membranes often have pores or channels which allow compounds with molecular weights of less than 100 daltons to pass through (DiPalma et al., 1990). Usually, larger molecules are excluded from this mode of transport except in the more highly porous tissues such as kidney and liver (Schanker, 1961). Because most substances encountered in forensic toxicology situations are of higher molecular weights, this route of transport assumes a less important position.

1.2.6. Special Transport as a Mechanism of Transport

There exist a number of special transport systems that aid in the transport of endogenous compounds across membranes. These processes may occur against a concentration gradient with the expenditure of energy (active transport) or may not require energy and be unable to move substances against concentration gradients (facilitated transport). Both processes involve carrier proteins to which substances bind in order to be transported through a membrane (Schanker, 1962). Such mode of transport is faster than simple diffusion and, in the case of active transport, may proceed against a concentration gradient. These mechanisms become important in those instances where a toxic substance has chemical or structural similarities to endogenous chemicals, which rely on mechanisms of special transport for normal physiological uptake, and can thus utilise the same systems.

1.2.7. Endocytosis as a Mechanism of Transport

Phagocytosis for solids and pinocytosis for liquids are two specialised transport processes in which the membrane invaginates or flows around a substance allowing for its quicker transport across membranes. In the lungs, endocytosis is a common mechanism of transport with several compounds relying on lung phagocytosis for transport (Hodgson et al., 1987).

1.3. Alternative Fluids & Matrices

1.3.1. Amniotic Fluid

Collection of amniotic fluid is an invasive procedure which can be harmful to the foetus. This specimen has traditionally been used to monitor foetal development and to detect abnormalities (Cooper et al., 1982). There are toxicological cases where such collection is unavoidable in order to establish the exposure of the foetus to harmful drugs and other toxic substances. Furthermore, in cases where a drug does not readily cross the placenta and must be administered directly into the amniotic fluid, drug level monitoring in this fluid can be useful.

In one recent report amniotic fluid collection and analysis from pregnant women who smoked cigarettes showed presence of nicotine and its metabolite, cotinine (Luck et al., 1984a). The measured levels of these two analytes in amniotic fluid were higher than the ones measured in the serum of the mothers thus indicating a significant prenatal exposure to these harmful chemicals.

1.3.2. Bile

Bile production is carried out by liver cells (hepatocytes) in minute channels termed canaliculi (Smith, 1973). It is in the canaliculi that the primary hepatocyte secretion is modified through the addition or removal of water and electrolytes. According to Dittmer in 1961, the composition of the final product has been found to be species specific. Nutritional and other balances have also been found to alter the composition of bile. In general, the largest portion of bile is water (97%). On average, bile acids account for 1.5% of bile. The remaining portion is made up of several other

ions such as Na^+, K^+, Cl^-, HCO_3^-, proteins, cholesterol and phospholipids (Klaassen, 1974; Klaassen et al., 1984).

Biliary excretion is perhaps the most important contributing source to faecal excretion of xenobiotics and even more important for the excretion of their metabolites. The liver is in a very advantageous position for removing toxic agents from blood after absorption from the gastrointestinal tract, because blood from the tract passes through the liver before reaching the general circulation. Thus, liver can extract compounds from blood and thereby prevent their distribution to other parts of the body. Furthermore, the liver is the main locus of biotransformation of toxicants, and the metabolites thus formed may be excreted directly into bile. Xenobiotics and/or their metabolites entering the intestine with bile may be excreted with faeces or when the physicochemical properties are favourable for reabsorption an enterohepatic recirculation may ensue.

Brauer classified different classes of chemicals excreted in bile based upon their bile to plasma ratios (Brauer, 1959; Brauer et al., 1954). It was suggested by these research reports that chemicals excreted in bile should be categorised into three groups. Substances with a bile to plasma ratio approximately equal to unity belong to class A. Class A substances include Cl^-, Na^+, K^+, and glucose. Compounds with bile to plasma concentration ratios between 10 and 1000 belong to class B. This class includes most bile acids, bilirubin, and several xenobiotics. Finally, class C compounds were defined as those whose bile to plasma concentration ratio is less than one. This class includes cholesterol, sucrose, albumin, phospholipids and other macromolecules.

Empirical data suggest that "most xenobiotics for which biliary excretion is an important route of elimination are class B compounds" (Klaassen et al., 1984). These compounds are commonly referred to as cholephils. From the early work of Brauer and colleagues (1954), Wheeler (1972), and Williams and colleagues (1965), a number of chemical characteristics required for elevated biliary excretion are identified.

Accordingly, substances of lower molecular weight (less than 300) are excreted into the bile in less than 5% of the dose. By iodinating p-aminobenzoic acid, an increased biliary excretion occurs due to the significant increase in molecular weight (Williams et al., 1965). The presence of polar groups appears to facilitate biliary excretion as well. For example, cardiac glycosides which are not ionised but are polar compounds are excreted in the bile in increased amounts (Cox et al., 1959; Caldwell et al., 1971). Conjugation is also regarded as important in the biliary excretion of substances. In particular, conjugation of a chemical or its metabolites with glucuronic acid increases its biliary excretion. Glucuronides of bilirubin, morphine, glutethimide, phenolphthalein and menthol are all known to be excreted in the bile in significant levels (Smith et al., 1966). Several possible explanations on how glucuronidation can enhance biliary excretion have been offered by these and other authors (Hearse et al., 1969). Finally, comparative studies have been performed in order to examine how biliary excretion varies between compounds which possess different lipid-to-water partition coefficients (Meyer-Brunot et al., 1968). These studies found that "the most lipophilic derivative was present in an approximately 100-fold concentration in bile, whereas the least lipophilic substance did not accumulate in the bile."

Biliary excretion has been established as a significant elimination pathway for many drugs in humans (Rollins et al., 1979). This pathway is unique since it offers multiple routes for drug disposition and elimination: enterohepatic recirculation, intestinal metabolism or faecal elimination. Due to the relative inaccessibility of bile in healthy humans, little information on the biliary excretion of drugs in humans exists (Carruthers et al., 1978) and many studies had to be conducted using animal models (Lemos, 1996).

Nonetheless, there have been reports where human bile has been collected following cholecystectomy (Russele et al., 1973) and several drugs have been identified and their concentrations determined in this specimen (Ayliffe et al., 1965; Caldwell et al., 1976; Douglass et al., 1974; Kaye, 1976; Terhaag et al., 1978; Christians et al., 1991). Barbiturates and opiates have been extensively analysed in bile (Gottschalk et al., 1980) and detailed data on these two drug group are, therefore, not presented herein. Other than opiates and barbiturates, benzodiazepines have also been identified in bile in significantly higher concentrations than in blood (Gottschalk et al., 1980; McIntyre et al., 1994). In 50% of the diazepam cases, drug bile concentrations were even higher than liver concentrations (see table 1). As table 2 demonstrates, antidepressant drugs have all shown several-fold higher concentrations in bile than in blood. Drugs such as imipramine, trimipramine, doxepin, sertraline and norsertraline showed higher concentrations in bile than in liver (Gottschalk et al., 1980; Dal Cortivo et al., 1963; Demorest, 1983; Levine, 1986; Robinson, 1979; Taylor, 1982; Kincaid, 1990). Other drugs such as ethchlorvynol, methaqualone and chlorpromazine have also been identified in bile in concentrations higher than blood concentrations and in some cases

(ethchlorvynol, methaqualone, isoniazid, acebutolol, metoprolol, mexiletine and phenmetrazine), bile concentrations were higher than liver concentrations (Gottschalk et al., 1980; Cravey et al., 1968; Kalin et al., 1994; Kempton et al., 1994; Levine et al., 1990; LoDico et al., 1992; Poklis et al., 1983; Rogers et al., 1993; Rohrig et al., 1987; Stajic et al., 1984; Tracqui et al., 1992; Winek et al., 1988). Data on these drugs are presented in table 3. Finally, table 4 presents data on cocaine and three metabolites (ecgonine methyl ester, benzoylecgonine and cocaethylene). In all the cases reported (Agarwal et al., 1996), bile concentrations were higher than the blood concentrations for the parent drug as well as for the metabolites. It is noteworthy that in several cases, cocaethylene was found negative in blood but was present in bile. This further emphasises the advantage of analysing bile as a routine toxicological specimen. If bile samples had not been analysed, the presence of cocaethylene would have been missed in several cases.

From the literature survey, it becomes apparent that a routine analysis of bile could prove useful because most drugs are found in this specimen in significantly higher amounts than in blood. More importantly, a drug present in low concentrations in a system may go undetected if blood is the only specimen analysed (e.g. cocaethylene, colchicine). However, there is a much smaller chance of this happening if bile is also collected and analysed.

As part of the experimental work presented in this thesis, bile was evaluated as an analytical specimen for the detection and quantification of dextropropoxyphene and its major metabolite norpropoxyphene (see chapter 2).

Table 1. Benzodiazepines in bile, blood & liver (average & range in mg/l or mg/kg).

Drug	No. of Cases	Bile	Blood	Liver
Chlordiazepoxide	2	4.0 (1.0-7.0)	2.5 (1.0-4.0)	5.5 (1.0-10)
Diazepam	4	2.8 (0.7-6.0)	0.9 (0.4-1.3)	2.1 (1.2-3.0)
Flurazepam	3	43 (30-64)	8.9 (5.5-11)	52 (9.0-130)

Table 2. Antidepressants in bile, blood & liver (average & range in mg/l or mg/kg).

Drug	No. of Cases	Bile	Blood	Liver
Amitriptyline	23	25 (0.3-171)	2.2 (0.1-17)	27 (0.1-323)
Amoxapine	1	61	18	150
Desipramine	3	39 (18-67)	5.6 (1.2-8.0)	48 (22-87)
Dothiepin	3	65 (12-157)	3.0 (0.9-7.4)	-
Doxepin	3	108 (81-148)	7.4 (1.9-11)	60 (6.7-95)
Fluoxetine	1	0.013	0.006	-
Imipramine	15	45 (1.7-171)	3.7 (0.3-10)	39 (1.3-140)
Maprotiline	1	161	6.2	-
Norsertraline	14	57 (0.1-236)	0.4 (0.02-3.0)	9.4 (0.4-42)
Nortriptyline	1	11	2.0	-
Sertraline	14	11 (0.2-35)	0.3 (0.02-1.3)	5.1 (0.1-34)
Trazodone	1	45	15	57
Trimipramine	1	2.4	1.3	1.8

Table 3. Miscellaneous drugs in bile, blood & liver (average & range in mg/l or mg/kg).

Drug	No. of Cases	Bile	Blood	Liver
Acebutolol	1	416	22	123
Chlorpromazine	3	80 (11-200)	1.37 (0.5-2.0)	115 (6.0-190)
Chlorprothixene	1	3.9	0.1	-
Diltiazem	1	180	11	-
Ethchlorvynol	8	382 (2.5-2000)	78 (1.3-180)	217 (1.8-620)
Flecainide	2	290 (160-419)	53 (13-94)	365 (180-550)
Isoniazid	1	900	43	650
Methapyrilene	2	25 (21-29)	8.05 (7.1-9.0)	34 (22-45)
Methaqualone	2	83 (40-125)	8.1 (8.0-8.3)	30 (25-36)
Methylphenidate	1	5.7	2.8	2.1
Metoprolol	1	254	4.7	6.3
Mexiletine	1	440	38	190
Phenmetrazine	6	4.37 (0.5-19)	0.78 (0.10-2.0)	4.23 (0.3-20)
Thioridazine	1	9.0	3.0	35

Table 4. Cocaine and metabolites in bile & blood (average & range in mg/l or mg/kg).

Drug	No. of Cases	Bile	Blood
Benzoylecgonine	50	12 (0.18-59)	2.43 (0.06-26)
Cocaethylene	13	0.55 (0.39-3.68)	0.08 (0.0-6.44)
Cocaine	50	0.73 (0.02-6.13)	0.15 (0.02-2.18)
Ecgonine methyl ester	50	3.30 (0.11-18)	1.21 (0.07-9.66)

1.3.3. Breast Milk

Extensive work has been carried out on the use of breast milk as an alternative biological sample in order to calculate the approximate dose of a drug administered to the mother that is delivered through this route to breast-feeding infants (Atkinson et al., 1988). The calculated levels were also related to maternal plasma drug concentrations (Levy et al., 1975; Kanako et al., 1979).

Drugs of abuse including morphine, tetrahydrocannabinol and phencyclidine have all been identified in breast milk of addict mothers (Pons et al., 1994) strongly suggesting that mothers using these substances should not breast-feed.

Studies on the transfer of nicotine and its metabolite cotinine into breast milk of smoking mothers have confirmed a rapid transfer rate from serum to milk (Luck et al., 1984b). Ideally, mothers should not smoke during the breast-feeding period but if they do, this should be delayed for as long as possible after the last cigarette in order for nicotine to be eliminated. This is usually not a very time-consuming process given the short elimination half-life of nicotine (approximately 100 minutes).

1.3.4. Cerebrospinal Fluid

Despite the difficulty of sampling cerebrospinal fluid, there exists a body of literature concerning drug monitoring in this fluid (i.e. lumbar puncture is a very invasive technique which can only be performed by highly trained personnel).

Several central nervous system (CNS) active drugs (psychoactive drugs, anticonvulsant drugs, etc.) have been monitored in cerebrospinal fluid in order to determine their passage rates through the blood-brain barrier and to correlate drug concentrations and clinical efficacy and toxicity (Morselli et al., 1977; Houghton et al, 1975). Close attention has been paid to drug distribution in cerebrospinal fluid due to the severity of the situations that the drugs cause or aim to cure and because of the unique permeability properties of the blood-brain barrier.

1.3.5. Cervical Mucus

There is not a significant amount of information on the determination of drugs and/or their metabolites in cervical mucus. Published reports, which have been based on animal models, have qualitatively demonstrated the transfer of drugs from the male reproductive tract to the female one during mating (Hales et al., 1986).

There seems to be an increased interest in the role of smoking in cervical cancer. Studies have been conducted evaluating the presence of the primary tobacco component, nicotine, in cervical mucus of smokers. One such study has reported both nicotine and its major metabolite, cotinine, present in the uterine cervix and suggested that more tobacco constituents could probably reach the cervical mucus (Sasson et al., 1985).

1.3.6. Faeces

There are published reports examining the use of faeces as an analytical matrix for the determination of drug pharmacokinetic and metabolic pathways (Midgley et al, 1983; Bernareggi, 1993; Van Gijn et al., 1993). Faecal analysis has been determined to be of importance in toxicokinetic studies of xenobiotics in order to determine factors such as metabolism and routes of elimination (Hologgitas et al., 1980; Meuldermans et al., 1983; Hege et al., 1984; de Kok et al., 1992). Furthermore, measurement of drugs and toxins from mushrooms and/or their metabolites in faeces aids in the determination of their biliary excretion and enterohepatic recirculation (de Kok et al., 1992; Jensen et al., 1993). Due to the inaccessibility of the human biliary tract, these determinations would, otherwise, be very difficult to achieve.

1.3.7. Foetal Blood

Just like amniotic fluid above, foetal blood collection can be damaging to the foetus. Nevertheless, there have been several reports offering information on the concentration of drugs and/or their metabolites in this fluid (Kafetzis et al., 1983; Brown et al., 1990; Pacifici et al., 1995). Drug concentration determination in foetal and maternal blood can help identify those drugs that produce a therapeutic effect on the mother but do not cross the placenta easily (Sperling et al., 1992). In this manner it was determined that some drugs reach a concentration equilibrium in foetal and maternal blood (Jordheim et al., 1980; Kafetzis et al., 1983) whereas others are not readily transferred through the placenta (Brown et al., 1990).

1.3.8. Hair

Starting in the late 1970s through the 1980s and into the 1990s, a very large volume of work has been published throughout the world on the use of hair as an analytical specimen, primarily due to the advantages this specimen may offer compared to blood or urine (Katz et al., 1988; Moeller et al., 1993a). As described by Uematsu in 1993, the main advantage of hair analysis is that evidence of one's profile of drug abuse may be obtained, covering a much more extended period of time (from weeks to months) compared to blood or urine (from hours to days). Furthermore, by choosing hair over blood or urine, sampling becomes a much easier and less invasive procedure that can be performed by almost any person.

In the late 1970s and 1980s, the analytical community's interest focused on the application of established detection techniques in hair analysis for drugs of abuse. Hair was evaluated as a specimen for determining opiate abuse histories and substances such as morphine, heroin and their metabolites were successfully identified (Baumgartner et al., 1979; Klug, 1980; Püschel et al., 1983; Marigo et al., 1986). In 1981, Smith et al., looking for methods to detect acidic drugs in hair, adopted a commercially available RIA for barbiturates and were able to detect phenobarbital in a single hair weighing less than one half of a milligram. Moreover, in the 1980s, several other drugs - including methamphetamine, nicotine, amitriptyline, nortriptyline, cocaine and phencyclidine – were also identified in hair (Baumgartner et al., 1981; Baumgartner et al., 1982; Ishiyama et al., 1983; Offidani et al., 1989) in an effort to determine if patients were under long-term usage of drugs. Single hairs from suspected methamphetamine users

were also used in a study which successfully detected amphetamine and methamphetamine by GC-MS (Suzuki et al., 1984a).

In the early and mid 1990s, more drugs were identified in hair (Marsh et al., 1994) and a plethora of specialised forensic tests involving comparisons between hair and other biological specimens was published (Cone, 1990; Wang et al., 1994; Bermejo Barrera et al., 1995; DuPont et al., 1995; Kintz, 1996). The study by Cone which appeared in 1990 compared morphine and codeine levels in plasma, urine, saliva and hair from two males with a history of intravenous heroin abuse. The author of that study reported that in the limited population studied (n=2), there appeared to exist a dose-response relationship between dose administered and measured drug levels in hair, and that there existed a minimum of a 7-day time delay between the drug administration and its appearance in hair (presumably due to the growth time required for the hair shaft to grow to a height above the skin surface sufficient for sampling).

Furthermore, tests were developed for specific drugs and their metabolites in hair of known drug users as reported by various research teams throughout the world (Goldberger et al., 1991; Nakahara et al., 1992; Cone et al., 1993; Moeller et al., 1993b; Kintz et al., 1994a; Kintz et al., 1994b; Nakahara et al., 1994a; Nakahara et al., 1994b; Couper et al., 1995a; Couper et al., 1995b; Goullé et al., 1995; Gwent et al., 1995; Gygi et al., 1995; Jurado et al., 1995; Kikura et al., 1995a; Kikura et al., 1995b; Kintz et al., 1995a; Kintz et al., 1995b; Magura et al., 1995; Marsh et al., 1995; Moeller et al., 1995; Nakahara, 1995; Selavka et al., 1995a; Selavka et al., 1995b; Staub, 1995; Strano-Rossi et al., 1995; Kauert et al., 1996; Kintz et al., 1996a; Nakahara et al., 1996; Sakamoto et al., 1996). In this way, in heroin, cocaine and methadone abuse cases, 6-

acetylmorphine, benzoylecgonine and EDDP were identified, respectively, among other metabolites. Moreover, published reports showed for the first time that substances like cocaine, an otherwise labile substance subject to rapid metabolism and breakdown in solution, may be retained in hair specimens for 3000 years under favourable conditions (Springfield et al., 1993).

In addition, research was conducted on the presence of drugs of abuse in hair of neonates whose mothers were known drug abusers and drugs such as heroin, nicotine, benzodiazepines, cocaine and amphetamines were identified in the neonates' hair (Kintz et al., 1992; Kintz et al., 1993; Koren, 1995). This research indicated that at least in the case of nicotine, a significant correlation existed between the drug levels in the neonates' hair and in the hair of their mothers.

As more information on the anatomy and physiology of hair (e.g. Harkey, 1993; Wilk et al., 1995) and its response to cosmetic treatments (Dawber, 1996; Pötsch et al., 1996a) reached the forensic community and models for the mechanisms behind drug incorporation into the hair matrix were proposed (e.g. Henderson, 1993; Harkey, 1995; Cone, 1996; Pötsch et al., 1996b), the scientific community's attention was drawn to other issues regarding the use of hair as a reliable analytical specimen in forensic toxicology.

Firstly, questions were raised regarding the apparent patterns of racial bias in hair assays as a result of the increased binding of drugs to melanin (Kidwell, 1990; Reid et al., 1994; Green et al., 1996a; Gygi et al., 1996; Joseph et al., 1996; Gygi et al., 1997). Published research started to include hair colour and/or race information

(Kidwell et al., 1990; Goldberger et al., 1991; Cone et al., 1991; Mieczkowski et al., 1993) in an effort to allow for any racial bias patterns to emerge and be studied.

Secondly, research was published which indicated that hair originating in different parts of the human body contains substantially different amounts of drugs (Ballabanova et al., 1989a, 1989b; Mangin et al., 1993; Nakahara et al., 1993; Offidani et al., 1993a). The slower growth rate of pubic hair as described by Trotter in 1963 and suspected environmental contamination from perspiration and/or sweat were offered as reasons for the concentration differences observed.

It was also at this time that reports appeared in the literature which reviewed the current state-of-the-art in hair analysis for drugs of abuse as used in forensic cases (Bost, 1993; Cassani et al., 1993; Moeller et al., 1993a; Welch et al., 1993a; Goullé et al., 1997) and related or compared international and inter-laboratory performance on these analyses (Welch et al., 1993b; Kintz, 1995; Jurado et al., 1996; Sniegoski et al., 1996; Cassani et al., 1997) in an effort to validate their results.

In the late 1990s, the scientific community remained focused on the use of hair as an analytical specimen and more work on this subject was published. Comparison of hair to other biological specimens remained a topic of interest (Kidwell et al., 1997; Kronstrand et al., 1998) as were the limitations of this specimen in regards to environmental contamination (Blank et al., 1995; Wang et al., 1995), reports of irregular hair growth (Sachs, 1995), and the melanin bias in hair (Gygi et al., 1997). Hair was used for the detection of more drugs and their metabolites (Nakahara et al., 1997; Sachs et al., 1998) and the use of more advanced analytical techniques in hair studies (Uhl, 1997; Keller et al., 1998; Tagliaro et al., 1998) has been offered in the literature in an

effort to strengthen the status and admissibility of this specimen as evidence in courts of law (Huestis, 1996; Moeller, 1996).

1.3.9. Interstitial Fluid

The human skin functions as a barrier for many chemical compounds. Some drugs possess physicochemical and pharmacokinetic properties which allow permeation through the skin. Such drugs are often delivered transdermally (i) in skin diseases (topical administration), (ii) in the form of patches (low-concentration, constant-release of nicotine, theophylline, etc.) and (iii) in neonatal therapy where the neonate's immature stratum corneum allows drugs that otherwise have low rates of transdermal penetration to be absorbed as part of a successful therapy (Schenplein et al., 1971; Harpin et al., 1983; Evans et al., 1985; Pichini et al., 1996).

Collection and analysis of interstitial fluid have been used in the past as a means of verification of drug penetration and to determine the feasibility of this administration route (Barrett et al., 1994). In this way the penetration into skin blister fluid of antibiotics (Schreiner et al., 1978; Soldberg et al., 1983), psoralens (De Wolff et al., 1986) and drugs effective in the treatment of dermatosis has been established.

Interstitial fluid collection is traditionally a very invasive technique although recently there have been reports published of a new, non-invasive method for it in infants which relies on a transcutaneous collection system (Murphy et al., 1990). The traditional collection method used in adults is the so-called 'suction blister fluid.' This involves the creation of suction blisters in the skin through the use of a vacuum pump and the withdrawal of the interstitial fluids with a needle and syringe (Herfst et al., 1980).

The use of interstitial fluid to establish the pharmacokinetic profile of drugs in the human body is gaining momentum especially since published reports appeared describing situations where certain drugs are present in the interstitial fluid only after one mode of administration (e.g. systemic) but not after another (e.g. topical application) because of their poor penetration through the skin (Haneke, 1990; Haneke, 1992; Laugier et al., 1994).

1.3.10. Nail

A part of the human skin, nail (see figure 1) and its immediate surroundings have been extensively studied (Forslind et al., 1975; Fleckman, 1985; Dykyj, 1989; Zook, 1990; Ditre et al., 1992; McCarthy, 1995; Hirai et al., 1995). Traditionally, nail has been used as an analytical specimen in studies examining the efficacy of antifungal agents such as itraconazole (Gauwenbergh et al., 1988; Willemsen, et al., 1992), fluconazole (Hay, 1992; Faergemann et al., 1996) and terbinafine (Matsumoto et al., 1995; Schatz et al., 1995; Nandwani et al., 1996) as reviewed by Zaias et al. in 1996.

The main purpose of nail in man is to provide protection of the finger's terminal phalanx (Samman, 1978). It is also there to help in touch and for picking up small objects as well as for scratching. The nail plate consists of keratin and is derived from an invagination of the epidermis. Zaias has reported as early as 1963 that this invagination is first visible in the 9-week embryo and that the nail is virtually formed by the 20th week (see figure 2).

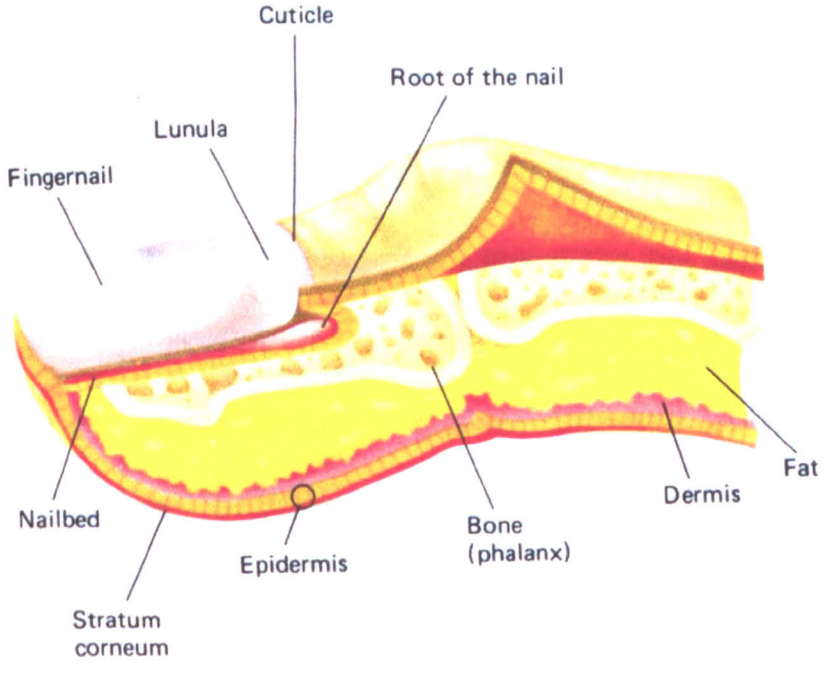


Figure 1. The nail unit (Copyright © 1990 by Saunders College Publishing).

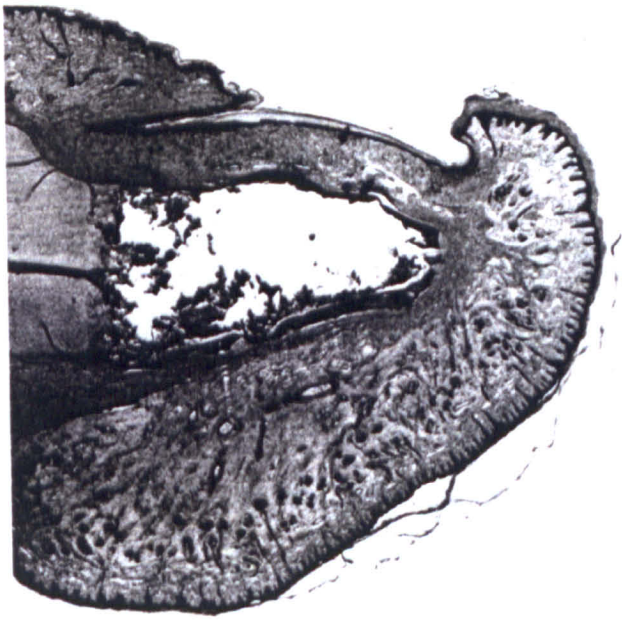


Figure 2. Longitudinal section through the distal phalanx of a full term foetal toe (Copyright © 1978 by Peter D. Samman).

The nail fold consists of a roof, a floor and lateral walls, whilst the nail bed represents that part of the terminal phalanx which lies below the exposed nail plate. There has been some debate regarding the area of the nail fold and nail bed which takes part in the formation of the nail plate. Zaias et al. in 1968 described that the nail plate is derived from the matrix and that there was some probability that a small part of the nail roof of the nail fold also takes part in the formation of the nail plate. The nail bed was not considered to take part in the formation of the nail plate. These observations of Zaias et al. were based on experiments on the squirrel monkey, a primate with flat nail very similar to that of a human (see figure 3a).

Experiments on human subjects, however, showed that there was some activity in the nail bed and so the formation of the nail in the squirrel monkey was somewhat different from that in humans (Norton, 1971). As early as 1954, the opinion was put forward that the nail is not formed from one source but in three layers which were termed 'dorsal,' 'intermediate' and 'ventral' nails (Lewis, 1954). The intermediate nail being the main portion is derived from the greater part of the matrix. The ventral nail is derived from the nail bed distal to the lunula while the dorsal nail is derived from the roof and a small portion of the floor of the nail fold (see figure 3b).

These two models of nail formation oppose the original theory on nail formation as described by Pinkus in 1927 quoting Boas (1894). This theory described three parts of the nail bed. Firstly, the proximal part extends as far forward as the distal margin of the lunula which is considered to be the nail forming or fertile part of the nail bed. Secondly, the part on which the nail plate rests and which takes no place in the formation of the nail (sterile part). This part extends from the distal margin of the

lunula to the line where the anterior edge of the nail separates from the bed (also known as the onychodermal band). Lastly, the 'sole horn' (sohlenhorn) part which brings horny substance to the nail but does nor form any part of it (see figure 3c).

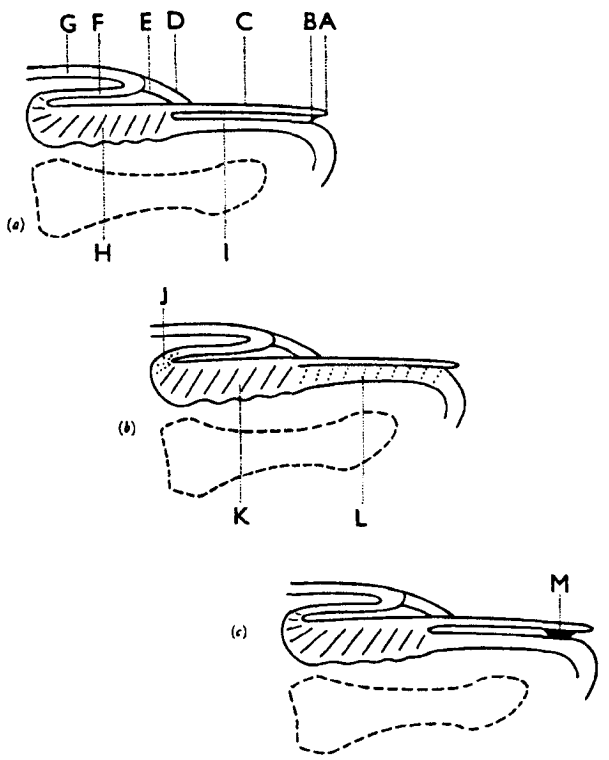


Figure 3. Nail formation – antero-posterior section (Copyright © 1978 by Peter D. Samman).

a. Traditional theory
b. Theory of Lewis
c. Theory of Boas
A. Free margin of nail
B. Hyponychium
C. Nail plate
D. Cuticle
E. Eponychium
F. Roof of the nail fold
G. Skin overlying posterior nail fold
H. Matrix
I. Nail bed
J. Matrix of dorsal nail
K. Matrix of intermediate nail
L. Matrix of ventral nail
M. Solenhorn

More recent research (Dawber et al., 1984; Samman, 1986; Zaias, 1980; Schel et al., 1997) allows for a more complete picture on the structure of the nail unit to be described (see figure 4).

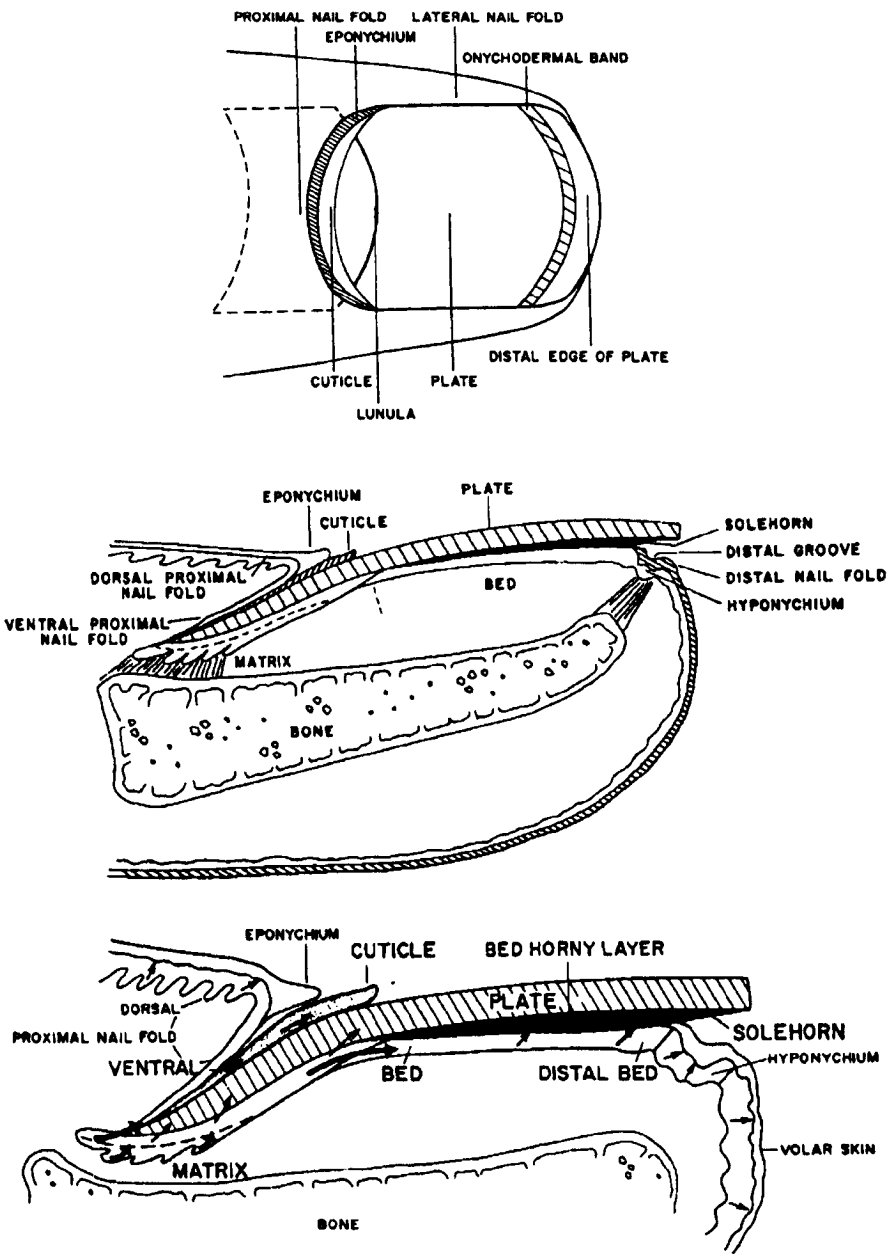


Figure 4. Dorsal and sagittal views of the nail unit (Copyright © 1997 by W.B. Saunders Company).

According to these reports the nail unit consists of six main components:

1. The portion responsible for generating the nail, the nail matrix.
2. The actual product, the nail plate.
3. The portion responsible for ensheathing comprised of the visible cuticle (or eponychium) and the true cuticle.
4. The portion responsible for support, the nail bed and the phalanx.
5. The portion responsible for anchoring, the ligaments between the phalanx and the distal and lateral grooves.
6. The portion responsible for framing, the nail walls (or nail folds).

Research on the structure and behaviour of the nail unit is ongoing with the interest of dermatologists remaining high. Recent reports addressed issues regarding the presence of vascular networks surrounding the nail (Wolfram-Gabel et al., 1995) as well as the suspected regeneration of the nail bed (Ogo, 1987).

Nails have been used for the determination of voluntary or involuntary exposure to substances. Harrison and Clemena (1972) have shown that by mass spectrometry it is possible to estimate the quantity of many trace elements in human finger nail clippings. Pounds et al. also published a method for the analysis of arsenic in nail in 1979. Furthermore, analytical protocols for the qualitative and quantitative determination of several drugs have been offered including amphetamines (Suzuki et al., 1984b; Suzuki et al., 1989; Cirimele et al., 1995) and cocaine and its metabolites (Tiess et al., 1994; Ropero-Miller et al., 1998; Engelhart et al., 1998). Finally, post-mortem nail clippings

of a three-month old infant tested positive for cocaine and confirmed in this way prenatal drug exposure (Skopp et al., 1997).

As part of the experimental work presented in this thesis, nail clippings were evaluated as an analytical specimen for the detection and quantification of cannabinoids (see chapter 3), benzodiazepines (see chapter 4), opiates (see chapter 5) and methadone (see chapter 6).

1.3.11. Peritoneal Fluid

The use of peritoneal fluid, just like interstitial fluid, to establish the pharmacokinetic profile of drugs in the human body is gaining momentum, especially since published reports appeared describing situations where certain drugs are absorbed into the peritoneum when administered intraperitoneally but not when given intravenously (Somani et al., 1982).

Peritoneal fluid as an analytical specimen for drugs is capable of demonstrating the speed of absorption of a drug as well as the attainment of necessary therapeutic levels at the site of action. This sample, however, can only be collected by highly trained personnel using very invasive techniques.

1.3.12. Perspiration (Sweat)

The small volume of perspiration collected has been a limiting factor in the past for use of this biological specimen in drug monitoring, due to the inherent requirement of a highly sensitive method of analysis (Haeckel et al., 1993). The recent advent of sweat patches was instrumental in developing this field of study, resulting in applications to drug abuse monitoring, amongst others. More recently, perspiration-

based drug tests such as DrugWipe™ have been promoted vigorously by their developers primarily because of the possibility of rapid drug screening. There now exists a volume of published research which uses perspiration in the analysis of amphetamines (Suzuki, et al., 1989), barbiturates (Smith et al., 1981), cannabinoids (Kintz, 1996), cocaine and its metabolites (Cone et al., 1994; Kintz, 1996; Kidwell et al., 1997), opiates (Cone et al., 1994), and benzodiazepines (Kintz, 1996).

Finally, the recent consideration of skin perspiration as a pharmacokinetic factor of drug incorporation into hair by means of a possible transfer of drugs into the medulla promises to keep the scientific community's interest increased in this biological specimen.

1.3.13. Saliva

Since the mid 1970s, there has been strong interest amongst the analytical community, manifested by a significant number of published reports, on the use of saliva (also known as oral fluid) as an alternative to blood for the monitoring of several drugs, as reviewed by Drobitch et al. in 1992, Schramm et al. in 1992 and Cone in 1993.

Saliva is, at least in part, an ultrafiltrate of interstitial fluid containing the unbound (free) component of drugs (Schramm et al. in 1992). Therefore, it could be a potentially useful sample for monitoring the state of intoxication of the donor.

There have been numerous reports on the use of saliva for monitoring therapeutic drugs and other chemicals (Danhof et al., 1978; Paxton, 1979; Mucklow, 1982; Idowu et al., 1982).

Particular interest has also been expressed for the application of saliva analysis as part of a road-side testing scheme to identify potentially intoxicated drivers (Peel et al., 1984; Starmer et al., 1988).

An extensive body of published research exists which offers analytical protocols based on saliva for the detection of barbiturates (Inaba et al., 1975; Smith et al., 1981; Sharp et al., 1983; van der Graaff et al., 1986), methaqualone (Sharp et al., 1983), amphetamines (Wan et al., 1978; Kajitani et al., 1989; Suzuki et al., 1989), cannabinoids (Just et al., 1974; Thompson et al., 1987a; Maseda et al., 1986; Gross et al., 1985), benzodiazepines (Kangas et al., 1979; de Gier et al., 1980; Hallestrom et al., 1980; Sharp et al., 1983), cocaine and its metabolites (Hamilton et al., 1977; Thompson et al., 1987b; Cone et al., 1988; Cone et al., 1989), opiates (Sharp et al., 1983; Cone, 1990), methadone and its metabolites (Kang et al., 1982) and phencyclidine (Cook et al., 1983; McCarron et al., 1984). More recently, reports have appeared which allow for the simultaneous determination of several groups of drugs in saliva (Wang et al., 1994).

Saliva's popularity as an analytical specimen for drugs-of-abuse is primarily due to the ease with which this sample may be obtained by non specialised personnel from all sorts of subjects including the elderly and children, using non-invasive collection methods which also respect the subject's privacy. Furthermore, by monitoring drug levels in saliva, the toxicologist measures the free or unbound fraction of the drug in plasma which is usually secreted into saliva by the salivary gland and is responsible for the drug's pharmacological action (Pichini et al., 1996).

1.3.14. Seminal fluid

The human seminal fluid and the male gametes in particular have been found to be susceptible to effects from xenobiotics which could potentially be the source of father-mediated teratogenicity (Friedler, 1985). As a result of the potential severity of the effects observed, studies have been conducted which examined the presence of drugs in the male genital tract (Malborg, 1978; Pichini et al., 1994).

Initially, the drugs monitored in semen were restricted to antimicrobial agents such as cephalosporins and quinolones used in the treatment of diseases related to the male reproductive system in order to verify the achievement of bactericidal concentrations with the lowest possible incidence of adverse effects (Armstrong et al., 1968; Milingos et al., 1981; Naber et al, 1991). These initial studies, however, did not examine the effects, if any, of drugs on the physiology and morphology of sperm.

Studies offering examination of the physiomorphological effects of drugs on semen were conducted for salicylates, nicotine, mesalazine, β-adrenergic blockers such as propranolol, and xanthines such as caffeine (Swanson et al., 1978; Rimerman et al., 1979; Kershaw et al., 1987; Kjaergaard et al., 1989). These preliminary studies have indicated that some of these agents (e.g. propranolol, salicylates, nicotine) have negative effects on sperm motility and count (Porat-Soldin et al., 1992; Mahajan et al., 1984), whereas others (e.g. caffeine) appear not to affect sperm to any significant degree (Beach et al., 1984).

Potential benefits from the analysis of seminal fluid are not limited to the physiomorphological effects of drugs on sperm itself but also extend to women's exposure to drugs excreted in semen and possibly to the outcome of pregnancy. It has

been argued in the literature that these benefits outweigh the invasiveness of the collection of this specimen, which include violation of a subject's privacy and negative psychological impact, and consideration of the toxicology of drugs excreted in seminal fluid should be standardised as part of the drug development process (Pichini et al., 1994).

1.3.15. Skeletal Muscle

A small amount of research has been performed on skeletal muscle as an alternative biological specimen for drug detection and quantification apart from its traditional use in cases where other specimens are unlikely to be available, for example plane crash fatalities, in which muscle is usually analysed for the presence of carbon monoxide.

Preliminary results published in the literature appear to be encouraging for the analysis of ethyl alcohol as well as for many commonly occurring basic drugs. In many cases, the muscle to blood drug concentration ratios are close to unity (Garriott, 1991).

More research could provide the necessary support for inclusion of muscle amongst the routinely collected forensic samples in cases of medico-legal interest.

1.3.16. Tear Fluid

The recent advent of highly sensitive analytical techniques has made possible the measurement of minute quantities of drugs in tear fluid (Van Haeringen, 1985). It has been reported in the literature that tear fluid's constant composition and homogeneity - better than those of saliva - could render it a valuable alternative biological specimen for drug analysis (Haeckel et al., 1993).

Tear fluid has been sampled and analysed for drugs administered against a variety of ocular diseases in an effort to establish the delivery of the required drug dose at the site of action. A large volume of research has been presented on the analysis of tear fluid for the detection and quantification of azithromycin and its metabolites (Tabbara et al., 1998; Raines et al., 1998), norfloxacin and its analogues (Konishi et al., 1998; Akkan et al., 1997; Green et al., 1996b; Borrmann et al., 1992), dexamethasone phosphate and its metabolites (Baeyens et al., 1997) and cyclosporin (Phillips et al., 1994).

This research did not always succeed in correlating the drug tear to plasma unbound drug concentration ratios mainly due to fluctuations of the tear fluid's pH (Van Haeringen, 1985). As described in section 1.2.2. above, a specimen's pH in conjunction with a drug's pK_a or pK_b determine the amount of the drug present in the specimen of interest. Upon closer examination of the literature, it appears that only acidic drugs with high pK_a in their non-ionised form are found in tears in predictable quantities. This disadvantage of tear fluid renders this specimen problematic for routine drug monitoring.

1.3.17. Vitreous Humor

There has been increased interest in the use of vitreous humor as an analytical fluid for the determination of drugs (Bermejo Barrera et al., 1992; McKinney et al., 1995; Samuel et al., 1994).

This specimen has been sampled and analysed for drugs administered against a variety of ocular diseases in an effort to establish the delivery of the required drug dose at the site of action. A large volume of research has been presented on the analysis of

vitreous humor for the detection and quantification of ofloxacin and its analogues (Cekic et al., 1998; Donnenfeld et al., 1997; Perkins et al., 1995) and azithromycin (Oday et al., 1994) among other drugs mainly important for ophthalmologic reasons.

Furthermore, vitreous humor has gained popularity among forensic toxicologists as an analytical specimen during death investigations. Methods have been developed and published on the analysis of vitreous humor for several commonly abused drugs. Methods have been offered for the detection and quantification of opiates (Pragst et al., 1999; Winek et al., 1999; Maurer, 1998), benzodiazepines (Robertson et al., 1998; McIntyre et al., 1994), cocaine and its metabolites (McKinney et al., 1995; Fernandez et al., 1994), phenothiazines (Avis et al., 1996), amphetamines (Crifasi et al., 1996) as well as fluorocarbons such as chlorodifluoromethane (Kintz et al., 1996b). The literature also provides extensive reviews on the use of vitreous humor as an alternative biological sample (Maurer, 1998; Mangin et al., 1991).

The frequency of vitreous humor sampling in cases of forensic interest will probably increase as more research is conducted on this matrix and correlations between blood and vitreous humor drug levels are determined.

1.4. Conclusions

The survey of the current state-of-the-art presented above clearly shows that the international analytical community has been highly interested in the determination of drugs in biological fluids and matrices other than the traditional ones (i.e. blood and urine) for quite some time.

The extent to which alternative fluids and matrices are used in standard operating procedures depends on several factors. The high costs associated with the training of specialised sample collection personnel as well as with the clean-up, analysis and storage of various biological materials are significant factors. Despite these inhibiting factors, laboratories around the world have been involved in alternative biological fluid and matrix analyses either because of interest or need. Traditionally, all analytical techniques available to the analyst at the time were implemented for optimisation of drug detection in any accessible biological specimen. However, as more sensitive techniques became available for the detection and quantification of drugs in blood, the use of alternative biological samples assumed a secondary role.

Recently, drug monitoring in specimens other than blood or urine has been considered again and in many instances successfully performed. Often this work has grown from needs other than forensic ones, for example, in clinical drug monitoring as described in section 1.1.

Drug monitoring in nonconventional biological specimens remains in its early stages but offers significant advantages e.g. extended drug retention, non-invasive sampling, etc. As part of the world-wide effort to evaluate alternative biological samples in routine drug analysis, the present work was conducted on the use of bile for the detection and quantification of dextropropoxyphene (chapter 2), and the use of nails for the detection and quantification of cannabinoids (chapter 3), benzodiazepines (chapter 4), opiates (chapter 5) and methadone (chapter 6).

2. DEXTROPROPOXYPHENE ANALYSIS IN BILE

2.1. Introduction

Although the use of bile as an analytical specimen in forensic laboratories is well established, little work has been done on the extraction of bile samples using current solid-phase techniques. As part of this thesis, experiments were conducted to develop a gas chromatographic method based on solid-phase extraction (SPE) for the simultaneous analysis of dextropropoxyphene and its major metabolite, norpropoxyphene, in bile. Dextropropoxyphene was selected as a model compound for a general extraction procedure for basic drugs in bile.

Dextropropoxyphene (see figure 5) is a regularly prescribed analgesic drug that is frequently found in cases of medico-legal interest in the UK. It is a synthetic oral analgesic that has been available for consumption since the late 1950's. It is usually prescribed for the symptomatic relief of moderate pain (Pearson, 1984). This drug has been used in the treatment of heroin addicts (Woody et al., 1980) and has been described as an abused substance since 1970 (Ng et al, 1993).

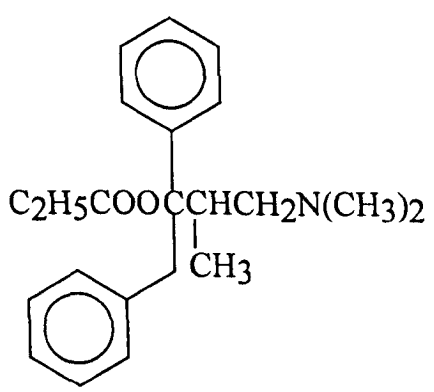


Figure 5. The structure of dextropropoxyphene.

Dextropropoxyphene is principally metabolised to norpropoxyphene via *N*-demethylation (see figure 6). Pharmacokinetic data indicates that norpropoxyphene has a significantly longer elimination half life (36 hours) than the parent compound (12 hours) (Nickander et al., 1984). Animal studies show that norpropoxyphene is partially responsible for some toxic effects of dextropropoxyphene in humans such as cardiotoxicity (Nickander et al., 1984).

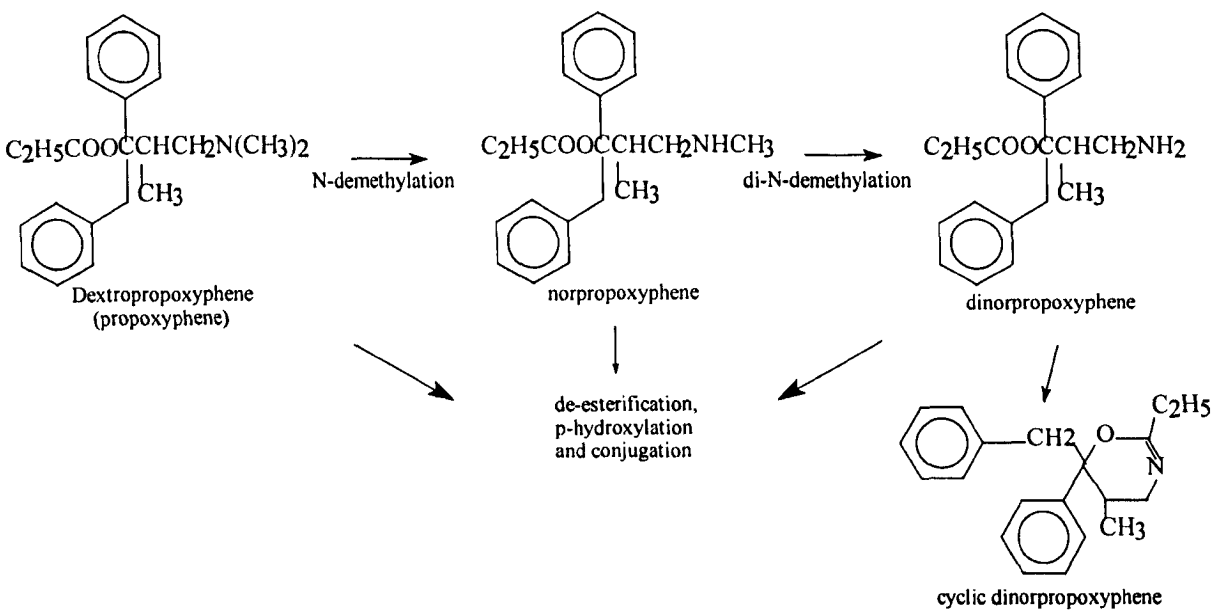


Figure 6. The metabolism of dextropropoxyphene.

In the UK, dextropropoxyphene has been implicated in a significant number of drug-related fatalities, often in combination with paracetamol (Distalgesic, Coproxamol). Therefore, it has assumed a position of particular significance in both clinical and forensic toxicology.

The present study examined the use of bile as an analytical specimen in those forensic cases in which dextropropoxyphene was suspected to have been a contributing factor.

2.2. Methods & Materials

2.2.1. Standards & Reagents

All chemicals and reagents used were of HPLC grade or better. Propoxyphene HCl was obtained from Eli Lilly & Company Ltd. (Basingstoke, UK). The maleate salt of d-norpropoxyphene was obtained from SIGMA Chemical Co. (Dorset, UK). Triphenylamine, used as the internal standard, was obtained from Fluka AG (Switzerland).

2.2.2. Samples

Following procedures in accordance with the ethical standards of the responsible regional committee on human experimentation, gall bladders were removed during autopsy (see figure 7) and transported to the forensic laboratory in Glasgow in small glass containers. Bile was then obtained by pouring after incision at the top portion of the gall bladder. Bile samples (1 ml) were diluted 1:50 in 100 mM phosphate buffer (pH 6.0) and the mixtures were vortexed. The pH of each sample was adjusted to 6.0 ± 0.5 with 100 mM monobasic or dibasic sodium phosphate and internal standard (triphenylamine) was added to 5 ml of the diluted bile samples.

Figure 7. A gall bladder.

2.2.3. SPE

The 200 mg Clean Screen® Extraction Columns were used for the analyses. The columns were conditioned with 3 ml of methanol followed by 3 ml of de-ionised water and 1 ml of 100 mM phosphate buffer (pH 6.0). The bile samples were then applied to the SPE columns at a rate of 1-2 ml/min. Following sample application, the columns were washed with 3 ml of de-ionised water followed by 1 ml 1.0 M acetic acid and 3 ml of methanol. The columns were then dried for 5 minutes under full vacuum. The analytes were eluted from the SPE column with 1 x 3 ml dichloromethane: isopropanol: ammonium hydroxide (78:20:2 v/v/v) at the rate of 1-2 ml/min. Subsequently, the eluents were concentrated via evaporation to dryness on a hot plate set at 60°C and the residues were reconstituted in 100 µl of methanol before analysis by gas chromatography equipped with a flame ionisation detection system (FID-GC).

2.2.4. FID-GC

A 1.0 µl aliquot of each sample was injected using an HP 7673 Injector (injector temperature: 275° C) through a Chrompak CP-SIL 5 CB (30m x 0.25 mm) of an HP

5890 Series II gas chromatograph equipped with a flame ionisation detection system (FID temperature: 310° C). Helium (10ml/min) was the carrier gas.

The column temperature was initially held at 80°C for 2 min and then programmed to rise to 215°C at a rate of 20°C/min and to 285°C at 5°C/min. The temperature was then held at 285°C for the final 2 minutes of the chromatographic analysis. Identification was achieved by comparison of retention times (t_R) and quantification was based on the ratio of the peak areas of analytes to the internal standard with the aid of standard calibration curves similar to the ones presented in figure 8 and figure 9.

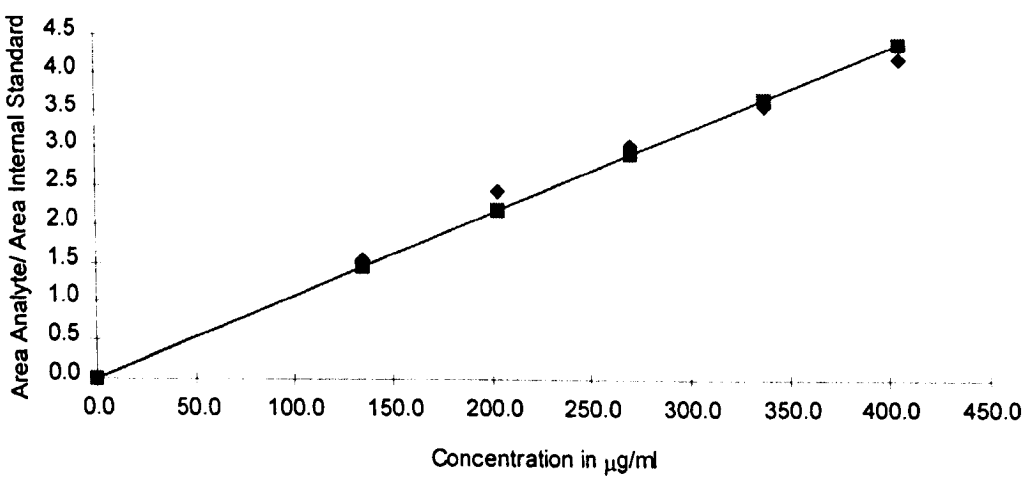


Figure 8. A standard curve used for the quantification of dextropropoxyphene in bile (r^2=0.9941).

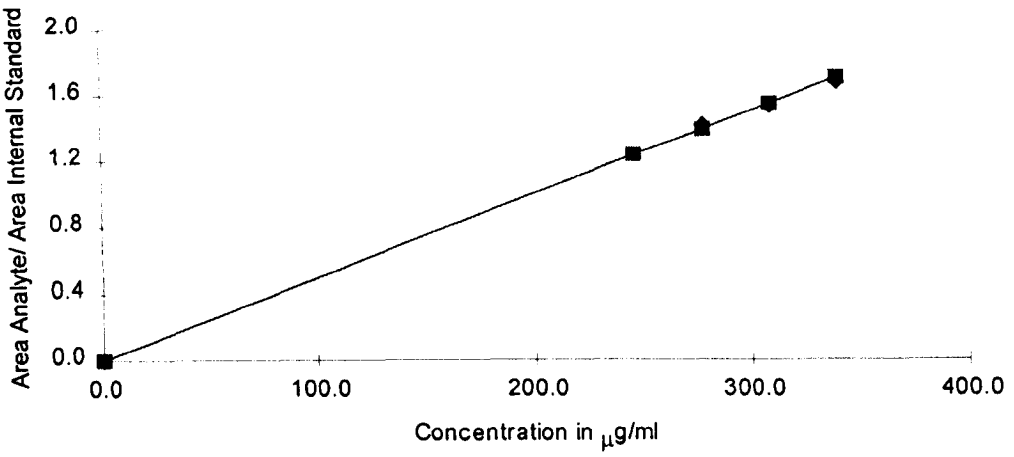


Figure 9. A standard curve used for the quantification of norpropoxyphene in bile (r^2=0.9996).

2.2.5. Method Validation

Drug-free bile samples were spiked with different amounts of dextropropoxyphene and norpropoxyphene standards and analysed as described above. In this way, the method's limits of detection and extraction recoveries were calculated for the two analytes.

2.3. Results & Discussion

The retention times for dextropropoxyphene and norpropoxyphene were 9.7 and 17.9 minutes, respectively, whereas the internal standard eluted at 13.1 minutes (see figure 10).

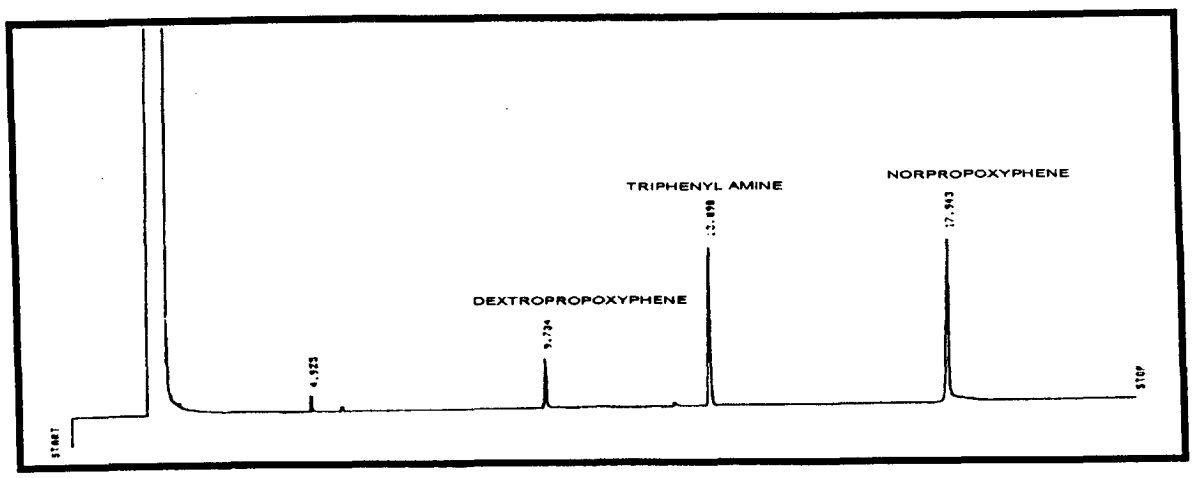


Figure 10. A GC-FID chromatogram of a biliary extract showing the dextropropoxyphene peak at 9.7 min, the triphenylamine peak at 13.1 min (internal standard) and the norpropoxyphene peak at 17.9 min.

The calculated recoveries based on spiked samples for dextropropoxyphene and norpropoxyphene were 90% and 92%, respectively. Calibration curves were linear for each analyte (r^2> 0.99 in each case). The method detected quantities as low as 1 μg of dextropropoxyphene and 4 μg of norpropoxyphene if 1 ml of bile was used to determine the limits of detection.

The combination of a solid phase extraction scheme with a gas chromatographic separation proved to be a valuable one in which endogenous biliary components were found not to interfere with the elution of dextropropoxyphene and norpropoxyphene. The two analytes separated well under the conditions described herein. The solid phase extraction technique for propoxyphene and norpropoxyphene described provided a rapid and clean extract which could be used in a high throughput setting. It also provided a reliable method that can be used for drugs-of-abuse testing.

2.4. Conclusions

This study offered a protocol involving the analysis of bile for the presence of dextropropoxyphene and its major metabolite, norpropoxyphene, by gas chromatography equipped with a flame ionisation detection system.

Bile has been established as a useful forensic specimen especially due to its long-term retention and high levels of drugs observed therein. The main advantage of bile analysis is its ability to detect drug presence even when blood and/or urine drug levels are very low or non-existent. Based on the results of this study and taking into account the advantages described above, bile was suggested as a useful analytical specimen for dextropropoxyphene determination (see annexe I). Further work is required in order to develop models for drug partition/distribution in bile that will allow for correct correlations of concentrations to physiological effects.

3. CANNABINOID ANALYSIS IN NAIL

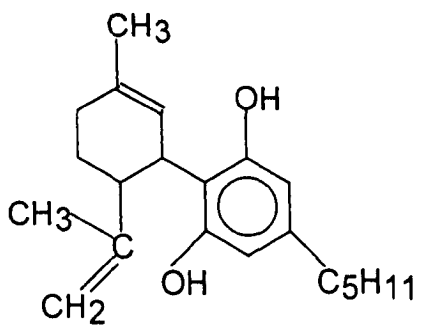
3.1. Introduction

Marijuana (*Cannabis sativa)* is the most popular recreational drug around the world apart from alcohol. In many cultures, cannabis use is a national tradition that is passed from one generation to the next in coffee shops and cafés. In most western cultures cannabis possession and use are illegal. The term *marijuana* usually refers to a mixture of the plant's leaves and flowering tops. There are 30 or more active components of the drug which are all derivatives of 2-(2-isopropyl-5-methylphenyl)-5-pentylresorcinol. They are all collectively known as cannabinoids (Moffat, 1986). Some significant cannabinoids are presented in figure 11 and include (-)-*trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC, THC), 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THCCOOH), cannabidiol (CBD), cannabinol (CBN), (-)-*trans*-Δ^8-tetrahydrocannabinol (Δ^8-THC) and Δ^9-tetrahydrocannabinolic acid (THCOOH).

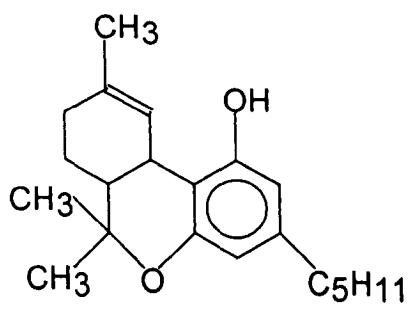
THC is considered to be the primary psychoactive constituent of *Cannabis sativa*. It is contained in various parts of the plant. It is usually administered orally or by smoking. The usual dose ranges from 5 to 20 mg and results in sedation, euphoria, hallucinations and temporal distortion (Baselt et al., 1995). About 80% of the THC contained in a cigarette is lost and cannot be recovered from the smoke (Mikes et al., 1971).

In 1981, Perez-Reyes et al. reported that in 6 subjects who smoked one cigarette each containing 8.8 mg of THC for 10 minutes, the peak plasma THC concentrations ranged from 46 to 188 μg/l (Perez-Reyes et al., 1981). In another 6 male subjects who

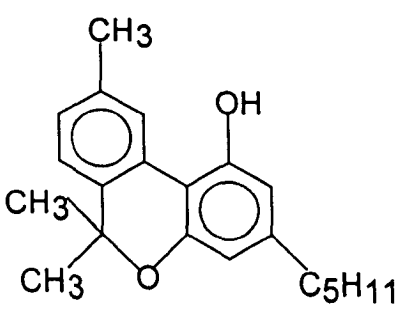
smoked cigarettes containing 15.8 mg of THC for 11 minutes, peak plasma THC concentrations occurred at an average time of 0.14 hours and ranged from 50 to 129 μg/l (average: 84 μg/l) whereas peak THCCOOH concentrations occurred at 2.4 hours and ranged from 15 to 54 μg/l (average: 25μg/l) (Huestis et al., 1992).



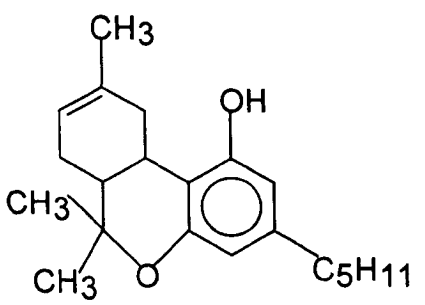
Cannabidiol



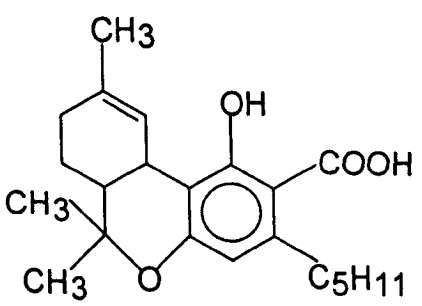
Δ^9–Tetrahydrocannabinol



Cannabinol



Δ^8–Tetrahydrocannabinol



Δ^9–Tetrahydrocannabinolic acid

Figure 11. Significant cannabinoids.

Intravenous infusion of 4 to 5 mg of THC in 7 subjects resulted in an average plasma THC concentration of 62 μg/l occurring at 20 minutes and an average plasma THCCOOH concentration of 14 μg/l occurring at 40 minutes (Wall et al., 1981).

Oral administration of THC resulted in lower peak plasma levels occurring at later times as compared to smoking or intravenous injection of the drug. Oral ingestion of 20 mg of THC resulted in an average peak plasma THC concentration of 6 μg/l occurring at 60 to 180 minutes (Lemberger et al., 1972; Hollister et al., 1981).

Elimination of THC takes place in several phases, including the initial (alpha) distribution phase in which the blood concentration falls rapidly, usually attributed to partition into tissues, followed by a slower (beta) excretion phase in which tissue depots are cleared. There is also a significant enterohepatic recirculation of THC and its metabolites. The beta elimination half-life of THC varies depending on the frequency of use. In infrequent users it ranges from 20 to 57 hours (Hunt et al., 1980; Wall et al., 1981; Agurell et al., 1986) while in frequent users it ranges from 3 to 13 days (Johanssson et al., 1989). In 1985, Peat et al. reported that the elimination half-life of THCCOOH was 33 hours on average in infrequent marijuana users and 40 hours on average in frequent marijuana users (Peat et al., 1985).

Despite both the volume of work on the distribution of cannabis in the human body and the frequency of cannabis abuse throughout the world, especially by younger people, there has been no report to date offering a methodology for the detection of cannabis in nail specimens.

The present study established fingernail clippings as an analytical specimen for the detection and quantification of cannabinoids by radioimmunoassay (RIA) and gas chromatography - mass spectrometry (GC-MS) in a forensic context.

3.2. *Methods & Materials*

3.2.1. Standards and Reagents

All chemicals and reagents used were of high-performance liquid chromatography grade. Δ^9-tetrahydrocannabinol (THC), Δ^9-tetrahydrocannabinol-d_3 (THC-d_3) and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THCCOOH) were obtained from SIGMA (Dorset, UK). 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid-d_3 (THCCOOH-d_3) was obtained from Radian Corporation (Austin, TX). The derivatising agent *N,O,-bis*(trimethyl-silyl)trifluoroacetimide (BSTFA) catalysed with 1% trimethylchlorosilane (TMCS) was obtained from Pierce (Rockford, IL).

3.2.2. Samples

The sets of nail clippings examined were obtained with consent from 23 cannabis users participating in the Edinburgh Drug Addiction Study (EDAS) in Edinburgh, Scotland. The procedures followed were in accordance with the ethical standards of the responsible regional committee on human experimentation. At the time of sampling, our human subjects provided answers to a short questionnaire regarding their use of cannabis and other drugs. This information is listed in table 5 except when answers were not provided in which case the information is marked "N/A" (not

answered). Nail clippings were collected from all of the subjects' fingers, pooled together and stored in plastic bags at room temperature. The specimens were then transported to the forensic toxicology laboratory in Glasgow where they were analysed as described below.

3.2.3. Sample Decontamination for RIA

The sets of nail clippings obtained from our human subjects were washed once with 10 ml of 0.1% sodium dodecyl sulphate solution (SDS) for 15 minutes using a CAMLAB TransSonic T 310 sonicator. The resulting wash solution was discarded by pouring. The nail clippings were then sonicated in distilled water (10 ml) three times for 15 minutes each time and the resulting washes were also discarded by pouring. Methanol (3 x 10 ml) was subsequently used to sonicate the sets of nail clippings three times for 15 minutes each time. The methanolic washes were collected in separate vials and evaporated to dryness on hot plates set at 60°C under a stream of nitrogen. The residues were then screened for cannabinoids by radioimmunoassay using the Cannabinoids Double Antibody procedure by Diagnostic Products Corporation (Gwynedd, UK) after being reconstituted in phosphate buffer (pH 7.4).

Table 5. Cannabis and other drug usage by subjects participating in the cannabis study as reported on the self-reporting questionnaires at the time of sampling.

Sample	Weekly cannabis consumption		Onset of consumption	Other drugs and length of time taken
	Ounces (oz.)	British Pounds (£)	(MM/YY)	Drug (time)
EDAS-02	N/A*	N/A	N/A	N/A
EDAS-03	N/A	N/A	N/A	N/A
EDAS-04	N/A	N/A	N/A	N/A
EDAS-05	N/A	N/A	N/A	N/A
EDAS-07	N/A	N/A	N/A	N/A
EDAS-08	N/A	N/A	N/A	N/A
EDAS-09	¾	45	Years	Dc† (years), T (years)
EDAS-11	¼	30	Years	AZT (years), D (years), Dc (years), T (years)
EDAS-12	¼	20	Several years	D (2 years), Dc (2 years)
EDAS-13	¼	25	Years	D, Dc
EDAS-14	½	45	Years	None
EDAS-15	¼	25	Several years	Dc, M, T
EDAS-16	¼	30	Several years	Dc, T
EDAS-17	1	100	Several years	D (years), M (years), T (years)

Table 5. (cont.)

EDAS-18	1	90	2 years	D (2 years), M (2 years)
EDAS-19	1	100	Years	D (years), Dc (years)
EDAS-21	N/A	N/A	N/A	N/A
EDAS-22	N/A	N/A	N/A	N/A
EDAS-23	N/A	N/A	N/A	N/A
EDAS-24	N/A	N/A	N/A	N/A
EDAS-109	N/A	N/A	N/A	D, M
EDAS-112	N/A	N/A	N/A	D, Dc
EDAS-114	N/A	N/A	N/A	D, M

* N/A, not answered.

† D: Diazepam; Dc: Dihydrocodeine; M: Methadone; T: Temazepam.

3.2.4. Sample Extraction for RIA

Once the methanolic washes had tested negative for the analytes of interest, the washed sets of nail clippings were weighed after being allowed to air dry overnight. In order to evaluate different extraction methods, two different sample preparation procedures were tested. In the first procedure, washed test nail clippings of known weights were ground in liquid nitrogen using a mortar and pestle. This gave unsatisfactory results because an average of 20% of the sample was lost in the process. In the second procedure, alkaline hydrolysis was used. This procedure was subsequently adopted as the extraction method in this study and all of the following studies described in this thesis. The procedure involved the incubation of the washed sets of nail clippings in the presence of 1 ml of 1M sodium hydroxide solution (NaOH) at 95°C for 30 minutes. After the resulting hydrolysates had cooled to room temperature, 3 ml of methanol were added. This methanol addition was carried out so that our experimental protocol would follow our laboratory's standard operating procedure for the detection of cannabinoids in blood by radioimmunoassay as closely as possible. Calibration curves were prepared in a similar manner. The resulting solutions were vortexed for 5 minutes and centrifuged for 20 minutes at 3000 rpm before being evaporated to dryness in their entirety on hot plates set at 60°C under a stream of nitrogen. The nail hydrolysate residues were then reconstituted in phosphate buffer (pH 7.4) for radioimmunological screening for cannabinoids.

3.2.5. RIA

The Cannabinoids Double Antibody procedure by Diagnostic Products Corporation (Gwynedd, UK) was used for the radioimmunological screening. The COBRA II Auto-Gamma counter (Packard, UK) was used for the measurement of the antibody-bound fraction in the pellet obtained after precipitation and centrifugation. All measurements were done in duplicate and the results obtained were the average of the two readings. Blank solutions, spiked solutions, and standard solutions (0, 10, 25, 50, 100, and 200 ng/ml, respectively) were analysed with the samples. Figure 12 shows a typical standard curve that was constructed using standard solutions and used for the quantification of the specimens.

3.2.6. Sample Decontamination for GC-MS

The sets of nail clippings obtained from the human subjects were washed once with 10 ml of 0.1% SDS solution for 15 minutes using a CAMLAB TransSonic T 310 sonicator as before. The resulting wash solution was discarded by pouring. The sets of nail clippings were then sonicated in distilled water (10 ml) three times for 15 minutes each time and the resulting washes were discarded by pouring as well. Methanol (10 ml) was subsequently used to sonicate the nail clippings three times for 15 minutes each time. The resulting methanolic washes were collected in separate vials and evaporated to dryness on hot plates set at 60°C under a stream of nitrogen. Internal standards (THC-d_3 and THCCOOH-d_3) were added to each methanolic residue, and the mixtures were once again evaporated to dryness on a hot plate set at 60°C under a stream of

nitrogen. The methanolic residues were then derivatised for 20 minutes using BSTFA catalysed with 1% TMCS at 95°C and subjected to GC-MS analysis.

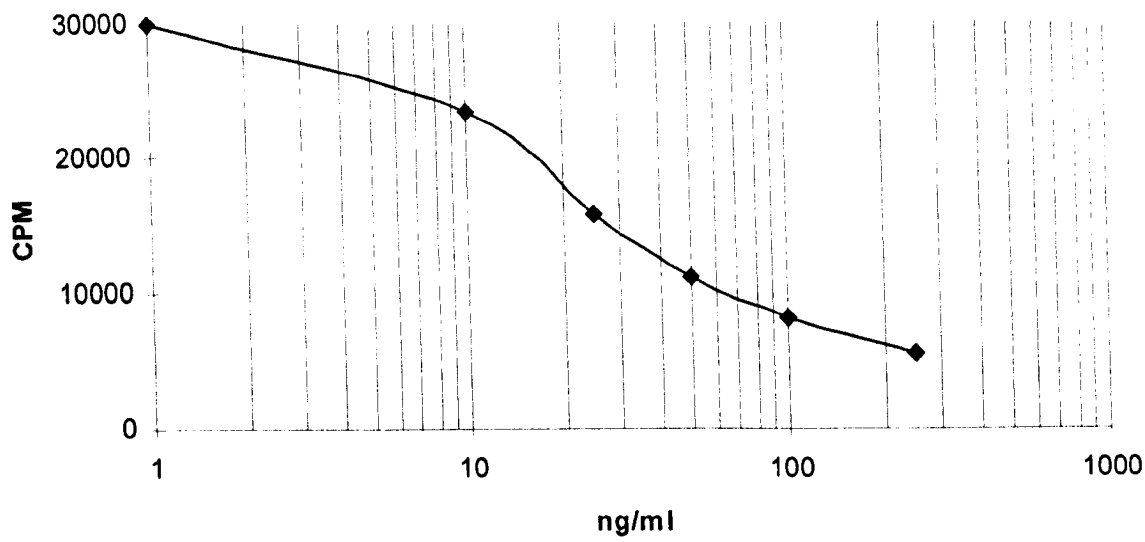


Figure 12. Standard curve for cannabinoid determination by RIA.

3.2.7. Sample Extraction for GC-MS

Once the methanolic residues had tested negative for the analytes of interest, the washed sets of nail clippings were weighed after being allowed to air dry overnight. Having rejected grinding of the nail clippings in liquid nitrogen using a mortar and pestle (see Section 3.2.4 above), alkaline hydrolysis was used as before to prepare samples. This involved the incubation of the washed sets of nail clippings in 1 ml of 1M sodium hydroxide solution at 95°C for 30 minutes in the presence of internal standards (THC-d_3 and THCCOOH-d_3). After the resulting hydrolysates had cooled down to room temperature, 5 ml of ethyl acetate were added and the mixtures were

agitated for 10 minutes on a mechanical rock-n-roller. The organic layer from each mixture was then transferred into a clean screw-cap vial and evaporated to dryness on a hot plate set at 60°C under a stream of nitrogen. The nail hydrolysate residues were then derivatised as before using BSTFA catalysed with 1% TMCS and subjected to GC-MS.

A separate study was performed on three sets of nail clippings in order to test the effect of pH on the extraction of the major cannabinoids (THC and THCCOOH) from nail clippings. These three sets of nail clippings were treated in exactly the same way as described above with the exception that, at the time of extraction with ethyl acetate, the pH was made acidic following the addition of 3M hydrochloric acid (HCl).

After extraction, the organic layers from these nail clipping hydrolysates were evaporated to dryness on a hot plate set at 60°C under a stream of nitrogen as before. These three nail extract residues were then derivatised following the exact procedures as described above using BSTFA catalysed with 1% TMCS and subjected to GC-MS.

3.2.8. GC-MS

A 1.0-µl portion of each sample was injected on an HP-1 crosslinked dimethyl silicone capillary column (30m x 0.25mm, 0.25-µm film thickness) in a FISONS 8000 Series gas chromatograph coupled to a FISONS MD 800 mass spectrometer. The injector was a FISONS AS 800 (injector temperature: 225°C) and splitless injection was employed with a split-valve off-time of 1 minute. Helium was used as the carrier gas.

The initial column temperature was 150°C and was programmed to 300°C at a rate of 10°C/min immediately after injection. The column was subsequently maintained at 300°C for 10 minutes.

The capillary column was inserted via a heated transfer line (250°C) directly into the ion source at a temperature of 200°C. The mass spectrometer was operated in the selected ion recording mode with electron impact ionisation at an electron energy of 70 eV and was auto-tuned daily according to the manufacturer's recommendations.

For THC and THCCOOH, qualitative and quantitative analyses were obtained using the selected ion recording mode and comparison of retention times (t_R) and relative abundances of confirming ions with THC-d_3 and THCCOOH-d3, respectively. Quantitative results were obtained in the selected ion recording mode after determination of the response factor of THC and THCCOOH against THC-d_3 and THCCOOH-d3.

3.2.9. Method Validation

There exists no method currently which tests intact nail matrix spiked with cannabinoids. Therefore, the limits of detection and extraction recoveries of cannabinoids were determined after dissolution of the nail in sodium hydroxide. Ten different nail hydrolysates in NaOH (each equivalent to 10 mg of nail) were spiked with cannabinoids and then analysed as usual in order to determine the extraction recovery of the method. Furthermore, three nail hydrolysates in NaOH (each equivalent to 10 mg of nail) were spiked with decreasing amounts of cannabinoids and then analysed as usual in order to determine the limit of detection of the method.

3.2.10. Spiking Experiments

Studies were performed to test for possible environmental contamination of nail by cannabinoids which originated from sources external to the human body. Drug-free nail clippings (10 mg) were obtained from volunteers who did not use cannabinoids or who had not been in the vicinity of cannabis users when they were using the drug. These sets of nail clippings were placed in separate vials in the presence of 2000 µl of THC and THCCOOH methanolic solutions (1ng/µl, respectively) and were allowed to incubate at room temperature for 1, 3, 5, 7 and 14 days undisturbed. Upon completion of each incubation period, the nail clippings were transferred from their incubation vials into new, clean vials using clean surgical forceps. The sets of nail clippings were then decontaminated and analysed for cannabinoids by the methods described earlier in order to establish whether or not any detectable transfer from the solution into the nail matrix had taken place.

3.3. Results & Discussion

The spiking experiments described above indicated that no detectable absorption took place from a solution containing cannabinoids into the nail matrix even after a two-week incubation period at room temperature.

Table 6 shows the ions monitored for THC, THC-d_3, THCCOOH and THCCOOH-d_3 along with their respective retention times (t_R).

Figure 13 shows a selected ion chromatogram of one of the nail clipping sets (EDAS-18). Figure 14 shows a selected ion chromatograph of cannabinoid standards THC, THC-d_3, THCCOOH and THCCOOH-d_3.

Under the analytical conditions used in the study, there was no interference in the cannabinoids tested due to any extracted endogenous materials present in nail.

Limits of detection and extraction recoveries are presented in table 7. By this method, concentrations of 0.1 ng/mg for both THC and THCCOOH could be detected if a minimum of 10 mg of nail was used for extraction. These detection limits were determined by decreasing the concentrations of the drugs spiked in the hydrolysate of a 10-mg nail sample. Extraction recoveries were determined for THC and THCCOOH by spiking drug-free nail clipping hydrolysates with known quantities of the two compounds, subjecting them to the extraction method, and then analysing them using the instrumental method described earlier. The extraction recoveries calculated were greater than 81% for the two compounds.

Table 6. Retention times (t_R) and selected ions for THC, THC-d_3, THCCOOH and THCCOOH-d_3.

Compound	**t_R (min)**	**Selected Ions**[†]
THC	9.56	371, **386**
THC-d_3	9.53	389
THCCOOH	13.36	371, **488**
THCCOOH-d_3	13.34	491

[†] The ions in bold typeface were used for quantification.

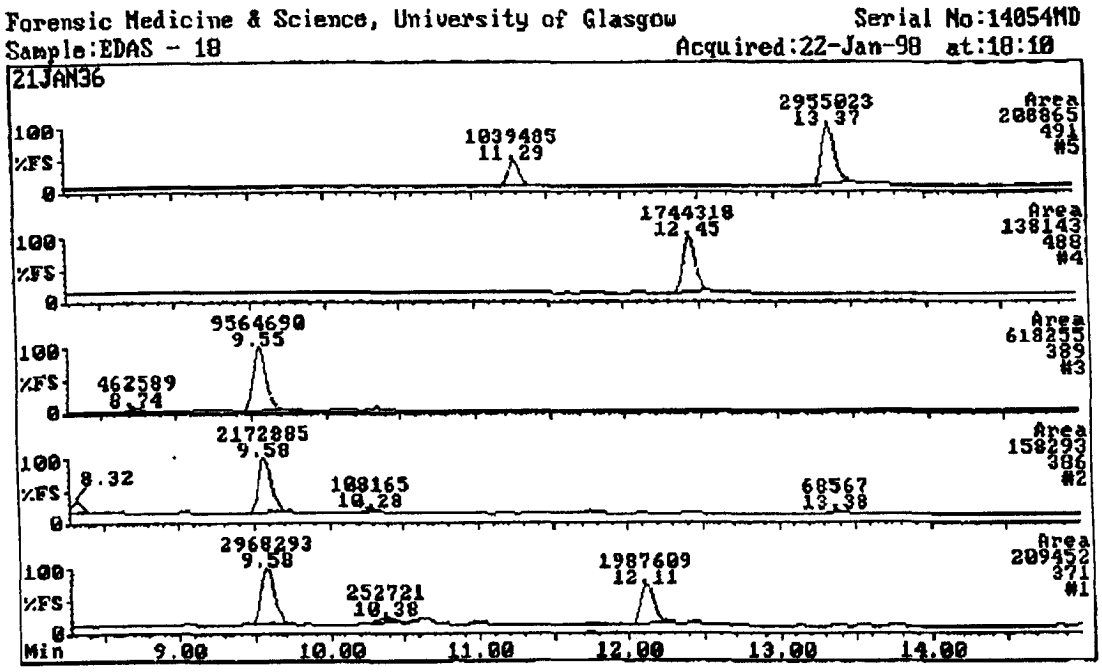


Figure 13. Selected ion chromatogram from a THC-positive nail sample. The determined concentration was 3.39 ng/mg.

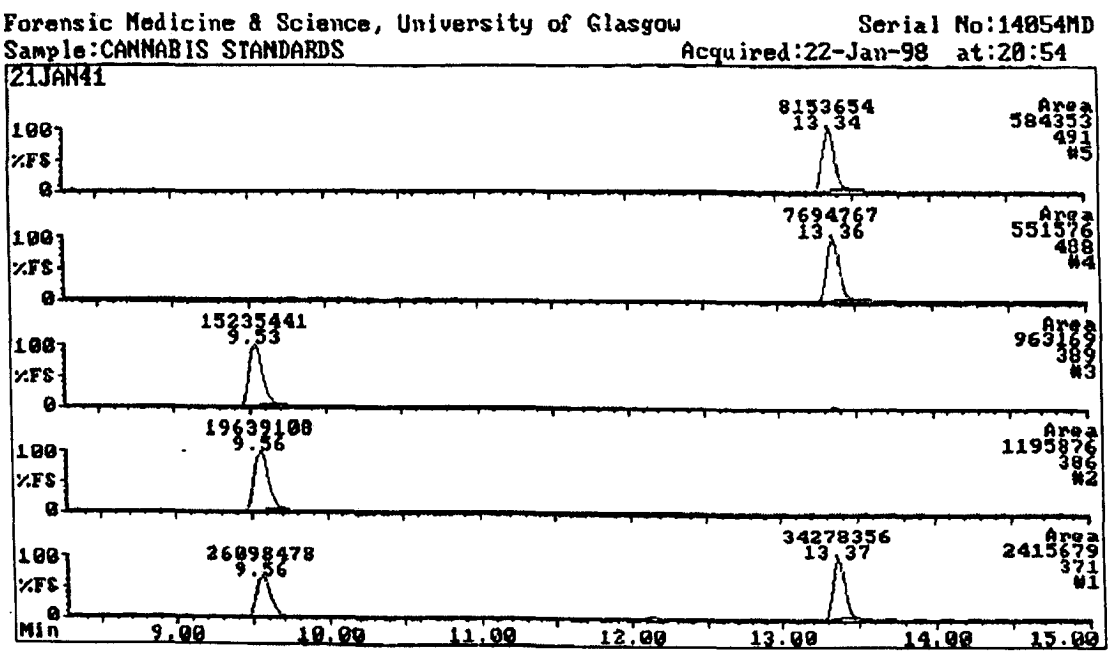


Figure 14. Selected ion chromatogram of cannabinoid standards used in the determination of THC and THCCOOH in nail by GC-MS.

Cannabinoids were found to be present in all six cases that were analysed for cannabinoids by RIA (see table 8). The cannabinoid concentration determined ranged from 0.23 ng/mg to 2.80 ng/mg with an average cannabinoid concentration of 1.03 ng/mg.

Using GC-MS, THC was detected in 11 out of the 14 nail clipping hydrolysates which were extracted at basic pH (see table 9). THCCOOH was not detected in any of these nail clipping hydrolysates. The concentration of THC ranged from 0.13 ng/mg to 6.97 ng/mg with a mean THC concentration of 1.44 ng/mg.

Figure 15 presents the level of THC measured in each of our samples by GC-MS plotted against the monetary value of cannabis used (in GBP, £) as reported by each participant on his/her questionnaire at the time of sampling. The variations of THC levels among individuals who declared to have consumed the same amounts of cannabis may be attributed to the non-homogeneity of the street cannabis available in Scotland, the different cannabis consumption patterns of our volunteers and to the different length of cannabis abuse within the population studied.

The major metabolite of THC, THCCOOH, was not detected in any of the 14 sets of nail clippings extracted under basic pH. However, it was detected in two out of the three fingernail clipping hydrolysates extracted under acidic pH. The concentration of THCCOOH determined ranged from 9.82 ng/mg to 29.7 ng/mg with an average THCCOOH concentration of 19.8 ng/mg. The parent drug, THC, was not detected in the three nail hydrolysates extracted under acidic pH. The GC-MS results for this group of samples are presented in table 10.

Table 7. Limits of detection and extraction recoveries of THC and THCCOOH obtained with 10 mg of drug-free nail spiked with THC and THCCOOH.

Compound	Limit of Detection (ng/mg)	Extraction Recovery (%)
THC	< 0.1	89.4
THCCOOH	< 0.1	81.1

Table 8. Cannabinoid concentration in nail clippings determined by RIA.

Sample	Weight of sample (mg)	Cannabinoid concentration (ng/mg)
EDAS-02	6.46	0.23
EDAS-03	8.07	2.80
EDAS-04	10.40	0.92
EDAS-05	10.58	1.14
EDAS-07	18.35	0.66
EDAS-08	21.51	0.45

Table 9. Concentration of THC in nail clippings determined by GC-MS.

Sample	Weight of sample (mg)	THC concentration (ng/mg)
EDAS-09	3.51	0.53
EDAS-11	4.25	6.97
EDAS-12	12.25	0.57
EDAS-13	6.17	N/D*
EDAS-14	2.79	0.13
EDAS-15	4.77	N/D
EDAS-16	6.18	0.14
EDAS-17	6.67	0.45
EDAS-18	5.18	3.39
EDAS-19	6.96	1.04
EDAS-21	2.68	0.65
EDAS-22	4.39	N/D
EDAS-23	2.53	0.32
EDAS-24	7.11	1.61

* N/D, not detected.

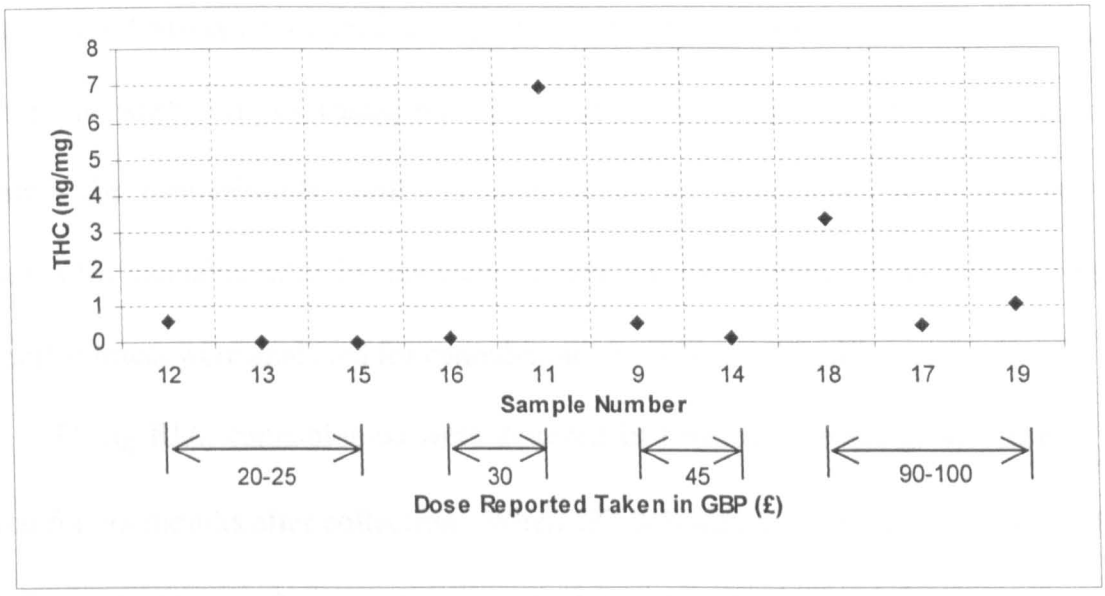


Figure 15. THC levels in ng/mg measured by GC-MS in each of our samples plotted against the monetary value (in GBP, £) of cannabis used by each participant as declared on his/her questionnaire at the time of sampling.

Table 10. Concentration of THCCOOH in nail clippings extracted under acidic pH and determined by GC-MS.

Sample	Weight of sample (mg)	THCCOOH concentration (ng/mg)
EDAS-109	5.81	9.82
EDAS-112	9.08	29.67
EDAS-114	9.21	N/D*

* N/D, not detected

The new analytical procedure presented herein has shown that the sonic wash protocol used always produced a negative cannabinoid result for the third and final methanolic wash. In 14 cases, both the second and third methanolic washes were negative for cannabinoids, while in eight cases all three methanolic washes were negative for cannabinoids. In this way, superficial contamination was removed before the nail extracts were analysed for cannabinoids by RIA or GC-MS.

Using RIA, cannabinoids were detected in fingernail clippings which had been stored for six months after collection. When GC-MS was used, Δ^9-tetrahydrocannabinol (THC) and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THCCOOH) were detected nine months after the samples were collected.

The growth of fingernails is known to be 3 to 5 mm per month (Le Gros Clark et al., 1938; Bean, 1953; Hamilton et al., 1955; Pounds et al., 1979; Heikkilä et al., 1996; Ropero-Miller et al., 1998). Considering that drugs are deposited into the root of the nail via the blood stream and progress towards the nail tip as the nail grows, the administration of drugs to an individual could be dated back at least 3 to 5 months for fingernails. However, the exact time of drug consumption is likely to be difficult to establish at this early stage of nail analysis for drugs of abuse because of the influence on nail growth of other factors such as sex, age, race and season (Pounds et al., 1979; Hamilton et al., 1955).

3.4. Conclusions

This study developed and validated a protocol involving the analysis of human nails for the presence of Δ^9-tetrahydrocannabinol (THC) and 11-nor-Δ^9-

tetrahydrocannabinol-9-carboxylic acid (THCCOOH) by RIA and GC-MS. The cannabinoid concentration determined by RIA ranged from 0.23 ng/mg to 2.80 ng/mg with an average cannabinoid concentration of 1.03 ng/mg. By GC-MS, the concentration of THC ranged from 0.13 ng/mg to 6.97 ng/mg with a mean concentration of 1.44 ng/mg and the concentration of THCCOOH determined ranged from 9.82 ng/mg to 29.7 ng/mg with an average concentration of 19.8 ng/mg.

Nail clippings are potentially more easily collected than blood or urine and are especially useful because of their long-term retention of drugs. The main advantage of nail analysis is its ability to detect long-term drug abuse for time periods from months to years. Moreover, the sample size required to detect drug exposure by nail analysis is very small (2.5-25 mg) and nail samples are collected by non-invasive procedures, are easy to store, allow for increased drug stability and are potentially less likely to suffer from any melanin race bias (Garside et al., 1998).

Based on the results of the study and taking into account the advantages described above, nails have been suggested as a potentially useful specimen for the detection of past cannabis use in cases of medico-legal interest (see annexes II and III).

4. BENZODIAZEPINE ANALYSIS IN NAIL

4.1. Introduction

Benzodiazepines are a group of similarly structured compounds used as sedative hypnotic and anticonvulsant drugs because of their anxiolytic, sedative and muscle relaxant properties (Harvey, 1985). Diazepam (Valium, Valrelease) and several other benzodiazepines are among the most widely prescribed drugs in the world (Finkle et al., 1979; Jones et al., 1985; Klotz et al., 1988) with short, intermediate or long term duration of action (Greenblatt et al., 1983; Cohen, 1983). Unfortunately, these highly effective psychoactive drugs are also subject to abuse, misuse, tolerance and dependence (Gerd et al., 1984; Busto et al., 1986; Haefely, 1986).

Diazepam (see figure 16) is administered as an antianxiety agent, muscle relaxant or anticonvulsant in single doses of 2 to 20 mg and up to 40 mg daily (Baselt et al., 1995).

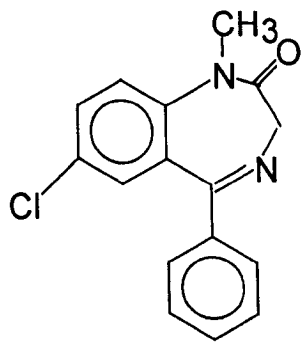


Figure 16. The structure of diazepam.

When ingested, diazepam undergoes extensive metabolism to a metabolite at least as active as the parent compound, nordiazepam. Further metabolism takes place and the two compounds, diazepam and nordiazepam, are converted to temazepam and

oxazepam, respectively (see figure 17). It has been reported in the literature (Duthel et al., 1992) that the serum of humans who did not receive any medication contained diazepam and some metabolites possibly due to its natural presence in some foods.

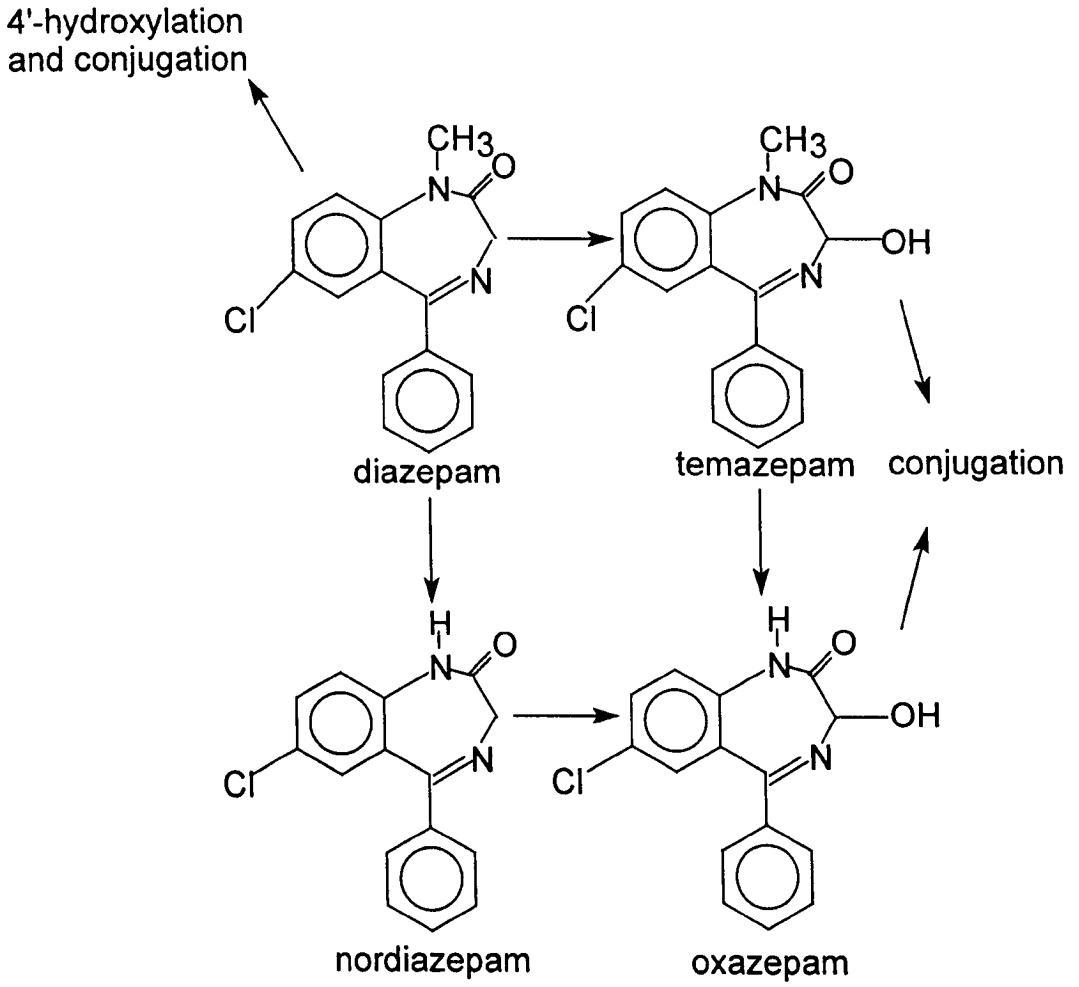


Figure 17. The metabolism of diazepam.

In 1989, Greenblatt et al. studied diazepam levels in 48 healthy individuals after a single oral diazepam dose of 10 mg. The peak plasma diazepam concentrations, average 0.406 mg/l, occurred within 0.5 to 2.5 hours and ranged from 0.253 to 0.586 mg/l. In another study (Kaplan et al., 1973), a single oral 10 mg dose of diazepam

resulted in an average peak blood diazepam concentration of 0.148 mg/l 1 hour after administration. After 24 hours, diazepam levels had declined to 0.037 mg/l. Nordiazepam, the major metabolite of diazepam, was also detected in blood after 24 hours at a peak concentration of 0.029 mg/l.

The levels of diazepam in the blood of individuals after chronic administration of the drug have been extensively studied. Chronic daily oral administration of 30 mg diazepam have resulted in steady-state plasma diazepam average concentration of 1.03 mg/l (range: 0.70 - 1.50 mg/l) and nordiazepam average concentration of 0.43 mg/l (0.35 - 0.52 mg/l) as reported by van der Kleijn in 1971. Studies on the administration of diazepam to schizophrenic patients have revealed that after a chronic daily 1 mg/Kg oral diazepam dose, steady-state serum levels for diazepam ranged from 2 to 4 mg/l, for nordiazepam ranged from 1 to 2 mg/l, for temazepam ranged from 0.1 to 0.6 mg/l and for oxazepam ranged from 0.05 to 0.40 mg/l (Tada et al., 1985).

When diazepam was administered intravenously (as opposed to orally) to 6 individuals at a dose of 20 mg, peak serum diazepam concentrations averaged 1.60 mg/l at 15 minutes declining to 0.44 mg/l at 2 hours. Nordiazepam levels remained below 0.10 mg/l for the 3 days following the injection (Hillestad et al., 1974). One study reported a peak serum diazepam concentration of 10 mg/l in a patient receiving frequent intravenous injections of diazepam as a means of controlling alcohol withdrawal symptoms (Kelly et al., 1979).

The half-lives of diazepam and nordiazepam have been estimated at 21-37 hours and 50-99 hours, respectively (Kaplan et al., 1973). However, obesity appeared to

prolong the half-lives to 51-122 hours for diazepam and 91-182 hours for nordiazepam (Abernethy et al., 1983).

Despite the commonality of benzodiazepine use and abuse around the world, there has been no report to date offering a methodology for the detection of these sedative hypnotics in nail specimens.

The present study established the use of nail clippings as an analytical specimen for the detection and quantification of diazepam, the most common of the benzodiazepines. This was achieved by means of gas chromatography equipped with an electron capturing detection system (ECD-GC) which could be applied to forensic cases in which diazepam was suspected to have been a contributing factor.

4.2. Methods & Materials

4.2.1. Standards and Reagents

All chemicals and reagents used were of high-performance liquid chromatography grade. Diazepam and prazepam (used as internal standard) were obtained from SIGMA (Dorset, UK).

4.2.2. Samples

The nail clippings examined in this study were obtained with consent from 6 benzodiazepine users participating in the Edinburgh Drug Addiction Study (EDAS) in Edinburgh, Scotland. The procedures followed were in accordance with the ethical standards of the responsible regional committee on human experimentation. At the time

of sampling the participants were asked to provide answers to a short questionnaire regarding their use of benzodiazepines and other drugs. This information is listed in table 11. Nail clippings were collected from all the subjects' fingers, pooled together and stored in plastic bags at room temperature. The samples thus packed were transported to the forensic toxicology laboratory in Glasgow for analysis.

Table 11. Drug usage by EDAS subjects participating in the benzodiazepines study as reported on the self-reporting questionnaires at the time of sampling.

Sample	Drugs taken
EDAS-101	Diazepam, Dihydrocodeine, Methadone
EDAS-107	Diazepam, Temazepam, Dihydrocodeine
EDAS-111	Diazepam, Amphetamine, Dihydrocodeine
EDAS-113	Diazepam, Amphetamine, Methadone
EDAS-115	Diazepam, Temazepam
EDAS-123	Diazepam, Dihydrocodeine, Propranolol

4.2.3. Sample Decontamination for ECD-GC

The sets of nail clippings obtained from EDAS volunteers were sonicated once with 10 ml of 0.1% SDS solution for 15 minutes using a CAMLAB TransSonic T 310 sonicator. The resulting wash solution was discarded by pouring. The sets of nail clippings were then sonicated in distilled water (10 ml) three times for 15 minutes each

time and the resulting washes were discarded by pouring as well. Methanol (10 ml) was subsequently used to sonicate the nail clippings three times for 15 minutes each time. The three methanolic washes were collected in separate vials and evaporated to dryness on a hot plate set at 60°C under a stream of nitrogen. The methanolic residues were analysed for the presence of benzodiazepines by ECD-GC after reconstitution in ethyl acetate.

4.2.4. Sample Extraction for ECD-GC

Once the methanolic washes had tested negative for the analytes of interest, the washed sets of nail clippings were weighed after being allowed to air dry overnight. Having rejected grinding of the nail clippings in liquid nitrogen using a mortar and pestle as a sample preparation method, alkaline hydrolysis was used as before. This involved the incubation of the washed nail clippings in 1 ml of 1M sodium hydroxide solution at 95°C for 30 minutes in the presence of internal standard (prazepam). After the resulting hydrolysates had cooled down to room temperature, borate buffer (pH 9.3) was added and the drugs were extracted into a toluene:heptane:isoamyl alcohol mixture (76:20:4 v/v/v) using a mechanical rock-n-roller. The organic layer from each mixture was then transferred into a clean screw-cap vial and evaporated to dryness on a hot plate set at 60°C under a stream of nitrogen. The nail extract residues were then reconstituted in ethyl acetate and subjected to ECD-GC.

4.2.5. ECD-GC

A 1.0-µl portion of each sample was injected on an HP-5 crosslinked dimethyl-silicone capillary column (30m x 0.32mm, 0.25-µm film thickness) mounted in a Hewlett-Packard 5890 Series II gas chromatograph equipped with an electron capture detection system. The injector temperature was 275°C and the detector temperature was 300°C. Helium was used as the carrier gas.

The initial column temperature was 100°C and was maintained at that for 1 minute after injection. The temperature was then programmed to rise to 200°C at a rate of 30°C/min and then to 280°C at a rate of 10°C/min. The column was subsequently maintained at 280°C for 15 minutes.

Qualitative and quantitative analyses of diazepam were obtained by comparison of retention times (t_R) and the use of standard curves similar to the one presented in figure 18.

4.2.6. Method Validation

As no method is currently available to test intact nail matrix spiked with diazepam, the limit of detection and extraction recovery were determined after dissolution of the nail in sodium hydroxide. Ten different nail hydrolysates in NaOH (each equivalent to 10 mg of nail) were spiked with diazepam and then analysed as described earlier in order to determine the extraction recovery of the method. Furthermore, three nail hydrolysates in NaOH (each equivalent to 10 mg of nail) were spiked with decreasing amounts of diazepam and then analysed as usual in order to determine the limit of detection of the method.

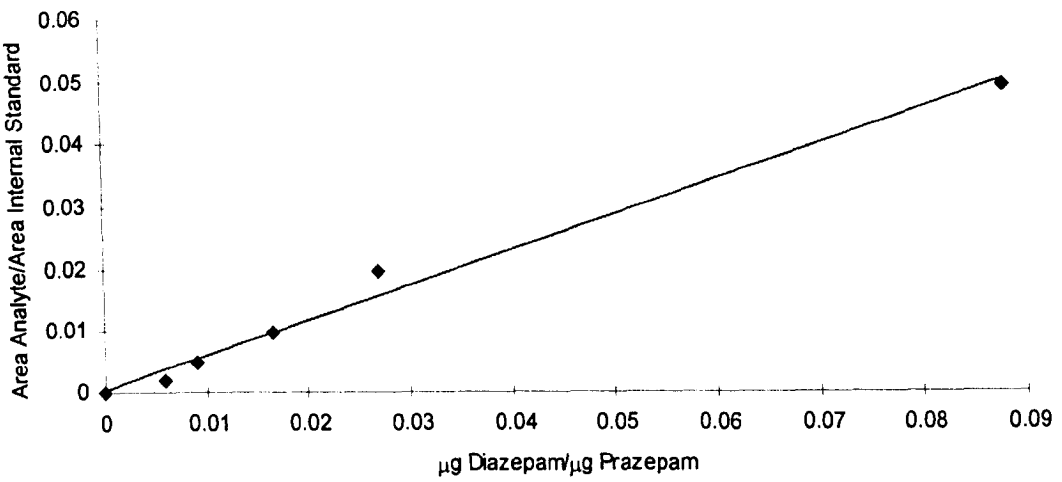


Figure 18. A standard curve used for the quantification of diazepam in nail (r^2=0.9938).

4.3. Results & Discussion

The retention times for diazepam and prazepam (used as internal standard) were 13.8 and 16.5 minutes (see figure 19). Calibration curves were used for the quantification of this analyte and were linear (r^2> 0.99).

The limit of detection and extraction recovery calculated are presented in table 12. Following the experimental method presented in this study, concentrations of 0.01 ng/mg for diazepam could be detected if a minimum of 10 mg of nail was used for extraction. These detection limits were determined by decreasing the concentrations of the diazepam spiked in the hydrolysate of a 10-mg nail sample. The extraction recovery was determined for diazepam by spiking drug-free nail clipping hydrolysates with known quantities of the compound, subjecting them to our extraction method, and then analysing them using the instrumental method described earlier. The extraction recovery thus calculated was greater than 87% for diazepam.

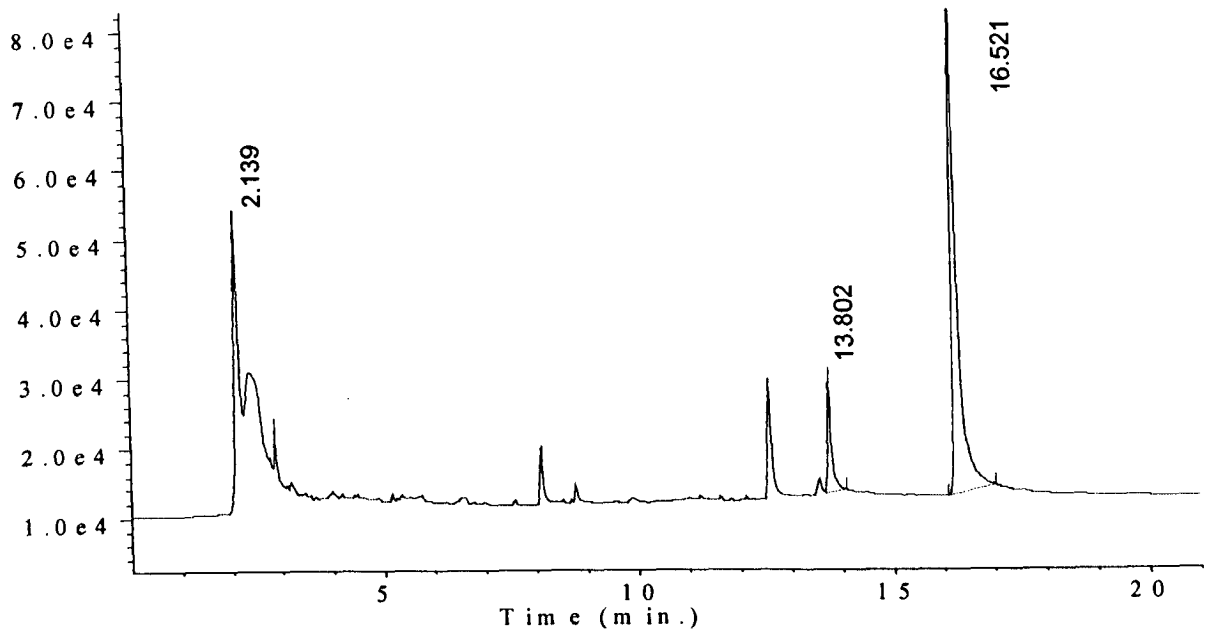
Using ECD-GC, diazepam was detected in all 6 nail clipping hydrolysates which were hydrolysed and extracted as described above. The concentration of diazepam ranged from 4.37 to 87.8 ng/mg with a mean concentration of 25.7 ng/mg (see table 13).

The new analytical procedure presented here has shown that the sonic wash protocol used always produced a negative diazepam result for all three methanolic washes in all 6 cases. In this way, superficial contamination, although less likely with tablet-form medication than with cannabis products, was removed before the nail extracts were analysed for diazepam by ECD-GC. Finally, using nail clippings as an analytical specimen permitted the positive identification of diazepam even after 9 months of sample storage at room temperature.

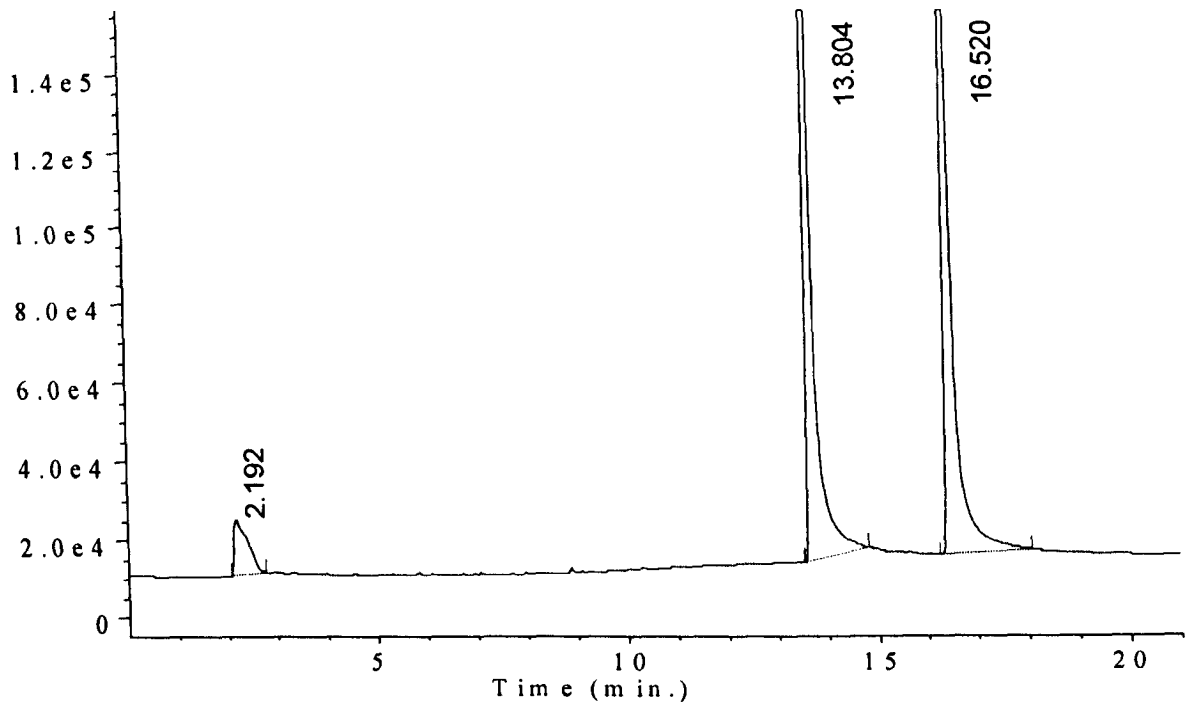
The combination of a nail hydrolysis scheme with a ECD-GC proved to be valuable, and endogenous nail components were found not to interfere with the analysis of diazepam. The analytical protocol for diazepam described here provides a rapid and clean extract that can be used in a high throughput setting. It also provides a reliable method that can be used for drugs-of-abuse testing.

Table 12. Limit of detection and extraction recovery of diazepam obtained with 10mg of drug-free nail spiked with diazepam.

Limit of Detection (ng/mg)	**Extraction Recovery (%)**
< 0.01	87.3



(A)



(B)

Figure 19. ECD gas chromatogram from (A) a diazepam-positive nail sample (determined concentration: 9.38 ng/mg) and (B) a mixture of standards.

Table 13. Concentration of diazepam in nail clippings determined by ECD-GC.

Sample	Weight of sample (mg)	Diazepam concentration (ng/mg)
EDAS-101	3.64	87.78
EDAS-107	4.68	13.32
EDAS-111	11.45	4.37
EDAS-113	8.25	9.31
EDAS-115	9.17	30.11
EDAS-123	7.04	9.38

4.4. Conclusions

This study offered a protocol involving the analysis of human nails for the presence of diazepam by ECD-GC. Using ECD-GC, diazepam was detected in all 6 nail clipping hydrolysates in concentrations ranging from 4.37 to 87.8 ng/mg (average: 25.7 ng/mg). Unfortunately, due to the lack of diazepam-use information from the donors' questionnaires, a correlation of the levels measured to the doses taken was not possible as part of this pilot study.

As mentioned earlier in this dissertation, nail clippings are potentially more easily collected than blood or urine and are especially useful because of their long-term retention of drugs. The main advantage of nail analysis as compared to blood or urine analysis remains its ability to detect long-term drug abuse for time periods from months to years. Moreover, the sample size required to detect diazepam exposure by nail analysis is small (3.64-11.45 mg) and nail samples are collected by non-invasive

procedures, are easy to store, allow for increased drug stability and are less likely to suffer from any melanin race bias (Garside et al., 1998).

In conclusion, the study described an analytical protocol using nails for the detection of past diazepam exposure by ECD-GC which has been offered to the analytical community (see annexe II).

5. OPIATE ANALYSIS IN NAIL

5.1. Introduction

In Scotland, excluding cannabis, heroin predominates in drug misuse accounting for almost half of the new individual notified addicts (49 %) and being involved in at least 30 deaths per annum according to recent official epidemiological data (Drug Misuse Statistics for Scotland, 1997). Injecting is still the predominant route of use in those cases where heroin is involved. 47% of heroin users still inject whereas only 4% take heroin orally and 32% smoke the drug (Drug Misuse Statistics for Scotland, 1997).

Heroin or diacetylmorphine (diamorphine) has been extensively studied and is known to be rapidly deacetylated in the body to 6-acetylmorphine (see figure 20). This metabolite undergoes further hydrolysis to morphine at a slower rate (Baselt et al., 1995). Morphine analysis is frequently used in forensic toxicology to determine the use of heroin (Tagliaro et al., 1998b).

To determine the usefulness of the nail as an analytical specimen in a forensic environment where radioimmunoassay (RIA) and high-pressure liquid chromatography (HPLC) are used for routine opiate analysis, nail clippings from known heroin users were examined for the presence of morphine by the two aforementioned techniques.

5.2. Methods & Materials

5.2.1. Standards and Reagents

All organic solvents were high-pressure liquid chromatography grade, and all chemicals were reagent grade. Morphine hydrochloride was purchased from CARLO ERBA (Milan, Italy). Sodium dodecyl phosphate (500mM), sodium hydroxide (1M) and sodium phosphate buffer (50 mM) were prepared from HPLC grade reagents in deionised water.

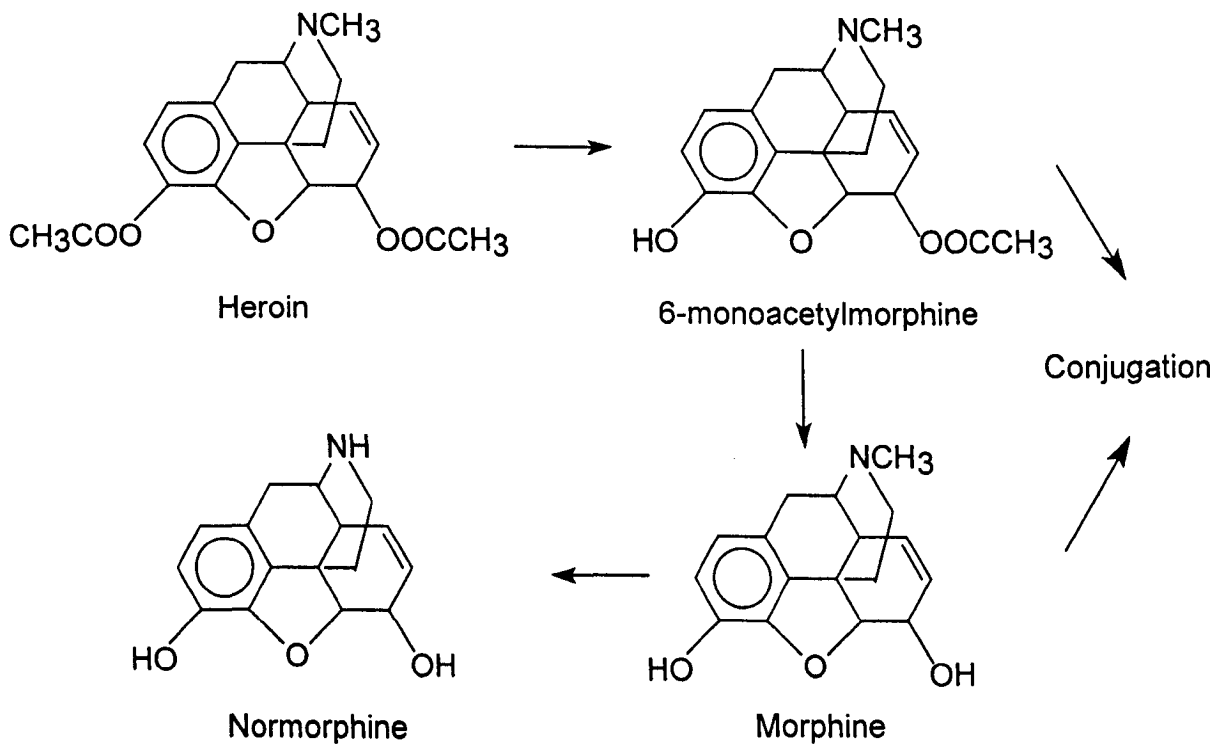


Figure 20. The metabolism of heroin.

5.2.2. Samples

Nail clippings (3.0 to 96.0 mg) were collected from consenting adults attending the clinics of the Glasgow Drug Problem Service in Glasgow, Scotland. The procedures followed were in accordance with the ethical standards of the responsible regional committee on human experimentation. At the time of sampling, the participants were asked to provide answers to a short questionnaire regarding their drug use patterns. Nail clippings were generated using commercially available cosmetic nail clippers. The nail clippings of each participant were pooled and stored in a plastic bag at room temperature until the time of analysis.

Demographic and epidemiological data for each participant are shown in table 14. The study population comprised 22 Caucasian males and 4 Caucasian females. Their average age was 26.5 years and ranged from 19 to 33 years.

In addition, 5 sets of fingernail clippings from individuals who were unlikely to be heroin or morphine users (i.e., laboratory personnel, postgraduate research students and academics) were obtained and used as controls.

A conventional drug screening using radioimmunoassay (RIA) followed by a confirmatory analysis based on high-pressure liquid chromatography (HPLC) was performed on the nail specimens at the Institute of Forensic Medicine, University of Verona, Verona, Italy.

5.2.3. Sample Decontamination

Using an ELMA-SINGEN HTW ultrasonication bath, the nail clippings were sonicated once in 10 ml of 500 mM sodium dodecyl sulphate (SDS) for 15 minutes and three times in 10 ml of deionised water for 15 minutes each time and all four resulting

washes were discarded. Next, there were 3 sonications in methanol for 15 minutes each and these washes were collected, evaporated to dryness at 50° C under a stream of pressurised air, reconstituted in 200 µl of 50 mM sodium phosphate buffer (pH 5.0) and mixed by vortex. A 50 µl aliquot was then screened by RIA for morphine. In those cases in which the third methanolic wash tested positive for morphine, a further (fourth) sonication in methanol for 15 minutes was conducted and the resulting wash was collected and screened by RIA for methanol as before. Only when the final methanolic wash (third or fourth, as required) tested negative for morphine did the analysis of the nail clippings proceed.

Table 14. Demographic and epidemiological data of the human subjects participating in the opiate study as reported on the self-reporting questionnaires at the time of sampling.

Sample	**Sex**	**Age (years)**	**Heroin consumption rate in GBP (£) or weight per time interval***	**Other drugs taken*,†**
1	Male	24	£20/day	M
2	Male	29	£30-100/day	Cn, D, M, T
3	Male	27	£60-80/day	Cn, Co, D, M
4	Male	23	£60-80/day	Cn, Co, D, M, T
5	Male	19	£10/day	M
6	Male	33	£100-150/day	Ap, Cn, D, M, T
7	Male	21	£10/day	Ap, Cn, M
8	Male	28	£10/day	Cn, D, M, T
9	Male	22	£10/day	D, M, T
10	Male	26	£20-30/day	Ap, Cn, M, T
11	Male	21	£80/day	Ap, Cn, Co, Dc, M, T
12	Male	32	£10/day	Cn, M
13	Female	23	£30/day	D, M
14	Male	32	£40-60/day	Cn, Co, D, M, T

Table 14. (cont.)

15	Female	25	£30-40/day	Ap, Cn, D, M, T
16	Male	31	£20/day	Cn, D, M
17	Male	19	£10/day	M
18	Male	24	£60/day	Ap, Co, M, T
19	Male	28	£30/day	Ap, Cn, Co, D, M, T
20	Male	27	£30/day	Cn, D, Dc, M, T
21	Female	33	£10/day	Cn, D, Dc, M
22	Male	24	1.5 g/day	Cn, Co, D, Dc, M, T
23	Male	31	£40/day	Ap, Cn, D, M, P
24	Male	31	£60/day	Ap, Cn, Co, D, M, T
25	Female	29	£40/day	At, Cn, D, Dc, M, T
26	Male	28	£20/day	Ap, Cn, D, M, T

*As reported on the questionnaires during sample collection at the clinics of Glasgow Drug Problem Service.

†At: Amitriptyline; Ap: Amphetamines; Cn: Cannabis; Co: Cocaine; D: Diazepam; Dc: Dihydrocodeine; M: Methadone; P: Paracetamol; T: Temazepam.

5.2.4. Sample Extraction and RIA

The decontaminated sets of nail clippings were allowed to air dry overnight and weighed. Alkaline hydrolysis was chosen as the extraction method for this study. This involved the incubation of the nail clippings for 60 to 120 minutes at 60° C in the presence of 1 ml of 1M sodium hydroxide solution. The resulting nail hydrolysates were then evaporated to dryness on a hot plate set at 50° C under a stream of pressurised air. Once dry, the residues were dissolved in 4 ml of deionised water, the pH was adjusted to 7.0, and the samples were sonicated for one hour and transferred to TOXI•TUBES A. The volume was brought up to the 5-ml mark using deionised water and the extraction tubes were agitated for 10 minutes. The extraction tubes were then centrifuged at 3500 rpm for 15 minutes and the resulting organic layer was stored. To each extraction tube containing the aqueous phase, 1 ml of dichloromethane: dichloroethane: heptane (19:18:63 v/v/v) was added and the extraction tubes were again agitated and centrifuged as before as part of a second extraction scheme. The organic layers from the two extractions were combined and evaporated to dryness under a stream of pressurised air. The dry residues were reconstituted in 200 µl of 50 mM sodium phosphate buffer (pH 5.0) and mixed by vortex. A 50 µl aliquot of the resulting solution was then analysed for morphine using the Coat-A-Count® Morphine solid-phase ^{125}I radioimmunoassay by DPC and a Packard Instrument RIA counter (Downers Grove, IL). The RIA kits contained antiserum which, according to their manufacturers, is highly specific to free (unconjugated) morphine with low crossreactivity to 6-acetylmorphine (1%), codeine (0.06%), morphine-3-glucuronide (0.3%) and morphine-6-glucuronide (0.05%). The standard curve was prepared from morphine hydrochloride

in 50 mM sodium phosphate buffer (pH 5.0) at concentrations of 0.0, 7.8, 15.6, 31.2, 62.5, 125.0 and 500.0 ng/ml.

5.2.5. HPLC

Aliquots of the nail extracts were filtered using micro-filters (0.45 μm) attached to syringes and the filtrates (diluted 1:1 with 50 mM sodium phosphate buffer) were analysed for morphine by HPLC following a published method (Tagliaro et al. 1988) reviewed below.

The HPLC instrument used for this analysis consisted of a Spectra-Physics AS 300 autosampler, a PRLP-S polymeric column (150 x 4.6 mm) operated at 63.8° C, a Jasco 880-PU pump, a BIO-RAD Electrochemical Detector and a Spectra Physics Chromjet integrator. During our sample analysis, morphine standards of known concentrations, blanks and spikes were also run and the morphine retention time was monitored.

5.2.6. Method Validation

To date, there is no published method available for spiking the intact nail matrix with morphine. Thus, the limits of detection and extraction recoveries of morphine were determined by spiking the solution obtained after hydrolysis of drug-free nail in sodium hydroxide. Ten different nail hydrolysates in NaOH (each equivalent to 10 mg of nail) were spiked with morphine and then analysed as usual in order to determine the extraction recovery of the method. Furthermore, three nail hydrolysates in NaOH (each equivalent to 10 mg of nail) were spiked with decreasing amounts of morphine and then analysed as usual in order to determine the limit of detection of the method.

5.2.7. Spiking Experiments

Studies were performed to test for possible contamination by morphine originating from a source external to the human body. Drug-free nail clippings (10 mg) were obtained from volunteers who did not use heroin or morphine. These sets of nail clippings were placed in separate vials in the presence of 10 ml morphine solution (10 μg/ml) and were allowed to incubate undisturbed at room temperature for 24 hours.

Upon completion of each incubation period, the nail clippings were transferred from their incubation vials into new, clean vials using clean surgical forceps. The sets of nail clippings were then decontaminated and analysed for morphine by the methods described above in order to establish whether or not any detectable transfer from the solution into the nail matrix had taken place.

5.3. Results & Discussion

The spiking experiments described above indicated that any detectable absorption of morphine from the morphine solution into the nail matrix was successfully removed by the decontamination protocol employed.

The decontamination protocol produced contamination-free nail clippings. In almost two thirds of the cases (65.4 %), the SDS wash, the three water washes and three methanol washes were sufficient in removing any superficial contamination and to produce a negative third methanol screen for morphine. In the remaining one third of the cases (34.6 %), a fourth methanol sonic wash was necessary before a negative methanol screen for morphine was obtained.

The results of the RIA screening and HPLC confirmation for the nail extracts and blanks are summarised in table 15 together with data regarding their final methanolic wash (either the third or the fourth wash, as required). As illustrated in table 15, the decontamination protocol employed in this study produced contamination-free nail clippings by the third (65.4% of the cases) or fourth (35.4% of the cases) methanolic wash and, had the nail samples not been decontaminated in this way, the final results would in most cases be heavily influenced. All blank samples and their methanolic washes tested negative for morphine by RIA and HPLC.

Hydrolysates of fingernail clippings were extracted using TOXI•TUBES A and liquid-liquid extraction and then assayed by RIA and HPLC for the presence of morphine. A typical RIA calibration curve showing the range of linearity of the technique is presented in figure 21 and chromatograms of analyses of a blank, a standard and a nail extract are shown in figure 22. Positive RIA results were obtained with nail clippings from 25 of the 26 heroin users. The levels ranged from 0.06 to 4.69 ng/mg with an average morphine concentration of 1.67 ng/mg. Using high-pressure liquid chromatography, morphine was positive in 22 of the 26 nail samples. The mean morphine level by HPLC was 2.11 ng/mg with a range from 0.14 to 6.90 ng/mg.

Figure 23 presents the level of morphine seen in each of the samples by both RIA and HPLC plotted against the monetary level of heroin used (in GBP, £) as reported by each participant on his/her questionnaire at the time of sampling. The noticeable variations of morphine levels among individuals who declared to have consumed the same amounts of heroin might be due to several factors which are discussed below.

In regard to the limit of detection of the method, concentrations as low as 0.05 ng/mg morphine could be detected if the equivalent of at least 10 mg of nail was used for extraction, with a signal-to-noise ratio of 5 (see table 16). This detection limit was determined by decreasing the concentration of the drug spiked in nail hydrolysate equivalent to 10 mg of nail. An extraction recovery was determined for morphine by spiking hydrolysate of drug-free nail clippings with known quantities of morphine, subjecting them to the extraction method and then analysing them using the instrumental methods described earlier. The extraction recovery thus calculated was 80.5 % for morphine by RIA and 86.3% for morphine by HPLC (table 16).

The relatively small size of the sample population examined in the present study (N=26) does not allow for the construction of proper dose-response relationships. The heterogeneity of the street heroin available in Scotland may also be an important factor. Having to rely on the self-reporting figures of what the participants think they have consumed as opposed to conducting experiments under controlled dosage parameters adds at least two potential sources of errors (what the participants think they have consumed and what the participants declare to have consumed based on what they believe their answers should be). Variations in the heroin consumption patterns of the volunteers and the varying length of heroin use within this group may also be significant factors in determining morphine concentrations in nail. Finally, the mechanism(s) of substance incorporation into the nail matrix are not yet understood and there may be substantial inter-individual variation due to physiological, biochemical and pharmacokinetic factors.

Table 15. Morphine levels in the final methanolic wash (third or fourth, as required) measured by RIA and in the nail extracts screened by RIA and confirmed by HPLC*.

Sample	Sample weight (mg)	Wash 3 (ng/ml)	Wash 4 (ng/ml)	RIA Screening (ng/mg)	HPLC Confirmation (ng/mg)
1	22.8	7.81	NEG	1.10	1.30
2	47.0	3.87	NEG	4.10	6.60
3	61.0	NEG	N/R	0.80	0.90
4	16.0	NEG	N/R	1.80	1.80
5	35.1	3.36	NEG	1.40	2.00
6	16.9	2.98	NEG	1.90	1.40
7	11.0	NEG	N/R	1.50	1.90
8	7.0	7.96	NEG	3.20	3.90
9	10.9	NEG	N/R	1.60	1.30
10	13.3	NEG	N/R	0.69	0.51
11	15.3	NEG	N/R	1.24	1.54
12	35.4	NEG	N/R	0.32	0.00
13	96.0	NEG	N/R	2.70	6.90
14	36.9	NEG	N/R	2.05	2.90
15	17.5	9.82	NEG	3.38	2.68
16	8.8	3.76	NEG	1.53	1.22
17	30.0	NEG	N/R	0.39	0.45

Table 15. (cont.)

18	9.2	NEG	N/R	1.16	0.50
19	23.4	NEG	N/R	0.62	0.57
20	29.2	NEG	N/R	0.06	0.00
21	15.4	5.79	NEG	4.69	4.51
22	6.3	NEG	N/R	1.39	0.00
23	3.0	NEG	N/R	0.00	0.00
24	38.5	NEG	N/R	0.32	0.14
25	48.8	NEG	N/R	0.91	0.72
26	31.6	1.09	NEG	2.80	2.59

*NEG: Negative morphine screen by RIA; N/R: 4th methanolic wash not required.

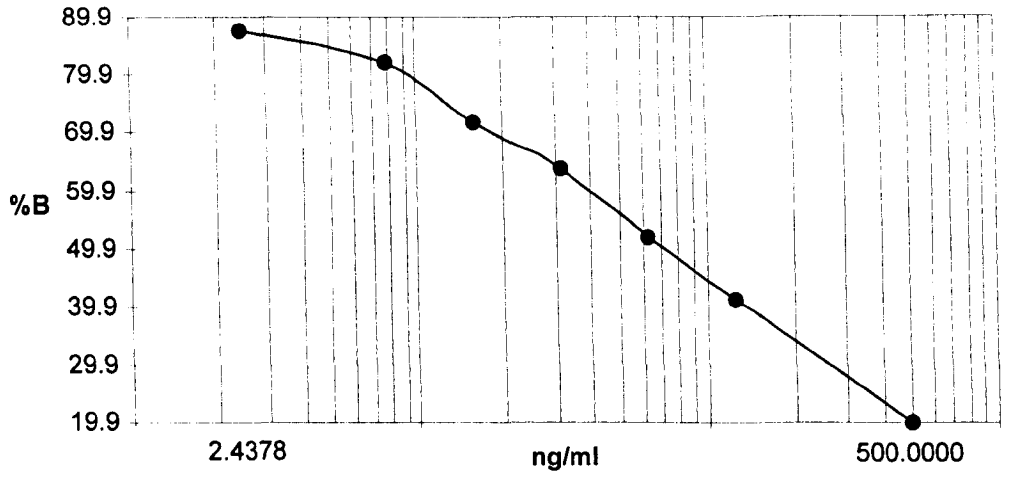


Figure 21. A typical RIA calibration curve generated using morphine calibrators showing the range of linearity for this assay and used for the initial morphine screening of nail extracts.

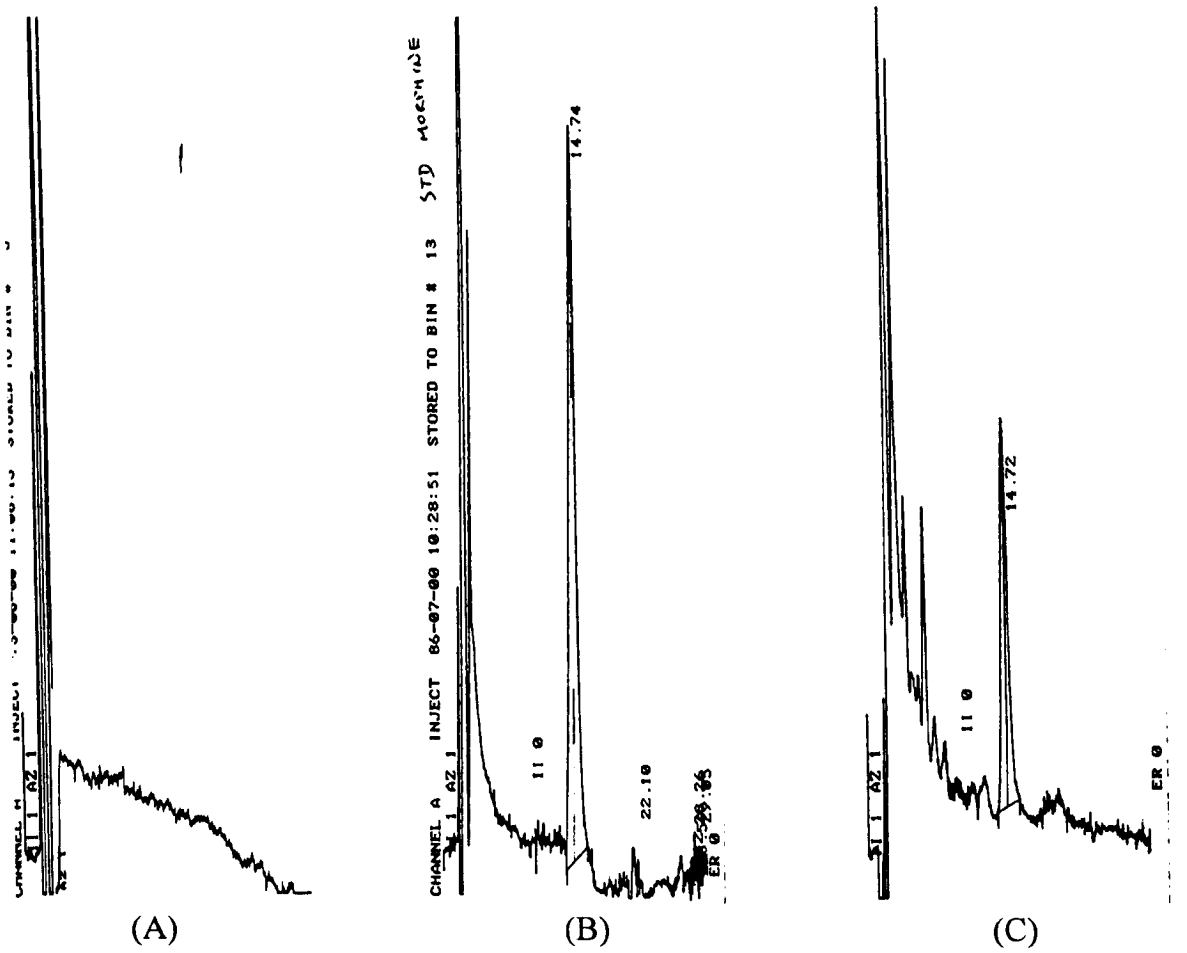


Figure 22. High-pressure liquid chromatograms of (A) a drug-free nail extract, (B) a standard morphine solution and (C) a nail extract from a participant (determined concentration: 1.30 ng/mg).

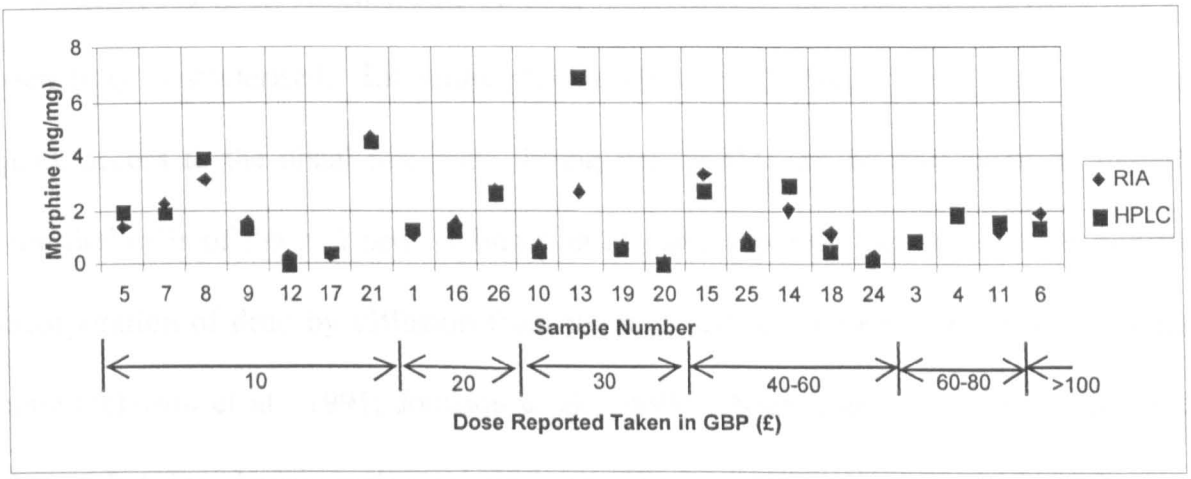


Figure 23. Morphine levels in ng/mg measured by RIA and HPLC in each of our samples plotted against the monetary value (in GBP, £) of heroin used by each participant as declared on his/her questionnaire at the time of sampling.

Table 16. Morphine limits of detection and extraction recoveries by RIA and HPLC obtained with 10mg of drug-free nail spiked with morphine.

Morphine by:	**Limit of Detection (ng/mg)**	**Extraction Recovery (%)**
RIA	< 0.05	80.5
HPLC	< 0.05	86.3

Although there is a significant portion of the scientific literature on the detection of drugs in nails, the mechanisms of drug incorporation into this matrix have not yet been fully characterised. Literature reports exist which suggest that drugs may gain quick access to the distal nail plate during nail production by incorporation into the cornified cells of the nail bed. Johnson et al. have reported that there exists minimal incorporation of drug by diffusion from the nail bed to the ventral portion of the nail plate (Johnson et al., 1991; Johnson et al., 1993). Norton and Zaias have separately shown that drug incorporation occurs in the lunular germinal matrix as the nail grows from the base of the nail along the distal axis (Norton, 1971; Zaias, 1967). There exist other potential sources of drug entry into the nail such as environmental contamination, and contamination from sweat, saliva, sebum, urine, etc. Finally, the chemical properties of the drugs in consideration could also play a protagonistic role in their incorporation into the nail. Further studies are needed to determine the actual mechanism(s) of drug incorporation into the nail matrix and the roles played by physiological, biochemical and pharmacokinetic factors.

The addiction history of heroin users in forensic toxicology cases is often determined by morphine analysis in hair. In this study nail clippings were successfully evaluated as analytical specimens. The nail decontamination protocol proposed herein consistently produced negative morphine screenings for the final methanol wash. Morphine was determined in decontaminated nail clippings by RIA (mean: 1.67 ng/mg) and confirmed by HPLC (mean: 2.11 ng/mg).

The nail matrix offers several advantages to the forensic toxicologist. Drugs remain trapped in the nail matrix for extensive periods of time thus allowing the

determination of exposure to drugs for periods from months to years. Furthermore, the collection of nail clippings is a non-invasive procedure as compared to the collection of blood or urine and only a small sample size is required as demonstrated in this study (mean sample size: 26.4 mg). Nails are easily stored in plastic bags at room temperature, allow for increased stability of drugs and are less likely to suffer any melanin race bias (Garside et al., 1998). It is because of these advantages that more work needs to be done on drug analysis in the nails. Based on the work presented in this chapter, nails should be considered a powerful analytical specimen for the detection of past heroin use in forensic cases.

5.4. Conclusions

This study offered a protocol involving the analysis of human nail for the presence of morphine. The presence of morphine in the nail clipping hydrolysates was initially screened by RIA and confirmed by HPLC. Positive RIA results were obtained with nail clippings from 25 of the 26 heroin users. The levels ranged from 0.06 to 4.69 ng/mg with an average morphine concentration of 1.67 ng/mg. Using high-pressure liquid chromatography, morphine was positive in 22 of the 26 nail samples. The mean morphine level by HPLC was 2.11 ng/mg with a range from 0.14 to 6.90 ng/mg.

As mentioned before, nail clippings are potentially more easily collected than blood or urine and are especially useful because of their long-term retention of drugs. The main advantage of nail analysis as compared to blood or urine analyses remains its ability to detect long-term drug abuse for time periods from months to years. Moreover, the sample size required to detect morphine presence by nail analysis was very small

and nail samples are collected by non-invasive procedures, are easy to store, allow for increased drug stability and are potentially less likely to suffer from any melanin race bias (Garside et al., 1998).

In conclusion, based on this work and understanding of these specimens, nails were suggested as having the potential of becoming a powerful analytical specimen for the detection of past heroin use in forensic cases (see annexes IV and V).

6. METHADONE ANALYSIS IN NAIL

6.1. Introduction

Methadone (figure 24) was first synthesised in Germany during World War II as a morphine substitute (Baselt et al., 1995). It possesses pharmacological properties similar to those of morphine and when administered parenterally it is approximately equipotent (Goodman Gilman et al., 1990). Methadone also produces sedative effects with chronic use as a result of drug accumulation. This initially undesirable effect of methadone was not taken advantage of until 1965 when Nyswander and Dole began narcotic maintenance treatment of former heroin users by administering large daily oral doses.

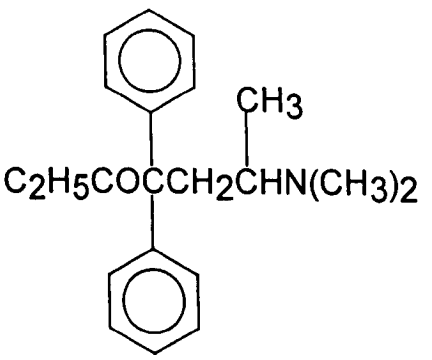


Figure 24. The structure of methadone.

In patients with low methadone tolerance, 50 mg or less of the drug have been known to be fatal whereas, in maintenance patients as much as 180 mg have been administered on a daily basis as part of their maintenance program.

Methadone undergoes extensive metabolism by N-demethylation (see figure 25) in the body and the unstable intermediate metabolites rapidly form 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenylpyrroline (EMDP) (Pohland et al., 1971). Further metabolism by hydroxylation with subsequent glucuronidation has been reported in the literature for all three compounds (Sullivan et al,. 1972a; Baselt et al., 1973).

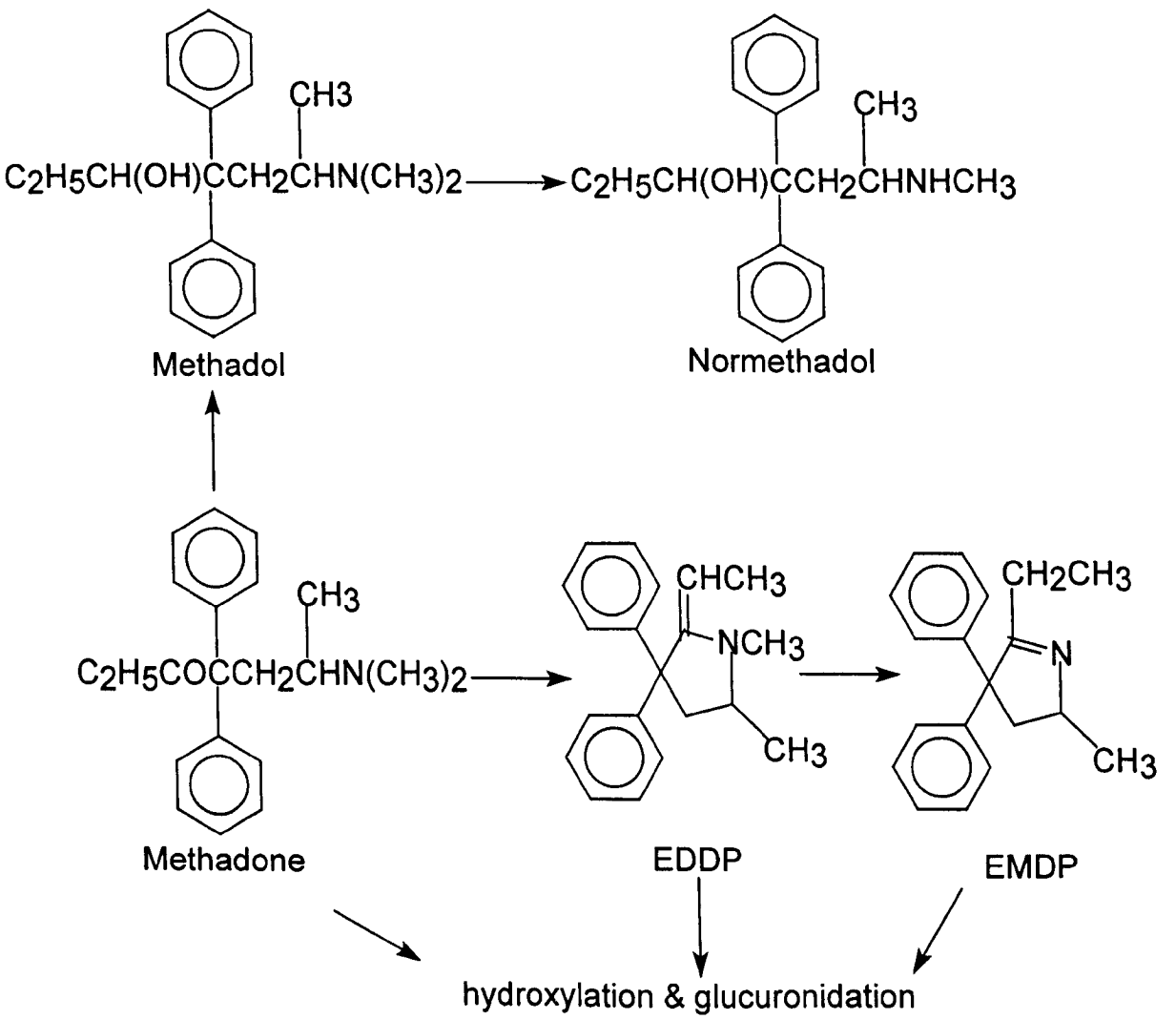


Figure 25. The metabolism of methadone.

Intramuscular injection of methadone at different parts of the body has been reported to cause a range of plasma levels. A single 10 mg methadone dose injected intramuscularly at the gluteal region has resulted in an average plasma methadone peak concentration of 0.034 mg/l at 50 minutes following the injection. The same dose injected into the deltoid region has resulted in an average plasma methadone peak concentration of 0.096 mg/l at 34 minutes after the injection (Grabinski et al., 1983).

A single 10 mg intravenous dose resulted in plasma concentrations as high as 0.50 mg/l immediately after injection but the levels decreased to 0.04 - 0.05 mg/l within two hours after injection (Inturrisi et al., 1987).

Finally, a 15 mg oral dose of methadone has resulted in peak plasma concentrations of 0.075 mg/l at 4 hours after ingestion which declined slowly to 0.030 mg/l at 24 hours after ingestion (Inturrisi et al., 1972a).

In tolerant subjects who received chronic methadone doses of 100 - 200 mg per day, the average peak plasma concentration was 0.83 mg/l at 4 hours and declined to 0.46 mg/l at 24 hours after the last dose (Inturrisi et al., 1972b). In 1991, Wolff et al. reported that, in maintenance patients plasma methadone concentrations increased by an average of 0.263 mg/l for every 1mg/Kg increase in oral dosage. A reduction of 50% in the plasma methadone concentration of maintenance patients has been reported as a result of omission of a single daily dose (Verebely et al., 1975). Bell et al., have reported in 1988 that, in order for a methadone maintenance program to be effective and for patients not to experience withdrawal symptoms, through plasma methadone levels should be at least 0.05-0.10 mg/l.

The analysis of biological samples for methadone and its major metabolites, EDDP and EMDP, have been extensively studied. All three compounds have been identified by gas chromatography equipped with flame ionisation detection without derivatisation (Inturrisi et al., 1972c; Sullivan et al., 1972b; Thompson et al., 1977; Greizerstein et al., 1983). Gas chromatography coupled to mass spectrometry has also been offered as an analytical technique for methadone detection (Sullivan et al., 1975; Kang et al., 1982; Baugh et al., 1991). Finally, several liquid chromatographic techniques have been offered in the literature (Rio et al., 1987; Wolff et al., 1991).

Blood and urine are not the only specimens for which analytical protocols for the detection of methadone have been offered in the literature. Methadone detection in alternative biological samples, namely hair, has also been published. In 1994 and again in 1995, Marsh et al. offered radioimmunological methods for methadone in hair and evaluated several decontamination procedures for this specimen. Furthermore, gas chromatography-mass spectrometry has recently been employed for the detection of methadone, EDDP and EMDP in human hair after solid phase extraction (Goldberger et al., 1998).

The present study was conducted in order to determine the usefulness of nail as an analytical specimen for methadone detection and quantification. The experiments were carried out using nail clippings from drug users already on a methadone maintenance program. Adaptations of a blood methadone enzyme immunoassay (EIA) method for screening and a gas chromatography-mass spectrometry (GC-MS) method for confirmation were evaluated.

6.2. Methods and Materials

6.2.1. Standards and Reagents

All organic solvents were high-pressure liquid chromatography grade, and all chemicals were reagent grade. Methadone was purchased from Alltech-Applied Science Labs (State College, PA) and methadone-d_3 was purchased from High Standard Products Corporation (Inglewood, CA). Sodium dodecyl phosphate (500mM) and sodium hydroxide (1M) were prepared from HPLC grade reagents in deionised water.

6.2.2. Samples

Nail clippings (0.18 - 16.33 mg) were collected from consenting adults attending the methadone maintenance clinics of the Edinburgh Drug Addiction Study (EDAS) in Edinburgh, Scotland. The procedures followed were in accordance with the ethical standards of the responsible regional committee on human experimentation. At the time of sampling, the participants were asked to provide answers to a short questionnaire regarding their drug use patterns. Nail clippings were obtained using commercially available cosmetic nail clippers. The nail clippings of each participant were pooled and stored in a plastic bag at room temperature until the time of analysis. Demographic and epidemiological data for each participant are shown in Table 17. The sample population studied comprised 29 Caucasians, 19 males and 10 females. Their average age was 34.3 years within a range of 22 to 47 years.

In addition, 5 sets of fingernail clippings from individuals who were unlikely to be methadone users (i.e., laboratory personnel, postgraduate research students and academics) were obtained and used as controls.

A conventional drug screen using enzyme immunoassay (EIA) followed by a confirmatory analysis based on gas chromatography-mass spectrometry (GC-MS) was performed on the nail specimens in the forensic laboratory in Glasgow.

6.2.3. Sample Decontamination

Using an ultrasonication bath, the sets of nail clippings were sonicated once in 10 ml of 0.1% sodium dodecyl sulphate (SDS) for 15 minutes and three times in 10 ml of deionised water for 15 minutes each time and each of the four resulting washes were discarded by pouring. Next, the samples were sonicated in methanol three times for 15 minutes each and these washes were collected in separate vials, evaporated to dryness on a hot plate set at 50° C under a stream of nitrogen, reconstituted in phosphate buffer (pH 7.0) and screened by enzyme immunoassay for methadone. Only when the final methanolic wash tested negative for methadone did the analysis of the nail clippings proceed.

Table 17. Demographic and epidemiological data of the human subjects participating in the methadone study as reported at the time of sampling.

Sample	Sex	Age (years)	Methadone dose and length of time dose taken*	Other drugs taken*,†
1	Female	35	50 ml/day	D
2	Male	32	65 ml/day - 2 years	At, Ap
3	Female	47	100 ml/day - 2 years	Ap, D
4	Female	32	100 ml/day - 2 years	At, Cn, T
5	Female	22	N/A‡	Ap, Cn, D, Dc
6	Female	33	140 ml/day	D, T
7	Male	29	N/A	Cn, D, Dc
8	Male	42	80 ml/day	D
9	Male	32	N/A	Cn, D, Dc
10	Male	29	N/A	Cn, D, Dc
11	Female	35	N/A	Ap, Cn, D, Dc
13	Female	34	N/A	Ap, Cn, Dc, T
14	Female	35	N/A	D, Mo
15	Male	31	N/A	Cn, D, H
16	Male	38	70 ml/day	Cn, D, Dc
17	Male	N/A	70 ml/day	Ap, Cn, D
18	Male	31	N/A	Cn, D, Dc

Table 17. (cont.)

19	Male	32	N/A	Ap, Cn, D, Dc, H
20	Female	37	65 ml/day	Cn, D
21	Female	47	90 ml/day	Cn, D
22	Male	43	140 ml/day	D
23	Male	30	80 ml/day	Cn, D
24	Male	23	60 ml/day	Cn, D
25	Male	37	N/A	Cn, Dc
26	Male	38	N/A	H
27	Male	31	N/A	Ap, Cl, Cn, Dc
28	Male	45	N/A	Cn, D, Dc
29	Male	36	50 ml/day	Cn, D
30	Male	26	N/A	At, Ap, Cn, D, Dc, LSD

*As reported on the questionnaires during sample collection at the clinics of Glasgow Drug Problem Service.

†At: Amitriptyline; Ap: Amphetamines; Cl: Chlorpromazine; Cn: Cannabis; Co: Cocaine; D: Diazepam; Dc: Dihydrocodeine; H: Heroin; LSD: Lysergic acid diethylamide; M: Methadone; Mo: Morphine sulphate; P: Paracetamol; T: Temazepam.

‡N/A: not answered.

6.2.4. Sample Extraction and EIA

Once the final methanolic washes had tested negative for methadone, the decontaminated sets of nail clippings were allowed to air dry overnight and weighed. Alkaline hydrolysis was chosen as the extraction method for this study. This involved the incubation of the sets of nail clippings for 30 to 40 minutes at 90° C in the presence of 1 ml of 1M sodium hydroxide solution. The resulting nail hydrolysates were then divided into two halves of 500 µl; one intended for methadone screening by EIA and the other for methadone confirmation by GC-MS. The aliquots intended for GC-MS confirmation were stored at 4° C until such confirmation was necessary. To the aliquots intended for methadone screening by EIA, 500 µl of phosphate buffered saline solution (1 x PBS solution) were added and the samples were subjected to enzyme immunoassay using the methadone microplate EIA forensic application by Cozart Bioscience Ltd (Oxfordshire, UK). The EIA analyses were carried out using a MARK-5 sample processor by DPC for all precision pipetting and Dynatech MRW and Dynatech MRX instruments for the rinsing and reading of the microplates, respectively. The EIA kits used had been tested for a wide range of non-related drugs at 10,000 ng/ml in serum with no cross-reactivity found according to their manufacturers, whereas low cross-reactivities have been reported to EDDP (0.01-0.69%) and EMDP (0.01-0.59%). Standard curves used in the analyses were prepared from methadone-containing protein matrices as supplied by the manufactures.

6.2.5. SPE

To the 500-µl aliquots intended for GC-MS confirmation, 50 µl of internal standard solution, containing 100 ng methadone-d_3 were added and the volume was

brought up to 1 ml with 100 mM phosphate buffer (pH 6.0). The samples were then vortexed and extracted using the 200-mg Clean Screen® solid phase extraction columns by Worldwide Monitoring Corporation (Congleton, UK). The extractions took place on a VARIAN Vac-Elut™ vacuum workstation using a Millipore® vacuum pump. The columns were initially conditioned using 3 ml methanol followed by 3 ml water and 1 ml 100 mM phosphate buffer (pH 6.0). The samples were then slowly applied at a rate of 1-2 ml/minute. The columns were then washed with 2 ml deionised water followed by 2 ml 100 mM hydrochloric acid, HCl, and 3 ml methanol and dried under maximum vacuum (≥ 10 inches Hg). Finally, the analytes were eluted using a mixture of dichloromethane: isopropyl alcohol: ammonia (78:20:2 v/v/v) which was freshly prepared on each day of analysis. The eluates were evaporated to dryness on a hot plate set at 40° C under a stream of nitrogen and the resulting residues were reconstituted in 100 µl ethyl acetate before confirmatory analyses took place by GC-MS.

6.2.6. GC-MS

A 2.0-µl portion of each sample was injected through an HP-5 capillary column (crosslinked 5% PH ME Silicone; 30m x 0.32mm, 0.25-µm film thickness) in a Hewlett Packard HP 5890 gas chromatograph coupled to a VG Analytical 70-250S double focussing mass spectrometer. The gas chromatograph injector temperature was 280°C and splitless injection was employed with a split-valve off-time of 0.7 minute. The mass spectrometer was operated at a resolution of 1000 and data was acquired and processed using a Mass Spectrometry Services (MSS) Maspec I Data System. Helium was used as the carrier gas at 5 psi. The column temperature was initially 180°C and was programmed to rise to 280°C at a rate of 10°C/min immediately after injection.

The capillary column was inserted directly into the ion source at a temperature of 200°C via a heated transfer line (275°C). The mass spectrometer was operated in the selected ion recording mode with electron impact ionisation at an electron energy of 70 eV and was tuned daily using PFK according to the manufacturer's recommendations. For methadone, qualitative and quantitative analyses were obtained using the selected ion recording mode and comparison of retention times (t_R) and relative abundance of confirming ions with methadone-d_3. Quantitative results were obtained in the selected ion recording mode after determination of the response factor of methadone against methadone-d_3.

6.2.7. Method Validation

As no method currently available tests intact nail matrix spiked with methadone, the limits of detection and extraction recoveries for methadone were determined after dissolution of the nail in sodium hydroxide. Ten different nail hydrolysates in NaOH (each equivalent to 10 mg of nail) were spiked with methadone and then analysed as usual in order to determine the extraction recovery of the method. Furthermore, three nail hydrolysates in NaOH (each equivalent to 10 mg of nail) were spiked with decreasing amounts of methadone and then analysed as usual in order to determine the limits of detection of the methods.

6.2.8. Results & Discussion

The decontamination protocol produced contamination-free nail clippings. In every case examined in this study, the SDS wash, the three water washes and three methanol washes were sufficient to remove any superficial contamination and to

produce a negative third methanol screen for methadone. In 90% of our cases, the second methanolic washes were also negative for methadone.

Table 18 shows the ions monitored for methadone and methadone-d_3 along with their respective retention times (t_R). A typical EIA calibration curve showing the range of linearity of the technique is presented in figure 26 and chromatograms of analyses of a nail extract and a standard mixture are shown in figures 27 and 28, respectively.

The results of EIA screening and GC-MS confirmation for the nail extracts and blanks are summarised in table 19. All blank samples and their methanolic washes tested negative for methadone by EIA and GC-MS.

Under the analytical conditions used in our experiments, there was no interference of methadone with any extracted endogenous material present in nail.

In regard to the limit of detection of our method, concentrations as low as 0.01 ng/mg methadone could be detected by EIA and better than 0.005 ng/mg methadone could be detected by GC-MS if the equivalent of at least 10 mg of nail was used for the extractions (see table 20). This detection limit was determined by decreasing the concentration of the drug spiked in nail hydrolysate equivalent to 10 mg of nail. An extraction recovery was determined for methadone by spiking hydrolysate of drug-free nail clippings with known quantities of the drug, extracting them and then analysing them using the instrumental methods described earlier. The extraction recovery thus calculated was 87.3 % for methadone by RIA and 90.2% by GC-MS (see table 20).

Table 18. Retention times (t_R) and selected ions for methadone and methadone-d_3.

Compound	**t_R (min)**	**Selected Ions***
Methadone	5.12	72.0814
Methadone-d_3	5.11	75.1002

*Accurate masses used in the magnetic sector instrument.

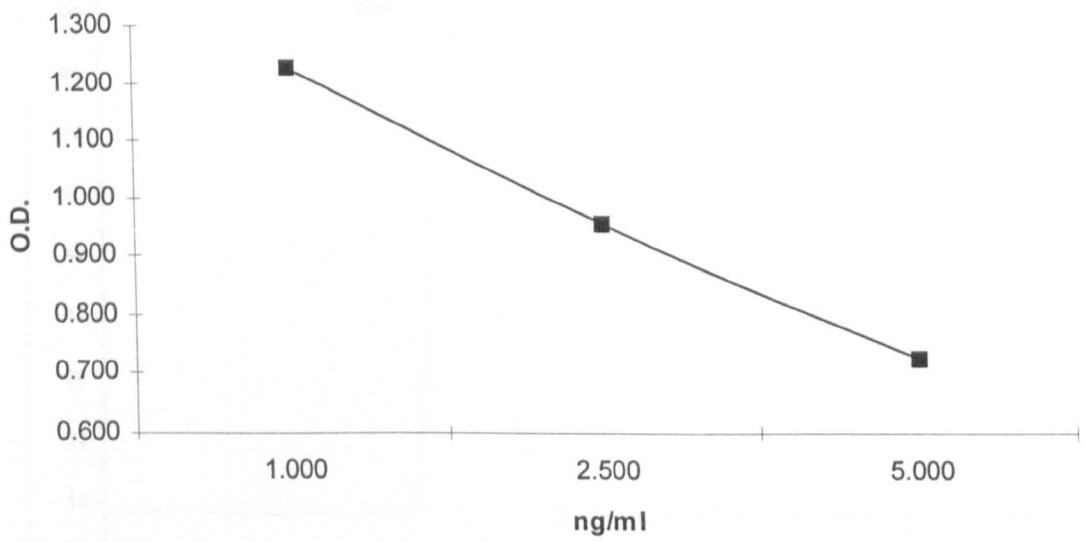


Figure 26. Standard curve used for methadone determination by EIA.

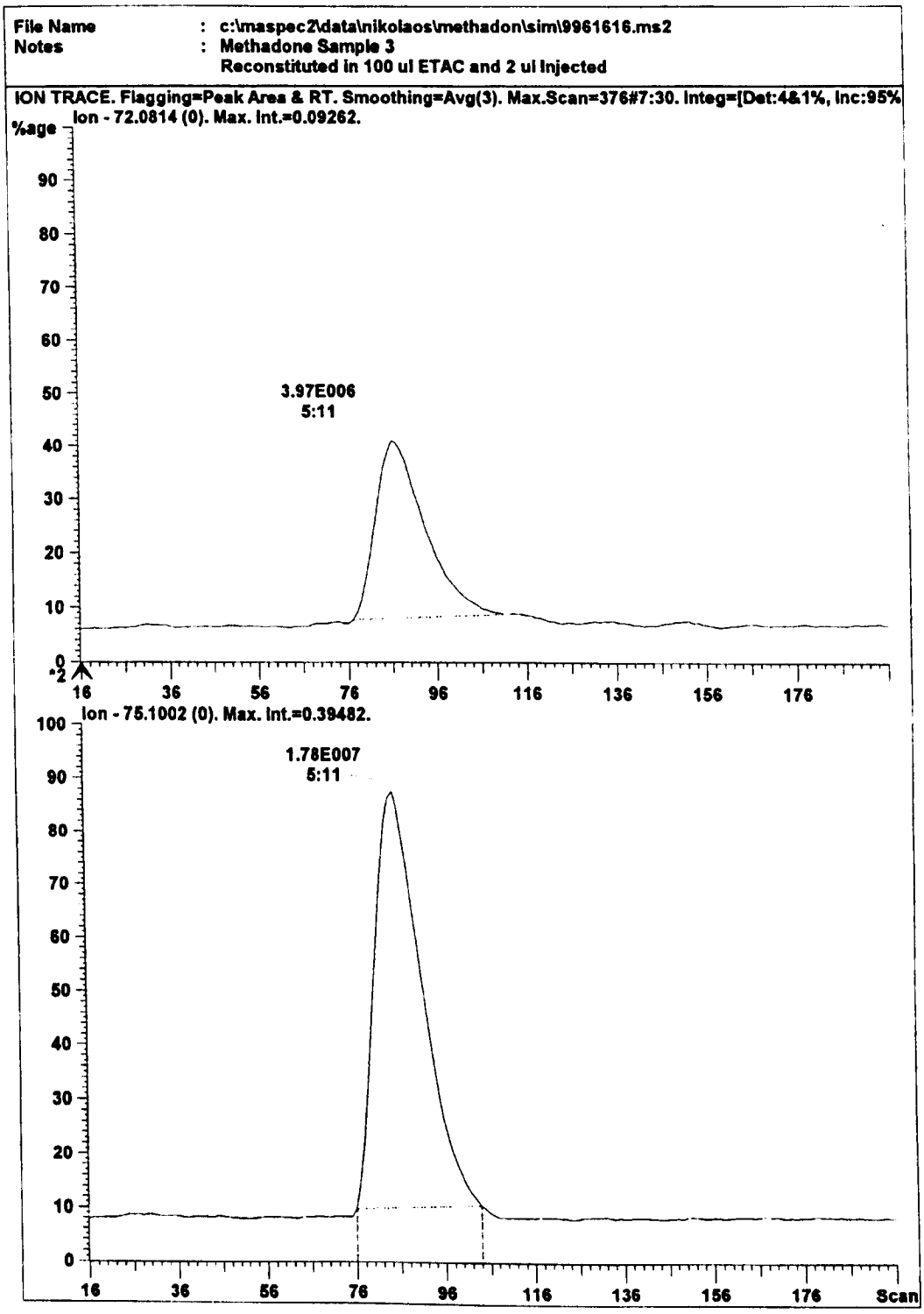


Figure 27. Selected ion chromatograms from a methadone-positive nail sample. The determined concentration was 15.7 ng/mg.

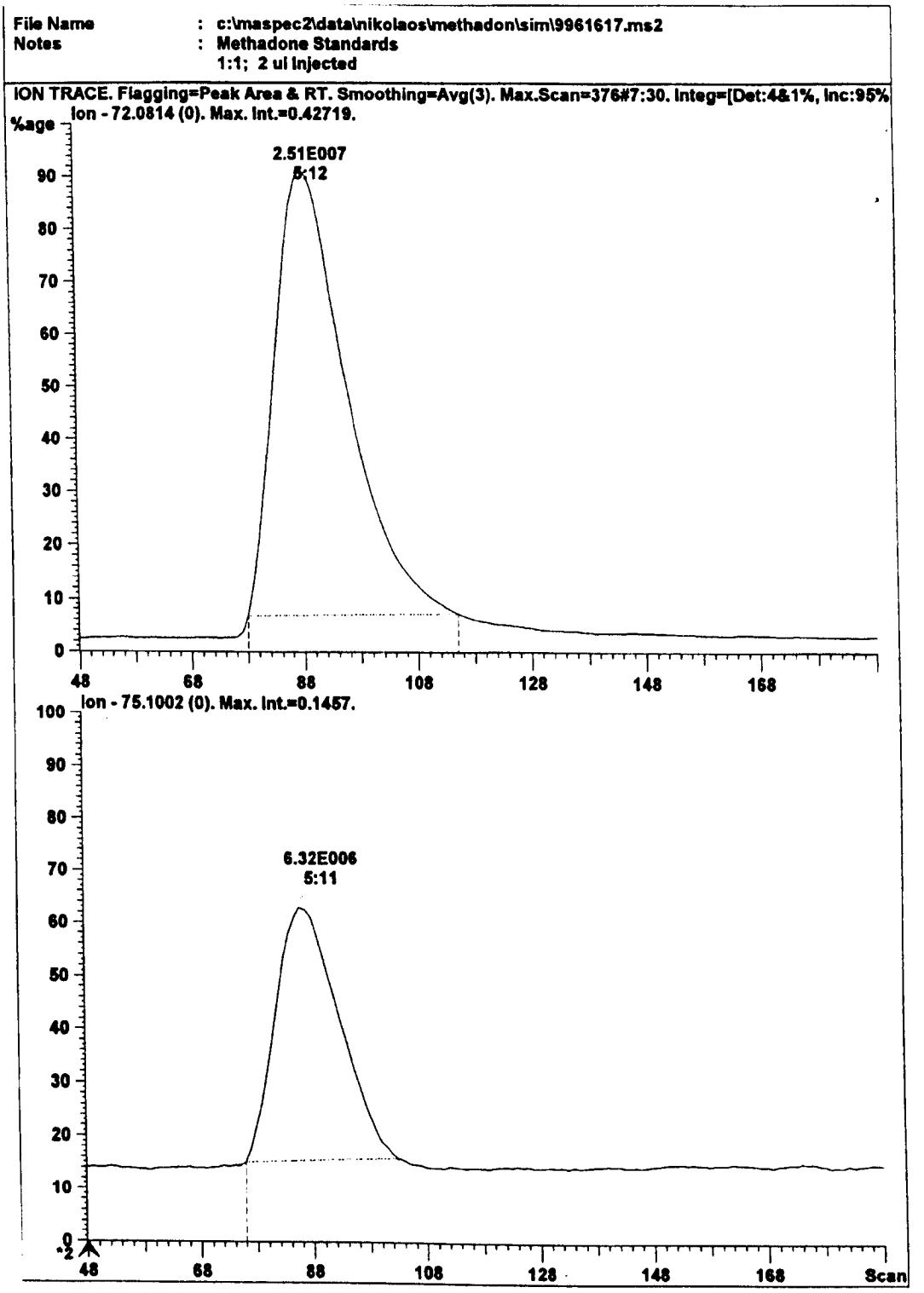


Figure 28. Selected ion chromatograms of the methadone standards used in the determination of the drug in nail by GC-MS.

Table 19. Methadone concentrations in nail by EIA and GC-MS.

Sample	Weight (mg)	EIA Screening (ng/mg)	GC-MS Confirmation (ng/mg)
1	5.58	15.8	25.5
2	2.12	60.4	97.6
3	7.07	39.7	15.7
4	0.79	89.5	92.6
5	15.58	3.0	2.43
6	7.09	8.6	0.55
7	6.28	9.8	13.9
8	0.18	577.8	362.5
9	5.89	14.1	12.9
10	9.54	3.7	1.43
11	10.40	5.8	4.93
13	7.33	10.6	32.2
14	4.27	9.70	7.04
15	9.25	9.06	9.53
16	15.29	4.0	5.76
17	12.17	2.95	1.15
18	11.28	4.44	4.63
19	7.90	1.15	N/D*

Table 19. (cont.)

20	6.43	7.77	7.64
21	3.97	2.19	0.81
22	8.26	5.32	3.08
23	5.38	12.6	15.1
24	16.33	1.59	0.88
25	5.58	5.63	4.53
26	6.47	7.14	1.98
27	8.06	4.56	N/D
28	9.26	2.81	0.51
29	9.01	N/D	11.6
30	6.31	N/D	17.5

*N/D: not detected.

Table 20. Methadone limits of detection and extraction recoveries by EIA and GC-MS obtained with 10mg of drug-free nail spiked with methadone.

Methadone by:	**Limit of Detection (ng/mg)**	**Extraction Recovery (%)**
EIA	< 0.01	87.3
GC-MS	< 0.005	90.2

The relatively small size of the sample population examined in the present study (N=29), although it allows for proper identification and quantification of methadone in the nail, does not allow for the construction of proper dose-response relationships. Furthermore, the study relied on participants consuming the daily dose prescribed to them rather than conducting experiments under controlled dosage parameters. This added at least two potential sources of error. Variations in the methadone consumption patterns of the volunteers and the varying length of methadone use within this group could also be factors of variation. Finally, the mechanism(s) of substance incorporation into the nail matrix are not yet understood and there may be substantial inter-individual variation due to physiological, biochemical and pharmacokinetic factors.

The compliance of methadone users participating in a long-term methadone maintenance program could eventually be determined by analysis in nail. In this study nail clippings were successfully evaluated as analytical specimens. The protocol for decontamination of the nail clippings used in this study consistently produced negative methadone screenings for the final methanol wash. Methadone was determined in hydrolysates of decontaminated nail clippings by EIA (mean: 32.8 ng/mg) and confirmed by GC-MS (mean: 26.9 ng/mg).

As stated earlier, the nail matrix offers several advantages to the forensic toxicologist. Drugs appear to remain trapped in the nail matrix for extensive periods of time thus allowing the determination of exposure for periods from months to years. Furthermore, the collection of nail clippings is a non-invasive procedure as compared to the collection of blood or urine and only a small sample size is required as demonstrated in this study (sample weight range: 0.18 - 16.33 mg). Nails are easily stored in plastic

bags at room temperature, allow for increased stability of drugs and are less likely to suffer any melanin race bias (Garside et al., 1998). Based on the work presented in this chapter, nail appears to have the potential of becoming a powerful tool in the analysis of methadone and the monitoring of patient compliance to their methadone maintenance programs.

6.3. Conclusions

The main achievement of this study was to offer the forensic toxicologist an analytical protocol to determine methadone presence in nail clippings from long term methadone users. This was achieved by initially screening nail clipping hydrolysates by EIA and confirming the presence of methadone by GC-MS. Methadone was determined in hydrolysates of decontaminated nail clippings by EIA (mean: 32.8 ng/mg) and confirmed by GC-MS (mean: 26.9 ng/mg).

The advantages of nail clipping usage as described elsewhere in this dissertation combined with the relative ease with which these samples may be analysed for methadone presence and with the relatively small sample size required, renders nail a useful analytical specimen for the detection of methadone presence and for monitoring patients' compliance to their methadone maintenance programs (see annexe VI).

7. CONCLUSIONS & FURTHER WORK

The work described in this thesis has achieved the goals set at the beginning of the research. Two nonconventional biological samples, bile and nail, were evaluated for various forensic applications.

Firstly, bile, a specimen which has been extensively used in the past for drug analysis, was used in the development of an analytical protocol for dextropropoxyphene, a drug frequently encountered in forensic cases in the United Kingdom and the rest of the world. A relatively easy preparatory procedure included a dilution of the bile in buffer by a 1:50 factor. A simple, rapid and easily automated solid phase extraction scheme was developed which could be employed in a high throughput of cases. The qualitative and quantitative analyses were carried out using a flame ionisation gas chromatograph - an instrument easily found in forensic laboratories around the world. The combination of a solid phase extraction scheme with a gas chromatographic separation proved to be a valuable combination in which endogenous biliary components were found not to interfere with the elution of dextropropoxyphene and norpropoxyphene, which separate well under the conditions used in these experiments.

The experimental results reported herein reinforce the status of bile as a useful forensic specimen especially due to its long-term retention and high levels of drugs observed therein. The main advantage of bile analysis is that it facilitates the detection of drug presence even when blood and/or urine drug levels are very low or negative. Based on the results of this study and taking into account the advantages described above, bile has been suggested as a useful analytical specimen for dextropropoxyphene

determination (see annexe I). However, further work is required in order to develop models for drug partition/distribution in bile that will allow for correct correlations of concentrations to physiological effects.

Regarding the second specimen examined, nail, very little information on its possible use as a forensic specimen existed when experimental work for this thesis began and nails were primarily used for elemental exposure detection and for the detection of drugs which target the nail as their primary locus for their therapeutic action. However, the work presented in this thesis has permitted nail to be examined as a forensic analytical specimen. The nail matrix offers several advantages to the forensic toxicologist. Drugs appear to remain trapped in the nail matrix for extensive periods of time thus allowing the determination of exposure to drugs for periods from months to years. Furthermore, the collection of nail clippings is a non-invasive procedure as compared to the collection of blood or urine and only a small sample size should be required as demonstrated here. Nails are easily stored in plastic bags at room temperature, allow for increased stability of drugs and are less likely to suffer any melanin race bias (Garside et al., 1998). It is because of these important advantages that experiments were carried out to evaluate the usefulness of this biological specimen in forensic and clinical drug analysis.

One of the issues which were addressed early in the nail study was that of the decontamination of the samples prior to analysis. This step in the methodology was significant since nails are prone to contamination from the environment due to their position and involvement in handling. Various decontamination procedures were evaluated but the decontamination protocol developed and eventually adopted included

the sonication of the nail clippings in SDS, water and methanol. The methanolic washes were always screened for analytes and the experiments proceeded only after the final methanolic washes tested negative for the analytes of interest.

After the decontamination protocol was established, various methods for the release of the drugs from the nail matrix were evaluated. The preferred method was alkaline hydrolysis which produced a manageable nail hydrolysate in every case.

Once protocols for the sonic decontamination and alkaline hydrolysis of nail clippings were adopted, studies on individual drugs were performed. Cannabis was the first substance to be examined in nail due to its popularity around the world, especially among young individuals. In chapter 3, protocols for the detection and quantification of cannabis in fingernail clippings of habitual cannabis users were developed and validated. The samples originated from the clinics of the Edinburgh Drug Addiction Study in Edinburgh, Scotland. The methods proposed formed a two-step forensic procedure: an initial screening for cannabinoids by radioimmunoassay and a confirmation of the two major cannabinoids, Δ^9-tetrahydrocannabinol and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid, by gas chromatography - mass spectrometry. Based on the results obtained, nail has been suggested as a potentially useful specimen for the detection of past cannabis use in cases of medico-legal interest (see annexes II and III). The study, however, is pharmacologically incomplete since it failed to establish a dose-response relationship necessary for the extrapolation of the dose taken based on the levels observed in the nail. This may be due to one or more of the reasons discussed in detail in chapter 3. In the same chapter, and upon closer examination of figure 15, an apparent increase in nail cannabinoid levels is observed in proportion to

the monetary value of cannabis declared to have been consumed by the sample population subjects. This trend could be verified and eventually developed into a dose-response relationship if a larger population was studied under controlled conditions in experiments involving accurate and precise quantities of cannabis consumption.

In chapter 4, nail clippings of known users were analysed for diazepam by ECD-GC. The structure of diazepam is such (the chlorine atom is electronegative) that it allows for easy detection by this detector. In this way a rapid and easily automated protocol for the detection of diazepam was developed. The combination of a nail hydrolysis with a gas chromatographic separation equipped with electron capturing detection proved to be valuable and endogenous nail components were found not to interfere with the elution of diazepam. Therefore, this protocol was suggested for the detection of diazepam in forensic cases (see annexe III).

The success of the initial studies involving nail clippings attracted an invitation from the Institute of Forensic Medicine of the University of Verona in Italy which analysed regularly for drugs in hair. A three-week research visit was arranged during which a method for the detection and quantification of morphine in nail clippings of heroin users was developed (chapter 5). The method involved screening for morphine by RIA and confirming the drug's presence by HPLC. Furthermore, a brief experiment involving nail analysis for drugs of abuse by capillary electrophoresis was performed with encouraging results (unpublished). Despite these successful results of the method, the work remains incomplete in that the analysis was limited to morphine and did not cover other opiates such as 6-acetylmorphine. The hydrolytic technique used in sample preparation essentially precludes the detection of this analyte and alternative approaches

need to be investigated. Furthermore, a dose-response relationship was not achieved as figure 23 indicates, and this could be due to one or more reasons discussed in chapter 5. The detection of morphine in nail clippings of known heroin users by RIA and HPLC has prompted the suggestion that nail clippings could eventually be developed to be a useful alternative to hair in analyses of morphine for forensic or clinical purposes (see annexes IV and V).

The final study presented herein evaluated the use of nail for the detection and quantification of methadone in clippings originating from chronic methadone users (chapter 6). Samples were decontaminated and hydrolysed as before and initial screening for methadone was performed by EIA. Confirmation of the methadone presence was achieved by GC-MS. The combination of nail as an analytical specimen with EIA and GC-MS as the analytical techniques proved very valuable as methadone was very easily detected and quantified without any chemical derivatisation. In this way methadone presence could be verified based on nail analysis and compliance of individuals to their methadone maintenance programs could be monitored. Based on this work, the use of nail clippings for the monitoring of methadone levels by past users and compliance by methadone maintenance program participants has been offered to the analytical community (see annexe VI). The methadone experiment conducted comprised a pilot study and there are ways to improve the technique. For example, due to instrumental or other limitations, the determination of methadone metabolites in nail clippings (EDDP, EMDP) was not pursued. Furthermore, a larger population would be required to develop a dose-response relationship. Nonetheless, the primary contribution

of the experimental results of this study remains the identification and quantification of methadone in the nail matrix.

In conclusion, the work described in this thesis, although validated and significant in its own right, allows for further experimentation. The constant need for accurate, reproducible and cost-efficient techniques for the detection of drugs requires the continuous assessment of existing techniques and the search for new alternatives in the field. The specimens examined as part of this dissertation can be subjected to further investigation.

Biliary use in the forensic context, although established for analysis of substances such as opiates, remains unused to its full potential. The dextropropoxyphene analysis presented in chapter 2 is only a small portion of the whole picture and can be extended to examine the differences (if any) that age, gender and billiary obstruction, among other factors, produce in the detection of these analytes in bile.

Similarly, nail analysis for drugs is still in its early stages. Further investigation must take place to correlate the levels observed in this specimen to levels of other biological matrices. Nail should be compared to hair so that the similarities and differences of the two specimens could be investigated. New techniques for the extraction of drugs from the nail matrix could be developed parallel to those offered for hair. These could include better hydrolysis techniques similar to the one offered by Offidani et al. in 1993b or visualisation of the drug binding site by microscopic or other techniques (Kalasinsky et al., 1993; Kalasinsky et al., 1994). Finally, the differences caused by variations in age and gender, among other factors, must also be addressed in

nail use for the detection and quantification of drug in this specimen. Methodologies offering accurate, reproducible and cost-efficient detection and quantification of many analytes at once should also be developed, validated and offered to the analytical community.

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9. APPENDICES

9.1. Appendix I.

FORMULAS FOR CALCULATIONS:

$$\text{Sample Ratio} = \frac{\text{Response of Drug in Sample}}{\text{Response of Internal Standard in Sample}}$$

$$\text{Standard Ratio} = \frac{\text{Response of Drug in Standard}}{\text{Response of Internal Standard in Standard}}$$

$$\text{Drug concentration (mg / l)} = \frac{\text{Sample Ratio}}{\text{Standard Ratio}} \times \frac{\text{Concentration of Drug in Standard}}{\text{Dilution Factor}}$$

$$\text{Drug concentration (ng / mg)} = \frac{\text{Sample Ratio}}{\text{Standard Ratio}} \times \frac{\text{Amount of Drug in Standard (ng)}}{\text{Weight of Nail (mg)}}$$

10. ANNEXES

Presentations & Publications

in support of this Dissertation.

10.1. Annexe I.

Biliary Analysis of Dextropropoxyphene and Norpropoxyphene by Solid Phase Extraction.

by Lemos, N. P. and R. A. Anderson

In

The International Association of Forensic Toxicologists

XXXV Annual Meeting Proceedings

pp. 695-698

Padova, Italy, 1997

Biliary Analysis of Dextropropoxyphene and Norpropoxyphene by Solid Phase Extraction

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Introduction and Aims

The use of bile as an analytical specimen in forensic laboratories is well established but, as yet, little work has been done on the extraction of bile samples using current solid-phase techniques. This study was conducted to develop a gas chromatographic method based on solid-phase extraction (SPE) for the simultaneous analysis of dextropropoxyphene and its major metabolite, norpropoxyphene, in bile. Dextropropoxyphene is a regularly prescribed analgesic drug that is frequently found in cases of medico-legal interest in the UK. Bile samples are diluted with phosphate buffer (pH 6.0) and triphenylamine is added as the internal standard. Bile is then extracted with 200 mg Clean Screen® Extraction Columns. The resulting extract is then analysed by GC-FID using a CHROMPAK CP-SIL 5 CB capillary column. The method is rapid, specific and reproducible and makes dextropropoxyphene analysis in bile readily available as a valuable analytical tool.

Background on Dextropropoxyphene

Dextropropoxyphene is a synthetic oral analgesic that has been available for consumption since the late 1950's. It is usually prescribed for the symptomatic relief of moderate pain (1). This drug has been used in the treatment of heroin addicts (2) and has been described as an abused substance since 1970 (3).

Dextropropoxyphene is principally metabolised to norpropoxyphene via *N*-desmethylation. Pharmacokinetic data indicate that norpropoxyphene has a significantly longer elimination half life (36 hours) than the parent compound (12 hours) (4).

Animal studies show that norpropoxyphene is partially responsible for toxic effects of dextropropoxyphene in humans as cardiotoxicity (4). In the UK, propoxyphene has been implicated in a significant number of drug-related fatalities, often in combination with paracetamol (Distalgesic, Coproxamol). Therefore, it is of particular significance in both clinical and forensic toxicology.

Background on Bile

Bile production is carried out by liver cells (hepatocytes) in minute channels termed

canaliculi. It is in the canaliculi that the primary hepatocyte secretion is modified through the addition or removal of water and electrolytes.
The composition of the final product has been found to be species specific. Nutritional and other balances have also been found to alter the composition of bile.
In general, the largest portion of bile is water (97%). On average, bile acids account for 1.5% of bile. The remaining portion is made up of several other species such as Na^+, K^+, Cl^-, HCO_3^-, proteins, cholesterol and phospholipids (5,6).

Background on Biliary Excretion

Biliary excretion is perhaps the most important contributing source to fecal excretion of xenobiotics and even more important for the excretion of their metabolites.
The liver is in a very advantageous position for removing toxic agents from blood after absorption from the gastrointestinal tract, because blood from the gastrointestinal tract passes through the liver before reaching the general circulation. Thus, liver can extract compounds from blood and thereby prevent their distribution to other parts of the body. Furthermore, the liver is the main locus of biotransformation of toxicants, and the metabolites thus formed may be excreted directly into bile. Xenobiotics and/or their metabolites entering the intestine with bile may be excreted with feces, or when the physicochemical properties are favorable for reabsorption, an enterohepatic recirculation may ensue.

Method of Analysis

Sample Preparation
Dilute bile sample (1 ml) 1:50 using 100 mM phosphate buffer (pH 6.0). Mix by vortex. Sample pH should be 6.0 ± 0.5. Adjust pH accordingly with 100 mM monobasic or dibasic sodium phosphate. Add internal standard (triphenylamine) to 5 ml of diluted bile.

Column Conditioning
SPE Column: 200 mg Clean Screen® Extraction Column

a. 1 x 3 ml methanol
b. 1 x 3 ml deionised water
c. 1 x 1 ml 100 mM phosphate buffer (pH 6.0)

Application of Sample
Apply the bile sample onto the SPE column at a rate of 1-2 ml/min.

SPE Column Washing
a. 1 x 3 ml deionised water

b. 1 x 1 ml 1.0 M acetic acid
c. 1 x 3 ml methanol

SPE Column Drying
Dry the SPE column for 5 minutes under full vacuum

Analyte Elution
Elute analytes with 1 x 3 ml dichloromethane:isopropanol:ammonium hydroxide (78:20:2 v/v/v) at the rate of 1-2 ml/min.

Sample Concentration
Concentrate sample by evaporating to dryness at 60°C. Reconstitute with 100 µl of methanol.

GC Conditions

Gas Chromatograph:	HP 5890 SERIES II
Detector:	FID
Automatic sampler:	HP 7673 INJECTOR
Column:	CHROMPAK CP-SIL 5 CB 30m x 0.25 mm
Carrier gas:	Helium (10 ml/min)
Oven temperature program:	80°C for 2 min, to 215°C at 20°C/min, to 285°C at 5°C/min,and held at 285°C for 2 min.
Injector temperature:	275° C
Detector temperature:	310° C

Quantitate using standard calibration curves.

Results

The retention times for dextropropoxyphene and norpropoxyphene were 9.7 and 17.9 minutes, respectively. The internal standard eluted at 13.1 minutes. The calculated recoveries based on spiked samples for dextropropoxyphene and norpropoxyphene are 90% and 92%, respectively. Calibration curves were linear for each analyte over the concentration range 0-100 mg/litre of bile ($r^2 > 0.99$ in each case).

Conclusions

The combination of a solid phase extraction scheme with a gas chromatographic separation proves to be a valuable combination in which endogenous biliary components were found not to interfere with the elution of dextropropoxyphene and norpropoxyphene, which separate well under the conditions described in this communication.
The solid phase extraction technique for propoxyphene and norpropoxyphene described herein provides a rapid and clean extract that can be used in a high throughput setting.

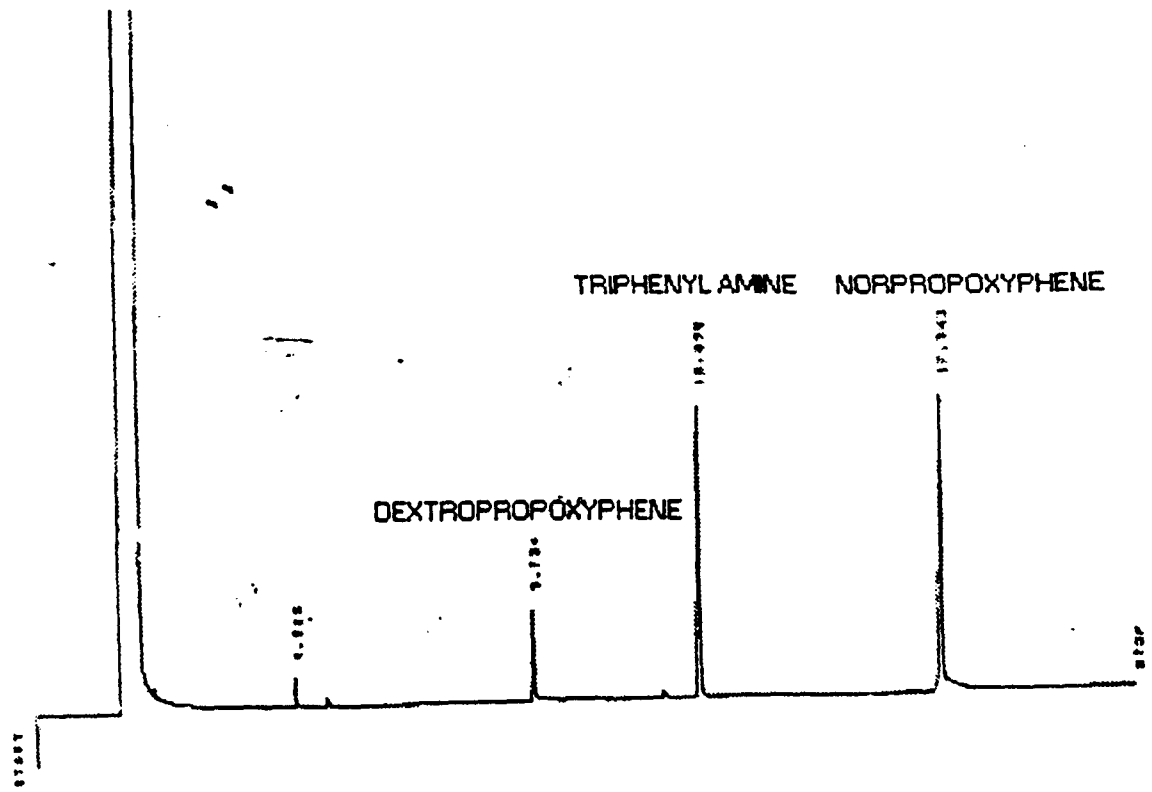

Figure 1: GC-FID Chromatogram of a Bile Extract

It also provides a reliable method that can be used for drugs-of-abuse testing.

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10.2. Annexe II.

Nail Analysis for Drugs of Abuse.

by Lemos, N. P., R. A. Anderson and J. R. Robertson

In

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Nail Analysis for Drugs of Abuse

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Abstract

Fingernail clippings were evaluated as analytical specimens for the detection and quantification of drugs of abuse. Fingernail clippings (2.5 – 25 mg) were obtained from consenting adults attending a drug clinic, together with information concerning the drugs they had used over the previous six months. Methods for the surface decontamination and extraction of the specimens were evaluated. The nail clippings were decontaminated by sonication in 0.1% sodium dodecyl sulfate (SDS) followed by sonication in distilled water and methanol. Whereas the SDS and water washes were discarded, the methanolic washes were analyzed for the presence of analytes. The nail clippings were then hydrolyzed in 1M NaOH and the hydrolysates were extracted with organic solvents prior to instrumental analysis. Extracts were analyzed by RIA, GC-MS or GC equipped with ECD. Positive RIA results were obtained with specimens from 6 known drug users. The average cannabinoid concentration in fingernail clippings determined by RIA was 1.03 ng/mg. Using GC-MS, the mean Δ^9-tetrahydrocannabinol concentration in fingernail clippings from a further 14 known cannabis users was 1.44 ng/mg. Finally, nail clippings from another 6 known drug users were analyzed for diazepam and its mean concentration was found to be 25.71 ng/mg. The limits of detection for the RIA method for cannabinoids and the GC-MS method for Δ^9-tetrahydrocannabinol were determined to be better than 0.1 ng/mg. The limit of detection of the GC-ECD method for diazepam was lower than 0.001ng/ml. The extraction recoveries for our methods were better than 81%. Based on these results, fingernails appear to be potentially useful biological specimens for the detection of past drug use in cases of medico-legal interest.

Introduction

Nails are horn-like plates that cover the dorsal surfaces of the fingers and toes. They consist mainly of a natural fibrous matrix of proteins interconnected via polypeptide chain cross-links. Nails are classified as hard keratin whereas stratum corneum is classified as soft keratin.[1-3] The nails of primates and humans developed with the ability to grasp and manipulate objects.

The use of nail as an analytical specimen is not a recent development in Forensic Toxicology. Pounds et al.[4] measured arsenic using neutron activation in the fingernails of five subjects in an effort to produce an alternative way of measuring the time of exposure to arsenic in cases of poisoning. When the amount of arsenic in fingernail was plotted against the days on which the clippings were taken, two arsenic peaks were observed. It was proposed that one peak (the larger) was the result of arsenic deposition into the nail root from the blood stream while the other, smaller peak was the result of exogenous contamination of the nail, probably from sweat. The sweat contamination was reportedly removed by scraping the underside of the nail.

More recently, amphetamines have been identified in nail clippings of drug abusers.[5] Nail clippings were collected from nine drug abusers, washed and dissolved via alkaline hydrolysis. Amphetamines were then extracted from nails using n-pentane, derivatized and analyzed by gas chromatography - mass spectrometry. The mean fingernail concentrations of methamphetamine and amphetamine were 4.75 ± 2.34 (range 0 - 17.7) and 0.14 ± 0.06 (range 0 - 0.40) ng/mg, respectively. These were found to be similar to the drug concentrations in hair from the same subjects. Average toenail methamphetamine and amphetamine concentrations were always higher than the corresponding fingernail concentrations of the same individuals. The authors offered the slower growth of toenails (1.1 mm/month) as compared to fingernails (3-5 mm/month) as a possible explanation for the consistent difference in drug levels between the two specimens. Cirimele et al.[6] also reported that several amphetamines were present in hair and using a similar extraction scheme as Suzuki et al.[5], they were able to identify amphetamine (AMP), 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphet-amine (MDMA) in one fingernail. The fingernail concentrations determined by gas chromatography - mass spectrometry were 12.0 ng/mg for AMP, 9.7 ng/mg for MDA and 60.2 ng/mg for MDMA. These results appear to be in agreement with the data of Suzuki et al.[7] where drug concentrations were determined by mass fragmentography.

Cocaine has also been reported present in nails. Tiess et al.[8] measured the hair and nail concentrations of cocaine of a man who died of an overdose using enzyme immunoassay and gas chromatography - mass spectrometry. The nail specimens were washed prior to analysis and the wash solutions were analyzed and found positive for cocaine (range 4.1 to 5.8 μg/mg). Cocaine concentrations in the fingernail extracts of this individual were 2.2 to 2.3 μg/mg. The toenails were also found to contain cocaine at concentrations that ranged from 6 to 16 ng/mg. The authors suggested that the deceased had recently handled cocaine because they found high concentrations of cocaine in the fingernail wash solutions.

Several cocaine metabolites were detected in nail clippings by J.D. Ropero-Miller et al.[9] Nails were cut into small pieces and washed with methanol. Methanolic reflux was then used to extract the drugs from the nail matrix and the extracts were analyzed by gas chromatography - mass spectrometry. Cocaine (0.25 to more than 10.0 ng/mg in the fingernail and 0 to more than 10 ng/mg in the toenail) and benzoylecgonine (0 to more than 10 ng/mg in both fingernail and toenail) were the predominant analytes in all cocaine positive nail specimens. Other cocaine by-products (anhydroecgonine methyl ester, ecgonine methyl ester, ecgonine ethyl ester, cocaethylene, norcocaine and norbenzoylecgonine) were also detected. Drug abuse patterns could be established based on these results. The detection of anhydroecgonine methyl ester corroborated the use of crack cocaine while the presence of cocaethylene suggested concomitant use of cocaine and ethanol. Furthermore, the presence of cocaine metabolites in the nail extracts indicated drug ingestion followed by metabolism and subsequent incorporation into the nail matrix.

Despite the frequency of cannabis and benzodiazepine abuse throughout the world, there has been no report to date offering a methodology on the detection of either cannabinoids or benzodiazepines in nail specimens. In this study, the results from our analyses using radio-immunoassay, gas chromatography - mass spectrometry and gas chromatography equipped with electron capturing detection of nail clippings which originated from known cannabis or benzodiazepine users are presented.

Materials and Methods

Reagents

All chemicals and reagents used were of HPLC grade. Δ^9-tetrahydrocannabinol (THC), Δ^9-tetrahydrocannabinol-d_3 (THC-d_3) and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THCCOOH) were obtained from SIGMA (Dorset, UK). 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid-d_3 (THCCOOH-d_3) was obtained from Radian Corporation (Austin, TX, USA). The derivatizing agent N,O,-bis(trimethylsilyl)trifluoroacetimide (BSTFA) catalyzed with 1% trimethylchlorosilane (TMCS) was obtained from Pierce (Luton, UK).

Samples

The nail clippings examined in this paper were obtained with consent from 29 known drug users participating in the Edinburgh Drug Addiction Study (EDAS) in Scotland, UK. At the time of sampling the participants were asked to complete a short questionnaire regarding their drug use habits. Based on the questionnaires, it was determined that cannabinoids and benzodiazepines were the two most frequently abused drug groups by our subjects (Figure I). The nail clippings were collected from the subjects' fingers using commercially available nail clippers (one per participant) and were stored in plastic bags at room temperature.

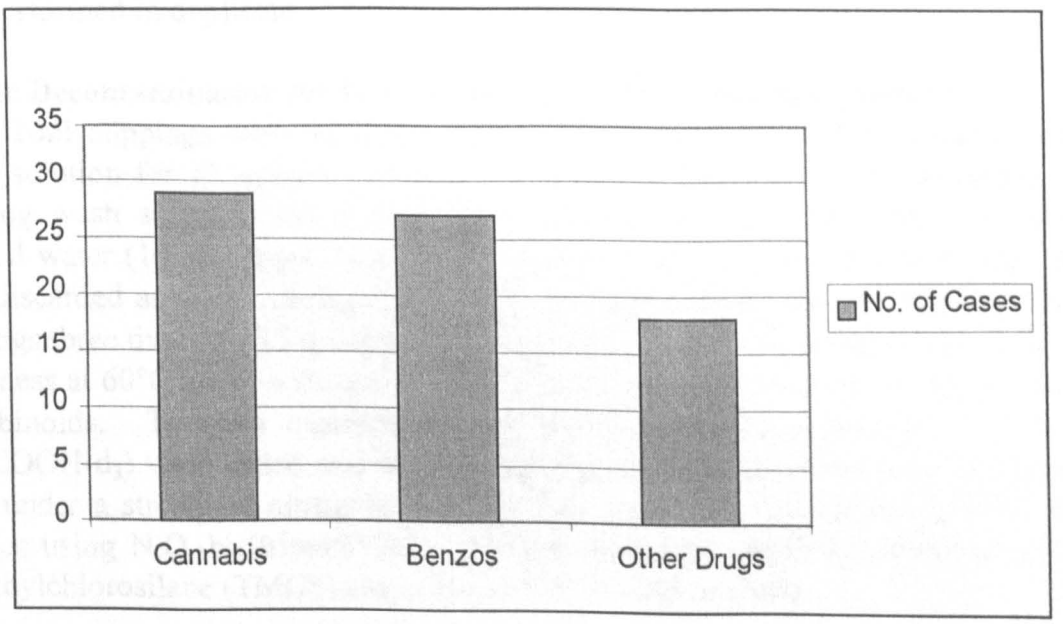

Figure I. Subjects' drug use during the six months preceding sampling based on self-reporting questionnaires.

Sample Decontamination for Radio-Immunoassay

Nail clippings were washed once with 10 ml of 1% sodium dodecyl sulfate (SDS) solution for 15 minutes using a CAMLAB Trans-Sonic T 310 sonicator. The resulting wash solution was discarded. The nail clippings were then sonicated in distilled water (10 ml) three times for 15 minutes each time and the resulting washes were also discarded. Methanol (10 ml) was subsequently used to sonicate the nail clippings three times for 15 minutes each time. The methanolic washes were evaporated to dryness at 60°C under a stream of nitrogen. The residues were then analyzed by

radio-immunoassay using the Cannabinoids Double Antibody procedure by Diagnostic Products Corporation after being reconstituted in phosphate buffer (pH 7.4).

Sample Extraction for Radio-Immunoassay

The washed nail clippings were weighed after being allowed to air dry overnight. Alkaline hydrolysis was used as the extraction method in this study. This involved the incubation of the washed nail clippings in the presence of 1 ml of 1M sodium hydroxide solution at 95°C for 30 minutes. After the resulting hydrolysates had cooled to room temperature, 3 ml of methanol were added. The resulting solutions were vortexed for 5 minutes and centrifuged for 20 minutes at 3000 rpm before being evaporated in their entirety to dryness at 60°C under a stream of nitrogen. The nail hydrolysate residues were then reconstituted in phosphate buffer (pH 7.4) for radio-immunoassay. Calibration curves were prepared in a similar manner.

Radio-Immunoassay

The Cannabinoids Double Antibody procedure by Diagnostic Products Corporation (Gwynedd, UK) was used for the radio-immunoassay analysis. The COBRA II Auto-Gamma counter (Packard, UK) was used for the measurement of the antibody-bound fraction in the pellet obtained after precipitation and centrifugation. Calibrators and controls were analyzed with the samples (blanks, spikes and six standard solutions: 0, 10, 25, 50, 100 and 200 ng/ml, respectively). All measurements were performed in duplicate.

Sample Decontamination for Gas Chromatography - Mass Spectrometry.

Nail clippings were sonicated once with 10 ml of 1% sodium dodecyl sulfate (SDS) solution for 15 minutes using a CAMLAB Trans-Sonic T 310 sonicator. The resulting wash solution was discarded. The nail clippings were then sonicated in distilled water (10 ml) three times for 15 minutes each time and the resulting washes were discarded as well. Methanol (10 ml) was subsequently used to sonicate the nail clippings three times for 15 minutes each time. The methanolic washes were evaporated to dryness at 60°C under a stream of nitrogen and analyzed to check for the presence of cannabinoids. To each methanolic wash residue internal standards (THC-d_3 and THCCOOH-d_3) were added and the mixtures were once again evaporated to dryness at 60°C under a stream of nitrogen. The residues were then derivatized at 95°C for 20 minutes using N,O,-bis(trimethyl-silyl)trifluoroacetimide (BSTFA) catalyzed with 1% trimethylchlorosilane (TMCS) and subjected to GC-MS analysis.

Sample Extraction for Gas Chromatography - Mass Spectrometry

The washed nail clippings were weighed after being allowed to air dry overnight. Alkaline hydrolysis was used as before. This involved the incubation of the washed nail clippings in 1 ml of 1M sodium hydroxide solution at 95°C for 30 minutes in the presence of internal standards (THC-d_3 and THCCOOH-d_3). After the resulting hydrolysates had cooled to room temperature, 5 ml of ethyl acetate were added and the mixtures were agitated for 10 minutes on a mechanical rock-n-roller. The organic layer from each mixture was then transferred into a clean screw-cap vial and evaporated to dryness at 60°C under a stream of nitrogen. The nail extract residues were then derivatized using N,O,-bis(trimethyl-silyl)trifluoroacetimide (BSTFA) catalyzed with 1% trimethylchlorosilane (TMCS) and subjected to GC-MS.

In order to test the effect of pH on the extraction of THC and THCCOOH from nail clippings, a separate study on 3 sets of nail clippings was performed. These 3 nail clipping sets were treated in exactly the same way as above with the exception that, at the time of extraction with ethyl acetate, the pH was acidic due to the addition of 3M hydrochloric acid, HCl. After extraction, the organic layer from these nail clipping hydrolysates was evaporated to dryness at 60°C under a stream of nitrogen as before. These 3 nail extract residues were then derivatized using N,O,-bis(trimethyl-silyl)trifluoroacetimide (BSTFA) catalyzed with 1% trimethylchlorosilane (TMCS) and subjected to GC-MS as before.

Gas Chromatography - Mass Spectrometry

A 1.0 µl portion of each sample was injected through an HP-1 crosslinked dimethyl silicone capillary column (30 m x 0.25 mm; 0.25 µm film thickness) of a FISONS 8000 Series gas chromatograph coupled to a FISONS MD 800 mass spectrometer. The injector was a FISONS AS 800 (injector temperature: 225°C) and splitless injection was employed with a split-valve off-time of 1 minute. Helium was used as the carrier gas.

The initial column temperature was 150°C and was programmed to rise to 300°C at a rate of 10°C/min immediately after injection. The column was subsequently maintained at 300°C for 10 minutes.

The capillary column was inserted directly into the ion source (200°C) via a heated transfer line (250°C). The mass spectrometer was operated in the selected ion recording mode with electron impact ionization at an electron energy of 70 eV and was auto-tuned daily according to the manufacturer's recommendations.

For THC and THCCOOH, qualitative and quantitative analyses were obtained using the selected-ion recording mode by comparison of retention times (t_R) and relative abundance of confirming ions with THC-d_3 and THCCOOH-d_3, respectively. Quantitative results were obtained in the selected-ion recording mode after determination of the response factor of THC and THCCOOH against THC-d_3 and THCCOOH-d_3.

Sample Decontamination for Gas Chromatography - ECD

Nail clippings were washed once with 10 ml of 1% sodium dodecyl sulfate (SDS) solution for 15 minutes using a CAMLAB Trans-Sonic T 310 sonicator. The resulting wash solution was discarded. The nail clippings were then sonicated in distilled water (10 ml) three times for 15 minutes each time and the resulting washes were also discarded. Methanol (10 ml) was subsequently used to sonicate the nail clippings three times for 15 minutes each time. The methanolic washes were evaporated to dryness at 60°C under a stream of nitrogen. The residues were then analyzed by GC-ECD.

Sample Extraction for Gas Chromatography - ECD

The washed nail clippings were weighed after being allowed to air dry overnight. Alkaline hydrolysis was used as the extraction method in this study. This involved the incubation of the washed nail clippings in the presence of 1 ml of 1M sodium hydroxide solution at 95°C for 30 minutes. After the resulting hydrolysates had cooled to room temperature, internal standard (prazepam) and borate buffer (pH 9.3) were added. The resulting solutions were extracted using toluene: heptane: isoamyl

alcohol (76:20:4). The organic layer was then evaporated to dryness at 60°C under a stream of nitrogen. The nail hydrolysate residues were reconstituted in ethyl acetate and analyzed by GC-ECD. Calibration curves were prepared in a similar manner.

Gas Chromatography - ECD

A 2.0 µl portion of each sample was injected through an HP-5 crosslinked 5% PH ME siloxane capillary column (30 m x 0.32 mm; 0.25 µm film thickness) of a Hewlett Packard 5890 SERIES II gas chromatograph equipped with an electron capturing detector (300°C). The injector temperature was 225°C and the purge valve on-time was 15 seconds. Helium was used as the carrier gas.

The initial column temperature was 100°C (1 min) and was programmed to rise to 200°C at a rate of 30°C/min and then to 280°C at 10°C/min. The column was subsequently maintained at 280°C for 15 minutes.

The qualitative and quantitative analyses of benzodiazepines were obtained by comparison of retention times (t_R) and standard curves.

Limits of Detection and Extraction Recoveries

No method currently tests for spiking the intact nail matrix with cannabinoids or benzodiazepines, therefore, the limits of detection and extraction recoveries were determined after dissolution of the nail in sodium hydroxide. Nail hydrolysate in NaOH (equivalent to 10 mg of nail) was spiked with different amounts of cannabinoids or benzodiazepines and then analyzed as usual.

Results and Discussion

Table I shows the ions monitored for THC, THC-d_3, THCCOOH and THCCOOH-d_3 along with their respective retention times (t_R).

Table I. Retention times (t_R) and Selected Ions for THC, THC-d_3, THCCOOH and THCCOOH-d_3.

Compound	t_R *(min)*	*Selected Ions*
THC	9.56	371, **386**
THC-d_3	9.53	389
THCCOOH	13.36	371, **488**
THCCOOH-d_3	13.34	491

Abbreviations: THC, Δ^9-tetrahydrocannabinol; THCCOOH, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid.

The ions **in bold** were used for quantification.

Figure II, III and IV show selected ion recording chromatograms of cannabinoid standards, extracted blank nails and an extracted set of nail clippings, respectively.

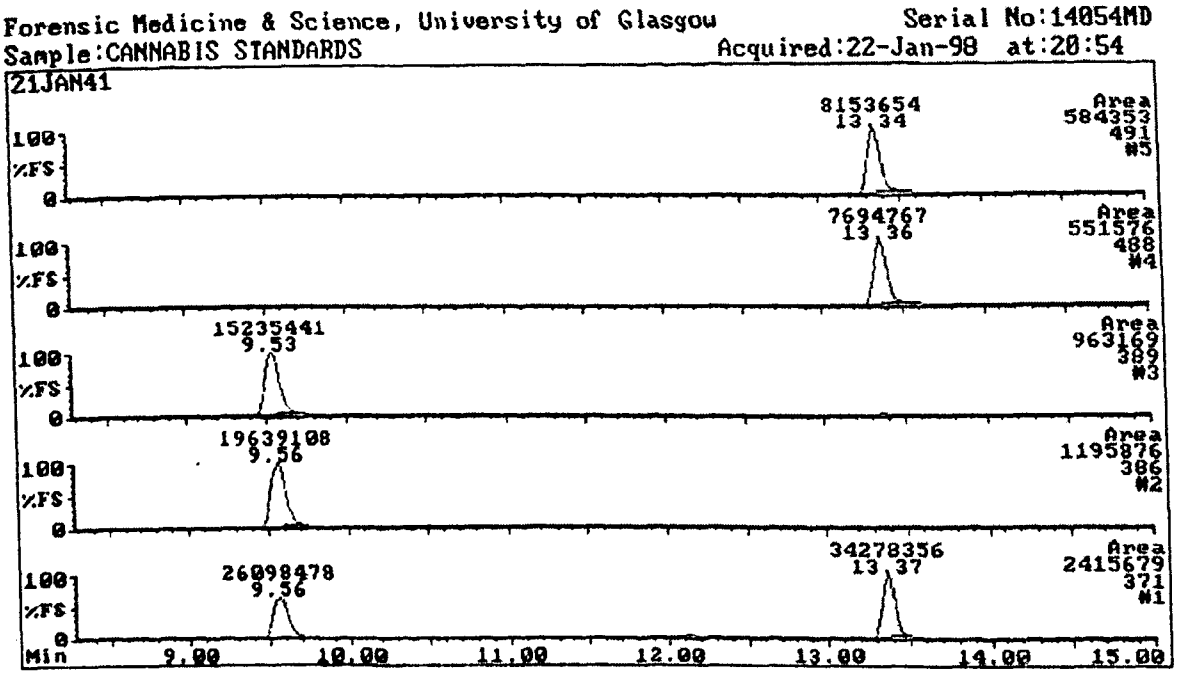

Figure II. Selected ion recording chromatogram of cannabinoid standards.

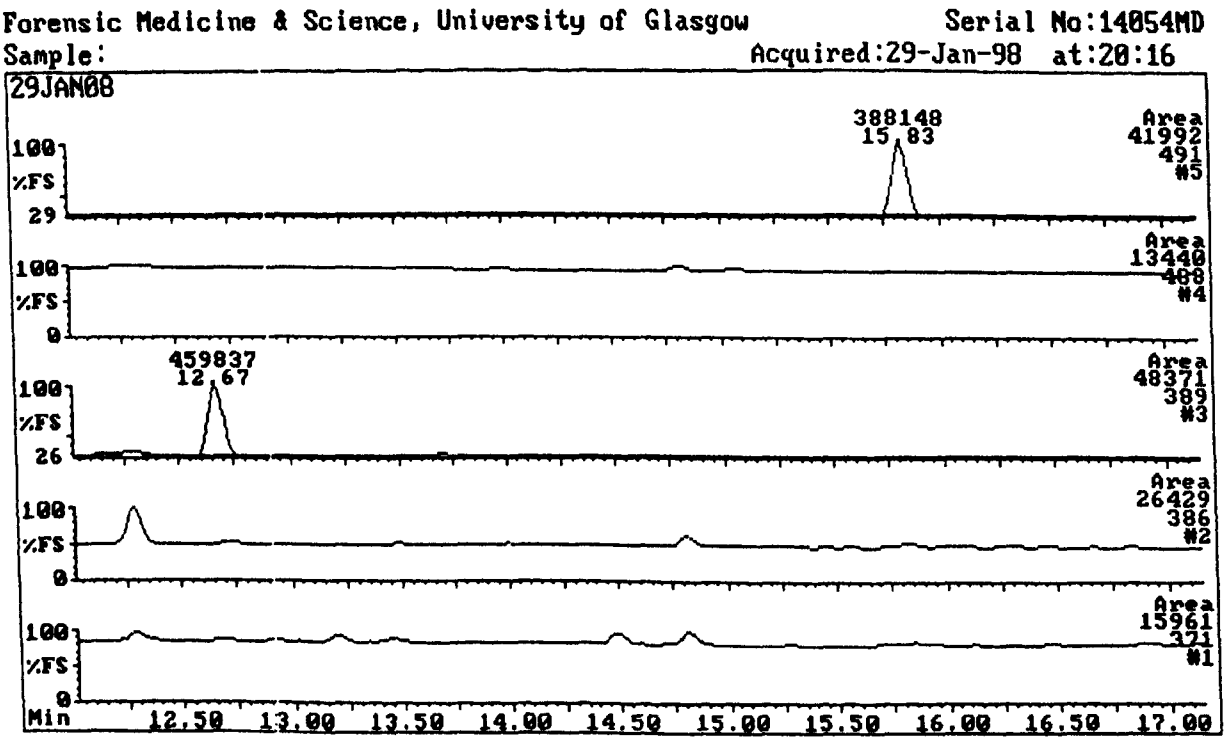

Figure III. Selected ion recording chromatogram of a blank nail extract.

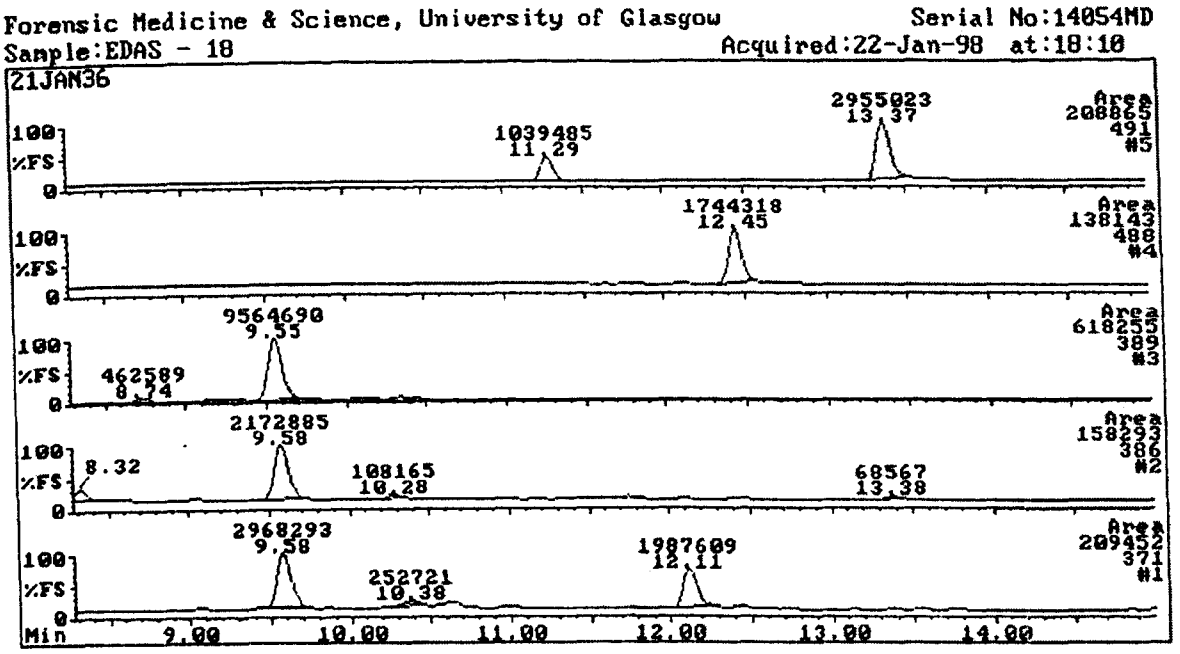

Figure IV. Selected ion recording chromatogram of an extracted set on nail clippings.

Figure V and VI show chromatograms of benzodiazepine standards and an extracted set of nail clippings, respectively.

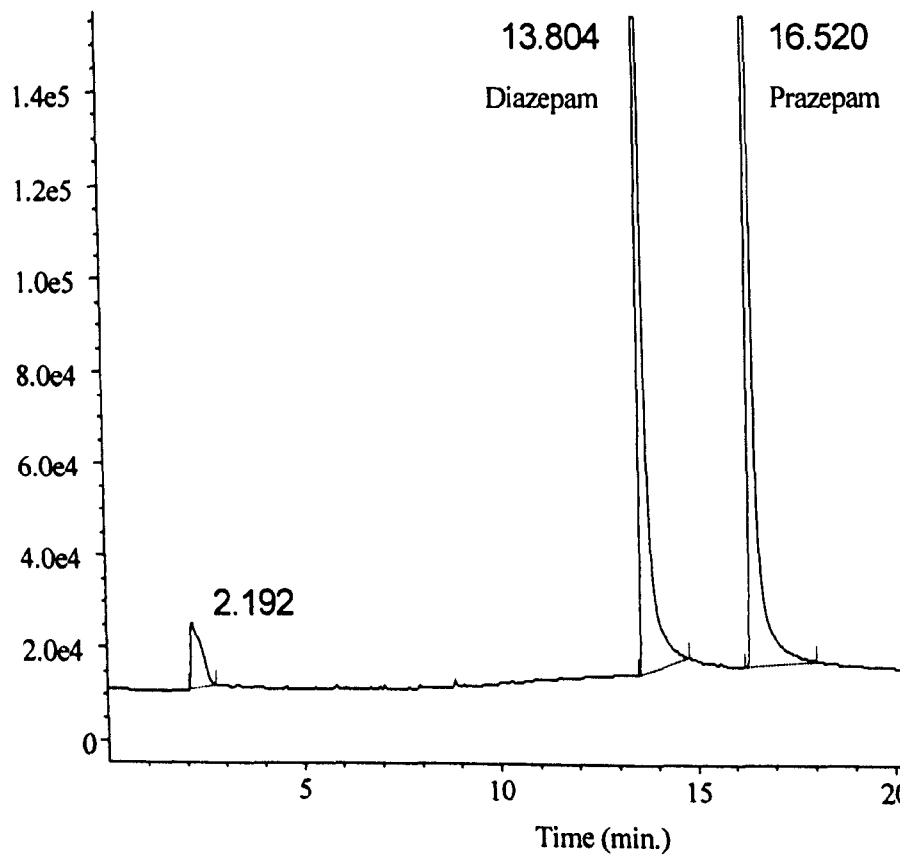

Figure V. GC-ECD chromatogram of benzodiazepine standards.

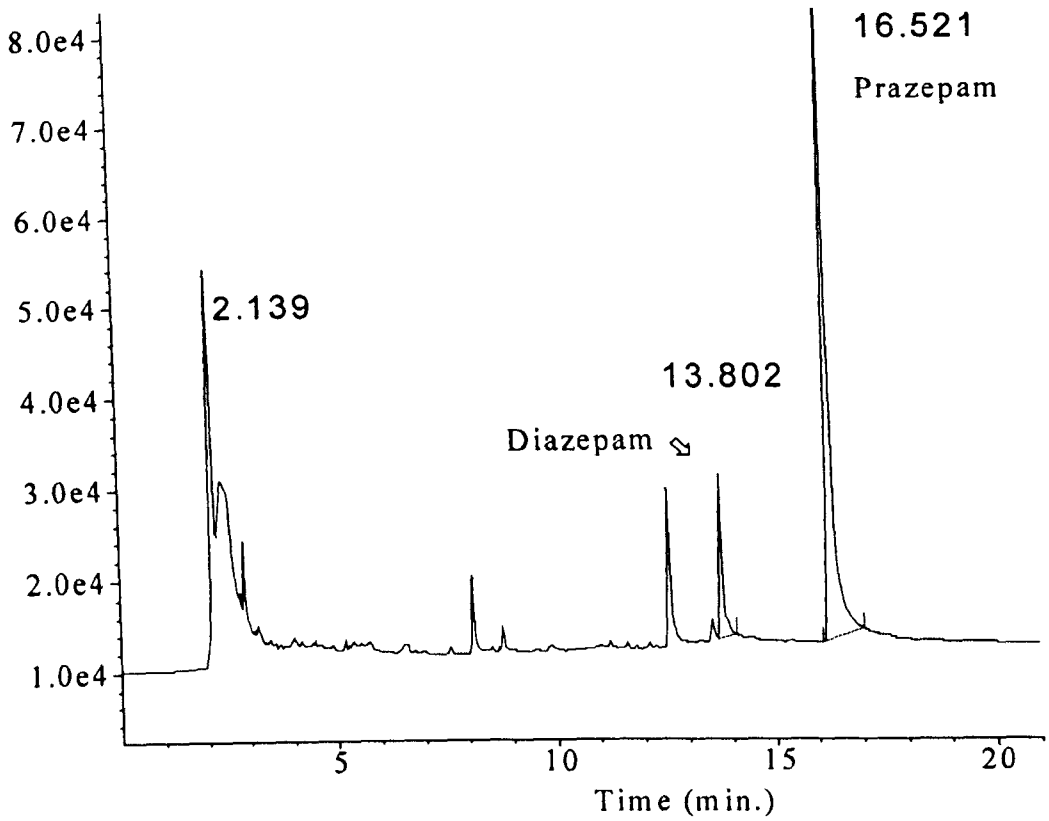

Figure VI. GC-ECD chromatogram of an extracted set of nail clippings.

Under the analytical conditions, there was no interference caused by any extracted endogenous material present in nails.
Cannabinoids were found to be present in all six cases which were analyzed for cannabinoids by radio-immunoassay (Table II). The cannabinoid concentration determined ranged from 0.23 to 2.80 ng/mg with an average cannabinoid concentration of 1.03 ng/mg.

Table II. Cannabinoids in nail clippings determined by RIA.

Case Number	*Weight of Nails (mg)*	*Cannabinoids (ng/mg)*
2	6.46	0.23
3	8.07	2.80
4	10.40	0.92
5	10.58	1.14
7	18.35	0.66
8	21.51	0.45

Using GC-MS, THC was detected in 11 out of the 14 nail clipping hydrolysates that were extracted at basic pH (Table III). THCCOOH was not detected in any of these nail clipping hydrolysates. The concentration of THC ranged from 0.13 ng/mg to 6.97 ng/mg with an average THC concentration of 1.44 ng/mg. This wide range of THC concentrations may be attributed to the non-homogeneity of the street cannabis available

in Scotland, the different cannabis consumption patterns of our volunteers as well as to the different length of cannabis abuse within our group of individuals.

Table III. THC in nail clippings extracted at basic pH and determined by GC-MS.

Case Number	*Weight of Nails (mg)*	*THC (ng/mg)*
9	3.51	0.53
11	4.25	6.97
12	12.25	0.57
13	6.17	N/D
14	2.79	0.13
15	4.77	N/D
16	6.18	0.14
17	6.67	0.45
18	5.18	3.39
19	6.96	1.04
21	2.68	0.65
22	4.39	N/D
23	2.53	0.32
24	7.11	1.61

Abbreviations: THC, Δ^9-tetrahydrocannabinol; N/D, Not Detected

The major metabolite of Δ^9-tetrahydrocannabinol (THC), 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THCCOOH), was not detected in those nail hydrolysates extracted under basic pH. However, it was detected in 2 out of the 3 fingernail clipping hydrolysates extracted under acidic pH. The concentrations of THCCOOH determined ranged from 9.82 to 29.67 ng/mg with an average concentration of 19.75 ng/mg. The parent drug, Δ^9-tetrahydrocannabinol (THC), was not detected in the three nail hydrolysates extracted under acidic pH. The GC-MS results for this group of samples are presented in Table IV.

Table VI. THCCOOH in nail clippings extracted at acidic pH and determined by GC-MS.

Case Number	*Weight of Nails (mg)*	*THCCOOH (ng/mg)*
109	5.81	9.82
112	9.08	29.67
114	9.21	N/D

Abbreviations: THCCOOH, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid; N/D, Not Detected

Using GC-ECD, diazepam was detected in all 6 sets of nail clippings analyzed. The concentration of diazepam ranged from 4.37 ng/mg to 87.78 ng/mg with an average concentration of 25.71 ng/mg. The GC-ECD results for this group of samples are presented in Table V.

Table V. Diazepam in nail clippings determined by GC-ECD.

Case Number	*Weight of Nails (mg)*	*Diazepam (ng/mg)*
101	3.64	87.78
107	4.68	13.32
111	11.45	4.37
113	8.25	9.31
115	9.17	30.11
123	7.04	9.38

Limits of detection and extraction recoveries are presented in Table VI. By our methods, concentrations of 0.1 ng/mg for both THC and THCCOOH could be detected if a minimum of 10 mg of nail was used for extraction. These detection limits were determined by decreasing the concentrations of the drugs spiked in a 10 mg nail sample. In the case of diazepam, concentrations of 0.001 ng/ml were detected by GC-ECD. Extraction recoveries were determined for THC, THCCOOH and diazepam by spiking drug-free nail clipping hydrolysates with known quantities of the two compounds, subjecting them to our extraction method and then analyzing for them using the instrumental methods described earlier. The extraction recoveries thus calculated were greater than 81% for the three compounds.

Table VI. Limits of detection and extraction recoveries of THC, THCCOOH and diazepam obtained with 10 mg of drug-free nail spiked with THC and THCCOOH or diazepam.

Compound	*Limit of Detection*	*Extraction Recovery (%)*
THC	< 0.1 ng/mg	89.4
THCCOOH	<0.1 ng/mg	81.1
Diazepam	<0.001 ng/ml	87.3

Abbreviations: THC, Δ^9-tetrahydrocannabinol; THCCOOH, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid

The new analytical procedure presented in this article has shown that the sonic wash protocol used always produced a negative cannabinoid result for the third and final methanolic wash. In 20 cases, both the second and third methanolic washes were negative for cannabinoids, while in 14 cases all three methanolic washes were negative for cannabinoids. In this way, superficial contamination was removed before the nail extracts were analyzed for drugs by RIA, GC-MS or GC-ECD.

Using RIA, cannabinoids were detected in fingernail clippings that had been stored for six months after collection. When GC-MS was used, Δ^9-tetrahydrocannabinol (THC) and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THCCOOH) were detected nine months after the samples were collected. In the case of diazepam, positive results were obtained by GC-ECD after nine months of storage at room temperature.

The growth of fingernails is known to be 3 to 5 mm per month.[4, 9-13] Considering that drugs are deposited into the root of the nail via the blood stream and progress

towards the nail tip as the nail grows, the administration of drugs to an individual could be dated back at least 3 to 5 months for fingernails. However, the exact time of drug consumption is likely to be difficult to establish at this early stage of nail analysis for drugs of abuse due to the influence on nail growth of other factors such as sex, age, race and season.[4, 11]

Conclusions

This study examined the analysis of human nails for the presence of Δ^9-tetrahydrocannabinol (THC), 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THCCOOH) and benzodiazepines. This was successfully performed by RIA, GC-MS and CG-ECD.

Nail clippings are potentially more easily collected than blood or urine and are especially useful due to their long-term retention of drugs. The main advantage of nail analysis is its ability to detect long-term drug abuse for time periods from months to years. Moreover, the sample size required to detect drug exposure by nail analysis is very small and can be as low as 2.5 - 5 mg. Nail samples are collected by non-invasive procedures, are easy to store, allow for increased drug stability and are less likely to suffer from any race bias.

Based on the results of this study and taking into account these advantages, nails are a potentially useful alternative to hair for the detection of past cannabis and benzodiazepine use in cases of medico-legal interest.

Acknowledgments

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10.3. Annexe III.

Nail Analysis for Drugs of Abuse: Extraction and Determination of Cannabis in Fingernails by RIA and GC-MS.

by Lemos, N. P., R. A. Anderson and J. R. Robertson

In

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Nail Analysis for Drugs of Abuse: Extraction and Determination of Cannabis in Fingernails by RIA and GC–MS

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Abstract

Fingernail clippings were evaluated as analytical specimens for the detection and quantitation of cannabinoids. Specimens were obtained from consenting adults attending a drug clinic, along with information concerning the drugs which they had used over the previous six months. Methods for the surface decontamination and extraction of the specimens were evaluated. Detergent, water, and methanol washes followed by alkaline hydrolysis and liquid–liquid extraction were selected for use in the study. Extracts were analyzed by radioimmunoassay (RIA) and gas chromatography–mass spectrometry (GC–MS) to detect and quantitate cannabinoids present in fingernail clippings. Positive RIA results were obtained from specimens from six known cannabis users. The mean cannabinoid concentration in fingernail clippings determined by RIA was 1.03 ng/mg. Using GC–MS, the mean Δ^9-tetrahydrocannabinol concentration in fingernail clippings from a further 14 known cannabis users was 1.44 ng/mg. Using GC–MS, the average 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid concentration in fingernail clippings from three known cannabis users extracted in acidic pH was 19.85 ng/mg. Based on these results, fingernails are potentially useful biological specimens for the detection of past cannabis use in cases of medicolegal interest.

Introduction

Nails, hair, and stratum corneum originate from a common type of skin cell. However, they all exhibit significant differences in their fully mature stages. Nails are hornlike plates that cover the dorsal surfaces of the fingers and toes, are derived from modified epidermal cells, and consist primarily of a natural fibrous matrix of proteins interconnected via polypeptide chain cross-links. Nails are classified as hard keratin, whereas stratum corneum is classified as soft keratin (1–3). Among all animals, primates are the only ones that possess nails as protective layers on their fingers and toes (4). The nail has evolved phylogenetically with the development of manual dexterity. From the claws of lower mammals, birds, reptiles, and other phyla, both hoof and nail have evolved divergently in higher species (5). The hooves of bovines and other quadrupeds are suited to the lifestyles of these animals, which graze and run. The nails of primates and humans developed with the ability to grasp and manipulate objects.

Nails have been used in substance analysis for several years. Pounds et al. (6) measured arsenic using neutron activation in the fingernails of five subjects who had been given therapeutic doses of arsenious oxide. This was done in an effort to produce an alternative way of measuring the time of exposure to arsenic in cases of poisoning. Fingernail clippings were taken prior to the start of the experiment and thereafter at intervals over a period of 180 days. When the amount of arsenic in fingernail was plotted against the days on which the clippings were taken, two arsenic peaks were observed. The authors suggested that one peak (the larger) was the result of arsenic deposition into the nail root from the blood stream and the other, smaller peak was the result of exogenous contamination of the nail, probably from sweat. Scraping the underside of the nail reportedly removed the sweat contamination of the nail.

Drugs and other deleterious substances remain in nails for long periods of time, thereby providing information about an individual's history of exposure to these substances. Amphetamines have been identified in nail clippings of drug abusers (7). Nail clippings were collected from nine drug abusers and then washed and dissolved via alkaline hydrolysis. Amphetamines were then extracted from nails using *n*-pentane, derivatized, and analyzed by gas chromatography–mass spectrometry (GC–MS). The mean fingernail concentrations of methamphetamine and amphetamine were 4.75 ± 2.34 (range 0–17.7) and 0.14 ± 0.06 (range 0–0.40) ng/mg, respectively. These were found to be similar to the drug concentrations in hair samples from the same subjects. Average toenail methamphetamine and amphetamine concentrations were always higher than the corresponding fingernail concentrations of the same individuals. The authors offered the slower growth of toenails (1.1 mm/month) as compared with fingernails (3–5 mm/month) as a possible explanation for the consistent difference in drug levels between the two specimens. Cirimele et al. (8) also reported that several

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amphetamines were present in hair, and using an extraction scheme similar to Suzuki et al. (7), they were able to identify amphetamine (AMP), 3,4-methylenedioxyamphetamine (MDA), and 3,4-methylenedioxymethamphetamine (MDMA) in one fingernail. The fingernail concentrations determined by GC–MS were 12.0 ng/mg for AMP, 9.7 ng/mg for MDA, and 60.2 ng/mg for MDMA. These results appear to be in agreement with the data of Suzuki et al. (9), where drug concentrations were determined by mass fragmentography.

Cocaine has also been reported present in nails. Using enzyme immunoassay and GC–MS, Tiess et al. (10) measured the hair and nail concentrations of cocaine in a man who died of an overdose. The nail specimens were washed before analysis, and the wash solutions were analyzed and found positive for cocaine (range 4.1–5.8 µg/mg). Cocaine concentrations in the fingernail extracts of this individual were 2.2–2.3 µg/mg. The toenails were also found to contain cocaine at concentrations which ranged from 6 to 16 ng/mg. The authors suggested that the deceased had recently handled cocaine because they found high concentrations of cocaine in the fingernail wash solutions. However, the efficiency of their washing protocol is questionable because substantially higher than expected cocaine levels were also found in the nail extracts.

Ropero-Miller et al. (11) detected several cocaine metabolites in nail clippings. Nails were cut into small pieces and washed with methanol. Methanolic reflux was then used to extract the drugs from the nail matrix, and the extracts were analyzed by GC–MS. Cocaine (0.25 to more than 10.0 ng/mg in the fingernail and 0 to more than 10 ng/mg in the toenail) and benzoylecgonine (0 to more than 10 ng/mg in both fingernail and toenail) were the predominant analytes in all cocaine-positive nail specimens. Other cocaine by-products (anhydroecgonine methyl ester, ecgonine methyl ester, ecgonine ethyl ester, cocaethylene, norcocaine, and norbenzoylecgonine) were also detected. Drug abuse patterns could be established based on these results. The detection of anhydroecgonine methyl ester corroborated the use of crack cocaine, and the presence of cocaethylene suggested concomitant use of cocaine and ethanol. Furthermore, the presence of cocaine metabolites in the nail extracts indicated drug ingestion followed by metabolism and subsequent incorporation into the nail matrix.

Despite the frequency of cannabis abuse throughout the world, especially by younger people, there has been no report to date offering a methodology on the detection of cannabis in nail specimens. In this study, the results from our analyses using radioimmunoassay (RIA) and GC–MS of nail clippings which originated from known cannabis users are presented.

Materials and Methods

Reagents

All chemicals and reagents used were of reagent or high-performance liquid chromatography grade. Δ^9-Tetrahydrocannabinol (THC), Δ^9-tetrahydrocannabinol-d_3 (THC-d_3), and 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THCOOH) were obtained from Sigma (Dorset, U.K.). 11-nor-Δ^9-Tetrahydrocannabinol-9-carboxylic acid-d_3 (THCOOH-d_3) was obtained from Radian Corp. (Austin, TX). The derivatizing agent *N,O-bis*(trimethylsilyl)trifluoroacetimide (BSTFA) catalyzed with 1% trimethylchlorosilane (TMCS) was obtained from Pierce (Luton, U.K.).

Samples

The nail clippings examined in this paper were obtained with consent from 23 cannabis users participating in the Edinburgh Drug Addiction Study (EDAS) in Scotland. At the time of sampling the participants were asked to provide answers to a short questionnaire regarding their use of cannabis and other drugs. This information is listed in Table I except when answers were not provided in which case the information is marked "N/A" (not answered). The nail clippings were collected from the subjects' fingers and stored in plastic bags at room temperature.

Sample decontamination for RIA

Nail clippings were washed once with 10 mL of 0.1% sodium dodecyl sulfate (SDS) solution for 15 min using a CAMLAB Trans-Sonic T 310 sonicator. The resulting wash solution was discarded. The nail clippings were then sonicated in distilled water (10 mL) three times for 15 min each time, and the resulting washes were also discarded. Methanol (10 mL) was subsequently used to sonicate the nail clippings three times for 15 min each time. The methanolic washes were evaporated to dryness at 60°C under a stream of nitrogen. The residues were then analyzed by RIA using the Cannabinoids Double Antibody procedure by Diagnostic Products Corp. (Gwynedd, U.K.) after being reconstituted in phosphate buffer (pH 7.4).

Sample extraction for RIA

The washed nail clippings were weighed after being allowed to air dry overnight. In order to evaluate different extraction methods, washed test nail clippings of known weights were ground in liquid nitrogen using a clean mortar and pestle with unsatisfactory results because an average of 20% of the sample was lost in the process. Alkaline hydrolysis was used as the extraction method in this study. This involved the incubation of the washed nail clippings in the presence of 1 mL of 1M sodium hydroxide solution at 95°C for 30 min. After the resulting hydrolysates cooled to room temperature, 3 mL of methanol was added. This methanol addition was carried out so that our experimental protocol would follow our laboratory's standard operating procedure for the detection of cannabinoids in blood by RIA as closely as possible. Calibration curves were prepared in a similar manner. The resulting solutions were vortex mixed for 5 min and centrifuged for 20 min at 3000 rpm before being evaporated in their entirety to dryness at 60°C under a stream of nitrogen. The nail hydrolysate residues were then reconstituted in phosphate buffer (pH 7.4) for RIA.

RIA

The Cannabinoids Double Antibody procedure by Diagnostic Products Corp. was used for the RIA analysis. The COBRA II Auto-Gamma counter (Packard, U.K.) was used for the measurement of the antibody-bound fraction in the pellet obtained after precipitation and centrifugation. A blank solution, a

Table I. Cannabis and Other Drug Usage by EDAS Subjects as Reported on the Self-Reporting Questionnaires at the Time of Sampling

Sample	Weekly cannabis consumption: Ounces (oz.)	Weekly cannabis consumption: British pounds (£)	Onset of current rate of consumption	Other drugs taken and length of time taken: Drug (time)
EDAS-02	1/2	45	years	methadone dihydrocodeine diazepam antiviral drugs
EDAS-03	N/A*	N/A	N/A	methadone diazepam
EDAS-04	N/A	N/A	N/A	diazepam temazepam inhaled steroids
EDAS-05	N/A	N/A	N/A	diazepam temazepam
EDAS-07	N/A	N/A	N/A	methadone diazepam
EDAS-08	1/8	7.50	years	methadone diazepam temazepam
EDAS-09	3/4	45	years	dihydrocodeine (years) temazepam (years)
EDAS-11	1/4	30	years	dihydrocodeine (years) temazepam (years) diazepam (years) AZT (years)
EDAS-12	1/4	20	several years	dihydrocodeine (2 years) diazepam (2 years)
EDAS-13	1/4	25	years	dihydrocodeine diazepam
EDAS-14	1/2	45	years	none
EDAS-15	1/4	25	several years	dihydrocodeine methadone temazepam
EDAS-16	1/4	30	several years	dihydrocodeine temazepam
EDAS-17	1	100	several years	methadone (years) diazepam (years) temazepam (years)
EDAS-18	1	90	2 years	methadone (2 years) diazepam (2 years)
EDAS-19	1	100	years	dihydrocodeine (years) diazepam (years)
EDAS-21	N/A	N/A	N/A	N/A
EDAS-22	N/A	N/A	N/A	N/A
EDAS-23	N/A	N/A	N/A	N/A
EDAS-24	N/A	N/A	N/A	N/A
EDAS-109	N/A	N/A	N/A	methadone diazepam
EDAS-112	N/A	N/A	N/A	diazepam dihydrocodeine
EDAS-114	N/A	N/A	N/A	methadone diazepam

* N/A, not answered.

spiked solution, and six standard solutions (0, 10, 25, 50, 100, and 200 ng/mL, respectively) were analyzed with the samples. Figure 1 a typical standard curve that was constructed using the six standard solutions (measured in duplicate).

Sample decontamination for GC–MS

Nail clippings were sonicated once with 10 mL of 0.1% sodium dodecyl sulfate (SDS) solution for 15 min using a CAMLAB Trans-Sonic T 310 sonicator. The resulting wash solution was discarded. The nail clippings were then sonicated in distilled water (10 mL) three times for 15 min each time, and the resulting washes were discarded as well. Methanol (10 mL) was subsequently used to sonicate the nail clippings three times for 15 min each time. The methanolic washes were evaporated to dryness at 60°C under a stream of nitrogen and analyzed for the presence of cannabinoids. Internal standards (THC-d_3 and THCOOH-d_3) were added to each methanolic wash residue, and the mixtures were once again evaporated to dryness at 60°C under a stream of nitrogen. The residues were then derivatized at 95°C for 20 min using BSTFA catalyzed with 1% TMCS and subjected to GC–MS analysis.

Sample extraction for GC–MS

The washed nail clippings were weighed after being allowed to air dry overnight. Having rejected grinding of the nail clippings in liquid nitrogen using a mortar and pestle as a sample preparation method, alkaline hydrolysis was used as before. This involved the incubation of the washed nail clippings in 1 mL of 1M sodium hydroxide solution at 95°C for 30 min in the presence of internal standards (THC-d_3 and THCOOH-d_3). After the resulting hydrolysates had cooled to room temperature, 5 mL of ethyl acetate were added and the mixtures were agitated for 10 min on a mechanical rock-n-roller. The organic layer from each mixture was then transferred into a clean screw-cap vial and evaporated to dryness at 60°C under a stream of nitrogen. The nail extract residues were then derivatized using BSTFA catalyzed with 1% TMCS and subjected to GC–MS.

In order to test the effect of pH on the extraction of THC and THCOOH from nail clippings, a separate study was performed on three sets of nail clippings. These three nail clipping sets were treated in exactly the same way as described here with the excep-

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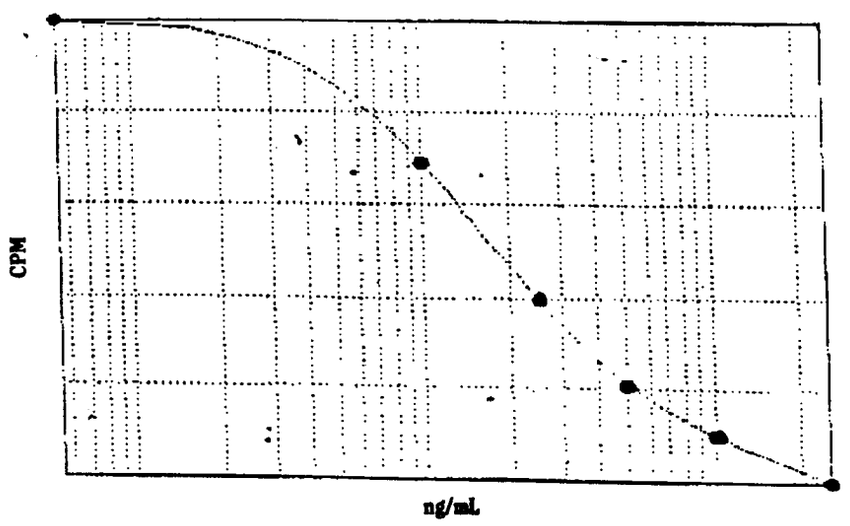
Table II. Retention times (t_R) and Selected Ions for THC, THC-d_3, THCOOH, and THCOOH-d_3

Compound	t_R (min)	Selected ions†
THC	9.56	371, **386**
THC-d_3	9.53	389
THCOOH	13.36	371, **488**
THCOOH-d_3 *	13.34	491

* Abbreviations: THC, Δ^9-tetrahydrocannabinol; THCOOH, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid.
† The ions in bold were used for quantitation.



STD #	CPM	Defined dose	%B/P	Calc. dose	% Diff
1	Det Lim	0.54712		0.53814	-1.64
2	23466	10.00000		10.00000	0.00
3	15809	25.00000		24.99966	0.00
4	11011	50.00000		49.99969	0.00
5	8151	100.00000		99.99917	0.00
6	5443	250.00000		250.00000	0.00

Figure 1. Standard curve for cannabinoid determination by RIA.

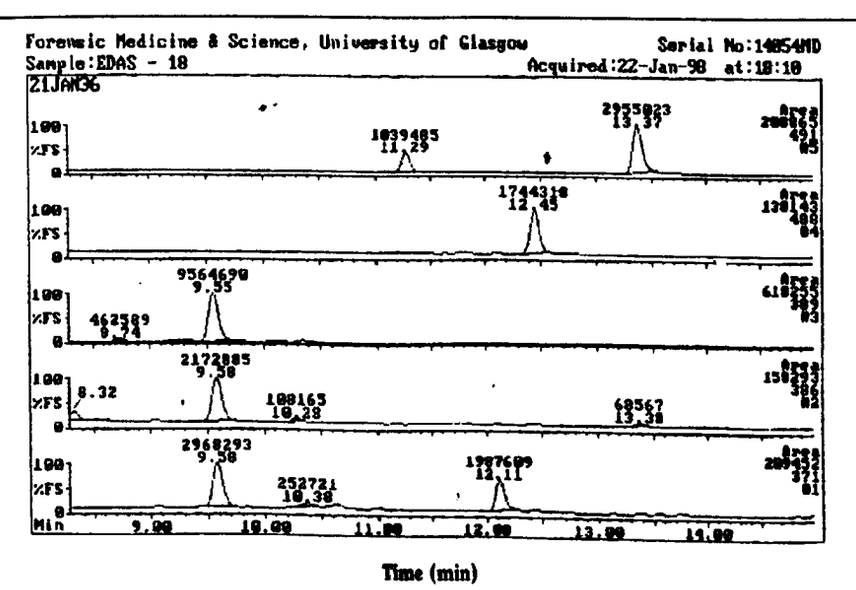


Figure 2. Selected-ion recording chromatogram from a THC-positive nail sample. The determined concentration was 3.39 ng/mg.

tion that, at the time of extraction with ethyl acetate, the pH was acidic following the addition of 3M hydrochloric acid (HCl). After extraction, the organic layer from these nail clipping hydrolysates was evaporated to dryness at 60°C under a stream of nitrogen as before. These three nail extract residues were then derivatized using BSTFA catalyzed with 1% TMCS and subjected to GC–MS as before.

GC–MS

A 1.0-µL portion of each sample was injected through an HP-1 crosslinked dimethyl silicone capillary column (30 m × 0.25 mm, 0.25-µm film thickness) of a FISONS 8000 series GC coupled to a FISONS MD 800 MS. The injector was a FISONS AS 800 (injector temperature: 225°C) and splitless injection was employed with a split-valve off-time of 1 min. Helium was used as the carrier gas.

The initial column temperature was 150°C and was programmed to rise to 300°C at a rate of 10°C/min immediately after injection. The column was subsequently maintained at 300°C for 10 min.

The capillary column was inserted directly into the ion source (200°C) via a heated transfer line (250°C). The MS was operated in the selected ion recording mode with electron impact ionization at an electron energy of 70 eV and was auto-tuned daily according to the manufacturer's recommendations.

For THC and THCOOH, qualitative and quantitative analyses were obtained using the selected-ion recording mode and comparison of retention times (t_R) and relative abundance of confirming ions with THC-d_3 and THCOOH-d_3, respectively. Quantitative results were obtained in the selected-ion recording mode after determination of the response factor of THC and THCOOH against THC-d_3 and THCOOH-d_3.

Limits of detection and extraction recoveries

No method currently tests intact nail matrix spiked with cannabinoids; therefore, the limits of detection and extraction recoveries of cannabinoids were determined after dissolution of the nail in sodium hydroxide. Nail hydrolysate in NaOH (equivalent to 10 mg of nail) was spiked with different amounts of cannabinoids and then analyzed as usual.

Results and Discussion

Table II shows the ions monitored for THC, THC-d_3, THCOOH, and THCOOH-d_3 along with their respective retention times (t_R).

Figure 2 shows a selected ion recording chromatogram of one of our nail clipping sets (EDAS-18). Figure 3 shows a selected ion recording chromatograph of our cannabinoid standards THC, THC-d_3, THCOOH, and THCOOH-d_3.

Under the analytical conditions, there was no interference caused by any extracted endogenous material present in nails.

Limits of detection and extraction recoveries are presented in Table III. By our method, concentrations of 0.1 ng/mg for both THC and THCOOH could be detected if a minimum of 10 mg of nail was used for extraction. These detection limits were determined by decreasing the concentrations of the drugs spiked in a 10-mg nail sample. Extraction recoveries were determined for THC and THCOOH by spiking drug-free nail clipping hydrolysates with known quantities of the two compounds, subjecting them to our extraction method, and then analyzing for them using the instrumental methods described earlier. The extraction recoveries thus calculated were greater than 81% for the two compounds.

Table III. Limits of Detection and Extraction Recoveries of THC* and THCOOH Obtained with 10 mg of Drug-Free Nail Spiked with THC and THCOOH

Compound	Limit of detection (ng/mg)	Extraction recovery (%)
THC	< 0.1	89.4
THCOOH	< 0.1	81.1

* Abbreviations: THC, Δ^9-tetrahydrocannabinol; THCOOH, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid.

Table IV. Cannabinoid Concentration in Fingernail Clippings Determined by RIA

Sample	Weight of sample (mg)	Cannabinoid concentration (ng/mg)
EDAS-02	6.46	0.23
EDAS-03	8.07	2.80
EDAS-04	10.40	0.92
EDAS-05	10.58	1.14
EDAS-07	18.35	0.66
EDAS-08	21.51	0.45

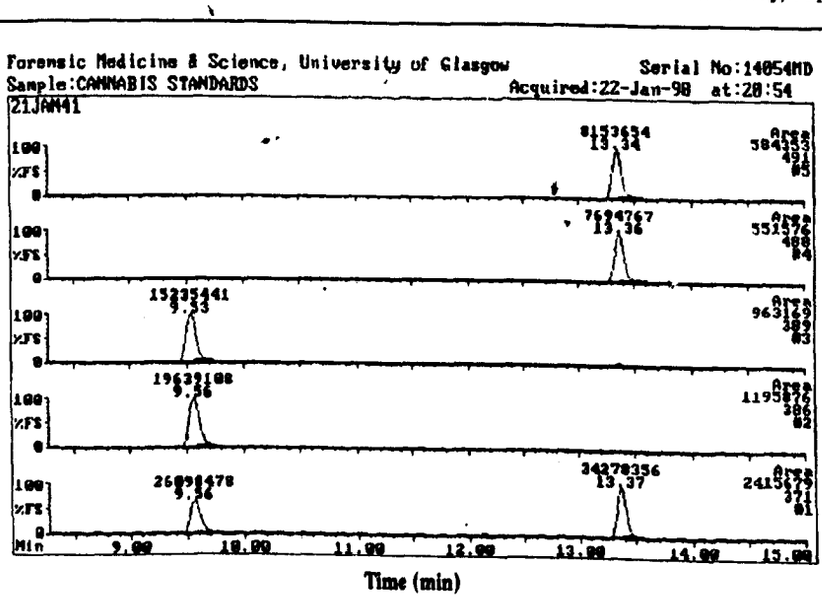


Figure 3. Single ion recording chromatogram of the cannabinoid standards used in the determination of THC and THCOOH in nails by GC-MS.

Cannabinoids were found to be present in all six cases that were analyzed for cannabinoids by RIA (Table IV). The cannabinoid concentration determined ranged from 0.23 to 2.80 ng/mg with a mean cannabinoid concentration of 1.03 ng/mg.

Using GC–MS, THC was detected in 11 out of the 14 nail clipping hydrolysates which were extracted at basic pH (Table V). THCOOH was not detected in any of these nail clipping hydrolysates. The concentration of THC ranged from 0.13 ng/mg to 6.97 ng/mg with a mean THC concentration of 1.44 ng/mg. This wide range of THC concentrations may be attributed to the non-homogeneity of the street cannabis available in Scotland, the different cannabis consumption patterns of our volunteers, or to the different length of cannabis abuse within our group of individuals.

The major metabolite of THC, THCOOH, was not detected in those nail hydrolysates extracted under basic pH. However, it was detected in two out of the three fingernail clipping hydrolysates extracted under acidic pH. The concentrations of THCOOH determined ranged from 9.82 to 29.67 ng/mg with an average concentration of 19.75 ng/mg. The parent drug, THC, was not detected in the three nail hydrolysates extracted under acidic pH. The GC–MS results for this group of samples are presented in Table VI.

The new analytical procedure presented in this article has shown that the sonic wash protocol used always produced a negative cannabinoid result for the third and final methanolic wash. In 14 cases, both the second and third methanolic washes were negative for cannabinoids, while in eight cases all three methanolic washes were negative for cannabinoids. In this way, superficial contamination was removed before the nail extracts were analyzed for cannabinoids by RIA or GC–MS.

Using RIA, cannabinoids were detected in fingernail clippings that had been stored for 6 months after collection. When GC–MS was used, THC and THCOOH were detected 9 months after the samples were collected.

The growth of fingernails is known to be 3–5 mm per month (6,11–15). Considering that drugs are deposited into the root of the nail via the blood stream and progress towards the nail tip as the nail grows, the administration of drugs to an individual could be dated back at least 3–5 months for fingernails. However, the exact time of drug consumption is likely to be difficult to establish at this early stage of nail analysis for drugs of abuse because of the influence on nail growth of other factors such as gender, age, race, and season (6,13).

Table V. Concentration of THC in Fingernail Clippings Determined by GC–MS

Sample	Weight of sample (mg)	THC* (ng/mg)
EDAS-09	3.51	0.53
EDAS-11	4.25	6.97
EDAS-12	12.25	0.57
EDAS-13	6.17	N/D
EDAS-14	2.79	0.13
EDAS-15	4.77	N/D
EDAS-16	6.18	0.14
EDAS-17	6.67	0.45
EDAS-18	5.18	3.39
EDAS-19	6.96	1.04
EDAS-21	2.68	0.65
EDAS-22	4.39	N/D
EDAS-23	2.53	0.32
EDAS-24	7.11	1.61

* Abbreviations: THC, Δ^9-tetrahydrocannabinol; N/D, not detected.

Table VI. Concentration of THCOOH* in Fingernail Clippings Extracted at Acidic pH and Determined by GC–MS

Sample	Weight of sample (mg)	THCOOH (ng/mg)
EDAS-109	5.81	9.82
EDAS-112	9.08	29.67
EDAS-114	9.21	N/D

* Abbreviations: THCOOH, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid; N/D, not detected.

Conclusions

To our knowledge, this is the first study involving analysis of human nails for the presence of THC and THCOOH. In the present work, this was successfully performed by RIA and GC–MS.

Nail clippings are potentially more easily collected than blood or urine and are especially useful because of their long-term retention of drugs. The main advantage of nail analysis is its ability to detect long-term drug abuse for time periods from months to years. Moreover, the sample size required to detect drug exposure by nail analysis is very small (2.5–25 mg) and nail samples are collected by noninvasive procedures, are easy to store, allow for increased drug stability, and do not suffer from any melanin race bias (unlike hair analysis).

Based on the results of the study and taking into account these advantages, nails are a potentially useful alternative to hair for the detection of past cannabis use in cases of medicolegal interest.

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10.4. Annexe IV.

Drug Analysis in Nail: Extraction and Determination of Morphine by RIA and HPLC in Fingernail Clippings of Heroin Users.

by Lemos, N. P., R. A. Anderson, R. T. A. Scott, F. Tagliaro and R. Valentini.

In

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Drug Analysis in Nail: Extraction and Determination of Morphine by RIA and HPLC in Fingernail Clippings of Heroin Users.

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LEARNING OBJECTIVES: This presentation will seek to inform participants of the advantages of nail specimens for forensic toxicological investigations and of the relative ease with which these specimens can be analysed, illustrated in this instance with a study on the morphine concentrations in the fingernails of heroin users.

This paper examines the use of nail as an analytical specimen for the detection of previous heroin consumption based on the analysis of morphine in fingernail clippings of known heroin users.

Samples: Fingernail clippings (3 - 96 mg) were obtained from 26 consenting adults attending the needle exchange clinics of the Glasgow Drug Problem Services in Glasgow, Scotland. At the time of sampling, the participants were asked to complete a short questionnaire pertaining to their drug use during the preceding 12 months. The nail clippings were generated using commercially available cosmetic clippers. The nail clippings from each participant were pooled and stored in a plastic bag at room temperature until the time of analysis.

Sample Decontamination: The nail clippings were sonicated once in 10 ml of 500 mM sodium dodecyl sulfate (SDS) and three times in 10 ml of deionized water for 15 minutes each time and the resulting washes were discarded. Finally, there were 3 to 4 sonications in methanol and these washes were collected and screened for analytes before trying to extract any drugs from the nail clippings. In almost two thirds of our cases (65.4 %) the SDS wash, the three water washes and three methanol washes were sufficient in removing any superficial contamination and to produce a negative methanol screen for morphine. However, in the remaining one third of cases (34.6 %), a fourth methanol sonic wash was necessary before a negative methanol screen for morphine was obtained.

Sample Extraction and Radioimmunoassay: The decontaminated nail clippings were allowed to air dry overnight and were then weighed. Alkaline hydrolysis was chosen as the extraction method for this study. This involved the incubation of the nail clippings for 60 to 120 minutes at 60° C in the presence of 1 ml of 1M sodium hydroxide solution. The resulting nail hydrolysates were then evaporated to dryness at 50° C under a stream of pressurized air. Once dry, the residues were dissolved in 4 ml of deionized water, the pH was adjusted to 7.0, and the samples were sonicated for one hour and transferred to TOXI•TUBES A. The volume was brought up to the 5-ml mark using deionized water and the extraction tubes were agitated for 10 minutes. The extraction tubes were then centrifuged at 3500 rpm for 15 minutes and the resulting organic layer was stored. To each extraction tube containing the aqueous phase, 1 ml of dichloromethane: dichloroethane: heptane (19:18:63) was added and the extraction tubes were again agitated and centrifuged as before as part of a second extraction scheme. The two organic layers from the first and second extractions were combined and evaporated to dryness under a stream of pressurized air. The dry residues were reconstituted in a 50 mM sodium phosphate buffer (pH 5.0) and vortexed. An aliquot of the resulting solution was then analyzed for morphine using the Coat-A-Count® Morphine solid-phase 125I radioimmunoassay by DPC. A set of controls and calibrators consisting of blanks, spikes and standards (0,0, 7.8, 15,6, 31,2, 62,5, 125,0 and 500,0 ng/ml) were also run with the nail extracts.

Confirmation by High Pressure Liquid Chromatography: An aliquot of the nail extract was filtered using a micro-filter needle attached to a syringe and the filtrate (diluted using 50 mM sodium phosphate buffer) was analyzed for morphine by HPLC. The HPLC instrument used for this analysis consisted of a Spectra-Physics AS 300 autosampler, a Polymeric column (63.8° C), a Jasco 880-PU pump, a BIO-RAD Electrochemical Detector and a *tsp* Chromjet integrator. During sample analysis, morphine standards of known concentrations, blanks and spikes were also run and the morphine retention time was monitored.

Results: Positive radioimmunoassay results were obtained with nail clippings from 25 of the 26 heroin users. The levels ranged from 0.06 to 4.69 ng/mg with an average morphine concentration of 1.67 ng/mg.

Using high pressure liquid chromatography, morphine was positive in 22 of the 26 nail samples. The average morphine level by HPLC was 2.11 ng/mg with a range from 0.14 to 6.60 ng/mg.

Conclusions: The addiction history of heroin users in forensic toxicology cases is often determined by morphine analysis. In this study, nail clippings were successfully and positively evaluated as analytical specimens . The nail decontamination protocol used consistently produced negative morphine screens in the final wash. Morphine was determined in nail clippings by RIA (average: 1.67 ng/mg) and confirmed by HPLC (average: 2.11 ng/mg). The use of nail clippings is now gaining more support in forensic toxicology laboratories world-wide especially due to their long-term retention of drugs and ability to detect drug abuse ranging in time from months to years. In addition, nails are collected by non-invasive procedures, only a small sample size is required, they are easy to store, they allow for increased stability of drugs and, because of their low melanin content, are less likely to suffer from race bias than hair specimens. Because of these advantages, nails have the potential of becoming a powerful alternative to hair for the detection of past heroin use in forensic cases.

Nail, Analysis, Opiates

10.5. Annexe V.

Analysis of Morphine by RIA and HPLC in Fingernail Clippings Obtained from Heroin Users.

by Lemos, N. P., R. A. Anderson, R. Valentini, F. Tagliaro and R. T. A. Scott

In

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Analysis of Morphine by RIA and HPLC in Fingernail Clippings Obtained from Heroin Users*

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Foot line (29 characters): MORPHINE ANALYSIS IN THE NAIL

ABSTRACT: Heroin is abused around the world and is frequently reported as the cause of death in overdose cases. Analysis of morphine in hair has been used in the past in forensic toxicology to study the addiction history of heroin addicts. The purpose of the present study was to evaluate the usefulness of the nail as an analytical specimen in the identification and quantification of morphine in fingernail clippings of known heroin users. Fingernail clippings were obtained from 26 consenting patients of the Glasgow Drug Problem Service. At the time of sampling, the participants provided answers to a questionnaire regarding their drug use patterns. Samples were decontaminated by sonication in SDS, deionized water and methanol and the methanolic washes were screened for analyte presence. The washed nail clippings were then hydrolyzed and extracted. RIA was used for the screening and HPLC for the confirmation of morphine. Positive RIA results were obtained with nail clippings from 25 of the 26 heroin users. The levels ranged from 0.06 to 4.69 ng/mg with a mean morphine concentration of 1.67 ng/mg. HPLC results were positive for 22 of the 26 nail samples. The mean morphine level by HPLC was 2.11 ng/mg with a range from 0.14 to 6.90 ng/mg. Based on these results, we suggest that nails have the potential of becoming a powerful alternative to hair for the detection of past heroin use in forensic cases.

KEYWORDS: forensic science, forensic toxicology, drug analysis, alternative specimens, nail, fingernail, heroin, morphine.

Introduction

In Scotland, excluding cannabis, heroin predominates in drug misuse accounting for almost half of the new individual notified addicts (49%) and being involved in at least 30 deaths per annum according to recent official epidemiological data (1). Injecting is still the predominant route of use in those cases where heroin is involved: 47% of heroin users inject whereas only 4% take heroin orally and 32% smoke the drug (1).

Heroin or diacetylmorphine or diamorphine has been extensively studied and is known to be rapidly deacetylated in the body to 6-acetylmorphine. This metabolite undergoes further hydrolysis to morphine at a slower rate (2). Morphine analysis is frequently used in forensic toxicology to determine the use of heroin (3).

Nail has been used in the past for the determination of voluntary or involuntary exposure to substances. Specifically, fingernail analysis has been used for the determination of arsenic exposure (4) as well as for the qualitative and quantitative determination of amphetamines (5-7), cocaine and its metabolites (8-10) and cannabinoids (11). To determine the usefulness of the nail as an analytical specimen in a forensic environment where radioimmunoassay and high-pressure liquid chromatography are used for routing opiate analysis, we examined nail clippings of known heroin users for the presence of morphine by the two aforementioned techniques.

Materials and Methods

Standards and Reagents: All organic solvents were high-pressure liquid chromatography grade, and all chemicals were reagent grade. Morphine hydrochloride was purchased from CARLO ERBA (Milan, Italy). Sodium dodecyl phosphate (500mM), sodium hydroxide (1M) and sodium phosphate buffer (50 mM) were prepared from HPLC grade reagents in deionized water.

Samples: Nail clippings (3.0 to 96.0 mg) were collected from consenting adults (N=26) attending the clinics of the Glasgow Drug Problem Service in Glasgow, Scotland. The procedures followed were in accordance with the ethical standards of the responsible regional committee on human experimentation. At the time of sampling, the participants were asked to provide answers to a short questionnaire regarding their drug use patterns. Nail clippings were generated using commercially available cosmetic nail clippers. The nail clippings of each participant were pooled and stored in a plastic bag at room temperature until the time of analysis. Demographic and epidemiological data for each participant are shown in Table 1. Our study population comprised of 22 Caucasian males and 4 Caucasian females. Their average age was 26.5 years and ranged from 19 to 33 years.

In addition, 5 sets of fingernail clippings from individuals who were unlikely to be heroin users (i.e., laboratory personnel, postgraduate research students and academics) were obtained and used as controls.

A conventional drug screening using radioimmunoassay followed by a confirmatory analysis based on high-pressure liquid chromatography was performed on the nail specimens at the Institute of Forensic Medicine, University of Verona, Verona, Italy.

Sample Decontamination: Using an ELMA-SINGEN HTW ultrasonication bath, the nail clippings were sonicated once in 10 ml of 500 mM sodium dodecyl sulphate (SDS) for 15 minutes and three times in 10 ml of deionized water for 15 minutes each time and all four resulting washes were discarded. Next, there were 3 sonications in methanol for 15 minutes each and these washes were collected, evaporated to dryness at 50° C under a stream of pressurized air, reconstituted in 200 µl of 50 mM sodium phosphate buffer (pH 5.0) and mixed by vortex. A 50 µl aliquot was then screened by RIA for morphine. In those cases in which the third methanolic wash tested positive for morphine, a further (fourth) sonication in methanol for 15 minutes was conducted and the resulting wash was collected and screened by RIA for methanol as before. Only when the final methanolic wash (third or fourth, as required) tested negative for morphine did the analysis of the nail clippings proceed.

Sample Extraction and Radioimmunoassay: The decontaminated nail clippings were allowed to air dry overnight and weighed. Alkaline hydrolysis was chosen as the extraction method for this study. This involved the incubation of the nail clippings for 60 to 120 minutes at 60° C in the presence of 1 ml of 1M sodium hydroxide solution. The resulting nail hydrolysates were then evaporated to dryness at 50° C under a stream of pressurized air. Once dry, the residues were dissolved in 4 ml of deionized water, the pH was adjusted to 7.0, and the samples were sonicated for one hour and transferred to TOXI•TUBES A. The volume was brought up to the 5-ml mark using deionized water and the extraction tubes were agitated for 10 minutes. The extraction tubes were then centrifuged at 3500 rpm for 15 minutes and the resulting organic layer was stored. To each extraction tube containing the aqueous phase, 1 ml of dichloromethane: dichloroethane: heptane (19:18:63) was added and

the extraction tubes were again agitated and centrifuged as before as part of a second extraction scheme. The organic layers from the two extractions were combined and evaporated to dryness under a stream of pressurized air. The dry residues were reconstituted in 200 µl of 50 mM sodium phosphate buffer (pH 5.0) and mixed by vortex. A 50 µl aliquot of the resulting solution was then analyzed for morphine using the Coat-A-Count® Morphine solid-phase ^{125}I radioimmunoassay by DPC and a Packard Instrument RIA counter (Downers Grove, IL). The RIA kits used contain antiserum which, according to their manufacturers, is highly specific to free (unconjugated) morphine with low crossreactivity to 6-acetylmorphine (1%), codeine (0.06%), morphine-3-glucuronide (0.3%) and morphine-6-glucuronide (0.05%). The standard curve was prepared from morphine hydrochloride in 50 mM sodium phosphate buffer (pH 5.0) at the concentrations of 0.0, 7.8, 15.6, 31.2, 62.5, 125.0 and 500.0 ng/ml.

High-Pressure Liquid Chromatography: Aliquots of the nail extracts were filtered using micro-filters (0.45 µm) attached to syringes and the filtrates (diluted 1:1 with 50 mM sodium phosphate buffer) were analyzed for morphine by HPLC following a published method (12). The HPLC instrument used for this analysis consisted of a Spectra-Physics AS 300 autosampler, a PRLP-S polymeric column (150 x 4.6 mm) operated at 63.8° C, a Jasco 880-PU pump, a BIO-RAD Electrochemical Detector and a Spectra Physics Chromjet integrator. During our sample analysis, morphine standards of known concentrations, blanks and spikes were also run and the morphine retention time was monitored.

Limit of Detection and Extraction Recoveries: To date, there is no published method available for spiking the intact nail matrix with morphine. Thus, the limit of detection

and extraction recoveries of morphine were determined by spiking the solution obtained after hydrolysis of drug-free nail in sodium hydroxide. Ten different nail hydrolysates in NaOH (each equivalent to 10 mg of nail) were spiked with morphine and then analyzed as usual in order to determine the extraction recovery of the method. Furthermore, three nail hydrolysates in NaOH (each equivalent to 10 mg of nail) were spiked with decreasing amounts of morphine and then analyzed as usual in order to determine the limit of detection of the method.

Results

Our decontamination protocol produced contamination-free nail clippings. In almost two thirds of our cases (65.4 %), the SDS wash, the three water washes and three methanol washes were sufficient in removing any superficial contamination and to produce a negative third methanol screen for morphine. However, in the remaining one third of the cases (34.6 %), a fourth methanol sonic wash was necessary before a negative methanol screen for morphine was obtained.

The results of our RIA screening and HPLC confirmation for the nail extracts and blanks are summarized in Table 2 together with data regarding their final methanolic wash (either the third or the fourth wash, as required). As it is illustrated in Table 2, the decontamination protocol employed in this study produced contamination-free nail clippings by the third (65.4% of the cases) or fourth (35.4% of the cases) methanolic wash and, had the nail samples not been decontaminated in this way, the final results would in most cases be heavily influenced. All blank samples and their methanolic washes tested negative for morphine by RIA and HPLC.

Hydrolysates of fingernail clippings were extracted using TOXI•TUBES A and liquid-liquid extraction and then assayed by RIA and HPLC for the presence of morphine. A typical RIA calibration curve showing the range of linearity of the technique is presented in Figure 1 and chromatograms of analyses of a blank, a standard and a nail extract are shown in Figure 2. Positive RIA results were obtained with nail clippings from 25 of the 26 heroin users. The levels ranged from 0.06 to 4.69 ng/mg with a mean morphine concentration of 1.67 ng/mg. Using high-pressure liquid chromatography, morphine was positive in 22 of the 26 nail samples. The mean morphine level by HPLC was 2.11 ng/mg with a range from 0.14 to 6.90 ng/mg.

Figure 3 presents the level of morphine seen in each of our samples by both RIA and HPLC plotted against the monetary level of heroin used (in GBP, £) as this was reported by each participant on his/her questionnaire at the time of sampling. The noticeable variations of morphine levels among individuals who declared to have consumed the same amounts of heroin might be due to several factors which are discussed below.

In regard to the limit of detection of our method, concentrations as low as 0.05 ng/mg morphine could be detected if the equivalent of at least 10 mg of nail was used for extraction with a signal-to-noise ratio of 5. This detection limit was determined by decreasing the concentration of the drug spiked in nail hydrolysate equivalent to 10 mg of nail. An extraction recovery was determined for morphine by spiking hydrolysate of drug-free nail clippings with known quantities of morphine, subjecting them to our extraction method and then analyzing for them using the instrumental methods described earlier. The extraction recovery thus calculated was 80.5 % for morphine by RIA and 86.3% for morphine by HPLC.

Discussion

The use of nail material for the determination of voluntary or involuntary exposure to substances is not a recent scientific development. In particular, exposure to arsenic by its detection in the nail of human subjects was among the first developments in this area. In one report, arsenic was determined in fingernails of five subjects who have been given therapeutic doses of arsenious oxide (4) in an effort to develop an alternative way for the determination of time of exposure to arsenic. Fingernail clippings from the five subjects were collected prior to the start of the experiment and thereafter at regular intervals over the following six months. When the measured amount of arsenic was plotted against the days on which the clippings were collected, two arsenic peaks were observed. Scraping the underside of the nail reportedly removed the second (smaller) arsenic peak. It was suggested that one peak (the larger) was the result of arsenic deposition into the nail root from the blood stream while the second, smaller peak was the result of exogenous contamination of the nail, probably from sweat.

Drugs remain in nails for extended periods of time thereby providing information on the history of drug exposure of an individual. Amphetamines were among the first drugs of abuse to be identified in nail clippings (5). In that study, the clippings were subjected to alkaline hydrolysis after washing. N-pentane was used to extract amphetamines from the nails and the extracts were derivatized and analyzed by gas chromatography - mass spectrometry. The mean concentrations of methamphetamine and amphetamine were 4.75 ± 2.34 (range 0.00 - 17.7) and 0.14 ± 0.06 (range 0 - 0.40) ng/mg, respectively. These were similar to the measured drug

concentrations in hair from the same individuals. Average toenail methamphetamine and amphetamine concentrations were always higher than the corresponding fingernail concentrations of the same subjects. The authors suggested that the slower growth of toenails (1.1 mm/month) as compared to fingernails (3 - 5 mm/month) provided a possible explanation for the consistent difference in drug levels between the two specimens. Furthermore, Cirimele et al. (6) reported that several amphetamines were present in hair and using a similar extraction method as Suzuki et al. (5), were able to identify amphetamine (AMP), 3,4-methylenedioxyamphetamine (MDA) and 3,4-methylenedioxymethamphetamine (MDMA) in one fingernail. The concentrations determined in this one sample by gas chromatography - mass spectrometry were 12.0 ng/mg for AMP, 9.7 ng/mg for MDA and 60.2 ng/mg for MDMA. These levels appear to be in agreement with the concentrations of Suzuki et al. where the drug analysis was carried out by mass fragmentometry (7).

Cocaine has also been measured in the nail. Nail and hair concentrations of cocaine in a man who died of an overdose were measured by enzyme immunoassay and gas chromatography - mass spectrometry (8). The nail specimens were washed prior to analysis and the wash solutions were analyzed and found positive for cocaine (range 4.1 to 5.8 μg/mg). The nail concentrations of cocaine also measured excessively high at 2.2 to 2.3 μg/mg. Toenail specimens were also positive for cocaine (range 6 to 16 ng/mg). The authors suggested that the deceased had recently handled cocaine because they found high concentrations of cocaine in the fingernail wash solutions. The washing protocol, however, would have removed the significant superficial contamination that was measured had it been more extensive and efficient.

Garside et al. have also been successful in determining several cocaine metabolites in nail (9). Nails were cut into small sections and washed with methanol. Methanolic reflux was then used to extract the drugs from the nail matrix and the solid phase extracts were analyzed by gas chromatography - mass spectrometry. Cocaine and benzoylecgonine (0.0 to more than 10 ng/mg in both fingernail and toenail for both drugs) were the predominant analytes in all cocaine positive nail specimens. Other cocaine by-products (anhydroecgonine methyl ester, ecgonine methyl ester, ecgonine ethyl ester, cocathylene, norcocaine and benzoylecgonine) were also detected in the nails. The pattern of drug abuse could be established based on these results: detection of cocaethylene suggested concomitant use of ethanol and cocaine and detection of anhydroecgonine methyl ester indicated use of crack cocaine. Moreover, the detection of cocaine metabolites in the nail indicated drug ingestion followed by metabolism and ensuing incorporation into the nail.

In a study examining eight postmortem specimens to determine cocaine and its metabolites in the nail, gas chromatography - mass spectrometry in the selected ion monitoring (SIM) mode was employed (10). All eight cases were positive for the parent drug as well as benzoylecgonine and ecgonine methyl ester. Cocaethylene and norcocaine were also found in some of the specimens. The same study reported the results of opiate analysis for a further eight cases. Morphine was found present in seven of the eight cases whereas 6-monoacetylmorphine and codeine were found positive in five cases. Finally, in two cases hydromorphine was also detected.

Cannabinoids have recently been identified in the nail (11). Fingernail clippings of known cannabis users were collected and decontaminated following a sonication protocol in sodium dodecyl sulfate, deionized water and methanol. The

methanolic washes were screened for analytes and only when they were negative, the authors proceeded with the drug extraction from the washed nail clippings. The decontaminated samples were subjected to alkali hydrolysis, extraction and analysis for cannabinoids by radioimmunoassay and gas chromatography - mass spectrometry. The average Δ^9-tetrahydrocannabinol concentration in fingernails was reported to be 1.4 ng/mg with a range from 0.1 to 6.9 ng/mg. The average concentration of Δ^9-tetrahydrocannabinol-9-carboxylic acid was 19.8 ng/mg with a range from 9.8 to 29.6 ng/mg.

The relatively small size of the population examined in the present study (N=26) does not allow for the construction of proper dose-response relationships. The heterogeneity of the street heroin available in Scotland may also be an important factor. Furthermore, having to rely on the self-reporting figures of what the participants think they have consumed as opposed to conducting experiments under controlled dosage parameters adds at least two potential sources of errors (what the participants think they have consumed and what the participants declare to have consumed based on what they believe their answers should be). Variations in the heroin consumption patterns of our volunteers and the varying length of heroin use within this group may also be factors of variation. Finally, the mechanism(s) of substance incorporation into the nail matrix are not yet understood and there may be substantial inter-individual variation due to physiological, biochemical and pharmacokinetic factors.

Although there is a significant portion of the scientific literature which reports on the detection of drugs in nails, the mechanisms of drug incorporation into this matrix have not yet been fully characterized. Literature reports exist which suggest

that drugs may gain quick access to the distal nail plate during nail production by incorporation into the cornified cells of the nail bed. Johnson et al. have reported that there exists minimal incorporation of drug by diffusion from the nail bed to the ventral portion of the nail plate (13, 14). Norton and Zaias have separately shown that drug incorporation occurs in the lunular germinal matrix as the nail grows from the base of the nail along the distal axis (15, 16). There exist other potential sources of drug entry into the nail such as environmental contamination, and contamination from sweat, saliva, sebum, urine, etc. Finally, the chemical properties of the drugs in consideration could also play a protagonistic role in their incorporation into the nail. However, further studies are needed to determine the actual mechanism(s) of drug incorporation into the nail matrix and the roles played by physiological, biochemical and pharmacokinetic factors.

The addiction history of heroin users in forensic toxicology cases is often determined by morphine analysis in hair. In this study nail clippings were successfully evaluated as analytical specimens. The nail decontamination protocol proposed herein consistently produced negative morphine screenings for the final methanol wash. Morphine was determined in decontaminated nail clippings by RIA (mean: 1.67 ng/mg) and confirmed by HPLC (mean: 2.11 ng/mg).

The nail matrix offers several advantages to the forensic toxicologist. Drugs remain trapped in the nail matrix for extensive periods of time thus allowing the determination of exposure to drugs for periods from months to years. Furthermore, the collection of nail clippings is a non-invasive procedure as compared to the collection of blood or urine and only a small sample size is required as demonstrated in this study (mean sample size: 26.4 mg). Nails are easily stored in plastic bags at

room temperature (11), allow for increased stability of drugs and are less likely to suffer any melanin race bias (9). It is because of these advantages that we suggest that more work be done in drug analysis in the nails. Based on our work and understanding of these specimens, we believe that nails have the potential of becoming a powerful alternative to hair for the detection of past heroin use in forensic cases.

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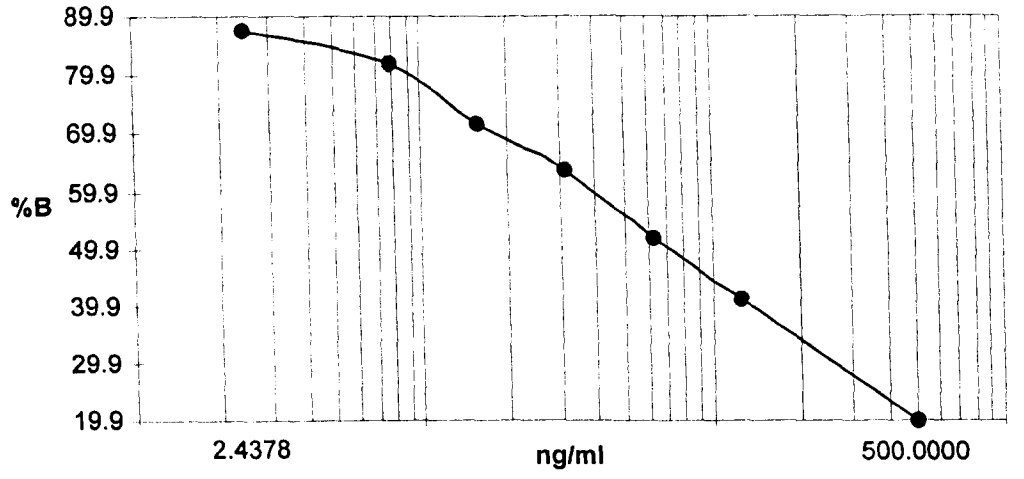

FIG. 1 – A typical RIA calibration curve generated using morphine calibrators showing the range of linearity for this assay and used for the initial morphine screening of nail extracts.

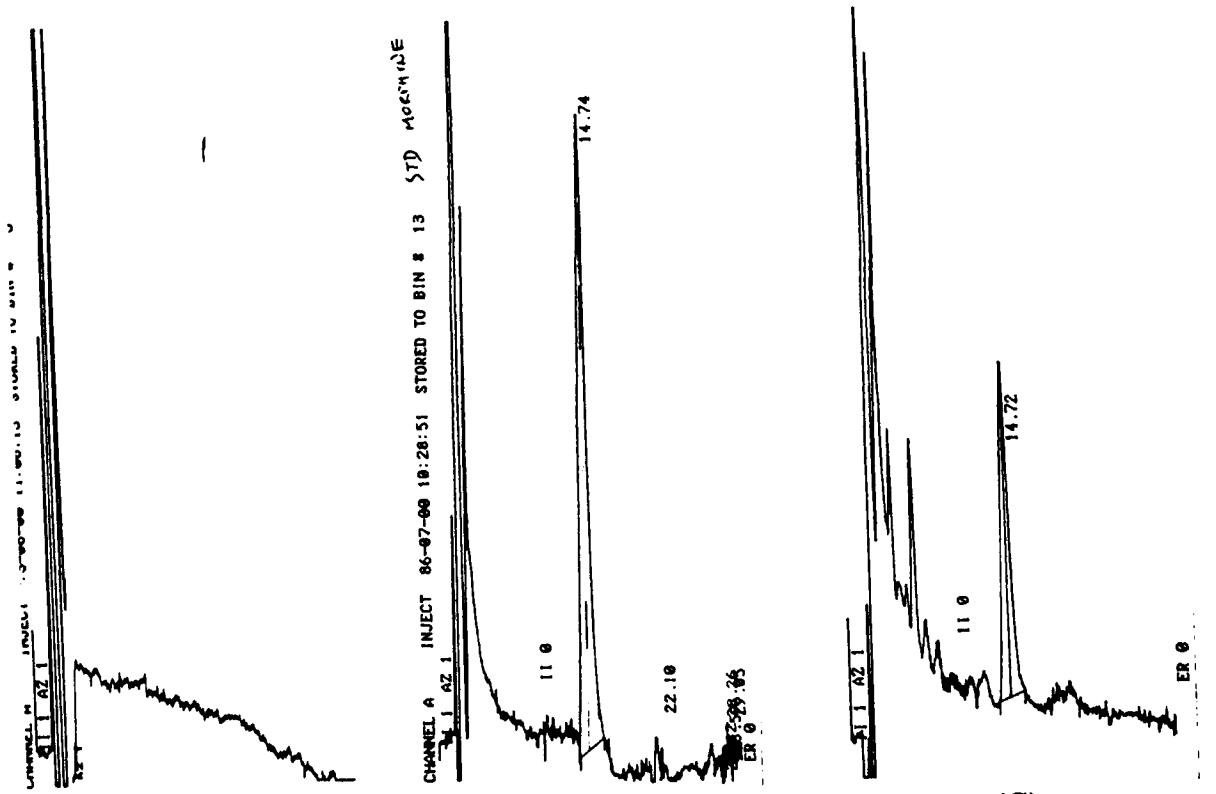

FIG. 2 – High-pressure liquid chromatograms of (A) a drug-free nail extract, (B) a standard morphine solution and (C) a nail extract from a participant (Sample 9: 1.30 ng/mg morphine).

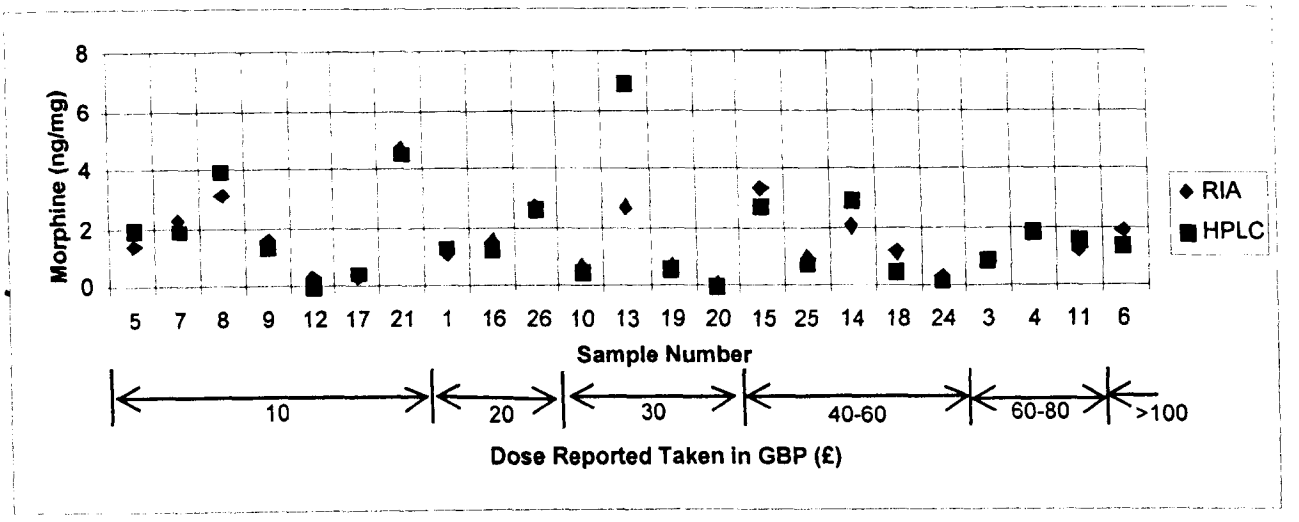

FIG. 3 - Morphine levels in ng/mg measured by RIA and HPLC in each of our samples plotted against the monetary value (in GBP, £) of heroin used by each participant as that was declared on his/her questionnaire at the time of sampling.

TABLE 1 – Subject demographic and epidemiological data.

Subject Number	Sex	Age*	Heroin consumption rate in GBP (£) or weight per time interval*	Other drugs taken*,†
1	Male	24	£20/day	M
2	Male	29	£30-100/day	Cn, D, M, T
3	Male	27	£60-80/day	Cn, Co, D, M
4	Male	23	£60-80/day	Cn, Co, D, M, T
5	Male	19	£10/day	M
6	Male	33	£100-150/day	Ap, Cn, D, M, T
7	Male	21	£10/day	Ap, Cn, M
8	Male	28	£10/day	Cn, D, M, T
9	Male	22	£10/day	D, M, T
10	Male	26	£20-30/day	Ap, Cn, M, T
11	Male	21	£80/day	Ap, Cn, Co, Dc, M, T
12	Male	32	£10/day	Cn, M
13	Female	23	£30/day	D, M
14	Male	32	£40-60/day	Cn, Co, D, M, T
15	Female	25	£30-40/day	Ap, Cn, D, M, T
16	Male	31	£20/day	Cn, D, M
17	Male	19	£10/day	M
18	Male	24	£60/day	Ap, Co, M, T
19	Male	28	£30/day	Ap, Cn, Co, D, M, T
20	Male	27	£30/day	Cn, D, Dc, M, T

21	Female	33	£10/day	Cn, D, Dc, M
22	Male	24	1.5 g/day	Cn, Co, D, Dc, M, T
23	Male	31	£40/day	Ap, Cn, D, M, P
24	Male	31	£60/day	Ap, Cn, Co, D, M, T
25	Female	29	£40/day	At, Cn, D, Dc, M, T
26	Male	28	£20/day	Ap, Cn, D, M, T

*As reported on the questionnaires during sample collection at the clinics of Glasgow Drug Problem Service.

†At: Amitriptyline; Ap: Amphetamines; Cn: Cannabis; Co: Cocaine; D: Diazepam; Dc: Dihydrocodeine; M: Methadone; P: Paracetamol; T: Temazepam.

TABLE 2 – Morphine levels in the final methanolic wash (third or fourth, as required) measured by RIA and in the nail extracts screened by RIA and confirmed by HPLC*.

Subject Number	Sample Weight Mg	Wash 3 ng/ml	Wash 4 ng/ml	RIA Screening ng/mg	HPLC Confirmation ng/mg
S 1	22.8	7.81	NEG	1.10	1.30
S 2	47.0	3.87	NEG	4.10	6.60
S 3	61.0	NEG	N/R	0.80	0.90
S 4	16.0	NEG	N/R	1.80	1.80
S 5	35.1	3.36	NEG	1.40	2.00
S 6	16.9	2.98	NEG	1.90	1.40
S 7	11.0	NEG	N/R	1.50	1.90
S 8	7.0	7.96	NEG	3.20	3.90
S 9	10.9	NEG	N/R	1.60	1.30
S 10	13.3	NEG	N/R	0.69	0.51
S 11	15.3	NEG	N/R	1.24	1.54
S 12	35.4	NEG	N/R	0.32	0.00
S 13	96.0	NEG	N/R	2.70	6.90
S 14	36.9	NEG	N/R	2.05	2.90
S 15	17.5	9.82	NEG	3.38	2.68
S 16	8.8	3.76	NEG	1.53	1.22
S 17	30.0	NEG	N/R	0.39	0.45

S 18	9.2	NEG	N/R	1.16	0.50
S 19	23.4	NEG	N/R	0.62	0.57
S 20	29.2	NEG	N/R	0.06	0.00
S 21	15.4	5.79	NEG	4.69	4.51
S 22	6.3	NEG	N/R	1.39	0.00
S 23	3.0	NEG	N/R	0.00	0.00
S 24	38.5	NEG	N/R	0.32	0.14
S 25	48.8	NEG	N/R	0.91	0.72
S 26	31.6	1.09	NEG	2.80	2.59
Blank 1	23.6	NEG	NEG	0.00	0.00
Blank 2	17.0	NEG	NEG	0.00	0.00
Blank 3	9.5	NEG	NEG	0.00	0.00
Blank 4	45.8	NEG	NEG	0.00	0.00
Blank 5	4.3	NEG	NEG	0.00	0.00
Mean S 1-26	-	-	-	1.67	2.11
Median S 1-26	-	-	-	1.40	1.40
Range S 1-26	-	-	-	0.06 - 4.69	0.14 - 6.90

*NEG: Negative morphine screen by RIA; N/R: 4th methanolic wash not required.

10.6. Annexe VI.

Methadone Analysis in Nail Clippings of Patients on a Methadone Maintenance Program.

by Lemos, N. P., R. A. Anderson and J. R. Robertson

In

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Methadone Analysis in Nail Clippings of Patients on a Methadone Maintenance Program

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This study offers an analytical scheme for methadone in fingernail clippings. Samples (0.79–16.33 mg) were collected from 28 consenting adults participating in a methadone maintenance program in Edinburgh, Scotland. At the time of sampling, participants provided answers to a questionnaire regarding the drugs they had used prior and during admission to the maintenance program. The nail clippings were stored in plastic bags at the time of collection and transferred to a forensic toxicology laboratory in Glasgow. There they were decontaminated by sonication in 0.1% sodium dodecyl sulphate (SDS) for 15 minutes followed by sonication in water three times for 15 minutes each and sonication in methanol three times for 15 minutes each. Whereas the resulting SDS and water washes were discarded, the methanolic washes were collected and screened for methadone by enzyme immunoassay (EIA). Once the methanolic washes had tested negative for methadone, the decontaminated nail clippings were hydrolysed in 1M NaOH. Aliquots of the hydrolysates were screened for methadone by EIA and confirmed by gas chromatography – mass spectrometry (GC-MS). The mean methadone concentration in fingernail clippings determined by EIA was 32.8 ng/mg nail. The mean methadone concentration in

fingernail clippings determined by GC-MS was 26.9 ng/mg. Hydrolysates of the equivalent of 10 mg of blank nail clippings were spiked with known concentrations of methadone and analysed by the proposed procedures. In this way, extraction recoveries and limits of detection of the two techniques for methadone in the nail were calculated. Based on our results, fingernails appear to be a potentially useful biological specimen for the analysis of methadone and the monitoring of patient compliance to their methadone maintenance programs.

Fingernail clippings, Analysis, Methadone