Analysis of Multiple Drugs In Small Blood Specimens and Meconium: Applications in Paediatric Toxicology

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by

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Summary:

This thesis deals with the quantitative analysis of multiple drugs in the neonate, in blood and in meconium as an alternative biological specimen in forensic toxicology and is also concerned with the dangers of transmission of drugs used and abused by the mother to the foetus and neonate. The aims of the work were to investigate methods for performing a full drug screen on small amounts of biological specimen and to carry out a survey of illicit drug use during pregnancy in the Glasgow area.

Following brief overviews of toxicology and the main problems facing the paediatric toxicologist, the development of analytical toxicology procedures to help to overcome them is summarised along with a more detailed examination of solid phase extraction (SPE) – theory, advantages and applications to biological samples.

The initial experimental work established the feasibility of performing a full drug screen on small samples of blood such as those obtained from neonates, having a volume of 1-2ml. This used a single SPE cartridge for the extraction of a mixture of acidic and basic drugs followed by end-step analysis with enzyme immunoassay (EIA) and gas chromatography-mass spectrometry (GC/MS). Representative drugs from each group were selected for evaluation purposes: butobarbitone, amylobarbitone, methaqualone, primidone, and phenytoin drugs for the acidic drug group and cocaine, ecgonine methyl ester, morphine, diazepam, and desmethyldiazepam for the basic drug group. These were chosen as examples of drugs commonly used in the UK. Four analytes (morphine, methadone, cocaine and benzoylecgonine) were used to compare two different SPE cartridges, Bond Elut

Certify® and Abselut®, which can be used to extract both acidic and basic drugs. The comparison involved four parameters: analyte recovery, lower limits of detection, presence of interferences in the extracts and analysis time required by each. Recoveries were in the range 60-100% and lower limits of detection were in the range 1-25 ng/ml and it was assessed that Bond Elut columns were better than Abselut columns. This method was applied to cases arising under the Road Traffic Act, which also provide very small samples for analysis and was found to be suitable for the intended purpose.

The background to drug abuse, especially in pregnancy is then reviewed, including information on the main groups of illicit drugs analysed in this thesis, on their history, chemistry, pharmacology, toxicology and maternal and foetal considerations. Then an overview is given of the serious complications that affect the foetus prenatally and the neonate due to the use of illicit drugs by the pregnant mother along with common congenital anomalies, which are the largest cause of infant mortality. Of particular relevance in the Glasgow area is the Neonatal Abstinence Syndrome due to opiate abuse. Subsequently, a brief overview is given of the structure of the placenta, its circulation, function and the mechanism by which drugs pass through it to the foetus. Finally, a review is made of conventional and alternative biological specimens used for analytical purposes from the mother/foetus/neonate, including blood, urine, hair, nail, amniotic fluid, breast milk, and meconium. Particular emphasis is placed on previous analytical methods and the advantages of meconium over the other specimens. Meconium gives a wide window of detection of drugs, ranging from 12 weeks of pregnancy till delivery, and displays high concentrations of drugs. Also it is easily collected and non-invasive.

The second experimental study investigated the use of the developed multi-drug extraction method for meconium. The target analytes were chosen as the most commonly used and abused drugs in the Glasgow area, which are known from the casework carried out in the Forensic Medicine Department. These drugs are cocaine, and its principal metabolite benzoylecgonine, morphine, codeine, methadone, diazepam, temazepam, tetrahydrocannabinol and tetrahydrocannabinol carboxylic acid. The drugs were extracted by SPE with Bond Elut Certify columns followed by qualitative and quantitative analysis by EIA and GC/MS. The recoveries ranged from 50-100% and limits of detection ranged from 1-100ng/gm. The method was tested initially on 25 cases from the Glasgow Royal Maternity Hospital.

An additional method study was required to develop the method to screen for amphetamine, methamphetamine and their analogues MDA, MDMA and MDEA, which could not be detected by the existing method. A new extraction procedure using ZD-Kleen® cartridges was explored. End-step analysis was also done by EIA and GC/MS. Recoveries ranged from 60-95%, with lower limits of detection ranging from 1-20ng/gm. This meant the method was sufficiently sensitive and specific and it was assessed on 12 real cases from the Royal Maternity Hospital.

The third study was a survey done on neonates born at Glasgow Royal Maternity Hospital in the period October 2000 till February 2001. This involved applying the developed methods to screen drugs of abuse in meconium (opiates and methadone, cocaine and its major metabolites, cannabinoids, benzodiazepines and amphetamines). This was the first survey done on meconium analysis in Glasgow to see the spread of drug used by pregnant mother and the drug concentrations, which are found in the neonate. For the survey, 450 cases were collected during this period. The percentages of cases in which drugs were confirmed were: morphine 38%,

codeine 29%, diazepam 18.5%, temazepam 12%, THC 10%, THCA 10%, methadone 4.5%, cocaine 2.5% and benzoylecgonine 2%. A number of polydrug addicted mothers were detected. These cases were also analysed for impurities found in street heroin, including acetylcodeine, 6-MAM, dihydromorphine, papaverine, and thebaine, as markers to distinguish between morphine derived from therapy and that derived from illicit heroin use. Nine cases were found to contain one or more of these markers.

The final experimental study investigated two types of hydrolysis of cannabinoid conjugates, using glucuronidase and alkali, to improve the sensitivity of the method. Several cases were encountered which gave positive results by EIA but which were not confirmed by GC/MS, because the cannabinoid metabolites are primarily in their conjugated forms. A comparison was made between these two methods of hydrolysis and between the methods with and without hydrolysis with respect to lower limits of detection and recoveries. The preferred method, using alkaline hydrolysis, was applied to some real case samples which had given positive results by EIA but negative results by GC/MS and this was found to increase the rate of confirmation.

The thesis ends with conclusions from the work and proposals for its future extension.

List of Abbreviations

AM	Amphetamine
ATP	Adenosine Triphosphate
BECC	Bond Elut Certify Columns
BEG	Benzoylecgonine
BSTFA	bis(trimethylsilyl)trifluoroacetamide
CSF	Cerebrospinal Fluid
d_3	tri-deuterated
ED	Electron capture detector
EDDP	2-ethylidene-1,5-dimethyl-3,3- diphenylpyrrolidine
EEG	Electroencephalogram
EI	Electron Impact
EIA	Enzyme Immunoassay
EMDP	2-ethyl-5-methyl-3,3-diphenylpyrrolidine
EME	Ecgonine
EMIT	Enzyme Multiplied Immunoassay Technique
EMDP	2-ethyl-5-methyl-3,3-diphenylpyrrolidine
FPIA	Fluorescence Polarisation Immunoassay
GC	Gas Chromatography
GC/ECD	Gas Chromatography with Electron Capture Detection
GC/FID	Gas Chromatography with Flame Ionisation Detection

GC/NPD	Gas Chromatography with Nitrogen-Phosphorus Detection
GIT	Gastrointestinal Tract
HPLC	High Performance Liquid Chromatography
IM	Intramuscular
IS	Internal Standard
IV	Intravenous
LC/MS	Liquid Chromatography/Mass Spectrometry
LD	Lethal dose
LD50	Lethal dose which cause death in 50% of the population
LLE	Liquid-Liquid Extraction
LSD	Lysergic acid diethylamide
MA	Methylamphetamine
6-MAM	6-Monoacetyl morphine
MDA	methylenedioxyamphetamine
MDEA	methlenedioxyethylamphetamine
MDMA	methylenedioxymethamphetamine
MS	Mass Spectrometry
NAS	Neonatal Abstinence Syndrome
NIDA	National Institute on Drug Abuse
PCP	Phencyclidine
PFPA	Pentafluoropropionic anhydride
Pk_a	pH at which 50% of the analyte charges and 50% not charges.

r^2	Linear Correlation Coefficient
RIA	Radioimmunoassay
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
t_R	retention time
THC-COOH	THC-11-nor-delta-9-Tetrahydrocannabinol-9-carboxylic acid
TLC	Thin Layer Chromatography
UK	United Kingdom
UV	Ultra Violet

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1. General Introduction and Aims

Toxicology is the study of poisons or toxic substances and their adverse effects on living organisms. In some contexts, toxicologists are also involved with the treatment of poisoning. A poison may be defined as any agent capable of producing a deleterious response in biological system, seriously injuring function or producing death[1]. Paracelsus (1493-1541) wrote: "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison and a remedy." In the UK, the most common causes of poisoning relate to drugs, including alcohol, while in other parts of the world, pesticides are the main cause of substance-related death[2]. It is generally accepted that the toxicity of a drug or substance depends on not only its toxic properties but also on the dose administrated.

Three specialised areas of toxicology are forensic, clinical, and environmental toxicology. *Forensic toxicology* is a hybrid of analytical chemistry and fundamental toxicological principles. It is concerned primarily with the medico-legal aspects of the harmful effects of the chemicals on humans and animals. The expertise of the forensic toxicologist is primarily invoked to assist in establishing the cause of death and elucidating its circumstances in a post-mortem investigation. *Clinical toxicology* designates an area of professional emphasis within the realm of medical science concerned with disease caused by or uniquely associated with toxic substances. Generally, clinical toxicologists are physicians who receive specialised training in emergency medicine and poison management. Efforts are directed at treating the patient poisoned with drugs or other chemicals and at the development of new techniques to treat these intoxications. *Environmental toxicology* focuses on the impacts of chemical pollutants found in the environment on biological organism.

There are many problems facing the clinical toxicologist in paediatric cases. One of the main problems is that only small blood samples are generally available, which is not enough for a full toxicological screen. Even in the event of fatalities, blood specimens recovered can be very small and tissue retention at autopsy is currently restricted.

Another problem facing clinicians is the wide use of illegal drugs by pregnant mothers, which is considered one of the serious problems facing the foetus as it causes severe morbidity and mortality, or may cause major congenital anomalies, affecting the foetus throughout the pregnancy and also the child after birth, causing physical, mental and behavioural disabilities. Proper clinical care of the neonate requires accurate, reliable and objective diagnostic tests to identify drug use by the mother, as self-reporting may be unreliable.

This thesis is concerned with some of the problems facing the paediatric toxicologist and the development of analytical toxicology procedures to help to overcome them. The aims can be stated as follows:

- to investigate and validate methods for the analysis of multiple drugs in small samples of blood, not exceeding 1ml, which is typically obtained for routine analysis from the newborn;
- to extend the applicability of the method to allow it to be applied to meconium samples from neonates, as this type of specimen is easily obtained, contains high concentrations of drugs and their metabolites and has a wide time window for detection of drugs;
- to apply the analytical procedures in an epidemiological survey of cases admitted to the Glasgow Royal Maternity Hospital, with the aim of knowing the true incidence of

drug abuse during pregnancy in the Glasgow area and the concentrations of drugs which are transmitted to the infants;

- to investigate alternative analytical procedures for the detection of cannabinoids and identify those which give the highest sensitivity i.e. the % of true positive cases identified.[3]

2. Review of Solid Phase Extraction and Analysis of Drugs from Biological Specimens:

2.1. Introduction

Reliable and reproducible isolation of toxicologically relevant compounds from biologically matrices is the first, the most important, and the most critical step in systematic toxicological analysis. Among different extraction methods currently used in analytical toxicology, solid phase extraction (SPE) has shown a dynamic development. Thus, various methodological and technical aspects of SPE have been comprehensively studied[4].

The use of SPE columns as an alternative to liquid-liquid extraction for the isolation of drugs started in the mid-1970's and gained popularity over recent years because of the reported excellent recoveries and ease of operation. Urinary drug analysis, whether for post mortem forensic purposes, doping control, pre-employment screening, or clinical purposes, is an expanding area of commercial interest for which such rapid, reproducible, analytical techniques are of great value[5].

SPE is widely used in forensic toxicological analysis, for both dedicated and general unknown procedures, mainly in the treatment of biological fluids of low viscosity, like urine or serum. It has also found application in the isolation of drugs from various other biological tissues and gives high purity biological extracts in comparison with liquid-liquid extraction (LLE)[6]. SPE has many advantages over LLE, which has many complications and disadvantages: for example, LLE needs more time and money spent on method development. The most common complications of LLE are that the extracts obtained are not clean and contain many impurities, the method can be non-reproducible, it can involve several sample handling steps, and can give low recoveries and sensitivities.

By contrast, the advantages of SPE are that it can result in higher sensitivity of detection; it produces clean extracts; as the analytes are retained and removed by elution, it is more reducible as it depends on specific molecular interactions. As a result, SPE is used for a wide range of drug analyses needed for systematic toxicology, and where there is a need for higher throughput due to automation. SPE extracts often cause less contamination of GC systems because they contain less extraneous material. Other advantages include elimination of time-consuming centrifugation and filtration steps and elimination of sample loss through emulsion formation[5].

For these reasons, SPE is considered a powerful technique for the pre-treatment of biological samples for clinical and toxicological drug analysis.

2.2. Characteristics of SPE:

The extraction procedure in SPE involves the interaction between a solid and a liquid phase, and is a physical extraction process, similar to liquid chromatography[7]. However, the aims of solid phase extraction are to isolate and then concentrate the compounds of interest, with good peak shape and height, and short retention times. The optimisation of extraction conditions by SPE depends on the properties of all three interacting factors - analyte, sorbent and eluant – which is the subject of numerous studies.

There are many different types of sorbents available: diatomaceous earth, silica, and bonded silica sorbents, in which there are a number of different types of column packing materials commercially available for drug extraction. These sorbents can be divided into three classes - non-polar, polar and ion exchange - according to the nature of the interactions between isolates, sorbents and solvents.

The majority of solid phase methods for the extraction of drugs from body fluids, which have been reported, involves the use of columns packed with non-polar adsorbent

materials. These consist of silica to the surface of which are bonded long hydrocarbon chains, principally C8 or C18, producing an essentially non-polar adsorbent phase similar to that present in a reversed phase HPLC column[8]. Most drugs have been extracted using non-polar interactions, using C8 or C18 bonded silica columns[9], and, although polar sorbents are more selective than non-polar sorbents, they are less commonly used[10,11]. Ion-exchange cartridges are potentially the most useful from a drug screening point of view. Several researchers have carried out extraction efficiency comparisons between the various types of sorbents available. The use of a granular support material as a column packing has been compared with XAD-2 resin and conventional solvent extraction methods for various drugs, the diatomaceous earth material giving superior results in terms of drug recovery and solvent volumes required[12].

Therefore, a solid phase extraction approach to sample preparation using bonded sorbents, as well as providing more selective screening methods for drugs liable to abuse, is rapid, simple, cheap, allows simultaneous extraction of multiple samples and is easily automated.

Recently developed cartridges contain both non-polar substituents and polar ion exchange substituents (mixed mode cartridges), which can be used for extraction of both acidic and basic drugs. Cartridges with either hydrophobic groups alone or with ion exchange groups alone can effectively bind only one type of substance. Taking into account that substances of toxicological interest can have acidic, neutral and basic properties and that preferably only one SPE column has to be used, a mixture of silica materials in one column has the desired selectivity potential. Such a mixed mode bonded silica, in which silanol groups are partially derivatised with medium length alkyl chains and partially with cation exchange substituents can exert at least two types of interactions. Screening procedures using this type of SPE material have been of increasing interest and SPE

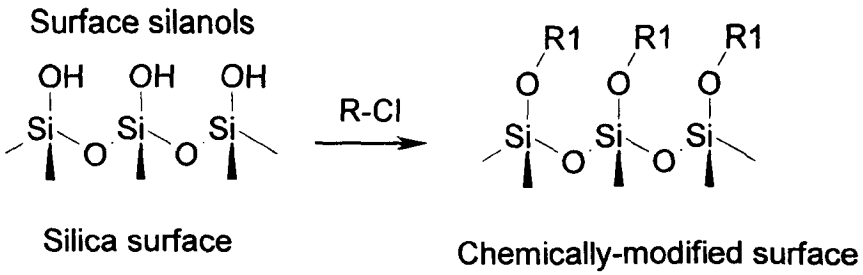
columns with mixed mode phases are now available from a number of manufacturers, for example Bond Elut Certify (Varian Sample preparation Products, Harbor City, CA, USA), Clean Screen DAU (World- Wide Monitoring Corp., Horsham, PA, USA), Isolute HCX (International Sorbent Technology, Hengoed, UK) and TSC (Merck, Darmstadt, Germany). Mixed-mode bonded silica can, at a suitable pH, retain acidic and neutral substances by hydrophobic interactions with the alkyl chains and retain the basic substances by interactions with the cation exchange groups. Differential elution can take place by a proper adjustment of the pH and the choice of solvents[13].

2.3. Sorbent Extraction Theory:

Sorbent extraction is a physical process that involves a liquid and a solid phase. The solid phase has a great potential for isolation of drugs. In theory, very selective extractions resulting in highly purified and concentrated isolates can then be achieved by choosing sorbents with an attraction for the isolate but not for the other sample components. The specific properties of a bonded cartridge are a result of the functional group covalently bonded to the silica substrate through reaction of the activated silica with organosilanes.

2.4. Synthesis:

Bonded silica is formed by the reaction of organosilanes with activated silica. The product is a sorbent with the functional group of the organosilane attached to the silica substrate through a silyl ether linkage. The intention is to create a surface whose principal properties are due only to the functional group, with minimum interactions from the silica substrate[7].



2.5. Physical Properties:

Bonded silica is a rigid material that does not shrink or swell in different solvents. This is a distinct advantage over polystyrene-based resins as a wide range of solvents can be employed for washing and eluting the isolates. The bonded sorbents equilibrate rapidly to new solvent conditions. The particles of the sorbent are usually irregularly shaped, nominally of diameter 50 microns, and allow rapid solvent flow through the sorbent bed. This does have certain disadvantages in terms of extraction reproducibility, particularly from batch to the same cartridges and certainly when cartridges are supplied by different manufacturers.

2.6. **Chemical Stability:**

The sorbents are stable within a pH range of 2 to 7.5. Above pH 7.5, the silica substrate is susceptible to dissolution in aqueous solutions and below pH 2, the silyl-ether linkage is labile. As a result, the functional groups bonded to the surface will begin to cleave, so altering the sorptive properties of the adsorbent bed. In practical terms, though, it is possible to use the beds over the whole pH range since degradation is a finite process and sorbents are usually only exposed to solvents for a short length of time.

2.7. Solvation and Conditioning:

To permit the sorbent to interact reproducibly with an isolate, the sorbent must be solvated and conditioned. This means, essentially, wetting the surface of the sorbent in order to create an environment suitable for analyte retention. Any solvent which will wet both the polar silica surface and the functional group can be used; most commonly methanol or acetonitrile is employed. The conditioning solvent must be miscible with the matrix of the isolate and the sorbent bed must not be allowed to dry out.

2.8. Retention and Elution:

Retention of the isolate is a function of the degree of attraction of a chemical species for the sorbent. Introducing a solvent to which the isolate is more strongly attracted than it is to the sorbent brings about elution. The eluant should elute the isolate from the bed in the smallest volume possible. Ammonia is used during SPE as better recovery rates are obtained than when sodium hydroxide is used[14].

2.9. Capacity and Selectivity:

The capacity of a given sorbent is defined as the total mass of a strongly retained isolate that can be retained by a given mass of the sorbent under optimum conditions. Capacities of silica vary, so it is necessary to consider capacity requirements of the isolate as well as any impurities likely to be present. Large columns may retain all the isolate but will then require large volumes of eluant to remove it.

Selectivity is the ability of the sorbent to discriminate between the isolate and all other sample matrix components. The selectivity is then a function of the chemical structure if the isolate, the properties of the sorbent and the composition of the sample matrix.

Maximum selectivity is achieved when a sorbent is chosen that interacts through functional groups common only to the isolate and not the other components of the matrix.

2.10. Analysis of Drugs in Biological Samples

There are many instrumental methods that have been used for drug analysis after the sample has been extracted by SPE. HPLC analysis of biological samples after SPE has focused primarily on plasma or serum[14-17], urine[18-20] and CSF[21]. These are all matrices which easily pass through SPE cartridges. Fewer methods have focused on the inherent problems associated with post mortem specimens, particularly blood, which is often haemolysed and degraded, frequently causing column blockage and reducing extraction efficiency and recovery.

Tebbett[22] first reported the extraction of morphine and codeine from whole blood. Subsequently a modification of Tebbett's method, which again involved sonification and dilution, incorporated the use of Bond Elut Certify™ columns (BECC). However neither method reported the isolation and detection of morphine conjugates[23].

Previous attempts to extract all types of drugs of abuse from a single aliquot of urine by SPE used lipophilic phases such as C18 or XAD-2, but these gave poor extraction efficiencies when selectively eluted, or else required matrix optimisation for each class, precluding a single extraction procedure. Many constituents present in the eluates interfered with the analysis when non-selective GC was used, but better results with clean extraction were obtained when GC-FID was used with a wide bore capillary column. The limit of detection was down to 100ng/ml, and recoveries averaged 93% for all drugs screened in urine over the concentration range 100-1000ng/ml[23, 24].

Recently, screening methods have been developed for acidic, neutral and basic drugs in urine using a single copolymer SPE column but this was not suitable for barbiturates.

However the use of Bond Elut Certify II gave good results. These cartridges contain a proprietary substituted silica sorbent that exhibits unique hydrophobic and ion-exchange extraction mechanisms and were first used to separate some drugs of abuse from urine (amphetamines, barbiturates and opiates)[17].

Determination of morphine in human blood is important for forensic toxicology, because of its frequent misuse. Also, it is one of the main metabolites of heroin, which has now become one of the most popular illicit drugs. Morphine is an amphoteric drug that may exist in different forms depending on the pH of the medium. There are several methods for the determination of morphine by GC and most of them need derivatisation.

BECC's contain a mixed mode bonded silica gel, which consists of hydrophilic and cation exchange functional groups. The columns have been successfully applied for the extraction of various classes of drugs from plasma and urine as well as whole blood[25].

In a subsequent chapter the use of BECC for extracting drugs from blood is described which, as mentioned, is one of the mixed mode columns used for extraction of acidic, neutral, and basic drugs in one run. The validation of the method of extracting multiple drugs from each group by this column from small (1 ml) blood samples is also described.

2.11. Components of Blood:

Blood consists of a pale-yellow, coaguable fluid called plasma, which contains suspended various blood cells including erythrocytes, granulocytes, monocytes, lymphocytes, and platelets. Blood plasma contains 9% of solids, 4/5 consisting of different proteins which are of three types - fibrinogen, albumin, and globulin – which are separated by different techniques including salt precipitation, electrophoresis, and ultracentrifugation.

The erythrocytes are considered to be slightly different cells compared to others, as the erythrocyte contains no nucleus or cytoplasmic organelles, and are responsible for the red colour of blood although they are pale reddish yellow in transmitted light. Their function transforming O_2, therby is giving energy and protecting against oxidising agents[26]. The relatively short life of erythrocytes is 120 days.

Granulocytes contain cytoplasmic granules and a segmented nucleus, with two masses connected by nuclear chromatin, These are highly mobile cells, whose function is to move towards infected sites and attack micro-organisms and subsequently envelope them by phagocytosis. They consist of three types - neutrophiles, eosinophiles and basophiles. Both eosinophiles and basophiles have phagocytic activity, but the phagocytic activity of eosinophiles is slower than that of basophiles. Eosinophiles, for example, have a diameter of 12-17μm

Lymphocytes are small cells present in the blood which range in size from 7-12μm, and which can be subdivided into two major groups: B-lymphocytes (B-cells) and T-lymphocytes (T-cells), both of which are different in origin and function. T-lymphocytes are involved in cellular immunity (e.g. against viruses, fungi and low grade intracellular pathogens such as mycobacteria) and so have a role in graft rejection, tumour rejection, delayed hypersensitivity, interaction with B-cells in production of antibodies against certain antigens, and suppression of B-cell function with production of eosinophiles. B-cells mature into plasma cells for the production of antibodies as immunoglobulins.

Platelets are small fragments of megakaryocytic cytoplasm, whose function is to produce energy, which are derived from the metabolism of glucose by the glycolytic pathway and tricarboxylic acid cycle. The energy is held as ATP within a metabolic pool that is

distinct from the storage pool of adenine nucleotides situated in the dense bodies. Platelets also play an essential role in the haemostatic mechanism.

Blood is a complex mixture of cellular entities suspended in a fluid medium. Blood may change in its chemical rather than anatomical composition, for example, changes in pH at any given point vary considerably, as does the oxygen tension. Finally, to make the situation more complex, there is the fact that the blood is only one-half of the circulatory system, the other half being the blood vessels[27].

For foetal blood, and amniotic fluid discussed later, there have been several reports offering information on the concentrations of drugs and/or their metabolites in this fluid[28, 29]. Drug 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[30]. In this manner it was determined that some drugs reach a concentration equilibrium in foetal and maternal blood[31, 32] whereas others are not readily transferred through the placenta[33].

3. Investigation of Solid Phase Extraction of Multiple Drugs from Small Blood Samples

3.1. Introduction

The aims of this investigation were to establish the feasibility of performing a full drug screen on a small sample of blood such as that obtained from a neonate or infant, typically having a volume of 1 millilitre. Most individual analyses in routine toxicology practice use a 1-millilitre aliquot of blood so for 6 analyses, single replicate only, a total volume of 6 millilitres is needed.

Three types of approach can be considered. In the first, smaller aliquots than usual of the blood sample are analysed for specific drugs or drug groups. This implies that the analytical techniques used must be more sensitive, in terms of limits of detection and quantification. Analytical instruments continue to develop with respect to their inherent sensitivity but there is no likelihood of an imminent quantum leap in sensitivity in sight at present. As an alternative, a larger percentage of the extract must be used for each test than is used routinely (typically, 2% of an extract is used for each gas chromatography analysis). If 0.1 ml blood is extracted, 20% of the extract must be used for each gas chromatography run in order to obtain the required sensitivity. This is made possible by injecting large sample volumes, using an appropriate inlet such as a programmed temperature vaporiser[34].

The second approach is to use a microextraction technique such as solid phase microextraction (SPME) or liquid micro-drop extraction. In these techniques, a 1 millilitre aliquot of biological specimen is used but the extraction technique takes out only 1-2% of the total analyte present and all of this extracted analyte is used in a single chromatographic run[35,36].

The third approach is to use a 1 millilitre aliquot of the biological specimen and extract all of the analytes of interest from this one aliquot, i.e. take several extracts from the same specimen. Some previous studies have used this approach for routine forensic toxicology, typically giving extracts containing acidic, basic or neutral drugs, respectively[36]. This approach readily lends itself to solid phase extraction techniques, which is the method adopted in the present study.

In this study, the previously-published method of Chen et al[37]. was adapted for use and evaluated using blood standards containing representative drugs from different drug groups, shown in Table 3.1.

Table 3.1. Drugs used for Method Development

Drug Group	Representative Drug(s)
Acidic Drugs	Amylobarbitone Barbitone Butbarbitone Methaqualone Phenytoin Primidone
Basic drugs	Amphetamine Cocaine Codeine Imipramine Methamphetamine Morphine Procaine Clonazepam Diazepam Nitrazepam

3.2. Experimental Section

3.2.1. Materials:

All chemical standards were purchased from Sigma-Aldrich (Dorset, UK). The solvents and concentrated ammonia were of HPLC grade supplied from BDH laboratories supplies

and Bond Elut Certify® columns with the capacity of 12x75mm test tubes were obtained from Varian (Harbor City, California, USA), and Abselut NEXUS columns were from Varian-Chrompack, Netherland BV Company (Herculesweg, Middelburg, The Netherlands). A Baker-20 SPE vacuum manifold system was obtained from International Sorbent Technology. Bistrimethylsilyltrifluoroacetamide (BSTFA) containing 1% by volume trimethylchlorosilane (TMCS) as catalyst was obtained from Pierce-Warriner (UK). Pentafluoropropionic anhydride (PFPA) was obtained from Sigma-Aldrich UK.

Phosphate buffer (0.1M) was prepared by dissolving 6.81g of potassium dihydrogen phosphate in 450ml of deionised water, adjusting the pH to 6.0 ± 0.1 with 1.0M potassium hydroxide, then making the total volume up to 500ml with de-ionised water.

A 0.01M acetic acid solution was prepared by adding 57.5 µL of glacial acetic acid to 100ml of de-ionised water.

Eluant (I) was prepared daily by mixing Chloroform to Acetone in the ratio 1:1v/v. Ammoniated ethyl acetate (2%) was used as elute (II) and prepared daily by adding 1ml of ammonia to 49ml of ethyl acetate. Both were sonicated for at least 5 minutes before use.

The Internal standard for GC was prepared by dissolving the appropriate amount of prazepam in methanol/ ethyl acetate (1:1) to make the solution equivalent to 200 µg/ml. Internal standards for GC/MS were deuterated morphine, cocaine, and benzoylecgonine purchased from Sigma-Aldrich, and deuterated methanol which was purchased from Radian Corporation (Austin, TX).

Instrumental enzyme immunoassays were carried out using kits from STC Technologies,Inc. (Bethlehem, Pennsylvania).

3.2.2. Instrumentation

(a) Gas chromatographic analyses were performed with a Hewlett-Packard Model 5890 Series II gas chromatograph equipped with a flame ionisation detector (FID, temperature: 300°C). Helium (10ml/min) was the carrier gas. The analytical column was a Hewlett-Packard (HP1 fused silica capillary column, 30m x 0.53 mm id x 1.5μm film thickness). A 1.0 μl aliquot of each sample was injected using a HP 7673 injector (injector temperature: 275°C) into the GC system described earlier. 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 then to 285°C at 5°C/min. The temperature was 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 peak areas of analytes to the internal standard with the aid of standard calibration curves similar to the one presented in Figure 3.1 below.

(b) Gas chromatography-mass spectrometry was carried out using a Hewlett-Packard 5890 Series II instrument connected to a mass spectrometer detector and equipped with a fused silica capillary column (HP5, 30m x 0.32 mm id, x 0.35 μm film thickness). Samples (volume 1μl) were injected *via* a split-splitless injector with an injection port temperature of 200°C and an initial split valve off time of 1 minute. Helium was used as the carrier gas at a flow rate of 1.5 ml/min. The initial column temperature was 200°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 1 minute. The mass spectrometer was a VG Analytical 70/250 S double-focussing mass spectrometer operated at a resolution of 1000 amu and data were recorded by a Maspec 1 data system. The spectrometer was used in the EI+ mode with an electron energy of 70 eV and source temperature 220°C. The instrument was tuned daily using

PFK according to the manufacturer's recommendations. Mass spectra were obtained by scanning from 600 to 40 amu at a scan rate of 0.3 s/scan.

3.2.3. Enzyme Immunoassay (EIA) Procedure:

The EIA method used was an Enzyme Linked Immunosorbent Assay (ELISA) from Standard Technology Company (STC). This assay involves the enzyme horseradish peroxidase, which produces a blue colour with its substrate.

All the reagents and samples were allowed to come to room temperature (15-30°C) before use. An aliquot of the extract after evaporation (200µl) was diluted 1:5 v/v in EIA buffer before use. All the samples, calibrators and controls were analysed in duplicate, using a 25 µl aliquot of each. For each drug assay, calibrators at four different concentrations were used, comprising a negative calibrator, one at the relevant cut-off value, and one each at medium and high concentrations. The calibration curve was checked before the results were accepted. After addition of the test sample (blood extract, calibrator or control) to the microplate well, enzyme conjugate (100 µl) was added and incubated for 30 minutes. Using a suitable plate washer, each well was washed six times with 300 µl of distilled water. Substrate Reagent (100 µl) was added to each well and incubation proceeded for 30 minutes at room temperature. Stopping reagent (100 µl) was then added to each well and the absorbency was measured within 15 min at dual wavelengths of 450 and 630 nm. All samples with an absorbance less than or equal to the Serum Cut-off Calibrator were considered to be positive, and if the absorbance was higher than that of the Cut-off calibrator they were considered to be negative.

Table 3.2: Cut-Off Values for EIA

Drug Group	Cut-Off (ng/ml)
Opiates	5
Methadone	25
Cocaine	10
Benzoylecgonine	10
Barbiturate	20

3.2.4. Solid Phase Extraction procedure:

The SPE procedure was evaluated using standard drug solutions prepared in blank blood and generally consisted of the steps described below.

(a) Sample preparation:

Aliquots of blood (1ml) in clean 20ml test tubes were spiked with different drugs representing the acidic (methaqualone, barbitone, amylobarbitone, phenytoin, butobarbitone and primidone), basic (morphine, cocaine, ecgonine methyl ester, diazepam and desmethyldiazepam) and neutral drug groups, at a concentration of 10μg/ml for each. Phosphate buffer (3ml, pH 6.0) was added to each tube, which was then vortex-mixed to make them homogeneous. An amendment to this procedure, in some cases, involved adding the phosphate buffer first to the tube before the blood specimen to assist in its dispersion.

(b) Column preparation:

SPE Columns (130 mg size) were used for the analyses. The columns were conditioned with 2 ml of methanol followed by 2 ml of 100 mM phosphate buffer (pH 6.0).

(c) Sample application:

The blood samples were then applied to the SPE columns at a rate of 1-2 ml/min.

(d) Column wash:

Following sample application, the columns were washed with 1 ml of de-ionised water followed by 0.5 ml 1.0 M acetic acid (pH 3.3). The columns were then dried for 4 minutes under full vacuum, then 50µl methanol was added under full vacuum to ensure complete removal of water.

(e) Analyte elution:

The analytes were eluted from the SPE columns with two eluants: one for acidic drugs, consisting of 1 x 4 ml acetone/chloroform (1: 1 v/v) and the second for basic drugs, consisting of 2 x 2 ml 2% ammoniated ethyl acetate, both at the rate of 1-2 ml/min.

(f) End step analysis:

Subsequently, the eluates were concentrated by evaporation under a stream of nitrogen on a hot plate set at 40°C. Extracts to be analysed by Enzyme Immunoassay (EIA) were evaporated until 400 µl remained. Extracts to be analysed by gas chromatography with a flame ionisation detection system (GC-FID) were evaporated under a stream of nitrogen at 40°C till 100µl remained, and for that analysis 100µl prazepam solution, previously prepared at a concentration of 200 µg/ml was used as internal standard. Extracts analysed by GC/MS were evaporated under nitrogen until 100µl remained then deuterated internal standards (cocaine d_3, benzoylecgonine-d_3, morphine d_3, and methadone-d_3,10ng of each) were added to each residue. The extracts for both GC and GC-MS were then derivatised with BSTFA catalysed with 1% TMCS for 20 minutes at 60°C. An additional evaluation was made of an alternative derivatising agent, pentafluoropropionic anhydride (PFPA) and these derivatives were compared with the trimethylsilyl derivatives with respect to their chromatographic properties and suitability for the developed method. For this procedure, the extracts, after evaporation, were dissolved in 100 µl of reagent containing

40% by volume of PFPA in ethyl acetate and then heated at 60°C for 30 minutes. After this time, the derivatised extracts were evaporated to dryness to remove the excess reagent and redissolved in 50 µl ethyl acetate prior to analysis.

3.2.5. Comparison of two types of SPE column:

A comparative study was made of two types of SPE column, Bond Elut Certify® and Abselut NEXUS®, for the simultaneous extraction of both acidic and basic drugs. This comparison was carried out using four test drugs only (methadone, morphine, cocaine and benzoylecgonine), by the same extraction method and the extracts were analysed by GC-MS as described above. The comparison between the two columns involved four aspects: recovery, lower limit of detection, cleanliness of the extracts, and analysis time for each. For measurement of recoveries, the internal standards were added after extraction and the analyte:internal standard ratios compared with those of unextracted standards.

3.2.6. Method Validation

Drug-free blood samples were spiked with different amounts of mixtures of drugs including barbiturates, cocaine, morphine, methadone, and ecgonine methyl ester and prazepam internal standard and analysed as described above. In this way, the limits of detection of the method and extraction recoveries were calculated for all the drug analytes of interest, which appear in figure 3.1. Identification was achieved by comparison of retention times (t_R) and quantification was based on the ratio of peak areas of analytes to the internal standard with the aid of standard calibration curves similar to the one presented in Figure 3.2 below.

3.2.7. Application to cases:

This method was applied to 12 cases resulting from the Road Traffic Act Section 4(which deals with cases of impaired ability to drive), each one consisting of blood not exceeding 1 ml (i.e. a small amount) and these were initially analysed as described before by EIA, screening for methadone, opiates, cocaine and benzoylecgonine as examples of basic drugs, and barbiturates as a representative acidic drug group.

3.3. Results and Discussion

The mixtures of acidic drugs (methaqualone, barbitone, amylobarbitone, phenytoin, butobarbitone and primidone), basic drugs (morphine, cocaine, ecgonine methyl ester, diazepam, and desmethyldiazepam) were extracted by SPE then analysed in sequence by EIA for four drug groups (cocaine, benzoylecgonine, morphine, and methadone) as example of common drugs found in some road accidents, by GC/FID, using prazepam as IS, then by GC/MS. The same methods were used for two types of SPE column, both of which were suitable for mixtures of basic and acidic drugs. The columns that gave good results were subsequently used for application to real road accident cases as an example of small samples (each one not exceeding 1 ml). For each instrumental method, the retention time for each drug was established, calibration curves were prepared, and lower limit of detection measured.

3.3.1. Gas Chromatography

The retention times for drugs obtained by analysing standards by GC-FID, are shown in Table 3.3.

Table 3.3 Retention times of drug standards in the GC-FID system.

Drug	*Retention time (min)*
Ecgonine methyl ester	**7.8**
Butobarbitone	**8.9**
Amylobarbitone	**9.2**
Methaqualone	**13.7**
Cocaine	**14.2**
Morphine	**14.5**
Primidone	**14.6**
Phenytoin	**16.0**
Diazepam	**17.5**
Desmethyldiazepam	**18.2**
Prazepam	**20.4**

The calculated recoveries based on analysis of spiked samples containing the representative drugs was as shown in Figure 3.3.

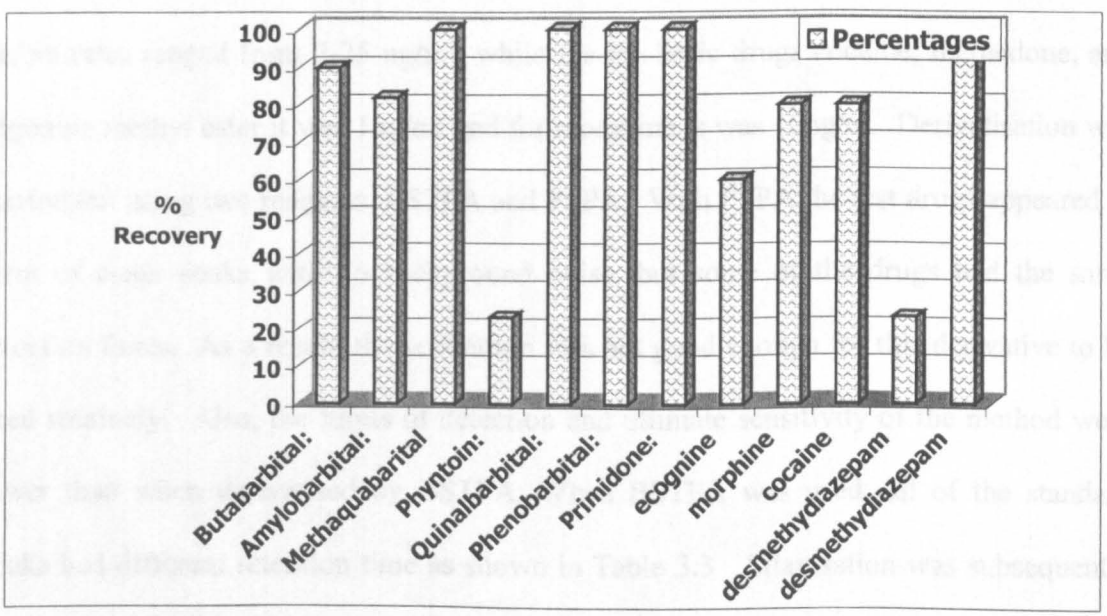


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Figure 3.1 Percentage recovery of drugs by SPE based on GC-FID analysis of extracts

Calibration curves for quantitative analyses were linear, with correlation coefficients (r^2) in the range 0.987-0.986 for all drugs. A typical example is shown in Figure 3.2.

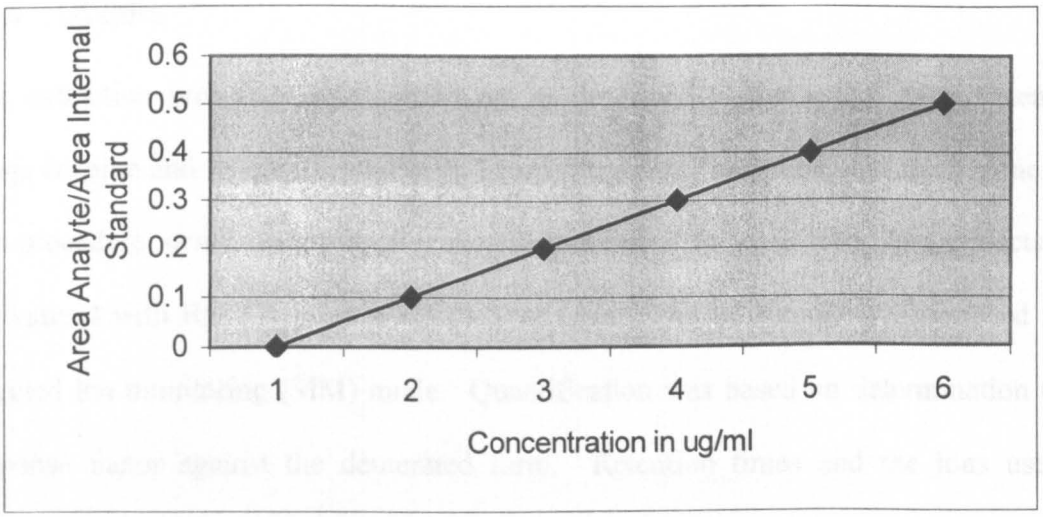


Figure 3.2 Typical calibration curve, in this example used for the quantification of cocaine in blood.

The lower limits of detection by this method varied from one drug to another so for barbiturates ranged from 2-25 ng/ml, while for the basic drugs cocaine, methadone, and ecgonine methyl ester it was 1ng/ml and for morphine is was 10ng/ul. Derivatisation was carried out using two reagents, BSTFA and PFPA. With PFPA the test drugs appeared in form of clean peaks with no background noise, but some of the drugs had the same retention times. As a result, the separation was not good enough for this derivative to be used routinely. Also, the limits of detection and ultimate sensitivity of the method were lower than when derivatised by BSTFA. When BSTFA was used, all of the standard peaks had different retention time as shown in Table 3.3 Silanisation was subsequently selected as the most appropriate procedure for the final method.

The combination of solid phase extraction with gas chromatographic separation proved to be a valuable one in which all the drug appeared with no significant interference either

with each other or with co-extracted materials and the eluates were clean extracts. This method was applied subsequently to other biological fluids.

3.3.2. GC/MS:

The extraction procedure was carried out as described earlier using, as representative drugs cocaine and its major metabolite benzoylecgonine, morphine and methadone. In this procedure, deuterated internal standards were used for each drug, and extracts were derivatised with BSTFA prior to GC/MS analysis. The instrument was operated in the selected ion monitoring (SIM) mode. Quantification was based on determination of the response factor against the deuterated form. Retention times and the ions used for quantification were as shown in Table 3.4. Calibration curves were also linear for GC/MS.

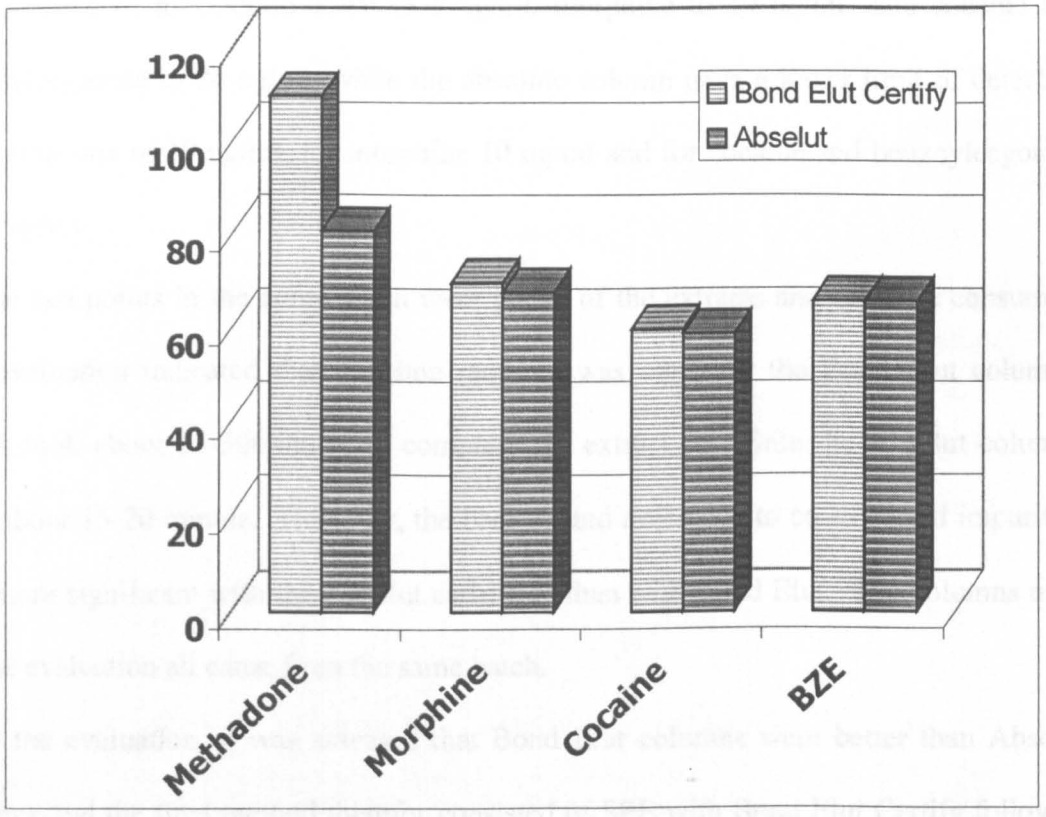
Table 3.4 Selected ions used for quantification and retention times for drug standards

drug	*Molecular ion*	*Retention time (min)*
cocaine	**303**	**7.2**
cocaine-d_3	**306**	**7.2**
benzoylecgonine	**361**	**8.2**
benzoylecgonine-d_3	**364**	**8.2**
morphine	**429**	**9.1**
morphine-d_3	**432**	**9.1**
methadone	**72**	**4.5**
methadone-d_3	**75**	**4.5**

2.5.1 Comparison between two columns:

GC/MS was used to compare two types of SPE column, both of which were suitable for simultaneous extraction of acidic and basic drugs, Bond Elut Certify as used and described above for the GC-FID procedure, and Abselut NEXUS columns. This comparison was carried for four drugs mentioned above, following the same extraction method and derivatisation using BSTFA before GC-MS analysis (using the instrumental conditions mentioned earlier). The comparison between the two columns involved four points (recovery, lower limit of detection, presence of interferences in the extracts and analysis time required by each). Each analysis was carried out in duplicate, in one set adding the IS before the extraction, and in the other adding the IS after the extraction. The former showed relative recoveries whereas the latter showed absolute recoveries.

The recoveries obtained were as shown in Figures 3.3 and 3.4. In general, lower recoveries, both relative and absolute were obtained with Abselut columns.



Figures 3.3 Recovery with IS added before the extraction.

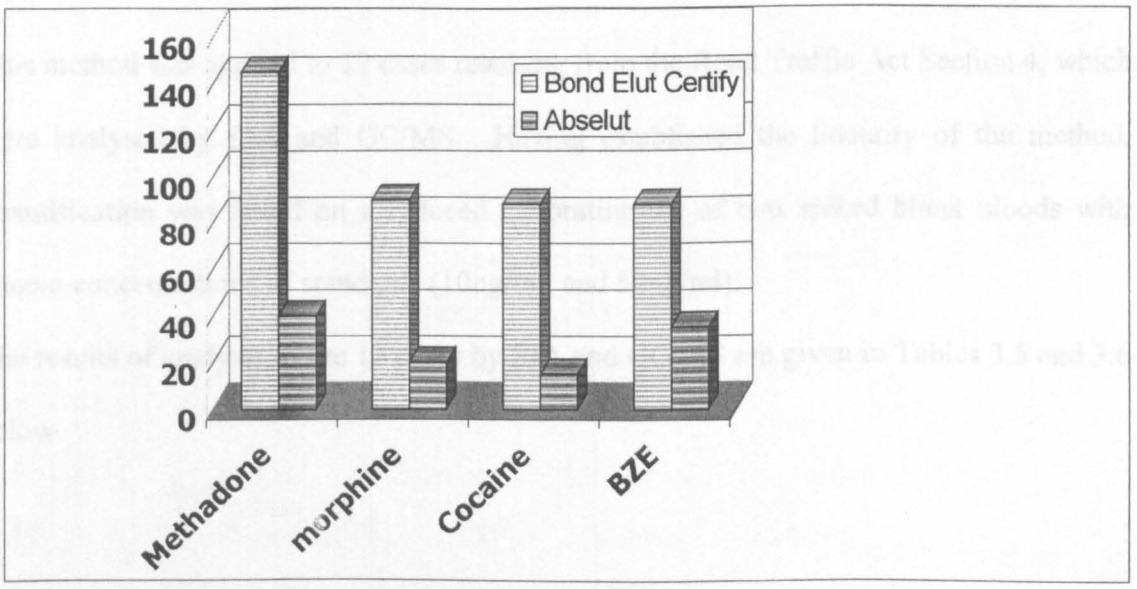


Figure 3.4 Recovery with I.S added after the extraction.

Comparison between the two column with respect to the lower limits of detection indicated that the Bond Elut Certify columns were generally more effective and permitted

the detection of methadone down to 1 ng/ml, morphine to 10 ng/ml and cocaine and benzoylecgonine to 20 ng/ml, while the absolute column gave a lower limit of detection for methadone of 10 ng/ml, for morphine 10 ng/ml and for cocaine and benzoylecgonine of 50 ng/ml.

The last points in the comparison were purity of the extracts and the time consumed. The evaluation indicated that the time required was more for the Bond Elut columns, which took about 25-30minutes to complete the extraction, while the Abselut columns took about 15-20 minute. However, the background noise due to co-extracted impurities was more significant with the Abselut cartridges than with Bond Elut. The columns used for the evaluation all came from the same batch.

From the evaluation, it was assessed that Bond Elut columns were better than Abselut columns and the final method therefor consisted of SPE with Bond Elut Certify followed by silanisation with BSTFA and analysis by GC/MS.

3.3.3. Application to real cases:

This method was applied to 12 cases resulting from the Road Traffic Act Section 4, which were analysed by EIA and GC/MS. Having established the linearity of the method, quantification was based on a reduced calibration set of two spiked blank bloods with known concentrations of standards (10ng/ml, and 50ng/ml).

The results of analysis of the 12 cases by EIA and GC/MS are given in Tables 3.5 and 3.6 below.

Table 3.5 Results of EIA analysis of 12 blood specimens from car drivers.

Case No	*Methadone*	*Opiate*	*Cocaine*	*Benzoylecgonine*	*Barbiturate*
1	negative	negative	negative	**positive**	**positive**
2	negative	negative	**positive**	**positive**	**positive**
3	negative	negative	negative	negative	**positive**
4	negative	negative	negative	negative	negative
5	negative	negative	**positive**	negative	negative
6	negative	negative	negative	negative	negative
7	negative	negative	negative	negative	negative
8	negative	negative	negative	negative	negative
9	**positive**	**positive**	**positive**	negative	negative
10	**positive**	**positive**	**positive**	negative	negative
11	negative	negative	**positive**	negative	negative
12	negative	negative	**positive**	negative	negative
Negative Control	negative	negative	negative	negative	negative

Table 3.6 Results of GC-MS analysis of 12 blood specimens from car drivers.

Cases	*Concentrations of detected drugs*			
	Methadone	*Morphine*	*Cocaine*	*Benzoylecgonine*
1	negative	negative	negative	89ng/ml
2	negative	negative	60ng/ml	120ng/ml
3	negative	negative	negative	negative
4	negative	negative	negative	negative
5	negative	negative	66ng/ml	negative
6	negative	negative	negative	negative
7	negative	negative	negative	negative
8	negative	negative	negative	negative
9	35ng/ml	215ng/ml	36ng/ml	negative
10	50ng/ml	125ng/ml	50ng/ml	negative
11	negative	negative	80ng/ml	negative
12	negative	negative	110ng/ml	negative
Control	negative	negative	negative	negative

3.4. Conclusions

SPE has been established as a useful technical procedure in analysis of mixture of drugs especially in small samples. It has been shown that analysis can be carried out using a single column, saving both time and expense, but at the same time giving good results and high sensitivity.

The survey of the current state-of-the art presented above clearly shows that the determination of drugs in blood can be carried out using small amounts of blood for both the analytic and confirmatory test, by EIA, GC/FID and GC/MS. This solves the problems which face the analyst when only limited material is available for testing, such as impairment testing in drivers, as well as in blood from the foetus or neonate. In the subsequent studies carried out for this project, these analyses were used to establish if mothers consumed drugs during pregnancy following further evaluation of the method to establish its applicability to meconium.

Subsequent Chapters review the use of illicit drugs with particular reference to the mother and foetus and then the biological specimens which have previously been used to assess maternal drug use during pregnancy.

4. Review of Illicit Drug Use

The work described in this thesis is largely concerned with the use and abuse of illicit drugs. Some background information on the pharmacology of these substances is relevant to setting the perspective by which the problems tackled during this project should be assessed and is presented below for three of the main groups of illicit drugs.

4.1. Cocaine

4.1.1. History

The world *coca* comes from *Aymara KhoKa* meaning *the tree*. Large quantities of cocaine and nicotine have been detected in 3,000- year old Egyptian mummies, although there is no known route by which coca might have been available in ancient Egypt.[38] It grows best on the moist, warm, slopes of mountains ranging from 1,500 to 5,000 feet, where the coca shrubs grow to heights of 6-8 feet. The trunk of the plant is covered by rough, somewhat glossy bark that has a reddish tint. It has greenish yellow leaves which are elliptical pointed at the apex and dark green in colour. The cocaine refining process is done by adding potassium permanganate to remove impurities, for example cinnamoylococaine is converted to ecgonine which is water soluble and easy to separate from cocaine. If the process is allowed to continue for too long, the cocaine itself is degraded and the yield drops. Norcocaine, which may be hepatotoxic, is formed at the same time.[39]

The first isolation of cocaine was in 1857 by Tschudi,[40] and many papers have been published on the effects of cocaine on humans. In the early 1880's, the Parke Davis[41] company began marketing a fluid extract containing 0.5 mg/ml of semipurified cocaine

and after that it was used in a variety of elements. Two events occurred in 1884 that significantly changed the pattern of cocaine use in the United States and Europe. The first was the publication of Freud's paper which discussed the benefits of cocaine use. The second was Koller's discovery that cocaine was a local anaesthetic, which first led to its use in opthalmological surgery, then to the treatment of morphine addiction and in 1870 to its use in haemorrhoid surgery and hay fever.[42]

4.1.2. Chemical Properties

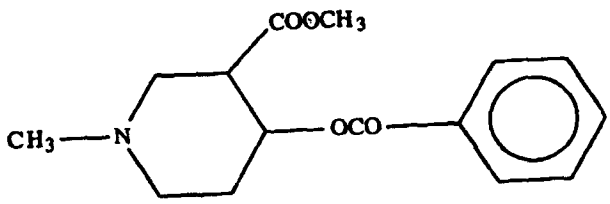


Figure 4.1 Chemical structure of cocaine

The free base has the formula $C_{17}H_{21}NO_4$ with a molecular weight of 303.4. The pure form has colourless crystals or white crystalline powder. It is odourless and has a bitter taste. Its melting point is 98°C. However it becomes volatile at 90°C. The pK_a at 15°C is 5.59[43]. It is soluble in water, acetone and ethyl acetate. The most important compound is cocaine hydrochloride, with a formula weight of 339.8. This has a slightly bitter taste. The melting point is 195°C and it is also soluble in chloroform, glycerol, and acetone. Solutions are stable for at least 21 days, provided the temperature is below 24°C and pH below 4.0.[44]

4.1.3. Routes of Administration

Cocaine is taken by many different routes: leaf chewing, snorting, injection and smoking. The average blood level is 38ng/ml one hour after administration by chewing. When snorted, the peak plasma concentration is proportional to the amount of cocaine ingested[45]

as it inhibits its own absorption but if taken in large amounts the time for absorption will be longer, but there is no difference between snorting and chewing. When taken by the intranasal route, the blood level is lower, especially in surgical applications (plastic surgery or otohumonagologists) as it is used in combination with epinephrine to maintain a dry field. The highest concentrations are obtained after intravenous administration, but this produces more complications, especially inflammation.

Genital or rectal application is used, especially in homosexuals and gives high blood level as it is highly absorbed by the transdermal route, hence its use in local anaesthesia.[46] The plasma level of cocaine in many children is found to be high due to the use of crack cocaine by their parents.(passive rout)

The last main route for absorption is by the gastrointestinal tract (GIT) and this is important in the body packer syndrome which is due to absorption of a large amount of packed cocaine after one or more packages is ruptured or leaked. These can be observed by abdominal X-radiography, CT scanning and barium contrast studies.[47]

4.1.4. Maternal / foetal Considerations

The effect of cocaine use by pregnant mothers is studied in animal models and it has been found that after 5 minutes of maternal infusion the foetal concentration reaches about 12% of that of the mother.[48] In the lactating mother, after 30 min-2 hour cocaine levels become 3-4 times higher in liver than in the blood; after 30-90 min. foetal brain concentrations were 50-90% those of the mothers and 1-5 times higher than blood; cocaine also appeared in the mother's milk. There is research on identification of cocaine and benzoylecgonine, along with cotinine and caffeine, in cord blood[49]

The human placenta has high affinity biniding sites for cocaine. In isolated perfused cotyledons of normal term human placenta, cocaine is passively, but rapidly transported without undergoing metabolic transformation. In the in-vitro model, the direction of transport depends entirely upon the relative concentrations of cocaine in the mother and foetus: when foetal levels are higher than maternal levels, equally rapid transport back across the placenta occurs. The results obtained with perfused human cotyledons suggest that the placenta may, under appropriate circumstances, act as a depot, initially absorbing, then slowly releasing, both cocaine and benzoylecgonine into foetal circulation. If the placenta acts as a buffer, then it may keep the infant from being exposed to large doses of cocaine taken as an IV bolus. On the other hand, storage and slow release by the placenta probably would ensure that the foetus is chronically exposed to low levels of drugs. The results of animal studies suggest that high maternal/ foetal cocaine ratios occur in humans as well as in animals. The ratio was 9/1: this high ratio may reflect cocaine-induced vasospasm and reduced flow to uterus.[50]

In cases of infant crib death it was found that the level of cocaine is higher in hair than in urine, and the amount found in foetus is due to cocaine metabolism in foetus as it cannot clear as quickly as the mother. Cocaine could be measured in saliva, vitreous, cerebrospinal fluid (CSF), breast milk, urine, amniotic fluid but gave the highest concentrations in meconium[51]

Neonatal exposure to cocaine, opiates and cannabis, can be detected in meconium by modified standard immunoassays. Comparing meconium to cord blood, it contains a greater number of cocaine metabolites than the blood, including norcocaine and benzoylecgonine. These same drugs can also be detected in hair and, if available, brain.[52] If a high concentration of benzoylecgonine can be detected with low or absent cocaine level, this suggests a pattern of long term exposure during pregnancy but no recent drug

abuse, and the opposite means recent exposure to cocaine. The presence of cocaethylene indicates that the mother was also consuming alcohol but as the cocaethylene has much longer half-life than cocaine, no cocaine may be detected, even though other cocaine metabolites will be present.[53]

4.1.5. Metabolism:

Cocaine is rapidly cleared from the blood stream with a steady state volume of distribution of 2 L/Kg. Elimination clearance is 2.0 L/minute and the half-life varies from 0.5-1.5 hours, all these measurements being done in volunteers. It may differ in that cocaine is stored, with changes in its rate of excretion, when cocaine consumption is measure in gram quantities, as in crack cocaine users, with small amounts of cocaine being excreted in urine.

In the absence of alcohol, benzoylecgonine (BEG) and ecgonine methyl ester (EME) are considered the principal breakdown products. EME is formed by hepatic esterase and plasma cholinesterase, but if these enzymes are deficient, cocaine is shunted via the BEG route. There is evidence that the cholinesterase level affects the toxicity of cocaine. The half lives of BEG and EME are much longer than that of cocaine, equalling 6 and 4 hours respectively. Benzoylecgonine can be detected in plasma for 24 hours but this differs according to route of administration. BEG is stable for as long as 3 years in urine samples with pH in the region of 3.5, but if increased to pH 9, 100% will have disappeared in 30 days.[54]

Neither of the metabolites is pharmacologically active, although results have shown that benzoylecgonine is cytotoxic and causes spasm of coronary and /or cerebral vessels. Spasm induced by cocaine is due to its adrenergic effect even although benzoylecgonine

is not an adrenergic agent, and the possibility exists that it causes spasm through other mechanisms such as calcium flux and calcium channel regulation.[55]

4.1.6. Foetal Metabolism:

The human placenta plays a role in protecting the foetus as it has cholinesterase activity and metabolises cocaine, possibly affording the foetus a degree of protection. In animal studies, newborns develop higher blood levels than adults receiving the same dose, and their plasma and tissue levels decline. Infants born to cocaine using mothers may have persistently elevated cocaine levels for days. However, there is a causal relationship between persistently elevated levels and prenatal stroke or intraventricular haemorrhage. The reverse occurs with its metabolites, as benzoylecgonine, which increases its activity in pregnancy.[56] The mother clears the metabolites much more quickly than the foetus. In a recent study by Meeker and Reynolds (1990)[57] it was found that the metabolite level in the mother is about 20-30 times higher than in the foetus, but there is low or no ecgonine methyl ester as the enzymatic pathway required to form this is poorly developed.

4.1.7. Pharmacological Effects

The effects vary according to the level in blood, so if it is rising it causes euphoria and mood elevation and if it increases above 5,000 ng/ml it causes seizure, respiratory depression or even death. In chronic users, a decrease in its level causes dysphoria. However, there is no level that can be considered either safe or toxic.[58]

Cocaine is lipophilic so it easily crosses the blood brain barrier giving high concentrations in the CNS, which has a high density of cocaine receptors in striatum. Cocaine, like some drugs of abuse causes its effects through disturbance of catecholamine systems (cocaine is a potent sympathomimetic agent). These also produce most of the complications as there are two types of receptor: B_1 receptors are found in the heart and when these receptors are

stimulated, another G-protein is activated and adenyl cyclase activity is increased. The results include increase in heart rate, contractility, and conduction velocity. B_2 receptors are found on bronchi and smooth muscle, and also exert their effects by activating a G-protein and adenyl cyclase. Stimulation of the B_2 receptors causes dilation of bronchi and blood vessels. When the receptors on vessels are stimulated, adenyl cyclase is inhibited, sympathetic flow increases catecholamines, leading to increased alfa-adrenergic stimulation of coronary vascular smooth muscle, leading in turn to vasoconstriction and ischaemia.[59] The oxygen demand is increased when cocaine is used, with changes in membrane potentials, which causes malignant ventricular arrhythmia. In chronic users, sudden death may occur. The concentration of cocaine increases in the heart 2-3 minutes after injection, causing changes in myocardium to produce contraction and necrosis.

Cocaine causes perforated septum, crack keratinitis (anaesthesia of the cornea also), erosion of the enamel of the upper front teeth (due to the teeth being bathed with acidic cocaine hydrochloride), and crack thumb, described in 1990. Crack smokers produce burns to the thumb with the serrated wheel of a lighter or torch, which present on the ulnar aspect of the thumb, and also contact with the heated crack pipe causes superficial burns on the palmer aspect of the hands.[60] Chronic users display blackened hyperkeratotic lesions on the palmer aspect of the hands, bite marks on the lips and tongue and also experience seizure activity as a terminal event.

Subcutaneous drug injection is more common amongst heroin than cocaine users, and cocaine users who elect to use this route generally experience fewer complications in the form of cellulitis, lymphangitis, lymphadenopathy, and oral infections which may required treatment with antibiotics or with severe cases surgery is required. Scleroderma is an uncommon disease.[61] Scleroderma and cocaine abuse share certain common

features. Vascular abnormalities are common in both, particularly in the heart, especially myocardial infarction.[62]

4.1.8. Cocaine related Deaths

The first case of death due to cardiac arrest was reported in 1986 and in 1987, 50 cases of cocaine toxicity were reported, four of which were fatal.[63] In 1994, the DAWN survey reported 3,981 but in the 1992 report, 3.220 and in late 1994 6,500 comprising 75% men, 56% in whites, 30% black, 12% Hispanics, 45% ruled accidental, 22.4% suicides 46% unknown in 26-34 year age group, 53% due to cocaine while in age 35.44 (49%).[58] In Scotland a recent survey of cases of drug related deaths no cases were determined as cocaine deaths in 1998, and around 1% cases in 1999, and no cases again had cocaine as the cause of death, but cocaine was recorded in 2% as a contributory cause along side other drugs in 2000.

4.2. Methadone

4.2.1. Introduction:

The discovery of methadone has been attributed to two German scientists, Max Bockmuhl and Gustav Ehrhart. The drug was first created in 1937 and was initially given the name Hoechst 10820 that was later changed to Polamidon.[64] The patent application for methadone was filed in September 1941. Although the structure of the new compound had no resemblance to morphine, its analgesic and spasmolytic properties were ascertained in 1942 when it was handed over to the military under the code name Amidon. Since then its use has not become very wide as it has significant side effect, but it was discovered that the side effects are due to high dosage.[65]

In 1965, it was introduced as a maintenance drug for heroin addicts. Methadone enjoyed little popularity, probably because in some individuals, its half-life can exceed 50 hours.

Today, it is the drug of choice for the treatment of heroin addiction and is frequently prescribed to cancer patients. It has been suggested that it may even normalise, a depressed immune function in heroin addicts, but its use must be controlled especially as it also faces big problems of addiction.[66] Despite this, early advertisements in the UK claimed little chance of addiction, and the general consensus was that it was better analgesic than morphine.

Home Office statistics on the numbers of methadone users found only 21 methadone addicts in 1955. By 1960 the number had risen to 60 cases, then in 1968 with the introduction of the Home Office Notification System, 297 methadone addict were registered, which in turn increased to 1687 cases in 1969 with the introduction of new drug clinics.

At present in the UK, most Health Authorities have a methadone prescribing service of some kind. At the present more than half of methadone adduct are known to the Home Office because methadone is a prescribed drug by law.

4.2.2. Prescription:

The principle use is for morphine/heroin addiction, but methadone is also used as a linctus for the treatment of coughing in terminal illness. It is prepared in form of liquid methadone, concentration 1mg/ml, which is the licensed medicine in the UK for treatment for of coughing in terminal illness and as a suppository, which is not preferred in the treatment of opiate addiction. The other form is a 5-mg tablet (Physeptone®). Most addicts prefer this as it is easily crushed and injected. The last form is injectable methadone, concentration 10mg/ml, used IV, IM or subcutaneously. The IV route is preferable to addicts as it produces a rapid effect.

4.2.3. Chemical Properties

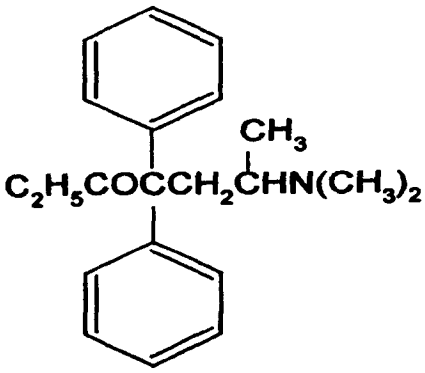


Figure 4.2 Chemical Structure of Methadone ($C_{12}H_{27}NO$)

Methadone (6-dimethylamino-4,4-diphenyl-3-heptanone) has a complex structure with a wide range of effects[67]. It was found that methadone has two active sites in its structure - the nitrogen atom and the phenyl groups. Its effect is principally on the peripheral nervous system and due to the two phenyl rings produces opiate-like actions and on the central nervous system, hence its use in opiate addiction to control withdrawal symptoms and relief of distress, but it may cause anxiety, and some psychological discomfort.

4.2.4. Absorption and Metabolism

Methadone is taken either orally or by injection. It is absorbed rapidly when taken orally the peak blood concentration being reached after 15-30 minutes. The volume of distribution is quite large, with high concentrations of the drug being found in the liver, lungs, kidney and spleen. The clearance rate is slow, but this is affected when taken with other drugs, which may increase toxicity, and with alcohol, which increases its metabolism leading to withdrawal symptoms, and with cocaine, leading to an increase in its excretion, so to decreased plasma levels.[68]

Methadone has two major metabolites, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrrolidine (EMDP), both of which are inactive. There are other minor pathways including hydroxylation to methadol, and N-demethylation to normethadol: there are more than eight metabolites known.

4.2.5. Pharmacokinetics:

The methadone causes development of tolerance in individuals with increases in the dose until it causes death, sometimes due to respiratory depression with increased level in plasma, and when the level decreases below 50ng/ml this leads to withdrawal symptoms. The main route of excretion is via the kidney into urine, mostly within 24 hours, with up to 33%, as unchanged methadone, 43% EDDP and 5-10% EMDP. The half-life ranges from 12-25 hours, although this changes when taken as a single dose or multiple dose.[69] The LD50 for methadone is 50mg/Kg, however tolerance may appear slowly compared to other opiates. Other pharmacokinetic data are: volume of distribution 5 L/Kg, clearance rate 2 ml plasma /min/Kg, plasma to whole blood distribution is 1:3, with 90% bound to protein.[67]

4.2.6. Legal Status

The Misuse of Drugs Act put methadone (Physeptone) under Schedule 2 as it is considered a Class A drug. This means that it is illegal to possess methadone without a prescription or other authority.

4.2.7. Methods of Analysis of Methadone

There is a method for the detection of methadone in the neonate, which will be mentioned. There are many methods used for its analysis in adults, but the more specific method is by GC/MS using SIM[70] also use GC/FID[71] extracted by L.L.E from plasma and urine, and from blood urine stomach content, bile and other tissue. Other use GC/ECD[72]

detects it from serum or extracted from plasma and urine by GC/MS[73] or use GC/NPD[74] All this method extract sample first by L.L.E, also SPE with LC-MS used by Verweij, et al 1996 [75] or followed by HPLC [76] and CE [77] from serum, urine, and hair.

4.2.8. Maternal/Foetal Considerations:

Maternal methadone levels correlate significantly with neonatal plasma methadone and metabolite levels at least during the first day of life. There are symptoms of CNS withdrawal which appear with decreasing infant plasma methadone levels. Measurement of the methadone level 16 hours after delivery found a maternal concentrations of 183ng/ml while in the infant it was 26ng/ml. Methadone levels decrease in the infant at the average rate of 0.2± 0.3ng/ml hour.[78]

4.2.9. Methadone Related deaths

There are many cases of death due to methadone reported in the 1994 DAWN survey, which reported 367 methadone related deaths, which represent 4.1% of all reported narcotic deaths in this year. This was a decrease percentage of death gradually declining from 7.5% in 1990, the cause of this is not known but may be due to an increase in the total number of methadone maintenance programmes.[79]

The autopsy findings often include bronchopneumonia, since the most common cause of death is respiratory depression. Chronic persistent hepatitis is a frequent finding in methadone related deaths. Inflammatory infiltrates, without evidence of necrosis, are commonly seen in the portal triads of heroin abusers. Opiate abusers enrolled in methadone programs are very likely to be infected with hepatitis C, with or without histological changes.[80] In Scotland in the last three years it was found that the proportion of methadone deaths is around 3% in 1998, and 1999, while it decreased to 1% in 2000.

However, methadone was also present in cases involving other drugs - around 10% in 1998, and 14% in 1999 and 2000.

4.2.10. Tissue Concentrations

Methadone tends to concentrate in the liver, but often blood levels equal or exceed hepatic levels when measured at autopsy.

4.3. Opiates

4.3.1. Introduction

The opiates are a group of drugs derived from the opium poppy (*papaver somniferum*).[81] Opium poppies can be seen on coins and in drawings that antedate written mention in the Greek literature by at least 1,000 years.[82] In Greece the poppy was called *opion*. The term was derived from the word for juice (*opos*). Translated into Latin, opion becomes opium. It was used for sleep, occasionally everlasting. The cup given to Socrates contained the standard solution used at the time for purposes of euthanasia and suicide, a mixture of hemlock and opium, also used as analgesics dates back as early as 7th century BC in the Assyrian medical tablets.[83]

Opium was used after that in medicine in Europe, then in 1868 when the first pharmacy act became law its use was restricted. In addition, after World War I, Britain implemented an international agreement to prohibit non-medical use of opiates due to the large number of post war addicts.[84] Opium poppies contain morphine and codeine, both used as pain killers. C.R. Wright, a researcher at St. Mary's Hospital was the first to synthesise heroin (diamorphine) from morphine. At the beginning it was used for treatment of morphine addiction due to ignorance of its liability for abuse.[85] After that a number of synthetic opiates were manufactured as pain killers, including pethidine, dipipanone and methadone. These are often referred to as *opioids* to distinguish them

from substances that come directly from opium. Heroin is considered to be one of the major problems affecting health and a Home Office Notification system was set up in 1968. In 1993, the number of registered addicts was 27,976. This estimation depends on the number of persons who register with their GP and is an under estimation - the number of actual opiate addicts may be greater than 125,000.

4.3.2. Prescription:

The most important use for opiates is as painkillers, but they are also used as cough suppressants and anti-diarhoea agents.[86,87] Table 4.1 shows the opiates/opioids commonly prescribed and their uses and names. In the U.K. morphine is favored by most doctors for the treatment of severe pain.

Table 4.1 Opiate-Based Medicines

Name	Proprietary name(s)	Treatment indication
Alfentanil	Rapifen®	Pain
Buprenorphine	Temgesic®	Pain, premedication
Codeine	Diarrest, Cocodamol®	Anti-diarrhoea, pain
Dextromoramide	Palfium®	Pain
Dextropropoxyphene	Co-proxamol, Distalgesic®	Pain
Diamorphine		Pain (IV), cough
Dihydrocodeine	Df118 Forte®	Pain
Diphenoxylate	Lomotil, Tropergen®	Anti-diarrhoea
Dipipanone	Diconal®	Pain
Fentanyl	Durogesic®	Pain
Loperamide	Imodium, Loperagen®	Anti-diarrhoea
Meptazinol		Pain
Methadone	Physeptone®	Pain, cough
Morphine	MST Continus, Kaolin®, Oramorph SR®, Sevredol®	
Nalbuphine	Nubain®	Pain, premedication
Oxycodone		Pain
Papaveretum		Pain
Pethidine	Pamergan®	Pain
Phenazocine	Narphen®	Pain
Phenoperidine		Pain
Pholcodine	Copholco, galenphol®	Cough

Tramadol	Zydol SR®	Pain

4.3.3. Chemical Properties

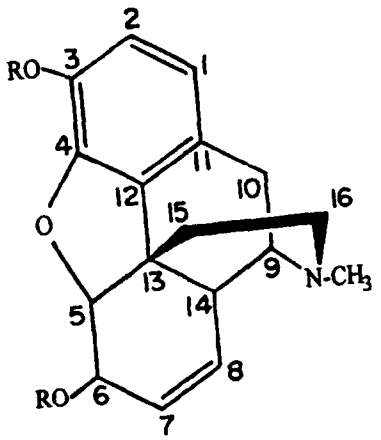


Figure 4.3 Chemical Structure of Opiates

Changing the group R1 (at position C3) and R2 (at C6) gives the structures of the three most important opiates: diamorphine, which contains COCH3 at R1 and R2; 6-monoacetyl morphine (6-MAM) which has COCH3 at R2 and H at R1; morphine in which H is present at both R1 and R2.[67]

4.3.4. Metabolism& Elimination:

(a) Morphine:

Once morphine enters the blood stream, it is quickly distributed throughout the body to the areas with the highest blood flow, so the highest levels can be measured in lung, kidney, liver, spleen and muscle.[88] Morphine also crosses the blood-brain and placental barriers. One third of morphine is protein bound and any change in this ratio (like in hepatic disease) causes an increase in the level of free morphine. Small amounts are excreted unchanged in the urine (10%) and about 70% is converted to glucuronide and

excreted through the kidney. Any disease affecting the kidney causes changes in elimination. Morphine enters the enterohepatic circulation and morphine excreted in bile had an average concentration of 312 mg/l.[89]

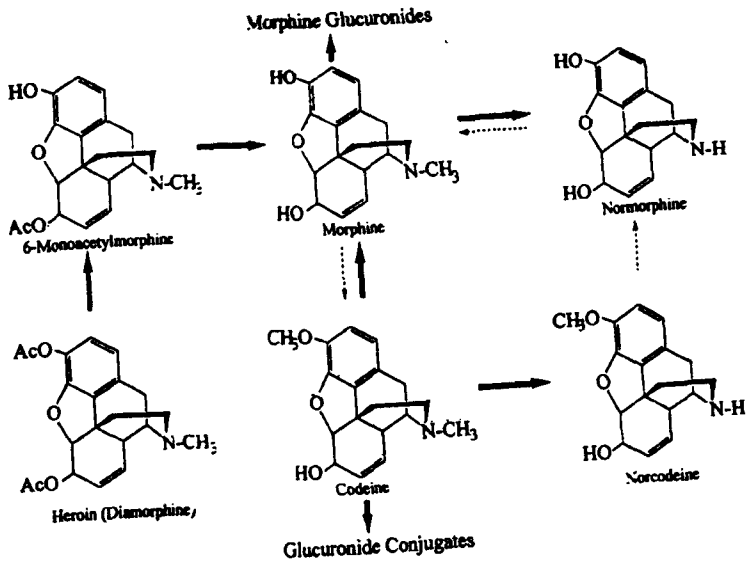


Figure 4.4 Metabolic Pathways of Opiates

Morphine is rapidly absorbed whatever the route of administration: if taken orally it is completely absorbed after 30-90 minutes, but the resulting plasma peak concentrations are only 10% of those obtained if taken IV. After absorption, morphine then undergoes metabolism in liver so its bioavailability is reduced. The degree to which morphine is subjected to extraction and metabolism by GIT is affected by many factors including age, liver disease, gender, food effects, disease state and genetic polymorphism. Because of these effects less than a quarter of a given dose enters the blood stream. The plasma levels after rectal administration are higher than with oral morphine, but less than IV levels. As morphine is not sufficiently fat soluble it is poorly absorbed through the skin in quantities sufficient to produce psychological effects, but other opioids, particularly fentanyl, sulfentanyl and meperidine are well absorbed.[90,91] Therapeutic plasma morphine concentrations are usually in the range of 0.01-0.07mg/l.

The elimination of morphine is best described as a biphasic process, in the first phase which take few minutes and rapidly disturbed through the tissues with the highest blood flow, transformed to its principal metabolite morphine 3-glucuronides which is done rapidly and small amount of M-6-glucuronides. The second phase take from one to eight hours, with two hours as the most widely accepted value, for the conversion of morphine, both metabolites are highly ionised and lipophilic so cross the blood brain barrier rapidly.[92]

(b) Diamorphine

Diamorphine is the active ingredient of heroin. It is rapidly absorbed and hydrolysed after about 3 minutes to 6-MAM in blood, then slowly to morphine. A small amount is converted to normorphine. All of the metabolites can be conjugated with glucuronic acid to form glucuronide derivatives. After intravenous administration of 4-5mg diamorphine the blood concentration declines to 0.01mg/l within 10 min. The estimated LD50 for diamorphine in man is 200mg. However, addicts can tolerate up to 10 times this amount and fatalities have been known after only 10mg. The peak heroin concentration in blood can occur within five minutes of intranasal or intramuscular administration. The mean elimination half life was 5-4 minutes after intranasal administration compared to 4-2 minutes after IM, and the elimination half life was longer for 6-MAM than for heroin when taken by inhalation and its peak declines within 30minutes of smoking.

Morphine and heroin are present also in saliva but saliva measurements must be taken with caution since the oral or intranasal use of these drugs may result in very high saliva levels due to contamination of the oral cavity.[93] A comparison between saliva, plasma, and urine disclosed that urine concentrations were 100 times greater than the levels in saliva and 16 times higher than in plasma. Heroin appears in saliva much more quickly

than morphine, but neither compound can be detected in saliva for much longer than 12 hours. The correlation between saliva and plasma levels of methadone appear to be excellent, and this drug can also be measured in CSF.[93,94]

4.3.5. Maternal/Foetal Considerations

It has been recognised for more than a century that morphine can be transmitted from the mother to her foetus through the placental barrier before birth and through breast milk after birth. Depending on their lipid solubility, narcotic agents passively diffuse across the placenta. In morphine users, the peak placenta levels were reached within a few minutes, and were half the maternal levels. After an hour, foetal levels were higher than those in the mother. The concentration in liver is quickly elevated, and quickly falls. Once the narcotic agents are taken up by the foetus, they are metabolised and excreted. They may be detected in amniotic fluid,[95] hair[96] and meconium.[97]

The results of animal studies suggest that morphine metabolites, chiefly morphine 3-glucuronide, enter the amniotic fluid more slowly than morphine itself[87]

4.3.6. Legal Status

The raw form of opium is not licensed for medicinal use and cannot be supplied, possessed or administered except in accordance with a Home Office Licence. The opiates are classified between class A (for morphine, heroin, methadone, fentanyl and pethidine) and Class B (for codeine and dihydrocodeine) except if taken by injection, when they are considered Class A drugs. Dextropropoxyphene and buprenorphine are Class C drugs. Dilute mixtures of codeine, morphine, opium are over-the-counter drugs used for many routine medical purposes such as coughs or diarrhoea[98].

4.3.7. Methods of Analysis of Morphine

Morphine can be analysed in many types of sample. Plasma is the most common one and most of the morphine is present in unconjugated form. About 30% is combined with plasma protein, but a lot of analyses are done using other biological samples as mentioned previously. Several instruments can be used for its detection including GC/MS, which is the most sensitive and specific method, especially when using SIM.[99] Also there are other methods used for determination of acetylcodeine, 6-MAM and other opiates in urine.[100].

Morphine can be detected by HPLC with UV detection[101] and by HPLC with elctrochemical detection[102] both of which do not require derivatisation. Many other methods which have been published used either GC[103] for extracts of morphine from blood and bile or GC-FID using blood and plasma[104], or blood, serum and saliva[105], or urine[106], or use SPE followed by HPLC for serum,[107] or more recently have used meconium, as mentioned previously.

4.3.8. Opiate Related Deaths

Medical examiners participating in the Federally sponsored DAWN program reported 5,876 opiate-related deaths in 1993, a 15% increase on the previous year, but this percentage has seen a sharp decrease since 1995, which may be due to methadone maintenance programmes, and this ratio has decreased till 1997. In Scotland the number of heroin-related deaths was at a maximum in 1999, but decreased in 2000. These deaths represent around 66% of all drug-related deaths in 1998, 95% in 1999 and 89% in all cases in which represent heroin intoxication, and heroin is one cause besides the presence of other drugs. Deaths due to morphine alone also maximised in 1999 then decreased slightly in 2000, representing about 13% of drug deaths in 1998 and 1999, decreasing to 10% in 2000.(unpuplished data- private communication).

5. Congenital Anomalies

5.1. Introduction

Substance abuse in women of childbearing age is a serious problem which affects the foetus prenatally and the neonate postnatally as it has long term effects, especially as it is often the case that these women are using multiple drugs (commonly involving alcohol, diamorphine and cocaine). The effects depend on the amount taken, type of drug abuse, gestation period during which they are used, duration and the method of use. Also, tolerance to opioids develops very rapidly in the foetus/neonate and has been demonstrated to occur within a few hours as it depends on the duration of opioid receptor occupancy. Therefore continuous administration of opioids may produce tolerance more rapidly than intermittent therapy.[108]

Congenital anomalies are the largest cause of infant mortality: the underlying factors are multiple, and include environmental, genetic mutifactors and idiopathic causes. Congenital anomalies also represent some of the serious complications which accompany drug abuse during pregnancy, as about 2-3% of malformations are caused by drugs and environmental chemicals, especially when exposure occurs during the organogenesis period (4-8th week of pregnancy) during which the embryo is most susceptible to factors interfering with development.[109]

The most common effect of opioids is neonatal abstinence syndrome (NAS), as these drugs crosses the placental barrier, causing definite behavioural and neurological effects in neonates and infants.[91,110]

5.2. Neonatal Abstinence Syndrome

This syndrome has special signs and symptoms and is common in neonates of pregnant addicts, using narcotics or on methadone maintenance. NAS varies from a condition displaying severe symptoms needing medical treatment to one with mild symptoms requiring observation only.[108]

NAS appears rapidly after birth if the mother herself suffers from withdrawal or may appear late if its cause is a drug with long elimination half life (for example, methadone or barbiturates). The duration of NAS depends on its severity, as acute cases last for 3-4 days and are then resolved by the 5-7th day after birth. In severe cases which require drug treatment, relapse occurs if treatment stops suddenly, as the signs disappear after 5-7 days of treatment but mild signs still continue for 2-4 months.[111]

The abstinence syndrome due to methadone appears in 70-90% of neonates in the first 3 days after birth, but in some cases may appear some days later (10-32nd day of life) due to slow clearance of methadone. NAS due to heroin addiction occurs in 50-80% of cases and lasts from a few days to several weeks. Also, there is no relationship between the amount taken by the mother and the severity of the syndrome.[112]

5.2.1. Common Drugs

NAS can be induced by all narcotics if taken during pregnancy. It is more prominent in morphine and heroin addicts. The use of methadone once daily for maintenance treatment of heroin addiction causes NAS as it has a long duration of action.[112]

The methadone has a low molecular weight, is lipophilic and is highly protein bound so it is excreted in bile and urine with only a small amount unchanged. Its level changes in pregnancy and it can pass easily through the placenta to the foetus, so it appears early, at the 14-16th week of gestation, with higher concentrations in plasma than in umbilical cord

or amniotic fluid. Excretion in urine along with its metabolites is greatest in the first days of life and then slowly decreases, with a time range from 7-21 days.[112]

5.2.2. Symptoms and Signs

Symptoms of CNS excitation such as irritability, jitteriness, coarse tremors, hypertonia, and myoclonic movement occur in 5-10% of these neonates. The EEG is normal except during seizures, and the neonates can display high pitched crying, seizures, hypothermia, hyperthermia and tachypnea.[112]

Respiratory distress, and vague autonomic symptoms, which include yawning, sneezing, sweating, stuffy nose, mottling, increased lacrimation, and fever, with tremor which start mild in the beginning, and occur when the infant is disturbed, but which progress to the point where they occur spontaneously without any stimulation, can also occur. High pitched cry, increase muscle tone and irritability develop with increased deep tendon reflexes and an exaggerated Moro reflex. The rooting reflex is exaggerated and neonates often suck frantically on their fists or thumbs.[108]

GIT disturbances arise in the form of incoordination in suckling, with vomiting, so the baby is always hungry and agitated, with loose stools. The baby can suffer from dehydration, low body weight and electrolyte imbalance, and may regurgitate frequently. Because of the uncoordinated and ineffectual suckling reflex, infants may develop loose stools, and may be susceptible to aspiration pneumonia, and respiratory alkalosis.[111]

5.2.3. Treatment

Treatment includes two main lines: general, and pharmacological. The general route depends on the severity of the symptoms, so in mild cases keeping the patient quite calm with no room light and with a decrease in movement is enough. If accompanied by hyperactivity, vomiting or diarrhoea, a follow up must be done by weighing the baby

every 8 hours, and supplying the baby with adequate caloric intake, especially in children with difficulty in suckling or swelling.[111]

The pharmacological route is initiated within 4-6 hours of birth for all infants who meet the scoring criteria for pharmacological intervention, and depends on drugs containing opium such as paregoric or tincture of opium, which control GIT symptoms with seizures or else involves non-opioids in the form of phenobarbital or benzodiazepines.[111]

A delay in initiation of an appropriate pharmacological intervention gives rise to a greater risk of increased morbidity [108]

5.3. Ocular Abnormalities

The development of the eye occurs in the gestation period from day 24 to 40. Any insults during this time result in ocular malformations.[109]

5.3.1. Development of the Eye

The eye develops in different steps. The appearance of early positive waves corresponds to the developmental of apical dendrites of pyramidal cells in the occipital cortex. These dendrites generate a summated excitatory postsynaptic potential through a highly synchronised afferent volley onto or near the soma of maturing deep lying pyramidal cells. This dendrite development continues over the first year of life and may contribute to the shortening of wave component latencies during the first months after birth. During the first few weeks after birth, the visual system is dominated by neurophysiologic activity from subcortical centres and with maturation during the first 2 months of life, significance synaplogenesis occurs.[113]

Intravenous drug abuse causes problems due to impurities in the drugs and can result in bilateral loss of vision due to particulate microembolisation to the retinal vessels, in a risk of HIV infection, and in an increased risk of acute bacterial and fungal endocarditis.[109]

5.3.2. Drugs Affecting the Eye

There are some drugs that have serious effects on the eye when taken during pregnancy which are summarised below.

(a) Lysergic Acid Diethylamide (LSD):

LSD is considered to be an hallucinogenic drug, inducing visual hallucinations and personality changes. It affects the offspring causing retinopathy, mydriasis and potential cycloplegia, leaving the pupil dilated and causing the retina to be more susceptible to retinal burns. This finding includes formal holes like lesions or a honeycombed macular region. In pregnancy it produces eye deformities, as it causes anterior subcapsular lens abnormalities which are pathologically similar to cataracts induced by x-rays. The lens epithelium is hyperplastic and the lens itself is widened posteriorly.

(b) Cannabis:

Cannabis is used along with other abused drugs and in some neonates causes chromosomal abnormalities that appear as breaks in the DNA strand and abnormal cells which is inheritable, with some destruction of the eye structure.

(c) Amphetamine;

Amphetamine produces some ocular abnormalities, such as microopthalmic eyes.

(d) Methadone:

A published study showed an increased incidence of strabismus in offspring of women on methadone maintenance, which increased with low birth babies and if large doses of methadone were taken, which may also cause nystagmus and ocular torticollis.

(e) Cocaine

Cocaine intoxication in neonates causes iris blood vessel abnormalities with dilation and tortosity of the vessels. It was found that these vascular anomalies occurred over 3-4 months without any loss of vision. Cocaine causes vasoconstriction, which presents in the premature infant as unilateral retinopathy, and funds abnormality when the mother used it during pregnancy.[109]

5.3.3. Drugs Affecting Power of Spectral Values

The effect of drugs on the eye of the neonate can known by measuring their electrophysiological responses in the brain: usually there are 2 types of electrophysiological waves: (P) for positive wave. (N) for negative wave, and it is measure in grades N1,N2, and N1 vision is better than N2.

If the latency is prolonged this means that the conduction is delyed, which means nerve damage, and the same if the amplitude is decreased. PS means near vision.

These substances produce different effects:

(a) Alcohol:

First trimester alcohol exposure results in significant prolongation of N1 and P1 latencies at 1 months of age, while in the 2nd and 3rd trimester it causes prolongation of the N2 wave at 4 months. Alcohol has also been correlated with an increase in N1-P1 amplitude (poor conduction through the optic nerve) at 4 months of age for both 2nd and 3rd trimester

use there was a decrease in N1 and P1 latencies at 18 months of age with the first trimester alcohol use.

(b) Cannabis:

The effects of cannabis are mostly related to 3rd trimester exposure, with little effect from first or second trimester exposure. Prolonged latency of P1 wave was found at 1 and 18 months with 3rd trimester exposure, however P1 latencies were shortened at age 8 with 3rd trimester use. There was also a decrease in N1-P1 amplitudes at 18 months with first trimester cannabis use

(c) Tobacco:

Tobacco is the only drug that has detectable effects at birth, as when it is used in the first trimester it gives prolonged N1 wave latencies at 4 months, prolonged P1 latencies at 18months and increased N1-P1 amplitudes at 18 months of age. Tobacco increases the latencies and amplitudes of N1-P1, but it is not possible to identify the relation between specific trimesters and effects because of high tobacco use across the trimesters[113].

5.3.4. Common Ocular Abnormalities with Drugs

(a) Micropthalmic eyes, with severe deficiency in visual function, which may extend to anopthalmia, especially if the parent uses multiple drugs like LSD, heroin and methadone.

(b) Anterior segment anomalies, including corneal opacities with vascularisation and shallow anterior chambers, which may develop into attacks of angle closure glaucoma.

(c) Iris colobomata, which is associated with colobmata of other structures of the lens and optic nerve.

(d) Lens abnormalities, including cortical degeneration with thickened capsules.

Retinal changes, ranging from those associated with an anencephalic absence of the nerve fibre layer, with loss of the ganglion cells and decreased optic nerve diameter, to retinal detachments which could not be explained by trauma or inflammation.[113]

5.4. Central Nervous System Malformation

Early development of the capacities in normal infants appears to follow CNS maturation and development, as specific learned experience, arousal, attention and state regulating capacities are fundamental neurobehavioral processes mediated by an adequately functioning CNS and form the basis for subsequent development.

CNS maturation is affected by neonatal CNS injury or intrauterine cocaine exposure at 4 months before birth.[114]

The most common drug affecting the CNS is cocaine, although the influence of cocaine exposure remains unknown, as research depends on maternal reporting of drug use or urine analysis which gives positive results only if the drug was taken shortly before the test. Its effects appear as some neonates suffering from stimulus seeking regardless of arousal, which appears after 4 months and not before, and behavioural disturbances[114]

This effect may be mediated by alterations in the mechanism of neurotransmitter synthesis and reuptake; especially dopamine. It is intriguing since both cocaine exposure and CNS injury produce short and long term alterations in CNS systems in the form of attention and arousal behaviours.[114]

In 1967 Auerbach and Rugowiski studied the effect of LSD in the mice embryo, and reported that 57% of them had multiple deformities including brain malformation with enlarged midbrain and improper closure of the hindbrain. Some deformities in jaw and eye position occurred when LSD was injected before day 7 of pregnancy, which

corresponds to the time when the neural structures are being formed. There may be destructive effects on the developing brain and eyes, causing progressive hydrocephalus with aqueduct stenosis. Abnormal cerebral convulsions were noted when the mother used LSD, cannabis and amphetamine during pregnancy, which may occur due to the combined effects, but not confirmation done on human.[109]

Several investigations have proven the effects of maternal alcohol or cannabis use during pregnancy in the form of disturbance of sleep or EEG patterns in children, especially with prenatal alcohol, including disturbed sleep architecture and continuity, altered power spectral values, and disruption in behavioural state and arousal.[113]

Methadone also produces disturbances in sleeping pattern, with CNS irritability in EEG's, and it has been that reported sudden infant death syndrome occurs which is accompanied by higher respiratory and lower mean heart rates.[112]

5.5. Body Stalk Anomaly

The body stalk anomaly is a fatal anterior abdominal wall defect affecting 1 in 14000 new-borns, which may be due to early rupture of the amnion. Prenatal diagnosis is by ultrasound scanning and is unequivocal, with absence of a free-floating umbilical cord and fusion of the placenta to the herniated viscera. Foetal malformation is often present and alpha-fetoprotein in maternal serum is commonly increased. It is confirmed by post-mortem.

This congenital anomaly commonly comes with kyphoscolosis and limb, cranial, facial, gastrointestinal, and genitourinary defects as well as an abnormal amniotic band. It occurs early in embryogenesis when body folding takes place, and the coelimic cavity is usually obliterated by the fourth week of development.

Cocaine use is commonly associated with birth defects, especially relating to the genitourinary tract, GIT, limbs, and causes destructive e and damaging effects on the CNS through mechanisms of foetal vasoconstriction and local haemorrhage. Cocaine also causes failure of both the cephalic and the caudal folding, and this would suggest a different chronological window in the vascular disruption during embryogenesis or a vascular segmental sparing of the effect of cocaine.[115,116]

6. The Placenta

6.1. Introduction

The placenta is an organ, which provides an essential connection between the mother and foetus: its integrity is crucial for foetal growth, development, and well being. Alternations in placental function may be due to maternal or foetal disorders or may be an intrinsic abnormality in the development of the placenta through the course of pregnancy.[1117]

6.2. Development of the Placenta

The ovum is fertilised in the fallopian tube and enters the uterine cavity as a morula, which rapidly sheds its surrounding zona pellucida and converts into a blastocyte. Its outer cell layer proliferates into cells which infiltrate between the trophoblastic cell mass, from which cells infiltrate between the endometrial epithelium. At this stage of development, the trophoblast consists of an inner cell layer(cytotrophoblast) and of an outersyncytial cell layer (the syncytiotrophoblast), these two tissue form the chorion, the cells degenerates with the trophoblast, to come into direct contact with the endometrial stroma, completed at 10^{th}-11^{th} postovulatory day. The stroma shows oedema and congestion which may be due to the action of some substance produced by the cells of implantation blastocyte. This congested and oedematous condition of the stoma provides nutritive material for the trophoblast, which now thickens rapidly at the region of contact. After 13 days of fertilization primary villi appear. At 15 days the villi enriched by an extra-embryonic mesenchyme to form secondry villi, which progress to tertiary villi enclosing primitive vasculo-blood islets. These islets are the original of the future villous circulation. At 21 days the fetoplacental circulation is established, at end of 3^{rd} month extensive growth of amniotic cavity flattens the ovular deciduas against the parital

deciduas, the uterine cavity is thus obliterated.[118] the villi on the side of the chorione toward the decidua basalis enlarge and become elaborately branched as pregnancy advances, toward the decidua capsularis, then degenerate between the third and the fourth month, leaving this side of the chorione smooth and almost non-vascular. Persisting villi then assume more branches to form the placenta which is supplied by intervillous maternal blood space, which gradually becomes subdivided into sinuses which reach the uterine side but not the foetal side.[119]At the end of 4th month and until term the placenta is fully formed with its blood supply.[118]At full term the placenta has the shape of a flattened cake and becomes thicker to meet the nutritional requirements though, doubtless, to compensate to some extent for thinning of the placental membrane separating the maternal and foetal blood streams, thus increasing its efficiency in transmission. After the placenta is made up of 15-20 convex areas covered by a very thin decidua basalis and separated from one another by irregular grooves. These convex areas are the so-called cotyledons. The foetal surface of the placenta is not subdivided and is covered completely by the vascular mesoderm of the chorione with its large umbilical vessels. The amnion is closely applied to the vascular mesoderm of the chorine with its large umbilical vessels. The amnion is closely applied to the chorine on the foetal side and in full term placenta is often fused with it, especially near the placental margin.[120]

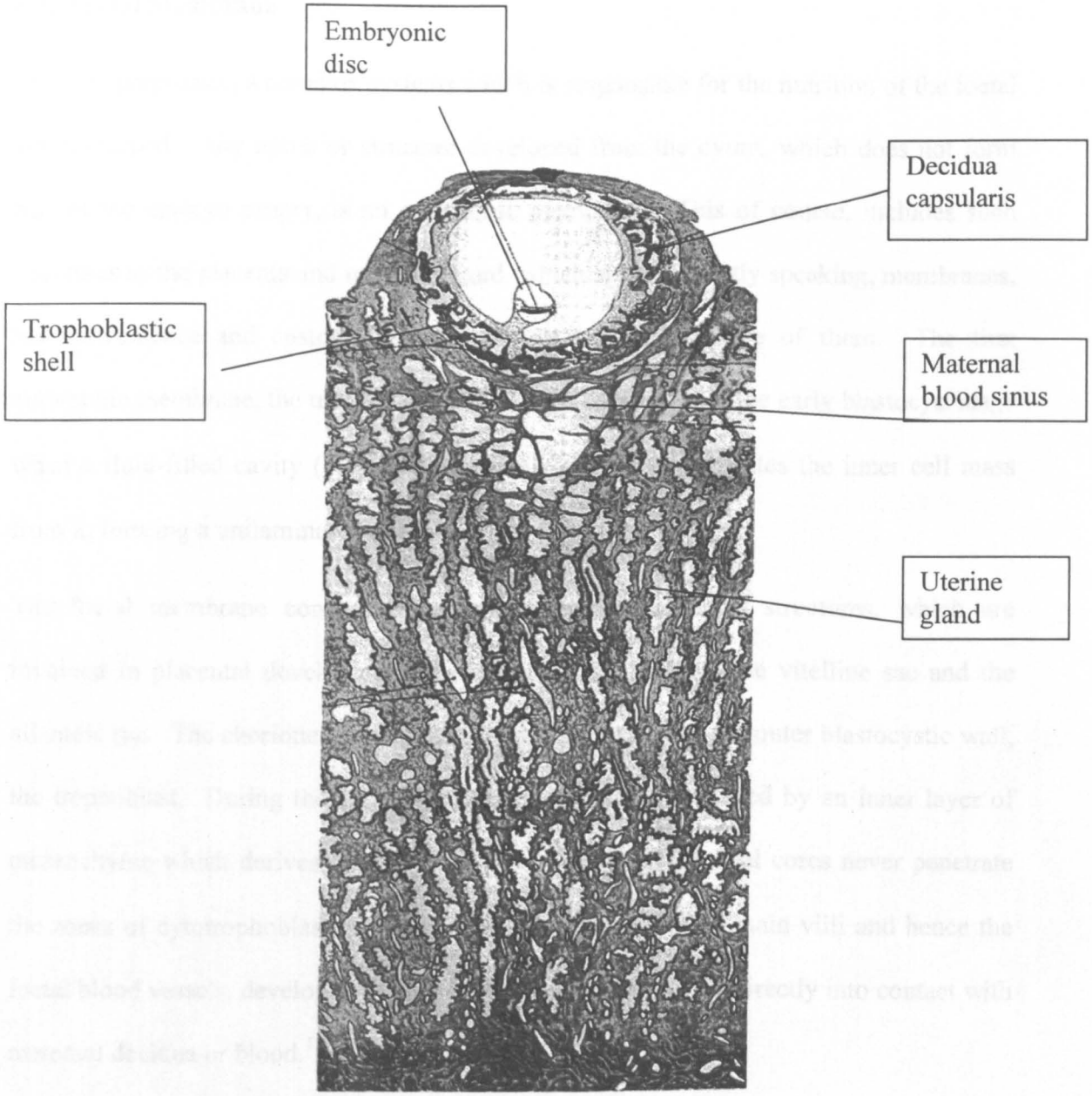


Figure 6.1 Relationship between the chorion and vessels(Human Embryology)[123].

6.3. Foetal Membrane

Through pregnancy, a complex systems which is responsible for the nutrition of the foetal are developed. Any tissue or structure developed from the ovum, which does not form part of the embryo proper, is an embryonic membrane. This of course, includes such structures as the placenta and umbilical cord, which are not, strictly speaking, membranes, but convenience and custom have sanctioned this broad usage of them. The first embryonic membrane, the trophoblast, becomes differentiated in the early blastocyst stage when a fluid-filled cavity (blastocoele) appears and partly separates the inner cell mass from it, forming a unilaminar foetal membrane.[121]

The foetal membrane consists of four different membranous structures, which are involved in placental development, the chorione, the amnion, the vitelline sac and the allantoic sac. The chorione is an epithelial layer derived from the outer blastocystic wall, the trophoblast. During the course of implantation it is completed by an inner layer of mesenchyme which derives from the embryo. The mesenchymal cores never penetrate the zones of cytotrophoblast at the decidual attachments of the main villi and hence the foetal blood vessels, developed in the mesenchyme, do not come directly into contact with maternal decidua or blood.[121,122]

The amnion, also consisting of epithelial layers derived from the embryonic ectoderm either by splitting or by folding, has no vessels. The amnion surrounds the embryonic foetus as a second inner sheet, but it never replaces the chorione of the yolk sac as the outermost embryonic membrane .

The yolk sac develops as an adenxal and structure of the embryonic midgut, and consists of a layer of endothermic epithelium accompanied by vascularised mesenchyme. The allantosis is the extra embryonic urinary bladder, which develops from the embryonic

hindgut as an extraembryonic sac. Its surrounding mesenchyme is richly vascularised, it is barely involved in transplacental exchange procession conclusion the following compositions of foetus

In conclusion, the following compositions of fetal membranes may establish the placenta:

*Chorionic placenta, first step of placental development in which as an unvascularised non-specialised part of the placenta.

*Choriovitelline placenta, chorionic membrane connected to the foetal circulation by vitelline vessels

*Chorioallantoic placenta chorionic membrane connected to the foetal circulation by allantoic vessels.

*Vitelline placenta yolk sac epithelium with vitelline vessels connecting it to the foetal circulation[122]

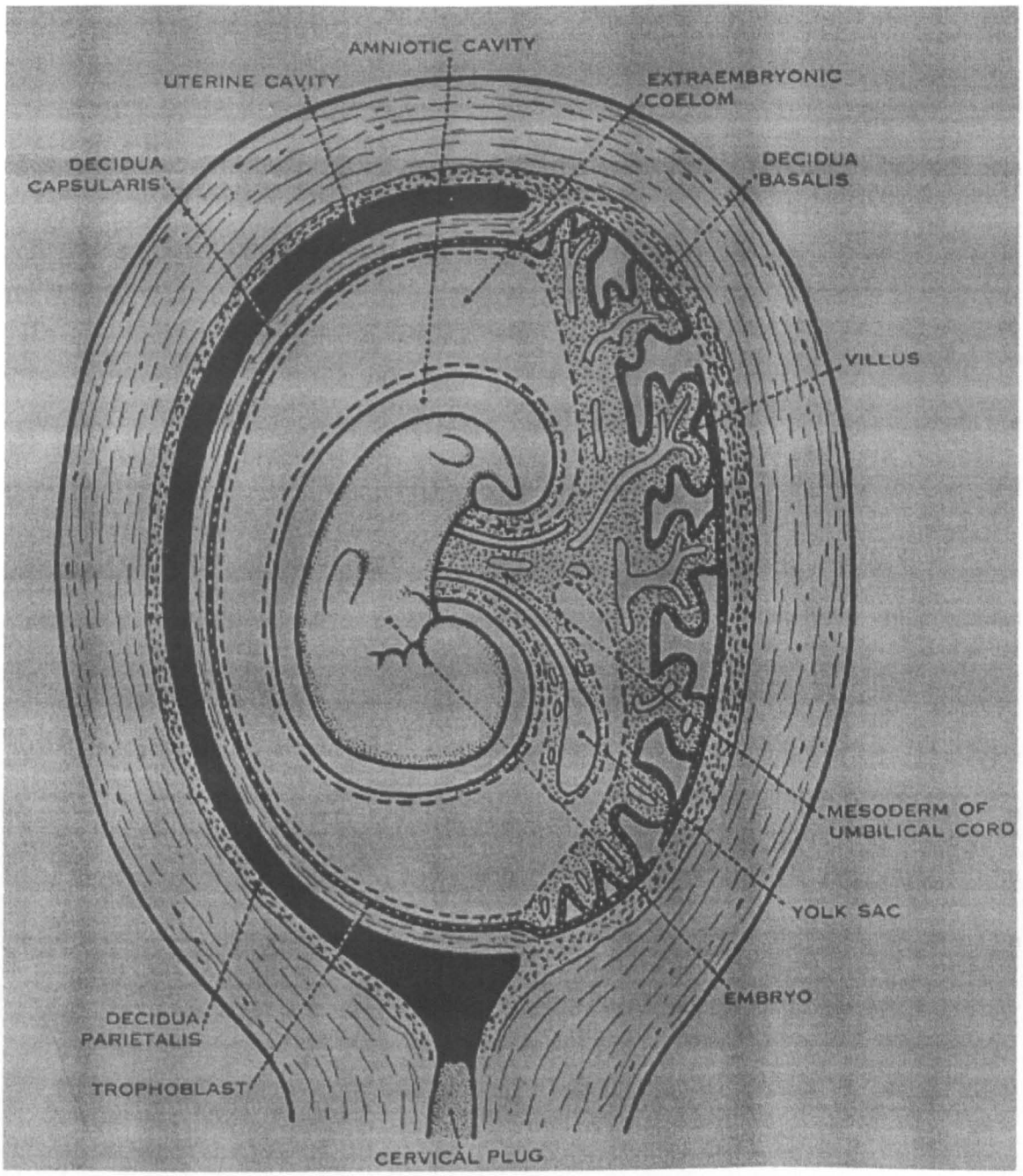


Figure 6.2 The embryo and its membranes.

(From Human Embryology. Hamilton, et.al. 1946)[123]

6.4. Anatomy of the Cervix

The cervix is the lower part of the uterus immediately posterior to the angle of the urinary bladder. This relationship is constant because the cervix is fixed by parametrium at the posterior angle of the bladder and project through the anterior wall of the vagina at the vaginal vault and has supravaginal and vaginal portions of equal length. The vaginal mucous membrane is continuous with that of the cervix, and the folds formed by the reflections of this mucous membrane at the front, back and sides of the cervix which are known as vaginal fornices. The cervix is roughly cylindrical and is about 3 cm long and 2.5 cm in diameter but this varies according to number of labours of the woman. The endocervical canal is visualised as an echogenic stripe surrounded by hypoechoic fibrous stroma, and its length is measured from the level of the internal os to its most caudal aspect in the fornix of the vagina. The epithelium covering the cervix is of two types, squamous epithelium which is stratified and non-keratinised under the effect of ovarian hormones, and columnar epithelium, which also covers the endocerivical canal. The majority are tall, thin, mucous secreting cells with basal nuclei, but few of them are ciliated. Typically, the amniotic fluid within the lower uterine segment has a funnel-shaped configuration and the apex of the funnel forms an acute angle at the internal os.[124]

6.5. Anatomy of the Uterus

The non-gravid uterus is pear-shaped and about 8-9 cm in length, divided into two distinct anatomic segments, a cranial corpus uterus with a smooth muscle wall and caudal cervix uteri composed of a fibrous tissue wall. The commonly-used term, fundus refers to the top of the corpus. The cavity of the uterine body is flattened anteroposteriorly and appears shield-shaped from the front view. It narrow toward the lower end, the isthmus grows rapidly in the early stages of pregnancy and produces most of the lower segment of the pregnant uterus. The uterine wall significantly increases in thickness during

pregnancy largely as a result of the physiologic hypertrophy of uterine blood vessels, which comes from uterine branch of the iliac artery on each side. Each uterine artery anastomises with the ovarian artery above and with the vaginal artery below. The utero-ovarian anastomosis forms a sinuous arterial channel along the lateral wall of the uterus within the broad ligament. The vessels penetrate all layers of the uterus and freely empty into plexuses at the sides of uterus and broad ligaments. These anastomosis corresponding to the physiological vascular changes of the pregnant uterus [125]

6.6. Placental Circulation

From the functional standpoint the most important features of the placenta are its maternal and foetal circulatory systems and the manner in which the two blood streams come into relation with one another. There is never, of course, any intermingling of foetal and maternal blood. The villi are supplied by an arterio-cappilary-venous system carrying foetal blood, and are bathed in maternal blood which flows in and out of the sinuses. The exact method of supply and drainage of the maternal sinuses is still controversial. The arteries opened into each cotyledonary sinus, usually near to or on the septa, while viens drained each sinus from its uterine surface or base. All venous openings are from the so-called circular, or border, sinus which is an irregular venous ring about 2.5 cm in diameter, located in the periphery of the placenta. Maternal arterial blood enters the fully formed placenta at the bases of the sinuses from enlarged coiled arteries of the endometrium. It then follows between the spongework of the villi to the foetal surface, where it spreads horizontally toward the border sinus through the more or less common intervillous spaces connecting all the sinuses between the surface chorine and foetal margins of the septa. Then the blood empties into the large endometrial venous plexuses of the region and then into the vein of the uterine wall.[127]

the maternal blood stream provide long distance and its energy source is the maternal heart, while foetal energy source is the foetal heart. This arrangement can be visualised as a system of two parallel lifts separated by a gate. There is the placental barrier boundary, which lets solutes across an inert membrane by simple diffusion or transfer under the combined influence of forces. The diffusion depends on the concentration difference, the barrier thickness, and surface area of the membrane that separates exchanging compartments.[124]

The foetal blood is usually supplied to the placenta by two umbilical arteries which, immediately anastomse before entering the organ, into a single arterial vessel. There is many branches, due to their exceptional location (a simultaneous blood supply from the arteries), which have a particular significance for the physiology of foetal blood circulation in the placenta.[126]

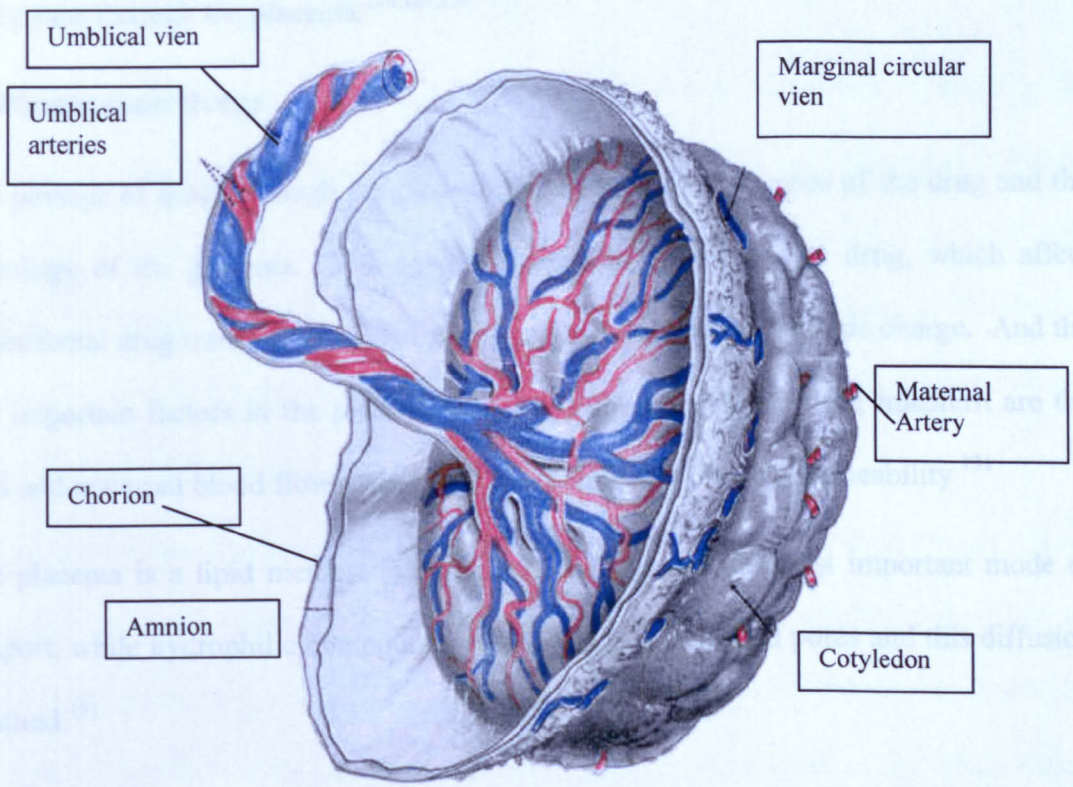


Figure 6.3 The normal placenta and the associated chorion at full term.

The umbilical cord normally contains two umbilical arteries and a single larger umbilical vein, covered by amnion and surrounded by clear gelatinous Wharton's jelly. The length is quite variable, with evidence suggesting that both cord length and spiralling depend on foetal activity. The arteries wind around the umbilical vein in a spiral fashion and carry deoxygenated blood from the foetus to the placenta; the direction of the cord twisting is not random.[127]

6.7. Physiology of the Placenta

The placenta is considered to be a membrane for exchange between mother and foetus so it is supplied with a double perfusion system: oxygen and other nutrients such as glucose and amino acids, oxygen and water are delivered from the maternal circulation. By contrast, CO_2, nitrogenous end products of foetal metabolism and hormones are removed by the same circulation, however most drugs of abuse are lipid-soluble and therefore rapidly pass through the placenta.[128,129,130]

6.8. Placenta and Drugs

The passage of drugs through the placenta depend on the properties of the drug and the physiology of the placenta. The most important properties of the drug, which affect mother/foetal drug transfere are: lipid solubility, molecular size and ionic charge. And the most important factors in the placenta which affect mother/feotal drug transferit are the foetal and maternal blood flow to placenta, placental structure and permeability [131]

The placenta is a lipid membrane so passive diffusion is the most important mode of transport, while hydrophilic compounds pass through water-filled pores and this diffusion is limited.[131]

Active transport may occur if the compound is closely related to an endogenous substance such as amino acids, for which carriers exist, and lipid soluble drugs will

equilibrate across the placenta rapidly. Hydrophilic compounds will equilibrate slowly and when the foetal blood concentration equals the maternal drug concentration a steady state is achieved during which constant rate maternal infusion will occur. The transport depends on foetal protein binding and blood pH relative to maternal parameters, placental bio-transformation and whether the foetus has the ability to eliminate the drug[131].

6.8.1. Lipid-Soluble Drugs

Cocaine, morphine and nicotine are examples of lipid-soluble compound that readily cross the placenta, then are found in amniotic fluid and new-born serum.

The concept of the placenta as a barrier to xenobiotic exposure is obsolete, the placenta does have the ability to selectively filter and metabolise drugs and chemicals. The placenta possesses cholinesterase activity which metabolises cocaine to its major metabolites, and other drugs are also affected.[132] There is genetic variation in cholinesterase activity that are ethnically specific.

Cocaine hydrochloride is water soluble, and its ability to cross human placentas in substantial amount has been demonstrated in spite of its water solubility, but the ability of metabolites to pass back into the maternal circulation appears limited due to their high polarity. Free base cocaine is lipophilic, hence the ability of the placenta to metabolise cocaine is related to the magnitude of exposure of the foetus to the drug, its metabolites, and the duration of exposure.

Foetuses of women who have lowered cholinesterase activity may be at increased risk of abnormalities from intrauterine cocaine exposure, because foetuses, regardless of maternal enzymes, do not possess cholinesterase activity. However, foetuses do demonstrate liver N-demethylase enzyme activity, which is important in the metabolision of many drugs.

6.8.2. Hydrophilic Compounds

Metabolites of abused drugs are frequently hydrophilic compounds and can not pass directly through lipid membranes, but they diffuse through the placenta via water-filled channels in the membrane: this passage is termed diffusion-limited transfer. The sizes of the channels vary with species.

Morphine-3-β-D-glucuronide, the major metabolite of morphine in man, has a molecular weight of 461, in late gestation, this metabolite does not cross the placenta in significant amount and if it injected into the foetus, it crosses into the maternal circulation very slowly, the permeability surface area of drug increases as the foetus ages increase during late gestation.

Benzoylecgonine, a hydrophilic inactive metabolite of cocaine has greater permeability than morphine glucuronide as it has a smaller size.[129]

6.8.3. Peptides

Placenta permeability to peptides depends on molecular size and lipid solubility. Also, cationic molecules cause structural changes in the placenta associated with increased permeability. Placental transfer of peptides is reduced by peptidases and histamine increases permeability to proteins, for example, oxytocin can cross the placenta[133].

6.8.4. Amino acids

Placenta concentrations of amino acids are higher than the foetal levels, so they are transported against the concentration gradient so transport to foetus need carrier-mediated, and energy. As the amino acids are required for neurotransmitter synthesis so their deficiency results in mental retardation in infants. A prolonged decrease in maternal amino acid concentrations causes an increase in placental amino acid transport, by

inducing synthesis of new carriers. Hormonal regulation of amino acid transport is inhibited by the effect of alcohol and nicotine causes foetal growth retardation. [131]

6.9. Foetal:Maternal Drug Concentration Ratios

The ratio of foetal to maternal blood drug concentrations is used to make judgements about the rapidity and extent of placental drug passage.

Under steady state conditions, the total drug concentration on both sides of the placenta need not be equal if there are foetal and maternal differences in protein binding and pH, for it is the unbound and unionised drug that equilibrates across the placenta. Placental drug biotransformation would allow lower foetal levels relative to maternal concentrations, but placental drug metabolism is negligible for most compounds. The foetal blood concentration of an unbound, unionized drug is less than the maternal concentration when foetal elimination is present and it significant appears that placental passage of drugs, including peptides, is determined by the physical, and chemical properties of the drug and the physiology of the placenta. Foetal blood concentrations of drugs administered to the mother depends on the time since maternal drug administration, dose, placental blood flow, permeability, plasma protein binding, blood pH, placenta biotransformation and foetal elimination[125].

Drug abuse has a serious effect on foetal development and pregnancy outcome either due to effects of the drug on placental perfusion and foetal oxygenation or to direct drug action. There are various methods used to assess drug exposure in neonates, including cord blood, amniotic fluid, neonatal urine, meconium, and hair sample. Complete understanding of the pharmacokinetics of these drugs in the maternal-foetal is needed.[134].

6.10. Maternal-Foetal Drug Disposition

The factors that influence the rate of drug transfer from mother to foetus depend on:

. *Thickness and surface area of the placental membrane.

. *Molecular size

. *Lipid solubility of the drug molecule.

. *The plasma protein binding of the drug.

. *Relative pH of the blood of the mother and foetus.

. *Rate of placental blood flow especially for lipid soluble compounds.

When taken intravenously, drugs rapidly rise in concentration in foetal blood until an equilibrium is reached, and as the drug continues to be cleared from the maternal plasma, the diffusion gradient is reversed and the foetal drug concentration will begin to fall. So the foetal level depends on rate of placental transfer and drug elimination in the mother. This happens with meperidine, methadone, cocaine and methamphetamine. Foetal drug levels were found to be similar to maternal levels for meperidine and cocaine but lower for methadone and THC[135].

Drug elimination is a big problem in the neonate, especially after removal of the placenta. In the case of repeated drug exposure a steady state plasma drug concentration depends on many factors: half life of the drug, placental permeability, foetal maternal drug elimination and plasma protein binding.

6.11. Foetal Drug Elimination

Foetal drug elimination depends on the presence of drug-metabolising enzymes in the foetal liver which are capable of catalysing the N-dealkylation of meperidine and methadone and glucuronidation of morphine. This is proved by the presence of certain metabolites in foetal plasma that are pharmacologically active and explain the adverse effects in the foetus. Foetal renal clearance is also important for drug elimination,

especially for the polar conjugates such as morphine 3-glucuronide, which can be detected in foetal urine[135].

Excretion of drugs into amniotic fluid appears late as it comes from foetal urine but concentrations exceed those in the maternal and foetal plasma. Some drugs accumulate in amniotic fluid even with complete diversion of foetal urine suggesting other sources of drug transfer such as diffusion across the chorioallantoic membrane. For lipid-soluble drugs, back-diffusion across the chorioallantoic membrane is the major pathway, and provides the final route of drug elimination. For less lipid-soluble drugs, and especially for polar metabolites, the final route of elimination is probably by foetal swallowing of the amniotic fluid.

6.11.1. Excretion of Drugs Into Meconium

Metabolites formed by the foetal liver may be excreted in the bile and deposited in meconium. Small amounts come from foetal swallowing of amniotic fluid. Because meconium is not normally excreted in uteri, it accumulates throughout the entire gestation period. Metabolites of cocaine, morphine and cannabinoids can be detected in meconium from babies of chronic drug users throughout the second half of gestation[134].

6.11.2. Excretion of Drug Into Foetal Hair

Metabolites can also be excreted into foetal hair, for example the concentration of benzoylecgonine in neonatal hair is linearly correlated with self-reported cocaine use. Hair reflects drug exposure during the last 3 months of pregnancy as it grows in this period. Hair grows approximately 1-1.5 cm/month, so it is possible to section the hair to assess the time of drug exposure[134].

7. Review of Biological Samples Used for the Analysis of Drugs in Pregnancy

7.1. Introduction

Drug abuse during pregnancy has become a problem of epidemic proportions. The National Institute on Drug Abuse (NIDA) has estimated that over five million pregnant women are drug addicts, with significant increases in the use of crack cocaine and its complications to the mother and foetus, such as increase in stillbirths, meconium stained amniotic fluid, premature rupture of the membranes, maternal haemorrhage (due to *abruptio placenta* or *placentae previa.*), foetal distress with increases in morbidity and mortality, asphyxia, prematurity, low birth weight, infection pneumonia, congenital malformations, cerebral infarction, drug withdrawal and increased risk of AIDS.[136]

Cocaine, if accompanied by methamphetamine can cause maternal hypertension, subarachnoid and intracerebral haemorrhage, premature labour, small head size, reduced birth weight, ruptured uterus and increase in foetal death with some behaviour disturbance.[137]

Exposure to opiates causes many problems, apart from that of withdrawal symptoms (Abstinence Syndrome), including irritability, tremors, hyperactivity and seizures. Birth defects and other congenital anomalies are thought to be related to foetal exposure to drugs, chemical agents, alcohol, or other xenobiotics.[138]

Many other problems appear later in childhood, including delays in physical growth, mental affection, learning disability, and sudden infant death syndrome. Maternal history, relating to the amounts of drugs used, frequency of use and how long the

mother used them, can help in prevention but is not accurate most of the time for fear of consequences that may stem from admission of use. Laboratory tests, which must be very sensitive, specific and capable of producing a broad drug screen with many confirmatory tests, also can help.[137]

Currently, identification of the drug-exposed newborn with toxicological screening has become of importance to allow follow-up of the infants by medical and social services. Detection can be done by analysis of blood, urine, meconium, hair, nail and breast milk. The problem which is encountered in newborn blood analysis is the small sample available and difficulty in obtaining it in many countries (this may be solved in United Kingdom as blood is collected from every newborn in hospital to do routine analysis). [139]

Frank et al (1998)[140], found that foetal urine analysis gives misdiagnosis of about 24% of self reported cases of maternal cocaine abuse, with low sensitivity and difficulties in collecting the sample. As a result, the focus must move to alternative methods of analysis. Hair and amniotic fluid have problems in collection. Amniotic fluid and gastric lavage cause many complications, including infection as one of the serious problems.[141]

Ostrea et al 1989[69] found that circulation in the foetus of drugs of abuse and their metabolites result in the accumulation of high concentrations of the drugs in meconium. Maynard et al, 1991[142] made a comparison between meconium and urine analysis and found meconium more specific and sensitive.[143]

Meconium which is a relatively recently used as a biological sample, is capable of being a very sensitive indicator of maternal drug use, easy to collect by non-invasive methods and containing high concentration of drugs and their metabolites. It allows

for a wider window of detection than urine as most drugs accumulate in meconium until delivery. Quantitative analysis of meconium provides further information on the amount, time and duration of foetal exposure.[139, 144]

7.2. Meconium

Unlike drug epidemics of earlier decades, the current drug epidemics have affected many women of childbearing age, so intrauterine drug exposure remains a major health concern because of the associated high incidence of prenatal complications and the high morbidity and mortality rates of the newborn. [136,137,138]

Clinicians have recognised the importance of identifying drug dependent mothers in the prenatal and post-partum periods to provide appropriate treatment and adequate follow up. At first, clinicians depended on maternal urine tests in conjunction with patient history, but these two routes fail to identify a significant number of drug using women. Researchers have explored other methods for identifying drug-exposed infants. Recently, attention has focused on testing meconium instead of urine.[145]

Meconium, the dark green, viscous, first stool of a newborn, is considered to be a mixture of secretions from foetal intestinal glands and amniotic fluid, produces a green-stained amniotic fluid. It consists of a collection of debris and is mainly of water, but also contains mucopolysaccharides, lipids, proteins, fatty material from the vernix caseosa, bile acids and salts, epithelial cells, cholesterol and sterol precursors, blood group substances, squamous cells, residual amniotic fluid, enzyme, languor and various intestinal secretions.[141]

The amount of meconium can be estimated by gross examination and has been classified as thin (light), moderate, or thick (heavy). Thick meconium, which has a "pea-soup" quality, is associated with a significantly increased risk of meconium aspiration syndrome. Recently a more objective assessment of meconium content of amniotic fluid, called the "meconium crit", is measured directly like a hematocrit and has been shown to be linearly related to meconium concentration.[144]

Meconium is seen in approximately 5% of amniotic fluid specimens obtained by amniocentesis. In general, meconium staining early in pregnancy is usually not caused by haemolytic disease but is usually due to a transient episode of foetal comprise. It does not indicate a poor foetal outcome. However, meconium staining in general is accompanied by a high foetal mortality rate.[145]

In human foetuses, meconium usually begins to accumulate in the bowel at approximately 16 weeks gestation (second trimester) and is not excreted until delivery. The foetal liver produces water-soluble metabolites that accumulate in the intestine and in urine. The urine is excreted into the amniotic fluid, which is ingested by the foetus. Thus drugs and their metabolites can accumulate in the meconium, since the foetus does not normally excrete stools *in utero*, until 1-5 days after birth. The meconium accumulates throughout gestation, with higher concentrations of drug metabolites than in urine, which is excreted continuously *in utero*.[107,142,146.]

Drug metabolites have been detected in the urine and plasma of both the human and animal newborn. However, it is difficult to determine the metabolic fate of drugs *in utero*. Each of the major metabolic pathways can be promoted by placental and/or foetal enzymes and although the reaction rate appears to increase with gestation age,

the presence of a metabolite in the foetus does not necessarily reflect the ability of the foetus to metabolise the drug.[147]

Drugs reach the foetus by passive diffusion of the small molecules of soluble drugs across the placenta, which was once thought to be a protective barrier for the foetus disallowing passive diffusion of drugs. The molecular size, the degree of ionisation, the hydrophilicity of the drug, the blood flow to the placenta and the degree of protein binding to maternal and or foetal plasma proteins affect the rate of drug transfer through the placenta. [148]

Meconium analysis has advantages over urinalysis mentioned by many researchers such as Ostrea et al.[149], who showed that meconium contained higher concentrations of drugs than neonatal urine.

Ryan et al.[150] stated that meconium testing was more likely than urine testing to identify an infant with intrauterine cocaine or opiate exposure, detecting an additional 33 % or 12 %, respectively. Maynard et al.[142] found that meconium was useful sample for detection because it was easier and more reliable to collect than urine and was easy to incorporate into routine procedures. Meconium is more sensitive than urine for the detection of cocaine and methadone exposure that occurred during the last two trimesters of pregnancy. The amount collected is usually sufficient for complete analysis including confirmation tests. The major advantage of meconium analysis is in extending the window of detection of drug use to approximately the last twenty weeks of gestation compared to urine, which provides information about recent drug exposure only (2-3 days before birth.).[151]

Meconium has the disadvantage that it is not a homogenous sample and therefore must be mixed thoroughly before analysis.[151]

Meconium is usually passed by the full term newborn within 24-48h, after which transition from a blackish green to yellow colours indicates the beginning of passing of neonate stools. Infants with low birth weight (less than 1 Kg, which is the major problem of the foetus of an addict mother) have been shown to pass their first meconium between three and five years after birth. Thus, meconium collection can be missed because of delayed passage and also may not be available until soon after birth for early detection of intrauterine death syndrome.[152,153]

Meconium is an unfamiliar matrix, being a sticky material that is more difficult to work with than urine. Furthermore, processing of meconium for analysis requires weighing and extraction steps that are not needed for urine.[154].

7.2.1. Previous Methods of Meconium Analysis

Meconium analysis has been carried out using different instruments, including gas chromatography (GC), gas chromatography/mass spectrometry (GC/MS), high performance liquid chromatography (HPLC) and by immunoassay using enzyme multiplied immunoassay technique (EMIT), radioimmunoassay (RIA) and fluorescence polarised immunoassay (FPIA). It can also be analysed by colour tests or thin layer chromatography (TLC). Many drugs are detected in meconium including nicotine, opiates, cocaine, amphetamine, phencyclidine (PCP), cannabinoids, methadone, and alcohol. The sample of meconium must be prepared by adding concentrated hydrochloric acid, water, buffer or methanol, before doing any analysis.

7.2.2. Analysis of Drugs by GC and GC/MS

These methods generally need derivatisation of drugs extracted from biological samples and have some disadvantages, for example, they are time consuming,

expensive and need experience. However, but they are sensitive, provide broad and specific screening, and have been used for the detection of several drugs as outlined below.

Nicotine:

Nicotine is considered to be a serious problem, equivalent to drug abuse, as many women smoke heavily, especially in pregnancy: Approximately 26% of adult women smoke. Many adverse effects are associated with the foetus of a smoking mother and depend on the dose taken. They include facial deformities, oral clefts, congenital heart disease, limb reduction defects and gastro-enteritis[137]. Also, there is a relationship between smoking and pregnancy resulting in an adverse outcome like increased spontaneous abortion, abroptio placentae, placenta praevia, prenatal mortality prematurely and poor foetal growth. Also, there are some cardiorespiratory changes affecting foetal heart rate, decreased foetal movements and breathing, damage to the heart, umbilical cord and foetal vessels, decrease in intervillous placenta blood flow and alteration in foetal oxygenation. These can lead to reduction in postnatal height, and, in a few cases, development of cancer, or neonatal jaundice or infant allergy. Some research has found a relationship between smoking and sudden infant death syndrome.[137]

e.1.1.1.1 Mechanism of action

Nicotine transfers through the placenta and is found in higher concentrations in amniotic fluid than serum. It causes increased levels of cocaine and carboxyhemoglobin in the foetus, with vasoconstriction leading to decreased placental blood flow, hypoxia and under-nourishment of the foetus.[155]

Secondary effects on the umbilical vessels, which cause some abnormalities, lead to decreased production of prostaglandin and decreased capacity for vasodilatation, resulting in histological, morphological and biochemical changes in the placenta.

Cigarette smoke contains many compounds such as cyanide, which affects the metabolism of important elements such as calcium and vitamins such as B12 and C and possibly vitamins B6, B1 and A.[140.]

e.1.1.1.2 Methods of detection of nicotine in meconium:

Ostera et al, 1994 analysed the nicotine metabolites cotinine and trans-3-hydroxy cotinine in meconium by GC/MS using a cross-linked dimethyl silicone GC column. The instrument was used in the Selected Ion Monitoring (SIM) mode for the trimethylsilyl derivatives of cotinine (using the ions at m/z 98, 176, 119) and 3-hydroxycotinine (at m/z 294, 144, 75), with d3-cotinine as internal standard (IS). The amount of metabolites present related directly to the extent of maternal smoking.[156]

Cocaine

Clark et al, 1992[157] extracted cocaine from meconium by solid phase extraction followed by analysis by GC using an HP-5 phenylmethyl capillary column. The linearity was up to 10μg/g, with regression coefficients of 0.99 and 0.97 for cocaine and benzoylecgonine respectively.

Abusada, et al, 1993[158] analysed cocaine, ecgonine methyl ester, benzoylecgonine, and cocaethylene in meconium in the concentration range 0-1000 ng/g by GC/MS with an HP2 capillary column (12m x 0.2 mm x 0.33μm phase thickness). The injector temperature was 260°C and the column temperature started at 145°C for

4 min, rising to 210°C at a rate of 60°C/min, then to 280°C for 0.25min at the same rate.

Cocaine, benzoylecgonine, norcocaine and cocaethylene were extracted by Solid Phase Extraction (SPE), eluted by chloroform:isopropanol:ammonium hydroxide (78:20:2 v/v/v) followed by derivatisation by PFPA and HFIP and analysis by GC/MS. The lower limit of detection was 0.03 μg/g and lower limit of quantification was 0.1μg/g.[159]

Opiates

Wingert et al 1994 [160] used a methanol extraction of meconium followed by SPE then analysis by GC/MS equipped with an HP-1 or HP-5 capillary column. The oven temperature started at 80°C, rising to 190°C at a rate of 40°C/min. Moore et al 1995 [161] confirmed the presence of hydrocodone, hydromorphine, codeine and morphine by GC/MS. The column was DB5-MS (25m x 0.2mm ID x 0.33μm film thickness). The oven temperature started at 100°C, rising to 230°C at a rate of 25°C/min, then to 260°C at a rate of 3°C/min. The lower limit of detection was 5ng/g for each drug.

In 1995, a study was carried out to detect morphine glucuronide in meconium but as only small amounts of it are transmitted through the placenta to the foetus, the absence of morphine glucuronide is not an unusual finding. However, other opiates and their metabolites were present in significant amounts and differed from those found in urine as there is no need to hydrolyse meconium. Measurable amounts of morphine could be detected, but no report of monoacetylmorphine has been published.[136]

Amphetamines

Nakamura, 1992[162] detected amphetamine using GC/MS on an HP-ultra2 column (25m x 0.2mm ID x 0.33μm) with the oven temperature starting at 100°C for 30sec, rising to 220°C at a rate of 20°C/min. He could detect as little as 1ng/g, but found that the gestation age can affect the sample. ElSohly 1999[163] used liquid-liquid extraction (LLE) followed by SPE and derivatisation by pentafluoropropionic anhydride (PFPA). The limit of detection was 10ng/g.

Cannabinoids

Detection and analysis of cannabinoids in meconium is difficult, as they are present in small amounts. However, Moore, 1996 [147] and ElSohly 1999 [163] report a method involving hydrolysis by alkali or enzyme, then analysis by GC/MS after derivatisation by dimethyl-*tert*-butylsilyltrifluoroacetamide (MTBSTFA) and *bis*-trimethylsilyltrifluoroacetamide (BSTFA) on a DB-5 MS or HP-1 column (25m x 0.2mm ID x 0.33 μm film thickness) respectively. The limit of detection with alkaline hydrolysis was 2ng/g, with good recovery.

Phencyclidine

This drug crosses the placenta to the foetus and is present in urine and plasma. There are several methods for its extraction. Moriya, et al 1994 [146] detected the drug with a limit of detection of 20ng/g. Moore, 1996 [147] tried to decrease the limit of detection to 5ng/g by using GC/MS in the SIM mode but no improvement was reported, although immunoassay was used to detect other metabolites. Another method reported used extraction by SPE with elution by 2% ammonium hydroxide,

followed by GC/MS-SIM with a limit of detection of 20ng/g of meconium and 7ng/ml of urine.[148]

Methadone

Methadone and its metabolite EDDP was detected first by Winger et al, 1994 [27] using SPE and confirmation by GC/MS. Stolk et al, 1997 [164] used FPIA and HPLC. The assay was linear over the range 460-3680ng/g methadone and 1000-6000ng/g EDDP, and the limits of detection were 99 and 113ng/g respectively. In another report, the lower limit of detection was 30ng/g.[145]

Analysis of Multiple Drugs

Some studies tried to detect multiple drugs using SPE with different eluants, for example, Moore et al, 1995[161] analysed opiates, amphetamines and cannabinoids, each one analysed separately using an HP 1 column (25m x 0.02mm x 0.33μm) and quantification by GC/MS. The meconium was prepared by two different methods, the first one using 10ml of water and concentrated hydrochloric acid, and the second using methanol. The detection rate for cannabinoids by the first method was only 19.6%, while all the positive cases could be detected by the second one. The rate of negative results increased because of the use of an inefficient extraction procedure. There were false positive results but the screening is not a problem if confirmed by a different technique. This may be due to giving the patient some medical prescriptions especially those containing opiates, but other drugs were not confirmed due to cross-immunoreactive responses. For example, antibodies for tetrahydrocannabinol (THC) and its metabolites cross-react with non steroidal anti-inflammatory drugs (NSAID's) while amphetamine assays can give false positive results due to cross-reacting substances such as phenylamine and other endogenous

compound, which interfere with the polyclonal assay. GC/MS confirmation gave positive results in about 74.3% of cases showing positive in the initial screen.

Another published method used meconium specimens and maternal urine for cocaine, benzoylecgonine, cannabinoid metabolites, opiates and amphetamines. Drugs were extracted by SPE and derivitised with PFPA. The limits of detection were: amphetamine (1000ng/ml), THC (50ng/ml), 11-nor-delta-9-tetrahydro-cannabinol-9-carboxylic acid (THC-COOH, 15ng/ml), opiates (morphine, codeine, 300ng/ml), benzodiazepines (300ng/ml). The results for meconium compared to urine were more accurate by 25% and showed higher concentrations for cocaine than maternal urine.[149]

Moore[147] and ElSohly 1999[163]analysed opiates, and major metabolites, cocaine, benzoylecgonine, amphetamine and cannabinoids, each drug eluted by a specially-selected eluant.

7.2.3. High Performance Liquid Chromatography

HPLC can be used with electrochemical and diode array ultraviolet-visible detectors, but it is expensive, needs significant experience and time consuming but it is specific and gives accurate results.[137]

HPLC has been used for the analysis of meconium after SPE to detect cocaine, norcocaine and cocaethylene, using onto a C18 column (300mm x 3.9mm ID). The quantification limit was 0.1mg/kg and the limit of detection was 0.03mg/kg.[159]

Murphy et al, 1993[165] determined benzoylecgonine in meconium by HPLC. The calibration curves were linear over the range 0.05-5.0μg/g of meconium and the limit of quantification was 0.05μg/g [136]

Morphine and amphetamine have been detected by HPLC: in one method, the recoveries were 65.6% and 91.8% respectively and the lower limits of detection (LOD) were 0.5ug/g and 500ng/g respectively. This represents an increase in LOD for morphine of 100 times that obtained with GC/MS. HPLC is therefore considered less sensitive and specific than confirmation by GC/MS.[13] Another method for morphine and amphetamine also used reversed phase system based on a C18 stationary phase (150mm x 4.6mm ID x 5µm particle size), with a diode-array detector and Millennium® software for identification by matching UV spectra. The lower limit of detection was 0.5µg/g of meconium, the regression coefficient 0.987 and the recoveries 91.8% for amphetamine, 65.6% for morphine.[146]

Stolk et al, 1997[164] detected methadone using reversed phase system involving a C18 stationary phase and a mobile phase consisting of 530ml distilled water, 146µl triethylamine and 350µl phosphoric acid at pH 3.3. The assay was linear over the range 460-3680ng/g for methadone and 1000-6000ng/g for EDDP. The limits of detection were 99 and 113ng/g, respectively.

7.2.4. Immunoassay Techniques

Multiple drugs have been detected in meconium using the enzyme multiplied immunoassay technique (EMIT) which depends on an antigen or hapten labelled with an enzyme. It can be semi-quantitative and is relatively expensive. Screening of meconium without some degree of extraction is difficult and gives false results, especially with EMIT or fluorescence polarisation immunoassay (FPIA) due to turbidity in the meconium extract. Chen and Raisys[166] used EMIT for detection of benzoylecgonine, with a limit of detection of 400ng/g of meconium.

Moriya et al,[148] screened for amphetamine, cocaine metabolites, opiates and PCP in meconium with a combination of EMIT and SPE. The cartridges were eluted with chloroform:propanol (3:1 v/v) and the extracts were then evaporated to dryness, reconstituted with buffer (pH6) and methyl acetate. The lower limits of detection were 250 ng/g for benzoylecgonine, 770 ng/g for d-amphetamine, 110 ng/g for morphine and 100ng/g for PCP.

Wingert et al, 1994[160] determined cocaine, cannabis metabolites, opiates and methadone by EMIT.

Amphetamine, methamphetamine, cocaine metabolites, opiates and PCP were screened by EMIT using the Syvatest plus system. Meconium was considered positive for cocaine metabolites when the reaction rate for a specimen minus the reaction rate for the negative control exceeded 40 units.[145]

A screen for benzoylecgonine by EMIT had a lower limit of detection of 100μg/l, sensitivity of 0.6μg/g, a recovery of 61% and the cut-off was set to 0.6μg/g. [24]

Comparative analyses of maternal urine and meconium for cannabinoids, benzoylecgonine and opioids including methadone which was used to treat cocaine-addicted mothers were done by EMIT, TLC and GC/MS. The cut off for EMIT was: cannabinoids 50ng/ml, benzoylecgonine 300ng/ml, amphetamine 1000ng/ml and opiates 300ng/ml. The results showed that no case gave positive results with urine which did not also give the same with meconium, while urine analysis missed 25% of the positive cases. From these results, meconium is considered more sensitive than urine and to have an accuracy up to 33% above a urine test.[167,168]

Screening of amphetamine, benzoylecgonine, opiates and PCP was carried out by mixing the meconium with methanol and concentrated HCl, then analysing by EMIT, followed by confirmation using GC/MS. The detection limits were 100ng/ml for benzoylecgonine, 500ng/ml for methamphetamine and morphine, and 75ng/ml for PCP. This method had many advantages in that it took a short time, gave clear extracts with a low background and had better recoveries than methanol alone.[148]

Another method to screen for cocaine and benzoylecgonine using EMIT had a lower limit of detection in the range 300-400ng/g but the sensitivity by this method was low as only microgram levels of cocaine/gm of meconium could be detected after concentration to give the same result as GC/MS, so methanolic extraction of meconium followed by SPE and EMIT assay can be used to screen newborns for intrauterine exposure to cocaine.[169]

The other main type of immunoassay which is more popular for the analysis of drugs of abuse in meconium is radioimmunoassay (RIA): recent work has been based on this method.

Enrique et al, 1991[137] found that RIA analysis is an ideal method which depends on the radioactive label competing with the unlabelled drug for binding sites on specific antibodies, so it is highly sensitive and semiquntative, but requires experience, is expensive, detects only one drug at a time (time consuming) and, as with all immunoassays, cross-reactions with other compounds can occur. It has been used for the detection of benzoylecgonine and cocaine, opiates and cannabinoids.[169]

The meconium samples were collected, mixed with water and concentrated HCl and centrifuged. The supernatant was analysed for morphine (heroin metabolite) and benzoylecgonine (cocaine metabolite) using RIA kits. The recoveries were 84-97%,

and 70-105%, respectively for morphine and benzoylecgonine and 97% for cannabinoids.

Another modified method involved mixing the meconium with phosphate buffer and methanol and using the ultrafiltrate for the detection of morphine, cocaine, and cannabinoids by EMIT, and also mixing the meconium with water followed by RIA analysis, with the lower limit of detection being 15ng/0.1g of meconium.[136]

A more sensitive and specific method for determination of opiates, cocaine and cannabinoids was the result of 100% recovery, with no cross reactivity.[150]

Cannabinoids and amphetamines can be screened by RIA with a lower limit of detection of 2ng/gm.[170]

A comparison of urinalysis with meconium analysis by RIA found that the lower limit of detection used 15ng/0.1g meconium with detection of a higher percentage of cocaine exposures when meconium was used, 11.8% overall more than with urine.[171]

Immunoassay by FPIA is also considered to be very sensitive and to give good results. Many researchers have used this method. In 1994 Lewis [169] screened for cocaine, cannabinoids, amphetamine and opiates after adding acetic acid and methanolic buffer to meconium. After centrifuging, the supernatant was analysed by FPIA.

Franssen et al, 1994[107] used FPIA for the determination of morphine and amphetamine in meconium, with a lower limit of detection of 1.35μg/g for amphetamine and 0.35μg/g for morphine.

Another method involves the addition of acetonitrile, vortexing and centrifugation with subsequent analysis of the supernatant by FPIA for benzoylecgonine. Precision over the linear range of the method were 12% at 0.08μg/g and 6.5% at 4μg/g.[167]

FPIA was used for the determination of cannabis metabolites, cocaine and cocaethylene and also gave good results for methamphetamine and PCP.[172]

FPIA reagents were obtained from Abbott Diagnostics, which is considered better than the Roche On Line cocaine assay, and uses Kinetic Interaction of Microparticles in Solution (KIMS). This was examined as a potential methodology for determination of cocaine metabolites in meconium, as this technology has similar sensitivity to FPIA and is better than EMIT. KIMS is based on latex beads (1-5μm) in diameter, so it can be expected to interact with meconium lipid droplets and give false positive results, while FPIA does not interact with lipid, so a decreased incidence of false results is obtained. In that study it was found that meconium analysis is better than urinalysis. Also, FPIA is a good analytical technique for true cocaine exposure whereas the KIMS technique lacks the requisite specificity to be a useful screening tool and must be followed by confirmation by GC/MS. Positive results obtained by FPIA are an accurate indication of prenatal cocaine exposure.[173]

Some researchers have carried out analyses using simple tests such as colour, or spot tests. Ostrea, 1991[137] considered this approach to be simple and early tests depended on reaction of small amounts of sample which react with the test reagent to give a colour. Colour tests are considered to be easy, quick and inexpensive. They do not need extensive experience, but can give high rates of false positive or false negative results. Colour tests need high concentrations of the analytes in the test matrix.

7.2.5. Thin Layer Chromatography

Thin layer chromatography (TLC) needs extraction and concentration of the drug metabolites from the matrix prior to separation and detection, so its identification capability is limited. TLC has a broad spectrum for drug detection, so it has low specificity.[137]

Fumio Moriya et al, 1994 [148] used TLC for the identification of opiates after SPE, eluted by chloroform:isopropanol (9:1 v/v), evaporated under nitrogen then reconstituted in ethanol and an aliquot applied to TLC. The plate was developed with ethyl acetate:methanol:concentrated ammonium hydroxide (34:4:3 v/v/v). Spots were visualised with iodoplatinate spray, sensitive enough to detect down to 300ng/ml in urine.

Some research compared maternal urine and meconium using TLC. The maternal urine was analysed by TLC and confirmed by enzyme immunoassay, gas liquid chromatography and ultraviolet spectroscopy. The infant samples were analysed by enzyme immunoassay and confirmed by one of the same methods, or by the result of maternal urine sample. Quantitative data on these results were considered unreliable and eliminated from statistical analysis.[174]

7.3. Hair

Drugs which cross the placenta and their metabolites are found in foetal hair which grows during the 3rd trimester. Therefore hair analysis reflects drug exposure during this time period. The growth cycle of neonatal scalp hair is different from infants as all hair fibres except those located at the vertex region are lost between 8-12 weeks after birth. Also, the amount of hair is very small, so allowing only one test which is

commonly performed by radioimmunassay.[153] Hair provides a very sensitive indicator of drug use by a pregnant mother, especially if analysed by radio-immunoassay. Validation studies have shown hair analysis to have a sensitivity of 96% and a predictive value of 100%, and that it does not give false positive results.[137]

Each hair consists of the hair follicle, cuticle and shaft. The rate of hair growth is 1-2cm/month, on average. Deposition of drugs occurs during the growth of the hair shaft and the recent portion is near the scalp. Sectional analysis is performed monthly to provide information on the duration and time of drug use by the mother. Detection and quantification can be carried out, especially for chronic drug users, by many methods including collisional spectroscopy. The proximal segment of the foetal hair corresponds to the 12th week, approximately, before delivery which gives information about the concentration of cocaine and its metabolite benzoylecgonine in foetal hair which grows during the 3rd trimester.[137]

Drugs are incorporated into the hair follicle then grow into the cuticle and shaft. The concentration of drug varies according to period of exposure. Hair gives information on the duration and time of drug use, but it needs consent from the patient as the analysis needs a bundle of hair approximately 0.6 cm in diameter from the posterior scalp.[140] Hair analysis has some limitations as some precautions are needed in collection and the analysis is generally expensive. Hair is affected by many factors that modify the quantity and concentration of drugs including its length, any hair dye, bleach, cosmetic agents and environmental contamination. So foetal hair must be taken in hospital before the newborn reaches home.[137]

The mechanism of drug transport into hair is not well understood, but depends on the physiochemical properties of the drug such as melanin affinity, lipophilicity, and membrane permeability. Hydrophilic drugs such as cocaine and heroin in the parent form tend to be concentrated in the modulated sections of hair, more than blood. The ratio of cocaine and benzoylecgonine concentration in adult hair is 10.5 times higher than child hair, as foetal hair has no medulla.[175]

Several drugs can be detected in hair by radioimmunoassay, FPIA, and GC/MS including morphine, codeine, monoacetylmorphine, nicotine, cocaine and benzoylecgonine.[176] Exposure to cocaine and its metabolites is considered a serious problem and its detection depends on the time of exposure, as it accumulates early in gestation with a short half life and in small quantities. Urine analysis was the first biological sample used in its detection but presents many problems such as proper specimen collection, time of collection, urine pH, method of analysis, temperature, the degree of dilution, and limitation of detection of only one drug alone.[175] Meconium and hair are biological samples used as alternatives to urine for detection of cocaine and its metabolites, which are embedded into the hair shaft and remain for the life of the hair or until it is cut. As the hair of the neonate grows during the last 3 months of pregnancy, a positive neonatal hair test uncovers an addiction pattern in which the mother consumed the drug long after she become pregnant.[175] The time of hair collection is not critical, but its analysis first requires decontamination, which is usually done in several steps. Firstly, it is washed with several solvents (methanol, ethanol, dichloromethane, acetone, detergent, or warm water). Drugs are then extracted from the hair by incubation with methanol, ethanol, acid, and proteinase or

pronase. Then the extract is analysed by RIA (needs 2-5 mg hair) or by GC/MS (which needs 1-10 mg hair).[141]

The neonatal hair was not washed prior to analysis as external deposition of cocaine from amniotic fluid is an indicator of prenatal exposure[175]. The foetus swallows amniotic fluid at a rate of 0.5L/day and bathing in it causes toxins circulating in the amniotic fluid to reach the foetus via the transdermal route. Because of low keratinisation, foetal skin is readily permeable to exogenous substances, although external infection is not common. Some investigators prefer meconium to hair, although it is available only during the first 2-3 days of life, and on some occasions is not excreted even up to three days after birth. Hair testing is also considered to be very sensitive and to give good results, especially if the urine test gives a false negative result.[175] Cocaine detected in the proximal segment of the mother's hair corresponds to her drug exposure history, but in foetal hair gives a sensitivity up to 78% with GC/MS (compared to 74% for meconium) and 52% with FPIA. Urine analysis gives a sensitivity of 38% and can result in misclassification of more than 60% of cases. In these statistics, *sensitivity* is defined as the percentage of true positives which are detected.

In summary, analysis of hair samples from the neonate is often not practical because of the problems in collecting suitable specimens and surveys may as a result be unreliable and often not reproducible. In contrast, drugs accumulate in meconium and are deposited in the last half of gestation. The drugs are essentially trapped in the meconium due to being tightly bound to cell fragments in the matrix that effectively prevent diffusion.[173]

7.4. Nail

Nail is formed in the 2nd and 3rd trimesters of pregnancy as it grows from 3rd month of gestation and reach the fingertip at the 8th month. During this time drugs are incorporated into the nail from the circulation and by soaking in the amniotic fluid.[44] At delivery, the composition of the newborn nail reflects drug exposure during this period of gestation. The sample size is always sufficient to allow confirmation by RIA and GC/MS, as 10mg may be available, while hair usually does not exceed 1-5 mg. Hair grows in an intermittent cycle while nail grows continuously, so assisting epidemiological studies.

The common drugs which have been detected in nail are morphine, 6-acetyl-morphine, codeine, cocaine, and benzoylecgonine. The identification and quantification of these drugs has been done by GC/MS. The limit of detection was as low as 1ng/10mg of nail powder for 6-acetylmorphine, morphine and cocaine while for benzoylecgonine it was 2ng/10mg of nail powder and for codeine was 3ng/10mg-nail powder. No other drugs in this study were detected at this limit of detection.[153]

7.5. Amniotic Fluid

Amniotic fluid is the fluid that surrounds the foetus through the pregnancy. All the foetal food and secretions are present in it and it contains high concentrations of drugs. Amniotic fluid is collected by amniocentesis to show lung maturity.[141] More is available in premature rupture of membrane. Generally, analysis of amniotic fluid has not been shown to give any benefit over urine analysis.[143]

Cocaine, benzoylecgonine and ecgonine methyl ester have been screened in amniotic fluid by GC/MS. The average limit of detection was 5ng/ml. Cocaine and morphine have also been detected by fluorescence polarisation immunoassay using the Abbot TDX system.[177] Cocaine and its metabolites can be found in amniotic fluid after maternal use, due to foetal urination after 18-20 weeks gestation and the foetus may repeatedly exposed to cocaine and its metabolites by swallowing amniotic fluid. The concentrations of benzoylecgonine and cocaine in amniotic fluid ranged from 400 to more than 5000 ng/ml and up to 250ng/ml, respectively. Neonatal urine contains these drugs at $1/6^{th}$ of the amniotic fluid level. A comparison of drug levels in three biological samples - amniotic fluid, umbilical cord blood and neonatal urine - found that benzoylecgonine was present in all three samples but the concentration was higher in urine than amniotic fluid, and was lowest in umbilical cord blood. However, more cocaine was present in amniotic fluid than urine.[178]

Cocaine and its metabolites reach the foetus by foetal swallowing, through the umbilical cord, or by diffusion across the placental surface vessels, so swallowing is not the only route. Desmethylation of cocaine by the foetal liver, with subsequent deposition into the intestine, then results in accumulation in amniotic fluid. The recovery of cocaine from plasma and amniotic fluid was 100% whereas for norcocaine and benzoylecgonine it was 86% and for ecgonine methyl ester was 66%. The minimal detectable levels were 5, 10, 5 and 10ng/ml respectively.[145]

The presence of cocaine and metabolites in amniotic fluid did not appear to influence foetal oxygenation. Cocaine was found in the largest quantities after 72 hours at a concentration of 350ng/ml, followed by ecgonine methyl ester and benzoylecgonine,

with very low concentrations of norcocaine. Cocaine, benzoylecgonine and norcocaine can be detected in foetal and maternal blood up to 24 hours after administration at approximately 3% of the amniotic fluid concentration. Cocaine when present in amniotic fluid is metabolised primarily to ecgonine methyl ester, benzoylecgonine and norcocaine. This was *in vitro* but *in vivo* it was found that cholinesterase is present in amniotic fluid and is responsible for the metabolism of cocaine to ecgonine methyl ester. This also depends on temperature and pH: the higher the temperature and pH the faster the hydrolysis.[179]

7.6. Blood Analysis

Blood analysis is the longest established method used for detection of neonatal drug exposure, especially to morphine, which has been used increasingly in intensive care during the past 10 years. Morphine is also used in the newborn with respiratory distress syndrome as it reportedly stabilises the fluctuations in arterial blood pressure and decreases the risk of intracranial haemorrhage. Morphine elimination may be prolonged in the neonate, and some studies have also shown that clearance of morphine is reduced in preterm neonate. Analysis of blood and urine indicates recent drug exposure while drug detection in meconium is considered to be a marker of remote drug exposure. Little is known about the relationship of cocaine metabolites in meconium compared with blood.[137]

Esterase activity facilitates the breakdown of cocaine. There is a relation between meconium BZE level and cord pseudocholinestrase activity. If benzoylecgonine is present in cord blood this means recent drug use and usually BZE is present in lower amounts than in meconium, as catabolism in meconium is less complete than in

blood, which has no cocaine or little benzoylnorecgonine, while meconium contains all of these. This was taken to mean rapid metabolism in the blood in what looked like a relapsed cocaine-abusing mother. Foetal biological fluids can be used for detection of cocaine metabolism, and similar results were found in both cerebrospinal fluid and cord blood, about 1.24μg/ml. Cocaine metabolites in meconium indicate past exposure, and their presence is due to slow release or cycling through amniotic fluid. Chronic drug use results in excretion of benzoylecgonine for up to 3 weeks after last intake, which affects behaviour leading to depression of the infant. Also the neonatal irritable syndrome appears after 1 month when the mother uses codeine during pregnancy, which has similar pharmacokinetics to those found in cocaine metabolism. [180]

7.6.1. Previous Analytical Methods

There are several method for the detection of cocaine, benzoylecgonine and other metabolites in cord blood. These have been used to investigate the relationship between the amount and distribution of cocaine species. Measurements have been made of cholinesterase (which is important for hydrolysis of cocaine), catabolic capacity and correlation with metabolite levels.[137]

The cord blood has been analysed by HPLC using a C18 column and ultraviolet detection. Positive samples were confirmed by GC/MS for the presence of benzoylecgonine, norcocaine and cocaine.[180] BZE can be separated from umbilical cord by SPE. Also, pseudocholinesterase can be used to detect BZE in serum using the Du Pont ACA Discrete Clinical Analyser, which depends on photometric measurement of the conversion of the blue dye 2,6-dichlorophenolindophenol to a colourless compound when butylthiocholine is hydrolysed by pseudocholinestrase.

Calibration and verification were performed according to clinical standards. Recovery ranged from 71% to 90% with a coefficient of variation in the range 3.1 to 6.9% and limit of quantification of 0.10μg/g.[180]

7.7. Breast Milk

Drugs of abuse can transmitted through breast milk, so if the mother is a chronic user it is contraindicated for her to breast feed her child. Chasnoff et al (1989)[181] reported infant toxicity by exposure to cocaine through breast milk. Cannabinoids and alcohol are also transmitted via breast milk and are present for a longer time than in serum. If the mother take drugs intravenously, her child may be exposed to hepatitis B, C, and HIV.[5] Nicotine can transmitted with its metabolites in breast milk. Nicotine is present in milk in concentrations between 1.5 and 3.0 times the simultaneous maternal plasma concentration.[182]

7.8. Urine Analysis

Urine is the most common biological fluid used for drug abuse detection. It has some advantages including that it is easily collected by non-invasive procedures, exhibits high concentrations of drugs due to the concentrating ability of the kidney, large amounts may be collected, it is more easily analysed than blood, drug metabolites are stable, especially if it is frozen, and it can be used in all instrumental methods.[137,140]

Urine has some disadvantages, for example it can give false negative results, urine specimens can easily be exchanged by the donor with another, clean sample, it can easily be diluted or other things added, like salt, which interferes with the testing methods, and also, it gives information only about recent exposure to drugs.[137]

It is now uncommon to use infant urine as that is difficult to collect, needing a specially positioned bag to obtain it, need repetition to collect a suitable specimen, and a skin rash may occur if it is in contact with the skin. The amount collected is usually not enough for both a primary and confirmatory test and the time of sample collection is very important to be within the window of detection, as after a certain period false negative results can be obtained.[141]

The window of detection depends on many factors: individual drug metabolism, hydration status of the subject, route of administration, and frequency of use, as most drugs appear in urine if used only 48 to 72 hours before analysis. In chronic heavy users of cannabis positive results can be obtained for over 1month after quitting. No cross-reactions are found with the immunoassays for cocaine or cannabis, but this is not so for amphetamines or opiates, as different types of food contain ephedrine or phenylpropanolamine, which cross-react with RIA and so give false positive results. Therefore, a confirmatory test is necessary. For opiates, immunoassay does not differentiate between morphine and codeine, the presence of which is taken as conclusive when trying to separate street heroin use from prescribed pain medicaments, which often contain morphine as a constituent. Immunoassay gives positive results for opiates up to 66 hours after administration.[140]

Several drugs can be detected in urine by different methods, involving extraction by SPE and screening by GC/MS. For cocaine, the limit of detection was 5ng/ml urine. Cocaine concentrations in urine are the same in mother and foetus. Cocaine metabolites are found in high concentrations in meconium but the cocaine concentration is lower than its metabolites.[143]

Another study involved screening by enzyme multiplied immunoassay technique and confirmation by GC/MS. The specimens with internal standard added were treated with acetate buffer and added to conditioned SPE columns, which were washed and extracts were eluted with 6ml methylene chloride: isopropanol: concentrated aqueous ammonium hydroxide (80:20:2 v/v/v). The organic phase was evaporated and the residue reconstituted in 0.02ml acetonitrile. Extracts were treated with 0.02ml BSTFA (containing 1% TMCS) heated at 60°C for 30min, then analysed by GC/MS on an HP1 column (12m x 0.2mm i.d x 0.33μm film thickness). The injector port was at 250°C and the oven temperature started at 70 °C for 1 minute, rising to 220°C at a rate of 35°C/min, then to 280°C at a rate of 30°C/min. Full mass spectra were collected repetitively over the range 50-550 mass units. The limit of detection was as low as 7.5ng/g. The mean concentrations of ecgonine methyl ester and cocaine were highest followed by benzoylecgonine and norcocaine, then the lowest was cocaethylene.[168]

Susan, et al. (1994)[183] analysed neonatal urine by RIA, which recovered 31% of positive cases, with a high rate of false negative results, but metabolites of drugs are found in high concentration in the gastrointestinal tract of the foetus so it is preferable to analyse meconium.

Urine sample analysis was performed by immunoassay and GC/MS for morphine, methadone, barbiturates, phencyclidine, hydrochloride, amphetamine, methamphetamine, diazepam, benzoylecgonine, and ecgonine as metabolites of cocaine and the positive group of infants was compared with controls and the results for maternal urine samples.[184]

Maternal urine was screened and confirmed as follows: cocaine, identified as benzoylecgonine by FPIA and confirmed by GC/MS, cannabis metabolites by EMIT and confirmed by GC/MS, amphetamine, benzodiazepines and opiate by EMIT and GC. Urine samples of infants were analysed for cocaine by FPIA and confirmed by GC/MS. The EMIT screening cut-offs were as follows: amphetamine, 1000ng/ml, benzodiazepines, 300ng/ml, benzoylecgonine, 50ng/ml, THCOOH, 15ng/ml, cannabinoids, 50ng/ml, opiates, 300ng/ml.

Analysis of infant urine missed 25% of the infants which gave positive results with meconium, so meconium is considered a good sample to analyse for cocaine rather than urine.[149] Urine was also analysed by RIA, with a lower limit of detection of 2ng/mg.[170]

Maternal urine sample analysis required 50ml urine and infant urine samples were tested for cocaine, heroin, methadone and tetrahydrocannabinol by normal TLC and confirmed by EMIT. Sensitivity of TLC for urinalysis was 1mg/ml for opiate and cocaine and EMIT had a cut-off set at 0.3mg/l. TLC analysis of urine samples may give false negative results so other techniques are preferred.[181]

8. Extraction of Multiple Drugs of Abuse from Meconium Using a Single SPE Column:

8.1. Introduction:

Previous work with meconium has generally been concerned with the analysis of single drugs or drug groups, especially cocaine and its metabolites in studies carried out in the United States. For the present project, the ultimate intention was to carry out a survey of drug use amongst pregnant mothers in the Glasgow area, based on toxicological screening, and a method was required to permit this to be done with small samples of meconium. The method described in Chapter 3 of this Thesis, involving SPE with Bond Elut Certify™ columns followed by qualitative and quantitative analysis by EIA and GC/MS was suitable for this purpose. However, adaptations were required to take into account the differences between blood and meconium in the initial sample preparation and treatment steps.

The target analytes were chosen as the most commonly used and abused drugs in the Glasgow area, which are known from the casework carried out in the Forensic Medicine Department at Glasgow University. These were: cocaine and its principle metabolite benzoylecgonine, morphine, codeine, methadone, diazepam, temazepam, tetrahydrocannabinol (THC) and tetrahydrocannabinol carboxylic acid.

An additional procedure was also introduced separately for the analysis of amphetamines, and this is described in Chapter 9.

8.2. Materials and Instrumentation

8.2.1. Materials

All chemicals were purchased from SIGMA Company (Dorset, UK). The solvents and concentrated ammonia were of HPLC grade supplied by BDH laboratories supplies. Analytichem International. Bond Elut Certify™ columns were obtained from Varian (Harbor city, California, USA), and the Baker-20 SPE vacuum manifold system was from International Sorbent Technology.

Phosphate buffer (0.1M) was prepared by dissolving 6.81g of potassium dihydrogen phosphate in 450ml of de-ionised water, adjusting the pH to 6.0 ± 0.1 with 1.0M potassium hydroxide, then making the total volume up to 500ml with de-ionised water. Acetic acid solution (0.01M) was prepared by adding 57.5 µl of glacial acetic acid to 100ml of de-ionised water.

The SPE eluant was prepared daily by mixing chloroform and acetone in the ratio 1:1 v/v. Ammoniated ethyl acetate (2%) was used as eluant II and prepared daily by adding 1ml of ammonia to 49ml of ethyl acetate. Both were sonicated for at least 5 minutes before use.

Cocaine, benzoylecgonine, morphine, codeine, THC and THC-carboxylic acid were purchased from SIGMA, and deuterated materials were from Radian Corporation (Austin, TX).

Enzyme Immunoassay (EIA) was carried out using kits from Cozart Bioscience Ltd., Oxfordshire.

8.2.2. Instrumentation

Gas chromatography-mass spectrometry was carried out using a VG Micromass 70-250S double focusing magnetic sector instrument. The gas chromatograph (Hewlett Packard 5890 Series II) was equipped with a fused silica capillary column (HP5, 30m x 0.32 mm id, 0.3 μm film thickness). Samples (volume 1ul) were injected with an injection port temperature of 280°C. The column oven was programmed from 200-300°C. The mass spectrometer was operated at a resolution of 1000 in the EI+ mode (ionisation energy 70 eV, source temperature 220°C). The instrument was tuned daily using perfluorokerosene according to the manufacturer's recommendations. Mass spectra were obtained by scanning from 600 to 40 AMU at 0.3 s/scan and data were recorded with a Masspec 1 data system from Mass Spectrometry Services, Manchester, UK. Quantitative analyses were carried out in the SIM mode, using deuterated analogs of the target analytes as internal standards.

8.3. Methods:

8.3.1. Sample Collection

Samples of meconium were collected from diapers of newborn infants delivered at the Royal Maternity Hospital, Glasgow 9 these are real case samples). The meconium was recovered using a wooden spatula and stored in plastic tubes at –4°C until analysed. In addition, 30 samples of meconium were collected from neonates delivered in several hospitals in Egypt who were not exposed to drugs *in uteri.* These were used as controls.

8.3.2. Sample Preparation

Each meconium specimen was weighed (range from 0.5g - 0.8g) into a clean vial and centrifuged at 2000rpm for 10 minutes to bring it to the bottom. Drug standards were added and the samples were homogenized by vortexing and sonicating for 15 minutes. Methanol (3 ml) was added, and the tubes were vortexed well for 10 minutes then centrifuged at 3000rpm for 20 minutes. The upper layers were transferred to clean tubes and evaporated to dryness under nitrogen, initially at room temperature then with slight warming up to 40°C until completely dry. Once dry, the residues were dissolved in 700 µl methanol with vortexing. The extraction tubes were then centrifuged at 2000rpm for 10 minutes.

8.3.3. Sample Extraction

The solid phase extraction (SPE) procedure applied was as follows:

- the column was prepared by washing with 2ml methanol, followed 2 ml phosphate buffer (pH6);
- the sample was applied at low vacuum pressure;
- the column was washed with 1ml distilled water, followed by 0.5ml acetic acid (1.0M, pH 3.3).
- the column was dried at maximum pressure for 4 min, then 50 µl methanol was added and the vacuum reapplied for 1min.

Elution was carried out in two stages:

- 4ml of eluant A (chloroform:acetone 1:1 v/v);
- 2 x 2ml of eluant B (2% ammonia in ethyl acetate).

A 500 µl aliquot of eluant B was analyzed by EIA for opiates, cocaine, methadone, cannabinoids and benzodiazepines.

To the remainder of the eluant, the deuterated forms of each target analyte were added as internal standards at a concentration of 10ng/ul. The extracts were mixed well and evaporated to dryness under nitrogen at 40°C until completely dry, then 50µl BSTFA were added, the vials were capped and incubated at 60°C for 20 minutes prior to GC-MS-SIM analysis. Selected ions used for quantitative analyses are listed in Table 8.1 along with the retention times for each analyte.

Table 8.1: Selected Ions Used for GC/MS/SIM

Target analyte	Retention Time (min)	Selected ions (m/z)	Internal Standard	Selected ions (m/z)
Cocaine	6.21	303	$^2H_3$-cocaine	306
Benzoylecgonine	7.34	361	$^2H_3$-benzoylecgonine	364
Morphine	8.58	429	$^2H_3$-morphine	432
Codeine	11.68	371	$^2H_3$-codeine	374
Methadone	9.03	72	$^2H_3$-methadone	75
Diazepam	8.55	284	$^2H_5$-diazepam	289
Temazepam	9.33	372	$^2H_5$-diazepam	289
THC	9.15	386	$^2H_3$-THC	389
THC-COOH	10.15	488	$^2H_3$-	491

8.3.4. Limit of detection and extraction recoveries

These were determined by spiking blank meconium with the target analytes. Ten different meconium samples were spiked with standards and analyzed as described earlier in order to determine the extraction recovery of the method. Furthermore, ten

meconium samples were spiked with decreasing amounts of drug standards and then analyzed as before to determine the limits of detection of the method. To determine the extraction recoveries, the internal standards (deuterated form of each drug) were added to the eluates obtained by SPE and these extracts were compared with unextracted standards.

8.3.5. Application to Real Case Samples

The same method was then applied to 25 real cases from the Royal Maternity Hospital, Glasgow. Extracts were initially screened by EIA and then confirmatory quantitative analyses were carried out by GC/MS.

EIA Analysis:

The EIA method used was an Enzyme Linked Immunosorbent Assay (ELISA) from Cozart Company. This assay involves the enzyme horseradish peroxidase, which produces a blue colour with its substrate. 500 μL of elute B was evaporated till dryness under nitrogen then reconstituted in EIA buffer (1ml). This was then analysed for morphine, cocaine, methadone, cannabis, and benzodiazepines using EIA kits containing antisera highly specific to free (unconjugated) form of parent drug and with a low cross reactivity to codeine. The method followed the same steps previously mentioned. The calibration curve was prepared from standards containing the analytes at concentrations of 0, 10, 100 and 500 ng/ml.

GC/MS

A 1.0ul aliquot of each standard and each extract was injected into the GC/MS instrument. Identification was achieved by comparison of retention times (t_R) and selected ion of each and quantification was achieved by comparison of the peak area

ratios from SIM chromatograms of extracts with those of standards. Meconium standards for quantification were prepared by spiking blank meconium at two concentrations (5 and 50 ng/g). Each concentration was prepared in triplicate and the average result was taken.

8.4. Results and Discussion

8.4.1. Method Validation

The developed method was satisfactory: blank meconium gave negative results and the target analytes were successfully extracted from spiked meconium samples and quantified. Specimen chromatograms and mass spectra of extracted standards are shown in Figures 8.1- 8.3.

Calibration curves for the target analytes were linear and had linear correlation coefficients (r^2) in the range 0.902 to 0.978. The calibration ranges chosen for the analytes were selected from the available literature data.

Recoveries for the analytes ranged from 50-100% (Table 8.2) but were considered acceptable if the method could be generally applied for the intended purpose. The lower limit of detection for each analyte is summarised in Table 8.3.

The reproducibility of the method was established by analysing five replicates of standards at five concentrations (10, 20, 30, 40 and 50ng/ml) for morphine and codeine, following the same method of extraction. The morphine and cocaine calibration curves, created using the means of these replicates, are shown in Figures 8.4 and 8.5. The stability of the extracts was evaluated by analysing the same samples immediately inject after extraction, and same samples are injected after two days, after one week and after two weeks. This data was used to measure the mean,

standard deviation, and relative standard deviation for each analyte and the results are show good reducibility in Table 8.4.

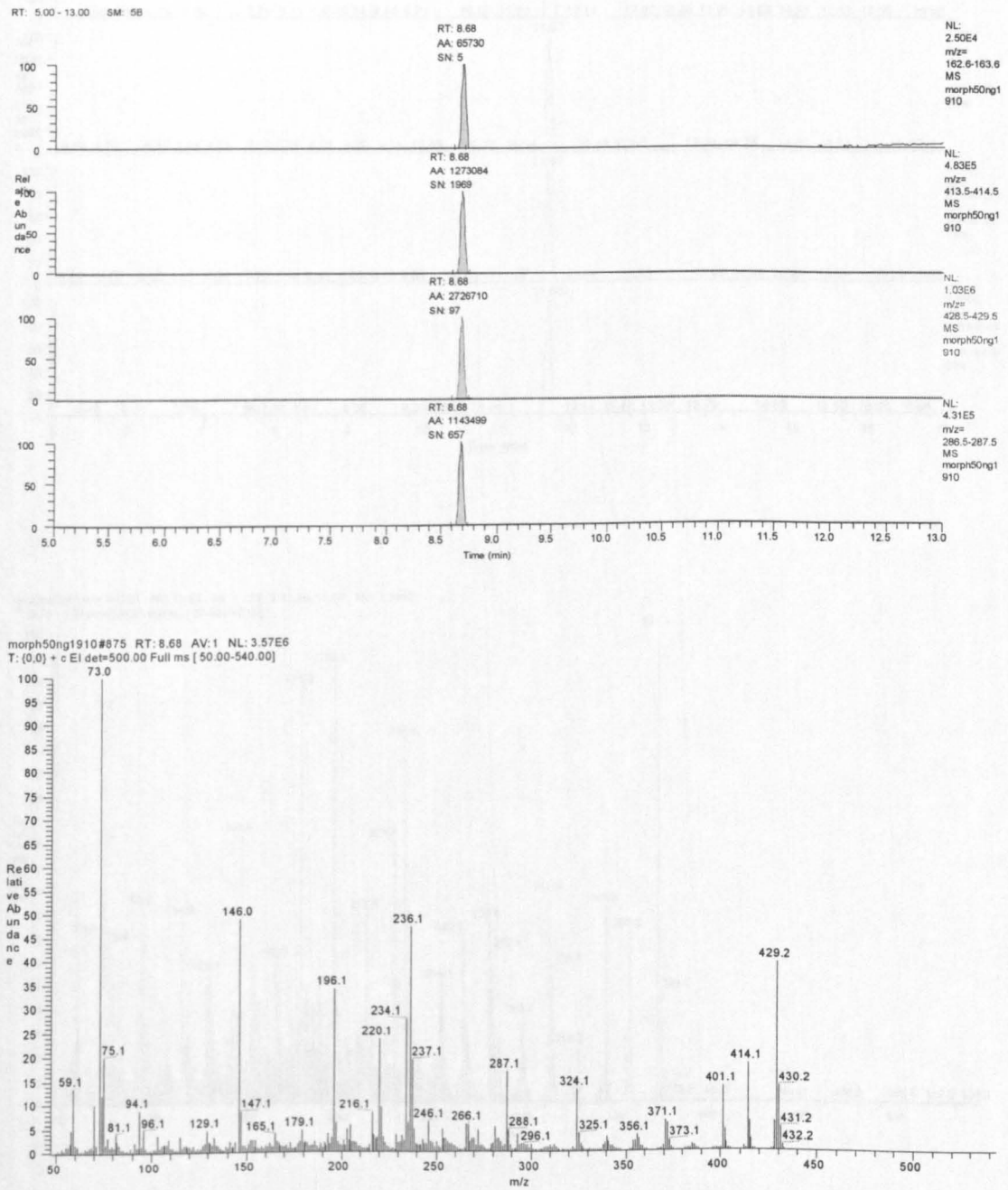
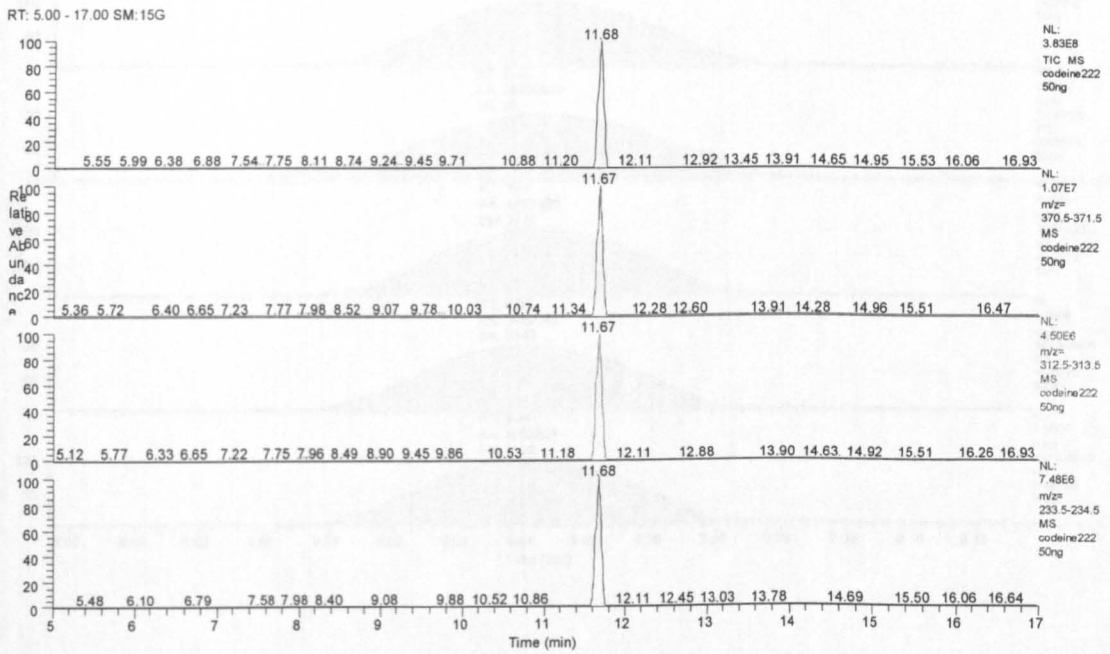


Figure 8.1 SIM Chromatograms and Mass Spectrum for Morphine.



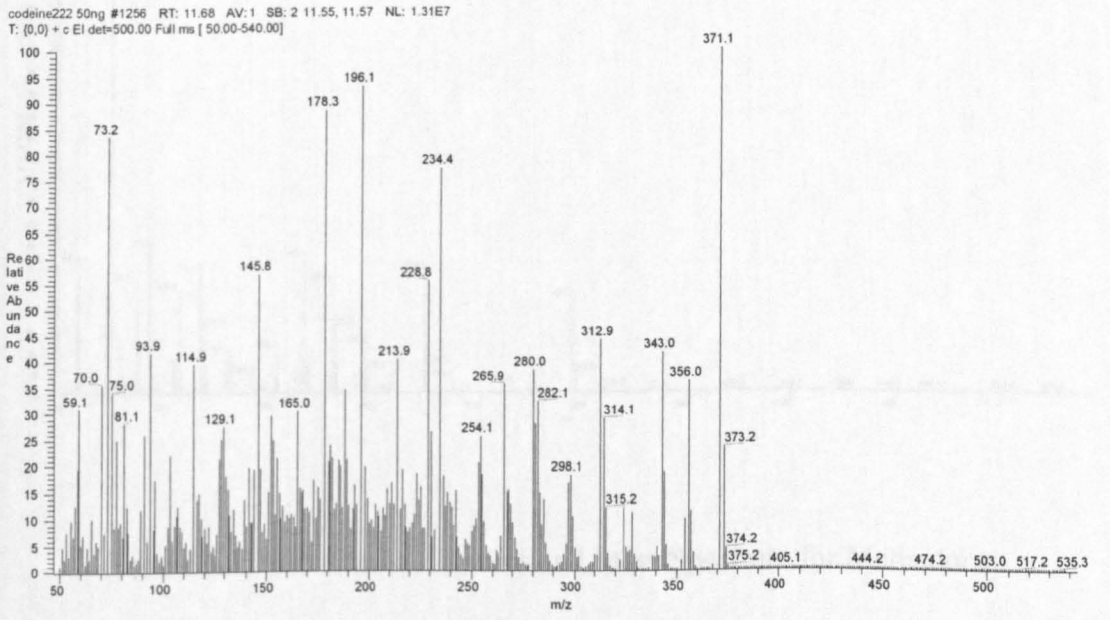
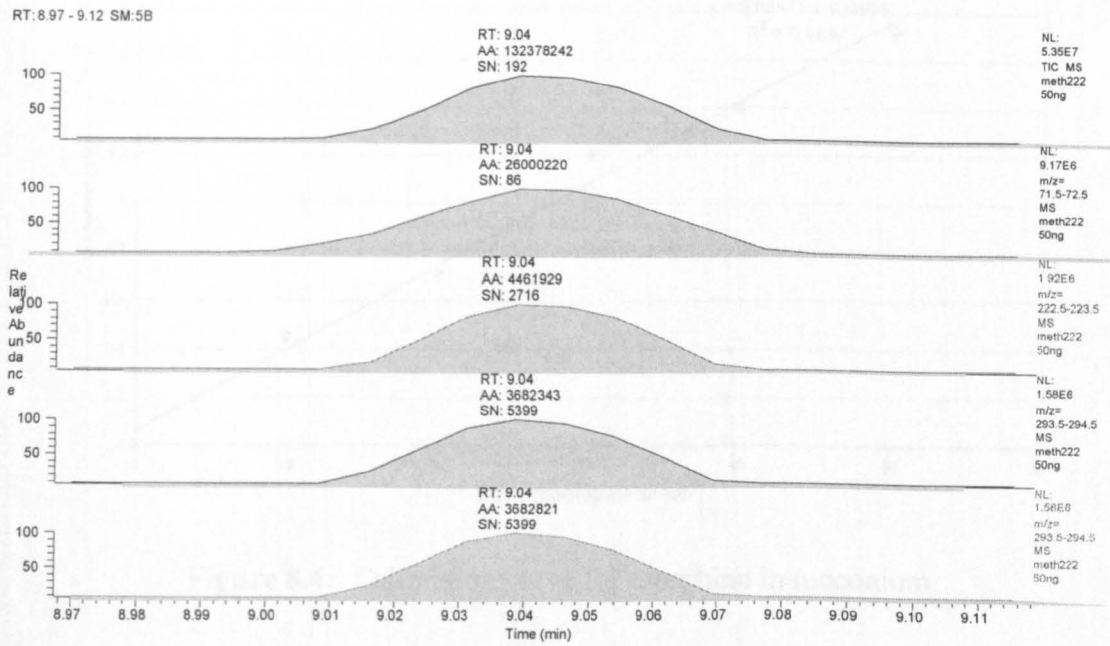


Figure 8.2 SIM Chromatograms and Mass Spectrum for Codeine.



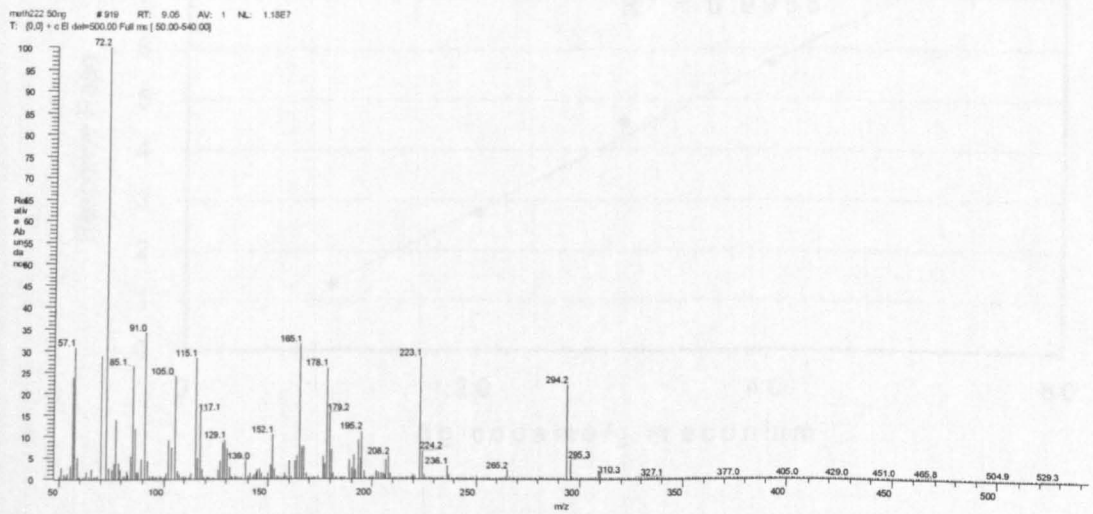


Figure 8.3 SIM Chromatograms and Mass Spectrum for Methadone.

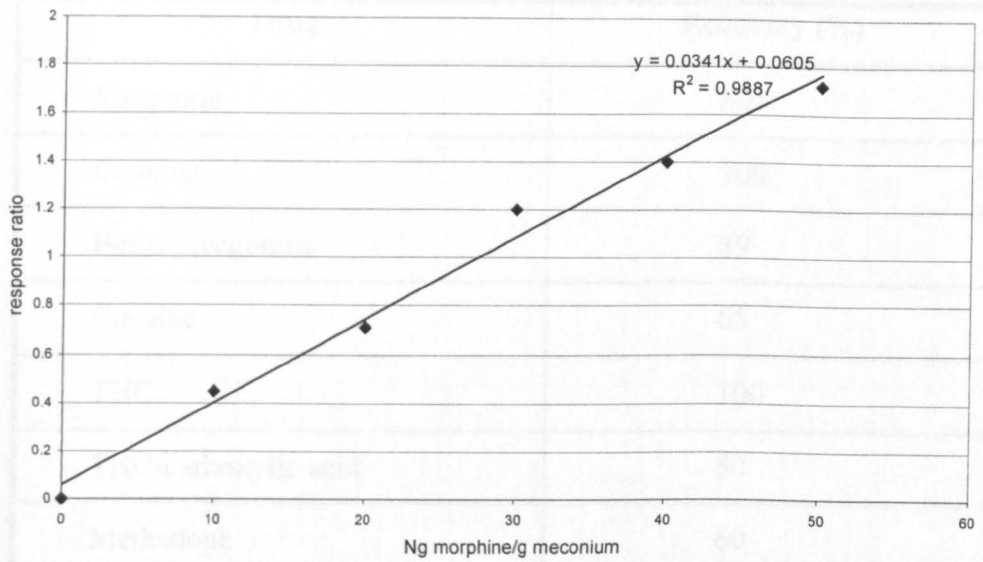


Figure 8.4: Calibration curve for morphine in meconium

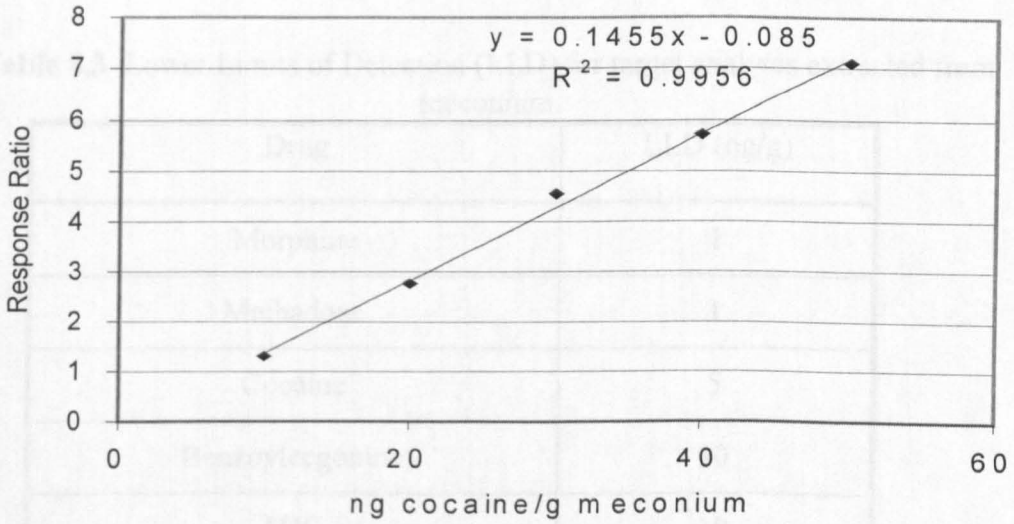


Figure 8.5 Calibration curve for cocaine in meconium

Table 8.2 Recoveries for target analytes extracted from meconium

Drug	Recovery (%)
Morphine	60
Codeine	100
Benzoylecgonine	89
Cocaine	65
THC	100
THC-carboxylic acid	50
Methadone	60
Diazepam	80
Temazepam	70

Table 8.3 Lower Limits of Detection (LLD) for target analytes extracted from meconium

Drug	LLD (ng/g)
Morphine	1
Methadone	1
Cocaine	5
Benzoylecgonine	10
THC	10
THC-carboxylic acid	20
Codeine	25
Temazepam	25
Diazepam	25

Table 8.4 Mean, standard deviation and relative standard deviation for each analyte

Days after extraction	drug	Conc. ng/g	mean	SD	RSD
None	codeine	10	4.10	0.100	2.44
	morphine		1.65	0.119	2.34
	codeine	30	7.49	0.195	2.60
	morphine		3.31	0.199	6.01
	codeine	50	9.80	0.117	1.20
	morphine		4.20	0.158	3.76
Two	codeine	10	6.60	0.332	5.02
	morphine		1.98	0.148	7.40
	codeine	30	8.64	0.174	2.01
	morphine		3.78	0.172	4.55
	codeine	50	11.2	0.135	1.21
	morphine		6.40	0.178	2.78
Seven	codeine	10	5.46	0.305	5.58
	morphine		1.94	0.125	6.46
	codeine	30	3.78	0.172	4.55
	morphine		8.46	0.480	5.67
	codeine	50	11.5	0.146	2.46
	morphine		5.10	0.186	2.45
Fourteen	codeine	10	4.44	0.890	5.15
	morphine		1.875	0.083	4.45
	codeine	30	7.66	0.114	1.49
	morphine		3.18	0.179	5.63
	codeine	50	9.20	0.122	2.44
	morphine		5.30	0.148	2.84

8.4.2. Application to Real Cases

The developed method was applied to 25 case samples which were collected at the Royal Maternity Hospital, Glasgow, from neonate on the first day after delivery using special stool vials with integral spoons in their caps. The specimens were kept frozen until collected and analysed.

The samples were first analysed by EIA: some of those which were positive for opiates, cocaine, methadone and benzodiazepines gave nominal concentrations which were outside the calibration range, while the two cannabinoid positives were both within the calibration range. The results of EIA analysis are given in Table 8.5.

The samples were confirmed and quantified by GC/MS, after adding the IS to the remaining eluate. Morphine was detected in 23 of the case samples, the other two being negative. The concentration range was 10-1070 ng/g meconium and these are plotted in Figure 8.6 below. It can be seen that two cases are significantly higher than the others. It became apparent later that most mothers in the Royal Maternity Hospital were given diamorphine as a sedative prior to Caesarean section or when the mother required it. The two high cases may therefore represent mothers who had used heroin during pregnancy.

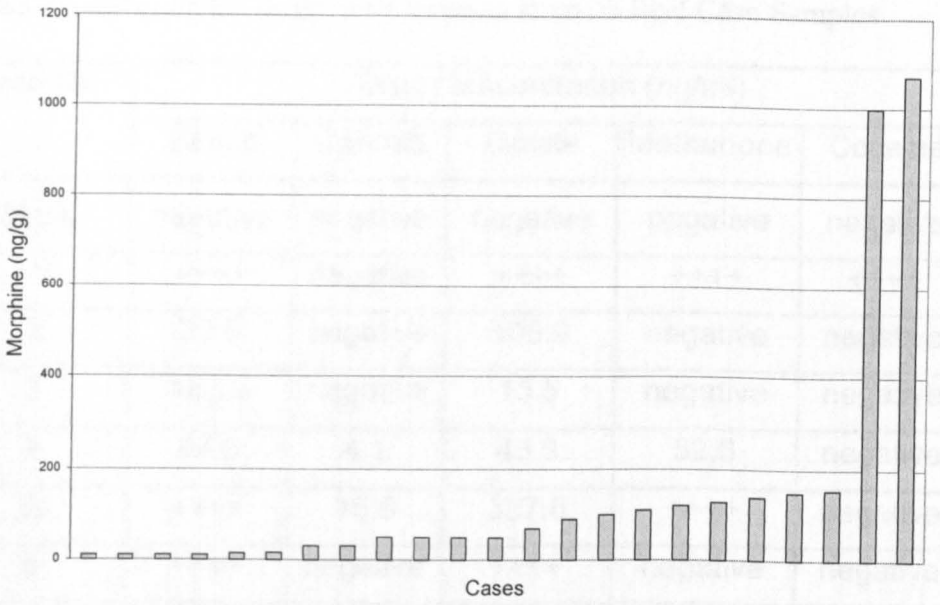


Figure 8.6 Histogram of morphine concentrations in meconium from hospital cases.

Table 8.5 Results of EIA Analysis of Extracts from 25 Real Case Samples

Case No.	Drug Concentration (ng/ml)				
	Benzo	Cannab	Opiate	Methadone	Cocaine
Blank	negative	negative	negative	negative	negative
1	++++	negative	++++	++++	++++
2	20.5	negative	105.9	negative	negative
3	183.6	negative	13.5	negative	negative
4	47.6	4.1	43.3	52.6	negative
5	++++	15.6	387.6	++++	negative
6	++++	negative	++++	negative	negative
7	negative	2.5	82.8	66.1	negative
8	negative	negative	132.8	negative	negative
9	negative	negative	243.4	negative	negative
10	negative	negative	46.6	negative	negative
11	34.9	negative	109.6	negative	negative
12	negative	negative	68.8	negative	negative
13	23.8	negative	59.6	negative	negative
14	negative	negative	57.3	negative	negative
15	17.4	negative	60.9	negative	negative
16	negative	negative	88.5	negative	negative
17	negative	negative	56.6	negative	negative
18	negative	++++	60.9	negative	negative
19	negative	negative	48.7	negative	negative
20	negative	negative	62.1	negative	negative
21	negative	negative	++++	negative	negative
22	negative	negative	72.9	negative	negative
23	-negative	negative	negative	negative	negative
24	negative	negative	negative	negative	negative
25	negative	negative	++++	negative	negative

++++ Nominal concentration above the upper calibrator concentration

Table 8.6 Results of GC/MS Analysis of Extracts from 25 Real Case Samples

Drugs	Diaz	Tem	Mor	Cod	Meth	Coca	BZE	THC	THCA
Blank	-	-	-	-	-	-	-	-	-
1	114	-	100	80	319	20	-	-	-
2	147	-	128	380	1100	-	-	-	-
3	1281	-	998	-	-	-	-	-	-
4	680	432	1070	-	2190	-	-	599	-
5	632	912	120	-	237	-	-	1010	-
6	70	-	30	30	-	-	-	-	-
7	-	-	110	60	51	-	-	520	154
8	-	-	150	-	-	-	-	-	-
9	-	-	50	-	-	-	-	-	-
10	-	-	140	40	-	-	-	-	-
11	23	223	10	-	-	-	-	-	-
12	-	-	15	40	-	-	-	-	-
13	81	501	14	50	-	-	-	-	-
14	-	-	10	-	-	-	-	-	-
15	25	125	10	60	-	-	-	-	-
16	-	-	10	30	-	-	-	-	-
17	-	-	156	-	-	-	-	-	-
18	-	-	50	15	-	-	-	175	122
19	-	-	70	60	-	-	-	-	-
20	-	-	90	110	-	-	-	-	-
21	-	-	50	40	-	-	-	-	-
22	-	-	30	20	-	140	-	-	-
23	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	-	-	-
25	-	-	50	10	-	-	-	-	-

(Diaz = Diazepam, Tem = Temazepam, Mor = Morphine, Cod = Codeine, Meth = Methadone, Coca = Cocaine, BZE = Bonzoylecgonine, THC = Tetrahydrocannabinol, THCA = THC carboxylic acid metabolite, - = Negative sample)

8.5. Conclusions:

The use of drugs by the pregnant mother can be detected by meconium analysis. Multiple drug analytes can be extracted by the SPE technique, screened using EIA and confirmed by GC/MS. The use of meconium analysis is now gaining more support in forensic toxicology laboratories worldwide especially in the United States, due to the long-term retention of drugs in meconium and the possibility of detecting maternal drug abuse over periods ranging in time from beginning of early pregnancy (12 weeks) till labour. In addition, meconium samples are collected by non-invasive procedures, only a small sample size is required, they are easy to store, and they are not affected by contamination, like nail, or by race bias, as with hair. Because of these advantages, meconium has the potential of becoming a powerful alternative to other biological samples for the detection of drug abuse in forensic cases.

The method was considered suitable for use in a proposed survey of newborn infants but additional methodological developments were first carried out to permit the inclusion of amphetamines in the study.

9. Extraction of Amphetamine and its Analogues from Meconium Using a Single SPE Column

9.1. Introduction and Aims

Recently drug abuse during pregnancy has become an epidemic problem and has serious effects on neonate. As mentioned before in previous chapters, a suitable method to extract multiple drugs from blood and meconium has been developed, but unfortunately this method can not be used for the amphetamine group (amphetamine, methamphetamine and their analogues), perhaps because of the derivatisation method or due to losses in the evaporation stage. As they are widespread illicit drugs, there has been some research published on estimating amphetamines in infant hair[185] and in meconium, and this involved liquid-liquid extraction with analysis by GC/MS.[136] Recently also Solohy et al., 1999[163] used liquid-liquid extraction, but there is no published method of extraction by SPE.

The aim of this part of the project was to extend the methodology available for the extraction of meconium by SPE to amphetamines and provide a suitable method for the proposed survey of newborn infants in the Glasgow area. The background to amphetamines will first be reviewed.

9.2. History of Amphetamines

Amphetamine along with its analogues (for convenience referred to below as "amphetamines") is a synthetic stimulant known since 1885. Its synthesis increased during the 1930's, when there were concerns that the supply of naturally occurring ephedrine might not be sufficient to meet the needs of asthma sufferers. So attempts

were made in several labs based to develop new synthetic routes based on the phenylisopropylamine molecule, which is considered as the starting point. Some of the first syntheses of different types of amphetamine and methamphetamine were by Ogata (with the brand name Methedrine)[186]

First amphetamine was used in treatment of schizophrenia seizures, which is unfortunate, bearing in mind that amphetamine itself induces psychosis. Amphetamines have been used in treating barbiturate overdoses, caffeine mania, smoking, multiple sclerosis, myasthenia gravis, head injury, cerebral palsy, migraine, urticaria, seasickness, dysmenorrhea, arterial colic, obesity, irritable colon, radiation sickness and other seemingly unrelated conditions, including loss of libido[187]. It was also used for the treatment of morphine addiction.[188].

Amphetamine is a potent CNS stimulant and the first case of amphetamine-related death was reported a few years after its introduction. There are serious complications associated with it: stroke, rhabdomyolysis and psychotic behaviour are the most common. Also cardiomyopathy is more common than with cocaine, while myocardial infarction is less common[189]. It was introduced in the 2nd World War to soldiers to overcome the effects of fatigue. Its use then spread in the UK, especially in London's West End clubs, especially amongst the youth which first used tablets called drinamyl (street name "purple heart"). The government tried to withdraw these tablets but was unable to stop amphetamine abuse[190].

Amphetamine abuse has caused social problems in many countries, even more than heroin, as abuse of these substances is growing rapidly, and is now a simple economic fact.[189] Amphetamine abuse is a relatively minor complication related to other drug abuse and accounted for less than 1% of drug emergency room calls and

less than 2% of drug related deaths in the DAWN survey for 1990 whereas cocaine represented 22%, so a suitable method for their determination is necessary (National Institute on Drug Abuse, 1992).

Methamphetamine is the N-methyl derivative of amphetamine and is used for treatment of obesity. Amphetamine and methamphetamine are abused especially in the United States, Sweden and Japan. They are classified as Class B, Schedule 2 drugs in the Misuse of Drugs Act (1971) in the UK. Methamphetamine use has increased since 1970's as it gives better sensations than amphetamine but is associated with strange and violent behaviour. There is evidence that methamphetamine is more commonly abused than cocaine, especially in cigarette smokers and in white women[191,192].

The ring substituted amphetamines, in particular 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxy-ethylamphetamine (MDEA) are abused for their hallucinogenic properties. MDMA along with MDA and MDEA are Class A, Schedule 1 drugs in the Misuse of Drugs Act (1971) and are most stringently controlled. Most common in the United Kingdom are ecstasy tablets, which may contain amphetamine, MDA[193, 194], MDEA[195,196] and paramethoxyamphetamine (PMA)[197] in addition to or in place of MDMA. These can cause severe toxicity and death but there is not enough information available about their toxicity. A survey done in 1996 reported that the most significant rise in drug use over last decade was in the dance drugs especially amphetamine[190], LSD and Ecstasy. Another study found amphetamine to be one of the most popular illicit stimulants in the UK, even preceding cannabis[198].

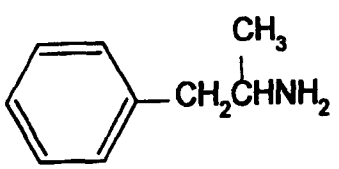
Ecstasy was also introduced in the 1st World War to suppress soldiers' appetites[199], then spread in U.S in psychotherapy treatment.[200] In the 1980's it spread amongst the addicts and at the end of this decade became known in the UK, especially during dance events or raves[196]. There is an estimated 500,000 people using ecstasy each weekend[201]. A survey done by Forsyth[202], which interviewed 135 participants in the Glasgow rave scene, found that they use other drugs beside ecstasy. Another survey by Hammersly et al.[203] on 209 individuals found that they used ecstasy once but also they used at least one other illicit drug. Another study was done by Handy et al[204] on 389 participants in the Cardiff area of South Wales where polydrug use and experimentation was prevalent. All of these studies concluded that there has been a change in the pattern of drug abuse since 1990.

9.3. Illicit Manufacture of Amphetamines

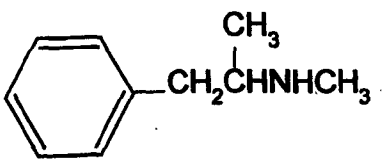
In 1958, the annual legal production of amphetamine was 75,000 pounds. By 1970, it had risen to over 200,000 pounds, enough to make 10 billion 5 mg tablets. . Consumption today is difficult to gauge. As the indications for medicinal amphetamine use became fewer and fewer, and more complications were recognised, legal production fell off. So illegal labs began to fill the void. In 1980, 150 illicit methamphetamine (as opposed to ice) labs were seized in the United States and by 1989 that number had risen to 650[205]. The first illicit drug syntheses used phenyl-2-propanone (P2P) as the precursor and then, when P2P itself became a controlled substance, clandestine chemists were also forced to synthesize it. P2P can be synthesized in a number of ways. Either (-) ephedrine or (+) pseudoephedrine can be converted to methamphetamine by reductive dehalogenation using red phosphorus as

a catalyst. If (–) ephedrine is used as the starting point, the process generates methamphetamine, while pseudoephedrine produces dextromethamphetamine. Regardless of the isomer produced, contaminants will be present.[206]

9.3.1. Chemistry of Amphetamines



Amphetamine



Methamphetamine

Figure 9.1 Chemical Structures of Amphetamine ($C_9H_{13}N$) and Methamphetamine ($C_{10}H_{15}N$)

Amphetamine is commonly available as the sulphate, a white crystalline salt, first synthesized in 1887. The molecular weight of amphetamine base is 135, the melting point is 300°C and pK_a is 9.9.[207, 208] The d-isomer of amphetamine has 3-4 times the central activity of the l-form.

Methamphetamine hydrochloride was first prepared in 1919 and forms white crystals or crystalline powder. The name "ice" is often used for methamphetamine because of the crystalline nature of the salt. Possible chemical designations for methamphetamine include N-dimethylbenzenthanamine, d-N,Ndimethylphenethylamine, and d-deoxyephedrine. The drug has been sold under many proprietary names (Deoxyn, Hiropon, Isophewn, Methedrine, to name a few). Its formula is $C_{10}H_{15}N$, and its molecular weight is 149.2. It has a melting point of 170-175°C and pKa of 10.1[208]. The low melting point permits it to be smoked, regardless of the crystal size. The crystals have a bitter taste and are soluble in water, alcohol, chloroform, and freon. Methamphetamine is not soluble in ether. Manipulation of the amphetamine's phenyl ring yields fenfluramine, a widely

prescribed anorectic. Manipulation of the side chain has led to the synthesis of a series of compounds with varying degrees of sympathomimetic activity.

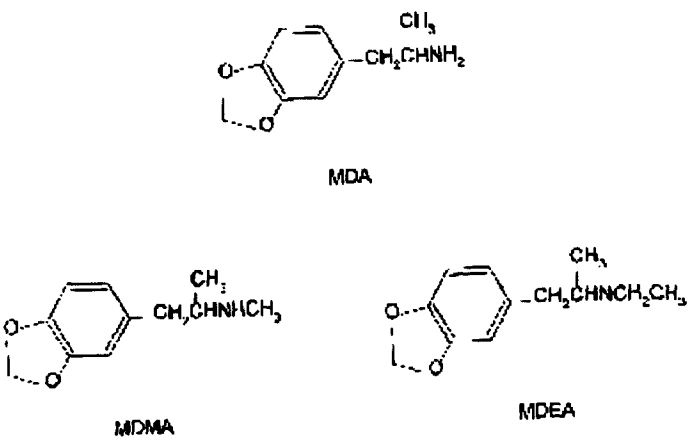


Figure 9.2 Chemical Structures of MDA ($C_{10}H_{13}NO_2$), MDMA ($C_{11}H_{15}NO_2$) and MDEA ($C_{12}H_{17}NO_2$)

MDA, MDMA and MDEA are ring-substituted amphetamines that have stimulant and hallucinogenic qualities in addition to unique pharmacological and psychological properties. MDMA was first synthesised in the early part of the 20th century. Similar to amphetamine, the d-isomer of MDMA has higher central activity than the l-isomer. The molecular weight of MDA is 179, melting point 187-188°C, while MDMA has a MW of 193, its melting point is 148-153°C, and MDEA has a MW of 204, melting point 201-202°C.

9.4. Routes of administration:

Amphetamine is commonly administered as the sulphate salt in single oral doses of 5-15mg, and occasionally in sustained-release form. It may be self administered either orally or by intravenous injection. Methamphetamine can be swallowed, injected, smoked, or snorted. In spite of the publicity accorded to ice smoking, most

users still prefer to inject it intravenously or take it orally. Preference for the intravenous route seems to be increasing[209]. A study done in 1988 found that when volunteers took 10 mg orally it produced plasma concentrations of 30-ng/ml one hour later[210].

Methamphetamine has a long half-life, if taken by smoking or injection: the mean plasma half-life is 11.1 hours for smoked and 12.2 hours for injected MA[211]. Peak blood levels range from 50-100ng/ml. Amphetamine concentrations were much lower reaching peak values of only 4 ng/ml after 3.3 hours. Methamphetamine levels in saliva were very high after smoking, but saliva amphetamine levels were negligible[211]. In other studies, larger doses of amphetamine have been given intravenously, but with comparable results. Volunteers given 160-200 mg of amphetamine intravenously had one-hour plasma concentration of 269ng/ml[212].

9.5. Disposition in the body

9.5.1. Amphetamine

Amphetamine is readily absorbed after oral administration, followed by deamination to phenylacetone, which is oxidised to benzoic acid and excreted as conjugates. A small amount is converted to norephedrine by oxidation. Approximately 30% of the dose is excreted unchanged in urine in 24 hours. However this is greatly dependent on the urinary pH. Acidic urine may increase the amount of unchanged dose excreted to 74% or it may decrease to 1% in alkaline urine. Amphetamine is also a metabolite of methamphetamine, benzphetamine[213] and selegiline[214].

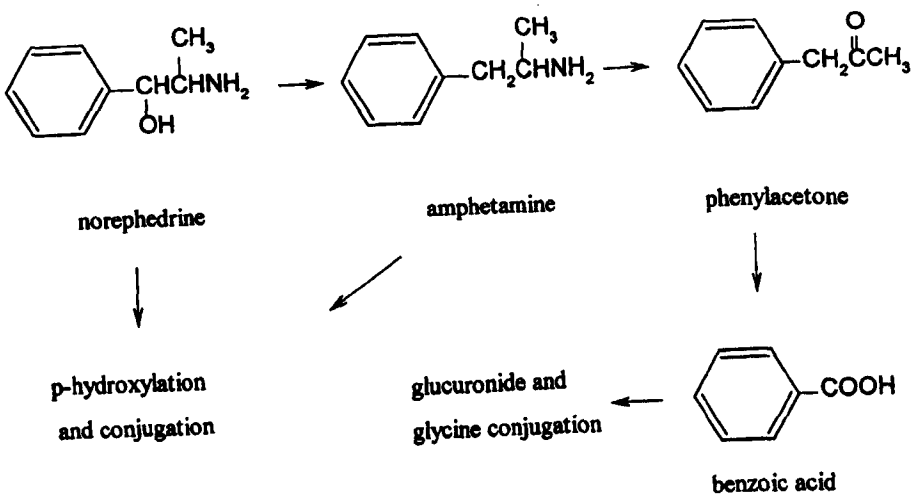


Figure 9.3 Metabolic Pathways of Amphetamine.

9.5.2. Methamphetamine

Methamphetamine is cleared from the blood by different routes. As mentioned before roughly 20% is demethylated to form amphetamine, and ephedrine derivatives which are psychoactive.[215, 216,] and this considered the minor route.(Figure 9.4). These metabolites are converted by deamination, to phenylacetone. Current NIDA regulations prohibit the reporting of methamphetamine in a urine specimen unless the methamphetamine level exceeds 500 ng/ml, and there is more than 200 ng/ml of amphetamine present. Oral doses appear to have little effect on either metabolism or peak blood levels. The saliva concentration is slightly higher than the concentration found in plasma with the average saliva to plasma ratio of 7:8[217]. In other studies MA was found in nail, hair, and meconium[218]. In urine, acidic pH decreases its excretion[213].

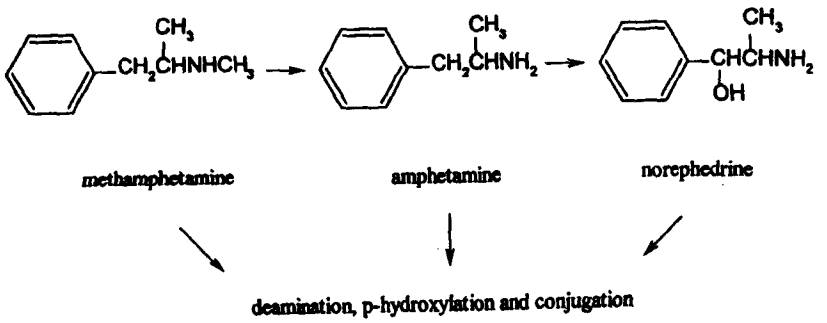
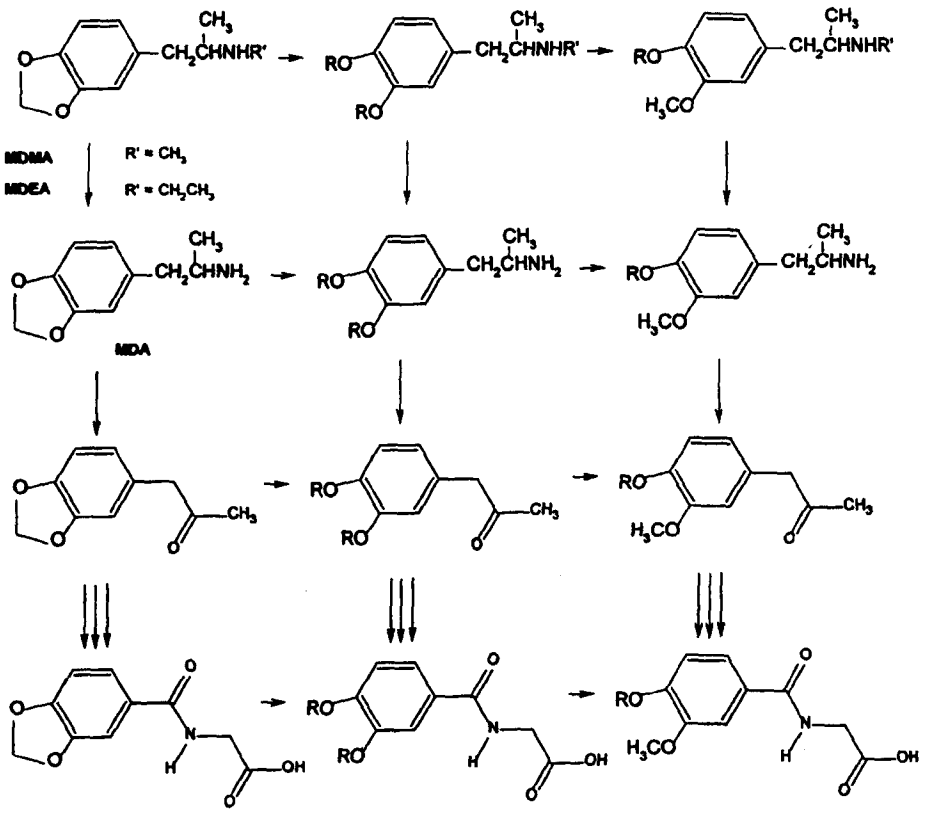


Figure 9.4 Metabolic Pathways of Methamphetamine.

9.5.3. Ring substituted Amphetamines

There is little information about the metabolism of MDMA and only limited data can be found in Clarke[214] or Baselt[219]. MDMA mainly undergoes N-demethylation to MDA and about 65% is excreted unchanged in urine along with 7% as MDA and this happens within 3 days[220]. The ring substituted amphetamines undergo two overlapping metabolic pathways, O-dealkylation of the methylenedioxy group to form dihydroxy compounds, then methylation of the hydroxy groups and degradation of the side chain to N-desalkyl and deamino-oxo metabolites. Further metabolism forms glycine conjugates of the corresponding 3,4-disubstitued benzoic acids (hippuric acids).



(phase I: R = H, phase II: R = sulphate or glucuronic acid)

Figure 9.5 Metabolic pathways of MDA, MDMA and MDEA

9.6. Toxicity

9.6.1. Amphetamine

The plasma concentration of amphetamine is usually below 0.1mg/L in therapeutic doses. In chronic users it is accompanied by weight loss, hallucination, and paranoid psychosis[221] with increased heart rate and blood pressure. The lethal dose in non-tolerant adults is 200 mg but death is rarely caused by it. If the concentration reaches 0.2-0.3 mg/L this can cause severe toxicity, while if it reaches 0.5 mg/L it can cause

fatalities[214]. Tolerance occurs so that when 100 mg amphetamine is taken orally on a daily basis, a steady blood concentration of 2.0-3 mg/L is maintained.[219]

In seven patients with evidence of amphetamine toxicity, blood levels ranged from 0.105-0.56 mg/L [210]. Different studies have been done on post mortem cases in which the cause of death was amphetamine to measure the concentrations of drug in different tissues as shown in Table 9.1.

Table 9.1 Fatal levels of amphetamine (mg/L or mg/Kg) reported in the literature.

Reference	Blood	Brain	Liver	Kidney	Urine
204	0.5-41 (n=11)	2.8-3.0 (n=2)	4.3-7 (n=11)	3.2-52 (n=6)	25-700 (n=8)
183	1.54	n/a	n/a	n/a	n/a
212	2.44	5.50	11.7	3.85	33.4
213	0.25-2.6 n=3	n/a	n/a	n/a	n/a
214	0.45-2.18 n=6	n/a	n/a	n/a	n/a
215	n/a	n/a	n/a	n/a	<0.5-320

n/a-matrix not analyzed.

Amphetamine and related compounds are responsible for a number of deaths either directly (adverse drug reactions, overdose) or indirectly (traffic accidents, suicides).[222] The use of amphetamines impairs mental function and causes a number of fatalities from road traffic accidents[223-224].

9.6.2. Methamphetamine

Therapeutic doses of methamphetamine range from 0.01-0.05 g, and chronic use also causes paranoid psychosis. Overdoses cause anxiety, hallucinations, cardiac arrhythmia, convulsion and severe cases result in coma and death. Similar to

amphetamine, deaths resulting from methamphetamine overdose are rare. The minimal lethal dose in non-tolerant adults is estimated at 1 gram.[214] In 4 cases of death after intravenous injection of the drug, blood methamphetamine ranged as high as 0.8mg/L.

9.6.3. Ring- substituted amphetamines

A literature search reveals very little data on the toxicity of MDA, MDMA and MDEA. The estimated lethal dose of MDA for non-tolerant adults is 0.5g. Symptoms of MDA overdose include tachycardia, hyperthermia, muscular rigidity, convulsions and coma. A non-fatal overdose of MDA resulted in an admission urine concentration of 131mg/L of MDA.[219] The victim, a one-year-old child, presented with muscle rigidity and seizures and was unconscious. There are many studies reporting the level of MDA in postmortem specimens including blood, urine, bile, and liver. The concentration ranges were 6-26, 46-175, 5-9, and 8-17 mg/L or mg/kg respectively.[225,226]

MDMA, MDEA and other amphetamines cause hyperthermia[227,228], disseminated intravascular coagulation[193], rhabdomyolysis[229,230], cerebral oedema[231] and cardiac arhythmias. Hepatotoxicity [232,233] and sudden cardiac death[234] are commonly cited in cases of fatal and non-fatal toxicity following MDMA use. Two unusual studies have been reported. One concerns a 17 year-old female who collapsed after taking two tablets of 'specked dove', a combination of MDMA, heroin and cocaine[234]. She was unconscious, unresponsive and hypothermic with a core temperature of 32.4°C. No postmortem toxicological results were reported in this case. The second was reported by Jorens [235] about a suicidal ingestion of MDEA and heroin by a 25 year-old male who took 40 tablets (approximately 4g) of MDEA and 12g of heroin, two hours prior

to admission. Toxicological analysis indicated high serum levels of both drugs but no classical signs and symptoms of intoxication appeared. Full recovery followed symptomatic treatment. The authors concluded that the opposite pharmacological properties of two drugs prevented the patient's death. Survival following a massive overdose of MDMA was reported in a 30 year-old male who took 50 ecstasy tablets, 10 tablets of oxazepam and 5 units of alcohol in 4-5 hours[236].

The unpredictable nature of ecstasy is reflected in the fact that thousands of people have consumed ecstasy and, while a very few deaths have been reported following the ingestion of just one ecstasy tablet, survival after consuming 50 ecstasy tablets has also occurred. Two possible explanations for the unpredictable responses are contamination of the ecstasy or genetic predisposition.

In addition to the short-term risks from the toxic effects of MDMA, further research is needed on the long-term effects on the brain, liver and heart. Sample and Johnstone[237] are currently studying the long-term effects of ecstasy use on damage to the nerve cells.

MDMA has been implicated in the onset of both psychological and behavioural complications. Researchers have reported an apparent association between MDMA use and serotonergic alterations.[238,239] Other studies have found a correlation between suicide and low levels of cerebrospinal fluid 5-hydroxyindoleacetic acid, the primary metabolite of serotinin[240,241], considered to be more dangerous than acute toxicity[242].

Recorded cases admitted to accident and emergency departments due to the use of MDMA at nightclubs increased to more than 48 in 15 months[243] while the use of ecstasy tablets was doubled. Polydrug use was common with around 40% having

consumed ecstasy. Adverse effects vary from feeling dizzy or weak (n=15, 31.3%) to feeling excessively hot, cold, feverish or shivering (n=7, 15%). There were six episodes which were classified as severe adverse effects. These included delirium in two cases, seizures in three cases and unconsciousness (coma) in one case, but there are no toxicological data nor was any confirmation done.

In the United States the exact number admitted to accident and emergency departments due to ecstasy use with related symptoms is not known. Monitoring these cases would present a clearer picture of the toxic effects of ecstasy. Full toxicological screening of admission urine and blood samples would help rectify the lack of available toxicity data for the ring-substituted amphetamines and would be of great benefit to the toxicologist. Many studies have measured ring substituted amphetamines in blood from postmortem cases and in these cases the concentrations range from 0.19 to 4.1 mg/L for MDMA,[193, 222] and from 0.04 to 0.49 mg/L for MDA, with two cases not having detectable levels, [222, 244] while levels of MDEA ranged from 0.09-33.0 mg/L with 3 cases not detected. [222,241,244,245] Some studies measured these analytes in urine[246,247]. A recent study on amphetamine was done in 2001 by Praisler et al.[248]

9.7. Tissue disposition

The level of methamphetamine after injection is higher in liver than blood. Also the concentration of amphetamine was 8 times higher[249].

Methamphetamine has been measured in breast milk at 10 and 42 days after delivery and it was demonstrated that the concentrations were much higher in the woman's breast milk than in her plasma[250]. There is very little evidence that amphetamine

found in mother's milk harms the foetus, although some studies found there was a relation between this and premature and low birth weight foetuses[251]. Very little data is available on the disposition of amphetamine in the foetus.

Environmental exposure to abused drugs is an increasing reality, especially in the inner city. Studies have repeatedly shown that, at inner city hospitals, 10% or more, of children presenting for treatment at hospital emergency rooms are positive for one or more abused drugs[252,253]. As the ability to detect nanogram quantities of drugs improves, drugs are being detected in more sick children. The mere fact that these drugs are present, however, is not sufficient reason to implicate them as a cause of illness or death in adults or in children.

9.8. Materials & Methods

9.8.1. Materials

All chemicals were purchased from SIGMA Company (Dorset, UK). The solvents and concentrated ammonia were of HPLC grade supplied by BDH laboratories supplies. ZD clean cartridges (20 ml) were from UTD™ and were obtained from Varian (Harbor city, California, USA), and the Baker-20 SPE vacuum manifold system was from International Sorbent Technology.

Phosphate buffer (0.1M) was prepared by dissolving 6.81g of potassium dihydrogen phosphate in 450ml of de-ionised water, adjusting the pH to 6.0 ± 0.1 with 1.0M potassium hydroxide, then making the total volume up to 500ml with de-ionised water. Acetic acid solution (0.01M) was prepared by adding 57.5 µl of glacial acetic acid to 100ml of de-ionised water.

The SPE eluant was prepared daily by mixing dichloromethanol- Isopropanol alcohol (IPA)-concentrated (78:20:2). Both were sonicated for at least 5 minutes before use.

Amphetamine, Methamphetamine, MDA, MDEA, MDMA were purchased from SIGMA, and deuterated materials were from Radian Corporation (Austin, TX).

Enzyme Immunoassay (EIA) was carried out using kits from Cozart Bioscience Ltd., Oxfordshire.

9.8.2. Instrumentation

Gas chromatography - mass spectrometry was carried out using a VG Micromass 70-250S double focusing magnetic sector instrument or a Finnigan-Thermoquest Trace quadrupole instrument. The gas chromatograph (Hewlett Packard 5890 Series II) was equipped with a fused silica capillary column (HP5, 30m x 0.32 mm id, 0.3 μm film thickness). Samples (volume 1ul) were injected with an injection port temperature of 280°C. The column temperature was initially held at 80°C for 2 min and then programmed to rise to 285°C at 5°C/min. The temperature was held at 285°C for the final 2 minutes of the chromatographic analysis. The mass spectrometer was operated at a resolution of 1000 in the EI+ mode (ionisation energy 70 eV, source temperature 220°C). Quantitative analyses were carried out in the SIM mode, using deuterated analogues of the target analytes as internal standards.

9.8.3. Methods

9.8.3.1. Sample collection

Eleven samples of meconium were collected from diapers of newborn infants delivered at the Royal Maternity Hospital, Glasgow (these are different from the

samples referred in paragraph 8.3.1). The meconium was recovered using a wooden spatula and stored in plastic tubes at –4°C until analysed. 30 samples of meconium were collected from neonates delivered in several hospitals in Egypt who were not exposed to drugs in uteri. These were used as controls. (as mentioned in page 106)

9.8.3.2. Sample preparation

Each meconium specimen was weighed (range from 0.5g - 0.8g) into a clean vial and centrifuged at 2000rpm for 10 minutes to bring it to the bottom. Drug standards were added and the samples were homogenized by vortexing and sonicating for 15 minutes. Methanol (3 ml) was added, and the tubes were vortexed well for 10 minutes then centrifuged at 3000rpm for 20 minutes. The upper layers were transferred to clean tubes and evaporated to dryness under nitrogen, initially at room temperature then with slight warming up to 40°C until completely dry. Once dry, the residues were dissolved in 700 µl methanol with vortexing. The extraction tubes were then centrifuged at 2000rpm for 10 minutes.

Half of the extract was analysed by EIA, and the other half followed the SPE method.

9.8.3.3. EIA analysis:

The EIA method used was an Enzyme Linked Immunosorbent Assay (ELISA) from Cozart Company. This assay involves the enzyme horseradish peroxidase, which produces a blue colour with its substrate. 500 µL of elute B was evaporated till dryness under nitrogen then reconstituted in EIA buffer (1ml). This was then analysed for amphetamines using EIA kits containing antisera highly specific to free (unconjugated) forms of the parent drugs. The method followed the same steps

previously mentioned. The calibration curve was prepared from standards containing the analytes at concentrations of 0, 10, 100 and 500 ng/ml.

9.8.3.4. Sample Extraction:

The solid phase extraction (SPE) procedure applied was as follows:

- The deuterated Internal Standards were added to the other half of the meconium extract and mixed well;
- the column was prepared by washing with 3ml methanol, followed by 3 ml water and 1 ml phosphate buffer (pH6);
- the sample was applied at low vacuum pressure;
- the column was washed with 3ml distilled water, followed by 1 ml ethyl acetate and 3 ml methanol;
- the column was dried at maximum pressure for 5 min;
- the analytes were eluted with 3 ml of eluant (dichloromethane: IPA: concentrated ammonium hydroxide, 78:20:2 v/v/v)

9.8.3.5. Derivatisation

The SPE extract was evaporated to dryness without heating and 50 µl of a mixture of PFPA: ethyl acetate (2:1 v/v) was added. The resulting solution was heated at 60°C for 20min. At the end of this time, the reagent was evaporated to dryness without heating till dryness, then 50µl of ethyl acetate was added.

9.8.3.6. GC/MS Analysis

The derivatised extracts were analysed by GC/MS in the SIM mode. The column oven temperature was programmed from 80°C-250°C at a rate of 10°C/min. with a

final hold time at 250°C of 2 minutes. Selected ions used for quantitative analyses are listed in Table 9.2 along with the retention times for each analyte.

Table 9.2 Retention Times and Selected Ions for Amphetamines.

Drug	Selected Ions (m/z)	Retention time (min)
Amphetamine	**190**	5.75
d_3-Amphetamine	**193**	5.75
Methamphetamine	**204**	7.08
MDA	**162**	9.77
d_6-MDA	**168**	9.77
MDMA	**204**	11.05
d_5-MDMA	**209**	11.05
MDEA	**218**	11.44
d_5-MDEA	**223**	11.44

9.8.3.7. Method Verification

Recoveries were determined by spiking blank meconium with the target analytes. Ten different meconium samples were spiked with standards and analysed as described earlier in order to determine the extraction recovery of the method. The internal standards (deuterated form of each drug) were added to the eluates obtained by SPE and these extracts were compared with unextracted standards. Furthermore, ten meconium samples were spiked with decreasing amounts of drug standards and then analysed as before to determine the limits of detection of the method.

9.8.4. Application to Real Case Samples

The same method was then applied to 11 real cases from the Royal Maternity Hospital, Glasgow. Extracts were initially screened by EIA and then confirmatory quantitative analyses were carried out by GC/MS.

9.9. Results and Discussion

9.9.1. Method Verification

The developed method was satisfactory: blank meconium gave negative results and the target analytes were successfully extracted from spiked meconium samples and quantified. Specimen chromatograms and mass spectra of extracted standards are shown in Figures 9.6- 9.9.

Calibration curves for the target analytes were linear and had linear correlation coefficients (r^2) in the range 0.897 to 0.991. The calibration ranges chosen for the analytes were selected from the available literature data. Representative calibration curves for amphetamine and methamphetamine are shown in Figures 9.10 and 9.11

Recoveries for the analytes ranged from 60-96% (Table 9.3) but were considered acceptable if the method could be generally applied for the intended purpose. The lower limit of detection for each analyte is summarised in Table 9.4.

Table 9.3 Recovery of Target Analytes

Drug	Recovery (%)
Amphetamine	90.0
Methamphetamine	60.0
MDA	95.9
MDMA	83.9
MDEA	87.2

Table 9.4 Lower Limit of Detection for Target Analytes.

Drug	L.L.D (ng/gm)
Amphetamine	1
Methamphetamine	2
MDA	5
MDMA	1
MDEA	10

The reproducibility of the method was established by analysing five replicates of standards at three concentrations (10, 30 and 50ng/g) for amphetamine and methamphetamine, following the same method of extraction. The stability of the extracts was evaluated by analysing the same samples immediately after extraction, after three days, after one week and after two weeks. This data was used to measure the mean, standard deviation, and relative standard deviation for each analyte and the results are given in Table 9.5. The R.S.D. ranged from 1.21- 6.55. From these results it was found that the reproducibility for this method for both amphetamine and methamphetamine were good.

Table 9.5 Reproducibility of Method for Amphetamine and Methamphetamine

Date	Drug	Conc. (ng/g)	Drug/I.S (Average of 3)	Std.dev.	Relative standard deviation
First day	AM	10	3.23	0.269	0.175
	MA		4.34	0.250	2.55
	AM	30	8.54	0.597	5.98
	MA.		10.4	0.326	3.13
	AM	50	9.80	0.117	1.20
	MA		14.2	0.158	3.76
3 days	AM	10	3.57	0.250	0.59
	MA		2.72	0.049	1.81
	AM	30	7.22	0.128	1.77
	MA		9.33	1.55	6.55
	AM	50	10.3	0.885	5.34
	MA		14.2	0.8507	5.51
1 Week	AM	10	2.88	0.064	2.21
	MA		2.83	0.061	2.14
	AM	30	8.76	0.576	6.60
	MA		8.01	0.443	5.53
	AM	50	10.6	0.885	5.34
	MA		13.9	0.851	5.51
2 Weeks	AM	10	2.93	0.084	2.90
	MA		3.52	0.172	4.88
	AM	30	9.13	0.493	5.40
	MA		8.20	0.100	1.21
	AM	50	10.8	0.885	5.34
	MA		14.1	0.851	5.51

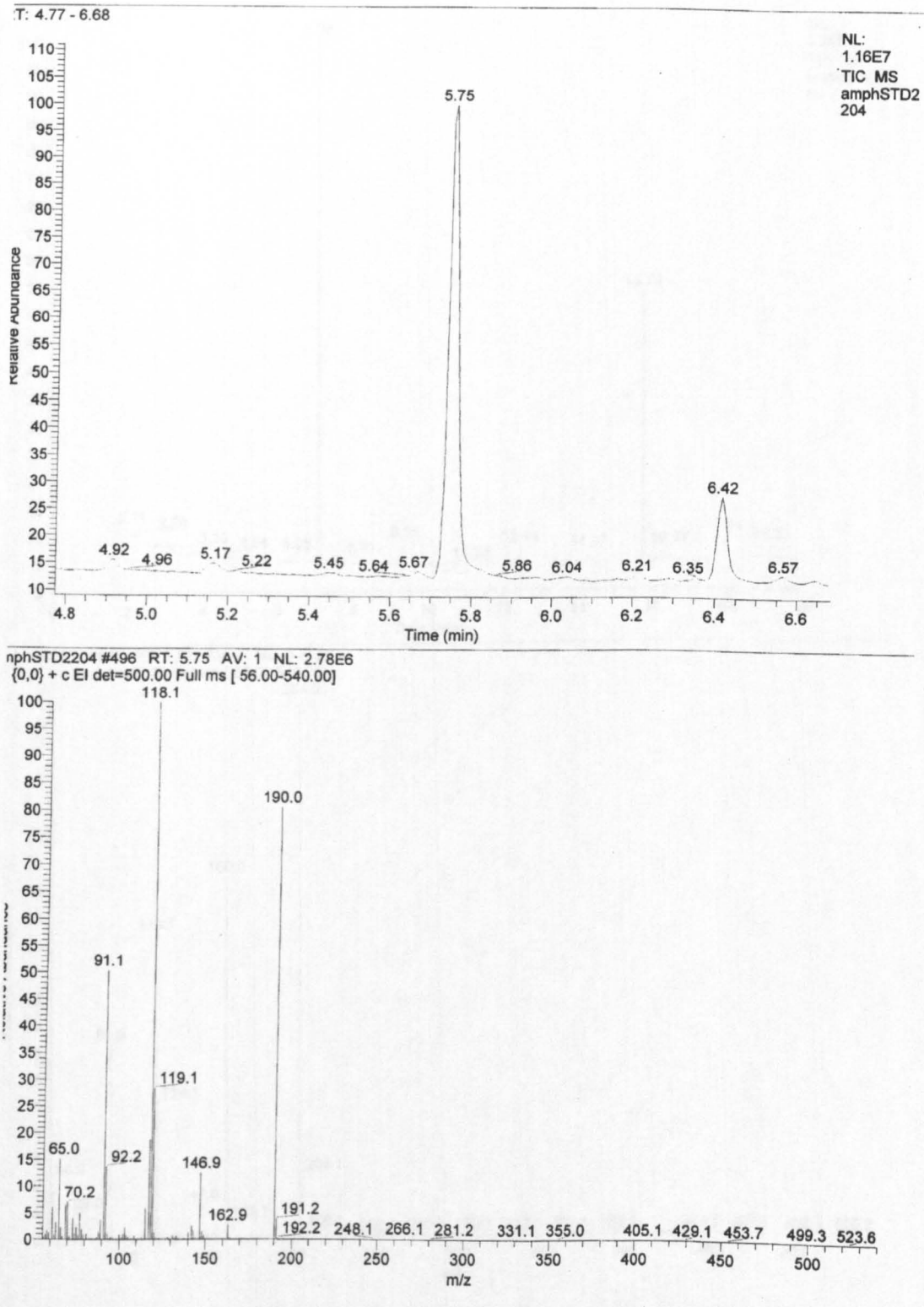


Figure 9.6 TIC Chromatogram and Mass Spectrum of Amphetamine PFP derivative.

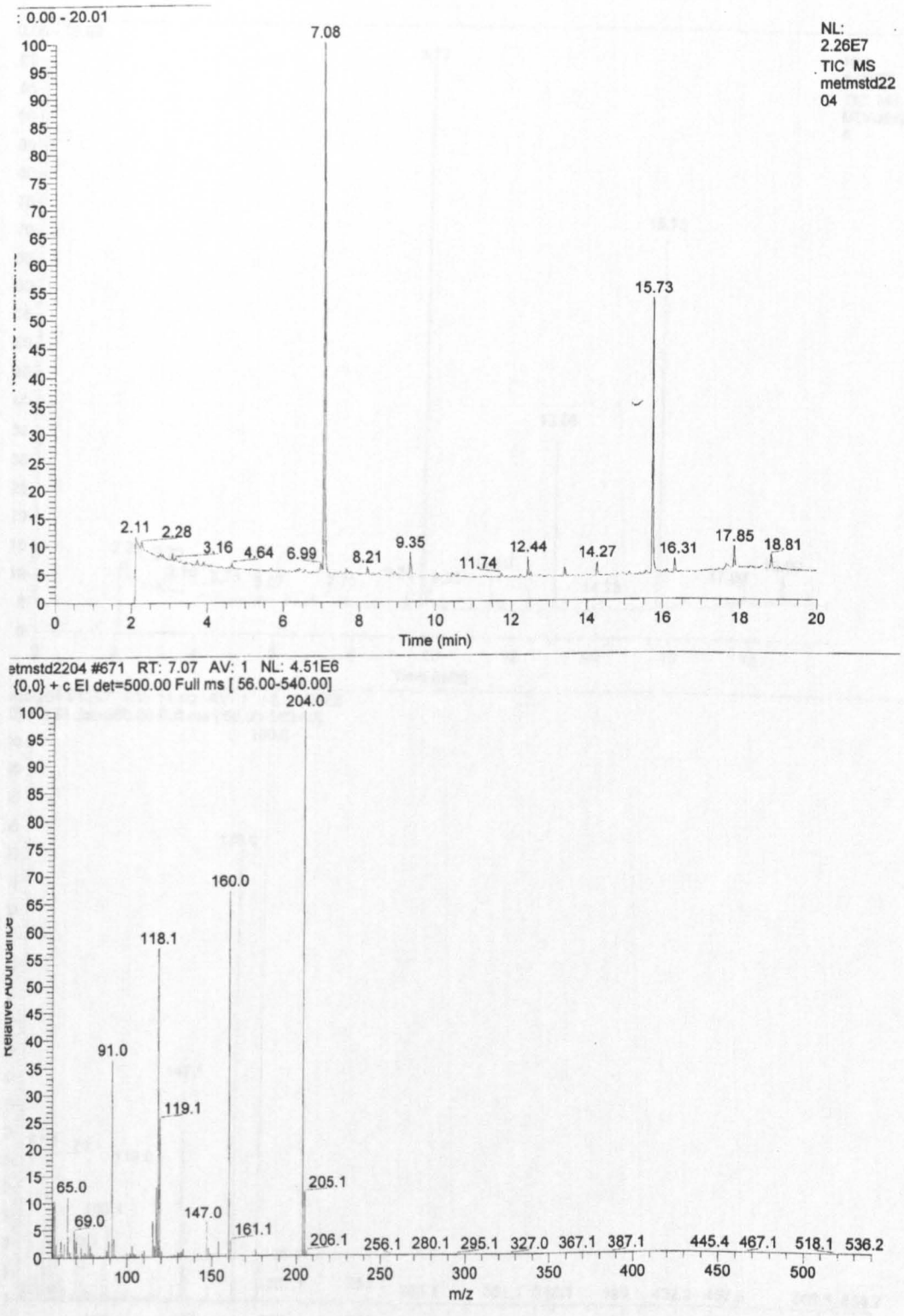


Figure 9.7 TIC Chromatogram and Mass Spectrum of Methamphetamine PFP derivative.

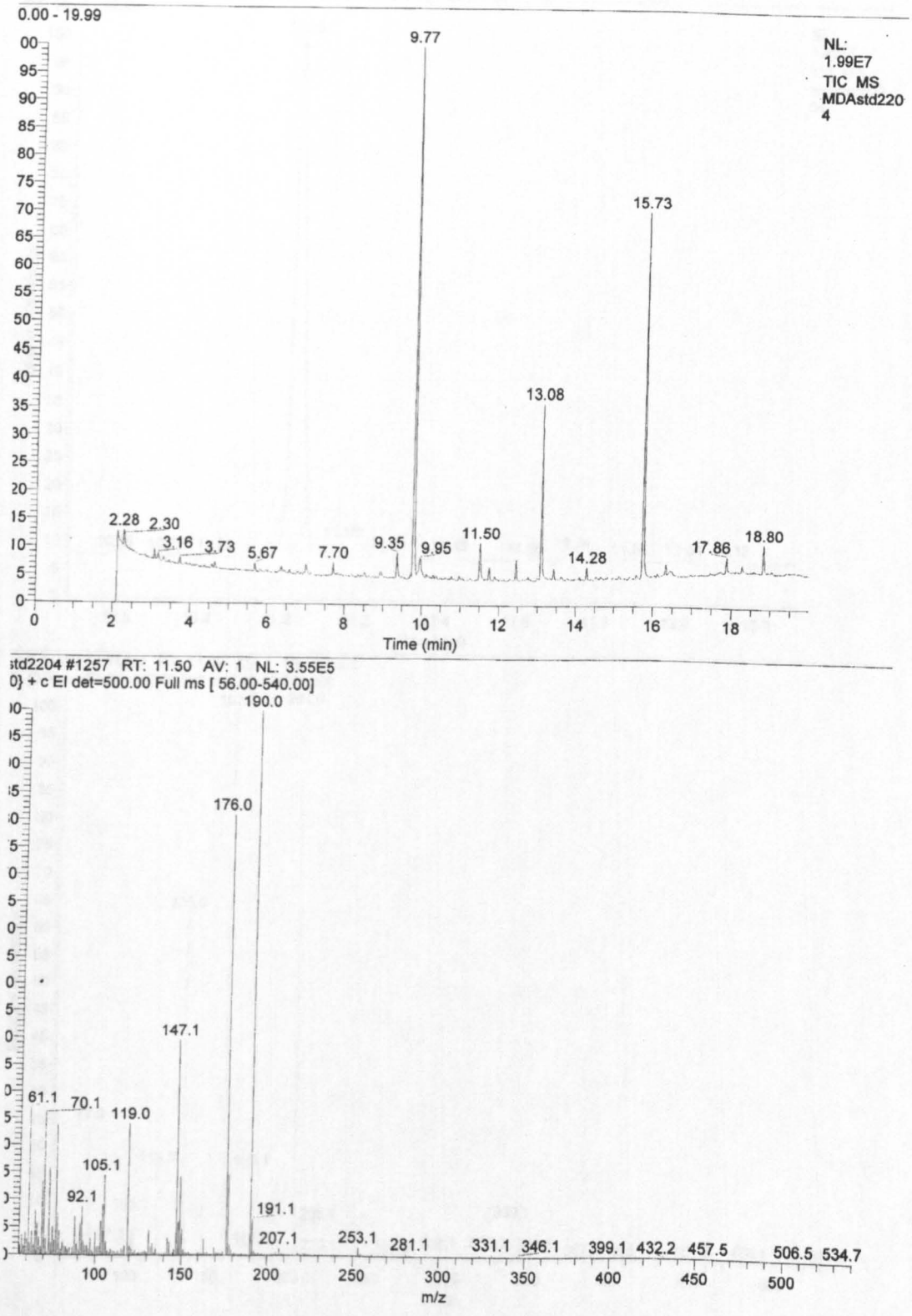


Figure 9.8 TIC Chromatogram and Mass Spectrum of MDA PFP derivative.

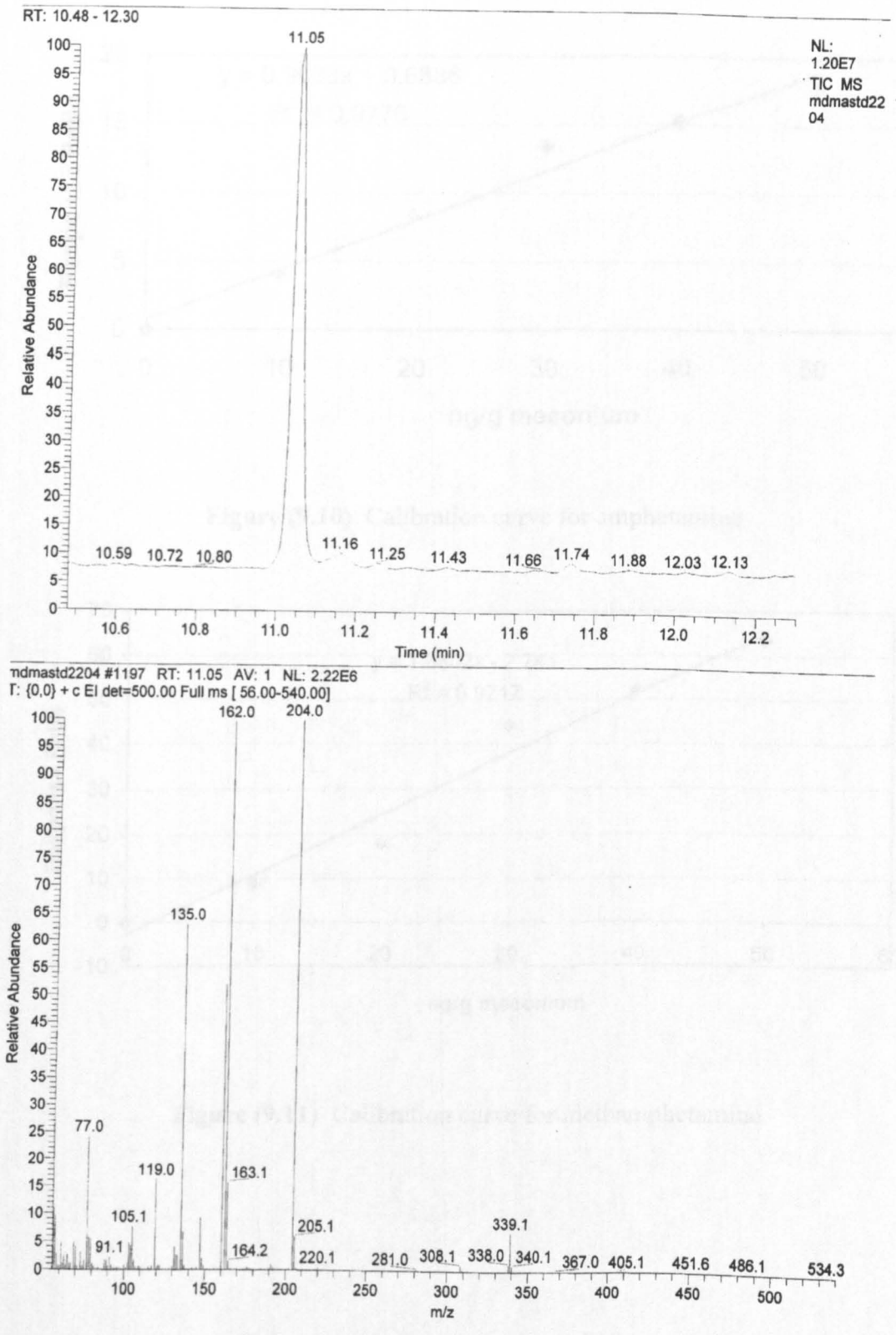


Figure 9.9 TIC Chromatogram and Mass Spectrum of MDMA PFP derivative.

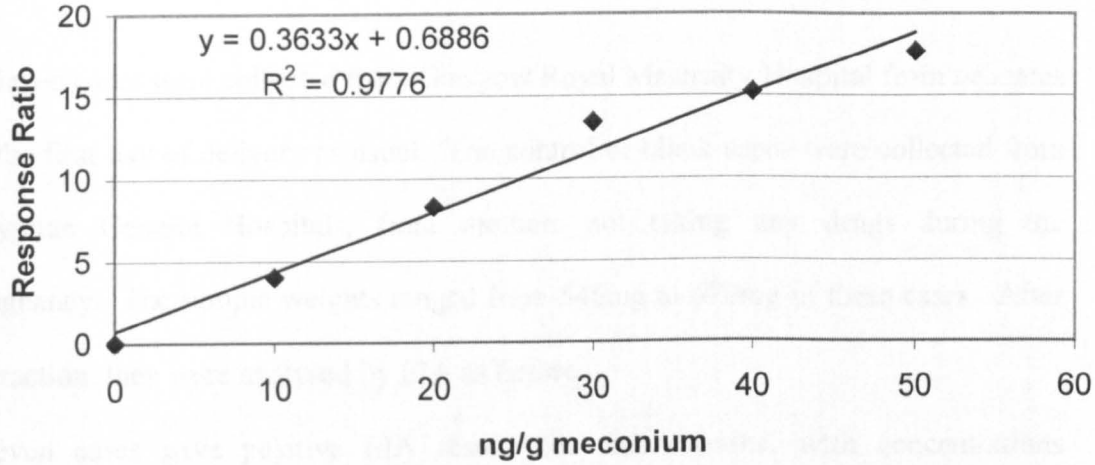


Figure (9.10) Calibration curve for amphetamine

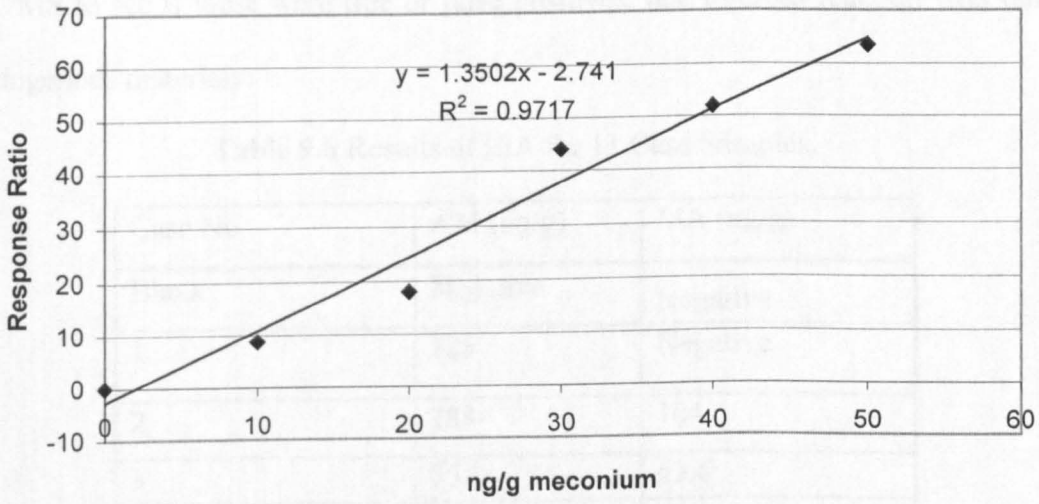


Figure (9.11) Calibration curve for methamphetamine

9.10. Application to real cases

Eleven cases were collected from Glasgow Royal Maternity Hospital from neonates in the first day of delivery as usual. The control or blank cases were collected from Egyptian General Hospitals, from mothers not taking any drugs during the pregnancy. The sample weights ranged from 546mg to 670mg in these cases. After extraction, they were analysed by EIA as before.

Seven cases gave positive EIA results for amphetamine, with concentrations ranging from 1-204ng/gm, and nine cases gave positive results for methamphetamines with the Cozart kits used (Table 9.6). Confirmation was done by GC/MS to see if these were true or false positives, due to cross reaction with other endogenous materials.

Table 9.6 Results of EIA for 11 Case Samples.

Case No.	AM (ng/g)	MA (ng/g)
Blank	Negative	Negative
1	129	Negative
2	188	104
3	55.1	63.6
4	58.5	Negative
5	133	41.6
Case 6	Negative	41.3
Case 7	Negative	44.9
Case 8	Negative	34.7
Case 9	27.4	84.0
Case 10	Negative	70.0
Case 11	43.5	74.0

The remaining analyses for confirmation by GC/MS were carried out after adding the I.S. at a concentration of 20ng/g, and spiked meconium standards were prepared at two concentrations (25, 100 ng/g) for quantification. Three replicates were prepared at each concentration and the average result was used. The results are given in Table 9.7. Specimen mass spectrometry traces are given in Figures 9.12-9.13

Table 9.7 Results of Analysis of Real Cases by GC/MS.

Case No.	AM (ng/gm)	MA (ng/gm)	MDA (ng/gm)	MDMA (ng/gm)	MDME (ng/gm)
1	4.76	2	10	NA	4
2	204	6	2	3	1
3	1	NEG	NEG	NEG	NEG
4	5.6	NEG	2	NEG	NEG
5	7.1	NEG	6.1	NEG	NEG
6	2.8	NEG	2	NEG	NEG
7	25.6	NEG	47.8	NEG	NEG
8	NEG	NEG	NEG	NEG	NEG
9	NEG	NEG	NEG	NEG	NEG
10	NEG	NEG	NEG	NEG	NEG
11	1	NEG	NEG	NEG	NEG

(NEG=negative)

From these results it appears that most of the cases which gave positive results by EIA gave negative results by GC/MS, and it is concluded that the EIA results were false positives due to cross-reaction with endogenous materials.

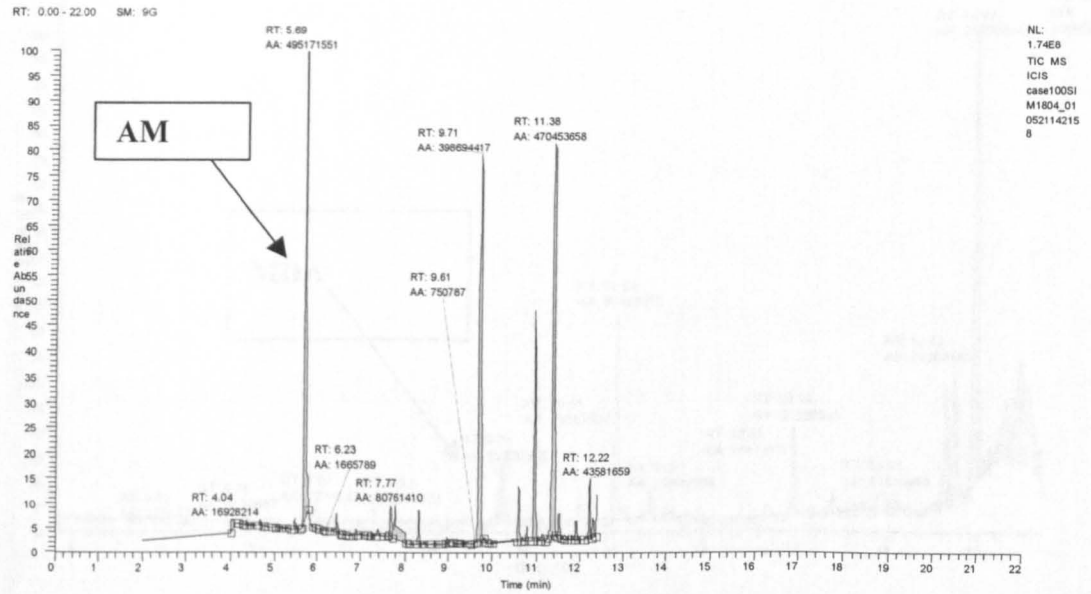
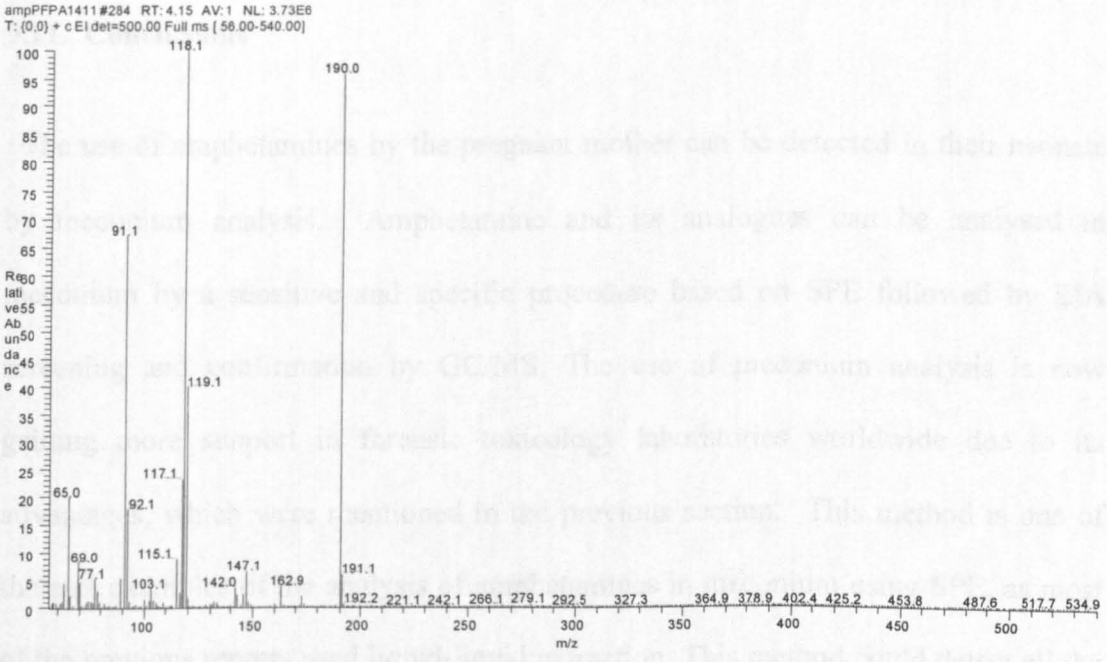


Figure (9.12) Total Ion Current Chromatogram for Meconium Extract in Case 2



Figure(9.13) Mass Spectrum of Amphetamine in Case 2

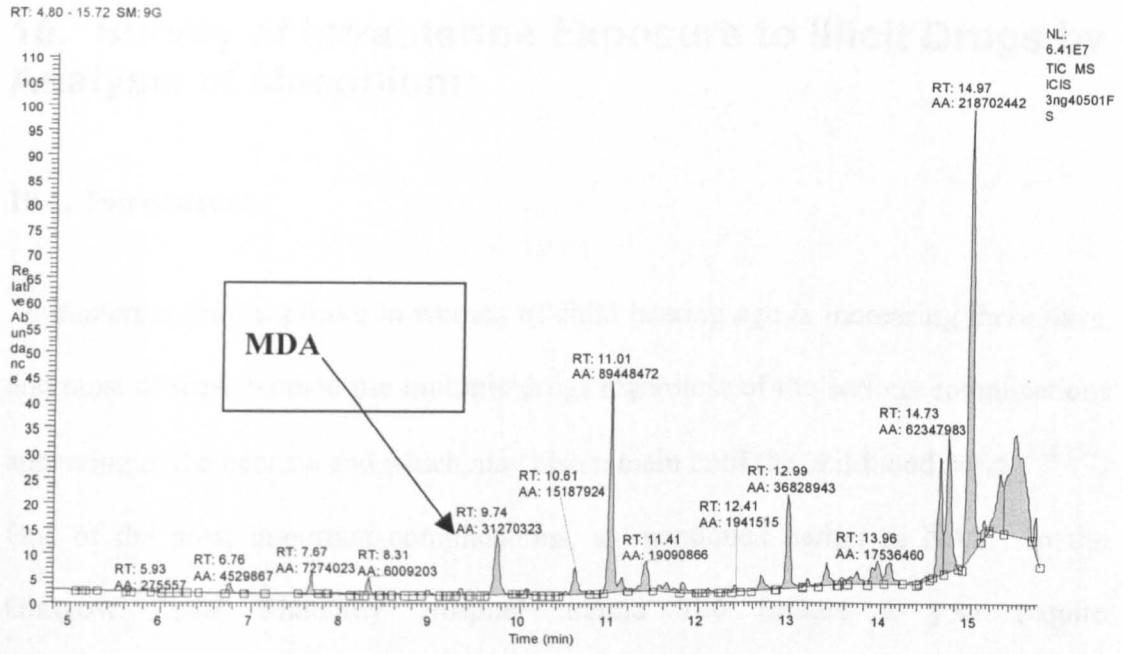


Figure 9.14 Total Ion Current Chromatogram for Meconium Extract from Case 9.

9.11. Conclusions

The use of amphetamines by the pregnant mother can be detected in their neonate by meconium analysis. Amphetamine and its analogues can be analysed in meconium by a sensitive and specific procedure based on SPE followed by EIA screening and confirmation by GC/MS. The use of meconium analysis is now gaining more support in forensic toxicology laboratories worldwide due to its advantages, which were mentioned in the previous section. This method is one of the rare examples of the analysis of amphetamines in meconium using SPE, as most of the previous reports used liquid-liquid extraction. This method could detect all the important target analytes in the amphetamines group. It is important not to misdiagnose the false positives due to cross reaction in EIA analysis and to use a GC/MS confirmatory step after immunoassay.

10. Survey of Intrauterine Exposure to Illicit Drugs by Analysis of Meconium:

10.1. Introduction:

Intrauterine drug exposure in women of child bearing age is increasing these days, and most of these women use multiple drugs regardless of the serious complications appearing in the neonate and which may also remain until the childhood period[254-256]. One of the most important complications, as mentioned earlier is NAS. In the Glasgow Royal Maternity Hospital around 120 infants a year require pharmacological management for severe Neonatal Abstinence Syndrome (NAS).[257]

The Maternal Lifestyle Study was set up in the United States as a prospective multi-site longitudinal study on the association of drug use during pregnancy with acute neonatal events and long term neurodevelopment child outcome, conducted under the auspices of the National Institute of Child Health and Human Development (NICHD). A fiscal and scientific collaboration was developed with the National Institute on Drug Abuse, the Administration on Children, Youth and Families, and the Center for Substance Abuse Treatment to design and fund the study.

The accurate identification of prenatal drug abuse is critical for 2 reasons:

1- To fully understand the nature and magnitude of the problem.

2- To determine appropriate medical and psychosocial interventions.

Accurate information regarding illicit drug using during pregnancy is difficult to obtain and depends on the maternal interview or on laboratory analyses. The reliability of maternal interview regarding drug use is adversely affected by many factors, such as the mother's mistrust of health care givers and fear of the mother for

her infant, but is routinely used in spite of its limitations.[255, 256] Ostrea et al.[254] found that only 11% of the mothers admitted to using illicit drugs. Frank et al.[255], found that self-reporting misclassified 24% of cocaine users identified by urine toxicology. Other methods used to identify prenatal cocaine exposure vary and include self-administered questionnaire, intake history, and urine testing of mother and infant and testing of infant hair and meconium, resulting in widely disparate estimates of rates of cocaine use by pregnant women.[257, 258, 259]

There are many studies that have used meconium and other specimens or mother interview to screen drugs during pregnancy and their transmission to the foetus. Those involving cocaine have been in the majority. Estimates of cocaine exposure vary according to the population. The 1988 National Maternal and Infant Health Survey, reported cocaine use is 0.4%.[260] More recent studies depending on urine tests reported users as 1.1% of the total, [261].

A number of studies suggest that meconium is the most useful specimen for the detection of prenatal cocaine exposure.[160, 254, 257-263] Lewis et al.[264] found that meconium detected 2.7 times more instances of cocaine use than urine. Casanova et al.[265] argued that the methods used for meconium measurement differed in analytical sensitivity and specify from the urine screening methods, precluding a direct comparison of the performance of urine *versus* meconium as matrices of detection of cocaine. It has even been suggested that serial analysis of meconium specimens may reflect both the chronology and degree of drug exposure. It was suggested that meconium collected 0-10 hours after birth reflects gestational exposure up to 20 weeks, that collected after 11 to 20 hours reflects 21-30 weeks gestation, and 21 to 36 hours more than 30 weeks gestation[266].

Biomarkers of *in utero* cocaine exposure in the neonate include measurement of tissues including lung, analysis of biological fluids like blood, urine, hair and meconium and, recently, in gastric aspirate and amniotic fluid. Urine has been the most widely used specimen. Ostrea el al.[138,262] reported meconium to be more suitable than urine for detecting foetal exposure to cocaine, but metabolites can be detected not more than 96-120 hours after the last cocaine use.

Some investigators have advocated the use of quantitative analysis of drugs in meconium, as a mean of estimating degree of drug exposure during gestation. Ostrea et al[267] reported that the concentration of nicotine metabolites in meconium was directly related to the degree of active smoking by the mother. In a similar manner, Stolk et al[268] observed a correlation between maternal methadone dose and the concentrations of methadone and EDDP, its primary metabolite detected in meconium.

Meconium is not formed by the foetus until the twelfth week of gestation,[259] thus meconium analysis cannot be used to detect illicit drug use before this period. The study done by Morales et al, showed that any drugs received by the mother during labor can be detected in meconium.[269] If opiates or cocaine are used during the first trimester of pregnancy, they can be detected by radioimmunoassay with a high degree of sensitivity and specificity. No false positive results were recorded for cocaine in meconium, in contrast to hair analysis. But meconium gave around 10.5% false positives for opiates, perhaps due to cross reactivity to various substances.However, when GC/MS confirmation was done, the opiates were identified to be codeine, instead of morphine, and this was consistent with the history in the mother of having taken a prescribed codeine-containing analgesic for false

labour pains 4 days before delivery. This example clearly reinforces the need for GC/MS confirmation of any positive laboratory tests result for drugs particularly if opiates are found[269].

Accurate identification of prenatal drug exposure is likely to be improved with GC/MS confirmation and when the meconium assay is used in conjunction with a maternal hospital interview. However the use of GC/MS may have different implications for research than for public policy.

The analysis of drugs in meconium and interpretation of test results are complex processes. There are some problems which affect the result of meconium analysis, especially at the current stages of development of this new and useful technology. It is clear that there are matrix and tissue effects, so special techniques must be used to avoid false negative and false positive results, so a confirmatory test is required[259,264,270]

In other surveys done on meconium, the specimens were collected from more than one diaper and pooled if the quantity of meconium was small, as often occurs in preterm infants, refrigerated, batched and transferred rapidly to the laboratory where it was analysed, by EMIT. Screening was carried out for cocaine, opiates, cannabinoids, amphetamine, and phencyclidine with the cut-off at the limit of detection of the assay, being 50ng/g for cocaine, opiate, and amphetamine metabolites, 5ng/g for cannabinoids, and 3ng/g for PCP. Then confirmation was done by GC/MS, although amphetamine and PCP were discontinued because of low prevalence.[163] The cases were considered negative if the mother denied using cocaine and opiate during the interview and EMIT was negative. Maternal use of

alcohol, marijuana and nicotine during the pregnancy were treated as background variables in both the exposed and unexposed groups[271].

False negative results may occur if the immunoassay does not cross react with the appropriate analytes or if the analyte is not free to react with the antibody, i.e., if it is the glucuronidated metabolite. Moore et al[272] found that the concentration of hydrocodone, hydromorphine and codeine increased markedly after acid hydrolysis with a more moderate increase noted in the morphine concentration. This is in contrast to a report by Becker et al.[273] in which hydrolysis of meconium was not found to significantly increase the morphine concentration. Also, foetal maturity was found to be a factor in the detection of methamphetamine in meconium from guinea pigs after intraperitoneal injection of the drug at different gestation ages.[162] Furthermore, gestation age was determined to be an important consideration in interpreting quantitative methamphetamine results.

Lastly, one must address the feasibility of performing these tests in a clinical or research setting. Meconium analysis is available for mass screening with an enzyme immunoassay kit, or by radioimmunoassay[149]. The cost of analysis per specimen approximates the cost of urine toxicology. On the other hand the analysis of hair for drugs is restricted to a few commercial laboratories, and the cost of analysis is substantially higher than meconium analysis[274].

In spite of that there are many questions which remain to be answered about the disposition of drugs in meconium. Could the presence of a particular analyte be a marker for drug exposure at a certain gestational age? How important is enzymatic or acid/base hydrolysis of the meconium sample to accurate and sensitive drug measurement? Foetal maturity also affects the efficiency of phase II metabolism and

the production of glucuronide and other metabolites. It is not yet known which are the most appropriate drug analytes to measure in meconium. It is clear that cocaethylene and benzoylecgonine should be measured for cocaine exposure[165], and El Sohly et al.[275] have recently described the importance of 11-hydroxy-Δ^9-tetrahydrocannabinol and 8,11-dihydroxy-Δ^9-tetrahydrocannabinol for detection of marijuana exposure.

There is also uncertainty about possible reabsorption of drugs from the foetal gastrointestinal tract during gestation, and foetal metabolism is different from that of the adult and is affected by gestation age. Meconium is also not a homogenous matrix, and as the drugs diffuse over time so mixing of meconium must be done. Another important confounding factor is the possible contamination of the meconium by urine, which may affect the concentration of drugs in meconium.

The importance of using both meconium and maternal self-report is to identify mothers who deny use but who did use as evidenced by positive meconium confirmation[276].

The discovery of polydrug use in pregnant mothers was reported[277]. It is becoming increasingly clear that women who use cocaine during pregnancy are also likely to use other illicit drugs. Only 13% of the studies reported that cocaine is the only drug used.[278] Chasnoff found that 32% of the positive meconium specimens were positive for at least 2 drugs,[261] and only 2 % of the mothers reported that they used cocaine and no other drugs, and mothers were found to be 49% times more likely to use another drug. The cocaine problem has truly become a problem of polydrug use.

This has implications for the development of improved methods to identify drug-exposed infants, especially those exposed to multiple drugs. There are also

implications for developmental follow up studies because little is known about the effects of drug interactions on child behaviour and development[261]. Finally the problem of cocaine use has been redefined as one of polydrug use, which has implications for the fields of drug toxicology, pediatrics, and child development[278].

Recently some studies have been done comparing the results of maternal interview with hair and meconium analysis, regarding their sensitivity, specificity and false negative results in each opiate, cocaine and cannabinoids analysis. A sample of hair was obtained for drug analysis from each subject at the beginning of the survey, at the end of the second trimester of pregnancy, and within 24 hours after delivery. Meconium collection started from the first meconium until the third postnatal day, which was collected, frozen and analyzed for cocaine, opiates and cannabinoids. Also, this study was accompanied by an interview with the mother[279].

There was a consistency in the detection of drugs in meconium and hair from the first to the subsequent samples for cocaine and opiate. If the first meconium sample was positive it would be positive for all the samples, and the same was also true for hair analysis. The maternal interview showed the lowest sensitivity in detecting cocaine and opiate exposures (65% and 67% respectively) but it had the highest sensitivity in detecting cannabinoid exposure (58%). Both hair and meconium analyses had high sensitivity for detecting cocaine or opiate exposures. Hair analysis had a sensitivity of 100% for cocaine and 80% for opiate detection. However it had a false positive rate of 13% for cocaine and 20% for opiate, probably as a result of passive exposure. Meconium analysis had a sensitivity of 87% for cocaine and 77% for opiate detection, but unlike hair analysis it had no false positive test results for

cocaine. Both hair and meconium analyses had low sensitivity in detecting cannabinoid exposure, most probably because of the sporadic use of cannabis[280].

The principal drawback of hair analysis is its potential for false positive test results associated with passive exposure to drugs. Maternal interview is a time consuming test of low sensitivity. The high sensitivity of meconium analysis and ease of collection make this test ideal for prenatal drug screening[278].

In that study, meconium was analysed for cocaine and for benzoylecgonine, opiates (morphine and morphine glucuronides) and cannabinoids (11-nor-delta-9-tetrahydro-cannbinol-9-carboxylic acid) by radioimmunoassay according to previously published procedures.[5] Cut-off concentrations were 25ng/ml for cocaine and opiates and 15 ng/ml for cannabinoids. The inter-assay coefficient of variation was 12.6% for cocaine, 4.8% for morphine, and 11.9% for cannabinoids. Positive meconium test results were confirmed by gas chromatography/mass spectrometry[281].

In detection of cocaine, Maternal interview showed a low sensitivity and a false negative rate of 55%. On the other hand, hair analysis showed a false positive rate of 13% in contrast to false positive rates of 0% for both maternal interview and meconium analysis[278,281,282].

From these previous studies, it is clear that a survey on meconium would be useful to detect drug abuse during pregnancy in Glasgow, as no previous studies have been done in this area. In a recent quotation in the Sunday Times newspaper on 25 March 2002, Dr Laurence Gruer, Chairman of the Advisory Council on the Misuse of Drugs, March 2002 underlined the size of the problem. We do not have good data on the scope of the problem of drug use during pregnancy. This is one of the reasons for the survey and why it is desirable to extend it to other areas of Scotland.

"Up to 50,000 children in Scotland are being raised by parents addicted to heroin, cocaine and other hard drugs. A generation of children will be damaged - physically and emotionally - by their parents' habits."

The purpose of the present study was to know the exact number of drug-using mothers in Glasgow and to estimate the concentrations of drugs transmitted to their foetus, using the method previously developed to screen multiple drugs in meconium. The method developed can be applied to opiates, including morphine and codeine, cocaine and its metabolite benzoylecgonine, methadone, diazepam and temazepam, as examples of the benzodiazepine group, tetrahydrocannabinol (THC), and tetrahydrocannabinol-9-carboxylic acid (THC-carboxylic acid). The cases which were positive for methadone, morphine, and codeine were also analysed for 6-acetylcodeine, to see if the mother had used street drugs (indicator of addict mother) as well as 6-MAM, diamorphine, papaverine, thebaine, and dihydrocodeine. These drugs are the most popular drugs in UK.

10.2. Materials and Methods

10.2.1. Materials

All materials were as mentioned in Chapters 8 and 9 .

10.2.2. Instrumentation

Gas chromatography - mass spectrometry was carried out using a VG Micromass 70-250S double focusing magnetic sector instrument or a Finnigan-Thermoquest Trace quadrupole instrument. The gas chromatograph was equipped with a fused silica capillary column (HP5, 30 m x 0.32 mm id, 0.3 μm film thickness). Helium was

used as the carrier gas. Samples (volume 1 µl) were injected with an injection port temperature of 280°C. The initial column temperature was 200°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 1 minute, this program was for all drugs screened except amphetamine. The column temperature was initially held at 80°C for 2 min and then programmed to rise to 285°C at 5°C/min. The temperature was held at 285°C for the final 2min.

10.2.3. Method

(a) Sample collection

in addition to the samples collected earlier(p106,137) samples of meconium were collected from diapers of newborn infants delivered at the Royal Maternity Hospital, Glasgow, from the period of October 2000 until February 2001. The total number was 450 cases. The meconium was recovered using a wooden spatula and stored in plastic tubes at –4°C until analysed. In addition, 30 samples of meconium were collected from neonates delivered in several hospitals in Egypt who were unlikely to have been exposed to drugs *in uteri.* These were used as controls.

(b) Sample preparation:

Each meconium specimen was weighed (range from 0.5g - 0.8g) into a clean vial and centrifuged at 2000rpm for 10 minutes to bring it to the bottom. Drug standards were added and the samples were homogenised by vortexing and sonicating for 15 minutes. Methanol (3 ml) was added, and the tubes were vortexed well for 10 minutes then centrifuged at 300 rpm for 20 minutes. The upper layers were transferred to clean tubes and evaporated to dryness under nitrogen, initially at room

temperature then with slight warming up to 40°C until completely dry. Once dry, the residues were dissolved in 700 µl methanol with vortexing. The extraction tubes were then centrifuged at 2000rpm for 10 minutes.

(c) Sample Extraction:

The solid phase extraction (SPE) procedures applied were the two methods mentioned in previous chapters; the first one was for all target analytes except amphetamine, which was as follows:

- the column was prepared by washing with 2ml methanol, followed 2 ml phosphate buffer (pH6);
- the sample was applied at low vacuum pressure;
- the column was washed with 1ml distilled water, followed by 0.5ml acetic acid (1.0M, pH 3.3).
- the column was dried at maximum pressure for 4 min, then 50 µl methanol was added and the vacuum reapplied for 1min.

Elution was carried out in two stages:

- 4ml of eluant A (chloroform:acetone 1:1 v/v);
- 2 x 2ml of eluant B (2% ammonia in ethyl acetate).

The second method, for amphetamine was as follows:

- The columns were prepared by applying 3 ml methanol, 3 ml water and 1 ml phosphate buffer (pH 6).
- Then the sample was applied under low vacuum.

- The column was washed by 3-ml water, 1-ml acetic acid (pH 3.3), and 3-ml methanol.
- The column was dried under maximum vacuum for 5 minutes.
- The analytes were eluted by daily-prepared eluant: 3-ml dichloromethanol: IPA: concentrated ammonia in the ratio of (78: 20: 2).
- The eluate was evaporated under nitrogen without heat till completely dry.
- 50 µl PFPA: ethyl acetate 2:1 v/v was added and the tubes heated at 60°C for 20 minutes.
- The derivatised extracts were evaporated again under nitrogen without heat until dryness and the residue reconstitutes in 50µl ethyl acetate and then injected into the GC/MS.

A 500µl aliquot of eluate B was analysed by EIA for opiates, methadone, benzodiazepines, cannabinoids, amphetamines and methamphetamines. To the remainder of the eluate, the deuterated forms of each target analyte were added as internal standards. The extracts were mixed well and evaporated to dryness under nitrogen at 40°C until completely dry, then 50µl BSTFA were added, the vials were capped and incubated at 60°C for 20 minutes prior to GC-MS-SIM analysis. Selected ions used for quantitative analyses are listed in Table 9.1 along with the retention times for each analyte.

Table 10.1: Selected Ions Used for GC/MS/SIM

Drug	Ions selected	Internal standard	Ions selected	Rt (min)
Cocaine	303	$^2H_3$-cocaine	306	6.21
BZE	361	$^2H_3$-BZE	364	7.34
Morphine	429	$^2H_3$-morphine	432	8.58
Codeine	371	$^2H_3$-codeine	374	7.33
Methadone	72	$^2H_3$-methadone	75	6.03
Diazepam	284	$^2H_5$-diazepam	289	8.55
Temazepam	372	$^2H_5$-diazepam	289	9.33
THC	386	$^2H_3$-THC	389	9.15
THC-COOH	488	$^2H_3$-THC	389	10.15
AM	190	$^2H_3$-AM	193	5.75
MA	204	$^2H_3$-MA	193	7.08
MDA	162	$^2H_5$-MDA	168	9.77
MDMA	204	$^2H_5$-MDMA	209	11.05
MDEA	218	$^2H_5$-MDEA	223	11.44

(d) Analysis by GC/MS

The analysis by GC/MS was done using SIM as mentioned before after SPE extraction. The quantitative method used blank meconium with known concentrations of analytes (1ng/g, 10ng/g) and by these known concentrations the unknown concentration in positive sample can be calculated, using a calibration curve for each drug separately. All the positive samples for opiates by EIA were analysed for morphine and codeine. In cases which were positive for cocaine, the analytes were cocaine and its major metabolite in meconium, benzoylecgonine. Cases positive for cannabinoids during screening were analysed for THC and THCA. Lastly, for cases which were positive for methamphetamine and amphetamine the problem of small

amounts of specimen remaining, as at least another 400-500mg of meconium was needed for analysis. So in 65 cases no meconium was available for analysis, while the other 121 cases were analysed.

10.3. Results and Discussion

10.3.1. Sample Sizes

The samples were weighed, and the weights are summarized in the following table:

Table 10.2 Weights and appearance of each sample.

Number of case	Total Weight (mg)	Weight used (mg)	Special character
Case 1	1980	532.	
Case 2	1532	544.	Given as blank
Case 3	963	522	
Case 4	853	552	
Case 5	659	553	
Case 6	873	547	
Case 7	591	491	Green lung appearance, all the sample
Case.8	957	613	Slight yellow
Case 9	1568	601	
Case 10	915	620	
Case 11	845	616	Bright green
Case 12	329	149	All the sample
Case 13	950	506	
Case 14	350	216	All the sample
Case 15	892	523	Stool appearance
Case 16	566	466	All the sample
Case 17	910	511	Yellowish in colour
Case 18	634	529	All the sample
Case 19	537	537	
Case 20	650	420	All the sample
Case21	510	451	All the sample, greyish brown
Case 22	1250	617	
Case 23	895	623	
Case24	212	174	All the sample
Case25	3380	539	solid
Case26	621	348	All the sample
Case 27	795	504	

Case28	500	499	All the sample
Case 29	778	445	
Case 30	4323	559	
Case 31	497	254	All the sample
Case32	836	551	All the sample
Case 33	3604	562	Stay in freeze for a month.
Case 34	3915	589	Stay in freeze for a month.
Case35	1980	573	
Case 36	471	202	All the sample
Case 37	8480	593	Hard sticky
Case 38	637	278	The entire sample.
Case 39	1514	526	
Case 40	1077	528	
Case 41	1166	525	Yellow green water stool.
Case 42	3261	537	Yellow brown
Case 43	347	189	All the sample
Case 44	4443	572	Hard sticky
Case 45	200	74	All the sample
Case 46	888	560	Yellow brown.
Case 47	4389	577	
Case 48	594	340	All the sample
Case 49	783	533	Yellow brown, the entire sample.
Case 50	409	258	All the sample.
Case 51	167	41	All the sample.
Case 52	162	47	All the sample.
Case 53	940	566	All the sample.
Case 54	4339	544	Mucoid has two different green colours.
Case 55	353	90	All the sample
Case56	9533	568	
Case 57	938	513	
Case 58	2097	579	
Case 59	637	507	All the sample.
Case 60	1181	548	
Case 61	783	521	All the sample
Case 62	959	573	
Case 63	570	320	All the sample
Case 64	2827	537	
Case65	5017	562	Sticky, difficult to weight.
Case 66	791	543	
Case 67	1509	558	Sticky. Difficult to weight.
Case 68	597	517	
Case 69	2671	592	
Case 70	840	551	
Case 71	1916	550	
Case 72	1469	577	
Case 73	505	520	Almost all the sample.

Case 74	626	538	Sticky, light green.
Case 75	810	553	
Case 76	729	504	
Case 77	1042	579	
Case 78	765	410	
Case 79	641	410	All the sample used.
Case 80	775	486	
Case 81	1197	552	Yellow brown.
Case 82	722	334	
Case 83	423	380	
Case 84	456	253	All the sample used
Case 85	492	449	All sample used
Case 86	722	477	All samples used.
Case 87	1088	577	
Case 88	945	527	
Case 89	7250	578	
Case 90	3093	589	
Case 91	576	428	All the sample used, has green star.
Case 92	1944	520	
Case 93	3544	575	
Case 94	2638	554	
Case 95	597	467	All samples used.
Case 96	1618	592	Move as one sample.
Case 97	2122	575	
Case 98	789	375	All sample used
Case 99	1827	565	
Case 100	570	450	Yellowish brown.
Case 101	813	519	All the sample used
Case 102	474	115	All sample used
Case 103	844	532	Slight yellowish green
Case 104	1384	575	Has green star
Case 105	900	509	
Case 106	9401	547	
Case 107	452.	327	All the sample used.
Case 108	1038	576	
Case 109	4030	573	
Case 110	1528	568	Contain mucous
Case 111	3540	577	Contain yellow mucous
Case 112	1062	552	
Case 113	711	551	Yellow brown in colour
Case 114	643	476	
Case 115	252	248	Used all the sample
Case 116	533	476	Used all the sample
Case 117	911	521	Smooth watery stick on wall
Case 118	640	435	Used all the sample
Case 119	1749	577	

Case 120	1328	574	
Case 121	582	521	Watery , all sample used
Case 122	360	312	Green brown, all sample used
Case 123	605	360	Green brown, all sample used
Case 124	545	409	Green brown, all sample used
Case 125	1188	577	Yellow brown, all sample used
Case 126	527	377	Yellow brown, all sample used
Case 127	605	375	All sample used
Case 128	820	541	Used all the sample
Case 129	556	511	Used all the sample
Case 130	455	377	Used all the sample
Case 131	733	526	Used all the sample
Case 132	462	319	Used all the sample
Case 133	611	454	Used all the sample
Case 134	867	562	
Case 135	1219	550	Soft sticky to wall
Case 136	964	540	
Case 137	2058	560	
Case 138	953	562	Yellow brown
Case 139	1132	550	
Case 140	547	365	Smooth, sticky, used all sample
Case 141	2586	567	
Case 142	672	550	Bright green(MOHABBA)used all
Case 143	2143	564	
Case 144	3005	565	
Case 145	640	503	
Case 146	1120	563	
Case 147	522	395	Sticky, used all the sample
Case 148	3531	566	
Case 149	634	494	
Case 150	655	514	Almost all the sample
Case 150	655	514	Almost all the sample
Case 151	3089	549	
Case 152	381	370	All the sample
Case 153	906	532	
Case 154	572	422	All the sample used
Case 155	8626	578	
Case 156	742.	500	
Case 157	560.	434	Green in colour, soft, all used
Case158	1065	547	Green brown
Case 159	558	409	Nearly all sample used
Case 160	579	444	
Case 161	569	531	All the sample used
Case 162	532	466	Brown yellow, soft, all sample used
Case 163	806	515	Green brown in colour
Case 164	666	518	Green brown, all sample used

Case 165	221	150	All sample used
Case 166	420	312	All sample used
Case 167	482	347	All sample used
Case 168	251	122	Yellow brown, all sample used
Case 169	250	121	All sample used
Case 170	549	475	Green in colour, all sample used
Case 171	988	566	
Case 172	2453	566	
Case 173	232	144	All sample used
Case 174	212	136	All sample used
Case 175	310	223	All sample used
Case 176	320	254	Green brown in colour, nearly all used
Case 177	813	547	Yellow brown in colour
Case 178	1302	570	
Case 179	3222	565	
Case 180	1789	537	Green brown
Case 181	2069	548	
Case 182	7300	564	
Case 183	4567	575	
Case 184	3989	572	
Case 185	1420	569	
Case 186	813	529	
Case 187	672	449	
Case 188	892	536	
Case 189	877	506	
Case 190	910	550	
Case 191	320	252	All sample used
Case 192	614	545	Nearly all sample used
Case 193	5202	545	
Case194	2102	547	
Case195	964	535	
Case196	571	475	All sample used
Case197	539	420	All sample used
Case198	3297	547	
Case199	513	409	All sample used
Case200	846	526	Yellow brown in colour
Case 201	601	522	All sample used
Case 202	874	544	
Case 203	666	465	Nearly all sample used
Case 204	998	500	
Case 205	837	528	
Case 206	642	448	From two container, green lungy in appearance
Case 207	1136	513	Green in colour
Case 208	412	393	All sample used, green brown
Case 209	587	439	All sample used

Case 210	1643	529	
Case 211	499	404	All sample used
Case 212	754	538	
Case 213	789	540	
Case 214	449	340	All sample used
Case 215	699	537	
Case 216	978	520	
Case 217	512	407	All sample used
Case 218	602	421	
Case 219	510	360	Green brown, all sample used
Case 220	107	91	All sample used
Case 221	410	349	All sample used
Case 222	350	226	All sample used
Case 223	813	540	Yellow brown
Case 224	469	380	Nearly all sample used
Case 225	500	340	Nearly all sample used
Case 226	350	267	All sample used
Case 227	515	456	Nearly all sample used
Case 228	459	339	
Case 229	433	341	All sample used
Case 230	410	305	All sample used
Case 231	971	527	
Case 232	1216	534	
Case 233	1169	552	Green brown
Case 234	1303	521	Yellow brown
Case 235	1255	528	
Case 236	2390	533	
Case237	2732	554	
Case238	710	534	
Case 239	9449	552	
Case240	6920	55.	
Case241	699	553	Green brown in colour
Case 242	702	532	
Case 243	3250	555	
Case 244	963	546	
Case 245	699	543	Nearly all sample used
Case 246	678	533	
Case 247	783	540	Yellow brown in colour
Case 248	633	540	All sample used
Case 249	549	436	All sample used
Case 250	5163	554	
Case 251	659	530	Yellow green in colour
Case 252	5102	453	All sample used
Case 253	564	466	Has part white green in colour, all sample used
Case 254	839	504	Nearly all sample used
Case 255	381	285	All sample used

Case 256	437	326	Green in colour, all sample used
Case 257	310	254	All sample used
Case 258	1065	511	
Case 259	1461	547	
Case 260	773	530	Green watery in consistency
Case 261	954	554	
Case 262	310	245	All sample used
Case 263	1261	552	
Case 264	622	497	Brown, watery, nearly all used
Case 265	613	533	
Case 266	732	535	Green brown in colour
Case 267	451	332	All sample used
Case 268	452	307	All sample used
Case 269	7302	545	
Case 270	499	490	All sample used
Case 271	312	253	All sample used
Case 272	350	235	All sample used
Case 273	307	266	Green brown in colour, all used
Case 274	401	396	All sample used
Case 275	200	164	All sample used
Case 276	422	376	Green brown in colour, all used
Case 277	608	434	Green brown, nearly all used
Case 278	370	301	Green brown , all used
Case 279	362.	310	Green brown, all used
'case 280	2063	557	
Case 281	510	477	All sample used
Case 282	4255	551	Green brown in colour
Case283	1296	554	
Case 284	964	531	
Case 285	639	466	Soft, green in colour
Case 286	5162	550	
Case287	934	520	
Case 288	499	466	All sample used
Case 289	898	548	Water in consistency, brown in colour
Case 290	1353	557	
Case 291	592	479	Bright green, all sample used
Case 292	459	363	Green brown, all sample used
Case 293	500	409	Green, all sample used
Case 294	320	228	All sample used
Case 295	340	258	All sample used
Case 296	1210	543	
Case 297	382	311	Present with case 296 in same envelope, all sample used
Case 298	2063	542	
Case 299	621	508	Green colour, nearly all sample used

Case 300	1581	564	Has some white area
Case 301	1277	536	
Case 302	873	544	Brown green in colour
Case 303	1802	507	
Case 304	2201	548	
Case 305	982	536	Green in colour
Case 306	782	503	Soft in consistency
Case 307	202	167	All sample used
Case 308	863	524	
Case 309	10327	544	
Case 310	4831	544	
Case 311	320	300	All sample used
Case 312	812	544	
Case 313	341	296	All sample used
Case 314	520	489	
Case 315	290	230	
Case 316	80	76.	All sample used
Case 317	870	530	
Case 318	223	148	All sample used
Case 319	205	172	All sample used
Case 320	434	378	Yellow brown in colour, all used
Case 321	192	163	Yellow brown, all used
Case 322	264	223	All sample used
Case 323	290	240	Yellow brown, all sample used
Case 324	3201	555	
Case 325	1147	557	Has area white yellow
Case 326	978	542	Green brown, soft
Case 327	4102	552	
Case 328	893	562	Green colour, soft
Case 329	3617	549	
Case 330	5101	553	Soft
Case 331	2650	553	Light dark green in colour
Case 332	551	473	All used
Case 333	597	535	Yellow brown, nearly all used
Case 334	531	467	All used
Case 335	392	332	All used
Case 336	461	376	All sample used
Case 337	609	471	All sample used
Case 338	530	434	Green brown, all used
Case 339	573	441	All used
Case 340	350	256	Yellow brown, all used
Case 341	1100	529	
Case 342	1680	515	Present in brown envelope
Case 343	831	526	
Case` 344	1230	556	Yellow green
Case 345	1093	540	
Case 346	1100	533	

Case 347	3577	542	Has small yellow mucous area
Case 348	462	407	Green colourful used
Case 349	291	225	All sample used
Case 350	509.	453	All sample used
Case 351	349.	264	All sample used
Case 352	4119	548	Green yellow in colour
Case 353	750	515	
Case 354	863	526	
Case 355	601	535	
Case 356	1463	550	
Case 357	2320	543	
Case 358	1793	558	
Case 359	2633	547	
Case 360	763	554	Nearly all the sample
Case 361	733	560	Nearly all the sample
Case 362	402	382	All sample used
Case 363	863.	535	
Case 364	763.	548	
Case 365	1201	550	Yellow green in colour
Case 366	5093	552	
Case 367	3681	553	
Case 368	852	549	Brown green in colour
Case 369	871	556	Brown green in colour
Case 370	720	551	
Case 371	5926	416	Nearly all the sample used
Case 372	5208	365	
Case 373	510	372	
Case 374	420	396	All samples used.
Case375	1210	542	
Case376	867	529	
Case377	1812	550	
Case378	601.	409	Nearly all samples used.
Case379	5312	550	
Case380	749.	547	
Case381	916.	546	
Case382	1571	550	
Case383	2166	547	
Case384	1210	551	
Case385	5691	557	
Case386	210.	169	All samples used.
Case387	512.	494	All sample used, alternation yellow foci.
Case388	410.	305	
Case389	310.	225	
Case390	450.	364	
Case391	1007	543	
Case392	1352	546	

Case393	710.	552	Yellow brown colour
Case394	871.	546	
Case395	520.	313	
Case396	1144	547	
Case397	560	364	
Case398	340	294	All sample used
Case399	491	367	
Case 400	320	254	

10.3.2. Results of EIA:

The results of analysis of cases by EIA are given in Table 10.3

Table 10.3 Results of EIA Analysis Of 400 Cases

Case No	Benzo	Cann	Opiate	Metha-done	AM	MA	Cocaine
Blank	-	-	-	-	-	-	-
1	++++	4.1	++++	++++	--	-	++++
2	21	-	106	-	-	-	-
3	184	-	14	-	-	-	-
4	48	-	43	-	-	-	-
5	++++	16	388.	++++	-	-	-
6	++++	27	++++	91	-	-	-
7	26	3	83	-	-	-	-
8	39	-	133	++++	-	-	-
9	118	7	243	-	-	-	-
10	-	-	47	-	-	-	-
11	35	-	109	-	-	-	-
12	-	-	69	-	-	-	-
13	24	-	59	-	-	-	-
14	-	-	57	-	-	-	-
15	17	-	61	-	-	-	-
16	-	-	89	-	-	-	-
17	-	-	58	-	-	-	-
18	-	+++++	60	--	-	-	-
19	128	-	48	-	-	-	-
20	-	-	62	-	-	-	-
21	-	14	-	-	-	-	-
22	--	-	73	+++++	-	-	-
23	23	-	70	+++++	-	-	-
24	-	++++	-	-	-	-	-
25	11	-	-	-	128	-	-
26	118	-	-	-	-	-	-
27	-	14	-	-	-	-	-
28	370	-	-	-	-	107	-
29	12	-	43	-	-	57	-
30	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-
32	-	32	83	-	-	53	-
33	-	-	18	-	-	31	-
34	-	-	94	-	-	44	11
35	-	-	++++	-	-	-	-
36	-	-	23	--	-	-	-
37	-	-	34	--	-	-	-
38	-	-	-	-	-	30	11
39	-	-	152	-	-	-	-

40	-	-	-	-	-	-	-
41	44	-	-	-	-	40	-
42	-	--	-	-	-	-	-
43	-	++++	-	-	-	-	++++
44	-	++++	-	-	-	-	++++
45	-	13.	52	-	-	-	++++
46	-	++++	-	-	-	-	++++
47	-	17	103	-	-	29	-
48	-	9	++++	-	-	-	-
49	159	++++	-	-	188	104	-
50	34	28	23	-	-	-	-
51	-	++++	++++	-	-	-	-
52	-	9	-	-	-	-	-
53	-	3	127	-	-	33	-
54	-	++++	-	-	-	-	-
55	-	17	66	-	-	-	108
56	202	11	-	-	-	-	31
57	-	++++	-	-	-	46	17
58	-	++++	-	-	-	-	-
59	45	+++++	-	-	-	-	-
60	-	-	-	-	-	42	-
61	15	-	-	-	-	48	-
62	-	-	-	-	-	33	-
63	-	-	226	-	-	-	-
64	-	-	-	-	-	-	-
65	--	-	-	-	-	31	-
66	-	--	-	-	-	67	-
67	-	-	42	-	-	-	-
68	-	-	-	-	-	-	-
69	-	++++	-	-	55	64	-
70	-	-	-	-	-	55	-
71	-	8	63	-	-	41	-
72	-	-	-	-	-	-	-
73	-	-	-	-	-	58	-
74	-	-	-	-	-	33	-
75	-	-	-	-	-	-	-
76	-	39	58	-	-	31	-
77	-	-	-	-	-	44	-
78	-	-	-	-	-	-	-
79	-	-	-	-	-	-	-
80	-	-	-	-	-	36.240	-
81	-	-	-	-	-	106.184	-
82	-	41.196	-	-	-	-	-
83	--	-	-	-	-	68.456	-
84	-	-	-	-	-	-	-
85	-	-	-	-	-	-	-
86	-	-	-	-	-	49	-

87	-	-	++++	-	-	71	-
88	-	-	-	-	-	-	-
89	-	-	--	-	-	35	-
90	-	-	259	-	-	36	-
91	-	-	10	-	--	47	-
92	-	-	45	-	-	45	-
93	-	-	-	-	-	-	-
94	-	-	-	-	-	47	-
95	-	-	-	-	-	32	-
96	-	-	16	-	-	-	-
97	-	6	-	-	-	-	-
98	-	-	37	-	-	42	-
99	-	-	12	-	-	-	-
100	-	-	-	-	-	37	-
101	13	20	-	-	-	38	-
102	-	-	-	-	-	-	-
103	-	-	203	-	-	37	-
104	33.868	50.000	++++	+++++	-	26	-
105	-	-	-	-	-	-	-
106	-	-	-	-	-	26	-
107	-	-	-	-	-	60	-
108	-	-	13	-	-	-	-
109	-	-	-	-	-	-	-
110	-	-	27	-	-	-	-
111	-	-	-	-	-	-	-
112	-	-	-	-	-	-	-
113	-	-	31	-	-	44	-
114	-	-	132	-	-	-	-
115	-	-	-	-	-	-	-
116	-	-	-	-	--	-	-
117	-	-	-	-	29	49	-
118	-	-	105	-	-	32	-
119	-	-	62	-	--	-	-
120	-	-	-	--	-	-	-
121	-	-	162	-	-	-	-
122	-	-	13	-	-	-	-
123	-	+++++	-	-	--	63	-
124	-	-	-	-	--	-	-
125	-	--	-	-	-	37	-
126	-	-	23	-	-	31.770	-
127	399	4	342	24	-	-	-
128	-	-	-	--	-	-	-
129	-	-	-	-	-	26	-
130	-	-	--	-	-	-	-
131	-	--	--	-	-	41	-
132	-	25	179	14	-	-	-
133	-	-	++++	-	-	68	-

134	-	-	336.	-	-	-	-
135	-	-	76	-	-	62	-
136	-	-	-	-	-	32	-
137	-	-	-	-	-	-	-
138	-	-	-	-	-	41	-
139	-	-	-	-	-	-	-
140	-	-	88	-	-	-	-
141	-	-	-	-	-	26	-
142	53	-	359	-	58	-	-
143	-	-	-	-	-	-	-
144	-	-	-	-	-	-	-
145	-	-	76	-	-	-	-
146	18	-	94	-	-	36	-
147	-	-	--	-	-	-	-
148	-	-	-	-	-	-	-
149	-	-	-	-	-	65	-
150	-	-	-	-	-	34	-
151	-	-	-	-	-	-	-
152	-	-	20	-	-	82	-
153	-	-	95	-	133	42	-
154	-	+++++	-	-	-	43	-
155	-	-	462	-	-	-	-
156	-	19	78	-	-	-	-
157	-	-	-	-	-	45	-
158	-	-	-	-	-	43	-
159	-	13	-	-	-	-	-
160	11	-	194	-	-	-	-
161	-	-	63	-	-	28	-
162	-	-	102	-	-	108	-
163	-	-	177	-	-	49	-
164	-	-	-	-	-	42	-
165	-	-	-	-	-	-	-
166	-	26	127	-	-	46	-
167	-	6.619	-	-	-	-	-
168	-	-	-	-	-	-	-
169	18	18	134	-	-	-	-
170	-	-	-	-	-	53	-
171	50	-	217	-	-	51	-
172	-	-	-	-	-	-	-
173	-	-	-	-	-	-	-
174	-	-	-	-	-	-	-
175	-	-	270	-	-	-	-
176	-	--	223	--	-	33	-
177	-	--	-	-	-	95	-
178	-	-	-	-	-	-	-
179	-	-	68	-	-	76	-
180	-	-	474	-	-	31	-

181	-	-	-	--	-	-	-
182	-	-	53	-	-	-	-
183	-	-	89	-	-	-	-
184	-	20	-	-	-	-	-
185	36	-	116	-	-	122	-
186	-	-	--	-	-	39	-
187	-	-	85	-	--	37	-
188	-	-	-	--	--	38	-
189	-	-	41	-	--	-	-
190	-	-	-	-	-	54	-
191	-	-	35	--	--	34	-
192	-	-	116	-	-	28	-
193	-	--	-	-	-	-	-
194	-	-	-	-	-	33	-
195	-	-	-	-	-	32	-
196	17	-	-	-	-	93	-
197	21	-	10	-	-	64	-
198	-	-	22	-	-	-	-
199	77	16	83	-	-	43	-
200	54.034	-	394.987	-	-	117	-
201	-	-	48.707	-	-	-	-
201	-	2.623	104.347	-	-	57	-
203	-	-	-	-	-	-	-
204	-	-	-	-	-	-	-
205	-	-	62	-	-	57	-
206	17	18	-	-	-	105	-
207	-	-	-	-	-	97	-
208	-	-	-	-	-	79	-
209	-	-	-	-	-	55	-
210	-	-	16	-	-	49	-
211	-	-	27	-	-	-	-
212	-	-	-	-	-	-	-
213	15	-	88	-	-	79	-
214	-	-	31	-	-	26	-
215	24	-	78	-	-	65	-
216	-	-	88	-	-	38	-
217	-	-	-	-	-	-	-
218	-	-	179	-	-	25	-
219	--	-	-	-	-	-	-
220	-	15.899	37	-	-	-	-
221	-	-	75	-	-	-	-
222	-	-	58	-	-	-	-
223	-	-	-	-	-	38	-
224	17	-	91	-	-	33	-
225	-	-	34	-	-	-	-
226	-	25	-	54	-	25	-
227	-	-	-	-	-	41	-

228	-	-	43	-	-	26	-
229	-	-	-	-	-	-	-
230	-	-	93	-	-	-	-
231	-	-	-	-	-	59	-
232	20	-	116	-	-	44	-
233	-	-	-	-	-	45	-
234	-	-	-	-	-	67	-
235	-	5	21	-	-	47	-
236	-	-	30	-	-	-	-
237	31	-	-	-	-	-	-
238	-	-	-	-	-	-	-
239	-	-	13	-	-	51	-
240	-	-	16	-	-	-	-
241	-	-	-	-	-	50.	-
242	-	-	-	-	-	-	-
243	40	-	+++++	-	-	194	-
244	-	-	123	-	-	-	-
245	-	--	-	-	-	-	-
246	-	-	63	-	-	46	-
247	-	-	-	-	-	38	-
248	38	-	+++++	-	-	-	-
249	-	-	-	-	-	74	-
250	-	-	-	-	-	-	-
251	10	-	38	-	-	-	-
252	-	-	22	-	--	-	-
253	-	-	-	-	-	-	-
254	29	-	115	-	-	92	-
255	17	-	91	-	-	39	-
256	-	-	100	-	-	-	-
257	-	-	13	-	-	31	-
258	-	-	-	-	-	-	-
259	-	-	36	-	-	-	-
260	-	-	-	-	-	114	-
261	42	-	157	-	-	62	-
262	-	-	14	-	-	26	-
263	-	-	-	-	-	35	-
264	-	-	+++++	-	-	41	-
265	-	-	-	-	-	45	-
266	-	-	19	-	-	-	-
267	107	-	-	-	27	84	-
268	-	-	-	-	-	-	-
269	-	-	16	-	-	27	-
270	20	-	-	-	-	101	-
271	-	-	-	-	-	-	-
272	-	-	11	-	-	-	-
273	-	-	-	-	-	66	-
274	-	- -	21	-	-	-	-

275	-	-	-	-	-	-	-
276	-	-	-	-	--	47	-
277	-	-	-	-	-	-	-
278	-	-	56	-	-	37	-
279	-	-	24	-	43	34	-
280	-	+++++	11	-	-	42	-
281	-	-	207.545	-	-	-	-
282	-	+++++	-	-	-	61	-
283	105	5	+++++	+++++	-	-	-
284	-	-	37	-	-	-	-
285	-	-	-	-	-	37	-
286	-	-	-	-	-	-	-
287	-	-	-	-	-	-	-
288	-	+++++	-	-	-	-	-
289	-	-	-	-	-	70	-
290	-	-	-	-	-	-	-
291	13	-	92	-	-	34	-
292	-	-	131	-	-	46	-
293	-	-	12	-	-	34	-
294	-	+++++	-	-	-	32	-
295	-	-	-	-	-	-	-
296	+++++	-	+++++	+++++	-	37	194
297	-	-	109	-	-	47	-
298	-	-	-	-	-	-	-
299	-	-	-	-	-	-	-
300	-	-	18	-	-	-	-
301	-	-	-	-	-	-	-
302	13	-	143	-	-	97	-
303	-	-	424	-	-	88	-
304	-	-	-	-	-	-	-
305	-	-	55	-	-	36	-
306	-	-	65	-	-	36	-
307	-	-	-	-	-	45	-
308	14	-	+++++	-	-	64	-
309	-	-	-	-	-	-	-
310	-	21	38	47	-	-	-
311	-	-	269	-	-	-	-
312	-	-	-	-	-	31	-
313	63	-	+++++	-	-	158	-
314	-	-	64	-	-	54	-
315	+++++	+++++	169	95	-	-	-
316	23	-	-	-	-	-	-
317	14	35	244	-	-	42	-
318	-	-	448	-	-	41	-
319	-	-	-	-	-	-	-
320	28	-	-	-	-	45	-
321	-	-	157	-	-	-	-

322	-	19	-	-	-	26	-
323	-	-	-	--	-	-	-
324	+++++	-	+++++	+++++	-	-	-
325	-	-	58	-	-	-	-
326	-	-	404	-	-	35	-
327	-	-	-	-	-	28	-
328	-	-	-	-	-	72	-
329	-	-	+++++	-	-	-	-
330	-	-	-	-	-	-	-
331	-	-	-	-	-	35	-
332	-	-	-	-	-	26	-
333	-	-	+++++	-	-	58	-
334	-	-	-	-	-	54	-
335	-	-	-	-	-	-	-
336	-	-	-	-	-	58	-
337	-	-	-	-	-	30	-
338	-	-	-	-	-	32	-
339	-	-	-	-	-	33	-
340	-	-	-	-	-	215	-
341	-	-	+++++	-	-	-	-
342	74	+++++	+++++	+++++	-	31	-
343	-	-	-	-	-	38	-
344	-	-	+++++	-	-	68	-
345	19	-	++++	+++++	-	27	-
346	-	-	+++++	-	44	74	-
347	-	-	-	-	-	-	-
348	-	+++++	-	-	-	61	-
349	-	-	-	-	-	35	-
350	-	-	38	-	-	-	-
351	61	-	76	-	-	32	-
352	-	-	33	-	-	68	-
353	10	-	-	-	-	29	-
354	-	-	36	-	-	-	-
355	-	6	23	-	-	29	-
356	-	-	-	-	-	29	-
357	-	-	--	-	-	-	-
358	-	-	95	-	-	-	-
359	-	29	+++++	-	-	34	-
360	-	-	-	-	-	-	-
361	-	-	-	-	-	35	-
362	-	-	-	-	-	42	-
363	-	-	26	-	-	32	-
364	28	23	56	-	-	-	-
365	28	34	-	-	-	-	-
366	16	35	-	-	-	-	-
367	16	67	88	-	-	-	-
368	16	-	++++	-	-	-	-

369	20	-	91	-	-	-	-
370	24	-	36	-	-	-	-
371	17	-	45	-	-	-	-
372	21	-	88	-	-	-	-
373	26	-	33	-	-	-	-
374	21	-	33	-	-	-	-
375	-	-	-	-	-	34	-
376	-	-	-	-	-	-	-
377	-	-	-	-	-	-	-
378	-	16	-	-	-	-	-
379	-	-	-	-	-	-	-
380		-	+++++	-	-	40	-
381		-	-	-	-	33	-
382		-	-	-	--	-	13
383		-	31	-	-	125	-
384		-	-	-	-	-	-
385		3	-	-	-	29	-
386		-	-	-	-	-	-
387	25	5	-	-	-	-	-
388		-	23	-	-	-	-
389		-	35	-	-	-	-
390		-	-	-	-	-	-
391		-	-	-	-	-	-
392		41	-	-	--	-	-
393		-	47	-	-	34	-
394	-	-	-	-	-	-	-
395	42	-	-	-	-	-	-
396	159	-	-	-	-	-	-
397	-	-	-	-	-	-	-
398	+++++	-	-	-	-	-	-
399	-	-	-	-	-	-	-
400	-	-	-	-	-	-	-

Out of scale = (++++), Negative = (-)

From these, a summary of the final results of EIA for drugs is given in Table 10.4

and Figure 10.1:

Table 10.4 Number of positive cases by EIA and percentages of each drug group.

Drug Group	Number of positive cases	Percentages (%)
Opiates	194	48.5
Benzodiazepines	81	20.3
Cannabinoids	68	17
Methadone	17	4.3
Cocaine	12	3
AM	9	2.3
MA	186	46.5

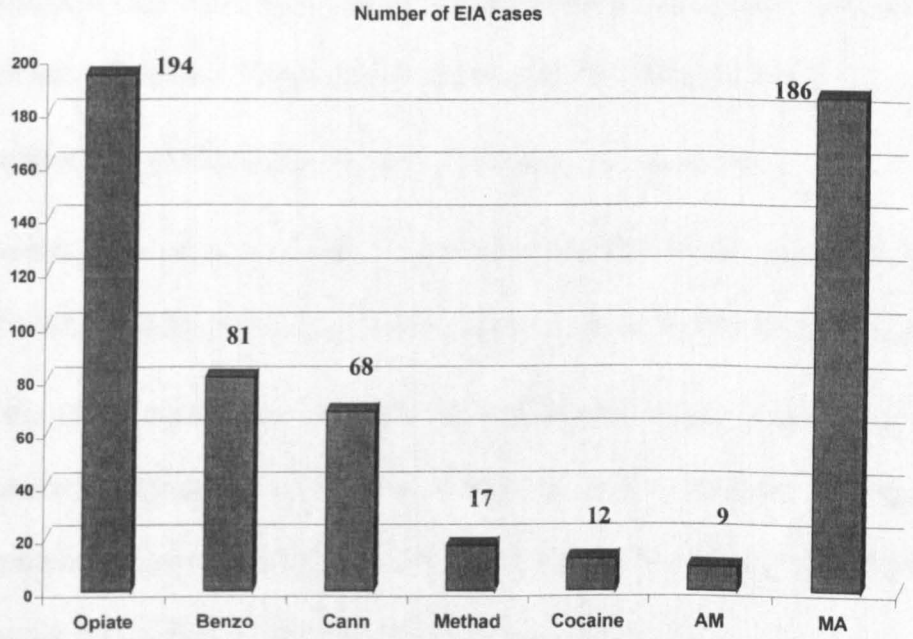


Figure 10.1 Number of positive cases of each drug group by EIA.

10.4. Drugs used in Hospital:

The EIA results indicated high percentages of the cases to be positive for both opiates and methamphetamine, so confirmation by GC/MS was carried out with reference back to the hospital files, when it was found that many of the mothers had been treated with drugs as follows:

❑ Diamorphine Injection 5 mg (201cases);

❑ Diamorphine injection 10 mg (291cases);

❑ These represent about 52.8% in all cases born in October and 45.5% in December;

❑ Temazepam tablets also given to (200cases) which represent 18.8% of all cases born.

10.5. Results of GC/MS analysis

It was also found that the results for methamphetamine were different if Cozart or Technological kits (which used occasionally in some cases) were used, so GC/MS analysis was carried out for all positive cases with the following aims:

1- Confirmation of EIA results as shown in Table 10.3 and 10.4;

2- Quantification of all the cases if possible by GC/MS. These results are given in Table 10.5 and the percentages agreeing with EIA are shown in Table 10.6.

3- In the cases which were positive for methamphetamine, to apply the method described in Chapter 9 to detect and measure all the important members of the amphetamine group (AM, MA, MDA, MDMA and MDEA) and result of its quantify by GC/MS. These results are given in Table 10.7.

4- In opiate-positive cases, which were found also positive for methadone, and codeine, and they are 9 cases, to distinguish between morphine derived from illict heroin and that originating in pharmaceutical diamorphine by analysing for impurities found in illicit heroin including 6-acetylcodeine, 6-monoacetylmorphine, dihydromorphine, dihydrocodeine, papaverine, and

thebaine. This was done by injecting standards of each of these substances to establish their retention times (given in Table 10.8). The results of the analysis of case samples are given in Table 10.9.

5- Addict mothers are commonly poly-drug users. The most common cause of death in substance-related deaths in forensic medicine is due to a combination of drugs. The study of Chasnoff et al 1988,[256] as mentioned before, reported that 32% of the cases which are drug-positive are positive for at least 2 drugs. In this survey an indication of the number of drug addicts can be obtained by taking notice of the methadone-positive group as these are most probably on a maintenance program, so in this study an additional aim was to try to establish the number of polydrug users during pregnancy, in those cases positive for methadone. The results are shown in Table 10.10.

Table 10.5 Results of GC/MS Analysis

Case No	Drug Concentration (ng/g meconium)									
	Diaz	Temaz	Morph	Cod	Metha	Coc	Benzo	THC	THCA	AM
Blank	-	-	-	-	-	-	-	-	-	-
Case1	114	-	100	80	319	20	11	-	-	-
2	147	128	38	110	-	-	-	-	-	-
3	-	4560	380	1600	-	-	-	-	-	-
4	1281	-	998	-	-	-	-	-	-	-
5	680	432	1070	-	2190	-	-	599	-	-
6	632	912	120	-	237	-	-	1010	-	-
7	70	-	30	30	-	-	-	-	-	-
8	-	-	30	80	-	-	-	-	-	-
9			110	60	510	-	-	-	-	-
10	-	-	150	-	-	-	-	-	-	-
11	-	-	50	-	-	-	-	-	-	-
12	-	-		40	-	-	-	-	-	-
13	230	223	10	40	-	-	-	-	-	-
14	-	-	15	40	-	-	-	-	-	-
15	240	51	14	50	-	-	-	-	-	-
16	-	-	10	50	-	-	-	-	-	-

17	-	-	14	50	-	-	-	-	-	-
18	-	-	10	50	-	-	-	-	-	-
19	-	-	10	60	-	-	-	-	-	-
20	-	-	10	30	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-
22	-	-	-	40	181	-	-	-	-	-
23	-	-	-	-	-	-	1	-	-	-
24	-	-	645	30	-	-	-	250	400	476
25	-	-	50	30	-	-	-	-	-	-
26	18	115	-	-	-	-	-	-	-	-
27	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-
29	78	100	30	20	-	-	-	-	-	-
30	-	-	-	-	-	-	-	-	-	-
31	-	-	-	-	-	-	-	-	-	-
32	-	-	50	15	-	-	-	175	180	-
33	-	-	70	60	-	-	-	-	-	-
34	-	-	90	110	-	-	-	-	-	-
35	-	-	50	40	-	-	-	-	-	-
36	-	-	30	20	-	-	-	-	-	-
37	-	-	50	10	-	-	-	-	-	-
38	-	-	-	-	-	-	-	-	-	-
39	-	-	50	10	-	-	-	-	-	-
40	-	-	30	10	-	-	-	-	-	-
41	53	273	-	-	-	-	-	-	-	-
42	-	-	30	10	-	-	-	-	-	-
43	-	-	70	10	-	-	-	175	180	-
44	-	-	20	-	-	-	-	150	180	-
45	-	-	30	10	-	-	-	100	-	-
46	-	-	50	30	-	-	-	750	-	-
47	-	-	70	20	-	-	-	125	120	-
48	-	-	20	500	-	-	-	200	-	-
49	560	766	160	20	-	-	-	175	160	240
50	510	1003	50	54	-	-	-	500	-	-
51	-	-	50	50	-	-	-	300	-	-
52	-	-	50	70	-	-	-	-	-	-
53	-	-	-	-	-	-	-	800	-	-
54	-	-	50	-	-	-	-	525	-	-
55	554	130	-	-	-	-	-	100	-	-
56	220	-	-	-	-	-	-	-	-	-
57	-	-	-	-	-	-	-	-	320	-

58	-	-	-	-	-	-	-	-	-	-
59	-	-	-	-	-	-	-	50	-	-
60	-	-	-	-	-	-	-	-	-	-
61	670	3330	-	-	-	-	-	-	-	-
62	-	-	-	-	-	-	-	-	-	-
63	-	-	32	30	-	-	-	-	-	-
64	-	-	50	10	-	-	-	-	-	-
65	-	-	-	-	-	-	-	-	-	-
66	-	-	-	-	-	-	-	-	-	-
67	-	-	357	119	-	-	-	-	-	-
68	-	-	-	-	-	-	-	-	-	-
69	-	-	-	-	-	-	-			100
70	-	-	-	-	-	-	-	-	-	-
71	-	-	132	10	-	-	-	-	-	-
72	-	-	-	-	-	-	-	-	-	-
73	-	-	-	-	-	-	-	-	-	-
74	-	-	-	-	-	-	-	-	-	-
75	-	-	-	-	-	-	-	-	-	-
76	-	-		614	-	-	-	-	-	-
77	-	-	-	-	-	-	-	-	-	-
78	-	-	-	-	-	-	-	-	-	-
79	-	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-	-
81	-	-	-	-	-	-	-	-	-	-
82	-	-	-	-	-	-	-	400	1120	-
83	-	-	-	-	-	-	-	-	-	-
84	-	-	-	-	-	-	-	-	-	-
85	-	-	-	-	-	-	-	-	-	-
86	-	-	-	-	-	-	-	-	-	-
87	-	-	-	1325	-	-	-	-	-	-
88	-	-	-	-	-	-	-	-	-	-
89	-	-	-	-	-	-	-	-	-	-
90	-	-	70	58	-	-	-	-	-	-
91	-	-	20	-	-	-	-	-	-	-
92	-	-	30	13	-	-	-	-	-	-
93	-	-	-	-	-	-	-	-	-	-
94	-	-	-	-	-	-	-	-	-	-
95	-	-	-	-	-	-	-	-	-	-
96	-	-	-	13	-	-	-	-	416	-
97	-	-	-	-	-	-	-	-	-	-
98	-	-	30	110	-	-	-	-	-	-

99	-	-	40	-	-	-	-	-	-	-
100	240	250	-	-	-	-	-	300	785	-
101	-	-	-	-	-	-	-	-	-	-
102	-	-	-	-	-	-	-	-	-	-
103	-	-	40	80	-	-	-	-	-	-
104	1810	-	1442	58	1434	-	-	500	375	-
105	-	-	-	-	-	-	-	-	-	-
106	-	-	-	-	-	-	-	-	-	-
107	-	-	-	-	-	-	-	-	-	-
108	-	-	48	16	-	-	-	-	-	-
109	-	-	-	-	-	-	-	-	-	-
110	-	-	23	16	-	-	-	-	-	-
111	-	-	-	-	-	-	-	-	-	-
112	-	-	-	-	-	-	-	-	-	-
113	-	-	48	237	-	-	-	-	-	-
114	-	-	100	24	-	-	-	-	-	-
115	-	-	-	-	-	-	-	-	-	-
116	-	-	-	-	-	-	-	-	-	-
117	-	-	-	-	-	-	-	-	-	
118	-	-	48	24	-	-	-	-	-	-
119	-	-	48	32	-	-	-	-	-	-
120	-	-	-	-	-	-	-	-	-	-
121	-	-	48	55	-	-	-	-	-	-
122	-	-	160	16	-	-	-	-	-	-
123	-	-	-	-	-	30	25	6200	268	-
124	-	-	-	-	-	-	-	-	-	-
125	-	-	-	-	-	-	-	-	-	-
126	-	-	48	16	-	-	-	-	-	-
127	394	100	210	-	162	-	-	4530	2050	-
128	-	-	-	-	-	-	-	-	-	-
129	-	-	-	-	-	-	-	-	-	-
130	-	-	-	-	-	-	-	-	-	-
131	-	-	-	-	-	-	-	-	-	-
132	-	-	2080	554	268	-	-	5467	2320	-
133	-	-	160	1013	-	-	-	-	-	-
134	-	-	128	40	-	-	-	-	-	-
135	-	-	5785	356	-	-	-	-	-	-
136	-	-	-	-	-	-	-	-	-	-
137	-	-	2147	736	-	-	-	-	-	-
138	-	-	-	-	-	-	-	-	-	-
139	-	-	-	-	-	-	-	-	-	-

140	-	-	2484	1092	-	-	-	-	-	-
141	-	-	-	-	-	-	-	-	-	-
142	704	25.9	351	204	-	-	-	-	-	56
143	-	-	-	-	-	-	-	-	-	-
144	-	-	160	79	-	-	-	-	-	-
145	-	-	640	94	-	-	-	-	-	-
146	39	119	369	10	-	-	-	-	-	-
147	-	-	-	-	-	-	-	-	-	-
148	-	-	-	-	-	-	-	-	-	-
149	-	-	-	-	-	-	-	-	-	-
150	-	-	-	-	-	-	-	-	-	-
151	-	-	-	-	-	-	-	-	-	-
152	-	-	41	40	-	-	-	-	-	-
153	-	-	10	2	-	-	-	-	-	71
154	-	-	-	-	-	-	-	1301	308	-
155	-	-	20	40	-	-	-	-	-	-
156	-	-	10	20	-	-	-	1717	1717	-
157	-	-	-	-	-	-	-	-	-	-
158	-	-	-	-	-	-	-	-	-	-
159	-	-	-	-	-	-	-	1063	923	-
160	400	167	3	-	-	-	-	-	-	-
161	-	-	-	-	-	-	-	-	-	-
162	-	-	-	-	-	-	-	-	-	-
163	-	-	383	140	-	-	-	-	-	-
164	-	-	-	-	-	-	-	-	-	-
165	-	-	-	-	-	-	-	-	-	-
166	-	-	-	-	-	40	31	-	462	-
167	-	-	-	-	-	-	-	-	1423	-
168	-	-	-	-	-	-	-	-	-	-
169	200	-	10	-	-	-	-	-	-	-
170	-	-	-	-	-	-	-	-	-	-
171	100	3910	43	-	-	-	-	-	-	-
172	-	-	-	-	-	-	-	-	-	-
173	-	-	-	-	-	-	-	-	-	-
174	-	-	-	-	-	-	-	-	-	-
175	-	-	40	21	-	-	-	-	-	-
176	-	-	4	-	-	-	-	-	-	-
177	-	-	-	-	-	-	-	-	-	-
178	-	-	-	-	-	-	-	-	-	-
179	-	-	13	-	-	-	-	-	-	-
180	-	-	-	-	-	-	-	-	-	-

181	-	-			-	-	-	-	-	-
182	-	-	5	-	-	-	-	-	-	-
183	-	-	-	-	-	-	-	-	-	-
184	-	-	-	-	-	-	-	-	303.8	-
185	400	5450	-	6.2	-	-	-	-	-	-
186	-	-	-	-	-	-	-	-	-	-
187	-	-	-	-	-	-	-	-	-	-
188	-	-			-	-	-	-	-	-
189	-	-	3	-	-	-	-	-	-	-
190	-	-	-	-	-	-	-	-	-	-
191	-	-	13	3	-	-	-	-	-	-
192	-	-	-	-	-	-	-	-	-	-
193	-	-	-	-	-	-	-	-	-	-
194	-	-	-	-	-	-	-	-	-	-
195	-	-	-	-	-	-	-	-	-	-
196	-	4087	-	-	-	-	-	-	-	-
197	131	1088	-	34	-	-	-	-	-	-
198	-	-	-	2.5	-	-	-	-	-	-
199	203	1163	-	20	-	-	-	1120	112.5	-
200	1256	2775	14	15	-	-	-	-	-	-
201	-	-	14	10	-	-	-	-	-	-
202	-	-	-	530	-	-	-	2100	287.5	-
203	-	-	-	-	-	-		-	-	
204	-	-	-	-	-	-	-	-	-	-
205	-	-	14	50	-	-	-	-	-	-
206	46.6	1050	-	-	-	-	-	400	1250	-
207	-	-	-	-	-	22	22	-	-	-
208	-	-	-	-	-	-	-	-	-	-
209	-	-	-	-	-	-	-	-	-	-
210	-	-	15	50	-	-	-	-	-	-
211	-	-	165	-	-	-	-	-	-	-
212	-	-	-	-	-	-	-	-	-	-
213	26	-	-	-	-	-	-	-	-	
214	-	-	-	-	-	-	-	-	-	
215	4	300	4	-	-	-	-	-	-	
216	-	-	-	-	-	-	-	-	-	-
217	-	-	-	-	-	-	-	-	-	-
218	-	-	-	-	-	-	-	-	-	-
219	-	-	-	-	-	-	-	-	-	-
220	-	-	-	-	-	-	-	2250	113.75	-
221			5	11	-	-	-	-	-	-

222			-	-	-	-	-	-		-
223					-	-	-			-
224	5	26.3	1.4	-	-	-	-			-
225			-	-	-	-	-			-
226					-	-	-	1394	112.5	256
227					-	-	-			-
228	-	-	-	-	-	-	-	-	-	-
229	-	-	-	-	-	-	-	-	-	-
230	-	-	-	-	-	-	-	-	-	-
231	-	-	-	-	-	-	-	-	-	-
232	-	-	-	-	-	-	-	-	-	-
233	-	-	-	-	-	-	-	-	-	-
234	-	-	-	-	-	-	-	-	-	-
235	-	-	-	-	-	-	-	-	-	-
236	-	-	-	-	-	-	-	-	-	-
237	63.1	15	-	-	-	-	-	4800	150	-
238	-	-	-	-	-	-	-	-	-	-
239	-	-	43	11	-	-	-	-	-	-
240	-	-	-	-	-	-	-	-	-	-
241	-	-	-	-	-	-	-	-	-	-
242	-	-	-	-	-	-	-	-	-	-
243	52	14	128.6	5	-	-	-	-	-	-
244	-	-	28.6	-	-	-	-	-	-	
245	-	-	-	-	-	-	-	-	-	-
246	-	-	64.3	35	-	-	-	125	-	
247	-	-	-	-	-	-	-	-	-	-
248	133	32.3	61.6	154	-	-	-	-	-	
249	-	-	-	-	-	-	-	-	-	-
250	-	-	-	-	-	25	15	-	-	-
251	271	24.9	-	-	-	-	-	-	-	-
252	-	-	-	136.4	-	-	-	-	-	-
253	-	-	-	-	-	-	-	-	-	-
254	61	32	15	6.6	-	-	-	-	-	-
255	472	13.8	48.6	17.2	-	-	-	-	-	-
256	-	-	33	-	-	-	--	-	-	
257	-	-	17	106.1	-	-	-	-	-	-
258	-	-	-	-	-	-	-	-	-	-
259	-	-	-	-	-	-	-	-	-	-
260	-	-	-	-	-	-	-	-	-	-
261	103	11	-	101	-	-	-	-	-	-
262	-	-	28.6	-	-	31	-	-	-	-

263	-	-	-	-	-	-	-	-	-	-
264	-	-	63	217.2	-	-	-	-	-	-
265	-	-	-	-	-	-	-	-	-	-
266	-	-	28.6	-	-	-	-	-	-	-
267	17	5	-	-	-	-	-	-	-	28
268	-	-	-	-	-	-	-	-	-	-
269	-	-	29	12.1	-	-	-	-	-	-
270	90	25	-	-	-	-	-	-	-	-
271	-	-	-	-	-	-	-	-	-	-
272	-	-	28.6	35.4	-	-	-	-	-	-
273	-	-	-	-	-	-	-	-	-	-
274	-	-	14.3	33	-	-	-	-	-	-
275	-		28.6	35.4	-	-	-	-	-	-
276	-	-	-	-	-	-	-	-	-	-
277	-	-	-	-	-	-	-	-	-	-
278	-	-	71.4	25.3	-	-	-	-	-	-
279	-	-	-	-	-	-	-	-	-	-
280	-	-	41	195	-	-	-	-	-	-
281	-	-	-	-	-	-	-	-	-	-
282	-	-	460	-	-	-	-	18	-	-
283	350	-	2100	240	2400	-	-	2100	240	-
284	-	-	30	600	-	-	-	-	-	-
285	-	-	-	-	-	-	-	-	-	-
286	-	-	-	-	-	-	-	-	-	-
287	-	-	-	-	-	-	-	-	-	-
288	-	-	-	-	-	-	-	1022	175	-
289	-	-	-	-	-	-	-	-	-	-
290	-	-	-	-	-	-	-	-	-	-
291	495	14	-	-	-	-	-	-	-	-
292	-	-	-	-	-	-	-	-	-	-
293	-	-	-	-	-	-	-	-	-	-
294	-	-	-	-	-	-	-	-	590	-
295	-	-	-	-	-	-	-	-	-	-
296	93	10	105	75	140	62	40	-	-	-
297	-	-	5	46	-	-	-	-	-	-
298	-	-	-	-	-	-	-	-	-	-
299	-	-	-	-	-	-	-	-	-	-
300	-	-	6	-	-	-	-	-	-	-
301	-	-	-	-	-	-	-	-	-	-
302	51.2	267	72	-	-	-	-	-	-	-
303	-	-	8	207	-	-	-	-	-	-

304	-	-	-	-	-	-	-	-	-	-
305	-	-	-	94	-	-	-	-	-	-
306	-	-	11	-	-	-	-	-	-	-
307	-	-	-	-	-	-	-	-	-	-
308	950	1900	16	-	-	-	-	-	-	-
309	-	-	-	-	-	-	-	-	-	-
310	-	-	5	-	33	-	-	188	300	-
311	-	-	16	-	-	-	-	-	-	-
312	-	-	-	-	-	-	-	-	-	-
313	450	-	450	-	-	-	-	-	-	-
314	-	-	79	-	-	-	-	-	-	-
315	61	-	57	-	33	-	-	241	350	-
316	2	-	-	-	-	-	-	-	-	-
317	60	-	31	-	-	-	-	-	160	-
318	-	-	-	-	-	-	-	-	-	-
319	-	-	-	-	-	-	-	-	-	-
320	-	-	-	-	-	-	-	-	-	-
321	-	-	19	-	-	-	-	-	-	-
322	-	-	-	-	-	41	40	943	-	-
323	-	-	-	-	-	-	-	-	-	-
324	-	-	-	-	-	-	-	-	-	-
325	-	-	-	-	-	-	-	-	-	-
326	-	-	21.2	48	-	-	-	-	-	-
327	-	-	-	-	-	-	-	-	-	-
328	-	-	-	-	-	-	-	-	-	-
329	-	-	-	-	-	-	-	-	-	-
330	-	-	-	-	-	-	-	-	-	-
331	-	-	-	-	-	-	-	-	-	-
332	-	-	-	-	-	-	-	-	-	-
333	-	-	98	195	-	-	-	-	-	-
334	811	-	6	-	23	-	-	-	-	-
335	-	-	133	31.5	-	-	-	-	-	-
336	-	-	-	-	-	-	-	-	-	-
337	-	-	-	-	-	-	-	-	-	-
338	-	-	-	-	-	-	-	-	-	-
339	-	-	16	-	-	-	-	-	-	-
340	-	-	-	-	-	-	-	-	-	-
341	-	-	500	15	-	-	-	-	-	-
342	1520	1380	98	-	305	-	-	240	-	-
343	-	-	-	-	-	-	-	-	-	-
344	-	-	62	14	-	-	-	-	-	-

345	521	133	-	-	240	-	-	-	-	-
346	-	-	415	106	-	-	-	-	-	-
347	-	-	-	-	-	-	-	-	-	-
348	-	-	-	-	-	-	-	500	119	-
349	-	-	-	-	-	-	-	-	-	-
350	-	-	-	10	-	-	-	-	-	-
351	129	825	-	-	-	-	-	-	-	-
352	-	-	370	15	-	-	-	-	-	-
353	1110	-	-	-	-	-	-	-	-	-
354	-	-	-	578	-	-	-	-	-	-
355	-	-	680	28	-	-	-	265	262	-
356	-	-	-	-	-	-	-	-	-	-
357	-	-	-	-	-	-	-	-	-	-
358	-	-	94	-	-	-	-	-	-	-
359	-	-	-	131	-	-	-	1265	-	-
360	-	-	-	-	-	-	-	-	-	-
361	-	-	-	-	-	-	-	-	-	-
362	-	-	-	-	-	-	-	-	-	-
363	-	-	13	30	-	-	-	-	-	-
364	948	-	15	-	-	-	-	-	161	-
365	240	-	-	-	-	-	70	-	171	-
366	300	-	-	-	-	-	-	70	476	-
367	247	1530	11	-	-	-	-	-	-	-
368	478	-	6	-	-	-	-	-	-	-
369	472	-	138	-	-	-	-	-	-	-
370	372	-	3	-	-	-	-	-	-	-
371	633	-	40	-	-	-	-	-	-	-
372	677	55	-	-	-	-	-	-	-	-
373	369	-	66	-	-	-	-	-	-	-
374	197	-	-	-	-	-	-	-	-	-
375	-	-	-	-	-	-	-	-	-	-
376	-	-	-	-	-	-	-	-	-	-
377	-	-	-	-	-	-	-	-	-	-
378	-	-	538	-	-	-	-	-	-	-
379	-	-	-	-	-	-	-	-	-	-
380	-	-	-	-	-	-	-	-	-	-
381	-	-	-	-	-	-	-	-	-	-
382	-	-	-	-	-	-	-	-	-	-
383	-	-	-	-	-	-	-	-	-	-
384	-	-	-	-	-	-	-	-	-	-
385	-	-	538	190	-	-	-	-	-	-

386	-	-	-	-	-	-	-	-	-	-
387	458	545	500	71	-	-	-	-	-	-
388	-	-	-	-	-	-	-	-	-	-
389	-	-	-	-	-	-	-	-	-	-
390	-	-	-	-	-	-	-	-	-	-
391	-	-	-	-	-	-	-	-	-	-
392	-	-	500	119	-	-	-	-	-	-
393	-	-	-	-	-	-	-	-	-	-
394	-	-	-	-	-	-	-	-	-	-
395	417	545	-	-	-	55	40	-	-	-
396	458	-	-	-	-	-	-	-	-	-
397	-	-	-	-	-	-	-	-	-	-
398	458	1318	-	-	-	-	-	-	-	-
399	-	-	-	-	-	-	-	-	-	-
400	-	-	-	-	-	-	-	-	-	-

Diaz=Diazepam, Temaz=Temazepam, Morph=Morphine, Methad=Methadone,

Benz=Benzoylecognine

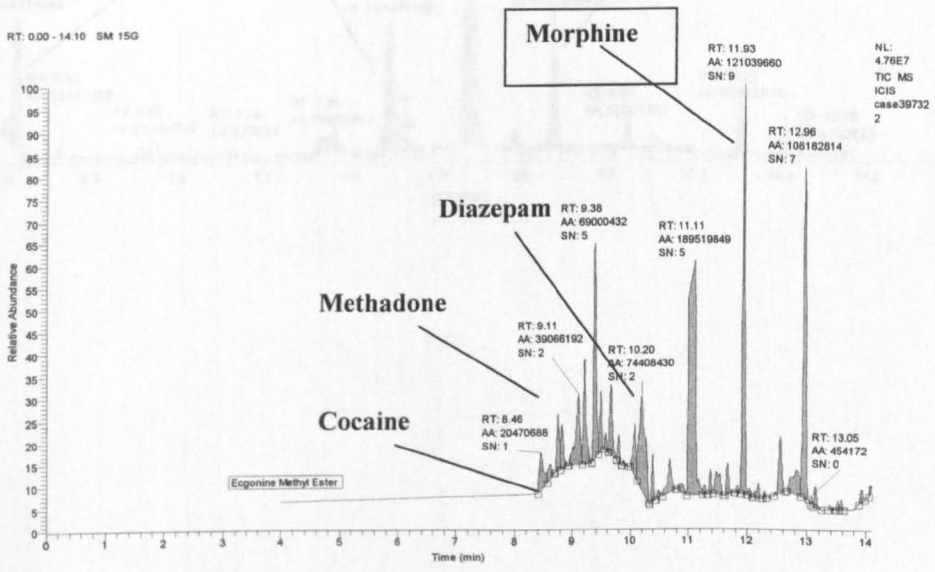


Figure 10.2 Chromatogram of case 296 by GC/MS Equipped by (HP1 column)

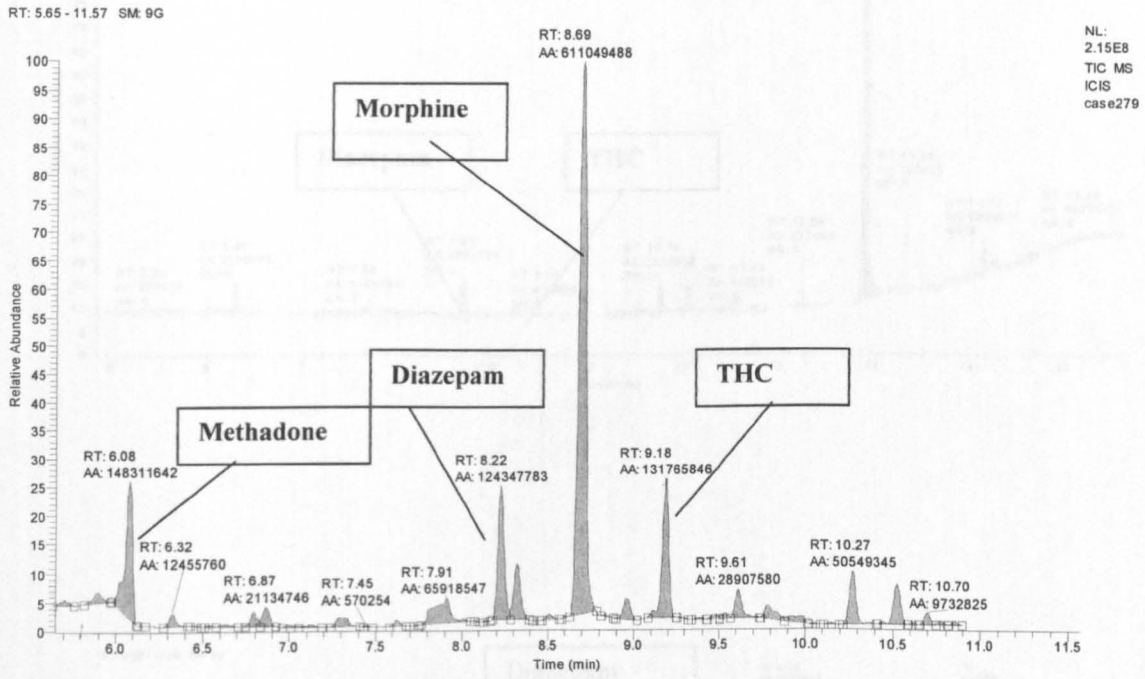


Figure 10.2b Chromatogram of case 296 by GC/MS equipped by ((HP5 column)

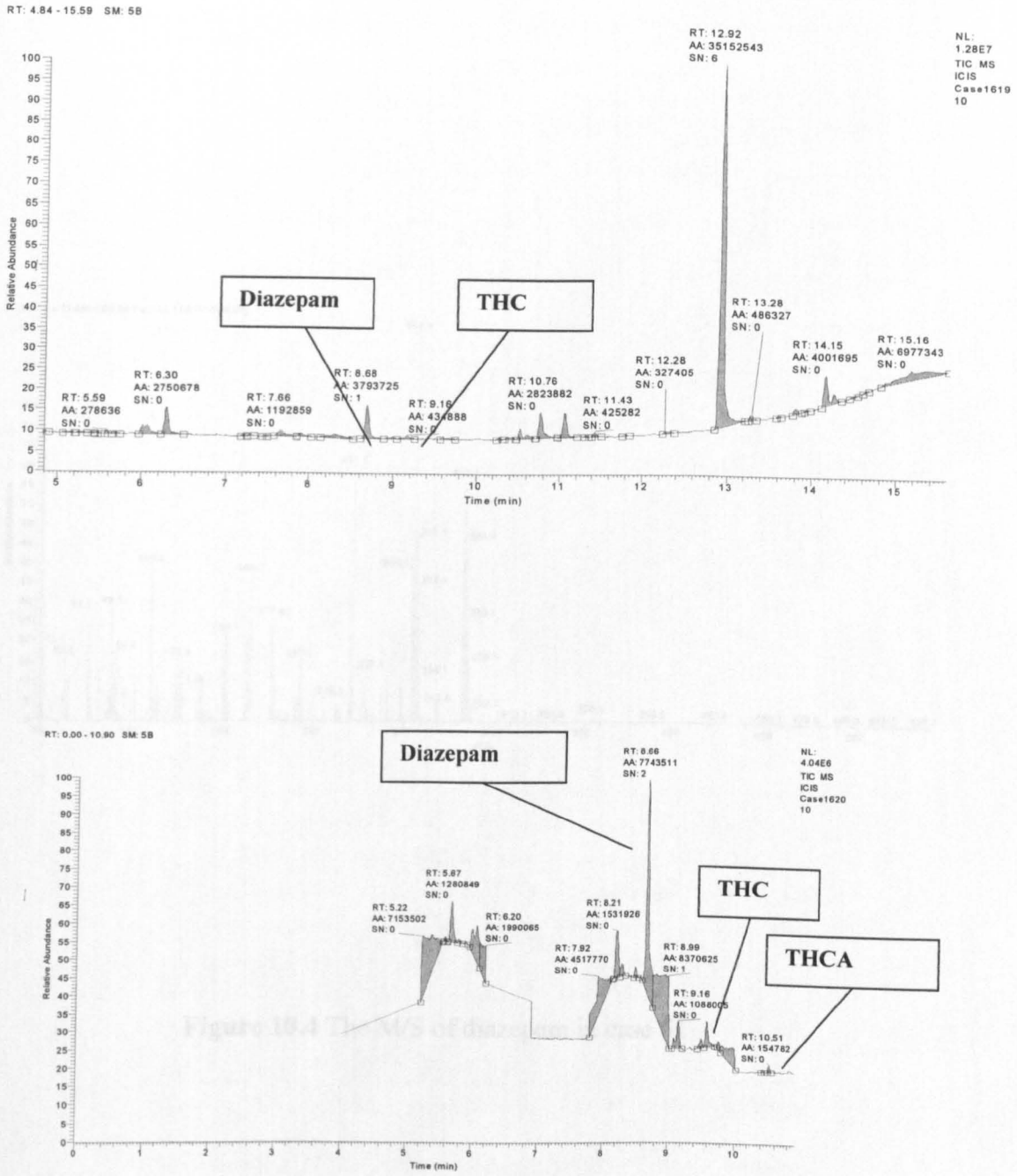


Figure 10.3 Full scan and SIM of case 61

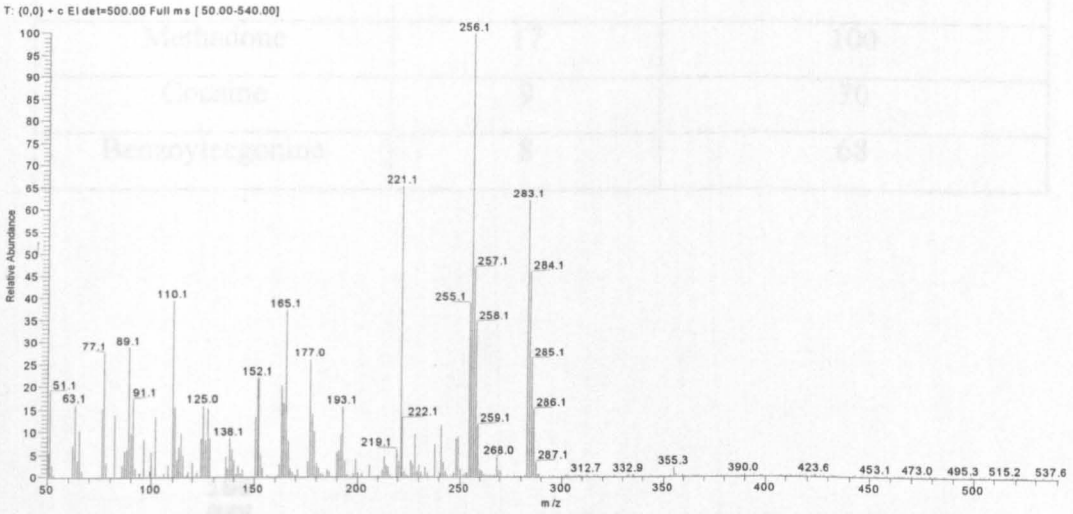
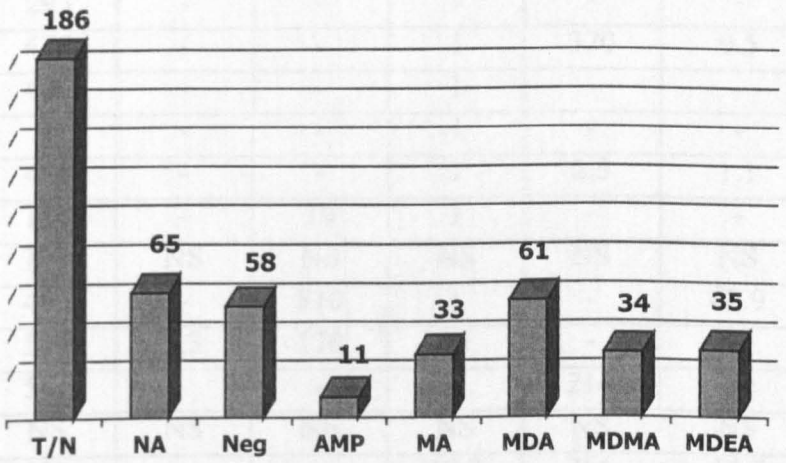


Figure 10.4 The M/S of diazepam in case 61

Table 10.6 Percentages of cases in which EIA results are confirmed by GC/MS.

Drug	Number of Cases	Percentage Confirmed (%)
Morphine	155	80
Codeine	116	60
Diazepam	70	80
Temazepam	48	59
THC	41	68
THCA	40	67
Methadone	17	100
Cocaine	9	70
Benzoylecgonine	8	68



T/N = total number NA= not analysied Neg= negative

Figure 10.5 Number of cases of amphetamines confirmed by GC/MS.

Table 10.7 Results of GC/MS for cases positive for amphetamines.

case	weight	Concentrations of drugs (ng/g meconium)				
		AM	MA	MDA	MDMA	MDEA
25	462	55.3	620	25.8	19.3	25.3
28	494	1	-	-	-	-
29	275	-	-	-	-	-
32	NS	NS	NS	NS	NS	NS
33	500	63.2	670	15.4	-	55.3
34	505	-	-	-	-	-
38	NS	NS	NS	NS	NS	NS
41	510	-	90	61.5	-	-
47	507	2.2	44	6.7	16.7	20
49	251	-	6.8	1.5	-	-
53	NS	NS	NS	NS	NS	NS
57	417	2.86	-	8.7	-	-
60	502	-	-	1	-	-
61	NS	NS	NS	NS	NS	NS
62	500	-	-	-	-	-
65	494	-	60	1.5	0.8	34.2
66	213	-	60	1.5	-	1.6
69	491	-	-	-	-	-
70	409	-	66	1	375	0.8
71	497	-	-	1.5	-	-
73	284	-	-	1	-	-
74	293	-	-	-	-	-
76	477	-	-	1	370	0.5
77	496	-	-	1	-	-
80	78	-	-	1	-	-
81	498	-	-	1	2.5	1.1
83	131	-	19	1	-	-
86	NS	NS	NS	NS	NS	NS
87	499	-	810	1	-	42.9
89	503	3.3	170	2.1	-	-
90	547	-	-	1	214	-
91	NS	NS	NS	NS	NS	NS
92	532	-	-	10.3	366	44.7
94	563	-	-	28	750	41.3
95	NS	-	-	-	-	-
98	504	-	-	1	583	39.5
100	632	-	-	1	5.2	-
101	NS	NS	NS	NS	NS	NS

103	397	-	0.6	1	0.8	1.5
104	472	-	-	-	-	-
106	499	-	-	-	-	-
107	NS	NS	NS	NS	NS	NS
113	542	-	-	1	-	-
117	212	-	-	-	-	-
118	NS	NS	NS	NS	NS	NS
123	NS	NS	NS	NS	NS	NS
125	419	-	-	1.5	4000	-
126	NS	NS	NS	NS	NS	NS
129	NS	NS	NS	NS	NS	NS
131	NS	NS	NS	NS	NS	NS
133	NS	NS	NS	NS	NS	NS
135	513	-	150	2.1	8.3	-
136	496	-	-	-	-	-
138	494	-	-	2.1	-	-
141	497	-	-	-	-	-
142	99	-	-	200	-	-
146	NS	NS	NS	NS	NS	NS
149	189	-	-	36	-	-
150	NS	-	-	30.8	-	-
152	NS	NS	NS	NS	NS	NS
153	433	-	-	-	250	-
154	NS	NS	NS	NS	NS	NS
157	NS	NS	NS	NS	NS	NS
158	503	-	-	-	-	-
161	NS	NS	NS	NS	NS	NS
162	NS	NS	NS	NS	NS	NS
163	314	-	2	1	18.2	8.2
164	NS	NS	NS	NS	NS	NS
166	NS	NS	NS	NS	NS	NS
170	86	-	-	-	-	-
171	507	-	-	-	-	-
176	NS	NS	NS	NS	NS	NS
177	274	5.5	11	6.2	3.6	76.3
179	503	-	-	-	-	-
180	507	-	-	-	-	-
185	503	-	-	-	-	-
186	420	-	-	-	199	-
187	122	7.1	14	10.8	390	0.3
188	327	7.5	16	5.1	872.7	0.5

190	432	-	-	-	-	-
191	NS	NS	NS	NS	NS	NS
192	NS	NS	NS	NS	NS	NS
194	502	-	-	-	-	-
195	317	-	-	-	-	-
196	NS	NS	NS	NS	NS	NS
197	NS	NS	NS	NS	NS	NS
199	NS	NS	NS	NS	NS	NS
200	NS	NS	NS	NS	NS	NS
201	185	NS	NS	NS	NS	NS
205	501	7.3	15	-	981.8	-
206	264	-	-	-	-	-
207	501	-	-	-	-	-
208	NS	NS	NS	NS	NS	NS
209	some	-	3	-	5.5	0.1
210	506	-	-	-	-	-
213	376	4.6	-	-	-	-
214	NS	NS	NS	NS	NS	NS
215	276	-	30	1	8.3	6.1
216	505	-	2	1	140	1.3
218	255	-	-	-	-	-
223	464	-	-	1	150	-
224	NS	NS	NS	NS	NS	NS
226	NS	NS	NS	NS	NS	NS
227	some	NS	NS	NS	NS	NS
228	185	-	-	-	-	-
231	501	-	2	1	16.7	0.3
232	500	-	1	1	250	-
233	500	-	-	-	-	-
234	505	-	-	54.4	-	-
235	499	-	110	1.02	-	16.8
239	433	-	-	-	-	-
241	329	-	-	-	-	-
243	505	-	-	-	-	-
246	169	-	-	-	8.9	-
247	189	-	-	56.4	5.5	-
249	NS	NS	NS	NS	NS	NS
254	some	NS	NS	NS	NS	NS
255	NS	NS	NS	NS	NS	NS
257	NS	NS	NS	NS	NS	NS
260	358	72.9	-	21.5	-	-

261	508	-	-	-	-	-
262	NS	NS	NS	NS	NS	NS
263	508	-	-	-	-	-
264	241	-	-	-	-	-
265	498	-	-	-	-	-
276	NS	-	-	-	-	-
269	504		-	-	-	-
270	NS	NS	NS	NS	NS	NS
276	NS	NS	NS	NS	NS	NS
278	NS	NS	NS	NS	NS	NS
279	NS	NS	NS	NS	NS	NS
280	527	-	-	-	-	-
282	NS	NS	NS	NS	NS	NS
285	84	-	-	-	-	-
289	318	-	-	-	-	-
291	NS	NS	NS	NS	NS	NS
292	NS	NS	NS	NS	NS	NS
293	NS	NS	NS	NS	NS	NS
294	NS	NS	NS	NS	NS	NS
296	505	-	-	-	-	-
297	NS	NS	NS	NS	NS	NS
302	345	-	-	-	-	-
303	322	-	-	-	-	-
305	504	-	-	-	-	-
306	326	-	-	-	-	-
307	NS	NS	NS	NS	NS	NS
308	296	-	-	-	-	-
312	505	-	-	82.1	1.7	36.8
313	NS	NS	NS	NS	NS	NS
314	155	-	-	-	-	-
320	NS	NS	NS	NS	NS	NS
322	NS	NS	NS	NS	NS	NS
326	343	-	-	-	-	-
327	532	-	44	0.8	5	5
328	521	-	300	1	-	62.8
331	505	-	-	0.5	-	52.6
332	NS	-	-	1	-	-
333	NS	NS	NS	NS	NS	NS
334	NS	-	-	-	-	-
336	185	-	-	0.5	-	51.3
337	125	-	91	0.5	-	52.6

338	122	-	12	0.5	-	-
339	155	-	54	0.5	-	50
340	198	-	1	0.5	981	55.3
342	502	-	5	1.5	134.5	0.8
343	112	-	1	13.8	7.3	8.4
344	502	-	-	1.5	-	-
345	506	-	-	-	7.3	8.9
346	522	-	-	-	-	-
348	NS	NS	NS	NS	NS	NS
349	NS	NS	NS	NS	NS	NS
351	NS	NS	NS	NS	NS	NS
352	506	-	1	1	90.9	0.8
353	379	-	-	-	-	-
355	101	-	-	-	-	-
356	507	-	-	-	-	-
359	507	-	-	-	-	-
361	NS	NS	NS	NS	NS	NS
362	NS	NS	NS	NS	NS	NS
363	352	-	-	-	-	-
375	504	-	-	-	-	-
380	337	-	-	-	-	-
381	503	-	34	1.5	3.6	-
383	504	-	-	-	-	-
385	501	-	-	-	-	-
393	439	-	-	1.5	-	-

NS=No sample remain (-)=negative

Table 10.8 Retention times and selected ions of markers of illicit heroin.

Drug	Retention Time	Ion Selected	Quantitation ion
Papaverine	14.62	339	339
6-MAM	12.62	399,340,287	399
6-Acetylcodeine	12.05	341,282	341
Thebaine	11.68,11.78	383,353	383
Dihydromorphine	13.23	268.369.268	369
Acetylcodeine	10.95	341,281,228	281
Dihydrocodeine	11.12	373,282,315	373
6-MAM-d3	12.61	402,343,290	402
Morphine		429,414	429
Codeine		371,343	371
Methadone	9.13	294,72,164	72

Table 10.9 Results of analysis for markers of illicit heroin.

Case No	Papaverine	6-MAM (ng/g)	6-AC	Thebaine	DHM	AC	DHC
5	N	N	N	P	N	N	N
104	N	85.3	P	P	N	N	N
2	N	N	P	P	N	P	N
22	P	0.6	N	P	N	N	N
23	N	81.9	P	P	P	N	P
283	N	73.4	P	P	N	P	N
296	N	73.1	N	P	N	N	P
310	P	1.1	N	P	N	N	P
315	N	73.6	P	P	N	N	P

.N=negative **P=positive**

6-AC=6-Acetylcodeine **DHM**=Dihydromorphin **AC**= Acetylcodeine

DHC= Dihydrocodeine

Table 10.10 Summary of results for markers of illicit heroin.

	positive	Negative
Number of cases	9	
6-MAM	7	2
6-acetylcodeine	5	4
Dihydromorphine	2	7
Papaverine	2	7
Thebaine	8	1
Dihydrocodeine	4	5

Table 10.11 Number of cases involving more than one drug.

Number of cases	Positive drugs
2	5
12	4
3	2

10.5.1. Conclusion:

This survey is the first one to have been done in Glasgow using meconium, and shows the percentages of mothers who used drugs during pregnancy. In this study all the drugs of abuse in Uk were analyzied, compared with other studies were only one or a maximum of three drugs were analyzed. Also, in this study high percentages of drugs abuse were identified compared to the previous studies. For example there were 39% and 10% positive cases for opiates and cannabinoids respectivelly. The least percentages was found to be associated with cocaine abuse, this is in contrast

with the previous American studies which showed that most of their cases were positive for cocaine. Also all the 3.5% which are positive for methadone were polydrug addict to more than one drugs or their metabolites. The concentration of drugs screened ranged from low to very high, which points out to the risks associated with drug use by pregnant mothers as high concentrations of drugs were measured in the meconium from their neonates. Most of these drugs cause severe morbidity with major complication and mortality, which may affect the neonate throughout its life. Precautions must be taken with respect to all drugs given to the mother in hospitals, as it is clear that these can reach the foetus, and should be avoided except in critical cases.

11. Hydrolysis of Cannabinoids in Meconium.

11.1. Introduction

The survey reported in the previous chapter produced several cases which were positive by immunoassay and negative by GC/MS. One explanation of these differences is that the cannabinoids in meconium are significantly conjugated with glucuronic acid and an additional hydrolysis step is needed in the method before extraction and GC/MS analysis. The aim of this final study was to determine the effect on the incidence of confirmed cannabinoid positives by including a hydrolysis step in the method. The background to cannabis use and abuse will first be reviewed.

11.1.1. History of Cannabis Use and Abuse

The hemp plant and its products have been a valuable source for man's commercial, medical, religious and recreational use for thousands of years. Different parts can be used for different purposes. Its fibre can be made into rope, twine and bags, and the biologically active constituents can be used as relaxants and mild intoxicants. Its oil is used in painting and varnishing.

The cannabis plant is called *Cannabis Sativa L.* or Indian hemp. It is a tall annual bushy plant present especially in high temperate and tropical zones of the world and is probably the oldest non-food plant ever cultivated. It contains over 400 chemical compounds amongst which more than 60 cannabinoids have been identified. The most important products are marihuana, consisting of the dried leaves and stems, and hashish, consisting of the dried buds.

Cannabis plant is dicœcious i.e. it has male and female plants, in which the female yields more but less potent resin than the male.[283] Generally, the content of active

constituents decreases in the following order: bracts, flowering tops, leafy tops, small leaf, large leaf, and stem. No cannabinoids are found in the roots or seeds. The concentration of cannabinoids in the plant is a function of genetic and environmental factors (type of soil, water, temperature, growing space). Other factors include the time of collection (maturity of the plant) and the treatment of the sample taken (drying, storage, extraction and analysis). Marihuana, bhang, ganja, kif and macoha are the commonly used names for cannabis or its products in various countries. Differences in names refer to differences in the mixture of the leaves and flowering tops of the plant. For example, marihuana consists of the dried and crumbled stems, leaves and seed pods of the female plant. Bhang consists of specially dried leaves and flowering shoots of both male and female plants, wild or cultivated, while ganja refers to a specially cultivated hemp plant that contains an unusually large quantity of active materials.

Meanwhile, hashish or charas are names often applied to the pure unadulterated resin from the top of the finest female plants of Indian hemp, which also contains a very high concentration of active constituents. Less common and strongest of all, is cannabis oil or hash oil, a liquid prepared from the resin.

Under the Misuse of Drugs Act (1971), the terminology for materials derived from *Cannabis Sativa L.* are specifically defined. Under British law, only the name "cannabis" is used and there is no reference to "marijuana" or "marihuana".

The first use for cannabinoids was in medicine as antiseptic and analgesic in China and India. Then its use spread throughout the Middle East, Africa and Eastern Europe, in treatment of pain, asthma, dysentery, the promotion of sleep, suppression of nausea, vomiting and the abolition of convulsion and spasm.[284] Western medical practitioners accepted its spread several years after the return of Napoleon's army

from Egypt. In the late 19th Century it began to lose its superiority in medicine. British doctors continued to have the right to prescribe cannabis (as a tincture for oral administration) until 1971.

In the beginning of 20th century, it became one of the serious social problems throughout the world. It became an illegal drug in many countries. In the United Kingdom under the Misuse of Drugs Act 1971, cannabis was deemed to have no valid medical use and was classified under Schedule 1. In the United States, some drug companies synthesised new analogues of tetrahydrocannabinol (THC) with the aim of developing some clinically useful preparations. Delta-9-THC has been prepared and used as an antiemetic during cancer chemotherapy which has proved unresponsive to other drugs, and this is the best known and accepted therapeutic use of cannabinoids.[285] The use of cannabinoids in cancer chemotherapy as an anti-emetic represents, in essence, the use of a drug with a relatively undefined mechanism of action to treat the side effects of other drugs, also with relatively undefined mechanisms of action, which are being used to treat cancer, a disease or series of diseases the precise nature of which remains enigmatic.

11.1.2. Routes of Administration:

Marihuana and hashish are usually smoked but are sometimes eaten in cookies and (rarely) used in drinks. The effects depend heavily on the situation (group effects), the personality of the user, the route of administration and of course, the dose. Smoking involves parts of the plant which are dried, chopped and rolled up into a cigarette and smoked, often combined with tobacco. Cannabis can be smoked in a pipe, brewed into a drink or cooked in foods. Cannabinoids are rapidly absorbed on inhalation from smoked cannabis preparation, with a bioavailability of 20-45%.

Its effect is obtained within seconds and become fully apparent in a matter of minutes. Its absorption is very slow when taken orally and concentrations reached are 25-30% of those obtained by smoking the same dose due to the fact that some of it is degraded by metabolism in the liver before reaching the circulation (first pass metabolism). The onset of effects of 11-hydroxy-delta-9-THC is delayed by a period ranging from 0.5-2 hours, and its duration is prolonged due to continual slow absorption from the gut.

Conversion of THC precursor acids to active THC during marihuana smoking may increase the potency of a cigarette, and a similar process may occur during the baking of brownies and other forms of cooking with marihuana plant material.[286] The prolonged heating may also destroy THC. One study on smoking cigarettes found that 50% of the THC is absorbed by the lungs if the smoking is done in 10 minutes and each inhalation was retained for 30 seconds with no side-stream loss[287].

THC is insoluble in water, but highly soluble in lipids. It is rapidly distributed throughout the body, reaching first the tissues with a high blood supply (brain, lungs, liver, adrenals, kidney, ovaries, and testes). High concentrations reach the brain within 15 minutes, which coincides with the onset of maximum psychological and physiological effects. A plateau is reached after 2-4 hours.

When taken orally the maximal effect is reached after 1 hour and lasts 5-6 hours because of continued absorption from the gut, but its effects may persist more.[288]. Lemberger et al. [289], by administration of radiolabelled THC, Cone et al.[290] and Law et al.[291] showed the pharmacokinetic differences between smoking and oral administration.

Cannabinoids also cross the placenta enter the circulation, and penetrate into breast milk.

Generally, the effects produced by ingested THC or ingested marihuana extract are comparable to those produced by nearly one third the amount of smoked and inhaled THC or marihuana.[292] Perez-Reyes et al.[293] found that it is the rate of THC absorption, and the duration of its action which are affected by chosen routes of administration.

11.1.3. Chemistry:

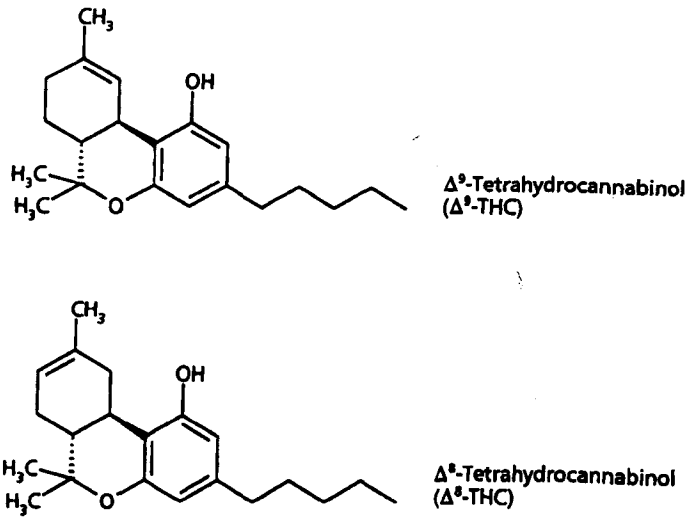


Figure 11.1 Chemical structures of THC isomers

The chemical formula is $C_{21}H_{26}O_2$. The molecular weight is 310.42 and carbon content is 81.25%[174]. THC is unstable, degraded by heat, light, acids and atmospheric oxygen, properties that account for the well known tendency of cannabis preparations to lose potency on storage[292].

11.1.4. Metabolism

Cannabinoids are highly lipid soluble and accumulate in fatty tissues, being slowly released back into other tissue and organs, so it is slowly eliminated from the

body. In chronic use it accumulates in body and continues to reach the brain over a longer period.

THC, the primary psychoactive component of marijuana is rapidly and extensively metabolised by humans. The major metabolic pathway involves the initial reaction of allylic hydroxylation in the lungs or in the liver, by different enzymes, at the C-11 or C-8 position in humans to give the major metabolite 11-hydroxy-Δ^9- THC, which is more potent than Δ^9- THC itself. Major lung metabolites are usually hydroxylated derivatives of the cyclohexane ring system.[294] This also is slowly excreted over days or weeks, in the urine and faeces. There are large interindividual differences in rates of metabolism, which is slow in old persons and in liver disease.[295]

The hydroxylation forming 11-hydroxy-Δ^9-THC is followed by further oxidation to 11-nor-9-carboxy-Δ9-tetrahydocannabinol (THC-COOH), the major metabolite found in blood and urine. Conjugation of glucuronic acid with THC and its hydroxylated and carboxylated metabolites may occur, generating water-soluble compounds to facilitate urinary excretion. Glucronic acid conjugates of THCCOOH and THC were identified in human urine. In addition, it was shown that a dihydroxylated metabolite is excreted as a glucronoide conjugate in human urine following the inhalation of marijuana smoke. These metabolites are present in trace amounts in urine, so are ignored in forensic analytical literature.

Agurell et al.[296] developed a method to identify and measure accurately nonlabelled delta-9-THC in the plasma of cannabis smokers. The results showed that within 10 minutes of smoking a cigarette containing 10mg of delta-9-THC, peak plasma levels of 19-26ng/ml were reached and later declined rapidly to < 5ng/ml within 2 hours. The method was based on the chemistry of the phenolic group

common to all cannabinoids, cannabinoid-like drugs and their metabolites. Rosenfed et al[297] developed and applied a mass fragmentographic assay for delta-9-THC in plasma of human volunteers. Results obtained were similar to those reported by Agurell et al[296].

Metabolism of THC involves two phases in plasma: an initial rapid phase representing metabolism by the liver and redistribution from the blood to the tissues. The second, slow, phase represents release from the tissues with subsequent metabolism. THC is essentially insoluble in water and is protein bound. Its terminal plasma half life is 28-56 hours and has been seen to be significantly shorter in daily users than in nonusers. Urinary excretion and biliary excretion (reflected a day later in the faeces) were found to be greatest during the initial 24 hours, then gradually tapering off. Law et al.[291] pointed out that following a single, moderate, oral dose of cannabis metabolites were detected in plasma samples for up 5 days and in urine samples for at least 12 days.

11.1.5. Pharmacodynamics:

There are natural specific receptors in human brain and spleen and natural (endogenous) substances have been identified which bind to the receptors to exert their effects.[298] Pharmacological actions of cannabis are highly subjective and are affected by a number of factors such as the quality of the drug, the dosage, the experience and expectations of the user and his environment. Its actions in man affect almost all systems.[299]

Table 11.1 Effects of Cannabinoids.

Central nervous system	
Psychological effects	Euphoria (high), dysphoria, anxiety, depersonalisation, precipitation/aggravation of psychotic states.
Effects on perception	Heightened sensory perception, distortion of space and time sense, misperceptions, hallucinations.
Sedative effects	Generalised CNS depression, drowsiness, sleep, additive effects with other CNS depressants.
Effects on cognition and psychomotor performance	Fragmentation of thoughts, mental clouding, memory impairment of performance especially in complex and demanding tasks.
Effect on motor function	Increased motor activity followed by inertia and incoordination, ataxia, dysathria, tremulousness, weakness, muscle twitching
Analgesic effects	Similar in potency to codeine but by a nonopioid mechanism
Antiemetic effects, increased appetite	In acute doses, effect reversed with larger doses or chronic use
Tolerance	To most behavioural and somatic effects, including the high with chronic use
Dependence, abstinence syndrome	Rarely observed but has been produced experimentally following prolonged intoxication: symptoms include disturbed sleep, decreased appetite, restlessness, irritability and sweating.
Cardiovascular system	
Heart rate	Tachycardia with acute dosage, bradycardia with chronic use
Peripheral circulation	Vasodilatation, conjunctival redness, postural hypertension
Cardiac output	Increased output and myocardial oxygen demand.
Cerebral blood flow	Increased acutely, decreased with chronic use.
Respiratory system	
Ventilation	Small doses stimulate, large doses depress.
Bronchodilatation	Coughing, but tolerance develops.
Airway obstruction	Chronic smoking
Eye	Decreased intraocular pressure
Reproductive system	
Males	Antiandrogenic decreased sperm count and sperm motility (chronic use)
Females	Suppression of ovulation, complex effects on prolactin secretion, increased obstetric risks (chronic use)
Immune system	Impaired bactericidal activity of macrophages in lung and spleen (chronic use).

The psychomimetic activity due to inhalation or ingestion of low doses (25μg/kg) of delta-9-THC may cause euphoria, relaxation, talkativeness, bouts of hilarity and sensory enhancement. Large doses (250μg/kg) will give an intense hallucinogenic experience, perceptual distortion, temporal disintegration, and confusion and can be distressful.

Death is rare with overdose of oral cannabis. The minimum fatal dose by oral ingestion of charas, ganja and bhang was 2, 8 and 10 g/kg of body weight respectively.[300] The lethal dose of delta-9-THC, administered intravenously is 30mg/kg of body weight[301].

Agurell et al.[302] pointed out the pharmacokinetics of delta-8-THC in man after smoking and the relationship of blood levels to physiological and psychological effects. It was shown that the increase in heart rate was well correlated with the delta-8-THC plasma levels but the alterations in mental performance were more delayed and prolonged than the peak plasma level.

Hollister et al.[303] used 11 volunteers to address the question of whether THC levels reflected the state of cannabis intoxication. The volunteers were given about 19mg THC by smoking, 20mg THC orally and 5mg THC intravenously. The THC plasma levels were measured by GC/MS up to 4 hours after administration. They concluded that the relationship between pulse rate and plasma concentration of THC was similar to that reported by Agurell et al.[302], but they saw no clear-cut relationship between plasma concentrations of THC and the degree of intoxication, in contrast to the case for ethyl alcohol.

Cannabis is not physically addictive. Thus abstinence or abrupt cessation of intake will not produce distinct withdrawal symptoms. The relationship between different doses and effects of cannabis can be briefly summarised. In relation to impairment

of motor and mental performance, the dose and effect relationship was studied in the following scenario. Marihuana was administered by smoking (1.7g, 1.3% THC) to 36 subjects, producing significantly more speed errors in a driving simulator.[304] In another study,[305] smoking 3.3-6.6 grams cannabis by ten experienced and ten naïve subjects, produced equal impairment in driving performance. Delta-9-THC taken orally in doses of 350, 400 and 450 µg/kg by 54 subjects, produced impairment of attention and concentration capacities, prolongation of reaction time, increased frequency of wrong and inadequate responses and smooth automation disturbances.[306] Smoking of only 18mg delta-9-THC by 58 subjects produced general impairment in roadside sobriety tests.[307] Ssmoking 6.3mg THC by 3 professional and 3 private pilots led to a deterioration in flying performance,[308] while smoking of a low dose of 0.09mg/kg of THC by 10 certified pilots caused significant deterioration in instrument flying ability in a flight simulator.[309] Smoking of 19mg THC by 10 private pilots resulted in performance decrements in a flight simulator.[310]

11.1.6. Users

Lindgren et al[311] determined the plasma concentrations of THC given by smoking and IV administration in groups of light and heavy users of marihuana. Passive inhalation of cannabis smoke can result in absorption of THC without active use of cannabis, and this depends on the amount of cannabis smoked by active user, the duration and frequency of exposure, the room size and ventilation conditions and the individual metabolic factors of the passive inhaler. A study done by Morland et al.[312] showed that the highest concentration of plasma THC reached by passive inhalation was 13ng/ml which corresponds to the concentration measured in plasma

approximately 30 minute after smoking of 15mg of THC. To avoid unfair penalisation of passive smokers a cut-off level is generally set up.

11.1.7. Stability of cannabinoids in body fluids:

A number of studies have been published on the stability of THC in body fluids during storage. Wong et al.[313] evaluated the effects of long term storage on the concentrations of THC in blood and serum stored between –20 and 5°C. They found that THC concentrations in blood and serum samples had decreased markedly after 17 and 19 weeks respectively. In contrast, THC did not change in meconium after more than 3 months in the deep freeze at –20°C.

Another study was done by Johnson et al[314]about the stability of THC , 11-hydroxy-THC and THC-COOH in blood and plasma stored at -10 °C, 4°C and room temperature, with the result that it could be stored for up to 17 weeks without loss of analytes. However, the concentration was lower after 26 weeks at room temperature for THC and 11-hydroxy- THC, but the concentration of THCCOOH did not change.

Levine et al.[315] studied the stability of THC in dry form, chloroform, hexane, and carbon tetrachloride and found that it deteriorated about 10% each month, while is remain stable at ethanol solution at room temperature more than 75 days, and this period could be increased up to one year at 0-5°C.

THC is a lipophilic molecule and tends to bind readily to hydrophilic surfaces. Its concentration will be greatly reduced if stored in certain types of containers. This is supported by results obtained from Garrett and Hunt[316] who reported absorption of THC to rubber stopers, but in unsilanized glass, its absorption was apparently prevented by proteins in blood that bind over 97% of THC. However, in the study cited above, Johnson et al.[317] found no absorption to rubber stoppers.

11.1.8. Methods of analysis of cannabinoids in biological specimens.

In recent years, the prevalence of marijuana use and the passage of legislation regulating its use have mandated the development of analytical procedures for detection of THC and its metabolic products in biological matrices. The most common biological samples used were urine, with testing procedures based on immunoassay or thin layer chromatography, and blood. These procedures, however, are limited for forensic science use, as they are not specific for the analytes due to false results caused by cross reactivity which occurs with other cannabinoid metabolites which cannot be measured[318]. In contrast, the presence of Δ9-tetrahydrocannabinol and its metabolites in blood can often be related to recent use of cannabis, and may be indicative of drug effects[319].

The most sensitive and specific technique for the analysis of cannabinoids, gas chromatography- mass spectrometry, provides the necessary technology to detect the very low concentrations of Δ^9-tetrahydrocannabinol and its metabolites often present in biological matrices. A number of these procedures including various extraction and derivatisation methods were recently reviewed[320].

The urinary concentrations of THC hydroxylated metabolites may however be important to the forensic and clinical community for the determination of accurate temporal relationships between marijuana smoking and the effects of the drug. Because cannabinoids and their metabolites are excreted as glucronic acid conjugates, hydrolysis of the bound compound is required to determine their concentration by GC/MS. Cleavage of the glucuronide bond can be accomplished chemically using alkaline conditions or enzymatically, using β-glucuronidase. Base is the preferred method in most laboratories because of time and cost constraints.

Hydroxylated metabolites, however, form ether bonds with the glucuronic acid. The ether bonds are not susceptible to cleavage under alkaline conditions. Source species from which glucuronidase is obtained include mollusk, bacteria, cattle and limpets[321].

Many methods have been used for the determination of cannabinoids including thin layer chromatography[322,323] radioimmunassay[324,325] EMIT[326,327] and FPIA.[328,329]. HPLC[330,331] and gas chromatography[332,333] which can used to confirm the identification of cannabinoids. Only GC/MS provides the sensitivity, selectivity and specificity necessary for confirmation of positive results generated by screening methods and for quantitative data needed in clinical studies on metabolic profiling and for evaluation of human performance[314]. Many studies have shown that cannabis metabolites can be screened more effectively by hydrolysing the sample. McBurney et al[334] used bacterial β-glucuronidase (Escherichia coli) for hydrolysis. The linearity ranged from (0-100ng/ml) in plasma and urine. Limits of detection ranged from 0.5-1.5ng/ml in urine and from 0.6-2.1ng/ml in plasma depending on the analyte.

Several methods have been used to analyse cannabinoids in meconium and most commonly these have included a hydrolysis step. β-glucuronidase enzyme was used by El Solhy et al.[335] and alkali by Moore et al.[336] When using hydrolysis by enzyme, El Solhy[328] mentioned that the recovery is very low and also there are many peaks which interfere with the peaks of THC and THCA.

In this chapter is reported the effects of hydrolysis of cannabinoids on the cases which gave negative results by GC/MS but positive results by EIA, and also a comparison was made between two methods of hydrolysis applied by previous workers.

11.2. Experimental Section

11.2.1. Materials and Methods:

All chemicals including β-glucuronidase were bought from SIGMA (Dorest, UK). Solvents and concentrated HCl were of HPLC grade supplied by BDH Laboratory Supplies.

Phosphate buffer (0.1M, pH 6.8) was prepared by dissolving 6.81g of potassium dihydrogen phosphate in 450ml of de-ionised water, adjusting the pH to 6.8 ±0.1 with 1.0M potassium hydroxide, then making the total volume up to 500ml with de-ionised water.

E-Coli enzyme for hydrolysis (in vials of 500.000 units from Sigma) was dissolved in 20ml water to give a concentration of 25,000 units/ml. A volume of 0.2 ml was used for each sample. The THC glucuronide and THC glucuronide-d_3 were from Alltech-Applied Science Labs (Carolean Industrial Drive, State College, PA).

Saturated potassium phosphate (monobasic) was prepared by dissolving potassium phosphate in 500 ml de-ionized water and shaking well until no more salt dissolved.

Hydrochloric acid (1.0 M) was prepared by diluting 83.3ml concentrated HCl to 1L with de-ionised water. Hydrochloric acid 100mM was prepared by adding 4.2 ml concentrated HCl to 400 ml deionised water. This was diluted to 500ml with de-ionised water and stored at 25°C in glass or plastic tubes. To prepare 7M hydrochloric acid, 294 ml concentrated HCl was diluted to 500ml with deionised water.

0.2N NaOH: 2 gm of Na OH was dissolved in 250cm^3 water or methanol, and to prepare 1N Na OH: 40 gm of Na OH was dissolved in 1 Litter H_2O.

Potassium hydroxide (11.8M) was prepared by dissolving 600 gm potassium hydroxide in 500-ml deionised water.

11.2.2. Instrumentation

The instrumentation used was as described in the previous chapter.

11.2.3. Methods

(a) *Sample collection:*

Meconium samples for analysis were collected anonymously from all infants born at Glasgow Royal Maternity Hospital, from the period of October 2000- until February 2001 and its count 450 cases(these were the same samples mentioned in paragraph 10.2.3). Control samples were collected from neonates delivered at hospitals in Egypt who were unlikely to have been exposed to drugs in utero. The meconium was collected from diapers of the newborn infants using a plastic spoon and stored in a plastic vial at –4°C until analyzed.

(b) Sample preparation:

Each meconium specimen was weighed (usual range from 0.5 g-0.8 g for the controls while the real cases according to weight which arrived) then put into a clean vial and centrifuged to bring the sample to the bottom. The specimens were mixed with 20 µl of IS of THC-d_3 glucuronide, THC-d_3, and THCOOH-d_3 to give10ng/g, mixed well for 5 minutes and vortexed for 15 min

(c) Hydrolysis of cannabinoids:

1.1.1.1.1 1. Enzymatic Hydrolysis:

◊ 4-ml methanol was added, mixed well, and the suspension was centrifuged at 3000rpm/20 min.

◊ The supernatant was taken, and evaporated under nitrogen at 50°C, until completely dry.

◊ 1 ml saturated potassium phosphate monobasic solution was added and 10 ml chloroform, then shaken for 20 min.

◊ The dark top aqueous layer was removed, and then the chloroform was evaporated under nitrogen at 50°C.

◊ The residue was dissolved in 1 ml of 0.1M phosphate buffer (pH 6.8) and 0.2 ml of β-glucuronidase previously prepared was added.

◊ The samples were put in an oven at 37°C overnight (16h).

◊ The mixture was acidified with 0.5ml of 1N HCl, and 10 ml of hexane/ethyl acetate (9-1) was added, and shaken for 5 min.

◊ The top organic layer was transferred to another tube, and 1.5ml of 1M NaOH was added.

◊ The top organic layer containing neutral analytes was transferred into another tube for determination of THC and 11-OH-THC.

◊ The bottom layer containing acidic analytes (THCCOOH and 8β,11-di-HO-THC) was filtered through a cotton plug to remove the semi-solid material.

1.1.1.1.2 2. Fraction containing neutral analytes:

◊ 4 ml of 0.2N NaOH in methanol solution was added to the organic extract

containing the neutral analytes, and shaken for 2 min.

◊ The top hexane layer was carefully removed and discarded.

◊ 1ml of 1 M HCl and 3 ml deionised water was added to the bottom methanol layer, and shaken for 1min.

◊ The top layer was separated and transferred to another tube.

◊ 3ml of hexane/ethyl acetate (9-1) was added to the bottom layer, shaken/1min.

◊ The solvent was evaporated under nitrogen, 50µl of BSTFA was added and the tube heated at 70°C/20min.

◊ The derivatised extract was injected into the GC/MS instrument: the column temp was initially set at 220°C/0.5min, then raised to 275°C at 30°C/min, held for 5.0min. and finally raised to 290°C at 30°C/min, and held for 8.0min.

◊ The target analytes were Δ^9-THC and 11-OH-Δ^9-THC

1.1.1.1.3 3. Fraction containing acidic analytes:

◊ 2 ml 1 N HCl was added to the aqueous phase.

◊ 4ml hexane/ethyl acetate (9-1) was added, shaken /2 minute.

◊ The top organic layer was transferred to a clean tube.

◊ The solvent was evaporated at 50°C under N2.

◊ The residue was derivatised with 80µl of BSTFA at 70°C/30minute.

◊ Analysis was by GC/MS: the column temp was initially set at 200°C, held for 0.5min. then increased to 280°C at 30°C/min. and held for 12 min.

◊ The target analytes were 11-nor-Δ^9-THC-9-COOH.

(d) Hydrolysis by Alkali:

◊ The meconium was weighed and spiked with THC-d_3 and THCOOH-d_3.

◊ 4-ml methanol was added, mixed well, and the supernatant centrifuged at 3000rpm/15min.

◊ 0.5ml potassium hydroxide 11.8M was added; left to stand 15 minute.

◊ It was centrifuged 5 min, and the supernatant was transferred to a clean tube.

◊ 3ml de-ionized water was added, then 6ml of hexane/ethyl acetate (9-1).

◊ Allowed to stand for 15 min. and the organic layer was discarded.

◊ 1 ml of HCl 7N was added, followed by 8ml hexane-ethyl acetate (9-1).

◊ Mixed well, and centrifuged; the supernatant was then evaporated to dryness.

◊ The residue was reconstituted in 100µl ethanol, transferred to autosampler vials, re-evaporated to dryness.

◊ The sample was derivitized with 50µl of BSTFA.

◊ Analysis by GC/MS: the oven temp started at 100°C held/1 min., then programmed to a final temperature of 310°C at 30°C/min., then held for 6.6min.

11.2.4. Application to cases

The two hydrolysis methods were applied when possible (depending on whether enough of the same sample remained) to cases which gave positive results by EIA and negative results by GC/MS when hydrolysis was not done.

11.3. Results and Discusion:

The developed method was found to be satisfactory. The retention times of the target analytes were determined by analysing standards and are given in Table 11.1 and Figure 11.2.

Table 11.2 Retention times and selected ions for each analyte.

Drug	Retention time (min)	Selected ions
THC	7.65	371, 386, 389
THCOOH	8.17	371, 488, 491

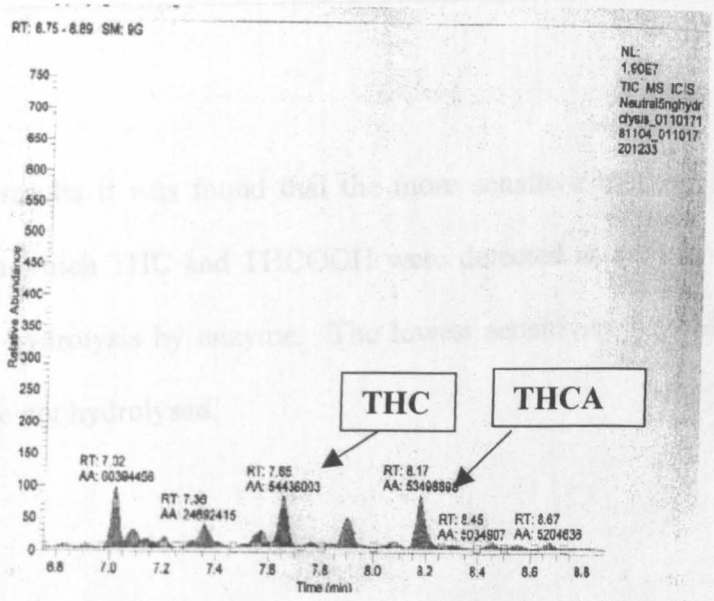


Figure 11.2 Chromatogram of THC and THCA Standards.

For each analyte the calibration curves were linear. The THC and THCOOH calibration curves gave correlation coefficients (r^2) of 0.890 and 0.891 respectively for enzymatic hydrolysis and 0.851 and 0.951 respectively for alkaline hydrolysis. The recovery and lower limits of detection of each method were calculated (Table 11.2) and a comparison made of the sensitivity and specificity of the two methods of hydrolysis and the previous method without hydrolysis.

Table 11.3 Comparison of methods used for analysis of cannabinoids in meconium

Method	Recovery (%)	LLD
Not hydrolysed	100	100 ng/gm
Enzymatic hydrolysis	45	35ng/gm
Alkaline hydrolysis	75	1ng/gm

From these results it was found that the more sensitive method involved alkaline hydrolysis in which THC and THCOOH were detected at very low concentrations, followed by hydrolysis by enzyme. The lowest sensitivity was obtained when the samples were not hydrolysed.

11.4. Application to cases:

The method was applied to 21 authentic cases collected in the Royal Maternity Hospital, Glasgow from neonates as described earlier. These were cases which were previously analysed but without hydrolysis in the survey and gave positive results by EIA for cannabinoids. The samples weighed 546mg (±50mg), except in some cases in which the remainder was less than that. So in these cases the small sample weight allowed hydrolysis by one method only, which by preference was alkaline hydrolysis.

The results of these analyses are summarised in Table 11.3.

Table 11.4 Results of analysis for cannabinoids in meconium, with and without hydrolysis.

Case No	EIA (ng/g)	No Hydrolysis		Alkaline Hydrolysis		Enzymatic Hydrolysis	
		THC	THCA	THC	THCA	THC	THCA
Case 1	4.1	N	N	+	+	N	N
Case 27	14.190	N	N	N	+	X	X
Case 47	17.727	125	120	+	+		+
Case 54	++++	525	N	+ (321)	+ (185)	+ (122)	++ (89)
Case 59	++++	50	N	N	+	N	N
Case 65	N	N	N	N	N	N	N
Case 71	7.766	N	N	N	+	N	N
Case 104	50.000	500	375	+ (321)	+ (210)	N	N
Case 156	19.080	1717	1717	+ (687)	+ (174)	X	X
Case184	20.192	N	303.8	N	+++ (237)	N	N
Case 202	2.623	2100	287	+ (824)	+ (97)	+ (531)	+ (58)
Case 282	++++	18	N	+ (12)	+ (16)	N	+ (12)
Case 342	++++	240	N	+	+	N	+
Case 355	++++	265	262	+	+	X	X
Case 359	28.929	1265	N	+ (564)	+ (123)	+ (98)	+ (55)
Case 364	23.12	N	161	+	+	X	X
Case 365	33.54	N	171	+	+	+	+
Case 366	34.98	70	476	+	+	+	N

Case 367	67.22	N	N	+	+	X	X
Case 385	3.183	N	N	N	+	N	N
Case 392	41.071	N	N	+	+	+	+

+= Positive N=negative X=not analysed ++++=out of scale in EIA

A summary of the results of analysis of the 21 cases is given in Table 11.4.

Table 11.5 Summary of the results of hydrolysis of cases

Number	Types of analysis	%
21	Positive by EIA	100
14	Positive by EIA and negative by GC/MS when no hydrolysis was used.	67
20	Positive by alkaline hydrolysis	100
6	Not analysed by enzymatic hydrolysis	29
9 (out of 15)	Positive by Enzymatic hydrolysis	60

11.5. Conclusions

This study showed that the alkaline hydrolysis of cannabinoid conjugates in meconium gives good results and the inclusion of this extra step gives a more sensitive method which can discover some cases which previously gave negative results. At least it can be concluded that hydrolysis of meconium for detection of cannabinoids is recommended.

12 Conclusions and Further Work

12.1 Conclusions

The work described in this thesis has achieved the goal of screening multiple drugs in neonates and has overcome the problems resulting from the small sample sizes available from the neonate regardless of the type of biological sample used. Two non-conventional biological samples were examined: blood and meconium. The methods which were produced were subsequently and successfully used to survey the incidence of illicit drug use in pregnancy in the Glasgow area.

Firstly, as is known, there are routine analyses that are performed for all newborn infants in hospital. Usually this requires blood analysis, but only 1 ml of blood can be collected and the initial study was designed to find a method to analyse multiple drugs using this small amount of blood. The selected approach here was to use the small sample available and to extract all of the target analytes from it, rather than subdividing the sample and using the aliquots for sub-groups of the target analytes. By using a single SPE column, it was possible to do this and extract a mixture of acidic and basic drugs in an efficient way. The evaluation and validation of this process were carried out using examples of the most common drugs in the Glasgow area, which are also those which are most likely to be used by mothers during pregnancy.

A simple and rapid solid phase extraction scheme was developed which could be employed for a high throughput of cases. The qualitative and quantitative analyses were carried out using gas chromatography with flame ionisation detection - an

instrument easily found in forensic laboratories around the world. Comparison was made between two SPE columns, both of which are used in the analysis of acidic and basic drugs. The method gave good recoveries f the target drugs and the method validation parameters were acceptable. The method was then applied to 13 samples of blood from cases arising under the Road Traffic Act Section 4, as examples of a situation in which only small samples are available. These were analysed using an enzyme immunoassay technique and the positive samples were confirmed by gas chromatography- mass spectrometry. It was concluded that the method was suitable for the intended purpose, and the approach was then developed to permit it to be applied to an alternative specimen, meconium.

The second part of the study was based on using meconium, with a view to carrying out a survey of drug use during pregnancy. Meconium has many advantages, as drugs accumulate in it from the 16^{th} week of pregnancy till delivery, so giving a wide window of detection with high concentrations of drugs. It is increasingly considered to be the most suitable biological specimen for the detection of drugs in the neonate. Suitable sample pretreatment was investigated to allow the solid phase extraction procedure to be used. The extracts could then be analysed by enzyme immunoassay and confirmed by gas chromatography- mass spectrometry to detect opiates, cannabinoids and cocaine plus its metabolites. The method was specific and sensitive with recoveries ranging from 60% to 100% for some drugs. All the target drugs could be analysed except the amphetamine group which was analysed separately using another SPE column. This method was also sensitive for amphetamine, methamphetamine and the ring-substituted analogues MDA, MDMA, and MDEA. This work was the first instance of the use of meconium for general

screening purposes based on a general/universal extraction and analytical process. Studies carried out by previous workers primarily used a conventional approach and used multiple analyses for groups of target analytes.

The method was applied in the first survey which has been done in Glasgow on all neonates born at the Royal Maternity Hospital from period of December 2000-February 2001, covering all the commonly-used illicit drugs and their metabolites. Out of 450 cases collected, there was sufficient sample in 400 cases for the analyses to be done, including qualitative screening by enzyme immunoassay and quantitative confirmatory analysis by gas chromatography-mass spectrometry. The results of the survey confirmed that there is a significant number of mothers using drugs and also confirmed the dangers to the foetus and neonate of drug use by the pregnant mother as potentially harmful concentrations of drugs were found, ranging from 1ng/gm to 1281ng.gm. It was also very interesting that 17 cases were positive for more than one drug and 2 cases were positive for 4 drugs. This was in agreement with observed trends in other populations, in which polydrug use is very common. The study also made some new developments in this area by attempting to distinguish between the origin of morphine detected in meconium. In Glasgow, it is routine clinical practice to administer diamorphine to mothers prior to Caesarian section, leading to the presence of morphine in meconium but also to the problem of distinguishing this pharmaceutically-derived material from illicit street heroin. The traditional approach is to analyse for impurities present in illicit heroin, including other opium alkaloids such as papaverine and also by-products of heroin synthesis such as 6-acetyl-morphine. This was carried out for all cases that were positive for morphine,

codeine, and methadone. Nine cases were found to contain one or more of these marker substances, providing some evidence of illicit heroin use.

The final study presented here was designed to investigate further cases which gave positive EIA results but which were not confirmed by GC/MS. One of the most likely explanations is that the cannabinoid metabolites in the meconium are largely conjugated with glucuronic acid and that the sample pre-treatment should involve a hydrolysis step. Two methods of hydrolysis were tried, using glucuronidase and alkali and a comparison between the two found that alkaline hydrolysis gave better results. Subsequent application to the same cases which had been analysed without hydrolysis found that most cases which gave negative GC/MS results without hydrolysis, were subsequently found, after hydrolysis to contain cannabinoids, especially 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid. This has important implications for future surveys.

12.2 Future Work:

this study has confirmed the usefulness of meconium analysis, but there are still many aspects of analysis of meconium, which have not yet been done. Firstly, the analysis of sequential specimens of meconium from the same neonate, up to three diaper collections, for morphine to see if there are differences in concentration affected by the time of gestation.

The analyses could also be extended to many other target analytes such as metabolites of methadone and other metabolites of cocaine, and see the concentrations are affected by time or not.

The analysis of benzodiazepines and other drugs could be investigated using other instruments such as LC-MS, which can detect small amounts of analyte. This has been applied to other biological samples like blood and urine but not to meconium. This technique would also allow the detection of intact polar metabolites such as glucuronide conjugates without the need for hydrolysis.

Lastly a study cold be done to compare the concentrations of drugs in meconium, hair and nail in the neonate and determine if there is variation in drug concentrations and between the three samples.

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