THE ANALYSIS OF DRUGS AND SOLVENTS IN FORENSIC TOXICOLOGY BY COMBINED GC- AND LC-MS

by

ABDEL-KADER HAMDI BATTAH,

M.B., B.S., M.Sc.,

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Dedicated to my parents, my wife and my children Khairat and Humdi.

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LIST OF ABBREVIATION

ACN	Acetonitrile
CI	Chemical ionization
DEA	Diethylamine
DETMDS	Diethyltetramethyldisilazane
DHS	Dynamic head space
DMF	Dimethylformamide
EDMS-I	Ethyldimethysilyl-Imidazole
EI	Electron impact
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HMDS	Hexamethldisilazane
HPLC	High performance liquid chromatography
I.Std	Internal standard
LC-MS	Liquid chromatography-mass spectrometry
LTIC	Limited total ion current
$M^{+\cdot}$	Molecular ion
M.Wt	Molecular weight
M3G	Morphine-3-glucuronide
MAM	Mono-acetylmorphine
MS	Mass spectrometry
MTBSTFA	N-methyl-N-(tert-butyldimethylsilyl)trifluoro -acetamide
MeOH	Methanol
m/z	Mass to charge ratio
MeOH	Methanol
PEG	Polyethylene glycol
PSP	Plasmaspray
PSP(+)/PSP(-)	Plamaspray positive/negative ion mode
SIR	Selective ion recording
TEA	Triethylamine
TIC	Total ion current
TLV	Threshold limit value
TMS-I	Trimethlylsilyl-Imidazole
TSP	Thermospray
TSP(+)/TSP(-)	Thermospray positive/negative ion mode
VSA	Volatile substance abuse

A C K N O W L E D G E M E N T S

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S U M M A R Y

The work described in this thesis was in the field of Forensic Toxicology, which is the study and practice of toxicology for legal purposes. Three different application areas were considered: (a) the analysis of drugs in blood by gas chromatography-mass spectrometry (GC-MS) following isolation from the biological matrix by solid phase extraction procedures, (b) the analysis of paint solvents in blood by gas chromatography (GC) and GC-MS following dynamic headspace elution from blood, and (c) an evaluation of Thermospray/Plasmaspray (TSP/PSP LC-MS) liquid chromatography-mass spectrometry as the basis of drug screening techniques in forensic toxicology.

(a) In the first of these areas, a sensitive, specific and reliable method was developed for the analysis of basic drugs in blood, using morphine and buprenorphine as model compounds which are also drugs commonly abused in the Glasgow area. A novel extraction procedure was used, based on a commercially available chemically-modified silica containing surface-bound benzenesulphonylpropyl groups, which served as a cation exchange resin. Several methods for the initial treatment of the biological matrix were examined and the one selected involved absorption of the blood sample on diatomaceous earth and solvent elution of the crude extract containing any drugs present in the sample. This extract was then purified using the cation exchange resin: conditions suitable for the efficient retention of basic drugs and subsequent elution were also

examined and developed. The extraction efficiencies for morphine were 92±5% and 95±4% at concentrations of 35 and 560ng/ml blood, respectively, for buprenorphine 83±6% and 87±5%, respectively, and for other drugs were better than 85%. at concentration of 0.5 and 8ng/ml

The end-step analytical technique chosen for this method was GC-MS, because of analytical and legal requirements with respect to sensitivity and specificity. The polar nature of the model compounds, and of many other drugs likely to be encountered in this field, required chemical modification of the substances prior to gas chromatography. A comparison was made of three silyl ether derivatives - the trimethylsilyl (TMS), ethyldimethylsilyl (EDMS) and tert-butyldimethylsilyl (MTBS) ethers. The conditions required for derivative formation were examined, including the choice of silyl donor reagent, solvents, temperature of reaction and time to completion. The EDMS donor, diethyltetramethyldisilazane, was selected on the basis of the mild reaction conditions required for the test compounds and also because this reagent caused less interference during GC-MS analysis. EDMS ethers gave satisfactory gas chromatographic behaviour and the presence of prominent ions at high mass was shown to be advantageous for specific and sensitive detection by SIR-MS with little background interference. The developed method was considered to be useful for both clinical and post-mortem blood samples containing morphine and buprenorphine down to the low picogram/millilitre level, and therefore adequate for the intended purpose.

The method was subsequently applied to 13 samples submitted for analysis to the Department of Forensic Medicine and Science and found to be satifactory.

(b) In the second area of application, dynamic head space (DHS) elution was used for the analysis of paint solvents in blood. Volatilised solvents were trapped on a Tenax-GC cartridge and were subsequently analysed by GC-MS. The extraction efficiency of this method for C_8-C_{12} n-alkane hydrocarbons, which were present in the paint materials, was better than 90% and sensitivity down to pg/ml levels was obtained.

Solvent extraction procedures for these hydrocarbons were evaluated using high purity solvent. The extraction efficiency was better than 85%. Analysis of extracts by GC or GC-MS suffered from interference from the solvent front which reduced the sensitivity.

The DHS method was applied to a pilot study for occupational monitoring of a group of painters to assess the presence of paint solvents in their blood. Two venous blood samples were collected at the beginning and at the end of a working week from each subject. They showed the presence of several solvents similar to those present in paint material and the levels in the second series of samples were higher than those of the first series. The differences between the levels in the two series were statistically significant for n-nonane, n-undecane and alkylbenzenes. The levels in the first sample indicated incomplete clearance of these solvents from the body during the weekend, and the second samples indicated

solvent uptake during the working week. Solvent contamination in the extraction system was tackled by several approaches but still hindered the accurate estimation of solvent levels in blood.

(c) In the third area of application, the operating parameters which control the sensitivity of the mass spectrometer using the TSP/PSP LC-MS interface were evaluated. These included the effects of the probe temperature and discharge voltage on sensitivity and mass spectral fragmentation pattern and the effects of the mobile phase constituents on sensitivity and mass spectral peak stability. Solvent systems containing ammonium acetate buffer and an organic modifier such as acetonitrile produced the best results in plasmaspray LC-MS.

Three model HPLC-MS analyses were developed for mixtures of basic drugs, barbiturates and opiates using both the plasmaspray positive and negative ion modes. During the development of the mobile phases, the optimization of chromatography by organic modifiers was assessed. The quality of chromatography obtained was not always as good as expected in conventional HPLC, but the combination of chromatographic and mass spectral data could be used for identification and quantification purposes.

A compilation of PSP mass spectra of drugs commonly encountered in forensic toxicology was produced. These mass spectra provided mostly molecular weight information with little structural information.

CHAPTER ONE

FORENSIC TOXICOLOGY

1.1 INTRODUCTION

Toxicology can be defined as the study of the adverse effects of chemical agents on the biological system [1]. This avoids the traditional definition, "the basic science of poison", where a precise definition of a poison is difficult to establish. In general a poison is any chemical substance that is capable of producing harmful effects on the living organism [2]. The group of simple chemicals and harmful plant poisons which were the causes of poisoning in previous times has expanded tremendously in recent years to include the new hazardous products - therapeutic drugs, illicit drugs, industrial chemicals and pollutants. All share the potential of causing harm as well as the possibility of misuse and abuse. Forensic Toxicology is the study and practice of toxicology for legal purposes [3]. It involves ascertaining the circumstances surrounding those cases where drugs or other poisons are involved, for example, motoring offences where drug-taking is implicated or suspected, fatalities following an overdose, poisoning where there is a suspicion of criminal intent [4] and the investigation of sudden or suspicious deaths in which there is no obvious cause of death.

In addition, the toxicologist can play a major role

as a member of a health care team and, through vigilance, can help to improve the general well-being of the community. By compiling accumulated data it is possible to monitor drugs of abuse, solvent abuse in the general public, persons under a rehabilitation programme, drug doping in sport and exposure to chemicals in industry and the environment.

The toxicologist may also became involved in clinical toxicology, in cases of acute overdose or suspected poisoning. Although treatment should be supportive in all cases to start with, quick identification of poisons which require specific treatment (by antidotes) will greatly influence the morbidity and prognosis of the outcome. Examples of these poisons are methanol, ethylene glycol, opiates, salicylates, paracetamol, cyanide and organophosphorus pesticides. The results of the analysis can be used for prognostic purposes as well as the diagnosis.

Finally, as a scientist, the forensic toxicologist also shares in the collective responsibility for the advancement of knowledge by continuing scientific research, as well as disseminating his knowledge through other teaching and educational obligations.

1.2 METHODOLOGICAL APPROACH

Most forensic toxicological analyses present a great challenge by virtue of the variety of analytes and the quality and quantity of the available sample material,

which is frequently limited, especially in putrefied, mummified or severely burned bodies. The following stages can be included to determine the nature of the analyte and the toxicological significance attributed to it.

1.2.1 Isolation and purification of the toxic substance from the biological material. This can be a tedious procedure, especially if the poison is unknown. A search for an unknown poison, can be an infinite task. Comprehensive screening for poisons in one procedure would be ideal but is not possible. Toxic substances can be classified into the following groups [5].

(i) Gaseous and volatile substances

(ii) Metals

(iii) Toxic anions

(iv) Non-volatile organic substances such as organic acids and bases, neutrals, amphoteric substances and quaternary ammonium compounds.

So, for example, in cases of suspected drug overdose, the most commonly encountered drugs are usually screened. Limiting factors can be time, amount of sample and instrumentation availability. The literature continually supplies new specific or general screening procedures.

1.2.2 Identification of the isolated toxic substance or substances by one or more of the analytical tools available, followed by corroborative tests to confirm the identity.

1.2.3 Measuring quantitatively the identified material in the biological sample.

1.2.4 Reporting the analytical findings. This may include an assessment of the sensitivity, specificity and reproducibility of the applied procedure.

1.2.5 Interpretation of the results. To reach a correct and accurate interpretation, the following inter-related factors have to be collectively considered.

(i) Analytical results: A negative result does not mean that the sample is absolutely free from all poisons. Rather it means that it is negative for a particular analyte or that if this is present, it is below the detection limit of the assay. Sample storage can also affect the result, when measuring cyanide in blood. The quantity of cyanide recovered will vary when storage is at room temperature, in the fridge or in the freezer [6]. Similar effects are seen in the analysis of volatiles. Other factors affecting storage are exposure to light and the presence of preservative in the sample [7].

(ii) Factors relating to the patient or deceased, such as age, sex, weight, recent and past medical history, especially relating to intake of drugs. Tolerance to a drug may have developed - a condition in which the body cells protect themselves against toxic substances by developing a resistance to them [8]. This state of decreased responsiveness occurs as a result of exposure, usually long term, to a given drug or its congeners [9].

(iii) Pharmacokinetic factors. These include:

- time and amount of drug taken, taking into consideration that in drug overdose, the drug does

not necessarily follow the same pharmacokinetics as at therapeutic levels [10];

- route of drug intake: blood levels in fatal cases are usually lower following intravenous injection than when administered by other routes. This reflects the importance of the rate of drug entry to the systemic circulation rather than the final concentration [9];

- distribution between different metabolic compartments, which can reflect the time of drug intake and survival time;

- presence of more than one drug, with the possibility of interactions between them which can affect the pharmacologic response as potentiation. Additive synergistic or antagonistic effects may occur, such as the synergistic effect on the toxicity of barbiturates caused by the presence of alcohol [11], which can increase the effective toxicity of each drug when present in combination.

(iv) Metabolic factors, which are affected at the same time by the route of administration, the presence of multiple drugs, leading to enzymatic induction or inhibition and whether the analyte is the parent drug or a metabolite and the relative quantities of the parent and the metabolites. The parent:metabolite ratio may be of value to indicate the time of drug intake and to differentiate acute from chronic overdose.

In the case of heroin, morphine and 6-monoacetyl

morphine are the major and minor metabolites observed respectively. Heroin is usually not detected but may be inferred as the drug administered from the presence of the metabolites.

(v) Previously recorded results. Many compiled data collections are available indicating therapeutic, toxic and fatal levels of substances in biological tissue [12-16]. Pharmacology and pharmacopoeia textbooks [17,18], as well as compilations from the toxicologist's own laboratory and other forensic toxicology departments are available for consultation during interpretation. Particular consideration should be given to the type of sample and the method of analysis used if a valid comparison is to be obtained.

(vi) Other factors. The clinical presentation, histopathological findings and the scene of the incident in fatal cases must be considered when trying to establish whether the fatality was primarily due to the toxic agent, or caused by medical complications of poisoning, or merely associated with that toxic agent, where the death (suicidal, homicidal or accidental) could be directly or indirectly from the influence of that agent. For example, a traffic accident can be caused by driving under the influence of drugs or a drowning caused by incoordination and loss of control following volatile solvent abuse [19]. Detailed discussions of interpretation criteria can be consulted in several excellent text books now available [4,9,12].

1.3. METHODOLOGICAL DEVELOPMENT

Forensic toxicology is a powerful science which has always rapidly adopted the most sensitive tools that modern technology offers, to be able to cope with a wide range of analytes with varying degrees of potency. An overview of these techniques will be presented. Details of the available techniques have been published [12,20,21] as have historical overviews of forensic toxicology [22,23].

The methodological approach adopted early in this century was the laborious technique known as Stas-Otto extraction to isolate organic poisons from biological tissue and fluids, followed by identification of the isolate by a series of colour tests, for example, Trinder's test for salicylate is a rapid spot test procedure. These chromogenic reagents are utilized as visualizing reagents (Dragendroff and iodoplatinate) for thin layer chromatography (TLC) and for post-column reactions for high performance liquid chromatography (HPLC) detection in recent years. Reliable spectrophotometers were developed during the 1940's. They were utilized for identification and quantification of barbiturate and anti-malarial drugs [22] and later, in the 1960's, were applied to other classes of drugs such as benzodiazepines and antidiabetic drugs. During this period the extraction procedure was modified to include the acid hydrolysis of the sample and purification of the extract by back-extraction procedures, following pH

adjustments, into an organic solvent. Solvent extraction is laborious, tedious and time and material consuming. It is being replaced in recent years by various types of solid phase extraction material which are commercially available. Solvent extraction is still used for tissue homogenates. As methods become more sensitive, the need to use liver (high concentrations of drugs, large amount of sample) decreases. Thin layer chromatography was a major advance in the 1950's, for the separation of drugs with a variety of developing systems and visualizing methods. The relative mobilities of substances on TLC plates (R_f values) are used to give a tentative indentification. Recent valuable collections of R_f values for standardized TLC systems are available for accurate and precise identification [12,24,25].

In the 1960's, gas chromatography (GC) was applied to analytical toxicology. It offered sensitive detection of a small amount of sample, as well as specificity and accuracy in quantitation. Added specificity and sensitivity were obtained by the use of a specific detector such as the nitrogen-phosphorus detector (NPD), the electron capture detector (ECD) and the mass spectrometer connected to the gas chromatograph (GC-MS). The latter offers the highest sensitivity and unequivocal identification. Also during this period, column chromatography advanced to become HPLC, which offers a wide range of separation and detection capabilities for analytical toxicology. A recent development is the

availability of instruments with a dynamic interface between a liquid chromatograph and the mass spectrometer (LC-MS).

Immunoassay procedures were developed in the past two decades and are based on immune response antibodies. Detection based on radiolabelled analyte, or enzyme substrate or a fluorescence marker, started to offer sensitivity and rapidity when screening for drugs in a small sample of biological material. Recent immunoassay procedures can offer specificity and quantitation at a very low concentration [26]. These procedures frequently do not require extensive sample preparation and can be automated to handle the increasing load on the analysts. Their importance is in their ability to screen for a group of drugs and therefore to act as a presumptive test. Positive results should be confirmed by another method.

The present study was designed to cover several aspects of forensic toxicology in three major areas.

(a) A relieable method was developed for the anlaysis of basic drugs, using morphine and buprenorphine as model compounds which are also drugs more commonly abused in the Glasgow area. A novel extraction procedure was used, based on a commercially available chemically-modified silica containing surface-bound benzenesulphonylpropyl groups, which served as a cation exchange resin. Extracts prepared in this way were subsequently treated to form chemical derivatives and analysed by GC-MS.

(b) Occupational exposure to solvents in paint.

Monitoring hazardous occupational exposure to chemicals is one of the forensic toxicology services. The acute toxicity of solvent in cases of volatile solvent abuse is well known. Also, the accumulation of solvent in biological systems due to chronic exposure is becoming a recognized hazard. Methods were developed for the analysis of paint solvents at low levels in blood and a pilot study was conducted with a workforce of painters to isolate, identify and quantify the solvents in their blood and to monitor changes in the course of the working week.

(c) Evaluation of thermospray and plasmaspray LC-MS as a technique for forensic toxicology. Factors influencing the production of ions in an LC-MS ion source were investigated and optimised. The thermospray and plasmaspray mass spectra of representative members from several commonly encountered groups of drugs were recorded under a range of conditions.

CHAPTER TWO

MASS SPECTROMETRY AND CHROMATOGRAPHY

2.1 INTRODUCTION

This chapter provides a brief background to the principals of mass spectrometry, chromatography and the interfacing of these two systems, which are related to the work presented in later chapters. More detailed descriptions are given in References 27-29.

2.2 MASS SPECTROMETRY

Mass spectrometry (MS) has become one of the most powerful analytical methods for the structural identification of organic compounds [27,29-32]. At present the mass spectrometer is an indispensible tool in the fields of organic chemistry, pharmacology, biochemistry, pollution studies and toxicology, including forensic toxicology.

It is an instrument that produces ions from a molecule, separates these ions as a function of their mass-to-charge (m/z) ratio and records and displays the relative abundance of these ions. The mass spectrometer may be conveniently divided into five basic components as shown in Figure 2.1.

2.2.1 Sample Inlet Systems

The interior of a mass spectrometer is under a high vacuum (10^{-5}-10^{-6} Torr) in order to minimize the number of collisions undergone by ions and thereby maximize the

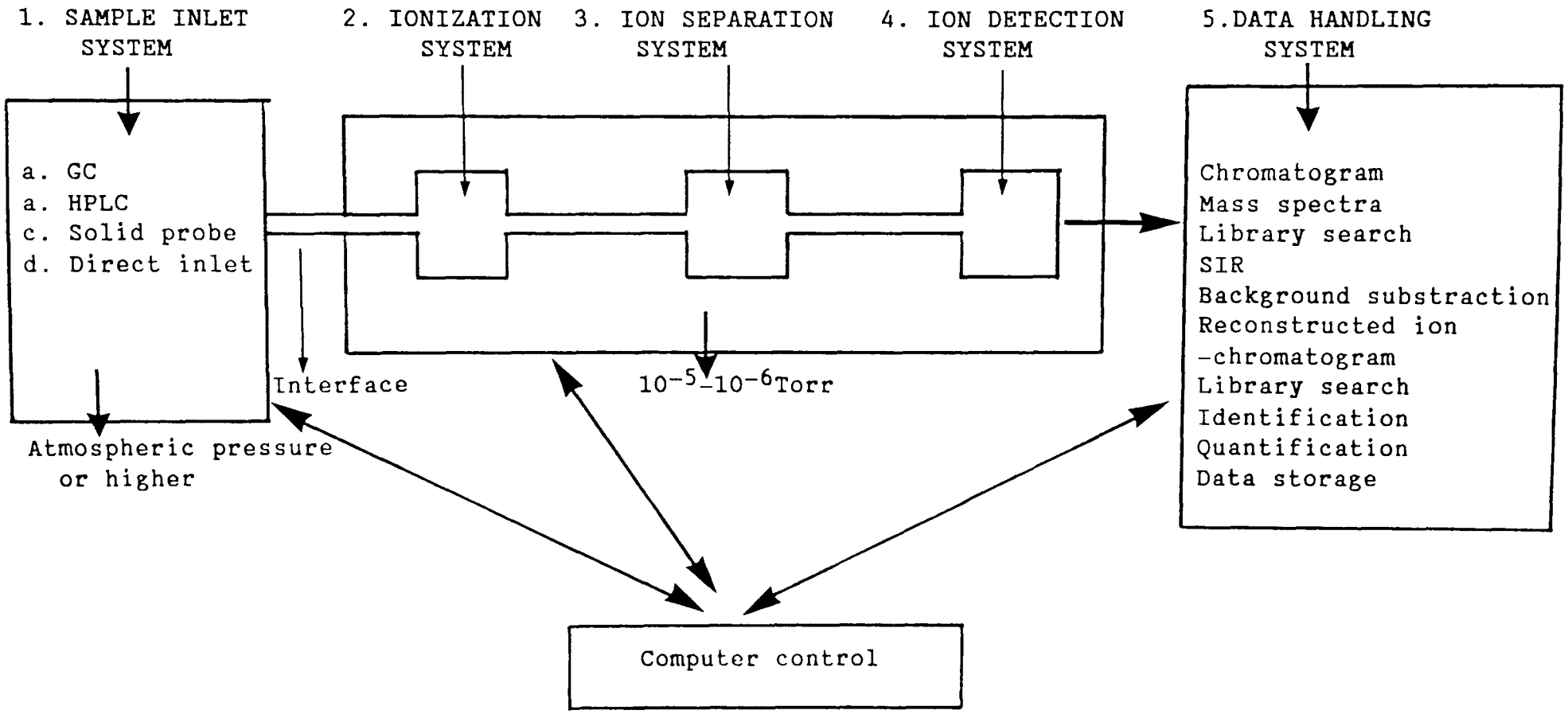


Figure 2.1 The basic components of mass spectrometer.

number of ions reaching the detector. The inlet system must enable the sample to be introduced without loss of vacuum and must be capable of handling gas, liquid or solid samples. A mass spectrometer can have one or more of these inlets.

(I) Direct Insertion Probe

The solid sample is placed in a small glass tube at the end of the probe, then the probe assembly is inserted through a vacuum lock into the ion source. The sample is vaporized directly into the source, either by the heat of the source or by a probe heater, to give optimum volatilization of the sample without producing thermal decomposition. The probe temperature can be programmed to vapourise higher boiling components if the sample is a mixture. The advantage of the probe is that it allows rapid analysis for a pure sample. Samples containing a mixture of compounds require a combination of chromatography with mass spectrometry to obtain pure mass spectra.

(II) Direct Inlet

These usually take the form of a heated, evacuated reservoir connected to the mass spectrometer via a molecular leak capillary. It is either employed for the introduction of liquid samples, such as a reference compound, or for gas samples where a precision calibrated chamber with a set of valves is used for accurate pressure and volume ratio measurements.

(III) GC Interface

This will be discussed in Section 2.4.2

(IV) HPLC interface

This will be discussed in Chapter 5

2.2.2 Ionization Systems (Ion Source)

Mass spectrometry requires that the molecules for analysis first be ionized. The ion source system (Figure 2.2) incorporates ion lens plates to focus the ion beam as well as extracting and accelerating the ions into the analyser. Two types of ionization will be discussed, namely, electron impact (EI) and chemical ionization (CI):-

2.2.2.1 Electron Impact

Electron impact is the most widely used method of ionization. The vaporized sample molecules are bombarded with a stream of electrons which will ionize and fragment the molecules. The fragmentation of the molecule is reduced by using lower electron energies so that, although there is less total ionization current, there is a larger proportion of the fragments in the higher mass ions. Operation at low electron energy can lead to great variation in fragmentation pattern. However, the majority of EI work has been done at 70eV, where the fragmentation pattern at this relatively high electron energy does not vary so much. The excess energy absorbed causes fragmentation of sample molecules to produce both positive and negative ions which can be represented by the following equations:

I. Positive Ions

(i) formation of the molecular ion

Parent molecule ABCD + e^- $\longrightarrow$ $ABCD^+$ + $2e^-$ Equation 1.

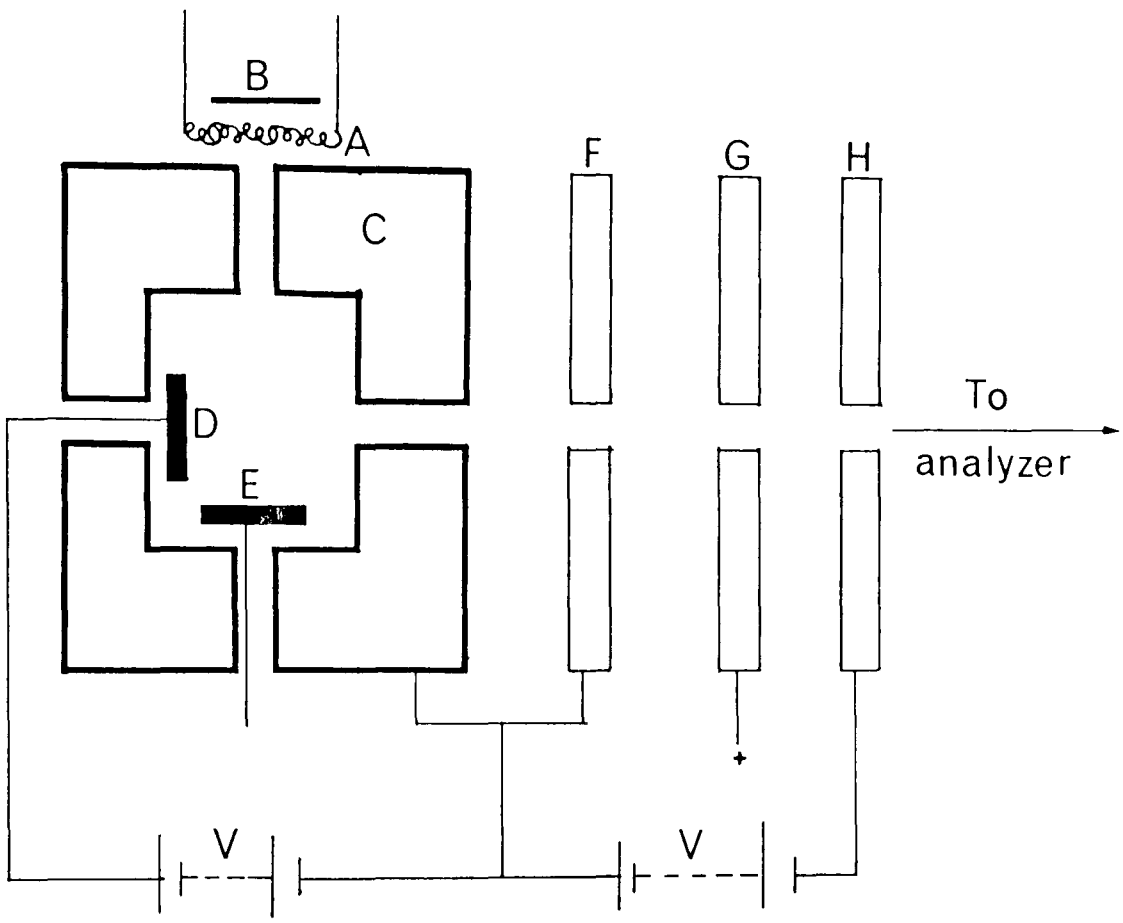


Figure 2.2 Schematic diagram of an EI ion source.

A..Filament	B..Filament shield
C..Source block	D..Repeller
E..Trap	F..Source slit
G..Focussing plates	H..Accelerating slit

(ii) fragmentation of the molecular ions

$ABCD^{+\cdot} \longrightarrow A^+ + BCD.$ or $A. + BCD^+$ Equation 2.

$\longrightarrow AB^+ + CD.$ or $AB. + CD^+$ Equation 3.

$\longrightarrow ABC^+ + D.$ or $ABC. + D^+$ Equation 4.

$AB^+ \longrightarrow A^+ + B$ or $A + B^+$ Equation 5.

where $ABCD^{+\cdot}$ represents a radical ion

A., etc., represent a radical.

AB^+, etc., represent an even-electron ion.

II. Negative ions

(i) production of an ion pair

$AB + e^- \longrightarrow A^+ + B^- + e^-$ Equation 6.

(ii) capture of an electron with dissociation

$AB + e^- \longrightarrow A + B^-$ Equation 7.

(iii) capture of an electron

$AB + e^- \longrightarrow AB^-$ Equation 8.

Ions with a life time more than 10^{-5} sec will be detected intact. Those with very short life times, less than 10^{-6} sec will decompose in the source of the mass spectrometer and will not be detected. The ions of intermediate life time (10^{-5}-10^{-6}sec) are accelerated from the source of the mass spectrometer with mass (M_1) but decompose to a smaller ion of mass (M_2) before reaching the detector. In the case of a magnetic sector instrument, a broad metastable peak (M*) is observed at a non-integral m/z value represented by the relationship:

$$M^* = \frac{M^2}{M_1} \text{ for } M_1^+ \longrightarrow M_2^+ + M \text{ (neutral)} \qquad \text{Equation 9.}$$

The presence of a metastable ion can confirm a

hypothesis for a fragmentation reaction.

2.2.2.2 Chemical Ionisation

Chemical ionisation is one of the 'soft ionization' techniques, where the molecular ion or quasimolecular ion is obtained and fragmentation is kept to a minimum, which is not always obtainable in electron impact. The sample is mixed with a large excess of a reagent gas (R), such as methane or isobutane, which is ionized by electron impact to give the primary ion R^+. This reacts with further reagent gas molecules to produce a number of chemically-reactive secondary ion species. The secondary ions subsequently react with sample molecules (M) by a transfer of a proton to produce new ions with a mass one greater than the molecular weight. An adduct of the molecule with the reagent gas can also be formed. These reactions are represented in the following equations:

$$R + e^- \xrightarrow{2e^-} [R^+] \xrightarrow{R} (R + H)^+ \qquad \text{Equation 10.}$$

$$(R + H)^+ + M \longrightarrow (M + H)^+ + R \qquad \text{Equation 11.}$$

$$\longrightarrow (M - H) + RH_2 \qquad \text{Equation 12.}$$

$$M + [R + H]^+ \longrightarrow [M + (R + H)]^+ \qquad \text{Equation 13.}$$

Using a mass spectrometer source equipped with electron impact and chemical ionisation facilities, both spectra can be recorded alternatively throughout the analysis, thus providing both molecular weight and fragmentation information from a single analysis.

Mostly positive ions are generated. Negative ions are 3 or 4 orders of magnitude less numerous than the positive ions. However, an increase in negative ion

sensitivity of several orders of magnitude can be obtained using negative ion CI for compounds with high electron affinity.

Other soft ionization techniques include field desorption, field ionization, atmospheric pressure ionization and fast atom bombardment. Each increases the range of sample types which may be studied by mass spectrometry.

2.2.3 Ion Separation Systems

After leaving the ion chamber in the source, the ions are accelerated with an acceleration potential (V) of about 8KV into the mass analyser where they are separated according to their m/z ratios by employing a magnetic sector or quadrupole filter. The ions follow path of radius (r). Ions of different m/z may be brought to focus at a detector (Figure 2.3) by variation of the magnetic field (H) according to the following equation:

$$m/z = \frac{H^2 r^2}{2V} \qquad \text{Equation 14.}$$

Ions of all m/z ratios can be obtained by scanning the magnetic field (Equation 14.) at a fixed value of r & V. Alternatively the mass spectrum may be scanned by varying V while the magnetic field is held constant. In the double focussing mass spectrometer, an electrostatic analyser (ESA) is added before the magnetic analyser but after the ion source to provide an initial energy focus to the ions leaving the source by restricting the range of their initial velocities. It consists of two curved plates with a high voltage (approximately 500V) between them. They are usually driven with one positive and the

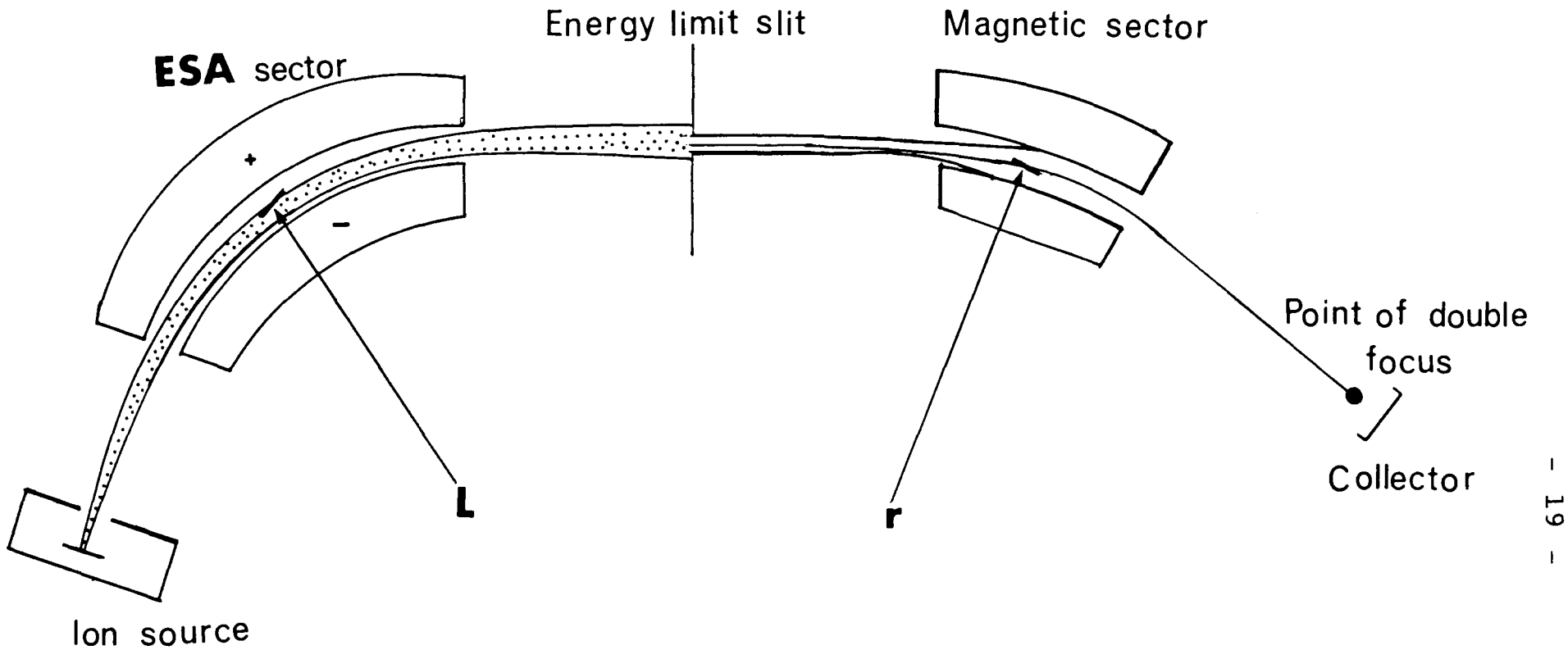


Figure 2.3 Schematic diagram of an Electrostatic analyzer (ESA) sector and Magnetic analyzer sector of a double focussing mass spectrometer. L and r are the ion path radii in ESA and magnetic sectors respectively.

other negative with respect to ground so as to give zero mean potential along their centreline. The ions will pass through the ESA if

$$\frac{mv^2}{L} = \frac{Ez}{d} \qquad \text{Equation 15.}$$

where m is the ion mass, v is the ion velocity, z is the charge on the ion, L is the ESA mean radius, E is the differential ESA voltage and d is the ESA gap. This leads to:-

$$L = \frac{2d}{Ez} \quad (\tfrac{1}{2}mv)^2 \qquad \text{Equation 16.}$$

$$L = \frac{2d}{Ez} \quad U \qquad \text{Equation 17.}$$

where U is the kinetic energy of the ion, which means that ESA is an energy analyzer. The beam passing from the ESA through the energy limiting slit can be input to the magnetic analyzer.

The equation of motion of an ion passing through the magnetic sector (Equation 14) can be restated as

$$m/z = \frac{H^2 r^2 d^2}{E\ L} \qquad \text{Equation 18.}$$

The resolution (R) is the ability of the mass spectrometer to separate adjacent masses by a given amount and is expressed by the following equation:

$$R = \frac{M_1}{\Delta M} \qquad \text{Equation 19}$$

where M_1 is the mass at which resolution is to be measured and ΔM is the peak width for a 10% valley between the peaks. The ion beam must be double focussed to perform high resolution mass spectrometry.

2.2.4 Ion Detection and Amplification

Detection is usually by an electron muliplier or by a conversion dynode and photomultiplier. The current generated is amplified typically by a factor of 10^6. The output is recorded by the data handling system.

2.2.5 Data Handling Systems

Most modern mass spectrometers are equipped with computer systems which can control the instrumental parameters of the mass spectrometer as well as process the vast amount of data it generates, especially when the mass spectrometer is connected to a chromatograph [27,33-35]. It allows manipulation of the data, including background subtraction, library matching and plotting of chromatograms and spectra.

2.3 CHROMATOGRAPHY

All forms of chromatography involve the separation of compounds as a result of a partition between two different phases, one mobile and the other stationary, and by exploiting differences in their physical or chemical properties. Each compound in a mixture partitions to a different degree between the two phases so that, as they are carried along over a bed of the stationary phase, a separation occurs. The longer this process is allowed to continue, the greater the separation achieved until the components emerge from the bed one by one into a detector. Although there are many forms of chromatography available, only high performance liquid chromatography (HPLC) and gas chromatography (GC) will be discussed.

2.3.1 GC and HPLC

The basic components of GC and HPLC instruments are listed in Table 2.1.

Table 2.1: Basic components of GC and HPLC instruments.

	GC	HPLC
1.	Carrier gas source	Solvent reservoir
2.	Sample inlet	Pump
3.	Column	Injector
4.	Detector	Column
5	Oven	Detector
6.	Recorder	Recorder

The chromatogram obtained in both systems (Figure 2.4) contains the analytical data for the components of a mixture. Qualitative information appears in the characteristic retention time of each compound. Quantitative information is contained in the peak height or area. A chromatogram is also a valuable measure of the performance efficiency of the chromatographic system which produced it. The retention time (or retention volume in HPLC) is a characteristic of a given sample/ chromatographic system combination expressed in absolute terms. When it is related to a non-retained sample (usually solvent) it is called the relative partition coefficient or capacity factor (k'):

$$k' = \frac{t_a - t_0}{t_0} \qquad \text{Equation 20.}$$

Where t_1 and t_0 are as defined in Figure 2.4.

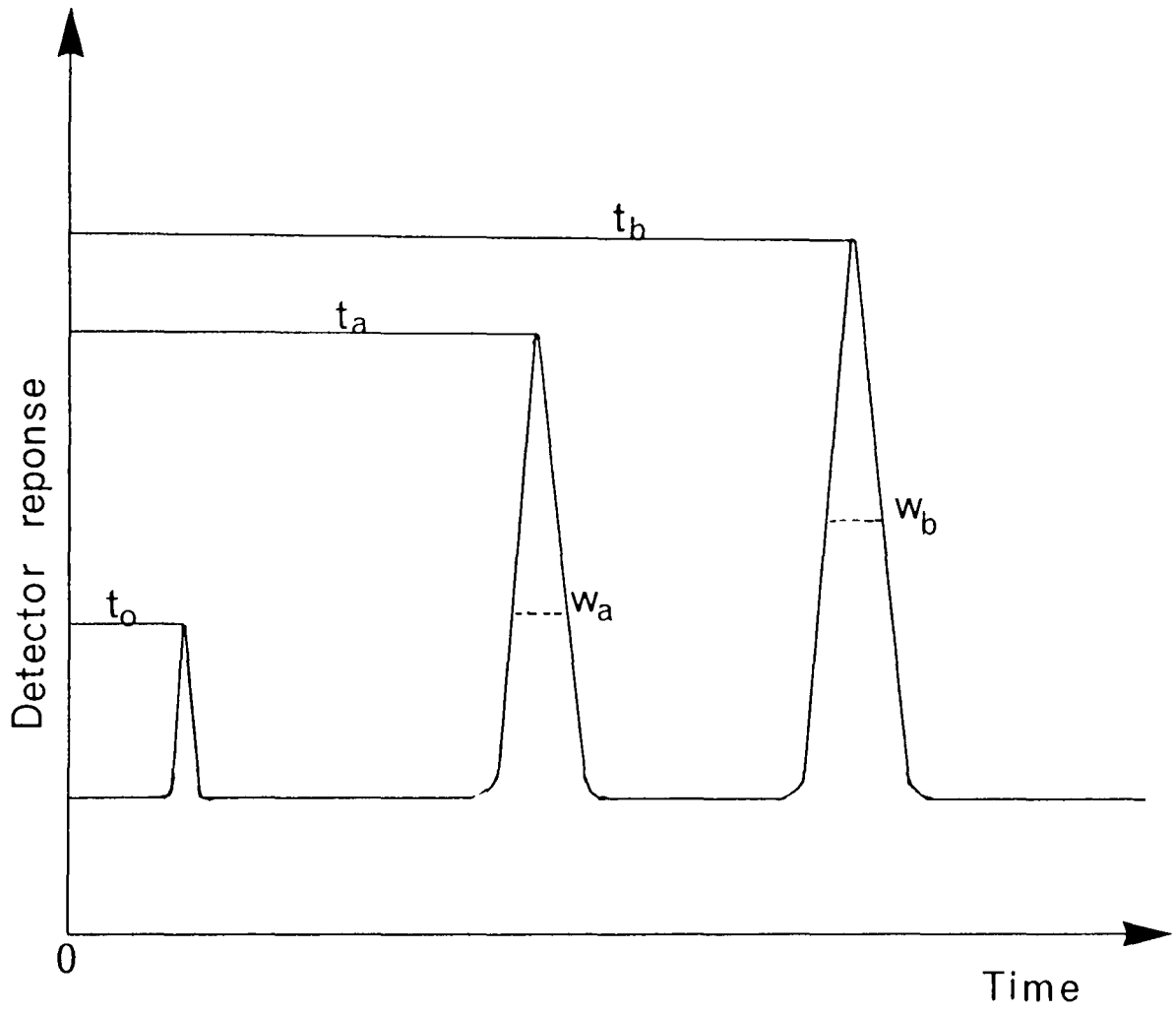


Figure 2.4 Schematic diagram of GC and HPLC chromatogram showing the measureable parameters. t_0=elution time of the unretained solute, t_a and t_b=retention time of peaks a and b, W_a and W_b=peak width at half height of peak a and b.

The separating ability of the column for components a and b is called the resolution (R):

$$R_{ab} = \frac{t_b - t_a}{W_b + W_a} \qquad \text{Equation 21.}$$

and the separation factor r_{ab} is given by:

$$r_{ab} = \frac{k'_b}{k'_a} = \alpha \qquad \text{Equation 22.}$$

Where k'_a and k'_b are the capacity factor for components a and b respectively and α is the selectivity factor. The column efficiency is measured by (h), the "height equivalent to a theoretical plate" (HETP):

$$h = \frac{\text{column length}}{N} \qquad \text{Equation 23.}$$

where N is the number of theoretical plates, which measures the column efficiency in producing narrow peaks and is given by:

$$N = 5.54 \left(\frac{t_a}{W_a}\right)^2 \qquad \text{Equation 24.}$$

The linear velocity of the mobile phase ($\bar{u}$) is given by:

$$\bar{u} = \frac{\text{column length}}{t_0} \qquad \text{Equation 25.}$$

These parameters can be calculated and then used as a guide to the operation of the column and for determining the changes that can be made to achieve optimum performance for a given analysis. They are also dependent on the specific mobile phase and stationary phase used and the physical characteristic of the column. The column parameters are described in the Van Deemeter equation:

$$h = A + B/\bar{u} + C\bar{u} \qquad \text{Equation 23.}$$

where $\bar{u}$ is the flow rate of the mobile phase and A, B and C are constants which are related to the physical processes occurring within the column. A full discussion of the equation is given in the literature [36-38].

2.3.2 GC Columns

GC columns mostly fall into two distinct categories: packed and capillary columns.

2.3.2.1 Packed Columns

Packed columns have, in general, low efficiencies and separating capabilities but have a higher capacity for the sample material. Table 2.2 presents a comparison between essential parameters of packed and capillary GC columns.

Table 2.2: Essential parameters of capillary and packed GC columns.

Parameter	Packed	Capillary
Length (m)	2-5	10-50
I.D. (mm)	2-4	0.2-0.5
Thickness of stationary phase (µm)	1-10	0.05-5
Capacity per peak	10µg	100ng
Total plates	up to 5000	up to 150,000
Carrier gas	All	H_2, He
Carrier gas flow rate (ml/min)	20-50	0.5-5
Carrier gas pressure (p.s.i)	10-40	3-15

Gas chromatography can be subdivided according to the stationary phase into:

I. Gas-solid chromatography

The stationary phase is an active solid which is

used for separation of low molecular weight materials. An example of such phases is Tenax-GC which is used both as a chromatographic phase and as an absorbent for volatile substances prior to analysis. Carbon monoxide in blood is commonly measured using another solid phase, molecular sieve 5A.

II. Gas-liquid chromatography

The stationary phase (liquid) is coated on a support material. The latter should have a very large surface area and uniform particle size, free from fines, and should be mechanically robust and chemically inert. The most common support particles are formed from diatomites.

The stationary phase is selected to suit the analysis required from non-polar, moderately polar and polar liquid phases such as SE-30, OV-17 and OV-225 respectively. Higher efficiency and lower column bleed is obtained with a low loading of the stationary phase (weight percent of the support material), in the range 1-8%.

2.3.2.2 Capillary columns

Capillary columns are available in glass, stainless steel and fused silica. The first two are not frequently used now due to the fragility of the glass and the presence of active sites on the stainless steel. The coating of the stationary liquid phase inside the capillary column can be in three forms [39]:

(i) Wall coated open tubular (WCOT)

(ii) Support coated open tubular (SCOT) where the film

of the stationary phase is stabilised by support particles adhering to the inner surface of the column.

(iii) Porous-layer open tubular (PLOT)

Chemical bonding of the stationary phase to the column (chemically bonded columns) provides stability of the phase and the columns will retain their efficiency longer than non-bonded columns.

Fused silica columns are flexible and, with an external coating of polyimide polymer, are extremely rugged. Both polar and non-polar stationary phases with high efficiency are available. They have superior resolution which can separate complex mixtures with narrow peaks, considerably enhancing the detection limit. Derivatization of polar compounds and non-volatile compounds might be required to convert them to more volatile and easily chromatographed compounds. Active protons such as those found in acids, amines, alcohols and phenols are reacted to form more inert ester, ether or silyl derivatives.

2.3.3 HPLC Columns

Conventional HPLC columns are usually constructed from straight, high quality, polished stainless steel or glass tubing with the following dimensions: 10-30cm long, 4.5-5mm I.D and 6.3mm O.D. They are usually packed with stationary phase under high pressure. The stationary phase consists of rigid, porous particles 3-10μm in diameter. Small particles allow rapid diffusion of solutes between mobile and stationary phase and provide high column efficiency within the pressure capabilities of

the HPLC pump. An adequate flow rate can be obtained without exceeding the pressure limits of the column which might distort the packing material. Microbore columns (narrow-bore packed columns) are available for microcolumn HPLC [40] but their application in forensic toxicology is limited.

2.3.4 Separation mechanisms in liquid chromatography

(i) Liquid-solid (adsorption) chromatography

This is based on the competition for sites on the active adsorbent surface of the stationary phase (e.g. silica gel), between molecules of the sample and those of the mobile phase or its components. The mobile phase (eluent) is usually less polar than the stationary phase. Such systems are described as normal phase.

(ii) liquid-liquid (partition) chromatography

The stationary liquid phase is coated on an inert support. It must be immiscible with the mobile phase. Partition is called normal phase, when the stationary phase is more polar than the eluent; eluant systems use non-polar organic solvents in which the sample has limited solubility, modified by a good solvent for the sample which would, if present in excess, cause rapid elution of the components from the column.

(iii) reversed phase chromatography

In reversed phase chromatography the mobile phase is more polar than the stationary phase. The most common example of this type of stationary phase is chemically bonded silica with C_{18} hydrocarbon substituents. Mobile phases are based on water to which a water-miscible

organic solvent is added to modify the elution characteristics of the sample.

(iv) Ion exchange chromatography

The sample interacts with anionic or cationic groups on the solid stationary phase. An anion exchanger possesses positively charged sites while a cation exchanger bears negatively charged sites. The degree of sample retention is decided by the pH of the mobile phase, the concentration of buffer solution in the mobile phase and the presence of any counter ions which could compete with the sample for the charged sites on the ion exchange surface. Secondary mechanisms can also be present, such as hydrophobic interaction, and these should be considered when selecting the eluant.

(iv) Steric exclusion chromatography

The separation relies on the different rates of diffusion or permeation of molecules of different size through the porous matrix of the stationary phase. Large molecules, unable to enter the pores, elute first, followed by progressively smaller species.

2.4 INTERFACING CHROMATOGRAPHY WITH MASS SPECTROMETRY

General detectors of GC and HPLC are not within the scope of this presentation. They have been extensively discussed in text books [38,41-45]. The use of mass spectrometry, interfaced with GC and HPLC, as a detection system, is presented. HPLC-MS is discussed in Chapter 5.

2.4.1 Gas Chromatography-Mass Spectrometry

The combination of GC with mass spectrometry

(GC-MS) has achieved a considerable importance in the field of chemical analysis and detection [26,46,47]. It is now a well-established, routinely-used technique. The chromatography provides the separation of substances, while MS provides universal detection and unequivocal identification, especially in the field of forensic toxicology. Detection limits are in the range 10^{-9}-10^{-12}g by SIR, which cannot be obtained by many other detection methods.

2.4.2 GC-MS Interfaces

The eluent leaving the GC column is at atmospheric pressure while the MS ion source operates at 10^{-5} Torr. So for the direct connection of GC and MS, the volume or pressure of the carrier gas must be reduced to avoid destroying the high vacuum condition. The ideal interface device would remove all the chromatrographic mobile phase and transfer all the solute to the ion source without degrading chromatrographic elution. A comparison of GC-MS interfaces is presented in Table 2.3 and schematic diagrams of them are shown in Figure 2.5

2.4.2.1 Capillary column direct interface

The flow rate of a capillary column is in the range 0.5-3ml/min, which can be accommodated by the vacuum system of the MS while still maintaining a suitable pressure in the ion source, so it is possible to introduce the fused silica capillary column directly into the ion source. Fused silica tubing can also be used to interface glass or metal capillary columns. All of the sample eluted from the GC enters the MS ion source.

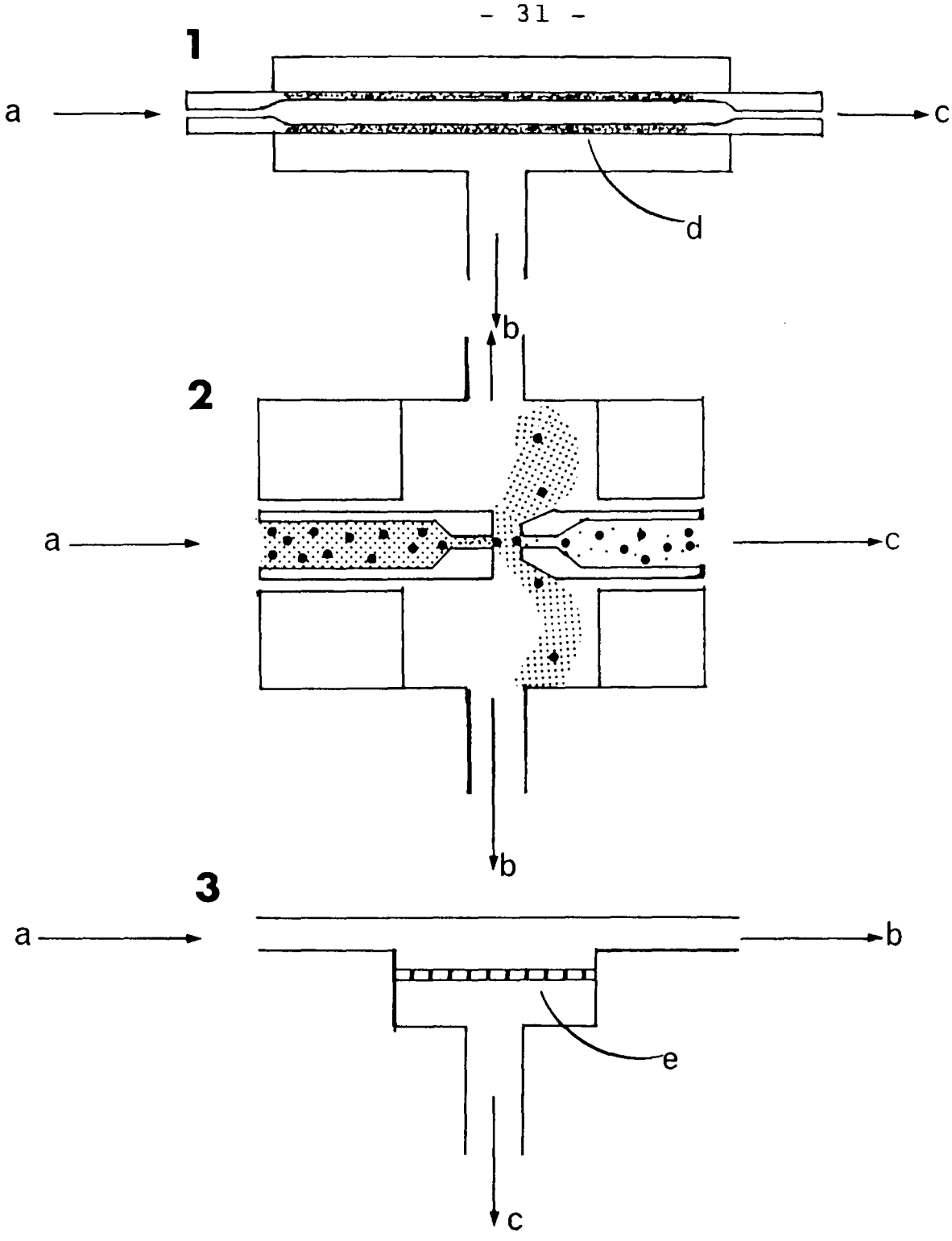


Figure 2.5 Schematic diagram of GC-MS interfaces. 1..Effusive, 2..Jet separator and 3..Membrane separator. a is the effluent from the GC column, b is vacuum pump, c is ion source, d is sintered glass and e is silicone rubber membrane.

TABLE 2.3: Comparative performance of GC-MS interfaces [27]

Classification	Efficiency X	Yield %	Delay sec.	Y	Inert	Carrier Gases
Ideal	Infinite	100	0	1	yes	All
Direct Split	1	1-100*	1	1	Yes	All
Jet	100	40	1	1-2	Yes	He,H_2
Effusive	100	50	1	1-2	Maybe	He,H_2
Permeable membrane	1000	80-95	Var-iable	3	Maybe	Inor-ganic
Direct	1	100		1	Yes	He,H_2

X Concentration in MS source/concentration in GC peak
Yield % = quantity in MS source/quantity in GC peak x 100.
Y = peak width in MS/peak width from CC
* = split ratio

2.4.2.2 Packed column interfaces

The flow rate of a packed column (20-50ml/min) is too high to be introduced directly into the MS. One of the interfaces described below is required:

(i) Effusive interface

The effluent of the GC passes into a tube of porous glass enclosed in a vacuum. The light carrier gas (helium) will preferentially pass through the sintered glass of ultrafine porosity into the vacuum region and be pumped away. The ability of a molecule to pass through the small pores in the glass is inversely proportional to its mass, so few sample molecules of high molecular weight compounds will be lost. This interface is unsuitable for small molecular weight compounds and it has a large dead volume which broadens the chromatographic peak shape.

(ii) Jet orifice interface

The effluent from the GC passes though a small

jet orifice where the gas stream increases in velocity and passes into a vacuum region in the direction of a collector orifice. This is about 0.5mm apart from and directly in line with the first orifice, and is connected directly to the ion source. The molecules with greater momentum (and lower diffusion coefficient) pass into the orifice while the lighter molecules with less momentum (higher diffusion coefficient) such as the helium atom are preferentially pumped away in the vacuum region. This type of interface can be used for capillary column interfacing as well, if make-up gas is added to the column effluent before the separation.

(iii) Permeable membrane interface

The GC effluent is passed over one surface of a silicone rubber membrane, while the other side is open to the ion source vacuum. The organic molecules dissolve on its surface and permeate through into the vacuum region of the MS. The carrier gas is vented to atmosphere.

(iv) Direct split interface

The effluent of the GC is split in the interface (by venting a portion to atmosphere) to accommodate the pumping capacity of the MS. The remainder enters the MS source directly.

Other techniques combining chromatography with mass spectrometry include supercritical fluid chromatography-mass spectrometry (SPC-MS) [48], while techniques for sample component discrimination without chromatography include the recently developed techniques of tandem mass spectrometry (MS-MS) and Fourier transform mass

spectrometry (FTMS) [32,48]. They provide high mass capabilities and low detection limits.

2.5 DATA ANALYSIS

The data generated by GC or LC-MS are utilized for both identification and quantitation of the analyte.

2.5.1 Identification

A mass spectrum of a compound under a specific set of experimental conditions is as unique as a fingerprint. Identification of the unknown spectrum can be performed by one or more of the following methods.

2.5.1.1 Fundamental interpretation of the fragmentation pattern to propose a chemical structure for the compound [29,49-52].

2.5.1.2 Comparison of the mass spectrum with libraries of reference spectra including:

I. Eight Peak Index (Mass Spectrometry Data Centre)[53].

II. Environmental Protection Agency - National Institute of Health (EPA-NHS) mass spectral data base [54].

III. Wiley-NBS (National Bureau of Standards) mass spectral data base for computer library search [55]. The computer can perform rapid and accurate library matching, although the basis for comparison can be complex. Both data storage space and search time are considered for the type of search. Three types of comparison - purity, mixture and reverse fit - can be used. A purity search makes a direct comparison between the unknown and the

library. A mixture search looks for library entries which match a subspectrum of the unknown. A reverse search looks for library entries which best contain the unknown.

IV. Smaller libraries which are constructed by the user from commonly encountered drugs found in previous analyses [56].

V. Other textbooks and publications [12,51,57].

VI. The most common and reliable method is by running an authentic standard at the same time and under the same conditions.

2.5.2 Quantification

Quantitative analysis using mass spectrometric detection was enabled by coupling it with gas chromatography which provides the separation necessary for reliable quantitation. The vast data generated during acquisition is stored in the data system for further manipulation. The principal requirements for quantitation are that the ions chosen are specific to the compounds quantified and they should preferably be the principal ions in the mass spectrum of the analyte. More than one ion can be monitored for each compound of interest. However, the detection limit becomes increasingly poor as the number of ions is increased. Accurate quantitation requires incorporating an internal standard (I.Std). The most effective I.Stds. in GC-MS are isotopically labelled analogues of the substance being analysed. Such compounds will have the same chemical and physical properties as the analyte, and are effective monitors of sample extraction

and detector efficiency as the ratio of the analyte response (peak area or height) in the sample or reference standard to the I.Std response during quantitation will compensate for variation in extraction losses or other variables during the analysis. Quantitation by mass spectroscopy can be performed by one of three scanning modes:

I. Repetitive scanning mode. This includes the computer-reconstructed total ion current (TIC) chromatogram or reconstructed ion chromtograms for specific masses as discussed above. This is the least sensitive mode and is avoided where a low detection limit is required. However, it is highly specific since the characteristic mass spectrum of the analyte is obtained in addition to the retention time.

II. Scanning over a limited mass range. This is similar to the above method but higher sensitivity could be obtained while lower numbers of masses are scanned.

III. Selective ion recording (SIR). This mode provides the highest sensitivity and can give a thousand-fold increase in detector response over the full scan mode. However, it can be the least specific when only one mass is being monitored. It is advisable to monitor two or three ions, characteristic of a specific compound. They will share same retention time, peak shape and will be represented in known relative abundances, which decreases the possibility of unexpected interference.

CHAPTER THREE

MORPHINE AND BUPRENORPHINE

3.I INTRODUCTION AND LITERATURE REVIEW

3.I.1 INTRODUCTION

Morphine is used clinically to produce analgesia. It is obtained from opium where it is the major alkaloid and constitutes about 10% of the opium weight [58,59]. Opium is obtained from the milky exudate of the incised unripe seed capsules of the poppy plant papaver somniferum. Major areas of opium production are currently Afghanistan, Pakistan and south-east Asian countries such as Burma, Thailand and Laos [60,61].

Morphine and related drugs produce their major effects on the central nervous system (CNS) and the bowel. The effects include analgesia, drowsiness, change in mood, respiratory depression, decreased gastro-intestinal motility, nausea, vomiting and alterations in the endocrine and autonomic nervous systems. A detailed pharmacological discussion is given in the literature [62]. Normal therapeutic analgesic doses of morphine range from 10-20mg given parenterally. Peak plasma levels are recorded after 30-40 minutes and the effects last 4-6 hours. The corresponding blood and serum levels range from 10-100ng/ml [62].

Buprenorphine is a relatively new drug with mixed

agonist and antagonist opiate action. It is 25-40 times more potent than morphine at the therapeutic dose of 0.4-0.8mg sublingually and 0.3-0.6mg parenterally. Its concentration in plasma peaks at two hours after oral ingestion and persists for more than a day [62,63]. After intravenous injection at 6µg/kg, buprenorphine concentrations are in the range 2-50ng/ml within the first three hours [64]. Little is known about the toxicity of buprenorphine in man. The median lethal single doses (LD 50's) for buprenorphine in mice were 24-29mg/kg, 90 to 97mg/kg and 260 to 261mg/kg for intravenous, intraperitoneal and oral administration respectively [63].

Morphine is important from both a medical and a legal viewpoint as a major drug of abuse which displays in users the effects of tolerance, addiction and withdrawal syndrome. Tolerance leads to decreased intensity of the pharmacological effects and shortened duration of action. Cross tolerance is the extension of this phenomenon to all drugs belonging to the class and can develop between all opioids [8,65]. A habitual morphine user can take 20-30 times the therapeutic dose.

In contrast buprenorphine shows a much lower physical dependence liability than morphine [63]. It was tested and used for substitution of morphine in heroin addicts [66,67]. However, buprenorphine does show withdrawal symptoms, albeit milder than those seen in other opiate withdrawal. The marked symptoms appear after two weeks of withdrawal of buprenorphine. Recently buprenorphine was reported to be abused [68].

3.I.2 FATALITIES AND MORPHINE TISSUE LEVELS

The correlation between levels of morphine in various tissues and fatal poisoning has been discussed frequently in the literature. Histological and pathological criteria for the diagnosis of death due to intravenously administerd narcotics have been described [69].

The survival periods of 22 acute heroin fatalities were divided into short, intermediate and long term after the last heroin injection according to different microscopic features in relation to the morphine level in blood [70] (Table 3.I.1).

Table 3.I.1 Acute heroin fatalities: morphine levels in blood and microscopic changes in relation to survival time [70].

Survival Time	Blood Level µg/ml	Microscopic Changes
Short Term < 3 hours	0.1-0.93	In the lung: minimal to extensive haemorrhages and oedema
Intermediate term 3 - 24 hours	0.03-0.1	varying degree of leukocytic infiltration ranging from focal and mild reaction to a picture indistinguishable from diffuse lobar pneumonia
Long term > 24 hours	0.06	pulmonary responses from organizing pneumonia to interstitial fibrosis. Most tissue showed some degree of arterial granulomas and polarizable foreign materials.

The authors in this study refer to characteristic criteria of refractile crystalline granulomas in the lung and skin,

increased lymphocytic infiltrate in the portal and periportal areas and benign lymphoid hyperplasia. These are not diagnostic for morphine fatalities [71]. However, morphine blood levels tend to be lower with longer survival periods which is explained by the metabolic removal of morphine, but others found these levels are not always consistent [71].

The minimum lethal tissue concentration of morphine was reported as 0.2-0.3µg/g in blood and muscle [72]. This is not always true. In a study of 114 cases of death due to intravenously administered narcotics [71], morphine was the only drug found in 35% of the cases at concentrations ranging from zero to 1.4µg/ml with a median of 0.06µg/ml. The value of the median was lowered when morphine was present in combination with alcohol or sedative and hypnotic drugs to 0.04 and 0.03µg/ml in blood respectively.

Current views on the mechanism of action of morphine and other opiates are based on the presence of receptor sites at the point of action [62,73]. The majority of opiate receptors are located in the brain. The brain is the site where most, if not all, the effects of morphine originate. A comparison between blood levels of morphine and tissue levels at different sites of the brain in 21 cases where the death was attributed to narcotics overdose and in which the levels were in the fatal concentration range mentioned above, showed that in more than half of the cases, the highest concentrations

were found in the brain stem and the thalamus [74]. These are the sites where many of the adverse effects of morphine originate, for example, the location of the respiratory centre is in the brain stem, and respiratory arrest is nearly always the cause of death in morphine overdose [62,70,75].

Morphine distribution in various tissues was compared in two cases of heroin-induced suicide [76]. The blood concentrations of morphine were almost similar (0.64 and 0.63µg/ml), but wide variation was found for the other tissues. High tissue and blood levels of morphine were reported in an exhumed body where the death was due to an overdose of injected morphine [77]. An unusually high blood morphine level of 8µg/ml in a heroin addict [78], and an even higher level of 120µg/ml in a case of a sudden death of a heroin body packer [79] have been reported. In the latter case, several condoms containing heroin, caffeine and acetylcodeine which were being smuggled inside the digestive tract had spilt their contents into the stomach and leaked into the peritoneum.

Post-mortem blood morphine levels in 16 cases of heroin-related death, ranging from 0-4.7µg/ml, were compared with those of patients receiving morphine as an analgesic on life-support machines, which ranged from 0-0.39µg/ml [80]. A high incidence of cases was observed where morphine had unquestionably contributed to the cause of death but at low concentrations, similar to those seen in patients receiving morphine for therapeutic purposes.

This study shows the disadvantages of setting rigid therapeutic, toxic and fatal levels for morphine in blood. The wide range of morphine levels in post-mortem tissue quoted in the literature illustrates the importance of considering the post-mortem pathological findings at autopsy as well as other pertinent circumstances at the time of death, such as the history of drug use, tolerance, presence of other drugs and absence of other significant causes of death for the proper interpretation of observed morphine levels.

3.I.3 METABOLISM AND EXCRETION

About one third of morphine and 96% of buprenorphine circulating in blood at therapeutic levels is bound to plasma protein [62]. The free morphine accumulates in the parenchymatous tissues of spleen, kidneys, lung and liver. A very small percentage passes the blood-brain barrier but heroin and 6-monoacetyl morphine pass the barrier more easily than morphine due to their higher lipid solubility [62]. Heroin is rapidly hydrolysed in blood (half life is less than 20 minutes) [81] to monoacetyl morphine (MAM), which in turn is hydrolysed to morphine.

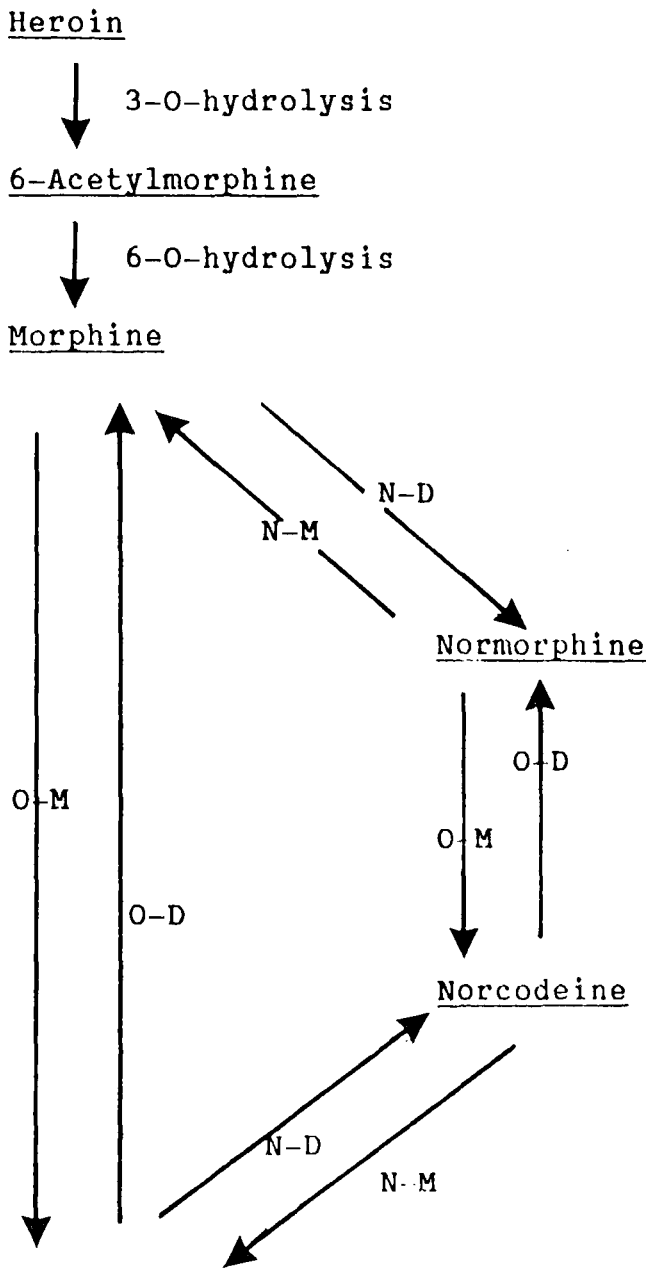
Detoxification of morphine and buprenorphine occurs by conversion to water-soluble molecules through conjugation with glucuronic acid to morphine-3-glucuronide and morphine-6-glucuronide (M3G and M6G) and buprenorphine-3-glucuronide, respectively, or with

sulphate to morphine-3-sulphate [81,82]. An illustration of the metabolic pathways is shown in Figure 3.I.1. Minor biological reactions occur in the formation of codeine and N-dealkylation of morphine to normorphine and buprenorphine to N-desalkylbuprenorphine [63].

The main dose of morphine (85-90%) is excreted in urine in small amounts as free morphine and in larger amounts as the conjugated forms. Also, 7-10% of the administered dose is excreted in the faeces as a result of biliary excretion of the conjugate from the liver [62]. Most (90%) of the urinary excretion takes place within 24 hours, although traces can be detected for well over 48 hours. Enterohepatic circulation may account for the persistent excretion of M3G in urine. The presence of MAM as well as morphine in urine indicates heroin intake [83]. While buprenorphine-3-glucuronide and the N-dealkylated form can be detected in urine, most of the buprenorphine is excreted unchanged in faeces.

3.I.4 SAMPLING AND INTERPRETATION

Almost all biological tissues have been used for the analysis of morphine. Each method has taken into account the availability of tissues and fluids and preference with respect to the ease of sampling and analysis or the importance of the selected medium for the final toxicological interpretation. For example, hair is considered a convenient non-invasive sample for investigating past chronic heroin intake [84].



Urinary excretion product

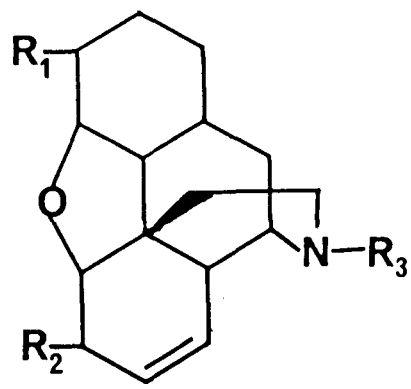
6-Acetylmorphine
6-Acetylmorphine-3-glucuronide

Morphine-6-glucuronide
Morphine-3-glucuronide
Free morphine
Morphine-3-sulphate

Free normorphine
Normorphine conjugates

Free norcodeine
Norcodeine conjugates

Free codeine
Codeine conjugates



Name	R_1	R_2	R_3
Heroin	CH_3CO	CH_3CO	CH_3
6-Acetylmorphine	H	CH_3CO	CH_3
Morphine	H	H	CH_3
Codeine	CH_3	H	CH_3
Normorphine	H	H	H
Norcodeine	CH_3	H	H
Morphine-3-glucuronide	Gluc	H	CH_3
Morphine-6-glucuronide	H	Gluc	CH_3

Figure 3.I.1 Heroin (diamorphine) and morphine metabolic pathways in man and their excretion products in urine. N-D=N-demethylation, N-M=N-methylation.O-M=O-methylation, O-D=O-demethylation.

Cerebrospinal fluid (CSF) [71,85] and vitreous humor are useful post-mortem samples, as free morphine can be detected and the samples are free from contamination.

Bile and urine are helpful in drug screening procedures, since high concentrations of morphine are present and persist for a few days after discontinuation of the drug intake [71]. However, morphine concentrations in urine show no correlation with lethality [72]. Morphine can be detected in urine after therapeutic codeine intake: both morphine and M3G appear in urine as well as codeine and its glucuronide. The ratio of these two metabolites is a helpful indication of codeine intake [86] within 24 hours [4].

The blood concentration of a drug usually presents the best correlation with its pharmacological effects, so blood, plasma and serum are considered as the specimens of choice [81]. The blood level can also be indicative of recent heroin usage and this is consistent with the idea that death from heroin injection is a result of a pharmacological response. High ratios (50-100%) of unconjugated morphine to the total (morphine and morphine glucuronide) suggest a rapid death after opiate injection, while low ratios may be due to a longer interval between injection and death or to the accumulation of morphine glucuronide in chronic use of opiate [87].

The inclusion of different media for the toxicological analysis of morphine as well as clinical and pathological findings will support the final

interpretation. The following paragraphs give some helpful remarks for this consideration.

The presence of traces of morphine in the bile and its absence in the urine in a fatality due to morphine or heroin injection suggest a prior morphine intake which is not related to the most recent injection. The absence of morphine in both urine and bile in a very abrupt death could be explained by the excretion into bile and urine being precluded due to a sudden and intense cardiovascular collapse with subsequent lack of perfusion of the liver and kidneys [88]. With a longer survival time after the injection, both fluids could have high concentrations of the drug. The presence of morphine in urine and absence in bile suggests that this injection was the first for several days [70,71].

3.I.5 MORPHINE AND BUPRENORPHINE ANALYSIS

3.I.5.1 Extraction From Biological Material

(a) Solvent Extraction

Drugs of the opiate type are polar. Their hydrophilic nature necessitates a rigorous extraction procedure to isolate them from biological media with sufficient purity for the identification and quantification of the isolate by various chromatographic methods.

At the biological pH (pH 6-7.5) the morphine and buprenorphine molecules have a low lipophilicity due to protonation of the amine group. The pK_a values are 8.0

and 9.9 for amine and phenol group, respectively, and log P (octanol/pH 7.4) is -0.1 [12]. To increase the apparent lipophilicity of morphine, biological samples should have a pH in the range 8.5-10 so that the free base can be extracted into an organic solvent. This pH range gives the optimum extractability [84]. Various buffering and ion pairing reagents have been utilized for that purpose (Tables 3.I.2 and 3), but none of these shows particular superiority over the others.

Previously, most methods depended on solvent-solvent extraction (Tables 3.I.2 and 3) where the aqueous biological sample was mixed with a suitable internal standard and the mixture was adjusted to the required pH with a buffer or an ion-pairing reagent. Extraction was performed with an organic solvent (Tables 3.I.2 and 3), usually twice with 3 to 4 times the volume of the aqueous layer. The organic solvent collected after centrifugation was back-extracted with an acid solution (e.g 0.05 M sulphuric acid) for further purification from endogenous material in the biological sample. Then the acid portion was alkalinized to the previous pH and extracted again, usually twice, into an organic solvent which was finally concentrated for the subsequent chromatographic method.

The organic solvent should be efficient in extracting the drug from the biological material and should be readily volatile. Most methods depend on chloroform with the addition of an alcohol (e.g isopropanol or butanol) to increase the polarity of the

Table 3.I.2: Summary of representative methods for the analysis of morphine in biological samples.

Ref	Matrix	Quantity ml	Buffer	pH	Extraction	Derivative	Method of Analysis	Recovery %	Detection Limit
90	Plasma	1	4M NaOH	14	Ethyl acetate*	PFB TFA	GC-MS GC-ECD		5ng/ml
91,92	Blood	0.5	0.8M	8.9	Ethyl acetate:propanol (9:1 v/v)*,**		HPLC,ECD	81	1ng/ml
93	Blood	0.1-0.2	Phosphate buffer	8.7-9	Acid aluminium oxide column ethyl acetate	PFP	GC-ECD	83	1ng
94	Blood	0.5	$NaHCO_3$		Chloroform:isopropanol (4:1 v/v)	TFA	GC-MS SIR		1.5ng/ml 1-5ng/ml
95	Plasma	1	Ammonium Chloride	9	Extrelut Column* isobutanol:DCM# (5:95 v/v)	PFP	GC-ECD	98	1ng/ml
96	Plasma, Urine	1	0.5M Ammonium Sulphate & Ammonia	9.3	Sep-Pak C_{18} Cartridge,purified over another similar cartridge		HPLC UV	84-90	5ng/ml
97	Blood Urine	10	2M NaOH	9	Chloroform:isopropanol*		HPLC-Fluorescence	80	10
98	Blood, Tissue & Urine	2	Phosphate buffer	9.9	Toluene:hexane:isoamyl alcohol (78:20:2 v/v/v)*	TFA	GC-MS SIR	55-60	20ng/ml

DCM: dichloromethane.
* included back extraction step
** tissue hydrolysis was included
*** different solvent systems were tested

Table 3.I.2 (continuation)

Ref	Matrix	Quantity ml	Buffer	pH	Extraction	Derivative	Method of Analysis	Recovery %	Detection Limit
99	Plasma	0.5	0.2M Borate	9	Bond-Elut C_{18}		HPLC-ECD	95	3ng/ml
83	Urine	1	Saturated Ammonium Chloride Solution	9.5	C_{18} Cartridge	PFP	GC-MS SIR		2ng/ml
59	Serum CSF	0.1-1	0.05M borax	9	Bond-Elut C_{18}*	TMS	GC-MS CI-SIR	90-95	
100	Biological Tissues Fluid	5-10	Na_2CO_3 -$NaHCO_3$	9	1.Toxi-Tube 2.Chloroform-2-propanol (4:1 v/v)		HPLC-Fluorescence	77-90	60pg on column
101	Urine	10	Ammonium Chloride	9	Isobutanol:DCM*	TFA PFP,HFP	GC-MS	58	2ng on column
102	Urine		Extrelut		2-propanol-DCM (15% v/v)		HPLC-Fluorescence	71-78	1ng/ml
103	Plasma	0.4	$NaHCO_3$ 10MNaOH	9.3	Clin-Elute-CE101 Cartridge		HPLC-ECD	91	2ng/ml
104	Urine Plasma	1	0.5M Ammonium Chloride	9.3	Sep-Pak C_{18} Cartridge		HPLC-Fluorescence -ECD	>80	1ng/ml

Table 3.I.3: Summary of the main methods used for the analysis of buprenorphine, based on solvent extraction.

Ref	Matrix	Quantity ml	Buffer	pH	Extraction	Derivative	Method of Analysis	Recovery %	Detection Limit
105	Plasma	1	0.2M NaOH	12.4	Ether*	HFB,TMS	GC-MS SIR		2ng/ml
106	Plasma	2	Carbonate	9.4	Toluene:2-butanol* (8:2 v/v)	PFP	GC-MS SIR	30	150pg/ml
107	Urine Faeces	1	K_2PO_4	10	Ethyl acetate:heptane (4:1) ***	PFP	GC-ECD	44-80**	10ng/ml
108	Serum	2	1M NaOH		Ether		HPLC-UV	98-100	2ng on column
109	Urine	10	20% NaOH	7.5	Bond Elute C_{18} Ether ***		TLC HPLC-UV	21-35	7.5ng/ml
64	Plasma	1	0.5N ammonium hydroxide		Ether		HPLC-ECD	88-119	0.2ng/ml
110	Plasma Urine	1	0.1M Borate	9.1	Benzene*		HPLC-ECD	88-104	10ng/ml 1ng/ml

* Acid back extraction purification step was included.
** Several solvents and/or combinations were tested.
*** enzyme hydrolysis was included.

extracting solvent. In a study to select a solvent system for morphine extraction from biological tissue, chloroform:isopropanol (4:1 v/v) was found to be the best amongst solvent systems tested because of its extraction efficiency (which was over 92%) and its relatively high volatility [89]. A solvent:sample ratio of 3:1 was recommended. In general, more polar solvents will give high extraction efficiencies, less adsorption to the glass surfaces and render the adjustment of the medium pH less critical, but on the other hand will extract more of the endogenous material. Low polarity solvents require critical optimization of the medium pH and, although they give lower efficiencies, the extract is usually cleaner with respect to matrix interference. Solvent extraction procedures require rather long analysis times, large volumes of solvent and are complicated by emulsion formation and the need for several centrifugation steps.

For total morphine estimation the extraction procedure has to be preceded by hydrolysis of the biological sample to free the morphine and normorphine from their conjugates. This is accomplished by enzyme hydrolysis (glucuronidase and sulphatase enzymes), under the optimum pH, temperature and incubation time conditions for these enzymes. About 80% recovery can be obtained after the reaction has proceeded for 40 hours [89]. Alternatively, acid hydrolysis can be carried out using concentrated hydrochloric acid (sample:acid 10:1 v/v). The mixture is heated or refluxed in boiling water for

30 min or autoclaved under more than one atmospheric pressure. The effect of temperature and the amount of hydrochloric acid added were found to be interdependent [89]: more acid must be added to a sample hydrolysed at lower temperatures to obtain equivalent recovery in the same time. Although acid hydrolysis was more efficient and faster than enzyme hydrolysis, acid hydrolysis of the sample might destroy heat-labile drugs present in the matrix, including morphine itself.

(b) Solid Phase Extraction

Solid phase extraction (SPE) methods have started to replace conventional solvent extraction procedures for most drugs in urine, plasma and other clear biological fluids. They have proved to be efficient, require relatively short analysis times, have low solvent consumption, have no solvent emulsion problems and do not require centrifugation for the procedure. The extracts are sufficiently clean for most chromatographic techniques.

Solid phase extraction is a physical extraction process that involves a partition between a liquid and a solid phase, where the solid phase has a greater attraction for the isolate than the medium in which the drug is dissolved.

Various materials which have been used in SPE for the analysis of narcotics were reviewed elsewhere [85,111]. Here, some examples of SPE materials applied to morphine analysis are presented.

XAD-2 is a neutral styrene-divinylbenzene

copolymer [112] having the capacity to adsorb organic material from aqueous solution. In one method, the buffered sample is mixed with a slurry, followed by washing of the resin and extraction with an organic solvent. The material can also be used in prepacked columns and a clean up step into acid solution can be included [113,114].

Diatomaceous earth (Extrelut and Clin-Elute) has been applied to the extraction of various drugs [115]. The sample is first applied to a prepacked column of the material. The aqueous layer is adsorbed on the Extrelut, leaving the drug of interest buffered to its optimum pH for the extraction and elution with a small aliquot of organic solvent. These materials were applied for morphine extraction from plasma [95,103] and urine [102]. The eluate was back extracted into acid and re-extracted over Extrelut as the first step.

Silica can be modified by chemically bonding different functional groups on the surface, providing a wide range of selective properties for the extraction procedure. A summary of the principles of different modes of SPE on these materials has been described [116]. The most common sorbents applied to morphine extraction have been the reversed phase packing materials (silica bonded to long-chain hydrocarbons), for example C18 (octadecyl) silica [83,85,99,104].

The sorbent has to be solvated before applying the buffered sample. Large amounts of sample can be applied

and concentrated in this way, followed by washing and finally elution of the isolate in a small fraction of solvent and buffer for further identification. Optimization of the conditions in each step is usually required for each drug.

The extraction of morphine and buprenorphine from whole blood using bonded phases has not yet been reported. The nature and constituents of blood require prior treatment before application to these phases.

3.I.5.2 Analytical Techniques

(a) Immunoassay Methods

Immunoassay methods are the most commonly applied techniques for the screening of drugs, including morphine and buprenorphine, in biological samples. Various types of immunoassays for the analysis of narcotics were reviewed [85]. They were applied to pharmacokinetic studies of morphine and the study of morphine distribution between different body fluids in post-mortem material [75,76,78,111] as well as the measurment of buprenorphine levels in plasma [106,117-119] and buprenorphine kinetics [106,118]. The results of immunoassay procedures were also compared with routine chromatographic methods for screening purposes [75,111,114,120-122].

An immunoassay procedure does not require extraction and purification of most biological fluids. It is experimentally simple, sensitive and readily automated, thus allowing a large number of assays to be carried out at one time. The recent commercial radioimmunoassay (RIA)

method (Coat-A-Cont) [26,123] quotes a sensitivity of 0.12ng/ml and 0.3ng/ml of free morphine and buprenorphine, respectively, in an assay which uses 25µl of urine. A radioreceptor assay for opiates including morphine and buprenorphine has been introduced [124]. It is based on the principle that opiates in CSF or plasma compete with ^{3}H-buprenorphine for binding to opiate receptors in the rat brain. Its sensitivity reached 100 pg of morphine and buprenorphine. The drug has to be extracted from the plasma or CSF to remove the interference in the binding caused by plasma protein and sodium ions.

In spite of the benefits of immunoassays mentioned above, they should be used in conjunction with other, chromatographic, procedures due to their cross reactivity as a result of binding of the antibodies to morphine, its metabolites and other chemically related compounds in the sample. This cross reactivity is determined by the reactive group to which the antibodies were originally raised by either modification of the N-methyl or 6-hydroxyl group on the morphine molecule [121]. The cross reactivity is beneficial in forensic toxicology as a presumptive screening procedure for a drug and its metabolites. The commercial Coat-A-Cont kit for RIA is very specific for morphine. In a study of the relative cross reactivity for morphine and 23 other structurally-related drugs [122], it was found that the relative cross reactivities for related drugs in spiked blood samples were negligible. Due to the specificity of

this method for morphine it is considered to be a quantitative analysis for morphine rather than a general screening procedure for opiates. Any positive result obtained in any of the immunoassay methods should be confirmed: samples with negative results can be excluded.

(b) Chromatographic Techniques of Analysis

A summary of various chromatographic procedures utilized for morphine analysis will be presented. Reviews of methods for morphine and other opiates analysis are also available in the literature [58,81,85,112].

(i) Thin Layer Chromatography

Thin layer chromatography (TLC) is commonly employed for screening and detection of drugs including morphine and buprenorphine. Recently, valuable collections of R_f values were published for a wide range of drugs and different systems of elution [12,24]. The more recent publication presented standardized systems for reliable and accurate identification of unknown drugs.

Different visualizing techniques were applied: spraying of chromogenic reagents [125], converting morphine to a fluorophore by reacting it with dansyl chloride to achieve higher sensitivity [126] or by adding anti-morphine antibodies in TLC-immmunoassay [127]. The latter suffers from lack of specificity for morphine due to cross reactions as in other immunoassays and it is not easy to set up. In general, TLC is relatively rapid, reliable and economical but lacks sufficient sensitivity for morphine and related drugs in a small amount of sample at low concentration.

(ii) High Performance Liquid Chromatography

Ultraviolet (UV) spectroscopy is another screening procedure. It is an inexpensive, nondestructive method but it lacks sensitivity due to the low extinction coefficient of morphine and lacks the specificity required for most of the narcotic drugs. It utilizes the ability of the phenol moiety of morphine and structurally- related drugs to absorb UV light. Combining UV to HPLC compensates for the lack of specificity in UV spectroscopy, by virtue of the separation of the compounds and the possibility of using the retention time for identification [108.109,128,129].

The sensitivity of HPLC was improved by converting the morphine molecule to a fluorophore (pseudomorphine) by a precolumn reaction with potassium hexacyanoferrate [102] or dansyl chloride [84,100,104] or by postcolumn reaction [97].

Chemiluminescence (CL) detection with postcolumn reaction creates the possibility of monitoring HPLC effluent for morphine and similar drugs which can be oxidised to produce pseudomorphine [130]. This process is blocked in the presence of a substitute group (methyl for codeine and acyl in heroin) at the C-3 position hydroxyl group. The process requires careful selection of pH, buffer, the nature and concentration of the oxidizing material and the flow rate of the reactant to produce CL in the flow cell. The sensitivity once the parameters are optimized can reach 0.7 pg per injection.

Electrochemical detection provides invaluable sensitivity for HPLC detection of morphine and related drugs. When the free phenolic oxygen is available for oxidation, sensitivity reaches 1-10 ng/ml of biofluid [64,91,92,103,104,110].

HPLC analysis usually does not require rigorous cleaning procedures or derivatization of polar drugs, so morphine and its metabolites M3G and M6G could be detected simultaneously [91,104].

The tedious optimization of the operating condition for fluorometric derivatization and detection, the frequent contamination of the electrodes in ECD, the interferences from the matrix and the huge influence of the mobile phase composition have decreased their extensive application in routine assays.

(iii) Gas Chromatography(GC)

Gas chromatography is extensively employed as a screening procedure for morphine and other narcotics as well as for confirming the positive results of the preliminary screening methods (TLC, UV, RIA). Both packed and capillary columns have been used, the latter providing better resolution and sensitivity. Due to the polarity of morphine, the extracts for morphine analysis have to be derivatized before application to the GC column, to decrease adsorption on the column. This leads to an improvement in sensitivity and better chromatography. Silylation by the addition of two trimethylsilyl groups (TMS) at the two hydroxyl groups is a commonly used

derivatization procedure. Commercial reagents, ready to use for quick silylation directly after the addition and warming of the mixture, have been applied to morphine, reproducibly giving one stable derivatization product. Representative of these reagents are Tri-Sil Z (a mixture of trimethylsilyl-imidazole in pyridine) [74,88], BSA (N,O-[bistrimethylsilyl]-acetamide) [72,85,131], BSTFA (N,O-[bistrimethylsilyl]-trifluoro-acetamide) [59, 85] and MSTFA (N-methyl-N-[trimethylsilyl]-trifluoro- acetamide) [98,132]. These are all powerful TMS donors.

Acetyl derivatives for morphine have been reported using acetic anhydride [114,132], trifluoroacetic anhydride (TFA) [81,90,94,101], pentafluoropropionyl anhydride (PFPA) [83,95,101,133,134] and for buprenorphine [107,135], heptafluoropropionyl anhydride (HFPA) [101], heptafluoro-benzyl anhydride (HFBA) [85,136] and hepta-fluorobutyryl anhydride [101].

Derivatization with acetic anhydride produced one product for morphine, namely diacetyl morphine. Its stability was tested up to 72 hours at room temperature and was found satisfactory [101]. Derivatization with TFA gave both 3,6-di-TFA morphine and 3-TFA morphine and heptafluorobutyrylation similarly produced two morphine derivatives; both showed spontaneous breakdown to morphine. Pentafluoropropionylation gave only 3,6-di-PFP morphine but was not stable; it transformed to 3-PFP morphine and morphine [101]. Buprenorphine was converted to a stable product for GC analysis by a mild acid

hydrolysis which resulted in ring formation between the side chain and methoxy group after the loss of methanol [107,135]. The phenolic hydroxyl group is derivatized as described above.

Any of the above derivatization procedures for morphine is suitable for detection by the general flame ionization detector (FID) [72,74,131,132] where an aliquot of the product is directly injected, especially the silylated derivative which is liable to hydrolyse on exposure to moisture. Sensitivity is improved by using the selective nitrogen phosphorus detector (NPD) [114,137]. Care is taken to remove solvents containing nitrogen which will contaminate the detector. Following acetylation reactions which give stable products, the excess of the reagent is either evaporated or the products are extracted into a suitable solvent. Fluoroacyl derivatives have a strong electron affinity and are readily detected by an electron capture detector (ECD) which can take the sensitivity to low nanogram levels [85,95,136].

iv. Mass Spectrometry

Detection by mass spectrometry coupled to gas chromatography (GC-MS) offers an insight into the molecular structure, provides sufficient sensitivity and offers the best available technique for unequivocal identification of opiates in biological material. The use of deuterium-labelled compounds as internal standards would be preferred, but their availability is quite limited.

All the morphine derivatives suitable for GC analysis can be detected by mass spectrometry [83,90,101, 131,134]. Selective ion recording (SIR) enhances the sensitivity of detection by a factor of 10^2-10^3 or more when monitoring the effluent of a GC column [138] by spending more time monitoring one or more of the characteristic masses, which usually represent the most intense mass or masses for each compound, compared to the sensitivity in full scan mode. Specificity can be increased by scanning more than one mass for the compound of interest in SIR and comparing their intensity ratios and the retention time with those of standards to provide identification and quantification of the analyte.

Mass spectrometry in the SIR mode has been applied to morphine and related drugs in both electron impact (EI) [94,98,105,106,133] and chemical ionization (CI) [81,85, 88,136], modes of ionisation: sensitivities down to 1ng/ml of sample were obtained for morphine and 150pg/ml for buprenorphine [106].

In the present study solid phase extraction procedures based on ion-exchange materials were evaluated for the extraction of basic drugs. Morphine and buprenorphine were used as model substances of current importance in forensic toxicolgy. Extracts were analysed by GC-MS following derivatisation with a novel silylation reagent.

3.II EXPERIMENTAL

3.II.1 MATERIALS AND REAGENTS

^{3}H-Morphine with a specific activity of 962 GBq/mmol (26Ci/mmol) was obtained from Amersham International PLC (U.K.). Its radioactivity purity was greater than 98% by TLC, on a precoated silica gel plate (Merck), developed in methanol:ammonia (100:1.5, v/v). The plate was monitored with a Panax Radio-TLC-Scanner. Solvents were of glass distilled grade. Solvents involved in derivatization processes were dried by boiling under reflux over calcium hydride and then distilled. These were toluene, acetonitrile (ACN), pyridine, benzene and dimethylformamide (DMF). All drugs were from the Department of Forensic Medicine and Science collection, individually supplied by the manufacturers. N-Methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide (MTBSTFA), hexamethyldisilazane (HMDS), and dimethylchlorosilane (DMCS) were purchased from Pierce and Warriner (U.K.) Ltd. Ethyldimethylchlorosilane was purchased from Lancaster Synthesis Ltd. (U.K.). Analytichem Bond Elut® cartridges were obtained from Jones Chromatography (U.K.) and Lipidex 5000 was from Packard Limited,U.K. Outdated whole blood was obtained from the Blood Transfusion Unit, Western Infirmary, Glasgow, and used for the preparation of spiked standards. Whole blood for preliminary assessment of morphine extraction was obtained from two rabbits weighing about 4.5kg each which were injected intramuscularly with

morphine (2mg) and ^{3}H-morphine (74000 dPm) in water (2ml). At 20 minutes after the injection, blood (20ml) was taken from the ear vein of each rabbit over a period of 30 minutes and collected in heparinised containers. These were stored at 4°C.

(a) Instrumentation

Two magnetic sector mass spectrometers were used. The first was a VG model 16F instrument operated at 4 KV. It was connected to a Perkin-Elmer model Sigma 3B gas chromatograph fitted with a capillary column (Chrompack CP-Sil 5, 25m x 0.32mm ID with a 0.4µm film thickness). The injector oven temperature was 300°C. Samples were applied using a split/splitless injection (Grob type) procedure in which the split valve was closed for 1 min following injection. The fused silica capillary interface to the mass spectrometer was kept at 250°C. The spectrometer was operated under the following conditions: multiplier 3 KV, emission 200 µA, electon energy 70 eV, scan range in full scanning mode 600-20 (exponential down scan), scan rate 1.5sec/decade and inter-scan delay 1sec.

The second instrument was a VG model 70-250S operated at 8 KV. It was connected to a Hewlett Packard model HP5980 gas chromatograph equipped with an OV-1 fused silica capillary column (25m x 0.2mm I.D. phase thickness 0.2µm from Hewlett Packard).

(b) Glassware

All glassware was cleaned in detergent, washed with water, dried, silanized with 10% DMCS in toluene and then rinsed in methanol.

3.II.2 APPLICATION OF MORPHINE TO BOND-ELUT SCX CARTRIDGES

Bond-Elut SCX cartridges (3ml capacity) with the cation-exchange functional group benzenesulfonylpropyl, were attached to a Vac-Elut box, which can hold up to 10 cartridges. Vacuum was applied using a water pump and the cartridges were conditioned as recommended by the manufacturer with 2ml methanol followed by 2ml of water and 1ml of the solvent in which the analyte was dissolved. Aqueous solutions containing morphine and ^{3}H-morphine were prepared from 1ml of water, 1ml of ^{3}H-morphine (2775 Bq/ml in methanol) and 8ml of an unlabelled morphine standard (1.18µg/ml in methanol). The resultant final concentration was 0.94µg/ml, morphine and 277 Bq/ml ^{3}H-morphine. Aliquots (5ml) were applied to conditioned columns. The vacuum pressure generated by the water pump was kept between 5-10PSI and the cartridge was not allowed to dry out between successive applications. The sorbent was washed with methanol (2-5ml) and drained by passing air for 30 sec. Final elution was performed with one of the following eluents to establish the best solvent to elute morphine from the sorbent which would be effective in a small volume.

(a) 0.2-1ml of the silanising reagent HMDS:DMCS: Pyridine (2:1:3 v/v/v).

(b) 0.5ml of warm derivatizing solution as in (a).

(c) 1ml of 10% ammonia in methanol (v/v).

(d) 5% of triethylamine (TEA) in methanol (v/v).

(e) 1ml of 10% TEA in methanol (v/v).

(f) 1ml of 10% TEA in ACN:MeOH 1:1 (v/v).

(g) 2ml of 10% TEA solution (v/v).

(h) 1ml of 10% diethylamine (DEA) in ACN:Meth 1:1 (v/v).

The following washing steps were added: acetone and pyridine before elution with the derivatizing solution and ACN/methanol before elution with solvents containing them. The solvents in the different stages were collected separately. Assessment of losses in each step and of the recovery in the final elution stage were performed as described in the next section.

3.II.3 RADIOACTIVITY COUNTING AND QUENCH CORRECTION

Quench correction was required for the calculation of the efficiency of the radioactivity measurements performed on a Packard Tricarb 2000 Scintillation Counter. Quenching experiments were carried out for each of the media in which the radioactivity required to be estimated. Each experiment was conducted as follows: to 6 vials, each containing 4ml of scintillation fluid (Ecoscint), was added 0.1ml of the ^{3}H-morphine solution. They were well mixed and were kept in darkness for 30 mins before measuring the radioactivity. Then vials with similar values were chosen for each experiment. To each vial, in duplicate, 0.2ml of the sample or its corresponding blank solvent was added. Nothing was added to two vials. The radioactivity was measured again. The scintillation counter was set on a preset time of 2min or preset count of 2000 counts. Both

were corrected to give finally the counts per minute (CPM). Efficiency was calculated by the following formula

Efficiency Percentage = q/a x 100 ..... Equation 3-1.

where q and a are the radioactivity measurements of the quenched and unquenched samples respectively.

The total radioactivity of each sample was calculated for the total volume of that sample, taking into consideration the Efficiency Percentage.

3.II.4 APPLICATION OF BLOOD TO BOND-ELUT COLUMNS

Assessment of a reasonable mode of application of blood samples to the selected sorbent (Bondelut SCX) was performed using rabbit whole blood. A 1ml aliquot of blood was utilized for each of the following experiments :

(a) Direct application of the blood diluted with 1ml of water.

(b) Direct application of the blood diluted with 5ml of water.

(c) The blood was diluted in 10ml of water and the solution was ultrasonicated for 20 min

(d) The blood was deproteinised by adding 1ml of blood drop-wise into 10ml of methanol in an ultrasonic bath and leaving there for 20 min, followed by centrifugation for 15 min at 3500 RPM. The solvent layer was applied to the column.

The sorbent was conditioned as described in the previous section before applying the sample. The radioactivity of the effluent fractions was estimated as described in Section 3.II.3.

3.II.5 MORPHINE RECOVERY BY SOLVENT EXTRACTION

The following experiments were conducted to select a quick and efficient solvent extraction procedure for morphine, which would provide an extract suitable for application to a Bond Elut SCX cartridge.

(a) Protein precipitation

Three experiments were performed as follows:
in a 15ml screw-capped test tube 1ml of rabbit blood was added drop-wise into 6ml of methanol in an ultrasonic bath and ultrasonicated for 20 min. The methanol layer was collected after centrifugation and the residue was resuspended in another 6ml of methanol. The mixture was vortex mixed for 30sec then the solvent layer was separated as above and added to the first methanol supernatant.

(b) Protein Precipitation Under Basic Conditions

Five experiments were conducted as described in (a) with the difference that 0.5ml of 20% ammonia in water was added to the methanol before addition of the blood.

(c) Ammonium Carbonate/Ethyl Acetate

Five experiments were performed, each of which was conducted by adding freshly crushed fused anhydrous ammonium carbonate to the blood. About 0.5-0.8g were required to saturate 1ml of blood. It was extracted twice with 6ml of ethyl acetate by vortex mixing for 30 sec then the solvents were collected after centrifugation.

(d) 1M borate buffer (pH9) was added to 1ml of blood. Four experiments were performed. Each sample was

extracted twice with 6ml of ethyl acetate as described in (c).

The extraction efficiencies for each experiment were determined by estimation of the radioactivity of each extract as described in Section 3.II.3. They were calculated using the following formula:

Percent Extraction efficiency = (calculated activity in the extract/the activity present in 1ml of blood) x 100.
..... Equation 3.2

The radioactivity present in the blood was estimated as described in Section 3.II.3, where 1ml of blood was diluted into 20ml of water. Blanks of human and rabbit blood were similarly treated before the preparation of the sample for counting.

3.II.6 APPLICATION OF BLOOD EXTRACT TO SCX CARTRIDGE

The recovered solvent extracts of the previous Section (5b) were applied to conditioned SCX columns, washed with ACN/MeOH (5ml) and eluted with 10% TEA in ACN/MeOH(2ml). The radioactivity was estimated in the eluate collected after the application, washing and elution steps.

3.II.7 EXTRACTION WITH LOW MOLARITY BUFFERS

For these experiments human whole blood was the medium used for the evaluation of low molarity (0.1M) buffers. A working standard of morphine and ^{3}H-morphine at concentrations of 1.55µg/ml and 2775Bq/ml of blood,

respectively, was prepared. The following buffers were assessed for the extraction: 0.1M ammonia and 0.1M borate in water and 0.1M ammonia in MeOH. Extraction was conducted in a 15ml screw-capped test tube where 1ml of blood and 1ml of the buffer were mixed. The blood was extracted twice by vortex mixing for 30 sec with 6ml of the extracting solvent (ethyl acetate:isopropanol 9:1, v/v). The collected solvents layers were applied to SCX cartridges as described in the previous section. Radioactivity measurements were carried out for each step.

3.II.8 SELECTION OF A SILYLATING REAGENT

Morphine (5µg) was placed in a screw-capped 1 dram glass vial. Eighteen samples were prepared and were divided into three groups. Each group was derivatized with one of the following reagents:

(a) Derivatizing solution prepared from HMDS, DMCS and pyridine in the ratio of 2:1:3 v/v/v.

(b) Tri-Sil Z (trimethylsilylimidazole in pyridine, 1.5mEq/ml)

(c) MTBSTFA in pyridine (1:1, v/v).

100 µL of the reagent was added to each vial. The closed vials were kept on a heating block at 60°C for 15 min. Four vials of each group were evaporated to dryness on the heating block under a gentle stream of nitrogen. The remaining vials were kept intact. The residues were reconstituted in 100 µl of hexane or ethyl acetate, two vials for each group. The products were analysed by GC-MS

using the VG-16F instrument with the GC oven temperature programmed from 200-300°c at 8°/min and left at the upper temperature for 3 min. Total ion current (TIC) chromatograms were obtained for each sample.

3.II.9 CONDITIONS FOR SILYLATION WITH MTBSTFA

Several 1 dram vials were prepared containing 23µg of morphine. The following conditions were assessed to force the silylation with MTBSTFA to completion to give 3,6-bis-MTBS-morphine.

(a) The effect of the solvent: 6 vials were divided into 3 groups. The morphine in each group was reconstituted in 50 µl of one of the following solvents: acetonitrile, dimethyl formamide or pyridine. This was followed by the additon of 50 µl of MTBSTFA. The vials were placed on a heating block at 60°C for 30 min.

(b) Time required to complete the reaction: several samples were derivatized as in (a) using DMF as the solvent for the reaction. The samples were kept on the heating block for 15, 90 or 150 min before analysis.

(c) Solvent and reagent ratio: the ratio of MTBSTFA present to solvent required to optimize the derivatization was assessed by preparing duplicate samples with 10-100% of MTBSTFA in DMF. The reagent (100µl) was added to each vial and the vials were kept on the heating block for 1 hr before the analysis.

(d) Effect of temperature: morphine was derivatized with 100 µl of 60% MTBSTFA in pyridine. Two duplicate

samples were kept in heating blocks for 1 hr at temperatures of 60° and 100°C, respectively.

(e) Effect of catalyst: four morphine samples were derivatized with 100 µl of 40% MTBSTFA in pyridine and 20µl of BF_3 etherate gas were added to two vials directly with the derivatizing reagent, and after 30 min on a heating block to the other vials. All samples were kept at 60°C for 1 hr. Each sample was diluted with 1ml of water and extracted into 4ml of chloroform. The solvent was evaporated to dryness under a stream of nitrogen and the residue was redissolved in 100 µl of the same solvent.

The product of each of the above experiments was analysed by GC-MS as described in Section 3.II.8. The areas of GC peaks measured on reconstructed mass chromatograms for the masses m/z 342 and 456, representing the (M-57) ion for the mono- and the bis-MTBS morphine derivatives, respectively, were taken for comparison of the silylation conditions.

3.II.10 INVESTIGATION OF THE INCOMPLETENESS OF THE REACTION

These experiments were conducted to find out why the derivatization of morphine with MTBSTFA did not go to completion to give 3,6-bis-MTBS-morphine and to establish which of the two hydroxyl groups was silylated in mono-MTBS-morphine.

(a) Sample preparation: 40 mg of morphine were dissolved in 2ml of pyridine and were derivatized with 1ml

of MTBSTFA. The mixture was kept over a heating block for 30 mins, then the volume of the solvent was reduced under a stream of nitrogen. A preparative TLC plate (20 x 20cm, silica gel, 2.5 mm thick) was first washed with the mobile phase (15% methanol in chloroform, v/v). It was air dried and reactivated in an oven at 60°C overnight. The residue of morphine derivative was applied in a line on the TLC plate. The plate was developed with the same solvent and dried. The chromatographic bands were localized by spraying the edge of the plate with acidified iodoplatinate reagent. The corresponding area of the silica gel of each product was scraped off separately. The silica was transferred to a glass filter funnel containing a plug of silanized glass wool and the products were eluted with 15% methanol in chloroform (v/v).

(b) Morphine base was prepared by dissolving morphine HCl in water. The solution was alkalinized with 0.1M ammonia and extracted into ethyl acetate:isopropanol (9:1, v/v).

(c) GC-MS

Each of the isolated products was analysed by GC-MS. The operating conditions were as described in Section 3.II.8. The purity of each isolate was assessed and its mass spectrum was obtained. The possible site of the MTBS group in the monoderivative of morphine was inferred with the help of its mass spectrum (see discussion).

(d) UV spectroscopy

The ultraviolet spectra of free morphine, mono-MTBS- morphine and 3,6-bis-MTBS-morphine were taken in a neutral solvent (methanol) and in an alkaline solvent (10% sodium hydroxide solution) against the corresponding blank solution in a Hewlett Packard HP8451A diode array UV/VIS spectrophotometer. The spectra were recorded from 190-450nm.

(e) NMR spectroscopy

NMR spectra of morphine and its derivatives were recorded. About 5mg of the bis-MTBS-morphine was dissolved in deuterated chloroform. Free morphine base and mono-MTBS-morphine were dissolved in deuterochloroform to which a few drops of deuteromethanol were added.

(f) Derivatization of Mono-MTBS-morphine

Two samples, each of about 25µg of mono-MTBS-morphine, were re-derivatized, the first with 100µl of 40% MTBSTFA in pyridine and the other with 100 µl of Tri-Sil Z. They were kept on a heating block at 60°C for 1 hr before analysis of the products by GC-MS.

(g) Derivatization of Codeine

Codeine (5µg) was derivatized with 40% MTBSTFA in DMF (100µl). The sample was kept at 60°C for 1 hour after which, 1µl of the mixture was analysed by GC-MS.

(h) Derivatization of 6MAM

6-Monoacetylmorphine was prepared from diamorphine as described in the literature [139]. An aliquot of the product was derivatized with 40% MTBSTFA in acetonitrile (100µl) at 60°C for 1 hour and 1ul was analysed by GC-MS.

3.II.11 SILYLATION WITH ETHYLDIMETHYLSILYL-IMIDAZOLE (EDMS-I)

EDMS-I was assessed as a new derivatizing reagent for morphine and buprenorphine in the following experiments.

(a) Reaction Time

Three samples of 5µg of morphine and 3µg of buprenorphine in 1 dram vials were prepared. 50µl of acetonitrile and 50µl of EDMS-I were added to each vial. They were kept on a heating block at 60°C for 0, 15, and 30 min, respectively, before GC-MS analysis.

(b) Amount of Reagent

100µl of a solution of the reagent in acetonitrile at concentrations of 20, 40 and 60% by volume were added to morphine and buprenorphine samples in 1 dram glass vials. The vials were warmed for 15 min at 60°C before GC-MS analysis.

(c) Stability of the Product

Three samples were derivatized with 40% EDMS-I in acetonitrile. The products were examined by GC-MS every other day for two weeks and compared qualitatively and quantitatively with freshly prepared samples.

3.II.12 SYNTHESIS OF EDMS-I

Synthesis of ethyldimethylsilyl-imidazole was conducted in two steps.

(a) Preparation of Diethyltetramethyldisilazane (DETMDS)

A 250ml three neck round bottom flask (RBF) was used for the reaction. The middle neck was connected to a

venting tube containing calcium oxide granules. A thermometer was fitted through the second neck to monitor the reaction temperature and a gas leak was fitted to the third neck permitting small bubbles of ammonia to pass through the reaction mixture. The flask was placed in a liquid paraffin bath on a stirrer hot plate. Stirring of the mixture was accomplished with magnetic stirrer bars in both the flask and the paraffin bath. Liquid ammonia was placed in a 500 ml RBF which was cooled in a bath of acetone and dry ice. Ammonia gas effluent was passed through two Dreschel bottles, the first containing a layer of liquid paraffin to monitor the flow rate and the other (empty) bottle was to prevent the solvent from the reaction mixture sucking back. Dry toluene (80ml) was added to the reaction flask and the flask was purged with ammonia gas for a few minutes at 50-60°C before the addition of 10g of dimethylethylchlorosilane. The temperature was maintained 50-60°C with continous stirring for 45 min. The reaction was stopped by disconnecting the ammonia supply.

(b) Preparation of EDMS-I

Imidazole (4.64g) was added to the reaction mixture from (a) above. The mixture was refluxed for 12 hrs with continous stirring. Venting was through a drying tube containing calcium oxide granules. After cooling and settling, the clear layer was transferred to a distillation apparatus and the bulk of the toluene was recovered. The remaining solvent which contained the

product was transferred to a three neck flask in a microdistillation apparatus. A gas leak tube to admit a fine stream of nitrogen bubbles into the solvent layer was fitted to the first neck and a thermometer was placed in the second. A vacuum pressure generated by a rotary pump was applied via the receiver adapter. The distillate was collected through a 3-neck rotating receiver. The bulk of the product (8ml) was recovered at 105°C and a pressure of 10mmHg.

EDMS-I was kept at 4°C in a Pierce reacti-vial fitted with a screw cap containing a teflon valve for sampling without exposure to air.

3.II.13 DERIVATIZATION OF BLOOD EXTRACTS

A standard solution of morphine in whole blood was prepared at a concentration of 0.46 µg/ml. To extraction tubes were added 1ml of this sample or 1ml of blank blood. Nalorphine (100µl of a solution containing 2.5µg/ml) was added as an internal standard (I.Std) to each sample and these were extracted as described in Section 3.II.7. The extract residues were treated with 50ul of 40% EDMS-I in acetonitrile and the vials were placed on a heating block at 60°C for 15 min before analysis. The analysis of the products was performed on the VG-16F GC-MS instrument using full scan and SIR modes as described earlier. Monitoring in SIR was on the molecular ions at m/z 457.2468 and 483.2625 of di-EDMS-morphine and di-EDMS-nalorphine respectively. The dwell time was 80msec per channel and the interchannel

delay (ICD) was 20msec. The SIR channels were calibrated and tuned using PFK reference masses at m/z 454.9729 and 492.9697. Aliquots of the following samples (1µl) were analysed to look for interference at the morphine peak: blank of blood extracts, solvent, EDMS-I solution and sample residues.

3.II.14 PURIFICATION OF THE DERIVATIVES

Derivatized blood extracts were purified from the excess derivatizing agent over Lipidex-5000 as follows: about 1g of the Lipidex material was packed under gravity flow into a silanized Pasteur pipette containing a plug of silanized glass wool as the bed support. The column was washed with 5ml of MeOH:water:chloroform (90:10:20, v/v/v) to remove possible contaminants, then washed successively with 1ml of MeOH, 2ml of acetone and 1ml of ACN:dimethoxy-propane (DMP) (8:2, v/v) to remove traces of water from the material. The derivatized sample was diluted with 1ml of the latter solution and applied to the on top of the column. The column was washed with a further 1ml of the same solvent and the combined filtrates were received into 1ml dram vial. The solvent was evaporated at 60°c under a stream of nitrogen. The residue was redissolved in 50µl of ethyl acetate and analysed by GC-MS.

3.II.15 HIGH RESOLUTION SIR

High resolution SIR mass spectrometry was evaluated as a means of reducing interference in the analysis of

morphine as the bis-EDMS derivative. The VG model 70-250S instrument was tuned on mass 454.9728 of PFK to a resolution of 1000, then the peak height was reduced to 10% by closing the source exit slit. The peak was reduced by a further 50% using the collector slit and to give a resolution of 10000. The collector was opened slightly to obtain a flat peak top. The samples prepared in Section 3.II.13 were reanalysed under these conditions.

3.II.16 CHEMICAL IONIZATION MASS SPECTROMETRY

Isobutane chemical ionization mass spectrometry was evaluated as a means of obtaining an analysis of morphine and nalorphine free from troublesome interferences. The following parameters were adjusted to optimize sensitivity: the emission current, the electron energy setting and the isobutane pressure in the source. Morphine and nalorphine (5µg each), in a 1 dram vial, were derivatized with EDMS-I (50µl) as described earlier and 1µl was injected into the GC-MS instrument. Full spectra were recorded and gas chromatographic peak heights were measured using reconstructed accurate mass chromatograms for masses 457, 458 and 354 for morphine and 483, 484 and 380 for nalorphine, following adjustment of each parameter tested.

3.II.17 GC-CI-SIR OF EDMS-MORPHINE

The following masses 458.2706 and 484.2685 for bis-EDMS derivatives of morphine and nalorphine, respectively, were set for SIR. The isobutane reagent gas

flow rate gas was set to give a source pressure of 3.5×10^{-5} torr. The electron energy and emission current were set to 70 mV and 0.5 mA, respectively. The analyses were conducted using samples prepared as in Section 3.II.13 to look for any interference. Another experiment was conducted under similar conditions as described above, to assess the sensitivity of CI-SIR analysis. Monitoring was conducted this time on the masses 354.1889, 380.2045 and 536.3559 which were the most abundant masses of morphine, nalorphine and buprenorphine EDMS derivatives, respectively.

3.II.18 EXTRELUT EXTRACTION

Diatomaceous earth (Extrelut) was washed in methanol and ethanol and was dried at room temperature. About 1g of the material was packed in a 10ml disposable plastic syringe with gentle tapping. This type of column was evaluated as a substitute for the initial solvent extraction process in the following manner: blood samples (1ml) and 100µl of nalorphine I.Std solution were mixed with 1ml of 0.1M ammonia in test tubes. The samples were then applied to Extrelut columns held in a Vac-Elut box and allowed to stand for 5 min. The columns were divided into two groups: the first group was washed initially with 4ml of hexane. Both groups were then placed over preconditioned Bond-Elut SCX columns via luer adapters and each was eluted with 6ml of the extraction solvent (ethyl acetate:isopropanol 9:1, v/v). The vacuum pressure was

maintained at 5-10PSI. Once the elution had stopped, the Extrelut columns were discarded. Washing and elution from SCX and subsequent derivatization were completed as previously described.

Blank blood samples and blood standards (0.46µg/ml morphine and 16ng/ml buprenorphine) were prepared in this way. The extracts were analysed by GC-MS on the VG 70-250S instrument in the SIR mode. The recovery of morphine, nalorphine and buprenorphine from blood using both extraction procedures was calculated by comparing the peak areas of the extracts with those of non-extracted standards prepared in methanol at an equal concentration which represent 100% recovery according the formula:

Percent Extraction Efficiency = (peak area of the blood extract/the peak area of the equivalent standard in MeOH) x 100 ..... Equation 3.3

3.II.19 DERIVATIZATION WITH HMDS

Silylation with hexamethyldisilazne with no added catalyst was assessed by derivatizing morphine, nalorphine and buprenorphine samples with 50% HMDS in acetonitrile. The vials were kept at 60°C for 15 min. Samples (1µl) were analysed by GC-MS in both SIR and full scan modes. The extent of the silylation was measured by comparison with similar samples prepared in HMDS:DMCS:pyridine 2:1:3 (v/v/v).

3.II.20 SYNTHESIS OF DETMDS

Diethyltetramethyldisilazane was prepared as described for the preparation of EDMS-I, but on a larger

scale, using 30g of dimethylethylchlorosilane and 150ml of dry redistilled benzene. Benzene was used as a substitute for toluene. The following procedures were conducted to isolate the product EDTMDS: the bulk of the solvent containing the product was decanted from the reaction flask into a round bottom flask. The remainder of the reaction mixture was filtered through a filter paper placed in a Buchner funnel. Vacuum was generated by a water pump, so this step was conducted very quickly to minimize exposing the product to hydrolysis. The collected clear solvent was placed in a distillation apparatus and the bulk of benzene was removed by distillation. The remaining fluid (25ml) was transferred to a smaller distillation apparatus where the benzene residue was removed by distillation at 78°C. The product (14ml) was recovered at 172°C and stored at 4°C.

3.II.21 DERIVATIZATION WITH DETMDS

Experiments were conducted as in Section 18 to select conditions for morphine and buprenorphine silylation based on DETMDS. DETMDS was not soluble in acetonitrile at room temperature. Its solubility in acetonitrile containing a small amount of toluene was tested by increasing the ratio of the latter gradually until solubility was obtained. A solution was finally prepared from 40% of DETMDS in a solvent composed of acetonitrile and toluene 7:3 (v/v). It was stored at 4°C in a screw capped reacti-vial with a teflon sampling

valve. This mixture was used for the derivatization process with heating of the samples at 60°C for 15 min as in the preceeding experiments.

DETMDS was assessed for its ability to derivatize other polar drugs commonly encountered in forensic toxicology containing either hydroxyl or amino groups. Samples of each drug (1µg) were derivatized as described. The mass spectra were recorded and the major ions of the spectra of the drugs were tabulated.

3.II.22 RECOVERY AND REPRODUCIBILITY

Five replicate samples of blood spiked with morphine at concentrations of 0.56µg/ml and 35ng/ml as well as buprenorphine at concentrations of 8ng/ml and 0.5ng/ml were extracted using the final modification of the extraction process, with Extrelut and hexane washing step included. Similar non-extracted morphine, nalorphine and buprenorphine standards were prepared in methanol. The residues were silylated with 50µl of 40% DETMDS in acetonitrile:toluene 7:3 (v/v). Analysis was conducted by SIR mass spectrometry at a resolution of 1000, using two masses (442.2233 and 457.2468) for morphine, two (468.2390 and 483.2625) for nalorphine and one (464.2620) for buprenorphine. The data system control of acquisition was set to monitor the three groups of ions during retention time intervals bracketing the three peaks of interest. In this way, only two ions were monitored at any time and sensitivity was increased. The dwell time for each mass

was 80 msec and the interchannel delay was 20msec. The extraction efficiency at each concentration was calculated using the formula given in Equation 3-3. The reproducibilities of the injection technique and the extraction were also estimated.

3.II.23 COMPARISON OF THE EXTRACTION PROCEDURES

Blood extracts from the developed method were compared with those from solvent extraction [80] and with the eluate from Extrelut. In these analyses, 1ml of blood containing morphine at a concentration of 0.46µg/ml was extracted. The derivatized extracts were compared by SIR mass spectrometry of the EDMS derivatives.

3.II.24 CALIBRATION CURVE

Morphine and buprenorphine were added to whole blood at a concentration of 1.2µg/ml and 16.5ng/ml, respectively, and the solution was serially diluted in blood for the construction of a calibration curve. The preparation and analysis of the samples was conducted as described in Section 3.II.22. The curve was constructed by plotting the morphine or buprenorphine concentration versus the ratio of response (peak area of morphine over the peak area of the I.Std). Linear regression analysis was used to get the best straight line.

3.II.25 EXTRACTION OF OTHER BASIC DRUGS

The final extraction procedure was assessed for the extraction of other opiates and basic drugs. Blood was

spiked with drugs as shown in Table 3.III.15. The blood samples (volume 1ml) were prepared as described in Section 3.II.22. GC-MS analysis was conducted using an oven temperature programme from 120°C to 300°C at 8°C/min with a final isothermal period of 3 minutes. To measure the extraction efficiencies drug standards were prepared in methanol at the same concentrations. For the comparison, 1ml of standard was evaporated to dryness and derivatized in the same manner as the blood extracts. Recoveries were calculated by comparing the peak areas in the TIC traces for the extracts and the non-extracted standards. Retention Indices were calculated using the retention times of n-alkanes (C_{16}-C_{28}) run under the same conditions.

3.II.26 APPLICATION OF THE EXTRACTION PROCEDURE

During the course of this work, there was an opportunity to analyse 13 samples (blood or urine) that were submitted to the Forensic Medicine Department to be analysed for morphine or buprenorphine. The origin of these samples was either from Road Traffic Act or post-mortem cases. The samples were prepared and analysed by GC-MS as described in Section 3.II.22. Also, syringes were submitted for analysis and were prepared by washing each syringe with 2ml of methanol, which was evaporated to dryness and the residue was derivatized directly with 50ul of 40% DETMDS in acetonitrile:toluene (7:3, v/v) at 60°C for 1 hour.

3.III R E S U L T S

3.III.1 APPLICATION OF MORPHINE TO BOND-ELUT COLUMNS

An aqueous solution containing morphine and ^{3}H-morphine was used for preliminary assessment of Bond-Elut columns with cationic active groups (SCX) before proceeding to biological samples, where the behaviour of morphine with respect to retention during application and washing as well as elution were studied. Conditioning of the sorbent with methanol and water was sufficient to reactivate the cation exchange sulphonic acid group. The applied morphine was completely adsorbed on the sorbent. There was no radioactivity detected in the eluate during the application stage or during washing with different solvents such as methanol, acetone, acetonitrile and pyridine. The elution recoveries with the solvents tested are listed in Table 3.III.1. Inclusion of a solvent containing acetonitrile for washing and elution led to a higher recovery of morphine. There was no significant difference in recovery between solvents containing ammonia, DEA or TEA in the final elution stage. A small volume (0.5ml) of a solution containing 10% TEA or DEA in methanol recovered more than 94% of the applied morphine and full recovery was obtained when the sorbent was eluted with 2ml of the same eluent.

Table 3.III.1 Recovery of ^{3}H-morphine from Bond-Elut SCX columns using different eluting solvents

Eluting Solvent	Recovery
1. derivatizing reagent mixture. 0.5ml (HMDS:DMCS:pyridine 2:1:3)	76%
2. warm derivatizing reagent 0.2ml	21%
3. warm derivatizing reagent 0.5ml*	93%
4. warm derivatizing reagent 1ml	92%
5. 0.5ml* 5% TEA in methanol (v/v)	94%
6. 0.5ml* 10% TEA in methanol (v/v)	96%
7. 1ml* 10% TEA in methanol/acetonitrile (1:1 v/v)	99%
8. 2ml* 10% TEA in methanol/acetonitrile (1:1 v/v)	100%
9. 1ml* 10% DEA in methanol/acetonitrile (1:1 v/v)	99%
10.2ml* 10% Ammonia in methanol/acetonitrile(1:1 v/v)	100%

*methanol/acetonitrile 1:1 v/v wash was included

3.III.2 APPLICATION OF BLOOD TO BOND-ELUT COLUMNS

The red blood cells (RBC) in a whole blood sample should be disrupted before applying the sample to the selected solid phase sorbent. Dilution of the blood sample with water to haemolyse the RBC and a combined process of denaturation and protein precipitation with methanol were tried. Ultrasonication was applied to enhance the disintigration of the RBC into fine particles. Application of the blood which had been haemolysed with water to the column resulted in partial clogging of the sorbent, requiring high vacuum pressure, better than 50kPa/m^2(369mmHg), for even partial elution. Washing the sorbent with methanol was not efficient and the final eluates were coloured and turbid. The degree of colouration and turbidity were variable in extracts of blank samples prepared simultaneously,

therefore quench correction and radioactivity estimation could not be carried out accurately. Methanolic precipitation produced a clear solvent after centrifugation, which was easily applied to the column. The washing and eluting of the sorbent also produced clear eluates. However, the efficiency of methanol precipitation for morphine extracion was only about 12%. There was no loss of the applied morphine in the washing eluate and all the applied radioactivity was recovered upon elution.

Samples prepared without sufficient centrifugation, or those which were not decanted carefully onto the column, produced coloured final eluates due to haemoglobin-derived pigments. Protein precipitation with methanol worked as a solvent extraction for the sample prior to the application but in poor yield, so alternative preliminary solvent extraction procedures were assessed.

3.III.3 SOLVENT EXTRACTION OF MORPHINE

The extraction procedures were based on utilizing 1ml of blood: efficiencies for the solvents tested are presented in Table 3.III.2. Methanol as a protein precipitating agent and an extracting solvent had poor efficiency for morphine recovery, which was about 15%. The efficiency improved to about 70% when the sample was alkalinized with ammonia solution. However, the methanolic extract of the akalinized sample was slightly coloured, resulting in coloured eluate from the sorbent.

Table 3.II.2: Efficiency of morphine extraction from blood with different solvent/buffer combinations

Solvent	Buffer	Solvent Amount	Average Extraction Efficiency(%)
Methanol	none	2 x 6 ml	15
Methanol	20% aqueous ammonia	2 x 6 ml	67
Ethyl acetate	ammonium carbonate	2 x 6 ml	12
Ethyl acetate	1M borate buffer	2 x 6 ml	48

Buffering with ammonium carbonate and extraction with ethyl acetate was complicated by emulsion formation. Also, the separation of the solvent was difficult and both the extraction efficiency and reproducibility were poor. Buffering the sample with 1M borate solution gave no problems with emulsions and produced clear solvent extracts with around 50% extraction efficiency.

3.III.4 APPLICATION OF BLOOD EXTRACTS TO BOND-ELUT COLUMNS

The solvent extracts described in the previous section, where the blood was aklalinized with ammonia, were applied to Bond-Elut SCX columns. 80% of the applied morphine was lost on application. The rest was lost with the wash eluate. The inability of morphine to absorb to the cation-exchange sorbent was due to the pH and ionic strength of the buffer used for the extraction. Adjusting the pH of the solvent was not practicable and dilution of the solvent with methanol, or dilute hydrochloric acid in methanol, failed to permit morphine adsorption. Low

molarity buffers were tried as replacements for the high molarity buffer to permit interaction between morphine and the sorbent.

3.III.5 EXTRACTION WITH LOW MOLARITY BUFFER

Due to the limited recovery of morphine and the high quenching obtained with rabbit blood, the latter was replaced with spiked human whole blood. The results of ethyl acetate:isopropanol extraction are listed in Table 3.III.3.

Table 3.III.3: Morphine extraction efficiency and elution recovery of extracts on Bond-Elut SCX cartridges.

	Buffer used during extraction		
	0.1M ammonia in H_2O	0.1M ammonia in MeOH	0.1M borate in H_2O
Extraction Efficiency *	94.5 ± 3	92 ± 6	85 ± 7
Application Loss	0	0	0
Washing Loss	0	0	0
Elution Recovery **	99 ± 5	98 + 6	85 ± 6

* Extraction with 2 x 6ml ethyl acetate:isoproponal (9:1 v/v)
** Elution with 2ml 10% TEA in ACN:MeOH (1:1 v/v).

The extraction efficiency for morphine when the sample was buffered with 0.1M ammonia was more than 95% and with borate buffer the efficiency was about 85%. The solvent extracts and the eluates for samples in which the buffer was prepared in methanol were slightly coloured.

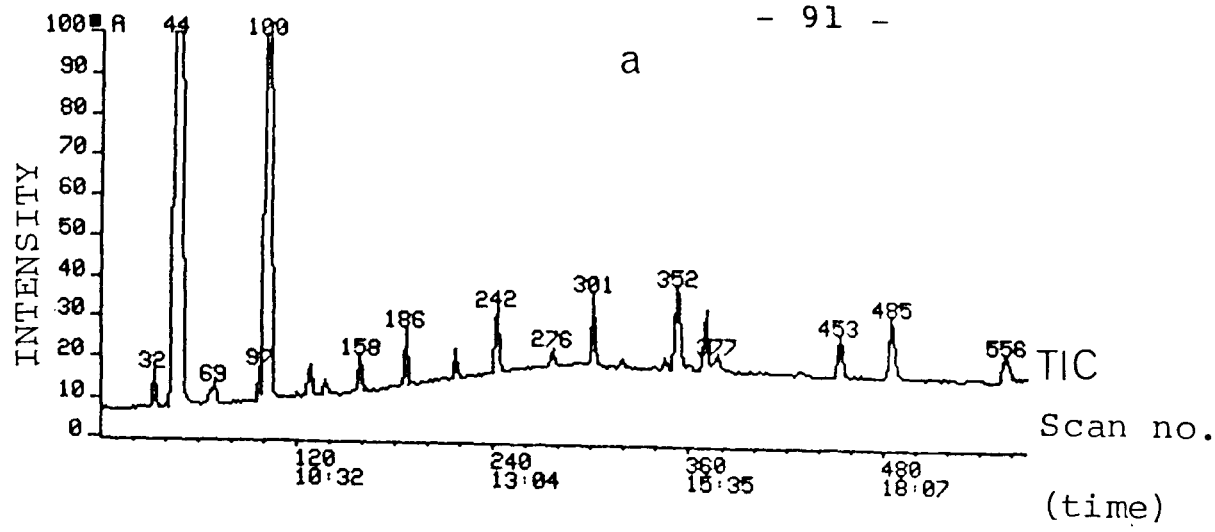
There was no loss of radioactivity on application of the solvent to the SCX sorbent or on washing the sorbent. All the applied radioactivity was recovered in the final eluate in 2ml of 10% TEA in ACN/MeOH. The ammonia buffer in water was selected for preparation of extracts for initial assessment of the end step (GC-MS) analysis. Solvent extracts of this type were subsequently replaced with a solid phase extraction procedure based on diatomaceous earth (Extrelut).

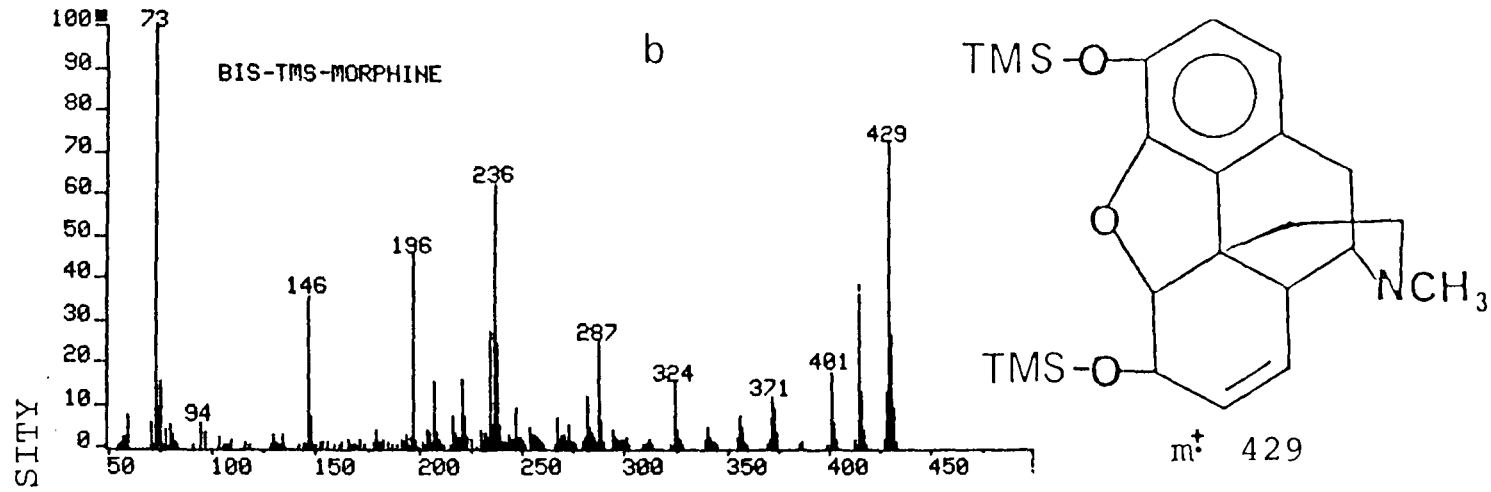
3.III.6 SELECTION OF A SILYLATING REAGENT

Two reagents for the preparation of trimethylsilyl ethers were tried for the derivatisation of morphine prior to GC and GC-MS. These were a solution of HMDS:DMCS:pyridine (2:1:3 v/v/v) and TMS-I in pyridine (1.5meq/ml, Tri-Sil Z). Both TMS donor reagents produced a single product, bis-3,6-TMS-morphine, shown in Figure 3.III.1.

In contrast, MTBSTFA resulted in two products for morphine which were identified by GC-MS. The major peak was the bis-3,6-MTBS-morphine and the smaller peak mono-MTBS-morphine. Their mass spectra are shown in Figures 3.III.2b and 2c, respectively.

The chromatograms (Figures 3.III.1a and 3.III.2a) obtained when the products were injected in the silylating reagent showed good chromatographic peaks for the derivatized morphine but several unrelated siloxane peaks from the silylating reagent were present. This





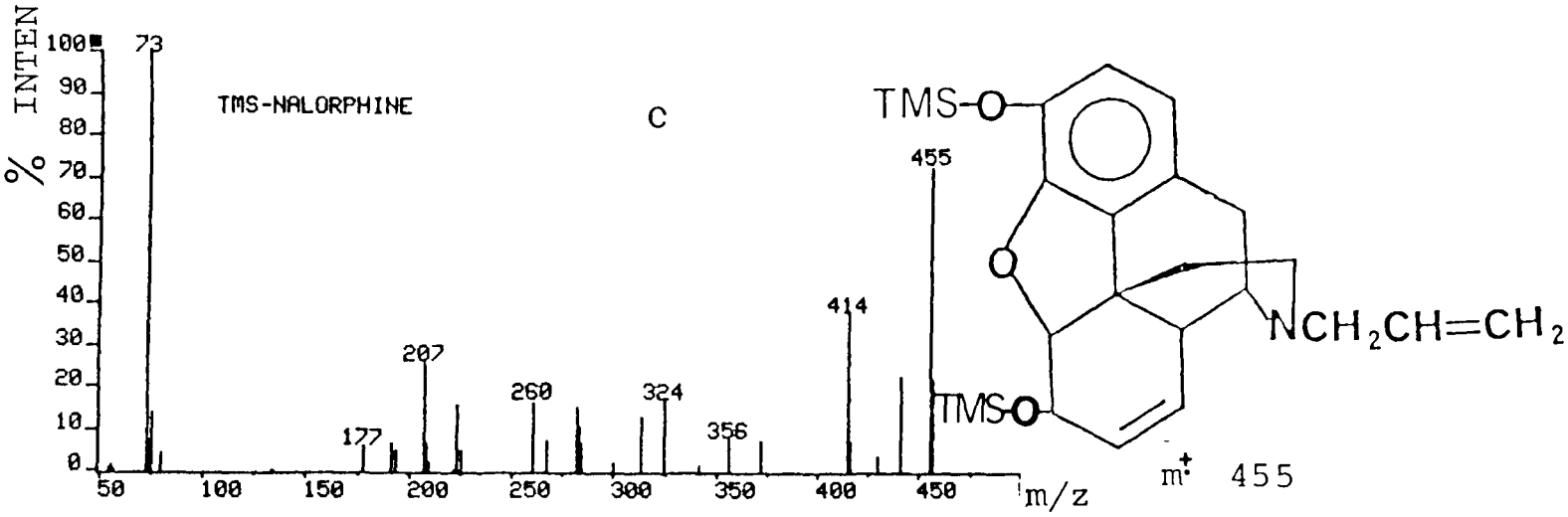
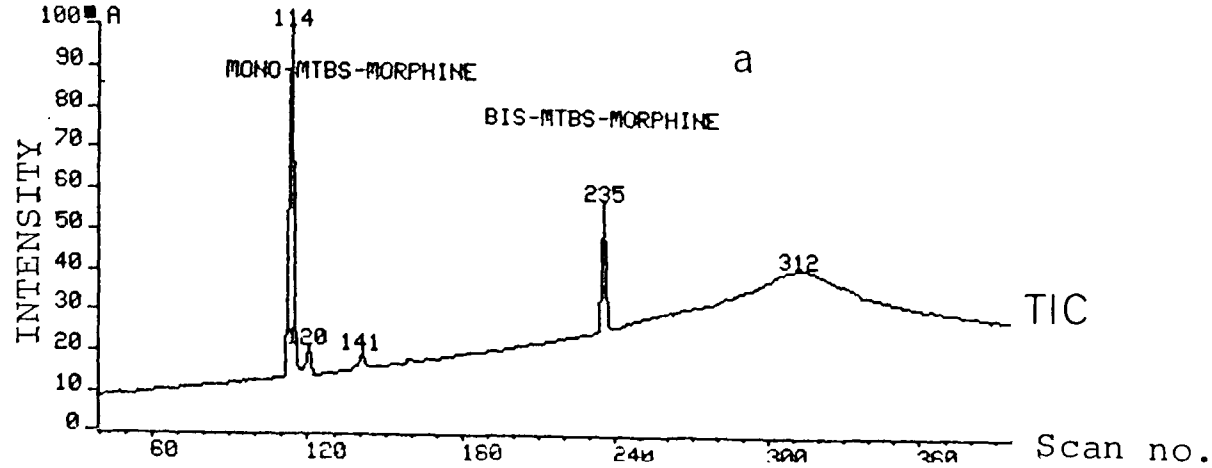


Figure 3.III.1 (a) TIC trace of morphine and nalorphine TMS derivatives (scan nos. 44 and 100 respectively) where 100ng of each was applied on column. The oven temperature was operated from 200° to 300°C at 8°C/min and the injector oven temperature was at 300°C.

(b) and (c) are the EI mass spectra of morphine and nalorphine TMS derivatives respectively.



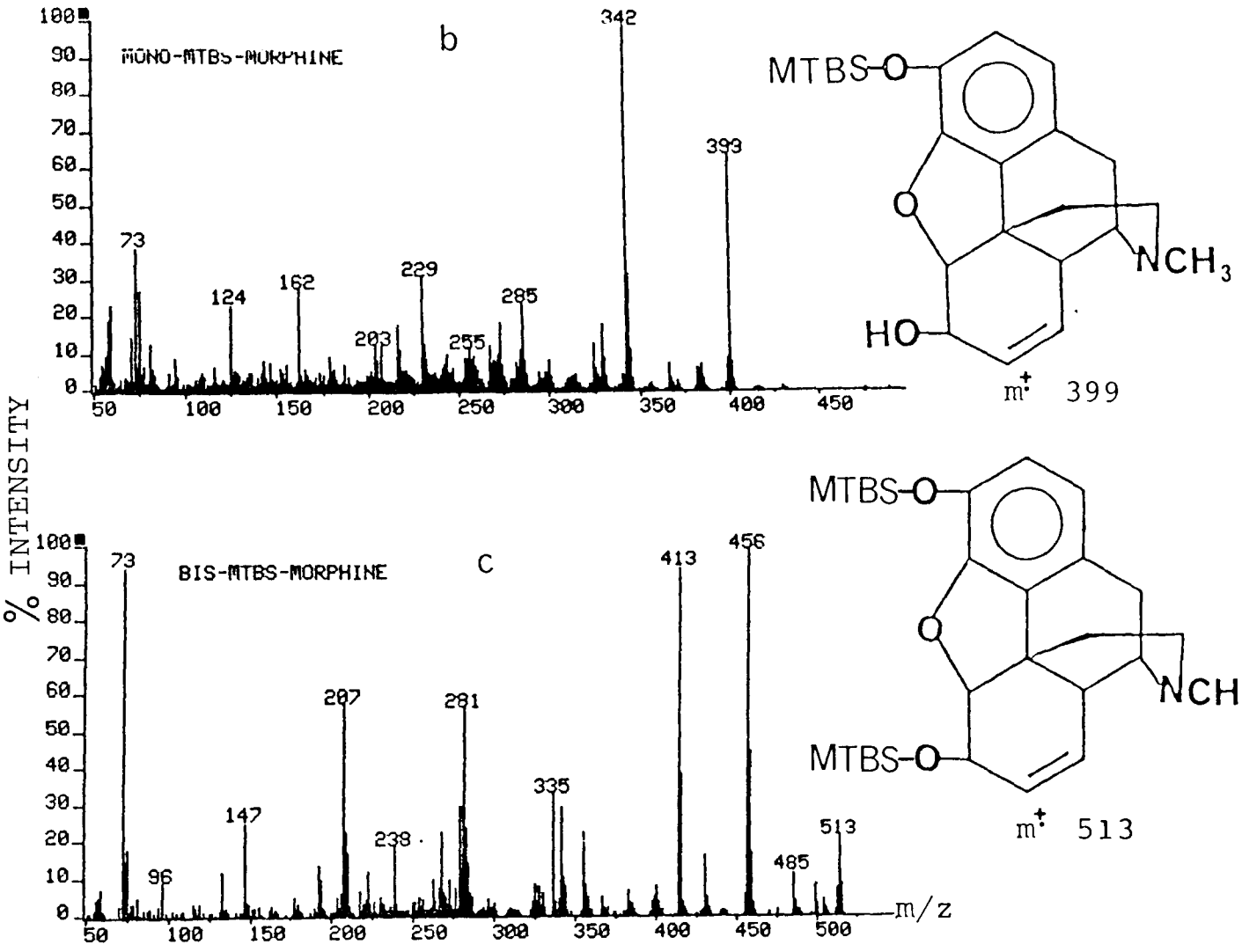


Figure 3.III.2 (a) TIC trace of morphine mono and bis-MTBS derivatives (scan nos. 114 and 235 respectively) where 5ug of morphine was derivatized with 100ul of 50% MTBSTFA in acetonitrile at 60°C for 1 hr and 1ul was analysed by GC-MS. The oven temperature was programmed from 200° to 300°C at 8°C/min and the injector oven temperature was at 300°C.

(b) and (c) are the EI mass spectra of mono and bis-MTBS-morphine respectively.

necessitated purifying the product from the excess derivatising reagent. The morphine silyl-ethers were hydrolysed when the products were evaporated to dryness and nothing was recovered intact in either hexane or ethyl acetate. The MTBS morphine derivatives were stable after evaporation. The chromatograms obtained when the residues were redissolved in hexane or ethyl acetate were cleaner and showed fewer siloxane peaks. Hexane redissolved about 5% of the product while ethyl acetate redissolved about 70%, as indicated by comparison with the peak areas obtained following injection of the products in the reagent. These experiments showed that MTBS derivatives were resistant to hydrolysis and that the products could be purified from the excess reagent.

Further experiments were conducted with MTBSTFA to try and obtain complete silylation of morphine, yielding bis-MTBS-morphine as the only product.

3.III.7 SILYATION WITH MTBSTFA

(a) Solvent: reagent combinations

The ions at m/z 342 and 456 represent the loss of the tertiary butyl group (57amu) from the molecular ions at m/z 399 & 513 of mono- and bis-MTBS-morphine respectively. They are the base peaks in the mass spectra, constituting 1.6% and 3.2%. of the total ion current (TIC), respectively. These TIC values were used to calculate response ratios for the comparison of different reaction conditions. The effect of different solvents used

in the derivatization reaction is shown in Table 3.III.4.

All of the reactions which were conducted in the three solvents produced a mixture of both MTBS-morphine derivatives. Acetonitrile was the poorest solvent for the production of bis-MTBS-morphine. DMF produced 30% of the monoderivative under the conditions tested.

(b) Reaction Times

The reaction time required to reach equilibrium was determined by leaving the derivatized samples in the heating block at 60^{o}C for different lengths of time (Table 3.III.5) and was found to be about 1 hour. After this time, the ratio of mono-MTBS-morphine/bis-MTBS-morphine produced was about 0.25 and was constant at the temperature and ratio of reagent/solvent used.

(c) Reagent:solvent ratio

The ratios of morphine-MTBS derivatives produced by varying the reagent:solvent ratio are listed in Table 3.III.6. The total volume of the mixture was 100µl for each sample. The results show that with lower reagent ratios in the mixture, the ratio of mono/bis derivatives decreased to about 12%. Subsequent reaction studies were conducted using 40% v/v reagent to keep it in excess without reducing the yield of the bis-derivative.

e) Effect of Temperature

A reaction temperature of 100^{o}C was found to produce a higher ratio of bis-to mono-product than was obtained at 60^{o}C. Also, the total yield of derivatives was slightly higher at the upper temperatures (Table 3.III.7).

Table 3.III.4: Effect of different solvents on MTBS-morphine products.

	peak area (TIC)		
SOLVENT	MTBS - morphine		Ratio *
	Mono-	bis-	
ACN	189084	12131	15.6
DMF	4663	15288	0.31
Pyridine	10794	13576	0.46

* Ratio = $\frac{\text{mono}}{\text{bis}}$

Table 3.III.5: Effect of reaction time on MTBS-morphine products.

	peak area (TIC)		
REACTION TIME (min)	MTBS - morphine		Ratio *
	Mono-	bis-	
15	1072	1873	0.57
60	1925 increasing	7861 increasing	0.24
90	2967	9176	0.23
150	2318	9024	0.25

Table 3.III.6: Effect of the ratio of the derivatizing reagent to solvent on MTBS-morphine derivatives.

	peak area (TIC)		
VOL ** %	MTBS - morphine		Ratio *
	Mono-	bis-	
100	17884	1161	15.4
80	5162	7973	0.65
60	2473	6477	0.38
50	1995	8518	0.23
40	1413	19671	0.13
20	901	7727	0.12
10	477	3452	0.14

** Vol% = (volume of reagent/volume of DMF) x 100

Table 3.III.7: Effect of temperature on MTBS-morphine products.

	peak area (TIC)		
TEMPERATURE	MTBS - morphine		Ratio*
	Mono-	bis-	
60°C	3218	7072	0.45
100°C	3340	9285	0.36

f) Effect of Catalyst

The products had to be extracted from their reaction media when catalyst was added as direct injection of the mixture containing the silylated product was not practicable. No peaks could be detected due to an overloaded chromatogram. Each sample was diluted with 2ml of water to destroy the excess reagent and was then extracted with chloroform. The extraction efficiencies in chloroform were about 90% and 10% for the bis- and mono-MTBS-morphine derivative respectively. The silylated products were found to be stable during this extraction procedure. The addition of the catalyst (BF_3 etherate) at the same time as the MTBSTFA or after the reaction had been running for 30 minutes, blocked the derivatization process (Table 3.III.8).

Table 3.III.8: Effect of addition of catalyst on MTBS-morphine products

		peak area (TIC)	
CATALYST	Time of Addition	MTBS - morphine	
		Mono-	bis-
BF3	0 min	0	0
	30 min	0	0

3.III.8 INVESTIGATION OF MTBS-MORPHINE PRODUCTS

The mixture of mono- and bis-MTBS-morphine derivatives was investigated by TLC to produce a system with sufficient separation between the two products, so that they could be easily separated on a preparative TLC plate. A solvent system of 15% methanol in chloroform showed two spots at Rf values 0.4 and 0.54. They corresponded to mono and bis-MTBS-morphine and they had bluish and pinkish colours, respectively with iodoplatinate reagent. Preparative TLC was used to isolate milligram quantities of the two products for further examination.

(a) GC-MS

An aliquot of each fraction obtained from the preparative TLC plate was analysed by GC-MS. The results of GC-MS of the separated products confirmed that the material with Rf value 0.4 was mono-MTBS-morphine with traces of the bis derivative, while the other was bis-MTBS-morphine with traces of the mono-derivative.

(b) Ultraviolet Spectrophotometry

The UV spectra of free morphine and its MTBS-products in both neutral and alkaline solutions had absorption maxima as presented in Table 3.III.9. Bis-MTBS-morphine solution became opaque upon the addition of 10% NaOH solution which suggested the product had hydrolysed. It was filtered through a silanized glass wool before the UV spectrum was taken. Both MTBS-morphine products in 10% NaOH were extracted into chloroform and were checked for their integrity on GC-MS. There was no significant hydrolysis of either of these products.

Table 3.III.9 UV absorption maxima of morphine and its MTBS derivatives

	UV Absorption Maxima (mu)	
	MeOH	10% NaOH
Free morphine	286	300
Mono-MTBS-Morphine	286	298
Bis-MTBS-Morphine*	288	300

* slightly turbid

(c) NMR SPECTROSCOPY

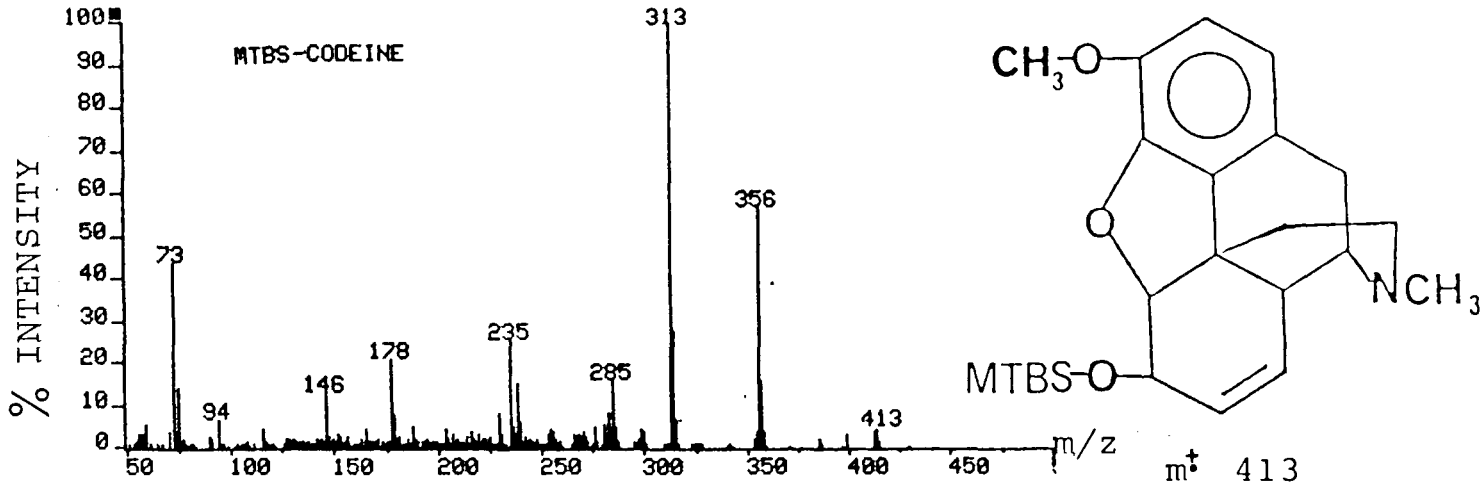
NMR spectra obtained for free morphine and its silylated products showed that the morphine nucleus was intact and that no structural rearrangements had taken place in the mono-MTBS derivative which could explain th failure to achieve complete derivatisation. Also the spectrum of the mono-derivative was not helpful in locating the silylated hydroxyl group.

(d) Derivatization of Mono-MTBS-Morphine

Further derivatization of this product with MTBSTFA produced both mono and bis-MTBS-morphine, while derivatization with HMDS produced mono-MTBS-morphine and MTBS-TMS-morphine. In both cases the mono-MTBS-morphine did not completely derivatize to a bis-derivative.

(e) MTBS-Codeine

The mass spectrum of the MTBS-derivative of codeine is shown in Figure 3.III.3. It corresponds to 6-MTBS-codeine. The derivatization was not complete under the condition used (40% MTBSTFA in DMF, v/v, with heating at $60^{o}C$ for 1 hour). The ratio of the free codeine to the derivatized product was 0.08:1.



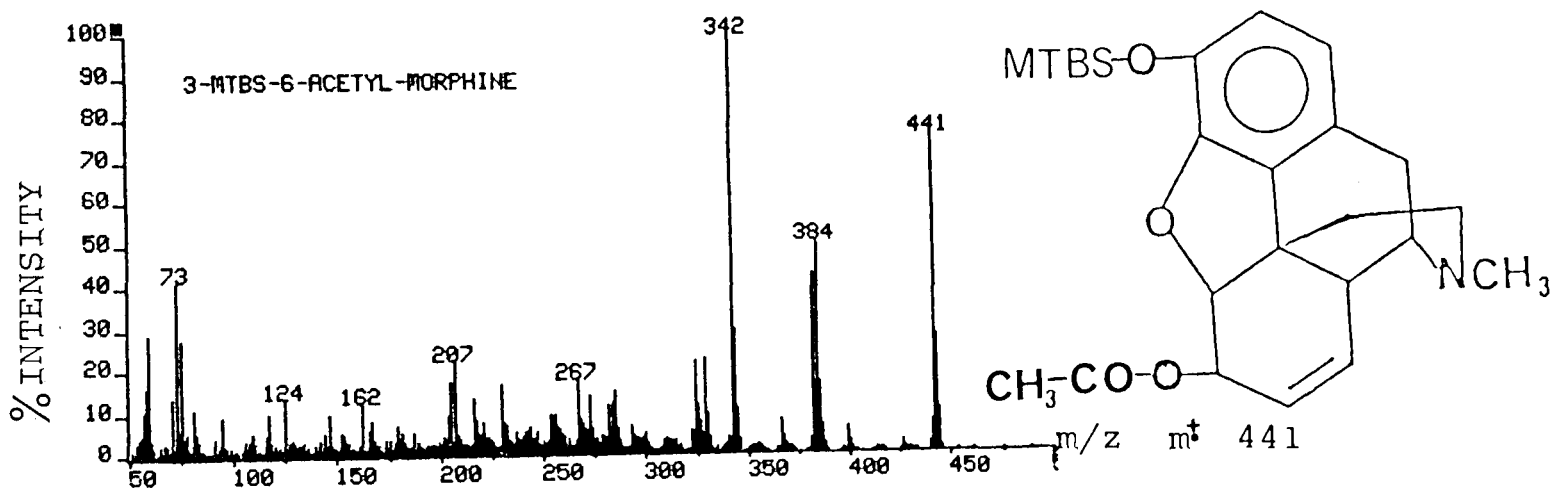


Figure 3.III.3 (above) EI mass spectrum of 6-MTBS-codeine.

Figure 3.III.4 (below) EI mass spectrum of 3-MTBS-6-monoacetylmorphine.

(f) MTBS-derivative of 6MAM

An aliquot of 6MAM was derivatized with 40% MTBSTFA in acetonitrile (100µl) at 60°C for 1 hour and 1µl was analysed by GC-MS. The spectrum of MTBS-6MAM is shown in Figure 3.III.4. None of the free monoacetylmorphine was detected in the chromatogram.

The interpretation of the results in (a) to (f) above is considered in the Discussion, Section 3.IV.5.

3.III.9 SILYLATION WITH EDMS-I

Derivatization of morphine and buprenorphine with EDMS-I was complete for morphine on mixing with reagent at room temperature. Warming the mixture on a heating block did not increase the yield of morphine product while buprenorphine required heating for 15 minutes to obtain complete derivatization (Table 3.III.10).Variation of the derivatizing reagent in the solvent (acetonitrile) was tested in the range 20-50% by volume and was found to have no effect on the amount of products (Table 3.III.11). The ratio of 40% EDMS-I in acetonitrile (v/v) was chosen for a routine derivatization solution.

Derivatization of morphine and buprenorphine with EDMS-I gave one product for each which had good chromatographic peaks (Figure 3.III.5). A smaller peak (about 1/10th of the main buprenorphine peak) eluted earlier than the buprenorphine product and corresponded to a degradation product of the derivatized buprenorphine. The buprenorphine degradation was investigated and was

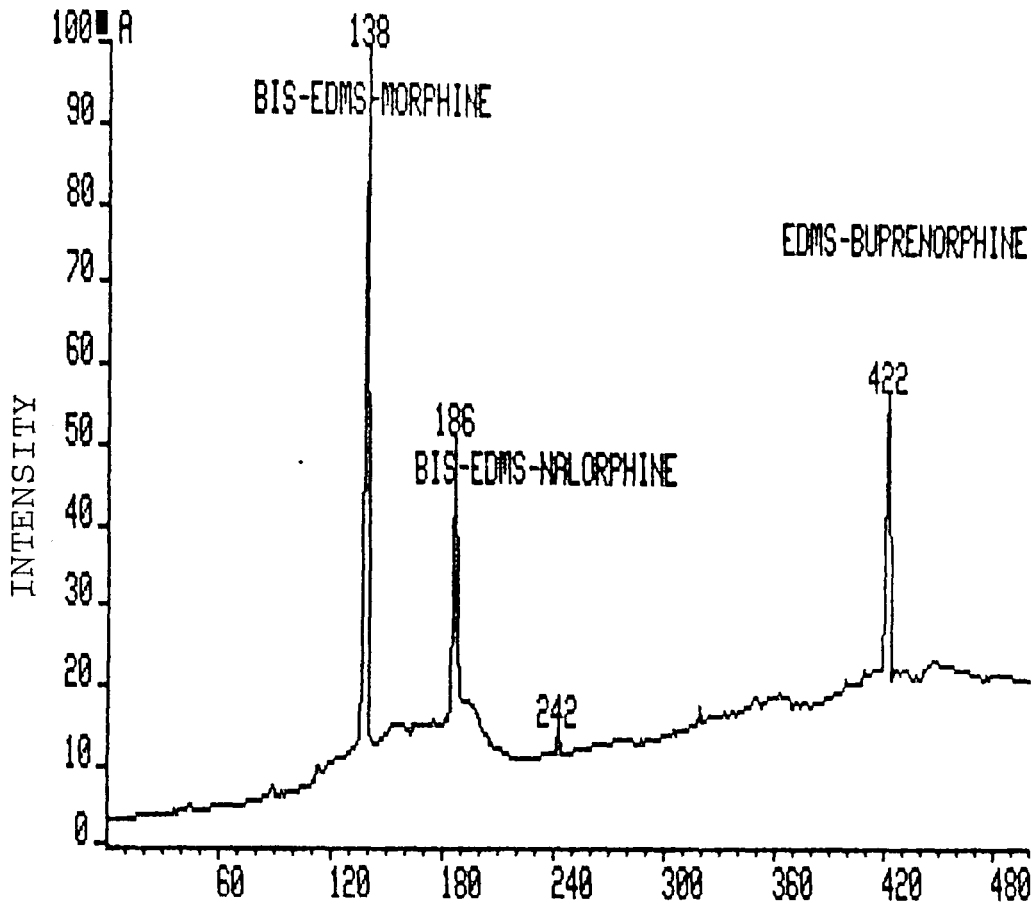


Figure 3.III.5 TIC trace of a standard of 100ng of morphine, 50ng of nalorphine and 100ng of buprenorphine as EDMS derivatives obtained under EI mass spectrometry. The oven temperature was programmed from 200° to 300°C at 8°C/min with a final isothermal period of 3 min and the injector oven temperature was at 300°C.

The scan range was 50-600, the scan rate was 1sec/decade and the interscan delay was 1 sec.

Table 3.III.10: Effect of reaction time at 60°C on the formation of morphine and buprenorphine EDMS derivatives

Time (min)	Peak Area	
	m/z 457	m/z 464
0	12547	1460
15	13158	4980
30	13646	4621

Table 3.III.11: Effect of EDMS-I concentration in ACN on morphine and buprenorphine EDMS products.

EDMS-I/ACN Ratio (% v/v)	Peak Area	
	m/z 457 *	m/z 464 **
20	11022	5100
40	11216	5238
50	11300	5360

* molecular ion of bis-EDMS-morphine
** M-57-32 for EDMS-buprenorphine

found to be related to the injector temperature and conditioning of the GC column. Smaller amounts of the product were obtained by lowering the injector temperature from 300°C to 220°C, but this also resulted in an overall loss of response and poorer chromatography of the main components. Priming the GC column with concentrated EDMS-buprenorphine also decreased the formation of the degradation product.

For morphine, the single product corresponded to bis-3,6-EDMS-morphine. The mass spectrum is shown in Figure 3.III.6a: the base peak was the molecular ion

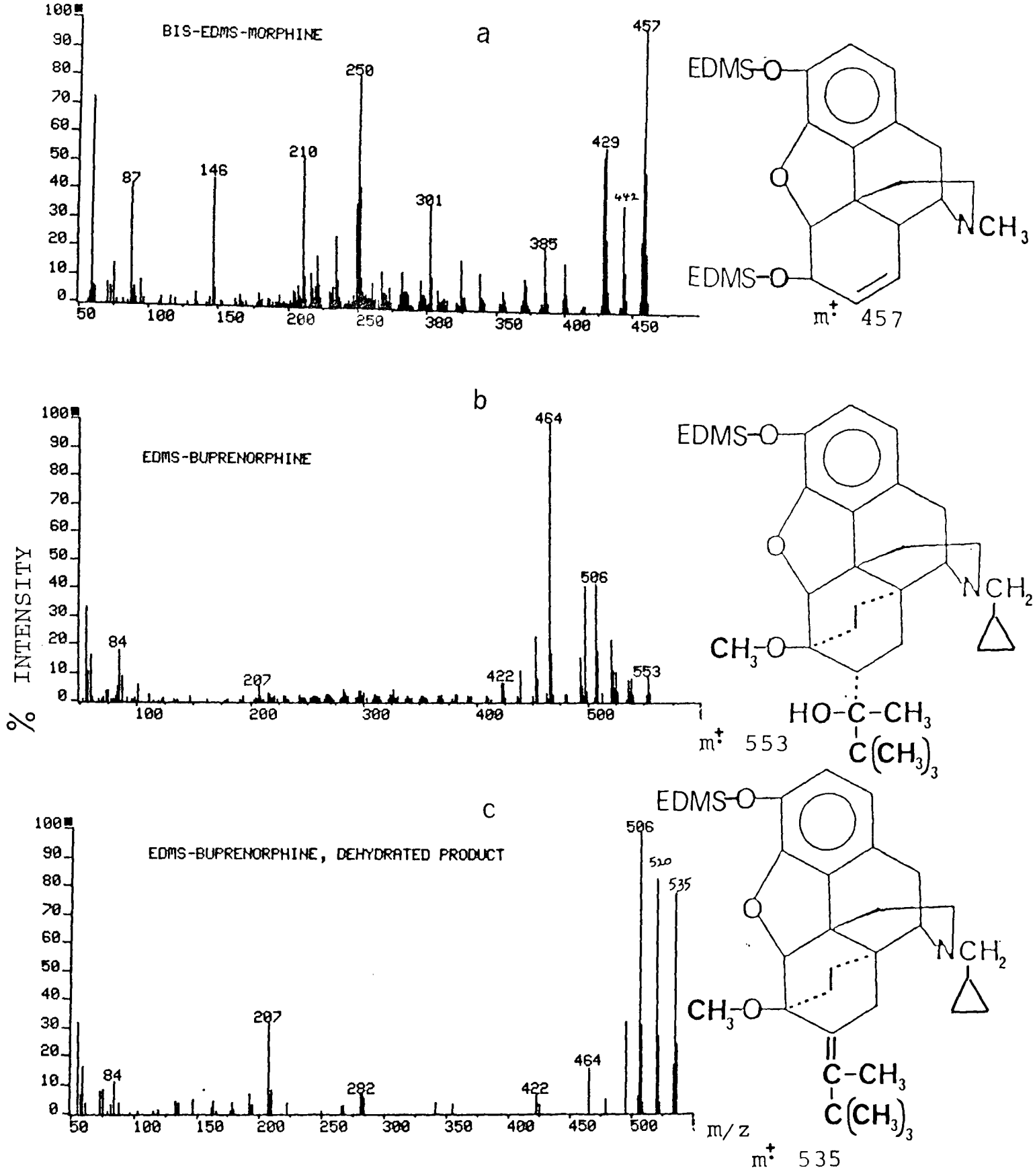


Figure 3.III.6 Electron impact mass spectra of bis-EDMS-morphine, EDMS-buprenorphine and the degradation product of EDMS-buprenorphine.

(m/z 457). The EDMS derivative of buprenorphine was 3-EDMS-buprenorphine, its mass spectrum is shown in Figure 3.III.6b. Its base peak was at m/z 464, representing the loss of the tertiary butyl group (57amu) and methanol (32amu) from the molecular ion at m/z 553. The buprenorphine degradation product, its spectrum is shown in Figure 3.III.6c, formed by loss of water from 3-EDMS-buprenorphine.

3.III.10 STABILITY OF THE PRODUCTS

The products were kept in the silylating reagent for extended periods. Both EDMS-morphine and EDMS-buprenorphine were found to be stable over a two-week test period and no hydrolysis or degradation occurred. However, there was a noticeable increase in the reagent-derived siloxane peaks following the injection of the product in the reagent and these peaks increased with the age of the sample. The glass insert of the injector liner became dirty after only a few injections. Trials to get rid of the reagent by drying it under a stream of nitrogen and redissolving the residue in hexane, ethyl acetate or acetonitrile showed that the products had completely disappeared, presumably by hydrolysis.

Similar results were obtained when morphine and nalorphine EDMS derivatives were purified over a column packed with Lipidex-5000. Morphine and nalorphine EDMS-products could not be detected by SIR within their retention time windows when an aliquot (1µl) of the residue

was analysed by GC-MS. Both compounds appeared in the next injection when a blank or ACN/reagent was introduced. This indicated that these products were hydrolysed in the purification procedure and rederivatized in the inlet by subsequent injection of the reagent.

3.III.11 DERIVATIZATION OF BLOOD EXTRACTS

Extracts of blood samples spiked with morphine at a concentration of 0.46µg/ml were prepared by mixing with nalorphine and 0.1M ammonia buffer and extracting with ethyl acetate:isopropanol. The latter was passed over a conditioned Bond-Elut SCX column followed by elution with 10% ammonia in methanol/acetonitrile. The final eluate was evaporated and the residue derivatized with EDMS-I in acetonitrile.

Since it was not possible to purify the derivatized drug from the excess reagent, the samples were analysed directly by injecting an aliquot of the reaction mixture into the GC-MS. The peaks of morphine and buprenorphine EDMS-derivatives eluted with RRT's of 0.87 and 1.52, respectively, relative to EDMS-nalorphine. They were sharp and symmetrical. Early eluting peaks started to tail if the oven temperature was started at a temperature higher than 240^{o}C. A ghost peak appeared in the solvent blank at the same retention time as morphine after the injection of a positive sample. Two injections of ACN/EDMS-I were required to get rid of the interference. Blank blood extracts showed an interfering peak eluting at the same

retention time as EDMS-morphine which could affect area measurements of the morphine peak. These also showed an elevated base line compared with blank solvents or reagents. Other peaks appeared at regular intervals, the mass chromatogram of m/z 457 in both blanks correponding to siloxane material from the reagent and/or the column stationary phase. Examination of this interference by full scan GC-MS did not show a peak at the retention of morphine in the blank sample due to masking by high background. What was observed, however, was a large peak of cholesterol-EDMS at the end of the run which produced an ion at m/z 457. This required the column to be held at the upper temperature of 300^{o}C, even if only morphine analysis was required, to remove possible subsequent interference in the next sample.

High resolution SIR and chemical ionization SIR were subsequently tried as methods of eliminating the interference, although the extraction method had to be modified to get rid of the cholesterol.

The results of EI-SIR at high resolution (10,000) of the samples prepared for the previous work, still showed an interfering peak in the blank blood sample extract, but not in solvent and derivatizing reagent blanks.

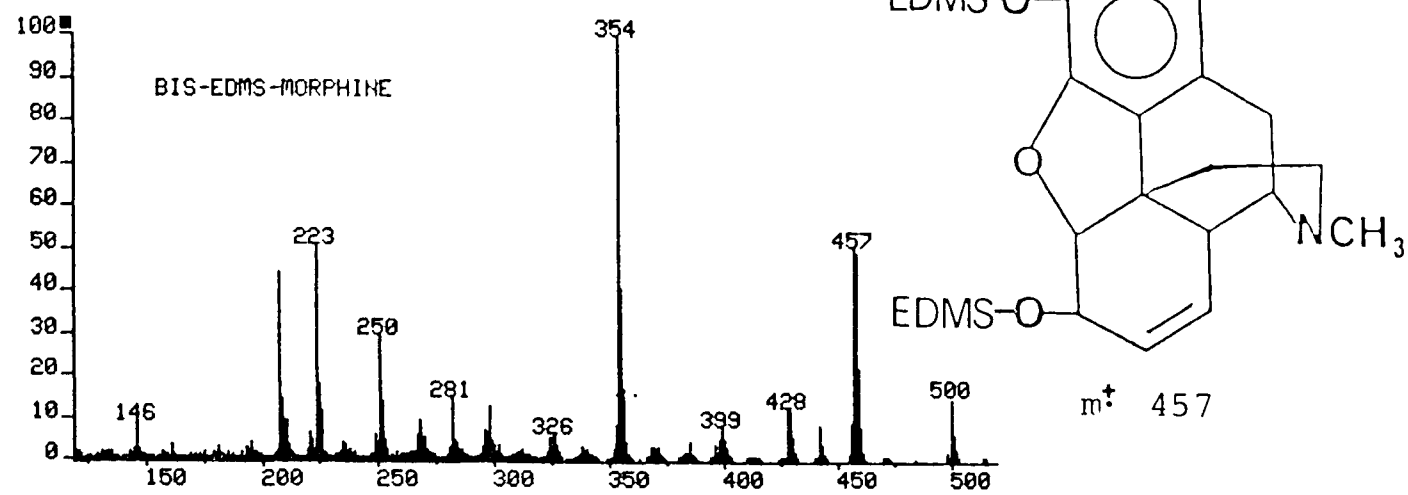
During the evaluation of chemical ionization mass spectrometry, three of the parameters which modify CI sensitivity were optimised: the results are summarised in Table 3.III.12.

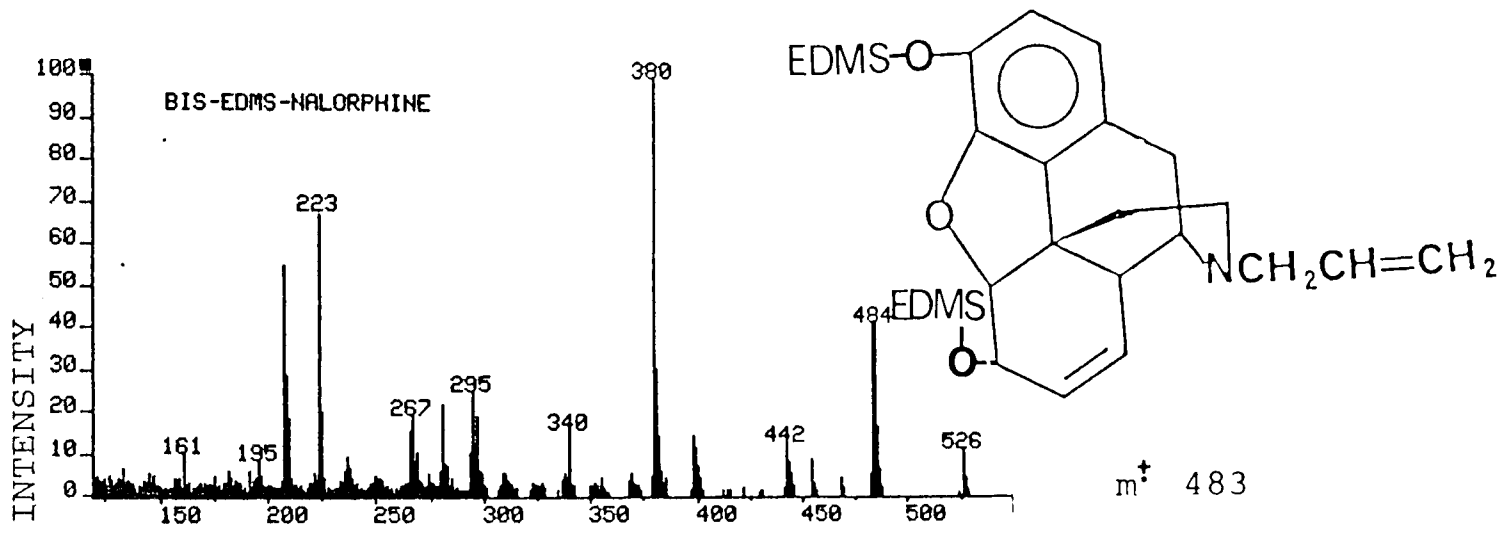
Table 3.III.12: Effect of variation of emission current, electron energy (eV) and reagent gas pressure on CIMS sensitivity and fragmentation patterns for the EDMS-derivatives of morphine and nalorphine

	Peak heights of mass chromatograms						
	Morphine			Nalorphine			
		m/z			m/z		
Emission* Current (mA)	457	458	354	483	484	380	Noise Level
0.2	1651		1964	1324	861	990	clean
0.5	5017	4138	7248	3578	3212	5038	1000
2.0	8696	7315	11790	5971	4937	7364	1200
eV ** (Volts)							
50	5054	4320	6018	3500	2766	3388	800
70	4076	3779	7317	2767	2627	4711	clean
90	4616	3833	5535	2973	2970	3023	720
Gas Pressure ***							
1.3×10^{-5}	4941	3600	4987	3845	2595	3154	600
3.5×10^{-5}	5207	4517	6791	3804	3134	4543	613
4×10^{-5}	4414	4300	7200	300	4100	3240	680
5×10^{-5}	4092	7802	4487		2737	4398	845
7×10^{-5}	4253	3525	8255		2409	4437	1300

* gas pressure was 3.8×10^{-5} Torr and eV 50V,
** gas pressure was 3.8×10^{-5} Torr and emission was 0.5mA,
*** eV was 50v and emission was 0.5mA.
Noise Level = level of the base line.

The CI mass spectra of morphine and nalorphine bis-EDMS-derivatives as well as EDMS-buprenorphine are shown in Figure 3.III.7 and their GC-MS chromatogram is showin in Figure 3.III.8. The most intense ions were at m/z 354 and 380, respectively, representing the loss of ethyldimethyl silanol from the quasimolecular ions $(M + H)^+$ of morphine and nalorphine EDMS derivative, respectively. The most intense ion of EDMS-buprenorphine was m/z 536 representing the loss of water from the





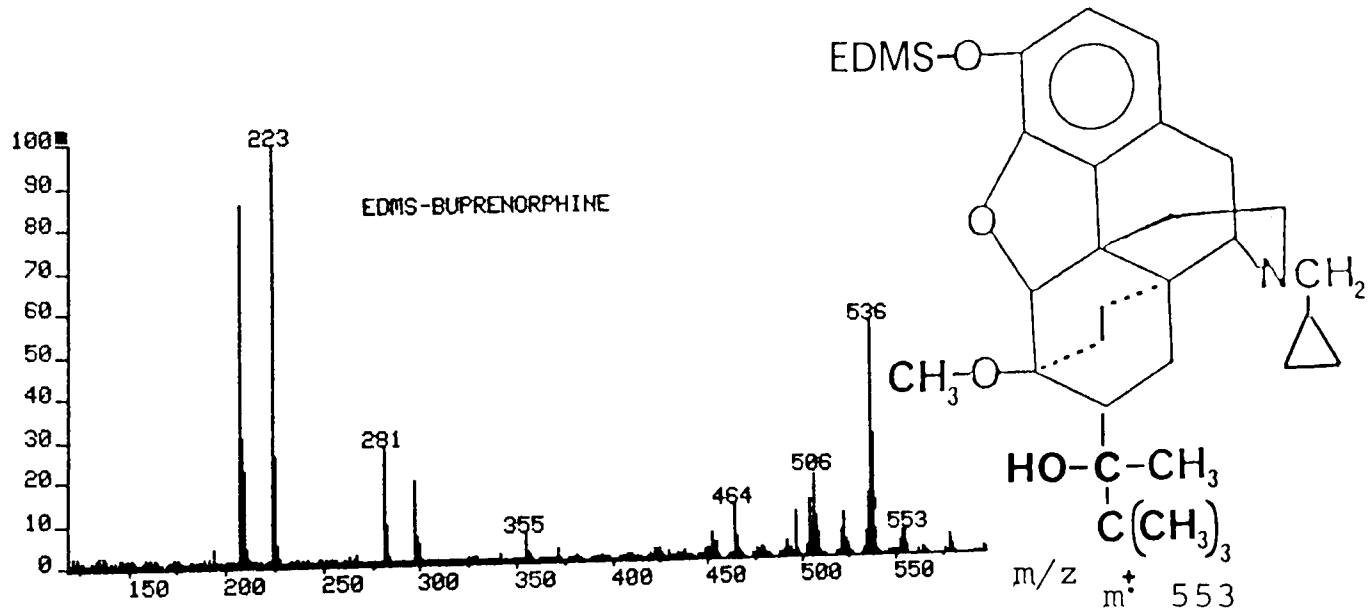


Figure 3.III.7 Chemical ionization mass spectra of bis-EDMS-morphine bis-EDMS-nalorphine and, EDMS-buprenorphine.

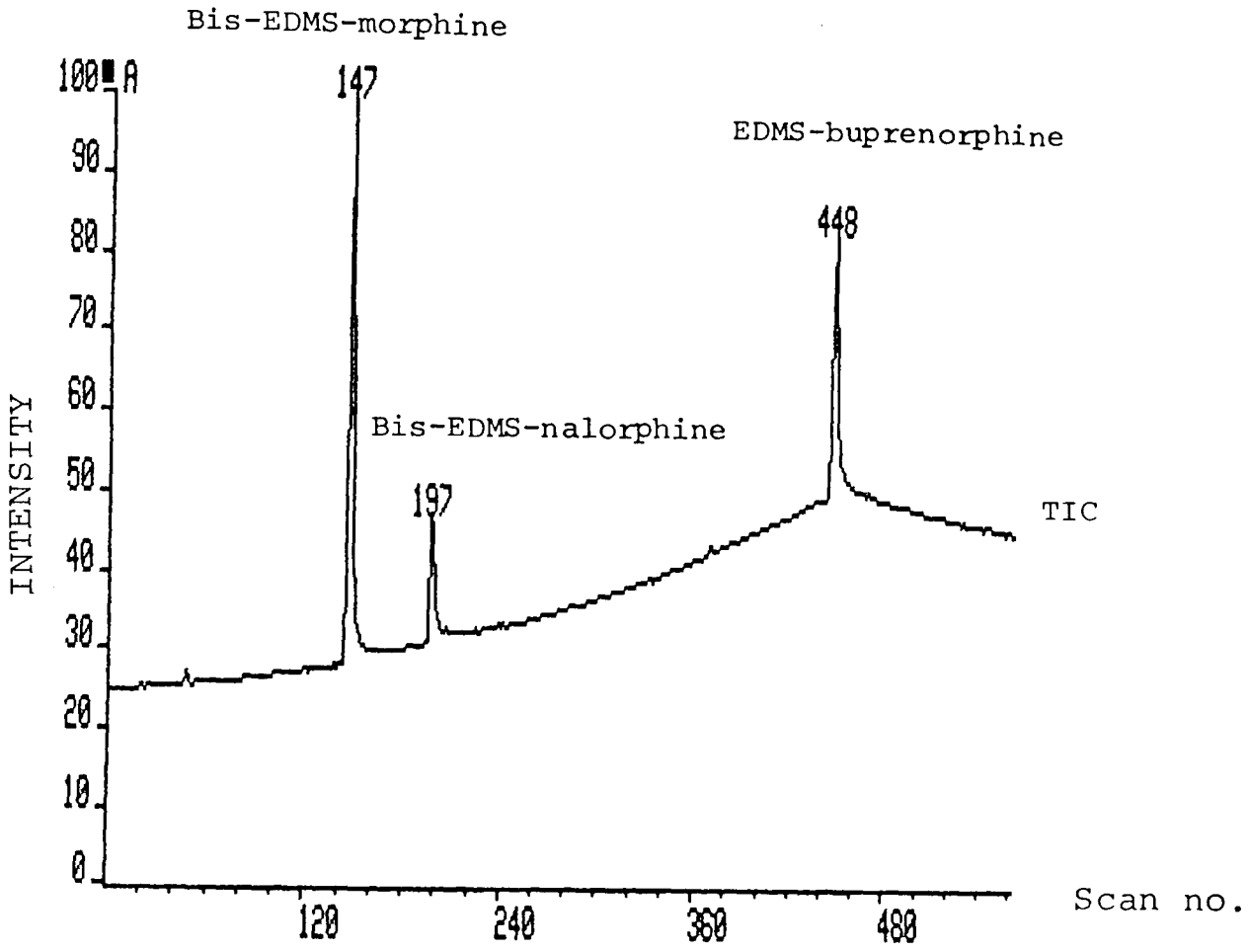


Figure 3.III.8 TIC trace of a standard of 100ng of morphine, 50ng of nalorphine and 100ng of buprenorphine as EDMS derivatives obtained under CI mass spectrometry. The oven temperature was programmed from 200° to 300°C at 8°C/min with a final isothermal period of 3 min and the injector oven temperature was at 300°C.

The scan range was 50-600, the scan rate was 1sec/decade and the interscan delay was 1 sec.

quasimolecular ion. Other ions (m/z 207, 223, 281, 297 and 355) were background ions in the mass spectrum. Variation of the parameters such as gas pressure affected the relative intensity of (M + H) and the ion representing the loss of silanol from the quasimolecular ion. During CI-MS, the background contained ions at almost every m/z value over the acquisition scan range, including m/z values of interest for SIR, although there were no chromatographic peaks eluting at the retention times of morphine or nalorphine. CI-SIR was conducted by monitoring the most abundant masses of the EDMS derivatives of morphine, nalorphine and buprenorphine, 354.1889, 380.2045 and 536.3559, respectively. Sensitivities down to the 20pg level were obtained easily without background interference (Figure 3.III.9) with the possibility of better detection after cleaning the ion source. There was no chromatographic interference with the morphine peak from blank blood extracts, making this extraction and derivatization procedure usable for morphine analysis under CI-SIR-MS conditions.

3.III.12 EXTRELUT EXTRACTION

The solvent extraction procedure which was the first stage of the developed method was later replaced by solid-phase extraction on a column containing 1g Extrelut. The blood, internal standard and added buffer required about 5 minutes to absorb completely on the Extrelut and avoid the risk of the aqueous phase eluting

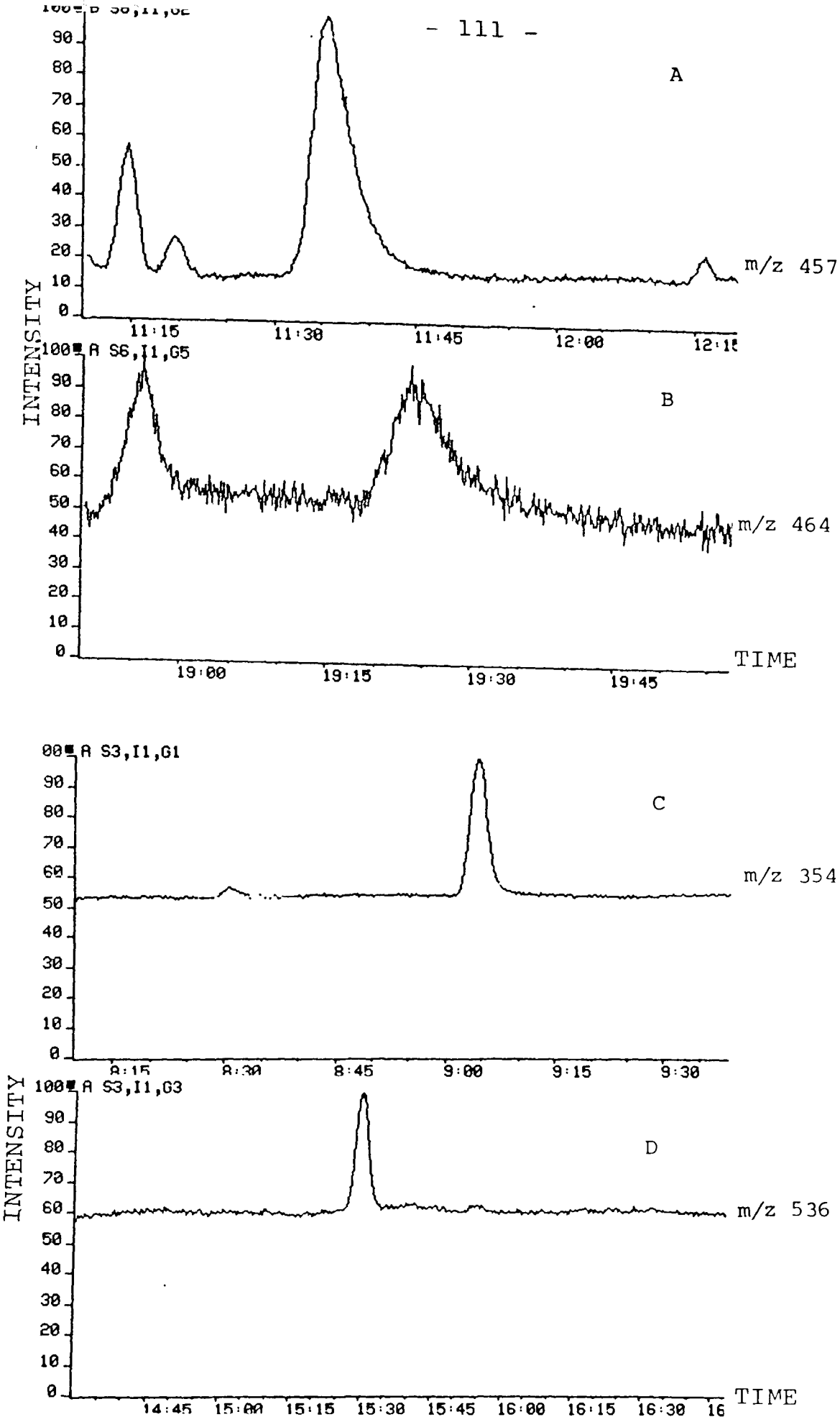


Figure 3.III.9 SIR traces for morphine (A and C at 200 and 20pg respectively) and buprenorphine (B and D at 1 and 20pg respectively) EDMS derivatives obtained by EI (A and B) as well as CI (C and D) SIR for the masses 457.2468, 464,2620, 354.1889 and 536.3559 respectively.

with the organic solvent. The final recoveries for morphine and nalorphine, which were measured by SIR, were 96% and 98%, respectively, while for buprenorphine the recovery was 94% with direct elution and 89% when a hexane wash was added. Blank blood extracts where a hexane wash was included did not show an interfering peak at the morphine retention time and in addition the base line and the cholesterol peak were significantly lower than the other blanks for which the hexane wash was omitted.

3.III.13 DERIVATIZATION WITH HMDS AND DETMDS

Solutions of HMDS or DETMDS were prepared in acetonitrile and an assessment was made of their ability to derivatize morphine, nalorphine and buprenorphine. A solution of 50% HMDS in acetonitrile gave complete derivatization of examined drugs after heating the sample for 15 min at 60^{o}C.

The products bis-TMS-morphine, bis-TMS-nalorphine and TMS-buprenorphine gave good quality chromatographic peaks. Derivatization using HMDS alone was complete when compared with the mixture HMDS:DMCS:pyridine. A decomposition product from the buprenorphine derivative, corresponding to about 5% of the main peak, represented the dehydrated form of TMS-buprenorphine and appeared at RRT of 0.84 relative to TMS-buprenorphine. The siloxane peaks were significantly reduced compared to those formed with other TMS-donor reagents, but a significant degree of interference would still occur on monitoring the molecular

ion at m/z 429 in SIR, at the low levels of morphine usually encountered, because this is also a prominent ion in the siloxanes. The corresponding reagent DETMDS was expected to produce derivatives in a similar reaction to HMDS but because of their increased molecular weights, they would be less prone to background interference.

DETMDS was not soluble in acetonitrile at room temperature and addition of 30% by volume of toluene to the acetonitrile regained the homogeneity of the mixture to ensure proper sampling for GC-MS.

The chromatogram obtained was free from interference, as with HMDS, except for the early peaks of grease material (artefacts from the synthesis). Morphine could be easily monitored at m/z 457 without interference. The glass injector liner was not contaminated as rapidly as with EDMS-I, and the occurrence of ghost peaks was negligible.

The reagent concentration in the solvent was tested over the range 5-50% by volume for the optimum derivatization of morphine, nalorphine and buprenorphine. The results are summarised in Table 3.III.13.
No significant differences were observed for morphine or nalorphine but the nature and quantity of buprenorphine products was affected. EDMS-buprenorphine peaked with reagent containing 40% by volume of DETMDS, which also produced the least amount of the dehydrated product.

Working reagent was subsequently prepared with 40% by volume of DETMDS in solvent. Routinely, 50µl were added to sample residues for derivatization.

Table 3.III.13: Effect of DETMDS concentration (% by volume) in ACN on morphine, nalorphine and buprenorphine derivatization

VOL % DETMDS	Peak areas *			
	Morphine m/z 457	Nalorphine m/z 483	Bup-H_2O ** m/z 506	Bup m/z 464
5	6100	27088	1448	208
20	63528	27840	1085	2590
30	59920	28466	1397	4510
40	64060	30400	1144	4824
50	63206	31118	1120	4415

* Reactions were carried out at 70°C for 15 minutes
** Bup-H_2O is the dehydrated product of EDMS-buprenorphine

3.III.14 RECOVERY AND REPRODUCIBILIY

The extraction efficiency and reproducibility (as S.D.) for morphine in blood were 91.6±4.9% and 94.8±4.2% at 35 and 560ng/ml, respectively. For buprenorphine these were 82.8±5.2 and 87.1±4.2 at 0.5 and 8ng/ml, respectively. For the internal standard (nalorphine) at 250ng/ml these were 97.2 ± 4.2. The results are shown in Table 3.III.14. The ratio of the two peaks monitored for morphine (m/z 457 and 442) was about 3.5, which could be used to confirm the presence of morphine. The lower mass peak was used for quantitation if the morphine peak was overloaded at m/z 457. Good reproducibility of multiple injections of a single sample was best obtained by using a 1µl Hamilton syringe and keeping the needle in the injector port for 7 seconds at 300°C. The reproducibility of the retention time was

Table 3.III.14 Extraction recoveries of morphine, buprenorphine and nalorphine from blood.

	Extraction Efficiency %				
	Morphine (ng/ml)		Buprenorphine (ng/ml)		Nalorphine (I.Std.)*
Sample Number	35	560	0.5	8	
1	94.2	99.1	80.2	84.5	95.4
2	98.3	88.8	89.8	93.8	102.6
3	85.1	95.3	81.4	84.4	97.3
4	89.5	97.4	76.1	84.4	91.2
5	91.9	94.2	86.2	88.9	98.8
Mean	91.8	94.9	82.7	87.2	97.1
S.D**	4.95	3.9	5.3	4.1	4.2
C.V.(%)#	5.4	4.1	6.4	4.7	4.3

* present at 250 ng/ml
** S.D. = standard deviation.
\# C.V. = coefficient of variation, percent.

excellent. The sensitivity of the method for morphine and buprenorphine EDMS derivatives tested were 200pg and 1pg on-column, respectively, at a multiplier setting of 5Kv. Under these conditions the signal to noise ratio was more than 3:1 for buprenorphine (Figure 3.III.9). Lower detection limits could be obtained for morphine. In both cases the limiting factor was the chromatography, where the peaks started to broaden due to adsorption on the column of both drugs at low concentration.

3.III.15 CALIBRATION CURVES

The peak ratio of drug standard (morphine or buprenorphine) to the internal standard was plotted against its concentration. Curves for morphine and

buprenorphine concentrations ranging from 35 to 1140ng/ml and 0.5 to 16.5ng/ml, respectively, were linear (Figure 3.III.10). The regression equations (Y = A + BX) and the correlation coefficients (r) were as follows:-

	A	B	r
Morphine	0.11	0.0061	0.99
Buprenorphine	0.01	0.045	0.98

where Y is the peak area ratio, and,

X is the concentration of drug in the blood.

3.III.16 EXTRACTION OF OTHER BASIC DRUGS

Recoveries obtained by a single determination for basic drugs extracted from blood by the modified procedure are listed in Table 3.III.15. A GC-MS trace showing their separation is presented in Figure 3.III.11 and their mass spectra are shown in Figure 3.III.12. Fragments above 3% of the base peak in spectra of the above compounds and examples of other compounds examined in this study are listed in Appendix 1. Recoveries were more than 80% for all drugs tested. None of these drugs interfered with morphine.

3.III.17 DRUG DERIVATIZATION WITH DETMDS

The spectra of additional derivatized drug standards, where DETMDS in acetonitrile was added to each drug at 60^{o}C for 15 minutes and 1ul was analysed by GC-MS, are shown in Figure 3.III.13. Ion abundances and possible sites of derivatization are listed in Table 3.III.16. Derivatization was complete for the drugs

tested except desmethyldiazepam, lorazepam and testosterone where the free drugs could be detected (up to 41% for testosterone).

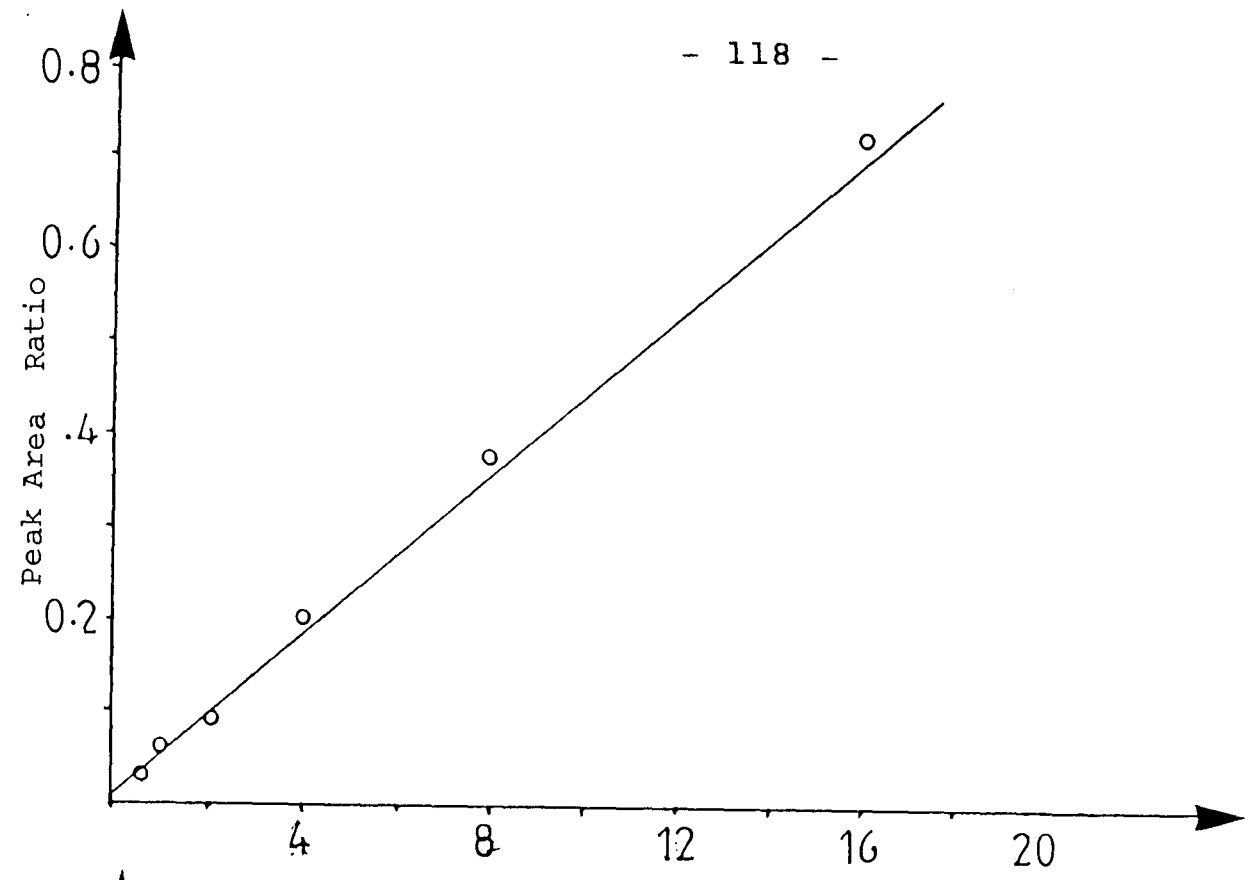
3.III.18 COMPARISON OF EXTRACTION METHODS

GC-MS chromatograms obtained for extracts by solvent extraction or by the modified procedure were comparable in respect of the clarity of base line and the presence of other extractable material. However, the recovery of morphine by solvent extraction was only 65%. The Extrelut eluate residue (before SCX clean-up) produced a GC-MS chromatogram with a high base line and large peaks of cholesterol and other unrelated materials. The recovery was more than 90%, similar to the fully modified procedure.

3.III.19 ROUTINE APPLICATION

The results of the analysis of autopsy samples using the modified procedure are listed in Table 3.III.17, including measurements of other drugs and alcohol.

Full scan GCMS was performed to verify the presence of other drugs for cases numbered 3, 5, 9 and 13. The findings are included in Table 3.III.17.



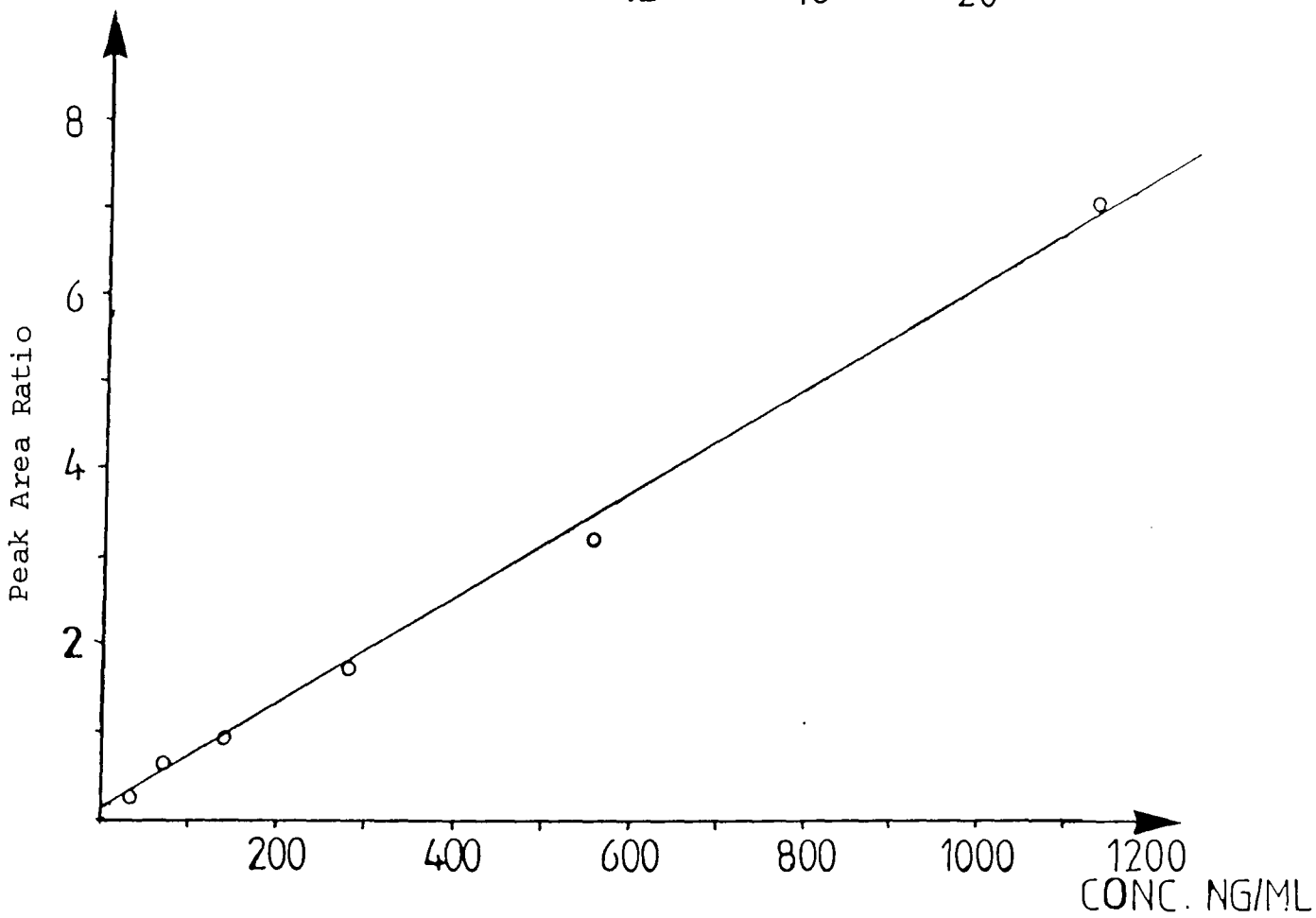


Figure 3.III.10 Standard calibration curves for buprenorphine (above) and morphine (below). The blood sample concentration is plotted against the peak area ratio (Std/I.Std).

Table 3.III.15 Extraction efficiencies, retention indices (RI) and significant ions in the mass spectra of selected drugs analysed as ethyldimethylsilyl ethers.

	Conc. µg/ml	M.wt	pK_a*	RI**	Ext.Eff. %	Derivative	Significant Ions(% base peak)
Caffeine	1.90	194	14	1803	94	None	194(100),109(51),55(28),67(23),89(21)
Dextromethorphan	1.02	271	8.3	2180	89	None	271(100),59(87),150(71),270(70), 214(44),171(35),212(26),213(26)
Methadone	0.76	309	8.3	2187	93	None	72(100),75(23),117(23),87(16),59(10), 129(9),131(8)
Amitriptyline	0.50	277	9.4	2242	88	None	58(100),202(3.2),203(2.6)
Pentazocine	0.92	285	8.5	2434	87	O-EDMS	303(100),70(81),69(66),110(49),258(48), 259(47),260(40),200(30)
Dipipanone	0.84	349	8.5	2528	93	None	112(100),113(12),110(6),59(6),297(6)
Codeine	0.70	299	8.2	2616	95	O-EDMS	385(100),178(72),210(54),57(46), 59(44),243(17),229(37),71(29)
Morphine	0.40	285	8.0, 8.9	2772	95	bis(O-EDMS)	457(100),250(96),210(72),59(92), 87(74),146(62),229(36),228(34)
Nalorphine	0.50	311	7.8	2878	96	bis(O-EDMS)	483(100),87(85),442(75),484(51), 454(50),338(48),236(45),274(42)
Dextromoramide	0.79	392	N.A.	2927	85	None	100(100),265(50),128(44),56(15), 208(14),98(12),266(14),55(10)

* Values of pK_a are from Ref[12]. M.Wt is molecular weight. RI is retention index.
** Retention Index measured under conditions given in Section 3.II.25.

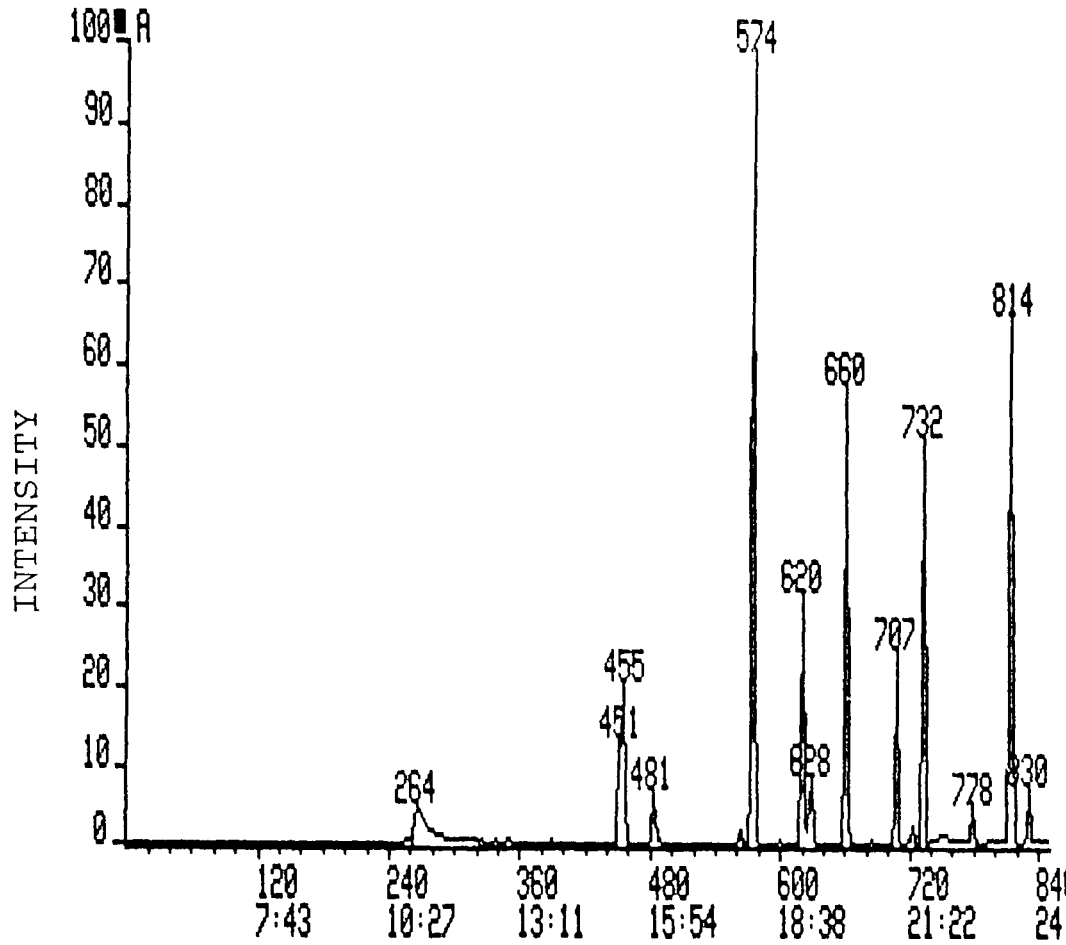
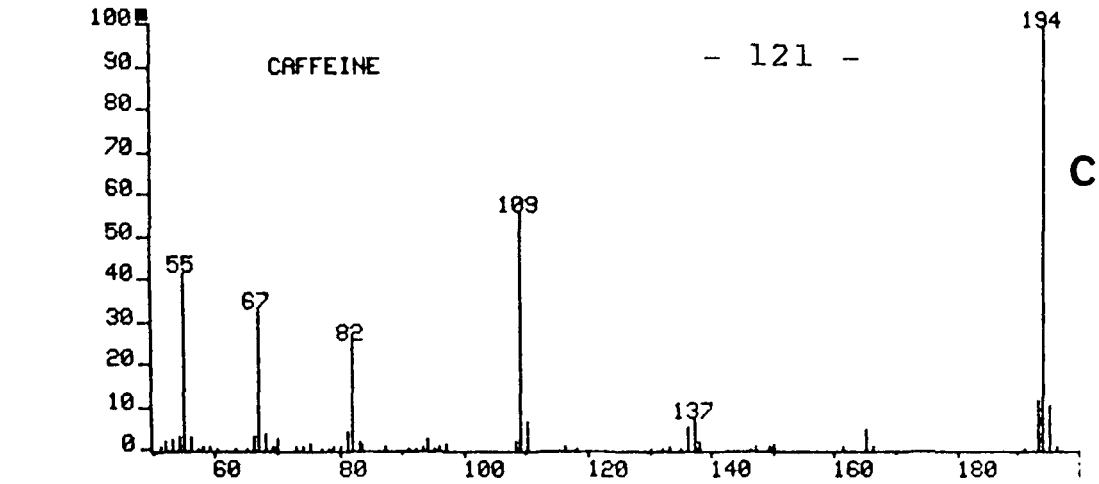
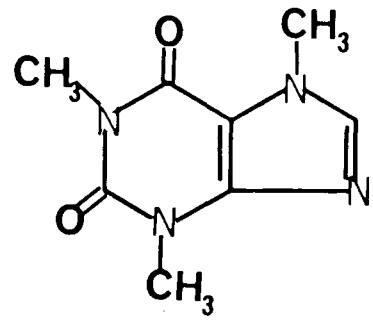


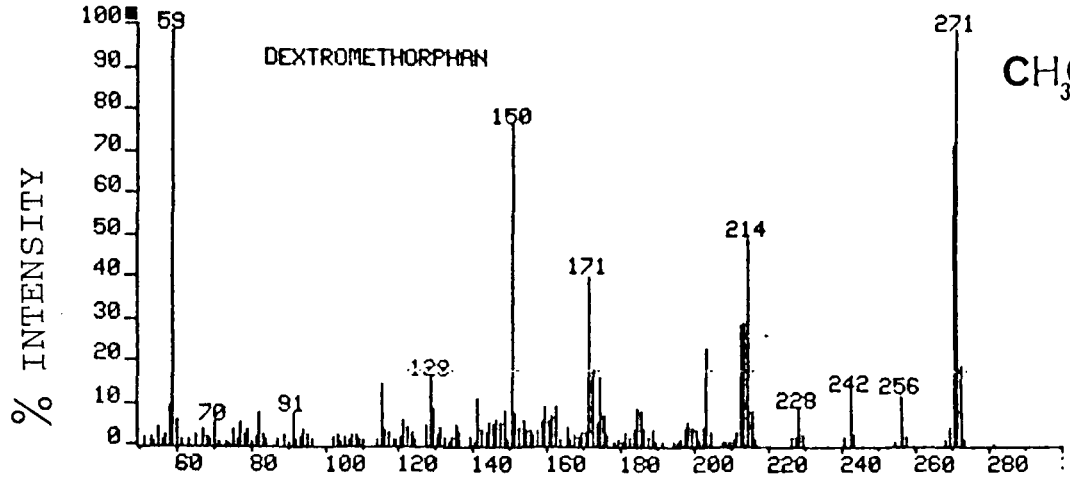
Figure 3.III.11 TIC trace of a spiked blood extract of the drugs presented in Table 3.III.15. The drugs appeared at the following scan numbers: caffeine at 264, dextromethorphan at 451, methadone at 455, amitriptyline at 481, pentazocine at 574, dipipanone at 620, EDMS-codeine at 660, morphine at 707, EDMS-nalorphine at 732 and dextromoramide at 814. The oven temperature was programmed from 120^{o} to 300^{o}C at 8^{o}C/min with a final isothermal period of 3 min and the injector oven temperature was at 300^{o}C.

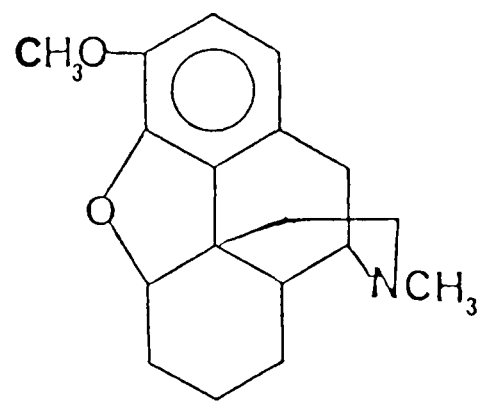
The scan range was 50-600 with a scan rate of 1sec/decade and interscan delay of 1sec.



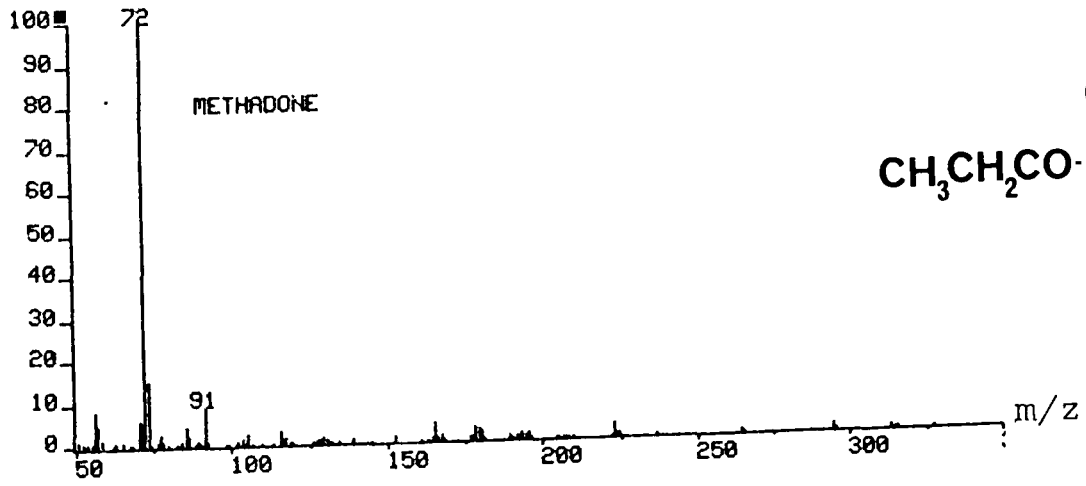


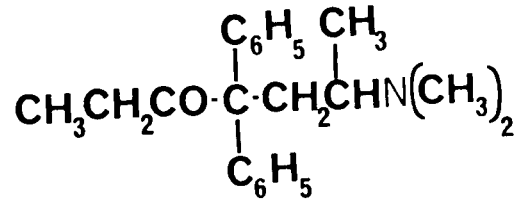
m^{+} 194





m^{+} 271





m^{+} 309

Figure 3.III.12 EI mass spectra of the drugs presented in Table 3.III.15 as EDMS derivatives.

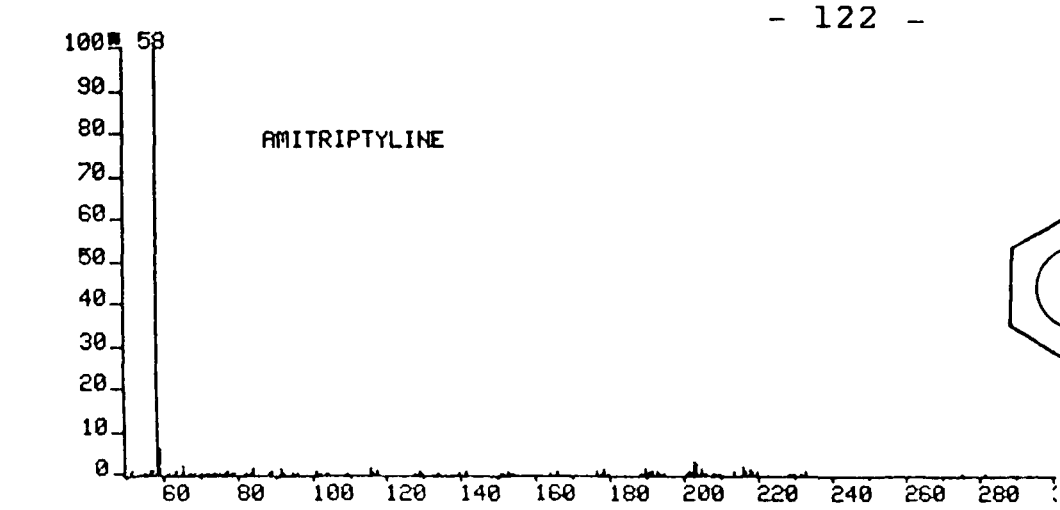
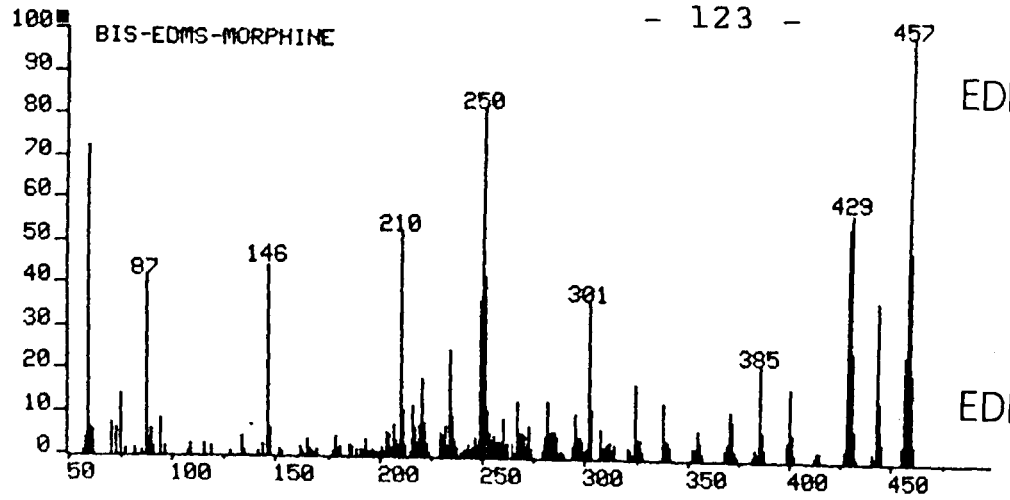
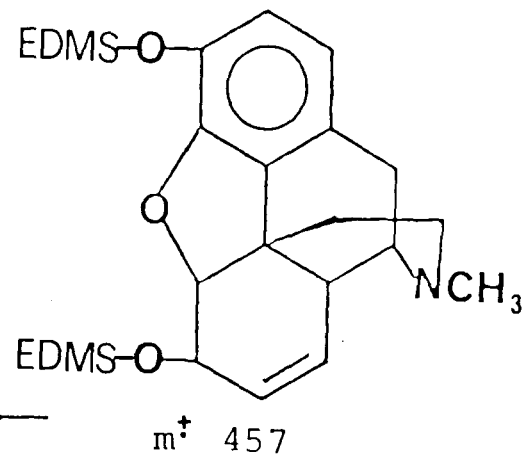
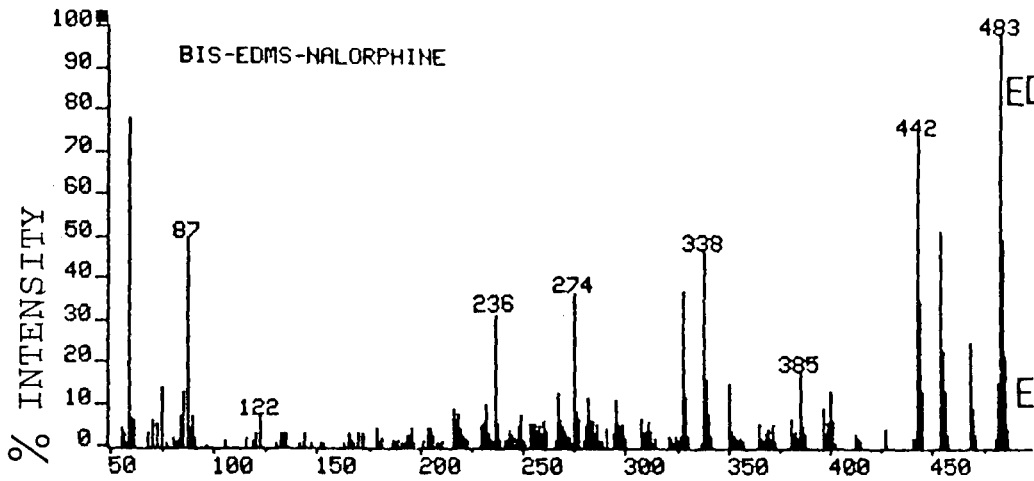


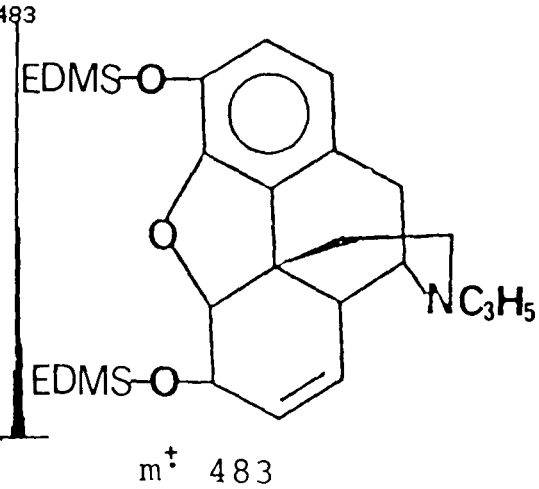
Figure 3.III.12 Continued.



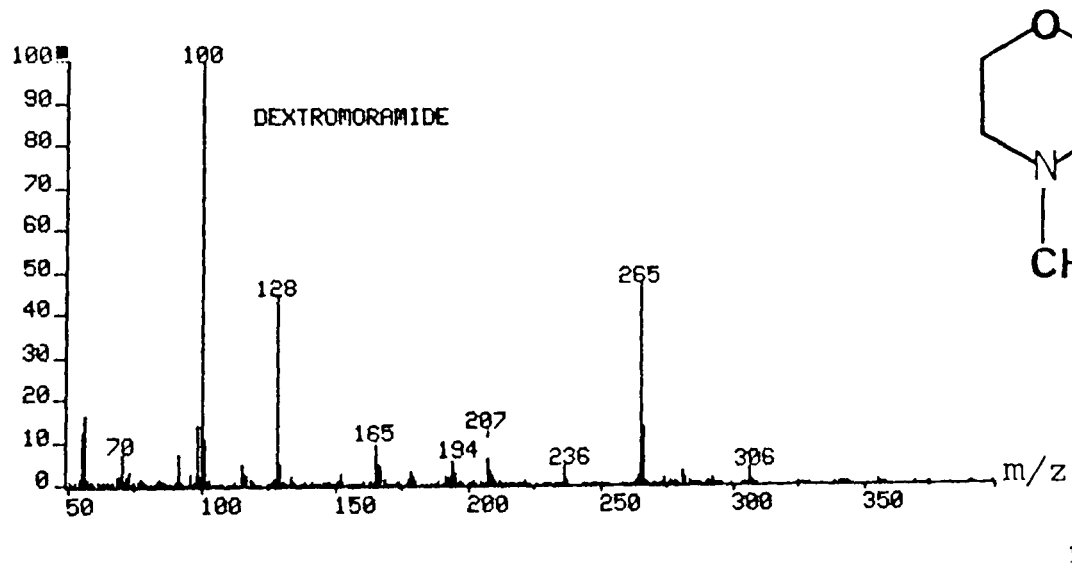


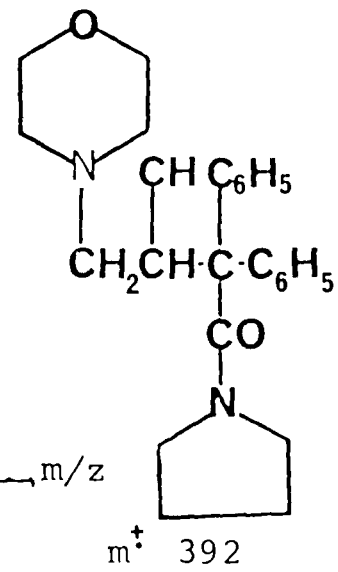
m+ 457





m+ 483





m+ 392

Figure 3.III.12 Continued.

Table 3.III.16 GC-MS data for selected drug EDMS derivatives.

	M.Wt (free drug)	Derivative	derivative m^+ m/z	%	RI*	Significant Ions (% base peak)
Desmethyldiazepam	270	N-EDMS	355	86	2467	59(100),355(86),356(55),327(24), 357(42),87(29),91(22),341(12)
Etorphine	411	O-EDMS O-EDMS	497	21		72(100),410(81),207(80),354(33), 162(34),161(34),354(33),250(37)
Lorazepam	320	O-EDMS	492	5	2760	59(100),347(91),459(52),458(44),87(44) 133(27),460(21),75(13)
Monoacetylmorphine	327	O-EDMS	413	100	2709	413(100),354(80),59(63),301(58), 204(33),414(37),355(27),75(23)
Temazepam	300	O-EDMS	386	18	2718	357(100),283(60),256(58),255(40), 59(58),359(48),257(52)258(35)
Cannabidiol	314	O-EDMS O-EDMS	486	12	2473	418(100),419(44),59(31),365(29),87(28), 420(21),315(17),352(16)
Testesterone	288	O-EDMS	374	44	2830	143(100),345(79),59(72),75(70), 374(44),270(39),91(31),115(32)
Oxazepam	285	O-EDMS N-EDMS	457	100	2596	457(100),358(76),359(64),429(50), 460(41),87(5),133(33),355(30)
Lormetazepam	384	O-EDMS	420	4	2788	391(100),393(76),317(44),392(30), 291(25),394(21),59(21),289(20)
Flupenthixol	434	O-EDMS	520	5	2960	229(100),98(40),230(30),97(27),125(15), 221(13),59(18),70(17).

* Recorded using conditions described in Section 3.II.25.

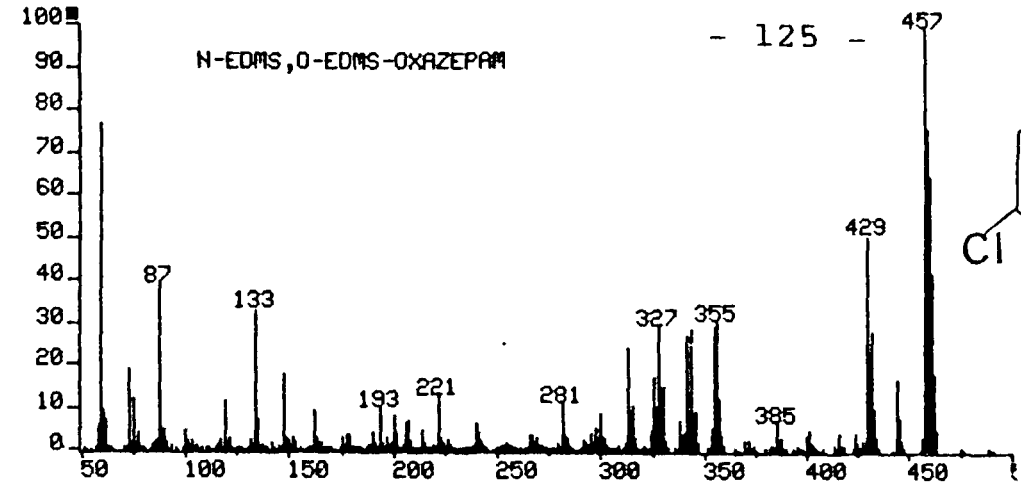
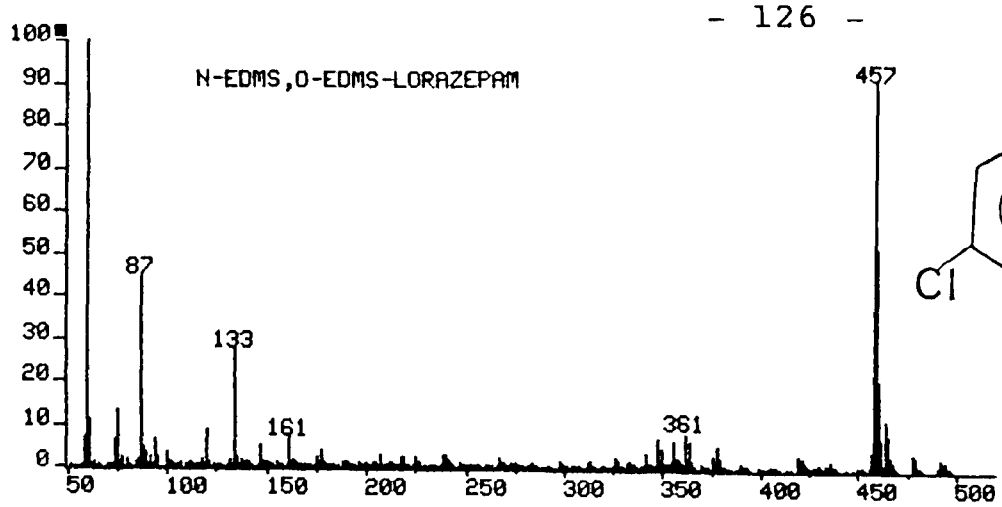
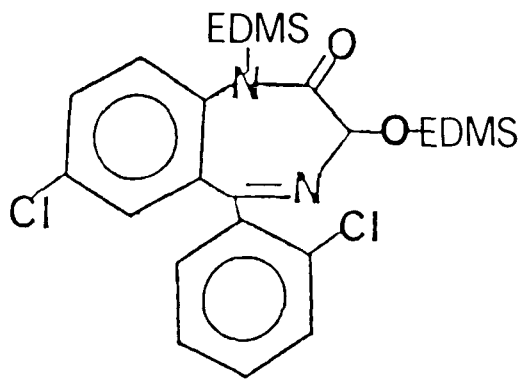
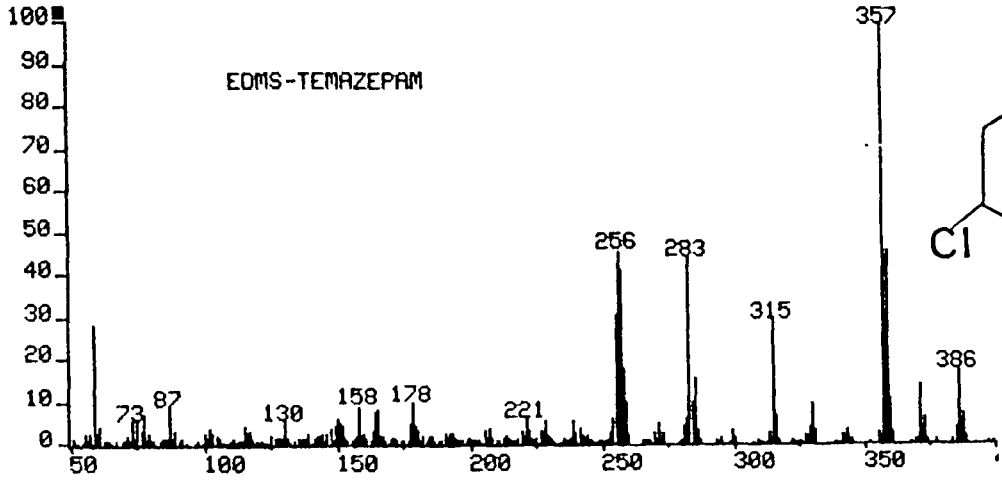


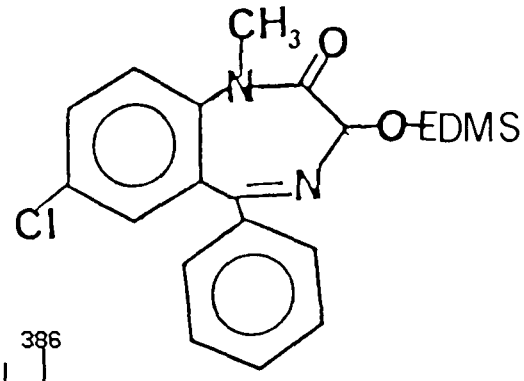
Figure 3.III.13 EI mass spectra of the drugs presented in Table 3.III.16 as EDMS derivatives.





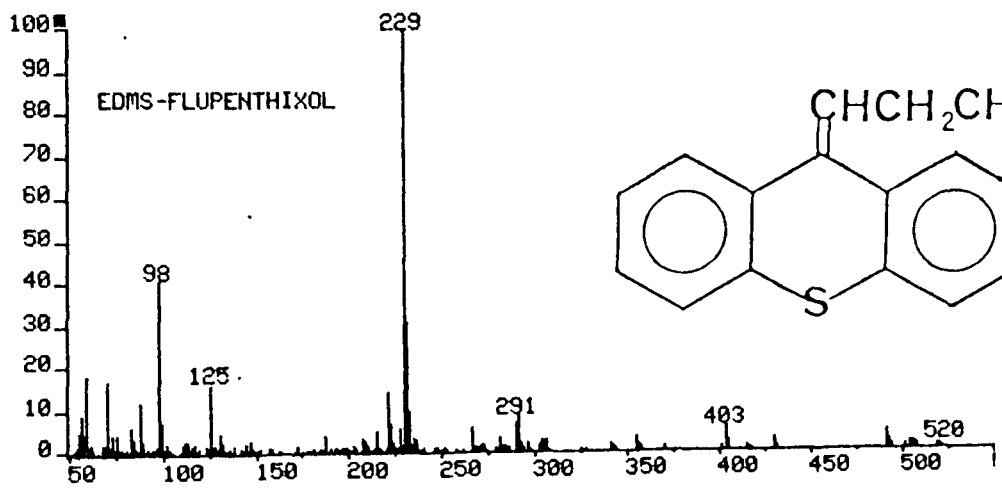
m^+ 492

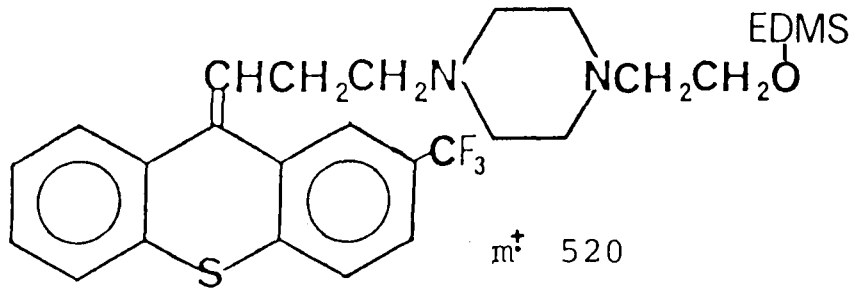




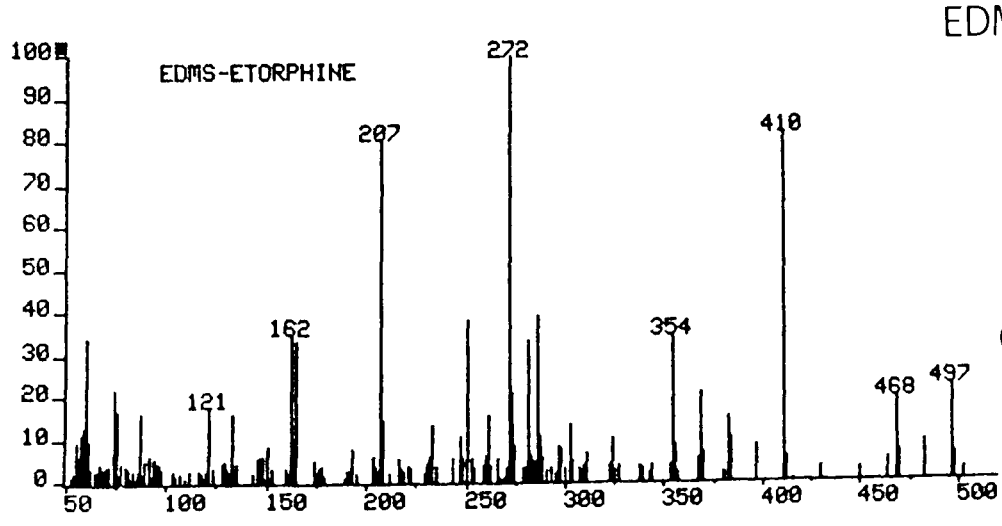
m^+ 386

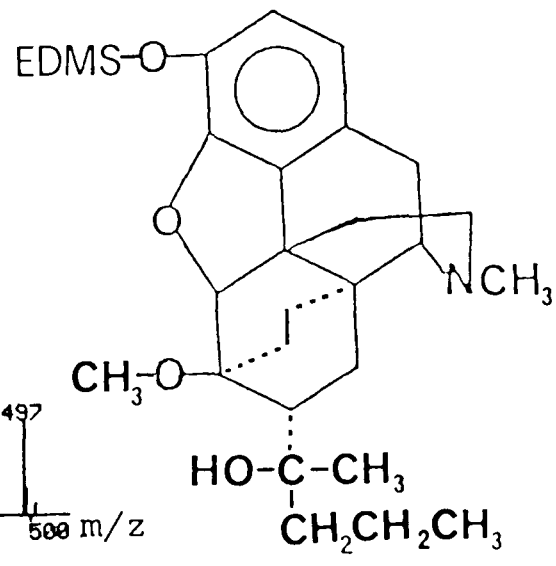
% INTENSITY





m^+ 520





m^+ 497

Figure 3.III.13 Continued.

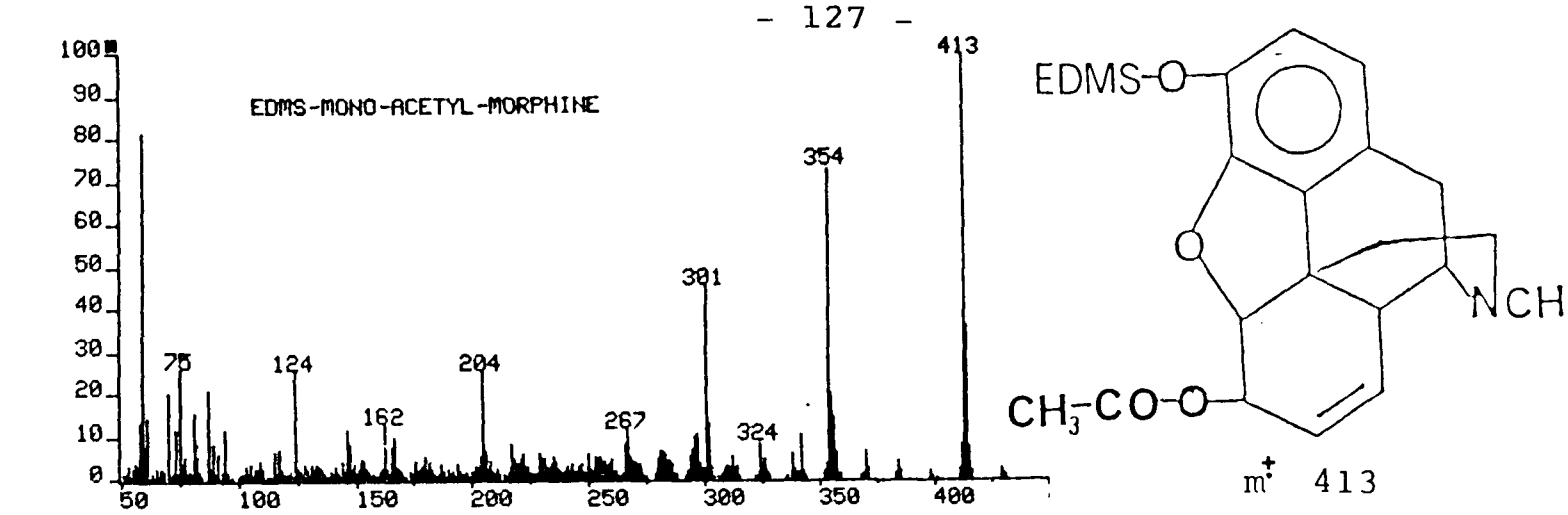


Figure 3.III.13 Continued.

Table 3.III.17: Results of routine application of the modified procedure: summary of post-mortem findings and causes of death where applicable.

REF NO.	AGE	Blood: Opiates µg/ml	Blood: Alcohol mg/100ml	Blood: Other Drugs µg/ml	CAUSE OF DEATH	POST-MORTEM FINDINGS	REMARKS
1	27	Morphine 0.72	151	N.D	Pulmonary oedema due to morphine intoxication.	Oedematous lung, with petechial haemorrhages on the surface.	Heroin addict. Consumed 250mg heroin per day. Found lying in toilet, syringe & needle beside him.
2	25	Morphine 0.19	N.D	temazepam 5	Temazepam and heroin overdose	Lung oedema. Scattered areas of haemorrhage. Occasional refractile material. Moderate fatty liver changes with lymphocytic infiltrate of the portal tract.	Had defined needle tract with marked inflammatory processes, several hours old.
3	21	Morphine 0.53	216	N.D	Heroin intoxication	Little regurgitated material in the small bronchi. Fresh needle marks and punctures.	Syringe sticking out of arm was witnessed. Syringe positive for phenobarbitone, amitriptyline, methaqualone, codeine, morphine, diazepam and caffeine.
4	19	Morphine 2.0	N.D	N.D	Morphine intoxication, bronchopneumonia, drug-induced fibrosing alveolitis were contributing factors	Food fragments in the air passages,pneumonic consolidation. Marked oedema and enlarged lymph nodes below the carina.	Hodgkin's disease, nodular sclerosing type. Prescribed morphine sulphate 100mg twice daily. At time of death there were 6 tablets missing.
5	23	Morphine 0.33	6	Temazepam 0.78, phenobarbitone 0.66	Inhalation of regurgitated vomitus due to morphine intoxication	Portion of food was present in air passages. Enlarged lymph nodes in hilar region and porta hepatis and enlarged spleen.	Opiate abuser. Syringe was witnessed sticking in groin region. Syringe positive for morphine, diamorphine, mono-acetylmorphine, temazepam, phenobarbitone,codeine, and acetylcodeine.

Table 3.III.17: Continued

REF NO.	AGE	Blood: Opiates µg/ml	Blood: Alcohol mg/100ml	Blood: Other Drugs µg/ml	CAUSE OF DEATH	POST-MORTEM FINDINGS	REMARKS
6.	24	bupren-orphine 0.65	N.D	N.D	Drug overdose	Mild congestion in the lungs	Heroin addict. Temgesic(buprenor-phine)& 5 syringes found beside him. Syringe wash positive for buprenorphine.
7.	20	Morph-ine 0.73	34	N.D	Hanging	Typical ligature marks around his neck with no internal damage.	Drug abuser. Found hanged and suspended from tree.
8.	NA	Bupren-orphine negative	N.D	Diazepam 0.49		NA	Data not available (NA).
9.	NA	Morphine 1.7 Bupren-orphine 5.1 ng/ml	N.D	Temazepam 4.8 desmethyl-diazepam 0.74 diazepam 0.18 monoacetyl-morphine		NA	Sample of 'urine' positive for cannabis & amphetamine by RIA
10.	NA	Negative	N.D		Drowning	NA	NA
11.	NA	Bupren-orphine 1.4	N.D		NA	NA	NA
12.	NA	Morphine 0.078	N.D	Methadone Diazepam			Two syringes were negative for morphine.
13.	NA	Morphine Positive	N.D	Codeine	Clinical case.		Morphine peak was 3 times larger than codeine peak. Person admitted morphine intake.

N.D = not detected

3.IV DISCUSSION

3.IV.1 INTRODUCTION

In forensic toxicology whole blood samples in various states of decomposition are the most frequently supplied specimens for drug screening. Ever increasing demands for the analysis of such samples necessitate the handling of multiple samples simultaneously rather than on an individual case basis. The appearance of commercially available SPE sorbents has produced literature reports of efficient and reproducible methods for the recovery of morphine from biological fluids other than blood. Also, ten samples or more can be prepared at the same time, providing a partial solution for the analysis of these samples. The extracts are often sufficiently clean for analysis by HPLC but further purification is required for other methods of analysis such as GC or GC-MS.

SPE with cationic exchange sorbents, for example, benzenesulphonylpropyl bonded silica, has not previously been applied to the extraction of drugs from autopsy samples. In the present study, a commercially available phase of this type (Bond-Elut SCX from Analytichem) was assessed for the extraction of basic drugs from blood, taking morphine and buprenorphine as prototypes. The basic properties of most drugs in common use allow them to be isolated from endogenous matrices by ion exchange chromatography [112].

3.IV.2 SPE EVALUATION

Before applying biological material to the Bond-Elut cartridges, the method parameters affecting sample application, retention, sorbent washing and elution were optimized using an aqueous morphine standard with a concentration range similar to that encountered in biological samples within the clinical or forensic contexts. Tritium-labelled morphine was added to the morphine standard in small amounts as a tracer and the behaviour of the two substances in the experimental procedure was assumed to be identical [140]. The radioactivity could be easily detected by liquid scintillation counting, which allowed very small amounts of radioactivity to be measured with accuracy. The detection of the tracer provided information about the behaviour of unlabelled morphine in a fast and sensitive manner for preliminary assessment of the method under development.

Solvation of the sorbent with methanol and water was sufficient to condition the phase and ionise the SCX functional group, which has a cationic exchange activity once it is charged. Both the sorbent and the isolate should be charged for a strong interaction to occur and to provide adequate retention of the solute [116]. The aqueous methanolic morphine standard was completely retained on the sorbent, evidenced by no loss of radioactivity on washing with methanol.

Adsorbed species are conventionally eluted from a

cation exchange sorbent by five methods [116]. Following the normal practice for SPE methods, elution is carried out with the minimal amount of solvent, to maintain as high a concentration of the analyte as possible in the eluent.

(a) A buffer with a pH which is 2 units lower than the pK_a of the sorbent neurtralizes the charge of the sorbent. However, sulphonic acid is a strong acid (pK_a 0.69) and this approach is unavailable in the present instance as the sorbent is destroyed by eluents with a pH below about pH 2.

(b) A buffer with a pH which is 2 units higher than the pK_a of the cationic solute (morphine pK_a values are 8 and 9.9 for amino and phenol groups, respectively) will neutralize the charge on the isolate.

(c) A high ionic strength buffer (>0.1M) can be used. The high concentration of cations in the buffer will compete with the cationic analyte for sites on the sorbent, promoting elution of the isolate.

(d) An eluent containing competitive cations or bases, for which the sorbent has an extremely high affinity, will displace the analyte.

(e) Combinations of the above four techniques can be used.

In the present study, an alternative procedure was first tried using the derivatizing reagent mixture of HMDS:DMCS:pyridine (2:1:3 v/v/v), after the sorbent had been dehydrated by passing acetone. HMDS as a TMS donor

should react with the active sites of the sorbent to form the TMS ester and further derivatize the morphine for subsequent gas phase analysis. This type of approach has been used in the analysis of organophosphorus pesticide metabolites following their adsorption on an anion exchanger, by on-column reaction with a diazoalkyl reagent [141]. However, only 76% of the radiolabel was recovered. The application of a warm solution to facilitate the reaction did not improve the recovery in the eluate while increasing the volume to 1ml increased the recovery to 92%. The recovery was improved after the addition of acetonitrile to the washing and elution solution to disrupt the non-polar interaction between the sorbent and morphine. It must be recalled that the SCX sorbent has a high potentiality for non-polar interaction due to the presence of the propylbenzene substituents on the surface [116] and, especially after reaction with the silylating reagent, the morphine molecule has a large non-polar skeleton. Using larger volumes of the derivatizing mixture is not practicable as the eluate would have to be concentrated and such solvents are not easily volatilized.

Triethylamine (TEA), diethylamine (DEA) and ammonia fall into category (d) above: they are volatile, give basic solutions in water or methanol and can break the interaction between the sorbent and morphine. One millilitre of 5-10% v/v solution of any of these bases in MeOH/ACN recovered more than 99% of the radioactivity and could be evaporated quickly.

3.IV.3. DIFFICULTIES IN RADIOACTIVITY COUNTING

The efficiency of liquid scintillation counting is usually dependent on many factors such as the presence and concentration of quenching substances, the nature of the sample and the variable conditions under which the analysis is conducted, particularly where several media containing different solvents were used, giving extracts which also varied in colour in each extraction procedure. These factors were difficult to control or compensate for, albeit efforts were made to include a blank sample in parallel with every analysis. The counting efficiency was estimated by comparing the counts per minute of the quenched blank sample with the count obtained before the blank was added. The actual count is corrected with the quench correction factor obtained under the same conditions. This method works as an internal standard method and is valid for colour quenching as well as for chemical and dilution quenching [140]. However, the figures obtained by radioactivity counting were considered as an approximate result for each experiment conducted. The final results of the extraction procedure were subsequently obtained by GC-MS analysis.

3.IV.4 APPLICATION OF BLOOD TO BOND-ELUT COLUMNS

Solid phase extraction cartridges contain silica particles of average diameter 40um, held in place by a porous fit. Particulate samples, e.g. homogenates, cannot readily be applied and viscous samples such as whole blood

must usually be pretreated before application to the cartridge. In autopsy samples, the problem is further complicated by the nature of the blood, which may be partially clotted and/or haemolysed. In most cases even Hb-stained plasma cannot be prepared. In this study, three different approaches were tried - haemolysis plus deproteination, solvent extraction and Extrelut extraction. Haemolysis and protein precipitation was disappointing, both in terms of morphine recovery and the suitability of the extract for application to the cartridge; the first was due to binding of the drug to the protein pellet, from which no successful procedure was found to dislodge it, and the second was due to the presence of protein microparticulates which failed to settle even with centrifugation. Ultracentrifugation was considered too complex for a routine procedure. However, direct application of blood on bonded silica after 20-30 minutes of ultrasonication has been described for morphine [142] and anti-inflammatory drugs [143]. The eluates were analysed by HPLC. The authors did not mention similar problems.

Extrelut extraction provided an easy solution for direct blood application. Blood buffered with low molarity buffer (0.1M/L ammonia in water) was applied to a column packed with Extrelut, after which the eluate was passed directly through the Bond-Elut SCX cartidge for purification. The diatomaceous material has the capability of absorbing the aqueous media and will act as

a filter for the blood material. The Extrelut was eluted with ethyl acetate:isopropanol 9:1 (v/v) which has an excellent extraction efficiency for morphine in biological matrices [91,92]. This combination produced a polar eluting solvent which also eluted undesirable endogenous material from the blood. These extracts, when analysed on GC-MS, produced huge peaks of cholesterol, high base lines and interferences with the morphine peak due to the endogenous material (Figure 3.IV.1A). The addition of a non-polar solvent (hexane) wash prior to the elution step with the eluting solvent, removed some of the non-polar lipids while polar drugs such as morphine and buprenorphine, which have poor solubility in hexane were not affected significantly. The chromatogram of the fully modified procedure extract (Figure 3.IV.1B) had a low base line, lower peaks of cholesterol and no peaks interfering with morphine.

In comparison with solvent extraction, the modified method has a high extraction efficiency, saves both time and solvents and avoids multiple extraction and centrifugation procedures. The purity of the extracts and the chromatograms were similar.

3.IV.5. DERIVATIZATION OF MORPHINE

The polarity of morphine due to the presence of two hydroxyl groups leads to losses during GC by adsorption to the active surfaces on the system. The GC column is normally coated with non-polar to moderately-polar phases

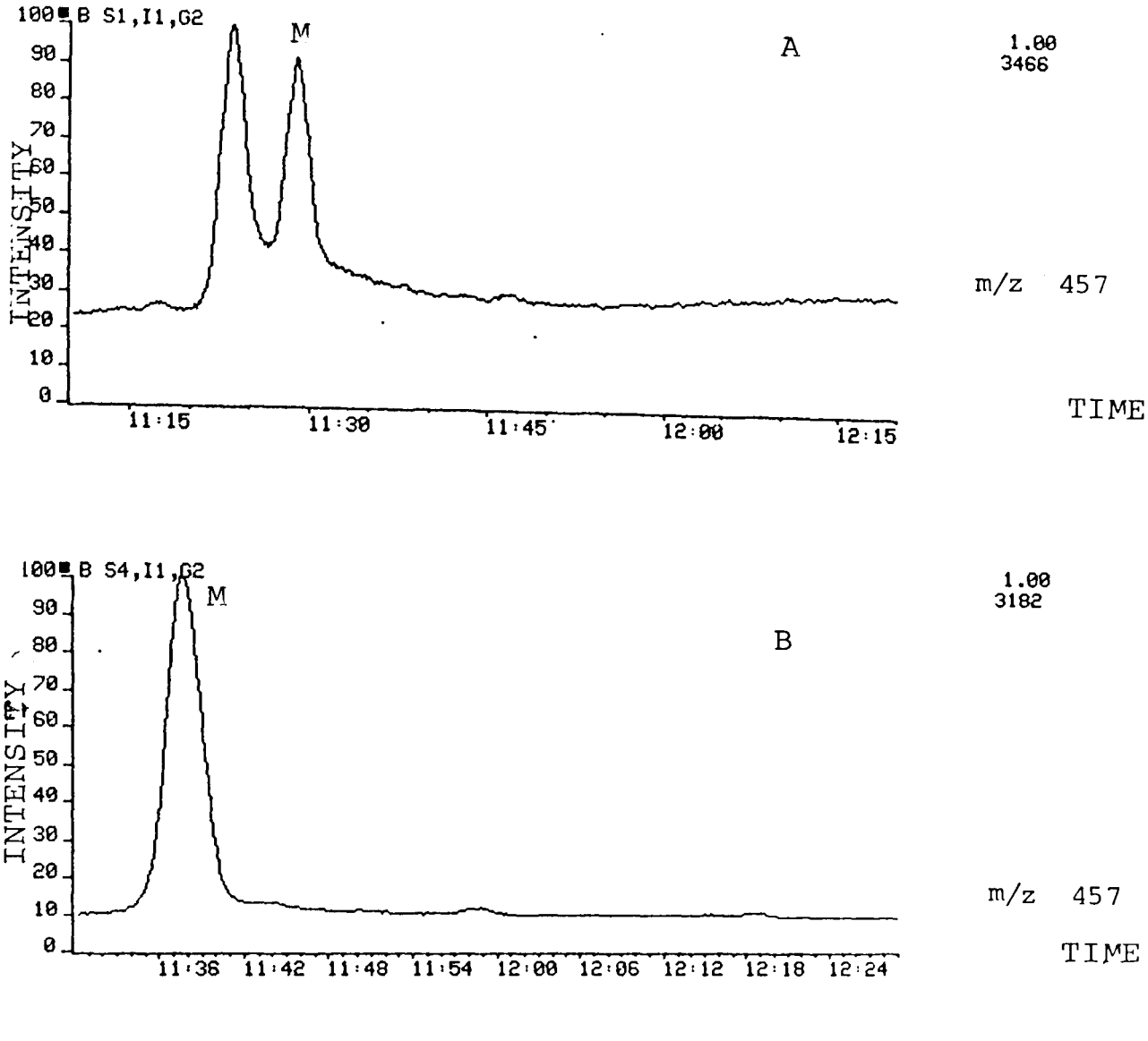


Figure 3.IV.1 GC-MS traces for spiked blood extracts of morphine (M) monitored by SIR to m/z 457.2468. The samples were prepared according to the developed method without (A) and with (B) hexane washing step. The retention time of morphine (as the EDMS derivative) was 11 min 29 sec and 11 min 38 sec respectively in the two GC runs.

such as dimethyl silicone or methylphenyl silicone, for example SE-30 or OV-17, which are widely employed for general drug screening. Morphine has to be derivatized at these hydroxyl groups to obtain a good quality chromatographic peak, in terms of peak shape and absence of tailing. From the literature review, silylation using the common TMS donor reagents reproducibly gives one derivatization product which in turn results in better sensitivity and reliability for quantification. Acetylation of morphine produced similar results, but acylation with higher molecular weight groups such as pentafluoropropionyl or heptafluorobutyryl each produced two products [101] which were unstable and had to be purified from the excess reagent.

During the present work, trimethylsilylation of morphine produced results similar to those described in the literature when the sample was injected on the gas chromatograph in the reagent. The reaction was essentially complete on dissolution for morphine whereas sample heating was required for buprenorphine. Silyl ethers are sensitive to moisture [144]. Removal of the excess reagent under a stream of dry nitrogen resulted in loss of the derivative, presumably by hydrolysis. This could be explained by exposure to atmospheric water and by the instability of the phenolic silyl ether when removed from the reagent [144].

The molecular ion of bis-TMS-morphine (m/z 429) was the most intense peak in the mass spectrum (excluding the

trimethylsilyl ion at m/z 73). However, monitoring of this mass in SIR suffered from interference from siloxane peaks and column bleed from the stationary phase, both of which increase when the sample is injected on-column in the reaction mixture. The net result of this interference was to produce confusing results and unreliable detection when morphine was present in samples at low levels (less than 1ng on-column).

On the other hand, the MTBS products of morphine were stable on exposure to moisture, and the derivatized morphine could be purified from the excess reagent. Bis-MTBS-morphine had a good chromatographic peak with the most abundant mass m/z 456 obtained by loss of a tertiary butyl group (57 a.m.u) from the molecular ion (m/z 513), Table 3.IV.1, which could be monitored without significant interference from the background ions which were mostly odd number masses. For this reason efforts were made to obtain complete derivatization of morphine as the bis-MTBS-morphine product, to increase the sensitivity and reproducibility of the assay, which are not expected to be acceptable when more than one product is formed.

The correct choice of solvent can influence the rate of a reaction, where the chief requirement of any solvent is that it should dissolve both reagent and sample without reacting with either [144]. The three solvents tested for the reaction were polar and should have promoted a faster reaction. However, none of them succeeded as a solvent to obtain complete reaction. The

Table 3.IV.1. Fragmentation pattern of morphine and buprenorphine silyl ethers.

Substance	Parent Ion	Mass Loss	Composition of fragment	Product ion m/z
1. Bis-EDMS-morphine (Electron Impact)	457 ($M^{+\cdot}$)	15	$\dot{C}H_3$	442
	457 ($M^{+\cdot}$)	29	$\dot{C}_2H_5$	428
	457 ($M^{+\cdot}$)	57	$\dot{C}_2H_5N$	414
	400 ($M^{+\cdot}$ - 51)	15	$\dot{C}H_3$	385
	457 ($M^{+\cdot}$)	87	$\dot{C}_4H_{11}Si$	370
	370 ($M^{+\cdot}$ - 87)	15	$\dot{C}H_3$	355
	457 ($M^{+\cdot}$)	104	$\dot{C}_4H_{12}OSi$	353
	353 ($M^{+\cdot}$ - 104)	29	$\dot{C}_2H_5$	324
	457 ($M^{+\cdot}$)	156	$\dot{C}_8H_{16}OSi$	301
	457 ($M^{+\cdot}$)	207	$\dot{C}_8H_{23}O_2Si_2$	250
2. Bis-EDMSi-morphine (Chemical Ionization)	458 ($M^{+\cdot}$)	15	$\dot{C}H_3$	442
	500 ($M^{+\cdot}$ + 43)		C_3H_7	500
	514 ($M^{+\cdot}$ + 57)		$\dot{C}_4H_9$	514
	457 ($M^{+\cdot}$)	29	$\dot{C}_2H_5$	428
	458 $(M + H)^+$	104	$\dot{C}_4H_{12}OSi$	354
	354	57	$\dot{C}_3H_7N$	297
	354	87	$\dot{C}_4H_{11}Si$	267
3. Bis-MTBS-morphine	513 ($M^{+\cdot}$)	15	$\dot{C}H_3$	498
	513 ($M^{+\cdot}$)	57	$\dot{C}_4H_9$	456
	456 ($M^{+\cdot}$ - 57)	15	$\dot{C}H_3$	440
	456 ($M^{+\cdot}$ - 57)	43	$\dot{C}_2H_5N$	413
	513 ($M^{+\cdot}$)	114	$\dot{C}_6H_{15}Si$	399
	513 ($M^{+\cdot}$)	131	$\dot{C}_6H_{15}SiO$	382

Table 3.IV.1. Continuation 1.

Substance	Parent Ion Mass Loss		Composition of fragment	Product ion m/z
4. Bis-EDMS-buprenorphine	553 ($M^{+\cdot}$)	15	$\dot{C}H_3$	538
	553 ($M^{+\cdot}$)	18	H_2O	535
	538 ($M^{+\cdot}$ - 15)	18	H_2O	520
	535 ($M^{+\cdot}$ - 18)	15	$\dot{C}H_3$	520
	535 ($M^{+\cdot}$)	32	CH_3OH	521
	521 ($M^{+\cdot}$ - 32)	15	$\dot{C}H_3$	506
	553 ($M^{+\cdot}$)	57	$\dot{C}_4H_9$	496
	496 ($M^{+\cdot}$ - 57)	32	CH_3OH	464
	496 ($M^{+\cdot}$ - 57)	18	H_2O	478
	553 ($M^{+\cdot}$)	101	$\dot{C}_6H_{13}O$	452
	452 ($M^{+\cdot}$)	15	$\dot{C}H_3$	438
	452 ($M^{+\cdot}$ - 101)	29	$\dot{C}_2H_5$	422
5. EDMS-buprenorphine (Electron Impact)	553 ($M^{+\cdot}$)	15	$\dot{C}H_3$	538
	553 ($M^{+\cdot}$)	18	H_2O	535
	538 ($M^{+\cdot}$ - 15)	18	H_2O	520
	535 ($M^{+\cdot}$ - 18)	15	$\dot{C}H_3$	520
	553 ($M^{+\cdot}$)	32	CH_3OH	521
	521 ($M^{+\cdot}$ - 32)	15	$\dot{C}H_3$	506
	553 ($M^{+\cdot}$)	57	$\dot{C}_4H_9$	496
	496 ($M^{+\cdot}$ - 57)	32	CH_3OH	464
	496 ($M^{+\cdot}$ - 57)	18	H_2O	478
	553 ($M^{+\cdot}$)	101	$\dot{C}_6H_{13}O$	452
	452 ($M^{+\cdot}$ - 101)	15	$\dot{C}H_3$	438
	452 ($M^{+\cdot}$ - 101)	29	$\dot{C}_2H_5$	422

Table 3.IV.1. Continuation 2.

Substance	Parent Ion Mass Loss		Composition of fragment	Product ion m/z
6. EDMS-buprenorphine (Chemical Ionization)	554 $(M + H)^{+\cdot}$	18	H_2O	536
	596 $(M + 43)^{+\cdot}$		C_3H_7	596
7. EDMS-buprenorphine (dehydrated form)	535 $(M^{+\cdot})$	15	$\cdot CH_3$	520
	535 $(M^{+\cdot})$	29	$\cdot C_2H_5$	506
	535 $(M^{+\cdot})$	41	$\cdot C_3H_5$	494
	535 $(M^{+\cdot})$	57	$\cdot C_4H_9$	478
	535 $(M^{+\cdot})$	71	$\cdot C_5H_{11}$	464
	535 $(M^{+\cdot})$	87	$\cdot C_4H_{11}Si$	448
	535 $(M^{+\cdot})$	103	$\cdot C_4H_{11}SiO$	432

yield of bis-product produced was higher in DMF than pyridine. Acetonitrile produced mainly the mono product which could be explained by poor solubility of the reagent in the solvent, similar to the results obtained when the reagent was used without any solvent. Lower mole fractions of reagent in the derivatisation mixture down to 10% by volume, promoted the formation of the bis-derivative.

Increasing the temperature and time of the reaction (to 100^{o}C) to increase the rate of the reaction and to enable complete derivatisation of the hindered hydroxyl group failed to do so, and although the total yield increased, the ratio of products could not be improved. The addition of a Lewis acid catalyst such as boron trifluoride etherate or a base such as aluminium oxide had no value.

The failure of the reaction to go to completion can be explained by at least three possibilities: firstly, the mono-derivative has a bulky tertiary-butylsilyl group which could cause steric hindrance to the other hydroxyl group and inhibit its silylation; secondly, a structural rearrangement, such as polymerization of the molecule, could occur at the free hydroxyl group of mono-MTBS-morphine; and thirdly, the reaction could reach a thermodynamic equilibrium at a certain point where it can not proceed further, rather than because it is rate (kinetically) limited.

The experiments which were conducted to investigate

the above possibilities (GC-MS and NMR analysis) confirmed that these products were mono-MTBS-morphine and bis-MTBS-morphine. The mass spectral analysis of the mono-MTBS-morphine corresponds to derivatization of the hydroxyl group at C-3, similar to mono-PFP and mono-HFB-morphine [101,145]. The major fragments obtained for 3-MTBS-morphine in this study are illustrated in Figure 3.IV.2. The routes of fragmentation were similar to those described for the morphine EI mass spectrum [146].

The ultraviolet spectra were not helpful in localizing the site of derivatization, although it might be assumed that if the derivatization occurred on the phenolic hydroxyl group it should prevent the 'red' or bathochromic shift to a longer wavelength in an alkaline solution [147]. The maximum UV absorption of free morphine in aqueous acid is 285nm and in aqueous alkali it is 298nm [12]. Free morphine and its two MTBS-products showed absorption around 300nm in alkaline solution which could be explained by the presence of free morphine due to partial hydrolysis, although GC-MS analysis showed no significant hydrolysis of products after solvent back extraction of the alkaline solution. However, free morphine if present could have been lost by absorption to the GC column and therefore not detected. This was not further investigated.

Derivatization of mono-MTBS-morphine and codeine (where the hydroxyl group at C-3 is replaced by methoxy

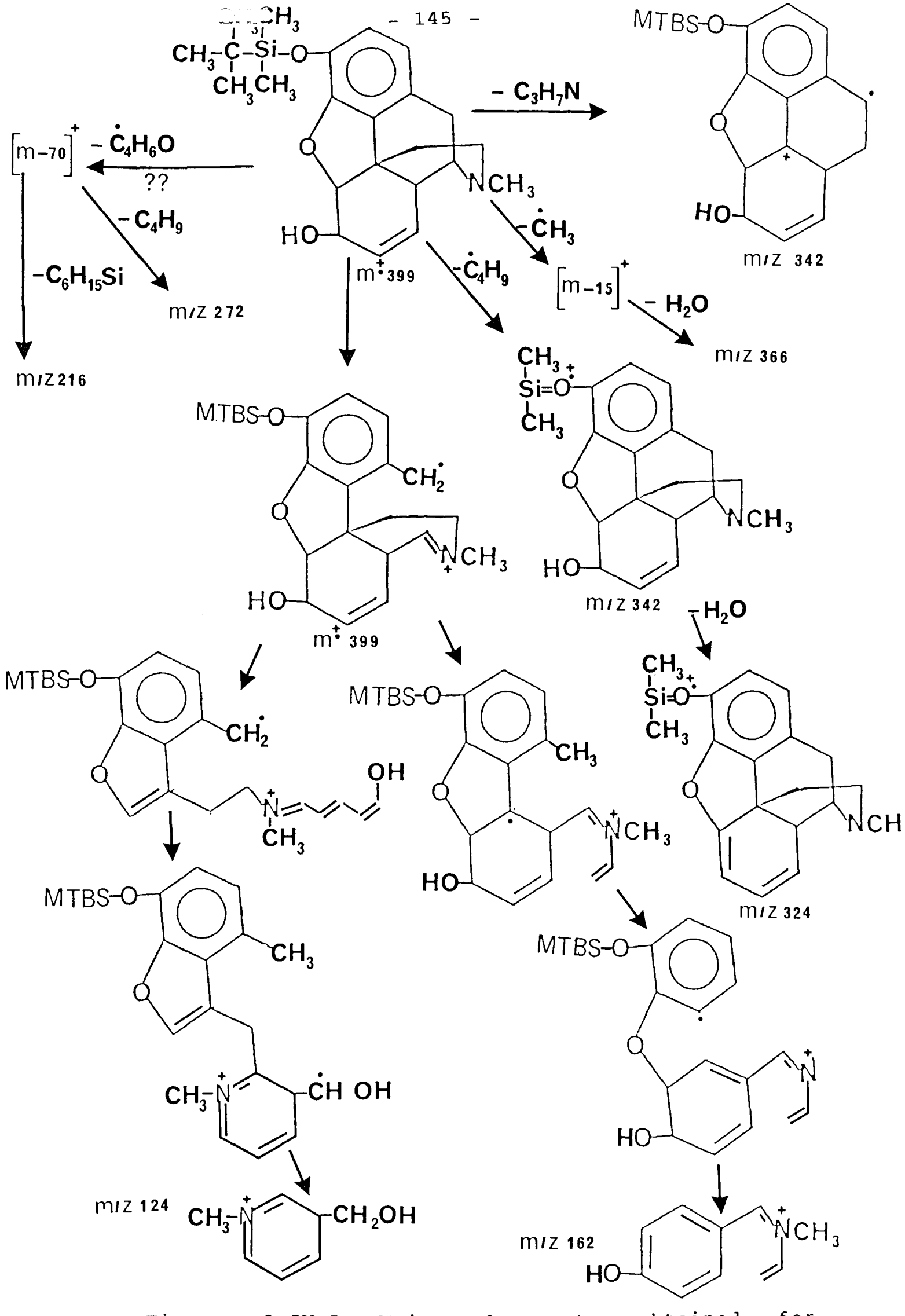


Figure 3.IV.2 Major fragments obtained for 3-MTBS-morphine during EI mass spectrometry, modified from Reference [146]. ?? is a tentative pathway.

group), with MTBSTFA produced bis-MTBS-morphine and 6-MTBS-codeine, respectively. In both cases about 10% of the products were underivatized. However, 6-MAM was completely derivatized by MTBSTFA at the C-3 position. These reaction studies, where codeine and mono-MTBS-morphine derivatization did not reach completion at C-6 position while 6-monoacetyl morphine was easily derivatized at C-3 position, indicate that the incompleteness of the derivatization is due to a thermodynamic equilibrium state and is not a kinetic limitation. In conclusion, although the products are stable, the presence of more than one product was not favourable for a good analytical method and a new reagent (EDMS-I) was assessed.

3.IV.6. ETHYLDIMETHYLSILYL-IMADAZOLE DERIVATIZATION

Derivatization with EDMS-I has not been reported for morphine and buprenorphine. It has an extra methylene group compared to the TMS group. Trimethylsilyl-imidazole has strong silyl donor ability [144]. The reactions and the products obtained with EDMS-I were similar to those from TMS-I. The product of morphine derivatization was bis-3,6-EDMS-morphine and the molecular ion (m/z 457) was the most abundant ion in the mass spectrum. It could be monitored in SIR without interference from the background siloxane ions. Derivatization of buprenorphine occurred only at the phenolic hydroxyl group, as the other group was sterically hindered. The most abundant ion at

mass 464 occurred by the loss of a neutral group (methanol) plus the tertiary butyl group (57 a.m.u.) from the molecular ion (m/z 553). This fragmentation reaction (Table 4.IV.1) did not occur in the degradation product of EDMS-buprenorphine, where the loss of a water molecule from the side chain produced a double bond (Figure 3.III.6) between C-7,1 which resulted in stronger bonding of the methoxy and tertiary butyl groups with less possibility of cleavage. The mass spectrum of the degradation product (Figure 3.III.6) is quite different from buprenorphine - the high mass ions are intense and therefore ideal for SIR. If it could be formed in 100% yield it would be useful. The dehydration of buprenorphine was due to a high injector temperature; a temperature of 300^{o}C did not compromise the results, produced better chromatography and resulted in a higher detector response compared with lower temperatures. This product can be eliminated by mild acid hydrolysis and ring formation between the side chain and methoxy group after losing methanol [107,109]. Also, more recent analysis carried out on a new capillary column (Chrompack CP-Sil 5) showed a much smaller dehydration product peak, suggesting that the by-product observed was at least partially due to the presence of active sites on the column surface.

Due to the liability of the above silyl derivatives to hydrolyse on exposure to air after removal of excess reagent by evaporation (as discussed earlier), samples were injected in the reaction mixture. Acetonitrile was

employed as a solvent for the reaction instead of pyridine, which is usually used for silylation, because pyridine tailed badly on the chromatogram and could interfere with the analysis of early eluting drugs if the method were to be extended to a general drug screen. Although the quantity of EDMS-I in the solvent (30%, v/v) was suffient for derivatization of both morphine and buprenorphine (40%, v/v) was employed to cover possible presence of extractable material in the blood which might consume the reagent. The products were found to be stable when kept in the reaction mixture for periods of up to 2 months, although background interference from siloxane material increased during this time. The presence of increasing siloxane material could be due to deterioration of the reagent at room temperature and to possible evaporation of the solvent, care being taken to avoid condensation of moisture in the storage vials.

3.IV.7. DERIVATIZATION OF BLOOD EXTRACT

Trimethylsilylation reagents can be placed in the following approximate order of silyl donor ability [144,148]:

TMSI > BSTFA > BSA > MSTFA > TMSDMA > TMSDEA > MSTA > TMCS (with base) > HMDS

Samples analysed by GC-MS were injected directly in the reaction mixture, as evaporation of excess reagent (EDMS-I) in a stream of nitrogen was not practicable, due to low volality of the reagent (BP 105°C/10mmHg) [149].

When this was tried, it was found that EDMS-morphine was hydrolysed similar to the TMS ether. The injection of this reagent into the GC-column was troublesome for the following reasons: firstly, there was a detrimental effect on the column due to head of the column contamination; secondly, there was a rapid accumulation of solid debris in the glass injector liner from the reagent itself and from co-extracted material which required frequent cleaning and silanization of the injector liner; thirdly, morphine 'ghost' peaks were observed, which required two blank injections of reagent mixture to clear the system and the occurrence of these peaks could be explained by rederivatization of the debris in the injector port; fourthly, the derivatization of extractable lipids such as cholesterol required prolongation of the final isothermal time at the upper temperature in the GC oven programme.

Purification of the silylated product on a column of hydroxyalkoxypropyl Sephadex (Lipidex-5000) was aimed at getting rid of excess reagent. This gel material has hydroxyl groups to react with the excess reagent and has been used by previous workers for purification of steroid TMS derivatives from excess TMS-I reagent [150]. In the experiment performed only free morphine and nalorphine (internal standard) were found in the eluate. This could be explained if, the EDMS derivatives of both drugs were hydrolysed on drying or on removing them from the reagent mixture. In the quoted method [150], HMDS was included in

the eluting solvent (hexane:pyridine:HMDS:dimethoxypropane 97:1:2:10 v/v/v/v) as a weak TMS donor [144] which could protect the derivatized molecule from hydrolysis.

This purification procedure would be too complex for a routine extraction and analytical method and was not further continued.

Extract derivatization was conducted using DETMDS instead of the analogous EDMS-I. The reaction conditions were similar in terms of reaction temperature and time as well as the stability of the product. However, the chromatograms obtained where DETMDS was used were relatively free from the siloxane peak material. Also, there was no contamination of the injector port and no significant carryover of the sample to the next chromatogram.

3.IV.8. HIGH RESOLUTION MASS SPECTROMETRY

High resolution mass spectrometry for quantitative analysis was discussed earlier [Chapter 2]. It was assessed in the present work as a method for EDMS-morphine analysis without interferences at the mass monitored, where by increasing the resolution of the mass spectrometer the cross-channel contribution between one mass and another in SIR is reduced and the background interference from other components is reduced accordingly. At 10,000 resolution, the interfering peaks could still be seen at low levels, which is explained by the fact that instrument can still register the signal

from a closely adjacent mass if the sides of the adjacent peak fall within the window used to monitor the target peak [151], even if the accurate mass ion is not shared with the background spectrum.

3.IV.9. CHEMICAL IONIZATION MASS SPECTROMETRY

As discussed in Chapter 2, CI is a soft ionization technique causing a low degree of fragmentation. The protonated molecular ion obtained by this method of ionization has greater relative abundance than the molecular ion obtained by EI. On this assumption EDMS-morphine coud be monitored at its protonated ion (m/z 458) and the interfering background ions, if resulting from fragmentation of a higher molecular weight molecule, should decrease or disappear. Analysis of blood samples and blanks by isobutane CI SIR showed that no interfering peak was present at the retention time of morphine.

Chemical ionization SIR also had excellent sensitivity, where detection down to the 20pg level was easily obtained by monitoring the most abundant ions of the morphine, nalorphine and buprenorphine EDMS derivatives.

In conclusion, CI-SIR can be used as a sensitive method for the above drug without interferences. Another potential benefit of CI is the use of negative ion CI for morphine and related drugs by derivatizing them with an electron-capturing moiety such as the heptafluorbenzyl[a]

groups. Higher sensitivity is expected for such derivatives under negative ion CI and background interferences would be lower. However, this approach was not studied during the present work.

3.IV.10 ESTABLISHMENT OF THE PROCEDURE

The fully developed extraction procedure produced excellent recovery for morphine and buprenorphine as well as a clean extract free from interferences. The calibration curves for these two drugs were linear over the range likely to be encountered in routine application. Reliable quantitation was obtained due to the excellent reproducibility of the method at high and low levels for these drugs.

The SIR mode of scanning where only one or two ions are scanned at one time and an analysis free from interferences have improved the detection limit as described earlier to levels better than quoted in the literature (Tables 3.I.2 and 3.I.3). The extraction efficiency of this method was also good for other basic drugs which are commonly encountered in forensic toxicology. By careful selection of retention time windows and the most intense ions, an SIR-based drug screening procedure could be developed to include a wide range of drugs which can be extracted using the developed extraction procedure.

The derivatisation reagent DETMDS produced good silylating ability for most of the examined compounds on

both nonhindered hydroxyl and amino groups. Drugs which were not completely silylated using DETMDS can be completely silylated using EDMS-I if the EDMS derivative is required. However, DETMDS would be suitable for the purpose of general drug screening in routine applications.

3.IV.11.2 ROUTINE APPLICATION

The levels of morphine in blood in cases where morphine or heroin was abused or taken over a prolonged time, were higher than 0.19ug/ml (Table 3.III.17). These values were in accordance with the reported minimum lethal level of 0.2ug/g morphine in tissue [72].

Rapid death was observed in cases numbered 1, 3 and 5 in Table 3.III.17 in which the deceased was witnessed just less than one hour before injecting heroin. In these cases, other agents such as alcohol or additional drugs contributed to the rapidity of death since even such high levels have been observed in heroin-associated fatalities whose deaths were due to other causes. However, the pathological findings and the circumstances of death are required for an accurate interpretation of the cause of death.

In case number 13, the morphine level was three times higher than that of codeine, which could reflect morphine intake, rather than codeine, if the urine sample was collected within the first 24 hours after dosing with morphine [4]. Although the exact time of drug intake was not known, however, the person had admitted taking morphine.

The buprenorphine concentration (0.65ng/ml) in blood in case number 6. was a low level. Levels of 2-50 ng/ml were reported within the first 3 hours of buprenorphine injection at a dose of 6µg/kg [64]. The cause of death in case No.6 was attributed to drug overdose due to the buprenorphine finding and the circumstances of death, although the length of time which had elapsed between intake and death was not known. The level of free buprenorphine in the urine in case No. 9 (5.1ng/ml) is not helpful on its own for interpretation. However, similar values (3.7 and 14.4ng/ml) were found after 72 hours in two patients receiving 4mg buprenorphine daily [109].

The finding of buprenorphine in the above cases and in the syringes of drug abusers supported previous reports of an increase in buprenorphine abuse [68].

CHAPTER FOUR

4.I ANALYSIS OF SOLVENTS

4.I.1 INTRODUCTION AND LITERATURE REVIEW

The importance of volatile solvent analysis in biological media in the field of forensic toxicology emerges in two major aspects. The first is the monitoring of volatile substance abuse (VSA) either in clinical practice or as part of a post-mortem investigation where VSA is suspected. The second is occupational monitoring where solvents are used during routine daily practice. A volatile substance is one which is capable of readily changing from a solid or liquid form to vapour; that is, one having a high vapour pressure and low boiling point [152]. Many solvents exhibit appreciable volatility under the conditions of use and consequently the persons who use them will be exposed to their vapours. The present study was concerned with the second of these categories, in particular the monitoring of occupational exposure to solvents in painters.

4.I.2 VOLATILE SUBSTANCE ABUSE

VSA is a term used to mean the deliberate inhalation of vapours given off by certain solvents and propellants solely for the purpose of intoxication. Other synonymous terms are glue sniffing, solvent sniffing and solvent abuse [153-157]. The epidemiology of VSA,

problems associated with it, clinical presentation and methods of abuse, have been extensively described in the literature [158-164], to mention a few. The abused solvents are available mostly in household products [163,165,166] and include xylene toluene, chlorinated hydrocarbons, white spirit, petrol, etc.

Solvent abusers experience short-term effects which are mostly reversible. The clinical picture starts with an excitatory phase of delirium followed by signs of CNS depression [165]. Early on, there is a feeling of euphoria, mental confusion, ataxia and dysarthria. Bizarre impulsive behaviour leads to trauma during this period which is a frequent source of morbidity and mortality [155,159]. Continuous inhalation may lead to coma, convulsion and cardiorespiratory arrest [163,164], and long term effects such as ataxia, encephalopathy and epilepsy have been reported [163,164,167,168]. Possible autopsy findings have also been reported [159,169].

4.I.3 OCCUPATIONAL MONITORING OF VOLATILE SOLVENTS

In contrast to VSA, workers are exposed to low levels of volatile compounds over a prolonged time and exposed to possible irreversible long-term hazards during their work practice. The hazards and toxicity of both aromatic and aliphatic solvents have been frequently reviewed [170-174]. Painters are one example of such workers; they are exposed to a wide range of solvents present in the various types of paint or paint removal

material. The main route of exposure is through the lungs with possible additional solvent absorption through the skin. Several factors influence the quantity of solvents entering the body, such as air concentration of solvents and duration of exposure in the workplace [175-178], which are, in turn, affected by the working material, working conditions, safety precautions and working methods. Other factors include pulmonary ventilation [179-182], solubility of the solvent in blood and tissue (partition coefficient) [183,184], the physique of the worker, metabolism and presence of drugs or alcohol in blood [185-187].

4.I.4 METHODS OF OCCUPATIONAL MONITORING

Occupational monitoring of exposure to volatile solvents can be conducted by one of the following procedures:

(a) Atmospheric air monitoring [188-194]

A threshold limit value (TLV) is one of the main regulatory controls of solvent in the workplace. A TLV is defined by the American Conference of Governmental Industrial Hygienists (ACGIH) as the maximum airborne concentration of a substance that represents conditions under which it is believed that nearly all workers may be exposed daily without adverse effects [172]. Three categories of TLV are described -

(i) Time Weighted Average (TWA). A value for a normal 8 hour work day and 40 hour work week.

(ii) Short-Term Exposure Limit (STEL). A value for a short time.

(iii) Ceiling (TLC-C). A value that should not be exceeded even briefly.

(b) Biological Monitoring

In this type of monitoring a marker is traced in either breath, urine or blood. The pharmacokinetics, metabolism and physical properties of the solvent of interest should be considered before selecting a biological medium. So a properly designed biological monitoring method should provide a better indication of the absorbed dose and a better estimate of the risk than air monitoring. An ideal biological monitoring protocol for a solvent or a mixture of solvents can correlate exposure with effect, detect changes before significant damage occurs, is specific and occurs shortly after exposure [195].

The literature is not scarce in the reporting of single biological markers or compilations of related data [196-198]. Body fluids used in the past are as follows:

(i) Breath Analysis

The technical and theoretical aspects of breath analysis have recently been reviewed [199]. Breath is an easily-obtained specimen for monitoring substances with sufficient volaltility [155,178]. Proper alveolar samples are collected in a plastic bag, glass tube [181,200], tube packed with adsorptive material [189] or by cryogenic

trapping. These samples are then analysed by GC or GC-MS. Direct analysis by exhaling into an infra-red detector [201], gas chromatograph [201] or mass spectrometer [202,203] has been used.

(ii) Urine Analysis

Urine analysis is used for detection of the volatiles [188,204] or their metabolites, for example benzoic acid [205] and its glycine conjugate hippuric acid(HA) for toluene monitoring [206,297], methylhippuric acids (MHA) for xylenes [178,207,208] and mandelic acid for styrene monitoring [186]. Limitations are present on the use of some of these metabolites as markers for monitoring. For example, HA can be present as a normal dietary metabolite [206,209] and the wide range of normal levels present in urine will decrease the significance of toluene monitoring at low levels of exposure to toluene. However, previous studies have shown that HA in the majority of urine samples collected at the end of the work shift was correlated to exposure even when the toluene concentration in the air did not remain constant during the working day [180,209-211]. Another factor leading to a limitation is the metabolic formation of HA and MHA which are affected by the availability of glycine in the rate limiting step [212]. Glycine can be consumed by the presence of high concentrations of toluene or xylene in blood or the presence of drugs such as salicylates [213]. Adding to these is a considerable variation of excretion of metabolites among individuals, as with mandelic acid

[181] where its excretion pattern is also modified in the presence of alcohol [185,186,209].

(iii) Blood Analysis

The blood level of a solvent usually shows the best correlation with the atmospheric concentration, the amount absorbed (regardless of exposure route), the degree of retention and severity of the effect [195,214]. The literature is rich in references to monitoring different types of solvents in blood and correlation with other biological media and atmospheric exposure [178-181,188, 209,212,215]. Blood biochemical and haematological parameters have also been studied for the purpose of monitoring [176,210,216-220] but due to the wide range of normal values for these parameters encountered in control groups, the correlation with the exposed group was rarely significant for the early detection of effects before occurrence of pathological changes.

4.I.5 OCCUPATIONAL HAZARDS OF SOLVENTS IN PAINT

Painters are usually exposed to a wide range of solvents present in the different types of paints. The degree of exposure is influenced by several factors as discussed in Section 4.I.3. Concern over the long-term health hazards to painters has emerged through the literature, especially after the appearance of clinical symptoms related to the work which are collectively described as "neuroasthenic syndrome" [221,222] or "painter's syndrome" [221,223]. The symptoms are related

to memory loss such as poor short-term memory and an array of neuropsychological deficits, as well as symptoms related to personality changes which include depression, anxiety, pre-occupation with somatic concerns, hostile feelings and fatigue. These symptoms were found significantly higher than in other groups of workers [221,224] as well as by experimental exposure of volunteer workers to various solvents used in paint material [190,222,225], where the solvents were within the TLV levels in most of the studies. Other reported hazards are peripheral neuropathy due to exposure to hexane [226], elevation of morbidity due to diseases of the respiratory and genito-urinary systems [227], glumerulonephritis [228], severe morbidity due to elevation of carboxyhaemoglobin to levels that stress the cardiovascular system on exposure to methylene chloride [229,230], encephalopathy and presenile dementia [221,231] as well as elevation of mortality due to malignant neoplasms [232].

At present, the neuroasthenic syndrome in a patient who has been exposed to solvents for at least 10 years, is recognized as a work-related risk when other diseases have been excluded reasonably well [222] and the resultant chronic toxic encephalopathy is accepted as an occupational disease entitling workers to compensation in Finland, Sweden, Norway and Denmark [231].

4.I.6 ANALYTICAL PROCEDURES FOR VOLATILES ANALYSIS

Analysis of volatile solvents from biological samples can be done by one of the following procedures:-

(a) Solvent Extraction

A low boiling point solvent is used to extract the volatiles from biological [233-236] or non-biological material [191,215]. The limitations of such a method are the dilution of the volatile analytes, loss of the more volatile analytes during the concentration step, the masking of early eluting peaks by the solvent and adverse effects on the detection limit due to solvent impurities, which may also cause chromatographic interference.

(b) Direct Aqueous Injection

A biological fluid such as blood [237] is injected directly into the GC. This method is routinely used for blood alcohol analysis and can be suitable for VSA samples, but might not have sufficient sensitivity for occupational monitoring.

(c) Head Space Techniques

The techniques and applications of head space analysis have been recently reviewed [209,238-242]. Two types of head space technique are commonly used for volatile enrichment followed by GC or GC-MS analysis.

(i) Static Head Space Analysis

The biological material, which is placed in a closed vessel, comes to equilibrium with its vapour at a controlled temperature. The sample in equilibrium within the closed system is normally a solution of the volatile

component of interest (i) in a liquid solvent. In an 'ideal' solution, the partial vapour pressure of the volatile component Pi can be expressed by Raoult's Law as [241]:-

$$Pi = Poi \times Xi \quad \text{..... Equation 4.1}$$

where Poi is the vapour pressure of pure component i and Xi is the mole fraction of component i in the solution. In most cases, however, molecular interactions exist in solution which leads to the expression:-

$$Pi = Poi \times Xi \times Ai \quad \text{..... Equation 4.2}$$

where Ai is the activity coefficient of component i. The activity coefficient Ai depends on the nature of the component i and other components in the mixture, the mole fractions of all the components, the temperature and to a small extent on the pressure. For the dilute solutions used in trace analysis, the vapour pressure of the solute tends to vary linearly with its concentration, hence, the activity coefficient becomes a constant.

$$Fi = Ci \times Pi \quad \text{..... Equation 4.3}$$

where Fi is the chromatographic peak area and Ci is a constant which depens on the detector being used. Such behaviour is known as Henry's Law. Combining Equations 4.2 and 4.3 the following relation is produced:-

$$Fi = Ci \times Poi \times Xi \times Ai \quad \text{..... Equation 4.4}$$

or $$Xi = Fi/ (Ci \times Poi \times Ai) \quad \text{..... Equation 4.5}$$

where 1 / (Ci x Poi x Ai) is a constant called the calibration factor which has to be determined experimentally. A volume of the head space is sampled for

chromatography. This technqiue is employed extensively for VSA samples [154,155,230,242-245] as well as biological monitoring [189,212,216] where relatively high levels of volatiles are encountered.

(ii) Dynamic Head Space Analysis

The sample is exposed to a continuous flow of gas, often passed through it as a stream of fine bubbles. As in static head space analysis, volatile substances partition into the gas, but because this is constantly being replaced, equilibrium is not achieved and the sample is purged or 'stripped' of volatiles. The stripped volatiles are collected in a suitable trap which may be a cold trap, a solid adsorbent or a solid support coated with a liquid stationary phase. This technique has been extensively used for the analysis of volatiles and the metabolite profile in blood [246-248], urine [249,250] and milk [251] as well as volatiles in water [192,252-254], sediments [248] and other media [239,255] to name a few.

In the present study dynamic head space analysis was evaluated for the measurement of volatile solvents present in painters and to assess solvent uptake as a pilot project for a future comprehensive study. This would include environmental and biological monitoring accompanied by clinical evaluation for the assessment of the hazards of paint solvents to these workers.

4.II E X P E R I M E N T A L

4.II.1 MATERIALS AND REAGENTS

N-alkanes, C_8 to C_{14} (Analar grade) and diethyl ether (Aristar grade) were individually purchased from BDH. Other solvents were glass-distilled grade. Tenax-GC (35-60 mesh) was purchased from Altech (Lancashire, U.K.).

4.II.2 INSTRUMENTATION

Gas chromatography used a Pye-Unicam model 204 gas chromatograph fitted with a glass capillary column (Chrompak CP-Sil 5, 25m x 0.5mm I.D. with a 0.8µm film thickness) and a flame ionization detector. GC-MS was carried out using the mass spectrometers described in Chapter 3.II. The scan parameters in the two mass spectrometers in full scanning mode were as follows:-

	16F	70-250S
Scan range (exponential down scan)	250-20	200-35
scan rate	1.0 sec/decade	1.0 sec/decade
Interscan delay	1.0 sec	0.3 sec

4.II.3 CHROMATOGRAPHIC PARAMETERS

The volatiles of interest which are present in paint are aliphatic hydrocarbons in the range C_8 to C_{12}. A solvent extraction procedure was assessed for these hydrocarbons, but before that, several factors which influence chromatography were examined, including the

injection technique, analytical column and GC operating conditions including the carrier gas flow rate.

4.II.3.1 INJECTION MODE

Two modes were assessed

(i) Direct injection mode on an SGE splitless injector.

(ii) Groß split/splitless mode in which the split valve was closed for 1 min following injection.

In both modes a helium pressure of 12 p.s.i. was applied at the head of the column to give a constant carrier gas linear velocity of 26.3cm/sec. The oven temperature was programmed from 40-180°C at 12°C/min. The injector and detector temperatures were 200°C and 220°C, respectively. Nitrogen make-up gas flow rate was 30ml/min. A standard mixture of C_8 to C_{12} n-alkanes in diethyl ether was prepared with individual concentrations of about 20µg/ml. Aliquots injected varying from 0.5-10µl were tested for their ability to produce reasonable chromatographic peaks and base lines. The base line in both cases was not acceptable, so a packed pre-column was added before the capillary column. Other solvents such as n-pentane, acetone, methanol and petroleum spirit were similarly examined.

4.II.3.2 PREPARATION OF PACKED COLUMNS

Two empty glass columns (1.8 and 3m x 4mm I.D.) were thoroughly cleaned with detergent and rinsed with water followed by acetone, then were filled with ethanolic potassium hydroxide (0.2% w/v) overnight to clean the inner surface of the columns. That layer was removed with

methanol and the columns were silylated with DMCS in toluene (10% v/v) followed by rinsing with methanol (150ml) and acetone (50ml) and were dried under a stream of nitrogen. Each column was packed with 3% SE-30 on Gas Chrom Q (80-100 mesh) and was conditioned from 40-300°C at 2°/min and kept at 300°C for 16 hours under a flow of the carrier gas of 30ml/min. The top of the 1.8m packed column was connected to the injector and the other end was connected via a $^{1}/_{4}$ x $^{1}/_{16}$ x $^{1}/_{16}$" T-piece to the capillary column and to an on/off vent valve. The flow through this valve was controlled by a needle valve and was set at 40ml/min. The flow rate in the capillary column was 5ml/min. Aliquots (5-10µl) of standard mixture were injected while the initial oven temperature and time were changed to optimize chromatography and response after closing the venting valve. The solvent void time for diethyl ether and the retention time of octane were determined by connecting the packed column to the detector directly at the same carrier gas flow rate and at an oven temperature of at 40°C.

The 1.8m packed column was replaced by the 3m column to offer better resolution of the early eluting peaks from the end of the solvent front. Similar tests were conducted as above.

The following parameters were then selected: the venting valve was closed at 3 min with helium flow rate at 40ml/min through the packed column and the column oven temperature was programmed from 40°C to 180°C at

12°/min, following an initial isothermal period of 3 min.

The reproducibility of retention time and response (peak height) were determined by repeated injection of 10ul of the standard mixture, at a detector attentuation of 1 x 10^2.

4.II.4 BLOOD EXTRACTION

A stock standard solution was prepared by adding 100ul of each of the C_8-C_{12} n-alkanes to methanol/acetone (1/1 v/v) in a 50ml volumetric flask. A blood sample was prepared by adding 100µl of the stock solution to 25ml of whole blood to give a concentration of about 8µg/ml of each. The blood sample (1ml) was extracted with 3ml of diethyl ether, acetone or dichloromethane, by vortex mixing for 30 secs in a 15ml screw-capped test tube. Another blood sample was extracted using diethyl ether as above, but the blood was saturated first with freshly crushed rock of ammonium carbonate. Then the mixture was centrifuged at 3000 rpm and an aliquot of the solvent (2ml) was collected into a 5ml tapered receiving vessel which was graduated in 0.1ml units. The solvent volume was reduced to 0.1ml under a stream of nitrogen at room temperature.

An aliquot of the residue (10µl) was injected into the GC as described in Section 4.II.3.2. Extraction efficiency was determined as in shown Equation 3-2, by comparing the peak height of the sample with that of similar standards prepared in diethyl ether. A solvent of

a similar volume was reduced as in the extraction procedure and was run on the GC to look for any interferences from each of the extracting solvents or from the blank blood. The reproducibility of the extraction was determined using ten replicate samples at concentration of 8µg/ml. These samples were prepared using Aristar grade diethyl ether as an extraction solvent and compared with similar standards on the GC. The above blood standard (8µg/ml) was diluted serially in blood down to 15ng/ml and 1ml of each was extracted, as described above, to determine the detection limit and to construct a calibration curve.

4.II.5 ANALYSIS BY GC-MS

I. Two-Column Arrangement

Analysis by GC-MS was aimed at improving the sensitivity of detection. The same column arrangement was reconstructed on the VG-16F mass spectrometer. The end of the capillary column was connected to the capillary interface and the vent at the end of the packed column was temporarily connected to the packed column interface (jet separator) to the mass spectrometer where the helium flow rate was 30ml/min. The GC oven was operated from 40°C (3 mins isothermal) to 180°C at 12°/min. The retention times of the solvent and n-octane were determined then the connection to the packed column interface was replaced by an on/off valve with needle flow rate controller similar to the GC set-up. However,

reasonable peaks could not be obtained with this arrangement under the condtions described so analysis was conducted on the packed column alone and, later, by the desorption and trap method followed by capillary column analysis.

II. Analysis on the Packed Column

The prepared packed column (3% SE-30, 3m) was connected via the packed column interface containing a jet separator and dump valve. The helium flow rate was set at 30ml/min. Separation of the standard mixure was conducted with an oven temperature programmed from 80°C (1 min isothermal) to 180°C at 12°/min. The reproducibility of response was determined by repeated injection (5µl) of a standard mixture containing 2µg/ml of each of the C_8 to C_{12} n-alkanes in diethyl ether. The detection limit was determined by serially diluting the above standard and applying 5ul of each standard solution. Analysis was by SIR of mass 57 for hydrocarbons and 68 for limonene (I.Std.). The channel dwell time was 100msec and the interchannel delay was 30msec.

III. Desorption and Trap Analysis

III.1 Preparation of Tenax-Column: Straight stainless steel columns (23cm x 4mm I.D) were cleaned with detergent, rinsed with water followed by acetone and dried in an oven at 100°C for 3 hours. Each column was packed with Tenax-GC (0.5g) and conditioned from 50°C-280°C at 5°/min and kept for 2 hours at 280°C under a helium flow rate of 30ml/min.

A Tenax-GC column was placed in a desorption oven (Figure 4.II.1) at 250°C, composed of a steel tube (22cm x 7.5mm I.D), insulated with glass tape and wound with a heating cord (Electrothermal Ltd.) which was controlled by a variable transformer. The carrier gas (He) flow rate was set at 20ml/min and passed to a cold trap (25cm x 0.5mm I.D stainless steel tubing) in a 6 port valve (Valco Ltd.). The collected volatiles in the cold trap (cooled under liquid nitrogen) were then transferred to the GC column (CP-Sil-5 CB WCOT fused silica column, 25m x 0.32μm I.D and 1.2 μ, film thickness), by electrically heating the sample loop which raised the temperature to approximately 100°C in 15 sec.

III.2 Testing the Desorption System

The optimum flow rate of the carrier gas through the Tenax column and cold trap was determined by injecting on to the hot column 1μl of a standard containing a mixture of C_8-C_{12} n-alkanes at a concentration of 32μg/ml. The column was desorbed for 15 min, and then hydrocarbons collected in the cold trap were transferred to the GC column, which was operated from 40°C to 180°C at 8°/min with the helium flow rate at 3ml/min. The reproducibility of the system was determined by repeated injections of the above standard at a flow rate of 20ml/min and a desorption time of 15 min.

III.3 Solvent Extracts

An aliquot of diethyl ether (10μl-1ml), representing a blood extract, was tested for applicability

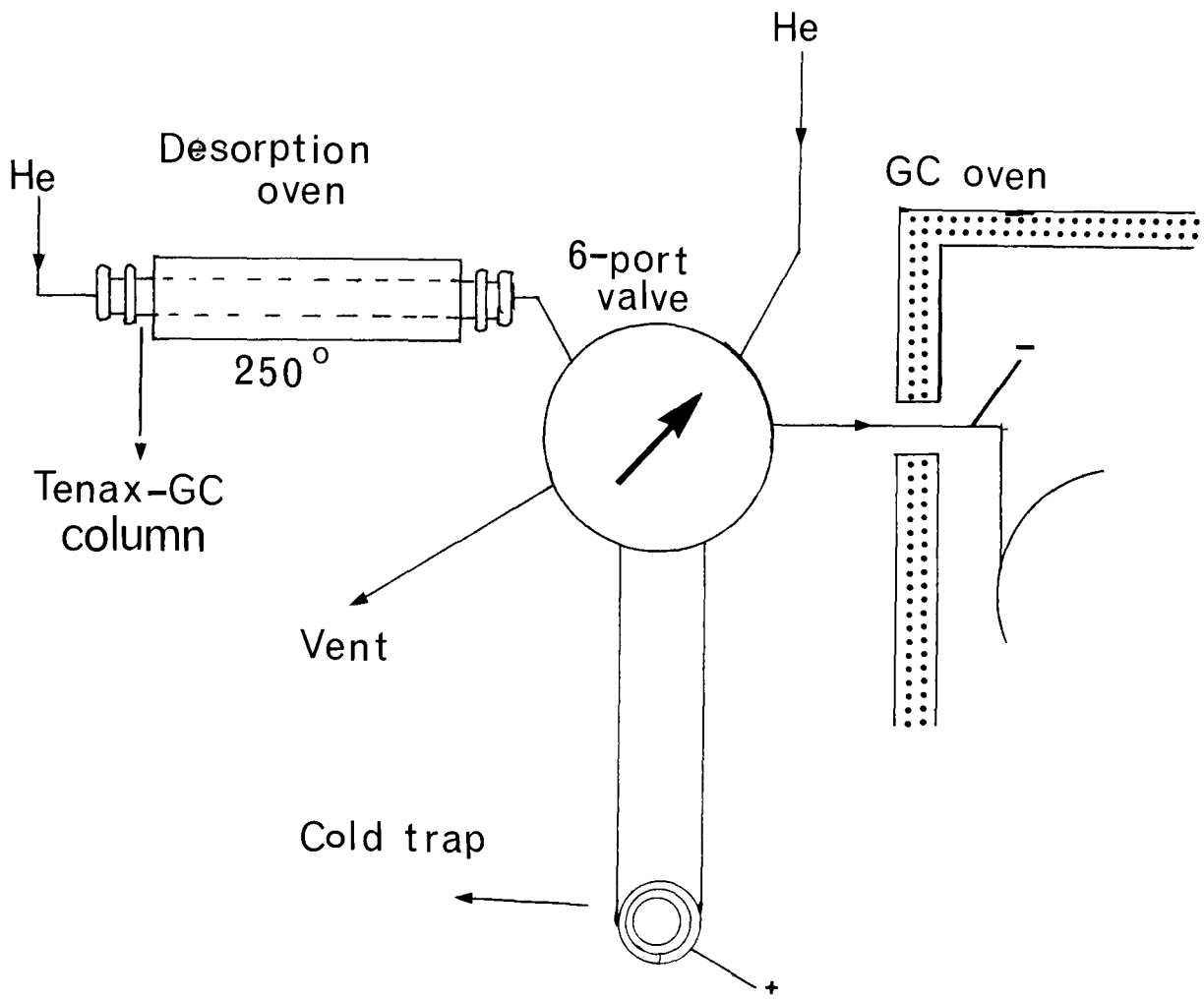


Figure 4.II.1 Schematic diagram of the desorption and inlet system used to transfer volatiles from the Tenax-GC column to the analytical GC column via a cold trap held in a 6-port valve. Electrical contacts for heating of the cold trap were at the points indicated as + and -.

on the Tenax-GC column. The ether layer was first eluted at room temperature and later the Tenax column was heated and the hydrocarbons were collected in the cold trap. Large volumes of ether were difficult to evaporate within a reasonable time. Eliminating the dilution effect of solvent extraction and improving sensitivity did not succeed using such a system. Dynamic head space analysis was evaluated to substitute the solvent extraction method.

4.II.6 DYNAMIC HEAD SPACE (DHS) ANALYSIS

I. Preparation of Standard Solutions

A working internal standard of limonene was prepared at a concentration of 32µg/ml in reboiled distilled water by dilution of a stock solution in solvent, and a working standard solution of each n-alkanes from C_8 to C_{12} was prepared in blood at a concentration of 32ng/ml. The elution flasks were cleaned with detergent after use followed by soaking in chromic acid overnight to get rid of organic material then washing in detergent and rinsing with distilled water. All glassware and pipettes were kept in an oven at 100°C until needed to reduce the surface condensation of volatiles from the laboratory environment.

II. Extraction Method

The apparatus used for DHS extraction was previously described [256,257]: a schematic diagram is presented in Figure 4.II.2. The extraction procedure was conducted as follows: the elution flask and condenser

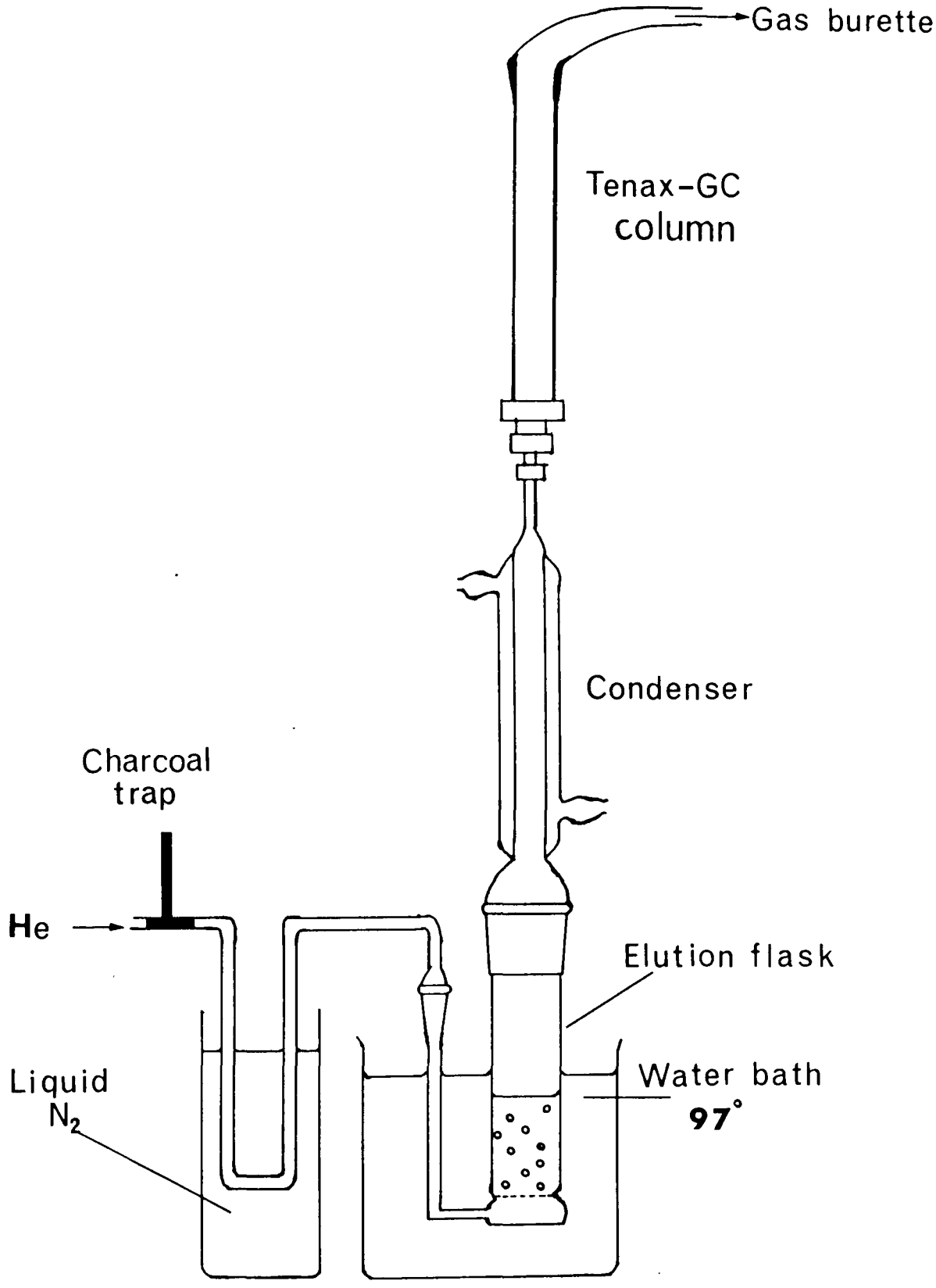


Figure 4.II.2 Schematic diagram of the apparatus used to strip solvents from blood and collect them in a Tenax-GC column.

were purged with helium at a flow rate of 100ml/min at 97°C in the water bath for 5 min before use. The helium supply was cleaned before use with a charcoal trap (Chrompack U.K.). A conditioned Tenax-GC column was then connected to the condenser outlet using a Swaglock coupling with Vespel ferrules. A sample of whole blood (1ml) was then transferred to the elution flask and 10µl of the internal standard solution was added and mixed with the blood. The flask was then connected to the condenser with the aid of retaining springs. Helium elution was then started for a few seconds to allow the foam formation in the sample to reach the middle of the flask, then the flask was lowered into the hot water bath to allow the blood to coagulate. Elution was then resumed at a flow rate of 40ml/min. The helium was collected quantitatively in the gas burette. The Tenax-GC column was removed at the end of the elution and sealed with two Swagelock blanking pieces fitted with Vespel ferrules. The sealed traps were kept at room temperature until analysis. Each sample was analysed as described in Section 4.II.5.III.

III. Source of Contamination in the Analysis

Large interefering peaks of aromatic and aliphatic hydrocarbons appeared in the blank samples of both extraction and desorption system. The source of contamination was investigated by collecting volatiles from about 800ml of helium at each of the possible sources.

III.1 Desorption System: the carrier gas to both the analytical column and desorption system were tested by

collecting volatiles from the helium in the cold trap under liquid nitrogen for each individually. A charcoal trap (Chrompack U.K. Ltd.) was placed in the common supply stream to try and eliminate the contamination and all accessible tubing was replaced by new tubing which was cleaned by flaming it under a flow of nitrogen at 100ml/min. Connections were made with the shortest possible lengths of tubing.

Later, the contamination was eliminated from the supply line by incorporating another cold trap immediately before the inlet system.

III.2 Extraction System: The background impurities in helium were collected in a clean Tenax-GC column from the following points:

i. Apparatus blank sample collected with the usual extraction procedure.

ii. At the exit of the charcoal trap.

iii. Before entrance to the charcoal trap.

iv. From the helium cylinder at the regulator exit.

The new tubing and charcoal trap did not eliminate the contamination. A cold trap of 8m x $^{1}/_{8}$" copper tubing was made and later was substituted by another trap made from a 10m x 0.5mm I.D stainless steel coiled tube which was placed under liquid nitrogen at the time of extraction.

IV. Determination of Stripping Volume

The volume of helium which was required to produce efficient extraction of C_8-C_{12} n-alkanes from blood

was determined by extracting 1ml of blood containing 32ng/ml of each hydrocarbon and 32ng of limonene (I.Std) as described above, with helium volumes of 400, 600, 800, 1000 or 1200ml. The extraction efficiency was estimated by comparing the peak areas (as in Equation 3-2) of each of the hydrocarbons with those obtained with a similar non-extracted standard in diethyl ether where 1µl was applied directly to the desorption system. The reproducibility of the extraction was measured by repeated extraction of the above blood sample using an elution volume of 800ml.

4.II.7 ANALYSIS OF PAINT MATERIAL

An aliquot (1g) of paint used by the painters under study was placed in a 6ml hypovial which was then closed with a rubber cap and an aluminium crimp seal. It was placed in a heating block at 40°C for 20 minutes and 10µl of the head space was taken by a warm glass gas-tight syringe and applied to the Tenax-GC column. This was desorbed and analysed as described above.

4.II.8 ANALYSIS OF BLOOD FROM PAINTERS

Blood samples (3ml each) were collected from 7 painters on two occasions. The first collection was at the beginning of the working day on Monday and the second was at the end of the working day on Friday. The samples were received in a lithium-heparin plastic tube and were kept at 4°C until analysis. The analysis was conducted

as described earlier by DHS extraction with an elution volume of 800ml. The analysis by GC-MS on the VG-70-250S instrument was by the full scanning mode. Reboiled distilled water from a sampling vial and a blank apparatus extraction were analysed at the same time.

Quantitation was peformed by measuring the peak areas in reconstructed ion chromatograms of the following masses: m/z 57 for C_6-C_7, 71 for C_8-C_{12}, 68 for limonene (I.Std), 91 for toluene and xylenes, and 105 for higher alkyl benzenes. The ratio of each response to the I.Std was taken. The ratios of the two groups of samples were compared by Student's T-test. Quantitation of the amount of hydrocarbon present in the blood sample was carried out for those substances whose ratios showed significant differences between groups by comparing with standards prepared in blood.

4.III R E S U L T S

4.III.1 INJECTION MODE

The direct injection mode on the capillary column produced a base line which remained high, even with a small injection volume (0.5µl), and the solvent peak obscured early-eluting peaks. This was more prominent in subsequent samples following repeated applications. The hydrocarbons were dissolved in other solvents, such as n-pentane, acetone, methanol and petroleum spirit, to try and reduce this problem but there was no improvement. However, the peaks were of good quality and the resolution was good.

The Groß split/splitless mode of injection permitted large volumes of solvent to be introduced while still bringing the baseline back to the origin after the solvent front, but the peaks were relatively small especially the octane and nonane peaks, indicating both inefficient solute transfer to the column and negative sample bias with respect to more volatile components.

4.III.2 PACKED AND CAPILLARY COLUMNS

It took more than 5 min for the baseline to return to the origin after injecting 5µl of diethyl ether at a carrier gas flow rate of 40ml/min and oven temperature of 40°C. While both packed columns offered retention of applied hydrocarbons, the longer column permitted a slightly bigger margin of separation between the solvent

front and the octane peak although in both columns this peak appeared on the tail of the solvent peak. The venting valve at the end of the packed column was closed after 3 min while the oven was at 40°C, allowing reasonable clearance of the solvent front. However the tail of the solvent front used to reappear after closure of the vent valve, especially with larger volumes of solvent injection (10µl) and the early-eluting peak (octane) eluted on the solvent peak tail (Figure 4.III.1).

The reproducibility of retention time and response are listed in Table 3.III.1.

Table 3.III.1: Reproducibility of analysis of n-alkane standards.

Analyte	Rt(min)	C.V.(%) (Rt)	C.V.(%) of response	RRt.
Octane	3.7	12.1	14	0.50
Nonane	4.9	11.2	14	0.67
Decane	6.7	7.4	13	0.92
Undecane	8.0	1.3	12	1.10
Dodecane	9.9	1.2	11	1.35
Limonene	7.3	5.2	14	1.0
Tetradecane	12.1	3.6	8	1.65

C.V.(%) is the coefficient of variation. RRt is the relative retention time.

4.III.3 BLOOD EXTRACTION

The extraction efficiency of C_8-C_{12} alkanes and the internal standard from a blood sample using dichloromethane or acetone ranged from 35-62% and the diethyl ether extraction efficiency was 60-70%. In general, higher efficiencies were obtained for the higher hydrocarbons. Diethyl ether extraction where ammonium carbonate was included produced efficiency better than 80%

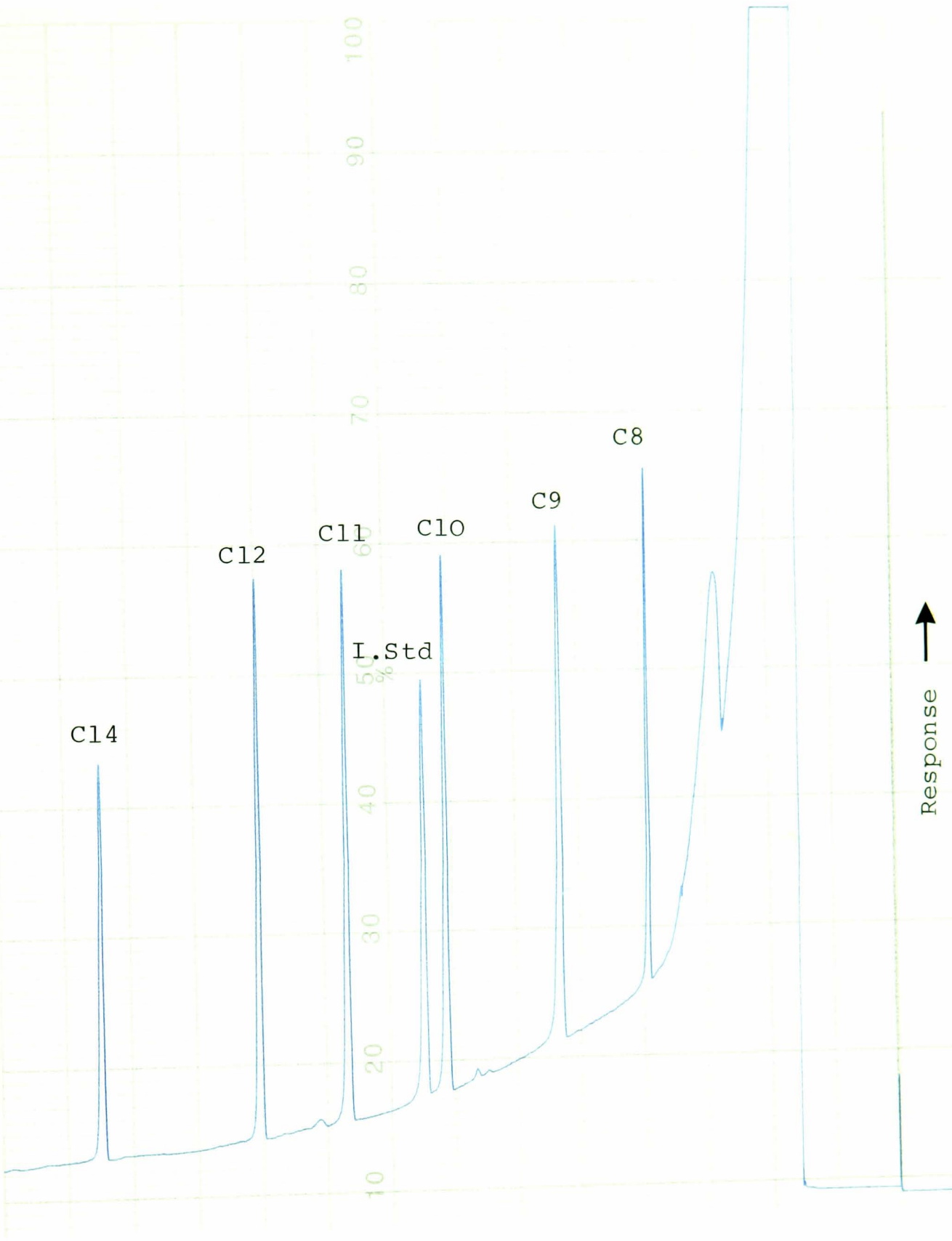


Figure 4.III.1 GC trace of n-alkane hydrocarbons C_8-C_{12} where 10µL of solvent containing the standards were applied at the inlet of the two-column arrangement and the vent valve at the end of the packed column was closed after 3min. The oven temperature was programmed from 40° to 180°C at 12°/min after a 3min isothermal period at 40°C.

for the hydrocarbons examined (Table 4.III.2). The small interfering peaks early in the chromatogram, which were most prominent in the diethyl ether blank, became negligible, when Aristar grade solvent was used instead of Analar grade. Two small unidentified peaks, present in extracts of blank blood, eluted after nonane and decane, respectively, without interference.

Table 4.III.2: Extraction efficiency of n-alkanes from 1ml of blood by 3ml of diethyl ether.

	Octane	Nonane	Decane	Undecane	Dodecane	Limonene
Ext.Eff	70	91	88	81	83	81
C.V.(%)	18	17	12	11	11	14

The GC detection limit for the extracted blood was approximately 125ng/ml for each hydrocarbon with peak-to-noise ratios of 3:1. The calibration curve (for nonane) was linear over the range 125ng to 8ug/ml with the regression equation:

$$Y = 1.78 \times 10^{-4} + 0.73X \quad \text{..... Equation 4.5}$$

where Y is the response ratio and X is the concentration. The correlation coefficient (r) was 0.98

4.III.4 ANALYSIS BY GC-MS

4.III.4.1 Two-Column Arrangement

The solvent front started to elute from the packed column (3mm) after 28 secs and octane at 1:48 minutes, so the vent valve was closed at 1:30 minutes without loss of the early eluting peak, but after closure of the vent valve, residual solvent led to a rise in the base line due

to loss of the split ratio. Peak separation was good but peaks had a broad shape due to band broadening in the pre-column. Attempts to increase the flow rate to sharpen the peak were limited by the adverse effect on the vacuum pressure in the mass spectrometer. However, sharp peaks could not be obtained through the capillary interface by this arrangement.

4.III.4.2 Packed Column Analysis

The solvent front in the packed column was eliminated by the jet separator valve in the packed column interface, which was opened after 1:5 minutes from the start. There was still a prominent solvent peak tail at this time and the first peak (octane) eluted on it. The peaks were well separated and had a reasonable peak shape for a packed column. The repeated application of 5µl of 2µg/ml standard solution in diethyl ether showed reproducible response where the coefficient of variation ranged from 3.7-9% (Table 4.III.3) and that of retention time varied from 6-11%.

Table 4.III.3: The reproducibility of response and retention time in the analysis of n-alkanes by GC-MS with a 3m packed column.

	Coefficient of Variation (%)					
	Octane	Nonane	Decane	Undecane	Dodecane	Limonene
Height	3.7	7	6	5.8	3	6
RT(min)	11.8	9.5	8.2	7.3	6.5	8.6
Ratio(r)	3	5	3	3	6	1

Ratio: Peak height of standard over the peak height of I.Std.

Although the solution had a similar concentration of each hydrocarbon, the chromatogram showed the phenomenon of increasing peak size with increasing molecular weight, due to a corresponding increase in the efficiency of the separator.

The detection limit obtained by SIR for a standard solution in diethyl ether (5µl) was 15ng/ml (75pg on-column). Application of a larger sample volume to improve detection led to an increase in the solvent tail which in turn led to the octane and part of the nonane peak being obscured.

4.III.4.3 Analysis by Desorption and Trap Method

The flow rate of helium, which was optimum for eluting the hydrocarbons from the Tenax-GC while enabling them to be collected efficiently in the cold trap, was 20 ml/min for 15 minutes. A lower flow rate was not sufficient to elute decane, undecane and dodecane effectively. The reproducibility (coefficient of variation) under these conditions for six replicate applications of 1µl standard solution containing 32ng of each hydrocarbon and internal standard ranged from 4.5-7.5%. Higher flow rates produced poor reproducibility and inefficient trapping. Application of a larger volume of ether (10µl) on the Tenax-GC required 10 minutes to clear the solvent from the column, which was not reproducible since it led to blocking of the cold trap by freezing of the residue of diethyl ether. So a larger sample volume required a longer time and caused more frequent blockage of the cold trap.

4.III.5 DYNAMIC HEAD SPACE ANALYSIS

4.III.5.1 Source of Contamination in the Analysis

Severe contamination by aliphatic and aromatic hydrocarbons was found in both extraction and desorption systems. Examples of the aromatic materials were benzene, toluene, xylenes (o, m and p-xylene) benzonitrile and alkylbenzenes (C_2-C_4 substrated benzenes); other contaminants included straight and branched alkanes, dichloromethane, ether, chloroform and acetone. All appeared within the normal temperature programme run of the column.

The source of contamination was from almost all parts of the two systems. Charcoal traps were found to be useless with this huge source of contamination. Cleaning the carrier gas by passing it through a narrow diameter steel tube (0.5mm I.D.) immersed in liquid nitrogen was sufficient as a temporary solution at the time of analysis, although later in the study this trap started to be inefficient in the extraction system even though the tubing was kept in a closed circuit without exposure to the atmosphere.

4.III.5.2 Helium Stripping Volume

The volume of helium which stripped more than 90% of the hydrocarbons in the blood sample was 800ml (Figure 4.III.2). Cooling the condenser led to decreased recoveries, especially for the higher hydrocarbons. None of the hydrocarbon broke through the Tenax column within the range of helium volumes examined. The amount of water

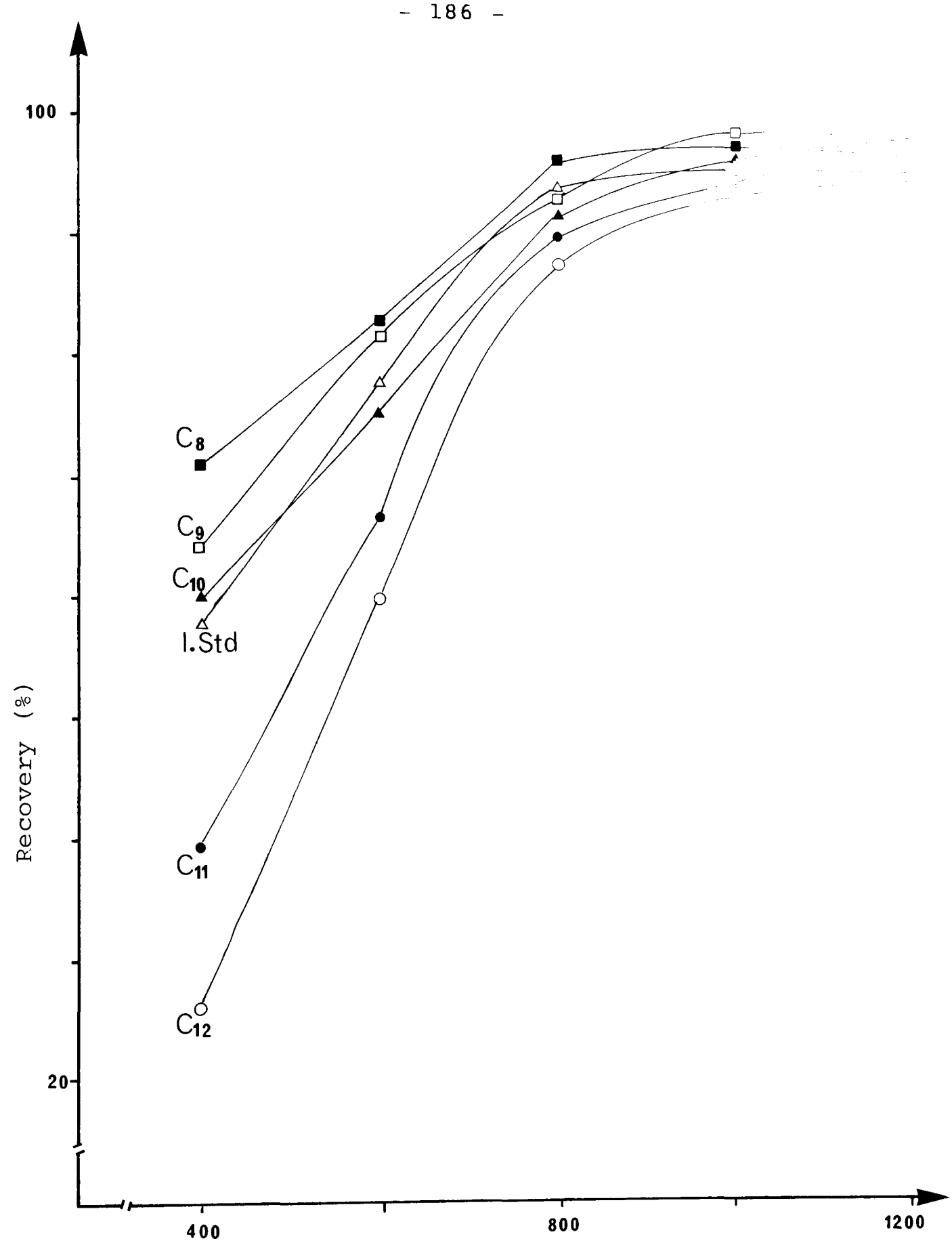


Figure 4.III.2 Recoveries of n-alkane hydrocarbons (C_8-C_{12}) and limonene (I.Std) from blood using different helium stripping volumes.

vapour entering the Tenax-GC column was very little and did not have a detrimental effect on the procedure, especially if the trap was flushed with helium for 5 minutes before heating to desorb the volatiles. The reproducibility (coefficient of variation) of the procedure (extraction and desorption) determined by five replicate samples ranged from 5 - 11% for different materials.

4.III.6 PAINT CONSTITUENTS

Both aliphatic and aromatic hydrocarbons were present in the paint material, these constituents are presented in figure 4.III.3 and listed in Table 4.III.4.

4.III.7 ANALYSIS OF PAINTERS' BLOOD

The chromatograms obtained for the blood samples showed numerous peaks of both aromatic and aliphatic hydrocarbons. At the same time these constituents were present to a lesser extent in the blank samples. Table 4.III.5 presents the ratio of the most prominent peaks to the internal standard (peak areas) for each blood sample in the two groups and for the blank vial sample. The ratios for the blank were least for C_9-C_{11} n-alkanes and ethyldimethylbenzene and less than any of the ratios for the first blood collection. The ratios of these C_9 to C_{12} hydrocarbons, trimethylbenzene isomers and ethyldimethylbenzene showed higher values in the second group for each individual case, but only nonane and

ethyldimethylbenzene showed a probably significant increase by student's t-test (unpaired), when the means of each group were compared. For the others, the significance was not proven. Toluene, xylenes, hexane and heptane did not follow any special pattern. Another significance test (Paired t-test) [258] was carried out on the ratios observed for each person taken at the beginning and at the end of the week (Table 4.III.5). This test showed a significant difference for the following substances: nonane, undecane, dodecane, trimethylbenzene and ethyldimethylbenzene, where the threshold of significance was $P \leqslant 5\%$.

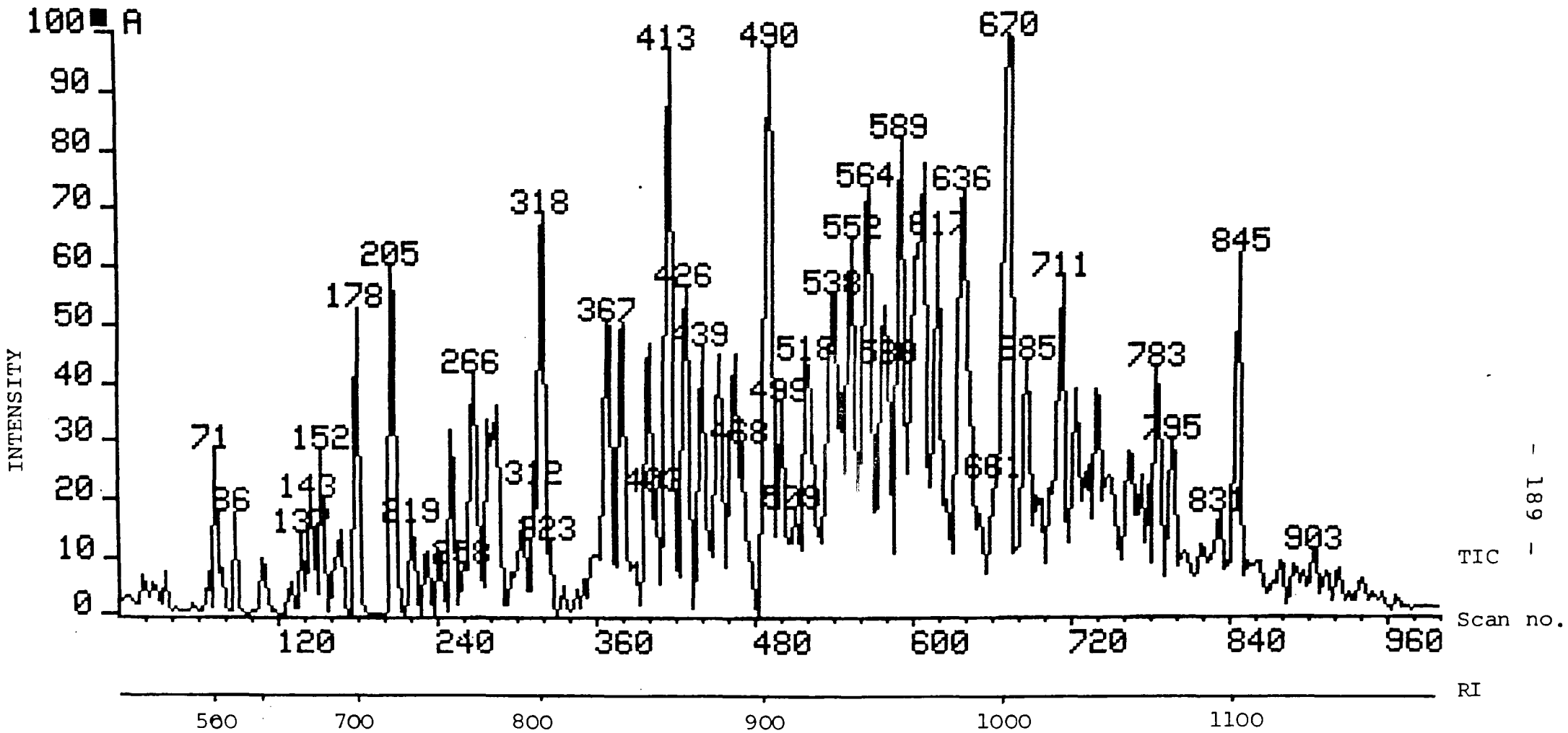


Figure 4.III.3 GC-MS analysis of a paint headspace sample. Condition were described in Section 4.II.5.III.2 The identity of the major peaks is presented in Table 4.III.4.

Table 4.III.4 Constituents of the paint material used by the painters under study.

RETENTION INDEX	C O M P O U N D	SCAN NUMBER
A.	ALIPHATIC HYDROCARBONS	
500	n-pentane	71
550	methylpentane	
560	2-butanone	
600	n-hexane	
630	methylpentane	
658	cyclohexane	
667	2,4-dimethylpentane	
677	3-ethylpentane	
681	1,2-dimethylcyclopentane	
695	4,4-dimethyl-2-pentane	
700	n-heptane	178
722	1,2,3-trimethylcyclopentane	
745	2-methylheptane	
758	3-methylheptane	
764	methylcycloheptane	
783	1-methyl-2-ethylcyclopentane	
800	n-octane	318
846	2,4-dimethylheptane	
858	2,5,5-trimethyl-1-hexane	
900	n-nonane	490
916	3-methyloctane	
927	3-ethyl-3-heptane	
932	4-ethylheptane	
938	3-ethylheptane	
951	1-methyl-3-isopropylcycloheptane	
960	isopropylcyclohexane	
971	isobutylcyclopentane	
983	3-methylnonane	
990	4-methylnonane	
1000	n-decane	670
1023	pentylcyclohexane	
1050	5-methyl-2-decene	
1077	4-methyl decane	711
1100	n-undecane	845
1109	2,3,7-trimethyl-4-octane	
1130	butylcycloheptane	
1144	2,4,6-trimethyloctane	
1200	n-dodecane	
1224	4-methyl-1-undecene	
1230	4-methylpentylcyclohexane	
B.	AROMATIC HYDROCARBONS	
635	benzene	250
732	toluene	397
875	p-xylene	414
890	m-xylene	450
920	o-xylene	

Table 4.III.4 Continued

RETENTION INDEX	C O M P O U N D	SCAN NUMBER
985	trimethylbenzene	589
988	trimethylbenzene	634
1010	1,2-diethylbenzene	685
1058	sec-butylbenzene	
1082	sec-butylbenzene	
1140	4-ethyl-1,3-dimethylbenzene	735
1152	4-ethyl-1,2-dimethylbenzene	
1158	4-ethyl-2,3-dimethylbenzene	
1270	1,3-diethyl-5-methylbenzene	
1281	4-phenybutan-1-ol	
1290	2-methyl-1-butenyl-benzene	

Table 4.III.5: Results of analysis of paired blood samples from painters.

RATIO OF ANALYTE TO I.STD *														
n-hexane	n-heptane	n-octane	n-nonane	n-decane	n-und-ecane	n-dod-ecane	toluene	m-xylene	p-xylene	o-xylene	C_3^- benzene	C_3^- benzene	$C_{\bar{3}}$ benzene	$C_{\bar{4}}$ benzene
Case: 1														
1.87	0.23	1.0	0.11	1.6	0.4	1.3	1.0	1.3	1.9	0.5	0.2	0.2	0.9	0.27
1.37	0.46	0.92	0.44	1.7	0.6	0.9	1.5	1.5	2.6	1.0	0.8	0.5	2.3	1.1
Case: 2														
0.65	0.38	0.35	0.06	1.2	0.26	1.0	0.9	0.9	1.6	0.4	0.2	0.2	0.9	0.41
1.2	0.27	1.1	0.51	2.6	0.7	0.2	1.2	1.7	3.1	1.1	0.9	0.7	2.2	0.96
Case: 3														
2.51	0.19	0.67	0.07	1.2	0.3	0.7	0.2	1.5	2.6	0.7	0.3	0.3	1.0	0.37
4.1	0.35	2.4	0.24	4.0	0.5	2.3	2.1	3.6	4.6	1.2	0.6	0.5	1.7	0.42
Case: 4														
0.78	0.63	0.56	0.18	1.3	0.6	0.4	6.1	1.7	2.3	1.0	0.7	0.3	1.0	0.54
0.75	0.12	0.5	0.34	1.5	0.8	0.9	0.6	0.9	1.8	0.7	0.7	0.8	1.9	1.1
Case: 5														
3.8	0.53	2.2	0.14	2.9	0.5	1.9	1.8	2.5	3.2	0.9	0.6	0.6	1.8	0.67
1.16	0.33	1.2	0.5	2.6	1.1	1.4	1.5	4.0	6.5	1.9	1.3	0.7	3.7	1.86
Case: 6														
2.7	0.91	0.86	0.32	2.0	0.7	1.0	4.0	4.0	6.2	2.6	1.6	1.6	3.8	0.91
1.6	1.56	1.1	1.0	2.6	1.5	1.5	5.4	4.1	5.6	2.4	2.0	1.9	4.0	1.85
Case: 7														
4.3	0.32	0.3	0.2	2.5	0.6	1.6	2.3	3.9	6.7	1.6	0.9	0.9	3.3	1.6
5.0	3.4	2.1	1.4	1.9	0.4	0.9	0.5	7.9	9.9	4.5	1.9	2.1	5.9	2.0
Blank														
1.6	0.22	0.14	0.03	0.3	0.1	0.5	1.7	3.2	6.4	1.1	0.2	0.3	1.4	0.23
P value x 100% **			1.3	21	4.4	99					1.6	1.9	5.3	4.7

* Results are given for paired samples in each case. Upper lines represent results for samples taken at the beginning of the week, and lower line these taken at the end of the week.
** P is the probability value.

4.IV DISCUSSION

4.IV.1 INTRODUCTION

The volatile analytes which represent an exposure hazard to painters and which were of interest to this project have molecular weights in the range 100-200. They are therefore in a critical position which is dissimilar to the one occupied by low molecular weight volatiles which can easily be analysed by simple headspace methods [155,225,243,245,259,260] or direct analysis of a solution by GC [237].

At the same time they require special attention during a solvent extraction procedure because at the end of the extraction the solvent cannot be evaporated to complete dryness, otherwise all the analyte will be lost. The volume can only be reduced to a reasonable size, to minimize the dilution of the analyte, which might result in a procedure with insufficient sensitivity.

4.IV.2 OPTIMIZATION OF CHROMATOGRAPHIC PARAMETERS

In GC analysis of this type of extract, several factors have to be controlled to produce good chromatography characterized by sharp, narrow and reproducible peaks. These will yield improved resolution which benefits qualitative and quantitative analysis. These factors include:

1. Analytical Column
2. Injection Mode

3. Detection Method

4. Operating conditions, including flow rate of the carrier gas and temperature of the injector, column oven and detector ovens as discussed in Chapter 2.

Capillary columns in general have very good chromatographic efficiency. A non-polar phase such as CP-SIL 5 (a chemically-bonded dimethylsilicone) is a suitable choice for non-polar hydrocarbons, especially with a film thickness of 0.8-1.2μ, which is preferable for the range of hydrocarbons of interest and provides better separation of low boiling point compounds and increased sample capacity.

Although sharp peaks were obtained for higher hydrocarbons in the direct injection (splitless) mode, this was not suitable for the present procedure since the solvent peak tail obscured octane and nonane which led to irreproducibility in quantification, especially at low concentrations.

The Groß split/splitless mode of injection produced sharp peaks without the solvent peak obscuring early eluting peaks. However, this mode is not suitable either since the low molecular weight hydrocarbons were lost after the split was opened, similar to the solvent, resulting in reduced peak height. In both modes of injection described above, the solvent should focus the analyte at the head of the column. However, the early peaks were broad in the direct injection mode because the

'solvent effect' was not operating, while in the other mode part of the analytes were lost with the solvent.

A packed pre-column was also evaluated. The aim of connecting the packed column, containing a stationary phase (SE-30) which was similar to the capillary column phase, was to permit application of larger samples and provide retention of analyte while eluting the solvent. This technique was tried during this study with an inexpensive home-made system similar to that used in multi-dimentional GC and column switching which are now commercially available. Warming of the column oven helped eliminate the bulk of the solvent within a reasonable time. Where the n-alkane solution was applied in 10µl of diethyl ether and the split valve was left open for 3 min, the shape of the early eluting peaks was not adversely affected. However, this technique affected the reproducibility of the retention times of both octane and nonane (Table 4.III.1).

To improve on this would require very reproducible timing of injection and closing the vent which would best be obtained by automation. The relatively high coefficient of variation of the response (8-14%) is not a hindrance to using such a system since the response can be corrected with the presence of an internal standard (limonene) which has similar behaviour. Tetradecane was tested as an internal standard but was found to be impractical due to its low volatility and also because it was later found to be present in the background.

4.IV.3 SOLVENT EXTRACTION

In selecting a suitable solvent for extraction of the test hydrocarbons the following criteria were desirable. The chosen solvent needed to be a very volatile, high grade solvent with very low concentrations of volatile impurities as well as being a good solvent for the analytes. N-alkanes C_9 to C_{12} are relatively soluble in the selected solvent, diethyl ether (Table 4.IV.6), which provided good solubility and extracted the hydrocarbon effectively from blood samples. The addition of ammonium carbonate to the blood sample produced higher extraction efficiency which can be explained by the salt saturating the aqueous phase and therefore increasing the ether/water partition coefficient [261].

Solvent extraction was performed in non-silanized glass tubes. If silanized tubes were used, the extraction efficiency was lower due to non-polar interactions between the silanized glass and the hydrocarbons. This problem was encountered when such glass tubes from the general laboratory store were used inadvertently. Silanization was removed by washing the glass in 5M sodium hydroxide solution.

The high coefficient of variation for both octane and nonane(18%) is due to their high volatility relative to the other hydrocarbons (Table 4.IV.6) causing variable losses during the solvent volume reduction. Further solvent reduction at room temperature would result in

Table 4.IV.6: Physical properties of C_8 to C_{12} aliphatic and aromatic hydrocarbons.

Compound	B.P C^o	Density g/cm^3	M.P C^o	m.wt.	Solubility w/al/eth	TLV (TWA, ppm)
octane	125	0.70	-56	114	i/v/s	500
nonane	150	0.71	-53	128	i/v/s	200
decane	174	0.73	-24	142	i/v/s	
undecane	195	0.74	-25	156	i/v/s	
dodecane	216	0.74	-9	170	i/v/s	
toluene	110	0.88	-94	92	i/v/s	100
o-xylene	144	0.86	-25	106	i/v/s	100
m-xylene	139	0.86	-47	106	i/v/s	100
p-xylene	138	0.86	13	106	i/v/s	100
1,2,3-trimethyl-benzene	176	0.89	-25	120	i/v/s	100
1,2,4-trimethyl-benzene	169	0.87	-43	120	i/s/s	
1,3,5-trimethyl benzene	164	0.86	-44	120	i/v/s	
methylethyl-benzene	161	0.86	-95	134	i/v/s	
1,2-diethyl-benzene	183	0.88	-31	134	i/v/s	
1,3-diethyl-benzene	181	0.86	-83	134	i/v/s	
1.4-diethyl-benzene	183	0.86	-42	134	i/v/s	
propylbenzene	159	0.86	-99	120	i/v/s	
butylbenzene	183	0.86	-88	134	i/v/s	

* Solubility in water/alcohol/ether: v = very soluble; s = soluble; i = insoluble [263,264].
** TLV (TWA) is time weighted average TLV

further losses and even lower reproducibility. Similar extraction procedures, using n-pentane as an extracting solvent for petroleum fuel in blood have been described [244,262] where the coefficient of variation ranged from 5-24%. This procedure might still be used for routine application with inclusion of an internal standard to correct for losses. The sensitivity of the method that was obtained by GC analysis using flame ionisation

detection was not acceptable for occupational monitoring where lower levels might be present. Slight improvements could be made by recovering all the diethyl ether extract by freezing the centrifuged mixture, when the solvent could be recovered by decanting it without risking contamination from blood components.

4.IV.4 ANALYSIS BY GC-MS

Analysis of the extract by GC-MS was aimed towards enhancing the sensitivity of the procedure. The two-column arrangement could not be duplicated successfully on the mass spectrometer where the source pressure requirements limited the higher carrier gas flow rates through to produce good chromatography, similar to that obtained by GC analysis. Packed column GC-MS provided good chromatography: the bulk of the solvent and the excess of the carrier gas were eliminated by the jet separator. Analysis by SIR improved the sensitivity down to 15ng/ml and there was no interference in the peaks of interest. The reproducibility of such procedures was very good especially with an internal standard to correct for variation. However, the retention times of octane and nonane showed a higher variation relative to the others caused by slight variation in the opening time of the separator valve, as discussed in Chapter 2. The disadvantages of applying larger volumes of sample extract were the masking of early eluting peaks, which would limit the detection of small quantitites of analyte passing into

the source, and the danger of exceeding the limits on the source pressure that can be tolerated by the mass spectrometer.

4.IV.5 DESORPTION AND TRAP

The aim of using a trap column packed with Tenax-GC was to permit a large volume of extract to be applied where by passing the carrier gas the diethyl ether would elute at room temperature as it is highly volatile, and the less volatile analytes would be retained. The analytes were then transferred by desorption at high temperature and a relatively high flow rate to be collected in a cold trap, followed by transfer of the analytes to the capillary column. Care was taken with the geometry and plumbing of the cold trap in order that the analyte would be concentrated as a sufficiently narrow band in the inlet section of the column.

Tenax-GC is an organic polymer material and was selected due to its catalytic inertness, toleration of high temperatures (300°) without deterioration [192] and excellent retention characteristics for volatiles which can be reversibly desorbed [192,239,251,265]. In this assay the limiting factor for the flow rate during the desorption stage was the narrow diameter of the cold trap which resulted in a high linear velocity through the trap and inefficient trapping at high flow rates. This had to be weighed against the desorption time, so that all analytes would be sufficiently desorbed and trapped within a reasonable time. An assay of this type showed excellent

reproducibility. However, the final limiting factor for its application was the impracticality of applying larger volumes of extract (to improve sensitivity and reduce the coefficient of variation observed by reducing the diethyl ether extract) which required long periods to purge the solvent and resulted in blockage of the cold trap by the diethyl ether, if insufficient time was allowed for the solvent to be eluted.

4.IV.6 DYNAMIC HEAD SPACE

Dynamic head space elution methods have been extensively applied for extraction of volatiles from fluids and biological matrices (see Section 4.I.5) where it proved to be reliable. Effective stripping of a blood sample elutes the volatiles into a Tenax-GC column as in the above procedure, after which the volatiles are totally transferred to the analytical column. Also, they are transferred in a narrow band, without the presence of a solvent front which usually masks early eluting peaks. Hence the sensititivy will be greatly improved. To ensure high reliability and sensitivity, the extraction and analytical systems should be freed from contamination as much as possible.

4.IV.6.1 Sources of Contamination

The contaminating materials which were present in the tubing and regulators of both systems included aromatic hydrocarbons such as benzene, toluene, p, m and o-xylene, alkylbenzenes and aliphatic hydrocarbons (both unsaturated and saturated) in the range C_5 to C_{14}.

Most of these materials are present in paint and possibly in blood obtained from painters. The origin of these materials is thought to be from atmospheric organic pollutants which might originate inside the laboratory, especially xylenes, toluene, acetonitrile, dichloromethane and other solvents, or which might be released in the vicinity of the laboratory and circulated through the laboratory suite via the ventilation system, for example, car exhaust, or from general building work in progress in the Department, including painter work, during the period of study [191,193,266,267]. These contaminants entered the extraction system while the tubing was opened and exposed to the atmosphere. Although parts of the apparatus were exchanged with shorter lengths of clean tubing, the contaminating peaks were still present. This necessitated either changing all tubing and regulators and re-starting with a clean cylinder, or else, as a temporary solution inserting a cold trap just before the helium supply point to the manifold supplying the inlet systems. Attempts to use adsorbent traps containing activated charcoal failed as these were soon overloaded and became themselves a source of contamination. Cold traps using wide-bore tubing did not trap the contaminants very efficiently, but a long piece of tubing (10m x 0.5mm i.d.) provided a good trap. However, after repeated cooling and bringing up to room temperature while it was not in use, it later started to fail and to allow contaminant peaks to break through once more.

4.IV.6.2 Helium Stripping Volume

The extraction procedure was similar to that described for volatiles in blood samples from fire fatality cases [246,247,256]. Cooling of the condenser with cold water was discontinued, which led to higher recoveries for the higher hydrocarbons (C_{10} to C_{12}). When blood samples were being extracted, water vapour did not hinder the completion of the procedure but larger quantities of water collected in the Tenax column when aqueous samples were extracted. If the water was not sufficiently purged with helium at ambient temperature before thermal desorption, it frequently led to blockage of the capillary tubing of the cold trap. Similar results occurred when collecting air pollutants during humid weather on Tenax material [193]. Water can be selectively eluted from the Tenax-GC column due to the retention characteristics of the latter, such that hydroxylated compounds including water, lower aliphatic alcohols and aliphatic carboxylic acids are eluted before other non-polar and/or lower boiling compounds [239].

A stripping volume of helium of 800ml plus heating of the sample to 97°C with foam formation, provided efficient recovery for C_8 to C_{12} hydrocarbons. Heating of the sample to this temperature reduced the volume of helium required for C_{10} to C_{12}, which have higher boiling points and require more than 1.5L of helium to strip them from water at ambient temperature [252]. None of the added hydrocarbons broke through the Tenax-GC column with this volume, which is in agreement with

previous workers [252] and shows the retentive ability of the Tenax-GC material.

The efficiency of the extraction, reproducibility of the analytical procedure and its sensitivity should make it applicable to the analysis of blood samples for occupational hygiene monitoring of paint volatiles or others which share the same characteristics.

4.IV.7 ANALYSIS OF BLOOD FOR SOLVENTS

The aim of this part of the project was to develop a reliable and sensitive procedure for detection and quantitation of solvent in painters' venous blood. This procedure will be used for a future comprehensive study of a painter work force including physical and psychological examination, environmental monitoring and estimation of solvents in blood during the study period. During the development of the analytical procedure, factors which can influence solvent intake by the body, such as age, weight, smoking history, the state of health and atmospheric solvent levels were not evaluated. Also, an appropriate control group was not established. However, two groups of samples, at the beginning and end of a working week, were included to estimate the uptake. The work exposure and physical effort during this week could be described as moderate, where the painting was indoors, ventilation was natural and there were no protective devices.

Identified volatile peaks in chromatograms from both groups of blood samples were materials similar to those in a paint sample and to those present in the method

blank. Because of the small numbers in each group and the background interference, significant differences could not be shown between the two groups when their means were compared (unpaired T-test). However, the differences in the two values for each individual (paired T-test) were significant for nonane, undecane and alkylbenzenes, but an accurate estimation of their levels in blood could not be obtained due to the interference. An estimation of nonane and n-undecane levels in blood for the first group was 10 ± 6ng/ml and 18 ± 6ng/ml, respectively, and the second group was 41 ± 27ng/ml and 31 ± 15ng/ml, respectively. The levels in the first group were slightly higher than the background interferences. Alkylbenzenes were not estimated partly due to the unavailability of standards. However, these values give an indication of an uptake of aromatic and aliphatic hydrocarbon during the course of the working week.

In a previous experimental study on volunteers, the half life of white spirit was estimated to be between 46-48 hours [183] which could mean that the low levels of solvent in the first group were due to incomplete clearance of these solvents from the body. However, the modified method described provides sufficient sensitivity for the estimation of solvents in blood in future studies. In view of the problems encountered it will be necessary to clean or replace the extraction system, ensure that samples are collected in clean glass vials and to perform the analysis in an isolated place away from solvents and contamination.

C H A P T E R F I V E

5.I LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY

5.I.1 INTRODUCTION AND LITERATURE REVIEW

Mass spectrometry, GC and HPLC are fundamental techniques in the field of forensic toxicology. The establishment of dynamically coupled GC-MS provided an invaluable technique in this and other fields. During recent years, fruitful efforts have been applied to the coupling of HPLC and mass spectrometry (LC-MS), with the aim of providing a technique with the same status as GC-MS. Successful LC-MS coupling would have the following potential benefits:

I. HPLC has advantages over GC in that compounds are not exposed to excessive heat, so heat-labile compounds can be safely analysed.

II. Less sample cleaning is required.

III. Can be applied in analysis of polar compounds since derivatization is not usually necesssary in HPLC.

IV. LC-MS will compensate for the lack of versatility of conventional HPLC detectors.

V. LC-MS will complement the important role played by GC-MS.

5.I.2 METHODS OF INTERFACING LC AND MS

Direct on-line combination of LC and MS was previously considered as an unapproachable ideal due to

the apparent incompatibility of the two techniques. Mass spectrometry requires high vacuum and ionization of molecular species in the gas phase, while HPLC is intended for the analysis of relatively non-volatile compounds in liquid media. An ideal LC-MS interface should have the following criteria:-

I.. No significant compromises in the various operating conditions of both systems.

II. A high transport efficiency, that is, most or all of the analyte leaving the LC column should enter the mass spectrometer.

III. Should keep the integrity of a chromatographic profile.

IV. The performance of the interface should remain the same for the transmission of volatile, non-volatile and thermally labile compounds and they should not be chemically modified by the interface in an uncontrolled manner.

Several LC-MS interfaces have been developed successfully. However, none have satisfied the above criteria as successfully as a good GC-MS interface. They were recently reviewed in the literature [268-273] and a brief description of these methods will be presented. At present, the most commonly accepted LC-MS interfaces can be divided into two main categories, mechanical transfer interfaces and aerosol-based interfaces.

5.I.2.1 MECHANICAL TRANSFER INTERFACES

This category includes the moving belt

interface [278-286] and, earlier, the moving wire interface [287]. The concept is that liquid from the column effluent passes either by direct deposition or spray deposition [277], onto a moving polymer belt which passes through successive vacuum locks, pumped mechanically, prior to entering the mass spectrometer ionization source. Solvent vapour is removed in these evacuated areas, in conjunction with thermal irradiation to enhance the rate of solvent evaporation. The analyte is then removed from the belt in the ion source region by a flash vaporization process and the vaporized molecules may be ionized by electron impact or chemical ionization processes. Alternatively the sample on the belt surface is bombarded with the fast atoms for FAB ionization. The solvent capacity of the belt system is 1-1.5ml/min for volatile, non-polar solvents and 0.4-1ml for solvents having intermediate volatility, while that of the wire system was much lower. The disadvantages of the moving belt interface include a restriction on solvents in reversed phase systems, thermally labile compounds may be decomposed in vaporization from the belt interface, non-volatile ionic species must be derivatized for their ready removal from the belt and finally the possibility of cross contamination if the analyte is not completely removed from the circulating belt. The response of the system is in the region of 1-10ng of analyte. The moving belt interface has been recently reviewed by Arpino [288].

5.I.2.2 AEROSOL BASED INTERFACES

Several types of aerosol generation interface have been developed. A properly designed system has the following advantages over the moving belt interface:

I. Minimal sample cross-contamination.

II. Minimal peak broadening.

III. Solvent removal by rapid evaporation of the solvent from the aerosol droplet surface is faster than that in the moving belt interface.

IV. It has a simpler mechanical design.

5.I.2.2.1 DIRECT LIQUID INTRODUCTION(DLI)

In this interface [289-293], the effluent from HPLC is split and only part of the stream which can be tolerated by the mass spectrometer pumping system is introduced, or else the entire effluent of microbore HPLC with a low flow rate (1-10µl/min) is introduced directly to the mass spectrometer [294-301]. Splitting of the effluent is the main disadvantage of this interface as the transport efficiency is reduced. Also, solvent does not significantly evaporate from the aerosol droplets which produces CI spectra where the solvent molecules act as reagent gas.

5.I.2.2.2 ATMOSPHERIC PRESSURE IONIZATION(API)

Three types of LC-MS interfaces will be summarized:

I. Heated Pneumatic Nebulizer

This is a probe-type interface [271,302]. It takes full advantage of the large solvent vapour throughput tolerated by an API source and provides routine operation

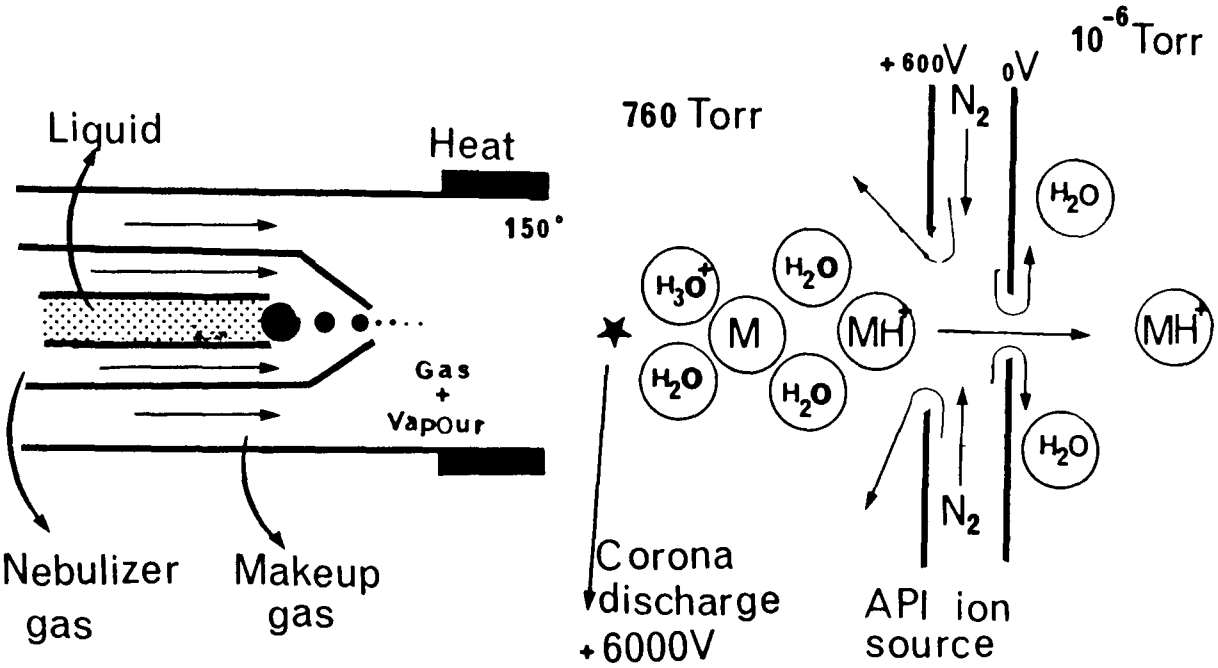
with an HPLC flow rate up to 2ml/min, including reversed phase systems. The effluent from HPLC passes through the central microbore tube (Figure 5.I.1) of the probe while the nebulizer gas and make-up gas are introduced coaxially into the heated nebulization region (150°C). The nebulized droplets are desolvated and produce a dry vapour of solvent and analyte molecule by the combined effects of the heat and gas flow. Ionization of these molecules is initiated by a corona discharge producing CI of the analyte by the solvent ions. These ions are focussed through a dry nitrogen curtain gas and then pass through a micro-orifice into the analyser which is in a high vacuum region.

II Electrospray

In this interface [271,303] a fine mist of charged droplets is produced by injection of the sample into nitrogen gas at atmospheric pressure through a metal capillary tube (Figure 5.I.2) at a potential of several kilovolts relative to the surrounding chamber walls. Dispersion of the mist results from repulsion forces on the charged surface of the droplets which overcome its surface tension. Then the emitted ions pass into the high vacuum of the mass spectrometer to the analyser. Optimum HPLC flow rates for operation are in the range of 5-10µl/min and CI spectra are produced.

III Monodisperse Aerosol Generation (MAGIC)

In this interface [270,304] a nebulizing gas is directed orthogonal to the flow of HPLC effluent and very



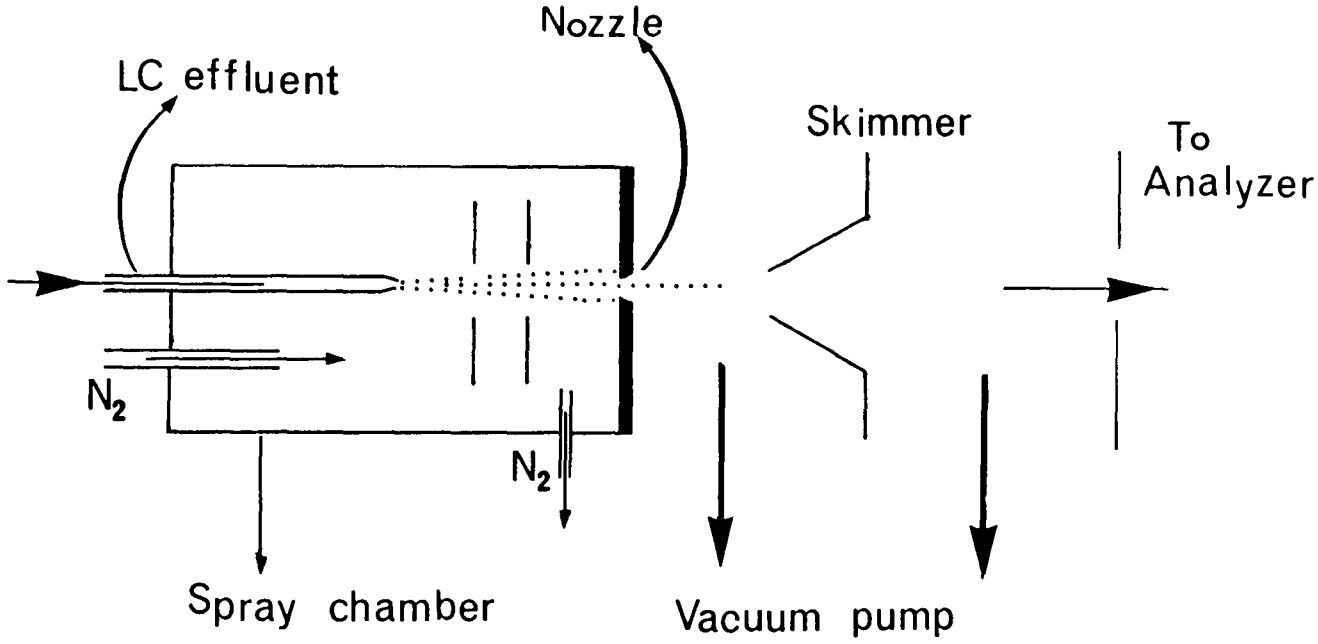


Figure 5.I.1 (Above) Schematic diagram of pneumatic nebulizer LC-MS interfase with an atmospheric pressure ionization (API) source.

Figure 5.I.2 (Below) Schematic diagram of electrospray LC-MS interfase.

uniformly-sized droplets are generated. A dispersion gas is used to prevent clustering of the drops as they are passed through a desolvation region on their way to the mass spectrometer ion source (atmospheric pressure) (Figure 5.I.3). The aerosol beam enters the high vacuum region of the mass spectrometer via an aerosol beam separator. Optimal operation occurs at flow rates of 0.1-0.5ml/min and both EI an CI spectra can be obtained.

5.I.2.2.3 THERMOSPRAY

I. Introduction

The thermospray (TSP) system, developed by Vestal [305], has achieved widespread use recently due to the simplicity of its operation where solvent removal and ionization of the sample occur in one process. In this system (Figure 5.I.4) eluent from HPLC is pumped through a capillary tube which is heated by passing a current through it. The eluent emerges from the capillary in a form of an expanding jet of vapours and droplets. The aerosol jet subsequently enters the thermospray chamber. Ions formed are sampled via the orifice in the sampling cone assisted by the lateral expansion of the jet that occurs as it traverses the ion source. The excess of solvent is removed directly via the pumping line. A TSP interface permits up to 2ml/min of polar solvent to be introduced to the mass spectrometer. One restriction is that the solvent should contain at least 20% by volume of an aqueous solution of ammonium acetate for ionization to be possible [306].

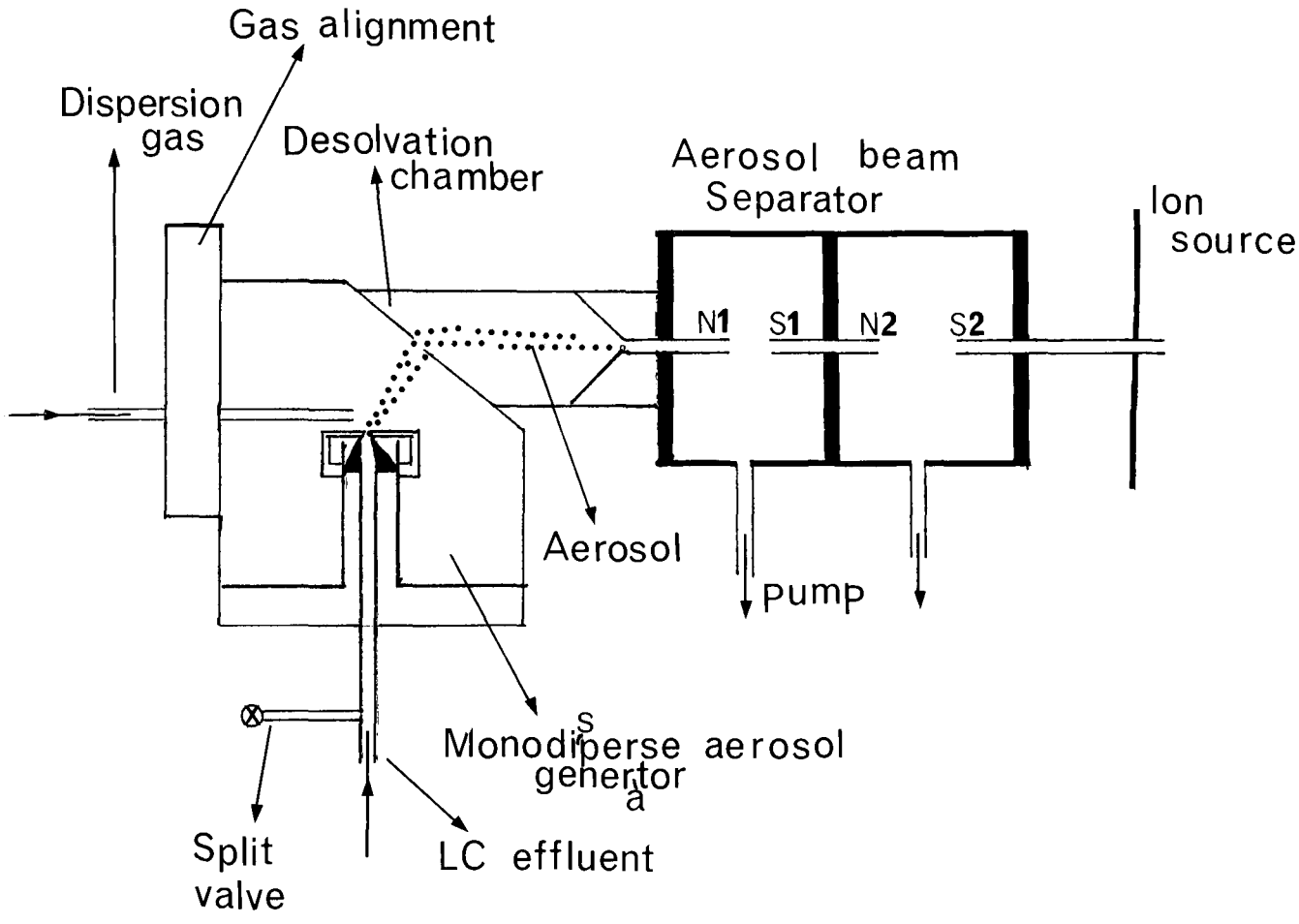


Figure 5.I.3 Schematic diagram of monodisper aerosol generator (MAGIC) LC-MS interfase. N1 and N2 a nozzle 1 and 2, S1 and S2 are skimmer 1 and 2.

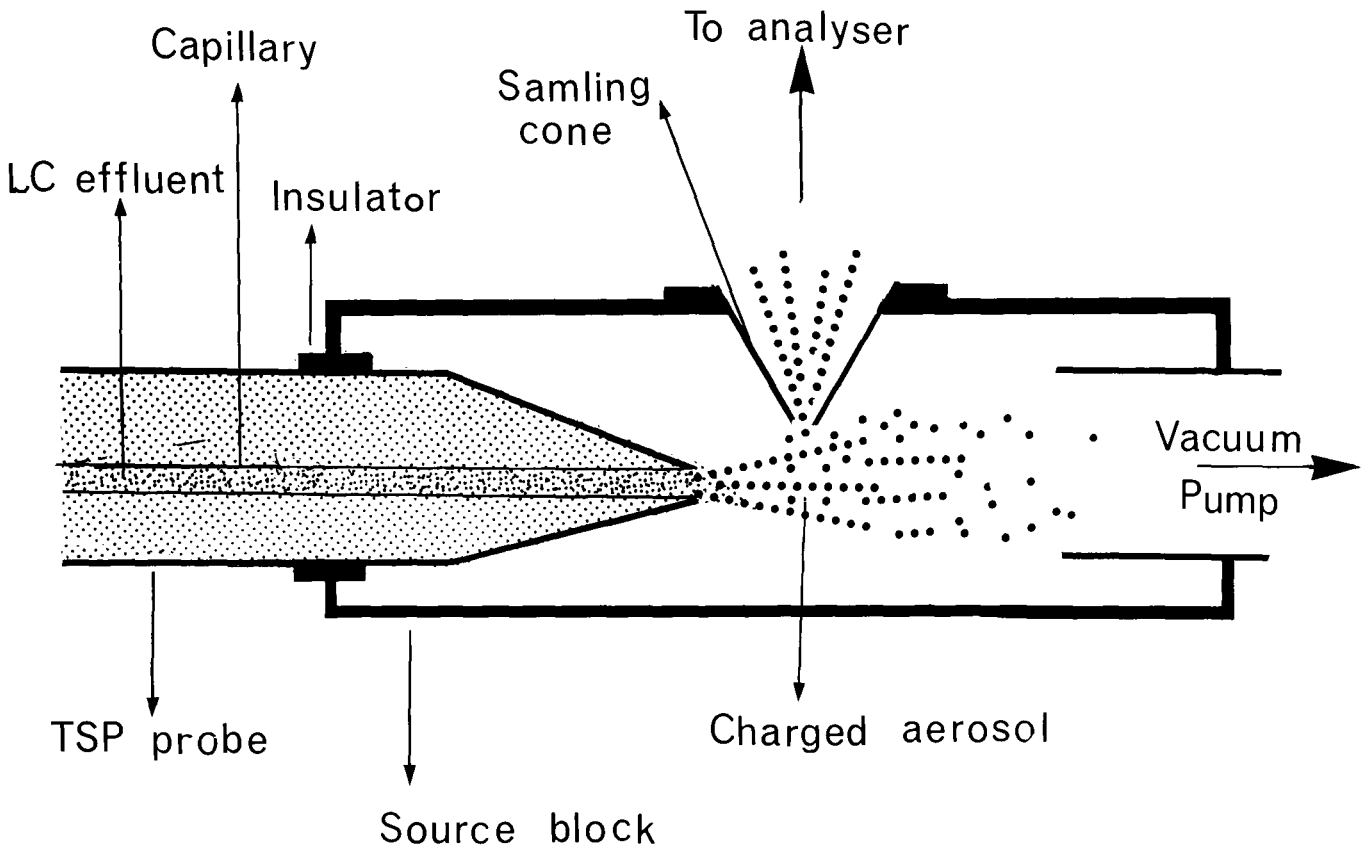


Figure 5.I.4 Schematic diagram of Thermospray (TSP) LC-MS interfase.

II. Ion Generation in Thermospray

1. Thermospray Ionization

Two distinct stages are involved in TSP mass spectra [307,308]. The first stage is the formation of the primary gaseous ions out of the microdroplets produced by the vaporizer. The mechanism of their formation is believed to be as follows. Complete varporization of the liquid at the rate at which it is supplied to the vaporizer produces a superheated mist carried in a supersonic jet of vapour. The droplets of the mist are charged positively or negatively according to random statistical fluctuations in the distribution of these charges in the droplets [304,309]. Molecular ions clustered with a few solvent molecules evaporate from the superheated droplets assisted by the high local electrical fields generated by the charge on the droplet.

In the second stage, new product ions are produced in a gas-phase ion/molecule reaction by removing the primary TSP ions [304,309,310]. This gas-phase ion/molecule reaction was found to have a strong influence on the relative ion intensities in TSP mass spectra in the presence of ammonium acetate [307], and the gaseous molecules were protonated by transfer from the ammonium ion [311]. The mass spectra have characteristics similar to those produced by ammonia CI, and exhibit both $[MH]^+$ and $[MNH_4]^+$ pseudo-molecular ions. The former is produced at the expense of the latter and their formation is dependent on the proton affinity of the sample molecule with respect to ammonia.

2. Plasmaspray(PSP) Ionization

Ions in PSP are achieved by initiating a glow discharge inside the ion source using the thermospray probe tip as an electrode (Figure 5.I.5). The probe is maintained as a cathode at a voltage of approximately -600V with respect to the source block (anode) and is operated with a discharge current of 0.5mA [312]. All analytes and solvents entering the ion source must pass through the cathode dark space which surrounds the probe tip. This region of the discharge is especially rich in energetic ions, neutrals and metastable species [313] which are primarily responsibile for the PSP ionization. In this mode there is no restriction on the mobile phase constituents for ionization to be possible and it is an energetic process in which more fragmentation of the molecule appears than in TSP.

3. Thermospray with Filament-on Ionization

This uses an external source of ionization such as a conventional electron filament, and is therefore called called "filament-on" TSP [314-316]. It eliminates the need for the presence of water and buffer in the mobile phase, which are required for true TSP ionization, hence normal phase and gradient elution HPLC eluents can be applied directly to the mass spectrometer without post-column addition [317] of the buffer. This method also increases the possibility of molecular fragmentation.

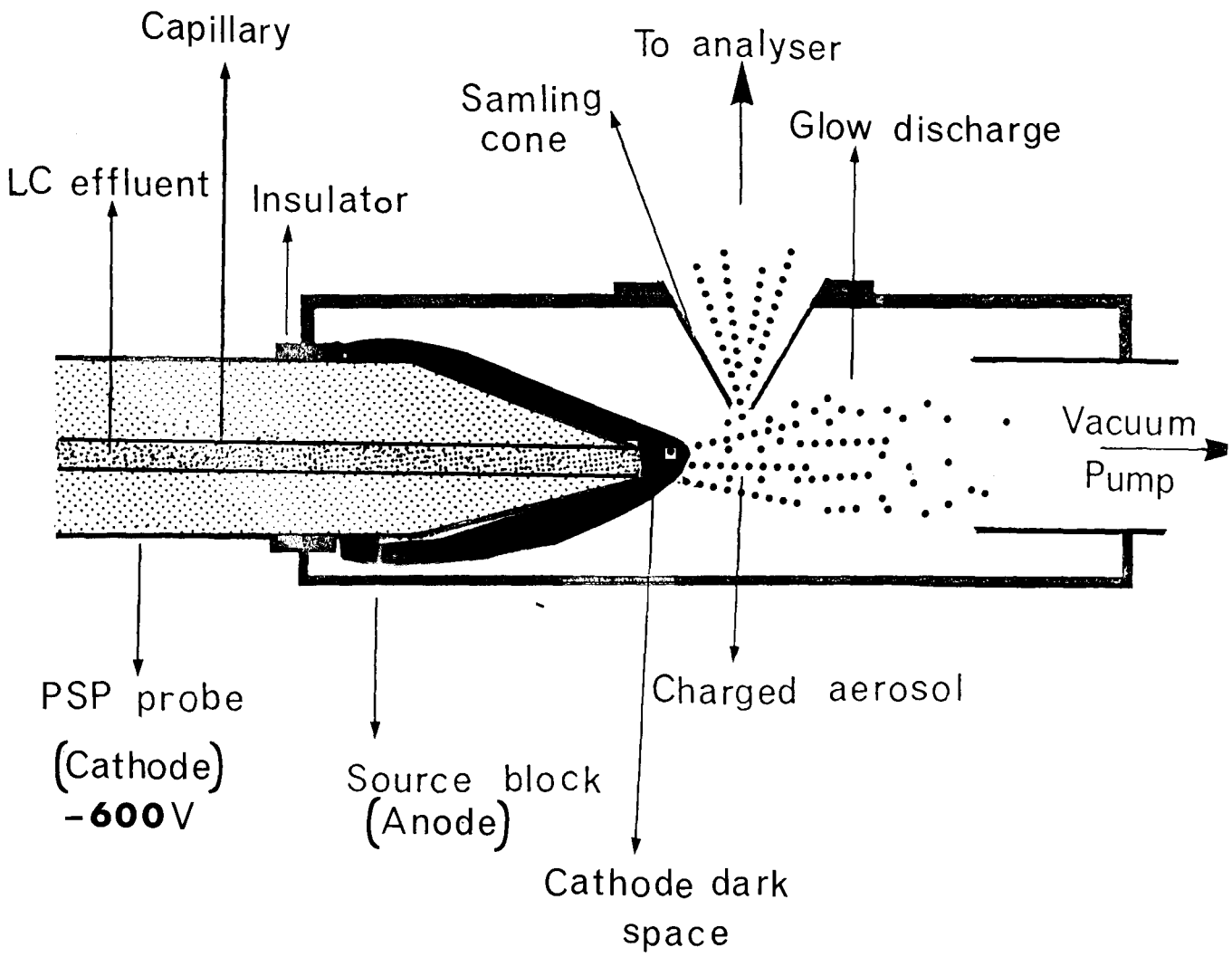


Figure 5.I.5 Schematic diagram of Plasmaspray (PSP) LC-MS interfase.

5.I.3 APPLICATIONS OF LC-MS

Although none of the developed LC-MS interfaces are as universal as the established GC-MS interface, an understanding of the benefits and limitations of each interface has permitted users to take full advantage of their specific capabilities. The literature contains many practical applications of LC-MS (Table 5.I.1). These applications include a large set of substance classes that are difficult to investigate using conventional GC-MS, such as very polar and thermally labile compounds as well as those with high molecular weight and low volatility, especially with the availability of different ionization methods which could be used in these interfaces [308,348]. Specific examples include the separation of therapeutic agents and their metabolites, such as glucuronide conjugates, from biological fluids [319,321,323,326], other thermally labile biologically-active compounds from their media [297,334,340,344] and metabolites of explosives from biological fluids [340]. Most of the reviewed methods using LC-MS interfaces provide qualitative and quantitative procedures for specific analytes with very good sensitivity. In general terms, the sensitivity of the detection has been determined to be compound-dependent and difficult to predict, especially in TSP-MS. However, impressive full scan data can be acquired at 10-100 ppt (parts per trillion) under TSP conditions [316]. The more recently developed PSP should provide higher sensitivity and stability compared to TSP [312].

Table 5.I.1 References to LC-MS applications using Thermospray (TSP) Moving Belt (MBI) and Direct Liquid Introduction (DLI) interfaces for different types of analyte.

Number		T S P	M B I	D L I
1	Therapeutic Agents			
	Antibiotic	318		
	Antiviral	319		
	Amphetamines	314	282	
	Diuretic	320		
	Analgesic			321,322
	Others	299,323-327	274,275,281	289,291,294
2	Steroids and their Conjugates	327,328	329	302
3.	Pesticides and Herbicides	317,330-333	274,279,292, 295,334	300,335-337
4.	Environmental Hazards - Dyes, Explosives, Polynuclear Aromatic Hydrocarbons	316,318,339	383	340
5.	Fatty Acids	315,341,342		293
6.	Plant and Fungal Products	334,343,344		297
7.	Organic Acids	284,345		
8.	Enzymes and Peptides	346,347		

In this study both TSP and PSP were assessed as an LC-MS interface for detection of the commonly encountered drugs in forensic toxicology. These drugs have established HPLC methods. Positive results by HPLC could then be confirmed and the nature of the unidentified peaks could be established using LC-MS, similar to the situation of using GC and GC-MS. Initially conditions were assessed which determine TSP and PSP sensitivity, such as operating temperature of the source and probe in TSP, as well as the applied voltage and mobile phase constituents.

5.II EXPERIMENTAL

5.II.1.1 MATERIALS AND REAGENTS

Methanol (MeOH) and acetonitrile (ACN) were HPLC (glass distilled) grade solvents. Water was purified in a MIKI water purification system (Millipore, U.K.). Ammonium acetate was Aristar grade obtained from BDH, U.K.

5.II.1.2 INSTRUMENTATION

An LKB model 2150 HPLC pump was used for solvent delivery. It was connected to a VG Thermospray/Plasmaspray probe by a switching valve, either directly via stainless steel tubing (25cm x 0.1mm I.D) which by-passed the HPLC column (subsequently referred to as "loop only injection"), or via an HPLC column (25cm x 4.6mm I.D) packed with Hypersil ODS, 5um spherical silica, obtained from HPLC Technology, U.K. An in-line low-dispersion filter with a 2µm frit (Hewlett Packard, U.K.) was placed at the inlet of the TSP probe. Mobile phases were degassed with helium before use using a 2µm solvent filter. Samples were introduced by a 25µl SGE syringe via a Rheodyne injection valve (Model 7125S) fitted with a 20µl sample loop. The LC-MS interface was a VG Thermospray/Plasmaspray model. The stainless steel capillary tubing of the probe (0.1mm I.D or 0.25mm I.D) tube was silver-soldered to the probe frame. The probe was isolated from the HPLC tubing by a deactivated fused silica tube (1m x 0.1mm I.D) which acted as a filter at the same time (Figure 5.II.1). The interface was

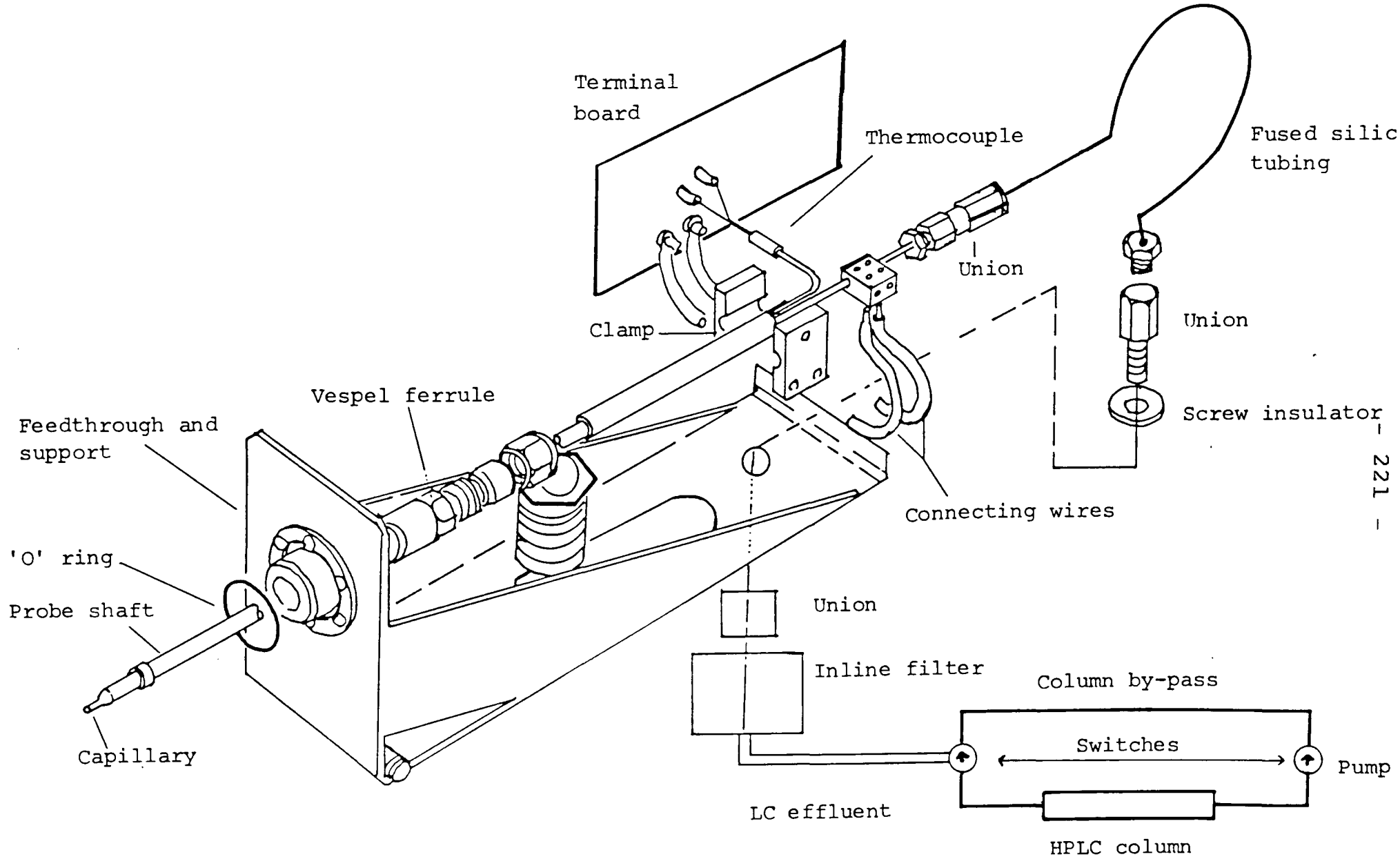


Figure 5.II.1 Thermospray/Plasmaspray probe assembly.

connected to the model VG 70-250S mass spectrometer with minor modifications, by replacing the EI/CI source with a special TSP source and attaching a line to remove excess solvents connected to a high-temperature rotary vacuum pump.

5.II.2 SAMPLE PREPARATION

Stock drug standard solutions were prepared in MeOH at approximately 10µg/ml each. Polyethylene glycol (PEG) stock solution was prepared by dissolving approximately 2g of PEG 200, 400, 600, 800, 1000 and 1200 in MeOH/0.1M ammonium acetate buffer in water (1:1, v/v) in a 100ml volumetric flask giving a final concentration of 2% w/v for each PEG.

5.II.3 CALIBRATION OF THE MASS SPECTROMETER

The mass spectrometer was initially tuned on the ammonium acetate ions at m/z 78 or 59 in the positive or negative modes, respectively, at the start of each period of work. The peak intensity was maximized by adjusting the temperature of the probe in the range 200-220°C for TSP or 270-300°C for PSP ionization. The flow rate of mobile phase, containing 0.1M ammonium acetate buffer:MeOH (1:1, v/v) was set to 1ml/min. The source temperature was set at 250°C, the multiplier at 2.5KV and the accelerating voltage at 6KV. The source pressure was monitored by a Pirani gauge, normally showing 5×10^{-4} mbar, and an Edwards digital pressure meter. Mass

calibration of the spectrometer was carried out using PEG as a reference as described below.

5.II.3.1 FULL SCAN MODE

The stock solution of PEG was diluted 100 times in mobile phase and filtered over Millex-HV4, 0.45µm filter before use. An aliquot of 20µl (containing 4µg of each PEG) was introduced to the mass spectrometer via loop only injection. The acquisition range was from 600-70 at 1 sec/decade, with an interscan delay of 0.3 sec.

5.II.3.2 SELECTIVE ION RECORDING MODE

A fresh solution of PEG in mobile phase (0.005% w/v) was introduced continuously via the by-pass to the probe. Tuning of the mass spectrometer was done on a reference mass from PEG nearest to the selected masses for SIR. During calibration, the computer selected the nearest upper and lower masses from PEG, which bracketed the masses chosen for the analyte, as references for calibration.

5.II.4 OPTIMIZATION OF RESPONSE

The following parameters influencing the response of the mass spectrometer when operated with the TSP/PSP LC-MS interface were studied:

1. Probe temperature.
2. Source temperature.
3. Probe discharge voltage in PSP.
4. Mobile phase composition.

5.II.4.1 PROBE TEMPERATURE

The effect of probe temperature in both TSP and PSP

modes on sensitivity and on the fragmentation patterns of the analyte molecules were evaluated in the following experiments:

I. A standard solution of amitriptyline (2mg/100ml) in mobile phase (0.1M ammonium acetate:MeOH, 1:1, v/v) was introduced to the mass spectrometer via the column by-pass and probe at 1ml/min. The plasmaspray emission in the positive ion mode (PSP+), was set at a current of 0.5mA, the source temperature at 270°C and the multiplier voltage at 4KV. The probe temperature was started at 150°C and raised to 340°C in 10C° steps and approximately 50 scans were recorded at each temperature. The scans obtained at each temperature were averaged and the spectra were compared for the intensity of the fragments obtained such as m/z 78 and the molecular ion of amitriptyline as well as for the sensitivity of detection based on the reconstructed TIC and mass chromatograms for ions in the mass spectra of the buffer and analyte.

II. The same experiment as above was repeated using TSP in the positive ion mode [TSP(+)] while the multiplier voltage was set at 5KV.

III. A standard solution of amylobarbitone (1.35mg/100ml) was prepared as above, and the above experiments (1 and 2) were repeated for both PSP and TSP using the negative mode of ionization (PSP(-) and TSP(-), respectively).

IV. A mobile phase of 0.1M ammonium acetate:MeOH (1:1, v/v) was introduced as above. The effect of the

probe temperature in PSP(+) at settings of 240°, 270°, 300° and 330°C was assessed with respect to sensitivity and fragmentation of the following drugs: cannabidiol, doxepin, propranolol, buprenorphine, flupenthixol, indomethacin, morphine and triazolam. An aliquot of 20µl containing 200ng of sample was introduced for each drug individually at each temperature. The peak shape, background noise in the chromatogram and sensitivity were recorded.

5.II.4.2 SOURCE TEMPERATURE

The effect of source temperature on mass spectral peak shape and fragmentation was assessed by repeating experiment number 4 above. The probe was set at 300°C and the following source temperatures were tested: 240°, 270°, 300° and 320°C.

5.II.4.3 PLASMASPRAY DISCHARGE VOLTAGE

The effect of the PSP discharge voltage on sensitivity of detection and on the fragmentation patterns of analyte molecules was assessed by repeating the above experiment. The source temperature was set at 270° and the voltage at the probe tip was increased in steps. Morphine and triazolam were used as test compounds at each PSP discharge setting.

5.II.4.4 MOBILE PHASE CONSTITUENTS

The mobile phase constituents used for liquid chromatography were studied to assess their effect on sensitivity using the PSP LC-MS interface, with buprenorphine at 10µg/ml as a model compound under the following conditions: flow rate 1ml/min, multiplier

voltage 3KV, source temperature 270° and probe temperature 270-280°.

The following mobile phases were assessed:

i. Individual constituents: water, MeOH and ACN.

ii. Ammonium acetate buffer in water: 0.01-0.3M.

iii. 0.15M buffer:MeOH (1:1, v/v).

iv. 0.15M buffer:ACN (1:1, v/v).

v. 0.15M buffer:MeOH:ACN (2:1:1, v/v:v).

Five replicate samples of 20µl of buprenorphine were introduced in each mobile phase. The sensitivity was assessed by comparing the peak areas of m/z 450 ($[M + H]^+-H_2O$) for buprenorphine. The peak shape, background interference, fragmentation pattern and HPLC pump pressure were observed.

5.II.5 REPRODUCIBILITY OF RESPONSE

The reproducibility of response in the mass spectrometer using the LC-MS interface in the PSP(+) mode was tested by repeated introduction of buprenorphine and doxepin (20µl aliquots containing 200ng of each drug) under the conditions described in Section 5.II.4.4. Both peak heights and areas were compared.

5.II.6 SENSITIVITY

The sensitivity was tested in both full scan and SIR modes using buprenorphine as a model compound, under the following conditions: flow rate 1ml/min, mobile phase 0.1M ammonium acetate:MeOH (1:1, v/v), multiplier voltage

5KV, source temperature 270°C, and probe temperature 240-250°C, scan range in full scanning mode was 150-600, scan rate 1 sec/decade and interscan delay 0.3 sec. Acquisition in SIR was on a single ion, mass 450, the dwell time 80msec and interchannel delay 20msec. The detection limit was determined by introducing an aliquot of buprenorphine solution containing 0.3-160ng via a 20µl sample loop and the direct connection (column by-pass) route, under both modes of acquisition.

5.II.7 DRUGS AMENABLE TO LC-MS

Several drugs representing different groups of compounds which are commonly encountered in forensic toxicology were studied. Each drug was prepared in mobile phase at concentrations of approximately 10µg/ml where 20µl of each drug was individually introduced via the direct connection. The mass spectra of these drugs in full scan PSP(+) mode (as in Section 5.II.6) were obtained to assess the usefulness in the forensic field of the LC-MS interface and the information which might be obtained from their fragmentation patterns.

5.II.8 HPLC-MS

The applicability of the TSP/PSP LC-MS interface for on-line connection and introduction of the HPLC effluent was assessed using the following three models of separation:

1. Mixture of basic drugs.

2. Mixture of sedative/hypnotic drugs and anticonvulsants.

3. Mixture of opiates.

5.II.8.1 BASIC DRUG MIXTURE

This was a mixture of basic drugs composed of oxazepam, diazepam, pentazocine, codeine, caffeine, dextromoramide, dextromethorphan, amitriptyline, pethidine, dipipanone and methadone, each at a concentration of approximately 10µg/ml. Separation was performed on the ODS-column with a mobile phase based on a mixture of ammonium acetate buffer, MeOH and ACN. The ratios (v/v) of the mobile phase constitutents and the concentration of the buffer, without pH adjustment, were varied to obtain optimum chromatography and flow rate within the capabilities of both HPLC column and vacuum system.

The following mobile phases were tested (ratios are v/v):

1. Methanol:water (80:20).
2. Methanol:0.05M buffer (80:20)
3. Methanol:0.1M buffer (80:20).
4. Methanol:0.1M buffer(90:10).
5. Methanol:ACN:0.2M buffer (80:10:10).
6. Methanol:ACN:0.2M buffer (65:20:15).
7. Methanol:ACN:0.2M buffer (50:30:20).

The interface and source temperatures were both set at 270°C and the mass spectrometer was operated in the PSP(+) full scanning mode with a mass range of 150-600.

The multiplier voltage was at 4KV. Comparison of chromatograms was performed using limited total ion current (LTIC) chromatograms and reconstructed ion chromatograms of the protonated molecular ions $[M + H]^+$ of the analytes.

5.II.8.2 HYPNOTIC AND ANTICONVULSANT DRUGS

Similar experiments to those detailed above were performed for a mixture of hypnotic/sedative and anticonvulsant drugs, composed of primadone, phenobarbitone, butobarbitone, amylobarbitone, glutethimide, phenytoin, methaqualone, carbamazepine and quinalbarbitone. The mobile phase 0.1M buffer:ACN:MeOH, (110:50:50, v/v/v) was a modification of a system described in the literature for such drugs [349], obtained by substituting ammonium acetate buffer for phosphate buffer. The pH was adjusted to pH5 with acetic acid. The flow rate was 1ml/min and other parameters were as described in the previous section. Acquisition was performed in full scan mode and both PSP(+) and PSP(-) mode spectra were obtained.

5.II.8.3 SEPARATION OF OPIATES

A mixture of morphine, morphine-3-glucuronide (M3G), nalorphine and buprenorphine, at a concentration of 10µg/ml each was made as a model for this type of separation. Experiments were performed as in the previous two sections, based on a similar mobile phase with the addition of diethylamine to obtain the optimum chromatogram and a suitable flow rate with reasonable pressures in the HPLC pump and ion source. Initially the

effect of probe temperature on the M3G fragmentation was assessed in the PSP(+) mode via the by-pass inlet for temperatures between 120-300°. The chromatography was then performed while the probe temperature was at 120-200° to locate M3G and the other drugs.

5.II.9 CLEANING THE PROBE AND SOURCE

The probe was cleaned at the end of each working day by passing water via the column by-pass connection at 150-200° for 15-30 minutes using the TSP mode while the mass spectrometer was on the standby position. A partially blocked probe was cleared as described in the literature [350]. The probe was removed from the mass spectrometer and citric acid solution (0.1M in distilled water) was passed through at a flow rate of 0.1-1ml/min according to the extent of blockage. The vaporizer temperature was slowly increased from 100-150° to permit as high a flow rate as possible to be used within the pressure limit of the system, until the back pressure dropped to the original level at that setting typically 50bar at a flow rate of 1ml/min of water in a probe of 0.1mm I.D. at 200°C. This procedure was followed by washing with distilled water. The probe tip was also polished under a flow of water. A completely blocked probe was replaced by a new one.

The metal sampling cone and the inside of the source block were polished with fine grade emery paper, followed by ultrasonication for 3 minutes in acetone, followed by air drying before installation.

5.III R E S U L T S

5.III.1 CALIBRATION OF THE MASS SPECTROMETER

Calibration tables for the mass spectrometer, which were stored in the computer data system in several calibration files, were found to be usable over a long period of time for the full scanning mode, while a new calibration was needed each day when analyses were performed by SIR. Although the introduction of PEG was *via* the direct connection and the system was thoroughly rinsed with the mobile phase, it used to interfere with subsequent analyses and required the source temperature to be raised to 300°C for ½-1 hour after the process of calibration to eliminate high molecular weight PEG condensed on the source block.

During full scan acquisition starting from low mass, solvent ions at low mass were the major background ions and no well-defined peaks could be seen in the TIC chromatogram for analytes at low concentration. Examples of these masses ions originating from the mobile phase constituents are listed in Table 5.III.1. These ions were used for initial tuning of the mass spectrometer. The problem of high noise levels was avoided by stopping the down scan at a high mass such as 150, or by generation of a limited total ion current (LTIC) chromatogram for masses above 200, for example.

Table 5.III.1 Major solvent ions generated by plasmaspray.

1.	Positive Ions	
	Masses	Origin
	19,37,55,73	$[(H_2O)_n + H]^+, n = 1-4$
	33,65,97	$[(CH_3OH)_n + H]^+, n = 1-3$
	42,83	$[(CH_3CN)_n + H]^+, n = 1-2$
	77,78	$[Acetate + H]^+$
	95	$[Acetate + NH_4]^+$
	100	$[(CH_3CN)_2 + NH_4]^+$
2.	Negative Ions	
	Masses	Origin
	59	$[CH_3COO]^-$
	119	$[(CH_3COO)_2 + H]^-$

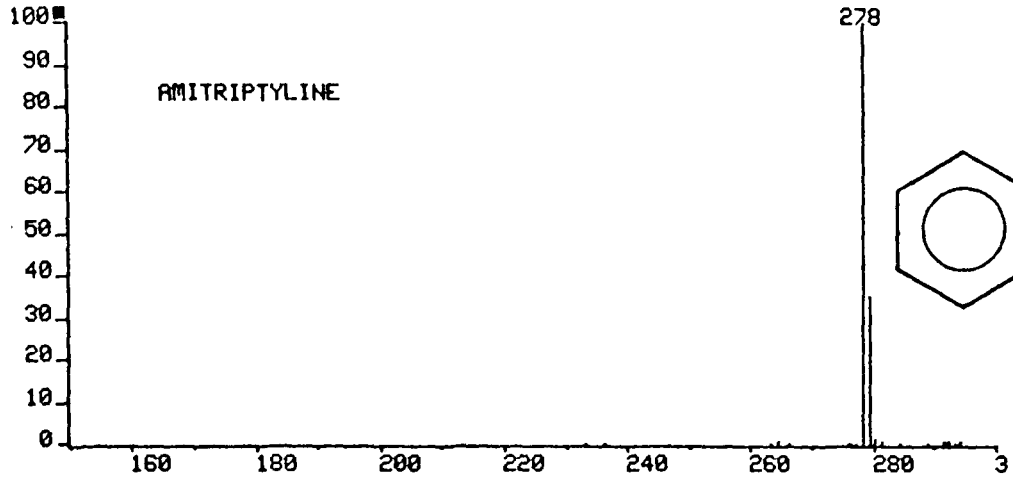
5.III.2 EFFECTS OF PROBE TEMPERATURE

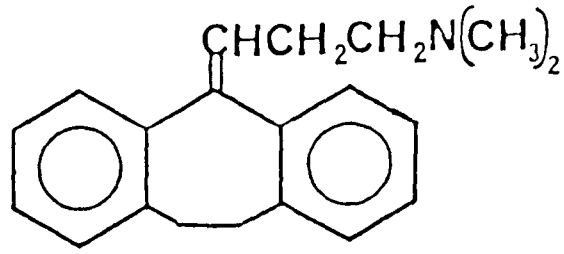
During the experiments which were conducted to study the influence of variation of the operating temperature of the TSP/PSP probe of the LC-MS interface, the probe temperature showed a profound effect on the mass spectral peak shape and intensity. In general, the peaks obtained at the optimum temperature for an analyte were more stable and smoother in the PSP mode than those obtained in the TSP mode of operation, and the background noise level was much lower in the former. Only positive ions were produced in significant amounts in the continuous analyte introduction experiment with amitriptyline, while both positive and negative ions were produced from amylobarbitone. The sensitivity for the latter was 10 times higher in the negative ion mode. The

chromatogram obtained using PSP(-) was free from background noise from the mobile phase constituents, which had a high intensity level in the positive ion mode. The mass spectra of amylobarbitone and amitriptyline are shown in Figure 5.III.1. These two drugs did not show any differences in the fragmentation patterns as a result of changing the probe temperature, which significantly influenced the sensitivity in both TSP and PSP modes.

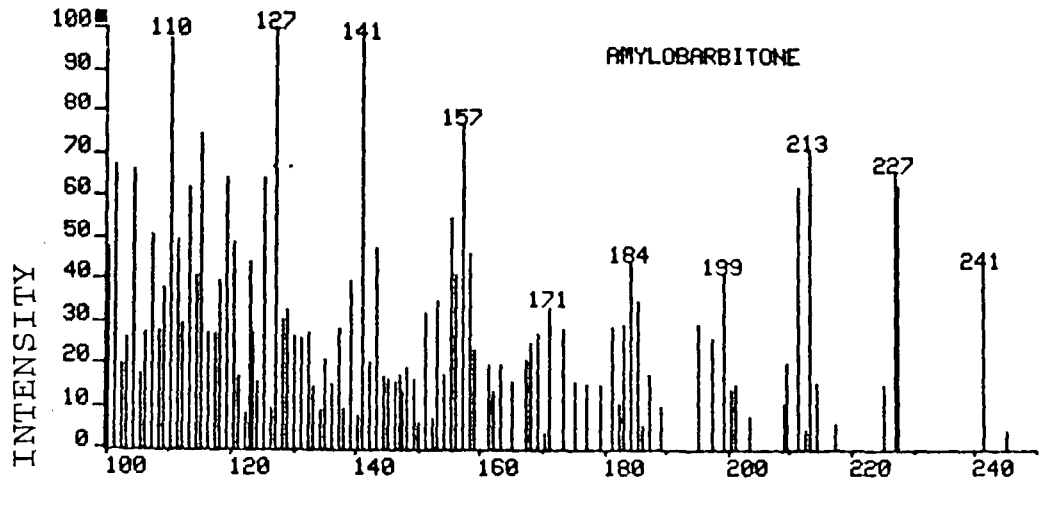
The amitriptyline experiment was conducted by continuous introduction of amitriptyline solution as a concentration of 2mg/100ml in the mobile phase *via* the by-pass connection while the probe temperature was raised from 180°C to 300°C in 10° steps and approximately 50 scans were acquired at each temperature. In PSP(+) mode the protonated molecular ion (m/z 278), which was the most intense analyte ion, showed the maximum intensitiy between 220-230° and a gradual loss of intensity outside this range down to 210° and up to 260°C, followed by a sharp drop after 260°C. The protonated molecular ion of ammonium acetate (m/z 78) did not follow the same pattern as the analyte: its highest response occurred where the intensity of m/z 278 for amitriptyline dropped (above 260°C) followed by a gradual drop with further increases of temperature (Figure 5.III.2a).

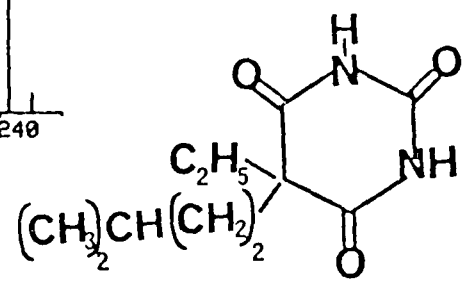
During TSP operation, the maximum response for m/z 278 was over a narrow range 190-200°C with a sharp drop at higher and lower temperatures. The response to m/z 78 corresponded to that of m/z 278 with a wider range over 190-230°C (Figure 5.III.2b). However, at lower





M Wt 277





M Wt 226

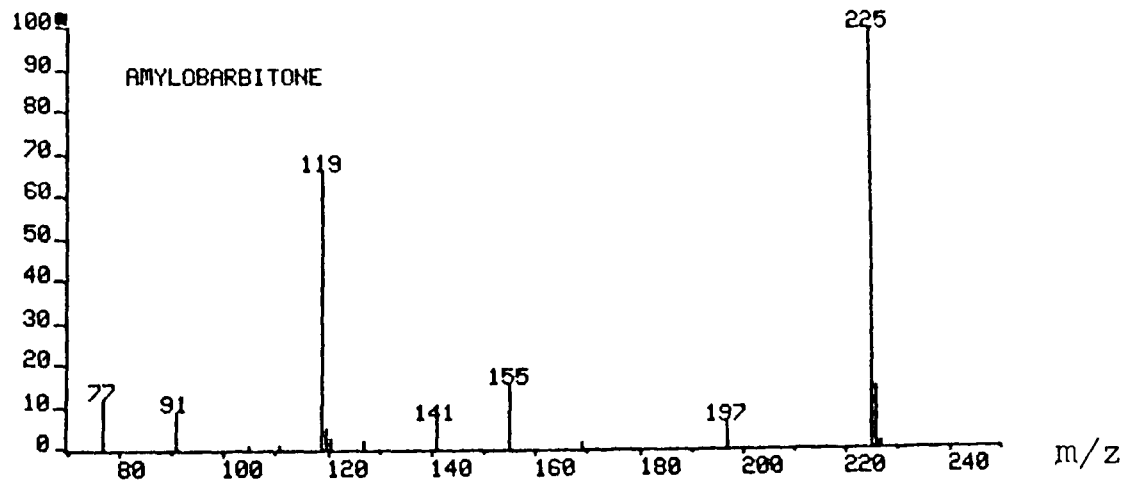


Figure 5.III.1 Plasmaspray-generated mass spectra of amitriptyline and amylobarbitone obtained in positive (a and b) and negative (c) ionization modes. The mobile phase was methanol:ammonium acetate buffer (0.1M), 1:1, v/v and the TSP/PSP probe temperature was 220°C.

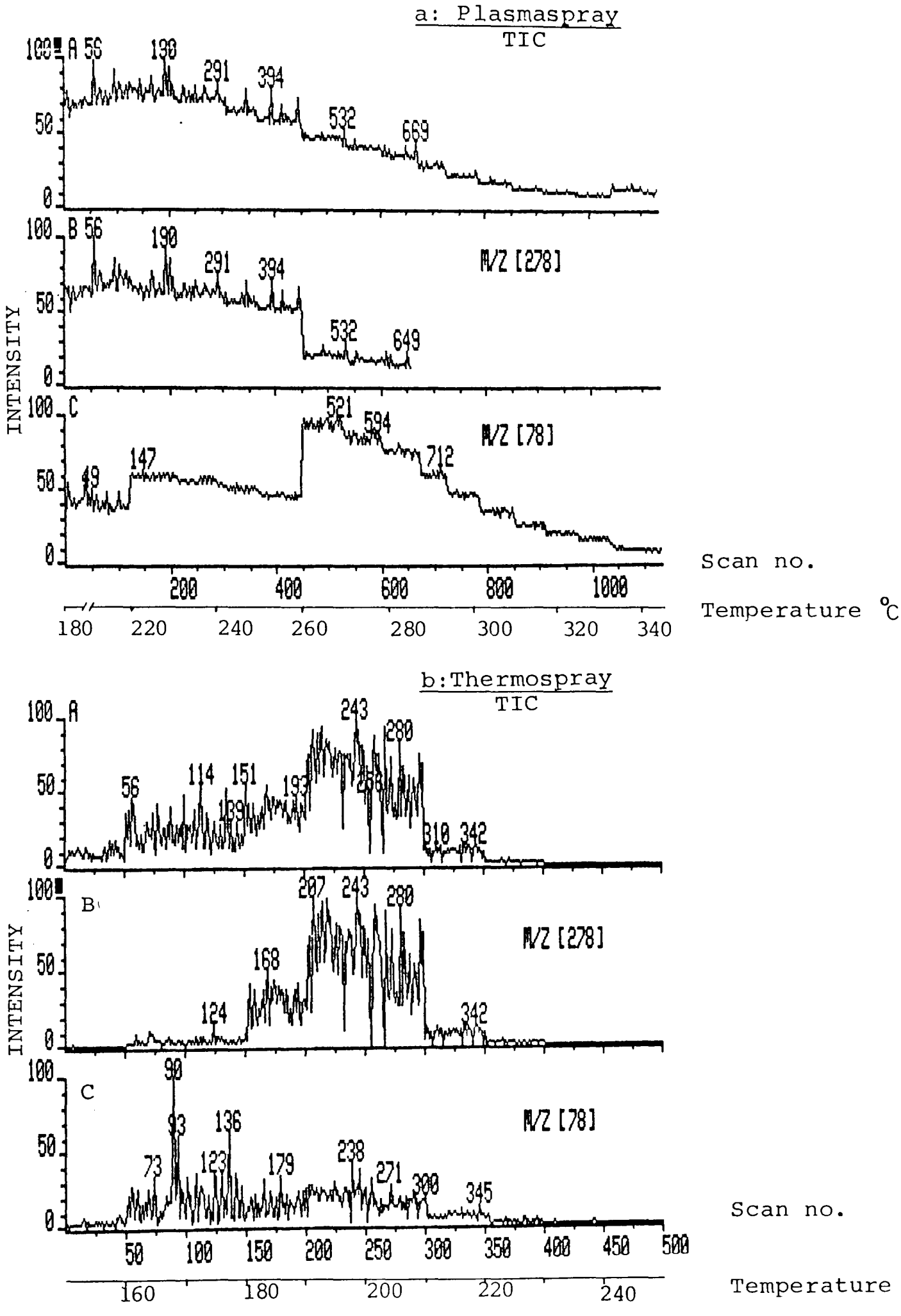


Figure 5.III.2 Effect of probe temperature on ionization of amitriptyline in methanol/ammonium acetate buffer (0.1M 1:1, v/v) by (a) PSP (+) LC-MS and (b) TSP(+) LC-MS. Amitriptyline was introduced continuously at 2mg/100ml in the mobile phase while the probe temperature was increased in steps of 10 C^{o}. The traces show: A the total ion current chromatogram, B and C: mass chromatograms for the quasi-
[illegible] ine and ammonium acetate at m/z 278 and m/z
current

temperatures the response was erratic, resulting in a chromatogram full of spikes and noise.

The amylobarbitone experiment was conducted in a similar way to the amitriptyline experiment and both PSP(+) and PSP(-) were evaluated. The quasi-molecular ions (m/z 227 and m/z 225, respectively) and the buffer ions (78 and 119, respectively) followed the same pattern, where they maximized at 200-210°C, followed by a gradual drop as the temperature was increased to 250°C (Figure 5.III.3). The latter was followed by a marked drop in response. The response using TSP mode was erratic and unstable. For this reason, examination of the effect of probe temperature on ionisation of amylobarbitone was not continued in that mode.

Following these tests, eight drugs were introduced separately (200ng each) via the by-pass connection at four different probe temperatures (240°, 270°, 300° and 330°C). Table 5.III.2 presents the peak areas from reconstructed ion chromatograms of the most intense ion for each drug obtained at the four different probe temperatures evaluated. It shows that the tested compounds did not share the same probe temperature for optimum sensitivity. However, very high temperatures (300°C or higher) dropped the sensitivity for all drugs, although they were detected to a varying degree over the wide range of temperature. The behaviour of ammonium acetate, represented by the intensity of the ion at m/z 78, was similar to that described earlier. Ion

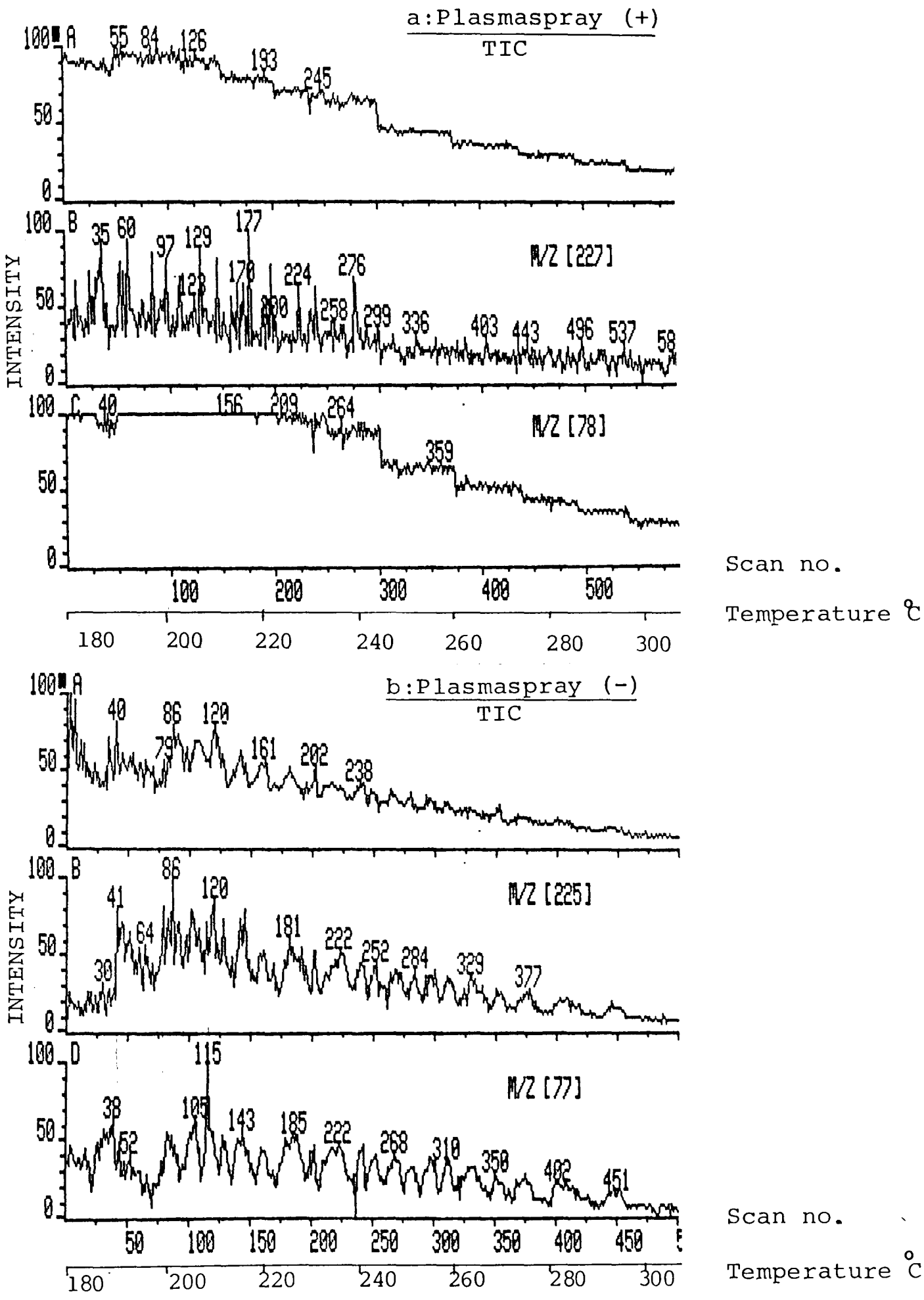


Figure 5.III.3 Effect of probe temperature on ionization of amylobarbitone in methanol/ammonium acetate buffer (0.1M 1:1, v/v) by (a) PSP(+) LC-MS and (b) PSP(-) LC-MS. Amylobarbitone was introduced continuously at 1.3 mg/100ml in the mobile phase while the probe temperature was increased in steps of 10 C^{o}. The traces show: A the total ion current chromatograms, B and C: mass chromatogram for the quasi-molecular ions of amylobarbitone and ammonium acetate.

intensities tended to increase at lower probe temperatures with a simultaneous increase in the irregularity of the base line (Figure 5.III.4).

The mass spectra of these drugs are presented in Figure 5.III.5, and Table 5.III.3 summarises their major fragment ions.

Table 5.III.2 Effect of probe temperature on the sensitivity of detection of eight representative drugs by PSP(+) LC-MS.

C O M P O U N D	Ion Used m/z	P e a k A r e a at probe temperature C° of			
		240	270	300	330
Cannabidiol	315	28167	13728	7079	4853
Doxepin	280	5732	29597	19249	15002
Propranolol	260	47136	20135	18036	11739
Buprenorphine	450	176	2095	1594	530
Flupenthixol	435	10958	28338	3456	2639
Indomethacin	220	7853	3455	1387	360
Morphine	286	26487		8148	6435
Triazolam	343	5402	3734	2344	1146

The source temperature was 270°C and the mobile phase was composed of 0.1M ammonium acetate buffer:MeOH (1:1, v/v)

Table 5.III.3 Summary of major ions in the mass spectra of eight representative drugs obtained by PSP(+) LC-MS.

COMPOUND	M.Wt	Base peak and other prominent ions (m/z)
Cannabidiol	314	315,316
Doxepin	279	280,281
Propranolol	259	260,261
Buprenorphine	467	450,468,396
Flupenthixol	434	435,436,437,407,405,393
Indomethacin	358	220,162,176,174,190,204,358,312
Morphine	285	286,268
Triazolam	343	343,245,309,275,311,236

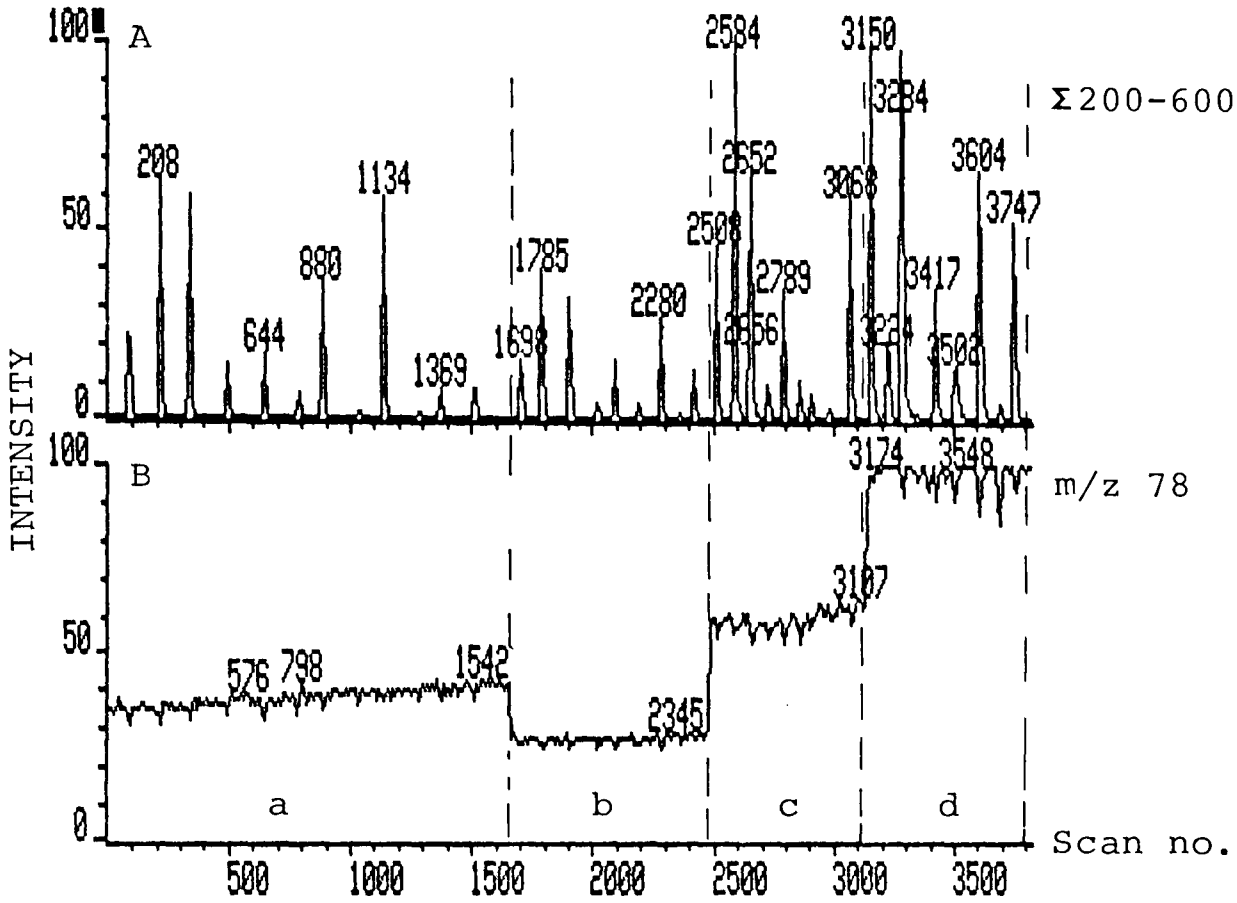
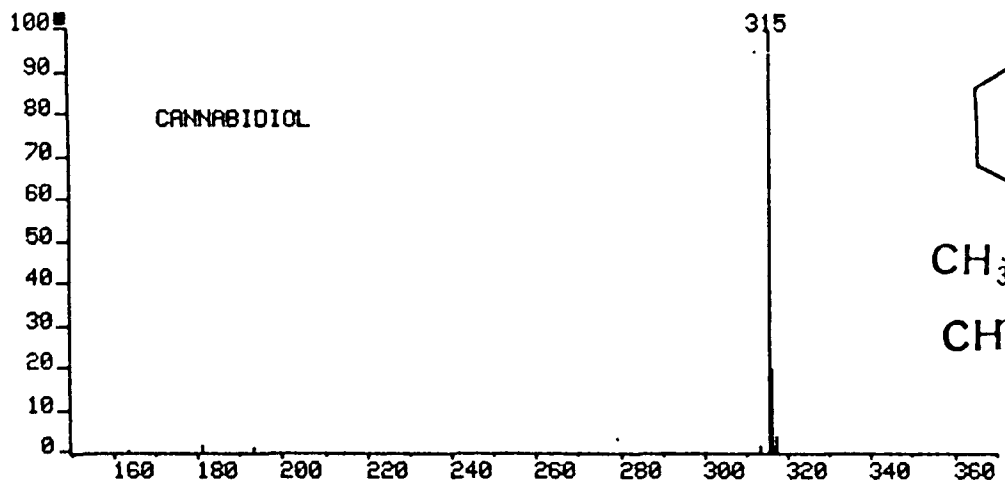
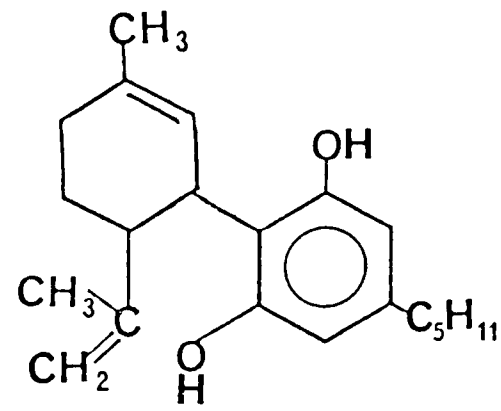
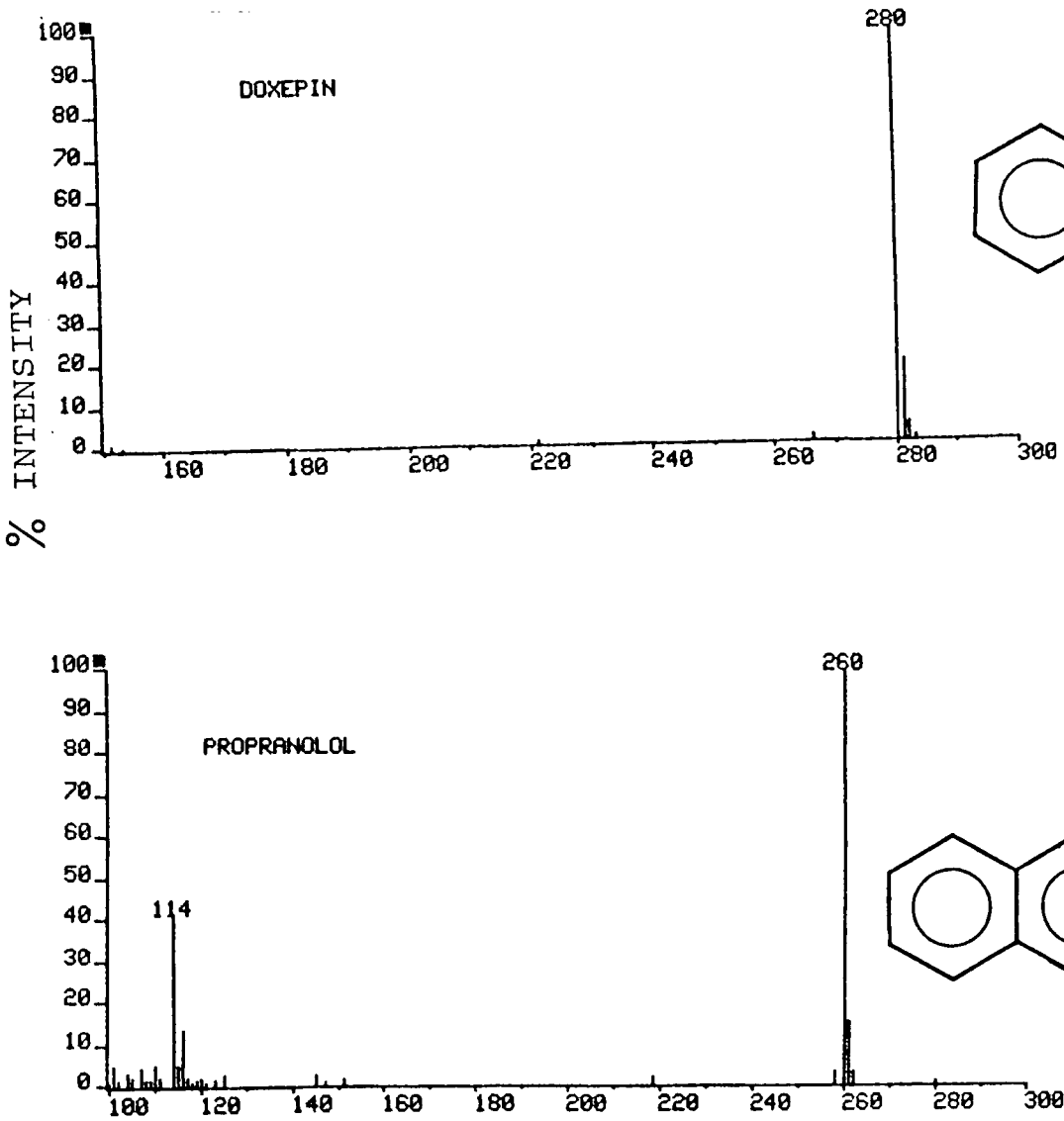


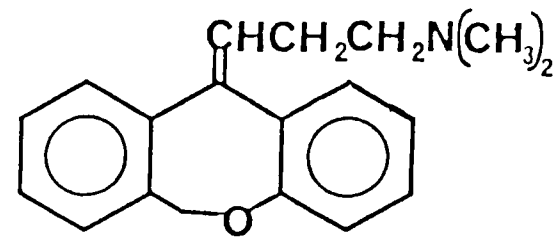
Figure 5.III.4 Effect of probe temperature on sensitivity of detection of 8 representative drugs by PSP(+) LC-MS. An aliquot (200ng) of each drug in Table 5.II.2 was injected via the column by-pass inlet at each of the temperatures (300°, 330°, 270° and 240°C represented by a,b,c and d respectively). The traces show the partial ion chromatogram (sum of masses 200-600) and a mass chromatogram for m/z 78, from the ammonium acetate buffer. The mobile phase was methanol:ammonium acetate buffer (0.1M), 1:1 v/v.





M Wt 314

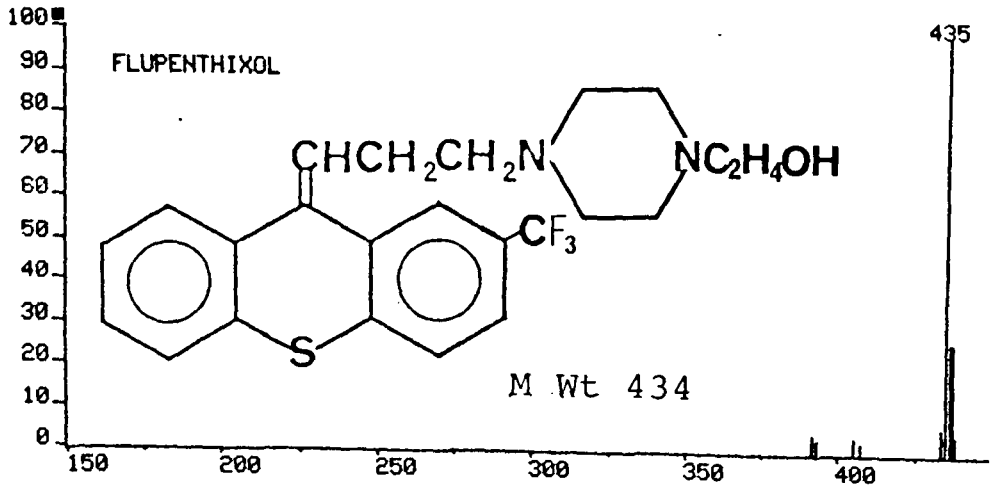




M Wt 279

M Wt 259

Figure 5.III.5 Mass spectra of representative drugs obtained in PSP(+) LC-MS mode. The mobile phase was methanol:ammonium acetate buffer (0.1M), 1:1 v/v, and the TSP/PSP probe temperature was 270°C.



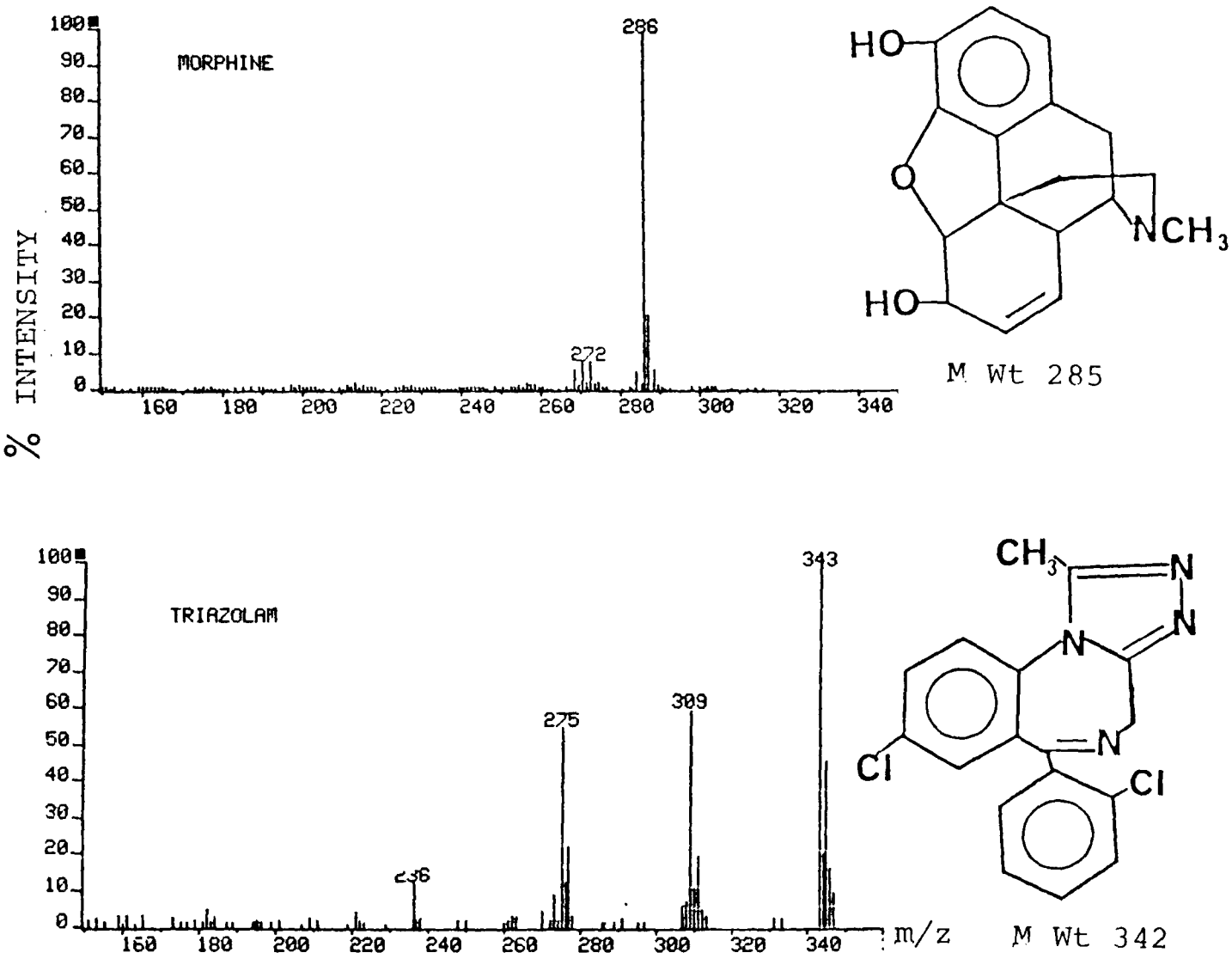
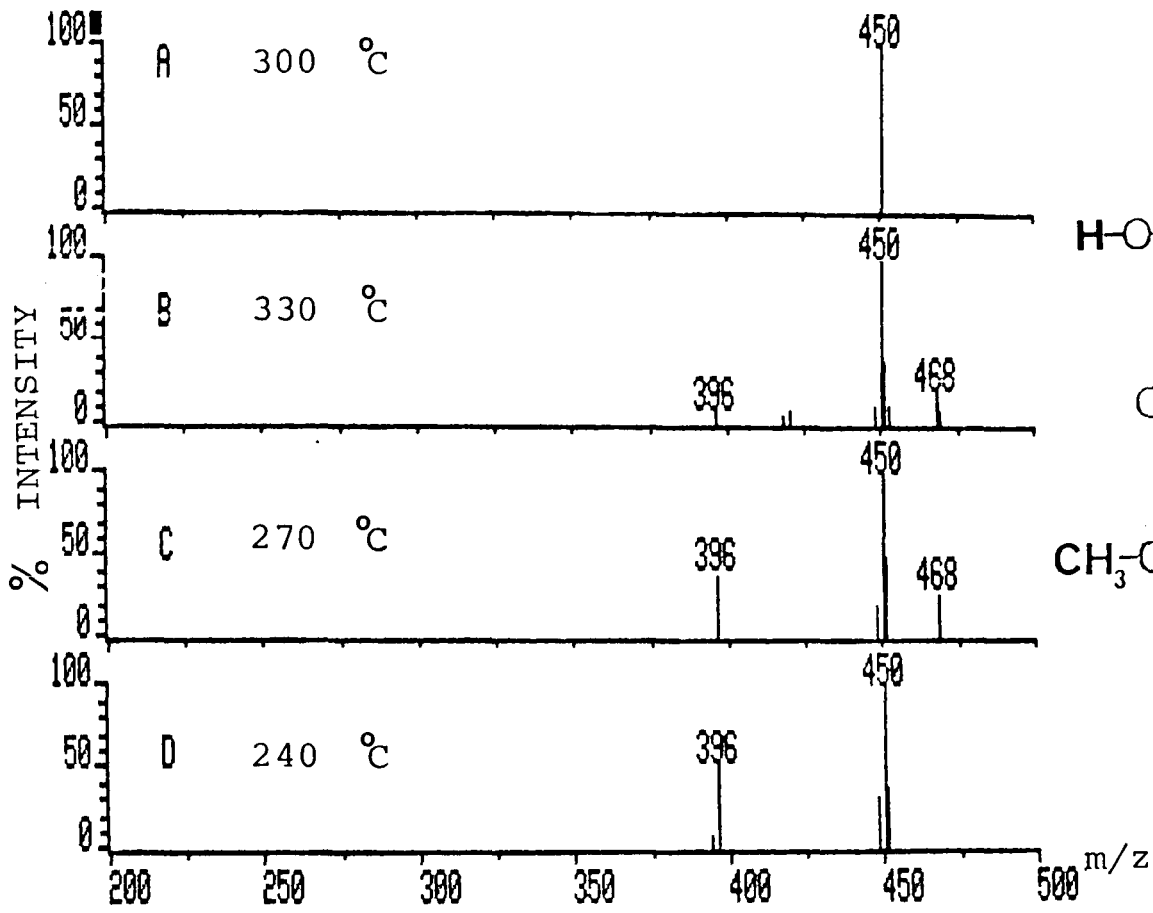


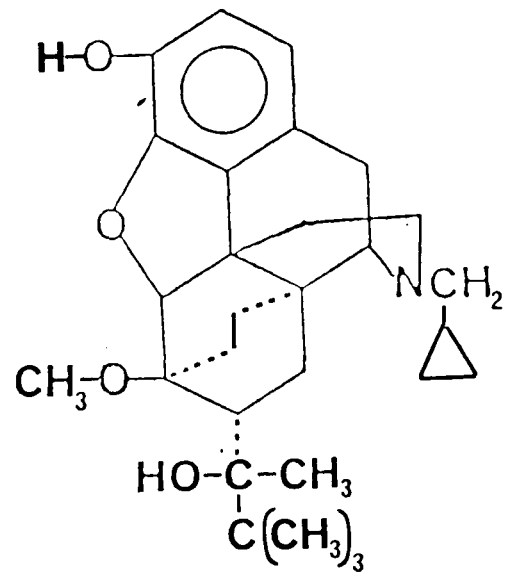
Figure 5.III.5 Continued.

The most significant changes in fragmentation patterns resulting from different probe temperatures were noticed for indomethacin and buprenorphine where their protonated molecular ions tended to disappear at high temperatures while, in addition, the intensities of the fragment ions tended to vary as shown in Figure 5.III.6.

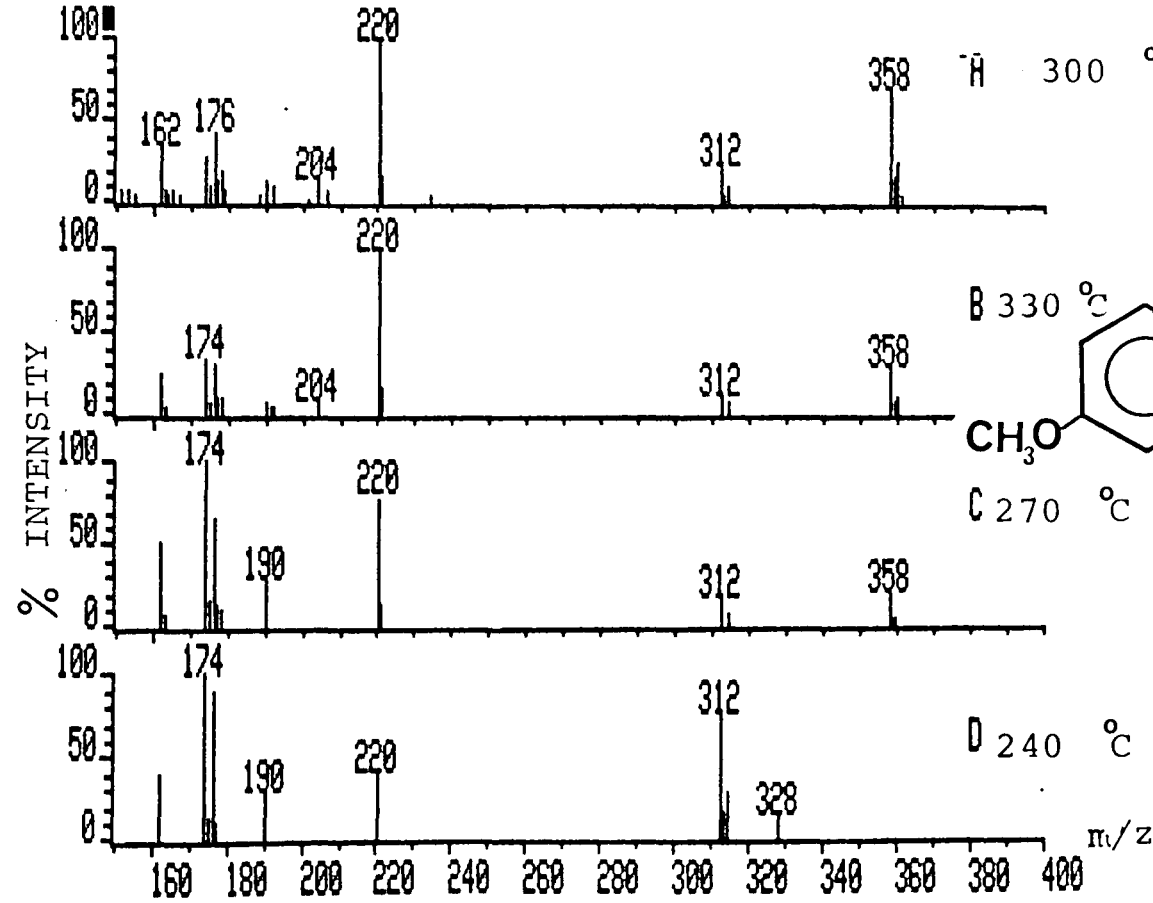
5.III.3 EFFECTS OF SOURCE TEMPERATURE

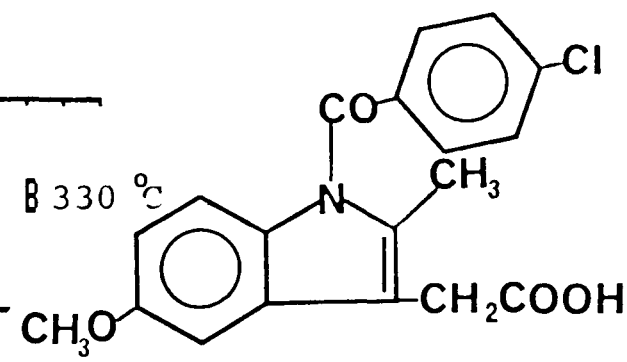
The mass spectrometer source block temperature was increased stepwise between 240-320°C during the operation of the LC-MS interface. At each source temperature mass spectra were recorded for 8 model drugs and these were examined and evaluated for the response, fragmentation pattern and peak shape of reconstructed ion chromatograms. The effect of source temperature on sensitivity did not follow a specific pattern. Peak areas from computer-reconstructed ion chromatograms of the most intense ion of each of the examined drugs are presented in Table 5.III.4. The results show that the best sensitivity was obtained at a source temperature of 270°C, which was almost general for the test compounds. There were no noticeable changes in the fragmentation patterns at the four different temperatures examined. Also, an obvious effect was noticed on the "peak" shape where there was less tailing with higher source temperatures, although the peaks were similar to those obtained in flow injection analysis and were not actual chromatography peaks. However, a temperature of 270°C was reasonable for both sensitivity and peak shape.





Buprenorphine
M Wt 467





Indomethacin
M Wt 457

Figure 5.III.6 Effect of probe temperature on the mass spectra of buprenorphine and triazolam obtained in PSP(+) LC-MS mode. The mobile phase was MeOH: ammonium acetate buffer (0.1M), 1:1 v/v, and the TSP/PSP probe temperature was at 300°, 330°, 270° and 240°C represented by A, B, C and D respectively.

Table 5.III.4 Effect of source temperature on the sensitivity of detection of 8 representative drugs by PSP(+) LC-MS.

COMPOUND	Peak area of the ion used (m/z) at source temperature °C			
	240	270	300	320
Cannabindiol	11449	12232	7472	10573
Doxepin	2816	27179	18894	14118
Propranolol	18720	25143	17275	28381
Buprenorphine	1899	1353	1573	1206
Flupenthixol	3949	4587	3398	4146
Indomethacin	2107	2958	1355	2724
Morphine	10427	10291	8132	10952
Triazolam	2075	2697	2174	2451

Probe temperature was 300°C and mobile phase was 0.1M ammonium acetate buffer:MeOH (1:1, v/v)

5.III.4 DISCHARGE VOLTAGE IN PSP MODE

In the normal PSP operation, a discharge voltage of 350V is generated at the probe tip. The PSP module has an analogue control which allows the discharge voltage to be increased in 6 increments, up to 600V. Such an increase showed higher sensitivity in response (Figure 5.III.7) with no apparent change in the fragmentation pattern of morphine or triazolam. However, the increase in sensitivity was obtained at the expense of ion beam stability where the TIC chromatogram was full of spikes in the background and on the sample chromatographic peaks, resulting in irreproducible response and a decrease in signal to noise ratio (Figure 5.III.7).

5.III.5 EFFECTS OF MOBILE PHASE CONSTITUENTS

The aim of this experiment was to assess the effect of changing the mobile phase composition on both TSP and

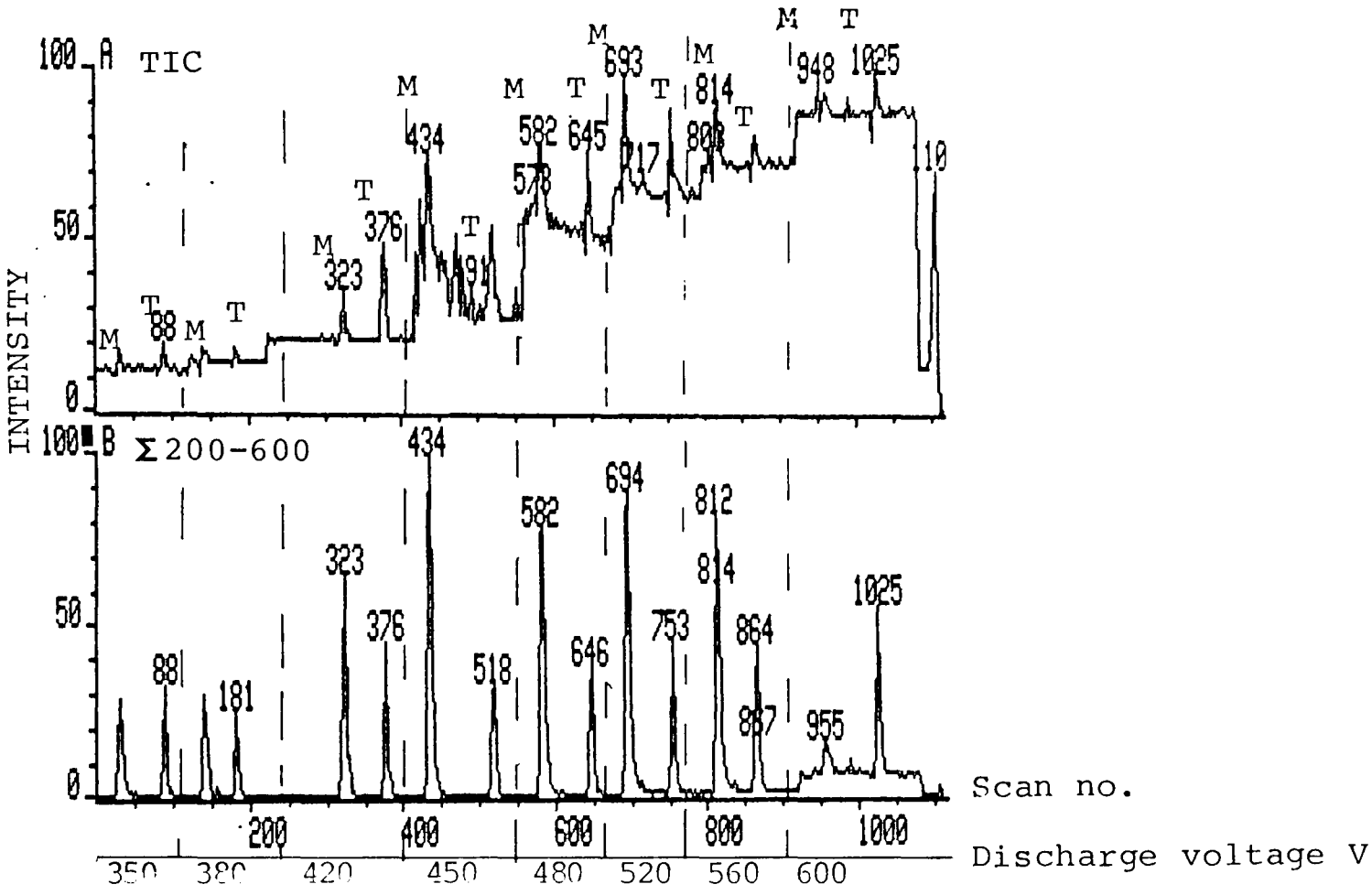


Figure 5.III.7 Effect of PSP discharge voltage on sensitivity of detection of morphine and triazolam. The traces show the intensities of the total ion current (A) and partial ion current above mass 200 (B) as a function of PSP discharge voltage from 350 to 600V, where 200ng of morphine (M) and triazolam (T) were introduced separately at each setting via the column by-pass inlet. The mobile phase was methanol:ammonium acetate buffer (0.1M), 1:1 v/v, and the TSP/PSP probe temperature was 270°C.

PSP modes, but due to the difficulties occurring in the TSP mode such as mass spectral peak instability and poor response, the experiment was conducted on PSP mode only.

The results of using different mobile phases based on ammonium acetate buffer, MeOH and ACN on the sensitivity of detection of buprenorphine in PSP(+) mode are listed in Table 5.III.5.

Table 5.III.5: Effect of different mobile phase constituents on mass spectral peak profile and on response and reproducibility of LC-MS of buprenorphine.

Mobile Phase	# Average Peak Area	C.V%	Peak * Stability	Pump Pressure 'Bar'
Methanol	2200	9.5	Poor	55
Water	2941	3.1	Poor	95
0.01M Buffer**	8822	24	Unstable	93
0.05M Buffer	13300	17	Good	92
0.10M Buffer	15004	17	Better	92
0.15M Buffer	21381	4	Best	92
0.20M Buffer	20823	15	Good	92
0.30M Buffer	21792	14	Good	94
0.15M Buffer:MeOH (1:1, v/v)	21000	12	Good	125
0.15M Buffer:ACN (1/1, v/v)	86936	14	Best	78
0.15M Buffer:ACN: MeOH (1:1:1, v/v:v)	56741	10	Good	95
Acetonitrile	6711	20	Unstable	45

** Buffer:Ammonium acetate,
C.V%: coefficient of variation for 5 replicate injections.
* Peak profile of ion at m/z 450.
# Based on area under peak in computer-reconstructed ion chromatograms for $[MH-H_2O]^+$

They show a significant influence on sensitivity in general. The increase in sensitivity of detection was for both buprenorphine and mobile phase constituents, but the sensitivity for buprenorphine has far exceeded that of the other mobile phase constituents. Buprenorphine peaks

started to become visible in the TIC chromatogram when ACN was included in the mobile phase. As the buffer concentration increased the protonated molecular ion of ammonium acetate (m/z 78) started to reach full-scale intensity on the detector and meaningful measurements could not be taken to compare its response with that of the analyte.

However, increasing the strength of the buffer was accompanied by increased sensitivity (Figure 5.III.8) up to a buffer concentration of 0.15M. At the same time, the reproducibility of response based on peak area improved. Higher buffer concentrations gave a full-scale response in the TIC chromatogram and worsened the reproducibility, although there was no significant change in the sensitivity. Methanol increased the back pressure on the HPLC pump while ACN reduced the back pressure. The major ion obtained for buprenorphine [$(MH-H_2O)^+$ at m/z 450] was obtained in all mobile phases. However, the improvement of sensitivity obtained at higher ammonium acetate concentrations permitted other ions to be detected as well, such as m/z 396 representing the loss of the N-alkyl group.

5.III.6 REPRODUCIBILITY OF RESPONSE

The work described in previous sections showed that the peak area reproducibility varied according to the operating conditions. In this experiment the reproducibility was tested after fixing all variables such

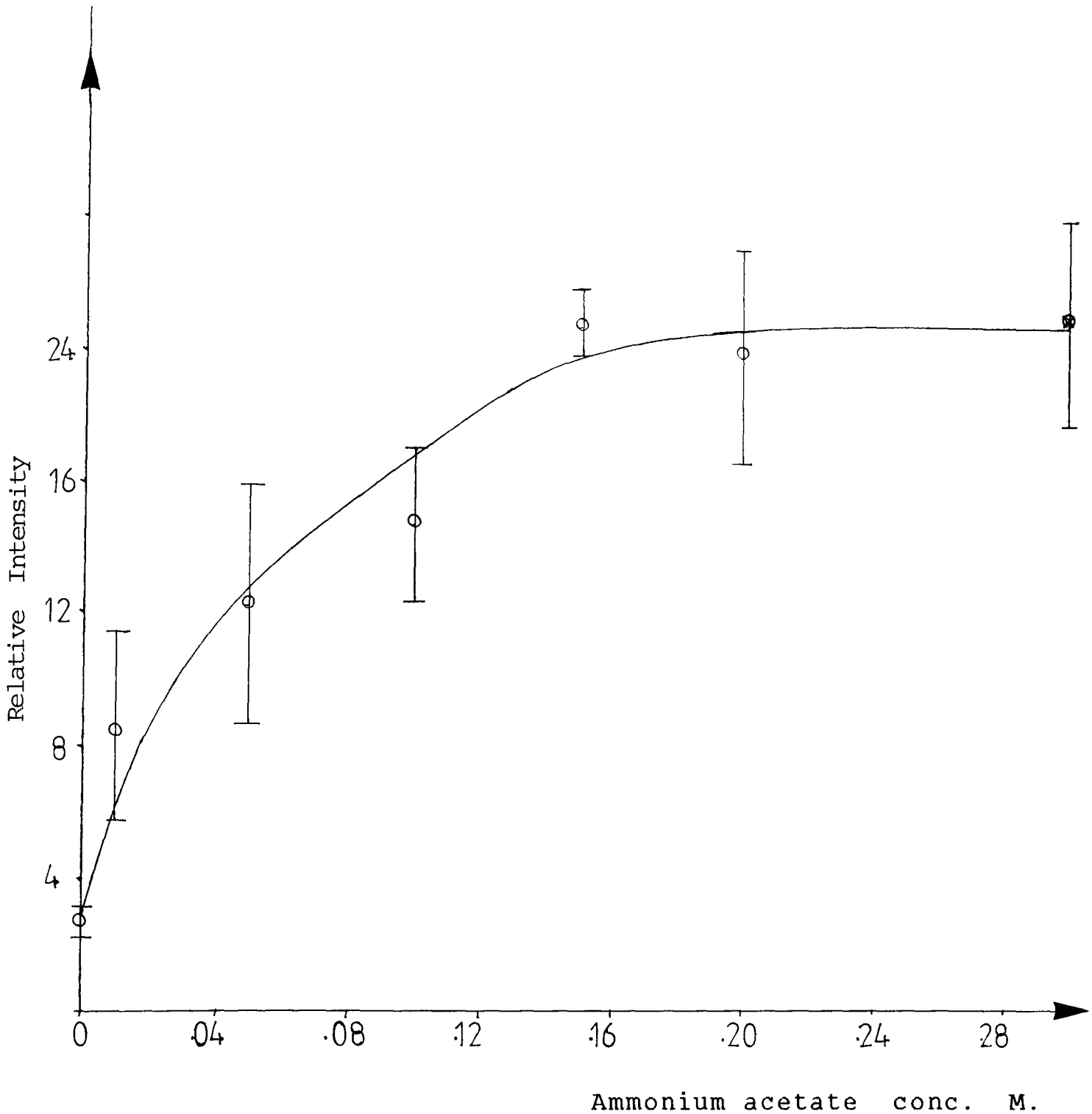


Figure 5.III.8 Ammonium acetate buffer concentration in the mobile phase versus the mass spectrometer response to 5 replicate injections of 200ng of buprenorphine introduced in each mobile phase via the PSP LC-MS interfase.

as probe temperature, mobile phase composition, source temperature and flow rate. The coefficients of variation for seven replicate injections of buprenorphine (200ng each) were 7 and 18% for peak area and height, respectively, and were 9 and 17%, respectively, for fourteen replicate injections of doxepin (200ng each) (Figure 5.III.9).

5.III.7 SENSITIVITY

The lowest amount of buprenorphine introduced to the mass spectrometer *via* the by-pass connection and the PSP LC-MS interface was 1.2ng, where a full scan spectrum was acquired. Only the base peak was observed at lower concentrations. However, this experiment was conducted after prolonged use of the interface, when both the source block and the probe were dirty, leading to loss of sensitivity and instability of the ion beam. Because of this, the detection limit could only be demonstrated down to 0.3ng by SIR and the experiment could not proceed further.

5.III.8 DRUGS AMENABLE TO LC-MS

Several drugs representative of those which are commonly observed in forensic toxicology were introduced separately to the mass spectrometer at 200ng each *via* the TSP/PSP LC-MS interface to evaluate their spectra using the PSP(+) mode of ionization. The mobile phase was MeOH:ammonium acetate buffer (0.1M), 1:1 v/v, at a flow

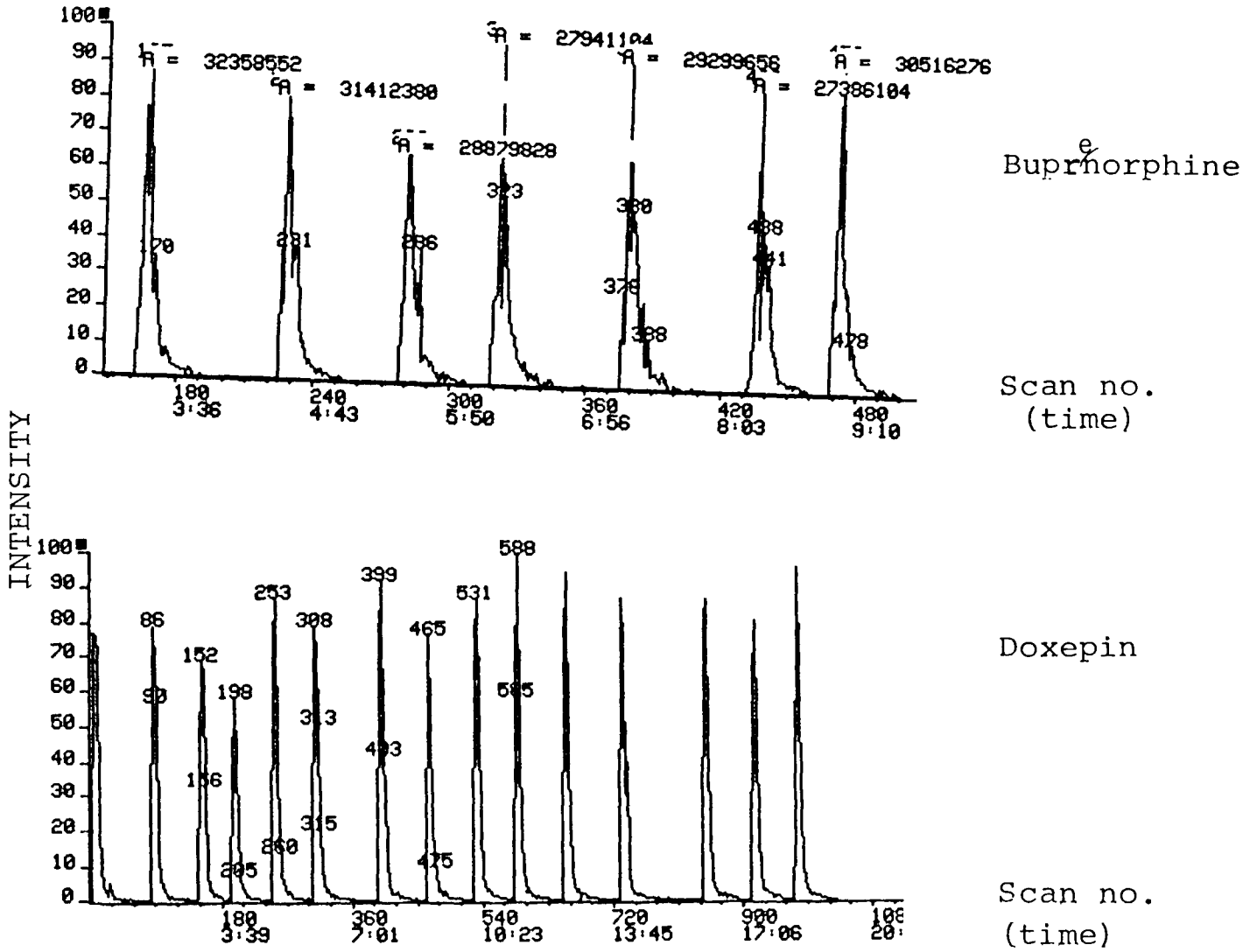


Figure 5.III.9 Reconstructed partial ion chromatograms (above mass 200) showing the response reproducibility on the TSP/PSP LC-MS interface for two model drugs, buprenorphine (above) and doxepin (below) where several aliquots of 200ng of each were introduced *via* the column by-pass inlet. The LC-MS interphase was operated on PSP(+) mode and the mobile phase was methanol:ammonium acetate buffer (0.1M), 1:1 v/v, and the TSP/PSP probe temperature was 230°C.

rate of 1ml/min and probe temperature of 270°C. The most abundant ions obtained for these compounds are listed in Table 5.III.6 and examples of their mass spectra are shown in Figure 5.III.10.

Table 5.III.6 Drugs, their molecular weights (M.Wt) and ions obtained by PSP(+) LC-MS.

C O M P O U N D	M.WT	Base Peak and Other Ions (m/z)
ß-Hydroxyethyl Theophylline	224	225,226
Caffeine	194	195
Carbamazepine	236	237,238
Chlordiazepoxide	299	284,300,273,237,294
Clobazam	300	301,303,318,267
Clonazepam	300	301,181,303,318,267,222,237
Codeine	299	300,282,317
Dextromethorphan	271	272
Dextromoramide	392	393
Diamorphine	369	370,328,310,286,268
Diazepam	284	285,287,286
Dipipanone	349	350
Flurazepam	387	388,390
Lorazepam	321	303,305,307,271,321,163,253
Lysergide (LSD)	323	324,325
Methadone	309	310,311
Morphine	285	286,286,303
Morphine-3-glucuronide	461	286,462,303,268,194,444,227
Nalorphine	311	312,313,314,294,272
Naproxen	230	231,248,185,232,233
Oxazepam	286	269,271,287,268
Pentazocine	285	286,287
Pethidine	247	248
Temazepam	300	301,303,267,286,237
Theophylline	180	181
Verapamil	454	456,212,291,305,196

These spectra provided molecular weight information on the majority of the compounds examined, especially for those where little or no fragmentation occurred and where scant structural information could be obtained. The base peaks of most of these compounds were the protonated molecular

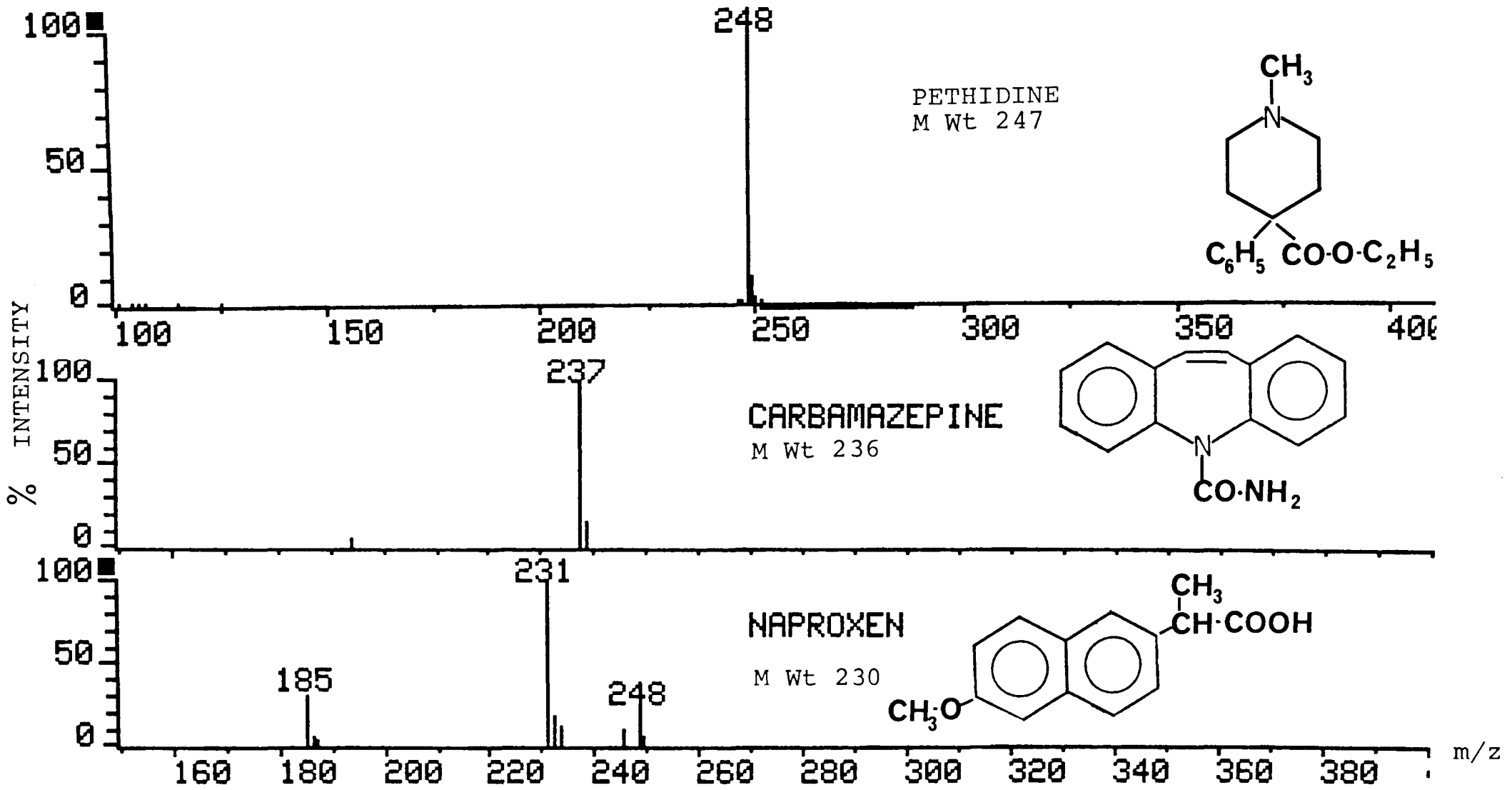


Figure 5.III.10 Examples of mass spectra of drugs generated by PSP(+) ion mode LC-MS. The mobile phase was methanol:ammonium acetate buffer (0.1M), 1:1 v/v, and the TSP/PSP probe temperature was 270°C.

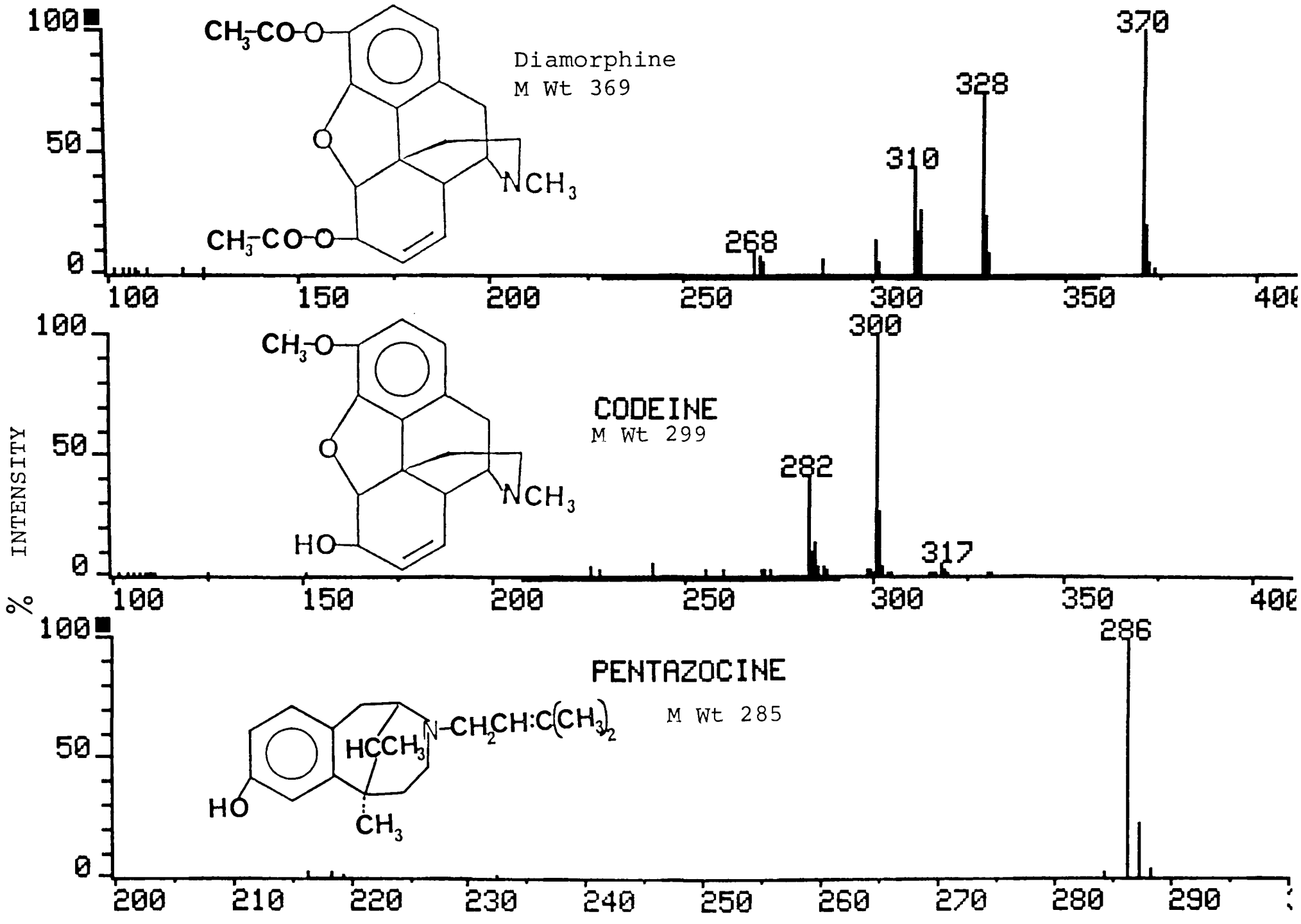


Figure 5.III.10 Continued.

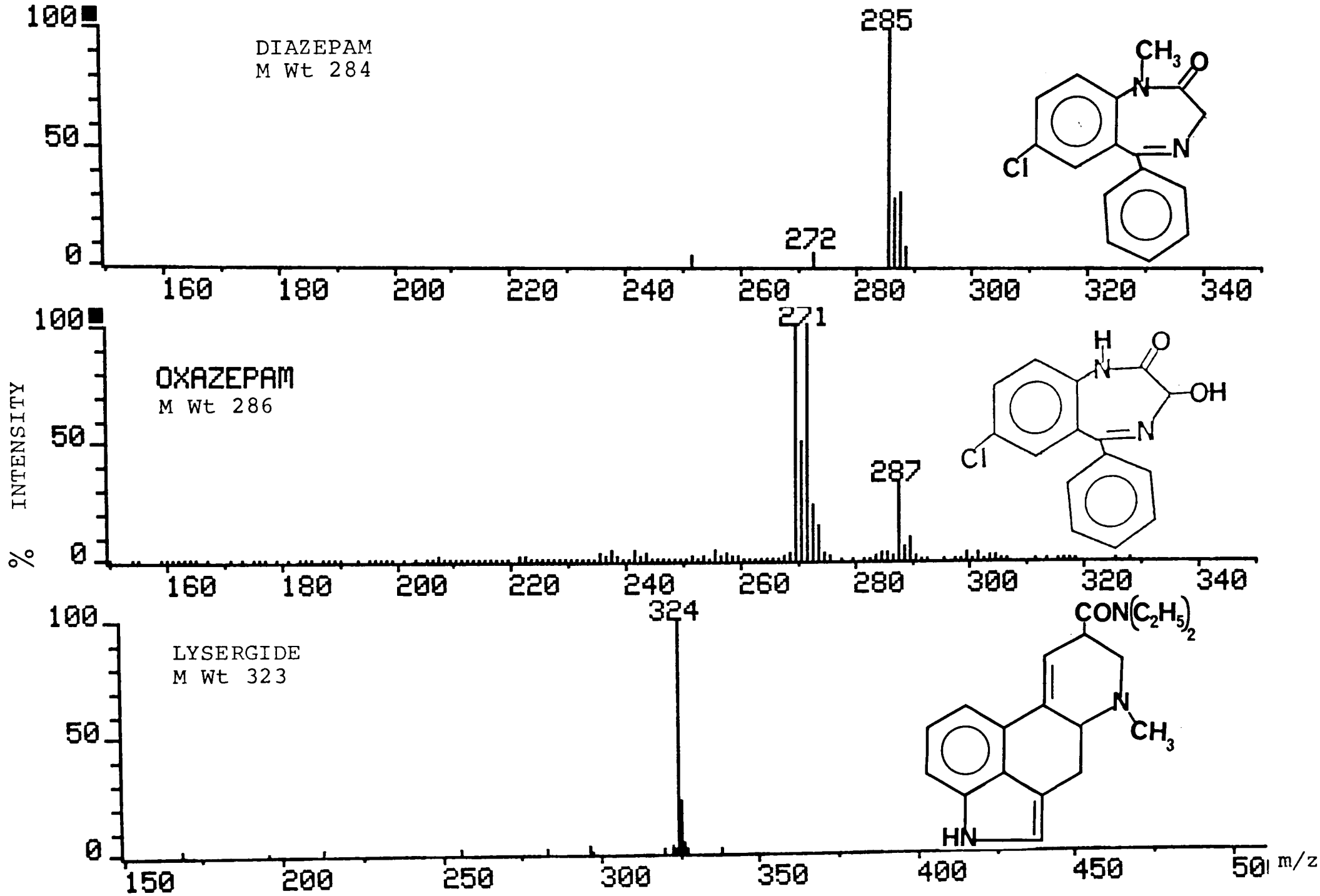


Figure 5.III.10 Continued.

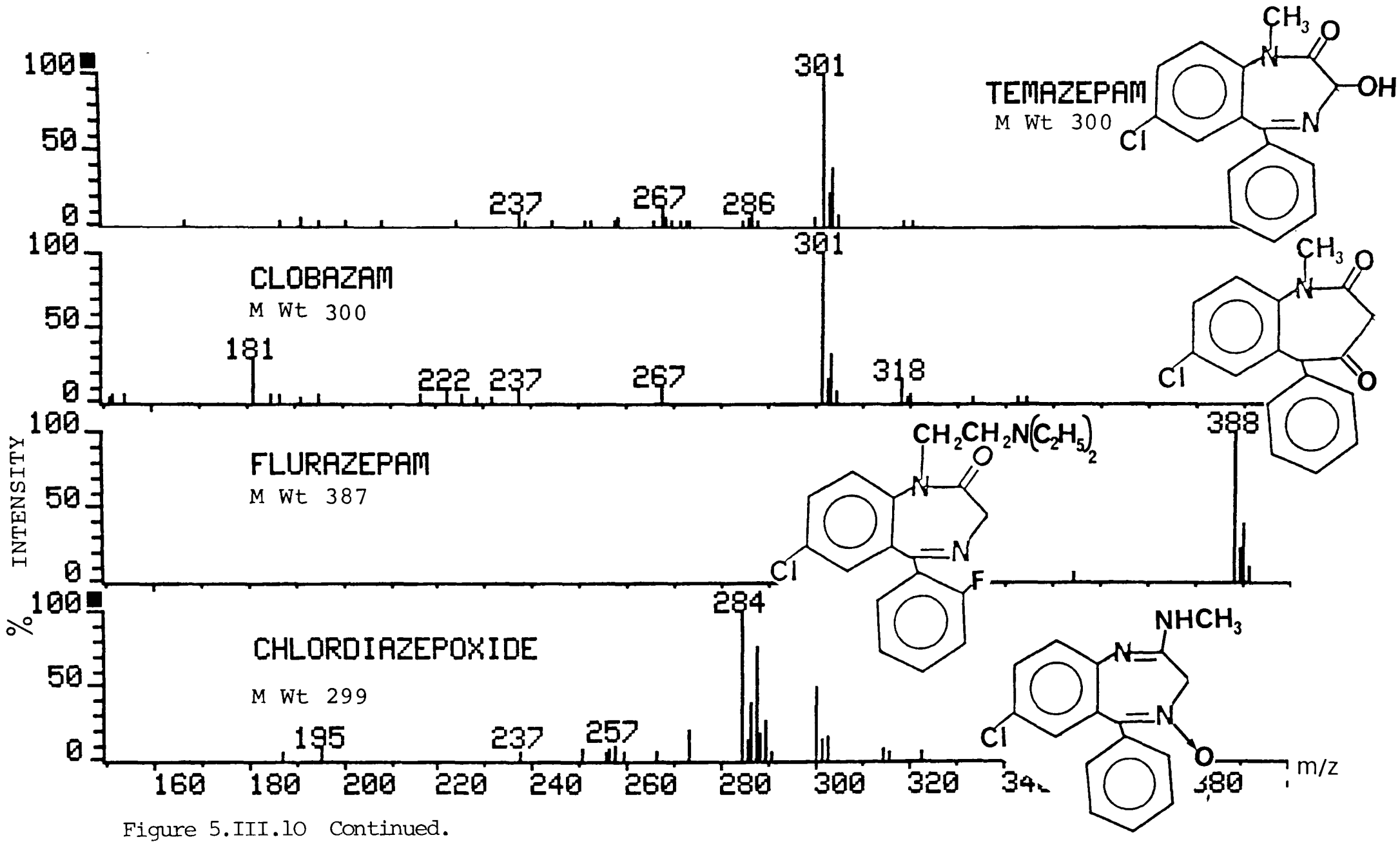


Figure 5.III.10 Continued.

ions $[MH]^+$: only for lorazepam was this ion less than 2% of the base peak. Other important characteristic ions were $[MH-H_2O]$ for nalorphine, codeine and clonazepam as well as oxazepam and lorazepam where this ion was also the base peak. Chlordiazepoxide yielded a base peak ion which corresponded to loss of oxygen from the protonated parent molecule.

5.III.9 HPLC-MS

5.III.9.1 BASIC DRUGS

Development trials were carried out to optimize a mobile phase based on aqueous ammonium acetate buffer and an organic modifier of MeOH and/or ACN for the separation of a mixture of basic drugs. The results obtained are listed in Table 5.III.7 and an example of a separation is shown in Figure 5.III.11, for which the mobile phase was constituted from MeOH:ACN:0.2M ammonium acetate buffer 65:20:15, (v/v:v). The following points were observed during this experiment:

I. The HPLC column added an extra back pressure on top of that generated by the flow resistance in the probe and the back pressure caused by the column was slightly lower with organic solvents than with water, and a flow rate of 1-1.2ml/min could be obtained. An upper pressure limit of 200 bar was used to prevent damage to the HPLC column.

II. The inclusion of ammonium acetate buffer produced better chromatography as well as a better response. However, drugs such as amitriptyline, methadone,

Table 5.III.7 Effect of different mobile phase constituents on capacity factor, chromatographic peak shape and response during PSP(+) LC-MS of basic drugs.

MOBILE PHASE v/v/v	Flow Rate (ml/min)	Pump Pressure (Bar)	Capacity Factor (k') of Drug No: * 1	2	3	4	5	6	7	8	9	10	11	REMARKS
MeOH water 30:20	1	175	1.1	3.6	3.6	nil	nil	nil	nil	nil	nil	nil	nil	Intensity is very low except for caffeine
MeOH:0.05 buffer 80:20	1	174	1.0	3.4	3.7	4.7	4.9	7.2	10.8	diff	diff	diff	diff	Slightly improved response and separation
MeOH:0.1M buffer 80:20	1	175	0.9	2.0	3.3	3.5	4.2	6.9	9.4	14.4	diff	diff	diff	Improved response and better peak shape
MeOH:0.1M buffer 90:10	1.2	180	0.4	0.5	1.0	1.6	2.6	3.1	3.3	6.1	diff	diff	diff	Further improvement of response and peak shape
MeOH:ACN:0.2M buffer 80:10:10	1	171	0.4	9.6	1.1	2.1	2.6	3.0	3.3	6.0	diff	8.4	6.3	Better response and peak shape, the last 4 peaks have severe tailing
MeOH:ACN:0.2M buffer 65:20:15	1	165	0.8	2.0	2.6	3.6	5.3	9.0	11.38	diff	diff	diff	diff	Better separation, slight peak tailing
MeOH:ACN.0.2M buffer 50:20:20	1	158	0.3	0.8	1.4	1.8	2.1	3.1	3.7	5.5	diff	8.4	6.6	Better response,but poorer separation
MeOH:ACN:0.2M buffer 50:30:20	1	155	0.3	0.8	0.8	0.8	1.1	2.2	3.8	4.7	diff	9.3	7.9	Poor separation

diff = diffused, nil = not observed, *1 = caffeine, 2 = oxazepam, 3 = pentazocine, 4 = diazepam, 5 = codeine, 6 = pethidine, 7 = dextromoramide, 8 = methadone, 9 = amitriptyline, 10 = dextromethorphan, 11 = dipipanone.

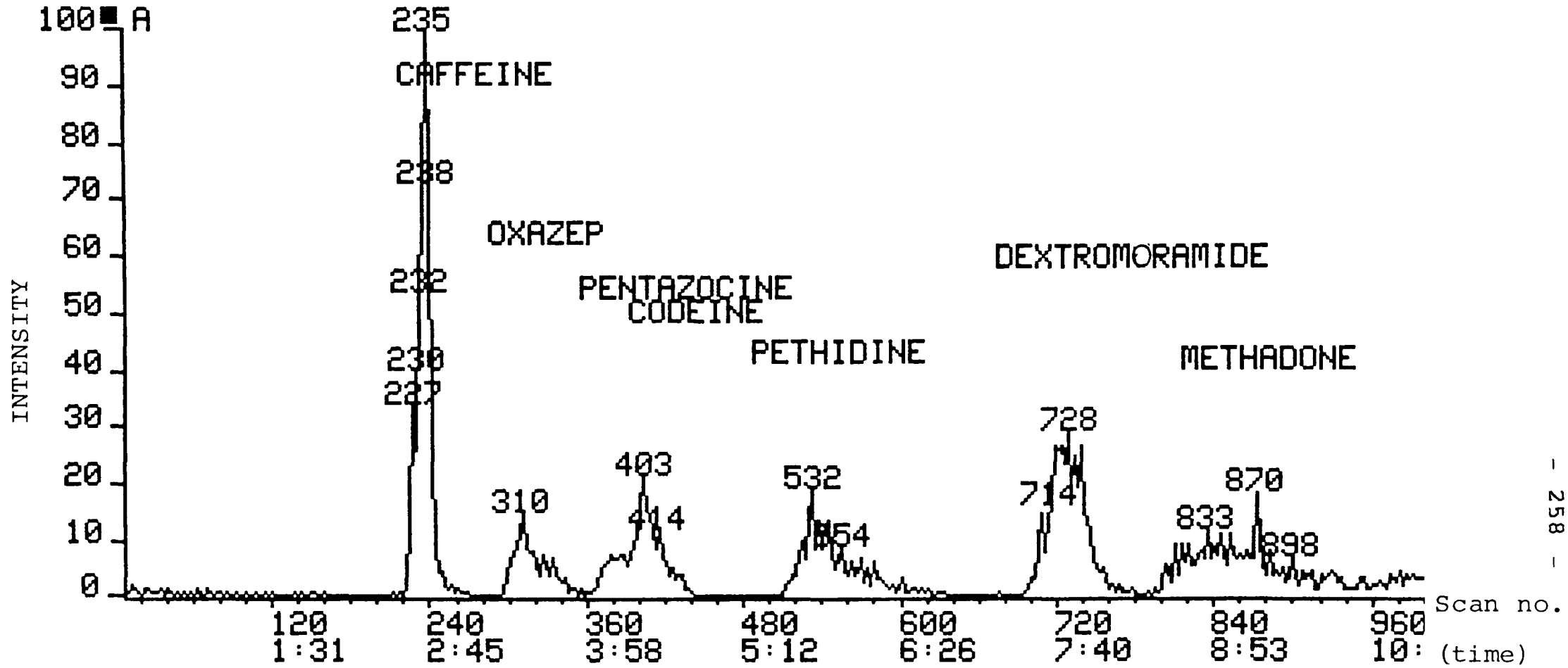


Figure 5.III.11 Computer reconstructed total ion current chromatogram obtained by HPLC-MS of a mixture of basic drugs (200ng of each drug on column). The LC-MS interfase was operated in PSP(+) ion mode, the mobile phase was methanol:acetonitrile:ammonium acetate buffer (0.2M), 65:20:15 v/v/v, pH 7.6, the flow rate was 0.9ml/min, The chromatography was performed on an ODS-Hypersil 25cm X 4.6mm ID column and the TSP/PSP probe temperature was 270°C.

dipipanone and dextromethorphan eluted either as severely tailing or diffused peaks.

III. The pH of the mobile phases ranged from pH 7.4-7.7 due to the ammonium acetate buffer.

5.III.9.2 HYPNOTIC AND ANTICONVULSANT DRUGS

Both positive and negative plasmaspray ionization modes were tried. The sensitivity to barbiturate drugs in PSP(+) LC-MS was very poor, so optimization of the mobile phase was based on PSP(-) mode, where only drugs which generate negative ions were monitored. A reasonable separation was obtained using a mobile phase of MeOH:ACN:0.1M ammonium acetate buffer 5:5:11 (v/v:v), adjusted to pH 5.5 with acetic acid (Figure 5.III.12). The flow rate could not be increased above 1ml/min within the pressure limit of the column due to the viscosity of the mobile phase.

Mass spectral fragment ions of the drugs examined via the TSP/PSP LC-MS interface in both positive and negative ion mode are listed in Table 5.III.8. The chromatogram obtained in negative ion LC-MS showed that the response for barbiturate drugs was very high but was poor for non-barbiturate drugs such as methaqualone, glutethimide and carbamazepine. However, the chromatograms obtained in the negative ion mode for barbiturates had less background noise and less interference than those in the positive ion mode, which would result in better detection.

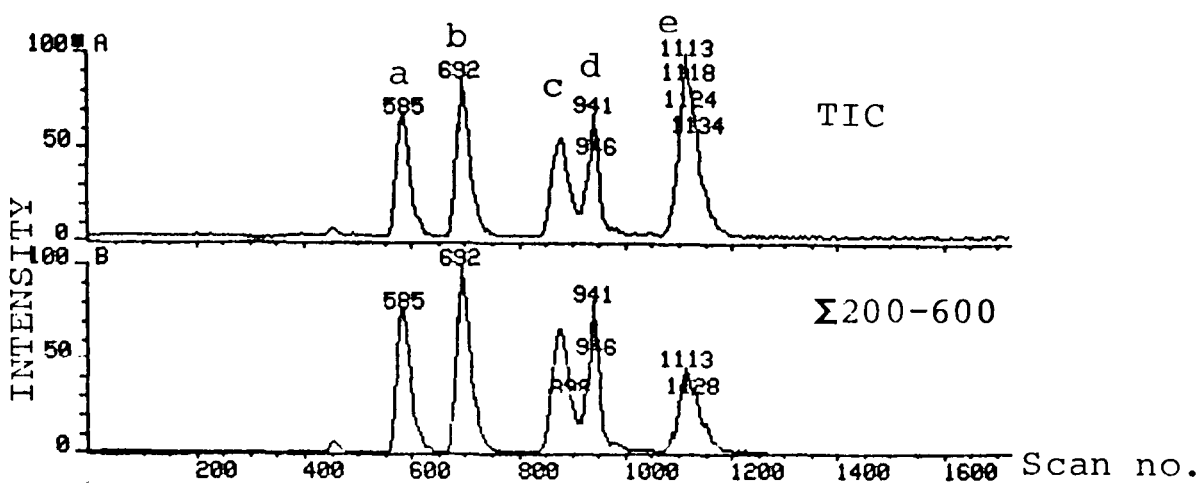


Figure 5.III.12 Computer reconstructed total ion current chromatogram obtained by HPLC-MS of a mixture of phenobarbitone (a), butobarbitone (b), phenytoin (c), amylobarbitone (d) and quinalbarbitone (e) (200ng of each drug on column). The LC-MS interfase was operated in PSP(-) ion mode, the mobile phase was methanol:acetonitrile:ammonium acetate buffer (0.2M), 5:5:11 v/v/v, adjusted to pH 5.5 with acetic acid; the flow rate was 1ml/ min. The chromatography was performed on an ODS-Hypersil 25cm X 4.6mm ID column and the TSP/PSP probe temperature was 230^{o}C.

Table 5.III.8 Prominent ions in the PSP(+) and PSP(-) LC-MS spectra of sedative and anticonvulsant drugs.

C O M P O U N D	M.Wt	Base Peak and other ions	
		Positive Mode	Negative Mode
Phenobarbitone	232	233,114,207,234 178,188,219	231,156,203,232
Amylobarbitone	226	227,158,184	225,197,155
Carbamazepine	236	237,194,238,180	193,194,207
Quinalbarbitone	238	239,240.241 169,170	197,237,167
Phenytoin	252	253,182,254,183	251,252,197
Methaqualone	250	251,168,252	N D
Glutethimide	217	218,219,168,154	N D

N D: not detected.

5.III.9.3 SEPARATION OF OPIATES

I. Effect of Probe Temperature on Morphine-3-glucuronide (M3G)

The effect of variation of the probe temperature between 120-300°C on the sensitivity and fragmentation pattern of M3G was clearly demonstrated. In this instance, the samples were introduced *via* the column by-pass inlet. Although the highest response measured on the TIC chromatogram was obtained at a probe temperature of 250°C, only fragment ions corresponding to free morphine in the form of $[MH]^+$ and $[MH]-H_2O$ were observed. At lower probe temperatures, the protonated molecular ion of M3G started to appear with an intensity of 10% and 20% of the base peak (m/z 286) at probe temperatures of 200° and 150°C, respectively. Other ions present were those of morphine, and m/z 444, representing loss of H_2O from the parent molecule and

m/z 194, the ammonium adduct of the glucuronide fragment (Figure 5.III.13). However, the appearance of the protonated M3G molecule corresponded to an overall loss of sensitivity.

II. Mobile Phase With Acidic Buffer

In this experiment the PSP probe was operated at temperatures of 180-200°C in order to locate morphine and M3G. The mobile phases examined were similar to those described in Section 5.III.9.1 but the pH of the mobile phases ranged from pH 3.4-4.5, adjusted by the addition of acetic acid. The following remarks can be made on the results:

1. Mobile phases containing only water and MeOH were insufficient to elute the analyte and give a good response within a reasonable time.

2. Mobile phases based on ammonium acetate buffer with acetic acid adjustment of the pH had improved the response and the presence of organic solvents in the range of 20-30% (v/v) in such mobile phases provided reasonable separation of morphine, M3G and nalorphine. A separation obtained using a mobile phase of MeOH:ACN:0.05M buffer at pH 4.0 with a flow rate of 0.8ml/min is shown in Figure 5.III.14, which could be used for identification and quantification, but which still showed peak tailing, especially for M3G. A sharper peak shape could not be obtained by using a higher flow rate, which was limited by the viscosity of

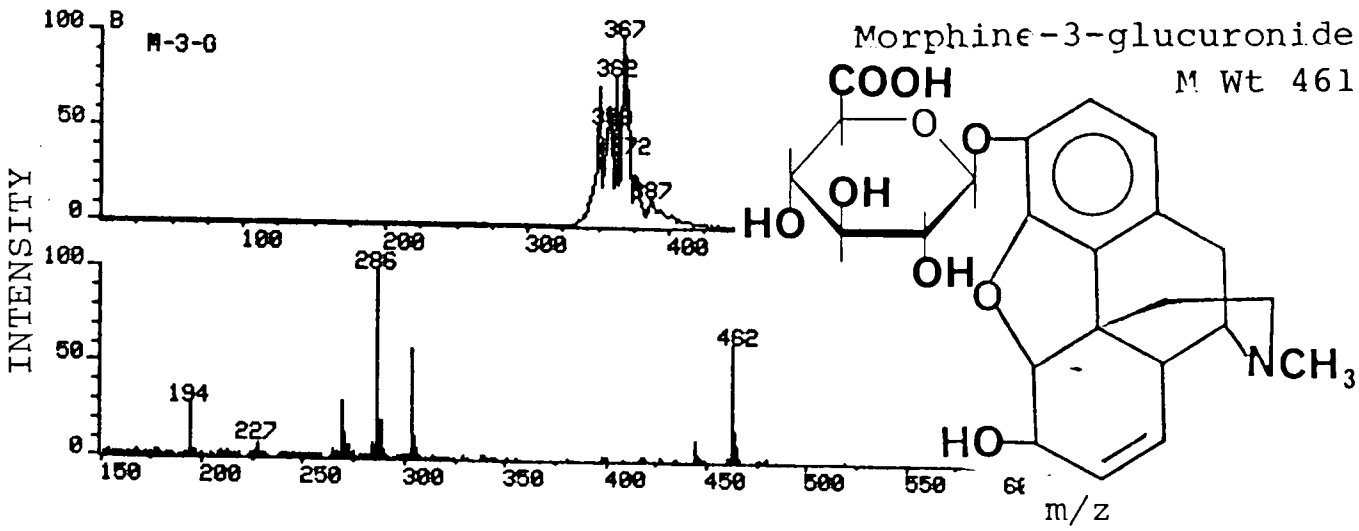


Figure 5.III.13 LTIC trace (above mass 200) and morphine-3-glucuronide (M3G) mass spectrum generated in the PSP(+) ion mode. The sample of 200ng of M3G was introduced via the column by-pass inlet. The mobile phase was methanol:ammonium acetate buffer (0.1M), 1:1 v/v, and the TSP/PSP probe temperature was 200°C.

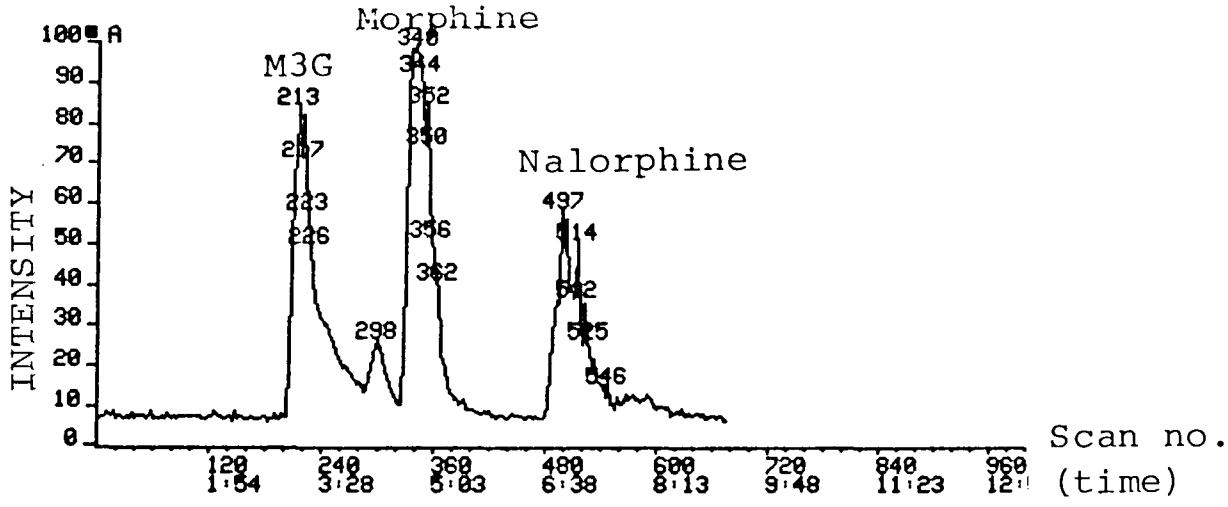


Figure 5.III.14 Computer reconstructed total ion current chromatogram from HPLC-MS of a mixture of M3G, morphine and nalorphine (200ng of each on column). The LC-MS interphase was operated in PSP(+) ion mode, the mobile phase was methanol:acetonitrile:ammonium acetate buffer (0.05M), 8:15:77 v/v/v, adjusted to pH 4.0 with acetic acid; the flow rate was 1ml/min. The chromatogr-araphy was performed on an ODS-Hypersil 25cm X 4.6mm ID column and the TSP/PSP probe temperature was 200°C.

the mobile phase containing acetic acid, which resulted in a high back pressure. However, the addition of ACN permitted a flow rate of 0.8ml/min to be used for the separation with a pump pressure of 188 bar. Acetonitrile/buffer mobile phases permitted higher flow rates but peak tailing was more pronounced.

III. Mobile Phases Containing Diethylamine

Further work was conducted to optimize the mobile phase for the separation of morphine, nalorphine and M3G based on solvent constituents as described above with the addition of diethylamine (DEA) as a volatile base to compete with the drugs for acidic sites on the silica surface caused by the presence of unsubstituted hydroxyl groups. The amount of the DEA added to the mobile phase, based on a buffer of ammonium acetate and organic solvent, was critical. Increasing the concentration of DEA was gradually accompanied by decreases in retention time and loss of resolution. Concentrations in the range 0.1-0.5% of DEA in the buffer produced early elution of M3G, morphine and nalorphine with narrow separation, usable for identification and quantification purposes on reconstructed ion chromatogram of each compound, but the peak tailing was not eliminated. Increasing the concentration of DEA to more than 0.5% led to elution of the above drugs with the solvent front, but gave good sharp peaks for late-eluting drugs such as buprenorphine, which normally require gradient elution for best results. The

use of DEA in water-only based mobile phase gave similar results as those in the previous sections, namely that the response was very poor and that analyte could not be readily detected.

Both ammonium acetate and DEA resulted in a slightly basic mobile phase, which, after passing through the HPLC column to the LC-MS interface caused a gradual increase in the back pressure, after only a few runs. This partial blockage necessitated gradual lowering of the flow rate so as not to exceed the pressure limit of the HPLC column.

5.III.10 CLEANING OF SOURCE AND PROBE

Blocking of the capillary tubing in the probe was rarely caused by insoluble particles due to the presence of the in-line filter and the fused silica column. The major cause of blocking was the use of basic mobile phase which dissolved silica material from the HPLC column. The heated probe precipitated these materials after evaporation of the solvent. A solution of citric acid was efficient in clearing the partial blockage caused by this type of precipitate. However, caution had to be exercised when using this solution since prolonged application with excessive heat occasionally resulted in perforation of the capillary tubing. Once complete blockage had occurred, the tubing was replaced with a new length of capillary, fixed in place with silver solder, which was a relatively easy procedure. Careful application of the solder was

required to avoid "dry" joints for proper conductivity. A recently available modification of the probe in which the capillary can be screwed into the probe should be easier and faster to repair. Also, a new modification of the source by the addition of a desolvation chamber resulted in reduced frequency of source cleaning. The source block was easily removable without interference from the source assembly, resulting in an easier and faster cleaning procedure. The probe tip used to get dirty quickly after switching to PSP mode due to the deposition of pyrolysed material. This charred material appeared in mobile phases containing organic solvent, especially when ACN was included. The net effect was the appearance of unstable, spikey and irreproducible peaks with loss of sensitivity. Consequently, the use of such solvents in the PSP mode necessitated cleaning both the source block and the probe tip at the end of the working day. Also, careful attention was required to monitor the temperature of the probe, which changed as a result of changing the solvent unless the probe control was manually adjusted.

5.IV D I S C U S S I O N

5.IV.1 INTRODUCTION

Recent applications of the TSP LC-MS interface suggest that such an interface has special advantages. These include the ability to interface a conventional HPLC to a mass spectrometer, where up to 2ml/min of aqueous eluent can be introduced. It provides very mild ionization conditions while the latest modification on this interface, the PSP ionization mode, should provide both ionization and structural information for the analyte without the need for an external source of ionization or restriction in the mobile phases as in the TSP mode. Understanding the limitations and the operational parameters controlling the TSP/PSP interface is a pre-requisite for full advantage to be taken of the interface for routine applications.

5.IV.2 EFFECT OF PROBE TEMPERATURE

The mass spectral peak profiles in the PSP mode were smoother and more stable than those obtained in the TSP mode, which was a direct result of the generation and ionization processes. The ions were transported in an aerosol jet of droplets and vapour and sampled at the sampling orifice. The plasmaspray was operated at higher temperatures which resulted in a finer aerosol jet and consequently more homogenous sampling. The high operating temperature also resulted in more sensitivity for analytes

of low volatility and lowered the dependence on the high volatility components of the mobile phase. Together, these led to lower background noise and hence a better detection limit.

However, very high probe temperatures resulted in lower sensitivity to analytes, which might be due to sample pyrolysis [351]. Furthermore, prolonged operation at high temperature could cause both deposition of solids within the capillary, leading to blockage and coating of the tip with pyrolysed, non-conducting material leading to an interruption in the plasma discharge. Neverthless, relatively high probe temperatures could be used to induce fragmentation without significant loss of sensitivity [312,352]. This was not true for all drugs examined: indomethacin and buprenorphine showed only minor variations while flupenthixol and triazolam gave the same mass spectra at different temperatures examined. Others - morphine, amitryptiline and cannabidiol - were resistant to significant fragmentation. The different sensitivities obtained for the limited range of drugs examined at various probe temperatures, indicate that no fixed probe temperature can be applied for optimum response for all analytes. However, the temperature in PSP mode is not as critical as in TSP mode and this interface can be used for screening for the presence of a limited group of drugs with a wide range of sensitivities that might be low or nil for an unknown. Moreover, there is no single test compound or test mixture that would be ideal for optimizing the probe temperature.

Initial tuning of the mass spectrometer on the protonated ammonium acetate ion is commonly used in TSP mode. Throughout the limited work carried out in this mode such tuning was satisfactory (Figure 5.III.2(b)): the intensity of $[MH]^+$ of amitriptyline followed that of the buffer ion when the probe temperature was varied. This can be explained by the fact that TSP ionization is similar to conventional CI, which depends on the reagent gas, ion/molecule reaction rate constant, sample concentration and reaction time to yield the analyte ions [305,351]. This tuning maximizes the yield of buffer ions and hence the (secondary) analyte molecule ions. Such tuning is not always valuable in PSP mode (Figure 5.III.3), which does not depend on solvent ions for optimum response but depends on the volatility of the analyte as discussed earlier.

5.IV.3 EFFECT OF SOURCE TEMPERATURE

A symmetrical peak is an important requirement for good chromatography. The source temperature should be kept at a high temperature to maintain the volatilized analyte molecules emerging from the probe in the vapour phase, otherwise low volatility high molecular weight material tends to condense on the source block, which will be followed by gradual re-evaporation leading to peak tailing. This was evident when PEG was introduced to the mass spectrometer, where a high source temperature was needed to clear the deposited high molecular weight

material from the source block. This was less obvious for the examined drugs. However, no thermal degradation was observed at the source temperatures examined beyond what resulted from the probe temperature.

5.IV.4 EFFECT OF DISCHARGE VOLTAGE

Increased sensitivity was observed by raising the discharge voltage and this could be explained by increased ionization of the analyte by the glow discharge. However, the irreproducibility and instability of response which was also observed with higher discharge voltages might be because the rate of ionization was not as stable and homogeneous as under normal operation. Higher voltages are not required during normal PSP operation and there is no added value with respect to structural information for the analyte. Also, the increase in noise level accompanying the rise in analyte response did not improve the detection limit.

5.IV.5 EFFECT OF MOBILE PHASE CONSTITUENTS

The literature has frequently addressed the effects of ammonium acetate buffer [334,353] and mobile phase constituents on thermospray ionization and response. Plasmaspray ionization depends on glow discharge (discharge ionization) [312,354,355] where the response should be independent of the mobile phase constituents for its optimum response. However, the results presented here contradict the earlier work, at least in respect of the

examined drugs. A significant improvement in response was produced by the introduction of increasing amounts of ammonium acetate buffer, similar to TSP [334,339] but in this case, the optimum response occurred at higher levels of buffer concentration than those expected for TSP. These findings suggest that both modes of ionization (TSP and PSP) might be occurring simultaneously, especially if the probe tip was dirty and glow discharge ionisation was inhibited. Similarly, organic modifiers have enhanced the ionisation efficiency and ion beam stability, both of which were higher in the presence of ACN than MeOH. Mobile phases containing either solvent were better than an aqueous solution alone. These results could be explained by an easier ejection of the ions from the droplets of a highly organic solvent system than from an aqueous system. The decrease of the droplet surface tension with the increase of probe temperature will further lower the barrier to desorption of the ions from the droplet as described for TSP ionization [306]. However, the optimum solvent system for TSP/PSP ionisation should be determined together with its suitability for optimum chromatographic separation of the analytes of interest.

5.IV.5 SENSITIVITY AND REPRODUCIBILITY

The sensitivity obtained for buprenorphine (1.2 and 0.3ng by full scanning and SIR, respectively) by PSP(+) LC-MS was more or less similar to the detection limit

quoted for TSP, although better and poorer sensitivities have been reported for different groups of compounds, ranging from pg-ng levels. It is important to recognize that TSP ionization can be particularly sensitive for certain analytes but frustratingly insensitive for others [323]. Plasmaspray ionization should be better due to its mode of operation and several compounds should be assessed to define their detection limit before a conclusion is drawn on the overall sensitivity. However, sensitivity and reproducibility can be further improved by careful optimization of the operating parameters such as probe temperature, flow rate and mobile phase constitutents as well as by using a clean source and sampling cone, clean probe tip with clearly opened probe capillary and a smooth flow from the HPLC pump. These usually affect the ability of the ionization and ion sampling processes to produce a stable ion beam.

5.IV.7 DRUGS AMENABLE TO LC-MS

The spectra obtained under PSP(+) mode show that PSP is a soft ionization method for most of the examined drugs (Table 5.III.6) where the protonated molecular ion was either the base peak or even the only ion. This could be used to obtain molecular weight information. Other drugs showed significant fragmentation which could aid in structural elucidation of the molecule. This was similar to fragmentation found in TSP [322,347,353] where the fragmentation occurs due to thermal degradation.

Similarly, a common fragmentation process was the loss of water from the protonated parent molecule or a daughter ion [329]. A few compounds such as morphine, codeine and nalorphine showed ammoniated adduct ions at less than 10% of the base peak. The glucuronic acid fragment of M3G also formed an adduct with ammonia. These occurrences resemble gas phase reactions in CI where the protonation or ammoniation of the molecule depends on its proton affinity (PA). Protonated species are observed when the PA exceeds that of ammonia, and adduct ions are observed when the PA is less than that of ammonia [324]. The presence of both products in the PSP mode of operation suggests that the ionization process was a mixture of PSP and TSP ionization.

The selection of positive or negative ions is an important deciding factor for sensitivity. For example, barbiturates gave very low sensitivity PSP(+) but very good sensitivity in the PSP(-) mode: similar results were reported for barbiturates using TSP ionization [339,356]. High sensitivity in negative ion MS can be obtained for compounds which have a high electron capturing affinity, such as organochlorine pesticides [337]. This mode has also been used for the identification of steroid glucuronides using TSP [327].

Therefore, in a case where a negative result was obtained in the analysis of an unknown compound, it is important to consider that the operating conditions such as the probe temperature or the mode of ionization

(positive or negative) were possibly not suitable for that compound. It would be ideal if a mass spectrometer with an LC-MS interface could be operated in PSP(+) and PSP(-) alternatively, similar to GC-MS, but this is not possible due to the incompatibility of tuning the mass spectrometer in both modes at the same time.

The spectra obtained in PSP(-) for barbiturates were dominated by the presence of $(M-1)^-$ as the base peak. Other fragments resulted from the loss of alkyl groups from the parent molecule. For example, in the spectrum of amylobarbitone m/z 225 is $(M-1)^-$ and ions at m/z 155 and 197 result from the loss of the isopentyl and ethyl groups from parent molecule, respectively. However, a more comprehensive drug list should be examined using different modes to compile libraries for the drugs commonly encountered in forensic toxicology, to be used as a reference for identification of unknowns.

5.IV.8 HPLC-MS

From the previous discussion, several points have to be considered before introducing the HPLC effluent to the mass spectrometer via a TSP/PSP LC-MS interface, for efficient and trouble-free operation. Firstly, only volatile buffers and volatile ion pair reagents can be used, if required, to prevent precipitation of involatile material inside the probe. Secondly, although the use of this interface in PSP mode should be applicable to all solvents, the optimum response requires the presence of

ammonium acetate buffer with an organic modifier. Thirdly, albeit this interface can permit up to 2ml/min of mobile phase to be introduced to mass spectrometer, the presence of an HPLC column was the limiting factor, because the back pressure generated by the column and by the resistance in the probe capillary should not exceed the packing pressure of the column, which is usually 200 bar, otherwise the packing material would be distorted.

The aims of using LC-MS were to take full advantage of its application as described earlier and to complement routine HPLC applications for further identification and unequivocal confirmation. A special interest was taken in drug metabolites, sush as morphine glucuronides, where the presence of 6-monoacetyl morphine-3-glucuronide could confirm a previous heroin intake [81].

However, modification of an HPLC procedure to suit this LC-MS interface was not always easy. The mobile phase for hypnotic and anticonvulsant drug separation was modified by direct exchange of the non-volatile buffer with ammonium acetate [349]. LC-MS application was hampered by the limitation on the mobile phase flow rate due to high back pressure, and one mode of ionization [PSP(-) or PSP(+)] was insufficient to detect all the applied drugs.

It was not possible to obtain as good a chromatographic separation of morphine and its metabolite M3G as in previously described methods [96,357]. The pH of the LC-MS mobile phase could not be lowered to pH 2.1

using ammonium acetate/acetic acid, because the latter is a weak acid with a pK_a of 4.8 at 25° [358], whereas in the published method phosphate buffer was used. Another problem was that a single probe temperature could not be used to give optimum response for morphine and M3G simultaneously and thus to identify them from their intact protonated molecular ion. The diethylamine could be used to produce reasonable chromatography of the basic drugs. Even if incomplete separation of drugs was present, the known analytes could be quantified or distinguished by monitoring the abundance of selected ions in the mass spectra. However, basic mobile phases containing DEA, partially dissolved the silica material in the column, followed by deposition and partial blockage of the probe which limited the flow rate. Changes in the flow rate will lead to irreproducible retention times which otherwise could be used with the molecular weight or full mass spectrum for identification.

Identification of drugs without chromatography could be done by loop only injection in a manner similar to flow injection analysis, where biological fluids such as gastric lavage and urine could be tested without extensive purification. Such identification requires the existence of a library of drug spectra, although complex mixtures might be confusing.

The above presentation has shown that, although TSP LC-MS is the most popular interface [359] and is suited to conventional HPLC, which can be further expanded to normal

phase using PSP mode, none of the available LC-MS interfaces is as effective as the established GC-MS interface. Also, the work has shown that only by understanding the parameters controlling the LC-MS interface can all its advantages be utilized.

CHAPTER SIX

CONCLUSIONS

In this study, work was carried out in three areas of Forensic Toxicology to develop methods which can be used for routine analyses of drugs and solvents in biological samples, either clinically derived or from autopsy. As time allowed these were applied to samples from real cases.

In the first study, a solid phase extraction procedure was evaluated for the extraction of basic drugs from whole blood samples. This method was based on the extraction of the sample through two different colums of solid phase material. The first column was packed with Extrelut (diatomaceous) material where the buffered blood sample was absorbed by the Extrelut. The drugs were eluted into a second column containing a cation exchange sorbent (Bond-Elut SCX) where the drugs were adsorbed, purified and finally recovered. The extracts were analysed following preparation of EDMS derivatives by capillary GC-EIMS using full scan and SIR acquisition modes. A clean extract was obtained. The extraction efficiency and reproducibility (as S.D.) for the model drug morphine in spiked blood samples were 92±5% and 95±4% at 35 and 560ng/ml, respectively. For buprenorphine these were 83±5% and 87±4% at 0.5 and 8ng/ml, respectively. For other drugs evaluated, the recovery was better than 80%.

This method was very sensitive. Buprenorphine could be detected by SIR-MS down to the low picogram level. The procedure was mainly applied for routine analysis of morphine and buprenorphine as well as other basic drugs. It had no limitations on its application to post-mortem blood. However, further application on a larger scale to the analysis of these drugs is needed to correlate the results with those obtained with other established procedures.

The silylating reagents EDMS-I and DETMDS demonstrated the ability to derivatize several drugs where both N-EDMS and O-EDMS derivatives were obtained. The extra methylene group in the product compared to the analogous TMS derivatives can increase the RRT or retention index of a drug as an aid to separation from interferences and identification of unknown substances. Derivatization using this reagent is rapid, similar to the analogous TMS donor reagents. It can be applied for routine GC and GC-MS analysis. However, to make identification easy and rapid, a library of retention indices and mass spectra of EDMS-drug derivatives for the commonly encountered drugs in forensic toxicology is required. Once these are prepared, an SIR procedure could be constructed to include a wide range of these drugs in separate groups by careful selection of their retention time windows and their representative masses. This procedure could be used as a screening method for the selected drugs without losing the sensitivity and the

quantification ability of SIR-MS.

In the second study, two extraction methods were assessed for the analysis of paint solvents in blood, where C_8 to C_{12} n-alkane hydrocarbons were used as model compounds due to their presence in the paint materials. The first method was based on solvent extraction using high purity diethyl ether to extract blood samples after saturating the latter with ammonium carbonate. The extraction efficiency of such a procedure ranged from 70-91% with a reproducibility (S.D.) of 11-18%. The volume of the final extract recovered was reduced to 0.1ml and aliquots were analysed by GC or GC-MS. This approach limited the sensitivity of the method, which in turn limited its application for occupational monitoring where low blood levels of solvents are expected.

The other method was based on stripping the solvents from the blood sample by a dynamic head space technique (DHS). When the elution flask was placed in a water bath at 97^{o}, an extraction efficiency better than 90% was produced. The recovered solvents were trapped on a Tenax-GC column and were subsequently transferred in total to the GC column. The chromatogram was free from interference from a solvent front or other extraction solvent residues. The absolute sensitivity is determined by the instrument on which the analysis is performed and where GC-MS is used a sensitivity down to the pg level can be obtained. However, a major problem existed in the use

of this technique in an open laboratory environment and the presence of high background levels of contaminating solvents in the extraction and desorption system limited the determination of the sensitivity limit, even after these systems were cleaned. In future work, such systems should be reconstructed with new components and kept in an isolated room away from laboratory and atmospheric solvent contamination.

The DHS method was applied to a pilot study for occupational monitoring of a group of painters to assess the presence of paint solvents in their blood. Two venous blood samples were collected at the beginning and at the end of a working week. They showed the presence of several solvents similar to those present in paint material. The levels in the second series of samples were higher than those of the first series. The differences between the levels of the two samples were statistically significant for n-nonane, n-undecane and alkylbenzene. The levels in the first sample indicated incomplete clearance of these solvents from the body during the weekend, while the higher level in the second samples indicated the uptake during the working week. However, a clean extraction system and clean glass sample vials for blood samples are required for accurate results. Once the analysis is free from contamination, in a future study the uptake of a solvent can be quantified and its hazards can be assessed. Other parameters will also be needed for accurate assessment. Examples of parameters which can

affect solvent uptake include details of work practice, physical status, past and present clinical history, smoking habit, alcohol and drug intake as well as including a matching control group.

In the third study a thermospray/plasmaspray (TSP/PSP) LC-MS interface was assessed for its application to HPLC-MS in forensic toxicology using the plasmaspray ionization mode. Initially the operating parameters which control the sensitivity of the interface were evaluated. The probe temperature was critical for sensitivity and the extent of the fragmentation pattern of a compound. Although, during the PSP mode of operation the sensitivity was not severely reduced over a wide range of operating temperatures for a similar group of drugs, when an optimum sensitivity is required, the probe temperature has to be individually optimized for that particular drug. According to the literature, another benefit of plasmaspray mode operation over that of thermospray is that it has no restriction on the solvent used for LC-MS but it was nevertheless found that the sensitivity can be influenced according to the solvent system used. Mobile phases containing ammonium acetate buffer produced better sensitivity and peak stability which indicate that the ionization mechanism which occurred was a mixture of thermospray and plasmaspray ionization. Inclusion of organic modifier also improved the sensitivity further and acetonitrile was better than methanol in producing such an effect. This was probably due to the easier ejection of

the ions from the droplets coming out from the probe tip when they were composed of a mixed organic/aqueous solvent system rather than from a purely aqueous system. Another benefit of acetonitrile was the reduction of the back pressure on the HPLC pump which was generated from the column and the resistance inside the heated probe. This back pressure limited the flow rate used in HPLC-MS, which was in the range of 1ml/min, although the efficiency of the vacuum system can permit up to 2ml/min of solvent to be introduced to the mass spectrometer.

Mobile phases containing ammonium acetate buffer or diethylamine produced slightly basic solvent systems which, once passed through the substituted silica stationary phase in the HPLC column, resulted in slight dissolution of this material which precipitated in the heated probe. This was the main cause of probe blockage and required frequent maintenance. Also, the presence of acetonitrile in the solvent system led to rapid loss of sensitivity due to the charring of the source and the probe tip which required frequent dismantling and cleaning. HPLC-MS was evaluated using three model mixtures of basic drugs, barbiturates and opiates. The optimum chromatography obtained had poorer quality than that of conventional HPLC using, for example, phosphate buffer, but using the mass spectrometer as an HPLC detector, chromatography can be useful for identification and quantification purposes.

The mass spectra obtained were mainly useful for molecular weight information and gave little structural

Both negative and positive ion mass spectrometry can be used in plasmaspray, separately, but not alternately during acquisition. This limits the universality of mass spectrometric detection using the TSP/PSP interface for a screening procedure for unknown drugs where these might only be detected with sufficient sensitivity using one mode of ionization only, such as barbiturates, which require the negative ion mode.

Another benefit of this interface, and where it can be used for a quick identification, is when clean samples suspected of containing only a few drugs are analysed using the by-pass inlet of the LC-MS. The compilation of a library of mass spectra acquired under specified conditions for the commonly encountered drugs in forensic toxicology could make these identifications rapid and reliable.

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Appendix 1 Presents the listing of fragments above 3% of the base peak obtained in EI or CI of the following compounds:

1. Bis-TMS-morphine.
2. Bis-EDMS-morphine.
3. 3-MTBS-morphine.
4. Bis-MTBS-morphine.
5. Bis-EDMS-morphine (CI).
6. Bis-EDMS-nalorphine.
7. Bis-EDMS-nalorphine (CI).
8. EDMS-buprenorphine.
9. EDMS-buprenorphine (CI).
10. EDMS-dehydrobuprenorphine
11. EDMS-monoacetylmorphine.
12. MTBS-codeine.
13. EDMS-codeine.
14. O-EDMS-pentazocine.
15. BIS-EDMS-etorphine.
16. O-EDMS-temazepam.
17. O-EDMS-lormetazepam.
18. O-EDMS-flupenthixol.
19. N-EDMS-desmethyldiazepam.
20. Bis-O-EDMS-cannabidiol.
21. O-EDMS-testosterone.
22. Dextromethorphan.
23. Methadone.
24. Caffeine.
25. Dextromoramide.
26. Dipipanone.
27. Amitriptyline.

BIS-TMS-MORPHINE		BIS-EDMS-MORPHINE		3-MTBS-MORPHINE		BIS-MTBS-MORPHINE	
Mass	% Base	Mass	% Base	Mass	% Base	Mass	% Base
59	7.3	58	3.2	55	6.4	56	3.7
70	5.7	59	71.8	57	9.3	57	4.7
73	100.0	59	9.2	58	17.1	59	7.0
74	8.5	60	6.0	59	19.4	73	93.9
75	15.2	61	5.2	70	13.2	74	14.0
77	4.2	70	6.8	73	39.8	75	17.5
79	5.0	73	5.6	74	6.1	77	3.1
81	3.1	75	13.6	75	26.9	79	3.0
94	5.5	87	41.2	81	12.8	81	4.8
96	3.4	88	3.2	82	6.2	96	8.5
129	3.1	89	5.9	94	8.9	115	3.1
146	35.6	94	7.8	96	9.5	133	11.4
147	7.3	133	4.0	96	7.0	146	16.4
179	3.9	146	44.3	115	7.3	147	24.7
196	45.9	147	5.9	124	21.2	148	3.5
197	7.1	165	3.3	133	8.7	177	4.4
203	3.9	179	4.1	142	7.5	191	13.0
205	3.6	193	3.3	146	7.6	192	3.1
207	15.2	203	5.4	162	27.1	193	8.9
208	3.4	205	4.4	163	6.0	203	4.6
216	7.5	207	6.6	165	6.6	205	5.8
217	3.3	208	3.2	179	10.7	207	56.9
220	16.2	210	51.7	181	6.1	208	22.4
221	7.5	211	10.2	186	7.0	209	16.4
229	4.0	216	11.1	191	10.1	216	6.2
232	3.4	217	6.1	193	8.7	220	3.8
234	27.5	219	6.7	203	12.9	221	11.6
235	5.4	220	17.8	204	7.1	222	4.7
236	62.5	221	7.5	205	8.9	225	3.7
237	24.7	229	5.0	207	59.5	229	4.6
238	5.3	230	4.5	208	16.8	231	3.0
246	9.1	231	6.7	209	12.3	238	18.2
253	4.5	232	5.0	216	18.8	239	3.0
266	6.5	234	24.6	217	11.6	249	3.1
267	6.9	235	9.3	220	6.4	251	3.5
269	3.1	236	3.3	229	32.2	253	4.3
270	3.1	246	4.2	230	14.2	255	4.0
272	5.2	248	35.7	231	11.9	262	9.0
281	12.1	249	7.7	232	9.5	263	3.4
282	4.8	250	81.2	241	6.6	265	5.8
283	3.5	251	41.7	242	7.4	266	6.8
287	25.1	252	10.3	243	11.0	267	22.4
288	6.3	253	5.4	246	6.0	268	5.8
293	4.1	254	3.5	253	10.1	269	4.8
324	15.5	255	4.8	254	8.3	272	9.3
325	4.1	257	3.2	255	12.4	276	6.9
340	4.3	260	8.5	256	9.0	278	29.2
355	4.5	262	3.1	257	10.0	279	11.7
356	7.4	264	3.1	258	6.8	280	3.4
357	4.4	266	7.4	259	7.1	281	56.1
371	12.1	267	12.5	266	10.5	282	23.6
372	9.3	268	5.0	267	16.4	283	13.8
373	3.5	269	4.9	268	8.4	284	5.5
401	18.0	270	4.8	269	9.2	285	4.8
402	5.5	272	6.7	270	8.0	295	3.9
414	38.6	280	4.4	271	10.6	323	3.6
415	13.1	281	12.5	272	19.7	324	7.1
416	4.2	282	5.3	273	7.7	325	7.8
428	13.2	283	5.8	281	17.2	326	3.6
429	72.9	284	5.8	282	9.8	327	7.4
430	27.1	286	4.7	283	14.2	329	6.0
431	8.9	295	9.8	284	10.6	335	32.6
		296	4.8	285	24.6	336	8.6
		297	4.5	286	8.9	337	3.5
		298	3.6	299	8.9	339	4.0
		301	36.0	324	15.1	340	3.8
		302	10.9	325	7.0	341	29.0
		307	6.1	329	18.0	342	9.9
		312	3.5	330	9.1	343	7.3
		314	3.1	342	100.0	355	22.3
		324	17.4	343	31.1	356	7.9
		325	4.1	344	11.4	357	4.8
		326	4.1	366	7.3	366	4.8
		338	12.8	382	6.4	382	6.3
		339	4.0	384	7.1	385	3.1
		340	3.2	398	8.7	397	3.4
		354	6.1	399	57.8	398	4.6
		355	3.3	400	21.9	399	7.3
		369	3.7	401	8.3	401	3.2
		370	10.6			413	94.5
		371	8.6			414	38.4
		385	21.5			415	15.4
		386	6.1			416	3.3
		398	4.0			429	16.2
		399	15.9			430	6.7
		400	16.5			431	4.7
		401	5.9			454	4.2
		428	54.4			455	5.4
		429	57.9			456	100.0
		430	25.3			457	44.8
		431	7.0			458	16.7
		442	37.0			485	11.0
		443	14.0			486	3.6
		444	4.2			498	7.8
		455	3.7			503	3.8
		456	24.6			512	7.1
		457	100.0			513	21.2
			49.8			514	7.8
			20.3				
			3.4				

BIS-EDMS-MORPHINE (CI)

Mass	% Base
61	5.8
63	6.6
67	40.0
68	7.7
69	56.2
70	26.4
71	44.0
72	7.2
75	24.5
79	10.8
81	19.2
82	4.6
83	22.9
84	8.5
85	39.3
86	4.5
87	14.0
89	6.3
95	6.8
97	7.0
105	47.4
106	6.9
107	5.3
146	5.1
207	43.1
208	12.0
209	8.1
210	8.4
220	5.4
223	49.8
224	14.3
225	9.4
248	5.8
250	26.1
251	13.3
252	4.4
266	5.0
267	8.0
268	4.3
269	4.8
281	12.2
282	4.4
295	5.5
296	5.0
297	11.9
298	4.8
324	5.6
326	6.0
353	9.2
354	100.0
355	42.1
356	19.4
357	4.2
385	5.7
396	5.1
398	5.2
399	9.4
400	6.7
428	17.5
429	16.9
430	7.3
442	12.1
443	5.0
456	12.8
457	56.0
458	55.3
459	27.4
460	10.3
500	21.5
501	9.0

BIS-EDMS-NALORPHINE

Mass	% Base
56	3.8
59	77.9
60	6.1
61	5.7
70	5.7
73	5.1
75	13.5
84	7.0
85	12.5
87	49.2
88	3.8
89	6.8
122	6.7
165	3.1
179	3.9
195	4.1
203	4.2
205	3.7
216	8.8
217	5.8
218	7.7
219	3.9
229	4.6
230	4.9
231	5.5
232	9.9
236	31.7
237	5.4
243	3.4
247	4.4
248	7.6
253	5.2
254	3.8
255	5.0
256	3.7
257	4.6
260	5.5
261	3.7
266	7.8
267	12.7
268	5.9
269	4.8
270	3.6
274	36.4
275	7.8
276	6.1
280	4.8
281	11.2
282	5.5
283	5.9
286	5.0
290	3.8
295	10.9
296	5.1
297	4.7
298	5.3
307	6.2
308	3.1
309	3.3
310	6.0
312	3.5
327	37.1
328	11.3
338	46.8
339	16.2
340	7.6
350	15.1
351	3.7
364	5.2
369	4.2
371	5.2
380	6.0
383	3.2
385	17.6
386	4.7
387	3.1
396	9.2
398	5.5
399	13.0
400	13.1
401	5.7
426	3.8
442	74.8
443	34.9
444	12.9
454	52.0
455	23.0
456	13.0
468	25.1
469	9.0
482	15.7
483	100.0
484	50.3
485	21.9
486	3.8
484	

BIS-EDMS-NALORPHINE (CI)

Mass	% Base
61	9.5
63	9.3
67	70.6
68	14.0
69	100.0
70	46.1
71	77.4
72	10.7
73	5.5
75	26.4
79	18.9
80	4.5
81	36.0
82	9.3
83	41.0
84	17.4
85	68.6
86	11.2
87	12.9
89	5.6
93	4.7
95	12.0
96	5.5
97	14.0
98	4.8
105	49.7
106	6.1
107	5.8
109	7.0
123	4.8
207	85.3
208	25.1
209	16.1
223	95.0
224	29.2
225	17.9
267	4.6
281	19.4
282	5.2
295	4.4
297	11.2
380	17.9
381	5.4
483	7.4
484	

EDMS-BUPRENORPHINE

Mass	% Base
55	58.8
56	9.0
57	20.3
59	29.1
73	3.6
75	5.6
83	10.2
84	30.1
87	16.1
101	9.9
110	4.3
207	4.2
216	3.9
281	4.5
295	3.9
297	3.9
299	3.4
325	4.4
422	6.5
424	6.1
438	11.8
452	21.3
453	8.5
464	100.0
465	48.3
466	16.8
492	14.9
493	4.9
494	5.1
496	40.3
497	15.7
498	3.5
506	41.4
507	17.8
508	4.7
520	21.2
521	10.0
522	4.0
524	9.7
535	7.5
536	3.5
538	7.4
553	8.0

EDMS-BUPRENORPHINE (CI)

Mass	% Base
61	3.6
63	2.4
67	29.1
68	6.5
69	43.2
70	21.1
71	34.2
72	5.5
73	3.0
79	7.7
81	15.0
82	4.3
83	18.9
84	10.6
85	30.9
86	3.9
93	2.4
95	6.4
96	3.3
97	6.9
98	6.8
99	2.1
100	2.0
101	2.0
105	4.4
107	2.4
109	4.1
111	3.2
112	2.2
121	2.2
123	3.1
137	2.2
193	3.1
207	88.7
208	28.5
209	20.4
210	3.2
223	100.0
224	32.9
225	23.1
226	3.9
267	2.0
281	24.4
282	7.6
283	5.4
297	15.6
298	4.8
299	3.4
355	4.4
371	2.0
452	3.1
464	6.3
465	2.4
496	5.4
504	7.1
505	4.4
506	10.2
507	4.9
508	2.8
520	3.1
521	2.5
522	5.2
534	2.7
535	7.6
536	29.6
537	15.3
538	6.6
553	2.7
554	3.2
578	2.2

EDMS-BUPRENORPHINE, THE DEHYDRATED FORM

Mass	% Base
55	71.1
56	7.2
57	17.3
59	35.4
73	5.1
75	7.7
84	35.9
87	13.1
110	9.0
147	3.9
205	4.1
207	9.6
208	3.1
209	3.4
216	4.3
267	3.4
281	5.1
282	4.6
283	4.9
284	4.2
297	3.6
299	4.1
301	4.5
349	5.1
356	4.1
422	11.7
423	4.1
424	7.2
432	3.4
448	5.4
464	23.5
465	6.8
478	7.7
480	4.1
492	4.1
494	40.1
495	12.2
504	6.8
506	100.0
507	41.1
508	9.3
520	88.3
521	35.8
522	7.2
534	22.9
535	77.6
536	30.6
537	6.9

EDMS-MONOACETYLMORPHINE

Mass	% Base
59	16.3
59	8.9
70	4.1
75	7.1
81	3.8
87	4.7
124	7.2
146	4.6
162	7.1
165	3.1
166	3.6
204	18.2
205	4.1
207	5.8
216	5.7
217	3.2
220	3.3
229	3.8
230	3.2
231	3.8
250	3.4
253	3.8
260	4.0
266	7.0
267	10.3
268	4.2
269	3.1
280	3.9
281	5.3
282	4.5
283	4.6
284	3.5
285	3.5
294	4.4
295	4.2
296	9.4
297	9.8
301	44.5
302	10.6
312	4.6
324	10.9
325	3.4
338	5.4
342	11.0
353	3.3
354	74.3
355	22.0
356	17.1
357	3.4
370	6.3
384	4.5
412	7.4
413	100.0
414	35.5
415	8.0

MTBS-CODEINE

Mass	% Base
59	5.0
70	3.5
73	43.4
74	3.5
75	13.5
94	6.1
115	3.7
146	12.6
165	3.9
178	20.4
179	7.4
188	4.9
204	4.1
207	3.1
216	3.5
229	8.1
235	25.2
236	4.5
238	15.1
239	5.6
254	3.7
255	3.5
266	3.1
270	3.6
276	4.6
280	5.4
281	3.1
282	8.0
283	3.6
285	15.3
286	4.7
298	4.1
299	3.5
313	100.0
314	27.7
315	6.5
355	3.5
356	56.3
357	15.6
358	3.6
412	3.5
413	4.2

EDMS-CODEINE

Mass	% Base
55	11.2
56	6.4
57	46.5
58	3.9
59	44.6
59	6.9
60	3.6
61	3.4
69	5.8
70	10.1
71	29.4
73	6.6
75	9.0
77	4.0
83	4.4
85	17.2
87	27.3
89	6.4
91	4.1
94	10.7
99	4.7
115	7.4
127	4.7
128	3.3
141	4.0
143	3.7
144	4.4
146	29.1
147	6.4
152	6.5
153	4.1
163	3.2
165	6.6
175	3.4
178	71.9
179	27.4
180	4.0
181	5.1
185	4.0
188	14.7
189	4.8
190	5.7
196	3.7
199	3.4
204	3.3
205	3.3
207	4.6
210	54.5
211	9.5
212	4.0
214	9.0
216	4.2
221	3.4
223	3.9
225	4.3
229	37.2
230	9.2
235	7.1
238	3.3
239	4.5
240	3.3
242	3.9
248	37.6
249	9.3
250	3.2
253	3.3
254	4.9
255	4.9
266	6.4
268	4.2
269	4.0
270	3.0
280	11.3
281	6.8
282	11.1
283	4.0
285	3.2
295	3.9
298	3.2
299	3.5
313	21.3
314	5.0
327	12.3
328	8.1
329	3.3
342	3.1
356	6.6
357	20.1
358	5.3
370	15.2
371	3.4
383	4.2
384	20.9
385	100.0
386	28.6
387	7.1

O-EDMS-PENTAZOCINE

Mass	% Base
59	14.2
68	3.1
69	14.4
70	18.9
71	5.8
84	3.1
87	8.3
110	18.4
113	11.2
115	4.0
128	3.5
129	4.0
138	3.5
141	3.4
157	4.0
163	3.5
164	14.7
164	3.6
171	4.3
178	19.6
189	4.2
193	4.6
199	3.0
201	5.7
203	4.7
215	10.8
216	3.8
217	5.7
220	3.3
229	16.9
230	5.3
231	10.2
232	19.7
233	10.9
234	4.8
243	3.9
244	4.3
245	23.6
246	18.0
247	9.4
248	5.4
258	51.6
259	51.6
260	43.9
261	12.2
271	5.6
272	4.3
273	5.2
274	10.7
286	6.9
289	9.9
302	26.2
303	100.0
304	30.1
305	6.8
316	22.1
317	5.1
328	12.9
329	4.7
342	10.6
354	3.3
356	60.7
357	18.2
358	4.2
369	4.4
370	44.6
371	36.4
372	9.7

O-EDMS-[illegible]

Mass	% Base
55	9.4
56	5.6
57	10.6
58	12.4
59	33.4
60	3.4
61	8.9
67	4.1
70	3.7
71	3.2
73	21.7
75	16.6
77	4.1
80	3.6
87	15.9
89	4.5
91	5.8
94	5.2
95	3.9
96	4.0
121	16.9
128	3.9
129	4.4
132	3.5
133	16.0
135	4.2
145	5.2
147	5.8
148	6.0
150	8.2
162	34.3
163	9.5
164	32.9
174	4.7
177	3.2
193	7.4
203	5.5
205	3.5
207	80.1
208	14.3
216	5.4
217	3.1
221	3.7
222	3.1
230	4.0
231	5.7
232	12.9
235	3.4
243	4.9
247	10.2
248	5.2
250	37.6
253	5.1
259	3.6
260	5.8
261	15.5
262	3.5
266	4.9
272	100.0
273	20.7
274	8.1
280	3.1
281	33.0
282	5.8
283	4.7
284	4.2
286	38.6
287	10.3
288	5.3
296	7.8
297	7.6
300	3.1
302	13.1
303	4.4
309	3.2
311	6.5
322	3.2
323	9.6
327	3.2
327	3.4
337	3.4
343	3.5
353	3.4
354	33.6
355	8.1
367	4.9
368	20.7
369	6.3
382	15.1
383	6.1
384	9.8
396	8.0
410	81.5
411	27.9
412	5.3
464	4.8
468	18.9

O-EDMS-TEMAZEPAM

Mass	% Base
58	3.5
59	58.1
60	4.1
61	5.6
75	12.3
77	10.8
87	19.8
89	3.3
102	6.4
115	4.9
125	3.5
130	9.4
151	4.5
152	4.2
164	9.5
165	10.3
177	7.4
178	13.6
179	3.1
179	6.4
180	4.0
193	4.2
205	4.8
219	4.7
221	6.8
226	3.0
228	6.4
239	6.9
241	3.5
253	3.9
254	8.0
255	40.3
256	58.0
257	52.3
258	27.1
259	14.3
271	4.8
282	6.4
283	60.6
284	14.3
285	22.4
286	4.1
329	3.8
343	3.0
357	100.0
358	34.9
359	48.2
360	12.6
361	3.1
371	14.8
372	3.6
373	5.3
385	3.9
386	17.7
387	5.9
388	6.4

O-EDMS-LORMETAZEPAM

Mass	% Base
57	3.4
58	5.6
59	97.1
60	7.9
61	7.8
75	20.8
77	4.8
87	31.6
89	3.8
93	4.5
100	3.5
102	6.1
111	5.8
115	4.8
117	3.3
125	5.0
130	4.0
138	3.1
150	3.0
151	3.2
163	4.1
164	3.7
165	5.9
176	3.1
177	11.6
178	14.5
179	3.2
179	6.6
180	3.5
181	4.2
182	4.9
190	3.4
193	3.8
207	3.0
219	3.3
226	3.6
239	4.8
253	8.9
254	5.1
255	9.6
256	5.8
269	4.3
288	3.7
289	27.6
290	16.5
291	34.0
292	12.3
293	12.8
294	3.0
317	51.6
318	10.3
319	34.5
320	5.6
321	5.9
363	3.7
385	5.4
391	100.0
392	31.6
393	77.1
394	22.7
395	16.9
396	3.7
405	13.6
406	3.8
407	9.4
420	3.6

O-EDMS-FLUPENTHIXOL

Mass	% Base
55	4.6
56	8.5
58	4.0
59	18.0
70	16.9
73	3.8
75	3.8
82	5.9
87	11.7
97	27.5
98	40.6
99	7.0
125	15.6
130	3.9
186	3.5
214	4.8
221	13.5
222	6.3
227	5.0
229	100.0
230	30.2
231	8.9
234	3.0
265	5.1
289	6.2
291	8.1
355	3.0
403	5.1
491	4.2
505	3.6

N-EDMS-DESMETHYLDIAZEPAM

Mass	% Base
51	4.2
58	5.7
59	100.0
60	7.2
61	4.0
65	4.1
75	6.5
77	7.0
87	29.1
88	3.2
89	3.9
90	3.1
91	21.6
92	3.8
100	3.4
103	3.8
116	3.8
117	3.3
130	3.7
146	8.8
150	3.1
163	4.6
190	5.1
207	4.2
227	11.3
228	3.8
229	5.3
241	3.4
269	3.8
313	5.9
327	24.3
328	6.1
329	8.5
341	11.8
342	4.2
343	4.7
355	85.9
356	54.6
357	42.9
358	19.9
359	4.5

BIS-O-EDMS-CANNABIDIOL

Mass	% Base
55	6.7
59	57.9
60	6.3
61	3.6
67	3.5
68	19.9
75	9.5
81	3.3
87	46.5
88	6.2
89	4.1
93	6.8
107	6.3
109	5.6
121	3.6
133	5.2
215	3.0
245	3.8
258	5.1
296	4.6
314	3.2
315	18.7
316	5.2
335	3.4
347	13.0
348	3.6
349	5.6
352	16.9
353	5.9
361	3.0
363	7.5
365	32.0
366	12.7
367	5.1
378	5.2
379	15.4
380	7.4
389	4.2
403	5.6
417	8.3
418	100.0
419	47.1
420	22.8
421	5.5
458	4.0
471	4.6
486	11.7
487	4.6

O-EDMS-TESTOSTERONE

Mass	% Base
53	7.8
55	19.6
58	5.2
59	82.4
60	6.4
61	22.0
65	4.0
67	15.2
69	3.4
73	6.3
75	69.7
76	5.7
77	16.7
78	3.4
79	26.1
80	3.4
81	14.6
87	49.9
88	4.4
89	12.6
91	31.0
92	5.3
93	22.6
94	3.9
95	12.6
101	17.6
103	4.0
105	24.4
106	4.8
107	13.5
109	4.1
115	31.9
116	4.7
117	8.7
119	14.4
120	4.6
121	9.8
122	5.7
123	7.5
124	10.8
129	6.2
130	4.0
131	14.1
132	3.3
133	11.4
134	6.5
135	5.1
136	4.7
143	100.0
144	21.8
145	19.3
146	9.6
147	27.7
148	9.5
149	6.7
155	3.2
157	6.5
158	4.2
159	7.0
161	4.6
162	3.1
171	9.9
173	4.1
174	5.1
175	3.4
183	6.8
185	6.8
187	4.2
195	3.0
197	5.6
199	3.8
210	4.7
211	5.0
213	6.5
226	13.4
227	10.3
228	8.9
229	7.9
242	5.3
251	5.0
252	4.0
253	6.4
255	5.0
269	13.8
270	39.2
271	7.3
318	16.9
319	3.9
331	9.2
332	5.7
345	79.5
346	25.5
347	5.6
359	14.0
360	3.3
374	44.2

DEXTROMETHORPHAN

Mass	% Base
55	4.0
58	9.2
59	98.2
60	6.0
67	3.2
70	5.0
75	3.4
77	4.9
79	3.2
82	7.4
91	7.5
94	3.4
115	14.1
116	3.3
117	3.1
121	5.6
122	3.9
127	4.7
128	15.8
129	8.8
131	4.1
135	4.4
136	3.0
141	11.0
142	3.6
143	3.1
144	5.3
145	4.8
146	6.0
147	5.2
148	7.8
150	76.5
151	7.2
152	4.1
153	5.8
154	3.2
155	3.7
157	3.5
158	5.9
159	9.3
160	5.6
161	7.0
162	9.3
165	4.2
170	3.1
171	40.2
172	17.9
173	5.3
174	16.2
175	7.0
181	3.0
183	3.4
184	8.4
185	9.1
186	3.7
188	3.4
197	3.4
198	5.3
199	4.1
200	3.3
202	3.8
203	22.8
211	3.1
212	28.6
213	29.2
214	49.4
215	8.1
228	8.7
242	12.3
256	11.7
269	3.8
270	72.2
271	100.0
272	18.9

METHADONE

Mass	% Base
56	11.2
57	6.5
70	8.7
71	8.6
72	100.0
73	23.2
77	4.4
85	9.0
86	3.5
91	17.2
105	6.5
115	8.5
117	4.9
152	3.3
165	15.2
166	3.0
167	3.4
178	10.4
179	9.4
180	6.3
193	3.9
195	4.6
223	13.6
294	9.8

DEXTROMORAMIDE

Mass	% Base
55	3.6
56	5.2
70	3.1
91	4.7
98	11.9
100	79.7
101	6.1
115	6.5
129	44.3
139	4.9
165	14.8
166	7.1
167	6.2
178	5.7
179	4.6
193	6.1
194	11.4
195	4.6
207	3.1
236	8.3
264	4.3
265	100.0
266	29.1
306	9.6

DIPIPANONE

Mass	% Base
55	3.5
56	5.7
57	3.3
69	4.4
91	7.6
105	3.6
110	12.0
111	3.4
112	100.0
113	19.4
115	4.6
117	3.3
165	8.8
178	6.6
179	6.4
180	4.4
223	8.9
334	16.3
335	4.0

AMITRIPTYLINE

Mass	% Base
58	100.0
59	5.1
189	2.1
202	4.9
203	3.7
215	3.5
217	2.4

CAFFEINE

Mass	% Base
55	41.1
56	3.1
67	32.6
68	3.5
81	4.1
82	25.1
109	55.4
110	6.4
136	5.1
137	6.9
165	4.7
193	11.3
194	100.0
195	10.2