Application of Solid Phase Microextraction (SPME) Technology to Drug Screening and Identification

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Abstract

Benzodiazepines, tricyclic antidepressants and opiates are involved in poisoning episodes and fatalities. Solid phase microextraction (SPME) is a new extraction technique, which uses a fused silica fibre coated with a polymeric coating to extract organic compounds from their matrix and directly transfer analytes into a gas chromatograph (GC) by thermal desorption in a GC injector.

This thesis reports for the first time, the development of a specific, sensitive and rapid procedure for identifying and quantifying such drugs in *post-mortem* matrices using direct immersion SPME and gas chromatography-mass spectrometry electron impact ionisation selected ion monitoring (GC-MS-Ei-SIM). SPME conditions such as extraction temperature, extraction time, salt addition and pH were investigated and optimised by extracting some of the drugs from a prepared plasma matrix. The most appropriate fibre coating for most drugs was found to be polyacrylate (PA), followed by Carbowax/divinylbenzene.

The results also showed that sodium chloride could be added to the samples for a considerable increase of the extraction yield. The enhancement factors achieved by addition of sodium chloride ranged from 1.4 for bupivacaine to 7 fold for diazepam. The detection limits were 0.27 mg/L for bupivacaine, 0.42 mg/L for diazepam, 0.008 mg/L for meperidine, 0.0048 mg/L for amitriptyline, 0.02 mg/L for clomipramine and 0.003 mg/L for chlorpromazine. The calibration curves of the drugs were reproducible and linear over the concentration range of 1-7 mg/L.

In addition, the method was applied to blood and urine samples from sixteen *post-mortem* cases where the cause of death was suspected to be drug poisoning. This method was used here for the first time on this type of sample. Drugs were detected in all the samples by this method. The results showed that the developed SPME method is useful for the extraction of the majority of antidepressants, benzodiazepines, analgesics and local anaesthetics in *post-mortem* samples. However, there were a few limitations for the extraction of some of the drugs of interest such as morphine, cocaine, nordiazepm, tramadol and fluphenazine. The

probable causes, for example the heat instability of benzoylecgonine, are discussed and some suggestions for solving these problems are offered.

This thesis also brings together:

a) All the reported results of *post-mortem* redistribution of drugs.

b) Data of stability of drugs in *post-mortem* materials.

c) Data of site- and time-dependant concentration differences.

d) Recommendations, from a variety of sources, on *post-mortem* sampling procedures.

Finally a protocol for the drug extraction from *post-mortem* tissue samples is offered and further investigations that research may follow are explained.

Preface

On graduation with a Pharm.D. (Doctorate of pharmacy) in Pharmacy from Isfahan University of Medical Sciences in 1986, I began to work as an academic staff at Yazd University of Medical Sciences. Following an offer of a scholarship to me by Yazd University of Medical Science, I came to Manchester in September 1997. After studying, for three months, Academic English and six months of the MSc courses on toxicology, I started my research for this thesis in July 1998. During my research, one paper was published in the Annals of Clinical Biochemistry 2001; **38**: 541-547. In addition, I had poster or oral presentation in the 25th International Symposium on Chromatography; 1-5th October 2000; London, Pathology 2000; 15-17th May 2000; Birmingham, the 9th International Congress of Toxicolgy; 8-12th July 2001; Brisbane (Australia), and the 6th International Convention of Islamic Medical Association of North America; 27-31st July 2001; Tehran (Iran).

Declaration

I declare that no portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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I would like to dedicate this project to my wife, Nasrin, and my son, Ali, who have been very patient with me enduring many hours of frustration and desperation.

Abbreviations

amu	Atomic mass unit	**m/z**	Mass-to-charge ratio
ASPEC	Automated sample preparation with extraction columns	**MS**	Mass spectrometry
CAR	Carboxen	**NAD**	Nicotinamide adenine dinucleotide
CV	Coefficient of variation	**NPD**	Nitrogen-phosphorus detector
CW	Carbowax	**PA**	Polyacrylate
DI	Direct immersion	**PDMS**	Polydimethylsiloxane
DVB	Divinylbenzene	**PFK**	Perfluorokerosene
ECD	Electron capture detector	**REMEDi**	Rapid emergency drug screening
ED50	Median effective dose	**rpm**	Revolutions per minute
EI	Electron impact	**RIA**	Radioimmunoassay
EMIT	Enzyme multiplied immunoassay technique	**SFE**	Supercritical fluid extraction
FID	Flame ionisation detector	**SIM**	Selected ion monitoring
G6PDH	Glucose 6-phosphate dehydrogenase	**SIR**	Selective ion recording
GC	Gas chromatograph (y)	**SPE**	Solid phase extraction
HEETP	Height equivalent to one effective theoretical plate	**SPME**	Solid phase microextraction
HPLC	High pressure liquid chromatography	**SD**	Standard deviation
HS	Head space	**SE**	Standard error
IS	Internal standard	**TD50**	Median toxic dose
LOD	Limit of detection	**TIC**	Total ion chromatogram
LOQ	Limit of quantification	**TLC**	Thin layer chromatography

The Aims of the Thesis

It is the major objective of this thesis to establish solid phase microextraction (SPME) as a variable sample preparation technique for screening, identification, and quantification of a wide range of different drug groups in *post-mortem* matrices. It is also a main purpose of this thesis to investigate the effects of parameters such as pH, temperature and addition of sodium chloride, which govern sensitivity and time efficiency of the technique. While SPME is primarily an equilibrium extraction method, quantitative extraction can be performed if the proper controls of extraction conditions, such as, heating temperature and pH have been selected. The further aim of this study is to compare the SPME-GC-MS results with the conventional methods.

Chapter 1

Introduction

1. Introduction

Most analytical procedures involving chromatography have several steps including sampling, sample preparation, separation, quantitation, statistical evaluation, and interpretation[1]. Each of these steps is critical for achieving correct results. The sampling steps include deciding where to obtain samples that properly describe the object or problem being characterised, and selecting a method to obtain samples in the requisite amounts[2].

Although some samples are inherently ready for analysis, a sample preparation step is essential to isolate the target compounds from a sample matrix, because most analytical instruments cannot handle the matrix directly[2]. Sample preparation, in its simplest form, may involve dilution, filtration, evaporation, or centrifugation[3]. However, in the preparation of more complex samples and samples with low levels of constituent compounds, is important to obtain certified analytical data. This may involve sample clean-up, sample concentration, or changing the physical form of the sample as to make it more amenable to the analytical technique of choice[2]. For example, before the determination of components present in *post-mortem* samples, they must be isolated from the matrix and then concentrated[4].

During the separation step, the clean, isolated mixture, containing target analytes, is divided into its constituents, typically by means of a chromatographic or electrophoretic technique[2].

The identification of the compounds can be based on a retention time, using gas or other chromatography. It may also be combined with more sensitive instruments, such as mass spectrometers, to eliminate possible ambiguity in detection and, in particular, to facilitate quantification where there is risk of interference from other compounds derived from matrices[5,6].

Statistical evaluation of the results provides both an estimate of the concentration of the targeted compound in the sample and the range within which its concentration lies. The data will then indicate certain decisions, which may include a move to take more samples for further investigation of the object or the problem[7].

It is important to notice that analytical steps follow one after another, and the next one cannot start until the previous one has been completed. Therefore, the slowest step generally determines the overall speed of the analytical process, and improving the speed of a single step may not cause an increased throughput. To enhance the overall performance of analysis, all steps have to be considered. Errors arising in any step, including sample preparation can cause poor performance of the procedure as a whole[2].

Modern instruments, such as gas chromatograph/mass spectrometers (GC/MS), can separate and quantify complex mixtures and automatically perform analysis and evaluation of the results. In spite of the advances in the separation and quantification methods, many extraction procedures are based on classical methods of liquid/liquid or liquid/solid extraction, using separating funnels and Soxhlet extractors and have not changed too much over the past century[2, 8].

The most commonly used technique has been liquid extraction, based on the fact that organic compounds can usually be removed from a matrix by extraction with a water-immiscible solvent. This method relies largely on the relative solubility, diffusion, partitioning, viscosity, or surface tension of the components of interest and the solvent[3].

At first sight, this may appear simple and easy, but, to obtain high recoveries, it is often necessary to repeat the extraction process several times and then to bulk the fractions together before analysis[3]. It is also true for solid-liquid extraction, where samples have to be continually extracted, to achieve the desired efficiency[9]. So, while traditional sample preparation methods can be very effective, they also are extremely time and labour intensive, use multiple-step procedures, which make them prone to loss of analytes, and use large amounts of toxic organic solvents. These characteristics make such methods very difficult to integrate with sampling and sample preparation[10].

Two essential considerations have driven the development of modern alternative extraction methods:

1) The need for faster analysis. Automation of both analytical measurement and data handling has decreased analysis time from hours and sometimes days. Thus, in most

cases, sample preparation or extraction has become the major rate-determining step in the analysis[10].

2) The concerns about the hazardous nature of many commonly used solvents and the risks and increasing cost of their disposal[10].

Recent years have seen considerable efforts to develop alternative and improved methods for extraction, and to make them commercially available. This has caused a number of systems to be developed for both liquid-liquid and liquid-solid extraction.

1.1. Extraction methods

The precision of any assay depends on the precision of the instruments and on the non-instrumental parts of the method. In order to increase later precision, the first approach is to increase the precision of sample preparation[10]. In order properly to detect and quantify drugs in medical or autopsy materials, the analytes should be extracted from the matrices before analysis[4]. Chromatographic methods require extensive purification of the sample to get rid of matrix contaminants and to concentrate the analytes into a small volume of suitable solvent[11].

1.1.1. Solvent extraction

Solvent extraction is defined as the process of separation of one compound from a mixture by dissolving it into a solvent in which it is readily soluble, but the other constituents of the mixture are not soluble, or are at least less soluble than the analyte[3].

The separation process may involve a mixture of two or more solids, a solid and a liquid, or two or more liquids[10]. The extraction of an analyte from one phase into a second phase is dependent upon two main factors of solubility and equilibrium. The principle by which solvent extraction is successful is that "like dissolves like". That is, to remove a polar solute from a solution, a polar solvent should be used and to remove a non-polar solute from a solution, a non-polar solvent should be used[3].

Un-ionised solutes are more easily extracted into non-polar organic solvents, and the less polar the solute the more efficient is the extraction process. The major problem

for extraction of polar solutes into polar solvents is the miscibility of polar solvents with water, which is the main matrix for many samples[12].

Not all drugs can be extracted efficiently by standard solvent extraction methods. For example, morphine is almost insoluble in diethyl ether and is only sparingly soluble in chloroform[4]. However, it has amphoteric characteristics and the pH of the sample is extremely important[13]. For maximizing the extraction of common drugs, modified solvent extraction procedures have been developed, in which solvent composition and the pH and ionic strength of the aqueous phase have been optimised[4]. Chloroform mixed with 10%(v/v) propan-2-ol has been used to extract a range of drugs from a buffered sample[14].

Extraction at around pH8-9 isolates many unconjugated drugs, the so-called basic drug screen. Some basic drugs including narcotic analgesics, benzodiazepines, methadone, local anaesthetics and phenothiazine tranquillisers can be extracted by a basic drug screening method. However, amphetamines require a pH of 10 or more[15].

A wide range of solvents has been used for maximizing recovery and purification of sample from matrix compounds, especially lipids[14,15,16,17].

Popular solvents comprise chloroform, dichloromethane and mixtures of these with each other and with ethanol, propan-1-ol, diethyl ether, diisopropyl ether or hexane[14,15,18,19]. N-butyl acetate has been used for a wide range of basic drugs[20].

To identify which solvent performs best in which system, a number of chemical properties can be calculated to predict the efficiency and success of an extraction[21].

1) The distribution/partition coefficient (K_D)

Generally the extraction process involves the separation of a dissolved substance, known as the solute, from a liquid, solid or gaseous sample by using a suitable solvent. This process is reliant upon the relationship described by Nernst's distribution law, which is also known as the partition law. The Distribution Coefficient (K_D) is the equilibrium ratio between the concentrations of a solute in each of two immiscible phases. The two phases may be a solid and a liquid, a liquid and a liquid, or a gas and a liquid. The solute (S) will distribute itself between the

two phases depending on the affinity and solubility of the solute for each phase. The ratio of the solute concentration in each phase will be constant.

$$(K_D) = [S]_E / [S]_O \quad (1.1)$$

Where, K_D is the distribution coefficient, and $[S]_O$ represents the concentration of the solute in one of the phases (sample) and $[S]_E$ represents the concentration of the solute in the second phase (solvent), which has been used to extract the solute from the original sample matrix. Therefore, the greater the solubility of the solute in the solvent the greater $[S]_E$ and hence the greater is the value of K_D. A distribution coefficient value is characteristic for a certain solute and solvent system and it is dependent on temperature.

2) The distribution ratio (D)

The distribution ratio (D) is the ratio of the concentration of all the types of the solute in each phase. For example, with a substance, HS, which may have partially dissociated, the distribution ratio would be:

$$D = [HS]_E / ([HS]_O + [S^-]_O) \quad (1.2)$$

Assuming that one of the phases is an aqueous phase containing an analyte that is an acid the acidity constant (K_a) for the ionisation of the analyte is:

$$K_a = [H^+]_O [S^-]_O / [HS]_O \quad (1.3)$$

And, therefore, $$[S^-]_O = K_a [HS]_O / [H^+]_O \quad (1.4)$$

From equation 1.1:

$$[HS]_E = K_D [HS]_O \quad (1.5)$$

Substitution of equations 1.4 and 1.5 into equation 1.2 gives:

$$D = K_D / (1 + (K_a / [H^+]_O)) \quad (1.6)$$

This equation implies that when $[H^+]_O$ is very much larger than K_a, then $D = K_D$, and D is maximal. If K_D is large then the analyte is extracted into the extracting solvent; but when $[H^+]_O$ is very much smaller than K_a, then $D = K_D [H^+]_O / K_a$, which will be small and extraction will not occur.

3) The percentage extracted

While the distribution ratio (D) is independent of the ratio of the volumes of the phases used, the amount of the solute extracted is dependent upon the volume ratio. The fraction of the solute extracted is equal to the amount of the solute in the extraction phase divided by the total amount of the solute. The equation is:

$$\%E = ([S]_E V_E/([S]_E V_E + [S]_O V_O)) \times 100 \quad (1.7)$$

V_E and V_O are the volumes of the extracting phase and the sample phase, respectively.

$$\%E = 100D / (D +(V_O/V_E)) \quad (1.8)$$

If equal volumes were used, so $V_O = V_E$, then:

$$\%E = 100D/(D+1) \quad (1.9)$$

The simplest apparatus required for liquid-liquid extraction is a separating funnel. The sample and the solvent are both placed in the separating funnel. The liquids are then shaken together, carefully releasing the pressure when necessary by inverting the funnel and opening the tap. The liquid phases are then left to settle, with the dense phase on the bottom. The separated phases can then be collected separately. The extraction process may be repeated many times using fresh solvent each time to extract the majority of the solute from the solution. The separate extracts would then be combined and if the analyte concentration in the final solution is too low, some of the solvent could be evaporated to reduce the volume in which the analyte is dissolved[3].

1.1.2. Solvent-free extraction

The operating principle of any method of sample preparation is to partition analytes between the sample matrix and an extracting phase. Sample preparation techniques, which use little or no organic solvent can be classified according to the extraction phases as gas, membrane or sorbent.

1.1.2.1. Gas phase extraction methods

Gas phase sample extraction methods include supercritical fluid extraction, static headspace sampling, and purge-and-trap. The two last have, as a common feature,

the partitioning of analytes into a gas phase. Non-volatile high molecular weight compounds are eliminated during the partitioning thereby preventing contamination of the separating column. Headspace sampling has been widely applied for volatile compounds because the extracting phases, such as air, helium or nitrogen, are compatible with most of the analytical instruments, especially gas chromatographs[22].

In the static headspace procedure, a sample is allowed to equilibrate with its headspace gases and then a small and precise amount of the headspace is directly injected into an injection port of a gas chromatograph. This method is the simplest and the most frequently used solvent-free technique, but the method is low in sensitivity[10].

A commonly used dynamic headspace method (purge-and-trap), for the analysis of volatile organic compounds, has two steps. The first one is to allow a carrier gas purge through an aqueous sample to remove volatile organic compounds from the matrix. The second step is to collect these compounds using a cold or a sorbent trap. The method has some disadvantages such as foaming and carryover of other analytes from previous determinations. Also, the flow rate is not compatible with the separation instruments[23].

The headspace approach, in combination with thermal desorbtion, may be applied for the analysis of less volatile compounds. A high temperature results in the analytes being thermally desorbed from the matrix so enhancing the gas phase concentration. However, thermally unstable analytes and a high moisture content in the gas phase often prevent the use of this method[24].

Another gas phase sample preparation method uses a liquid, such as compressed carbon dioxide, as an extracting phase that is able to remove less volatile compounds at room temperature. The technique is called supercritical fluid extraction (SFE). A fluid is said to be in its supercritical state when both its temperature and pressure are above their respective critical values. At this point, which is called the critical point, the supercritical fluids can be seen as intermediates between gases and liquids. The method has been rapidly developed and has some advantages because supercritical fluids have both gas-like mass transfer and liquid-like solvating characteristics.

However, it needs an expensive high-pressure fluid delivery system and a high purity gas source[25,26].

1.1.2.2. Membrane extraction methods

Membrane extraction consists of two concurrent processes: extraction of analytes from the sample matrix by the membrane, and extraction of analytes from the membrane by a stripping phase. This method has been developed for mass spectrometry over the last three decades. In the past, nitrogen-stripping gas was used to transfer the permeated analytes from the surface of a flat polymeric membrane to a bed of activated charcoal[27]. The compounds were desorbed into a gas chromatograph for analysis. Although many early methods used supported membrane sheets, most recent developments have focused on the use of hollow fibres. Hollow fibre membrane modules are simpler to make because a hollow fibre is self-supporting. In comparison with membrane sheets and headspace methods, hollow fibres provide a higher ratio of surface area to volume for the stripping gas, which allows a more efficient extraction. Membrane extraction can be directly combined with mass spectrometry or gas chromatography to perform continuous monitoring[28,29,30].

Membrane extraction is not limited to the analysis of volatile compounds; indeed, using higher temperatures, or microporous membranes with various pore diameters, it can achieve extraction of higher molecular weight compounds. The membrane extraction method has been used for the analysis of semi-volatile compounds using a high pressure stripping gas[31].

1.1.2.3. Sorbent extraction methods

Sorbent materials have been used to extract organic compounds from various matrices including water and soil[32,33]. A sorbent with a strong affinity towards organic materials will retain and concentrate these materials from a very diluted aqueous or gaseous sample[32]. Many sorbents are specified for the extraction of different groups of organic compounds with different degrees of selectivity[34].

An alternative approach to liquid-liquid extraction is solid phase extraction (SPE). From 1978, when the first commercial SPE columns were introduced, the use of SPE

techniques for the separation of drug materials from different biological samples has increased[35,36,37,38,39]. SPE columns can cope with urine, blood and tissue extracts. SPE also permits a degree of automation of the extraction using such instruments as the Gilson ASPEC[38,40].

Solid phase extraction is a physical extraction method that involves a liquid phase and a solid phase. During sorbent extraction, three sets of interactions must be considered. The first of these, sorbent/isolate interactions, causes retention of the isolate on the SPE columns. The second one, matrix/isolate interactions, also affects isolate retention by causing the isolate to be unavailable to the sorbent. The last one, sorbent/matrix interactions, is competitive with the first interaction and causes isolate elution. There are three common extraction mechanisms used in solid phase extraction; non-polar, polar and ion-exchange[9,41].

Three factors determine which extraction mechanism is most suitable for any individual sample.

The first of these is the isolate properties. Functional groups on the compound to be isolated, isolate solubility and its ionisation characteristics can influence retention on a sorbent. The second factor is matrix characteristics. For example, if the matrix is aqueous, an isolate with non-polar or ionic functional groups can generally be extracted using non-polar or ion-exchange sorbents, respectively. The last factor is the aim of extraction. The final analytical procedure may dictate the need for a certain type of elution solvent. For example, if GC is to be used, the elution solvent should be non-aqueous and volatile[9].

The wide variety of bonded silica sorbents are classified in general groups based on their most important properties (polar, non-polar and ion-exchange) and the functional groups of the sorbent[23].

Solid phase extraction is simple and uses relatively little solvent. The sensitivity is much better relative to liquid-liquid extraction, since by choosing a proper sorbent the distribution constant for a particular analyte will be much higher[10].

In spite of the obvious advantages of SPE, it has some serious disadvantages. SPE methods are prone to contamination by materials in the column, for example, phthalate elasticisers. Sorbent particles can also be transferred to the extracts during

extraction[10]. SPE requires more skill than solvent extraction, and is less useful for batch extraction[10]. However, this problem can be removed by use of the Vac Elut™ vacuum manifold system[40]. A column can only be used once and they are not cheap. Furthermore, very different recoveries, up to compete failure of the extraction, were reported for the same or different batches of columns, especially for cation exchange and mixed-phase columns. Solid and oily components in a sample matrix may plug the SPE cartridge or block pores in the sorbent causing it to become overloaded[4].

Dispersing a minute quantity of the extracting phase on a fine rod, which has been made of fused silica or other appropriate materials, has decreased the limitation of solid phase extraction.[42]. Earlier uses of small amounts of the liquid phase in microextraction methods has shown improved performance over the large volume approach. Although full removal of target analytes from the sample matrix was not obtained, the high concentrating ability and selectivity of the method allowed direct analysis and high sensitivity of the extracted mixtures. The difficulty in handling small volumes of solvents was a major limitation of this method. Application of sorbent materials permanently attached to the fibre addressed this limitation and permitted reuse of the same extraction phase many times[42,43]. The resulting technique was called solid phase microextraction (SPME).

1.2. Solid Phase Microextraction

Solid phase microextraction (SPME), introduced by Belardi and Pawliszyn[42] in 1989, is an excellent alternative to the other extraction methods, and is an adsorption/desorption and solvent-free extraction technique that incorporates sampling, extraction, concentration and sample introduction into a single step[10]. The method saves preparation time, solvent purchase and disposal costs. It can improve precision, linearity and the limit of detection for selected substances[44,45,46,47].

Initially SPME was mainly used for the extraction of volatile and semi-volatile compounds in water, air and foods[48,49,50,51,52]. Since then, the interest in this method has continued to grow very rapidly. This new extraction technique is based on the partitioning of the analyte between a phase immobilised on a fused-silica fibre and the matrix (air, water, plasma, etc). After equilibration, the absorbed analytes are

thermally desorbed by exposing the fibre in the injection port of a gas chromatograph or are redissolved in a solvent using high pressure liquid chromatography (HPLC)[10]. The technique was made practical by mounting the fibre in a syringe-like device. Initially, SPME was used to prepare samples for gas chromatography[53,54,55,56], but in 1995 Chen and Pawliszyn described an interface for SPME/HPLC[57].

SPME was originally used for the extraction of analytes from foods and water[48, 49], but, in recent years, has been applied to the extraction of some drugs from various biological samples, such as, air[58], whole blood[59], urine[60,61], hair[62,63], breath[64,65] and saliva[66].

1.2.1. SPME fibre assembly and sampling

The SPME device consists of a 1cm length of fused silica fibre, coated on the outer surface with a stationary phase and bonded to a stainless steel plunger in a holder that resembles a modified microlitre syringe. Two types of fibre assemblies are commercially available: one of them is for use in the manual holder and the other one is for use in a holder for an autosampler[67].

The SPME process has two steps: first, partitioning of analytes between the sample matrix and the fibre coating, followed, in the second step, by desorption of the analytes into the analytical instrument. In the first step, the fibre is drawn into the needle; the needle is passed through the septum, which seals the sample vial and the plunger is depressed to lower the fibre into either the sample or the headspace above the sample. This allows partitioning of analytes between the matrix and the fibre coating[68].

In the second step, the fibre is retracted into the needle, and then the needle is withdrawn from the sample vial and inserted through the injection port septum of the gas chromatograph. The plunger is again depressed which exposes the fibre and allows desorption of the analytes to take place[68].

1.2.2. Fibres (Coatings)

All manufactured SPME fibres contain a fused silica core. The diameter of fused silica is 110μm for the most of the fibres, although the diameter of the pre-coated

core used with HPLC fibres is 160μm. The length of a fibre is 1 cm, however, longer fibres of up to 2 cm can be manufactured[10].

Three categories are used to describe the coating's stability: non-bonded, crosslinked and bonded. Non-bonded phases are stabilised, but do not contain any crosslinking agents. These phases are not solvent-resistant and tend to swell in organic solvents. However, they can withstand some polar organic solvents, such as methanol and acetonitrile, but have less thermal stability than bonded fibre coatings[68].

Partially crosslinked fibre coatings contain crosslinking agents, such as vinyl groups. The phase crosslinks with itself, producing a more stable coating than the non-bonded type. These coatings are more solvent resistant in comparison with non-bonded coatings and so have better thermal stability[69]. Most of them can be used with a variety of solvents, but special care must be taken to prevent the coating being stripped off the fused silica core because of coating swelling[67,68].

Bonded fibre coatings are similar to partially crosslinked coatings that contain crosslinking agents. The difference is that the coating is also crosslinked to the fused silica. These fibre coatings are very resistant to most organic solvents and have good thermal stability. Furthermore, they can be rinsed in organic solvents with minimum swelling[68].

Fibre coatings are generally classified by their polarity and thickness. The most common non-polar coating is polydimethylsiloxane (PDMS), which is similar to the OV-1 and SE-30 gas chromatograph column phases. Three different film thicknesses of PDMS, 7, 30, and 100μm, are available. A thicker coating extracts more analyte, but the extraction time is longer than for a thinner coating[69].

The more polar coatings are polyacrylate and Carbowax. These fibres are completely solvent resistant and durable. However, the fibres may be oxidised at high temperatures (more than 250°C) and so the carrier gas must be free of oxygen. The polyacrylate fibre will turn to dark brown when exposed to temperatures more than 280°C, but this process is normal and does not affect the performance of the fibre. However, if the fibre turns charcoal black, it has been oxidised and is not suitable for further use[69].

Carbowax has been used as a moderately polar phase in GC. However, it has some disadvantages that need to be addressed. Firstly, Carbowax tends to swell or dissolve in water. Swelling of the fibre leads to stripping of the fibre surface when the fibre is retracted into the sheath. Secondly, Carbowax is sensitive to oxygen at temperatures of more than 220°C. When oxidation occurs the fibre turns dark, becomes powdery, and can be easily removed from the fused silica. To reduce this problem, some precautions can be taken[68]:

1) Use of appropriate carrier gas purifiers or 99.99% (V/V) helium.
2) Use of an injector port temperature in the range of 180-240°C.
3) Not conditioning fibres for longer than 1 hour at temperatures above 220°C.
4) Use of desorption times of less than 5 minutes.

Divinylbenzene (DVB) is a porous polymer that can adsorb analytes and physically retain them. Because DVB is a solid particle, it should be suspended in a liquid phase to coat it onto the fibre[70]. The blend of DVB and Carbowax increases the polarity of the fibre and its ability to extract analytes of a wide range of molecular weights. When Carboxen is mixed with PDMS it creates a bipolar phase for smaller analytes[71].

The efficiency of the extraction process is dependent on the distribution constant, K_f. This is a characteristic parameter that describes the selectivity of the coating towards the analyte versus other matrix components. The affinity of the fibre for an analyte is the most important factor in SPME[43].

Based on the principle of "like dissolves like", fibres with different properties or thickness may be selected for different analytes[43]. Among the different coatings that are commercially available are polydimethylsiloxane (PDMS) with thicknesses of 100, 30 and 7μm, polydimethylsiloxane/divinylbenzene (PDMS/DVB) at thicknesses of 65 and 85μm, stable flex™ PDMS/DVB at 65 μm thickness, polyacrylate at 85μm thickness, Carboxen™/polydimethylsiloxane (CAR/PDMS) at 75μm thickness, and Carbowax®/divinylbenzene (CW/DVB) at 65 μm thickness[71].

1.2.3. Extraction modes

Three basic types of extraction can be performed using SPME: direct immersion (DI), headspace configuration and a membrane protection approach[10].

In the direct immersion mode, the coated fibre is inserted directly into the sample and the analytes are transported directly from the sample matrix to the fibre coating[10]. To facilitate rapid extraction, some level of agitation, vibration, and rotation of the microfibre is required in order to transport analytes from the bulk of the solution to the fibre[72].

In the headspace mode, the analytes need to be volatile in order to reach the coating. This modification serves primarily to protect the fibre coating from damage by high molecular weight and other nonvolatile interfering agents present in the sample matrix, such as proteins. This headspace mode also allows modification of the matrix, such as a change of the pH, without damaging the fibre[10]. The extraction approach of direct immersion-SPME (DI-SPME) has to be used if the volatility of drugs, such as cocaine, cannot be increased by the other methods[73].

The extraction mode should be selected based on the matrix, the volatility of the analyte and its affinity for the matrix. HS-SPME is generally preferred because of its faster equilibration times. The selectivity of HS-SPME is higher when complex samples are analysed. Clean aqueous samples such as aqueous solutions can be extracted using the DI mode, especially if semi-volatile and non-volatile analytes have to be extracted[10]. For very complex samples the fibre can be protected using membrane protection[74]. For aqueous samples, very polar compounds such as strong acids and bases are very difficult to extract. The adjustment of the pH value and the addition of salt can be considered in extracting these compounds, which have a high affinity for the matrix[75].

1.2.4. Extraction time and extraction temperature

Extraction time is mainly determined by the agitation rate, coating thickness, temperature and distribution constant (K_f) of an analyte between the coating and matrix. Coating thickness increases not only the amount of analyte extracted, but

also the equilibrium time. The amount of analyte extracted increases with K_f, but the equilibrium time becomes longer as well[76]. The analyte that has the higher affinity toward the coating, reaches equilibrium later[68].

An increase in extraction temperature translates to increased diffusion coefficients and a decreased distribution constant, both leading to faster equilibrium times. If sensitivity is high enough at a higher temperature, increasing the temperature can lead to faster determinations[76]. In general, the highest possible temperature at which the analyte is stable should be used. In HS-SPME, an increase in extraction temperature also causes an increase of analyte concentration in the headspace, and helps to facilitate faster extraction[68]. The extraction times were reported to take from four to sixty minutes, depending on agitation rate, distribution constant (K_f) of the analyte and temperature[61,77,78].

1.2.5. Experimental parameters that affect extraction efficiency

Several factors have been shown to affect extraction efficiency. These are agitation, additives, pH, temperature, extraction time, and fibre stationary phase: each is dealt with in more detail here.

1.2.5.1. Agitation

Several agitation methods such as magnetic stirring[79,80], fibre movement[81], sonication[82], vibration[83] and rotation[72] of the fibre have been reported. Magnetic stirring, in which a small magnetic bar is put into the sample vial, was most commonly used in both HS-SPME and DI-SPME experiments[80].

Equilibration times for the analysis of volatile analytes are short and frequently limited only by the diffusion of analytes in the coating. Analytes sufficiently volatile to be brought into the headspace may have relatively fast extraction times, even when no agitation is used. For this reason, in all reports for the extraction of amphetamines using HS-SPME no agitation was used[63,84,85,86,87,88]. However, in other cases agitation was required to facilitate mass transport between the sample and the fibre[60,61,77,78,89,90].

1.2.5.2. Additives

Generally the presence of an electrolyte, e.g. sodium chloride, can influence both SPME direct immersion and headspace absorption[68]. It may decrease the solubility of hydrophobic compounds in the aqueous phase. It is dependent on the water molecules "preferring" to solvate the salt ions rather than the neutral molecules. This is often referred to as "salting in" and has been used widely to increase the sensitivity of analytical methods[91].

Water has proved to be a very effective additive to facilitate the release of analytes from the matrix and it is often used to accelerate extraction[79]. Volatile analytes are often liberated from solutions in which they are dissolved by the introduction of an electrolyte such as sodium chloride[91]. The resultant escape of gas is a consequence of the attraction of the salt ions for the water molecules[91]. Salting in phenomena may also occur in solutions of liquids in liquids and solids in liquids. In general, adding salts, for example, NaCl, K_2CO_3 and NaF, increased the yield of the extraction[45,73,92,93]. Saturation with salt can be used to lower the detection limits of the determination and also to normalise randomly variable salt concentrations in natural matrices, especially urine[68].

1.2.5.3. pH adjustment

Sample pH affects the association equilibrium in aqueous media and its adjustment can improve sensitivity for basic and acidic analytes. For instance, a decrease in pH causes a concentration increase of neutral (uncharged) species of acidic compounds present in the sample and so increases the amount extracted. For full conversion of acidic species to neutral forms, the pH should be at least two units below the pK of a given analyte. For the basic analytes, the pH must be larger than pK+2 [76]. For example at pH12.4 the results of SPME for the extraction of amphetamine (pKa=9.9) methamphetamine (pKa=10.1) and dimethamphetamine (pKa=9.8) were optimum[94], whereas, at pH5.5 and 6 the results of SPME were favourable for diazepam, oxazepam, prazepam and nordiazepam[77,78].

1.2.5.4. Heating

An increase in extraction temperature translates to an increased diffusion coefficient and decreased distribution constant, both leading to shorter equilibrium times. These effects can be applied to optimise the extraction time for a given coating. If the sensitivity is high enough at a high temperature, increasing the temperature can lead to faster determinations[76]. In general, if the extraction rate is of major concern, the highest temperature, which still provides satisfactory sensitivity without pyrolysis, should be used[68]. In headspace SPME, an increase in extraction temperature also leads to an increase of analyte concentration in the headspace, and helps to facilitate faster extraction[76]. An internally cooled fibre SPME device can eliminate the sensitivity loss[79].

1.2.5.5. Extraction time and sample volume

The extraction time has a direct effect on SPME analysis. The fibre must reach equilibrium with its surroundings so that the maximum amount of the analyte can be extracted. Once equilibrium is reached, any increase in the time of extraction will not affect the extracted mass. In direct immersion SPME, the volume of the sample is much larger than the volume of the stationary phase, so that the mass of an analyte extracted at the equilibrium point is generally not affected by the volume of the sample, but is affected by the volume of the stationary phase[10]. However, in headspace SPME analysis the volume of the headspace affects the amount absorbed by the fibre. If the headspace volume is larger than the sample volume, the amount of analyte extracted by the fibre decreases, because the analyte concentration in the headspace is diminished[63].

1.2.5.6. Fibre stationary phase

Several kinds of coatings have become commercially available, namely polyacrylate (PA), polydimethylsiloxane (PDMS), polydimethylsiloxane/divinylbenzene (PDMS/DVB) and Carboxen/PDMS fibres. Each fibre type has an individual type of stationary phase, thickness and, therefore, operating condition[76]. Care must be taken when handling any SPME fibres as they are easily contaminated and touching a fibre onto skin causes contamination with fatty acids[95].

In SPME analysis, each analyte has a different affinity/solubility for a stationary phase and therefore the detection of an analyte is dependent on the type of stationary phase used. It has been shown that polyacrylate fibre coatings preferentially absorb polar compounds when compared to PDMS fibre coatings[96]. If the volume of the stationary phase is changed, i.e. its thickness, then both the amount of analyte extracted and the time to reach equilibrium are altered. A decrease in the thickness of the stationary phase will decrease both the mass of analyte absorbed and the equilibrium time[76].

1.2.6. SPME analysis by GC

The majority of the fibre analyses have been performed by gas chromatography. Typically, analytes trapped in SPME fibres are thermally desorbed in the injection port of a gas chromatograph[76]. Several different types of injectors have been used in combination with SPME. These include programmable, on-column, and split/splitless injectors. To optimise desorption, the depth of the fibre in the injector should be adjusted so that the fibre is exposed to the hottest part of the injector port[68].

It is important in GC analysis that the method takes into account a cryofocusing step at the beginning of each run. Desorption is dependent on the boiling points of the analytes and the thickness of the stationary phase of the fibre. A cryofocusing step, where the temperature of the column is adjusted at a temperature usually 90°C below the boiling point of the lowest boiling analyte, is important to produce sharp peaks in gas chromatography[47].

The temperature of thermal desorption is also important. It should always be slightly above that of the analyte with the highest boiling point. This temperature allows all the analytes to desorb thermally from the fibre, producing the greatest sensitivity and preventing carry over between injections[76].

Autosamplers for the automation of SPME-GC are commercially available. In the autosamplers the sample vials are not only sampled, but are also agitated at the same time. Automated SPME has some advantages in comparison with manual SPME analysis. It improves precision and also provides a faster sample preparation[45].

1.2.7. Basic principle of SPME

The physicochemical laws of classical liquid-liquid equilibrium control SPME extraction, because of the physicochemical properties of the polymer[76]:

$$Drug_{aq} \xleftrightarrow{k_f} Drug_{fibre} \tag{1.10}$$

$$K_f = \frac{C_f}{C_{aq}} \tag{1.11}$$

Where K_f is the equilibrium constant of liquid-liquid equilibrium, C_f is the equilibrium concentration of analyte in the stationary phase of the fibre (the coating) and C_{aq} is the equilibrium concentration of the analyte in the aqueous matrix[76].

$$K_f = \frac{\frac{n_f}{v_f}}{\frac{n_{aq}}{v_{aq}}} = \frac{n_f v_{aq}}{v_f n_{aq}} \tag{1.12}$$

Therefore:

$$n_f v_{aq} = K_f v_f n_{aq} = K_f v_f (n_o - n_f) \tag{1.13}$$

Hence,

$$n_f = \frac{K_f \, v_f n_0}{v_{aq} + K_f v_f} \tag{1.14}$$

Where n_f is the number of molecules in the fibre at equilibrium, n_0 is the initial number of molecules of analyte in the sample, v_f is the volume of the fibre, and v_{aq} is the volume of the aqueous sample. According to equation 1.14, it is obvious that the number of molecules in the fibre, n_f, is linearly dependent on the initial number of analyte molecules, n_0, in the sample. Salts, pH and temperature influence the

equilibrium[76]. Different models of sorption and the dynamics of the sorption using polyacrylate fibres are discussed in appendix A.

1.3. Analytical Methods

Various analytical methods have been described for the determination of drugs. They include thin layer chromatography, immunoassay, high-pressure liquid chromatography, gas chromatography and mass spectrometry.

1.3.1. TLC and Immunoassay

Thin layer chromatography (TLC) is a common method for the identification of drugs, which works by eluting analytes through a solid stationary phase and separating them selectively[97]. TLC is a very versatile analytical method. The equipment required is cheap, compact and easy to use[98]. TLC provides a single system, which can identify a wide range of drugs including most basic drugs[4]. Generally, it has poor sensitivity, low specificity and speed and may be cumbersome[10]. TLC cannot produce a quantitative answer. In addition, an unequivocal identification of some drugs cannot be achieved using this method alone[4].

An immunoassay method can be used to detect drugs in body fluids[97]. The basic mechanism of immunoassay is the antigen-antibody reaction[10]. This method is sensitive and specific for the detection of drugs in urine, blood and organ extracts[97]. Though they are rapid and simple to use, immunoassay measurements lack absolute specificity and positive results require the application of other methods as confirmatory tests[98].

Though these two aforesaid methods can detect particular drug groups, for example benzodiazepines in small amounts of clinical materials, they cannot identify an individual member of a group for instance diazepam in benzodiazepines[4].

TOXI-LAB drug detection systems (from Microgen Bioproducts Limited)) are designed for the separation, detection, and identification of a broad spectrum of

drugs. TOXI-LAB A drug detection system is a rapid thin-layer chromatographic method for the separation, detection, and identification of basic and neutral drugs including analgesics, stimulants, tranquillisers and antidepressants. TOXI-LAB A may be used to screen for the presence of drugs in biological fluids and other samples in solid or liquid form[99].

For forensic testing purposes, the TOXI-LAB A system provides only a preliminary analytical test result. A more specific alternative method must be used in order to obtain a confirmed result[100].

In the TOXI-LAB A technique drugs are extracted from biological matrices using TOXI-TUBES A which contain a mixture of solvents and buffering salts that extract basic and neutral drugs. The solvent extracts are concentrated by heat and evaporation, depositing the unknown drugs onto discs of chromatographic media. The dried discs are then inserted into the centre openings of TOXI-GRAMS A[100,101]. The loaded chromatograms are developed by placing them in developing chambers containing small volumes of organic solvents. Elution of the unknown and standard drugs from the discs, and the resulting position (Rf) of these drugs on the chromatograms, occurs during migration of the developing solvent. Detection of the unknown and standard drug spots is achieved when the chromatograms are dipped into chromogenic reagents. Identification is based on matching a drug spot in the unknown zone with an adjacent standard drug spot having the same Rf, size, shape, and colour characteristics[100].

TOXI-LAB B drug detection system is a rapid thin layer chromatographic method for the separation, detection, and identification of barbiturates and other hypnotics (acidic and neutral drugs). The principle of TOXI-LAB B is as the same as TOXI-LAB A[98].

Emit® II plus assay is a homogeneous enzyme immunoassay technique used for the analysis of specific compounds in human urine. The assay is based on a competition between the drug in the specimen and the same drug labelled with the enzyme glucose-6-phosphate dehydrogenase (G6PDH) for antibody binding sites[98]. Enzyme activity decreases upon binding to the antibody, so the drug concentration in the specimen can be measured in terms of enzyme activity. Active enzyme converts

nicotinamide adenine dinucleotide (NAD) to NADH, resulting in an absorbance change that is measured spectrophotometrically[102,103]. Endogenous serum G6PDH does not interfere because it uses NADP rather than NAD[104] and the coenzyme NAD functions with the bacterial enzyme employed in the assay. All specimens that show positive results by this method should be confirmed by the GC-MS method[98].

1.3.2. High pressure liquid chromatography

High-pressure liquid chromatography (HPLC) has been used for the detection of drugs[105]. HPLC requires that a mobile liquid containing the analyte mixture passes through a column of fine, preferably spherical, particles. As the analytes pass through this column of particles, they interact selectively with the particles according to the physico-chemical principles, resulting in the components of the mixture being separated from each other. The particles are almost always based on pure silica for the analysis of drugs of abuse. Because the silica particles contain free surface siloxane groups (Si-OH), they absorb on their surface a large amount of water, which is mainly held by hydrogen bonding. If the mobile phase is composed of a non-polar solvent such as isooctane or diethylether, then any analyte will partition between this solvent and the water held on the silica surface. This is called normal partition chromatography. However, should the surface of the silica be pre-treated, so that it possesses a strongly lipophilic or ionic character, then the chromatography is referred to as reversed-phase or ion-exchange chromatography, respectively[106].

The main disadvantages of HPLC are capital outlay, the skills needed in its operation and interpretation of the data, and a mass spectrometric detector is required to confirm positive detection[106].

The REMEDi HS system from Bio-Rad Laboratories is an automated HPLC system, which uses a scanning ultraviolet (UV) detector to identify a broad spectrum of drugs. It can detect and quantify over 800 drugs and metabolites in serum and in urine[107]. Four cartridges are used in this system; two cartridges initially separate drugs from proteins, salts and so on, and the other two cartridges produce an analytical differentiation of drugs, based on their characteristic retention times[108,109].

1.3.3. Gas Chromatography

There are several different means of introducing samples into a mass spectrometer, such as gas chromatographic inlets, cold inlet, hot inlet systems, liquid chromatographic inlets and direct introduction via a probe. The choice of inlet system used is governed by the physical properties of the sample under investigation, for example volatility, purity and complexity. Gases or compounds that are very volatile at room temperature are introduced into the mass spectrometer using a cold inlet. In the hot inlet, the compounds may be heated to about 300°C to be volatilised and then led along a heated line to the ion source[110]. Compounds that are not sufficiently volatile to be introduced through the hot or cold inlet systems may be inserted directly into the ion source by means of a probe passing through a vacuum lock. At the low pressures of about 10^{-7}-10^{-6}torr inside the ion source and with heating, most of the compounds are sufficiently volatile to yield good mass spectra[111].

The effluents from gas chromatographic columns consist of carrier gas admixed with the compounds being investigated. Carrier gas flow rates of 50mL/min are common in gas chromatography with a packed column, but such quantities of gas cannot be introduced directly into the ion source because large increases in pressure would result. Consequently, it is necessary to remove as much carrier gas as possible, ideally without diminishing the amount of sample going into the source. Differentiation between carrier gas and the sample can be effected by molecular separators. The ion sources of quadrupole mass spectrometers, when evacuated with highly efficient pumps, are able to cope with high carrier gas flow rates without the need for a molecular separator[111].

Gas chromatography is a separation process in which the components of a sample (solute molecules) are repetitively equilibrated between a stationary liquid phase and a gaseous mobile phase (carrier gas). Sample molecules having a greater affinity with the stationary liquid phase move into the gaseous phase less frequently than the sample molecules having a lower affinity, and, consequently, the former take longer to pass through the chromatographic column[112]. The retention characteristics of a compound are therefore dependent on its distribution between the mobile and

stationary phases and their relative affinity towards each other. Its equilibrium distribution between the two phases is reflected by the distribution constant K_D expressed as the ratio of the weights of solute in equal volumes of the liquid and gas phases[110]:

$$K_D = \frac{C_s}{C_m} = \frac{\text{Concentration of solute per unit volume in liquid phase}}{\text{Concentration of solute per unit volume in gas phase}} \quad 1.15$$

K_D is a true equilibrium constant, and only the solute, liquid phase and temperature govern its magnitude.

$$K_D = \frac{\text{Weight solute in liquid phase/ volume of liquid phase}}{\text{Weight solute in gas phase/ volume of gas phase}} \quad 1.16$$

So

$$K_D = \frac{\text{Weight solute in liquid phase}}{\text{Weight solute in gas phase}} \times \frac{\text{Volume of gas phase}}{\text{Volume of liquid phase}} \quad 1.17$$

$$K_D = k\beta \quad 1.18$$

Where k = partition coefficient (partition ratio) and β = phase ratio.

Column efficiency describes the rate at which the solute molecules spread out as they travel through the chromatographic column. The chromatographic peak width (w) is a measure of column efficiency and is reflected in the number of theoretical equilibrium steps that have occurred within the column.

Since the actual separation ability of the column is related to the amount of time a solute spends in the liquid phase, the adjusted retention time, t'_R, should be used.

$$t'_R = t_R - t_m \quad 1.19$$

Where

t'_R = amount of time a solute spends in the liquid phase

t_R = uncorrected solute retention time

t_m= unretained peak (gas hold-up time)

The gas-hold-up time is found by injecting a non-retained compound such as methane or butane into the column. It is equivalent to the volume of carrier gas required to conduct a nonsorbed component through the column.

This value is used in calculating the number of effective theoretical plates N:

$$N = 16\left(\frac{t'_R}{w_b}\right)^2 = 5.54\left(\frac{t'_R}{w_{0.5}}\right)^2 \quad 1.20$$

Where w_b is the idealised width of the peak at the baseline and $w_{0.5}$ is the width of the peak at idealised half height. The same units must be used for the t'_R and w measurements[113].

To compare columns of different lengths the number of theoretical plates per metre of column length is often used. More often, however, the inverse of this value, the length of column occupied by one theoretical plate is used[113].

$$H = L/N \quad 1.21$$

Where H is the height equivalent to one effective theoretical plate (HEETP) and L is the column length.

Obviously, smaller values of H indicate higher column efficiencies and greater powers of separation. As much as the column temperature affects the values of N, the compound and the nature of the carrier gas also affect it[113].

In a gas chromatograph with a column of very high efficiency, such as narrow-bore capillary columns, plate heights less than 0.1mm and plate numbers of up to one million can be attained. Standard capillary columns, as used in analytical practice,

have plate numbers of about 50000 to 150000. Packed columns usually do not have more than 2000 to 5000 theoretical plates[114].

Analysis of defined compounds is obtained by comparing the retention time of a suspected sample with that of a reference standard. Reproducibility of retention times is affected by factors such as variation from apparatus to apparatus, day-to-day influences, effect of the injection technique and even variations between different operators[11].

Usually the reference compound data collection is not expressed in absolute retention times, as they may show large variations because of some variable involved, including gas type and flow rate, temperature and stationary phase. To minimise such influences, the concepts of relative retention times and retention indices, have been used. The first approach, relative retention time (RRT), is to express retention times as a quotient of the retention time of a reference standard obtained under the same conditions[115].

The second approach is the use of retention indices (RI), in which n-alkanes are used as standards. Retention index is a measure of the retention of a solute relative to normal alkanes at a given temperature on a particular stationary phase. Retention indices normalise instrument variables so that retention data can be effectively compared for different chromatographic systems. Retention indices are useful when comparing relative elution orders of various solutes for a given column and conditions. Retention indices are also useful for comparing the retention behaviour of two columns with the same description. The retention index for a compound can be calculated using equation 1.22 under isothermal temperature conditions[116].

$$I = 100y + 100(z-y)\{\frac{\log t'_r(x) - \log t'_r(y)}{\log t'_r(z) - \log t'_r(y)}\} \qquad 1.22$$

Where

t'_r=adjusted retention time

x=solute of interest

y=normal alkane with y number of carbon atoms eluting before solute x

z=normal alkane with z number of carbon atoms eluting after solute x

A gas chromatograph consists of an injection port, an oven, a chromatographic column and a detector as shown in figure 1.1. There are several inlet systems to allow introduction of the sample into the GC, such as split (closed) injectors, splitless (open) injectors, combined split/splitless injectors and on-column injection modes[117].

The most common variety of injection system is the combined split/splitless injector due to its flexibility. It is a hot injection system with the standard operating temperature of between 200°C and 300°C[118]. Introduction of the sample into the high temperature environment causes vaporisation of the components allowing gaseous phase transfer onto the capillary column. As the concentration of many samples easily exceeds the capacities of the most capillary columns, limiting the amount of sample reaching the column by "splitting" it prior to column introduction prevents column overloading. The injector splits the sample into two unequal portions, normally with the smaller fraction going to the column and the larger fraction being vented. Typical split ratios range from 1:10 up to 1:300. However, the use of split injections is not applicable for trace level analysis, as this often requires as much sample as possible to be introduced onto the column to allow detection of the analytes. The entire sample can be introduced onto the column using a splitless injector where no sample is purged via the split valve[119].

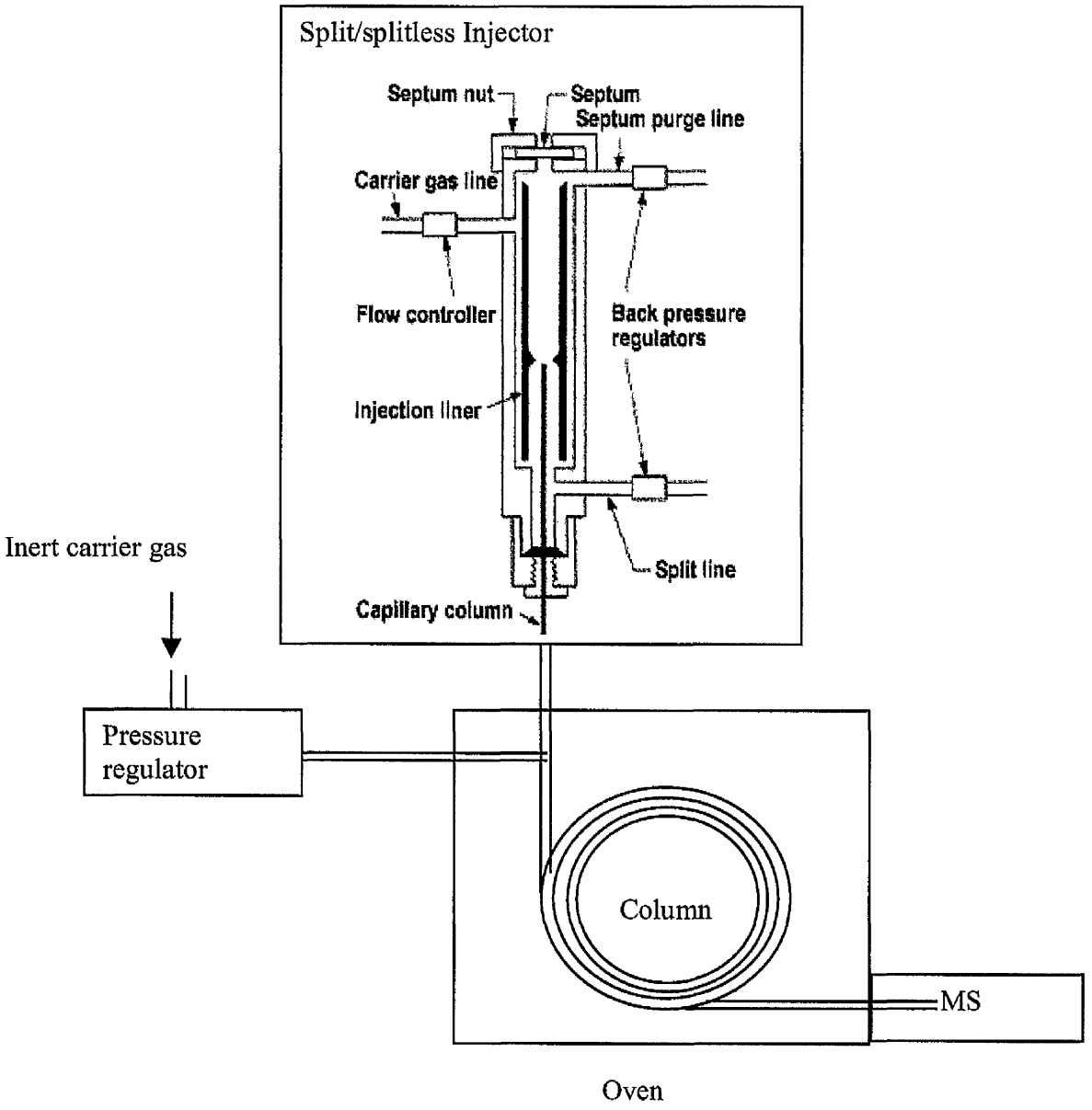


Figure 1.1. Schematic diagram of a split/splitless gas chromatograph inlet system

On-column injection uses a syringe to deposit the sample directly into the column, therefore, it eliminates vaporisation processes as the mechanism of sample introduction into the column. Cold trapping involves having the initial column temperature low enough to condense the sample components. This effectively narrows the band widths so that the full efficiency of the column can be utilised[113].

A gas chromatograph can be equipped with a wide range of detectors including flame ion detector (FID), thermal conductivity detector (TCD), electron capture detector (ECD), nitrogen-phosphorus detector (NPD) and flame photometric detector (FPD)[110]. FID is the most popular detector used in gas chromatography. It is an ionisation detector that is sensitive to most organic compounds. Carrier gas exiting from the column is mixed with hydrogen and burned at the tip of the jet. This combustion process generates ions. An electrode collects these ions and the resulting electrical current is amplified to provide the chromatographic signals[117].

The nitrogen-phosphorus detector (NPD) is extremely sensitive to compounds containing phosphorus or nitrogen[20]. The NPD functions in nearly the same way as the flame ionisation detector except for the presence of an alkali salt bead above the flame in the combustion chamber. A rubidium silicate bead is electronically heated to 600-800°C by application of a current. Alkali metal ions are emitted from the bead, and these ions interact with the column effluent[120,121]. The ECD is an ionisation detector that is specific to compounds containing highly electronegative atoms. It is primarily used for the analysis of halogenated compounds due to the extreme sensitivity for these compounds. A radioactive source of electrons, which emits β-particles, is used to bombard the column effluent passing through the detector. The most common source is ^{63}Ni supported by a metallic foil[117].

The TCD can detect the compounds that have different thermal conductivities from the carrier gas. The detector consists of two heated cells each with a heated filament whose resistance to current flow is electronically monitored. The carrier gas, along with eluting compounds, will pass through one cell while the other cell (the reference cell) will receive only carrier gas that has not passed through the column. The difference between the currents of the two cells is the signal that is sent to the recorder[11].

The FPD is an optical detector specific for sulphur or phosphorus-containing compounds. The carrier gas exiting the column is mixed with air (oxygen) then introduced into a hydrogen flame. The eluting sample is decomposed to chemiluminescent species, which emit light at specific wavelengths. A filter is used to screen out all other wavelengths so that only the wavelength of interest reaches the

photomultiplier tube. A current generated by the photomultiplier tube provides the chromatographic signal[117].

One of the most important parts of every gas chromatograph is the column. A gas chromatograph can be fitted with two completely different kinds of columns, packed columns and capillary columns. Packed columns are made by packing stainless steel or glass tubes with inner diameter of 1-5mm with a support and coating. These columns are sturdy and easy to use. It is essential to repack the head of the column frequently[110]. The main disadvantage of the column is the poor separation efficiency; therefore it does not allow high resolution or steep peak profiles for high signal-to-noise ratios in trace analysis[115].

Capillary columns are made of glass tubes, preferably of a fused-silica material, with inner diameters of about 30-500 μm[119]. Rarely stainless steel and nickel capillary columns are used. Column lengths are between 1-100m. Capillary columns have three distinct "layers" or parts. They are the fused silica tubing, the outer resin coating, and the stationary phase. Fused silica tubing is synthetic quartz containing typically less than 1ppm of metallic oxide impurities. A very low concentration of these oxides is required so that the activity of the fused silica surface is minimised. An inactive tubing surface is essential to chromatograph active compounds, such as alcohols, amines and acids. The active surface of the column usually causes the absorption of active compounds or peak's tailing. The outer surface of fused silica is coated with polyamide. It fills flaws in the tubing, which prevents the further increase in defects, and it acts as a waterproof barrier to prevent corrosion of the outer surface of the tubing[114].

Capillary stationary phases are polymers that are deposited on the inner walls of the tubing in a thin and uniform film. The most common stationary phases are polysiloxanes. The type and the amount of substitution on the polysiloxane backbone distinguish each phase and its properties. The most elementary phase is the dimethylpolysiloxane. The interactions between solutes and the stationary phase are limited to dispersive forces. Thus, solute elution occurs in the order of increasing solute boiling points. Compounds having very similar or equal boiling points require a different stationary phase for separation. Changing the functional groups on the

polysiloxane backbone can make different stationary phases. The stationary phase selectively is controlled by the substitution of phenyl, cyanopropyl, or trifluopropyl groups in place of some of the methyl groups on the polysiloxane backbone. These substitutions enable the solutes to engage in dipole, acidic or basic interactions in addition to dispersive interactions[115].

Polyethylene glycol is the other type of stationary phase used in the capillary columns. Carbowax 20M is the most widely used for gas chromatography. The major disadvantage of Carbowax phases is their extreme sensitivity towards oxygen, especially at high temperature. Phase solubility in water and low molecular weight alcohols along with a high lower-temperature limit are other disadvantages of the capillary columns coated with Carbowax 20M[114].

GC is widely used for the analysis of drugs, both in toxicology and pharmacoloy due to its ability to perform the separation of components in complex mixtures and ease of combining quantitative and qualitative analyses. The other advantage of GC is good reproducibility, which cannot be always obtained in other chromatographic methods[5].

One of the disadvantages of GC is that some drugs such as the amphetamines and some of the benzodiazepines are not stable at the high temperature required for GC; therefore they have to be derivatised[4,122]. The confirmation of detected compounds usually requires the specificity of mass spectrometry[5].

1.3.4. Mass Spectrometry

Mass spectrometry (MS) is a powerful and sensitive analytical technique that is used to elucidate and/or confirm the structural properties of molecules under investigation[123].

A mass spectrometer is an instrument that supplies energy to sample molecules and converts these molecules, mostly in the gaseous state, into ions and neutral particles. These ions can be either positively or negatively charged. The ions are separated according to their mass-to-charge ratio. A record of the ions formed and their relative abundances are displayed as a mass spectrum. Mass spectrometry has the advantage of sensitivity, requiring as little as a picogram quantitiy of sample for

analysis. If the analyte is known, mass spectrometry may be used quantitatively. Mass spectrometers are still high cost instruments and require skilled support for their operation and maintenance[124].

Mass spectrometry has been applied in many different fields such as environmental analysis, agriculture, forensic science, pharmacology, pharmacy, geochemistry, archaeology and biochemistry[124].

The origins of the mass spectrometer can be traced back to the discovery by Erich Goldstein in 1886 of "positive rays". During the course of his study of these rays, which were produced in a gas at low pressure when subjected to an electrical discharge, Goldstein observed faint streams of light behind a perforated cathode. He assumed that the light was a result of rays of some kind that travelled in the opposite direction from the cathode rays and he called them "Kanalstrahlen" (canal rays). Subsequently in 1898 Wilhelm Wien showed that a beam of "positive rays" could be deflected by using magnetic fields. In 1958, Paul *et al.* introduced the type of mass analyser called the "quadrupole mass spectrometer", which could analyse the mass-to-charge ratio of ions directly[111].

There are now many different types of mass spectrometer that may be used to study organic molecules[10]. Most modern instruments incorporate a variety of inlet systems, ionisation modes and analyser types. Despite the large number of options available the instruments tend to have some features in common: the essential parts of a mass spectrometer are presented in Figure 1.2.

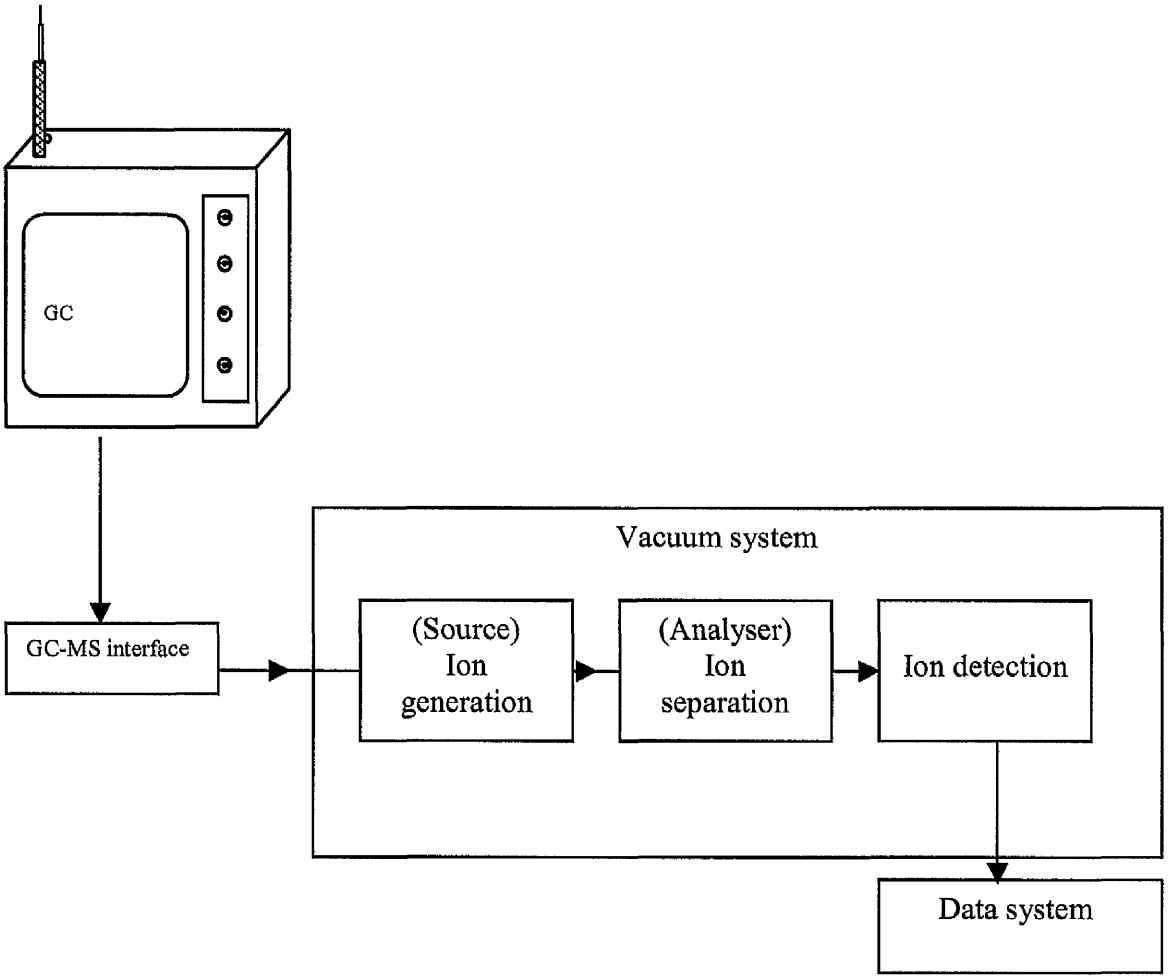


Figure 1.2. Block diagram of the essential features of a mass spectrometer

1.3.4.1. Vacuum system

Mass spectrometers must normally operate under high vacuum to prevent damage to the electron ionisation source filament and reduce the danger of electrical breakdown in high voltage regions of the instrument. The high vacuum also minimises ion molecule reactions by increasing the mean free path of ions[123].

1.3.4.2. Ion source

The region of the mass spectrometer where ions are generated is called the ion source. The ion source is usually enclosed in a small chamber in which the sample is

ionised (the ion chamber). Several distinct methods of converting organic molecules to ions are known including electron impact ionisation (EI), chemical ionisation (CI), fast atom bombardment (FAB) and field ionisation/desorption (FI/FD)[123].

The chemical ionisation involves the collision of two bodies, an ion and a molecule. The ions are formed by electron impact ionisation of a reactant gas in the source. The reactant gas ions collide with molecules under investigation and a chemical ionisation can accur in which the molecules are ionised. Drugs which do not yield molecular ions under electron impact can yield excellent quasi-molecular ions under chemical ionisation[124].

In FAB, a beam of ions such as xenon can be produced by ionising xenon atoms and accelerating the resulting ions through an electric field. The resulting ions are directed through a xenon gas chamber when charge-exchange occurs to give fast atoms. This beam of atoms is made to strike a metal plate coated with the sample. This technique is a low-energy process, which leads to very little fragmentation and is ideally suited to thermally labile and involatile compounds[8].

Field ionisation/desorption utilises the fact that when a very high positive electric field is formed by a sharp blade or thin wire at a high positive potential, this field induces electron tunnelling through a potential energy barrier in the molecule and forms positively charged ions. In field desorption, the sample in the gas phase passes close to the electric field and so causes ionisation. In field desorption, the sample is coated on to the electrode and it is useful for analysis of thermally labile and non-volatile compounds[124].

1.3.4.2.1. Electron impact ionisation

The commonest method of ionisation is by means of electron impact (EI) in which electrons are obtained from a heated filament in a vacuum and are accelerated. The electron impact source is constructed around the ion chamber into which gaseous sample molecules are introduced. Electrons are spontaneously emitted from the heated filament and are accelerated across the ion chamber by an electrical potential gradient. Standard mass spectra are obtained at 70eV, because maximum ion yield is obtained near this value[124]. A volatilised sample molecule (M) and an electron with

energy greater than the ionisation energy of M will react if they pass close enough for the electron to impart its energy to the sample molecule. Assuming that the mass spectrometer is tuned to analyse positive ions, negative ions will be discharged at the positive repeller and along with neutral species will be pumped away. Positive ions formed by collisions between gaseous sample molecules (M) and electrons are extracted and focused into a beam, which enters the mass analyser.

$$M + e^- \rightarrow M^{+\bullet} + 2e^- \qquad 1.23$$

The extent of ionisation differs among substances for a given electron energy and is found to be a maximum for electron energies in the range of 50-100eV. For most of the organic molecules, the first ionisation energy is in the range of 7-13eV, which causes a large excess of energy to be transferred to the molecular ion ($M^{+\bullet}$). The excess energy imparted to the $M^{+\bullet}$ may be distributed throughout the ion causing bond-breaking, a series of reactions, and the formation of fragment ions.

$$M^{+\bullet} \rightarrow A^+ + B^\bullet \qquad 1.24$$

or

$$M^{+\bullet} \rightarrow C^{+\bullet} + D \qquad 1.25$$

Where $B^\bullet$ is a radical, $C^{+\bullet}$ is an odd electron species and D is a neutral molecule.

The fragment ions A^+ and $C^{+\bullet}$may undergo further fragmentation, depending on the amount of internal energy they possess, while successive loss of radicals is seldom encountered[122].

Electron impact ionisation is accompanied by the generation of low levels of negative ions, which are formed by the molecule's capturing an electron (equation 1.26).

$$M + e^- \rightarrow M^- \qquad 1.26$$

This process is 100-fold less probable than electron removal and, consequently, negative ion mass spectrometry by electron ionisation is inherently less sensitive than positive ion mass spectrometry. To monitor negative ions the repeller plate and acceleration potentials must be reversed in polarity[122].

1.3.4.3. Analysers (Ion separation)

Generally, mass spectrometers can be classified on the basis of their method of separation of the charged ions into three types; "magnetic field deflection", "time of flight" and "quadrupole". In the magnetic field deflection mass spectrometer, by varying the magnetic field, ions of different mass may be focused on a final collector, thus enabling the mass spectrum of a compound to be obtained. In the time of flight spectrometer, sample ions are accelerated through a potential. This gives them a velocity dependent on their mass and they will arrive at a detector at different time intervals depending on their velocity and mass. The ion trap detector is derived from the quadrupole instrument. The unique feature of the ion trap detector is that sample ionisation and mass analysis occur in the same region[124].

A quadrupole mass analyser consists of two pairs of precisely parallel rods, which are located between an ion source and a detector as shown in Figure 1.3. Two opposite rods have an applied potential of (U+Vcos (t)) and the other two rods have a potential of –(U+Vcos (t)), where U is a dc voltage and Vcos (t) is an ac voltage. The applied voltages affect the trajectory of ions travelling down the light path centered between the four rods. For a given dc and ac voltage combination, only ions of a certain mass-to-charge ratio pass through the quadrupole analyser and all other ions are thrown out of their original path. A mass spectrum is obtained by monitoring the ions passing through the quadrupole analyser as the voltages on the rods are varied. Such an instrument does not require the ions to be accelerated to high energies before separation according to their individual mass-to-charge ratios[123].

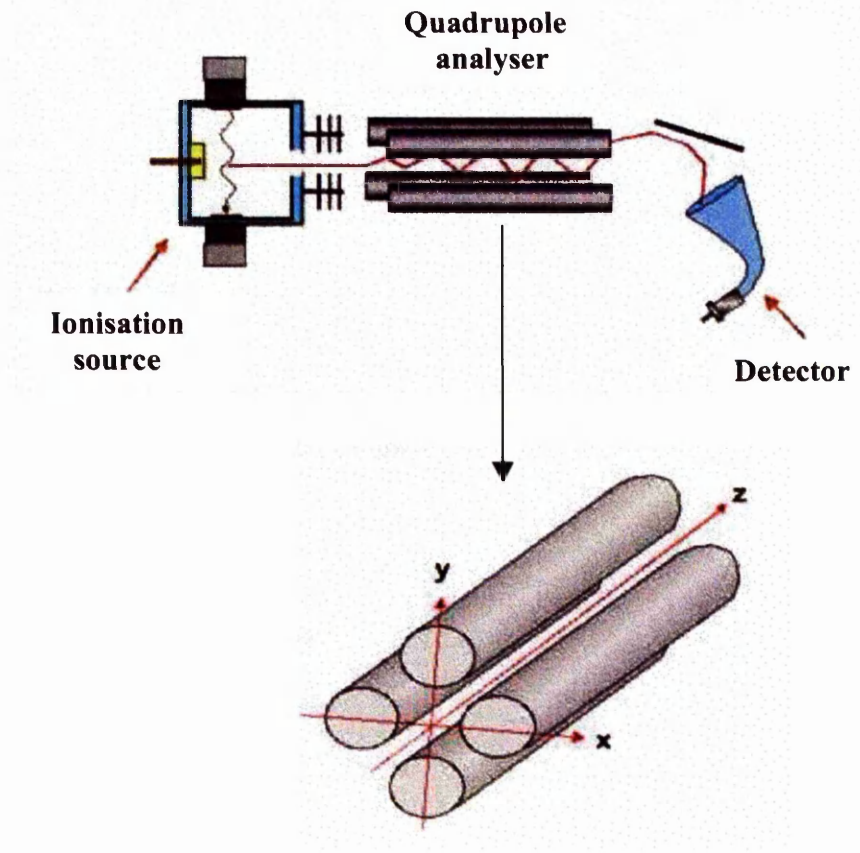


Figure 1.3. Diagram of a quadrupole mass spectrometer

The analytical usefulness of the quadrupole analyser is based on the rapid scan speeds that may be achieved by computer control of the quadrupole electric fields. A complete mass spectrum can be scanned within a few milliseconds, thereby allowing the detection of compounds eluting in narrow bands (i.e. eluting in 1-5 seconds) from chromatographic capillary columns[111]. Enhanced sensitivity may also be achieved by allowing the quadrupole analyser to monitor a specified ion, or suites of ions, eluting from a chromatographic column. This kind of scanning, which yields an ion chromatogram, is known as selective ion monitoring (SIM)[8].

1.3.4.4. Data collection and mass calibration

The full potential of any mass spectrometer is not be realised without a computerised data system. If the amount of a sample is very small, the human operator cannot react rapidly enough to get a representative mass spectrum during the limited period in which the compound evaporates in the ion source. The computer can obviate this

problem by controlling continuous repetitive scanning of the mass spectrometer from the time that the sample is introduced into the source. The spectra of interest can be recalled from the data stored in the computer[125].

1.3.4.4.1. Data collection and manipulation

All recent mass spectrometers are linked to some form of data system, which can be utilised in several different ways. Typically a modern data system is capable of three basic operations: (1) control of the scanning of the mass spectrometer, (2) data acquisition from the mass spectrometer and (3) data processing. In the past, spectra were recorded on ultraviolet sensitive chart paper and masses were manually assigned to each peak. This was extremely time-consuming, limiting the number of analyses that could be carried out by an operator[124]. Data systems are not only able to carry out this, but can also manipulate the data as required. A data system can process previously acquired data while it is acquiring new data[111].

In normal operation, the data system will record the mass and intensity of ions as the spectrometer is scanning. A suitable reference calibration compound such as heptacosafluorotributylamine, which is suitable for the mass to charge ratio (m/z) 40-600 and used most often with quadrupole mass spectrometers, is used to calibrate the mass scale[123].

For an individual scan, the sum of the intensities of all the ions can be recorded. This is known as the total ion current (TIC). The data system can subtract spectra from each other to produce good quality spectra, free from background interference. The data system can also plot the variation in intensity of individual ions with time, to produce what is known as a single-ion or mass chromatogram. This is particularly useful when attempting to detect a specific drug in a mixture. It is also useful for detecting different drugs after all the spectra have been recorded, but it is not particularly sensitive[111].

If a particular drug is suspected, the alternative technique is selected ion monitoring (SIM), which is also known multiple-peak monitoring (MPM) and multiple-ion detection (MID). In SIM mode of operation, instead of recording the whole

spectrum, the mass spectrometer monitors and records only the signals from those ions sufficient to characterise the drug of interest[126].

Typically three different ions would be monitored and recorded. These ions would commonly include the molecular ion, where it is intense, and the other major fragment ions of diagnostic value. The absolute intensities of the ions are recorded and they enable the calculation of the drug concentrations with the use of a suitable internal standard. The advantage of this method is that it is notably much more sensitive than whole spectrum recording, because of the increased time spent on the monitoring of each particular ion[124].

To obtain a complete mass spectrum, nanogram amounts of drugs are normally required, whereas picogram or even femtogram quantities of the drugs can be measured using selected ion monitoring methods[5]. Selected ion monitoring is particularly easy to perform using quadrupole and ion trap mass spectrometers, since the electric fields can be rapidly and reproducibly switched, in a repetitive manner, between the ions being monitored[123].

1.3.4.4.2. Mass calibration

The ideal calibration compound would be reasonably volatile for easy inlet to the ion source and give a mass spectrum of closely spaced, large peaks covering a wide mass range; the m/z values would be quite distinct from the m/z values of the sample. The commonest calibration compounds are perfluorokerosene (PFK), which is most useful for magnetic sector instruments operating in the mass range m/z 30-800, and heptacosafluorotributylamine that is suitable for the quadrupole mass spectrometers in the mass range of 40-600. For calibration to high mass, fomblin oil (a polyfluoroether) gives reference ions up to m/z 3000[124].

The information acquired by the computer is a time/intensity file. The process of converting the time/intensity file to a mass spectrum (mass/intensity) is known as "mass calibration"[111]. It relies upon the acquisition of data from a reference calibration compound, the mass spectrum of which is stored in the computer as a mass/intensity file called a reference table. Matching and equating the acquired time/intensity file and the reference table produce a mass/time conversion file, called

a calibration table. It can be used to convert any subsequently acquired time/intensity files to mass/intensity files[124]. The process of mass calibration is shown as a flow chart in Figure 1.4.

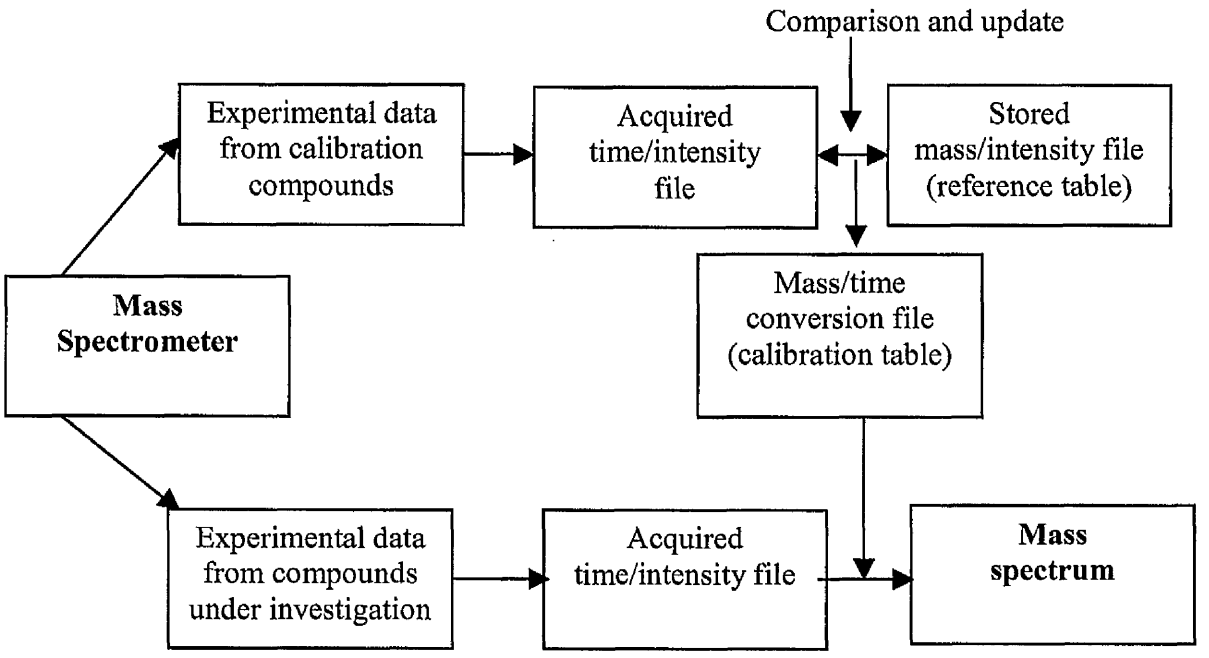


Figure 1.4. Mass calibration

1.3.4.4.3 Mass Spectral Libraries

Detection of an unknown drug may be achieved by comparison of its mass spectrum with those in a spectral library[123]. Published electron impact spectra are usually obtained at 70ev[126].

Recent data systems usually include library search amenities, including a spectral library, so that it can be carried out automatically when an unknown compound is present. Such libraries are commercially available[127,128], although searches of hard copy spectral listings can also be performed manually[129]. Special libraries, which contain only the compounds likely to be of interest, can be searched more

quickly[130,131]. It is also advantageous to the analyst to build up an individual library, which contains the compounds most likely to be encountered.

When spectra are searched manually, the analyst has to obtain the unknown spectrum in the same format as the hard copy spectra and must compare the spectra using his experience to obtain the best match. The use of a data system to perform this process is rapid and more objective than manual searching[124].

The result of a library search is a list of library matches in order of their fitness. The fitness factor normally is listed on a range of 0-1000 for each match and it is a measure of the similarity between the spectrum of the unknown compound and the library spectrum. It considers differences in intensities between matching ions in the unknown and library spectra[132]. There are two basic forms of search, named forward search and reverse search. In the forward search, all the peaks in the unknown spectrum are compared with all the peaks in the reference spectrum. This does well for pure compounds. The reverse search only takes into account the peaks present in the reference spectra. It is mainly useful for mixtures[111]. To make the searching of a large library, which may include 50000 spectra, sufficiently fast, a pre-search can usually be performed. This procedure selects the library spectra that contain at least some of the largest peaks of the unknown spectrum. This can result in the decrease of the numbers of spectra to several hundred spectra. It also leads to more precise and limited checking of all peaks and relative intensities. As a result the best matches, usually of 6-10 compounds, can be displayed in order of their fitness factors. The results may include some related information such as molecular weight and molecular formulae with a list of the peaks of the spectra[126].

1.4. Pharmacology of Poisoning and Fatality

Forensic toxicology is that discipline which combines analytical chemistry with essential toxicological principles to deal with medico-legal aspects of the toxic effects of drugs and chemicals on humans. The role of forensic toxicology is to help establish cause and effect relationships between exposure to a drug or chemical and the toxic or lethal effects that result[133].

To confirm a cause and effect relationship unequivocally, the forensic toxicologist relies on specific, highly sensitive, analytical methods that can efficiently isolate, identify, and quantitatively determine the toxic compounds in question from biological fluids and tissues[133].

Accidental and intentional poisonings constitute a major cause of morbidity and mortality in the world[133]. There is no way to determine accurately the exact incidence of poisoning, since not all cases of poisoning are reported or detected. Although it is reported that at least 5000 to 10000 Americans die from poisoning each year, it is quite possible that there is another group of victims, in number equal to or more than those reported, who die each year from unreported poisonings[134]. For example, in a car fatality, the victim may have taken a drug such as an antihistamine, which caused drowsiness while driving. The cause of death in this case would probably be reported as a car accident rather than a drug-related accident.

Median effective dose (ED50) is the dose at which 50% of individuals show the particular effect. Similarly, the dose needed to produce a particular toxic effect in 50% of animals is called the median toxic dose (TD50). If the toxic effect is death of the animal, a median lethal dose (LD50) may be experimentally defined. Therapeutic index relates the dose of a drug needed to produce a desired effect to the dose that produces an undesired effect. In animal studies, the therapeutic index is usually defined as the ratio of TD50 to the ED50 for some of the therapeutically related effects[135].

The dose-response relationship relating to ED50 and LD50 is shown in Figure 1.5.

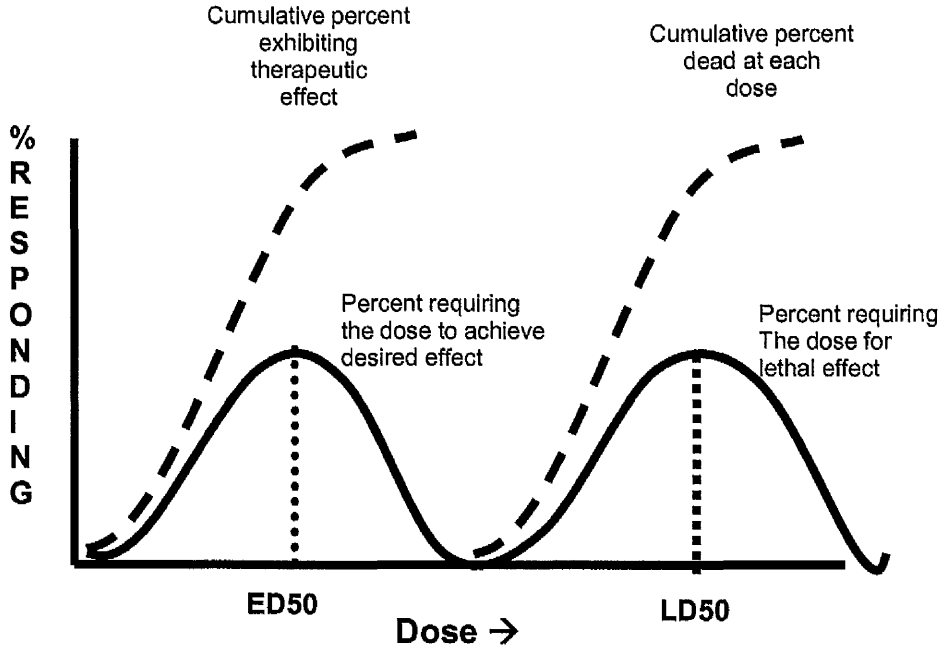


Figure 1.5. Dose-response relationship

Individuals may differ considerably in their responsiveness to a drug. Also a single individual may respond differently to the same drug at different times during the course of treatment[135].

Quantitative differences in drug response are common and clinically important. An individual patient may be hyporeactive or hyperreactive to a drug, in that the intensity of the effect of a given dose of the drug is decreased or increased in comparison with the most usual effect in the population at large[135].

With some drugs, the intensity of response to a constant dose may change during the course of therapy. In these cases responsiveness usually diminishes as a result of continuous drug administration, producing a state of relative tolerance to the effect of the drug. When responsiveness decreases rapidly after administration of a drug, the response is said to be subject to tachyphylaxis[135].

Occasionally, individuals show an unusual or idiosyncratic drug response, which is one that is seldom observed in most of the patients. These idiosyncratic responses

are usually caused by genetic differences in metabolism of the drug or by immunological mechanisms, including allergic reactions[135].

Antagonists reduce or block the actions of some drugs by several different mechanisms. Chemical antagonists may oppose the actions of a drug by binding to it and, thereby, inactivating it. For example, protamine may be used clinically to antagonise the effects of heparin. Physiological antagonists can work against the actions of a drug by opposing a regulatory pathway. For example, the effects of glucocorticoid hormones on the concentration of blood glucose can be physiologically countered by insulin[136].

Acute alcohol administration may inhibit metabolism of other drugs. This inhibition may be caused by alteration of metabolism or changes to hepatic blood flow. This acute alcohol effect may contribute to the commonly recognised danger of mixing alcohol with other drugs when performing activities that need some skill, specially driving. Phenothiazines, tricyclic antidepressants and sedative-hypnotic drugs are the most important drugs that interact with alcohol by this mechanism[136].

1.4.1. Mechanisms of toxicity and characteristics of opioid poisoning

All opioid derivatives have the potential to produce severe toxicity, which is dependent on the dose and route of administration. The mechanisms by which they produce their toxic effects are similar. These effects are related to the different actions of these drugs upon various opiate receptors in the central nervous system[137].

Acute opioid toxicity may result from a variety of situations. These include intentional, accidental, or therapeutic overdose of prescribed medicines. Whatever the reason, the toxicological effects are basically the same. The most common characteristics of acute opioid toxicity are central nervous system depression, leading to coma at maximum level, respiratory depression, pulmonary oedema, hypothermia, miosis, bradycardia, hypotension, decreased urinary output and decreased gastrointestinal motility[136].

Signs and symptoms associated with acute opioid overdose usually begin within 20 to 30 minutes of oral ingestion and within a few minutes of parenteral administration.

The most important effects involve opioid action on the central nervous system, which causes nausea and vomiting as the first symptoms noted[135].

The most obvious and severe toxic effect of opioid poisoning is central nervous system depression. The extent of this central nervous system depression and its duration will vary according to the opioid involved, the quantity, and route of administration. For a large overdose, the victim rapidly lapses into coma and is not arousable by verbal or painful stimuli[136].

In acute overdose, respiration is severely depressed to a rate as low as 2 to 4 inspirations per minute[138]. Cyanosis becomes apparent and many victims have a frothy pulmonary oedema. In humans, death from an acute opioid overdose is almost always from respiratory arrest. When there is a high concentration of the drug in the medulla and brain stem there is decreased sensitivity of the brain stem respiratory centres to increases in the partial pressure of carbon dioxide and, in the medulla, there is depression and slowing of the rhythm of respiration. Respiratory depression is complicated by bradycardia and hypotension. Pinpoint pupils (miosis) are usually considered to be the classical sign of narcotic poisoning. When mydriasis accrues, the victim's prognosis is grave[138,139]. The opioids include codeine, dihydrocodeine, morphine, diphenoxylate, meperidine, pentazocine, and propoxyphene[135].

1.4.2. Mechanisms of toxicity and characteristics of benzodiazepine poisoning

Benzodiazepines have a high therapeutic index and are the safest sedative-hypnotic drugs as the gap between therapeutic dose and lethal or toxic dose is extremely wide[136]. Most toxic effects of benzodiazepines result from their sedative action on the central nervous system. At extremely high doses, neuromuscular blockade may occur. Also, after intravenous injection, peripheral vasodilation causes a fall in blood pressure, and shock may result[140]. Respiration is not markedly affected, even with hypnotic doses of most of the benzodiazepines. Some derivatives might decrease alveolar ventilation (leading to decreased P_{O2} and increased P_{CO2}) and induce CO_2 narcosis in persons with pre-existing compromised respiratory functions[141]. Benzodiazepines, however, potentiate the respiratory depressant effect produced by other sedative drugs when taken concomitantly[141,142]. Most fatalities associated with

benzodiazepine overdose after oral ingestion have occurred in persons who ingested ethanol or another central nervous system depressant coincidentally[143]. Intravenous administration of benzodiazepines has greater associated risks of life-threatening hypotension and respiratory depression leading to death than have other routes of drug delivery[144]. Signs and symptoms of benzodiazepine overdose include mild toxicity (ataxia, drowsiness), moderate toxicity (respond to verbal stimuli, coma stage 0-1), and severe toxicity (patient responds only to deep pain, respiratory depression, hypotension, coma stage 1-2). Benzodiazepines include nitrazepam, flunitrazepam, flurazepam, diazepam, lorazepam, oxazepam, alprazolam, bromazepam, chlordiazepoxide, clobazam, clorazepate, loprazolam, temazepam, clonazepam and midazolam[136].

1.4.3. Mechanisms of toxicity and characteristics of tricyclic antidepressant poisoning

Tricyclic antidepressants are referred to as tricyclic compounds because of their chemical structures, which contain three rings[136]. A newer compound, maprotiline, is a tetracyclic compound, but, for simplicity, all of the drugs are here referred to as tricyclic antidepressants. Toxicities with tricyclic antidepressants result from both accidental and deliberate overdoses. Unlike poisoning with antipsychotic drugs, toxicity with tricyclic antidepressants is potentially life threatening. Furthermore, most poisonings involve multiple ingestions with at least one other drug, often alcohol, diazepam, propoxyphene or codeine. Thus, the poisoning symptoms may be potentiated and additional problems might be encountered[145].

It should be remembered that many patients who take tricyclic antidepressants and/or antipsychotic drugs have a high risk for suicide[146]. So, it is a rule of prescribing that the quantity of tricyclic antidepressants dispensed should be limited to a one-week supply[147].

Tricyclic antidepressants decrease the action of acetylcholine centrally and peripherally. The majority of toxic symptoms can be explained by this action. For example, chorea is believed to result from an imbalance in acetylcholine and dopamine levels at receptor sites. Myoclonus is caused by reduced serotonin uptake with its resultant increase within the synapse. Respiratory dysfunctions and

disturbances in body temperature result from direct action of tricyclic antidepressants on the respiratory centre (in the medulla) and the thermoregulatory site (in the hypothalamus), in addition to anticholinergic effects such as decreased sweating[136].

The usual characteristics of tricyclic antidepressant toxicity include hypothermia, respiratory depression, seizures, disorientation, agitation, coma, pyramidal signs, arrhythmias, cardiac arrest, hypotension, mydriasis, tachycardia, vasodilation, urinary retention and decreased gastrointestinal motility[148,149,150,151]. Tricyclic and related antidepressant drugs include imipramine, amitriptyline, mianserin, trazodone, amoxapine, clomipramine, doxepin, nortriptyline, trimipramine, maprotiline, dothiepin and loxapine[136].

1.4.4. Mechanisms of toxicity and characteristics of antipsychotic poisoning

Several drugs, used to treat severe psychotic disorders, are involved in overdoses. In addition to their use in mental disorders, they are all used as antiemetics, tranquillisers and cough suppressants. These drugs, including chlorpromazine, promazine, trifluoprazine, mesoridazine, thioridazine, perphenazine, fluphenazine, trifluoperazine, haloperidol, thiothixene and loxapine, are known as antipsychotics, neuroleptics, major tranquillisers, psychotropics and ataractics[136].

Antipsychotic overdoses are common; serious morbidity and mortality to pure antipsychotic overdoses are low[152]. However, many antipsychotic drug poisonings represent mixed poisonings, involving a combination of different drugs. There are some reasons for the large number of poisonings. First, antipsychotic drugs are widely prescribed. Patients who have mental illnesses receive these drugs and so they are widely available. Also these patients are at greater risk than the general population for suicide[153].

The mechanism of antipsychotic drug toxicity is by inhibition of dopamine receptors in the basal ganglia. Blockade of the receptors causes decreased neuronal cell firing. Synthesis of catecholamines in the central nervous system may also be inhibited[136]. Central effects of the drugs include sedation, muscle relaxation, lowering of seizure threshold, anxiolytic activity and depression of vasomotor reflexes. Overdoses of

antipsychotic drugs result in a variety of signs and symptoms that involve actions on the central, extrapyramidal, autonomic nervous system and the cardiovascular system[147,154].

The hypothalamus modulates a multitude of psychological functions including control of the vasomotor and temperature regulating systems. These drugs inhibit these regulatory systems causing vasodilation and orthostatic hypotension. Hypothermia or hyperthermia might result from these drugs and could be fatal[153].

1.5. Review of published work related to extraction and detection of targeted drugs

1.5.1. Benzodiazepines

Benzodiazepines are an important class of sedative, hypnotic, and anticonvulsant drugs widely prescribed throughout the world for the treatment of anxiety, epilepsy and insomnia[136]. In spite of their low toxicity compared with many drugs that act upon the central nervous system, benzodiazepines are often involved in drug intoxication and they are liable to abuse and can affect human skilled performance[155].

Benzodiazepines are frequently involved in drug intoxication or traffic accidents. In 1978 about 200 deaths were reported from them in England and Wales, therefore screening and quantification of benzodiazepines from different specimens is widely indicated for medical and legal purposes[156].

One or more benzodiazepines were found in 10% and 2% of autopsy cases, respectively[156]. A study (1993), in Scandinavia, indicates that benzodiazepines were responsible of 60% of deaths among drug addicts[157].

1.5.1.1. Detection Methods

A large number of analytical methods have been used for the determination of benzodiazepines. They can be measured by thin layer chromatography and immunoassay methods[158], but these methods cannot always identify the benzodiazepines found. Identification can be performed using GC/MS methods[4].

Benzodiazepines can be screened in urine as benzophenones. However, triazolam, alprazolam and clobazam do not produce benzophenones at all and, since many benzodiazepines produce the same benzophenone, positive results must be confirmed by another method[60].

Gaillard *et al.* (1993) were able to detect most benzodiazepines in plasma at pharmacological and toxic concentrations by dual channel gas chromatography, using electron capture and nitrogen-phosphorous detectors. GC-NPD and GC-ECD have been used for quantitative analysis of benzodiazepines without derivatisation[159]. The methods were reproducible enough to permit reliable quantification of plasma diazepam, nordiazepam, oxazepam, lorazepam, chlordiazepoxide, temazepam, midazolam, alprazolam, clobazam, norclobazam, adinazolam, flunitrazepam, bromazepam, triazolam, nitrazepam and clonazepam[159].

GC-NPD, using packed columns, has been used for the determination of basic drugs including benzodiazepines. The method employed liquid-liquid extraction using N-butyl acetate and analysed drugs without derivatisation. The sensitivity of the method was sufficient to be usable for the purpose of clinical and forensic toxicology[20].

Capillary gas chromatography has been applied for the assay of underivatised nitrazepam and clonazepam in plasma. The method allowed accurate assay of these drugs in plasma, at low concentrations. The detection limit was about 1ηg of nitrazepam or clonazepam per ml of plasma[160].

Chen *et al.* (1994) used capillary GC-NPD for the determination and measurement of basic drugs including some benzodiazepines. The method applied solid phase columns for extraction of analytes from biological matrices. The basic drugs could be detected at concentrations of 100-200ηg/ml[39]. Louter *et al.* (1997) applied GC-NPD using automated on-line solid phase extraction, using an ASPEC system, which was connected to a laboratory-built loop-type interface[38].

Guan *et al.* (1999) used GC-ECD and solid phase microextraction for the detection and quantification of benzophenones in urine[60]. GC-ECD and GC-NPD have been used for the quantitative analysis of benzodiazepines in plasma[161]. Prazepam was

used as an internal standard and flunitrazepam, diazepam, fludiazepam, prazepam, midazolam, flurazepam and nimetazepam have been detected.

The identification and quantification of some benzodiazepines and their unconjugated metabolites by electron ionisation capillary GC/MS of their trimethylsilyl derivatives was described by Drouet-Coassolo *et al.* in 1989[162]. Negative chemical ionisation was preferable to electron ionisation, and much more sensitive than positive chemical ionisation with linear ranges of 50-2000 μg/l for a number of benzodiazepines. GC/MS was used as the reference method of choice, although there were problems with some kinds of analytes such as nitrazepam.

1.5.1.2. Extraction Methods

The analysis of drugs in biological fluids usually requires the preparation of samples for all forms of chromatography. The sample preparation causes the best selectivity in the determination[10]. These methods include protein removal, enzymatic digestion, ultrafiltration, dialysis, liquid-liquid extraction, solid phase extraction (SPE) and, recently, solid phase microextraction (SPME)[10].

Most benzodiazepines are weak bases and so they can be extracted to some extent below pH12. A commonly used pH is 9-9.5. However, their considerable differences in pKa lead to a requirement for the extraction to be optimised by changing the pH and the organic solvent[89].

Extractants used for liquid-liquid extraction have included benzene, benzene/isopentanol, tert-butyl alcohol, benzene/isoamylalcohol, benzene/dichloromethane, dichloromethane, hexane/dichloromethane and N-butyl acetate[20,161-163].

In liquid-liquid extraction a large loss of volatile substances, such as amphetamines, takes place during the concentration of the extracts, where the solvent is evaporated under a gentle stream of nitrogen[164].

An alternative approach is solid phase extraction. Solid phase extraction columns and discs in a wide range of sizes, materials and capabilities are available which can cope with various kind of matrices, such as urine, plasma, whole blood and tissue extracts[41].

Louter *et al.* (1997) extracted four benzodiazepines from plasma using C-18 cartridges. They used water-methanol (9:1v:v) in order to remove salts and polar matrix constituents[38].

Mixed-phase Bond Elut Certify® columns, one kind of SPE column, were used by Chen *et al.* (1994), who extracted basic drugs from plasma, urine and whole blood samples successfully. Whole blood samples required deproteinisation. These samples were sonicated in an ultrasonic bath for 15 minutes at room temperature. The urine samples required washing with 1 ml of a 20% (v/v) solution of acetonitrile in water after the washing step with 1 ml deionised water[39].

Zweipfening *et al.* (1994), also using Bond Elut Certify® columns, extracted analytes including some benzodiazepines from whole blood samples. They deproteinised blood samples using a sonic bath and centrifuge. The elution step was performed using two different solvents to separate acid-neutral and basic analytes. The extracts were to some extent clear and did not contain much material which would contaminate the injection port of the GC. They concluded that this makes the method very useful for routine analyses in the toxicological laboratory[165].

The extraction of analytes, including some benzodiazepines, using an ASPEC and Bond Elut Certify® columns was reported by Chen *et al.* (1993). They compared the results of both manual and automated procedures and found that the reproducibility of the automated method was better than that of the manual procedure. Furthermore, the automated method considerably reduced the amount of labour required[40]. There are many reports of the use of different kinds of solid phase columns and one of them found C2 columns to be superior to C18, C12, and C8 columns for extraction of seven benzodiazepines[38]. However, Gaillard *et al.* (1993) obtained similar results using C8 columns eluting with 0.5% (v/v) acetic acid in methanol[159].

Solid phase extraction allows the preparation of smaller volumes than with liquid-liquid extraction without the problem of possible formation of an emulsion. It is faster and cheaper than liquid-liquid extraction, which allows the automation of extraction, with reproducibility and recoveries at least the same as liquid-liquid extraction[166].

Reubsaet *et al.* (1998) extracted oxazepam, nordiazepam, flunitrazepam, diazepam, and alprazolam from urine and plasma using solid phase microextraction (upon a polyacrylate fibre). They used sodium chloride in order to increase the yield of extraction. The detection limits were in the range of 0.01-0.48 μmol/l[61]. Carbowax/divinylbenzene was used as an SPME fibre coating by Luo *et al.* (1998). They extracted the benzodiazepines from urine and plasma. These drugs were extracted by SPME using a direct immersion method and were detected by a gas chromatograph-mass spectrometer[78].

PDMS fibres were used by Guan *et al.* (1999) for the extraction of benzodiazepines from urine[60]. These drugs were extracted by a direct immersion method. Seno *et al.* (1997) extracted prazepam, midazolam, fludiazepam, flunitrazepam and flurazepam from urine using direct immersion solid phase microextraction[89]. However, trazolam, estazolam, alprazolam and etiozolam were not extracted. Mullett *et al.* (2002)[167] also described the use of solid phase microextraction for direct extraction of oxazepam, temazepam, nordiazepam and diazepam[167]. The method was linear with an average linear coefficient value of 0.999.

1.5.2. Opiates

Opiates are the drugs which are structurally related to morphine. Opioids are compounds that have morphine-like activity, but may not be structurally related to morphine. Opiates are used clinically in the relief of moderate to severe pain and have a wide range of pharmacological effects and different potency for the production of analgesia, sedation, respiratory depression, decreased gut motility and cough suppression[168].

1.5.2.1. Detection Methods

Capillary GC, either alone or in combination with MS, has been used to detect morphine and other opiates in biological fluids. GC/MS is a method, which is completely capable of the unequivocal identification of individual opiates[4].

Giavanni and Rossi (1994) applied GC/MS for simultaneous analysis of cocaine, heroin and their metabolites from small amounts of urine. In this method, MS was performed in the electron-impact selected-ion-monitoring mode. Derivatisation was

necessary to change the polar hydroxyl groups of the opiates into their non-polar trimethylsilyl derivatives, so as to improve their chromatographic behaviour and increase sensitivity. Cocaine contains no active group that can be derivatised in this way with bis-trimethyl-silyl trifluoro acetamide[36].

Assay of cocaine, heroin and their metabolites using GC/MS in hair, plasma, saliva and urine was reported by Wang *et al.* (1994). The determination of analytes was performed by GC/MS operating in the selected-ion-monitoring mode[169]. Another GC/MS method, using an ion trap detector, was applied to the assay of opiates by Bogusz *et al.* (1996)[35].

Zweipfening *et al.* (1994) applied GC/MS to the analysis of whole blood and detected cocaine, morphine and codeine. This method was very useful for quantitative and semi-quantitative applications[165]. The use of GC/MS for the determination of cocaine and its metabolites has been reported by Lillsunde *et al.* (1996)[170].

1.5.2.2. Extraction Methods

Chromatographic methods need some form of extraction to separate the opiates from the matrix. Traditionally, this has been performed by liquid-liquid extraction after mixing the sample with a buffer at a selected pH and ionic strength[17].

Dawling *et al.* (1990) used N-butyl acetate for the extraction of a wide range of basic drugs from blood. Cocaine and codeine were extracted by N-butyl acetate[20].

Lillsunde *et al.* (1996) used dichloromethane/toluene for the extraction of basic drugs and ethyl acetate for cocaine and its metabolites[170].

Wang *et al.* (1994) applied SPE columns (clean screen DAU, 200mg, 10ml) for the extraction of cocaine, heroin and their metabolites from plasma, saliva, hair and urine. SPE techniques were used in which the pH was adjusted to minimize hydrolysis of heroin and cocaine[169].

Bakerbond SPE columns have been used by Giovanni *et al.* (1994) for detection of cocaine, heroin and their metabolites in urine only[36].

Bogusz *et al.* (1996) examined four different kinds of mixed-phase SPE columns in order to compare the extraction efficiencies and the chromatographic purity of the

extracts. They showed that the absolute recoveries of opiates from blood or serum were very different for all mixed-phase SPE columns[35].

Zweipfening *et al.* (1994) used a Bond Elut Certify column and GC-NPD for the analysis of whole blood samples. Certain opiates, including codeine and morphine, were studied in this paper. Clean extracts and highly reproducible recoveries were obtained[165]. Extraction using Bond Elute Certify was automated by ASPEC for the screening of drugs including cocaine and codeine[40].

Myung *et al.* (1999) used solid phase microextraction in combination with GC-NPD for the determination of meperidine in human urine. Sodium chloride was used to increase the yield of the extraction[171]. Methadone in human hair has been determined using solid phase microextraction and GC-MS by Lucas *et al.* (2000)[172].

Another study applied a headspace SPME (with a PDMS fibre coating) and a GC-FID (flame ionisation detector) for the detection of meperidine in whole blood and urine samples[173]. Chiarotti and Marsili (1994) used a PDMS fibre coating and GC-MS for the analysis of methadone in urine samples. They selected pH7.7 as the best pH for the extraction of the drug from urine, using the direct immersion mode[174].

1.5.3. Antidepressants

Antidepressant drugs are used in the treatment of patients with depression. They are commonly involved in voluntary poisonings because large amounts are prescribed to patients who suffer from major depression and because of their usefulness in some psychiatric disorders, which involve an elevated risk of suicide[136,155].

1.5.3.1. Detection Methods

Ulrich *et al.* (1996) applied capillary GC, with nitrogen-phosphorous-selective detection, for determination of amitriptyline, nortriptyline and their hydroxylated metabolites in serum[175]. Abernethy *et al.* (1984) described the determination of trimipramine and its metabolites by GC-NPD as a sensitive and specific method[176]. Jourdil *et al.* (1997) applied capillary GC-NPD for the determination of the second-generation antidepressants fluoxetine and medifoxamine in plasma. The method was useful in identifying and determining the concentration of these drugs[177].

Measurement of basic drugs, including most antidepressants, using a glass column GC-NPD was reported by Dawling *et al.* (1990). They concluded that the sensitivity of the method was more than adequate for the purposes of clinical and forensic toxicology[20]. Determination of the presence of the antidepressants using capillary GC-NPD was reported by Chen *et al.* (1994) and Torre *et al.* (1998) [39,120].

Capillary gas chromatography with flame-ionisation detection was applied by Lee *et al.* (1997) to the analysis of whole blood samples containing amitriptyline, chlorimipramine, imipramine and trimipramine[178].

Namera *et al.* (1998) have used gas chromatography-mass spectrometry for the analysis of mianserin, maprotiline and setiptiline in whole blood. They used a PDMS fibre coating to extract the drugs from samples using the headspace mode[179].

1.5.3.2. Extraction Methods

Hexane/dichloromethane/isoamylalcohol[177], hexane[16], hexane/isoamylalcohol[18,180] (98:2 v:v), N-butyl acetate[19], and hexane/isoamylalcohol (97:3 v:v)[16] have been used for the liquid-liquid extraction of antidepressant drugs. Jourdil *et al.* (1997) decreased the proportion of hexane and increased the proportion of a polar solvent, dichloromethane, to break up emulsions[177].

Solid phase extraction columns have been universally used, manually or in automated procedures, for the extraction of antidepressants. Torre *et al.* (1998) used the mixture water/acetonitrile/methanol (2:3:2 v:v:v) for the complete elution of benzodiazepines because they are commonly administered concurrently with tricyclic antidepressants. They described a sample cleaning procedure, which made the columns reusable up to three times without significant cross contamination[120].

Lee *et al.* (1997) applied headspace solid phase microextraction for the detection of tricyclic antidepressants in whole blood. Targeted drugs included amitriptyline, chlorimipramine, imipramine and trimipramine. They described SPME as a sample extraction method without a requirement for organic solvent and with low background noise[178].

Ulrich and Mantens (1997) also described the use of solid phase microextraction for antidepressant drugs. The drugs they studied were imipramine, desimipramine,

amitriptyline, trimipramine, doxepin, nortriptyline, mianserin, maproteline, clomipramine and desmetylclomipramine. They found SPME to be a suitable alternative to traditional liquid-liquid and solid phase extractions for sample preparation of both basic and lipophilic drugs in plasma. The therapeutic plasma levels were too low for this approach because the analytical recoveries were very low. It seems to be a useful method for toxicological cases only. The sensitivity could be improved by optimising the rate of stirring, the dimension of the fibre and increasing the extraction time. However, the main advantage of SPME was the short time required for sample preparation[90].

A PDMS fibre coating and GC-NPD have been applied for the extraction and assay of clozapine (a neuroleptic drug) in human plasma[181]. Ulrich *et al.* (1999) investigated the influence of the concentration of proteins, triglycerides and salt on the extraction yield[181]. Koster *et al.* (1998) determined the concentration of lidocaine in plasma using direct immersion SPME combined with gas chromatography. They developed the SPME method for the determination of the total amount of lidocaine in plasma. Protein binding was reduced by acidification and subsequently the sample was deproteinised with trichloroacetic acid[93].

Jinno *et al.* (2001)[182] also applied solid phase microextraction combined with gas chromatography for the detection of tricyclic antidepressant drugs in urine[182]. The drugs they studied were amitriptyline, imipramine, nortriptyline and desipramine.

1.6. Drug Screening

Generally, there is no single method, which provides all the analyses required for a comprehensive service. It is important to employ two stage testing for most drugs. A screening test followed by a confirmatory test[5]. Most analyses commence with a screening test for the detection of unknown substances. They usually employ immunoassay techniques, which do not need the preparation of the sample and are easy to perform[4]. Various kinds of chromatography can also been used for screening tests. However, it is usually necessary to extract drugs from different matrices and to

concentrate the analytes into a small volume of a suitable solvent before undertaking chromatographic characterisation[5].

For screening purposes, GC with a packed column is the best choice for compounds with intermediate and low volatilities. GC fitted with a capillary column can be used as a confirmatory test for some drugs such as opiates and amphetamines[5].

All positive results obtained by screening tests require to be assured by confirmatory tests defined by The American Association of Forensic Science/Society of Forensic Toxicology (AAFS/SOFT) protocol[6] (Table 1.1)

Table 1.1: Summary of AAFS/SOFT guidelines for confirmatory analyses. Tests used as confirmatory analysis:

1. Should be based on a physical or chemical principle different from that of the first test.
2. Should offer a higher degree of specificity for the analyte than the first test.
3. Should have a detection limit equivalent to, or lower than, the first test.
4. Should employ mass spectrometry whenever possible.
5. Should employ a different derivative if the same technique, such as gas chromatography, is used.
6. Should employ a different technique if quantitation is used as confirmation.
7. May use a specimen different from the one employed in the first test.
8. May use a second aliquot of the same specimen if necessary.
9. Must not use the original extract.

1.7. Quantitative analysis

Several methods for quantitation are used, such as external standard and internal standard methods. For the analysis of drugs, the internal standard method is most frequently used.

An internal standard is a known compound added in a known amount to a sample and its accompanying calibrator. The principle reason for the use of an internal standard is to compensate for any variation in extraction or analysis between calibrator and the sample under investigation. The compound employed, as an internal standard must have the following qualities:

1. It should not already be present in either the calibrator or the sample under investigation.
2. It should not be a likely metabolite of the drug to be quantified.
3. It should be stable during the entire analysis.
4. It should have extraction and chromatographic properties as closely similar to the analyte as possible.

The following is a chromatogram without the use of an internal standard.

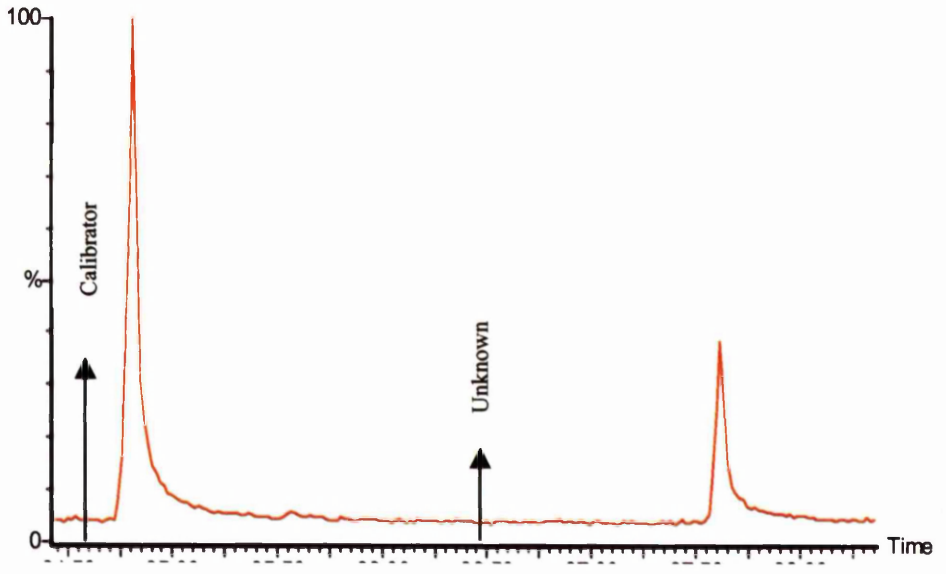


Figure 1.6. Quantitation without the use of an internal standard

The calculation appears below:

Concentration of calibrator = C_{Cal}

Peak area of calibrator = A_{Cal}

Peak area of unknown = A_{Unk}

Hence, concentration of unknown (C_{Unk}) = $A_{Unk} \times C_{Cal} / A_{Cal}$

This simple method is basically one in which the analysis can be regarded as calibrated by the calibrator using the factor C_{Cal} / A_{Cal}. This method is simple, but it suffers from all the variations of extraction, volume injected and on-column losses. It is essential, for accurate and precise work, to use an internal standard. The following is a chromatogram, which includes an internal standard.

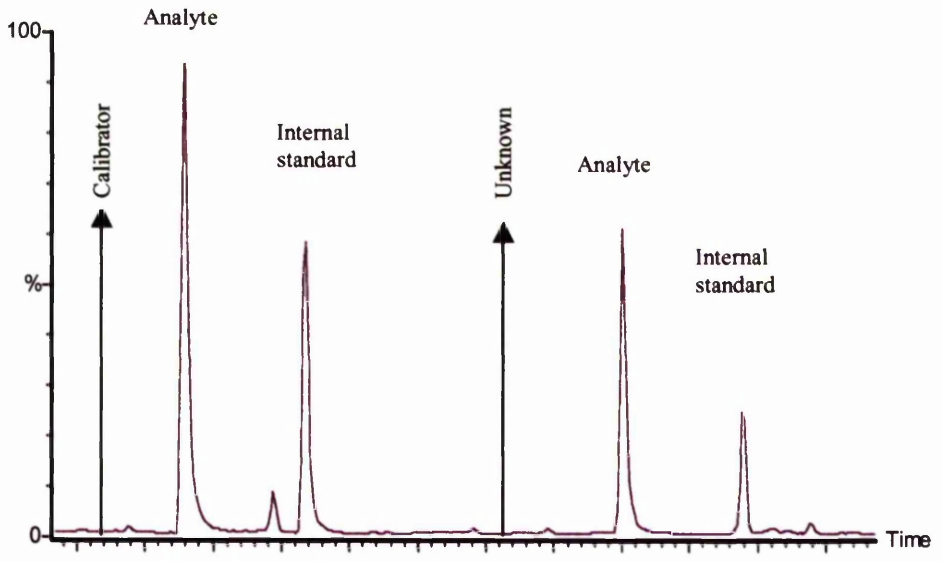


Figure 1.7. Quantitation using an internal standard

The calculation appears below:

Concentration of calibrator = C_{Cal}

Peak area of calibrator = A_{Cal}

Peak area of internal standard in calibrator = IS_{Cal}

Peak area of unknown = A_{Unk}

Peak area of internal standard in unknown = IS_{Unk}

The "standard ratio" (SR) i.e. response factor of the calibrator = $IS_{Cal} \times C_{Cal} / A_{Cal}$

Hence, concentration of unknown (C_{Unk}) = $A_{Unk} \times SR / IS_{Unk}$

It assumes that exactly the same amount of internal standard has been added to the unknown sample and the calibrator and that both have been treated in exactly the same way, i.e. have been processed in identical ways before and during analysis[183].

Chapter 2

Materials and Methods

2. Materials and Methods

2.1. Reagents

Deionised water was obtained from an Elgacan C114 deioniser and distilled water from an Aquatron A45 distillation apparatus. Methanol, ethanol, dichloromethane, diethylamine and N-butyl acetate, all of HPLC grade, were obtained from Sigma-Aldrich England (Gillingham, Dorset).

Sodium hydroxide, sodium chloride, sucrose, perchloric acid, glycine, citric acid and disodium phosphate dihydrate ($Na_2HPO_4.2H_2O$), all of the analytical grade, were obtained from BDH (Lutterworth, Leics).

"Pure" samples of benzoylecgonine, clobazam, codeine, diazepam, dihydrocodeine hydrochloride, heroin, mianserin, midazolam, morphine, pseudoephedrine, nortriptyline hydrochloride, promethazine, meperidine and thioridazine were purchased from Promochem Ltd (Herts, England). Certificates of analysis, purity and ideal storage conditions of the drugs are presented in Appendix B.

"Pure" samples of bupivacaine hydrochloride, loxapine succinate, procyclidine hydrochloride, nitrazepam, prazepam, oxazepam, chlordiazepoxide, flunitrazepam, clonazepam, cocaine, amitriptyline hydrochloride, clomipramine hydrochloride, dothiepin, diethyldothiepin, imipramine, trimipramine, protriptyline, doxepin, haloperidol, chlorpromazine and trifluoprazine were purchased from Sigma-Aldrich, England (Gillingham, Dorset). Certificates of analysis, purity and ideal storage conditions of the drugs are presented in Appendix B.

From these data the maximum impurity in all drugs was less than 1.2%, as determined by the manufacturers using two analytical techniques in most cases. In no instance were additional compounds, i.e. impurities, detected in the GC-MS. Therefore, any impurities are likely to be non-reactive/non-drug compounds.

2.1.1 Preparation of buffers

Glycine/NaOH buffers (pH9-12) were prepared by adding appropriate amounts of 0.1 mol/L NaOH to 0.1 mol/L glycine and citric acid/ phosphate buffers (pH4-8) were prepared by adding appropriate amounts of 0.1 mol/L citric acid to 0.2 mol/L disodium phosphate as shown in Table 2.1.

Table 2.1: The amounts of stock solutions required to make up buffer solutions of desired pH

pH	4	5	6	7	8	9	10	11	12
Glycine 0.1 mol/L						88.5mL	62.5mL	51mL	46mL
NaOH 0.1 mol/L						11.5mL	37.5mL	49mL	54mL
Citric acid 0.1 mol/L	62mL	49mL	37.5mL	19mL	5mL				
Disodium phosphate 0.2 mol/L	38mL	51mL	62.5mL	81mL	95mL				

2.1.2. Preparation of standard solutions

The stock solutions of individual drugs were prepared by dissolving or diluting the appropriate amount of each drug in methanol to make solutions of 1 g/L for each drug. Working standard solutions (50, 100, 200, 400 and 800 mg/L) of the drugs were prepared in methanol. During the study, identical amounts of working solutions were added to drug-free plasma in order to keep the amount of methanol in the samples constant and so standardise the effect of the organic solvent on the yield by extraction. All solutions were stored in the dark at 4°C.

2.1.3. Preparation of glassware

All glassware and magnetic stirring bars were washed with detergent (Decon 90) followed by rinsing with copious amounts of deionised water. This was followed by rinsing the glassware with a mixture of dichloromethane/methanol (1:1 v:v) in a

sonic bath for ten minutes. They were allowed to dry inverted at room temperature. Glassware was then capped until required for use.

2.2. Equipment

1) An SPME fibre holder and the SPME fibres below:

   100 μm polydimethyl siloxane (PDMS)

   85 μm polyacrylate (PA)

   65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB)

   carboxen^{TM}/polydimethylsiloxane (CAR/PDMS)

   65μm Carbowax®/divinylbenzene (CW/DVB)

   were purchased from Supelco (distributed by Sigma-Aldrich, Gillingham, Dorset, England).

2) Samples were stirred using a magnetic stirrer (Stuart scientific magnetic stirrer hotplate SM26) with a Teflon-coated stirring bar.

3) Measurement of pH was made with a Corning 140 pH meter.

4) The GC-MS instrument used was a Top Carlo Erba gas chromatograph in combination with a Fisons MD 800 mass spectrometer.

5) Sample screw top glass vials (5mL) fitted with Minort valves were supplied by Thames Restek (Windsor, Berks, UK).

6) A Hewlett-Packard HP-5 fused silica capillary column (30m × 0.32mm i.d., 0.25 μm film thickness) was purchased from GC2 Ltd (Wythenshawe, UK).

7) Samples were centrifuged using a MSE Cenataur2 centrifuge.

8) A Hamilton 701 syringe applied for solvent flush injections.

2.2.1. Instrumentation

The working standard solutions of the targeted drugs were injected into the GC fitted with an HP-5 capillary column and detected using mass spectrometry (quadrupole) to give the results listed in Table 2.2.

Table 2.2. Retention time (RT) of targeted drugs obtained using direct injection of 1µl of working standard solutions

Analyte	RT/min	Analyte	RT/min
Diazepam	34.4	Methadone	30
Nitrazepam	38.9	D-propoxyphene	30.7
Prazepam	37.3	Codeine	33.6
Chlordiazepoxide	35.6, 37.4	Cocaine	31
Flunitrazepam	36.9	Heroin	36.2
Oxazepam	32.9	Meperidine	22.7
Clonazepam	39.9	Dihydrocodeine	32.9
Medazepam	31.6	Morphine	NR
Loprazolam	NR	EDDP	28.9
Ketazolam	34.4	EMDP	28
Benzoyl ecgonine	NR	MDA	20.6
Midazolam	34.9	Methamphetamine	10.2
Clobazam	34.4	Nortriptyline	31
Amitriptyline	30.9	Protriptyline	31.7
Clomipramine	33.9	Desipramine	31.5
Mianserin	30.8	Doxepine	31.1
Dothiepin	33.7	Loxapine	34.6
Butriptyline	30.8	Thiothixen	NR
Imipramine	31.3	Haloperidol	40.8
Trimipramine	31.1	Trimeprazine	32.4
Chlorpromazine	35.1	Trifluoperazine	36.6
Bupivacaine	32.2	Fluphenazine	NR
Thioridazine	43.2	Disopyramide	NR

NR=no response

Chromatographic separation was achieved on the HP-5 fused silica capillary column. It was non-polar and so was suitable for separation of the drugs of interest. The samples were injected in the splitless mode, using the cryogenic focusing method for one minute, and the splitter was opened after one second. The chromatographic conditions were as follow:

Injection port:	250°C or 300°C (Depending on the fibres)
Column:	45°C for 2 minutes, 45°C to 290°C at a rate of 6°C/min hold 5 minutes at 290°C (In order to elute the heaviest contaminants)
GC-MS interface	300°C

Ultra pure Helium CP grade (B.O.C. Gases, Guildford, UK) was used as a carrier gas with a constant on-column pressure of 8 psi.

The mass spectrometer was operated in electron impact mode at 70eV and tuned to heptacosafluorotributylamine (a reference calibration compound) to achieve the best sensitivity. The mass range scanned was from 45 to 600 atomic mass units (amu). A computer equipped with Masslynx software, which was also devoted to data acquisition and processing, controlled the whole procedure including the various operatings of the gas chromatograph and mass spectrometer. Mass spectrometer data were collected in full scan mode; searching the related spectra in commercial libraries NIST, LIBTX and Wiley identified the drugs. The determination of concentrations was achieved by calculating the peak area ratios of the drugs to the internal standard and comparing with a calibrator. The mass spectrometric conditions were as follow:

Electron energy	+70ev
Multiplier	400-600
Source temperature	200°C
Interface temperature	300°C
Low mass resolution	7-12
High mass resolution	12-13
Ion energy	1
Ion energy ramp	1
Scan time	1 sec
Inter-scan delay	0.1 sec

The detector was turned off for the first three minutes of the sample run and the first seven minutes of the working standard solution to prevent detector overloading from the solvent peak.

Improved sensitivity was achieved when the mass spectrometer was operated in selected ion monitoring (SIM) mode. The relevant ions are listed in Table 2.3.

Table 2.3. Selected ions

Compound	Ions (m/z)
Amitriptyline	58, 202 and 215(±0.5)
Bupivacaine	84,140 and 243(±0.5)
Chlordiazepoxide	205, 220 and 247(±0.5)
Chlorpromazine	58, 272 and 318(±0.5)
Clomipramine	58, 192 and 268(±0.5)
Codeine	115, 162 and 299(±0.5)
Diazepam	221, 256 and 283(±0.5)
Dihydrocodeine	185, 199 and 301(±0.5)
Diphenhydramine	58, 73 and 165(±0.5)
Dothiepin	58, 202 and 221(±0.5)
D-propoxyphene	58, 193 and 208(±0.5)
Haloperidol	123, 192 and 224(±0.5)
Medazepam	165, 242 and 270(±0.5)
Meperidine	103, 172 and 247(±0.5)
Mesoridazine	98, 126 and 185(±0.5)
Prazepam	91, 269 and 295(±0.5)
Procyclidine	84, 105 and 204(±0.5)
Thioridazine	98, 126 and 370(±0.5)
Trifluoprazine	70, 248 and 266(±0.5)

Acquisition was first performed in full scan mode to identify the compounds in a *post-mortem* sample. Then, acquisition was performed in the selected ion-monitoring (SIM) mode in order to enhance sensitivity. Three of the most abundant

ions were selected for each compound, one of them for quantitation of the drug and the remaining two for confirmation.

Acquisition was most sensitive in the SIM mode, but detection of multiple masses decreased the sensitivity. To maintain adequate sensitivity, the masses were grouped into three or four time periods, for example, masses 58, 84, 103, 140, 172 and 202 into the first time period, masses 58, 192, 221, 256 and 272 into the second time period, and masses 123, 192, 248, 266, 269 and 295 into the third period.

2.2.2. Data handling

Data acquisition and the control of the whole procedure, including the operation of the gas chromatograph and mass spectrometer, was performed by a computer equipped with Masslynx software. The Masslynx library system was used to identify unknown spectra by comparison with databases of known spectra. The result of a library search was a list of library compounds or "hits" whose spectra give the best match with the unknown spectrum. The result of the library search included the compound list, which gives a textual listing of the best hits, the hits, which show the unknown spectrum followed by the spectra of the best fits, and the chemical structure of the currently selected compound.

The library search process had two parts, the pre-search and the main-search. The pre-search was a quicker search, which was designed to select a number of likely candidates from the library. These candidates were then passed through to the main-search where a more rigorous and lengthy comparison took place. The results of the main-search were then displayed in the hit list.

The library pre-search file contained a spectrum for each library entry, which has been reduced to the eight most intense mass-weighted peaks. The unknown spectrum was also reduced to its eight most intense mass-weighted peaks and then compared to the library pre-search file. The most likely candidates were the compounds that had the greatest number of matching peaks with the unknown compound. A list of most likely candidates was passed to the main-search.

In the main-search, the search spectrum was compared to each of the possible candidates from the library and the results of this comparison were presented. The

reverse fit value was computed for each hit. It showed how likely it was that the search spectrum contained the library entry. In this case the search spectrum might be a mixture of compounds. Any peaks, which were present in the library spectrum, but were not present in the search spectrum, decreased the reverse fit value.

Besides the standard libraries, NIST, LIBTX, and Wiley, an individual collection of spectra was used. The individual library, containing the compounds most likely to be encountered including benzodiazepines, local anaesthetics, antidepressants and analgesics, was built up during this study.

2.2.3. Structure and use of the SPME fibre and holder

The fibre holder was constructed and operated in a similar way to a gas chromatograph syringe. The SPME device consists of a 1cm length of fused silica fibre, coated on the outer surface with a stationary phase and bonded to a stainless steel plunger in a holder that looks like a modified microlitre syringe (Figure 2.1).

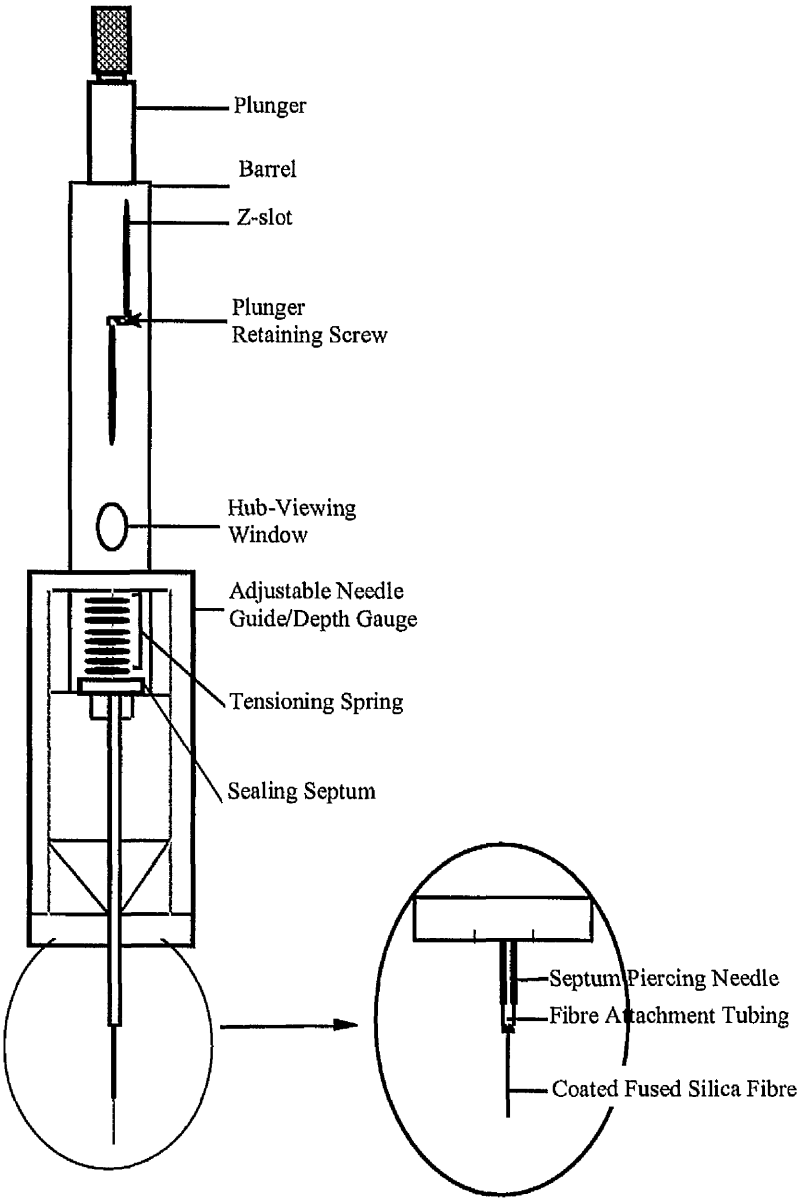


Figure 2.1. Diagram of manual SPME fibre holder and assembly

The SPME process had two steps: partitioning of analytes between the sample matrix and the fibre coating, followed by desorption of the analytes into the analytical instrument. In the first step, the fibre was drawn into the needle, the needle passed through the septum which sealed the sample vial and the plunger was depressed to lower the fibre into either the liquid sample or the headspace above the sample. This allowed partitioning of analytes between the matrix and the fibre coating.

In the second step, the fibre was drawn into the needle, the needle was withdrawn from the sample vial and was inserted though the injection port septum of the gas chromatograph. The plunger was again depressed, which exposed the fibre and allows desorption of the analytes to take place. Figure 2.2 illustrates an SPME set-up for direct extraction and GC analysis.

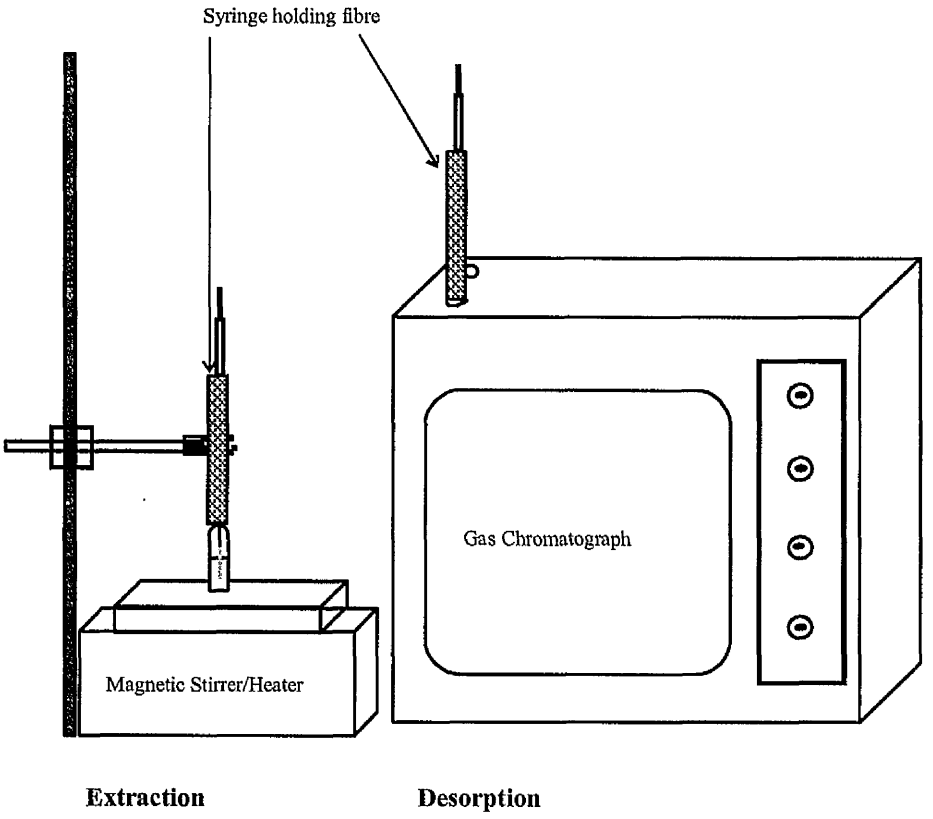


Figure 2.2. SPME set-up for direct extraction and GC analysis

In use, the prepared sample was contained in a 5 ml glass vial with a small stirrer magnet and a septum cap. The vial was heated in a beaker of water, at 60°C for 45 minutes (Figure 2.3). With the clean fibre retracted, the sheath was passed through the septum and the fibre was exposed. At the end of the equilibration time, the fibre was retracted and the sheath withdrawn. The GC injection port septum was then penetrated by the sheath and the fibre exposed to allow desorption (Figure 2.2).

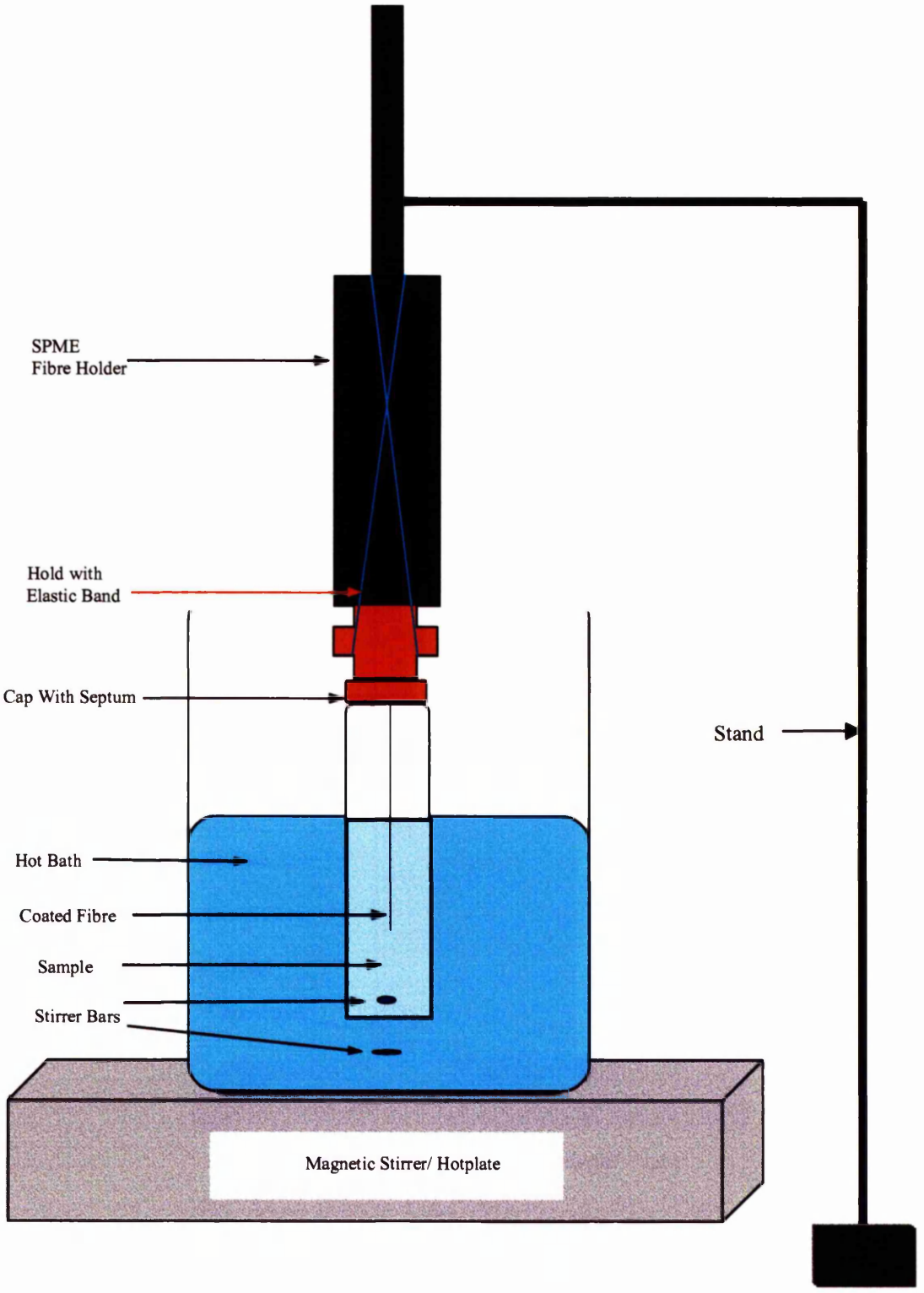


Figure 2.3. Schematic illustration of the SPME extraction method

2.3. Preparation of spiked plasma for coating selection

A sample (10 μL) of the working standard solutions (100 mg/L) of the targeted drugs was added to 1 mL of drug free plasma then 1.5 mL of 1 mol/L perchloric acid was added. This was followed by vortex mixing for 3 min and sonication for 5 min to complete deproteinisation. After centrifugation at 3000 rpm for 10 min, 2 mL of the supernatant was transferred into a 10 mL beaker containing 2 mL of the appropriate buffer (pH4, 7 or 10). The fibre was placed into the sample solution and the extraction took place for 20 min.

A working internal standard solution of prazepam (10 μL; 100 mg/L) in methanol was added to 1 mL urine. Centrifugation at 3000 rpm was performed for 5 min and the supernatant was transferred to a 10 mL beaker. The solution was buffered to the desired pH (4, 7 or 10) with 3 mL of the appropriate buffer and the pH was adjusted at the pH meter, using 1 mol/L NaOH or 1 mol/L perchloric acid. The solution was transferred to a 5 mL glass vial with a magnetic stirrer bar. The fibre was placed into the sample solution, where extraction was allowed to take place for 45 min.

2.4. Extraction mode selection

To select the extraction mode, drug free plasmas, spiked with seven of the targeted drugs each at the concentration of 1 mg/L, were prepared as described above (2.3) and extracted using the headspace-SPME mode. The fibre was introduced into the GC injector and desorbed for four minutes. The chromatogram obtained from that is shown in Figure 2.4. None of them was detected at 1 mg/L spiked plasma.

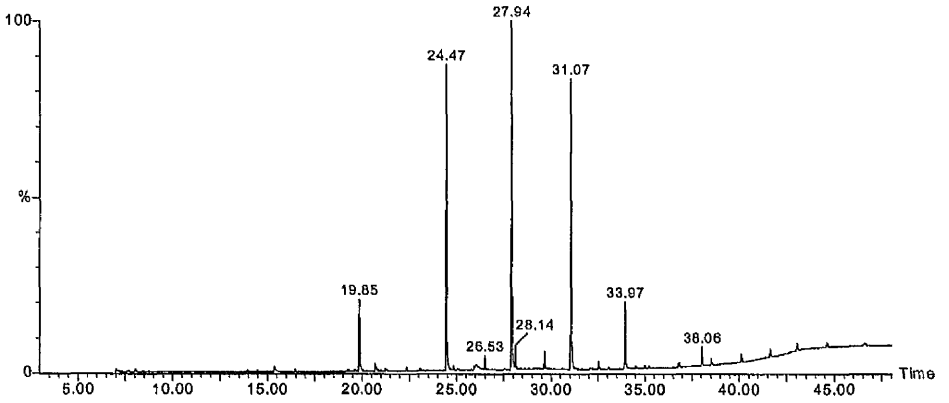


Figure 2.4. SPME-GC total ion chromatogram of the drug-free blood sample spiked with the drugs at the concentration of 1 mg/L

Direct immersion mode was selected for the extraction of the samples. In order to treat the very complex *post-mortem* blood samples, perchloric acid was used to deproteinise the blood. Finally, sonication of the blood samples was applied to complete the deproteinisation.

2.5. Preparation of samples for the determination of equilibration time

An aliquot of the working standard solutions (10 µL of 100 mg/L) of each drug was added to 1 mL of drug free plasma. Then, 1.5 mL of 1 mol/L perchloric acid was added to the vial, followed by vortex mixing for 3 min and sonication for 5 min to complete deproteinisation. The sample was centrifuged at 3000 rpm for 10 min and the pH was adjusted to pH7. The fibre was placed into the sample solution for extraction to take place.

2.6. Preparation of samples for investigation of pH effect

The targeted drugs have different P^{ka} values therefore partitioning of the drug between the sample and the fibre was strongly affected by pH. To determine the effect of pH on the extraction yield, sample vials were prepared with the pHs 4, 5, 6, 7, 8, 9, 10 and 11. The PA fibre was exposed for 45 minutes at 60°C and then analysed using GC-MS.

2.7. Preparation of samples for investigation of sodium chloride effect

In the literature addition of NaCl, K_2CO_3 and NaF were reported to increase the yield of extraction. The presence of an electrolyte can influence both SPME direct immersion and headspace absorption. Saturation with salt (eg. sodium chloride) has been used to lower the detection limits and also to normalise randomly variable salt concentrations in natural samples, especially urine.

Working standard solutions (10 μL of 100 mg/L) of members of drug groups were added to 1 mL of drug free plasma. Then, 1.5 mL of perchloric acid was added and this was followed by vortex mixing for 3 min. The sample was sonicated for 5 min and then centrifuged at 3000 rpm for 10 min. A 2 ml of the supernatant was transferred into a 5 mL beaker containing 2 mL of the pH7 buffer with 5 mol/L NaCl.

2.8. Urine sample preparation

The working internal standard solution (100 mg/L prazepam in methanol; 10 μL) was added to 1 mL urine. Centrifugation at 3000 rpm was performed for 5 min and the clear supernatant was transferred to a 10 mL beaker containing 5 mol/L NaCl. The solution was buffered to the desired pH (4,7 or 10) with 3 mL of the appropriate buffer and the pH adjusted at the pH meter using 1 mol/L NaOH or 1 mol/L perchloric acid. The solution was transferred to a 5 mL glass vial with a magnetic stirrer bar. The fibre was placed into the sample solution, where extraction was allowed to take place for 45 min.

2.9. Plasma and blood sample preparation

The working internal standard solution of prazepam in methanol (10 μL of 100 mg/L) was added to 1 mL of plasma and 1.5 mL of 1 mol/L perchloric acid was then added. This was followed by vortex mixing for 3 min. The sample was sonicated for 5 min to complete deproteinisation and then centrifuged at 3000 rpm for 10 min. The clear supernatant (2 mL) was transferred into a 5 mL beaker, containing 2 mL of

the appropriate buffer with 5 mol/L NaCl. The pH was then adjusted and the extraction achieved as for the urine samples.

2.10. Conditioning and maintenance of fibres

2.10.1. Conditioning of new fibres

New fibres were conditioned by exposing them in the injection port of the gas chromatograph. The injection port temperature was set according to Table 2.4, with a column installed and carrier gas adjusted to the desired flow rate. The splitter was open. The needle was inserted into the injection port and the fibre exposed for the time recommended by the manufacturer. After this, the fibre was retracted and the needle was removed from the injection port. The column was then conditioned for an additional 30 minutes at the upper temperature of the program.

2.10.2. Testing the conditioned fibres

The injection port was adjusted to the desired operating temperature and the gas chromatograph oven was cooled to 45°C. While the split injection port valve was closed, the conditioned SPME fibre was inserted into the injection port. The fibre was desorbed for five minutes after the gas chromatograph had been started. There would usually be some initial extraneous peaks, primarily from the glue used to attach the fibres. These peaks usually diminished after two periods of conditioning. When the peaks did not diminish after three desorptions, the fibre was exposed in deionised water for 10 minutes while stirring.

Table 2.4. Temperature and conditioning recommendations for GC use

Needle coating	Film thickness	Maximum temp.	Recommended Operating temp.	Conditioning temp.	Time (hour)
PDMS	100μm	280°C	200-270°C(250)	250°C	1
PDMS/DVB	65μm	270°C	200-270°C(250)	260°C	0.5
polyacrylate	85μm	320°C	220-310°C(300)	300°C	2
CAR/PDMS	75μm	320°C	240-300°C(300)	280°C	0.5
CW/DVB	65μm	265°C	200-260°C(250)	250°C	0.5

Temp. = temperature

Severely contaminated fibres, usually after 10 application runs, were cleaned as described below depending on the fibre phase coating. Non-bonded fibres such as polydimethylsiloxane (100 μm) were treated, using thermal cleaning only, by inserting at their maximum temperature i.e. 280°C for 1-2 hours, or 10-20°C less than the maximum temperature overnight. Use of a solvent might remove the phase from the fibre. Water-miscible solvents can clean partially bonded fibres, such as PDMS/DVB, CW/DVB, polyacrylate and CAR/PDMS. They were wiped three times on a tissue soaked with methanol before thermal conditioning as above. Chlorinated solvents can cause the epoxy adhesive to dissolve and the fibre to loosen and were not used on any fibres.

2.10.3. Daily routine cleaning

Before use, fibres were routinely cleaned by immersing them in deionised water, stirred with a bar, at 60°C for 5 min. Then they were briefly reconditioned in the GC injector for 3 min as instructed by the manufacturer. Each day a fibre blank was run, in order to check the baseline and determine the extent of any laboratory contamination, followed by a system blank.

2.11. Method validation parameters

2.11.1. Linearity

Calibration curves of the drugs were generated by least-squares linear regression. They were constructed by plotting the peak area ratios of the drugs to internal standard ($A_{analyte}/A_{IS}$) at the concentrations 1, 2, 5 and 7 mg/L, which were extracted from plasma using a polyacrylate fibre at pH7 and 60°C.

The equation of a straight-line relationship between variables x and y is y=a +bx, where "a" and "b" are constants. The first, a, is called the intercept and the second, b, is called the regression coefficient. The following test was used to check the linear relationship between the concentrations and peak area ratios[184]. The sum of the squares of the deviations from the line were calculated by:

$$\sum(y_i - \bar{y})^2 - b^2\sum(x_i - \bar{x})^2$$

where

$\bar{y}$ is the mean of the peak area ratios

$\bar{x}$ is the mean of the concentrations

The variation of y about the line called the residual variance, is

$$S^2 = \frac{1}{n-2}\sum(y_i - \bar{y})^2 - b^2\sum(x_i - \bar{x})^2$$

Where n-2 is called the degree of freedom.

Then, the standard error of b, SE(b), and the t distribution are given by:

$$SE(b) = \sqrt{\frac{S^2}{\Sigma(x_i - \bar{x})^2}}$$

$$t = \frac{b}{Se(b)}$$

The t calculated for the drugs was compared with the standard t distribution Table[184].

2.11.2. Precision

Precision of the assay was expressed as the relative standard deviation of the concentration values obtained for low, medium and high control samples, after

repeated analyses. The precision was calculated after analysis of the three replicates on the same day (within-day precision) and after repeated analysis over several days (between-day precision).

2.11.3. Limits of detection and quantification

The limit of detection (LOD) may be defined as the smallest quantity of analyte, which can produce a response significantly different from that of a blank. The limit of quantification (LOQ) may be defined as the smallest quantity of analyte, which can be quantified with acceptable precision.

Limits of detection and quantification were determined by diluting successively to the lowest point of calibration. The limits of detection and quantification were defined as the signals equal to the blank signal plus ten and three times of the standard deviation, respectively. The value of calculated intercept (a) was used as an estimate of the blank signal[185]. Therefore, the limits of detection and quantification were calculated as below:

LOD= a +3×standard deviation

LOQ= a +10×standard deviation

2.11.4. Solvent flush injection (sandwich method)

Solvent flush injection was applied for the injection of the authentic compounds in methanol. Using this method, injection-to-injection reproducibilities were as good as ±1% peak height variation. This method was as follows:

1) The syringe was repeatedly filled and flushed with solvent to wet the needle and barrel.

2) The needle was immersed in the desired solvent and the plunger was retracted until a value of 1.2 µl (needle volume is about 0.8 µl for a Hamilton 701 syringe, so the flush volume was 2 µl).

3) The syringe was removed from the solvent and the plunger was retracted until an air pocket was observed on the syringe end of the needle.

4) The syringe was placed into the sample solution and an appropriate amount of the sample was drawn into the syringe.

5) The syringe was removed from the sample solution and the plunger was drawn back until the sample was in the barrel between two air pockets (see Figure 2.5).

6) The exact sample size was read and the sample was ready for injection.

Since solvent was behind the sample, this ensured that sample was flushed into the injection without "hang up". After the penetration of the septum of the injection port the sample was injected rapidly.

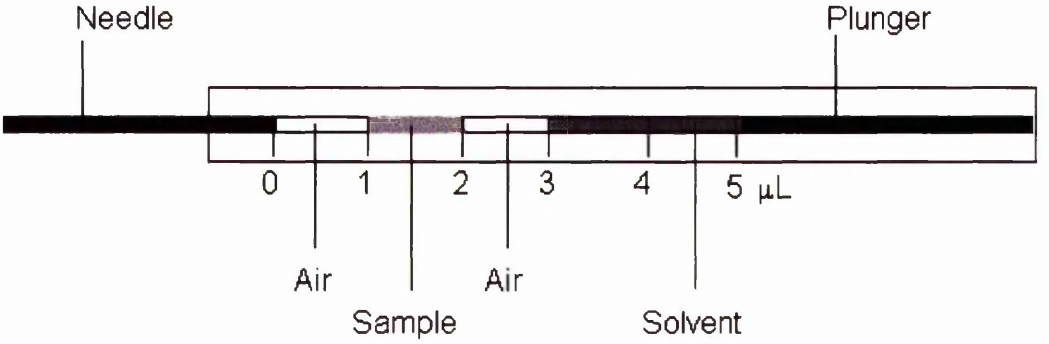


Figure 2.5. Solvent flush injection

2.12. Final selected protocol

2.12.1. Extraction

2.12.1.1. Extraction from blood for drug detection

1. Label clean tubes for blank and test.
2. Pipette 1 mL of drug-free serum into the blank tube.
3. Pipette 1 mL of *post-mortem* blood sample into the test tube.
4. Add 10 µL of 0.1 mg/mL of internal standard (prazepam) solution in methanol to all of the tubes.
5. Add 1.5 mL of 1 mol/L perchloric acid to all tubes.
6. Cap all the tubes and vortex mix all of them for 3 min.

7. Sonicate all the tubes for 5 min.
8. Centrifuge the tubes for 10 min at 3000 rpm (MSE Centaur2).
9. Transfer clear supernatant (2 mL) into a 5 mL beaker.
10. Add 2 mL of appropriate buffer (of pH4, 7, or 10), which contains 5 mol/L NaCl.
11. Adjust the pH with 1mol/L perchloric acid and 1 mol/L NaOH using pH meter.
12. Transfer the solutions to appropriately labelled 5 mL screw top glass vials.
13. Put a magnetic stirrer bar into each vial and cap the vials.
14. Place the selected fibre into the sample vial and extract the sample for 45 min at 60°C using the magnetic stirrer.

2.12.1.2. Extraction from urine for drug detection

1. Label clean and empty tubes for urine test.
2. Pipette 1 mL of *post-mortem* urine sample into the test tube.
3. Add 10 μL of 0.1 mg/mL of internal standard (prazepam) solution in methanol to all of the tubes.
4. Centrifuge the tubes for 5 min at 3000 rpm (MSE Centaur2).
5. Transfer clear supernatant (2 mL) into a 5 mL beaker.
6. Add 2 mL of appropriate buffer (of pH4, 7 or 10), which contains 5 mol/L NaCl.
7. Adjust the pH and extract as for blood samples.

2.12.1.3. Extraction from blood for drug quantification

1. At the start of the extraction, a fresh solution of working standard solution/s must be made up by diluting the stock solution with methanol.
2. Label clean tubes for each of the blank, calibrator and test.
3. Pipette 1 mL of drug-free serum into the blank and calibrator tubes.

4. Pipette 1 mL of *post-mortem* blood into the test tube.
5. Add 10 µL of 0.01 mg/mL of internal standard (prazepam) solution in methanol to all the tubes.
6. Add appropriate amount of working standard solutions of the detected drugs into the calibrator tubes.
7. Add a volume of methanol into the test tubes so as to keep the amount of methanol in the test and calibrator tubes the same and so as to standardise the effect of organic solvents (i.e. methanol) on the extraction yield.
8. Add 1.5 mL of 1 mol/L perchloric acid to all tubes.
9. Cap all the tubes, vortex mix them for 3 min, sonicate them for 5 min and centrifuge all of them for 10 min at 3000 rpm (MSE Centaur2).
10. Transfer clear supernatant (2 mL) into a 5 mL beaker, add 2 mL of the most suitable buffer from the detection procedure, and then add 5 mol/L NaCl.
11. Adjust the pH, then extract the calibrator, blank and test at precisely the same extraction time and temperature.

2.12.2. Gas Chromatography and Mass Spectrometry

Instruments: 8000 Top Carlo Erba gas chromatograph in combination with a Fisons MD 800 quadrupole mass spectrometer.

Column: Hewlett-Packard HP-5 fused silica capillary column (30 m×0.32 mm i.d., 0.25 µm film thickness).

Carrier gas: Ultra pure helium with an on-column pressure 8 psi.

Detection: Mass spectrometer in EI^+ mode at 70eV.

For screening, TIC was used and SIM mode was used for quantitation of positive samples.

Samples were diluted when necessary.

2.12.3. Calculation

1. Calculate peak area ratios of the targeted drugs to internal standard (prazepam) in the calibrator and unknown.
2. Calculate the standard ratio (SR) using the following equation:

$$\mathbf{SR=(IC/DC)\times CC}$$

3. Then calculate drug concentration (CT) in the *post-mortem* samples using:

$$\mathbf{CT=(DP/IP)\times SR}$$

In which:

IC=peak area of internal standard in calibrator;

DC=peak area of drug in calibrator;

CC=concentration of drug in calibrator;

DP=peak area of drug in *post-mortem* sample;

IP=peak area of internal standard in *post-mortem* sample;

Chapter 3

Results and Analysis of the Results

3.1. Results of experiments using different coatings and pHs

Members of each drug group were extracted from spiked plasma using the most frequently used fibre coatings (PA, CW/DVB and PDMS) and different pHs as described in section 2.3. The chromatographic retention time, pH and the fibre used for the extraction of targeted drugs are listed in Tables 3.1, 3.2 and 3.3.

Table 3.1. Retention time (min) of some benzodiazepines (1 mg/L) obtained using different fibres and pHs

Analyte	pH	Fibre		
		PA	CW/DVB	PDMS
Diazepam	7	34.44	NA	NA
Nitrazepam	4	NA	38.93	NA
	7	NR	NR	NR
	10	NA	NR	NA
Prazepam	7	37.35	NA	NA
Oxazepam	4	NR	NR	NR
	7	NR	NR	NR
	10	NR	NR	NR
Chlordiazepoxide	4	NA	35.59,37.34	NA
	7	35.5,37.5	35.59,37.35	NA
Flunitrazepam	4	NR	36.83	NA
	7	NR	36.8	NR
Medazepam	4	31.59	NA	NA
	7	31.63	NA	31.6
Ketazolam	7	34.44	NA	NR
Midazolam	4	NA	34.98	NA
	7	NA	34.9	NR
Clobazam	4	NA	34.33	NA
	7	NA	34.4	NR
Clonazepam	4	NR	NR	NR
	7	NR	NR	NR
	10	NR	NR	NR

NR = no response NA = not analysed

Table 3.2. Retention time (min) of some analgesics and local anaesthetics (1 mg/L) obtained using different fibres and pHs

Analyte	pH	Fibre		
		PA	CW/DVB	PDMS
Codeine	7	NR	NA	NR
	10	NR	33.61	NR
Cocaine	7	NR	31	NR
	10	NR	NR	NR
Heroin	7	NR	NR	NR
	10	NR	NR	NR
Meperidine	7	22.74	NA	NA
	10	22.7	NA	NA
Dihydrocodeine	7	NR	NA	NA
	10	NR	32.93	NA
Methadone	7	30.04	NA	NA
D-propoxyphen	7	30.77	NA	NA
Bupivacaine	7	32.2	NA	NA

NR = no response NA= not analysed

Table 3.3. Retention time (min) of some antidepressants (1 mg/L) obtained using different fibres and pHs

Analyte	pH	Fibre		
		PA	CW/DVB	PDMS
Amitriptyline	7	30.87	NA	30.87
Clomipramine	10	33.96	NA	NA
Mianserin	7	30.88	NA	NA
Dothiepin	7	33.65	NA	NA
Butriptyline	7	30.78	NA	NA
Imipramine	7	31.3	NA	NA
Trimipramine	7	31.08	NA	NA
Nortriptyline	7	NR	NR	NR
Protriptyline	7	31.67	NA	NA
Doxepin	7	31.1	NA	NA
Loxapine	10	34.62	NA	NA
Haloperidol	7	40.85	NA	NR
Trimeprazine	7	32.36	NA	32.4
Chlorpromazine	7	35.13	NA	NA
Trifluoperazine	7	36.6	NA	NA

NR = no response NA = nat analysed

3.2. Investigation of temperature effects

The samples prepared as described in section 2.3 at pH7, were heated at five different temperatures (room temperature, 35, 50, 60 and 75°C) and the fibre was exposed for 20 minutes. The adsorbed amounts of six targeted drugs are shown in Table 3.4.

The extraction rates were found to increase over a temperature range from room temperature up to approximately 75°C. However, it proved difficult to stabilise the temperature at 75°C, so 60°C was selected and used for the SPME extraction of the *post-mortem* samples for quantitation analysis.

TABLE 3.4. Effect of temperature on peak area responses of various drugs (polyacrylate fibre)

Temperature	Analyte					
	Methadone	Imipramine	Bupivacaine	Diazepam	Prazepam	Trifluoperazine
Room Temp.	260	746	48	622	1895	1981
35° C	234	626	198	1655	4229	3788
50° C	368	1527	643	3030	7592	6943
60° C	1138	4253	2002	4703	10351	7321
75° C	1660	6810	3627	5831	13187	9305

3.3. Determination of the extraction time

Based on the previous published studies the following extraction times were chosen.

To determine the effect of extraction time, a set of vials containing equal amounts of five targeted drugs were prepared as described in section 2.5. They were extracted for eight different times (5, 10, 15, 20, 25, 35, 45 and 60 minutes). Immediately after the vial was heated at 60°C, the fibre was passed through the septum and the extraction started. The masses absorbed by the PA fibre from the spiked plasma for the different times are shown in Figure 3.1.

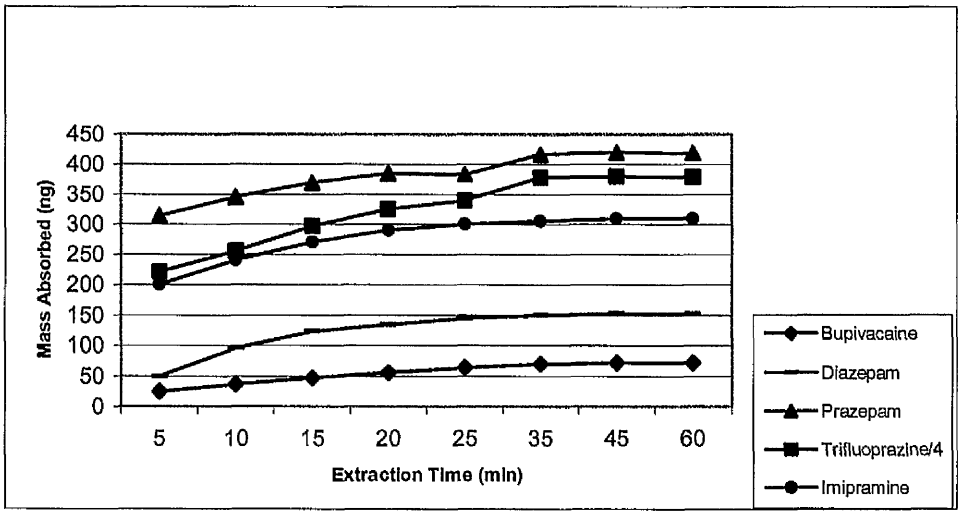


Figure 3.1. Time profiles of the mass absorbed by the polyacrylate fibre from spiked human plasma

The adsorbed amounts of bupivacaine, prazepam, diazepam, imipramine and trifluoprazine reached a maximum within about 35 minutes. In the following experiments 45 minutes extraction time was chosen for quantitative studies to optimise the extraction yield and enhance precision.

A four-minute desorption time at 300°C for PA fibre and 250°C for the other fibres was chosen because shorter times lead to incomplete desorption that was reflected in carryover to subsequent samples.

3.4. Investigation of pH effects

Members of each drug group were extracted from spiked plasma using different pHs (range 4-11) as described in section 2.6. The peak area responses of six targeted drugs are shown in Table 3.5 and Figure 3.2. Optimum pHs are shown in bold.

TABLE 3.5. Effect of pH on peak area responses of various drugs (polyacrylate fibre, 60°C)

pH	Analyte					
	Methadone	Imipramine	Bupivacaine	Diazepam	Prazepam	Trifluoprazine
pH4	--	35	–	711	2305	–
pH5	49	107	14	843	2532	543
pH6	62	97	11	922	2843	871
pH7	142	370	54	**990**	**2868**	2531
pH8	854	4170	350	664	1922	3824
pH9	1358	7307	**400**	801	2305	**6614**
pH10	2854	8172	358	702	1982	4674
pH11	**3387**	**9216**	293	656	1621	5847

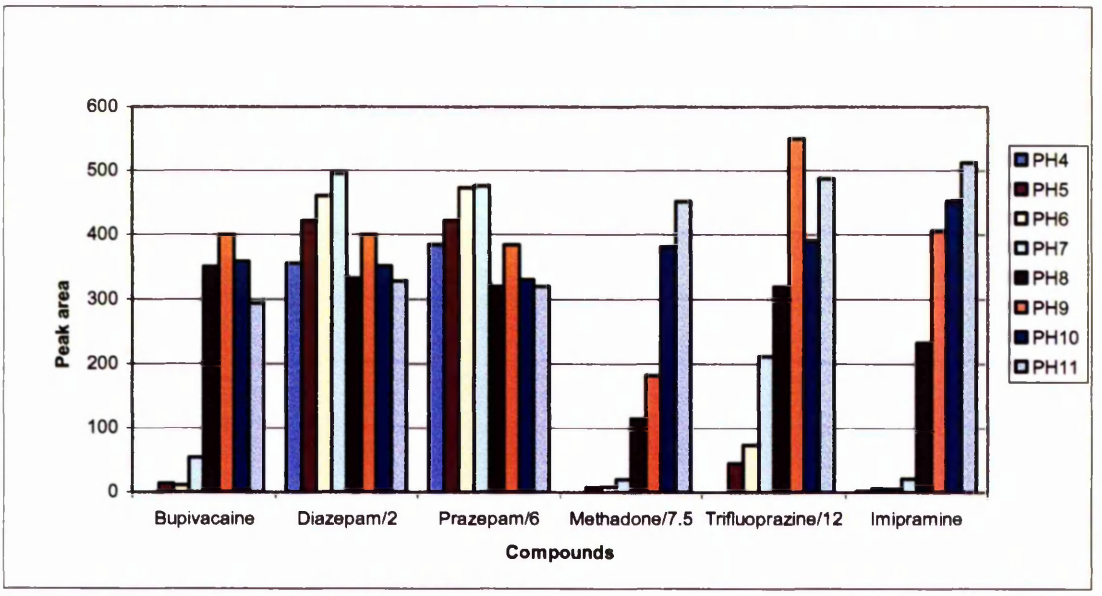


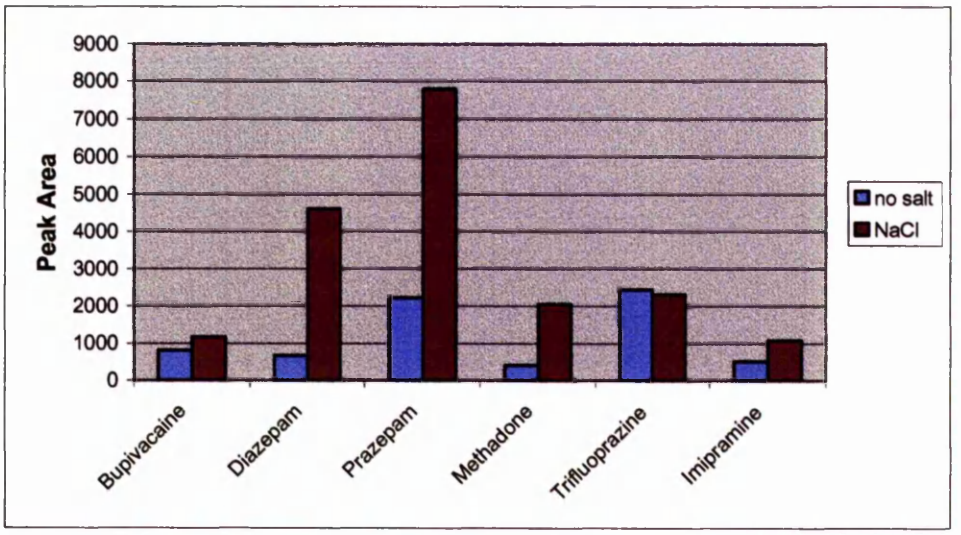
Figure 3.2. Effect of pH on the peak area responses of the drugs (polyacrylate fibre, 60°C)

The optimum pH for the extraction of diazepam and prazepam was pH7. Trifluoprazine and bupivacaine were optimally extracted at pH9 and methadone and imipramine at pH11. Prazepam showed similar extraction rate throughout the range of pH values used (Table 3.5). pH values of 7 and 10 for extraction of the samples were therefore selected.

3.5. Investigation of sodium chloride effects

Members of each drug group were extracted at pH 7 with and without sodium chloride using a polyacrylate fibre as described in section 2.7.

It was observed that addition of sodium chloride solution (5 mol/L) enhanced the extraction of methadone, bupivacaine, imipramine, diazepam and prazepam. The enhancement factors achieved by addition of sodium chloride ranged from 1.4 for bupivacaine to 7 fold for diazepam. The only drug less well extracted after the addition of sodium chloride was trifluoprazine. Therefore, a sodium chloride concentration of 5mol/L was used for all further extractions. The results are shown in Figure 3.3.



	Bupivacaine × (10)	Diazepam	Prazepam	Methadone × (5)	Trifluoprazine	Imipramine
no salt	811	656	2224	416	2428	517
NaCl	1160	4596	7791	2059	2300	1072

Figure 3.3. The effect of addition of sodium chloride on the extraction of the some of the drugs

3.6. Linearity

The linearity studies described in section 2.11.1 are presented here in graphical form for the drugs bupivacaine, diazepam, trifluoprazine, meperidine, clomipramine, amitriptyline and chlorpromazine.

Concentration (mg/l)	Peak Area Ratio (drug/IS)	Peak Area Ratio (drug/IS)	Peak Area Ratio (drug/IS)	Bupivacaine (Mean)	SD	SE
1	0.440	0.550	0.460	0.483	0.06	0.03
2	0.600	0.687	0.780	0.689	0.09	0.05
5	1.808	1.915	2.092	1.938	0.14	0.08
7	3.114	3.019	3.220	3.118	0.10	0.05
Concentration mg/l	Peak Area Ratio (drug/IS)	Peak Area Ratio (drug/IS)	Peak Area Ratio (drug/IS)	Diazepam (Mean)	SD	SE
1	0.524	0.549	0.530	0.534	0.01	0.008
2	0.905	0.962	0.948	0.938	0.03	0.017
5	1.350	1.390	1.420	1.387	0.04	0.02
7	1.980	1.890	1.930	1.933	0.05	0.03
SD= standard deviation SE= standard error IS=internal standard						

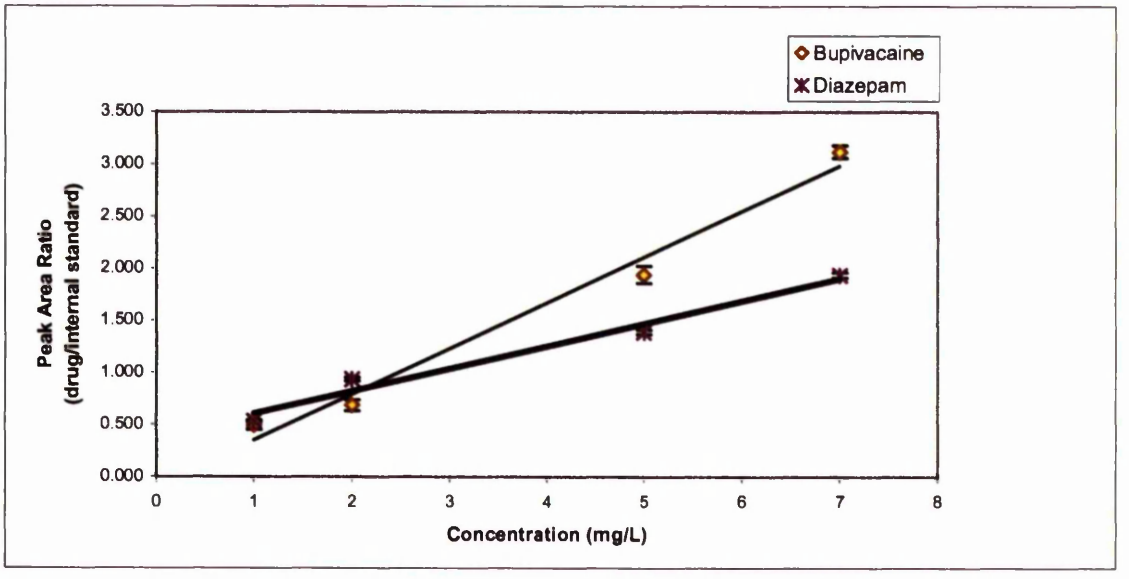


Figure 3.4. Linearity of SPME extraction and GC-MS responses for bupivacaine and diazepam over the range 1-7 mg/L

The equations for the curves were: $y = 0.44 x - 0.09$ for bupivacaine and $y = 0.215 x + 0.39$ for diazepam. There were significant effects of the concentration on the peak area ratio (with the confidence intervals greater than 99.9%). The detection limits (at a signal to noise ratio of 3) and quantitation limits were 0.27 and 0.69 mg/L for bupivacaine and 0.42 and 0.49 mg/L for diazepam. The between-day coefficients of variation (%CV) for bupivacaine were 12, 12 and 3 % (for the concentrations of 1, 2 and 7 mg/L) and for diazepam were 2, 3 and 3 % (for the concentrations of 1, 2 and 7 mg/L).

Concentration (mg/l)	Peak area ratio (drug/IS)	Peak area ratio (drug/IS)	Peak area ratio (drug/IS)	Meperidine (Mean)	St.Dev	SE
1	0.013	0.015	0.017	0.015	0.0020	0.0012
2	0.030	0.030	0.035	0.032	0.0029	0.0017
5	0.062	0.067	0.069	0.066	0.0036	0.0021
7	0.096	0.099	0.099	0.098	0.0017	0.0010

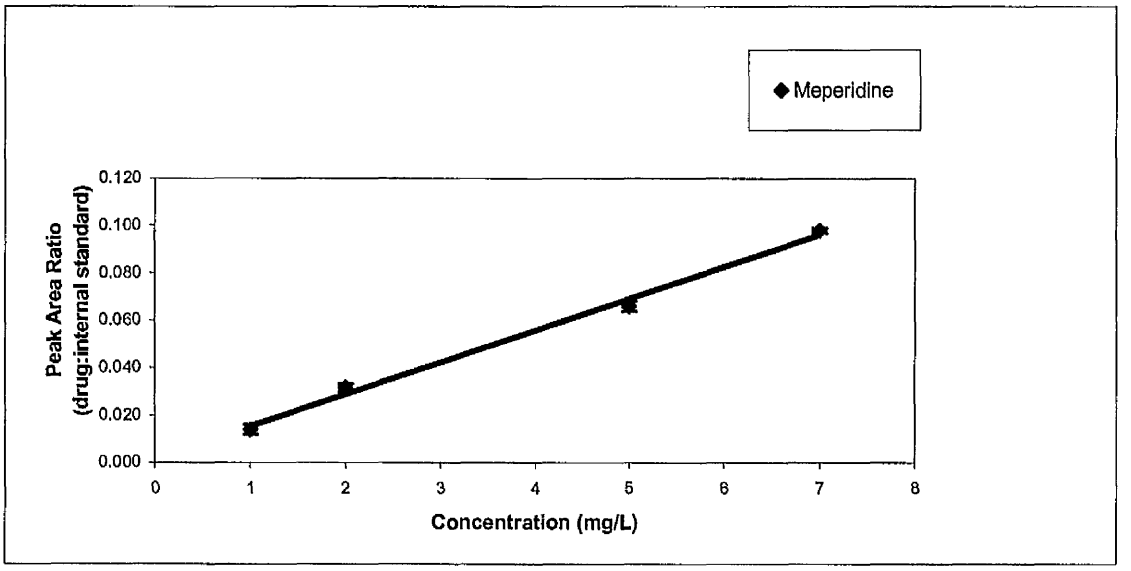


Figure 3.5. Linearity of SPME extraction and GC-MS response for meperidine over the range 1-7 mg/L

The equation for the curve was: $y = 0.0135\ x + 0.002$ for meperidine. There was a significant effect of the concentration on the peak area ratio (with a confidence interval greater than 99.9%). The detection limit (at a signal to noise ratio of 3) and quantitation limit were 0.008 and 0.022 mg/L. The between-day coefficients of variation (%CV) were 13, 1 and 2 % (for the concentrations of 1, 2 and 7 mg/L).

Concentration (mg/l)	Peak area ratio (drug/IS)	Peak area ratio (drug/IS)	Peak area ratio (drug/IS)	Clomipramine (Mean)	SD	SE
1	0.012	0.009	0.01	0.010	0.0016	0.0009
2	0.011	0.013	0.014	0.013	0.0015	0.0009
5	0.073	0.074	0.079	0.075	0.0032	0.0019
7	0.114	0.119	0.118	0.117	0.0026	0.0015
Concentration (mg/l)	Peak area ratio (drug/IS)	Peak area ratio (drug/IS)	Peak area ratio (drug/IS)	Amitriptyline (Mean)	SD	SE
1	0.025	0.026	0.029	0.027	0.0021	0.0012
2	0.038	0.040	0.041	0.040	0.0015	0.0009
5	0.095	0.095	0.099	0.096	0.0023	0.0013
7	0.143	0.150	0.151	0.148	0.0044	0.0025

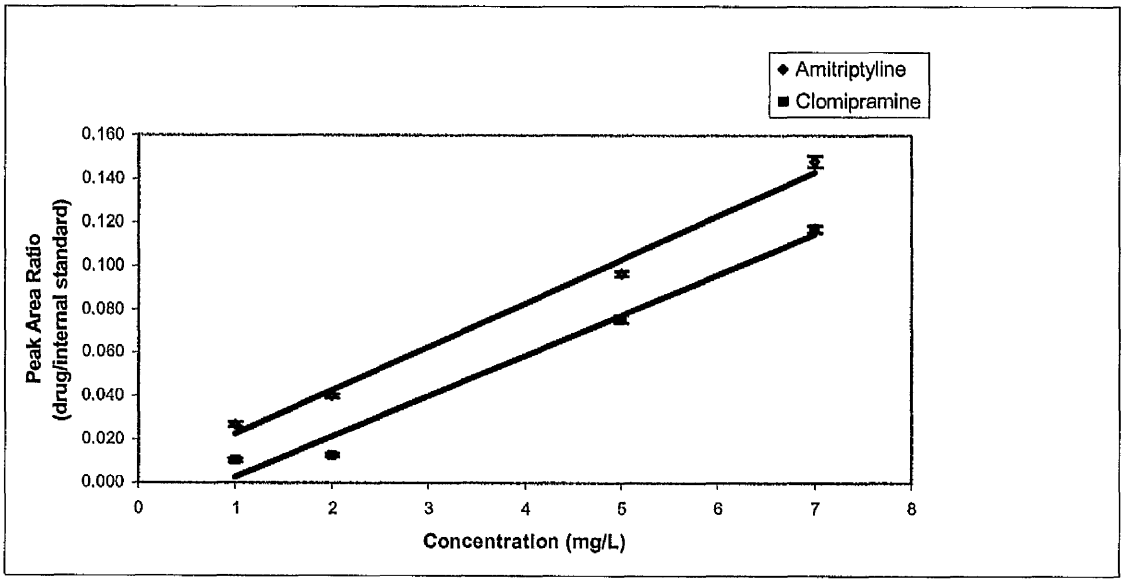


Figure 3.6. Linearity of SPME extraction and GC-MS responses for clomipramine and amitriptyline over the range of 1-7 mg/L

The equations for the curves were: $y = 0.02\ x - 0.0003$ for amitriptyline and $y = 0.0185\ x - 0.016$ for clomipramine. There were significant effects of the concentration on the peak area ratio (with confidence intervals greater than 99.9%). The detection limits (at a signal to noise ratio of 3) and quantitation limits were 0.0048 and 0.015 mg/L for amitriptyline and 0.02 and 0.03 mg/L for clomipramine. The between-day coefficients of variation (%CV) for amitriptyline were 8, 4 and 3 % (for the concentrations of 1, 2 and 7 mg/L) and for clomipramine were 16, 11 and 3 % (for the concentrations of 1, 2 and 7 mg/L).

Concentration (mg/l)	Peak area ratio (drug/IS)	Peak area ratio (drug/IS)	Peak area ratio (drug/IS)	Chlorpromazine (Mean)	SD	SE
1	0.003	0.003	0.004	0.003	0.0005	0.0003
2	0.005	0.006	0.007	0.007	0.001	0.0006
5	0.020	0.021	0.023	0.022	0.0015	0.0009
7	0.031	0.030	0.035	0.033	0.0026	0.0015

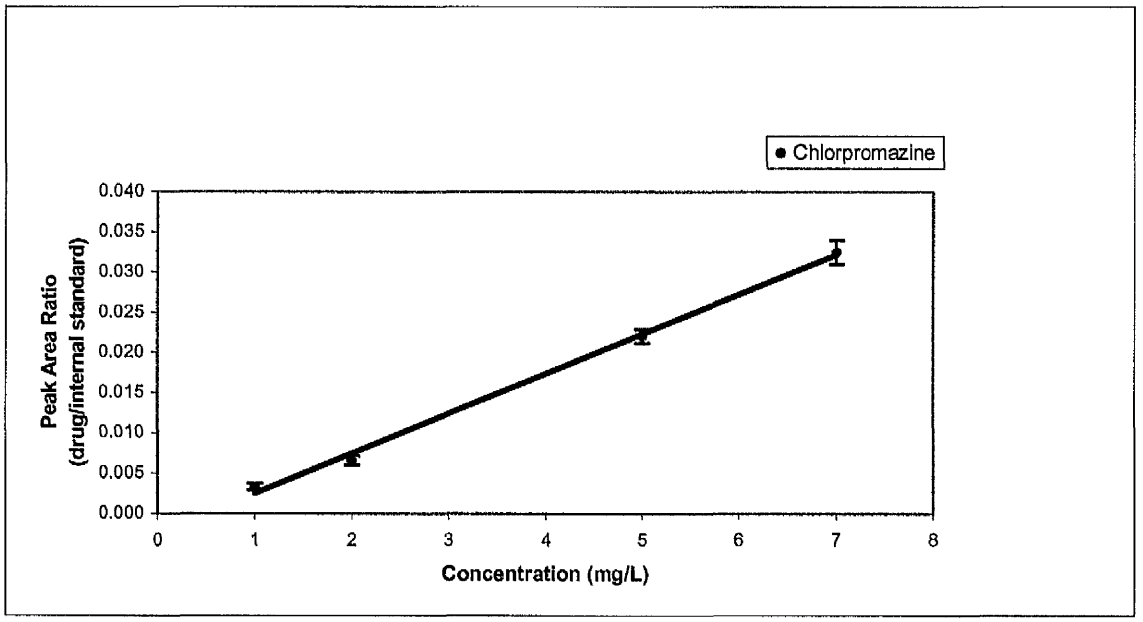


Figure 3.7. Linearity of SPME extraction and GC-MS response for chlorpromazine over the range of 1-7 mg/L

The equation for the curve was $y = 0.0046 x - 0.003$ for chlorpromazine. There was a significant effect of concentration on the peak area ratio (with a confidence interval greater than 99.9%). The detection limit (at a signal to noise ratio of 3) and quantitation limit were 0.003 and 0.03 mg/L. The between-day coefficients of variation (%CV) were 16, 7 and 8 % (for the concentrations of 1, 2 and 7 mg/L).

3.7. Application of SPME-GC-MS to *post-mortem* samples

The developed SPME-GC-MS method was applied to the detection and quantification of the targeted drugs in sixteen *post-mortem* cases (urine and blood

samples). They had previously been analysed in the toxicology laboratory of Guy's Hospital and Manchester Royal Infirmary (MRI) using Toxi A, Toxi B (thin layer chromatography methods), EMIT (enzyme multiplied immunoassay technique), Remedi (automated HPLC) and HPLC.

Case 1) A 30-year-old man was reportedly found dead. He had made numerous suicidal attempts and he had access to dothiepin and alcohol at the last attempt. His blood sample was taken from peripheral blood vessels; the urine and blood samples were prepared as explained in sections 2.8 and 2.9.

Firstly, the samples were analysed using full scan mode to identify the compounds present. The concentration of dothiepin in blood was out of the linear range and so, for the quantitation of dothiepin, the blood sample was diluted.

The chromatogram obtained from the blood sample is shown in Figure 3.8. Figures 3.9, 3.10 and 3.11 show the EI-mass spectra of dothiepin, medazepam and prazepam (internal standard) extracted from the blood sample at pH10 using a polyacrylate fibre.

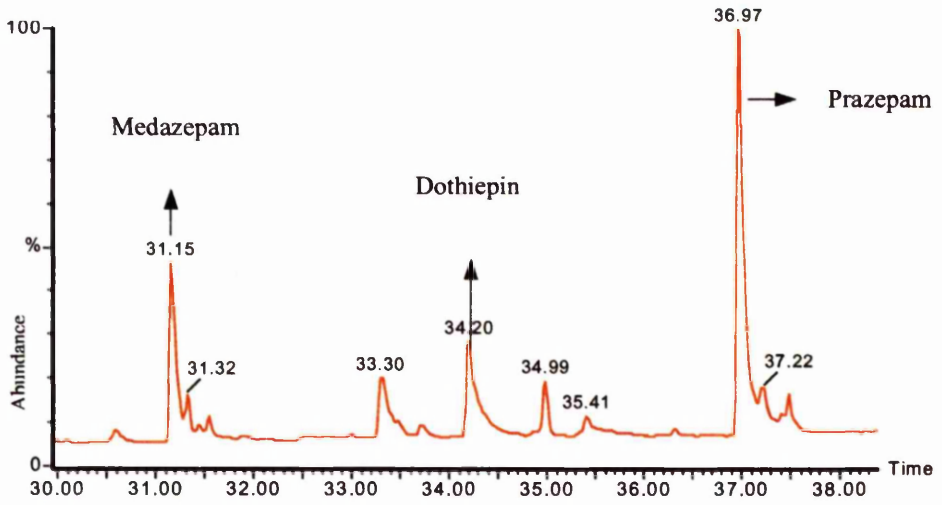


Figure 3.8. SPME-GC total ion chromatogram of the blood sample (case 1) spiked with prazepam (0.1 mg/L; I.S.)

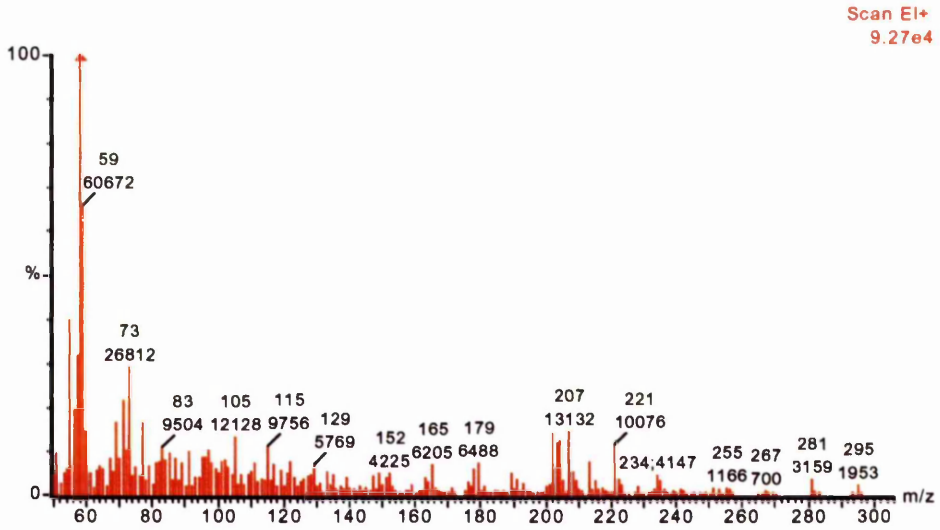
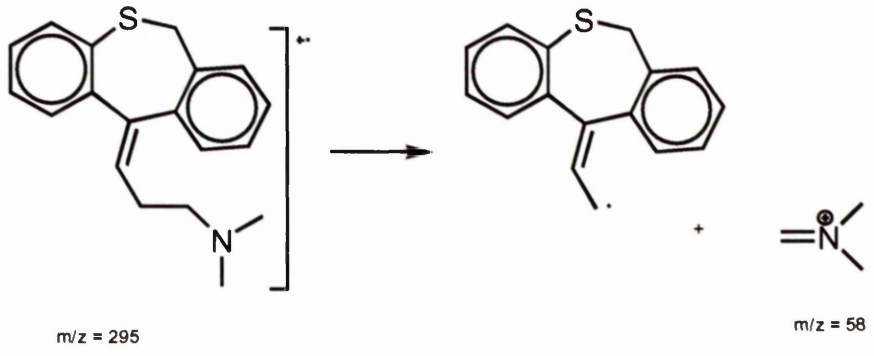


Figure3.9. EI-mass spectrum of dothiepin extracted by SPME from case 1 blood at pH10

Identification of dothiepin depends upon a combination of chromatographic and mass spectrometric data. The use of chromatographic retention time is necessary because the dothiepin molecular ion undergoes a preferred and facile fragmentation whereby the basic side chain is extruded as the principal observed ion upon electron impact.



The co-generic radical is resonance stabilised, but not observed in positive mode detection. The combination of retention time, signal at m/z= 58 and absence of other signals is taken as positive identification for dothiepin.

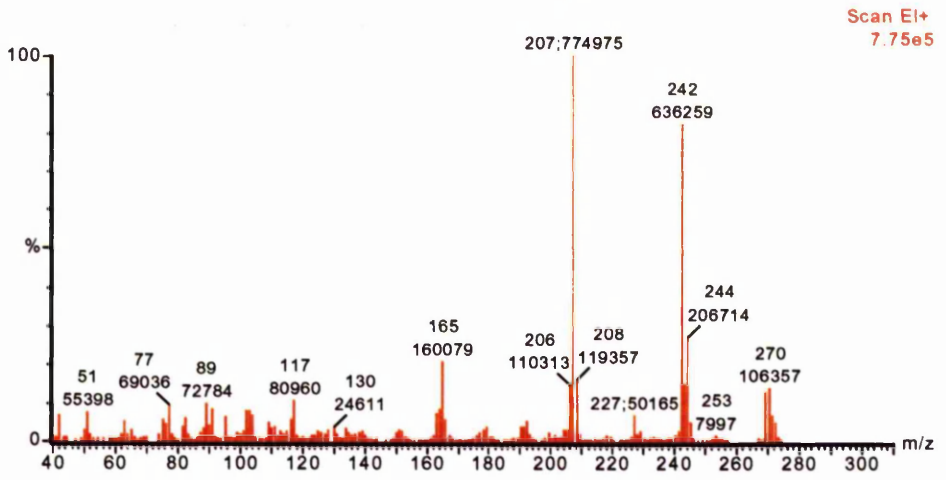
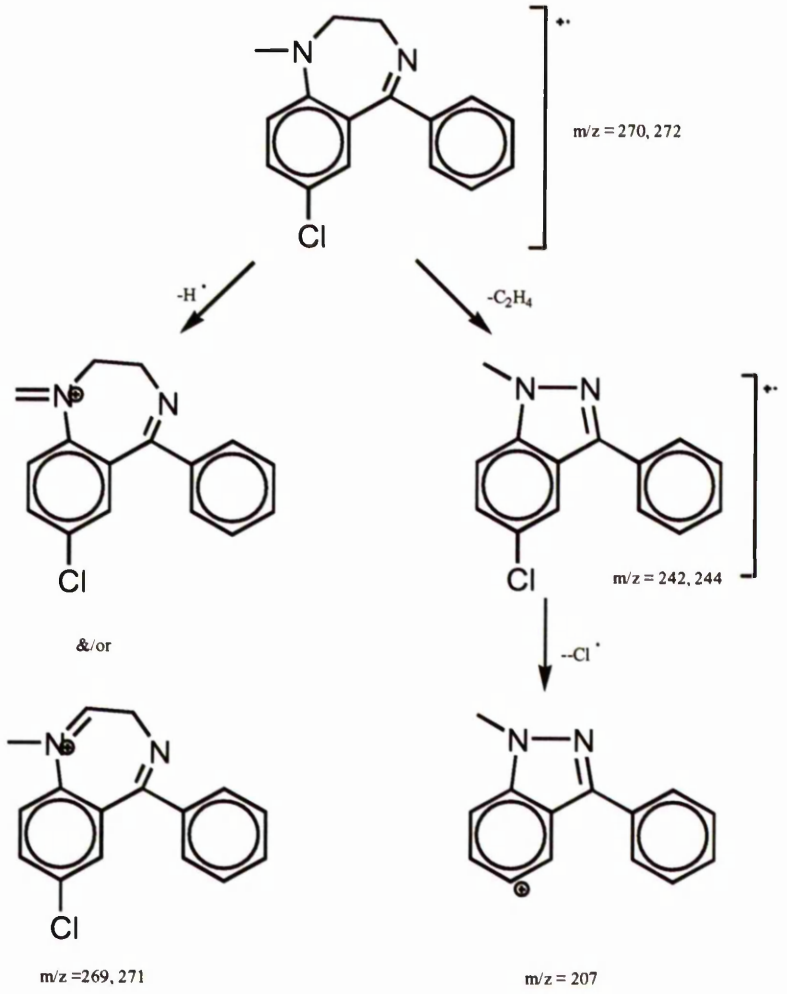


Figure 3.10. EI-mass spectrum of medazepam extracted by SPME from case 1 blood at pH10

The spectrum is a good match with that from an authentic sample of medazepam. The molecular ion appears at m/z 270 and 272 corresponding to a monochloro compound showing satellites due to ^{35}Cl and ^{37}Cl isotopes. The signals at m/z 242 and 244 also show the monochloro isotopic relationship.

The pattern of signals in the molecular ion region is complicated due to the presence of signals corresponding to [M-1] ions. Loss of a hydrogen radical generates a stabilised immonium ion as shown in the scheme below. The signals at m/z 242 and 244 arise by loss of a fragment likely to be C_2H_4: loss of such fragments to generate five-membered heterocyclic structures in a rearrangement process is common in benzodiazepines. The signal at m/z 207 does not show the typical chlorine isotope pattern hence must arise by extrusion of $Cl^{\bullet}$ from m/z 242/244.



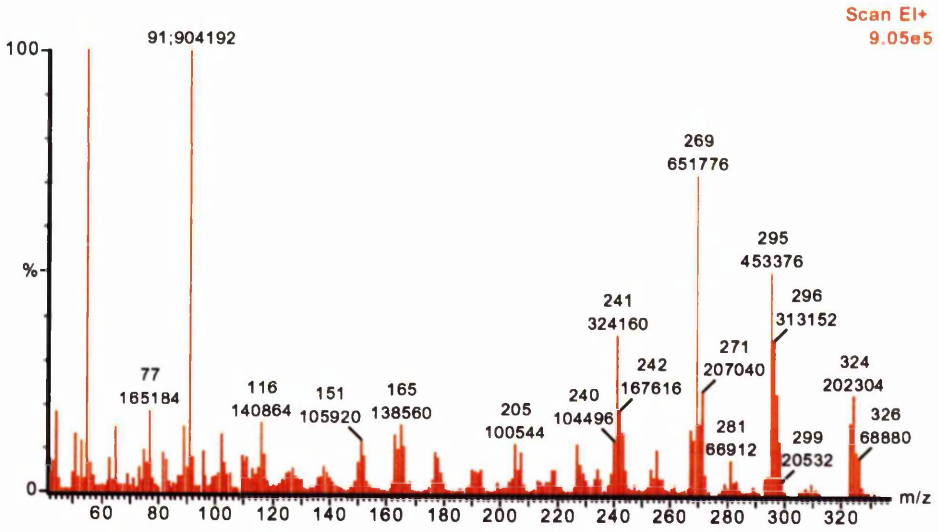
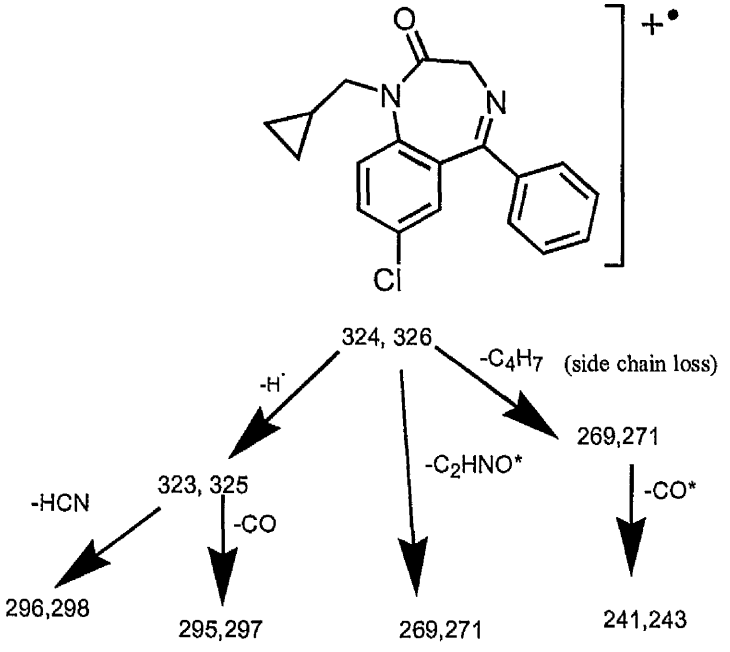


Figure 3.11. EI-mass spectrum of prazepam (IS) extracted by SPME from case 1 blood at pH10

The molecular ion of prazepam undergoes similar extrusion to medazepam with loss of H· and ring contraction of the heterocyclic system. Extrusion of HCN is a characteristic fragmentation of heterocyclics and seen here.



*= ring contraction

Dothiepin and medazepam were detected in the blood at concentrations of 2200 µg/L and 6.7 µg/L, respectively, using SPME-GC-MS (SIM). Dothiepin was present in the blood at a concentration of greater than 1000 µg/L reported with serious toxicity[186]. The major effects of ingestion of an excessive amount of dothiepin are cardiac arrhythmias, central depression and coma[136]. These findings are probably consistent with a diagnosis of death due to the toxic effects of an excessive amount of dothiepin.

Case 2) A 36-year-old man was reportedly found dead at home. His usual medication was fluphenazine decanoate. His blood sample was taken from peripheral blood vessels and a urine sample had been taken; the urine and blood samples were prepared as explained in the sections 2.8 and 2.9. The blood sample was analysed to identify the compounds present in the blood and then the drugs were

quantified using SPME-GC-MS (SIM). Dextropropoxyphene, amitriptyline, dothiepin, thioridazine and mesoridazine were detected in the blood.

Total ion chromatogram, mass chromatograms of selected ions of 208 and 202 (for propoxyphene and amitriptyline, respectively) and EI-mass spectra of D-propoxyphene, amitriptyline, mesoridazine and thioridazine are shown in the following figures (3.12, 3.13, 3.14, 3.15 and 3.16). The selected ions were 58, 202 and 215 for amitriptyline, 58, 202 and 221 for dothiepin, 98, 370 and 126 for thioridazine, 58, 208 and 193 for dextropropoxyphene and 91, 269 and 295 for prazepam.

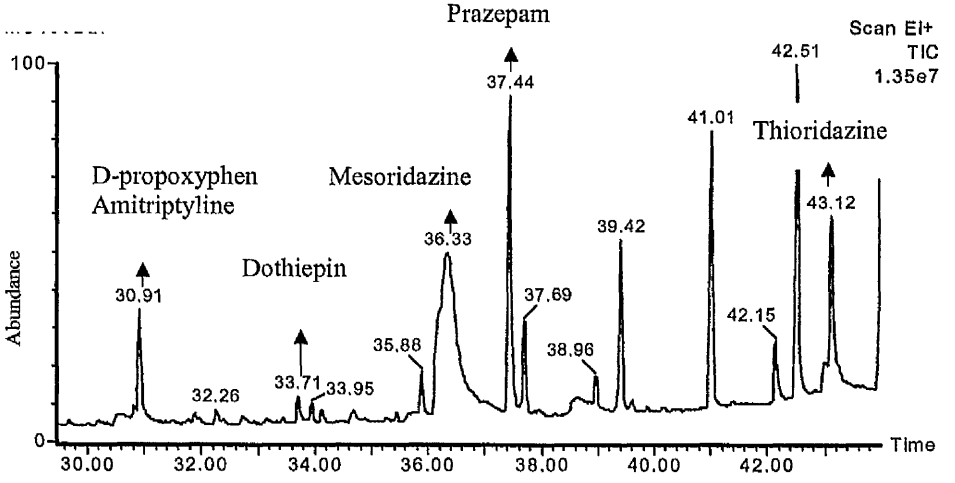


Figure 3.12. SPME-GC total ion chromatogram of the blood sample (case 2) spiked with prazepam (0.1 mg/L; I.S.)

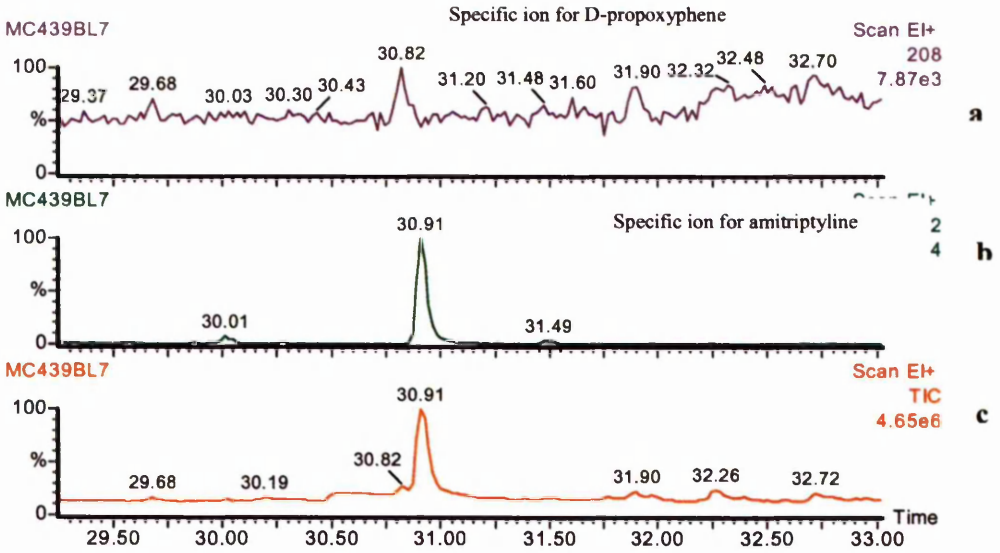


Figure 3.13. Total ion chromatogram of blood (case 2) after extraction by SPME procedure (c), selected ions for D-propoxyphene and amitriptyline (a and b)

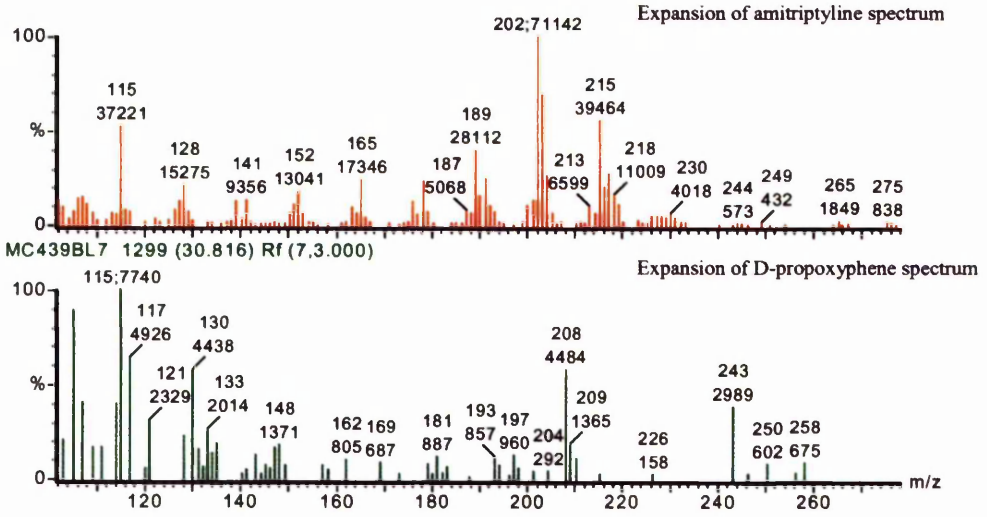


Figure 3.14. EI-mass spectra of amitriptyline and D-propoxyphene extracted by SPME from case 2 blood at pH7

The EI-MS for dothiepin and prazepam showed good matches to the spectra of authentic samples. The fragmentation pathways for these compounds are described in case 1.

D-propoxyphene and amitriptyline chromatograph very close to each other, but the deconvolution afforded by the data system incorporated in the GC-MS system allows each to be separately identified.

Identification of amitriptyline and propoxyphene depend on a combination of chromatographic (retention time) and mass spectrometric data. Amitriptyline and propoxyphene molecular ions undergo facile fragmentations whereby the basic side chain is extruded as the intense ion observed at m/z=58 upon electron impact. The combination of retention time, signals at m/z=58 and 202 for amitriptyline and m/z= 58 and 208 for propoxyphene are taken as positive identifications for amitriptyline and propoxyphene.

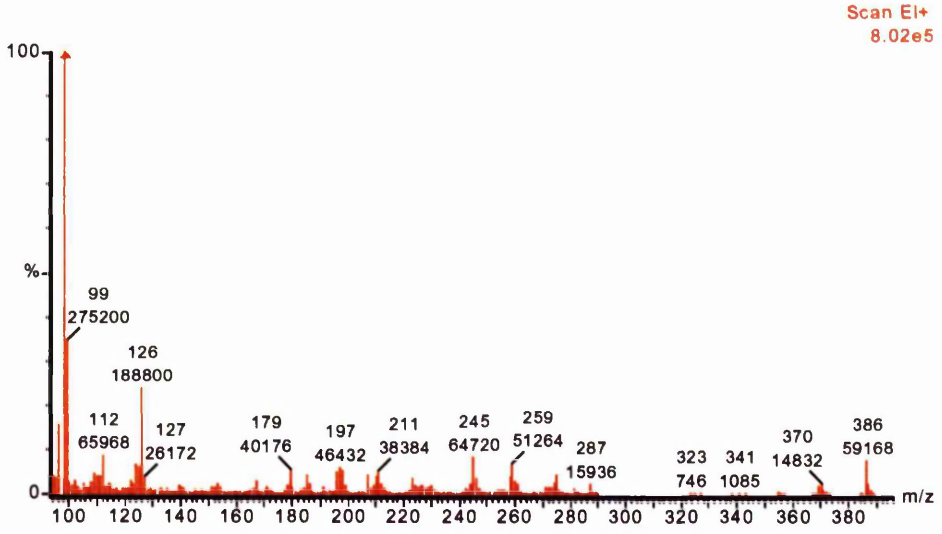


Figure 3.15. EI-mass spectrum of mesoridazine extracted by SPME from case 2 blood at pH7

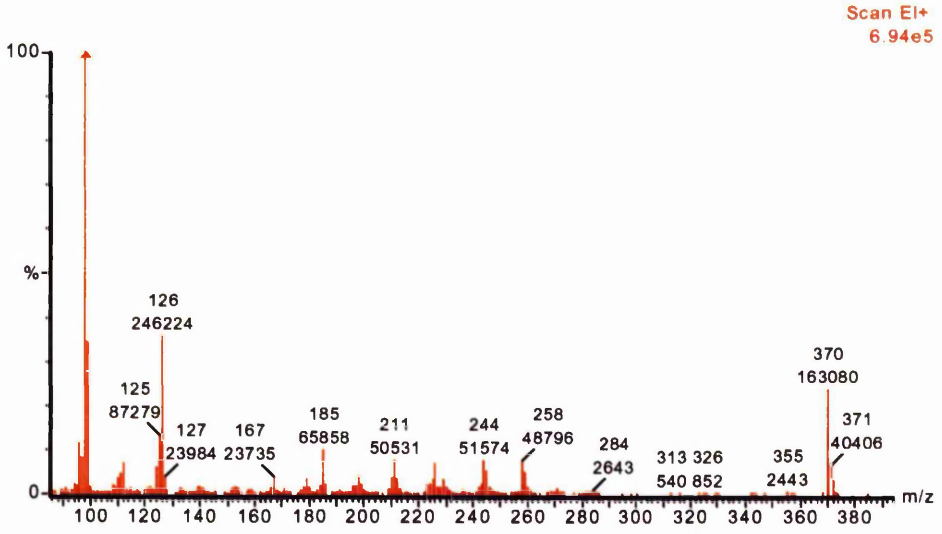
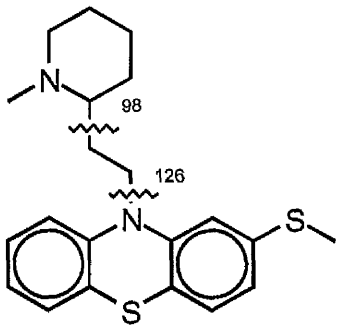


Figure 3.16. EI-mass spectrum of thioridazine extracted by SPME from case 2 blood at pH7

Thioridazine was identified by its mass spectrum that showed a good match to the NIST library. Fragmentation occurs in the basic side chain to give intense ions at m/z= 98 and 126 as shown on the following structure.



A signal at m/z= 370 corresponded to the molecular ion. This conclusion was confirmed by the identification of the metabolite mesoridazine that gives a very similar fragmentation pattern and spectrum with a molecular ion at m/z= 386.

Dextropropoxyphene-37 μg/L, amitriptyline-150μg/L, dothiepin-900μg/L and thioridazine-1000μg/L, were present in the blood. Only dothiepin was near to the concentration of 1000 μg/L reported to have toxicity[186].

Case 3) A 72-year-old man was found dead at home, with an empty co-proxamol container. His blood and urine samples were taken. The samples were prepared and analysed using full scan detection to identify the compounds present. Dextropropoxyphene was found by analysing the sample at pH7 using a polyacrylate fibre. Analysis of the blood sample at pH10 identified diphenhydramine and norpropoxyphene as shown in Figure 3.17. EI-mass spectra of norpropoxyphene and diphenhydramine are shown in Figures 3.18 and 3.19.

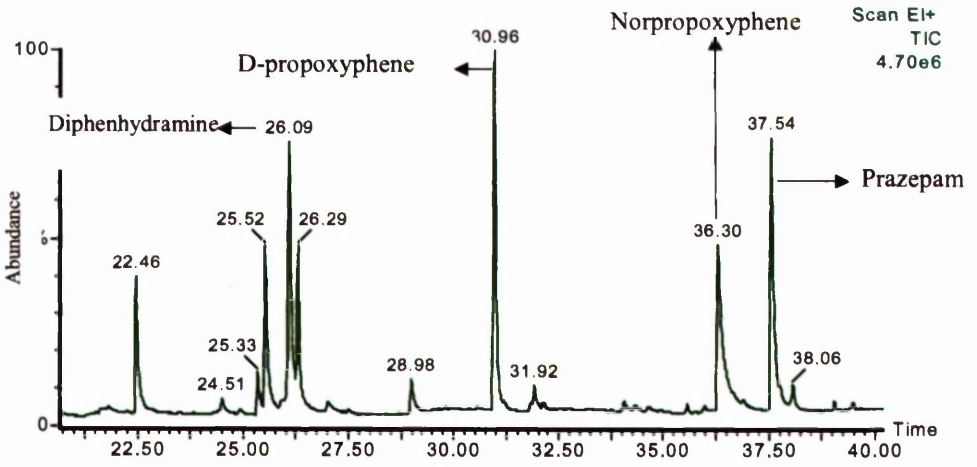


Figure 3.17. SPME-GC total ion chromatogram of the blood sample (case 3) spiked with prazepam (0.1mg/L; I.S.)

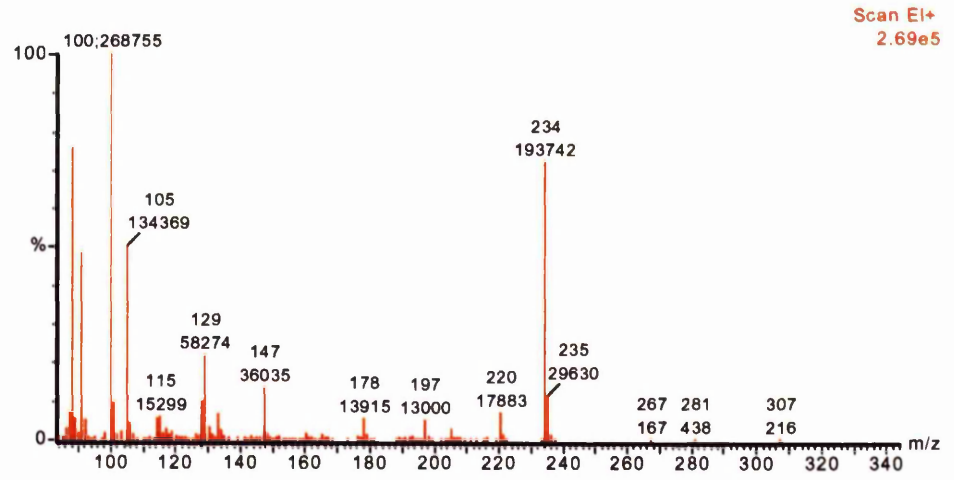


Figure 3.18. EI-mass spectrum of norpropoxyphene extracted by SPME from case 3 blood at pH10

The EI-mass spectra of D-propoxyphene and prazepam were identical to those from authentic samples and other case studies described herein. The identifications were confirmed by comparable chromatographic retention times. Norpropoxyphene has a molecular mass of 339; the molecular ion is too unstable to be detected. Instead, a facile decomposition occurs to generate fragments of 105 and 234.

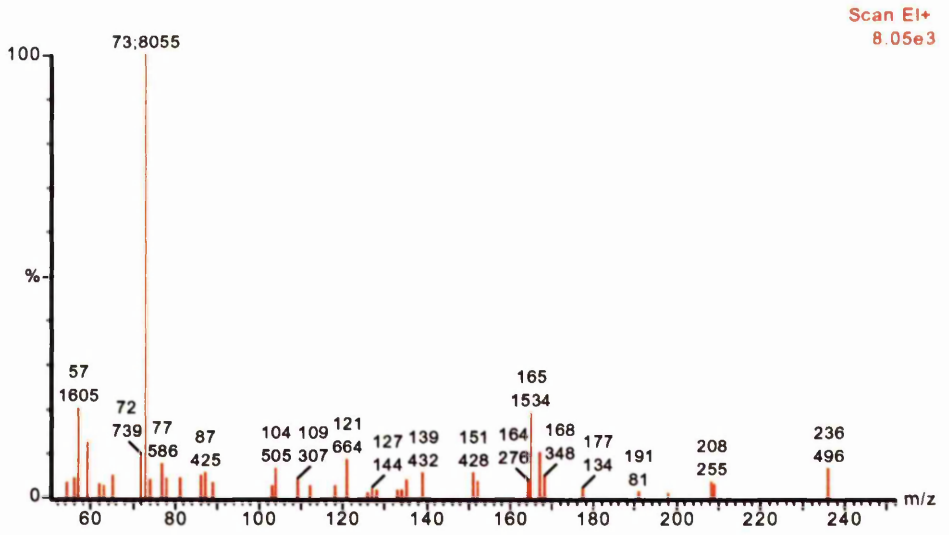
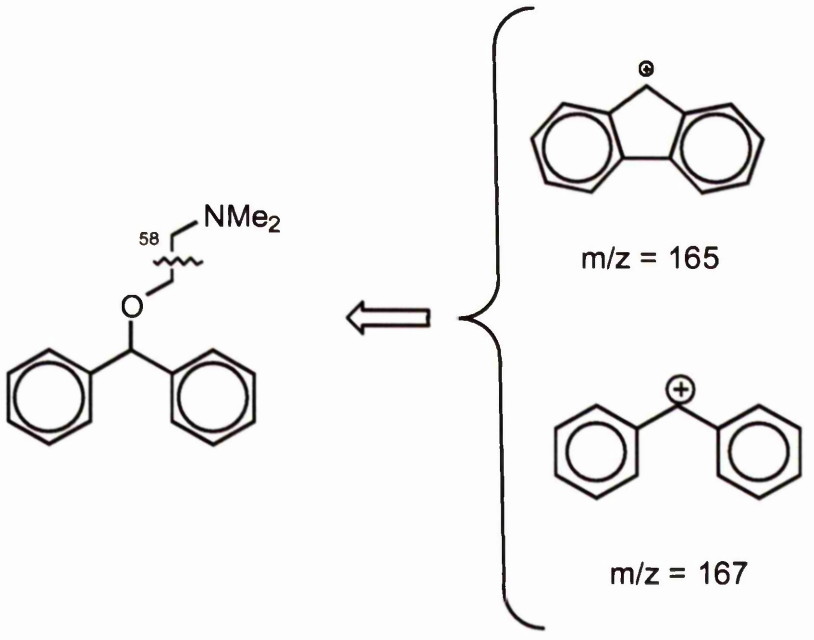


Figure 3.19. EI-mass spectrum of diphenhydramine extracted by SPME from case 3 blood at pH10

Diphenhydramine has the structure:



This molecule gives an intense m/z=58 ion in the EIMS because of the facile extrusion exhibited by dimethylamino moieties in basic drugs. Also observed are fragments of m/z=165 and 167 assigned from the diphenylmethenium moiety.

Dextropropoxyphene was present at a concentration more than 1 mg/L, which is the concentration with serious toxicity[186]. The dextropropoxyphene concentration in the blood was 9 mg/L and so it is consistent with a diagnosis of death due to the toxic effects of a hyperdose.

Case 4) A 42-year-old woman was admitted to hospital with a suspicion of pethidine overdose. A peripheral blood sample was taken for analysis. The blood sample was prepared as before. The analysis of the sample was performed using full scan mode and the drugs were quantified using SPME-GC-MS (SIM). Dothiepin and pethidine were found in the sample. Figure 3.20 shows the total ion chromatogram of the sample extracted at pH10.

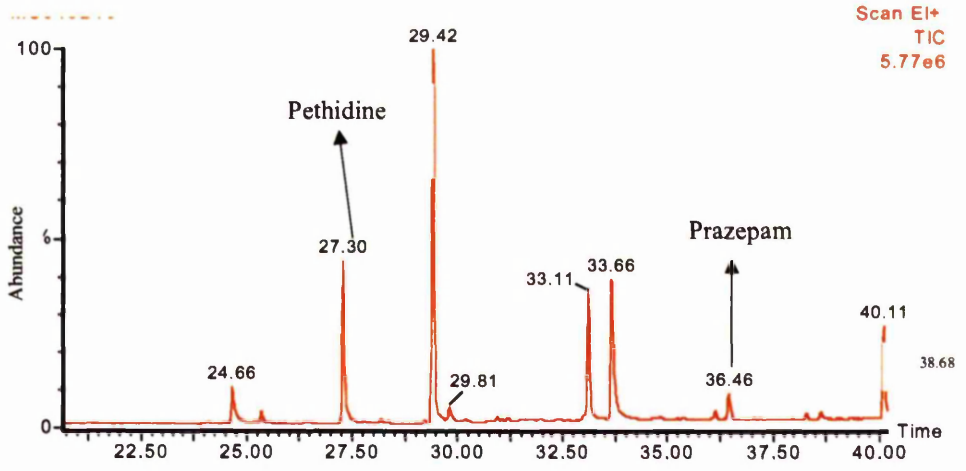


Figure 3.20. SPME-GC total ion chromatogram of the blood sample (case 4) spiked with prazepam (0.1mg/L; I.S.)

Pethidine was detected by analysing the blood sample at pH10 using a polyacrylate fibre. Dothiepin was detected by polyacrylate fibre at pH7. Figure 3.21 represents the chromatogram of the extracted sample at pH7. EI-mass spectrum of pethidine extracted from blood at pH10 is shown in Figure 3.22.

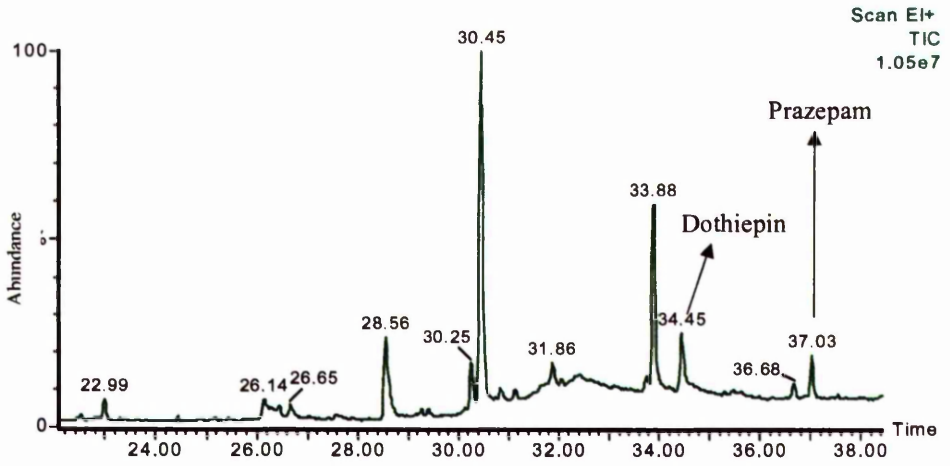


Figure 3. 21. SPME-GC total ion chromatogram of the blood sample (case 4) spiked with prazepam (0.1mg/L; I.S.)

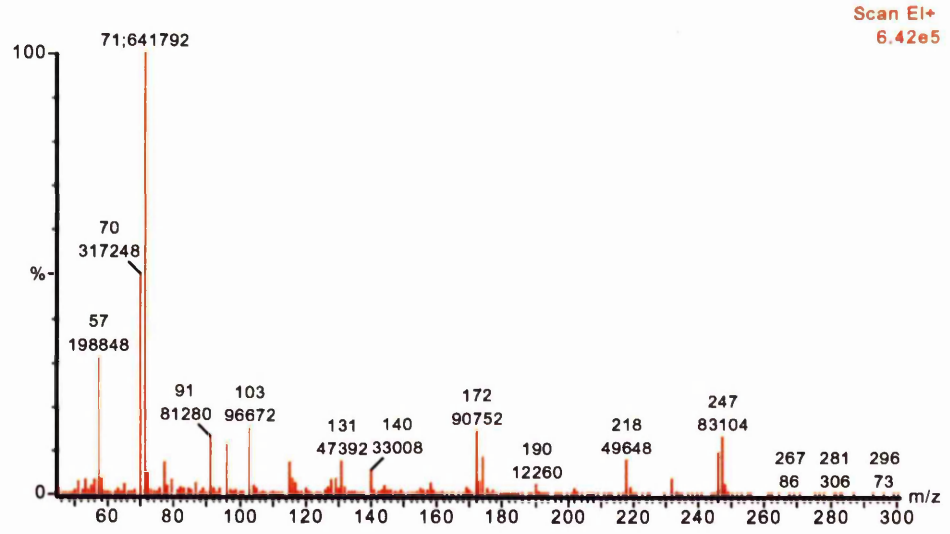
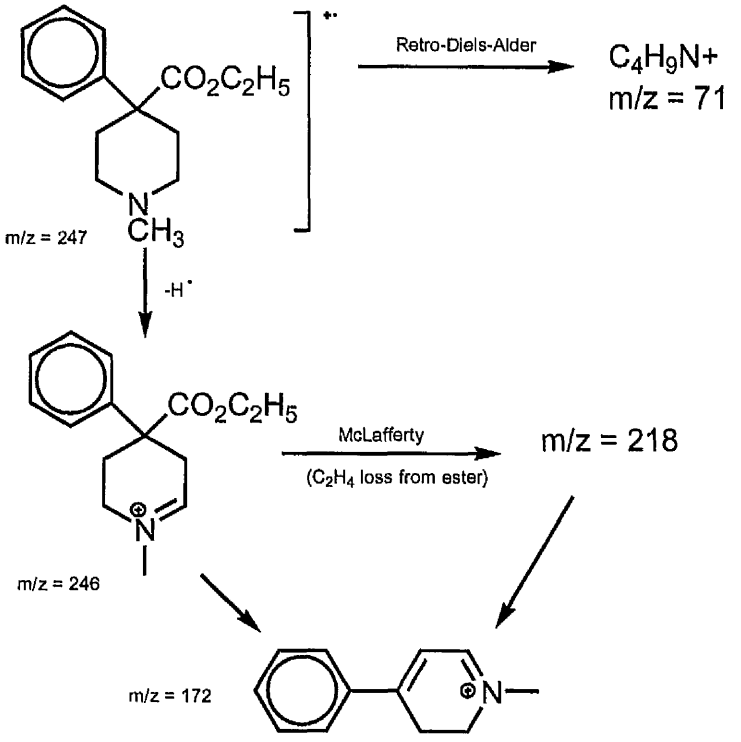


Figure 3.22. EI-mass spectrum of pethidine extracted by SPME from case 4 blood at pH10

The spectrum of dothiepin is a good match with laboratory data and there is correspondence with the retention time. Dothiepin was identified by its mass spectrum and retention time. The spectrum of pethidine is a good match with that from an authentic sample of pethidine and to the NIST library. The molecular ion appears at m/z=247. Loss of a hydrogen radical generates an ion as shown in the scheme following. It then undergoes a type of McLafferty fragmentation that generates an ion with m/z= 218[111].



Dothiepin, 320 µg/L, and pethidine, 4.3 mg/L were present in the blood. The excessive amount of pethidine in the blood was probably the cause of death.

Case 5) A 55-year-old man was found decomposed. He had a history of suicide threats. His urine sample was taken and prepared as explained in section 2.9, and analysed using SPME-GC-MS (TIC). Codeine, dothiepin and paracetamol were found to be present in the urine. Total ion chromatograms obtained from the urine sample at pH10 and pH7 are shown in Figures 3.23 and 3.24, respectively. EI-mass spectra of codeine and paracetamol from the urine sample, after extraction at pH10, are shown in Figures 3.25 and 3.26.

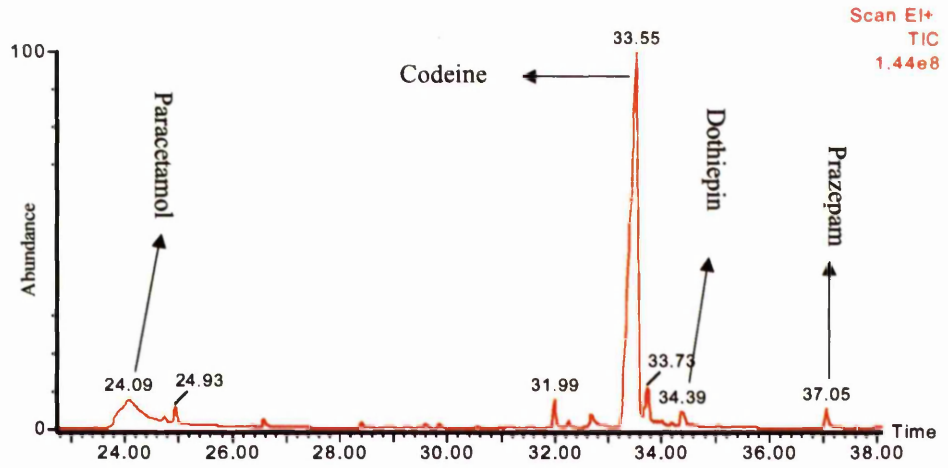


Figure 3.23. SPME-GC total ion chromatogram of the urine sample (case 5) spiked with prazepam (0.1mg/L; I.S.) at pH10

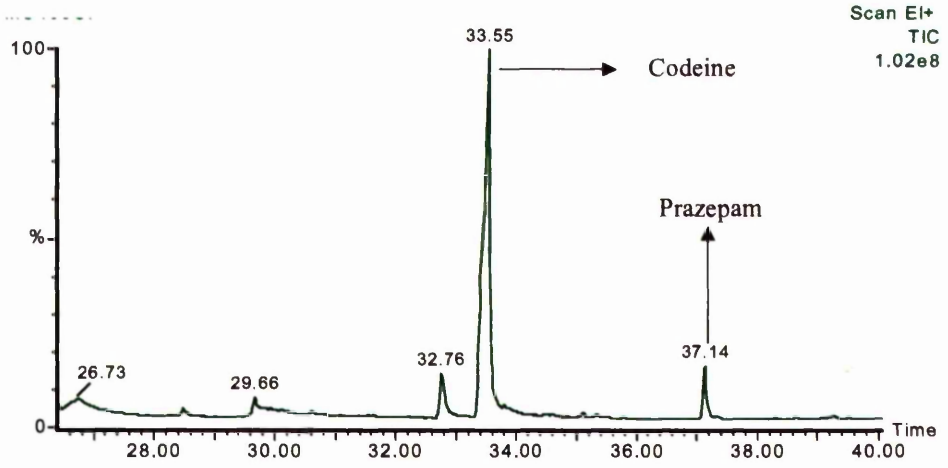


Figure 3.24. SPME-GC total ion chromatogram of the urine sample (case 5) spiked with prazepam (0.1mg/L; I.S.) at pH7

The EI-MS for dothiepin showed a good match to the spectrum of the authentic sample. The fragmentation pathway for the compound is as described in case 1.

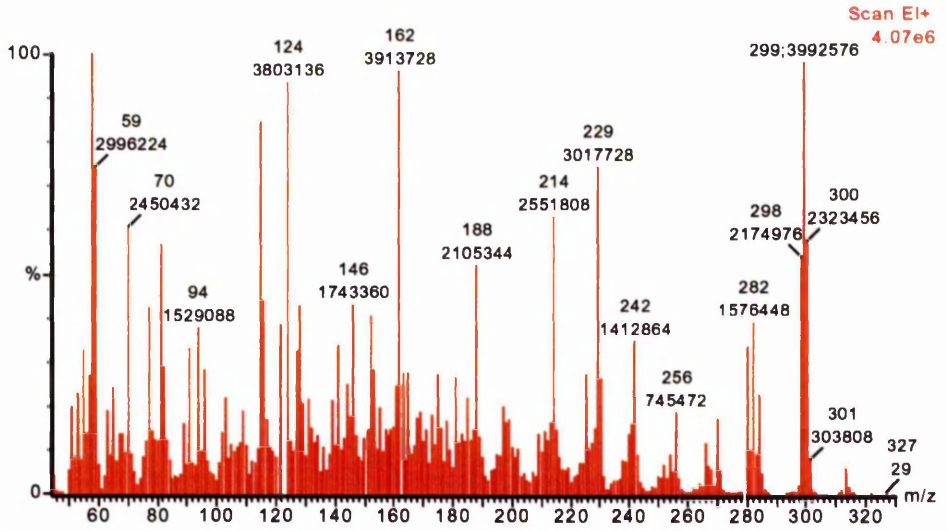


Figure 3.25. EI-mass spectrum of codeine extracted by SPME from case 5 urine at pH10

Codeine undergoes a series of complex fragmentations reflecting the complexity of the molecular ion. The mass spectrum obtained matches the standard spectrum in the NIST library.

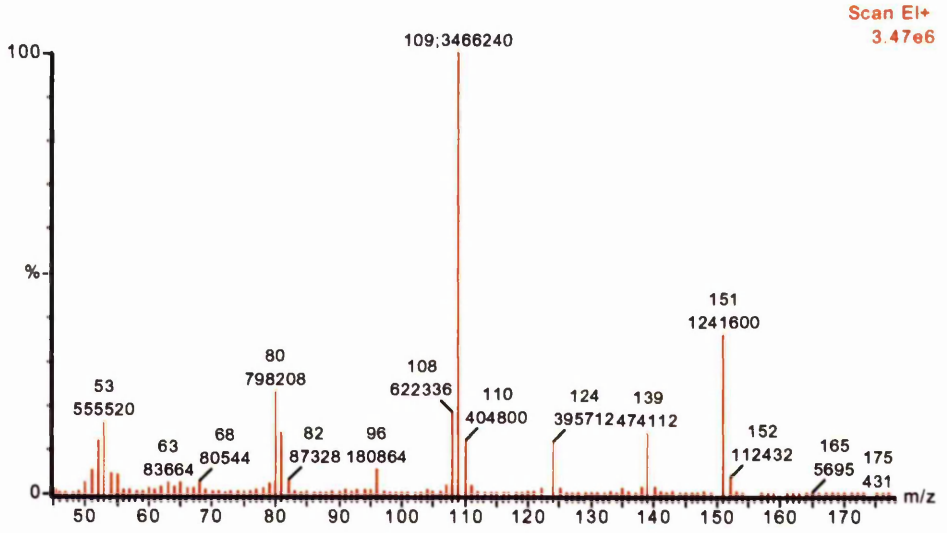
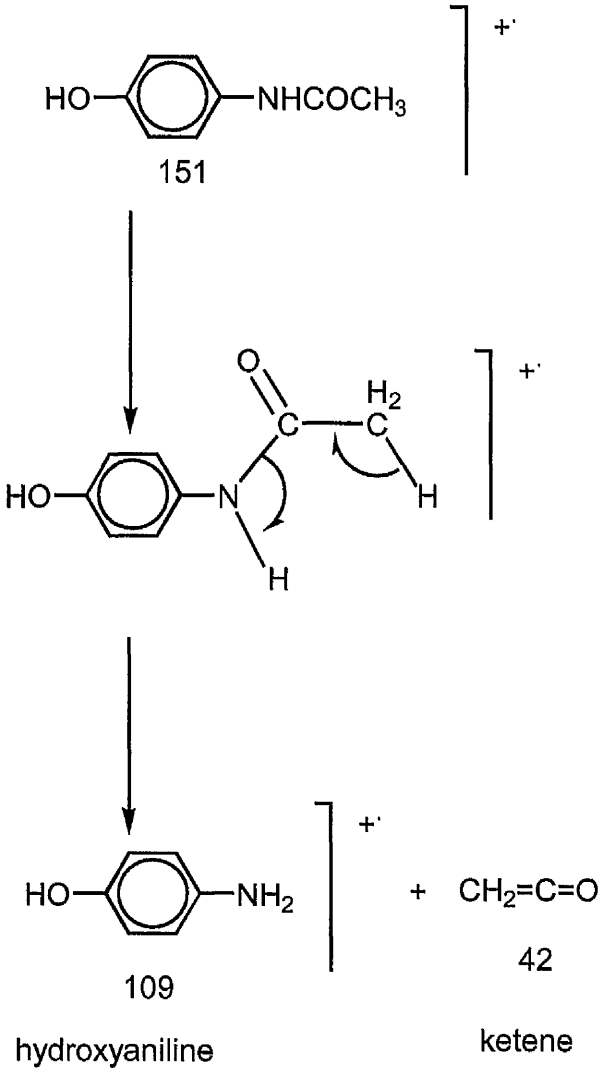


Figure 3.26. EI-mass spectrum of paracetamol extracted by SPME from case 5 urine at pH10

The extrusion of ketene (CH_2=C=O) from acetyl derivatives of aromatic amines (i.e. paracetamol) is commonly observed and thought to proceed via a type of McLafferty rearrangement/cyclic extrusion process as follows[111]:



Case 6) A 39-year-old man was reportedly found deceased at home with empty medication containers. He had made previous attempts on his life and had suffered from psychiatric disorders. He had access to procyclidine, disulfiram, diazepam, trazodone and depixol®(flupenthixol). A blood sample has been taken, prepared and analysed using full scan mode to identify the compounds present. Amitriptyline, diazepam and procyclidine were found in the urine and blood. They were detected at pH7 using a polyacrylate fibre.

Total ion chromatograms obtained from urine and blood samples are shown in Figures 3.27 and 3.28, respectively. EI-mass spectra of diazepam and procyclidine, extracted using a polyacrylate fibre at pH7, are shown in Figures 3.29 and 3.30.

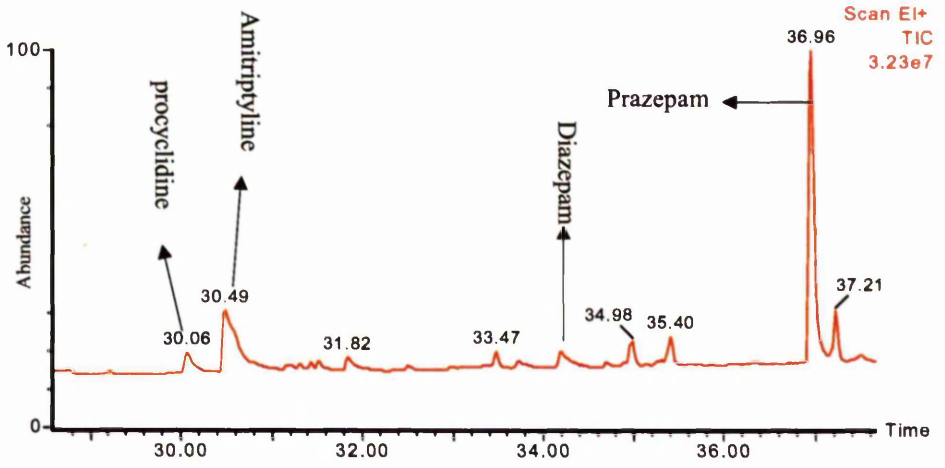


Figure 3.27. SPME-GC total ion chromatogram of the urine sample (case 6) spiked with prazepam (0.1mg/L; I.S.)

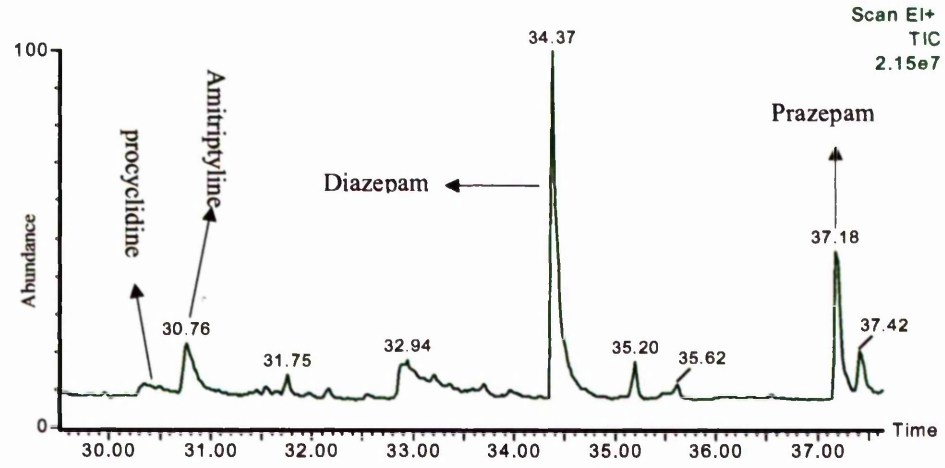


Figure 3.28. SPME-GC total ion chromatogram of the blood sample (case 6) spiked with prazepam (0.1mg/L; I.S.)

The EI-MS for amitriptyline showed a good match to the spectrum of the authentic sample. The fragmentation pathway for the compound is as described in case 2.

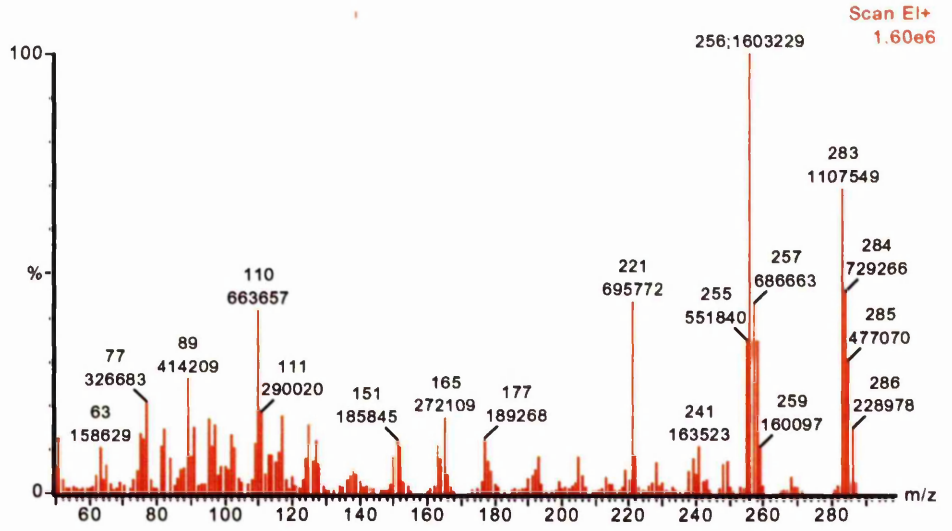
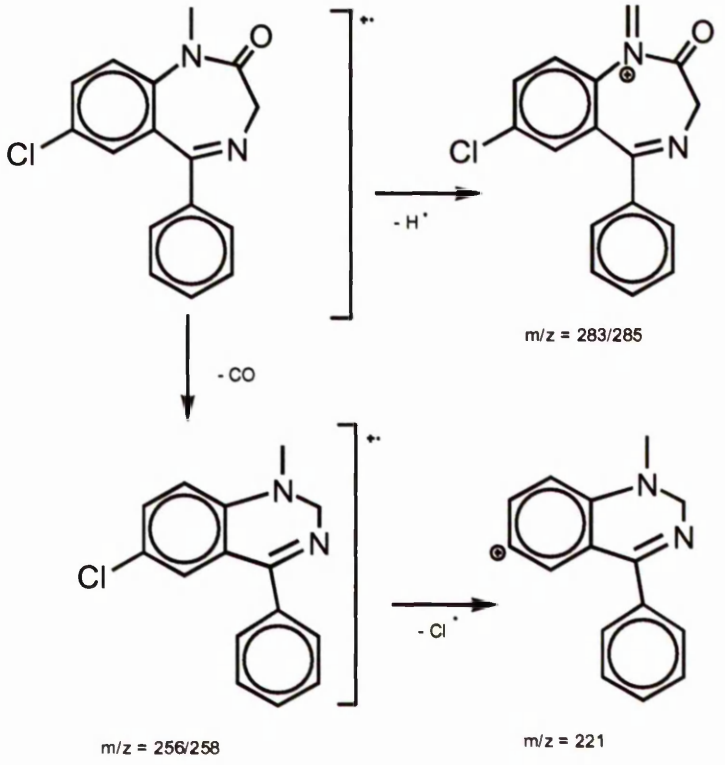


Figure 3.29. EI-mass spectrum of diazepam extracted by SPME from case 6 blood at pH7

The spectrum of diazepam shows several interesting features including isotope patterns corresponding to monochlorination and ring contraction processes associated with the diazepine system.



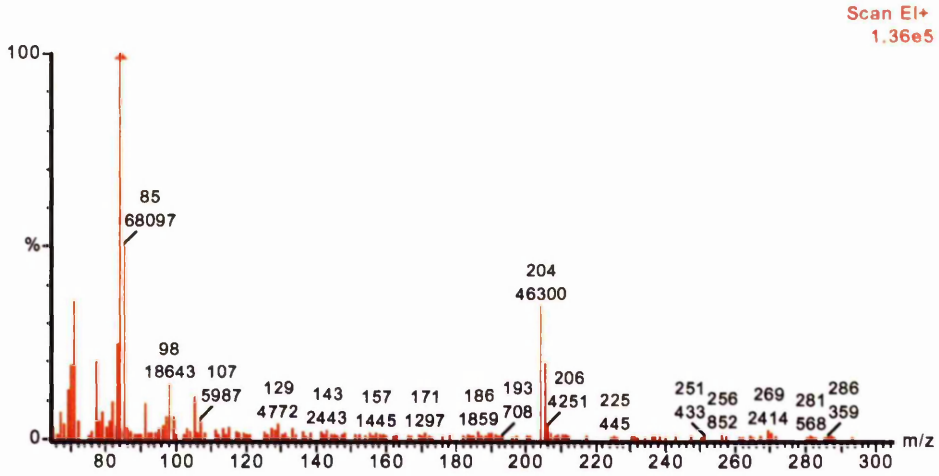
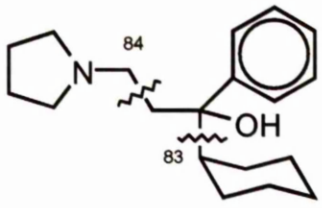


Figure 3.30. EI-mass spectrum of procyclidine extracted by SPME from case 6 urine at pH7

Procyclidine fragments as shown in the diagram below to give ions at m/z= 83 and 84.



The concentrations of amitriptyline, procyclidine and diazepam were 1780 µg/L, 1400 µg/L and 1850 µg/L, respectively. The reported concentrations of procyclidine, amitriptyline, and diazepam with serious toxicity were 1 mg/L, 2 mg/L and 5 mg/L, respectively[186]. Procyclidine was present at a concentration of more than 1mg/L, which is the concentration with serious toxicity.

Case 7) A 76-year-old man was reportedly found dead. He had suffered from severe depression and had previous suicidal attempts. He had access to dihydrocodeine, tramadol, captopril, chlordiazepoxide, aspirin, ibuprofen and gastrocote at his last attempt. His blood sample was taken from peripheral blood vessels. The urine and blood samples were prepared as explained in sections 2.8 and 2.9. Dothiepin, diazepam, and chlordiazepoxide were found in the blood at pH10. The chromatogram obtained from the blood sample is shown in Figure 3.31. Figure

3.32 shows the EI-mass spectum of chlordiazepoxide from the blood sample after extraction at pH10 using a polyacrylate fibre.

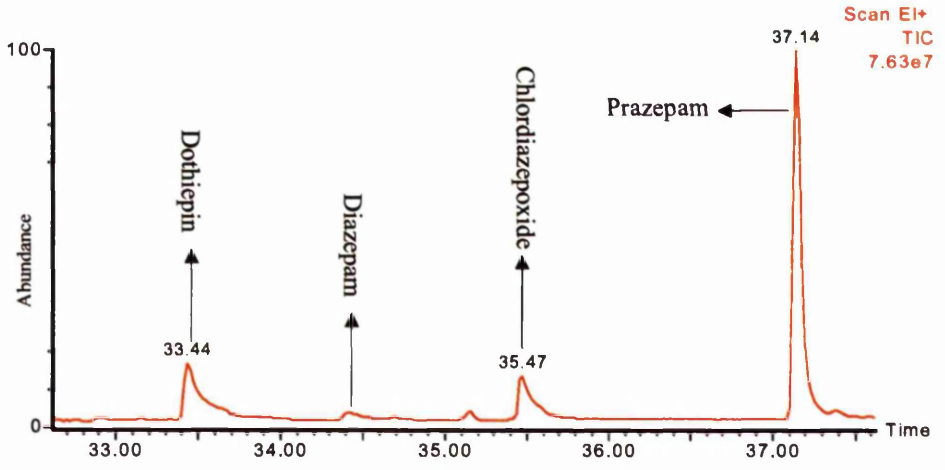


Figure 3.31. SPME-GC total ion chromatogram of the blood sample (case 7) spiked with prazepam (0.1mg/L; I.S.)

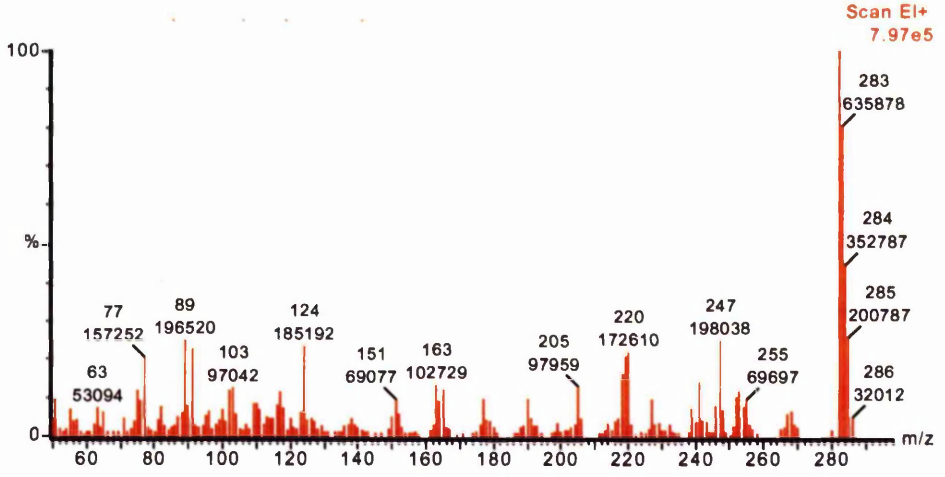
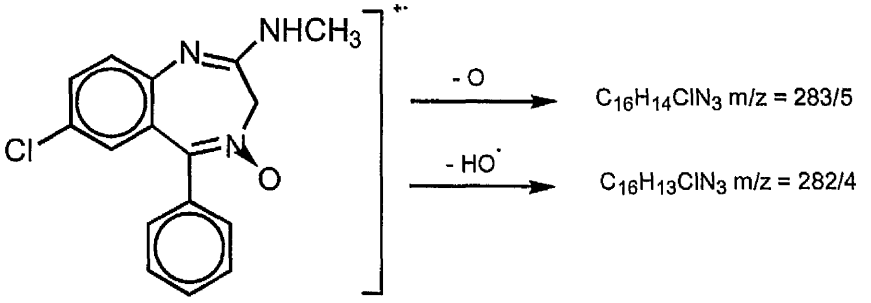


Figure 3.32. EI-mass spectrum of chlordiazepoxide extracted by SPME from case 7 blood at pH10

The EI-MS for dothiepin and diazepam showed good matches to the spectra of the authentic samples. The fragmentation pathways for the compounds are shown in cases 1 and 6.

The complex pattern of peaks at 282-285 in the mass spectrum of chlordiazepoxide arises by losses of oxygen and hydroxyl radicals.



Dothiepin, diazepam, and chlordiazepoxide were found in the blood at the concentrations of 360 μg/L, 86 μg/L and 340 μg/L, respectively. None of them were present at or above the concentration with serious toxicity, which has been explained by Flanagan[186].

Case 8) A 43-year-old man was found dead at home. He had access to nefopam (acupan®), nytol®, zoton® and numerous other drugs. His blood sample was taken from peripheral blood vessels. The urine and blood samples were prepared as explained in sections 2.8 and 2.9. The samples were analysed in full scan mode to identify the compounds present: diphenhydramine and nefopam were detected from blood at pH10 using a polyacrylate fibre.

The chromatogram obtained from the blood sample is shown in Figure 3.33. EI-mass spectrum of nefopam, extracted using a polyacrylate fibre at pH10, is shown in Figure 3.34.

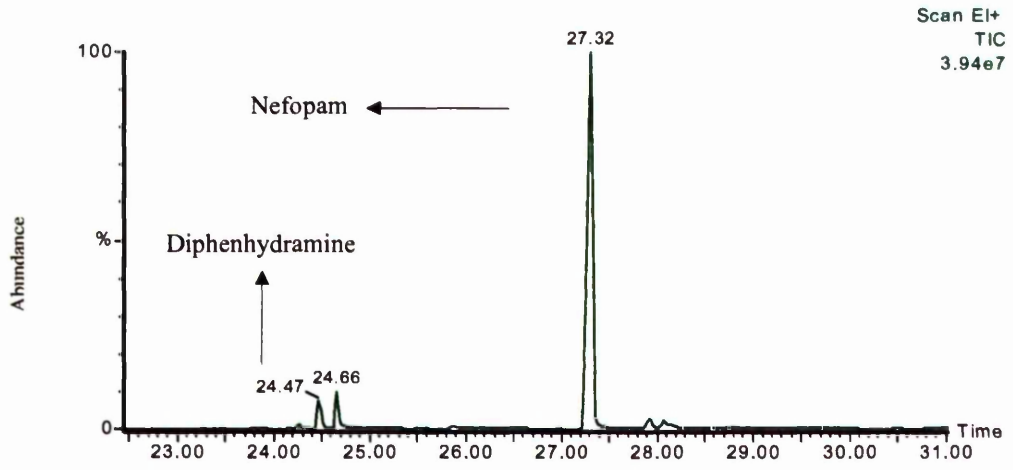


Figure 3.33. SPME-GC total ion chromatogram of the blood sample (case 8) spiked with prazepam (0.1mg/L; I.S.)

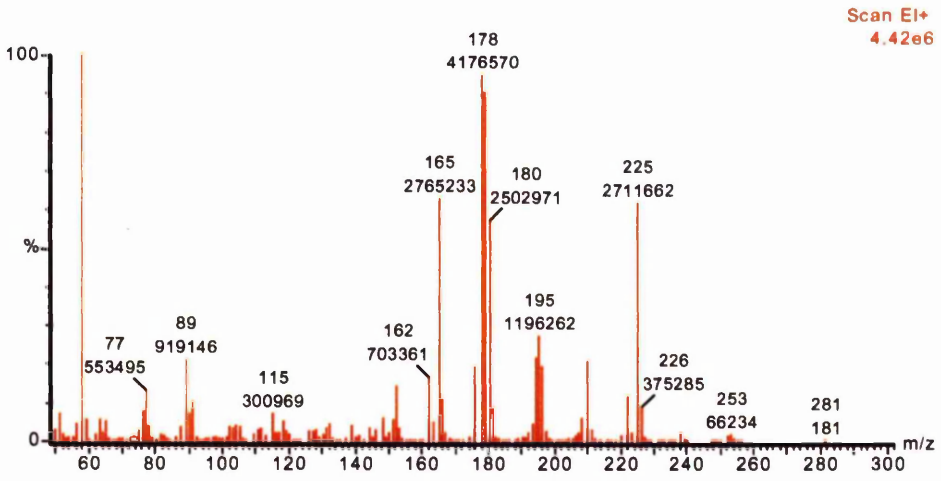
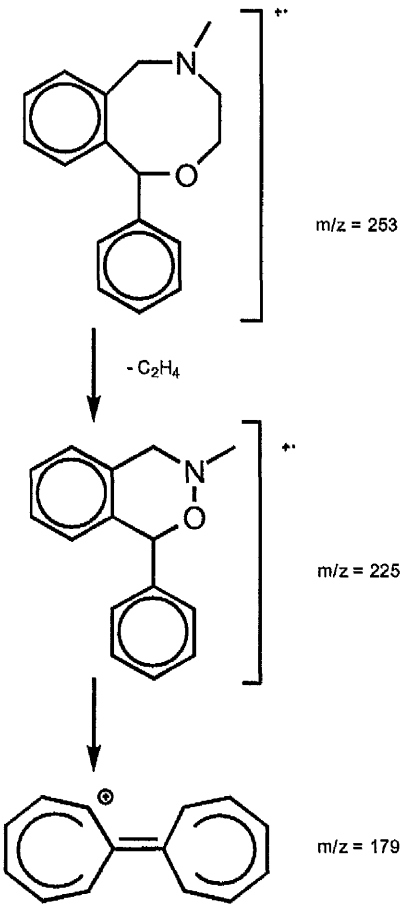


Figure 3.34. EI-mass spectrum of nefopam extracted by SPME from case 8 blood at pH10

The EI-MS for diphenhydramine showed a good match to the spectrum of the authentic sample. The fragmentation pathway for the compound is shown in case 3.

The spectrum of nefopam shows several extrusions from the heterocyclic ring to generate ions at 225 and 178-180.



Case 9) A 28-year-old was found dead in a car with an empty syringe in his hand. Blood and urine samples were taken. The urine sample was analysed and only diazepam was found. However, diazepam, chlordiazepoxide, procyclidine and nefopam were found in blood at pH7. The chromatograms obtained from the urine and blood samples after extraction at pH7 are shown in Figures 3.35 and 3.36, respectively.

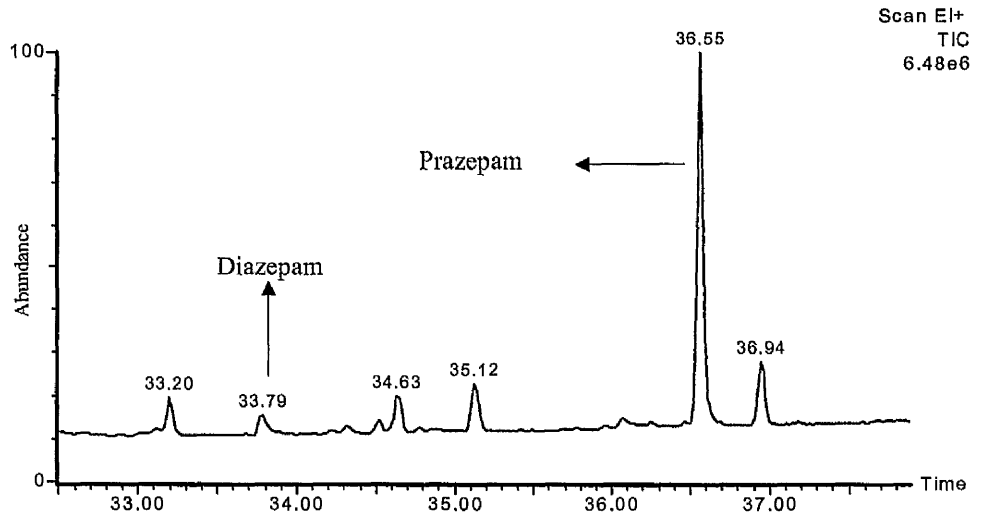


Figure 3.35. SPME-GC total ion chromatogram of the urine sample (case 9) spiked with prazepam (0.1mg/L; I.S.)

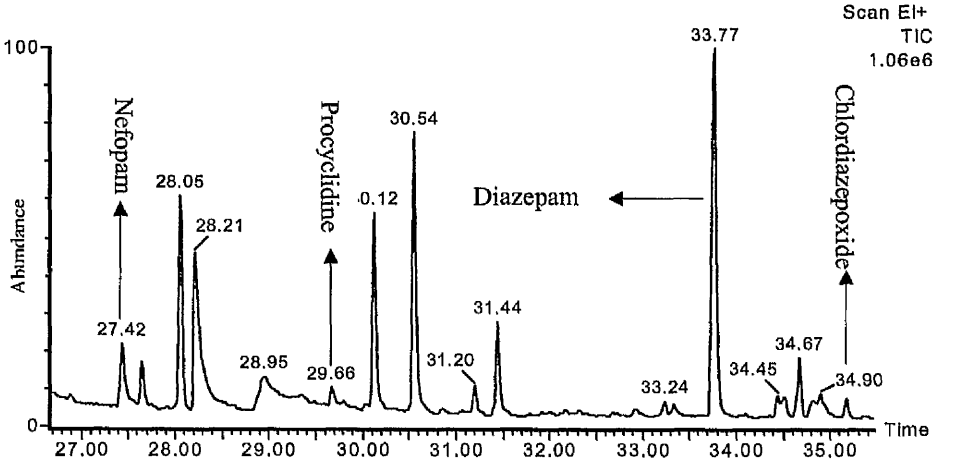


Figure 3.36. SPME-GC total ion chromatogram of the blood sample (case 9) spiked with prazepam (0.1mg/L; I.S.)

The EI-MS for diazepam, chlordiazepoxide, procyclidine, and nefopam showed good matches to the spectra of authentic samples. Fragmentation pathways for these compounds are described in the other cases.

The concentrations of diazepam, chlordiazepoxide and procyclidine in the blood were 0.28 mg/L, 47 μg/L and 19 μg/L, respectively. None of them were present at or above the concentration with serious toxicity, which has been explained by Flanagan[186].

Case 10) A-50-year-old man was reportedly found dead. He had access to omeprazole, paracetamol and dihydrocodeine. Peripheral blood and urine samples were taken. The samples were prepared and then analysed in full scan mode to identify the compounds present. Chlordiazepoxide, diazepam, nordiazepam and propoxyphene were found in the blood at pH10 using a polyacrylate fibre. The chromatogram obtained from the blood sample is shown in Figure 3.37. EI-mass spectrum of nordiazepam, extracted from the blood at pH10 using a polyacrylate fibre, is shown in Figure 3.38.

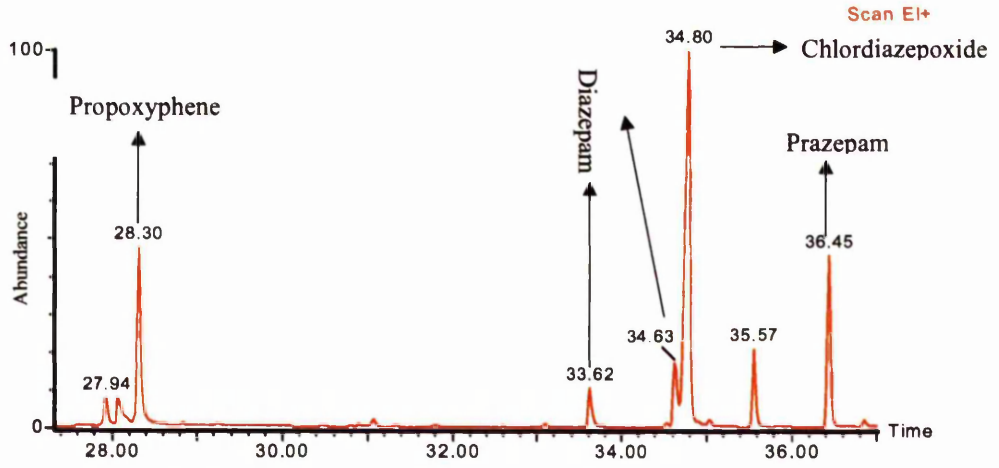


Figure 3.37. SPME-GC total ion chromatogram of the blood sample (case 10) spiked with prazepam (0.1mg/L; I.S.)

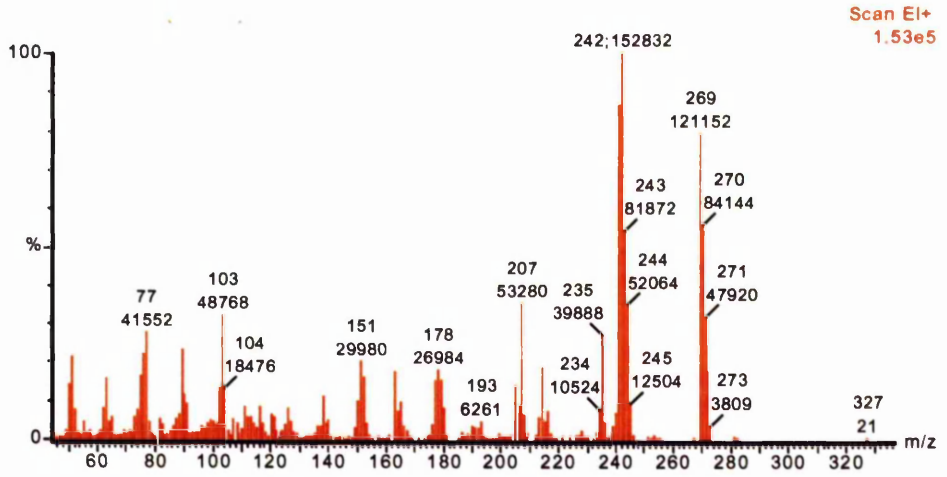


Figure 3.38. EI-mass spectrum of nordiazepam extracted by SPME from case 10 blood at pH7

The EI-MS for diazepam, chlordiazepoxide and propoxyphene showed good matches to the spectra of authentic samples and NIST library. Fragmentation pathways for these compounds are described in the other cases. The detection of diazepam is confirmed by the identification of the metabolite nordiazepam that gives a similar fragmentation pattern.

The concentrations of propoxyphene, diazepam and chlordiazepoxide were 590 µg/L, 740µg/L, and 960µg/L, respectively. None of them were present at or above the concentration with serious toxicity, which has been explained by Flanagan [186].

Case 11) A 27-year-old man suffered sudden unexpected death. He had taken procyclidine, diazepam, chlorpromazine and zopiclone. Blood and urine samples were taken. They were prepared as explained before and then analysed to identify the compounds present. Chlorpromazine and procyclidine were found in the urine using a polyacrylate fibre. Procyclidine was found in the blood after extraction at pH10 using a polyacrylate fibre.

The chromatograms obtained from the urine and blood samples using a polyacrylate fibre are shown in Figures 3.39 and 3.40, respectively. Figure 3.41 shows the EI-mass spectrum of chlorpromazine extracted from urine at pH7 using a polyacrylate fibre.

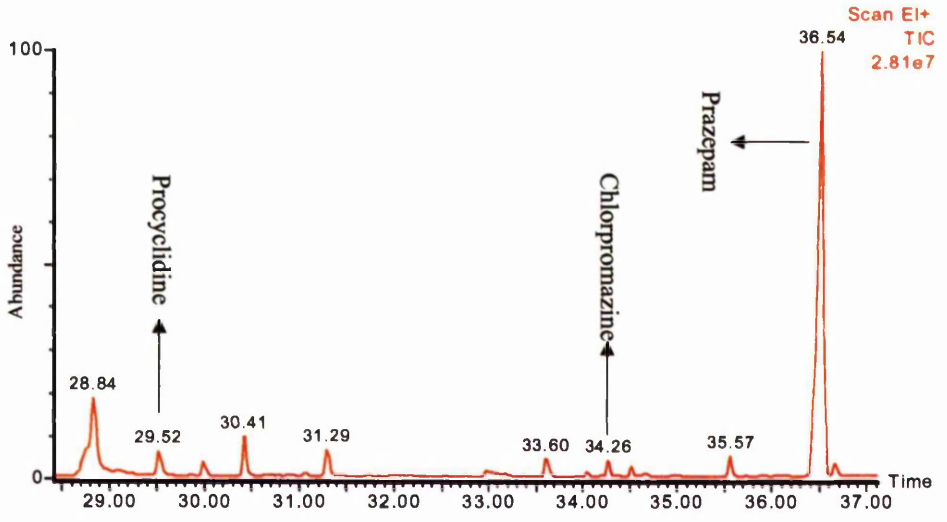


Figure 3.39. SPME-GC total ion chromatogram of the urine sample (case 11) spiked with prazepam (0.1mg/L; I.S.)

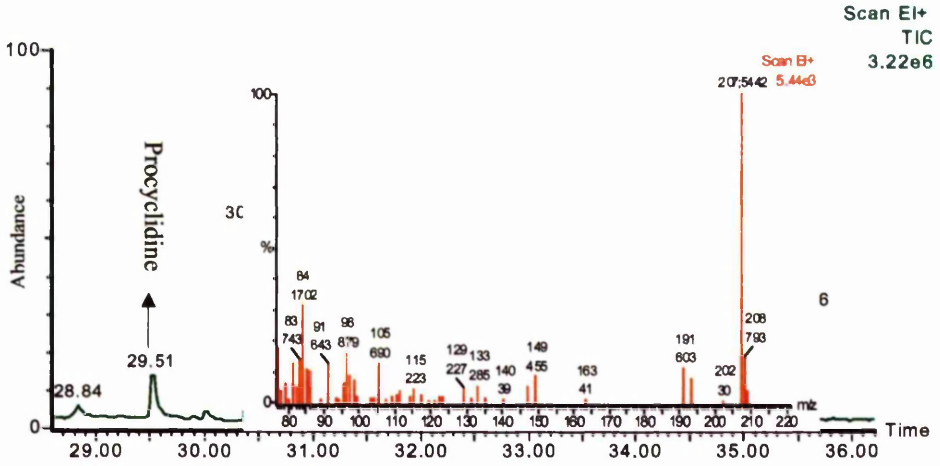


Figure 3.40. SPME-GC total ion chromatogram of the blood sample (case 11) spiked with prazepam (0.1mg/L; I.S.)

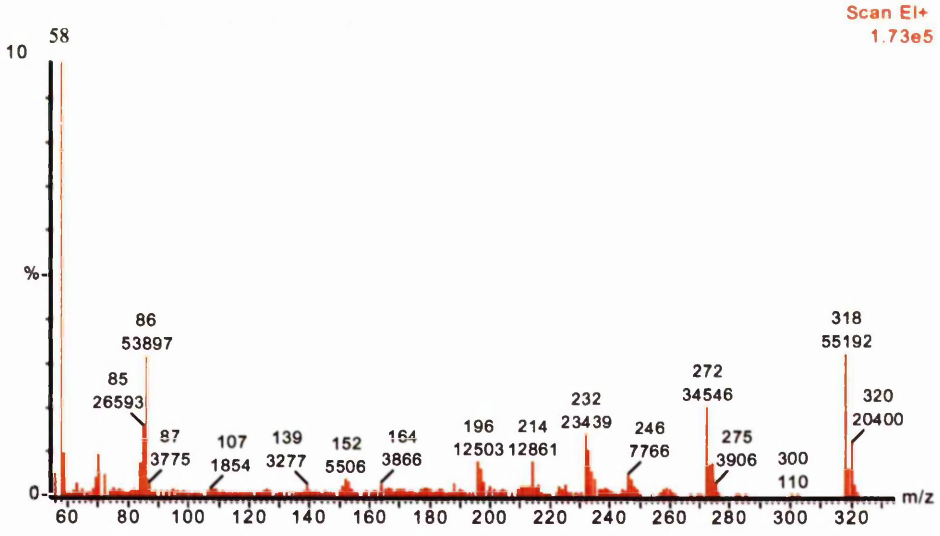


Figure 3.41. EI-mass spectrum of chlorpromazine extracted by SPME from case 11 urine at pH7

The EI-MS for procyclidine showed a good match to the spectrum of authentic sample. The fragmentation pathway for the compound is described in case 6.

Identification of chlorpromazine depends upon a combination of chromatographic retention time and spectrometric data. The chlorpromazine molecular ion undergoes a facile fragmentation whereby the basic side chain is extruded as the intense observed ion (m/z=58) upon electron impact. Procyclidine was quantified in the

blood at pH10 and the concentration was 0.63 µg/L, which was within the therapeutic concentration range[186].

Case 12) An 18-year-old man was found dead in bed at home. Blood and urine samples were taken. The samples were prepared and analysed using the full scan mode to identify the compounds present. Dihydrocodeine and dicyclomine were found in the blood sample at pH10.

The chromatogram obtained from the blood sample at pH10 is shown in Figure 3.42. EI-mass spectra of dihydrocodeine and dicyclomine are shown in Figures 3.43 and 3.44.

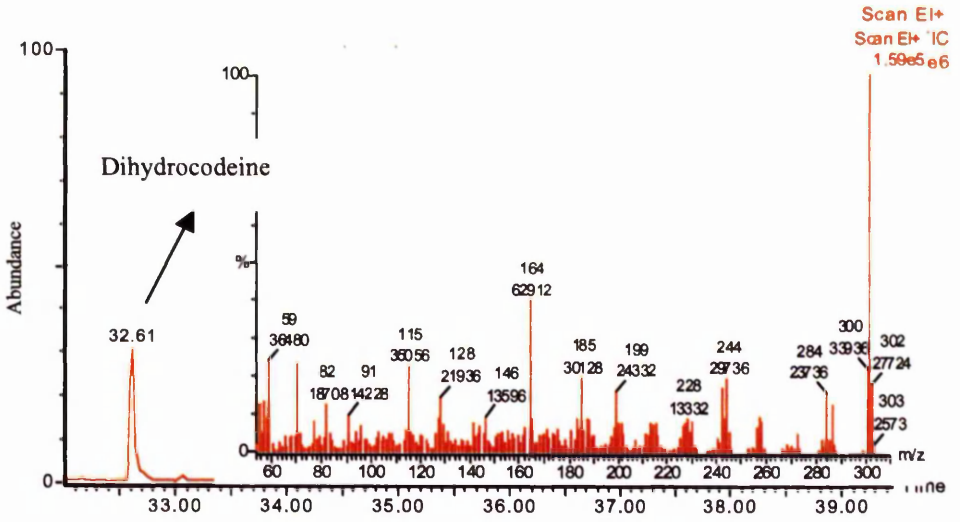


Figure 3.42. SPME-GC total ion chromatogram of the blood sample (case 12) spiked with prazepam (0.1mg/L; I.S.)

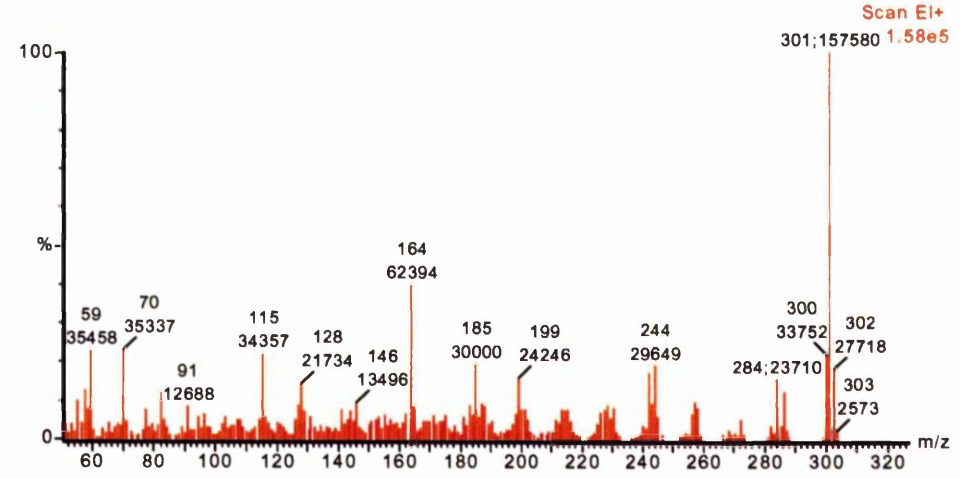


Figure 3.43. EI-mass spectrum of dihydrocodeine extracted by SPME from case 12 blood at pH10

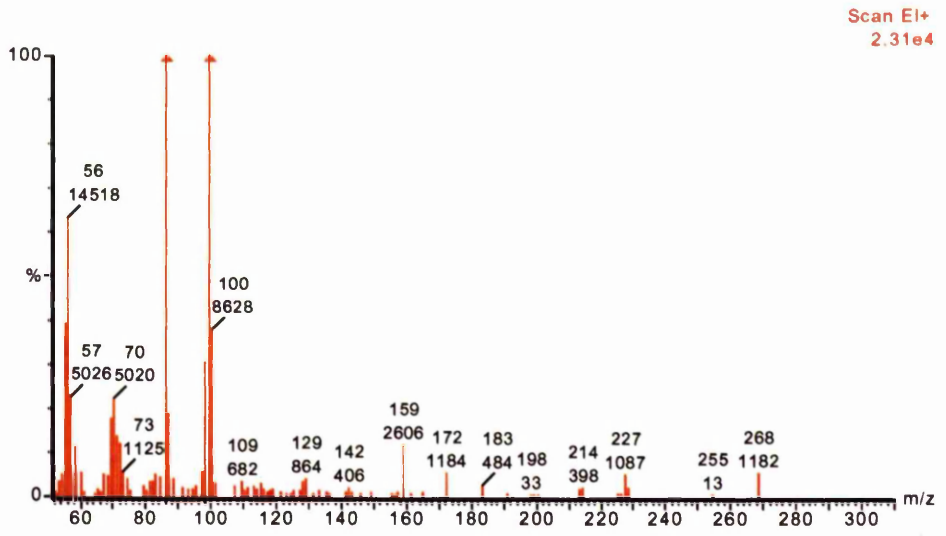
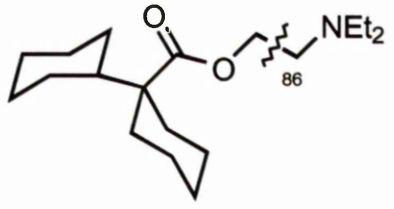


Figure 3.44. EI-mass spectrum of dicyclomine extracted by SPME from case 12 blood at pH10

Dihydrocodeine was confirmed by a very similar fragmentation pattern to the codeine, chromatographic retention time, and spectrum with a molecular ion at m/z= 301. The dicyclomine molecular ion undergoes a facile fragmentation whereby the basic side chain (m/z=86) is extruded as the intense observed ion upon electron impact.



Dihydrocodeine concentration in the blood sample was 460 µg/L, which was within the therapeutic concentration range[186].

Case 13) A 46-year-old man was found dead at home. There was no obvious natural cause of death. He had access to diazepam and olanzapine. A blood sample was taken from peripheral blood vessels. Dothiepin and diazepam were found in the blood sample using a polyacrylate fibre at pH7. The total ion chromatogram

obtained from the blood sample at pH7 using a polyacrylate fibre is shown in Figure 3.45.

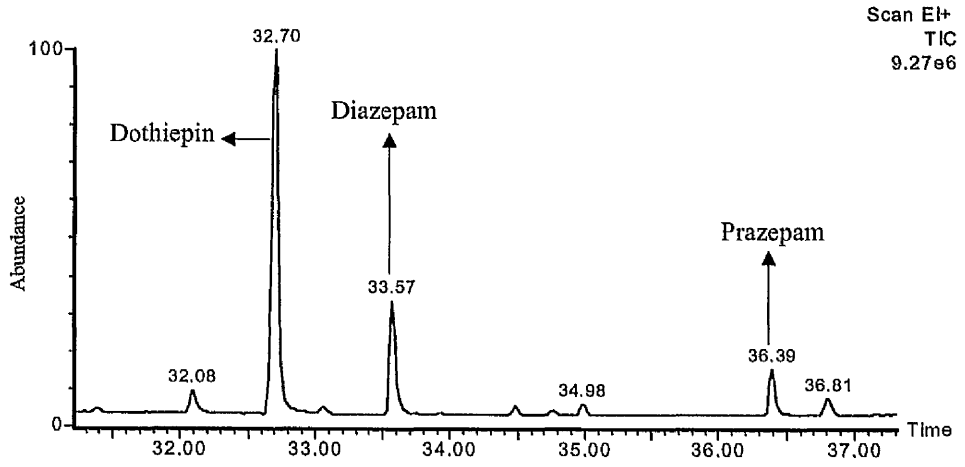


Figure 3.45. SPME-GC total ion chromatogram of the blood sample (case 13) spiked with prazepam (0.1mg/L; I.S.)

The EI-MS for diazepam and dothiepin showed good matches to the spectra of the authentic samples and NIST library. Fragmentation patterns for these compounds are described in the other cases.

Dothiepin and diazepam concentrations were 5.6 mg/L and 950 μg/L, respectively. Dothiepin was present in the blood at a concentration of greater than 1000 μg/L reported with serious toxicity[186]. The major effects of ingestion of an excessive amount of dothiepin are cardiac arrhythmias, central depression and coma[136]. These findings are probably consistent with a diagnosis of death due to the toxic effects of an excessive amount of dothiepin

Case 14) A 73-year-old man was found in bed at home with empty tablet containers. Thirty-five tablets were found in his stomach at autopsy. He had access to codeine and dihydrocodeine. Blood and urine samples were taken. Dihydrocodeine, codeine, nefopam and diphenhydramine were found in blood at pH 10. The chromatogram obtained from the blood sample after extraction at pH10 is shown in Figure 3.46.

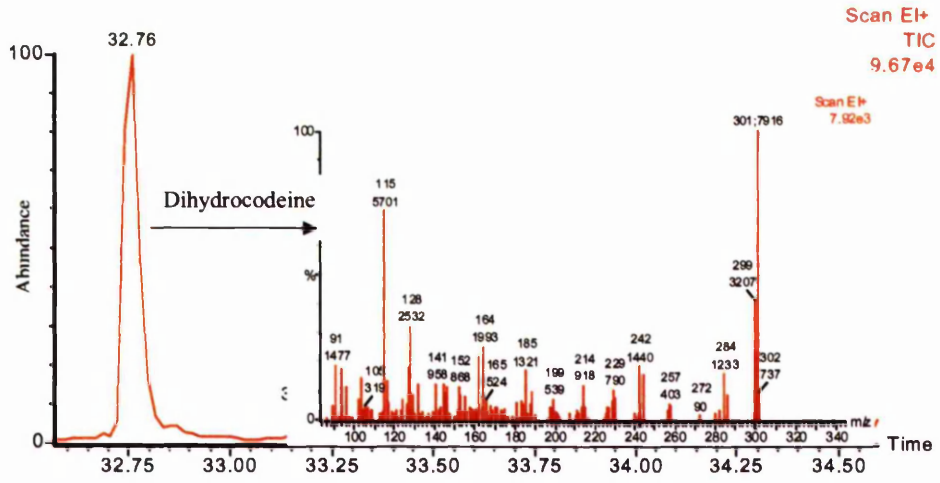


Figure 3.46. SPME-GC total ion chromatogram of the blood sample (case 14) spiked with prazepam (0.1mg/L; I.S.)

The EI-MS for codeine, dihydrocodeine, nefopam, and diphenhydramine showed good matches to the spectra of authentic samples and NIST library. Fragmentations pathways for these compounds are described in the other cases.

The dihydrocodeine concentration in the blood was 3.1 mg/L which suggests that death was due to the toxic effects of a hyperdose[186].

Case 15) A 55-year-old woman was admitted to the hospital after taking chlorpromazine, zopiclone, carbamazepine and strong lager. She had taken multiple overdoses in the past. A peripheral blood sample was taken for analysis. The blood sample was prepared as before. Carbamazepine and 3-hydroxy chlorpromazine were found in the sample. Total ion chromatogram, extracted from the blood sample at pH10 using a polyacrylate fibre, and EI-mass spectrum of carbamazepine are shown in Figures 3.47 and 3.48.

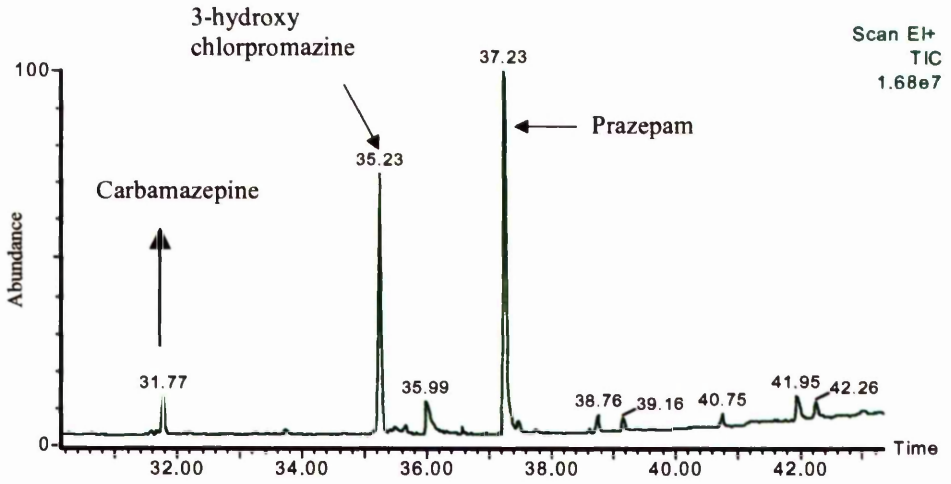


Figure 3.47. SPME-GC total ion chromatogram of the blood sample (case 15) spiked with prazepam (0.1mg/L; I.S.)

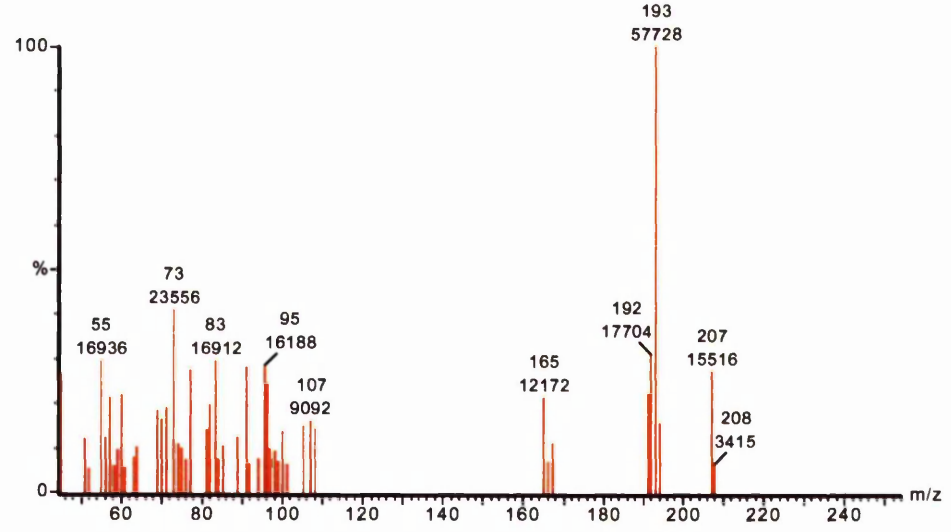
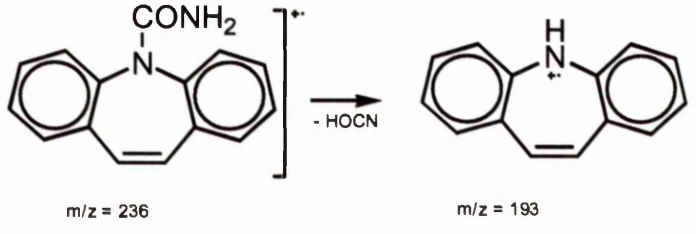


Figure 3.48. EI-mass spectrum of carbamazepine extracted by SPME from case 15 blood at pH10

The EI-mass spectrum of carbamazepine is a good match with that from an authentic sample of carbamazepine. The signal at m/z= 193 arises by a fragmentation as shown below:



Case 16) A 37-year-old man was reportedly found dead at home. He had made several suicidal attempts on his life. Ampules of morphine were found nearby. His blood sample was taken from peripheral blood vessels. The urine and blood samples were prepared as before. The total ion chromatogram of the sample extracted at pH10 is shown in Figure 3.49.

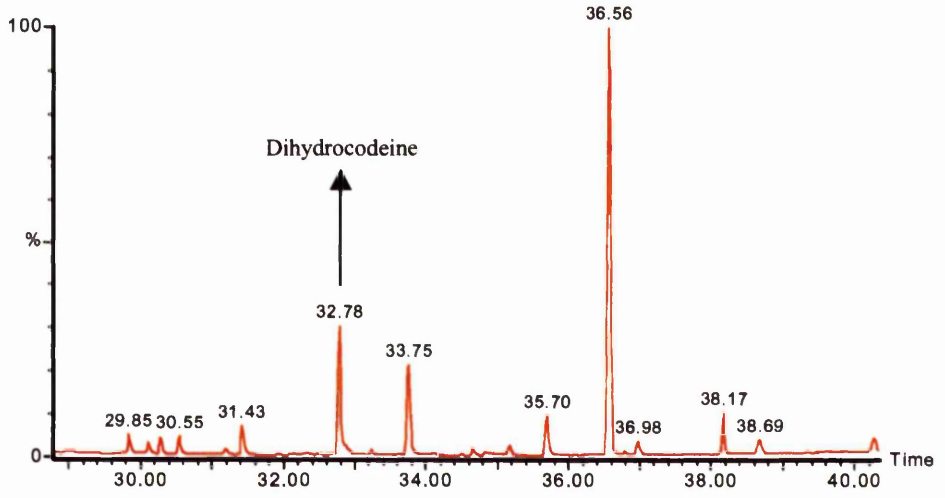


Figure 3.49. SPME-GC total ion chromatogram of the blood sample (case 16) spiked with prazepam (0.1mg/L; I.S.)

The EI-MS dihydrocodeine showed a good match to the spectrum of the authentic sample and NIST library. The fragmentation pathway for the compound is as described in the case 12.

Chapter 4

Discussion

4.1. Introduction

The aims of this section are to discuss the selection of the capillary column, optimisation of the parameters related to SPME, comparison of the parameters with the current knowledge available in the literature and to find out the effects of these parameters on the extraction.

A further aim is to compare the SPME-GC-MS results from sixteen *post-mortem* cases with the results of the other methods, which had been applied by the toxicology laboratories of Guy's Hospital and Manchester Royal Infirmary (MRI). The final aim is to explain the problems of *post-mortem* sampling, suggest a protocol for drug extraction from *post-mortem* tissue samples and give suggestions for future investigations that research may follow.

4.2. Capillary column selection

There were several stages in the development of the solid phase microextraction method. The development of solid phase microextraction methods is dependant upon the use of capillary gas chromatography as a separation method coupled to a quadrupole mass spectrometer for detection and quantification.

The first stage, prior to the start of the SPME study, was to assess the sensitivity of the coupled gas chromatography-mass spectrometry system towards the targeted drugs.

Unfortunately, there is not a consistent and comprehensive set of rules for selecting the best stationary phase of a capillary column for a separation. There are a few guidelines that might be helpful: the most important one is the use of a phase with a polarity that best matches that of the solutes (e.g. non-polar phase for non-polar solutes). Another guideline is to use the least polar phase that will provide satisfactory separation. Non-polar phases exhibit superior lifetimes, better efficiencies and lower bleed levels than polar phases. Column bleed is the normal background signal caused by the elution of degradation products from a column's stationary phase[117]. The best general-purpose phase is the 5% phenyl / 95% dimethyl polysiloxane followed closely by 100% dimethypolysiloxane.

Published literature references of similar analytes are a good source of column selection information. Literature from column manufacturers is another source of good quality information. The phase selection guide from Hewlett-Packard recommends the best stationary phase for the drugs of interest is HP-5, which contains 5% phenyl and 95% dimethylpolysiloxane and is stable in the temperature range of 60 to 325 °C.

Whereas SPME is a desorption-based technique, the on-column and "purge and trap" types of injection system are not suitable. Due to the low concentrations of the drugs, the split/splitless injection system operating as a splitless injector was used for transferring the analytes into the column.

4.3. Optimisation of the parameters

4.3.1. Coating selection

The choice of an appropriate coating is essential for the development of an SPME method. Supelco® provides several coatings, which include: polydimethysiloxane (PDMS), polyacrylate (PA), polydimethylsiloxane/divinylbenzene (PDMS/DVB), and Carbowax™/divinylbenzene (Carbowax/DVB). Typically, the chemical nature of an analyte of interest determines the type of coating used. The sensitivity of the fibre towards an analyte depends on the molecular weight and polarity of the analytes to be extracted.

In this study, the most frequently used fibre coatings were chosen to examine the extraction of the drugs. Retention times and the selection of the appropriate fibre for the extraction of the targeted drugs were shown in Tables 3.1, 3.2 and 3.3. In all of the cases, it was found that polyacrylate (PA) and then Carbowax®/divinylbenzene (CW/DVB) were the best fibres for the extraction of the drugs. Therefore, SPME on PA and CW/DVB were chosen to be applied to the *post-mortem* matrices.

In the literature, the use of PA and Carbowax/DVB fibres has been reported for the analysis of plasma and urine samples containing benzodiazepines. It was found that PA followed by Carbowax/DVB were the best fibres for the extraction of benzodiazepines. The extraction of antidepressants from blood and urine samples

was reported using PDMS and PA fibres[90]. However, the results of this study confirmed the findings of Spokert and Pragst (2000), who found that PA fibres, in comparison with PDMS, were better for the extraction of antidepressants[187] and Frison *et al.*(2001), who developed an SPME-GC-MS (SIM) method using PA fibres for the extraction midazolam from human plasma[188].

4.3.2. Extraction mode selection

Sample matrix, analyte volatility and analyte affinity to the matrix, control selection of the extraction mode. For very complex matrices that can damage the fibre coating or change its properties, the headspace mode is preferred. On the other hand, for aqueous matrices, it is usually difficult to apply the headspace SPME method with very polar analytes that have a high affinity toward the aqueous matrices.

Headspace-SPME mode was applied to the targeted drugs. Where the drugs were not extracted and detected using headspace mode, direct immersion mode was selected for the extraction of the samples. The *post-mortem* samples had to be pre-treated to avoid damaging the fibre coating. Perchloric acid with sonication was used to deproteinise the blood.

It was found that imipramine, methadone, diazepam, prazepam, trifluoprazine, meperidine and bupivacaine were not extracted using headspace mode at pH7 and 60°C. This is at variance with the findings of Lee *et al.* (1997) for the extraction of imipramine[178], and with the findings of Seno *et al.* (1995) for the detection of meperidine[173]. They detected imipramine and meperidine in blood samples using headspace analysis, but at 100°C in the presence of NaOH. This can be explained in that they improved the yield of the extraction by increasing the extraction temperature and changing the pH of the matrix. However, in this study as detailed below, the temperature could not be stabilised above a temperature of 60°C.

4.3.3. Temperature effect

The temperature affects both the sensitivity and the extraction kinetics. An increase in the extraction temperature causes an increased diffusion coefficient and decreased distribution constant, both leading to a faster equilibrium time. If sensitivity is high

enough at a higher temperature, increasing the temperature can lead to faster determination.

The extraction rate was found to increase over a temperature range from room temperature up to approximately 75°C. However, it proved difficult to stabilise the temperature at 75°C, so 60°C was used for the SPME extraction of the *post-mortem* samples.

In the literature, adsorption temperatures, for the direct extraction of the targeted drugs, were reported between room temperature for lidocaine, benzodiazepines and cocaine and 45°C for lidocaine and benzodiazepines[59-61,73,78].

4.3.4. Extraction time

SPME is an equilibrium process in which the analytes partition between the sample matrix and the stationary phase (coating). As explained, the equilibration time is determined by exposing the fibre coating to an aqueous solution containing the targeted analytes for a series of time periods until the amount extracted reaches a maximum. The extraction of the selected drugs reached equilibrium within 35 minutes. To obtain reproducible results a period of 45 minutes of extraction time was chosen, this is equal to the time needed for running a sample using the GC-MS system. Therefore a sample was being extracted to completion, while the previous sample was being subjected to the GC-MS analysis. In the literature, for the DI-SPME of the targeted drugs, extraction times between 15 min for methadone and 60 min for benzodiazepines were reported[78,174].

4.3.5. Salt addition

Using the well-known "salting in effect" can enhance extraction of the drugs. Addition of sodium chloride into the sample decreases the solubility of the drugs. This leads to an increase in the extracted amount of analyte by SPME fibres. As a result, the sensitivity can be increased for polar drugs. It was shown that the addition of sodium chloride increased the extraction yield of most of the drugs. In buffer solutions at pH7 the extraction yield was increased by a factor of 1.4-7 times by a 5 mol/L sodium chloride concentration. The results confirm the findings of Namera *et al.*[179], Luo *et al.*[78], and Seno *et al*[89].

4.3.6. pH effect

The targeted drugs have different p^{Ka} values and therefore the partitioning of the drugs between the sample matrix and the fibre was strongly affected by pH. In the literature, pH5.5 was used for the extraction of diazepam[189], pH7 for the extraction of benzodiazepines[89] and pH9.5 for the extraction of lidocaine[93]. It was found that the optimum pH for the extraction of diazepam and prazepam was pH7, for trifluoperazine and bupivacaine it was pH9 and for methadone and imipramine it was pH11. Since a pH less than 4 and more than 10 can damage the SPME fibre, the pH values 7 and 10 were selected for the extraction of *post-mortem* samples.

4.3.7. Linearity

The results of the statistical analysis of the relationship of peak area ratios to the concentrations of the standard drugs showed excellent linearity (confidence intervals were greater than 99.9%). This meant that a high degree of confidence could be placed in the quantitation of the drugs.

4.4. Application to *post-mortem* samples

Although some of the parameters have been investigated previously for developing SPME methods to extract drugs from clear and uncomplicated matrices (such as water, air and headspace), to date there are no reports of the use of SPME for the extraction of drugs from *post-mortem* samples. Also, none of the previous studies analysed samples contain dothiepin, thioridazine, mesoridazine and nefopam using SPME-GC-MS.

Blood and urine samples from the sixteen cases were analysed using the developed protocol. In each of these cases other methods including immunoassay, thin layer chromatography, radioimmunoassay (RIA), colorimetry and HPLC had been applied and positive results obtained. A summary of these findings is given in Table 4.17 (page 162) and each individual case is discussed below.

Case 1) Table 4.1 shows the comparison of the results obtained by SPME-GC-MS, ToxiA and HPLC. The performance of SPME-GC-MS was comparable to ToxiA and HPLC for dothiepin and superior to the other techniques for the detection

of medazepam. That is the first time SPME has been used for the extraction of dothiepin. The polarity of medazepam is lower than the other benzodiazepines and so it was not detected by HPLC.

Table 4.1. Comparison of case 1 results between SPME-GC-MS and other methods.

Case 1	SPME-GC-MS	ToxiA	HPLC
Medazepam	+	-	-
Dothiepin	+	+	+

+ = drug detected, - = drug not detected

Case 2) In Table 4.2 the results obtained by SPME-GC-MS and five other methods are tabulated. The results from SPME-GC-MS were comparable to HPLC and Remedi for thioridazine, and to HPLC for mesoridazine. The performance of SPME-GC-MS was superior to the other techniques for the detection of D-propoxyphene, amitriptyline and dothiepin. However, SPME-GC-MS could not detect opiates or fluphenazine, which were detected by EMIT and HPLC, respectively. Fluphenazine, opiates and benzodiazepines all generate good quality mass spectra in electron impact mode hence the absence of signal in SPME-GC-MS must relate to binding problems in the extraction (personal communication Dr V. Garner). That is the first report of the use of SPME for the extraction of thioridazine and mesoridazine.

Table 4.2. Comparison of case 2 results between SPME-GC-MS and other methods.

Case 2	SPME-GC-MS	EMIT	ToxiA	HPLC	RIA	Remedi
D-propoxyphene	+	-	-	-		-
Amitriptyline	+	-	-	-		-
Dothiepin	+	-	-	-		-
Thioridazine	+	-	Phenothiazines	+		+
Mesoridazine	+	-	-	+		-
Fluphenazine	-	-	-	+		-
Opiates	-	+	-	-	-	-
Benzodiazepines	-	+	-	-		-

+ = drug detected, - = drug not detected

Case 3) In Table 4.3 the results obtained by five techniques are shown. The results of SPME-GC-MS were comparable to ToxiA and HPLC for the detection of D-propoxyphene and diphenhydramine, and to HPLC for the detection of norpropoxyphene. However, neither SPME-GC-MS nor HPLC detected

paracetamol, which was detected by ToxiA and colorimetry. A derivatisation reagent such as bis(trimethylsilyl)trifluoroacetamide (BSTFA) could be used to enhance both selectivity and sensitivity of the determination of paracetamol[5].

Table 4.3. Comparison of case 3 results between SPME-GC-MS and other methods.

Case 3	SPME-GC-MS	EMIT	ToxiA	HPLC	Colorimetry
D-propoxyphene	+	-	+	+	-
Norpropoxyphene	+	-	-	+	-
Paracetamol	-	-	+	-	+
Diphenhydramine	+	-	+	+	-

+ = drug detected, - = drug not detected

Case 4) Table 4.4 shows the comparison of the results obtained by four techniques. The performance of SPME-GC-MS was comparable to ToxiA and HPLC for pethidine and superior to the other methods for the detection of dothiepin. SPME-GC-MS did not detect norpethidine, which was detected by ToxiA and HPLC. So far, there has been no report of the application of SPME for the extraction of norpethidine. There are some reports of detection of norpethidine using GC[190-193], therefore, it seems that the absence of signals is likely due to its binding to SPME fibres.

Table 4.4. Comparison of case 4 results between SPME-GC-MS and other methods.

Case 4	SPME-GC-MS	EMIT	ToxiA	HPLC
Pethidine	+	-	+	+
Norpethidine	-	-	-	+
Dothiepin	+	-	-	-

+= drug detected, -= drug not detected

Case 5) Table 4.5 compares the results obtained by SPME and five other techniques. The performance of SPME-GC-MS was comparable to ToxiA for the detection of codeine, dothiepin and paracetamol and superior to HPLC for the detection of dothiepin and paracetamol.

Table 4.5. Comparison of case 5 results between SPME-GC-MS and other methods.

Case 5	SPME-GC-MS	EMIT	ToxiA	HPLC	Remedi	Colorimetry
Codeine	+	Opiates	+	+	+	-
Dothiepin	+	-	+	-	+	-
Paracetamol	+	-	+	-	-	+

+ = drug detected, - = drug not detected

Case 6) In Table 4.6 the comparison of results obtained by SPME-GC-MS and four other methods are shown. The performance of SPME-GC-MS was comparable to HPLC, Remedi, and ToxiA for amitriptyline, to HPLC and Remedi for procyclidine, and to HPLC for diazepam. Nordiazepam and nortriptyline were not detected by SPME-GC-MS. However, they were detected by HPLC. As shown in Table 3.3 none of the fibres could extract nortriptyline and it was detected using direct injection of the methanolic solution; therefore, the absence of signal in SPME-GC-MS must relate to binding problems in the extraction. Levine *et al.*[194] found that nordiazepam was unstable under storage conditions. Reubsaet *et al.*[61] detected nordiazepam by SPME-GC, therefore, the absence of signal in the SPME-GC-MS probably is due to the long period of storage (more than seven months) of the sample before the analysis.

Table 4.6. Comparison of case 6 results between SPME-GC-MS and other methods.

Case 6	SPME-GC-MS	EMIT	ToxiA	HPLC	Remedi
Amitriptyline	+	-	+	+	+
Procyclidine	+	-	-	+	+
Diazepam	+	Benzodiazepines	-	+	-
Nordiazepam	-	-	-	+	-
Nortriptyline	-	-	+	+	-

+= drug detected, -= drug not detected

Using ToxiA, amitriptyline, nortriptyline and flupenthixol were found in the stomach contents.

Case 7) Table 4.7 shows the comparison of the results obtained by SPME-GC-MS and four other techniques. The performance of SPME-GC-MS was comparable to HPLC for the detection of chlordiazepoxide and superior to the other techniques for the detection of dothiepin and diazepam. Tramadol was detected by HPLC, but it was not detected by SPME-GC-MS. A study by Veselovskaia *et al.* showed that tramadol underwent a thermal destruction with the formation of water molecules and

a cyclohexane structure[195]. So the lack of signal with SPME may be related to its degradation at 290°C on the GC column.

Table 4.7. Comparison of case 7 results between SPME-GC-MS and other methods.

Case 7	SPME-GC-MS	EMIT	ToxiA	HPLC	Remedi
Tramadol	-	-	+	+	+
Dothiepin	+	-	-	-	-
Diazepam	+	-	-	-	-
Chlordiazepoxide	+	Benzodiazepines	-	+	-

+= drug detected, -= drug not detected

Case 8) Table 4.8 shows the comparison of results obtained by five techniques. The performance of SPME-GC-MS was comparable to ToxiA, EMIT, Remedi and HPLC for the detection of diphenhydramine, and superior to ToxiA, EMIT and Remedi for the detection of nefopam. This is the first report of using SPME for the extraction of nefopam.

Table 4.8. Comparison of case 8 results between SPME-GC-MS and other methods.

Case 8	SPME-GC-MS	EMIT	ToxiA	HPLC	Remedi
Diphenhydramine	+	-	+	+	+
Nefopam	+	-	-	+	-

+= drug detected, -= drug not detected
Nefopam was found in the stomach contents using ToxiA.

Case 9) Table 4.9 shows the comparison of the results obtained by SPME-GC-MS and five other techniques. The performance of SPME-GC-MS was comparable to HPLC for the detection of diazepam, and superior to all five other techniques for the detection of chlordiazepoxide, procyclidine, and nefopam. SPME-GC-MS did not detect morphine, cocaine, and benzoylecgonine, which were detected by radioimmunoassay, Remedi and HPLC, respectively. Morphine has two polar hydroxyl groups; therefore, a reliable method for the determination of morphine is based on derivatisation to a less polar and more stable compound. A study reported that most of cocaine hydrolysed to ecgonine methyl ester under 150-day storage[196]. Dugan *et al.* found that 37% of cocaine was degradated under -20°C storage in a 12-

month period[197],therefore, the absence of signal in the SPME-GC-MS probably is due to the long period of storage (more than seven months) of the sample before the analysis. Other studies[198,199] confirmed that a derivatisation step was necessary before gas chromatography of benzoylecgonine; therefore, the lack of signal was due to its thermal unstability. So, for the detection of benzoylecgonine some derivatisation reactions such as silylation, perfluoroalkylation or alkylation were recommended[199].

Table 4.9. Comparison of case 9 results between SPME-GC-MS and other methods.

Case 9	SPME-GC-MS	EMIT	ToxiA	HPLC	Remedi	RIA
Diazepam	+	Benzodiazepines	-	+	-	-
Chlordiazepoxide	+	Benzodiazepines	-	-	-	-
Procyclidine	+	-	-	-	-	-
Nefopam	+	-	-	-	-	-
Morphine	-	Opiates	-	-	-	+
Cocaine	-	+ (Borderline)	-	-	+	-
Benzoylecgonine	-	-	-	+	-	-

+= drug detected, -= drug not detected

Case 10) Table 4.10 shows the comparison of the results obtained by SPME-GC-MS, Remedi and HPLC techniques. The performance of SPME-GC-MS was comparable to HPLC and Remedi for chlordiazepoxide, and superior to the other methods for diazepam and propoxyphene. HPLC detected venlafaxine and desmethylvenlafaxine, which were not detected by SPME-GC-MS. The absence of signal in SPME-GC-MS for venlafaxine and its metabolite is likely to relate to its instability at 290°C or its binding to the fibre or column.

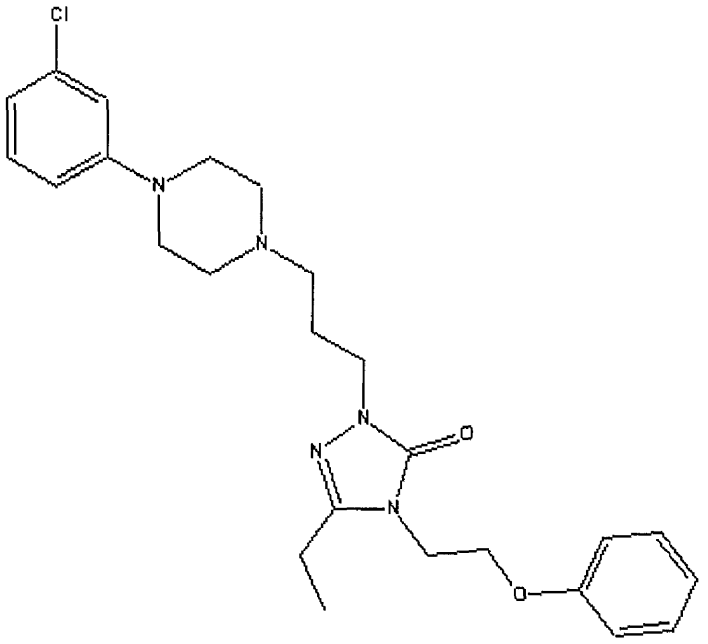
Table 4.10. Comparison of case 10 results between SPME-GC-MS and other methods.

Case 10	SPME-GC-MS	Remedi	HPLC
Chlordiazepoxide	+	+	+
Diazepam	+	-	-
Propoxyphene	+	-	-
Venlafaxine	-	-	+
Desmethylvenlafaxine	-	-	+

+= drug detected, -= drug not detected

Case 11) Table 4.11 shows the comparison of the results obtained by five techniques. The performance of SPME-GC-MS was comparable to HPLC and

superior to ToxiA, EMIT and Remedi for the detection of procyclidine and chlorpromazine. ToxiA detected nefazodone, which was not detected using SPME-GC-MS.



Nefazodone was not detected in this study. The chemical structure of nefazodone (as shown above) shows a piperazine moiety, which acts as a strong binding site. This binding site may interfere with the SPME extraction (personal communication Dr V. Garner).

Table 4.11. Comparison of case 11 results between SPME-GC-MS and other methods.

Case 11	SPME-GC-MS	ToxiA	EMIT	Remedi	HPLC
Procyclidine	+	-	-	-	+
Chlorpromazine	+	-	-	Chlorpromazine metabolite	+
Nefazodone	-	+	-	Nefazodone metabolite	-

+= drug detected, -= drug not detected

Case 12) Table 4.12 shows the comparison of the results obtained by five techniques. The performance of SPME-GC-MS was comparable to ToxiA, Remedi, and HPLC for the detection of dihydrocodeine, and superior to the other techniques for the detection of dicyclomine.

Table 4.12. Comparison of case 12 results between SPME-GC-MS and other methods.

Case 12	SPME-GC-MS	ToxiA	EMIT	Remedi	HPLC
Dihydrocodeine	+	+	Opiates	+	+
Dicyclomine	+	-	-	-	-

+= drug detected, -= drug not detected
Dihydrocodeine was detected in stomach contents using ToxiA.

Case 13) Table 4.13 shows the comparison of the results obtained by four techniques. The performance of SPME-GC-MS was comparable to HPLC and ToxiA for the detection of dothiepin, and to HPLC for the detection of diazepam. HPLC detected nordiazepam and desmethyldothiepin, which were not detected using SPME-GC-MS. Desmethyldothiepin was detected using gas chromatography; therefore, it seems that the absence of signal for desmethyldothiepin could be due to its binding to the SPME fibres (personal communication Dr V. Garner).

Table 4.13. Comparison of case 13 results between SPME-GC-MS and other methods.

Case 13	SPME-GC-MS	ToxiA	EMIT	HPLC
Diazepam	+	-	Benzodiazepines	+
Nordiazepam	-	-	-	+
Dothiepin	+	+	-	+
Desmethyldothiepin	-	-	-	+

+= drug detected, -= drug not detected
Dothiepin was found in stomach contents using ToxiA method.

Case 14) Table 4.14 shows the comparison of the results obtained by six techniques. The performance of SPME-GC-MS was comparable to HPLC and Remedi for the detection of codeine and dihydrocodeine and superior to the other methods for the detection of diphenhydramine and nefopam. RIA, ToxiA and Remedi detected morphine, which was not detected by SPME-GC-MS.

Table 4.14. Comparison of case 14 results between SPME-GC-MS and other methods.

Case 14	SPME-GC-MS	ToxiA	EMIT	Remedi	HPLC	RIA
Codeine	+	-	Opiates	+	+	-
Dihydrocodeine	+	+	-	+	+	-
Morphine	-	+	-	+	-	+
Nefopam	+	-	-	-	-	-
Diphenhydramine	+	-	-	-	-	-

+= drug detected, -= drug not detected

Case 15) Table 4.15 shows the comparison of the results obtained by three techniques. The performance of SPME-GC-MS was comparable to ToxiA and HPLC for the detection of chlorpromazine and carbamazepine. However, SPME-GC-MS did not detect zopiclone that was detected by HPLC. Zopiclone generates a good quality mass spectrum in electron impact mode. However, it was not detected using SPME-GC-MS. Comparison of the structure of zopiclone with chlorpromazine and dothiepin, which were detectable by the SPME-GC-MS, shows that it has a piperazine moiety and so offers additional basic binding sites. Such extra binding interferes with the SPME extraction (personal communication Dr V. Garner).

Table 4.15. Comparison of case 15 results between SPME-GC-MS and other methods.

Case 15	SPME-GC-MS	ToxiA	HPLC
Chlorpromazine	3-hydroxy chlorpromazine	-	+
Carbamazepine	+	Carbamazepine metabolite	+
Zopiclone	-	-	+

+= drug detected, -= drug not detected

Case 16) Table 4.16 shows the comparison of the results obtained by six techniques. The performance of SPME-GC-MS was comparable to ToxiA and Remedi for the detection of dihydrocodeine. However, SPME-GC-MS did not detected morphine and temazepam, which were detected by RIA and HPLC, respectively.

Table 4.16. Comparison of case 16 results between SPME-GC-MS and other methods.

Case 16	SPME-GC-MS	ToxiA	Remedi	EMIT	HPLC	RIA
Morphine	-	+	+	Opiates	-	+
Temazepam	-	-	-	Benzodiazepines	+	-
Dihydrocodeine	+	+	+	-	-	-

+= drug detected, -= drug not detected

The results of the analysis of *post-mortem* samples using the SPME procedure and the other methods are summarised in Table 4.17.

Table 4.17. Summary of the postmortem analysis results using the SPME procedure and the other methods

	SPME-GC-MS	EMIT	ToxiA	HPLC	RIA	REMEDI	Colorimetry
propoxyphene	2, 3, 10	[2], [3]	[2], 3	[2], 3, [10]		[2], [10]	[3]
norpropoxyphene	3	[3]	[3]	3			[3]
paracetamol	[3], 5	[3], [5]	3, 5	[3], [5]		[5]	3, 5
pethidine	4	[4]	4	4			
norpethidine	[4]	[4]	[4]	4			
tramadol	[7]	[7]	7	7		7	
carbamazepine	15		15	15			
medazepam	1		[1]	[1]			
amitryptyline	2, 6	[2], [6]	[2], 6	[2], 6		[2], 6	
nortriptyline	[6]	[6]	6	6		[6]	
dothiepin	1, 2, 4, 5, 7, 13	[2], [4], [5], [7], [13]	1, [2], [4], 5, [7], 13	1, [2], [4], [5], [7], 13		[2], 5, [7]	[5]
desmethyldothiepin	[13]	[13]	[13]	13			
nefopam	8, 9, 14	[8], [9], [14]	[8], [9], [14]	8, [9], [14]	[9], [14]	[8], [9], [14]	
venlafaxine	[10]			10		[10]	
desmethylvenlafaxine	[10]			10		[10]	
nefazodone	[11]	[11]	11	[11]		[11]	
codeine	5, 14	5, 14	5, [14]	5, 14	[14]	5, 14	[5]
dihydrocodeine	12, 14, 16	12, [14], [16]	12, 14, 16	12, 14, [16]	[14], [16]	12, 14, 16	
morphine	[9], [14], [16]	9, [14], 16	[9], 14, 16	[9], [14], [16]	9, 14, 16	[9], 14, 16	
thioridazine	2	[2]	2	2		2	
mesoridazine	2	[2]	[2]	2		[2]	
fluphenazine	[2]	[2]	[2]	2		[2]	
diphenhydramine	3, 8, 14	[3], [8], [14]	3, 8, [14]	3, 8, [14]	[14]	8, [14]	[3]
procyclidine	6, 9, 11	[6], [9], [11]	[6], [9], [11]	6, [9], 11	[9]	6, [9], [11]	
diazepam	6, 7, 9, 10, 13	6, [7], 9, 13	[6], [7], [9], [13]	6, [7], 9, [10], 13	[9]	[6], [7], [9], [10]	
nordiazepam	[6], [13]	[6], [13]	6, [13]	6, 13		[6]	
chlordiazepoxide	7, 9, 10	7, 9	[7], [9]	7, [9], 10	[9]	[7], [9], 10	
cocaine	[9]	9	[9]	[9]	[9]	9	
benzoylecgonine	[9]	[9]	[9]	9	[9]	[9]	
chlorpromazine	11, 15	[11]	[11], [15]	11, 15		11	
dicyclomine	12	[12]	[12]	[12]	[12]		
zopiclone	[15]		[15]	15			
temazepam	[16]	16	[16]	16	[16]	[16]	

Numbers are the case numbers of those tested. Numbers in brackets show the drug **not** detected.

The Table shows the drugs that were detected by the different methods. Summarising these results, SPME-GC-MS was the method that had the highest rate of detection over the range of drugs at 70.5%, compared to the next best, which was HPLC at 60.7%. These results are for drugs that were detected by at least one of the methods for each particular sample.

4.5. Detection of individual drugs

Fluphenazine (case 2) generates a mass spectrum as shown in figure 4.1. It was not detected in this study whereas chlorpromazine, thioridazine and dothiepin with the same chemical structure were all detectable. Analysis of the structure of fluphenazine shows a piperazine moiety and it thereby offers additional basic binding sites compared to the other compounds. Such extra binding interferes with the SPME extraction. A similar condition arises with zopiclone (personal communication Dr V. Garner).

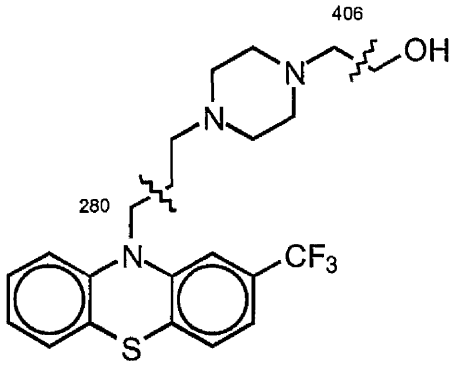
Cocaine was not detected by SPME-GC-MS in case 9. Cocaine in biological matrices is rapidly degraded at room temperature; it is a labile compound, which is easily hydrolysed[200]. In addition, it has been shown that cocaine is destroyed by an esterase in *post-mortem* samples[201]. It seems that the absence of signal for cocaine was due to degradation of cocaine under the long-term period of storage.

Morphine (cases 14 and 16) has two polar hydroxyl groups and a basic nitrogen moiety. Since the SPME-GC-MS method favours non-polar compounds, derivatisation is necessary to change the hydroxyl groups into non-polar derivatives to improve its chromatographic behaviour. Changing the column to a more polar column such as HP-17 may improve the detectability of morphine (personal communication Dr V. Garner).

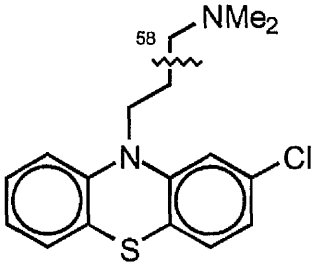
Medazepam and diazepam were detected by the SPME-GC-MS. However, temazepam (case 16) was not detected by SPME-GC-MS. On the other hand, HPLC detected diazepam and temazepam but did not detect medazepam. Medazepam is the least polar and temazepam is the most polar of the three compounds. So medazepam was favoured by the SPME-GC-MS method, but not temazepam: the reversed phase

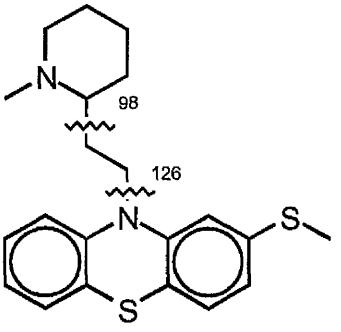
HPLC (more polar) system being preferred instead (personal communication Dr V. Garner).

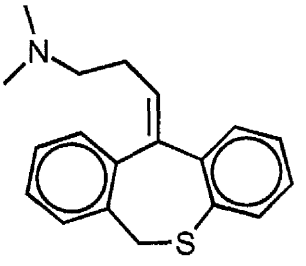
Paracetamol was detected by ToxiA in cases 3 and 5 and by SPME-GC-MS in case 5, but not case 3. This discrepancy may be explained by the presence of different paracetamol concentrations. In addition, paracetamol has a polar hydroxyl group and since the SPME-GC-MS method favours non-polar compounds, paracetamol is not detected at low concentrations. The limit of detection of paracetamol might be enhanced by the use of a derivatisation reagent such as bis(trimethylsilyl)trifluoroacetamide (BSTFA).



fluphenazine







chlorpromazine thioridazine dothiepin

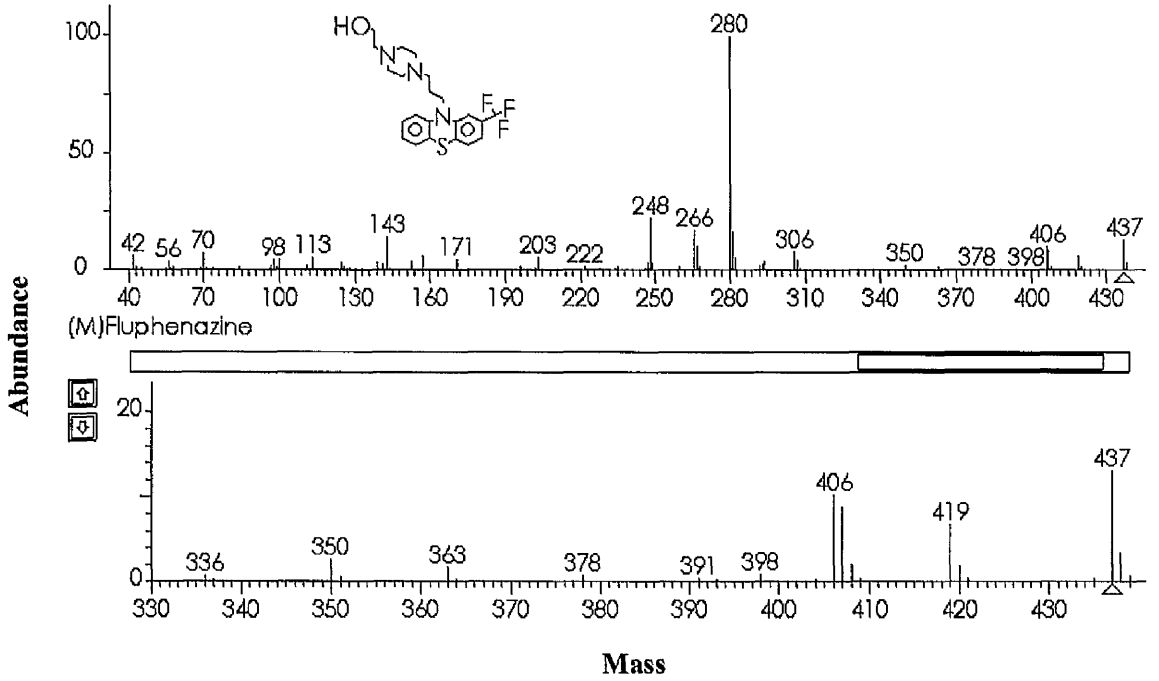


Figure 4.1. Comparison of fluphenazine with chlorpromazine, thioridazine and dothiepin

4.6. Autopsy procedure and sampling techniques

One of the common methods of suicide is self-poisoning with tranquillisers (benzodiazepines), antidepressants and/or analgesics (opiates). For toxicological analysis, different samples including blood, urine, stomach contents, brain, liver, kidney and intestine should be collected and stored in chemically clean containers[202].

4.6.1. Necropsy technique*

The first step in *post-mortem* investigation is a systematic examination of the external features. Hair distribution and colour, eye colour, external ears, ear passages, haemorrhages or colour change of the conjunctivae, lips, teeth and gums are examined carefully in an external examination. The mouth may contain foreign bodies, drugs and/or damaged teeth. Any cyanosis (blueness) in the lips and fingertips, deformities, injuries, swellings and any evidence of needle puncture should be noted. Weight and length of the cadaver should be measured and noted[202].

1. After appropriate external examination the cadaver is opened by a midline anterior incision. This is usually from sternal notch to symphysis pubis. The neck end of the incision may be extended, if required, to the point of the chin, along the side of the neck or outwards over both clavicles.

The anterior jugular vein is usually opened by the incision into the neck and air enters the vein. Movement of the head and neck draws air into the neck veins and the heart. Therefore, if an air embolism is suspected, the abdomen should be opened first and the inferior vena cava should be examined. An air embolism is confirmed by the presence of froth in the vein. The presence of a pneumothorax must be considered before the chest is opened[202].

2. The breast plate, comprising sternum and ribs on each side at the front of the chest, is removed after clipping through the bones. This exposes the heart and lungs. Another common method is a "Y-shape" cutting, which is to cut from behind each ear to a point above the manubrium and continue downwards. The method is usually applied for *post-mortem* examinations of infants or to avoid disfiguring the front of the neck[202].

.*This is based on the procedures described by Gresham and Turner[202], with slight modifications based on personal observation.

3. The intestines are removed. The intestines should be divided between paired ligatures and sampled from different parts of the intestines by cutting between further ligatures. When the body is opened, any fluid in the peritoneal cavity should be collected and cultured. The stomach should be opened up from cardia to pylorus and the whole content should be collected. In the case of poisoning with corrosive compounds the stomach lining should be examined[202].

The tongue and neck structures are loosened by the dissecting knife and the neck structures, heart, lungs, liver and kidneys are removed *en bloc* after cutting through the diaphragm on both sides. The aortic arch, thoracic and abdominal aorta, and the vena cava are included. For the identification of the appropriate side of the kidneys, the left ureter should be cut longer than the one on the right[202].

4. The pelvic organs and tissues are removed separately.

5. After a scalp incision the skull cap is sawn off and the brain removed. The cutting line should be an angled removal to reconstitute the head without unsightly sliding of the calvarium. The examination of the brain should be made immediately after weighing of the brain by the "wet-cutting" method, or suspending of the brain in formalin until fixed. Thinner and more accurate sections can be taken from the fixed brain tissue[203].

6. All organs are dissected free, weighed, measured and sectioned with a post-mortem knife. Blocks are taken for histological examinations. When the eyes are removed, the same colour artificial eyes should be replaced[202].

7. Dissected organs are returned to the body cavities and the external incisions sewn up.

8. Tissue samples for analysis can be taken during the dissection. Normally 100g samples of tissue are adequate for most toxicological analyses. Brain, lungs, hair, adipose tissue, liver and kidney may be sampled[203].

9. Percutaneous samples, taken with a trephine or possibly through a small incision, could be taken of lung, liver and kidney (probably with increasing difficulty). Puncture of the heart and urinary bladder or fluid-containing body cavities could be

performed with a hypodermic needle and syringe with aspiration before the body is dissected[202].

10. Fluids for drug analysis must be collected carefully in the ways described below. As described later in Section 4.5.2, inappropriate collection may cause contamination of the samples or render the results impossible to interpret.

11. In cases where the cause of death is not immediately apparent at necropsy, it is advisable to take tissue samples in case the need for toxicological analysis becomes obvious at a later stage in the investigation.

Blood and urine:

Blood samples should be taken from the external iliac vein. After the abdomen is opened the vein can be easily found. The blood samples should be taken into two separate containers, one of which should contain sodium fluoride (1.5%w/v) to prevent the ethanol concentration changes that occur in *post-mortem* samples after sampling[202]. Quantification of the drugs is often allowed by the analysis of the blood samples.

Urine samples are best taken by direct puncture of the exposed bladder[202]. They may also be obtained by the insertion of a urethral catheter before the necropsy starts. If the patient was catheterised shortly before death, the laboratory should be informed, because lignocaine or other local anaesthetics present in the lubricant gel may contaminate the urine[204].

Gastric contents:

The best samples of stomach contents are provided by either clamping or applying ligatures to the lower end of the oesophagus and the pylorus before the stomach is dissected free. In cases of suspected drug overdosage, ideally, all of the stomach contents should be submitted to the laboratory, otherwise a note of the total volume of the stomach contents should accompany the sample that is submitted[204].

If distinct tablets or capsules are observed in the stomach contents, these should be carefully extracted, and put in individual containers[203]. Identification of such materials can be carried out by reference to a computerised database of

pharmaceutical products, or by using the website of "the identification CD-ROM for tablets and capsules" TICTAC (http://www.tictac.org).

Vitreous humour:

Vitreous humour, which is behind the lens of the eye, can be sampled by puncturing the eyeball. It can usually be obtained intact even if a corpse has been extensively burnt or damaged, and does not suffer from the extensive *post-mortem* changes of blood. It is likely to be free from microorganisms, which can raise blood ethanol concentrations by up to 1500 mg/L, and may be useful for determining some drug concentrations when a satisfactory specimen of blood is not available or when only limited examination of the body is possible[205,206]. The vitreous humour should be replaced with an equal volume of saline solution to prevent the eye from collapse[202].

Cerebrospinal fluid:

This can be sampled by using a special needle inserted above the spine of the second vertebra and pushing it upwards in the midline. Opening the spinal canal anteriorly and aspirating by syringe is the other method of sampling of cerebrospinal fluid[207].

Liver and bile:

It is usual to take a portion of the right lobe of the liver since it should be uncontaminated with bile[203]. Bile is useful for some drug analyses, such as morphine and chlorpromazine, which are concentrated by the liver and excreted into the gall bladder. Direct sampling of bile into a bottle is recommended, since it is usually too viscous to be collected by a needle[203].

Lungs:

Entire lungs should be submitted for the diagnosis of death by inhalation of toxic gas or vapour. The specimen should be placed in a glass specimen jar or nylon bag and stored at 4°C prior to transport to the laboratory[203].

Brain:

A portion of brain (about 100g) should be taken. It may be useful in the investigation of death due to gases and volatile substances and some drugs e.g.

cocaine. The brain sample should be placed in a glass container or nylon bag and deep-frozen prior to transport to the laboratory[203].

Hair:

Hair specimens may be useful in the investigation of death related to drug abuse (particularly opiates and methadone). Analysis of hair (approximate rate of growth 1 cm per month) is able to provide useful information concerning the chronocity of drug abuse, which is valuable in the interpretation of *post-mortem* drug concentrations[204].

Containers and syringes:

Containers found at the scene are frequently useful for indicating the nature of any substance injected, ingested or inhaled. In some instances, however, substances found at the scene are not implicated in the fatality, highlighting the danger of relying entirely on circumstantial evidence without analysis of body fluids.

4.6.2. Special problems of *post-mortem* samples

There are large overlaps between the therapeutic and toxic concentration ranges quoted in a number of texts. This means it is difficult to conclude confidenly, which is a toxic dose. Some of the reasons are mentioned in Table 4.18. Flanagan[186] described a website (http://www.leeds.ac.uk/acb/annals/netwise) designed to assist in the interpretation of analytical toxicology results.

Table 4.18. Factors likely to contribute to the uncertainty of reference ranges for drugs

Pharmacokinetics of the drug
Age (neonates, children, adults, elderly)
Variations in the rate of metabolism
Idiosyncratic reaction and tolerance
Drug changes *post mortem*
Presence of other drugs
Post-sampling redistribution of drug between serum and cells
Post-sampling instability of drug
Differences in analytical methods employed
Presence of concomitant disease
Variations in sampling site
Variations in elapsed time between administration of the drug and death
Post-mortem redistribution of the drug
Chemical characterisations of collection syringes and needles

Containers:

New containers can be regarded as being drug-free and generally cause few problems. The use of plastic containers and disposable plastic pipette tips should be avoided because they commonly add plasticisers, in the form of phthalates, to specimens. Cleaning of the containers using chromic acid can cause some problems in chromium poisonings. Note, also, that containers used for collection of tissues and fluid for analysis must not contain even the slightest traces of any preserving fluids such as formol saline or formaldehyde. These may interfere with the analysis and may also react chemically with drugs and other analytes of interest and thereby make identification impossible[204].

Drug changes post mortem

Drugs and the concentrations of their metabolites in blood are interpreted by comparison with previously reported concentrations corresponding to therapeutic, toxic and fatal conditions[203]. This is not as simple as comparison of the results, because blood drug concentrations have been shown to change significantly during the *post-mortem* period and so the concentrations may be different depending on their sampling sites[208,209,210].

The concentrations of barbiturates in samples from the inferior vena cava and the femoral vein can differ by a factor of ten [211]. Concentrations of the tricyclic antidepressant amitriptyline can vary from site to site by a factor of four[212]. Similar results have been reported for digoxin[213], nortriptyline, desipramine and doxepine[214].

During drug absorption, there is distribution of the drug from the blood to the tissues and this distribution phase lasts for about thirty minutes to two hours for most of the drugs. During this period there can be a remarkable difference between arterial and venous drug concentrations. This might also be reflected in site differences in *post-mortem* blood drug concentrations, where a person has died during this absorptive period[210].

When bodies are recovered after some time, their decomposition can cause problems in obtaining reliable results. Decomposition can destroy some drugs, such as β_2-agonists, cocaine and nitrobenzodiazepines[201,215,216]. However, paracetamol, salicylates (including aspirin), morphine, diazepam and amitriptyline are much more stable[217].

Compounds that are susceptible to attack by esterases are changed rapidly *post mortem*. Examples of these compounds include cocaine and most prodrugs including those of the benzodiazepines[194,201]. Nitrobenzodiazepines such as flunitrazepam are rapidly converted to the 7-amino forms. Sulphur-containing drugs such as thioridazine are unstable *post mortem* and they are converted partly to the sulphoxide and sulphone forms[215,216]. This even occurs in blood samples frozen at –20°C. Therefore, biological samples must be taken aseptically to minimize any bacterial contamination, contain preservative such as fluoride and be frozen to less than -20°C to reduce losses[194].

Redistribution between cells and plasma/serum:

Most drugs and poisons are probably stable in biological material for many months, particularly if frozen, although there are some notable exceptions. For some analyses, special arrangements need to be made to prevent loss of analyte by degradation or other processes. A number of commonly analysed drugs are very unstable in blood, notably cocaine[218,219] (and its metabolite in urine[220]) and benzodiazepines, e.g. nitrazepam[221].

Temperature:

Several studies have addressed the effect of temperature on stability. One study[222] found that 11-nor-Δ-9-tetrahydrocannabinol-9-carboxylic acid was lost from urine on freezing below -16°C although benzoylecgonine, phencyclidine, codeine, morphine, amphetamine, metamphetamine and lysergic acid diethylamide were unaffected[197,222,223].

Site and time dependence of post-mortem blood sampling:

It is clear that there are site- and time-dependent changes in drug concentrations after death. Various studies have shown significant differences in drug concentrations between arterial and venous blood, between blood vessels in different parts of the body and between plasma and cells.

In one study, paracetamol concentrations were found approximately to double in peripheral (femoral) blood and to increase more than six fold in central (heart and inferior vena cava) blood over the 12-h period after death[224]. Gomez *et al.*[224] offered several possible explanations, for the redistribution of drugs to the central compartment, which included: (a) diffusion from specific tissue sites of higher concentration postingestion (such as liver or mesenteric portal vessels) to central vessels in close proximity; (b) diffusion of unabsorbed drug in the stomach to the heart and the inferior vena cava; and (c) collection of blood from the corpse over time may cause the blood to `circulate', i.e. suction with a syringe moves blood or blood fluid along central vessels from regions of higher concentration. Peripheral vessels may be relatively protected by distance from this effect.

Other studies have suggested further mechanisms of *post-mortem* redistribution of drug concentrations, such as cell death, changes in drug-binding proteins, changes in the permeability of inter-tissue barriers after death, and *post-mortem* changes in the pH and ionic strength of intra- and extracellular fluids[210,224-226].

The mechanisms whereby these *post-mortem* changes come about are not fully understood and are likely to be complex. Pounder[209], in a review, discusses these mechanisms and concludes: "For interpretive purposes, the ideal toxicological sample is a peripheral blood specimen obtained from a ligated vessel immediately after death. All autopsy specimens fall short of this ideal, but the more they do so, the more contentious will be the interpretation of the analytical results."

A great deal of work on *post-mortem* distribution has been reported in recent years and has included investigation of cimetidine[225], amiodarone[227], clozapine[228], digoxin[227], disopyramide[226], dothiepin[229], heroin[230,] imipramine[210,] lignocaine[226], midazolam[229], tricyclic antidepressants[214] and zopiclone[231]. The changes caused by *post-mortem* redistribution are less likely to cause problems with interpretation when the concentrations obtained by analysis are many times higher than therapeutic concentrations.

Pharmacokinetics:

Knowledge of pharmacokinetics is useful in interpreting drug concentrations in blood. Pharmacokinetic data obtained on adult volunteers administered therapeutic doses are not necessarily helpful in interpreting drug concentrations in blood from corpses after ingestion of excessive amounts. Possibly more helpful is pharmacokinetic data on long-term therapy and data on kinetics in overdose. However, these data should be applied with caution[217]. The usual subjects of pharmacokinetic reports are the hospitalized, poisoned patients whose airway, cardiac output, hydration status, renal and hepatic function, electrolyte and acid/base status had been well maintained. In these, the distribution, metabolism and elimination of drugs and poisons are likely to be very different from the common subject of the autopsy report, who has lain in a cramped position for 24 or more hours before dying with diminished cardiac output, falling blood pressure, decreased

tissue perfusion, impaired ventilation, tissue hypoxia, acidosis, progressive dehydration and renal and hepatic failure.

Interaction:

Interactions between drugs can cause both enhancement or reduction of effect and arise by a number of mechanisms described in any standard pharmacology text. These should be borne in mind when interpreting toxicology data[217]. The presence of more than one drug of the same class will usually result in a summation of effect, which may be very significant. Other combinations exhibit potentiation, where the effects of both drugs together are greater than the summation of the effects of each separately.

Tolerance:

Individuals respond differently to drugs not only from time to time, but from other individuals. The different responses may be due to age, race, the presence of disease and/or other undetermined factors. Tolerance is well-recognized in and by regular users of opiates and other drugs; it builds up over a period of time and is gradually lost during abstinence. It is said to have developed when it becomes necessary to increase the dose of a drug to obtain the effect previously obtained with a lower dose. Most tolerance is acquired tolerance and may be due to decreased efficacy at receptor sites or increased metabolism due to enzyme induction. Decreased efficacy at receptor sites allows individuals to tolerate much higher blood and tissue concentrations. There is commonly cross-tolerance between drugs with a similar structure, as shown by the substitution of methadone for heroin in the treatment of addicted individuals, and occasionally between drugs whose structures are dissimilar[136].

Effects of treatment in hospital:

The collapsed or unconscious patient who is transferred to hospital and survives for a period of time before dying often presents the most difficult investigative and interpretive problem. Treatment with intravenous fluids and other manoeuvres usually renders *post-mortem* blood and tissues devoid of detectable drugs. It is essential to have available as many of the *ante-mortem* blood and urine samples as possible in order to demonstrate the presence of a toxic agent. Hospitalization may

also complicate the interpretation of drug concentrations in those abusers in whom tolerance has developed.

Many drugs may be administered in the course of diagnosis or treatment of the unconscious patient. Unless a detailed record of all therapy is available to the laboratory, it is often impossible to determine which drug(s) is likely to be the causative agent[232].

4.6.3. Drug analysis of *post-mortem* tissue samples

In *post-mortem* drug analysis, the most commonly used sample matrix is whole blood. However, as previously explained, *post-mortem* changes can denature this matrix, thus biasing analytical findings. Vitreous humour is thought to be less affected by *post-mortem* changes and should have the potential to provide a more reliable estimation of *ante-mortem* drug concentrations. Some studies have been published comparing analysis of ethanol and benzodiazepines in blood and vitreous humour [233-235]. Scott and Oliver[235] have found some correlation, for temazepam and diazepam, between vitreous humour and blood (R^2=0.789 and 0.724, respectively).

Vitreous humour is taken by puncturing the eyeball, therefore, it might be possible to design a special SPME needle with a robust sheath, which has fine holes on the surface, for direct sampling and thus direct extraction of drugs from the vitreous humour. In the first step, the needle should puncture the cornea to reach the vitreous liquid for direct extraction. The cadaver temperature is far below the optimum temperature for extraction of 60°C and, therefore, the needle should remain exposured to the vitreous humour for a relatively long time to maximise the extraction. Finally, the needle should be withdrawn from the eye and then inserted through the injection port septum of a gas chromatograph. The same procedure, with the longer needle necessary, might be used for the direct extraction of urine from inside the urinary bladder.

A suggested protocol for drug analysis of tissue samples might be the procedure below:

1. A small amount of appropriate tissue sample is taken during the dissection, or prior to an autopsy, and is put in a glass specimen jar.

2. The sample of the tissue is cut off and transferred to a weighing boat and the weight is noted.
3. The tissue is cut up into small pieces with a scalpel and scissors and transferred to a labelled glass universal bottle.
4. Deionised water (10 mL) is added and the bottle is capped.
5. The tissue is frozen and homogenised using a percussion mortar.
6. Subtilisin A, a proteolytic enzyme, (10 mg) is added to the homogenised contents of the bottle. Alternatively, centrifugal filter devices (Centrifree® or Amicon® Ultra) can be used to separate proteins from the sample.
7. The bottle is recapped and incubated at 50°C for one hour.
8. The contents of the bottle are centrifuged and 2 mL of clear supernatant is transferred into a beaker.
9. Selected buffer (2 mL), which contains 5 mol/L NaCl, is added.
10. The pH is adjusted and the solution is transferred to an appropriately labelled 5 mL screw top glass vial.
11. The selected fibre is placed into the sample vial and the sample is extracted for 45 min at 60°C.

4.7. Future investigations

4.7.1. Autopsy studies

SPME needles are sufficiently rigid to penetrate tissues and organs and would probably be a satisfactory way to extract drugs from urine when inserted into the urinary bladder and from vitreous humour when inserted into the eye. The presence of proteins in tissues could contaminate the surface of the SPME fibres and prevent satisfactory sorption of the drugs. Similarly, food components present in the gastrointestinal fluids could prevent application of SPME needles directly to tissues and gut contents at autopsy. However, urine, vitreous and cerebrospinal fluids normally having low protein content may be amenable to the direct application of SPME for solute extraction.

4.7.2. Fieldwork

SPME fibres are simple to use, easily portable and extracted solutes are stable while absorbed onto the fibre. These factors indicate that SPME may be very suitable for fieldwork such as extraction of drugs and poisons directly from a cadaver. The presence of proteins, almost universal in biological tissues and fluids, is likely to be a major problem with this application.

At the sites of accidents and fatalities involving many diverse compounds, SPME may prove useful for extracting significant analytes from the air, water and soil, which surround the corpse.

SPME fibres may readily absorb significant environmental contaminants and therefore be amenable to use in analysis, e.g. of pesticides, industrial effluents, chemical weapons and vehicle exhaust components.

While there are a number of small, portable GC and HPLC instruments available for fieldwork, the limiting factor has always been sample processing. SPME simplifies sample processing significantly and is likely to make a major impact on "on-site" analysis.

If a delay were likely in returning the used SPME fibres to the laboratory for analysis, some method of stabilising the extracted analytes, e.g. liquid nitrogen, may need to be applied.

4.7.3. Biological compounds

Several papers have appeared in the recent literature demonstrating the application of SPME to the extraction and analysis of other biological compounds. This list could be extended in the future to include sugars, peptide and steroid hormones, nucleic acids, fatty acids, proteins, co-enzymes and vitamins.

4.7.4. Biological reactions

The study of biological, including enzyme, reactions usually involves the halting of the reaction in order to analyse the products. Reactive intermediates are usually lost in this process. SPME may allow the trapping of these reactive intermediates and their removal for analysis without significantly disturbing the overall reaction. SPME

may, therefore, be able to make a significant contribution to the investigation of biochemical mechanisms and disease states.

4.7.5. Pharmacology studies

SPME may allow useful investigation of both the pharmacokinetic and the pharmacodynamic properties of drugs in a way analogous to the investigation of biological reactions. It can be envisaged that, following a dose of a drug, the different components i.e. of free drug, bound drug, intermediate drug metabolites and final metabolite, may be absorbed onto the SPME fibre when further reaction would cease. This would allow quantitative analysis of each component and calculation of a number of parameters such as distribution constants.

4.7.6. Common non-drug poisons

A large number of inorganic and non-drug compounds are of significance in toxicological and forensic investigations. These include alcohol, lead, cyanide, oxalate, nitroprusside, thiocyanate and paraquat, all of which it may be possible to extract using SPME fibres.

4.7.7. Automation

While there are a small number of devices on the market for the automation of solvent and solid phase extraction techniques, all of them are based on a "batch principle" in which all the samples and calibrators are extracted together and then each in turn is injected onto a gas- or liquid-chromatograph. The available automatic device developed for SPME allows the extraction of calibrators and samples sequentially with each being injected onto the analyser as soon as it is extracted, and extraction of the next occurring while the previously extracted calibrator or sample is being analysed.

With all automated solvent and solid phase extraction methods there are considerable consumable costs and environmental issues associated with the solvents and the solid-phase columns or discs. SPME fibres, because they are re-usable many times, have insignificant cost implications and only minimal impact on the environment.

4.7.8. More selective coatings

Manufacturers are constantly developing new fibre coatings that possess greater selectivity, greater avidity and with a wider range of properties. These new coatings need to be exploited with imagination and investigated in novel applications.

4.7.9. Further miniaturisation

It is likely that fibres, and holders, of smaller physical dimensions will be developed allowing extraction of smaller samples. Ultimately, appropriately designed and constructed fibre devices may become available which could be inserted into a vein or tissue of a patient in order to measure directly the concentration of normal or disease-related components or a wide range of toxins.

Chapter 5

Conclusions

5. Conclusions

A direct immersion SPME-GC-MS method has been developed for the analysis of drugs in *post-mortem* samples. This is the first time that the solid phase microextraction method has been applied to extract the drugs of interest from such samples and the analytical procedure is detailed herein.

SPME is a simple and solventless extraction method for biological samples and it needs only a small volume. However, a careful optimisation of the SPME step is needed to obtain good reproducibility and sensitivity.

Outlined in this work is a successful and sensitive SPME method for the detection and quantification of the targeted drugs. Optimising some parameters, such as pH, addition of sodium chloride and extraction temperature, considerably enhanced the yield of extraction. The optimum conditions were provided by the use of a polyacrylate or Carbowax/DVB fibres, 45 minutes extraction time at 60°C, pH7 or 10 and four minutes desorption time. The best fibre for the extraction of the targeted drugs is polyacrylate and followed closely by Carbowax/DVB. Some of the currently available fibres are combinations of two or more coating materials, and a customised fibre containing polyacrylate, Carbowax and divinylbenzene, which is not currently manufactured, is recommended for use in future studies.

The results from this study show that the direct immersion SPME procedure is useful for the extraction of the majority of antidepressants, benzodiazepines, analgesics and local anaesthetics in spiked plasma and *post-mortem* samples. However, there are some limitations for the extraction of a few of the drugs of interest such as morphine, cocaine, nordiazepam, tramadol and fluphenazine. Probable causes of this include heat instability, deterioration during a long period of storage and retention of the drugs by the SPME fibres and/or the column's coatings. These problems can be solved by derivatising the drugs to stable derivatives or compounds that do not bind irreversibly to the SPME fibres.

Whereas cocaine was extracted and detected using Carbowax/DVB fibres at pH7 from spiked plasma, it seems that the cocaine existing in the *post-mortem* samples was broken down under storage. This could be due to an esterase present in the

plasma as suggested by Shannon *et al*[201]. In addition, it has been shown that some other drugs including benzodiazepines and β_2-agonists are altered under storage even at -20°C[194,216]. So, samples that are likely to contain cocaine and/or its metabolites should be analysed promptly and before long periods of storage.

In comparison to other sample preparation methods the chromatographic background is very low. Therefore the low m/z values such as 58, 91 and 98, which form the base peaks of many drugs, but are normally not usable because of high matrix burden, can be successfully used in quantitative GC-MS-SIM methods (personal communication, Dr V. Garner).

This study shows that the developed SPME method is useful for the detection and quantification of the drugs in *post-mortem* samples. It had the highest rate of detection over the range of drugs at 70.5%, compared to 60.7% for HPLC, 39.3% for ToxiA, 36.4% for REMEDI, 28.6% for colorimetry, 23.5% for EMIT, and 18.8% for RIA. It is potentially a method that could be employed on a regular basis because of its high detection rate, ease of use and lack of need of pollutants such as organic solvents or radioisotopes.

Chapter 6

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6. References

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Appendix A

Appendix A

A1. Sorption

Chromatography is a powerful tool for achieving separations. It is based upon partition and relative movement of phases. The retention of material on or in the stationary phase is often dependent upon some forms of sorption. However, SPME depends upon a simple partitioning and sorption on a stationary phase. If the polymer film is a liquid phase (such as polyacrylate coating), analyte molecules are absorbed into the film and the extraction process is the analyte partitioning between two liquids.

A1.1. Models of sorption

When a gas or vapour is brought into contact with a solid, part of it is taken up by the solid. The molecules that leave from the gas phase either enter the inside of the solid, or remain on the outside attached to the surface. The former phenomenon is termed absorption (or dissolution) and the latter is termed adsorption. When the phenomena occur simultaneously, the process is termed sorption. The solid that takes up the gas is called the adsorbent, and the gas or vapour taken up on the surface is called the adsorbate.

Molecules can attach themselves to surfaces in two ways. In physisorption (physical adsorption), there is a weak attraction of the adsorbate to the surface usually by van der Waals forces[236]. During the process of physisorption, the chemical identity of the adsorbate remains intact. In chemisorption (chemical adsorption), the adsorbate sticks to the surface of the solid by the formation of a covalent chemical bond. This interaction is much stronger than physisorption, and, in general, chemisorption has more stringent requirements for the compatibility of adsorbate and surface site than has physisorption.

The energy of adsorption depends on the extent to which the available surface is covered with adsorbate molecules. This is because the adsorbate molecules can interact with each other when they lie upon the surface. The extent of surface coverage is normally expressed as the fractional coverage θ:

$$\theta = \frac{\text{Number of occupied adsorption sites}}{\text{Total number of possible sites}} \qquad 1$$

The rate of adsorption $\theta^{\bullet}$ is the rate of change of surface coverage, and can be determined by observing the change of fractional coverage with time.

At any temperature, the adsorbate and the surface come to a dynamic equilibrium, that is, the chemical potentials of the free adsorbate and the surface-bound adsorbate are equal. The chemical potential of the free adsorbate depends on the pressure of the gas, and the chemical potential of the bound adsorbate depends on the coverage θ. Thus the coverage at a given temperature is a function of the applied adsorbate pressure. The variation of θ with pressure at a given temperature is called an adsorption isotherm.

Several adsorption isotherms have proven useful in understanding the process of adsorption. The simplest isotherm is based on the assumptions that every adsorption site is equivalent and that the adsorption and desorption rate is independent of whether or not nearby sites are occupied; this is called the Langmuir isotherm[236].

The dynamic equilibrium is

$$A + S \leftrightarrows A.S \qquad 2$$

Where A is the free adsorbate, S is the free surface and A.S is the substrate bound to the surface.

The rate of adsorption will be proportional to the pressure of the gas (P) and the number of vacant sites, N(1-θ), for adsorption. If the total number of sites on the surface is N, then the rate of change of the surface coverage due to the adsorption is:

$$\frac{d\theta}{dt} = k_a PN(1-\theta) \qquad 3$$

The rate of change of the coverage due to the adsorbate's leaving the surface (i.e. the rate of desorption) is proportional to the number of adsorbed species, Nθ:

$$\frac{d\theta}{dt} = k_d PN\theta \qquad 4$$

In these equations, k_a and k_d are the rate constants for adsorption and desorption, respectively, and P is the pressure of the adsorbate gas.

At equilibrium, the coverage is independent of time and thus the adsorption and desorption rates are equal.

$$k_aPN(1-\theta) = k_dPN\theta \qquad 5$$

Hence, the solution to this condition gives a relation for θ:

$$\theta = \frac{kP}{kP+1} \qquad 6$$

Where $k=k_a/k_d$

At pressure P, if y is the amount of gas adsorbed and y_m the amount of gas required for complete coverage as a monolayer. Then

$$\theta = \frac{y}{y_m} \qquad 7$$

Hence

$$y = \frac{y_m kP}{kP+1} \; or \; \frac{P}{y} = \frac{1}{ky_m} + \frac{P}{y_m} \qquad 8$$

Hence, a plot of p/y against p should give a straight line of slope $1/y_m$ and intercept $1/ky_m$. The Langmuir isotherm is generally applied to all cases involving chemisorption. Physisorptions usually give non-linear and sigmoid curves[236].

The Langmuir isotherm ignores the possibility that the initial monolayer may act as a substrate for further adsorption. The most widely used isotherm dealing with

multilayer adsorption was derived by Brunauer, Emmett and Teller (1938), and is called the BET isotherm[237].

If the rates of condensation and evaporation are assumed to be equal for the first layer, then:

$$a_1 P S_0 = b_1 S_1 e^{-E_1/RT} \qquad 9$$

Where

P=pressure

S_0=effective surface area for sorption of first layer

S_1=effective surface area for sorption of second layer

a_1=a constant, independent of area covered

b_1=a constant, independent of area covered

E_1=heat of adsorption of first layer, which is independent of area covered

R=the gas constant

T=the absolute temperature

If the evaporation and condensation properties of the second and higher layers of molecules of sorbate are assumed to be equivalent to those of the liquid state,

i.e. $E_2=E_3=\ldots\ldots=E_i=E_1$

Where E_1=the heat of liquification

and $b_2/a_2=b_3/a_3=b_i/a_i=g$

Where g is a constant.

Hence, for an infinite number of layers:

$$V = \frac{V_m C P}{(P_0 - P)\{1 + (C-1)(P/P_0)\}} \qquad 10$$

Where the number of layers is finite (=n)

$$V = \frac{V_m C(P/P_0)}{(1-P/P_0)} * \frac{1-(n+1)(P/P_0)^n + n(P/P_0)^{n+1}}{1+(C-1)(P/P_0)^n - C(P/P_0)^{n+1}} \quad 11$$

Where V_m=volume of sorbate when whole surface is covered with a monolayer

P_0=saturation pressure of sorbate

$$C = \frac{e^{(E_1-E_L)}}{RT}(approximately) \quad 12$$

The constant C represents the relative strengths of adsorption to the surface and condensation of the pure adsorbate.

The BET isotherm predicts that the amount of adsorption increases indefinitely as the pressure is increased since there is no limit to the amount of condensation of the adsorbate. It is found to describe adequately the physisorption at intermediate coverage (θ=0.8-2.0), but fails to represent observations at low or high coverage.

If the sorbate is water, of density=1, V and V_m can be replaced by r and r_m (weights). Hence, a plot of $p/r(p_0-p)$ against p/p_0 gives a straight line of slope $C-1/r_mC$ and intercept $1/r_mC$.

The Smith model (Smith, 1947)[238], makes similar assumptions to that of Brunauer, Emmett and Teller, but regain is regarded as of two types, one with normal heat of condensation and the other one bound by excessive forces. Hence, if r=regain, r_b=weight of bound water, and r_c=weight of condensed water, thus:

$$R=r_b+r_c=r_b+r_1\log_e(1-p/p_0) \quad 13$$

Where r_1=weight of normally condensed water required to saturate the first layer of the r_c fraction.

If the matrix of the gel swells, r must be replaced by r/[1+f(r)], which is approximately r/(1+r)=W (the weight fraction of sorbate in the swollen gel lattice).

Where $W=W_b+W_c=W_b-W_1\log_e(1-p/p_0)$

Hence, a plot of W against $-\log_e(1-p/p_0)$ should give a straight line once p/p_0 is large enough to complete saturation of binding sites of sorbate as fast as they appear as the gel swells.

The model of Hailwood and Horrobin (1946)[239] is a summation of Langmuirs' adsorption isotherm and a Raoult's law solution curve. The former predominates at low relative humidity and the latter at high relative humidity.

The equation is:

$$\frac{Mr}{1800} = \frac{\alpha h}{1-\alpha h} + \frac{\alpha\beta h}{1+\alpha\beta h} \qquad 14$$

Where h=relative humidity and α, β, and M are constants.

r is the total grams of water absorbed per 100 grams of dry polymer.

$$Log_e 100\alpha = \frac{-\Delta G^0_{sol.}}{RT} \qquad 15$$

$$Log_e \beta = \frac{-\Delta G^0_{chem.}}{RT} \qquad 16$$

And $-RTLog_e 100\alpha\beta = -\Delta G^0_{tot.}$

$\Delta G^0_{sol.}$=Gibbs free energy of solution

$\Delta G^0_{chem.}$=Gibbs free energy of chemical bonding

Curve fitting is done by putting equation 12 into the form:

$A+Bh-Ch^2=h/r$ 17

The constants A, B, and C cannot be independently determined. A criticism of this model is that it generates an equation, which is similar to a polynomial form. Such equations can be fitted to a very wide range of curves by selection of appropriate constants, but are of uncertain physical meaning where these constants cannot be measured independently of the model to which they are applied.

There are two distinct types of SPME coatings available. The most widely used PDMS is a liquid coating. Polyacrylate (PA) is a solid crystalline coating that turns into liquid at desorption temperatures. Both PDMS and PA extract analytes via absorption. The remaining coatings, including PDMS/DVB, Carbowax/DVB, and Carboxen are mixed coatings, in which the primary extraction phase is a porous solid[68]. These coatings extract analytes via adsorption rather than absorption. In adsorption, there are a limited number of surface sites where the process can take place. Absorption is a non-competitive process and therefore quantitative SPME analysis with the use of liquid coatings such as PA is usually unaffected by matrix composition. Also, the linear range of analyte uptake is typically very broad. On the other hand, adsorption is a competitive process and therefore extraction conditions affect the amount of analyte extracted by the fibre. These coatings can be expected to perform well for quantification provided that the concentration of analyte of interest is far below that required to saturate the surface. The sorption isotherm will then be almost linear and, since the deviation from linearity will be too small to be measured, the isotherm can be treated as linear.

Since most of the polymer coatings used are normal liquid polymers (such as polyacrylate coating that has been used for the quantitation of the analytes) diffusion is the only mass transport mechanism and so it determines the migration of the analyte molecules in the system[240]. The dynamics of this absorption process have been modelled by Louch *et al.* (1992)[241]. The amount absorbed as a function of time (the extraction profile) can be determined using Fick's second law of diffusion.

$$\frac{\partial C(x,t)}{\partial t} = D\frac{\partial^2 C(x,t)}{\partial^2 x} \qquad 18$$

Where D is the diffusion coefficient of the analyte and C (x,t) is the concentration of the analyte in the fibre at position x and time t. The mass absorbed into the fibre coating can be expressed as:

$$M = \int_a^b C_f(x,t)dx \qquad 19$$

Where C_f is the concentration of analyte in the stationary phase, a and b are the radius of the silica core and coated fibre, respectively, and (b-a) is the film thickness of the fibre coating.

In direct SPME, the fibre is immersed into the sample matrix to isolate target analytes. It is a two-phase system, which contains the fibre and sample matrix. For a two-phase system, at equilibrium, the mass balance of the system can be described as:

$$C_0V_s = C_fV_f + C_sV_s \qquad 20$$

Where C_0 is the initial concentration of the analyte in the aqueous phase, C_f and C_s are the concentrations of the analyte in the fibre coating and the aqueous phase, respectively, and V_f and V_s are the volumes of the stationary phase and the sample, respectively. When the two-phase system reaches equilibrium, we also have:

$$K = \frac{C_f}{C_s} \qquad 21$$

Where K is the partition coefficient of the analyte between the fibre coating and the aqueous phase. By combining equations 20 and 21, the amount of analyte, n, absorbed by the stationary phase, after the system reaches equilibrium, can be expressed as:

$$n = \frac{C_0V_fV_sK}{KV_f + V_s} \qquad 22$$

Equation 20 clearly indicates the linear relationship between the mass of analyte absorbed by the fibre coating and the initial sample concentration.

Appendix B

Appendix B

Appendix B details the purity and ideal storage conditions of the drugs investigated. This information was obtained from the certificates of analysis provided by the manufacturers. These certificates appear in this appendix.

Table B1: Chemical purities and storage conditions of standard compounds obtained from Sigma-Aldrich.

Compound	Chemical purity	Storage condition
Procyclidine hydrochloride	100%	2-8° C (methanolic solution)
Prazepam	> 99%	2-8° C (methanolic solution)
Oxazepam	99%	2-8° C (methanolic solution)
Nitrazepam	>99%	2-8° C (methanolic solution)
Loxapine	>99%	2-8° C (methanolic solution)
Flunitrazepam	>99%	2-8° C (methanolic solution)
Chlorpromazine hydrochloride	100%	2-8° C (methanolic solution)
Chlordiazepoxide hydrochloride	>99%	2-8° C (methanolic solution)
Bupivacaine	>99.9%	2-8° C (methanolic solution)
Clonazepam	>99%	2-8° C (methanolic solution)
Cocaine hydrochloride	>99%	2-8° C (methanolic solution)
Amitriptyline hydrochloride	>99%	2-8° C (methanolic solution)
Clomipramine hydrochloride	>98.8%	2-8° C (methanolic solution)
Trimipramine maleate	99%	2-8° C (methanolic solution)
Protriptyline hydrochloride	>99%	2-8° C (methanolic solution)
Haloperidol	>99%	2-8° C (methanolic solution)
Imipramine	99%	2-8° C (methanolic solution)

Note: Certificates of analysis are as supplied on the internet. Separate certificates were not given for the particular samples used.

Table B2: Chemical purities and storage conditions of standard compounds obtained from Promochem Ltd.

Compound	Chemical purity	Storage condition
Heroin	99%	Protect from light, store in freezer
Nortriptyline	99%	Refrigerate, protect from light
Promethazine	99%	Refrigerate, protect from light
Midazolam	99%	Refrigerate, protect from light
Clobazam	99%	Refrigerate, protect from light
Benzoylecgonine	99%	Refrigerate, protect from light
Pseudoephedrine	99%	Refrigerate, protect from light
Thioridazine	99%	Refrigerate, protect from light
Meperidine	99%	Refrigerate, protect from light
Morphine	99%	Refrigerate, protect from light
Mianserin	99%	Refrigerate, protect from light
Codeine	99%	Refrigerate, protect from light
Diazepam	99%	Refrigerate, protect from light
Dihydrocodeine hydrochloride	99%	Refrigerate, protect from light

SIGMA

Certificate of Analysis

TEST	LOT {SAMPLE} RESULTS
Product Name	Procyclidine hydrochloride
Product Number	P3794
CAS Number	1508765
Formula	$C_{19}H_{29}NO \cdot HCl$
Formula Weight	323.9
APPEARANCE	WHITE POWDER
SOLUBILITY	CLEAR COLOR LESS SOLUTION AT 50 MG/ML IN ETHANOL
PURITY†	100%
QC ACCEPTANCE DATE	MARCH 2002

David Feldker, Manager
Analytical Services

†Chemical purity was determined by TLC and perchloric acid titration.
The safety data sheet is available on website: http://www.sigma-aldrich.com.

SIGMA

Certificate of Analysis

TEST	LOT {SAMPLE} RESULTS
Product Name	Prazepam
Product Number	P3654
CAS Number	2955386
Formula	$C_{19}H_{17}ClN_2O$
Formula Weight	324.8
APPEARANCE	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS SOLUTION AT 50 MG/ML IN CHLOROFORM
ELEMENTAL ANALYSIS	70.2% CARBON 8.4% NITROGEN
IR SPECTRUM	CONSISTENT WITH STRUCTURE
PURITY†	GREATER THAN 99%

David Feldker, Manager
Analytical Services

†Chemical purity was determined by TLC.
The safety data sheet is available on website: http://www.sigma-aldrich.com.

SIGMA

Certificate of Analysis

TEST	SPECIFICATION	LOT {SAMPLE} RESULTS
Product Name	Oxazepam	
Product Number	O5254	
CAS Number	604751	
Formula	$C_{15}H_{11}ClN_2O_2$	
Formula Weight	286.7	
APPEARANCE	WHITE TO OFF-WHITE POWDER	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS SOLUTION AT 10 MG/ML IN CHLOROFORM:METHANOL (1:1)	CONFORMS
IDENTITY	CONSISTENT WITH STRUCTURE BY IR OR NMR	CONFORMS BY NMR
CARBON	61.3 TO 64.4%	62.7%
NITROGEN	9.5 TO 10.1%	9.5%
†PURITY	MINIMUM 98%	99%
SHELF LIFE SOP QC-12-006	3 YEARS	SEPTEMBER 2004
QC ACCEPTANCE DATE		SEPTEMBER 2001

David Feldker, Manager
Analytical Services

†Chemical purity was determined by TLC.
The safety data sheet is available on website: http://www.sigma-aldrich.com.

SIGMA

Certificate of Analysis

TEST	LOT {SAMPLE} RESULTS
Product Name	Nitrazepam
Product Number	N3889
CAS Number	146225
Formula	$C_{15}H_{11}N_3O_3$
Formula Weight	281.3
APPEARANCE	LIGHT YELLOW POWDER
SOLUBILITY	CLEAR LIGHT YELLOW SOLUTION AT 50 MG/ML IN GLACIAL ACETIC ACID
ELEMENTAL ANALYSIS	64.5% CARBON 15.2% NITROGEN
IR SPECTRUM	CONSISTENT WITH STRUCTURE
†PURITY	GREATER THAN 99%
QC ACCEPTANCE DATE	SEPTEMBER 1993

David Feldker, Manager
Analytical Services

†Chemical purity was determined by HPLC and perchloric acid titration.
The safety data sheet is available on website: http://www.sigma-aldrich.com.

SIGMA

Certificate of Analysis

TEST	LOT {SAMPLE} RESULTS
Product Name	Loxapine succinate salt
Product Number	L106
CAS Number	27833643
Formula	$C_{18}H_{18}ClN_3O \cdot C_4H_6O_4$
Formula Weight	445.9
Melting Point	151-153 deg C
Representative Melting Point	151-153 deg C
Analysis:	C18H18ClN3O + C4H6O4
HPLC	Mobile phase: 30% KH2PO4 (0.05 M)/CH3CN
HPLC	Column: uBondapak C18, 3.9 mm x 30 cm length
HPLC	Detector: UV at 254 nm
HPLC	Flow rate: 1.0 ml/min
Solubility	Soluble in water
†Purity	The purity of this lot is greater than 99%
QC Approval Date	6/19/89

David Feldker, Manager
Analytical Services

†Chemical purity was determined by HPLC.
The safety data sheet is available on website: http://www.sigma-aldrich.com.

SIGMA

Certificate of Analysis

TEST	LOT {SAMPLE} RESULTS
Product Name	Flunitrazepam
Product Number	F9261
CAS Number	1622624
Formula	$C_{16}H_{12}FN_3O_3$
Formula Weight	313.3
APPEARANCE	WHITE POWDER WITH A LIGHT YELLOW CAST
SOLUBILITY	CLEAR FAINT YELLOW SOLUTION AT 50 MG/ML IN CHLOROFORM
IR SPECTRUM	CONSISTENT WITH STRUCTURE
CARBON	61.5%
NITROGEN	13.5%
†PURITY	>99%
QC ACCEPTANCE DATE	MAY 1992

David Feldker, Manager
Analytical Services

Chemical purity was determined by TLC.
The safety data sheet is available on website: http://www.sigma-aldrich.com.

SIGMA

Certificate of Analysis

TEST	SPECIFICATION	LOT {SAMPLE} RESULTS
Product Name	Chlorpromazine hydrochloride	
Product Number	C0982	
CAS Number	69090	
Formula	$C_{17}H_{19}ClN_2S \cdot HCl$	
Formula Weight	355.3	
IDENTITY	PASS	PASS
MELTING RANGE	195 TO 198 DEG C	196.5 DEG C
LOSS ON DRYING	NMT 0.5%	0.2%
RESIDUE ON IGNITION	NMT 0.1%	PASS *
OTHER ALKYLATED PHENOTHIAZINES	NMT 0.5%	PASS *
ORGANIC VOLATILE IMPURITIES	METHOD I TESTING	PASS *
†PURITY	98.0% TO 101.5% (DRY BASIS)	100.0%
EXPIRATION DATE		NOVEMBER 2003
		* SUPPLIER DATA
	DOCUMENT # C0982/03/17/98/1	MEETS REQUIREMENTS OF USP 24
QC ACCEPTANCE DATE		NOVEMBER 2001

David Feldker, Manager
Analytical Services

†Chemical purity was determined by HPLC.
Safety data sheet is available on website: http://www.sigma-aldrich.com.

SIGMA

Certificate of Analysis

TEST	LOT {SAMPLE} RESULTS
Product Name	Chlordiazepoxide hydrochloride
Product Number	C2517
CAS Number	438415
Formula	$C_{16}H_{14}ClN_3O \cdot HCl$
Formula Weight	336.2
APPEARANCE	WHITE POWDER WITH A LIGHT YELLOW CAST
SOLUBILITY	SLIGHTLY HAZY FAINT YELLOW SOLUTION AT 200 MG PLUS 4 ML OF WATER
IR SPECTRUM	CONSISTENT WITH STRUCTURE
ELEMENTAL ANALYSIS	56.9% CARBON 12.4% NITROGEN
†PURITY	GREATER THAN 99%
	REVIEW DATE 10/31/02
QC ACCEPTANCE DATE	OCTOBER 1994

David Feldker, Manager
Analytical Services

†Chemical purity was determined by TLC.
Safety data sheet is available on website: http://www.sigma-aldrich.com.

SIGMA

Certificate of Analysis

TEST	LOT {SAMPLE} RESULTS
Product Name	Bupivacaine hydrochloride
Product Number	B5274
CAS Number	14252803
Formula	$C_{18}H_{28}N_2O \cdot HCl$
Formula Weight	324.9
APPEARANCE	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS SOLUTION AT 50 MG/ML IN WATER
QUALITATIVE TEST(S)	POSITIVE FOR CHLORIDE *
IR SPECTRUM	CONSISTENT WITH STRUCTURE
LOSS ON DRYING	5.3% *
†PURITY	>99.9%
	* SUPPLIER'S INFORMATION
QC ACCEPTANCE DATE	OCTOBER 2001

David Feldker, Manager
Analytical Services

†Chemical purity was determined by GC.
Safety data sheet is available on website: http://www.sigma-aldrich.com.

SIGMA

Certificate of Analysis

TEST	SPECIFICATION	LOT {SAMPLE} RESULTS
Product Name	Clonazepam	
Product Number	C1277	
CAS Number	1622613	
Formula	$C_{15}H_{10}ClN_3O_3$	
Formula Weight	315.7	
APPEARANCE	WHITE TO LIGHT YELLOW POWDER	LIGHT YELLOW POWDER
SOLUBILITY	CLEAR YELLOW SOLUTION AT 20 MG/ML IN ACETONE	CLEAR YELLOW SOLUTION AT 20 MG/ML IN ACETONE
IR SPECTRUM	CONSISTENT WITH STRUCTURE	CONSISTENT WITH STRUCTURE
†PURITY	NOT LESS THAN 98%	GREATER THAN 99%
SHELF LIFE SOP QC-12-006	14 YEARS	OCTOBER 2002
QC ACCEPTANCE DATE		OCTOBER 2000
		REVIEW DATE OCTOBER 2001

David Feldker, Manager
Analytical Services

†Chemical purity was determined by TLC.
Safety data sheet is available on website: http://www.sigma-aldrich.com.

SIGMA

Certificate of Analysis

TEST	SPECIFICATION	LOT {SAMPLE} RESULTS
Product Name	Cocaine hydrochloride solution	
Product Number	C8912	
CAS Number	50362	
Formula	$C_{17}H_{21}NO_4$	
Formula Weight	303.4	
APPEARANCE	WHITE TO OFF-WHITE POWDER	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS TO VERY FAINT YELLOW SOLUTION AT 50 MG/ML IN ETHANOL	CLEAR COLORLESS
IR SPECTRUM	CONSISTENT WITH STRUCTURE	CONFORMS
CARBON		67.4%
NITROGEN		4.6%
†PURITY		>99%
QC ACCEPTANCE DATE		OCTOBER 2000

David Feldker, Manager
Analytical Services

†Chemical purity was determined by TLC.
Safety data sheet is available on website: http://www.sigma-aldrich.com.

SIGMA

Certificate of Analysis

TEST	SPECIFICATION	LOT {SAMPLE} RESULTS
Product Name	Amitriptyline hydrochloride	
Product Number	A8404	
CAS Number	549188	
Formula	$C_{20}H_{23}N \cdot HCl$	
Formula Weight	313.9	
APPEARANCE	WHITE TO WHITE WITH A FAINT YELLOW CAST POWDER	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS TO FAINT YELLOW SOLUTION AT 50 MG/ML IN METHANOL	CLEAR COLORLESS
PURITY BY PERCHLORIC ACID TITRATION	NLT 98%	100%
†PURITY	NLT 98%	>99%
SHELF LIFE SOP QC-12-006	3 YEARS	MAY 2005
QC ACCEPTANCE DATE		MAY 2002

David Feldker, Manager
Analytical Services

†Chemical purity was determined by TLC.
Safety data sheet is available on website: http://www.sigma-aldrich.com.

SIGMA

Certificate of Analysis

TEST	SPECIFICATION	LOT {SAMPLE} RESULTS
Product Name	Clomipramine hydrochloride	
Product Number	C7291	
CAS Number	17321776	
Formula	$C_{19}H_{23}ClN_2 \cdot HCl$	
Formula Weight	351.3	
APPEARANCE	WHITE TO OFF-WHITE POWDER	WHITE POWDER WITH A FAINT YELLOW CAST
SOLUBILITY	CLEAR COLORLESS TO FAINT YELLOW SOLUTION AT 25 MG/ML IN WATER	CLEAR VERY FAINT YELLOW SOLUTION AT 25 MG/ML IN WATER
IR SPECTRUM	CONSISTENT WITH STRUCTURE	CONSISTENT WITH STRUCTURE
†PURITY	MINIMUM 98%	98.8%
SHELF LIFE SOP QC-12-006	10 YEARS	JULY 2004
QC ACCEPTANCE DATE		JULY 2002

David Feldker, Manager
Analytical Services

†Chemical purity was determined by TLC.
Safety data sheet is available on website: http://www.sigma-aldrich.com.

SIGMA

Certificate of Analysis

TEST	LOT {SAMPLE} RESULTS
Product Name	Trimipramine maleate salt
Product Number	T3146
CAS Number	521788
Formula	$C_{20}H_{26}N_2 \cdot C_4H_4O_4$
Formula Weight	410.5
APPEARANCE	WHITE POWDER
SOLUBILITY	CLEAR FAINT YELLOW SOLUTION AT 50 MG/ML IN CHLOROFORM
IR SPECTRUM	CONFORMS *
†PURITY	>99%
	* SUPPLIER'S INFORMATION
QC ACCEPTANCE DATE	DECEMBER 1999

David Feldker, Manager
Analytical Services

†Chemical purity was determined by TLC and perchloric acid titration.
Safety data sheet is available on website: http://www.sigma-aldrich.com.

SIGMA

Certificate of Analysis

TEST	SPECIFICATION	LOT {SAMPLE} RESULTS
Product Name	Protriptyline hydrochloride	
Product Number	P8813	
CAS Number	1225554	
Formula	$C_{19}H_{21}N \cdot HCl$	
Formula Weight	299.8	
APPEARANCE	WHITE TO WHITE WITH A YELLOW CAST POWDER	WHITE POWDER WITH A FAINT YELLOW CAST
SOLUBILITY	CLEAR COLORLESS TO LIGHT YELLOW SOLUTION AT 200 MG PLUS 4 ML OF WATER	CLEAR VERY FAINT YELLOW SOLUTION AT 200 MG PLUS 4 ML OF WATER
†PURITY	MINIMUM 99%	>99%
SHELF LIFE SOP QC-12-006		JANUARY 2004
QC ACCEPTANCE DATE		JANUARY 2002

David Feldker, Manager
Analytical Services

†Chemical purity was determined by TLC and silver nitrate titration.
Safety data sheet is available on website: http://www.sigma-aldrich.com.

SIGMA

Certificate of Analysis

TEST	SPECIFICATION	LOT {SAMPLE} RESULTS
Product Name	Haloperidol	
Product Number	H1512	
CAS Number	52868	
Formula	$C_{21}H_{23}ClFNO_2$	
Formula Weight	375.9	
APPEARANCE	WHITE TO OFF-WHITE POWDER	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS TO FAINT YELLOW SOLUTION AT 30 MG/ML IN CHLOROFORM	CLEAR COLORLESS
IR SPECTRUM	CONSISTENT WITH STRUCTURE	CONFORMS
CARBON	66.1 TO 68.9%	67.1%
NITROGEN	3.4 TO 4.0%	3.7%
†PURITY	NLT 98%	99%
SHELF LIFE SOP QC-12-006	3 YEARS	MARCH 2005
QC ACCEPTANCE DATE		MARCH 2002

David Feldker, Manager
Analytical Services

†Chemical purity was determined by TLC.
Safety data sheet is available on website: http://www.sigma-aldrich.com.

SIGMA

Certificate of Analysis

TEST	SPECIFICATION	LOT {SAMPLE} RESULTS
Product Name	Imipramine hydrochloride	
Product Number	I7379	
CAS Number	113520	
Formula	$C_{19}H_{24}N_2 \cdot HCl$	
Formula Weight	316.9	
APPEARANCE	WHITE TO WHITE WITH A FAINT YELLOW CAST POWDER	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS TO FAINT YELLOW SOLUTION AT 200 MG PLUS 4 ML OF WATER	CLEAR COLORLESS SOLUTION AT 50 MG/ML IN WATER
†PURITY	99%	102.6%
		TEST RESULTS AS OF JANUARY 1985.

David Feldker, Manager
Analytical Services

†Chemical purity was determined by perchloric acid titration.
Safety data sheet is available on website: http://www.sigma-aldrich.com.

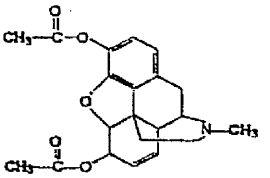
RADIAN INTERNATIONAL
A GAMES & MURDIE GROUP COMPANY

H-038
32453-07A

Certificate of Analysis

Heroin

7,8-Didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol diacetate



Catalog Number:	H-038
Solution Lot:	32453-07A
Expiration Date:	August 2001
Solvent:	Acetonitrile
Amount per Ampule:	1 mL
Storage:	Protect from light, store in freezer.
Handling:	We advise laboratories to use measured volumes of this standard solution before diluting to the desired concentration.
Intended Use:	For laboratory use only. This product is a quantitative standard useful for calibration, quality control, and other general applications requiring accurate solutions.

Component	Purity[1]	Prepared Concentration[2]	Analyzed Concentration[3]
Heroin	99%	1.000 ± 0.031 mg/mL	0.976 ± 0.022 mg/mL

Standard Solution Comparability

Standard Solution	*Lot Number*	*Concentration[3] (mg/mL)*	*% Difference from Target*
New Lot	32453-07A	0.976	-2.4
Previous Lot	30328-13A	0.982	-1.8

Standard Solution Homogeneity

Ampuling Position	*Concentration[3] (mg/mL)*	*Mean*	*% RSD*
Early	1.003		
Middle	0.959		
Late	0.966	0.976	2.4

1 Chemical purity was determined by chromatographic analysis. See following pages for more information.
2 The range of the prepared concentration is determined by statistical process control of our production and analysis systems with a 95% confidence.
3 Concentration values are determined by comparison to an independent calibration curve. We suggest using the prepared concentration value for dilutions. The concentration range is calculated from the distribution of multiple analyses of the new standard with a 95% degree of confidence.

Chemical purity was determined by GC/FID and HPLC.

Radian International LLC certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is ensured by exhaustive purity determinations and gravimetric preparation calibrated with NIST traceable weights. Precision is guaranteed by multiple weighings/triplicate analysis and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

Authorized Signature:

Joseph B. Rettinger, Quality Control Manager

Analytical Reference Materials
P.O. Box 201088 / Austin, TX 78720-1088 / (512)238-9974 / (800)848-7837 / FAX: (512)238-9129

RADIAN INTERNATIONAL LLC

N-907
27287-65B

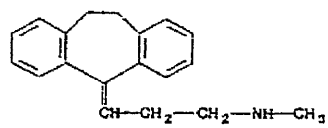
Certificate of Analysis

Radian International LLC certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is ensured by exhaustive purity determinations and gravimetric preparation calibrated with NIST traceable weights. Precision is guaranteed by multiple weighings/triplicate analysis and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

NOTE: *To ensure the accuracy of stock solutions, we advise laboratories to measure precise volume of standard solution from ampules before diluting to the desired volume.*

AUTHORIZED SIGNATURE: H.K. Yaser, Product Manager

COMPOUND DATA

Name:	NORTRIPTYLINE (3-(10,11-Dihydro-5H-dibenzo[a,d]-cycloheptan-5-ylidene)-N-methyl-1-propanamine)
Synonyms:	Avantyl, Pamelor
CAS #:	72-69-5
Structure:	
Chemical Formula:	$C_{19}H_{21}N$
Molecular Weight:	263.37
Compound Lot:	27287-65
Chemical Purity:	99%

STANDARD DATA

Catalog Number:	N-907
Solution Lot:	27287-65B
Concentration:	1.0 mg/mL
Solvent:	Methanol
Amount per Ampule:	1 mL
Storage:	Refrigerate. Protect from light.
Date Prepared:	June 1996
Expiration Date:	June 2000

Chemical purity was determined by GC/FID and HPLC.

8501 N. Mopac Blvd./P.O. Box 201088/Austin, TX 78720-1088/(512)454-4797/(800)848-7837(US & Canada)

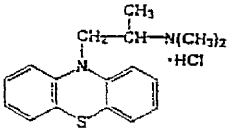
RADIAN INTERNATIONAL
A GAMES & MOORE GROUP COMPANY

P-044
30900-11

Certificate of Analysis

Promethazine·HCl

N,N,alpha-Trimethyl-10H-phenothiazine-10-ethanamine hydrochloride



Catalog Number:	P-044
Lot Number:	30900-11
CAS Number:	58-33-3
Chemical Formula:	$C_{17}H_{20}N_2S$ HCl
Molecular Weight:	284.43
Chemical Purity:	99%
Expiration Date:	April 2002
Storage:	Protect from light. refrigerate.
Handling:	See MSDS for handling instructions.
Intended Use:	For laboratory use only.

Spectral and Physical Data

Elemental Analysis

	%C	%H
Calculated	63.63	6.60
Analyzed	63.61	6.63

Chemical purity was determined by GC/FID and HPLC/UV.

Radian International certifies that this material meets or exceeds the purity value stated in this data sheet. Purity and identity are established using a variety of chromatographic and spectroscopic methods. The results of these analyses are included in this data package.

Authorized Signature:

Joseph B. Reninger, Quality Control Manager

Analytical Reference Materials

P.O. Box 201088 / Austin, TX 78720-1088 / (512)238-9974 / (800)848-7837 / FAX: (512)238-9129

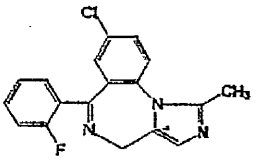
RADIAN INTERNATIONAL
A GAMES & MOORE GROUP COMPANY

M-908
30714-41C

Certificate of Analysis

Midazolam

8-Chloro-6-(2-fluorophenyl)-1-methyl-4H-imidazo[1,4]benzodiazepine



Catalog Number:	M-908
Solution Lot:	30714-41C
Expiration Date:	December 2001
Solvent:	Methanol
Amount per Ampule:	1 mL
Storage:	Protect from light, refrigerate.
Handling:	We advise laboratories to use measured volumes of this standard solution before diluting to the desired concentration.
Intended Use:	For laboratory use only. This product is a quantitative standard useful for calibration, quality control, and other general applications requiring accurate solutions.

Component	Purity[1]	Prepared Concentration[2]	Analyzed Concentration[3]
Midazolam	99%	1.000 ± 0.031 mg/mL	0.977 ± 0.015 mg/mL

Standard Solution Comparability

Standard Solution	*Lot Number*	*Concentration[3] (mg/mL)*	*% Difference from Target*
New Lot	30714-41C	0.977	-2.3
Previous Lot	30714-41B	0.996	-0.4

Standard Solution Homogeneity

Ampuling Position	*Concentration[3] (mg/mL)*	*Mean*	*% RSD*
Early	0.969		
Middle	0.978		
Late	0.983	0.977	0.7

1 Chemical purity was determined by chromatographic analysis. See following pages for more information.
2 The range of the prepared concentration is determined by statistical process control of our production and analysis systems with a 95% confidence.
3 Concentration values are determined by comparison to an independent calibration curve. We suggest using the prepared concentration value for dilutions. The concentration range is calculated from the distribution of multiple analyses of the new standard with a 95% degree of confidence.

Chemical purity was determined by GC/FID and HPLC.

Radian International LLC certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is ensured by exhaustive purity determinations and gravimetric preparation calibrated with NIST traceable weights. Precision is guaranteed by multiple weighings/triplicate analysis and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

Authorized Signature:

Joseph B. Rettinger, Quality Control Manager

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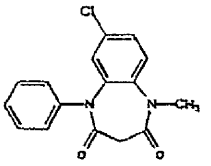
RADIAN™ INTERNATIONAL
Analytical Reference Materials

C-909
29554-11B

Certificate of Analysis

Clobazam

7-Chloro-1-methyl-5-phenyl-1H-1,5-benzodiazepine-2,4(3H,5H)-dione



Catalog Number:	C-909
Solution Lot:	29554-11B
Expiration Date:	October 2002
Solvent:	Methanol
Amount per Ampule:	1 mL
Storage:	Protect from light, refrigerate.
Handling:	We advise laboratories to use measured volumes of this standard solution before diluting to the desired concentration.
Intended Use:	For laboratory use only. This product is a quantitative standard useful for calibration, quality control, and other general applications requiring accurate solutions.

Component	Purity[1]	Prepared Concentration[2]	Analyzed Concentration[3]
Clobazam	99%	1.000 ± 0.031 mg/mL	1.001 ± 0.016 mg/mL

Standard Solution Comparability

Standard Solution	*Lot Number*	*Concentration[3] (mg/mL)*	*% Difference from Target*
New Lot	29554-11B	1.001	0.1
Previous Lot	29554-11A	1.004	0.4

Standard Solution Homogeneity

Ampuling Position	*Concentration[3] (mg/mL)*	*Mean*	*% RSD*
Early	0.986		
Middle	1.003		
Late	1.014	1.001	1.4

[1] Chemical purity was determined by chromatographic analysis. See following pages for more information.
[2] The range of the prepared concentration is determined by statistical process control of our production and analysis systems with a 95% confidence.
[3] Concentration values are determined by comparison to an independent calibration curve. We suggest using the prepared concentration value for dilutions. The concentration range is calculated from the distribution of multiple analyses of the new standard with a 95% degree of confidence.

Chemical purity was determined by GC and HPLC.

Radian International LLC certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is ensured by exhaustive purity determinations and gravimetric preparation calibrated with NIST traceable weights. Precision is guaranteed by multiple weighings/triplicate analysis and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

Authorized Signature:

Joseph B. Rettinger, Quality Control Manager

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RADIAN INTERNATIONAL LLC

B-004
30318-61B

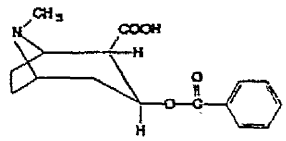
Certificate of Analysis

Radian International LLC certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is assured by exhaustive purity determinations and gravimetric preparation calibrated with NIST traceable weights. Precision is guaranteed by multiple weighings/triplicate analysis and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

NOTE: *To ensure the accuracy of stock solutions, we advise laboratories to measure precise volume of standard solution from ampules before diluting to the desired volume.*

AUTHORIZED SIGNATURE: H.K. Yaser, Product Manager

COMPOUND DATA

Name:	BENZOYLECGONINE (3-(Benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic acid)
Synonyms:	Ecgonine Benzoate
CAS #:	519-09-5
Structure:	
Chemical Formula:	$C_{16}H_{19}NO_4$
Molecular Weight:	289.33
Compound Lot:	30318-61
Chemical Purity:	99%
Melting Point:	198.5 - 199.5°C (Lit. = 195°C)

STANDARD DATA

Catalog Number:	B-004
Solution Lot:	30318-61B
Concentration:	1.0 mg/mL
Solvent:	Methanol
Amount per Ampule:	1 mL
Storage:	Refrigerate. Protect from light.
Expiration Date:	June 2002

Chemical purity was determined by GC/FID and HPLC.

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RADIAN INTERNATIONAL LLC

P-035
JBR-30317-43A

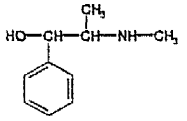
Certificate of Analysis

Radian International LLC certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is ensured by exhaustive purity determinations and gravimetric preparation calibrated with NIST traceable weights. Precision is guaranteed by multiple weighings/triplicate analysis and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

NOTE: *To ensure the accuracy of stock solutions, we advise laboratories to measure precise volume of standard solution from ampules before diluting to the desired volume.*

AUTHORIZED SIGNATURE: H.K. Yaser, Product Manager

COMPOUND DATA

Name:	(+)-PSEUDOEPHEDRINE ([1S,2S]-(+)-α-[1-(Methylamino)ethyl]benzenemethanol)
Synonyms:	d-Pseudoephedrine
CAS #:	90-82-4
Structure:	
Chemical Formula:	$C_{10}H_{15}NO$
Molecular Weight:	165.23
Compound Lot:	JBR-30317-43
Chemical Purity:	99%
Enantiomeric Purity:	98%ee
Melting Point	117 - 119.5°C (Lit. = 119°C)

STANDARD DATA

Catalog Number:	P-035
Solution Lot:	JBR-30317-43A
Concentration:	1.0 mg/mL
Solvent:	Methanol
Amount per Ampule:	1 mL
Storage:	Refrigerate. Protect from light.

Chemical purity was determined by GC/FID and HPLC.

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RADIAN INTERNATIONAL LLC

T-905
26424-57B

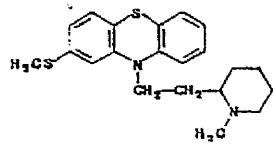
Certificate of Analysis

Radian International certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is ensured by exhaustive purity determinations and gravimetric preparation calibrated with NIST traceable weights. Precision is guaranteed by multiple weighings/triplicate analysis and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

NOTE: *To ensure the accuracy of stock solutions, we advise laboratories to measure precise volume of standard solution from ampules before diluting to the desired volume.*

AUTHORIZED SIGNATURE: _____
J.B. Rettinger, Quality Control Manager

COMPOUND DATA

Name:	THIORIDAZINE (10-[2-(1-Methyl-2-piperidyl)ethyl]-2-(methylthio)phenothiazine)
Synonyms:	Mellaril, Sonapax
CAS #:	50-52-2
Structure:	
Chemical Formula:	$C_{21}H_{26}N_2S_2$
Molecular Weight:	370.58
Compound Lot:	26424-57
Chemical Purity:	99%
Melting Point	68.5 - 71.0°C (Lit. = 72 - 74°C)

STANDARD DATA

Catalog Number:	T-905
Solution Lot:	26424-57B
Concentration:	1.0 mg/mL
Solvent:	Methanol
Amount per Ampule:	1 mL
Storage:	Refrigerate. Protect from light.
Expiration Date:	June 2002

Chemical purity was determined by GC and HPLC.

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RADIAN INTERNATIONAL LLC

M-035
30906-41A

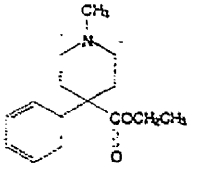
Certificate of Analysis

Radian International LLC certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is ensured by exhaustive purity determinations and gravimetric preparation calibrated with NIST traceable weights. Precision is guaranteed by multiple weighings/analyses and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

NOTE: *To ensure the accuracy of stock solutions, we advise laboratories to measure precise volume of standard solution from ampules before diluting to the desired volume.*

AUTHORIZED SIGNATURE: Kevan Yaser
H.K. Yaser, Product Manager

COMPOUND DATA

Name:	MEPERIDINE (1-Methyl-4-phenyl-4-piperidine carboxylic acid ethyl ester)
Synonyms:	Pethidine*, Mepergan*, Demerol*
CAS #:	57-42-1
Structure:	
Chemical Formula:	$C_{15}H_{21}NO_2$
Molecular Weight:	247.34
Compound Lot:	30906-41
Chemical Purity:	99%

STANDARD DATA

Catalog Number:	M-035
Solution Lot:	30906-41A
Concentration:	1.0 mg/mL
Solvent:	Methanol
Amount per Ampule:	1 mL
Storage:	Refrigerate. Protect from light.

Chemical purity was determined by GC/FID and HPLC.

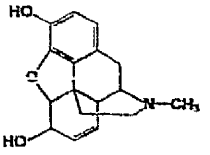
RADIAN INTERNATIONAL

M-005
31332-75B

Certificate of Analysis

Morphine

7,8-Didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol



Catalog Number:	M-005
Solution Lot:	31332-75B
Expiration Date:	November 2003
Solvent:	Methanol
Amount per Ampule:	1 mL
Storage:	Protect from light, refrigerate.
Handling:	We advise laboratories to use measured volumes of this standard solution before diluting to the desired concentration.
Intended Use:	For laboratory use only. This product is a quantitative standard useful for calibration, quality control, and other general applications requiring accurate solutions.

Component	Purity[1]	Prepared Concentration[2]	Analyzed Concentration[3]
Morphine	99%	1.000 ± 0.031 mg/mL	1.000 ± 0.011 mg/mL

Standard Solution Comparability

Standard Solution	Lot Number	Concentration[3] (mg/mL)	% Difference from Target
New Lot	31332-75B	1.000	0.0
Previous Lot	30898-03A	0.991	-0.9

Standard Solution Homogeneity

Ampuling Position	Concentration[3] (mg/mL)	Mean	% RSD
Early	1.000		
Middle	0.997		
Late	1.002	1.000	0.3

[1] Chemical purity was determined by chromatographic analysis. See following pages for more information.
[2] The range of the prepared concentration is determined by statistical process control of our production and analysis systems with a 95% confidence.
[3] Concentration values are determined by comparison to an independent calibration curve. We suggest using the prepared concentration value for dilutions. The concentration range is calculated from the distribution of multiple analyses of the new standard with a 95% degree of confidence.

Chemical purity was determined by HPLC/UV.

Radian International LLC certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is ensured by exhaustive purity determinations and gravimetric preparation calibrated with NIST traceable weights. Precision is guaranteed by multiple weighings/triplicate analysis and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

Authorized Signature:

Joseph B. Rettinger, Quality Control Manager

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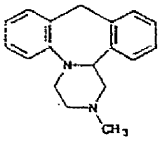
A DAMES & MOORE GROUP COMPANY

M-904
27287-90C

Certificate of Analysis

Mianserin

1,2,3,4,10,14b-Hexahydro-2-methyldibenzo[c,f]pyrazino[1,2-a]azepine



Catalog Number:	M-904
Solution Lot:	27287-90C
Expiration Date:	April 2002
Solvent:	Methanol
Amount per Ampule:	1 mL
Storage:	Protect from light, refrigerate.
Handling:	We advise laboratories to use measured volumes of this standard solution before diluting to the desired concentration.
Intended Use:	For laboratory use only. This product is a quantitative standard useful for calibration, quality control, and other general applications requiring accurate solutions.

Component	Purity[1]	Prepared Concentration[2]	Analyzed Concentration[3]
Mianserin	99%	1.000 ± 0.031 mg/mL	0.973 ± 0.004 mg/mL

Standard Solution Comparability

Standard Solution	*Lot Number*	*Concentration[3] (mg/mL)*	*% Difference from Target*
New Lot	27287-90C	0.973	-2.7
Previous Lot	27287-90B	0.978	-2.2

Standard Solution Homogeneity

Ampuling Position	*Concentration[3] (mg/mL)*	*Mean*	*% RSD*
Early	0.970		
Middle	0.972		
Late	0.977	0.973	0.4

[1] Chemical purity was determined by chromatographic analysis. See following pages for more information.
[2] The range of the prepared concentration is determined by statistical process control of our production and analysis systems with a 95% confidence.
[3] Concentration values are determined by comparison to an independent calibration curve. We suggest using the prepared concentration value for dilutions. The concentration range is calculated from the distribution of multiple analyses of the new standard with a 95% degree of confidence.

Chemical purity was determined by GC/FID and HPLC.

Radian International certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is ensured by exhaustive purity determinations and gravimetric preparation calibrated with NIST traceable weights. Precision is guaranteed by multiple weighings/triplicate analysis and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

Authorized Signature:

Joseph B. Rettinger, Quality Control Manager

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RADIAN INTERNATIONAL LLC

C-006
31546-79A

Certificate of Analysis

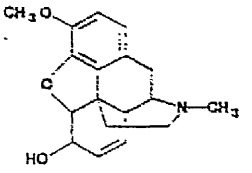
Radian International LLC certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is ensured by exhaustive purity determinations and gravimetric preparation calibrated with NIST traceable weights. Precision is guaranteed by multiple weighings/triplicate analysis and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

NOTE: *To ensure the accuracy of stock solutions, we advise laboratories to measure precise volume of standard solution from ampules before diluting to the desired volume.*

AUTHORIZED SIGNATURE: Kevan Yaser

H.K. Yaser, Product Manager

COMPOUND DATA

Name:	**CODEINE** (7,8-Didehydro-4,5-epoxy-3-methoxy-17-methylmorphinan-6-ol)
Synonyms:	Morphine Monomethyl Ether, Methyl Morphine
CAS #:	76-57-3
Structure:	
Chemical Formula:	$C_{18}H_{21}NO_3$
Molecular Weight:	299.37
Compound Lot:	31546-79
Chemical Purity:	99%
Melting Point:	158.5 - 159.5°C (Lit. = 154 - 156°C)

STANDARD DATA

Catalog Number:	C-006
Solution Lot:	31546-79A
Concentration:	1.0 mg/mL
Solvent:	Methanol
Amount per Ampule:	1 mL
Storage:	Refrigerate. Protect from light.

Chemical purity was determined by GC/FID and HPLC.

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RADIAN INTERNATIONAL LLC

D-907
27120-15A

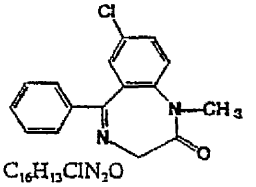
Certificate of Analysis

Radian International LLC certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is ensured by exhaustive purity determinations and gravimetric preparation calibrated with NIST traceable weights. Precision is guaranteed by multiple weighings/analyses and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

NOTE: *To ensure the accuracy of stock solutions, we advise laboratories to measure precise volume of standard solution from ampules before diluting to the desired volume.*

AUTHORIZED SIGNATURE: H.K. Yaser, Product Manager

COMPOUND DATA

Name:	**DIAZEPAM** (7-Chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepin-2-one)
Synonyms:	Valium, Valrelease
CAS #:	439-14-5
Structure:	
Chemical Formula:	$C_{16}H_{13}ClN_2O$
Molecular Weight:	284.74
Compound Lot:	27120-15
Chemical Purity:	99%
Melting Point:	132.5 - 133.5°C (Lit. = 131 - 135°C)

STANDARD DATA

Catalog Number:	D-907
Solution Lot:	27120-15A
Concentration:	1.0 mg/mL
Solvent:	Methanol
Amount per Ampule:	1 mL
Storage:	Refrigerate. Protect from light.

Chemical purity was determined by GC and HPLC.

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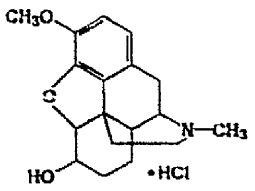
Analytical Reference Materials

D-019
32466-42A

Certificate of Analysis

Dihydrocodeine HCl

4,5-Epoxy-3-methoxy-17-methylmorphinan-6-ol Hydrochloride



Catalog Number:	D-019
Solution Lot:	32466-42A
Expiration Date:	August 2001
Solvent:	Methanol
Amount per Ampule:	1 mL
Storage:	Protect from light, refrigerate.
Handling:	We advise laboratories to use measured volumes of this standard solution before diluting to the desired concentration.
Intended Use:	For laboratory use only. This product is a quantitative standard useful for calibration, quality control, and other general applications requiring accurate solutions.

Component	Purity[1]	Prepared Concentration[2]	Analyzed Concentration[3]
Dihydrocodeine HCl	99%	1.000 ± 0.031 mg/mL (as free base)	0.970 ± 0.01 mg/mL (as free base)

Standard Solution Comparability

Standard Solution	Lot Number	Concentration[3] (mg/mL)	% Difference from Existing
New Lot	32466-42A	0.970	1.9

Standard Solution Homogeneity

Ampuling Position	Concentration[3] (mg/mL)	Mean	%RSD
Early	0.965		
Middle	0.985		
Late	0.961	0.970	1.3

[1] Chemical purity was determined by chromatographic analysis. See following pages for more information.
[2] The range of the prepared concentration is determined by statistical process control of our production and analysis systems with a 95% confidence.
[3] Concentration values are determined by comparison to an independent calibration curve. We suggest using the prepared concentration value for dilutions. The concentration range is calculated from the distribution of multiple analyses of the new standard with a 95% degree of confidence.

Chemical purity was determined by GC/FID and HPLC/UV.

Radian International LLC certifies that this standard meets or exceeds the specifications stated in this data sheet. Accuracy is ensured by exhaustive purity determinations and gravimetric preparation calibrated with NIST traceable weights. Precision is guaranteed by multiple weighings/triplicate analysis and comparison to previous lots (when available). Finally, homogeneity is demonstrated by random analysis of the ampuled standard.

Authorized Signature:

Joseph B. Rettinger, Quality Control Manager

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