An Evaluation of the Use of Supercritical Fluid Extraction Techniques to Recover Drugs From Biological Matrices

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

by

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Karen Scott.

TO MY PARENTS

Drugs and Materials

Tem/Temaz:	Temazepam
Diaz:	Diazepam
DMD:	Desmethyldiazepam
CDP:	Chlordiazepoxide
Triaz:	Triazolam
Praz:	Prazepam
Morph:	Morphine
6MAM:	6-monoacetylmorphine
DHC:	Dihydrocodeine
Meth:	Methadone
Cycl:	Cyclizine
d_3:	tri-deuterated
MeOH:	Methanol
DCM:	Dichloromethane
EtAc:	Ethyl acetate

Analytical Methods

HPLC:	High performance liquid chromatography
GC:	Gas chromatography
MS:	Mass spectrometry
FID:	Flame ionisation detector
EIA:	Enzyme-immumoassay
RIA:	Radio-immunoassay

Other

psi:	Pounds per square inch
NPM:	Needle puncture mark
IVDA:	Intravenous Drug Abuser
PM:	Post-mortem
RTD:	Road traffic drug

SUMMARY

The use of supercritical fluid extraction (SFE) was evaluated for the determination of drugs of Forensic interest. Three matrices were investigated. The first two (blood and vitreous humor) were compared to SPE and LLE methodology currently in use at the Department of Forensic Science and Medicine. The third matrix, hair, was assessed to determine its usefulness as a marker of past drug use.

Three types of drugs were investigated by SFE namely, benzodiazepines, morphine and methadone. These drugs were chosen due to their high frequency of occurrence in post-mortem cases in the West of Scotland. Successful methodology was developed for all three matrices and all three drug types, providing an efficient, reproducible alternative method to SPE and LLE, which reduced the environmental risks from organic solvents.

The developed methods were applied to the analysis of authentic forensic samples. In addition to comparing well with the results obtained with the conventional techniques, good correlation was obtained between blood and vitreous humor results for temazepam, diazepam, methadone and morphine. Thus, in cases where a body is badly decomposed or burned, vitreous humor can be used as an alternative post-mortem sample.

In addition to the determination of morphine, 6-monoacetyl morphine (6MAM) was used as a marker of heroin abuse. 6MAM was detected in all three samples matrices, thus confirming the use of heroin prior to death.

Hair analysis for all three types of drugs was carried out using a single extraction method. A wide range of concentrations was found for all drug types. As with blood and vitreous humor, 6MAM was detected and used as a marker of heroin abuse. From this, 61% of known heroin users were confirmed.

On the whole, the developed extraction methodologies provided an efficient alternative approach to drug analysis in Forensic Toxicology.

"We shall not cease from exploring
And the end of our exploring
Will be to arrive where we started
And know the place for the first time"

from 'Little Gidding': T. S. Elliot

1. SUPERCRITICAL FLUIDS

1.1 INTRODUCTION

One hundred and seventy five years ago Baron Charles Cagniard de la Tour discovered that above certain temperatures and pressures, some substances can no longer be classified as either liquids or gases but share the properties of both.[1] The ability to manipulate these properties for a variety of applications, by regulating pressure and temperature, is now being exploited by the analytical chemist. The most commonly used analytical tools which have resulted from the discovery of supercritical fluids are, supercritical fluid extraction (SFE) and supercritical fluid chromatography (SFC).

It is possible to describe a supercritical fluid (SF) by reference to Figure 1.1.[2] The areas of solid, liquid and gas are well defined by boundaries. Moving along the gas liquid boundary, both temperature and pressure increase. The liquid becomes less dense due to thermal expansion and as a result of increasing pressure, the gas becomes more dense.[3] At the critical point (C.P.) the densities of the two phases are identical and there is no distinction between the gas and liquid phases. The substance can now only be described as a fluid.

The critical point has co-ordinates of temperature and pressure as seen in Figure 1.1. These points are specific to a substance and are referred to as the critical temperature (T_c) and critical pressure (p_c). Other important critical parameters are the critical density (ρ_c) and the critical volume (V_c) for one mole of substance. The term *supercritical fluid* is used to describe substances above their critical temperature and pressure. Critical parameters of some of the substances used as supercritical fluids are shown in Table 1.1. Of these substances, the most commonly used supercritical fluid in analysis is carbon dioxide (CO_2).[4] This is due to its convenient supercritical temperature and pressure, its cheapness, its non-explosive nature and its non-toxicity.

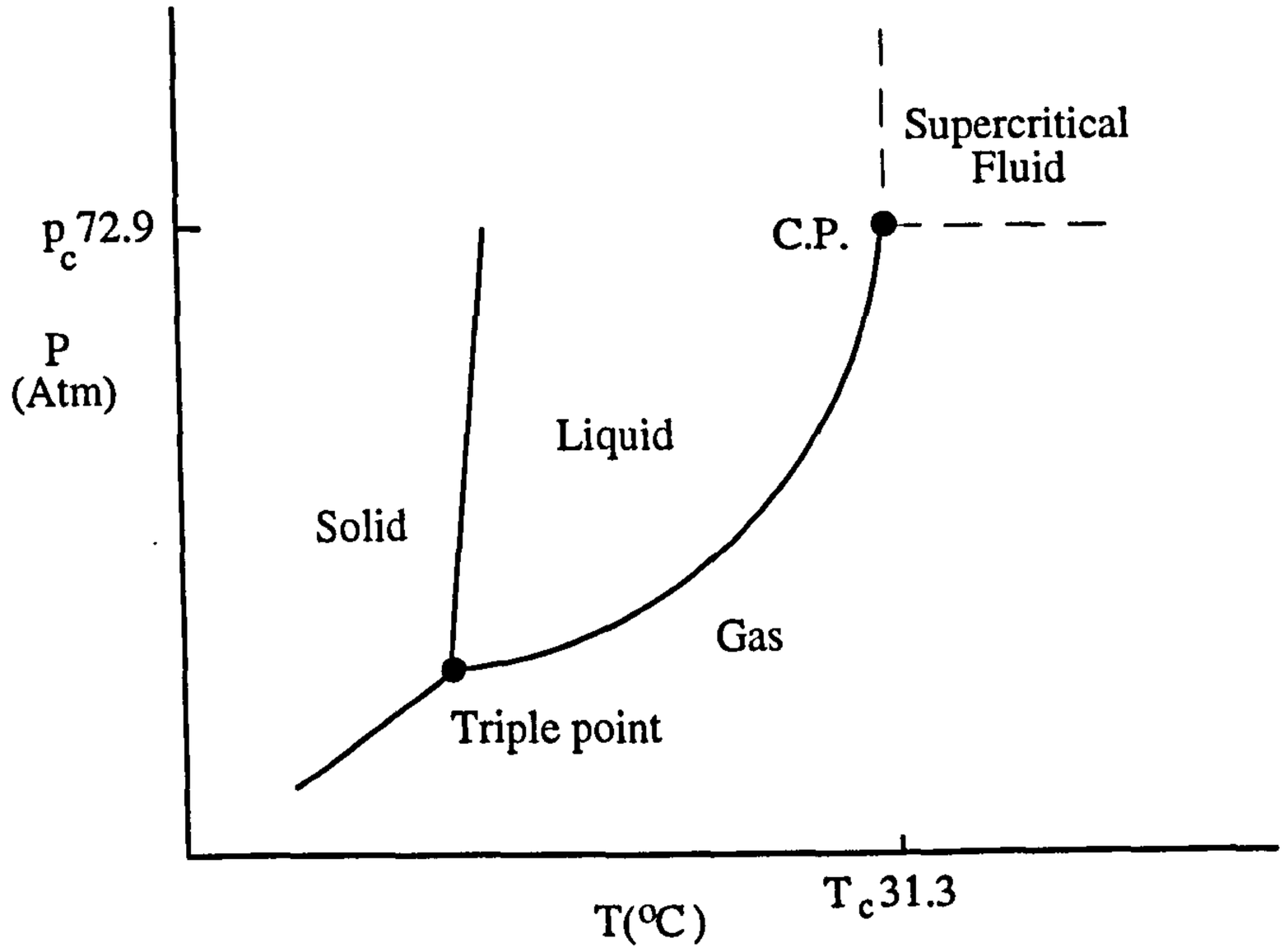


Figure 1.1: *Phase Diagram for Carbon Dioxide*

Table 1.1: *Physical Properties of Selected Supercritical Fluids*

Substance	T_c (°C)	p_c (atm)	ρ_c (g cm^{-3})
CO_2	31.3	79.2	0.47
N_2O	36.5	72.5	0.45
SF_6	45.5	37.1	0.74
NH_3	132.5	112.5	0.24
H_2O	374	227	0.34
n-C_4H_{10}	152	37.5	0.23
n-C_5H_{12}	197	33.3	0.23
Xe	16.6	58.4	1.10
CCl_2F_2	112	40.7	0.56
CHF_3	25.9	46.9	0.52

1.1.1 Properties of Supercritical Fluids

From a historical viewpoint, the solvating power of a supercritical fluid (SF) was first discovered more than a century ago by Hannay and Horgarth.[5] However, it is only in the past thirty years that their use as an analytical tool has been developed.

Supercritical fluids have several analytically desirable properties. Table 1.2 gives a comparison between gas, liquid and supercritical fluid phases for density, diffusivity and

dynamic viscosity.[4] It can be seen that SF's have densities comparable to liquids, dynamic viscosities comparable to gases and diffusivities between liquids and gases.

Table 1.2: *Comparison of Properties of Gases, Liquids and SCFs*[4]

Phase	Density ρ (g ml^{-1})	Diffusivity D (cm^2 s^{-1})	Dynamic viscosity η (g cm^{-1}s^{-1})
Gas	$\approx 10^{-3}$	0.01-1.0	0.5-3.5(x10^{-4})
SCF	0.2-0.9	3.3-0.1(x10^{-4})	0.2-1.0(x10^{-3})
Liquid	0.8-1.0	0.5-2.0(x10^{-5})	0.3-2.4(x10^{-2})

The combined gas-like mass transfer and liquid-like solvating characteristics of supercritical fluids, first led analytical chemists to use them as chromatographic mobile phases (supercritical fluid chromatography (SFC))[6] and later as fluids capable of removing species from solid samples (supercritical fluid extraction (SFE)). In both these techniques, probably the most important factor for consideration is the ability of the mobile phase (in SFC) or extractant (in SFE) to solubilise the analytes of interest. The solvent strength of a liquid is essentially constant regardless of the extraction conditions. However, the solvent strength of a supercritical fluid is dependant on the temperature and pressure used for the extraction.[7]

1.1.1.1 Solubility

By using supercritical fluid technology it is possible to exploit the unusual variations in solubility with pressure and temperature, which occur immediately above the critical point of a volatile solvent.[8] As stated previously, the solvent strength of a SF depends on the pressure and temperature used for the extraction. At constant temperature, extraction at lower pressures will favour less polar analytes and extraction at higher pressure will favour more polar and higher molecular weight analytes. This variation in solvent strength is highlighted in Figure 1.2 which shows the dependence of the Hildebrand solubility parameter on temperature and pressure for supercritical carbon dioxide (SF-CO_2).

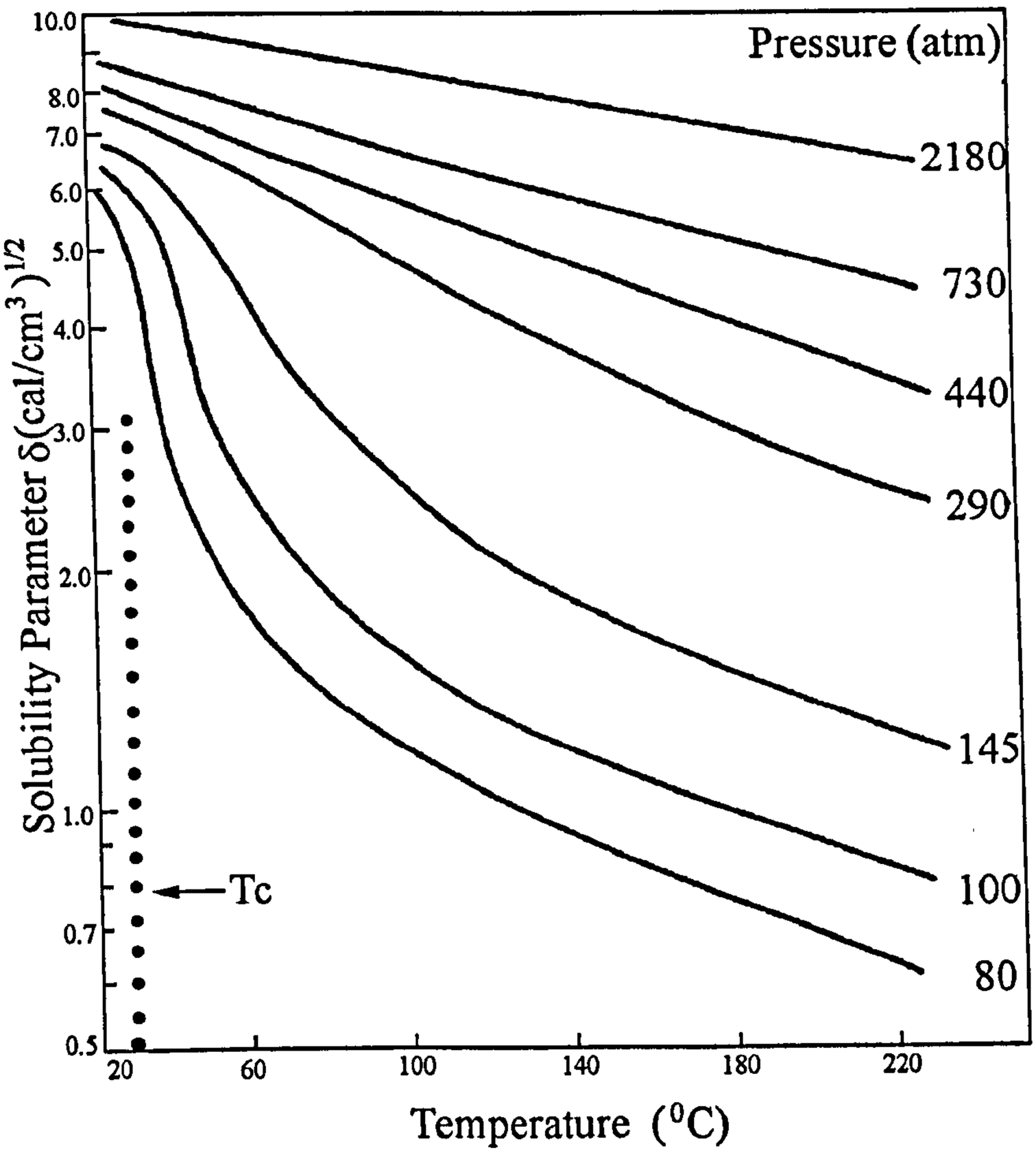


Figure 1.2: *Effect of Temperature and Pressure on the Hildebrand Solubility Parameter for SF-CO_2*[8]

It is essential that the analyte of interest dissolves in the supercritical fluid and thus without any knowledge of analyte solubility, the optimisation of analysis conditions can only be carried out on a trial and error basis. However, there are many models available which describe the solubility of analytes in SF's. The simplest of these is the solubility parameter.

1.1.1.2 *Solubility Parameter*

The dissolution of a solute in a supercritical fluid is a complicated phenomenon thus, the theoretical prediction of equilibrium solubility is often difficult. The maximum solubility which a solute can attain in a SF can be approximated by relating the solubility parameter (δ) of the gas to its critical and reduced state properties using Giddings equation:[9]

$$\delta = 1.25 p_c^{1/2} \frac{\rho_{rg}}{\rho_{rl}}$$

where: p_c = critical pressure

ρ_{rg} = reduced density of gas

ρ_{rl} = reduced density of liquid

For maximum solubility, the solubility parameters of the fluid and the solute should be identical. Therefore, by using the above expression we can estimate which supercritical fluid could be used, and at what density for a given solute.

Other methods which have been used to predict solubility data include equations of state,[10] Hildebrand solubility parameters[11] and the virial coefficient.[12] These approaches require some experimental data to compute unknown parameters and often utilise complicated equations. A simple empirical method was developed by Mitra and Wilson[13] which predicts solubility as a function of the prevailing temperature, pressure and density based on the fact that solubility is determined by a combination of temperature and density. However, other properties such as viscosity, diffusivity and polarity also change with temperature and pressure and these may affect the equilibrium solubility. Mitra and Wilsons' theory is based on a negative gradient linear relationship existing between temperature and density, *i.e.* if the density is raised, the temperature has to be lowered to keep the pressure constant.

1.1.2 Phase Behaviour for Supercritical Fluid-Solute Mixtures

Interpreting and extrapolating high pressure phase behaviour data can be a relentless task. This is a result of the complexity of high pressure phase behaviour of substances at or near their critical points, even in simple binary mixtures, where the components are chemically similar.[14] For a given practical application of SF technology, it is likely that not only will the mixture components be different in molecular size, but they will also differ in shape, structure and polarity. When the mixture components are so diverse, or when the mixture has numerous components, the resultant phase behaviour can be very complex.

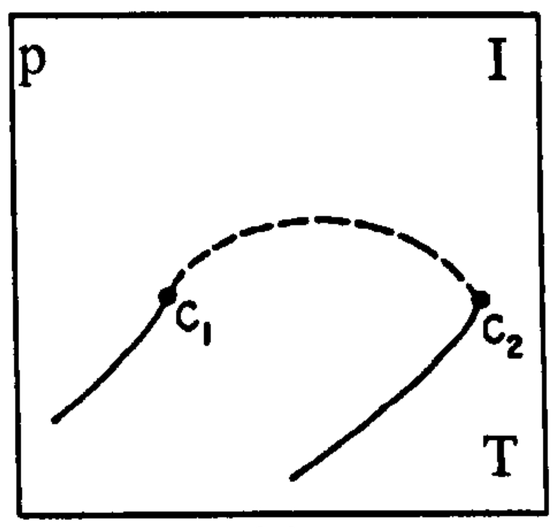
The addition of polar modifiers to CO_2 is well documented.[15] The modifier acts in two ways, firstly by interacting with the analyte/matrix complex to facilitate rapid desorption into the supercritical fluid and secondly by enhancing the solubility properties of SF-CO_2. The phase behaviour of binary systems is highly varied and much more complex than single component systems. Even simple binary compositions can undergo complicated phase transitions near the critical region. Gas-liquid or gas-gas immiscibility can occur at conditions exceeding the T_c or p_c of either pure component. As a result, inhomogeneous phases can occur. For mixed phases, the critical constants of temperature and pressure for the mixture can be approximated as the arithmetic mean of the critical temperatures and pressure *i.e.*:

$$T_c = x_a T_a + x_b T_b$$

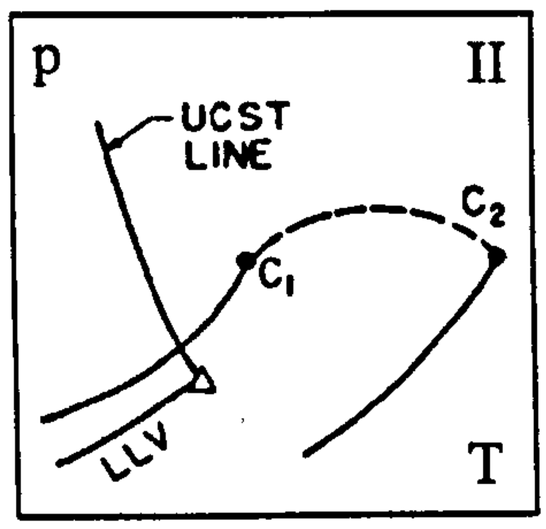
$$p_c = x_a p_a + x_b p_b$$

where x_a and x_b are the mole fractions, T_a and T_b are the critical temperature of a and b and p_a and p_b the critical pressures of components a and b respectively. More elaborate methods are based on the method of Cheuh and Prausnitz[16] for the critical temperature and Kreglewski and Kay[17] for the critical pressure.

A comprehensive treatment of phase diagrams as well as phase behaviour in binary and ternary systems has been published by McHugh and Krukonis.[18] Of the systems described, the simplest is concerned with a binary mixture of a single SF solvent and a single solute.[19] In 1970, Scott and van Konynenburg[20] demonstrated that virtually all experimentally observed binary phase diagrams can be qualitatively described using the van der Waals equation of state. From this, five classes of possible binary phase diagrams were derived as indicated in Figure 1.3. Points C_1 and C_2 represent the critical points of components 1 and 2 respectively. The dashed line depicts the critical mixture curve for the binary mixture, and the open triangles, represent the critical end points. Figure 1.3a is the simplest binary phase diagram. The critical-mixture curve runs continuously from the critical point of the heavier component to the critical point of the lighter component.



(a)



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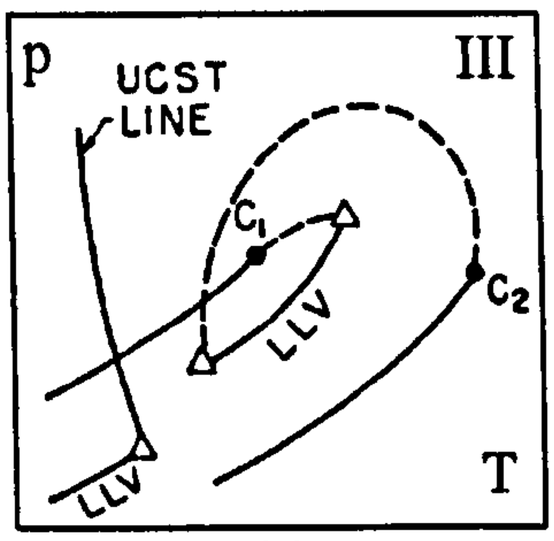

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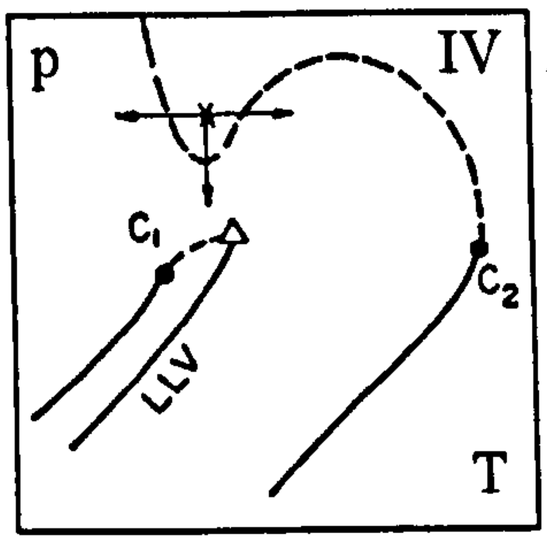
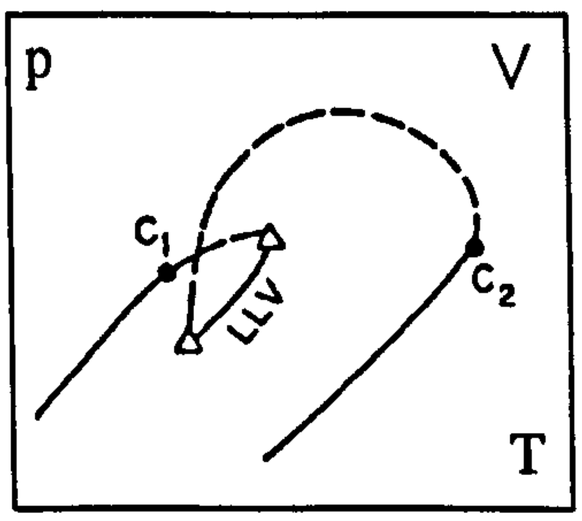


(d)



(e)

Figure 1.3: *Five Classes of Possible Binary Phase Diagrams*[18]

The use of these phase diagrams provides the basis for an understanding of the phase-equilibrium principles that are operative during SF solvent extraction of mixtures, and for simplicity they are drawn as two-dimensional p-T plots. By looking at the general three-dimensional p-T-x plot where x denotes the composition of the heavier component for type I binary mixtures (Figure 1.4), it can be seen how these p-T plots are formed. The p-T-x diagram is constructed by compiling a number of isothermal p-x plots as shown in figures 1.4c and 1.4d. Figure 1.4c is a plot at a temperature which is below the critical temperature of the two components. A vapour-liquid envelope is observed which intersects the pressure axis at two points: the vapour pressure of pure component 1 and the vapour pressure of pure component 2. At a fixed overall composition (denoted by x*) a single

vapour phase exists at low pressures. As the pressure is isothermally increased, the two-phase vapour-liquid envelope is intersected and a liquid phase now appears. The locus of points which separates the two-phase vapour-liquid region from the one phase vapour region is called the "dew-point curve". The concentration of the equilibrium vapour and liquid phases within the two-phase boundary of the vapour-liquid envelope is determined by a horizontal tieline.

As the pressure is increased further, the amount of liquid phase in the mixture increases and the amount of vapour decreases until only a small bubble of vapour exists. As the pressure is increased still further, the bubble of vapour disappears and only a liquid remains. The locus of points which separates the two-phase vapour-liquid region from the one phase liquid region is called the "bubble-point curve". This vapour-liquid envelope is shown at the front of the three-dimensional p-T-x plot (1.4a).

If the temperature is now increased to a temperature above the critical temperature of the component 1 (T_{c1}), the behaviour shown in figure 1.4d is observed. The vapour-liquid envelope is similar to that in figure 1.4c, however the envelope intersects the pressure axis only at the vapour pressure of component 2. As the temperature is greater than T_{c1}, the vapour-liquid equilibrium line for component 1 is never crossed. If an experiment is performed at an overall composition of x**, the vapour-liquid envelope is first intersected along the dew-point curve at low pressures and then at its highest pressure. The second intersection corresponds to the critical mixture point for this temperature and composition. If the overall composition is to the right of x** and the pressure is decreased, a bubble point is observed and if the overall composition is to the left of this point a dew point is observed. Again the three-dimensional plot at this temperatre (T_2) is shown in figure 1.4a.

In order to create the full critical curve, p-x diagrams are produced at higher and higher temperatures to determine the critical mixture points. These p-x diagrams are then assembled to form the three-dimensional p-T-x representation and the critical mixture curve plotted by joining the critical mixture points. Figure 1.3a is created from this by projecting the three-dimensional p-T-x diagram onto a two-dimensional p-T diagram.

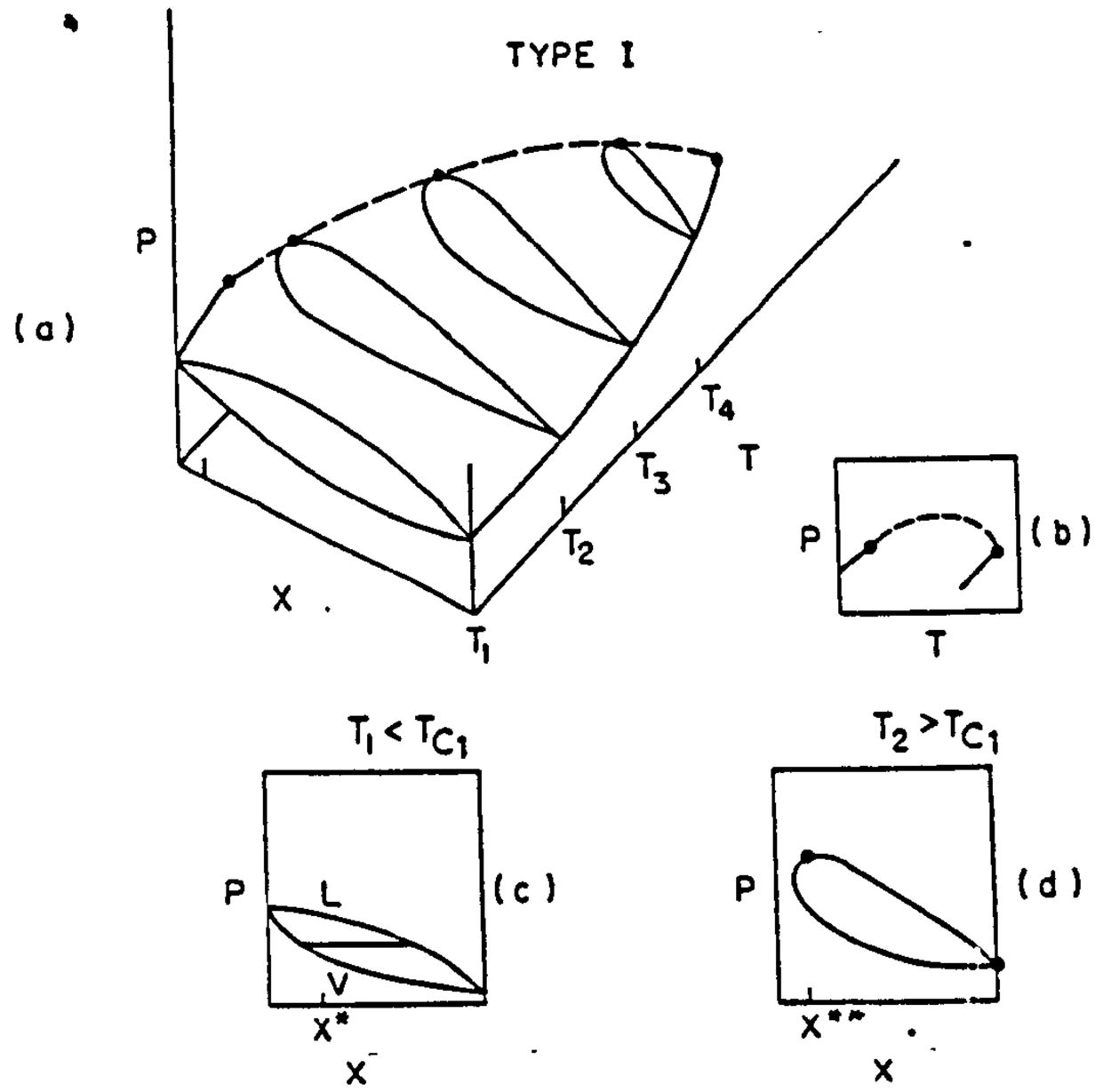


Figure 1.4: *p-T-x, p-T and p-x Diagrams for a Type I Binary Mixture*

The same principles apply when creating the other four binary phase diagrams. An in depth description of all five classes of binary mixture behaviour is provided by McHugh and Krukonis.[18]

1.2 Supercritical Fluid Extraction

The first step in biological sample analysis involves separating the compounds of interest from the matrix (*e.g.* blood, tissue, urine). At present, this is done mainly by liquid extraction techniques such as solid-phase extraction (SPE) and liquid-liquid extraction (LLE). These can prove to be time consuming and costly as they require high purity organic solvents.[21] The use of high purity solvents also means that there is a significant amount of toxic waste produced. Supercritical fluid extraction (SFE) which is a quicker, more convenient alternative method to liquid extraction techniques offers the following advantages.[22]

- Improved efficiency
- Non-toxicity and cost effectiveness of extraction fluid

- Extraction of thermally labile compounds
- Easy control of conditions
- Ease of separation of analytes from the supercritical fluid
- Possibility of direct analysis of complex matrices and thus reduced risk of sample contamination
- Potential of fractionation
- Compatibility with on-line methods
- Possibility of class sensitive extraction by choosing fluid polarity and density and/or the use of a modifier

1.2.1 Method Development in SFE

Before developing a method for SFE, it is essential that the analyst has an understanding of the composition of the matrix and the properties of the analytes.[23] Solubility data and correlations as discussed earlier, provide useful information for choosing initial SFE conditions. However, the determination of the optimum extraction conditions has been largely experimental for two reasons.[8] Firstly, analytical SFE often involves the recovery of a complex mixture of analytes rather than a single analyte. In such cases, the extraction must be optimised for several groups of compounds which complicates the prediction of conditions. Secondly, consideration of solubility only addresses part of the extraction problem. As the extraction of an analyte depends on its distribution between the SF and the sorptive sites on the sample matrix, the ability of the SF to compete with the analyte for the sorptive sites may be more significant than solubility considerations in determining the optimum extraction conditions.

Most SFE methods concentrate on the use of CO_2 due to its reasonable critical properties, its low toxicity and its chemical inertness. It is an excellent choice for non-polar organic compounds. However, it has a severe limitation in that its polarity is often too low to obtain efficient extractions, either because the analytes lack sufficient solubility or the extractant has poor ability to displace the analytes from the active matrix sites. As most of the work on SFE has used analytes spiked on inert supports, it has been assumed that CO_2 is the ideal leacher. However, in authentic samples, the matrix-analyte interactions

dramatically reduce the extraction efficiency which further reduces as the polarity of the analytes increases. There are however ways of getting round this problem as summarised in Figure 1.5.[24]

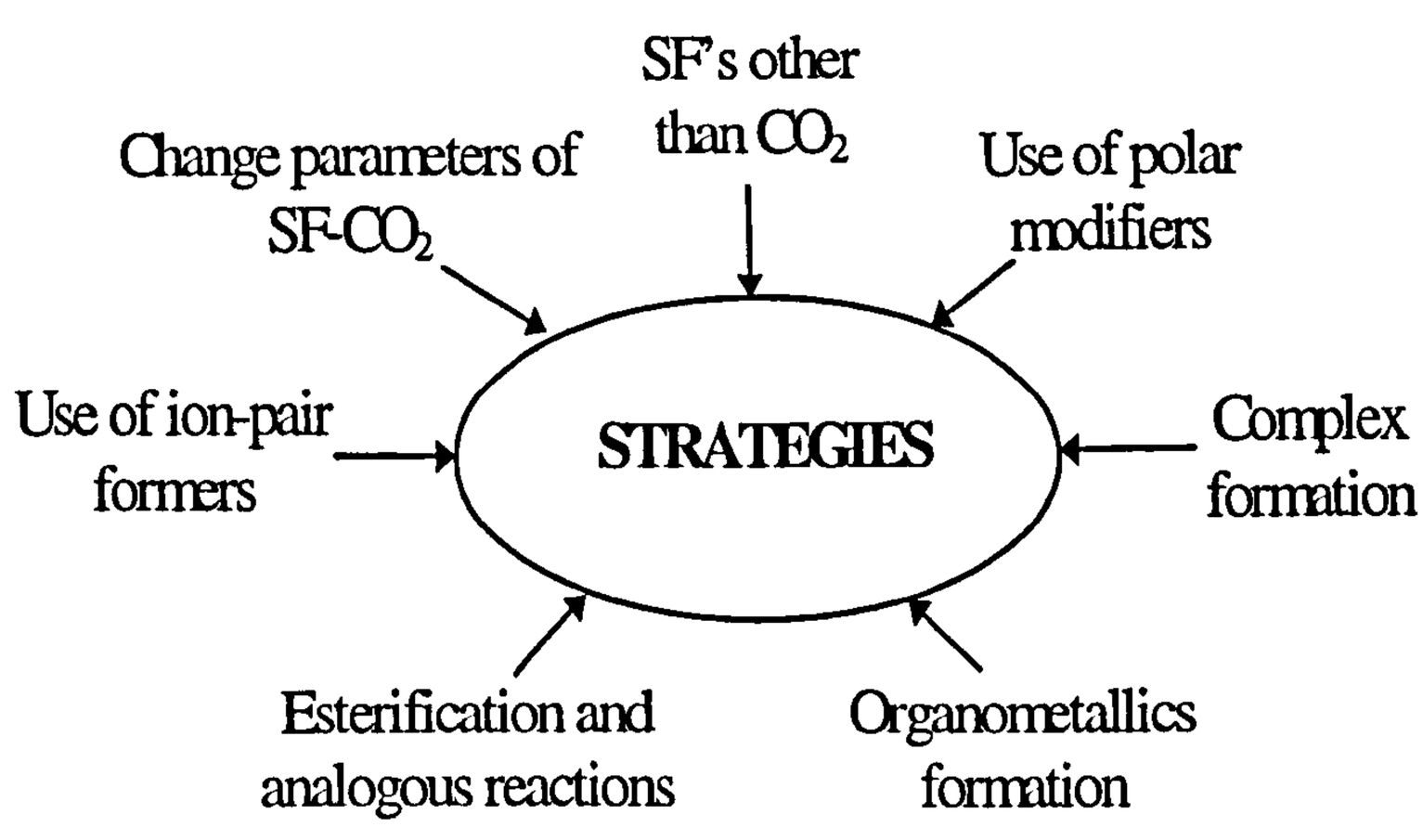


Figure 1.5: *Strategies for Facilitating SFE of Polar and Ionic Compounds*

The simplest of these strategies to apply, when SF-CO_2 at its most polar is ineffectual, would be to use an alternative supercritical fluid. However, in practice, many difficulties are encountered when using polar fluids (*e.g.* ammonia tends to dissolve pump seals and water has impractical critical parameters (T_c = 374 °C, p_c = 227 atm)). As a result of this, the extraction of polar analytes is usually carried out with the addition of polarity modifiers.

The combination of modifier and high temperature has been shown by Yang *et al*[25] to be highly effective in the extraction of polar analytes. The modifier can either be added directly to the sample in the extraction cell or mixed with the CO_2. In general, the latter is more effective since the modifier is continuously passed through the sample. However, as will be seen later, the addition of the modifier directly to the sample also has its advantages.

Although methanol is the most widely used modifier in SFE, a variety of organic compounds have been used including alcohols, propylene carbonate, 2-methoxyethanol and

methylene chloride.[26] The selection of modifiers and their concentration has been largely empirical as very little analyte solubility data exists for modified SFs. In addition, the competitive interactions between the modified SF and the target analytes with the sorptive sites on the matrix are poorly understood. Janssen *et al*[27] have evaluated methods of estimating critical properties and densities of mixed fluids.

The use of modifiers in SFE was first reported by Zhuze.[28] Probably the best known extraction using SFE is that of caffeine from coffee and tea.[29] Although caffeine is extremely soluble in CO_2, it cannot be extracted from coffee and tea leaves using CO_2 alone. The addition of a small amount of water to the CO_2 allows almost complete extraction thus, emphasising the importance of the use of modifiers.

1.2.2 Analytes

The choice of analyte is not a free variable, but is predetermined by the sample. Analysts are interested in qualitative presence and quantitative amounts of a small number of the compounds present in the sample. By adjusting the solubilizing power of CO_2 in SFE, we can selectively reduce the number of compounds extracted.[30] This is done by adjusting the temperature and/or pressure or by the addition of a modifier.

For samples in *e.g.* blood, serum and plasma, the non-polar nature of SF-CO_2 allows easy extraction of non-polar drugs *e.g.* nitroglycerine from water, salts, proteins, carbohydrates, peptides, amino acids and other interfering compounds in a biological matrix. In general, drugs of abuse are polar in nature and thus, the addition of a polar modifier to SF-CO_2 is essential to the extraction process.[31]

1.2.3 Matrices

SFE exhibits its best advantage when extracting analytes from solids or semi-solids as opposed to liquids, fluids and gases.[30] The primary limitation in extracting from a liquid or fluid sample matrix is the design of the extraction cell. The matrix is held within the extraction cell by porous frits, similar to those used for the end frits in HPLC columns. These frits hold small particles inside the column and allow fluids to pass through, thus

attempts to pass SF-CO_2 through a liquid will result in the liquid matrix passing through the frits. Successful SFE of liquids requires the liquid being mixed with a solid material *e.g.* diatomaceous earth so that the sample is no longer free flowing.

1.2.4 Choice of Extracting Fluid

A good solvent for extraction should:[21]

- Be selective: as the objective is to separate the analyte from a matrix, the solvent should be able to dissolve the desired analyte better than the other constituents of the matrix
- Have a high capacity for the analyte: this helps minimise the volume and time required to extract quantities suitable for analysis
- Be stable and unreactive to the solute and matrix under the conditions of the extraction
- Not be corrosive to the equipment
- Be relatively inexpensive
- Be non-toxic

From an extraction point of view, the important properties of supercritical fluids include the diffusion coefficient, the density and the viscosity. Faster diffusion leads to more efficient separations than liquids facilitate. The viscosity of a supercritical fluid falls between that of a gas and a liquid, which facilitates pumping and fluid flow. Enhanced passage of the solvent into the interstices of the matrix occurs as a result of low viscosity and the absence of surface tension.

1.2.5 Selectivity in Extractions

The extraction of analytes from adsorbing matrices involves three basic steps:[32]

1. Removal of compounds from the matrix surface.
2. Solvation of analytes in the fluid.
3. Mass transport of solubilized molecules to the bulk of the fluid to allow removal from the extraction vessel.

To optimise analytical SFE, it is necessary to define a rate determining step (RDS). Due to the chemical differences of each molecule and matrix, the analyte-matrix interactions are unique for each sample. Conditions have to be selected to emphasise the differences between any interferences present and the target analyte in order to achieve effective extraction.

Step three is least likely to introduce selectivity. The primary driving force here is molecular diffusion within the solid matrix and the rate is defined by the diffusion coefficient.[33] This is inversely proportional to the square root of the molecular weight of the solute at constant density of the fluid, therefore if this was the RDS, separation would only occur between species of substantially different weights.[34] These differences can be emphasised by adjusting the porosity of the extraction matrix.[35] However, in most cases the interferents do not differ substantially from the analytes in weight.

Step two has been extensively investigated.[36,37] To ensure dissolution of the analyte in the fluid, the cohesive energy (*i.e.* the energy which holds the structure of a substance together) must be overcome by the interaction of the molecules with the fluid. When this is the case the solubility properties of the solutes are defined by the solubility parameter (δ).[38]

$$\delta = \left(\frac{\Delta E_v}{V}\right)^{0.5} = \left[\frac{D(\Delta H_v - RT)}{M}\right]^{0.5}$$

where: ΔE_v = energy of vaporisation
V = molar volume
D = density
ΔH_v = heat of vaporisation
R = Gas constant (8.314 J K^{-1} mol^{-1})
T = temperature
M = molecular weight of solute or fluid

This same value of δ can however be calculated for a SF more conveniently by using Giddings equation as described in section 1.1.1.2.

As the solubility of analytes in a fluid is related to the difference between their solubility parameters, the more similar the solubility parameters are, the greater the solubility. It is

therefore feasible to design experiments where the analyte is soluble in the fluid, but the interferents are not. This is only possible if the analyte and the interferents have different solubility parameters.

The most promising separations are achieved by the presence of an additional phase in the system. A distribution constant K, can be defined, which is related to the differences in the solubility properties of the two phases.[39] There must be a substantial difference between the distribution constants and the solubility parameters corresponding to the analyte and interferences to ensure complete separation based on a low efficiency system such as a loosely packed extraction column.

In addition to determining a single type of analyte, the selectivity of SFE can be exploited by controlling the temperature and pressure, to perform a sequential extraction of various classes of analytes, providing they are of suitably different polarities. At a constant temperature, the extraction of non-polar analytes is favoured at low pressures, while the extraction of polar analytes requires increased pressure. Most extractions use a low temperature of the order of 50 °C and increasing pressure is used to extract increasingly more polar target analytes to obviate the risks to thermolabile compounds. However, Langenfeld *et al*[40] demonstrated that the influence of temperature is paramount to the efficiency of the extraction of polar compounds in environmental matrices.

Step one makes use of solid surface adsorbants. Separation occurs due to partitioning differences caused by different interactions between the target analyte and interactions with the surface. Interactions between the surface and the analyte can be in the form of weak adsorption through dispersive interactions, but can also involve sharing of electrons between the adsorbent and the analyte (chemisorption).[41] Interactions with a surface, are much more specific than with a liquid. Small differences in geometry, basicity or acidity of the molecules can differentiate between individual molecules. This means that molecules with similar solubility properties which differ in chemical or steric properties can be separated.

The other advantage is that the elution process can be designed to be selective since it proceeds through the formation of the activation complex. Figure 1.6 shows the energy requirements for the desorption process of an analyte from a solid surface. If partitioning is involved, with a liquid as the stationary phase, equilibrium is established immediately according to the partition coefficient (neglecting adsorption/desorption kinetics at the interface). If the process involved is desorption from a solid surface it is kinetically limited due to the energy barrier of desorption.

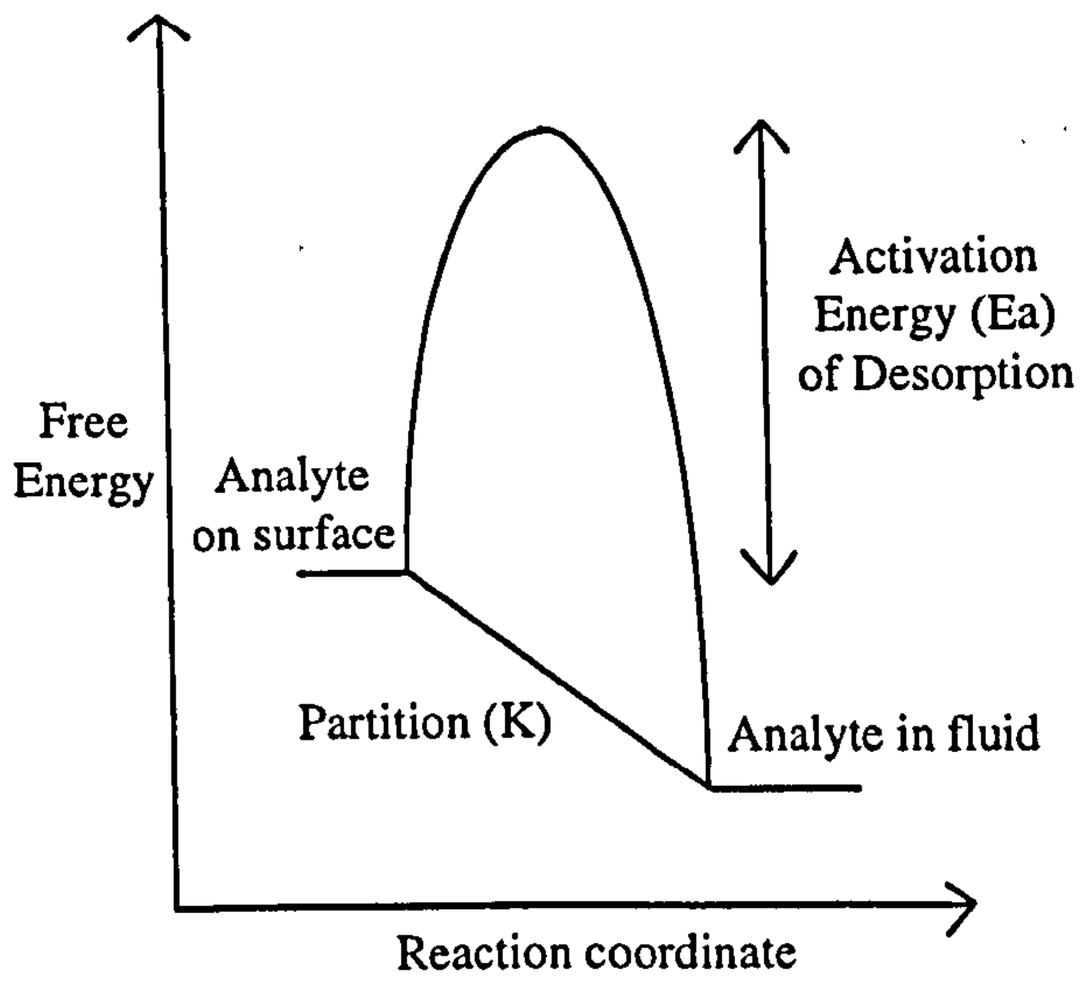


Figure 1.6: *Free Energy v Reaction Coordinate Indicating Energy Requirements for Desorption of Chemisorbed Species*[31]

The thermal energy easily overcomes the low energy barriers associated with weakly adsorbed species. However, strongly adsorbed species require that the activation energy (E_a) be reduced by selective interaction of solvent molecules with the matrix-solute complex in order to break the bonds. Instead of the solubility parameter of the eluting solvent being the most important parameter, its chemical/structural properties have more of an effect. It should be noted that it may be easier to remove the interferents first, leaving the analyte of interest on the extraction surface until harsher conditions are applied.

1.2.6 PARTICLE SIZE

Another factor effecting extraction recoveries is the particle size of the extracting media. Diffusion from the sample matrix is very important. It has been shown that the percentage recovery increases from 78% to 99% by halving the particle size.[21] An alternative way to increase the recovery would be to increase the extraction time by a factor of four. As one of the main advantages of SFE is its reduced time, this is extremely impractical.

1.2.7 EXTRACTION METHODOLOGY

SFE can be carried out in either a dynamic or a static mode.[8] However, most SFE methods are in the dynamic mode where the SF is passed continuously through the extraction cell. Pressure is controlled *via* a flow restrictor which maintains supercritical conditions inside the extraction vessel, but allows depressurisation into a collection device. Static SFE is carried out by pressurising the extraction vessel and extracting the sample without an outflow of the SF. After a pre-defined time, a valve is opened which allows the extracted analytes to be swept out into the collection device. It is here that the advantage of adding the modifier directly to the extraction vessel is important. A fixed amount of modifier can be added to the matrix and then the extraction vessel pressurised using only CO_2.

An advantage of dynamic SFE is that fresh SF is constantly passed over the surface of the matrix containing the analytes. However, this method uses more fluid than static SFE which is particularly important when the sample volumes are large.

1.2.8 OFF-LINE SFE VERSUS ON-LINE SFE

Off-line SFE is inherently simpler than on-line SFE, as only the extraction step needs to be optimised and the extract can be analysed by any appropriate method.[8] On-line SFE requires optimisation of both the SFE and the chromatographic conditions and unlike with off-line SFE, the sample extract is not available for subsequent analyses. The principle advantages of on-line SFE are, the elimination of sample handling between the extraction and chromatographic analysis and the potential to achieve maximum sensitivity by quantitatively transferring the extracted analytes into the chromatographic system.

1.2.8.1 Off-Line SFE

Off-line SFE has been extensively studied as a sample preparation technique for chromatographic analysis.[8] The analytes are most often collected in a few millilitres of a liquid solvent and analysis is carried out as it would be in a conventional liquid solvent extraction. It is adaptable to sample sizes ranging from milligrams to several grams and is equally applicable to the recovery of trace analytes or complex mixtures.[42] One important concern with off-line SFE is the efficacy of the analyte trapping/collection method as discussed in section 1.2.9.

1.2.8.2 On-Line SFE

The combination of SFE with a chromatographic technique is a logical extension of the process.[8] The variable solvating power of a SF provides the mechanism for the selective extraction of the components of interest from the sample matrix. It also provides the basis for an automated method where the sample preparation and final analysis can be instrumentally linked. On-line SFE is particularly attractive for small sample sizes and/or trace analysis.

1.2.9 SFE-Coupled Techniques

A variety of techniques have been used for both on and off line analysis with SFE.[22,43] The most common of these is GC[8] with both packed and capillary columns. Other methods which have been used include TLC[44], HPLC[45], SFC.[46] Selection of the best technique for coupling is based on the nature of the analytes *e.g.* if the analytes have sufficient vapour pressure, GC is a good choice due to high resolution per unit time and the possibility for the use of many types of detector.

1.2.9.1 Trapping Techniques for SFE-Coupled Techniques

For good results with chromatographic coupling, it is essential to trap and focus the analytes before chromatography commences. If this step was omitted, the resulting chromatogram would be a collection of flat broad peaks. The trapping methods used must be capable of quantitatively collecting all the analytes at relatively high flow rates.

Trapping can be external or on-column. External trapping can be done using either a cold trap or a sorbent resin. The extract is depressurised onto the trap and once the SFE has finished, the trap is heated and the analytes swept onto the chromatographic column. This method is preferable to on-column trapping as the fluid does not have to be vented and hence, there is no risk of contaminating the column or detector. However, if the primary objective is quantitation, on-column trapping is far superior. The use of an external cryogenic trap is particularly useful with SFE-GC where a FID is used. Modifiers *e.g.* MeOH, cause detector problems and since the trapping can be made selective, the MeOH can be removed prior to injection onto the column.

1.2.9.2 SFE-TLC

SFE-TLC provides a rapid and simple insight into the performance of an extraction. The strength in this technique is that the extract is deposited directly onto the TLC plate and therefore, detection is a static process.[47] Both one and two dimensional chromatography can be carried out.

There are several limitations with this technique including difficult quantitation and low resolution compared to SFE coupled with HPLC, GC or SFC. There is also the possibility that the high pressures involved in the SFE can cause problems with the TLC support material.

1.2.9.3 SFE-HPLC

Various off-line applications of SFE-HPLC have been reported *e.g.* investigating the effects of different extraction parameters on the amount of caffeine extracted from roast coffee beans.[48] Off-line SFE-HPLC can be carried out using a closed loop system with recycling and a trap column. The analytes are washed from the trap column, collected and injected into the HPLC system.

On-line SFE-HPLC was first reported by Unger and Roumeliots in 1983.[49] Their system consisted of two high pressure sample injection valves connected in series. The first of these valves acted as a switch valve to the loop and controlled the pressure over the

column. Two short microbore columns were placed between the valves to absorb the analytes and act as a sample loop for the second valve which served as an injector for the HPLC column.

1.2.9.4 SFE-GC

Most of the work on coupled SFE techniques concentrates on SFE-GC, although SFE-SFC is becoming more widespread. Examples of off-line SFE-GC include the analysis of polyaromatic hydrocarbons (PAH's) and polychlorinated biphenols (PCB's) which are well documented in the literature.[22] As samples for GC analysis tend to be volatile, the method by which the analytes are trapped is very important. Various trapping methods have been tried including solvent traps and sorbent traps (*e.g.* XAD-2, polyurethane foam and Tenax).[50]

On-line SFE-GC was first carried out by Hawthorne and Miller[51] when they successfully analysed automobile-exhaust organics. Since then, the number of applications reported on this technique has increased dramatically.

There are several possible modes of action for SFE-GC, such as quantitative extraction of analytes from a matrix, quantitative extraction and concentration of trace analytes and selective extraction at various solvating powers. The supercritical extract can either be directly pressurised inside a conventional split/splitless injection port or on the column itself. The best sensitivity is achieved where the sample is small since all the extracted analytes go directly onto the column. If the sample is large, the split mode can be used.

1.2.9.5 SFE-SFC

An obvious advantage of SFE is that it is an ideal way to introduce a sample into an SFC system. The injection solvent is the same as the mobile phase and therefore, the criteria for successful coupling of different techniques are fulfilled. An additional advantage that the technique has over SFE-HPLC/GC is that it is highly unlikely that sample components which are insoluble in the mobile phase will be introduced into the column.

There are many ways of combining SFE with SFC. Conventional packed columns, capillary columns and packed capillary columns have all been successfully used.[22]

Directly coupled SFE-SFC with packed columns was introduced in 1985 by Sugiyama *et al*[52] for the analysis of powdered coffee beans. No sample pretreatment was required. The system was set up with an injector valve containing an extract trap loop after the extraction column and another valve prior to the SFC column, which remained closed during the extraction step. Once extraction was complete, the valve prior to the column was opened and the contents of the extract trap loop flushed onto the column. The use of valve switching is very common in SFE-SFC and is well documented.[53]

SFE with capillary SFC has also been widely investigated. Gmur *et al*[54] investigated the optimisation of important instrumental parameters for the analysis of natural products. On-line SFE-capillary SFC had also been coupled with off-line FTIR for the separation and identification of PAH's in coal pitch. In this case the samples were collected on a bromide disc after the SFC.[55]

The use of packed capillary columns is advantageous as it provides higher loading capabilities and shorter analysis times than capillary columns. However, capillary columns give lower pressure drops, lower flow rates and higher efficiencies than packed column SFC.

1.2.10 Trapping Techniques in SFE

Three different trapping techniques are commonly used in off-line SFE for the collection of analytes.

- Liquid collection
- Dry deposition onto a solid surface
- Solid phase sorption

All three systems have their advantages and disadvantages with respect to choice of restrictor type, maximum flow rate and compatibility with various types of SF's, modifiers and analytes.[56]

One of the main problems in SFE, is the limitation in the decompression speed after the extraction due to difficulties in collecting the extracted analytes with good efficiency. In most systems, the maximum flow through the restrictor is in the range of 0.5-2.0 $mLmin^{-1}$ which is equivalent to a flow after the restrictor of 500-1000 $mLmin^{-1}$ of gas. Since it is desirable to minimise the extraction time, a maximum flow of 0.5-2.0 $mLmin^{-1}$ of SF leads to limitations in the size of the extraction vessel and thus limitations in the detection limits.

1.2.10.1 Liquid Collection

Liquid collection is the simplest method for collecting analytes from an SFE system. The restrictor is simply placed into a vial containing solvent as indicated in Figure 1.7. The analyte is trapped in the solvent while the decompressed SF vents to the atmosphere. The solvent used must be compatible with the analytes and with any modifier which is required for the extraction. A broad range of solvents have been used for SFE including methanol, methylene chloride, chloroform, acetone and hexane.[57] Due to the cooling associated with the decompression of CO_2, it is possible for the trapping solvent to freeze and for small pieces of ice to clog the restrictor tip. For this reason, the restrictor is often heated or the collection solvent maintained at a constant temperature. However the advantage of this cooling is that it prevents evaporation of the collection solvent which would occur due to the high gas flows.[8]

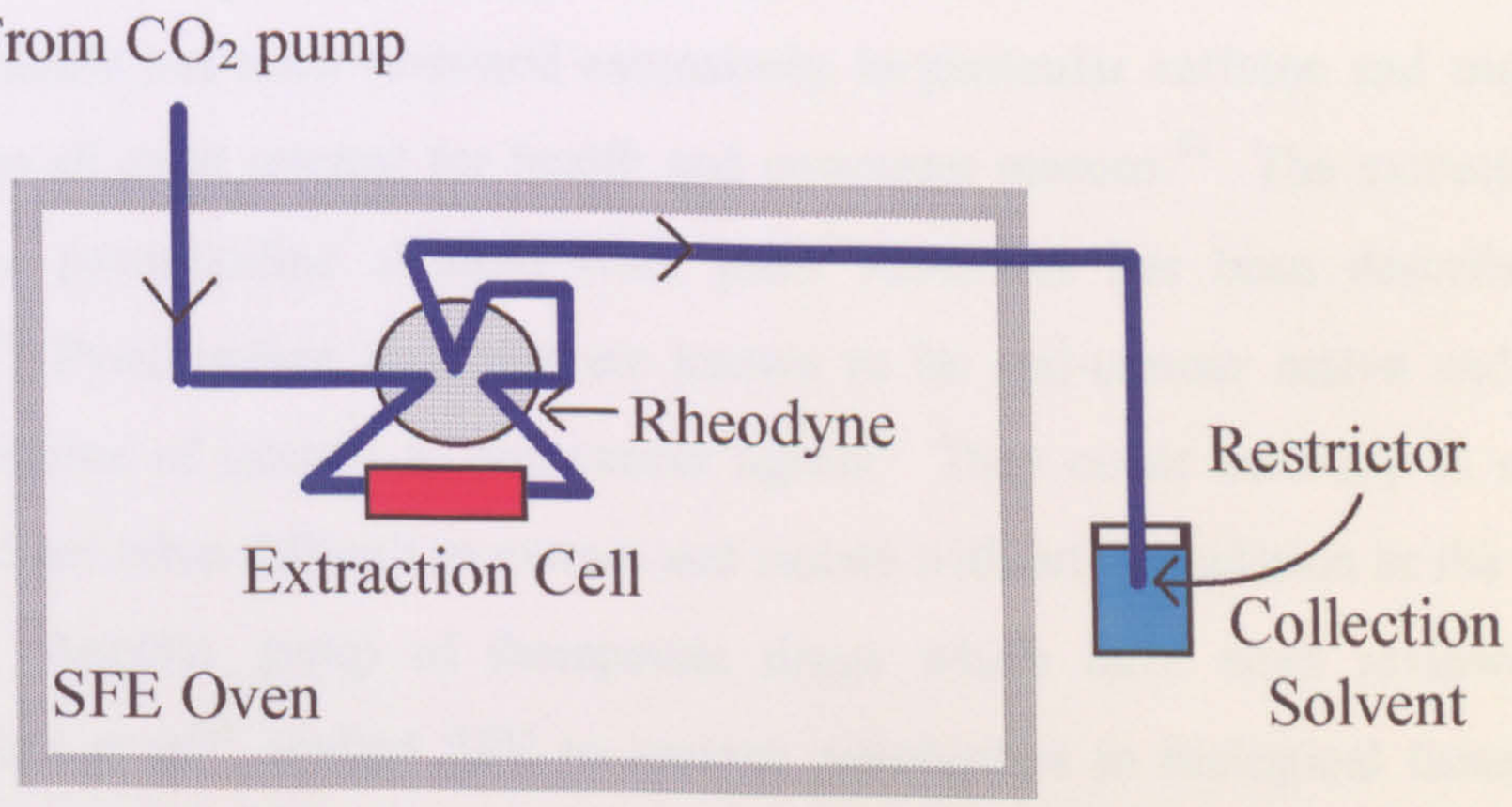


Figure 1.7: *Diagram of Liquid Collection Set-up*

1.2.10.2 Dry Deposition

Here, the surface of a solid is used as a support to collect the analytes of interest. The surface may be cooled cryogenically using liquid nitrogen or carbon dioxide or simply by the expansion of the SF at the flow outlet. Typical solid surfaces which have been used are glass vials, stainless steel beads and glass beads. Once trapped, the analytes are washed from the surface and collected for further analysis. With this method, faster flow rates (up to 4 $mLmin^{-1}$) may be used however, an additional washing step is required which is not necessary with liquid trapping.

1.2.10.3 Solid Phase Sorption

This is often a chromatographic packing material which traps by two mechanisms. The trap is cryogenically cooled again either by an external source or by the expanding SF and the analytes are absorbed onto the packing material. The analytes can then be washed from the packing material using a suitable solvent. As with dry deposition, flow rates up to 4 $mL\ min^{-1}$ can be used, again an additional wash step is required.

1.2.11 Applications of SFE

The applications of SFE, some of which have been mentioned in the course of this report, are very varied. In general the applications of SFE have been developed as faster, less solvent-intensive alternatives to traditional extraction schemes. Some of the categories of compounds have been analysed by SFE are summarised in Table 1.3.

Table 1.3: *Examples of Uses of SFE*

Explosives and propellants	Nitroglycerin Trinitrotoluene Dipropylphthalate
Polar compounds	Phenols Polyhydroxybenzoic acids
Flavours and fragrances	Red and black pepper Citrus oils
Fossil fuels	PAH's Coal and petroleum pitches
Environmental samples	Sediments/soils/sands Chlorinated materials
Pesticides and herbicides	4-nitrophenol
Natural products	Dairy produce
Fats and oils	Triglycerides Soyabean oil
Pigments and dyes	Carotene Porphyrins Metal chelates
Drugs	Pharmaceutical Drugs of abuse

1.2.11.1 SFE Of Drugs

Of the applications mentioned, the one of most relevance to this report is drugs. Several methods exist in the literature for the supercritical analysis of drugs but these are mainly concerned with chromatography.[58,59,60]

The SFE of alkaloids has been reviewed extensively in particular caffeine and nicotine, both of which are of great interest for health and consumer reasons.[61] The extraction of chemotherapeutic pyrrolizidine alkaloid from plant substrates has been described by Schaeffer *et al.*[62] Pyrrolizidine alkaloids are known to be anti-tumour active and more recently have become of interest as anti-cancer agents. They occur naturally in several plant species and are often difficult to extract and isolate without degradation or the use of toxic solvents. Another group of therapeutic drugs which have been reviewed are xenobiotics. Nam *et al*[63] applied SFE to various xenobiotics in biological tissues and fluids and achieved good recoveries of all but the polar solutes when CO_2 was used alone.

Methods also exist in literature for the subcritical extraction of morphinic alkaloids in urine[64], the supercritical fluid extraction of budesonide from blood plasma[65] and benzodiazepines from solid dosage forms[66] and more recently the extraction of drugs of abuse (morphine, cocaine) from drug addict hair.[67] As these drugs are mentioned later in this report, these papers will be reviewed where relevant.

In general, despite these examples, the extraction of pharmaceutical drugs and drugs of abuse using supercritical fluids is not well documented.

1.3 Instrumentation in SFE

1.3.1 Introduction

Inherently SFE is a simple process. The SF is delivered by a pumping system through a thermostated high pressure vessel, containing the sample of interest, and a flow restrictor to an appropriate analyte collection device. Due to this, the instrumentation for SFE has required little development since it was first introduced. Commercial systems are available which have addressed issues like automation, multiple extensions, flow restrictor plugging, analyte collection and interfacing.

A schematic representation of the instrumentation required for SFE is given in Figure 1.8. It consists of three basic sections. The first of these is a pumping system similar to that used in HPLC, the second is an extraction vessel which houses the matrices from which the analytes have to be extracted and the third is a pressure regulator.

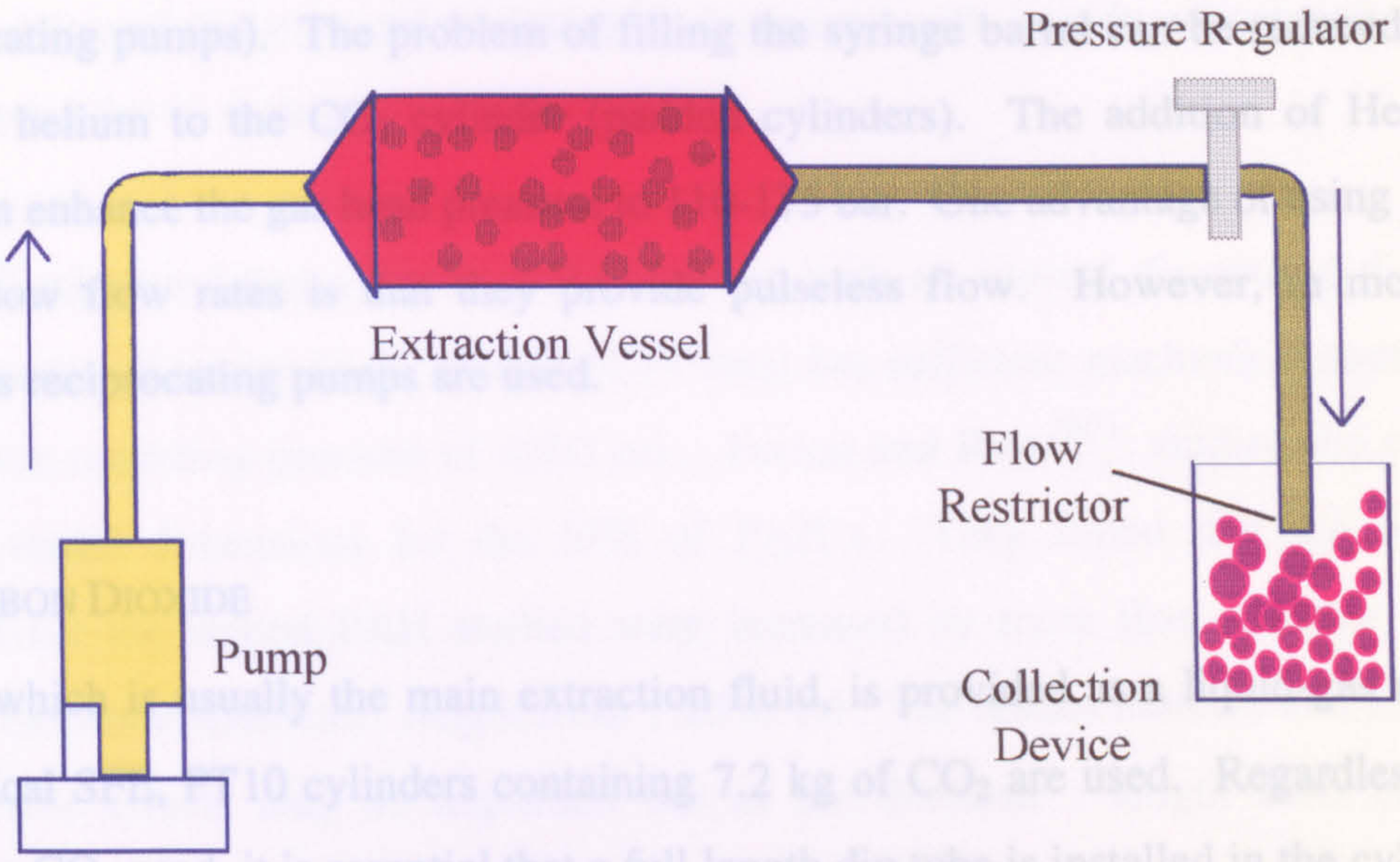


Figure 1.8: *Schematic Representation of the Main Components of a SF System*

Two other important components of the SF system are the collection device, used to trap the extracted analytes and a flow restrictor at the end of the outlet tubing which helps prevent complete depressurisation of the SF in the outlet tubing. A detector, generally UV, can also be used to monitor the extraction effluent.

1.3.2 SFE Pumps

The pumps used for SFE are derived from HPLC pumps, often with little or no modification. One useful adaptation is to cool the head of the CO_2 pump so that the CO_2 is in its liquid form and is thus highly compressed.[68] It is important to properly insulate the CO_2 pump cooling system to prevent excessive air moisture condensation and loss of cooling efficiency. Cut-to-fit polystyrene foam can be used with expanded polystyrene hoses for the cooling system tubing.

When high flow rates are being used, reciprocating pumps are preferred. As well as having the advantage of delivering unlimited volumes within a continuous flow, they can easily incorporate a gradient system. When syringe pumps are being used it is found that they take a long time to fill their cylinder if only the CO_2 vapour pressure displaces the liquid

into the pump. Thus, they can only be used with very low flow rates ($\mu Lmin^{-1}$ *c.f.* $mLmin^{-1}$ for reciprocating pumps). The problem of filling the syringe barrel can be reduced by the addition of helium to the CO_2 cylinder (padded cylinders). The addition of He to the cylinder can enhance the gas head pressure to 110-175 bar. One advantage of using syringe pumps at low flow rates is that they provide pulseless flow. However, in most SFE applications reciprocating pumps are used.

1.3.3 Carbon Dioxide

The CO_2, which is usually the main extraction fluid, is provided as a liquid-gas mixture. For analytical SFE, PT10 cylinders containing 7.2 kg of CO_2 are used. Regardless of the grade of the CO_2 used, it is essential that a full length dip tube is installed in the cylinder as it is the liquid CO_2 which is required for pumping. At room temperature CO_2 has a substantial vapour pressure which helps displace the liquid to the pump.

1.3.4 Modifiers

Organic modifiers can be introduced into the eluent in a number of ways. Cylinders doped with MeOH, 2-propanol and other solvents are commercially available. Alternatively, various mixtures of modifiers in CO_2 can be made by mixing the components together in a syringe pump or by having a two pump system and using on-line mixing which allows gradient eluent compositions.

1.3.5 Extraction Vessel

The high pressure sample vessels initially used for SFE were made using empty HPLC columns. Subsequently, vessels designed specifically for SFE have been introduced. Unlike HPLC, where only the inlet fitting is under pressure, the extraction column outlet fitting is also under pressure and must withstand the maximum operating pressure in general at least 4500 psi.

Conveniently, HPLC fittings can be used for SFE systems, but great care must be taken regarding the pressure resistance of such components. When a gas is compressed to a high

pressure, at a few hundred kg cm^{-3}, a considerable amount of energy is stored that could be explosively released if the vessel is damaged, compared with liquids, where only a little energy is stored due to their lower compressibility.

Saito *et al*[69] calculated that a typical HPLC column with an internal diameter (i.d.) of 4.6 mm and an outer diameter (o.d.) of 1/4" (6.35 mm) has sufficient mechanical strength for the maximum operating pressure of 4500 psi . Furton and Rein[70,71] studied the effect of extraction vessel dimensions for the SFE of PAH's. They found that the observed efficiencies for the largest PAH studied were increased by more than a factor of 2 by increasing the vessel diameter : length from 1:20 to 1:1. The results indicate that for longer cells inductive effects may be important but as the diameter : length ratio increases, diffusion controlled mechanisms dominate the extractability of PAH's.

Ong *et al*[72] designed an extraction cell for use with either liquid or solid samples which yielded an extraction efficiency of 98% for cholesterol spiked equine blood/serum. Another approach for liquid samples is to absorb the liquid onto a solid material prior to extraction. Hopper and King[73] reported the use of calcified diatomaceous earth to increase the permeability of the sample to the SF extracting solvent and absorb H_2O from the sample.

1.3.6 The Back Pressure Regulator

The device which characterises SF instrumentation is the back-pressure regulator (BPR). The BPR, pressurises the system and is where the main pressure drop of the system abruptly occurs. There are two different types of BPR currently used in SFE and SFC.

1. Simple restrictor
2. Mechanical or electronic feedback regulator

A simple restrictor is a capillary tube with an appropriate length for the required back-pressure and flowrate. This type of restrictor is used most commonly with open-tubular capillary SFC. It is easy to assemble and has a small dead volume. However, in order to

change the back-pressure, it is necessary to alter the flowrate as the back-pressure is produced only by flow resistance.

A mechanical/electrical feedback regulator is a complex device consisting of a pressure sensitive mechanism and a needle valve. The regulator can control back pressure irrespective of the mass flowrate of the fluid. Therefore, these BPR's are more convenient to use than simple restrictors for the precise examination of extraction yield versus pressure in SFE and other parameters which require measurement under the same mass flowrate. However, conventional BPRs have a high dead volume and thus, must be placed in line in the system post-detector.

An ideal BPR should have a low dead volume (<10 μl), high precision, pressure control independent of mass flowrate and an external reference signal for pressure/density programming. Saito *et al*[74] have developed a new style BPR based on a totally different operational system to that conventionally used. Flow resistance is created by changing the gap size between a needle valve and a valve seat. The system is based on high speed switching of the fluid flow by periodically opening and closing the flow path, and is used in conjunction with a pressure transducer and control circuitry for the regulation of back-pressure.

1.3.7 Detection in SFE

Various types of detector have been used for SFE and SFC including ultra-violet (UV), diode array (DAD), flame ionisation (FID), mass spectrometry (MS) and Fourier transform infra-red (FTIR). Although the detector is an integral part of a SFC system its use in SFE is advantageous as the extraction process can be monitored.

The most widely used type of detector in SFE is UV. With SFs and UV detectors, some noise is inherent due to density related changes in the highly compressible SF. However, this is counteracted by the fact that SF-CO_2 is transparent even at 190 nm, which is the short wavelength cut-off of most variable wavelength detectors. Thus, even compounds which are thought to have little or no UV absorbance, *e.g.* lipids, can be detected by UV at wavelengths of <200 nm. This advantage of SF-CO_2 is revoked by the addition of

modifiers to the SF system which generally have cut-off wavelengths >200 nm *e.g.* methanol → 205 nm, ethyl acetate → 255 nm, diethylether → 205 nm.

Although fixed wavelength UV detectors have been the detector of choice for analytical SFE, photodiode array detectors have also been used. By using multiwavelength photodiode array detectors, it is possible to monitor the UV spectra of an analyte as a function of extraction time.

To utilise any type of UV detector with a SF, it must be equipped with a high pressure flow cell which can withstand pressure of at least 4500 psi. Metal gaskets of lead or 24 ct. gold with sapphire windows are generally suited for the construction of high pressure flow cells. However, the UV cut-off wavelength of sapphire is ≈220 nm which sacrifices the excellent transparency of SF-CO_2. Although the tensile strength of quartz is several times lower than that of sapphire and thus the window must be substantially thicker, it is a good material for use when monitoring below 220 nm.

1.3.8 Flow Through the System

The CO_2 remains liquid throughout the pumping and compression zones and passes through small diameter metal tubing as it approaches the column (SFC) or extraction cell (SFE). A preheated injection valve before the column or extraction cell is useful so that supercritical conditions are applied immediately to the analyte matrix. The flow then passes through the column/extraction cell at a rate and temperature determined during method development. The mathematical product of the time period and flow rate yields a net volume of CO_2 analogous to partition coefficients and total retention volumes in other types of extraction and chromatography. Next, the SF containing the extracted analyte flows through additional capillary tubing (*via* the detector) until it reaches the restriction zone at the BPR. After leaving the extraction vessel, the fluid can return to liquid state. At this point retaining supercritical conditions for analyte solubility is unnecessary. Lower temperatures increase the density which in turn causes an increase in intrinsic saturation solubility. At the restrictor zone, the temperature usually increases significantly. Between the pump and the outlet of the restrictor the pressure is deliberately increased to a very high level. As the fluid passes out of the restrictor the conditions are no longer supercritical.

The pressure is lowered to 1 bar in a short period of time over a short linear distance. Joule-Thomson type cooling occurs which is compensated by heating the restrictor zone to avoid plugging and too rapid precipitation of the analytes, CO_2 or any other material present.

Finally the analyte either goes to waste or is collected for further analysis. There are three main types of collection device as described in section 1.2.8. All three methods are dependent on flow restriction. There is a risk of plugging due to co-extracted or extraneous materials due to the sudden decrease in pressure at the flow outlet. This can be prevented by the use of a post-extraction solvent flush.

1.4 Setting up of Instrumentation

A home-made SFE system was favoured over a commercial one, as many of the components were already available. A schematic representation of the instrumentation used for this study is shown in Figure 1.9. The system consisted of the following components.

- PT 10 CO_2 cylinder fitted with a full length dip-tube (7.2 kg of CO_2)
- Two Gilson 10SC pumps programmed *via* Gilson 715 software
- A Gilson 811B dynamic mixer
- A Gilson 805S manometric module
- A 7125 Rheodyne fitted with a 100 µl injection loop
- A Pye Series 104 GC oven
- A Kratos Spectraflow 757 UV detector
- A Rheodyne 7037 back-pressure regulator

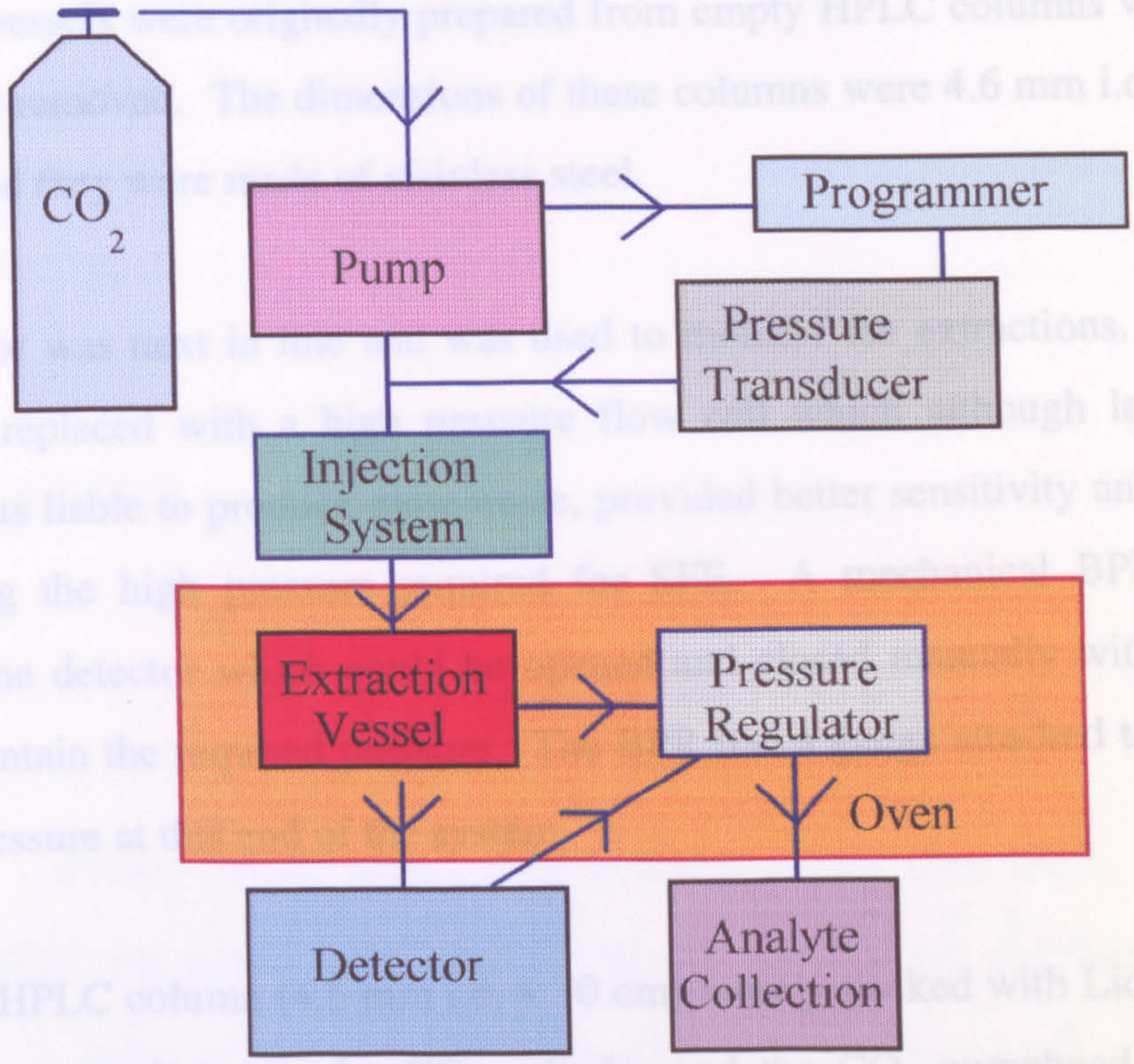


Figure 1.9: *Instrumentation Used for SFE*

The original PTFE (polytetrafluoroethylene) check valves in the CO_2-10SC pumphead were replaced with PVDF (polyvinyldifluoride) check valves as these are more resistant to extremes of heat and cold. This pumphead was chilled using a SFC3 refrigeration unit. The second pumphead was used with the PTFE check valves and was used to pump modifier.

The pressure at the pump end of the system (*i.e.* before the extraction cell) was measured by the Gilson 805S manometric module and monitored on the Gilson 715 software package. The software controller allows programming of up to four pumps. The CO_2 and modifier were premixed by the Gilson 811B dynamic mixer. An HPLC 7125 Rheodyne valve fitted with a 100 µl injection loop was used to apply the samples to the extraction vessel and this was placed in line between the dynamic mixer and the extraction cell which was housed within the GC oven. Ideally the Rheodyne should be placed inside the oven so that supercritical conditions are applied directly to the matrix containing the analytes, but with the initial set up used, this was not mechanically feasible.

The extraction vessels were originally prepared from empty HPLC columns which had the stationary phase removed. The dimensions of these columns were 4.6 mm i.d. (0.25" o.d.) 10 cm length and they were made of stainless steel.

The UV detector was next in line and was used to monitor the extractions. The original flow cell was replaced with a high pressure flow cell which although larger than the original and thus liable to produce more noise, provided better sensitivity and was capable of withstanding the high pressure required for SFE. A mechanical BPR was placed downflow of the detector which could be opened and closed manually with the use of a spanner to maintain the required pressure. The BPR had a gauge attached to it in order to monitor the pressure at this end of the system.

An additional HPLC column (4.6 mm i.d. x 10 cm) loosely packed with Lichoprep 60 was placed in the system between the CO_2 cylinder and the CO_2 pumphead. This was to prevent deposits from the CO_2 cylinder contaminating the system.

It is essential that the CO_2 cylinder is not allowed to be pumped dry as gaseous CO_2 will harm the check valves in the pumphead. For this reason in order to monitor the amount of liquid CO_2 remaining in the cylinder, it was placed on a set of bathroom scales.

The system which is described above was used for preliminary method development. Throughout the course of this study several modifications were made to the system however, these will be discussed where relevant in the following chapters.

"DEEPER

DEEPER

DEEPER... Now I'm

dwdy from you...I'm diding down the well,
pdst my platform out of thdt tunnel of dorkness
into d dedr blue sky dbove the tropicdl
sdvdnndh of Africd, the pldce of my
dredms, of my freedom..."

Irvine Welsh; MdrdbouStork Nightmdres

2. BENZODIAZEPINES

2.1 INTRODUCTION

Benzodiazepines were accidentally discovered in 1955 by Dr. Leo Sternbach in the Hoffman LaRoche laboratories.[75] Initially, it was thought that the newly discovered drug belonged to the quinazoline-*N*-oxide group of compounds. However, further investigation showed effects very different to those of the quinazolines. On administration, the new drug was found to have calming effects, produce muscle relaxation and prevent convulsions. Evaluation of the chemical structure of the drug showed it to be a benzodiazepine and it was given the name chlordiazepoxide. Chlordiazepoxide is now marketed under the name Librium® and is prescribed for the treatment of anxiety.

2.2 PRESCRIPTION

Since the development of chlordiazepoxide, hundreds of benzodiazepines have been synthesised and at present 16 are available by prescription in the United Kingdom.[76,77] These 16 drugs along with some of their proprietary names are shown in Table 2.1.

The benzodiazepines are used primarily in the treatment of anxiety. However, some are used as sleeping aids, some as antiepileptics and three are used as premedications.

2.3 CHEMICAL STRUCTURE

After the discovery of chlordiazepoxide, to determine which aspects of its chemical structure were related to its pharmacological effects, several other benzodiazepines were synthesised. Most were found to have effects similar to chlordiazepoxide however, one was found to be much more potent. This drug was given the name diazepam and was marketed as Valium®.

The basic structure of benzodiazepines is detailed in Figure 2.1 and the 10 of the prescribed benzodiazepines with this basic structure are indicated in Table 2.2.

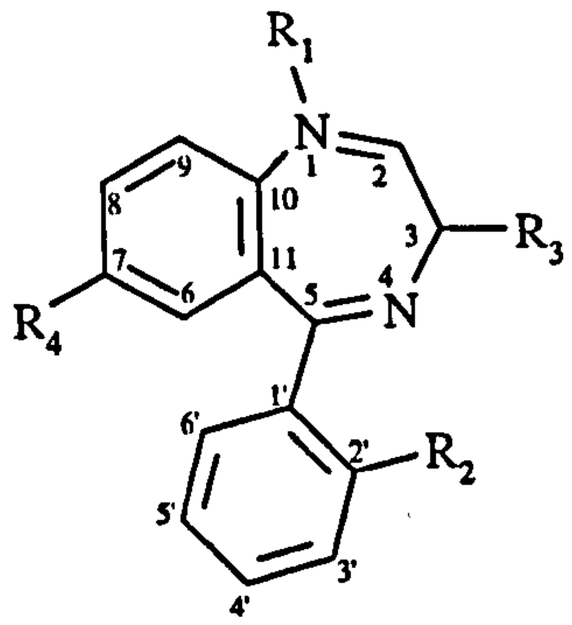


Figure 2.1: *Basic Structure of 1,4-Benzodiazepines*

Table 2.1: *Currently Prescribed Benzodiazepines*

Name	Proprietary Name(s)	Treatment	Action
Alprazolam	Xanax®	anxiolytic	PA
Bromazepam	Lexotan®	anxiolytic	IA
Chlordiazepoxide	Librium®, Tropium®	anxiolytic	PA
Clobazam	NA	anxiolytic, anti-epileptic	PA
Clonazepam	Rivotril®	anti-epileptic	-
Clorazepate	Tranxene®	anxiolytic	PA
Diazepam	Valium®, Valclair®, Diazemuls®, Heminevrin ®	anxiolytic, anti-epileptic, skeletal muscle relaxant, premed	PA
Flunitrazepam	Rohypnol®	hypnotic	IA
Flurazepam	Dalmane®	hypnotic	PA
Loprazolam	NA	hypnotic	IA
Lorazepam	Ativar®	anxiolytic, anti-epileptic, premed	IA
Lormetazepam	NA	hypnotic	IA
Midazolam	Hypnovel®	light sedative (IV)	-
Nitrazepam	Mogadon®, Remnos®, Unisomnia®	hypnotic	PA
Oxazepam	NA	anxiolytic	IA
Temazepam	Normison®	hypnotic, premed	IA

PA Prolonged action, IA Intermediate action,

The activity of benzodiazepines arises from the substituent on the 7-position of the 1,4-benzodiazepine structure.[78] This is generally a chloride or a nitro group (R_4). Substitution of chloride or fluoride on the ortho position of the 5-phenyl ring (R_2) usually decreases the half life and almost always increases the potency. Another developmental approach was to synthesise new 1,4-benzodiazepines which were extensively metabolised to desmethyldiazepam *i.e.* pro-drugs. Synthesis of the newest benzodiazepines has involved

annelation of the 1,2-diazepine position with a triazo (alprazolam) or an imidazo ring (midazolam).

Table 2.2: *Structural Groups of Classic Benzodiazepines*

Benzodiazepine	R_1	R_2	R_3	R_4
Clonazepam	H	Cl	H	NO_2
Clorazepate	H	H	COOH	Cl
Desmethyldiazepam*	H	H	H	Cl
Diazepam	CH_3	H	H	Cl
Flunitrazepam	CH_3	F	H	NO_2
Flurazepam	$C_6H_{14}N$	F	H	Cl
Lorazepam	H	Cl	OH	Cl
Lormetazolam	CH_3	Cl	OH	Cl
Nitrazepam	H	H	H	NO_2
Oxazepam	H	H	OH	Cl
Temazepam	CH_3	H	OH	Cl

* metabolite of diazepam

2.4 PHARMACOLOGY

There are four basic pharmacological properties of benzodiazepines.[75]

- Anxiolytic
- Sedative-hypnotic
- Anticonvulsant
- Muscle relaxant

The predominance or potency of these effects differs from drug to drug and therefore, the use of a specific benzodiazepine for a particular ailment depends on the acceptability of the other properties as side effects, *e.g.* in sufficient dosage, all benzodiazepines act as sedatives.

Research on the benzodiazepines shows that these drugs attach to specific receptors named the benzodiazepine receptors. All benzodiazepines shown to be capable of reducing anxiety are also capable of binding to the receptors. The more effective the anxiety relief the tighter the binding is found to be. Other drugs which are capable of reducing anxiety

e.g. meprobamate, barbiturates and alcohol do not bind to these receptors and hence it seems that the receptors are specific to benzodiazepines.

Scientists believe that the body itself may produce compounds which act on the benzodiazepine receptors and are thus working on isolating and identifying a natural anxiety-reducing compound.[79]

2.5 Metabolism

The metabolism of benzodiazepines is largely predictable. Classic benzodiazepines are metabolised by *N*-demethylation (*N*-methyl hydroxylation followed by hydrolysis) and by hydroxylation in the 3-position of the diazepine ring. This is known as phase I metabolism and is followed by conjugation with glucuronic acid (phase II metabolism). Other metabolic reactions do occur but these are minimal. In most cases, activity is maintained after *N*-demethylation (*e.g.* diazepam ⇒ desmethyldiazepam). After the hydroxylation step, the metabolites are rapidly conjugated and therefore do not accumulate in the blood. This means that *e.g.* oxazepam, the hydroxylation metabolite of diazepam, rarely achieves significant concentrations in the plasma after the administration of diazepam. Figure 2.2 indicates the metabolic routes of several benzodiazepines.

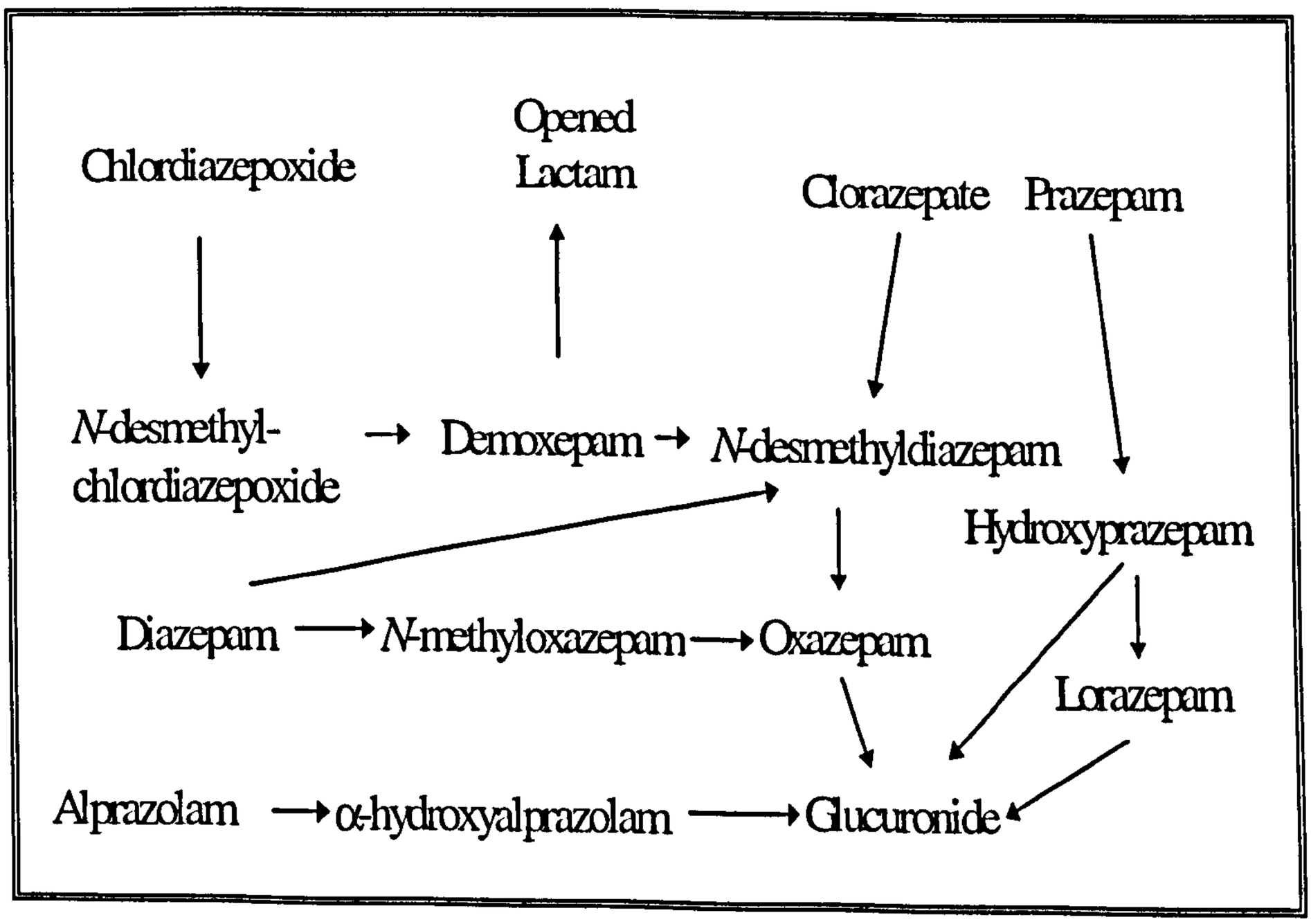


Figure 2.2: *Metabolic Route of Benzodiazepines*[79]

2.6 PHARMACOKINETICS

Benzodiazepines exhibit a two compartment pharmacokinetic model.[79] Central compartment redistribution is rapid with a second phase redistribution to adipose and peripheral tissue. With single dose administration, the redistribution phase determines the duration of action for each benzodiazepine, with lipid solubility being an important variable. Highly lipophilic benzodiazepines tend to be redistributed quicker than less lipophilic ones. With multiple dosing, once the adipose sites are saturated, the drug is not redistributed so quickly. Accumulation of the drug may occur for the more lipid soluble drugs which are extensively metabolised. Due to the possibility of active metabolites, and the large redistribution of benzodiazepines with high lipid solubility, the characterisation of benzodiazepines according to the elimination half life of the parent drug is not a reliable indication of the duration, onset or clinical effects of the drug. A better indicator of the therapeutic effect of benzodiazepines is the time which benzodiazepines exist at or above their therapeutic concentration at the intended receptor site.

Some of the pharmacokinetic data for the prescription benzodiazepines is given in Table 2.3.

Table 2.3: *Pharmacokinetic Data for Benzodiazepines*[80]

Drug	$t_{1/2}$ (hr)	VD (Lkg^{-1})	CL ($ml\ min^{-1}kg^{-1}$)	Distribution in blood	Protein binding
Alprazolam	6-20(12)	1	1	/	70
Bromazepam	8-19(12)	0.9	/	/	40-70
Chlordiazepoxide	5-30(15)	0.3-0.6	0.5	P:S 33	90-97
Clobazam	10-58(25)	1	0.5	/	85
Clonazepam	18-45	2-4	1	/	85
Clorazepate	2	0.5-2.5	0.1-0.3	P:WB 1.1	97
Diazepam	20-100(48)	0.5-2.5	0.3-0.5	P:WB 1.8	98-99
Desmethyldiazepam	25-200(40-100)	0.5-2.5	0.1-0.3	P:WB 1.7	97
Flunitrazepam	10-70(25)	4	2	P:WB 0.75	78
Flurazepam	2-3	/	/	/	97
Loprazolam	4-11(7)	/	/	/	/
Lormetazolam	10	5	4	/	90
Lorazepam	2	0.5-2.0	2-8(5)	/	95
Midazolam	9-24(14)	1-2	1	/	90
Nitrazepam	18-38(28)	2-3	1	/	85-88
Oxazepam	4-25(8)	0.5-2.0	1-2	P:WB 0.9	95
Temazepam	3-38(10)	1	1-2	P:WB 1.9	97

$t_{1/2}$ = elimination half life from blood (bracketed numbers = mean)

P:S; Plasma : Saliva

P:WB; Plasma : Whole Blood

2.7 Pharmacodynamics

Pharmacodynamically, a reverse loop hysterisis has been seen with some benzodiazepines. This means that while benzodiazepine concentrations remain constant or increase, the impairment of mental functioning improves over the time course of a few hours. It is unclear why this happens, but it is thought that receptor kinetics and adaption are more important in determining the pharmacodynamic effects of the benzodiazepines.[81]

2.8 Legal Status

Benzodiazepines are prescription only medicines. They were inserted into the Misuse of Drugs Act in 1985 and are controlled under Class C which makes them illegal to supply. However, it is legal to possess them in the medicinal form without a prescription.

2.9 Analysis of Benzodiazepines

Reviewing the previous literature on the analysis of benzodiazepines shows an increase in the reporting of SPE compared to LLE and HPLC and GC/MS compared to GC/FID and GC/ECD. In his handbook on benzodiazepines, Schütz reviewed the extraction methodology of the late 1970's and early 1980's.[82] Of 21 reviews, only 3 use HPLC as the method of choice for analysis and only one reference was made to the use of MS as a detector.

The most recent literature has been concerned with the GC/MS analysis of benzodiazepines with derivatisation. In 1996, Valentine *et al*[94] compared the use of HPLC versus GC/MS for the quantitation of urinary benzodiazepines. They conclude by stating that both methods can be used for the determination of benzodiazepines. However, HPLC offers some advantages including reduced analysis time and better determination of some of the benzodiazepines *e.g.* chlordiazepoxide. Table 2.4 summarises some of the more recent literature detailing the type of extraction and analysis.

Table 2.4: *Analysis of Benzodiazepines*

Year	Extraction Technique	Analysis Technique	Screen/ Quantitate	Sample Volume	Sample Type
1991[83]	GC/MS/MS	SPE	Q	10 ml	Urine
1991[84]	GC/NPD, HPLC	LLE	S/Q	1 ml	Serum
1991[85]	FPIA, HPLC	LLE	S/Q	1 ml	Serum
1994[86]	/	IA	S	/	Urine
1989[87]	GC/MS	SPE	Q	2 ml	Urine
1992[88]	HPLC	SPE	Q	1 ml	Blood
1994[89]	HPLC	LLE	Q	1 ml	Blood
1996[90]	HPLC	SPE	Q	1 ml	Serum/ Plasma
1995[91]	HPLC	LLE	S/Q	1 ml	Blood/SC/ Liver
1995[92]	GC/MS	LLE	Q	1 ml	Urine
1997[93]	HPLC	SPE	Q	1g/2 ml	Tissue/ Blood
1996[94]	GC/MS, HPLC	LLE	Q	3 ml, 1 ml	Urine

SC: stomach contents.

2.10 Supercritical Fluid Analysis of Benzodiazepines

As with most drugs of abuse there is a severe lack of literature available on the SFE of benzodiazepines. To date only one other group appear to have investigated the extraction of benzodiazepines using SF's. Lawrence *et al* used SFE to extract benzodiazepines from their solid dosage forms.[95] Their method used CO_2 with 2% MeOH as a modifier, a temperature of 65 °C and 100 atmospheres pressure with an extraction time of 15 minutes (5 minutes static, 10 minutes dynamic). The drugs analysed represented parent drugs and metabolites (*e.g.* oxazepam was readily extracted). In addition, by using GC/MS, no matrix components or contaminants were found to be extracted.

2.11 Current Methodology

The methodology used at present in the routine toxicology laboratory consists of SPE followed by HPLC with UV detection. The SPE method is detailed in section 2.11.1 and was used as a comparison method for SFE. The HPLC method used is described in Section 2.12.1.1.

2.11.1 SPE OF BENZODIAZEPINES

A 10 ml Plastipak syringe was plugged with a small amount of glass wool and $^3/_4$ filled with dichloromethane (DCM) washed Extrelut® (diatomaceous earth). Three types of vials were prepared as follows:

1) Standard vial containing: 100 µl of standard mix (temazepam, triazolam, chlordiazepoxide, desmethyldiazepam and diazepam, all 3-5 mg/100 ml), 100 ml of prazepam (3 mg / 100 ml), 0.9 ml blank blood, 250 µl 5% NH_3 and phosphate buffer (pH 7.4).
2) Blank vial containing: 100 µl of prazepam (3 mg/100 ml), 1 ml blank blood, 250 µl 5% NH_3 and 1 ml of phosphate buffer.
3) Sample vial containing: 100 µl of prazepam (3 mg/100 ml), 1 ml sample blood, 250 µl 5% NH_3 and 1 ml of phosphate buffer.

For each vial, an Extrelut® column was prepared and a labelled 8 ml vial placed under each column. 250 µl of 5% NH_3 were pipetted onto the top of each column and the vortexed contents of each vial pipetted onto the appropriate column. This was allowed to sit for 5 minutes before elution with diethylether under gravity. Approximately 7 ml were eluted and then evaporated to dryness under a stream of N_2. The dried residue was reconstituted in 180 µl of HPLC mobile phase.

The phosphate buffer added to the vials was prepared as follows:

A = 13.6 g of KH_2PO_4 / 500 ml H_2O

B = 4 g of NaOH / 500 ml H_2O

450 ml of A and 355 ml of B were mixed and the pH adjusted to pH 7.4 using either A or B.

2.12 HPLC OF BENZODIAZEPINES

2.12.1 SET-UP OF HPLC

2.12.1.1 EXPERIMENTAL

A Gilson HPLC system was tested to ensure that the analytical results produced for the separation of benzodiazepines were comparable to those produced in the routine

laboratory. The system consisted of a Gilson 305, 5SC pump, a Gilson 805 manometric module, and a Gilson 115 UV detector controlled *via* Gilson 715 software. The mobile phase (0.01M Na_2HPO_4 : MeOH (30 : 70 (v/v)) was prepared as follows and allowed to equilibrate in the HPLC system for 30 minutes before recycling.

1.78 g of $Na_2HPO_4.2H_2O$ (m.wt. 177.99) was dissolved in 1 L of distilled water (dH_2O). 120 ml of this was added to 280 ml of MeOH and degassed with helium for 10 minutes at room temperature.

The flowrate for the separation was 1.0 ml min^{-1} which gives a pressure of approximately 2500 psi through a 25 cm x 4.6 mm i.d. Hypersil ODS column. Injections were controlled *via* a 7125 Rheodyne fitted with a 20 µL loop. For the detection of benzodiazepines, the wavelength used was 254 nm.

Solutions of the eleven benzodiazepines listed in table 2.5 which had been prepared for the routine laboratory were analysed by HPLC. A 1 ml aliquot of each standard was pipetted into a vial, evaporated to dryness under N_2 at 60 °C and reconstituted in 1 ml of HPLC mobile phase. By reconstituting the standards in mobile phase, the solvent front and peak distortions are minimised. A 20 µl injection of each solution was made into the HPLC and the chromatograms recorded on a Asea Brown Boveri (ABB) SE 120 chart recorder.

2.12.1.2 Results and Discussion

The retention times and capacity factors obtained from the chromatograms of the eleven benzodiazepines, including the five routinely screened for (marked * in Table 2.5), with prazepam as an internal standard are shown in Table 2.5.

Table 2.5: *Retention Times of Selected Benzodiazepines.*

Drug	Retention time (R.T.) (minutes)	Capacity Factor (k')
Clonazepam	4.4	0.96
Nitrazepam	4.7, (6.9)	1.19 (2.21)
Clobazam	5.2	1.31
Loprazolam	5.6	1.49
Lorazepam	6.0, (9.9)	1.22 (2.67)
Triazolam *	5.7	1.11
Oxazepam	6.4	1.37
Temazepam *	6.8	1.52
Chlordiazepoxide *	7.1, (8.3)	1.63 (2.07)
Desmethyldiazepam *	7.7	1.85
Diazepam *	8.7	2.22
Prazepam	14.3	4.30

Each of the benzodiazepines chromatographed had separate retention times and capacity factors. However, for several of the drugs, the retention times and capacity factors were close *e.g.* loprazolam (5.6 min) and triazolam (5.7 min) and thus, it would be desirable to have a separate method in order to verify which drug was being detected in an unknown sample.

It was essential that great care was taken while preparing the mobile phase. The elution of benzodiazepines is extremely sensitive to the addition of excess methanol to the system. Inaccuracies of as little as 5 ml in a litre of mobile phase was found to cause co-elution of some of the more commonly encountered drugs *e.g.* temazepam and chlordiazepoxide.

2.12.2 Determination of Retention Time of Chlordiazepoxide, Nitrazepam and Lorazepam

2.12.2.1 Experimental, results and discussion

As can be seen in Table 2.5, the injections of chlordiazepoxide, nitrazepam and lorazepam, gave two peaks. Fresh standards of these drugs were prepared in methanol and these gave only one peak per drug (the retention time which is not in brackets). Of the three, chlordiazepoxide is the only one of relevance to this study, as nitrazepam and its

metabolites are chromatographed by a separate method and lorazepam is only analysed for in cases where the subject is known to have used the drug.

2.12.3 CHLORDIAZEPOXIDE STABILITY

The degradation peak of chlordiazepoxide elutes between desmethyldiazepam and diazepam and for the normal standard mix used (triazolam, chlordiazepoxide, temazepam, diazepam, desmethyldiazepam) does not affect the chromatography to any great extent so long as the optimal methanol volume is not exceeded. Chlordiazepoxide abuse at the time of this initial study was extremely rare and on the occasions where positives were observed, a fresh standard was prepared for quantitation purposes. However, the situation in 1997 has changed with reference to benzodiazepines and with the withdrawal of temazepam from the market, more chlordiazepoxide is found in case samples.

The structure of chlordiazepoxide is shown in Figure 2.3.

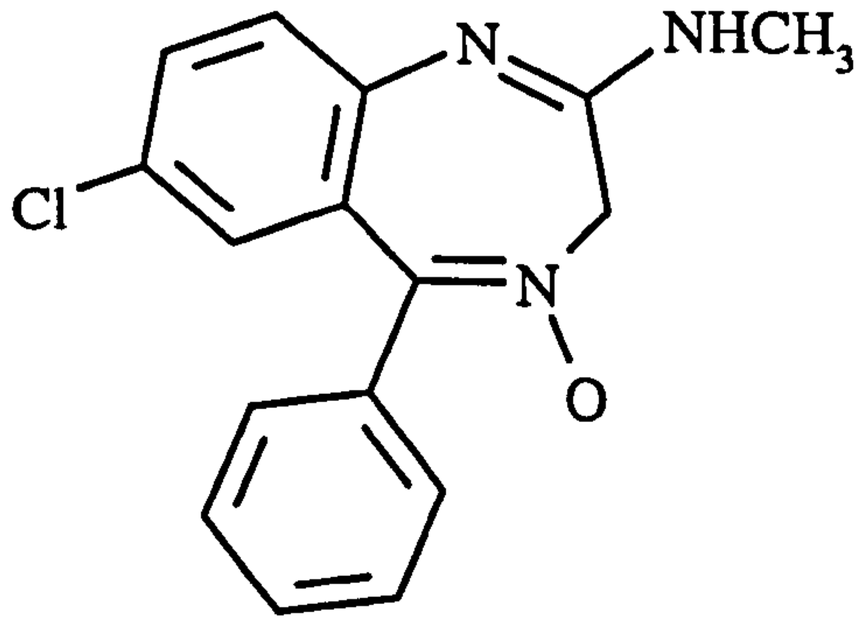


Figure 2.3: *Structure of Chlordiazepoxide*

2.12.3.1 EXPERIMENTAL

A short study was carried out to see how quickly the chlordiazepoxide decomposed. A standard mix containing all five of the standards used in the laboratory was prepared by pipetting 5 ml of each drug standard (5 mg/100ml) into a vial, evaporating to dryness under N_2 at 60 °C and reconstituting in 5 ml of MeOH. For injection onto the HPLC, aliquots of

this standard mix were pipetted into a vial, evaporated to dryness and reconstituted in the appropriate volume of mobile phase.

In addition, a fresh chlordiazepoxide standard was prepared by dissolving a 5 mg Librium tablet into 100 ml of methanol in a volumetric flask. The study was carried out over one week to determine how quickly the degradation peak appeared.

2.12.3.2 Results and Discussion

The relative peak heights of the five standards to prazepam were calculated from the traces obtained from HPLC injections on day one and day eight and are shown in Table 2.6.

Table 2.6: *Chlordiazepoxide Stability Study*

Peak	Retention time (minutes)	Relative height Day 1	Relative height Day 8
Triazolam	5.3	1.26	1.07
Temazepam	6.3	1.46	0.91
Chlordiazepoxide	6.7	1.14	0.20
Desmethyldiazepam	7.4	1.56	1.30
Impurity	7.8	0	0.74
Diazepam	8.1	1.20	1.13
Prazepam	13.4	1.00	1.00

Although none of the relative peak heights from days one and eight have remained constant, the one which has shown the most marked decrease is chlordiazepoxide. Over the eight days, an impurity peak has appeared which elutes between desmethyldiazepam and diazepam. By injecting each of the drugs on their own, it was confirmed that this peak was due to the chlordiazepoxide as each of the other drugs produced only one peak none of which corresponded to the impurity peak.

For the chlordiazepoxide on its own, initially (t=0 hours), only one peak was observed. At the end of the first day (t=2 hours) an additional peak was observed at 7.8 minutes with a height of 0.6 cm. After 18 hours the peak height had increased to 2.1 cm and after 4 days the peak height was 8.5 cm corresponding to a 1417% increase from t=2 hours. These results are shown graphically in Figure 2.4.

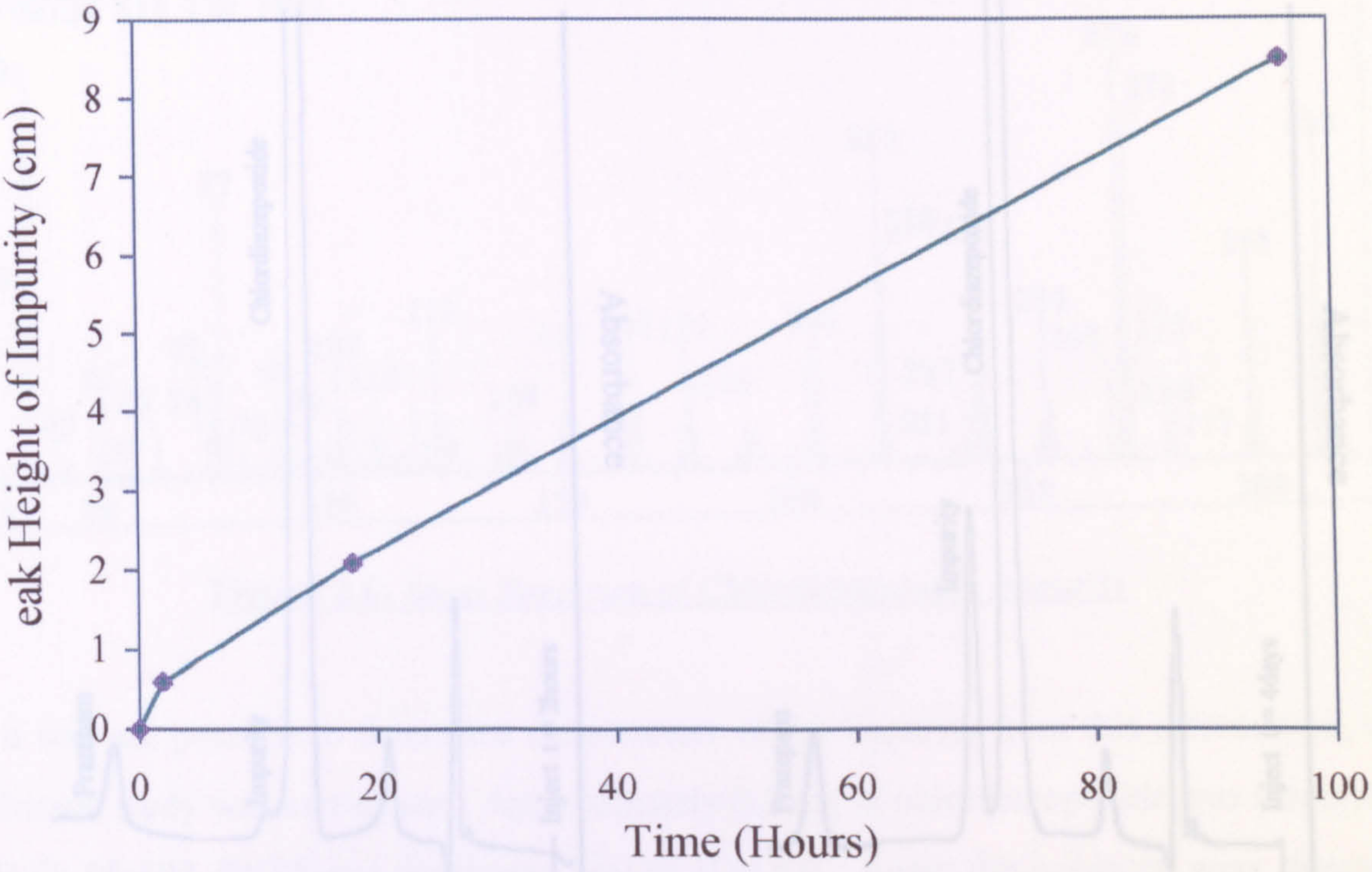


Figure 2.4: *Increase in Height of Chlordiazepoxide Impurity Peak with Time*

As chlordiazepoxide rapidly degrades, it is essential that if a case sample is positive, a new chlordiazepoxide standard is prepared prior to quantification. It should be noted that the relative height of the impurity peak to chlordiazepoxide could not be calculated as in order to measure the height of the impurity peak, the sensitivity of the detector had to be increased and thus, the chlordiazepoxide peak was off scale on the chart recorder.

The HPLC chart recorder traces of the chlordiazepoxide and its impurity on days one and four are shown in Figure 2.5.

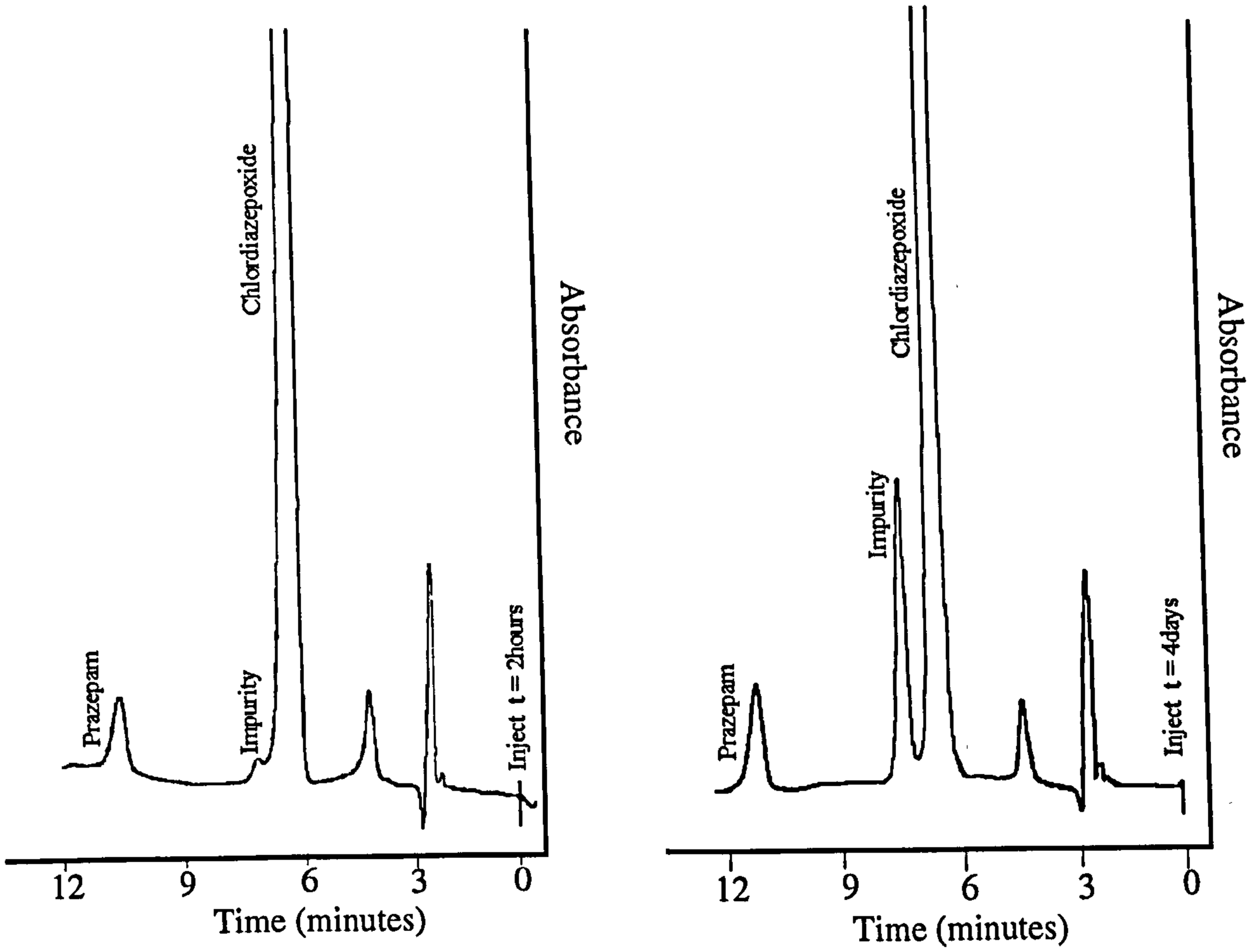


Figure 2.5. *Chromatograms of Chlordiazepoxide Peaks on Days One and Four*

2.12.4 IDENTITY OF CHLORDIAZEPOXIDE IMPURITY PEAK

2.12.4.1 EXPERIMENTAL

A fresh standard of chlordiazepoxide in MeOH was prepared (1 mg/5 ml). This was injected into HPLC and GC/MS immediately after preparation. Each day after, further injections were made on both instruments and the impurity peak monitored. The solution was made concentrated so that the impurity peaks mass spectrum could be obtained for quantification. Several small impurity peaks were observed in the chlordiazepoxide mass chromatogram. Only one of these was found to increase in area with time. After 28 days at room temperature, the impurity peak was larger than the chlordiazepoxide peak.

The mass spectrum obtained for the impurity peak was investigated in order to attempt to identify the impurity. The mass spectrum obtained is shown in Figure 2.6.

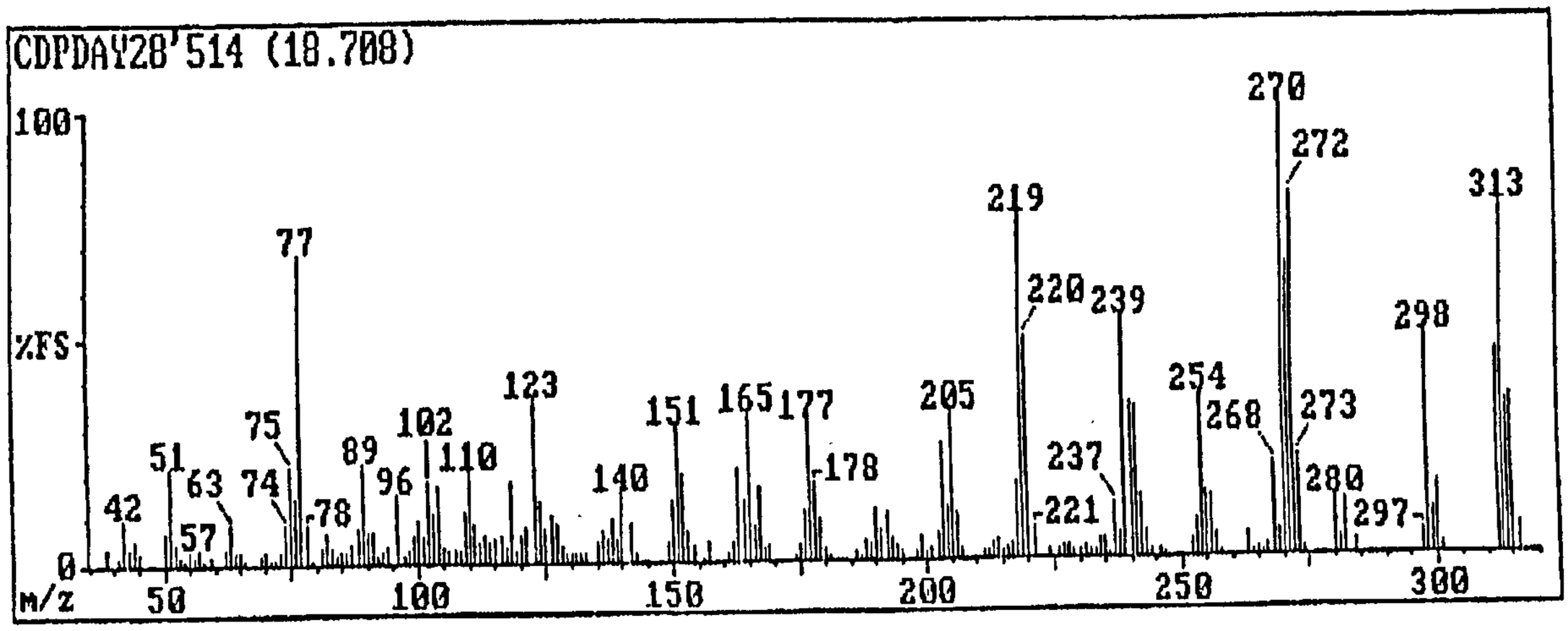


Figure 2.6: *Mass Spectrum of Chlordiazepoxide Impurity*

As it was not possible to determine the structure of the impurity from this information, an additional study was carried out. Approximately 0.5 mg of chlordiazepoxide was dissolved in H_2O, MeOH, EtOH and deuterated MeOH (D_3OD). Again the solutions were injected on day one and consecutive days thereafter, until the impurity peak mass spectrum could be ascertained.

The mass chromatograms for the chlordiazepoxide in H_2O and EtOH showed no impurity peak after 10 days. The chromatograms for chlordiazepoxide in MeOH and CD_3OD showed a peak at the same retention time that had been observed for the experiment previously carried out in MeOH. The mass spectra of these impurity peaks are shown in Figure 2.7, the mass spectrum for chlordiazepoxide is also given for comparison.

From these spectra alone, it was not possible to identify the impurity. It can be hypothesised that in some way the MeOH (CD_3OD) is adding into the chlordiazepoxide structure (from the m/z 313 in the MeOH which becomes m/z 317 in CD_3OD) possibly at the imino amide part of the diazepine ring.

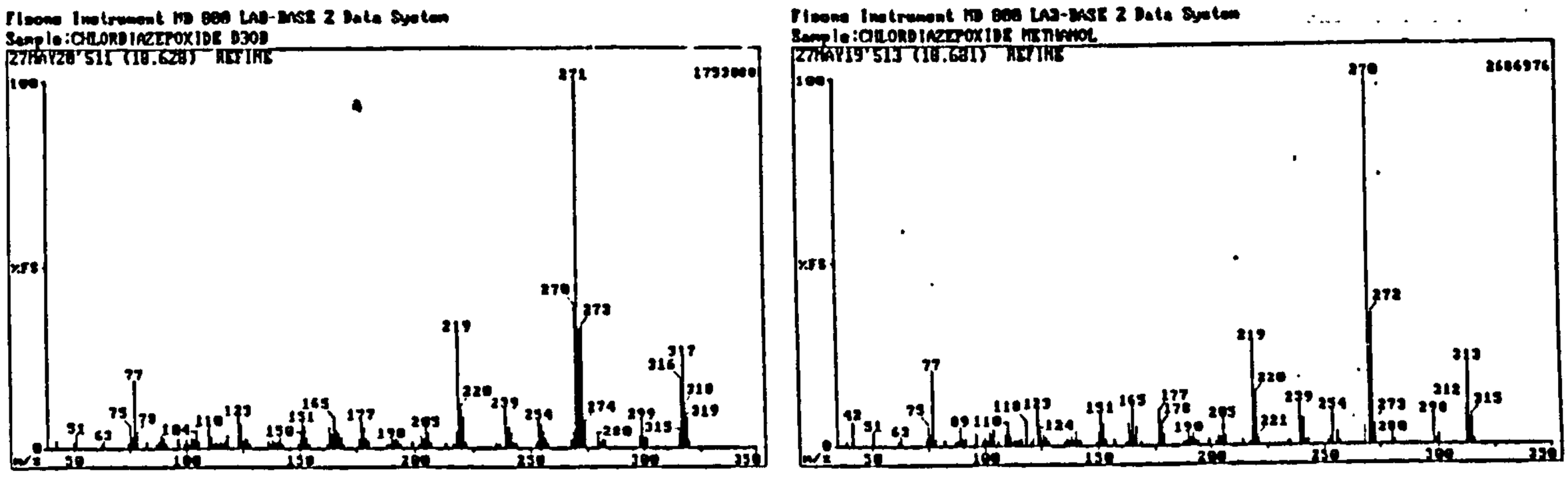


Figure 2.7: *Mass Spectra of CDP Impurity in MeOH and D_3OD*

2.12.5 Retention Time Reproducibility

2.12.5.1 Experimental

The remainder of this study was carried out using only the five benzodiazepines routinely screened for as highlighted in Table 2.5. Three to five injections of each of the standards were made in order to check the retention time reproducibility of the HPLC system for an equilibrated mobile phase (MP1). An injection of each standard was also made using a freshly prepared mobile phase (MP2) to ensure reproducibility in the mobile phase preparation. The second mobile phase was equilibrated in the system overnight.

2.12.5.2 Results and Discussion

The retention time reproducibility results are reported in Table 2.7, where x_1 and x_2 are the mean retention times for mobile phases 1 and 1+2 respectively. The relative standard deviation (RSD) for each set of means was computed using a calculator.

Table 2.7: *Injection Time Reproducibility*

Sample	MP1	MP2	x_1	x_2	RSD_1*	RSD_2
Triaz	5.3, 5.3, 5.2, 5.2, 5.3	5.2	5.3	5.3	1.04%	1.04%
Temaz	6.4, 6.3, 6.3	6.2	6.3	6.3	0.91%	1.29%
CDP	6.8, 6.8, 6.7, 6.7, 6.7	6.6	6.7	6.7	0.81%	1.12%
DMD	7.4, 7.4, 7.4, 7.4, 7.4	7.3	7.4	7.4	0.00%	0.55%
Diaz	8.1, 8.2, 8.1, 8.1, 8.1	8.2	8.1	8.1	0.55%	0.63%
Praz	13.6, 13.4, 13.4	13.4	13.5	13. 5	0.86%	0.74%

*For calculation of RSD see Appendix II.

From these results it is apparent that the reproducibility of the system is sufficient for use both on a daily basis and a weekly basis which requires changing of the HPLC mobile phase. There is little variation seen in the retention times of the peaks and the RSD values are low (all less than 1.3%).

2.13 Set up of SFE System.

2.13.1 Initial Set-up

The initial SFE set-up is described in Section 1.4. A start-up check list was devised as indicated below:

- Switch on the chiller unit, the detector and the GC oven
- Prime the modifier pump with the CO_2 cylinder closed
- Once the chiller is cold, check the system for leaks with CO_2 (***Do not pump CO_2 until the chiller is cold***)
- Once the oven is at the correct temperature, switch on the pumps and the mixer
- Adjust the pressure using the back-pressure regulator
- Place the extraction cell into the oven
- Put the analyte collection device in-line (in this case a 6 ml hypovial containing ≈2 ml of MeOH)
- Start the run using the Gilson 306 software
- Inject the matrix containing the analytes to be extracted into the rheodyne
- Switch the rheodyne valve from load to inject and ***remove the syringe***

After extraction:

- Switch the rheodyne valve from inject to load
- Remove the collection vial
- Evaporate the MeOH to dryness
- Reconstitute the residue in HPLC mobile phase
- Inject the sample onto HPLC

At the end of the day:

- Stop the flow.
- Remove and clean the extraction vessel.
- Isolate the two halves of the system by switching the rheodyne to the middle position and letting the pressure drop on the oven side.
- Unplug the chiller unit.
- Switch off the mixer, the oven and the detector.
- Turn off the CO_2.

2.13.1.1 Experimental

In order to check that the system had no mechanical or electronic faults and that there were no leaks, an ODS Hypersil (25 cm x 4.6 mm i.d., 5µ particle size) HPLC column was inserted into the GC oven. A 100 µl injection of a mixed solution of the five benzodiazepine standards was made under the following conditions:

1%-25% MeOH in CO_2 over fifteen minutes, hold for two minutes, back to 1% MeOH over one minute at 60 °C and 0.5 ml min^{-1}.

2.13.1.2 Results and Discussion

All six peaks were found to elute within the first few minutes and the peak shapes were very poor. However, on injecting blank MeOH, no peaks were observed other than a slight solvent front indicating that the system was functioning and improvement of the chromatography could be achieved with simple method development.

However, the purpose of this study was SFE and thus, as the system was functioning, an extraction vessel was prepared. An empty HPLC column (10 cm x 4.6 mm i.d.) was half filled with DCM washed Extrelut®. 200 µl of the benzodiazepine standard mixture was loaded into this and the column inserted into the oven which was set at 60 °C. At the flow outlet, a hypovial containing MeOH was attached, ensuring that the end was immersed in the MeOH. The vial was sealed using a butyl rubber septa and a disposable needle was inserted as a pressure release as shown in Figure 2.8.

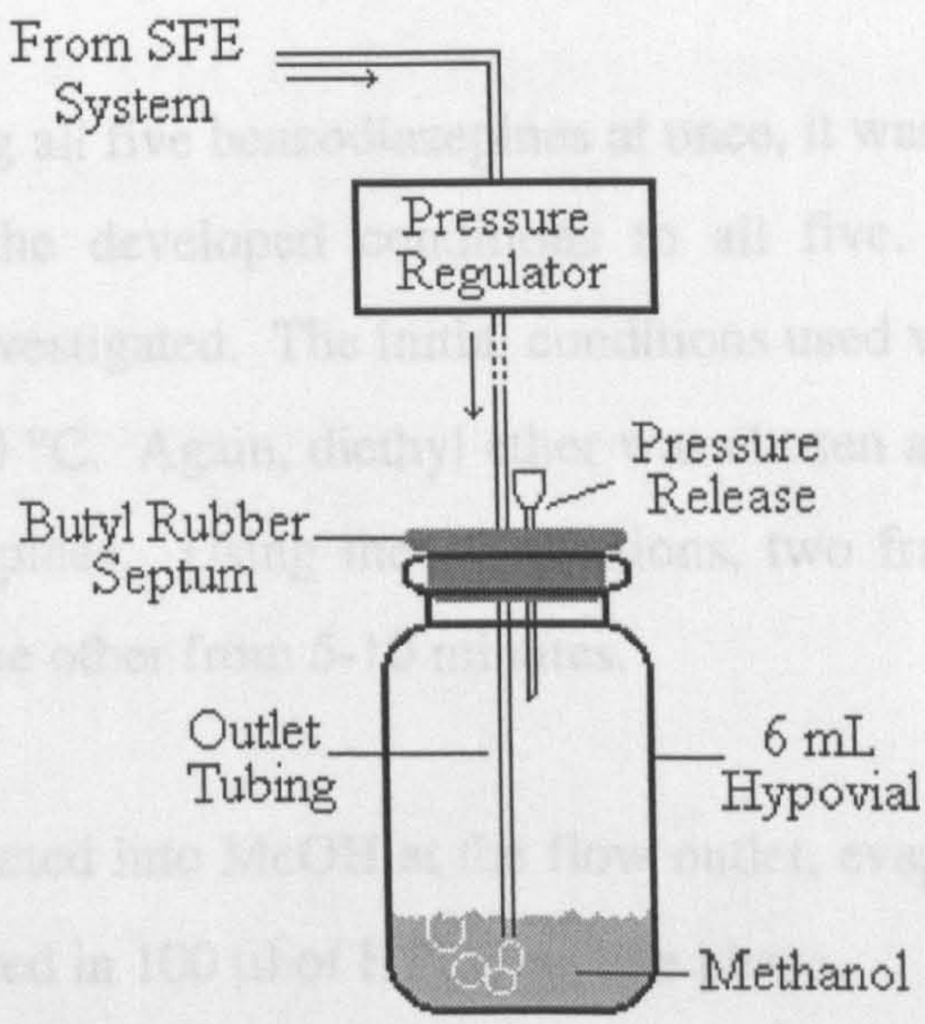


Figure 2.8: *Collection Device for SFE*

Initially 1% MeOH was pumped through at 1 ml min^{-1}, however, as the pressure increased, the flow had to be decreased to 0.5 ml min^{-1}. The percent of MeOH was increased stepwise to 25% over three minutes. Next, the ODS column was reinserted in place of the extraction vessel, and the extract injected. All previously detected peaks were found to elute.

These two experiments indicated that the SF system was functioning properly and thus was suitable for use for the SFE of benzodiazepines.

2.14 METHOD DEVELOPMENT: SFE OF BENZODIAZEPINES

Several benzodiazepines were investigated by SFE, namely triazolam, temazepam, diazepam, desmethyldiazepam, chlordiazepoxide and prazepam. These were initially extracted from methanol standards by loading 200 µl of the mixed drug standard onto DCM washed Extrelut®. The Extrelut® was chosen as an extraction media as this is the same as that used in the routine SPE method.

2.14.1 SFE OF TRIAZOLAM

2.14.1.1 EXPERIMENTAL

As opposed to extracting all five benzodiazepines at once, it was decided to investigate one drug and then apply the developed conditions to all five. Triazolam was the first benzodiazepine to be investigated. The initial conditions used were 5% diethyl ether, 1500 psi, 0.5 ml min^{-1} and 70 °C. Again, diethyl ether was chosen as a modifier as it is used in the SPE of benzodiazepines. Using these conditions, two fractions were collected, one from 0-5 minutes and the other from 5-10 minutes.

The extracts were collected into MeOH at the flow outlet, evaporated to dryness under N_2 at 65 °C and reconstituted in 100 µl of HPLC mobile phase.

A second extraction of triazolam was carried out using the same conditions except that the pressure was increased by closing the pressure regulator to a pressure of 3000 psi. For this extraction, the extract fractions were collected from 0-8 minutes and 8-15 minutes.

2.14.1.2 RESULTS AND DISCUSSION

HPLC of the first extraction fractions showed several peaks at the solvent front and one peak at 4.6 minutes. This peak was masking two other peaks at 4.2 minutes and 5.1 minutes. On injection of a standard solution of triazolam this was found to have a retention time of 5.4 minutes, thus no triazolam had been extracted. On HPLC of the second extraction extracts, the 0-8 minutes extract showed a peak at 5.3 minutes corresponding to triazolam. The recovery, as calculated by comparing the PH of the

extracted standard to that of an unextracted standard at the same concentration, for this part of the extraction was very low (<5%). The 8-15 minutes extract had no corresponding peaks.

2.14.2 SFE OF TEMAZEPAM

The number of toxicology cases in the period 1992-1996, which were positive for benzodiazepines was investigated and yielded the bar chart shown in Figure 2.9. No triazolam positive cases were found in this time period. In addition to this, it was found that the most commonly encountered benzodiazepine in toxicology samples in the period leading up to this study in 1994 was temazepam. For this reason it was decided to use temazepam instead of triazolam as a starting drug.

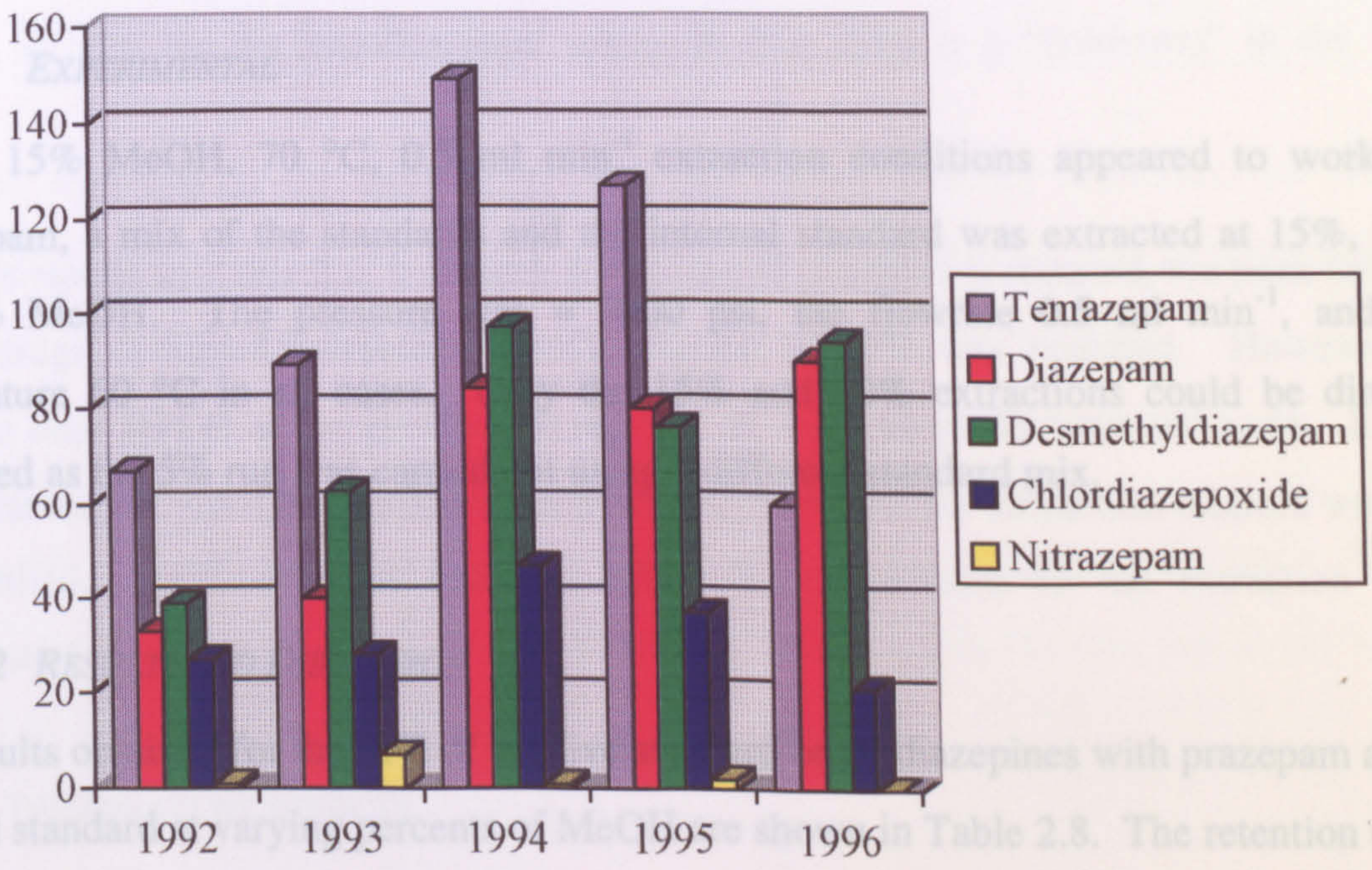


Figure 2.9: *Benzodiazepine Cases 1992-1996*

2.14.2.1 EXPERIMENTAL

The second set of conditions used for triazolam (*i.e.* 3000 psi, 5% diethyl ether, 0.5 ml min^{-1}, 70 °C) were used for the extraction of temazepam. The pressure was difficult to control and fluctuated throughout the extraction. For this reason all pressures quoted

development are an average of the initial and final pressures. All extractions were run for fifteen minutes unless otherwise stated.

2.14.2.2 Results and Discussion

At an average pressure (p_a) of 2502 psi, temperature at 70 °C and 0.5 ml min^{-1}, only a very small amount of temazepam was extracted (<1%). At the same temperature and pressure but with 15% diethyl ether, the recovery was still less than 5%. The modifier was changed from diethyl ether to methanol and this time the peak height of the extracted temazepam increased from 2.9 cm to 20.8 cm which corresponded to an approximate recovery of 100%.

2.14.3 SFE of the Standard Mix + Internal Standard

2.14.3.1 Experimental

As the 15% MeOH, 70 °C, 0.5 ml min^{-1} extraction conditions appeared to work for temazepam, a mix of the standards and the internal standard was extracted at 15%, 10% and 5% MeOH. The pressure was ≈ 3000 psi, the flowrate 0.5 ml min^{-1}, and the temperature 60 °C in all cases. Only the 15% and 10% extractions could be directly compared as the 5% run was carried out using a different standard mix.

2.14.3.2 Results and Discussion

The results obtained for the SFE of the five standard benzodiazepines with prazepam as the internal standard at varying percents of MeOH are shown in Table 2.8. The retention times (R.T.) are in minutes and the peak heights (P.H.) in cm.

Table 2.8: *Comparison of Extractions at Various % MeOH*

Drug	15% MeOH			10% MeOH			5% MeOH		
	R.T.	P.H.	PHR	R.T.	P.H.	PHR	R.T.	P.H.	PHR
Triaz	5.4	2.3	3.3	5.4	4.6	2.1	5.4	6.75	1.2
Temaz	6.4	2.5	3.6	6.4	4.4	2.0	6.4	5.1	0.9
CDP	6.8	0.9	1.3	6.9	1.8	0.8	6.9	1.8	0.3
DMD	7.5	1.2	1.7	7.5	4.8	2.2	7.4	7.6	1.4
Diaz	8.4	0.9	1.3	8.3	2.6	1.2	8.3	6.6	1.2
Praz	14.1	0.7	1.0	14.1	2.2	1.0	14.1	5.5	1.0

A blank extraction of MeOH was made to see if there was any carry over of the benzodiazepines within the system. Traces of all the benzodiazepines were observed by HPLC. A second blank extraction was carried out this time using diethyl ether. Again peaks were observed by HPLC. Finally, another MeOH blank was extracted. This time, no peaks were observed by HPLC. The carry over peaks indicate that either the extraction time is not long enough, that the conditions are not ideal for 100% recovery, that the drugs have an affinity for the stainless steel tubing or that there is a "cold-spot" in the SFE system.

From the results in Table 2.8, it appears that as the % modifier is reduced, the peak heights of the drugs extracted increases. The extraction at 10% was repeated. However on injection onto HPLC at the previous sensitivity of 0.05, all peaks were off scale. It was noted that the GC oven had been switched off and therefore, the extraction mixture was not supercritical. As a result of this, the effects of temperature on the extraction were investigated.

2.14.4 Temperature Effects.

2.14.4.1 Experimental

A series of extractions were carried out on the standard mix at ambient, 40 °C and 60 °C. The flow was set to 0.5 ml min^{-1} with MeOH as the modifier at 10%. A new extraction column was put in place. The pressure for the ambient extraction was ≈ 4500 psi which was too high and thus the pressure was decreased by opening the regulator until a pressure of ≈ 3500 psi was obtained for the other extractions. To ensure that there was no carryover

between extractions, blank diethyl ether was injected between each extraction. No carry over peaks were observed.

2.14.4.2 Results and Discussion

The % recoveries calculated for the extraction of the five benzodiazepines at various temperatures on a peak height basis are shown in Table 2.9.

Table 2.9: *Recoveries for Standard Benzodiazepines at Various Temperatures*

Drug	Unextracted		Ambient			40 °C			60 °C		
	P.H.	PHR	P.H.	%Rec	PHR	P.H.	%Rec	PHR	P.H.	%Rec	PHR
Triaz	12.3	1.07	14.4	117	1.5	9.5	77	1.51	1.6	13	1.6
Temaz	10.4	0.90	8.9	86	0.93	6.3	61	1.00	1.0	9.6	1.0
CDP	2.3	0.20	2.1	91	0.22	1.4	61	0.22	0.2	43	0.2
DMD	14.4	1.25	13.3	92	1.39	9.2	64	1.46	1.5	10	1.5
Diaz	13.0	1.13	10.9	84	1.14	8.0	62	1.27	1.2	9.2	1.2
Praz	11.5	1.0	9.6	83	1.0	6.3	55	1.0	1.0	8.7	1.0

It can be seen that as the temperature increases the % recovery decreases, but at low temperatures, the system is not operating in the supercritical mode. The benzodiazepines seem to be remaining absorbed on the Extrelut® at the higher temperatures.

The temperature in the SFE system was set to 50 °C and the extractions run at 10, 15 and 20% MeOH. The pressure and flow rate were left at ≈ 3500 psi and 0.5 ml min^{-1}. These conditions did not improve the extraction efficiency and it was thought that at the higher percents of MeOH that the system was again not supercritical.

2.14.5 Critical Temperature of CO_2 and MeOH Mixtures

As an estimation of the critical temperature at various % modifiers, a straight line graph can be plotted from T_c for CO_2 to T_c for modifier. Using the X-axis for % modifier from 0-100%, with the critical temperatures of the two components on the y-axes, the T_c of the mixture can be approximated. In practice, due to forces mentioned in section 1.1.2 of the SF introduction, these temperatures may not be supercritical and thus a temperature of 5 - 10 °C higher is suggested as a good starting point for method development.

The graph in Figure 2.10 shows the approximate critical temperatures for CO_2 and MeOH mixtures. So for example at 30% MeOH in CO_2, the approximate critical temperature would be 94 °C and the starting point for method development would be 99 °C.

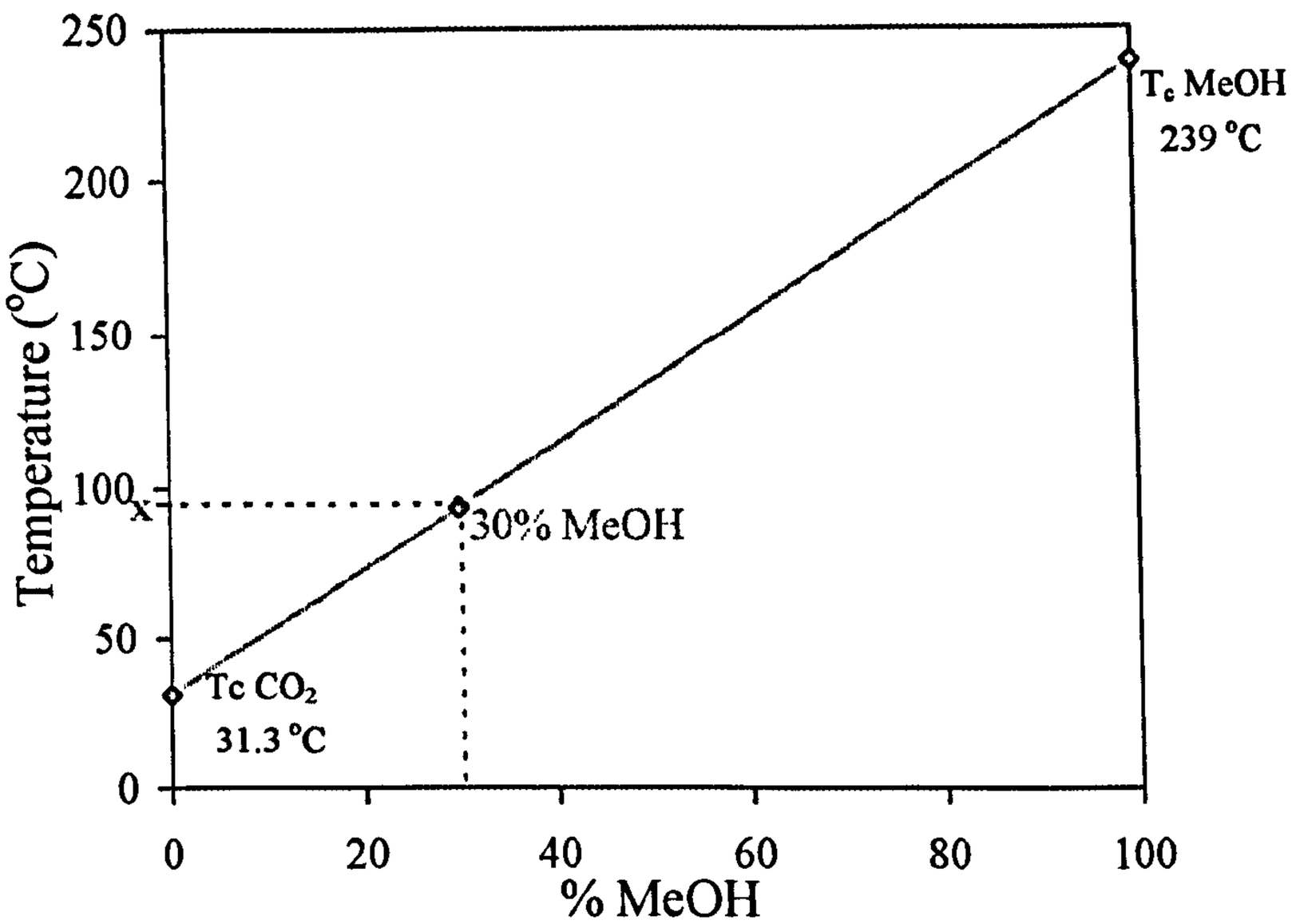


Figure 2.10: *Approximation of the Critical Temperature of MeOH in Supercritical CO_2*

2.14.6 CONTINUATION OF TEMAZEPAM METHOD DEVELOPMENT

Instead of trying to further continue this line of method development with all five benzodiazepines plus the internal standard, it was decided to once more investigate temazepam on its own. The conditions developed should extract the other benzodiazepines due to their similar structures.

2.14.6.1 EXPERIMENTAL

The initial conditions used for the temazepam extraction were:

- Flow rate: 0.5 ml min^{-1}
- % modifier: 20% MeOH
- Temperature: 70 °C

- p_i: 4017
- p_f: 4075
- p_a: 4046

The extraction was recorded and the profile indicated that the drug is extracting between 3 and 12 minutes as indicated in Figure 2.11.

Injection of unextracted temazepam onto the HPLC produced a peak height of 10.2 cm. Using the SFE conditions described, a peak height of 3.2 cm was obtained, corresponding to a recovery of 31%.

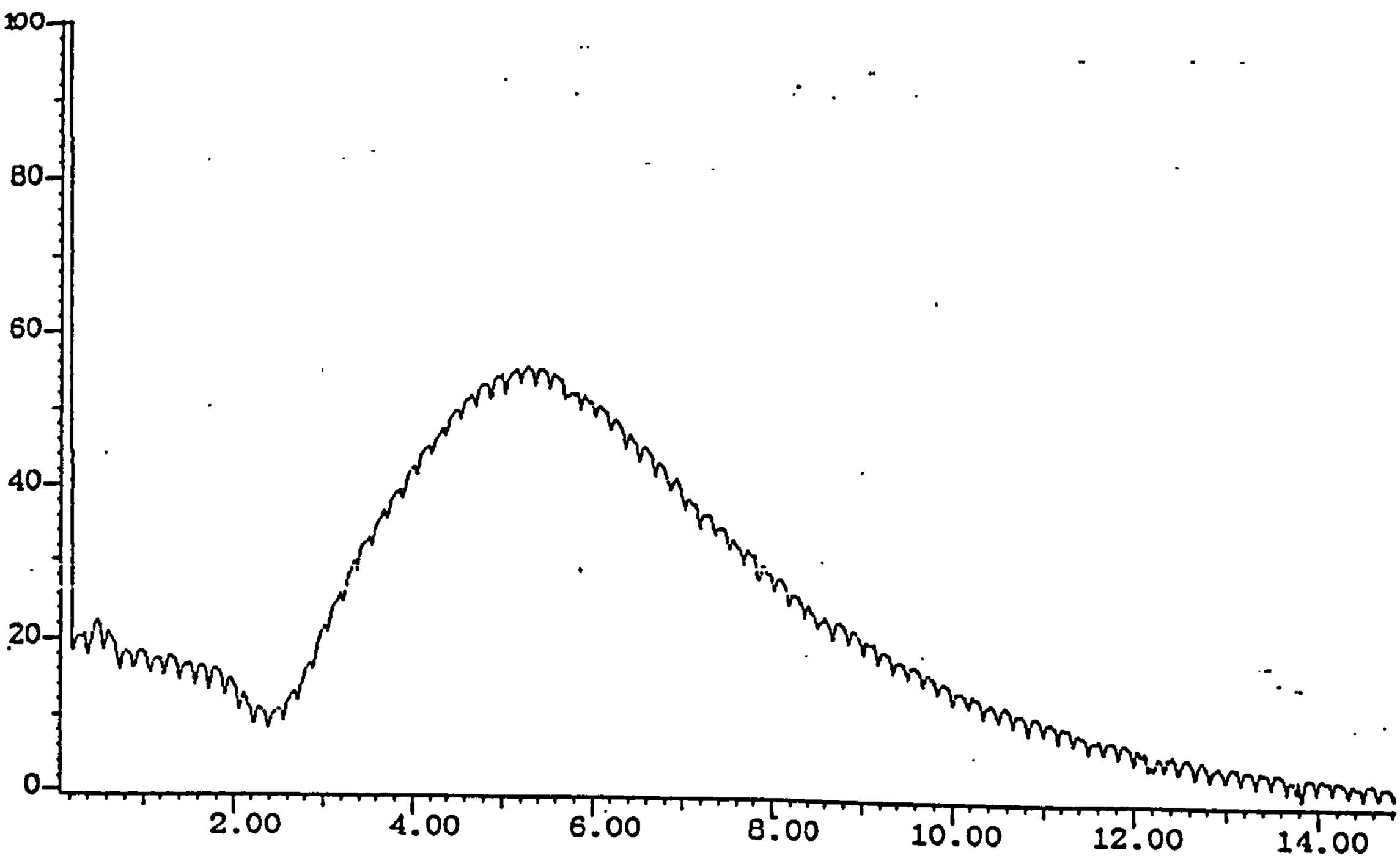


Figure 2.11: *Extraction Profile of Temazepam*

2.14.7 Optimisation of Temazepam Extraction.

Optimisation of SFE can be achieved in three ways:[96]

- Altering the modifier / % modifier
- Altering the temperature
- Altering the pressure

2.14.8 Effect of Altering the % Modifier.

2.14.8.1 Experimental

The percent of MeOH used for the extraction of temazepam was varied from 10-50% whilst maintaining the pressure at ~4000 psi and the temperature at 70 °C. The extractions were carried out as described previously.

2.14.8.2 Results and Discussion

The average pressures are indicated in Table 2.10 with the results obtained for the extraction of temazepam at various percent of MeOH.

Table 2.10: *Variation in % Recovery with Increasing % Modifier*

% modifier	% recovery	P (psi)
10	9.8	3894
20	18	4126
30	26	3828
40	59.4	3640
50	96	3718

As the % modifier is increased, the % recovery increases but again the conditions will not be supercritical (T_c at 50% MeOH ≈ 135 °C).

2.14.9 Reproducibility of Extraction Using MeOH (40%) as the Modifier

2.14.9.1 Experimental

Three extractions were carried out at 40% with all other parameters kept constant. The collected extracts were injected in duplicate ensuring that the 25 µl loop on the HPLC system was overfilled.

2.14.9.2 Results and Discussion

The results for the reproducibility test are shown in Table 2.11.

Table 2.11: *Extraction Reproducibility*

Injection	E_1	E_2	E_3
1	8.2 (97%)	7.5 (88%)	15.1 (>100%)
2	10.4 (>100%)	11.2 (>100%)	15.9 (>100%)

The extraction at 40% showed poor reproducibility by HPLC, both intra- and inter-extraction and therefore, a peak height injection reproducibility test was carried out on the HPLC.

2.14.10 HPLC System Reproducibility

2.14.10.1 Experimental

The temazepam standard in MeOH was injected five times onto the HPLC at a sensitivity of 0.5. The peak heights obtained were measured manually.

2.14.10.2 Results and Discussion

The peak heights obtained for the five injections of temazepam were:

8.4 cm, 8.8 cm, 8.4 cm, 9.8 cm, 8.4 cm.

These heights give a mean peak height of 8.76 and a RSD of 6.9%. It would appear that the 9.8 cm value is an outlier, however, more injections would be required in order to verify this. In practice only a maximum of three injections can be made per extraction due to the reconstitution volume and so the reproducibility of the HPLC system is unacceptable for this work.

2.14.11 TEMAZEPAM EXTRACTION FROM WHOLE BLOOD

2.14.11.1 EXPERIMENTAL

Despite the conditions at 70 °C and 40% MeOH being non-supercritical, these were yielding reasonably high recoveries and thus were used in an attempt to extract temazepam from a spiked blood standard.

A 1 ml aliquot of 38.4 μg ml^{-1} temazepam standard in methanol was evaporated to dryness under nitrogen, reconstituted in 1 ml of blank blood and sonicated for three minutes. A fresh extraction vessel was inserted into the GC oven and the system was set for 0.5 ml min^{-1}, 40% MeOH and 70 °C. A 100 μl injection of the blood standard was made followed by an injection of 100 μl of distilled water (dH_2O). The rheodyne was then switched back to the load position and flushed through with dH_2O until it ran clear and after one minute a further injection of 100 μl dH_2O was made in an attempt to spread the blood over the Extrelut® in the column.

The initial pressure was 3840 psi and the final pressure was 4640 psi. A second blood extraction was attempted. This time, a pressure drop of ≈ 1000 psi was seen across the system. On removing the column, the pressure drop was found to be due to the injected blood forming a clot in the extraction vessel, thus the injections of dH_2O were not spreading the blood, over the contents of the extraction vessel. In addition, the collection solvent turned pale brown in colour which was possibly due to the water carrying blood through to the collection vial. Two further extractions were carried out and the extraction vessel changed between each one to prevent blocking.

2.14.11.2 RESULTS AND DISCUSSION

Results for the first extraction and the two further extractions were obtained by HPLC and are reported in Table 2.12. The peak height of the unextracted temazepam was 10.9 cm.

Table 2.12: *Reproducibility for 3 Standard Blood Extractions*

	Extraction Recovery (Peak height (cm))		
Injection	Ext.1	Ext.2	Ext.3
1	53.2%* (5.8)	86.2% (9.4)	>100% (11.2)
2	99% (10.8)	80.7% (8.8)	>100% (13.4)
3	94.5 (10.3)	81.7 (8.9)	>100 (15.8)

* HPLC mobile phase not settled down, therefore, peak late.

In addition to the temazepam peak, the blood extract traces were showing a large impurity peak at approximately 14 minutes. This will probably co-elute with the internal standard (prazepam) once it is introduced to the system. An extraction of blank blood was carried out and the impurity peak was still present. One of the components of blood which could be responsible for the peak was cholesterol and thus, an injection of a cholesterol standard in mobile phase was made in order to determine if it had the same retention time. No peaks were observed after 20 minutes. An alternative possibility for the peak is the anticoagulants and preservatives added to blank blood at the hospital. As a solution of the hospital additives was not available, to determine if the peak was due to this, a post-mortem sample, which contained no additives and was known to be negative for benzodiazepines, was extracted. No corresponding peak at 14 minutes was observed and the MeOH collection solution showed no breakthrough blood.

The extraction of temazepam from spiked hospital blood did however yield good recoveries using the conditions described. It was decided to continue method development by altering the modifier to determine if the extraction could be made more effective using a different modifier.

2.14.12 Effect of Altering the Type of Modifier

As stated previously, the conditions with 40% MeOH are not supercritical. In order to make the conditions supercritical it was decided to use a different modifier, namely ethyl acetate (EtAc).

2.14.12.1 EXPERIMENTAL

The following conditions were set-up and both standards from MeOH and blood extracted.

- Flow rate: 0.5 ml min^{-1}
- Pressure: ≈ 3000 psi
- Temperature: 70 °C
- Modifier: 10% EtAc

2.14.12.2 RESULTS AND DISCUSSION FOR 70 °C

For both MeOH and blood standards, recoveries of >80% were achieved. In addition, the peak at 14 minutes was no longer observed and the solvent front was markedly cleaner, indicating better selectivity. In order to decrease the harshness of the conditions, the temperature was decreased to 50 °C with 10% EtAc as, at this temperature, the conditions are still supercritical. In addition, in order to decrease the extraction time, the flow rate was increased to 1.0 ml min^{-1}.

2.14.12.3 RESULTS AND DISCUSSION FOR 50 °C

At 10% EtAc, 50 °C, 1 ml min^{-1} (p = 2762 psi) the extraction recovery was only 9.6%. The EtAc was increased to 15% and the extraction was repeated (p = 3462 psi). This time, the % recovery was 82.4%.

2.14.13 EFFECT OF TEMPERATURE WITH ETAC (15%) AS THE MODIFIER

2.14.13.1 EXPERIMENTAL

The percent of EtAc was set to 15% and the effect of altering the temperature monitored whilst keeping the pressure constant.

2.14.13.2 Results and Discussion

At 60 °C, 15% EtAc (p = 2974 psi) the recovery was 100% but by increasing the temperature further, the recovery dropped to 37% at 70 °C, 20% at 75 °C and 27% at 80 °C. These results are plotted in Figure 2.12.

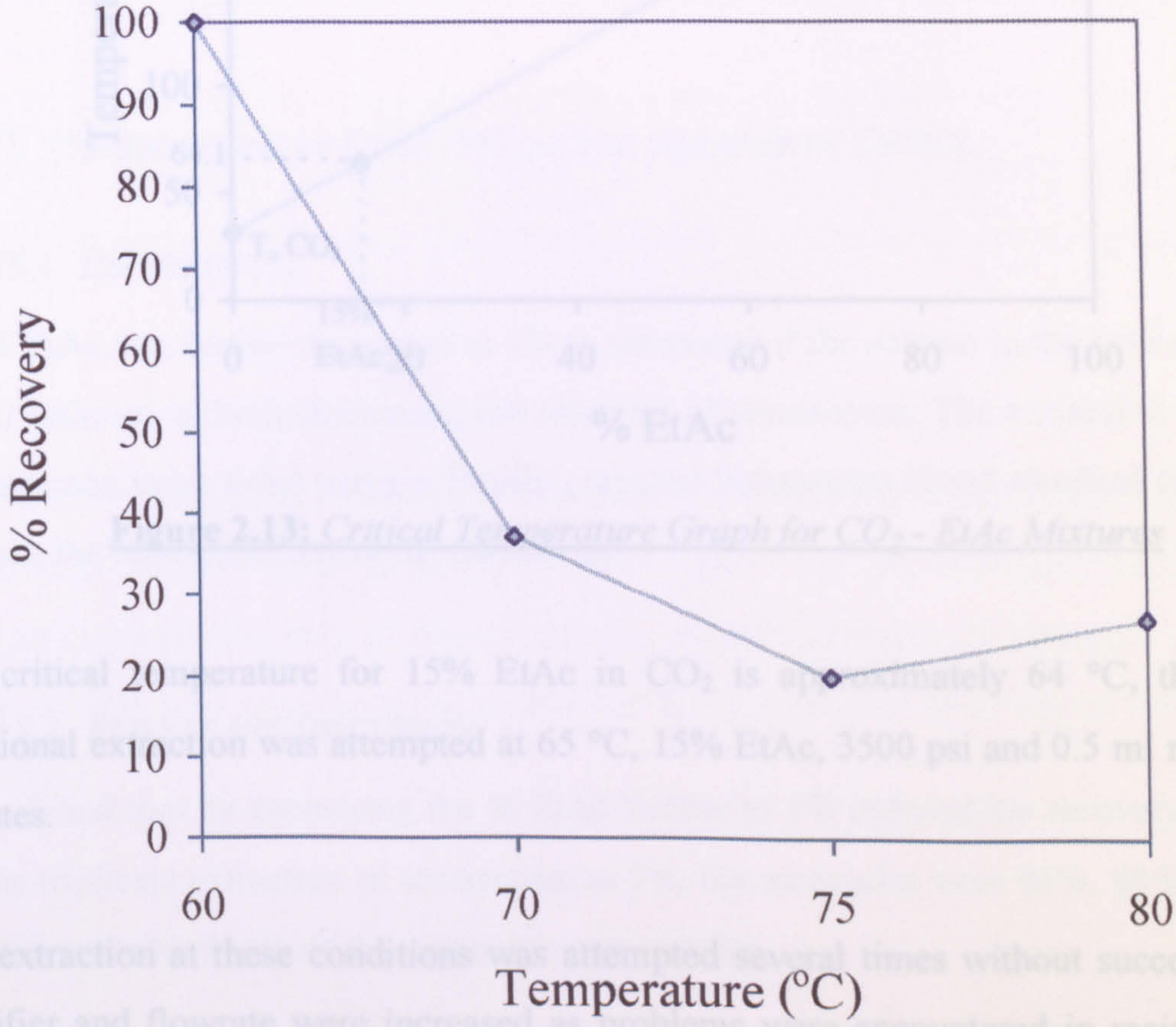


Figure 2.12: *% Recovery Vs Temperature for Temazepam at 15% EtAc*

The approximate critical temperature plot for EtAc in CO_2 is shown in Figure 2.13.

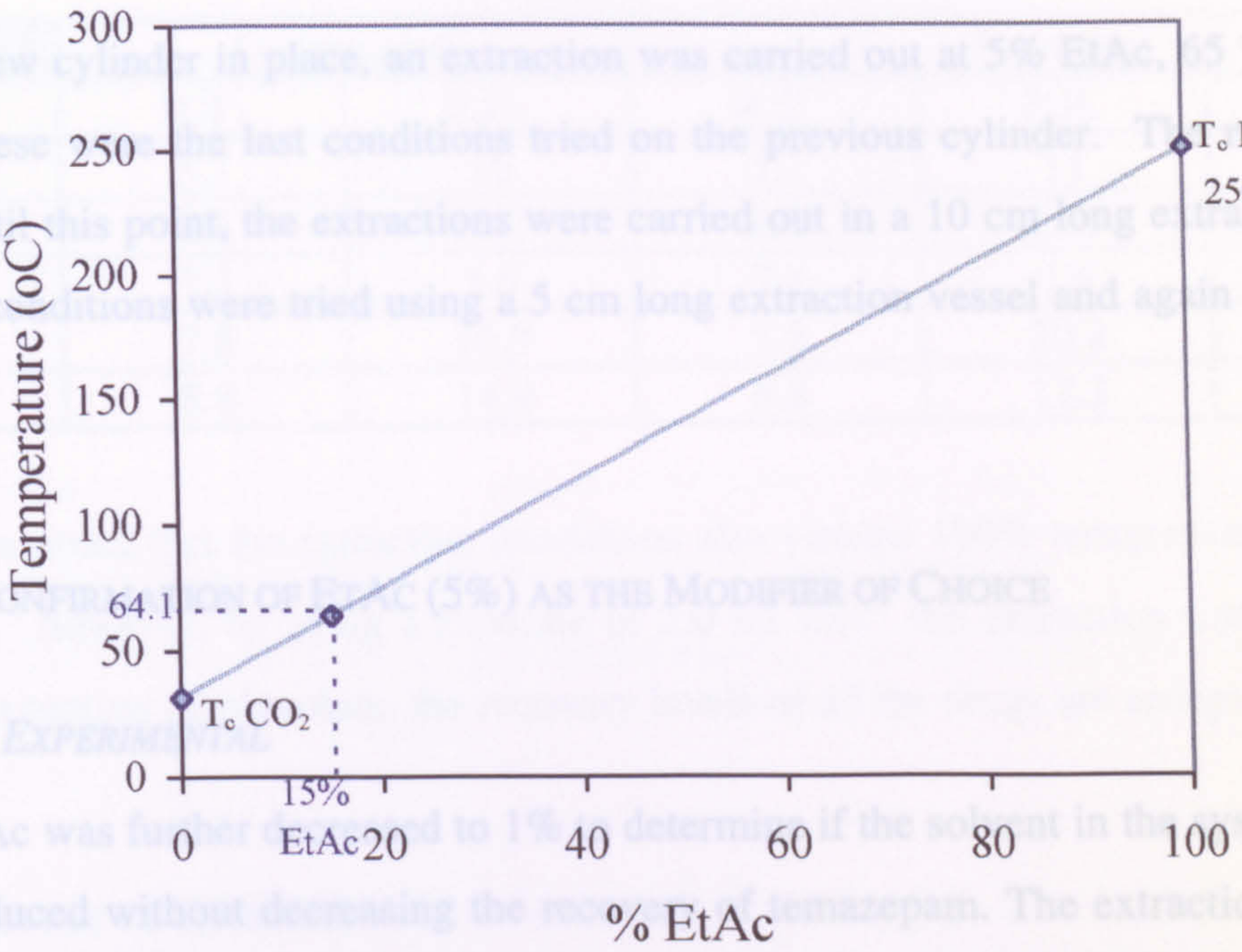


Figure 2.13: *Critical Temperature Graph for CO_2 - EtAc Mixtures*

The critical temperature for 15% EtAc in CO_2 is approximately 64 °C, therefore an additional extraction was attempted at 65 °C, 15% EtAc, 3500 psi and 0.5 ml min^{-1} for 20 minutes.

The extraction at these conditions was attempted several times without success. The % modifier and flowrate were increased as problems were encountered in maintaining the pressure in the system. The head pressure of the CO_2 cylinder was found to have dropped from ≈700 psi to ≈500 psi. This decrease in head pressure was enough to prevent sufficient pressurisation of the system, and it was thought that gaseous CO_2 was being pumped and thus a new cylinder was placed in the system.

Future cylinders were checked once the head pressure had dropped by ≈150 psi to determine if they required replacing. This method was found to be more effective than weighing the cylinders, and thus use of the scales mentioned in Chapter 1 was discontinued.

2.14.14 Alteration of Extraction Cell Length

With the new cylinder in place, an extraction was carried out at 5% EtAc, 65 °C and 2 ml min^{-1} as these were the last conditions tried on the previous cylinder. The recovery was 100%. Until this point, the extractions were carried out in a 10 cm long extraction vessel. The same conditions were tried using a 5 cm long extraction vessel and again the recovery was 100%.

2.14.15 Confirmation of EtAc (5%) as the Modifier of Choice

2.14.15.1 Experimental

The % EtAc was further decreased to 1% to determine if the solvent in the system could be further reduced without decreasing the recovery of temazepam. The extraction at 5% was also repeated three times using a freshly prepared temazepam blood standard (4.42 mg/L) to check the reproducibility of the conditions.

2.14.15.2 Results and Discussion

It was found that by decreasing the % EtAc further to 1% reduced the recovery to ≈ 20%. For the triplicate extraction of temazepam at 5%, the recoveries were 96%, 98% and 100% thus, the reproducibility of the extraction was suitable.

2.14.16 Addition of other Benzodiazepines to the System

2.14.16.1 Experimental

Using the same extraction conditions, diazepam and desmethyldiazepam were extracted on their own, however, the recoveries were very low. The standard mixture was then extracted to determine the effect of the other benzodiazepines on the extraction of temazepam.

2.14.16.2 Results and Discussion

The results for one extraction of the standard mix are shown in Table 2.13.

Table 2.13: *Effect of other Benzodiazepines on Temazepam*

	Unextracted		Extracted		
Drug	R.T.	P.H.	R.T.	P.H.	Recovery.
Triaz	5.7	16	5.7	11	69%
Temaz	6.8	11.7	6.8	11.6	99%
CDP	7.1	1.8	7.1	1.6	89%
DMD	7.8	16.9	7.8	20.2	120%
Diaz	8.8	14.9	8.8	17.2	115%

It should be noted that the extraction conditions also yielded 100% temazepam at a flow of 1.0 mlmin^{-1} however, by using a flowrate of 2.0 ml min^{-1} the extraction time is reduced. With the exception of triazolam, the recovery levels of all the drugs are acceptable.

2.14.17 Effect of Pressure

In order to investigate the effect of pressure on the extraction, it was decided to introduce the internal standard (prazepam) to the extractions. For the optimum conditions, it is essential that both the drug of interest and the internal standard are extracted to the same extent so the best conditions need not be those that give the highest recoveries of temazepam.

2.14.17.1 Experimental

1 ml of prazepam and 1 ml of temazepam were pipetted into a vial, evaporated to dryness under nitrogen and reconstituted in 1 ml of MeOH. 100 µl aliquots of this solution were extracted. The extraction was carried out five times at different pressures.

2.14.17.2 Results and Discussion

The following table (2.14) indicates the results obtained for the extraction of temazepam and prazepam at various pressures.

Table 2.14: *Effect of Pressure on Temazepam Recovery*

Mean Pressure (psi)	P.H. Temaz	PHR	%Rec. Temaz
1536	8.4	1.68	74%
2016	6.2	2.48	55%
2362	4.5	2.00	40%
3096	7.7	2.33	68%
3675	5.3	2.52	47%

The unextracted PHR was 2.06. A graph of pressure versus recovery was plotted Figure 2.14) and from this the pressure for the extractions was chosen as 3000 psi as the rate of change of PHR with pressure is less pronounced at this pressure and the recovery is relatively high.

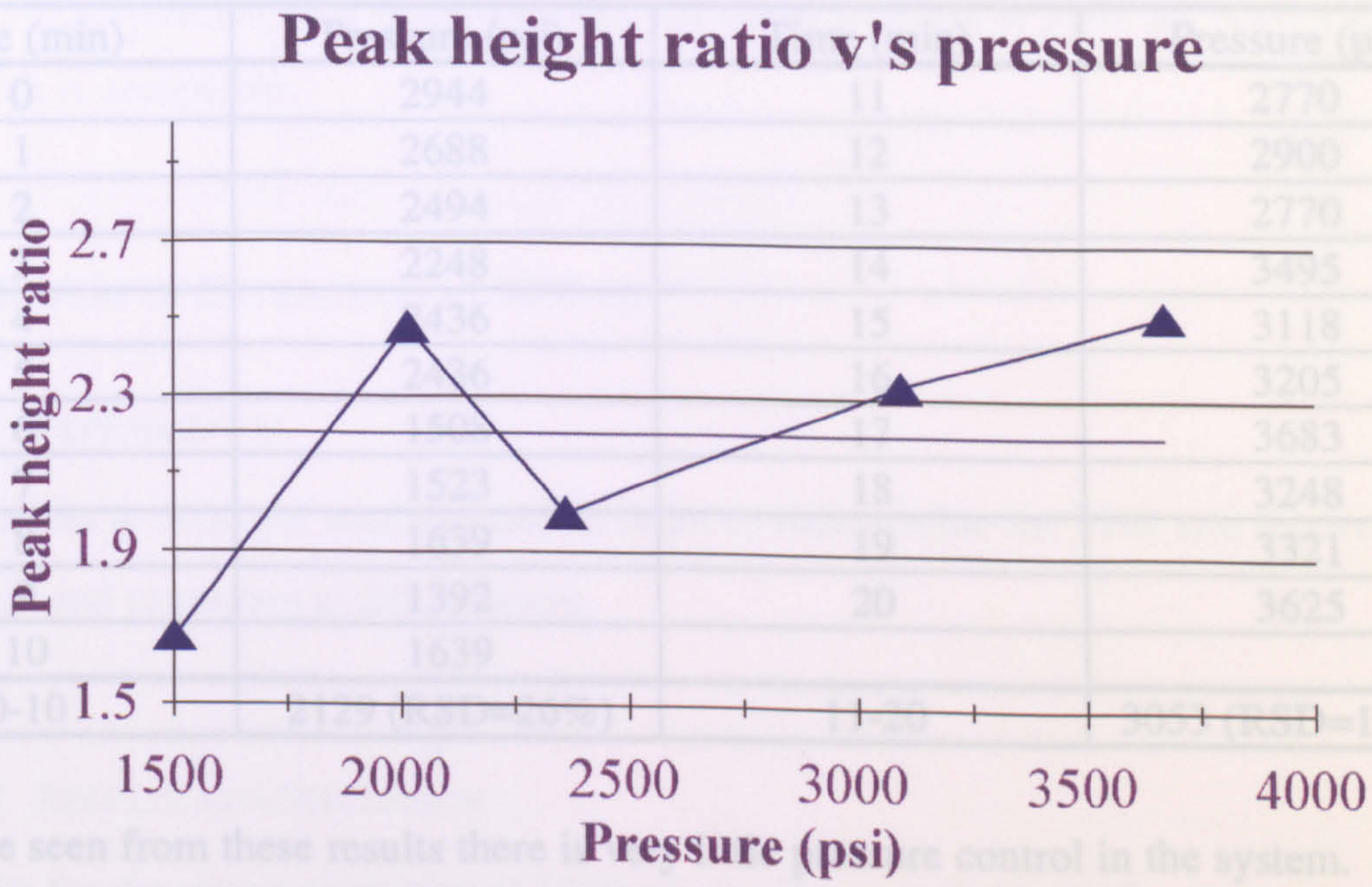


Figure 2.14: *Variation of PHR with Pressure*

2.14.18 VARIATION OF PRESSURE WITHIN THE SFE SYSTEM

2.14.18.1 EXPERIMENTAL

As the pressure appears to have such a pronounced effect on both the PHR and the % recovery of temazepam and prazepam, a study on the variation of a set pressure of 3000 psi for 20 minutes was carried out.

2.14.18.2 RESULTS AND DISCUSSION

The variation in pressure of the SFE system at a set pressure of 3000 psi is shown in Table 2.15.

Table 2.15: *Variation of Pressure set at 3000 psi*

Time (min)	Pressure (psi)	Time (min)	Pressure (psi)
0	2944	11	2770
1	2688	12	2900
2	2494	13	2770
3	2248	14	3495
4	2436	15	3118
5	2436	16	3205
6	1508	17	3683
7	1523	18	3248
8	1639	19	3321
9	1392	20	3625
10	1639		
0-10	2129 (RSD=26%)	11-20	3053 (RSD=19.5%)

As can be seen from these results there is very little pressure control in the system. As the CO_2 did not require changing, this variation was thought to be due to the check valves in the CO_2 pump which were changed and the experiment repeated. The repeated pressure results are given in Table 2.16.

Table 2.16: *Variation of Pressure Set at 3000 psi with New Check Valves*

Time (min)	Pressure (psi)	Time (min)	Pressure (psi)
0	2987	11	3045
1	2625	12	3089
2	2436	13	3002
3	2306	14	3132
4	2813	15	3045
5	2944	16	3045
6	3016	17	3060
7	2944	18	3045
8	2944	19	3074
9	2987	20	3161
10	3060		
0-10	2824 (RSD = 9%)	11-20	3070 (RSD = 1.5%)

The mean pressure for the twenty minutes was 2941 with a RSD of 7.5%. But, if five minutes re-equilibration time is allowed for, the mean pressure is 3043 psi with a RSD of 2.0% which is acceptable.

2.14.19 Repeat of Extraction at 3000 psi

2.14.19.1 Experimental

The extraction at 3000 psi was repeated in order to redetermine the PHR and recoveries of temazepam and prazepam at this pressure.

2.14.19.2 Results and Discussion

The results for the repeat extraction at 3000 psi are shown in Table 2.17.

Table 2.17: *Repeat of Extraction at 3000 psi*

	Temaz	Praz	PHR
Unextracted	11.3	5.5	2.055
Extracted	9.9 (88%)	5.2 (95%)	1.904

It can be seen that these results agree well with those obtained during the optimum pressure study and thus it was decided to use a pressure of 3000 psi for future extractions.

2.14.20 Effect of Temperature

2.14.20.1 Experimental

A temperature study at 3000 psi, 5% EtAc and 2 ml min^{-1} was carried out. Three extractions were made at each temperature and the initial and final pressures were noted. Previous studies have indicated that a temperature around 60 °C should be ideal and thus the temperature was varied around this value. It should be noted that precise control of the temperature gauge on the GC oven controller was difficult so the exact temperature measurements were made by placing a thermometer into the oven.

2.14.20.2 Results and Discussion

The results for the temperature study on temazepam and prazepam are shown in Table 2.18. For the runs at 62 °C, the EtAc was not pumping efficiently which explains the relatively low recoveries at this temperature. This was found to be due to faulty modifier pump check valves which were subsequently changed.

Table 2.18: *Temperature Study for Temazepam and Prazepam*

Temaz %Rec.	Mean (SD)	Praz %Rec.	Mean (SD)	PHR	Mean (SD)	Pressure (psi)
55 °C				**1.947**		
139	129 (61)	121	121 (31)	2.232	2.133 0.087)	3169
90		90		2.098		3067
160		151		2.070		2890
60 °C				**1.991**		
73	103 (26)	76	99 (21)	1.929	2.047 (0.103)	3133
123		117		2.092		3205
112		105		2.120		3212
62 °C				**2.152**		
41	37 (21)	44	41 (29)	2.022	2.332 (0.762)	3365
56		68		1.775		3179
14		10		3.200		3125
64 °C				**1.991**		
90	106 (14)	92	103 (96)	1.951	2.042 (0.082)	3227
114		110		2.066		3154
114		107		2.109		3104
66 °C				**2.055**		
72	107 (61)	73	105 (60)	2.025	2.043 (0.055)	3314
178		174		2.000		3053
71		69		2.105		2920

2.14.21 Effect of Collection Solvent and Continuation of Temperature Study

2.14.21.1 Experimental

It has been suggested in literature that MeOH is a poor collection solvent and that chloroform should give better recoveries.[97] For the rest of the temperature survey, the extracts were collected in both MeOH and $CHCl_3$ so that the recoveries for each could be compared.

2.14.21.2 Results and Discussion

The results for the extraction of temazepam and prazepam using chloroform and methanol as collection solvent at varying temperatures are shown in Tables 2.18a and 2.18b.

Table 2.18a: *Temperature Study Using Chloroform as a Collection Solvent*

Temazepam Recovery		Prazepam Recovery		Peak Height Ratio		Pressure
64 °C				**2.087**		
121	133 (67)	99	122 (68)	2.549	2.323 (0.20)	3118
73		69		2.225		3140
206		198		2.196		3089
65 °C*				**2.743**		
132	135	135	139	2.680	2.67	3003
137		142		2.650		3089
66 °C				**1.815**		
123	118 (25)	109	110 (18)	2.042	1.947 (0.13)	3161
141		128		2.000		3220
92		93		1.800		3170
70 °C				**2.087**		
17		15		2.467		3154

Table 2.18b: *Temperature Study Using Methanol as a Collection Solvent*

Temazepam Recovery		Prazepam Recovery		Peak Height Ratio		Pressure
64 °C				**2.087**		
111	78 (45)	107	75 (45)	2.173	2.210 (0.15)	3261
95		95		2.082		3118
27		23		2.375		3111
65 °C*				**2.743**		
101	104	119	110	2.341	2.644	3067
107		100		2.946		3060
66 °C				**1.815**		
127	109 (20)	118	103 (12)	1.915	1.895 (0.13)	3082
91		93		1.780		3183
92		93		1.800		3169
126		109		2.085		3214
70 °C				**2.087**		
2.3		2.9		2.000		3075

* For the run at 65 °C a more concentrated solution was injected (25 mg/L *c.f.* 5 mg/L) to see if a smaller injection volume would improve the reproducibility.

Although the intra-temperature results were not very reproducible, as seen from Figure 2.15, the optimum temperature with least variance is around 65 °C. With the temperature gauge on the GC oven programmer set to 65 °C, the temperature reading on a thermometer placed in the oven was found to be 64 °C which is satisfactory.

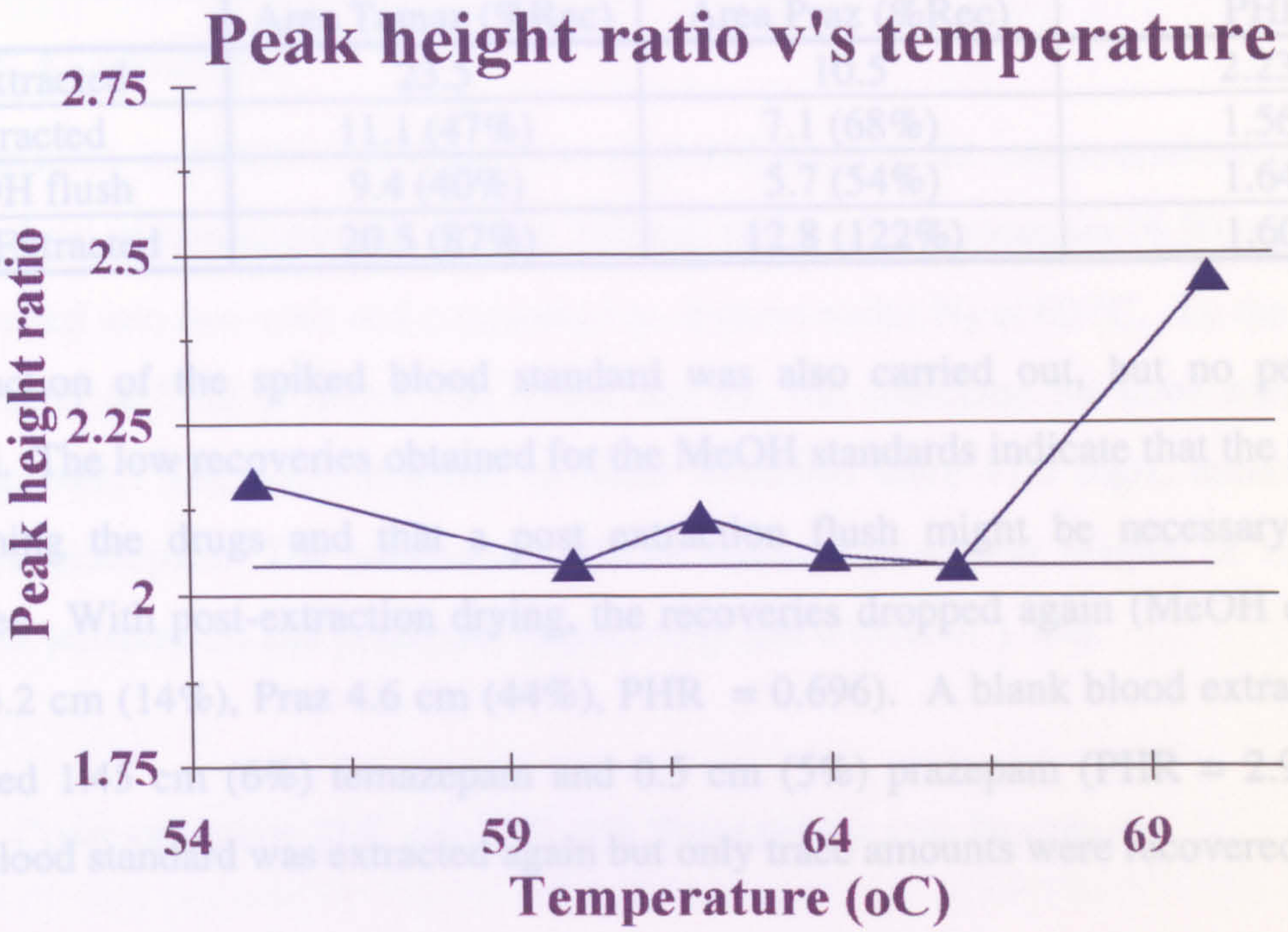


Figure 2.15: *Variation of PHR with Temperature*

2.14.22 Extraction of Temazepam and Prazepam from Blood

2.14.22.1 Experimental

Standards of temazepam and prazepam were prepared in blood, however post-restrictor blocking became problematic, possibly due to Joule-Thomson cooling of extracted water in the outlet tubing. A column containing dried silica was placed in-line before the extraction vessel in order to trap the water. An extraction of temazepam and prazepam in methanol was carried out in order to see whether or not the silica affected the extraction. This was followed by an injection of MeOH.

2.14.22.2 Results and Discussion

The results for the effect of a silica drying column on the extraction of temazepam and prazepam are shown in Table 2.19.

Table 2.19: *Effect of Silica on Temazepam MeOH Standard Extraction*

	Area Temaz (%Rec)	Area Praz (%Rec)	PHR
Unextracted	23.5	10.5	2.238
Extracted	11.1 (47%)	7.1 (68%)	1.563
MeOH flush	9.4 (40%)	5.7 (54%)	1.647
Total Extracted	20.5 (87%)	12.8 (122%)	1.602

An extraction of the spiked blood standard was also carried out, but no peaks were observed. The low recoveries obtained for the MeOH standards indicate that the silica may be retaining the drugs and that a post extraction flush might be necessary for high recoveries. With post-extraction drying, the recoveries dropped again (MeOH extraction, Temaz 3.2 cm (14%), Praz 4.6 cm (44%), PHR = 0.696). A blank blood extraction after this eluted 1.45 cm (6%) temazepam and 0.5 cm (5%) prazepam (PHR = 2.900). The spiked blood standard was extracted again but only trace amounts were recovered.

2.14.23 Alternative Method of Preventing Restrictor Plugging

2.14.23.1 Experimental

As an alternative to using a silica column to dry the extracts, the outlet tubing was coiled inside the oven and the remaining tubing which was outside the oven lagged. Again at this point, the CO_2 head pressure was dropping and thus the cylinder was replaced.

Several injections of temazepam and prazepam MeOH and blood standards were made into the SFE system.

2.14.23.2 Results and Discussion

From MeOH the recoveries were 24% and 60% for temazepam and prazepam respectively however, the blood extract showed recoveries of >100% for both drugs and a subsequent MeOH flush was negative indicating that the blood extract had removed the remaining temazepam and prazepam from the MeOH extraction. It was not possible to calculate the recoveries for the blood extract, as the concentrations were different to those of the MeOH standards.

2.14.24 Preparation of Standard Temazepam and Prazepam

2.14.24.1 Experimental

Fresh standards in MeOH and blood were prepared keeping the concentrations constant as follows. 4 ml of temazepam (5.16 mg / 100 ml) and 4 ml of prazepam (4.50 mg / 100 ml) were pipetted into two vials and evaporated to dryness under N_2 at 60 °C. To the residue, 5 ml of MeOH or 5 ml of blood was added and the vial sonicated for three minutes in an ultrasonic bath. The concentrations of these standards were 41.3 mg/L temazepam and 36.0 mg/L prazepam. The unextracted MeOH standard gave peak heights of 13.7 cm and 7.2 cm for temazepam and prazepam respectively and a PHR of 1.903.

2.14.25 Comparison of SFE and SPE for Temazepam and Prazepam

2.14.25.1 Experimental

A SPE was carried out on the blood standard for comparison with SFE. The method used was as described in Section 2.11.1 however, only 100 µl of the blood standard was extracted for a direct comparison with SFE.

2.14.25.2 Results and Discussion

The results obtained by SPE were temazepam 13.3 cm (97%) and prazepam 6.6 cm (92%), PHR 2.015. The SFE of the same blood standard gave 5.2 cm (38%) temazepam and 8.75 (122%) prazepam, PHR 0.594 with a MeOH flush yielding 0.75 cm (5%) temazepam and 0.4 cm (6%) prazepam indicating that the extraction set-up was still not robust.

2.14.26 Procedure for Drying of Blood Samples

Due to the many problems which were being encountered with the "wetness" of the blood samples, a literature search on the problem of water in SFE systems was carried out with suggestions found on removing the water prior to extraction by freeze drying followed by SPE[98] and cryogenic trapping.[99] Alternatively, a modifier could be selected with sufficiently strong antifreeze properties to counteract the Joule-Thomson cooling effect.[100] The most feasible of these, which would cause least disruption to the system, would be the

addition of antifreeze either as or in the modifier. However, as the extraction conditions used are mild and provide high recoveries, an alternative sample loading method was investigated.

Instead of injecting the samples *via* the rheodyne loop onto the Extrelut® in the extraction vessel (*i.e.* the extraction vessel was in the position of an HPLC column), the plumbing of the system was altered and the extraction vessel was placed over the loop position of the rheodyne. This has the following advantages:

- The extraction vessel can be changed without disturbing the equilibrium of the supercritical fluid
- Reduction in blockages in loop and connective tubing
- Reduced chance of contamination from sample to sample as the system will operate in a one vessel per extraction mode
- Reduce time, as a post extraction flush will not be required

Initial runs with this system gave poor recoveries, possibly as the drugs needed to be extracted twice, as the original vessel was still in line. The original extraction vessel was removed and replaced with a dead volume connector.

The first blood extraction with this system gave good recoveries (100%) with a PHR of 1.804 (*c.f.* 1.652) however, pressure fluctuations were still problematic throughout the course of the extraction. It was decided to dry the blood samples on the Extrelut® prior to insertion into the extraction vessel. Although this step is time consuming and allows for an increased chance of sample loss, the importance of these factors is counteracted by the following three points:

- Reduction in blockages (increased up-time)
- Multiple samples can be prepared and dried overnight
- Prazepam is added to the samples and thus any loss of sample is compensated for

The components for the initial extraction were weighed in order to monitor the sample loss. 0.19 g of Extrelut® and 0.10 g were weighed onto the boat and dried. 0.22 g of this were transferred to the column. Therefore, without taking into account losses due to drying of the sample, the recoveries should be 76%. The recoveries obtained were 95% for temazepam and 80% for prazepam giving a PHR of 2.051 (*c.f.* 1.729 for the standard). Taking into account the variability seen for the unextracted standards and errors which might occur in sample preparation of both the unextracted standards and the blood standards, this method was chosen for use on authentic forensic samples, validation pending.

2.14.27 Effect of Altering the Support Medium

2.14.27.1 Experimental

In addition to changing the temperature, pressure and % modifier in the extraction, the other variable which was investigated was the effect of the support medium. Until now all method development was carried out using Extrelut®. Two other support mediums, namely C_{18} and HCX were investigated for the extraction of all 6 benzodiazepines.

2.14.27.2 Results and Discussion

From Extrelut® all 6 benzodiazepines were extracted however, the other two support mediums were less successful. Using C_{18}, triazolam, temazepam, chlordiazepoxide, desmethyldiazepam and diazepam extracted but the recoveries were very low and the prazepam did not extract. Using the HCX support medium, none of the benzodiazepines extracted. For this reason the support medium was kept as Extrelut®.

2.14.28 Conclusions from Method Development

The final conditions developed for the extraction of temazepam and prazepam are as follows:

- Flow Rate: 2.0 $mlmin^{-1}$
- % EtAc: 5%
- Temperature : 65 °C
- Pressure: 3000 psi
- Run Time: 5 minutes
- Support Medium: Extrelut®

The SFE system was set to equilibrate at the above conditions and samples prepared as follows. A 100 µl aliquot of the sample to be analysed was loaded onto Extrelut® in a weighing boat and mixed thoroughly. This was allowed to dry at room temperature until a friable consistency was achieved and then loaded into an extraction vessel. The extraction vessel was placed into the equilibrated SFE system and the extraction commenced by switching the Rheodyne valve from load to inject, allowing the flow of supercritical fluid to pass through the extraction vessel. A Hypovial® containing MeOH placed at the flow outlet, trapped the extracted analytes.

2.15 Method Validation for Temazepam

2.15.1 High Concentration Calibration Curves

2.15.1.1 Experimental

The first part of the validation was carried out by producing high concentration calibration graphs for:

1) MeOH standards straight onto HPLC (Table 2.20)
2) MeOH extracted standards (Table 2.21)
3) Blood extracted standards (Table 2.22)

The range of concentrations was from 0-51.6 mg/L. For the unextracted standards, 100 µl of temazepam was evaporated to dryness and reconstituted in 100 µl of mobile phase and for the extractions, either 100 µl of MeOH standard or blood standard were extracted and the collected extract evaporated to dryness and reconstituted in 100 µl of mobile phase.

2.15.1.2 Results and Discussion

The peak heights of temazepam obtained for the calibration are shown in Tables 2.20, 2.21 and 2.22. The % recoveries were calculated by comparing the extracted PH's to the extracted PH for the relevant concentration. The equation for each of the best fit lines is given as $y = Bx + A$.

Table 2.20: *Unextracted Standards*

Concentration	Peak Height
0	0
10.32	3.1
20.64	6.15
25.80	8.4
41.26	12.7
51.60	17.05

$r^2 = 0.995$

A = -0.469

B= 0.332

Table 2.21: *MeOH Extracted Standards (average of 3)*

Concentration	Peak Height (% Recovery)
0	0
10.32	3.2 (103%)
20.64	5.8 (94%)
25.80	9.3 (111%)
41.26	12.6 (99%)
51.60	15.15 (89%)

$r^2 = 0.975$

A = 0.494

B= 0.291

Table 2.22: *Blood Extracted Standards (1 injection only)*

Concentration	Peak Height (% Recovery)
0	0
10.32	4.2 (135%)
20.64	6.0 (98%)
25.80	8.1 (96%)
41.26	8.5 (67%)
51.60	11.65 (68%)

$r^2 = 0.919$

A = 2.800

B= 0.163

At this point, the concentrations of the standards used were much higher than those seen from therapeutic and even overdose levels of benzodiazepines in case samples. It can be seen that in addition to the poorer correlation seen for the blood extracted standards the concentration range appears to have overloaded the range for extraction (*i.e.* a longer

extraction time would have to be used in order to extract this amount of temazepam). This is also the case for the 51.6 mg/L MeOH extraction.

In terms of the gradients (B) of the curves produced, it can be seen that as the matrix effects increase (*i.e.* MeOH to blood) compared with the unextracted standards, a decrease in the gradient is seen.

The injection reproducibility of the HPLC system was checked again. Ten injections of the 51.6 µg ml^{-1} standard were made and the peak heights were found to range from 16.9 cm to 18.3 cm (mean 17.4 cm, RSD 2.86%). A 20 µl loop was used and 50 µl was injected each time. This was compared to five 25-30 µl injections; range 16.0-18.5 cm, mean 17.2 cm, RSD 5.23%. Thus, the injection reproducibility could also contribute to the results obtained as the blood extraction results were based on only one injection.

2.15.2 Extractions with Internal Standard

2.15.2.1 Experimental

The MeOH unextracted and extracted calibrations were repeated this time including prazepam to compensate for the injection reproducibility. 0.5 ml of each standard dilution plus 0.5 ml prazepam was analysed in each case (N.B. prazepam on its own extracts 100%).

2.15.2.2 Results and Discussion

The results for the extractions with prazepam and temazepam are shown in Table 2.23.

Table 2.23: *Extraction with Temazepam and Prazepam*

[Temaz]	PHR Ext.	PHR Unext.	%Rec. Temaz	%Rec. Praz	% Error
0	0	0	na	na	na
5.16	0.633	0.313	76	38	+103
10.32	0.900	0.775	58	50	+11
12.9	1.077	0.923	39	33	+17
20.64	1.714	1.56	40	36	+10
25.80	1.78	2.20	42	52	-19

Unextracted: r^2: 0.990 Extracted: r^2: 0.967

A: -0.174 A: 0.316

B: 0.089 B: 0.060

Again at the higher concentrations, a loss of temazepam is apparent and the gradient is less than that of the unextracted standards.

2.15.3 Linearity of Extraction Above the Therapeutic Concentration

2.15.3.1 Experimental

A fresh standard of temazepam was prepared by weighing 1.28 mg of temazepam standard into a 100 ml volumetric flask and making up to the mark with MeOH to give a concentration of 12.8 mgL^{-1} This was sequentially diluted to give calibration solutions of 0, 1.28, 2.56, 3.84, 6.40, 12.8 mgL^{-1} of temazepam with 11.24 mgL^{-1} of prazepam in each. Each standard type was extracted by SPE and SFE for comparison.

2.15.3.2 Results and Discussion

The unextracted standard calibration was carried out in duplicate in order to check the reproducibility. The results are shown in Tables 2.24 and 2.25.

Table 2.24: *Unextracted MeOH Standards 1*

[Temaz] mg L^{-1}	Peak Height Ratio
0	0
1.28	0.164
2.56	0.342
3.84	0.559
6.40	0.973
12.80	1.892

$r^2 = 0.999$
A = -0.025
B = 0.151

Table 2.25: *Unextracted MeOH Standards 2*

[Temaz] mg L^{-1}	Peak Height Ratio
0	0
1.28	0.158
2.56	0.348
3.84	0.538
6.40	0.973
12.80	1.908

$r^2 = 0.999$
A = -0.036
B = 0.153

From these results it can be seen that a reproducible calibration graph can be easily constructed over this concentration range.

Using the system as described previously with the chart recorder, calibrations for MeOH SFE standards and blood SPE standards were constructed as shown in Tables 2.26 and 2.27.

Table 2.26: *Extracted MeOH 1 - SFE*

[Temaz] mg L^{-1}	Peak Height Ratio
0	0
1.28	0.193
2.56	0.409
3.84	0.717
6.40	1.047
12.80	2.014

$r^2 = 0.995$
A = 0.038
B = 0.156

Table 2.27: *Extracted Blood 1 - SPE*

[Temaz] mg L^{-1}	Peak Height Ratio
0	0
1.28	0.220
2.56	0.524
3.84	0.682
6.40	1.247
12.80	2.567

$r^2 = 0.998$
A = -0.044
B = 0.203

At this point there were no other graphs to compare these to, however, the correlation coefficients are high and in the case of the MeOH SFE extracts, the gradient is similar to that of the unextracted standards.

2.15.4 Continuation of Calibration with System Controller

2.15.4.1 Experimental

At this point, the system controller was modified so that the traces could be directly compared using the Gilson software. This also reduces the human error from manually measured peak heights. Injections of the unextracted MeOH standards and extracted blood and MeOH, SFE and SPE extracts were continued using the new set-up.

2.15.4.2 Results and Discussion

The unextracted MeOH standard calibration was repeated. As can be seen from the results in Tables 2.28, 2.29, 2.30, 2.31 and 2.32 a slightly different gradient is calculated using the system controller peak areas as opposed to manually measured peak heights.

Table 2.28: *Unextracted MeOH Using Gilson Software 1 (n=3)*

[Temaz] mg L^{-1}	Peak Area Ratio
0	0
1.28	0.103
2.56	0.248
3.84	0.384
6.40	0.679
12.80	1.331

$r^2 = 0.999$
A = -0.024
B = 0.107

During the course of the SFE extractions it was noted that the PTFE check valves in the modifier pump were allowing flow back of CO_2 into the solvent reservoir. These were replaced and left overnight, however, the same problem was noted the next morning. In order to check whether it was the combination of PTFE and EtAc, PVDE check valves were inserted into the modifier pump. These did not hold the flow at all. A fresh batch of PTFE check valves were inserted which worked well, and thus the problem was attributed to a faulty batch of check valves. As the CO_2 had been leaking throughout the night, a new cylinder was placed in the system.

Table 2.29: *Extracted MeOH - SFE (3 extractions, 3 injection each n=9)*

[Temaz] mg L^{-1}	Peak Area Ratio
0	0
1.28	0.139
2.56	0.304
3.84	0.570
6.40	0.758
12.80	1.565

$r^2 = 0.991$
$A = 0.015$
$B = 0.121$

Table 2.30: *Extracted Blood - SFE (2 extractions, 3 injections each n=6)*

[Temaz] mg L^{-1}	Peak Area Ratio
0	0
1.28	0.091
2.56	0.449
3.84	0.794
6.40	0.851
12.80	1.236

$r^2 = 0.827$
$A = 0.220$
$B = 0.086$

Table 2.31: *Extracted MeOH - SPE (1 extraction, 3 injections n=3)*

[Temaz] mg L^{-1}	Peak Height Ratio
0	0
1.28	0.127
2.56	0.232
3.84	0.403
6.40	0.804
12.80	1.406

$r^2 = 0.989$
$A = -0.017$
$B = 0.114$

Table 2.32: *Extracted Blood SPE (2 extractions, 3 injections n=6)*

[Temaz] mg L^{-1}	Peak Height Ratio
0	0
1.28	0.074
2.56	0.160
3.84	0.207
6.40	0.359
12.80	0.966

$r^2 = 0.981$
A = -0.065
B = 0.078

From these tables it can be seen that good correlation exists over the range 1.28-12.80 mg/L temazepam for the various extractions.

2.15.5 Intra- and Inter-Day Reproducibility

2.15.5.1 Experimental

To check the reproducibility of the temazepam extraction throughout the course of one day and from day to day, sequences of extractions of the same standard were extracted on days one and two. This experiment was also used to ensure that there was no carry over of the benzodiazepines in the system. 5 extractions of temazepam and prazepam were carried out on day one, and 6 on day two with blank extractions in between to ensure no carryover. The temperature in the oven was found to be 62 °C instead of 64 °C as previously noted using the thermometer in the oven.

2.15.5.2 Results and Discussion

The results for the extractions on day one are shown in Table 2.33.

Table 2.33: *Extraction of Temazepam and Prazepam; Day 1*

Extraction	Area Temaz	Area Praz	PAR
1	6102	9590	0.636
2	5005	9477	0.528
3	35583	57505	0.619
4	31208	63479	0.492
5	57152	113041	0.506
Unext.	501961	602912	0.833

$\bar{x} = 0.556$

RSD: 12%

NB: The sensitivity of the HPLC was turned up for the injections of extracts 3,4 and 5 and the unextracted standard.

The results for the extractions on day two are shown in Table 2.34.

Table 2.34: Temazepam and Prazepam Extraction; Day 2

Extract	Area Temaz	Area Praz	PAR
1	108258	140896	0.768
2	79817	123005	0.649
3	32316	51446	0.628
4	117640	190665	0.617
5	44081	61156	0.721
6	22548	39166	0.576
Unext.	501961	602912	0.833

$\bar{x}$: 0.660

RSD: 11%

The mean PAR over the two days was 0.613 with a RSD of 13.9%. Thus the variation from day to day is no greater than that seen within the same day.

Due to the slight variation in PAR observed between extractions, for any authentic samples extracted, the standard will have to be run in triplicate at least.

2.15.6 Alteration of Outlet Restriction

2.15.6.1 Experimental

Problems were being encountered due to blockages caused by precipitation of material within the flow outlet tubing. This was causing a reduction in the recoveries at the previously optimised conditions and was thought to be due to insufficient restriction being provided. In order to amend this, the pressure in the system was set to 1500 psi and the end of the outlet tubing crimped until an increase in pressure was observed. With this in place, extractions were carried out at 10% EtAc, 5% EtAc and then 10% EtAc again.

2.15.6.2 Results and Discussion

The results for the extractions at 5 and 10% EtAc are shown in Table 2.35.

Table 2.35: *Temazepam Extractions With Crimped Outlet*

% EtAc	Area Temaz (% Rec)	Area Praz (% Rec)	PAR
10/1	85455 (21)	251453 (51)	0.340
5	136636 (33)	305927 (61)	0.447
10/2	183388 (44)	333325 (67)	0.550
Unext.	414021	497680	0.832

The recoveries were still quite low, so to ensure that the drugs were not binding to matter which had adhered to the inside of the tubing, all of the tubing in the system was replaced.

Due to the recent problems with the system, the extraction conditions for benzodiazepines were modified slightly to 10% EtAc, 65 °C, 2.0 ml min^{-1} and 3000 psi. Despite the fact that the use of 10% EtAc decreases the selectivity of the extraction slightly, it was found to be better for the extraction of authentic forensic samples perhaps due to the increased matrix effects as compared to spiked blood samples.

2.15.7 VITREOUS HUMOR

2.15.7.1 DEFINITION

In addition to analysing blood samples, this study was also involved with the extraction of drugs from post-mortem vitreous humor samples. The vitreous humor is defined as a clear, avascular, gelatinous body which comprises two thirds of the volume and weight of the eye and fills the space bound by the lens, retina and optic disc.[101] It consists of approximately 99% water with the remaining components including collagen and hyaluronic acid which give it its specific physical character.

The gel like form of the vitreous is due to a loose mass of long chain collagen molecules capable of binding up to 200 times their own weight in water. The hyaluronic acid molecules are very large, loose bundles capable of binding up to 60 times their own weight in water. Combined with the collagen element, they account for the physical characteristics of normal vitreous.

The use of vitreous humor as an alternative to post-mortem blood is of particular importance when the body has undergone considerable decomposition or burning.[102] In comparison to post-mortem blood, which is often putrefied, vitreous humor is a relatively simple matrix as highlighted in Table 2.36. It is also much less susceptible to post-mortem changes. The transport of drugs across the blood / vitreous humor barrier is limited by the lipid solubility of the drug and its charge at physiological pH.[103]

Table 2.36: *Composition of Putrefied Blood c.f. Vitreous Humor*[102]

Putrefied Blood (pH 4-9)	Vitreous Humor (pH7-7.8)
Water (20-70%)	Water (98.0-99.7%)
Lysate	Glucose
Clots - denatured & bacterial	Hyaluronic acid
Denatured protein	Simple anions & cations
Fat droplets	Collagen
Steroids	
Putrefactive bases	
Protein microagglutinates	
Enzymes	

2.15.8 Analysis

Most studies concerning the analysis of vitreous humor have been concerned with time-since-death determinations[104] and it is only in the last decade that interest has been placed on the use of vitreous humor for the determination of drug levels.

Some of the drugs which have been investigated in vitreous humor include morphine,[105] flurazepam,[106] methadone,[107] and cocaine.[102] The methodology usually involves a solid phase extraction followed by the appropriate analysis technique.

2.15.8.1 Vitreous Humor Analysis for Benzodiazepines

In terms of this study, the 10% EtAc extractions were found to give slightly better recoveries from authentic forensic samples than the 5% EtAc. The method development for vitreous humor is taken as being the same as the MeOH standard method development. The reasons for this are twofold:

- No blank vitreous humor was available for direct comparison
- The matrix used to mimic vitreous humor was water, however as the samples are dried prior to extraction, the addition of water to the Extrelut® should make little difference to the matrix to be extracted.

2.15.9 EXTRACTIONS WITH ETAC (10%) AS THE MODIFIER

2.15.9.1 EXPERIMENTAL

A combination of various standards was extracted using 10% EtAc to ensure that the system was functioning and that the 10% EtAc produced high recoveries. The standards analysed are detailed below.

Extraction 1:Triazolam
Extraction 2: Temazepam
Extraction 3: Chlordiazepoxide
Extraction 4: Diazepam
Extraction 5: Desmethyldiazepam
Extraction 6: Triazolam + Temazepam
Extraction 7: Temazepam + Diazepam
Extraction 8: Temazepam + Desmethyldiazepam
Extraction 9: Triazolam + Temazepam + Desmethyldiazepam + Diazepam
Extraction 10: All 5 standards

2.15.9.2 RESULTS AND DISCUSSION

The results are shown in Table 2.37 on page 92.

Table 2.37: *Extractions at 10% EtAc*

Extracted						Unextracted				
Extract	Triaz	Temaz	CDP	DMD	Diaz	Triaz	Temaz	CDP	DMD	Diaz
1	1.233(123)	-	-	-	-	1.005	-	-	-	-
2	-	0.739(97)	-	-	-	-	0.762	-	-	-
3	-	-	1.968(109)	-	-	-	-	1.809	-	-
4	-	-	-	-	0.946(80)	-	-	-	-	1.188
5	-	-	-	0.715(111)	-	-	-	-	0.647	-
6	1.021(115)	0.816(108)	-	-	-	0.889	0.757	-	-	-
7	-	0.759(96)	-	-	0.904(82)	-	0.788	-	-	1.096
8	-	0.781(102)	-	0.789(122)	-	-	0.767	-	0.642	-
9	1.041(114)	0.723(92)	-	1.177(165)	1.237(117)	0.913	0.788	-	0.712	1.057
10	0.858(93)	0.988(127)	1.240(71)	0.706(82)	0.849(75)	0.922	0.775	1.747	0.861	1.125

The figures in brackets represent the % recoveries of each drug compared to the unextracted standards.

From Table 2.36 it can be seen that the extractions work well with good recoveries and thus the 10% EtAc was used for all future analyses.

2.16 METHOD VALIDATION FOR ALL FIVE STANDARDS

2.16.1 LINEARITY OF DIAZEPAM, DESMETHYLDIAZEPAM, CHLORDIAZEPOXIDE AND TRIAZOLAM METHANOL STANDARDS

2.16.1.1 EXPERIMENTAL

The linearity for diazepam, desmethyldiazepam, chlordiazepoxide and triazolam from methanol standards was calculated. Each of the results is an average of three extractions and three injections (n=9). The results obtained are shown in Tables 2.38, 2.39, 2.40 and 2.41.

2.16.1.2 RESULTS AND DISCUSSION

Table 2.38: *Linearity for Diazepam; Methanol Standards*

	Unextracted				Extracted			
Conc^{n} (mg/L)	Area Diaz	Area Praz	PAR		Area Diaz	Area Praz	PAR	
0	0	376610	0		0	126320	0	
0.55	74593	174663	0.427	r^2:0.997	18454	55613	0.332	r^2:0.988
1.10	111750	134850	0.829	A:0.128	37460	63283	0.592	A:-0.098
1.66	151680	137650	1.102	B:0.594	59210	65235	0.908	B:0.662
2.21	232840	160620	1.450		72608	51827	1.401	
2.76	269600	153270	1.759		189560	107760	1.759	

Table 2.39: *Linearity for Desmethyldiazepam; Methanol Standards*

	Unextracted				Extracted			
Conc^{n} (mg/L)	Area DMD	Area Praz	PAR		Area DMD	Area Praz	PAR	
0	0	374379	0		0	308326	0	
0.42	91825	386670	0.237	r^2:0.999	99775	368763	0.271	r^2:0.989
0.85	175928	384498	0.458	A:0.007	167417	273455	0.612	A:0.053
1.27	275638	399624	0.690	B:0.537	147918	161554	0.916	B:0.630
1.70	338065	370187	0.913		241397	215479	1.120	
2.12	424846	368959	1.151		532755	392761	1.356	

Table 2.40: *Linearity for Chlordiazepoxide; Methanol Standards*

	Unextracted				Extracted			
Conc^{n} (mg/L)	Area CDP	Area Praz	PAR		Area CDP	Area Praz	PAR	
0	0	309210	0		0	197976	0	
0.84	20760	299681	0.069	r^2:0.980	39487	329021	0.120	r^2:0.989
1.68	41073	315776	0.130	A:-0.044	69464	317427	0.219	A:0.042
2.52	70073	300417	0.233	B:0.112	109292	353057	0.310	B:0.101
3.36	95808	310848	0.308		159054	441691	0.360	
4.20	135004	299282	0.451		142440	300824	0.473	

Table 2.41: *Linearity for Triazolam; Methanol Standard*

	Unextracted				Extracted			
Conc^{n} (mg/L)	Area Triaz	Area Praz	PAR		Area Triaz	Area Praz	PAR	
0	0	418840	0		0	89123	0	
0.71	33598	435380	0.077	r^2:0.936	15887	227390	0.077	r^2:0.985
1.42	25112	172230	0.146	A:0.034	30040	219200	0.137	A:0.010
2.12	38077	151910	0.251	B:0.083	52818	265555	0.199	B:0.089
2.83	44259	163100	0.271		33474	138440	0.242	
3.54	58552	189810	0.308		36381	107300	0.339	

From these it can be seen that the linearity over the range studied is good and the gradients of both the unextracted and extracted standard curves are in good agreement for all drugs.

2.16.2 Linearity of Diazepam and Desmethyldiazepam Blood Standards

2.16.2.1 Experimental

For blood standards only diazepam and desmethyldiazepam were extracted as these and temazepam were the only benzodiazepines quantitated in the authentic blood samples as discussed in chapter 6.

2.16.2.2 Results and Discussion

The PAR's obtained for three extractions and three injections of diazepam and desmethyldiazepam blood standards are shown in table 2.42.

Table 2.42: *Linearity for Diazepam and Desmethyldiazepam; Blood Standards*

	Diazepam				Desmethyldiazepam		
Conc^n (mg/L)	Unext. PAR	Ext. PAR		Conc^n (mg/L)	Unext. PAR	Ext. PAR	
0	0	0		0	0	0	
0.55	0.427	0.180	r^2:0.919	0.42	0.237	0.380	r^2:0.881
1.38	0.852	0.430	A:-0.190	1.06	0.549	0.517	A:0.231
2.21	1.450	0.771	B:0.508	1.70	0.913	0.606	B:0.279
2.76	0.759	1.364		2.12	1.151	0.903	

Although the correlations are poorer for the blood standards than the MeOH standards, the gradients are still in good agreement.

2.16.3 Preparation of Fresh Standards and Calculation of Limits of Detection

2.16.3.1 Experimental

Fresh standard benzodiazepines were prepared at the following concentrations:

Temazepam: 3.44 mg/100 ml

Diazepam: 3.17 mg/100 ml

Desmethyldiazepam: 3.09 mg/100 ml

Prazepam: 2.52 mg/100 ml

The limit of detection was calculated for temazepam, diazepam and desmethyldiazepam using these standards by sequentially diluting the stock standards.

2.16.3.2 Results and Discussion

The limit of detection results obtained are shown in Table 2.43.

Table 2.43: *Linearity for Limit of Detection : 1*

[redacted]	Temazepam			DMD			Diazepam			[redacted]
Sol^{n}	mg/L	PAR		mg/L	PAR		mg/L	PAR		AUFS
1P	4.3	0.204		3.96	0.352		3.86	0.418		0.05
2P	2.15	0.099	r^2:0.996	1.98	0.178	r^2:0.993	1.93	0.203	r^2:0.999	0.05
3P	1.08	0.054	A:0.002	0.99	0.104	A:0.012	0.97	0.101	A:0.003	0.05
4P	0.54	0.035	B:0.046	0.50	0.067	B:0.086	0.48	0.062	B:0.107	0.05
5P	0.27	0.010		0.25	0.018		0.24	0.029		0.01
6P*	0.14	0.012		0.13	0.036		0.12	0.023		0.002

*The prazepam peak in 6P overloaded the detector and thus the PAR for these values was not taken into account for the linearity.

New standard dilutions were prepared using a more dilute prazepam standard. This time the concentrations of each drug were quoted in absolute nanogram values injected into the HPLC system as this way the limits of detection can be more easily converted for different extraction volumes. The results are shown in Table 2.44.

Table 2.44: *Linearity for Limit of Detection : 2*

[redacted]	Temazepam			DMD			Diazepam		
Sol^{n}	ng	PAR		ng	PAR		ng	PAR	
1	688	12.169		634	11.319		618	13.316	
2	344	7.470	r^2:0.989	317	6.996	r^2:0.987	309	7.825	r^2:0.993
3	68.8	1.265	A:0.174	63.4	1.038	A:0.117	61.8	1.232	A:0.084
4	34.4	0.733	B:0.018	31.7	0.578	B:0.018	30.9	0.669	B:0.022
5	6.88	0.121		6.34	0.169		6.18	0.125	
6	3.44	0.094		3.17	0.063		3.09	0.080	

The last solution (6) was diluted further as the limit of detection had not been reached, to yield the following nanogram values and PAR's for each drug:

Table 2.45: *Linearity for Limit of Detection : 3*

[redacted]	Temazepam		DMD		Diazepam	
Sol^{n}	ng	PAR	ng	PAR	ng	PAR
6a	3.44	0.087	3.17	0.076	3.09	0.084
6b	2.29	0.051	2.11	0.038	2.06	0.062
6c	1.72	0.043	1.59	-ve	1.55	0.049
6d	0.86	-ve	0.79	-ve	0.78	-ve

From this the limit of detection was taken as being 1.72 ng for temazepam, 2.11 ng for desmethyldiazepam and 1.55 ng for diazepam. In all cases, the drugs could still be distinguished clearly from the baseline noise.

2.17 Conclusions

It has been found that supercritical fluid extraction can be used successfully for the extraction of benzodiazepine drugs from a variety of matrices loaded onto Extrelut®. This method which was developed and consequently used for the extraction of Forensic blood and vitreous humor samples (see Chapter 6), is described below.

Samples were prepared by loading the 100 µl of sample blood or vitreous humor onto approximately 19 g of Extrelut®. To this, 100 µl of internal standard was added, mixed and allowed to dry. Once dry, the Extrelut® was transferred to an extraction cell which was sealed and placed into the SFE oven at 65 °C. The SFE system which was equilibrated at a flow of 2 ml min^{-1} and a pressure of 3000 psi was then switched to extract and the extracted analytes collected for 10 minutes into MeOH at the flow outlet.

The methodology was found to be reproducible and provide high recoveries of temazepam, desmethyldiazepam and diazepam (80-100%), the three most commonly encountered benzodiazepines in forensic specimens in the West of Scotland over the studied period. In addition, the method was found to be comparable to the currently employed SPE method but was less solvent intensive (2 ml of EtAc used per extraction *c.f.* ~15 ml of diethylether for SPE).

"Not poppy, nor mandragore,
Nor all the drowsy syrups of the world
Shall ever medicine thee to that sweet sleep
Which thou ow'dst yesterday."

William Shakespeare; Othello

3. OPIATES

3.1 INTRODUCTION

The opiates are a group of drugs derived from the opium poppy (*papaver somniferum*).[108] Their use as analgesics dates back as early as the 7th century B.C. with reference to the juice of the poppy in the Assyrian medical tablets.[109] By the 16th century opium was well established in Western European medicine and many of the physicians of the time stated that they could not practice medicine without it. At the beginning of the 19th century, the use of opium was still thought to be central to medicine however, its widespread use became restricted in 1868 when the first Pharmacy Act became law. In addition, after World War One, Britain implemented an international agreement to prohibit non-medical use of opiates due to the large number of post-war addicts.

Natural opiates which are found in the opium poppy include morphine and codeine, both of which are effective as painkillers. Diamorphine (heroin) was first synthesised from morphine in 1874[110] and in its pure form is a white fluffy powder which is more than twice as potent as morphine. It was initially thought to be a safe means of curing morphine addiction and its potential for abuse was largely ignored. A number of other synthetic opiates have been manufactured as pain-killers including pethidine, dipipanone and methadone, the latter of which will be discussed more fully in Chapter 4.

In order to monitor the size of the national heroin problem, a Home Office Notification System was set up in 1968. In 1993, the number of registered addicts was 27,976. However, as this is based on those users who register their addiction with their GP, this number is an under-estimation and the number of actual opiate addicts is believed to be greater than 125,000.

3.2 PRESCRIPTION

Opiates are generally prescribed as painkillers, however, they also have medical uses as cough suppressants and anti-diahorrea agents. Table 3.1 lists the opiates commonly

prescribed along with their proprietary names and uses. In the U.K., morphine is the favoured opiate by most doctors for the treatment of severe pain.

Table 3.1: *Prescription Opiates*[111,112]

Name	Proprietary Name(s)	Treatment
Alfentanil	Rapifen®	Pain
Buprenorphine	Temgesic®	Pain, premed.
Codeine	Diarrest®, Co-Codamol®	Anti-diarrhoea, pain
Dextromoramide	Palfium®	Pain
Dextropropoxyphene	Co-Proxamol®, Distalgesic®	Pain
Diamorphine	-	Pain (IV), cough
Dihydrocodeine	DF118 Forte®	Pain
Diphenoxylate	Lomotil®, Tropergen®	Anti-diarrhoea
Dipipanone	Diconal®	Pain
Fentanyl	Durogesic®	Pain
Loperamide	Imodium®, Loperagen®	Anti-diarrhoea
Meptazinol	-	Pain
Methadone	Physeptone®	Pain, cough
Morphine	MST Continus®, Kaolin®, Oramorph SR®, Sevredol®	Pain, cough, dyspnoea
Nalbuphine	Nubain®	Pain, premed.
Oxycodone	-	Pain
Papaveretum	-	Pain
Pethidine	Pamergan®	Pain
Phenazocine	Narphen®	Pain
Phenoperidine	-	Pain
Pholcodine	Copholco®, Galenphol®	Cough
Tramadol	Zydol SR®	Pain

3.3 Chemical Structure

The three opiates which are of interest to this study are diamorphine (3,6-o-diacetylmorphine), 6-monoacetylmorphine (6-MAM) and morphine ((5a*R*,5*S*,7a*R*,8*R*,9c*S*)-4a,5,7a,8,9,9c-hexahydro-12-methyl-8,9c-iminoethanophenanthro[4,5-bcd]furan-3,5-diol).

Methadone, which is also an opiate is discussed in Chapter 4. All three of the drugs which will be discussed here have the same basic chemical structure, which differs only in the R_1 and R_2 groups as indicated in Figure 3.1 and Table 3.2.

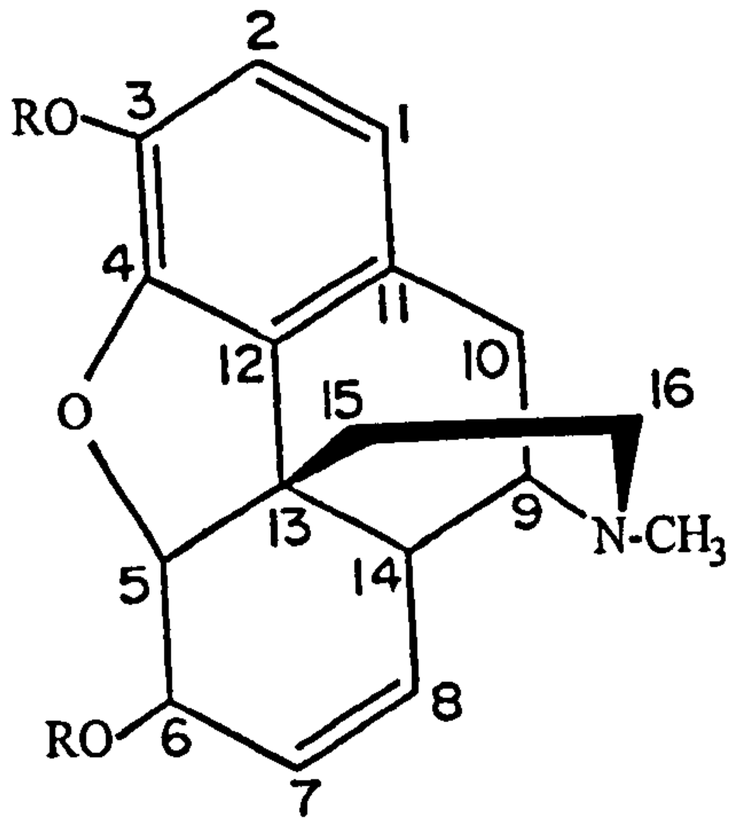


Figure 3.1: *Structure of opiates*

Table 3.2: *R_1 and R_2 Groups for Opiates of Interest*

Drug	R_1	R_2
Diamorphine	$COCH_3$	$COCH_3$
6MAM	H	$COCH_3$
Morphine	H	H

3.4 PHARMACOLOGY

Opiates appear to share some of the properties and mimic the action of some groups of the body's naturally occurring chemicals called peptides and in particular the endorphins, enkephalins and dynorphins. Binding studies have indicated that there may be as many as eight types of opiate receptors which in addition may all have sub-type receptors.[113] Opiates directly cause a large number of actions:

- Effects on the central nervous system
- Effects on the peripheral nervous system
- Induced histamine release-related effects
- Effects for which there is no identified and/or proven causal effect

The main pharmaceutical uses of opiates are due to their effects on the CNS. These include pain relief and cough reflex depression as well as some of the less desired side effects including nausea, vomiting, and respiratory depression which is the main cause of opiate related deaths.

Pain relief with opiates occurs through a number of pain reducing mechanisms and altered perception of painful stimuli. Opiates do not alter the sensitivity of the nerve endings to pain, but instead, interfere with the transmission of signals *via* the nervous system to the brain by four mechanisms:

- Decreasing the conduction along the nerves running between the nerve endings and the spine
- Preventing production of chemicals which allow signals to pass between the nerves and the spinal cord
- Stimulating production of serotonin (5HT) and noradrenaline which significantly reduces the pain signals reaching the brain
- Mimicking the action of endorphins at their receptor sites

The side effect which is responsible for the national opiate addiction problem is euphoria. Although the mechanism by which opiates produce a sense of euphoria is not yet agreed, it is thought to be partly *via* action on part of the brain called the locus cerulus, which is known to have a high concentration of opioid receptors.[114]

3.5 Metabolism

Diamorphine is the active ingredient of Heroin. Post-administration, it is readily absorbed and rapidly hydrolysed to 6-MAM in blood, then more slowly metabolised to morphine which is the major active metabolite. Normorphine is also formed to a lesser extent. All metabolites may be conjugated with glucuronic acid to form the glucuronide derivatives. Morphine itself undergoes considerable first pass metabolism and is widely distributed to the kidneys, liver, lungs and spleen. It does not however accumulate in the tissues. Figure 3.2 shows the major metabolic pathway of diamorphine.

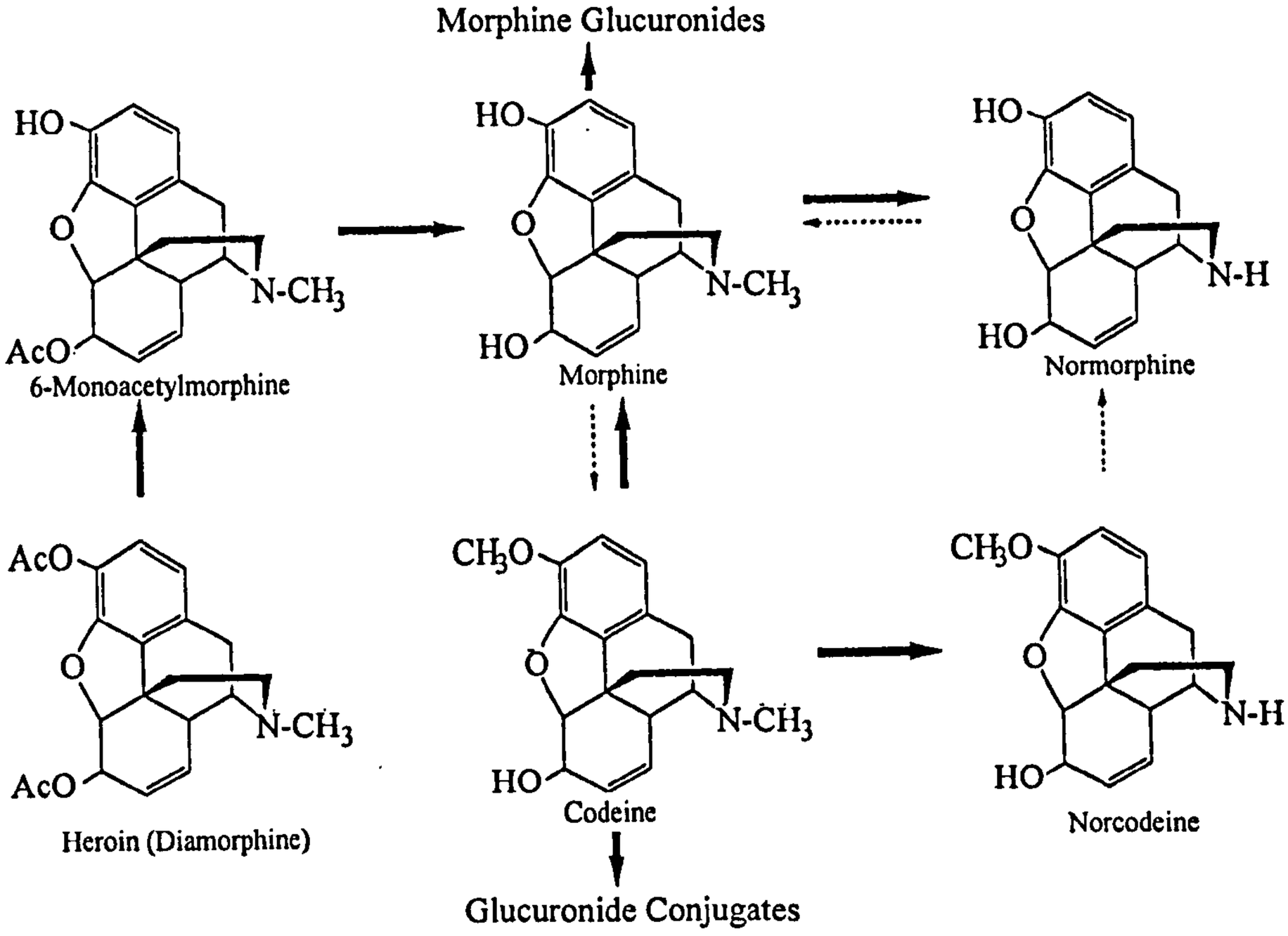


Figure 3.2: *Metabolic Pathway of Diamorphine*

3.6 PHARMACOKINETICS

Due to the short half lives of diamorphine and 6MAM, there is a lack of pharmacokinetic data on these drugs. The information available from Clarkes Identification of Drugs[115] is summarised in Table 3.3. As can be seen other than the half lives of diamorphine, and 6MAM, no pharmacokinetic data was available and the volume of distribution (V_D), clearance (Cl) and protein binding quoted for these drugs refer to morphine.

Table 3.3: *Pharmacokinetic Data for Opiates*

Drug	Half Life	V_D (L/kg)	Cl (ml/min/kg plasma)	Protein binding
Diamorphine	~3 min	-	-	-
6-MAM	~30 min	-	-	-
Morphine	2-3 hr	3-5	15-20	20-30%

Diamorphine is difficult to detect in plasma due to its rapid hydrolysis to 6MAM. An intravenous dose of 4-5 mg declines to a blood concentration of 0.01 mg/L within 10 minutes. Therapeutic morphine plasma concentrations are usually in the range 0.01-0.07 mg/L. The estimated LD_{50} for diamorphine in man is 200 mg however, addicts can tolerate up to 10 times this amount and fatalities have been known after only 10 mg.

3.7 Legal Status

Opium is one of the few drugs in the Misuse of Drugs Act which in its raw form, is not authorised for medicinal use and can only be supplied, possessed or administered in accordance with a Home Office Licence. It is also an offence to frequent a place used for smoking opium, to posses utensils used for smoking and preparing opium or to allow premises to be used for smoking or preparing opium.

Dextromoramide, dipipanone, fentanyl, heroin, methadone, morphine, opium and pethidine are all Class A drugs. Codeine and dihydrocodeine are Class B drugs unless prepared for injection in which case they are Class A drugs. Dextropropoxyphene and buprenorphine are Class C drugs. Dilute mixtures of codeine, morphine or opium used for the treatment of coughs or diarrhoea are available over the counter from pharmacies.

3.8 Analysis of Morphine

In reviewing the available literature on morphine analysis it was found that the tissue of choice for analysis was plasma. Here morphine is present in unconjugated form and there is little else in the matrix likely to interfere with the isolation of the drug, although up to 30% plasma protein binding is reported. However, plasma is seldom available in most postmortem cases and the tissue most commonly supplied is haemolysed, often putrefied, blood.

Direct analysis of body fluids and tissues is impractical and usually some form of cleanup or pre-concentration is required before chromatographic methods can be applied. RIA can be performed on unprepared samples but a simple extraction into MeOH is usually carried

out to eliminate matrix interference and increase sensitivity. Methods which have been used for the preparation of samples to be analysed for morphine include:

1. Protein precipitation, ultrafiltration and centrifugation (Curry, 1969)[116]
2. Liquid/liquid extraction (Jones *et al*, 1985[117] - urine: GC/MS; Basett, 1980[118] - plasma: GC-ECD; Todd *et al*, 1982[119] plasma/CSF: LC-EC; White, 1979[120] - blood: LC-EC)
3. SPE (Mule, 1974[121] urine: GC, Moore *et al*, 1984[122] - plasma: LC-ED; Fritschi *et al*, 1985[123] urine: GC/MS/SIM; Derko *et al*,[124] - urine: LC-UV)

In terms of the analysis of opiates, the method of choice has to be GC/MS due to its sensitivity and selectivity *via* the use of SIM. The use of GC/MS for opiates is well documented and recently a simultaneous method for the determination of acetylcodeine, monoacetylmorphine and other opiates in urine was reported by O'Neal and Poklis.[125] Other popular methods for the analysis of opiates are summarised in Table 3.4.

Table 3.4: *Opiate Analysis Methods*

Year	Analysis Technique	Extraction Type	Sample Type	Sample Volume	Derivatisation Required
1972[126]	HPLC-UV	n/n	n/n	n/n	No
1984[127]	HPLC-ED	n/n	Serum	n/n	n/n
1984[117]	GC/MS	n/n	n/n	n/n	Yes
1991[128]	GC	n/n	Blood/ Bile	n/n	Yes
1993[129]	GC/MS	On-line dialysis	Plasma/ Blood	n/n	Yes
1994[130]	GC/MS	SPE	Urine	0.5 ml	Yes
1994[131]	GC-FID	n/n	Blood stains	n/a	No
1995[132]	GC/MS	SPE	Saliva/ Blood/ Serum	n/n	Yes
1995[133]	Review	various	various	various	-
1995[134]	HPLC	SPE	Serum	200 µl	No

3.9 SFE Analysis of Morphine

The super/sub critical analysis of opiates has been reported by several authors.[135,136] Janicot *et al*[135] used CO_2 with an amine polar modifier for the successful separation of opium alkaloids from poppy straw extracts and Edder *et al*[136] reported the use of SF-CO_2 with a

polar modifier for the extraction of opiates from biological matrices. Indeed this second method was used as a starting point for the SFE of morphine as described in section 3.13.

3.10 Status of Cases

Figure 3.3 shows the number of toxicology cases in which morphine was found over the time period 1992 to 1997. It should be noted that this number will include therapeutically administered morphine as well as morphine arising from heroin related deaths.

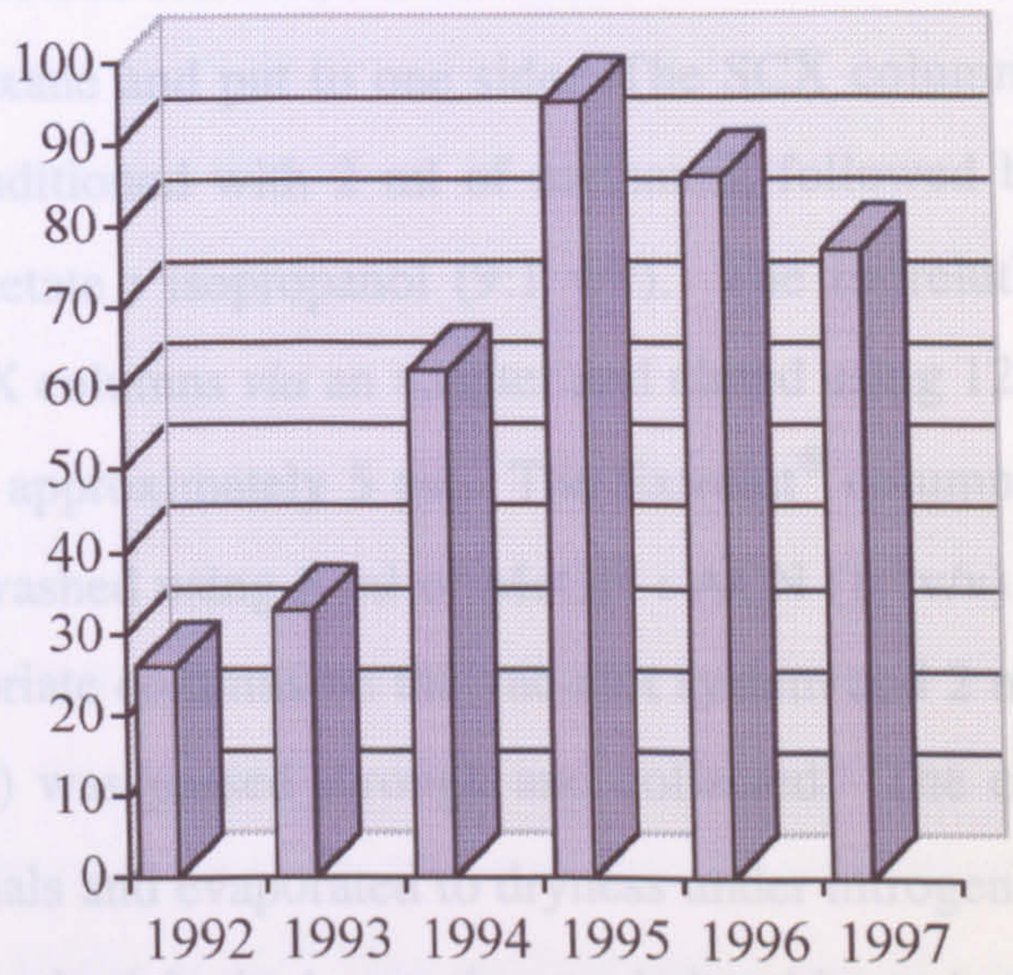


Figure 3.3: *Morphine Related Deaths*

It can be seen from this figure that over the period 1992 to 1995, there was a sharp increase in the number of morphine fatalities whereas from 1995-1997, the morphine related deaths appear to be declining in number. One explanation for this is the introduction of the methadone maintenance programmes. This will be discussed in more detail in the next chapter.

3.11 Current Methodology

At the time of this work the method of extraction employed in the routine laboratory made use of solid phase extraction as described below.

Samples and blanks were prepared by pipetting 1 ml of 0.1M ammonia buffer, 1 ml of sample or blank blood and 50 µl of d_3-morphine (1µg/ml) into a vial. In the case of standards, 50 µl of morphine (1µg/ml) was also added. Two extraction columns were required for each extraction. The first was prepared by plugging a small amount of glass wool into a 10 ml plastic syringe and filling to the 10 ml mark with EtOH / MeOH washed Extrelut®. The other extraction columns were Bond Elut SCX. In each case the extraction columns were labelled with the appropriate sample number.

The contents of the sample, standard and blank vials were pipetted onto the corresponding Extrelut® column and allowed to stand for 10 minutes. The Extrelut® columns were then washed with 5 ml of hexane and put to one side. The SCX columns were placed on the vac-elut system and conditioned with 2 ml of methanol, followed by 2 ml of water and finally 1 ml of ethyl acetate : isopropanol (9:1 v/v). The Extrelut® columns were then placed on top of the SCX columns *via* an adapter and eluted using 12 ml of EtAc: IPA (9:1 v/v) under a vacuum of approximately 5 psi. The Extrelut® columns were then discarded and the SCX columns washed using 5 ml of MeOH : ACN (1:1v/v). Labelled tubes were placed under the appropriate columns on the vac-elut system and 2 ml of 10% ammonia in MeOH : ACN (1:1 v/v) was passed through and collected. The collected extracts were transferred into small vials and evaporated to dryness under nitrogen. 50µl of BSTFA (1% TMCS) were added to each vial which was then sealed and heated at 90 °C for 15 minutes. For GC-MS analysis, 1 µl of the derivatised extract was injected.

3.12 GC/MS Methodology

The GC/MS for both morphine and methadone (chapter 4) was carried out using a Fisons GC 8000 series gas chromatograph with a MD 800 mass spectrometer. The GC was fitted with a HP1 capillary column (30 m x 0.25 mm x 0.25 µm film thickness).

For the analysis of morphine and 6MAM, the temperature programme was as follows:

Initial Temperature: 100 °C

Final Temperature: 300 °C

Temperature Ramp: 10 °C/min

Final hold Time: 5 minutes

Injector Temperature: 225 °C

The detector was run in the selected ion monitoring mode (SIR) and the mass to charge ratios (m/z) detected were 414 and 429 for morphine and 432 for d_3-morphine. The 6MAM was investigated at a later stage and the m/z which were used are discussed in section 3.14.

It should be noted that morphine peaks at m/z : 414 and m/z : 429 are only defined as being positive when the m/z ratio of 414/429 is between 0.4 and 0.85. The value obtained is usually close to 0.5, however alterations in the tuning of the instrument give some variation.

3.13 Experimental

Initially, a literature review on previous methodology was carried out. Several papers were noted to use CO_2 as an extractant for the quantitation of opiates as previously mentioned.[136,137] The first of these to be reviewed was the subcritical extraction method for morphine, published by Edder *et al.*[29] This method makes use of a ternary phase extractant which at the low temperatures used will be sub-critical and not supercritical. The conditions used for the extraction were as follows:

- Support medium: C_{18} silica
- Extraction phase: CO_2 : MeOH : Et_3N (90 : 8.5 : 1.5 (v/v))
- Pressure: 25 MPa (3625 psi)
- Temperature: 40 °C
- Time: 30 minutes
- Flowrate: 0.5 ml min^{-1}

3.13.1 Sub-Critical Fluid Extraction of Morphine

3.13.1.1 Experimental

Four extractions of morphine and d_3-morphine in MeOH were carried out using a variety of C_{18} supports. In each case, 100 µl of morphine and d_3-morphine (10 mg/L) were pipetted onto a C_{18} support and allowed to dry at room temperature. In addition to the MeOH extracts, an additional extraction (1/1) was carried out using a blood spiked standard (10 mg/L). Again 100 µl of the standard containing morphine and d_3-morphine were loaded onto the C_{18} support.

In their paper, Edder *et al*[136] suggest that a small amount of water added to the extraction vessel may aid the extraction and thus 10 µl of dH_2O was added to two of the four MeOH extracts. A further two blood standard extractions (2/1, 2/2)were carried out the following day, this time, one (2/2) had 10 µl of dH_2O added.

The actual conditions used were slightly modified from the original conditions used by Edder *et al*[136] as follows:

- Extraction phase: CO_2 : MeOH : Et_3N (90 : 8.5 : 1.5 (v/v))
- Pressure: 3500 psi
- Temperature: 45 °C
- Time: 30 minutes
- Flowrate: 1.0 ml min^{-1}

3.13.1.2 Results and Discussion

The extraction support phase was varied as only limited amounts of C_{18} from any one supplier was available. The phases used are detailed below.

MeOH Extraction 1: Shandon 5µ ODS

MeOH Extraction 2: As for Extraction 1 (10 µl dH_2O added)

MeOH Extraction 3: Spherisorb C_{18}

MeOH Extraction 4: Glass Appliances 5µ ODS

Blood Extractions: Hypersil ODS

The results obtained for the four MeOH extractions are shown in Table 3.5. Area based % recoveries were calculated for each of the monitored ions.

Table 3.5: *Morphine MeOH Extractions : Modification of method by Edder et al*

	Area % m/z:432	Area % m/z:429	Area % m/z:414	m/z ratio 414/429	m/z ratio 429/432
Area Unext.	*64431052*	*84183768*	*47162980*	0.560	1.307
Ext. 1	77	70	67	0.536	1.178
Ext. 2	180	147	145	0.553	1.064
Ext. 3	31	27	27	0.552	1.145
Ext. 4	42	30	30	0.570	0.926

The results obtained for the blood extractions on days one and two are shown in Table 3.6.

Table 3.6: *Morphine Blood Extractions*

	Area % m/z:432	Area % m/z:429	Area % m/z:414	m/z ratio 414/429	m/z ratio 429/432
Day One					
Ext. 1/1	176	172	151	0.529	1.243
Area Unext.	*64431052*	*84183768*	*47162980*	0.560	1.307
Day Two					
Ext. 2/1	348	314	400	0.576	1.077
Ext. 2/2	618	619	707	0.518	1.193
Area Unext.	*37501364*	*44641888*	*20241124*	0.453	1.190

Although in both cases (blood and MeOH) the % area recoveries vary widely from extraction to extraction, good comparison with the unextracted standard in terms of the 429/432 ratio is observed. Some of the variation in the areas can be attributed to the derivatisation procedure as highlighted in section 3.13.7.

3.13.2 EXTRACTION AT VARIOUS VOLUMES OF MORPHINE/D_3-MORPHINE

3.13.2.1 EXPERIMENTAL

The same conditions which were applied in section 3.13.1, were used for the extraction at various volumes of morphine and d_3-morphine from 0 to 1.0 ml. Both extracted and unextracted MeOH standards were run through the GC/MS.

3.13.2.2 RESULTS AND DISCUSSION

The results obtained for the subcritical extraction of various volumes of morphine and d_3-morphine are shown in Table 3.7.

The volumes of standard used cover a range from 1.1 to 11.0 mg/L of morphine hydrochloride. The case samples which have been received for analysis by SPE to date are in the concentration range 0.03 to 1.1 mg/L by SPE followed by GC/MS, thus a ten-fold dilution of these samples will have to be carried out in order to check the linearity over this range.

Table 3.7: *Subcritical Extraction at Various Volumes of Morphine and d_3-Morphine*

	Unextracted m/z ratio		Extracted m/z ratio		Apparent
Vol. (ml)	429/432	414/429	429/432	414/429	Conc^n
0	0.0033	13.89	0.0044	10.42	-ve
0.1	0.9298	0.582	1.1176	0.574	1.32
0.2	1.0457	0.597	1.2016	0.570	1.26
0.3	1.0337	0.628	1.1390	0.575	1.21
0.4	1.0116	0.632	1.1340	0.577	1.23
0.5	1.0578	0.602	Extract solidified		
0.75	1.0576	0.795	1.0475	0.551	1.19
1.0	1.0130	0.795	0.9504	0.678	1.03
Mean 429/432	1.021		1.098		
RSD 429/432	4.4%		7.9%		

As the volumes of morphine and d_3-morphine being analysed were the same, the m/z 429:432 ratio should remain constant. From the results obtained in this and the previous sections, it is noticeable that the 429/432 m/z ratio of morphine for unextracted standards varies from 0.93 to 1.44 on a day to day basis. For this reason it is essential that where possible, an unextracted standard is run on the same day as the extracted standards.

3.13.3 LINEARITY OF MORPHINE EXTRACTION

3.13.3.1 EXPERIMENTAL

An 11 mg/L morphine hydrochloride standard solution was diluted 1:10 to give a stock concentration of 1.1 mg/L. In real samples, the morphine being analysed will not be as the hydrochloride, therefore the concentrations were adjusted for morphine only, to give a stock solution concentration of 0.98 mg/L. Aliquots of this solution were pipetted into a vial with 200 µl of d_3-morphine, blown to dryness under a stream of nitrogen, reconstituted in 50 µl of BSTFA (1% TMCS) and derivatised for 15 minutes at 90 °C. The same volumes of morphine and d_3-morphine were also extracted by pipetting the appropriate volumes of morphine and d_3-morphine onto C_{18}, drying, extracting and derivatising as detailed above. The derivatised samples were then injected onto the GC/MS.

3.13.3.2 RESULTS AND DISCUSSION

The results for various concentration of morphine at constant d_3-morphine concentration are shown in Table 3.8.

Table 3.8: *Morphine Linearity over the range 0.049 to 0.98 mg/L*

		Unextracted m/z ratio		Extracted m/z ratio	
Vol. (µl)	Conc^n	429/432	414/429	429/432	414/429
0	0	0	0	0	0
50	0.049	0.256	0.702	0.224	0.681
100	0.098	0.553	0.581	0.475	0.589
200	0.196	1.144	0.535	0.957	0.632
300	0.294	1.698	0.519	1.567	0.559
400	0.392	2.310	0.513	2.354	0.521
500	0.490	2.754	0.504	2.962	0.498
600	0.588	3.216	0.504	3.186	0.558
700	0.686	3.620	0.501	3.589	0.603
800	0.784	4.036	0.501	3.964	0.540
900	0.882	4.590	0.497	4.479	0.503
1000	0.980	4.932	0.498	4.877	0.536

For the unextracted standards: r^2: 0.995, A: 0.165, B: 5.019.

For the extracted standards: r^2: 0.987, A: 0.110, B: 5.041.

The morphine m/z 414 and m/z 429 peaks for the 0.049 mg/L standard were still easily detectable by GC/MS as shown in Figure 3.4.

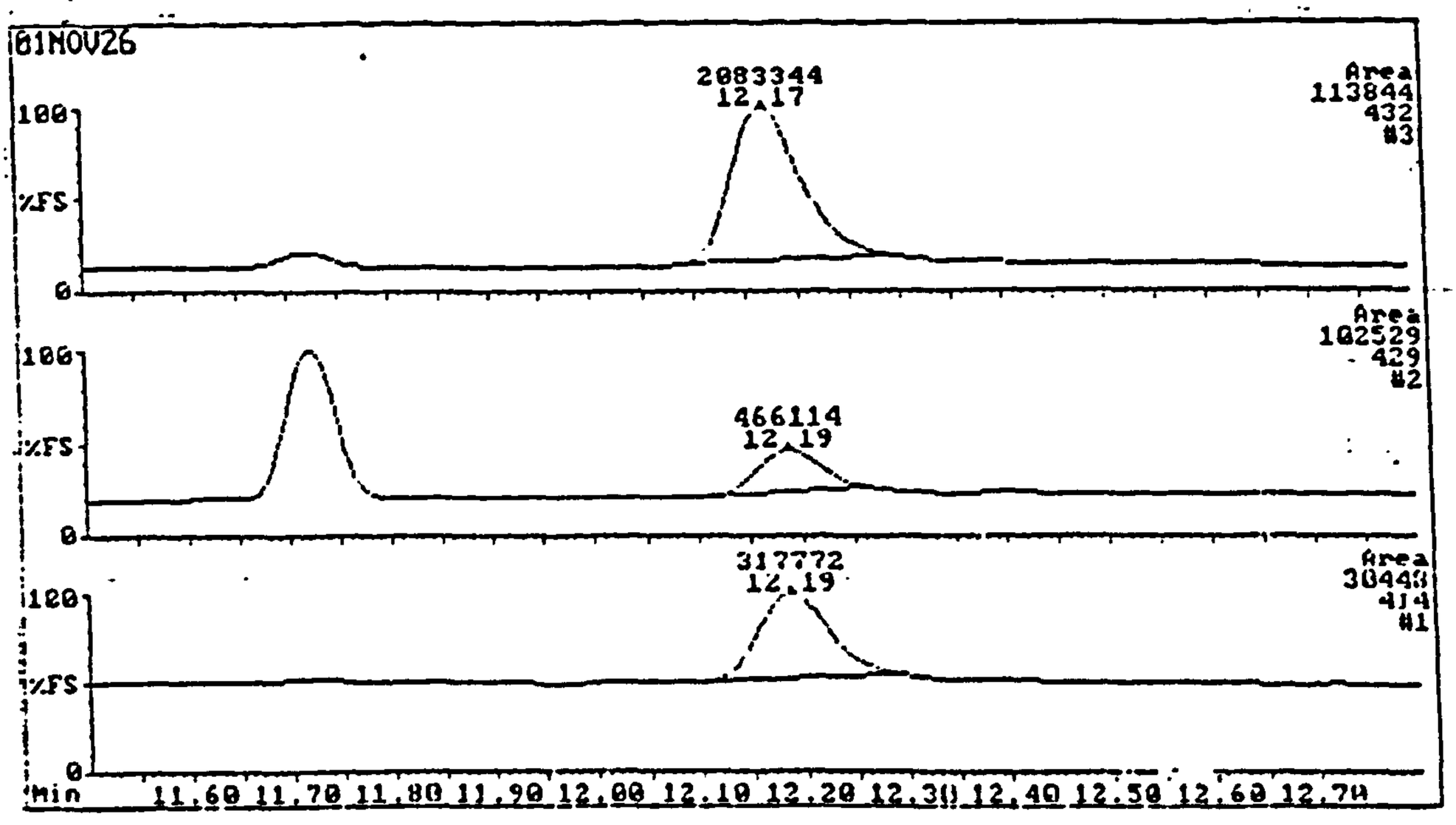


Figure 3.4: *Mass Chromatogram of 0.049 mg/L Morphine*

3.13.4 Supercritical Fluid Extraction Method

3.13.4.1 Experimental

An additional method for the supercritical fluid extraction of morphine was investigated.[137] This method was designed for the extraction of hair samples but it was attempted in a modified form for the extraction of morphine from MeOH and blood standards. The original method uses the following conditions:

- Temperature: 100 °C
- Flowrate: 2.0 ml min^{-1}
- Pressure: 25 Mpa (3625 psi)
- Modifier: MeOH:Et_3N:H_2O (2:2:1 v/v)

The conditions were maintained with the exception of the modifier which was kept as the MeOH : Et_3N used in the initial extraction method by Edder *et al.*[136]

Fresh standards of morphine and d_3-morphine were prepared in methanol as follows:

Morphine: 1.35 mg of morphine hydrochloride was weighed into a 100 ml volumetric flask and made up to the mark with methanol. As the morphine is as the hydrochloride, the actual concentration of morphine → 1.20 mg/100 ml

d_3-morphine: 1.01 mg of d_3-morphine were weighed into a volumetric flask and prepared as above.

For d_3-morphine the absolute concentration does not have to be calculated. It is only important that the concentration is approximately the same as the morphine concentrations being investigated and that it is kept constant for all samples and standards.

An extraction calibration curve with these standards diluted 1:10 was carried out. 1000 µL of d_3-morphine was added for each extraction.

3.13.4.2 Results and Discussion

The results for the calibration using the new extracted standards are shown in Table 3.9 and Figure 3.5.

Table 3.9: *Calibration with New Standards*

Volume added (µl)	Conc^n (mg/L)	429/432 m/z Ratio	414/429 m/z Ratio
0	0	$4.5*10^{-3}$	10.31
100	0.12	0.202	0.879
200	0.24	0.387	0.826
300	0.36	0.550	0.838
400	0.48	0.879	0.681
500	0.60	0.957	0.806
750	0.90	1.337	0.819
1000	1.20	1.762	0.768

r^2: 0.989
A: 0.070
B: 1.432

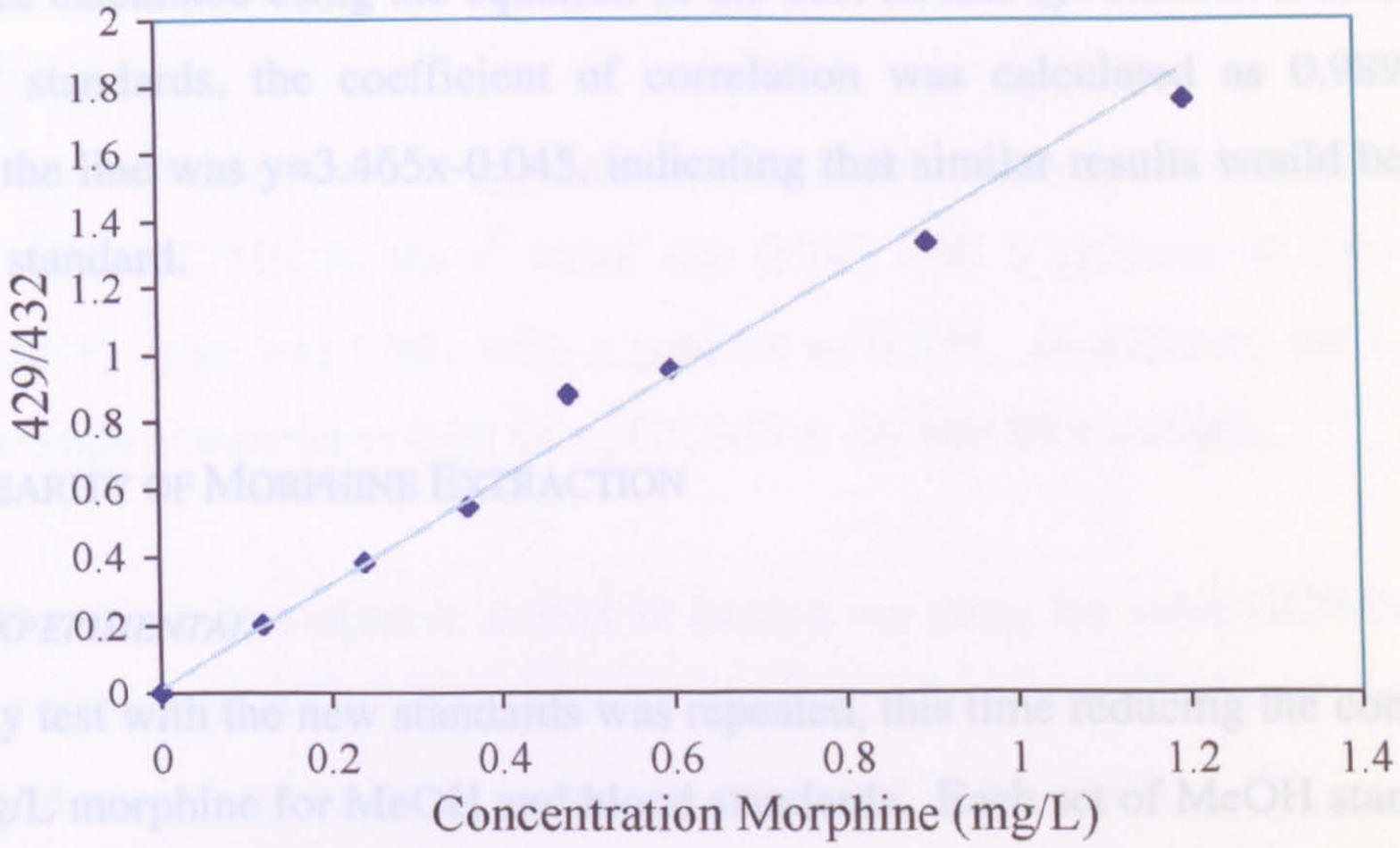


Figure 3.5: *Linearity of Morphine over the Range 0.12-1.20 mg/L*

The new standards yielded a better correlation coefficient for the extraction. The gradients of the two calibrations could not be directly compared as the amount of internal standard added was different (1000 µl *c.f.* 200 µl). In order to check that the two sets of standards were however providing reproducible results, the PAR's were plotted against each other as shown in Figure 3.6.

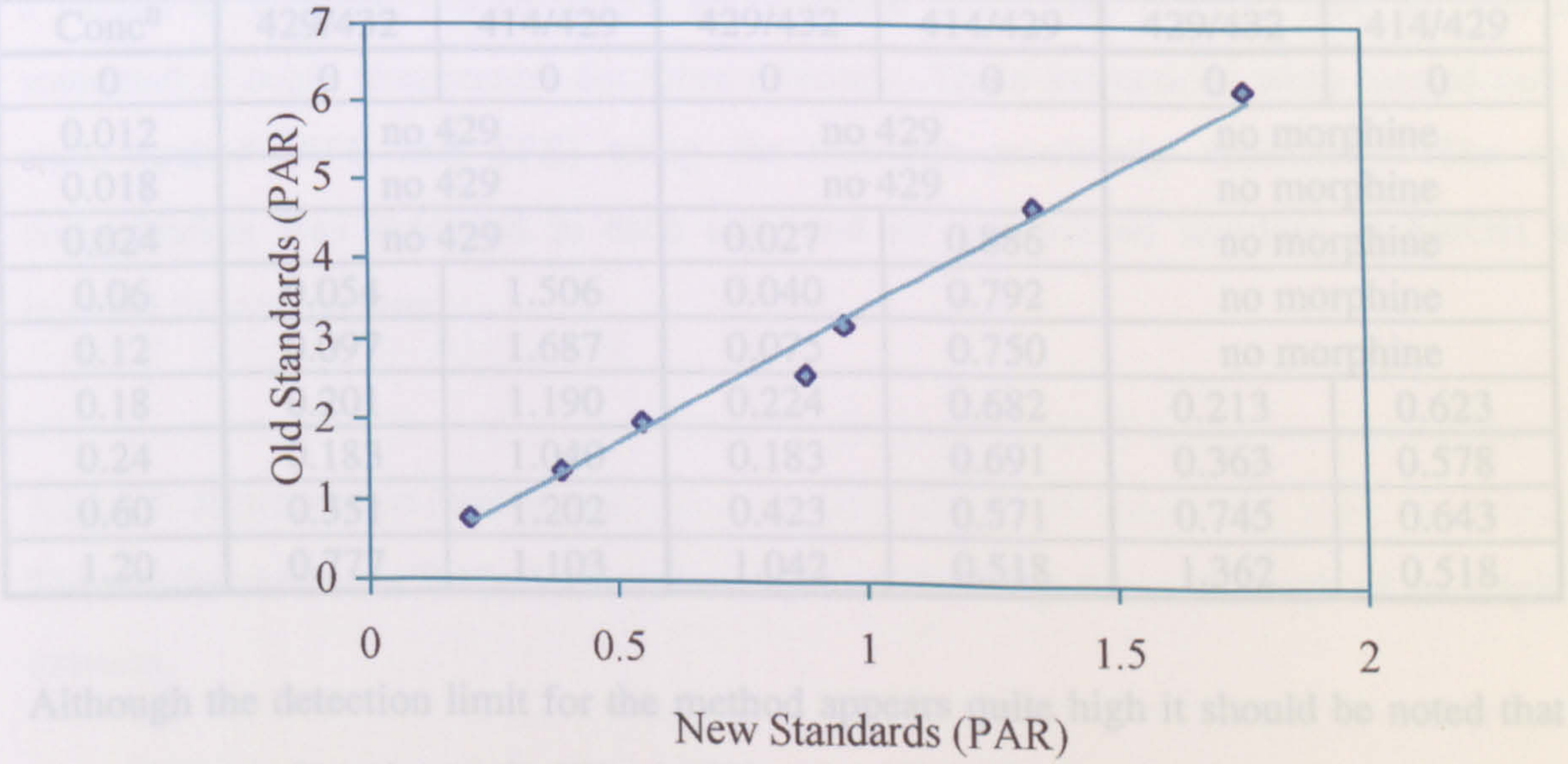


Figure 3.6: *Comparison of Old and New Morphine Standards*

As the concentrations were different for the two sets of standards, the points for the old standard were calculated using the equation of the best fit line (y=5.019x+0.165). For the two sets of standards, the coefficient of correlation was calculated as 0.989 and the equation of the line was y=3.465x-0.045, indicating that similar results would be obtained using either standard.

3.13.5 Linearity of Morphine Extraction

3.13.5.1 Experimental

The linearity test with the new standards was repeated, this time reducing the concentration to 0.012 mg/L morphine for MeOH and blood standards. Each set of MeOH standards was injected into both GC/MS's to ensure that both instruments were producing the same results.

3.13.5.2 Results and Discussion

The results obtained for the MeOH extractions on both GC/MS's and for the blood standards are shown in Table 3.10.

Table 3.10: *Linearity After One Hundred-Fold Dilution of Standards*

	GC/MS A: MeOH		GC/MS B: MeOH		Blood	
Conc[n]	429/432	414/429	429/432	414/429	429/432	414/429
0	0	0	0	0	0	0
0.012	no 429		no 429		no morphine	
0.018	no 429		no 429		no morphine	
0.024	no 429		0.027	0.886	no morphine	
0.06	0.054	1.506	0.040	0.792	no morphine	
0.12	0.097	1.687	0.075	0.750	no morphine	
0.18	0.201	1.190	0.224	0.682	0.213	0.623
0.24	0.183	1.040	0.183	0.691	0.363	0.578
0.60	0.351	1.202	0.423	0.571	0.745	0.643
1.20	0.777	1.103	1.042	0.518	1.362	0.518

Although the detection limit for the method appears quite high it should be noted that the extractions are based on only 100 µl of blood or MeOH being extracted instead of the 1 ml

used in SPE. Thus, if for example 500 µl were extracted, the detection limit should be decreased by a factor of five.

From the results in Table 3.10 it is apparent that the results from the two GC/MS's are not comparable. For GC/MS A, the r^2 value was 0.983 with a gradient of 0.604 and for GC/MS B, the r^2 value was 0.981 with a gradient of 0.839. In addition, the LOD using GC/MS B is 0.024 compared to 0.06 using GC/MS A for MeOH standards.

In theory, all method development should be carried out using the same GC/MS, however this was not always possible. The linearity (indicated by r^2)and limits of detection are however, good enough for use with the samples involved in this study.

3.13.6 Comparison of SFE and SPE

3.13.6.1 Experimental

A comparison between SFE and SPE was made to check that the same results were achieved for the extraction of blood using the two methods.

A stock blood standard was prepared by pipetting 5 ml of 1.2 mg/L morphine and 5 ml of the 1:10 diluted d_3-morphine internal standard into a vial and evaporating to dryness under a stream of nitrogen. Once dry, 5 ml of blank blood was pipetted into the vial and sonicated at room temperature for three minutes. Three extractions were carried out for each method (SFE and SPE) using the methods previously described. The same concentration was extracted in each case and an unextracted standard in MeOH also injected for comparison.

3.13.6.2 Results and Discussion

The results shown in Table 3.11 are averages of three injections for each of the triplicate extracts.

Table 3.11: *Comparison of SFE and SPE for the Extraction of Morphine from Whole Blood*

	m/z ratio 429/432	m/z ratio 414/429	
Unext. Std.	1.186	0.549	
SFE 1	1.124	0.555	x=1.137±0.01
SFE 2	1.152	0.554	RSD=1.24%
SFE 3	1.136	0.554	
SPE 1	1.292	0.561	x=1.218±0.09
SPE 2	1.242	0.549	RSD=7.24%
SPE 3	1.121	0.535	

SFE + unextracted: x=1.150±0.03, RSD=2.34%

SPE + unextracted: x=1.210±0.07, RSD=6.08%

Both methods were found to produce similar results with SFE yielding slightly better reproducibility.

3.13.7 Effect of Derivatisation Time

3.13.7.1 Experimental

The effect of derivatisation time on the areas of morphine and d_3-morphine was investigated. An unextracted standard was prepared by pipetting 100 µl of both morphine and d_3-morphine into a vial, evaporating to dryness and adding 50 µl of BSTFA (1% TMCS). After 15 minutes of derivatisation at 90 °C, an injection of the standard was made onto the GC/MS. The same standard was reinjected after a total derivatisation time of 1 hour.

3.13.7.2 Results and Discussion

The results for the effect of derivatisation on the peak areas of morphine and d_3-morphine are shown in Table 3.12. The last row shows the relative % increase in peak area obtained by increasing the derivatisation time from 15 minutes to 1 hour.

Table 3.12: *Effect of Derivatisation Time*

Time	m/z 432	m/z 429	m/z 414	m/z ratio 429/432	m/z ratio 414/429
15 min.	2696164	3090318	1607642	1.146	0.520
1 hour	84656264	100363992	58051708	1.186	0.578
1hr/15min	3140%	3248%	3610%		

Although the 414/429 and 429/432 m/z ratios are the same for the two derivatisation times, and thus would yield the same analytical results, an increase of greater than 3000 % is seen simply by leaving the standards derivatising for 1 hour as opposed to the stated 15 minutes. In practice this is not always practical as it is time consuming and in some cases, the samples were found to dry in the vials and thus, had to be reconstituted before injection. This step incurs a decrease in the recovery of the samples.

3.13.8 Conclusions on Method To Use

As both of the methods investigated yielded good results for the extraction of both MeOH standards and blood standards, it was decided to use the supercritical method as opposed to the subcritical method. The final conditions used for the extraction are detailed below:

Extractant Fluid: CO_2:MeOH:Et_3N (90:8.5:1.5)

Pressure: 3500 psi

Temperature: 100 °C

Support Medium: EtOH:MeOH washed Extrelut®

Flow Rate: 2.0 ml min^{-1}

Extraction Time: 20-30 minutes

Collection Solvent: MeOH

3.14 Introduction of 6MAM into the SFE

As the purpose of this study was to investigate heroin related deaths, it was decided to look at one of the more confirmatory metabolites of diamorphine namely, 6-monoacetylmorphine (6MAM), as a means of determining if the death was heroin or morphine related.

A concentrated solution of 6MAM was prepared by pipetting 5 ml of a 13.3 mg/L solution of 6MAM in MeOH into a vial, evaporating to dryness under N_2 and reconstituting in 50 µl of BSTFA (1% TMCS). This solution was then derivatised for 15 minutes at 90 °C and run full scan on the GC/MS using the morphine GC parameters to determine the best peaks to look for. The mass spectrum of the 6MAM peak is shown in Figure 3.6 and from this the ions m/z 340 and m/z 400 were initially chosen.

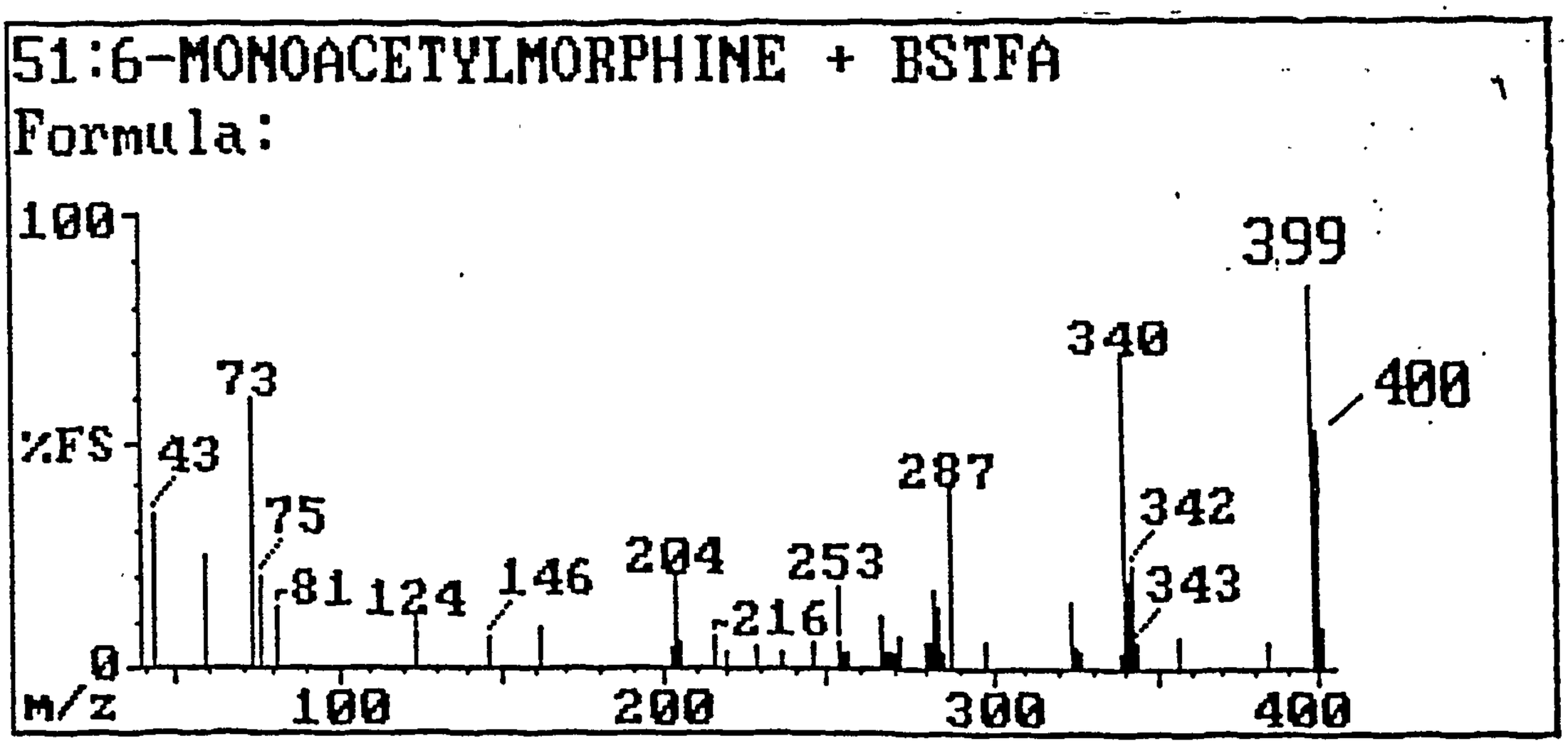


Figure 3.6: *Mass Spectrum of BSTFA Derivatised 6-Monoacetylmorphine*

3.14.1 Determination of m/z 340/400 Ratio for Verification Purposes

3.14.1.1 Experimental

A set of standards at varying concentrations of 6MAM from 0.67-2.66 mg/L were run through the GC/MS in order to determine the ratio of 340:400 for confirmation of positive 6MAM. d_3-morphine was included as an internal standard in order to plot a calibration curve for the concentrations investigated.

3.14.1.2 Results and Discussion

The results obtained are shown in table 3.13.

Table 3.13: *Calculation of 340/400 for 6MAM*

Vol. (µl)	m/z ratio 400/432	m/z ratio 340/400
0	0	0
50	0.135	2.34
100	0.371	2.32
150	0.513	2.28
200	0.686	2.10

r^2: 0.989
A: 0.024
B: 0.270

From this, the m/z ratio of 340/400 was estimated as 2.2.

This experiment was also carried out for extracted standards. However, the samples had to be left in the refrigerator for one week while routine maintenance was carried out on the GC/MS and on analysis, morphine peaks were observed. A second batch of 6MAM standards were extracted to ensure that:

1) The 6MAM was not breaking down to morphine during the extraction
2) The 6MAM was not effecting the extraction of morphine

3.14.2 Stability of 6MAM in the SFE System and its Effect on Morphine

3.14.2.1 Experimental

Two sets of standards were prepared as previously described. The first had the various concentrations of 6MAM and 100 µl of d_3-morphine and the second was as above but also had 100 µL of morphine added to each sample.

3.14.2.2 Results and Discussion

The results for the extractions are shown in Table 3.14.

Table 3.14: *Extraction of 6MAM With and Without Morphine*

	Without Morphine			
Conc^{n} (mg/L)	m/z ratio 340/400	m/z ratio 400/432	m/z ratio 429/432	m/z ratio 414/429
0	-	0	0	-
0.67	Sample dried in vial			
1.33	2.860	0.148	0	-
2.00	2.757	0.340	0	-
2.66	2.792	0.629	0	-
	With Morphine			
0	-	-	0.896	0.555
0.67	2.735	0.169	0.925	0.560
1.33	2.729	0.224	0.990	0.548
2.00	2.699	0.672	0.934	0.553
2.66	2.453	0.996	0.837	0.562

For the three points obtained for the 6-MAM without morphine: r^2: 0.986, A: -0.349, B: 0.361

For the extractions with morphine: r^2: 0.932, A: -0.220, B: 0.441

The presence of 6MAM does not appear to effect the extraction of morphine. In addition, no morphine was observed for the 6MAM and d_3-morphine extractions and thus, the extraction conditions are not causing the 6MAM to degrade to morphine.

3.14.3 Investigation of Morphine Glucuronide

In addition to investigating the 6MAM it was also decided to see whether or not the morphine glucuronide was being broken down to morphine during the SFE process. This degradation could result in artificially high results obtained by SFE compared to the SPE method which does not break down the glucuronide.

3.14.3.1 Experimental

A 200 µl aliquot of a 7.65 mg/L morphine glucuronide sample prepared in water, was loaded onto Extrelut® and extracted. The extract was dried under nitrogen, derivatised and injected onto the GC/MS.

3.14.3.2 Results and Discussion

Peaks for both morphine and d_3-morphine were observed. As no d_3-morphine had been added to the standard at any stage it was assumed that either the glucuronide standard was contaminated or the system was contaminated as had been observed previously for the benzodiazepines. The outlet tubing of the system was changed. The replacement tubing had a narrower internal diameter and thus provided additional restriction at the flow outlet. A second standard of morphine glucuronide from a fresh bottle was also prepared. This time the concentration was 5.76 mg/L

3.14.4 Extraction With Fresh Morphine Glucuronide

3.14.4.1 Experimental

A blank MeOH extraction was carried out to determine if there was any carry over in the remainder of the instrumentation. As this was negative, an extraction of 200 µl of the new morphine glucuronide standard was carried out. No peaks were seen for morphine or d_3-morphine thus, the glucuronide is not being broken down to free morphine by the extraction process and the results obtained from SFE and SPE should be directly comparable.

Several case samples were run to see whether the results which were being obtained were in agreement with those seen by SPE. The results are shown in section 6.4.1.2.

3.14.5 Check of SFE Conditions for the Determination of Morphine and 6MAM

3.14.5.1 Experimental

The following samples were prepared for extraction in order to check for carryover and as a final check on whether or not the 6MAM was being broken down to morphine and/or effecting the morphine results:

A) 6MAM: 0, 50, 100, 150, 200 µl + 100 µl d_3-morphine

B) 6MAM: 0, 50, 100, 150, 200 µl + 100 µl morphine + 100µl d_3-morphine

C) Morphine: 0, 50, 100, 150, 200 µl + 100 µl d_3-morphine.

3.14.5.2 Results and Discussion

The results from these sets of extractions are shown in Table 3.15 on page 132. As can be seen from the results there is significant carryover from extraction to extraction as indicated in bold. The system was again flushed out and an extraction of morphine and d_3-morphine was carried out successfully with no carryover 6MAM peaks.

In addition to flushing out the system, the outlet tubing was further investigated. The narrower bore tubing was not providing a sufficient restriction (which is indicated by the collection solution becoming cold). In order to achieve better restriction, it would be ideal to use GC capillary tubing as a restrictor. GC capillary tubing was housed inside a piece of stainless steel tubing and connected to the flow outlet. By using this method, it was easier to change the outlet tubing more frequently thus decreasing any contamination risk.

Table 3.15: *Extraction of Various Combinations of 6MAM, Morphine and d_3-Morphine*

Extract	m/z 340	m/z 400	Area m/z 432	Area m/z 429	Area m/z 414	m/z ratio 340/400	m/z ratio 400/432	m/z ratio 414/429	m/z ratio 429/432
Unext.	549321392	204081856	397746368	898508672	491006304	2.692	0.513	0.546	2.260
0A	3621994	1380645	41852044	**10536567**	**7218538**	2.623	0.033	**0.615**	**0.404**
50A	163448528	5794532	230482192	**25775954**	**23153804**	2.819	0.252	**0.685**	**0.252**
100A	30327132	11225196	35464572	**5296166**	**4167398**	2.702	0.317	**0.898**	**0.118**
150A	6040512	2203347	5472887	**2118614**	**1303325**	2.735	0.404	**0.787**	**0.153**
200A	30783052	1144720	21981780	**16529545**	**9341030**	2.762	0.507	**0.565**	**0.752**
0B	28778162	10498986	20302116	14252570	8287954	2.741	0.517	0.582	0.702
50B	341517408	128641472	268836000	220847232	124334520	2.655	0.479	0.563	0.821
100B	33823100	12904303	24311370	20363722	11423322	2.621	0.531	0.561	0.838
150B	839424	2366091	1266142	1370765	767017	2.819	0.663	0.560	1.083
200B	0	0	1103286	607415	363341	-	-	0.598	0.551
0C	**90431**	**29543**	1399696	751755	457306	**3.061**	0.021	0.608	0.537
50C	0	0	1256084	832716	485113	-	-	0.589	0.656
100C	**165382**	**64259**	462882	588471	332792	**2.574**	0.139	0.566	1.271
150C	**163436**	**488908**	1065298	1591055	878746	**3.34**	0.459	0.552	1.494
200C	**2460586**	**911905**	12521506	27181132	14803769	**2.700**	0.072	0.545	2.17

3.14.6 INVESTIGATION OF M/Z 399 PEAK FOR VERIFICATION PURPOSES

3.14.6.1 EXPERIMENTAL

In addition to the m/z 400 peak, the 6MAM also has a m/z 399 peak which is stronger than the m/z 400 peak. As opposed to the m/z 340 and m/z 400 being used for the quantitation of 6MAM, the m/z 399 and m/z 340 peaks were investigated. The following mixes of standards were analysed by GC/MS. Both unextracted and extracted standards were injected.

A) 6MAM only

B) 6MAM + d_3-morphine

C) Morphine glucuronide only

D) Morphine glucuronide + d_3-morphine

E) Morphine only

F) Morphine + d_3-morphine

3.14.6.2 RESULTS AND DISCUSSION

The results obtained for the six standard mixes are shown in Table 3.16.

Table 3.16: *Unextracted and Extracted Standard Mixes*

Unextracted									
Run	m/z 432	m/z 429	m/z 414	m/z 399	m/z 340	429/ 432	414/ 429	340/ 399	340/ 432
A	-	-	-	161349	141142	-	-	0.875	-
B	599159	-	26044	157900	147965	-	-	0.937	0.247
C	-	-	-	-	-	-	-	-	-
D	38703	-	-	-	-	-	-	-	-
E	8632	293200	137351	-	-	33.97	0.468	-	-
F	427511	345231	182786	-	-	0.808	0.529	-	-
Extracted									
A	-	-	-	-	-	-	-	-	-
B	31013	-	-	17501	16879	-	-	0.964	0.544
C	-	8279	8603	-	-	-	1.039	-	-
D	7228	13359	7163	-	-	1.848	1.284	-	-
E	-	9166	17840	-	-	-	0.855	-	-
F	13317	15139	6208	-	-	1.137	0.410	-	-

From these results it appears that the contamination problems have been reduced. The only carry over peak appears to be in the unextracted morphine run. d_3-morphine is observed at a low concentration. The extracted 6MAM yielded no peaks, however a high pressure blockage occurred during the extraction and thus some sample may have been lost. Peaks were seen at 414 and 429 for both glucuronide extracts. However, the ratio of the peaks were found to be 1.039 and 1.284 which means that they are not due to morphine. No corresponding peaks were seen in the unextracted glucuronide.

3.14.7 LINEARITY AND LOD FOR 6MAM

3.14.7.1 EXPERIMENTAL

The linearity and limit of detection were calculated for 6MAM. The initial stock standard had a concentration of 1.33 mg/100 ml. This was diluted 1:10 with methanol and this solution (1.33 mg/L) was used as the starting concentration for the linearity with 100 µL of d_3-morphine. Serial dilutions of this solution were made down to a concentration of 0.665 µg/L.

3.14.7.2 RESULTS AND DISCUSSION

The results obtained for the linearity of 6MAM are given in Table 3.17.

Table 3.17: *Linearity and Limit of Detection for 6MAM*

6-MAM (mg/L)	m/z ratio 340/432	m/z ratio 340/399
0.000665	-	-
0.00133	-	-
0.00655	0.00142	0.323
0.0133	0.00293	0.587
0.0655	0.0113	0.606
0.133	0.0282	0.627
0.655	0.1084	0.641
1.33	0.421	0.599

From these results the limit of detection (where no 399 ion is present) was taken as 1.33 ng/ml and the limit of quantitation as 6.65 ng/ml. The range of linearity is from 6.65 - 665 ng/ml ($r^2 = 0.999$, $y = 0.157 x + 0.003$, (y=Bx+A))

3.15 Conclusions

From the work carried out in this section the conditions chosen for the extraction of morphine and 6-monoacetylmorphine were as follows:

Support medium:	Extrelut®
Extractant Phase:	CO_2 : MeOH : Et_3N (90:8.5:1.5)
Temperature:	100 °C
Pressure:	3500 psi
Flowrate:	2.0 ml min^{-1}
Extraction Time:	30 minutes
Collection Solvent:	MeOH
Analysis Technique:	GC/MS (SIM)
Derivatisation:	BSTFA (1%TMCS) at 90 °C for 15 minutes

The samples were prepared as for the benzodiazepines by loading 100 µl of the samples onto Extrelut® and drying. The method was found to be reproducible and yield high recoveries. Again, the solvent usage was reduced, this time the total volume required per extraction was 6 ml (*c.f.* 27 ml for the SPE method). The results of case samples analysed using this methodology are given in Chapter 6.

"The difficulty lies not in the use of a bad thing but in the abuse of a very good thing."

Abraham Lincoln (1809-65)

4. METHADONE

4.1 INTRODUCTION

The discovery of methadone has been attributed to two German scientists Max Bockmühl and Gustav Ehrhart.[138] The drug was first created in 1937 and was initially given the name Hoechst 10820 which was later changed to polamidon. The patent application for methadone was filed in September 1941. Although, the new compounds' structure had no resemblance to morphine, its analgesic and spasmolytic properties were ascertained in 1942 when it was handed over to the military under the code name Amidon®. At this point its use was not exploited to a great extent due to side effects, which were later attributed to the dosage being too high.[139]

In 1947, Isbell *et al* published experimental work on methadone stating that morphine addicts responded very well to the properties of methadone, but unless the manufacture and use were controlled, then addiction to it would become a serious health problem.[140] Despite this, early advertisements in the UK claimed little chance of addiction and the general consensus was that it was a better analgesic than morphine.

In 1955, the Home Office were aware of only 21 methadone addicts with this figure rising to 60 in 1960.[141] In 1968 with the introduction of the Home Office Notification System, 297 methadone addicts were registered and an even larger increase was observed by 1969 with the introduction of new drug clinics (1687 addicts registered). Prior to the 1970's, addiction maintenance was mainly in the form of prescribing the abused drug to the patient at clinics. However, the dramatic increase in the number of heroin addicts throughout the 1970's and 1980's promoted a change from this to the prescription of oral methadone as a means of addiction maintenance.[142] At present in the UK, most Health Authorities have a methadone prescribing service of some kind. All GP's have the right to prescribe methadone and more than half of all methadone addicts known to the Home Office are *via* GP's.

4.2 PRESCRIPTION

Methadone is prescribed mainly for the treatment of opiate addiction, however it is available as a linctus which is used for the treatment of coughing in terminal illness. There are five preparations of methadone:

- Liquid Methadone 1mg/1ml

  This is the treatment of choice for opiate dependency.[143] It is available as a pre-prepared solution or as concentrate which is diluted at the pharmacy.

- Methadone Linctus 2mg/5ml
- This is a general medicine licensed in the UK for the treatment of coughing in terminal illness. It is not usually prescribed for the treatment of opiate dependency.
- Methadone 5mg tablets - Physeptone®

  This is not licensed for the treatment of opiate dependency and prescription is discouraged due to the possibility of crushing and injecting the tablets.

- Methadone Suppositories

  These are rarely used in the treatment of dependency again due to the possibility of injection

- Injectable Methadone 10mg/ml

  This is prescribed for subcutaneous or intramuscular injection. However, when obtained illicitly it is usually used intravenously.

4.3 CHEMICAL STRUCTURE

Methadone (6-dimethylamino-4,4-diphenyl-3-heptatone) has a relatively simple structure and has a complex and powerful range of effects on those who take it. The structure of methadone is shown in Figure 4.1.

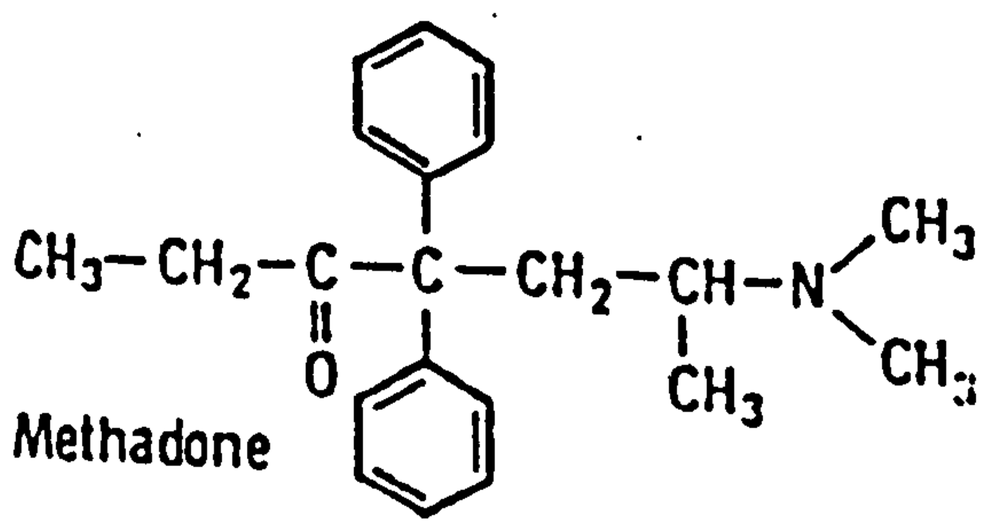


Figure 4.1: *Structure of Methadone*

Structurally, methadone appears to have two active regions:

- The nitrogen atom, which is thought to act on the peripheral nervous system
- The two phenyl rings, which are thought to be necessary for its opiate like action on the central nervous system.

4.4 Pharmacology

The pharmacology of methadone is very similar to that of the opiates (section 3.4). When used for the treatment of opiate addiction, the feeling "on methadone" is often described as an absence of withdrawal symptoms. The increase in anxiety and psychological discomfort experienced by many detoxification programme patients, compared to methadone maintenance patients, suggests that methadone contributes to a sense of relief from distress.

4.5 Metabolism

Methadone is rapidly absorbed following oral administration and is widely distributed in tissues with higher concentrations in the liver, lungs, kidneys and spleen than in the blood. It undergoes extensive first pass metabolism and the main metabolic reaction is *N*-demethylation, which results in a substance which instantly cyclizes to form methadones' two major metabolites, 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrrolidine (EMDP), neither of which is active. Hydroxylation to methadol followed by *N*-demethylation to normethadol also occurs to some extent. Other minor metabolic reactions also occur and there are at least 8 known metabolites.

The principle metabolic route of methadone is shown in Figure 4.2.

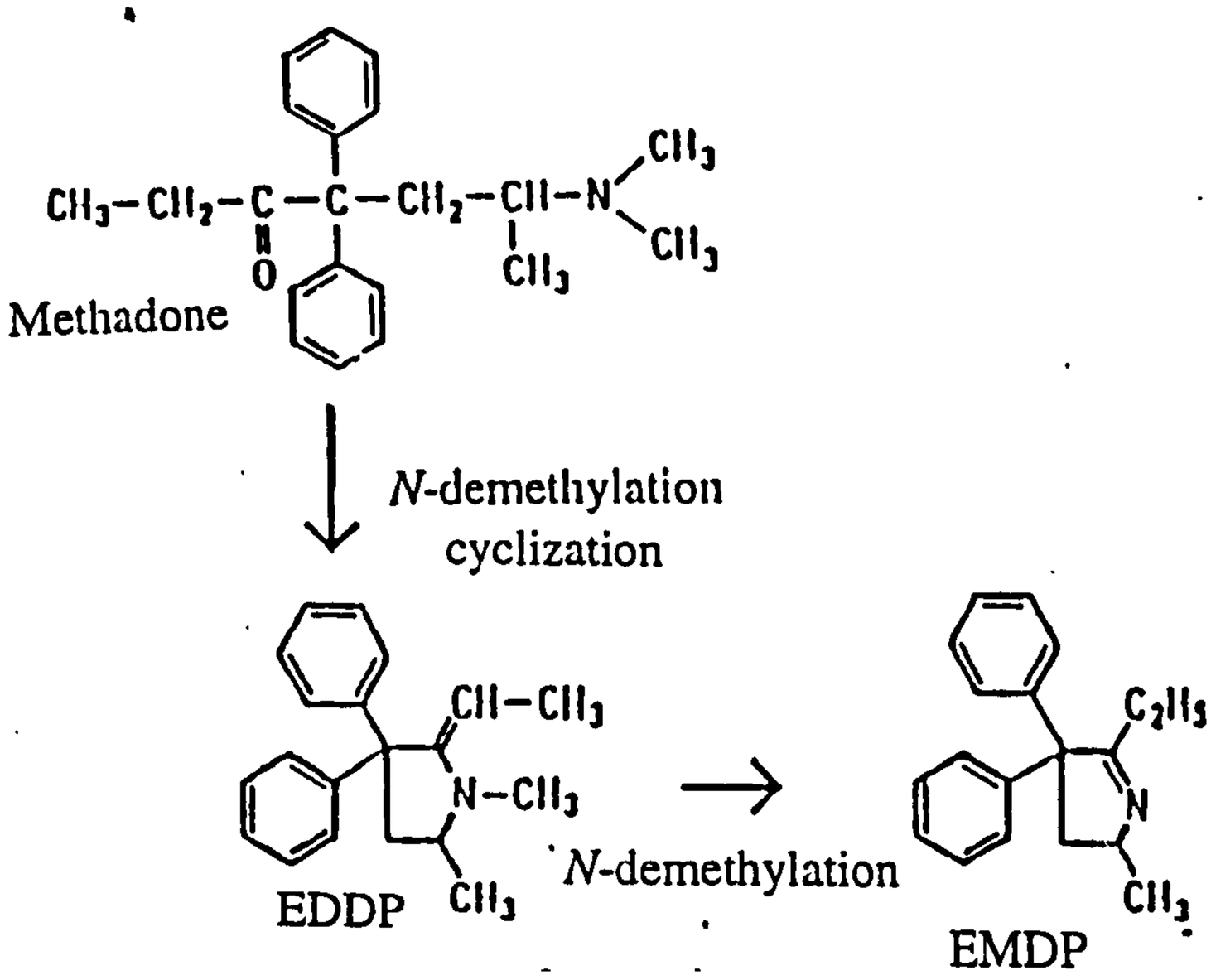


Figure 4.2: *Metabolic Route of Methadone*

4.6 PHARMACOKINETICS

In subjects on methadone maintenance, 20-60% of a dose is excreted in urine within 24 hours with up to 33% as unchanged methadone, 43% as EDDP and 5-10% as EMDP. The half life of methadone is very much dependant on the type of dosing due to its high affinity for tissue in the lungs, kidneys and liver. For a single dose, the half life of methadone is between 12-18 hours (mean 15 hours) and for multiple dosing the half life is between 13-47 hours (mean 25 hours).[144] The estimated LD_{50} for methadone is 50 mg, however tolerance to the drug may build up, albeit slowly compared to the other opiates, and doses of 200 mg or more may be withstood. Other pharmacokinetic parameters for methadone are given below:

V_D: 5 L/kg

Cl: 2 ml/min/kg plasma

Plasma : whole blood distribution: 1.3

Protein binding: up to 90%

4.7 Legal Status

Methadone (Physeptone) is a Class A drug which is listed under Schedule 2 of the Misuse of Drugs Act. This means that it is illegal to possess methadone without a prescription or other authority.

4.8 Analysis of Methadone

As with the other two groups of drugs, a literature review was carried out on previously used methadone extraction and analysis methods. As with the analysis for diamorphine and its metabolites, the method of choice for the analysis of methadone is GC/MS with SIM.

Table 4.1 provides a review of the analysis and extraction methods for methadone.

Table 4.1: *Methadone Analysis*

Year	Analysis Technique	Extraction Technique	Sample Type	Sample Volume
1972[145]	GC-FID	LLE	Plasma/ Urine	4/0.5-2 ml
1975[146]	GC-ECD	LLE	Serum	<2 ml
1977[147]	GC-FID	LLE	Blood/ Urine/ Stomach contents/ Bile/ Tissue	5/5/2.52.5 ml/ 2.5g
1977[148]	GC/MS	LLE	Plasma/ Urine	1 ml
1981[149]	GC-NPD	LLE	Blood/ Plasma/ Urine	1 ml
1982[150]	GC-MS	LLE	Plasma/ Saliva/ Urine	0.5/0.5/1 ml
1987[151]	HPLC	LLE	Blood/ Liver/ Brain	2 ml/250/500 mg
1996[152]	HPLC	SPE	Serum	5 ml
1996[153]	LC-MS	SPE	Blood	1 ml
1997[154]	CE	LLE	Serum/ Urine/ Hair	1/1 ml/50 mg

4.9 SFE Analysis of Methadone

No methodology was found to be available on the supercritical analysis of methadone or its metabolites other than for hair. This will be discussed in Chapter 5

4.10 Status of Cases

Figure 4.3 shows the number of toxicology cases in which methadone was found over the time period 1992 to 1997.

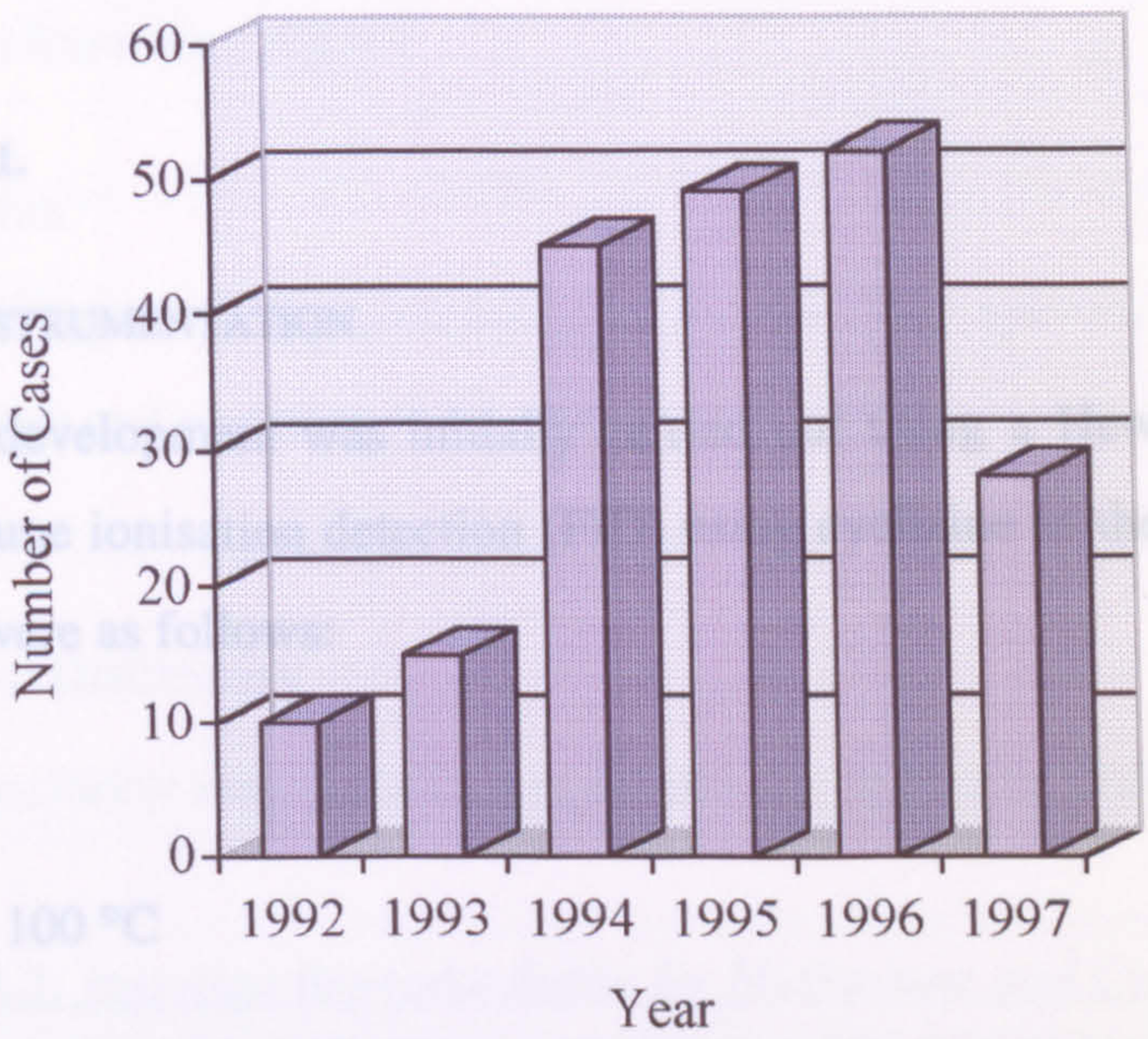


Figure 4.3: *Methadone Related Deaths*

In comparing this to the previous morphine related death figures, a trend between the decrease in morphine fatalities and the onset of methadone maintenance programmes is apparent.

4.11 Current Methodology

For the analysis of methadone, the routine method used in the Forensic Science Laboratory was liquid-liquid extraction as detailed below. This methodology was used as a comparison with SFE.

Into a screw capped test-tube, 1 ml of pH 10 carbonate buffer (1M Na_2CO_3, 1M $NaHCO_3$), 1 ml of blood, 100 µl of cyclizine standard and 5 ml of EtAc were pipetted. The test-tube was capped and rock and rolled for 15 minutes then centrifuged for 10 minutes. The EtAc layer was then transferred to a clean test-tube and 2 ml of 0.2 M HCl added. This test-tube was then rocked and rolled and centrifuged as before. The EtAc layer was then discarded. The acid layer was basified by the addition of 2 ml of pH 10 carbonate buffer. 3 ml of

EtAc were also added at this stage. The rock and roll, centrifuge step was again repeated. Finally, the EtAc layer was transferred to a vial and evaporated to dryness under N_2 and the residue reconstituted in 20 µl of EtAc. A 1 µl aliquot of this was injected onto GC.

4.12 Experimental

4.12.1 Analysis Instrumentation

Methadone method development was initially carried out using a Hewlett Packard 5890 Series II GC with flame ionisation detection (FID) using cyclizine as the internal standard. The GC conditions were as follows:

Column: HP5

Initial Temperature: 100 °C

Rate: 10 °C/min

Final Temperature: 300 °C

Injection Temperature: 280 °C

Detector Temperature: 300 °C

Equilibration Time: 0.2 minutes

Using these conditions, the methadone eluted at 19 minutes which meant that the run time was very long. For this reason, the initial temperature was changed to 120 °C which eluted the methadone at 15.4 minutes and the cyclizine at 13.9 minutes which is still too long. The run time was decreased by changing the conditions to the following:

Initial Temperature: 150 °C for 1 minute

Rate: 10 °C / minute

Final Temperature: 300 °C for 5 minutes

The injector and detector temperatures and the equilibration time were kept constant.

With these conditions, the methadone eluted at 12.8 minutes and the cyclizine at 11.6 minutes which was satisfactory.

4.12.2 GC Injection Reproducibility

4.12.2.1 Experimental

In order to check the injection reproducibility of the GC-FID system, five injections of methadone (10.4 µg/ml) and cyclizine (10.1 µg/ml) were made.

4.12.2.2 Results and Discussion

The injection reproducibility results for five injections are shown in Table 4.2.

Table 4.2: *Injection Reproducibility for Methadone and Cyclizine*

Methadone Area	Cyclizine Area	PAR (Meth/Cycl)
8306	10569	0.786
7142	9141	0.781
7210	6426	0.765
6779	8690	0.780
5414	7276	0.744

For the results in Table 4.2, the mean PAR was 0.771 with an RSD of 2.2% which is acceptable for this work at present.

4.12.3 Extraction of Methadone Using EtAc as a Modifier

4.12.3.1 Experimental

Extractions were carried out using ethyl acetate as a modifier at 0%, 1%, 5% and 10% in CO_2. For each percentage of EtAc, 100 µl of methadone (10.4 µg/ml) and cyclizine (10.1 µg/ml) in MeOH were loaded onto C_{18} and Extrelut® and extractions carried out from each support. The pressure for the extractions was 2500 psi, the temperature 60 °C and the flowrate 2.0 ml min^{-1}. The extraction time was 30 minutes. The collected extracts were evaporated to dryness under N_2 and reconstituted in 20 µl of EtAc.

4.12.3.2 Results and Discussion

The runs at 0, 1 and 5% were negative for both methadone and cyclizine. The run at 10% gave small peaks for both drugs from both Extrelut and C_{18}. The standard methadone and cyclizine had to be reconcentrated before being observed on the FID therefore, if the extraction efficiency is poor then no peaks would be observed even if they were present. The unextracted standards were concentrated 10:1 compared to the reconstitution of the extracted standards: 500 µl → 20 µl (5:1).

4.12.4 Extraction of Methadone by SFE using MeOH as a Modifier

4.12.4.1 Experimental

The modifier was changed to MeOH and extractions carried out at 0, 2, 4, 6, 8 and 10% MeOH in CO_2. These extractions were carried out using C_8 as a support medium. 500 µl of methadone (10.4 µg/ml) and cyclizine (10.1 µg/ml) were loaded onto the contents of a C_8 SPE column and dried at room temperature. The pressure for the extractions was 3500 psi, at a temperature of 60 °C and a flowrate of 2.0 ml min^{-1} for 30 minutes. The collected extracts were evaporated to dryness and reconstituted in 20 µl of EtAc.

4.12.4.2 Results and Discussion

The results for the extraction of methadone and cyclizine using MeOH as a modifier at various percentages in CO_2 are shown in Table 4.3.

Table 4.3: *Extraction of Methadone and Cyclizine Using MeOH as a Modifier*

Run	R.T. Methadone (min)	Area Methadone	R.T. Cyclizine (min)	Area Cyclizine
Unextracted	12.792	7738	11.559	9585
0% MeOH	/	/	/	/
2% MeOH	/	/	/	/
4% MeOH	/	/	11.570	1220
6% MeOH	/	/	11.590	483
8% MeOH	/	/	/	/
10% MeOH	/	/	11.603	1109

As can be seen from these results, the extraction conditions are not of use for the extraction of methadone or cyclizine.

4.12.5 EXTRACTIONS FROM C_8

4.12.5.1 EXPERIMENTAL

Four extractions were carried out using C_8 as a support medium. In each case the pressure was 2500 psi, the temperature 60 °C and the flowrate 2.0 ml min^{-1}. 10% MeOH was used as a modifier.

4.12.5.2 RESULTS AND DISCUSSION

The results are shown in Table 4.4.

Table 4.4: *Extractions from C_8 at 2500 psi*

Run	Area Methadone	R.T. Methadone	Area Cyclizine	R.T. Cyclizine	PAR (M/C)
1	1445	12.836	1032	11.597	1.400
2	5144	12.883	506	11.596	10.167
3	0	/	1604	11.582	0
4	2085	12.815	8578	11.571	0.243

From Table 4.4 it can be calculated that the PAR of the unextracted standard is (0.807), thus the method is not extracting methadone and cyclizine to the same extent. A requirement of the developed method is that the drug of interest (methadone in this case) and the internal standard (cyclizine) are extracted to the same extent and thus, further method development was necessary.

In addition, the GC traces were very dirty and thus the peaks seen at 12.8 and 11.6 minutes may not be due to methadone and cyclizine.

4.12.6 Determination of Interfering System Peaks

4.12.6.1 Experimental

Two EtAc blanks were extracted using these same conditions to determine if the peaks at 11.6 and 12.8 minutes could be due to the support medium. The extraction traces were still quite dirty but no peaks were seen which would coelute with methadone or cyclizine.

In order to clean up the extraction and determine how long the extraction would have to be run for, collections were made from 0-5 minutes, 5-10 minutes and then for 0-10 minutes for comparison.

4.12.6.2 Results and Discussion

The 0-5 minutes extract showed peaks at 11.566 minutes (PA:2066) and 12.802 minutes (PA:2102) which would correspond to cyclizine and methadone respectively. The PAR of methadone to cyclizine was 1.017. In total 9 peaks were integrated, with the trace being less noisy than previously seen.

The 5-10 minutes extract again had peaks at 11.547 minutes (PA:5398) and 12.802 minutes (PA:3633) and this time only 5 peaks were integrated and the PAR of methadone to cyclizine was 0.673.

The combined areas for 0-5 and 5-10 minutes were 7464 for cyclizine and 5734 for methadone giving a PAR of 0.768. This compares well to the unextracted standard ratio of 0.807.

The 0-10 minute extraction recovered less than the 5-10 minute extraction but more than the 0-5 minutes extraction with cyclizine at 11.552 minutes (PA: 4352) and methadone at 12.789 minutes (PA: 2383) and a PAR of 0.548 Nine peaks were integrated and there was more baseline noise than with the 0-5 minute extraction.

4.12.7 Extraction of Methadone using the Benzodiazepine SFE Conditions

4.12.7.1 Experimental

Methadone and cyclizine were extracted using the benzodiazepine extraction conditions but at a temperature of 58 °C Three extractions were carried out and injected onto the GC along with an unextracted standard at the same concentration.

4.12.7.2 Results and Discussion

Large peaks were observed at 14.7 minutes and 16.0 minutes but none corresponding to the methadone (12.811 minutes, PA 5731) and cyclizine (11.584 minutes, PA : 7687) of the unextracted standard (PAR : 0.746).

It was initially thought that the methadone and cyclizine had deteriorated as they had been loaded onto the C_8 for 4 days. Fresh standards were prepared on C_8 and these were extracted under the same conditions. However, the same two peaks were observed and no peaks at 11.6 or 12.8 minutes.

C_8 was extracted on its own to see if the peaks were due to the support medium. The peaks were again observed. However, as the peaks are not coeluting with the methadone or cyclizine its was decided to keep using the C_8 as a support medium as it had yielded the best results to date.

4.12.8 Extractions Using EtAc and C_8

4.12.8.1 Experimental

A 1 ml aliquot of each of the standards was loaded onto the contents of three C_8 SPE columns, which had been emptied into a weighing boat. Five extractions were carried out using this support.

(1) 10% EtAc, 1 ml min^{-1}, 65 °C, 3000 psi

(2-5) 5% EtAc: 2 ml min^{-1}, 65 °C

For extractions 1, 2, 3 and 4, the extraction vessels were filled. For extraction 5 there was only enough silica to half fill the extraction vessel. No methadone or cyclizine was extracted for any of the runs.

4.12.9 Extractions with EtAc and C_8 Continued

4.12.9.1 Experimental

The following extractions were run in duplicate with 100 µl methadone and cyclizine standards from C_8:

(1) 5% EtAc: 2 ml min^{-1}, 65 °C, 3000 psi

(2) 10% EtAc, 2ml min^{-1}, 65 °C, 3000 psi

(3) 15% EtAc, 2 ml min^{-1}, 65 °C, 3000 psi

Again, no methadone or cyclizine were recovered.

4.12.10 Extractions Using Alternative Supports

4.12.10.1 Experimental

The next set of duplicate extractions were carried out using various different supports at 5% EtAc:

(1) DCM washed silica gel

(2) Unwashed silica gel

(3) C8

(4) DCM washed Extrelut®

None of these extractions recovered methadone or cyclizine.

4.12.11 CHECK OF SFE SYSTEM

At this point the benzodiazepines extraction was checked to see if the system was functioning. The drugs were extracting but the recoveries were poor. Modifications to the system were made using the benzodiazepines (see section 2.15.6) and once it was functioning satisfactorily, method development of methadone was continued as follows.

4.12.11.1 EXPERIMENTAL

Two methadone and cyclizine extractions were carried out at 67 °C , 3000 psi and 5% EtAc and four at 10% EtAc.

4.12.11.2 RESULTS AND DISCUSSION

The results are shown in Table 4.5

Table 4.5: *Extractions at 67 °C, 3000 psi and 5% EtAc*

% EtAc	R.T. Methadone (minutes)	Area Methadone	R.T. Cyclizine (minutes)	Area Cyclizine	PAR
Std	12.785	40660	11.341	41472	0.980
5	12.773	21327	11.341	37754	0.565
5	/	0	11.618	16316	/
10	12.805	7423	11.365	13341	0.556
10	12.672	10970	11.369	14194	0.773
10	/	0	11.321	5758	/
10	/	0	11.346	6844	/

The first 10% EtAc, 3000 psi, 67 °C extraction gave the closest PAR to the unextracted standard with 27% and 34% recoveries for methadone and cyclizine respectively. The other two 10% EtAc extractions were unsuccessful.

4.12.12 SYSTEM FLUSH

After being left over the weekend, the SFE was flushed with EtAc to see if there was any remaining methadone or cyclizine in the system. The flush was very dirty with 23 peaks

observed and small peaks were seen corresponding to methadone (12.818 minute PA: 1540) and cyclizine (11.318 minutes PA: 1580).

4.12.13 New Retention Times for Methadone and Cyclizine

4.12.13.1 Experimental

The retention time of the methadone and cyclizine had changed due to the head pressure being altered as the flows had been switched off. The methadone was now eluting at 14.5 minutes and the cyclizine at 13.2 minutes. The column head pressure was set to 10 psi, the air at 40 psi, the H_2 at 20 psi and the carrier gas to 35 psi. The previous pressure had not been noted.

Three methadone and cyclizine extractions were carried out at 5% EtAc, 67 °C and 3000 psi, 3500 psi and 2500 psi.

4.12.13.2 Results and Discussion

The runs at 3000 psi and 2500 psi had high pressure errors in the middle of the extractions. However some peaks were observed for methadone and cyclizine. The results are shown in Table 4.6.

Table 4.6: *Extractions at 5% EtAc*

% EtAc	Pressure	R.T. Methadone	Area Methadone	R.T. Cyclizine	Area Cyclizine	PAR
Std	/	14.517	1714	13.248	2214	0.774
5	2500	14.528	16482	13.240	4373	3.769
5	3000	14.477	19785	13.197	14923	1.326
5	3500	/	0	/	0	/

4.12.14 Repeat of Extractions with New Pressure Regulator

4.12.14.1 Experimental

The pressure restrictor in the SFE system was replaced as it was not holding pressures above 2000 psi. With the new restrictor in place, extractions of methadone and cyclizine were carried out.

4.12.14.2 Results and Discussion

The following results were obtained at 5% EtAc and 3000 psi (Table 4.7).

Table 4.7: *Methadone Extraction Results with new Pressure Regulator*

	Methadone		Cyclizine		
Sample	Area	R.T. (min)	Area	R.T. (min)	PAR
Unext.	1714	14.517	2214	13.248	0.774
Ext. 1	2757	14.496	3508	13.216	0.786
Ext. 2	11003	14.472	22619	13.190	0.486

The recoveries were better than had previously been seen and in the case of the first extraction, the PAR was comparable to the unextracted standard.

4.12.15 Extractions at Various Pressures

4.12.15.1 Experimental

More methadone extractions were carried out at various pressures from 2500-4000 psi at 5% EtAc and 67 °C.

4.12.15.2 Results and Discussion

The results for the extraction of methadone and cyclizine at various pressures are shown in Table 4.8.

Table 4.8: *Recoveries of Methadone at Various Pressures*

	Methadone		Cyclizine		
p_a (psi)	Area	%Rec	Area	%Rec	PAR
HP error	1470	6.86	1463	6.88	1.005
3000	1737	8.11	5989	28.20	0.290
3000	0	0	0	0	-
4000	3682	17.19	2465	11.59	1.494
4000	0	0	0	0	
2500	0	0	2257	10.61	0
2500	0	0	0	0	-
Unext.	21422	-	21276	-	1.007

Once again it can be seen that the extraction method is not appropriate.

4.12.16 Investigation of pKa

The method development so far was carried out using the same modifiers, temperatures and pressure that had been used for the temazepam method development. The pKa values of the drugs being investigated was examined.

Methadone pKa: 8.3

Morphine pKa: 8.0, 9.9

Temazepam pKa: 1.6

Thus the pKa of methadone is more like morphine than temazepam so the effect of adding Et_3N which works well in the extraction of morphine was investigated.

4.12.17 Extraction using Morphine Conditions

4.12.17.1 Experimental

C_{18} was tried as a support instead of the Extrelut® used for the extraction of morphine at the following pressures: 3400, 3200, 2800, 3300, 2700, 3000 psi. 500 µl of methadone and cyclizine were extracted in each case. The extractions were run for 20 minutes at 65 °C, 90% CO_2: 9% MeOH: 1% Et_3N at 1 ml min^{-1}.

4.12.17.2 Results and Discussion

The results obtained are shown in Table 4.9.

Table 4.9: *Extraction of Methadone Using Morphine Modifier at Various Pressures*

Pressure	R.T. Cycl. (min)	Area Cycl.	% Rec Cycl.	R.T. Meth. (min)	Area Meth.	% Rec. Meth.	PAR
Unext	13.195	150387	/	14.463	183047	/	1.217
3400	13.186	26011	17	14.447	25794	14	0.992
3200	13.178	3798	2.5	14.442	3033	1.7	0.799
2800	13.166	7674	5	14.429	5332	3	0.695
3300	13.172	2636	1.7	14.430	2579	1.4	0.978
2700	13.177	1172	0.8	14.431	1200	0.7	1.024
3000	/	0	0	/	0	0	0

The effect of pressure on the PAR and recovery of methadone is shown graphically in Figure 4.4.

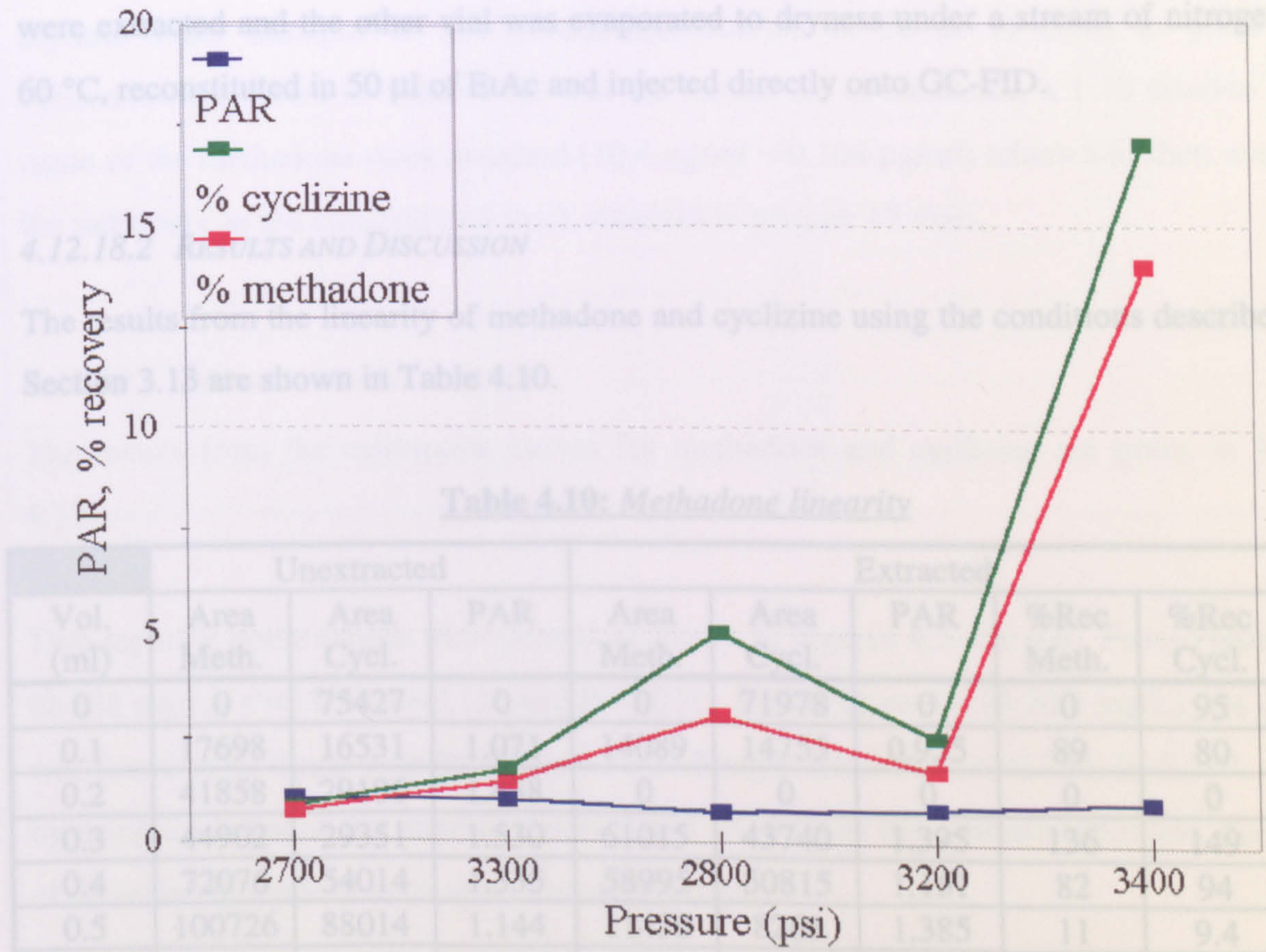


Figure 4.4: *Effect of Pressure on the PAR and Recovery of Methadone and Cyclizine*

In general the recoveries for the extraction are higher at higher pressures and the PARs are closer to that of the unextracted standard.

4.12.18 Morphine Modifier Methadone Extraction

4.12.18.1 Experimental

The conditions used for the extraction of morphine (3.13) were repeated for the extraction of methadone and cyclizine standards at various concentrations. MeOH and Et_3N (85:15 v/v) were mixed with CO_2 at 10%. The temperature for the extraction was 40 °C, the pressure 3600 psi and the flowrate 0.5 $mlmin^{-1}$. The extractions were run for 25 minutes. Seven vials were prepared in duplicate containing 1.0, 0.75, 0.5, 0.4, 0.3, 0.2 and 0.1 ml of methadone (10.4-1.04 µg/ml) and cyclizine (10.1-1.01 µg/ml) to ensure that the PAR of methadone : cyclizine remained constant at all concentrations and that the concentrations were linear over the range being studied. At each concentration the contents of one vial were extracted and the other vial was evaporated to dryness under a stream of nitrogen at 60 °C, reconstituted in 50 µl of EtAc and injected directly onto GC-FID.

4.12.18.2 Results and Discussion

The results from the linearity of methadone and cyclizine using the conditions described in Section 3.13 are shown in Table 4.10.

Table 4.10: *Methadone linearity*

	Unextracted			Extracted				
Vol. (ml)	Area Meth.	Area Cycl.	PAR	Area Meth.	Area Cycl.	PAR	%Rec Meth.	%Rec Cycl.
0	0	75427	0	0	71978	0	0	95
0.1	17698	16531	1.071	14089	14755	0.955	89	80
0.2	41858	29106	1.438	0	0	0	0	0
0.3	44902	29351	1.530	61015	43740	1.395	136	149
0.4	72076	54014	1.335	58995	50815	1.161	82	94
0.5	100726	88014	1.144	11450	8268	1.385	11	9.4
0.75	146326	130282	1.121	168548	121128	1.392	115	93
1.0	296935	264296	1.125	0	0	0	0	0

From these results it is obvious that the addition of the internal standard is of utmost importance. The PAR's are comparable in all cases where a result was obtained, but where the unextracted standard areas are linear over the range for both methadone (r^2=0.925) and cyclizine (r^2=0.915), no linearity is seen for the extracted standards using these conditions.

4.12.19 Linearity of Methadone with Cyclizine as the Internal Standard

4.12.19.1 Experimental

A calibration graph for methadone with cyclizine as the internal standard was carried out over the concentration range 187.2 µg/ml to 1.04 µg/ml. Two separate curves were drawn on from 208 - 10.4 µg/ml with cyclizine at 40.4 µg/ml and the other from 20.8 - 1.04 µg/ml with cyclizine at 4.04 µg/ml. For the first set of standards, 11 vials were prepared containing 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100 and 0 µl of methadone methanol standard with 200 µl of cyclizine. The contents of each vial was evaporated to dryness under nitrogen at 60 °C and reconstituted in 50 µl of EtAc to yield the concentrations shown in Table 4.10. For the second set of standards, a 1:10 dilution was made of the methadone stock standard (10.4 µg/ml →0.104 µg/ml) which was then used in the same way as the concentrated stock standard to prepare 17 vials.

4.12.19.2 Results and Discussion

The results from the calibration curves for methadone and cyclizine are given in Table 4.11.

Two separate linear ranges were plotted as shown in Figures 4.5 and 4.6. From Figure 4.5 (0-208 mg/L), r^2=0.989; A=0.110 and B=0.024. From Figure 4.6 (0-20.8 mg/L), the graph was found to be linear over the range 0.82-20.8 mg/L (r^2=0.981; A=0.754; B=0.299. Thus by using cyclizine as the internal standard and GC-FID as the analysis technique, the limit of detection of methadone is approximately 1 mg/L.

Table 4.11: *Calibration of Methadone with Cyclizine as the Internal Standard*

Methadone Conc^{n} (µg/ml)	Area Methadone	Area Cyclizine	PAR
208	193128	35148	5.495
187.2	215655	45211	4.770
166.4	209796	54360	3.859
145.6	211982	58894	3.599
124.8	109939	36884	2.981
104	95235	36122	2.636
83.2	66454	31957	2.079
62.4	78870	51204	1.540
41.6	57141	50441	1.133
20.8	20391	30837	0.661
10.4	20129	36228	0.556
0	0	20073	0
20.8	7397	1006	7.353
18.72	16979	2700	6.289
16.64	10832	1957	5.535
14.56	11967	2532	4.726
12.48	9558	2296	4.163
10.40	5512	1192	4.13
8.32	10575	3647	3.400
6.24	4717	1735	2.719
4.16	1720	717	2.400
2.08	485	492	1.586
1.04	1284	2249	0.971
0.832	532	947	0.562
0.624	744	1214	0.613
0.416	877	1438	0.610
0.208	1702	2032	0.835
0.104	1669	2510	0.665
0	0	2736	0

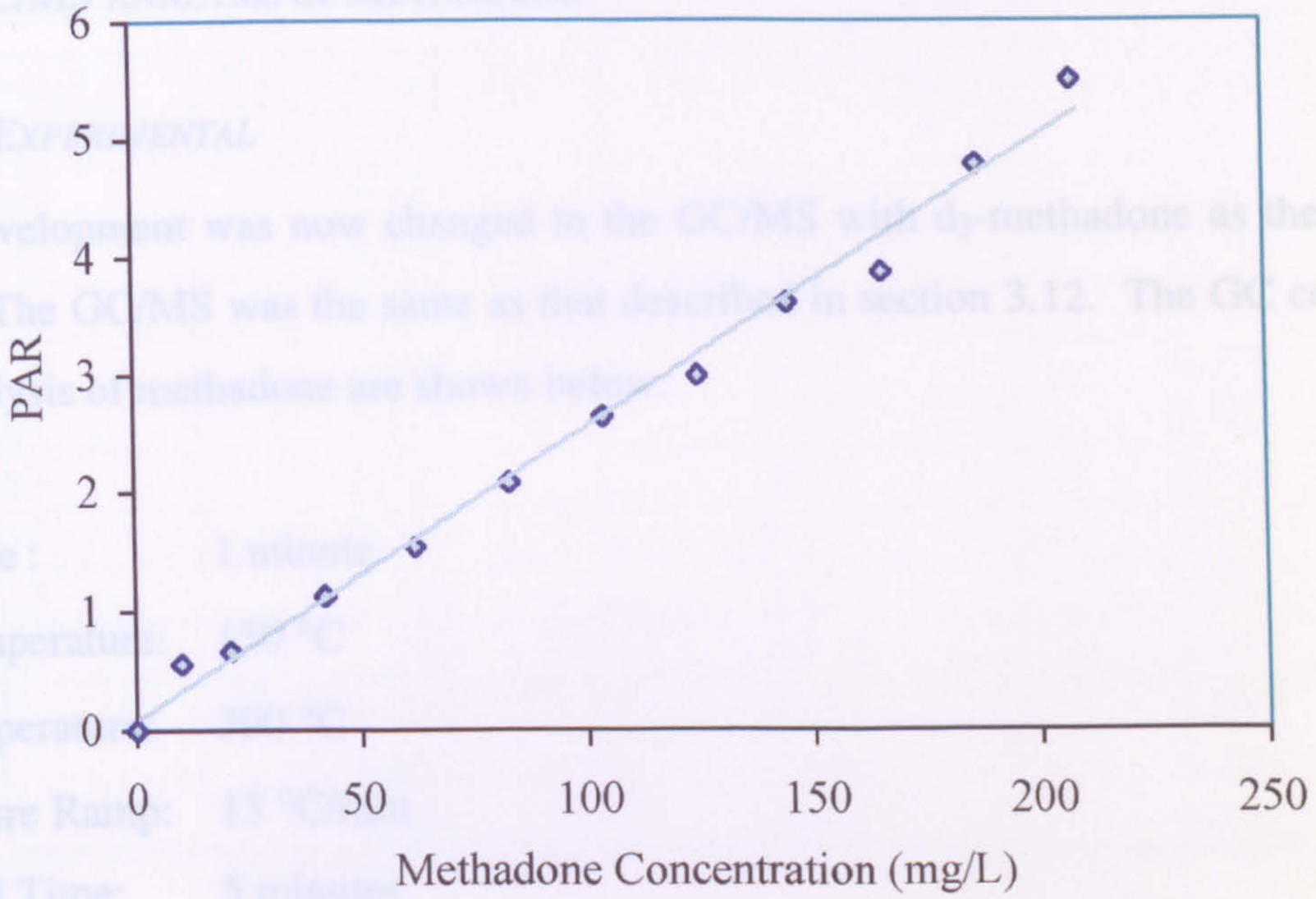


Figure 4.5: *Methadone Linearity Over the Range 0-208 mg/L*

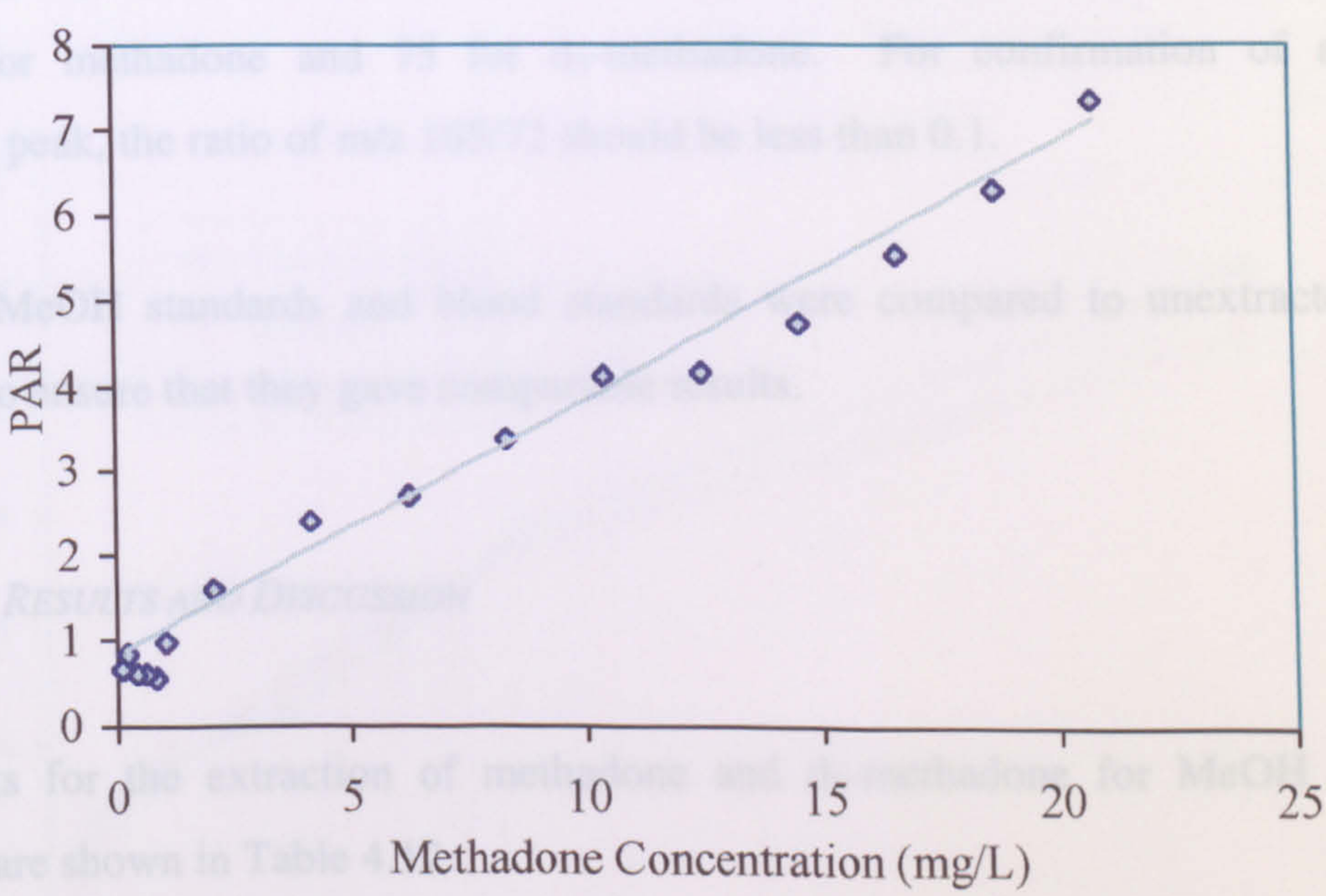


Figure 4.6: *Methadone Linearity over the Range 0-20.8 mg/L*

4.12.20 GC/MS ANALYSIS OF METHADONE

4.12.20.1 EXPERIMENTAL

Method development was now changed to the GC/MS with d_3-methadone as the internal standard. The GC/MS was the same as that described in section 3.12. The GC conditions for the analysis of methadone are shown below.

Initial Time : 1 minute
Initial Temperature: 150 °C
Final Temperature: 300 °C
Temperature Ramp: 15 °C/min
Final Hold Time: 5 minutes
Injector Temperature: 250 °C

The detector was run in the SIR mode and the mass to charge ratios (m/z) detected were 72 and 165 for methadone and 75 for d_3-methadone. For confirmation of a positive methadone peak, the ratio of m/z 165/72 should be less than 0.1.

Extracted MeOH standards and blood standards were compared to unextracted MeOH standards to ensure that they gave comparable results.

4.12.20.2 RESULTS AND DISCUSSION

The results for the extraction of methadone and d_3-methadone for MeOH and blood standards are shown in Table 4.12.

Table 4.12: *Methadone and d_3-Methadone MeOH and Blood Extractions*

Conc^{n} (mg/L)	Unext MeOH PAR	Ext. MeOH PAR	Ext. Blood PAR
0	0	0	0
0.0107	0.185	0.161	0.201
0.0153	0.204	0.216	0.226
0.0214	0.245	0.237	0.274
0.0535	0.345	0.398	0.398
0.107	0.515	0.503	0.503
0.153	0.623	0.619	0.619
0.214	0.759	0.727	0.782
0.535	1.129	1.132	1.249
1.07	1.568	1.614	1.473
r^2	0.930	0.942	0.887
A	0.307	0.302	0.340
B	1.290	1.327	1.222

From these results a calibration graph was plotted, however only the lower concentrations were linear. The highest concentration in each case was omitted from the calibration as at this value, the graph levelled off. The graph produced is shown in Figure 4.7.

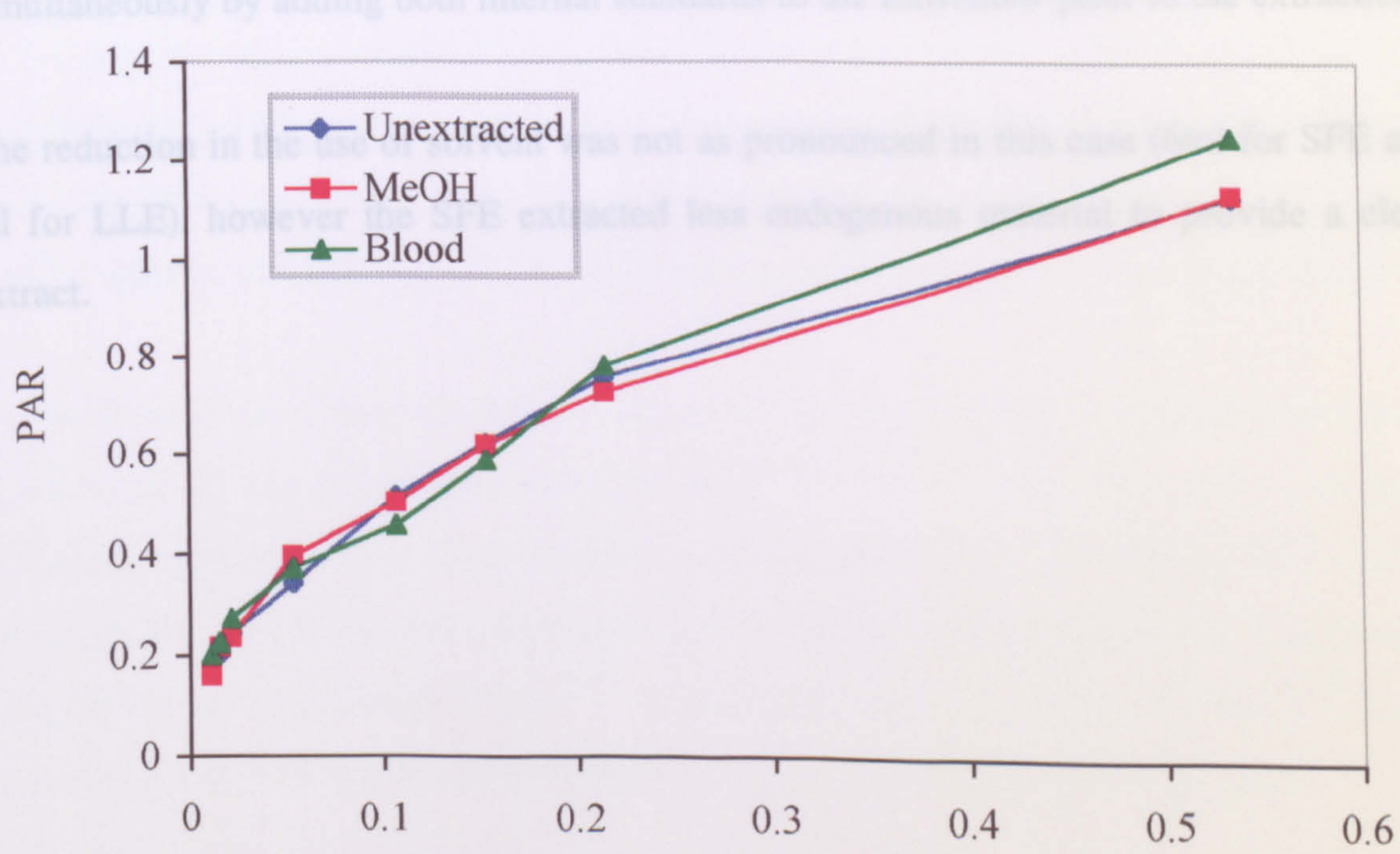


Figure 4.7: *Linearity of Methadone with d3-Methadone by GC/MS*

Using GC/MS, a 100 fold reduction in the limit of detection was found with the calibration being linear over the range 0.0107 to 0.214 mg/L (r^2 = 0.989, y=2.842x+0.177 unextracted; r^2 = 0.964, y=2.709x+0.186 MeOH extracted; r^2 = 0.986, y=2.749x+0.203 blood extracted).

4.13 Conclusions

Initially method development for methadone was carried out using GC-FID. Using this apparatus and the conditions developed for the extraction of morphine in Chapter 3, a limit of detection of approximately 1 mg/L was obtained. A the majority of samples being analysed for methadone will contain less than 1 mg/L, the methodology was changed and GC/MS was used as the analysis method. By this method, the limit of detection was decreased 100 fold to 0.01 mg/L.

The conditions for the extraction are the same as those developed for the extraction of morphine from blood and vitreous humor (p127) and indeed, both extractions can be run simultaneously by adding both internal standards to the Extrelut® prior to the extraction.

The reduction in the use of solvent was not as pronounced in this case (6ml for SFE and 8 ml for LLE), however the SFE extracted less endogenous material to provide a cleaner extract.

"Having put him to sleep on her lap
she called a man to shave off the seven
braids of his hair, and so began to
subdue him. And his strength left him."

Judges 16:19

5. HAIR ANALYSIS

5.1 INTRODUCTION

The analysis of hair is becoming increasingly accepted as a tool for the determination of use and abuse of drugs of forensic interest.[155] The importance of hair analysis stems from the fact that, at present, no toxicological method is available for the confirmation of long term drug use. Drugs pass into the hair from body fluids and remain there, thus each hair strand should provide a fixed record of past use.[156] Hair analysis cannot pinpoint the actual dose of drug consumed but, by carrying out a segmental analysis, it can provide information regarding the approximate period of time of drug usage which cannot be determined from blood or urine.

Since 1979, hair has been used to document chronic drug exposure.[157] To date more than 400 articles concerning hair analysis have been published with applications in clinical and forensic toxicology. The main practical advantage of testing hair, compared to blood or urine, is its longer surveillance window (weeks to months depending on length compared to 2-4 days for fluid analysis).[158] For practical purposes, the two compliment each other in that biological fluid analysis yields short term information, whereas hair analysis provides long term histories of drug use. The severity and pattern of an individuals drug use can also be determined without many of the problems associated with conventional analysis.

The accumulation of drugs in hair of drug users has been confirmed by several independent laboratories in the US, Japan and Europe.[159,160,161,162,163] with the drugs investigated including cocaine, heroin, amphetamines and phencyclidine. In addition to its obvious utility in the drug abuse area, hair analysis also offers applications in the monitoring of compliance of medication intake,[164] establishing toxic chemical exposure[158] and for monitoring the control of diabetes by measuring the glycosylation of hair proteins.[165]

The main advantages of hair analysis are summarised below.[166]

- The test cannot be thwarted by temporary abstention from drug use or adulteration of the sample.

- Hair can be collected under close supervision without embarrassment.
- A second sample can be collected and compared to the first by its appearance, by microscopic examination and by DNA analysis.
- The specimen and analytes are stable even under adverse environmental conditions and can be stored almost indefinitely without refrigeration.
- The test provides information on the temporal pattern and severity of drug use.

In conjunction with the wide detection window, hair analysis is therefore much more efficient than biological fluid analysis for the identification of drug abusers.

5.2 Structure of Hair

5.2.1 Introduction

Head hair grows at approximately 1 cm per month and the identification of a drug in hair depends on the length and type of hair. Its analysis can reveal drug use from one week to months prior to sampling[166] depending upon the length of the hair.

In order to interpret the results of hair analysis tests accurately, and to understand the appropriate role of hair analysis in drug abuse testing, it is necessary to have a basic understanding of hair biology.

5.2.2 Anatomy of Hair

Hair is a complex structure, the biology of which is only partly understood.[167] It is composed of cylindrical structures or shafts made up of tightly compacted cells that grow from small sac-like organs called follicles.[168] In man, the diameter of these shafts may range from 15-120 μm depending on the type of hair and the region of the body. Hair contains a family of sulphur-rich proteins known as keratins which form long fibrils in the shaft which become tightly bound through the replacement of S-H bonds with S-S bridges. These bridges produce a tough, highly stable structure.

5.2.3 Chemical Composition of Hair

Hair is essentially a cross-linked, partially crystalline, oriented polymeric network containing a number of functional chemical groups (*e.g.* acid, base, peptide) which have the potential to bind small molecules. Depending on the moisture content, human hair is between 65-95% protein, 5-35% water and 1-9% lipids. Mineral content is between 0.12-0.95% on a dry weight basis. Essential trace elements and heavy metals can also be found.[168]

The lipid material in hair is derived from sebum and apocrine secretions. It consists of free fatty acids, mono-, di-, and tri-glycerides, wax ethers, hydrocarbons and alcohols. Human hair contains relatively large amounts of side-chain amino-acids (*e.g.* glycine, threonine, cysteine).[169]

5.2.4 The Hair Follicle

This is a highly complex skin appendage consisting of both epithelial (matrix and outer root sheath) and dermal (dermal papilla and connective tissue sheath) tissues and may be considered as a miniature organ. There are approximately 80,000-100,000 hair follicles on the head and the number decreases with age.[170] The structure of the hair follicle is shown in Figure 5.1.

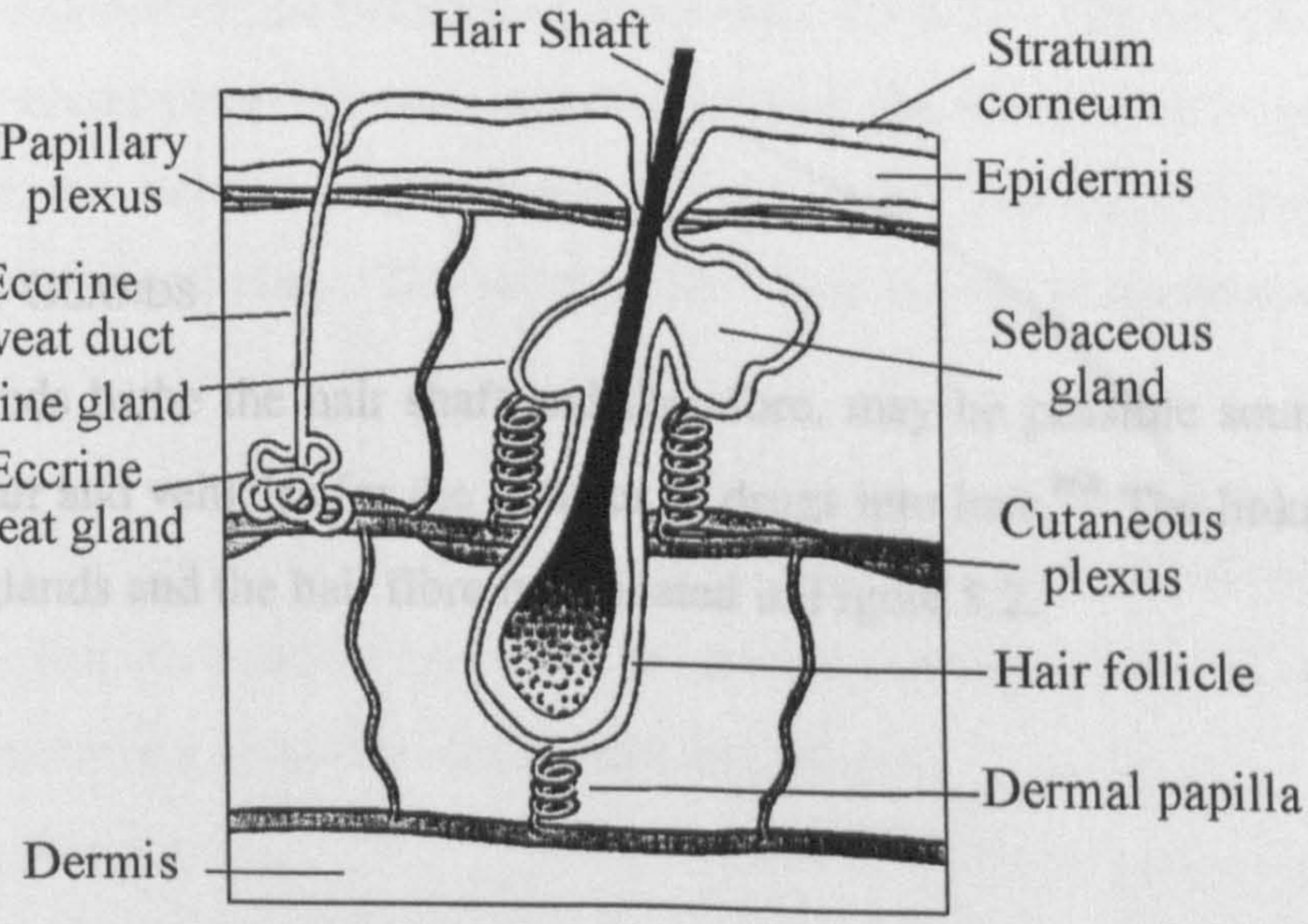


Figure 5.1: *Structure of the Hair Follicle*

The follicles are embedded in the epidermal epithelium of skin approximately 3-4 mm below the surface. They are closely related to the sebaceous and apocrine glands . In most body parts, the hair follicle and the sebaceous gland are fused both anatomically and functionally into a pilosebaceous unit. The ducts of both sebaceous and apocrine glands empty into the follicle.[171]

There are three functional zones along the hair shaft:[168]

- The innermost zone in and around the bulb is the site of biological synthesis of hair cells.
- The keratinogenous zone where keratinization of the cells occurs is directly above the bulb. Here hair undergoes hardening and solidification.
- The region of permanent hair. Here the hair shaft consists of dehydrated cornified cells which have formed fibrils fused by intercellular binding.

Hair growth begins in cells located in a germination centre (matrix) located at the base of the follicle. As the cells divide, increase in volume and elongate, they move up the hair shaft to the keratinogenous zone where melanin is synthesised. Long fibrils are formed through cross-linking of sulfhydryl groups in the amino-acids. Gradually the cells die, decompose by elimination of the nucleus and water, and coalesce into a dense "horny" mass.

5.2.5 Secretory Glands

Secretions by glands bathe the hair shaft and therefore, may be possible sources of trace elements in the hair and vehicles for the transfer of drugs into hair.[168] The linkage between various types of glands and the hair fibre is indicated in Figure 5.2.

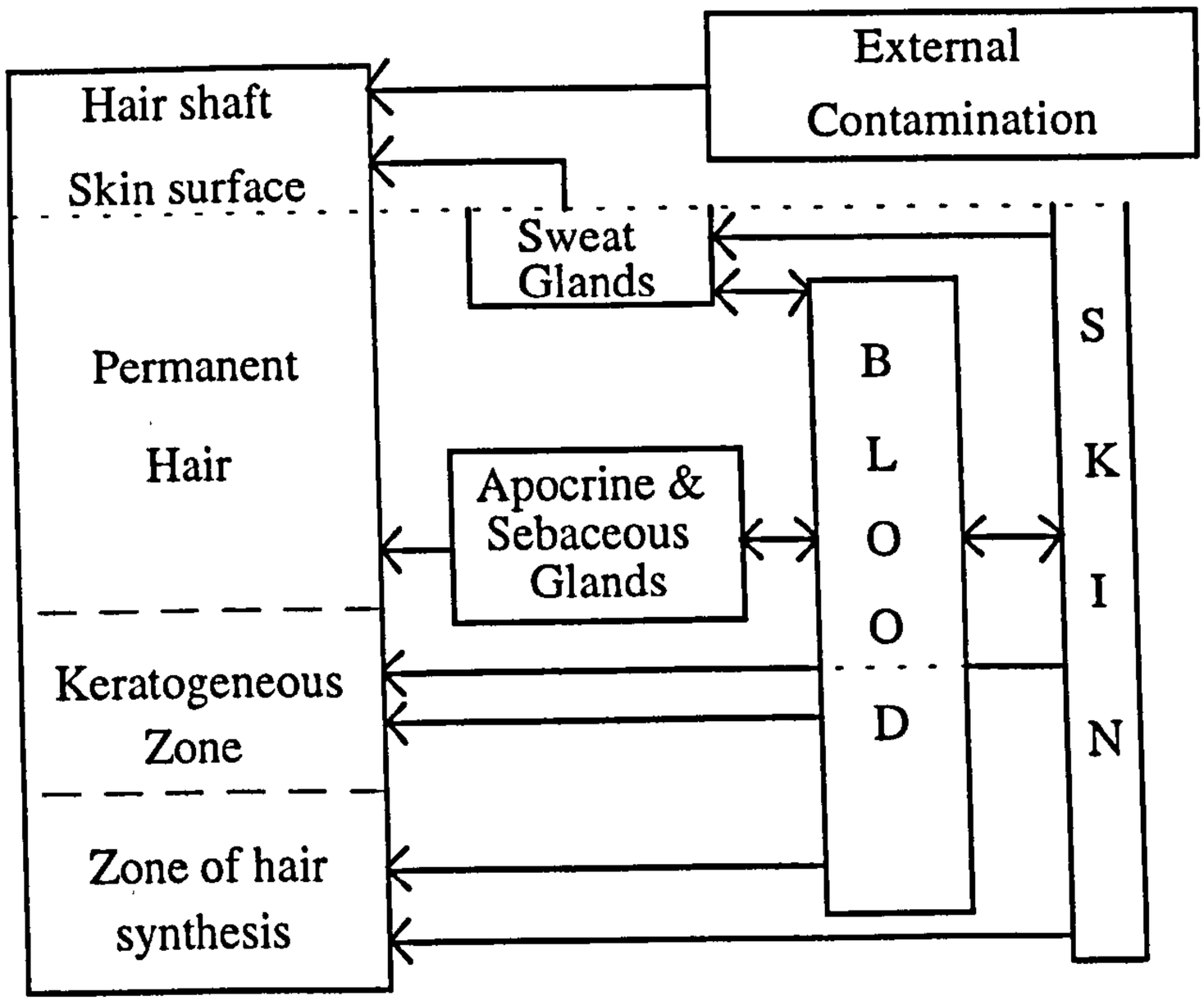


Figure 5.2: *Linkage Between the Secretory Glands and a Hair Fibre*[172]

5.2.5.1 SEBACEOUS GLANDS

These glands are present over the entire body surface except on the palms and the soles and dorsum of the feet.[168] They are located just below the surface of the skin and their ducts exit directly into the tunnel of the hair follicle. This means that the permanently formed hair shaft is bathed in serum just before it emerges from the skin. The exception to this is the beard follicle whose complimentary sebaceous gland has a duct which opens directly onto the skin surface. Sebaceous glands are at their largest and most numerous on the scalp, forehead, cheeks and chin. The sebum which they secrete gives unwashed hair its oily appearance.

The composition of the sebum depends on its anatomical area but in general consists of one third aqueous as free fatty acid, one third as combined fatty acid and one third as unsaponifiable material *e.g.* squalene, cholesterol and waxes.

5.2.5.2 APOCRINE GLANDS

These are localised in the axilla, external auditory meatus, eyelids and perennial region.[168] They are different from the sweat glands on the scalp in that they secrete by the separation of cytoplasm from secretion cells. They discharge directly onto the hair follicle rather than the skin surface. Apocrine gland secretions are oily, colourless, odourless substances about which little is known other than that their bacterial breakdown results in body odour.

5.2.5.3 SWEAT GLANDS (ECCRINE OR MEROCRINE)

These are distributed all over the body surface and originate in the dermal layer.[168] Their ducts exit near to the follicles but they are not directly in contact with them. They exist as simple tubules consisting of a coiled segment in the dermis and a straight duct which opens onto the epidermis.

The quantity and concentration of fluid secretions from sweat glands varies largely depending on the individual and the environment. The principal ingredients of the fluid are water, salts (sodium and potassium), urea, amino-acids, lactate and pyruvate.

5.2.6 THE DERMAL PAPILLA AND BLOOD SUPPLY TO THE FOLLICLE

The main source for the transport and delivery of blood elements to hair is the vascularized dermal papilla which contains a population of fibroblast-like cells and endothelial cells.[167] Due to the enormous amount of amino acids, lipids and carbohydrates required to produce hair and its sheaths, fenestrated vessels with thin diaphragm type membrane areas for accelerated transport are present.[173] As in many tissues the common source of energy in the hair follicle is glucose, which becomes phosphorylated and directed through the major pathways like Embden Meyerhof, the pentose cycle and the tricarboxylic cycle. The major activity of glucose-6-phosphatdehydrogease is found in the matrix and upper bulb region.

Most drugs undergo metabolic transformations including oxidative, reductive, hydrolytic and conjugative reactions in the body. The main site of drug transformation is the liver, but other tissues like the hair follicle may also participate. It was found that a mono-oxygenase system is operative in the hair follicles which is comparable to the

corresponding system in the liver. The fact that the follicle contains cytochrome P-450 may be interesting for toxification reactions. These findings present strong evidence that biotransformations in drugs of forensic interest may also occur within the hair follicle, prior to fusion in the hair shaft.[174]

Hair follicles are surrounded by two dense capillary networks, whose patterns are different in active and quiescent follicles.[168] The lower third of an actively growing follicle is surrounded by a rich vascular plexus composed of long parallel vessels connected by cross-shunts. The second network is located at the level of the sebaceous glands and extends to the skin surface. Therefore, both the growing hair bulb and the sebaceous glands are richly vascillarized. The two networks are connected by a simpler parallel system of larger vessels which descends along the sides of the follicle.

5.2.7 Structure of the Hair Shaft

Each shaft consists of three types of cell:

5.2.7.1 Outer Cuticle

This consists of a single layer of elongated overlapping individual cells each between 0.5-1.0 μm long.[168] Its function is to anchor the hair shaft in the follicle and protect the interior fibre. The cuticle can become damaged or destroyed by chemicals, heat light or mechanical injury and as a result becomes less intact towards the distal end of the shaft and may become frail and fall apart.

Assuming that drug substances are mainly located inside the hair fibre, the fact that the weight contribution of the cuticle depends on the fineness of the hair, may imply possible minor variations of the drug content in hair analysis as a function of the hairs diameter.

5.2.7.2 Central Cortex

The cortex forms the bulk of the hair shaft and is composed of long keratinized cells which form into long fibrils approximately 100 μm long.[168] They are held together by a special

chemical cement. Between the cells are very small air spaces called fusi. In the living portion of the hair roots, these are fluid filled but as the hair grows, the fluid dries out and is replaced with air. Pigment granules are also found in the cortex and their alignment give hair its colour. As with the skin and eyes, melanin is the main pigment. It is synthesised in special organelles known as melanosomes. These are located within the hair bulb in small bodies called melanocytes where the enzyme tyrosinase acts on tyrosine to produce melanin.

The associated proteins forming the cortex matrix consist of two large families of nonhelical proteins, the "high sulphur" and the "high glycine/tyrosine" proteins[167,175] The intermediate filaments of a cortex cell are embedded in a matrix of globular proteins with the interactions of the molecular chains. The space in the matrix for small molecules is limited by the globular proteins and it has been demonstrated for water molecules that the microfibrils or intermediate filaments absorb much more water than the interfilament associated proteins of the matrix.[176] In the polypeptide chains of the intermediate filaments and in the associated protein, a large number of binding sites (including hydrogen bonding, ionic, amide, disulphide and electrostatic) are present. Thus, different drug substances may be easily built in during the keratinization process of the cortical cells. Since the cortex makes up the main part of the hair fibre both by weight and volume, drug binding within the cortex is believed to dominate the analytical results, but this is not yet proven.[177]

5.2.7.3 Central Medulla

Medullar cells are initially loosely packed but as the hair grows they dehydrate leaving a series of vacuoles along the fibre axis.[168] In general, the number of medullar cells increases as the fibre diameter increases. In human hair the medullar cells comprise only a small percent of the mass of the hair and in some cases may be totally absent, continuous along the centre or discontinuous.

The hair medulla is difficult to isolate and has an insoluble fraction.[178] It seems to be rich in proteins and lipids and has been shown to have ε(-glutamyl)lysine cross linkages. It also contains cutrulline, has a very low cystine and sulphur content and consists of relatively large amounts of acidic and basic amino groups and hydroxyamino acids.[169] Drug

substances have been found in the medulla thus, it can be speculated that the type of medullation and the medullary index (ratio of width of medulla to hair diameter) in a hair sample may also have an influence on the analytical results.[167]

5.2.8 The Melanin Granule

Melanin granules are the cell components of melanocytes, which are situated at the apex of the dermal papilla.[167] They release branches filled with melanin granules during the anagen phase of the growth cycle. The matrix cells, which are to become cortex cells later, actively phagocytose the granules as they pass by the melanocyte region on their way upwards to the keratinization region. As the cortical cells harden during keratinization, the melanin granules become fixed between the ketatinous fibrils. Melanin granules are spread throughout the cortex but are more concentrated towards the periphery. They are observed in the medulla, are rarely present in the spaces between the cortical cells and are usually absent in the hair cuticles.[179]

Hair colour depends on the number of melanin granules present, their size, arrangement and distribution in the hair shaft and their melanin composition. When pigment is deficient or lacking, the hair appears grey or white.

Melanins are polymers which are basically composed of indole-5,6-quinone units which can occur in different stages of oxidation with units of 5,6-dihydroxyindole carboxylic acids present. The macromolecules are formed by condensation on active sites in a soluble protein matrix of the immature melanin granule within the melanocytes and they are extremely conjugated.[180] They act as unique biological absorbers. *In vitro* experiments demonstrate that even high concentrations of different drugs can be taken up by isolated granules.[167] The melanin affinity for drugs is dependent on the drugs chemical nature. The maximum affinity group is characterised by the presence of a series of highly conjugated double bonds as seen in chloroquinine. The loading of pigment granules also seems to be influenced by the quantity of pigment and the concentrations of the offered drug as well as the morphology and chemical composition of the granules themselves.[181] The molecular binding mechanism is not well known. It is believed to be a type of adsorption in which forces occur which may be due to charge transfer reactions, the melanin being the electron

acceptor, as well as electrostatic forces. For many drugs which are present as cations at physiological pH's with known melanic affinity, ionic attraction also occurs between the positively charged molecules and the anionic carboxyl groups of the melanin polymer.[182] This means that the measurement of drugs in hair may be biased by its pigmentation, thus the results should be interpreted in light of particular drug interactions with melanin polymers.

5.2.9 The Growth Cycle

Hair growth is cyclic in humans and is asynchronous and varies in its rate depending on the follicle type from 0.1-0.45 mm per day.[167] The rate shows vestiges of seasonal changes and is affected by endocrine dysfunctions, metabolic and genetic disorders, nutrition, hormones and drugs which target the follicular segments and interfere with the cell kinetic of hair growth.

5.2.9.1 Anagen Phase

The length of this phase determines the length and type of hair. The onset of the anagen phase is accompanied by an increase in the metabolic activity of the matrix cells located at the base of the follicle just above the papilla.[168] The bottom of the follicle is pushed more deeply into the dermis by continual cell division. New hair begins to grow as new cells elongate and form a thin filament then push their way upward into the follicular canal. Here the hair cells differentiate into cuticle, cortex and medullar types and the keratinization process begins.[183] It is thought that drugs and trace elements are incorporated into hair at this time. When radioactive compounds *e.g.* ^{35}S labelled cysteine are administered, appreciable radioactivity is seen in the keratinogenous zone but little activity is seen in the follicular bulb. When radioactive glucose is administered, the opposite is observed. This implies that the hair follicle receives nutrients from the vessels surrounding the bulb but other chemicals are incorporated at the keratinogeneous level.

5.2.9.2 CATAGEN PHASE

This is the transitional phase. Cell division stops and the base of the shaft becomes fully keratinized and forms the dry, white node characteristics of a club hair. The bulb begins to degenerate and the follicle becomes considerably shorter.[168]

5.2.9.3 TELOGEN PHASE

The follicle enters a quiescence or resting period in which the hair shaft stops growing and is retained in the upper portion of the follicular canal where it can be easily removed by pulling. The length of this period depends on the body area and the age of the individual. For scalp hair this period is relatively short (~ 10 weeks) compared to the general body surface (2-6 years).[184]

5.2.9.4 SUMMARY

Each hair follicle has its own growth cycle. The percent of hairs growing compared to the percent resting, varies depending on the area of the body. The longer the type of hair, the longer the growing phase. On the scalp, approximately 15% of the hairs are resting and the remaining 85% are growing. This process can be complicated by disease states, nutritional deficiencies and pregnancy.

5.2.10 RATE OF GROWTH

Hair usually grows at 1 cm/month. This is an oversimplification as not all hair is in the growing phase at any one time and the percent of follicles in the growing phase varies with anatomical location and age. The range of growth is from 0.6 cm/month to 3.36 cm/month. Hair type and anatomical region are the most important factors. Scalp hair grows faster than pubic or axillary hair which grows faster than beard hair. Caucasian hair grows faster than Asian hair. In general the rate decreases with age. All of these factors are important when considering where to take a sample of hair from. Usually hair in the vertex region of the scalp is chosen as the best specimen, as it has the largest amount of hair in the telogen phase and the fastest growth rate.[168]

Provided that exogenous factors have neither removed or added to the drug in the hair, the presence of a drug at a particular distance from the scalp should reflect that the drug was available to the hair at that time of formation and keratinization of that hair section. Thus, analysis of the hair segments as recording filaments at different distances from the scalp should provide retrospective information. If sufficient information on the rate of growth is available, a rough correlation between the distance of a section from the scalp and the time interval since that section was in the follicle can be made.[172]

New hairs take about three weeks to reach the surface of the scalp.[167] Thus, if a sample of hair is taken by cutting as opposed to plucking, the first segment of the hair strand was formed more than four weeks earlier and does not represent the drug uptake in the month prior to sampling.

5.2.11 SCALP HAIR V BEARD HAIR V PUBIC HAIR AS A SPECIMEN[168]

5.2.11.1 SCALP HAIR

This type of hair is the easiest to collect but there exists considerable variation in growth rates in the various regions which makes the choice of sampling area important. Scalp hair has the highest growth rate of all the hair types therefore is probably the most variable. Also, as the ducts of the sebaceous glands discharge directly into the hair follicles, the hair shaft is exposed directly to sebaceous secretions before it emerges from the skin. Scalp hair is exposed also to sweat and external contamination. Finally the chemistry and physiology of scalp hair may be modified by the use of cosmetic treatments whose impact on hair analysis is not yet fully understood (*e.g.* bleach, perm lotions).

5.2.11.2 BEARD HAIR

Beard hair is thick and its follicles in comparison to other types are enormous. It has the slowest growth rate. The fact that the hair shaft and sebaceous gland ducts exit from the skin in two separate channels means that this hair is less likely to be contaminated by sebaceous excretions. However, when beard hair is obtained by shaving, it may contain parts of the epidermis. External contamination is as likely here as it is with scalp hair.

5.2.11.3 PUBIC HAIR

This type of hair has the apparent advantage in that it may be less contaminated through environment exposure or cosmetic treatment. However, contamination due to urine is an important issue which must be considered. Hair in the axillae and pubic region is curled rather than straight which makes segmental analysis difficult. Pubic hair is exposed to sebum and sweat secretions and to the secretions of the apocrine glands which are present in significant numbers only in the axilla and pubic areas. These glands discharge their secretions directly into the follicle. Pubic hair grows slower than scalp hair and has a longer resting period.

5.2.12 SUMMARY

Hair is quite an attractive indicator in forensic science because it is easy to obtain and easily stored. Nevertheless, it is a very complex material which requires sophisticated analytical techniques and profound knowledge to interpret the results. For hair analysis, the formation and the morphology of the hair fibre are suggested to be key structures but much more information than already available is needed to answer the open questions and to evaluate hair analysis in an adequate manner.

5.3 TRANSFER OF DRUGS INTO HAIR

The precise mechanism(s) involved in the incorporation of drugs into hair, or the factors influencing it, have yet to be determined.[172] The generally accepted mechanism is one in which chemicals present in blood move via passive diffusion into the rapidly growing cells of the hair follicle.[185] As the cells die and fuse to form hair strands, the drugs become trapped.

It has been suggested that the passive diffusion of drugs into growing cells may be augmented by the drugs binding to intracellular components of the hair cells, *e.g.* pigment. This is especially so for amphetamine and methamphetamine which are chemically similar to tyrosine and DOPA (3,4-dihydroxyphenylalanine), the precursors of melanin. This mechanism is probably not too important as drugs may be found in the hair of albinos and only a small amount of drug is found in the melanin fraction of hair.[186] Bonding of the

drugs to hair could also be due to sulfhydryl containing amino acids, especially for drugs which can form stable direct covalent bonds *e.g.* divalent cations.

5.3.1 Simple Passive Transfer Model

This is the simplest model to describe drug binding in hair. Drugs move by passive diffusion from the bloodstream into the growing cells at the base of the follicle, then become tightly bound in the interior of the hair shaft during keratogenesis. The model is dependent on the concentration of drug in blood and thus the quantity of the drug administered. Figure 5.3 is a schematic representation of this model.

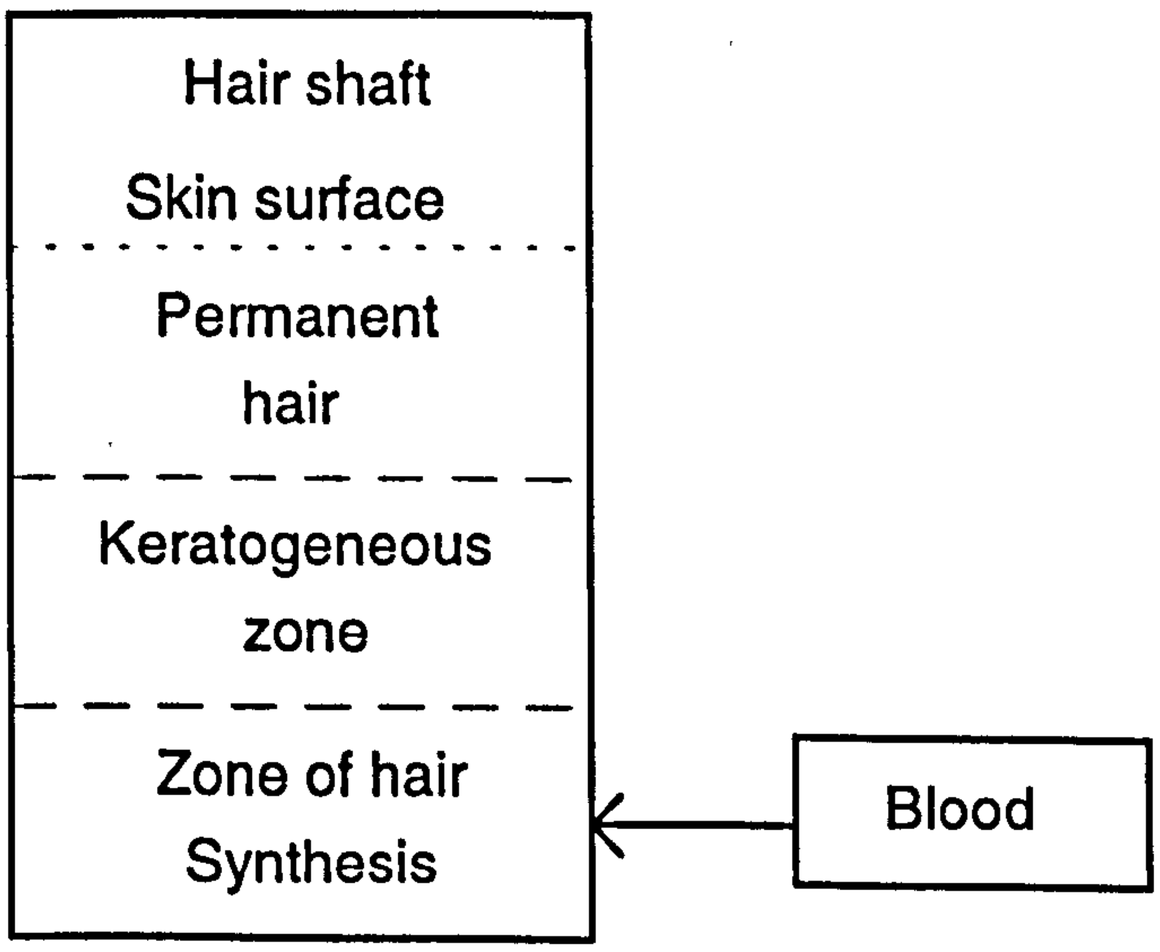


Figure 5.3: *Simple Passive Transfer Model* [172]

Hair is assumed to grow at a constant rate, thus the position of the drugs along the hair shaft can be correlated with the time that the drug was in the bloodstream. This model also forms the basis for segmental analysis. As techniques for hair analysis improve, newer findings suggest that this model is somewhat oversimplified.

5.3.2 Problems with the Simple Passive Transfer Model

Some elements/drugs have shown good correlation between the concentration of ingested drug[187] and the concentration of drug in hair, others correlate badly.[188] Although, some of

this can be put down to external contamination, it has been proposed that another possible method involved is "pooling".[172] This introduces the concept of multiple equilibria and steady states. This proposal is element and exposure dependent, but only after transfer from pool to pool *via* multiple equilibria and steady states. It has not yet been verified experimentally.

5.3.2.1 CORRELATION

Baumgartner *et al* have reported a positive correlation between self reported drug use and concentration in hair for heroin,[189] marijuana,[190] cocaine[191] and PCP (phencyclidene)[192] by RIA (radioimmunoassay). More recent studies using GC-MS have also shown a positive correlation *e.g.* Nakahara *et al*[193] found good correlation between methoxyamphetamine intake and hair concentration. In a two person study by Cone *et al*[194] good correlation between opiate dose and concentration in beard hair was found. Puschel *et al*[195] also analysed opiates in the hair of cancer patients on morphine and found poor correlation with the highest concentration corresponding to patients who had received the lowest doses. Poor correlation has also been shown for cocaine and its metabolites in hair, urine, blood and sweat.[172] However, in general with cocaine the concentration found in hair increases with dose. Intersubject variability has been found to be high, therefore the estimation of dose is very difficult to make. This variability cannot be assigned solely to plasma pharmacokinetics.

5.3.2.2 DRUG METABOLITE RATIOS

Perhaps the greatest challenge to the passive transfer model is the relatively high parent drug to metabolite ratio *e.g.* heroin is highly metabolic and 6-MAM which is a relatively short lived metabolite is found in high concentrations in hair.[196]

5.3.2.3 TIME COURSE OF DRUG INCORPORATION

Two assumptions are made for segmental analysis

1) Hair grows at a constant rate of ≈ 1 cm/month
2) No diffusion occurs along the hair shaft

Some investigators have shown that the presence of drug does correspond to the time of injection,[194] but not all. This leads to the belief that a more complex multicompartment model exists. For example, hair obtained after a single administration of deuterated cocaine showed that the position of the d^5-cocaine along the hair shaft did not always correlate with the time of drug injection.[172]

5.3.3 Multicompartment Model

A suitable model to describe the incorporation of drugs into hair would have to explain findings such as:

- Why drug and metabolite ratios in hair are different from those in blood.
- Why the time for drugs to appear in hair varies between subjects.
- Why the distribution along the hair shaft is not always consistent with what is predicted from hair growth.
- Why the drug and metabolite concentrations in hair vary to a great extent between subjects receiving the same dose.

5.3.4 Proposed Model

This involves three pools[172] as indicated in Figure 5.2, Section 5.2.5.

a) Blood during formation of the hair shaft.
b) Sweat and sebum after formation of the hair shaft.
c) External contamination after formation of the hair shaft and after hair has emerged from the skin.

In addition, the drugs and metabolites may be transferred from multiple body compartments or pools in the tissues surrounding the hair follicle.

5.3.4.1 Transfer via Sweat, Sebaceous and Apocrine Glands

Nearly all drugs of abuse have been found in sweat, often in concentrations higher than those found in blood.[172] In order to determine the contribution of drugs in sweat to drugs

in hair, Henderson carried out an experiment where subjects who had ingested cocaine under supervision held uncontaminated hair in their hands two hours after ingestion. Subsequent analysis showed significant amounts of cocaine in the hair, in some cases even after washing. In the experiment, the inter-subject variability was found to be high and therefore, this contribution is more significant for some individuals than others.

Contributions due to sweat and sebaceous excretions in the hair are not separable and therefore, inter-individual differences of drug levels in subjects consuming known amounts of a particular drug may be partially explained by this. This phenomena also goes some way to explaining the apparent band broadening seen in some individuals known to have used drugs over a certain time period, *i.e.* excretions in the sweat may explain why drugs are found in longer areas of hair than expected. If glandular secretions are significant routes for incorporation, interlaboratory variability in washings and extraction procedures could also result in variable findings.

5.3.4.2 Transfer from the External Environment

External contamination can come from a variety of media including air, water and cosmetic treatments and is possibly the reason why it is difficult to establish a baseline concentration for trace elements in hair.[172] For example, hair analysis cannot distinguish smokers from non-smokers. External contamination in addition to coating the hair can be intradermally transferred especially for highly lipid soluble drugs.

5.3.4.3 Transfer from Deep Compartments in the Skin

Skin has been shown to act as a reservoir for certain drugs, especially lipophiles.[172] More specifically this occurs in the epidermis and hypodermis. When drugs penetrate from the external environment across the skin, they are retained by the lipid layers of the stratum corneum and are released slowly into the bloodstream. Conversely, when drugs move from the systemic circulation to the surface they are retained primarily by the adipose tissue of the hypodermis and secondarily by the stratum corneum. Both the stratum corneum and the hypodermis are in intimate contact with the hair follicle and its associated glands and

the capillaries surrounding the hair bulb could maintain an equilibrium between these structures and any pool in the adjacent tissues.

5.4 Analysis

5.4.1 Quantitative Aspects of Hair Analysis

Figure 5.4 represents the change in concentration of a drug with time for various sample types.[158] For body fluid analysis (BFA), the area under the curve correlates to the amount of drug taken. The figure indicates that BFA can be used for a set time period and there is no way of monitoring whether the sample was taken on the ascending or descending part of the curve and thus there is no relation to amount taken (*i.e.* small amount recently or large amount some time ago). Therefore, this is not very practical for long term drug screening. With hair analysis, the drug recirculates in the bloodstream in direct proportion to concentration. In this respect, hair acts as an integrating monitor. The concentration in the bloodstream depends on the amount taken, the mode of administration, the metabolic rate, the excretion rate and the distribution coefficient.

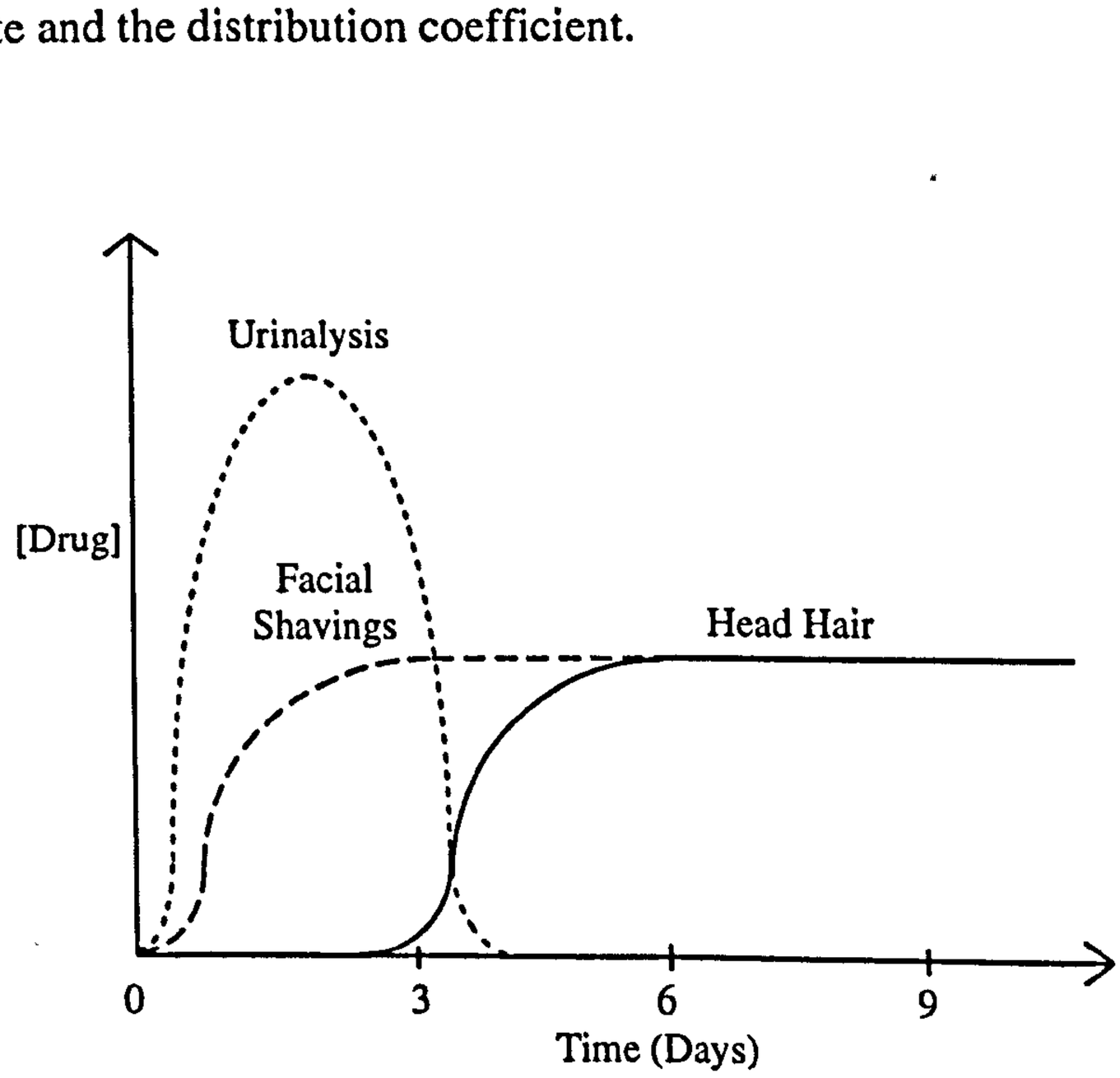


Figure 5.4: *Concentration of Drug Versus Time for Various Matrices*

The most important considerations in developing a hair analysis method are:[162]

- Washing and digestion (extraction).
- Measurement of the metabolite : drug ratio.
- Reproducibility of results with a second sample.

Wash procedures are used to remove external contamination and are based on the following properties with respect to exogenously applied drugs.

- Cocaine, opiates, amphetamines and marijuana (and their respective metabolites) do not bind strongly to keratin proteins and therefore are readily removed by washing.
- Metabolites are not formed by contact with hair.
- Deposition of endogenous and exogenous drugs in the hair involves three hair domains:

◊ *Surface domain*

This is readily accessible to exogenous drugs, *e.g.* from vapours and powders. However these will be loosely bound and readily accessible to even non hair-swelling solvents *e.g.* EtOH and are removable in this way.

◊ *Accessible interior domain*

This is located in the interior structures of the hair and offers moderate resistance to penetration of exogenous drugs. These are not easily removed by non-hair-swelling solvents but are readily removed by water. As this section of the hair also contains endogenous drugs these too may be removed. However the percentage of endogenous drug will be small in comparison to that found in the inaccessible interior domain. Therefore, washing will not have a significant effect on the overall result.

◊ *Inaccessible interior domain*

This is very rarely accessible to exogenous drugs and aqueous solvents under realistic conditions, and endogenous drugs cannot be easily removed from it with the use of solvents. In general, it is the largest of the three domains and depends

on the type of hair and its condition. Endogenous drugs are usually released from this domain by liquefaction of the hair specimen.

The high resistance of the inaccessible domain to exogenous drugs, their ready removal from the accessible interior domain and surface domain and the absence of metabolic formation by contact, all combine to greatly reduce the possibility of evidentiary false positives. In contrast, the probability of evidentiary false positives with BFA is greatly increased by active inhalation of drug vapours, by ineffective barriers to drug penetration in the lung and gastrointestinal tract, and by the formation of metabolites.

5.4.2 Wash Procedures

Two types of wash procedure are adopted in hair analysis:[162]

- Extended wash
- Truncated wash

Extended washes are continual washes which are carried out until a plateau drug concentration has been reached. The duration and number of washes is determined by the results obtained with a particular hair specimen and therefore, involves the analysis of wash solutions while the wash procedure is being performed.

Truncated wash procedures involve a fixed number of washes over a fixed duration. The results can be mathematically related to the extended criteria. This type of wash is used for mass production testing whereas, the extended wash procedure is used for individual forensic specimens.

In general, wash procedures consist of two steps. A solvent wash is carried out first (usually with an alcohol *e.g.* MeOH, EtOH) to ensure that and oils or greasy hair applications do not diminish the efficacy of the wash procedure and to remove loosely adhering drugs from the surface domain. This is followed by aqueous washings, which allow the hair to swell and thus allow penetration into the accessible interior domain. A final rinse in solvent may be advisable as this will facilitate drying of the hair.

5.4.3 Digestion / Extraction Procedures

The rate of extraction is firstly due to the solubility of the analyte in the supercritical fluid and secondly due to transfer of the mass of the solute outside of the matrix. There are four mechanisms of mass transport.

1. Diffusion of analyte across internal volume of sample.
2. Desorption from surface of sample.
3. Diffusion of analyte across boundary layer on surface of sample.
4. Transfer into core of supercritical fluid.

If step 1 is predominant then the extraction is dependant on particle size and therefore pulverisation will consequently increase the rate of extraction. If steps 2 or 3 are more important then the extraction will be improved by addition of a more polar modifier.

The typical analysis is therefore a four stage process:

1. Wash - elimination of contamination.
2. Pre-treatment *e.g.* elution by aqueous solvent, hydrolysis.
3. Extraction *e.g.* LLE, SPE.
4. Analysis *e.g.* GC/MS.

If solvent extraction procedures are used, complete extraction of the drugs from the hair cannot be guaranteed since the extraction efficiency depends on the physical properties of the hair. The amount of melanin present, may give rise to racial or colour biases especially if the major portion of the drug is in the melanin granules. In some digestion methods the melanin is removed and the drugs are not released from this fraction during digestion.[197]

Sample preparation techniques and procedures for hair analysis are often time consuming, costly and can require the use of toxic solvents. For example:[198]

- Acid hydrolysis requires an 18 hour incubation period.
- Enzymatic methods use β-glucuronidase and aryl-sulphatase which are expensive.

- The presence of 6-MAM is conclusive proof of heroin intake however 6-MAM is hydrolysed to morphine under alkaline conditions meaning that it is not possible to distinguish between drug abuse and medical intake.
- Direct methanolic extraction requires 5 hours sonication.

As an alternative to the current wet extraction methods, there has been an increasing interest in the use of SFE for the extraction of drugs from hair samples. By using an SFE system for the analysis of drugs washing, pretreatment, extraction and final analysis can be carried out using the same apparatus, thus minimising many of the potential problems associated with other techniques.[199]

5.4.3.1 Benzodiazepine Hair Analysis

The detection of benzodiazepines in human hair (the most abused pharmaceuticals world-wide)[200] appears mostly undocumented. At the time of this study, only five papers were found to report their detection. Diazepam was readily found in hair samples,[201,202] however, alprazolam and lorazepam were not detected in patients receiving therapeutic doses of these drugs.[203] Yegles *et al*[200] detected diazepam, desmethyldiazepam, oxazepam, flunitrazepam, lormetazepam and lorazepam in the hair of deceased polydrug addicts. Other benzodiazepines which have been detected include flunitrazepam and alprazolam.[204,205]

5.4.3.2 Opiates Hair Analysis

Hair analysis for opiates has been around for almost three decades however, it was not until 1985 that RIA findings could be supported by a second independent method as demanded by the courts.[206] Initially, it was assumed that the hair had to be totally dissolved and then, the drugs determined quantitatively in order to differentiate between heroin and codeine consumption. The hair samples were dissolved in NaOH and then treated in the same way as urine samples. However, not all illegal drugs can withstand NaOH treatment therefore, a method was developed whereby the hair was completely dissolved using enzymes and the solution extracted using Bond Elut Certify® columns. More recently, procedures have been developed where the hair is only partially dissolved. Several methods for this exist in the

literature. Of these, two are used routinely, one developed by Moeller and Fey[207] and the other developed by Kauert *et al.*[208]

5.4.3.3 Methadone Hair Analysis

Hair analysis with methadone is of extreme importance as it provides a means of monitoring the compliance of patients on methadone maintenance programmes and also allows for the detection of other non-prescribed drugs. In the case of urinalysis a positive morphine in urine may indicate that a small amount of heroin was used by the patient the night before the test or that the patient had been regularly using heroin but had thought it wise not to do so for a few days leading up to the test. By using hair analysis, the difference between these two patterns of use can be determined.

Hair analysis for methadone in maintenance clinics has been incorporated since the early 1990's.[209] Recent literature on the methodology usually involves a basic hydrolysis procedure with GC/MS as the favoured analysis technique.[210]

5.4.3.4 SFE Hair Analysis

The use of supercritical fluids in the extraction of drugs from hair was first reported in 1992 by Sachs and Uhl.[211]. The quantitative determination of opiates in hair using SFE was also first investigated by Sachs.[212] Hair was collected from sixteen subjects who had died after a fatal heroin overdose. Levels of morphine detected ranged from 0.17-1.54 (0.69) ng/mg and 6-MAM 0.15-14.90 (3.78) ng/mg. Cocaine and cannabinoids were also extracted using the same procedure. In 1994, Edder *et al*[213] also investigated the possible use of SFE for the extraction of opiates from drug addict hair samples while Morrison *et al*[214] investigated their use for the extraction of cocaine. Both types of drugs were extracted effectively with high recoveries. More recently, in 1995, a method for the supercritical fluid extraction of opiates form hair was also investigated by Cirimele *et al.*[198] They extensively investigated the effect of parameters such as temperature and modifier volume on the extraction recovery of morphine and 6-MAM. In 1996, Staub *et al*[199] applied a modified version of the method by Edder *et al*[213] to the extraction of opiates and

methadone. Three cases were successfully analysed for the presence of morphine 6-MAM and methadone.

5.4.4 Avoiding False Positives

5.4.4.1 Contamination

Hair analysis allows a trilevel reporting system to be used.

- No drug use.
- Drug use.
- Contaminated but no drug use.

This is useful in criminal justice type situations where neither drug use or being in the presence of drugs is permissible for parolees or probationers.

Contamination can be divided into two subsections

a) Trivial contamination

This is characterised by contamination levels exceeding the endogenous cut-off values by a small amount and is reported as being either zero or trivial. This situation may arise when non-drug user is constantly in the presence of drug users

b) Extensive contamination

This is characterised by contamination levels exceeding the cut-off levels by a large margin. This situation arises from individuals who are frequently exposed to high levels of drugs *e.g.* non-drug using drugs dealers or manufacturers.

5.4.4.2 Measurement of Metabolites

This is used in conjunction with the other methods to avoid evidentiary false positives. Metabolites can sometimes be formed by non-metabolic processes (*e.g.* the hydrolysis of cocaine to benzoylecgonine) therefore, the preferred method is to measure the metabolite :

drug ratios rather than simply the concentration of the metabolite. Thus, the use of the wash procedures is still very important and allows monitoring of the contribution that the hydrolysis process may make in the digestion. In the case of cocaine, a hydrolysis control is always included.[215]

With opiates, a number of metabolic approaches may be taken to distinguish between external contamination and drug use. Morphine is a metabolite of heroin and the presence of monoacetyl morphine has been reported in hair. The most convenient and sensitive metabolite procedure is based on the detection of morphine glucuronide. This involves the measurement of the increase in free morphine following hydrolysis by glucuronidase.

Due to the unfavourable signal to noise ratio of endogenous : exogenous cannabinoids in hair, wash procedures have no use. Instead, the use of cannabis is determined by the presence of 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid (Δ9-THCCOOH) which has not been found to be present in marijuana smoke.

It is important to realise that the determination of accurate metabolite : drug ratios as well as the endogenous cut-off levels, depend critically on effective wash procedures therefore, the measurement of metabolites is not a substitute for effective washings. Similarly, good wash procedures critically determine the reproducibility of results with a newly collected specimen. Reproducibility of results is a very important criteria for excluding external contamination since contamination is usually characterised by highly variable results.

5.5 Method Development For Hair Analysis

Method development of hair samples was carried out using hair samples obtained from persons known not to have used any drugs being investigated in this study. The hair samples were prepared and spiked with the appropriate standards as described in the following sections.

5.5.1 PREPARATION OF HAIR STANDARDS.

5.5.1.1 WASH PROCEDURE

In order to remove grease and cosmetic preparations, which might be present in the hair samples, they were first of all washed using distilled water (dH_2O), MeOH, dH_2O and $CHCl_3$. At each wash stage, the appropriate solvent was added to the hair and sonicated for 15 minutes at room temperature. The solvent was then decanted off and the next solvent added. Once the $CHCl_3$ wash had been carried out, the samples were dried overnight in an oven at 60 °C.

5.5.1.2 GRINDING PROCEDURE

In order to maximise the extraction from authentic forensic hair samples, it was decided to grind the hair samples to provide the largest possible surface area to particle size ratio. Several methods were attempted for this. The first method which was tried was the use of an electric razor. This method was found to be extremely time consuming and wasteful of sample. In addition, the best method for "fixing" the hair in order to facilitate shaving was to glue the ends of the hair to a piece of card meaning that this part of the hair was unavailable for analysis. Some of the case samples were to be segmented and this method would mean that the shortest length which could be analysed would have to be at least 5 cm long and thus, no roots could be ground for analysis.

As an alternative method of grinding, a Janke and Kunkel electric grinder was tested. However, when hair samples were placed in the grinding chamber, they wound round the central axis and did not grind. After consulting with the manufacturers, a star shaped cutter was purchased which when used with $CO_{2(s)}$ produced a fine powder which was similar to that achieved by the shaving method but was less wasteful of sample and less time consuming. Approximately 5 g of blank powdered hair were obtained using this method.

Additional hair for standards was obtained from the beard shavings of a person also known not to use any of the drugs of interest to this study. Although this hair was from a different source and may be contaminated with some skin fragments, the wash procedure developed

for it was more rigorous including two additional MeOH washes and a DCM wash. The results obtained from spiked samples were the same.

5.5.2 Hair Spiking Procedure

The powdered hair samples were spiked with MeOH standards of the drugs of interest as follows.

For a 1ng/mg standard, 100 µl of the standards to be used (*e.g.* temazepam, diazepam and desmethyldiazepam for the benzodiazepines in hair) at a concentration of 1 mg/L was added to the powdered hair (100 mg) in a glass vial. 500 µl of MeOH was added to ensure that all the hair was coated and the vial sealed and sonicated before being placed in an oven at 60 °C for 30 minutes. The vial was then sonicated again at room temperature for 10 minutes and left to soak overnight. The vial was then opened and the hair allowed to dry at room temperature.

Although the use of this method of spiking was not ideal as most of the drug would be on the surface of the hair and not incorporated into the hair structure, an additional wash step was not used as this would mean that the exact concentration of the drugs in the hair standards would not be known.

Internal standards were used for all extractions (prazepam for the benzodiazepines, d_3-morphine for opiates and d_3-methadone for methadone) and were added to the hair either prior to insertion of the hair into the extraction cell or at the modified static extraction step as discussed later.

5.6 Extraction Methodology

For the analysis of benzodiazepines, opiates and methadone in hair, the method reported by Cirimele *et al*[198] for the determination of opiates in hair was attempted and found to be successful. The next sections detail how this method was introduced to the system and describes the method development which was subsequently carried out.

5.7 METHOD DEVELOPMENT FOR THE EXTRACTION OF BENZODIAZEPINES FROM HAIR

5.7.1 PRELIMINARY EXTRACTIONS

5.7.1.1 EXPERIMENTAL

For this section the same three benzodiazepines which had been determined in blood and vitreous humor were investigated. Spiked hair samples were prepared at ~20 ng/mg by pipetting 100 µl of 20 µg/ml standards onto 100 mg of hair.

Once dried, these hair standards were extracted as follows:

Cleanup: CO_2 pumped for 20 minutes

Extraction: MeOH : Et_3N (85:15 v/v) introduced at 10% for 30 minutes.

Temperature: 100 °C

Pressure: 3500 psi

Flowrate: 2.0 ml min^{-1}

As before, the extracts were collected into MeOH in a HypoVial® at the flow outlet. The extracts were then evaporated to dryness under a stream of nitrogen and reconstituted in 100 µl of mobile phase. 25 µl was then injected onto the HPLC system using the previously described conditions (Section 2.12).

5.7.1.2 RESULTS AND DISCUSSION

Some pressure problems were encountered with the modifier, possibly due to the added water in the system. However, by opening and closing the pressure regulator throughout the extraction it was possible to keep the pressure reasonably constant.

The first run of temazepam from hair was done without the addition of prazepam to the extraction. An area of 367611 units was obtained for temazepam extracted from hair as compared to the unextracted standard at 357771 units corresponding to a recovery of 103%. This high recovery indicated that the extraction was sufficiently successful. It should also be noted that the initial extractions were carried out without the use of static

mode and the extractions were 15 minutes with CO_2 only and 25 minutes with CO_2: MeOH: Et_3N (90:8.5:1.5).

5.7.2 Extraction of Temazepam, Desmethyldiazepam and Diazepam

5.7.2.1 Experimental

Spiked benzodiazepine hair (105.2 mg) was prepared at 23.0 ng/mg temazepam, 20.2 ng/mg desmethyldiazepam and 26.2 ng/mg diazepam. Two aliquots of this hair (37.7 mg and 26.6 mg) were analysed using the aforementioned method with the static mode in operation as described later in section 5.8. No drugs were seen to elute in the CO_2 extracts. The prazepam was added with the modifier to the extraction cell. An unextracted standard of the benzodiazepines, at the concentration the hair was spiked at, was also injected onto HPLC in order to calculate the recovery of each of the drugs

5.7.2.2 Results and Discussion

The results obtained for the extraction of temazepam, desmethyldiazepam and diazepam are shown below in Table 5.1.

Table 5.1: *Extraction of Temazepam, DMD and Diazepam from Spiked Hair*

	Temazepam PAR	DMD PAR	Diazepam PAR
Std 1: 37.7 mg	0.197	0.155	0.320
Std 2: 26.6 mg	0.118	0.091	0.216
Unextracted	0.958	0.802	1.198
[Std 1]	13.2 ng/mg	10.9 ng/mg	19.6 ng/mg
[Std 2]	11.2 ng/mg	9.1 ng/mg	18.7 ng/mg
[Mean] % Recovery	12.2±1 ng/mg 53±4 %	9.97±0.9 ng/mg 49.5±4.5 %	19.14±0.4 ng/mg 73±1.5 %

It should be noted that the standards were added to the hair after the initial weight had been recorded but prior to weighing into the extraction vessel and thus, the weights reported will be for the hair in addition to the drugs and any residual MeOH. A check of the contribution of the weight of drug was carried out by adding 100 µl of temazepam to a blank hair sample which had been weighed at 32.6 mg. On reweighing, the hair weight was found to be 34.9 mg which corresponds to an increase of 2.3 mg. If this is taken into

account for all three drugs being added the approximate error in weight is 6.9 mg (6.6 %). Thus, the weight of the 1st standard will be 35.2 mg and the 2nd standard 24.9 mg making the recoveries for temazepam, DMD and diazepam 57 %, 53 % and 78 % respectively.

5.7.3 Effect of Temperature on the Extraction of Temazepam from Hair

5.7.3.1 Experimental

A temperature recovery study was carried out for temazepam and prazepam at 60, 70, 80, 90 and 100 °C. In addition to using MeOH:Et_3N:H_2O as a modifier, EtAc was attempted at each temperature. In each case 300 µl of modifier were added directly to the extraction vessel.

5.7.3.2 Results and Discussion

The results for the extraction of temazepam from hair using MeOH:Et_3N:H_2O (mod) and EtAc as modifiers are shown in Table 5.2.

Table 5.2: *Effect of Temperature and Modifier on the Extraction of Temazepam*

Run	Peak Height Temazepam	Peak Height Prazepam	Temaz/Praz	% Recovery Temazepam
Unext. Std.	10.30	7.15	1.44	n/a
60 Mod	5.10	4.90	1.04	49.5
60 EtAc	2.05	0.95	2.16	19.9
70 Mod	1.20	2.15	0.56	11.7
70 EtAc	5.65	3.20	1.77	54.9
80 Mod	1.10	1.30	0.85	10.7
80 EtAc	10.40	7.00	1.49	101
90 Mod	4.00	3.30	1.21	38.8
90 EtAc	4.10	1.60	2.56	39.8
100 Mod	0.35	0.25	1.40	3.4*
100 EtAc	2.80	1.75	1.60	27.2

* Extraction vessel leaked.

From the results in Table 5.2, the best extraction conditions appear to be 80 °C with EtAc as a modifier and 60 °C with MeOH:Et_3N:H_2O as a modifier. However, during the 100 °C

extraction with the latter modifier a leak occurred in the extraction cell and some of the modifier escaped.

5.7.4 Effect of Temperature on the Extraction of Benzodiazepines from Hair with EtAc as a Modifier

5.7.4.1 Experimental

The temperature study previously carried out for temazepam and prazepam only was carried out for temazepam, desmethyldiazepam and diazepam using EtAc as a modifier.

5.7.4.2 Results and Discussion

The results are shown in Table 5.3

Table 5.3: *Recoveries of Temazepam, DMD and Diazepam at Various Temperatures with EtAc as the Modifier*

Run	PAR Temazepam	PAR DMD	PAR Diazepam	Recovery Temazepam	Recovery DMD	Recovery Diazepam
53 °C	0.938	0.954	1.266	65/33	72/37	85/44
67 °C	0.541	0.579	1.043	37/13	44/15	70/25
77 °C	0.733	0.851	1.083	50/26	64/33	73/25
80 °C	2.307	2.453	1.493	159/57	185/69	101/38
100 °C	1.387	1.100	1.600	95/95	83/85	108/104
Unext.	1.453	1.327	1.483	n/a	n/a	n/a

Recoveries x/y: x = recovery based on PAR, y = recovery based on area

From these results it can be seen that for all three drugs the recoveries in terms of PAR and area are best at 100 °C.

5.7.5 Effect of Temperature on the Extraction of Benzodiazepines from Hair with MeOH:Et_3N:H_2O as a Modifier

5.7.5.1 Experimental

The experiment in section 5.3.4 was repeated using MeOH:Et_3N:H_2O as the modifier.

5.7.5.2 Results and Discussion

The results are shown in Table 5.4

Table 5.4: *Recoveries of Temazepam, DMD and Diazepam at Various Temperatures with MeOH:Et_3N:H_2O as the Modifier*

Run	PAR Temazepam	PAR DMD	PAR Diazepam	Recovery Temazepam	Recovery DMD	Recovery Diazepam
50	0.948	0.472	1.339	64/48	35/26	86/66
56	0.798	0.482	1.316	54/40	36/27	84/64
60	0.958	1.335	1.543	64/134	100/208	99/206
86	1.024	0.979	1.423	69/147	73/138	91/173
103	0.773	0.197	0.735	52/30	58/10	47/33
Unext.	1.490	1.340	1.560	n/a	n/a	n/a

This time the best recoveries appear to be at 60 °C, however, the extractions previous to the 60 °C extraction were very low in recovery and thus the high recoveries were possibly due to carryover. For this reason, the extraction at 86 °C was chosen to be the best for using the MeOH:Et_3N:H_2O modifier.

5.7.6 Extraction of an Authentic Hair Sample

5.7.6.1 Experimental

An extraction of an authentic hair sample was carried out using both 100 °C with EtAc and 86 °C with MeOH:Et_3N:H_2O. The extraction at 86 °C worked but the one at 100 °C with EtAc did not, indicating that the EtAc is not a strong enough modifier to remove the drugs from within the hair matrix.

The same case was extracted at 86 °C and 100 °C with the MeOH:Et_3N:H_2O for a comparison at these two temperatures. The results which were obtained at 100 °C were better fitting to results published in the literature and showed less endogenous material being extracted. Thus, although the recoveries appeared to be less at 100 °C, this temperature was chosen for running authentic samples as it was thought that the drugs being extracted were more likely to be from within the hair sample then that at 80 °C.

5.7.7 LINEARITY FOR THE EXTRACTION OF BENZODIAZEPINES FROM HAIR

5.7.7.1 EXPERIMENTAL

Sets of hair spikes were prepared for temazepam, desmethyldiazepam and diazepam over the concentration range of 0.1 to 100 ng/mg in order to determine the linearity over this range for the extraction method. All three drugs were extracted in the same run and samples were run in triplicate.

5.7.7.2 RESULTS AND DISCUSSION

The linearity results for the extraction of temazepam, desmethyldiazepam and diazepam for spiked hair samples is shown in Table 5.5.

Table 5.5: *Extraction of Temazepam, Desmethyldiazepam and Diazepam for Spiked Hair Samples*

Temazepam			Desmethyldiazepam			Diazepam		
Conc^{n} ng/mg	PAR		Conc^{n} ng/mg	PAR		Conc^{n} ng/mg	PAR	
103.2	15.612		95.1	14.564		92.7	17.394	
68.8	11.436		63.4	10.713		61.8	12.596	
34.4	5.741	r^2:0.996	31.7	4.871	r^2:0.996	30.9	6.489	r^2:0.997
17.2	3.102	A:0.170	15.9	2.703	A:0.083	15.5	3.410	A:0.259
8.6	1.258	B:0.155	7.9	1.305	B:0.157	7.7	1.594	B:0.190
3.44	0.461		3.17	0.444		3.09	0.582	
1.15	0.198		1.06	0.174		1.03	0.237	
0.57	-ve		0.52	-ve		0.52	-ve	
0.29	-ve		0.26	-ve		0.26	-ve	

From this, very good linearity was seen over the full range. The lower two concentrations in each case were not detected, thus based on a 50 mg hair sample, the limits of detection for the three drugs are as follows:

Temazepam: 1.15 ng/mg

Desmethyldiazepam: 1.06 ng/mg

Diazepam: 1.03 ng/mg.

5.8 Method Development for the Extraction of Morphine from Hair

5.8.1 Preliminary Check of Methodology

Three extractions of morphine spiked hair were carried out using the same methodology as was used for the initial extraction of temazepam.

All 3 CO_2 cleanup extracts were found to be negative for morphine. For the three modified extracts, the following results were obtained as shown in Table 5.6.

Table 5.6: *Preliminary Morphine Extractions*

Extraction	m/z ratio 429/432	m/z ratio 414/429
1	0.839	0.605
2	0.594	0.652
3	0.687	0.643

Extraction three yielded the largest areas which may be as a result of the extraction being run for a longer time due to a high pressure error during the extraction.

At this point the system was set up for use in the static mode. The Rheodyne set up for operation of the static mode is shown in Figure 5.5.

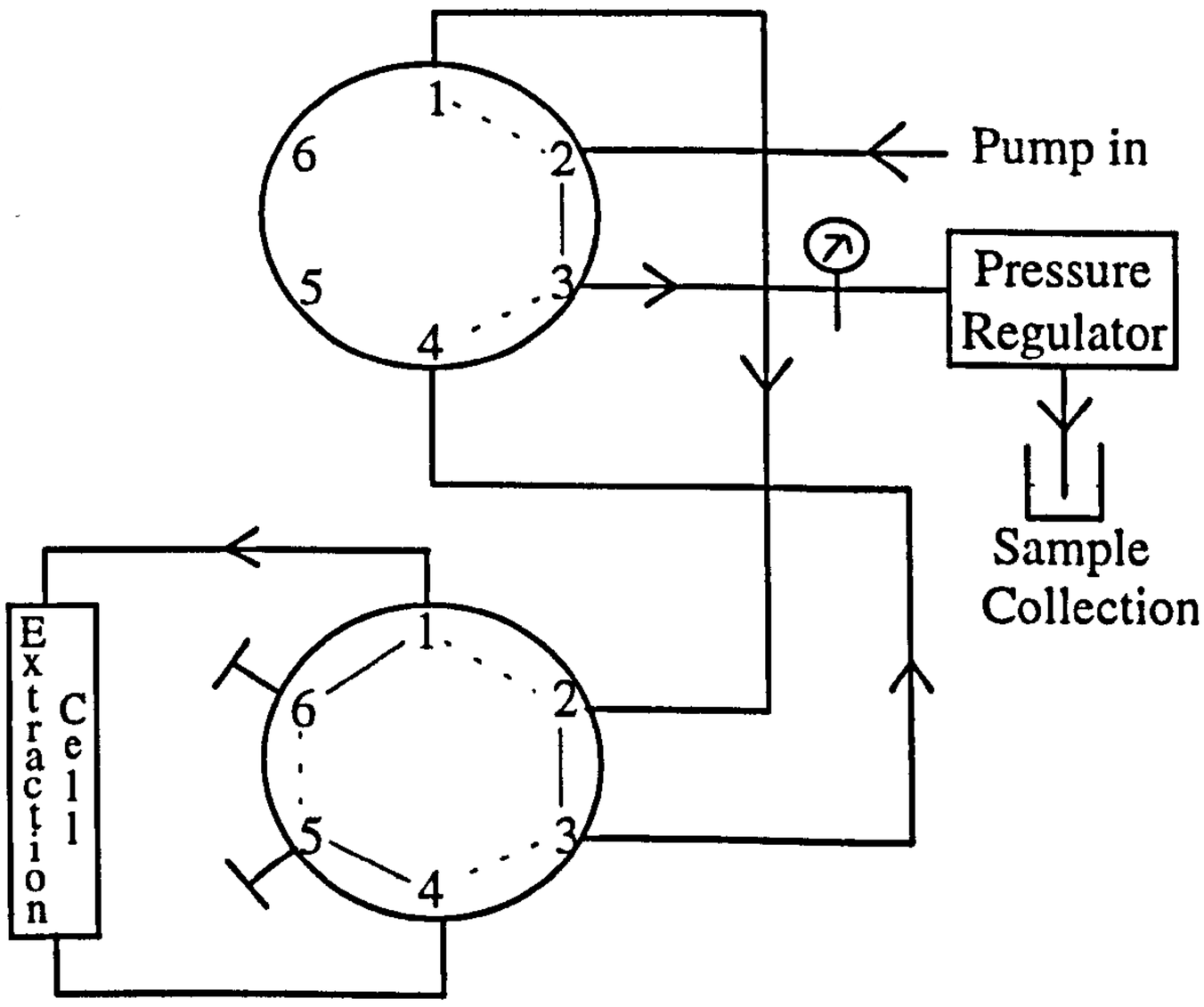


Figure 5.5: *Schematic Representation of Static SFE Set-up*

When the first (top) Rheodyne is at the load position, flow passes from position 2 to position 3 and bypasses the second Rheodyne. When the first Rheodyne is in the inject position, flow is diverted from position 2 to position 1 and then to position 2 in the second (bottom) Rheodyne. In order to carry out a static extraction, the Extraction cell is pressurised by placing the second Rheodyne in the inject position. Once the required pressure has been reached, the second Rheodyne is switched to the load position and flow is diverted back to position 4 in the first Rheodyne, *via* position 3 to the pressure regulator and the collection vessel. Once the static extraction has been carried out for the required length of time, the second Rheodyne is put to the load position and the supercritical fluid and any extracted material from the extraction cell, flushed out.

5.8.2 Effect of Temperature on the Extraction of Morphine from Hair (Internal Standard Added Pre-Extraction)

5.8.2.1 Experimental

A temperature investigation was carried out for the extraction of morphine from spiked hair. 10 spiked hair samples were prepared with 100 µl of morphine and d_3-morphine and extractions carried out in duplicate at 60, 70, 80, 90 and 100 °C.

5.8.2.2 Results and Discussion

The results are shown in Table 5.7. It should be noted that the hair was not added to the extraction column for the second extraction at 100 °C and therefore no result has been reported.

Table 5.7: *Temperature Recovery Study for Morphine Spiked Hair*

	Relative Recovery (Ext/Unext) n=3			Ratio Average	
Run	m/z 432	m/z 429	m/z 414	m/z ratio 414/429	m/z ratio 429/432
Unext. Std.	na	na	na	0.520	1.146
60/1	14	5	10	1.105	0.403
60/2	19	15	16	0.587	0.866
70/1	126	106	105	0.535	0.967
70/2	40	33	37	0.592	0.946
80/1	75	60	64	0.582	0.909
80/2	163	146	137	0.509	1.021
90/1	30	26	28	0.504	0.975
90/2	42	39	40	0.556	1.065
100/1	103	98	98	0.541	1.095

From these results, although the best recoveries appear to be at 80 °C, at 100 °C the recoveries are still high and the ratios more comparable to the unextracted standard.

5.8.3 Effect of Temperature on the Extraction of Morphine from Hair (Internal Standard Added Post-Extraction)

5.8.3.1 Experimental

This study was repeated but this time the internal standard was added post extraction to obtain absolute recoveries. It should be noted that when the internal standard is added directly to the weighed hair, losses in transferring the hair to the extraction vessel do not effect the results. However, transfer losses will decrease the recoveries when the internal standard is added post extraction.

5.8.3.2 Results and Discussion

The results are shown in Table 5. 8.

Table 5.8: *Temperature Study Results with d_3-Morphine Added Post-Extraction*

Run	m/z ratio 414/429	m/z ratio 429/432	% Recovery
Unext. Std.	0.498	1.252	n/a
60/1	0.754	0.114	9.1
60/2	0.858	0.095	7.6
70/1	0.703	0.158	12.6
70/2	0.651	0.194	15.5
80/1	0.678	0.166	13.3
80/2	0.660	0.146	11.7
90/1	4.750	0.008	-ve
90/2	0.702	0.148	11.8
100/1	0.586	0.229	18.3
100/2	0.847	0.196	15.7

Again from these results, the extraction at 100 °C gives the best recoveries. Based on these results and the results in Table 5.7, it would appear that the losses on transport are high and thus the internal standard should be added directly to the weighed hair.

5.8.4 Direct Comparison of Pre and Post Adding of Internal Standard

5.8.4.1 Experimental

A comparison of d_3-morphine added pre and post-extraction was carried out to compare the two on the same day in the same GC/MS run.

5.8.4.2 Results and Discussion

The results are shown in Table 5.9. These extractions were only run once in order to extract all samples within the same day.

Table 5.9: *Pre- and Post-Extraction d_3-Morphine Addition*

Run	m/z ratio 414/429	m/z ratio 429/432	% Recovery
Unext.	0.501	0.865	n/a
60Post	0.626	0.258	29.8
60Pre	0.433	0.101	11.7
70Post	0.518	0.103	11.9
70Pre	0.472	1.081	125
80Post	0.518	0.103	11.9
80Pre	0.662	0.417	48.2
90Post	0.473	0.857	99.1
90Pre	0.521	1.681	194
100Post	0.504	0.879	102
100Pre	0.513	0.946	109

The pre-extraction recoveries represent how well the PAR of the extracted and unextracted standards compare and the post-extraction recoveries represent the actual recoveries for the extraction. Again from this it was decided to stay with a temperature of 100 °C for the morphine hair extractions.

5.8.5 Introduction of 6MAM to Hair Extractions

5.8.5.1 Experimental

At this point, 6MAM was introduced to the system. A spiked hair sample was prepared as previously described by pipetting 100 µl of morphine (8.5 mg/L) and 100 µl of 6MAM (13.3 mg/L) onto 100 mg of hair. This sample was split into three portions which were then extracted using the developed conditions at 100 °C.

5.8.5.2 Results and Discussion

The results for the extraction of morphine and 6MAM from spiked hair samples are shown in Table 5.10.

Table 5.10: *Extraction of Morphine and 6MAM from Spiked Hair*

Extract	m/z ratio 340/399	m/z ratio 340/432	%Rec.	m/z ratio 414/429	m/z ratio 429/432	%Rec.
Unext.	0.875	1.304	na	0.543	1.097	na
1	0.792	1.216	93	0.468	0.855	78
2	0.843	1.143	88	0.410	1.139	104
3	0.894	1.197	92	0.525	0.991	90

From this it can be seen that the extraction conditions yield good recoveries of the 6MAM from spiked hair. In addition to this, a spiked 6MAM sample with no morphine was prepared and extracted as before to determine if the extraction was hydrolysing the 6MAM to morphine. The ratio of 340/399 was found to be 0.742 and the ratio of 340/432 1.079. No morphine was recovered.

5.8.6 Linearity of Morphine and 6MAM

5.8.6.1 Experimental

Morphine and 6MAM spikes were prepared at 0.085 ng/mg to 85ng/mg and 0.133 to 133 ng/mg respectively in order to determine if the extraction was linear over this range and to determine the limit of detection for each of the drugs. In each case the drugs were spiked onto approximately 50 mg of hair and the d_3-morphine concentration was approximately 5 ng/mg.

5.8.6.2 Results and Discussion

The results for the linearity and limit of detection for morphine and 6MAM are shown in Table 5.11.

Table 5.11: *Linearity and Limit of Detection for Morphine and 6MAM*

Concentration ng/mg	m/z ratio 414/429	m/z ratio 429/432	m/z ratio 340/399	m/z ratio 340/432
0.085.0.133	no peaks	-ve	no peaks	-ve
0.17/0.27	no peaks	-ve	0.698	0.084
0.34/0.53	1.239	-ve	0.853	0.135
0.85/1.33	0.653	0.264	0.842	0.279
2.13/3.33	0.649	0.634	0.796	0.446
4.25/6.65	0.558	1.147	0.742	0.983
8.5/13.3	0.524	2.367	0.831	1.992
17.0/26.6	0.613	4.597	0.739	3.675
25.5/39.9	0.598	6.861	0.856	7.268
34.0/53.2	0.499	10.394	0.816	9.883
85/133	0.515	25.468	0.875	19.684

For morphine, r^2=0.999 and the equation of the line is y=0.301x - 0.200. For 6MAM, r^2=0.987 and the equation of the line is y=0.152x + 0.211. The limits of detection for morphine and 6MAM were taken as 0.85 ng/mg and 0.27 ng/mg respectively for a 50 mg hair sample. Both sets of drug concentrations were found to be linear over the range studied.

5.9 Method Development for the Extraction of Methadone from Hair

At this point methadone method development was still being carried out using the FID and not GC/MS. This caused problems due to extracted endogenous material which the FID was sensitive to as highlighted in Figure 5.6. It should be noted that the methadone and cyclizine elute between 10 and 15 minutes and thus, unless they were present in high concentrations, they would not be seen above the background.

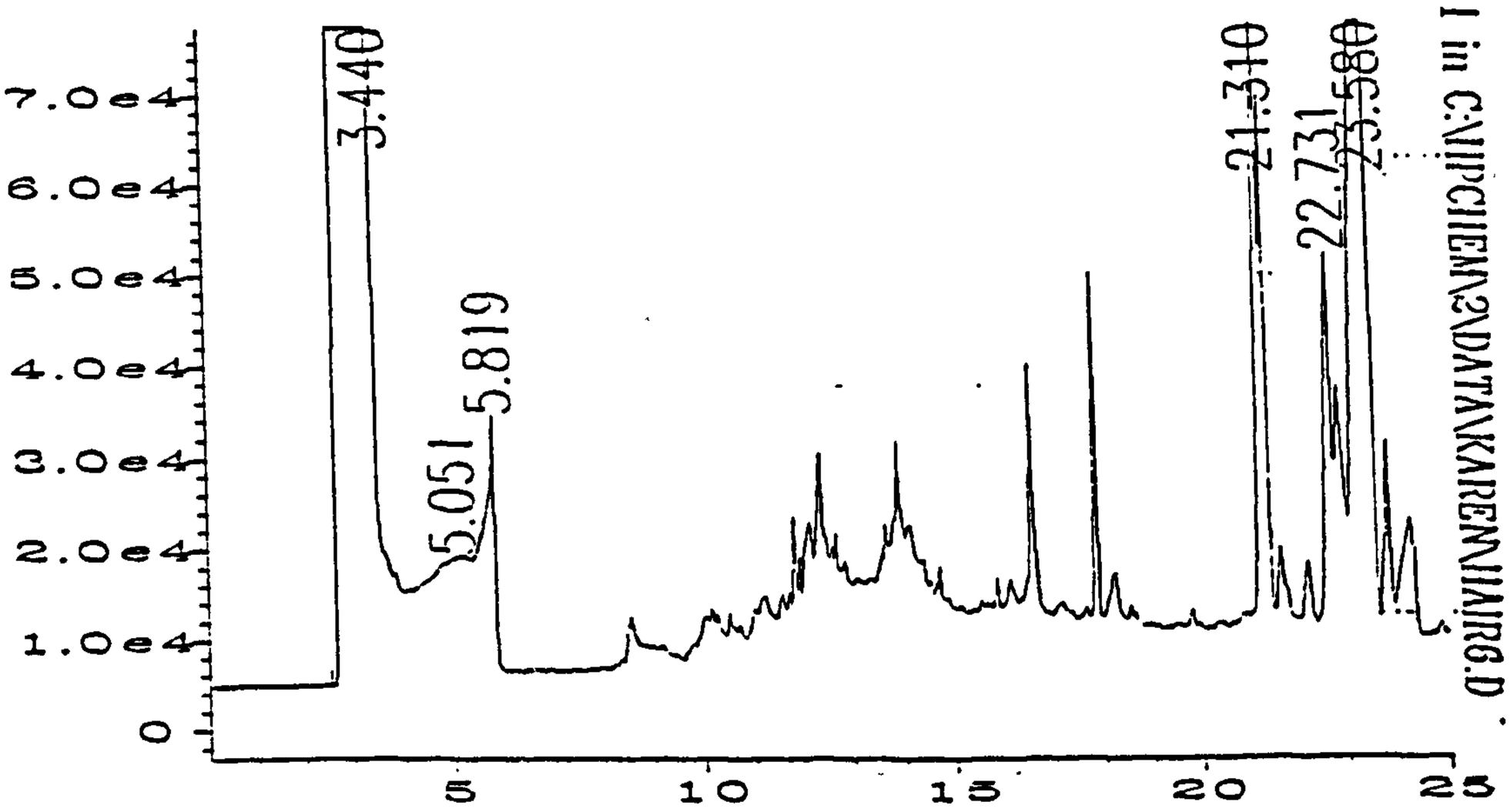


Figure 5.6: *GC/FID Trace of Background Noise for Methadone Extraction*

This endogenous material did not interfere with the morphine on the GC/MS or benzodiazepines on the HPLC/UV. At this point the modifier was being added using the second pump and not directly to the extraction cell. By extracting the modifier on its own, most of the endogenous peaks were still being observed and thus, method development for methadone was switched to the GC/MS. In addition, as the hair had already been spiked with cyclizine this was used as the internal standard and was not detected in any of the traces. Three extractions were carried out using the methodology described previously and methadone was found in all three modified hair extracts. As with the morphine extracts, the CO_2 cleanup extracts were negative.

When the internal standard for methadone was first attempted with d_3-methadone, it was discovered that the wrong internal standard had been ordered from Sigma (deuterated at the wrong part of the molecule). Two spiked samples were extracted and methadone was observed in both cases. However, the methadone peak will contain contributions from

both the methadone and the d_3-methadone and thus even area based recoveries could not be calculated.

As the method development for the benzodiazepines and for the morphine/6MAM had been successful and these drugs were both extracted efficiently by the same method, it was decided to use the method for all three types of drug. A recovery study was carried out over the range 0.112 to 71.68 ng/mg methadone. The results obtained are shown in Table 5.12.

Table 5.12: *Linearity of Methadone in Hair*

Concentration (ng/mg)	m/z ratio 72/75	m/z ratio 72/165
0.112	-ve	no 165
0.56	0.105	0.096
1.12	0.186	0.064
2.24	0.349	0.063
5.60	0.507	0.073
11.2	0.794	0.054
22.40	2.169	0.037
33.60	3.947	0.051
44.80	5.671	0.029
56.0	7.931	0.039

For methadone over this concentration range, the coefficient of correlation was found to be 0.982 with the equation of the line as y=0.135x - 0.599. The limit of detection for a 50 mg hair sample was 0.56 ng/mg.

5.10 Conclusions

For the extraction of benzodiazepines, morphine and methadone from hair samples, the SFE method developed by Cirimele *et al*[198] was successfully applied. Good reproducibility was found within extractions of the same concentration of spiked standard and high recoveries were obtained.

The actual conditions used for the extraction of the hair samples is detailed below:

In order to maximise the surface area, the hair samples were prepared by grinding under liquid nitrogen to produced a fine powder. The hair to be analysed was weighed and transferred to the extraction vessel. The extraction consisted of two stages. First of all a clean up step was employed to remove exogenous compounds from the surface of the hair. This consisted of running the SFE in the static mode for 10 minutes followed by a dynamic extraction for 15 minutes. The extraction cell was then removed from the SFE and 300 µl of modifier (MeOH:Et_3N:H_2O 2:2:1) and 100 µl of the internal standard added. Once the extraction cell had been replaced in the SFE system, a second static extraction was carried out for 15 minutes. This was followed by a dynamic extraction for 20 minutes and the analytes were collected in to MeOH at the flow outlet.

For all steps, the flowrate was 2.0 ml min^{-1}, the pressure was 3500 psi and the temperature 100 °C.

Linearity over a wide range was seen for benzodiazepines analysed by HPLC and for morphine and methadone analysed by GC/MS. The detection limits are low enough to permit detection of the drugs in hair even when the sample size is relatively small (temazepam: 1.15 ng/mg, desmethyldiazepam: 1.06 ng/mg, diazepam: 1.03 ng/mg, morphine: 0.85 ng/mg, 6MAM: 0.27 ng/mg, methadone: 0.1 ng/mg based on a 50 mg hair sample).

It terms of comparison with other methodology for the extraction of drugs from hair, the method offers several advantages. Firstly, all three drug types were extracted using the same method without degradation. In addition the total extraction time was 1 hour which makes the extraction much quicker than conventional methods *e.g.* acid hydrolysis requires an 18 hour incubation and methanolic extractions require 5 hours sonication.

"Of all the forms by which human nature may be overcome the most detestable is that of poison, because it can of all others be the least prevented by manhood or forethought."

Mr Justice Avory, 1924

6. CASE STUDIES

6.1 INTRODUCTION

The ultimate aim of any method developed for toxicological analysis is its application to authentic samples. Method development for the extraction of an analyte from a spiked sample is the best way of imitating an authentic sample however, in reality, other factors will come into effect when an authentic sample is put through the developed extraction process. Of particular importance to SFE is the effect that other analytes or matrix artefacts will have on the extraction efficiency of the analyte of interest.

6.2 CASE INFORMATION

For this study 48 post-mortem cases were investigated. Samples were obtained for suspected drug abuse cases from the pathologist in charge of the postmortem. Blood, vitreous humor and hair samples were requested and in most of the cases all three sample types were made available. Summary information on each of the cases investigated, including the samples made available is given in Table 6.1. A more detailed description of the circumstances surrounding the death is given in section 6.2.

Hair samples were taken from the posterior vertex and were about the thickness of a pencil. Initially the roots and tips of the hair samples were not kept aligned and this made sectioning of the hair very time consuming. After a visit to the mortuary it was decided that the easiest way to keep the roots of the hair together was to tie a piece of string round the centre of the sample which had been pulled and then place the tied hair sample in a glass universal container.

Blood and vitreous humor samples were also collected in glass universal containers however, no information was made available on the site of blood sampling and whether the vitreous came from one or two eyes.

Table 6.1: *Case Summary Information*

Case	Age/Sex	#Days Died-PM	Cause of Death (As PM report)	Suspected Drugs	Hair	Vit. Humor	Blood	# of Drugs	Alc
K96 01	24/F	12.1.96	a)PO & C b)Prothiaden and propanolol OD	propanolol, cannabis, prothiaden, speed, Valium®	✓	x	x	2	x
K96 02	26/M	6	a)I.G.C. b)Morphine and alcohol intox.	temazepam, procyclidine, Temgesic, heroin	✓	✓	✓	1 O	✓
K96 03	34/M	12.1.96	a)AFDL b)acute alcohol intox.	cocaine, heroin, alcohol	✓	✓	✓	3 O	✓
K96 04	23/M	12/1/96	a)PO & C b)Drug related death	Librium®, nitrazepam, DHC, oxazepam, diazepam, insulin	✓	x	✓	5 OB	✓
K96 05	36/M	16/1/96	a)Pneumonia b) IVDA	methadone, heroin, benzodiazepines	✓	x	x	1 M	x
K96 06	22/M	17.1.96	a)I.G.C.	nitrazepam, DHC, heroin, cannabis	✓	x	x	1 M	x
K96 07	34/F	3	a)PO & C b)Heroin and temaz. intox.	heroin, Diconal® temazepam, Librium®, methadone	✓	✓	✓	3 OB	x
K96 08	39/M	5	a)Septis syndrome b)Chronic IVDA	methadone, diazepam, cocaine	✓	✓	✓	3 BM	x
K96 09	29/M	16.2.96	a)Morphine and temaz. intox.	heroin	✓	x	x	2 BM	x
K96 10	38/M	6	a)PO & C b)Heroin intox.	alcohol, cannabis, heroin	✓	✓	✓	1 O	x
K97 11	27/F	21.3.96	a)I.G.C. b)Drug related death	heroin	✓	✓	✓	4 OB	x
K96 12	30/M	21.3.96	a)PO & C b)Morphine intox.	solvents, sulpiride	✓	✓	✓	1 O	x
K96 13	25/M	18.7.96	a)PO & C b) Drug related death	diazepam, heroin	✓	✓	✓	4 BM	✓
K96 14	25/M	6	a)PO & C b)Heroin intox.	none	✓	✓	✓	3 OB	✓
K96 15	35/F	9	a)PO & C; b)Methadone intox.	methadone, cannabis, heroin	✓	✓	✓	4 BM	✓
K96 16	30/F	29.7.96	a)PO & C; b)Heroin intox.	heroin	✓	✓	✓	2 O	x
K96 17	26/M	3	a)Morphine overdose	heroin, procyclidine, depixol, losac	✓	✓	✓	1 O	✓

Case	Age/Sex	#Days Died-PM	Cause of Death (As PM report)	Suspected Drugs	Hair	Vit. Humor	Blood	# of Drugs	Alc
K96 18	33/M	7	a)Morphine overdose	heroin, temazepam, methadone	✓	✓	✓	4 **OBM**	x
K96 19	32/M	3	a)I.G.C. b)Heroin intox.	heroin	x	✓	✓	1 **O**	x
K96 20	39/M	7	a)PO & C; b)heroin intox.	cannabis, heroin	✓	✓	✓	3 **OB**	x
K96 21	29/F	5	a)PO & C b)Heroin intox.	methadone, heroin, temazepam, diazepam	✓	✓	✓	5 **OBM**	x
K96 22	27/F	7	a)PO. b)Heroin and temaz intox.	methadone, heroin	✓	✓	✓	4 **OBM**	x
K96 23	17/M	4	a)I.G.C. b)Methadone, diaz., cannabis & alcohol intox.	DHC, Valium®, cannabis, alcohol	✓	✓	✓	4 **BM**	✓
K96 24	20/F	21.10.96	a)Methadone, diazepam & temazepam intoxication	methadone, temazepam, heroin, paroxetine, diazepam, ibuprofen, loprazolam	✓	✓	✓	4 **BM**	x
K96 25	25/M	7	a)I.G.C. b)Morphine & diaz.intox.	heroin, Valium®, Librium®, DHC, LSD, cannabis, temgesic, temazepam	✓	✓	✓	3 **OB**	x
K96 26	24/M	3	a)Asphyxia due to hanging	heroin	✓	✓	✓	0	x
K96 27	26/M	8	a)Morphine & temaz. intox.	heroin, DHC, temazepam, methadone	✓	✓	✓	4 **OB**	✓
K96 28	21/M	4	a)PO & C b)Morphine intox.	oxazepam, nitrazepam	✓	✓	✓	3 **OB**	x
K96 29	33/M	5	a)AGC b)Heroin and temaz. intox.	temazepam, heroin	✓	✓	✓	3 **OB**	x
K96 30	17/F	10.12.96	a)I.G.C.related b)Drug related death	heroin, temazepam, diazepam	✓	✓	✓	3 **B**	x
K97 01	37/F	12	a)Gastro-intestinal haemorrhage: b)Methadone intox	heroin, alcohol	✓	x	x	2 **M**	✓
K97 02	35/M	5	a)Methadone, heroin, temaz. & diaz. intox	diazepam, methadone	✓	x	x	5 **OBM**	x

Case	Age/Sex	#Days Died-PM	Cause of Death (As PM report)	Suspected Drugs	Hair	Vit. Humor	Blood	# of Drugs	Alc
K97 03	23/M	14	a)I.G.C. b)Methadone and TCE intox	methadone, Valium®	✓	x	✓	2 **M**	x
K97 04	24/M	12	a)PO & C b)Heroin intox	heroin, diazepam, Voltarol®, Remedeine®	✓	x	✓	1 **O**	x
K97 05	19/M	10	a)Hanging	None	✓	✓	✓	1	✓
K97 06	26/M	7	a)PO & C b)Heroin & temazepam intoxication	trazodone, cannabis, temazepam, Valium®	✓	✓	x	5 **OB**	✓
K97 07	40/F	8	a)PO&C b)Heroin intox.	heroin	✓	✓	✓	1 **O**	✓
K97 08	29/M	4	Unascertained	heroin, methadone	✓	✓	x	5 **OB**	✓
K97 09	32/F	6	a)PO & C b)Heroin & temaz. intox	alcohol, heroin	✓	✓	✓	2 **OB**	✓
K97 10	30/M	5	a)Drug related death	cannabis, cocaine, temazepam	✓	✓	✓	9 **OB**	x
K97 11	30/F	5	a)Chlor., diaz. & morphine (heroin) intox.	heroin, temazepam, diazepam	x	✓	✓	4 **OB**	x
K97 12	29/M	11	a)PO&C b)Heroin, diaz. & alcohol intox.	heroin, methadone, diazepam	✓	x	✓	3 **OB**	✓
K97 13	18/M	5	a)I.G.C.	methadone, heroin	✓	✓	✓	2 **M**	✓
K97 14	27/F	7	a)A.G.C.	heroin	✓	✓	✓	2 **OB**	x
K97 15	29/M	6	a)A.G.C b)Heroin intox.	alcohol, DHC, Coproxamol, Codydramol	✓	✓	✓	2 **O**	✓
K97 16	20/M	4	a)Heroin & diaz. intox.	heroin, amitryptiline	x	✓	✓	3 **OB**	x
K97 17	35/M	12.6.97	a)Alcohol, diaz. & heroin intox.	heroin, alcohol	x	✓	✓	2 **OB**	✓
K97 18	36/M	12.6.97	a)Heroin & methadone intox.	heroin	x	✓	✓	1 **M**	x

Abbreviations: M: male; **F**: female; **PO&C**: pulmonary oedema and congestion; **AFDL**: acute fatty degeneration of the liver; **A/IGC**: aspiration/inhalation of gastric contents; **DHC**: dihydrocodeine; **O**: opiate; **B**: benzodiazepines; **M**: methadone; #: number; Alc: alcohol; ✓ : detected; x: not detected

A graph of the range of ages, for each sex was plotted and is given in Appendix III.

6.3 Circumstances Surrounding Death and Other Relevant Information

The following section summarises the information available from the post-mortem and police reports for each of the cases examined.

K96 01

Multiple needle puncture marks (NPMs) on right side of neck (post-mortem), front of right shoulder, groin, and arms. History of taking cannabis and speed. Use of harder drugs denied. Diabetic (perhaps explains needle marks). Recently prescribed Valium®. On night of death swallowed most of the contents of Propanolol and Prothiaden bottles. Taken to hospital hypotensive and unresponsive. Given Gelofusion and Inotrapses to raise blood pressure and Boprenailin, adrenaline and Sluragon intravenously. Resuscitation proved unsuccessful. Died in hospital.

K96 02

History of long-standing schizophrenia. Known intravenous drug abuser (IVDA). Admitted injecting temazepam and temgesic 6 years ago. Denied the use of other drugs at this time. On Procyclidine for schizophrenia (5 mg, twice daily). Thought to have been supplied heroin. Two possible fine needle marks on right antecubital fossa. Died at home with syringe still in hand. Last seen previous evening.

K96 03

Known to have abused heroin for 14 years and also thought to abuse cocaine. Alcohol abuse for 5 years. Previous heroin overdose. Not on any medication at time of death. No evidence of needle puncture marks. Drinking heavily on day of death and complaining of stomach pains. Returned home 1900 drunk and left to sleep in the recovery position. Found dead in the morning 0855.

K96 04

Diagnosed diabetic. Severe insulin dependence. Recently admitted to hospital with diabetes ketoacidosis, but failed to go back for check ups. Received one months supply of insulin and Valium,® did not reattend clinic for more. Records show the deceased to be a registered drug abuser as late as one month prior to death. No evidence of needle puncture

marks. Complained of feeling unwell night before death but refused medical attention. Found dead the next morning in bed.

K96 05

Infected injection site on right arm. Thrombosed veins on left arm. Signs of injection in both sides of groin. Previous year admitted to hospital with abscesses and blood clots in legs and groin. General poor health due to drugs. On a series of programmes for 12 years to curtail drug problem. On methadone programme but failed to pick up last prescription. Admitted still abusing heroin and benzodiazepines. Last seen 16 days before being found dead at home. Hypodermic syringe, needles, sterilised pads, Abdine powders and heroin found in the vacinity.

K96 06

No fresh or old needle puncture marks identified. Thought to smoke heroin and cannabis but not an IVDA. Six months prior to death attended surgery with respect to drug dependency problem with heroin and cannabis. Administration of dihydrocodeine (DHC) since then. Thought to be overcoming drug problem. One month prior to death, suffered sleeping difficulties and prescribed nitrazepam. On night of death, went to bed at around 2130. Found dead in bed the next morning

K96 07

No petechial haemorrhages. Needle marks and scarred sinuses in groins. Recent NPM on right side. Both lungs oedematous and congested. Rigor beginning to wear off. In prison for three weeks, released two days prior to death. IVDA for 17 years. Abused heroin, Diconal and heroin/temazepam. Took overdose of heroin two years ago. Recently prescribed methadone and Librium.® On day of death, took drugs between 1400 and 1430 hours then became unwell. Found dead at 1610 hours with a syringe in close proximity.

K96 08

No petechial haemorrhages. Marked oedema in lungs. Needle puncture marks on both sides of neck. Evidence of old puncture marks at groin and possibly on arms. Signs of chronic IVDA on shins and ankles. Prescribed methadone (50 ml daily) and diazepam (15

ml daily). Complained of swollen testicles as the result of an assault and attended hospital. Swelling thought to be due to injection on the right side of the groin, but could have been due to assault. Further investigation found cellulitus of the abdominal wall, infection under the skin round the groin due to long term injection. Resulted in sceptic shock. Died in hospital. Thought to have been off heroin for about two years, but had started snorting cocaine.

K96 09

Body badly burned and hair singed. Needle and syringe clasped in right hand. 80% burns. No obvious signs of chronic drug abuse. No violence evident. History of drug abuse. Abused heroin regularly. Thrombosis in right leg prior to death. Appears to have overdosed and fallen onto a fire. Life pronounced extinct at 1410. Burning post mortem therefore cause of death is overdose and not fire (no CO in blood).

K96 10

Known drug abuser. Last seen alive in the afternoon three days before being found dead. May have been seen on video surveillance camera night before death. Needle and syringe in hand (still injected). History of alcohol abuse and smoked cannabis. Reported using "smack". No medical records of drug use, but treated for alcoholism and asthma. Venepuncture mark on back of left hand.

K96 11

No fresh needle marks although some old ones possibly. Taking drugs for 11 years and most recently heroin. Overdose seven years ago. One week before death given sick line from doctor for nervous debility. Released from prison 8 days before death. On day of death, said to have been "sniffing". Fell asleep at 1900. Found collapsed on the floor at 2030. Ambulance called but no signs of life.

K96 12

No sign of chronic IV use but one puncture mark on the left antecubital fossa perhaps due to resuscitation. Suffered from mental illness and was on medication. Diagnosed psychotic

on sulpiride. Known solvent abuser. Last thought to be alive at around 2130. Found two hours later. Needles, syringe and suicide note found in the vacinity.

K96 13

Died in hospital. Taken in as having problems breathing. Resuscitation carried out. Adrenaline, atropine, diazepam, streptokinase and intravenous fluids given. Condition deteriorated. Died at 2157. Four needle puncture marks in right antecubital fossa (two with bruising) three on front of right wrist, one on front of right ankle, one on right side of left ankle. Suggestion of scarring on both sides of groin. Being assessed for suitability for rehabilitation and treatment at a Drug Clinic. Extensive evidence of IVDA. No medication at time of death

K96 14

Possible needle marks on right forearm at the elbow and left arm just above the elbow. Fresh needle mark on the back of the left hand. Rigor mortis established. Lungs oedematous and highly congested. Last seen by family doctor two months previously suffering from anxiety neurosis. Seen at 1500 in apparently good health. Found dead at home at 2000 with syringe in close proximity.

K96 15

Petechiae confined to areas of hypostasis. Early decomposition setting in. Rigor mortis diminished. Lungs congested and oedematous. Right arm, three old healed puncture marks. History of drug abuse. Known drug abuser for eight years. Registered drug abuser. Not known what medication she was on at time of death. Released from prison day before death after a three month sentence. Overdosed on methadone at 1pm on day of release and admitted to hospital. Treated with Narcan and discharged at 2.30 p.m. At around 8 p.m., at friends house drinking heavily and smoking cannabis. Went to bed at 10 p.m. Found to be dead at 9 am following morning.

K96 16

No petechial haemorrhages. Rigor mortis diminished. Lungs congested and oedematous. Left front forearm, two healing puncture marks. Back of left forearm, 1 healing puncture

mark. Two puncate abrasions on back of left hand. On maintenance prescription in the form of painkillers to treat serious burns received three years earlier. Two months prior to death, admitted to hospital due to heroin overdose. Needle marks found on groin and arm. At 2pm on day of death, started drinking heavily. 8.30pm, returned home and continued drinking then fell asleep. Found dead in living room at 11.15pm

K96 17

Petechial haemorrhages noted in left anterior axillary fold and the adjacent axilla. Rigor mortis well established. Moderate to severe oedema and congestion in lungs. Possible recent puncture mark on left antecubital fossa. Past medical history of schizophrenia and known IVDA. Prescribed procyclidine, depixol and Losec. Referred to a Drugs Centre one month before death. Last seen by doctor two weeks before death and prescribed procyclidine. Collapsed suddenly at home after injection of heroin. Resuscitation attempts failed. Seized syringe from outside flat.

K96 18

No petechial haemorrhages. Rigor mortis wearing off. Moderate to severe oedema and congestion of the lungs. Recent puncture mark on radial border of left wrist. Pock mark scarring in groin region. Thought to have become involved with drugs eight years ago. Had drug counselling as part of probation. Abused heroin and temazepam. First medical history of drug abuse seven years ago. On methadone for one year, 45 ml daily. Last prescribed five months previously. Registered drug addict. On night of death went to friends at about 10.15 p.m. Went out for alcohol (20 minutes) then watched video till 0230 before going to bed. At 1430 the next day found dead (1-2 hours only). Found blood filled syringe on floor, shoelace round left wrist, spoon and open Abdine health drink sachet.

K96 19

A few petechial haemorrhages on the conjunctivae. Mouth filled with vomit. Limbs still rigid. Lungs showed haemorrhage and oedema consistent with death in cardiac failure with superimposed inhalation changes. Fresh needle puncture mark on dorsum of right hand plus some small similar old wounds. No fresh needle marks anywhere else on the body. Known IVDA. In prison for several weeks prior and released two days before death. First

note of IVDA four years before death. Attending drugs counselling since then. Known to have Hepatitis B. At around 1pm on night before death visited friends with heroin and appropriate drug paraphernalia, apparently under the influence of drugs. From then till midnight, smoked hash. Fell asleep around midnight. Found dead 0945 the next morning with syringe and needle in right hand.

K96 20

No conjuctival petechial. Possible needle puncture mark on back of right hand. No other fresh or recent NPMs. Rigor mortis diminished. Lungs markedly congested and oedematous. History of alcohol and drug abuse for 18 years. Previous overdose. Prescribed methadone one year prior to death. Only prescribed once as doctor felt he no longer needed it. Prescribed GNT spray for angina five months before death. Also on ventalin. Regular smoker of cannabis and heroin. Not known to inject over the past two years. On night of death, went to bed drunk. In morning found dead in bathroom with hypodermic syringe and other drug paraphernalia on the floor (~7am). Possible needle puncture mark on back of right hand. No other fresh or recent needle puncture marks.

K96 21

Hypostatic petechiae on right upper eyelid. Rigor mortis diminished. Lungs congested and oedematous. Lower limbs showed multiple old healing needle puncture marks of varying ages with associated purple and greenish bruising. Scabbed sinus in left groin with fresh needle puncture mark and blood dot at its base with tissue paper attached to it. Multiple skin popping marks round knees and calves of both legs. Long history of drug abuse. On methadone treatment and obtained prescription of 9 x 10 mg diazepam prescribed day before death which was consumed at the pharmacists. Three days before death found collapsed after injecting herself with drugs in the toilet. Recovered sufficiently to refuse treatment by the time the ambulance had arrived. Last seen alive 1700. Following morning found dead in room with trousers and pants at knees and syringe nearby. Been on several detox programmes but resumed habit on leaving. On diazepam on daily dispensing reducing course for temazepam addiction. Week before death requested methadone detox. Suffered seizures due to temazepam abuse and thrombosis in legs due to IV injection in

groins. Seized from the premises: paper fold containing white powder, used syringe, 2 syringes, spoon with substance thereon.

K96 22

Multiple fine petechiae on the upper eyelids and the congunctivae. Rigor mortis diminished. Lungs showed oedema on sectioning. Needle puncture marks on breasts, arms, thighs, shins, and ankles. History of drug abuse for nine years. At the time of death, was on methadone treatment. Last seen at 0930. Didn't pick up methadone from the chemists. At about 1215, witness became concerned and investigated bedroom. Found lying in a semicrouched position on the floor with needle and syringe inserted into left leg. Ambulance called, but found to be dead.

K96 23

No fresh needle marks on body. Two years prior to death, told doctor he had a problem with temazepam and was referred to drug abuse clinic. Failed to turn up. Overdosed on alcohol and diazepam seven months prior to death. Seen to take 5 double strength dihydrocodeine and 7 yellow Valium® tablets at party night before. Also seen to drink lager and smoke cannabis. Walked dog at 0430, and returned to flat. 1030 found dead. Small amount of blood emitting from nose.

K96 24

No recent needle puncture marks or evidence of old scars around groin areas. No recent needle puncture marks on arms. Known drug abuser but not intravenous injection. Said to have had a slight drug problem - taking temazepam when depressed. Suffered from anxiety, depression and panic attacks and prescribed Paroxetine 4/96. Not on any medication at time of death. Week before death admitted to taking Valium,® temazepam and smoking heroin. Night before death, having withdrawal symptoms. Returned home at 2100. Went to bed around 0300 next morning. Around 0940 found to be dead. Seized: 48 ml bottle of methadone mixture - empty, 5 mg diazepam tablets bottle - empty, 400 mg ibuprofen tablets, 400 mg ibuprofen tablets and 1 mg Loprazolam tablets.

K96 25

Face and conjunctivae congested with a few small petechial marks. Limbs still rigid. Lungs extremely congested and oedematous. Right arm showed small crusted wound over the medial aspect of the front of the elbow joint. No signs of recent or chronic drug abuse on arms. Both legs showed pitted scars on linear aspect of the shins consistent with healed ulcerated lesions associated with IVDA. No evidence of fresh needle marks. History of chronic IVDA. Recently in prison. Released day before death. Two months prior to death, given a prescription for Librium® and dihydrocodeine. Before this had been prescribed DHC on several occasions and admitted to having a £30 per day heroin habit. Five years prior to death noted to be using heroin and temgesic. Overdose seven years prior to death. Claims to have used LSD, cannabis, temazepam, DHC and temgesic. On release from prison, went home. Thought to be under the influence (Valium®). Later, left house to buy drugs. Returned home 0730 and went to bed. Last seen returning home around 1530 apparently well. Around 2140, witness found deceased slumped in a sitting position with string tied round right arm and needle lying next to him. Appeared to have been dead for several hours. Several drug related items were removed from the locus (not noted in police report).

K96 26

Ligature mark round neck. Fresh needle puncture mark on antecubital fossa of left arm. Admitted to prison. No drug use for one week although known to be an IVDA. Found hanging within cell at around at 1100. IV abuse of heroin (£60-80 per day). Moderate drinker and smoker.

K96 27

Eyes congested with some large petechiae in right sclera and conjunctivae. Rigor mortis worn off. Lungs congested and oedematous. Greenish discoloration of the abdomen due to decomposition. Possible recent puncture mark over radial border of left mid forearm. Known IVDA. Two weeks prior to death, found unconscious with syringe in arm. Day of death at 0020 returned home. At about 0900 found in room sitting on bed apparently dead. Large amount of blood and vomit on the floor. Syringe still injected in arm. Various items of drug paraphernalia seized from the locus. Admitted to hospital due to drug abuse for

first time ten years previously for IVDA of heroin. Continued to abuse heroin and other drugs. Admitted to drug dependency three and a half years prior to death. At this stage also suffering from epilepsy. On release continued to abuse methadone, dihydrocodeine, temazepam and heroin intravenously. Admitted to various hospitals due to overdoses. Attended psychiatric unit for drug abusers, but still continued to abuse drugs.

K96 28

Scleral haemorrhage in left eye. Many incorporated petechial haemorrhages within hypostasis on face and trunk. Rigor mortis passed off. Moderate congestion and oedema in lungs. Possible venepuncture mark on left antecubital fossa with associated bruising. No other venepuncture marks. Suffered from anxiety and had a drink and drug problem. Prescribed oxazepam and nitrazepam. Last seen 1700 hours. Three days later at 1400 found dead in bed with flex tied round left arm. Bloody fluid coming out of mouth and nostrils. Syringe and white substance found at the locus.

K96 29

No petechial haemorrhages. No recent puncture marks on either arm. Lungs oedematous. Four heeling needle puncture marks overlying right groin with faint bruising. Multiple recent and fresh puncture marks overlying left groin with surrounding bruising, several with overlying blood clots. Long history of drug abuse. Lunchtime, day of death, purchased temazepam and heroin. Seen to swallow 5 temazepam tablets then prepared heroin and injected himself. Went to sleep until around 1430 when witnesses noted blue coloration and vomit. Resuscitation attempts were unsuccessful.

K96 30

Possible old needle puncture mark on right forearm. Day before death purchased £10 bag of heroin and shared half then fell asleep. Woke up in morning and smoked other half then went back to sleep again. Found later on "foaming at the mouth". Resuscitation attempts failed. Known drug abuser for 2 years. Smoked heroin daily. Attempted suicide two years prior to death - temazepam. Previous overdose on diazepam - temporary coma and pneumonia. In months leading to death, attending Drug Dependency Unit. Seized 2 rolled cigarette papers and 1 piece of tinfoil with brown substance thereon.

K97 01

No fresh needle puncture marks on either arm. Known IVDA. Cirrhosis of the liver recently diagnosed. On methadone treatment at time of death. Aneurism due to IVDA on right femural artery requiring treatment. Last seen by doctor two weeks prior to death for methadone. Last seen alive three days prior to death heavily under the influence of alcohol. Found dead at home.

K97 02

No fresh needle puncture marks. Puckered scarring on left groin. Suspected hepatitis C. 15 year drug history. Attending various clinics for drug abuse with mixed results. Prescribed diazepam and methadone daily. Last seen alive 2200. Found dead 1430 following day. Syringes found in bedroom.

K97 03

Medical needle puncture marks on both sides of neck. Fresh needle puncture mark on radial aspect of right antecubital fossa. No evidence of old scarring or needle puncture marks. Drug abuser for 2/3 years. Attending rehabilitation and prescribed methadone and valium. Not known IVDA but police found fresh needle puncture mark on left arm.. Escorted home by police at 2000 as heavily under the influence, ambulance summoned. Attempts made at resuscitation. 2126 arrived at the hospital after having an apparent cardiac arrest. Never regained consciousness. Pronounced dead 2142.

K97 04

Foamy white froth in mouth. No petechial haemorrhages. Lungs congested and markedly oedematous. Fresh needle puncture mark at front of right elbow. Healing needle puncture marks on right arm above wrist crease and radial wrist crease. No needle puncture marks on left arm. Two possible needle puncture marks at right ankle. History of asthma and known to smoke heroin. On voltarol, remedeine and diazepam at time of death. Last seen alive 1600. 2300 following day, found fully clothed, dead on bed. Medication at time of death: Voltarol, Remedeine, Diazepam. Drug paraphernalia seized from locus.

K97 05

No petechial haemhorrages. ECG monitoring pads on chest and front of right elbow. Dressing over therapeutic puncture mark. Ligature mark on neck with several linear petechial bruises along the axis. Multiple healing excoriated abrasions with small circular scars over whole body due to scabies. Therapeutic puncture mark on front of right elbow. Multiple healing needle puncture marks on right forearm overlying vein. On left arm, outer side of elbow, needle puncture mark, four at the front of the elbow and one on the back of the hand. Rigor mortis diminished. Hypostasis on back of body. Lungs oedematous with congested lower lobes. Arrested day before death. Taken into custody and given 7 days in prison. Ate meal at 1630, heard talking to inmates in next cell at 1730 and found hanging in cell at 1930. Heroin addict suffering from withdrawal symptoms when last seen. Inmates next door heard scrapping of bed across floor and banging of pipes at ~1900. Found by guard on duty at 1930. Resuscitation attempts failed.

K97 06

Needle puncture mark due to post-mortem sampling on left side of neck. Front of right elbow, overlying antecubital fossa, needle puncture mark with medical dressing. Front of left antecubital fossa, fresh needle puncture mark with associated bruising. Rigor mortis diminishing. History of drug abuse. Last seen by doctor one month prior to death. Complained of sleeping difficulty said to be using cannabis, temazepam and Valium.® Asked for methadone, but told this was not available until blood tests had been carried out. Following tests, prescribed one months supply of trazodone. Given weeks supply but didn't pick up the rest. Night before death went drinking. Found the following morning still sitting on the couch. Called ambulance but resuscitation attempts unsuccessful. Life pronounced extinct 1010.

K97 07

No petechial haemorrhages. Front of right arm several circular skin popping scars. Multiple circular and oval skin popping scars over both lower limbs, thighs and lower legs. Right groin, scarred sinus with fresh blood clot related to needle puncture mark. Left groin, old healed scarred sinus overlying femoral vein. Rigor diminished. Lungs congested and oedematous. Ten year history of heroin IVDA. Admitted for detox

rehabilitation in previous year for two months. Following treatment, said only to smoke heroin occasionally. Went to bed 2300 and found following morning found at 0820 dead on living room floor. Syringe, spoons and Abdine powder nearby. Fresh injection site noted in left groin.

K97 08

No petechial haemorrhages. Mouth full of black fluid. Advanced signs of decomposition. Not possible to identify recent needle puncture marks in either antecubital fossa, groins or ankles. Rigor mortis worn off. Internal organs showed moderate decomposition changes. Moderate oedema in lungs. Registered drug abuser. Previous heroin overdose. Previously prescribed methadone. Released from prison one month prior to death. Last seen alive three days prior to death. On day found dead, strange smell coming from room. Found dead inside with trousers round ankles and used needle nearby. Life pronounced extinct at 1340. Due to decomposition, insufficient blood to carry out full drug screen. Overall findings consistent with drug related death, probably heroin.

K97 09

No petechial haemorrhages. Post-mortem needle puncture mark on left side of neck. Single tiny fresh needle puncture mark on left arm at the front of the elbow. Old scarred sinus in both groins overlying femoral veins. No fresh needle puncture marks on groins. Rigor mortis diminished. Lungs congested and oedematous. History of drug abuse. Heavy drinker. In prison for 11 days. On day before release was overheard saying she was going for a hit as soon as she got out. Following day arrived at train station at 0902. Found dead within cubicle of toilets at 1350. Syringe and needle, teaspoon and paper fold with white granules seized from locus. Drug dependant for 12 years.

K97 10

Petechial haemorrhages over mid chest and clavicles. No evidence of needle puncture marks or scars in groins. No fresh marks injuries or fresh/old needle puncture marks on arms. Lungs bulky, congested and oedematous. History of long term drug abuse. Known to abuse cannabis, cocaine and temazepam but not known to be an IVDA. Last seen alive 1700. Following night found dead in bed.

K97 11

No needle puncture marks in arms or groins. Limbs flaccid. Both lungs congested and oedematous. History of drug abuse. Known to have taken temazepam and had suffered seizures as a result therefore prescribed chlordiazepoxide at this point. At one point treated with diazepam to control seizures. Taking sleeping tablets and temazepam in the days leading up to death. Not seen by family since 9pm two days prior to death. Forced entry into flat and found her lying face down in the bedroom. Telephoned for ambulance but found to be dead 1910. When last seen, suspected to have been smoking heroin.

K97 12

Face and conjunctivae congested. Needle puncture marks on both sides of neck (PM). Circled needle puncture marks on both groins. Right arm, needle puncture mark in outer aspect of antecubital fossa (circled). Rigor mortis wearing off. Methadone addict. Known drug abuser for 10-15 years. Released from prison month before death. Prescribed 10 mg x 4 diazepam daily. Known to use heroin. 1740, forced entry and found deceased face down on bedroom floor unconscious. Tried resuscitation and phoned ambulance. Resuscitation unsuccessful. Seized 2 spoons, belt fastened as a tourniquet and brown resinous material in silver foil.

K97 13

No petechial haemhorrages. Rigor mortis worn off. No needle puncture marks. Lungs contained food, oedematous and congested. On methadone maintenance programme. Smoked heroin for approximately one year. Prescribed methadone but didn't return for repeat prescriptions. Three months prior to death recommenced on methadone maintenance programme and one month prior to death had daily amount increased from 50-60 mg. Taken at chemists. Last spoken to at 1245. 1545 witness checked on him and thought to be asleep. Checked again at 2200 before going to bed and still "sleeping". 0930 tried to wake him but couldn't. Second witness found him to be dead and covered in vomit.

K97 14

No petechial haemorrhages. PM needle puncture marks on neck. Two old pale circular scars on front of right elbow overlying vein. No recent needle puncture marks on either arm. Old circular scars overlying vein on left elbow. Lower limbs, innumerable small bruises, some overlying veins, many associated with overlying healing small needle puncture marks. Multiple old circular and oval slightly depressed scars. Front of left thigh, 2 cm above the medial edge of the kneecap, fresh needle puncture mark. Rigor mortis diminishing. Lungs congested and oedematous. Widespread aspiration of gastric contents into airways. Long history of drug and alcohol abuse. Attended drug rehabilitation but thought to have recently relapsed. Last seen by doctor one month prior to death. Day of death found by cleaner in toilets of health centre on all fours. Found needle and syringe inserted just above left knee.

K97 15

Intravenous line in right elbow. Possible tiny needle puncture mark at front of left elbow. Rigor mortis diminished. Hypostasis on back. Lungs congested and oedematous. Heavy drinker. Known to abuse painkillers. Previous overdose of co-dydramol. Day before death prescribed 100 co-proxamol in relation to back pain. Also recently prescribed dihydrocodeine and still on co-dydramol. Not known if IVDA or not. 1800 found kneeling with face on floor appeared to be dead but tried resuscitation anyway. Ambulance arrived 1806 and continued resuscitation to no avail. Found prescribed drugs and syringe in bin near deceased's right hand.

K97 16

Few petechiae in conjunctivae of left lower eyelid. No evidence of IVDA. Lungs congested and oedematous. Rigor still present in some parts of the body. Referred psychiatric hospital ~ five years prior to death and revealed drug and alcohol problem. Six months prior to death saw psychiatrist. Said to be depressed. Doctors prescribed amytriptyline 10 mg/day due to back pain. Night before death appeared to be under the influence of drugs. 0725 following morning found in living room, obviously dead. Dead some time. Rigor still evident. Drug paraphernalia seized from locus.

K97 17

Needle puncture mark in right anticubital fossa. No previous signs of IVDA. Rigor still present. Lungs congested. Said to be a binge drinker and heroin user. Six months prior to death, prescribed 4 week course of antidepressants. 1745, appeared to be under the influence of alcohol. 1750 bought some heroin (2x£10 bags) to share with friend. Prepared heroin, both injected at same time from the same solution. Deceased closed eyes almost immediately after, but thought to be OK. 1940 found apparently unconscious in the same position. Attempted basic first aid but no success. Called ambulance but found to be dead.

K97 18

Conjunctivae congested. Multiple needle puncture marks in right groin (medical ?). Small sinus in left groin consistent with chronic IVDA. Right groin bleeding with 5 needle puncture marks. Rigor still present. Lungs congested and oedematous. Heroin abuser for 13 years. 1015, awake in bed. Got up ~1100 and out at 1145. 1250 found lying face down on the living room floor with head in rubbish bag and jeans pulled down below groin area, syringe sticking out of groin. Not breathing and blood coming from mouth and nose. Tried resuscitation. Phoned ambulance. Arrived 1303. Taken to hospital but resuscitation unsuccessful. Found spoon and paper fold in kitchen.

6.4 Results and Discussion

6.4.1 Blood and Vitreous Humor

6.4.1.1 Benzodiazepines

6.4.1.1.1 Analysis of PM and RTD Blood Samples

In addition to the 48 post-mortem cases previously described, 15 post-mortem (PM) and road traffic drug (RTD) blood samples which had been analysed in the routine laboratory, were extracted by SFE. The results were then compared to those obtained using SPE for temazepam only. In both cases the analyses were carried out using the same standards (temazepam: 4.04 mg/100 ml, prazepam 4.89 mg/100 ml) for direct comparison of the results and the samples were extracted at approximately the same time.

In most cases the analyses were carried out in triplicate. The results are shown in Table 6.2 and a correlation graph of these results is shown in Figure 6.1. An example of the method used to determine the concentration of drug present in the extracts is given in Appendix I.

Table 6.2: *Case Blood Analysis for Temazepam*

Case	SFE [Tem] mg/L	SPE [Tem] mg/L
RTD1	3.17	3.96
RTD2	1.34	1.18
RTD3	8.04	7.57
RTD4	2.38	2.13
RTD5	1.85	1.89
RTD6	3.20	3.23
RTD7	1.08	1.22
PM1	3.08	3.42
PM2	2.01	2.41
PM3	1.97	2.07
PM4	2.52	2.65
PM5	2.04	2.26
PM6	4.51	4.51
PM7	1.89	1.58
PM8	5.02	5.94

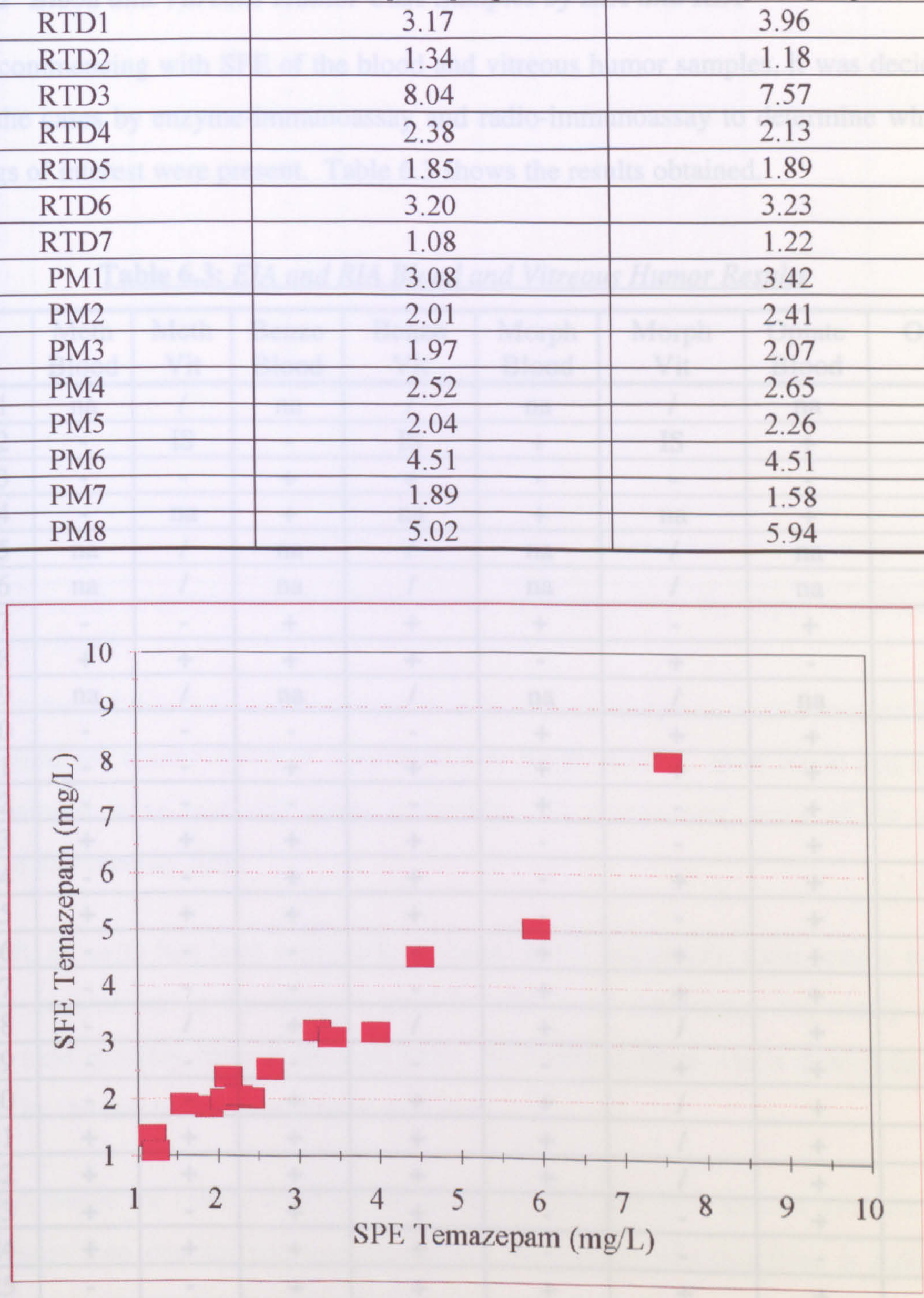


Figure 6.1: *Correlation Between SPE and SFE for RTD and PM Samples*

For the 15 cases analysed, good correlation was found between SFE and SPE. The equation of the best fit line through the points was calculated as y=0.97x + 0.06. For both methods to be extracting the temazepam to the same extent, the gradient of the line should be 1.0. From the equation of the line it is therefore apparent that there is no significant difference between the values obtained by the two techniques.

6.4.1.1.2 Blood and Vitreous Humor Case Samples by EIA and RIA

Before commencing with SFE of the blood and vitreous humor samples, it was decided to screen the cases by enzyme-immunoassay and radio-immunoassay to determine which of the drugs of interest were present. Table 6.3 shows the results obtained.

Table 6.3: *EIA and RIA Blood and Vitreous Humor Results*

Case	Meth Blood	Meth Vit	Benzo Blood	Benzo Vit	Morph Blood	Morph Vit	Opiate Blood	Opiate Vit
K96 01	na	/	na	/	na	/	na	/
K96 02	-	IS	-	IS	+	IS	+	IS
K96 03	-	-	+	+	-	-	-	+
K96 04	-	na	+	na	+	na	+	na
K96 05	na	/	na	/	na	/	na	/
K96 06	na	/	na	/	na	/	na	/
K96 07	-	-	+	+	+	-	+	-
K96 08	+	+	+	+	-	+	-	+
K96 09	na	/	na	/	na	/	na	/
K96 10	-	-	-	-	+	+	+	+
K96 11	-	-	+	+	+	+	+	+
K96 12	-	-	-	-	+	-	+	+
K96 13	+	+	+	+	-	-	+	+
K96 14	-	-	+	+	-	+	+	+
K96 15	+	+	+	+	-	-	+	+
K96 16	-	-	-	-	+	+	+	+
K96 17	-	-	-	-	+	+	+	+
K96 18	-	/	+	/	+	/	+	/
K96 19	-	-	-	-	-	+	+	+
K96 20	-	-	+	+	+	/	+	+
K96 21	+	+	+	+	+	/	+	+
K96 22	+	+	+	+	+	/	+	+
K96 23	+	-	+	+	-	-	+	+
K96 24	+	+	+	+	-	-	-	-
K96 25	-	-	+	+	+	+	+	+
K96 26	-	-	+	-	-	-	-	-

Table 6.3 continued

Case	Meth Blood	Meth Vit	Benzo Blood	Benzo Vit	Morph Blood	Morph Vit	Opiate Blood	Opiate Vit
K96 27	-	-	+	+	+	-	+	+
K96 28	-	n/l	+	n/l	+	n/l	+	n/l
K96 29	-	-	+	+	+	/	+	+
K96 30	-	-	+	+	-	/	+	+
K97 01	na	/	+	/	-	/	-	/
K97 02	+	/	+	/	+	/	+	/
K97 03	na	/	+	/	-	/	-	/
K97 04	-	/	+	/	+	/	+	/
K97 05	-	-	+	-	/	/	-	-
K97 06	-	-	+	+	+	/	+	+
K97 07	-	-	+	+	-	/	+	+
K97 08	-	-	+	+	-	/	+	+
K97 09	-	-	+	+	+	/	+	+
K97 10	-	+	+	+	+	/	+	+
K97 11	-	-	+	+	+	/	+	+
K97 12	-	/	+	/	+	/	+	/
K97 13	+	+	+	-	-	/	-	-
K97 14	-	-	+	+	+	/	+	+
K97 15	-	-	+	-	/	/	+	+
K97 16	-	-	+	+	/	/	+	+
K97 17	-	-	-	-	/	/	+	+
K97 18	+	+	-	-	/	/	+	+

+: Positive result as determined as a results of any deviation from the negative calibrator.
-: Negative result.

The cases from Table 6.3 which were positive for benzodiazepines were extracted by SFE to determine the concentration of benzodiazepine drugs present. Both blood and vitreous humor samples were extracted where applicable. For comparison, some of the samples were also extracted by SPE.

Although, all cases had been previously analysed in the laboratory, these results were not used for comparison as the samples used for this study were taken separately at post-mortem (and possibly from a different site). In addition, there was a time delay in the analysis by the two techniques of up to one year.

Table 6.4 shows the results obtained for blood by SFE and SPE and for vitreous humor. Where more than one analysis was carried out the number of extractions is indicated in brackets.

Table 6.4: *Benzodiazepine Blood SFE and SPE and Vitreous Humor Results*

Case	SFE Blood mg/L	SFE Vitreous mg/L	SPE blood mg/L	Blood EIA/*RIA* ng/mg	Vitreous EIA/*RIA* ng/mg
K96 02	Temaz: 1.22 Diaz: 0.18	Temaz: 1.19	NA	NA	NA
K96 03	Temaz: 0.20(2) DMD: 0.31 Diaz:0.17	*	NA	1255	181
K96 04	Temaz: 0.27(4) DMD: 0.53 Diaz: 0.49	*	Temaz: 0.22 DMD: 0.50 Diaz: 0.42	>2500	*
K96 07	Temaz: 0.72(6) DMD: 0.06 Diaz: 1.98	Temaz: 0.71(2) DMD: 0.42 Diaz: 0.63	Temaz: 0.60 DMD: 0.06 Diaz: 0.94	>2500	1015
K96 08	DMD: 0.14(3) Diaz:0.44	DMD: 0.17 Diaz: 0.39	DMD: 0.23 Diaz: 0.25	>2500	1751
K96 11	Temaz: 0.48 DMD: 0.47 Diaz: 1.01	- DMD: 0.39 Diaz: 0.74	Temaz: 0.35 DMD: 0.55 Diaz: 0.76	>2500	616
K96 13	DMD:0.55(3) Diaz: 0.47	- Diaz:0.47	NA	+	>2500
K96 14	DMD: 0.29(2) Diaz: 0.15	- Diaz: 0.15	NA	+	638
K96 15	DMD: 0.20 Diaz: 0.29	DMD: 0.37 -	DMD: 0.44 Diaz: 0.27	+	1368
K96 18	Temaz: 1.24 DMD: 1.54 Diaz: 0.08	*	Temaz: 0.99 DMD: 0.85 -	+	*
K96 20	DMD: 0.38 Diaz: 0.39	*	DMD: 0.51 Diaz: 0.44	+	***646***
K96 21	Temaz: 0.01 DMD: 0.39 Diaz: 0.55	- DMD: 0.35 -	- DMD: 0.54 Diaz: 0.77	+	***91***
K96 22	Temaz: 1.30 DMD: 0.68 -	Temaz: 0.76 DMD: 0.27 Diaz: 0.62	Temaz: 0.92 DMD: 0.46 -	+	***3353***
K96 23	DMD: 0.18 Diaz: 0.45	DMD: 0.025 Diaz: 0.068	DMD: 0.19 Diaz: 0.40	>2500	731
K96 24	Temaz: 0.25(2) DMD: 0.29 Diaz: 0.03	Temaz: 0.08 DMD: 0.10 Diaz: 0.01	Temaz: 0.20 DMD: 0.24 Diaz: 0.10	+	>2500
K96 25	DMD: 0.08 Diaz: 0.27	DMD: 0.07 Diaz: 0.10	DMD: 0.03 Diaz: 0.35	+	713
K96 26	Temaz: 0.06 DMD: 0.23	- DMD: 0.10	NA	+	-ve

Table 6.4: Continued

Case	SFE Blood mg/L	SFE Vitreous mg/L	SPE blood mg/L	Blood EIA/*RIA* ng/mg	Vitreous EIA/*RIA* ng/mg
K96 27	Temaz: 0.78 DMD: 0.22 Diaz: 0.08	Temaz: 0.35 DMD: 0.12 Diaz: 0.10	Temaz: 0.81 DMD: 0.18 Diaz: 0.08	>2500	1056
K96 28	Temaz: 0.11 DMD: 0.11 Diaz: 1.51	Temaz: 0.11 - Diaz: 1.25	NA	>2500	NA
K96 29	Temaz:0.36(3) DMD:0.52	Temaz: 0.17(2) DMD: 0.52	NA	+	*529*
K96 30	Temaz:0.23(3) DMD:0.20 Diaz: 2.62	Temaz:0.19(3) DMD:0.28 Diaz: 2.48	NA	+	*497*
K97 02	Temaz: 0.20 DMD: 0.44 -	*	NA	+	*
K97 04	Temaz: 0.38 DMD: 0.26 Diaz: 1.13	*	NA	+	*

* Vitreous not available for analysis

NA Not analysed

+ Some of the EIA/RIA results were for the laboratory samples. Where this was the case, + has been inserted as opposed to the value to indicate that the result was positive.

As can be seen by looking at the results obtained by HPLC and EIA/RIA, there is no correlation between the results. For this reason none of the EIA or RIA results were displayed graphically.

The results obtained by SFE for blood and vitreous humor were graphed to determine if any correlation existed as shown in Figure 6.2.

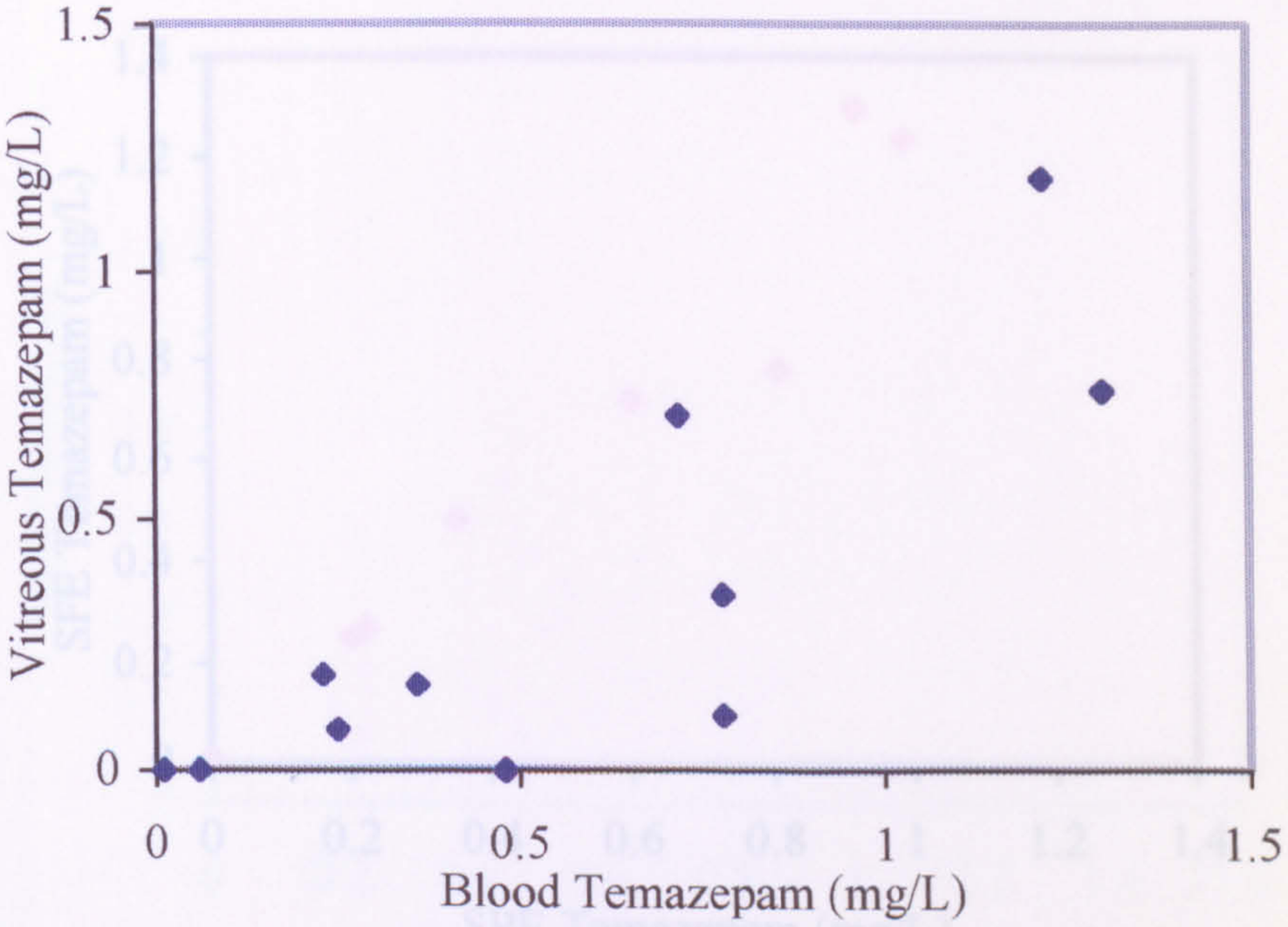


Figure 6.2: *Correlation between Blood and Vitreous Humor Temazepam Levels by SFE*

From this, the coefficient of correlation (r^2) for temazepam in blood and vitreous is 0.692 indicating that there is some correlation between the two extraction media. The gradient of the best fit line through the results was 0.75 indicating that the level of temazepam in vitreous is generally lower than the corresponding level in blood.

It should be noted, that in some cases, larger blood samples were analysed (up to 500 µL). Due to the small amount of vitreous humor available, only 100 µL were analysed at any one time. This means that in some of the samples where only a small amount of temazepam was present, the level may have been too small to be detected.

The correlation between the blood levels of temazepam determined by SFE and SPE was also graphed and is illustrated in Figure 6.3.

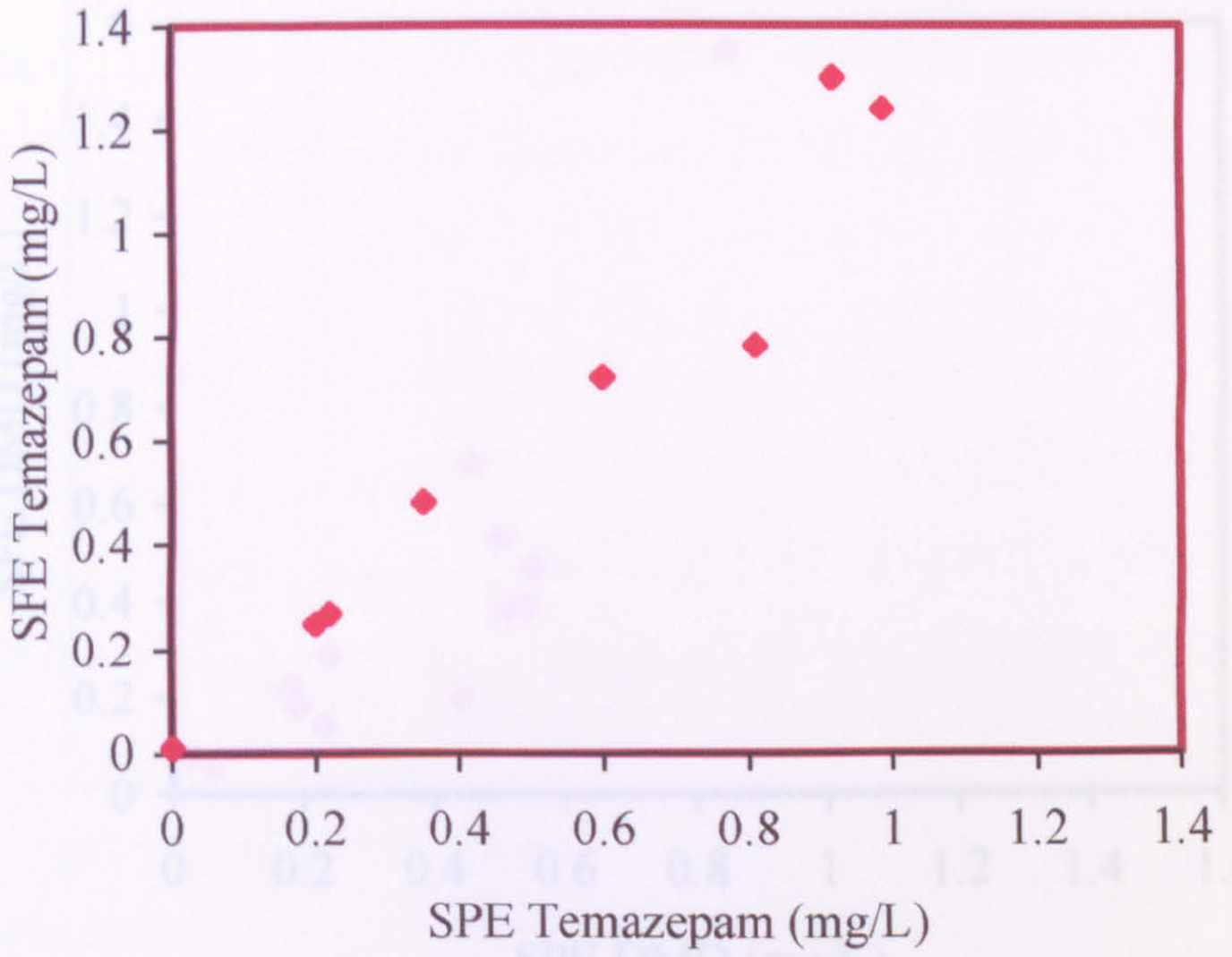


Figure 6.3: *Correlation between SFE and SPE Blood Temazepam Levels*

From this, the coefficient of correlation was found to be 0.949 with a gradient of 1.23 indicating that the SFE method is extracting more temazepam than the SPE method.

The blood versus vitreous and SPE versus SFE graphs for temazepam were repeated for desmethyldiazepam and diazepam as shown in Figures 6.4-6.7.

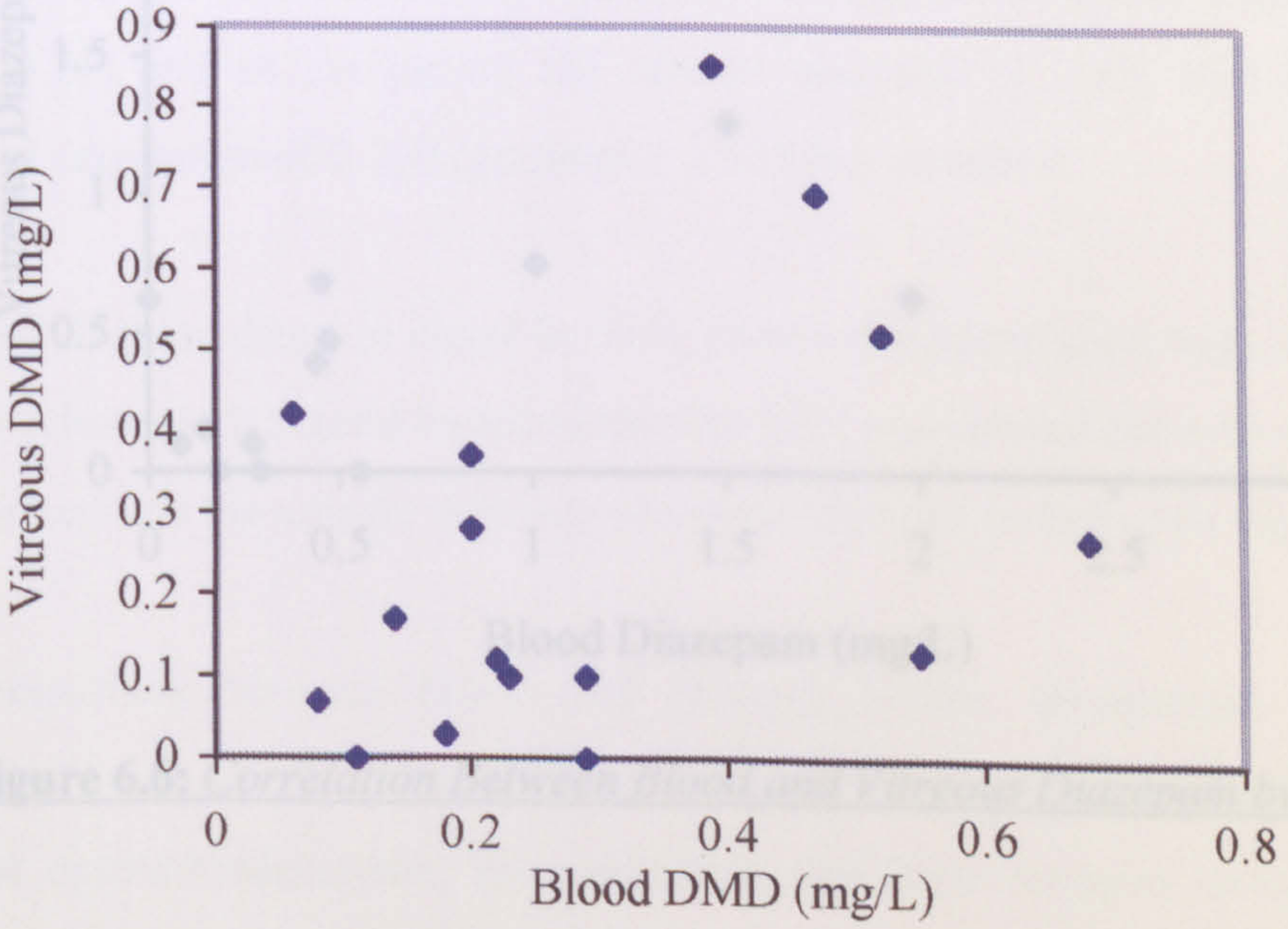


Figure 6.4: *Correlation Between Blood and Vitreous Desmethyldiazepam Levels by SFE*

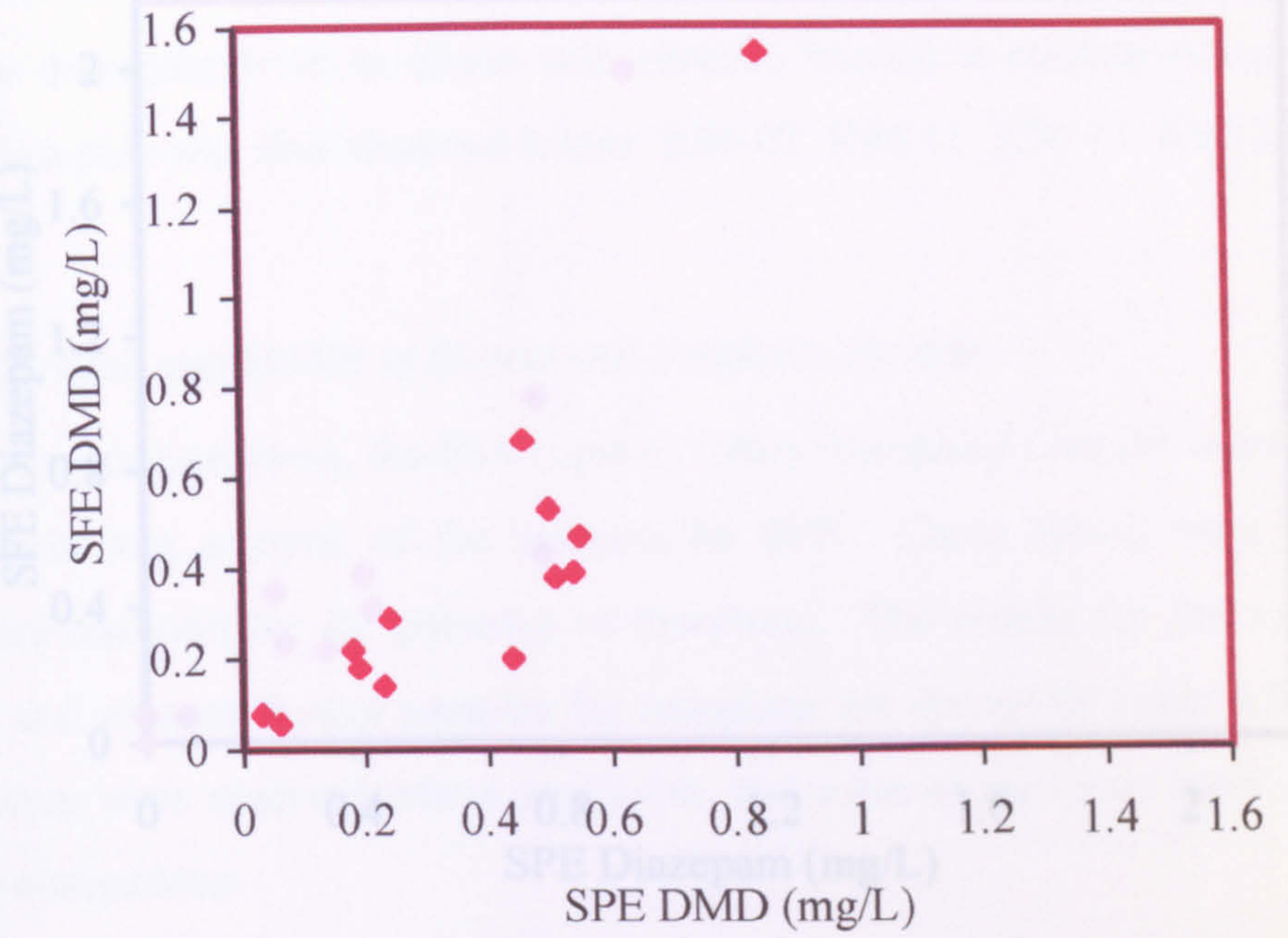


Figure 6.5: *Correlation Between SFE and SPE Blood Desmethyldiazepam Levels*

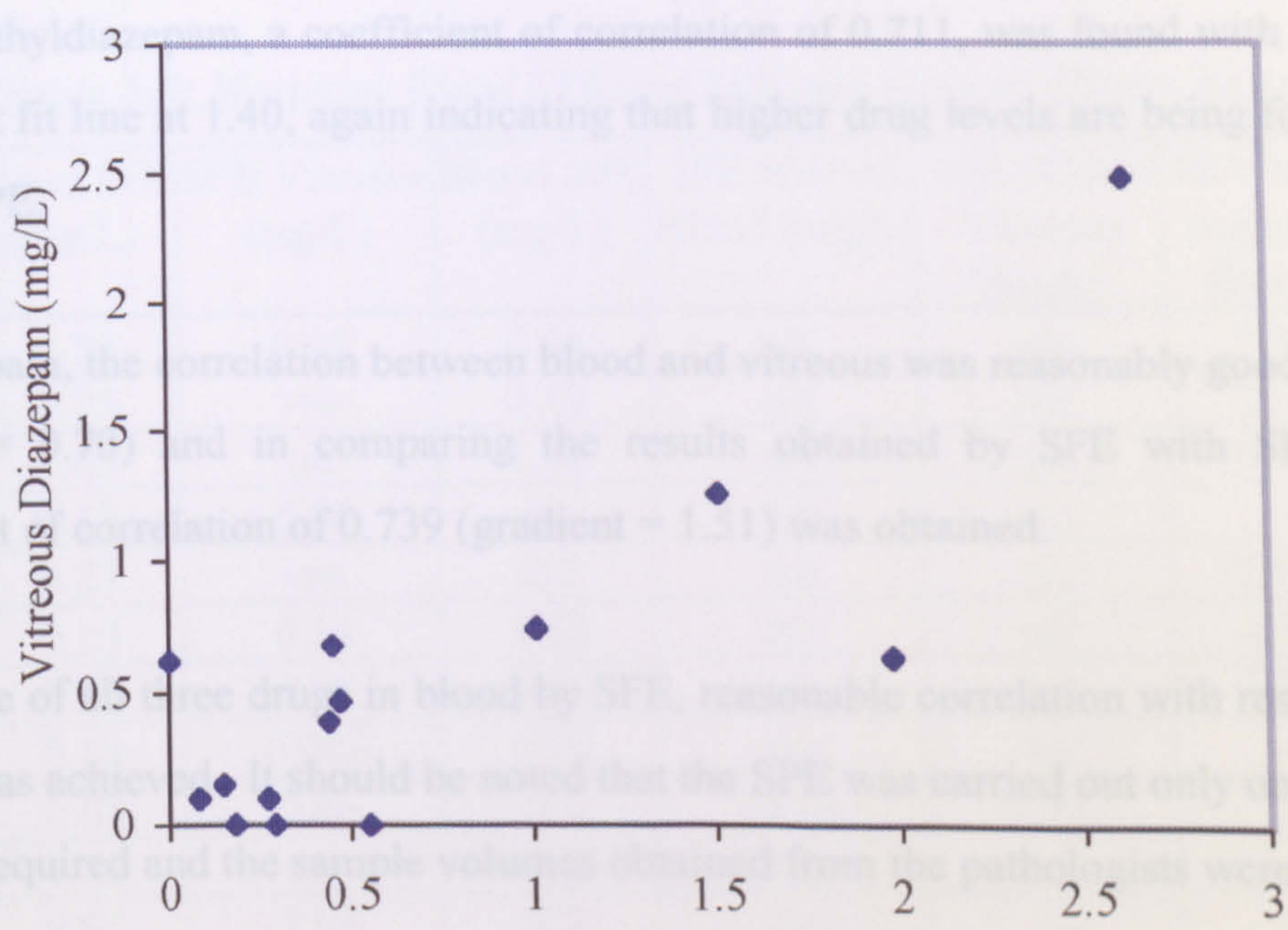


Figure 6.6: *Correlation Between Blood and Vitreous Diazepam by SFE*

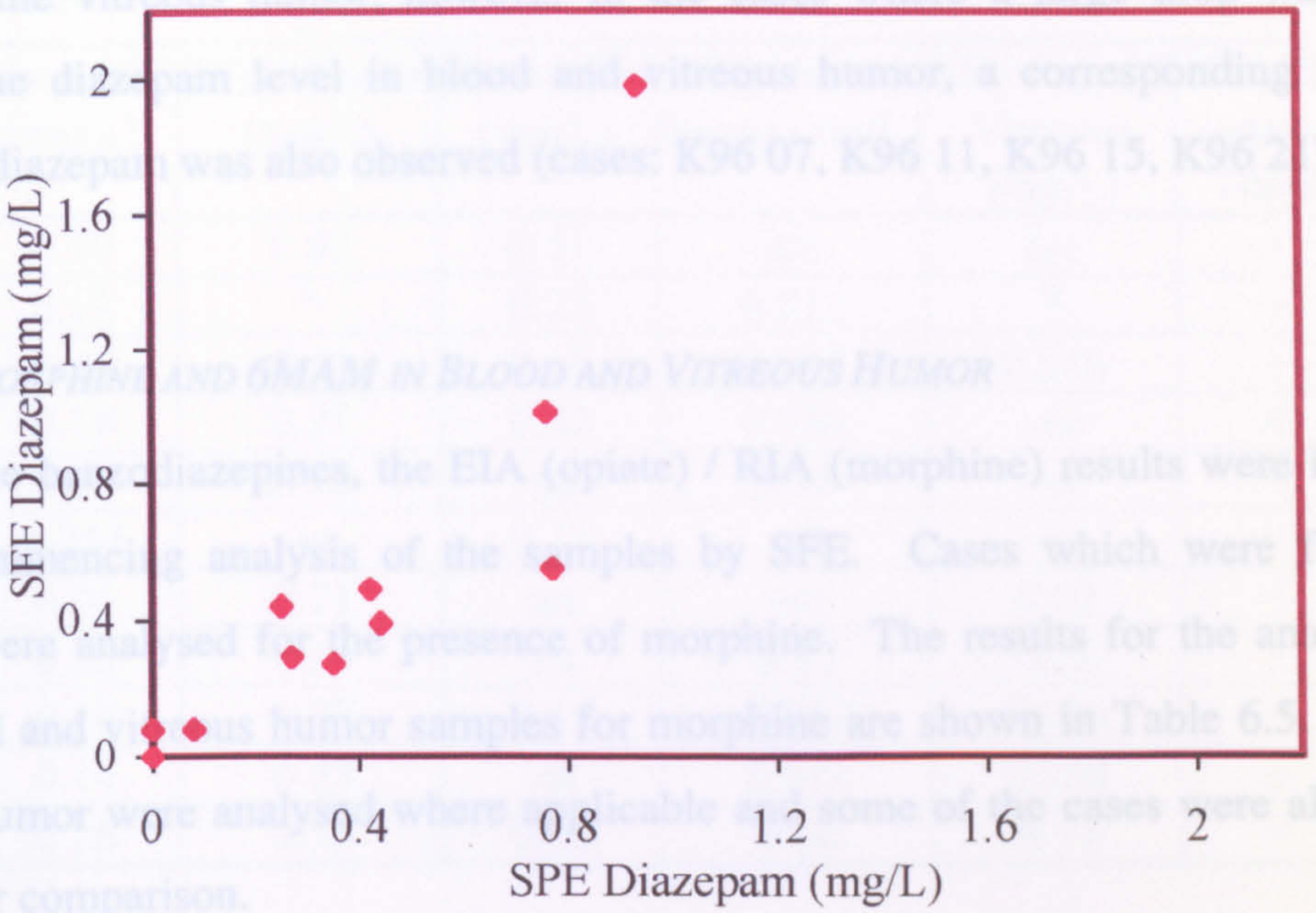


Figure 6.7: *Correlation Between SFE and SPE for Blood Diazepam Levels*

For the comparison between blood and vitreous humor for desmethyldiazepam, $r^2 = 0.143$ indicating that no correlation exists between the two media. In comparing SFE with SPE for desmethyldiazepam, a coefficient of correlation of 0.711, was found with the gradient of the best fit line at 1.40, again indicating that higher drug levels are being found by SFE than by SPE.

For diazepam, the correlation between blood and vitreous was reasonably good ($r^2 = 0.714$, gradient = 0.70) and in comparing the results obtained by SFE with SPE results a coefficient of correlation of 0.739 (gradient = 1.51) was obtained.

In the case of all three drugs in blood by SFE, reasonable correlation with results obtained by SPE was achieved. It should be noted that the SPE was carried out only once as 1 ml of blood is required and the sample volumes obtained from the pathologists were low.

For the comparison between blood and vitreous humor, temazepam and diazepam correlated well, with levels in blood being generally higher than the equivalent vitreous values. For desmethyldiazepam, no correlation was seen between blood and vitreous humor. It is not known whether or not any metabolic reaction of the benzodiazepines

occurs in the vitreous humor, however in the cases where a large drop was observed between the diazepam level in blood and vitreous humor, a corresponding increase in desmethyldiazepam was also observed (cases: K96 07, K96 11, K96 15, K96 21).

6.4.1.2 Morphine and 6MAM in Blood and Vitreous Humor

As with the benzodiazepines, the EIA (opiate) / RIA (morphine) results were investigated before commencing analysis of the samples by SFE. Cases which were found to be positive were analysed for the presence of morphine. The results for the analysis of the case blood and vitreous humor samples for morphine are shown in Table 6.5. Blood and vitreous humor were analysed where applicable and some of the cases were also analysed by SPE for comparison.

It should be noted that 6MAM analysis was not initially carried out and thus, some of the blood and vitreous samples were not analysed for 6MAM. Where this was the case n/c has been inserted.

Table 6.5: *Morphine Blood SFE and SPE and Vitreous Humor Results*

Case	Blood SFE (mg/L)	SFE Vitreous (mg/L)	Blood SPE (mg/L)	SFE 6MAM Blood (mg/L)	SFE 6MAM Vitreous (mg/L)	EIA/*RIA* (ng/mg) Blood	EIA/*RIA* (ng/mg) Vitreous
K96 02	0.79	0.45	0.68	n/c	n/c	728	na
K96 04	0.13	-ve	0.18	n/c	n/c	257	na
K96 07	1.05	na	1.06	n/c	na	646	-
K96 08	0.13	0.04	na	n/c	n/c	-	162
K96 10	0.23	-ve	0.3	n/c	n/c	237	291
K96 11	0.15	0.18	0.08	n/c	n/c	467	924
K96 12	0.25	0.08	0.14	n/c	n/c	399	359
K96 14	0.10	0.086	0.06	n/c	n/c	+	411
K96 16	0.62	0.46	0.6	0.13	0.032	+	1470
K96 17	1.35	1.03	na	n/c	n/c	+	383
K96 18	0.91	1.94	0.7	-ve	-ve	+	na
K96 19	0.42	0.69	na	n/c	0.73	+	375
K96 20	0.85	0.67	na	n/c	0.057	+	***6989***
K96 21	1.97	1.48	1.7	-ve	0.96	+	***10762***
K96 22	2.21	1.67	1.94	-ve	0.011	+	***7355***
K96 25	0.48	0.067	0.45	0.005	-ve	+	630
K96 27	0.19	0.12	0.26	-ve	0.048	268	1264
K96 28	0.39	na	0.34	n/c	na	321	na

Table 6.5: Continued

Case	Blood SFE (mg/L)	SFE Vitreous (mg/L)	Blood SPE (mg/L)	SFE 6MAM Blood (mg/L)	SFE 6MAM Vitreous (mg/L)	EIA/***RIA*** (ng/mg) Blood	EIA/***RIA*** (ng/mg) Vitreous
K96 29	0.79	0.62	0.51	n/c	n/c	+	***6794***
K97 04	0.13	*	0.23	0.012	*	+	*
K97 07	0.45	0.16	0.36	0.084	0.016	+	***6293***
K97 09	0.19	0.31	0.42	0.088	-ve	+	***6467***
K97 10	0.17	0.18	na	0.38	-ve	+	***5978***
K97 11	0.054	-ve	0.11	-ve	-ve	+	***4646***
K97 14	0.74	0.71	na	-ve	0.02	+	***7110***
K97 15	0.20	0.25	na	0.23	0.20	+	***5897***
K97 16	0.20	0.23	0.15	0.051	0.006	+	***6018***
K97 17	0.14	0.27	na	0.011	0.084	+	***4494***
K97 18	0.26	0.55	0.53	0.16	0.11	+	***5132***

A correlation graph of blood versus vitreous morphine by SFE was produced from these results and is shown in Figure 6.8.

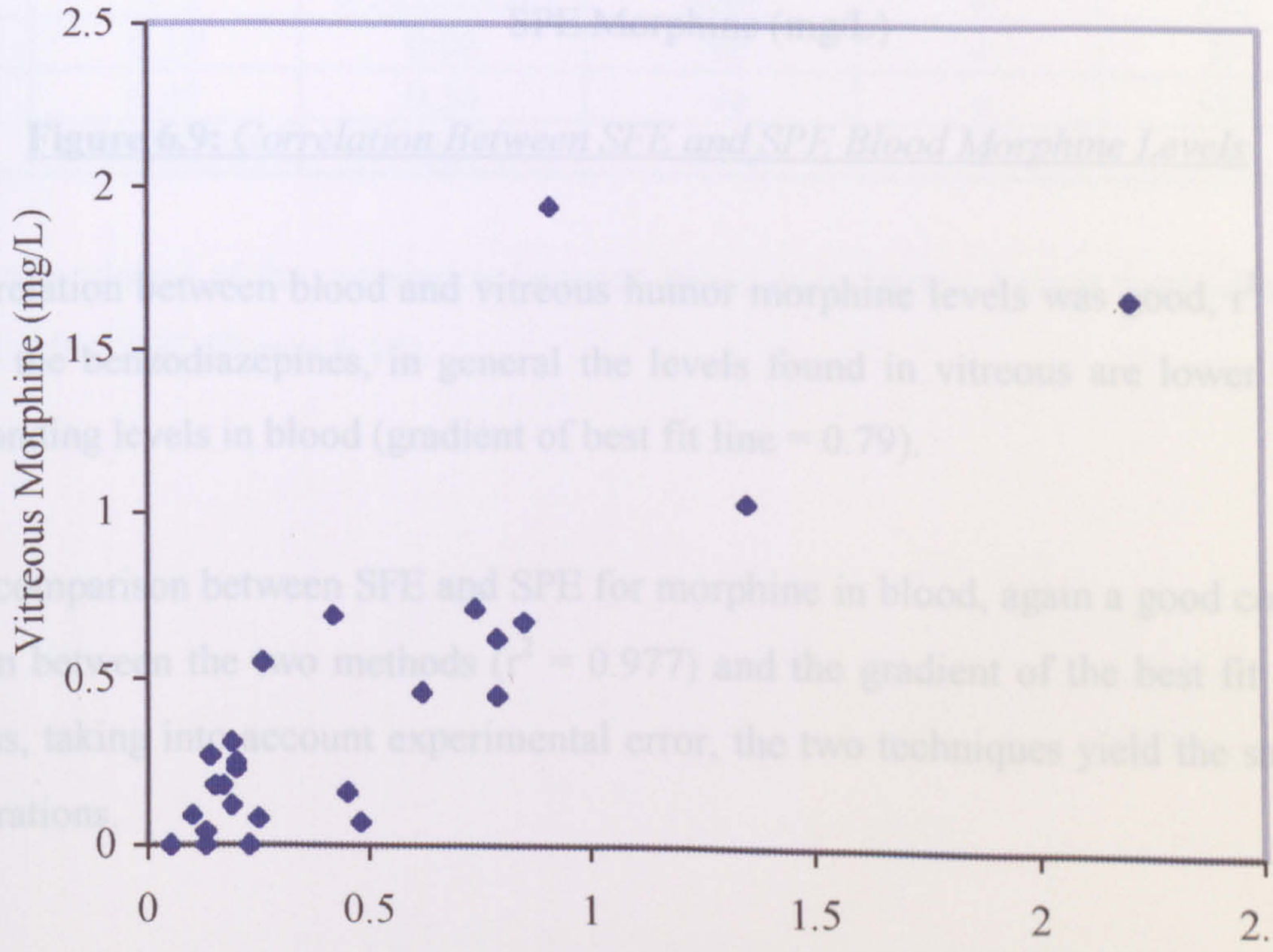


Figure 6.8: *Correlation Between Blood and Vitreous Humor Morphine Levels by SFE*

A graph of the results obtained by comparing SFE to SPE was also plotted as shown in Figure 6.9.

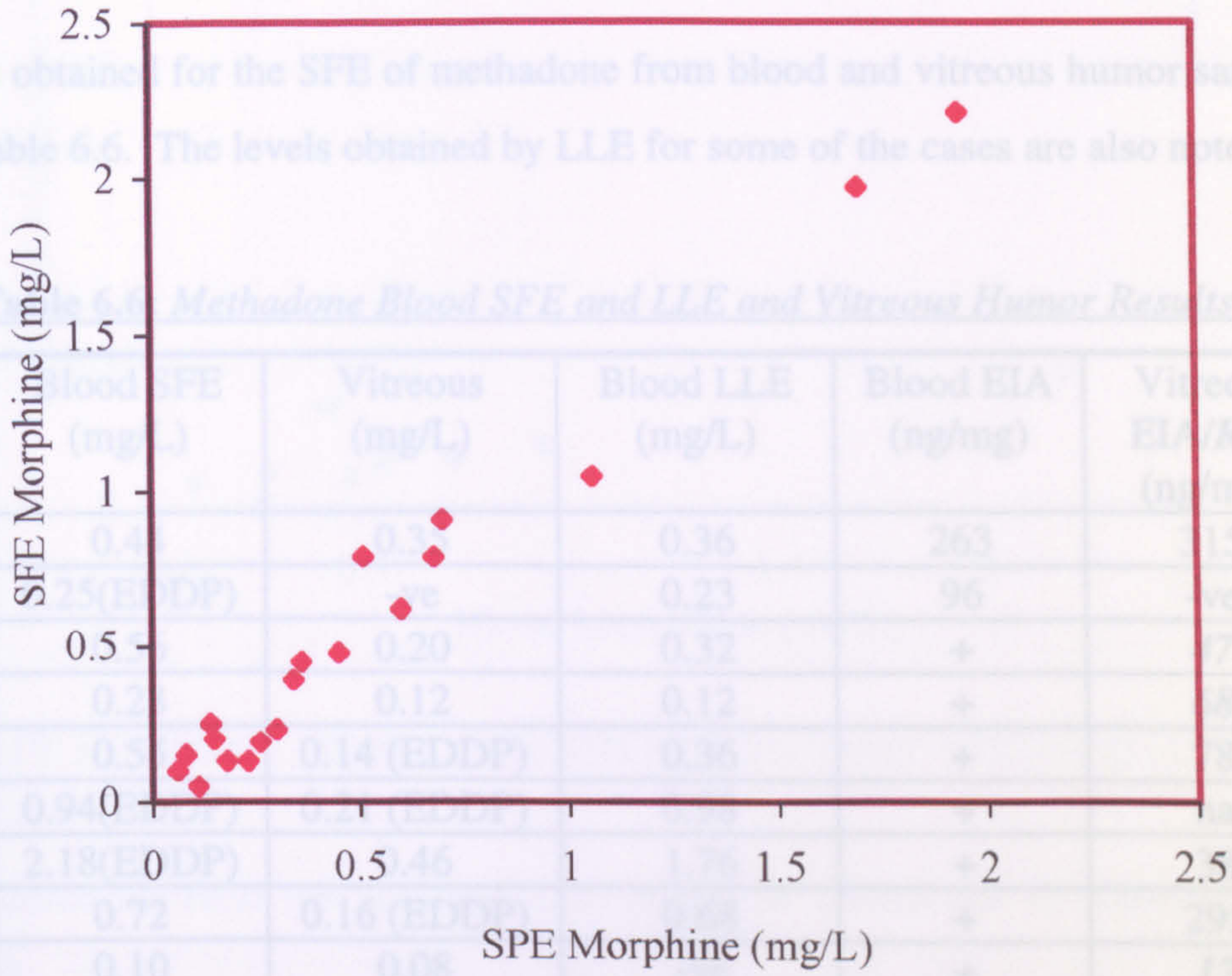


Figure 6.9: *Correlation Between SFE and SPE Blood Morphine Levels*

The correlation between blood and vitreous humor morphine levels was good, $r^2 = 0.712$. As with the benzodiazepines, in general the levels found in vitreous are lower than the corresponding levels in blood (gradient of best fit line = 0.79).

For the comparison between SFE and SPE for morphine in blood, again a good correlation was seen between the two methods ($r^2 = 0.977$) and the gradient of the best fit line was 1.13 thus, taking into account experimental error, the two techniques yield the same drug concentrations.

6.4.1.3 Methadone in Blood and Vitreous humor

The final drug to be analysed from blood and vitreous humor samples was methadone. As with the other two sets of drugs, EIA and RIA results were obtained. In addition to the methadone it was noted during the final stages of this study that EDDP was also being

extracted from blood and vitreous humor samples. As no method development was carried out for this metabolite it could not be quantitated. Where EDDP was detected in blood or vitreous humor it has been noted with the corresponding methadone level.

The results obtained for the SFE of methadone from blood and vitreous humor samples are given in Table 6.6. The levels obtained by LLE for some of the cases are also noted.

Table 6.6: *Methadone Blood SFE and LLE and Vitreous Humor Results*

Case	Blood SFE (mg/L)	Vitreous (mg/L)	Blood LLE (mg/L)	Blood EIA (ng/mg)	Vitreous EIA/*RIA* (ng/mg)
K96 08	0.44	0.35	0.36	263	315
K96 23	0.25(EDDP)	-ve	0.23	96	-ve
K96 21	0.56	0.20	0.32	+	***47***
K96 22	0.28	0.12	0.12	+	***68***
K96 24	0.55	0.14 (EDDP)	0.36	+	78
K97 03	0.94(EDDP)	0.21 (EDDP)	0.98	+	na
K96 13	2.18(EDDP)	0.46	1.76	+	38
K96 15	0.72	0.16 (EDDP)	0.68	+	295
K97 10	0.10	0.08	-ve	+	***17***
K97 13	0.41	0.34	-ve	+	***355***
K97 18	0.48(EDDP)	0.10 (EDDP)	0.30	+	***143***

A graph was plotted of the correlation between blood and vitreous humor methadone levels obtained using SFE (Figure 6.10).

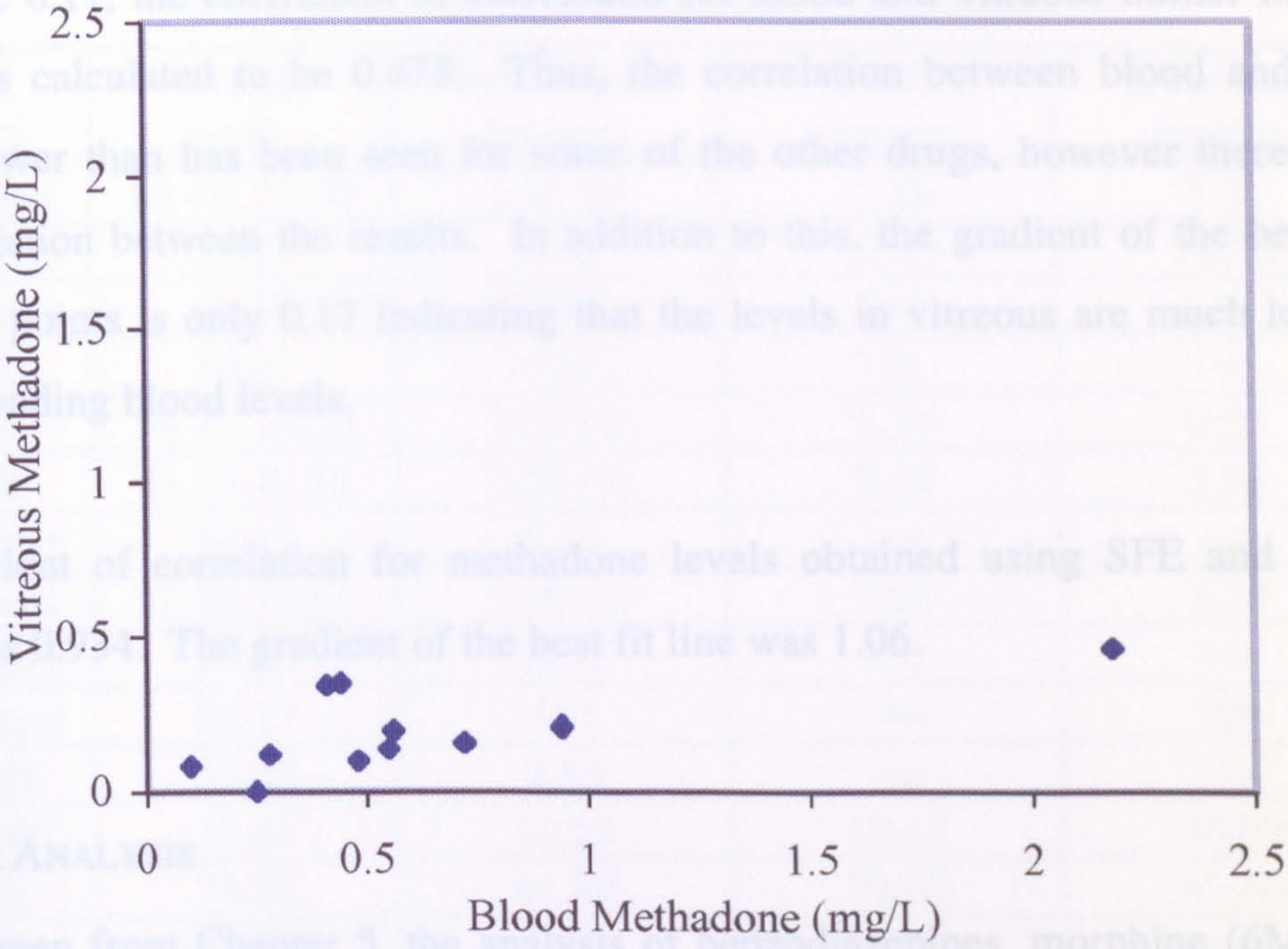


Figure 6.10: *Correlation Between Blood and Vitreous Humor Methadone Levels by SFE*

The correlation between the SFE and LLE results of methadone in blood is shown in Figure 6.11.

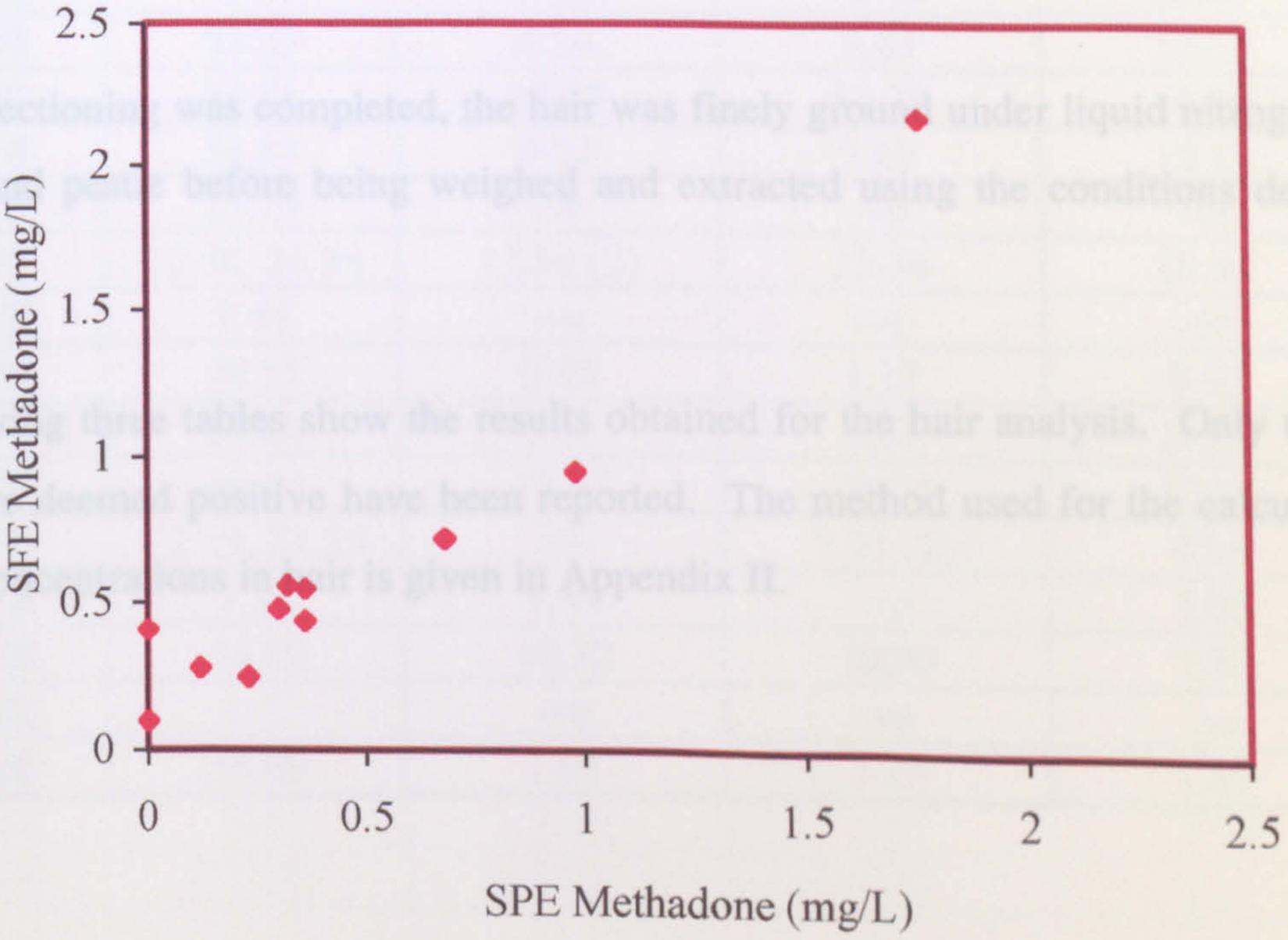


Figure 6.11: *Correlation Between SFE and LLE Blood Methadone Levels*

From Figure 6.11, the coefficient of correlation for blood and vitreous humor methadone by SFE was calculated to be 0.478. Thus, the correlation between blood and vitreous humor is lower than has been seen for some of the other drugs, however there is still a slight correlation between the results. In addition to this, the gradient of the best fit line through the points is only 0.17 indicating that the levels in vitreous are much lower than the corresponding blood levels.

The coefficient of correlation for methadone levels obtained using SFE and SPE was calculated as 0.934. The gradient of the best fit line was 1.06.

6.4.2 Hair Analysis

As can be seen from Chapter 5, the analysis of benzodiazepines, morphine (6MAM) and methadone from hair samples can be carried out using the same SFE method. Prior to being extracted, the hair samples were washed in MeOH, dH_2O and MeOH. Once dried, some of the samples were then sectioned. In most cases the hair samples were less than 6 cm long and the sectioning consisted of separating the roots from the bulk hair. In cases where the hair samples were longer than 6 cm, they were sectioned in 6 cm or 3 cm lengths. The sections are highlighted after the case numbers in the results tables below.

Once the sectioning was completed, the hair was finely ground under liquid nitrogen using a mortar and pestle before being weighed and extracted using the conditions defined in Chapter 5.

The following three tables show the results obtained for the hair analysis. Only the cases which were deemed positive have been reported. The method used for the calculation of the drug concentrations in hair is given in Appendix II.

Table 6.7: *Benzodiazepine Positive Hair Cases*

Case	Weight (mg)	Temazepam (ng/mg)	DMD (ng/mg)	Diazepam (ng/mg)
K96 01/**uncut**	50.46, 25.80	3.53 (2)	7.36	7.31
K96 02 **uncut**	29.85	8.44	15.99	14.16
K96 04/**uncut**	28.33	0.78	-ve	-ve
K96 05/**R**	16.27	-ve	-ve	-ve
K96 05/**1st3**	34.77, 39.14	1.04 (2)	1.36	0.95
K96 05/**B**	31.19,49.26	2.06 (2)	2.45	2.36
K96 07/**uncut**	37.49	9.15	-ve	-ve
K96 08 **uncut**	27.94,15.61	5.00 (2)	3.98	3.23
K96 09 **uncut**	26.83	23.55	-ve	-ve
K96 11 **uncut**	86.27	111	-ve	-ve
K96 16/**1st6**	32.35	2.33	-ve	-ve
K96 16/**2nd6**	19.67	10.23	12.13	16.09
K96 16/**B**	36.92	61.83	52.05	49.46
K96 16/**R**	12.68	-ve	-ve	-ve
K96 16 **uncut**	112.31	2.18	-ve	1.36
K96 21/**R**	20.03	-ve	-ve	-ve
K96 21/**1st6**	33.43	5.70	8.60	7.83
K96 21/**2nd6**	28.85	5.29	9.88	8.19
K96 21/**B**	28.85	56.79	34.58	38.24
K96 23/**R**	14.68	-ve	-ve	-ve
K96 23/**B**	22.15, 14.39	45.43 (2)	25.28	80.14
K96 24/**R**	16.44, 24.49	2.52 (2)	3.03	4.62
K96 24/**6**	45.37	23.89	18.78	20.08
K96 24/**5**	53.35	6.06	3.83	13.53
K96 24/**B**	54.81	37.05	57.38	16.04
K96 24/**B**	54.81	4.22	2.69	12.11
K96 29/**R**	25.14	-ve	-ve	-ve
K96 29/**3**	10.38, 26.94	13.50 (2)	12.84	10.81
K96 30/**R**	9.64	-ve	-ve	-ve
K96 30/**5**	49.11	0.93	1.17	0.85
K96 30/**B**	16.80	9.13	3.67	24.25
K97 01/**R**	46.04	13.34	13.24	17.18
K97 01/**B**	72.25, 21.35	-ve	7.91 (2)	4.96
K97 06/**R**	7.37	4.30	-ve	5.00
K97 06/**B**	14.80	10.32	10.00	7.30
K97 10/**R**		-ve	-ve	-ve
K97 10/**B**	38.09	1.98	2.29	1.67

R: Roots

B: Bulk

Table 6.8: *Morphine and 6MAM Positive Hair Cases*

Case	Weight (mg)	Morphine (mg/L)	6MAM (mg/L)	Morphine/ 6MAM
K96 13/**uncut**	51.75	10.18	0.78	13.05
K96 15/**R**	14.72	-ve	-ve	-
K96 15/**B**	14.72	13.4	63.6	0.21
K96 17/**uncut**	10.27	14.04	9.23	1.52
K96 18/**uncut**	12.14	55.24	42.75	1.29
K96 20/**R**	15.31	-ve	-ve	-
K96 20/**B**	43.10	-ve	-ve	-
K96 21/**R**	20.03	77.6	203.8	0.38
K96 21/**1st6**	33.43	18.2	22.6	0.81
K96 21/**2nd6**	28.85	40.5	64.0	0.63
K96 22/**R**	14.16	41.5	41.5	1.00
K96 22/**3**	34.68	15.8	-ve	-
K96 22/**B**	29.64	4.24	1.41	3.01
K96 24/**R**	30.90	0.65	2.07	0.31
K96 24/**6**	91.26	1.15	1.28	0.9
K96 24/**5**	53.53	1.57	2.62	0.60
K96 24/**B**	87.59	1.06	0.75	1.41
K96 25/**B**	22.67	10.37	5.56	1.87
K96 26/**R**	12.85	-ve	-ve	-
K96 26/**B**	83.26	99.1	16.61	5.97
K96 29/**R**	7.41	40.77	5.36	7.61
K96 29/**3**	8.71	46.82	-ve	-
K96 29/**B**	15.47	12.25	n/c	
K96 30/**R**	10.19	57.42	9.70	5.92
K96 30/**5**	84.05	18.7	1.43	13.08
K96 30/**B**	47.58	36.14	7.25	4.98
K97 03/**R**	17.58	10.53	5.80	1.82
K97 03/**B**	45.84	2.07	0.20	10.35
K97 05/**R**	20.93	9.14	8.49	1.08
K97 05/**B**	71.44	1.10	4.39	0.25
K97 07/**R**	15.95	-ve	-ve	-
K97 07/**3**	84.54	13.51	4.31	3.13
K97 07/**B**	61.11	5.44	7.69	0.71
K97 08/**R**	14.63	70.93	72.31	0.98
K97 08/**B**	30.00	2.97	8.08	0.37
K97 12/**R**	8.83	-ve	-ve	-
K97 12/**B**	56.72	-ve	-ve	-
K97 13/**R**	34.81	3.65	0.97	3.76
K97 13/**B**	112.84	1.43	1.48	0.97

Table 6.8: *Methadone Positive Hair Cases*

Case	Weight (mg)	Methadone (mg/L)	EDDP
K96 13/**uncut**	51.75	24.02	✓
K96 15/**R**	16.98	-ve	x
K96 15/**B**	14.72	20.20	✓
K96 17/**uncut**	10.27	1.26	x
K96 18/**uncut**	12.14	0.70	✓
K96 20/**R**	15.31	0.91	✓
K96 20/**B**	43.10	2.12	✓
K96 21/**R**	14.16	16.7	✓
K96 21/**1st6**	33.43	4.16	✓
K96 21/**2nd6**	28.85	-ve	x
K96 22/**R**	14.16	7.81	✓
K96 22/**3**	34.68	1.78	x
K96 22/**B**	29.64	2.42	x
K96 24/**R**	30.90	5.53	✓
K96 24/**6**	91.26	8.09	✓
K96 24/**5**	53.53	1.78	x
K96 24/**B**	87.59	-ve	x
K96 25/**B**	16.50	0.40	✓
K96 25/**R**	26.28	0.53	✓
K96 25/**B**	22.67	1.68	✓
K96 29/**R**	7.41	9.07	✓
K96 29/**3**	8.71	10.53	✓
K96 29/**B**	15.47	5.93	✓
K96 30/**R**	10.19	1.22	x
K96 30/**5**	84.05	-ve	x
K96 30/**B**	47.58	-ve	x
K97 03/**R**	17.58	63.93	✓
K97 03/**B**	45.84	11.92	✓
K97 05/**R**	20.93	-ve	x
K97 05/**B**	71.44	-ve	x
K97 07/**R**	15.95	1.90	x
K97 07/**3**	84.54	8.37	x
K97 07/**B**	61.11	-ve	x
K97 08/**R**	14.63	-ve	x
K97 08/**B**	30.00	-ve	x
K97 13/**R**	34.81	-ve	x
K97 13/**B**	112.84	-ve	x

As with the blood and vitreous humor samples, EDDP was detected but not quantitated.

✓ : Detected; x: not detected.

In addition to analysing the SFE hair extracts for the presence of drugs, the final MeOH hair wash was also analysed. All washes were found to be negative for the drugs being investigated.

6.5 Comparison of SFE Results with Case Histories

For this final part of this study, the cases histories were individually compared firstly with the results from blood and vitreous humor and secondly with the hair results. The first comparison relates to the circumstances surrounding the death of the deceased and the second related to their drug history over the past year. In each case, an example of how the comparisons were made will be given. This will be followed by a summary table for the remainder of the cases.

6.5.1 Comparison of Case History with Blood and Vitreous Humor Results

The case which will be discussed here is K96 22 which was found to be positive for temazepam (1.30 mg/L blood; 0.76 mg/L vitreous humor), desmethyldiazepam (0.68 mg/L blood; 0.27 mg/L vitreous humor), diazepam (none detected in blood; 0.62 mg/L vitreous humor), morphine (2.21 mg/L blood; 1.67 mg/L vitreous humor), 6MAM (none detected in blood; 0.011 mg/L vitreous humor) and methadone (0.28 mg/L blood; 0.12 mg/L vitreous humor). The case was that of a 27 year old female and from the post-mortem report it was ascertained that the cause of death was due to heroin and temazepam intoxication. The time between the death of the deceased and post-mortem was 7 days, indicating that a degree of decomposition will have occurred.

From the police report which accompanied the case, the information available implied that the cause of death was heroin related as a syringe was found *in-situ*. The deceased was also on a methadone maintenance programme although she failed to pick up her last prescription. No mention was made in the report of the use of benzodiazepines.

If no police report had been available, with only the blood results, it would not have been possible to say for definite that the case of death was due to heroin intoxication as no 6MAM was present in the blood. However, the analysis of vitreous humor shows that

death was in fact heroin related as a small amount of 6MAM was detected indicating that heroin had been used shortly before death.

Table 6.10 gives a brief summary of the cases which were positive, drugs which were detected, and expected and whether the results obtained agree or disagree with the drug history.

Table 6.10: *Comparison of Case Results with PM and Police Reports*

Case	Drugs Detected				Drugs Expected			Agree / Differ	Heroin Abuse Confirmed
	M	6M	Meth	Benzo	Heroin	Meth	Benzo		
K96 02	✓	n/c	x	✓	✓	x	✓	A	-ve*
K96 03	x	x	x	✓	✓	x	x	D	-ve
K96 04	✓	n/c	x	✓	x	x	✓	D	+ve*
K96 07	✓	n/c	x	✓	✓	✓	✓	D	-ve*
K96 08	✓	n/c	✓	✓	✓	✓	✓	A	-ve*
K96 10	✓	n/c	x	x	✓	x	x	A	-ve*
K96 11	✓	n/c	x	✓	✓	x	x	D	-ve*
K96 12	✓	n/c	x	x	x	x	x	D	-*
K96 13	x	x	✓	✓	✓	x	✓	D	-ve
K96 14	✓	n/c	x	✓	x	x	x	D	-*
K96 15	x	x	✓	✓	✓	✓	x	D	+ve
K96 17	✓	n/c	x	x	✓	x	x	A	-ve*
K96 18	✓	x	x	✓	✓	✓	✓	D	-ve
K96 19	✓	✓	x	x	✓	x	x	A	+
K96 20	✓	✓	x	✓	✓	x	x	D	+
K96 21	✓	✓	✓	✓	✓	✓	✓	A	+
K96 22	✓	✓	✓	✓	✓	✓	x	A	+
K96 23	x	x	✓	✓	x	x	✓	D	-
K96 24	x	x	✓	✓	✓	✓	✓	D	-ve
K96 25	✓	✓	x	✓	✓	x	✓	A	+
K96 26	x	x	x	✓	✓	x	x	D	-ve
K96 27	✓	✓	x	✓	✓	✓	✓	D	+
K96 28	✓	n/c	x	✓	x	x	✓	D	-*
K96 29	✓	n/c	x	✓	✓	x	✓	A	-ve*
K96 30	x	x	x	✓	✓	x	✓	D	-ve
K97 02	x	x	x	✓	x	✓	✓	D	-
K97 03	x	x	✓	x	x	✓	✓	D	-
K97 04	✓	✓	x	✓	✓	x	✓	A	+
K97 07	✓	✓	x	x	✓	x	x	A	+
K97 09	✓	✓	x	x	✓	x	x	A	+
K97 10	✓	✓	✓	x	x	x	✓	D	+ve
K97 11	✓	x	x	x	✓	x	✓	D	-ve*

Table 6.10: Continued

Case	Drugs Detected				Drugs Expected			Agree / Differ	Heroin Abuse Confirmed
	M	6M	Meth	Benzo	Heroin	Meth	Benzo		
K97 13	x	x	✓	x	✓	✓	x	D	-ve*
K97 14	✓	✓	x	x	✓	x	x	A	+
K97 15	✓	✓	x	x	x	x	x	D	+ve
K97 16	✓	✓	x	x	✓	x	x	A	+
K97 17	✓	✓	x	x	✓	x	x	A	+
K97 18	✓	✓	✓	x	✓	x	x	D	+

M: Morphine; 6M: 6MAM; Meth: Methadone; Benzo: Benzodiazepine.

+: heroin confirmed by presence of 6MAM in blood and/or vitreous

-: heroin not expected and not determined

-*: heroin not expected, morphine not detected but 6MAM not analysed for

+ve: heroin determined but not expected

-ve: heroin expected but not determined

-ve*: heroin expected but not determined however, morphine positive and 6MAM not analysed for

✓: expected or detected

x: : not expected or not detected

6.5.2 Comparison of Case Histories with Hair Results

As mentioned previously, the second comparison which was carried out was to use the results obtained by hair analysis to confirm past drug abuse. Case K96 24 was chosen as an example.

Case K96 24 was found to be positive for all three types of drug. As the hair sample was reasonably long, it was sectioned into three parts (roots (R), next 6 cm, final 5 cm) in addition to this some of the hair was left unsectioned as a bulk (B) sample. As can be seen from Tables 6.7-6.9, the sample contained the amounts of drugs shown in Table 6.11.

Table 6.11: *Summary of Hair Analysis Results for Case K96 24*

Section	Benzodiazepines (ng/mg)			Opiate (ng/mg)		Methadone (ng/mg)
	Temaz	DMD	Diaz	Morphine	6MAM	
Root	2.52	3.03	4.62	0.65	2.07	5.53
6 cm	23.89	18.78	20.08	1.15	1.28	8.09
5 cm	6.06	3.83	13.53	1.57	2.62	1.78
Bulk	4.22	2.69	12.11	1.06	0.75	-ve

The deceased in this case was a 20 year old female. From the past history it was seen that she was a known drug abuser whose drug problem was defined as "slight". She was said to have taken temazepam when depressed and had taken Valium® the week before her death. In addition, she was known to have smoked heroin. She was not known to be on a methadone maintenance programme, however an empty bottle of methadone mixture was seized from the locus.

From the information obtained from the hair analysis, it appears that the deceased had been using temazepam, diazepam, methadone and heroin in the year and month prior to death. The blood analysis showed the presence of methadone, temazepam, diazepam and desmethyldiazepam which were confirmed as being used over the past year by the hair analysis. No heroin was detected in blood or vitreous humor, thus without the use of hair analysis, this would have gone undetermined.

Although the detection of heroin in the hair of the deceased is not relevant in terms of the cause of death, in cases where the subject is still alive and drug abuse is suspected, hair analysis is more informative than blood analysis. In particular, the roots of the hair were found to contain all three types of drugs indicating use of these drugs within one month prior to death.

Table 6.12 contains summary information of all hair samples analysed and relates then to the information available from the PM and police reports.

The results obtained for benzodiazepines, morphine and methadone were compared to the deceased's drug history. In this respect the hair analysis is particularly interesting as findings may be compared to the drug history of the deceased several months prior to

death. In some cases, the results obtained supported the police report, however there were cases where drug use was not confirmed and indeed some cases where drugs were detected which were not mentioned in the deceased's drug history (*e.g.* methadone detected in the hair of a subject not known to be on a methadone maintenance programme).

Table 6.12: *Comparison of Case Hair Results with PM and Police Reports*

Case	Drugs Detected				Drugs Expected			Agree/ Differ	Heroin Abuse Confirmed
	M	6M	Meth	Benzo	Heroin	Meth	Benzo		
K96 01	x	n/c	x	✓	x	x	✓	A	-*
K96 02	x	n/c	x	✓	✓	x	✓	D	-ve*
K96 04	x	n/c	x	✓	x	x	✓	A	-*
K96 05	x	n/c	x	✓	✓	✓	✓	D	-ve*
K96 07	x	n/c	x	✓	✓	✓	✓	D	-ve*
K96 08	x	n/c	x	✓	x	✓	✓	D	-*
K97 09	x	n/c	x	✓	✓	x	x	D	-ve*
K96 11	x	n/c	x	✓	✓	x	x	D	-ve*
K96 13	✓	✓	✓	x	✓	x	✓	D	+
K96 15	✓	✓	✓	x	✓	✓	x	A	+
K96 16	x	x	x	✓	✓	x	x	D	-ve
K96 17	✓	✓	✓	x	✓	x	x	D	+
K96 18	✓	✓	✓	x	✓	✓	✓	D	+
K96 20		x	✓	x	✓	x	x	D	-ve
K96 21	✓	✓	✓	✓	✓	✓	✓	A	+
K96 22	✓	✓	✓	x	✓	✓	x	A	+
K96 23	x	x	x	✓	x	x	✓	A	-
K96 24	✓	✓	✓	✓	✓	✓	✓	A	+
K96 25	✓	✓	✓	x	✓	x	✓	D	+
K96 26	✓	✓	x	x	✓	x	x	A	+
K96 29	✓	x	✓	✓	✓	x	✓	D	+*
K96 30	✓	✓	✓	✓	✓	x	✓	D	+
K97 01	x	x	x	✓	✓	x	x	D	-ve
K97 03	✓	✓	✓	x	x	✓	✓	D	+ve
K97 05	✓	✓	x	x	x	x	x	D	+ve
K97 06	x	x	x	✓	x	✓	✓	D	-
K97 07	✓	✓	✓	x	✓	x	x	D	+
K97 08	✓	✓	✓	x	✓	✓	x	A	+
K97 10	x	x	x	✓	x	x	✓	A	-
K97 12	✓	✓	x	x	✓	✓	✓	D	+
K97 13	✓	✓	✓	x	✓	✓	x	A	+

For key see Table 6.10.

From Table 6.12, it can be seen that less than half of the cases correlate to the results expected from the PM and police reports. 8 of the 23 "known" heroin users had no morphine or 6MAM detected in their hair sample, however only two cases were detected where heroin abuse was not suspected.

For methadone, 7 cases (47% of total positives) were found to be positive where no mention of methadone maintenance was given in the police report. There were 5 cases where the deceased was previously on a maintenance programme where no methadone was detected.

The best correlation between the reports and the hair analysis results was for the benzodiazepines. Only 5 cases were found not to be positive where expected and only 4 false positives were determined (no record of previous use and positive hair result).

In terms of agreement of the comparison between the results by blood / vitreous humor and hair with the PM and police reports, there is little difference (39.5 % agreement for blood / vitreous humor and 35.5% agreement for hair). However, in terms of the determination of heroin abuse, hair analysis is more likely to detect the use of heroin (% agreement = 61%) than analysis of blood or vitreous humor (% agreement = 50%). (It should be noted that the cases where 6MAM was not analysed for have been omitted from the % agreement).

6.6 Conclusions

Post-mortem blood, vitreous humor samples were analysed for the presence of benzodiazepines (temazepam, desmethyldiazepam, diazepam), morphine and methadone using the SFE methods developed in Chapters 2, 3 and 4 respectively. Metabolites of morphine (6MAM) and methadone (EDDP) were also detected. All three SFE methods were found to successfully extract the drugs from the matrices and yielded results comparable to those determines using SPE and LLE techniques.

The usefulness of vitreous humor analysis as an alternative to blood was highlighted. Good correlations between blood and vitreous humor were seen for most of the drugs investigated indicating that vitreous humor can be used as an alternative sample when

blood is not available for analysis. Due to its relatively simple matrix, vitreous humor also produced cleaner extracts then blood.

As with the blood and vitreous humor analysis, the hair extraction method was found to successfully recover benzodiazepines, morphine, 6MAM and methadone. Although the correlations between the analytical results and the PM and police reports were relatively poor, it was possible to use 6MAM as a marker of past heroin abuse and in some cases confirm past drug use up to one year prior to death. The use of hair in post-mortem cases is of limited use, other than in the confirmation of drug histories, however the developed method would be useful in determining past drug use in other areas *e.g.* employee and parolee testing.

7. CONCLUSIONS FROM THESIS

The aim of this study was to develop extraction methodology using supercritical fluid technology for the determination of drugs of forensic interest in a range of biological matrices. Three types of drug were investigated:

1) Benzodiazepines
2) Morphine
3) Methadone

The first sample matrix to be investigated was blood. Successful method development was carried out and methodology was established for all three drug types. The benzodiazepines were extracted using SF-CO_2 with ethyl acetate as a modifier at 65 °C and 3000 psi. The extraction yielded >80% recoveries of temazepam, desmethyldiazepam and diazpam within 10 minutes.

For the extraction of morphine and methadone, it was found that both drugs extracted with >80% recoveries using the same conditions. SF-CO_2 was still used as the main extraction fluid and a mixture of methanol and triethylamine was added as a modifier. The extractions were run at 100 °C and 3500 psi. In addition to efficiently extracting morphine and methadone, an additional benefit of this extraction was that it also allowed for the extraction of 6-monoacetyl morphine, the primary metabolite of heroin. Recoveries and reproducibility for the extraction of 6MAM were good.

The second sample matrix to be investigated was vitreous humor. It was found that the methods which had been developed for the extraction of the drugs from blood were also ideal for the extractions from vitreous humor. This allowed direct comparison of the two sets of results obtained from blood and vitreous humor.

The final matrix to be investigated was hair. Hair analysis is becoming of increasing importance in the forensic field as it provides a readily available marker of past drug use.

Methodology was developed from existing procedures and all three types of drugs were successfully extracted using the same technique.

Extractions of post-mortem blood, vitreous humor and hair samples were carried out using the developed methods and were found to compare well to conventional extraction techniques.

A total of 48 cases were investigated and the following comparisons were made.

1) Vitreous humor samples were analysed and the results compared to those obtained for blood in order to determine the usefulness of this sample matrix. With the exception of desmethyldiazepam, all drugs investigated gave good correlations between blood and vitreous humor results, although the levels in vitreous humor were generally lower than the corresponding levels in blood.

2) In order to confirm that the developed SFE methods were efficient for the extraction of the drugs of interest from authentic samples (all method development was carried out using spiked samples) some of the cases were analysed by the conventional technique used in the routine laboratory (SPE for benzodiazepines and morphine, LLE for methadone). In all cases the two methods correlated well.

3) The results obtained for benzodiazepines, morphine and methadone were compared to the deceased's drug history to determine if correlations existed. Heroin abuse was confirmed by the presence of 6MAM in all three matrices. Hair analysis was found to be particularly useful as it provided a better match with the drug history than blood or vitreous humor.

It has been shown, that supercritical fluid extraction is a valuable tool for the analysis of drugs and their metabolites in a variety of matrices. The developed SFE methods are more efficient and less costly in consumables than conventional extraction techniques. For the analysis of hair, SFE has the additional advantage of a substantial reduction in extraction time, as the preparative steps can be included in the methodology.

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CALCULATION OF CONCENTRATION IN BLOOD / VITREOUS HUMOR SAMPLES

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

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

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

The Dilution Factor is used when the volume of the sample and standard are not the same *e.g.* 1 ml of standard and 100 µl of sample gives a dilution factor of 10.

CALCULATION OF CONCENTRATION IN HAIR SAMPLES

$$\text{Drug Concentration(ng / mg)} = \frac{\text{Sample Ratio}}{\text{Standard Ratio}} \text{x} \frac{\text{Concentration of Drug in Standard (ng)}}{\text{Weight of Hair Sample (mg)}}$$

RECOVERY STUDIES

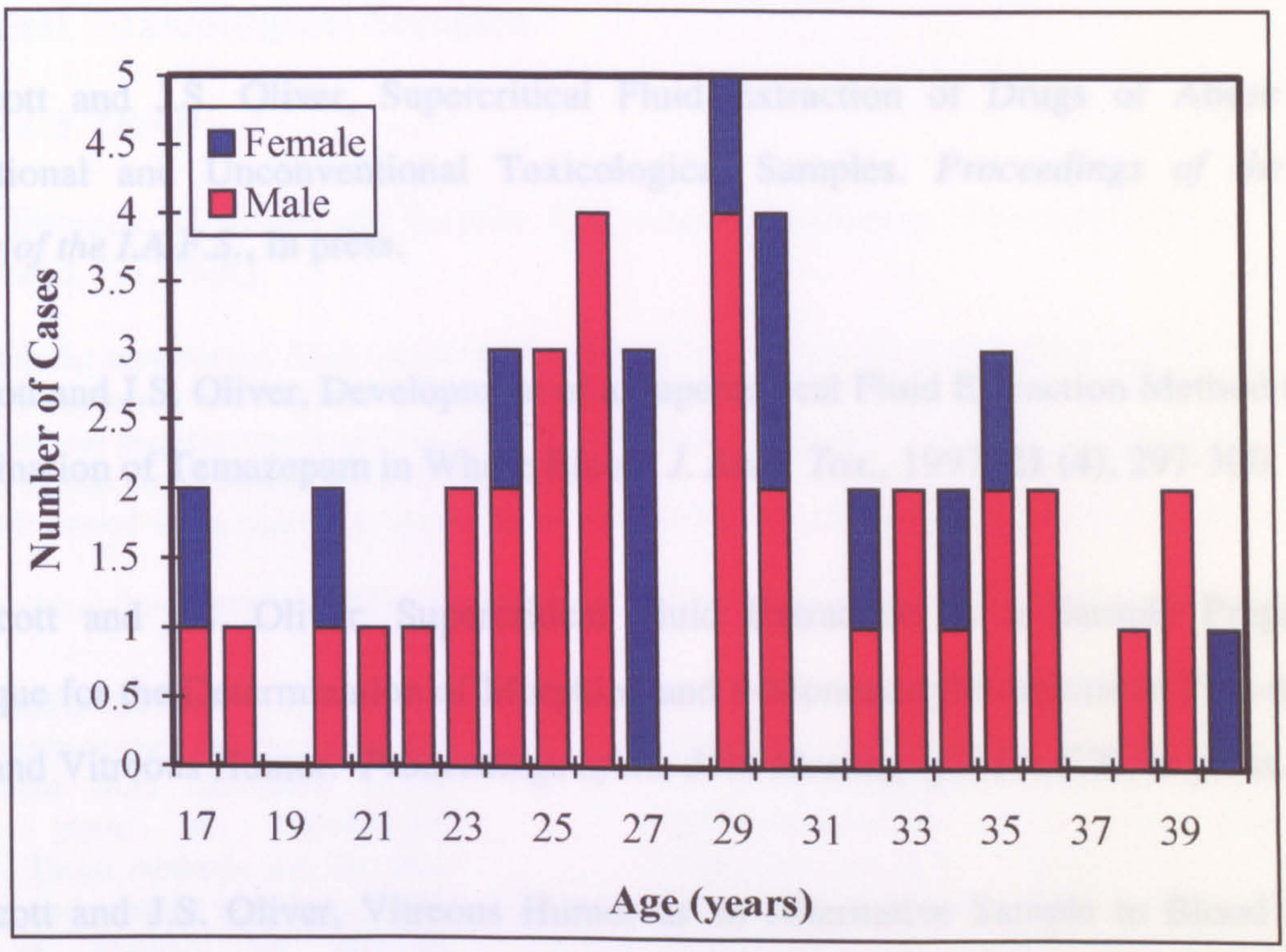
For recovery studies, the calculations were carried out as follows:

$$\% \text{ Recovery} = \frac{\text{Extracted Standard Ratio}}{\text{Unextracted Standard Ratio}} \text{x } 100$$

STANDARD DEVIATION:

In order to assess variability of data obtained, the relative standard deviation (RSD) was calculated according to the equation below;

$$RSD = \sqrt{\frac{\sum(x-\bar{x})^2}{\sum(n-1)}}$$



The distribution of age and sex of the cases investigated is shown in the above figure. It can be seen that the cases were predominantly male (72%) and that the most common age was 29 (mean 27).

The following papers have been published in support of this thesis.

K.S. Scott and J.S. Oliver, Supercritical Fluid Extraction of Drugs of Abuse from Conventional and Unconventional Toxicological Samples. *Proceedings of the 14th Meeting of the I.A.F.S.*, In press.

K.S. Scott and J.S. Oliver, Development of a Supercritical Fluid Extraction Method for the Determination of Temazepam in Whole Blood. *J. Anal. Tox.*, 1997, **21 (4)**, 297-300.

K.S. Scott and J.S. Oliver, Supercritical Fluid Extraction as a Sample Preparation Technique for the Determination of Morphine and 6-Monoacetylmorphine in Post-mortem Blood and Vitreous Humor. *Proceedings of the 35th Meeting of T.I.A.F.T.*, In press.

K.S. Scott and J.S. Oliver, Vitreous Humor as an Alternative Sample to Blood for the Supercritical Fluid Extraction of Morphine and 6-Monoacetylmorphine. *Accepted for publication in Medicine Science and the Law, Dec 1997.*

Supercritical Fluid Extraction of Drugs of Abuse from Conventional and Unconventional Toxicological Samples

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This article details the supercritical fluid extraction of benzodiazepines, morphine and methadone from post-mortem blood, vitreous humor and hair from suspected drug abusers.

A comparison between post-mortem blood and vitreous humor levels was made in order to investigate the use of vitreous humor as an alternative toxicological medium to blood.

Hair analysis was carried out in order to evaluate the use of hair in the determination of chronic drug abuse.

Keywords: Supercritical fluid extraction, hair, vitreous humor, blood, benzodiazepines, morphine, methadone.

Introduction

Most present methodology for the extraction of drugs of abuse from biological liquids involve solid phase or liquid-liquid extractions[1,2]. These methods can be time consuming, costly and wasteful of sample. With the increase in demand for efficient toxicological analyses the development of new extraction technology is of the utmost importance. Supercritical fluid technology has grown dramatically over the past decade, however despite this, its acceptance in routine forensic analysis has been limited[3,4,5].

In addition to speed and convenience of use, the main advantages of supercritical fluids include improved efficiency, non-toxicity and cost-effectiveness[6]. The possibility for direct extraction from complex matrices, in parallel with a reduced risk of sample contamination highlights the power of supercritical fluids as a tool for analysis.

As well as time of death related drug analysis, it can be important to obtain information relating to long term drug abuse. Although the analysis of hair has been used for this purpose[7,8,9], work is still required in this area in order to provide a validated, universally accepted method.

The supercritical fluid analysis of drugs of abuse in hair has also been reviewed[10]. The results obtained tend to be similar to those obtained by conventional digestion or methanolic extraction techniques which are time consuming and destructive. Here again the advantage of short extraction times with supercritical fluids is emphasised.

Experimental

Temazepam and prazepam were purchased from Wyeth Laboratory. Morphine, d3-morphine, methadone and d3-methadone were purchased from Sigma. Methanol, ethyl acetate and dichloromethane are HPLC grade (Lab Scan Analytical Sciences). Triethylamine is HPLC grade and Na_2HPO_4 GPR grade (BDH). BSTFA with 1% TMCS (Pierce) was used for the derivitisation of morphine and d3-morphine. The CO_2 is supplied by Air Products and comes in 25 kg cylinders fitted with a dip-tube. Extrelut® (Merck) is prewashed in dichloromethane. Collection vials for SFE are 6 mL HypoVial™ (Pierce) with butyl rubber septa (Pierce and Warriner). Columns for SFE were made using 3 cm x 4.6 mm i.d. stainless steel tubing. Water was deionised using a Milli-Q® water purification system (Millipore®)

The SFE system consists of two Gilson 306 pumps, two rheodyne valves and a Pye series 104 GC oven with pressure restrictor. All components were modified to suit the supercritical extractant. The CO_2 pumphead was refridgerated to maintain the flow of liquid CO_2 using a Gilson model SFC3 refridgeration unit. The extraction columns were placed over positions 1 and 4 in the second rheodyne and the extract collected by expansion into MeOH in a HypoVial™ at the flow outlet.

For the extraction of temazepam from blood and vitreous humor, the method used was that reported by Scott and Oliver[11]. This method proved ineffective in the extraction from hair. Methadone and morphine analysis was carried out using a triethylamine : methanol modifier which again was efficient in the extraction of these drugs from blood and vitreous humor but failed in the analysis of hair. Successful extraction of morphine from hair was reported by Cirimele, *et al*[10] and this method proved

effective in the extraction of all three drug types from hair.

Sample preparation

Blood and vitreous humor

Extrelut® was used as a support medium for blood and vitreous humor samples. Approximately 0.2 g of extrelut were placed in a plastic weighing boat and 100 µL of sample loaded, mixed and left to dry at room temperature. The contents of the boat were then transfered to an extraction cell.

Extraction conditions

Condition	Benzodiazepines	Morphine/Methadone
Temp:	65 °C	100 °C
Pressure:	300 psi	3500 psi
Modifier :	Ethyl Acetate	MeOH:Et_3N(85:15)
Flow:	2 mL/min	2 mL/min
Time:	20 min	30 min
Analysis:	HPLC	GC-MS

Hair

Hair samples were decontaminated by washing with H_2O then methanol, then again with H_2O. In order to maximise the surface area for extraction, the hair was ground using either a mortar and pestle with liquid nitrogen or an analytical mill (IKA, Labortechnik) with dry ice. Approximately 50 mg of decontaminated ground hair was then placed in an extraction cell.

Extraction conditions

Condition	All drugs
Temp:	100 °C
Pressure:	3500 psi
Modifier:	MeOH:Et_3N:H_2O (2:2:1)
Flow Rate:	2 mL/min
Cleanup:	Static 10 min Continuous 15 min
Extract:	Add 500 µL modifier Static 15 min Continuous 30 min

Results and Discussion

Blood and vitreous humor

Post-mortem blood, vitreous and hair samples were obtained for nine suspected drug abuse cases. In all nine cases, quantities of drug were found which could have contributed to death.

For the hair samples, the three wash steps were sufficient to remove any environmental contamination from the hair and results reported are from consumed drugs only.

Seven of the cases showed levels of morphine in blood from 0.07-1.20 mg/L (mean 0.57). Of these, five had positive vitreous humor, 0.24-0.44 mg/L (mean 0.35). In the case with a level of 0.07 mg/L blood, no morphine was detected in vitreous humor by GC-MS and for one of the cases no vitreous humor was available.

Only two of the cases showed positive methadone levels in blood and vitreous humor. In blood, the levels were 0.20 and 0.09 mg/L and in vitreous humor 0.19 and 0.32 mg/L respectively.

Six of the cases showed positive temazepam blood levels ranging from 0.13-1.22 mg/L (mean 0.54). Of these, three showed positive vitreous humor (in two, vitreous humor was not available and in one temazepam was not detected by HPLC). The positive measurements ranged from 0.18-0.54 mg/L (mean 0.31). Other benzodiazepines were found in three cases. For one of these cases vitreous humor was not available. One case showed 1.01 mg/L desmethyldiazepam (DMD) in blood and 0.93 mg/L in vitreous humor. The other case showed 0.47 mg/L DMD plus 1.00 mg/L diazepam in blood and 0.18 mg/L DMD in vitreous humor.

Hair

Four cases were positive for morphine in hair and the values ranged from 1.09-1.50 ng/mg (mean 1.31). The same cases were also positive for methadone. Methadone concentrations ranged from 1.29-2.91 ng/mg (mean 2.00). In hair only temazepam has been analysed for at present. Five cases showed positive temazepam ranging from 0.92-111 ng/mg (mean 28.35).

In order to determine if any relationship exists between blood and vitreous humor results, it will be necessary to review more cases. However, primary results have shown that in general, positive vitreous humor results are lower than the corresponding blood results.

The results obtained from hair analysis verify information available on previous drug histories *e.g.* four of the cases were receiving methadone treament prior to death. In two of these cases methadone was not detected in blood or vitreous humor. The hair analysis was positive.

Conclusions

This study has demonstrated the use of supercritical fluids in the extraction of drugs of abuse from blood, vitreous humor and hair. It provides a fast and efficient alternative to conventional extraction methods.

As only 100 µL of sample is required for blood and vitreous humor extractions, it is more economical in its use of sample than conventional procedures.

It is beneficial to find that the hair extraction method works for all three drug types investigated, resulting in a requirement for only

one extraction per case. Ordinarily, the digestion conditions for hair have to be carefully selected to ensure no artefact production. In the case of supercritical fluid extraction this is not an issue as the conditions used are relatively mild.

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Development of a Supercritical Fluid Extraction Method for the Determination of Temazepam in Whole Blood

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Abstract

A supercritical fluid extraction (SFE) procedure for the analysis of temazepam from whole blood was developed. Quantitative recoveries were obtained by high-performance liquid chromatography using prazepam as an internal standard and carefully monitoring the extraction temperature and pressure. The results were found to compare well with those obtained by solid-phase extraction techniques, but they also had the advantages of reduced solvent consumption and minimal sample handling. The application of this method to authentic forensic blood specimens showed the SFE method to be useful as an alternative procedure for the extraction of temazepam in the toxicology laboratory.

Introduction

In western Scotland in 1994, some 141 deaths in which temazepam was a contributory factor were reported. Between 1993 and 1995, an 80% increase in the number of drivers found with considerable levels of temazepam was observed (1). These figures reflect the increasing trend in temazepam abuse in Scotland.

At present, temazepam analysis from biological matrices involves a solid-phase extraction (SPE) method. The procedure used is a modification of that reported by Zweipfenning et al. (2). Disadvantages of this procedure include the need for high-purity organic solvents and the resultant generation of substantial quantities of waste solvent. In addition, the sample preparation can be tedious and time consuming with increased potential for contamination (3). As an alternative method of sample preparation, the use of supercritical CO_2 has been investigated.

In addition to speed and convenience, the main advantages of supercritical fluid extraction (SFE) are the improved efficiency, the nontoxicity, the cost effectiveness of the extraction fluid, and the possibility for direct analysis of complex matrices, thus reducing the risk of sample contamination (4).

In order to develop a method for SFE, the analyst must have an understanding of the properties of the analyte and the composition of the matrix (5). As the benzodiazepines were relatively polar, it was necessary to modify the CO_2 by the addition of an entrainer (ethyl acetate) in order for the extraction to be feasible. The solvating power of the supercritical fluid was optimized by varying the pressure and temperature to obtain the maximum recovery of temazepam.

Quantitation by the SFE method was achieved by the addition of prazepam as an internal standard. The optimum conditions for temazepam gave peak-area ratios comparable with those of unextracted standards as long as the temperature and pressure were carefully controlled.

The developed method was first used to assess SFE in comparison with SPE for the extraction of temazepam and prazepam standards from methanol and blood. It was then used to investigate the analysis of authentic forensic blood specimens, again by comparison with SPE.

Experimental

Materials

Temazepam and prazepam were supplied by Wyeth Laboratory (Hants, U.K.). Methanol, ethyl acetate, diethyl ether, and dichloromethane were high-performance liquid chromatographic HPLC/grade (Lab Scan Analytical Sciences, Dublin,

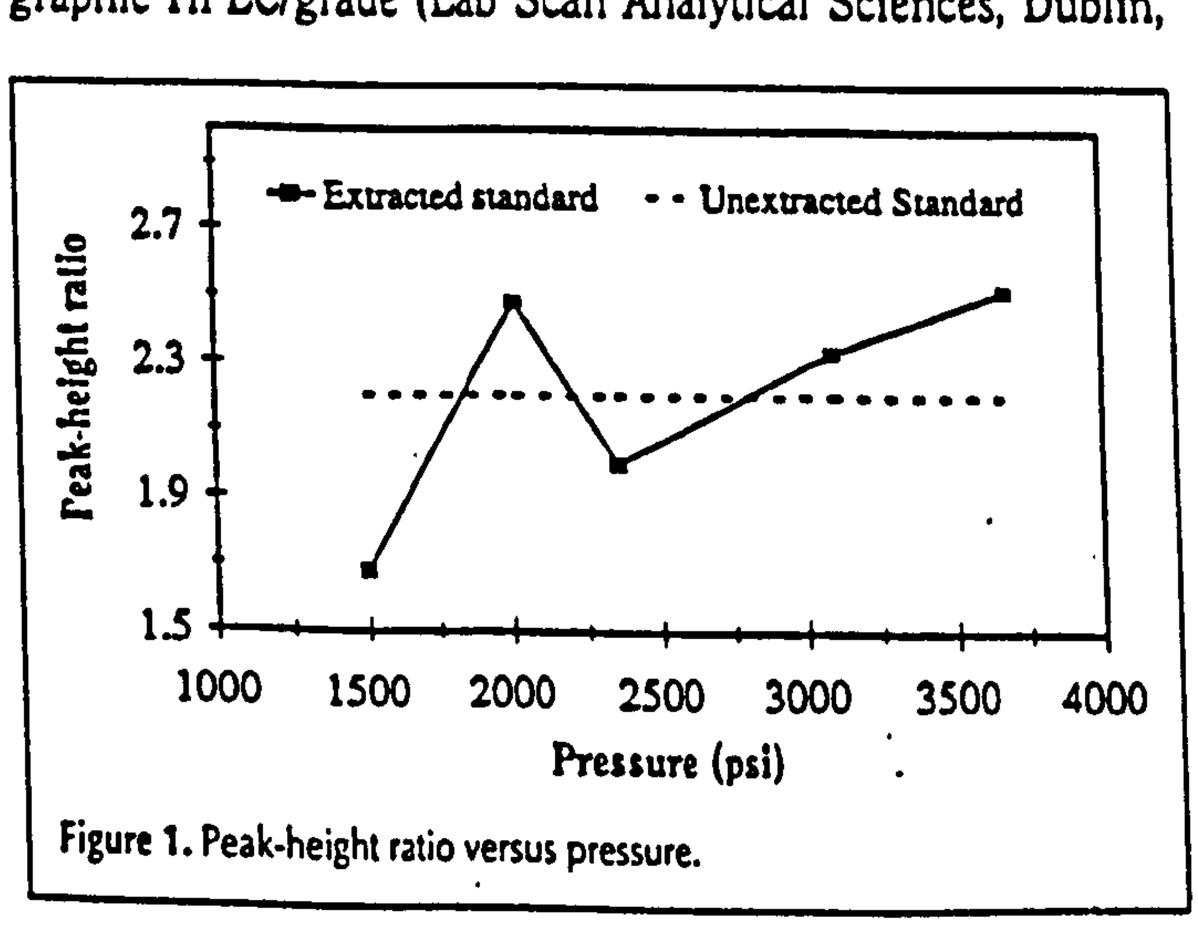

Figure 1. Peak-height ratio versus pressure.

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Ireland), and ammonia was analytical grade (Merck, Poole, U.K.). KH_2PO_4 was HiPerSolv grade, and Na_2HPO_4 was GPR grade (Merck). The CO_2 was supplied by Air Products (Walton-on-Thames, U.K.) in 25-kg cylinders fitted with a dip tube. Plastic syringes for SPE were 10-mL sterile Plastipak syringes (Becton Dickinson, Oxford, U.K.). Glass wool for SPE was silanized (Jones Chromatography, Mid Glamorgan, U.K.). Extrelut® (Merck) was prewashed using dichloromethane. The vials used for SPE were Anchor and Trident (FBG, London, U.K.) with screw caps (Merck). The vials used for SFE were 6 mL HypoVial™ (Pierce, Oud-Beijerland, The Netherlands) with butyl rubber septa (Pierce and Warriner, Chester, U.K.). Columns for the SFE were made using 3 cm × 4.6-mm internal diameter stainless steel tubing. Water for the HPLC mobile phase was deionized using a Milli-Q® water purification system (Millipore, Watford, U.K.).

Apparatus

The SFE system consisted of two Gilson (Middleton, WI) 306 pumps, a PYE Unicam (Cambridge, U.K.) series 104 GC oven with pressure restrictor, and a Spectroflow (Kratos, Manchester, U.K.) 757 UV detector. All components were modified to suit the supercritical extractant. The CO_2 pumphead was refrigerated with a Gilson model SFC3 refrigeration unit to maintain the flow of liquid CO_2. The extraction cell was placed over the loop position of a Rheodyne (Cotati, CA) 7161. The extract was collected by expansion into methanol in a HypoVial at the flow outlet. The extractant was CO_2–ethyl acetate (95:5) at 2 mL/min. The temperature and pressure were 65°C and 3000 psi, respectively. The extraction was monitored at a wavelength of 254 nm.

The HPLC system consisted of a Gilson 305 pump and a Gilson 115 UV detector. The column (25 cm × 4.6 mm) and guard column (2 cm × 4.6 mm) used were prepacked with Hypersil ODS (5 µm) (Capital HPLC Specialists, Bathgate, U.K.). The injector valve was a Rheodyne 7161 with a 20-µL sample loop. The mobile phase used was Na_2HPO_4–methanol (30:70, v/v). The flow rate was 1 mL/min, and the eluent was monitored at 254 nm.

Preparation of standards for comparison

A series of standards ranging from 0 to 12.8 mg/L temazepam with 11.24 mg/L prazepam were prepared in both methanol and blood. Both sets were prepared by adding the appropriate volumes of each drug stock standard (12.8 mg temazepam per liter methanol, 56.2 mg prazepam per liter methanol) to a vial, evaporating at 65°C under N_2, and reconstituting in 5 mL of either methanol or blood.

SPE procedure

The extraction columns were prepared by plugging the end of a 10-mL plastic syringe with glass wool, filling the syringe

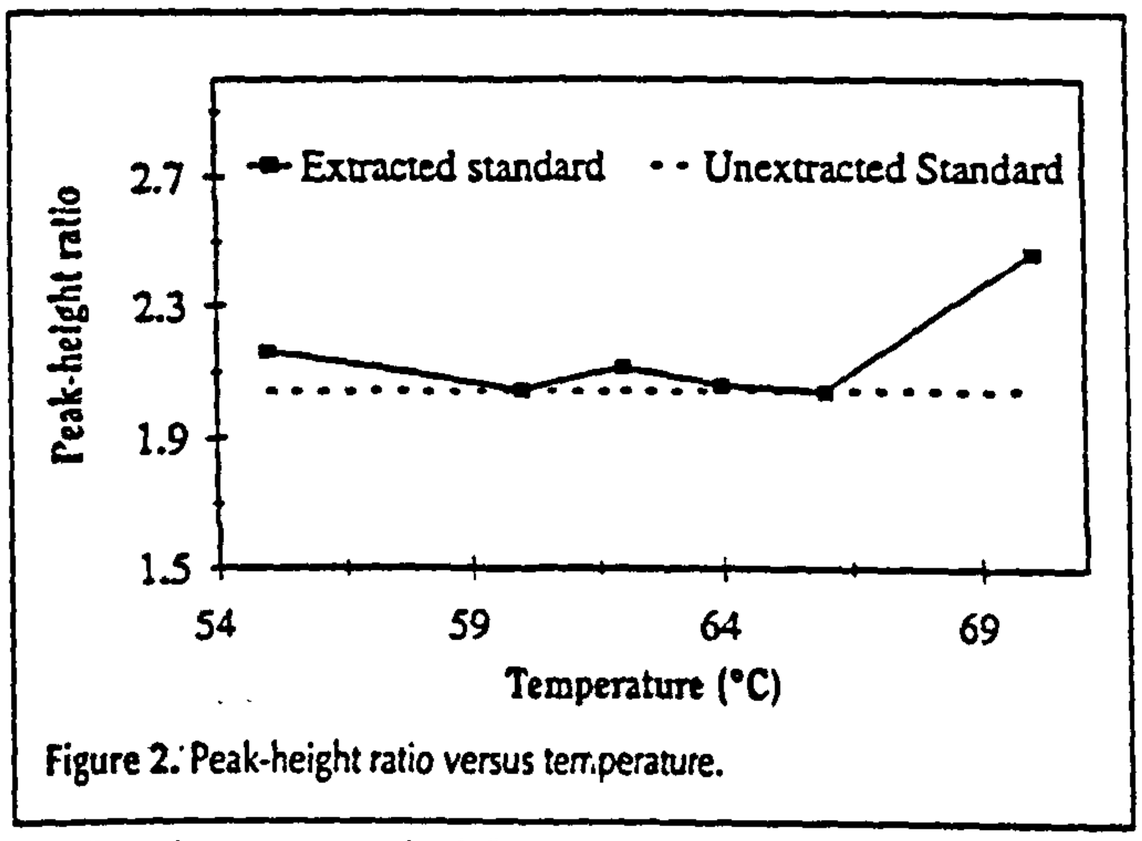

Figure 2. Peak-height ratio versus temperature.

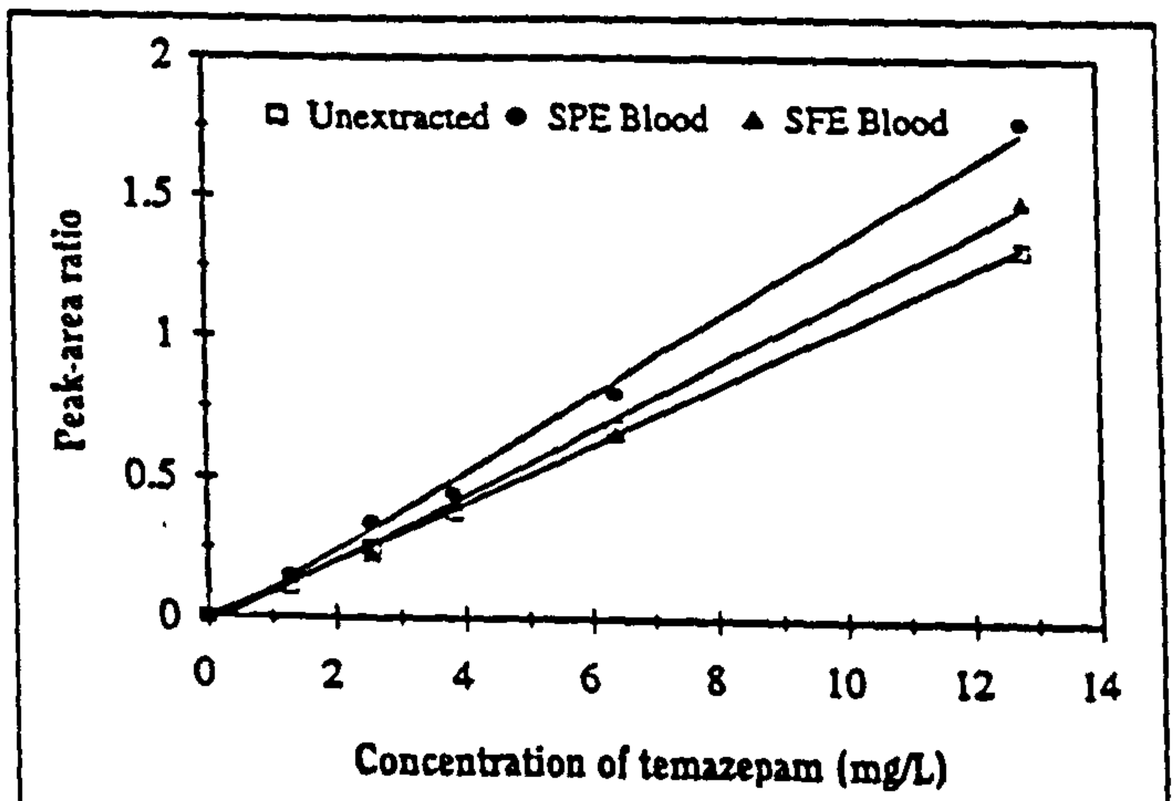

Figure 4. Peak-area ratio versus concentration for blood-extracted standards.

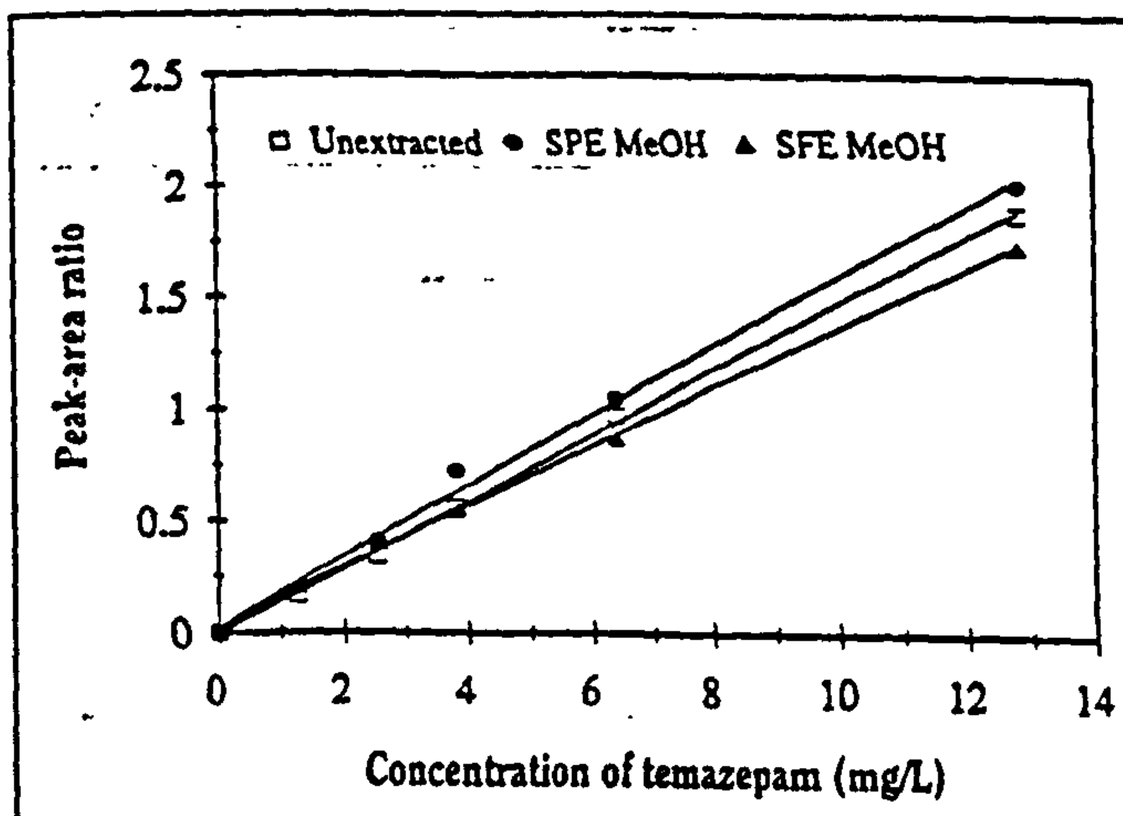

Figure 3. Peak-area ratio versus concentration for methanol-extracted standards.

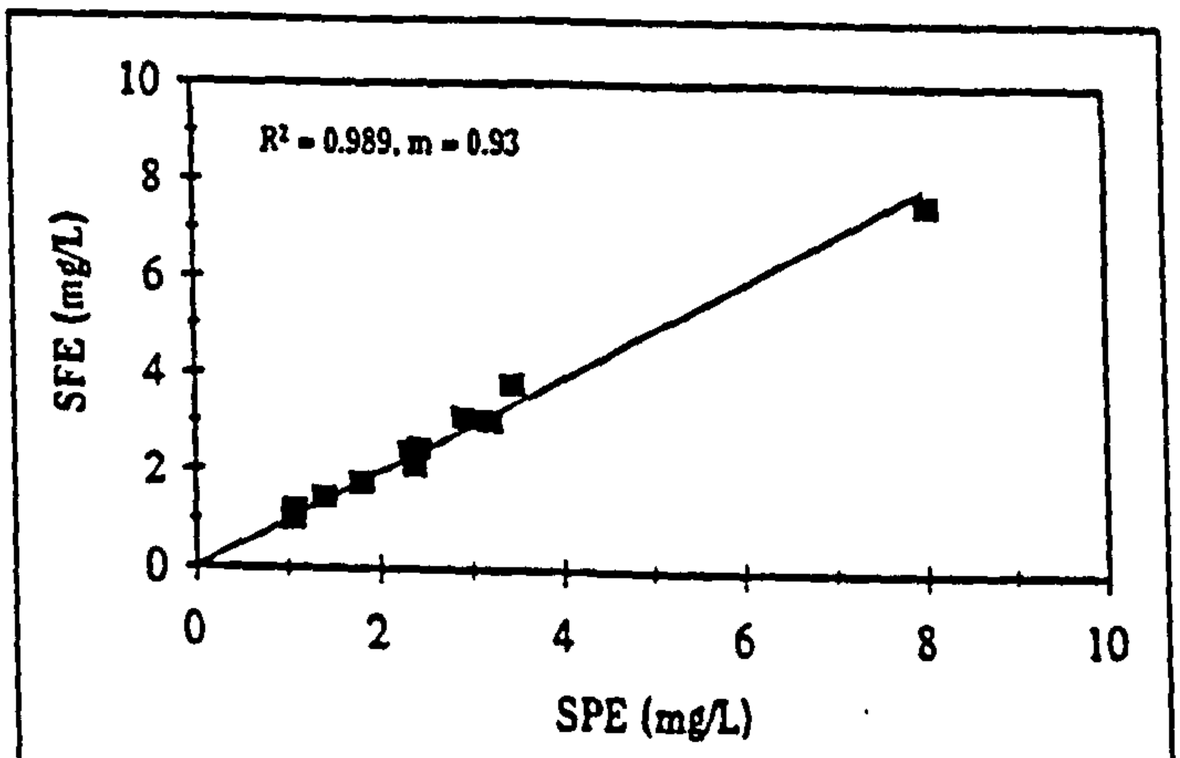

Figure 5. Comparison of SPE and SFE results for the analysis of forensic toxicological case samples.

with approximately 10 g of Extrelut, and pipetting 0.25 mL of a 5% NH_3 solution onto the top. Either 1 mL of standard blood, 0.9 mL of blank blood, and 100 µL prazepam standard (5 mg/100 mL in methanol) or 0.9 mL of sample blood and 100 µL prazepam (5 mg/100 mL) were pipetted into a clean glass vial. To this mixture, 1 mL of phosphate buffer (A, 13.6 g KH_2PO_4 in 500 mL H_2O; B, 4 g NaOH in 500 mL H_2O, 450 mL A and 355 mL B, pH adjusted to 4 using A or B) and 0.25 mL of a 5% NH_3 solution were added. The contents of the vial were mixed thoroughly, then pipetted onto the extraction column. After 5 min, the temazepam and prazepam were eluted under the influence of gravity using diethyl ether. Once 8 mL was collected, the eluent was evaporated to dryness at 65°C under nitrogen and reconstituted in 180 µL of HPLC mobile phase.

SFE procedure

Approximately 0.2 g of Extrelut was placed in a plastic weighing boat. To this solution, 0.5 mL of standard blood–methanol, 0.4 mL blank blood, and 100 µL prazepam (5 mg/100 mL) or 0.4 mL sample blood and 100 µL prazepam (5 mg/100 mL) were added. After mixing, the contents of the boat were allowed to dry at room temperature, until a friable consistency was achieved (approximately 1–2 h), before being transferred to an extraction column. It was not necessary to completely dry the samples but, when possible, the samples were left overnight. The column was sealed and placed over the loop position of the Rheodyne in the equilibrated SFE system. The extraction was started by switching the Rheodyne from load to inject and was carried out for 10 min. The collected extract was dried at 65°C under nitrogen and reconstituted in 50 µL of HPLC mobile phase.

Table I. Comparison of SPE and SFE Results for the Analysis of Forensic Toxicological Case Samples

Case	SPE (mg/L)	SFE (mg/L)	% Difference
RTD94 266	8.04	7.57	5.8
RTD95 032	2.38	2.13	10.5
RTD95 036	2.34	2.43	3.8
RTD95 039	3.20	3.06	4.4
RTD95 057	1.08	1.22	13.0
T94 1297	2.43	2.51	3.3
T94 1310	1.40	1.48	5.7
T94 1329	2.92	3.12	6.8
T95 117	1.81	1.77	2.2
T95 182	3.45	3.83	11.0
T95 241	1.07	1.02	4.7

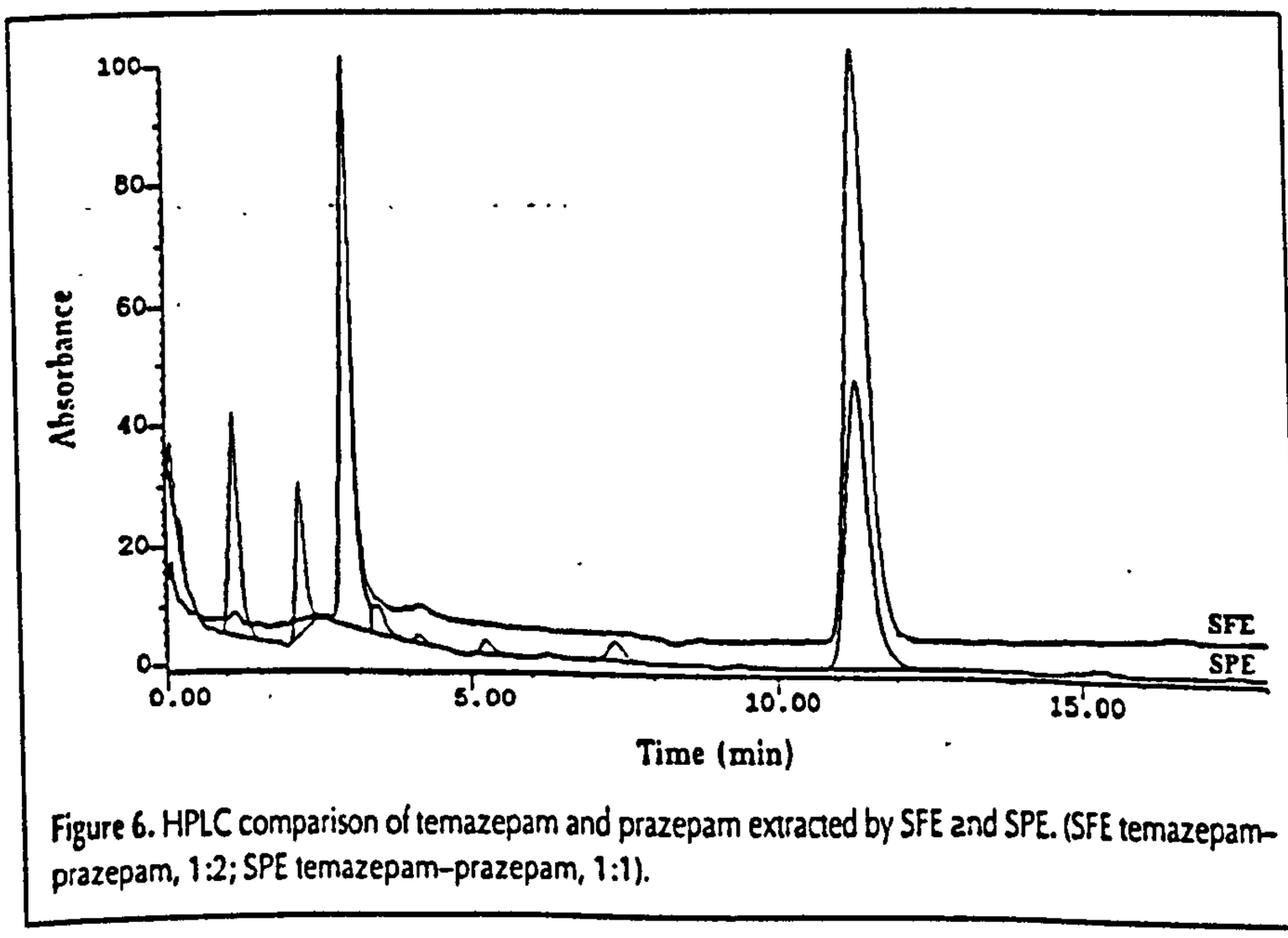

Figure 6. HPLC comparison of temazepam and prazepam extracted by SFE and SPE. (SFE temazepam–prazepam, 1:2; SPE temazepam–prazepam, 1:1).

Results and Discussion

Reproducibility problems were encountered when the extraction conditions were not carefully controlled; in particular, care was required in the control of pressure. Figure 1 shows how small fluctuations in pressure can affect the peak-height ratios. The pressure chosen for the extractions was 3000 psi. The rate of change in peak-height ratio with pressure for the extracted standards compared with unextracted standards was less pronounced.

Extractions were tried at various temperatures between 50 and 100°C. The temperature for the optimum recovery of temazepam was found to be around 60°C. Figure 2 shows the variation in peak-height ratio around this value, and, from this, an extraction temperature of 65°C was chosen.

In the absence of matrix interferences, the peak-area ratios obtained by SFE were found to be linear over the range 0–12.8 mg/L temazepam ($r^2 = 0.999$). Figure 3 shows that the peak-area ratios for both SFE and SPE compare well with those obtained for unextracted standards ($m_{unext} = 0.15$, $m_{SFE} = 0.134$, $m_{SPE} = 0.158$).

As SFE exhibits its best advantages when extracting analytes from solids or semi-solids (6), all blood samples were dried before analysis. This also avoids problems that may occur because of Joule-Thomson cooling as the supercritical fluid expands at the outlet. No matrix interferences from the blood were noted other than a slight decrease in the recovery of both drugs (80–100% compared with 90–100% from methanol). Again, the SFE was found to be linear over the range 0–12.8 mg/L temazepam as shown in Figure 4 ($r^2 = 0.995$). Comparison with unextracted standards and SPE was favorable ($m_{unext} = 0.11$, $m_{SFE} = 0.117$, $m_{SPE} = 0.131$). A comparative chromatogram of SPE and SFE extracted blood standards is given in Figure 5.

Investigation of the SFE and SPE methods on authentic forensic specimens showed that the methods correlate well. Table I and Figure 6 show the results obtained. The SFE method was also found to extract other benzodiazepines and their metabolites of forensic interest, including diazepam, nordiazepam, oxazepam, and chlordiazepoxide.

Conclusion

With careful control of temperature and pressure, both temazepam and prazepam can be extracted to the same extent from blood with recoveries of 80–100%. Reproducible results can also be achieved as long as the pressure fluctuations are kept to a minimum (= 150 psi).

The results obtained show that the SFE method can be used as an alternative procedure for the extraction of temazepam from forensic blood samples, with the potential for use as an extraction procedure for other benzodiazepines of forensic significance. The method offers a rapid, environmentally friendly approach that requires minimal sample handling.

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Supercritical Fluid Extraction as a Sample Preparation Technique for the Determination of Morphine and 6-Monoacetyl-morphine in Post-mortem Blood and Vitreous Humor.

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Abstract

The use of supercritical fluid extraction (SFE) was investigated for the detection of heroin abuse by quantifying levels of morphine and 6-monoacetyl-morphine (6-MAM) in post-mortem blood and vitreous humor samples. The levels achieved in each of the two toxicological specimens were compared on a case to case basis to determine if a correlation exists.

Blood/vitreous humor samples containing deuterated morphine as the internal standard, were loaded on Extrelut®, dried at room temperature and then extracted using supercritical carbon dioxide:methanol:triethylamine (90:8.5:1.5) at 100°C and 3500 psi for 20 minutes. The collected extracts were analysed by GC/MS.

Results show that, in general, the levels of morphine obtained from blood are higher than the corresponding levels in vitreous humor and that correlation between the two sample types is good. Preliminary studies for 6-MAM again show lower levels in vitreous humor than in blood, with the levels being lower than the corresponding morphine levels in both cases. However for 6-MAM, no correlation between blood and vitreous humor was found.

Introduction

Heroin (diamorphine) abuse in the West of Scotland has been on the increase for several years now and has only just started to decrease with the introduction of methadone maintenance programmes(1). At present confirmation of heroin abuse as a contributory factor to death, is achieved by the detection of morphine in the blood or urine and circumstantial evidence obtained from the examination of postmortem reports, police reports and statements from friends and relatives of the deceased. However, in some cases the information gathered may be insufficient to confirm the use of heroin prior to death. Therefore, by detecting the presence of the primary metabolite of diamorphine (6-monoacetylmorphine) in body fluids, namely blood and vitreous humor, the abuse of heroin could be verified.

Over the past few decades, supercritical fluid extraction (SFE) has been widely investigated in many fields of study including forensic toxicology(2,3). Although its use has not escalated in the routine laboratory, it has many advantages which may be exploited by the toxicologist. Due to difficulties in automating instrumentation for SFE, its advantages must be taken on a case to case basis. Extractions can be carried out quickly and efficiently with minimal sample handling. Unlike other types of extraction where selectivity is achieved using separate solvents, the same solvent can be used to selectively extract a wide range of sample types by simply varying the pressure, temperature or percentage of solvent in the supercritical fluid. Commonly used supercritical fluids include carbon dioxide and Xenon thus reducing and in some cases omitting the risks incurred on the environment by conventional liquid solvents.

Experimental

Sample preparation [2]

Approximately 0.2 g of Extrelut® were placed in a plastic weighing boat. To this, 200 µL of sample blood / vitreous humor and 100 µL of D_3-morphine were added. After mixing, the contents of the boat were allowed to dry at room temperature, until a

friable consistency was achieved (~1-2 hours), before being transferred to an extraction column. It is not necessary to completely dry the samples, but where possible, the samples were left overnight. The column was sealed and placed over the sample loop position of the Rheodyne in the equilibrated SFE system. The extraction was started by switching the Rheodyne from load to inject. The collected extract was dried at 65 °C under nitrogen then derivatised with 50 µL of BSTFA (1%TMCS) at 90 °C for 10 minutes before injecting onto the GC-MS.

SFE

The SFE system consisted of two Gilson 306 pumps and a PYE series 104 GC oven with pressure restrictor. All components were modified to suit the supercritical extractant. The CO_2 pumphead was refrigerated to maintain the flow of liquid CO_2 using a Gilson model SFC3 refrigeration unit. The extraction cell was placed over the loop position of a Rheodyne 7161. The extract was collected for 20 minutes by expansion into methanol in a HypoVial™ at the flow outlet. The extractant was CO_2 : methanol : triethylamine (90 : 8.5 : 1.5) at 2 mL/min. The temperature and pressure were 100 °C and 3500 psi respectively.

GC-MS conditions

Model:	Fisons GC8000 series with MD800 EI mode (70eV)
Column:	HP-1 fused silica widebore capillary (30 m, 0.53 mm i.d., 0.88 µm film thickness)
Temperature programme:	150°C-300°C @ 10°C/min, hold for 5 minutes
Injector temperature:	225°C
Interface temperature:	250°C
SIM:	Morphine m/z: 414, 429
	6-MAM m/z: 340, 399
	D_3-morphine m/z: 432

Cases

The information available on the 20 cases studied is summarised below:

- 13 males: Age 20-39 (mean 30); 7 females: Age 27-40 (mean 31)
- Cause of death: 11 respiratory failure, 4 aspiration of gastric contents. All drug related
- 12 known intravenous drug abusers
- 17 sudden deaths
- 17 with fresh needle puncture marks (not due to hospital treatment)
- 15 with drug paraphernalia at the place of death (e.g. syringe in close proximity/still injected, powders/tablets in the vacinity of the body, ligature still tied round arm)
- 18 poly drug deaths (8x2 drugs, 5x3 drugs, 3x4 drugs, 1x5 drugs, 1x9 drugs). Drugs found: benzodiazepines (n=15), methadone (n=4), paracetamol (n=2), norpropoxyphene (n=1), amphetamines (n=1), cocaine (n=1)
- 9 cases positive for alcohol

The morphine and 6-monoacetylmorphine levels found in each of the 20 cases are listed in Table 1. A plot of the concentration found in blood versus the concentration found in vitreous humor is shown in Figure 1. It can be seen that a correlation exists between the matrices for morphine (r^2 = 0.697). In addition the levels found in vitreous humor are in general less than the corresponding blood levels (gradient = 0.701). In contrast from Figure 2 it is apparent that no correlation exists between blood and vitreous humor for 6-monoacetylmorphine (r^2 = 0.006).

Case	Blood		Vitreous Humor	
	Morphine (mg/L)	6-MAM (mg/L)	Morphine (mg/L)	6-MAM (mg/L)
1	1.18	0.51	1.22	0.42
2	1.30	0.14	0.13	0.51
3	0.05	0.10	0.09	1.72
4	0.73	0.14	0.46	0.03
5	0.81	-ve	0.42	0.73
6	0.81	-ve	0.67	0.06
7	1.68	0.07	1.48	0.96
8	2.14	0.04	1.69	0.01
9	0.48	-ve	0.07	-ve
10	0.24	-ve	0.18	0.05
11	0.34	-ve	0.44	-ve
12	0.79	-ve	0.62	-ve
13	0.45	0.08	0.16	0.02
14	0.25	-ve	0.31	0.09
15	0.16	0.38	0.18	-ve
16	0.74	-ve	0.71	0.02
17	0.25	0.23	0.23	-ve
18	0.28	0.05	0.23	0.006
19	0.28	0.01	0.27	0.08
20	0.29	0.16	0.55	0.11

Table 1 Case Results

All 20 of the cases studied were positive for morphine, however from Table 1 it can be seen that three of the cases were not positive for 6-MAM in either blood or vitreous humor. In these instances, if corroborative information were not available, it would not be possible to state that the death was heroin related.

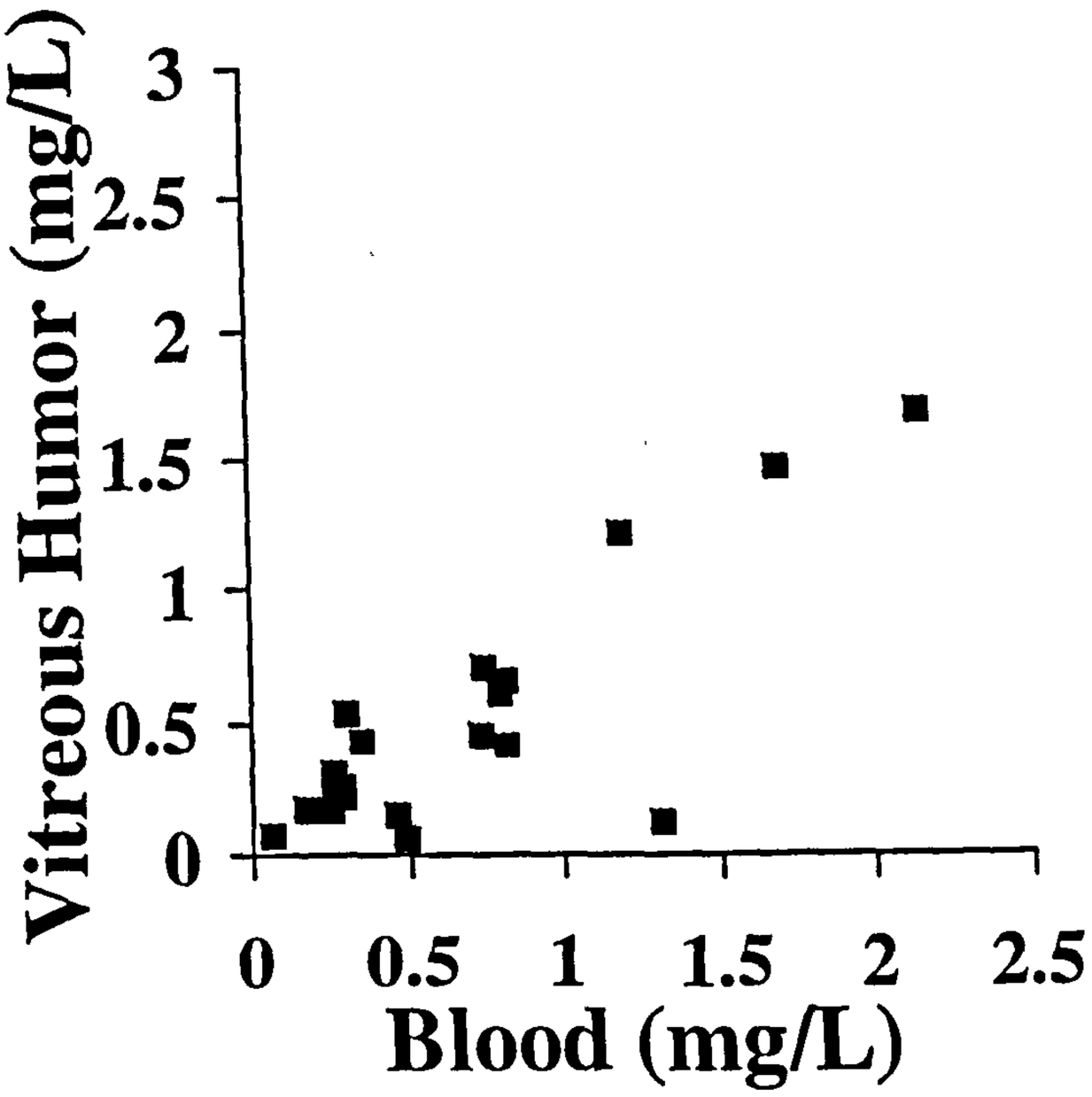

Figure 1: Morphine Blood v Vitreous Humor

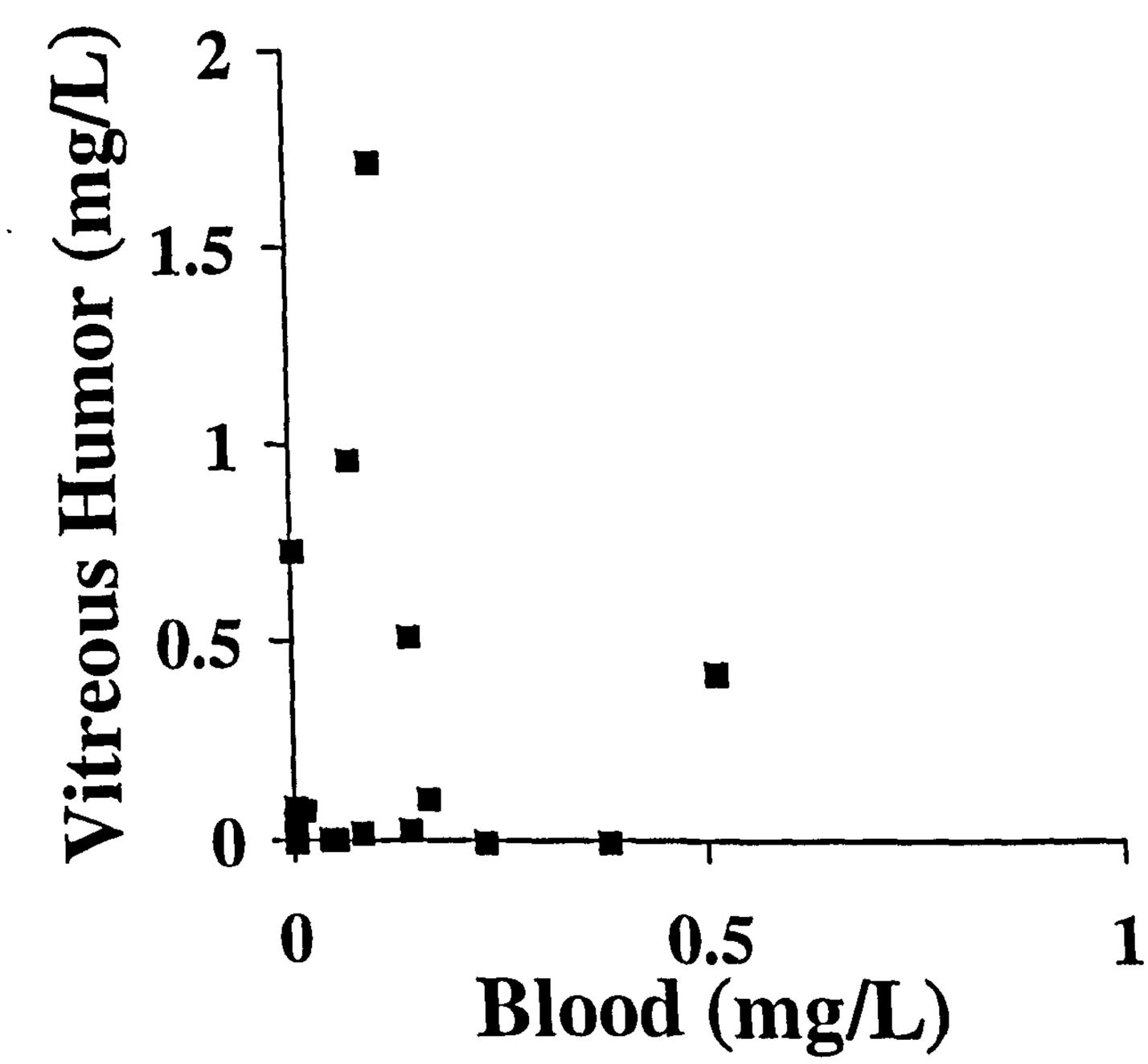

Figure 2: 6-MAM Blood v Vitreous Humor

Conclusions

In conclusion we have found SFE to be a useful tool in the analysis of morphine and 6-monoacetylmorphine from both blood and vitreous humor samples. This in addition to the information available on the deceased can be used to prove that death was contributed to by the use of heroin. The method has been found to compare well to conventional solid-phase extraction methods for morphine (r^2=0.99)[3] and therefore can be used in the toxicology laboratory. In addition we have shown that it is possible to use vitreous humor as a toxicological sample. This may be of use in cases where blood is not avalable for analysis.

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Vitreous Humor as an Alternative Sample to Blood for the Supercritical Fluid Extraction of Morphine and 6-Monoacetylmorphine

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Abstract

The use of vitreous humor as an alternative sample to blood was investigated for the detection of heroin abuse by quantifying levels of morphine and 6-monoacetyl-morphine (6-MAM) in post-mortem samples. The levels achieved in each of the two toxicological specimens were compared on a case to case basis to determine if a correlation existed. A total of 20 positive morphine cases were examined. In general the levels of morphine in blood were higher than in the corresponding vitreous humor samples, with some correlation existing. 6-MAM was found in 15 blood samples and 17 vitreous humor samples. Although no correlation was found between the levels of 6-MAM in blood and vitreous humor, the latter may still be used for verification of heroin abuse.

Introduction

The use of vitreous humor as an alternative sample matrix for the determination of drugs has been recently reviewed by several workers.(Bermejo *et al*, 1992, McKinney *et al*, 1994, Samuel *et al*, 1994) However, without further understanding of the relationship between the levels obtained in conventional sample types (i.e. blood, urine) the interpretation of analytical results for vitreous humor is difficult.

For several years deaths due to the abuse of heroin (diamorphine) in the West of Scotland have been on the increase. However, at present the confirmation of heroin

abuse as a contributory factor to death, depends heavily on the circumstantial evidence obtained from the examination of postmortem reports, police reports and witness statements. This information is verified by quantitation of morphine in the case blood or urine. In order to provide a better toxicological support for the circumstantial evidence it is necessary to look for a more specific diamorphine marker. The metabolic pathway of diamorphine is shown in Figure 1. From the half lives it is apparent that the routine detection of diamorphine is not practical. However, due to its longer half life, 6-MAM should provide a more practical diamorphine marker and thus it was chosen for this study.

A recent study on the comparison of supercritical fluid extraction and solid-phase extraction for the detection of morphine in postmortem blood samples showed excellent correlation between the two methods.(Allen e*t al*, 1997) Although the use of SFE in forensic toxicology is not widespread, it has many advantages which may be exploited by the toxicologist. In addition to speed and efficiency of extraction one of the main advantages of SFE is the potential for using a single solvent to selectively extract a wide range of sample types simply by varying the pressure or temperature of the supercritical fluid. Due to its practical critical temperature and pressure and low cost, carbon dioxide is the most commonly used supercritical fluid, thus the need for organic solvents is reduced.

Experimental

Materials

Morphine, 6-monoacetlymorphine and D_3-morphine were supplied by Sigma (Poole, U.K.). Methanol, dichloromethane were high performance liquid chromatographic (HPLC) grade, (Lab Scan Analytical Sciences, Dublin, Ireland). BSTFA (1% TMCS) used as the GC-MS derivatising agent was supplied by Pierce (Rockford, IL, USA). Triethylamine general purpose reagent (GPR) grade was supplied by Merck (Poole, U.K.). Extrelut® (Merck) was prewashed using dichloromethane. The collection

vials for SFE, were 6mL HypoVial™ (Pierce, Oud-Biejerland, The Netherlands) with butyl rubber septa (Pierce and Warriner, Chester, U.K.)

Apparatus

The SFE system is described elsewhere (Scott *et al*, 1997). The extraction was carried out for 20-30 minutes using CO_2-methanol-triethylamine (90:8.5:1.5) at 2.0 mL/min with a temperature of 100 °C and a pressure of 3500 psi. The analytes were collected by expansion into methanol at the flow outlet.

The GC/MS system consisted of a Fisons GC8000 series with MD800 EI mode (70 eV) fitted with a HP-1 fused silica widebore capillary column (30 m, 0.53 mm i.d., 0.88 µm film thickness). The temperature was programmed from 150 °C to 300 °C at 10 °C/minute and held for 5 minutes. The injector temperature was 225 °C and the interface temperature 250 °C. Selected ion monitoring was used with the following ions: morphine m/z 414 and 429, 6-MAM m/z 340 and 399, D_3-morphine m/z 432.

Sample preparation

Approximately 0.2 g of Extrelut® was placed in a plastic weighing boat. To this, 200 µL of sample blood / vitreous humor and 100 µL of D_3-morphine were added. After mixing, the contents of the boat were allowed to dry at room temperature, until a friable consistency was achieved (~1-2 hours), before being transferred to an extraction column. The column was sealed and placed over the loop position of the Rheodyne in the equilibrated SFE system. The extraction was started by switching the Rheodyne from load to inject. The collected extract was dried at 65 °C under nitrogen then derivatised with 50 µL of BSTFA (1%TMCS) at 90 °C for 10 minutes before injecting onto the GC-MS.

Cases

The information available on the 20 cases studied is summarised in Table I. Using only this information it should be possible to predict whether 6-MAM will be present in the blood and vitreous humor. For example in case 1, the deceased was a known intravenous heroin abuser who died suddenly. Fresh needle puncture marks were found on the body and a syringe was found in close proximity to the body. Therefore, in this case, we would expect to find traces of 6-MAM. In contrast with case 15, there was no evidence of heroin abuse and thus we would not expect to find morphine or 6-MAM.

Results and discussion

The levels of morphine and 6-monoacetylmorphine found in each of the 20 cases are listed in Table II. A plot of the morphine concentration found in blood versus the concentration found in vitreous humor is shown in Figure 2. It can be seen that a correlation exists between the matrices for morphine ($r^2 = 0.697$). For the 17 sudden death cases only, the correlation coefficient is 0.885, indicating a dependance on the rate of death. The levels found in vitreous humor are in general less than the corresponding blood levels (gradient = 0.701 (0.811 for sudden deaths)). In contrast from Figure 3 it is apparent that no correlation exists between the levels found in blood and vitreous humor for 6-monoacetylmorphine ($r^2 = 0.006$) even when the sudden deaths are taken separately ($r^2 = 0.03$).

All 20 of the cases studied were positive for morphine, however from Table I it can be seen that three of the cases were not positive for 6-MAM in either blood or vitreous humor (9, 11, 12) Therefore, in the absence of corroborative information, it would not be possible to state that the death was heroin related in these cases. On the other hand, the circumstantial evidence does not imply death related to heroin overdose in cases 2, 4, 15 and 19, yet the results show that these cases were in fact heroin related due to the presence of 6-MAM in the blood / vitreous humor.

Conclusions

SFE can be successfully used for the quantitation of 6-MAM in both blood and vitreous humor samples. The detection of this compound is useful in the confirmation of death due to heroin overdose. However, due to the short half life of 6-MAM it is necessary to review the case notes relating to a particular toxicology file before making a judgement as to the contribution of drugs to the death. The good correlation between blood and vitreous humor levels for morphine, indicates that this is an ideal matrix for use when blood is not available or is badly denatured. For the determination of 6-MAM, it has been shown that in general higher levels are measured in this matrix, however no correlation between the two matrices exists and thus further work will need to be carried out in order to determine the value of vitreous humor as an alternative for the quantitation of 6-MAM.

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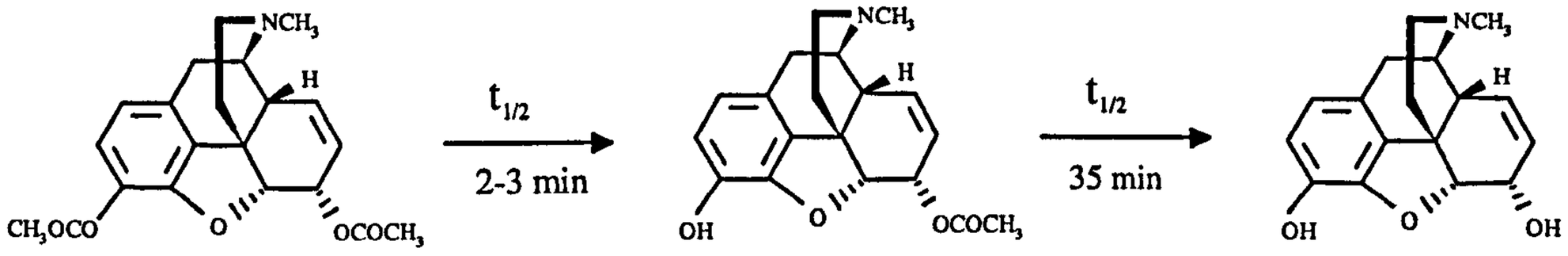
Table I. Summary of case information

Case	Sex/Age	Cause of Death	IVDA	Sudden death	Fresh NPM	DP at SOD	Other drugs	Alcohol
1	F/34	RF	✓	✓	✓	✓	1	x
2	M/39	DRD	✓	x	N/A	N/A	3	x
3	M/25	RF	x	✓	✓	✓	2	✓
4	F/30	RF	✓	x	x	x	1	x
5	M/32	AGC	✓	✓	✓	✓	x	x
6	M/39	RF	S	✓	✓	✓	2	✓
7	F/29	RF	✓	✓	✓	✓	4	x
8	F/27	RF	✓	✓	✓	✓	3	x
9	M/25	AGC	✓	✓	✓	✓	1	x
10	M/26	DRD	✓	✓	✓	✓	3	✓
11	M/21	RF	x	✓	✓	✓	2	✓
12	M/35	AGC	✓	✓	✓	x	2	✓
13	F/40	RF	S	✓	✓	✓	x	✓
14	F/32	RF	✓	✓	✓	✓	1	✓
15	M/30	DRD	x	x	x	x	8	x
16	F/27	AGC	✓	✓	✓	✓	1	x
17	M/29	RF	?	✓	✓	✓	1	✓
18	M/20	RF	x	✓	✓	✓	2	x
19	M/33	DRD	✓	✓	x	x	1	✓
20	M/36	DRD	✓	✓	✓	✓	1	x
	13M (20-29, 30) 7F (27-40, 31)	10RF, 4AGC, All DRD	13 IVDA	17/20 sudden deaths	17/20 with fresh NPM	15/20 with DP at SOC	18/20 +ve for other drugs	9/20 +ve for alcohol

Cause of death as on PM report: RF-respiratory failure due to pulmonary oedema and congestion; DRD-drug related death; AGC-aspiration of gastric contents.
IVDA-Person known to be an intravenous drug abuser immediately prior to death; S-known to smoke heroin
NPM-needle puncture mark
DP at SOD-drug paraphenalia at scene of death

Table II. Morphine and 6-MAM case results

Case	Blood		Vitreous Humor	
	Morphine (mg/L)	6-MAM (mg/L)	Morphine (mg/L)	6-MAM (mg/L)
1	1.18	0.51	1.22	0.42
2	1.30	0.14	0.13	0.51
3	0.05	0.10	0.09	1.72
4	0.73	0.14	0.46	0.03
5	0.81	-ve	0.42	0.73
6	0.81	-ve	0.67	0.06
7	1.68	0.07	1.48	0.96
8	2.14	0.04	1.69	0.01
9	0.48	-ve	0.07	-ve
10	0.24	-ve	0.18	0.05
11	0.34	-ve	0.44	-ve
12	0.79	-ve	0.62	-ve
13	0.45	0.08	0.16	0.02
14	0.25	-ve	0.31	0.09
15	0.16	0.38	0.18	-ve
16	0.74	-ve	0.71	0.02
17	0.25	0.23	0.23	-ve
18	0.28	0.05	0.23	0.006
19	0.28	0.01	0.27	0.08
20	0.29	0.16	0.55	0.11



Diamorphine 6-Monoacetylmorphine Morphine

Figure1

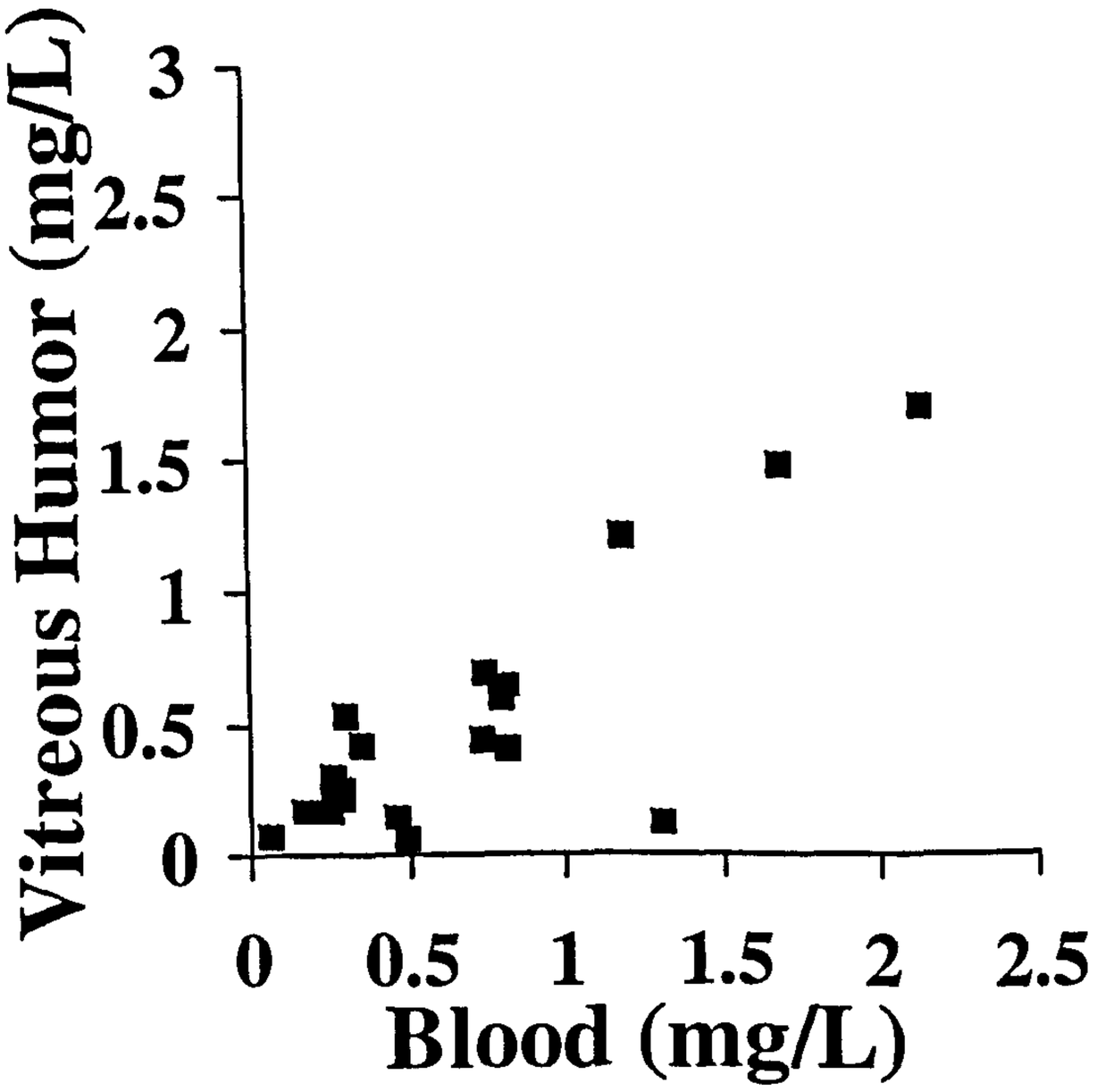


Figure2

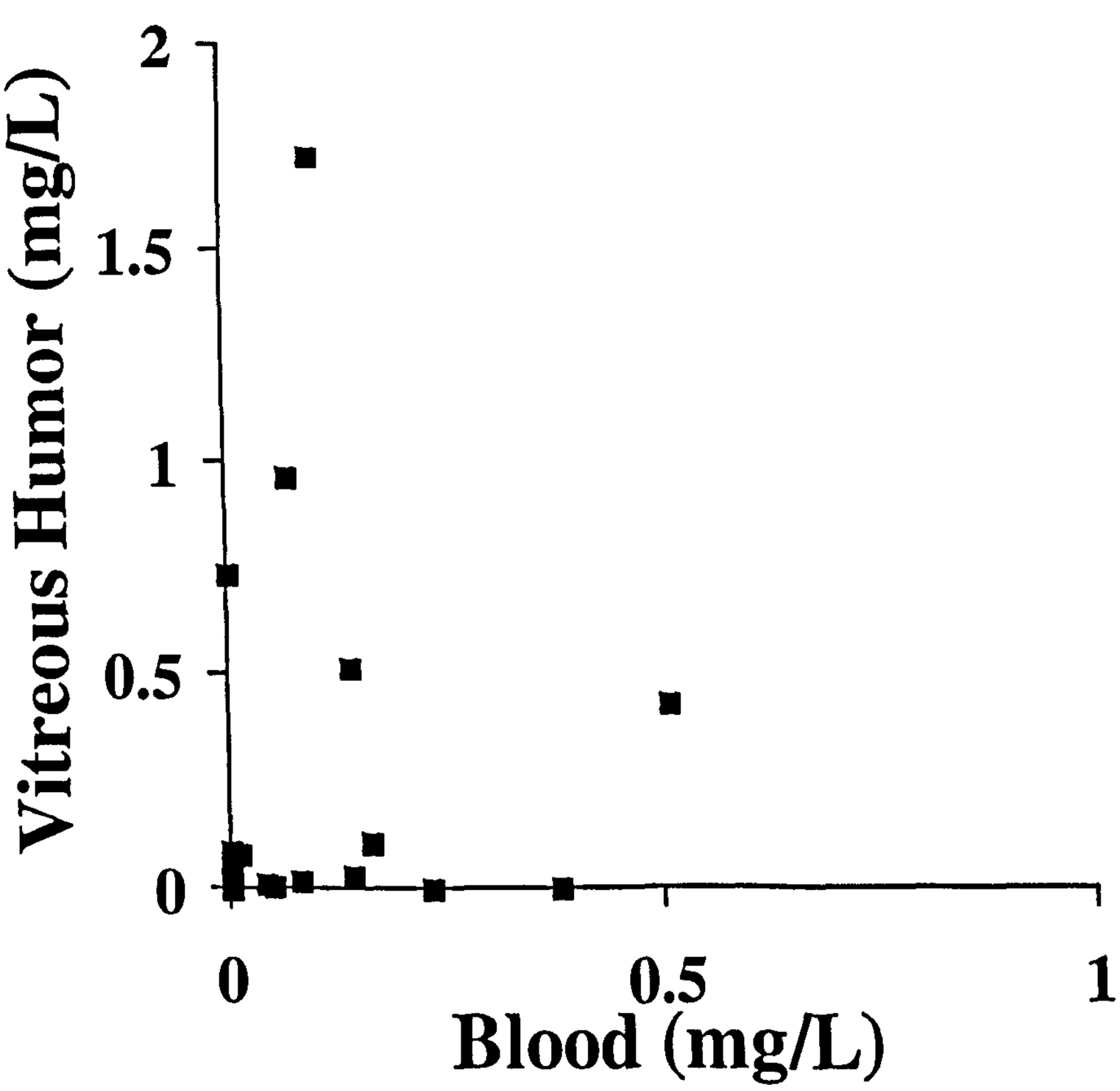


Figure3