THE ANALYSIS OF DRUGS

IN BIOLOGICAL FLUIDS

BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

Thesis submitted in accordance with the

requirements of the University of Glasgow

for the degree of Doctor of Philosophy

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TO LINDA

"In the welter of conflicting fanaticisms one of the few unifying forces is scientific truthfulness, by which I mean the habit of basing our beliefs upon observations and inferences as impersonal, and as divested of local and temperamental bias as is possible for human beings."

Bertrand Russell

History of Western Philosophy

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SUMMARY

A brief introduction to the practice of modern liquid chromatography is given, in particular to its use in the forensic sciences and toxicology.

The first compound examined was morphine. Its suitability for analysis by HPLC was reviewed and discussed. Its electrochemical and UV absorbance properties were then examined in detail in order to assist with the choice of a detection system. The electrochemical properties of several other narcotic analgesics were examined for comparison, and some assessment of the structure-electrochemical activity relationship was possible.

The selection of a suitable chromatographic system progressed through the examination of binary solvents and varying conditions of pH and ionic strength. Results from these studies suggested the use of ion pairing techniques. A detailed study of the effect of ion pairing agents on the retention of morphine resulted in the choice of one particular system, which was then characterised in terms of interference from other drugs, sensitivity and linearity of response. This was done for both UV and electrochemical detectors and electrochemical detection was selected as the technique of choice for further work. Nalorphine was selected as the internal standard.

A technique described in the literature for the extraction of morphine from biological fluids was examined. Several modifications were made to the technique to improve extraction efficiency. This modified procedure was then applied to the analysis of morphine in blood samples from clinical studies and

also post mortem sample in cases where opiate abuse was suspected. The clinical study monitored blood morphine levels in patients under intensive care who were receiving morphine for chronic pain. The effect of imparied renal function on morphine metabolism was considered, but no conclusive results could be drawn due to the concurrent use of haemofiltration in these patients.

The second group of compounds considered were all local anaesthetics. In particular bupivacaine, procaine and cocaine. These were of interest from the point of view of clinical studies, doping in the racing greyhound, and social drug abuse respectively. The electrochemical and UV absorbing properties of these compounds were examined, however the potentials required for oxidation of the local anaesthetics was however too high to permit electrochemical detection as a practical detection technique, and UV detection was used for further work. The selection of a suitable chromatographic system progressed through the examination of binary solvents and varying conditions of pH and ionic strength. Ion paired chromatography was examined as was the use of amine modifiers. In order to produce acceptable chromatography for a range of local anaesthetics however, it proved neccessary to use a reversed phase column of low lipophilicity. Using this column with a series of binary solvents it proved possible to separate a wide range of basic compounds, including several local anaesthetics, with high efficiency.

An extraction procedure was developed, based on liquid/solid extraction from an absorbent support (diatomaceous earth). This procedure was applied to blood, plasma and urine samples and found to provide extracts suitable for HPLC on the systems developed above. Plasma samples were received from a clinical study where patients were receiving bupivacaine abdominally (post-surgery). These were analysed by the above techniques and it was shown that the plasma levels obtained were high, in some cases approaching the toxic range.

Two screening procedures recommended for the isolation of drugs from greyhound urine were examined for their ability to detect bupivacaine, procaine and cocaine in spiked urine samples. Both proved suitable and it was noted that the presence of local anaesthetics in a urine sample could be detected by pre-race testing, and that the identity of the drugs could be confirmed by the HPLC technique developed earlier.

Greyhounds were then administered with the three compounds of interest, and the urine samples collected were tested for the presence of drugs. Bupivacaine was detected up to twelve hours post administration, procaine up to 48 hours post administration, and cocaine up to twenty four hours post administation.

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CHAPTER ONE

INTRODUCTION

1.1. HIGH PRESSURE LIQUID CHROMATOGRAPHY

1.1.1. Introduction

High Pressure Liquid Chromatography (HPLC) (also called High Performance Liquid Chromatography) has been in routine use as an analytical and preparative technique for about fifteen years, and its principles and practice have been well documented and reviewed (Snyder and Kirkland, 1978; Knox, 1982; Pryde and Gilbert, 1979; Krstulovic and Brown, 1982; Lawrence, 1981, Simpson, 1982; Majors et al., 1983).

Many applications have also been described in the literature including the analysis of lipids, steroids, carbohydrates, proteins, vitamins, pesticides, environmental contaminants, pharmaceuticals and the analysis of drugs and metabolites in biological fluids. Examples of all these applications are included in the above references.

Many of these areas have applications in the forensic sciences. Analysis and characterisation of trace evidence eg inks (Colwell and Karger, 1977), dyestuffs (Passarelli and Jacobs, 1975) and cosmetics (Reuland and Trinler, 1980).

There are also applications in the analysis of explosives (Dalton et al., 1975), and analysis of hydrocarbons (Doran and McTaggart, 1974), and plastics and polymers (Wheals , 1979).

The major forensic application of HPLC is however in drug analysis (Wheals, 1976; Saferstein, 1982).

This falls into two categories, the identification of drugs in questioned material, and the detection and identification of drugs in biological material.

The former includes the identification of the constituents of cannabis resin (Baker et al., 1980; Baker et al., 1983; Turner and Mahlberg, 1982), the identification of drugs, and by-products and adulterants in street drugs notably cocaine (Gill et al., 1984) and heroin (Trinler and Reuland, 1975). HPLC has also been used for the identification of components of psilocybe mushrooms (Vanhaelen-Fastre and Vanhaelen, 1984; Kysilka et al., 1985),

The identification of drugs in biological fluids is a larger field, as potentially any prescription drug and many more compounds besides might be present in a sample submitted for analysis.

HPLC has been shown in the literature to play an important part in this field. Many methods have been reported for the identification of compounds of forensic interest in biological fluids. Some of these are included in table 1.1..

HPLC may not be the most suitable technique for the analysis of all compounds. Solvents for example are more suited to analysis by GC (Ramsey and Flanagan, 1982; Oliver, 1982).

HPLC is of special interest for those compounds which are not suitable for analysis at the temperatures required for GC. As well as proteins, carbohydrates and high molecular weight compounds, this includes quaternary ammonium compounds, and some esters, amides and anhydrides which are temperature labile.

The option of using stationary and mobile phases of different

TABLE 1.1.

HPLC METHODS FOR THE ANALYSIS OF DRUGS OF FORENSIC INTEREST

Author	Date	Compounds	Class	Comments
Ray et al.	1985	propanolol	B blocker	RP-HPLC UV
Levine and Coplan	1985	fenprofen ibuprofen indomethacin naproxen and others	non-steroidal anti-inflammatories	RP-HPLC UV
Uges and Bloemhof	1984	pancuronium alcuronium pyridostigmine vercuronium tubocurarine	quaternary neuromuscular blocking agents	RP-HPLC UV
Gill et al.	1981	30 barbiturates	sedatives	RP-HPLC UV
Brodie et al.	1978	benzodiazepines	sedatives	RP-HPLC UV
Wurst et al.	1978	ergot alkaloids	hallucinogens	RP-HPLC UV
Law et al.	1984	Cannabinoids	hallucinogens	RP-HPLC Off-line radioimmunoassay detection

polarity also allows a range of very polar compounds (such as phenols, thiols, sugar conjugates, and free acids) to be analysed directly without the need for chemical alteration or derivatisation.

1.2. METHOD DEVELOPMENT

The selection of the appropriate technique has to be carefully considered for the analyte of interest.

Having decided on HPLC as the method of choice, the process of selecting suitable conditions for the assay is based on a consideration of the physical and chemical properties of the analyte.

1.2.1. Detection systems

The detection system must be suitable for measuring small quantities of the analyte. It should be as selective as possible, and compatible with the solvent system used.

The detection systems available to this study were ultra violet/visible (UV) absorbance detection, and electrochemical detection (ED).

UV detection was one of the first methods of on-line detection to be applied to liquid chromatography (Scott, 1977) and its principles and application have been well documented elsewhere (Skoog and West, 1980). It is a quantitative method by virtue of the relationship between concentration and absorption described by Beer and Lambert.

Electrochemical detection is a more recent innovation.

1.2.2. Electrochemical detection - principles

This method of detection is particularly suited to reversed phase HPLC as similar conditions are required for both, namely the use of an aqueous electrolyte-containing solvent.

The principle on which the electrochemical detector operates is to apply a potential difference across two electrodes lying in the stream of the eluent from the chromatographic column. If the potential is sufficient to cause an electrochemical reaction of any eluting compounds, the flow of electrons resulting from that reaction can be measured as a current, proportional to the amount of material passing through the flowcell.

The basis of the electronics is shown in figure 1.1.. One operational amplifier (OA1) connected through the reference electrode in a voltage follower circuit, supplies a constant potential relative to the reference electrode between the working electrode and the solution. A second operational amplifier (OA2), in a current-voltage converter configuration, amplifies any current resulting from a reaction at the working electrode (w), and converts this to a voltage proportional to that current which can be displayed digitally or connected to a chart recorder.

1.2.3. Organic electrochemistry

A wide range of compounds and functional groups can react electrochemically, both oxidatively and reductively. The easiest reactions to perform experimentally are oxidations, since such strict degassing and experimental procedures as those for reduction reactions are not required (Kissinger, 1982; Ryan, 1984).

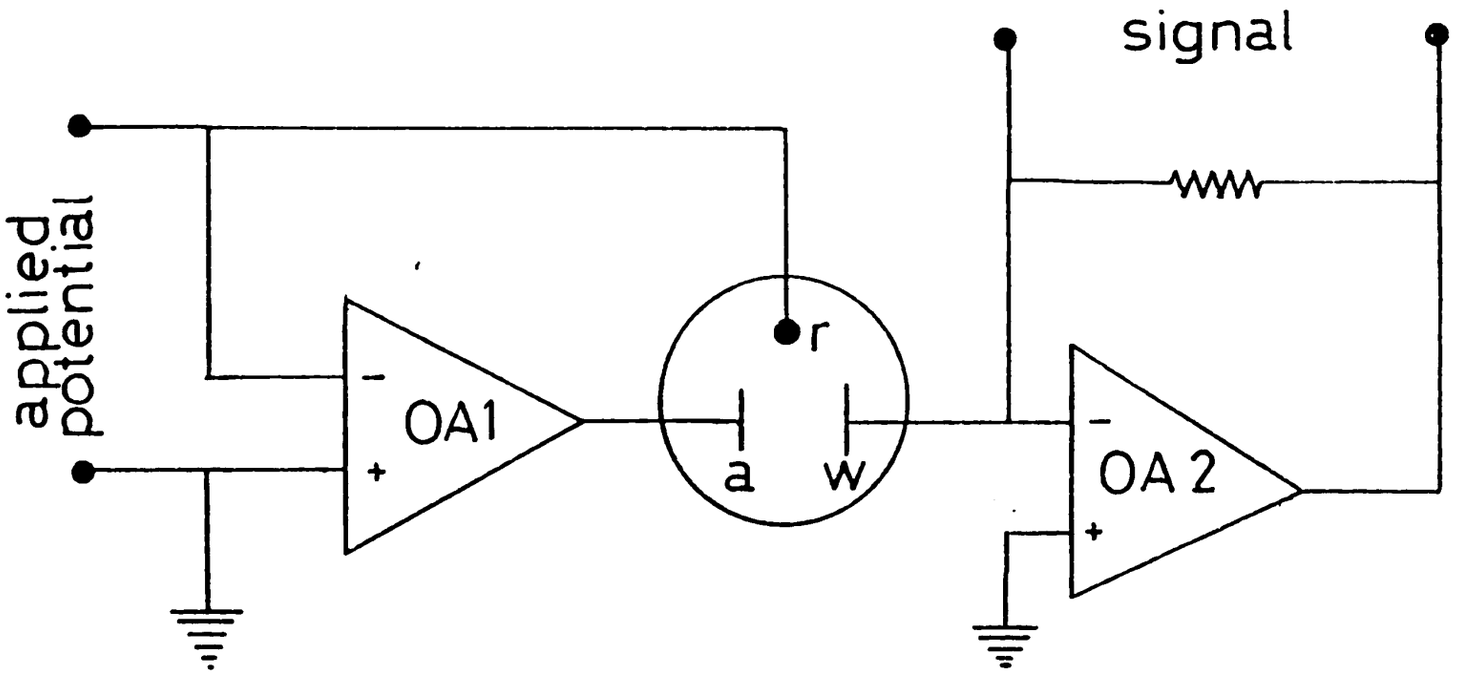


FIGURE 1.1.

BASIC CIRCUIT DIAGRAM FOR ELECTROCHEMICAL DETECTOR SHOWING VOLTAGE CONTROL CIRCUIT (OA1), SIGNAL PROCESSING (OA2), AND CELL WITH REFERENCE (r), AUXILIARY (a) AND WORKING (w) ELECTRODES.

Organic oxidations can be performed on most electron-rich compounds. Common reactions include phenol oxidative couplings, oxidation of primary and secondary alcohols to aldehydes and ketones respectively, Kolbe de-carboxylation of carboxylic acids oxidation of primary and secondary thiols, hydroquinones, indoles, and xanthines (Volke, 1983; Tomlinov, 1978). These two references list many more specialised and less common reactions known to occur.

Electrochemical detection is now being widely applied to a variety of compounds including drugs, environmental contaminants and biochemicals. Recent examples include the analysis of platinum complexes used in chemotherapy (Elfrink et al., 1985), the measurement of hallucinogenic indoleamines (Kysilka et al., 1985), Catecholamines, derivatives and metabolites (De Jong et al., 1985), and the detection of a wide range of compounds including phenothiazines (Kauffmann et al, 1985), analgesics, local anaesthetics, and anti-convulsant drugs, (Musch et al., 1985). Oxidative electrochemical detection has also been reported for benzodiazepines (Smyth et al., 1982).

Recent developments in the field of electrochemical detection include the use of electrochemically induced fluorescence in aromatic compounds to measure these compounds as they elute (Blatchford and Malcolme-Lawes, 1985), and the use of multifuctional detectors, incorporating UV, fluorescence and amperometric detection in a single flow cell (Schmidt and Scott, 1985)

1.2.4. Examination of electrochemical properties

By applying a sufficient potential to a suitable analyte in an electrolyte solution (to minimise mobility of the electrochemically generated intermediates) an unstable intermediate, usually a radical, can be produced. This may react with other components of the solution giving an hetrogeneous product, rearrange itself to give some new homogeneous product or decay back to give the starting material. The actual course of the reaction will depend on the nature of the compound and on other components in solution. There is no firm agreement as to whether the reaction need go to completion for current to flow or whether the formation of a radical itself is sufficient (Anderson et al., 1981)

If the potential applied to an electrolyte solution is gradually increased a point will be reached where the potential is sufficient to cause the electrochemical reaction to occur. Further increasing the potential will give a gradual increase in the current up to a point where the reaction will equilibrate and the current stabilises at a value dependent on the nature of the electrode surface, the temperature of the solution and upon whether the solution is stirred (Evans et al., 1983).

The most useful piece of information which can be obtained from this is the voltage at which maximum current is achieved, the E_p value (peak potential or full wave potential). The E_p value is usually selected as the working potential for electrochemical detection, as it represents the point at which the current/potential ratio is maximised. Increasing the potential beyond E_p does not give rise to any further increase in current.

Another parameter often quoted is $E_{p/2}$, which has no thermodynamic significance, but is useful in assessing the course of the reaction.

There are a number of methods for obtaining these current/voltage plots (Skoog and West, 1980).

One method is to use a developed chromatographic system with an electrochemical detector. Succesive injections of the analyte at increasing potentials can be made and the resulting detector response, plotted against potential can be used to construct a Hydrodynamic Voltammogram (HDV) (for an example see figure 2.21.).

When there is no suitable chromatography developd, an alternative is to use cyclic voltammetry (CV). This technique involves applying a constantly changing potential to a solution of the compound of interest in a three electrode cell similar to the design for the ED cell. At a pre-selected potential (the switching potential) the direction of voltage change is reversed and can be allowed to change until it reaches a second switching potential. This cycling of applied voltage will give rise to a cyclic voltammogram, (for an example, see figure 2.6.).

Oxidations occur when the voltage is changing in a positive direction, reductions when the voltage change is towards negative potentials (Evans et al., 1983).

More information about the nature of the reaction taking place can be obtained from CV than from HDV, including the number of steps involved and whether the reaction is reversible. Like HDV however a value for the full wave potential (E_p) can also be obtained.

It has been shown (Anderson et al., 1981; Kissinger, 1983 (i)), that there is good agreement between E_p values obtained by CV and HDV, providing the experiments are performed under comparable conditions of solvent, temperature, pH, and electrode material.

1.2.5. Principles of operation and practical considerations

The electrochemical detector relies on the three electrode design, used in most electroanalytical instruments (Plambeck. 1983). The controller supplies a constant potential difference between the working and auxilliary electrodes measured with respect to a third reference electrode. It constantly monitors the liquid junction potential at the working electrode and adjusts the potential applied accordingly to keep this at the pre-set value on the controller.

The resulting current is amplified and converted to an analog voltage which can be recorded as a graph of current against elution time/volume.

The electrochemical detector is selective to a certain extent. Other compounds present in the eluent which react at the applied potential will also be detected, non electroactive compounds will not be detected or interfere with the analyte of interest.

The potential provided must therefore be at a low enough voltage to exclude interference from co-eluting compounds, but be high enough to give a measurable current.

Two modes of electrochemical detection are recognised. One of these, coulometric detection, is where complete conversion of the analyte takes place. This is achieved using a porous graphite

electrode with a large surface area. This high rate of conversion is accompanied by an increase in background current, although a net improvement in sensitivity over the other design has been claimed (ESA, 1986). An additional advantage of coulometric detection is that electrode maintainance required is minimal.

The other approach is amperometric detection, wherein only a fraction (typically 5-20%, (BAS, 1980) of the analyte actually reacts on an electrode of limited surface area, the associated signal current produced is lower than with coulometric detection as is the background current. The signal-to-noise ratio and hence the sensitivity is similar to coulometric detection.

Glassy carbon is the material used most widely as the working electrode, however for specific applications the use of gold, mercury/gold or carbon paste electrodes may be advised (Ryan, 1983) Two designs of flow cell are commercially available for amperometric detectors.

(a) Wall jet electrode.

Figure 1.2.(b) shows the configuration of electrodes for this design. The eluent from the column travels through capillary tubing and impinges onto the electrode surface as a jet. The eluent and any products formed in the reaction then leave through the auxilliary electrode. This is designed to increase the amount of the compound which reacts (the conversion efficiency). However, as the flow of the jet onto the electrode surface causes some turbulance in the cell body, this leads to increased noise on the baseline. Another suggested advantage of

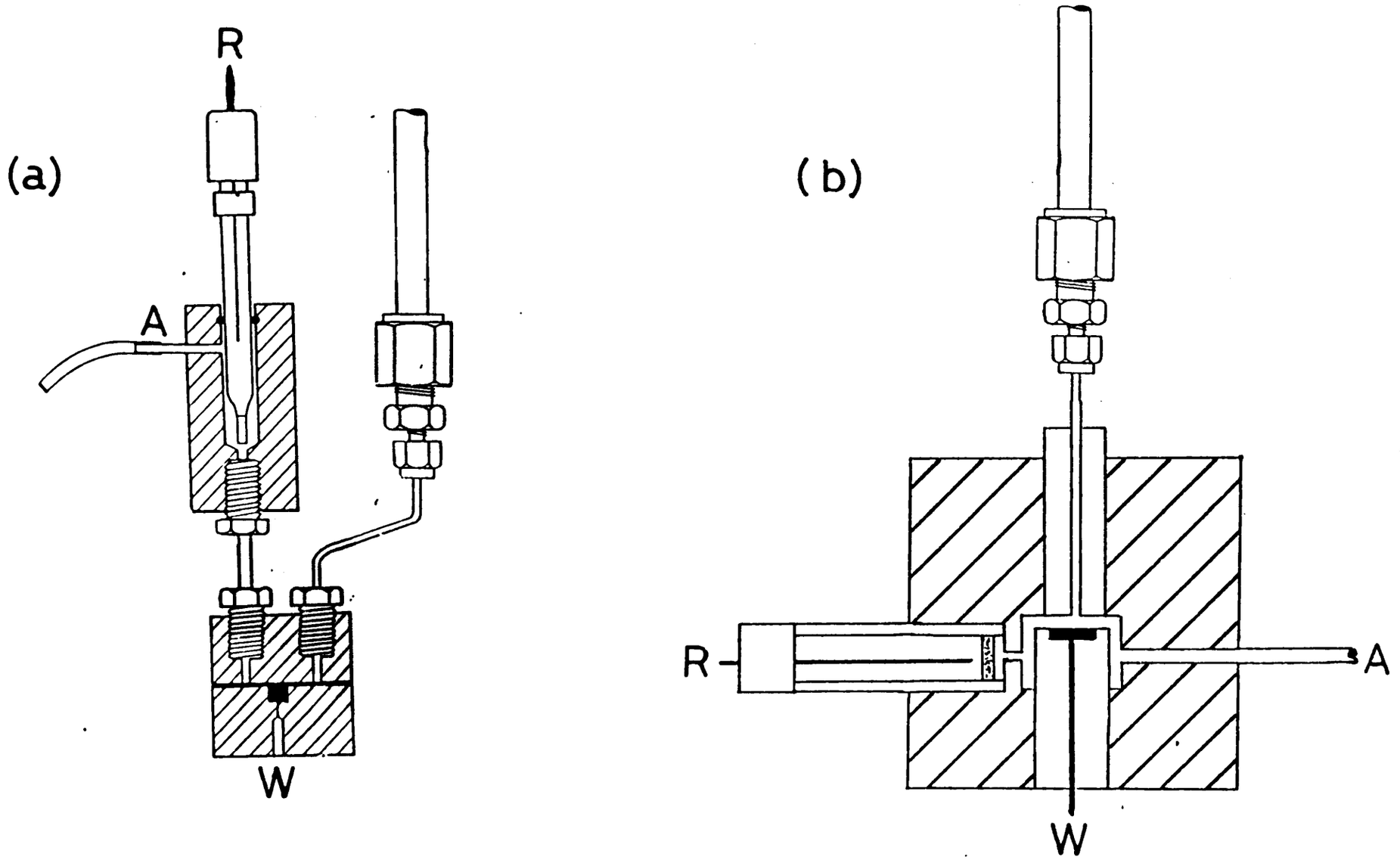


FIGURE 1.2.

(a) THIN LAYER FLOW CELL (BAS) SHOWING REFERENCE (R), WORKING (W) AND AUXILLIARY (A) ELECTRODES.
(b) WALL-JET ELECTRODE FLOW CELL, SHOWING REFERENCE (R), WORKING (W) AND AUXILLIARY (A) ELECTRODES.

this configuration is that the continuous flow of liquid onto the electrode surface helps to keep the electrode clean.

(b) Thin layer flow cell.

This is shown in figure 1.2.(a). The principle behind this design is that the smooth flow of the eluent over the electrode surface will limit the noise on the baseline and in this way lead to enhanced sensitivity. The extent of the reaction efficiency however relies on the diffusion of the analyte to the electrode surface where the reaction takes place.
This results in a lower conversion efficiency than the wall-jet system.

1.2.6. Conclusions and Selection of equipment

The sensitivity claimed for electrochemical detection and the prospect of a selective detector suitable for the measurement of some compounds of forensic interest prompted this investigation of its suitability for routine use.
At the outset of this work, coulometric detectors were not widely available, and amperometric detection had been selected for further examination.
There is no agreement as to which of the two cell designs provides the best sensitivity or ease of use. Some workers (Law, 1984) concluded that there was no significant difference.
Two commercially available flow cells, one of each type, were examined and found to be comparable in sensitivity and ease of use. The design of the thin layer flow cell was prefered because of its low volume and its modular design which could allow for

adaptation for microbore LC or for other electrode materials, eg gold, carbon paste if this proved neccessary. The control unit selected (Bioanalytical Systems, Anachem, Luton, Great Britain) gave a constant display of the current being measured and readily gave information about the potential applied and the background current. This was useful for troubleshooting purposes. It also had a facility to allow the use of two or more electrodes in series or in parallel, and an electronic self-test system. Its ease of operation and the wide range of facilities made this the unit of choice, for routine use and for troubleshooting.

1.3. SELECTION OF COLUMN AND SOLVENT

1.3.1. Introduction

Having examined the analyte and selected the most appropriate detection system, the chromatographic conditions may be dictated to some extent. If UV detection is being used, certain solvents with chromophores in the same region as the analyte may not be used (Snyder and Kirkland, 1978).

Although electrochemical detection has been applied to straight phase chromatography (Gunasingham and Fleet, 1983) the conditions required for electrochemical detection (aqueous based solvent system, high/low pH, water soluble charge carriers are more compatible with reversed phase HPLC (Kissinger, 1983 (ii)).

Most HPLC methods for drug analysis are performed on reversed phase materials (usually octadecylsilyl (ODS) (C-18)) (see table 1.1.). For this work the stationary phase material selected was

ODS Hypersil (Shandon Southern, Runcorn, Cheshire). This was chosen as it had been shown to be one of the most reliable in terms of batch-to-batch variation and quality of chromatography achieved, by work in this department (Eppel, 1980), and elsewhere (Smith et al., 1984 (i) and (ii)).

In addition, ODS-Hypersil was chosen as the stock reversed-phase material by the Home Office Forensic Science Service, in order to ensure good column reproducibility. It was considered convenient to follow suit in this respect, as many of the HPLC methods used in this department are based on assays developed by the Home Office Forensic Science Service.

Reversed phase HPLC is popular especially for the analysis of basic drugs, because of the phenomenon of peak tailing caused by the interaction of the basic nitrogen atoms with the silica packing material. This happens to a lesser extent with residual silanol sites on reversed phase materials and some of the techniques used to reduce this are considered in the course of this work. Of these the most widely used is ion pairing.

1.3.2. Ion pairing techniques

The principle of ion pairing has been used in conventional solvent/solvent extraction for many years. If a specific solute of interest is polar or ionised and therefore insoluble in a particular non-polar solvent, it can be made to dissolve by reducing the energy of interaction between the polar and non-polar moieties, to a level lower than the solvation energy. This can be done by adding to the solution a counter-ion of opposite charge to the solute with which it will form an ion pair which is overall

electrically neutral and effectively non-polar, making this new species much more soluble in the non-polar solvent.

Factors concerning the selection of the correct ion pairing reagent include the steric situation and nature of the charge in the solute molecule, how good a nucleophile or electrophile it is, the dissociation constant of the concommitant ion-pair and the polarity of the solvent.

The actual mechanisms of ion pair retention are still not agreed upon. Some workers (Horvath et al., 1977 (i); Horvath et al., 1977 (i) maintain that the ion-pairs are formed in solution and then partition onto the organic stationary phase.

The other popular mechanism supported by Knox (1976) and Bidlingmeyer (1979) is an ion exchange type, in which the ion pair reagent is first adsorbed onto the bonded phase thereby creating a charged surface, and partitioning of the sample then takes place between the charged surface and the mobile phase.

This effect was demonstrated by Knox (1976) by measuring how much ion pairing agent from the mobile phase was retained on the column. He found that a reversed phase packing material (SAS Silica) retained 13.0 mg of ion pairing agent per gram of packing material.

It seems likely that the actual mechanism in most cases is a mixture of these two proposals and will depend to a large extent on the concentration and composition of the mobile phase and the nature of the sample type and choice of counter ion.

Popular pairing reagents for cations include $H_2PO_4^-$, Br^-, ClO_4^- and more recently the sodium salts of pentane to nonane sulphonic acids.

Dissociation of the ion-pair in a separation can influence the symmetry of the chromatographic peaks.

Symmetrical chromatographic peaks are obtained if the degree of dissociation of the sample ion-pair is kept low and constant. This is acheived by maintaining a high and constant concentration of the counter-ion in the mobile phase.

One way of maintaining this constant concentration is to add a cation which will bring about a suitable ion pair extraction of the counter ion onto the stationary phase.

This use of amine modifiers in the mobile phase has been investigated by other workers (Gill et al., 1982; Kiel et al., 1985; De Ruyter et al., 1980), and it is proposed that these may improve the chromatography of basic compounds on reversed phase materials by blocking the residual silanol sites on the support.

When ion paired chromatography is to be used the solvent should have an aqueous base with an organic modifier to adjust its polarity (see below, 1.3.4.) to obtain the desired value for the extraction constant and hence capacity factor.

The pH must be adjusted to ensure dissociation of the sample ion from its own counter ion, but must not increase the dissociation of the solute ion-pair.

1.3.3. Optimisation of chromatography

In this study,once the mode of chromatography and the initial conditions had been selected, the control of retention of the analytes was achieved by controlling their ionisation through the pH of the solvent, The mode of chromatography through the use of solvent additives, and the strength or eluting power of the

solvent through control of the organic modifier.

Some quantitative measure of the polarity of the solvent is essential to the systematic optimisation of conditions, and the concept of polarity is therefore considered.

Solvents are chosen for HPLC by considering both the conditions enforced by the nature of the technique and those dictated by the analyte.

The former considerations include the temperature at which the separation takes place, the pressure required to force the solvent through the column and the method by which the eluting analyte is to be detected. This dictates that the solvent should be of low viscosity to allow practical flow rates and thus acceptable retention times, it must be a liquid at the temperature and pressure being used and is normally a liquid at atmospheric pressure although there are reports of carbon dioxide being used under super-critical conditions as an eluent for HPLC (Haddow et al., 1985). Trends in these two properties of viscosity and boiling point generally follow one another for most solvents, except where there is a large amount of hydrogen bonding, in solvents like water and the lower alcohols, or in compact molecules like cyclohexanes and aromatics, which have higher viscosities than predicted from their boiling points.

Those conditions dictated by the analyte include whether normal or reversed phase conditions should be used (Snyder and Kirkland, 1979), which in turn dictates the choice of solvent. It must be sufficiently non-reactive so that it will not react with the analyte in the course of the separation and it must have the correct solvency characteristics in order to elute the analyte

selectively from the injected mixture.

Good laboratory practice alone imposes considerations about the toxicity and flammability of the solvents being used, and makes the use of dimethyl sulphoxide, acrylonitrile and hydrogen sulphide undesirable where there are alternative approaches.

1.3.4. THE CONCEPT OF SOLVENT POLARITY

1.3.4.1. Introduction

In selecting a solvent for HPLC it is seldom the case that one wishes to maximise the solubility of the analyte in the mobile phase. This would merely result in eluting it from the column at a very early stage, probably with the solvent front. The usual objective is to vary the solvent strength of the mobile phase to allow the analyte to be selectively partitioned between the stationary and mobile phases and eluted on its own, separated efficiently from other components in the mixture in a reasonable time.

It is usually the case that analysis time should be as short as possible, normally under thirty minutes.

There are a number of physico-chemical parameters which dictate how the pure solvent or solvent mixture interact with the analyte. These include dispersion interactions, dipolar interactions (both dipole induction and dipole orientation), hydrogen bonding interactions and the Hydrophobic effect. Each of these can bring either a degree of ordering or disordering to the solvent and thus effect its interaction with the analyte. Having to consider each of these processes in turn when selecting a solvent system for

HPLC would be laborious and time consuming. For this reason several schemes have been suggested whereby the sum of these effects can be expressed in simple terms.

1.3.4.2. Solvent classification schemes

The polarity or strength of a solvent represents its ability to enter into all the types of interactions considered above and is defined as the sum over all the interactions. Several schemes have been proposed to do this. One scheme (Hildebrand, 1975) for classifying the solvating properties of a solvent is based on an empirical approach to the problem. Delta, the solubility parameter is related to the enthalpy of vapourisation. This arises from the fact that the process of vapourisation ie from liquid phase to gas phase is similar in principle to transfer between two liquid phases. Delta is easily calculated from boiling point data.

Another scheme (Rhorschneider, 1973) for classifying the solvating properties of a solvent is based on experimental data rather than theoretical considerations. Rohrschneider measured the partition coefficients for a number of solutes and by doing so illustrated the degree to which a range of some 84 solvents participate in the various interactions described earlier. This data was examined by Snyder in 1974 and further in 1978, and simpified to allow its application to solvent selection in liquid chromatography.

Each solvent can be described in terms of interactions with three test solutes.

The three solutes chosen were ethanol, dioxane and nitromethane, and were designed to show the proton donor, proton acceptor and

dipole properties respectively, of all the solvents examined.

Since subdivision of P' into individual polarity contributions is implicit in its derivation (Snyder, 1978; Snyder, 1979), the X_e,X_d and X_n (chi) values represent the fractional polarity to be ascribed to proton acceptor, proton donor and dipole interactions respectively for that solvent, assuming these to be the major interactions.

The solubility and hence the relative partitioning of an analyte between the stationary and mobile phases of a chromatographic system can thus be controlled by varying the individual Xe, Xn and Xd values and hence the polarity of the mobile phase.

This may be done mixing two solvents . The solvating properties of the resulting mixture will be proportional to the volume fractions of the constituent solvents (Snyder, 1978).

$$P'_{mix} = M_a P'_a + M_b P'_b + \ldots\ldots + M_i P'_i$$

Where M_{a-i} are the mole fractions of solvents a to i, and P'_{a-i} are the respective polarities.

If the mixture contains two solvents, a range of polarities ranging between the polarities of the pure solvent components can be obtained.

Taking the situation dealt with in this work where there are three solvents, acetonitrile, methanol and 0.01M KH_2PO_4 (assumed to be equivalent to water), mixtures of these solvents in all proportions can be represented in two dimensions as demonstrated in figure 1.3., as the mole fractions are interdependant (ie

$M_a+M_b+M_c = 1$). Thus a range of solvents with different X_e, X_d and X_n values contributed from the constituent solvents, but the same P' value (or solvent strength) can be prepared. The use of ternary rather than a binary solvent mixture thus allows a greater degree of fine tuning in the optimisation process.

The derivation of these parameters is found in Snyder, (1974) and (1978), and discussed in detail in Snyder, (1979).

1.3.5. TERNARY SOLVENT MIXTURES AND MIXTURE DESIGN STATISTICAL TECHNIQUES IN LIQUID CHROMATOGRAPHY.

1.3.5.1. Introduction and discussion

The use of three solvents in the mobile phase is best avoided where possible. This is due to the increase in complexity in optimising the mobile phase conditions when there is an additional degree of freedom. The strength of a solvent in terms of its position in an elutropic series can be represented by its polarity P' (Snyder, 1974; Snyder, 1978). The concept of polarity has been discussed above (1.3.4.). In summary it is a single term used to represent the sum of the contributions to net solvating properties of a solvent by the major molecular interactions. These are proton accepting, proton donating and dipolar interactions.

The polarity of a solvent mixture is determined by the relative proportions of its constituent pure solvents. Thus, using three pure solvents, several combinations of the substituents may have

the same polarity. Other properties of the solvent however, namely contributions from proton accepting and proton donating interactions (Xe, Xd and Xn respectively), may differ.

Finding the optimum solvent for a particular separation when three solvents are used is considerably more complex than with binary mixtures. The advantage of ternary solvent systems is in the broad range of variation in selectivity they offer compared to binary mixtures.

Random alteration of the solvent composition may provide a suitable solvent system, but this is rather hit and miss, and a more structured approach was considered essential if a ternary system was to be used.

A number of reports have appeared over the last six years on the optimisation of ternary solvent mixtures, based on statistical interpretation of a small number of trial solvents (Sachok et al., 1980; Glajch et al., 1982; Berridge, 1982; De Smet et al., 1984; Weyland et al., 1985; Colley, 1985).

An early report Glajch et al., 1982, outlined an approach to the problem. A recent review of this subject has noted that this early approach is still one of the most reliable (D'Agostino et al., 1985). In its simplest form, that is for less than five analytes, where the elution order does not change, the process is described below. The mathematical basis for the optimisation procedure is well established (Box and Wilson, 1951; Snee, 1979). The process can be considered in three stages.

Firstly the three starting solvents are selected. These may be pure solvents or solvent mixtures. These form the apices of the triangle shown in figure 1.3.. A series of trial solvents (e.g.

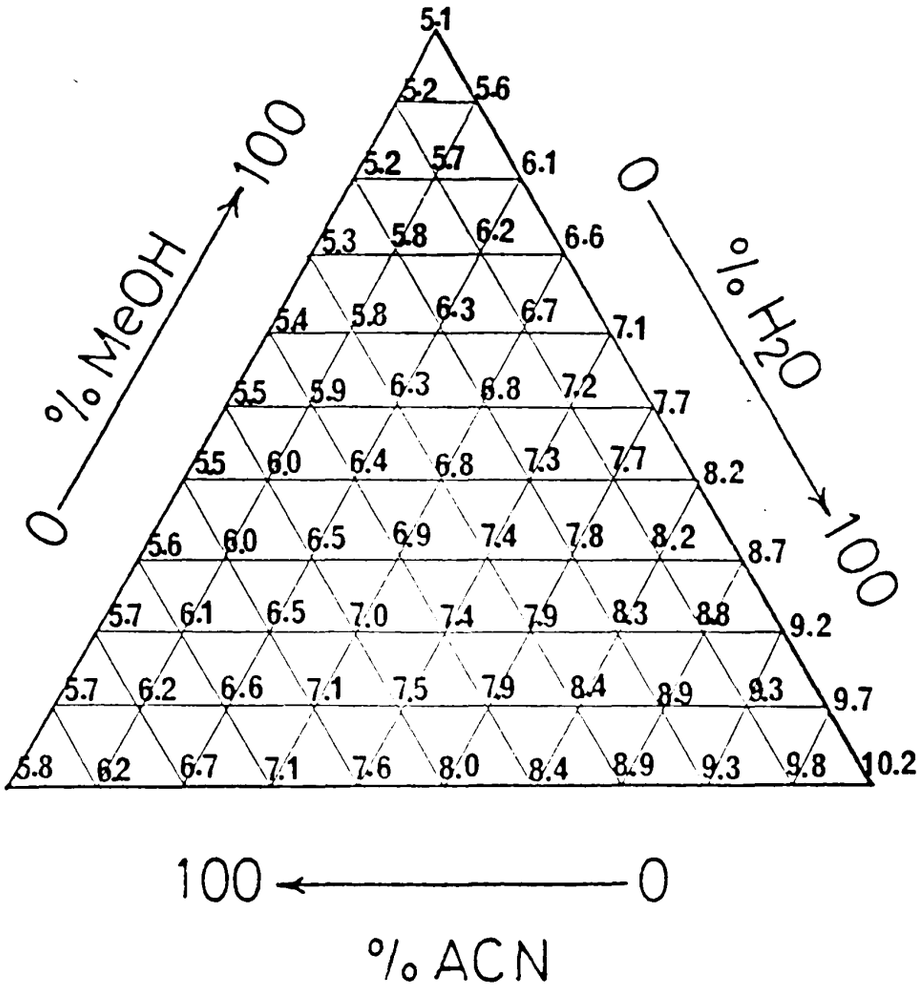


FIGURE 1.3.

PLOT OF POLARITY VALUES FOR TERNARY SOLVENT MIXTURES OF METHANOL, ACETONITRILE AND WATER, IN STEPS OF 10%.

7 and 10 have been used (Glajch et al, 1982, Snee, 1979), are examined experimentally.

The chromatograms produced are assessed in terms of efficiency, analysis time, capacity factor, resolution and peak shape. In order to procede further with the optimisation, all this data must be reduced to a single number, known variously as the chromatographic optimisation factor (COF), alpha value (α), chromatographic response function (CRF), relative resolution product (RRP) etc., depending on the algorithm used to assess the chromatograms. These have been reviewed by D'Agostino et al., 1985.

The COF chosen is usually designed to have a minimum or maximum value for the solvent with the optimum chromatographic properties. The COF as described by Glajch et al, 1982, approaches zero from the negative direction.

Most algorithms can be weighted in favour of one parameter, (e.g. resolution or efficiency) if required. There is no generally agreed algorithm for this reduction, and by the nature of the reduction process a lot of information about the chromatogram is lost.

The COF calculated for each of the trial points is fitted to a quadratic or polynomial curve fitting procedure which will produce an equation for the response surface of the solvent map.

The final stage is to solve the equation for the desired or optimum value of the COF used. This can be done by calculating the COF for a number of points on the solvent map and plotting the data (Goldberg and Nowakowska, 1984). Alternatively it can be done by using a simplex design (Box and Wilson, 1951; Deming and

Morgan, 1973; Berridge, 1982) to solve the equation for the desired COF value. This will in theory give the solvent map coordinates of the optimum solvent composition.

This process requires an extensive number of calculations which are readily performed on a computer. A number of commercially produced optimistation procedures have been produced, many with interactive HPLC and solvent switching systems to perform the whole procedure automatically (TAMED, CHEOPS, PESOS, ISOOPT, etc.).

In summary, the success of the procedure depends on the selection of the correct starting solvents, and the use of an appropriate COF. Additional complications can arise from the incorrect identification of peaks if the elution order changes between solvents.

This procedure has also been applied to the optimisation of other mobile phase conditions such as electrolyte concentration, ion-pair concentration and pH (Sachok et al., 1981; Goldberg and Nowakowska, 1985).

1.3.5.2. Conclusion

The establishment of such a system was considered to be beyond the scope of this study. However the advantages of ternary solvent systems were still considered worthy of some investigation, to determine if any significant improvement in the chromatography could be achieved without recourse to changes in hardware or the use of computers.

1.4. ASSESSMENT OF CHROMATOGRAPHIC PERFORMANCE

1.4.1. Introduction

The assessment of the performance of HPLC separations in this thesis has been made by calculating the following parameters and considering them in the context of each other. The relevent parameters are shown in figure 1.4..

1.4.2. Capacity factor

The capacity factor, K', assesses the ability of a column with a particular solvent system to retain the solute of interest, with respect to the eluent. See figure 1.4..
This is defined as

$$K' = \frac{Tr - Tr^{O}}{Tr^{O}} \qquad (1)$$

With typical flow rates of 1-3ml/min and a dead volume of about 2ml, Tr^{O} is typically 0.6 to 2 minutes. With analysis times of 20 minutes or less, acceptable K' values are in the range 0-10.

1.4.3. Separation

Where two or more solutes are present it is useful to be able to assess the degree of separation between them. This can be expressed by taking the ratio of the relevant capacity factors. This is defined as the selectivity, a.

$$a = \frac{K'_2}{K'_1} \qquad (2)$$

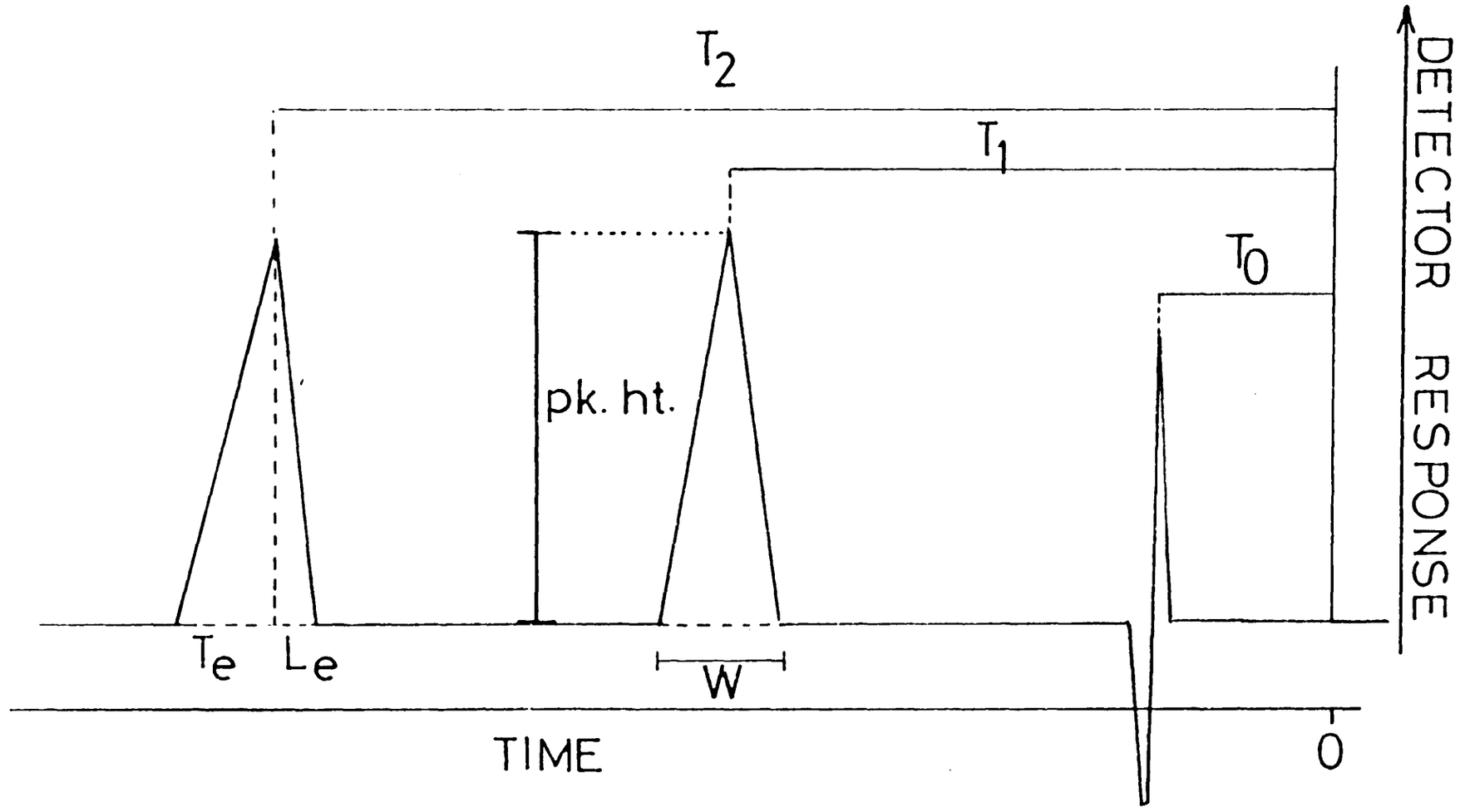


FIGURE 1.4.

MODEL CHROMATOGRAM SHOWING RETENTION TIME (T1 and T2), PEAK WIDTH (W), VOID TIME (To), PEAK HEIGHT (pk.ht.) AND TRAILING AND LEADING EDGES (Te and Le).

Thus in order for two components to be separated, a must be greater than 1.

1.4.4. Efficiency

The efficiency of a separation relates to the volume in which a given solute will elute with respect to its retention volume. This can be expressed quantitatively by the term

$$N = 16\left(\frac{Tr}{W}\right)^2 \qquad (3)$$

where N is the efficiency in number of theoretical plates. As the retention time increases, so also does the longitudinal diffusion of the solute through the column, and hence the band width increases also. As a consequence of this, for one particular compound, minor changes in elutropic strength of the solvent do not produce major changes in the efficiency of the chromatography. In order to produce significant changes in the efficiency it may be neccessary to change the selectivity of the separation, say by using a different solvent or a solvent additive. It may also be neccessary to change the stationary phase or the mode of chromatography eg to an ion paired separation.

1.4.5. Resolution

The goal of the chromatographer is to produce a system which will give adequate separation (a) of two or more components with a reasonable retention time (K') and good peak shape (N).
It is useful to be able to express the quality of the separation

in one expression and the term of choice is the resolution. The resolution between two adjacent bands is defined as the distance between the band centres divided by the average band width

$$R_S = \frac{(T2-T1)}{0.5(W_{T1}-W_{T2})} \qquad (4)$$

which by substitution in eq 1. and 3, gives rise to

$$R_s = (1/4) \times (a-1) \times (\sqrt{N}) \times (K'/1+K') \qquad (5)$$

This shows the empirical dependence of good chromatography on a separation (a) greater than unity, a high efficiency (N) and an acceptable value of capacity factor (K'). Control of resolution can be effected by altering any of these terms.

1.4.6. Peak assymetry

The peak shape is determined by the adsorption isotherm of the analyte on the column (Snyder and Kirkland, 1978). If the solubility of the analyte is substantially different in one of the phases, then the peak may be skew, with either a long leading or trailing edge. If mixed principles of separation are involved, e.g., the adsorption of the analyte on free silanol sites of the packing material, then the peak will tail. A measure of the degree of tailing is to drop a perpendicular line from the peak to the base line and measure the leading (Le) and trailing (Te) edges. The assymetry factor A_s is defined as the ratio of T_e/L_e.
A_s should always be considered in terms of the peak width, since a narrow, skew peak is usually prefered over a broad symmetrical peak.

1.5. EXTRACTION OF DRUGS FROM BIOLOGICAL FLUIDS

1.5.1. Introduction

The principle of selective extraction of compounds from complex matrices is well established.

The process is based on the partitioning of the analyte between two immiscible solvents by selection of appropriate solvents and conditions of pH. This is normally done by shaking the two phases together to allow exchange of the analyte between phases, but the practice of using a solid phase in place of one of the liquid phases is also known (Harkey and Stolowitz, 1984).

The selection of the appropriate conditions for the extraction is specific to the compound of interest (or compounds of similar chemical properties) and is discussed in detail for each analyte examined (2.3. and 3.1.7.).

Two methods of solid phase extraction (Absorption and Adsorption) are also considered. These are introduced in some detail at the appropriate point in the text (2.3.4. and 3.1.7.3.).

1.5.2. THE USE OF INTERNAL STANDARDS

1.5.2.1. Introduction

In an assay involving the extraction of a particular compound from some matrix, the accuracy and precision of the method must be considered before the method can be used to measure the compound of interest in samples containing unknown amounts.

1.5.2.2. Discussion

Accuracy is the proximity of the measured value to the true value, and precision is the ability of the method to produce the same answer on repeated examinations of the same sample.

In the assays developed in this study, the absolute recovery of the drug from the sample is calculated by comparison of the peak height produced by a known amount of drug (an unextracted standard) with the peak height produced by a known aliquot of the sample extract.

Accuracy therefore depends on the sample and the prepared standard behaving in the same way during the extraction.

Precision depends on several extracts from the same sample giving the same answer. There is apparently a link therefore between precision and accuracy.

In most cases the analysis was repeated four or five times, and a measurement of the precision was made. This is given in table and text as the standard deviation (SD) and also as the coefficient of variation (CoV), which is the standard deviation expressed as a percentage of the absolute recovery.

The accuracy of the extraction procedure is dependent on the precision, as the level in the sample is calculated from a comparison of peak heights of the standard with the peak height from the sample and calculated by simple proportion.

The assumption made is that the sample and the standard will behave similarly and the compound of interest will extract equally well from the sample and the standard.

Even when the greatest care is taken with the extraction procedure, fundamental differences of the sample material and

standard may make this assumption invalid. Even if both samples are treated identically throughout the extraction, factors which may effect the extraction efficiency include the nature of the sample and standard (both should be the same media eg blood or water or urine), the age of the sample and standard, the temperature at which the sample or standard is stored prior to analysis and the initial pH of the sample or standard.

It is accepted practice that the inclusion of a known amount of a similar compound in the sample and standard can be used to eliminate many of these problems.

The properties required of an internal standard are that it should resemble the analyte as closely as possible in all its properties, and that it should be possible to measure it simultaneously with the analyte. Most importantly it should be extracted by the same extraction procedure. If a suitable internal standard is available and is included in both the sample and standard prior to the extraction, any differences in the treatment of the two samples can be accounted for. Thus, whilst in replicate analysis, the extraction efficiency between samples might vary, the ratio of the analyte to internal standard between assays should be constant. Knowing that the amount of internal standard added in the first place was the same, the ratio of internal standard in the sample to the internal standard in the standard will give a measure of the difference in extraction efficiency between the two materials. This ratio can then be used to correct for differences in the relative amounts of analyte in the sample and standard.

1.5.2.3. Conclusion

The use of an internal standard whilst not altering the precision of the extraction efficiency should improve the percieved precision (ie lower the CoV) in the ratio of drug to internal standard compared to the Cov found in the absolute extraction efficiency. As was shown above, an improvement in precision will lead to an improvement in accuracy, therefore both precision and accuracy can benefit from the use of a suitable internal standards.

1.1.6. CONCLUSIONS ON CHAPTER ONE

The above review and discussion of the current practice of HPLC has shown that numerous applications exist in the field of the forensic sciences. There are a number of areas in which HPLC is the technique of choice, depending mostly on the nature of the analyte.

The principles of solvent theory have been outlined to illustrate the use of solvent mixtures for the selective elution of compounds from a chromatographic column.

The principle of the relatively new technique of electrochemical detection and associated voltammetric techniques have been introduced together with some aspects of the theory involved.

The use of specialised chromatographic techniques such as ion-pairing and automated or systematic method development have also been introduced, as have the parameters used in this work for the assessment of chromatographic performance.

These factors are all involved in the development of HPLC assays for two groups of compounds of forensic interest (morphine and the local anaesthetics), discussed in the following two chapters.

CHAPTER TWO

MORPHINE

2.1. LITERATURE

2.1.1. Introduction

Opium (Gr. 'juice'), the dried, compressed resin from the seed case of the poppy Papaver Somniferum (Papaveracaea) has been recognised as a painkiller for over 4000 years. Its narcotic and analgesic properties were known in early Egypt, and the practice of smoking or eating opium for its euphoric effect has since became widespread (Trebach 1982).

In 1803, a German chemist, F.W. Sertürner isolated from opium some bitter colourless crystals, "a soporific and narcotic principle of opium" (Doenicke, 1983). Although the structure of the material was not elucidated until 1925 (Gulland and Robinson 1925), this isolation of morphine was followed by attempts to manufacture derivatives and to test them for their analgesic properties (Sneader, 1985). The ultimate aim was to produce a powerful narcotic drug which did not produce tolerance or dependence in the user.

Many of these derivatives and structural analogues have subsequently been found to be more potent than the parent compound and a range of synthetic and semi-synthetic opiates are now available (Martindale, 1984). The other objective of producing a narcotic free from addictive properties has not yet been achieved

The synthesis in 1874 of diacetyl morphine (heroin) produced a

substance which was discovered to have the same effect as morphine but with an enhanced (x3-4) dose-response relationship, with less of the disagreeable side effects of morphine (esp. nausea, vomiting). This made it more popular with addicts (Trebach, 1982).

Both morphine and heroin are addictive substances and users are prone to psychological and physiological dependence. Tolerance develops rapidly with regular use, and can be extensive.

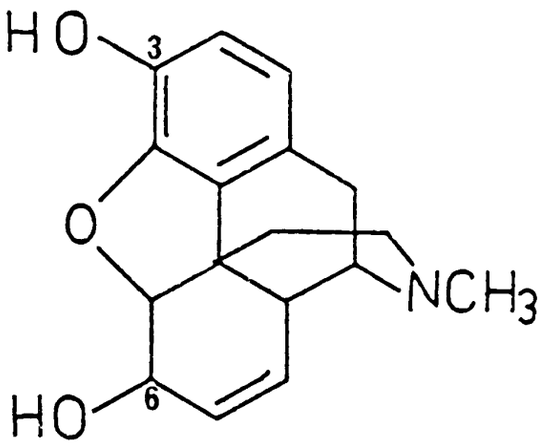
Detoxification or withdrawal from opiates can be a distressing and uncomfortable process lasting 2-3 days (De Ropp, 1958). Abrupt withdrawal however is seldom used in modern treatment of addicts, as methadone therapy is used to alleviate the unpleasant symptoms (Goodman and Gilman, 1980).

In the United Kingdom, morphine is the opiate favoured by most clinicians for severe pain. It is often used in conjunction with cocaine, or alternatively as Brompton Mixture, a mixture of heroin and cocaine, in the treatment of chronic severe pain.

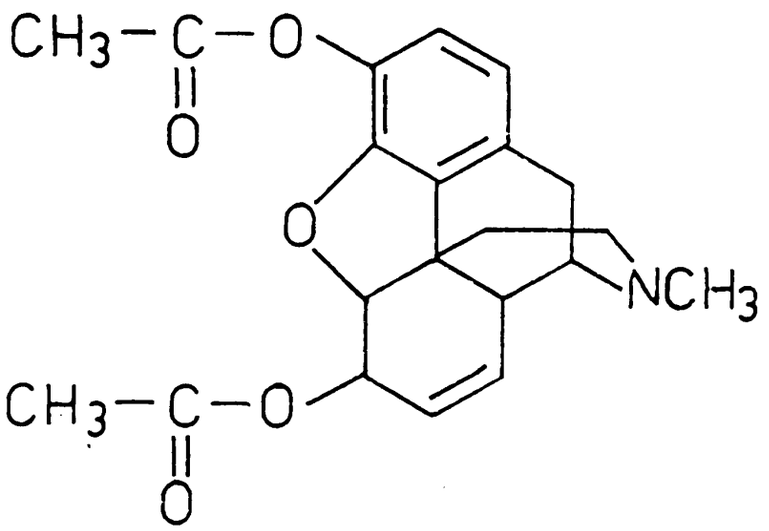
Heroin and morphine are also used for perioperative and postoperative pain, and as sedatives (Martindale, 1984). Heroin, however, is not available clinically in the United States.

Opiates are not naturally present in the body but have structural similarities to a group of endogeneous peptides called endorphins and enkephalins, discovered in 1978, which play an important role in the body's own control of pain relief (Blum, 1984). It is believed that morphine and its analogs act at the receptors for these compounds in the central nervous system.

The structures of morphine and heroin, its diacetyl derivative, are shown in figure 2.1..



MORPHINE



DIACETYL MORPHINE (DIAMORPHINE, HEROIN)

FIGURE 2.1.

STRUCTURES OF MORPHINE AND DIAMORPHINE

2.1.2. The increase in opiate abuse

Drug abuse as a significant social problem has only existed in Britain since the early 1960's, and drug use in Britain until then was exceptional only for its trivial extent (Edwards and Busch, 1981).

The indicator used as a measure of the extent of the problem is the number of addicts known to the Home Office. What percentage of the actual number of addicts this figure represents is widely disputed and estimates range from 96% to 20% (Edwards and Busch, 1981), although the current estimate accepted by the Home Office is 25% (Picardie and Wade, 1985).

The number of addicts known to the Home Office over the period 1958 to 1984 is given in table 2.1., and shown in figure 2.2. (Spear, 1969; Edwards and Busch, 1981; Home Office Statistical Bulletin, 1984)

The increasing black market availability of heroin reflected in the number of siezures of illicit opiates (Reports of the Chief Constable, Strathclyde Region, 1976-84) and the depressed economic climate are the two factors generally held to be responsible for the current increasing trend in heroin abuse. (C.U.R.R., 1982; Picardie and Wade, 1985).

As a result, there has also been an increase over the last five years in the demand for analyses for morphine both in post-mortem casework and in clinical accident/emergency admissions.

TABLE 2.1.

NARCOTIC ADDICTS NOTIFIED TO THE HOME OFFICE BETWEEN 1934 AND 1984.

Year	Known addicts (UK)
1934†	300
1958‡	442
1959	454
1960	437
1961	470
1962	532
1963	635
1964	753
1965	927
1966	1349
1967	1729
1968	2782
1969	2881
1970	2657
1971	2762
1972	2936
1973	3023
1974*	3252
1975	3425
1976	3474
1977	3605
1978	4116
1979	4787
1980	5107
1981	6157
1982	7962
1983	10235
1984	12489

† Spear, 1985
* Home Office Statistical Bulletin, 1984
‡ Edwards and Busch, 1981

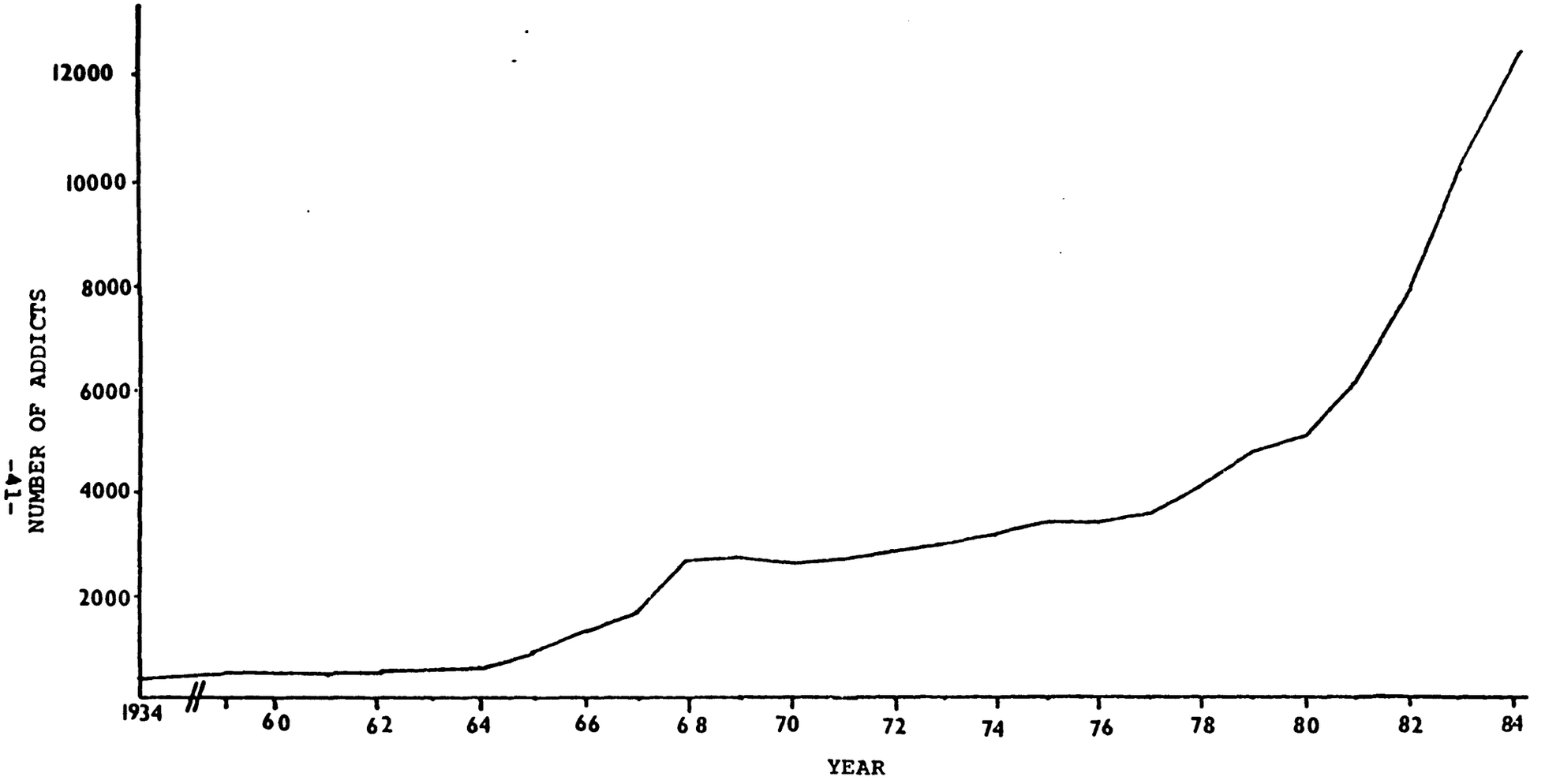


FIGURE 2.2.

NARCOTIC ADDICTS NOTIFIED TO THE HOME OFFICE BETWEEN 1934 AND 1984

2.1.3. Metabolism and Toxicity

Morphine and heroin (also called diacetyl morphine, diamorphine) are narcotic analgesic drugs. Heroin itself appears to be inactive but is immediately metabolised in the blood to monoacetylmorphine and then to morphine.

Morphine acts as an agonist with stereospecific and saturable binding sites or receptors in the brain and other tissues (Goodman and Gilman, 1980). These sites are widely but unevenly distributed throughout the central nervous system. It is believed that morphine, its analogs and antagonists occupy the sites normally occupied by β-endorphine and the enkephalins. These are endogeneous peptides which act as neurotransmitters or modulators of neurotransmission. They can be shown to be structurally similar to morphine. They act by interfering with the production of cyclic AMP, which in turn appears to alter some mechanism fundamental to neuronal function (Blum, 1984).

In man morphine produces analgesia, drowsiness, changes in mood and mental clouding without loss of consciousness. In some cases, under the correct conditions, users also experience euphoria similar to sexual orgasm, which is the initial attraction of the drug, and the motivation behind psychological dependence (Goodman and Gilman, 1980; Laurie, 1980).

Other effects are dilation of the pupils and dilation of the surface capilliaries causing flushes. Side effects to therapeutic dosing include constipation through peristaltic depression, vomiting and mild respiratory depression (Goodman and Gilman, 1978; Martindale, 1984).

Morphine levels in the body fluids can be used to indicate whether

the drug is present in therapeutic or harmful quantities. Peak levels of morphine of 1ug/ml in plasma have been reported following bolus doses prior to surgery (Vandenberghe et al, 1983). The therapeutic range reported by Stead and Moffat (1983) was 0.04 to 0.5 ug/ml, whilst the therapeutic range reported in Moffat (1986) is 0.01 to 0.07 ug/ml. This wide range (0.01 to 1ug/ml overall) is due to the fact that the body can develop tolerance to morphine requiring increased doses for the same effect. The estimated minimum lethal dose in man is 200mg, but addicts can tolerate up to ten times this amount. This makes the interpretation of blood concentrations of morphine difficult.

Patients recieving intensive care hospital treatment can tolerate prolonged high doses by bolus and infusion if respiration is applied.

Toxic effects follow from therapeutic effects. An increase in respiratory depression can lead to coma and so to death. An increase in smooth muscle relaxation can then lead to muscle spasm and convulsions.

Secondary effects from the drug include pneumonia through the anti-tussive effects of morphine coupled with the dangers of inhalation of gastric contents. Other drug associated illness is considered in 2.10.1.1.. The blood concentrations which cause toxicity vary due to tolerance as discussed above. Toxic reactions have been reported at blood levels of 0.5 to 5ug/ml (Stead and Moffat, 1983) and fatalities have occured at 0.2ug/ml (Felby et al., 1974).

Although the site of action of morphine is primarily in the CNS, only a small amount actually crosses the blood brain barrier.

Free morphine accumulates in the kidney, lung, liver and spleen. Recent evidence suggests that it may persist in these tissues for up to 21 days post withdrawal (Jones et al, 1984).

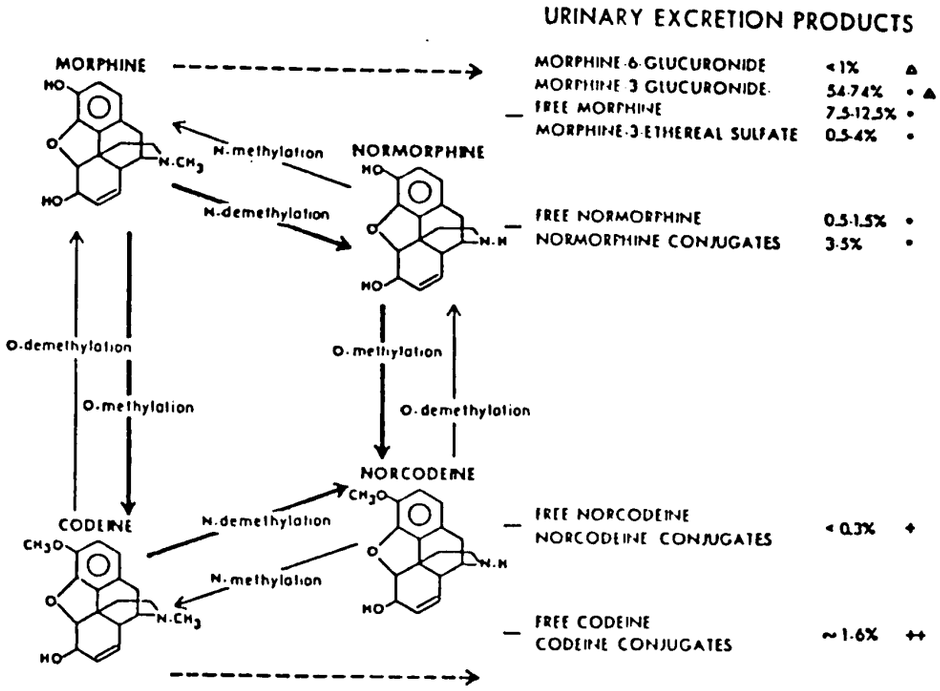
Morphine is eliminated from the blood fairly rapidly. It has a half-life of 2-3 hours (Goodman and Gilman, 1978). Absorption of the drug will depend on the route of administration. In terms of the onset and duration of respiratory depression, intravenous administration produces maximum effect within seven minutes. Death following intravenous administration can be rapid, and is most commonly due to pulmonary cedema (Polson et al., 1983). The reason for this is not known.

The delay to peak effects is 30 minutes following intramuscular injection, and up to 90 following subcutaneous administration. Adsorption through the mucous membranes occurs rapidly whilst oral dosing can take one to two hours for maximum effect.

The effect of a given oral dose is less than from an equivalent dose administered parenterally, as there is a significant amount of first pass metabolism in the liver.

The major metabolic pathway is well known (Boerner et al., 1975). Very little unchanged drug (less than 1%) is excreted in the urine. Morphine, administered either as morphine or heroin, is metabolised to small quantities of morphine-3-ethereal sulphate, codeine, norcodeine, and normorphine. Morphine and the last three metabolites mentioned are conjugated through the 3-OH and 6-OH positions and excreted in the urine. This is summarised in figure 2.3. (Boerner et al., 1975).

There is currently a controversy over the role of the kidney in the elimination of morphine. Recent studies in patients with



from Boerner et al., 1975

FIGURE 2.3.

MAJOR PATHWAYS FOR THE METABOLISM
AND EXCRETION OF MORPHINE

impaired renal function (McQuay and Moore, 1984; Moore et al., 1983) concluded that the renal clearance of the drug is the dominant route of elimination at low concentrations, and hepatic clearance of more importance at higher blood concentrations.

The immunoassay methods used in this study however are criticised because of possible interference from metabolites. The 6-glucuronide which has a reported cross reactivity of 1% with the best antibody available (Moore et al., 1984 (i)) is often present at levels 25 times that of morphine (Svensson et al., 1982). The ability of the kidney tubules to transport morphine actively was shown as early as 1969 (Watrous et al., 1969), and the gut wall has been shown to metabolise morphine also (Hanks and Aherne, 1985).

2.2. ANALYTICAL METHODS

2.2.1. Introduction

In order to meet the demand for analysis of morphine in biological fluids, the various analytical methods currently available were reviewed. The tissue of choice for analysis is plasma. In this material, morphine is present in the unconjugated form, and there is little else in the matrix itself likely to interfere with the isolation of the drug, although 30% binding to plasma proteins is reported. Plasma is seldom available in post-mortem cases however, and the tissue most commonly supplied is haemolysed, often putrified, blood. A recent review of literature (Stead and Moffat, 1983) suggests that

the range of concentrations likely to be encountered are greater than 0.01ug/ml. The highest level of morphine reported in the blood of an overdose victim, is 6ug/ml following accidental ingestion whilst attempting to smuggle heroin in balloons in the stomach (Connett, 1984). A suitable method should therefore be capable of detecting morphine concentrations within this range. Typical blood sample volume is 1ml, so the method should have an absolute sensitivity of greater than 10ng.

2.2.2. Microchemical methods

Qualitative determination of morphine in biological extracts by colour reactions and thin layer and paper chromatography have been reviewed (Mulé, 1974(i)). Several thin layer chromatography systems suitable for opiates are also included in Moffat, 1986. Additionally, Rf data for morphine is included in most TLC data collections (Rentoul and Smith, 1973; Sunshine,, 1974). The sensitivities of the methods are around lug on the TLC plate, which implies that a large sample volume would be required for sensitive detection limits. Photometric methods involving a simple extraction of the tissue and subsequent UV absorbance measurements (Mulé, 1964; Goldbaum and Williams, 1.,1964) were non-specific. These methods were also susceptible to interference from natural decomposition products and insufficiently sensitive for typically sized tissue samples. In summary, these methods were found to unsuitable for reliable or quantitative analysis of small amounts of morphine in biological extracts.

2.2.3. Fluorimetry

Fluorimetric methods based on the oxidative dimerisation of morphine to pseudomorphine (Kupferberg et al., 1964; Fry et al., 1974) have been described. Extracts from tissue samples were treated with potassium ferricyanide. Any phenols present may form homogeneous of heterogeneous dimers by a phenolic oxidative coupling reaction (Ternay, 1978) (see figure 2.8.). Pseudomorphine is formed by this reaction and is fluorescent with a strong, fairly specific emission at 440nm. The procedure has the advantage of being specific for opiates with a free 3-OH, and more selectivity could be achieved by first purifying the extract by TLC. It is however cumbersome and the potential yield of the $K_3Fe(CN)_6$ reaction depends to a large extent on co-extractives. Any other phenolic material in the extract however will lead to mixed dimer formation and may give rise to false negatives.

2.2.4. Immunoassay

Immunoassay techniques were developed in the 1960's to measure insulin in human plasma. Their potential was quickly realised and a radioimmunoassay (RIA) for morphine was produced in 1971 (Spector 1971). The theory is well reviewed (Mulé et al, 1974(i) and (ii)) where several immunoassay techniques for morphine (Radioimmunoassay, Free Radical Assay Technique, Enzyme Multiplied Immunoassay Technique and Haemagglutination-Inhibition) are assessed. The quality of the assay and its specificity depend on the antibodies available, but in most cases RIA is the method of choice. Although RIA is claimed to be more sensitive

than any other technique (100 femtomole/assay tube, Moore et al., 1984), it has the disadvantage of having limited rather than absolute specificity. The antibody of choice in the above study (Moore et al., 1984) has 100% cross reactivity with diacetyl morphine and presumably with monoacetyl morphine and some cross reactivity with the 6- and 3-glucuronides (Hanks and Aherne 1985).

2.2.5. Gas Chromatography

The advent of gas chromatography by James and Martin, circa 1952, and its subsequent popularity in the 1960's led quickly to a method of analysis for morphine (Lloyd et al.,1960). This involved a packed column with poor efficiency. Interference was common and unavoidable. Subsequent improvements in gas chromatography and the introduction of capillary columns has led to better resolution and improved sensitivity for most compounds including morphine (Schepers et al., 1982). A review of the development of GC with regard to the analysis of morphine is given in Wallace et al., 1974.

Morphine is a polar compound by virtue of its two hydroxyl functions (figure 2.1.). These require derivatisation in order to allow efficient chromatography without tailing on the type of polar column routinely in use (Schepers et al., 1982).

Evidence has also been published for the transacetylation of morphine in the presence of diacetyl morphine in the injector zone (Brochmann-Hanssen and Swendsen, 1962). Several ways of preparing derivatives have been described in the literature, including esterification using the appropriate acid anhydride. Trifluoroacetyl morphine has been produced in this way and is

suitable for electron capture detection (Mulé, 1974 (i)). Peralkylation (Corey, 1965) and carbamate formation (Hartvig and Vessman, 1974) have also been described.

The most popular method of derivatisation for tertiary alcohols is the formation of the corresponding silyl ether (Wilkinson and Way, 1969; Thenot & Horning, 1972; Clarke & Foltz, 1974) using, for example, hexamethyldisilazane in the presence of a catalyst, eg pyridine. The method of detection can vary depending on the derivative. Halogenated derivatives may be suitable for electron capture detection. Flame ionisation detection and nitrogen/phosphorus detection are suitable for all derivatives but may be too insensitive for the measurement of nanogram quantities. The mass spectrometer couples readily to a gas chromatograph and GCMS is the method of choice when absolute identification is required (Mcnair and Bonelli, 1969). Selective ion monitoring can be used to increase sensitivity (Jones et al., 1984; McFadden, 1973). The limited availability of these instruments due to their high cost makes GCMS procedures of less general interest. Furthermore, derivatisation procedures must be carefully selected, can be time consuming, and can involve the use of toxic reagents. The derivatisation step can also lead to sample loss and poor reproducibility.

2.2.6. High Pressure Liquid Chromatography

The wide range of columns, solvent systems and detectors available in modern liquid chromatography makes it a suitable technique for the analysis of most compounds, particularly those which are highly polar, non volatile or unstable at high

temperatures. An adsorption extraction method for morphine from biological fluids was published in 1970 (Fujimoto et al, 1970) using XAD-2 resin and eluting with organic solvents. The first High Pressure Liquid Chromatographic (HPLC) method for the separation of morphine from diacetyl morphine, and 6-moncacetyl morphine (Cashman and Thornton, 1972) used an alumina column with an ultra violet (UV) spectrophotometry detection system (254nm), and would separate the three compounds from each other but with poor efficiency and not without interference from common adulterants like procaine. An HPLC method for the determination of opiates in opium (Wittner, 1973) used an ion-exchange column, again with UV detection (254nm). The chromatography was much improved on previous reports, but gradient elution was required.

A report in 1975 (Trinler and Reuland, 1975) reviewed methods for the measurement of opiates by HPLC. It also described the first separation on reversed phase material (C_{18} corasil) with an isocratic solvent system, of morphine in street drug samples. Again UV detection (254nm) was used.

The first sensitive method for the determination of morphine in blood appeared in 1975 and used a porous silica column (Jane and Taylor 1975). Morphine was derivatised to the fluorescent dimer pseudomorphine which was then chromatographed and monitored by fluorescence detection at 400nm. Compounds whose fluorescent oxidation products may have given false positives are distinguished from morphine by their relative retention times. The inclusion of an internal standard improves the quality of this assay. The overall sensitivity is however dependent on the condition of the sample and the presence of co-extractants. A

mixed dimer of the internal standard and morphine will also lead to a decrease in sensitivity.

The prototype electrochemical detector (Kissinger et al., 1973) was noted to be most suited to the analaysis of phenols, thiols and primary amines. The first application of electrochemical detection (ED) to morphine analysis was reported by White (1979) using a silica column system and a purpose built detector and cell. Morphine from blood extracts was oxidised at a potential of 0.6 volts as it left the column. The absolute sensitivity of the method was reported as less than 1ng.

The reaction taking place is assumed to be the electrochemical oxidation of morphine to pseudomorphine, and is shown in figure 2.8. (Proksa & Molnar 1978). This is the same reaction accomplished by the use of potassium ferricyanide in fluorescence methods described above (2.2.3.).

This demonstrated the potential use of electrochemical detection in the sensitive determination of morphine in biological samples without derivatisation. It was of simple design and relatively low cost (the detector cost £100 to build).

A study in 1982 of morphine concentrations in the blood and cerebrospinal fluid (Todd et al., 1982), used a reversed phase system (μbondapak C18). A sensitivity of 20pg on column was claimed for this method, which makes it as sensitive as GC-MS-SIM methods reported elsewhere (Jones et al., 1984). Two subsequent studies on morphine used HPLC with electrochemical detection (LCED) to examine the pharmacokinetics of morphine in hospital patients (Vandenberghe et al 1982, Moore et al., 1984(ii)). Both these workers found it neccessary to use ion-pairing agents to

attain efficient chromatography of morphine. The detection limits reported were 3ng/ml and 0.3ng/ml respectively.

Other reports have been made where sensitive detection using UV has been claimed (Svenson et al., 1982, Uges 1984), including in the former case the identification of several conjugated metabolites and the tentative identification of the morphine-6-glucuronide. UV detection is essential for the measurement of metabolites as it is believed that the major metabolite morphine-3-glucuronide cannot be detected electrochemically, being conjugated through the 3-OH.

One report (Svenson et al., 1982) also demonstrates another advantage of HPLC over GC, namely the chromatography of conjugated glucuronide metabolites without the need for hydrolysis and subsequent derivatisation prior to analysis.

2.2.7. Conclusions

Of the quantitative methods described above for the analysis of morphine, GCMS is the method of choice, in terms of sensitivity and absolute identification of the drug. Sensitivity is further enhanced by the use of selective ion monitoring. This method, in use until recently in this department, was found to be sensitive, but occasional problems were encountered.

These included interference from a column bleed peak of the same mass as the base peak of the morphine derivative, and the presence of silicones in the eluent presumably from the septum (Anderson 1986).

The increasing demand for morphine analysis has prompted the development of a complimentary technique which can be used

selectively to measure morphine in a suitable extract.

Other gas chromatographic methods with flame ionisation detection (FID) and nitrogen/phosphorus detection (NPD) had been examined as alternatives.

Electron capture detection is reputedly more sensitive (McFadden, 1973) and this has been investigated, although problems were encountered with excess fluorinated derivatising agent in the prepared sample contaminating the detector and causing interference (Anderson, 1986).

The use of radioimmunoassay was considered. Minimal sample preparation and high sensitivity makes RIA a useful screening procedure for opiates, where large numbers of samples require analysis, as is the case with samples taken under the Road Traffic Act. In spite of the high quality of antibodies currently available for morphine, the still lack absolute specificity, particularly in their cross reaction with codeine and dihydrocodeine, confirmation of the presence of morphine following a positive RIA result must be made by independent means.

The many degrees of freedom available from HPLC make it suitable for the analysis of most compounds (Pryde and Gilbert, 1979; Lawrence 1981). The use of HPLC for the analysis of morphine has the advantage that the drug does not require derivatisation prior to analysis. HPLC has not been used extensively for morphine in biological fluids in the past due to the lack of sensitivity of the detectors available. Modern UV detectors and the development of electrochemical detectors hasnow made HPLC more popular for this application.

Reports in the literature, cited above, prompted the investigation

of LC as a suitable routine method for morphine analysis.

As sensitive procedures using both UV and electrochemical detection have been described, these techniques merit further comparative study.

2.3. SAMPLE PREPARATION

2.3.1. Direct analysis

The direct analysis of body fluids and tissues, particularly those of post mortem origin is impractical. These samples usually require some form of clean-up or preconcentration before chromatographic methods can be applied. Radioimmunoassay can be performed on unprepared samples, but a simple extraction into methanol is usually carried out to eliminate interference from the matrix and to increase sensitivity.

2.3.2. Protein precipitation, ultrafiltration and centrifugation

Following protein precipitation with tungstic acid, analysis of morphine in the supernatant has been reported by TLC and paper chromatography (Curry, 1969).

Whilst this will eliminate some interference from the matrix, protein precipitation does not concentrate the sample. Simple protein precipitation, ultrafiltration and centrifugation methods were considered unsuitable for this study, because of the complexity of the sample, and the need to concentrate rather than dilute the sample. This is important due to the low concentrations of morphine normally encountered in blood samples. Protein precipitation has been shown to be successful

where the analyte is encountered in microgram, rather than nanogram, quantities (Black and Sprague, 1978).

2.3.3. Liquid/Liquid extraction.

Liquid/liquid partition extraction is probably the most popular and widely used method of sample preparation available in the laboratory. By correct choice of the buffer and extraction solvent, a considerable degree of specificity can be achieved. Morphine is a basic drug with a pKa of 8.21 (Sunshine 1978). Extraction by partition extraction, following adjustment to a suitable pH, is the most popular method in published work to date. Some examples are contained in table 2.2.. A variety of solvents and buffers are cited and most methods require multiple back extractions.

The many problems associated with putrifying tissue are seldom considered in clinical publications. The problem of material co-extracting when higher aliphatic alcohols are present in the extracting solvent has been noted (Horning et al., 1974), but the degree of interference this causes will depend on the method of analysis being used. Liquid/liquid extraction procedures are the usual choice for sample preparation, as they have been well researched and there is a good understanding of the principles involved (Snyder, 1976 (i)). Careful selection of buffer and extraction solvent offer a range of selectivities.

2.3.4. Solid phase extraction techniques.

The principle of solid phase extraction is well known. As applied to the removal of compounds from blood these methods

TABLE 2.2.

SUMMARY OF SOME LIQUID/LIQUID EXTRACTION TECHNIQUES REPORTED TO BE SUITABLE FOR MORPHINE

SOURCE		SAMPLE	SOLVENT	RATIO	BUFFER	STEPS	RECOVERY (%)	METHOD	SENSITIVITY (ng/ml)
Hackett et al	(1975)	aq. Ref.	CH_3Cl	NA	Na_2CO_3	3	lt 10	GC	NG*
Mulé	(1969)	general	$EtCl_2$/IBA	9:1	base	3	NG	GC	NG
Jones et al	(1985)	urine	CH_3Cl/IPA	9:1	NH_4OH/NH_4Cl	3	NG	GCMS	NG
Jane & Taylor	(1975)	urine	CH_3Cl/IPA	9:1	NaOH	3	60-70	LCF	1.2
Kupferberg et al	(1964)	plasma	CH_3Cl/IBA	9:1	Na_2CO_3	1	NG	Fluor.	100
Baselt	(1980)	plasma	EtoAc	NA	NaOH	1	NG	GC-ECD	1
Horning et al	(1974)	plasma	EtoAc	NA	$(NH_4)_2CO_3$	3	90	GC	NG
		plasma	CH_2Cl_2	NA	$(NH_4)_2CO_3$	3	63	GC	NG
		plasma	C_6H_6	NA	$(NH_4)_2CO_3$	3	8	GC	NG
Todd et al	(1982)	plasma/CSF	CH_3Cl/IBA	95:5	Borate pH 8.9	3	78	LCED	1
Vandenberghe et al	(1982)	serum	CH_3Cl	9:1	phosph. pH 8.9	3	NG	LCED	3
Moffat	(1986)	blood/liver	CH_3Cl	NA	NH_4OH	3	NG	UV/TLC	NG
White	(1979)	Blood	EtoAc/IPA	9:1	Borate pH 9	3	81	LCED	NG

* NG = Not Given

fall into two categories, adsorption and absorption.

ADSORPTION extraction relies on the reversible binding of the compound of interest to a solid phase material. Examples of this include charcoal adsorption and XAD-2 extraction procedures. More recently a variety of adsorbent phase materials have been produced commercially. These include polar phases, silica, alumina and silica material with bonded phases like cyanide, phenol and quaternary ammonium functions. There are also some non-polar phases such as C-2, C-8 and C-18. The principle involved is as follows. The analyte, is prepared in a form in which it will absorb to the stationary phase. This may be done by the use of appropriate conditions of solvent, pH and the phase used.

The support is washed to elute extranaeous material and then with a suitable solvent to elute the compound of interest. There are several variations employed. Table 2.3. includes three examples of adsorbent extraction. The reported recoveries are high (84-100%), and extracts have been used for GC, LCUV and LCED. The eluates used are aqueous based however and carry over of the wash into the eluate can arise. Additionally the use of an inappropriate wash solvent might lead to loss of the analyte in the wash step. In spite of the success of the technique with plasma and urine samples, it has not been widely applied to the analysis of whole blood samples as these present their own problems with sample handling.

The use of solid phase adsorption techniques, in theory at least, adds further selectivity to the extraction procedure (Harkey and Stolowitz, 1984), and should make this approach ideal for sample

TABLE 2.3.

SUMMARY OF SOLID PHASE EXTRACTION METHODS REPORTED TO BE SUITABLE FOR MORPHINE

SOURCE		SAMPLE	SUPPORT	BUFFER	WASH SOLVENT	ELUENT	RECOVERY (%)	ANAL. METHOD	SENSITIVITY (ng/ml)
			adsorbent						
Mule	(1974)	urine	XAD-2	borax 0.12M	none	CH_3Cl/IPA	85-100%	GC	NG*
Svenson et al	(1982)	plasma	sep pak C_{18}	NH_3 pH9.3	as buffer	ACN/Phosph.	84	LCUV	5
Moore et al	(1984)	plasma	ODS bond elut	borate pH9	water	methanol	100	LCED	0.28
			absorbent						
Breiter	(1976)	blood	extrelut	pH9.5	none	CH_2Cl_2/IPA	100	GC/TLC	300
Todd et al	(1982)	plasma/CSF	clin elut	borate pH9	none	CH_3Cl/IPA	NG	LCED	1
Fritschi et al	(1985)	urine	extrelut	NG	none	CH_2Cl_2/IPA	NG	GCMSSIM	300
Derks et al	(1985)	urine	extrelut	NH_4Cl	none	CH_3Cl/IPA	NG	LCUV	2

*NG = Not Given

preparation for LC.

Methods for the extraction of whole blood, CSF, plasma and urine have been reported for ABSORPTION extraction. This technique relies on the absorption of one phase (the aqueous phase) onto an absorbent support usually wide pore kieselguhr, or diatomaceous earth (eg. Extrelut (Merck) and Clin-elut (Analytichem). An immiscible solvent with a high solubility for the analyte is then passed down the column. The technique is designed to mimic liquid/liquid extraction but has several advantages. Problems involving phase separation and emulsion formation are avoided. No wash step is required so there is less risk of sample loss. Table 2.3. includes a summary of four absorption procedures which have been used for the extraction of morphine from biological fluids. These extracts have been analysed by LCUV, TLC, and GC. Absorption procedures add no further selectivity to the extraction, but have a high capacity for the stationary aqueous phase, and limit sample breakthrough. It is also compatible with almost all solvents and all pH values.

Both the techniques have the advantage of being modular, reducing the risk of contact with potentially hazardous biological material. They may also improve the reproducibility of the extraction procedure by avoiding emulsion formation. Their main drawback is cost, the adsorbent cartridges cost about £0.50 each, and may be used only once. Absorbent cartridges can be packed in the laboratory more readily and cost around £0.10 each.

An examination of commercially available solid phase extraction techniques was conducted (Stewart et al, 1984). This study showed that for the extraction of basic compounds (procaine , pKa

8.4 was used as a model) from plasma, the most efficient approach was with the use of ODS cartridges, diatomaceous earth absorbent, and XAD-2 resin. These gave extraction efficiencies of 40%, 52% and 61% respectively. Methanol was used as the eluting solvent. Concentrations were measured using UV absorbance, so no indication of the selectivity of the extraction procedure is given. Other workers have examined the suitability of diatomaceous earth and XAD-2 resin for the extraction of several drugs (not including morphine) in post mortem blood (von Meyer et al, 1980). The extracts were examined by GC-FID, and the authors reported that high recoveries were achieved from both procedures, with marginally better recoveries with extrelut, particularly for basic drugs.

Although commercial automated sample preparation units are now available and several references exist for on-column extraction (AASP, WISP, Du Pont Prep), very few of these include applications for whole blood.

2.3.5. Conclusions

For most analyses involving HPLC, a clean-up step or concentration step are required. In general, it is accepted that sample clean up for LC, particularly with sensitive detection systems like electrochemical detection (ED) should be more selective than is required for GC (Dell, 1976). It has also been shown that LC column lifetimes are extended by the use of more rigourous sample clean up (Kabra et al., 1983). This applies particularly to post mortem blood samples, which are among the most complex matrices encountered in toxicology (Oliver, 1984).

For these reasons direct analysis of untreated post mortem blood was not considered.

Liquid/liquid extraction methods offer a wide variety of solvents and the option of multiple extraction steps to provide a clean, selective and efficient extract.

The method currently in use in the department for extracting basic drugs from blood and liver homogenate is based on that described by Horning et al., (1974). The extracts from this procedure had not been examined to assess their suitability for LCED.

Blood specimens which require analysis in the forensic context range from the almost perfect non-haemolysed blood, through grossly haemolysed and coagulated non-pipettable specimens, to putrid dark brown fluids. The presence of putrefactive amines common in post mortem tissue (Kaempe, 1969; Oliver and Smith, 1973; Oliver et al., 1977) may interfere with either the extraction or the analysis.

Solid phase adsorption methods are in increasingly common use in clinical applications for serum or urine analysis (Bond elut applications notes 1985), but very few applications for whole blood samples have been reported.

It was concluded that both absorption and adsorption extraction procedures should also be examined. With extraction procedures requiring several steps, clean up for example, the cost would be greater and some of the advantage of using solid phases lost. A suitable liquid extraction method may be further improved by adapting it to an absorption/adsorption procedure.

2.4. PHYSICAL AND CHEMICAL PROPERTIES OF MORPHINE.

2.4.1. Introduction

Morphine is a plant alkaloid with analgesic and narcotic properties. It occurs naturally in the opium poppy as a free base (pKa 8.21) and has a molecular weight of 285.33. It is used clinically as morphine sulphate pentahydrate (MW 758.76) and for the purposes of this work was used as morphine hydrochloride trihydrate (MW 375.8).

As liquid chromatography (LC) with two forms of detection are to be considered in this study i.e. ultra violet (UV) absorbance and electrochemical detection (ED), the relevant properties of morphine are examined.

2.4.2. UV/VISIBLE SPECTROPHOTOMETRY

2.4.2.1. Introduction

Electrons in bonding and non-bonding orbitals of saturated and unsaturated bonds and delocalised systems can change their energy states by the absorbance of electromagnetic radiation in the ultraviolet and visible regions. The pattern of absorbance is specific to the bonds present in the absorbing compound and different compounds display different absorbance maxima. The UV absorbance spectrum for morphine was measured to determine at which wavelength it would absorb, this could then be used as a selective wavelength for monitoring the eluent in HPLC.

2.4.2.2. Method

A standard solution of morphine (2mg/100ml) was prepared in 0.1N H_2SO_4 and placed in a 1cm quartz cell. The spectrum was scanned from 190 to 340nm. 0.1N H_2SO_4 in a matched cell was used as a blank. The instrument used was a scanning diode array UV/visible spectrophotometer (Hewlett Packard 8451A). The bandwidth was 2nm.

2.4.2.3. Results and discussion

The spectrum obtained is shown in figure 2.4.. Absorbance maxima were found at 212nm and 286nm. The extinction coefficients (A_1^1) were calculated and are shown in table 2.4..

The selective detection of small amounts of morphine is difficult, as it does not have a strong chromophore other than the ubiquitous aliphatic sigma-sigma* and aromatic pi-pi* transitions between 150 and 220nm (Williams and Fleming, 1980). As this is the region in which many solvents used in HPLC also absorb (Snyder and Kirkland, 1978) it is inaccessible on many instruments, and the sensitivity can be poor.

The other absorbance at 285nm is due to the transitions of delocalised electrons in the aromatic system. This is more specific and accessible with most spectrphotometers, however the extinction coefficient is low (A_1^1 = 60) which will limit the sensitivity.

2.4.2.4. Conclusion

The concentration of free morphine found in blood is low even in overdose cases (see 2.1.3.). In order to use UV

TABLE 2.4.

UV ABSORBANCE DATA FOR MORPHINE SOLUTION (2mg/100ml)

wavelength	Absorbance	A^{1}_{1}
220	1.359	680
286	0.119	60

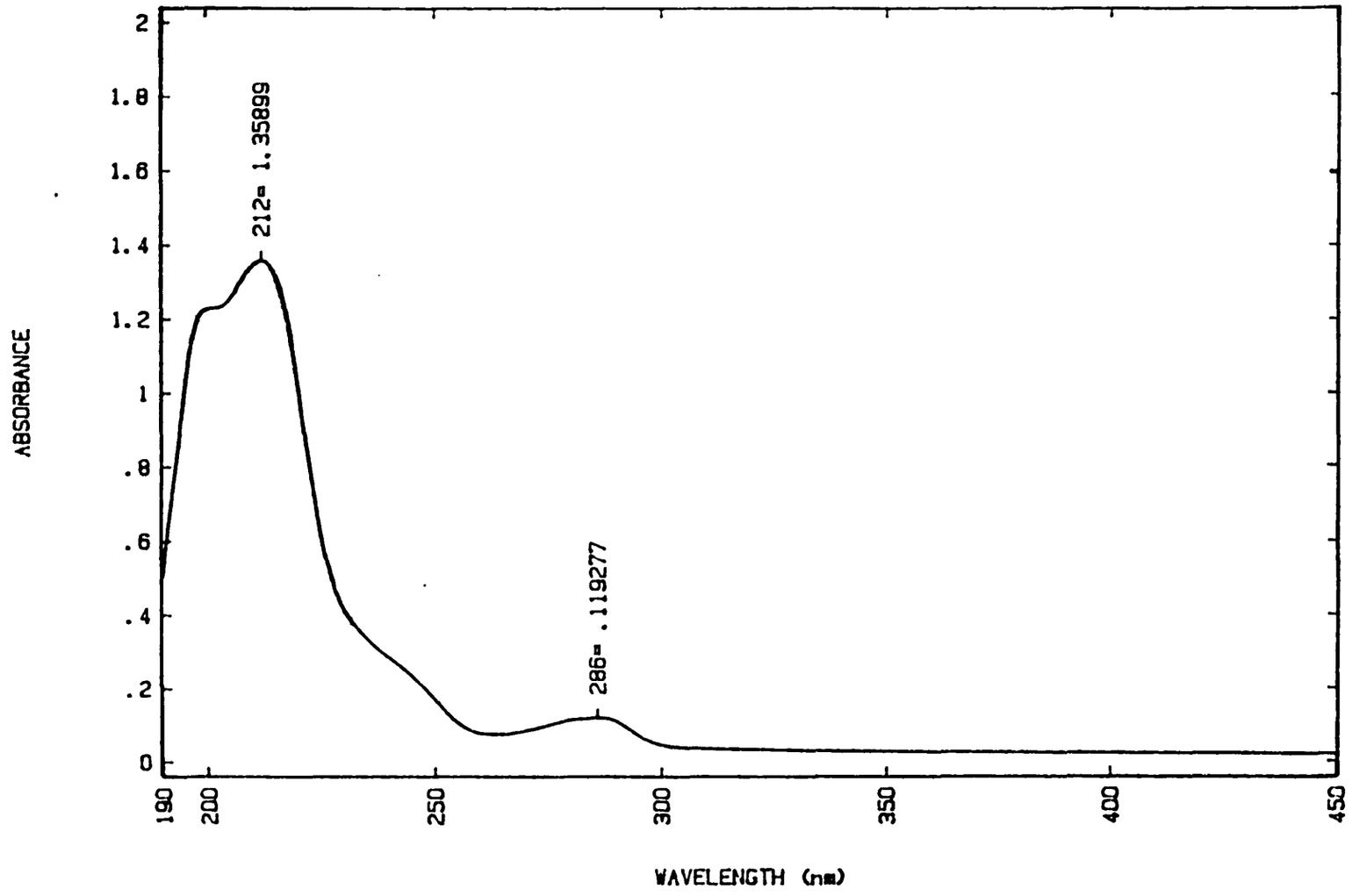


FIGURE 2.4.

UV/VISIBLE ABSORBANCE SPECTRUM FOR MORPHINE (2mg/100ml) IN 0.1N H_2SO_4

detection for the measurement of morphine in blood, it would probably be necessary to monitor at 212nm and larger sample volumes may be required.

2.4.3. ELECTROCHEMICAL PROPERTIES

2.4.3.1. Introduction

To monitor compounds electrochemically, sufficient potential difference must be applied to perform a reaction, which results in the uptake or production of electrons.

The applied potential should not however be so high as to produce a strong background signal from the mobile phase. The optimum level can be ascertained by performing hydrodynamic voltammetry (HDV) under the chromatographic conditions to be used in the assay. Initially, as the best conditions for chromatography had not been developed, cyclic voltammetry (CV) was used to determine a value for the E_p and $E_{p/2}$ for morphine to allow the choice of a suitable detector potential. Both these techniques are discussed in chapter one.

2.4.3.2. Design of electrochemical cell - Method

The cell design shown in figure 2.5.(a) was constructed on the principle of classical cyclic votammetric cells. The electrodes consisted of platinum wire (22ga., 7cm), coiled to allow the greatest surface area in a small volume. These were embedded in epoxy resin. Connections to the voltage generator were led from the bottom of the cell.

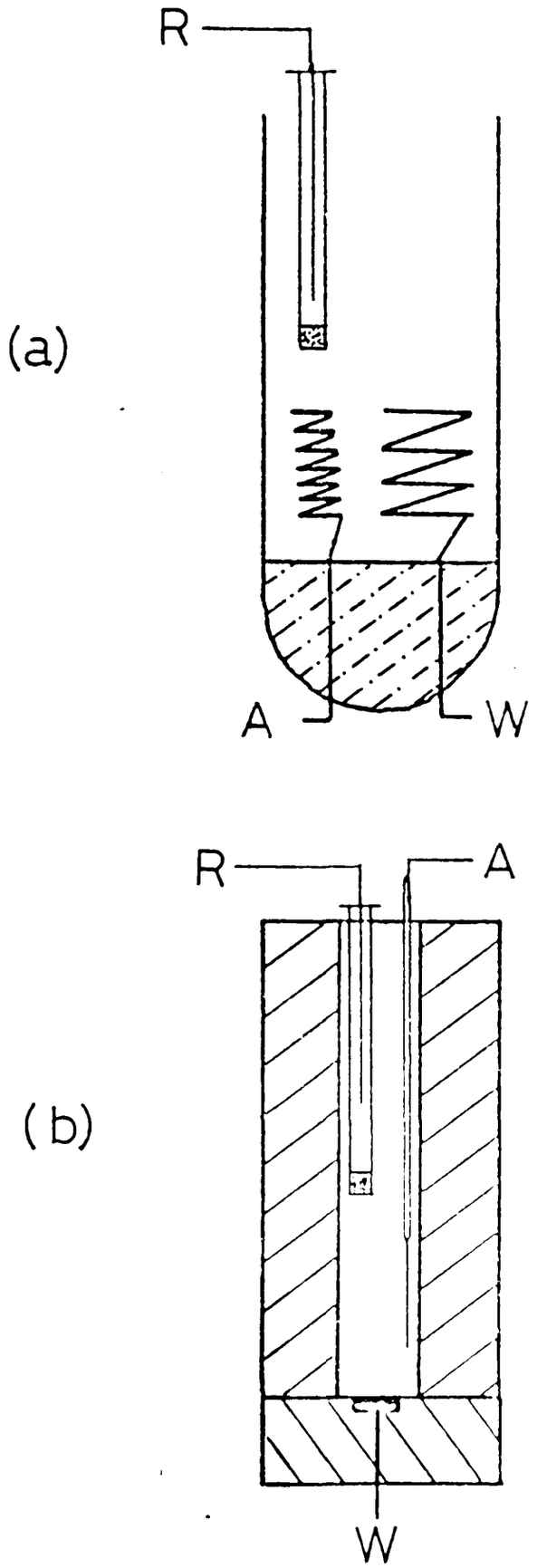


FIGURE 2.5.

CELL DESIGNS USED FOR CYCLIC VOLTAMMETRY

The control unit was a BAS CV-1A (Anachem, Luton, U.K.) instrument which provided the changing potential and also amplified the resulting current from the cell and produced an analog voltage for the chart recorder.

A Ag/AgCl (SCE) reference electrode (BAS) with E^{o}=217mV vs standard hydrogen electrode (SHE), was dipped into the open top of the glass tube. Background scans were performed on a variety of solutions under different conditions of scan rate.

2.4.3.3. Results and discussion

The cell gave a suitable scan window (±2.0volts) in aqueous solutions and buffers, however in the presence of acetonitrile or methanol, anomalous results were obtained on repeat scans. This appeared to be due to softening of the epoxy resin by these solvents. Reaction of the resin components or their depositing on the electrode surfaces may account for this behaviour.

2.4.3.4. Conclusion

The inconsistent results obtained and the obvious incompatability of the cell with some solvents often used in HPLC and CV, meant that no further work could be done with this cell. An alternative was sought.

2.4.4. MODIFICATION OF CELL DESIGN

2.4.4.1. Introduction

Following the incompatability of epoxy resin an alternative cell design was chosen. Anderson, (1981), noted that the most reliable methods of determining a working potential (WP) for LCEC by CV was to use the same electrode materials and conditions in the CV cell as were found in the HPLC cell.
A second cell design (figure 2.5.(b)) was considered based more closely on the flow cell used in the BAS-TL-5A LC detector cell.

2.4.4.2. Method

The body was made from a PTFE block which was found to be inert to methanol and acetonitrile. This was bored through the centre with a 10mm dia. hole and attached with four 1/2 inch self-tapping screws to a 5mm dia. glassy carbon electrode mounted in KEL-F polymer (BAS TL-5A). This was used as it is reported that electrochemical mechanisms are affected by the nature of the working electrode material (Albery and Hugget, 1984). A Ag/AgCl (SCE) reference electrode (BAS) with E^{o}=217mV vs standard hydrogen electrode (SHE), was dipped into the top of the PTFE body. A piece of platinum wire (22ga) was sealed in glass with the bottom 15mm exposed and used as the auxillary electrode.
The unit was sealed with a neoprene top to prevent atmospheric contamination, or temperature fluctuations.
Background scans were run in a series of buffer and acetonitrile or methanol containing solvents. A scan rate of 10mV/sec and sensitivity of 2 and 20 uA/V were used required (Plambeck, 1982; Anderson 1981).

2.4.4.3. Results and discussion

Good reproducibility was obtained for repetitive scanning, and a window of ± 1.5 volts was found for all the solvent systems tested. The cell appeared to be inert to all the solvents used. The voltage window for acetonitrile itself was ± 2.5 volts, but for the purposes of LCEC where practical limits are less than 1.2 volts, the switching potentials were set at around ± 1.5 volts.

2.4.4.4. Conclusion

The second cell design is more suitable for examining the electrochemical proporties of drugs, as it is constructed of the same materials as the cell used in the electrochemical detector, and is inert under test conditions. This cell was therefore used for all further CV experiments.

2.4.5. CYCLIC VOLTAMMETRY OF MORPHINE

2.4.5.1. Introduction

Morphine has electrochemical behaviour which has been examined both reductively (Kirkpatrick, 1947) and oxidatively (Proksa and Molnar 1978). The most suitable solvent for the conduction of a cyclic voltammetry (CV) experiment is highly purified and dried acetonitrile, containing an inert electrolyte such as tetrabutyl ammonium tetrafluoroborate (Evans et al., 1983), acting as a charge carrier. This is essential where the mechanism of the reaction is being studied and reaction with water

or ions from the electrolyte might interfere. This solvent also gives a window with a low background current within the limits -3.5 to +1.8 volts (Billon et al., 1982). In HPLC, potentials outwith ±1.5 volts are seldom of interest, as the associated background current at these values is too high to allow sensitive measurements.

2.4.5.2. Method

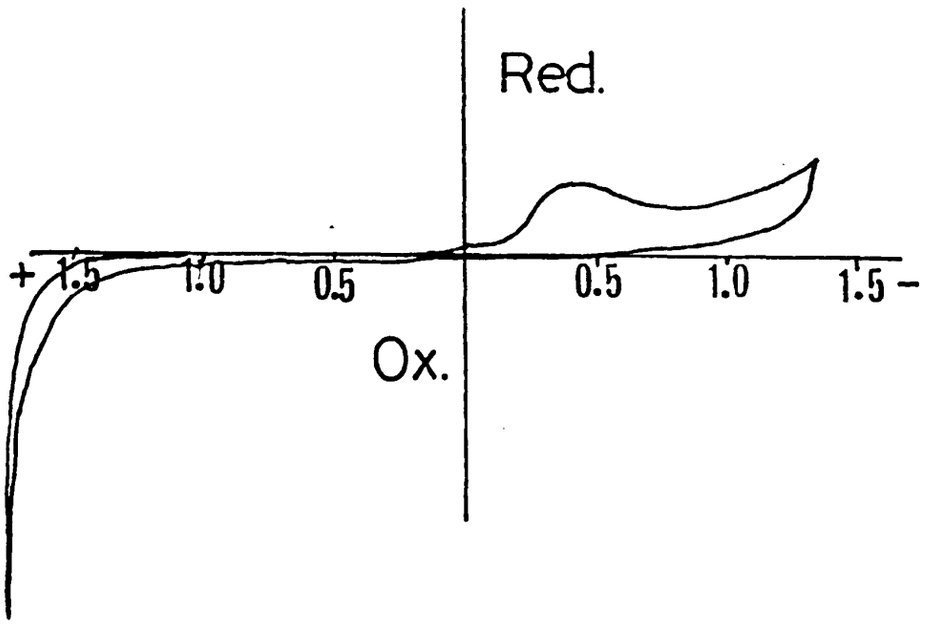
The solvent used, acetonitrile, 8%, in 0.01M KH_2PO_4, at pH 4.6, was based on the results of initial chromatography which had been developed (see 2.5.2.).

A blank run was performed using a solvent containing no morphine. This was scanned in single fast scan mode, oxidation-reduction, at a rate of 10mV/sec, and sensitivities of 20 and 200 uA/V were used.

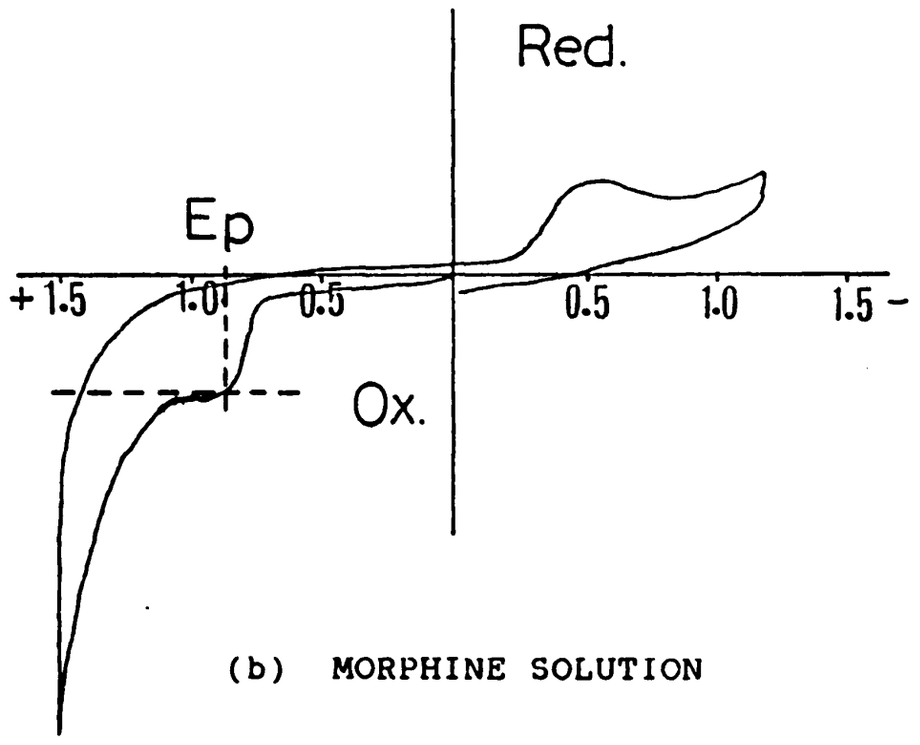
This was then replaced with a solution of morphine (10^{-3}M) in the same solvent, and scanned under the same conditions.

2.4.5.3. Results and discussion

The voltammograms for the blank solvent and the morphine containing solvent are shown in figure 2.6.. The E_p and $E_{p/2}$ values are +0.87V and +0.76V vs Ag/AgCl, respectively. This is in reasonable agreement with other reported values, noted in table 2.5.. Information on the peak current is also included but this is of little practical use for this application, as the steady state conditions in the CV cell differ greatly from the turbulent dynamic conditions found in the LCEC flow cell.



(a) BLANK SOLUTION



(b) MORPHINE SOLUTION

FIGURE 2.6.

CYCLIC VOLTAMMOGRAMS OF BLANK SOLUTION AND MORPHINE CONTAINING SOLUTION

TABLE 2.5.

COMPARISON OF RESULTS WITH

ELECTROCHEMICAL DATA FOR MORPHINE REPORTED ELSEWHERE

Author	method	E_p	$E_{p/2}$	WP
This work	CV	0.87	0.76	0.87
White, 1979	HDV	0.83	0.5	0.6
Moore et al., 1984	-	-	-	1.0
Vandenberghe et al., 1983	HDV	0.70	0.43	0.6
Todd et al., 1982	CV	0.6-0.8	-	0.79
Law, 1984(iii)	HDV	no plateau		0.85

2.4.5.4. Conclusion

Cyclic voltammetry performed under the conditions described appears to be a good method for screening compounds for electrochemical activity, and determining a reliable value for the E_p and $E_{p/2}$ values. This allows a reliable estimate of a suitable working potential for LCEC.

2.4.6. THE EFFECT OF pH ON ELECTROCHEMICAL RESPONSE OF MORPHINE

2.4.6.1. Introduction

Factors such as temperature, supporting electrolyte and pH may have an effect on the activation energy, rate and mechanism of the reaction (Proksa and Molnar 1982).

The temperature is generally ambient for HPLC separations and was not investigated. The effect of the pH which is important in the development of a suitable chromatographic system is investigated below.

2.4.6.2. Method

A series of solvents (8% acetonitrile in 0.01MKH_2PO_4) were prepared and the pH adjusted either with concentrated phosphoric acid or 60% KOH solution as required. Oxidation-reduction cyclic voltammetry was performed as before. The electrode was polished between experiments.

2.4.6.3. Results and discussion

The effect of altering the pH on the E_p value and on the response is shown in figure 2.7. and table 2.6..

As the pH decreases, the E_p and $E_{p/2}$ values increase, idicating that the reaction requires a higher activation energy to proceed.

The peak current is not signifigantly changed between experiments. This suggests that the peak current is limited by the mass transport of the analyte to the electrode surface and diffusion through the helmholtz layer.

For a given analyte the peak current will be a function of the electron transfer rate, electrode surface area and cell design (Anderson et. al. 1981; Evans et al., 1983). As the formation of a helmholtz layer requires unperturbed conditions (Crow, 1980), different behaviour may be found in hydrodynamic voltammetry, where the surface of the electrode is constantly being washed by the eluting solvent. In the case of HDV the limiting current is a function of mass transport to the electrode surface which in turn is a function of the flow rate.

A study of the usefulness of CV in estimating $E_{app.}$ (Anderson et al., 1981) showed that, in general, there was good agreement between E_p (CV) and $E_{plateau}$ (HDV) values. The study also confirms that both E_p and $E_{plateau}$ depend to some extent on the solution pH and the solution composition.

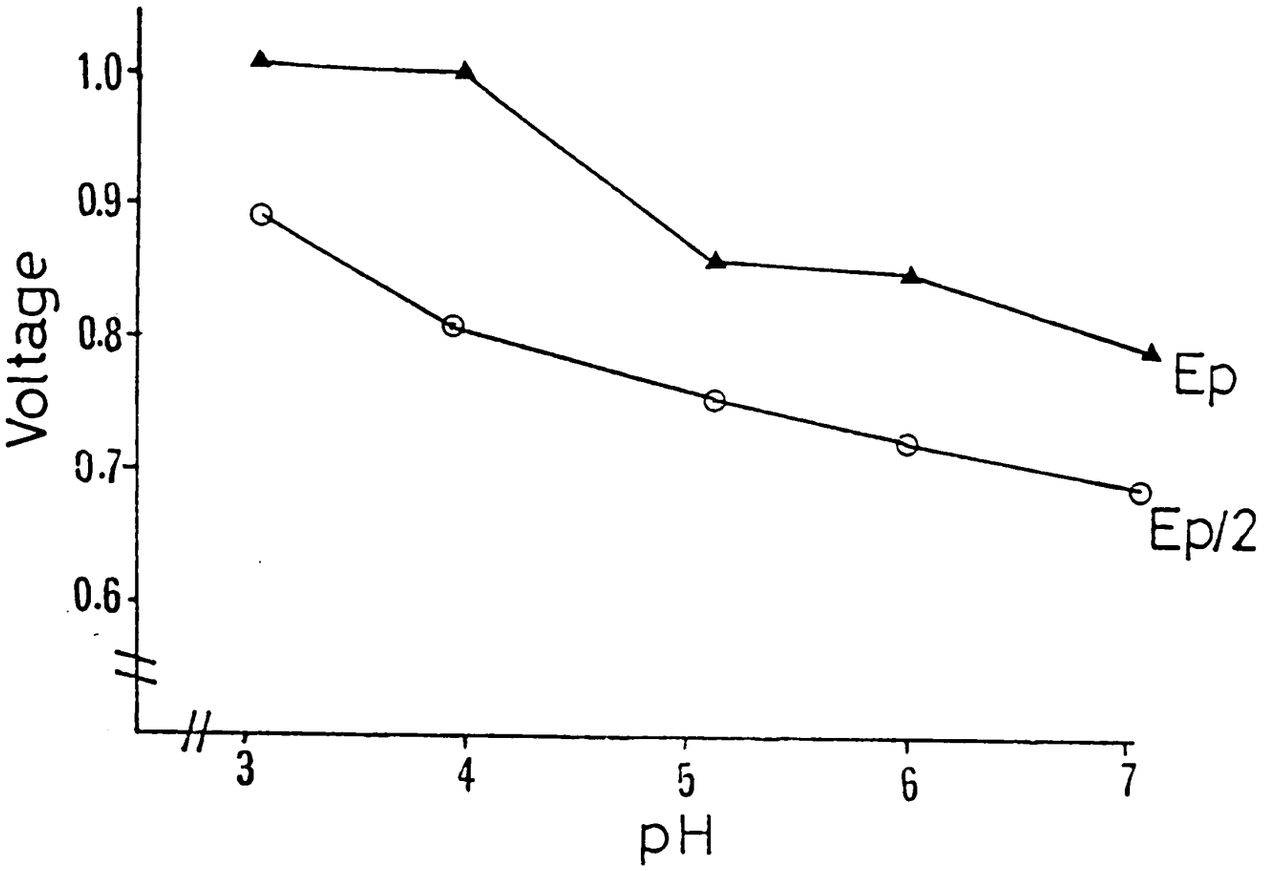


FIGURE 2.7.

VARIATION OF FULL AND HALF WAVE POTENTIALS FOR MORPHINE WITH pH OF SUPPORT ELECTROLYTE

TABLE 2.6.

EFFECT OF pH OF SUPPORT ELECTROLYTE ON THE FULL WAVE (E_p) AND HALF WAVE ($E_{p/2}$) POTENTIALS OF MORPHINE

pH	$E_{p/2}$	E_p	I_p(uA)
3.1	0.89	1.05	3.4
3.95	0.81	1.05	3.2
5.2	0.76	0.86	3.3
6.0	0.72	0.84	3.2
7.1	0.69	0.80	3.5

2.4.6.4. Conclusion

Care is required when selecting the applied potential of constant potential amperometric detectors for LC applications. To maximise analytical response, and minimise background interference, the applied potential should be held at the minimum value at which current reaches the limiting current plateau of the analyte. Cyclic voltammetry has been shown to be a suitable method for the determination of electrochemical activity. The $E_{p/2}$ value obtained for morphine is in good agreement with other reported values. The working potential chosen for initial LCED on the basis of this experiment was 0.870 volts.

It was decided to use this CV procedure to determine the electrochemical behaviour of other compounds to assess their suitability as internal standards or potential interferences in an LCED assay for morphine.

2.4.7. CYCLIC VOLTAMMETRY OF OTHER NARCOTIC ANALGESICS

2.4.7.1. Introduction

A selection of narcotic analgesic drugs were examined by cyclic voltammetry in order to assess any likely interference from other drugs at the chosen working potential of around 0.870 volts, and to examine any structure activity relationship between the compounds and their EC properties.

TABLE 2.7.

FULL WAVE AND HALF WAVE POTENTIALS RECORDED FOR A SELECTION OF NARCOTIC ANALGESICS

compound	E_p	$E_{p/2}$
morphine	0.87	0.76
nalorphine	0.84	0.73
papaverine	1.94	1.60
pentazocine	1.10	0.95
codeine	1.09	0.96
oxycodone	1.03	0.90
diamorphine	1.41	1.19
levorphanol	1.00	0.84

2.4.7.2. Method

The cell and scanning conditions were as above (2.4.5.2). Solutions containing the compounds of interest were prepared (10^{-3}M) in the support solution and the voltammograms recorded.

2.4.7.3. Results and Discussion

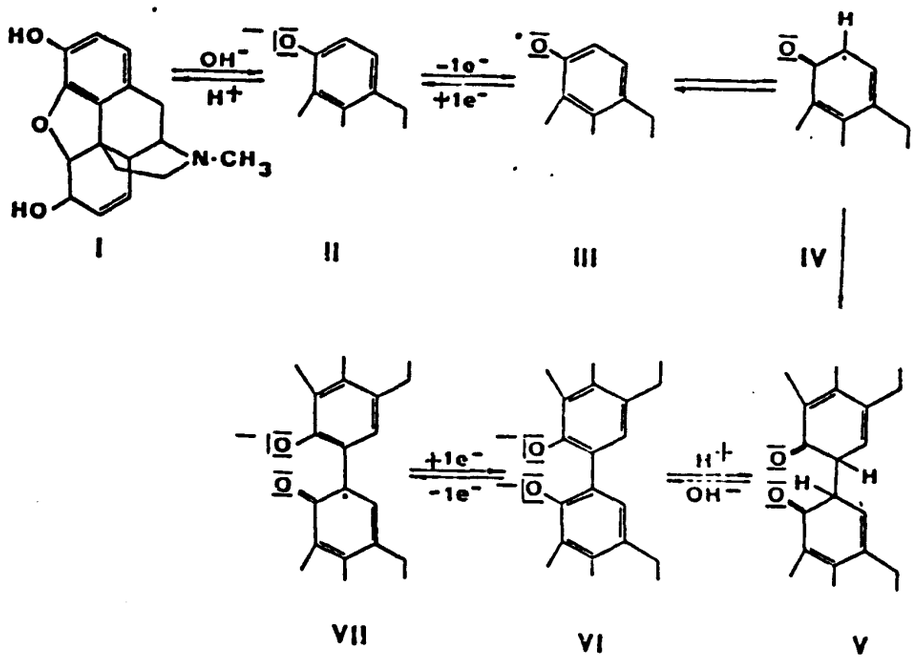
E_p values are recorded in table 2.7.. The structures are shown in figures 2.9. to 2.11..

The mechanism proposed for the electrochemical oxidation of morphine is a phenolic oxidative coupling (see figure 2.8.) (Proksa and Molnar, 1976).

This involves the initial loss of a proton, a one electron oxidation to a phenoxonium radical and subsequent dimerisation to pseudomorphine. This is a reaction of the EC type (Plambeck, 1982) representing an electrochemical step (E) followed by an intermolecular chemical step (C).

There is no firm agreement as to whether a reaction need go to completion in order for that reactant to be detected electrochemically. Some authors suggest that the formation of a short lived radical is all that is required (Wilson, 1984). There are no reports in the literature of isolation of the EC product following amperometric detection.

The reaction mechanism for morphine shown in figure 2.8., pertains at pH 13.3 where the phenoxide ion is readily formed, assisting with the formation of the radical. The reported E_p value is 0.3 volts, however the authors acknowledge that the reaction mechanism in acidic media differs (Proksa and Molnar, 1976), although they do not specify how. An E_p value of 0.44 volts



from Proska and Molnar, 1978

FIGURE 2.8.

REACTION SCHEME FOR PHENOLIC OXIDATIVE COUPLING IN ALKALINE SOLUTION

has been reported for morphine (White, 1979). This was also in alkaline media (pH 11.2).

The E_p values found in this study were higher than this, but all were measured in acidic media. It was shown in the section above, (table 2.6.) that the E_p value for morphine was dependent on the pH of the support sclution, and that higher potentials were required in acidic media.

This observation would support phenoxide ion formation as the rate determining step (RDS) for the electrochemical oxidation of morphine and its analog nalorphine (figure 2.10.), as the phenoxide ion will be less likely to form in acidic media.

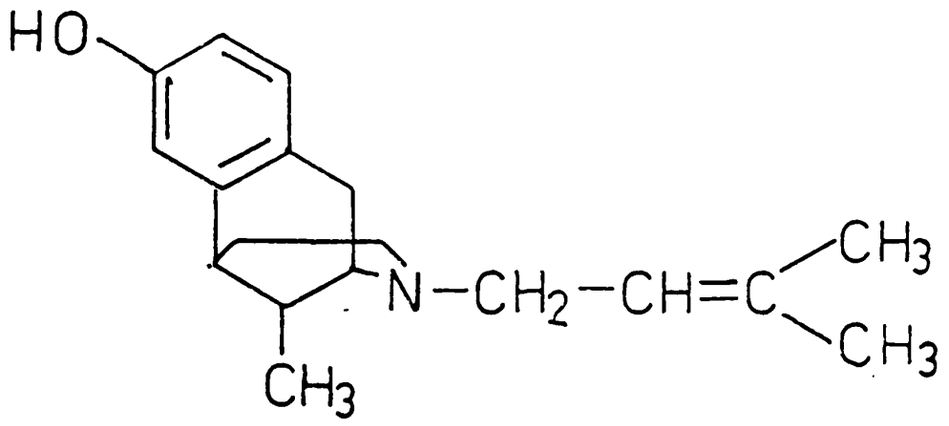
The analgesics whose electrochemical behaviour was examined here are discussed below in terms of their structure.

Deoxycatechols

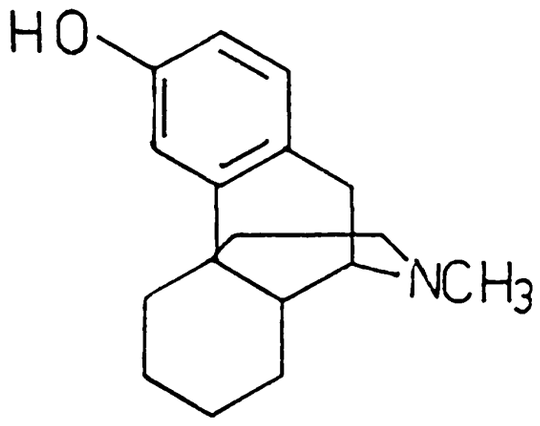
Compounds in this group (figure 2.9.) have a single free phenol and hence phenoxide ion formation is a likely RDS. It has been suggested that phenolic oxidative coupling would only occur if the phenol function was part of a catechol group (Petersen et al, 1980). The measurement of E_p values of 1.0 and 1.1 volts for levorphanol and pentazocine respectively discounts this contention as both are deoxycatechols, having only one phenolic hydroxy residue.

Catechols

Compounds in this group (figure 2.10.) have one free hydroxyl in addition to the second ortho oxygen and therefore phenoxide ion formation is also a likely RDS. As the E_p values



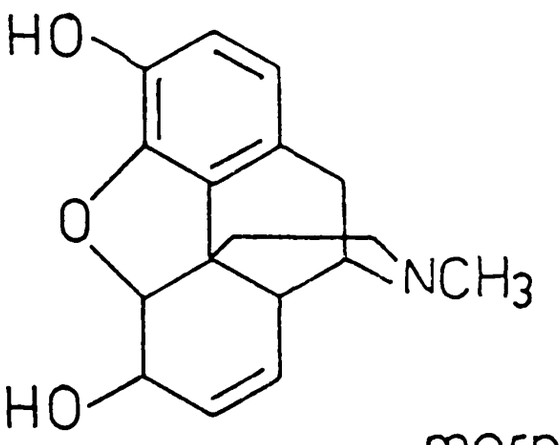
pentazocine



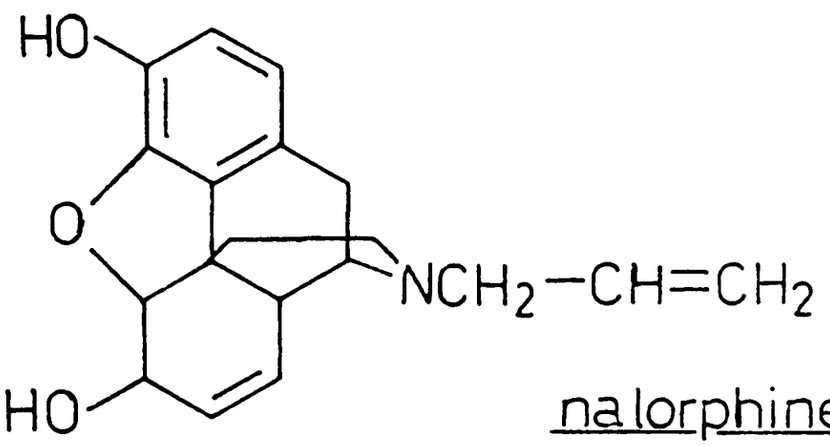
levorphanol

FIGURE 2.9.

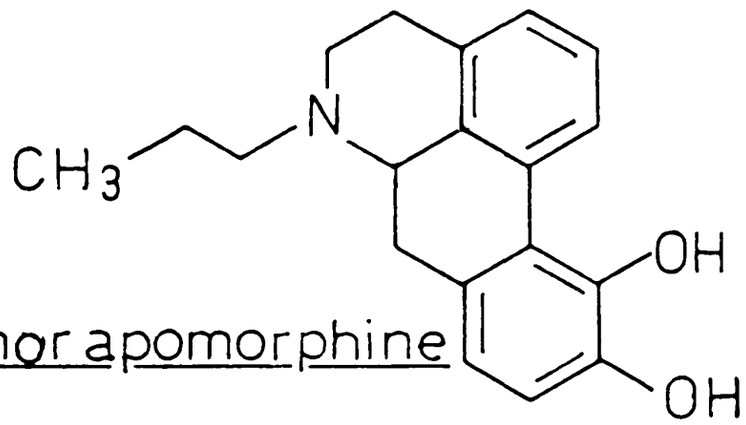
STRUCTURES OF TWO NARCOTIC ANALGESICS
CONTAINING DEOXY-CATECHOL FUNCTION (PHENOL)



morphine



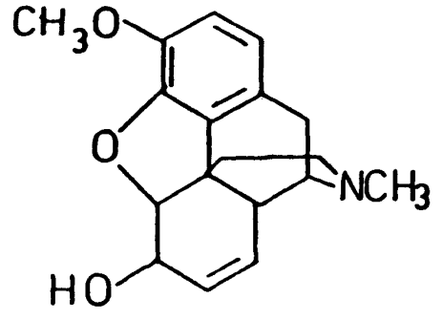
nalorphine



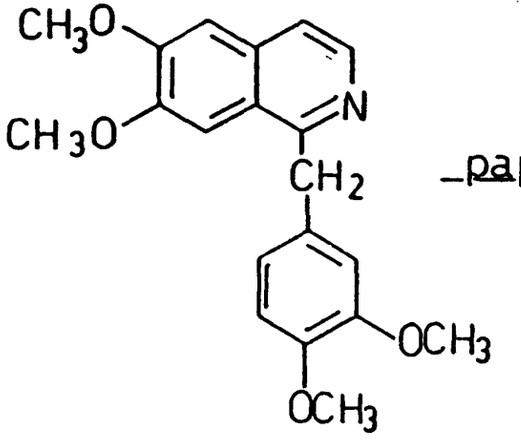
N-1-propyl norapomorphine

FIGURE 2.10.

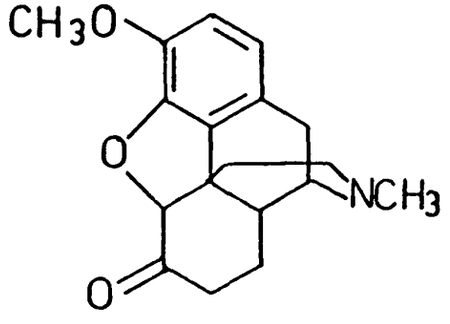
STRUCTURES OF THREE NARCOTIC ANALGESICS CONTAINING FULL CATECHOL FUNCTION



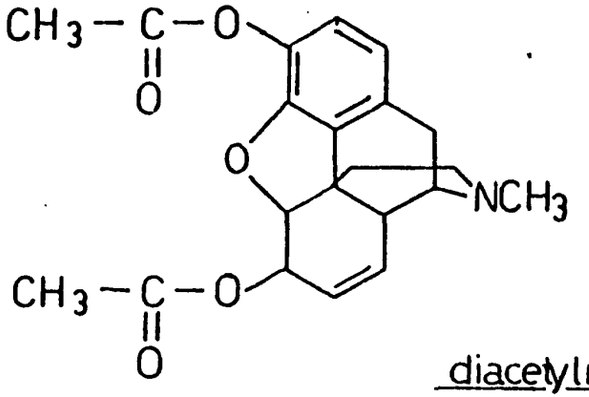
codeine



papaverine



oxycodone



diacetylmorphine

FIGURE 2.11.

STRUCTURES OF FOUR NARCOTIC ANALGESICS CONJUGATED THROUGH BOTH CATECHOL HYDROXYL GROUPS

for morphine and nalorphine (both catechols, although conjugated through one hydroxyl) are lower than for the deoxycatechols discussed above, the presence of the second oxygen may stabilise the radical as shown in figure 2.12.. This increased stability will assist in radical formation.

Assuming this to be the case, full catechols would be expected to have lower E_p values. This is indeed found to be so. The reported E_p value of 0.5 volts for N-1-propylnorapomorphine (Wilson, 1984) which has the structure shown in figure 2.10., containing an ortho biphenyl (catechol) is lower than that for either morphine or nalorphine. This is further confirmed by the report of the E_p for apomorphine itself as 0.52 volts (Bianchi and Landi, 1985). Other catechols including dopamine, 5-hydroxytryptamine, norepinephrine also have E_p values below 0.5 volts (Ishikawa et. al., 1982)

Bi-conjugated catechols

This group of compounds (figure 2.11.) includes codeine, oxycodone and diacetylmorphine. Codeine and oxycodone are both methyl ethers at one hydroxyl function. They are also conjugated through the second hydroxyl as in morphine. Formation of a phenoxide ion and the subsequent radical is therefore unlikely because of the energy required to break the CH_3-O bond.

An alternative mechanism is suggested.

The mechanism proposed for the reaction of these compounds is based on the reported electrochemical behaviour of anisyl alcohol (Lund 1957), where the ability of phenoxy ethers to form radicals, without involving initial phenoxide ion formation, was shown.

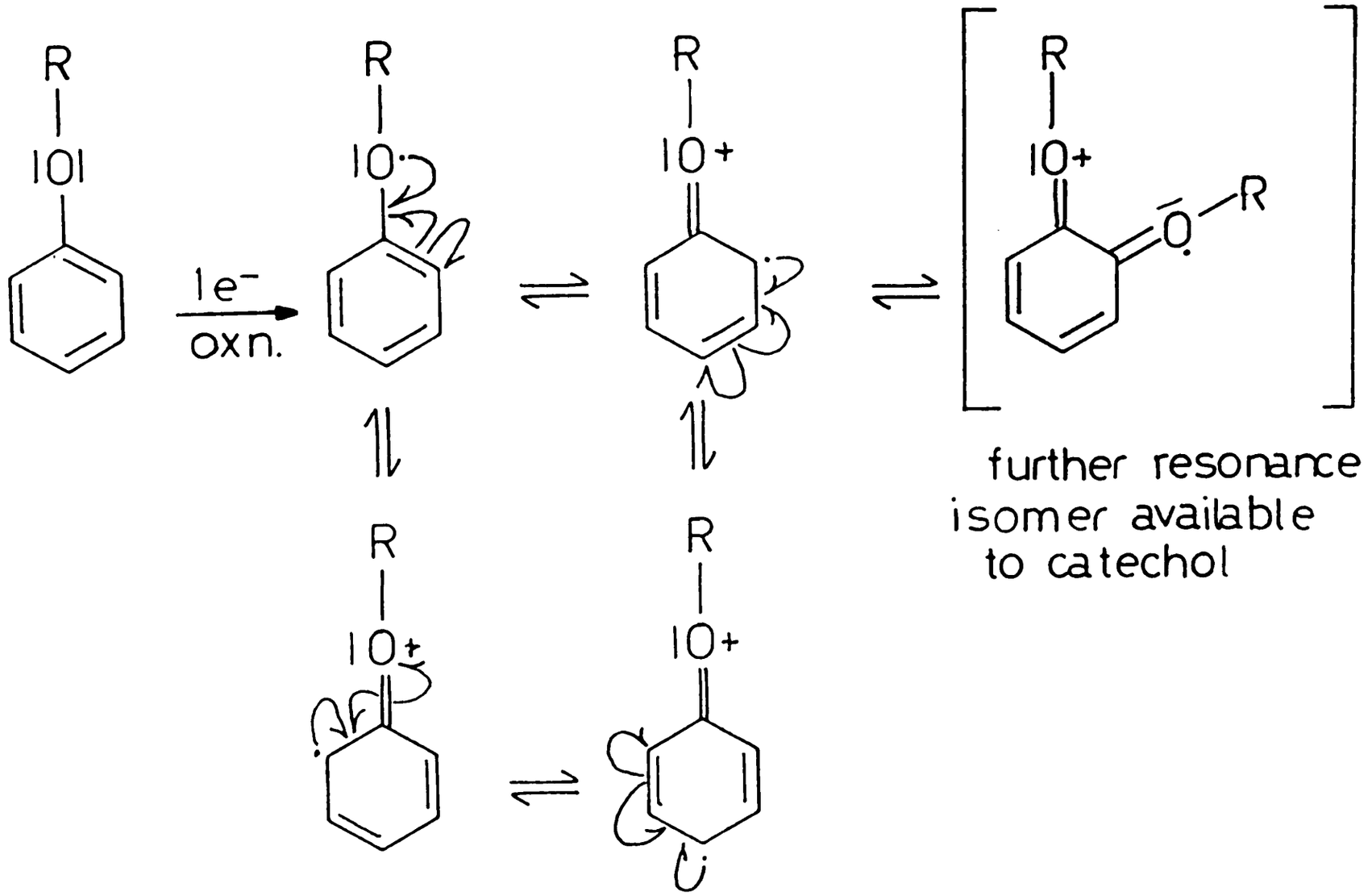


FIGURE 2.12.

DELOCALISATION FOR RADICAL RESULTING FROM LOSS OF ONE ELECTRON FROM THE AROMATIC SYSTEM OF A PHENYL ETHER

It was suggested there that the primary step in the reaction was the loss of an electron from the aromatic system.

Consideration of the electrons involved and the mechanism shown for POC (figure 2.8.), suggests that initial electron abstraction from the aromatic oxygen is most likely, as shown in figure 2.12.. The presence of a catechol system will make this radical more stable by the existance of an additional resonance isomer (figure 2.12.). This increased stability will make its formation easier. Any subsequent reaction of this radical is unlikely, as it would involve breaking a $C-O^+$ bond. This observation could support the contention that the formation of a radical is sufficient for electrochemical detection.

In the case of diacetylmorphine and papaverine, the second phenoxy function is an ester and an ether respectively. The same anisyl alcohol mechanism can be invoked here. E_p values have been reported for this compound (Musch et al., 1985; Wilson, 1984) although no mechanism has previously been proposed. The E_p value for diacetylmorphine is lower than for codeine or oxycodone. The additional delocalisation through the acetyl group available to diacetylmorphine may account for this.

Further chemical reaction is also possible in the case of diacetylmorphine, as subsequent acid catalysed hydrolysis of the ester may take place.

2.4.7.4. Conclusion

The above study shows that the widely held belief that ED is suitable only for free hydroxyls (Pryde and Gilbert 1980) is open to question, and ED might be more broadly applicable to drug

analysis than previously assumed. The E_p values for certain compounds can be lowered by altering the pH or temperature of the mobile phase (Proksa and Molnar 1976; Anderson et al., 1981).

The E_p values observed for these opiates may represent the relative degree of difficulty in forming the initial radical.

These results suggest that by judicious choice of potential and mobile phase conditions, many more molecules might be suitable for electrochemical detection. The mechanisms proposed will also account for a number of unexplained observations in the literature (Musch et al., 1985, Wilson; 1984).

A further implication is that morphine metabolites conjugated through the 3-OH (including codeine, morphine-3-glucuronide and morphine-3-ethereal sulphate) might after all be suitable for sensitive detection by ED.

2.4.8. CONCLUSIONS ON THE CHOICE OF DETECTION SYSTEMS

The above sections have demonstrated that morphine has both electrochemical and light absorbing properties which might be used to measure it eluting from an HPLC column.

The electrochemical properties of several other narcotic analgesics have also been examined and this information may be of use in future assessments of the likely EC properties of a substance, based on consideration of its structure.

Because of the difference in configuration of these two methods from the design actually used in HPLC detectors, an assessment of the comparative sensitivities has not been possible.

In summary, the relative merits of the UV and ED detection could

only be properly compared when a suitable HPLC system was developed.

Initial chromatographic development work was done using a UV detector as no electrochemical detector was available. Once an electrochemical detector was aquired it was decided to use this at a potential of 0.870 volts (from CV work, 2.4.5.) in order to gain experience with its use and to assess its suitability for routine use.

2.5. THE CHROMATOGRAPHIC BEHAVIOUR OF MORPHINE

2.5.1. CHROMATOGRAPHY WITH METHANOL AS THE ORGANIC MODIFIER

2.5.1.1. Introduction

The selection of a chromatographic system for morphine was begun as follows. A reversed phase system was chosen because of the interest in examining the suitability of ED. The initial chromatographic conditions described below were selected as an arbitrary starting point, within reasonable limits. The aim of the study was to produce a simple binary solvent system for rapid analysis with efficient chromatography and high selectivity and sensitivity. The subsequent alteration of the chromatographic conditions was then directed firstly towards finding an acceptable capacity factor (K'), secondly improving peak shape (W) and efficiency (N), and lastly optimising sensitivity and selectivity.

2.5.1.2. Method

When this investigation was begun, the electrochemical detector was not available, therefore UV detection was used. The monitoring wavelength of 212nm was chosen (see section 2.4.2.), however in practice the lowest wavelength which could be used was 220nm. This was the lowest wavelength at which measurements could be made before the cut-off point of the mobile phase was reached, and was considered to be a limit imposed by the use of the Cecil CE 212 detector being used for this work. The pump used was a Constametric I (Laboratory Data Control, Birmingham, U.K.), and sample introduction was through a Rheodyne

7125 six-port sample introduction valve, fitted with a 20ul loop. The loop was filled from a 25ul SGE syringe.

Several mobile phases with different ratios of methanol as the organic modifier in 0.01M KH_2PO_4 were made up and allowed to equilibrate on the column for 30 minutes. After this time reproducible chromatograms could be obtained. Methanol/water (50/50) was pumped through the column for twenty minutes between solvent changes.

The flow rate chosen was 3.0 ml/min. Injections of 25ul were made into a Rheodyne valve injector fitted with a 20ul loop. This ensured thorough wetting of the loop (Eppel 1980). A standard solution of 10^{-2}g/l of morphine HCl in distilled water/methanol (99:1) was used unless otherwise stated. Injecting distilled water between injections gave a measurement of the dead time of the system as 0.5 mins.

2.5.1.3. Results and discussion

All the solvents tested eluted morphine with such an early retention time that whilst the peaks were very sharp, they eluted just beyond the solvent front which made resolution from any other components difficult. The retention data are given in table 2.8.. Adjusting the flow rate to a lower level did increase the retention time but only at the cost of the peak shape and no improvement in the overall performance was noted. The flow rate was therefore maintained at 3ml/min.

TABLE 2.8.

VARIATION IN RETENTION BEHAVIOUR OF MORPHINE AS A FUNCTION OF METHANOL CONTENT OF THE MOBILE PHASE

% MeOH	RT	Pk W	K'	N
75	1.60	0.6	2.2	114
50	1.60	0.8	2.8	64
25	1.75	0.9	2.6	60
10	1.80	0.9	3.0	64
5	2.10	1.5	3.2	31

Dead time = 0.5 mins.

2.5.1.4. Conclusion

None of the broad range of solvents tested gave adequate resolution of morphine from the solvent front. One possible solution was to change the selectivity of the system by using a modifier with a different polarity and different selectivity.

2.5.2. INITIAL CHROMATOGRAPHY USING ACETONITRILE AS THE ORGANIC MODIFIER

2.5.2.1. Introduction

Acetonitrile (ACN) is a low boiling, low viscosity, organic solvent commonly used in HPLC. It has a higher polarity (Snyder scale 5.8) and different selectivity (Chi) values from methanol (Snyder, 1974 & 1978). For this reason it was decided to test several acetonitrile containing solvents for their suitability.

2.5.2.2. Method

The same monitoring wavelength (220nm) was used as was the same buffer, 0.01M KH_2PO_4. The HPLC equipment used was as in section 2.5.1.2.. The undernoted solvent systems were allowed to equilbrate on the column for 30 minutes as before and the retention behaviour of the morphine established. The flow rate used was 3.0ml/min. A morphine HCl standard of 10^{-2}g/l was used as before.

2.5.2.3. Results and discussion

Retention data for the various solvent systems is shown in table 2.9.. From the data presented it was apparent that the retention time of the drug on the column was a function of the percentage of acetonitrile in the mixture. The efficiency of the separation was relatively poor, around 100-200 theoretical plates. This was caused by poor peak shape with considerable tailing. The capacity factor however was in the desired region. The chromatography obtained using the solvent with 8% acetonitrile was considered to be the optimum, as it had a high efficiency with an acceptable retention time.

2.5.2.4. Conclusion

The results indicate the best conditions so far for the chromatography of morphine. The efficiency was however still slightly lower than would be suitable for the analysis of morphine in a complex sample. Having examined the effect of two of the more common organic modifiers, it was decided that the effect of pH should also be investigated.

2.5.3. THE EFFECT OF pH ON THE CHROMATOGRAPHIC BEHAVIOUR OF MORPHINE.

2.5.3.1. Introduction

Since morphine is a weak organic base (pKa=8.21) the form in which it exists in solution, either as the uncharged base or as the protonated conjugate acid, will determine to some extent its chromatographic behaviour.

TABLE 2.9.

VARIATION IN RETENTION BEHAVIOUR OF MORPHINE AS A FUNCTION OF ACETONITRILE CONTENT OF THE MOBILE PHASE

% ACN	RT	Pk W	K'	N
15	1.5	0.5	2.0	144
10	2.15	0.7	3.3	151
8	2.65	0.8	4.3	176
7	2.95	0.9	4.9	172
5	4.6	1.5	8.2	151
2	11.2	4.2	21.4	113

Dead Time = 0.5 mins

2.5.3.2. Method

A stock solution of the optimum mobile phase established above, 8% acetonitrile in 0.01M KH_2PO_4, was made up and adjusted to the desired pH using concentrated orthophosphoric acid or 60% potassium hydroxide solution. These solutions (250ml) were allowed to equilibrate on the column for 30 minutes prior to use.

Injections of $10^{-2}gl^{-1}$ morphine standard solution were made as before. Instrumental conditions were as described in section 2.5.1.2..

2.5.3.3. Results and Discussion

The capacity factor and efficiency for the morphine peak obtained with each of the solvents is shown in table 2.10. The resulting concentration of phosphate in the mobile phase is also given. The K' and N values are also plotted in figure 2.13. as a function of the pH.

It was noted that the capacity factor was fairly consistent below pH 4.5, but that the efficiency only began to improve below pH 4.5. The observed differences were small, but the trend was important in assessing the optimum conditions for the chromatography.

The fact that orthophosphoric acid was used to adjust the pH of the mobile phase led to a consequent change in the concentration of phosphate. The use of other acids to adjust the pH would have introduced other ions and complicated the interpretation of results. It was noted that the increase in efficiency which was obseved corresponded well with the increase in phosphate

TABLE 2.10.

EFFECT OF pH AND PHOSPHATE CONCENTRATION ON RETENTION BEHAVIOUR OF MORPHINE

pH	RT	W	K'	N	[phosphate]
2.5	2.15	0.7	3.78	151	0.024
3.5	2.20	0.9	3.88	95	0.012
4.5	2.20	1.0	3.88	77	0.010
5.5	2.50	1.3	4.56	59	0.010
6.5	2.90	1.5	5.44	60	0.010

Dead Time = 0.45 mins

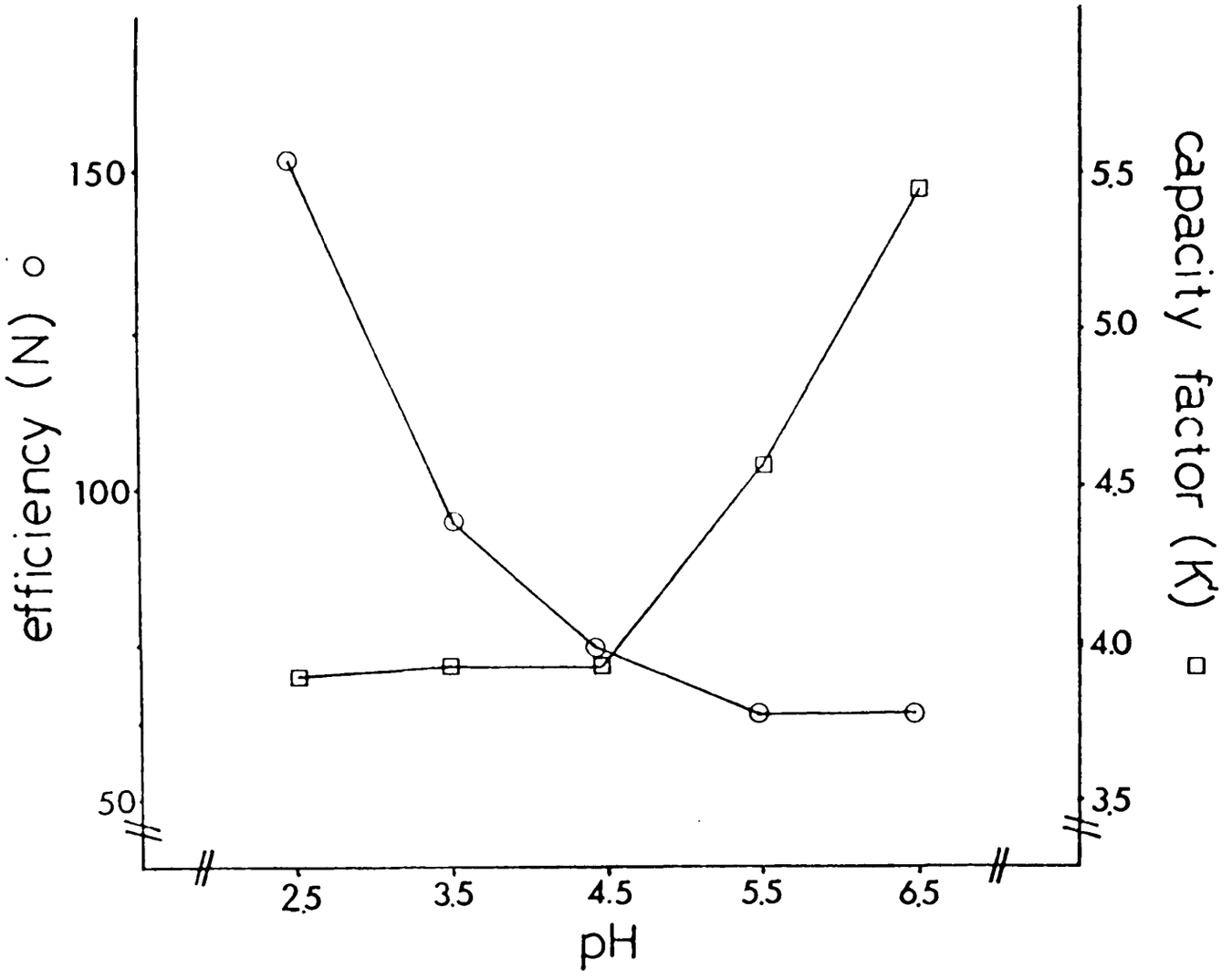


FIGURE 2.13.

VARIATION IN EFFICIENCY AND CAPACITY FACTOR OF MORPHINE WITH pH

concentration. This is not shown in figure 2.13., but table 2.10. shows that phosphate concentration was constant up to pH 4.5, wherafter the addition of orthophosphoric acid caused an increase in phosphate concentration. The efficiency, due mostly to a drop in peak width, was seen to increase only below pH 4.5.

This increase in efficiency suggests a hypothesis that the elution of morphine as the morphonium ion, may have occured via the formation of ion-pairs between the phosphate ion, $H_2PO_4^-$, and the morphonium ion, MH^+.

The principles of ion paired chromatography have been discussed in chapter one, and the use of phosphate as a pairing ion has also been noted (Wahlund and Sokolowski, 1978; **Snyder** and Kirkland, 1978)

If this is the case, increased $H_2PO_4^-$ concentration would increase k2 (EQ. 1, below), and shift the equilibrium to the right, by virtue of the common ion effect.

EQ. 1 $$[M] + [H^+] \underset{k4}{\overset{k1}{\rightleftharpoons}} [MH^+] + [H_2PO_4^-] \underset{k3}{\overset{k2}{\rightleftharpoons}} [MH^+H_2PO_4^-]^o$$

The degree of ion-pair dissociation will be less at higher phosphate concentrations, and should lead to improved peak shape as was observed.

The use of acidic pH could also improve peak shape by ensuring that k1 was high, and most of the morphine was present as morphonium ion.

From the Henderson-Hasselbach equation, the degree of protonation of morphine, can be shown to vary only slightly from pH 4.5 to 2.5 (see table 2.11.), and to a greater extent above 4.5 by virtue of its logarithmic nature.

TABLE 2.11.

VARIATION IN DEGREE OF PROTONATION OF MORPHINE WITH pH

pH	[Morphine]/[Morphonium ion]	% as MH^+
2.5	$1.95X10^{-6}$	100
3.5	$1.95X10^{-5}$	100
4.5	$1.95X10^{-4}$	100
5.5	$1.95X10^{-3}$	99.8
6.5	$1.95X10^{-2}$	98.1
7.5	$1.95X10^{-1}$	83.6
8.5	1.95	33.8
9.0	6.17	13.9
9.5	19.5	95.1

Calculated from Henderson-Hasselbach eqn.:

$$pH=pKa+\log[Morphine]/[Morphonium\ ion]$$

(morphine pKa = 8.21)

The fall in K' between pH 6.5 and 4.5, might be due to poor equilibration of the column when using a mobile phase which was insufficiently buffered (Knox, 1978), as the buffer region of this 0.01M KH_2PO_4/H_3PO_4 solution is either side of pH 2.5.

2.5.3.4. Conclusion

The improvement in efficiency was shown to correlate to both the increase in protonation with pH, and to the increase in phosphate concentration. It is most likely that pH, phosphate concentration and the buffer properties of the solution all have some effect on the chromatography of morphine. Of most interest was the effect of phosphate concentration, as this indicated the possible operation of an ion-paired effect which might be exploited to improve the efficiency of this separation.

2.5.4. THE EFFECT OF PHOSPHATE CONCENTRATION ON RETENTION OF MORPHINE

2.5.4.1. Introduction

In order to investigate the possible existance of an ion pairing effect, a series of solvents at constant pH but varying phosphate concentrations were examined.

2.5.4.2. Method

A series of mobile phases were prepared from solutions of different phosphate concentration; all were however 8% in acetonitrile. The pH was controlled by adding 200mg of 85%

orthophosphoric acid to each. As volumes of 250ml were used for each solvent, this increased the concentration of phosphate in each by approximately 0.007 molar with each 200mg of 85% orthophosphoric acid (H_3PO_4).

The mobile phase was allowed to equilibrate for 1 hour before use.

Other chromatographic conditions were as before (2.5.1.2.).

2.5.4.3. Results and discussion

The buffer capacity of each solvent examined was sufficient to maintain the pH within the range 2.5 - 2.7. The results are shown in table 2.12..

There was a trend showing an improvement in peak shape as phosphate concentration increased. No improvement in efficiency was noted above 0.02M phosphate.

The retention time did decrease also, but not sufficiently to account for the apparent increase in efficiency.

This gives qualified support to the above hypothesis that the mechanism by which morphine was chromatographed on this system was by some form of ion-pair formation.

At high concentration of phosphate however (greater than 0.27 molar), problems were encountered with phosphate crystallising around the seals on the pump and liquid joints. Higher back pressures from the column were also noted. By reducing the phosphate concentration to 0.017M, these problems were less severe and no great loss in efficiency was noted.

TABLE 2.12.

EFFECT ON THE RETENTION BEHAVIOUR OF MORPHINE OF PHOSPHATE CONCENTRATION

[Phosphate]	pH	RT	W	K'	N
0.008	2.51	2.5	1.0	4.0	100
0.012	2.54	2.3	0.8	3.6	132
0.017	2.55	2.3	0.7	3.6	172
0.022	2.55	2.2	0.6	3.4	215
0.027	2.69	2.2	0.6	3.4	215
0.107	2.71	*			

Dead Time = 0.5 mins

* unsuitable due to precipitation of phosphate

2.5.4.4. Conclusion

The apparent increase in efficiency with increasing phosphate concentration noted above was confirmed. This supports a theory involving ion-pair formation between phosphate and the morphonium ion. An example of the optimum chromatography achieved using 8% acetonitrile in 0.01M KH_2PO_4 adjusted to pH 2.5 is shown in figure 2.14.. This shows a relatively sharp peak for morphine separated from the solvent front. The efficiency was however only 172 theoretical plates, which was low by most standards and the suitability of a system like this for the analysis of biological extracts is not generally good. Before further attempts were made to improve the chromatography however, a typical extract obtained from the procedure corrently used routinely for the isolation of morphine from post mortem blood was examined for suitability on this system.

2.5.5. SUITABILITY OF THE SYSTEM FOR THE MEASUREMENT OF MORPHINE IN BIO-EXTRACTS

2.5.5.1 Introduction

The chromatographic system developed above was set up and an extract prepared from an expired transfusion blood sample by the procedure described by Horning et al. 1974. This procedure is examined in detail later (2.6.1.), but it was considered to be of assistance in the development of the assay to include this experiment here.

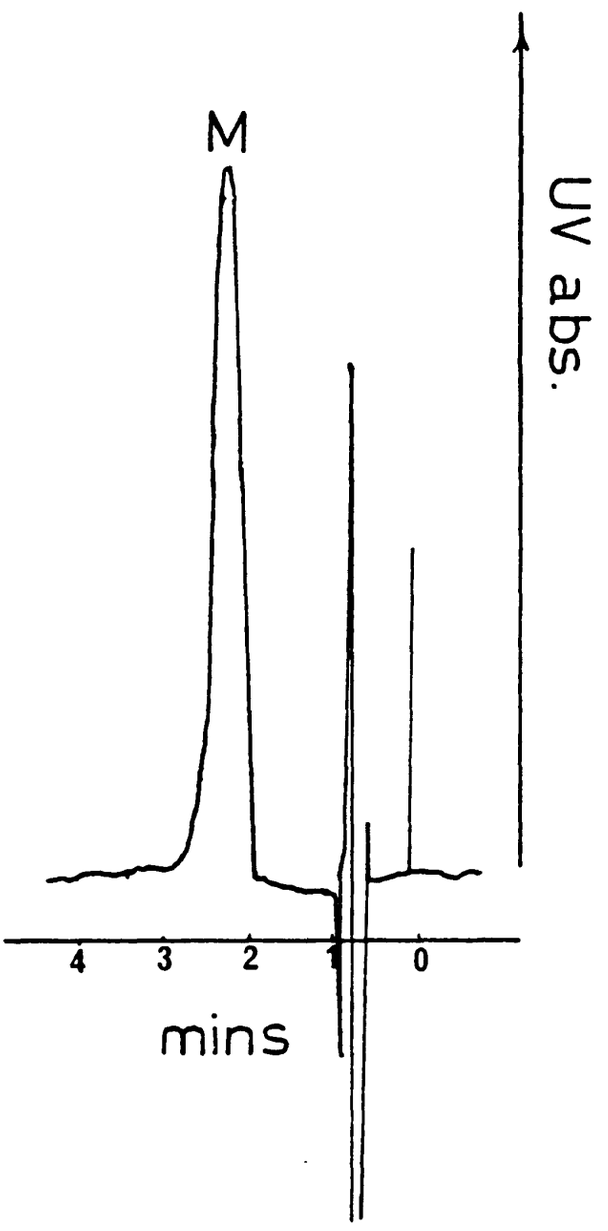


FIGURE 2.14.

CHROMATOGRAPHY OF MORPHINE RESULTING FROM 8% ACETONITRILE IN 0.01M KH_2PO_4 AT pH 2.5

2.5.5.2. Method

A blank blood sample and a sample spiked at a concentration of 10^{-3} g/l, morphine HCl, were extracted by the method described in section 2.6.1..

The extract was reconstituted in 100ul and 25ul injected as above. Detection was by UV monitoring at a wavelength of 220nm.

2.5.5.3. Results and discussion

A typical chromatogram is shown in figure 2.15..

The amount of interference was too great to allow either definite identification or sensitive detection. The morphine peak is hidden by the material eluting following the injection peak. The peaks were broad and badly tailed. The low acetonitrile concentration led to longer retention times for lipophilic material which had co-extracted. Peaks resulting from coextracted material continued to elute for up to 24 minutes.

2.5.5.4 Conclusion

The system as presented was found to be unsuitable for the examination of biological extracts. In addition to the fact that the morphine peak was quite broad, its capacity factor on the system was short in comparison to the later eluting peaks resulting from coextracted material in the extract. Ideally the drug peak should be the last to elute from the column. This suggests that a further change in the selectivity of the separation was required.

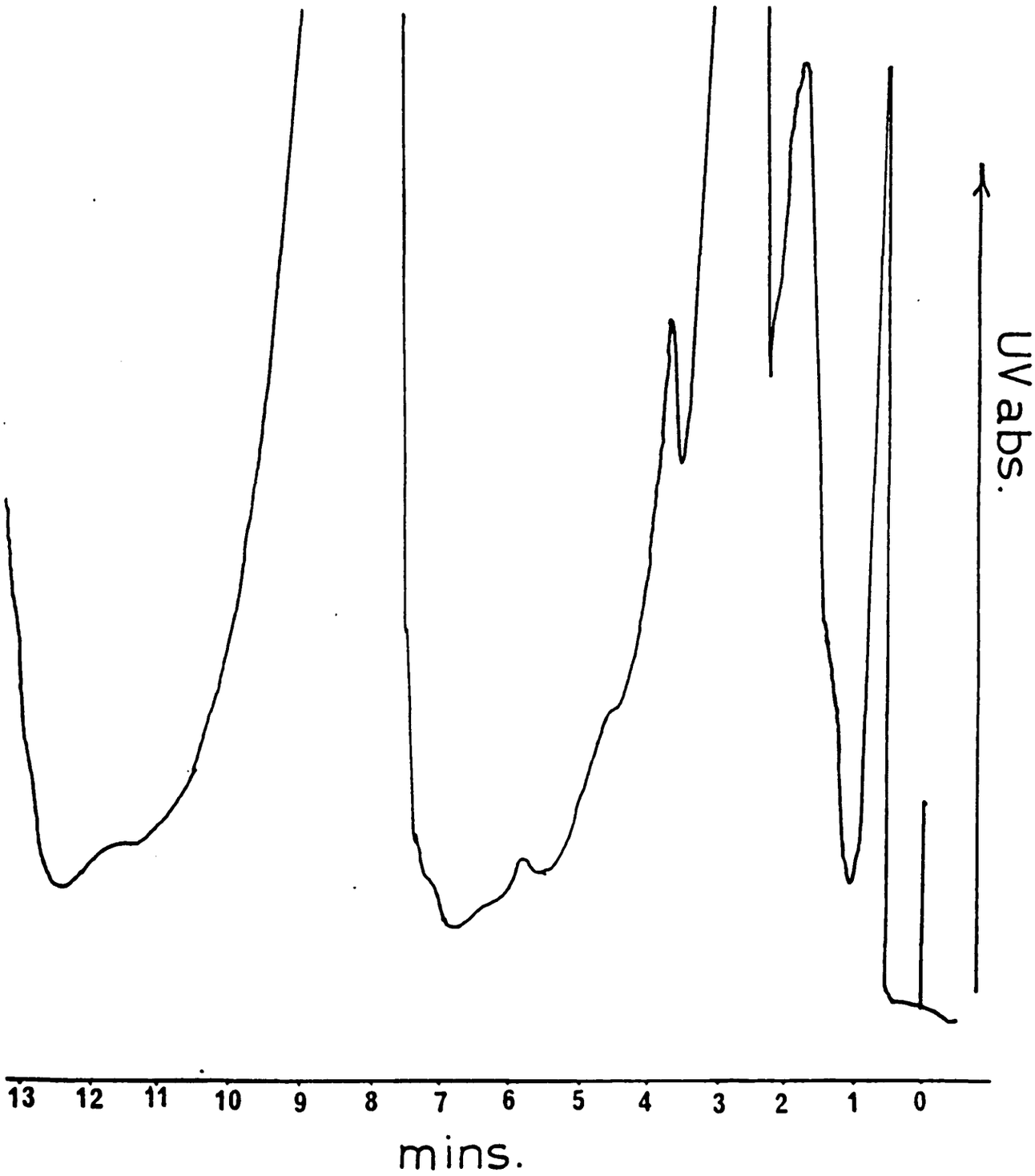


FIGURE 2.15.

AMMONIUM CARBONATE EXTRACT OF A SPIKED BLOOD SAMPLE RUN ON A 10cm ODS HYPERSIL COLUMN. 8% ACETONITRILE, 0.01M KH_2PO_4. pH 2.5.

2.5.6. STRATEGIES FOR IMPROVED CHROMATOGRAPHY

2.5.6.1. Introduction

The selectivity of the assay required improvement, as did the peak shape and the efficiency the LC method. The chromatography of ionisable substances such as amines often presents problems with respect to retention, efficiency and tailing, when in order to separate these compounds by adsorption chromatography, it is neccessary to use polar eluents, which reveal the inhomogeneity of the adsorbent surface (Snyder and Kirkland, 1978; Twitchett and Moffatt, 1978; Gill et al., 1984)

The presence of heterogeneous retention sites leads to mixed principles of separation. At low sample concentrations the stronger sites are preferentially combined with sample molecules. When these sites are not overloaded, normal elution of the sample bands is observed. For more practical sample sizes however these sites become saturated and this overloading results in band tailing. For later eluting compounds there is a greater concentration of the analyte on the stationary phase and therefore greater band tailing. There are several ways in which to avoid this.

The high lipophilic nature of the C_{18} chains means that only a low percentage covering of the support material is possible (typically 5-20%), and overloading can occur at low analyte concentrations. The remaining silanol groups can be deactivated by reacting them with a moiety of lower hydrophobicity. The most common reagent is chloro trimethyl silane. This is known as capping.

Another approach is to alter the selectivity of the solvent or to change the mode of chromatography, for example from reversed phase to normal phase, ion exchange or ion paired chromatography.
As a range of binary solvent mixtures have already been examined, the use of a deactivated packing material was considered, before changes were made to the mode of chromatography.
At this point the electrochemical detector became available and was used for the further development of the assay. The model used was a BAS LC-4B amperomètric detector. It was operated with a TL-5A glassy carbon working electrode at a potential of +0.870 volts as determined by the use of cyclic voltammetry in 2.4.5..

2.5.7. USE OF A CAPPED PACKING MATERIAL

2.5.7.1. Introduction

As ODS Hypersil has no capping performed on it, an ODS reversed phase material APEX ODS (Jones chromatography) was considered. The product specifications state that it is a fully capped reversed phase material, absorptive functions being eliminated by reacting the residual sites silanol sites on the C_{18} material with a small hydrocarbon function to give a "reproducible, single function separation" (Applications notes, Jones chromtography, Glamorgan, U.K.)

2.5.7.2. Method

The ability of the packing material to form a slurry in the methanol/sodium acetate mixture described in appendix A was

examined. This was found to be suitable, and a 10cm column was packed according to this procedure.

The 8% acetonitrile in 0.01M KH_2PO_4 at pH 2.6, mobile phase was used. Detection was by ED at a potential of +0.870V as before.

2.5.7.4. Results and Discussion

The results are noted in table 2.13., and compared with results from ODS Hypersil under identical conditions.

This indicates that there was no great difference in the retention properties of the two materials. This could be accounted for in several ways. The limiting factor for the efficiency may be the adsorption characteristics of the stationary phase materials themselves. The degree of capping of the Apex material may have been insufficient to accomodate all the analyte at the conditions being examined, therefore no difference between the materials would be apparent.

For practical purposes, this experiment demonstrates that there was no improvement in the peak shape or overall performance using a capped material. The slight apparent increase in efficiency was attributed to the reduced retention time.

Hypersil ODS was initially selected for reasons outlined earlier in chapter one and as there was minimal cost difference between the two materials, it was decided to continue using ODS Hypersil.

TABLE 2.13.

EFFECT OF USING END-CAPPED STATIONARY PHASE ON THE RETENTION BEHAVIOUR OF MORPHINE

Material	column	capped	k'	N	cost/10g
Hypersil ODS	100X4.6	No	3.8	154	£61.00
Apex ODS	100x4.6	Yes	3.7	162	£55.00

TABLE 2.14.

EFFECT OF PACKING PROCEDURES AND COLUMN LENGTH ON RETENTION BEHAVIOUR OF MORPHINE

column	packing proc.	k'	N
100x4.6	A	3.8	158
100x4.6	B	3.80	188
100x4.6	B	3.20	196
250x4.6	B	3.13	215

2.5.8. REVISED PACKING PROCEDURE FOR LC COLUMNS

2.5.8.1. Introduction

The packing procedure used for the HPLC column used in this study was that described by Eppel (1980), and was based on information supplied by the manufacturers of the packing material. In an attempt to improve the efficiency of the columns packed in the laboratory, the manufacturers were contacted for details of any changes to the recommended packing procedure. The procedure currently recommended is given in full in appendix B.
In addition it was decided to examine the effect of using a 250mm column as a longer column can give greater resolving power to a separation (Snyder and Kirkland 1978).

2.5.8.2. Method

The columns were packed according to the full procedure as outlined in appendix B. Acetone was used as the slurrying solvent and also used as the packing solvent. Changing to this solvent required changes to the seals in the packing pump, to ensure that they were compatible with acetone.
Other alterations to the procedure included the practice of inverting the column during packing.
Once the columns had been packed and the end fittings attached, they were conditioned with 50/50 methanol/water (500ml) before being washed with water (100ml) and methanol (100ml). Columns were left in methanol when not in use.
One 250mm and two 100mm columns were packed in this manner and the retention of morphine was examined on each. The retention

behaviour of morphine on a column packed by the procedure in appendix A is given for comparison.

2.5.8.3. Results and discussion

The capacity factor and efficiency of the chromatography of morphine on each of the columns is given in table 2.14.. For the 100mm columns there was no significant change in the capacity factor, but there was a slight change in the efficiency which was marginally improved.

For the 250mm column the increased length caused a consequently higher back pressure and the flow rate was changed to 2ml/min.

The efficiency of the separation was slightly increased over the shorter column, and although there was a small decrease in the capacity factor, the retention time of morphine had been increased without any consequent loss of efficiency. This was one of the aims of the optimisation and whether it was due to the greater resolving power of the column or the reduced flow rate, it was considered an improvement. This column operated at 2ml/min was used for all further developement work.

2.5.8.4. Conclusions

The use of the acetone packing procedure (B) appears to give improved chromatography, and the use of longer columns also increases the efficiency with no significant change in the capacity factor.

The efficiency was still relatively low and should be improved before the method was suitable for the analysis of biological extracts. For all future work procedure B was adopted.

Having demonstrated that the use of the deactivated packing material had not improved the chromatography of morphine, an alternative approach was considered.

The results obtained in section 2.5.3. suggested that morphine might be amenable to ion-pair formation, and so the use of ion-paired chromatography was examined.

A number of reports involving the use of ion pairing techniques have appeared in the literature (Vandenberghe et al., 1984; Moore et al., 1984) and these have demonstrated acceptable peak shape, better than has been achieved above. Other pairing ions with selectivities considerably different from phosphate were therefore considered.

2.5.9. THE USE OF ION-PAIRING AGENTS

2.5.9.1. Introduction

A discussion of ion-paired chromatography was given in chapter 1. Its use is recommended to reduce tailing of basic compounds in reversed phase chromatography (Gloor and Johnson, 1977; Knox, 1978; Wahlund and Sokolowski, 1978).

As morphine is a basic compound (pKa=8.21), it will readily become protonated in aqueous solutions below pH 8 (see table 2.11.). The pH range in which it would be completely unprotonated is outwith the stability range of the packing material (greater than pH 11), therefore the complete or partial protonation of morphine must be taken into consideration. The approach adopted for ion-pairing is to ensure complete protonation of the morphine by

using a mobile phase of pH less than 3.

From the discussion in 2.5.3.3. it can be seen that the region of buffer activity is in the pH range 2.5-2.7.. This pH will ensure complete protonation of the morphine (see table 2.11.).

Morphine as the morphonium ion can then be paired with an anion (figure 2.16.), and the chromatography of this uncharged moiety examined.

Earlier results indicate that morphine may have been pairing with phosphate to some extent (see sections 2.5.3. and 2.5.4.). Many anions have been reported in the literature as pairing ions for basic compounds including phosphate, bromide, chloride, perchlorate, picrate, dodecyl sulphate, methyl sulphonate and various sulphonic acids (Snyder and Kirkland, 1978; Gloor and Johnson, 1977).

It was decided to examine the suitability of alkyl sulphonic acids. These are recognised as good ion-pairing agents and their hydrophobicity increases with increasing chain length, imparting further selectivity to the separation.

2.5.9.2. Method

Heptane sulphonic acid (HSA) was added to the optimum mobile phase developed above (8% acetonitrile in 0.01M KH_2PO_4, pH2.5), at a concentration of 0.005M. This was allowed to equilibrate for 1 hour before any samples were introduced. After this time reproducible chromatography could be achieved. A standard solution of 10^{-2}g/l of morphine was used.

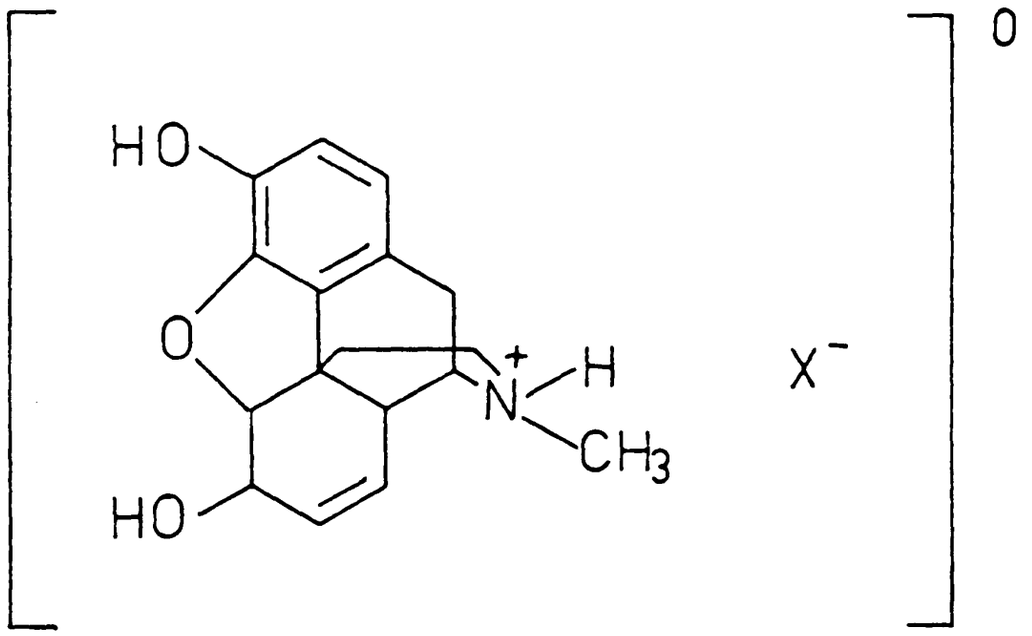


FIGURE 2.16.

FORMATION OF NEUTRAL ION-PAIR BETWEEN MORPHONIUM ION AND A SUITABLE ANION

2.5.9.3. Results and discussion

Morphine eluted from the column considerably later (25 minutes, K' = 18). The efficiency however was 400, a significant improvement on before. The peak was very broad (20 minutes) and the system as described was of little practical value for the analysis of morphine. Having confirmed however the operation of some ion paired effect in the chromatography of morphine, this approach was considered further.

2.5.9.4. Conclusions

The presence of the ion-pairing compound had increased the retention time of the morphine on column. It was decided to investigate whether the retention could be altered by varying the concentration of the organic modifier.

2.5.10. OPTIMISATION OF ION-PAIRED CHROMATOGRAPHY OF MORPHINE

2.5.10.1. Introduction

The retention of a moiety on column can be altered by changing the concentration or chain length of the ion-pairing agent (Bartha et al., 1984), and also by controlling the amount of organic modifier.

The concentration of the pairing ion will also effect the retention of the analyte (Gloor and Johnson, 1977).

2.5.10.2. Method

Two ion-pairing agents heptane sulphonic acid (HSA), and octane sulphonic acid (OSA) were examined in detail. A series of solvents with increasing acetonitrile concentrations were prepared. 0.01M KH_2PO_4 was used and the pH of the solvents was kept at pH2.5 to assist with the formation of morphonium ion and hence with ion pairing. Detection was by UV detection monitoring at 220nm, and ED at a potential of +0.870V connected in series in that order. The large volume of the reference compartment in the electrochemical cell would dilute the analyte and prevent its detection in the UV cell. Further, the electrochemical detector will not withstand the pressures created by attaching further flow cell after it.

The solvent was adjusted to the desired concentration in ion-pairing agent by the addition of a weighed amount of the respective sodium salt.

The mobile phase was allowed to equilibrate for one hour, and the retention behaviour of morphine investigated by the injection of 20ul 10^{-3}g/l morphine HCl.

This was done for a range of pairing ion concentrations. Ion pairing agents can be difficult to remove from the column after use. To prevent the interference of ion pairing agents from previous solvents the column were pumped through with 0.01M Ethylenediamine tetra acetate (EDTA) to remove the ion pairing agent. EDTA is highly water soluble and any residual material was eluted with a subsequent water wash (200ml) when changing between solvents with different ion pairing agents.

TABLE 2.15.

VARIATION OF MORPHINE RETENTION TIME WITH ACETONITRILE CONTENT AND OSA/HSA CONCENTRATION

OSA concentration	acetonitrile concentration (%)									
	8%		10%		15%		20%		25%	
	Rt	K'	Rt	K'	Rt	K'	Rt	K'	Rt	K'
0.005	27.9	17.6	22.8	14.2	14.3	8.5	7.2	3.8	4.4	1.9
0.01	30.6	19.4	26.0	16.3	16.8	10.2	9.0	5.0	4.7	2.1
0.02	36.0	23.0	28.4	17.9	19.0	11.7	11.3	6.5	5.7	2.8

HSA concentration	acetonitrile concentration (%)									
	8%		10%		15%		20%		25%	
	Rt	K'	Rt	K'	Rt	K'	Rt	K'	Rt	K'
0.005	26.3	16.5	16.9	10.3	8.4	5.0	4.6	2.0	3.5	1.4
0.01	27.8	17.5	18.9	11.6	10.8	6.2	5.4	2.6	4.3	1.9
0.02	30.0	19.0	21.9	13.6	13.0	7.7	6.7	3.5	4.6	2.0

Dead Time = 1.5min

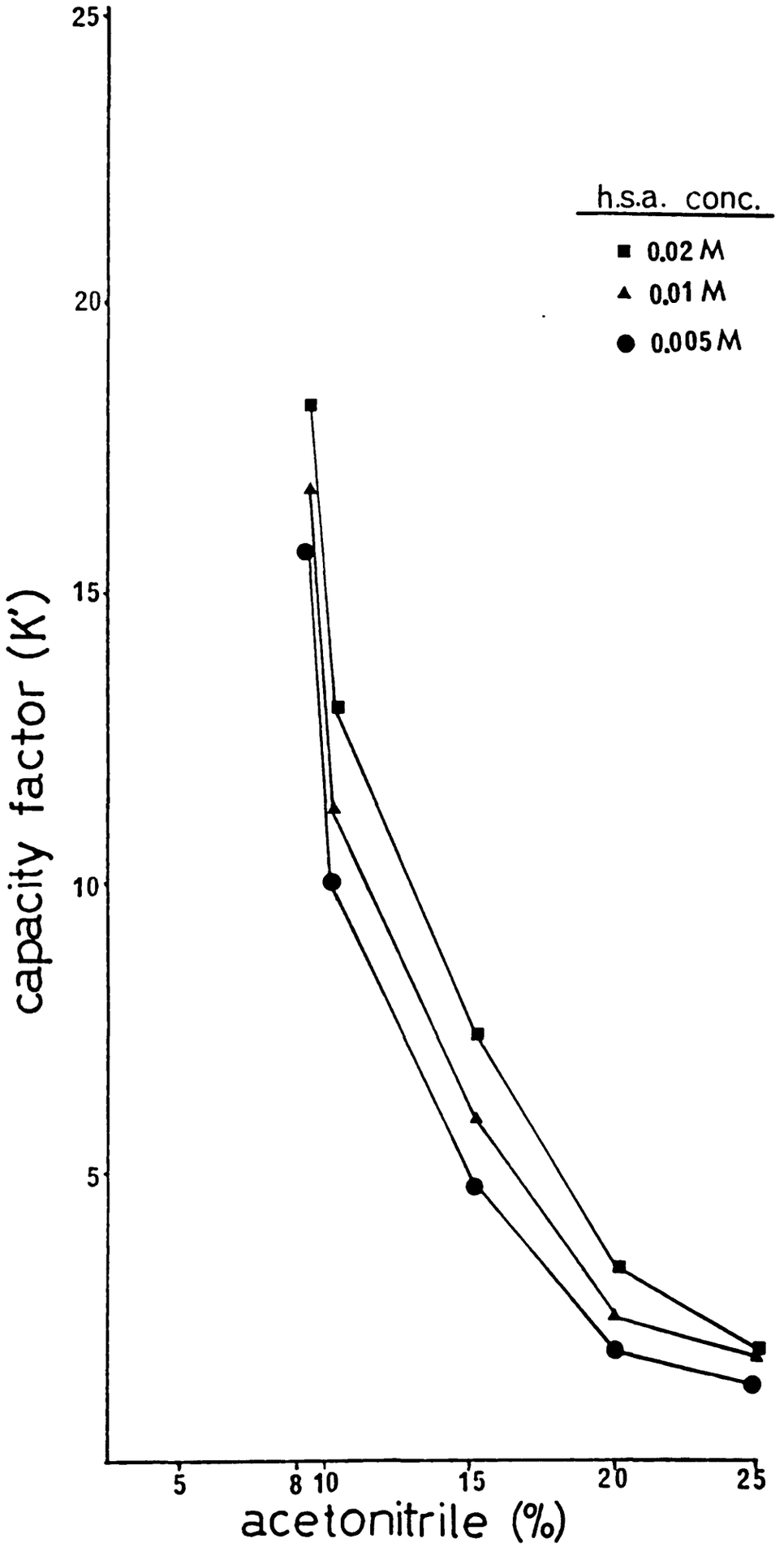


FIGURE 2.17.

VARIATION IN CAPACITY FACTOR OF MORPHINE FOR GIVEN CONCENTRATION OF HEPTANE SULPHONIC ACID AND ACETONITRILE CONTENT OF THE MOBILE PHASE

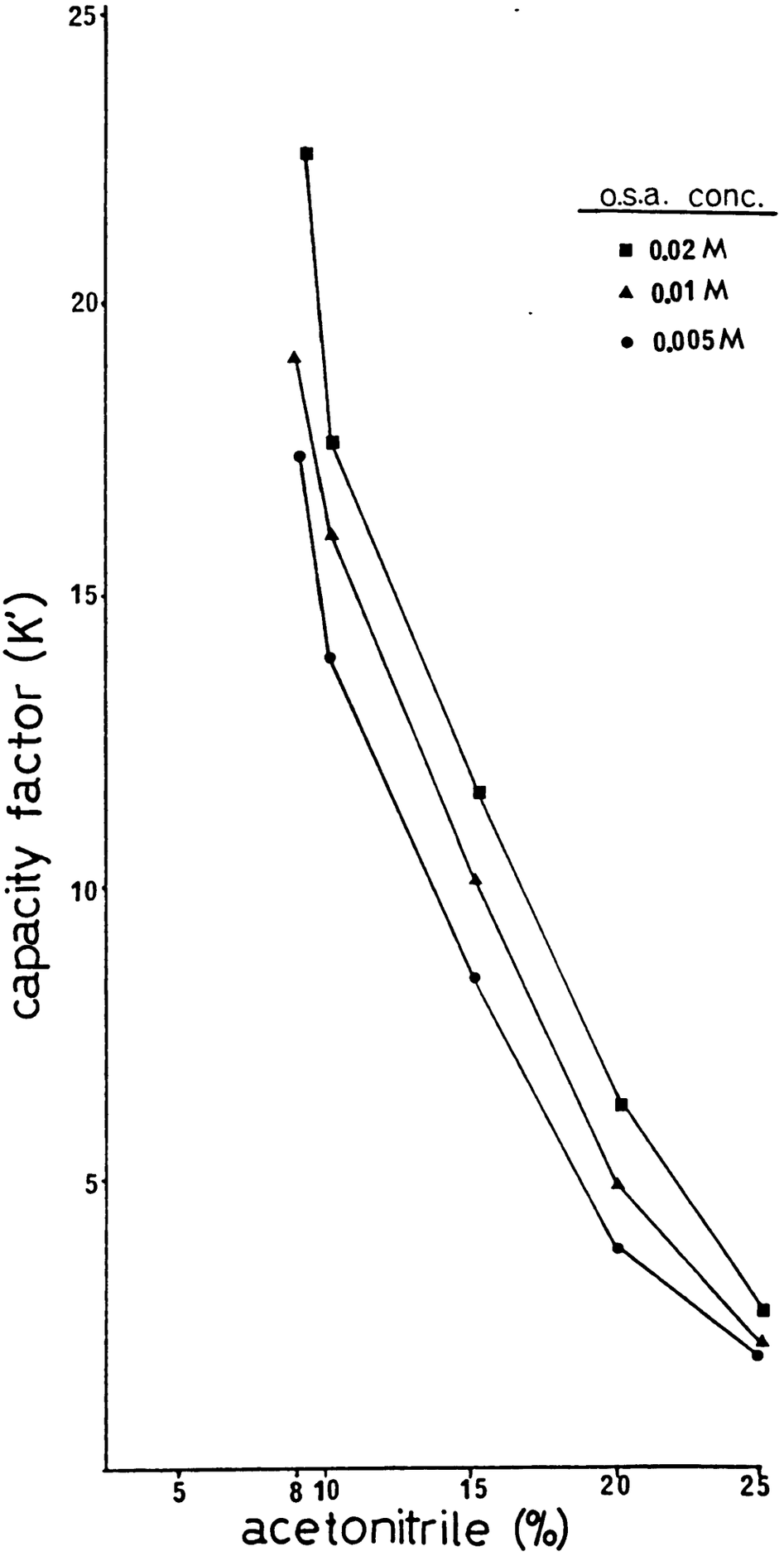


FIGURE 2.18.

VARIATION IN CAPACITY FACTOR OF MORPHINE FOR GIVEN CONCENTRATION OF OCTANE SULPHONIC ACID AND ACETONITRILE CONTENT OF THE MOBILE PHASE

2.5.10.3. Results and discussion

Retention times and capacity factors are shown in table 2.15., and graphically in figures 2.17. and 2.18..

(a) Heptane Sulphonic Acid

As the concentration of ion-pair increases, so does the degree of retention. This could be due to k2 being shifted further to the right in EQ. 1 above (2.5.3.3.). As this change in retention follows the trend observed for phosphate in section 2.5.4. above, it would support the hypothesis of ion-pairing between morphine and $H_2PO_4^-$ in that case.

Increasing the concentration of the pairing ion also resulted in increased retention time/capacity factor of morphine by a small amount. In the presence of the ion pairing agent at any of the concentrations examined, by far the greatest control of retention was achieved by altering the acetonitrile content of the mobile phase.

(b) Octane Sulphonic Acid

The results follow the pattern for HSA. The increase in retention is greater, due to the greater hydrophobicity of the $[OSA^-MH^+]$ ion-pair. Again higher concentrations of ion pairing agent increased the retention time of morphine by a small extent, however a wide range of capacity factor could be achieved by appropriate choice of the acetonitrile content of the mobile phase.

The observed increase in retention time with ion pair reagent concentration agrees with observations which show that smaller pairing ions require very large concentrations before the critical micelle concentration is reached (Gloor and Johnson, 1977). After that point the retention decreases with increasing ion-pair concentration.

2.5.10.4. Conclusion

The use of ion-pairing agents improves the peak shape for morphine. The morphine peak can be retained on the column for a longer period of time without the spreading of the peak observed when binary solvent mixtures were used (1.5.1. and 1.5.2.). The alkyl chain length has a more noticeable effect on the retention of morphine than on the peak shape or efficiency. With the wide range of capacity factors produced from different concentrations of acetonitrile it was expected that a suitable solvent could be found for the analysis of morphine in extracts from biological samples

2.5.11. THE PRESENCE OF SYSTEM PEAKS WITH ELECTROCHEMICAL DETECTION

2.5.11.1. Introduction

In the above experiments a large peak (marked S) appeared with every injection on the trace from the electrochemical detector, and to a slight extent on the UV detector.
Sample ED traces are shown in figure 2.19.(a - c) for mobile phases which were 20% acetonitrile in 0.01M KH_2PO_4 at pH 2.5

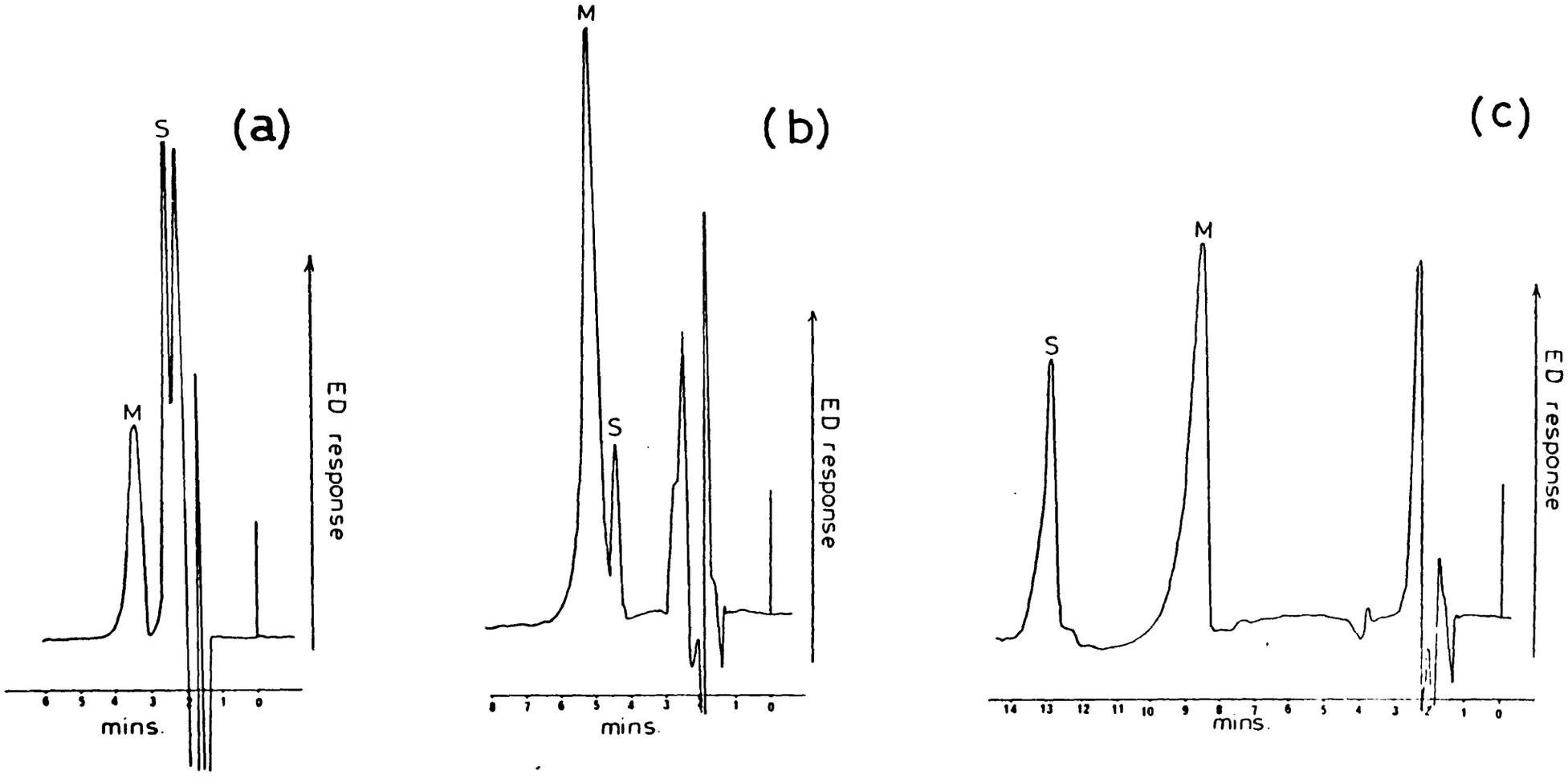


FIGURE 2.19.

TRACES SHOWING THE POSITION OF PEAK DUE TO MORPHINE (M) AND SYSTEM PEAK (S) FOR (a) PENTANE SULPHONIC ACID (10^{-2}M). (b) HEPTANE SULPHONIC ACID (10^{-2}M) AND (c) HEPTANE SULPHONIC ACID (10^{-2}M) IN 20% ACETONITRILE IN 0.01M KH_2PO_4 AT pH3

and 10^{-2}M in pentane, heptane and octane sulphonic acids respectively.

This peak was not immediately apparent with pentane sulphonic acid, as it appeared just after the solvent front. With heptane sulphonic acid, the peak was very much more apparent and overlapped to some extent with the morphine peak. With octane sulphonic acid the peak appeared sufficiently after the morphine peak not to interfere. In order to rule out the possibility of contamination, the identity of this peak was investigated.

2.5.11.2. Method

The HPLC system was set up with a mobile phase as follows. 20% acetonitrile in 0.01M KH_2PO_4 at pH 2.5. Sufficient octane sulphonic acid was added to make the resulting solution 10^{-2}M. This mobile phase was chosen as it gave good resolution of morphine from the solvent front, good peak shape and the fact that the morphine peak and the system peak were well separated.

A variety of reagent blanks and sample types were examined by injection into the sample loop.

2.5.11.3. Results and Discussion

A peak in the position shown in figure 2.19.(c) was found after the introduction of samples of water, mobile phase, air, helium, nitrogen, 0.001M NaOH, 0.01M HCl. The peak height was not reproducible, but the peak was always present to some extent. It was assigned as a "system peak".

This is the name given to peaks which are inherent in the system

and are caused by equilibration or re-equilibration of the mobile phase after some disturbance. Although these peaks are often present in published chromatograms, they are rarely identified or commented upon.

A separation of amino acids gave rise to four peaks (Levin and Grushka, 1986) which were identified as corresponding to the four constituents of the mobile phase. Of these, the latest eluting was identified as heptane sulphonic acid, a constituent of the mobile phase.

The mechanism proposed here for this phenomenon is that following the injection of any material onto the column, any component of the mobile phase which is adsorbed onto the top part of the column (all are to a greater or lesser extent) will be displaced. The equilibrium concentrations on the top of the column will immediately be restored by adsorbing material out of the mobile phase.

The displaced material will be carried by the mobile phase, to which it will be in disproportion, out of the column. The degree to which each component is retained by the column will determine at what time the system peak elutes. There should be the same number of system peaks as species present in the mobile phase.

Water, phosphate, acetonitrile and potassium will have negigible retention on the column and these will indicate the dead time. Alkyl sulphonic acids however having hydrophobic tails will be significantly retained by the non-polar stationary phase. The degree of retention which should increase with hydrophobicity and hence chain length, the shorter chained species being retained less and eluting earlier (Bartha et al., 1984).

TABLE 2.16.

EFFECT OF NATURE OF ION-PAIRING AGENT ON THE RETENTION OF MORPHINE (M) AND THE SYSTEM PEAK (S)

pairing agent	M/S*	DT	RT	K'
C_5SO_4H	S	1.3	2.4	0.85
	M	1.3	3.2	1.44
C_7SO_4H	S	1.3	4.4	2.38
	M	1.3	5.0	2.85
C_8SO_4H	S	1.3	12.9	8.92
	M	1.3	8.7	5.69

* Morphine or System peak

This is confirmed in the results shown in table 2.16. for the K' values of the system peak caused by pentane, heptane and octane sulphonic acid mobile phases shown in figures 2.19.(a - c).

2.5.12. CONCLUSION - SELECTION OF A SUITABLE SOLVENT FOR THE CHROMATOGRAPHY OF MORPHINE

From the data in the above experiments a solvent was chosen for further investigation in an attempt to find a system suitable for the sensitive and selective measurement of morphine in biological extracts.

Figures 2.17. and 2.18. provide information concerning the capacity factor and retention time of morphine achieved using various strengths of heptane and octane sulphonic acids.

The solvent selected contained 20% acetonitrile and octane sulphonic acid (10^{-2}M). This system had a capacity factor of 5.7 for morphine and an efficiency of around N=600.

Solvents containing heptane sulphonic acid were rejected because of the proximity of the system peak to the morphine peak. An example of the chromatography achieved with this solvent is shown in figure 2.20.. Also shown in this figure is nalorphine which was later selected as an internal standard. This is discussed below in section 2.5.15.. The other chromatographic conditions used in further sections was as follows. A Gilson 302 single piston pump (5mm pump head) operated at 2ml/min. Sample introduction was through a Rheodyne 7125 six-port injection valve. The detector was a BAS LC-4B amperometric detector

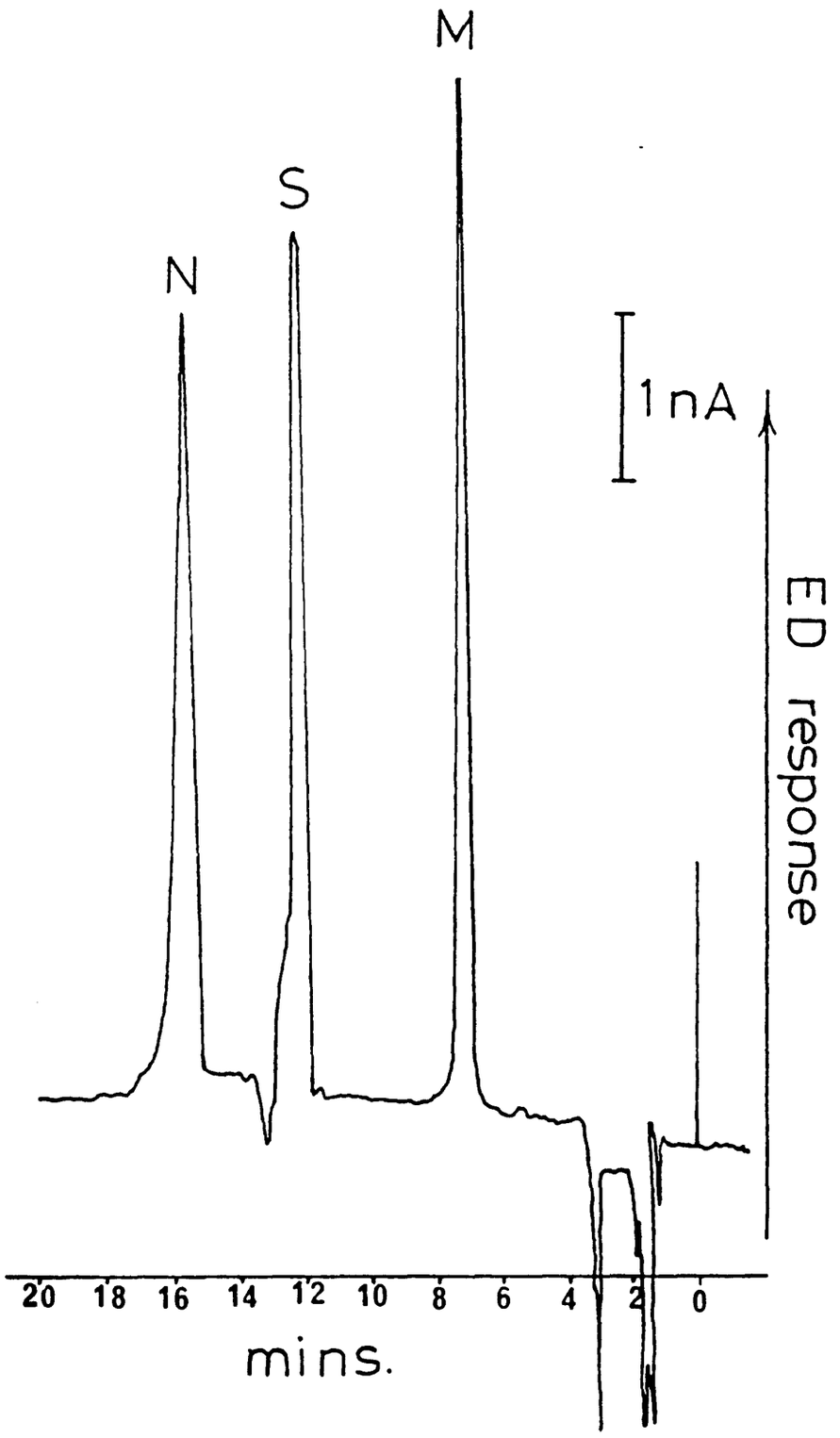


FIGURE 2.20.

OPTIMUM CHROMATOGRAPHY FOR MORPHINE ACHIEVED WITH 20% ACETONITRILE IN 0.01M KH_2PO_4 AT pH 3 0.01M OCTANE SULPHONIC ACID

operated at +0.870 volts (oxidation) with the signal filter and sensitivity settings set as required.

2.5.13. HYDRODYNAMIC VOLTAMMETRY OF MORPHINE

2.5.13.1. Introduction

Hydrodynamic voltammetry (discussed in chapter one) is a technique used to study the electrochemical properties of a substance when it can be eluted from an HPLC column.

Having selected a suitable solvent system for morphine, using a potential +0.870 volts predicted by cyclic voltammetry (a steady state technique) (see section 2.4.5.), the suitability of this working potential for morphine was examined under the conditions of the analysis, by recording hydrodynamic voltammograms (HDV's).

2.5.13.2. Method

Two HDV's were conducted under the same chromatographic conditions but with the voltage changing in opposite directions to gauge the effect this might have on the observed E_p value. Fresh solvent was prepared each time, and the electrode was polished before each HDV was measured.

The first was performed starting from 1.2 volts and decreasing the potential. The second from 0.3 volts and increasing the potential.

Successive injections of a 10^{-3}g/l standard were made and the potential altered between each injection. The background current was allowed to equilibrate between changes in potential, before

the sample was injected. Chromatographic conditions were as described in section 1.5.12.

2.5.13.3. Results and discussion

The HDV data is shown as percentage of maximum response (%MR) (table 2.17.), as the values for the current produced were not recorded. The data is plotted in figure 2.21. and the E_p values are indicated.

From the two sets of results it is evident that the pattern of the voltammetry changes considerably depending on the direction of voltage sweep.

With increasing potential the observed E_p was 0.76 volts compared to 1.05 volts for decreasing potential. The implication is that irreversible contamination of the working electrode occurs at potentials greater than one volt, resulting in larger potentials being required to ensure the adsorption of the analyte on to the surface. This contamination comes probably from constituents or impurities in the mobile phase.

It would be reasonable to assume that more complex mobile phases would contain more potential contaminants and this may account for the discrepancy observed between these values and the value obtained by CV (+0.870 volts). Various values of E_p have been recorded for morphine by CV and HDV in different solvents (See table 2.5.). Also noted is the observation by Gill et al., (1984) of no plateau.

RESPONSE DATA FOR HYDRODYNAMIC VOLTAMMETRY EXPERIMENTS WITH VOLTAGE SCAN UP AND VOLTAGE SCAN DOWN

HDV 1 potential scan down		HDV2 potential scan up	
voltage	%MR	voltage	%MR
1.20	88	0.30	0
1.15	97	0.40	0
1.10	100	0.50	0
1.00	89	0.55	0
0.95	66	0.60	7
0.90	44	0.65	43
0.85	25	0.70	79
0.80	11	0.75	96
0.75	3	0.80	92
0.70	1	0.85	100
0.65	0	0.90	99
0.60	0	0.95	97
0.55	0	1.00	92

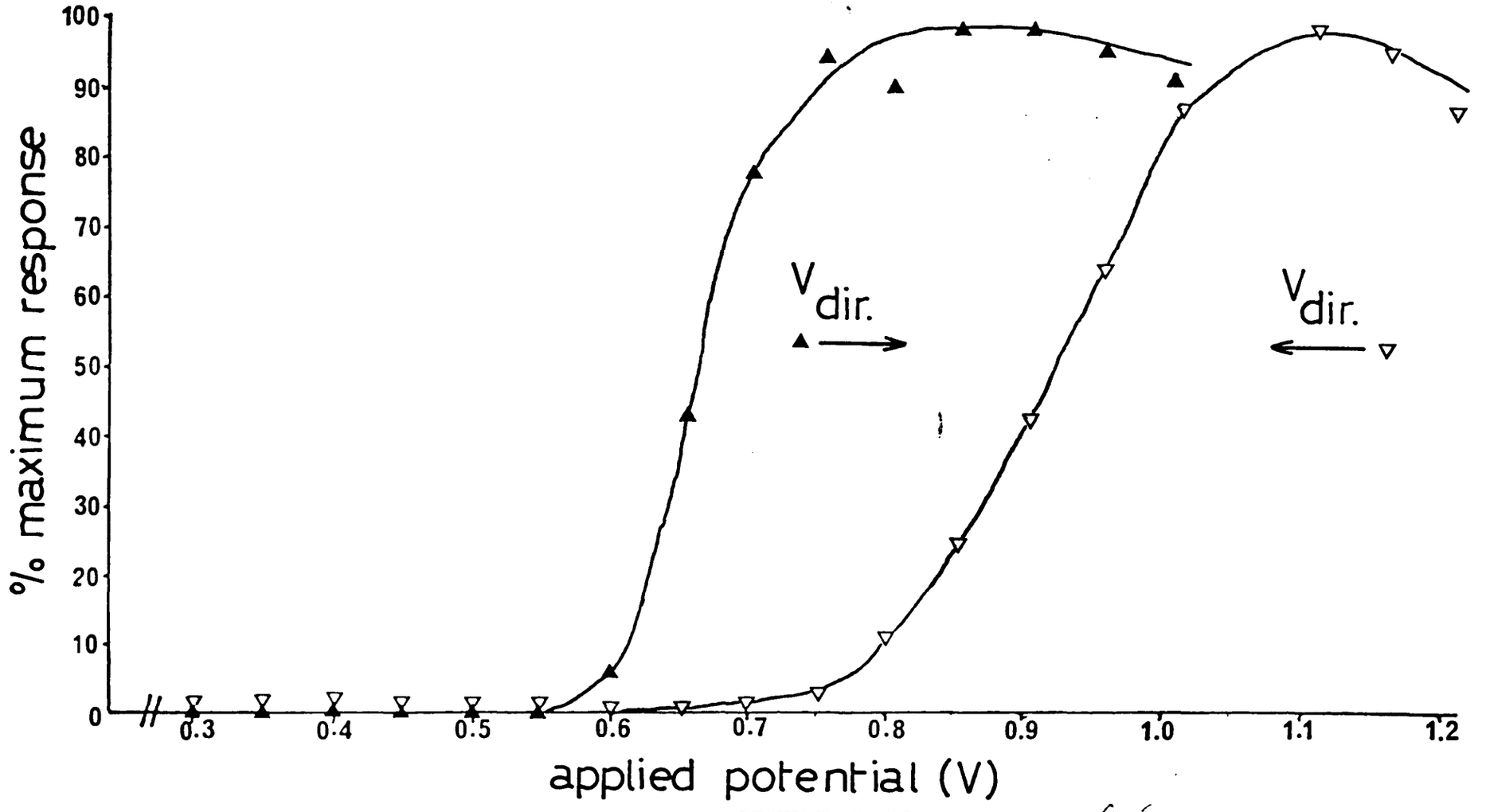


FIGURE 2.21.

HYDRODYNAMIC VOLTAMMOGRAMS FOR MORPHINE RESULTING FROM INCREASING AND DECREASING CHANGES IN APPLIED POTENTIAL

2.5.13.4. Conclusion

This experiment demonstrated that the method by which the full wave potential (E_p) of morphine is measured can influence that measurement. Contamination of the electrode is unavoidable in the case of analysis of complex extracts, and this may effect the ability of the detector sensitively to detect the analyte. This suggests the need for both an internal standard of similar electrochemical properties to the analyte and also regular analysis of standard solutions in order to check the sensitivity of the detector.

As a result of this experiment, the potential selected for further work was +0.870 volts, obtained by cyclic voltammetry and used in investigations up to this point.

It was noted in practice that if, over a period of several days the response of the detector fell, it could be regained to some extent without polishing by increasing the applied potential to +1.000 volt.

2.5.14. COMPARISON OF SENSITIVITIES OBTAINED FROM UV AND ED DETECTORS

2.5.14.1. Introduction

The suitability of UV absorbance and electrochemical detectors has been described above (2.2.6. and 2.4.). A comparison was therefore made of the two detection systems in order to assess which would be most suitable for the detection of low levels of morphine.

2.5.14.2 Method

In order to make a valid comparison of two different modes of detection, the detectors were operated under identical conditions. They were attached in series and standards of decreasing concentration were analysed. The detection limit was defined as the settings at which signal to noise ratio fell below 75%. Other parameters were set at values normally used in this assay. The UV detector (Kratos spectroflow) was operated at 212nm as described in 2.4.2.. For the electrochemical detector (BAS LC-4B, with TL-5A thin layer flow cell), a potential of +0.870V was used. The ion-paired solvent system developed above (2.5.12.) was used.

A series of standard solutions of morphine were prepared, ranging from 10^{-2} to 10^{-5}g/l. These were analysed on the above system.

2.5.14.3. Results and discussion

The linearity of response for both detectors is shown in table 2.18. and figure 2.22. Because of the wide range of concentrations being examined, the units of current are in nA as read from the digital display. Absorbances are calculated from peak height and presented as Abs x 10^{-3}, (mAbs).

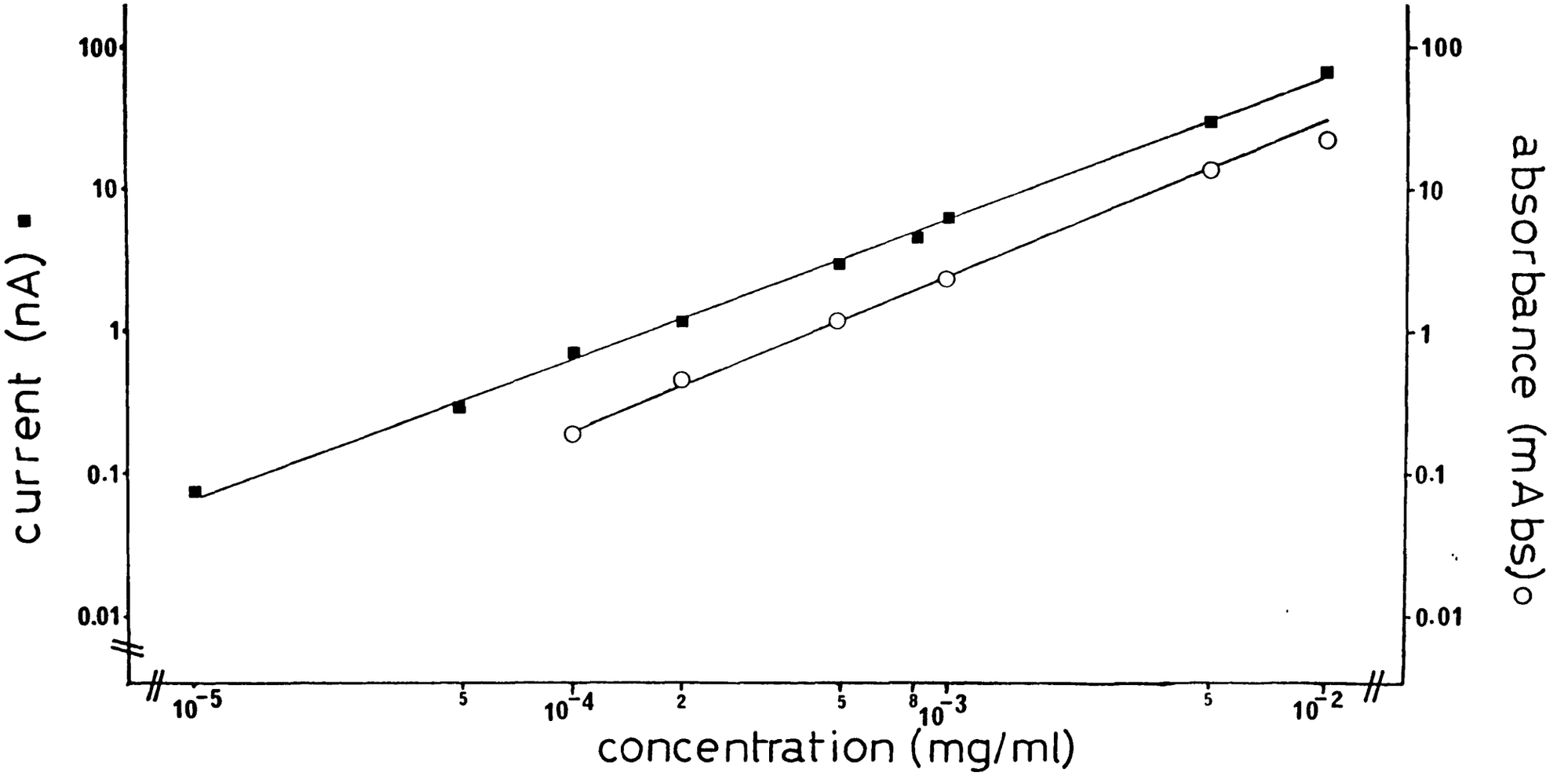
ED is reported to have a greater linear range than other forms of detection. This is not evident from these results as both display a linear response with correlation coefficients greater than 0.99 (UV 0.9923, and ED 0.9999) within the range of interest, although the final point (for 10^{-2}g/L) did not lie on the best fitted line (see figure 2.22.).

TABLE 2.18.

RESPONSE OF UV AND ELECTROCHEMICAL DETECTORS TO A RANGE OF MORPHINE CONCENTRATIONS

Concentration (g/l)	$ ng o/c	UV (mAbs)	ED (nA)
10^{-5}	0.2	-*	0.08 (S/N 4:1)
$5x10^{-5}$	1.0	-*	0.30
10^{-4}	2.0	0.2 (S/N 3:1)	0.62
$2x10^{-4}$	4.0	0.5	1.20
$5x10^{-4}$	10.0	1.2	3.05
$8x10^{-4}$	16.0	-‡	4.48
10^{-3}	20.0	2.3	6.20
$5x10^{-3}$	50.0	13.4	29.45
10^{-2}	100.0	21.2	64.30

* Not detected.
‡ omitted
$ nanograms of morphine on column



LINEARITY OF RESPONSE TO MORPHINE FROM UV AND
ELECTROCHEMICAL DETECTORS OVER A RANGE OF CONCENTRATIONS

The poorer correlation seen for UV detection may result from measuring error when measuring the small peaks produced by the UV detector.

The sensitivity of the two methods is also compared. The ED is shown to be ten times more sensitive (for a similar signal to noise ratio) than UV detection, under optimum conditions.

The response of the ED varies from day to day, and the results shown above represent the optimum, achieved by using a freshly polished electrode, shortly after the equilibration of a fresh solvent. Typical day to day variation is evident in the study discussed in section 2.9.3. (esp. table 2.27.).

For normal operation, a signal of 0.5nA for a 10^{-4}g/l standard could be expected.

2.4.14.4. Conclusion

Because of its greater sensitivity, the ED detector was used on its own in this study for the examination of extraction procedures, and subsequently for the analysis of extracts obtained from authentic samples.

2.5.15. SELECTION OF AN INTERNAL STANDARD

2.5.15.1. Introduction

A series of drugs commonly encountered in forensic casework were analysed on the system selected above in order to check any possible interference with the morphine assay.

These drugs would have to be basic to pass the extraction step,

TABLE 2.19.

RETENTION BEHAVIOUR OF OTHER DRUGS ON MORPHINE HPLC SYSTEM

Drug	K'	UV_{220}	$ED_{1.000V}$
primidone	2.0	+	+
methyprylone	3.3	+	-
morphine	4.5	+	+
phenobarbitone	5.5	+	-
butabarbitone	6.5	+	-
butalbital	6.1	+	-
nalorphine	9.0	+	+
codeine	11.5	+	-
phenytoin	15.0	+	-
amylobarbitone	19.0	+	-
glutethimide	20.1	+	-
oxycodone	22.1	+	-
quinalbarbitone	22.3	+	
benzoylecgonine	ne		
bupivacaine	ne		
butacaine	ne		
caffeine	ne		
cannabinol	ne		
cannabidiol	ne		
chlordiazepoxide	ne		
cinchocaine	ne		
cocaine	ne		
cyclizine	ne		
desmethyldiazepam	ne		
diamorphine	ne		
diazepam	ne		
dihydrocodeine	ne		
dipipanone	ne		
ecgonine	ne		
levorphanol	ne		
lignocaine	ne		
lorazepam	ne		
mepivacaine	ne		
methyldopa	ne		
nicotine	ne		
nitrazepam	ne		
noscapine	ne		
papaverine	ne		
pentazocine	ne		
phenazocine	ne		
prilocaine	ne		
procaine	ne		
delta-9-THC	ne		

*ne = not eluted with K' below 30

however a range of basic, neutral and acidic drugs were examined. Also of interest was the possibility of another compound having suitable chromatography on the same system, such that it might be used as an internal standard if the extraction scheme developed had poor recovery.

2.5.15.2. Method

Standard solutions (10^{-2}g/l) of the drugs were prepared in 5% methanol in distilled water. A drop of HCl or KOH was added to increase solubility as required. 20ul of the solution was injected as before. The eluent was monitored by UV and ED detection.

2.5.15.3. Results and discussion

The traces for those drugs which chromatographed are shown in figure 2.23.. Details of all drugs tested for interference are found in table 2.19.. It is evident that the system described will be selective for morphine and nalorphine, both of which chromatograph with similar efficiency, differing only in the character of the side chain on the basic nitrogen atom (see figure 2.10.). Those opiates lacking a free hydroxyl, have no electrochemical behaviour at this potential (+0.870 volts) as demonstrated previously (2.4.7.). They also eluted later than morphine and nalorphine and had much broader peaks. The obvious choice of compound for use as an internal standard is nalorphine. It has a good efficiency, electrochemical properties at this voltage, and has a similar pKa value to morphine, suggesting that it is likely to extract from biological matrices

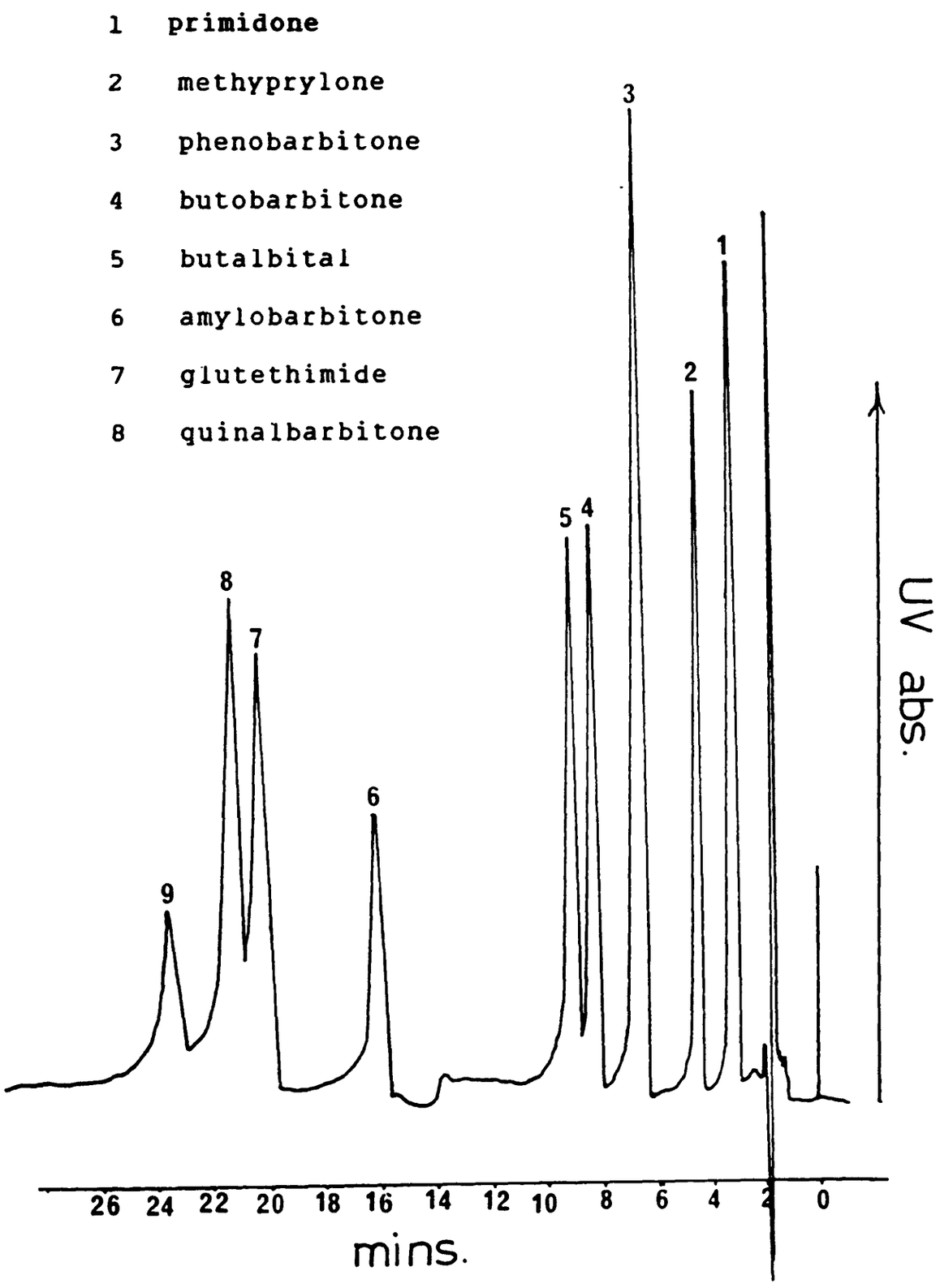


FIGURE 2.23.

CHROMATOGRAPHY OF A RANGE OF ACIDIC DRUGS ON THE HPLC SYSTEM DEVELOPED FOR MORPHINE

under the same conditions.

The system as described coincidentally provides a good separation for several acidic drugs (figure 2.23.). This separation is possible on reversed phase material without the use of ion-pairing agents (Battah, 1986). These acidic compounds are free from interference with the silanol sites on the stationary phase and will therefore chromatograph well on reversed phase columns with a very wide range of solvent systems and without the requirement for an ion pairing agent.

Whilst these acidic drugs would be unlikely to extract under the same conditions as morphine, and generally have no electrochemical properties, the use of this system with dual detectors (ED and UV) would provide a single system for the measurement of all those compounds whose capacity factor is quoted in table 2.23..

The one other compound which appears to display electrochemical properties at this potential is primidone. Most acidic sedatives are highly oxidised and would not be expected to have further oxidative behaviour. Primidone however can be prepared by the electrolytic reduction of phenobarbital (Boon et al., 1952; Bogue and Carrington, 1953).

The reverse process, shown in figure 2.24., is a likely mechanism to account for this observation.

2.5.15.4. Conclusion

The reversed phase ion paired HPLC method for morphine described above appears to be quite selective for morphine and its N-allyl analogue, nalorphine. Other similar compounds (codeine and oxycodone have the same skeleton, but lack the free hydroxyl,

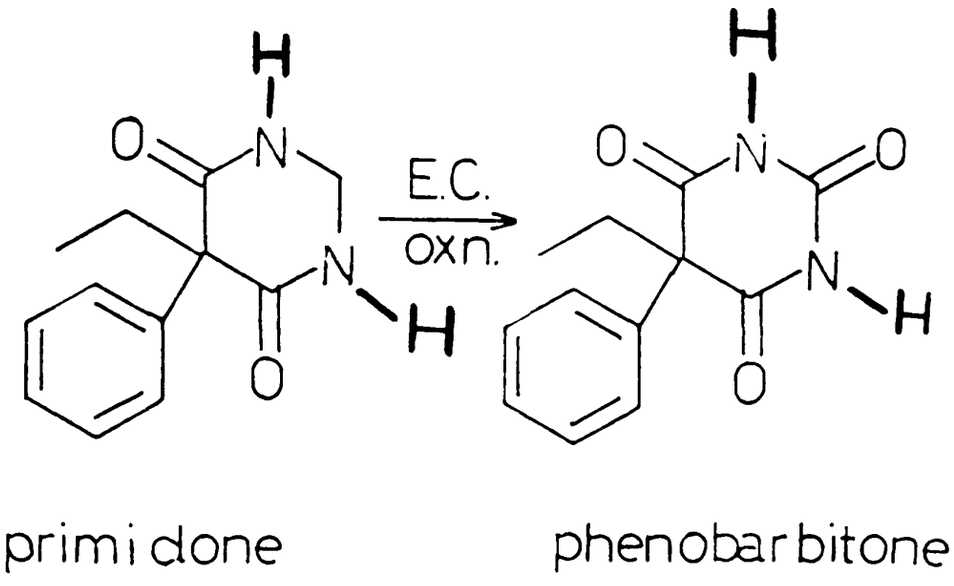


FIGURE 2.24.
ELECTROCHEMICAL OXIDATION OF PRIMIDONE
TO PHENOBARBITONE

the reason both for their poor chromatography and lack of electrochemical activity at 0.870 volts.

It has been shown that electrochemical detection has a high degree of selectivity for morphine. Nalorphine is the most appropriate internal standard for this assay.

2.5.16. CONCLUSIONS ON MORPHINE CHROMATOGRAPHY

The above sections have illustrated the developement of a suitable HPLC system for the chromatography of morphine and a likely candidate as an internal standard, nalorphine.

The original aims (1.5.1.1.) of producing an efficient, rapid and sensitive chromatographic system for morphine have been achieved but only with the use of an ion paired chromatographic system.

This has the dissadvantage that it may lead to lower detection limits caused by electrode fouling, and has introduced the presence of a system peak.

It has however avoided changing the nature of the stationary phase, and has produced a system with sensitivity in the range required to measure levels of morphine likely to be encountered in forensic applications - particularly in biological fluids.

2.6. SAMPLE PREPARATION FOR MORPHINE ANALYSIS BY HPLC

2.6.1. AMMONIUM BICARBONATE EXTRACTION PROCEDURE

2.6.1.1. Introduction

Methods for the extraction of morphine from biological fluids were reviewed earlier (2.3.).

For most analyses involving HPLC, a clean-up and/or concentration step is required.

The method currently favoured for extracting basic drugs from blood and liver homogenate is based on that described by Horning et al., (1974). The extracts from this procedure were examined earlier to assess their suitability for LCED (2.5.5.). Having altered the conditions to a system where efficient chromatography is possible with a higher acetonitrile content (20% cf. 8%), the suitability of this extract was examined again.

2.6.1.2. Method

A variety of samples were spiked with a known amount of morphine (10^{-4}g/l, unless otherwise stated).

These were water, plasma (obtained from centrifugation of expired transfusion blood), fresh transfusion blood and a sample of the latter which had been left exposed to the air, resealed and left for about two weeks.

An appropriate blank was analysed in tandem with all samples to check for interference.

These were extracted using the following procedure:

Sample (1ml) and nalorphine HCl, (10^{-4}g/l, 1ml) as internal standard were added to a 15ml screwcap tube.

This was saturated with freshly ground ammonium carbonate (approx 1g). Ethyl acetate (8ml) was added, the tube was sealed with a PTFE lined cap. The contents were thoroughly mixed on a vortex mixer (approx. 20secs), and the tube centrifuged at 3000 rpm until two layers formed.

The ethyl acetate was aspirated and transfered to a second 15ml screwcap tube containing 3ml 0.1 N H_2SO_4.

The contents were vortex mixed (approx. 20secs) and centrifuged at 3000 rpm.

The ethyl acetate layer was aspirated and discarded.

The aqueous residue was saturated with freshly ground ammonium carbonate (approx 1g). Ethyl acetate (8ml) was added and the contents vortex mixed (approx. 20secs).

The ethyl acetate layer was aspirated and transfered to a tapered centrifuge tube. This was evaporated to dryness under a stream of Oxygen Free Nitrogen (OFN) at 50°C.

The residue was reconstituted in 100ul of the mobile phase and 20ul injected into the HPLC.

HPLC conditions were as described in section 2.5.12..

2.6.1.3. Results and discussion

Problems encountered with this extraction procedure included the formation of an emulsion in the tube after vigourous mixing. This occured only with blood samples, not with plasma. It was unpredictable and seemed to vary from extraction to extraction. The formation of an emulsion may have been due

either to an excess of ammonium carbonate, or to the particle size of the ground material. It was difficult, especially with coagulated, viscous or putrified blood samples, to determine when the saturation point was reached.

Ammonium carbonate was purchased in rock form as the polymer, ammonium carbamate ($NH_2CO_2NH_4$) with a surface covering of ammonium bicarbonate (NH_4HCO_3) (Parkes, 1939). This was finely ground immediately prior to use, to expose the carbamate and added to water where the following reaction takes place, releasing ammonium carbonate, and forming a buffered solution.

$$NH_2CO_2NH_4 + H_2O \longrightarrow 2[NH_4^+] + [CO_3^{2-}] + 2H^+ + 2OH^-$$

The reagent must be ground just before use, otherwise the outer layer of ammonium bicarbonate reforms by loss of ammonia, and the buffer will not form.

The grinding of ammonium carbonate was done manually with a pestle and mortar. The reagent was unpleasant to use in the open laboratory, due to the release of ammonia.

The absolute extraction efficiencies for morphine ($M_{eff.}$) and nalorphine ($N_{eff.}$) were calculated by comparison of peak heights with an unextracted standard. The results are given in table 2.20.. The relative extraction efficiency ($M_{eff.}/N_{eff.}$) is also calculated. This is found to be quite consistent for all sample types, ranging from 1.00 ± 0.06 to 0.92 ± 0.09, for aqueous standards and aged blood respectively. The extraction was performed five times.

TABLE 2.20.

EXTRACTION EFFICIENCIES FOR MORPHINE AND NALORPHINE FROM A VARIETY OF MATRICES USING THE PROCEDURE DESCRIBED BY HORNING ET AL, 1974

sample type	$M_{eff.}$	$N_{eff.}$	Rel. eff.
Aqueous std.			
	75	76	0.99
	72	68	1.06
	69	76	0.91
	80	79	1.01
	83	79	1.05
n=5			
average (X)	75.8	75.6	1.00
S.D.	5.7	4.5	0.06
CoV	7.5	5.9	6.1
Plasma std			
	67	69	0.97
	50	52	0.96
	45	44	1.02
	48	53	0.91
	54	58	0.93
n=5			
average (X)	52.8	55.2	0.96
S.D.	8.5	9.2	0.04
CoV	16.3	16.7	4.2
Fresh blood			
	45	48	0.94
	49	46	1.06
	27	33	0.82
	37	40	0.93
	40	42	0.95
n=5			
average (X)	39.6	41.8	0.94
S.D.	8.4	5.8	0.09
CoV	21.2	14.0	9.35

TABLE 2.20. contd.

sample type	$M_{eff.}$	$N_{eff.}$	Rel. eff.
Aged blood			
	15	18	0.83
	20	22	0.90
	23	27	0.85
	32	33	0.97
	38	36	1.06
n=5			
average (X)	25.6	27.2	0.92
S.D.	9.2	7.5	0.09
CoV	36.2	27.4	9.9

Blank samples

The blank water and plasma samples were found to contain no interfering peaks in the region of morphine and nalorphine. Some of the fresh blood samples did however produce late eluting peaks, and the aged blood samples produced many more peaks in the region of the chromatogram of interest.

Aqueous standards

The extraction efficiencies for morphine and nalorphine ($E_{morph.}$ and $E_{nal.}$ respectively) for the aqueous standards were 75.8% ± 5.7, and 75.6% ± 4.5 respectively. The relative recovery was 1.00 ± 0.06. There were no interfering peaks present.

Plasma

Extracts from the plasma samples were clean and there were few or no other peaks in the chromatogram. The absolute extraction efficiencies were $M_{eff.}$ = 52.8% ± 8.5, and $N_{eff.}$ = 55.2% ± 9.2%. There were no interfering peaks present.

Whole blood

In extracts from whole blood samples there was some interference from small peaks in the same region of the chromatogram as morphine was eluted, thus making reliable quantitation difficult. The low extraction efficiency ($M_{eff.}$ = 39.6% ± 7.5, $N_{eff.}$ = 41.8 ± 5.3), meant that the procedure had a poor sensitivity.

Whole blood samples (fresh and aged) were susceptible to emulsion formation by this procedure which probably accounts for the low absolute recovery.

This was initially thought to be caused by the ammonium carbonate not being sufficiently ground. Whilst this was thought to be true in general, the principle factor in emulsion formation was found to the degree of mixing of any blood/ammonium carbonate/ethyl acetate mixture.

Slow mixing on a rocking rolling mixer did not achieve homogeneity of the liquid part. Vigourous mixing was required for this and emulsification followed rapidly.

Emulsion formation was independent of the volume of solvent used. The emulsion could occasionally be broken up by stirring and recentrifuging the sample. This was unreliable and never completely successful.

As the relative recoveries for the extraction of fresh blood and aged blood were similar (0.94 ± 0.09, and 0.90 ± 0.09 respectively), the difference in absolute efficiencies is probably due to matrix effects.

Aged blood samples appeared to have more coextracted material in them (both from the appearance of the extract and the appearance of the chromatogram).

It was difficult to consistently reproduce the degree of aging in the blood samples, and there was considerable sample to sample variation, confirming the need for an internal standard.

2.6.1.4. Conclusion

In spite of the low recoveries compared to published reports (Horning et al. 1974, see table 2.2.), this was considered a good general method for the analysis of morphine in a variety of samples. The object of further work would therefore be to improve the efficiency and selectivity of the procedure, and if possible to simplify it.

In order to avoid problems arising from inconsistencies between aged samples, it was decided that further developement work should be done on freshly prepared blood standards, one to three days old, and the optimum extraction procedure then applied to aged or post mortem blood samples.

2.7. IMPROVEMENTS TO THE EXTRACTION PROCEDURE

2.7.1. Introduction

The main problems with the above extraction procedure were sample loss through emulsion formation, poor recovery and carry over of material into the final extract. These problems were investigated separately . Firstly methods were examined to avoid emulsion formation, then attempts to improve the efficiency of the extractionby the use of other solvents and changes in solvent volume. Finally methods of keeping particulate material out of the final extract were investigated.

For the purposes of the following study, fresh blood was used to prepare standards shortly before analysis (1-2 days).

2.7.2. EMULSION FORMATION

2.7.2.1. Introduction

As the ammonium carbonate appeared to be the main cause of this problem, alternative methods of adjusting the pH to a suitable value were sought. Those solutions examined were either in routine use in the laboratory or had been used in methods published elsewhere reviewed in 2.3..

2.7.2.2. Method

Various alkaline solutions were used to alter the pH of the sample. Volumes of 1ml were chosen to keep the volume of the aqueous part as small as possible to improve the yield of the extraction.

This investigation was carried out on blood samples rather than aqueous standards or plasma.

Sample (1ml), buffer (1ml), nalorphine (10^{-4}g/l, 1ml) and ethyl acetate (8ml) were mixed thoroughly on a vortex mixer (approx. 20 secs). The mixture was centrifuged at 3000rpm until separated (approx. 2 mins). The ethyl acetate was aspirated and transfered to a second tube containing 3ml 0.1N H_2SO_4. The rest of the procedure was as before (2.6.1.2.). Each extraction was performed in duplicate and the mean is given.

2.7.2.3. Results and discussion

In the absence of solid ammonium carbonate, no emulsions were formed, even with badly haemolysed blood.

The extraction efficiency for morphine and nalorphine are recorded

TABLE 2.21.

EFFECT OF VARIOUS pH MODIFIERS ON THE EXTRACTION EFFICIENCY OF MORPHINE FROM FRESH BLOOD

Modifier	Meff.	Neff.	M/N
0.1M NaOH	24.6	25.2	97.6
1M NaOH	42.1	43.1	97.6
1M phosphate, pH9	* /	/	/
0.1M Borate buffer pH9	43.4	43.2	100.0
1M Borate buffer pH9	62.5	64.1	97.5
1M Borate buffer pH8.5	60.1	62.0	96.9
1M Borate buffer pH9.5	61.4	61.8	99.3

*Unsuitable for analysis

in table 2.21.. Phosphate buffer was found not to give very good recovery, the exact value was not determined due to interference. NaOH on its own did give a relatively clean extract, but the efficiency was poor. This may be due to deprotonation of the phenolic group on morphine by the strongly alkaline NaOH solution pH 11-12. This would make the morphine negatively charged and hence more soluble in the aqueous phase. As morphine is amphoteric in nature the pH range used for the extraction obviously requires to be carefully controlled or buffered.
Borate buffers gave the best extraction efficiency, and of these the more concentrated worked notably better.
There was no appreciable difference between borate buffers pH 8.5 to 9.5.

2.7.2.4. Conclusion

Emulsion formation was successfully avoided, and the extraction efficiency improved considerably. The buffer selected for further work was 1M borate buffer, pH 9.0, which gave a mean efficiency of 62.5%.

2.7.3. SOLVENT SELECTIVITY

2.7.3.1. Introduction

Some of the interfering peaks still appeared in the chromatograms. Whilst these appeared sporadically, older samples were notably worse. Alternative extraction solvents were used in an attempt to improve the selectivity of the extraction.

2.7.3.2. Method

The extraction procedure was carried out as above. Various solvents were used in place of ethyl acetate. Most of these were selected on the basis that they had been reported in the literature as giving good recoveries for morphine (tables 2.2. & 2.3.). Extractions were performed in duplicate and the mean is given.

2.7.3.3. Results and discussion

The recoveries of morphine and nalorphine are shown in table 2.22..

Although there is evidence of improved extraction efficiency for morphine with the ethyl acetate/isopropyl alcohol solvent (67.4%), it was noted that a large amount of co-extracted material which, whilst not interfering with the assay, increased the length of the run time to about 45 minutes.

The coextraction of material other than the analyte is a greater problem when mixed solvents or salting-out procedures are used. This can improve the efficiency but usually reduces the selectivity of the extraction procedure.

2.7.3.4. Conclusion

The use ethyl acetate whilst having a lower apparent efficiency (60.4%) than the mixed solvents examined, gave a better quality extract and was therefore retained, and modifications were made to the volume of solvent used in the extraction procedure.

TABLE 2.22.

EFFECT OF VARIOUS EXTRACTION SOLVENTS
ON THE EXTRACTION EFFICIENCY OF MORPHINE FROM FRESH BLOOD

Solvent	Meff.	Neff.	M/N
EtOAc	60.4	62.1	97.1
CH_2Cl_2	11.0	10.2	107.8
$CHCl_3$:IPA (9:1)	60.4	61.5	98.2
EtOAc:IPA (9:1)	67.4	69.2	97.4

2.7.4. SOLVENT VOLUME

2.7.4.1. Introduction

No attempt had been made to examine if the extraction efficiencies observed could be improved by increasing the volume of the extraction solvent.

The transfer of a solute between two media is limited by its partition coefficient and the relative volumes of the two immiscible solvents (Alberty and Daniels, 1976). The pH may also play an important part in this partition if the analyte is ionisable. The effect of the pH has been examined above.

Having shown that ethyl acetate is a suitable solvent and decided to continue with its use, the relative volume of the solvent was changed to assess the effect this might have on the extraction efficiency.

2.7.4.2. Method

In an effort to further improve the extraction efficiency, two measures were taken. The volume of the aqueous part in the first step of the extraction was reduced by using 100ul of internal standard (10^{-3}g/l), instead of 1ml.

The ethyl acetate extraction was performed twice with two volumes of 5ml. These were then combined in the second tube. The rest of the procedure was as before (2.7.3.2.).

2.7.4.3. Results and discussion

The results are shown in table 2.23.. The absolute recovery for morphine was found to be 64.6% ± 5.9 (n=5). This represents an improvement on the previous absolute recovery (60.4%) and there was no significant change in the relative efficiency which is 0.94 ± 0.06 (cf. 0.98).

Performing two extractions did increase the analysis time, but the increase in extraction efficiency could be significant if only small sample volumes were available, or if low levels were present.

2.7.4.4. Conclusion

Replacing ammonium carbonate with a buffer, in the second extraction step was considered, but this would have involved increasing the volume of the aqueous phase and was considered a disadvantage, so this was not investigated.

Using two extraction volumes in place of one, and decreasing the volume of the aqueous fraction has increased the efficiency of the procedure.

The use of 100ul of internal standard solution in place of 1ml was also incorporated into the general procedure.

2.7.5. CARRY OVER OF MATERIAL

2.7.5.1. Introduction

The presence of greasy semi-solid matter in the dried down residue caused some concern, as continually injecting this onto the column was found to lead to a build-up in column back

TABLE 2.23.

EFFECT OF CHANGES IN SOLVENT VOLUMES ON THE EXTRACTION EFFICIENCY OF MORPHINE FROM FRESH BLOOD

	Meff.	Neff.	Rel. eff.
	74	72	1.03
	66	73	0.90
	63	68	0.93
	59	65	0.89
	61	65	0.94
n=5			
Average (X)	64.6	68.6	0.94
S.D.	5.9	3.78	0.06
CoV	9.1	4.8	5.5

pressure. Some of the extracts also appeared to contain a white crystalline residue, thought to be ammonium sulphate formed in the final extraction step. This may either be slightly soluble in the ethyl acetate, or result from some of the aqueous layer being carried over to the evaporation stage. Extracts containing this material gave rise to a large negative peak in the chromatogram just after the system peak. The problems of carbonate in the sample and the presence of the so called "carbonate dip" peak have been reported by other workers (Sundén et al., 1984; Okada and Kuwamoto, 1984). Whilst of no real interest in itself, it can interfere with the analyte peak, or in this case with the internal standard.

The optimum efficiency of the morphine on this HPLC system was found to be N = 600. Whilst an improvement on previous solvent systems used, this is low by most standards, but is limited by the interaction of basic compounds like morphine with the stationary phase. Any deterioration in efficiency which might be caused by a pre-column, was considered unacceptable. Whilst the use of a pre-column would protect the analytical column, the build up in back pressure would not be avoided. In addition, as the columns used in this study were packed in the laboratory, it was just as easy to repack the top of the analytical column as to do likewise to a short precolumn.

Other methods of preventing carry over of material were therefore considered. Three methods are in general use for preventing aqueous carry over from

emulsions or organic liquids containing small amounts of water.

The first, freezing the residual water in a dry-ice/acetone bath

and pouring off the solvent, was tried but was considered uneccessarily complex and the solvent still had to be filtered to remove particles of ice.

The other methods were therefore considered. These are the use of phase separating filter paper and filtering the organic solvent through anhydrous sodium sulphate. It was expected that more of the solvent would be retained by the second procedure, and that this would consequently lower the extraction efficiency. For that reason the use of phase separating filter paper was considered.

Phase separating filter paper is prepared by treating ordinary filter paper with a silicone wax in the presence of a catalyst (Whatman applications notes, 1984). This process makes it impermeable to aqueous solvents, but allows less polar organic solvents to pass through. The use of phase separating filter paper is avoided for some toxicological studies, particularly where GC and GCMS are being used. The catalysts used in the impregnating process and silicones of medium carbon number washed from the paper, can give rise to spurious peaks on GC (Whatman product notes, 1982). The degree of interference in LCED caused by these effects was checked before the phase separating paper was used in this assay.

It was first established that no interference would arise from the filter paper.

2.7.5.2. Method

Ethyl acetate (20ml) was filtered through fluted circles (10cm) of Whatman PS-1 filter paper, and collected.

This was evaporated to dryness at 50°C under OFN, and reconstituted in 50ul of the mobile phase. 20ul were injected into the HPLC as before.

As no peaks with K' over 2 were noted it was possible to use the phase separating paper in the extraction procedure.

Four blood samples were extracted as before (2.7.4.2.). The final ethyl acetate layers were aspirated and, prior to being transfered to a tapered centrifuge tube, two were first filtered through pre-wet PS-1 filter paper. The other two samples were not filtered. Each sample was evaporated to dryness under a stream of OFN at 50°C.

The residue was reconstituted in 100ul of the mobile phase and 20ul injected into the HPLC.

2.7.5.3. Results and discussion

The absolute extraction efficiency of morphine for those cleaned up using phase-separating filter paper was 50 and 59% (54.5% av.). For those without, this was higher 61 and 66 (63.5% av.).

Some loss in efficiency would be expected due to a small amount of the ethyl acetate being retained in the filter paper. Pre-wetting the filter paper with ethyl acetate should prevent this to some extent.

The physical appearance of the extract was improved and none of the waxy material or salt noted previously was present in the filtered extracts.

In the chromatogram there was no evidence of the carbonate dip, and there was a generally better appearance to the chromatogram.

2.7.5.4. Conclusion

As no interference from the paper itself was encountered, it was decided that the loss in efficiency of the extraction could be justified in terms of improved column lifetime and extract purity when phase separating filter paper was used.
A phase separating step was therefore incorporated into the procedure.

2.7.6. POSSIBLE USE OF SINGLE STEP EXTRACTION

2.7.6.1. Introduction

As the extracts from the above procedure were very clean, it was decided to examine the quality of the extract proiduced if the back extraction into 0.1N H_2SO_4 was ommitted from the extraction procedure.

2.7.6.2. Method

The procedure described below represents the first part of the extraction procedure described in 2.7.5.2..
Sample (1ml, 10^{-4} g/L morphine), 1M pH9 borate buffer (1ml), internal standard (100ul) and ethyl acetate (8ml) were added to a 15ml screwcap tube and mixed thoroughly on a vortex mixer (approx. 20 secs). The sample was then centrifuged at 3000rpm and the ethyl acetate layer removed. This was filtered through pre-wet phase separating paper and then evaporated to dryness under OFN at 50°C. The residue was reconstituted in 100ul and 20ul were injected.

2.7.6.3. Results and discussion

The extract was found to be unsuitable for the analysis of whole blood samples due to the presence of a small number of large peaks in the early part of the chromatogram. Extracts obtained from plasma samples were however suitable for analysis and gave absolute extraction efficiencies of 84% ± 5.2 (n=4).

2.7.6.4. Conclusion

The acidic back extraction was found to be necessary with whole blood samples to provide an extract sufficiently clean for LCEC on this system.

Plasma samples were however rapidly and conveniently extracted by this procedure. Although plasma is rarely encountered in forensic applications, this may be of use for clinical studies.

2.7.7. SUMMARY OF OPTIMISED EXTRACTION PROCEDURE

2.7.7.1. Introduction

The extraction procedure examined initially (2.6.1.2.) has been substantially modified, and the absolute extraction efficiency for whole blood samples (fresh) has been improved from 41.8% ± 4.1, to a maximum of 69% ± 4.0.

A compromise was accepted in the use of phase separating filter paper which gave cleaner extracts and slowed the rate of column fouling, but lowered the efficiency of the extraction.

The procedure chosen for routine use is summarised below.

Sample (1ml), pH9 1M Borate buffer (1ml) and internal standard

(100ul) were added to a 15ml screwcap tube. Ethyl acetate (8ml) was added, the tube was sealed with a PTFE lined cap. The contents were thoroughly mixed on a vortex mixer (approx. 20secs), and the tube centrifuged at 3000 rpm.

The ethyl acetate was aspirated and transfered to a second 15ml screwcap tube containing 3ml 0.1 N H_2SO_4.

The contents were vortex mixed (approx. 20secs) and centrifuged at 3000 rpm.

The ethyl acetate layer was aspirated and discarded.

The aqueous residue was saturated with freshly ground ammonium carbonate (approx 1g). Ethyl acetate (8ml) was added and the contents vortex mixed (approx. 20secs). The ethyl acetate layer was aspirated and passed through pre-wet PS-1 filter paper into a tapered centrifuge tube. This was evaporated to dryness under a stream of OFN at 50°C.

The residue was reconstituted in 100ul of the mobile phase and 20ul injected into the HPLC system described in section 1.5.12..

Sample chromatograms obtained from a blank blood sample, a standard sample (10^{-4}g/L) and an extract from an authentic post mortem sample, are shown in figure 2.25.

The error obtained in the calculated level in an unknown sample when using this extraction procedure followed by HPLC is low (S.D. = 5.5%, as measured in section 2.7.4.).

Some disadvantages of the procedure are that it is time consuming, using three or more successive extraction steps, and requires the use of ammonium bicarbonate which is unpleasant to use in the open laboratory and has to be freshly ground before use.

The possibility of transferring this procedure to a solid phase extraction method was considered.

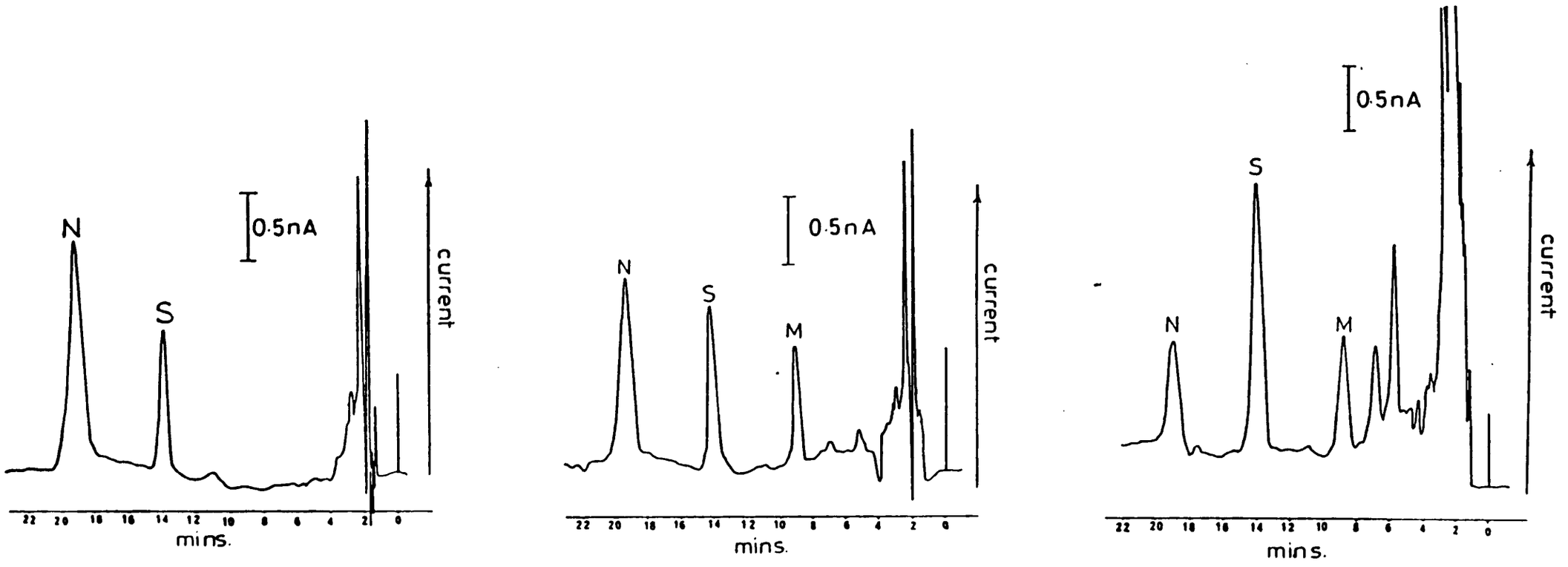


FIGURE 2.25.

CHROMATOGRAMS RESULTING FROM EXTRACTS FROM (a) BLANK BLOOD, (b) STANDARD BLOOD SAMPLE (10^{-4}g/L) AND (c) AUTHENTIC POST MORTEM BLOOD SAMPLE

2.8. OTHER EXTRACTION PROCEDURES

2.8.1. Introduction

Examples in the literature of rapid sample preparation using adsorbent or absorbent materials and their advantages over liquid/liquid extraction (reviewed above 2.3.4.), prompted investigation of their suitability for post-mortem blood samples.

2.8.2. ADSORPTION EXTRACTION PROCEDURES

2.8.2.1. Introduction

The use of activated silica supports with a variety of bonded phases of varying selectivities, has introduced a high degree of selectivity to sample preparation (Sep Pak application notes 1984; Harkey and Stolowitz, 1984; Stewart et al., 1984; Bond elut applications notes; 1985). Methods reported for the extraction of morphine were reviewed above (2.3.4.), and it was decided that the approach most suitable for the extraction of basic drugs was the use of a C_{18} cartridge. It was considered reasonable that the optimum conditions for the control of ionisation and elution would be similar to those found for simple liquid/liquid extraction as examined in section 2.3.3..
These conditions were therefore used as a starting point for the investigation of solid phase adsorbance procedures.

2.8.2.2. Method

Commercially prepared C_{18} (Sep Pak) cartridges were activated as recommended by the manufacturers, by washing with

methanol (4ml) and water (4ml).

The sample (1ml, 10^{-4}g/L morphine) was mixed with 1M pH9 borate buffer (1ml) and internal standard (100ul), in a sample tube. This was thoroughly mixed on a vortex mixer, and loaded onto the cartridge under reduced pressure from a water suction pump.

The cartridge was washed with a wash solvent and morphine eluted in an eluting solvent.

The solvent was evaporated to dryness under OFN and the residue reconstituted in 100ul of the mobile phase.

2.8.2.3. Results and discussion

The chromatograms were assessed for recovery of morphine where possible, and for the suitability of the extract for analysis by LCEC. This is recorded in table 2.24..

The only extracts which were suitable for analysis were those from the plasma sample, and whilst the morphine peak was quite clear at this concentration (10^{-4} g/L), there were a number of smaller peaks in the same region which may have interefered at lower concentration.

Some interference was noted even in the reagent blanks. Thorough pre-washing of the cartridges with the elution solvent did not completely eliminate this, although it did appear to cut down the interference.

Having one of the phases in a sealed cartridge, whilst increasing the ease of sample handling, made it difficult to assess when all the interstitial aqueous phase had been removed, prior to the elution step. It was found that the amount of interference was reduced if the cartridge was thoroughly dried under reduced

TABLE 2.24.

RECOVERY OF MORPHINE FROM SEVERAL MATRICES WITH ADSORBENT CARTRIDGES USING DIFFERENT WASH AND ELUTING SOLVENTS

Sample	Wash	volume	Eluent	Recovery %	Suitability
Aq. blk.	water	2	EtOAc	some interference.	
	1M pH 9 borate	2	EtOAc	some interference	
Aq. Std.	water	2	EtOAc	low	poor
	pH 9 borate 1M	2	EtOAc	84%	good
Plasma	water	2	EtOAc	low	poor
	pH 9 borate 1M	2	CH_2Cl_2	* -	poor
	pH 9 borate 1M	2	EtOAc	80%	?good
Blood	water	2	EtOAc	-	poor
	pH 9 borate 1M	2	EtOAc	-	poor
	pH 9 borate 1M	4	EtOAC	-	poor
	pH 9 borate 1M	2	$CHCl_3$/IPA 9:1	-	poor
	pH 9 borate 1M then $CHCl_3$	2 2	EtOAC	-	poor

* - = not suitable for LCEC

pressure, after the wash step and prior to elution of the analytes. Even so, some aqueous material often appeared in the eluate and this had to be removed by phase separating paper. This reduces the extraction efficiency as demonstrated above (section 2.7.5.).

The fact that a single extraction step is used probably adds to the poor suitability of this method for whole blood.

One of the advantages of these cartridges is their facility for rapid sample preparation. This benefit is lost by the requirement of a second clean up extraction.

The possibility of using a second cartridge for this clean up step was considered, but was not investigated.

2.8.2.4. Conclusion

It had been proposed that the increased selectivity of the adsorbent might make a single extraction sufficient. This was found not to be the case and a subsequent clean up step would be required for extracts from whole blood samples in order for them to be suitable for LCEC.

These extraction procedures were rapid and convenient for handling samples of this type. The extracts obtained however were not suitable for LCEC.

2.8.3. AN ALTERNATIVE ADSORPTION EXTRACTION PROCEDURE

2.8.3.1. Introduction

The procedures used above for the extraction of morphine using bonded phase cartridges gave poor results. It was assuned that this was due to the lack of selectivity of the process, as when liquid/liquid extraction was used a thorough clean up step was required.

One method of altering the selectivity is to include an ion pairing agent in the eluent. This was done to some effect in optimising the HPLC of morphine (2.5.9.). A method described for the isolation of morphine from plasma using a sulphonic acid extraction procedure on ODS (Bond elut) cartridges (Moore et al., 1984 (ii)) was investigated.

2.8.3.2. Method

The procedure was followed directly from the above reference. An ODS bond elute cartridge was attached to a vacumn line. The cartridge was primed with methanol (2ml) and water (2ml).

The sample (1ml, 10^{-4}g/L morphine), 0.2M pH9 borate buffer (1ml), and 0.2M heptane sulphonic acid (1ml) were mixed thoroughly on a vortex mixer. The mixture was applied to the top of the cartridge and sucked through under reduced pressure.

the cartridge was washed through with distilled water (6ml), and when dry, the morphine was eluted with methanol (2ml).

The methanol was evaporated to dryness under OFN at 65^{o}C and reconstituted in 100ul of mobile phase. 20ul were analysed by LCED.

2.8.3.3. Results and discussion

The procedure was examined for suitability with plasma samples and whole blood samples. As before, plasma samples gave a suitably clean extract with an absolute extraction efficiency for morphine of 74% ± 6 (n=4).

This is comparable with other methods described above but the recovery is short of that reported in the literature (greater than 95%, Moore et al., 1984(ii)).

Blood samples analysed in this way gave contaminated extracts which were unsuitable for LCEC.

2.8.3.4. Conclusions

In the above sections two adsorbent phase cartridges have been examined in normal operation and with the use of an ion pairing agent in an attempt to improve the selectivity of the extraction procedure. Although neither proved suitable for preparing clean extracts from whole blood samples, the bond elut cartridges were preferred, as they retained less of the aqueous layer after drying, and none appeared to be carried over in the eluting solvent. It appears that bonded phase cartridges are of little use for the analysis of whole blood samples other than when plasma may be obtained.

A second clean up step would be required, offsetting some of the advantages gained in their use.

2.8.4. ABSORBENT EXTRACTION PROCEDURES

2.8.4.1. Introduction

The literature includes two descriptions of the extraction of basic drugs from blood (Breiter et al., 1975, von Meyer et al., 1975). The material used in these reports was extrelut, a diatomaceous earth absorbent. Its ability to produce an extract suitable for LCEC was examined.

2.8.4.2. Method

Short extrelut columns were prepared using glass syringes. A frit was formed with a plug of glass wool or glass fibre matting at the bottom. The pipette was tap-filled with extrelut material. The capacity of the columns was found by loading them with increasing volumes of blood/water (1:1).
The maximum recommended loading is that which leaves the bottom 10% of the packing material dry (Christensen, 1984).
Sample (1ml, 10^{-4}g/L morphine) and buffer (1ml), were mixed thoroughly on a vortex mixer in a sterile polypropylene syringe.
This was then applied to the top of a prepared extrelut column.
It was allowed to equilibrate for 10 minutes, and then eluted with a solvent (2ml). The solvent was collected and evaporated to dryness under OFN at 65^{o}C.
The residue was reconstituted in 100ul of the mobile phase and 20ul injected. The sample types and solvents used and recoveries obtained are shown in table 2.25..

TABLE 2.25.

RECOVERY OF MORPHINE FROM SEVERAL MATRICES WITH ABSORBENT CARTRIDGES USING DIFFERENT BUFFERS AND ELUTING SOLVENTS

sample	buffer	solvent	recovery
aqueous standard	0.1M NaOH	ethyl acetate	74%
	0.1M NaOH	CH_2Cl_2/IPA (9:1)	76%
	0.1M Borate (pH 9)	ethyl acetate	72%
	0.1M Borate (pH 9)	CH_2Cl_2/IPA (9:1)	60%
plasma	0.1M NaOH	ethyl acetate	69%
	0.1M NaOH	CH_2Cl_2/IPA (9:1)	* -
	0.1M Borate (pH 9)	ethyl acetate	72%
	0.1M Borate (pH 9)	CH_2Cl_2/IPA (9:1)	-
blood	0.1M NaOH	ethyl acetate	-
	0.1M NaOH	CH_2Cl_2/IPA (9:1)	-
	0.1M Borate (pH 9)	ethyl acetate	-
	0.1M Borate (pH 9)	CH_2Cl_2/IPA (9:1)	-

* - unsuitable for analysis by LCEC

2.8.4.3. Results and discussion

The loading of the columns was established as 2ml sample per 6ml extrelut material. The wash step in the adsorbent extraction procedure examined above (sections 2.8.2. and 2.8.3.), is avoided here by assuming that the water which comprises the aqueous stationary phase will retain all material not desired in the extract by a judicious choice of pH and solvent. Recoveries of around 70% were attained for extraction of morphine from aqueous standards. Blank aqueous samples produced no interference.

With one combination of solvent and buffer (borate buffer and ethyl acetate), it was possible to measure morphine in a plasma sample without interference (absolute recovery = 72%).

One other combination of solvent and buffer produced an extract which was suitable for analysis by LCEC, but the presence of a shoulder on the morphine peak made quantitation difficult.

None of the combinations of extracting solvent and buffer produced a suitable extract from whole blood samples. This confirms results obtained in the single step liquid/liquid extraction procedure.

On inspection of the results table it was evident that the mixed dichloromethane/isopropyl alcohol extraction solvent produced interfering peaks in all the biological samples extracted. As little interference was noted in the reagent blanks (aqueous standards) it was assumed that this had arisen from the biological material itself.

Because of the poor quality of the extracts from whole blood samples, this procedure was not tested on aged blood samples nor

used in routine analysis.

The plasma extracts obtained by this procedure were relatively clean.

2.8.4.4. Conclusion

The interference noted in extracts from blood samples was unfortunately in the region of the morphine peak, and the selectivity of the LC system would require to be altered in order to make these extracts suitable for analysis without further clean-up.

2.8.5. DISCUSSION AND CONCLUSIONS ON SOLID PHASE EXTRACTION TECHNIQUES

The results obtained in this section are discussed below. The attractive aspects of solid phase extraction techniques are the speed of sample preparation, the high efficiencies reported for optimised extraction procedures, and the convenience for sample handling. These must be balanced against the cost, selectivity of the procedure and the quality of the extract.

With samples of a relatively simple matrix (plasma, serum, aqueous standards), these procedures have been shown above to have a generally suitable for LCED. In cases where the sample matrix is complex, and heavily contaminated with a wide range of putrefactive material, the selectivity required is much narrower. This has not been achieved for single step extraction in the above study.

Further extract purification is required. Subsequent classical liquid/liquid extraction would nullify some of the advantages gained from the use of these rapid procedures.

The use of a second adsorbent/absorbent step as a clean up procedure is a possibility, but this would increase the cost, and would require an extensive study to optimise this extraction.

Other alternatives would be to incorporate an in-line sample purification step using pre-columns, column switching and backflushing. These were not available for this study and they would also require extensive optimisation.

As a suitable liquid/liquid extraction procedure had been optimised (2.7.7.), it was decided to concentrate on further characterising this method, and its examining its application to authentic samples.

2.9.1. CONCLUSIONS ON THE EXTRACTION PROCEDURE OF CHOICE

2.9.1.1. Introduction

The liquid/liquid extraction procedure optimised in section 2.7. and summarised in 2.7.7., was found to be suitable for all sample types examined, and was therefore used as a general method. For plasma samples which were occasionally supplied, the method of choice was the extrelut procedure described in section 2.8.4..

2.9.2. CALIBRATION CURVE FOR PREPARED MORPHINE STANDARDS

2.9.2.1. Introduction

An examination of a series of spiked samples was made to establish whether the recovery using the optimised method, described above, was linear over a range of concentrations.

2.9.2.2. Method

A standard solution of morphine (10^{-2}g/l) was prepared in expired transfusion blood. Other standards were prepared by series dilutions. These samples were extracted in duplicate and the mean result calculated against the 10^{-4}g/l standard, by peak height ratios.

2.9.2.3. Results and Discussion

The concentration was calculated by comparison of peak heights based on the mean M/N level for the 0.1ug/ml standard (1.035). The calculated concentrations are given in table 2.26. and shown plotted against the prepared concentration in figure 2.26..

The correlation coefficient is 0.9923 and the correlation is linear over the range examined.

2.9.2.4. Conclusions

Most concentrations encountered clinically or post-mortem would be expected to fall within this range (2.1.3.).

The linearity of this correlation means that a single point calibration should be sufficient to allow an accurate measurement of morphine in an unknown sample.

TABLE 2.26.

CALIBRATION CURVE FOR MORPHINE IN BLOOD ANALYSED USING THE DEVELOPED METHOD

Sample conc. ug/ml	Morphine nA	Nalorphine nA	M/N	Conc. found ug/ml
5.0	163.8	3.2	51.2	4.94
	207.4	4.2	49.4	4.77
	189.6	3.8	49.9	4.82
	147.6	2.9	50.9	4.92
n=4				
Average (X)				4.86
S.D.				0.08
CoV				1.7%
1.0	35.3	3.5	10.1	0.98
	27.8	2.8	9.9	0.96
	24.6	2.4	10.3	0.99
	44.3	4.0	11.1	1.07
n=4				
Average (X)				1.00
S.D.				0.05
CoV				4.8%
0.8	29.8	3.5	8.5	0.82
	17.7	2.1	8.4	0.81
	20.0	2.4	8.3	0.80
	32.7	3.8	8.6	0.83
n=4				
Average (X)				0.82
S.D.				0.013
CoV				1.6%
0.5	8.9	1.8	4.9	0.47
	13.4	2.6	5.2	0.50
	15.4	3.1	5.0	0.48
	17.2	3.3	5.2	0.50
n=4				
Average (X)				0.49
S.D.				0.015
CoV				3.0%

TABLE 2.26. (contd.)

Sample conc. ug/ml	Morphine nA	Nalorphine nA	M/N	Conc. found ug/ml
0.1	4.3	4.0	1.08	0.104
	3.6	3.5	1.03	0.099
	3.3	3.2	1.03	0.099
	3.8	3.8	1.00	0.097
n=4				
Average (X)				0.100
S.D.				0.003
CoV				3.0%
0.05	1.6	3.4	0.47	0.045
	1.2	2.1	0.57	0.055
	1.7	3.0	0.57	0.055
	1.6	2.9	0.55	0.053
n=4				
Average (X)				0.052
S.D.				0.008
CoV				15.4%
0.01	0.2	2.4	0.08	0.007
	0.2	3.4	0.06	0.006
	0.1	1.9	0.05	0.005
	0.3	3.0	0.01	0.009
n=4				
Average (X)				0.007
S.D.				0.002
CoV				25.3%
n=28				
Average (X)		3.08		
S.D.		0.66		
CoV		21.6%		

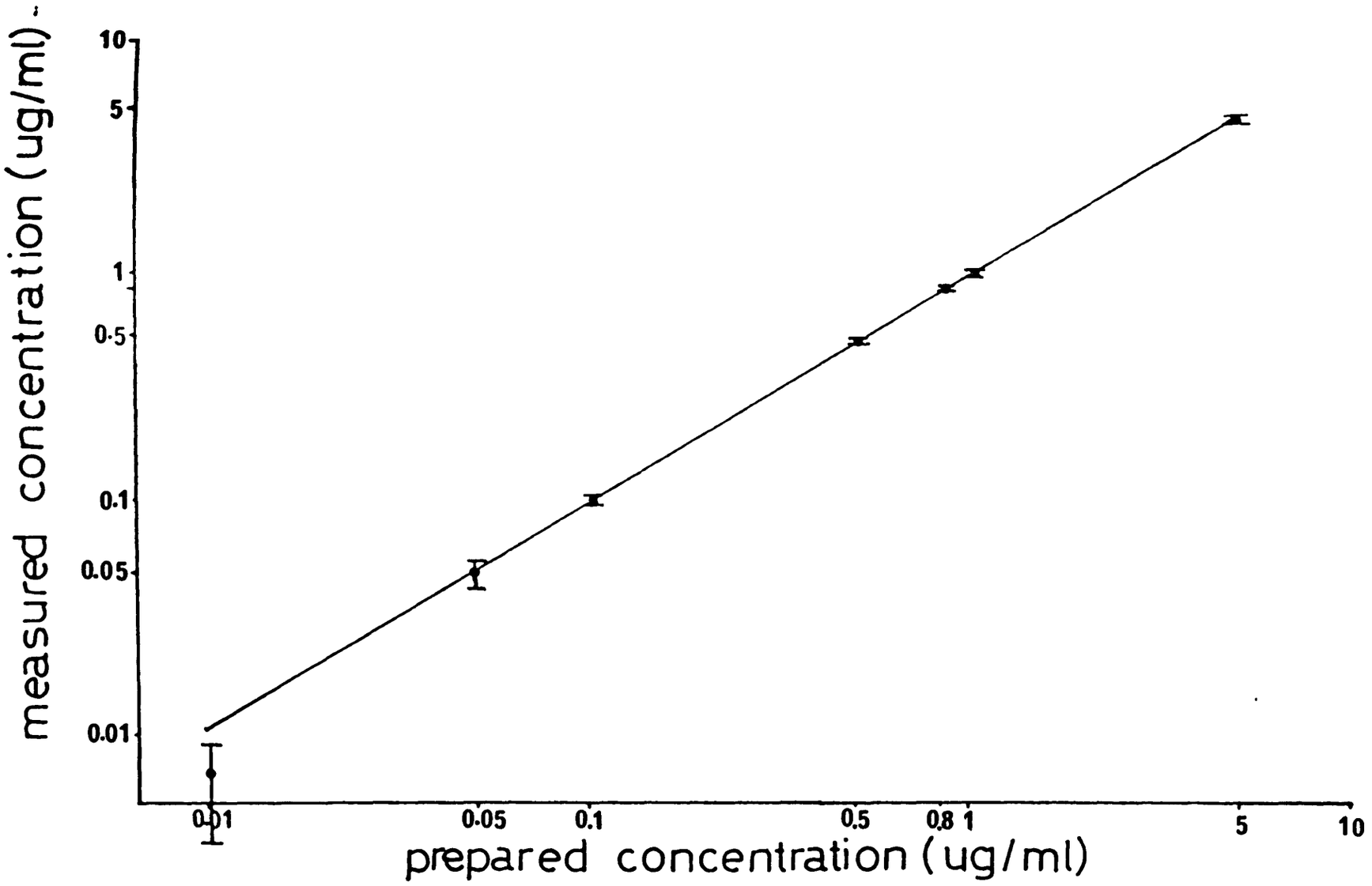


FIGURE 2.26.

CALIBRATION CURVE FOR MORPHINE IN BLOOD

2.9.3. EFFECT OF SAMPLE AGE ON EXTRACTION EFFICIENCY

2.9.3.1. Introduction

As interference with badly haemolysed samples had been encountered throughout the work, and typical post mortem samples are of this type, the effect of aging of a blood sample on the recovery of morphine was investigated.

2.9.3.2. Method

A sample of expired transfusion blood was obtained and a standard of 10^{-4}g/l was prepared. The sample was analysed immediately and left open to the atmosphere for about one day. It was stored at room temperature for the duration of the study. Using the extraction procedure developed above and outlined in 2.7.7., the sample was extracted at intervals for a period of three weeks.

A qualitative assessment of the blood sample was made at intervals. The blood sample used to prepare the standard was expired transfusion blood, containing preservative which may limit the decomposition of the blood to some extent.

As there is some day-to-day variation in the response of the detector, the absolute and relative extraction efficiencies shown in table 2.27. were calculated by analysing unextracted standards of morphine and nalorphine immediately prior to the extract.

The efficiencies of extraction for each sample was calculated by comparison of the peak height with an unextracted standard. 20ul of 10^{-3}g/L is equivalent to 20ng on column. 100% recovery from 1ml of 10^{-4}g/L is equivalent to 100ng. This extract was

reconstituted in 100ul and 20ul analysed. This is equivalent to 20ng on column also.

The calculation of the concentrations was based on the assumption that the day to day variation in the relative peak heights of morphine and nalorphine (see table 2.27.) was significant.

For this reason the concentration was calculated on the basis of the recovery from the sample rather than simply on peak heights

The day 0 sample was used as a standard.

2.9.3.3. Results and discussion

The blood sample started as a mobile red fluid, free from particulate matter and odour.

By day three it was beginning to darken in colour.

By day nine it was visibly more viscous and was beginning to smell characteristically of amines.

By day fourteen it appeared as a dark reddish brown fluid with clumps of material present although no large clots.

By day twenty one it was considerably more viscous and gas was heard to escape when the tube was opened. The odour was much stronger and the sample was sluggish and difficult to pipette.

These changes in the physical properties of the blood may account for the observed trends in the extraction efficiencies discussed below.

Two effects are noted in the extraction efficiencies found for the sample. Firstly the overall decrease in absolute extraction efficiency for morphine is considerable with sample age, falling from 70% (day 0) to 36% (day 21). A similar trend is noted for

TABLE 2.27.

EFFECT OF SAMPLE AGE ON THE RECOVERY OF MORPHINE AND NALORPHINE FROM BLOOD

	10^{-3}g/l Std soln. 20ng o/c			10^{-4}g/l extract ‡100% =20ng o/c				
Day	*$M_{ht.}$	$N_{ht.}$	M/N	$M_{ht.}$	$N_{ht.}$	$M_{eff.}$	$N_{eff.}$	Conc.$
0	101	80	1.26	71	59	70	74	0.100
1	96	81	1.19	69	57	72	70	0.109
2	80	76	1.05	53	51	66	67	0.104
5	85	72	1.18	50	40	59	56	0.112
7	76	69	1.10	45	42	59	61	0.103
9	104	87	1.19	56	49	54	56	0.102
11	94	88	1.07	47	48	50	55	0.096
14	100	84	1.19	50	46	50	55	0.096
19	72	57	1.26	32	29	44	51	0.091
21	88	76	1.16	32	32	36	42	0.091
n=10								
Average (X)			1.17			56.0	58.7	0.100
S.D.			0.07			11.5	9.5	0.007
CoV(%)			6.18			20.5	16.2	7.04

‡ 100% recovery = 100ng, reconstituted in 100ul. 20/100ul injected.

* Peak heights in mm., sensitivity 10nA fsd.

$ concentrations calculated from extraction efficiencies wrt Day 0 sample.

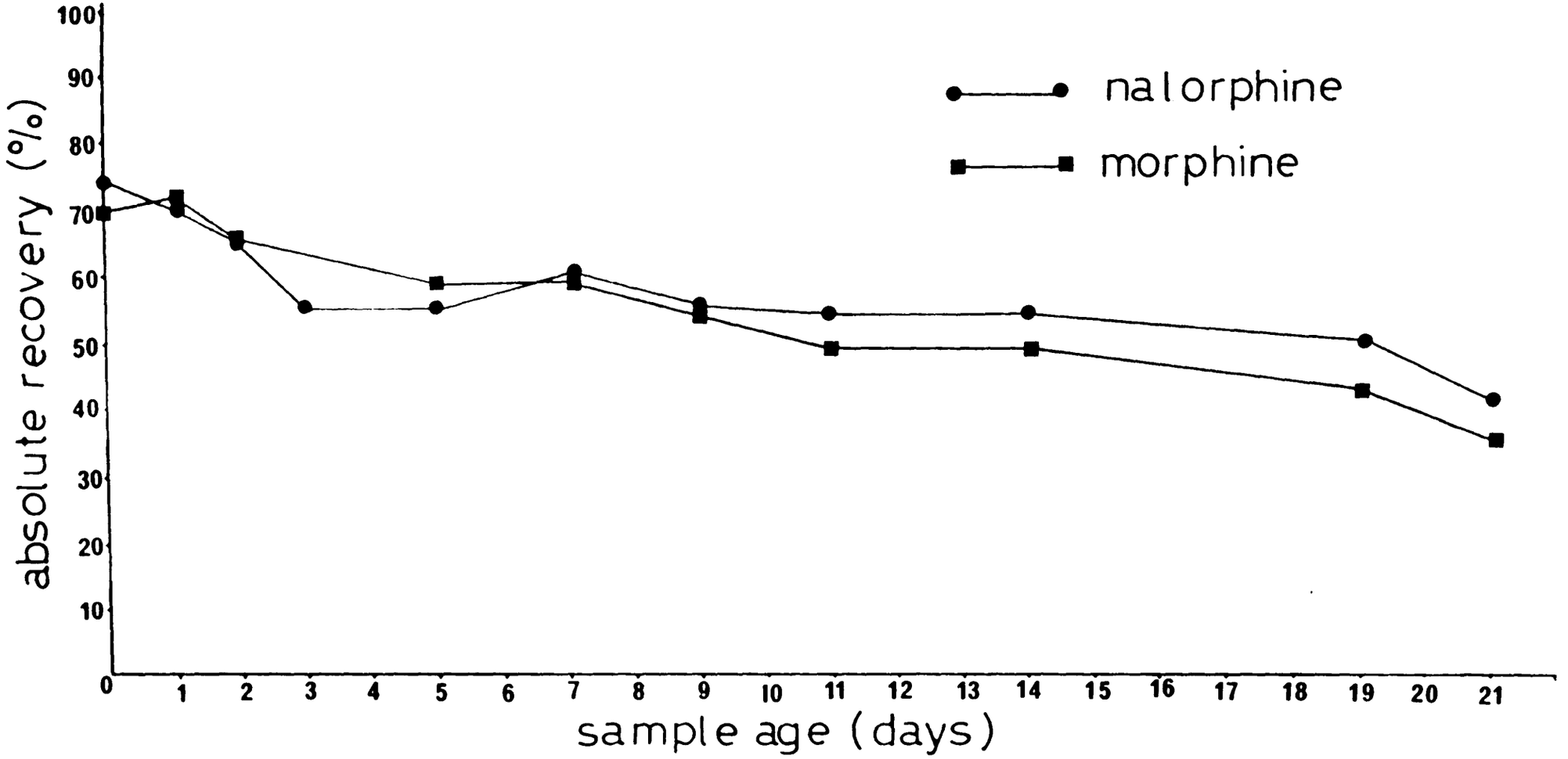


FIGURE 2.27.

CHANGES IN ABSOLUTE RECOVERY OF MORPHINE AND NALORPHINE FROM A PUTRIFYING BLOOD SAMPLE WITH SAMPLE AGE

the recovery of nalorphine. This is shown in figure 2.28..

The fact that the recovery for both the drug and the internal standard drop, suggested that physical changes to the matrix had made the extraction less efficient.

Secondly, whilst standard deviation on the calculated morphine level is only ± 7% throughout the three weeks of this study, a week-by-week analysis shows that there is a gradual decrease in the measured morphine concentration (calculated with respect to the Day 0 sample) from 0.106g/L in the first week to 0.093g/L in the third week. This is shown in table 2.28. and figure 2.29..

This suggests that in addition to the loss of efficiency due to matrix effects, the amount of morphine in the sample is decreasing. The fall in the relative recovery of morphine could be ascribed to microbial degradation.

It has also been noted that morphine in cadavers can be converted to the dimer, pseudomorphine, and may be found as this on exhumation (Curry, 1969). No indication is given of the rate of this conversion but this process may account for some of the loss of morphine observed.

An element of the change might also be accounted for by errors in the measurement of the blood aliquot for analysis. Transfer of the sample to the extraction tube was done with a Gilson automatic pipette (1ml) (Scotlab Instrument Sales, Glasgow, U.K.). As the blood became more sluggish, pipetting became more difficult and greater care had to be taken with this step.

TABLE 2.28.

MEAN WEEKLY ABSOLUTE AND RELATIVE RECOVERY OF MORPHINE FROM BLOOD

		relative recovery		absolute recovery ($M_{eff.}$)	
Age in wks	n	X	S.D.	X	S.D.
1	4	0.106	0.005	66.8	5.7
2	3	0.100	0.004	54.3	4.5
3	3	0.093	0.003	43.3	7.1

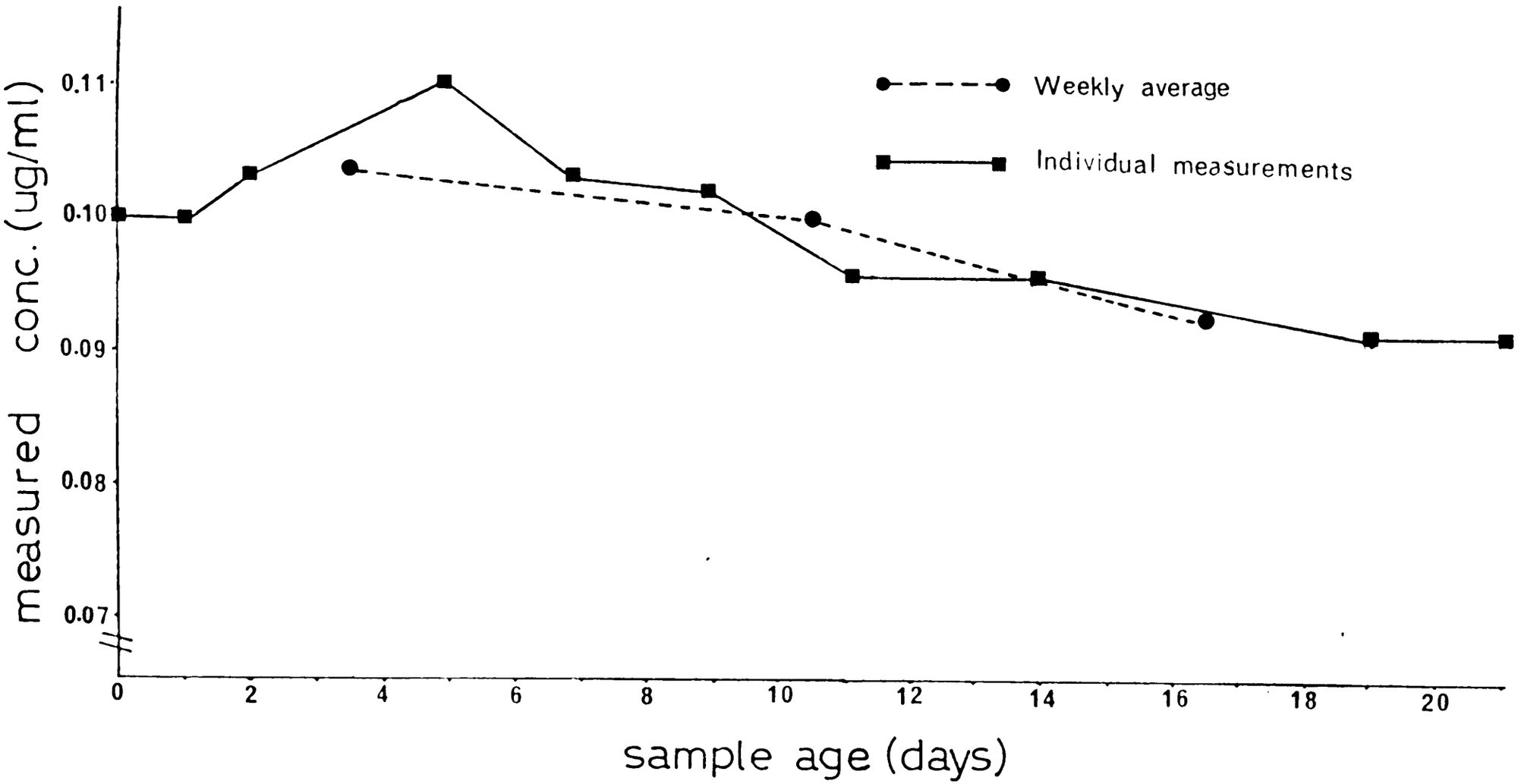


FIGURE 2.28.

CHANGE IN MEASURED CONCENTRATION OF MORPHINE WITH AGE OF BLOOD SAMPLE

2.9.3.4. Conclusion

It has been shown that the effect of the age and degree of putrefaction of a blood sample will have an effect on the both the absolute recovery and the recovery relative to an added internal standard of morphine in a blood sample.

It was also noted that the physical condition of a three week old blood sample prepared from transfusion blood (which contains preservatives) was considerably better than many one week old post-mortem specimens. How closely this prepared specimen resembles real post mortem blood can only be assumed.

2.10. APPLICATIONS OF THE METHOD TO AUTHENTIC SAMPLES

2.10.1. POST MORTEM ANALYSIS

2.10.1.1. Introduction

As discussed earlier, the assay for morphine in post-mortem blood was developed to cope with the increased demand for analysis (2.1.2.). This increase is seen as a direct result of the increase in drug use in the UK.

The lore of drug culture has been extensively reviewed (Laurie, 1978; Trebach, 1982; Winek, 1974; Picardie and Wade, 1985). The lifestyle of a person habituated or addicted to opiates, generally leads to contact with the medical profession either in an attempt to obtain drugs, or as a result of drug related health problems (Cherubin, 1967).

These include conditions associated with poor injection hygeine eg. cellulitis, thrombophlebitis, septicaemia, hepatitis, tetanus, malaria (Doame, 1924; Louria et al, 1967) and more recently AIDS (Hunsmann, 1985; Winterton et al., 1985).

Other problems, common but not associated with injection are endocarditis, pulmonary complications and overdose. Inhaling the drug carries the same degree of risk of these latter ailments as injecting.

Overdose from heroin is recognised as being of two types.

Immediate overdose, resulting from a bolus injection of morphine/diacetylmorphine causing pulmonary oedema or respiratory arrest through pulmonary smooth muscle spasm, and leading to

death. This occurs within five to ten minutes, and the deceased is often found with the needle and syringe still in place. (Polson et al., 1983).

The other form, delayed overdose, is a result of heroin metabolites, morphine, monoacetylmorphine, methylmorphine, causing CNS respiratory depression, coma and death. (Perry, 1975).

Overdose is usually a result of the drug user buying drug material of an unusually high purity. The purity of street heroin varies between 0 and 100% (Polson et al., 1983).

The metabolism of morphine and heroin have been discussed earlier (2.1.3.). The detection of morphine in a blood sample therefore can only imply the use of either morphine or heroin. Differentiation between the two would require determination of the specific heroin marker, 6-monoacetyl morphine, the initial metabolite of diacetyl morphine.

This metabolite is acetylated through the 6-OH position of morphine.

Assays for this metabolite have been reported (Christiansen, 1985; Derks et al., 1985).

2.10.1.2. Samples for analysis

Samples provided for this study came from post-mortem examinations where the pathologist had reason to suspect opiate use. This suspicion may have arisen from the police, the deceased's medical record, unusual pulmonary changes, recent injection marks or lack of an adequate cause of death.

Samples were generally received within two to five days of death, and analysed as soon as possible thereafter.

2.10.1.3. Limits of the assay

The quantitative cut off point for the assay was taken as $1X10^{-5}$g/l, where the CoV was found to be 25%.(see section 2.9.2.). Below this level, suspected positives could often be seen at $5X10^{-6}$g/l, and confirmation of this result would be required by repeating the analysis on a larger sample or by the use of radioimmunoassay or GCMS procedures.

As the lower end of the therapeutic range is taken as $5X10^{-5}$g/l (Stead and Moffat, 1983), concentrations below this are unlikely to be encountered if morphine played a significant role in the cause of death.

The procedure used routinely for the assay was that developed in the first part of this chapter, the chromatographic conditions are summarised in section 2.5.12, and the extraction procedure in section 2.7.7..

2.10.1.4. Collection of data

Over the period between January 1984 to December 1985, twenty-four samples analysed for morphine (by the above method) gave positive results. Details of many of these cases are not available, as they came from several different pathologists and procurators fiscal in Scotland. Because of this lack of background, only limited conclusions can be drawn from the results. In addition, the samples were preselected in as much as they came from cases where the pathologist already suspected opiates were involved.

This laboratory was one of the first in Scotland to provide a chromatographic method for morphine in blood. As a result in the

TABLE 2.29.

BLOOD MORPHINE LEVELS IN PERSONS DYING MORPHINE RELATED DEATHS AS ANALYSED IN THIS STUDY

case #	year	area	sex	level (mg/L)
1	1984	Glasgow	F	0.1
2		Edinburgh	F	0.9
				0.6
3		Glasgow	M	0.12
4		Glasgow	M	0.26
5		Edinburgh	M	0.40
6		Edinburgh	F	15.4
7		Edinburgh	M	0.7
8		Edinburgh	?	0.01
9		Edinburgh	M	0.36
10		Edinburgh	F	0.25
11		Edinburgh	F	0.31
12		Edinburgh	M	0.50
13	1985	Glasgow	M	0.25
14		Aberdeen	M	0.14
15		Glasgow	M	0.017
16		Glasgow	M	0.34
17		Glasgow	M	0.43
18		Glasgow	M	0.51
19		Glasgow	M	0.05
20		Edinburgh	M	0.6
21		Edinburgh	M	0.2
22		Glasgow	M	4.7
23		Glasgow	M	0.18
24		Glasgow	F	0.30
				n=25
				X=1.11

early part of 1984, almost all suspected opiate deaths were sent to Glagow University Forensic medicine department.

Since then other hospital laboratories have acquired this facility, and the number of samples from outwith Strathclyde Region has declined. The Department of Forensic Medicine and Science at Glasgow University still provides the sole Forensic Toxicology service for the Glasgow area.

An examination of the data (table 2.29.), shows that the number of positive results for morphine in the Glasgow area has increased from four cases in 1984 to nine cases in 1985. In the five months up until May 1986 however, only one positive result had been found.

An increase in morphine related death would be expected in line with the increasing number of people abusing the drug. The data available here does not allow any conclusions to be drawn on the scale of the increase in morphine related death. In addition, the recent increase in awareness of pathologists to the drug problem in Glasgow, and the availability of a suitable assay has prompted an increase in the number of requests for morphine determination.

2.10.1.5. Interpretation of Results

The interpretation of blood concentrations of drugs which produce physiological dependence is difficult. Tolerance, that is a decreased response to the same dose, has been seen to develop soon after the administration of the first dose of morphine or heroin (Martin and Fraser, 1961).

This requires the addict to increase his dose to achieve the same

degree of euphoria as was experienced with the first dosage. The degree of tolerance will increase with increasing dose . As a result of this, concentrations which would be considered fatal in the average person are often encountered without seemingly ill effects in those who have acquired a tolerance for the drug (Goldstein et al., 1974). There is therefore, considerable overlap in the accepted ranges of therapeutic, toxic and fatal concentrations of morphine in blood (Stead and Moffat, 1983). The concentrations given for these three ranges are 0.04 to 0.5, 0.5 to 5 and greater than 0.2mg/L respectively. This information is in good agreement with that reported elsewhere (Felby et al., 1974), where fatal concentrations were found above 0.2mg/L. The role of morphine in the cause of death may be either causitive or subsidiary. The toxicological findings can confirm or refute this to some degree. High levels (above 0.2mg/L) may be sufficient to cause respiratory arrest, but levels below this may lead to unconsciousness, inhalation of vomit and suffocation or subsequent pneumonia. As the survival times will be longer in deaths resulting from pneumonia, the morphine concentration might be low or undetectable. The presence of morphine can however give the pathologist a likely explanation for any of the above findings.

2.10.1.6. Discussion of results

The range of morphine concentrations found in these samples varies from 0.01 to 15.4 mg/L (mean 1.12). This is in agreement with levels reported in the literature and the findings in the post mortem examination are consistent with death from morphine

overdose. The level found in case 6 is extremely high, and the circumstances suggest that this arose from the injection of heroin. In this case, two of deceased were found together (numbers 5 and 6).

A high blood morphine concentration reported recently (Connett, 1984), caused by the absorption of heroin from capsules in the stomach, was still less than that found in sample number 6. The lowest level is just above the cut off point of the assay and is unlikely to be the sole cause of death. The inhalation of vomit during unconsciousness can lead to suffocation or if the victim survives this, pneumonia and subsequent death. Similar to the behaviour noted in the case of barbiturate poisoning (Oliver, 1986).

Two representative cases were studied.

2.10.2. CASE 1

2.10.2.1. Medical and social history

A young (24) unemployed single male, was a known drug addict and had been registered as such for over two years.

This had first come to light when he was admitted to hospital suffering from hepatitis B.

He was reffered to a drug clinic as an out patient, but never attended.

2.10.2.2. Circumstances

The man left his girlfriend's house one evening at 10.45pm. He had been drinking, but not heavily. He gave no

indication of feeling unwell.

At about 12.45pm the following day, he was found dead in the living room of his own flat. The police searched the house. A needle and a syringe containing traces of blood and a white substance, were found in the kitchen. An empty blister pack (6 blisters) marked "MST continus 100" was also found. There was vomit in the toilet.

The body was removed to the city mortuary where a post-mortem examination was carried out four days later.

2.10.2.3. Pathology

Relevant features at post mortem were the presence of eight fresh needle punctures in the right antecubial fossa, and one on the left side.

Internally, both lungs were intensely congested and oedemateous, but there was no evidence of inhalation of gastric contents. There were also numerous small firm nodules throughout both lungs measuring up to 0.5cm in diameter. Similar nodules were noted in the liver and spleen.

2.10.2.4. Bacteriology and Virology

Tissue samples were examined for tuberculosis and hepatitis, but were negative for both.

2.10.2.5. Toxicology

A blood sample was screened for alcohol, barbiturates and morphine, while a urine sample was screened for alcohol and basic drugs.

The following results were obtained:-

13 milligrammes of alcohol per 100ml of blood

43 milligrammes of alcohol per 100ml of urine

4.7 microgrammes of morphine per millilitre of blood.

All other analyses gave negative results.

The syringe and the empty packet were also submitted for analysis. The packet was identified as having contained morphine sulphate tablets (MST, manufactured by NAPP). Each capsule would contain 100mg of morphine sulphate.

The white material was oily and resembled vaseline in appearance. Some of this was dissolved in methanol and analysed by HPLC for the presence of morphine, and by GC-NPD for the presence of heroin. All analyses gave negative results.

The blood residue in the syringe was washed out with distilled water and extracted by the method described in section 2.5.12.. This extract was analysed by HPLC for the presence of morphine, and by GC-NPD for the presence of heroin.

The extract was found to contain 0.05 milligrammes of morphine.

No heroin was found in the extract.

2.10.2.6. Discussion and Conclusion

The blood concentration of morphine was well within the fatal range (Stead and Moffat, 1983) and is sufficient to have caused death by CNS respiratory depression.

This morphine which was detected could have come from the administration of morphine or heroin. The absence of any packet

of heroin in the flat, and the presence of the MST package, together with the finding of morphine in the syringe but not heroin, suggests that the deceased may have crushed and injected the MST tablets.

The nodules noted in the lungs and all the other organs were examined microscopically but their exact nature was never determined. Although very different from the typical talc granulomas seen they were nevertheless considered to be somehow related to chronic drug abuse. The death was finally certified as morphine intoxication.

2.10.3. CASE 2

2.10.3.1. Medical and Social History

A young (18) female had a history of drug misuse and had been an in-patient in a psychiatric hospital receiving treatment for this condition. She was suffering from depression and had been prescribed temazepam tablets by her general practitioner.

2.10.3.2. Circumstances

In a fit of depression the girl had taken a large number of temazepam tablets and a quantity of Malibu liquor, in an apparent suicide attempt. She had been found lying outdoors in a semi-conscious state and was admitted to hospital as a casualty patient. The next day she discharged herself, and went to see some aquaintances whom she had met during a stay in the psychiatric hospital. Both these persons were known drug addicts.

The circumstances thereafter are vague. Having told the two aquaintances that she wished to kill herself, they obtained a quantity of heroin and one of them injected her with some of this. She then "fell asleep" and was heard to be snoring. Some time later, apparently unconscious, she was removed from the bed where she had been lying and was laid on some cushions. Later that night she was found to be "blue", and unrousable and she was taken to hospital, where she died shortly after admission.
The cause for her admission to hospital was stated to be the effects of an overdose of temazepam.

2.10.3.3. Pathology

The main findings at post mortem were the precence of several recent needle punctures, at the elbows and at the wrist. Internally there was severe congestion and oedema of the lungs.

2.10.3.4. Bacteriology and Virology

At the request of the pathologist samples from the deceased were examined for hepatitis. All samples were found negative.

2.10.3.5. Toxicology

Blood and urine samples were retained by the pathologist. The presence of morphine was detected in the blood by radioimmunoassay. A sample of the blood was subsequently sent to this department for confirmation and quantification of this result. The sample was extracted and analysed by LCED.
A concentration of 0.9 microgrammes of morphine per millilitre of blood was found.

2.10.3.6. Conclusion

This level is within the overdose range and the cause of death was certified as morphine (heroin) poisoning.

2.10.3.7. Discussion

Police enquiries in this case resulted in the apprehension for murder of both of the girls who allegedly injected the deceased with heroin. As a result, a second postmortem and toxicological examination were carried out for the defendants' solicitors. The blood sample was analysed by LCED for morphine and by GCMS for temazepam.
The results of this analysis were as follows:-
0.6 microgrammes of morphine per millilitre of blood
0.92 microgrammes of temazepam per millilitre of blood

The second post mortem confirmed the findings of the first, but also disclosed the presence of pneumonia in the lungs, this being considered to be the prime cause of death, it was further considered that the pneumonia developed as a result of inhalation of vomit whilst intoxicated by a combination of temazepam and heroin. The pathologists added that the cause of death was probably pneumonia caused by the combination of these two drugs.

Both the Crown and Defence analyses had been carried out by the same technique on two different blood samples from the deceased. The levels found were 0.9 and 0.6 microgrammes of morphine per millilitre of blood. Allowing the standard deviation in relative extraction efficiency of 13%, this still leaves some of the error

unaccounted for. The question of this error was raised in court. The explanation given was that the samples had been taken one month apart, and were respectively four weeks and eight weeks old when taken. The advanced putrefaction caused by pathogens present in the mortuary would lessen the extraction efficiency in an older sample.

In addition it is known that morphine is often converted to pseudomorphine in cadavers, and the amount of morphine in the sample may indeed have been less by the time of the second analysis.

It should also be noted that it was suggested in court that the second sample may have been simply scooped out of the abdominal cavity, as no heart blood was available at the time of that post mortem. This was however not substantiated.

2.10.3.8. Conclusion

It was held by the court that one of the deciding factors in the death of the girl was the administration of heroin in sufficient quantities to cause respiratory depression leading to death. As a result, both defendants were found guilty on the reduced charge of culpable homicide.

2.10.4. HOSPITAL STUDY.

2.10.4.1. Introduction

A study was carried out on critically ill patients receiving morphine for sedation. The object of the study was to examine morphine kinetics in the severely ill, and relate this to the level of sedation, analgesia and the severity of illness. Approval for the study was given by the local Medical Ethics Board. Morphine use is becoming more prevalent in intensive care medicine since the withdrawal of two other popular agents, Althesin and Etomidate, because of side effects (Editorial, Lancet, 1984, British National Formulary, 1985).

Morphine kinetics have been well researched, but usually for single dose administration (Moore et al., 1983), and there is little reported information on results of infusions lasting more than 72 hours (Don et al., 1975).

The study of critically ill patients receiving morphine by infusion, allows the assesment of morphine kinetics in relation to the sedative effect of the drug after long term administration.

Many problems arise with the interpretation of results under these conditions. The patients welfare must take precedence at all times and the study cannot be allowed to influence the treatment received by the patient. This in turn means that a great number of variables must be taken into account when considering the results.

Variables interfering with the steady state morphine levels included haemodialysis and haemofiltration in patients with Acute Renal Failure (ARF) and occasional bolus injections of morphine in all patients as required.

Problems involved in relating blood morphine levels to the level of sedation, were the additional administration of midazolam (by bolus and infusion) and other sedative drugs (esp. fentanyl), administered either for surgery or to give the patient relief from pain.

Because of the time scale of the study, samples were taken infrequently (three times daily) and were often not taken immediately before a change in infusion rate, when the information would have been of most use. Lastly, most of the levels found in the study were at the low end of the therapeutic range, and hence subject to larger error.

2.10.4.2. Protocol

Patients admitted to the Intensive Therapy Unit (ITU) were sedated using a combination of opiates and benzodiazepines. These are normally given by infusion, as this causes less ventilatory depression than a bolus injection. Additional bolus injections however, were given as required. Blood samples were taken through an indwelling venous catheter two or three times a day, and more often when the infusion was stopped, to allow calculation of the half life. The samples were stored at -20^{o}C until analysis.

At the time the samples were taken, an assessment was made of the patient's sedation level and sickness score.

2.10.4.3. Sedation and Sickness scoring

This was a subjective assesment by the clinician (all assesments were made by the same clinician), using a ten point

linear analogue scale. On this scale zero represented no response to verbal or tactile stimuli, and ten represented full alertness (Bion et al., 1986).

The severity of the illness was measured using a sickness score (Bion et al. 1985), based on the APACHE II sickness scoring system. The higher the score, the poorer the prognosis; scores of 19 and above are usually associated with subsequent death.

The blood samples were analysed as described above. The analysis was performed in duplicate where there was sufficient sample.

2.10.4.4. Results

Eleven patients were examined. Details of their medical history are given in table 2.30..

A record of the infusion rate of morphine over the previous four hours, the sickness score and the sedation score were abstracted from the record charts and are given in appendix C, along with the measured morphine concentration. Elimination half-lives were calculated where sufficient samples were taken during the decay period. This was done by linear regression analysis, using the method of least squares.

2.10.4.5. Selection of data

Correlations were not examined in cases where there were insufficient data points (ie data for less than three infusion rates).

The level of sedation would be affected by the presence of any other sedative drugs, and could potentially be affected by intermittant dialysis. For that reason the sedation score

TABLE 2.30.

MEDICAL HISTORIES OF THE ELEVEN HOSPITAL PATIENTS IN THIS STUDY

No.	Age	sex	Wt.	Diagnosis	inf. period	survived?
1	68	M	80	Post operative sepsis, choleostasis, ARF	160	No
2	61	M	80	Acute haemolysis, ARDS, ARF	56	Yes
3	55	M	80	Road Traffic Accident, Head injury, Flail chest, ARF	108	No
4	43	F	55	Septic shock, Peritonitis	62	Yes
5	56	F	80	Necrotising fasciitis	117	Yes
6	53	M	80	Subphrenic abscess	205	Yes
7	38	M	68	Intestinal infarction	69	Yes
8	64	F	70	Road Traffic Accident, Ruptured diaphragm and liver	81	Yes
9	22	F	45	ARDS, Pulmonary Hodgkins' disease ,stg. IV	210	No
10	24	M	75	Road Traffic Accident, Pulmonary necrosis	326	No
11	52	M		ARDS, Acute pancreatitis	528	Yes

corresponding to any samples taken within the period FOUR HOURS after administration of another drug or dialysis, were discarded. More data were available to correlate the infusion rate with the blood morphine concentration, as the latter was assumed to be independent of the presence of other drugs, whereas sedation was not.

2.10.4.6. Discussion

The patients record chart was examined and details were taken at points where steady state morphine levels were operating. Steady state kinetics were assumed to be operating where the infusion rate had been constant for four hours prior to sampling. For patients receiving dialysis, levels were not considered until four hours after dialysis.

The details of interest were the patient's sickness score, the level of sedation, the blood morphine concentration and the corresponding infusion rate.

Each patient was first considered in turn.

The data collected was examined to establish the degree of correlation between the following sets of parameters;

Infusion rate and Sedation level

Blood morphine concentration and Sedation level

Sickness score and Sedation level

Infusion rate and blood morphine concentration

Correlation coefficients (r) were calculated using a best straight

TABLE 2.31.

TEST OF CORRELATION BETWEEN SEDATION LEVEL, INFUSION RATE, BLOOD MORPHINE LEVEL AND SICKNESS SCORE FOR EACH PATIENT

patient number	Correlation Coefficients sed./sick.	sed./I.R.	sed./Conc.	I.R. Conc.
1	+0.19	-0.79	-0.48	+0.76
2		insufficient data		
3	+0.67	-0.65	-0.45	+0.16
4		insufficient data		
5		other drugs used		+0.92
6	-0.49	-0.23	-0.14	+0.96
7	-0.76	-0.43	-0.54	+0.94
8		insufficient data		
9		other drugs used		+0.87
10		other drugs used		+0.86
11		insufficient data		

TABLE 2.32.

COMPARISON OF ELIMINATION HALF LIVES FOR MORPHINE IN PATIENTS UNDER STUDY

Patient number	morphine T/2 (mins)	highest blood concentration (mg/L)	corresponding infusion rate (mg/hr)
1	320	0.039	2
	420	0.037	2
2	65	0.108	5
3	280	0.078	5
4	60	0.015	3
5	113	0.135	7.5
6	60	0.093	5
7	50	0.082	5
8	*	0.046	3
9	90	0.332	15
10	90	0.388	15
11	160	0.077	4

* no data available

$ dialysis performed during decay

line fit, linear regression analysis programme, run on a PET microcomputer (Commodore).

The results of these comparisons are shown in table 2.31..

No consistent correlation is seen between measured blood morphine level and the degree of sedation. Indeed, some patients appeared to remain sedated for several days after receiving any sedative drug and when there was no detectable morphine in the blood.

This observation is supported by a similarly low correlation between infusion rate and sedation.

There is evidence (Martin and Fraser, 1961) that tolerence to the euphoriant effects of morphine can develop shortly after the onset of administration (within to 2-3 days). If the same applies to the sedative effects, this would result in a corresponding drop in the level of sedation for a given dose (or blood concentration) with time. The extent to which tolerence develops is difficult to assess from the data available in this study. There are many variables which will alter the sedation level of the patient, including the administration of other sedatives and the continuous change in the level of the patients illness, which is later shown to affect sedation. This made the assessment of the degree of tolerence developed by the patients impossible on the basis of the results from this study.

The only set of data showing consistent correlation were infusion rate and blood morphine concentration. These showed a high correlation - r values were greater than 0.7 for all the patients. The lowest correlation (r= 0.71 and 0.80) for infusion rate and blood morphine concentration was found in the two patients (numbers one and two respectively) who had Acute Renal failure (ARF).

There was a significant difference in the elimination half-life of the drug once infusion was stopped (table 2.32.), between those patients with ARF and those without.

It was proposed that these observations might support the current theory that the kidney played a major role in the elimination of morphine (Moore et al., 1983; Ball et al., 1984). The differences between these two groups (ARF and normal) were examined.

2.10.5. EFFECTS OF ACUTE RENAL FAILURE

2.10.5.1. Introduction

The patients were considered as two groups: those with and those without renal failure.

Within these two groups all the data were then considered collectively, in order to eliminate bias in mean blood levels for infusion rates with a small number of data points.

The degree of correlation was calculated as before.

2.10.5.2. Results and Discussion

The results are shown in figure 2.29..

The comparison of the data was made only up to infusion rates of 5mg/hr, as this was the highest rate of infusion administered to patients with ARF.

No weight correction was done, as the patients were not usually in a suitable condition to be weighed when admitted, and only approximate weights were known.

The data was treated in the following manner. A best straight

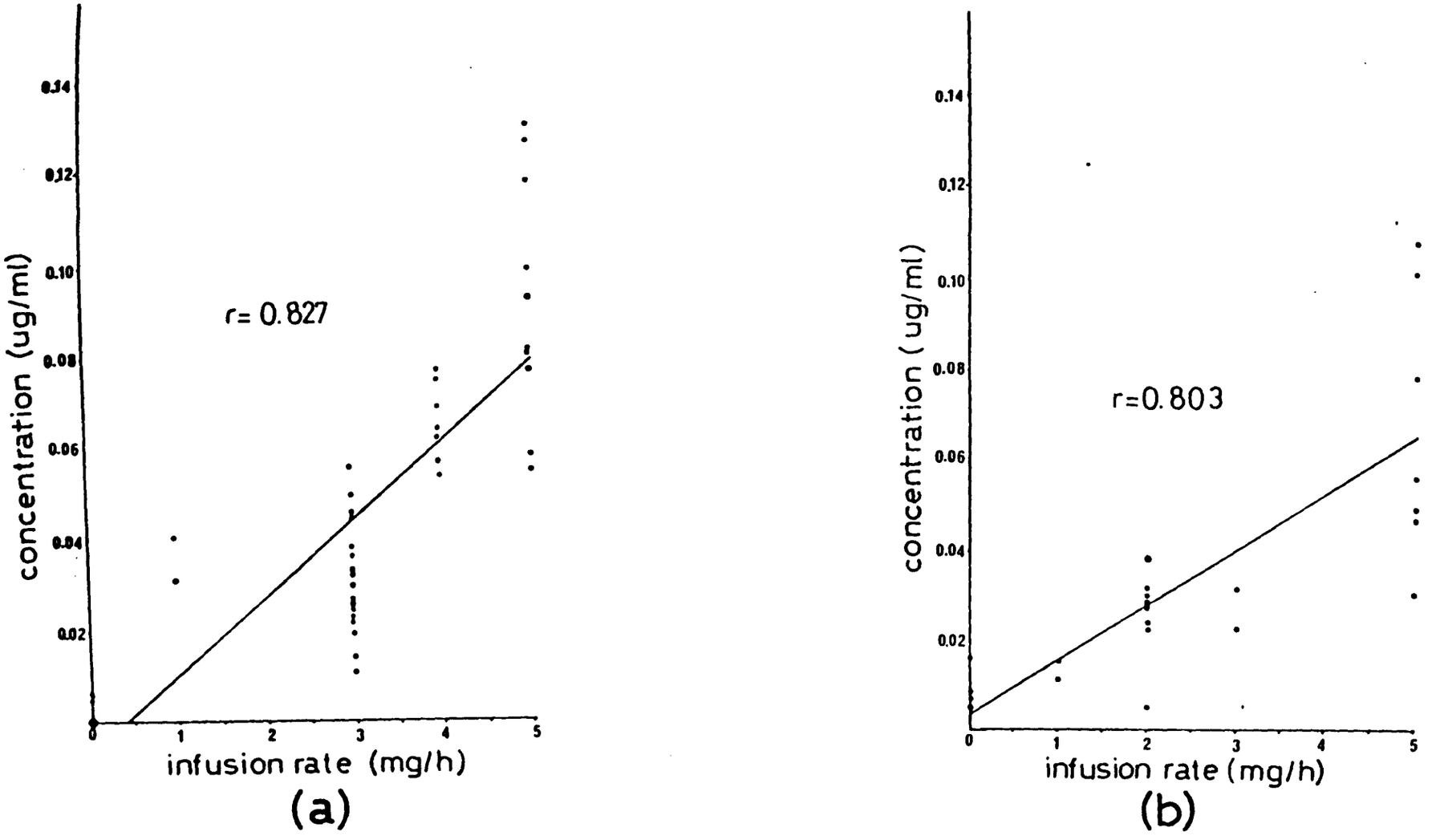


FIGURE 2.29.

CORRELATION OF MEASURED BLOOD MORPHINE CONCENTRATION WITH INFUSION RATE FOR (a) NORMAL PATIENTS AND (b) THOSE SUFFERING FROM ACUTE RENAL FAILURE

line was calculated for all the data in each of the groups. The alternative, to calculate the best straight line for the mean blood level at each infusion rate, would not have given sufficient weight for infusion rates with more data points.

It was shown that the correlation coefficients were 0.934 and 0.803 for normal and ARF patients respectively. A test of significance, student's T-test (Langley, 1970) was done on each of the points where there was sufficient data (table 2.33.). No significant difference was found for any of these points at a probability level of 5%.

There was no evidence of the accumulation of morphine in the blood of those patients with ARF, as has been observed elsewhere (Ball et al., 1985). The gradient of the slope is actually less in patients with ARF. The regression coefficients were 0.021 and 0.012 for normal and ARF patients respectively. This implies that patients with renal failure were eliminating morphine more rapidly than patients with normal renal function.This unlikely finding was ascribed to the fact that those patients with ARF were, in addition to receiving intermittant dialysis, also receiving continuous haemofiltration on an Amicon^{r} haemoperfusion unit.

It was proposed that this procedure was removing the sedatives from the blood. Other information provided by this comparison is that in patients with normal renal function, no detectable morphine would be found in the blood at infusion rates below 0.5mg/hr, and that infusion rates of greater than 2.5mg/hr would be required to produce blood levels equal to that quoted as being the lower end of the therapeutic range (Stead and Moffat, 1983)

TABLE 2.33.

T-TEST DATA FOR THE RELATIONSHIP BETWEEN BLOOD MORPHINE CONCENTRATION AND INFUSION RATE WITH RESPECT TO STATUS OF RENAL FUNCTION

	Infusion rate								
	0	1	2	3	4	5	7.5	8	10	15
ARF										
n	5	2	10	2		7				
mean	8.2E-3	0.013	0.028	0.028		0.067				
SD	4.5E-3	2.8E-3	9.4E-3	6.3E-3		0.029				
Normals										
n	5	3		16	7	13	4	2	14	8
Mean	1.8E-3	0.036		0.033	0.065	0.092	0.099	0.134	0.172	0.320
SD	2.5E-3	5.5E-3		0.012	8.7E-3	0.025	0.024	0.134	0.051	0.043
DoF	8	3		16		18				
t	2.21	4.80		0.78		1.77				
t(5%)	2.36	4.3		2.13		2.11				

2.10.6. ANALYSIS OF HAEMOPERFUSION FLUID

2.10.6.1. Introduction

To support the above hypothesis that morphine was being removed from the blood of anuric ARF patients by haemofiltration, it would have to be shown that morphine was present in the haemofiltrate.

2.10.6.2. Method

Three separate samples of amicon fluid were received from one of the patients in the trial (number two). These were extracted using the general procedure described earlier, and analysed by LCED.

2.10.6.3. Results and discussion

All three samples gave peaks corresponding to morphine. The calculated levels were 0.015, 0.101 and 0.008ug/ml.
This shows that haemofiltration does remove some of the morphine from the blood. The haemofiltrate is little different from serum; the filter retaining only blood cell and the larger plasma proteins. A standard liver function test is that of antipyrine clearance. When antipyrine is administered concurrently with the use of haemoperfusion or dialysis, misleadingly low clearance values will be obtained (Maddocks et al., 1976). In this connection it is important to remember that the clearance of compounds by the Amicon cannot be measured by the product of the filtrate/blood concentration ratio and the filtration volume as would be done for urine (Stevens 1984). This is because the

filtrate in this case is more similar to serum and - unlike the situation encountered with renal clearance - the volume will not effect the concentration.

The observed increase in elimination half life can be accounted for by the fact that the role of the kidney in morphine elimination is an active transport system, whereas the Amicon device is a simple filter.

2.10.6.4. Conclusion

It is clear that haemoperfusion is removing morphine from the circulating blood of those patients receiving this treatment. Although a recent review has considered the use of haemoperfusion in the treatment of poisoning (de Broe et al., 1986), no quantitative study of the removal of drugs by haemoperfusion has been made, but such a study would lead to a better understanding of the pharmacological needs of patients with renal failure. The increased elimination half life may implicate the rôle of the kidney in morphine metabolism, but none of the patients meet the requirements of a control experiment, and no firm conclusions can be drawn.

2.10.7. CONCLUSIONS ON THE CLINICAL ASPECTS OF THE STUDY

2.10.7.1. Introduction

Further analysis of this data was carried out by the Clinical Shock Study Group and the Clinical Pharmacology Unit where the study was conducted (Bion et al.,1986).

2.10.7.2. Discussion

Whilst there was no consistent relationship between blood morphine concentration and the level of sedation in the patient, they were able to show that within a given blood morphine concentration range there was a significant relationship between the level of sedation and the sickness score of the patient (figure 2.30.).

Not much is known of the effect of high morphine concentrations on the critically ill, but there are fears that it may lower their resistance to infection (Tubaro et al., 1983). Clinicians are therefore anxious to keep the morphine concentrations as low as absolutely neccessary for a given level of sedation.

One major concern was the high blood morphine concentrations - up to 6.75ug/ml - reported in the literature (Ball et al.,1985). These levels - above the mean fatal level reported earlier (section 2.1.3. and 2.10.1.4.) - were found in patients with ARF. The highest level found in this study in patients with ARF was 0.108ug/ml. This large discrepancy was attributed to two factors.

Firstly the use of continuous haemoperfusion in this study. This has been shown to remove morphine from the blood, and the extent to which this operates will depend on the volume of filtrate removed.

The other is the method of measuring morphine. The high levels reported in Ball's study were measured by radioimmunoassay (RIA). Recent reports suggest that the chronic administration of morphine may result in a substantial increase in the blood concentrations of morphine metabolites, particularly morphine-6-glucuronide (Joel

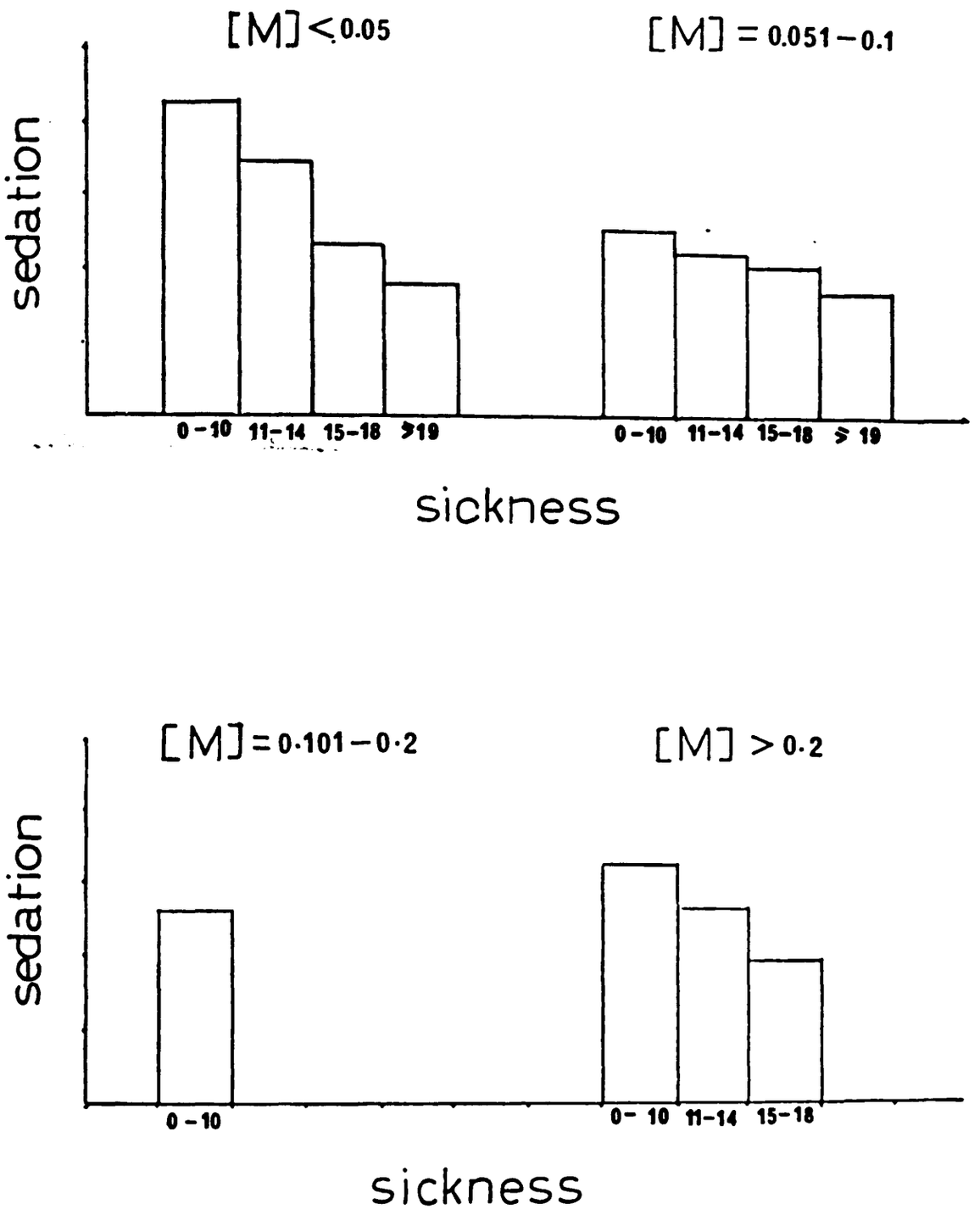


FIGURE 2.30.

CORRELATION OF SEDATION SCORE TO SICKNESS LEVEL FOR GIVEN BLOOD MORPHINE CONCENTRATION [M]

et al., 1985). High levels of this metabolite, which has a reported 1% cross reactivity with the morphine antibody, would cause a significant amount of interference with an RIA assay.

2.10.7.3. Conclusions

The study did not provide a direct correlation of sedation to blood morphine concentration or infusion rate, either on the same patient or between patients.

This finding leaves the appropriate infusion rate for a given degree of sedation a matter for the judgement of the clinician. This assesment should be made on the basis of the sickness of the patient and constant monitoring of the clinical signs. The rôle of a morphine assay in therapeutic drug monitoring is therefore limited. One possible application would be to ensure that high levels of morphine do not accumulate in patients with metabolic disorders such as acute anuric renal failure or liver dysfunction

2.11. CONCLUSIONS ON CHAPTER TWO

This chapter may been considered in three main parts. Firstly, the consideration of appropriate methods for the measurement of morphine in post mortem blood and the selection of a technique based on these considerations.

Having selected HPLC as the most appropriate method in terms of convenience, accessibility and ease of use, the appropriate conditions for the analysis of morphine were selected by iterative improvements to the conditions selected at each stage. In the

course of this process some consideration as to the chemical processes taking place both with respect to the chromatography and to the electrochemical detector were made. This assisted with a wider understanding of the processes involved and gave an insight into the advantages, dissadvantages and limitations of the technique.

Secondly, methods for the isolation of morphine from complex matrices, in particular putrified blood, was considered.

Successive alterations to the extraction procedure resulted in an overall improvement in the quality of the extract, and made it suitable for use with the LCED system developed. Alternative extraction procedures which had potential advantages in terms of sample handling and rapid sample preparation were also considered to some extent and found to be less compatible for use with putrified blood than with plasma.

Thirdly, the application of the method to the analysis of samples encountered in the field of forensic toxicology was demonstrated.Morphine was detected in significant quantities in blood samples from cases involving possible opiate use. The levels found were within the range expected.

The method was also applied to the measurement of morphine in intensive care patients. This showed that there was considerable overlap between the therapeutic and fatal ranges, as suggested by the literature. No significant difference in blood morphine level was noted between patients with acute renal failure. this may however have been due to the fact that these patients were receiving arteriovenous haemoperfusion to remove body waste. It was also that the filtrate from this procedure contained morphine.

the method was shown to be suitable for the application for which it was developed, and has since been incorporated into the routine procedures currently in use.

CHAPTER THREE

LOCAL ANAESTHETICS

3.1. LITERATURE

3.1.1. Introduction

Local anaesthetics are drugs which block nerve conduction when applied locally in the appropriate concentrations, to nerve tissue (Goodman and Gilman, 1980). Many compounds will do this however and those which are clinically acceptable must give controlled reversible anaesthesia at concentrations which will not be toxic in the systemic circulation.

Local anaesthesia was first achieved through hypothermia by applying crushed ice or volatile solvents topically (Tobin, 1980; Sneader, 1985).

In 1884 Sigmund Freud had been examining the psychotropic effect of cocaine, an alkaloid found in the plant erythroxylum coca. His co-worker, Carl Koller, noticed the numbing effect this compound had on the tongue and shortly afterwards published the first report of the use of cocaine as a local anaesthetic.

The local anaesthetic properties of other natural products were examined over the ensuing years. Most were only mildly anaesthetic and either toxic at the doses required to produce anaesthesia or had undesirable side effects. The discovery in 1900 of the vasoconstrictor properties of adrenaline allowed the surgeon to localise the drug in the area where surgery was to be performed. This in turn allowed the use of those milder

anaesthetics whose use had previously been avoided due to their poor or short acting analgesic properties. One of the more popular of these compounds because of its low toxicity was procaine (novocaine), synthesised in 1904 by Einhorn.

After the First World War, several new compounds with local anaesthetic activity were synthesised. The most effective of these was lignocaine (lidocaine, xylocaine) which is now in widespread use as a dental anaesthetic.

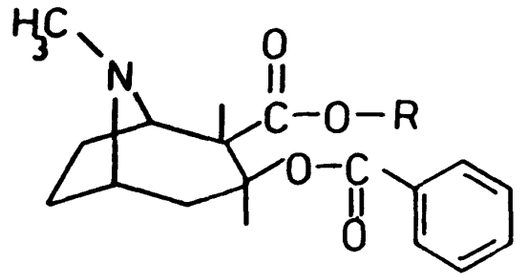
The most recent advance in local anaesthesia was the synthesis in 1957 of several lignocaine analogues, in which part of the side chain was cyclised. Two of these new compounds, mepivacaine (carbocaine) and bupivacaine (marcain), proved to be longer acting and more powerful than any other previously known agent and could produce nerve blocks lasting up to eight hours (Sneader, 1983).

The structures of the relevant compounds are given in figure 3.1..

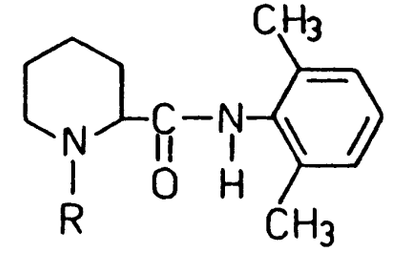
3.1.2. Medical use of local anaesthetics

The advantage of local, as opposed to general, anaesthesia is found where the surgeon can best benefit from the patients cooperation and consciousness. Examples include labour and childbirth, arthroscopy, ophthalmology and dental surgery. There are currently eleven local anaesthetics available for various applications in the UK (MIMS, 1986). Those applied systemically rather than topically include procaine, lignocaine, bupivacaine and mepivacaine. Cocaine is not included in MIMS.

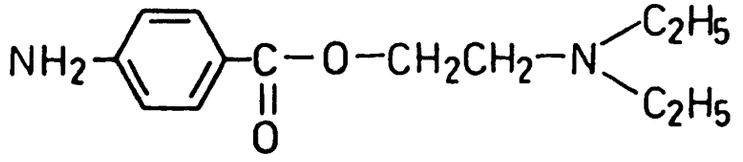
Procaine Hydrochloride is available as a local anaesthetic and also as a substituent of the topical anti-infective procaine Penicillin G (Depocillin). The rôle of procaine in this



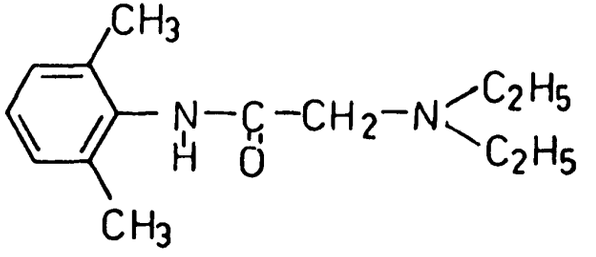
R = CH_3 – cocaine
R = H – benzoyl ecgonine



R = CH_3 – mepivacaine
R = C_4H_{10} – bupivacaine



procaine



lignocaine

FIGURE 3.1.

STRUCTURES OF SOME RELEVANT COMPOUNDS

preparation is simply to form a poorly water soluble compound with penicillin and thus to slow the rate of dispersion of the antibiotic into the tissues.

Lignocaine is used as a local anaesthetic and as an anti-arrhythmic for treating cardiac arrest or to treat status epilepticus (BNF, 1986). As this can be self administered, poisonings do occur.

Other local anaesthetics with the exception of cocaine are used almost exclusively for surgical purposes and hence the incidence of self poisoning with these compounds is rare.

Bupivacaine has recently been used in a project in analgesia following abdominal surgery (Blyth et al., 1985). The clinicians were interested in assessing the degree of systemic absorption of the drug from the abdominal cavity. These samples were sent to this department for analysis.

3.1.3. NON MEDICAL USE OF LOCAL ANAESTHETICS

3.1.3.1. Drug abuse

Although most local anaesthetics can stimulate the CNS at high dosage, this usually occurs at blood concentrations in the toxic range. For this reason "recreational" abuse of local anaesthetics is rare. The notable exception to this rule is cocaine, which has a pronounced effect on the cerebral cortex, producing euphoria even at low concentrations.

Cocaine has a long history of use as a stimulant and euphoriant. At first thought to be a cure for opiate addiction, it soon became

clear that cocaine could be as addictive as morphine. Psychological dependence can develop and tolerance to the convulsant effects has been reported (Goodman and Gilman, 1980). The use of cocaine in the UK has until recently been small and restricted to the professional classes (Henman et al., 1986). This pattern is currently changing as the American cocaine market is saturated and the surplus drug is exported to Europe. This is reflected in the increase in seizures of the drug by the police and customs and excise (ISDD report, 1983; and supplement, 1984).

3.1.3.2. Doping in sport

The practice of affecting a competitors performance with drugs is not new. Messengers in South America have for centuries carried a supply of coca leaves which were chewed to relieve fatigue. Cocaine was one of the most popular early drugs used in cycling and marathon running (Woodland, 1980).

Local anaesthetics in general can be used to relieve pain thus allowing athletes to compete beyond limits where their bodies would normally not permit them. Local anaesthetics are listed as proscribed substances by the International Olympic Committee (IOC report, 1982). On medical grounds it is potentially dangerous for an athlete to compete whilst injured, as the risk of further injury is greater. Various local anaesthetic drugs, notably bupivacaine (marcain), are advertised in the sports medicine press (Physician and Sports medicine, 1984), for the treatment of sports related injuries. The extent to which these are used during sporting events is not known.

The above restrictions on local anaesthetics apply in horse racing

and greyhound racing also. In the latter, the use of all drugs is forbidden.

As the dose/effect relationship of drugs in a dog is not widely known, it is likely that the administration of any drug could act to the detriment of the dogs performance. This may be done maliciously or with the intention of masking a dogs potential in order to improve its odds in subsequent races.

For this reason it is likely that the use of local anaesthetics in these sports is more widespread than in human sport.

Details of the incidence of doping in horse racing in the UK between 1970 and 1981 have been reported (Moss and Haywood, 1984). Local anaesthetics, represented by procaine and lignocaine, accounted for 5.2% of all doping.

Greyhound racing is a major spectator sport in Great Britain, second only to football. It is also a popular gambling sport accounting for approximately £900M (18%) of the £5 billion staked each year. For this reason it is important that a doping control program should exist to detect any attempted doping and to discourage attempts at doping. This is done in the United Kingdom by the provision of a pre-race testing laboratory at those race tracks licenced by the British administrative body, the National Greyhound Racing Club (NGRC). Thin Layer Chromatography (TLC) following solvent/solvent extraction is used to detect barbiturates, neutral drugs and alkaloids (Bogan and Smith, 1968). The Fujiwara test is used to detect chloral drugs. This track side testing is backed up by a fully equipped analytical laboratory at the University of Glasgow, Department of Forensic Medicine and Science where confirmatory analysis is carried out.

TABLE 3.1.

DRUGS FOUND IN THE URINE OF RACING GREYHOUNDS
AS REPORTED TO THE AORC IN 1984

Drugs	Times reported	% of total
Caffeine	35	18.9
Chlorbutanol	2	1.1
Dexamethasone	1	0.5
Dimethyl sulphoxide	4	2.2
Naproxen	1	0.5
Nikethamide	10	5.4
Oxyphenylbutazone	33	17.8
Phenylbutazone	53	28.6
Procaine	20	10.8
Salicylic acid	7	3.7
Sulphamethoxazole	1	0.5
Unspecified	18	9.7

Countries included: USA, Australia, New Zealand, Mexico.

Full details of those drugs which are detected in greyhound racing in the UK are not released by the NGRC, however figures provided by the Association of Official Racing Chemists (AORC, 1985) for Canada, Australia, New Zealand and America are shown in Table 3.1. In summary it can be seen that local anaesthetics, represented by procaine, accounted for 10.8% of all doping in greyhound racing in 1984 in those countries mentioned. The NGRC expressed an interest in studying the suitability of the current screening procedure to detect local anaesthetics.

3.1.3.3. Conclusions

From the broad applications of local anaesthetics discussed above, both in sport, medicine and drug abuse, it was deemed to be of interest to develop methods for the isolation, detection and measurement of these compounds in biological fluids. Those particularly of interest are bupivacaine, procaine and cocaine. Also of interest is the major human metabolite of cocaine, benzoyl ecgonine.

3.1.4. METABOLISM AND TOXICITY

3.1.4.1. Introduction

The structure activity relationships of local anaesthetics has been reviewed (Büchi and Perlia, 1971). The properties considered necessary for anaesthetic activity include sufficient lipid solubility to cross the phospholipid nerve membrane. Once inside the nerve they block the conduction of a nerve impulse by

altering the cell's permeability to sodium and preventing depolarization (Albert, 1973; Goodman and Gilman, 1980).

Local anaesthetics all have certain similar structural features. These include hydrophobic and hydrophilic domains separated by an alkyl chain. The hydrophilic region is a secondary or tertiary amine and the hydrophobic region is an aromatic residue. In most cases the linkage between the two includes an ester or amide bond. The type of bond involved, will determine to a large extent the persistence of the drug. In humans the ester type local anaesthetics (eg cocaine, procaine) are short acting, the ester being rapidly hydrolysed by plasma esterases. Amide-type local anaesthetics (lignocaine, bupivacaine) are less readily broken down and are therefore longer acting. Metabolism of these compound proceeds via oxidative dealkylation and hydroxylation prior to excretion (Bouche and Lhoest, 1976). The main toxic effect caused by high doses of local anaesthetic is excitation of the Central Nervous System (CNS) which will produce, by degrees, yawning, restlessness, excitement, nausea and vomiting.

These may be followed by depression, muscle twitching, convulsions, respiratory failure, coma and death (Martindale, 1984).

This wide range of effects illustrates the difficulty in assessing the correct dose of the drug required for a desired effect. In addition, certain individuals display hypersensitivity to these drugs in the form of anaphylaxis or cardiac depression (BNF, 1986). The metabolism and excretion patterns of these compounds in man were therefore considered.

TABLE 3.2.

SUMMARY OF THERAPEUTIC, TOXIC AND FATAL CONCENTRATIONS OF SOME LOCAL ANAESTHETICS†

compound	body fluid	therapeutic (all concentrations in ug/ml)	toxic	fatal
cocaine	urine	0 - 3	-	38 - 118
	blood	0.12 - 0.3	0.25 - 5	gt. 0.9
procaine	blood	12.7	96	gt. 21
bupivacaine	blood	0.6	gt. 0.4	-
mepivacaine	blood	2-5	-	gt. 9.8
lignocaine	blood	-	-	gt. 14

gt. = greater than.

† for sources, see text.

A summary of the therapeutic, toxic and fatal levels is given in table 3.2..

3.1.4.2. Cocaine

Cocaine can be taken orally, intranasaly or injected intravenously or intramuscularly. Some addicts can inject up to a gram a day. Typical of ester-type local anaesthetics cocaine has a short plasma half life of 0.7 to 1.5 hours. The main metabolites are inactive. These include benzoyl ecgonine, methyl ecgonine, ecgonine and recent reports include the isolation of an active metabolite, norcocaine (Chinn et al., 1982). 4-9% of the dose can be excreted unchanged. This will depend on the route of administration, orally or nasally administered cocaine may be hydrolysed prior to absorption. 16-55% is excreted as benzoyl ecgonine and 30-60% as ecgonine methyl ester (Moffat, 1986). Levels of cocaine in the urine following the use of cocaine applied topically to the nasal mucosa of 0 to 3ug/ml have been recorded (Wallace et al., 1976). Concentrations found in urine from fatal overdose cases are 38 to 118ug/ml for cocaine and 15-185ug/ml for benzoyl ecgonine (Chinn et al.,1984). As cocaine is psychologically addictive and tolerance to the toxic effects can develop there is considerable overlap between therapeutic, toxic and fatal blood concentrations, making interpretation difficult. The reported ranges are 0.12-0.3ug/ml, 0.25-5ug/ml and greater than 0.9ug/ml respectively (Stead and Moffat, 1984). Blood concentrations of benzoyl ecgonine in 5 fatal cases ranged from 1.0 - 7.4ug/ml (Chinn et al., 1984). The wide variation reported in the amount of drug excreted unchanged in the urine may

be due to hydrolysis of the cocaine during storage. This has been shown to be significant, and precautions should be taken to guard against it.

A qualitative study of cocaine in the racing greyhound (Lewis, 1984) showed that most of the dose was excreted as benzoyl ecgonine and could be detected for up to eight hours post administration. No cocaine was found in the urine.

3.1.4.3. Procaine

Procaine is a short acting ester type local anaesthetic with a half-life of around 0.1 hours. It is rapidly hydrolysed in man to p-aminobenzoic acid. Only about 2% is excreted unchanged in the urine. Procaine has a relatively low toxicity and steady state blood concentrations 12.7ug/ml have been reported for therapeutic treatment. Following accidental administration of 4g of the drug to a patient a plasma concentration of 96ug/ml was recorded. The patient survived (Wikinski et al., 1970). Fatalities have however been recorded above 21ug/ml (Stead and Moffat, 1984).

Toxic effects in the horse have been reported as CNS stimulation excitability and pawing at the ground (Dunlop et al, 1935; Reidenberg 1972).

3.1.4.3. Bupivacaine, mepivacaine and lignocaine

These compounds are amide type local anaesthetics (see figure 3.1) with correspondingly longer half lives of 1-3 hours, 1-2hours and 2-3 hours respectively (Moffat, 1986). Metabolism of these compounds in man proceeds by oxidative dealkylation and

subsequent hydroxylation prior to excretion. The metabolites are sufficiently polar to be excreted without conjugation (Bouche and Lhoest, 1976).

With bupivacaine, less than 10% of the dose is excreted unchanged. Therapeutic blood concentrations of 1.1ug/ml have been reported (Wilkinson and Lund, 1970), and concentrations of 0.6ug/ml were found in maternal blood during labour. Toxic reactions including muscular rigidity and convulsions have been reported at concentrations of greater than 4ug/ml. No information has been found for fatal levels. Mepivacaine has a similar toxicity to bupivacaine, and similarly, less than 10% is excreted unchanged. Therapeutic blood levels of 2-5ug/ml have been reported and fatalities have occured at levels greater than 9.8ug/ml. (Stead and Moffat, 1984).

Lignocaine has given rise to toxic reactions at 6ug/ml and fatalities have occurred at concentrations of greater than 14ug/ml. Less than 5% of the dose is excreted unchanged.

3.1.5. ANALYTICAL METHODS

3.1.5.1. Introduction

Ideally when screening samples for the presence of local anaesthetics, it would be possible to detect or confirm the presence of several of the drugs using one assay.
The literature however shows that most analytical methods are developed for the measurement of one drug and possibly its metabolites.

3.1.5.2. Chemical and Colorimetric Tests

Various presumptive tests have been developed for the rapid identification of cocaine in questioned material (Baker and Gough, 1979). Generally however, these are not sufficiently sensitive to be applied to biological materials.

3.1.5.3. Thin layer chromatography

Thin layer chromatography (TLC) has been applied to the identification of local anaesthetics in biological extracts. Comprehensive data collections for the identification of drugs by TLC normally include Rf values for local anaesthetics. Stead et al. (1982) investigated the TLC behaviour of 794 drugs in eight TLC systems. This improves the chances of correct identification by allowing comparison of Rf values of an unknown compound on different systems. The above study included Rf data for 20 local anaesthetics, among them procaine, cocaine, lignocaine and bupivacaine.

A recent publication (Moffat, 1986) includes a review of TLC systems for local anaesthetics and recommends a system from Stead et al, (1982), of silica gel plates, sprayed with KOH in methanol and dried. The plates are developed in acetone. A number of locating agents, including acidified potassium iodoplatinate (PIP), are recommended.

The Handbook of Analytical Toxicology (Sunshine, 1969) also includes Rf data for these compounds on several systems.

An earlier study of TLC for a large range of basic drugs (Rentoul and Smith, 1973; Oliver 1971) recommended silica gel plates, developed in chloroform:methanol, 3:1. These studies include Rf data for procaine, cocaine and lignocaine. All Rf values are in the acceptable range, however no Rf data are given for benzoyl ecgonine, bupivacaine or mepivacaine. Procaine, cocaine and lignocaine all gave positive reactions with acidified potassium iodoplatinate as the locating agent. This TLC system is used in the screening procedures for local anaesthetic drugs in greyhound urine (Bogan and Smith, 1968).

In addition there are reports of various TLC systems used in the investigation of one drug in particular, notably for cocaine in biological fluids (Wallace et al., 1975; Lewis, 1980).

All the above TLC systems provide suitable methods for screening for the presence of most local anaesthetic drugs. Detection limits are around one microgram on the plate when a suitable locating reagent is used.

The Rf values may be affected by the presence of other material in the extract and the colour produced with the locating reagent may depend on the amount of material on the plate. TLC is best used

therefore, to detect the presence of a compound whose identity can then be confirmed by other means.

3.1.5.4. Immunoassay

Several immunoassay methods have been reported for cocaine in a variety of samples including hair (Valente et al., 1981), blood, plasma and urine (Roche, 1978). An RIA method for the measurement of procainamide in serum has also been reported (Gallagher et al., 1980).

Immunoassay methods have the advantage that they can be applied directly to biological samples and require little or no sample preparation. Occasionally a methanol extraction is used in order to assist with sample handling and to reduce matrix interference. The disadvantage of the method is the high degree of cross reactivity of some antisera with metabolites and structural analogs and the complete lack of cross reactivity with other major metabolites (eg methyl ecgonine, ecgonine, Budd, 1981). RIA methods for other local anaesthetics have not been developed.

Radioimmunoassay techniques are among the most sensitive reported (Mule, 1974, Moffat, 1986). The fact that they are not absolutely specific can be an advantage as this allows RIA to be used as a screening procedure for the drug and its metabolites. The operator should however be aware that the method is not specific and that some confirmatory anaytical technique should be applied.

For any method other than RIA, where the sample contains material either from a biological extract or adulterants, some chromatographic technique should be applied.

3.1.5.5. Gas Chromatography

Gas chromatography (GC) has been extensively used to measure local anaesthetics in a variety of samples (Gudzinowicz and Gudzinowicz, 1979). A number are summarised in table 3.3.. Modern capillary columns giving high resolution allow the analysis of a large range of compounds on a single column (Ardrey and Moffat, 1981). GC is best suited to the analysis of non-polar, volatile, heat stable compounds. Local anaesthetics are generally amides or esters, and as such are prone to dissociation at temperatures often encountered in GC injectors or detectors. Decomposition of the drug may lead to multiple peaks or reduced sensitivity (Gudzinowicz and Gudzinowicz, 1979). Additionally when using GC care must be taken that the analyte is in the correct form for analysis, and that an appropriate solvent is used. As is shown later, one of the extraction procedures considered leaves any base in the extract in the form of the hydrochloride salt. It has been shown that only a narrow temperature range will permit the dissociation of this to the free base without the hydrolysis of the amide or ester.

A review of GC methods for the analysis of cocaine and its metabolites in biological fluids (Lindgren et al., 1981) shows that the analysis of cocaine is popular by GC, but simultaneous determination of its major metabolite, benzoyl ecgonine, is not possible, as this is an amino acid which requires derivatisation, usually methylation, back to cocaine, before GC analysis. Conversion to the propyl ester has also been reported (Wallace et al., 1976; Von Minden and D'Amato, 1977).

GCMS is the method of choice for absolute identification. Both

TABLE 3.3.

GC METHODS FOR THE IDENTIFICATION OF LOCAL ANAESTHETICS

Reference	date	compounds examined	sample	technique
Nelson et al.	1959	Lignocaine and metabolites	plasma/urine	GC-CI-MS
Beckett and Moffat	1968	procaine prilocaine lignocaine benzocaine cocaine	urine	GC-FID
Wallace et al.	1976	cocaine benzoyl ecgonine	urine	GC-FID
von Minden and D'Amato	1977	cocaine benzoyl ecgonine	urine	GC-FID
Kogan et al.	1977	cocaine benzoyl ecgonine	plasma/urine	GC-NPD/ECD
Naito et al.	1977	lignocaine mepivacaine	brain/liver/ muscle	GC-FID
Park et al.	1980	mepivacaine tetracaine bupivacaine etidocaine lignocaine	plasma	GC-NPD

TABLE 3.3. CONTINUED

Reference	date	compounds examined	sample	technique
Chinn et al.	1980	cocaine and metabolites	plasma	GC-CI-MS
Gettings et al.	1981	tocainide lignocaine	plasma/serum	GC-FID
Ardrey & Moffat	1981	1318 drugs inc. 20 locals	-	GC-FID/NPD
Baselt	1982	cocaine cocaine benzoyl ecgonine	plasma urine	GC-NPD GC-FID
Liu et al.	1983	lignocaine	blood/urine	GC-CI-MS
Matsubaru et al.	1984	cocaine benzoyl ecgonine methyl ecgonine	blood/urine	GC-CI-SIM
Marko et al.	1985	pentocaine	serum	GC-FID

electron impact (EI) and chemical ionisation (CI) mass spectrometry have been reported for cocaine (Chinn et al, 1982; Matsubara et al., 1984). Mass spectrometry is not widely available however and when it is, it is more efficient to use it for difficult one-off analyses rather than for routine screening.

3.1.5.6. High Pressure Liquid Chromatography

HPLC is well suited to the analysis of non-volatile or thermally labile compounds. Compounds containing low energy bonds, such as amides or esters in local anaesthetics and $C-N^+$ bonds in quaternary amines, are particularly suited to this technique. As a result, many procedures have been developed for the LC assay of individual local anaesthetics. A number are summarised in table 3.4..

Most of the methods examined use reversed phase columns. These are well suited to the analysis of biological extracts, retaining any highly lipophilic material irreversibly and allowing the chromatography of smaller ionised or ionisable molecules. In addition, reversed phase columns are compatible with a wide range of aqueous based solvent systems and are relatively stable between pH 2 and 9 (Snyder and Kirkland, 1978).

The use of highly contaminated extracts can shorten the lifetime of a column, and pre-columns should be used for these samples.

Local anaesthetics are basic by virtue of their secondary or tertiary amine functions. Examples from the literature and experience with morphine chromatography (see chapter 2) has shown that peak tailing problems associated with hydrophilic interactions can be overcome with the use of the correct mode of

chromatography, eg ion pairing.

Most of the reports in table 3.4. which include examples of chromatography exhibit either broad peaks (Width) or asymmetrical peaks. Various approaches have been adopted to overcome this, for example the use of ion pairing agents which has been reviewed earlier (1.3.2.).

Gill et al., (1982) have described a method for modifying the chromatography by including an alkyl amine in the mobile phase. They suggest that the amine competitively occupies the residual silanol sites on the stationary phase surface and thus reduces peak tailing. This was then used to good effect in improving the chromatography of phenyl ethylamines. The inclusion of N-hexylamine in the mobile phase has been applied to the analysis of some 35 local anaesthetic compounds (Gill et al., 1984). The authors noted however that the range of polarities encountered made it impractical to analyse all the drugs with one solvent system.

UV absorption detection is the method chosen in most cases for monitoring local anaesthetics. The advent of linear diode array and multiple wavelength detectors also improves the selectivity of UV detection (Fell et al., 1983) through complete capture of spectra or absorption ratioing at selected wavelengths. This has not been applied specifically to local anaesthetics.

Although the electrochemical properties of local anaesthetics have not been investigated in great detail, a report on the measurement of lignocaine in biological fluids by HPLC with electrochemical detection (Halbert and Baldwin, 1984) did include some cyclic voltammetry on lignocaine and bupivacaine. The full wave

TABLE 3.4.

HPLC METHODS FOR THE IDENTIFICATION OF LOCAL ANAESTHETICS

Reference	date	compounds examined	sample	column	Detection
Trinler and Reuland	1975	cocaine	street drugs	ODS	LCUV @ 254nm
Khalil and Shelver	1976	procaine	pharmaceuticals	ODS	LCUV @ 254nm
Jatlow et al.	1978	cocaine and metabolites	urine	ODS	LCUV @ 235nm
Evans and Morarity	1980	lignocaine cocaine and metabolites	plasma/tissue	ODS	LCUV @ 235nm
Warsaszkiewicz et al.	1981	lignocaine	pharmaceuticals	CN	LCUV @ 254nm
Jane et al.	1981	cocaine and others	street drugs	RP-2	LCUV @ 279
Nachtmann and Gstrein	1982	procaine	pharmaceuticals	RP-8	LCUV @ 220nm
Noggle and Clarke	1983	procaine cocaine mepivacaine lignocaine and others	street drugs	ODS	LCUV @ 254nm

TABLE 3.4. CONTINUED

Reference	date	compounds examined	sample	column	Detection
Ohtake et al.	1983	procaine	urine	D-814	LCRI/UV @ 270nm
Lindberg and Pihlajamaki	1984	bupivacaine	serum	RP-8	LCUV @ 210nm
Wiegand and Chou	1984	bupivacaine	plasma	ODS	LCUV @ 254nm
Halbert and Baldwin	1984	lignocaine bupivacaine	blood/serum	silica	LCED @ 1.2V
Gill et al.	1984	data for 30 locals	street drugs	ODS	LCUV @ 230nm

potentials (E_p) recorded were high (greater than 1 volt) and were found to be dependent on the pH and the acetonitrile content of the support solution. They did show that ED might be suitable for these compounds under the correct mobile phase conditions, but the authors concluded that the high potentials recommended (1.2 volts) meant that these compounds were not ideal candidates for LCED.

A recent report (Musch et al, 1985), has indicated that a number of local anaesthetics, including procaine and lignocaine, were detectable by ED, but the potential required was 1.2V. The method was not applied to the analysis of biological extracts.

An examination of the structures of local anaesthetics suggests that the aromatic amine function might be responsible for the electrochemical activity. Reactions of primary and secondary aromatic amines have been reviewed (Adams, 1969; Ryan, 1984). They all involve radical formation at the anilinium nitrogen and subsequent delocalisation and dimerisation. Examples of reactions for para substituted anilines (eg procaine) and secondary aromatic amines (eg bupivacaine, lignocaine) are shown in figures 3.2. and 3.3..

Isocratic liquid chromatography appears to be a rapid method of analysis which might be applied to a range of local anaesthetic compounds.

The literature suggests that at least two detection methods might be suitable also.

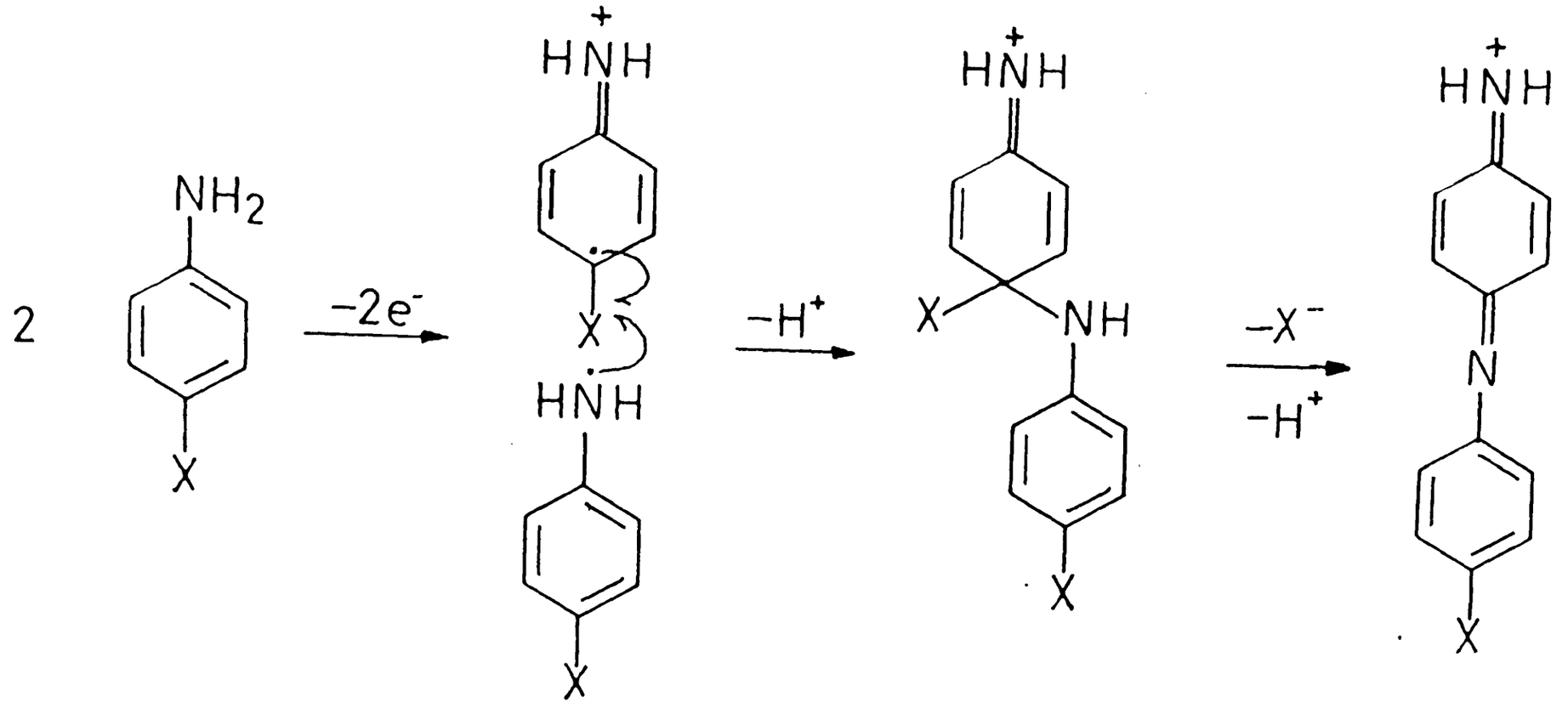


FIGURE 3.2.

ELECTROCHEMICAL OXIDATION OF PRIMARY AROMATIC AMINES

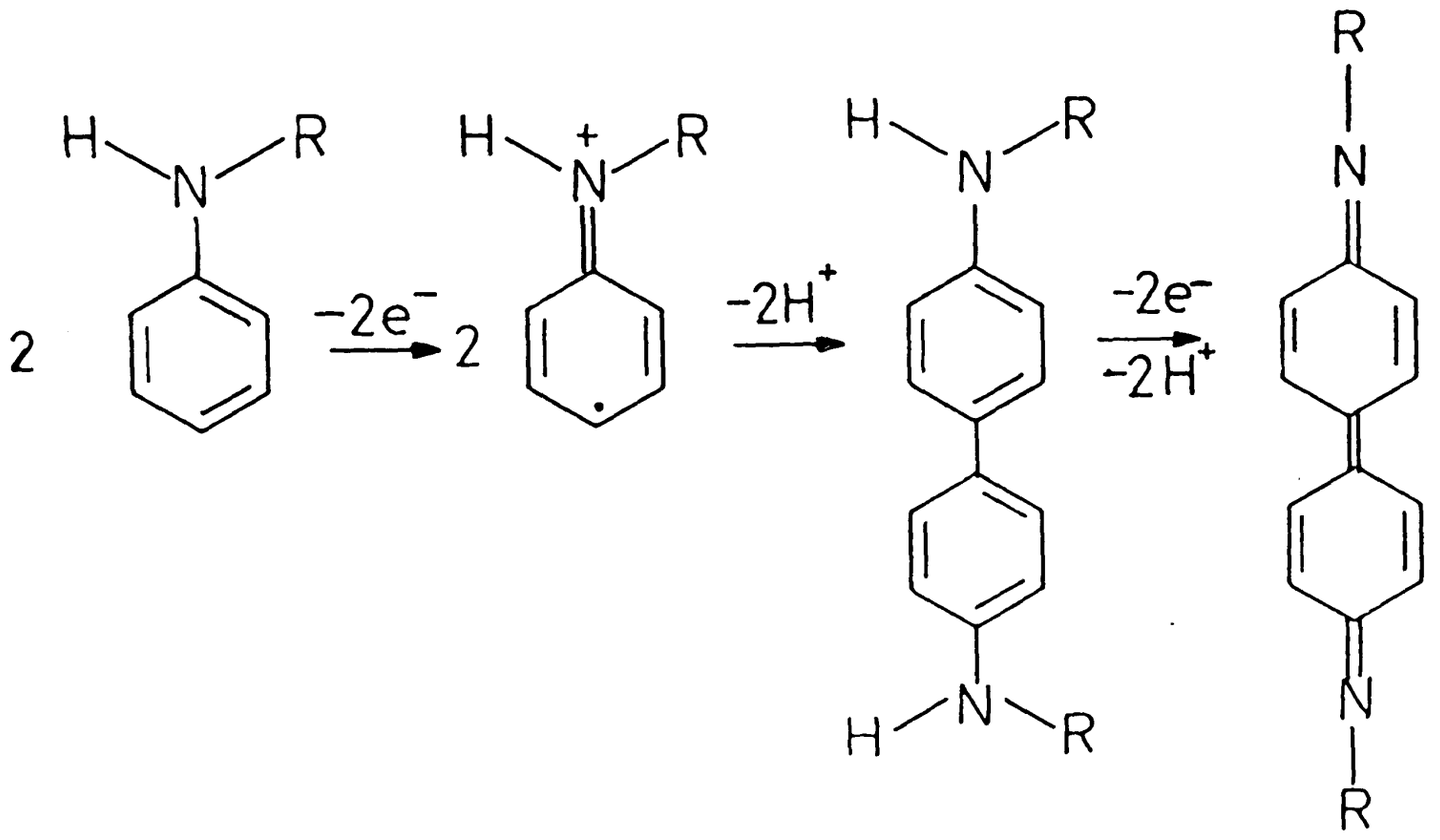


FIGURE 3.3.

ELECTROCHEMICAL OXIDATION OF SECONDARY AROMATIC AMINES

3.1.5.7. Conclusions

The above techniques have been considered with a view to selecting a screening method for the presence of drugs in biological fluids, and to consider appropriate confirmatory techniques.

Immunoassay is sensitive but has not generally been applied to the detection of local anaesthetics. The use of mixed antisera would provide a specific screening procedure for local anaesthetics. This however would prevent the detection of other drugs in a sample unless a comprehensive series of RIA screens was used. TLC is cheap, rapid and a much more general method for screening biological extracts for the presence of exogeneous compounds. For this reason it was prefered to RIA and the TLC system described by Rentoul and Smith (1973) was selected for use in this work as it is currently in routine use in the department. It has been shown over many years to be suitable for the detection of most common drugs (Smith, 1972; Smith 1978).

For confirmatory analysis, an isocratic HPLC method may offer a suitable assay in terms of selectivity, convenience and speed. Individual properties of the solvent, column or solvent additives can be altered to allow the separation of a range of analytes from each other and from other material in an extract. For this reason the development of an HPLC assay for these compounds was considered. The compounds of most interest (see 3.1.3.) were procaine, for its doping applications, bupivacaine from a clinical point of view and cocaine and its metabolite benzoyl ecgonine, from the point of view of drug abuse. The ideal HPLC method should therefore be suitable at least for these compounds.

3.1.6. SAMPLES AND SAMPLE PREPARATION

3.1.6.1. Introduction

The samples most commonly submitted for toxicological analysis for local anaesthetics are of biological origin - blood, urine or plasma. These arise from cases where the drugs may have been administered either in therapeutic, toxic or overdose levels in any of the circumstances discussed earlier (see 3.1.3.)

3.1.6.2. Blood and Plasma

Plasma as a medium is preferred to whole blood, as whole blood is prone to decomposition and changes in its gross properties which make analysis more difficult . Information on protein binding is available for bupivacaine and mepivacaine, suggesting that 90% and 77% respectively are bound to plasma proteins (Moffat, 1986).

This suggests that the drug is not concentrated in the erythrocytes, and that blood concentrations and plasma concentrations should not differ greatly. The degree of protein binding will not neccessarily prevent the extraction of the drug from the sample, as the extent of binding is likely to change with pH and the solvent conditions.

Plasma samples also contain less material likely to interfere in the assay than does whole blood.

Therapeutic concentrations of the drug in blood or plasma will often be quite low. For clinical purposes, with respect to therapeutic drug monitoring (TDM), it is important to monitor blood or plasma concentrations to ensure that the drug level is

sufficient for therapeutic effect, but is not allowed to accumulate to toxic levels.

3.1.6.3. Urine

Urine is the sample of choice in dope testing. This is due to the ease of collection and also the relatively large volume available. In clinical and post mortem situations urine is often not available or difficult to collect.

Unchanged drug is often found in the urine in addition to the metabolites. In the case of cocaine, Chinn et al., (1984), reported several cases where high levels of cocaine were found in the urine, but cocaine was not detected in the blood. Hydrolysis of cocaine in stored urine samples has also been noted however (Baselt, 1983; Fletcher and Hancock, 1983).

One problem with urinalysis is that metabolites are often converted to the conjugated form in order to improve their water solubility prior to excretion. In order to extract and measure these conjugated metabolites, the sample must be hydrolysed using for example acid/base hydrolysis, Helix Pomaciea or Subtilisin Carlsberg prior to analysis.

Local anaesthetics and their metabolites are generally sufficiently polar to be excreted without conjugation.

Quantitation of drug concentrations in urine is done relatively easily by the addition of an internal standard prior to extraction. The results obtained however are of little practical use, unless the total volume of urine over the excretion period is known, or the drug concentration is related to some internal marker such as creatinine clearance (Duarte, 1980).

In metabolism studies where the total volume of urine is not known, the concentration of the drug is often normalised with respect to the creatinine concentration of the urine. Lactate and creatinine are the products of anaerobic activity in the muscles. They are normally present in low but constant concentration in human blood, when normal renal function is present. Creatinine is excreted by the renal tubules at a constant rate, independent of the glomerular filtration rate (GFR). Relating the urinary drug concentration to the creatinine concentration gives an accurate indication of the blood drug concentration. In the dog however, creatinine is excreted by a weak proximal tubule secretory mechanism. Studies on creatinine clearance in the dog have shown that a very wide range exists, making creatinine clearance an unreliable marker (Bovee and Joyce, 1979). This is further complicated in the racing greyhound, as blood creatinine is likely to vary considerably during bursts of anaerobic activity such as racing.

3.1.7. SAMPLE PREPARATION

3.1.7.1. Introduction

Direct analysis is suitable only for immunoassay methods where the technique is sensitive enough not to require preconcentration of the analyte, and not susceptible to interference from the sample matrix. Preconcentration of the drug improves the sensitivity of the assay, and the removal of extraneous material from the sample improves the selectivity.

Centrifugation or ultra filtration are occasionally used prior to an extraction step to assist with sample handling.

Extractions are generally of two types, liquid/solid or liquid/liquid. The application of these to the isolation of local anaesthetics is considered below.

3.1.7.2. Liquid/Liquid partition extraction

As with extraction procedures for morphine reviewed earlier, most reports favour liquid liquid extraction.

Local anaesthetics are basic drugs and are therefore extracted from aqueous alkaline samples into organic solvents. They are likely to be extracted in most basic drug screens. Some of these, both general and specific procedures are summarised in table 3.5.. In general, alkaline pH is used with chloroform based solvents. The pH of the extraction should be carefully controlled and the extraction procedure should be as rapid as possible as some of the ester type local anaesthetics are prone to hydrolysis even on short exposure to base (Fletcher and Hancock, 1981; Baselt et al., 1983).

Many methods have been described for the isolation of cocaine and in some cases its metabolites. As benzoylecgonine is a water soluble amino acid, it is not extracted well into pure solvents. Salting out procedures have been used (Koontz et al, 1973) but were shown to cause coextraction of other endogeneous material from the urine (Wallace et al, 1976). This suggests that mixed solvents would be require for the extraction of benzoylecgonine.

A thorough study (Wallace et al., 1975; Wallace et al., 1976) conducted on the extraction of cocaine and benzoyl ecgonine from

TABLE 3.5.

EXTRACTION PROCEDURES SUITABLE FOR LOCAL ANAESTHETICS

Reference	date	compound/s	sample	buffer	solvent	recovery (%)
Beckett and Moffat	1968	basic drugs	urine		CH_2Cl_2 diethyl ether	
Bogan and Smith	1968	basic drugs	urine	NH_4OH	EtOAc	NG
Asling et al.	1969	bupivacaine mepivacaine	plasma	NaOH	diethyl ether	NG
Wallace et al.	1975	cocaine benzoyl ecgonine	urine	none	$CHCl_3$:EtOH 8:2	90-95 65-70
Horning et al.	1976	basic drugs	urine blood	$NH_4(CO_3)_2$	EtOAc	87-104
Linberg and Pihlajamaki	1978	bupivacaine	plasma	NaOH	Hexane/IPA/$CHCl_3$ 3/6/1	92
Jatlow et al	1978	cocaine and metabolites	urine	9.5	$CHCl_3$:EtOH 8:2	
Park et al.	1980	mepivacaine bupivacaine etidocaine lignocaine tetracaine	plasma CSF blood	NaOH	diethyl ether	NG

TABLE 3.5. CONTINUED

Reference	date	compound/s	sample	buffer	solvent	recovery (%)
Chinn et al.	1980	cocaine and metabolites	blood brain liver urine kidney	KH_2PO_4	toluene/heptane/IAA	NG
Lewis	1980	cocaine	urine	pH3-4	$CHCl_3$	NG
		benzoyl ecgonine	urine	pH9-10	$CHCl_3$/IPA 95:5	NG
Hill et al.	1982	basic/acidic drugs	urine	ion-paired	$CHCl_3$/IPA 3:1	NG
Sundlof et al.	1982	procaine	urine	NH_4OH	ether/hexane/IAA	80
Delbeke et al.	1983	basic/acidic drugs	urine	various	benzene cyclohexane	
Wiegand et al.	1984	bupivacaine	plasma	NaOH	hexane	95

biological fluids showed that the recovery of cocaine and benzoyl ecgonine were relatively pH independent over the range pH 5.5 to 9.5. The recovery of cocaine fell at pH above 10. Various solvents were examined and an optimum solvent of 20% ethanol in chloroform was found. This gave recoveries of 90-95% for cocaine and 65-70% for benzoyl ecgonine. Other workers have reported this solvent mixture to work very well (von Minden and D'Amato, 1977; Jatlow et al., 1978). The solubility of cocaine and benzoyl ecgonine in other solvents has been shown to be poor.

A study in 1980 (Lewis, 1980) of cocaine in the racing greyhound used separate extraction procedures for cocaine and benzoyl ecgonine. These are discussed later.

Two methods are reported for the isolation of procaine from the urine of the racing greyhound (Sundlof et al. (1984). These involve back extractions and the use of benzene as the extracting solvent which was unacceptable on grounds of toxicity.

Extraction procedures for bupivacaine include those described by Wiegand et al. (1978) and Lindberg and Pihlajamaki (1984). Both are performed at alkaline pH using NaOH to adjust the pH.

A small number of purpose designed extraction schemes for drug screening in urine have been described. The method developed by Bogan and Smith (1968) for the analysis of acidic, neutral and basic drugs in greyhound urine uses an initial extraction at basic pH into ethyl acetate, to remove basic compounds from the sample. A subsequent extraction at low pH removes neutral and weakly acidic drugs. Some of the interfering compounds encountered in these extracts have been characterised and identified (Oliver et al., 1978). This procedure does however

use a large amount of solvent (200ml/sample) and requires several subsequent extraction steps which might reduce the efficiency of the procedure. No specific details of the recovery of local anaesthetics are given.

A more recent report (Hill et al., 1982) describes a single step extraction procedure for the analysis of basic neutral and weakly acidic drugs in greyhound urine. This is performed at pH7 and the basic drugs are extracted by formation of an ion pair complex with dioctyl sulphosuccinate (DOSS). This appears to be a very rapid method and requires only 5ml of extracting solvent. It has been shown to recover cocaine, but no information is given regarding procaine, bupivacaine or benzoyl ecgonine.

3.1.7.3. Liquid/solid extraction

A small number of applications of liquid/solid extraction procedures which are or may be suitable for the analysis of local anaesthetics have been reported (table 3.6.). Of these, most are for plasma samples and are for one drug in particular rather than a range.

Two of these procedures use Extrelut, a diatomaceous earth absorbent. One is a general extraction method for drugs in blood or urine (Breiter et al, 1978) and no data is given specifically for local anaesthetics.

The other is designed specifically for the extraction of cocaine and its metabolites from urine. High recoveries are reported.

Another two procedures use bonded phase extraction cartridges.

One of these procedures (Ohtake et al., 1983) is designed for drug screening in urine from racehorses. No information is given

TABLE 3.6.

SOLID PHASE EXTRACTION PROCEDURES SUITABLE FOR LOCAL ANAESTHETICS

Reference	date	compound/s	sample	buffer	solvent	recovery
Absorption on extrelut diatomaceous earth						
Breiter et al.	1976	basic/acidic drugs	blood urine	pH 9	IP ether MeOAc (7:3)	86-102
Matsubara et al.	1984	cocaine methyl ecgonine benzoyl ecgonine	urine	pH9	$CHCl_3$:IPA 9:1	83 38
Stewart et al.	1984	procaine	plasma	NaOH	CH_2CL_2	51
Adsorption extraction on C-18 cartridge.						
Ohtake et al.	1983	basic/acidic drugs	urine	neutral	$CHCl_3$	NK
Halbert et al.	1984	bupivacaine lignocaine	serum	NH_4OH	acetonitrile	76
Stewart et al.	1984	procaine	plasma	NaOH	CH_2Cl_2	40

NK - not known

regarding recoveries, but the procedure is reported to be suitable for the detection of procaine.

The other procedure (Halbert and Baldwin, 1984) gave a recovery of 76% and the extract was analysed by HPLC.

In addition to these specific reports, a review (Stewart et al, 1984) examined six solid phase extraction methods of both types. several basic and acidic drugs were extracted from plasma and aqueous standards and the recoveries measured by UV spectrophotometry. Among those drugs examined was procaine, and the recoveries ranged from 15% on a silica adsorbent phase through 16% on a cyano phase and 39% on a C-18 phase, to 51% on an absorbent stationary phase and 61% on XAD-2. This work also demonstrated the different recoveries observed when the drug was present in different matrices.

Automated preconcentration methods such as direct injection of a large volume of urine onto a precolumn and subsequent adsorption/elution using column switching have been reported. These are still in the development stage, expensive and unsuitable for routine analysis as yet.

3.1.7.4. Conclusions

For clinical studies or post mortem analyses, blood or plasma are the most suitable samples, whilst urinalysis provides a good indicator of drug administration in sport.

As all these applications are of interest in the present study, two approaches are required. Firstly, a drug screening procedure suitable for the extraction of a broad range of drugs from a large sample, probably urine. Secondly an extraction procedure

applicable to smaller samples, typically blood and plasma, but ideally suitable for confirmatory analysis on small volume urine samples.

For screening urine samples two procedures were considered for further study. The first is that used routinely for the analysis of drugs in greyhound urine, based on the procedure described by Bogan and Smith (1968). This study will examine the suitability of that method for the detection of local anaesthetic drugs in urine. Secondly, a procedure described by Hill et al. (1982), using ion-pair extraction to provide a single step extraction procedure for acidic, basic and neutral drugs is examined as it is reportedly very rapid, cheap and efficient.

For blood and plasma samples, it was decided to investigate the method described by Lindberg and Pihlajamaki (1984). This had been used effectively for bupivacaine (recovery = 92%), but has not been assessed for other local anaesthetics.

Experience gained in the extraction of morphine and the reports cited above (Matsubara et al., 1984; Stewart et al., 1984) suggested that liquid/solid extraction on diatomaceous earth might be suitable for the extraction of at least some of the compounds of interest from all sample types.

3.2. UV ABSORBANCE AND ELECTROCHEMICAL PROPERTIES OF LOCAL ANAESTHETICS

3.2.1. Introduction

In order to select an appropriate detection system, suitable for a range of local anaesthetics, the UV absorbance and electrochemical properties of some of the local anaesthetics were considered.

3.2.2. UV/visible spectrophotometry

Examination of the UV visible spectra of the local anaesthetics will show wavelengths at which these compounds will absorb most strongly. This was done for the compounds of interest in the hope of finding a strong chromophore common to all. Other local anaesthetics were also considered for their possible use as internal standards.

3.2.2.1. Method

Standard solutions of between 2 and 4 mg/100ml were prepared in 0.01N H_2SO_4. The UV spectra of each was recorded in turn. The absorbance maxima were noted and the gravimetric absorbance coefficient for a 1mg/100ml standard solution in a 1cm cell (A^1_1) at these maxima calculated (Moffat, 1986). The spectra were recorded against a 0.01N H_2SO_4 blank in a 1cm glass cell. The instrument used was an HP8451A scanning diode array spectrophotometer, scanning from 190 to 400nm, bandwidth 2nm.

3.2.2.2. Results and Discussion

The absorbance maxima are listed in table 3.7., with the calculated A_1^1 values.

The UV spectra are shown superimposed in figure 3.4. This demonstrates that all the compounds examined have absorbance maxima below 220nm and that the greatest number have maximum absorbance at 200nm. Absorbance at this wavelength is caused by sigma-sigma* transitions and is common to most organic molecules. This wavelength is best avoided if alternatives are available as this region of the spectrum is inaccessible to some UV detectors, and with some mobile phase solvents and additives.

Further examination of the spectra shows that most of the compounds demonstrate a second absorbance maximum at around 230nm. Exceptions to this however are cinchocaine, bupivacaine, mepivacaine and lignocaine which have no maximum in this region.

Further maxima are also seen in the region 260 to 280nm and above. The absorbances are poor however and are not coincident. Cinchocaine, for example, peaks at 322nm.

In selecting an appropriate wavelength for UV monitoring, the maximum number of compounds should be detectable with the highest extinction coefficient. This suggests the use of monitoring at 200nm. If this were not possible, monitoring at 230nm would provide greater selectivity and similar sensitivity.

Bupivacaine however has poor absorbance at this wavelength ($^{230}A_1^1 = 75$).

It was decided that if UV detection was to be used, a wavelength of 230nm should be used to monitor the eluent. This would however reduce the sensitivity towards bupivacaine, mepivacaine,

TABLE 3.7.

UV ABSORBANCE MAXIMA FOR VARIOUS LOCAL ANAESTHETICS.

compound	Lambda Max.	A^{1}_{1}	$^{230}A^{1}_{1}$
amethocaine	200	467	431
	230	431	
	312	68	
	282	55	
amylocaine	236	451	427
	202	375	
	276	47	
benzocaine	200	733	693
	228	697	
	274	92	
bupivacaine	200	408	75
	264	5	
butacaine	200	351	319
	230	319	
	280	59	
cinchocaine	206	934	297
	248	637	
	322	224	
cocaine	236	379	360
	202	368	
	276	37	
lignocaine	210	394	60
	264	14	
mepivacaine	202	614	125
procaine	230	425	425
	200	388	
	280	95	

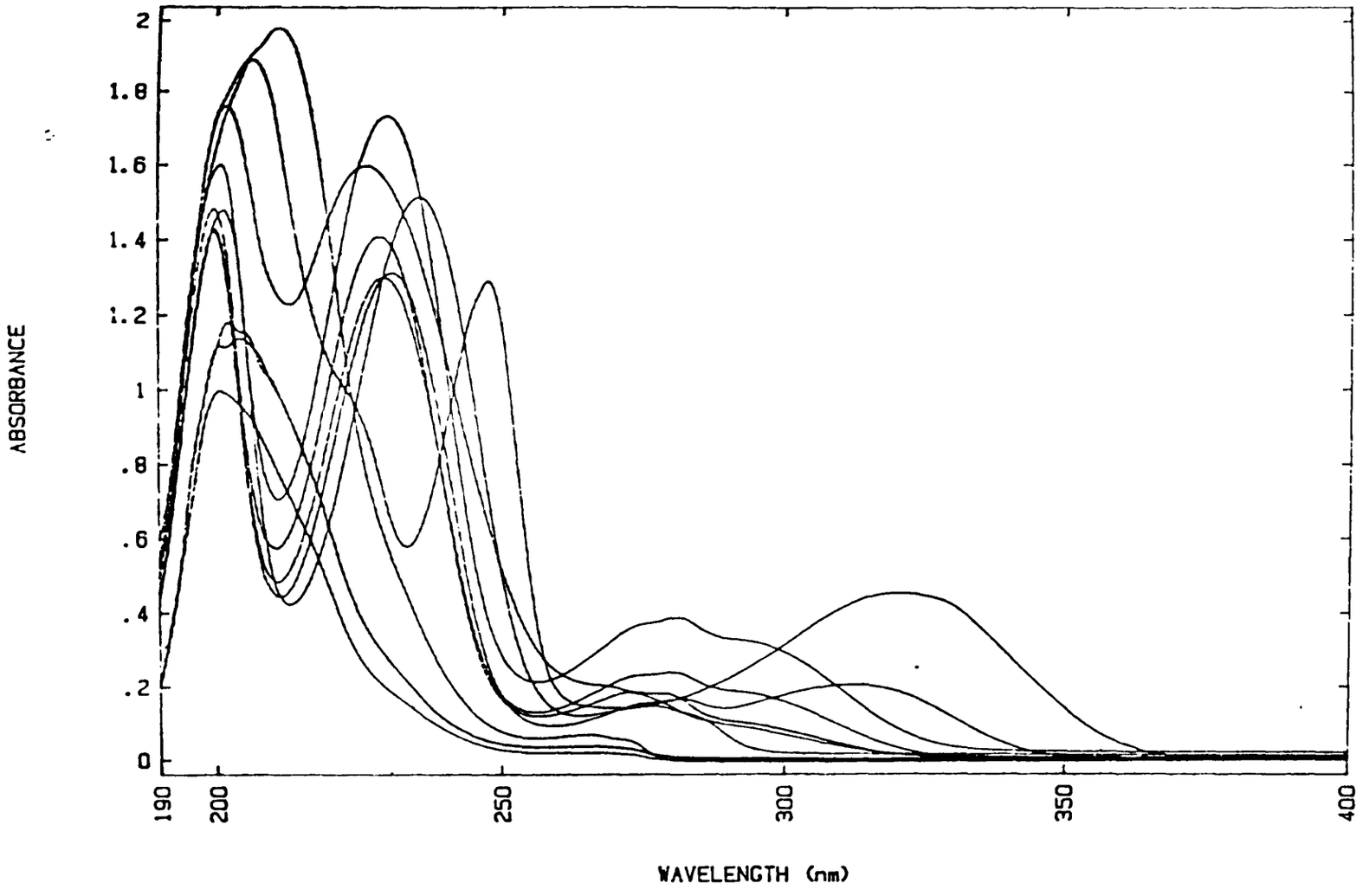


FIGURE 3.4.

SUPERIMPOSED UV SPECTRA FOR THOSE LOCAL ANAESTHETICS LISTED IN TABLE 3.7.

lignocaine and cinchocaine, as suggested by the $^{230}A_1^1$ data in table 3.7.. If these compounds were of special interest, reducing the monitoring wavelength towards 200nm would improve the sensitivity.

3.2.2.3. Conclusions

Having assessed the appropriate wavelengths for the detection of the local anaesthetics of interest, and noted the problems associated with the detection of bupivacaine, mepivacaine, lignocaine and cinchocaine, it was decided also to investigate the possibility of using electrochemical detection to screen for a range of local anaesthetics.

3.2.3. Electrochemical properties

3.2.3.1. Introduction

The use of electrochemical detection has been reported for lignocaine, bupivacaine (Halbert and Baldwin, 1984; Musch et al., 1985), procaine, amylocaine, tetracaine and benzocaine (Musch et al., 1985). This has been reviewed earlier (3.1.5.6.). It has been demonstrated above that UV detection, whilst suitable for the detection of all the local anaesthetics, did not have a common wavelength giving good response to all the drugs. It was decided therefore to examine, by the use of cyclic voltammetry (CV), the electrochemical properties of some of the local anaesthetics to determine if ED would be suitable.

3.2.3.2. Method

Cyclic voltammetry was performed under the same conditions as described for morphine (2.4.5.). The support electrolyte solution (8% acetonitrile in 0.01M KH_2PO_4, was also the same. The pH used was adjusted to 3, as it has been demonstrated that pH in this range was required to give acceptable chromatography for basic compounds.

Samples were scanned oxidation-reduction with a switching potential of ± 1.5 volts. The electrode was polished between successive experiments.

3.2.3.3. Results and discussion

The compounds examined were procaine, bupivacaine and cocaine. The results are shown in table 3.8.. Voltammagrams for procaine and bupivacaine are shown in figure 3.5. An electrochemical response for bupivacaine was observed at 1.25V, slightly higher than reported (Halbert and Baldwin, (1984), Ep=1.0). A small oxidative wave was noted for procaine at 0.65 volts. A wave at 1.35 volts was noted for cocaine.

The proposed reaction mechanism for bupivacaine and procaine is radical formation in the aniline system (see 3.2.3.3. and 3.1.5.6.). It is assumed that mepivacaine would have a similar activity.

Cocaine however does not contain an aromatic amine and the activity observed here was assumed to result from reactions at the benzoyl group. Reactions at this group were noted earlier with some of the narcotic analgesics (2.4.7.).

Insufficient material was available to permit the analysis of

TABLE 3.8.

Ep VALUES (Volts) MEASURED FOR LOCAL ANAESTHETICS

compound	Ep	Ep/2
bupivacaine	+1.25	+1.05
cocaine	+1.35	+1.10
procaine	+0.65	+0.55

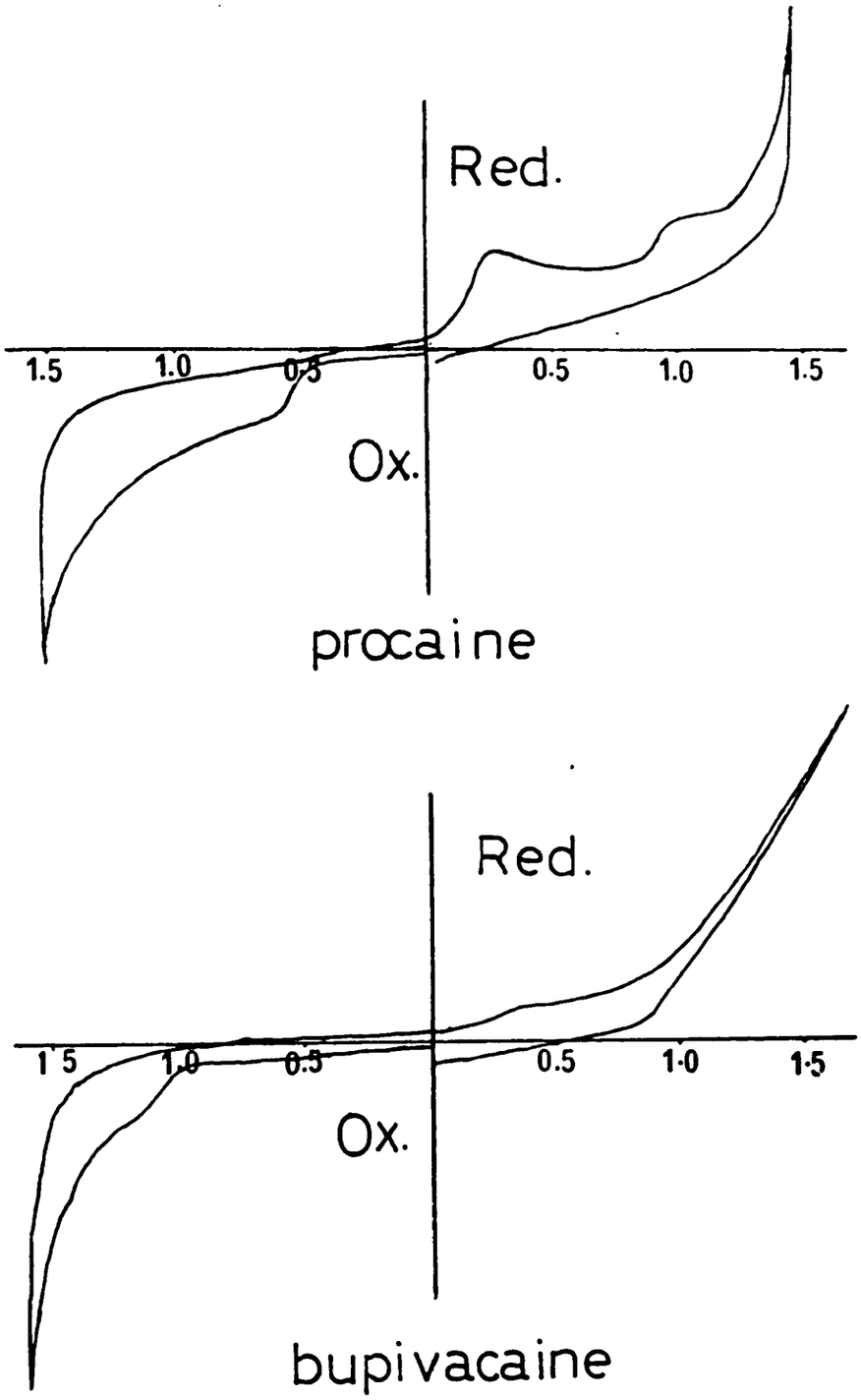


FIGURE 3.5.

OXIDATION-REDUCTION CYCLIC VOLTAMMOGRAMS RECORDED FOR PROCAINE AND BUPIVACAINE

benzoyl ecgonine which could have tested this theory.

Further examination of the electrochemical behaviour of procaine produced the voltammogram shown in figure 3.6..

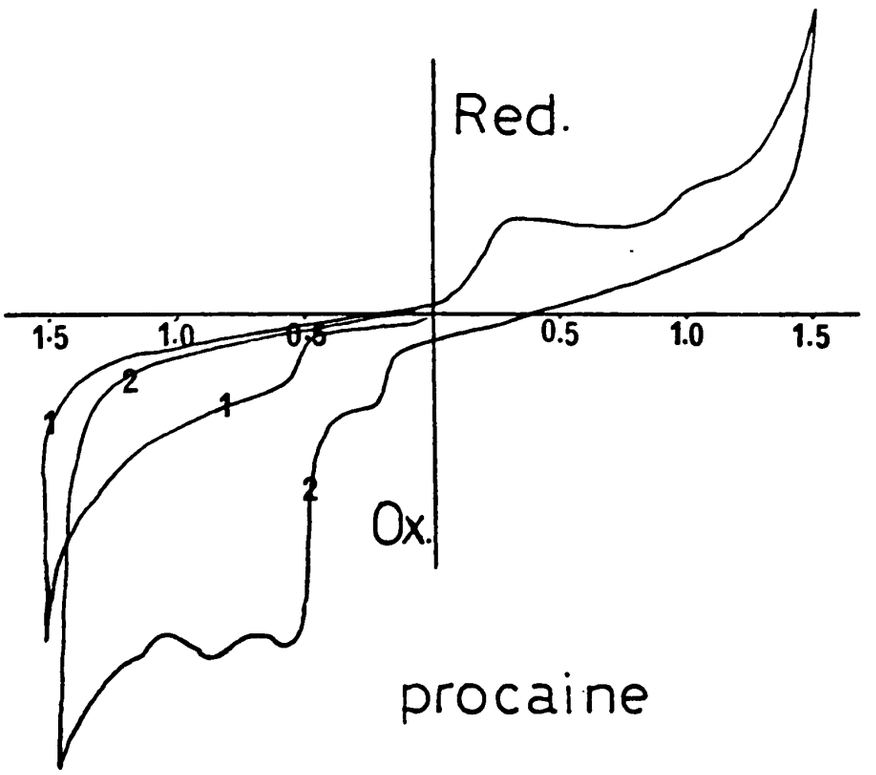
Following oxidation reduction cycling, subsequent oxidation produced the behaviour shown. The same pattern was observed in reduction-oxidation cycling. This implies that a reductive product of procaine (possibly arising from the wave noted at -0.25 volts (figure 3.6.)) has electrochemical activity at low oxidative potentials. This behaviour was similarly observed for bupivacaine and xylidine (2,6,dimethyl aniline). This suggested the involvement of the aromatic amine, but no suitable reaction scheme is apparent, given that electron rich compounds such as aromatic amines are not expected to be so readily reduced (Tomlinov, 1972).

3.2.3.4.Conclusions

The EC activity of local anaesthetics occurs mostly at high potential. These are at the upper end of the potential range suitable for ED. Further cyclic voltammetry might be carried out to investigate the cycling effect noted for bupivacaine and procaine. The use of multiple electrode coulometric detectors might allow the detection of these reduction products at lower oxidative potentials (Roston et al, 1984).

3.2.4. Summary

Both UV and electrochemical detectors have been shown to be suitable for the measurement of local anaesthetics by HPLC. The potentials required for ED are high, which might lead to rapid



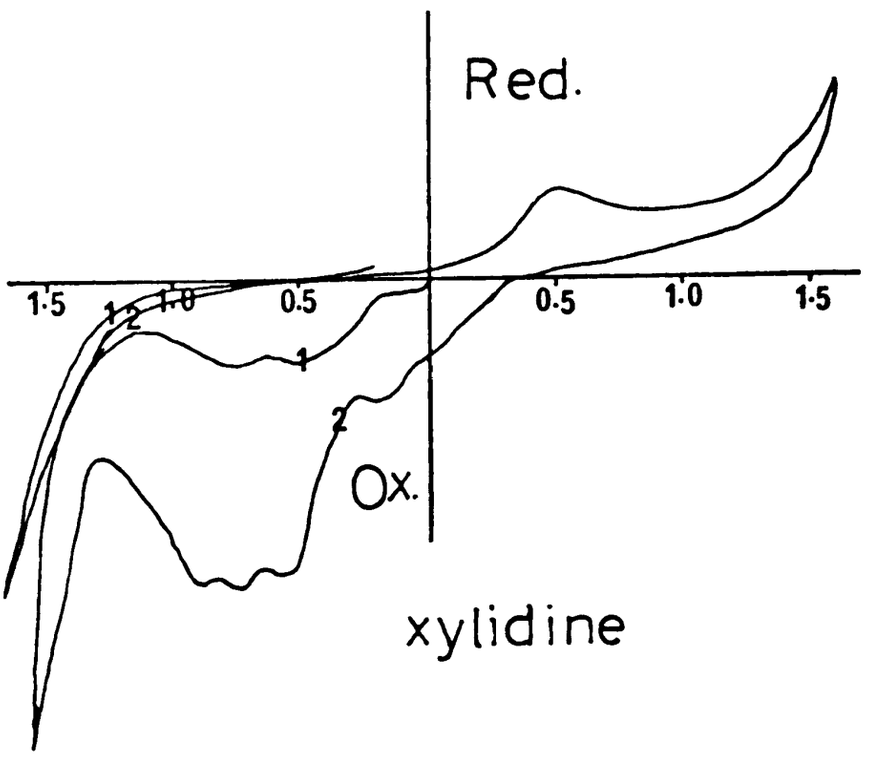


FIGURE 3.6.

OXIDATION-REDUCTION-OXIDATION CYCLIC VOLTAMMOGRAMS RECORDED FOR PROCAINE AND XYLIDINE

electrode fouling, and as large number of compounds will react at this potential, some of the selectivity of the detector is lost. There is an additional possibility of interference from the other components in the mixture and a larger background current from the mobile phase leading to greater noise. This confirmed the observation by Halbert and Baldwin, that local anaesthetics did not make ideal candidates for electrochemical detection.

For these reasons it was decided that UV monitoring at 230nm should be used as the method of detection for an HPLC assay for local anaesthetics. The sensitivity at this wavelength is poorer for bupivacaine, mepivacaine, lignocaine and cinchocaine but should impart some degree of selectivity to the assay.

The usefulness of cyclic voltammetry in assessing the suitability of electrochemical detection for a specific compound has been demonstrated. In addition, an understanding of the mechanism involved in the reaction might suggest the suitability or otherwise for compounds not previously considered for electrochemical detection.

3.3. CHROMATOGRAPHY

3.3.1. Introduction

Bupivacaine was used as a model compound in an attempt to develop an HPLC assay suitable for several local anaesthetics. It was chosen for two reasons. There were a number of samples resulting from a hospital study on bupivacaine in abdominal surgery which required analysis. Secondly, bupivacaine is a relatively new and increasingly popular local anaesthetic and therefore a potential doping agent in sport. No rapid screening method for the drug in urine has been reported.

On the basis of the investigation of the electrochemical and UV properties of these compounds (3.2.), it had been decided that UV detection at 230nm should be used for monitoring the eluent.

The behaviour of bupivacaine in binary methanol/0.01M KH_2PO_4 and acetonitrile/0.01M KH_2PO_4 solvent systems was investigated to determine if these would provide acceptable chromatography and if so, whether that system would be suitable for the analysis of other local anaesthetics.

3.3.2. THE USE OF METHANOL AS ORGANIC MODIFIER

3.3.2.1. Method

Binary methanol/0.01M KH_2PO_4 mixtures were prepared and the chromatography of bupivacaine was examined. The HPLC system used was as follows. A single piston pump (Gilson 302 with 802C manometer) operated at 2 ml/min. Sample introduction was through a Rheodyne 7125 6-port injection valve with a 20ul

loop, using an 25ul glass syringe (SGE).

A 25cm ODS Hypersil column (Shandon Southern, packed as in appendix B) was used. The detector was a variable wavelength UV monitor (Spectroflow 757 (Kratos) or PU4025 (Phillips)), set at 230nm. The absorbance and chart recorder settings were adjusted as neccessary. High pressure fittings were stainless steel, low pressure fittings were PTFE or polyethylene..

A 25cm ODS Hypersil column was used. This material was preferred for reasons given earlier. Longer columns are capable of producing longer retention times and greater resolution without a drop in efficiency. Once the best binary solvent mixture had been found, the effect of adjusting the pH was also investigated. This had been shown to be important when chromatographing morphine (2.5.3.).

3.3.2.2. Results and discussion

The retention data obtained are given in table 3.9.. Increasing the methanol content of the mobile phase caused a decrease in the retention time of bupivacaine. At a concentration of 100% methanol however, the drug was not eluted.

It was noted that decreasing the pH of the mobile phase from 6.4 to 3.0, caused a reduction in retention time, but also gave rise to sharper peaks. This was also noted in the chromatography of morphine and is due to improved solubility of the analyte in the mobile phase.

The optimum retention behaviour obtained was with a 70/30 methanol 0.01M KH_2PO_4 mobile phase, adjusted to pH 2.5 with concentrated orthophosphoric acid (K'=1.9; N=123) (figure 3.7.).

TABLE 3.9.

EFFECT OF METHANOL CONTENT AND pH ON CHROMATOGRAPHY OF BUPIVACAINE

% Methanol	RT	K'	Pk W	N
20	not eluted			
50	16.0	8.4	16	16
70	9.5	4.6	4	94
90	5.3	2.1	2.8	57
100	not eluted			

% Methanol	pH	RT	K'	Pk W	N
70	4.6	9.5	4.6	4	94
70	3.0	5	1.9	1.8	123 *

* Chromatogram shown in figure 3.7.

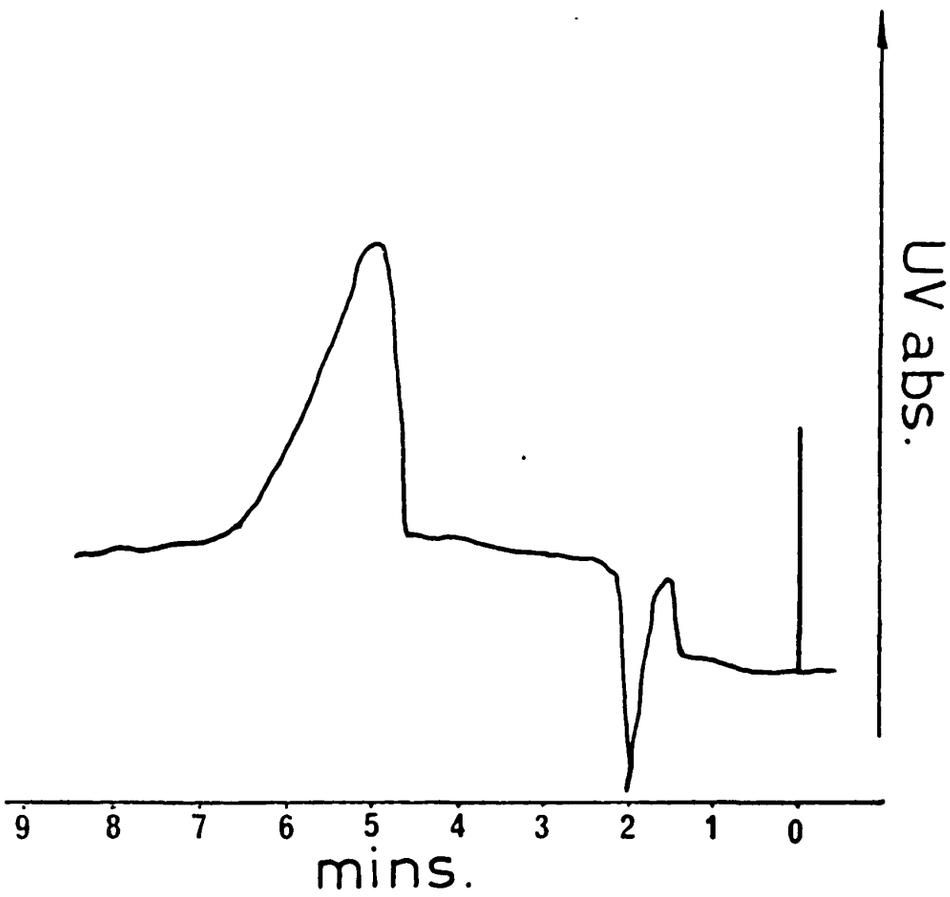


FIGURE 3.7.

CHROMATOGRAM OF BUPIVACAINE USING 250mm ODS HYPERSIL COLUMN WITH 70% METHANOL IN 0.01M KH_2PO_4 AT pH 3.0 AT 2ml/min

The peak was however quite broad and tailed badly (W=1.8mins; A_s=3.75). It was therefore not considered acceptable for analytical work.

3.3.2.3. Conclusions

The chromatography achieved with methanol as the organic modifier was not satisfactory. It was decided that a solvent containing acetonitrile should be investigated, as its selectivity properties are different from methanol.

3.3.3. USE OF ACETONITRILE AS THE ORGANIC MODIFIER

3.3.3.1. Introduction

Solvents with the same polarity have similar net solvating properties, but may have different selectivities depending on the extent of their proton donating, proton accepting and dipolar interactions (Snyder, 1974 and 1978).

A similar capacity factor was required, therefore the 0.01M KH_2PO_4/acetonitrile mixture with the same polarity as the above 0.01M KH_2PO_4/methanol mixture was used.

3.3.3.2. Method

The composition of an acetonitrile containing solvent with the same polarity was calculated using the equation discussed earlier (1.3.4.2.) and was found to be 81.2% acetonitrile. The effect of using lower pH was also investigated, as this had been

shown to improve the peak shape when methanol was used as the modifier.

Other HPLC conditions were as before (3.3.2.1.).

3.3.3.3. Results and discussion

The results are shown in table 3.10.. The 81.2% acetonitrile solvent gave a similar retention time to the methanol solvent for bupivacaine, but also gives a slightly less tailing peak. The peaks were still broad however, so the acetonitrile content was increased to 91.5%. This reduced the retention time and also improved the efficiency. Increasing the acetonitrile content further to 95% resulted in the non-elution of bupivacaine.

The effect of pH on the chromatography is similar to that of methanol sovents. The retention time is decreased as is the peak width. In this case however there is a net fall in the efficiency.

Optimum chromatography was obtained with the 91.5% solvent, at pH 3.0 (figure 3.8.).

3.3.3.4. Conclusion

The solvent giving the best chromatography so far (91.5% acetonitrile/ buffer) was rejected on the grounds that the efficiency was still low and the acetonitrile content was very high. Solvents with high organic modifier content should be avoided as they are likely to elute any lipophilic material present in extracts, causing late peaks and long term baseline fluctuation.

Similar problems of tailing and poor efficiency were encountered

TABLE 3.10.

EFFECT OF ACETONITRILE CONTENT AND pH ON CHROMATOGRAPHY OF BUPIVACAINE

% Acetonitrile	RT	K'	Pk W	N
81.2	10.3	5.1	3.4	147
91.5	8.5	4	2.2	239
95	not eluted			

% ACN	pH	RT	K'	Pk W	N
91.5	4.6	8.5	4	2.2	239
91.5	3.0	6.3	2.7	1.8	196 *

*Chromatogram shown in figure 3.8.

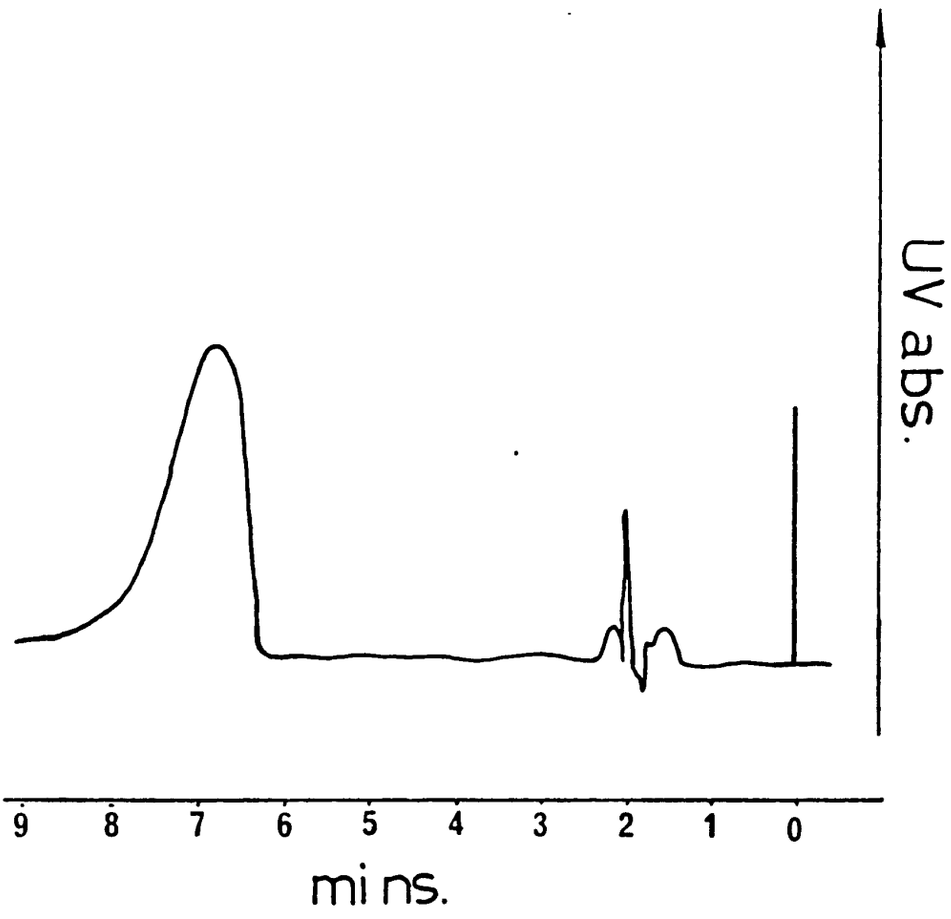


FIGURE 3.8.

CHROMATOGRAM OF BUPIVACAINE USING 250mm ODS HYPERSIL COLUMN WITH 91.5% ACETONITRILE IN 0.01M KH_2PO_4 AT pH 3.0 AT 2ml/min

in the chromatography of morphine. In that case tailing was avoided by the use of ion-paired chromatography. The suitability of that technique to the chromatography of bupivacaine was therefore examined.

3.3.4. ION-PAIRED CHROMATOGRAPHY OF BUPIVACAINE

3.3.4.1. Introduction

Ion-paired chromatography is a popular means of preventing peak tailing in liquid chromatography (Gloor and Johnson, 1977). In the chromatography of morphine, N-alkyl sulphonic acids have been shown to provide a suitable ion-pair (2.5.10). Local anaesthetics are nitrogeneous bases and have similar pKa's (table 3.21.) and might be expected to form similar ion-pairs. Pentane sulphonic acid (PSA) was chosen for this study as it was expected to give an increase in retention of the compounds, which could thereafter be further increased if neccessary by replacing PSA with a sulphonic acid with a longer alkyl group.

3.3.4.2. Method

A 91.5% acetonitrile/0.01M KH_2PO_4 solvent, 10^{-2}M in pentane sulphonic acid was prepared and allowed to equilibrate on column for one hour, after which reproducible chromatography was achieved. Other HPLC conditions were as described in 3.3.2.1.. The acetonitrile content of the mobile phase was then adjusted and the effect on the chromatography was noted. The pH was kept at pH3.0 as this had been shown assist ion-pair formation in the case

of morphine (2.5.10.2.).

3.3.4.3. Results and Discussion

The retention behaviour is recorded in table 3.11.. A sample chromatogram is shown in figure 3.9.. A comparison of these results with the results obtained without ion-pairing agents (tables 3.9. and 3.10), shows that even with solvents containing a relatively low percentage of acetonitrile (eg 50%), short retention times are achieved. This strongly suggests that ion-pairs are formed and their solubility in the mobile phase is greater than that of the unpaired drug. This is the opposite effect to that observed when ion-paired chromatography was examined for morphine, where the formation of ion pairs increased the solubility of the drug in the stationary phase and suggests that there are fundamental differences in the nature of the ion pairs formed and the mechanism of the separation.

Although the retention time is decreased, the peak width is still excessively broad, suggesting that the ion-pairs may still be interacting with the silanol sites on the stationary phase. This implies that reducing the acetonitrile content further would only make the peak broader without improving its shape.

The retention behaviour of some other local anaesthetics was examined on the system using the solvent containing 50% acetonitrile. Their retention behaviour is noted in table 3.12.. The results show that most compounds elute earlier than bupivacaine and the retention times were very short (K' lt. 1). Increasing the chain length of the ion-pairing agents would increase the retention time, but as seen with morphine this does

TABLE 3.11.

EFFECT OF ACETONITRILE CONTENT ON ION PAIRED CHROMATOGRAPHY* OF BUPIVACAINE

% Acetonitrile	RT	Pk W	K'	N
91	2.1	1.3	0.75	42
81.4	2.8	1.4	1.33	64
75	3.0	1.5	1.50	64
70	3.0	1.6	1.50	56
50	5.0	2.8	3.20	51

*Concentration of pentane sulphonic acid constant at 10^{-2}M

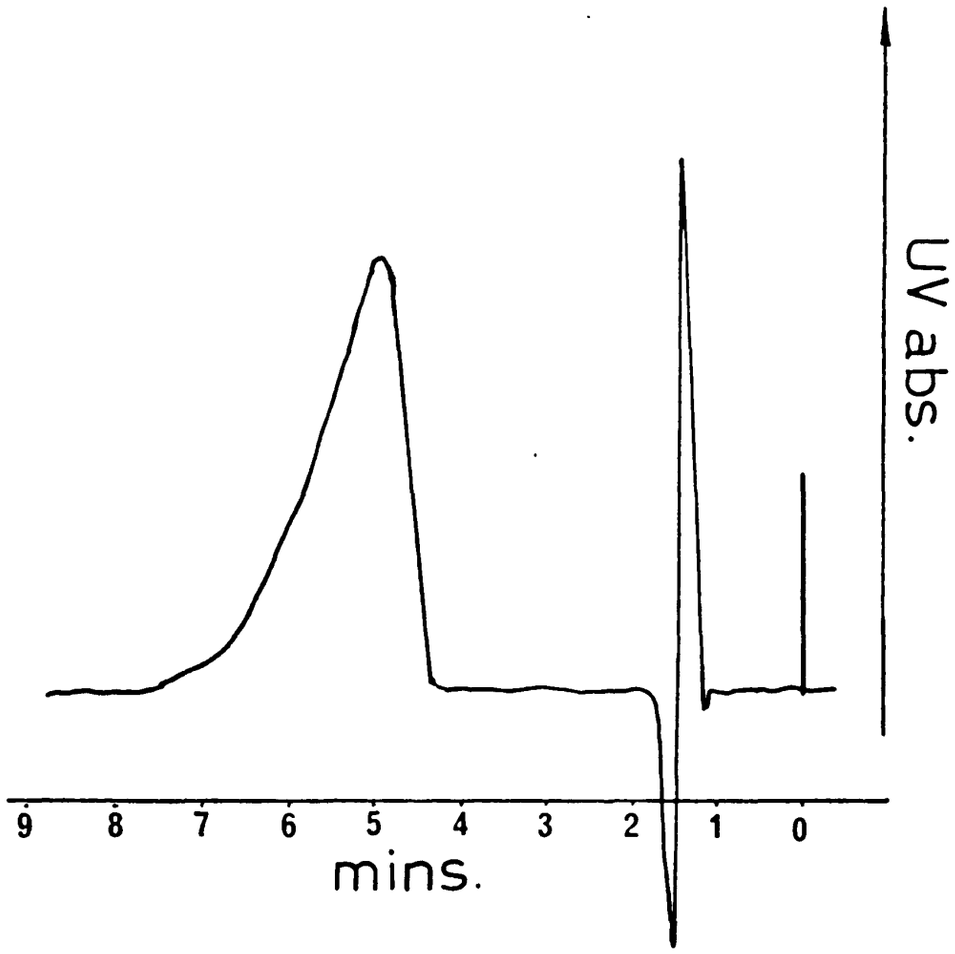


FIGURE 3.9.

CHROMATOGRAM OF BUPIVACAINE USING 250mm ODS HYPERSIL COLUMN WITH 50% ACETONITRILE IN 0.01M KH_2PO_4 AT pH 3.0, ALSO 10^{-2}M PENTANE SULPHONIC ACID AT 2ml/min

TABLE 3.12.

CHROMATOGRAPHY OF OTHER LOCAL ANAESTHETICS ON AN ION PAIRED SYSTEM (SEE 3.3.4.).

compound	Rt	W	K'	N
Bupivacaine	5.0	2.8	3.20	51
cocaine	2.8	1.2	1.33	87
butacaine	1.9	0.8	0.58	90
lignocaine	1.8	0.8	0.50	81
procaine	1.6	0.8	0.33	64

not significantly alter the peak shape or the degree of tailing (2.5.11.).

3.3.4.4. Conclusions

The small variability in retention over a large range of acetonitrile concentration and the poor efficiencies achieved, suggested that ion-paired chromatography might not be suitable for these compounds. For this reason ion-paired chromatography was not considered further and other techniques were therefore considered.

3.3.5. OTHER TECHNIQUES TO REDUCE PEAK TAILING

3.3.5.1. Introduction

A recent report (Gill et al., 1984) reviewed above (3.1.5.6.), describes the use of liquid chromatography to examine a range of local anaesthetics in street drug samples. This was performed on a C-18 column (ODS Hypersil), modified by including hexylamine in the mobile phase.

The capacity factors for lignocaine, mepivacaine and procaine are all very low however (table 3.13.), and this solvent is therefore unlikely to be suitable for the analysis of these compounds in biological extracts. No details of the efficiency of the separation, or the degree of peak tailing were given in that report, although an earlier publication (Gill et al., 1982) had demonstrated an improvement in peak shape for phenylethylamines using hexylamine in the mobile phase.

TABLE 3.13.

RETENTION DATA FOR LOCAL ANAESTHETICS USING MOBILE PHASE MODIFIED WITH HEXYLAMINE COMPARED WITH DATA FROM GILL ET AL., 1984

	experimental results				Gill et al. 1984
Compound	RT	W	N	K'	K'
amethocaine	25.5	4.2	589	16	16.25
amylocaine	12.0	3.6	178	7	7.19
benzocaine	30.0	3.3	1322	19	20.06
benzoyl ecgonine	10.5	3.3	162	6.0	5.68
bupivacaine	12.6	3.3	282	7.4	7.19
cocaine	7.2	3.6	64	3.8	2.68
lignocaine	3.3	1.2	64	1.2	0.79
procaine	1.5	0.9	44	0	0

void time = 1.5minutes

This approach was investigated to assess the quality of the chromatography for bupivacaine and to determine if alterations to the mobile phase might make the method suitable for the investigation of biological extracts.

3.3.5.2. Method

The mobile phase reported by Gill et al. (1984) was prepared. This was methanol/water/aqueous phosphoric acid (1% v/v)/hexylamine; 30:70:100:1.4 v/v/v/v. Other HPLC conditions were as described in 3.3.2.1.. A range of local anaesthetics were examined on this system and their capacity factors are compared with those reported by Gill et al. (1984). The calculated efficiencies are also given.

3.3.5.3. Results and discussion

The capacity factors achieved for the same mobile phase (table 3.13.) are similar to those reported, given that a 25cm column was used instead of 16cm as described by Gill et al. (1984). The efficiencies achieved for bupivacaine did represent a significant improvement on binary mobile phases investigated earlier (N = 282 cf. 197). The peak shape achieved for bupivacaine was much more symmetrical, although not significantly sharper (figure 3.10.). The capacity factor for bupivacaine was however quite large (K' = 7.4) with a correspondingly wider peak (W = 4mins). It was felt that this wider peak would compromise the sensitivity of the chromatography for this application, and so the methanol content was increased to see if an earlier elution time would give an increase in efficiency and peak width.

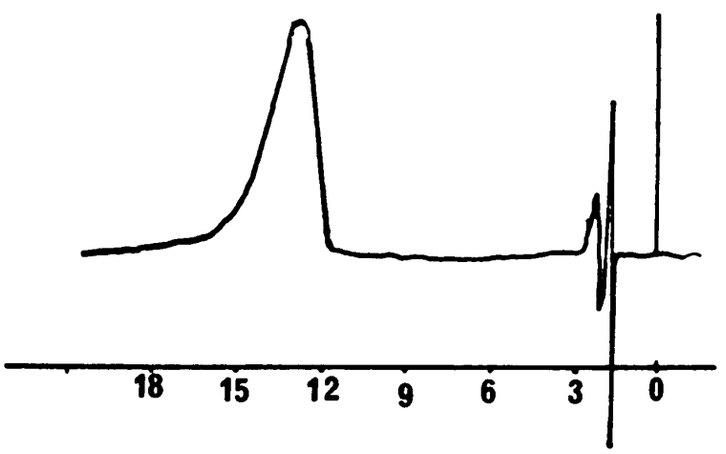


FIGURE 3.10

CHROMATOGRAM OF BUPIVACAINE USING 250mm ODS HYPERSIL COLUMN WITH METHANOL/WATER/H_3PO_4 (1% v/v)/ /HEXYLAMINE; 30:70:100:1.4 v/v/v/v.

Increasing the methanol content of the mobile phase to 30% (from 15%) at the expense of water, caused a decrease in the retention time, however there was no major improvement in the peak width or efficiency (figure 3.11.) and the peak appeared more tailed. This suggested that the change in selectivity of the column caused by the inclusion of hexylamine was not compatible with efficient chromatography of bupivacaine.

3.3.5.4. Conclusions

The use of hexylamine in the mobile phase does give further control of the retention properties of the solvent and in this case does extend the suitability of a simple isocratic mobile phase to a large number of local anaesthetics. The peak for bupivacaine is however still quite broad and was not improved sufficiently over earlier binary mobile phases to make this the method of choice for quantitative analysis of complex samples.

Having examined binary mobile phases and ion-paired chromatography, other approaches which might be considered are the use of ternary mobile phases, elevated column temperatures or a change of the stationary phase. The use of gradient elution is another possibility but it is outwith the scope of this study as it requires the use of more complex equipment and makes the method no more convenient than temperature programmed GC.

Of these alternatives, ternary mobile phases, as discussed in (1.3.5.), were considered as they involved no alteration to the HPLC hardware.

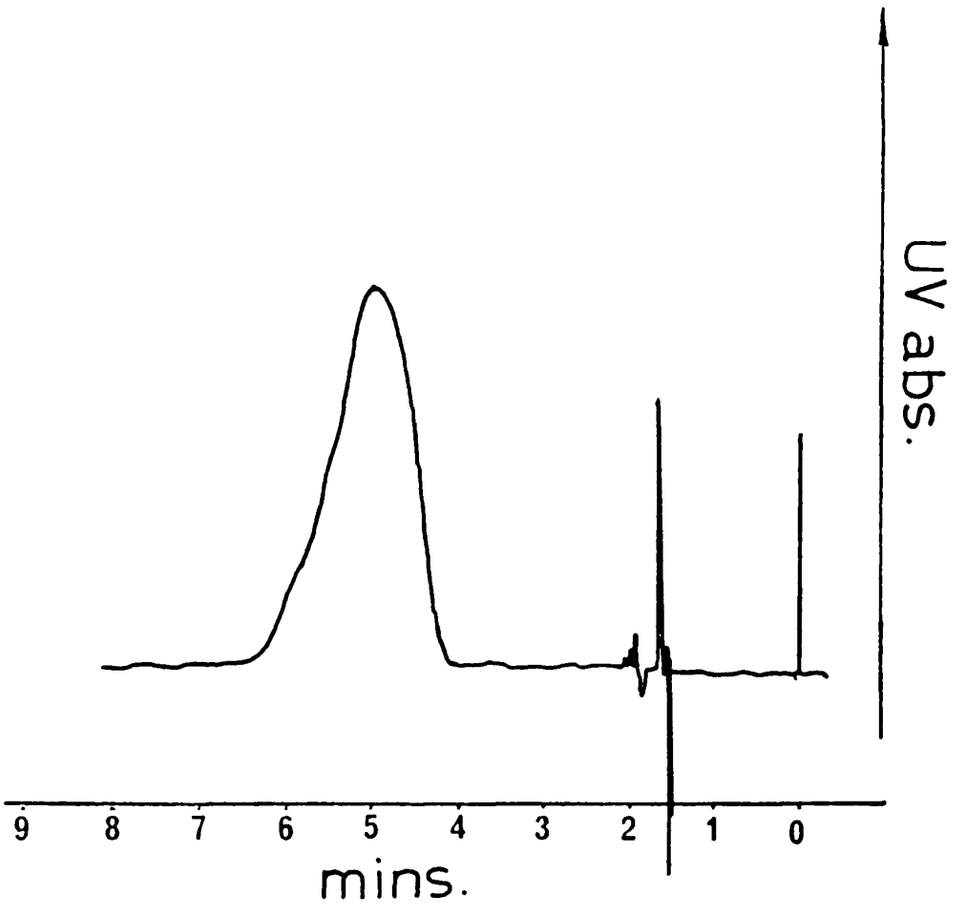


FIGURE 3.11.

CHROMATOGRAM OF BUPIVACAINE USING 250mm ODS HYPERSIL COLUMN WITH METHANOL/WATER/H_3PO_4 (1% v/v)/ /HEXYLAMINE; 60:70:70:1.4 v/v/v/v.

3.3.6. A SIMPLE STRUCTURED APPROACH FOR TERNARY SOLVENT OPTIMISATION

3.3.6.1. Introduction

On consideration of the complex assumptions required for a reliable mathematical optimisation procedure, it was decided to use the concept of the solvent map, but to assess the chromatography semi-intuitively, based on the efficiency, capacity factor and peak shape achieved. This allowed trends in chromatographic improvement to be corelated with the relative solvent composition.

3.3.6.2. Method

The approach adopted was as follows. Figure 3.12. and table 3.14 show the solvents selected for initial investigation. These were chosen to represent the range of polarities 5.1-10.2 available from these three solvents. The table also demonstrates the variation in Xe, Xd and Xn values (Chi values) representing the degree of proton acceptor, proton donator and dipole interactions respectively of the solvent mixtures.

Fourteen solvents were examined in all and the retention data for bupivacaine in each is noted in table 3.14..

Other HPLC conditions were as noted in 3.3.2.1.

3.3.6.3. Results and Discussion

Of the fourteen solvents examined, only four gave measurable retention data for bupivacaine. This is shown in table 3.14.. This would be insufficient for any further

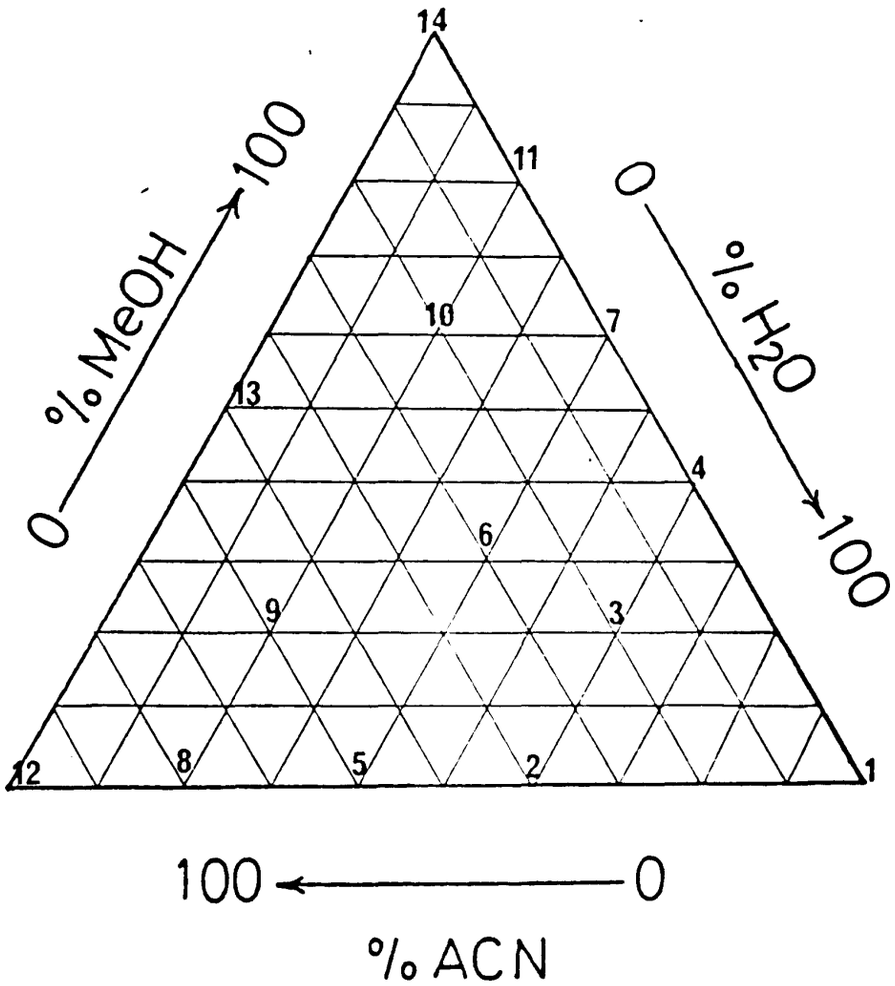


FIGURE 3.12.

COMPOSITIONS OF FOURTEEN SOLVENTS SELECTED FOR STRUCTURED SOLVENT OPTIMISATION WITH THREE SOLVENTS (FOR DETAILS OF SOLVENT STRENGTHS SEE TABLE 3.14.)

TABLE 3.14.

SOLVENTS SELECTED TO DETERMINE OPTIMUM SOLVENT FOR BUPIVACAINE

#	Pure solvent % ACN	MeOH	Buffer	P'	Xe	Xn	Xd	Rt	W	K'	N
1	0	0	100	10.2	0.37	0.25	0.37	not eluted			
2	40	0	60	8.44	0.35	0.32	0.33	not eluted			
3	20	20	60	8.3	0.38	0.30	0.32	not eluted			
4	0	40	60	8.2	0.41	0.27	0.31	not eluted			
5	60	0	40	7.6	0.33	0.33	0.31	not eluted			
6	30	30	40	7.4	0.39	0.32	0.30	6.6	1.8	4.5	215
7	0	60	40	7.1	0.44	0.29	0.28	not eluted			
8	80	0	20	6.7	0.32	0.39	0.29	10.4	3.6	7.7	133
9	60	20	20	6.5	0.36	0.36	0.28	4.1	1.0	2.4	269
10	20	60	20	6.3	0.42	0.32	0.26	10.8	3.9	8.0	123
11	0	80	20	6.1	0.46	0.30	0.25	not eluted			
12	100	0	0	5.8	0.31	0.42	0.27	not eluted			
13	50	50	0	5.5	0.40	0.37	0.25	not eluted			
14	0	100	0	5.1	0.48	0.31	0.22	not eluted			

= number in figure 3.12.

optimisation under the scheme described by Glajch et al, 1982, considered above, (1.3.5.) and illustrates the importance of having three suitable starting solvents. It might however provide three solvent mixtures to allow the process to be repeated with more success.

A few qualitative observations were made on the results.

The optimum solvent for bupivacaine probably lies in between points 6,9 and 8. Of these three, solvent (number 9) obtained by the method described above, did provide a more efficient example of chromatography than achieved by the use of binary solvent mixtures (K'=2.4, N=269). This is shown in figure 3.13.. The efficiency is similar to that achieved with the use of the hexylamine containing solvent (3.3.5.), however the capacity factor is lower.

As demonstrated, the use of other solvents could increase the capacity factor only at the expense of the efficiency. This was not acceptable.

In summary, the degree of improvement over previous solvents was small and was not considered sufficient to make this chromatographic system acceptable for biological extracts.

Increasing the methanol content of the mobile phase at the expense of acetonitrile increased the retention time (see point 9 to point 10). The exact identity of the optimum solvent could be calculated by the use of an iterative procedure, taking the three solvents 6,9 and 8 as the apices for a second run of fourteen solvents as performed above. If this was performed until the improvements in the efficiency were negligible, this should give the optimum separation of bupivacaine for a ternary methanol,

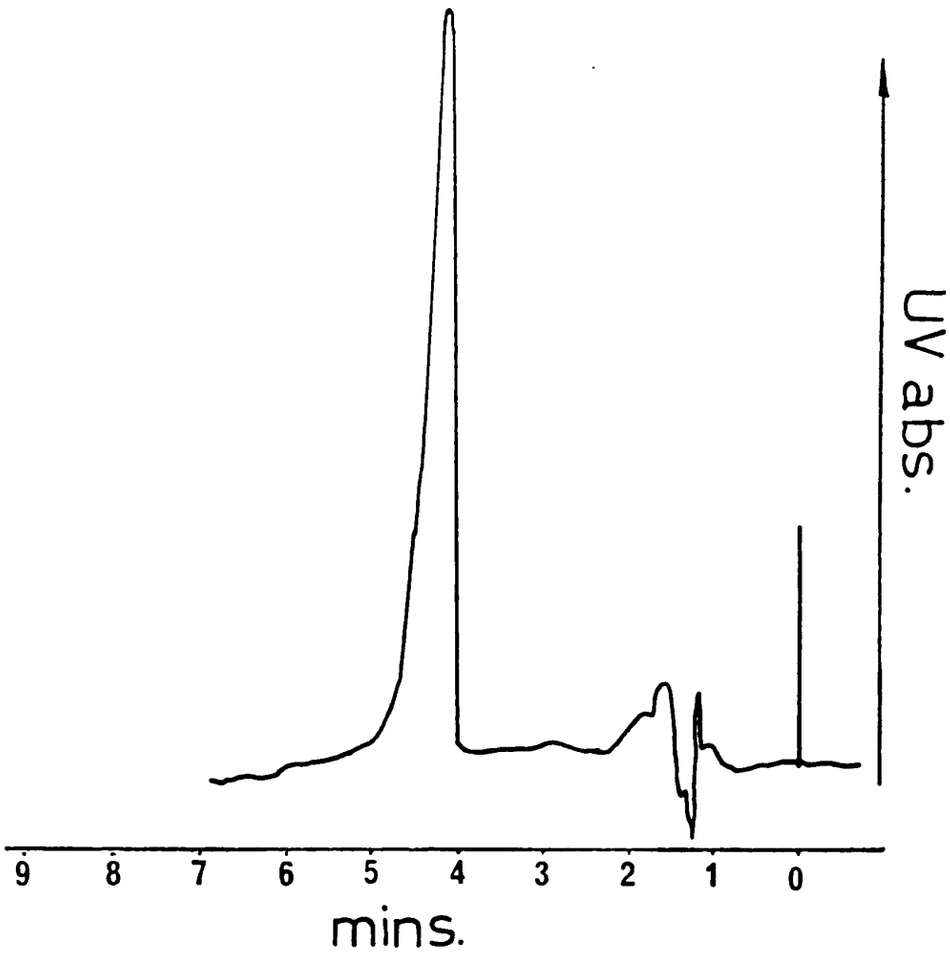


FIGURE 3.13.

CHROMATOGRAM OF BUPIVACAINE USING 250mm ODS HYPERSIL COLUMN WITH ACETONITRILE/METHANOL/BUFFER 60/20/20, AT pH3 AND 2ml/min. (SOLVENT No. 9, TABLE 3.14. AND FIGURE 3.12.)

acetonitrile, water solvent.

This is obviously a long and tedious process with several disadvantages. The most notable is the choice of the three optimum solvents at the end of each iterative run and the diminishing returns in improvement of efficiency on successive runs. There is also the requirement of a subjective choice about when the drop in capacity factor outweighs any improvement in efficiency.

3.3.6.4. Conclusions

Most reports in the literature avoid the use of ternary solvent sytems because of the demonstrated difficulties in optimising the eluent. Computer assisted optimisation has been reviewed in chapter one (1.3.5.). Most of these methods require interactive computer control of the chromatograph because of the solvent changes and repeat injections required. The calculation of the response surface and the subsequent simplex search procedure used by most methods, are complex and best handled by a computer. The initial selection of three suitable starting solvent mixtures is however essential to the success of any optimisation method and the approach used above for bupivacaine may be useful for this preliminary step.

These optimised procedures are still in the development stage and until agreement is reached on the correct algorithm to assess the chromatograms in terms of resolution, retention time and efficiency, the optimisation of ternary solvents will be a tedious process.

The examination of these ternary solvent systems has demonstrated the effect of changing the solvent selectivity. A comparison with the optimisation of binary solvent mixtures examined earlier has shown that the chromatography has been improved. The capacity factor is still quite low however and the efficiency is not greatly improved on previous solvent systems. It was considered unlikely that the variation in selectivity afforded by the use of a ternary solvent system was sufficient alone to improve the chromatography of bupivacaine to a level acceptable for the examination of biological extracts.

No further work was done on ternary solvent systems and some of the other alternatives examined in section 3.3.5. were considered. The improvement in efficiency gained by the greater range of selectivities available from ternary mixtures is significant. This suggests that another change in the selectivity of the separation might improve the chromatography by the required degree.

3.3.7. REVIEW

Several approaches for the optimisation of mobile phases for local anaesthetics have been considered, however none has represented a significant improvement on efficiency or peak shape. Each of the methods was assessed for the degree of tailing and assymmetry of the peak (table 3.15.). Most of the methods exhibit this to a greater or lesser extent. The best results were obtained with a mobile phase phase containing hexylamine which is believed to modify the selectivity of the

stationary phase (Gill et al., 1982) and a ternary solvent mixture, which was directed towards changing the selectivity of the mobile phase. This led to the conclusion that the ODS hypersil column used for all the above chromatography is not ideally suited for the analysis of local anaesthetics, due to its particular selectivity. ODS hypersil was chosen as the standard reversed phase material for HPLC assays in this department, following work by Eppel (1980) and Smith et al., 1984), showing it to have a reliable batch to batch reproducibility and to be suitable for a wide range of assays. A further factor was the decision by the Home Office to use this material in all their laboratories (Gill, 1984), in an attempt to standardise their HPLC assays. It was considered convenient to follow this decision, in order to allow the direct use of methods suggested or reported by Home Office Central Research Establishment.

Following the poor results produced above, it was decided that the use of an alternative stationary phase might be neccessary to achieve the aims of this study, namely to provide efficient chromatography for a range of local anaesthetics.

The literature survey (3.1.5.6. and table 3.4.) showed that a variety of stationary phases had been used for the chromatography of local anaesthetics. These included C-2 and C-8 reversed phases, as well as polar phase materials such as CN and silica.

It was decided that another reversed phase material should be examined, as these are reported to be more suited to the analysis of biological extracts. It was decided to examine the suitability of a C-8 stationary phase to determine if the change in selectivity of the column would make any significant change in the chromatography.

TABLE 3.15.

COMPARISON OF CHROMATOGRAPHIC PARAMETERS FOR SEVERAL MOBILE PHASES

mobile phase	K'	W	N	A_S	section
methanol/buffer* 70/30, pH3	1.9	1.8	123	3.75	3.3.2.
acetonitrile/buffer 91.5/8.5, pH3	2.7	1.8	197	3.5	3.3.3.
acetonitrile/buffer 50/50 10^{-2}M PSA, pH3	3.2	2.8	51	4.3	3.3.4.
methanol/water/H_3PO_4 (1% v/v) /hexylamine; 30:70:100:1.4 v/v/v/v.	7.4	4.0	282	2.0	3.3.5.
acetonitrile/methanol/buffer 60/20/20 pH3	2.4	1.0	269	4.0	3.3.6.

*buffer = 0.01M KH_2PO_4 solution.

3.3.8. USE OF C-8 STATIONARY PHASE.

3.3.8.1. Introduction

The HPLC systems examined above were not considered to be suitable for the analysis of biological extracts because of the peak width or the degree of tailing of the drug peak. The HPLC system described for bupivacaine by Lindberg and Pihlajamaki, (1984) is based on a C-8 column packed with Lichrosorb RP-8. As this material was held in stock in the department, a 25cm column was prepared using the packing procedure described in appendix B, having first assessed its ability to form a slurry in acetone.

3.3.8.2. Method

Using this column and the mobile phase recommended (28% acetonitrile in 0.05M KH_2PO_4, Lindberg and Pihlajamaki, 1984), the chromatography of bupivacaine was examined. Other HPLC conditions were as described in 3.3.2.1.. The retention behaviour of several related compounds was examined on the same system.

3.3.8.3. Results and discussion

The chromatographic data are shown in table 3.16.. A capacity factor for bupivacaine of K' = 3.5 and an efficiency of N = 2916 were noted. It was also noted that the peak width was very narrow (W = 0.4 mins), and it proved dififcult to accurately assess the assymmetry of the peak. The efficiency using this system is improved by an order of magnitude over the efficiency noted with any of the other mobile phase systems considered

TABLE 3.16.

CHROMATOGRAPHIC BEHAVIOUR OF A RANGE OF LOCAL ANAESTHETICS ON THE SYSTEM DESCRIBED BY LINDBERG AND PILAJAMAKI, (1984)

compound	Rt	W	K'	N
procaine	2.2	0.2	0.83	1936
lignocaine	2.5	0.4	1.1	625
mepivacaine	2.6	0.4	1.16	676
benzoyl ecgonine	3.7	0.5	2.05	853
cocaine	4.4	0.5	2.66	1239
bupivacaine	5.4	0.4	3.5	2916
cinchocaine	12.9	1.3	9.75	1575

void time = 1.2 minutes

previously (table 3.15.). It also appears to provide an efficient separation for a variety of other local anaesthetics including cocaine and cinchocaine (figure 3.14.). The other local anaesthetics investigated elute earlier. This implies that the selectivity of the C-8 stationary phase is more suited to these compounds than the ODS material used in all previous chromatography. There is very little tailing, the peaks being almost symmetrical.

3.3.8.4. Conclusions

For a given compound in solvents of different strength, the capacity factor may vary, but the peak shape and efficiency remain more or less constant. This implies that if a particular separation is tailing badly, this will not be solved simply by altering the organic content of the mobile phase. This affords only a small change in selectivity and whilst the solvent strength (P') can be adjusted to control the degree of retention, the subsequent changes in the individual solvent interactions (Xe, Xe and Xn) are not generally sufficient to improve the degree of tailing. Greater control of these individual contributions to P' is possible with the use of ternary solvent systems, but these have proved difficult to optimise.

To make significant changes to the quality of the chromatography (i.e. efficiency, capacity factor and assymmetry), alternatives such as ion-pairing or changes to the stationary phase may be required.

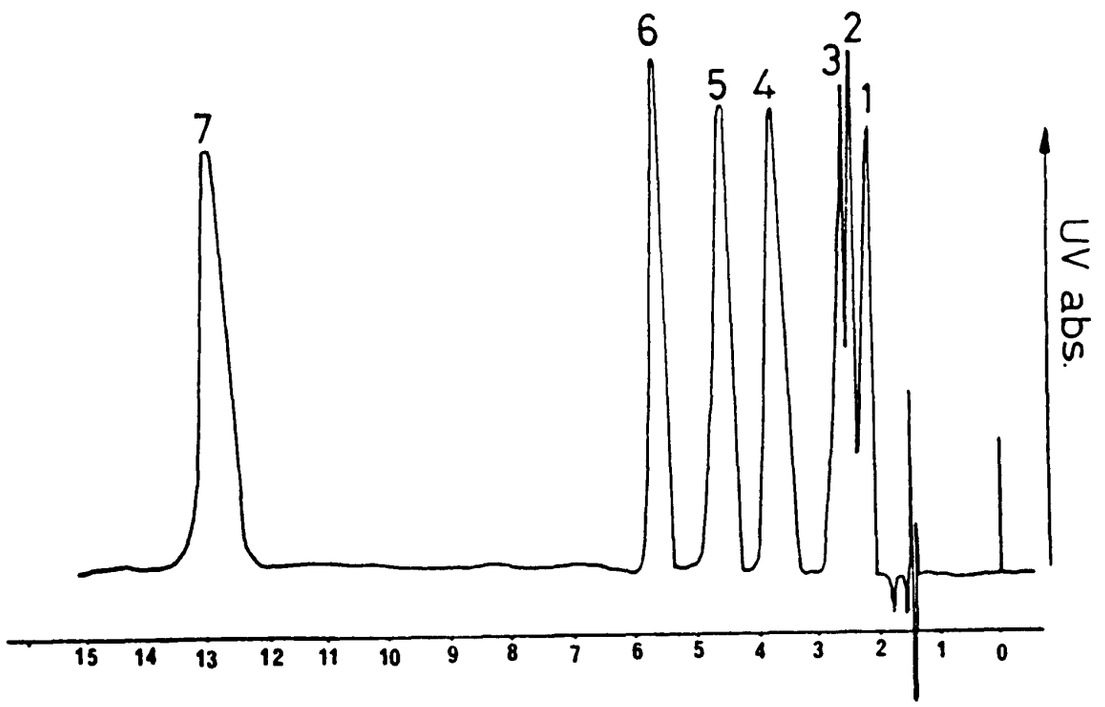


FIGURE 3.14.

CHROMATOGRAM OF SEVEN LOCAL ANAESTHETICS (1: PROCAINE, 2: LIGNOCAINE, 3: MEPIVACAINE, 4: BENZOYL ECGONINE, 5: COCAINE, 6: BUPIVACAINE, 7: CINCHOCAINE) USING 250mm LICHROSORB RP-8 COLUMN WITH 28% ACETONITRILE IN 0.05M KH_2PO_4 AT pH3 AND 2ml/min.

3.3.9. THE EFFECT OF ACETONITRILE CONTENT ON THE CHROMATOGRAPHY OF BUPIVACAINE AND RELATED COMPOUNDS USING A C-8 COLUMN.

3.3.9.1. Introduction

Having established that the use of a stationary phase of lower lipophilicity gave good chromatography of bupivacaine and some other compounds, it was decided to investigate whether altering the acetonitrile content might allow one HPLC system for a range of local anaesthetics.

3.3.9.2. Method

A series of acetonitrile/0.05M KH_2PO_4 solvents from 50% to 10%) were prepared and allowed to equilibrate on the column for one hour, after which time reproducible chromatography was achieved. Other HPLC conditions were as in 3.3.2.1..
Standard solutions of 10^{-3}g/L of the compounds of interest were used unless otherwise stated.

3.3.9.3. Results and discussion

The chromatographic data are given in table 3.17. (data for 28% solvent are shown in table 3.14.) and shown graphically in figure 3.15..
These results show that using acetonitrile/0.05M KH_2PO_4 solvents on a C-8 column, it is not possible to produce one system which will be suitable for the analysis of the compounds of interest.
The quality of the chromatography in general on these systems was sufficient to make the use of a C-8 column the method of choice

TABLE 3.17.

VARIATION IN CHROMATOGRAPHIC BEHAVIOUR OF LOCAL ANAESTHETICS WITH ACETONITRILE CONTENT

10% acetonitrile

compound	Rt	W	K'	N
procaine	5.4	0.8	3.5	729
lignocaine	6.4	0.7	4.3	1338
mepivacaine	7.3	0.8	5.1	1332
benzoyl ecgonine	9.8	1.1	7.2	1270
cocaine	NOT ELUTED			
bupivacaine	NOT ELUTED			
cinchocaine	NOT ELUTED			

20% acetonitrile

compound	Rt	W	K'	N
procaine	2.8	0.4	1.3	784
lignocaine	3.4	0.4	1.83	1156
mepivacaine	3.5	0.4	1.91	1225
benzoyl ecgonine	6.0	0.6	4.00	1600
cocaine	8.0	0.9	5.7	1264
bupivacaine	9.7	0.6	7.1	4181
cinchocaine	38	2.5	31	369

40% acetonitrile

compound	Rt	W	K'	N
procaine	K' LESS THAN 1			
lignocaine	K' LESS THAN 1			
mepivacaine	K' LESS THAN 1			
benzoyl ecgonine	K' LESS THAN 1			
cocaine	2.7	0.6	1.25	324
bupivacaine	3.0	0.2	1.5	3600
cinchocaine	4.6	0.8	2.8	529

50% acetonitrile

compound	Rt	W	K'	N
procaine	K' LESS THAN 1			
lignocaine	K' LESS THAN 1			
mepivacaine	K' LESS THAN 1			
benzoyl ecgonine	K' LESS THAN 1			
cocaine	K' LESS THAN 1			
bupivacaine	2.3	0.2	0.9	2116
cinchocaine	2.8	0.6	1.3	348

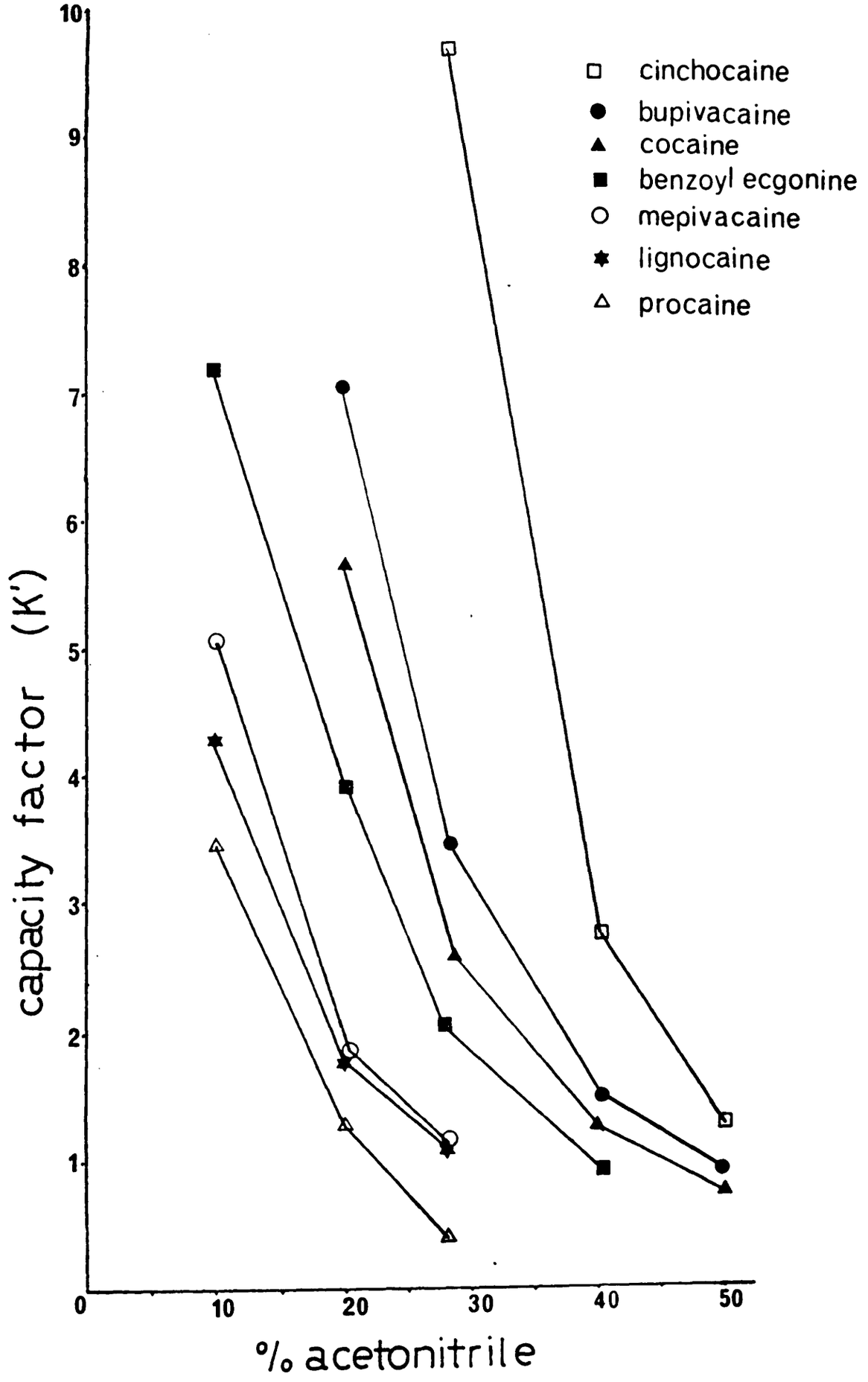


FIGURE 3.15

VARIATION IN CAPACITY FACTOR FOR SEVEN LOCAL ANAESTHETICS WITH ACETONITRILE CONTENT OF THE MOBILE PHASE

for the analysis of these compounds.

From the above experiment three solvent systems were chosen, for three groups of compounds. These are as follows:

SYSTEM 1:- For bupivacaine and cinchocaine, the system described by Lindberg and Pihlajamaki, (1984), 28% acetonitrile in 0.05M KH_2PO_4 adjusted to pH3.

SYSTEM 2:- For cocaine, bupivacaine and benzoyl ecgonine, 20% acetonitrile in 0.05M KH_2PO_4 adjusted to pH3.

SYSTEM 3:- For procaine, mepivacaine and lignocaine, 10% acetonitrile in 0.05M KH_2PO_4 adjusted to pH3.

Other HPLC conditions were as described in 3.3.2.1..

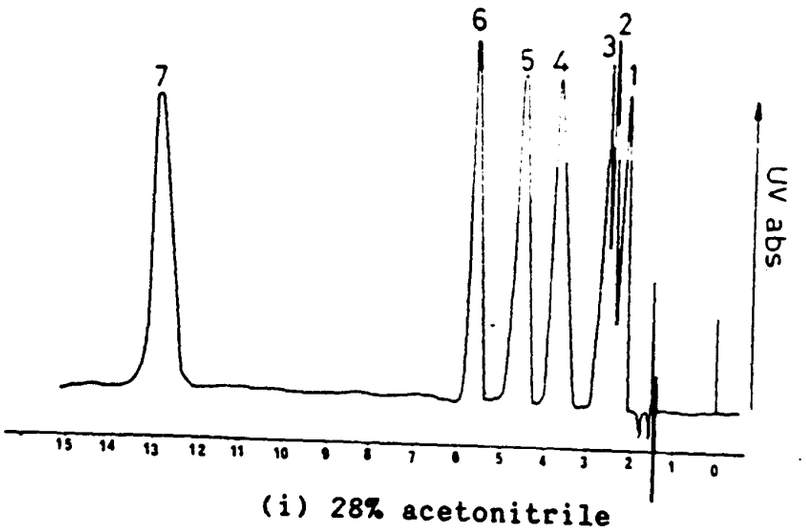
Sample chromatograms from each system are shown in figure 3.16..

3.3.9.4. Conclusions

Although a single system for a wide range of local anaesthetics has not been found, suitable chromatography on similar systems has been achieved for the compounds of interest.

Not considered in this study was the use of elevated column temperature, which is known to allow earlier elution of compounds with high capacity factors (Gant et al., 1979).

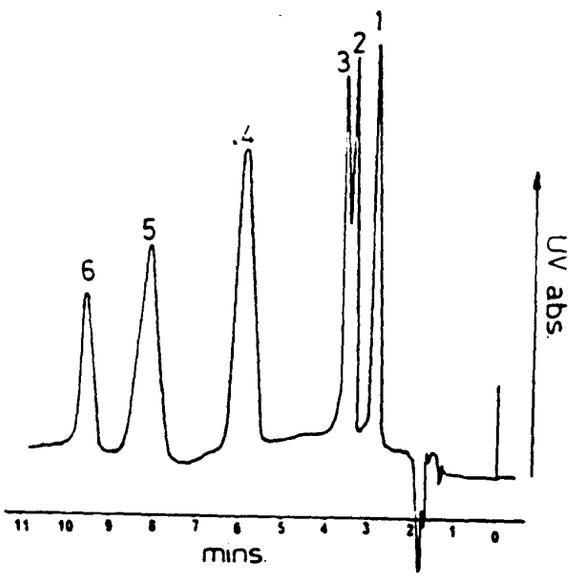
The process of changing to methanol based solvents, the use of ion-pairing agents, ternary solvent systems etc. with this C-8 column could now be investigated in an attempt to provide one HPLC



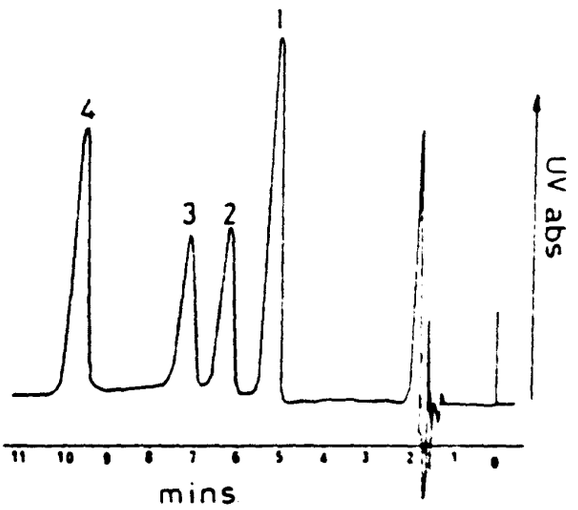
(i) 28% acetonitrile

KEY

1: PROCAINE
2: LIGNOCAINE
3: MEPIVACAINE
4: BENZOYL ECGONINE
5: COCAINE
6: BUPIVACAINE
7: CINCHOCAINE



(ii) 20% acetonitrile



(iii) 10% acetonitrile

FIGURE 3.16.

CHROMATOGRAMS FOR SEVEN LOCAL ANAESTHETICS
IN SOLVENTS WITH VARYING ACETONITRILE CONTENT

system suitable for a larger range of local anaesthetics. Similarly, the fact that the compounds of interest elute in different strengths of the same solvent mixture suggest that it would convert readily to a gradient system.

The chromatography achieved was however efficient and the compounds of interest displayed very little tailing or assymmetry. The linearity of the response to the compounds of interest was examined and possible interference in the assay from other common drugs was assessed, before suitable extraction procedures for each were investigated

3.3.10. LINEARITY OF DETECTOR RESPONSE

3.3.10.1. Introduction

The linearity of the detector response to increasing amounts of drug, is essentially a property of the detector, up to the point at which the HPLC column becomes overloaded with sample (Snyder and Kirkland, 1978). For this reason only one of the detectors used previously, the PU4025 UV spectrophotometer (Phillips), was selected for further work. Its linearity was investigated for cocaine, procaine, bupivacaine and benzoyl ecgonine using one of the HPLC systems described above which was known to separate all these compounds (system 2, (3.3.9.3.) and table 3.17.).

3.3.10.2. Method

The HPLC system used was as follows. A single piston pump (Gilson 302 operated at 2 ml/min, with 802C manometer). Sample introduction was through a Rheodyne 7125 6-port injection valve with a 20ul loop, using an 25ul glass syringe (SGE). A 25cm Lichrosorb RP-8 column (Merck, Darmstadt) packed as in appendix B. The detector was a variable wavelength UV monitor PU4025 (Phillips), set at 230nm. The absorbance and chart recorder settings were adjusted as neccessary. High pressure fittings were stainless steel, low pressure fittings were PTFE. Drug standards were prepared by serial dilution and the absorbance was calculated from the peak heights. The absorbances are given in table 3.18. and linearity plots are shown in figure 3.17..

3.3.10.3. Results and discussion

All the drugs gave linear calibration over the region of interest, however that noted for procaine was poorer than for the other compounds examined. Deviations from linearity arise from stray light, which in turn is a measure of the instruments optical quality.

The absorbance is determined by the concentration of the drug, and the A_1^1 value for the compound, as in the Beer-Lambert equation, Absorbance = $A_1^1 Cl$.

Samples containing concentrations above the upper boundary of this region would probably require to be diluted and reanalysed. The absolute detection limits were calculated as 2ng o/c (20ul of 10^{-4}g/L) for procaine, cocaine and benzoyl ecgonine, and 10ng o/c (20ul of $5x10^{-4}$ g/L) for bupivacaine. This is due to the

TABLE 3.18.

ABSORBANCE DATA FOR ASSESSING LINEARITY OF
OF HPLC METHODS

compound	amount o/c	absorbance
cocaine	2ng	1.6×10^{-4}
	10ng	6.4×10^{-4}
	20ng	1.3×10^{-3}
	100ng	6.6×10^{-3}
	200ng	0.013
	1ug	0.060
benzoyl ecgonine	2ng	2.4×10^{-4}
	10ng	7.2×10^{-4}
	20ng	1.4×10^{-3}
	100ng	7.4×10^{-3}
	200ng	0.015
	1ug	0.075
procaine	2ng	1.2×10^{-4}
	10ng	8.0×10^{-4}
	20ng	1.6×10^{-3}
	100ng	8.8×10^{-3}
	200ng	0.015
bupivacaine	2ng	2.4×10^{-5}
	10ng	1.6×10^{-4}
	20ng	2.8×10^{-4}
	100ng	1.3×10^{-3}
	200ng	2.6×10^{-3}

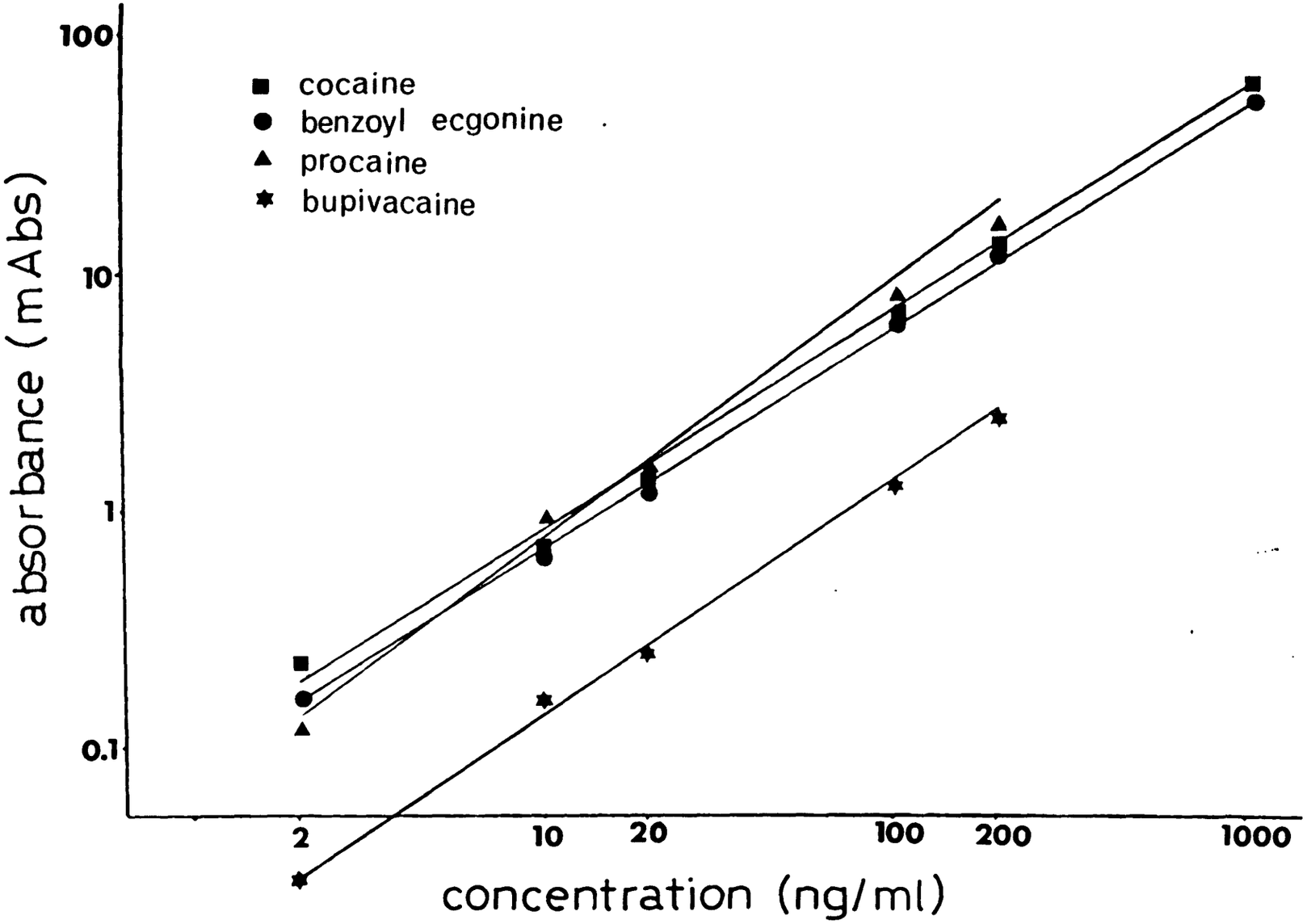


FIGURE 3.17.

LINEARITY OF UV ABSORBANCE DETECTOR FOR FOUR LOCAL ANAESTHETICS

poorer absorbance of bupivacaine at 230nm (see 2.3.2.). Assuming 100% extraction efficiency from biological samples, this is within the range of sensitivity required for the analysis of biological samples for therapeutic to fatal levels (3.1.4. and table 3.2.)

3.3.10.4. Conclusions

It has been shown that the linearity of the detector response within the region of interest is good for the particular detector examined. It was therefore decided that this detector should be used exclusively for further work in this study.

3.3.11. EXAMINATION OF OTHER COMPOUNDS FOR INTERFERENCE

3.3.11.1. Introduction

A selection of drugs commonly encountered in forensic toxicological analysis were examined to determine their elution behaviour on the HPLC systems developed for the local anaesthetics.

3.3.11.2. Method

Standard solutions of the drugs (10^{-2}g/L) were prepared and examined on HPLC systems consisting of the pump and detector as described in 3.3.2.1., and the Lichrosorb RP-8 column with mobile phase ranging from 10% to 40% acetonitrile. The chromatographic behaviour of a series of local anaesthetics and narcotic analgesics, benzodiazepines and acidic anti-depressant drugs was examined. The retention data were recorded for those

compounds which eluted in less than 30 minutes. These are given in table 3.19 (I - IV).

3.3.11.3. Results and discussion

As the retention data for bupivacaine, cocaine, benzoyl ecgonine and procaine (table 3.16.) were not recorded at the same time as these other drugs, there is a slight difference in the capacity factors as demonstrated by the position of bupivacaine which was included in the present study.

This data can therefore only be used to indicate compounds eluting in the same region of the chromatogram as the compound of interest. In summary, on the 10% system, procaine had a similar K' to prilocaine. And if the identification of an unknown compound was required, prilocaine should be compared for interference.

Also included in table 3.19 (I), is a list of GC retention indices on a non-polar SE-30 or OV-1 type column for the compounds examined (from Ardrey and Moffat, 1981). A comparison of the GC retention indices shows that these compounds could be discriminated against by GC however (procaine RI = 2018, prilocaine RI = 1825.

This indicates that the selectivity of the GC separation of the drugs is different from the selectivity of the HPLC method. Which would give added specificity to a cross-check method incorporating GC and HPLC.

Similar comparisons can be made for other compounds.

From the capacity factors recorded in table 3.19.(I -IV), and the chromatograms in figure 3.18. it can be seen that all the drugs examined were separated in at least one of the solvents examined.

Benzocaine (peak 13) is unusual in that it demonstrates the

TABLE 3.19.

COMPARISON OF CHROMATOGRAPHIC PERFORMANCE OF SOME COMMON DRUGS ON SOLVENT SYSTEMS USED FOR LOCAL ANAESTHETICS

I

Solvent: 40% acetonitrile in 0.05MKH_2PO_4 at pH 3
flow rate : 2
Column: 25cm Lichrosorb RP-8
Wavelength: 210nm
Dead Time: 13

compound	Rt	W	K'	N	RI
GENERAL BASIC DRUGS					
1 morphine	15	-	-	-	2454
2 nalorphine	16	-	-	-	2577
3 codeine	19	4	0.5	361	2376
4 oxycodone	19	3	0.5	641	2524
5 prilocaine	22	2	0.7	1936	1825
6 levorphanol	23	3	0.8	940	2234
7 diamorphine	24	3	0.9	1024	2614
8 amylocaine	29	3	1.2	1495	1600
9 bupivacaine	33	3	1.5	1936	2273
10 pentazocine	34	3	1.6	2055	2275
11 papaverine	33	5	1.5	670	2825
12 amethocaine	34	4	1.6	1156	2219
13 benzocaine	36	3	1.8	2304	1555
14 butacaine	43	4	2.3	1849	2457
15 cinchocaine	48	5	3.0	1475	2701
16 dextromoramide	55	5	3.2	1936	2940
17 dipipanone	94	7	6.2	2885	1474
BENZODIAZEPINES					
nitrazepam	24	2	0.7	2304	
lorazepam	37	2	1.6	5476	
chlordiazepoxide	40	2	1.9	6400	
desmethyldiazepam	48	3	2.4	4096	
diazepam	62	3	3.4	7260	
ACIDIC DRUGS					
primidone	21	1	0.6	7056	
methyprylone	25	1	0.9	10000	
phenobarbitone	27	2	1.1	2916	
butobarbitone	32	2	1.5	4096	
butalbital	34	2	1.6	4624	
amylobarbitone	34	2	1.6	4624	
glutethimide	36	2	1.8	5184	
quinalbarbitone	49	5	2.8	1536	

TABLE 3.19. contd.

COMPARISON OF CHROMATOGRAPHIC PERFORMANCE OF SOME COMMON DRUGS ON SOLVENT SYSTEMS USED FOR LOCAL ANAESTHETICS

II

Solvent: 30% acetonitrile in 0.05MKH_2PO_4 at pH 3
flow rate : 2
Column: 25cm Lichrosorb RP-8
Wavelength: 210nm
Dead Time: 13

compound	Rt	W	K'	N
GENERAL BASIC DRUGS				
1 morphine	19		0.4	-
2 nalorphine	20		0.4	-
3 codeine	22	4	0.7	484
4 oxycodone	22	5	0.7	310
5 prilocaine	27	4	0.9	729
6 levorphanol	29	3	1.1	1495
7 diamorphine	38	3	1.7	2567
8 amylocaine	40	5	1.9	1024
9 bupivacaine	46	5	22.3	1354
10 pentazocine	48	5	2.4	1475
11 papaverine	50	8	2.6	625
12 amethocaine	62	5	3.4	2460
13 benzocaine	54	5	2.9	1866
14 butacaine	71	5	4.1	3226
15 cinchocaine	95	9	5.8	1782
16dextromoramide	114	9	7.2	2567
17dipipanone	227	18	15.2	2544
BENZODIAZEPINES				
nitrazepam	35	4	1.7	1225
lorazepam	71	8	4.4	1260
chlordiazepoxide	81	9	5.2	1296
desmethyldiazepam	105	11	7.1	1458
diazepam	131	9	9.1	3390
ACIDIC DRUGS				
primidone	26	3	1.0	1202
methyprylone	35	3	1.69	2177
phenobarbitone	38	4	1.71	1444
butobarbitone	49	5	2.77	1537
butalbital	58	5	3.46	2153
amylobarbitone	60	6	3.61	1600
glutethimide	61	6	3.69	1653
quinalbarbitone	92	10	5.57	1354

TABLE 3.19. contd.

COMPARISON OF CHROMATOGRAPHIC PERFORMANCE OF SOME COMMON DRUGS ON SOLVENT SYSTEMS USED FOR LOCAL ANAESTHETICS

III

Solvent: 20% acetonitrile in 0.05MKH_2PO_4 at pH 3
flow rate : 2
Column: 25cm Lichrosorb RP-8
Wavelength: 210nm
Dead Time: 13

compound	Rt	W	K'	N
GENERAL BASIC DRUGS				
1 morphine	21	2	0.61	1764
2 nalorphine	25	3	0.92	1111
3 codeine	28	5	1.15	502
4 oxycodone	32	6	1.46	455
5 prilocaine	37	5	1.85	876
6 levorphanol	53	5	3.07	1798
7 diamorphine	59	7	3.53	1137
8 amylocaine	80	8	5.15	1600
9 bupivacaine	95	9	6.31	1783
10 pentazocine	107	10	7.23	1832
11 papaverine	137	15	9.54	1334
12 amethocaine	165	15	11.69	1936
13 benzocaine	99	9	6.62	1936
14 butacaine	180	15	12.9	2304
13 cinchocaine	NOT ELUTED IN UNDER 30 MINUTES			
14 dextromoramide	NOT ELUTED IN UNDER 30 MINUTES			
15 dipipanone	NOT ELUTED IN UNDER 30 MINUTES			
BENZODIAZEPINES				
nitrazepam	74	7	6.98	1788
lorazepam	184	19	13.1	1500
chlordiazepoxide	229	19	16.6	2324
desmethyldiazepam	347	37	25.7	1407
diazepam	430	35	32.1	2415
ACIDIC DRUGS				
primidone	40	5	2.07	1024
methyprylone	57	6	3.38	1444
phenobarbitone	64	5	3.92	2621
butobarbitone	98	6	7.46	4268
butalbital	124	10	8.37	2460
amylobarbitone	130	10	9.00	2704
glutethimide	143	13	10.0	1936
quinalbarbitone	240	20	17.46	2304

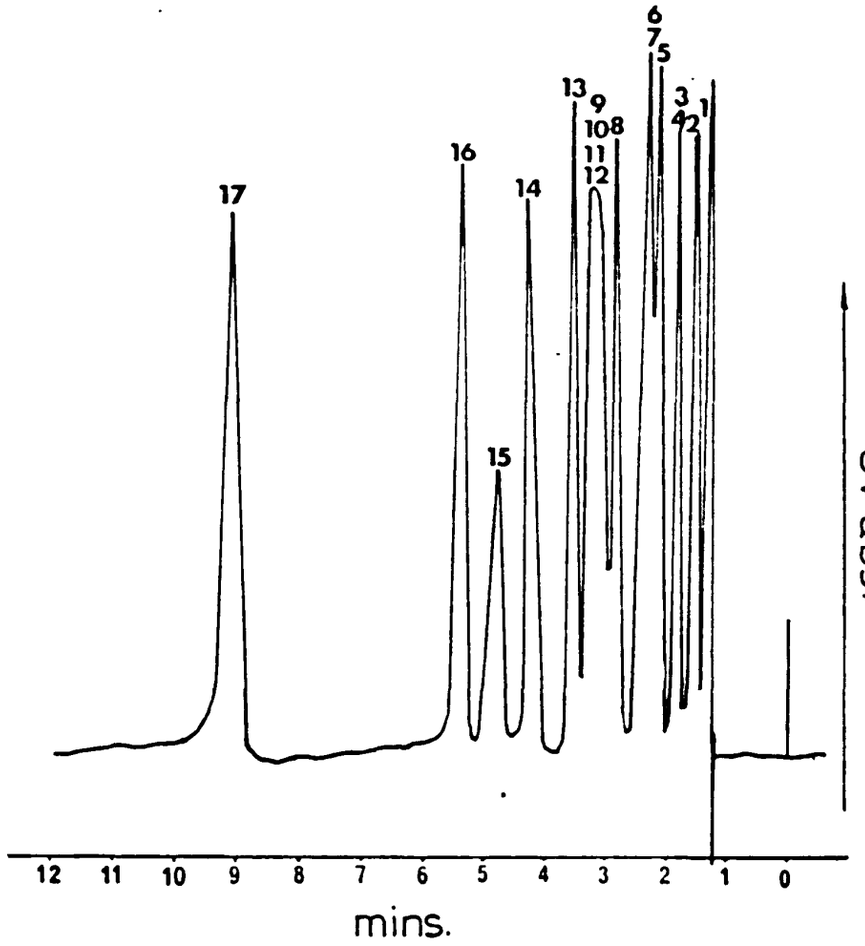
TABLE 3.19. contd.

COMPARISON OF CHROMATOGRAPHIC PERFORMANCE OF SOME COMMON DRUGS ON SOLVENT SYSTEMS USED FOR LOCAL ANAESTHETICS

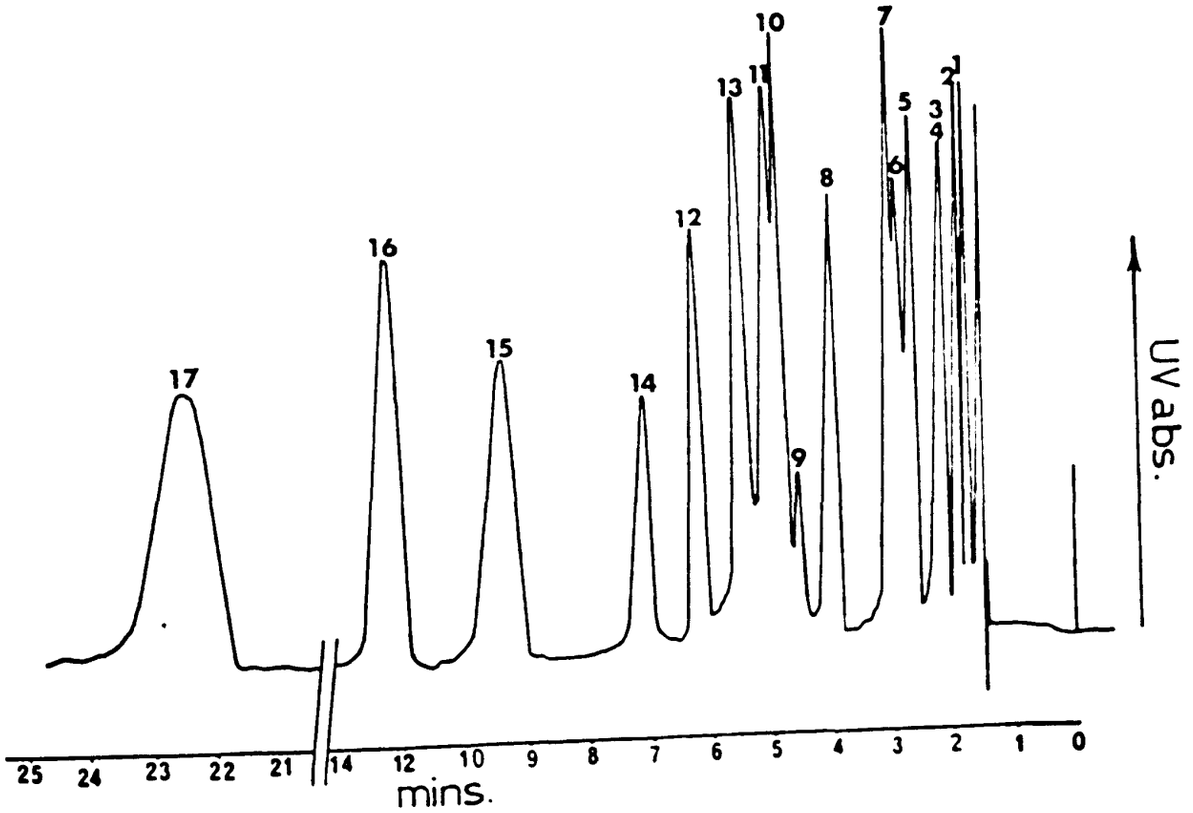
IV

Solvent: 10% acetonitrile in 0.05MKH_2PO_4 at pH 3
flow rate : 2
Column: 25cm Lichrosorb RP-8
Wavelength: 210nm
Dead Time: 13

compound	Rt	W	K'	N
GENERAL BASIC DRUGS				
1 morphine	27	6	1.07	324
2 nalorphine	37	6	1.85	608
3 codeine	45	7	2.46	661
4 oxycodone	51	11	2.88	344
5 prilocaine	56	7	3.31	1024
6 levorphanol	117	9	8.00	866
7 diamorphine	155	14	10.9	1961
8 amylocaine	180	17	12.8	1793
9 bupivacaine	205	24	14.8	1167
10 papaverine	305	34	22.5	1287
11 amethocaine	NOT ELUTED IN UNDER 30 MINUTES			
12 pentazocine	NOT ELUTED IN UNDER 30 MINUTES			
13 benzocaine	173	12	12.3	3325
14 butacaine	NOT ELUTED IN UNDER 30 MINUTES			
15 cinchocaine	NOT ELUTED IN UNDER 30 MINUTES			
16 dextromoramide	NOT ELUTED IN UNDER 30 MINUTES			
17 dipipanone	NOT ELUTED IN UNDER 30 MINUTES			
BENZODIAZEPINES				
nitrazepam				
lorazepam				
chlordiazepoxide	NO DATA COLLECTED			
desmethyldiazepam				
diazepam				
ACIDIC DRUGS				
primidone	65	9	4.0	834
methyprylone	100	13	6.7	946
phenobarbitone	119	14	8.2	1156
butobarbitone	210	22	15.2	1457
butalbital	296	35	21.8	1144
amylobarbitone	NOT ELUTED IN UNDER 30 MINUTES			
glutethimide	NOT ELUTED IN UNDER 30 MINUTES			
quinalbarbitone	NOT ELUTED IN UNDER 30 MINUTES			



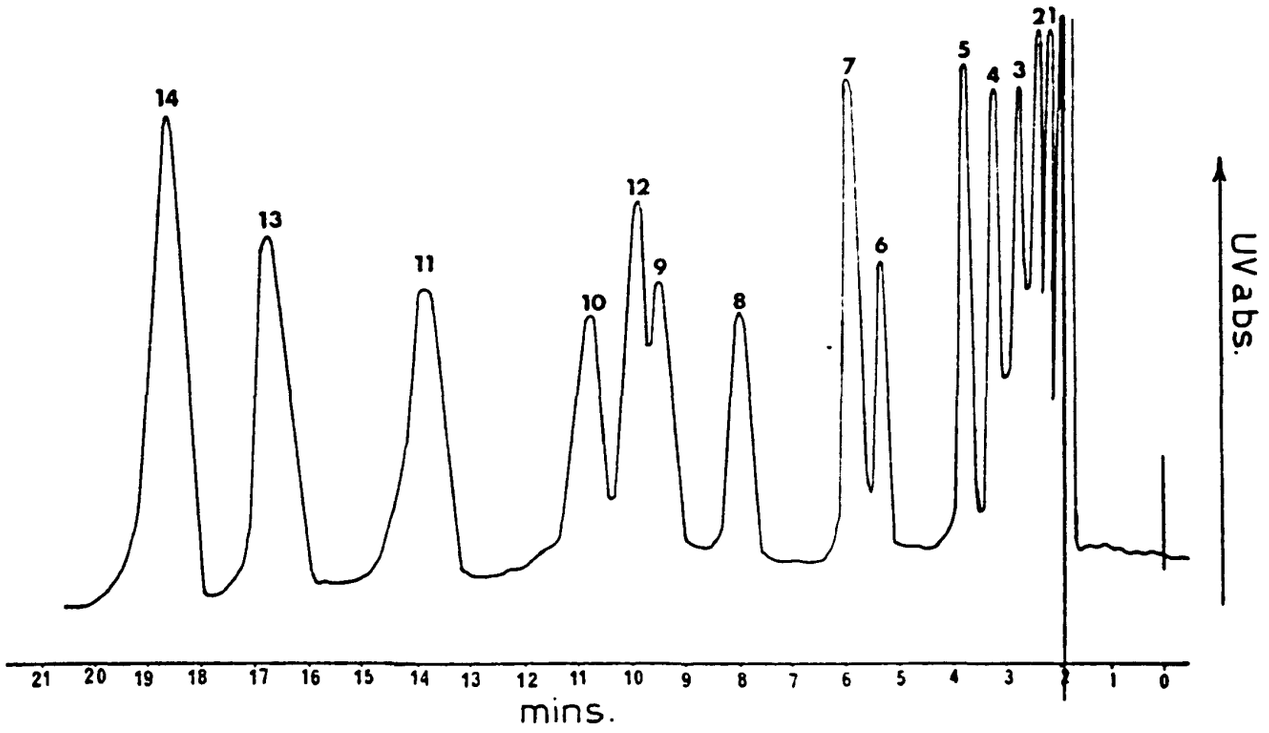
(I) 40% ACETONITRILE IN 0.05M KH_2PO_4 AT pH3 AND 2ml/min.



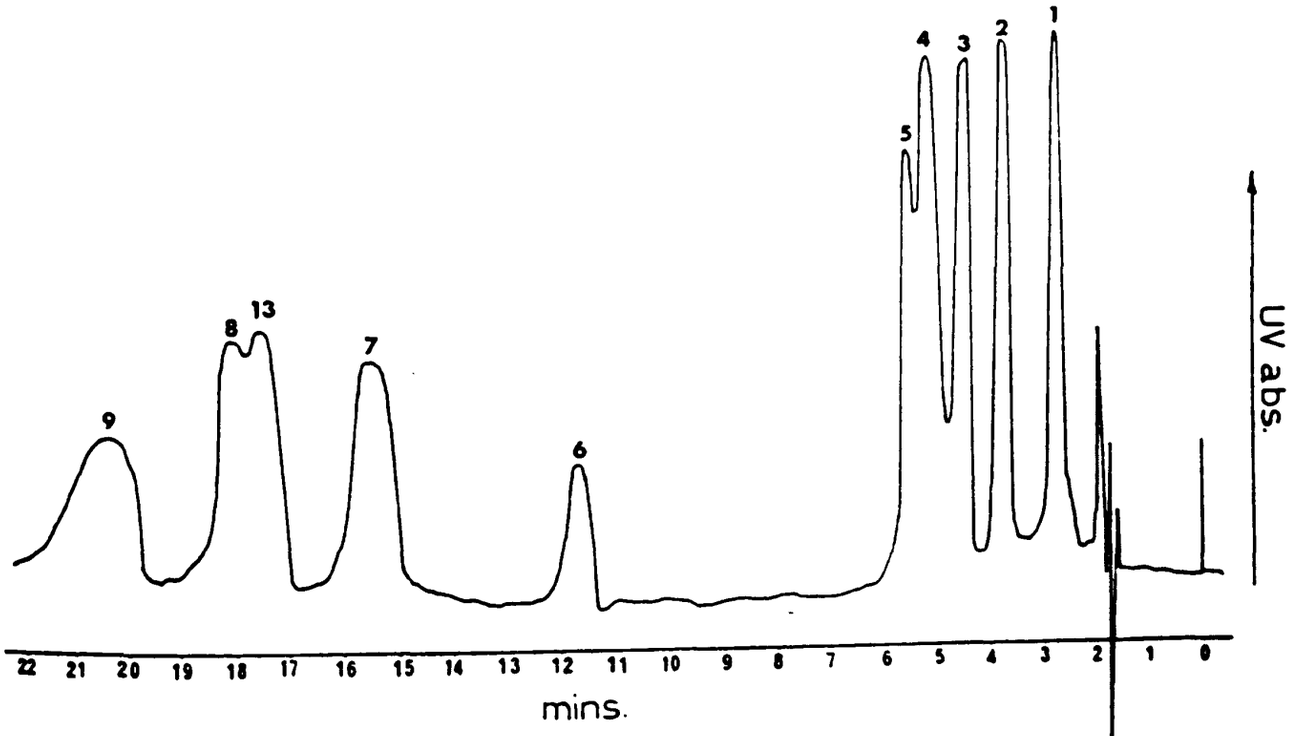
(II) 30% ACETONITRILE IN 0.05M KH2PO4 AT pH3 AND 2ml/min.

FIGURE 3.18.

CHROMATOGRAMS OF SEVENTEEN COMMONLY OCCURING BASIC DRUGS ON THE CHROMATOGRAPHIC SYSTEMS DESCRIBED IN TABLE 3.19..



(III) 20% ACETONITRILE IN 0.05M KH_2PO_4 AT pH3 AND 2ml/min.



(IV) 10% ACETONITRILE IN 0.05M KH_2PO_4 AT pH3 AND 2ml/min.

FIGURE 3.18. contd.

CHROMATOGRAMS OF SEVENTEEN COMMONLY OCCURING BASIC DRUGS ON THE CHROMATOGRAPHIC SYSTEMS DESCRIBED IN TABLE 3.19..

phenomenon of peak cross-over, its position relative to the other compounds changing throughout the series of solvents examined.

The separation achieved for the acidic drugs and the benzodiazepines was not as good has been achieved elsewhere (see Eppel, 1980 and 2.5.15. respectively), illustrating the effect that changing the selectivity of the stationary phase can have on the chromatography of a group of similar compounds.

Also noted was the lower efficiencies obtained for the narcotic analgesics compared to the local anaesthetics. Of these compounds the best efficiency achieved was for papaverine which implicates the involvement of the free hydroxyl groups in codeine, morphine and nalorphine in the phenomenon of peak tailing, as in papaverine, the hydroxyl groups are in the form of methyl ethers. The fundamental differences between the chromatographic behaviour of morphine and the local anaesthetics was noted earlier (2.3.4.)

Overall the conclusion drawn from this experiment was that the less lipophilic C-8 phase is less selective than the C-18 phase as it allows a wider range of compounds to be chromatographed with the same solvent system. This should be compared with the examination of potential interferants in the study of morphine where very few compound would chromatograph on the same system (2.5.15.).

This has both advantages and disadvantages. It suggests that a C-8 stationary phase might provide a suitable chromatographic system for screening samples for the presence of a wide range of drugs. It also suggests that more compounds might elute in the same area of the chromatogram and greater care should be taken to

ensure the identity of the peaks. This is analogous to the use of an OV-17 type column for screening samples by gas chromatography (Ardrey and Moffat, 1981). A number of different compounds might have the same retention index, and an independent analytical technique may be required for confirmatory analysis. For a routine confirmatory method, shorter chromatographic run times are preferred.

3.3.11.4. Conclusion

It has been shown that the lower selectivity obtained from a C-8 column allows a large number of compounds to be identified on one system over a longer period of time. A comparison with GC retention indices reported suggests that the use of a C-8 column might provide a complimentary screening procedure for unknown drugs. This might be investigated further.
The possibility of interference makes this method less selective than (say) the system developed for morphine (2.5.5.) but the increase in the number of compounds which can be analysed on one system must be considered against this.

3.3.12. CONCLUSIONS ON DEVELOPMENT OF CHROMATOGRAPHY FOR LOCAL ANAESTHETICS

The above study has shown the evolution of suitable chromatographic systems for the measurement of the compounds of interest (bupivacaine, procaine, cocaine and benzoyl ecgonine) and others. In the course of this development it proved neccessary to change to use a stationary phase of different selectivity. The resulting improvement in the quality of the peak shape and the chromatography made this the method of choice.

These systems were then ready to be applied to the analysis of the compounds of interest in suitable extracts from biological fluids.

3.4. EXAMINATION OF DRUG SCREENING PROCEDURES FOR LOCAL ANAESTHETICS IN GREYHOUND URINE

3.4.1. Introduction

General screening procedures have been reviewed above (3.1.7.2.). The applicability of the method which is in general use (Bogan and Smith, 1968) has not been examined specifically for its ability to detect local anaesthetics. It is generally accepted that an important part of an effective doping control programme must include checking and updating of the methods used (Smith, 1972).

It was therefore decided to examine this method and an alternative procedure also reviewed earlier, to assess the suitability of both for this application.

3.4.2. SUITABILITY OF THE TLC SYSTEM FOR THE CHROMATOGRAPHY OF LOCAL ANAESTHETICS.

3.4.2.1. Introduction

The Rf values of several local anaesthetics on the TLC system recommended by Bogan and Smith (1968), (Rentoul and Smith, 1971) were examined and compared with data tabulated therein. The suitability of the locating procedures recommended was also examined.

3.4.2.2. Method

Using stock solutions (made up in ethanol) of 1g/L and 0.1g/L of the drugs indicated in table 3.20., 1ug and 10ug spots were applied, 1cm from the bottom of a 10cm silica TLC plate. The plates were then developed in a chloroform:methanol (3:1) solvent system in a paper lined tank for a distance of 6-8cm. The plates were removed and air dried for 2-5 minutes before being subjected to the following locating tests.

1. Examination under UV light to show the position of fluorescing material. Non-flourescing material was also visible on these TLC plates as they contained a fluorescent indicator which was quenched where this material was present

2. The plates were sprayed with concentrated HCl, under which phenothiazine type drugs show up as pink spots (Oliver, 1971).

3. They were finally oversprayed with acidified potassium iodoplatinate solution (PIP) (prepared as in appendix D) to show the position of any basic nitrogen-containing or alkaloidal material.

3.4.2.3. Results

The response of the drugs to these detection systems is shown in table 3.20.. The Rf values obtained were compared with those reported elsewhere for this system (Rentoul and Smith, 1973). Most were found to compare well.

It was noted that when the plates were sprayed with iodoplatinate following spraying with HCl, the response was notably poorer than if only acidified iodoplatinate spray had been used.

No reaction was noted for some of the 1ug or 10ug spots.

This was contrary to the reaction reported in Rentoul and Smith, (1973) and elsewhere (Oliver, 1971). It was also noted that the intensity of the spots would develop slowly over a period of 2-8 hours.

If the acid pre-spraying step was ommitted, 10ug and 1ug spots could all be clearly seen immediately after spraying.

It was found that this developing process could be accelerated by warming the TLC plates over a hot plate for a few minutes.

It was therefore concluded that the acid was responsible for inhibiting the developement of these spots, and that heating or leaving the plates for a period of time would allow the evaporation of HCl as vapour.

After the full intensity of the spots had developed it was noted that response of bupivacaine was poorest, giving a faint brown spot, compared to the darker blue/purple spots for other compounds.

All drugs were visible at levels of 1ug on the TLC plate.

Subsequent overspraying with concentrated HCL caused the spot to fade. On warming, the spots would re-appear. Using procaine as an example, as much as 40ug on the plate remained undetected if it was presprayed with acid.

TABLE 3.20.

TLC OF VARIOUS LOCAL ANAESTHETICS

compound	Rf	Rf*	UV	KPtIO4	HCl	later
amethocaine	0.52	0.72	+	++	-	+-
benzocaine	0.79	0.66	+	-	-	-
bupivacaine	0.73	$	+	+	+	+
butacaine	0.60	0.55	+	++	+--	+-
cinchocaine	0.45	0.53	+	+	+	+
cocaine	0.54	0.68	+	++	++	++
lignocaine	0.84	0.7	+-	+	-	+
metycaine	0.48	$	+	+	+	+
prilocaine	0.67	$	+	+-	-	-
procainamide	0.1	0.22	+	+	-	+--
procaine	0.39	0.40	+	++	-	+-

* data for same solvent system, Oliver, 1971

key:-
++ strong positive
+
+- positive
+--
- negative

$ No data given

3.4.2.4. Discussion

The minor differences observed in the Rf values between this and other studies can be attributed to differences in the silica used on the plates, and minor changes in the solvent composition.

Acidified potassium iodoplatinate spray is used to detect alkaloids, amines and organic nitrogen compounds including quaternary ammonium compounds. The various colours reactions produced have been discussed by Stead et al. (1982). It is known that factors such as the amount of drug, pH and amount of locating reagent sprayed onto the plate may affect the hue and intensity of the spot.

The exact mechanism of the reaction is not known, but it is likely that it involves complex formation involving iodide, the platinum ion and the electrons on the basic nitrogen.

Several possible causes of the acid masking of certain compounds were considered. Several properties of the compounds are given in table 3.21 (the properties of caffeine are given for comparison).

A comparison of the pKa values showed no consistent relationship

An examination of the structures showed that there was no relationship between the degree of substitution on the basic nitrogen and acid masking. It had been thought that the use of concentrated acid on an ester might cause hydrolysis of the peptide bond, however, no consistent relationship was found between acid masking and the type of linkage (i.e. ester or amide). In addition this would be irreversible and the colour reaction would not be expected to return.

TABLE 3.21.

COMPARISON OF PROPERTIES OF LOCAL ANAESTHETIC DRUGS IN RELATION TO ACID MASKING WITH TLC SPRAYS.

compound	masking	pKa	amine substitution	linkage	amine coordination
benzocaine	-	2.8	no amine	ester	no amine
amethocaine	Y	8.5	3ry	ester	chain
butacaine	Y	9.0	3ry	ester	chain
lignocaine	Y	7.9	3ry	amide	chain
prilocaine	Y	7.9	2ry	amide	chain
procainamide	Y	9.2	3ry	ester	chain
procaine	Y	9.0	3ry	ester	chain
bupivacaine	N	8.1	3ry	amide	piperidine
cinchocaine	N	7.5	3ry	amide	pyridine
cocaine	N	8.6	3ry	ester	piperidine
metycaine	N	-	3ry	ester	piperidine
caffeine	N	14	4 x 3ry	-	pyrimidine

The only consistent factor in those drugs examined which did not show acid masking was the incorporation of the basic nitrogen in a ring system (e.g. cocaine, bupivacacine, caffeine). This might implicate some steric effect in the reaction causing the colour change. This point was not developed any further.

3.4.2.5. Conclusions

The TLC system investigated has been shown to produce acceptable Rf values for the compounds investigated.

The practice of acid pre-spraying TLC plates prior to the use of acidified iodoplatinate can however, mask even large amounts of some compounds. On the other hand, acid spraying enhances the intensity of the spot with some ring nitrogen compounds, e.g. bupivacaine and caffeine, and allows the identification of phenothiazines. The compromise settled upon was to continue pre-spraying the plates with acid, mark the position of any spots indicating phenothiazines, and then spray with acidified potassium iodoplatinate. Any spots which immediately developed were noted and the plates were gently warmed over a hotplate for two to three minutes to allow the development of any spots which were inhibited by acid prespraying.

On the basis of this study the TLC system described above incorporating these changes was retained for the examination of extracts obtained from screening procedures for these drugs in urine.

3.4.3. EXTRACTION PROCEDURES (I) - Bogan and Smith, (1968)

3.4.3.1. Introduction

The extraction procedure in routine use for urinary drug screening in the racing greyhound is based closely on that described by Bogan and Smith, (1968). This was used to extract several urine samples which had been spiked with known concentrations of bupivacaine, procaine and cocaine and its metabolite benzoyl ecgonine.

3.4.3.2. Method

A bulk urine sample (1L) was collected from a drug-free greyhound and the pH measured. This was then divided into aliquots of 50ml and spiked with known concentrations (10^{-3}g/L) of the drugs of interest. Each of these spiked samples were extracted as below. A drug-free sample was also extracted.
The routine extraction procedure in its current form is as follows:

Urine (20ml) and 20% ammonia solution (10ml) were added to a 100ml separating funnel. Ethyl acetate (50ml) was added and the flask was shaken thoroughly. The aqueous layer was removed and kept for subsequent analysis for acidic drugs. 10% H_2SO_4 (10ml) was added to the ethyl acetate and shaken thoroughly. The pH of the aqueous layer was checked with pH paper, to ensure it was acidic (below 4). The acid layer was transfered to a second separating funnel and ethyl acetate (50ml) added. This was shaken thoroughly and made alkaline by the addition of 20% ammonia solution (about 20ml). The pH of the aqueous layer was checked

to ensure that it was alkaline (above pH8). This layer was discarded and the ethyl acetate was filtered through anhydrous sodium sulphate to remove any residual water, collected in an evaporating basin and evaporated to dryness on a steam bath. A few drops of 25% HCl were added to the final organic extract to prevent the evaporation of volatile bases (if present) by the formation of the HCl salt.

The residue was reconstituted in ethyl acetate (5ml). Half (2.5ml) was removed and kept for confirmatory anlysis by GC or GCMS.

The remainder of the extract was evaporated to a small volume and spotted onto a TLC plate. The appropriate drug was spotted alongside as a standard. The plate was developed in the solvent system described above (3.2.2.1.) and examined under UV light, sprayed first with concentrated HCl and then with potassium iodoplatinate. The plates were warmed gently over a hotplate and the Rf values were noted as the spots appeared.

3.4.3.3. Results and discussion

The results of the TLC analysis of each of the extracts are shown in table 3.22.. The TLC plates showed the presence of spots corresponding to the drugs added, at a concentration of 10^{-3}g/l. The response of bupivacaine and benzoyl ecgonine was poor, suggesting that they might have a lower recovery from the sample. The recovery was not measured.

The extracts were relatively clean. Other staining was noted at the foot of the plates (Rf lt. 0.2), but no distinct dark spots.

The analysis time for this extraction procedure (including the

TABLE 3.22.

RESULTS OF URINARY DRUG SCREEN PERFORMED BY THE METHOD DESCRIBED BY BOGAN AND SMITH, 1968

compound	TLC screen	GC (EtOAc)	GC(MeOH)
procaine	++	+	++
cocaine	++	+ -	++
bupivacaine	+-	+	++
benzoyl ecgonine	+-	-	-

acidic extraction) was about one hour and four samples could be extracted, conveniently, at the same time.

Confirmatory analysis was carried out by gas chromatography as described below. (3.4.5.)

3.4.3.4. Conclusions

The screening procedure currently in use for the detection of drugs in the urine of racing greyhounds has been shown to be capable of detecting those local anaesthetics examined, at concentrations of 1ug/ml in a urine sample.

3.4.4. EXTRACTION PROCEDURES (II) - Hill et al., (1982)

3.4.4.1. Introduction

An alternative urinary screening method developed specifically for the identification of acidic, basic and neutral drugs in greyhound urine was examined in order to assess its efficiency and to compare it with the method currently in use, examined above. This procedure is based on a single step extraction procedure using ion-pair formation. It is performed at pH7 where it is assumed that basic compounds will be sufficiently protonated to form ion-pairs, and that weakly acidic and neutral drugs will be unionised. Both neutral species should then partition into the organic solvent.

3.4.4.2. Method

In addition to the spiked samples prepared previously, two barbiturate drugs, phenobarbitone and pentobarbitone, were extracted from spiked urine samples as follows.

Urine (9ml), 1.25mM dioctyl sulphosuccinate (1ml) and chloroform/isopropanol 3:1 (5ml) were added to a 15ml screwcap test tube. This was capped and mixed on a rocking/rolling mixer for 10 minutes.

The tube was centrifuged at 3000rpm for approximately five minutes or until the layers were separated. The upper aqueous layer was aspirated and discarded. The organic solvent was passed through a glass pasteur pipette containing some glass wool, to remove particulate matter. This was collected in a glass centrifuge tube and evaporated to dryness under a stream of oxygen-free nitrogen.

The residue was reconstituted in 30ul of ethyl acetate. As this extract would normally contain acidic, neutral and basic drugs, 10ul were spotted onto two 10cm TLC plates, using a micropipette. One plate was developed in the solvent system examined above for basic drugs (3.4.2.), and the other in a chloroform/acetone, 9/1 solvent system (Rentoul and Smith, 1971; Oliver, 1971) suitable for a range of acidic drugs. The plates were sprayed with saturated mercurous nitrate solution which will indicate the presence of barbiturates and phenylbutazone metabolites (Oliver, 1971).

The remaining 10ul was retained for confirmatory GC analysis.

3.4.4.3 Results and Discussion

A summary of the results is shown in table 3.23..

All the local anaesthetics examined were detected on the TLC plates at concentrations of lug/ml. The poorest response was for bupivacaine and benzoyl ecgonine which were just visible. Their visualisation was made easier by the presence of a drug standard run alongside the extract on the same plate.

The extracts did contain interfering material and produced long yellow or white "candle flame" streaks on the TLC plates. The blank however did not contain distinct dark spots which might have been confused for drugs.

The procedure was very rapid and as many as 20 samples were extracted and the TLC analysis completed in one hour. It should also be noted from the data in table 3.23. that this single step extraction procedure was effective in extracting two barbiturates. Both gave positive results at lug/ml. No further analysis of acidic or neutral drugs was considered as part of this study.

The extracts were readily reconstituted in ethyl acetate or methanol.

3.4.4.4. Conclusions

This alternative extraction procedure has been shown to be effective in extracting the local anaesthetic drugs of interest from a spiked urine sample. It apppears to be as effective as the other procedure examined in spite of the fact that a smaller urine sample was used.

In addition this procedure has been shown to be effective in

TABLE 3.23.

RESULTS OF URINARY DRUG SCREEN PERFORMED BY THE METHOD DESCRIBED BY HILL ET AL., 1982.

compound	TLC screen	GC (EtOAc)
procaine	++	+
cocaine	++	+
bupivacaine	+-	+
benzoyl ecgonine	-	-
phenobarbitone	++*	+
pentobaritone	++*	+

* developed in chloroform/acetone, 9/1 solvent system and sprayed with saturated aqueous mercurous nitrate. (Rentoul and Smith, 1971; Oliver, 1971)

extracting two barbiturates in the same fraction as the basic drugs. Whilst this makes the extraction less specific, it makes it easier to perform. A full comparison of both methods follows in section 3.4.6..

3.4.5. THE USE OF GAS CHROMATOGRAPHY AS A CONFIRMATORY TECHNIQUE

3.4.5.1. Introduction

The use of gas chromatography for the measurement of local anaesthetics was reviewed earlier (3.1.2.5.). It is however the method currently used for the confirmatory analysis of drugs in urine extracts, although its suitability has not been examined specifically for this application.

For this reason the extracts obtained by both extraction procedures described above were examined by this technique.

3.4.5.2. Method

Gas chromatography was performed on a capillary gas chromatograph (Packard Instruments, model 427). The column eluent was split and the instrument was equipped with a flame ionisation detector and a nitrogen/phosphorus detector (model 612). The injection system used was a Grob type splitless injector. The column was a CP-Sil 5 fused silica capillary column (SGE). The extracts were analysed on a temperature programmed run. Two minutes at 110°C after which the purge valve was opened, then a temperature rise from 110° to 300°C at 8°C a minute. There was a final plateau of five minutes at 300°C.

The injector and detector temperatures used were both 250°C.

Standard solutions of the drug were analysed to determine the retention index. The remainder of the residue was evaporated to dryness and reconstituted in ethyl acetate (50ul). 1ul was injected into the injection port of the gas chromatograph.

The results obtained from both extracts are considered seperately below. The run time inluding thermal re-equilibration of the system took about forty minutes.

3.4.5.3. Results and discussion

Bogan and Smith, 1968

The presence of procaine, bupivacaine and cocaine were all confirmed in the relevant extracts (table 3.22.) by the use of retention indices and subsequent comparison with unextracted drug standards.

Benzoyl ecgonine was not detected, but it is known not to chromatograph under these conditions without derivatisation (Lindgren, 1981).

It was noted that the peaks obtained for the drugs were quite small on both NPD and FID. It also appeared that the dried extract did not reconstitute very well in the 50ul of ethyl acetate used as the GC injection solvent.

A more suitable solvent was therefore sought. Methanol was considered as an alternative. The extract appeared to dissolve more readily and the peak heights improved significantly (by a factor of 3 or 4).

It was thought that this phenomenon might be caused by the

practice of converting any free base in the extract to the hydrochloride salt in the last step of the extraction. HCl salts have a poor solubility in ethyl acetate (Albert, 1976). Methanol is a more readily ionised solvent and therefore a better solvent for these ionisable species. Methanol can contain a relatively large proportion of water however which is not good for the GC column, and may lead to a decrease in column lifetime and performance. This problem could be avoided by reconstituting the extract in alkali and back-extracting the free base into ethyl acetate or some other organic solvent. This however will add an additional step to the sample preparation procedure for GC.

Hydrochlorides can be readliy dissolved in water or dilute acid, and in aqueous form can conveniently be analysed by reversed phase liquid chromatography.

Aqueous samples cannot be analysed directly by GC.

Hill et al., 1982

The gas chromatograms obtained from these extracts contained a number of additional peaks on both the FID and NPD traces. The presence of the drugs of interest were confirmed in the relevant extracts (table 3.23.). This applied also to pentobarbitone and phenobarbitone.

The residue appeared to reconstitute readily in ethyl acetate. There was no apparent improvement in peak height if methanol was used to reconstitute.

No attempt was made to measure the extraction efficiency of this procedure.

3.4.5.4. Conclusions

Confirmation of positive results is possible with GC, but care must be taken that the sample is not lost through the use of an inappropriate solvent for reconstitution, if the drug is extracted as the hydrochloride salt. This factor may lower the detection limit of the assay as a whole, both by TLC and GC.

A detailed study of solubility of these salts in different solvents would be useful.

In this case it was not possible to directly analyse for the major metabolite of cocaine, benzoyl ecgonine by GC under the conditions described, derivatisation being required.

This illustrates an additional advantage that a general HPLC method for these local anaesthetics would have over GC, as no derivatisation is required.

3.4.6. COMPARISON OF EXTRACTION PROCEDURES

3.4.6.1. Discussion

Both extraction procedures have been shown to extract a range of local anaesthetics from urine samples. The extraction efficiencies were not calculated. A possible area of sample loss in the method described by Bogan and Smith, (1968) is the poor solubilty of hydrochloride salts in ethyl acetate, demonstrated above. The formation of lipophilic ion-pairs involving dioctylsulphosuccinate avoids this complication.

The ion-pair extraction method was very much quicker and

convenient, being a single step extraction. It was more economical also, requiring only 5ml of solvent, compared to 200ml in the other method.

The sensitivity of the ion-pair method is probably lower, as only 1/6 of all the basic material in the extract is applied to the basic TLC plate. One half of the basic extract is applied in the method described by Bogan and Smith. Hills method uses 9ml of urine, however this could be scaled up to 20ml relatively easily. No quantitative measure of the extraction efficiency was made for either process.

The extracts from the ion-pair method were less clean and contained more interfering material. Spots resulting from drugs were more clearly visible if 20cm TLC plates were used.

3.4.6.2. Conclusions

The method described by Bogan and Smith was preferred on grounds of quality of the extract for TLC. It was also considered to be more suitable for racetrack testing, as the interpretation of the TLC plates was more straightforward. This is a major factor in testing procedures, as the personnel employed at the racetrack are not always technically qualified. Both procedures could detect all three local anaesthetics and benzoyl ecgonine when present in urine at concentrations of 1ug/ml.

For convenience and rapid extraction of acidic or basic compounds, a scaled-up version of Hills method was preferred for confirmatory analysis. This single step procedure was rapid and has been shown to extract basic and acidic drugs at levels of 1ug/ml in a 9ml urine sample. It also avoids subjecting the analytes to

extremes of pH. The rate of hydrolysis of cocaine has been shown to be significant at high pH even over periods of several minutes (Fletcher and Hancock, 1981; Baselt, 1983).

The GC method described above was capable of detecting all the compounds of interest except benzoyl ecgonine and the existence of possible problems involving the reconstitution of the sample was demonstrated.

For the reasons outlined earlier (3.1.5.7), the suitability of an HPLC method was to be considered. A suitable HPLC method could also be used to confirm the identity of a drug suggested by its GC retention index.

Both urine extracts were examined for their compatibility with the HPLC methods developed earlier, before consideration was made of extraction procedures for other samples.

3.4.7. SUITABILITY OF URINARY DRUG SCREEN EXTRACTS FOR CONFIRMATORY HPLC ANALYSIS

3.4.7.1. Introduction

In previous sections (3.3.) HPLC methods were developed for the analysis of bupivacaine, mepivacaine, lignocaine, cocaine, benzoyl ecgonine, cinchocaine and procaine.

Extracts obtained by the extraction procedures examined above (Bogan and Smith, 1968; Hill et al., 1982) were analysed to determine if the extracts could be applied directly to HPLC.

3.4.7.2. Method

A urine sample was obtained and spiked with a known concentration (1ug/ml) of bupivacaine. This was extracted by both the procedures described above. The residues were reconstituted in the respective mobile phases (200ul) prior to injection into the HPLC system. The HPLC conditions were as described in System One, section 3.3.9.3..

3.4.7.3. Results and discussion

Both extracts gave very dirty chromatograms. There were peaks present in the region where the drugs were expected, but there were also other smaller peaks and shoulders on the peaks of interest.

The extracts from Hills method, was considerably more dirty and peaks continued to elute for up to 40 minutes after injection.

The urine extracts are known from TLC screening to contain the drugs of interest. The HPLC method directly applied to these extracts was able to confirm a peak in the region of interest, but other extraneous material interfered with this and it took some time for all the extract to elute from the column. To eliminate this interference it was concluded that further clean-up would be required. Alternatively, another extraction procedure for urine could be found.

3.4.7.4. Conclusions

Although the extracts produced from the drug screening procedures have been shown to be suitable for local anaesthetics by GC, neither of the extracts were suitable to be directly

applied to HPLC analysis. It was concluded that further sample clean up would be required, if HPLC was to remain the method of choice.

3.5. EXTRACTION PROCEDURES FOR THE ISOLATION OF LOCAL ANAESTHETICS IN PLASMA

3.5.1. LIQUID/LIQUID EXTRACTION OF BUPIVACAINE FROM PLASMA

3.5.1.1. Introduction

The HPLC system (System One, 3.3.9.3.) selected for bupivacaine was that described by Lindberg and Pihlajamaki, (1984). The extract from the urinary screening procedure has been shown to be unsuitable for direct analysis by HPLC (3.4.7.). An alternative extraction procedure applicable to blood, plasma and urine was sought.

The extraction procedure described in Lindberg and Pihlajamaki, (1984), for the extraction of bupivacaine from plasma was reviewed earlier (3.1.7.2.). It had a reported extraction efficiency of 92% and the extract had been shown to be suitable for analysis by HPLC. This therefore seemed to provide appropriate conditions on which to base a general method for local anaesthetics in biological fluids.

For practical reasons several modifications were made to the described procedure. Dichloromethane was used as the extracting solvent as it was easier to evaporate than hexane-containing solvents, and was also expected to have a good solubility for most

of the drugs of interest as it has a low polarity (P' = 3.1, Snyder, 1974) which is compatible with the extraction of the unionised lipophilic drugs. Its selectivity is also likely to be good as it has a low solubility for water (0.17% weight). 1ml of plasma, instead of 0.5ml, was used. This was done because larger volumes of plasma were available, and also to enhance the sensitivity of the assay.

Extraction efficiency was calculated by comparison of peak heights in the extracted standard with an unextracted standard of the appropriate strength. To show the improvement in precision achieved by the use of an internal standard, the ratio of internal standard to drug is also given in terms of extraction efficiencies.

3.5.1.2. Method

A plasma sample spiked with bupivacaine was prepared (10^{-3}g/L). Plasma (1ml) was placed in a 15ml screwcap test tube. 0.1M NaOH (1ml) and 1ug of cinchocaine (0.1ml of 10^{-2}g/L) as internal standard were added. The extraction solvent, dichloromethane (10ml) was added and the mixture was shaken thoroughly on a vortex mixer for approximately 30 seconds, then placed on a rocking-rolling mixer for 10 minutes. This was then centrifuged to separate the layers. The organic layer was removed and filtered through phase separating paper (Whatman PS-1) before being evaporated to dryness at 50°C under OFN.

The residue was reconstituted in 100ul of mobile phase and 20ul injected into the HPLC.

3.5.1.3. Results and discussion

This extraction procedure was easy to perform and provided a clean extract suitable for HPLC. No emulsion formed. An extraction efficiency of 76.8% ± 8.8 (n=5) was achieved for bupivacaine and 53.4% ± 6.6 for cinchocaine. The ratio of cinchocaine to bupivacaine was 0.69 ± 0.017 (CoV=2.4%).

The recovery is less than that reported by Lindberg and Pihlajamaki (1984), (91%), but the small error relative to the internal standard, is comparable. Extracts from blank plasma samples showed no interference and no peaks eluted after cinchocaine.

The removal of the upper aqueous phase occasionally left a semi-solid residue requiring the use of the phase separating paper. It was decided to investigate the possibility of transfering this procedure to a solid phase method as this could remove the requirement for the filtering step, and eliminate the problem of phase separation which might be a source of sample loss (see section 2.7.5.).

3.5.2. THE USE OF ABSORPTION/ELUTION EXTRACTION TECHNIQUES

3.5.2.1. Introduction

The advantages of solid phase extraction methods have been considered earlier for local anaesthetics (3.1.7.3) and the adaptation of liquid/liquid extraction methods to solid phase extraction techniques can often be fruitful in terms of ease of sample handling, prevention of emulsion formation, phase

separation problems and time required for the analysis. A limited degree of success had been experienced with solid phase extraction techniques for morphine from plasma (see 2.8.4.). It was decided to investigate whether the most promising of these, namely absorbent extraction on diatomaceous earth, would be suitable for the extraction of local anaesthetics from biological fluids.

3.5.2.2. Method

5ml glass syringes were prepared as described earlier (2.8.4.) and filled with 6 to 7ml of extrelut material. A plasma sample spiked with bupivacaine (10^{-3}g/L) was used. Plasma (1ml), 0.1M NaOH (1ml) and cinchocaine internal standard ,1ug (0.1ml of 10^{-2}g/L solution) were added to a 2ml disposable syringe and mixed thoroughly by inversion. This mixture was applied to the extrelut column and allowed to absorb for 3-5 minutes. The drug was then eluted with dichloromethane. 4ml of the eluent was collected in a glass sample tube (8ml) and evaporated to dryness. The residue was reconstituted in 100ul of the mobile phase and 20ul were injected into the HPLC.
HPLC conditions were as in System One, section 3.3.9.3.. The mobile phase was 28% acetonitrile in 0.05M KH_2PO_4, adjusted to pH 3.

3.5.2.3. Results and discussion

The chromatograms produced from these plasma extracts were of an acceptable quality. An example is shown in figure 3.19.. The procedure was easy to perform and no problems were

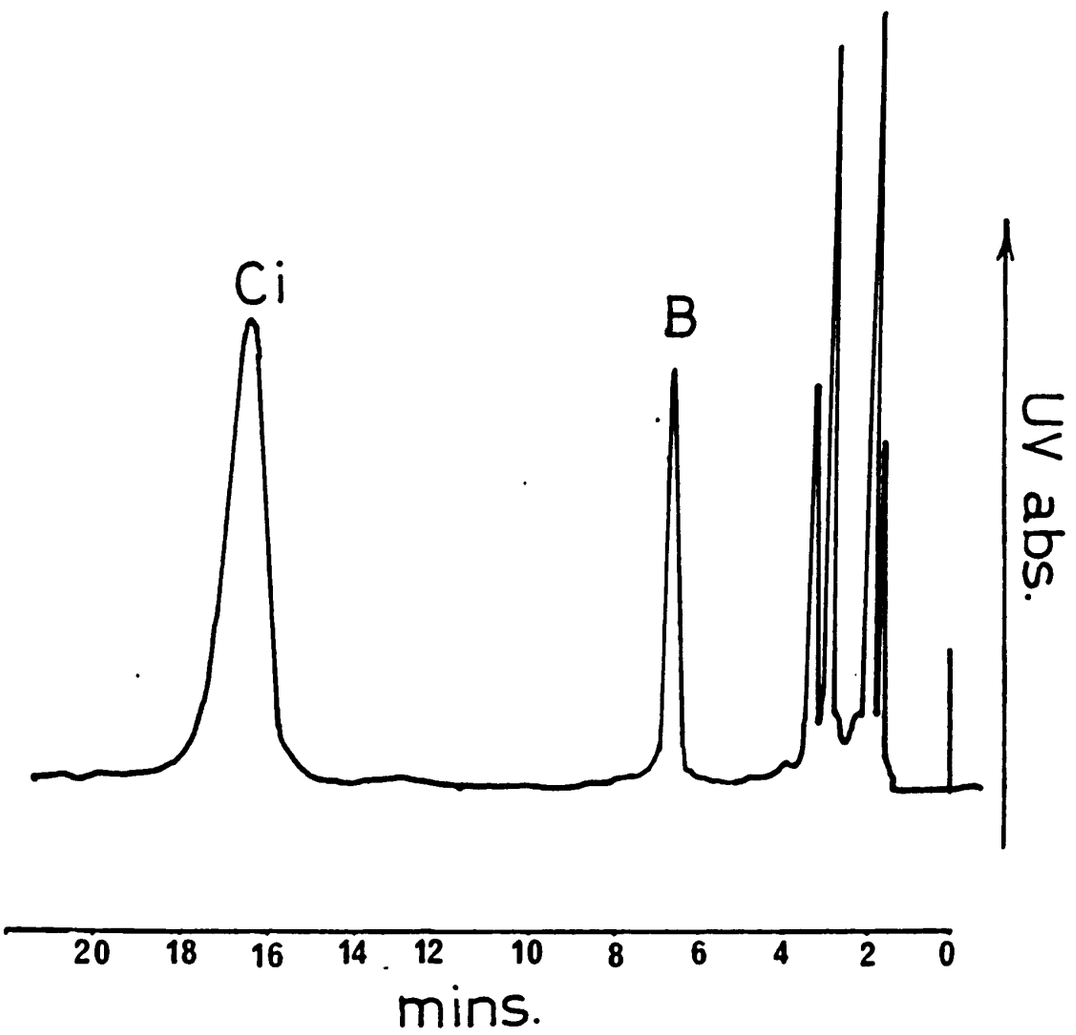


FIGURE 3.19.

CHROMATOGRAM OF EXTRELUT® PLASMA EXTRACT CONTAINING BUPIVACAINE AND CINCHOCAINE

TABLE 3.24.

EXTRACTION EFFICIENCY OF BUPIVACAINE FROM PLASMA USING EXTRELUT EXTRACTION METHOD

	Bupivacaine Eff.	Cinchocaine Eff.	Ceff/Beff
	85	55	0.65
	70	42	0.62
	83	55	0.66
	100	60	0.60
X	84.5	53.0	0.63
SD	12.3	7.7	0.024
CoV(%)	14.6	14.5	4.3

encountered. Sample preparation took approximately 10 to 15 minutes, an improvement over the 20 to 25 required for liquid/liquid extraction, most of the time being saved by the omission of the centrifugation step.

The chromatographic run time was up to 21 minutes and no peaks eluted after the internal standard. The extraction was performed four times and the results are given in table 3.24. and discussed below. The absolute recovery of bupivacaine was found to be 84.5% ± 12.3. This compares well with the previous procedure (3.5.1.) where the absolute recovery was 76.8% ± 8.8 (n=5).

The CoV is 14.6%, which is high. The recovery of the internal standard (cincocaine) was 53.0% ± 7.7, and the ratio of drug to internal standard is 0.63 ± 0.024 (CoV=4.3%). This illustrates the improvement in precision obtained with the use of an internal standard in this assay.

3.5.2.4. Conclusions

This simple, one step, extraction procedure is suitable for extracting bupivacaine from plasma quickly and efficiently, with reproducible recovery. The use of an internal standard has been shown to give a relative error of 4.3%, an improvement over the absolute error of 12.3%. It was decided to investigate the elution volume of the drug from the extrelut column to determine the suitability of the arbitrary volume (4ml) used initially.

3.5.3. EFFECT OF SOLVENT VOLUME ON EXTRACTION PROCEDURE

3.5.3.1. Introduction

The elution volume of the drug and internal standard was investigated by collecting the eluent in small fractions and analysing each separately.

3.5.3.2. Method

An extrelut column was packed and the sample prepared and applied as before. An aqueous standard of bupivacaine, 1ug/ml was used. Dichloromethane was applied to the top of the column and collected in 400ul fractions. These were evaporated to dryness and the residues reconstituted in 100ul of the mobile phase. These were analysed in turn on the HPLC system described above and the recoveries in each fraction were calculated.

3.5.3.3. Results and discussion

The recoveries are shown in table 3.25.. All the detectable drug and internal standard were eluted in the first four fractions. The cumulative recoveries (66% and 49% for bupivacaine and cinchocaine respectively) were lower than that calculated in the previous experiment (84.5% and 53% respectively), indicating that sample loss may occur in the reconstitution step. It has been reported that the elution pattern from extrelut columns can vary depending on the rate of elution (Christiansen, 1984). The percentage of the compound not recovered may be bound to plasma proteins and not recoverable simply by solvent extraction.

TABLE 3.25.

EFFECT OF ELUTION VOLUME ON THE RECOVERY OF DRUG AND INTERNAL STANDARD

fraction #	Cumulative volume (ml)	Recovery (%) bupivacaine	cinchocaine
1	0.4	34	24
2	0.8	24	19
3	1.2	8	5
4	1.6	trace	1
5	2.2	none detected	
6	2.6	none detected	
		66%	49%

3.5.3.4. Conclusions

Both bupivacaine and cinchocaine were eluted in the first 1.6ml, but it was decided that in future 4ml of the eluate would be collected to ensure maximum absolute and relative recovery. This volume had proved convenient to collect and evaporate to dryness.

3.6. EXTRACTION PROCEDURES FOR BUPIVACAINE IN OTHER BIOLOGICAL FLUIDS

3.6.1. Introduction

Plasma samples are easier to handle and to extract than whole blood as they are less prone to putrefaction or changes in consistency than is whole blood. Problems associated with these changes have been reviewed and were investigated in the case of morphine (see 2.9.3.). Plasma is generally available only from clinical samples where there is ready access to a centrifuge. Other sample types have been discussed above, but normal post mortem samples include blood and occasionally urine. For drug testing in sport, whether human or animal, urine is invariably supplied. The extrelut extraction procedure developed above was examined for compatability with these other sample types.

3.6.2. WHOLE BLOOD

3.6.2.1. Introduction

Blood samples are generally in various states of putrefaction or decomposition when presented for analysis. For the purposes of this study only fresh blood samples were examined. Based on previous experience (2.9.3.), extraction efficiencies for older samples might be expected to be lower, and the extracts may contain other interfering material. As the object of this study was to extend the applications of the extrelut method rather than assess its limits, aged blood was not investigated initially. Before this method is applied to post mortem blood however, these effects should be investigated.

3.6.2.2. Method

Expired transfusion blood was spiked with a known concentration of bupivacaine (10^{-3} g/L unless otherwise stated). Blood (1ml) was mixed with 0.1M NaOH in a 2ml disposable syringe. Cinchocaine ,1ug (100ul of 10^{-2}g/L solution) was added as an internal standard. This was thoroughly mixed and applied to the top of an extrelut column prepared as described earlier (2.8.4.2.). This was allowed to equilibrate for 3-5 minutes, and then the eluted with dichloromethane. 4ml were collected. The solvent was evaporated to dryness under oxygen free nitrogen (OFN) and reconstituted in 100ul of mobile phase. 20ul were injected into the HPLC. HPLC conditions were as described in 3.3.2.1..

3.6.2.3. Results and Discussion

The chromatograms produced from these extracts were of an acceptable quality. An example is shown in figure 3.20.. The use of blood on the extrelut extraction columns increased the time required for absorption of the sample. In some cases the sample would not absorb and the extraction had to be repeated. It was found that the sample would absorb more readily if the extrelut column was loosely packed.

The use of blood also made the rate of elution from the column slower. 4ml were collected in approximately 25 minutes, getting slower towards the end. The speed of elution also depended to a large extent on the density of packing of the columns. Reports in the literature suggest that elution from the sample can be made easier and the efficiencies improved if the blood is diluted prior to application to the column (Breiter et al., 1978; Christiansen, 1984). The chromatographic run time was 16 minutes although some peaks eluted after the internal standard. The extraction was performed four times and the results are given in table 3.26. and discussed below. The absolute recovery of bupivacaine was found to be 63.8% ± 7.3. This is lower than from plasma, where the absolute recovery was 84.5%. The CoV is 11.4%, which is lower than for plasma (14.6%). The recovery of the internal standard cinchocaine was 42.0% ± 4.5 (CoV=10.8%), and the ratio of internal standard to drug is 0.66 ± 0.04 (CoV=5.5%).

The fact that the relative recovery is similar to that noted for plasma (0.63) would suggest that the drop in absolute recovery may be due to matrix effects rather than binding or decomposition of the drug in the sample.

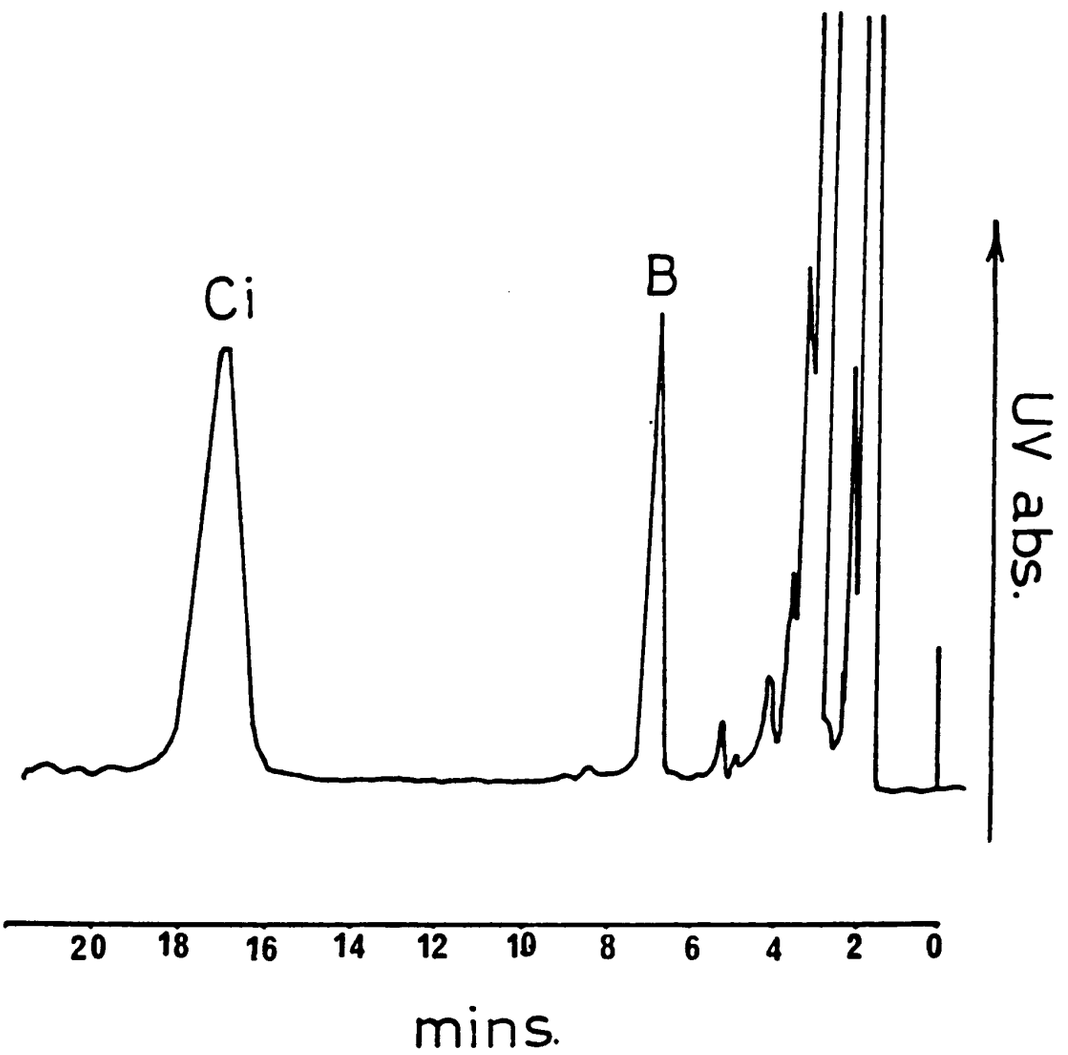


FIGURE 3.20.

CHROMATOGRAM OF EXTRELUT® BLOOD EXTRACT CONTAINING BUPIVACAINE AND CINCHOCAINE

TABLE 3.26.

RECOVERY OF BUPIVACAINE AND CINCHOCAINE FROM BLOOD USING EXTRELUT EXTRACTION METHOD

Bupivacaine Eff.(%)	Cinchocaine Eff.(%)	C/B
54	38	0.66
67	43	0.64
71	48	0.68
63	39	0.62
X 63.8	42.0	0.66
SD 7.3	4.5	0.04
CoV(%) 11.4	10.8	5.5

No interference was found in the blank sample.

3.6.2.4. Conclusions

The extrelut extraction method has been shown to be suitable for extracting bupivacaine and cinchocaine from an alkaline blood sample. Further examination of the extraction procedure with respect to sample age, speed of elution, density of packing and absorption time might give greater control over the recoveries, and improve on the variation observed in extraction efficiency.

3.6.3. URINE

3.6.3.1. Introduction

Urine samples are occasionally obtained at post mortem in addition to other samples. In drug testing in sport (human or animal) the sample supplied is invariably urine. Two methods for screening urine samples for basic drugs using TLC have been examined above (sections 3.4.3. and 3.4.4.). Both were shown to be suitable for the extraction of a range of local anaesthetics, including bupivacaine, from urine. The extracts were however found to be unsuitable for direct analysis by HPLC (3.4.7.).

3.6.4. THE USE OF EXTRELUT EXTRACTION PROCEDURE AS A CLEAN UP STEP FOR EXTRACTS OBTAINED FROM THE ROUTINE DRUG SCREEN

3.6.4.1. Introduction

The extracts from both screening procedures were shown to be unsuitable for direct analysis by HPLC (3.4.7.). The use of extrelut to remove the interfering material was investigated.

3.6.4.2. Method

A urine sample spiked with bupivacaine (10^{-3}g/L), was extracted by the procedures described by Hill et al. (1982) and Bogan and Smith (1968). The extracts were then reconstituted in 1ml of water. This was treated in the same way as plasma samples in the above section. It was mixed with 0.1M NaOH (1ml) and applied to the top of a 6ml extrelut column prepared as described earlier (2.8.4.2.), then allowed to absorb for 2-3 minutes. No internal standard was used. The column was eluted through with dichloromethane and 4ml were collected. The eluate was evaporated to dryness at 40°C under OFN and the residue reconstituted in 100ul of the mobile phase. 20ul were injected into the HPLC. HPLC conditions were as described in System One, section 3.3.9.3..

3.6.4.3. Results and discussion

The chromatogram produced by this procedure was less clean than that resulting from the extraction of blood or plasma samples. This may be because the initial extraction procedure has the effect of concentrating the interfering material in the

extract as well as the analyte, from 10 or 20 ml of urine into 1ml of material to be analysed. Consequently both the drug peaks and the interference peaks were larger. This was avoided to some extent by diluting the residue by a greater factor, adjusting the absorbance setting on the UV detector or by using only a small fraction of the initial extract for extrelut clean-up. The extracts still contained a number of other peaks, although no interference was found in the region of the drug peak in the blank samples examined.

3.6.4.4. Conclusions

The HPLC system described for bupivacaine has been shown to be suitable for the analysis of extracts from blood and plasma prepared by the extrelut procedure. It has also been shown that the presence of bupivacaine in an extract from a general drug screen can be confirmed by this procedure. The extract was found to be dirty and to take a long time for all the co-extractants to elute (run time up to 40 minutes). In addition, as the extraction efficiency of the screening procedure was not measured, the overall extraction efficiency for the procedure was not known. For this reason it was decided to determine whether the extrelut procedure used above for plasma and whole blood could be applied to a small volume of urine (1ml) to give a clean extract suitable for HPLC.

3.6.5. EXTRELUT EXTRACTION PROCEDURE APPLIED TO A SMALL VOLUME URINE SAMPLE.

3.6.5.1. Introduction

A urine sample spiked with a known concentration of bupivacaine (10^{-3}g/L) was analysed in tandem with a blank sample, to determine if the extrelut procedure would provide a suitable extract for bupivacaine in urine samples.

3.6.5.2. Method

Greyhound urine was obtained and spiked with a known concentration of bupivacaine (10^{-3} g/L). This sample and a blank sample were treated as follows. Urine (1ml) and 0.1M NaOH (1ml) were added to a 2ml disposable syringe. Cinchocaine (1ug) was added as an internal standard. This was mixed thoroughly and applied to the top of a 6ml extrelut column prepared as described earlier (2.8.4.2.). The sample was allowed to absorb for 2-3 minutes and then eluted through with dichloromethane (4ml). The eluate was evaporated to dryness at 40^{o}C under OFN and the residue reconstituted in 100ul of the mobile phase. An aliquot of 20ul were injected into the HPLC. HPLC conditions were as described in 3.3.2.1..

3.6.5.3. Results and discussion

The chromatograms produced from these extracts were of an acceptable quality. An example is shown in figure 3.21.. The urine samples adsorbed readily and the solvent eluted through rapidly, 4ml being collected in 2-3 minutes. The chromatographic

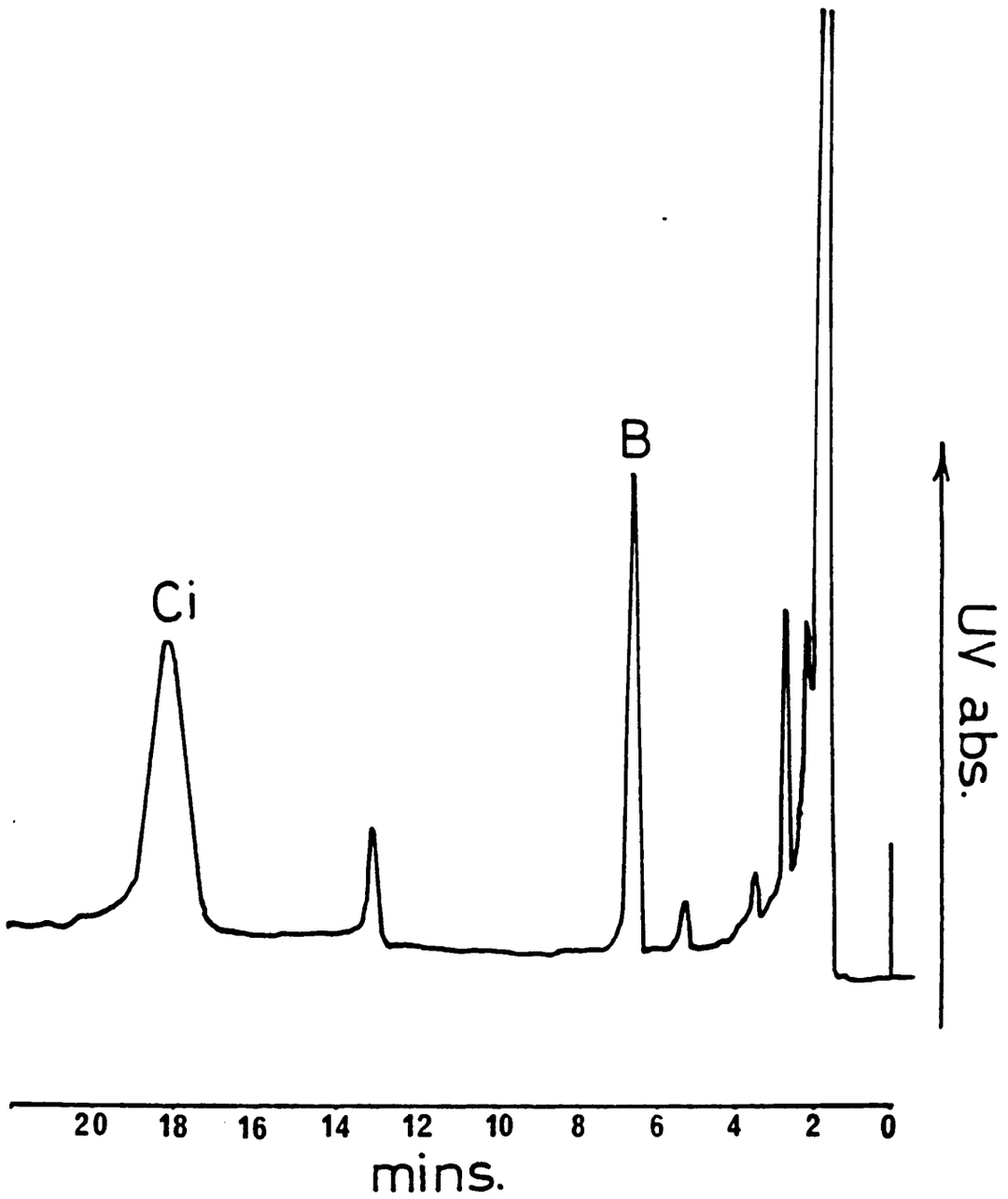


FIGURE 3.21.

CHROMATOGRAM OF EXTRELUT® URINE EXTRACT CONTAINING BUPIVACAINE AND CINCHOCAINE

TABLE 3.27.

RECOVERY OF BUPIVACAINE AND CINCHOCAINE FROM URINE USING EXTRELUT EXTRACTION METHOD

	Bupivacaine Eff.	Cinchocaine Eff.	C/B
	80	52	0.65
	95	56	0.59
	102	59	0.57
	75	48	0.64
X	88.0	53.8	0.61
SD	12.6	4.8	0.04
CoV(%)	14.3	8.9	6.3

run time was up to 20 minutes, although one small peak eluted after the internal standard. The extraction was performed four times and the results are given in table 3.27. and discussed below. The absolute recovery of bupivacaine was found to be 88.0% ± 12.6. This is comparable to the efficiency for plasma and is better than for whole blood. The CoV is 14.3%, which is rather high.

The recovery of the internal standard was 53.8% ± 4.8, and the ratio of internal standard to drug is 0.61 ± 0.04 (CoV=6.3%). This illustrates the improvement in precision achieved by the use of an internal standard in this assay.

3.6.5.4. Conclusions

The extrelut extraction method provides a suitable procedure for the analysis of bupivacaine in 1ml urine samples. This could be used to confirm the identity of bupivacaine in a small urine sample, if its presence had been suspected in the extract from the drug screen.

3.6.6. APPLICATION OF THE METHOD TO DOPING CONTROL IN THE RACING GREYHOUND

3.6.6.1. Introduction

Having established that levels of bupivacaine in biological samples as low as 2ng on column can be detected (3.3.10.), it was decided to apply this method to authentic samples obtained from a dog to which bupivacaine had been administered.

The metabolism and elimination of bupivacaine in man has been examined and has been studied. It has been shown that up to 6% of the drug is excreted unchanged (Reynolds, 1971) and various metabolites have also been identified. The excretion of the drug in the racing greyhound has not been examined.

3.6.6.2. Method

A 40Kg male greyhound retired from racing was given a deep intramuscular injection of bupivacaine HCl (Marcain), 250mg in the hind quarters. Over the following 24 hours it was placed in a mesh bottomed metabolic cage and the urine was collected. Thereafter urine was collected for the next 24 hours when possible. The samples were pooled in two 12 hour fractions. The dogs response to the injection was also noted. The urine samples were analysed by the method described by Bogan and Smith, and 1ml was extracted separately by the extrelut procedure described in 3.5.2.2. for confirmatory HPLC analysis.

3.6.6.3. Results and discussion

The dog accepted the injection without any complaint, either from the injection or the loss of feeling induced by the drug. The muscle tissue in the hind quarters is quite dense and not heavily enervated. The dogs behaviour as reported by the kennel staff was not noticably different, and no impairment in walking or whilst exercising was noted. The best method of assessing the effect of a drug on the dogs performance would be to race it over a timed distance. This was not possible within the constraints of the animal licence, and was against the policy of

the institute where the dogs were kenneled. The dogs were unwilling to urinate in the metabolic cages and generally only did so when allowed out for exercise. For this reason total urine collection was not always possible. This in turn meant that the absolute excretion rates could not be measured, and any quantitative assessment was impossible.

The results of the analysis were as follows. The TLC screen indicated the presence of bupivacaine in the 0 to 12 hour sample, after its administration to the animal.

The TLC plate for the 12-24 hour sample indicated the presence of some material, but this did not correspond to the Rf value for bupivacaine and probably represents the presence of some metabolites. These were not identified. The presence of these spots in the TLC screen would however have been sufficient to require further investigation, resulting in the dog being withdrawn from racing.

Bupivacaine was identified by HPLC in the 0-12 hour sample at a concentration of 1.4ug/ml, and in the 12-24 hour sample at a concentration of 0.3ug/ml. The presence of the drug was confirmed by GCMS. The concentrations found were significant, but the total excretion cannot be assessed without knowing the total urinary volume. Relating the concentrations to creatinine levels was not considered for the reasons discussed earlier (3.1.6.3.).

3.6.6.4. Conclusions

The result of greatest importance in the above experiment is the detection of bupivacaine in the 12 and 24 hour samples from

the greyhound. Bupivacaine was identified by HPLC and further analysis by GCMS confirmed the presence of the drug. The therapeutic duration of the drug for muscular anaesthesia is around 12 hours, so within the period in which it was effective, the drug would be detected. This is of special importance as the administration of a therapeutic dose to the dog made no noticeable difference to its gait and the dog may have passed a veterinary inspection. The effect of the local anaesthetic may only become apparent at racing speed, and as there are only tenths of a second difference between placings, this would be significant. In addition to the effects on the dogs speed, any loss of sensation in one of the limbs might lead to imbalance and to the dog injuring itself by falling or stumbling.

Further work might be done to identify the other metabolites of the drug. More rigourous control of the dogs, eg immobilisation and bladder catheterisation, would be required for a full investigation into metabolism and excretion patterns, and an assesment of how soon after administration detectable drug was identified in the urine.

Creatinine excretion, and its application to the normalisation of drug levels in the racing greyhound is currently under investigation in this department.

3.6.7. PLASMA BUPIVACAINE LEVELS IN PATIENTS RECEIVING BUPIVACAINE DURING SURGERY

3.6.7.1. Introduction

Bupivacaine is popular as a local anaesthetic in general surgery because because of its persistance (being an amide). This section illustrates the use of the method established above for the detection of bupivacaine in plasma samples obtained from hospital patients receiving the drug for post operative pain.

The route of administration is important as it may affect the rate of absorption of the drug. Fast absorption from the site of administration, coupled with the persistance of the drug may lead to high plasma levels, perhaps in the toxic range (3.1.4.).

The aim of the following study was to assess the degree of absorption of the drug into the systemic circulation following one particular route of administration.

3.6.7.2. Method

A double blind study was conducted involving a number of patients in a west coast Scottish hospital who were receiving general peritoneal surgery. The site of the surgery was washed with bupivacaine solution (20ml 0.5%, Marcain plain) before the peritoneum was closed.

A control group had the wound washed with saline.

Blood samples were taken from the patients at intervals of 10, 30 and 60 minutes following administration.

These were centrifuged immediately to obtain plasma, which was stored frozen (-20°) until the analysis was performed.

TABLE 3.28.

BUPIVACAINE LEVELS IN HOSPITAL PATIENTS

patient no.	blood concentration (ug/ml) 10mins	30mins	60mins
1	1.3	2.0	2.8
2	7.7	8.7	9.6
3	5.6	5.2	4.8
4	2.2	2.5	4.2
5	1.5	2.6	2.9

The extraction procedure used was that described in section 3.5.2., and the HPLC conditions were as described in System One, section 3.3.9.

Plasma concentrations were calculated by comparison of peak heights with an extracted standard.

3.6.7.3. Results and discussion

The extracts provided good chromatograms (see figure 3.19.) and the calculated levels are shown in table 3.28..

All patients who received bupivacaine were given the same dose. The plasma concentrations were at the top end of the therapeutic levels quoted in Moffatt (1986), 1-2ug/ml, with toxic reactions including muscular rigidity and convulsions being noted at 4ug/ml. Additionally, in four of the five cases reported, the plasma concentrations had not peaked by the time the 60 minute sample was taken. As no later samples were taken, the peak concentration is not known, but the results indicate that a significant amount of the drug is being absorbed into the systemic circulation.

A full assessment of the clinical findings is not yet available, but initial results (Blythe et al., 1985) have shown that there was no significant correlation between the administration of bupivacaine or placebo, and the amount of analgesic required by the patient to relieve post-operative pain.

The findings above suggest that the bupivacaine is being dispersed from the site of pain, and is therefore not acting locally.

3.6.7.4. Conclusions

The use of bupivacaine douche to relieve post operative pain has been shown to be ineffective in patients receiving peritoneal surgery.

The measurement of plasma levels of the drug are important as they indicate that a significant amount of the drug is absorbed into the general circulation from the site of the surgery, at levels in the toxic range in some cases.

The degree of absorption of the drug is however not predictible.

If future studies of this nature were performed, blood samples should be taken for a longer time period to establish the peak concentration following this route of administration.

3.7. THE ISOLATION OF OTHER LOCAL ANAESTHETIC COMPOUNDS FROM BIOLOGICAL FLUIDS.

3.7.1. Introduction

The success of the extrelut extraction procedures for rapid extraction of bupivacaine from small volumes of biological fluids prompted the investigation of this procedure for some other local anaesthetics for which HPLC methods had been developed earlier. The compounds considered were procaine because of its popularity as a doping agent in greyhound racing (Sundlof et al., 1982; Hartford Courant, 1985), and cocaine because of its increased availability and its potential for use as a doping agent (Lewis, 1982).

3.7.2. PROCAINE

3.7.2.1. Introduction

Procaine is a short acting local anaesthetic which is rapidly broken down in most mammals by serum and liver cholinesterases (Goodman and Gilman, 1980). The greyhound however possesses very little serum cholinesterase and therefore most of the drug is excreted unchanged in the urine. This of course aids in detection, as urinary concentrations are consequently quite high. Procaine is occasionally used on its own as a short acting local anaesthetic but more often as procaine penicillin G.

This is a popular veterinary and medical preparation used as a topical anti-infective. The procaine salt is lipophilic and hence less water soluble. This localises the counterion, penicillin G, in the area surrounding a wound where infection is likely to set in.

Anaesthesia resulting from the procaine may reduce the running performance of the dog, or make it more susceptible to accident and injury as discussed above for bupivacaine. Procaine has also been reported as having CNS stimulating activity in some mammals (Dunlop, 1935; Sundlof et al., 1983).

The effectiveness of the screening procedures for procaine and the use of extrelut extraction prior to HPLC was examined.

3.7.2.2. Method

As the HPLC analysis was performed on a different solvent system, an alternative internal standard with a suitable

capacity. As shown earlier (3.3.9.) lignocaine and mepivacaine chromatographed well on the same system as procaine (10% acetonitrile in 0.05M KH_2PO_4, adjusted to pH3). Both mepivacaine and lignocaine have similar $^{230}A_1^1$ to procaine (see table 3.7.). Mepivacaine however has a higher capacity factor (see table 3.17.) and was selected as an internal standard. Blood, plasma and urine samples were spiked with a known concentration of procaine (10^{-3} g/L, unless otherwise stated). These samples were extracted as for bupivacaine.

Sample (1ml) and 0.1M NaOH (1ml) were added to a 2ml disposable syringe with 1ug of mepivacaine. This was mixed thoroughly by inversion and applied to the top of a 6ml extrelut column prepared as described earlier (2.8.4.2.). The sample was allowed to absorb for 2-3 minutes before being eluted with dichloromethane. 4ml of eluate were collected and evaporated to dryness at 40°C under OFN.

The residue was reconstituted in 200ul of mobile phase and 20ul injected into the HPLC. HPLC conditions were as described in system three, section 3.3.9.3..

3.7.2.3. Results and discussion

The chromatograms produced from the blood and plasma samples were similar and of good quality. An example, that for the plasma sample, is shown in figure 3.22. Determination of the extraction efficiencies was possible. Similar problems as before were encountered with the extraction procedure for whole blood, namely the increased elution time. For plasma and whole blood the chromatographic run time was up to twenty minutes and no peaks

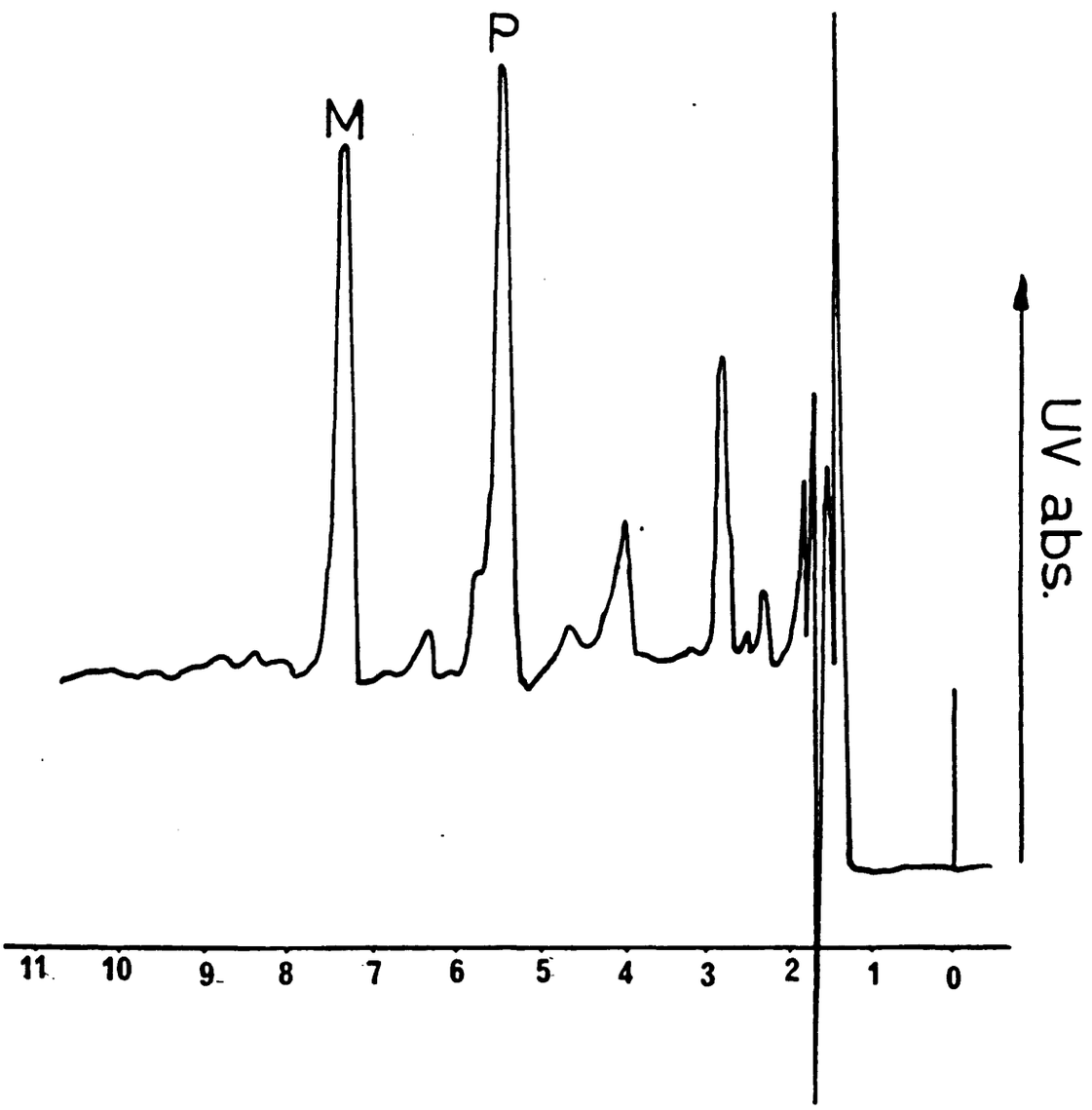


FIGURE 3.22.

CHROMATOGRAM OF EXTRELUT® PLASMA EXTRACT CONTAINING PROCAINE AND MEPIVACAINE

eluted after the internal standard. More peaks were present than had been observed in the analysis of bupivacaine extracts.

The extracts from the urine sample contained a peak corresponding to procaine (see figure 3.23 (a)). The blank samples extracted in tandem however contained a number of large peaks eluting in the same region as procaine.

This made any unequivocal identification of procaine impossible. These additional peaks were thought to appear as a result of the lower concentration of acetonitrile in the mobile phase. With the previous mobile phase described for bupivacaine, these peaks would have eluted in the same region as procaine (ie K' lt. 1) and did not interfere with bupivacaine.

The extraction was performed four times and the results for plasma, urine and whole blood are given in table 3.29. and discussed below.

In plasma, the absolute recovery of procaine was found to be almost complete, 95.5% ± 4.4. The CoV was 4.6%, which is good. The recovery of the internal standard (mepivacaine) was 79.3% ± 7.3 , and the ratio of internal standard to drug was 0.83 ± 0.07 (CoV=8.1%). This suggests that the use of an internal standard in this assay would actually reduce its precision, as the drug of interest was almost completely recovered whilst the internal standard was not.

For whole blood, the absolute recovery of procaine was lower, 76.3% ± 7.6. The CoV was 10.0%. The recovery of the internal standard (mepivacaine) was 64.5% ± 10.7, and the ratio of internal standard to drug was 0.84 ± 0.06 (CoV=7.4%).

As with the analysis of bupivacaine, the whole blood was less easy

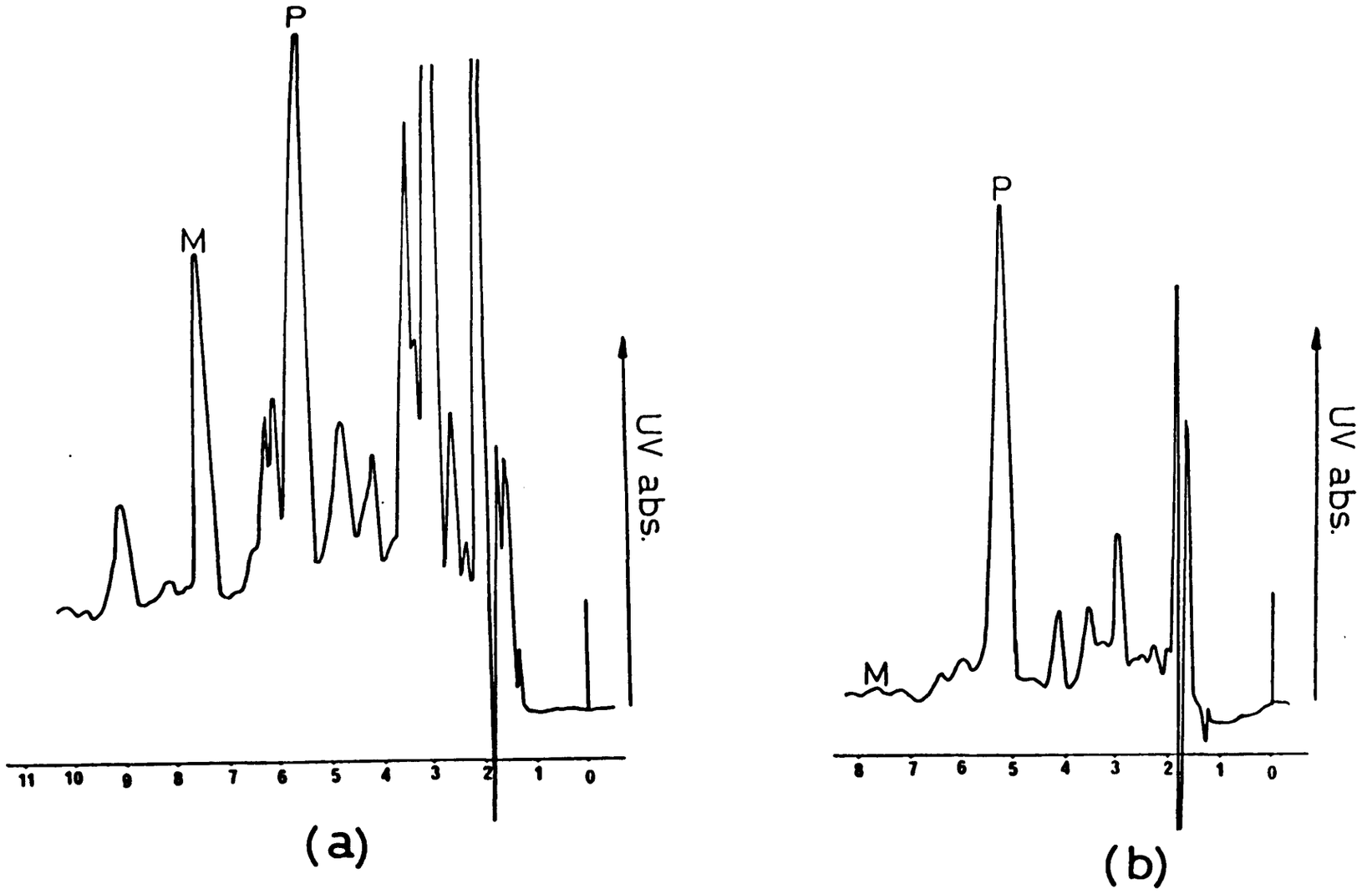


FIGURE 3.23.

CHROMATOGRAM OF EXTRELUT® URINE EXTRACT CONTAINING PROCAINE AND MEPIVACAINE. MONITORED AT (a) 230nm AND (b) 280nm

TABLE 3.29.

RECOVERY OF MEPIVACAINE AND PROCAINE FROM PLASMA AND BLOOD WITH DICHLOROMETHANE

plasma

	recovery of procaine	recovery of mepivacaine	mepivacaine / procaine
	92	84	0.91
	94	76	0.81
	102	87	0.85
	94	71	0.75
X	95.5	79.3	0.83
SD	4.4	7.3	0.07
CoV(%)	4.6	9.2	8.1

blood

	recovery of procaine	recovery of mepivacaine	mepivacaine / procaine
	83	71	0.86
	75	63	0.84
	81	74	0.91
	66	50	0.76
X	76.3	64.5	0.84
SD	7.6	10.7	0.06
CoV(%)	10.0	16.6	7.4

urine

	recovery of procaine	recovery of mepivacaine	mepivacaine / procaine
	98	-	-
	103	-	-
	93	-	-
	93	-	-
X	96.8	-	-
SD	4.8	-	-
CoV(%)	4.9	-	-

to extract on the extrelut columns.

This suggests that for whole blood, the use of an internal standard improves the precision and should be used.

Examination of the UV spectrum of procaine showed a significant absorbance maximum at 280nm (table 3.7.). The urinary extracts were re-analysed under the same conditions but monitoring the eluent at 280nm (figure 3.23 (b)).

Whilst this added selectivity to the method in that the procaine was identified in the spiked samples, and no interference was noted in the blank, it was also noted that the internal standard did not absorb at this wavelength. The absolute recovery of procaine from the urine samples was 96.8% + 4.8 (CoV=4.9%) (table 3.29.), this is comparable to the recovery from plasma. It was concluded therefore that the measurement of procaine in urine could be performed without the use of an internal standard, and still have an acceptable level of precision and accuracy.

3.7.2.4. Conclusions

The extrelut extraction procedure has been shown to extract procaine almost completely from plasma and urine samples. The recovery from whole blood was lower, probably due to matrix effects.

Interference in the urine extract prevented the use of the internal standard but it was decided that this was not essential for the accurate measurement of procaine in these samples, and for the further analysis of plasma and urine samples, the extraction efficiency was assumed to be 97% (± 4%), and no internal standard was used.

It is recommended that the internal standard be retained for the analysis of whole blood.

3.7.3. STABILITY OF PROCAINE IN STORED URINE SAMPLES

3.7.3.1. Introduction

It had been suggested that procaine, being an ester, might undergo auto-hydrolysis in urine samples if stored for any length of time. The rate of this hydrolysis may increase if the storage media was alkaline. This auto-hydrolysis has been widely reported for cocaine (Fletcher and Hancock, 1981; Baselt, 1983; Garrett and Seyda, 1983). The aim of this experiment was to determine the rate of hydrolysis of procaine in a stored urine sample.

3.7.3.2. Method

A survey of some 23 urine samples recieved in the department was conducted to determine the mean pH of a greyhound urine sample, this was found to be 9.1. A urine sample at this mean pH was spiked with procaine (1ug/ml). This was stored at room temperature and analysed at intervals for two weeks. The samples were analysed by HPLC as described above without the use of an internal standard and monitored at 280nm (3.7.2.2.). The procaine concentration was calculated against a freshly prepared extracted standard on each occasion.

3.7.3.3. Results and discussion

Alkaline conditions are known to increase the rate of hydrolysis of cocaine in stored samples (Fletcher and Hancock,1981; Baselt, 1983; Garrett and Seyda, 1983).

The calculated concentrations for the spiked sample are shown in table 3.30. This shows that the concentration of procaine falls significantly over the first three days to 48% of its original concentration, and that after 7 days less than 20% remained. This drop in the level of the drug in the sample may be due to a combination of hydrolysis of the drug due to the pH and to the action of bacteria in the sample.

This however implies that after being stored for one week the amount of procaine in the sample could have fallen by greater than 80%. This level may be below the detection limit, and so a confirmatory analysis might be negative. Against this, it was noted that the levels found in urine samples were rather high due to the low metabolism of the compound, and that even a 90% reduction in the level found in an authentic sample (see 3.7.4.) would still leave a level of lug/ml or greater.

3.7.3.4. Conclusions

Samples being analysed at the racetrack are taken less than 30 minutes before extraction, therefore no significant hydrolysis will take place. Samples sent to this department for analysis are generally analysed within a week of being taken. Although the concentration might drop in this period as shown above, that drop should not be sufficient to prevent the detection of the drug in most cases. This might be prevented by the

TABLE 3.30.

STABILITY OF PROCAINE IN A STORED URINE SAMPLE

Day	pH	concentration (ug/ml)
0	7.9	0.94
1	7.6	0.80
2	7.4	0.62
3	-	0.48
5	8.1	0.41
7	8.5	0.19
9	9.0	0.17

inclusion of a preservative (eg sodium fluoride) or a buffer in the sample collection bottles. It would also be useful if the original positive extract obtained at the racetrack was available for confirmatory analysis.

3.7.4. APPLICATION OF THE METHOD TO DOPING CONTROL IN THE RACING GREYHOUND

3.7.4.1. Introduction

Procaine is one of the drugs most often detected in greyhound screening overseas (3.1.3.2.). Information on the incidence in this country is not available. However, in most incidences where this compound is found it is argued either that procaine levels arose as a result of therapeutic treatment of the animal with procaine penicillin G for a bite or scratch, or that procaine came from contaminated food (NGRC, 1986). Greyhounds are often fed on meat rejected as unfit for human consumption. This may be because the animal was diseased prior to death, or because it was treated with drugs before its death. These drugs may include procaine penicillin G. A study in the United States showed that because procaine is only slightly metabolised by the dog, even small amounts of the drug ingested in the food may appear in detectable levels in the urine (Dunlop, 1935; Sundlof et al., 1978). To ensure that the method developed above would detect procaine satisfactorily in urine from a dog which had been administered with the drug, the following experiment was carried out.

3.7.4.2. Method

A 38Kg greyhound bitch was administered with 840mg (2.8ml of 300mg/ml suspension) of procaine penicillin G. This corresponds to 337mg of procaine. This was given as a deep intra-muscular injection into the hind quarters of the animal. Over the following 24 hours it was placed in a mesh bottomed cage and the urine was collected. Thereafter urine was collected for a further 24 hours when possible. The samples were collected in twelve-hourly fractions. The dogs response to the injection was also noted. The urine samples were analysed by the screening method described by Bogan and Smith, (1968), and by the screening method described by Hill et al, (1982). 1ml was extracted separately by the extrelut procedure for confirmatory analysis by HPLC.

3.7.4.3. Results and discussion

The results were as follows All samples up to 24-36 hours were positive for the presence of procaine by TLC following both screening procedures.

This was confirmed by HPLC in all cases.

Levels of 24, 11, 2 and 0.2ug/ml were found in the 0-12, 12-24, 24-36 and 36-48 hour samples respectively.

The concentrations are of interest from the point of view of their high levels. No quantitative assessment of the fraction excreted unchanged was possible as a total urine collection was not made, and the volume was not known. The samples were analysed within one day of being collected, so the extent of any hydrolysis may be between 0 and 20%, depending on whether the decomposition is due

to alkaline conditions or microbial action.

No noticeable deterioration in the greyhounds condition was noted by the kennel staff, and the dog may have passed a veterinary inspection. As noted with bupivacaine, this indicates the importance of detecting these drugs in the pre-race chromatography testing, as any incapacity caused by the drug might only become visible at racing speed. It is considered that the injection of this drug would have to be repeated every twelve to fifteen hours to maintain any therapeutic effect.

3.7.4.5. Conclusions

It has been demonstrated that the adminstration of procaine to a greyhound would be detected by the TLC screen even in the 36 hour period following any therapeutic effect.

It was also shown that the HPLC system is capable of confirming the presence of the drug in the urine. The findings in section 3.7.3. however indicate that the sample should be analysed as soon as possible after collection, otherwise the drug level in the sample may fall.

3.7.5. COCAINE

3.7.5.1. Introduction

Cocaine is a CNS stimulant which also has local anaesthetic properties. It is seldom used medically and is not included in MIMS. It is taken as a euphoriant and is psychologically addictive. Overdose of this compound may cause death through cardiac or respiratory depression. Toxic, therapeutic and fatal levels in biological fluids have been discussed in section 3.1.4..

The incidence of cocaine use in the UK is believed to be increasing, and its use as a stimulant makes it a likely candidate for doping of greyhounds (Lewis, 1982), although to date no positive cases have been identified.

An HPLC method developed above has been shown to provide suitable chromatography for the measurement of cocaine and its major metabolite in man, benzoyl ecgonine (System Two, 3.3.9.3.). The applicability of the extrelut extraction procedure was therefore examined for these compounds.

3.7.5.2. Method

As shown earlier (3.3.9.) bupivacaine chromatographed well on the same system as cocaine and benzoyl ecgonine and so was selected as the internal standard. Its absorbance at 230nm was less than for cocaine and the reported absorbance for benzoyl ecgonine (3.2.2.). In addition, the peak eluted later than cocaine, so band broadening may lead to reduced sensitivity. It was found neccessary to use a 2ug internal standard.

Plasma samples were spiked with a known concentration of cocaine and benzoyl ecgonine (10^{-3} g/L, unless otherwise stated). These samples were extracted as for bupivacaine.

Plasma (1ml) and 0.1M NaOH (1ml) were added to a 2ml disposable syringe with 2ug of bupivacaine (0.1ml of $2x10^{-2}$g/L solution). This was mixed thoroughly by inversion and applied to the top of a 6ml extrelut column prepared as described earlier (2.8.4.2.). The sample was allowed to absorb for 2-3 minutes before being eluted with dichloromethane. 4ml of eluate were collected and evaporated to dryness at 40°C under OFN.

The residue was reconstituted in 200ul of mobile phase and 20ul injected into the HPLC. HPLC conditions were as described in System One, 3.3.9.

3.7.5.3. Results and discussion

The initial extracts obtained did not appear to contain measurable quantities of cocaine or benzoyl ecgonine. The elution volume was therefore increased from 4 to 8ml. The extracts obtained gave acceptable chromatography. The extraction efficiencies were calculated and are shown in table 3.31.. The recovery of bupivacaine was high 75.3% ± 5.8 (CoV=7.7%) and was similar to that found earlier (3.5.2.). The recovery for cocaine was 38% ± 10.8 (CoV=28.4%) and for benzoyl ecgonine, 20.3% ± 5.7 (CoV=28%).

These recoveries were poor and there was a large error in the absolute recovery of both compounds. The relative extraction efficiency of each compound to the others is shown (table 3.31).

TABLE 3.31.

RECOVERY OF BUPIVACAINE, COCAINE AND BENZOYL ECGONINE FROM PLASMA WITH DICHLOROMETHANE

	recovery of			bup.	bup.	coc.
	bup.	coc.	b.e.	coc.	b.e.	b.e.
	80	50	24	1.60	3.33	2.08
	80	44	26	1.82	3.07	1.69
	73	27	17	2.70	4.29	1.58
	68	31	14	2.19	4.86	2.21
X	75.3	38	20.3	2.08	3.88	1.90
SD	5.8	10.8	5.7	0.48	0.81	0.29
CoV	7.7	28.4	28	23	20.9	15.4

however the degree of correlation between any of these is poor, the CoV being large in each case.

With liquid/liquid extraction, the degree of partition is determined by an equilibrium based on the polarity of the two solvents and the solubility of the analyte in each.

With solid phase absorption/elution extraction the solvent is continually being replaced, and the extraction procedure is non-equilibrium. The degree of partition will be determined by the polarity of the two solvents, the solubility in each of the analyte and the volume of the eluting solvent used. In theory this should enable the elution of all analytes and the internal standard in some finite volume of an appropriate eluting solvent.

3.7.5.4. Conclusions

Increasing the volume of the eluate has been shown to be ineffective in producing an acceptable extraction procedure for these compounds.

For this reason it was decided to investigate the use of another solvent for extracting bupivacaine, cocaine and benzoyl ecgonine.

3.7.6. THE USE OF ANOTHER SOLVENT FOR THE EXTRACTION OF COCAINE, BENZOYL ECGONINE AND BUPIVACAINE FROM BIOLOGICAL FLUIDS.

3.7.6.1. Introduction

Extraction procedures for cocaine and benzoyl ecgonine from biological fluids have been reviewed earlier (3.1.7.2.). It was noted that mixed solvents or the use of salting out procedures

had been shown to be suitable but that both had their disadvantages.

Several reports of the isolation of cocaine and benzoyl ecgonine from biological fluids use the solvent system described by Wallace et al., 1975, (von Minden and D'Amato, 1977; Jatlow et al., 1978). It was decided to investigate the application of this solvent (20% ethanol in chloroform) to the extrelut procedure.

3.7.6.2. Method

Samples of blood, plasma and urine were spiked with equal concentrations (10^{-3}g/L) of cocaine and benzoyl ecgonine.

Sample (1ml) and 0.1M NaOH (1ml) were added to a 2ml disposable syringe. Bupivacaine (2ug) was added as an internal standard. This was mixed thoroughly by inversion and applied to the top of a 6ml extrelut column prepared as described earlier (2.8.4.2.). The sample was allowed to absorb for 2-3 minutes and then eluted through with ethanol/chloroform (20:80). The first 6ml of the eluate were collected. The eluate was evaporated to dryness at 40^{o}C under OFN and the residue reconstituted in 100ul of the mobile phase. 20ul were injected into the HPLC. HPLC conditions were as described in System Two, 3.3.9.3..

Appropriate blanks were extracted in tandem with the spiked samples.

3.7.6.3. Results and discussion

The chromatograms produced from these extracts were of an acceptable quality. An example of an extract from a plasma sample is shown in figure 3.24.. No problems were experienced

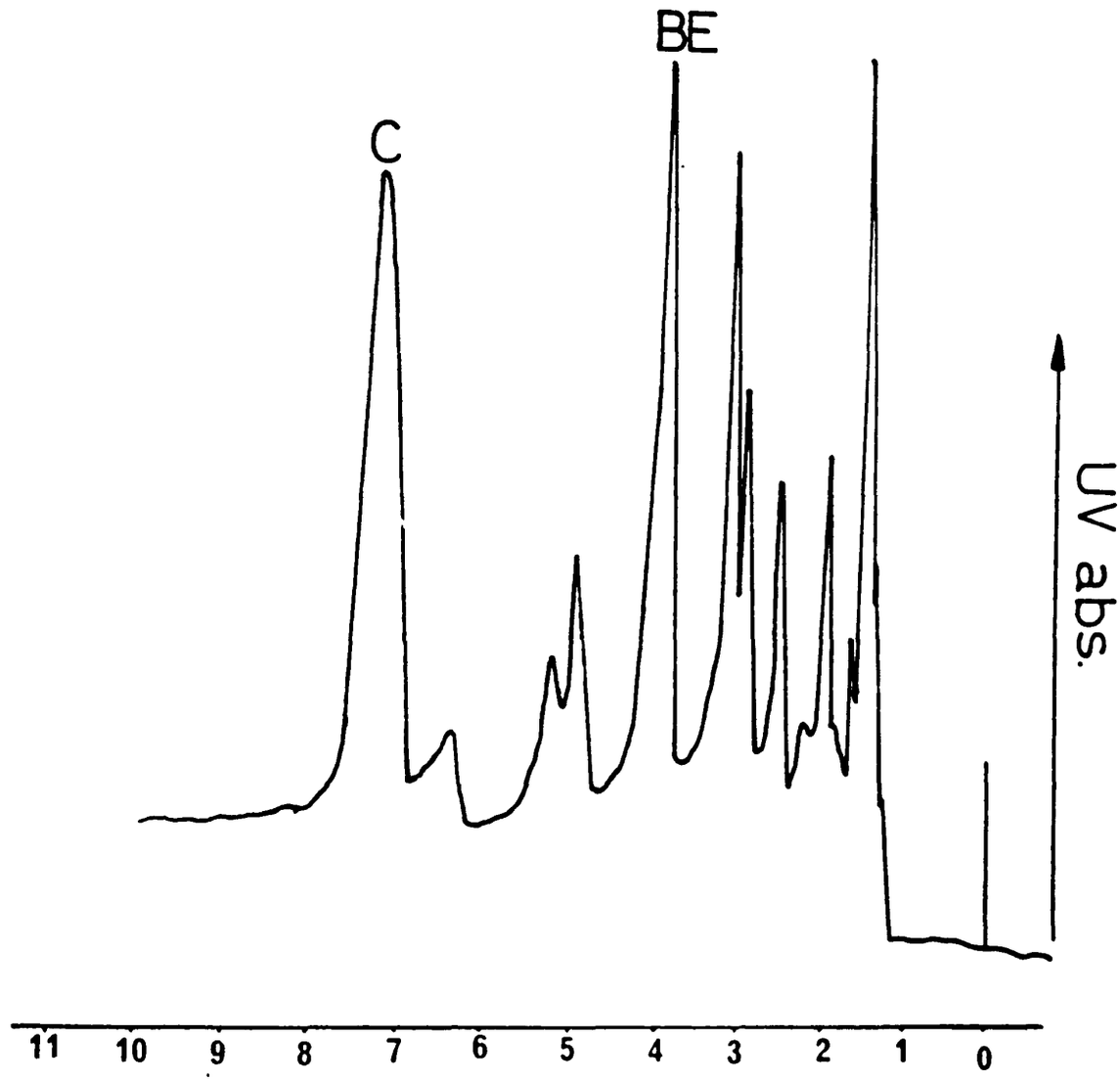


FIGURE 3.24.

CHROMATOGRAM OF EXTRELUT® PLASMA EXTRACT CONTAINING BUPIVACAINE, COCAINE AND BENZOYL ECGONINE

with the extraction of plasma or urine samples.

As experienced previously with blood samples, the sample took a considerable time to absorb onto the column. When the eluting solvent was added to the columns, the blood turned to a brownish colour suggesting that the blood proteins were being denatured probably by the presence of the alcohol in the extracting solvent and becoming coagulated. This change in the blood sample prevented the solvent from eluting, and so analysis of whole blood by this procedure proved impossible.

For plasma and urine samples the extraction was performed four times and the results are given in table 3.32. and 3.33., and discussed below.

The chromatographic run time was up to 25 minutes.

Plasma

For plasma samples the absolute recovery of bupivacaine was found to be 80.3% ± 8.8 (CoV=11.0%). This compares well with the efficiency achieved when dichloromethane was used as the extracting solvent (75.3% ± 5.8, CoV=7.7%).

The recovery of cocaine was 74.3% ± 5.9 (CoV=8.0%), and the ratio of internal standard to drug was 1.08 ± 0.06 (CoV=5.3%). Both the relative and absolute recoveries were improved with the use of this eluting solvent.

The recovery of benzoyl ecgonine was 55.8% ± 5.9 (CoV=10.5%), and the ratio of internal standard to drug was 1.44 ± 0.14 (CoV=10.0%). The coefficient of variation is quite high for both these compounds. As with cocaine, both the relative and absolute recoveries were improved with the use of this eluting solvent.

TABLE 3.32.

RECOVERY OF BUPIVACAINE, COCAINE AND BENZOYL ECGONINE FROM PLASMA WITH CHLOROFORM/ETHANOL

	recovery of			bup.	bup.	coc.
	bup.	coc.	b.e.	coc.	b.e.	b.e.
	80	72	49	1.11	1.63	1.47
	88	82	62	1.07	1.42	1.32
	68	68	53	1.00	1.28	1.28
	85	75	59	1.13	1.44	1.27
X	80.3	74.3	55.8	1.08	1.44	1.34
SD	8.8	5.9	5.9	0.06	0.14	0.09
CoV	11.0	8.0	10.5	5.3	10.0	6.9

These results indicate that a precise measurement of cocaine and benzoyl ecgonine in plasma and urine samples can be obtained using this method. The use of an internal standard did improve the precision of the procedure.

Urine

The recoveries from the urine samples were similar but slightly lower (table 3.33.). An example of the chromatogram is given in figure 3.25.. The absolute recovery of bupivacaine was found to be 78.3% ± 9.8.

The recovery of cocaine was 64.3% ± 10.2, and the ratio of internal standard to drug was 1.22 ± 0.04 (CoV=3.6%).

Whilst the recovery was slightly lower than obtained for plasma (64.3% cf. 74.3%) and the error on the absolute recovery was greater (CoV=15.9% cf. 8.0%), the error relative to the internal standard was similar (CoV=3.6% cf. 5.3%). This illustrates the improvement in precision obtained by the use of an internal standard in this assay.

The recovery of benzoyl ecgonine was 42.3% ± 6.9, and the ratio of internal standard to drug was 1.86 ± 0.10 (CoV=5.2%).

As with cocaine the recovery was slightly lower than for plasma (42.3% cf. 55.8%) and the error on the absolute recovery was greater (CoV=16.0% cf. 10.5%). The error relative to the internal standard was however improved (CoV=5.2% cf. 10.0%).

These differences between the recoveries from plasma and urine samples probably arise from the fact that the rate of elution from the extrelut columns was much greater with the urine samples than with plasma samples. The lower error noted in the measurements

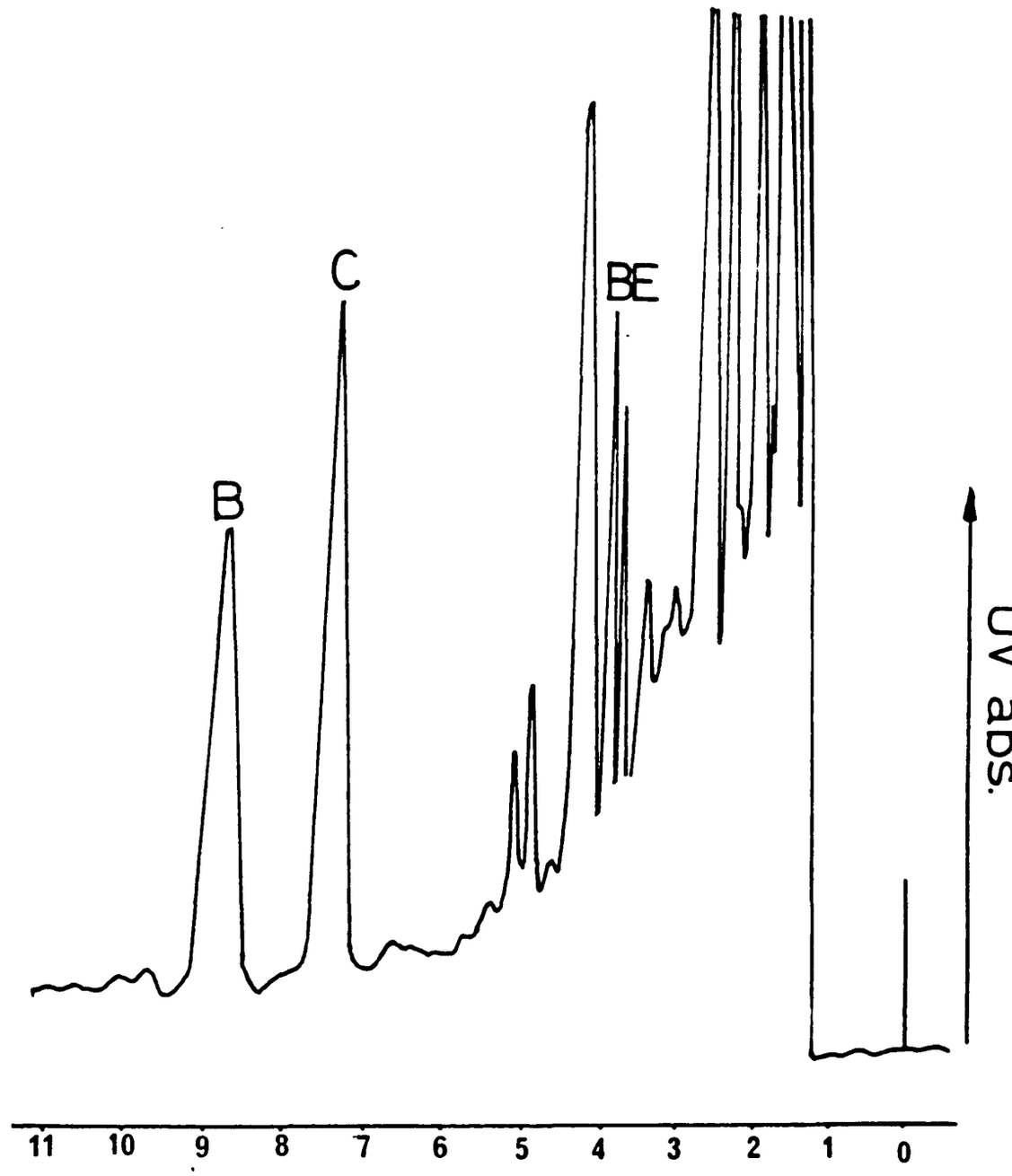


FIGURE 3.25.

CHROMATOGRAM OF EXTRELUT® URINE EXTRACT CONTAINING BUPIVACAINE, COCAINE AND BENZOYL ECGONINE

TABLE 3.33.

RECOVERY OF BUPIVACAINE, COCAINE AND BENZOYL ECGONINE FROM URINE WITH CHLOROFORM/ETHANOL

	recovery of			bup.	bup.	coc.
	bup.	coc.	b.e.	coc.	b.e.	b.e.
	84	72	44	1.17	1.91	1.63
	89	74	51	1.20	1.75	1.45
	71	57	39	1.24	1.82	1.46
	69	54	35	1.27	1.97	1.38
X	78.3	64.3	42.3	1.22	1.86	1.48
SD	9.78	10.2	6.9	0.04	0.10	0.11
CoV	12.5	15.9	16.0	3.6	5.2	7.2

on the urine samples and the fact that the internal standard to drug ratio is higher in the urine sample than in the plasma sample may suggest that there is differential binding of the cocaine to the plasma proteins in the plasma sample.

3.7.6.4. Conclusions

In summary, the use of bupivacaine as an internal standard was shown to improve the precision of the assay for cocaine and benzoyl ecgonine in plasma and urine.

It was decided that this method was sufficient to provide a precise estimation (within the limits shown above) of the concentration of cocaine and benzoyl ecgonine in plasma and urine samples. The method was however not suitable for whole blood samples.

The use of the appropriate solvent demonstrates how the extraction efficiency can be improved. It was also shown that the HPLC method developed above was suitable for the detection of small amounts of cocaine and benzoyl ecgonine in plasma and urine extracts.

3.7.7. APPLICATION OF THE METHOD TO DOPING CONTROL IN THE RACING GREYHOUND - COCAINE

3.7.7.1. Introduction

Cocaine as a popular stimulant drug is an obvious choice to attempt to enhance a greyhounds performance (Lewis, 1980). The effect that this has on the dog has not been noted, but is

likely to be in the form of CNS and cerebral stimulation, leading to excitability. A study has been made of the excretion pattern of orally administered cocaine in the greyhound, and this concluded that no unchanged cocaine was excreted (Lewis, 1980). The extraction procedures used in that report were performed at notably low pH, and it was thought that the administration of cocaine to the greyhound might merit further investigation.

3.7.7.2. Method

A 36Kg greyhound bitch was fasted overnight prior to the oral administration of 40mg of cocaine HCl in a gelatine capsule. This was chosen as it was the least toxic route, and absorption of the drug will be slower. Assuming the lack of plasma esterases to be significant, rapid absorption such as occurs following intravenous injection or absorption through the mucous membranes, might cause an accumulation of toxic levels of cocaine in the blood.

The animal was kept in a metabolic cage for 24 hours and urine was collected at 0,3,7,24 and 30 hours. The dog did not urinate in the cage and urine was collected by allowing the animal out and collecting urine by the "free-catch" method. This resulted in an incomplete urine collection. Blood samples were taken from the neck at 0,1,2,3,4,6, and 24 hours and collected in heparinised tubes.

All samples were stored under refrigeration and analysed within 8 hours of collection. Blood was centrifuged at 3000rpm to obtain plasma. The extraction procedure used on all samples was that described in section 3.7.6.. The samples were also extracted by

the routine screening procedure (Bogan and Smith, 1968) and that described by Hill et al, (1982) and analysed by TLC. Further confirmatory analysis was done by GCMS.

3.7.7.3. Results and discussion

The results of the analyses of the urine samples were as follows. Both screening procedures gave positive results by TLC for the first three urine samples (3, 7 and 24 hours). There were also other spots, one corresponding to benzoyl ecgonine with Rf = 0.12. The confirmatory HPLC procedure gave the following concentrations. The 3, 7 and 24 hour urine samples had concentrations of 0.9, 1.5, and 2.6 ug/ml respectively. Peaks corresponding to benzoyl ecgonine were noted in all four samples, but the presence of other peaks in the same region made quantitation difficult (see figure 3.25.). The levels calculated were 1.2, 1.5, 1.5 and 0.3 ug/ml respectively.

No cocaine was found in the 30 hour urine sample by TLC or HPLC. HPLC did show a small peak corresponding to benzoyl ecgonine. The presence of cocaine in the 3, 7 and 24 hour samples was confirmed by GCMS. GCMS also showed the presence of the cocaine metabolite, methyl ecgonine, in the samples. This has been reported as a major metabolite in dog urine elsewhere (Matsubara, 1984). No benzoyl ecgonine was available as a standard during the course of the study however, so its behaviour on the HPLC system is not known.

The plasma samples were extracted by the same method and showed some evidence for the presence of cocaine and benzoyl ecgonine at concentrations below 0.01ug/ml. No attempt was made to quantify this.

These results illustrate one of the advantages of urine analysis over other body fluids, namely its tendency to concentrate drugs and metabolites. The urinary concentrations cannot be realistically interpreted without knowing the total urinary volume. The finding of cocaine in the urine directly contradicts the report by Lewis, (1980). In that report 80mg of cocaine administered orally gave rise to detectable levels of benzoyl ecgonine up to eight hours post administration, but no cocaine was found in the extract.

This is attributed to the use of innappropriate conditions for the extraction of cocaine in that report. The author states that an extraction at pH 3-4 into chloroform should remove any cocaine in the sample. Wallace et al., (1975) concluded that cocaine would be extracted efficiently only at pH between 7 and 10. The basic extraction used by Lewis to isolate benzoyl ecgonine would be more likely to extract the cocaine. The subsequent derivatisation of the benzoyl ecgonine back to cocaine for purposes of GC analysis, would mask the presence of any cocaine originally in that extract.

The third sample which contains the highest concentration of the drug was darker in appearance and was probably a lot more concentrated than those samples collected during the day. This is due to the fact that the sample was collected in the morning following a period of 18 hours in the metabolic cage. The greyhound would not urinate in the cage and this sample represents the metabolic waste from that 18hour period. As the samples were refrigerated after collection ($4^{o}C$) and analysed within several hours of collection it is proposed that the degree of conversion of cocaine to benzoyl ecgonine will be small. This situation may

differ in the case of samples sent for confirmatory analysis wher the degree of hydrolysis may be significant. The ability of the TLC analysis to detect benzoyl ecgonine and the HPLC analysis to confirm its presence is therefore important.

3.7.7.4. Conclusions

The HPLC experiment described above, by avoiding derivatisation, simplifies the measurement of cocaine and its metabolite benzoyl ecgonine in biological extracts. Although no quantitative assesment of the percentage of the drug excreted unchanged has been made, it is evident that a significant proportion of the drug is excreted unchanged as cocaine, probably due to the dogs lack of plasma cholinesterase. This is sufficient to allow, in this case, the detection of the parent drug in the urine for up to 24 hours post administration. This is well after the time of any likely stimulating effect.

3.8. CONCLUSIONS ON THE ANALYSIS OF LOCAL ANAESTHETICS IN BIOLOGICAL FLUIDS BY HPLC

In this study local anaesthetics were considered to be suitable candidates for analysis by HPLC. Several HPLC methods were examined in turn, and it was shown ultimately that suitable chromatography could be achieved for a range of these compounds using a reversed phase of intermediate lipophilicity.

The procedure developed for the extraction of these drugs from biological fluids, namely solvent extraction on an absorbent phase, has been shown to be rapid and convenient and to give clean extracts. It is applicable to plasma, blood and urine, giving extracts suitable for analysis on the chromatographic systems described. The methods were applied to authentic samples obtained from clinical studies and from doping studies and were shown to be suitable for both applications.

The results from the clinical study indicated that bupivacaine was being rapidly and extensively absorbed from the site of application, with important consequences for further studies of this nature.

The presence of unchanged bupivacaine, procaine and cocaine in the urine of greyhounds administered with these drugs confirms that the administration of these drugs to the racing greyhound would not only be detected in the screening procedures currently in use, but showed that trace amounts (below the sensitivity of the TLC screen) could be confirmed at low levels in small samples.

Further development of these solid phase extraction procedures might improve recoveries and allow the extension of the method to cover a wider range of drugs.

APPENDIX A

PACKING PROCEDURE RECOMMENDED BY EPPEL, 1980

NOTE: MASK AND GLOVES SHOULD BE WORN.

1 A 25cm column requires 4.0g of ODS Hypersil packing material. Weigh out packing material into a 20ml glass vial. Add 15ml of 0.1% sodium acetate/methanol 20/80, stopper and shake thoroughly for 2 minutes.

2 Place in an ultrasonic bath for 5 minutes.

3 Place the column in 5M nitric acid, and place in the ultrasonic bath for 15 minutes. Remove and wash with distilled water, then with methanol.

4 Prime packing pump with 0.1% sodium acetate/methanol 50/50, Attach bottom end fittings to column and attach to reservoir and packing pump.

5 Bring up to pressure and check for leaks. Remove from pump and drain.

5 Remove packing material from ultrasonic bath and shake thoroughly. Add quickly to the reservoir and attach to the packing pump.

6 Bring up to pressure (4-5Kpsi) and allow 500ml of packing solvent to flow through column.

7 Remove from the packing pump and attach top fittings. attach to HPLC pump and pump through 150ml methanol/water 50/50 conditioning solvent. Follow this with 100ml water and 100ml methanol.

APPENDIX B

PACKING PROCEDURE RECOMMENDED BY SHANDON SOUTHERN PRODUCTS ltd, 1984

NOTE: MASK AND GLOVES SHOULD BE WORN.

1 A 25cm column requires 4.0g of ODS Hypersil packing material. Weigh out packing material into a 20ml glass vial. Add 15ml of acetone, stopper and shake thoroughly for 2 minutes.

2 Place in an ultrasonic bath for 5 minutes.

3 Place the column in 5M nitric acid, and place in the ultrasonic bath for 15 minutes. Remove and wash with distilled water, then with methanol.

4 Prime packing pump with acetone, attach bottom end fittings to column and attach to reservoir and packing pump. ENSURE PACKING PUMP IS FITTED WITH KEL-F SEALS AND WASHERS

5 Bring up to pressure and check for leaks. Remove column from pump and drain.

5 Remove packing material from ultrasonic bath and shake thoroughly. Add quickly to the reservoir and attach to the packing pump. The column should be inverted immediately and remain that way during packing.

6 Bring up to pressure (5-6Kpsi) and allow 500ml of packing solvent to flow through column. (This takes about half as long as with procedure A.)

7 Remove from the packing pump and attach top fittings. attach to HPLC pump and pump through 150ml methanol/water 50/50 conditioning solvent. Follow this with 100ml water and 100ml methanol.

APPENDIX C

DETAILS ABSTRACTED FROM PATIENTS CHARTS IN HOSPITAL MORPHINE STUDY (2.10.4.)

patient number	Infusion rate	sickness score	sedation score	blood morphine level
1	0	17	6.1	0.016
	0	17	6.4	0.005
	0	19	7.1	0.007
	0	19	5.5	0.008
	1	13	3.7	0.015
	1	15	3.5	0.011
	2	19	4.5	0.039
	2	15	3.3	0.039
	2	15	4.5	0.028
	2	17	4	0.031
	2	19	3	0.024
	2	19	3.7	0.033
	2	17	4	0.006
	2	15	3.7	0.029
	2	17	3.5	0.028
2	5	20	5	0.102
	5	*	4.1	0.047
	5	*	*	0.108
3	0	24	6.5	0.005
	2	20	6.5	0.023
	3	25	7.5	0.032
	3	22	5	0.023
	5	20	6	0.078
	5	20	4.5	0.031
	5	19	0.5	0.056
	5	20	2	0.050
4	3	*	4	0.011
	3	*	3	0.015
5	0	9	6.4	0
	0	8	5.8	0
	3	3	9.2	0.030
	5	7	4.2	0.081
	7.5	9	5	0.077
	7.5	5	4.5	0.079
	7.5	7	4.9	0.118
	7.5	6	5.5	0.120
	7.5	7	4.4	0.135

patient number	Infusion rate	sickness score	sedation score	blood morphine level
6	0	11	4.6	0.005
	3	8	4.5	0.056
	3	8	6.5	0.050
	4	10	5.8	0.064
	4	9	3	0.075
	4	13	2.2	0.062
	4	12	2	0.069
	4	12	2	0.054
	5	7	3.5	0.093
	5	11	5.2	0.093
7	0	2	10	0.004
	0	0	8.6	0
	3	2	10	0.029
	3	0	9	0.034
	3	4	8.5	0.037
	3	7	7.5	0.033
	3	7	7.6	0.035
	3	7	7.4	0.045
	4	*	*	0.057
	5	*	*	0.082
	5	*	*	0.058
	5	*	*	0.055
8	3	5	10	0.027
	3	6	9	0.039
	3	2	8.2	0.046
	3	4	8.5	0.025
	3	8	8.5	0.023
9	5	*	*	0:071
	5	*	*	0.100
	5	*	*	0.105
	8	*	*	0.143
	8	*	*	0.124
	10	*	*	0.112
	10	*	*	0.095
	10	*	*	0.145
	10	*	*	0.169
	10	*	*	0.248
	10	*	*	0.239
	10	*	*	0.211
	10	*	*	0.222
	15	*	*	0.322
	15	*	*	0.299
	15	*	*	0.280
	15	*	*	0.349

patient number	Infusion rate	sickness score	sedation score	blood morphine level
10	5	*	*	0.078
	5	*	*	0.119
	5	*	4.4	0.128
	5	10	5.2	0.132
	10	*	*	0.162
	10	*	*	0.138
	10	*	*	0.109
	10	*	*	0.154
	10	*	*	0.180
	10	*	*	0.230
	15	*	*	0.253
	15	*	*	0.312
	15	*	*	0.388
	15	*	*	0.358
11	1	10	1	0.032
	1	10	1	0.032
	1	14	2	0.042
	4	10	2	0.077

APPENDIX D

PREPARATION OF ACIDIFIED IODOPLATINATE SPRAY FOR TLC

Chloroplatinic acid (1gram) was dissolved in water (10ml). A solution of potassium iodide (10grams) in water (350ml) was then added. The final volume was made up to 500ml with a solution of hydrochloric acid:water, 3:2.

APPENDIX E

SUPPLIERS OF CHEMICALS AND EQUIPMENT USED IN THE STUDY

Solvents: ethyl acetate,, dichloromethane, chloroform, methanol and isopropyl alcohol, (all HPLC grade), and acetonitrile (HPLC grade S), Rathburn solvents ltd., Walkerburn, Scotland.
Water was singly distilled in the department.
Pentane, heptane and octane sulphonic acids, dioctyl sulphosuccinate and para-amino benzoic acid were supplied by Sigma chemicals, Poole, Dorset, UK.
All drug standards were supplied gratis to the Department of Forensic Medicine and Science courtesy of the relevant drug companies, and benzoyl ecgonine was supplied courtesy of Strathclyde police forensic science laboratory, Pitt Street, Glasgow.
Thin layer chromatography plates (5 and 10cm) with background fluorescence (Merck), Extrelut diatomaceous earth (Merck), Whatman PS-1 phase-separating filter paper and ammonium carbonate (rock form) (BDH) were obtained from Macfarlane Robson, Ltd, Glasgow.
All other reagents were Analar grade by Koch Light Chemicals or BDH and were obtained from the Chemical Store, Glasgow University.
Sep Pak C-18 cartridges were obtained from Waters inc., Edinburgh, UK.
Bond elut C-18 cartridges were supplied by Crawford Scientific, Strathaven, Scotland, UK.

UV spectra were recorded on a Hewlett Packard HP8451A scanning diode array spectrophotometer, supplied by Hewlett Packard, Edinburgh, UK.
The Bioanalytical Sysyems CV-1A cyclic voltammeter was loaned by the department of Chemistry, Glasgow University and was supplied by Anachem ltd, Luton, UK.
The Gilson 302 single piston pump and 802C manometer, the Bioanalytical Systems (BAS) LC-4B electrochemical detector and various HPLC fittings and accessories were supplied by Scotlab Instrument Sales ltd., Bellshill, Glasgow, UK.
The Spectroflow 757 (Kratos) UV monitor was supplied by Severn Analytical, Bristol, UK.
The Phillips PU4025 UV monitor was supplied by Pye Unicam, Cambridge, UK.
Servoscribe flat bed chart recorders were supplied and serviced by Burke Electronics ltd, Glasgow, UK.

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