A PRELIMINARY STUDY OF THE METABOLISM OF MORPHINE IN PATIENTS WITH LIVER FAILURE

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by

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To my family and friends

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ABSTRACT

The Renal Hypothesis, which suggests that patients with kidney failure do not metabolise morphine, has been refuted by work carried out in this department and this study is continued in this thesis. Several solid phase extraction methods for preconcentrating morphine from plasma are assessed and a method which is fast, and gives 90-95% extraction efficiency, is used. An improved synthesis of N-trideuteromethylnormorphine, for use as internal standard, is described, and optimum conditions for derivatisation of morphine using pentafluoroproprionic anhydride are established.

The metabolism of morphine in patients with chronic liver failure is investigated by GCMS.

The GCMS SIM methodology for the determination of morphine, originally carried out using an HP 5982A instrument, is transferred to an HP MSD and to an MS80RFA magnetic sector instrument. The SIM function of the MS80 is shown to be unreliable, and the fault to lie in the proprietory DS90 software.

Preliminary results for one patient with chronic liver failure are interpreted as showing that morphine metabolism in such patients is not impaired.

CHAPTER ONE

MORPHINE ANALYSIS BY MASS SPECTROMETRY

A major problem with many of the samples presented for drug analysis is the minute amounts of the material of interest they contain. Because of its sensitivity and specificity mass spectrometry is considered a very powerful instrumental method for their chemical analysis. An exceedingly small amount of sample can be used and the resulting mass spectra will often provide unambiguous identification or structural analysis of the unknown compound. The use of a variety of ionisation techniques means that a large range of biochemically and biomedically important compounds can now be analysed by this method. In combination with a gas chromatograph, mass spectrometry can be further enhanced, making gas chromatography - mass spectrometry (GCMS) one of the most powerful analytical techniques currently available for the analysis of trace amounts of organic compounds. There have been a number of recent developments in GCMS including the direct coupling of a capillary gas chromatographic column to the mass spectrometer, the development of chemical ionisation and other specialised ionisation techniques, and the use of isotopically labelled internal standards in samples using quantitative selected ion monitoring (QSIM).

The two main advantages in using capillary columns over packed columns are the increased sensitivity of the capillary columns and the improved separation of components in a complex mixture. Chemical ionisation (CI) mass spectrometry has the advantage of concentrating the ion current in the mass spectrum into a relatively small number of intense peaks, including the pseudomolecular ion (MH^+). This can give greatly enhanced sensitivity over electron impact ionisation (EI) using QSIM, and provided

there are a few other characteristic ions in the CI spectrum, adequate specificity can be maintained.

When analysing for an known compound by mass spectrometry, a stable isotopically labelled analogue of the compound, makes an excellent internal standard. This is because the analogue behaves almost identically to the analyte through the extraction, chromatographic separation and the ionisation processes, so that the weight ratio of the two remains unaffected by these procedures.

Morphine is a powerful and commonly used analgesic. The detection of morphine and its metabolites, at low levels, in a very complex biological matrix such as blood, is a particularly difficult task. Mass spectrometry is an exceedingly specific and sensitive method to enable the detection and measurement of morphine in this situation. This thesis is concerned with the analysis of morphine, by GCMS, in blood taken from patients suffering from both renal or hepatic dysfunction and from a control group of normal subjects.

There has been renewed interest in the metabolism of morphine in recent years and and considerable controversy relating to the established views of the processes and sites involved. The first section of this thesis looks at the structure of morphine, its metabolism and some of the metabolites. Recently there has been a great deal of interest in the metabolites particularly as one is suggested to have even greater analgesic qualities than morphine itself. This section of the work also surveys some of the recent studies of morphine metabolism and the range of conclusions drawn from them. The previously accepted view that the liver was the main site of morphine metabolism was contested by a group who proposed the Renal Hypothesis - a view, refuted by many other groups, that led to the earlier study of morphine carried out in the Chemistry department of the University of Canterbury (UC).

The second section of this thesis describes the GCMS facilities used, in the Chemistry department of the University of Canterbury and in the Chemistry Division of the Department of Scientific and Industrial Research

Chemistry Division of the Department of Scientific and Industrial Research (DSIR), for this work. In it the development of instrumental and extraction methods is described. The use of solid phase extraction (SPE) methods was investigated for the first time in this department, and found to be an exceedingly efficient and rapid method of extracting morphine from plasma. As a number of mass spectrometers was used in the course of this project a number of different derivatisation techniques was employed to gain maximum sensitivity from each one.

N-deuteriomethylnormorphine (D_3-morphine) is routinely used as the internal standard of choice in the analysis for morphine, and attempts to effect improved synthesis of this compound became a part of this project.

As the project progressed significant problems were encountered in getting any sensible results from the MS80 mass spectrometer in this department. Investigations, carried out to determine where the problems lay, proved frustrating and time consuming. Eventually it was shown that there was a serious fault in the software supplied with the instrument and since the establishment of this fact took a major part of the project time, a brief outline of this work is included in the thesis.

The third section describes the on-going work in a study of morphine pharmacokinetics in patients in chronic renal failure and hepatic failure. This has been the continuation of a study carried out by Graeme Wright (University of Canterbury) and Dave Winter (DSIR) in conjunction with Kelvin Lynn and his team at the Christchurch Clinical School of Medicine. The results of this study have been previously described.[1,2] In this study the plasma morphine concentrations in four patients with renal failure and in three healthy volunteers were measured using CI QSIM, and morphine pharmacokinetics of the two groups compared. This work established that renal failure does not impair morphine metabolism in the way suggested in the Renal Hypothesis. For the present thesis the samples from the remainder of the renal failure patients were to be analysed and the study was to go on to look at a group of

liver failure patients. Unfortunately the time for this section of the study became severely limited and consequently this section is now restricted to only three patients.

CHAPTER TWO

MORPHINE KINETICS IN PATIENTS WITH KIDNEY AND LIVER FUNCTION DISEASE

2.1 INTRODUCTION

Morphine is an alkaloid isolated from opium and is considered to be the most valuable of all analgesics. It is used widely to provide pain relief in acute and chronic disorders and in surgical procedures. Morphine metabolism and excretion are now reasonably well understood for normal healthy patients but there still remain uncertainties as to how morphine is handled in patients with renal or hepatic dysfunction. Recent studies in patients with renal failure have shown that the active metabolite morphine-6-glucuronide (M6G) may accumulate producing analgesia and other side effects. Morphine metabolism in patients with hepatic disease is not affected until there is severe hepatic dysfunction. Adverse effects experienced by patients with lesser degrees of hepatic impairment probably result from altered pharmacodynamics*[1] rather than disturbed kinetics.[3]

2.2 MORPHINE METABOLISM

Morphine is considered to be a high extraction drug, having a first-pass extraction ratio of 0.6-0.8,*[2] with a low plasma binding (15-30 %), and is

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[1] Pharmacodynamic effects are the physiological changes made by the drug in the body

[2] Drugs absorbed form the gastro-intestinal (GI) tract into the blood stream go, via the hepatic portal vein, to the liver where they may be metabolised. The first-pass effect is the loss of the drug from the bloodstream as

detoxified by conjugation with glucuronic acid.[4,5] The glucuronide conjugates were considered to be pharmacologically inactive but recently there has been much interest in their possible analgesic activity which will be discussed later. The glucuronides are excreted by the kidney. Some non-renal elimination also occurs via the faeces and via the enterohepatic recirculation.*[3]

To appreciate morphine metabolism its structure-activity relationships must be understood. In relation to its activity and metabolism the three most important positions on the morphine molecule are:

- the phenolic hydroxyl at position 3
- the allylic hydroxyl at position 6
- the N atom

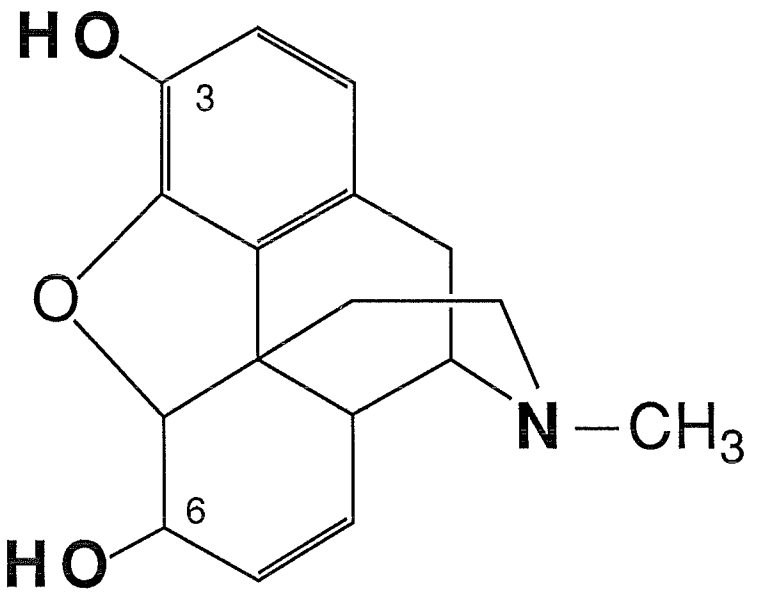

Figure 1: **Morphine**

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it passes through the liver. In some cases, the first-pass effect results in virtually complete elimination of the administered drug before it gains access to the general circulation.

[3] Drugs that are secreted into the bile re-enter the intestine and if reabsorbed pass again to the liver creating a cycle termed enterohepatic recirculation. The effect of this cycle is to increase the persistence of the drug in the body and, if the concentrations of the drug are high enough at its receptor sites, to prolong its action.

Morphine's analgesic activity can be affected significantly by alterations at any of the three sites indicated. Alteration to the structure changes the pharmacological activity, with important clinical consequences, most of which have been recognised for quite some time.[6]

Both hydroxyl groups can be converted to ethers or esters (e.g. heroin (diacetylmorphine)). Depending on which hydroxyl group is altered, the changes give opposite clinical effects. Additions at the phenolic group reduce pharmacological activity by more than 90%, but modifications at the allylic hydroxyl increase activity with the compound produced being two to four times more potent than morphine as an analgesic after parenteral dosing in standard tests. However, some substitutions at the 6-OH position, e.g. conjugation with long-chain aliphatic acids, decrease activity because of steric hindrance and other factors. Short chain fatty acid substitutions, e.g. 3,6- dibutanoylmorphine, have been used to increase the lipophilicity and therefore potency of morphine [7,8] The tertiary nitrogen is especially important in morphine's analgesic activity, with modifications which make the nitrogen quarternary (e.g. the N-oxide) greatly reducing analgesic potency as a result of reduced penetration into the central nervous system (CNS). Replacement of this methyl group not only reduces analgesic activity but produces compounds (e.g. nalorphine) which antagonise the actions of morphine.

The principal metabolic pathways for morphine are glucuronidation, sulphonation and N-dealkylation. Morphine is metabolised in man primarily through conjugation with uridine diphosphoglucuronic acid to form morphine-3-glucuronide (M3G). Some is glucuronidated in the 6-position (M6G) or demethylated to normorphine.[9] In humans more than 90% of an administered dose of morphine is excreted in the urine as metabolites and approximately 10% as free (unconjugated) morphine. M3G is the major metabolite in several mammals[10] and accounts for 54-74% of the excretion products in man.[11] Until recently it was thought that M6G was a minor metabolite because less than 1% of the dose was found, in this form, in the urine of previously addicted males.

More recent studies, based on plasma samples, indicate that this figure is far too low.[12] Morphine-3-ethereal sulphate accounts for approximately 5% of the morphine dose in human subjects with approximately the same amount of normorphine or normorphine-6-glucuronide.

Only M3G and M6G have been identified in human plasma, though M6G only recently. Studies, using high performance liquid chromatography (HPLC), of the plasma of cancer-pain patients on high doses of morphine have shown levels of M6G higher than morphine and approximately 10% of the M3G concentration.[13] These findings were confirmed using differential radioimmunoassay (RIA).[14] Plasma concentration of the metabolites increases quickly after injection with both glucuronides peaking after about 70 minutes. M6G concentrations relative to morphine were higher after oral administration. Levels were approximately equivalent at all times in six patients given a 6 mg oral dose of a delayed-release preparation, (MST, Morphine Continus, Napp Lab). Peak concentrations of morphine M3G and M6G occurred about four hours after dosing[15] as expected for a delayed release preparation.[16]

The analgesic conjugates have been studied in experimental animals with the work focussed on M3G and M6G, the metabolites occurring in humans. M3G has no analgesic activity. However, M6G is three to four times more potent than morphine as an analgesic after subcutaneous injection in mice and 45 times more potent after intracerebroventricular injection. The difference in potency ratio between these two routes of administration arises because morphine free-base enters the CNS more rapidly than the glucuronide.[17] This ability of M6G to penetrate the blood-brain barrier unchanged was confirmed using radioactively labelled M6G.[18] The analgesic activity does not appear to be due to the hydrolysis of the conjugate in the brain, or elsewhere, as only conjugated morphine was found in the brains of rats after intraperitoneal M6G injection.[17] There is always a danger in extrapolating results from animals to man as even different animal species react differently to doses of morphine, e.g. morphine is shown to be stimulatory in cats. M6G is thought to act on the opiate

receptors because it is antagonised by nalorphine and shows a cross tolerance to morphine.[17] The fact that M6G has been shown to pass the blood-brain barrier*[4] is in itself puzzling as this is a water soluble metabolite. It raises a number of questions including, perhaps, the necessity to revise the current concept of the blood-brain barrier itself. Morphine-6-ethereal sulphate has actions similar to M6G, while nalorphine-6-sulphate has been shown to be a more potent antagonist than nalorphine itself.[18]

2.3 MORPHINE DISPOSITION DURING RENAL OR HEPATIC FAILURE

A number of studies using non-specific analytical methods have produced conflicting results for the metabolism of morphine in certain disease states. Patients in renal failure frequently appear to be more sensitive to the effects of morphine than patients with normal renal function.[19-24] Because renal failure patients exhibited adverse drug reactions, such as an unexpected degree of respiratory depression and prolonged duration of drug action, from a therapeutic dose of morphine, it was suggested that morphine disposition (i.e. distribution and elimination) was altered in some way by renal failure, even though the liver was considered to be the main site of opioid metabolism. Current evidence suggests that these adverse reactions are due to the accumulation in plasma of the morphine glucuronides, especially M6G. Aitkenhead's[25] team administered morphine IV to eleven patients with normal renal function and nine with chronic renal failure and measured the morphine plasma concentrations with HPLC. They showed that mean values of terminal

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4 The blood brain barrier is a physical difference in the endothelial cells of the capillaries of the central nervous system. These are tightly packed together preventing molecules from passing between them. The effect of this is that only lipid soluble substances can be carried through the cell membranes and enter the CNS.

elimination and total body clearance were similar in the two groups and concluded that elimination of unchanged morphine was not significantly impaired in patients with chronic renal failure, but thought that the accumulation of M3G probably occurred.

It was also suggested that during renal failure the increased sensitivity to morphine could be caused, at least partly, by changes in the drug binding processes. Morphine, like most drugs, normally binds reversibly to plasma proteins.[26] Unusually high or low plasma levels of unbound and active drug can be caused if changes to this binding, caused by altered concentrations or altered binding capacity of the protein, occur. Alterations in protein binding are usually of little clinical significance except for those drugs that have a very high binding, e.g. phenytoin (~90%) Total serum proteins and albumin concentrations are often lowered by renal syndrome (e.g. nephrotic syndrome) and morphine binding was shown to be dependent on these concentrations but not on the severity of renal failure as measured by creatinine clearance.[26]

The suggestion that the kidney was a site of morphine metabolism became known as the Renal Hypothesis and it was formalised as the result of a number of studies, including one using a "specific" RIA for unconjugated morphine. This work, which was carried out by a group of researchers from the Radcliffe Infirmary and the Pain Relief Research Unit, Oxford,[27] had been interpreted as demonstrating an important role for the kidney in the metabolism of morphine. The Oxford group[28] had also reported on morphine disposition in a group of fifteen end stage renal failure patients during and after renal transplantation. They showed that morphine elimination did not occur until the transplant began to function. The same group[4] demonstrated reduced morphine clearance in patients with renal dysfunction, the clearance being related linearly to creatinine clearance. The Renal Hypothesis had been challenged by groups who doubted the specificity of the RIA method[29-32] even though the RIA results obtained at Oxford had been reported as having a good correlation with an HPLC method used by the same group.[27] It seems likely that studies implying

morphine accumulation in renal failure used RIA's that suffered from cross-reactivity with M6G with the result that the elevated concentrations measured were the sum of the morphine and the M6G. This could be an indication of M6G as a major active metabolite of morphine and of the limitations of RIA.

The Renal Hypothesis has been shown to be inconsistent with evidence obtained from a highly specific GCMS study carried out by the Canterbury group[1] in which they assessed the elimination of morphine and morphine glucuronides, in patients in renal failure, after the intramuscular (IM) administration of Papaveretum.*[5] Details of this study are reported later.

It is the traditional medical view that because morphine is metabolised in the liver it is not a suitable analgesic for patients with liver disease because its use may result in excessive sedation and precipitate encephalopathy (i.e. the general disturbance of brain function) in such patients. Recently it has been shown that in liver disease, morphine metabolism was altered in only minor ways.[33,34] This suggests that any exceptional effects may be due to morphine sensitivity, i.e. dynamic rather than kinetic effects. Drug metabolism is only affected with very severe liver failure sufficient to alter the liver's synthetic function. When the liver is badly diseased, as in cirrhosis, a phenomenon called shunting occurs. Blood cannot flow through the diseased liver and is diverted into the systemic circulation, bypassing the liver. In severe liver disease shunting is so great that the liver is totally bypassed. The effect of this is that compounds absorbed from the gut are not detoxified and build up in the blood stream. Bacteria from the gut produce endotoxins which would normally be metabolised in the liver and the levels of these also rise; the resulting

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*[5] Papaveretum (Omnopon®, Roche) is the most commonly used opioid in New Zealand hospitals.[35] It is considered superior to morphine in providing analgesia and sedation.[36,37] It is a standardised mixture of four opium alkaloid hydrochlorides containing the equivalent of anhydrous morphine 47.5-52.5%, anhydrous codeine 2.5-5%, noscapine 16-22% and papaverine 2.5-7%.[38]

encephalopathy is a good indication of the degree of shunting. The brain does not function because the liver is not detoxifying these compounds which are now available to cross the blood-brain barrier. Two other criteria indicative of hepatic failure are based on two synthetic functions of the liver, namely the production of albumin and of vitamin K clotting factor. Impairment of these synthetic functions correlate very well with impairment in enzyme function necessary for drug metabolism.

It may be that metabolism of morphine is extrahepatic. Patwardhan *et.al.*,[34] reported that the disposition and elimination of morphine were unaffected by moderate to severe cirrhosis. They postulated that since intra or extrahepatic shunting, or both, in cirrhosis did not significantly impair morphine clearance, significant extrahepatic morphine conjugation might occur in normal subjects and patients with cirrhosis. This site is unlikely to be the intestine, since drugs with low lipophilicity, like morphine, are not subject to gut wall glucuronidation[3] with this inability of the gut wall to metabolise morphine being supported by *in vivo* data.[39] Although rabbit kidney tubules are able to metabolise morphine to glucuronide under certain circumstances[40] and perfused rat kidney can actively excrete morphine[41] these findings do not substantiate the idea of significant renal glucuronidation in humans, so there is no clear evidence for an alternative organ of metabolism.

2.4 RENAL FUNCTION DOES NOT IMPAIR MORPHINE METABOLISM

The work at Canterbury reported a pharmacokinetic study of unchanged morphine in four patients with renal failure (one anephric) and three healthy volunteers after the IM administration of Papaveretum. The patients were on haemodialysis (Appendix I) and were studied on a non-dialysis day. The pharmacokinetics of morphine glucuronides of two patients were also studied.

Papaveretum (0.25 mg/kg body weight) was injected into the deltoid muscle of the fistula arm in the patients. Blood was obtained from a venous cannula (Appendix I) in the contralateral arm immediately before injection and at intervals up to twenty-four hours. The serum was separated and stored at -20°C. The extraction process for morphine was based on established methods.[9,42] Analyses were performed on a Hewlett Packard 5982A GCMS using chemical ionisation with ammonia reagent gas. Calibration standards were prepared by adding standard weighed mixtures of morphine and N-trideuteriomethyl-normorphine (D_3-morphine) to plasma from "pre-injection blood" followed by the extraction and derivatisation procedure used for the samples.

Results of this study showed that $t_{\frac{1}{2}}$ of absorption and $t_{\frac{1}{2}}$ of elimination were significantly shorter in the patients with renal failure than in the controls. The difference in mean clearance and the volume of distribution corrected for body weight did not reach significance so it was not possible to establish which component(s) was responsible for the decrease in the elimination half life. The concentration-time curve of the morphine-glucuronides, measured in the two kidney failure patients, showed the expected plateau. The results suggested that patients with renal failure eliminate morphine more rapidly than normal subjects and it was noted that the anephric patient had the shortest half life. The finding of a shorter $t_{\frac{1}{2}}$ was not unexpected because cardiac output is greater in patients with renal failure.[1,43] These results supported those of Sawe *et. al.*,[31] who used HPLC to measure morphine and its two glucuronides in a study reported at about the same time.

The serum extracts contained several compounds with GC retention times close to that of morphine. GCMS, or high performance gas chromatography - mass spectrometry (HPGCMS), in SIM is the only analytical method available which can unambiguously identify and quantitate morphine in such extracts. The results suggested that immunoassay identifications of morphine in plasma were not likely to be accurate because these methods do not rely on separation but require that co-extractants do not cross-react. In renal failure patients the

retention of water-soluble metabolites, potentially immunologically cross-reactive, could be a significant problem. Many nephrologists have observed delayed recovery from the effects of opioids in patients with renal failure. This study did not explain these anecdotal observations, but Woolner *et. al.*, have shown that patients on haemodialysis undergoing vascular access procedures and patients with normally functioning kidneys having other peripheral surgery had similar recovery times after premedication with IM Papaveretum.[35] This study concluded that renal failure did not impair the elimination of morphine.

2.5 THIS WORK

2.5.1. Renal Failure

This work, as all ready mentioned, is a small section of an ongoing study. The previous Canterbury study established that renal failure does not impair the metabolism of morphine[1] and that its pharmacokinetics are not distinguishable from those in control subjects. There are, however, a number of aspects of morphine metabolism which remain to be clarified. In stable patients renal failure does not seem to interfere with clearance, but patients with renal failure do sometimes present who appear to have morphine toxicity. These patients exhibit respiratory distress, excessive sedation, and prolonged duration of analgesia.

Continuing the renal failure study would require many analyses, so developing a faster method of extraction would be advantageous. One aspect to be considered is the role of the water soluble metabolites. Especially interesting is the 6-glucuronide for which there is increasing evidence of enhanced analgesic effect. Clearance of M6G is via the kidneys as it is water-soluble and yet there is evidence of it passing the "blood brain" barrier. This is an area of controversy as some groups feel that the most likely explanation for the analgesic effect of M6G is its back-dissociation to the free drug in circulation even though this is in opposition to the findings of Shim et. al.[17] who reported

finding only conjugated morphine in the brains of rats after injection with M6G. The role and function of these metabolites requires further study. Another explanation may be changes to the morphine receptors and/or the binding to these - changes in renal failure and the accumulation of metabolites may alter the function of the main receptors. An example of this is the effect of sodium on endorphin receptors. The Canterbury group would like to mount a study where the clinical effects of morphine and its blood levels are measured at the same time to examine the relationship between these. A study of this sort has been done[4] but not using GCMS.

2.5.2. Hepatic Failure

More urgently, the Canterbury group would like to know that they could safely administer morphine to liver failure patients who needed it. They have a number of prospective subjects and would look at the metabolism of morphine given intravenously (IV), where after entry to the system it goes straight to the brain; and orally, as there may be metabolism in the gut wall. They have samples ready from two liver failure patients, who have received morphine orally, and IV.

2.6 THIS PROJECT

Not all the patient samples taken for the previous study were used. It was planned, for this thesis, to look at the pharmacokinetics of unchanged morphine in the remainder of the patient samples and at least two of the previously studied patient samples. The latter is important because it is necessary to find out what effect long term freezing has on the plasma samples. The rest of the project was to study the pharmacokinetics of unchanged morphine in the two patients with reversible liver failure and to use these results to decide if the analytical method was suitable for the larger study planned.

However, because of continuing equipment problems, only two patient studies were able to be undertaken (the results of which are discussed in a later chapter). As the project progressed it became obvious that there was a major problem in the SIM software of the MS80 and establishing sufficient proof of this to convince the manufacturer to make changes became a significant part of this thesis.

CHAPTER THREE

DEVELOPMENT OF INSTRUMENTAL AND EXTRACTION METHODS

3.1 THE MASS SPECTROMETERS

It was planned to do the work for this thesis on the Department's Kratos MS80 RFA mass spectrometer but difficulties in installing this instrument meant that the early work was carried out on a Hewlett- Packard 5982A GCMS (HPGCMS). The latter part of the project was carried out at the DSIR (Chemistry Division, Christchurch) on a Hewlett- Packard 5970B MSD (MSD).

3.1.1 The Hewlett-Packard GCMS

This quadrupole instrument has an ion source configured for either EI or CI operation. A "dodecapole", i.e. four pairs of turnable electrodes or "blades" between the cylindrical quadrupole rods, forms the mass filter. An electric field is produced by the radio frequency and direct current voltages applied to the rods. The blades modify this field so that it approximates that which would be generated by a hyperbolic rod assembly. This machine is pumped differentially by two high capacity oil diffusion pumps each of which is backed by a mechanical rotary pump.

Admission of samples is by either one of the two gas chromatograph capillary column inlets, or through a heated direct insertion probe (DIP). The machine is tuned using perfluorotributylamine (PFTBA) using the batch inlet probe and and the GC sample introduced using the on-column (OC) injector. Most of the work for this thesis done on the HPGCMS used an OCI-3 on-column injector (SGE Pty) which had been previously fitted. The GC is

direct-coupled to the mass spectrometer source through a heated transfer oven constructed in this department. If the CI reagent gas is also the carrier gas it is admitted through the chromatographic column inlets, otherwise it is admitted via the DIP inlet.

On this mass spectrometer, mass spectra are recorded, with a light-beam oscilloscope on light sensitive paper, over a mass range of 3-1000 μ In selected ion monitoring mode up to four separate ions can be monitored, using the hardware multiple ion detector, producing selected ion chromatograms recorded on a conventional chart recorder connected to that detector.

It is important, in SIM mode, to ensure the mass spectrometer is set to monitor the correct mass/charge values, and that it does not drift from these values. Each channel of the HP5982A's multiple ion detector (MID) supplies a tuning voltage of 0-10 volts to control the mass over the range 0-1000 μ Up to four masses are selected by adjusting four ten-turn potentiometers on the MID's front panel with the selected masses being displayed on a digital voltmeter (DVM). Because there had been so much difficulty in setting these masses a new DVM had been attached to the MID and two resistors inserted in each mass selection potentiometer circuit to reduce the mass range covered by the potentiometers. For the purposes of my work considerable improvements had all ready been made to the instrument but the drift from the set masses was a constant problem.

The first study of morphine metabolism carried out by this department[44] used the HPGCMS. The instrument was only just sensitive enough to cope with the levels of morphine being determined and the drift in the MSD proved a major problem. For these reasons the present study was designed around the MS80. This mass spectrometer is controlled by the computer system which corrects for magnet drift by sweeping the electrostatic analyser (ESA) across the set mass peak. A lock mass system can also be used to correct for any long term drift.

3.1.2 The MS80 GCMS

The MS80 is a magnetic sector instrument. Resolving power in excess of 25,000 is provided by the double - focussing analyser system and rapid mass measurement can be made to an accuracy better than 5 ppm. The double - focussing geometry enables the instrument to determine accurately the atomic composition of ions in the mass spectrum, making it possible to distinguish between different combinations of atoms of the same nominal mass. The complete GCMS instrument comprises a Carlo Erba Mega gas chromatograph and temperature controlled interface, the mass spectrometer and a Data General DG30 computer, using Kratos DS90 software. The GC column is coupled directly to the mass spectrometer source.

A special feature of this department's MS80 is a storage oscilloscope, which is attached to the mass spectrometer outlet and runs parallel with the data system monitoring the output as the ESA or magnet scans. This modification proved to be invaluable when it came to verifying the suspected problems in the software.

3.1.3 The MSD

The DSIR's instrument is a Hewlett-Packard 5970B mass selective detector direct-coupled to an HP5890GC with an HP77673A automatic sampler and an HP59940 Chem-station (HP-UX-Series). It is an EI only quadrupole mass spectrometer with about the same sensitivity as the MS80 in comparable mode.

3.2 THE ADVANTAGES OF SELECTED ION MONITORING

A variety of methods are available for focussing ions at the detector of the MS after they have left the source. The method used depends on what information is required from the sample, with the most commonly used form of

data acquisition, on a magnetic sector mass spectrometer, being magnet scanning. The magnetic field is allowed to decay from a high to low value so that ions of decreasing $\frac{m}{z}$ values are focussed at the collector. This produces a complete spectrum of mass versus intensity for all ions focussed between the limits of the field scanned. This method is typically used for GCMS acquisition in either high resolution (HRP) or low resolution (LRP). For accuracy it relies on the reproducibility of the magnet scan and can process a mass range of up to 3000 amu. Since this technique is most often used for identifying unknowns, and this project involved quantitating a known substance, morphine, this technique was only used to establish working parameters.

The quantitative analyses were carried out in selected ion monitoring (SIM) mode. This technique is used when specific compounds with previously determined mass spectra are being analysed, as it is sufficient to determine only selected ions for each analyte. SIM on the MS80 is achieved by switching the electrostatic analyser and accelerating voltages to focus selected ions at the collector. The magnetic field is maintained at the same value for each ion monitored. Much lower detection limits are achieved since most of the detection time is spent monitoring these ions. For a given value of the magnetic field, the mass focussed at the collector is inversely proportional to the accelerating voltage; e.g. if mass 100 is focussed at 4 kV, then mass 200 is focussed at 2 kV. Generally the sensitivity decreases as the source kV decreases, but typically, a mass range factor of 3 is acceptable, e.g. 100-300, 300-900, or 30-90 amu.

In SIM the magnet stays at the field required to focus the lowest mass at full ESA voltage and the ESA steps down to each of the other higher masses selected. Although sensitivity of detection increases as the number of masses monitored decreases, the specificity of the analysis also decreases. It is therefore very important that the ions being monitored are as characteristic as possible of the substance being analysed for. It is usual to

select at least three characteristic ions including the parent ion, a major fragment as close in mass as possible to the parent ion (since switching the ESA over a large mass range can cause a decrease in sensitivity) and one other. For many substances it is also useful to monitor the M+1 ion; the M:M+1 ratio can be accurately calculated as the percentage abundance of the isotopes is well established, so this ratio serves as a useful check on the presence of interfering ions.

A considerable number of difficulties arose with the SIM mode of our particular MS80 and these are discussed in chapter 4.

3.3 DEVELOPMENT OF THE ANALYTICAL METHOD

3.3.1 Extraction Methods

The earlier study[2] used liquid-liquid extraction to isolate the morphine from plasma. This method is time consuming and interest had been shown in this department in solid phase extraction (SPE) which appeared to have a number of advantages. SPE is a sample preparation technique, based on the principles of liquid-solid chromatography, in which the sample is drawn, under vacuum, through a disposable column which has been prepacked with a suitable sorbent. The advantages of SPE are that it is rapid, suitable for very small samples, offers a wide variety of sorbents allowing for selected extraction of specific substances from complicated matrices (e.g. blood), and it is capable of a high extraction efficiency. The stages of the method are: conditioning the column by aspirating with an appropriate solvent followed by water or buffer, adsorbing the analyte on the solid phase by passing the sample through the column, eluting for interferents which may have bonded to the column, and eluting the analyte with a suitable solvent.

In the work for this thesis an SPE method based on Bond -Elut C_{18} columns was used. A number of SPE methods for extracting morphine from

plasma had been reported.[36,37,20,38] The techniques investigated were those with the highest reported recoveries associated with the most straightforward procedures. A number of modifications of these methods, especially variations of the interferent elutions, was tried from aqueous solutions and from plasma. The morphine recovery, by comparison with a known amount of D_3-morphine added at the end of the extraction process, ranged from 20-95%.

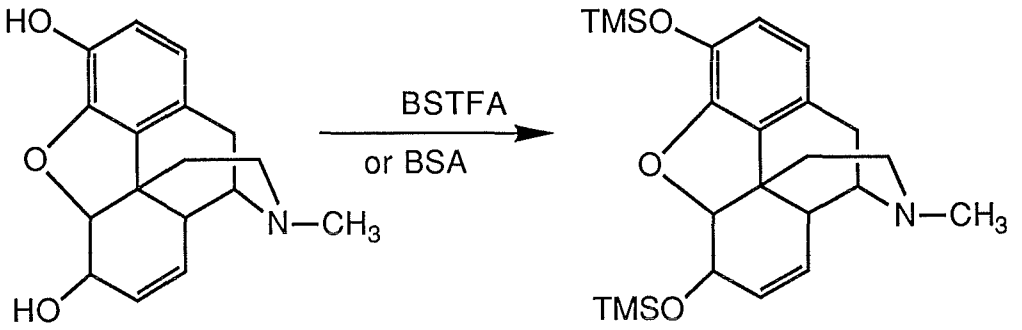
The following technique, the least complicated procedure tried and one of the two which gave 95% recovery, was adopted for this project. The column was conditioned with methanol and water. The sample, buffered to pH 8.5 using a carbonate buffer, was drawn through the column, the interferents were eluted with water after which the column was air dried, and the morphine eluted with two aliquots of a chloroform /isopropanol mixture. Analysis of morphine recovery was carried out on the HPGCMS.

At various times considerable difficulty was experienced in getting the plasma samples to run through the Bond-Elut columns. Dr John Livesey, at Princess Margaret hospital in Christchurch,[39] was approached for assistance in this matter. His experience showed that the problem arose as the result of fibrin formation in the plasma. It was his impression that anticoagulants merely slowed down the clotting process, rather than preventing it, and that most plasma samples would precipitate a degree of fibrin. The anticoagulant used had some effect, with heparin plasma being worse than EDTA plasma. He concluded that the fibrin formation was time-dependent and was not prevented by conventional freezing and he made a number of suggestions for dealing with the problem. A number of these were tried with some improvement being found when the plasma samples were mixed with a 1 mg mL^{-1} solution of disodiumEDTA. It was suggested[40] that where adsorption/extraction procedures were necessary it was important to remove the coagulating material beforehand and that extensive centrifuging had been found to be the most useful way of achieving this. Subsequently all

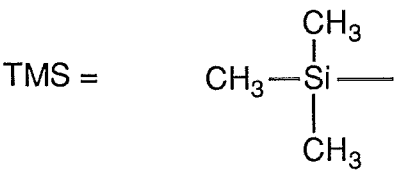
plasma samples in this work were centrifuged for at least one hour before use, and ran through the cartridges without difficulty provided a rotary oil pump was used to provide the vacuum.

3.3.2 Derivatisation Techniques

Morphine has both acidic (OH) and basic (N) centres and undergoes specific interactions with most GC columns leading to poor peak shape and broad peaks. The GC behaviour of morphine is improved by derivatisation which inactivates the -OH groups by converting them to non-polar moieties such as trimethylsilylethers (TMS). N,O-Bis-(trimethylsilyl)-acetamide (BSA) and N,O-Bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) have been recommended as derivatising agents[41] for morphine and were found to be successful in the earlier study (Fig 2).



morphine TMS-morphine



BSA - N,O-bis-(trimethylsilyl)-acetamide

BSTFA - N,O-bis-(trimethylsilyl)-trifluoroacetamide

Figure 2: **Trimethylsilylation of Morphine**

The TMS derivatives were formed by reacting the dry residue of the plasma extracts with either BSA or BSTFA for one hour at 60^0C immediately prior to GCMS analysis. Some GC columns appeared to be adversely affected by these derivatising agents and at various times a further step of blowing down the derivatised sample, under nitrogen, and reconstituting in ethyl acetate was included. The earlier study had shown that once derivatised, the samples must either be used within a few hours, or frozen in liquid nitrogen until required. If these precautions were not taken the TMS derivatisations decomposed with dramatic drop in sensitivity. In this work, samples derivatised in this way were used within 4 h or frozen in liquid nitrogen.

For the work done at the DSIR a new technique had to be employed as the DSIR did not want either of the above agents introduced into their MS source. The method (received from Gracefield DSIR, Wellington) was an adaption by A.W Missen,1977, of an established technique.[42] In this method the residues are derivatised in a mixture of ethyl acetate and pentafluoroproprionic anhydride (PFPA), dried under nitrogen and reconstituted in heptane (Fig 3).

Samples derivatised in this way were found to hydrolyse very quickly (on one occasion over the course of 15 mins). It was suggested that the problem lay in inadequate drying of the heptane even though it had been distilled over phosphorus pentoxide, and as it was felt that further treatment of the heptane was not likely to solve the problem a number of variations of the above technique were tried.

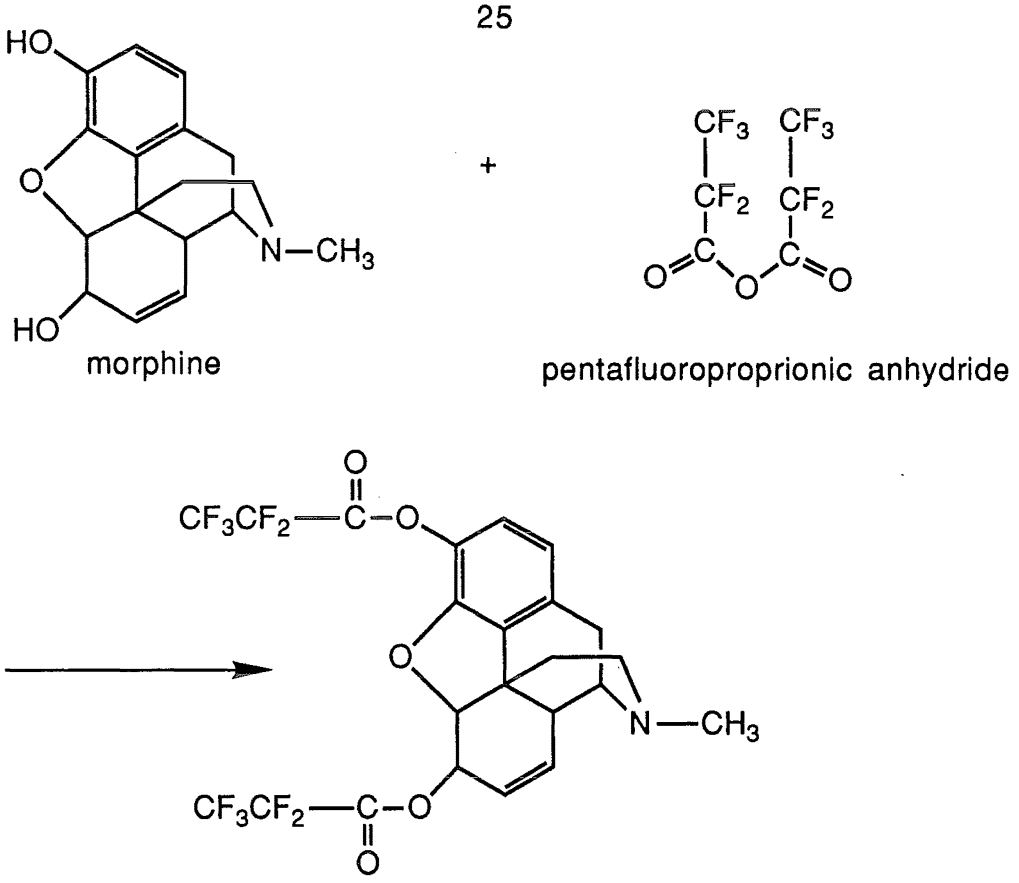


Figure 3: **Derivatisation with Pentafluoroproprionic Anhydride**

A trial was set up in which samples were derivatised as normal but were reconstituted in the following solvents after blowing down: heptane, ethylacetate, 50% heptane with 50% EtOAc, and PFPA. A sample was included that was derivatised in 100 µl of EtOAc and 100 µl of PFPA and injected without further treatment. An autosampler programme was used to sample each vial twice, with a heptane blank in between, followed by a repeat injection after a two hour delay. The best sensitivity was obtained for the sample derivatised in the mixture of EtOAc and PFPA. The sample reconstituted in heptane had decomposed before its first injection (Fig 4).

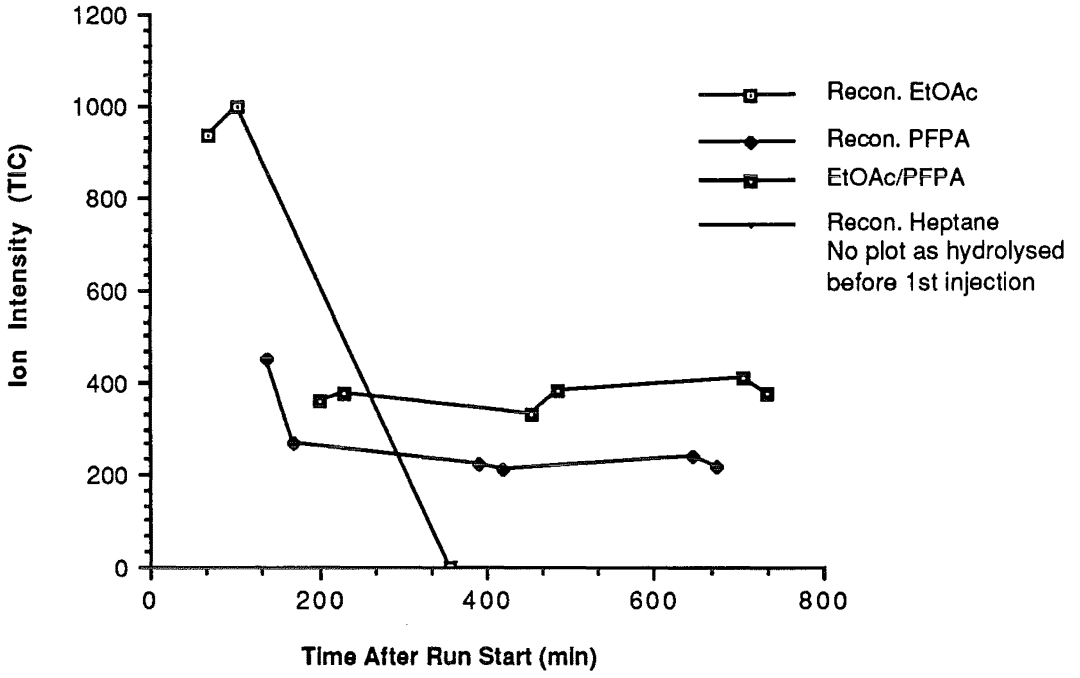


Figure 4: **Comparison of Derivatisation Methods**

200 µL of solution was required for the automatic sampler vials with 1 µL of this used at each injection. Using the PFPA level in the above derivatisation technique (at ~ a $1x10^4$ excess over morphine) meant injecting ~0.5 µL of PFPA at each sampling with consequent contamination of the source and possible septum and column damage. A second investigation, using the following three concentrations of the derivatisation reagent were used to ascertain the minimum amount of PFPA that could be used in a derivatisation process which did not involve a second drying down and reconstitution.

1. 150 µl EtOAc : 50 µl PFPA
2. 190 µl EtOAc : 10 µl PFPA
3. 199 µl EtOAc : 1 µl PFPA

The second method resulted in satisfactory sensitivity (Fig 5) using a minimum of PFPA and was consequently adopted for this project. This method also had the advantage of removing the second drying down and reconstitution, giving a faster derivatisation and eliminating one possible source of analyte loss during sample preparation. Samples derivatised in this way would last for more than a week, under normal refrigeration, before hydrolysing.

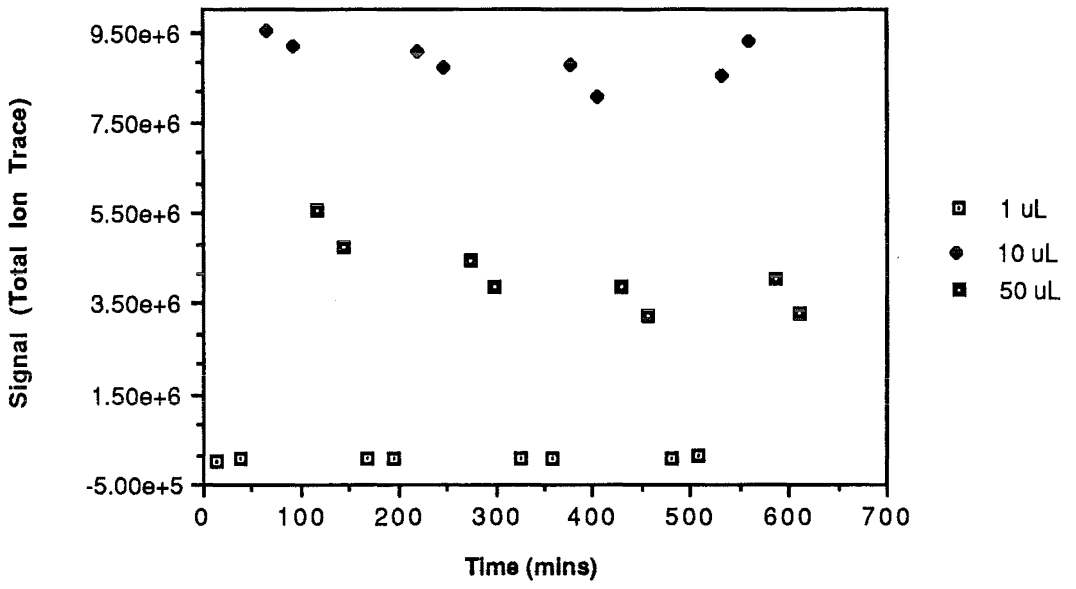


Figure 5: **Comparison of PFPA Concentrations**

CHAPTER FOUR

ESTABLISHING RELIABLE SELECTED ION MONITORING PROCEDURES

4.1 INTRODUCTION

The major problem encountered in this thesis was obtaining reproducible ratios from the SIM measurements. In attempting to resolve this problem the TMS derivatives of D_0 and D_3- morphine were used, rather than the PFPA derivatives used later. The characteristic ions of TMS-D_0-morphine (429 and 414) and TMS-D_3-morphine (432 and 417) were monitored, and in the absence of interfering compounds the elution of morphine from the GC should show as a sharp peak in the intensity of all four ions with almost identical retention times (D_3-morphine (D_3) elutes very slightly after D_0-morphine (D_0)). The ratios 432/429 and 417/414 measured either as areas or maximum heights, should be identical within experimental error. Examples of typical D_0 and D_3 morphine spectra (in both EI and CI) are shown (Figs 8-11, pp. 43-46). Since CI did not give a greater morphine sensitivity EI was used for this project.

In this study, a great deal of work was required to establish why the ratios obtained from standard solutions for both pairs usually varied widely from day to day or during a day, or even from injection to injection for the same sample. Often the two ratios were quite different from the same GC run. The situation was made more difficult by the fact that sometimes reproducible ratios were obtained for several consecutive days, though when that happened the ratios frequently differed greatly from those calculated from known concentrations of standard solutions. On one occasion an excellent calibration curve, slope 1.00, correlation coefficient 0.998, was obtained at

the 100 ng mL^{-1} concentration; the next day solutions made from the same standards gave results which were unusable.

Although in hindsight the source of these problems is fairly obvious, it was much more difficult to discern while the work was in progress. Such variable results could arise from the following sources:

(i) D_0-morphine and D_3-morphine reference samples of variable purity and errors in making up standard solutions and subsequent dilutions to lower levels

(ii) Unreliable derivatisation chemistry - particularly differences between D_0-morphine and D_3-morphine.

(iii) Malfunctioning of the SIM software - such malfunction could occur in the instrument control, data acquisition or data analysis function, or in more than one of these.

Because the DS90 software is proprietary software, supplied by Kratos without source files, it is not possible to make changes to it. At an early stage in this work discussion was initiated with Kratos about the possibility of a fault in the software, but for many months the company insisted that the problem was only a local one and could not therefore lie in the SIM subroutines. In fact, nearly 12 months after we first brought our problems to their attention, and largely as a result of the work described here, the company acknowledged that the software was faulty. A further six months later they provided a version of DS90 which appears to have overcome these malfunctions.

To compound the problem, attempts were made to investigate the source of these difficulties using (1) a Finnigan Ion Trap Detector on loan to the DSIR for several weeks, and (2) the HPMSD shortly after its installation at the DSIR. In retrospect it was a mistake to have used these instruments before they had been adequately checked, or real expertise in their operation had been acquired. Their use only added to the confusion.

The work carried out during this study to establish beyond doubt that the software was at fault occupied a major part of the thesis time. For that reason it is described here, together with a brief discussion of the fault and its origin.

4.2 D_O-MORPHINE AND D_3-MORPHINE STANDARDS AND SOLUTIONS

The stock standard solutions were made by weighing 1 mg of morphine on the Cahn electro-balance, correct to four decimal places, and making up to the mark in a 1mL silanised volumetric flask. Dilutions were made from these standard solutions. All solution measurements were made using solid displacement pipettes (Socorex Pty. Ltd.) The percentage deuterium incorporation in the D_3-morphine was obtained from the mass spectra of D_0 and D_3 by evaporation from the DIP and integration of the total ion currents of the molecular ion region. Using the intensity data from D_0 and D_3 and the Labdet [53] programme the percentage incorporation can be calculated.

The initial work used the very small amounts of D_0-morphine and D_3-morphine which had been left from Dr Winter's work. When these ran out a new sample of D_0 was obtained by extraction from the commercial hydrochloride (Pharmaceutical Sales and Marketing Ltd.) followed by recrystallisation and sublimation; D_3 was synthesised as described later. The first D_3 made was later discarded when it was shown to contain some other material, not D_0 and as yet unidentified. The use of this D_3 confused the issue even further.

Before calibration curves can be obtained it is necessary to establish that solutions that are theoretically 1:1 mixtures of D_0 and D_3 morphine give 1:1 ratios of the monitored ions when run in SIM, or at least give consistent ratios. By the time this work was being tried on the MS80 the D_3-morphine being used was the first of the material synthesised in the department. SIM ratios of the monitored ions were not 1:1 and on more than one occasion appeared to be approximately

2:1. Because the SIM results were so variable from run to run a series of full scan (LRP) GC runs were carried out and the 432/429 ratios extracted. The run statistics table for each run was checked to ensure that the ion intensities were not saturated and the areas for the two masses were used to calculate the ratio. The results of this indicated that the D_3-morphine was not at the level it was thought to be.

As an additional check a 1:1 mixture of D_0:D_3 benzotropine hydrobromide (theoretical ratio:0.956) was also used and run under SIM conditions as follows:

Lock mass 304.9822 , start mass 292.9822, masses monitored: 307.1936 and 310.2124

The measured ratios of 0.94 and 0.95 were so close to the theoretical ratio that this was also taken as an indication that the problem was with the D_3-morphine. At this time Version 4 of the software arrived and the benzotropine system was not investigated further.

To further check the purity of the D_3-morphine the following investigation was carried out. D_0:D_3 1:1 samples from those that had been used at the University of Canterbury (UOC) and from the DSIR were derivatised in the following combinations. Six injections were made on the ITD from each sample. Sample 2 was included to check the D_0 being used.

	Ratios	
	Theoretical	Measured
1 D_0 (UOC) : D_3 (UOC)	1.095	2.01
2 D_0 (DSIR) : D_3 (UOC)	1.012	1.15
3 D_0 (DSIR) : D_3 (DSIR)	1.054	1.14

This showed conclusively that the newly synthesised D_3 was contaminated.

Using D_3 solutions provided by the Christchurch DSIR a calibration curve was obtained, from SIM runs, at the 100 ng level on the MS80. The theoretical ratio of the D_0:D_3 mixture was 1.124 and these runs gave an average ratio of 1.16. The same 1:1 standard was used to check the perfluorokerosene (PFK) levels required to give a reasonable lock mass peak and on that occasion ratios ranged from 1.16-1.23 (average 1.19). These results showed that the D_3 now being used was pure. The following day another run was tried with a set of newly diluted samples from the same concentrated standards with the range of ratios now being 2.02-2.08. This result was taken to show that a mistake in sample dilution had occurred so these samples were checked on the ITD with resulting ratios of 1.013. It was confusing and disconcerting that solutions that had produced such a good calibration curve could now be seemingly so inconsistent. The 1 mg mL^{-1} standards derivatised and evaporated from the DIP gave ratios averaging 1.21 from full scans; the same as the calibration curve. These solutions were diluted ten-fold, with the mass spectrometer technician checking each preparation, and run both from the DIP and by GC using LRP full scans and both of these methods gave ratios close to the 1.124 mass ratio value. Since the MS80's integration function was not operating at that time, these ratios were obtained by expanding the peaks on the chromatogram traces, photocopying these, cutting out the peaks and weighing the paper cut-outs. When samples of the same solutions were derivatised and run in SIM the ratios ranged from 1.85-1.91. As a further check these solutions were now run by high pressure liquid chromatography (HPLC) against the standards of the Government analyst, at the DSIR, and proved to be the concentrations expected. Increasingly it appeared that there was a problem with the SIM software but it was extremely difficult to prove.

It was decided finally that Dr Winter should try his 1:1 standard (theoretical ratio 0.96) on the MS80. The first injection gave a ratio of 0.95 and

the second 0.50. The machine was retuned and recalibrated. For the next injections the ratios ranged from 0.4-1.2.; evidence at last that there was a definite fault in the SIM software. This was the first occasion on which such large variations in intensity ratio had been obtained from repeat injections of the same sample over a period of an hour or two. These results forced us to the conclusion that in spite of some consistent sets of ratios, such as those from the benzotropine study, there was a fault in the SIM software.

For the part of this work done on the MSD totally fresh standards were used. The DSIR (Gracefield, Wellington) provided 1.3 mg of commercial D_3-morphineHCl pre-weighed and shipped in a 10 mL volumetric flask and the D_0 standard was made from the D_0 previously recrystallised and sublimed.

4.3 DERIVATISATION CHEMISTRY

It seemed highly unlikely that the derivatising agent was selectively derivatising the D_0-morphine, but the possibility had to be ruled out. Underivatised mixtures were run on the DB-5 column and from the probe with no conclusive results.

4.4 SOFTWARE

DS90 acquires low resolution SIM data by switching the ESA voltage sequentially to the values required to focus the ions required at the collector. The ESA is calibrated using a known compound (normally perfluorokerosene) and ramping the voltage from full value to a value low enough to cover the range of masses required. The calibration results can be inspected manually to ensure that the calibration has been successful. Early versions of the software had frequent calibration problems, which were not always apparent on inspection, and some of the initial SIM problems arose from this. However, the version in use when serious SIM work for this project began had supposedly overcome the calibration difficulties. At this stage, Version 3.2 of

the software was installed, but again SIM proved to be impossible, this time because the software changed the magnet current at the start of each experiment; Version 3.3 overcame this aberration and Kratos were confident that this version would solve all the SIM problems.

The ESA voltage and magnet reference voltage on the MS80 are set by the data system via 18-bit digital-to-analog converters (DACs). During the SIM calibration process the DAC value corresponding to any m/z value is calculated and used in the subsequent SIM scans. This calibration is only satisfactory if the magnet current does not drift from its value during calibration. Kratos claim that SIM measurements at low resolving power (with peaks about 500 mmu wide in this study) can be satisfactorily carried out with the magnet in field control, because the magnet drift in field control, over several hours, should be much less than the width of the mass peaks. In fact this is a doubtful claim, at least at our installation, and two ways of overcoming this drift are provided. The magnet may be operated in current control, which is less convenient but does reduce drift, and the SIM software lock mass facility can be used.

Attempts to assess whether the software was functioning correctly were hampered by the fact that no visual indication of ion current is provided by the data system during SIM scans other than the total ion chromatogram, produced on the VDU after considerable manipulation of the acquired signal by the software. To overcome this, and enable the ion current to be observed during the switching sequences of the SIM experiment, a digital storage oscilloscope, constructed in this department, was connected to the mass spectrometer head amplifier in parallel with the data system. In this way, at any time during a SIM run the ion signal being received from the mass spectrometer can be observed directly.

A SIM experiment file, set up for this study, is shown in Fig 6. Option T sets the time taken for the instrument to switch the ESA through one

```
DS90                              EDIT EXPERIMENT FILE

A) Experiment file: MORPH2      [V]      T) Cycle time:      .80   seconds
B) Component file:  MORP
D) Delete Experiment file                P) ESA Settling time:       20   ms
E) Component file Editor                 M) Magnet settling time:     0   ms
Z) List Experiment files
V) Verify Groups                         R) Dwell Times? Fixed
J) Edit Group                            S) Sweep:        1500   ppm
K) Disable Group                         F) Lock Dwell:    100   ms
L) Display Masses                        G) Lock Sweep:   1500   ppm

Group    Start      End    Lock  Mass   Start Mass   Masses   Status
    1     3:00     7:00    .404.9758     390.0000        3   Enabled
```

```
DS90                                GROUP MASSES

                MASSES                Rel      Dwell     Mass
     Parent Ion       Daughter        Int%      Time     Status

1      414.1128          -.-           100       .20     Enabled    1) Current Group:  1
2      417.1317          -.-            90       .20     Enabled    2) Select Next Group
3      430.1078          -.-            10       .20     Enabled
                                                                     R) Restore Group

                                                                     Y) Enable all masses
                                                                     Z) Disable all masses

                                                                     E) Enable a mass
                                                                     D) Disable a mass
```

Figure 6: **SIM Experiment File**

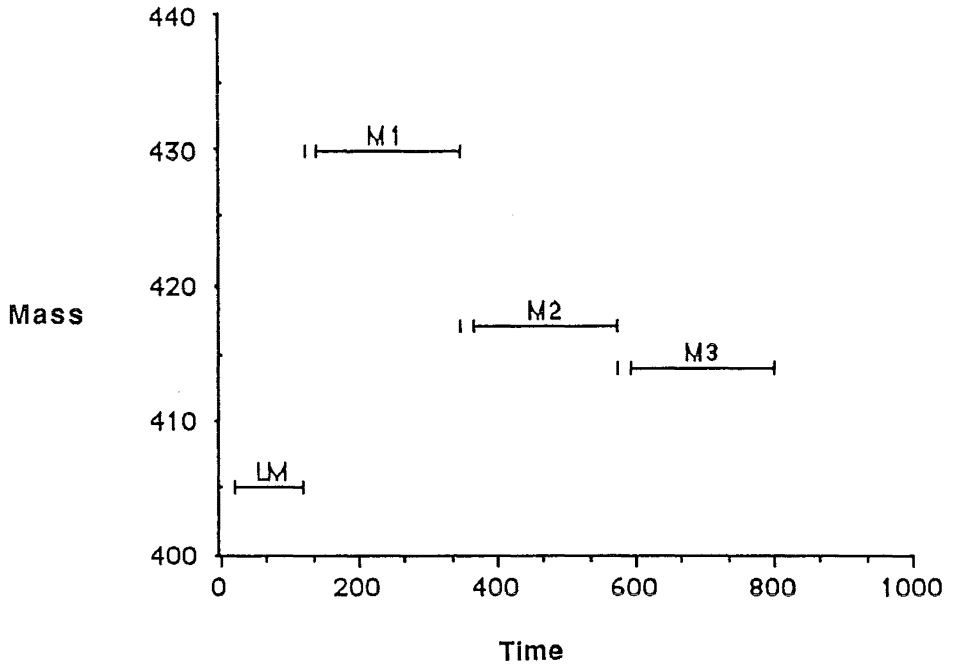
complete cycle of masses and return to its initial value. Option P sets the time allowed between switching to a new mass and beginning intensity measurement for that mass. Option M allows the magnet time to switch to a new field setting and settle - it was set to zero in this work because all measurements were made at constant magnetic field or current. Option R was always set to "fixed", thus ensuring that all masses were measured for the same time. Option S sets the ESA voltage sweep over each mass; this value may be zero, in which case the system switches to a single ESA voltage corresponding to the calculated peak centroid and measures the ion beam intensity for the whole dwell time at that voltage. Options F and G set the

dwell and sweep for the lock mass, if required - setting both to zero disables the lock mass function. Once these parameters have been entered the data system calculates the ESA voltage profile required - the profile for the file shown is illustrated in Fig 7 and the times calculated for each step shown in Table 1.

Table 1 : Electrostatic Analyser Cycle

Function	Time/ms
Settling time	20
Lock mass dwell	100
Monitored masses dwell	206.6

Fig 7: **Electrostatic Analyser Cycle**



It is clear that choosing inappropriate parameters could lead to unsatisfactory SIM results. Major likely sources of problems are:

i) Cycle time too long, so that too few cycles are acquired during a GC peak. It is generally assumed that at least 5, and preferably 8 complete

cycles should be acquired during elution of a GC peak and for this work, after some initial tests, a minimum of 8 cycles per peak was used. If too few cycles are acquired it is possible that the D_0 and D_3 peaks are "seen" differently as there are not enough points to reliably define the peak shape.

ii) ESA settling time too short. Although Kratos recommend 20 ms for the Mark II HV supplies on our MS80, they do so only because this was felt to be "probably adequate". The larger the voltage swing, the longer the settling time required, and some work was done to establish appropriate times.

iii) Sweep on monitored masses. Several enquiries to Kratos have failed to elicit any information about what intensity information is acquired during the sweep process. Tests done in this work suggest that the data system simply stores the largest A/D sample from the pre-processor interface. If this is so, too large a sweep could cause problems by including masses close to but not identical in mass to the selected ion. Too narrow a sweep could miss the selected mass completely, or measure only the edge of the mass peak. Clearly the optimum sweep will be a function of the MS resolution, and Kratos state that at low resolution (1000, 10% valley) no sweep is required; the system will focus the selected mass accurately enough to make a sweep unnecessary.

iv) In selecting the lock mass it is usual to choose the next significant PFK peak below the start mass. The lock mass is used by the data system to constantly update the ESA voltages required to focus the selected mass peaks, and thus compensate for magnet drift. It is, of course, essential in high resolution SIM measurements, but Kratos claim that, even in field control, SIM runs at 1000 resolving power do not require a lock mass. The advantage of this, of course, is that time in the SIM switching cycle is not spent monitoring the lock mass. Because so much difficulty was experienced in obtaining

sensible SIM results, most of the work in this thesis was done using a lock mass. In hindsight this proved to be an unfortunate decision, although early tests with and without a lock mass seemed equally unreliable.

4.4.1. SIM Calibration

The DS90 software allows the SIM calibration to be checked visually by stepping sequentially through the masses to be monitored using the keyboard. This was done with earlier versions of the software using the following PFK masses: 149.99, 161.99, 167.99, 180.99. A lock mass of 130.99 was used. Early versions of the software often showed that the calibration was inaccurate, with set masses significantly displaced from the calibration point; the lock mass often showed the greatest error. Version 4 of the software appeared to have eliminated the problem.

4.4.2. Lock Sweep

In early attempts to use the lock mass function, lock sweep values of around 50 - 200 ppm were used. These are much smaller than the peak widths characteristic of resolutions employed in this work, (~ 1000 ppm) and it appeared that the software could not correctly determine the peak centroid using such small sweep values. Variations of the lock sweep were tried:

D0:D3 Theoretical Ratios	Lock sweep (ppm)	Ratios (range)
0.9776	100	0.42-0.84
0.9776	350	0.32-1.34
0.9776	800	0.29-0.48
1.1244	1500	1.85- 2.07

Later work used values of at least 1500 ppm. However the lock mass sweep value seemed to have little effect on the variability of the D0:D3 ratios.

4.4.3. Lock Dwell

When the early SIM problems with morphine were first noticed it was decided to try and establish good working parameters using an alternative chemical system. Pentamethylbenzene (PMB) was chosen because of its relatively short retention time on the GC column. Two ions, 133 and 148, of the PMB were monitored by LRP giving an intensity 133:148 of 1.88 (range 1.8 - 2.0) over 8 runs.

The SIM results (sweep 600, lock mass 130.99, lock sweep 800) were as follows:

lock dwell	ratio
30	1.73-2.75
200	1.52-3.79

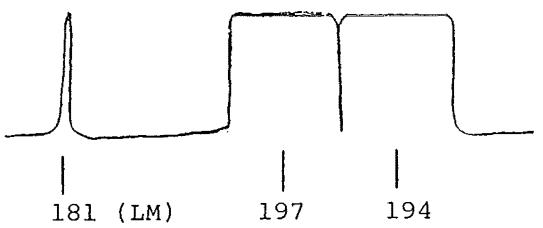
These and other results indicated that the lock dwell time was not critical.

4.4.4. Settling time

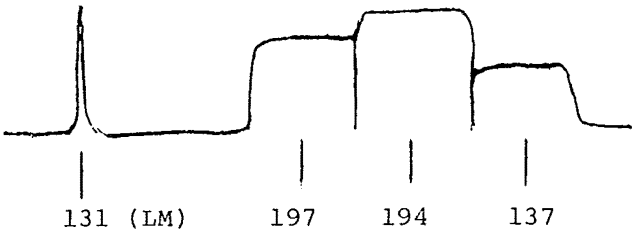
A caffeine investigation was running concurrently with the morphine work. A 1:1 mixture of D_0- and D_3-caffeine (D_0:D_3 = 1.028) was used to investigate the settling time. A lock mass of 180.98 and a settling time of 20 ms was used with the following results:

Run 1: monitoring m/z 197 and 194. D_0:D_3 measured 1.014 (an average of 5 runs)

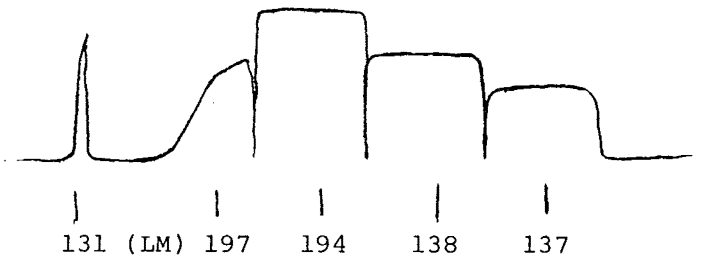
Visual inspection of the storage scope during the SIM runs showed :



Run 2: monitoring 197, 194 and 137. D_0:D_3 0.753 (6 runs) :



Run 3: monitoring 197, 194, 138, 137. D_0:D_3 0.631 (5 runs)



This and related experiments established that a large change in mass (requiring a large switch in ESA voltage) caused the intensity of the first mass monitored after the change to be unreliable, almost certainly as a result of the inadequate settling time. Results similar to those above were obtained even with a settling time of 50 ms; settling times longer than this become a problem because settling then takes up a large part of the cycle, with a reduction in the time spent on each mass.

Although many of the measurements to determine the source of this problem were made using ions only 2 amu apart, most used a lock mass which is often 15-20 amu away from the first measured mass. Since it seemed that the Mk II power supplies might need longer settling times than desirable, some experiments were made using a dummy mass to reduce the total settling time. This suggestion was taken up by Kratos, who now recommend this as standard practice in instruments fitted with these power supplies, using relative dwell settings to vary the settling times. The principle is illustrated in the following example.[54]

When switching over a wide mass range it is advisable to use a dummy high mass because SIM starts at the high mass and works down; the large swing at the start of the cycle causes problems. The Relative dwell can then be used together with specified intensities so that a short time is spent on the dummy mass and longer equal times on the masses being monitored; e.g. if the relative intensity of the dummy mass is set at 100% and for the other masses at 20%, then each set mass will be monitored five times longer than the dummy mass.

In a programme with a dummy mass, four set masses, a lock mass dwell of 100 ms and a settling time of 20 ms the following would occur. The total dwell time on the 5 masses for a 1 s cycle would be: 1000 - 100 - (6 x 20) = 780 ms The total dwell on all ions is 1 (dummy) + (5 x 4) (set masses) = 21 units. Thus the dwell on the dummy is 780/21 38 ms and the dwell on each set mass is 186 ms. The time for the swing from the lock mass to the first monitored mass will be 77 ms (i.e. 20 + 20 + 37 ms)

Although this approach will presumably improve the reliability of SIM quantitation, it was not used further in this work because the HPMSD had become available. The evidence from the work summarised above overwhelmingly suggested that the problem lay in the software, and Kratos accepted that conclusion. Over a period of several months Kratos located and corrected the following problems:

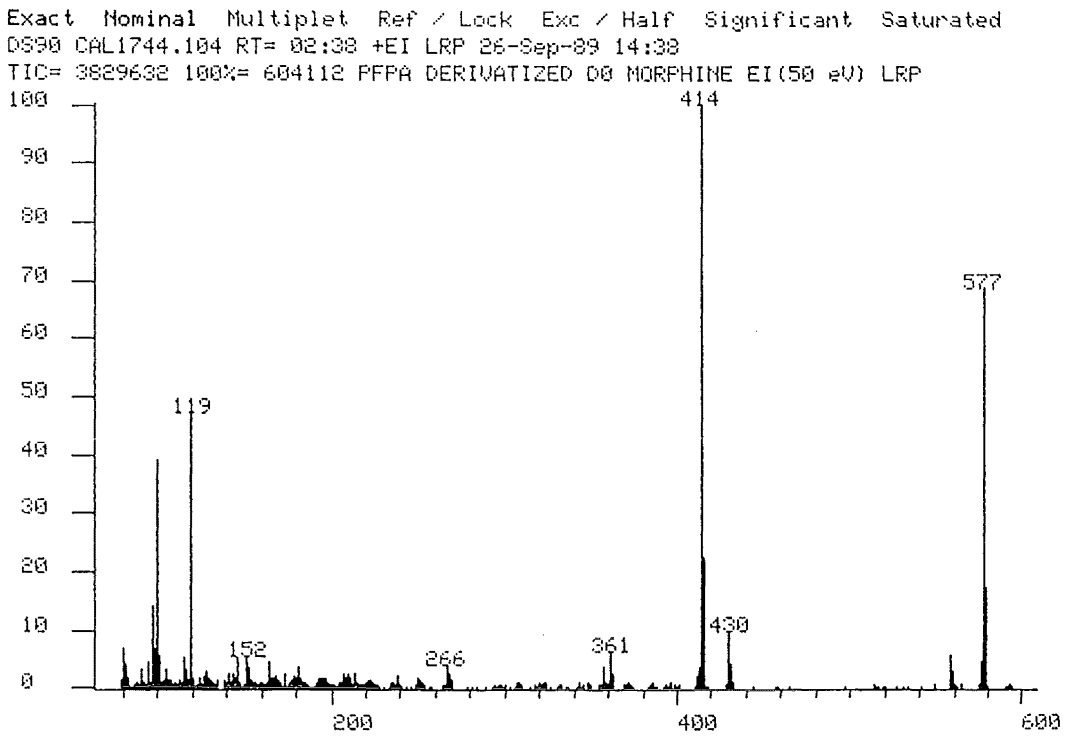
i) If lock sweep values of less than 500 ppm were used the lock mass function either did not work or worked incorrectly.

ii) In the calibration routines the intensity tolerances (pre-set in the software) were too small; calibrations failed frequently, but failures were often reported as successes.

iii) The lock mass drift correction worked only when the number produced by the calculation was odd. In version 4 of the software[55] an odd number was required to be generated by the lock mass correction algorithm for a correction to be made - an even number resulted in no correction.

Version 5 of DS90, supposedly incorporating all of these changes became available only a few weeks before completion of this work. Brief tests suggest that SIM is now producing satisfactory results for the D_0/D_3-morphine pair m/z 414/417, but time did not allow any extensive tests to be carried out.

This part of the thesis proved to be frustrating and time consuming. Because assessment of all possible sources of the problem had to be carried out over the same period, concurrently with synthesis of D_3-morphine, and because each new version of the software had to be installed, tested and the SIM performance checked, it was very difficult to test one possibility at a time. Furthermore, the fact that the software sometimes performed superbly made it difficult to justify the conclusion that it was faulty.



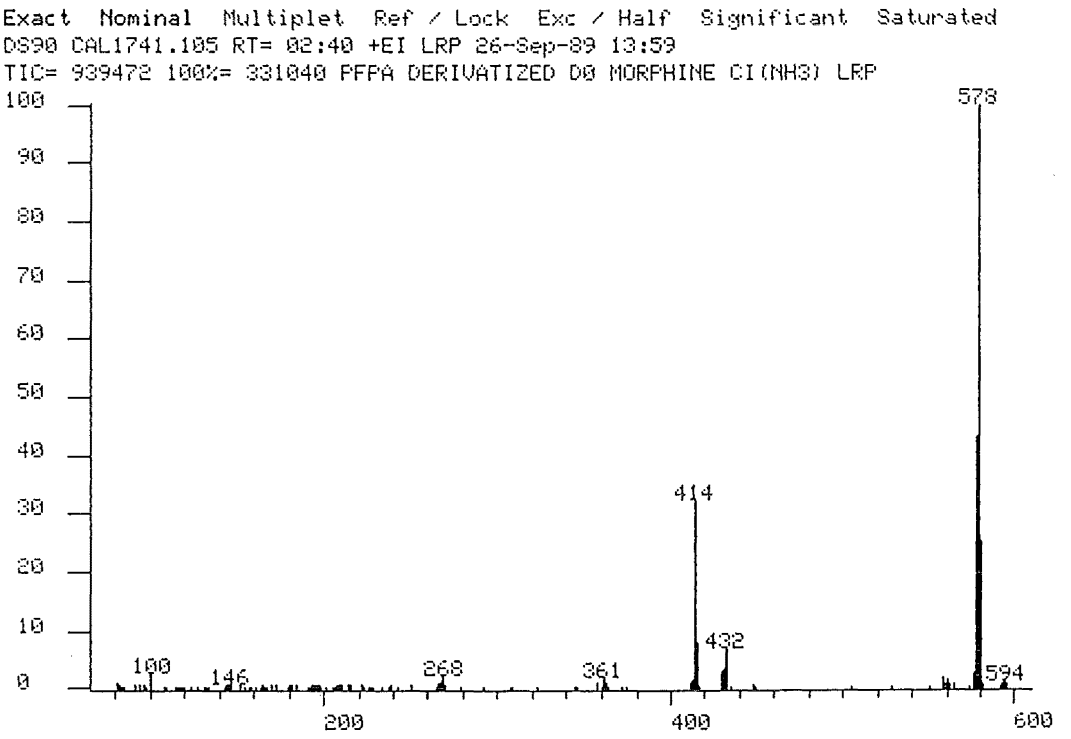
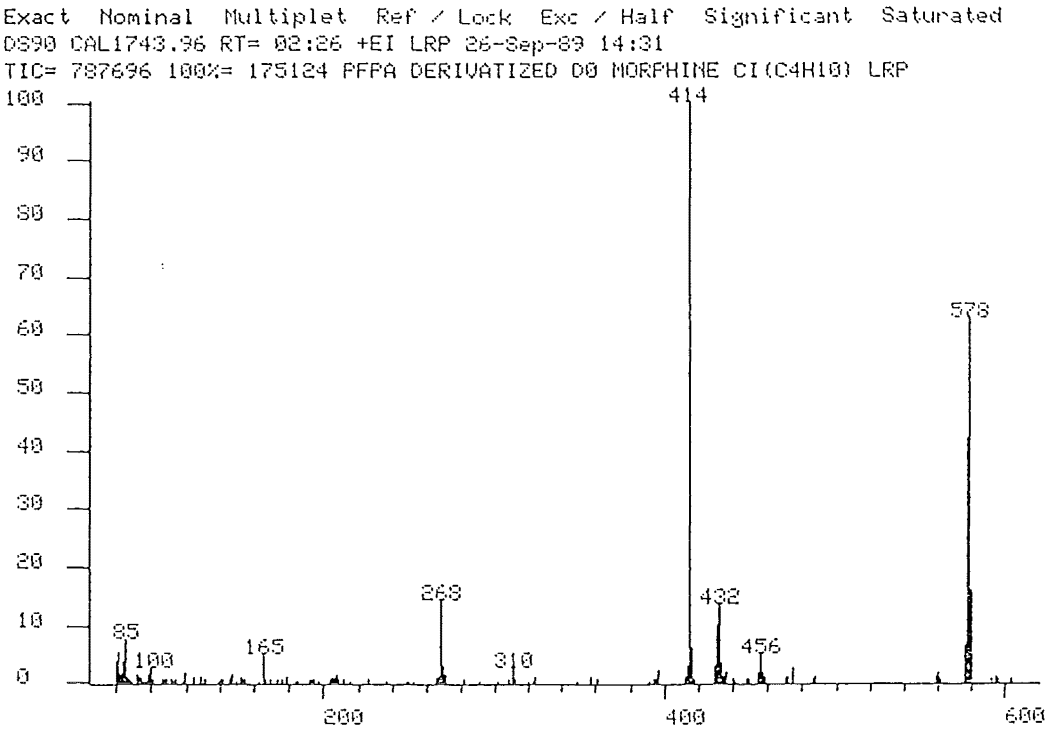


Figure 8a and 8b



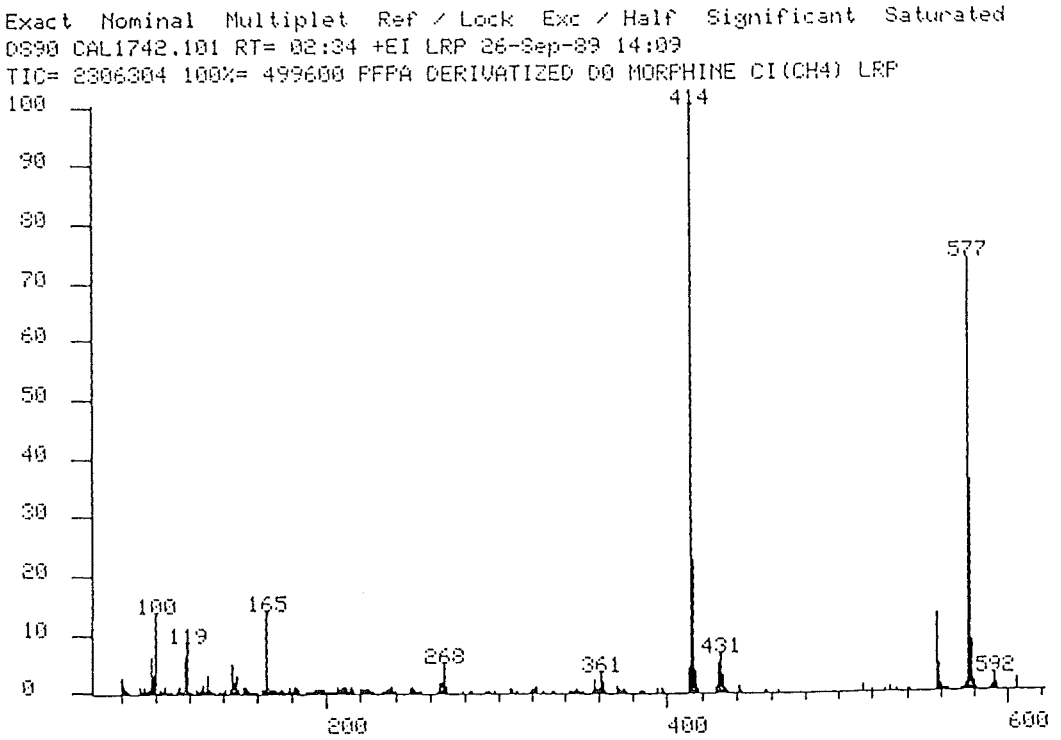
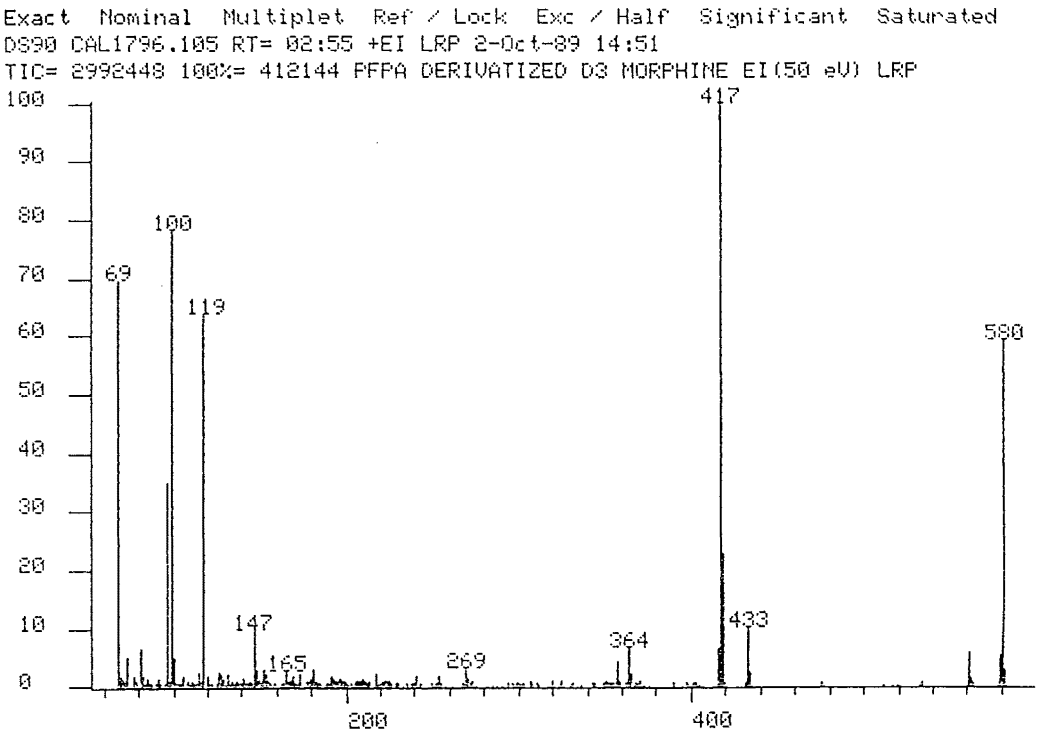


Figure 9a and 9b



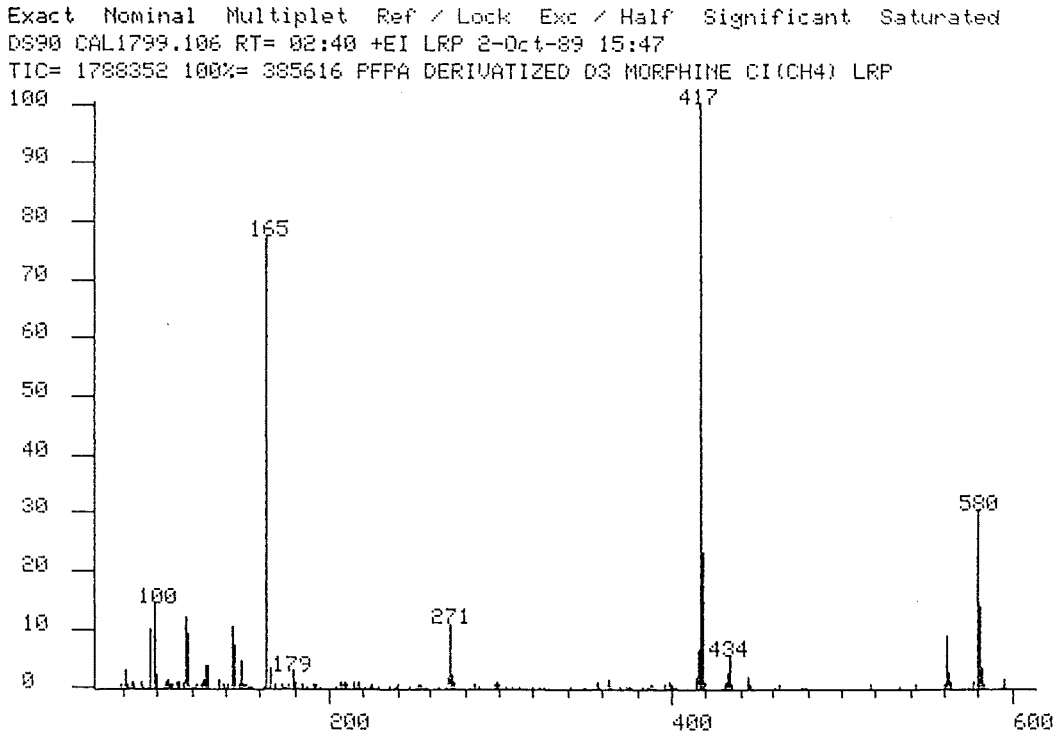
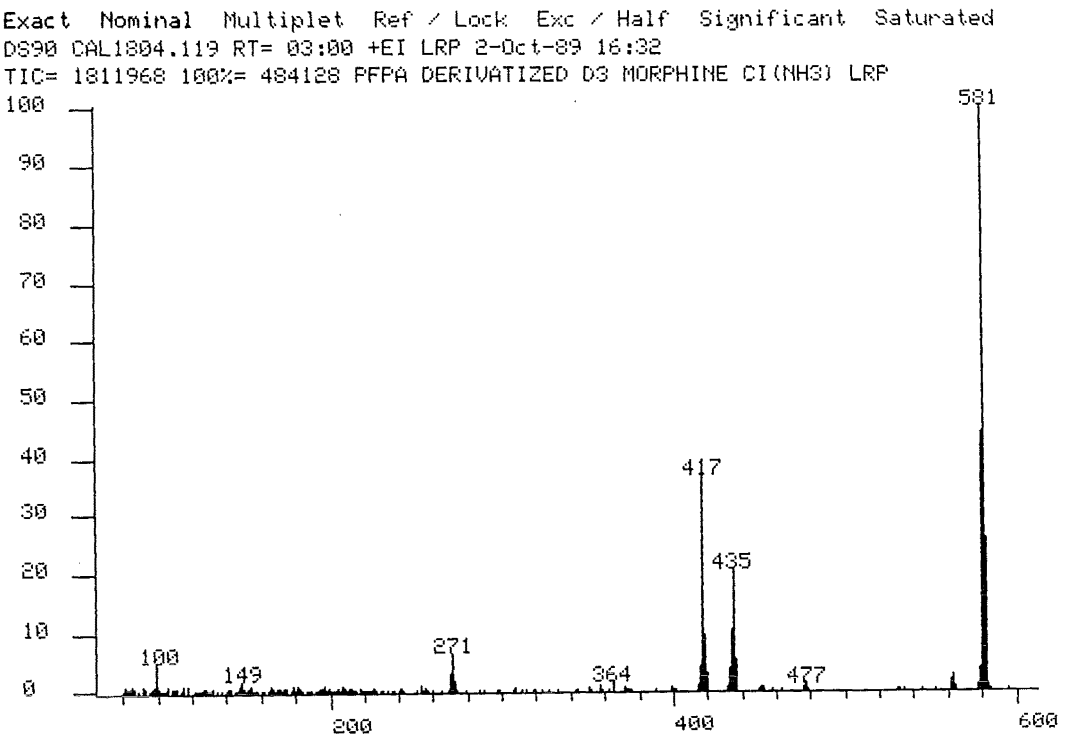


Fig 10a and 10b:



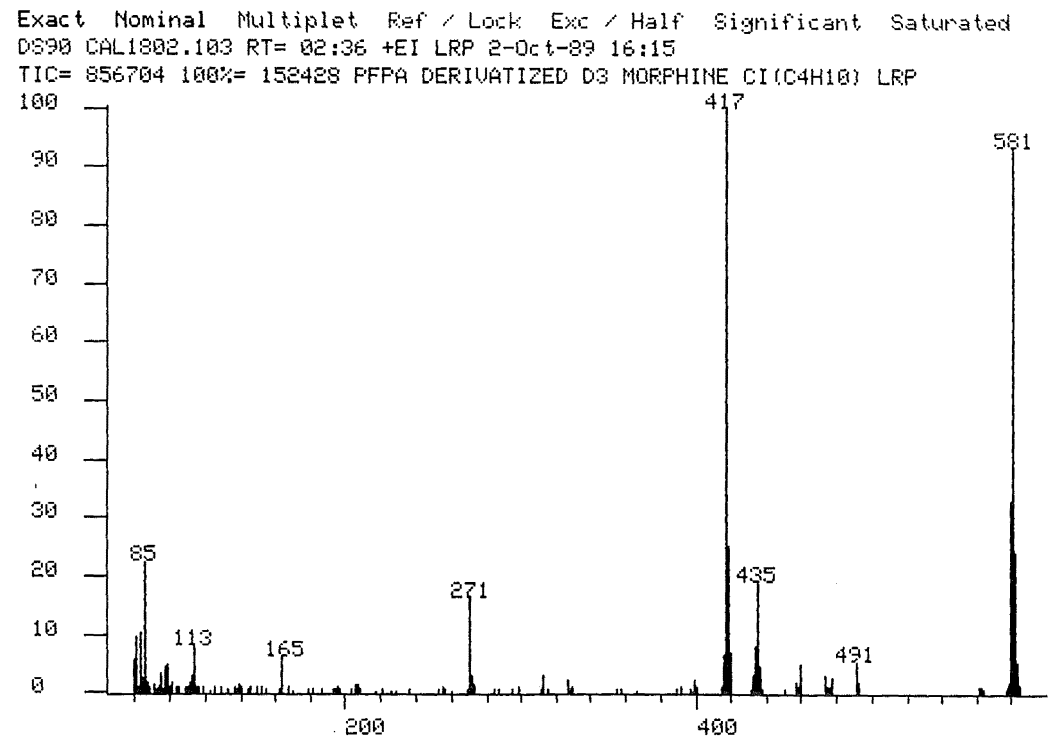


Fig 11a and 11b:

CHAPTER FIVE

CALIBRATION CURVES AND THE INTERNAL STANDARD

5.1 PREPARATION OF N-TRIDEUTERIOMETHYLNORMORPHINE

During the extraction of morphine from plasma loss of the drug almost certainly occurs, so that the concentration established in the final analysis is not the concentration actually present in the original plasma sample. This problem can be overcome by the addition of a known amount of internal standard at the start of the extraction process and for the analysis of morphine, N-trideuteriomethylnormorphine (D_3- morphine) was used.

The preparation of D_3-morphine became a section of work for this thesis. The method developed was an adaptation of methods used for the N-demethylation of morphine. The N-demethylation of morphine to normorphine has received little attention and the few methods reported were very similar. The procedure of Abdel-Monem and Portoghese[56] for the preparation of normorphine involved the hydrolysis of N-3,6-tricarbophenoxynormorphine to N-carbophenoxynormorphine, its chromatography and crystallisation, followed by cleavage in ethanoic potassium hydroxide to give an overall yield of ~ 40%. Rice *et. al.*[57] in their refinement of this procedure used aqueous hydrazine to cleave the same intermediate carbamate which they found unnecessary to isolate and purify before proceeding. They found the analytically pure normorphine product precipitated from the hydrazine mixture in 95% overall yield. Montzka *et. al.*, using a procedure similar to Portoghese,[58] reported the N-demethylation of morphine, in 75% yield, using 2,2,2-tricloroethylchloroformate to produce trichlorocarboethoxynormorphine. This was converted to normorphine by zinc reduction in high yields but Rice reported very poor yields (< 40%) from his attempts to repeat this work.

Previous attempts in this department to prepare D_3-morphine had been made by P. A. Gnad,[44] using a modification of Portoghese's method, with yields between 9 and 37%. It was decided to repeat this work but, because of the unavailability of phenylchloroformate, the 2,2,2-trichloroethylchloroformate was tried first. B. M. Clark, in this department, had previously prepared the 2,2,2,-trichloroethoxynormorphine intermediate, as a yellowish-brown gum. This was reduced using lithium aluminium deuteride (LAD) to produce N-trideuteriomethylnormorphine as a white powder. As repeated attempts at crystallisation were unsuccessful, the product was chromatographed on a C-18 reverse-phase column eluting with a 50:50 methanol/water mixture. A combination of the appropriate fractions, identified by t.l.c., gave D_3-morphine in 12% yield (based on the carbamate) (Fig 12) Clark's previous preparation, using

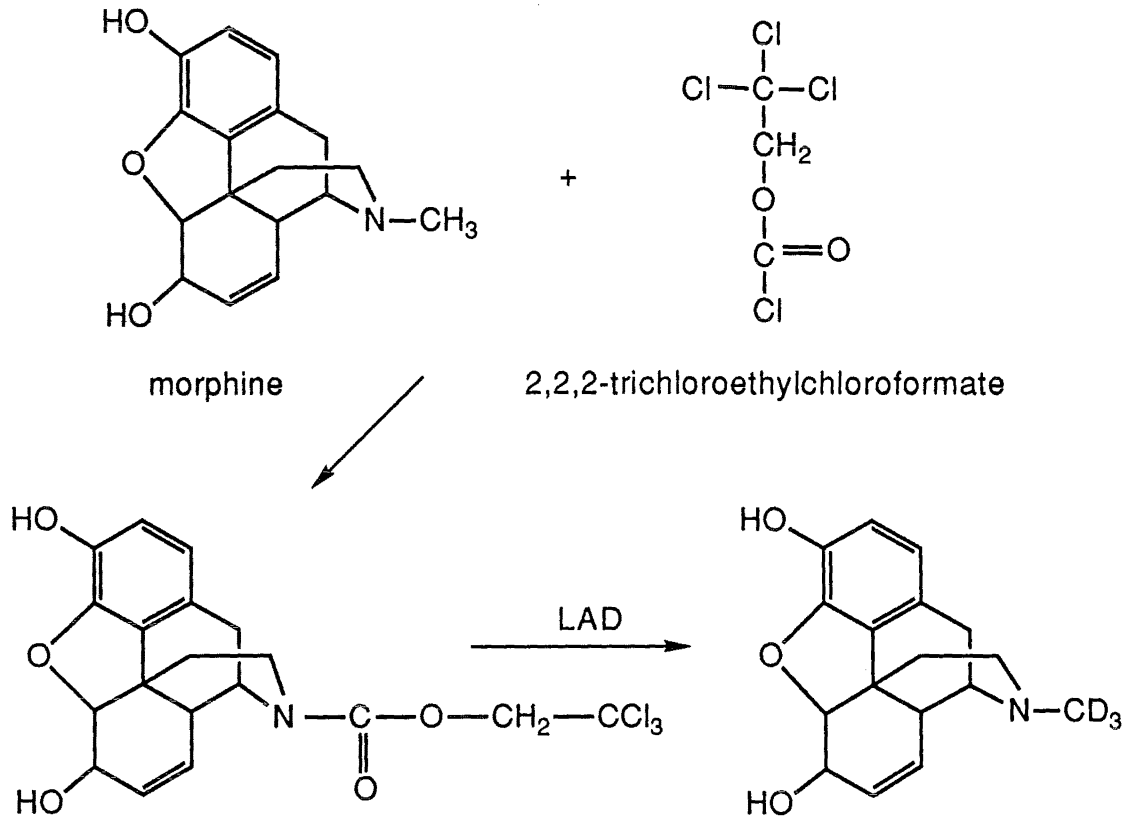


Figure 12: **Preparation of D3-Morphine (Scheme I)**

the same method and batch of carbamate, had yielded 4% (also based on the carbamate).

It was thought that the problems in extracting the D_3-morphine from these preparations and the low yields, might be due to impurity of the carbamate intermediate. It had been shown by mass spectrometry that this material was actually a mixture of the carbamate and chloroethanol. For the second preparation, using the Montzka method, the trichlorocarboethoxynormorphine was made and recrystallised to produce large white cuboid crystals in 39% yield. A second attempt gave a similar yield. LAD reduction of this intermediate produced, after recrystallisation from methanol/water, white needle-like crystals of D_3-morphine in 22% yield. Purifying the carbamate had made the D_3-morphine more readily recoverable without the necessity of the chromatographic procedures.

With the subsequent arrival of the phenylchloroformate starting material the 2,2,2-trichloroethoxynormorphine reaction was not further pursued. The Gnad method was followed to prepare the N-carbophenoxynormorphine and produced a brown viscous oil. This material was chromatographed twice on a silica gel column and yielded a creamy-yellow solid. Attempts to recrystallise this material from hot chloroform and methanol were not successful. This product was set aside. The second preparation of N-carbophenoxynormorphine produced a deep yellow oil which was distilled under high vacuum as suggested by Rice[57]. This removed the phenol and yielded the carbamate as a golden yellow solid which was reduced immediately with LAD. Attempts to recrystallise this product were also unsuccessful and it was purified by chromatography using a C-18 reverse-phase column. The solid product recrystallised from methanol/water to give D_3-morphine in 35% yield. This product was sublimed in a cold-finger apparatus yielding anhydrous D_3-morphine, shown by mass spectrometry using the DIP to have a greater than 99% deuterium incorporation (Fig 13).

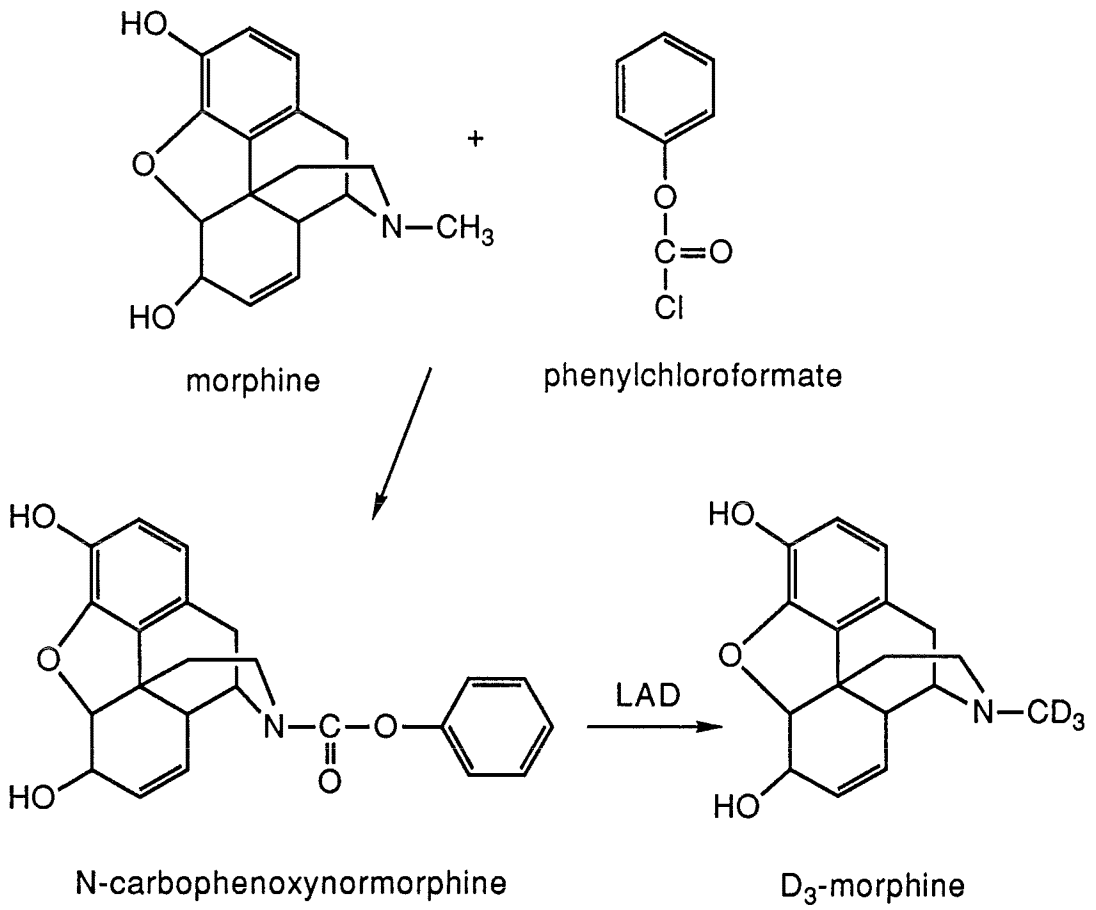


Figure 13: **Preparation of D3-Morphine (Scheme II)**

Nalorphine has been produced from morphine in a 70% overall yield[59] using vinyl chloroformate. Olofsen *et. al.*[60] report the synthesis of noroxymorphone from oxymorphone in 98% yield (an increase of ~80% over previously used methods) using vinyl chloroformate. The increased yields are attributed to the enhanced electrophilicity of the acyl carbon attached to an electron withdrawing -OCH=CH_2 moiety. If this compound were available it would be interesting to attempt to use it to produce D_3-morphine by modification of the previous reactions.

5.2 QUANTITATIVE GCMS ANALYSIS

Quantitation of morphine was based on M^+ ion intensities from TMS-D_0-morphine (m/z 414, 429) and TMS-D_3-morphine (m/z 417, 432) for the MS80 and from PFPA-D_0-morphine (m/z 414,577) and PFPA-D_3 (m/z 417, 580). The resulting ion intensity ratios, measured as ratios of the heights of the peaks -

m/z 429 : m/z 432, m/z 414 : m/z 417 and m/z 577 : 580 were used, with the calculated weight ratios, to construct calibration graphs. The calibration graphs were constructed, using standard solutions of D_0 and D_3-morphine, to establish that the relationship between the mass spectrometer ratios and the mass ratios was linear and to define errors in the analysis. The previous study had shown no significant difference between the calibration curves derived from aqueous morphine solutions and those from morphine extracted from plasma. For this study the standard aqueous solutions were used. The calibration graph obtained for the MS80, with a total $D_0 + D_3$ concentration of 100 ng mL^{-1} of solution, used the weight ratios (D0:D3) 0.25, 0.50, 1.00, 2.00, and 4.00. Two calibration graphs were obtained for the MSD. The first at the 500 ng mL^{-1} concentration used the weight ratios (D_3:D_0) 0.25, 0.5, 1.00, 2.00 and 4.00 and the second at 30 ng mL^{-1} used 0.20, 0.50, 1.00, 2.00 and 3.00. The preparation of the standard samples was as follows. Methanol standard solutions of D_0- and D_3-morphine were diluted to prepare stock solutions at the 100 ng mL^{-1}, 2 ng mL^{-1} and 1 ng mL^{-1} level. From these stock solutions the following ratios were measured into silanised Reacti-vials:

For the calibration curve at the 500 ng mL^{-1} concentration using the 100 ng mL^{-1} stock solution and for the 100 ng mL^{-1} curve using the 2 ng mL^{-1} solution:

D_0:D_3 1:4 (20 µL and 80 µL)

1:2 (33 µL and 66 µL)

1:1 (50 µL and 50 µL)

2:1 (66 µL and 33 µL)

4:1 (80 µL and 20 µL)

For the 30 ng level the following ratios were prepared from the 1 ng mL^{-1} solution:

D_0:D_3 1:5 (10 µL and 50 µL)

1:2 (20 µL and 40 µL)

1:1 (50 µL and 50 µL)

2:1 (40 µL and 20 µL)

3:1 (45 µL and 15 µL)

Using the ion intensity ratio to weight ratio data (Appendix II) a linear least squares procedure was used to find the regression line for the calibration graphs. The regression analyses were carried out by treating the mass ratios from the standard samples as the independent variable and the measured ion intensity ratios as the dependent variable.[61] Calibration curves are shown for the m/z 414 : m/z 417 ion pair at each of the concentrations used and also for the m/z 577 : 580 ion pair at the 30 ng mL-1 and the 500 ng mL-1 concentrations (Figs14 to 18, pp. 56-60).

5.3 STATISTICAL ANALYSIS OF THE CALIBRATION CURVES

The MINITAB statistical package[62] was used to fit the calibration curves, by the method of least squares, to the plots of Peak Height Ratio against Mass Ratio. From these plots it is clear that the data points display considerable heteroscedasticity, i.e. the spread of the points increases with the value of the D_0:D_3 Mass Ratio. Much of this is due to the method of integration of the areas under the peaks. For this reason the weighted least squares technique provided in MINITAB was used (see MINITAB Reference Manual, page 109). For each D_0:D_3 Mass Ratio, the sample standard deviation of the Peak Height Ratio observations was calculated, and the reciprocal of this standard deviation was used as the weight applied to these observations. Samples containing D_0 and D_3 in the ratio of 4:1 were excluded as calibration standards at the 30 ng level because in the previous study such samples were found consistently to give points below the least-squares line on the 60 ng mL^{-1} calibration graph. This deviation was attributed to the M+3 peak m/z 432 of TMS_2-D_0-morphine

contributing significantly to the intensity of the M^+ ion of the TMS_2-D_3-morphine (m/z 432) in a mixture with a large proportion of D_0-morphine. Curvature in a calibration graph can also be caused by isotopic impurity of the labelled compound. In the 1:4 D_0:D_3 morphine case the D_3 morphine will show a low intensity due to the presence of a small amount of unlabeled TMS-D_0-morphine. Calibration curves were also fitted at the 30 ng concentrations using only the values up to 2:1. These are given below. They showed very little difference from those for the full range of concentrations.

The standard deviation of the estimate of the slope of the regression equation is given in parentheses under the estimated slope. For all the regressions the slope of the line was significantly different from unity at a significance level well below 1%. The value of the coefficient of correlation (R^2) is also given.

414:417 Ion Pair

30 ng.

Peak Height Ratio = 0.16975 + 0.67970 Mass Ratio
(0.02028)

$R^2 = 0.988$

100 ng.

Peak Height Ratio = 0.03181 + 1.03236 Mass Ratio
(0.01225)

$R^2 = 0.997$

Up to 2:1 Ratios

Peak Height Ratio = 0.15057 + 0.71231 Mass Ratio
(0.02123)

$R^2 = 0.990$

500 ng.

Peak Height Ratio = 0.06998 + 0.92697 Mass Ratio
(0.01872)

$R^2 = 0.989$

577:580 Ion Pair

30 ng.

Peak Height Ratio = 0.10812 + 0.77920 Mass Ratio
(0.05215)

$R^2 = 0.941$

Up to 2:1 Ratios

Peak Height Ratio = 0.10654 + 0.77686 Mass Ratio
(0.02301)

R2 = 990

500 ng

Peak Height Ratio = 0.02609 + 1.04409 Mass Ratio
(0.00947)

$R^2 = 0.998$

Since the closeness of the Peak Height Ratio to the Mass Ratio is of interest, tests on the null hypothesis that the slope of each regression line is 1, against the alternative that it is not one, were also carried out.

	t-statistic	Degrees of Freedom	Conclusion
414:417 Ion Pair			
30ng.	-15.794	14	Reject at 1% level
<3 : 1	-13.551	11	Reject at 1% level
100ng	2.642	23	Reject at 5% level Accept at 1% level
500ng	-3.901	27	Reject at 1% level
577:580 Ion Pair			
30ng.	-4.234	14	Reject at 1% level
<3:1	-9.697	11	Reject at 1% level
500ng	4.656	27	Reject at 1% level

Thus while it is clear that several of the samples, especially those at the 100 and 500ng level, had calibration curve slopes that were very close to 1, the hypothesis that the true slopes are exactly 1 cannot be statistically supported.

The important criterion of linearity is satisfied.

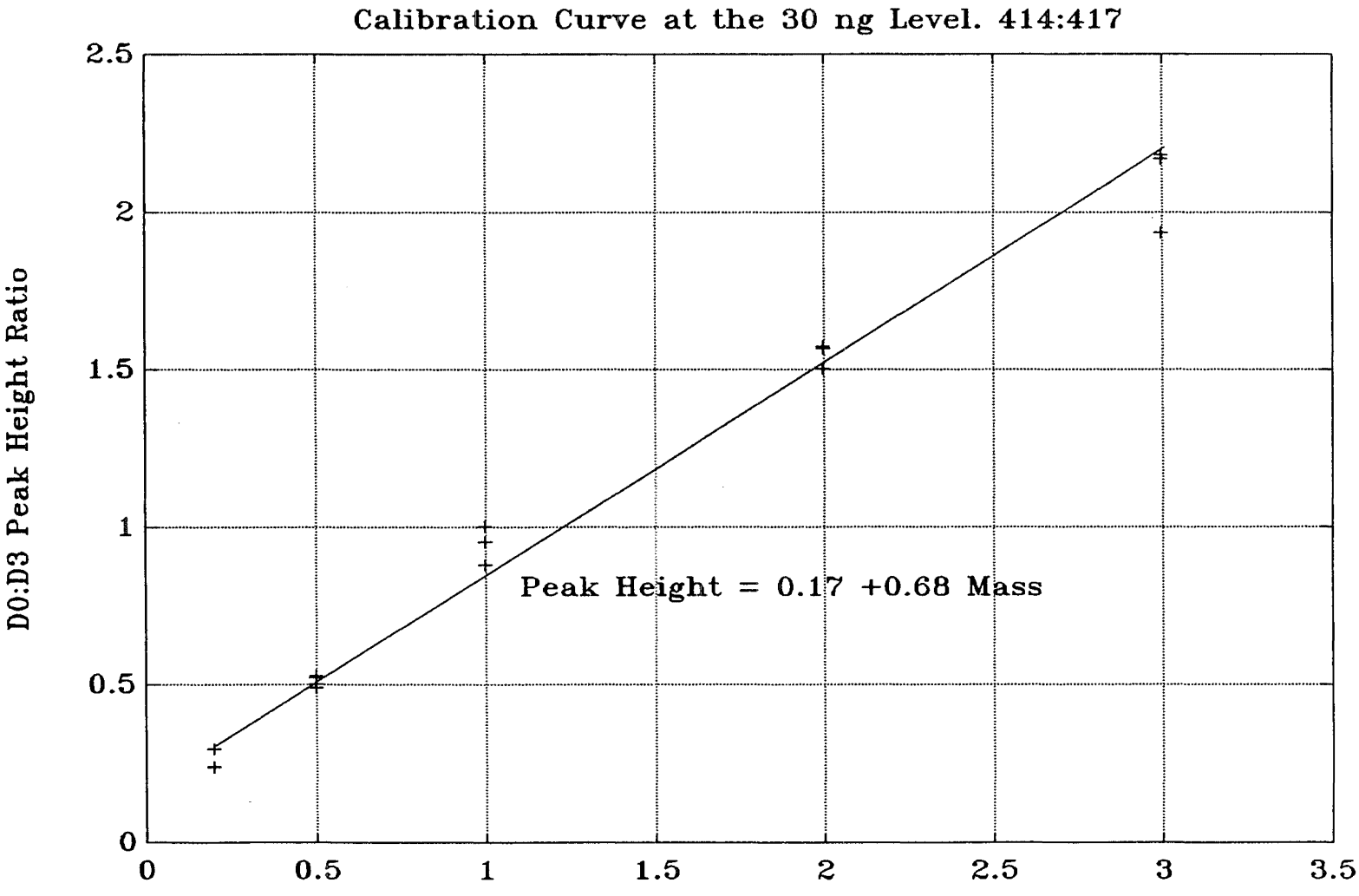


Figure 14 D0:D3 Mass Ratio

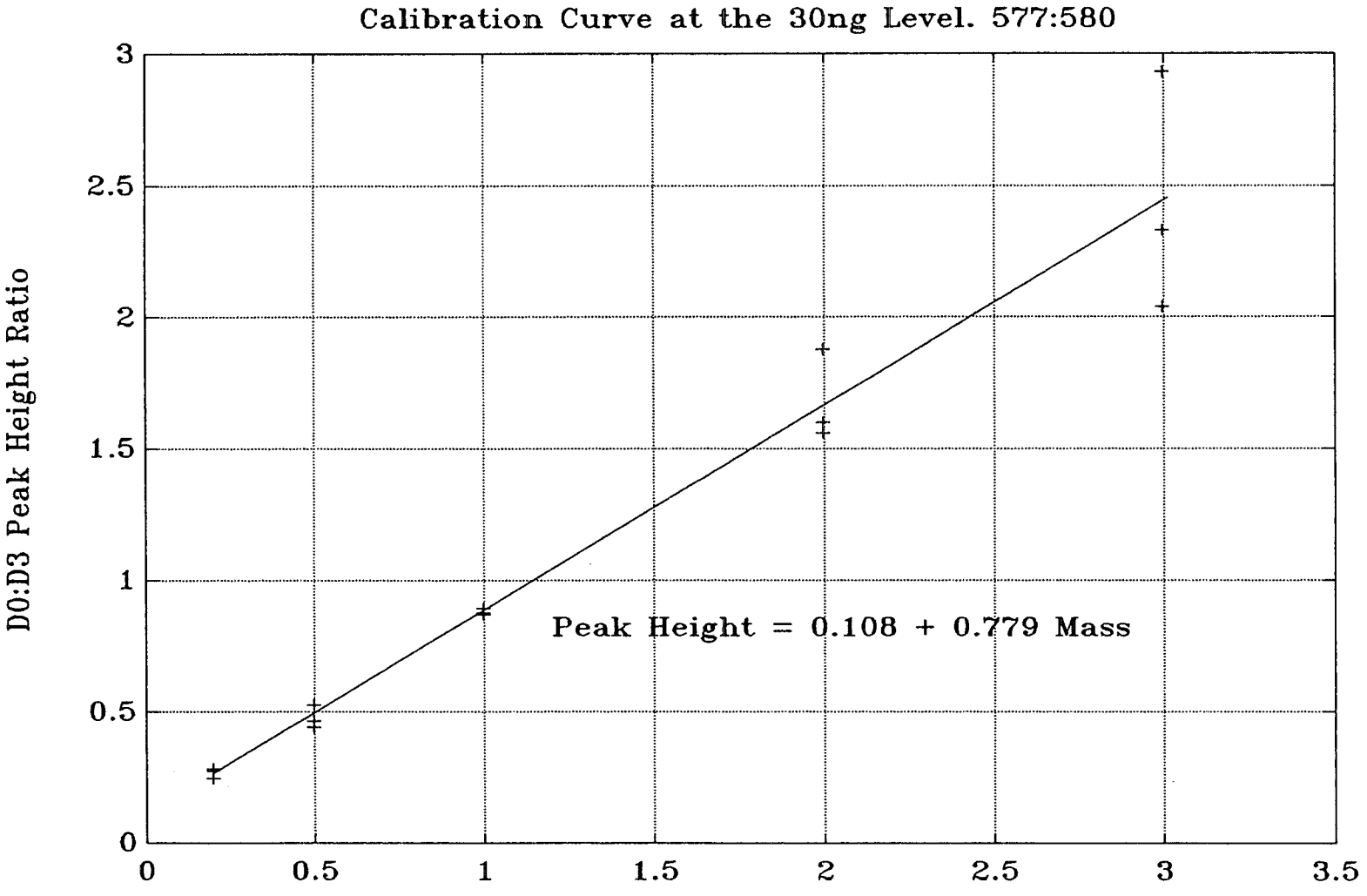


Figure 15 D0:D3 Mass Ratio

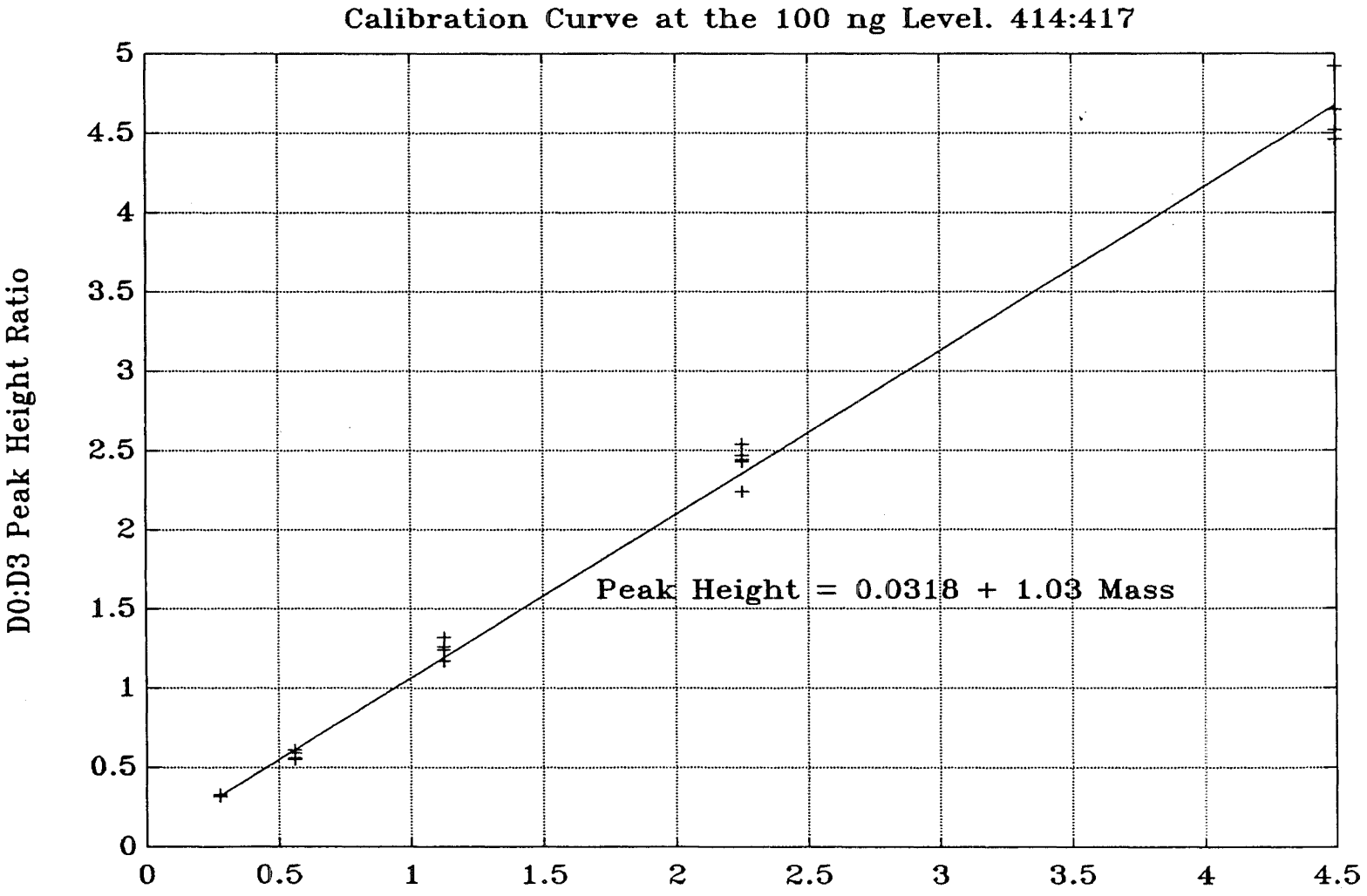


Figure 16 D0:D3 Mass Ratio

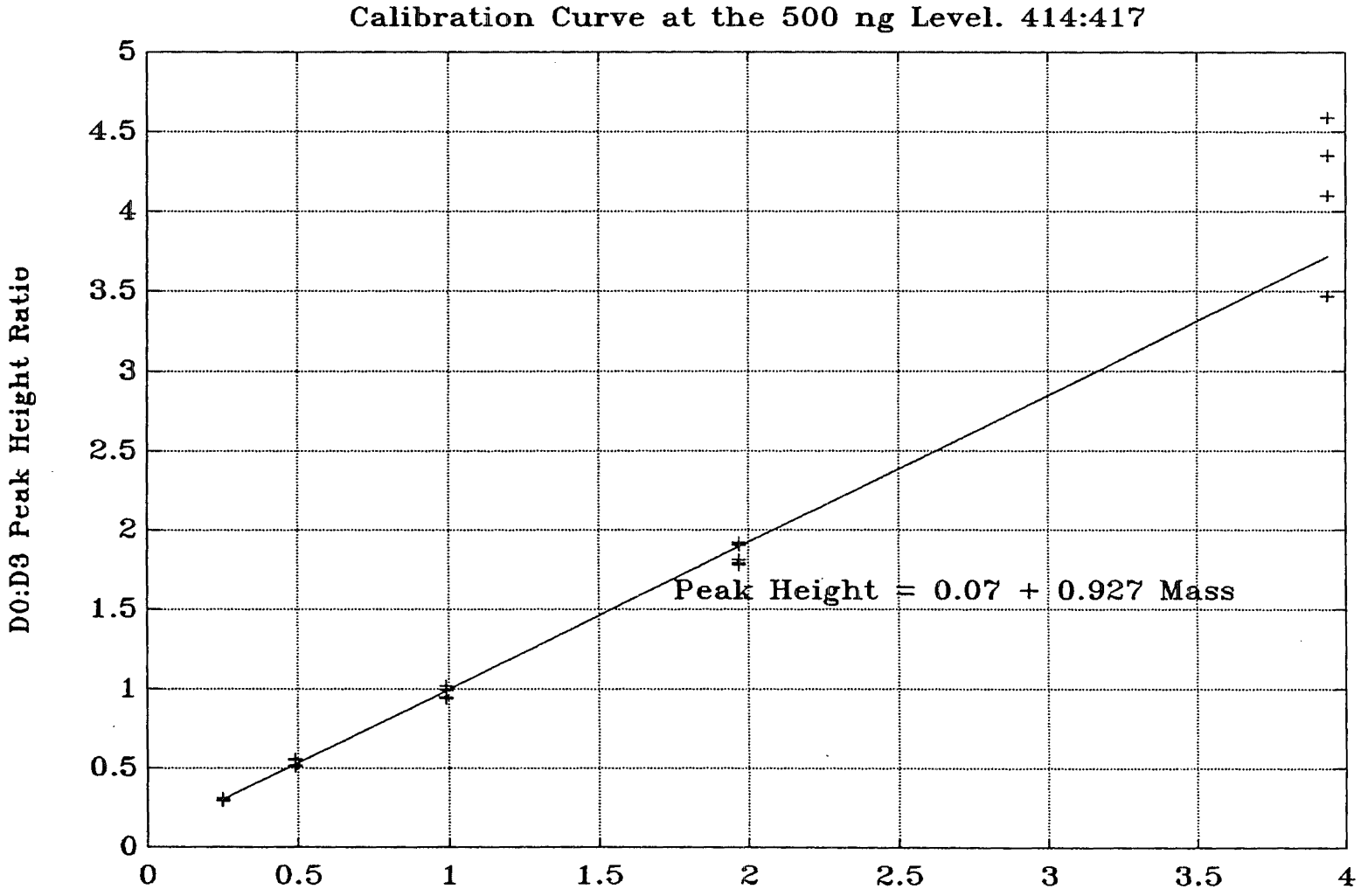

Calibration Curve at the 500 ng Level. 414:417
D0:D3 Peak Height Ratio
Peak Height = 0.07 + 0.927 Mass
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
D0:D3 Mass Ratio

Figure 17

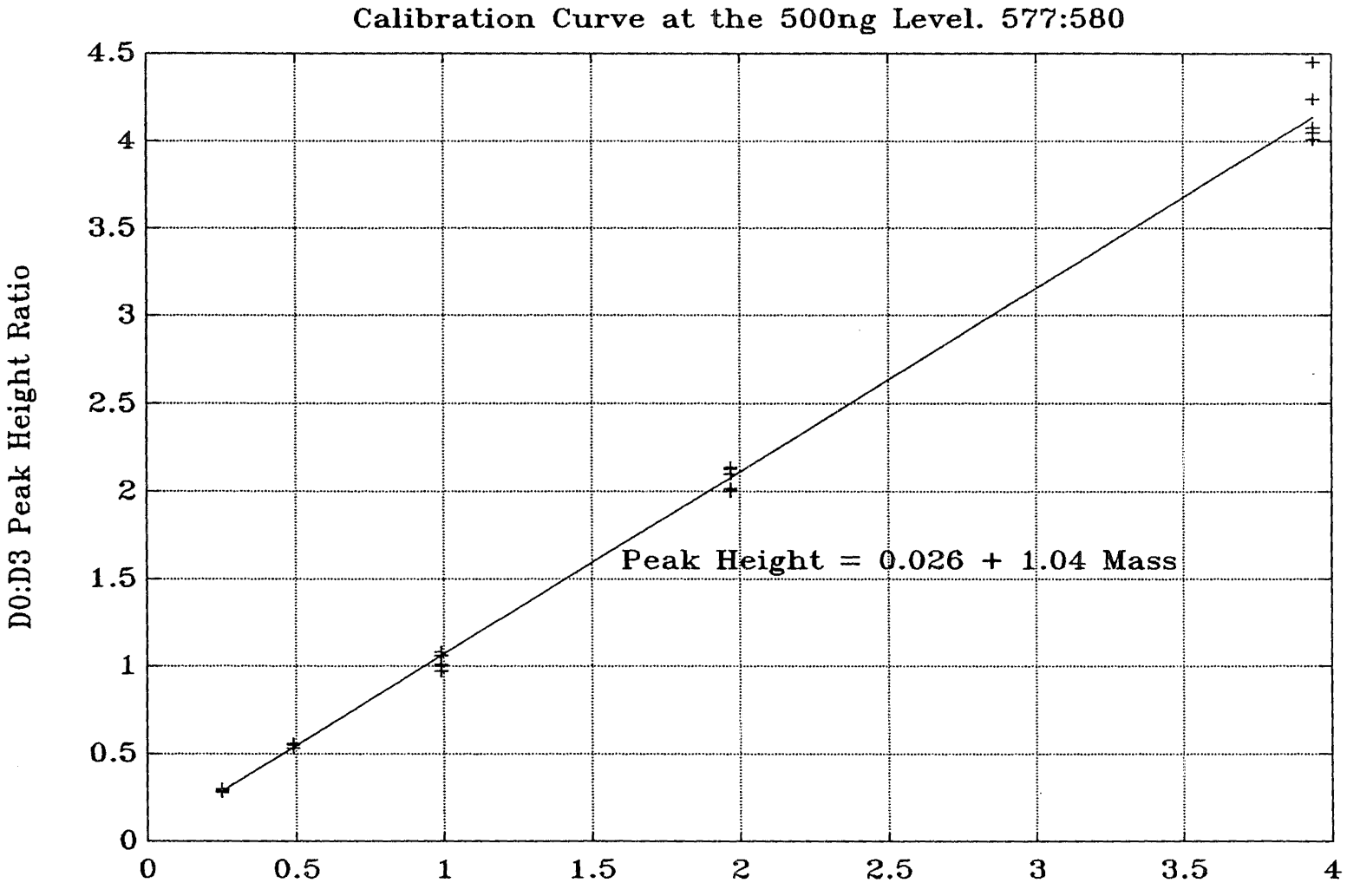


Figure 18 D0:D3 Mass Ratio

CHAPTER SIX

PATIENT STUDIES

6.1 PATIENTS AND NORMAL SUBJECTS

For the earlier Canterbury study the patients were four men with renal failure, of whom one was anephric. All four were on maintenance dialysis (Appendix I). Because the liver was believed to be the major site of morphine metabolism it was important to establish that the renal failure patients were free of liver disease. Normal serum concentrations of the enzyme aspartate aminotransferase (AST) in all these patients was taken as an indication of their freedom from liver disease as the concentration of this enzyme is normally high in liver but low in blood - liver cells damaged by disease release this enzyme causing abnormally high AST serum concentrations. Serum albumin concentrations were in the normal range for all patients except one. Morphine in plasma binds to this protein and it had been suggested[26] that reduced albumin concentrations during renal failure might cause unusually high unbound plasma concentrations. Renal failure patients are frequently also anaemic and all the patients studied had significantly lower than normal haemoglobin concentrations. One of the consequences of anaemia is an increase in cardiac output[43] and hence a raised circulatory level as a compensatory mechanism to maintain tissue oxygenation. Higher than normal blood flows were therefore indicated by the reduced haemoglobin concentrations observed in these patients.

The two liver failure patients were both chronic alcoholics. They both had very low serum albumin levels showing that albumin was not being synthesised in their livers, significantly raised bilirubin levels associated with the liver being unable to excrete this substance and high prothrombin ratios

indicative of an inability to synthesise the vitamin K clotting factor. If the liver is severely damaged it is unable to excrete a number of substances, including oestrogen, and it is quite common for chronic alcoholics to exhibit physiological changes, such as the development of female characteristics and spider naevi,*6 associated with high oestrogen levels. Both these patients exhibited changes symptomatic of increased oestrogen levels. Since albumin levels are low and albumin plays a major role in maintaining the osmotic pressure of the blood there is a loss of fluid from the blood into the abdominal cavity causing the oedema (a fluid filled abdomen) noticeable in both these men.

Because of the very limited time available for this study samples from only three patients were looked at. These patients were one of the men from the original renal failure study and two males suffering from irreversible liver failure. Important characteristics of and laboratory data for these patients are:

Patient One (renal failure)

A sixty year old male (weight 80 kg) who had been on dialysis for two months. The cause of his renal failure was Polycystic kidney disease, and he had no other medical problems.

Patient Two (hepatic failure)

A fifty-six year old male suffering from chronic alcoholic liver disease (cirrhosis) with portal hypertension and oesophageal varices (caused by portal hypertension). He had had a heavy alcohol intake for 30 years but no history of hepatic encephalopathy. The clinical evidence of his liver disease was numerous spider naevi, mild jaundice, oedema, ascites and urine sodium excretion of zero.

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*6 Raised web-like veins found in both men and woman with abnormally high oestrogen levels.

Patient Three (hepatic failure)

A forty-six year old male suffering from chronic alcoholic liver disease (cirrhosis) with portal hypertension, peripheral neuropathy and alcoholic cardiomyopathy (The latter two secondary to the thiamine deficiency i.e. beri beri). The clinical evidence of his liver disease with bilateral Dupuytren contractures was spider naevi, gynaecomastia, atrophic testes, female hair distribution, pale nails, bruises, oedema and ascites. This patient developed hepatic encephalopathy in hospital before the morphine study but responded to treatment for this. A liver scan showed severe parenchymal liver disease and splenomegaly. His red cell thiamine phosphate level was low (consistent with beri beri).

Normal Subjects

The normal subjects were three male physicians, aged 34-41 years, all of whom were free of disease and were not taking regular medication.

Samples were extracted for all 3 patients, but in the final morphine determination analyses were carried out for only patients One and Two.

The dosage of morphine administered to achieve analgesia depends on the degree of analgesia required, the patient's reponse and the route of administration. Parenteral dosing of ~0.1-0.15 mg kg^{-1} of body weight achieves plasma concentrations of ~0.05 $\mu g\ mL^{-1}$ with the plasma concentrations peaking in 20-90 minutes. This dosage will relieve severe to moderate pain.

6.2 CLINICAL PROTOCOL

The study was approved by the Canterbury Hospital Board Ethical Committee, and all subjects gave informed written consent. All medical procedures were performed by staff at the Christchurch hospital. For the renal failure study papaveretum (0.25 mg/ kg body weight) was injected into the

deltoid muscle in the upper arm. In the patients this was the fistula arm (Appendix I). Morphine was administered orally as an elixer (20 mg) or IV (10 mg) over 2 minutes to the liver failure patients. Blood was obtained from a venous cannula in the contralateral arm immediately before injection and at intervals over the next twenty four hours. Plasma was separated from the blood samples collected and stored at -20°C until analysed.

In both the patients and the healthy volunteers the range of clinical responses following the Papaveretum injection included somnolence, elation, hyperactivity, and vomiting.

6.3 ANALYSIS OF THE SERUM SAMPLES

Serum samples from the patients were analysed for morphine by EI GCMS, using the procedures which have been described. The analyses of the samples from the healthy volunteers, used in the previous Canterbury study, were accepted for this work.

The time available, after resolution of the SIM problems, to complete this thesis was only a few days. It was therefore decided to limit the objectives to:
i) determining whether the serum samples frozen for more than two years gave the same plasma morphine concentration levels as those used within a few days. This is important because patients often present with interesting conditions but not always at the time that a GCMS analyses can be carried out.
ii) To determine the shapes of the serum morphine curves for IV and oral morphine administration for liver failure patient Two.
To do this required making assumptions about the shapes of the curve in order to know how much D3 to add to get the D3:D0 ratio within the calibration graph range of 3:1 - 1:3. Because it had been suggested that liver disease does not markedly affect plasma morphine levels it was assumed that the curves would follow the shapes of those for normal subjects.

A further complication arose at the commencement of this limited series of measurements - the HP MSD failed and could not be repaired in time for use. Hence, although all the analytical development work had been done on the MSD, the MS80 had to be used. There was not time to re-run calibration samples on the MS80, and it was therefore decided to assume a calibration curve slope of 1.00. This might mean that the levels reported are in error by up to 25%, but the shape of the curves will not be affected. Reproducibility was established for the MS80 prior to the measurements by running a selection of samples, from the complete set for patient One, using SIM. For each sample 4 to 7 injections were made and the means and standard deviations for these sets of data were:

Data set	Mean	S.D.
1	4.67	0.171
2	4.73	0.135
3	0.76	0.026

For each patient, six blood samples were analysed for both unchanged and glucuronidated (conjugated) morphine. Total morphine was analysed for by incubating the plasma sample with the enzyme β-glucuronidase at 37°C for 24 h, and conjugated morphine calculated as the difference between total and unchanged morphine.

Fig 19 shows the plasma morphine concentration versus time curves (reproduced from the literature) for healthy subjects who had been given IM morphine.[2] On this graph curves 1-3 are for three subjects who each received 10 mg of morphine sulphate. Morphine concentrations were measured by RIA. Curve 4 represents the mean measured plasma morphine concentrations for six subjects who each received 5.75 mg of N-methyl-^{14}C morphine sulphate for each square metre of body surface area. Fig 20 shows the the morphine and morphine glucuronide concentrations for patient One, as determined in the

Fig 19: **Morphine metabolism in normal subjects**

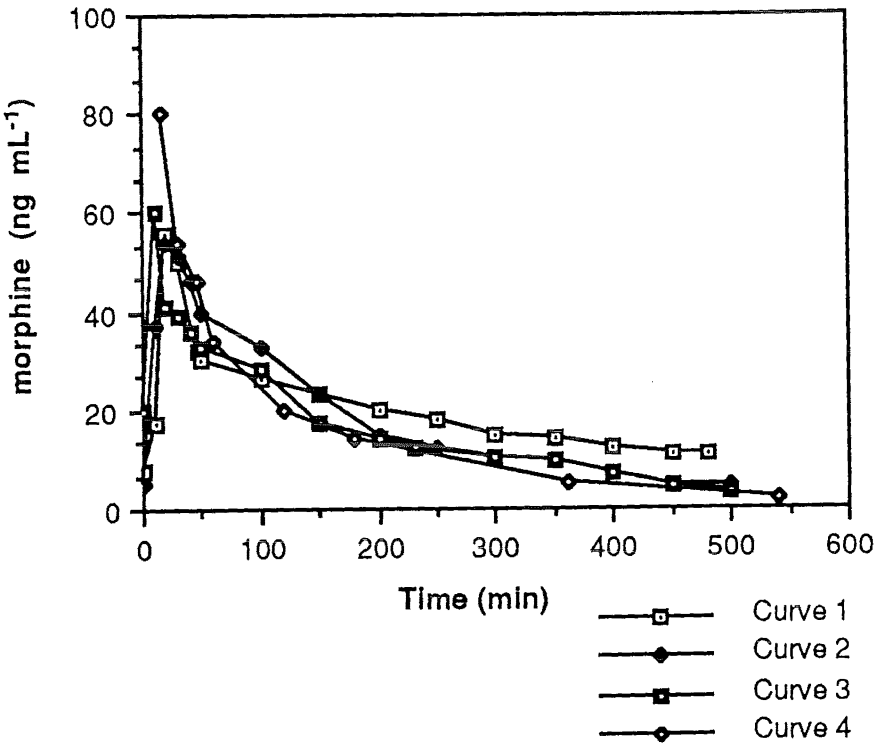
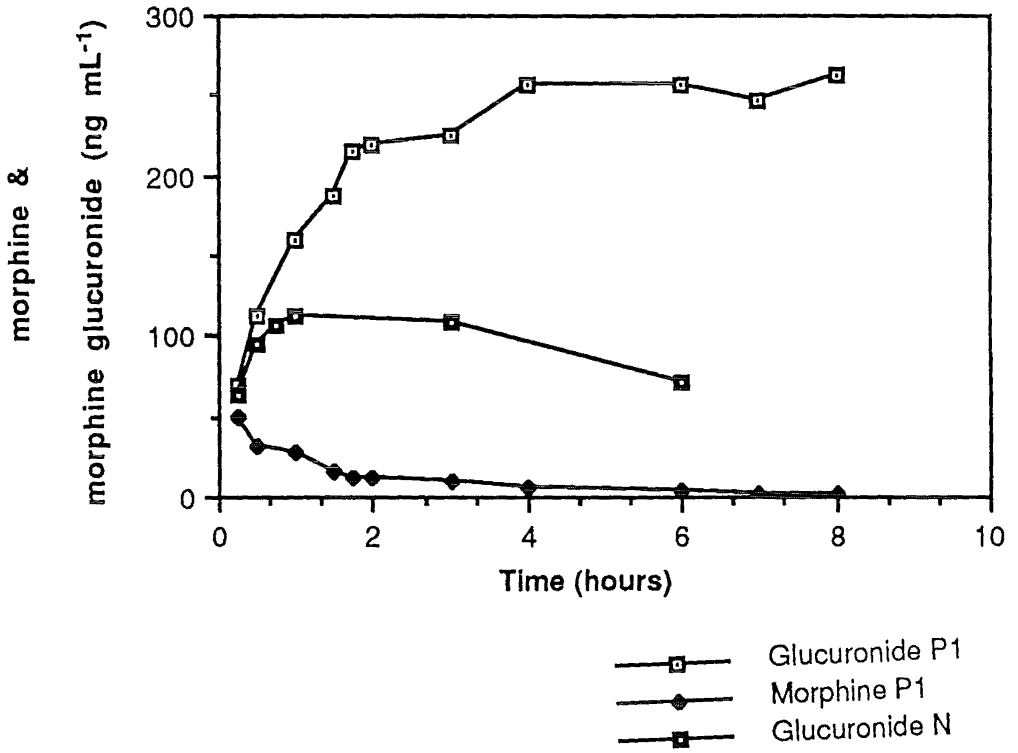


Fig 20: **Morphine and morphine glucuronide metabolism**



previous study, along with mean morphine glucuronide concentrations (reproduced from the literature) for six healthy subjects. A comparison of these graphs shows clearly that the morphine clearance in patient One was not different from that expected in healthy subjects, but his glucuronide clearance shows the impairment expected in renal failure. From the renal failure samples run during this determination (Fig 21) it can be seen that (within the limits of this study) the plasma morphine levels and the shapes of the curves are the same. The samples do not appear to have been affected by being frozen for three years.

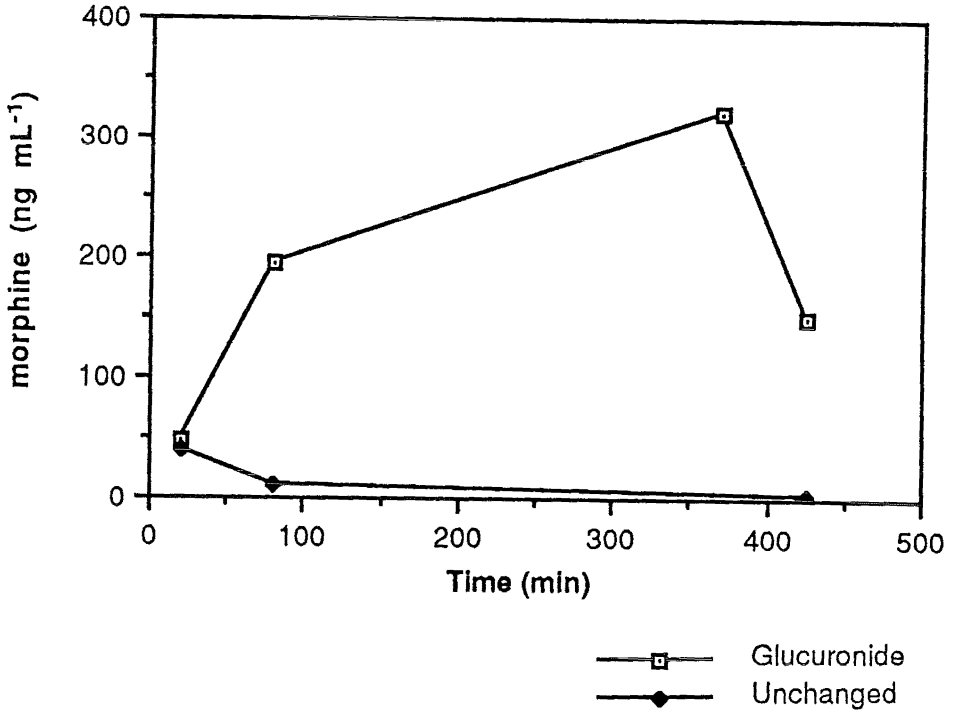


Fig 21: **Renal failure IM administration**

The results of this work, shown in figure 22 appear to establish that liver failure has not dramatically affected the metabolism of morphine after IV administration. The curve for the unchanged morphine appears to follow that of healthy subjects, but the glucuronide curve peaks at a later time. Since morphine is a high intermediate clearance drug with a plasma clearance approximating predicted liver blood flow,[63] it is expected that changes in liver function might produce alterations to morphine kinetics. The results in figure 22 and the work of Patwardhan *et. al.*[34] which found no significant difference between healthy volunteers and cirrhotic patients in systemic plasma clearance of morphine after single IV doses, strongly indicate that the changes are minimal.

Studies in healthy volunteers show that morphine is well absorbed from the gastrointestinal tract with about 80% of the dose of oral morphine being recovered in the urine collected during the 48 hours after administration. It is difficult to interpret the curves (Fig 23) for patient Two following oral the administration of morphine. The curve for glucuronidated morphine is similar to that for IV administration but the curve for unchanged morphine is quite different and the levels are low. There was no control group for comparison with the patient data after oral administration. Sawe *et.al.* found that ~35% of an oral dose of morphine reaches the systemic circulation as unchanged drug and that it reached peak plasma concentrations within a short period of time[63] Fig 23 shows a similar rapid appearance of morphine in the blood of patient Two. Selection of the samples appears to have been good for the IV administration but a sample at ~3 h after dosage would have been better for the oral administration, as it is difficult to tell, with this data, where the conjugated morphine concentrations peak.

It is apparent that the liver failure patient is metabolising morphine, although more slowly than a healthy subject, and since the liver is supposed to be the major site of morphine metabolism this raises the question as to where this process is occuring. As previously mentioned in this thesis, there is considerable evidence for gut wall metabolism of morphine in many animals but

Fig 22: **Liver failure - IV administration**

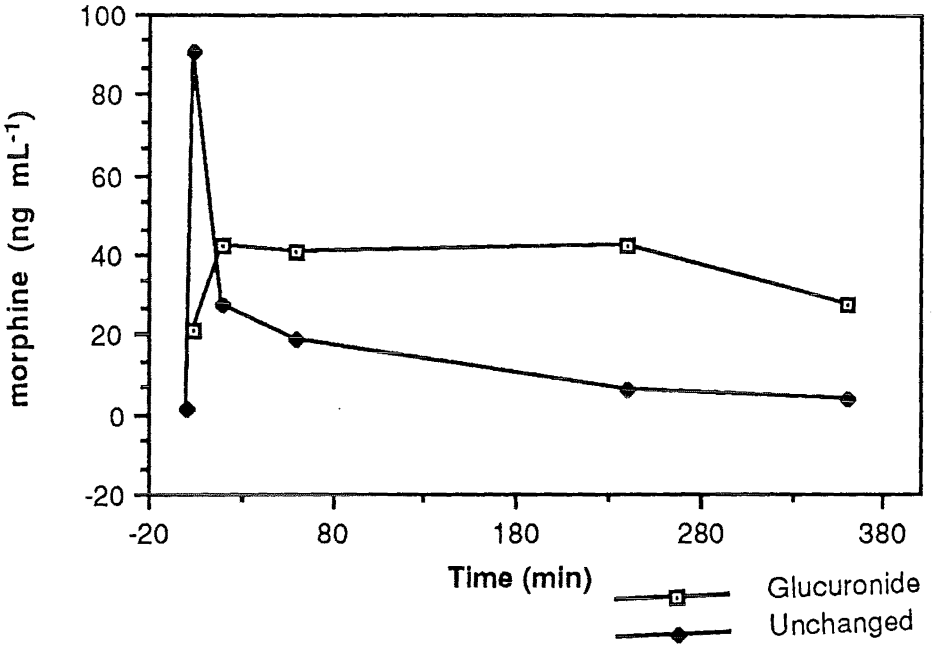
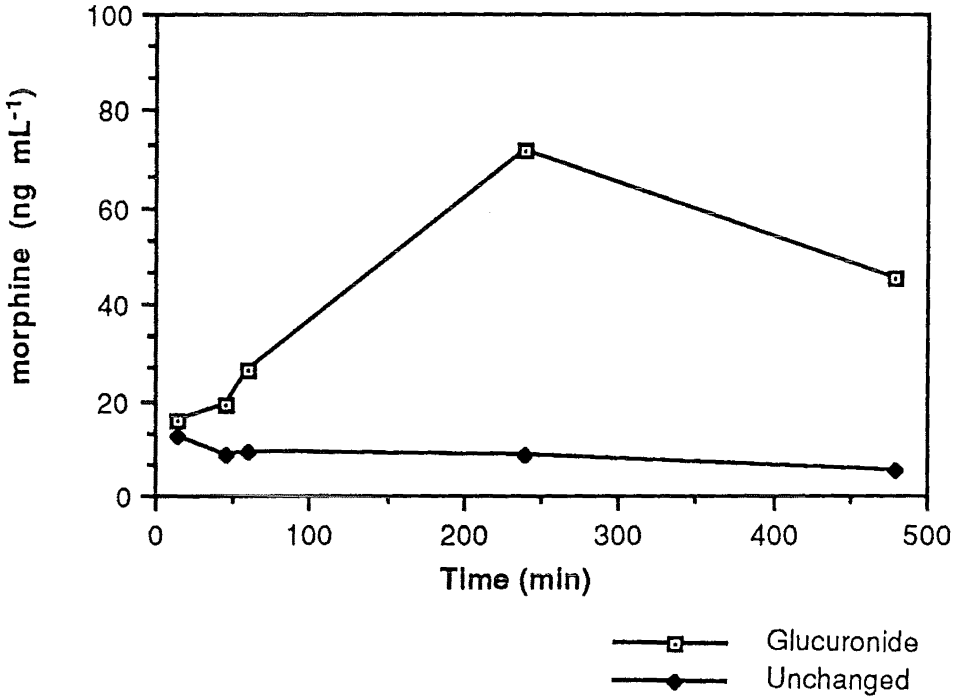


Fig 23: **Liver failure - oral administration**



this site has not been demonstrated in humans. It is possible that with the unavailability of the normal site the body is able to make use of a little-used alternative or possibly the morphine is being metabolised in pathways other than glucuronidation e.g. N-demethylation or sulphonation.

It is not possible to draw substantiated conclusions from the little data this study has been able to obtain, but it does seem that the established view that liver failure does not impair morphine metabolism is upheld. What data has been obtained in this work would indicate that the further larger study planned would be worth pursuing.

6.4 CONCLUSION

The work described in this thesis has established the following:

(i) The Solid Phase Extraction techniques used have a high extraction efficiency and produce samples pure enough for good quantitative morphine determination.

(ii) The problems with the MS80 SIM function were identified and corrected.

(iii) Analysis of the renal failure sample supported the findings of the previous study that renal failure does not impair morphine metabolism. It also showed that plasma could be frozen for four years without affecting the morphine levels detected by the analytical process.

(iv) Analysis of the liver failure sample indicated that metabolism of morphine was not the same as for normal subjects and that further study would be useful.

EXPERIMENTAL

GLASSWARE

All glassware was initially cleaned by soaking in a chromic acid bath (70 mL saturated sodium dichromate solution and 2 L of concentrated sulphuric acid) for several hours and subsequently rinsed thoroughly several times with hot water before being oven dried. All glassware used for drug extraction procedures, including Reacti-vials (Pierce Chemical Co., Rockford, Ill.), was silanised by soaking overnight in a solution of dimethyldichlorosilane in toluene (1:20) followed by a methanol wash and oven drying. After use all glassware was boiled in "Decon 90" solution (Decon Laboratories Ltd., Hove, England) for 2 h, allowed to soak for a minimum of 2 h, thoroughly rinsed with distilled water, and oven dried before resilanising.

EXTRACTION OF MORPHINE FROM MORPHINE HYDROCHLORIDE

Morphine hydrochloride (1.0 g) was dissolved in 20 mL water and a solution of sodium bicarbonate (0.5 g) in 20 mL of water added. The morphine appeared as a white precipitate almost immediately. The mixture was cooled in iced water and stirred occasionally. The precipitate was collected in a sintered glass funnel, washed with cold water and dried. Yield: 0.585 g (66%); m.p. 254°C (dec) (Lit 254°C (dec) Merck).

MORPHINE AND N-TRIDEUTERIOMETHYLNORMORPHINE

The crude compounds[44] were purified by repeated recrystallisation from methanol : water followed by sublimation at 190°C under vacuum.

EXTRACTION OF MORPHINE FROM PLASMA

(i) Unchanged Morphine

The C-18 Bond-Elut columns (Analytichem International, Harbour City, CA, U.S.A.) were conditioned by drawing through, under vacuum, 1 mL of methanol followed by 1mL of distilled water and the vacuum turned off. The sample, plasma or aqueous standards (1mL), buffered to pH 8.5 with a carbonate buffer (1 ml, 5:1 w/w $NaHCO_3$:Na_2CO_3), was added to the reservoir, the vacuum turned on and the sample drawn through the column. With the vacuum still on the column was eluted for interferents by drawing through 1 mL of water and air drying under vacuum for 2 min, then turning the vacuum off. The Vac Elut cover was removed and the needles wiped with a tissue. The collection rack containing silanised glass tubes was inserted and the cover replaced. The morphine was eluted by adding 600 µL of the elution solvent (9:1 v/v chloroform:isopropanol) to the column and letting it stand for 1 min. The vacuum was turned on to collect the eluent and then turned off and released. A second elution of 200 µL was done in the same way. The eluents were transferred to 1 mL Reacti-vials and evaporated under nitrogen at room temperature. The residue was derivatised by the appropriate procedure immediately prior to analysis by GCMS.

(ii) Total Morphine

An appropriate volume of standard D_3-morphine solution was added to plasma (1 mL) in a 10 mL silanised centrifuge tube, stoppered, thoroughly mixed and left to stand for 1 h. The plasma was buffered at pH 5.0 with acetate buffer (~0.2 M, 1 mL), 2000 Fishman units of β-glucuronidase (Sigma G-0750) added and the mixture incubated in a water bath at 37°C for 24 h. The procedure for unchanged morphine was then followed.

DETERMINATION OF EXTRACTION EFFICIENCY

An appropriate volume of standard D_0-morphine solution was added to 1 mL aliquots of drug free plasma. The spiked samples were thoroughly mixed, left to stand 1 hour and extracted by the procedure for unchanged morphine. An appropriate volume of standard D_3-morphine solution was added to the extracted material prior to derivatisation.

DERIVATISATION TECHNIQUES

1. The plasma extract was placed in a 1 mL Reacti-vial, and evaporated to dryness at 70-80°C under nitrogen. The residue was reacted with N,O-Bis(trimethylsilyl)acetamide (BSA, 50 µL) or N,O-Bis(trimethylsilyl)-fluoroaacetamide (BSTFA, 50 µL) in a sealed vial for 1 hour at 60°C.

2. The extract was placed in a 1 mL React-vial and evaporated to dryness under nitrogen at room temperature. 190 µL of ethyl acetate (AR) and 10 µL of pentafluoroproprionic anhydride (PFPA) was added and the mixture heated in a sealed vial at 90°C for 15 mins.

PREPARATION OF 2,2,2-TRICHLOROCARBOETHOXY-NORMORPHINE

Morphine hydrochloride (1 g) and potassium carbonate (9 g) were stirred for 10 mins in 100 mL of chloroform. To this suspension was added quickly 5.23 g (3.4 mL) of 2,2,2,-trichloroethylchloroformate (reacts readily with atmospheric water) and the mixture refluxed under dry conditions for 60 h at 80°C. The resulting red solution was cooled, 40 mL of water added and the golden coloured chloroform phase separated and concentrated *in vacuo*. 60 mL of methanol were added, the solution treated with 40 mL of an aqueous solution of potassium hydroxide (2.25 g) and potassium bicarbonate (4 g) and stirred under nitrogen for 24 h after which the the solution was acidified with

conc HCl and concentrated *in vacuo* to remove the methanol. A yellow oil was visible in the aqueous fraction. The aqueous material was extracted with five 20 mL aliquots of chloroform and concentrated *in vacuo* to produce a very viscous deep golden coloured oil. This was crystallised from a minimum of hot methanol and water to produce 2,2,2-trichlorocarboethoxynormorphine as large cuboid white crystals (0.552 g) in 39% yield. m.p. 195-197°C (Lit. 196-198°C, *Tet. Let.*, (1944), **14**, 1326).

PREPARATION OF N-TRIDEUTERIOMETHYLNORMORPHINE (Scheme I)

Lithium aluminium deuteride (LAD) (0.552 g) was added to 100 mL of tetrahydrofuran (THF) which had been dried over sodium wire immediately prior to use. A solution of N-2,2,2-trichlorocarboethoxynormorphine (0.552 g) in 35 mL dry THF was added and the mixture refluxed for 20 h. The mixture was cooled, 22 mL of ethyl acetate added, refluxed 30 mins and the pale golden coloured mixture cooled. A solution of sodium potassium tartrate (4.14 g) in 44 mL of HCl (2 M) was added and the mixture refluxed a further 4 h. The cooled mixture was concentrated *in vacuo* to remove the THF. The remaining white aqueous layer, containing some white crystals and a yellow powder around the sides of the flask was extracted with three 30 mL aliquots of ether. The aqueous fraction was saturated with sodium carbonate and extracted with three 30 mL aliquots of an isopropanol/chloroform mixture (IPA:$CHCl_3$,1:3). The combined organic fractions were dried over magnesium sulphate and concentrated *in vacuo* to produce white crystals and a little brown oily material. Recrystallisation from the minimum of hot methanol/water yielded white needle-like crystals of D_3-morphine (0.076 g, 22%) m.p. 251-253°C dec (Lit. 254°C dec. Merck). Deuterium incorporation was shown by mass spectrometry to be greater than 98%.

PREPARATION OF N-CARBOPHENOXYNORMORPHINE

Anhydrous morphine (1.04 g, 3.64 mmol) and finely divided potassium bicarbonate (6.17 g, 61.64 mmol) were suspended in chloroform (100 mL). To this was added rapidly phenylchloroformate (4.72 g, 3.12 mL, 30.16 mmol), the mixture vigorously stirred, and refluxed for sixty hours. The chloroform was decanted from the mixture. The remaining inorganic material was dissolved in ~30 mL of water, added to the chloroform and shaken well. The chloroform layer was collected from a separating funnel, washed with ~25 mL of water followed by ~25 mL HCl (2 M), dried over magnesium sulphate and evaporated *in vacuo*. The resulting deep yellow oil, was distilled under high vacuum to remove the phenol, to produce N-carbophenoxynormorphine as a golden yellow solid (2.16 g, 3.42 mmol) in 93.8% yield.

PREPARATION OF N-TRIDEUTERIOMETHYLNORMORPHINE (Scheme II)

N-carbophenoxynormorphine (2.16 g) was dissolved in 120 mL of dry THF, immediately added to a solution of LAD (2.16 g) in THF (400 mL) and refluxed in a nitrogen atmosphere for 20 h. Ethyl acetate (80 mL) was added to the cooled solution which was refluxed for a further 30 min. A solution of sodium potassium tartrate (15 g) in HCl (2 M, 160 mL) was added and the mixture refluxed for 4 h. The THF was removed *in vacuo* , the aqueous layer extracted with three 120 mL aliquots of ether, made alkaline by the addition of sodium carbonate and extracted with four aliquots of IPA:$CHCl_3$ (1:3). Removal *in vacuo* of the solvent from the combined extracts left a pinkish brown solid. As attempts to recrystallise this material from hot methanol/water were unsuccessful ~0.5 g was chromatographed on a C-18 (10 g) reverse phase column using methanol in ether with gradual increases in the methanol percentage. The product (identified by t.l.c.) was found in the 60%,80%, and 100% methanol fractions. These were combined and recrystallised from 50:50 MeOH/H2O to

produce white needle-like crystals of D_3- morphine (0.3665 g, 35% yield based on the 1 g morphine) Deuterium incorporation was shown by mass spectrometry to be greater than 99%.

DETERMINATION OF PURITY OF MORPHINE STANDARDS BY HPLC

To determine if the D0-morphine and D3-morphine standards were at the calculated concentrations they were run against the Government Analyst's standards, by HPLC, at the DSIR. The results are tabulated below:

MorphineHCl (std) M_r 375.8 (97% pure) 0.96 mg mL^{-1} ≡ 0.931 mg mL^{-1}

Counts 1013127 = 108821 cts mg^{-1}

MorphineHCl (std) 0.74 mg mL^{-1} ≡ 0.718 mg mL^{-1}

Counts 863526 = 1202683 cts mg^{-1}

MorphineSO_4 (std) M_r 379.4 (99% pure) 1.02 mg mL^{-1} ≡ 1.01mg mL^{-1}

Counts 1130217 = 1119027 cts mg^{-1}

Average = 1136641 cts mg^{-1} (s = 5%)

Samples:

D_0- morphine (std) 1.0135 mg mL^{-1}

Counts = 1231787 ⇒ 1.0837 mg mL^{-1}

D3-morphine (DSIR std) 0.978 mg mL^{-1}

Counts = 1356330 ⇒ 1.1933 mg mL^{-1}

Calculated: D0:D3 (wt) = 1.036

Measured: D0:D3 (HPLC) = 0.908

GAS CHROMATOGRAPH PARAMETERS

The analyses are carried out on a Carlo Erba gas chromatogram using a DB5 column. For each run the GC is held at the injection temperature of 80^{o}C for 1 min, ramped ballistically to 280^{o}C where it is held for 10 mins, then ramped ballistically to 285^{o}C to help clean the column. The helium carrier gas flows at 3 mL min^{-1}. The source temperature of the mass spectrometer was 200^{0}C.

APPENDIX I

Haemodialysis is the procedure used to remove the waste products of metabolism from the blood of patients who have impaired or failed renal function. The blood is passed through an artificial membrane surrounded by the dialysate (dialysis fluid), and returned to the patient. Waste products such as urea, creatinine and uric acid diffuse out of the blood through the dialyser membrane and into the dialysate which is adjusted to the individual requirements of each patient. An anticoagulant is required to prevent the blood from clotting.

Haemodialysis requires repeated reliable vascular access. This used to be by means of an external artificial arteriovenous shunt (Fig 24) the ends of which were disconnected, during dialysis, and joined to the appropriate parts of the dialyser. These shunts had a tendency to clotting and their presence meant the limb could not be immersed in water. The commonest access in use today is the subcutaneous arteriovenous fistula (Fig 25) The radial artery of the non-dominant arm is joined, usually side by side, to the vein at the wrist causing the vein further up the arm to increase in size and develop a thicker wall. This enlarged artery-like vein provides easy access to the bloodstream both to withdraw blood and then to return it after it has passed through the dialyser.

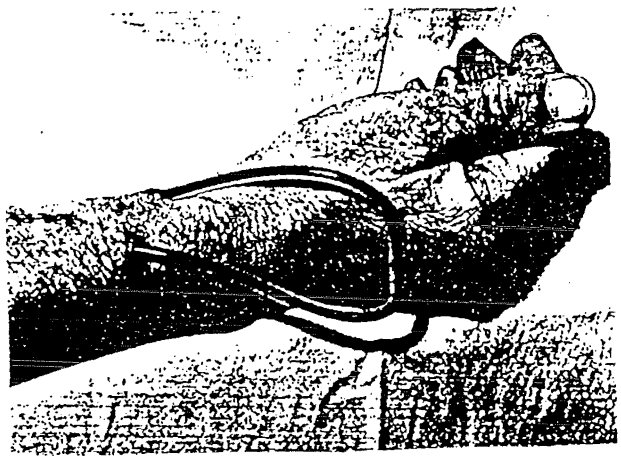


Figure 24: **External arteriovenous shunt**

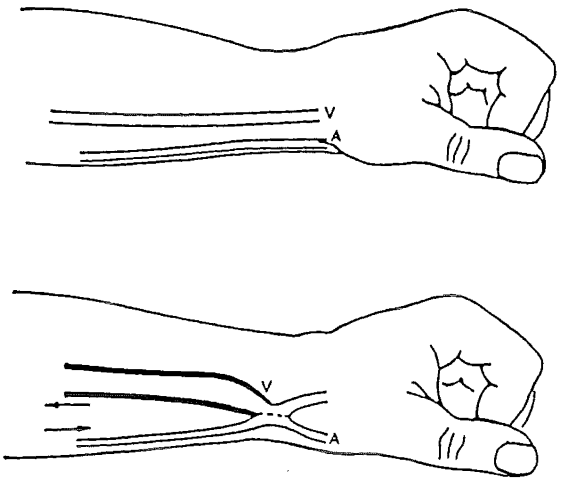


Figure 25: **Subcutaneous arteriovenous fistula**

APPENDIX II

Data for Preparation of Calibration Curves

Calibration Curve at the 500 ng mL^{-1} Concentration

Weight Ratio	Ion Intensity Ratios			
	577:580		414:417	
0.25	0.29	0.29	0.31	0.31
	0.30	0.28	0.31	0.29
	0.29		0.30	
0.49	0.56	0.53	0.56	0.52
	0.55	0.53	0.55	0.52
	0.56	0.53	0.55	0.51
0.99	1.06	0.97	1.02	0.94
	1.08	1.00	1.00	0.94
	1.06	1.01	0.99	0.95
1.97	2.02	2.10	1.81	1.91
	2.01	2.13	1.78	1.90
	2.00	2.14	1.79	1.92
3.94	4.08	4.45	3.47	4.10
	4.01	4.24	3.47	4.35
	4.05	4.24	3.47	4.59

Calibration Curve at the 30 ng mL^{-1}

	414:417	577:580
0.1996	0.241	0.273
	0.239	0.245
	0.298	0.280
0.499	0.489	0.441
	0.502	0.527
	0.522	0.527
	0.527	0.465
0.998	1.002	0.892
	0.879	0.877
	0.953	0.868

1.996	1.502	1.878
	1.573	1.560
	1.566	1.599
2.995	2.170	2.932
	2.181	2.038
	1.935	2.332

Curve at the 100 ng mL^{-1} Concentration

0.281	0.32
	0.32
	0.33
	0.32
	0.33
0.562	0.56
	0.55
	0.59
	0.59
	0.61
1.124	1.26
	1.24
	1.17
	1.17
	1.32
2.249	2.24
	2.43
	2.44
	2.54
	2.47
4.498	4.92
	4.52
	4.46
	4.52
	4.65

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